

## AN ABSTRACT OF THE THESIS OF

Carrie Lewis for the degree of Master of Science in Botany and Plant Pathology  
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Title: Pathways of Infection of *Phytophthora ramorum* in Rhododendron.

Abstract approved: **Redacted for privacy**

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Jennifer L. Parke

*Phytophthora ramorum*, a plant pathogen, is the cause of sudden oak death and ramorum blight and shoot die-back. It has a wide host range including many native forest species and common nursery plants. The lack of knowledge regarding infection biology of *P. ramorum* limits our understanding of its ecology and epidemiology. Pathways of infection were investigated in *Rhododendron* 'Nova Zembla' using tissue culture plantlets and greenhouse-grown container plants planted in artificially-infested potting medium, or inoculated with a zoospore suspension or mycelial plugs. The presence of the pathogen in plant tissue was determined by isolation onto selective medium and PCR analysis. Histological examinations of tissue samples were performed with fluorescence, scanning electron and scanning laser confocal microscopy. Inoculated roots, stems and leaves were examined to identify pathways by which *P. ramorum* infects and colonizes rhododendrons. Results indicate that roots can be infected by *P. ramorum* without causing root rot. Below-ground infections arising from artificially infested potting medium resulted in infection of above-ground stems and leaf petioles. *P. ramorum* was found in the primary xylem of below-ground and above-ground stem tissue. Inoculation of roots with zoospore inoculum resulted in inter- and intracellular penetration of root tissue. Cysts appeared to aggregate at wound sites and near root primordia. Examination of inoculated leaves revealed that *P.*

*ramorum* does not require stomata for leaf infection. *P. ramorum* spread from inoculated leaves into petioles and stems via the vascular tissue (primary xylem).

These results indicate that *P. ramorum* may be present but not cause obvious symptoms in certain plant tissues. This may contribute to difficulties in detection of infected plants, a requirement for limiting the long-distance spread of the disease with infested nursery stock.

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Pathways of Infection of *Phytophthora ramorum* in Rhododendron

by  
Carrie Lewis

A THESIS

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degree of

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Master of Science thesis of Carrie Lewis presented on September 9, 2005.

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Dean of the Graduate School

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Carrie Lewis, Author

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Pathways of Infection and Colonization by *Phytophthora ramorum* in  
Rhododendron

## Chapter 1

### General Introduction

*Phytophthora ramorum* is a recently discovered plant pathogen first observed in Europe in 1993 causing a twig blight on *Rhododendron* and *Viburnum*. It was eventually described as a new species of *Phytophthora* (Werres et al. 2001). Meanwhile, in the early to mid-1990's, large numbers of dead and dying *Lithocarpus densiflorus* (tanoak), coast live oak, canyon live oak and black oak were observed in the San Francisco Bay Area of California. In 2000, researchers at UC Davis attributed the cause of the tree disease, popularly known as Sudden Oak Death, to an unknown *Phytophthora* species (Rizzo et al. 2002). Clive Brasier of the UK Forestry Commission recognized that the Californian *Phytophthora* was morphologically identical to the new European *Phytophthora*, and DNA tests confirmed that they were indeed the same species. A few months later *P. ramorum* was isolated from rhododendron container plants in a California nursery, followed soon after by Oregon's first report of an outbreak in forests in southern Oregon. By 2004 *P. ramorum* had been detected in horticultural nurseries in California, Oregon, Washington, and British Columbia, leading to shipments of infected nursery stock to 21 states and British Columbia. Currently *P. ramorum* is established in the wild in 14 counties in California and in one county in Oregon (COMTF).

The host range for *P. ramorum* is broad and diverse, distinguishing it from most other *Phytophthora* species, which usually have more limited host ranges (Erwin et al. 1983; Erwin and Ribeiro 1996). The host list for *P. ramorum* includes trees, shrubs, and herbaceous species representing native forest vegetation and horticultural crops. Susceptible forest species include members of Fagaceae, Ericaceae, some conifers including Douglas-fir, redwood, grand fir and yew, while common woody ornamental nursery crops are *Rhododendron*, *Viburnum*, *Pieris*, *Kalmia*, and *Syringa*. Both this pathogen and the host species it naturally infects are subject to state, federal and international quarantine (ODA 2003; USDA-

APHIS 2005). There are currently 38 "officially proven" hosts and 37 "officially associated" hosts. Proven hosts are hosts for which Koch's postulates have been completed. Associated hosts are species that have been found to be naturally infected with *P. ramorum* and for which Koch's postulates have not yet been completed (USDA-APHIS 2005); these species are regulated only as nursery stock. This list continues to grow as new species infected with *P. ramorum* are discovered.

Disease symptoms associated with *P. ramorum* are also very diverse and they vary depending on the host species. The three distinct sets of symptoms associated with *P. ramorum* have been called "Sudden Oak Death", "ramorum leaf blight", and "ramorum dieback" (Davidson et al. 2003; Hansen et al. 2002; Hansen et al. 2005). The disease symptoms characteristic of "Sudden Oak Death" result from lethal bole cankers in the bark, cambium, and outer xylem that expand and girdle the stem and kill the tree. These cankers often ooze and bleed. Tanoaks and certain oaks in the red oak subgenus exhibit these symptoms (Rizzo et al. 2002). Disease symptoms characteristic of "ramorum blight" are foliar blighting and shoot dieback typical of symptoms exhibited by many non-oak host species. These symptoms are less severe than cankers and include leaf spots and blotches. In extreme cases, juvenile and mature plants with ramorum blight symptoms can be killed. Additionally, some species can exhibit both sets of symptoms, for example, *P. ramorum* can produce foliar symptoms as well as lethal bole cankers on tanoaks (Davidson et al. 2003; Rizzo et al. 2002).

*Phytophthora ramorum* is classified as an Oomycete belonging to a major line, or kingdom of eukaryotes called Stramenopiles. It is not considered a true fungus; it is more closely related to brown algae. *P. ramorum* produces several asexual reproductive structures important for pathogen spread and survival, including sporangia, zoospores, and chlamydospores. Sporangia produce zoospores, bi-flagellate spores which in many other *Phytophthora* diseases are considered the main infective propagule, capable of moving to other plants through water. Chlamydospores are thick-walled resting structures that may contribute to survival

during periods of extreme temperatures or other adverse conditions (Erwin et al. 1983; Erwin and Ribeiro 1996). Chlamydospores of *P. ramorum* in plant tissue survive several months in infested leaves (Fichtner et al. 2005; McLaughlin et al. 2005; Shishkoff and Tooley 2004). Both sporangia and chlamydospores can be produced on (or in) leaf surfaces and may be dispersed through windblown rain, stream water, irrigation water, contaminated soil, infested potting media, and in infected plant debris (Colburn et al. 2005; Davidson et al. 2005; Fichtner et al. 2005; Jeffers 2005; Linderman and Davis 2005; Shishkoff and Tooley 2004).

*Phytophthora ramorum* is heterothallic and requires two different mating types for sexual reproduction (Erwin et al. 1983). Mating type A1 predominates in Europe while mating type A2 predominates in North America. However, the A1 mating type has been detected in several nurseries in Oregon, Washington and British Columbia (Hansen et al. 2003), and an A2 isolate was discovered in Belgium (Werres and De Merlier 2003). Different molecular techniques have also shown that two distinct populations exist (Bonants et al. 2005). These findings suggest that *P. ramorum* may be an exotic species new to N. America and Europe having been introduced to each continent separately (Ivors et al. 2003). Because of geographic separation of the two mating types, sexual reproduction (oospore production) within this organism has only been observed under laboratory conditions. There is concern, however, that crossing of the two mating types A1 and A2 might create new, potentially more virulent or more competitive offspring since A1 and A2 differ in virulence and symptom expression (Hansen et al. 2003).

*Phytophthora ramorum* is a species with a wide temperature range (Davidson et al. 2002; Davidson et al. 2005), reaching optimal growth at 20°C (Werres et al. 2001). Moisture is also important for survival, spread, and infection of *P. ramorum* as evidenced by natural infections of forests limited to coastal "fog belts" of California and Oregon where moisture from the ocean and winter rainfalls are high.

Any new discovery or detection of *P. ramorum* is subject to state and federal regulations and quarantines. Efforts to control *P. ramorum* include treatment with

fungicides, quarantine and destruction of infected nursery stock, prevention of shipments of *P. ramorum* host stock outside of “infested” counties, and cutting, slashing and burning of infested areas of forests. To date these efforts have not completely eliminated new forest infections or shipments of infected plants (Garbelotto et al. 2002; Goheen et al. 2002; ODA 2003; Rizzo and Garbelotto 2003; Rizzo et al. 2005). This can be attributed, in part, to our incomplete understanding of the life cycle and biology of this formidable pathogen.

Local spread of *P. ramorum* commonly occurs from movement of infected plant material, dispersal by rain and irrigation water, and possibly human activities (Kaminski et al. 2005; Kelly and Meentemeyer 2002; Tjosvold et al. 2002).

*Phytophthora ramorum* has been dispersed over long distances through shipments of infected nursery stock. Additionally, research is currently underway to determine the survivability of *P. ramorum* in the sapwood of infected trees, implying that infected logs, firewood, and woodchips stored under moist conditions might serve as reservoirs of inoculum (J. Parke, personal communication). Long-distance spread resulting from shipment of infected nursery stock is likely to be the greatest threat to native forests across the country where susceptible tree hosts are naturally distributed. Among the susceptible tree hosts are red oaks, including the northern red oak (*Quercus rubra*) and southern red oak (*Q. falcata*), both dominant species in the eastern mixed deciduous forests of North America. *P. ramorum* continues to expand its geographic and host range, and the potential for the establishment of new infections to areas outside the current range is high as the risk of long-distance transmission from infected nursery crops is a distinct possibility (Davidson et al. 2005).

The pathways of infection for *P. ramorum* are not well understood. *P. ramorum* is reported to be a pathogen of aerial plant parts only (Davidson et al. 2005; Rizzo 2003). Bleeding cankers on Fagaceae are believed to result from infection through the bark from zoospores or sporangia. Cankers do not appear to originate beneath the soil line, nor have root infections been observed on any species. *P. ramorum*

propagules do survive in soil and in potting media, however (Colburn et al. 2005; Davidson et al. 2005; Fichtner et al. 2005; Jeffers 2005; Linderman and Davis 2005; Shishkoff and Tooley 2004). Part of my research addresses the possibility that *P. ramorum*-infested potting media can lead to disease through root infection of container-grown rhododendrons. Another aspect of my research concerns the anatomy of infection and means of pathogen spread from inoculated leaves into stems. Understanding the pathways of infection and manner of spread within plants is crucial for controlling the disease and for detecting the pathogen in nurseries.

## Literature Cited

- Bonants, P., Verstappen, E., Weijacha, K., de Vries, I., and Ivors, K. 2005. Molecular identification and detection of *Phytophthora ramorum*. In: Proc. Sudden Oak Death Sci. Symp., 2nd, P.J. Shea and M. Haverty, (eds). Pacific Southwest Res. Stn., For. Serv., USDA. Albany, CA. (In press).
- Colburn, G.C., Sechler, K., and Shishkoff, N. 2005. Survivability and pathogenicity of *Phytophthora ramorum* chlamydospores in soil. *Phytopathology* 95:S20.
- COMTF. 2005. A disease chronology of *Phytophthora ramorum*, cause of sudden oak death and other foliar diseases. <http://www.suddenoakdeath.org/>.
- Davidson, J.M., Rizzo, D.M., Garbelotto, M., Tjosvold, S., and Slaughter, G.W. 2002. *Phytophthora ramorum* and sudden oak death in California: IV. Transmission and survival. Pages 741-749 In: Proc. Fifth Symp. Oak Woodlands: Oak Woodlands in California's Changing Landscape R.B. Standiford, D. McCreary and K.L. Purcell, (eds). Gen. Tech. Rep. PSW-GTR-184. Pacific Southwest Res. Stn., For. Serv., USDA, Albany, CA.
- Davidson, J.M., Werres, S., Garbelotto, M., Hansen, E.M., and Rizzo, D.M. 2003. Sudden oak death and associated diseases caused by *Phytophthora ramorum*. *Plant Health Progress*. doi:10.1094/PHP-2003-0707-01-DG.
- Davidson, J.M., Wickland, A.C., Patterson, H.A., Falk, K., and Rizzo, D.M. 2005. Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. *Phytopathology* 95:587-596.
- Erwin, D.C., Bartnicki-Garcia, S., and Tsao, P.H. 1983. *Phytophthora: its Biology, Taxonomy, Ecology, and Pathology*. APS Press, St. Paul, MN.
- Erwin, D.C., and Ribeiro, O.K. 1996. *Phytophthora Diseases Worldwide*. APS Press, St. Paul, MN.
- Fichtner, E.S., Lynch, S., and Rizzo, D.M. 2005. Summer survival of *Phytophthora ramorum* in forest soils. *Phytopathology* 95:S20.
- Fichtner, E.S., Lynch, S., and Rizzo, D.M. 2005. Seasonal survival of *Phytophthora ramorum* in soils. *Phytopathology* 95:S20.
- Garbelotto, M., Rizzo, D.M., and Marais, L. 2002. *Phytophthora ramorum* and sudden oak death in California: IV. Chemical control. Pages 811-818 In: Proc. Fifth Symp. Oak Woodlands in California's Changing Landscape R. Standiford, D. McCreary and K.L. Purcell, (eds). Gen. Tech. Rep. PSW-GTR-184. Pacific Southwest Research Station, Forest Service, USDA, Albany, CA.

