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Title: Histological Examination of *Phytophthora ramorum* in *Notholithocarpus densiflorus* Bark Tissues

Abstract Approved

Everett M. Hansen

Colonization of *N. densiflorus* tissues by *P. ramorum* is not well understood. The pathogen is able to colonize nearly all tissues of this host but it is unclear how a tree is ultimately killed. Because this is such a destructive invasive pathogen, it is important to investigate its pathogenic strategy. Microscopic investigation of xylem colonization has been conducted, but colonization of bark tissues, which has always been regarded as an important part of infection, has not been investigated previously. In this research, *P. ramorum* infected *N. densiflorus* bark tissues were examined using various microscopic techniques to try to better understand the importance of bark infection in killing a tree. Host responses to infection were detected by various methods in conjunction with examining *P. ramorum* colonization, to try to better understand why the host is unsuccessful in stopping the pathogen. Results of this work indicate that the pathogen can colonize nearly all *N. densiflorus* bark tissues but that phellogen and parenchyma of the inner bark are the most frequently and densely colonized. A last piece of this work investigated the role of elicitins in pathogenicity during this interaction. These low molecular weight proteins have been implicated as having a role in pathogenicity in this and other phytopathogenic *Phytophthora*

species. A fluorescent antibody was used to label elicitins in infected plant tissues to determine where they are produced, and what effect, if any, they appear to have on host cells. Elicitin labeling caused hyphal cell walls to fluoresce in plant tissues, contributing to an understanding of bark colonization more than to an understanding of the role of elicitins in promoting infection. Findings of this research show that nearly all bark tissues are capable of being colonized, that this host responds to infection in several ways, and that elicitins are present in cell walls of hyphae in infected plant tissues.

Histological Examination of *Phytophthora ramorum* in *Notholithocarpus densiflorus* Bark Tissues

by
Molly M. Botts

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Molly M. Botts, Author

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Histological Examination of *Phytophthora ramorum* in *Notholithocarpus densiflorus* Bark Tissues

1. Introduction

1.1 Background

The disease best known as sudden oak death has been the cause of extensive mortality of tanoak, *Notholithocarpus densiflorus* (Hook. And Arn.) Rehder Manos, Cannon, and Oh, through much of coastal California since 1995 (Rizzo et al., 2002) and in Curry County, Oregon since 2001 (Goheen et al., 2002). The causal agent of this disease, *Phytophthora ramorum* Werres, Cock, and Man int'l Veld, is a water mold, belonging to the phylum Oomycota in the kingdom Stramenopila. *Phytophthora* and its sister genus *Pythium*, in the family Pythiaceae, are genera well-known for their plant pathogen members, many of which cause root and crown rots, but others which cause foliar blights, stem cankers, fruit rots and other diseases.
Phytophthora ramorum was discovered in the U.S. and Europe around the same time, being native to neither place and causing extensive damage in both places. However, to what region of the world the organism is native remains unknown. In addition to infecting *N. densiflorus*, this organism is capable of infecting at least 44 other species in 35 genera native to California and Oregon, including three *Quercus* species and other prominent forest species such as *Umbellularia californica*, *Rhododendron macrophyllum*, *Vaccinium ovatum*, and *Arbutus menziesii* (http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/). In addition to these native species, the wide host range of *P. ramorum* includes numerous nursery plant species and varieties.

Although *P. ramorum* is capable of infecting coast live oak (*Quercus agrifolia)*, black oak (*Q. kelloggii)*, and Shreve's oak (*Q. parvula* var. *shrevei)*, these oak species are apparently less susceptible to the disease than *N. densiflorus*, with a smaller percent dieback occurring in coast live oak and California black oak after plant dip inoculations (Hansen et al, 2005), and smaller lesions developing within inoculated logs and stems in coast live oak and California black oak (Rizzo et al, 2002; Hansen et al, 2005).

The most apparent symptom of infection on *N. densiflorus* is the presence of lesions on the bole of the trunk, only visible after the outer bark has been peeled away. It has, therefore, always been speculated that trees are killed due to extensive death of bark and cambium tissues and the disruption of phloem transport. However, we now know that *Phytophthora ramorum* is capable of infecting the sapwood of *N. densiflorus* trees as well as reducing hydraulic conductivity (Parke et al, 2007; Collins et al, 2009). The colonization of both *Rhododendron macrophyllum* twigs and *N. densiflorus* sapwood by *P. ramorum* has been investigated microscopically (Pogoda and Werres, 2004; Parke et al, 2007), but colonization of *N. densiflorus* bark tissues has not yet been investigated. Within the context of the broad question asking how a *N. densiflorus* tree is killed, we address and may begin to answer more specific questions. I will list the specific objectives of this research here, followed by a description of my research approach to the questions presented by each of my objectives. Following this, I will provide a more detailed review of the literature relating to each objective. The remainder of this chapter and each subsequent chapter will address each objective in the order they are listed below.

1.2 Objectives

1. To determine how *Phytophthora ramorum* colonizes the bark of *N. densiflorus*.

2. To determine the ways in which *N. densiflorus* responds to infection by *Phytophthora ramorum*, and how the pathogen may be affected by them.

3. To determine the role that the pathogen proteins known as elicitins have, if any, in pathogenicity in this interaction.

1.3 Research Approach

The question of how a *N. densiflorus* tree is killed is important and the answer, so far, is unclear, because *P. ramorum* infects leaves, shoots, bark, and wood. The pathogen has the ability to overcome or bypass the physical boundaries presented on stems, leaves and bark, such as trichomes and cuticles on leaves, and the heavily suberized

and lignified cork of the outer bark. Moreover, once inside these tissues, *P. ramorum* is capable of causing extensive damage despite any defenses induced in the host upon infection. This combination of abilities is achieved by few pathogens, even among situations where the pathogen and host have not coevolved. Considering the complex of both preformed and induced defenses, particularly in the bark, that are common to most trees, it is clear that there is something unique about this pathogen and/or this host that allows this destructive interaction to occur. This system suggests a unique, and perhaps less definitive answer to how the tree is ultimately killed.

We cannot discount the fact that infection of all of these tissues will contribute to creating stress in the tree and therefore, contribute to its overall death, or at least the rapidity with which the tree dies. However, another way to think about this question is to consider whether infection of one of these tissues alone could cause the tree to die. It may be difficult to determine specifically what causes the tree to die, but as the number of non-native plant pathogen epidemics increases, the investigation of this question may become more valuable as this situation and likely others in the future, call for a new way of approaching/understanding plant pathogenesis.

1.3.1 Colonization of *N. densiflorus* bark tissues

Although the infection of leaves and young stems occurs and is certain to contribute to the overall stress of an infected tree, it is clear that it does not occur frequently or severely enough to kill a large tree. Infection of woody tissues is now also known to be a significant feature of *N. densiflorus* infection by *P. ramorum*; infecting the ray parenchyma, vessels, and fiber tracheids, and disrupting sapflow in the outermost sapwood (Parke et al., 2007). The question still to be answered, however, is of the importance of bark infection in killing the tree. The first and most obvious way to gain a better understanding of the significance of bark infection is by looking at the infected tissues microscopically and trying to understand the strategy of the pathogen. What specific tissues within the bark are colonized and what is the frequency/density of hyphae within those tissues? Are sieve tube elements being obstructed or is the cambium being severely damaged? Does it appear that cells are killed before they are colonized? Is the pathogen present in dead cells such as those in the cork, or sclereids

and fibers of the inner bark? How does *P. ramorum* enter bark tissue and how does it then move from cell to cell? These questions were investigated primarily using fluorescence microscopy, and staining with calcofluor for cellulose, a major component of *Phytophthora* cell walls. In addition, immunolabeling of *P. ramorum* elicitins was performed using a fluorescently labeled antibody to illuminate hyphae. Confocal microscopy and Scanning Electron Microscopy were used supplementarily to look at tissues in a more three-dimensional way so that growth of hyphae between cells could be better tracked, and so that hyphae could be visualized more clearly by removing some of the distortion that can be caused by autofluorescence of bark tissues.

1.3.2 Host Responses to Infection

In addition to observing pathogen localization and movement in *N. densiflorus* bark, it is also important to look at activities of the host in response to infection, particularly defense responses. The first challenge of the pathogen is getting into the plant which means, for this host, first penetrating or bypassing a preformed defense, the heavily suberized and lignified cork tissue of the outer bark. In conjunction with observing hyphal entry into these tissues, as mentioned earlier, we looked for evidence of disruption of the cork tissues by the pathogen. Once the pathogen is inside bark tissue, it is clear that it is effective in causing extensive lesions (Hansen et al, 2005) despite any host defense responses that are presented. We know that *N. densiflorus* responds to infection in the bark, because of the presence of "bleeding" on the surface of the bark in infected areas and the wetness and discoloration of lesions, but we do not know what this material is or how to interpret the production of it. It is worthwhile to look more closely at what kind of host defense responses are occurring in bark tissue and where, particularly in relation to where the pathogen occurs. Major defense responses that are typically known to occur in bark in response to pathogen challenge include the production of phenolics; callose deposition; and wound periderm formation. We will look for these responses in bark tissue and look for evidence that the pathogen is hindered by them. Phenolics were stained using the vanillin HCl method and callose was stained with calcofluor. Wound periderm was observed without staining, using autofluorescence of tissues. We could then ask

specific questions during observation. If hyphae were present outside the edge of the region of phenolic production, did they appear to be less damaged than those within that region? Was the pathogen able to penetrate a wound periderm or cells filled with callose? It is important to note that all of these responses are known to be triggered by pathogen challenge in bark and all can be effective in deterring and/or preventing further infection in many interactions. However, they are also all responses known to be triggered by injury, so they do not suggest that the tree is responding specifically to pathogen attack.

Specific recognition of pathogens by plants can trigger what is known as a hypersensitive response (Graham and Graham, 1999; Morel and Dangl, 1997; Levine et al., 1996). This is a defense response that occurs rapidly in response to pathogen challenge, and is characterized by the production of superoxides, hydrogen peroxide, and hydroxyl ions that lead to plant cell death followed by pathogen cell death. When a strong hypersensitive response occurs, it is typically very effective in blocking the pathogen (Graham and Graham, 1999; Morel and Dangl, 1997; Levine et al., 1996). There is evidence to suggest that a partial hypersensitive response may occur during the interaction between *N. densiflorus* and *P. ramorum* (Manter, 2007). Investigation into this possibility will be performed through inoculation of leaves from *N. densiflorus* sprout cuttings, followed by sampling of the leaves periodically over a several hour time course and staining for superoxides using Nitroblue Tetrazolium.

1.3.3 Elicitin Production

The last of my objectives concerns a class of small molecular weight proteins known as elicitins that are produced only by *Phytophthora* and *Pythium* species. Their major presumed biological role is to function as sterol transport proteins, but they were first discovered and have been best studied for their ability to act as elicitors of host defense in incompatible disease interactions (Kamoun et al, 1993; Keller et al, 1996; Sasabe et al, 2000; Tyler et al, 2002). However, it has been demonstrated that host recognition and a subsequent partial hypersensitive response can be triggered even in compatible interactions (Kamoun, 1999). There is, additionally, evidence to suggest that these proteins may have a role in pathogenicity (Manter, 2007; Fleischman et al,

2005; Tyler 2002). The tools have now been developed to label elicitins using a fluorescently labeled antibody for *Phytophthora ramorum* elicitins (Manter lab, Ft. Collins). To better understand the role that these proteins play, if any, in the host pathogen interaction between *P. ramorum* and *N. densiflorus*, I will employ these tools on infected bark samples and observe them microscopically to determine where elicitins are produced, whether they are secreted into cells, and, if so, whether or not any detectable reaction by the plant cells can be seen. I hope that the collective results of these studies will improve our overall understanding of the mode of pathogenesis of *P. ramorum* on *N. densiflorus*.

1.4 Literature Review

1.4.1 Colonization of host tissues by *Phytophthora* species, with emphasis on *Phytophthora* species that colonize woody tissues

Most tree canker diseases caused by *Phytophthora* species are known to preferentially colonize the cambium and neighboring inner phloem and outer xylem tissues (Tippett et al., 1983; Robin, 1992; Davison et al., 1994; Scott, 2009). During later stages of infection they may move further into the phloem or xylem, depending on the *Phytophthora* species (Davison et al., 1994; Tippett and Hill, 1984; Robin, 1992; Brown and Brasier, 2007). Pogoda and Werres (2004) discussed the histopathology of *Rhododendron* stems wound inoculated with *P. ramorum*. Hyphae could be found in all tissues except the cambium, primary phloem and epidermis, but damage to the first two tissues in preparation made this difficult to determine. The pattern of infection changed moving from a healthy zone to an infected zone, suggesting a timeline of infection where colonization occurs first in the cortical parenchyma, pith parenchyma, and xylem followed by colonization of the pith rays and phloem and formation of chlamydospores. Overall, hyphae were most frequent in tracheids of the xylem. Much less discoloration of xylem, phloem, and pith ray tissues was observed compared to cortical parenchyma, cambium and collenchyma tissues which exhibited severe discoloration. Cell collapse was most severe in the cortical parenchyma and cambium but also occurred in groups of cells of the phloem. No other tissues exhibited cell collapse. They also describe that a more shriveled/shrunken appearance

to a stem appeared to be correlated with heavier discoloration in phloem tissues as well as a greater degree of cell collapse in the cortical parenchyma and cambium. Hyphae grew intercellularly and intracellularly in the pith and cortical parenchyma, but mostly intracellularly in other tissues. Chlamydospores were most frequent in the cortical parenchyma.

The authors suggested that *P. ramorum* first attacks xylem, followed by cambium and phloem, though it is unclear since damage to the cambium may have occurred in sample preparation. We must consider also that these are wound inoculations, where an actively growing culture of *P. ramorum* in agar was placed on the top of an excised *Rhododendron* stem tip. So, the timeline of infection here does not necessarily indicate the order in which tissues become infected in a natural infection situation. Transverse movement, it is suggested, likely occurs through the dilated pith rays as these cells were, indeed, the first to show discoloration, and hyphae in the phloem are not present until after this discoloration is seen. Hyphae were mostly intercellular in tissues which, is known for many other interactions between *Phytophthora* species and their hosts. It is also suggested that *P. ramorum* prefers to invade living cells rather than damaged ones. Many *Phytophthora* species are known to require living cells (Panstruga, 2003 and Grenville-Briggs, 2005; as cited by Kamoun). Next in the *P. ramorum Rhododendron* interaction, cell discoloration is followed by cell collapse and tissue disorganization. This could occur due to a hypersensitive response induced by elicitors of the pathogen or it could be due to the activity of a toxin. In either case, the death of cells may make contents more available to the pathogen. Several *Phytophthora* species are known to form haustoria within plant cells, ranging in appearance from "simple button-like structures to digit-like structures of increasing lengths" (Coffey and Wilson, 1983).

Histopathological studies of *Phytophthora lateralis,* the closest known relative of *Phytopthora ramorum*, in roots of *Chamaecyparis lawsoniana,* or Port-Orford Cedar, showed that hyphae grew inter- and intracellularly in the phloem, but were most concentrated in sieve cells and parenchyma cells of the functional phloem in the most susceptible plants (Oh and Hansen, 2007).

Many different modes of entry have been reported for *Phytophthora* species, including penetration between epidermal cells, directly through epidermal cells, through stomata of leaves, through wounds (Oh and Hansen, 2007; Coffey and Wilson, 1983). There has been limited investigation of *Phytophthora ramorum* entry into tissues, but Florance, (2003) reports, without documentation, on hyphae entering a lenticel on a *N. densiflorus* stem and hyphae entering a stomate of an *Umbellularia californica* leaf. Parke and Lewis (2007) report on the apparent attraction of *Phytophthora ramorum* zoospores to root primordia and wounds on the roots of *Rhododendron* plants grown in infested potting media, demonstrated by microscopic images showing the aggregation of germinating zoospore cysts in these areas. A histological study of *Phytophthora lateralis* on Port-Orford Cedar roots revealed that hyphae of the pathogen are capable of directly penetrating epidermal cells when entering the host plant, but may also enter between them (Oh and Hansen, 2007).

Consistent with the theory that *P. ramorum* may move radially through rays are the findings of Parke et al. (2007) with the discovery that *P. ramorum* infects sapwood of *N. densiflorus*. Here also, hyphae are abundant in ray parenchyma and it is hypothesized that they may gain access to sapwood tissues by traveling through rays from bark tissue. Hyphae are also found in vessels and fiber tracheids of the sapwood, penetrating a little over 3 cm deep into it, on average. Abundant tyloses form in the vessels of infected tissues and sapwood conductance is disrupted. Infection does not appear to spread further in the sapwood than in the bark. *Phytophthora ramorum* was also found to infect the sapwood of *Quercus* and *Fagus* species (Brown and Brasier, 2007).

In conducting histological studies on bark tissues, it is important to understand the basic anatomy of bark (Figs 1-3). I will provide brief definitions of bark regions and cell types, their functions, and how they were delimited and/or identified. The term "bark" will be used to describe all tissues outside of the vascular cambium. The "outer bark" is defined as all tissues from the innermost periderm outward. Periderm is composed of three tissues, one of which is the phellogen or cork cambium, a meristematic tissue from which the other two tissues arise. The phellogen produces the phelloderm to the inside, and the phellem or cork to the outside.

The phellogen arises from cortical tissue in the young stem and the entire periderm is formed within the first year of growth in most trees, replacing the epidermis as the outer protective layer of the stem. As a tree ages, it may produce multiple periderms which will then be layered alternately with secondary phloem tissue layers. When multiple periderms are present, the term "rhytidome" becomes synonymous with "outer bark". The phellogen may be one to a few layers in width, consisting of thin-walled cells that appear oblong in transverse section. The overall appearance of the phellogen is similar to that of the vascular cambium. Phellem, the tissue produced to the outside of the phellogen, is comprised of several layers of heavily suberized and lignified dead cells, appearing oblong and radially shortened in transverse section. This layer serves as a skin and as protection from extreme weather conditions, pests, and pathogens. The phelloderm is comprised of radially arranged parenchyma cells, polygonal to rounded in shape with intercellular spaces that vary in size and abundance among species. Their cell walls may or may not be thickened. The outermost layer(s) of phelloderm may contain chloroplasts and perform photosynthesis in some thin-barked species (Alekseev et al, 2007; Shigo, 1994). Both phellem and phelloderm cells may contain "resinous" substances and phellem is also known to sometimes contain callose (Chang, 1954; Litvay and Krahmer, 1976).

The "inner bark", also sometimes called "active bark" is defined as the region that extends from the vascular cambium to the edge of the innermost periderm. This region is composed of secondary phloem, only the innermost region of which contains conducting phloem. Cell types of the inner bark include sieve tube elements and their associated companion cells (in Angiosperms), sclereids, fibers, ray parenchyma, and phloem parenchyma. The sieve tube element is the phloem conducting cell. It is tubular and elongated and possesses sieve areas on its elongated walls as well as its end walls. A sieve area is a region containing specialized pores through which materials may pass. The end walls of sieve elements that connect the cells in strands are known as sieve plates and may have one or more sieve areas. While sieve elements contain some organelles, the majority of cellular functions for the sieve element take place in the adjacent and much smaller companion cell, connected to the sieve tube element by plasmodesmata. These two cell types occupy the vast majority

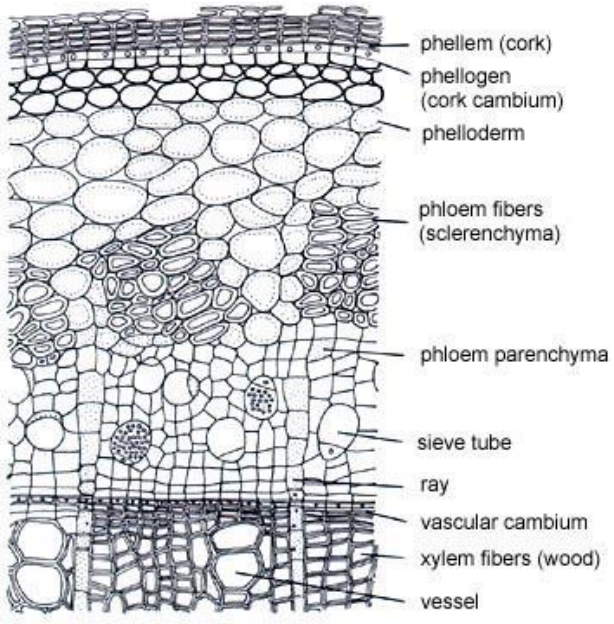
of area within the functional phloem, with some interspersed fibers and other parenchyma.

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. These can be distinguished from one another by their shape and arrangement when looking at transverse, tangential and radial sections. In tangential and radial sections, phloem parenchyma are vertically elongated and arranged in parallel strands. In tangential sections, bundles of ray parenchyma are vertically elongated and tapered at both ends and the cells within them appear oval to round. In a radial section, ray parenchyma cells are arranged in parallel rows of rectangular, horizontally elongated cells. In a transverse section, phloem parenchyma look oval to round but do not appear to be in rows, and ray parenchyma appear slightly vertically elongated (when inner to outer bark arrangement is vertical). The ray parenchyma in these sections demonstrate their ray shape; the rays increasing in width, in terms of increase in cell numbers, as you move from inner bark to outer bark. Phloem parenchyma can store a variety of compounds including sugars, lipids, and defense compounds, and can transport them locally. Ray parenchyma mainly serve to compensate for xylem girth increases but can also transport materials locally.

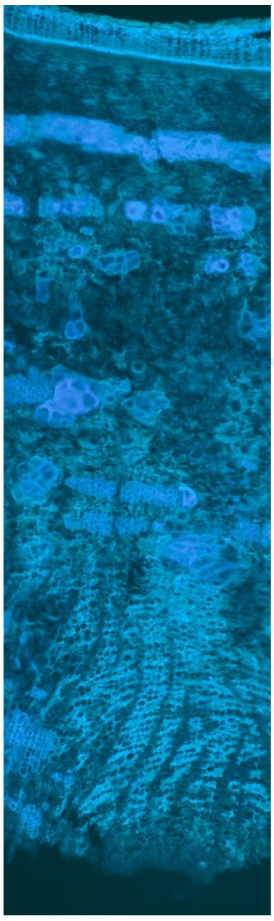
Sclereids and fibers are the only two types of cells with secondary walls in *N. densiflorus* bark, also known as sclerenchyma tissues, so they are the only ones we will mention here. These cells usually have heavily lignified secondary walls and are dead at maturity. Fibers in *N. densiflorus* have smaller diameters than sclereids and are elongated, whereas sclereids in *N. densiflorus* are polygonal to rounded. Sclereids can also be distinguished from fibers by the high density of pits in cell walls which can be seen with UV fluorescence. Both cell types usually occur in clusters but can occur singly also. Fibers originate from the vascular cambium and sclereids arise from parenchyma cells. Sclerenchyma tissues provide structural support to bark tissue.

Bark anatomical characteristics of coast live oak that have been suggested to be important for infection are bark thickness and the presence of unweathered brown areas in bark fissures, presumably a result of recent bark expansion (Swiecki and

Bernhardt, 2006). Bark thickness and presence of these unweathered brown areas in fissures are both positively correlated with the presence of disease symptoms. The combination of variables measured that correlated with presence of disease symptoms or disease progression are consistent with a model that suggests that vigorous, fast-growing coast live oak trees with dominant canopy positions are more likely to become infected than suppressed and stressed trees (Swiecki and Bernhardt, 2006).



www.apsnet.org/.../PhotosN-R/phelloderm.htm



Figures 1 and 2. Left: Figure 1. Diagram of a typical bark cross section: tissues and cell types of the inner and outer bark. Right: Figure 2. A calcofluor stained *N. densiflorus* bark cross section: an uncolonized region of a bark sample from a *P. ramorum* infected *N. densiflorus* tree.

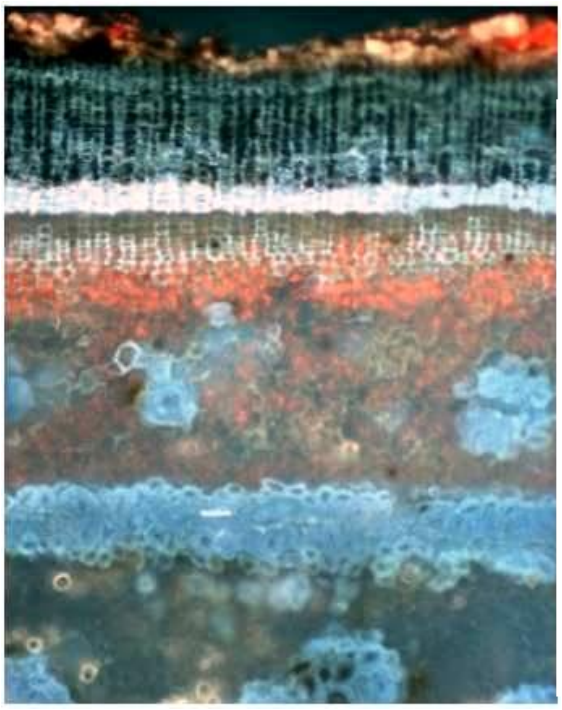


Figure 3. Autofluorescence of a *N. densiflorus* bark cross section: autofluorescence of uninfected *N. densiflorus* bark. Photo by Peter Kitin

1.4.2 Host Responses to Infection

There are a wide variety of defense strategies employed by plants. These may be preformed defenses or induced upon attack by herbivores or pathogens, and they may be specific to the organism that is attacking or general for all attackers. Preformed defenses include thorns, a cuticle on a leaf surface or cork on bark surfaces, as well as resin ducts containing volatile secondary compounds. Volatiles are particularly useful compounds because they deter potential attackers from a distance without any damage having to be inflicted on the plant. Common secondary defense compounds produced in the bark of trees include alkaloids, terpenes, and phenolics. Many secondary defense compounds can, however, be induced as well. In either case, these chemicals usually either act as contact poisons or as inhibitors of herbivory or attack. This depends both on the compound and the particular organism that may challenge the plant.

The antimicrobial activity of extracts from various conifer heartwoods, often similar to defense chemicals of the bark, has been tested on *P. ramorum* (Manter, Kelsey and Karchesy, 2007). Many compounds had an effect on the growth of the pathogen, but some did not. Also, the levels of nine phenolic compounds produced in response to *P. ramorum* infection were measured for the less susceptible tree species, coast live oak (Ockels et al., 2007). Three phenolic compounds were induced at higher levels than the others and two of these compounds were demonstrated to have antimicrobial activity against this pathogen as well as three other *Phytophthora* species. The production and/or storage of secondary defense compounds is an energy demanding process and although some secondary defense compounds serve as precursors to structural polymers, such as lignin, the production of defenses usually must come at a cost to growth and/or reproduction (Herms and Mattson, 1992; Levin, 1976). We will use the vanillin-HCl method for staining of tannins and observe where they are localized in relation to pathogen hyphae.

Callose is a polysaccharide made up of glucose molecules connected by β-1,3 linkages. These polysaccharides may also be termed β-1,3 glucans. Callose occurs both in various cells and tissues of healthy, wounded, and pathogen infected plants. In a healthy plant, callose usually occludes the sieve pores of sieve elements during the fall and winter months while the plant is dormant, as well as sieve pores of older, non-conducting sieve elements (Fink, 1999). Callose may also be present in trichomes, root hairs, epidermal cells, lactifers, differentiating tracheids, and cork cells (Fink, 1999; Litvay and Krahmer, 1976).

The occlusion of sieve pores with callose also occurs with pathogen introduction, and can occur within minutes; being among the most rapid of plant defense responses. In all cases where callose is produced in response to pathogen infection, it is effective in helping to wall off the pathogen by making regions where it is deposited more difficult to penetrate (Fink, 1999). Callose is produced in other kinds of cells in response to small puncture wounds, produced either by wounding or pathogen infection, and may be deposited specifically in pits, plasmodesmata, or uniformly around the inside of the cell wall (Fink, 1999). Deposition of callose in pits and plasmodesmata is also a first step in the formation of papillae, or wound plugs.