- Goheen, E.M., Hansen, E.M., Kanaskie, A., McWilliams, M.G., Osterbauer, N., and Sutton, W. 2002. Eradication of sudden oak death in Oregon. *Phytopathology* 92:S30.
- Hansen, E.M., Sutton, W., Parke, J.L., and Lindermann, R.G. 2002. *Phytophthora ramorum* and Oregon forest trees - one pathogen, three diseases. Sudden Oak Death, a Science Symposium: The State of Our Knowledge, Monterey, CA.
- Hansen, E.M., Reeser, P., Sutton, W., and Winton, L.M. 2003. First report of A1 mating type of *Phytophthora ramorum* in North America. *Plant Dis.* 87:1267.
- Hansen, E.M., Parke, J.L., and Sutton, W. 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. *Plant Dis.* 89:63-70.
- Ivors, K., Hayden, K., Bonants, P., Rizzo, D.M., and Garbelotto, M. 2003. AFLP and phylogenetic analyses of North American and European populations of *Phytophthora ramorum*. *Mycol. Res.* 108:378-392.
- Jeffers, S. 2005. Recovery of *Phytophthora ramorum* from soilless mixes around container-grown ornamental plants. *Phytopathology* 95:S20.
- Kaminski, K.S., Wagner, S., Werres, S., Beltz, H., Seipp, D., and Brand, T. 2005. Infectivity and survival of *P. ramorum* in recirculating water of nurseries. In: Proc. Sudden Oak Death Sci. Symp. 2nd. P.J. Shea and M. Haverty, (eds). Pacific Southwest Res. Stn., For. Serv., USDA. Albany, CA. (In press).
- Kelly, M., and Meentemeyer, R.K. 2002. Landscape dynamics of the spread of sudden oak death. *Photogramm. Eng. Remote Sensing.* 68:1001-1009.
- Linderman, R.G., and Davis, A. 2005. Survival of *Phytophthora ramorum* in potting mix components or soil and eradication with aerated steam treatment. *Phytopathology* 95:S20.
- McLaughlin, I., Sutton, W., and Hansen, E.M. 2005. Survival of *Phytophthora ramorum* in tanoak and rhododendron leaves. *Phytopathology* 95:S20.
- ODA. 2003. Final report on *Phytophthora ramorum* in Clackamas County, Oregon. Oregon Department of Agriculture.  
<http://oda.state.or.us/plant/ppd/path/SOD/030624SODrpt.pdf>. [Cited on July 20, 2005]:



- Rizzo, D.M., Garbelotto, M., Davidson, J.M., Slaughter, G.W., and Koike, S.T. 2002. *Phytophthora ramorum* and sudden oak death in California: I. Host relationships. Pages 733-740 In: Proc. Fifth Symp. Oak Woodlands in Californias Changing Landscape, San Diego, CA. Gen. Tech. PSW-GTR-184 R. Standiford, D. McCreary and K.L. Purcell, (eds). Pacific Southwest Res. Stn. For. Serv. USDA. Albany, CA.
- Rizzo, D.M. 2003. Sudden Oak Death: Host plants in forest ecosystems in California and Oregon. <http://sod.apsnet.org/> [Cited July 20, 2005].
- Rizzo, D.M., and Garbelotto, M. 2003. Sudden oak death: endangering California and Oregon forest ecosystems. *Front. Ecol. Environ.* 1:197-204.
- Rizzo, D.M., Garbelotto, M., and Hansen, E.M. 2005. *Phytophthora ramorum*: integrative research and management of an emerging pathogen in California and Oregon forests. *Annu. Rev. Phytophology* 43:309-335.
- Shishkoff, N., and Tooley, P.W. 2004. Persistence of *Phytophthora ramorum* in nursery plants and soil. *Phytopathology* 94:S95.
- Tjosvold, S.A., Chambers, D.L., Davidson, J.M., and Rizzo, D.M. 2002. Incidence of *Phytophthora ramorum* inoculum found in soil collected from a hiking trail and hikers' shoes in a California park. Sudden Oak Death Sci. Symp: The State of Knowledge. Monterey, CA.
- USDA-APHIS. 2005. List of hosts and plants associated with *Phytophthora ramorum*. [http://www.aphis.usda.gov/ppq/ispm/pramorum/pdf\\_files/usdaprlist.pdf](http://www.aphis.usda.gov/ppq/ispm/pramorum/pdf_files/usdaprlist.pdf). [Cited Aug 21,2005]
- Werres, S., Marwitz, R., Man in't Veld, W.A., Cock, W.A.M.d., Bonants, P.J.M., Weerd, M., Themann, K., Ilieva, E., and Baayen, R.P. 2001. *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. *Mycol. Res.* 105:1155-1165.
- Werres, S., and De Merlier, D. 2003. First detection of *Phytophthora ramorum* mating type A2 in Europe. *Plant Dis.* 87:10.

## Chapter 2

### Root Infection

#### 2.1 Introduction

*Phytophthora* species can generally be grouped by the types of diseases they cause into two categories: root pathogens that cause root rot and collar rot, and foliar pathogens that cause bole cankers, leaf blight and tip dieback. *Phytophthora ramorum* has been considered a foliar pathogen having only aerial biology (Davidson et al. 2005; Werres and De Merlier 2003). It causes both lethal bole cankers in certain forest trees (Rizzo et al. 2002) and a leaf blight and shoot tip dieback on other hosts, including nursery crops (Davidson et al., 2003; Parke et al, 2003; Tjosvold et al., 2005).

The soil ecology of *P. ramorum* has been investigated recently. It has been found on the shoes of hikers (Tjosvold et al. 2002), recovered from soil in hiking trails (Cushman and Meentemyer, 2005), shown to persist in soil and potting media (Fichtner et al. 2005; Linderman and Davis 2005; McLaughlin et al. 2005; Shishkoff and Tooley 2004), and was recovered from potting mix (Jeffers 2005;). Research has also shown that *P. ramorum* is able to survive in infested leaf litter (Davidson et al. 2005; Fichtner et al. 2005; McLaughlin et al. 2005) which can serve as inoculum for new infections (Davidson et al. 2005). Detached leaves buried in soil for up to 6 months can still yield sporangia upon wetting (Fichtner et al. 2005). This ability to survive in below-ground substrates is cause for concern because most nursery stock is shipped as potted container plants. The possibility for long distance transmission of *P. ramorum* with infested potting media appears to be high. This is especially true if infested plants appear to be asymptomatic. The objective of my work was to examine the potential significance of inoculum in potting media and to determine if *P. ramorum* can infect plants through roots. I chose to work with rhododendron because it is commonly shipped as a potted container plant, it is susceptible to *P. ramorum* infection, and it is important

economically for Oregon because it comprises a large portion of the stock produced and sold by the nursery industry.

## 2.2 Methods and Materials

### 2.2.1 Organisms

#### 2.2.1.1 Pathogen

*Phytophthora ramorum* isolate 03-74-N11-A was obtained from Dr. Nancy Osterbauer of the Oregon Dept. of Agriculture. Isolate 03-74-N11-A was originally isolated from an infected rhododendron cv. Unique in an Oregon nursery in 2003. It is mating type A1, European genotype (Everett Hansen, personal communication). Cultures of this isolate were maintained on modified V-8-CMA (100 mL clarified V8 juice broth, 1200 mL water, 23.4 g cornmeal agar) at room temperature (19°-20°C). A reference isolate MYA 3240 was deposited at the American Type Culture Collection.

#### 2.2.1.2 Host

##### 2.2.1.2.1 Whole Plants

Rhododendron cv. Nova Zembla plants were obtained from Bear Creek Nursery, Scio, OR. The 18-month-old plants were propagated from rooted cuttings and potted in 4"x 4" pots containing Douglas-fir bark medium.

##### 2.2.1.2.2 Tissue Culture Plantlets

Tissue culture plantlets (stage 2, without roots) of rhododendron cv. Nova Zembla were donated by Microplant Nurseries, Inc., Gervais, OR. To initiate root formation, each plantlet was dipped for 30-60 s in liquid rooting concentrate 'Dip 'n Grow' (Astoria Brand, Clackamas, OR) containing 1% indol-3-butyric acid and 0.5% 1-napthalene acetic acid. Sterile technique under a laminar flow hood was used. The plantlets were then potted into cell packs containing rooting media consisting of autoclaved potting medium, vermiculite or perlite. Each cell pack was inserted inside a plastic bag, the bag was sealed, and then placed on a heated germination mat (65-70° F) under artificial light for approximately one month.

### 2.2.2 Inoculum Preparation

Water used in all inoculum preparations was reverse osmosis purified water (Barnstead/Thermolyne, Dubuque, IA). V-8 broth was prepared by mixing V-8 juice (Campbell Soup Co. Camden, NJ) with  $\text{CaCO}_3$  and water (1 part V-8: 4 parts water; 1g  $\text{CaCO}_3$  per 100 mL V-8). Broth (167 mL) was mixed with fine grade vermiculite (333 mL) and placed in a 1500 mL Erlenmeyer flask. A total of 5 flasks were prepared, each containing approximately 500 mL of the V-8/vermiculite mixture. Each flask was shaken well, plugged with cotton, covered with foil and autoclaved for 40 min. After 24 h, the flask was autoclaved again. Agar plugs (6mm diam) with mycelium from the actively growing edge of three-week-old *P. ramorum* colonies were removed and placed into the flasks using sterile technique. Each flask was inoculated with 15-16 plugs, covered, and allowed to incubate at room temperature (18-20°C) for one month.

#### 2.2.2.1 Infested Potting Media

In a clean plastic tub, 500 mL of the prepared V-8/vermiculite inoculum was mixed with 1 L of standard greenhouse potting mix (#3 OBC, Canby, Oregon). In another tub, plain vermiculite was mixed at the same ratio of 1:2 with potting mix to serve as non-infested control medium.

#### 2.2.2.2 Zoospore Preparation

A zoospore suspension was prepared from two-week-old actively growing agar plate cultures of *P. ramorum*. For each plate, 5 mL of sterile water was added to the agar surface, the surface was gently scraped with a sterile rubber policeman to dislodge zoosporangia, and the liquid was poured off into a separate sterile Petri dish. The process was repeated with an additional 5 mL aliquot of sterile water. To stimulate the release of zoospores from zoosporangia, the Petri dish containing the suspension was chilled at 4°C for 60 min, and then allowed to sit at room temperature (18-20°C) for another 60 min. The suspension was then filtered through nylon phytoplankton netting with 35  $\mu\text{m}$  mesh openings (Aquatic Eco-systems, Inc., Apopka, FL) to remove chlamydospores, zoosporangia, and most

hyphal fragments. Two small aliquots of the suspension were placed in a hemocytometer for quantification of zoospores. Once the concentration was determined, the suspension was diluted with water to a final working concentration of  $6 \times 10^4$  zoospores  $\text{mL}^{-1}$ .

## 2.2.3 Inoculation

### 2.2.3.1 Whole plants

Rhododendron plants in 4" pots were removed from their pots, and the potting medium carefully teased from their roots. Each plant was repotted back into the same pot using 200 mL per pot of the prepared infested vermiculite/potting medium mixture. Control plants were repotted using the same technique but with the non-infested vermiculite/potting medium mixture. All plants were repotted carefully so that none of the lower leaves touched the potting medium or the edge of the pots. Plants were placed into separate plastic tubs grouped by treatment and kept in a growth chamber at 21°C. Each tub was filled with tap water to just below the level of potting medium in the pots and the water level maintained for a period of 7 days. The water was then removed and the plants left to dry for a period of 14 days, after which they were flooded again for another 7 day period. Care was taken while adding water to the tubs to ensure that plants were only watered from below in order to avoid splash dispersal of the pathogen. There were four replicate plants in each of two treatments, *P. ramorum*-infested and non-infested controls, and the experiment was conducted twice.

### 2.2.3.2 Tissue Culture Plantlets

Under a laminar flow hood, several plantlets rooting in potting medium, vermiculite, or perlite were removed from their plastic bags and placed in a Petri dish of water. The roots of the plantlets were cleaned by agitation to dislodge potting medium, then using a stereomicroscope any remaining medium was carefully teased off the newly formed roots. Sections (5 cm) of the rooted portion of the plantlets were removed and placed into small beakers containing either a

zoospore suspension or water and allowed to incubate on a lab bench at 18-20°C for 48 h. Two stems rooted in each of the three different media were placed in beakers containing zoospores or water for a total of 12 stems. The experiment was conducted twice.

## 2.2.4 Analysis

### 2.2.4.1 Isolation

All isolations were performed with plant material placed on paper towels, with small sections removed with sterilized forceps and scalpels. Tissues were then plated onto PAR selective medium (17 g CMA, 10 mg pimarinic acid, 10 mg rifampicin, 250 ampicillin), and maintained at 18-20°C until morphological structures of *P. ramorum* could be identified. Root tissues were collected from whole plants, washed to remove potting medium, sub-samples collected, rinsed in a beaker of DI-water, surface sterilized in 10% bleach rinsed again with DI-water and plated onto PAR. The exposure time in the bleach was at least 3 min. for the first half of the samples, and then was reduced to 30 s. for the remaining half of the samples.

### 2.2.4.2 Polymerase Chain Reaction (PCR) Assay

Tissue samples of above-ground stems, leaf petioles and below-ground stems and roots were collected for diagnostic PCR analysis and stored in 2 mL microfuge tubes in the freezer (-20°C) until processed. DNA was analyzed using multiplex polymerase chain reaction (amplification of more than one DNA target), and amplification was conducted using the internal transcribed spacer (ITS 4 and 5) region of rDNA. The diagnostic primers and methods used were designed for *Phytophthora lateralis* (Winton and Hansen 2001) and are effective in amplifying *P. ramorum* (Hansen et al. 2005).

### 2.2.4.3 Microscopy

#### 2.2.4.3.1 Fluorescence

Tissue samples of stems, petioles and below-ground tissues were placed in containers with screw-on caps containing 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2), the caps loosened and placed under vacuum at 20-25 psi for 30-45 min, after which the caps were re-tightened. The samples were taken through an alcohol dehydration process consisting of 2 h in 50% EtOH, 5 h in 70% EtOH, overnight in 95% EtOH, then transferred to 1:1 plastic infiltration solution: 95% EtOH, under vacuum at 20-25 psi. After 12 h, samples were vacuum infiltrated with full-strength plastic infiltration solution. Tissue was embedded in glycol methacrylate plastic (Technovit 7100, Energy Beam Sciences, Agawam, MA), sectioned (4-5  $\mu$ m thick) on an AO 820 rotary microtome with a steel knife and mounted on glass slides. Each slide was flooded with 0.01% Calcofluor White M2R (Tinopal) (Sigma Chemical, St. Louis, MO) for 10 min, rinsed with DI-water and allowed to dry. Polymount mounting medium (Fischer Chemical, Fairlawn, NJ) was used to affix cover-slips. Slides were examined using a Zeiss Axiostar epifluorescence compound microscope with either DAPI (excitation 350 nm) or Calcofluor filters specific for excitation of 425 nm. Images were collected using a Micropublisher 3.3 RTV digital camera and Q-Capture Pro imaging software.

#### 2.4.3.2 Confocal Microscopy

Roots from tissue culture plantlets were removed from stems, floated in 0.01% Calcofluor White M2R for 10 min, rinsed with DI-water and placed on a slide with a drop of DI-water. To obtain the best images possible, the roots were gently separated with a probe under a stereomicroscope before placement of a cover slip. The samples were viewed using a Zeiss LSM 510 Confocal Laser Scanning Microscope, a Diode 405 laser, and images were collected using Zeiss LSM 510 imaging software.

## 2.3 Results

### 2.3.1 Artificially Infested Potting Medium

Symptoms on plants grown in *P. ramorum*-infested potting medium were observed 3 weeks after inoculation. These included wilted upper leaves, discolored lower leaves and necrotic lesions on stems just above the soil line. After 4 weeks, the upper leaves had collapsed and necrotic lesions had advanced several cm upward from the soil line, some into the lower petioles (Fig. 1). Control plants showed no wilting, discolored lower leaves or stem necrosis (Fig. 2).

At harvest, internal stem necrosis was observed in the lower above-ground portions of all the plants grown in infested potting medium and all below-ground stem portions (Fig. 3). Roots of plants grown in infested medium were discolored with intact, but fragile cortical tissues unlike typical rotted roots that are soft and disintegrated. Inoculated roots also had a distinctive odor compared to the control root masses, which were neither discolored, nor fragile and did not have an odor (Fig. 4).

Isolation of above-ground plant parts onto selective medium indicated that *P. ramorum* was present in middle, lower and below-ground stems, and lower petioles. The highest frequency of recovery came from below-ground stem tissue, which had the largest amount of discoloration. *P. ramorum* was not recovered from any of the upper stems or leaf blades, but was recovered from both fine roots and large roots. Diagnostic PCR analysis performed on stems, petioles, and roots supported the isolation data confirming the presence of *P. ramorum* (Table 1) except for fine roots which were negative in isolation. This was most likely due to lengthy surface sterilization.

Microscopic evaluations of thin sections of stem tissues indicated that hyphae were present in the pith, primary and secondary xylem (Fig. 5 and 6), cambium and phloem of the above-ground stem tissues. The cortex was the only tissue without visible hyphae; instead I observed numerous chlamydospores (Fig. 7). This is



consistent with histological observations of rhododendron cuttings inoculated with mycelial plugs of *P. ramorum* (Werres and Pogoda, 2004).

Examination of the leading edge of the infection indicated only hyphae in primary xylem tissues, visible in both vessels and tracheids (Fig. 5).

Table 1. Frequency of recovery from isolation onto PAR selective medium.

Frequency of Recovery from Tissue Samples <sup>a</sup>		
Tissue Sampled	<u>Inoculated</u>	<u>Non-Inoculated</u>
Upper Stem	0/14 = 0%	not sampled
Middle Stem	33/37 = 89%	0/29 = 0%
Lower Stem	37/38 = 98%	0/28 = 0%
Upper Leaf	0/40 = 0%	0/29 = 0%
Middle Leaf	4/41 = 10% <sup>b</sup>	0/40 = 0%
Lower Leaf	5/39 = 13% <sup>b</sup>	0/41 = 0%
Below-ground Stem	33/33 = 100%	0/7 = 0%
Large Roots	11/22 = 50%	0/7 = 0%
Fine Roots	0/7 = 0%	not sampled

<sup>a</sup> Not all tissues from each of the 4 plants per inoculation treatment were sampled.

<sup>b</sup> Positive results on middle and lower leaves were from petioles only.

### 2.3.2 Tissue Culture Plantlets Inoculated with Zoospores

Plantlets inoculated for 24 h were mounted whole and examined with a stereomicroscope. Germinating cysts were aggregated in large masses along the stem, and on sections of the roots. Examinations with a compound microscope revealed lateral roots emerging through the epidermis creating gaps in the main root around the newly formed root, and large numbers of germinating cysts were observed aggregating at those junctures. Plantlets inoculated for 48 h were

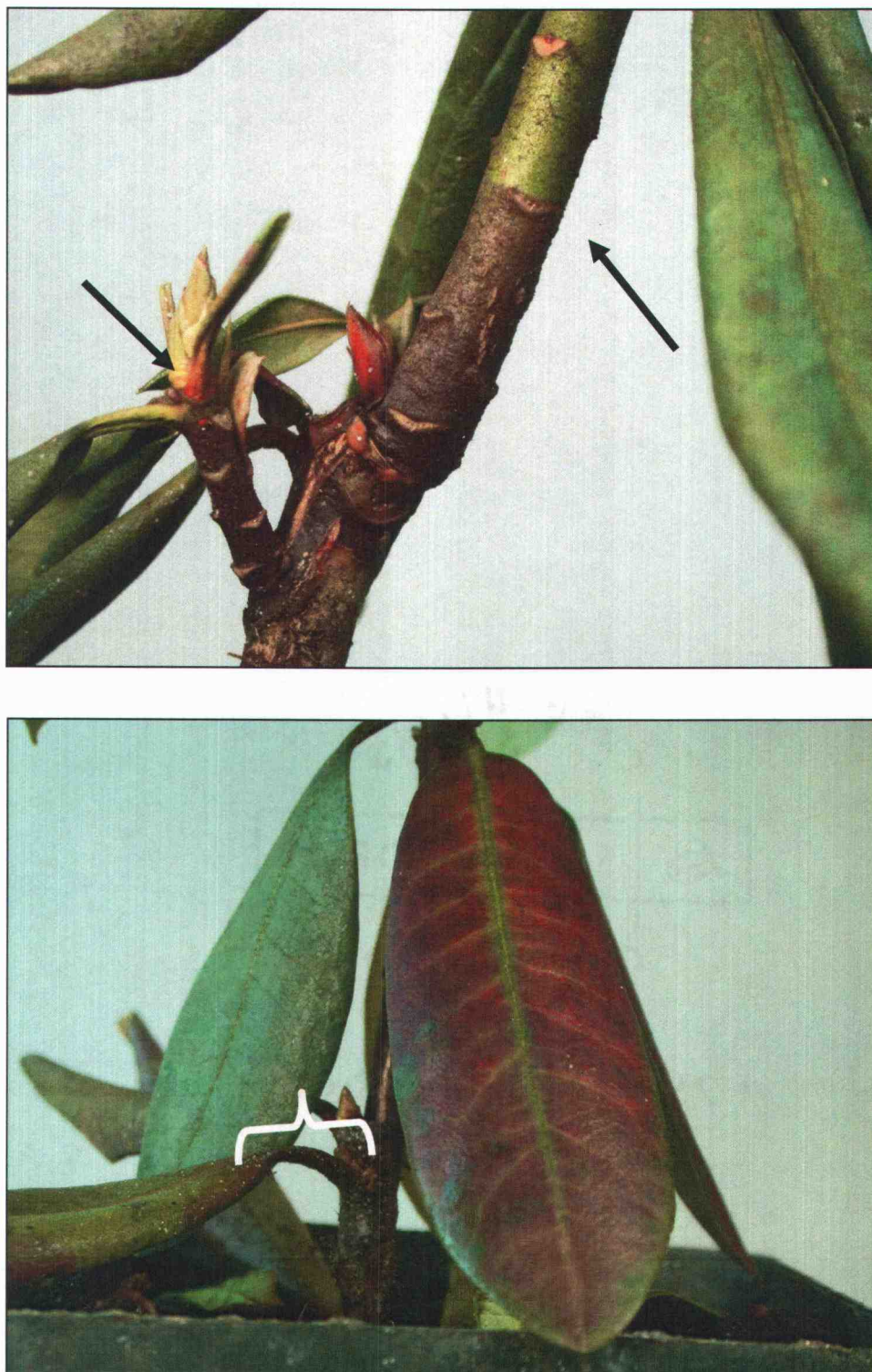


Fig. 1. Middle (top) and lower (bottom) stems one month after inoculation with artificially infested potting media. Lesion (arrows) advancing upward from below soil line. Stem necrosis advancing into petiole (brace) of lowest leaf and discoloration of lower leaves.

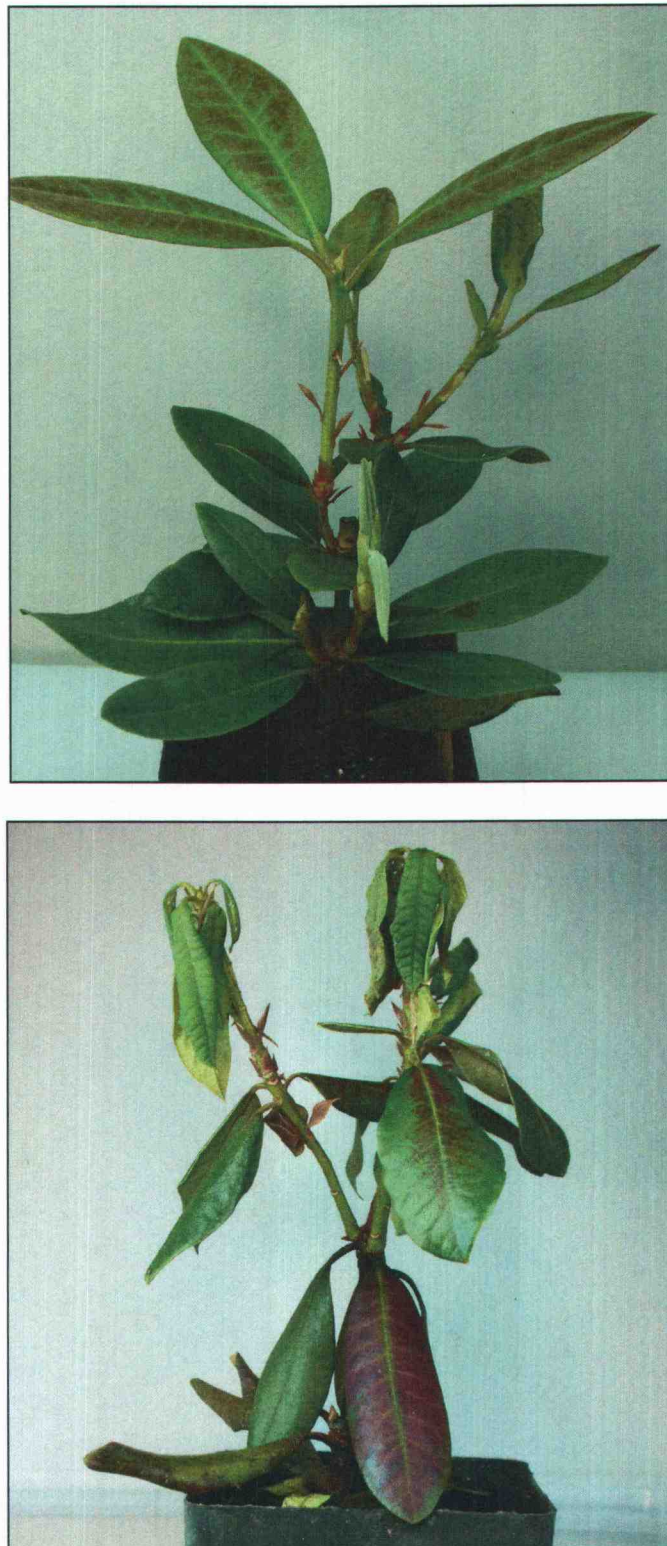


Fig. 2. Non-inoculated control (top) and *P. ramorum*-inoculated (bottom) plants one month after inoculation with infested potting media. Upper leaves of inoculated plants collapsed. Lower leaves were discolored.