Papilla formation is considered a specific response to pathogen infection, not just a wound response, controlled by a series of highly regulated cellular processes (Fink, 1999). A last way callose is produced as a defense response occurs in parenchyma cells surrounding vessel elements. This usually occurs in their interior walls, closest to the vessels, particularly in pits, "by the fusion of electron-transluscent globules into variously shaped aggregates with a layered or marbled appearance" (Fink, 1999; Mueller and Beckman, 1988; Muller et al, 1994).

When specific recognition of a pathogen by a plant occurs, there is an important response that sometimes occurs that, in many cases, renders the plant immune to the pathogen. This reaction, well-documented in the literature, is known as the hypersensitive response (Tyler, 2002; Graham and Graham, 1999; Morel and Dangl, 1997; Levine et al, 1996). During this reaction, a molecule within the plant cell, encoded by a resistance gene (R), acts as a receptor for a molecule of the pathogen, encoded by an avirulence gene (avr). This avr gene often encodes a cell wall protein or structural component, or a secreted compound that may often be important for survival or pathogenicity (Dangl and Jones, 2001; Dixon et al., 2000). In either case, it is often among the first molecules a plant cell encounters upon pathogen introduction. Once the R gene product binds or interacts with the avr gene product, allowing for recognition, a signaling cascade is triggered within the plant cell that leads to the hypersensitive response.

At first, an ion flux occurs, with an efflux of hydroxide and potassium ions and an influx of hydrogen and calcium ions (Heath, 2000; Morel and Dangl, 1997; Graham and Graham, 1999). Next, there is an oxidative burst, with the production of reactive oxygen species including superoxide ions, hydrogen peroxide, and other reactive oxygen species which leads to plant cell death and death of the pathogen within that cell (Heath, 2000; Morel and Dangl, 1997; Graham and Graham, 1999). After the hypersensitive response occurs in one cell, it is triggered in surrounding cells within a certain radius and may also lead to systemic responses, such as salicylic acid and phytoalexin production, making distal points better protected against the pathogen. Some of the hydrogen peroxide that is produced in the hypersensitive response is directed to the cell wall and effectively cross links glycoproteins and callose, making

cell walls more impenetrable and thus providing another method by which this response can effectively stop the pathogen (Heath, 2000; Morel and Dangl, 1997; Graham and Graham, 1999). There is evidence to suggest that a partial hypersensitive response can occur when the interaction between the R gene product and avr gene product do not interact as closely or when there is not as much of one of the products produced (Kamoun et al., 1999). As noted earlier, there is evidence that a partial hypersensitive response may occur in *N. densiflorus* in response to *P. ramorum* infection (Manter, 2007).

Wound periderms, produced in response to mechanical or pathogenic wounding, are anatomically similar in appearance to a normal periderm (Biggs, 1993). They usually form inside of the wounded tissue and connect, at some point, to the regular periderm. Cell walls may be a bit thinner than a normal periderm, cells may be more irregularly arranged, and phellem cells may contain soluble purple pigments rather than insoluble brown pigments in a normal periderm (Fink, 1999; Mullick, 1977). Wound periderms have been demonstrated to serve as effective barriers against pathogens (Fink, 1999).

Although this response will not be investigated here, in a five year study following disease progress in symptomatic trees, callus formation was observed by external observation on a small proportion of both tanoak and coast live oak trees (Swiecki and Bernhardt, 2006). It was not certain whether or not the callus encircled entire cankers or extended to the cambium. Callus is a proliferation of undifferentiated parenchyma cells and is a well-known response to wounding in trees (Fink, 1999).

1.4.3 Elicitin Production

Recognition of *Phytophthora* species by hosts and non-hosts is known to occur in a multitude of interactions. Triggers of host recognition include cell wall fragments, elicitins, arachidonic acid, glycoproteins and others (Tyler 2002). Elicitins, as mentioned earlier, have been most studied for their ability to trigger non-host defense responses. However, we also know some to be sterol transporters (Mikes et al, 1998). This is thought to allow these organisms, which are not capable of producing their own sterols and on which they are dependent for their survival and the completion of their life cycle, to acquire them from their host. The *P. capsici* elicitin, capsicein, is

known to have phospholipase activity (Nespoulous et al., 1999). Some elicitins are secreted and others have sequences that suggest cell wall anchoring, including a *P. ramorum* elicitin (Tyler, 2002; Meijer et al, 2006).

Elicitins are small enough to pass through a plant cell wall, they have been proved capable of moving systemically through a plant (Tyler, 2002; Devergne et al, 1992), and there is some evidence that elicitins can enter cells independently of the pathogen (Tyler, 2002-refers to his own unpublished work). Secretion of the elicitin quercinin was demonstrated in the roots of *Quercus robur* seedlings infected with *P. quercina* using fluorescent antibody to track its localization (Brummer et al, 2002). Fluorescence clearly illustrated that quercinin was present in hyphal cell walls (Brummer et al, 2002). Elicitins have been demonstrated to bind to transcription factors that bind to promoters of both light-regulated genes and defense regulated genes (Tyler, 2002, refers to his own unpublished work). The combination of this information suggests a possible role for these proteins in pathogenicity. And now, as I will explain below, we have reason to question whether elicitins produced by *Phytophthora ramorum,* in particular, may be involved in pathogenicity.

A study by Manter et al., 2007, measured declines in photosynthesis, in response to inoculation of stems of *Rhododendron* plants with *P. ramorum,* prior to any visible damage to tissues or any measurable decrease in hydraulic conductivity that could be caused by necrosis of tissues due to disease. This suggests that somehow, tissues distal to the point of inoculation respond to introduction of the pathogen. The most likely explanation for this is that some molecule of the pathogen is translocated through tissues and either acts as a toxin on these tissues, or else acts as an elicitor of host defense, causing a partial hypersensitive response.

This same study shows that exposure of purified elicitins to three host plant species and one non-host, resulted in reduced chlorophyll fluorescence, and increased ethylene production and hydrogen ion (H^+) uptake in leaves of all plants, though all of these responses occurred to a greater degree in the non-host plants. These changes are consistent with a hypersensitive response and may suggest an explanation for the initial photosynthetic declines measured in *Rhododendron* in the first part of the study. Induction of a partial hypersensitive response has, in fact, been thought to contribute

to pathogenicity in one situation, aiding the pathogen in accessing host materials (Qutob et al, 2002). In addition, more recent research by Manter et al (2010) demonstrates the existence of correlations between measures of virulence of different isolates and levels of elicitin production. Due to the collective evidence that suggests elicitins contribute to pathogenicity, further investigation into the activity and role of these proteins in this interaction is merited.

2 Methods

2.1 Histology

2.1.1 Log Inoculations

Two log inoculations studies were conducted. All logs for both studies came from Curry County, Oregon outside of the *P. ramorum* quarantine area. All logs were inoculated with *P. ramorum* isolates 2018.1 and 2027.1 which were originally isolated from infected *N. densiflorus* trees in Curry County, Oregon. All cultures used for inoculation were grown on cornmeal agar. For the first study, 12 *N. densiflorus* logs were collected in early January 2007 and stored in plastic bags at room temperature with ends sealed with tree wax. These were inoculated five weeks later in mid February, and harvested seven weeks after that in early April. For the second study, seven *N. densiflorus* logs were collected in early June 2008, inoculated in mid June, and harvested six weeks later in late July.

Each log in both studies was inoculated at three points; the first with isolate 2018.1, the second with isolate 2027.1, and the third with a control. Controls in the first study were sterile agar plugs and controls in the second study were *P. gonapodyides* cultures, isolate 9616. *P. gonapodyides* was chosen to serve as a control because it is ubiquitous in forests where sudden oak death occurs (Hansen, unpublished data) but only occasionally causes disease on *N. densiflorus*. The symptoms of infection by *P. gonapodyides* are the same as those of *P. ramorum* on *N. densiflorus*, with similar bleeding cankers on the bole and similar lesions visible when outer bark tissues are peeled away. So, in observing how *P. gonapodyides* infects bark tissues compared to *P. ramorum*, we may gain insight as to how the pathogenic strategies or abilities of the latter make it more successful.

Logs were inoculated in three different ways. For the first study, all logs were wound inoculated using a cork borer to create a wound to the cambium, then an agar plug with an actively growing *P. ramorum* culture was placed on the wound. In the second study, three of the logs were inoculated by this method, three were inoculated by a pinhole wound method and one log was non-wound inoculated. In the pinhole wound

method, a small pinhole wound was created using a dissecting needle and the agar plug was placed above the wound on the surface of the bark. In the non-wound method, the agar plug was simply placed on the surface of the bark. In all cases, the agar plug was covered with damp cheesecloth and foil was duct-taped over the area to help retain moisture at the inoculation site. The logs were placed in bags, the bags were sealed with duct tape to retain moisture and they were left for seven weeks at room temperature.

2.1.2 Sprout Cutting Inoculations

Four sprout inoculation studies were conducted; the first included 12 inoculated sprouts and two controls, the second included 48 inoculated sprouts and two controls, the third included 21 inoculated sprouts and six controls, and the fourth included five inoculated sprouts and no controls. The first study was conducted in January 2009 and the second, third, and fourth were conducted in April and May, 2009. All sprouts were collected from Curry County, Oregon outside of the quarantine area, collected either from the base of trees or the tips of branches. Zoospore suspensions were made up of mixtures of isolates 2018.1 and 2027.1 in the first and third of these studies. The second study used 16 different isolates each one from a different tree located in a total of 11 sites in Curry County, Oregon. The fourth study included two of these isolates as well as one other isolate collected from a 12th site in Curry County, Oregon. For studies one and three, controls were placed in containers with 100ml water, and for study two, controls were not placed in water but were simply uninoculated sprouts.

P. ramorum cultures were grown on V8 agar (66mL clarified V8 and 15g/L bactoagar) for 2-3 weeks until many sporangia had formed on the surface. Approximately 5 mL deionized water was poured on top of each plate and a rubber policeman was used to scrape the surface of the agar in order to detach sporangia and suspend them in the water. Water was poured off of each plate (# of plates used varied by study) and collected into one beaker. Sporangial suspensions were then incubated at 4°C for 1-1.5 hrs., then at room temperature for 45 minutes.

At this point an estimate of the number of zoospores in the solution was made. To do this, the suspension was mixed on a stir plate for about one minute, a small amount was pipetted into a 2ml tube with a lid, and vortexed for 20 seconds to cause zoospores to encyst. A small amount of this suspension was pipetted between a hemacytometer and its cover slip to cover the surface completely. This was repeated on the other side of the hemacytometer. Zoospores were counted and summed in five of the 9 grid boxes for each side of the hemacytometer; the four corner grids and the center grid. The two sums from each side were averaged and multiplied by 2000 to give an estimate of zoospores/ml. Concentrations were adjusted to 10^4 for inoculation.

For studies one, three, and four, 100 ml of the suspension was poured into 1025ml beakers filled with sprout cuttings. For study two, stems were placed in individual 20 ml tubes and 10ml of zoospore suspension was added to each. In all cases, the solution reached approximately 1.5-2cm above the cut ends of the sprouts. In studies one, two, and four, cuttings were left in zoospore suspensions for two weeks before removing for sampling. In study three, two inoculated samples and one control were sampled at 2 hr. intervals and subsequently stained for superoxides with nitroblue tetrazolium as described later.

2.1.3 Field Collections

Field collections were made in late May, 2008. Five infected trees were sampled at three sites, and two control trees were sampled at one of the sites. Both control trees were healthy, having no signs of infection; one having a DBH of approximately 20cm and the other of 36cm. Sites were initially identified with aerial survey by Mike McWilliams, of the Oregon Department of Forestry (ODF), by locating discolored *N. densiflorus* crowns and marking GPS points. ODF field crews performed follow-up ground surveys, taking samples from symptomatic trees at the GPS locations to later confirm or deny the presence of *P. ramorum*. Samples were isolated from bark along lesion margins and plated on CARP, a *Phytophthora* selective medium containing 10 ppm natamycin, 200 ppm Na-ampicillin, and 10 ppm rifamycin. An additional piece of bark from the cankered area was taken and both were brought back to the Hansen lab for identification. Cultures were identified by hyphal growth pattern and

sporangial characteristics by Paul Reeser, research assistant of the Hansen lab at Oregon State University. Pieces from the second bark sample, within or along the lesion margin, were analyzed by PCR by Wendy Sutton, research assistant of the Hansen lab at OSU, as an additional form of identification.

Of the five trees sampled, three were trees that had green crowns, one had a dead crown, and 3 had fading crowns, but all had been confirmed positive for *P. ramorum* that year and all still had well-defined lesions. The DBH of these trees were 10, 20, 28, 36, and 46 cm, approximately. One sample each was taken from three of the trees, two samples were taken from one tree, and 6 samples were taken from the last tree. This was the 10cm DBH tree, which had a green crown and excellent symptoms, with very clear boundaries around lesions and wet cankers up to 1m above the ground. Areas to be sampled were identified by removing bark with a hatchet to reveal cankers symptomatic of *P. ramorum* infection.

Samples were removed directly adjacent to an uncovered lesion margin using a chisel to cut an approximately square or rectangular section of intact bark, going all the way to the cambium, that would contain the lesion margin along with healthy and damaged tissue on either side of it. By looking at the removed bark in cross section, the lesion margin could still be seen clearly to confirm that the piece included what was expected. After collection in the field, all samples were immediately immersed and stored in a 50:50 EtOH and glycerol solution to kill and fix cells but also preserve autofluorescence.

2.1.4 Sampling of Logs

Logs were initially scored a short depth into the bark with a scoring tool both lengthwise and horizontally from each inoculation point to reveal lesion margins.

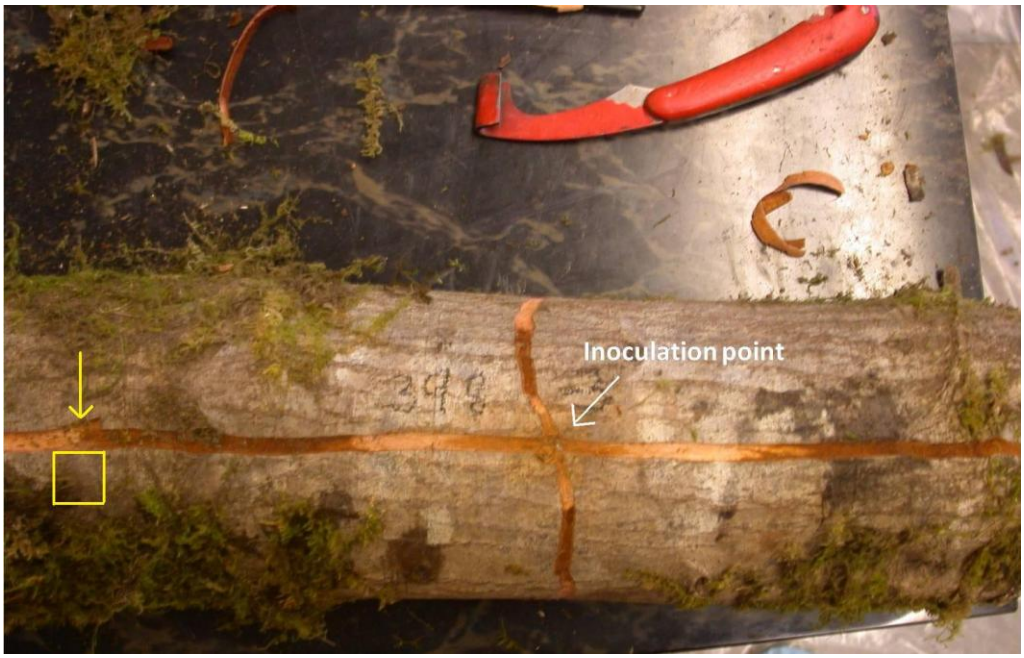


Figure 4. A *P. ramorum* inoculated region of a *N. densiflorus* log: log has been scored lengthwise and crosswise from the inoculation point (white arrow). Yellow arrow shows a lesion margin and yellow box shows an example of where a bark sample would have been taken.

For study one, edges of lesion margins were then measured and recorded, and logs were cut with a miter saw into "cookies". Sapwood discoloration was then observed in the cookies. The length of discoloration was determined using the cookie furthest from the inoculation point that still showed discoloration and estimating that 2mm were lost for each saw cut. It was then estimated which cookie contained the bark lesion margin (this could often be confirmed visually) by stacking the cookies on top of one another and again estimating that 2 mm were lost for each saw cut. A section of intact bark was then cut out of the cookie that should have been along the lesion margin and included all bark and a small section of sapwood, using a chisel. Each sample that was taken was divided into two parts. A small piece of this sample right along the lesion margin was cut away with a razor blade and put in FAA and the rest of the sample was immersed and stored in a 50:50 solution of ethanol and glycerol. Small pieces from the cookies were also cut using a scalpel on or within the lesion margin, where the bark had been scored and the lesion margin could be clearly seen. These were plated on CARP, to confirm that *P. ramorum* was actively growing there.

Small pieces of wood from the cookie furthest from the inoculation point that still showed discoloration were also plated on CARP to look for actively growing *P. ramorum*. *Phytophthora ramorum* was identified by its characteristic hyphae and growth pattern.

In the second study, logs were not cut into cookies and samples were taken directly adjacent to where the lesion edge was revealed by scoring. Again one piece of bark was put into FAA and another into a 50:50 EtOH glycerol solution, only this time there was no wood included in the samples. Additional small pieces were again plated on CARP to confirm that either *P. ramorum* or *P. gonapodyides* was growing there. Two to three samples were taken from all inoculated log sections except for one where there was virtually no lesion so only one sample could be taken. *P. gonapodyides* was identified by hyphal and growth pattern characteristics.

2.1.5 Sampling of Sprout Cuttings from Studies One, Two, and Four

One sprout from each zoospore inoculation container was destructively sampled and plated onto CARP to ensure that it was infected with *P. ramorum.* The lesion margin usually extended a distance up the stem from the water line of the zoospore suspension. For study one, an approximately 1.5cm sample was taken for microscopy, cut with a razor blade with the lesion margin in the center, so that about 0.75 cm was healthy looking and green and the other 0.75 cm was unhealthy and dark brown.

For study two, 30 inoculated samples were used for microscopy and the other 18 samples were stained with Nitroblue Tetrazolium (NBT) (protocol below in host defense response methods). Each sample taken for microscopy for this study and all samples from study four were cut into three pieces, each about 0.5 cm. long. The first piece extended from the lesion margin down towards the cut end, so that all tissue was discolored and brown. The second section extended from the lesion margin up so that all of the section was green and healthy looking. The third section extended from just above the second section up, again meaning the whole section was green and healthy looking. The three sections were cut at angles in certain places so that the top and

bottom of the sample could be determined later (Fig. 5). All samples for both studies were placed immediately in FAA (50% EtOH (95%), 5% glacial acetic acid, 10% formalin (37% formaldehyde), and 30% dH_2O). Samples from study one were later embedded in PEG (protocol below).

Figure 5. Diagram showing how sprout cuttings were divided in study two: The low section is colored black to demonstrate that that is the section that was within the lesion margin while the other two were beyond it. To the left would be toward the bottom of the stem and to the right is toward the top of it.

2.1.6 Sample Dehydration and PEG embedding Protocol

Approximately 15 bark samples were selected for embedding from field collections and sprout inoculations. These were originally stored in a 50:50 solution of EtOH and glycerol. From this, they were put directly through a series of dilutions of 1500 Polyethylene Glycol (PEG) carried out at 50°C. Samples were in each dilution for one day and step four was carried out twice, using new PEG the second time. Samples were embedded through the following series:

1. 70% dH_2O and 30% 1500 PEG
2. 50% dH_2O and 50% 1500 PEG
3. 75% 1500 PEG and 25% dH_2O
4. 100% 1500 PEG

All sprout samples from study one were embedded in a lower molecular weight PEG so that they would not be brought above 40°C, helping to prevent the possibility of protein degradation, a concern for elicitin labeling later. To dehydrate, samples in FAA were washed in deionized water two times for 30 minutes each. They were then transferred through an ethanol series as follows:

1. 50% EtOH and 50% dH_2O

2. 75% EtOH and 25% dH_2O
3. 90% EtOH and 10% dH_2O
4. 100% EtOH.

All embedding steps were carried out at 40°C, samples were in each dilution at least 2 hrs, and step three was carried out twice, using new PEG the second time. Samples were embedded through the following series:

1. 50% 1000 PEG and 50% EtOH.
2. 75% 1000 PEG and 25% EtOH.
3. 100% 1000 PEG.

2.1.7 Sectioning of Samples and Microscope Specifications

Samples in FAA and in the 50:50 EtOH, glycerol solution were washed for at least 2 hrs in either PBS or water prior to sectioning on the microtome. Embedded samples were not washed prior to sectioning. Samples were sectioned using a sliding mictrotome to between 10 and 60 μm, depending on the purpose for which the samples were being used. Some samples were hand sectioned with razor blades as well, to approximately 40 to 80μm. The primary microscope used for both fluorescence and light microscopy was a Zeiss Axioskop.

2.1.8 Calcofluor Staining for Cellulose

Fixed material, some embedded and some not, was used for calcofluor staining. Recipe for stain and methods for staining were modeled after Mueller et al (2004). Microscopic sections were washed thoroughly in dH_2O before staining. Sections were transferred to a clean, dry petri dish and each covered with 1-2 drops of 0.05% calcofluor white in Tris 0.5M, pH 8. They were allowed to stain for 5 minutes, flooded with dH_2O and swirled to rinse for several seconds. The water was drawn out of the dish with a pipette. Sections were washed three more times in this way before mounting on slides. Sections were mounted in dH_2O and viewed under the microscope using either a filter cube with excitation at 390-420nm and emission at

450nm or another with excitation at 345nm and emission at 425nm (UV). Plant cell walls and *Phytophthora* cell walls will both stain by this method due to the presence of cellulose (Erwin and Bartnicki-Garcia, 1983) and appear a bright whitish blue under these filters.

2.1.9 Aniline Blue Staining for hyphae

Aniline Blue stain was prepared according to the methods described by Ruzin, 1999. This was a 0.005% solution of aniline blue in a 0.15M K_2HPO_4 at pH 8.2. Sections were placed on slides and then 1-2 drops was placed on each section. Tissues were allowed to stain for 10-15 min., then were viewed with fluorescence microscopy using a UV filter with excitation at 345nm and emission at 425nm. Aniline blue can stain cellulose, callose, and other β-1,3 glucans (Ruzin, 1999; YuanTih and YuLing, 2004)

2.1.10 Confocal Microscopy

A Zeiss LSM510 META Confocal Microscope was used to view hyphae in bark tissues in a more three-dimensional way, after calcofluor staining and elicitin staining.

2.1.11 Scanning Electron Microscopy

An FEI Quanta 600F Scanning Electron Microscope was used to view hyphae and other structures in bark tissues three-dimensionally. SEM samples included three *P. ramorum* infected field samples, two *P. ramorum* inoculated log samples, one *P. ramorum* inoculated sprout cutting sample, one *P. gonapodyides* inoculated log sample, and one uninfected field control.

2.2 Host Responses to Infection

2.2.1 Staining for Tannins by the Vanillin-HCl Method

Methods for tannin staining are modeled after those described by Gardner (1975). Tannins are a group of high molecular weight phenolics that are capable of protein

coagulation (Fink, 1999). Samples used for tannin staining were all from trees rather than sprout cuttings, but included both field samples and inoculated log samples, and uninfected tissues as controls. All material was initially fixed in 50:50 EtOH and glycerol but some had been embedded and some had not. All samples were sectioned on the microtome to between 20 and 60µm. After sectioning, 1-2 drops of saturated alcoholic vanillin were dropped on each section. Then 1 drop of concentrated HCl was added to each section. Slides were viewed with light microscopy and the staining of tannins was indicated by a bright red color.

2.2.2 Staining for Callose with Calcofluor

These methods are the same as described above for cellulose staining. That this is a useful stain for callose has been described by Hughes and McCully, 1975.

2.2.3 Detection of a Hypersensitive Response

Three small *N. densiflorus* seedlings, approximately 20-40 cm tall, and two small tobacco plants, approximately 10 cm tall, were inoculated. Four to six leaves and the stem of each *N. densiflorus* seedling were inoculated with *P. ramorum*, half of the inoculations with isolate 2018.1 and half with isolate 2027.1. Pinhole wounds were made in all leaf and stem inoculation points directly before each was covered with an agar plug containing a culture of *P. ramorum*, covered with damp cheesecloth, and wrapped with parafilm.

Tobacco plants were inoculated with *P. cryptogea* and *P. infestans* to serve as controls as these *Phytophthora* species are known to produce elicitors of a hypersensitive response in tobacco cells (Platt, 1999 and Blein et al., 1991). Five leaves of one of the tobacco plants were inoculated with *P. cryptogea*, isolate 145, growing on 1/3 V8 and five leaves of the other tobacco plant were inoculated with *P. infestans*, isolate 98-289-1, growing on rye agar. Agar plugs containing these cultures were placed directly on the tops of leaves followed by a couple of drops of water.

Three leaves or two leaves and one stem from the three *N. densiflorus* seedlings inoculated with *P. ramorum* were sampled every 2 hours for 12 hours and one last

time at 24 hours post-inoculation, using scissors or hand clippers (sterilized between each sampling). One leaf from each inoculated tobacco plant was sampled every 2 hours for 12 hours post-inoculation, using scissors (sterilized between each sampling). All tissues were immediately stained for superoxides with NBT as described below.

Methods for staining superoxides were modeled after those described by Venisse et al., 2001 and Wang and Higgins, 2006. Samples used for this procedure included tanoak stem sections from sprout cuttings inoculated in zoospore suspensions of *P. ramorum*; tanoak leaves from live seedlings wound inoculated with agar plugs of *P. ramorum,* and with *P. lateralis* as controls; and tobacco leaves from live plants non-wound inoculated with agar plugs of *P. cryptogea* and *P. infestans* as controls. Stem sections or leaves were removed from sprout cuttings or live plants, respectively, and were vacuum infiltrated with 0.1% NBT in 10mM potassium phosphate buffer, pH 7.8 for 20 minutes. They were then placed in boiling 95% EtOH for 10 minutes to stop the reaction and clear chlorophyll from tissues. The samples were then hand sectioned and viewed under a dissecting microscope and light microscope.

2.2.3 Observation of Wound Periderm

Wound periderm could be seen with autofluorescence using fluorescence microscopy. Wound periderm appeared as a layer of cells in the inner bark that resembled cork tissue due to the shape of cells and whitish fluorescence of lignin in the cell walls.

2.3 Elicitin Production

Methods for immunofluorescent labeling of elicitins were modeled after those used by Brummer et al (2002). Samples in FAA were washed overnight in phosphate buffer saline (PBS), pH 7.4 and sectioned with a microtome. To prevent non-specific labeling, sections were first treated with a blocking solution containing 3% BSA in PBST (PBS with 0.2% Tween-20) for 30 minutes at room temperature. Next, they were incubated in a 1:500 diluted solution containing the anti- elicitin rabbit serum in PBS and 1% BSA for 2 hrs at 37°C. The sections were then washed well in PBST 4 times for 1-2 minutes each time followed by incubation in a 1:200 diluted solution of CY2 labeled goat anti-rabbit IgG for 1 hr. at 37°C. They were again washed well four

times with PBST as before. Sections were observed under the microscope using a filter cube with excitation at 450-490 nm and emission at 515nm.

3 Results

3.1 Inoculation Results

Lesions developed around points inoculated with *P. ramorum* in 8 of the 12 logs inoculated in the first study. It was assumed that inoculations were unsuccessful in the remaining four logs, so these logs were not sampled or included in any data reported beyond here. In addition, there was one log that appeared to be covered in one large lesion, so lesion margins could not be identified and this log was also excluded from further analysis. The pathogen was recovered in culture from each bark lesion that developed around the remaining 14 inoculation points on seven logs inoculated with *P. ramorum* in the first study. The pathogen was also recovered from the discolored sapwood behind each inoculation point except for one. Successful recovery from bark for this inoculated region indicated that the inoculation was successful. *P. ramorum* was not recovered in culture from any of the control points pseudo-inoculated with sterile agar plugs.

For the first study, average lesion length in bark was 23.4 cm and average length of discoloration in the sapwood was 9.1 cm. Lesion length in the bark was greater than the length of discoloration in the sapwood in nine inoculations and sapwood discoloration length was greater in the remaining five. The longest bark lesion was 58cm and the greatest length of sapwood discoloration was 25cm. Results of a paired t-test report that bark lesion length is significantly greater than the length of sapwood discoloration, with a p-value of 0.016 within a 95% confidence interval.

Of the 14 inoculation points on seven logs inoculated with *P. ramorum* in the second study, the pathogen was recovered in culture from each bark lesion that developed around an inoculation point, except in two cases. Both of the failed recoveries were from the non-wound inoculated log and there were no lesions in the bark here, so these were presumed to be unsuccessful inoculations. *P. gonapodyides* was recovered in culture from each of the seven bark lesions that developed around or under an inoculation point.

In the first sprout cutting inoculation study, *P. ramorum* was recovered in culture from a cutting in each inoculation container. Nearly all reisolations from study two were also successful.

3.2 Colonization of *N. densiflorus* bark tissues

3.2.1 Characteristics of hyphae within bark tissues

A total of 16 *P. ramorum* infected samples, including six infected field samples, seven inoculated log samples, and three inoculated sprout cutting samples were stained with calcofluor and viewed with fluorescence microscopy. Two *P. gonapodyides* infected controls and two uninfected controls were also stained and compared to *P. ramorum* infected samples. Additional infected samples were stained but findings were not recorded as hyphae were not seen in these samples. In successfully stained *P. ramorum* infected samples, calcofluor stained hyphae with similar characteristics to *P. ramorum*. Coenocytic hyphae with diameters that varied along their length, pinching at branch points, and sizes ranging from approximately 0.5-10μm in diameter were considered good candidates for *P. ramorum*. *P. ramorum* hyphae are reported to reach up to 8 μm in diameter in culture (Werres et al, 2001), and although they ranged here from 0.5-10μm, candidate hyphae in these tissues usually fell within the diameter range of 2-7μm. Hyphae with consistently small diameters (1.5μm or less) and/or frequent crosswalls were also encountered, but the occurrence of these will not be reported except to illustrate morphological differences from what were considered candidate *P. ramorum* hyphae and when they co-occurred with *P. ramorum* hyphae. Most samples in which characteristic hyphae were observed by calcofluor staining were from inoculated logs or infected field samples. Hyphae were most clearly visible with calcofluor staining in tissues where plant cell walls were not fluorescing or had diminished fluorescence, and this was more frequently the case in inoculated log and infected field samples. The lack of fluorescence was presumably due to the breakdown of cellulose in damaged, dead, or dying cells.

Two inoculated log samples and one pseudo-inoculated log control sample were stained with aniline blue and viewed with UV fluorescence. Aniline blue staining

caused coenocytic hyphae, 2-6µm in diameter to fluoresce but did not stain plant cell walls. This was one way in which localization of hyphae in certain plant tissues could be confirmed.

Observing samples with scanning electron microscopy allowed for three dimensional, high resolution, visualization of hyphae in bark tissues. Samples used for SEM included one *P. ramorum* inoculated log sample, two *P. ramorum* infected field samples, one *P. ramorum* inoculated sprout cutting, one *P. gonapodyides* inoculated log sample, and one field control. Crosswalls could not be seen with SEM, however other morphological characteristics such as hyphal diameter, variability of hyphal width, and pinched branch points were still useful and could be compared to what had been seen with calcofluor staining and what was seen with elicitin immunostaining. Hyphae seen in these samples ranged from about 0.5-8µm, but were usually within the range of 2-6µm.

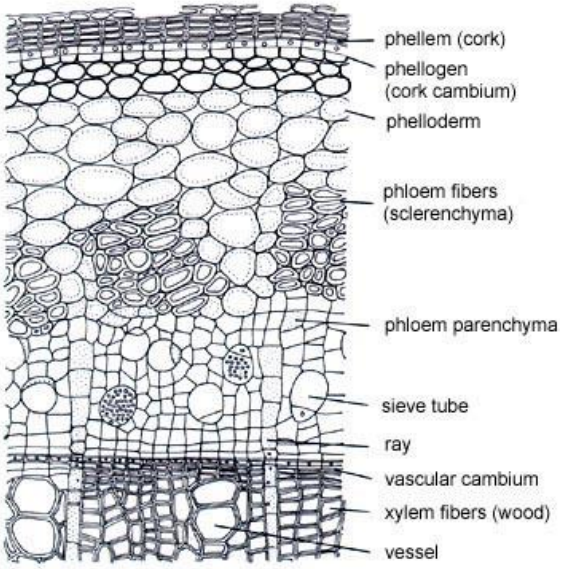
Thirty-eight *P. ramorum* infected samples were treated by the immunofluorescent elicitin labeling procedure, but only eight samples can be confirmed to have been successfully stained. All of these were inoculated sprout cuttings. The 38 samples treated included 27 inoculated sprout cutting samples, nine inoculated log samples, and two infected field samples. Four uninfected sprout cutting samples, two *P. gonapodyides* inoculated log samples, and two field controls were also treated by this method. Elicitin labeling caused hyphal cell walls to fluoresce green using a filter with excitation at 450-490nm and emission at 515nm. Although fluorescence of cell walls was achieved through calcofluor staining, the use of the elicitin antibody allowed us to be more confident that hyphae that were observed were those of *P. ramorum*. Hyphal diameter ranged from 3-6µm in these tissues. Further details of elicitin immunostaining are described in section 3.4.

3.2.2 Overview of colonization and plant cell damage

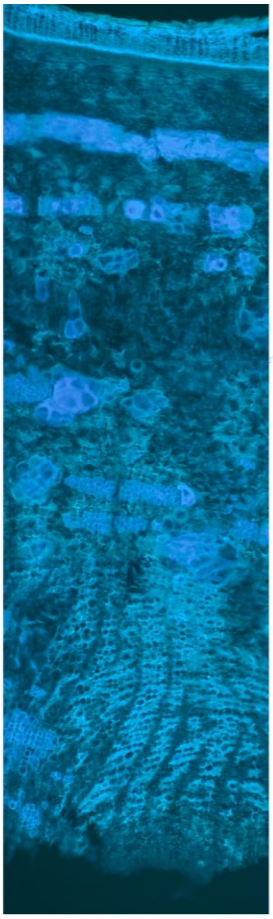
Hyphae characteristic of *P. ramorum* were seen in all outer and inner bark tissues of inoculated log and infected field samples, excluding fiber and sclereid tissues. Characteristic hyphae were also seen on the surface of bark/stem tissues in all sample types. Colonization of all tissues was primarily intracellular. Scanning electron

microscopy allowed for visualization of hyphal movement from cell to cell. Hyphal walls appeared to fuse with plant cell walls or membranes in some places but in other places, it appeared as though hyphae were traveling through the pits of cell walls (figs 6 and 7). Hyphae were seen either entering or growing out of lenticels as well as directly penetrating bark surfaces (figs 27-30).

In most inoculated log and infected field samples, plant cells in infected tissues were misshapen, cell arrangement was disorganized, and tissues were discolored, compared to controls (figs 8 and 9). Often a layer of sclereids and/or fibers, viewed in transverse section, appeared to be a boundary between damaged, discolored tissues and healthy tissues (fig 10). Plant cells did not appear as damaged in inoculated sprout cutting samples as in the inoculated log and infected field samples. Here, cell wall shape was usually retained and tissues were not as discolored. However, it was evident in these infected tissues that plasma membranes had pulled away from cell walls and their shape had become distorted, compared to controls. This occurred in most cells in an infected area regardless of whether or not hyphae were seen in them (figs 11 and 12). Figures 1 and 2 from Chapter 1 have been reinserted here to be used as a reference when specific tissues are referred to in the text.



www.apsnet.org/.../PhotosN-R/phelloderm.htm



Figures 1 and 2. Left: Figure 1. Diagram of a typical bark cross section: tissues and cell types of the inner and outer bark. Right: Figure 2. A calcofluor stained *N. densiflorus* bark cross section: an uncolonized region of a bark sample from a *P. ramorum* infected *N. densiflorus* tree.

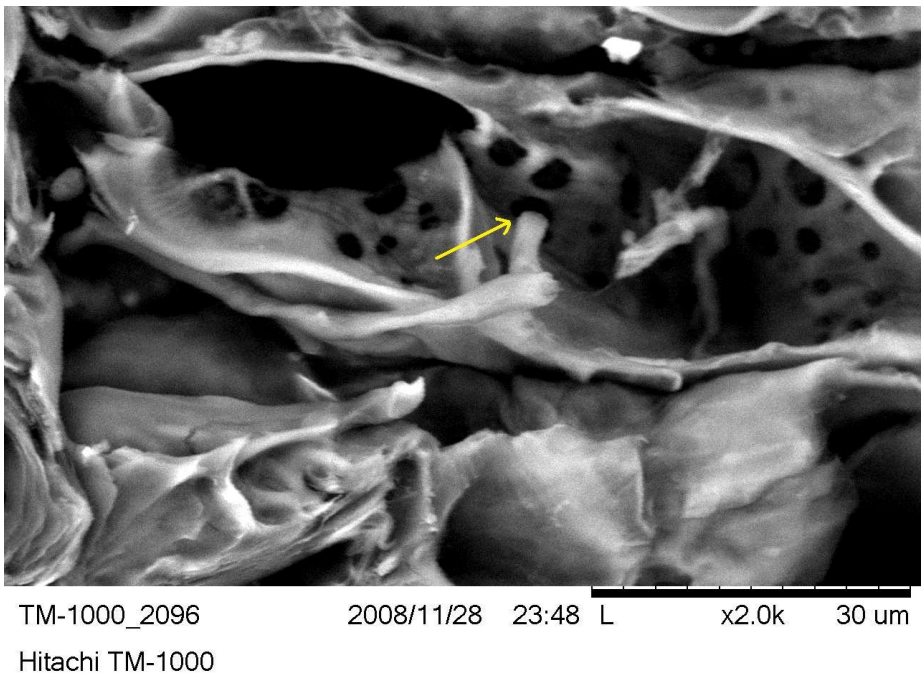


Figure 6. Hyphae growing through plasmodesmata: A scanning electron micrograph of a cross section of a *P. ramorum* infected bark sample collected from an infected *N. densiflorus* tree. A hypha appears to be growing into the plasmodesma of a bark parenchyma cell.

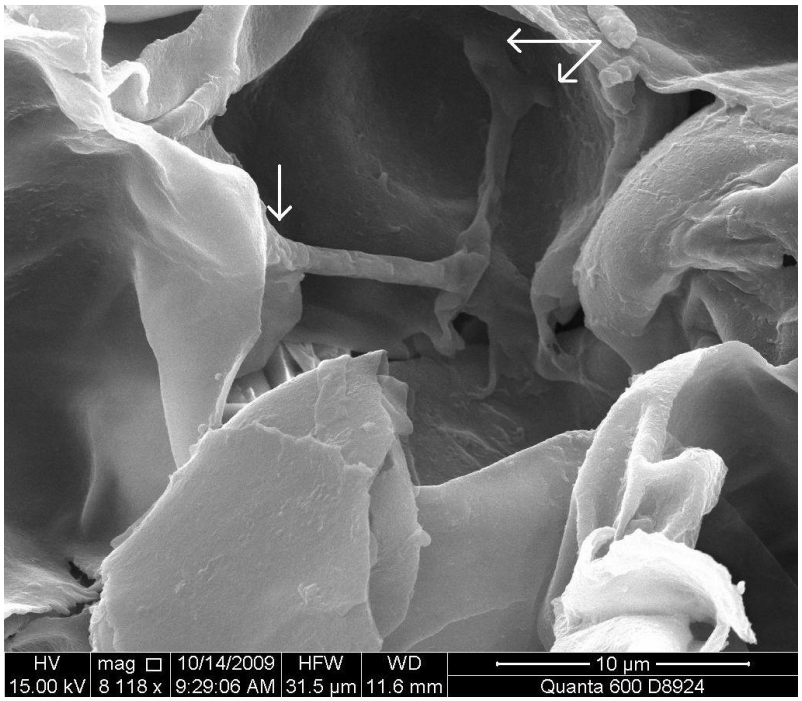


Figure 7. Fusion of hyphae with plant cell wall: A scanning electron micrograph of a cross section of a *P. ramorum* infected bark sample collected from an infected *N. densiflorus* tree: Hyphae appear to fuse with the cell wall parenchyma of the inner bark.

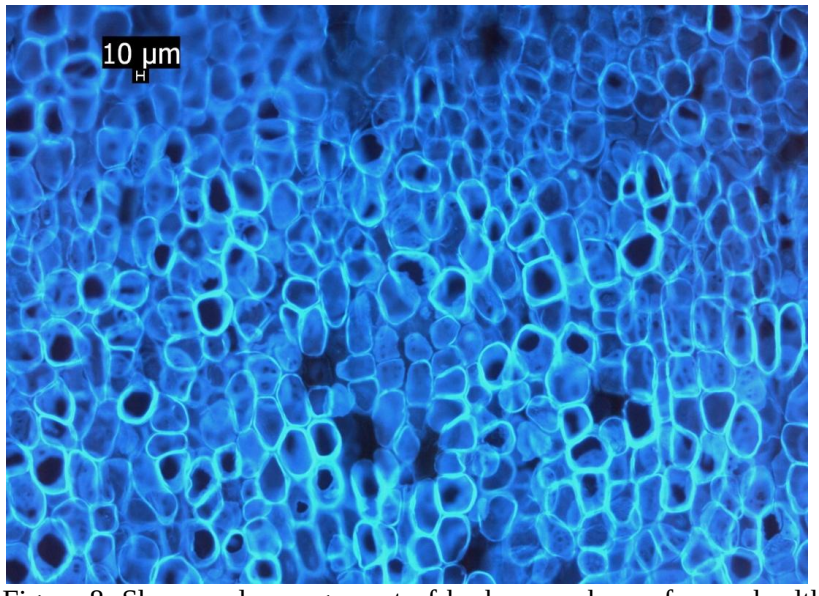


Figure 8. Shape and arrangement of bark parenchyma from a healthy field collected sample: Fluorecence micrograph of a cross section of a calcofluor stained bark sample. Cell shape and cell arrangement are retained and cell walls are intact with strong fluorescence from calcofluor staining.

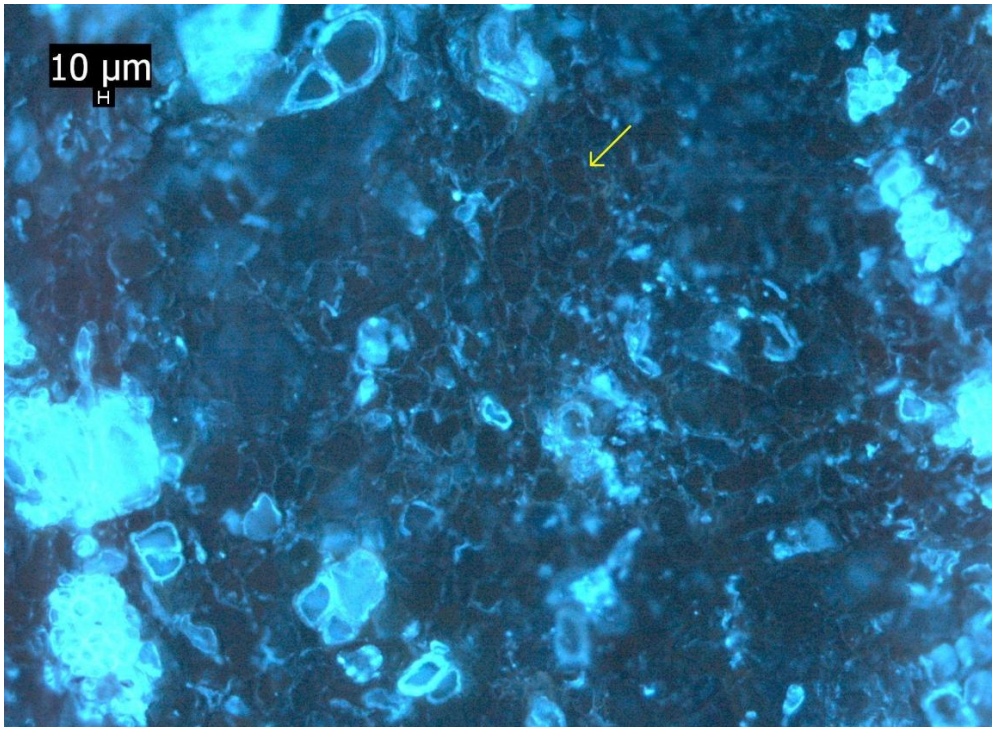


Figure 9. Shape and arrangement of bark parenchyma from a *P. ramorum* infected field collected sample: Fluorescence micrograph of a cross section of a calcofluor stained bark sample. Disorganization of bark parenchyma tissue can be seen: cell shape is distorted, parenchyma tissue is discolored and there is diminished fluorescence of cell walls (yellow arrow) with calcofluor staining.

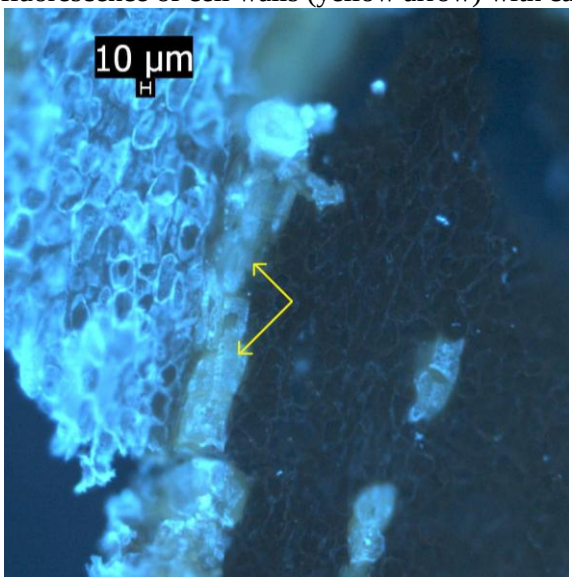


Figure 10. Boundary between damaged, discolored, tissue and healthy tissue in *P. ramorum* infected bark: Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. ramorum* inoculated region of a *N. densiflorus* log showing a boundary between healthy and damaged tissue with a layer of sclereids as the dividing line between them (yellow arrows).

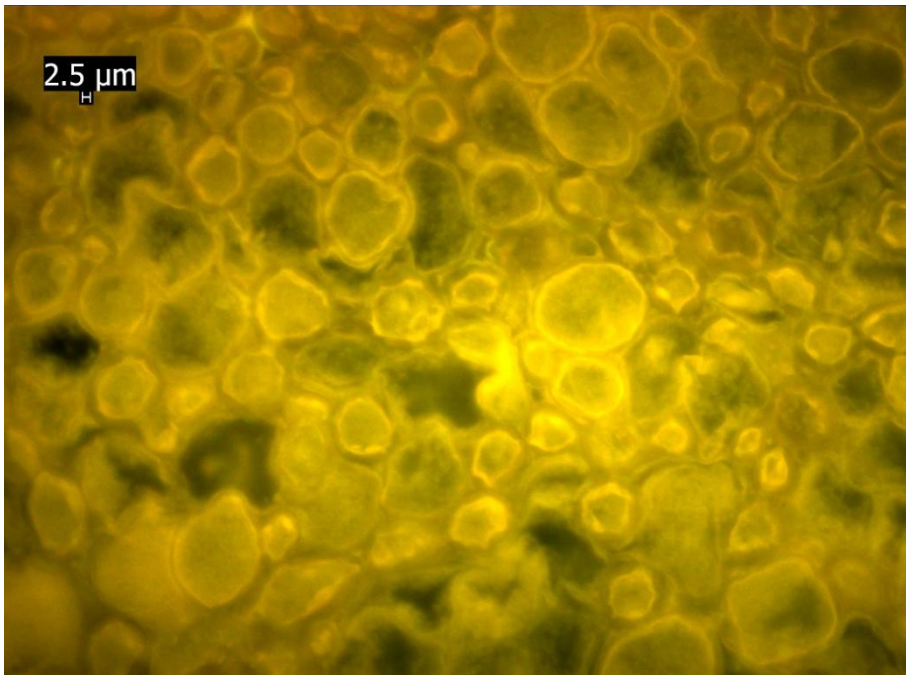


Figure 11. Shape and arrangement of bark parenchyma from a healthy sprout cutting: Fluorescence micrograph of the bark of an elicitin immunostained uninfected sprout cutting control sample. Most cells appear turgid and do not show discoloration.

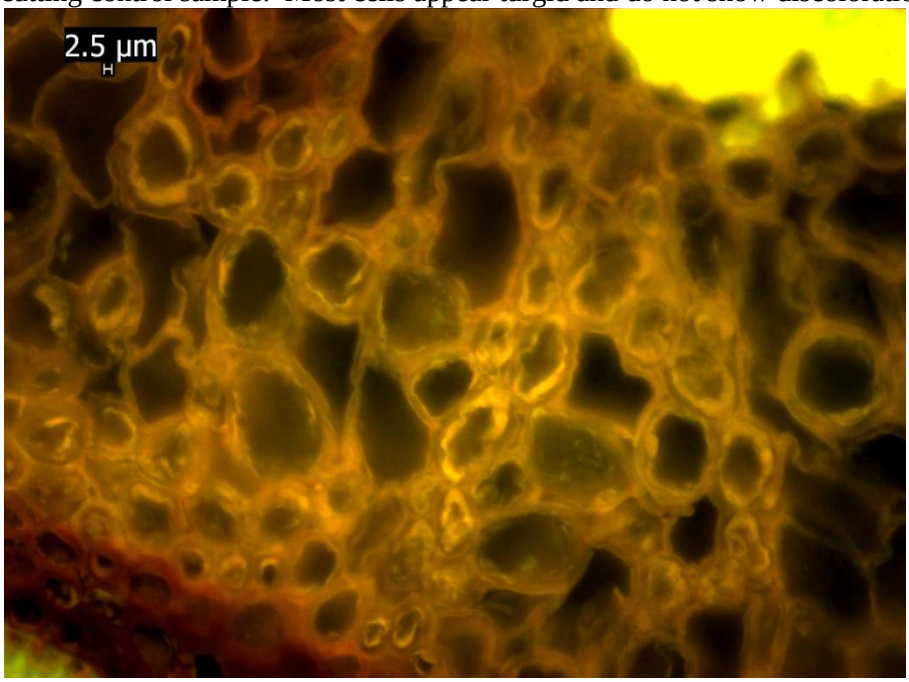


Figure 12. Shape and arrangement of bark parenchyma in *P. ramorum* infected sprout cutting sample: Fluorescence micrograph of the bark of an elicitin immunostained *P. ramorum* inoculated sprout cutting sample. Plasma membranes have separated from cell walls, membrane shape has become distorted, and cells are discolored.

3.2.3 Colonization of the phelloderm and parenchyma of the inner bark

Hyphae were seen most frequently in the phelloderm and parenchyma cells in the outer part of the inner bark in all sample types, but exclusively in these tissues in inoculated sprout cutting samples. Parenchyma cells in the outer part of inner bark is probably largely, if not all, cortical parenchyma in sprout cutting samples, a primary plant tissue. In other, older samples, these parenchyma cells probably contain a higher percent of parenchyma that arose from the vascular cambium, but there is likely still some cortical parenchyma here, too. In calcofluor stained tissues, these were also the tissues in which diminished plant cell wall fluorescence was most typical, but results from SEM, aniline blue staining, and elicitin labeling showed a similar pattern. Hyphae in these tissues grew vertically, horizontally, and radially (figs 13, 14, 17 and others). Hyphae grew primarily intracellularly in these tissues but, also grew intercellularly in places (fig. 16). Aniline blue staining allowed for observation of hyphae in the absence of plant cell wall fluorescence (fig 17).

The second most common place that hyphae were seen was in parenchyma tissues just inside of the first layer of sclereids and fibers encountered going from the outer to inner bark, beyond the usually discolored, unhealthy looking region. Again, hyphae here were primarily intracellular and grew in every direction.

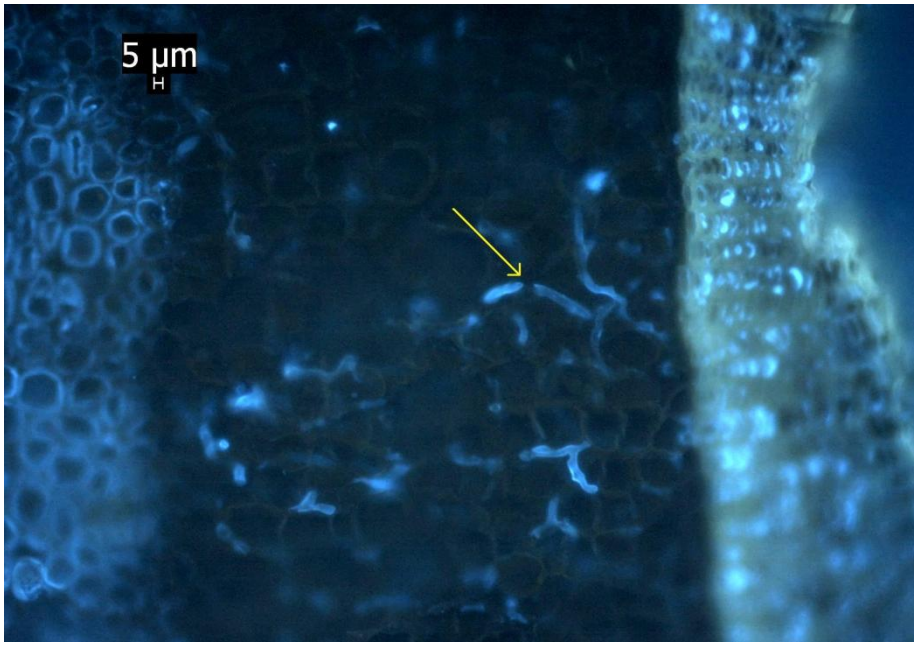


Figure 13. Longitudinal section of *P. ramorum* infected bark with hyphae growing from cell to cell: Fluorescence micrograph of a calcofluor stained bark sample from a *P. ramorum* inoculated region of a *N. densiflorus* log. Hyphae in phelloderm and parenchyma of the outer part of inner bark extend radially and vertically; in one place from cell to cell (arrow).

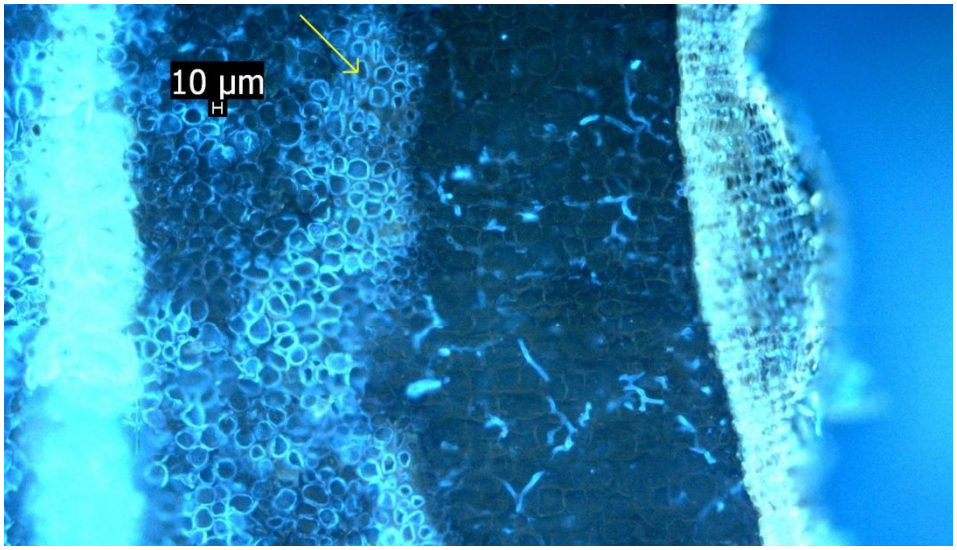


Figure 14. Longitudinal section of *P. ramorum* infected bark with hyphae growing radially and vertically: Fluorescence micrograph of a calcofluor stained bark sample from a *P. ramorum* inoculated region of a *N. densiflorus* log. Hyphae are in phelloderm and parenchyma of the outer part of inner bark. Cells colonized by hyphae are discolored but cell wall fluorescence is maintained in many of the cells lacking visible hyphae to the left.