Fig. 3. Cross sections of stems. Middle stem of non-inoculated control (top), inoculated middle (center), inoculated below-ground stem with roots attached (bottom).





Fig. 4. Non-inoculated (top) and inoculated (bottom) roots after rinsing to remove potting medium.



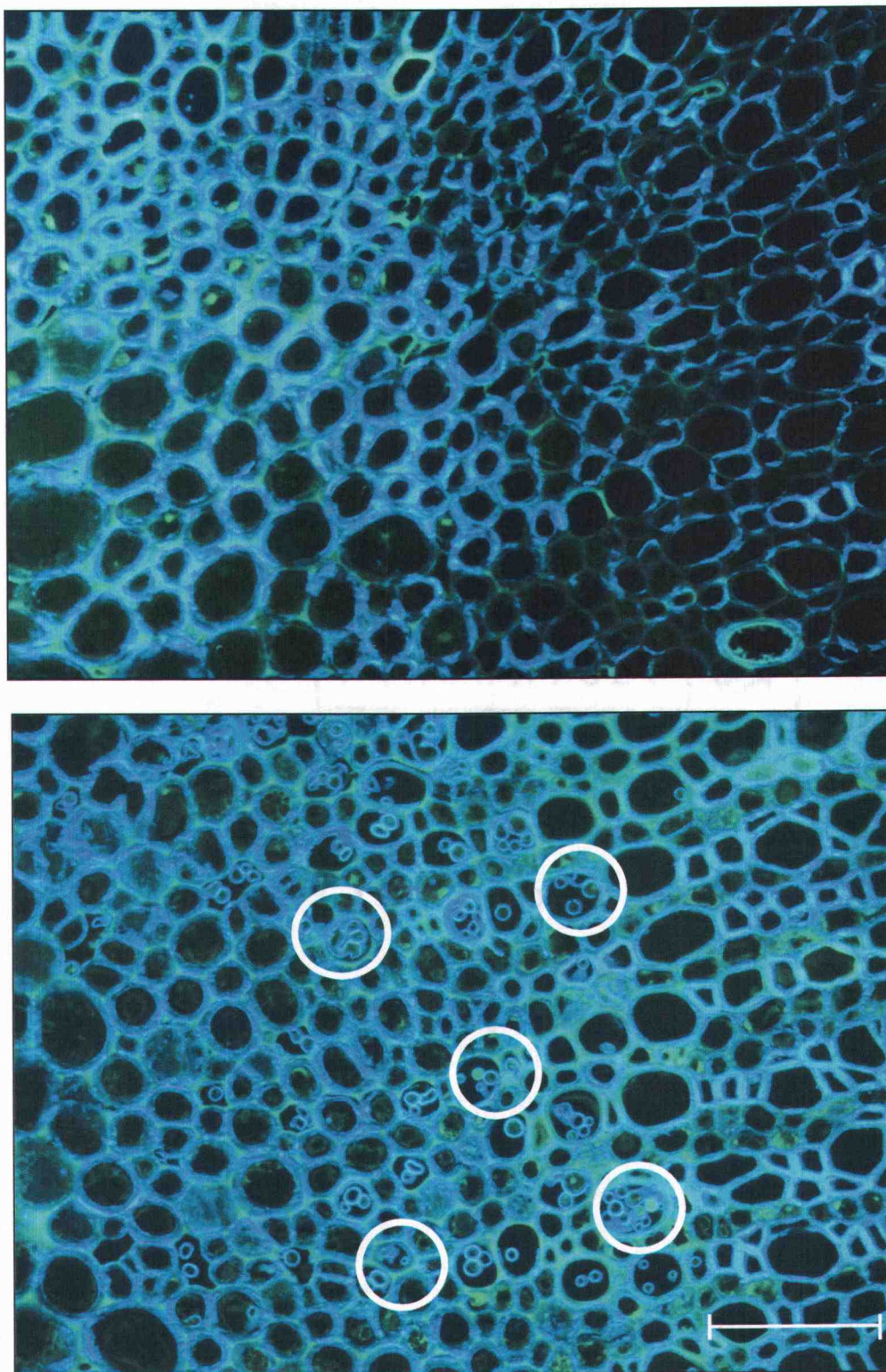


Fig. 5. Middle stem cross sections of primary xylem from non-inoculated (top) and inoculated (bottom) plants. At the leading edge of the infection, *P. ramorum* hyphae were found in primary xylem cells (circles) only. Bar = 50  $\mu$ m



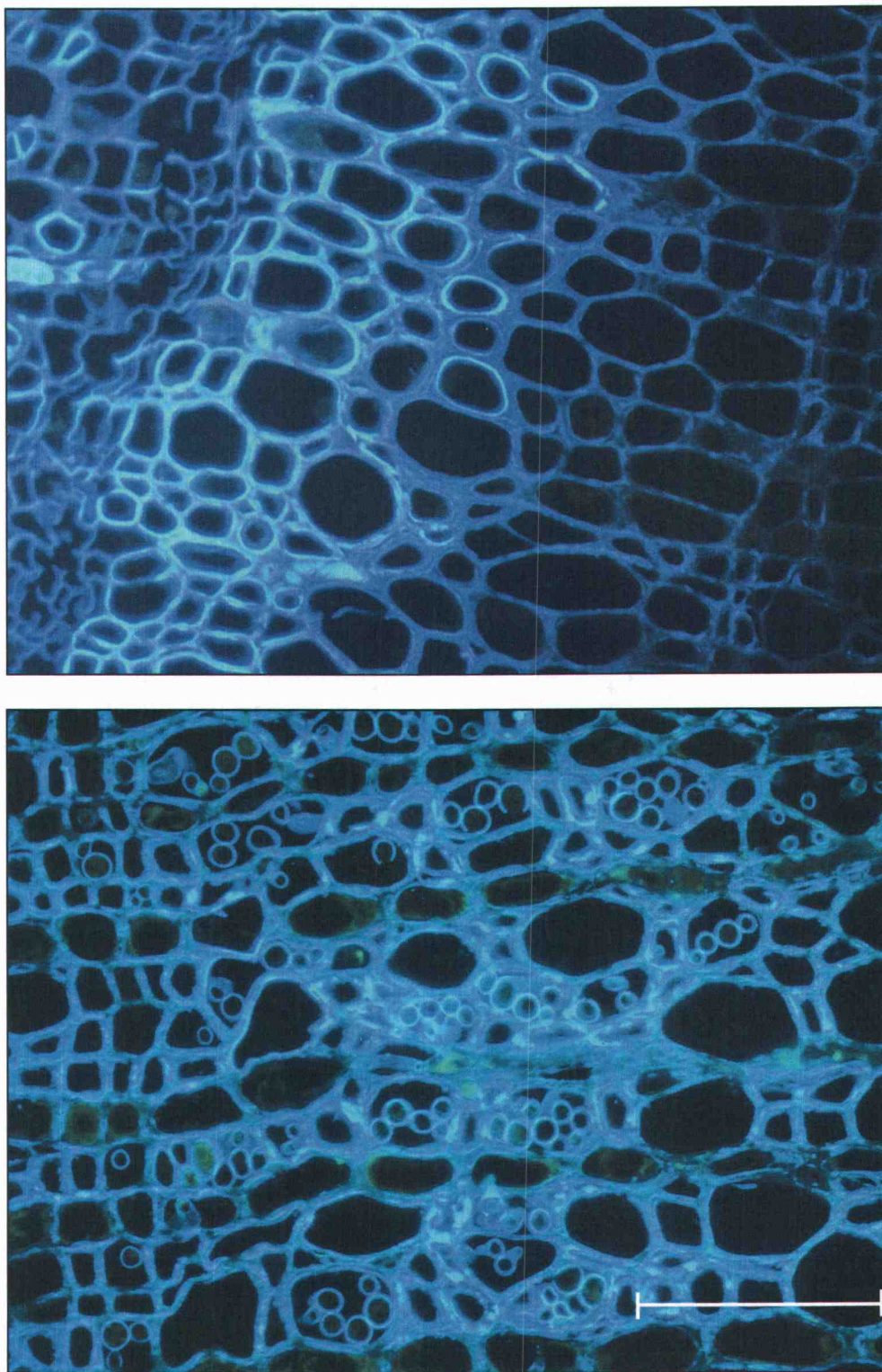


Fig. 6. Lower stem cross sections of secondary xylem from non-inoculated (top) and inoculated (bottom) plants. *P. ramorum* hyphae were found colonizing large sections of secondary xylem cells. Bar = 50  $\mu$ m



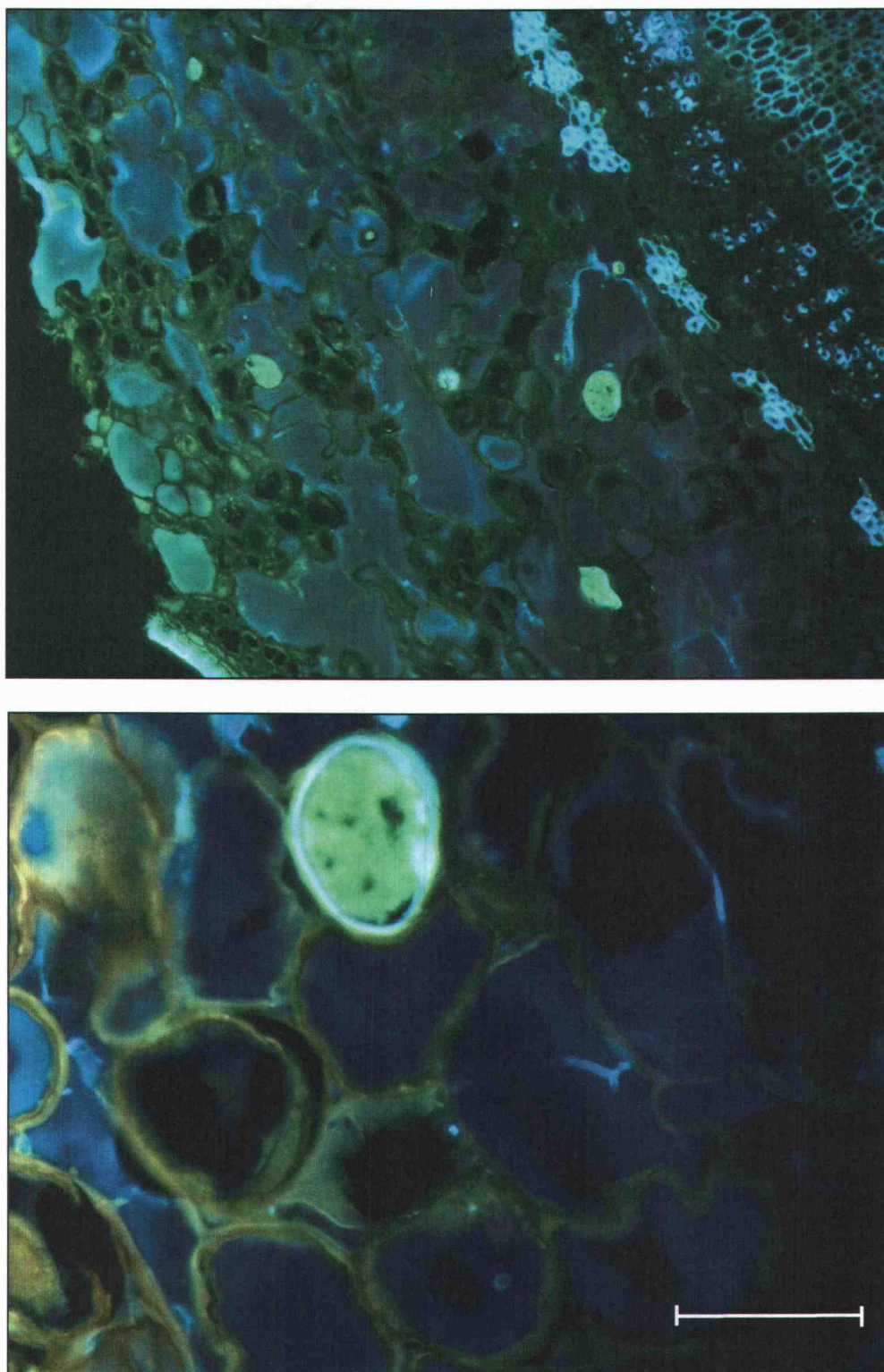


Fig. 7. Lower stem cross sections showing cortical cells from inoculated plant. Cortex with chlamydospores (upper). Bar = 200  $\mu\text{m}$ . Single chlamydospore (bottom). Bar = 50  $\mu\text{m}$



examined using both compound and stereomicroscopes. Whole roots and thin sections of roots and stems all showed the same attraction of zoospores to the junctures of emerging laterals, and to root primordia. Zoospores had encysted and emerging germ tubes were oriented in the direction of the gap in cortical cells created by the emerging laterals, in the direction of the root primordia, and could be observed penetrating both tissues.