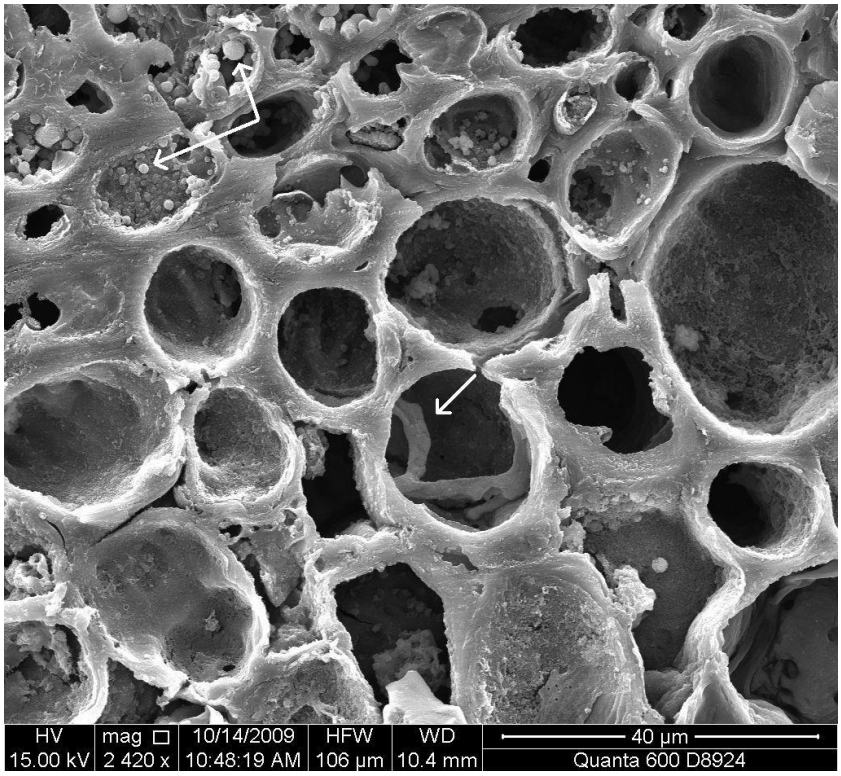


Figure 15. Hyphae in parenchyma in a *P. ramorum* infected sprout cutting: Scanning electron micrograph of a cross section of a bark sample from a *P. ramorum* inoculated sprout cutting. A hypha is seen in a parenchyma cell of the inner bark (center arrow). Globules present in cells of the phelloderm (top left arrows)

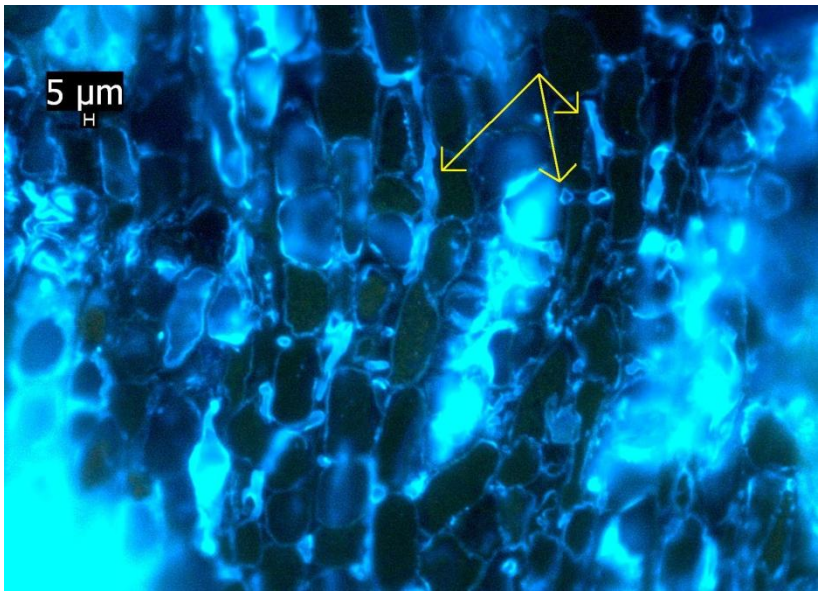


Figure 16. Hyphae growing intercellularly: Fluorescence micrograph of a longitudinal section of a calcofluor stained *P. ramorum* infected bark sample collected from an

infected *N. densiflorus* tree. Hyphae are growing primarily intercellularly (arrows). There appears to be some distortion of cell shape and diminished fluorescence of cell walls. Cells appear to be filled with a reddish brown material.

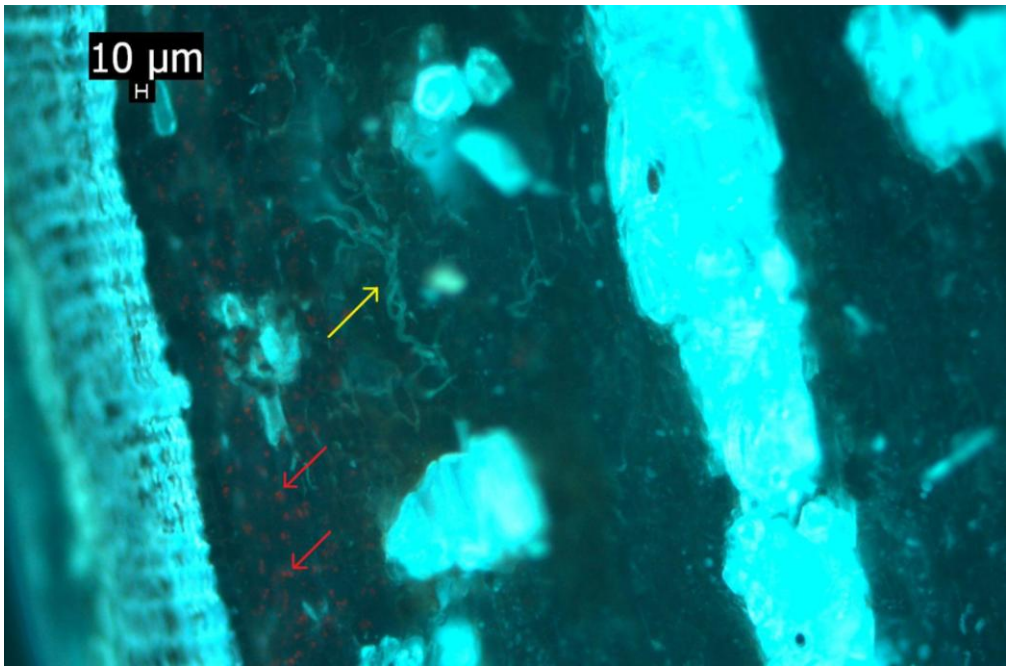


Figure 17. Aniline blue stained hyphae in *P. ramorum* infected bark: Fluorescence micrograph of a cross section of an aniline blue stained bark sample from a *P. ramorum* inoculated region of a *N. densiflorus* log. Fluorescence of hyphae in phelloderm and parenchyma of the outer part of inner bark (yellow arrow). without plant cell wall fluorescence. Chloroplasts fluorescing red can be seen in phelloderm (red arrows).

3.2.4 Colonization of Innermost Bark Tissues

Tissues of the innermost bark, in the region of conducting phloem, were seen colonized in only three samples. In the first sample, a hypha was seen inside of a sieve element (fig 18), in the second, hyphae were seen more frequently in ray parenchyma than any other cells in these tissues, and in the third sample, the cambium was most densely colonized (figs 19 and 20). Of these three samples, the third was most densely colonized overall, with most cells in the cambium and directly adjacent tissues of the inner phloem and outer xylem, housing at least one hypha. Diameters of hyphae in this sample reached 7 µm. The sample was an inoculated log from the second inoculation study. The direction of hyphal growth in the outer xylem was almost exclusively in the vertical direction whereas the direction of growth in the

cambium and inner bark of this sample was primarily vertical, but also horizontal and radial (figs 19 and 20). Inner bark and cambium tissues were heavily discolored here but cell walls in the xylem retained a bright blue fluorescence. Although the xylem was not investigated thoroughly in these studies, hyphae were seen in xylem vessels and xylem ray parenchyma (fig 21) in addition to the differentiating cells in the outermost xylem.

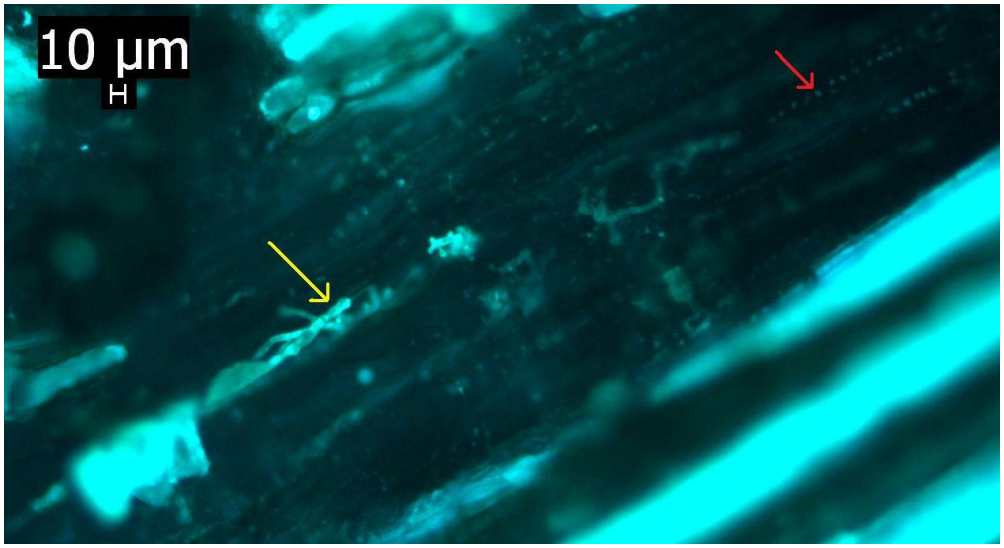


Figure 18. Aniline blue stained hyphae in a sieve tube element: Fluorescence micrograph of a longitudinal section of an aniline blue stained bark sample from a *P. ramorum* inoculated region of a *N. densiflorus* log. Hyphae in inner bark in a sieve tube element (yellow arrow). There is no plant cell wall fluorescence. Sieve areas fluoresce due to presence of callose (red arrow).

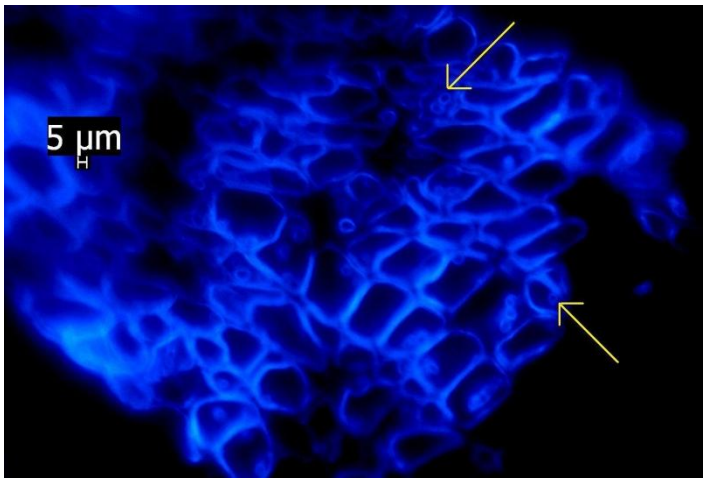


Figure 19. Hyphae in outer xylem: Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. ramorum* inoculated region of a *N. densiflorus* log. Hyphae are densely colonizing cells just inside the cambium in the outer xylem growing longitudinally (arrows).

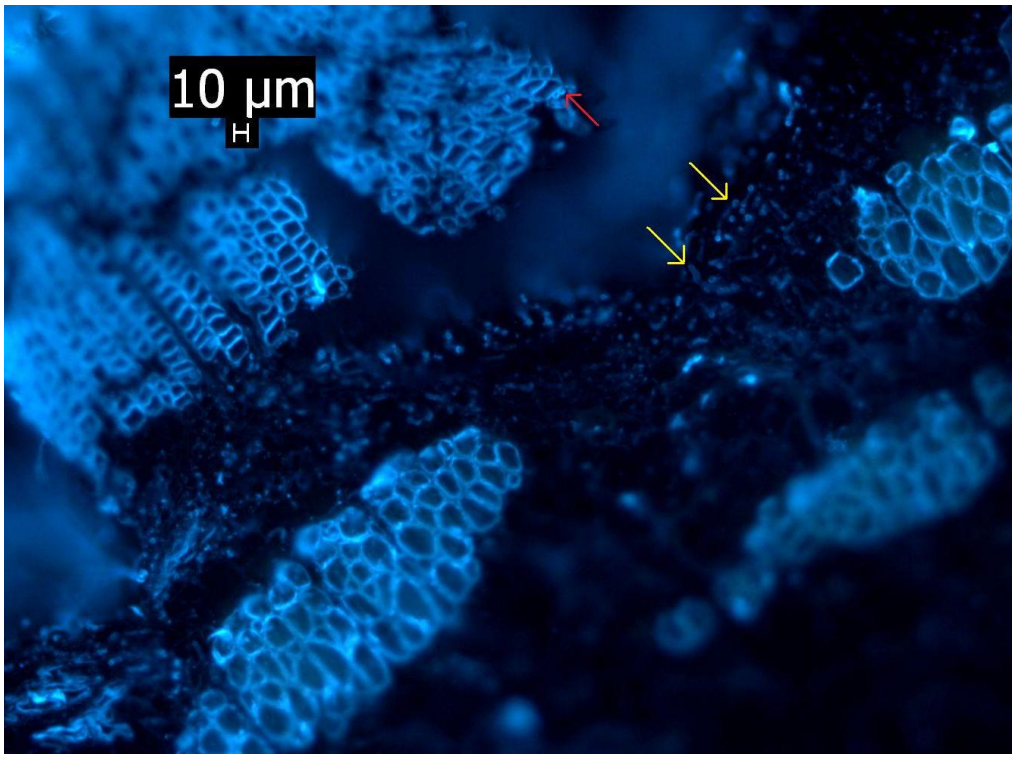


Figure 20. Hyphae in cambium and neighboring outer xylem and inner phloem. Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. ramorum* inoculated region of a *N. densiflorus* log. Hyphal colonization of cambium and adjacent inner phloem and outer xylem. All white fluorescent bodies like those pointed to with yellow arrows are hyphae in cambium/inner bark. Red arrow points to hyphae in outer xylem. Tissues were separating from each other in most places.

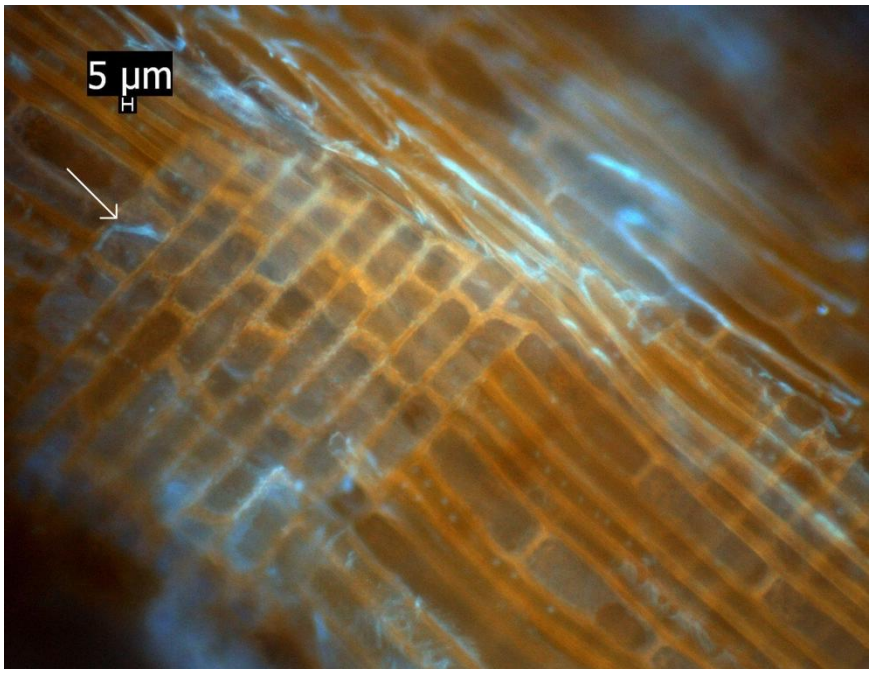


Figure 21. Hypha in a xylem ray: Fluorescence micrograph of a longitudinal section of a calcofluor stained *P. ramorum* infected bark sample collected from an infected *N. densiflorus* tree. A hypha is growing in a xylem ray (arrow).

3.2.5 Colonization of phellem and bark surfaces

Hyphae were seen in cork tissues with scanning electron microscopy (fig 22). They were also thought to be seen with calcofluor staining, but because of the small size of these cells and their bright autofluorescence, it was difficult to determine with this method whether or not these were hyphae characteristic of *P. ramorum* and, in places, whether or not they were hyphae at all (fig. 23). In some cases, characterstic hyphae could be seen with calcofluor staining in between layers of cork that had partially separated from each other (fig. 24). A material that stained blue-white with calcofluor was present in cork cells of some inoculated log and infected field samples which further complicated the ability to identify characteristic hyphae (fig. 25). A material could also be seen inside of cork cells with SEM (fig 26). The presence of callose in phellem cells has been reported and is considered a likely candidate for the substance in cork cells that stained brightly with calcofluor in this research (Litvay and Krahmer, 1976). Other materials such as suberin may also be present in cork cells and may account for some of the material that was seen in SEM samples. Direct penetration of the bark surface in all sample types appeared to occur, but hyphae could

never be traced to tissues directly inward of penetration sites (figs. 27, 28, and 29). Characteristic hyphae were also in and around lenticels either going into or coming out of them. Entry into bark through lenticels would mean bypassing the cork altogether (fig 30). Clusters of hyphae were often seen around lenticels, in both inoculated log and infected field samples but most of the time, these were hyphae with consistently small diameters and were not thought to be *Phytophthora* hyphae (fig. 31). Sprout cutting stems did not yet have lenticels. Sporangia with all of the morphological characteristics of *P. ramorum* sporangia were also observed in one calcofluor stained sample. Sporangial characterstics here were compared to those described by Werres et al, 2001 for *P. ramorum*. This was an infected field sample and sporangia appeared to be lodged in crevices of cork tissue (figs. 32, 33, and 34). What appeared to be encysted zoospores were seen on the surface of an inoculated sprout cutting sample and on the surface of a field collected sample (figs 35 and 36). A hypha grew out from the region where the zoospore was seen on the surface of the field collected sample and appeared to grow in a corkscrew pattern at its tip (fig 37).

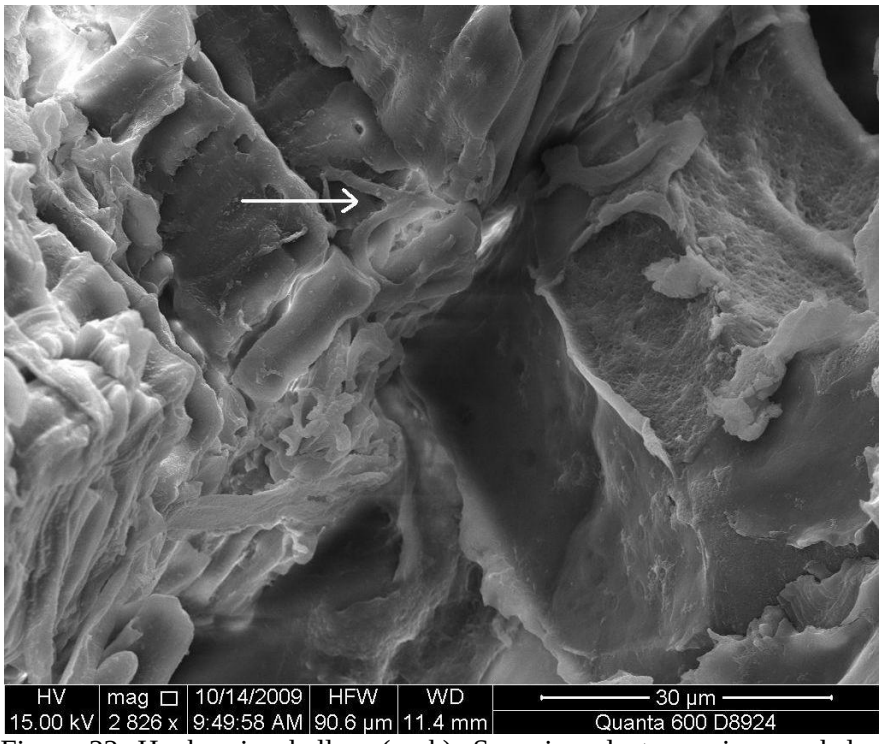


Figure 22. Hyphae in phellem (cork): Scanning electron micrograph looking into a bark crevice of a bark sample from a *P. ramorum* inoculated region of a *N. densiflorus* log. Hyphae in cork tissue (arrow).

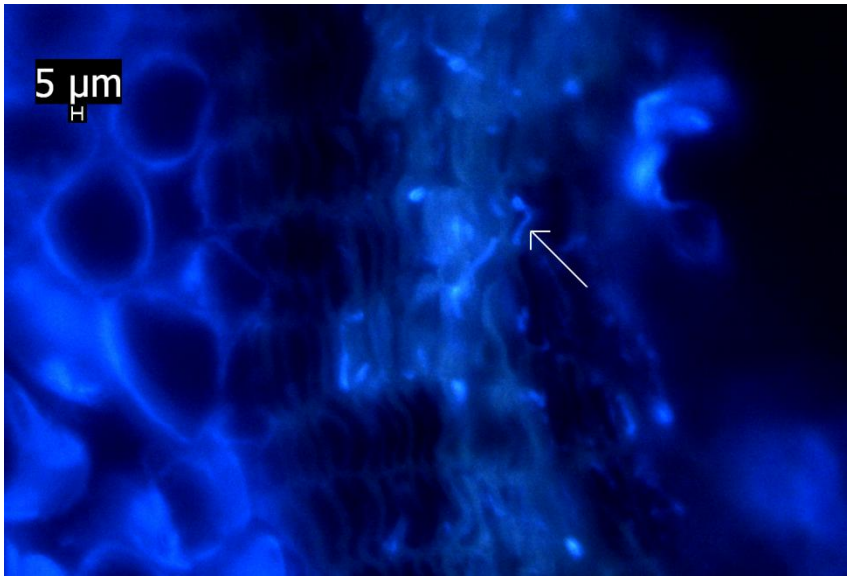


Figure 23. Possible hyphae in cork: Fluorescence micrograph of a cross section of a calcofluor stained, *P. ramorum* infected bark sample collected from an infected *N. densiflorus* tree. Hyphae in cork tissues (white arrow).

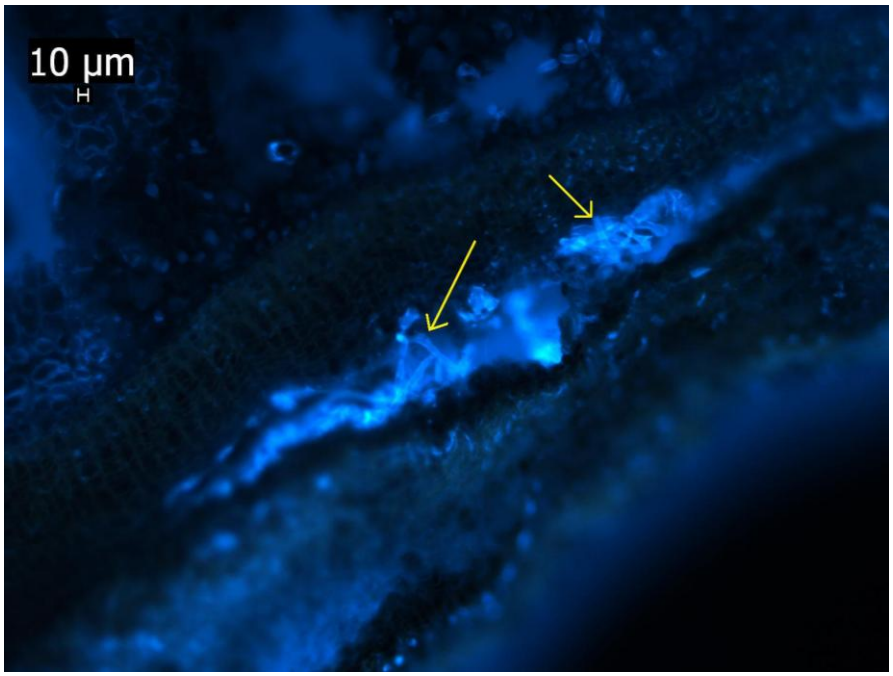


Figure 24. Hyphae in between cork layers: Fluorescence micrograph of a longitudinal section of a calcofluor stained, *P. ramorum* infected bark sample collected from an infected *N. densiflorus* tree. Hyphae growing in between layers of cork (arrows).

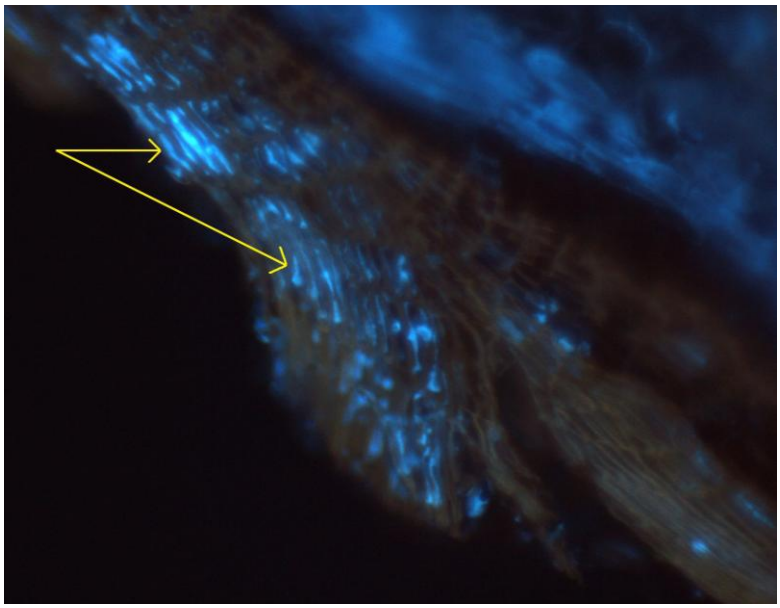


Figure 25. Calcofluor stained material in cork tissue: Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. gonapodyides* inoculated region of a *N. densiflorus* log. Cork cells are filled with a material that stains brightly with calcofluor.

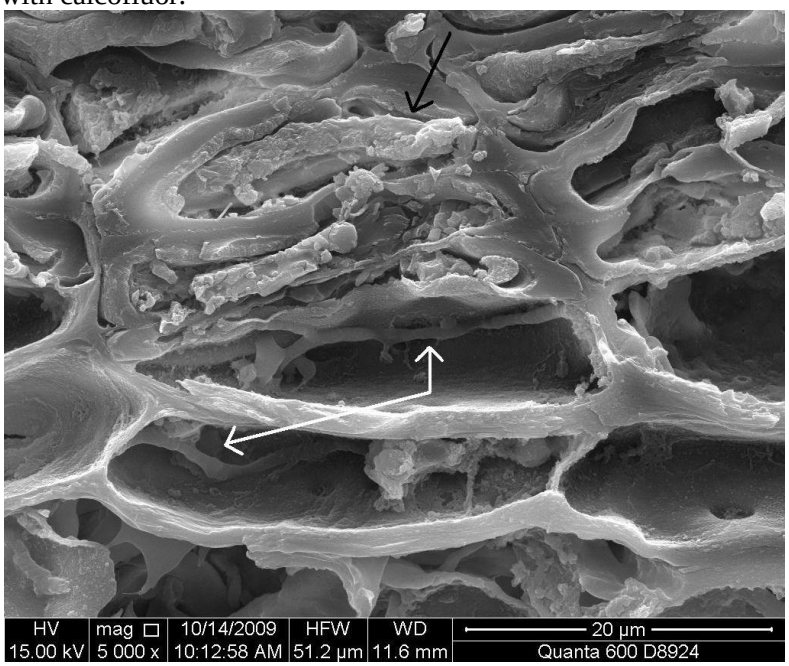


Figure 26. *P. gonapodyides* hyphae in phellogen: Scanning electron micrograph of a cross section of a bark sample from a *P. gonapodyides* inoculated region of a *N. densiflorus* log. Cork cells appear to be filled with some material (black arrow). Hyphae growing in phellogen (white arrows).

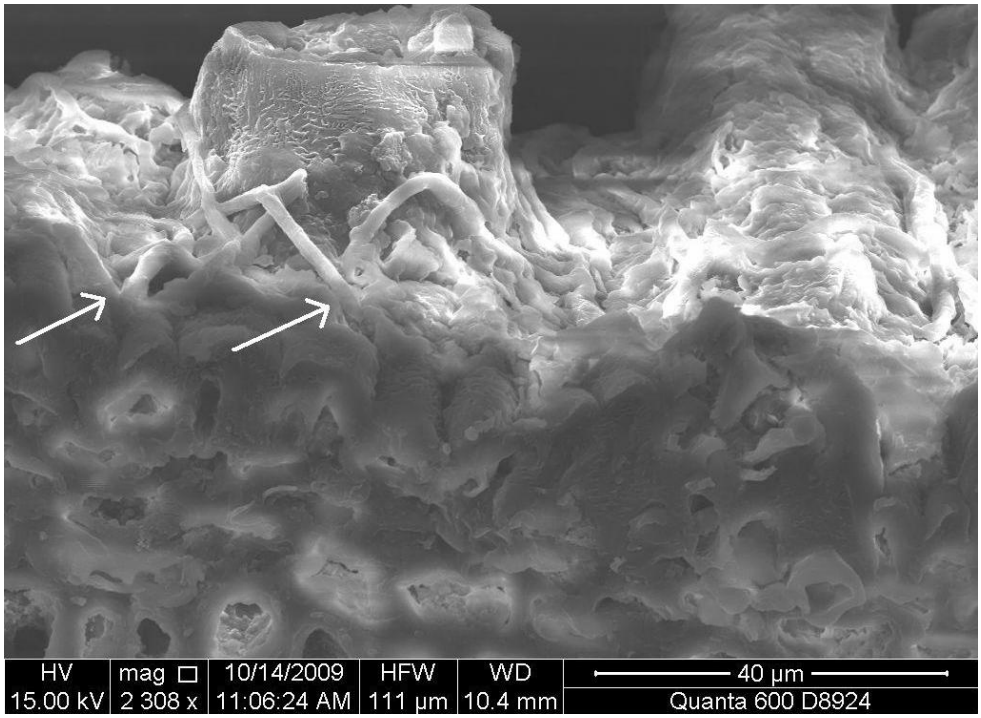


Figure 27. Hyphae disappear into bark surface of a sprout cutting: Scanning electron micrograph of a cross section and surface view of a bark sample from a *P. ramorum* inoculated sprout cutting. Hyphae on bark surface appear to disappear into cork tissues in a couple of places (arrows), suggesting that they penetrate the cork directly. Only one or two layers of cork cells present in young stem.

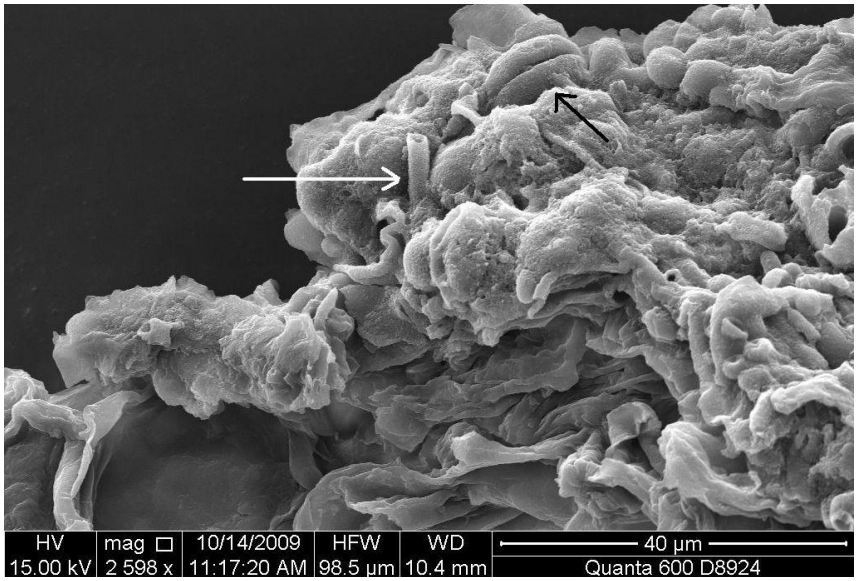


Figure 28. Hyphae disappear into bark surface of a sprout cutting 2: Scanning electron micrograph of a surface view of a bark sample from a *P. ramorum* inoculated sprout cutting. Hypha on bark surface seems to disappear into cork tissue (white arrow),

suggesting that it penetrates the cork directly. Guard cells of a stomate are seen on the surface (black arrow).

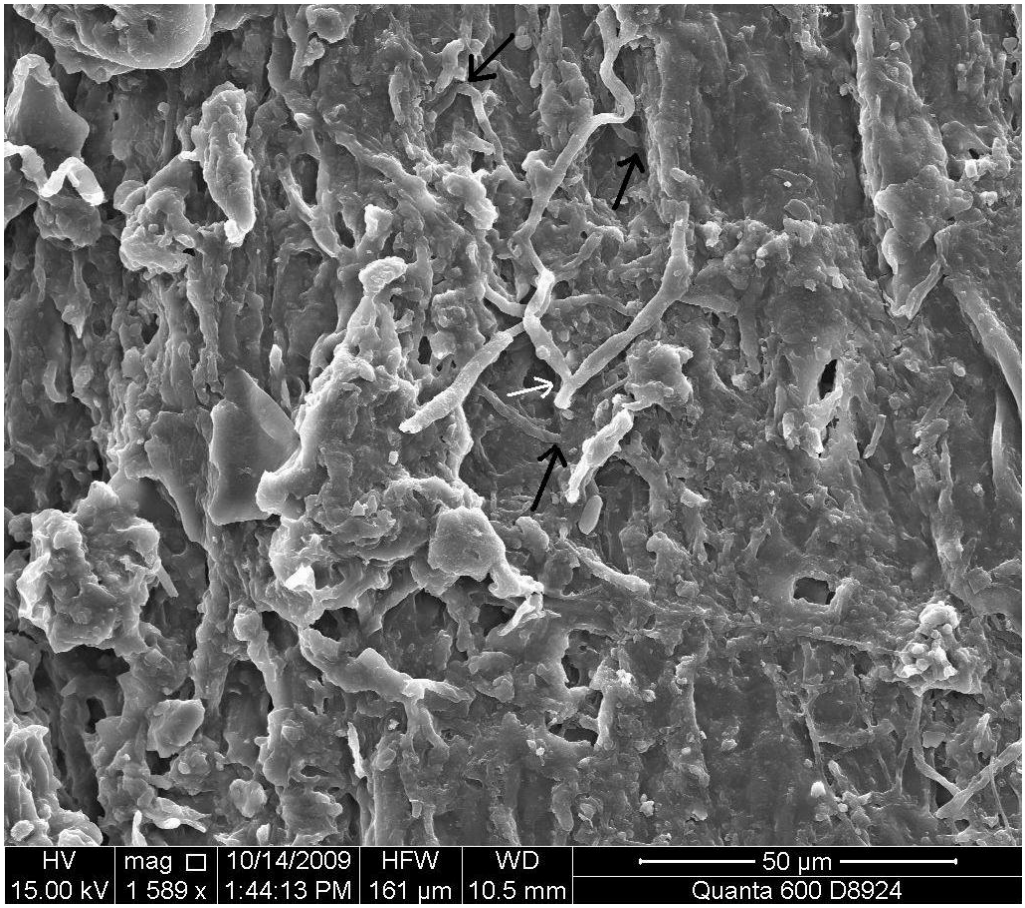


Figure 29. Hyphae disappear into bark surface of an infected field collected sample: Scanning electron micrograph of the surface of a bark sample collected from a *P. ramorum* infected *N. densiflorus* tree. Hyphae are seen growing on the bark surface and seem to disappear into it in a couple of places, suggesting direct penetration (black arrows). Pinching at a branch point can clearly be seen in one place (white arrow).

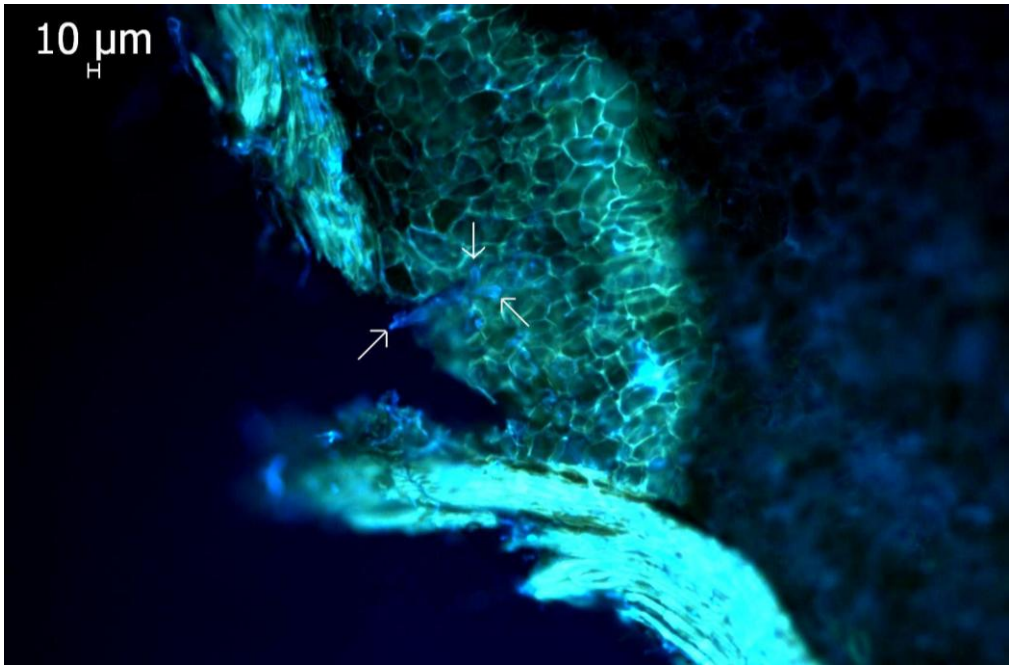


Figure 30. Hyphae going into or coming out of a lenticel: Fluorescence micrograph of a longitudinal section of a calcofluor stained, *P. ramorum* infected bark sample collected from an infected *N. densiflorus* tree. Hypha going into or coming out of a lenticel and growing inside bark tissues (arrows).

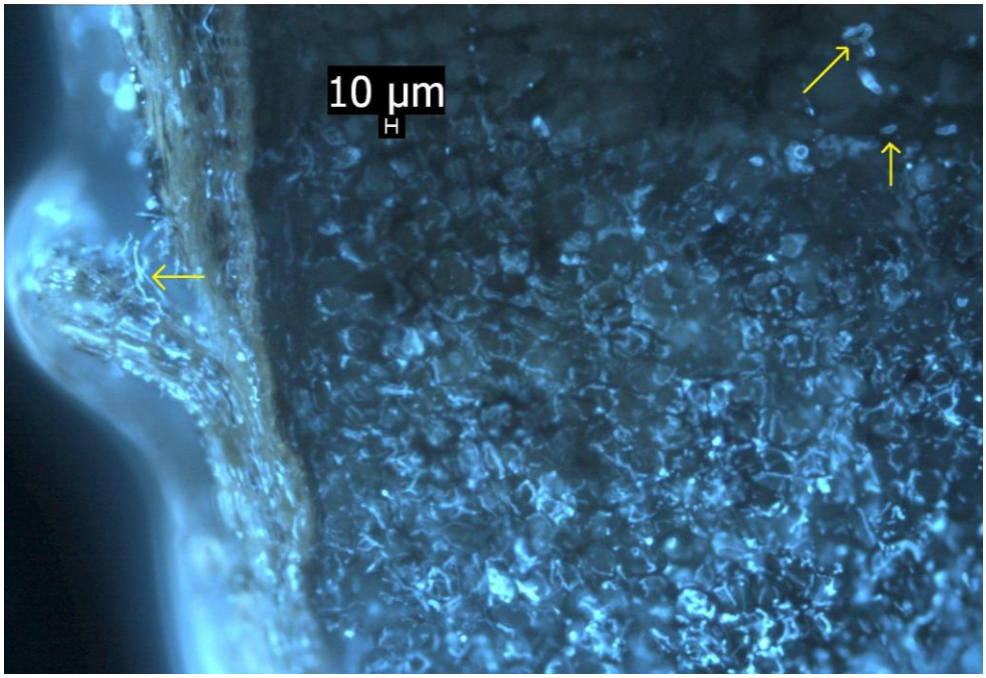


Figure 31. Non-*Phytophthora* hyphae: Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. gonapodyides* inoculated region of a *N. densiflorus* log. Hyphae that are not believed to be *Phytophthora* hyphae in a lenticel

(left arrow) and heavily colonizing parenchyma tissues and hyphae that are believed to be *P. gonapodyides* hyphae (upper right corner with arrows).

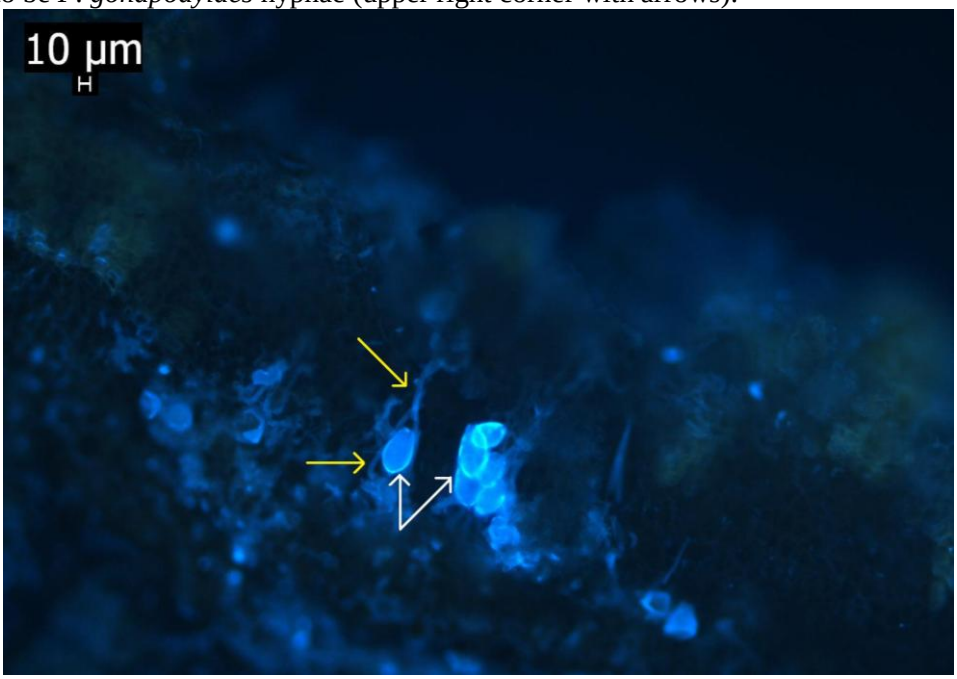


Figure 32. Sporangia in a bark crevice: Fluorescence micrograph near the surface of a longitudinal section of a calcofluor stained, *P. ramorum* infected bark sample collected from an infected *N. densiflorus* tree. Sporangia characteristic of *P. ramorum* are lodged in a bark crevice in cork tissue. Hyphae growing right next to a sporangium but it is not clear whether they germinated from it.

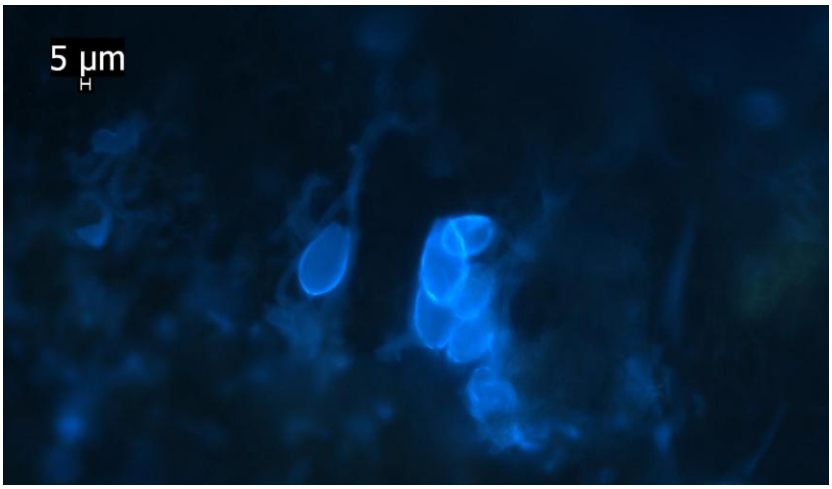


Figure 33. Sporangia in a bark crevice 2: Fluorescence micrograph near the surface of a longitudinal section of a calcofluor stained, *P. ramorum* infected bark sample collected from an infected *N. densiflorus* tree. Sporangia characteristic of *P. ramorum* are viewed more closely. Hyphae growing right next to a sporangium but it is not clear whether they grew from it.

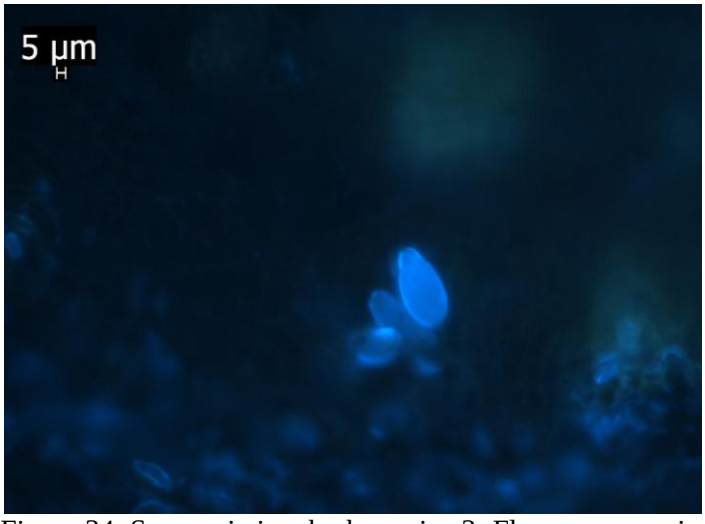


Figure 34. Sporangia in a bark crevice 3: Fluorescence micrograph near the surface of a longitudinal section of a calcofluor stained, *P. ramorum* infected bark sample collected from an infected *N. densiflorus* tree. Sporangia characteristic of *P. ramorum* are lodged in a bark crevice in cork tissue. Sporangia have retained their papillae. These sporangia were in the same sample as those shown in figs 31 and 32, seen a short distance away.

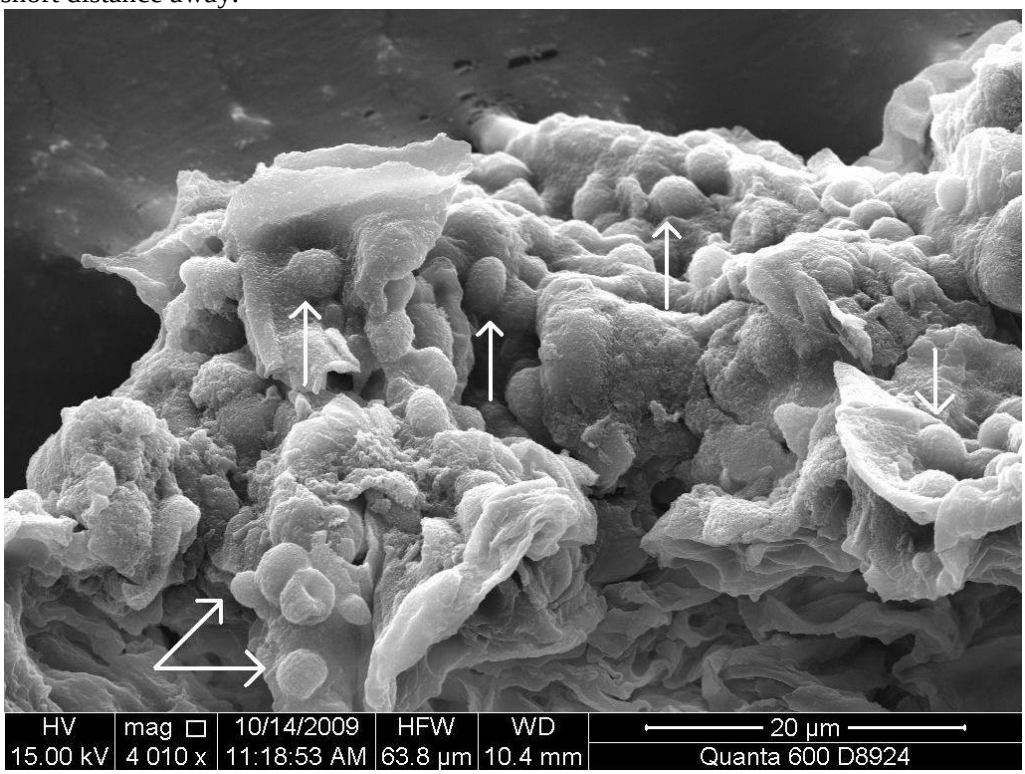


Figure 35. Zoospores on surface of inoculated sprout cutting: Scanning electron micrograph of a surface view of a bark sample from a *P. ramorum* inoculated sprout cutting. Multiple encysted zoospores are present on the surface (arrows).

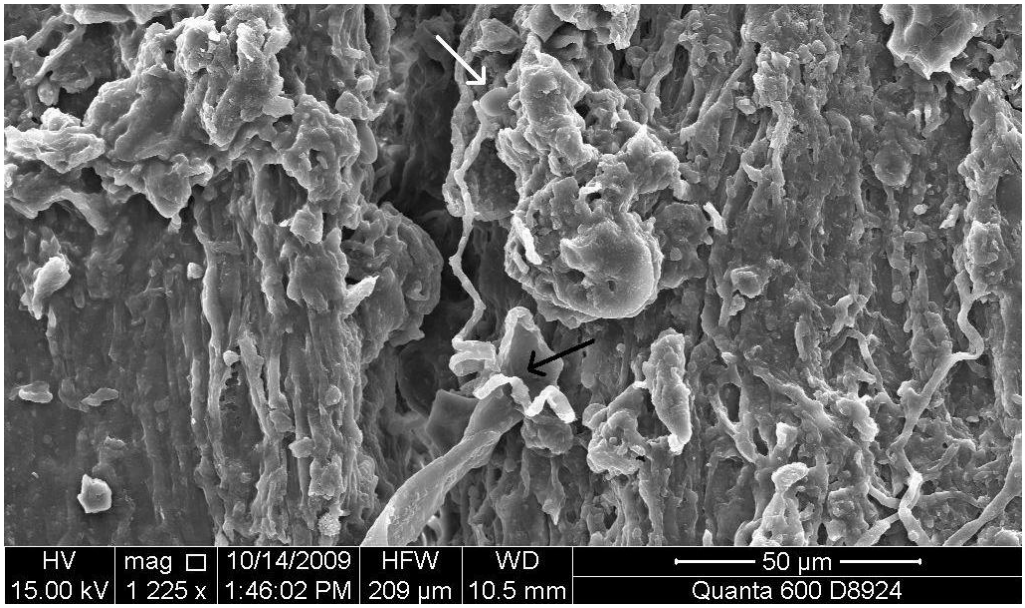


Figure 36. Possible zoospore next to hypha on surface of field collected bark sample: Scanning electron micrograph of the surface of a bark sample collected from a *P. ramorum* infected *N. densiflorus* tree. Hypha is seen growing on the surface next to a bark crevice growing in a corkscrew pattern at its tip (black arrow), originating from a place where there is what could be a zoospore cyst (white arrow).

3.2.6 *Phytophthora gonapodyides* bark colonization

P. gonapodyides infected controls possessed hyphae with similar characteristics to those seen in *P. ramorum* infected tissues. This species has coenocytic hyphae within a similar diameter range to *P. ramorum*, but *P. ramorum* was not recovered in culture from areas inoculated with *P. gonapodyides*. Hyphal diameter was 2-8µm and hyphae were seen most commonly in the phelloderm and outer part of inner bark. With scanning electron microscopy, hyphae were usually only seen singly, but in two places, multiple hyphae were seen colonizing a single cell (fig. 36). A couple of times, hyphae were seen in and around lenticels, in one place either going into or coming out of one (fig. 37). In two places in one sample, at the interface between phelloderm and inner bark parenchyma, there was a small area where there appeared to be a lack of any plant cell material, only an abundance of hyphae in a gap within the plant tissue (fig. 38). This may simply have been due to a separation of plant tissues from each other, but the pattern of the surrounding cells suggests this was not the case and, rather, that the plant tissue had been consumed by the hyphae. Hyphae were not seen in the innermost bark of any *P. gonapodyides* infected samples. Host

defense responses will be discussed in the next section but we will mention that the only host defense response that was seen in *P. gonapodyides* infected tissues was production of tannins.

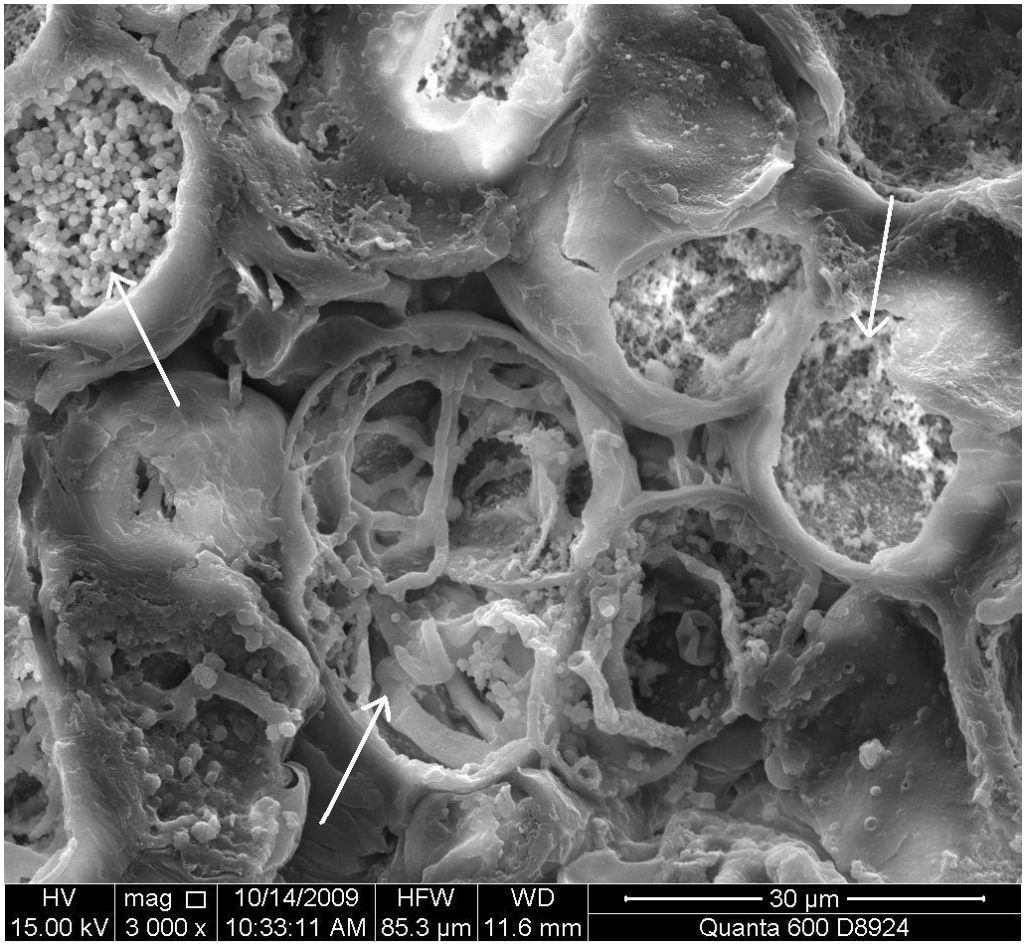


Figure 37. Multiple *P. gonapodyides* hyphae in a bark parenchyma cell: Scanning electron micrograph of a cross section of a bark sample from a *P. gonapodyides* inoculated region of a *N. densiflorus* log. Multiple hyphae are seen in one cell (center), small globules in a cell at the top left and amorphous material in cells top right.