Fluorescence microscopy of thin sections of roots, both cross-sections and longitudinal sections showed that germ tubes from germinating cysts were oriented toward the root tissues (Fig 8). Examinations of root tips revealed that hyphae originating from germinating cysts penetrated both inter- and intra-cellularly, and did not appear to be oriented towards a certain area or cell type (Fig. 9). Examination of the inoculated portion of stem in both cross-section and longitudinal sections showed that zoospores had aggregated at root primordia and wounds (Fig. 10). The germinating cysts were seen within wounds, and hyphae were observed colonizing all the tissues inside and adjacent to the wounds. One of the inoculated plantlets had a wound along its stem. In cross-section this portion of the stem showed that large numbers of zoospores had entered the wound. Those zoospores had encysted, germinated, and mycelium was colonizing the internal stem tissue (Fig. 11).

Laser scanning confocal microscopic examinations of tissue culture plant roots supported the light microscopy observations. Although penetration at the root cap was observed, there were also many additional sites along the roots where germ tubes from germinating cysts were again found penetrating both inter- and intra-cellularly. Additionally, when inoculated plantlets were allowed to incubate for one week, confocal microscopy of the roots revealed the production of sporangia of varying sizes (Fig.12). All the sporangia were empty and had released their contents.

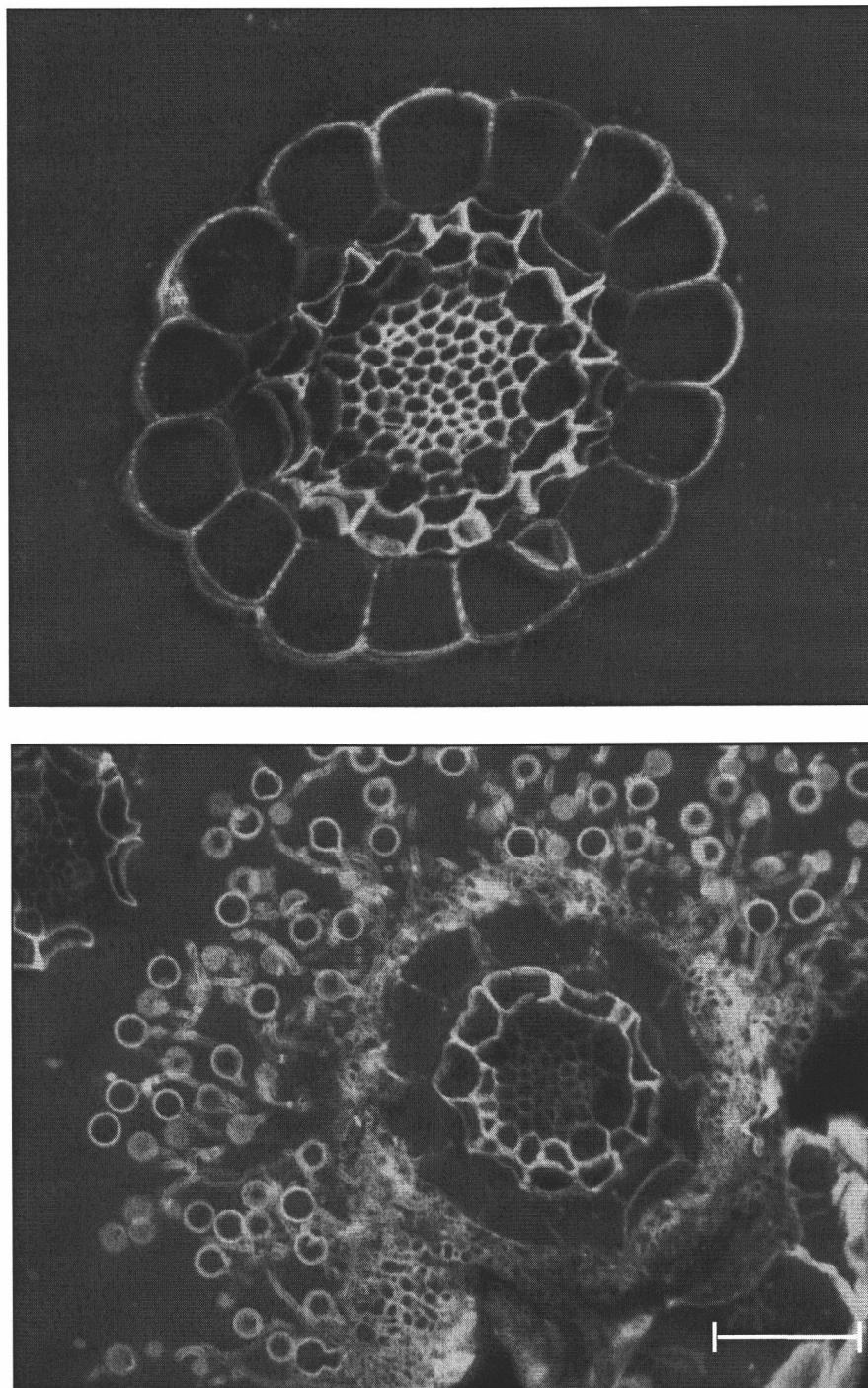


Fig. 8. Cross sections of roots from tissue culture plantlets, non-inoculated (top) and inoculated (bottom). At 48 h post inoculation, germ tubes from germinating cysts are oriented toward root tissue (bottom). Bar = 50  $\mu$ m

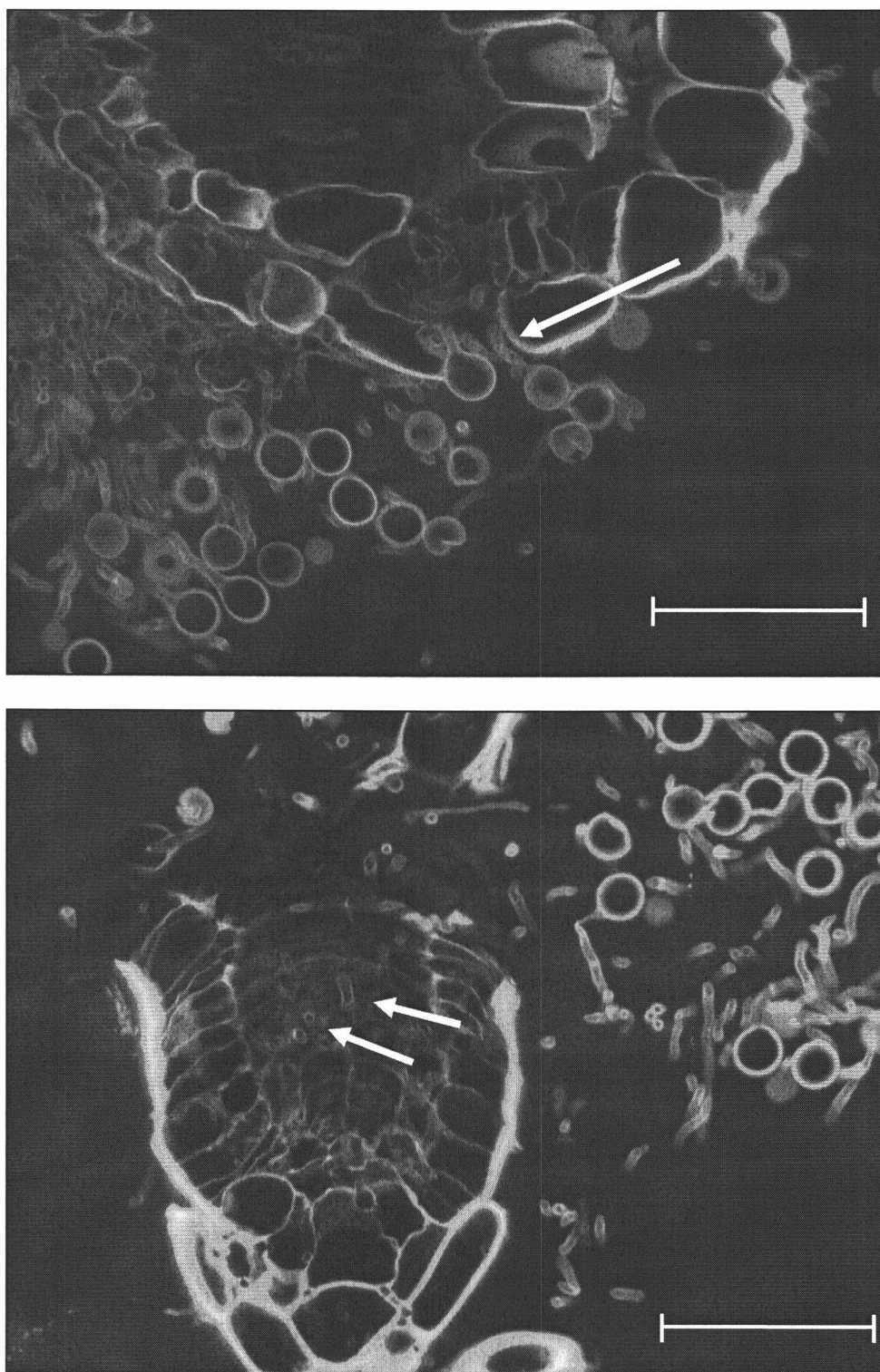


Fig. 9. Longitudinal sections of inoculated root tip. Germ tubes (arrows) from germinating cysts are penetrating root epidermal cells inter- (top) and intra-cellularly (bottom). Lower image shows germ tubes penetrating the root tissue, as some cysts were removed during sectioning process. Bar = 50  $\mu$ m

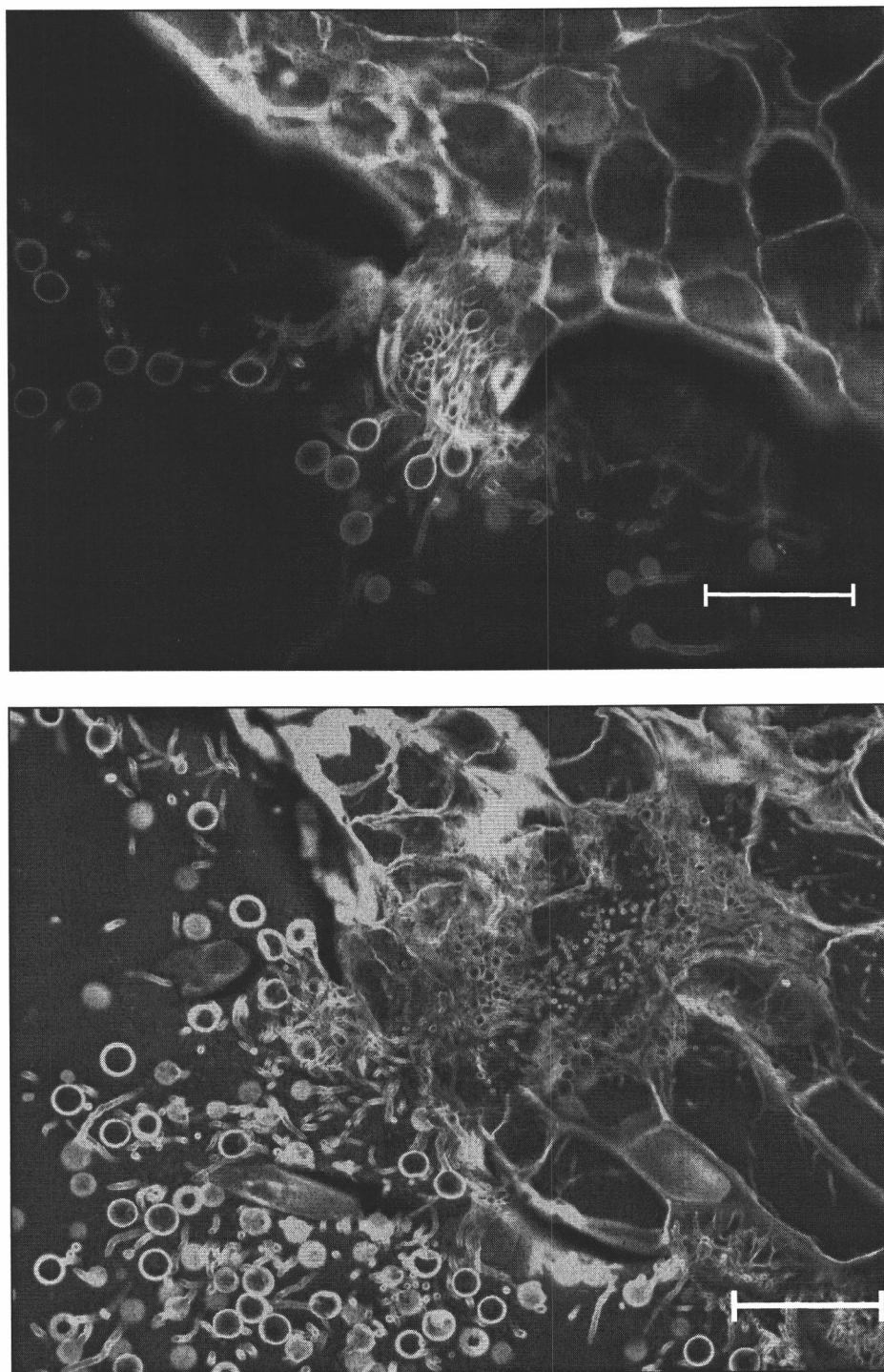


Fig. 10. Longitudinal sections from inoculated, rooted portion of tissue culture plantlet stems. Germ tubes from germinating cysts were oriented towards and growing in the direction of root primordia (top) and a wound (bottom). Bar = 50  $\mu$ m



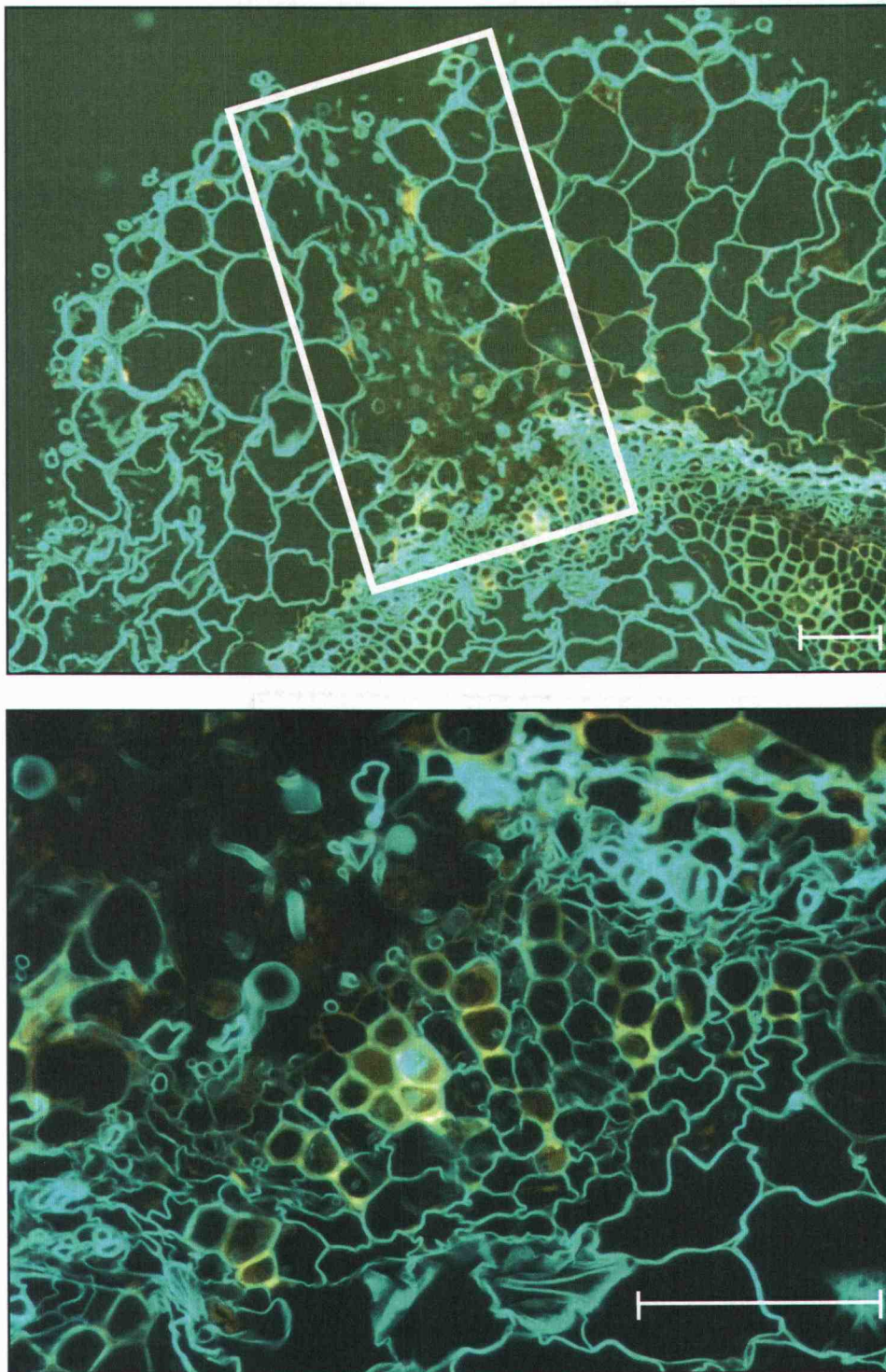


Fig. 11. Cross section from rooted portion of inoculated tissue culture plantlet stem. Wound in stem (boxed area) extends from epidermis into vascular tissue (top). Germinating cysts and mycelium colonizing wound and internal tissues (bottom). Bar = 50  $\mu\text{m}$

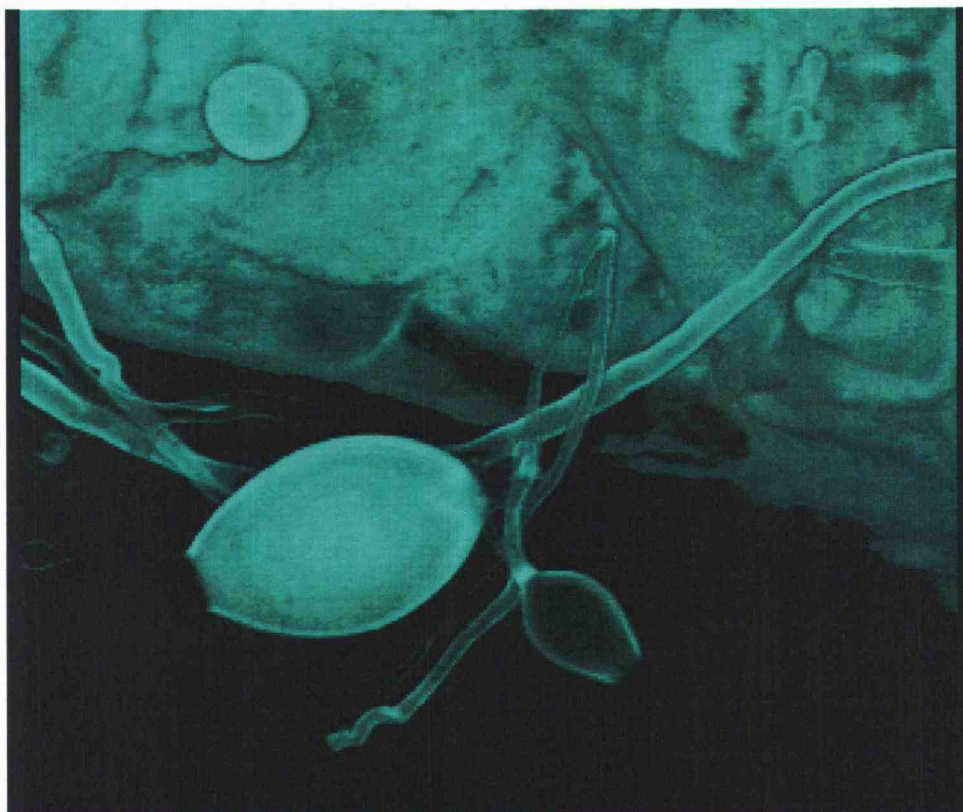


Fig. 12. Inoculated tissue culture plantlet root with cyst, hyphae and sporangia. Bar = 10  $\mu$ m

## 4 Discussion

*P. ramorum* appears to be similar to several other rhododendron-infecting *Phytophthora* species (*P. cactorum*, *P. citricola*, *P. hevea*, and *P. parasitica*) that can cause foliar blight and dieback as well as infect roots (Benson and Jones, 1980). The below-ground phase for dieback-causing *Phytophthora* species is poorly understood, but *P. parasitica* and several other foliar *Phytophthora* species may be recovered in mid-winter, when temperatures approach freezing, from stems, roots, and pine bark mulch in which container-grown plants are grown (Benson and Hoitink 1986), even when they cannot be recovered from attached leaves. These sites may provide a refuge for “foliar” *Phytophthora* spp. during environmental conditions unfavorable for disease development. Infection of above-ground tissues by *P. parasitica* occurs during moist, warmer conditions following splash dispersal of soilborne inoculum onto leaves or from growth from infected plant roots or stems (Kuske and Benson 1983). It is not known if *P. ramorum* behaves similarly.

The experimental conditions for the infested potting medium experiment used were similar to those used by other researchers to establish conditions conducive for disease development by other root-infecting *Phytophthora* species on rhododendrons and other hosts (Erwin and Ribeiro 1996). Periodic saturation or flooding typically is used to stimulate disease development (Matheron and Mircetich 1985) either to provide suitable conditions for zoospore release or pre-dispose the host, or both.

The soil phase of the disease cycle for *P. ramorum* and its epidemiological significance under field conditions in oak or tanoak woodlands is still largely unknown. Although *P. ramorum* can persist in soil in infected leaves (McLaughlin et al., 2004; Fichtner and Rizzo, 2005), there is no direct evidence that this serves as primary inoculum for root infection. Root infection for these native species has not been investigated thoroughly, but patterns of disease suggest that soil inoculum could be important in splash dispersal onto above-ground plant parts (Davidson et

al. 2005; Hansen et al. 2005). In addition to this work with rooted cuttings of rhododendron, root infection has only been demonstrated for artificially inoculated camellia (Shishkoff, 2005) and *Rhododendron macrophyllum* grown from seed (Parke et al. 2005).

In these experiments, disease developed after the potting medium was artificially infested with *P. ramorum*, but there is evidence that *P. ramorum* has occurred naturally in commercial container media. *P. ramorum* was detected in the medium from around container-grown rhododendrons in Oregon (N. Osterbauer, *unpublished*), and it also has been baited from several potting medium samples collected from container-grown camellias shipped from a *P. ramorum*-infested nursery in California to South Carolina (Jeffers 2005). It is not known if *P. ramorum* in the potting media resulted from irrigation runoff or infected aboveground plant parts that fell to the potting medium surface, or if the infested potting media could have served as primary inoculum for plant infections. In either case, it would seem that infested potting media could be an important means of transmitting the pathogen, potentially over large distances, with asymptomatic nursery plants. Inoculum in potting media may escape detection because there is no requirement for testing potting media as part of the routine nursery inspection, sampling, and certification procedures required by the Emergency Federal Order (USDA-APHIS 2005). The results presented here indicate there is potential for disease transmission from infested potting media to plants. Further research is needed to verify that disease transmission occurs in the field to determine if there is a need to monitor potting media for the presence of *P. ramorum* as part of the routine nursery sampling procedures required by the Emergency Federal Order.



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## Literature Cited

- Benson, D.M., and Hoitink, H.A.J. 1986. *Phytophthora* dieback. In: Compendium of Rhododendron and Azalea Diseases. Pages 12-15. D.L. Coyier and M.K. Roane eds. St. Paul, MN. APS Press.
- Davidson, J.M., Wickland, A.C., Patterson, H.A., Falk, K., and Rizzo, D.M. 2005. Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. *Phytopathology* 95:587-596.
- Erwin, D.C., and Ribeiro, O.K. 1996. *Phytophthora* Diseases Worldwide. APS Press, St. Paul, MN.
- Fichtner, E.S., Lynch, S., and Rizzo, D.M. 2005. Summer survival of *Phytophthora ramorum* in forest soils. *Phytopathology* 95:S20.
- Fichtner, E.S., Lynch, S., and Rizzo, D.M. 2005. Seasonal survival of *Phytophthora ramorum* in soils. *Phytopathology* 95:S20.
- Hansen, E.M., Parke, J.L., and Sutton, W. 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. *Plant Dis.* 89:63-70.
- Jeffers, S. 2005. Recovery of *Phytophthora ramorum* from soilless mixes around container-grown ornamental plants. *Phytopathology* 95:S20.
- Kuske, C.R., and Benson, D.M. 1983. Survival and splash dispersal of *Phytophthora parasitica*, causing dieback of rhododendron. *Phytopathology*. 73:1188-1191.
- Linderman, R.G., and Davis, A. 2005. Survival of *Phytophthora ramorum* in potting mix components or soil and eradication with aerated steam treatment. *Phytopathology*. 95:S20.
- Matheron, M.E., and Mircetich, S.M. 1985. Influence of flooding duration on development of *Phytophthora* root and crown rot of *Juglans hindsii* and Paradox walnut rootstocks. *Phytopathology* 75:973-976.