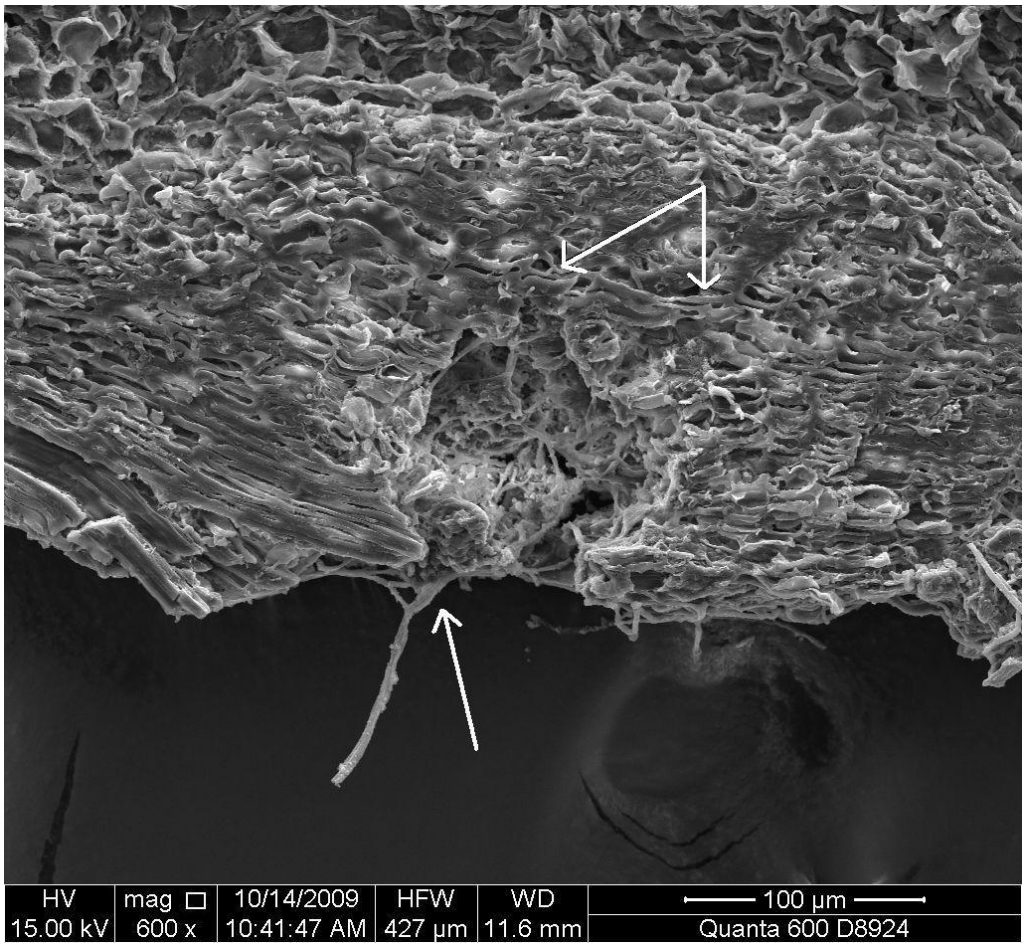


Figure 38. *P. gonapodyides* hyphae going into or coming out of a lenticel: Scanning electron micrograph of a cross section of a bark sample from a *P. gonapodyides* inoculated region of a *N. densiflorus* log. Hyphae are seen going into or coming out of a lenticel (bottom). Cells inside the lenticel have thick secondary walls (upper arrows).

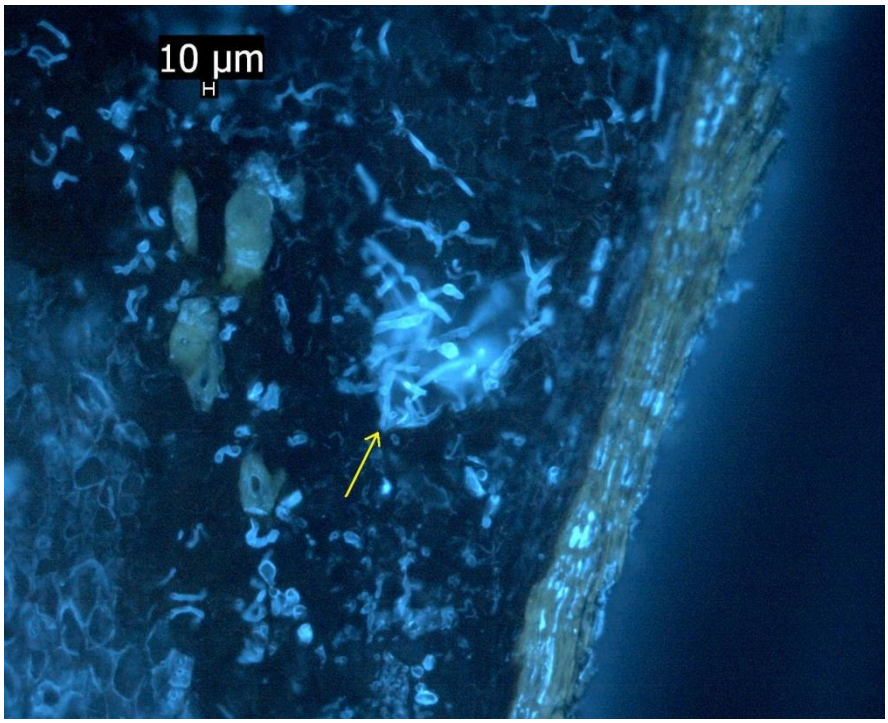


Figure 39. Many *P. gonapodyides* hyphae in a gap where there is a lack of any plant cell material: Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. gonapodyides* inoculated region of a *N. densiflorus* log. Many hyphae are present in a gap where there is a lack of any plant cell material (arrow).

3.3 Host Responses to Infection

3.3.1 Callose Production

Staining of callose by calcofluor occurred in all sample types including uninfected samples with the staining of sieve areas in conducting phloem in both infected and control samples (figs. 39, 40, and 41). In addition, small callose depositions were seen in parenchyma cells of the inner bark of both infected and uninfected tissues from inoculated log and infected field samples. The frequency and density of these depositions, however, was much greater in infected samples. Only a few scattered, single cells in control samples show this response, with all of the surrounding cells lacking depositions. In some infected samples, most cells in infected areas were full of these depositions, both within and around infected tissue (fig. 42 and 43). In some samples, the deposits could all be brought into the same plane of focus within individual cells (fig. 43). The frequency and density of the deposits varied among infected samples, and they were not present in most infected samples. These

depositions were not present in any of the three sprout cutting samples that were viewed with calcofluor staining though they occurred in both inoculated log and infected field samples. In one infected field sample, crosswalls of parenchyma cells in the outer part of inner bark stained brightly with calcofluor staining (fig. 44). When viewing samples with SEM, the plasmodesmata of one of the samples that stained positively for callose by caclofluor appear to be plugged by a bright material (fig. 45).

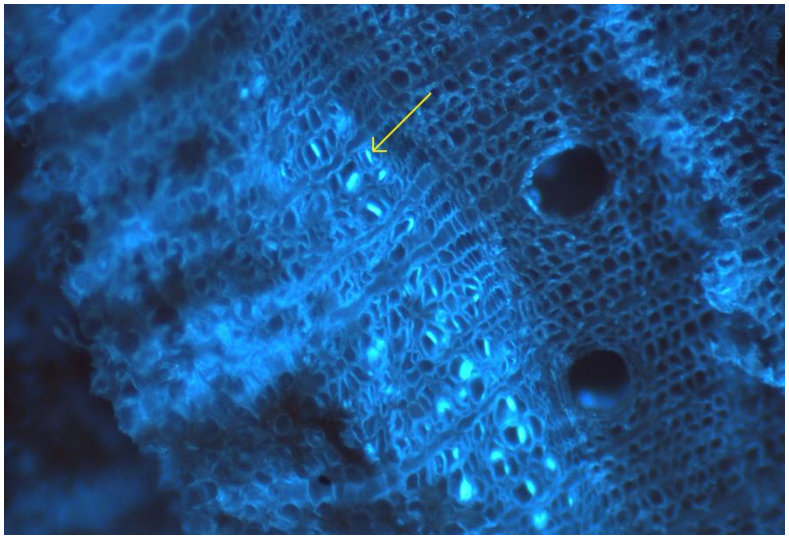


Figure 40. Callose in sieve tube elements (cross section): Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. ramorum* infected *N. densiflorus* tree. Sieve areas of sieve elements fluoresce brightly with calcofluor staining due to the presence of callose. Fluorescence of sieve areas was also seen in uninfected control samples.

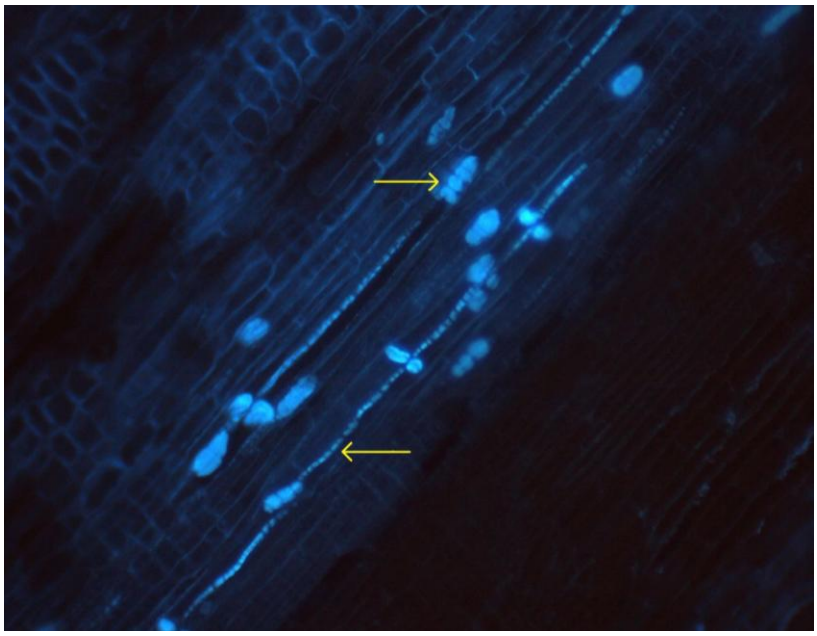


Figure 41. Callose in sieve tube elements (longitudinal section): Fluorescence micrograph of a longitudinal section of a calcofluor stained bark sample from a *P. ramorum* infected *N. densiflorus* tree. Sieve areas of sieve elements fluoresce brightly with calcofluor staining due to the presence of callose (sieve areas in a sieve plate- top arrow; sieve areas on longitudinal walls- bottom arrow).

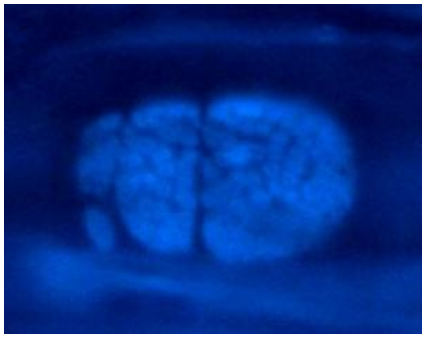


Figure 42. Callose in sieve pores of a sieve plate: Fluorescence micrograph of a longitudinal section of a bark sample from an uninfected, live *N. densiflorus* control tree. Sieve pores of sieve plates fluoresce with calcofluor staining due to the presence of callose.

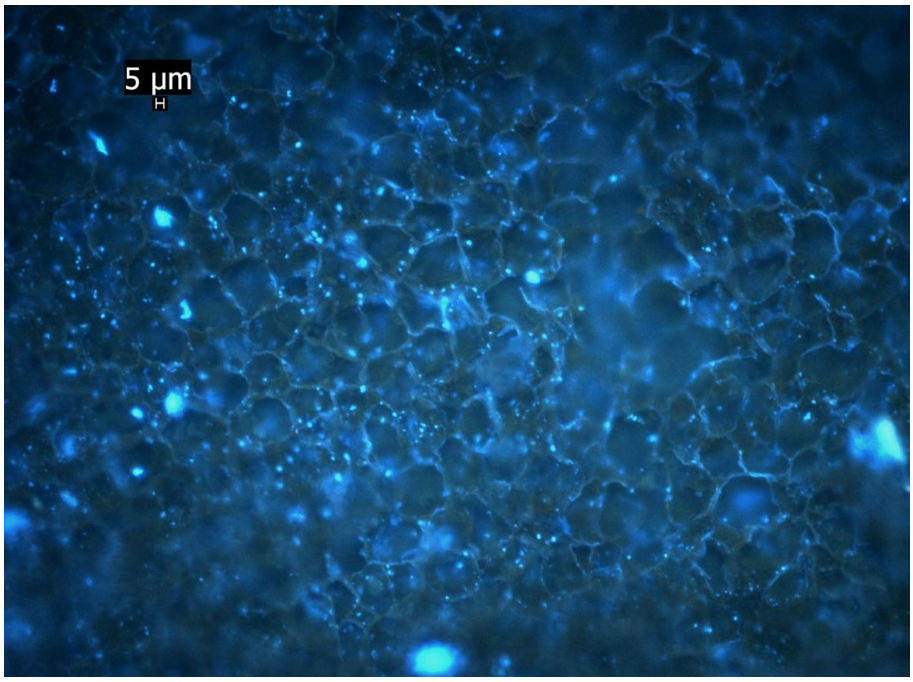


Figure 43. Callose production in *P. ramorum* infected bark tissue: Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. ramorum* inoculated region of a *N. densiflorus* log. Whitish fluorescent flecks in parenchyma of the inner bark are callose depositions.

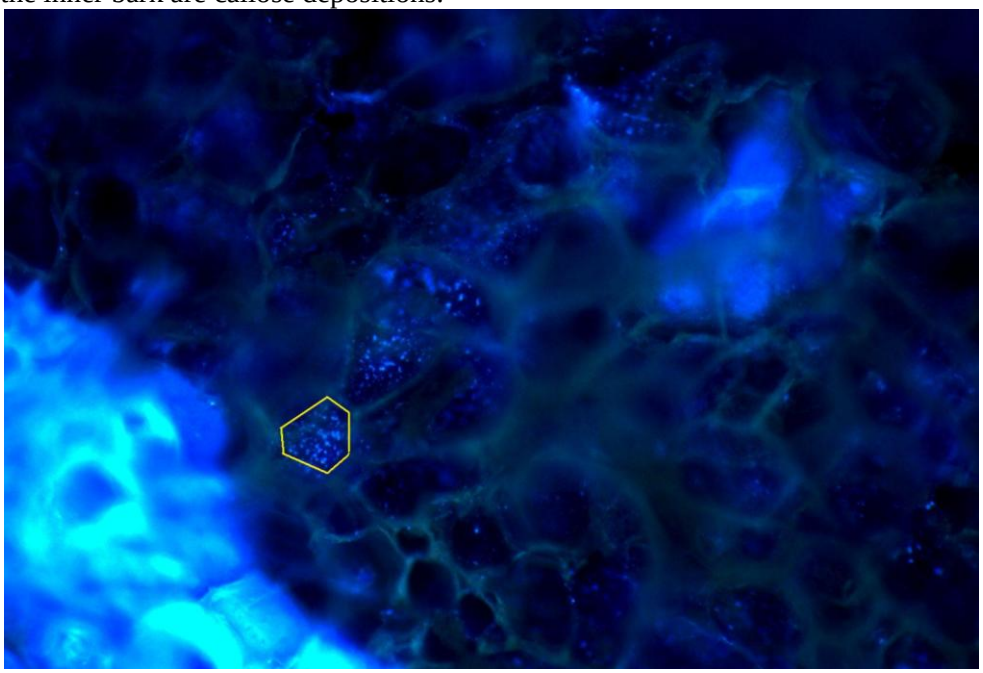


Figure 44. Callose in cell wall of bark parenchyma: Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. ramorum* infected *N. densiflorus* tree. Fluorescent flecks in parenchyma of the inner bark are callose

depositions. Flecks appear to be in the same plane of focus within individual cells suggesting that they are all in the cell wall (one such cell is outlined in yellow).

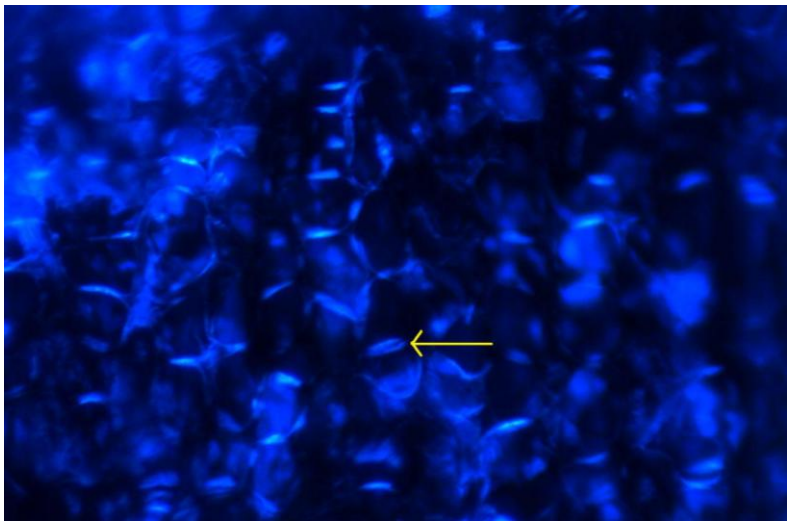


Figure 45. Thickening of bark parenchyma crosswalls: Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. ramorum* infected *N. densiflorus* tree. Bright staining of thickened crosswalls in parenchyma of inner bark tissue suggests callose or additional cellulose deposition (arrow). The cork, to the outside, is up in this picture.

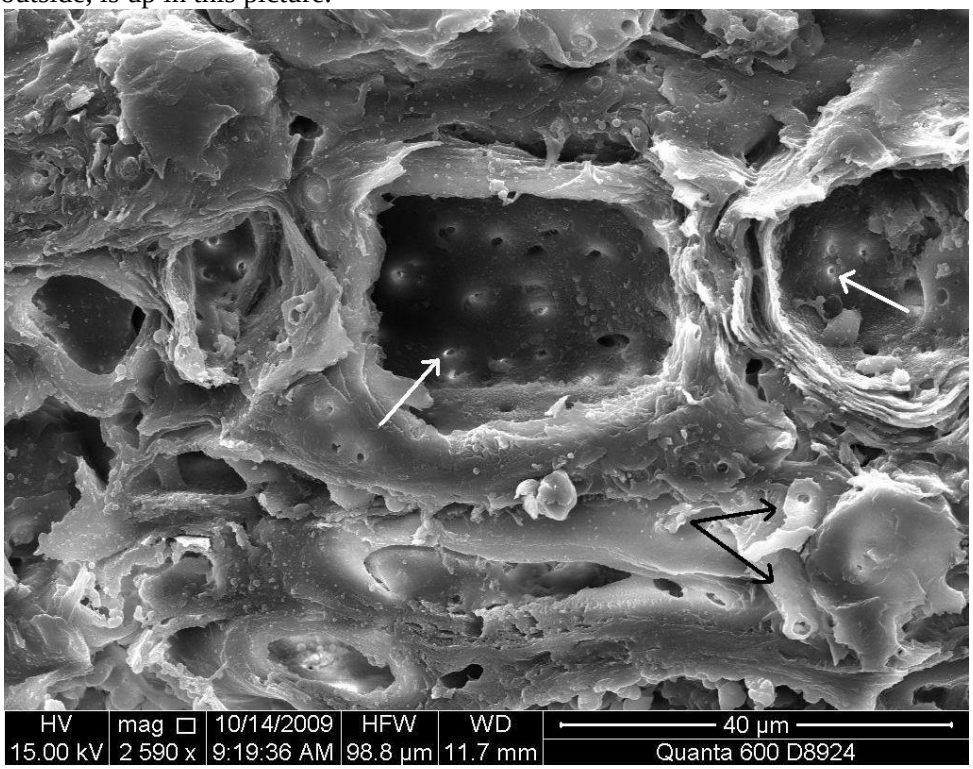


Figure 46. Thickenings of plasmodesmata in bark parenchyma. Scanning electron micrograph of a cross section of a bark sample collected from a *P. ramorum* infected

N. densiflorus tree. Some plasmodesmata of parenchyma cells in the inner bark appear to be plugged with something (white arrows). May be two hyphae below these cells (black arrows).

3.3.2 Staining for tannins

Globules inside cells that appeared beige in color with UV fluorescence stain bright red with the vanillin HCl method for staining tannins (figs. 46 and 47). Additionally, tissues that were discolored, appearing brown with normal light, but red-brown to brown with UV light, often contained a globular material that was this same color and also consistently stained for tannins (figs. 48 and 49). Presence of tannins most often occurred in the phelloderm and parenchyma cells of the outer part of the inner bark, but occurred in all tissues at times, including the xylem. Tannin staining could always be seen under normal light and part or all of the response could be seen with UV fluorescent light. Control samples also stained positively for tannins, but usually there were just small pockets of stained material in a few places throughout tissues. The production of tannins was greater in infected tissues and was usually widespread throughout an infected area (fig. 49). In the two places where characteristic hyphae were seen growing predominantly intercellularly, surrounding cells were filled with a dark red material in one (fig. 16) and a creamy colored opaque material in the other, though the latter was a *P. gonapodyides* infected sample. Phelloderm cells and parenchyma cells of the inner bark were frequently filled with an abundance of small globules or an amorphous material (figs. 15 and 36). These cells were sometimes colonized by hyphae.

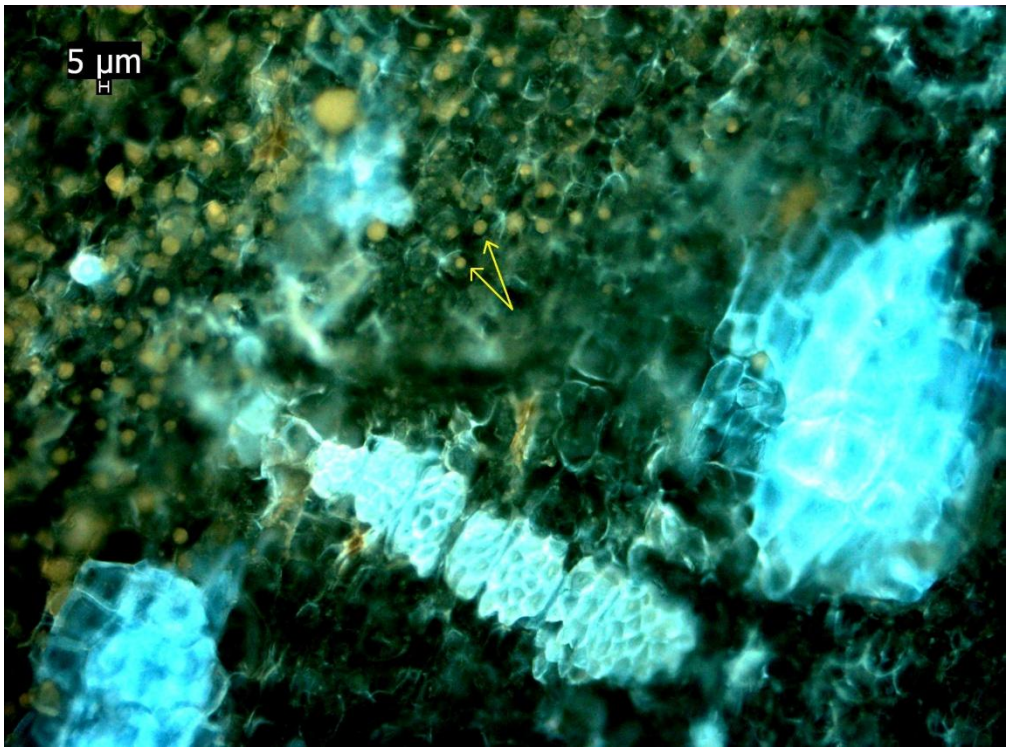


Figure 47. Unstained tannins: Fluorescence micrograph of a cross section of an unstained bark sample from a *P. ramorum* infected *N. densiflorus* tree. Transition zone between parenchyma cells in the inner bark filled with tannins (light brown globules pointed out with arrows) and cells lacking them.

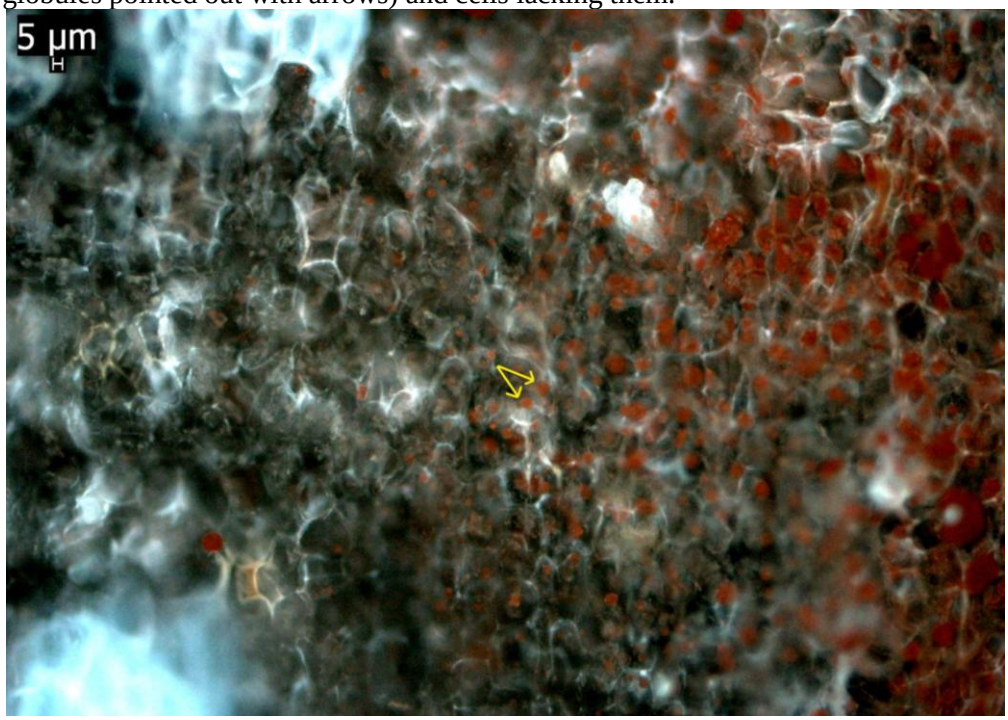


Figure 48. Stained tannins seen with fluorescence. Fluorescence micrograph of a cross section of a bark sample stained by the vanillin-HCl method from a *P. ramorum* infected *N. densiflorus* tree. Transition zone between parenchyma cells filled with tannins (red globules) and cells lacking them in the inner bark.

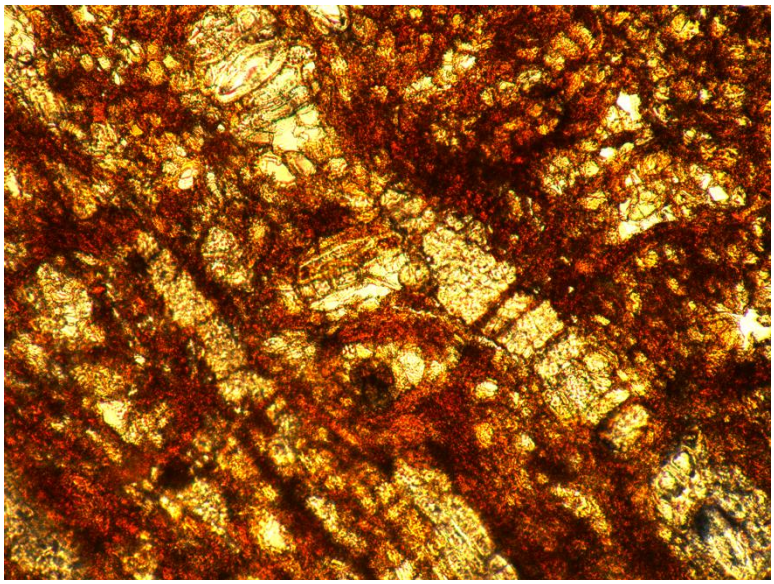


Figure 49. Unstained tannins seen with light microscopy: Light micrograph of a cross section of an unstained inner bark sample from a *P. ramorum* infected *N. densiflorus* tree. Parenchyma cells have a brown coloration and fibers and sclereids are transluscent.

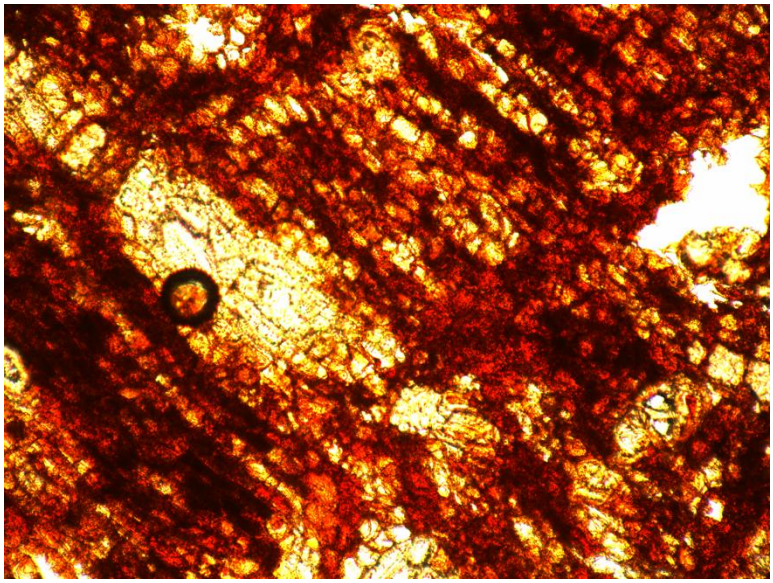
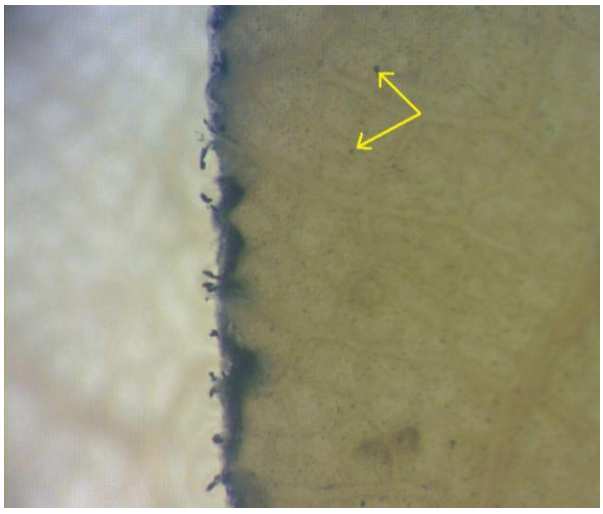
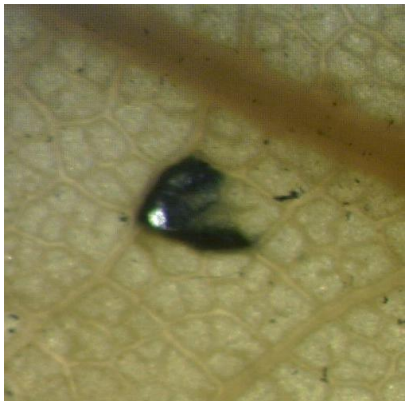


Figure 50. Stained tannins seen with light microscopy. Light micrograph of a cross section of a bark sample stained by the vanillin-HCl method from a *P. ramorum* infected *N. densiflorus* tree. Parenchyma cells now appear red due to the presence of tannins and fibers and sclereids are still transluscent.

3.3.3 Detection of a Hypersensitive Response

Staining of all *N. densiflorus* tissues for a hypersensitive response after challenge with *P. ramorum* was unsuccessful. Blue staining occurred in these tissues only in places where there was wounding and not where the tissue had been challenged by the pathogen (figs. 50 and 51). One leaf from each tobacco plant challenged with either *P. cryptogea* or *P. infestans* both successfully stained for a hypersensitive response (figs 52 and 53).





Figures 51 and 52. Left: figure 51. Light micrograph of Nitroblue tetrazolium stained *N. densiflorus* leaves 6 hrs after inoculation with *P. ramorum*: Cut edge of a leaf staining blue with NBT representing production of superoxides. Hairs and debris on leaf surface also stain blue but this also occurs in uninoculated controls (arrows). Right: figure 52. Light micrograph of Nitroblue tetrazolium stained *N. densiflorus* leaves 6 hrs after inoculation with *P. ramorum* 2: Blue staining with NBT where puncture wound was made for inoculation.

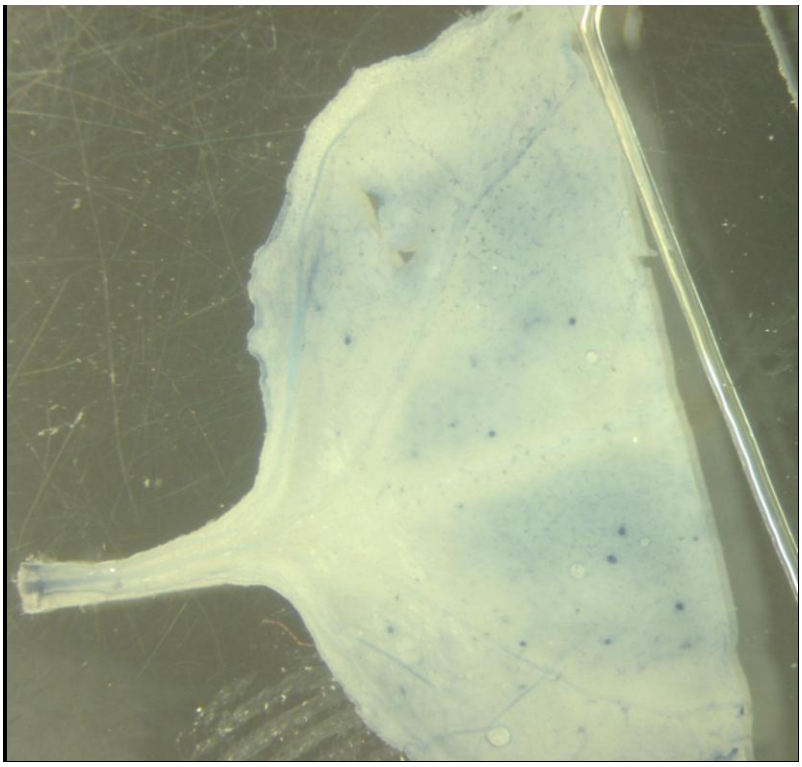


Figure 53. Superoxide staining in a *P. infestans* infected tobacco leaf: Light micrograph of a nitroblue tetrazolium stained *Nicotiana benthamiana* leaf 4 hrs after inoculation with *P. infestans.* A blue color is present in much of the leaf tissue after nitroblue tetrazolium staining, indicating the production of superoxides, an indicator of a hypersensitive response.

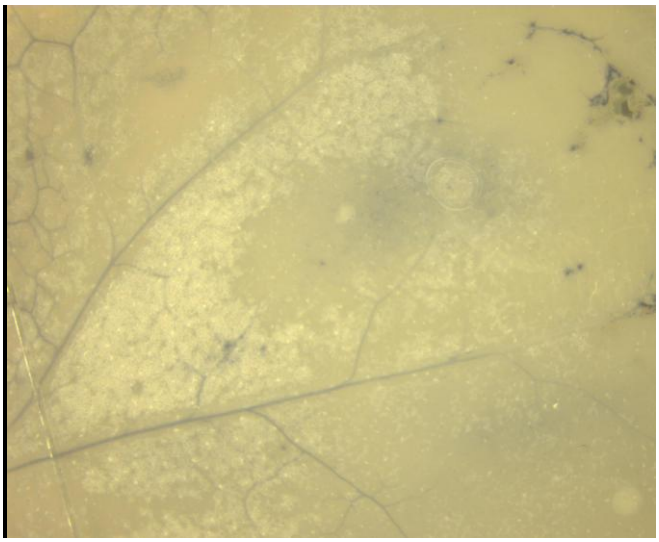


Figure 54. Superoxide staining in a *P. cryptogea* infected tobacco leaf: Light micrograph of a nitroblue tetrazolium stained *Nicotiana benthamiana* leaf 6 hrs after inoculation with *P. cryptogea.* A blue color is present in some leaf tissue after nitroblue tetrazolium staining, indicating the production of superoxides, an indicator

of a hypersensitive response. Staining at top right is from minor wounds that formed from handling with forceps.

3.3.4 Wound Periderm Production

There was one sample in which a periderm that was believed to be wound periderm was seen. This was an infected field sample from a 10cm DBH tree. A meristematic layer made up of highly oblong cells could be seen, at least in some regions, and cells of the meristem and those that appeared to have arisen from it had walls that fluoresced white under UV light (fig. 54). Cells that appeared to have arisen from the meristem were not small and highly oblong in cross section like cells in cork of outer periderms, but were more similar in shape and size to the cells in the surrounding parenchyma and/or phelloderm. A white fluorescent material that looked like webbing or a matrix also appeared inside of cells (fig. 55). This webbing was seen at other times and places, both in infected and control samples. However, the webbing in other areas could usually only be seen with calcofluor staining or, if it was seen with autofluorescence, the intensity of the white fluorescence was not as strong as it was in this periderm. Cell walls of cells in this "wound periderm" also appeared thicker than those of cells in the surrounding tissues and of those in similar regions in healthy tissues. There were other places in bark tissues of inoculated log samples where a similar kind of cell wall thickening was seen, most often occurring behind lenticels (figs. 30 and 37). This may be a normal feature of cells just inside lenticel openings. Cells on either side of the wound periderm were filled with the material that consistently stained positively for tannins (discussed in the next section), but cells to the outside of the periderm had a more uniform presence of this material and it contained more of a creamy tan colored material uniformly filling the cells whereas the material to the inside was in globules and more of a red-brown color. Cell walls of the cells to the outside of the periderm were no longer fluorescing with white autofluorescence.

Because a second periderm was seen in one field control sample, from a 36cm DBH tree, it was possible to compare this to the "wound periderm" that was seen. The second periderm in the control sample appeared more or less the same as most outer

periderms, with a well developed cork layer of flattened, lignified cells, and no webbing (fig. 56).

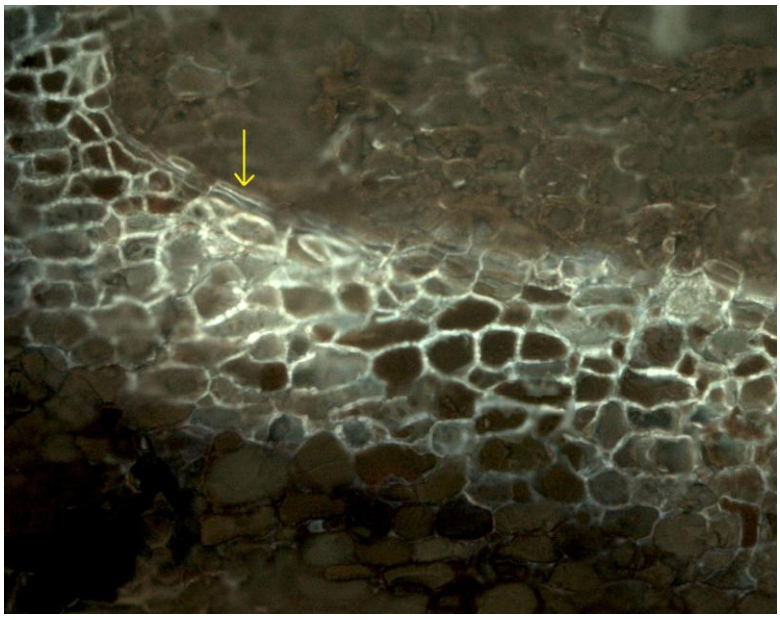


Figure 55. Wound periderm: Fluorescence micrograph of a cross section of an unstained bark sample from a *P. ramorum* infected *N. densiflorus* tree. A wound periderm has begun to form (white fluorescent cells) in parenchyma of the inner bark arranged in a radial/longitudinal direction here. Outer bark is to the left. Oblong white fluorescent cells along the top are meristematic cells (arrow), giving rise to the other tissues of the periderm.

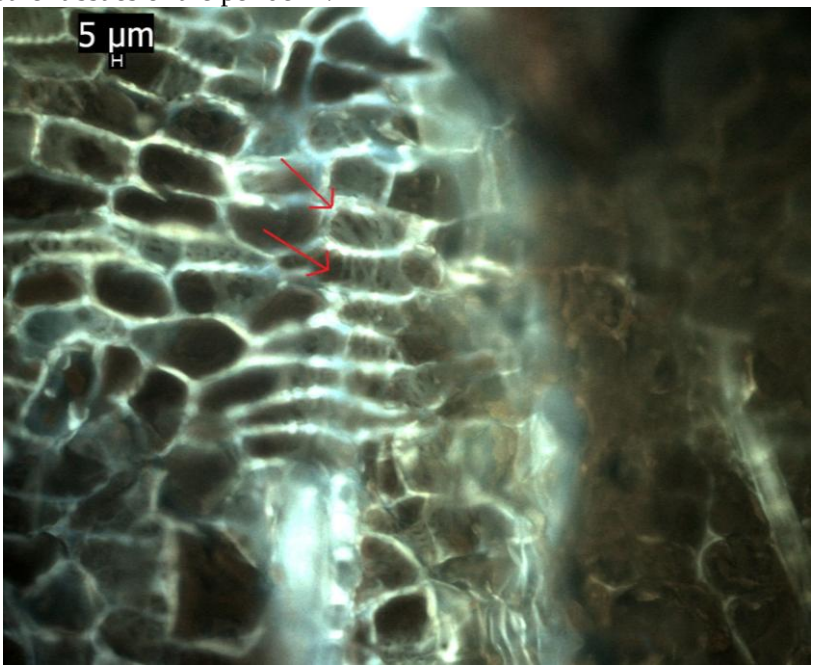


Figure 56. Wound periderm 2: Fluorescence micrograph of a cross section of an unstained bark sample from a *P. ramorum* infected *N. densiflorus* tree. A wound periderm has begun to form in parenchyma of the inner bark (white fluorescent cells) arranged in a longitudinal direction here. Webbing (arrows) can be seen inside cells of the wound periderm and fluoresces white with autofluorescence suggesting deposition of additional secondary wall material. Oblong white fluorescent cells to the right are meristematic cells giving rise to the periderm.

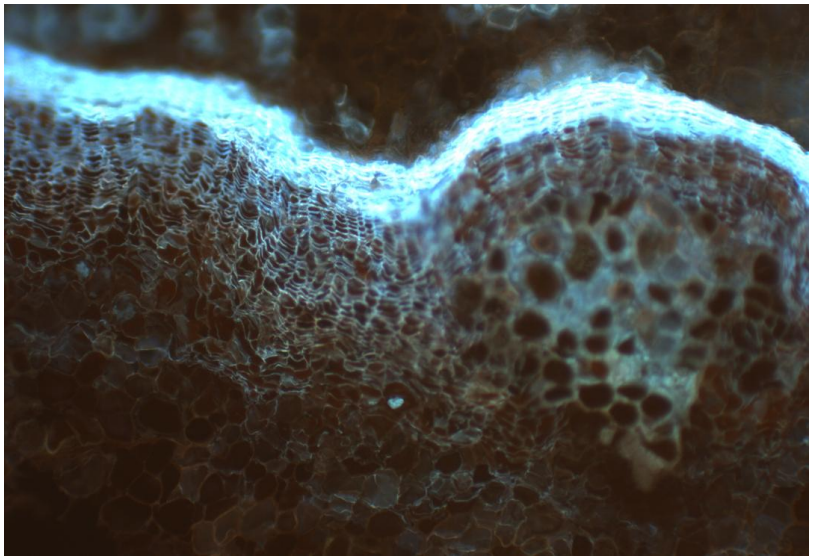


Figure 57. Secondary periderm: Fluorescence micrograph of a cross section of an unstained bark sample from an uninfected *N. densiflorus* control tree. A mature second periderm has formed, with well developed cork cells fluorescing bright white due to heavy lignification of the secondary cell wall.

3.4 Elicitin Production

Hyphae were only successfully stained in 8 of the 38 infected samples to which the elicitin labeling procedure was applied, and all were young sprout cuttings. Three of these samples were from the first inoculation study where samples included about ¾ cm of tissue inside the lesion margin and ¾ cm of tissue outside of it, but hyphae were never seen beyond the lesion margin. Four samples were from the fourth inoculation study and all were samples from the bottom section that was taken, where the entire sample was within the lesion margin but the sample ended at the lesion margin. The last sample from which hyphae were successfully illuminated by the immunofluorescence method was from the second inoculation study but was also a bottom sample, where all tissues were within the lesion margin. The procedure was also applied to samples from both log inoculation studies as well as infected field

samples but hyphae were not successfully stained in these samples. Other methods of staining confirmed that hyphae were present in these samples.

A large fraction of the hyphae illuminated by this method were hyphal tips as seen in figures 57 and 60. Hyphae could be seen growing from cell to cell but breakdown of plant cell walls was not evident (fig. 60). In some places, multiple hyphae were illuminated by this method (figs 58 and 59). No hyphae were observed in *P. gonapodyides* infected controls or any other controls, however, the only tissues successfully stained by this method were inoculated sprout tissues which included no *P. gonapodyides* controls. Green fluorescence occurred in other forms in these tissues including a uniform green fluorescence inside of some cells and small bright green flecks in some tissues, but this kind of fluorescence also occurred in controls, so they were not considered to represent a successful immunofluorescence response (figs 58 and 61).

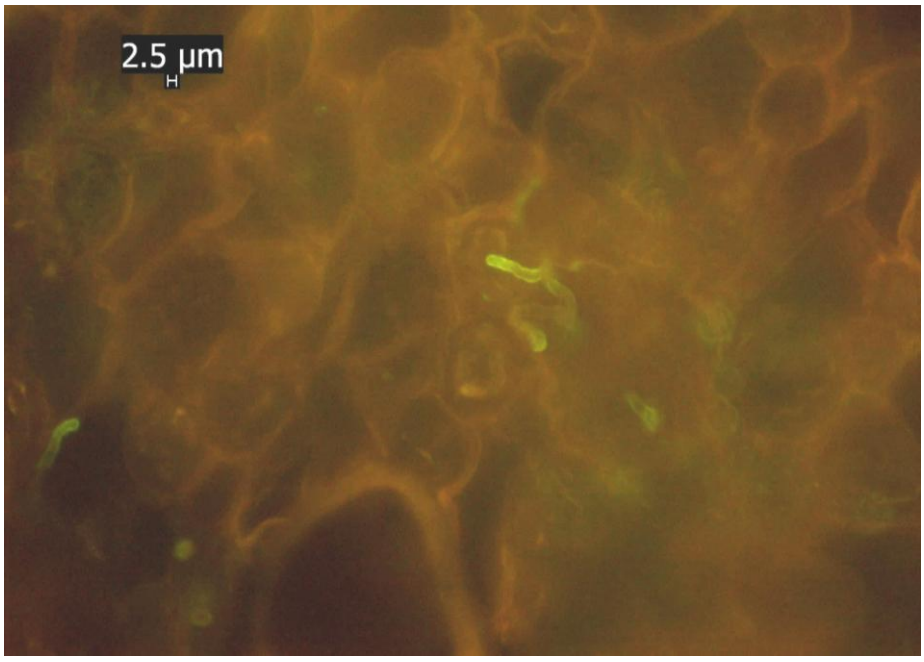


Figure 58. Elicitin production in hyphal cell wall: Fluorescence micrograph of a cross section of an elictin immunostained bark sample from a *P. ramorum* inoculated sprout cutting. Image taken with a filter having excitation at 450-490 nm and emission at 515 nm. Hyphae fluoresce green with elicitin immunostaining suggesting the production of elicitin. Hyphal tips can clearly be seen.

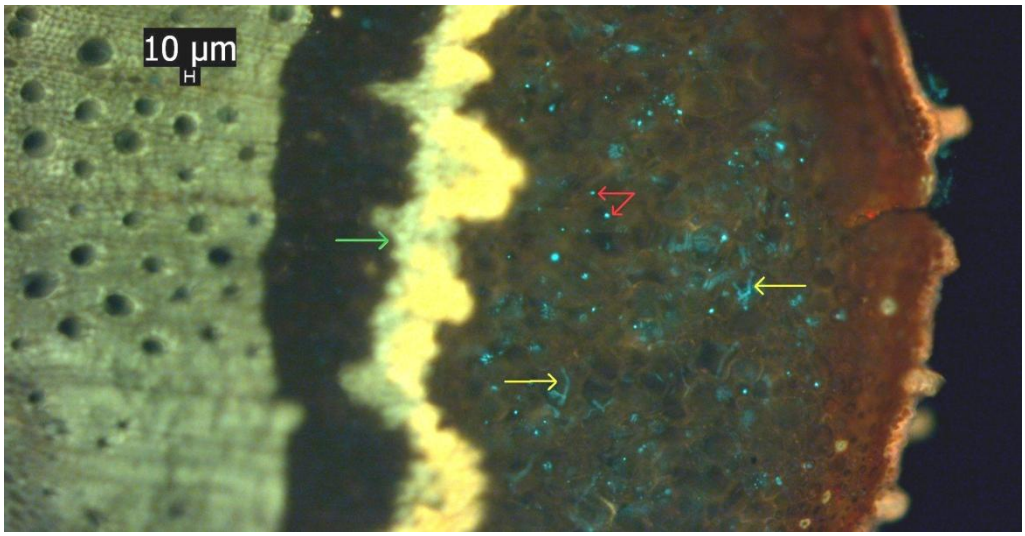


Figure 59. Elicitin immunostained hyphae: Fluorescence micrograph of a cross section of an elictin immunostained bark sample from a *P. ramorum* inoculated sprout cutting. Image taken with a filter having excitation at 450-490 nm and emission at 515 nm. Several hyphae are seen in the parenchyma and phelloderm of inner bark (yellow arrows) but are not seen inside the layer of fibers (green arrow) that separates the inner bark from innermost bark containing conducting phloem. Hyphae fluoresce green with elicitin immunostaining but appear blue here due to alteration in imaging software in order to maximize contrast. Fluorescence suggests the production of elicitin. Lots of green flecking (red arrows) is also present which is not regarded as being a positive immunofluorescence response.

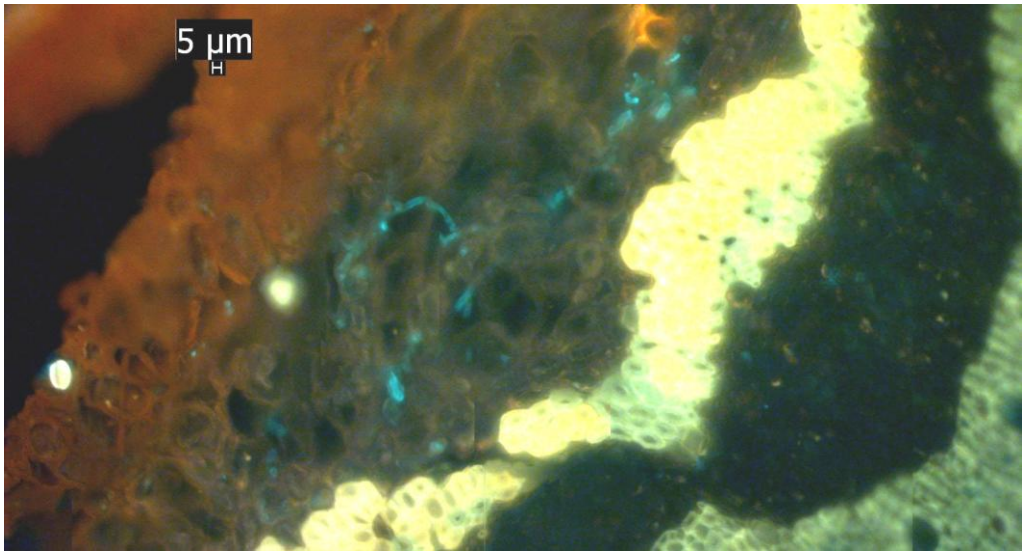


Figure 60. Elicitin immunostained hyphae 2: Fluorescence micrograph of a cross section of an elictin immunostained bark sample from a *P. ramorum* inoculated sprout cutting. Image taken with a filter having excitation at 450-490 nm and emission at 515 nm. Hyphae fluoresce green with elicitin immunostaining but appear blue here due to image alteration. Fluorescence suggests the production of elicitin. Many

hyphae are seen in the parenchyma and phelloderm of inner bark but are not seen inside the layer of sclereids that separates the inner bark from innermost bark containing conducting phloem.

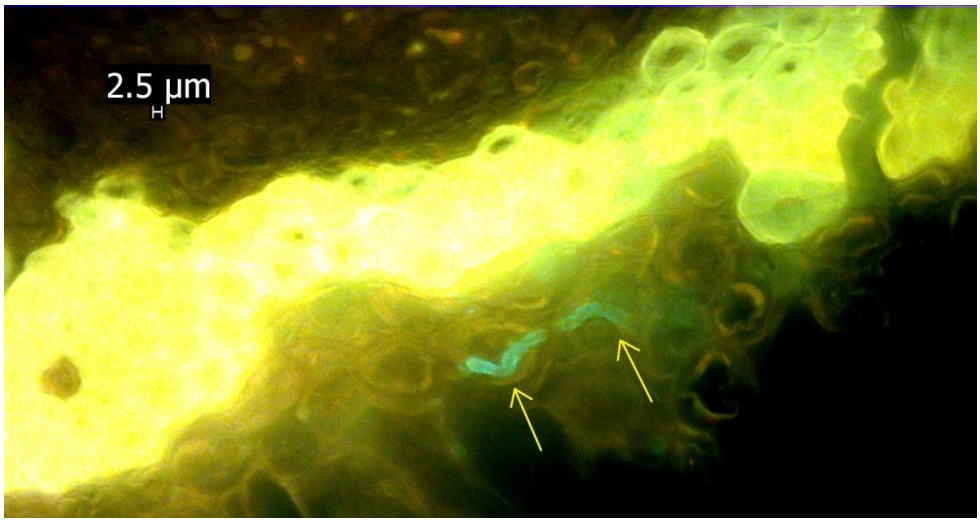


Figure 61. Elicitin stained hyphae growing cell to cell: Fluorescence micrograph of a cross section of an elictin immunostained bark sample from a *P. ramorum* inoculated sprout cutting. Image taken with a filter having excitation at 450-490 nm and emission at 515 nm. Hyphae fluoresce green with elicitin immunostaining, but appear bluish here due to image alteration. Fluorescence suggests production of elicitin. Hypha is seen moving from cell to cell (red arrows) and hyphal tip can clearly be seen.