- McLaughlin, I., Sutton, W., and Hansen, E.M. 2005. Survival of *Phytophthora ramorum* in tanoak and rhododendron leaves. *Phytopathology* 95:S20.
- Parke, J.L., Roth, M., and Choquette, C. 2005. *Phytophthora ramorum* disease transmission from infested potting media. in: Proc. Sudden Oak Death Sci. Symp., 2nd. P.J. Shea and M. Haverty, (eds). Pacific Southwest Res. Stn., For. Serv., USDA. Albany, CA. (In press).
- Rizzo, D.M., Garbelotto, M., Davidson, J.M., Slaughter, G.W., and Koike, S.T. 2002. *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. *Plant Dis.* 86:205-214.
- Shishkoff, N., and Tooley, P.W. 2004. Persistence of *Phytophthora ramorum* in nursery plants and soil. *Phytopathology* 94:S95.
- Tjosvold, S.A., Chambers, D.L., Davidson, J.M., and Rizzo, D.M. 2002. Incidence of *Phytophthora ramorum* inoculum found in soil collected from a hiking trail and hikers' shoes in a California park. Sudden Oak Death Sci. Symp: The State of Knowledge. Monterey, CA.
- USDA-APHIS. 2005. List of hosts and plants associated with *Phytophthora ramorum*.  
[http://www.aphis.usda.gov/ppq/ispm/pramorum/pdf\\_files/usdaprlist.pdf](http://www.aphis.usda.gov/ppq/ispm/pramorum/pdf_files/usdaprlist.pdf).  
 [Cited Aug 21,2005]:
- Werres, S., and De Merlier, D. 2003. First detection of *Phytophthora ramorum* mating type A2 in Europe. *Plant Dis.* 87:10.
- Winton, L.M., and Hansen, E.M. 2001. Molecular diagnosis of *Phytophthora lateralis* in trees, water, and foliage baits using multiplex polymerase chain reaction. *For. Pathol.* 31: 275-283.

## Chapter 3

### Foliar Infection and Spread

#### 3.1 Introduction

*Phytophthora* diseases are generally characterized as root rots, foliar blights, or stem cankers and are not considered vascular wilt pathogens (Erwin et al. 1983; Erwin and Ribeiro 1996). Little is currently known about the infection pathways of *Phytophthora ramorum* and mode of spread within plants. It is clear that *P. ramorum* has different infection biology depending on the host. While symptoms on certain members of the oak family may be evident on the bole and also on the leaves, it is not clear where infections were initiated. Woody shrubs are infected on buds, leaves and twigs notwithstanding the evidence presented in Chapter 2 on the potential for root infection of rhododendron (Davidson et al. 2005; Rizzo et al. 2002). However it is not known which tissues are colonized and how the pathogen spreads within the plant from foliar infections.

Descriptions of the spread of *P. ramorum* in plant tissues vary depending on the host. General symptoms on ornamental nursery hosts (Tjosvold et al. 2005) include “irregular necrotic leaf lesions, rather than distinct spots. Leaf infections can develop down the petiole and into twigs”. Tjosvold et al. also noted that “infections can move up or down a branch into a leaf base” and that “infected leaves often fall off before the lesion reaches the petiole.” There have been other reports of necrosis following the midrib of inoculated leaves and the disease progressing quickly into stems of the host (Hansen et al. 2005; Lewis and Parke 2005). This vertical spread in stem tissues and necrosis along leaf midribs hints that *P. ramorum* may spread through the vascular tissue (Davidson et al. 2005; Tjosvold et al. 2004), but experimental evidence for this is lacking. An extensive histological evaluation of *P. ramorum* colonization of rhododendron stems was conducted by Pogoda and Werres (2004), however this study utilized a somewhat artificial inoculation method. Detached segments of rhododendron stems were inoculated by placing mycelial plugs on the cut ends.

The goal in the following experiments was to better understand the infection biology of *P. ramorum* in rhododendron using more natural inoculation methods. Specific goals were to determine if stomata are required for infection on foliar surfaces, to identify which tissues are colonized by advancing infections, and to determine if *P. ramorum* colonizes vascular tissues.

## 3.2 Materials and Methods

### 3.2.1 Organisms

#### Pathogen and Host

The same pathogen isolate and the same host cultivar described in Chapter 2 were used.

### 3.2.2 Inoculum Preparation

#### 3.2.2.1 Zoospore Suspension/Preparation

A zoospore suspension was prepared as described in Chapter 2 section 2.2.2.

#### 3.2.2.2 Agar Plugs

Agar plugs (6mm diameter) were removed from the edge of actively growing three-week-old *P. ramorum* colonies grown on modified V-8 agar (see recipe Chapter 2 section 2.1.1.).

### 3.2.3 Inoculation

#### 3.2.3.1 Initial Infection of Leaf Surfaces

Experiments were conducted to observe the process of leaf penetration by zoospore/cyst inoculum of *P. ramorum*. Leaves were removed from greenhouse grown plants, placed in plastic bags, then transported to the lab. Approximately 15 mature leaves were chosen to achieve similar age and size from among several different plants. With a sterile scalpel, 1-cm squares were removed from the center portion of the leaf blades. The squares did not include leaf margins or mid-ribs. Cut sections were floated in a beaker of the zoospore suspension. Approximately

10 squares were inoculated; half were floated abaxial (upper) side up, the other half adaxial (lower) side up. Leaf squares used for the non-inoculated control were treated the same, but floated on the surface of sterile water. They were allowed to incubate at room temperature (18-20°C) for 2.5 h. Leaf squares were then removed, fixed in F.A.A. (5 mL 40% formalin, 5 mL 100% acetic acid, 80 mL 70% ethanol), processed through an alcohol dehydration in preparation for critical point drying, and viewed with a scanning electron microscope. Only the lower surfaces of the leaf squares, exposed to the inoculum, were observed. This experiment was conducted once.

### 3.2.3.2 Penetration of Detached Leaves from Tissue Culture Plantlets

Tissue culture plantlets are more succulent and tender than greenhouse-grown potted plants, and the initial infection process was examined on these plants for comparison of the infection process. Leaves were collected from tissue culture plantlets growing in Magenta GA-7 plant culture boxes (Plant Media, Dublin, OH). Each leaf ranged in size between 5 mm-8 mm. Using sterile technique, leaves were excised from the plantlets and floated in a beaker of zoospore suspension, or water. Ten leaves were inoculated with *P. ramorum* while six leaves were used for non-inoculated controls. Similar to the above experiment, half were floated abaxial side up, the other half adaxial side up. Incubation and processing were as previously described. This experiment was conducted twice. The second trial differed from the first trial in that 12 inoculated leaves and 8 control leaves were incubated in a time-series, and removed from the inoculum at 1.0, 1.5, 2.0, and 2.5 h.

### 3.2.3.3 Spread of Infection within Whole Plants from Foliar Inoculation

To follow the spread of infection arising from foliar inoculation, disease progress was observed on mature greenhouse-grown plants inoculated with mycelial plugs following leaf wounding. Two leaves of each plant were inoculated, one young leaf from immature growth at the top of the plant and one mature leaf from older growth at the bottom. Each leaf was wounded with a sterile push pin, and an agar plug was placed on the wound with the mycelium side down. After inoculation, a

plastic bag was placed over each inoculated leaf (Fig.13). Plants were grouped by treatment (control or inoculated), placed into clean plastic tubs and kept in a growth chamber at 20°C. All plants were watered with care so that no water or soil was splashed onto any parts of the plants. These plants were watered just enough to keep the potting medium moist; they were not flooded. There were three treatment groups: two *P. ramorum* treatments for which mycelial plugs were used as inoculum, and one control group for which plain agar plugs were used. There were two inoculation sites. Leaves were inoculated on the mid-vein (4 plants) or on the side of the leaf blade (called mid-blade) (4 plants) (Fig.14). Two control plants were wounded and treated with plain agar plugs, each at one of the two sites. This experiment was conducted three times, in October, February, and March.

### 3.3 Results

#### 3.3.1 Initial Infection of Leaf Surfaces

##### 3.3.1.1 Penetration of Detached Mature Leaves

Rhododendron leaves characteristically have large numbers of stomata on the lower leaf surface and very few on the upper surface. Large numbers of encysted zoospores were observed on both upper and lower surfaces of these detached mature leaves. The majority of cysts had germinated, and some had multiple germ tubes. Numbers of germinated versus non-germinated cysts were not quantified, but it appeared that the frequency of germination was similar regardless of which leaf surface was inoculated. Initial observations of mature leaves inoculated for 2.5 h showed no association between adhesion sites of cysts, or orientation of growing germ tubes in relation to stomata (Fig. 15), and germ tubes appeared to penetrate the leaf surface directly through the cuticle (Fig. 16 and Fig. 17).

Germ tubes of cysts were observed growing in apparently random directions on the leaf surface, and many of them had a structure on the tips that resembled a swelling but with a “fuzzy” appearance (Fig. 18). Inoculated leaves examined after 4 or more hours of incubation time (data not included) did not reveal any further differentiation of those structures. After 6 hours of incubation time the “fuzzy”



Fig. 13. Mature greenhouse grown *Rhododendron* cv. "Nova Zembla", inoculated with mycelial plugs following wounding. One young leaf (top) and one mature leaf (bottom) were inoculated and covered with a plastic bag.



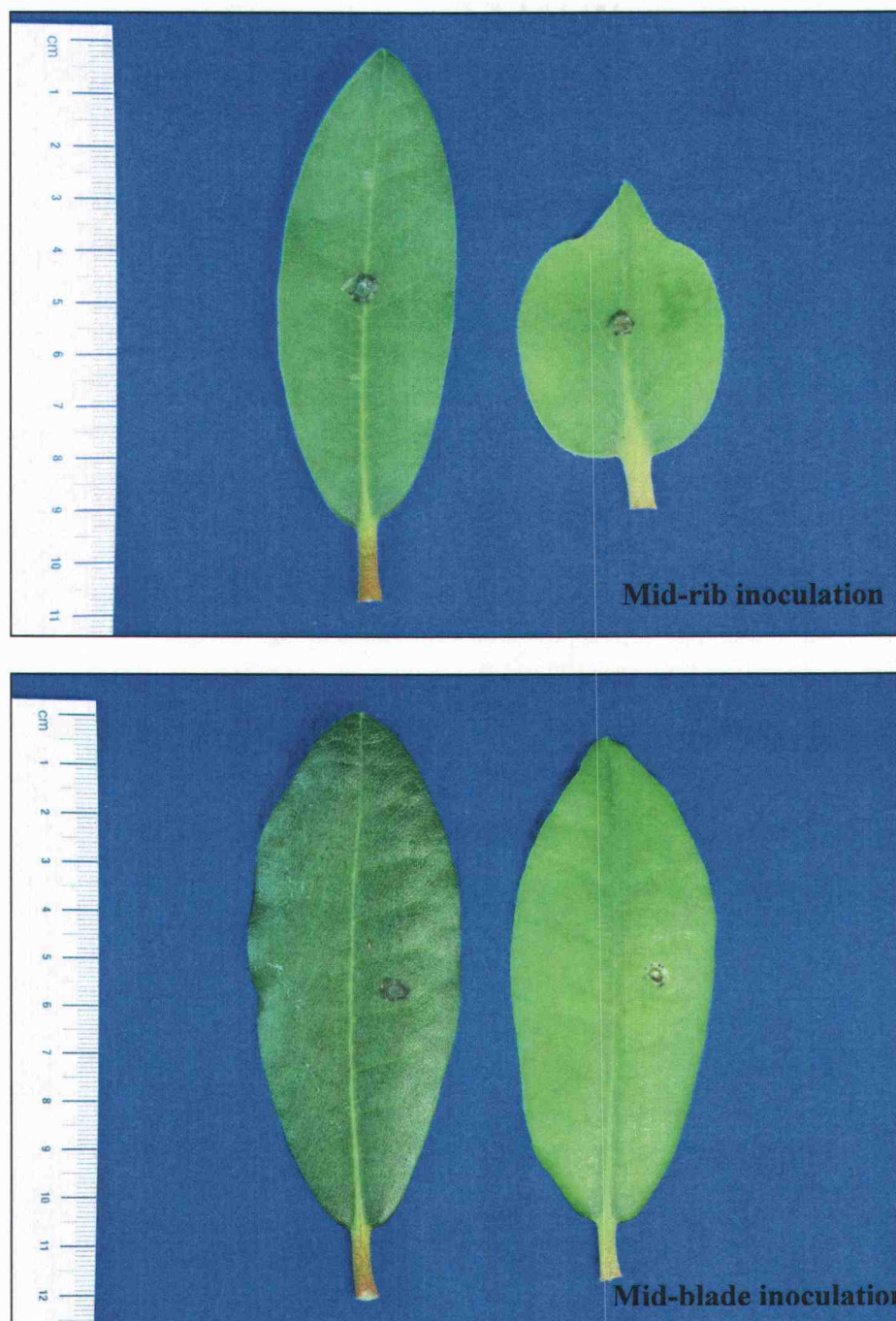


Fig. 14. Leaves of control plants demonstrating inoculation method using wounding and agar plugs, mid-rib (top) and mid-blade (bottom). Two leaves from each plant were inoculated, one mature leaf (left leaves) and one young leaf (right leaves).