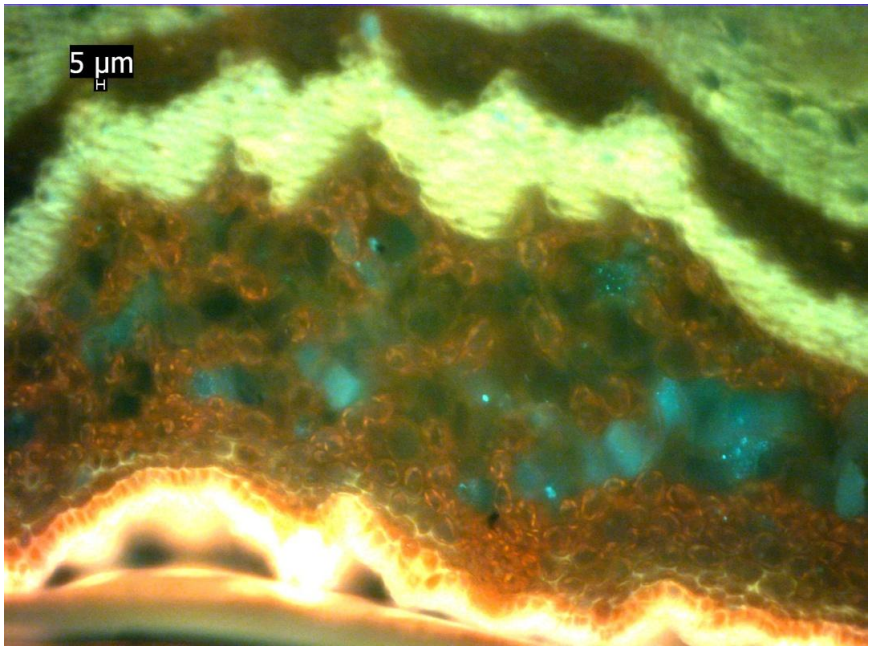


Figure 62. Fluorescence from elicitin immunostaining in control tissues: Fluorescence micrograph of a cross section of an elictin immunostained bark sample from an uninfected sprout cutting control. Image taken with a filter having excitation at 450-490 nm and emission at 515 nm. A general green staining as well as green flecking is seen, but appears blue here due to image alteration. Staining does not represent a successful immunofluorescence response.

4 Discussion

4.1 Colonization of *N. densiflorus* bark tissues

Calcofluor staining, aniline blue staining, scanning electron microscopy, and elicitin immunostaining allowed for observation of hyphal colonization of bark tissues, demonstrating that nearly all tissues are capable of being colonized. Hyphal diameter and overall appearance of most hyphae was usually consistent with that of *Phytophthora ramorum* in culture. Because samples were taken at lesion edges and the pathogen was recovered in culture from them, it seems that the likelihood that characteristic hyphae belonged to *P. ramorum*, is high. Hyphae grew primarily intracellularly, which is consistent with what was seen in the *P. ramorum*, *Rhododendron* interaction, as well as many other *Phytophthora* host interactions (Pogoda and Werres, 2004; Erwin and Bartnicki-Garcia, 1983; Keen and Yoshikawa, 1983; Klarman and Corbet, 1974).

Damage to plant cells in infected tissues was evident; with cell collapse, discoloration, and tissue disorganization in inoculated log and infected field samples, and with some discoloration and plasmolysis in inoculated sprout cutting samples. Inoculated log tissues were more damaged overall, so the difference between infected and control tissues in inoculation studies was sometimes not very dramatic. However, when comparing infected field samples to field controls, the difference in cell damage between the two was quite dramatic. Discoloration, cell collapse, and tissue disorganization were also reported in infected *Rhododendron* tissues, and were thought to occur in the order just listed (Pogoda and Werres, 2004). It was even suggested that cell damage could be due to a hypersensitive response (Pogoda and Werres, 2004). Collapse of cortical cells was also reported in the interaction of *P. lateralis* and *Chamaecyparis lawsoniana* (Oh and Hansen, 2007).

Hyphae were seen most frequently in the phelloderm and parenchyma of the outer part of inner bark, in *P. ramorum* infected tissues. Difficulty in distinguishing hyphae in other inner bark tissues, where plant cell walls fluoresced brightly from calcofluor staining, may have contributed to a perception that these tissues were more densely colonized. However, examination of samples with aniline blue staining, scanning

electron microscopy, and elicitin immunostaining supported these findings, showing that these are tissues in which hyphae are present in greatest densities. Calcofluor stained cellulose tissue and aniline blue likely stained β-1,3 glucans such as laminarins in hyphal cell walls. Both of these polysaccharides are known to be present in *Phytophthora* cell walls (Erwin and Bartnicki-Garcia, 1983). It is important to remember that in most inoculated log samples, a wound was made to the cambium so the pathogen should have had direct access to any tissues from the outer bark to the cambium. 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 and Hansen, 2007). This was markedly different from what was seen in resistant seedlings, where hyphae did not enter the vascular tissues (Oh and Hansen, 2007). Colonization of *Rhododendron* tissues by *P. ramorum* suggests colonization of pith, cortical parenchyma of the phloem, and xylem, followed by pith rays and phloem.

The most obvious reason that the phelloderm may have been preferentially colonized is that many of the cells in this region in uninfected, fresh samples appeared to contain chloroplasts, suggesting that these tissues perform photosynthesis. Carbohydrates from carbon sequestered during photosnythesis in these tissues could serve as a food source to *P. ramorum*. Chloroplasts are seen 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.

Another possible reason that parenchyma of the outer part of inner bark were more frequently and more densely colonized is that many parenchyma cells of the inner part of inner bark are smaller and walls of many cells are thicker than in the outer part of inner bark. So, it may be more difficult for the pathogen to get through this increased amount of cell wall material. So, this could mean that the pathogen "prefers" the tissues at the interface of inner and outer bark because of greater ease in getting through cell wall material and because, aside from conducting phloem, there may be more reward in terms of usable energy since cells are larger here. As mentioned in chapter one, parenchyma cells often serve as storage cells for various materials including starches and lipids. However, it may also be that all tissues are colonized at

the same time and that the pathogen takes longer to get through the inner part of inner bark and/or that the pathogen is able to sustain itself in those tissues for longer because of the abundance of carbohydrates and nutrients present in the conducting phloem and cambium. Furthermore, because materials move through these tissues through long conduits, the pathogen may not have to move as far through them. Some inoculated logs were collected in the winter when phloem tissues may be dormant, but field samples were collected in spring and other inoculated logs were collected in the summer when phloem tissues should be actively conducting.

Results of the first log inoculation study show that the pathogen was colonizing the bark and the wood around almost every inoculation point, but microscopy samples were taken from bark lesion edges to capture the leading edge of pathogen infection and minimize the frequency of colonization by saprophytic fungi. We can recall, also, that usually bark lesions extended further than sapwood discoloration. So, perhaps this is also the pattern that is seen with colonization in the inner parts of inner bark and cambium so that if samples had been taken closer to inoculation points, colonization of conducting phloem and cambium tissues may have been encountered more frequently. The sample in which the cambium and adjacent inner phloem and outer xylem were heavily colonized, exhibited as high a density of hyphae as was seen in any sample, which demonstrates that the pathogen can be very successful in the inner bark. Therefore, although heavy colonization was only seen in these tissues once, it would probably not be accurate to draw the conclusion that these tissues are not preferentially colonized by *P. ramorum*.

N. densiflorus sprout cuttings were never colonized very densely, but observation with SEM, calcofluor, and with elicitin immunofluorescence in these samples, showed a pattern of colonization similar to that seen in log inoculations, with the highest frequency of hyphae in the parenchyma of the outer part of inner bark. This parenchyma is probably largely cortical parenchyma, if not all cortical parenchyma, and it would be interesting to know what portion of bark samples from larger materials are cortical parenchyma. Any cortical parenchyma that existed in older bark tissues (several years) should be only present in the outermost part of the inner bark. It is possible that cortical parenchyma, arising from the meristem rather than the

vascular cambium, are physiologically different from parenchyma that arise from the vascular cambium, and these differences make them more attractive to *P. ramorum*. The only other tissues in which hyphae were seen in inoculated sprout cutting samples were the outer bark and xylem, and colonization of the conducting phloem and cambium was never observed. Observation of more infected *N. densiflorus* samples, particularly more samples closer to the initial point of infection, is necessary to make a well-informed statement about which tissues are preferentially colonized in this particular species interaction.

The one inoculated log sample in which hyphae in the inner part of inner bark were seen most frequently in the ray parenchyma and were traveling primarily in the radial direction gives some suggestion that the pathogen may use rays to move from phloem to xylem. Again, these 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. This may be a preferred conduit for the pathogen to get to xylem, and would be consistent with the theory just described regarding the greater density of hyphae in the outer part of inner bark. Although colonization of xylem tissues was not investigated here, hyphae were seen in rays in outer xylem in one sample also, again traveling primarily in the radial direction; and hyphae were seen in vessel elements in several places. Furthermore, histological examination of *N. densiflorus* xylem tissue and *Rhododendron* phloem and xylem tissues infected with *P. ramorum*, suggest that ray parenchyma likely serve as such a conduit (Parke et al, 2007; Pogoda and Werres, 2004).

It is worth noting that the sample in which hyphae were seen inside of a sieve tube element was a sample stained with aniline blue. There were not many samples for which this technique was applied but aniline blue can stain cellulose, but is also known for staining β-1,3 glucans such as callose and laminarins (Ruzin, 2001; YuanTih and YuLing 2004). *Phytophthora* species do possess β-1,3 glucans including laminarins which cross-link cellulose in their cell walls (Erwin and Bartnicki-Garcia, 1983) It did not appear that cellulose did stain in these tissues as plant cell walls, even in the very inner bark, did not stain. However, sieve areas, which contain callose, were stained, so it is more likely that this formulation of aniline

blue stained β-1,3 glucans. Perhaps cell walls stained too brightly in calcofluor stained tissues for hyphae to ever have been seen in sieve tube elements elsewhere. Further application of this staining technique may help to elucidate the uncertainty regarding the frequency with which these cells and tissues are colonized. Most *Phytophthora* species that cause stem cankers preferentially colonize the cambium and often the neighboring phloem and/or xylem tissues (Scott, 2009; Davison et al., 1994; Tippett et al., 1983; Robin, 1992). Histological studies on *P. ramorum* infected *Rhododendron* twigs suggest, however, that the xylem is first colonized followed by cambium and phloem tissues (Pogoda and Werres, 2004) however, this may have been an artifact of the inoculation method. Tips of *Rhododendron* stems in this study were removed and cultures of the pathogen were placed on the top of the cut stem. This gave the pathogen direct access to all tissues so the pattern of colonization here would not necessarily represent what would be seen in a natural infection situation. It seems likely thus far, that the pattern of colonization by *P. ramorum* in *N. densiflorus* is likely different from that seen in *Rhododendron* and may be unique compared to colonization patterns of most *Phytophthora* species in vascular tissues.

Inoculated log samples, infected field samples, and sprout cutting samples were all useful for understanding how *P. ramorum* enters the bark of a *N. densiflorus* tree. We saw, in both inoculated log and infected field samples, numerous examples of characteristic hyphae either entering or growing out of lenticels. More commonly, hyphae not charactistic of *P. ramorum,* that either stained blue rather than bluish white with aniline blue, had crosswalls, or had consistently small diameters, were seen densely colonizing lenticels and the areas around them. *P. ramorum* hyphal penetration of *N. densiflorus* lenticels is supported by the work of Florance (2003) though the only justification that they were *P. ramorum* hyphae was that they came from *P. ramorum* infected tissue. It is not suggested from the research reported here that lenticels are a preferential means of entry for *P. ramorum*; a larger number of examples with clear evidence that the pathogen is going into rather than coming out of lenticels is necessary before any conclusions can be drawn regarding this subject. Preferential entry into *Picea abies* (Norway Spruce) bark tissues through lenticels for the bark beetle species *Pityogenes chalcographus* has been reported (Rosner and Führer, 2002). Possible reasons for this preference are discussed and include the

possibility that volatiles may be more easily detected over lenticels, cells are more loosely arranged with largely intercellular spaces making tissues easier to colonize, and that there are fewer resin canals in the tissues behind lenticels. Intercellular spaces behind lenticels were not measured but may be an area worth further investigation. It is not likely that *P. ramorum* would be attracted to volatiles as these would either serve as species identification, and the two are not coevolved, or as an indicator of tree stress which is not thought to be sought by *P. ramorum.* It has, in fact, been suggested that tree vigor is positively correlated with likelihood of *P. ramorum* infection (Swiecki and Bernhardt, 2006), though this may have more to do with a dominant canopy position and the likelihood of spores landing on these trees. What appeared to be direct penetration of bark surfaces and sprout stem surfaces was also seen in several places. However, further work to better track hyphae after they appear to enter *N. densiflorus* bark is recommended. Direct penetration of epidermal cells has also been seen by the closest relative of *P. ramorum*, *P. lateralis,* during infection of roots of *Chamaecyparis lawsoniana* as well as in the interaction of *P. infestans* and potato on leaf surfaces (Oh and Hansen, 2007; Coffey and Wilson, 1983).

Related to the subject of host penetration, sporangia characteristic of *P. ramorum* were seen in a bark crevice of an infected field sample with hyphae growing nearby. This is a significant finding because of only eleven infected field samples from five trees, each sample smaller than 8cm on any side, with probably no more than 3 x 3 cm of actual tissue observed from all of those samples combined, seven sporangia were encountered. It seems unlikely that sporangia would be encountered if they weren't present in considerable densities on bark. Furthermore, unless sporangia were in a protected area like a bark crevice, it seems likely that most would be removed from tissues due to either rain in the field, the EtOH solution samples were placed in after collection, or the process of embedding samples in PEG.

In addition to sporangia, zoospores were seen on bark surfaces, not only in inoculated sprout cutting samples, where we might expect to see many of them, but also on the surface and in bark crevices of infected field samples. In one place, a hypha seemed to originate from the site of a zoospore though direct germination, again, was not seen.

These may not have been zoospores, but their morphological similarity to what were believed to be zoospores in inoculated sprout cutting samples, and the size similarity of all of these to what is known for *P. ramorum* zoospores, gives some support to the possibility that they were. If zoospores were present, they would have to have been produced from sporangia in the field. Zoospore production is typical for Phytophthora root pathogens, but not for *Phytophthora* species affecting tissues above ground. If the presence of zoospores could be confirmed, it would be very interesting and important to consider in the context of understanding how this disease is spread in the field.

Observation of sprout cuttings with the fluorescent antibody label for elicitins attached, illuminated hyphae that had diameters and overall appearance consistent with those of *P. ramorum* and with what was seen with calcofluor staining and SEM. However, further application of this method to other *Phytophthora* and *Pythium* species is recommended to determine the specificity of this antibody to *Phytophthora ramorum* elicitins. As mentioned before, hyphae in these samples were only observed in the parenchyma cells of inner bark tissues; cells of the outer bark, which was only one or two cell layers thick at this growth stage; and once in a xylem vessel. Xylem tissues were not scrutinized to the extent that bark tissues were in these samples but they were usually observed in every sample, so that if they had been more heavily colonized than phloem tissues, this would have been detected. These samples were taken at or beyond lesion margin edges so, the possibility still holds that other tissues were infected but that the pathogen did not grow as rapidly in these tissues and, therefore, was not present in them at the leading edge of infection.

Microscopic observation of *Phytophthora gonapodyides* infected *N. densiflorus* bark tissues yielded certain results that were not seen in *P. ramorum* infected bark tissues. This was the only time when multiple hyphae were seen colonizing a single cell. It is possible that these were not *P. gonapodyides* hyphae as most were in the lower range of the correct diameter, most 2μm or less, and it seems more likely that a saprophyte would demonstrate this behavior rather than a facultative saprophyte, like most *Phytophthora* species, that usually requires living cells for survival. It does not seem likely that a cell colonized by multiple hyphae would be capable of remaining alive

for very long. If these were, in fact *P. gonapodyides* hyphae, it may be that this organism expresses stronger saprophytic behavior than *P. ramorum*. It is believed to have coevolved with *N. densiflorus* so, the host may detect its presence more easily than it does *P. ramorum*, and *P. gonapodyides* may then have to be better adapted to killing and then consuming bark cells so that the host does not kill it first.

Because hyphae characteristic of *Phytophthora* were also seen in and around lenticels in *P. gonapodyides* infected tissues, it is possible that this species may use lenticels to enter the tree. Furthermore, this pathogen was not seen directly penetrating bark surfaces so a mode of entry through lenticels may, in fact, be important for this pathogen. The possibility that volatiles may be used by *P. gonapodyides* as attractants to lenticels, as they were for the bark beetle *P. chalcographus*, would also be an interesting subject to investigate in trying to understand whether or not this species is more tolerant of dead or dying tissues than *P. ramorum*.

Another unique observation that was made in *P. gonapodyides* infected tissues was in one sample where, in a couple of places, there was a lack of plant cell material where multiple hyphae were present. The arrangement of surrounding cells did not suggest that the tissue had separated from itself but, rather, that the hyphae had consumed the plant tissue in these areas. Brown and Brasier, 2007, refer to the presence of "lagoons" in *Phytophthora* infected bark tissues of *Fagus* and *Quercus* species, referring to canals that often extended into the wood and were commonly filled with a clear, pale pink, or orange liquid that turned red to brown upon exposure to air. These lagoons commonly existed behind bleed spots that were seen on the surface. No such canals were identified in *N. densiflorus* bark during log sectioning or in sample observation, but they were also never specifically looked for. Because they were not identified and because multiple hyphae were seen in them, it seems more likely that they were formed by hyphal consumption of plant tissue.

4.2 Host Responses to Infection

Callose was successfully stained in both uninfected and infected tissues by calcofluor in sieve areas of sieve tube elements. 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, but 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 defense 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). In infected samples, because small depositions of the material were seen, we believe it is likely that callose was deposited in plasmodesmata around the walls of these parenchyma cells. When the depositions were viewed very closely, they could usually all be brought into the same plane of focus within a single cell, which supports this hypothesis as they would all be more or less in the same plane on a cell wall in a cross section or longitudinal section. The reason for the presence of callose in pits of parenchyma cells of field controls is not clear because these samples were placed directly in EtOH and glycerol solution and cells should not have had any time to produce this response from wounding. It may be that small amounts of callose in plasmodesmata of bark parenchyma cells are normal. Although only three sprout cutting samples were viewed with calcofluor staining, a callose response was not seen in any of them. It may be that because these samples were only infected for two weeks and the sample was taken from the leading edge of infection, they had not yet produced a callose response. However, callose deposition can occur within minutes and is regarded as one of the first defense responses that can occur. So, it may also be that the young stems of *N. densiflorus* are not physiologically equipped to produce this response at their young age. Indeed, since these young sprouts should be actively growing, it may be that little energy is allocated to defense at their young age.

Another defense response which was seen in all sample types was the production of tannins. Cells within the discolored region of a lesion consistently stained positively for tannins by the Vanillin HCl method. It, in fact, seems likely that the discoloration in these regions may have been due to the production of tannins. Discoloration before staining simply appears brown under normal light but appears a red-brown to brown under UV light. Cells in discolored regions often appeared misshapen, some cells were filled or partially filled with small globules that were this same red-brown to

brown color under UV light, and walls of cells did not fluoresce a whitish color under UV light as cell walls in non-discolored regions or control tissues did. After staining, all cell material in the discolored region turned a bright red under normal light and under UV light, some dark red material could be seen in patches throughout the discolored tissue region. The rest of the contents remained the red-brown to brown color under UV light that they were before staining. In one infected field sample, tissues within the lesion margin did not appear red-brown to brown and before staining, cells contained large globules that appeared light brown in color under UV light. These cells were not misshapen and cell walls retained a whitish fluorescence under UV light. After staining, the globules appeared a bright red color under UV light and under normal light. It is interesting that, in most samples, only some of the material that stained positively for tannins under normal light, also showed a color change under UV light. This may suggest that there are different kinds of tannins in these tissues with different extinction coefficients so that some of them fluoresce under UV light and others do not. Because hyphae were seen in high densities in these discolored tissues that consistently stained positively for tannins, it would not seem that these compounds are very effective deterrants of *P. ramorum.*

The periderm seen in one sample, possessing thickened cell walls that fluoresced white with UV fluorescence but which were not thickened to the extent of walls in cells of the cork, was thought to be a wound periderm. It did not have the appearance of a normal, mature outer periderm nor of a secondary periderm as the cells that seemed to have arisen from the meristematic layer were not small and highly oblong like those in normal cork and the periderm was arranged more or less perpendicular to the bark surface. The "webbing" that was present may have been either material reinforcing the primary cell wall or else deposition of secondary cell wall material, made up of lignin. In either case, this would suggest that this periderm was actively differentiating and because secondary periderms were never seen in a tree this small, a 10cm DBH tree, and it was with *P. ramorum* actively growing in the immediate area, it is highly likely that this was a wound periderm. The fact that wound periderms were not seen in other samples may suggest that this response can only be produced in a living tree. However, it may be that they were produced in other samples, but were not captured where samples were taken. Physiological factors that contribute to

overall tree health as well as tree age may also play a part in whether or not wound periderm can be produced. It is unclear whether the pathogen was colonizing tissues on both sides of this wound periderm as this sample was not stained with calcofluor before it was discarded and the wound periderm was not found again when going back to the bark piece from which it came, which may also indicate that the periderm was not fully developed. Although the occurrence of hyphal colonization of tissues around the periderm was unknown, it is clear that this is not a defense response capable of effectively stopping the pathogen from killing a tree.

Results from NBT staining for superoxides as an indication of a hypersensitive response suggest that *P. ramorum* does not induce this response in *N. densiflorus*. Positive controls from tobacco leaves challenged with *P. cryptogea* and *P. infestans* suggest that the protocol used for staining was effective. Previous work by Manter et al (2007) suggests that *N. densiflorus* may produce a hypersensitive response after exposure to elicitins from *P. ramorum*. It may be that this is, in fact, the case, and that a response was not seen with challenge by the pathogen because elicitins were not present in high enough quantities for a visible response to be detected. Application of purified elicitins to *N. densiflorus* tissues followed by periodic staining for NBT may afford these results. Other possibilities are that a hypersensitive response was produced but disappeared during one of the two hour time intervals between sampling. It may also be that a hypersensitive response was not yet produced during the first 12 hours after pathogen introduction and that it is produced later, between the 12 and 24 hour sampling times or after the 24 hour sampling time. However, most studies involving detection of a hypersensitive response find that it occurs within the first 12 hours (Morel and Dangl, 1997; Levine et al, 1996; Bozsó et al, 2005; Venisse et al, 2001).

4.3 Elicitin Production

As was already discussed, elicitin immunofluorecent labeling stained hyphae contributing to histological examination of *P. ramorum* in *N. densiflorus* bark tissues. More specifically, though, hyphal cell walls were illuminated by immunostaining. This suggests that elicitins are localized in cell walls of *P. ramorum*, which is

consistent with findings of Meijer et al (2007) showing that at least one *P. ramorum* elicitin is associated with the cell wall. Another finding that may have importance in concurrence with this, is that a large fraction of hyphae that were seen with elicitin immunostaining were hyphal tips. This was a difficult finding to assess and quantify, but it is estimated that at least 40% of hyphae that were seen were hyphal tips, based on observation of over 100 hyphae. Although these findings do not necessarily suggest 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 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 *Q. robur* roots (Brummer et al, 2002). Systemic movement of elicitins was suspected to contribute to early declines in photosynthesis in the research of Manter et al (2007). 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. The presence of these proteins in cell walls suggests that they either play a role in cell wall function or are secreted for some reason. The reason it was difficult to quantify and assess the fraction of hyphae that were seen were hyphal tips was mostly due to difficulty in clarifying whether or not something was a hyphal tip. Also, there was never a hypha that could be seen continuously for more than distance of 25 μm so it is unclear how far back from a hyphal tip elicitin immunofluorescence was seen. Additionally, samples were taken from lesion edges so we might expect to see a lot of hyphal tips. I do feel confident in saying, however, that a much higher proportion of hyphal tips were seen by this method than by calcofluor staining or SEM.

The fact that hyphae were illuminated by this method only in inoculated sprout cuttings is interesting. The pathogen isolates used in the first sprout cutting inoculation study, which included some successfully stained samples, were also used in log inoculation studies, so unsuccessful immunostaining should not be attributed to differences among isolates. A total of 24 sprout cutting samples and 12 inoculated log and infected field samples were treated and observed by this procedure. So, in

addition to the failure with inoculated log and infected field samples, 16 sprout cutting samples were also not successfully stained.

Samples that were successfully stained on one day were sometimes not successful on another day or did not fluoresce as brightly and, when several samples were labeled at the same time, being subjected to all of the same conditions, some would be successfully stained and some would not. It was determined that when more primary antibody solution was added to wells, hyphal fluorescence was brighter, in those samples that were successfully stained. However, it was obvious that there were other undetermined factors involved in the variability in success. If it is the case that elicitins are only produced at the growing tips of hyphae, it may be that where sections were taken within samples was often not exactly at the leading edge of pathogen growth. Since all inoculated log samples and presumably all infected field samples had been infected for at least six weeks, it may also be that elicitins are only produced during the early stages of infection so that there is some length of time after which they are no longer produced. If this is the case, it would also make sense in the context of these proteins having a role in pathogenicity.

4.4 Concluding Remarks

By merging pieces of this research together, we can understand *N. densiflorus* infection by *P. ramorum* in a new way. Heavy colonization of bark tissues suggest that bark infection in the interaction of *P. ramorum* and *N. densiflorus* is likely an important contributor to tree death. We already know that this pathogen is capable of overcoming this host, but we now see that bark is likely an important part of this process. However, we also found here that the host reacts to infection in several ways. It appears that the pathogen can enter host tissues through lenticels and bark crevices and can also directly penetrate and colonize cork tissues, although more work is recommended to determine whether lenticel entry is preferable. Once inside the bark, the pathogen appears to colonize phelloderm and parenchyma of the inner bark most densely. This may be for reasons of preference or it may be that colonization of conducting phloem and cambium were seen infrequently due to sampling technique. Inner bark tissues were seen colonized, including the colonization of a sieve element.

It may be that calcofluor staining, a method by which many samples were stained, is not optimal due to the bright fluorescence of plant cell walls that occurs. Aniline blue staining may be a more useful stain, particularly in these tissues.

A wide variety of host responses to infection appeared to occur, including the production of tannins, production of callose, and formation of wound periderm. There is a little evidence that suggests production of phenolic compounds may deter the pathogen and help confine it to intercellular spaces, but certainly do not deter them completely. Additional research in this area is necessary to understand how these compounds affect the pathogen. Callose production as a response to infection was seen with small depositions of callose in cells in and around infected areas and may also have been seen as a thickening of crosswalls in one sample. Wound periderm formation was seen in one sample, but it is unclear how this affected the pathogen. Since it is clear that none of these defense responses are capable of stopping the pathogen, they may contribute to tree death by using energy and making it weaker. Specific recognition of *P. ramorum* by *N. densiflorus* causing a hypersensitive response does not appear to occur in this interaction. This is not surprising considering that the two species are not coevolved, but further research is recommended to determine whether there may still be a partial hypersensitive response that has gone undetected in these studies.

Elicitin immunostaining yielded unexpected results that were more useful for looking at bark colonization than understanding the role of these proteins in pathogenicity. However, the large fraction of hyphal tips that were seen, the fact that elicitins appeared to be localized in cell walls, and the fact that elicitin immunofluorescence was only seen in samples that had been infected for two weeks, all suggest that they may have some importance for pathogenicity. This research has uncovered much information regarding *P. ramorum* infection of *N. densiflorus* and interactions between the two species, but it is clear that more research is necessary to draw conclusions in many of the areas investigated in this research. The author hopes this research will contribute to a growing knowledge base toward understanding how to manage invasive forest pathogens and lay the groundwork for future studies in this or related areas.

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