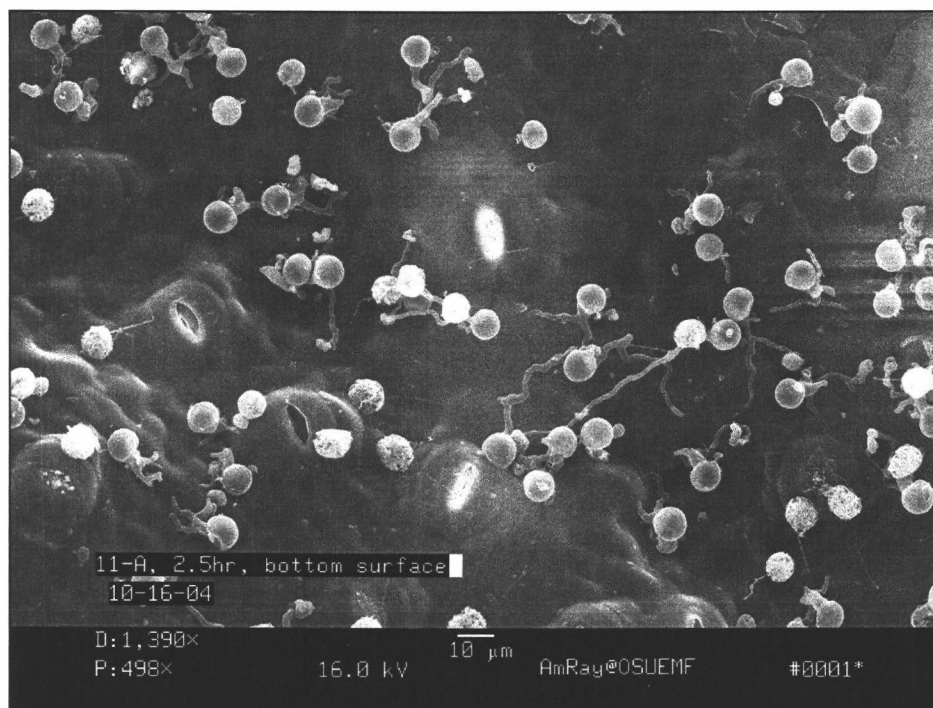


Fig. 15. Lower surface of mature leaf 2.5 h post-inoculation. Encysted zoospores are germinating and growth of emerging germ tubes does not appear directed towards stomatal openings.

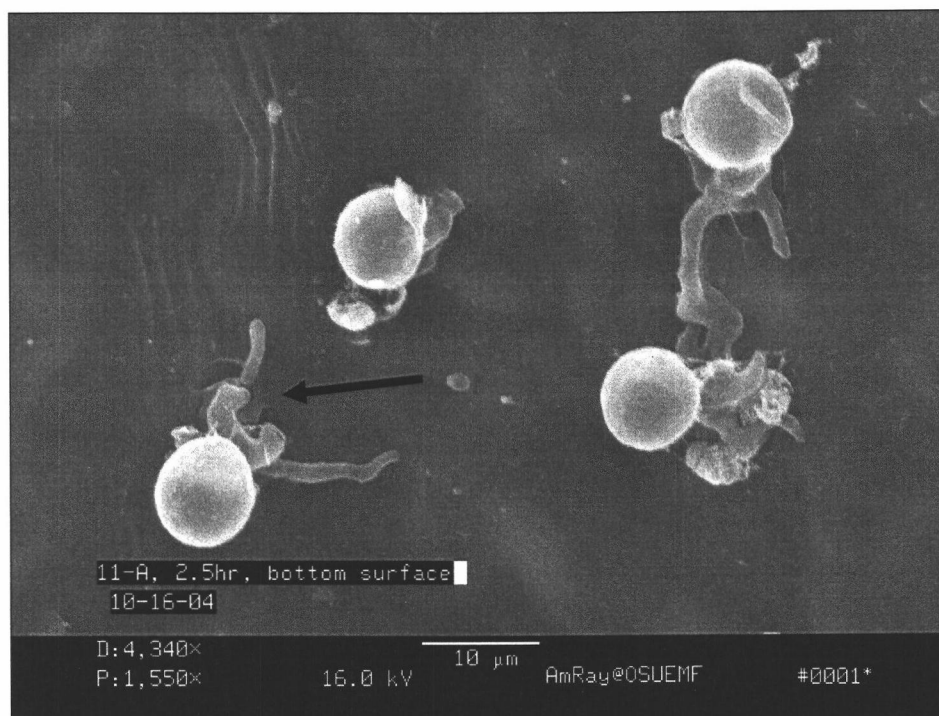


Fig. 16. Lower surface of mature leaf 2.5 h post-inoculation. Germinating cyst directly penetrating cuticle (arrow).

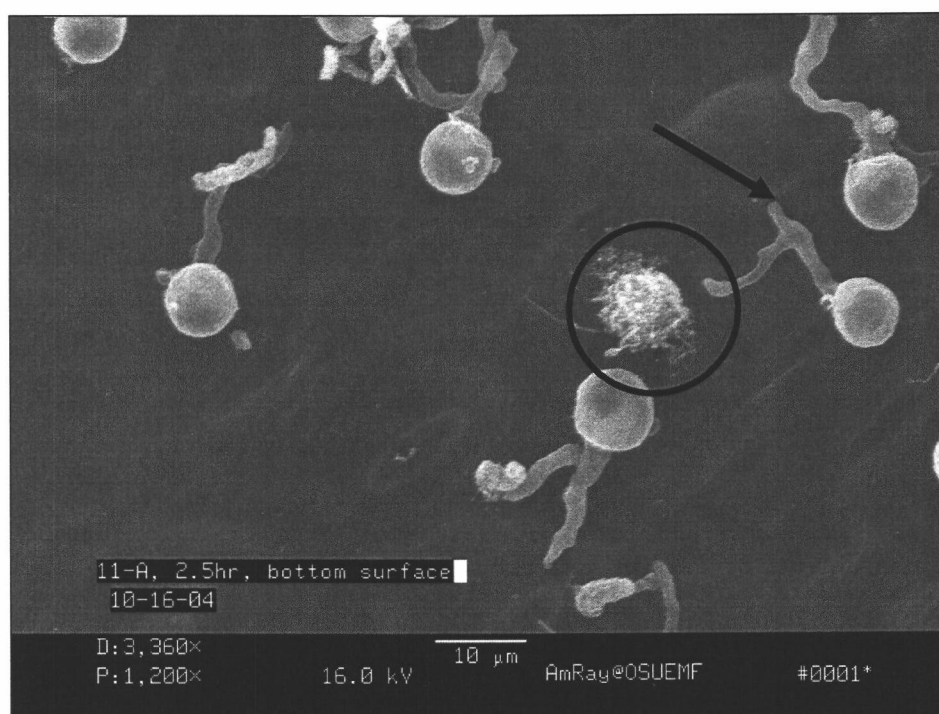


Fig. 17. Lower surface of mature leaf 2.5 h post-inoculation. Hyphae from germinating cyst directly penetrating guard cell (arrow), degrading cyst (circle).



Fig. 18. Lower surface of inoculated mature leaf with germ tube of germinating cyst producing "fuzzy" appendage (arrow).

structures were no longer visible as it appeared that all hyphae emerging from germinating cysts continued to elongate and grow along the surface of the leaf.

Some cysts appeared to be in various states of deterioration. Of the degrading cysts, some had not germinated; of those that had, the emerging germ tubes appeared similar to those of healthy-appearing cysts (Fig. 19).

### 3.3.1.2 Penetration of Detached Leaves from Tissue Culture Plantlets

Similar to mature leaves, young plantlet leaves have very few stomata on the upper surface, and numerous stomata on the lower surface. Unlike results obtained with mature leaves, SEM images indicated that cysts adhering to the lower leaf surface were generally associated with stomata. Cysts were usually present adjacent to or directly on top of stomatal openings (Fig. 20), and germ tubes could be observed penetrating directly into the stomata (Fig. 21). Cysts not associated with stomates had much longer germ tubes than cysts that were associated with stomates. Growth patterns were similar to those observed on mature leaves, as were the formation of “fuzzy” structures on germ tube tips.

### 3.3.1.3. Time Study of Detached Tissue Culture Leaves

In most areas, encysted zoospores were observed adjacent to, or directly on top of stomates. After 1.0 h of incubation, multiple germ tubes approximately 1-2  $\mu\text{m}$  in length were visible (Fig. 22). At 1.5 h growth had progressed, germ tubes were up to 30  $\mu\text{m}$  in length, and the first “fuzzy” structures were observed (Fig. 23). At 2.0 h germ tubes were not longer but had increased in width. Additionally the “fuzzy” structures were more robust (Fig. 24), and some cysts had collapsed while others had begun to degrade (Fig. 25). At 2.5 h both the germ tubes and the “fuzzy” structures had increased in length without any visible differentiation (Fig. 26). Zoospores appeared to have been attracted to wounds, supporting data collected from thin sections of inoculated rooted stems. An SEM image of a group of encysted zoospores shows that they aggregated near a crack in the cuticle rather than near stomata (Fig. 27).

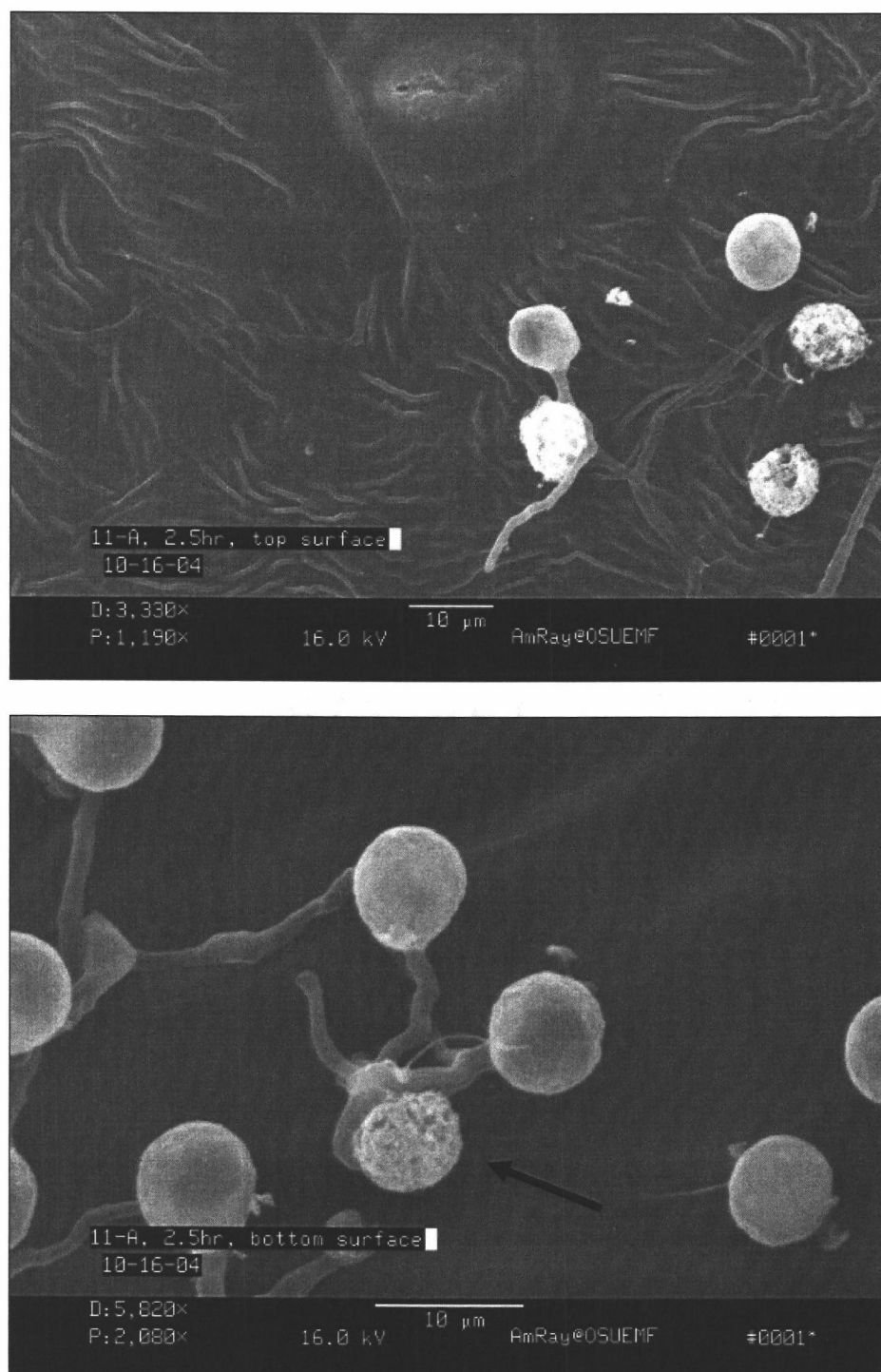


Fig. 19 Upper (top) and lower surface (bottom) of mature leaves 2.5 h post inoculation. Growth of germ tubes from germinating cysts not directed towards stomata (white arrow) (top); some degradation is apparent (black arrows).

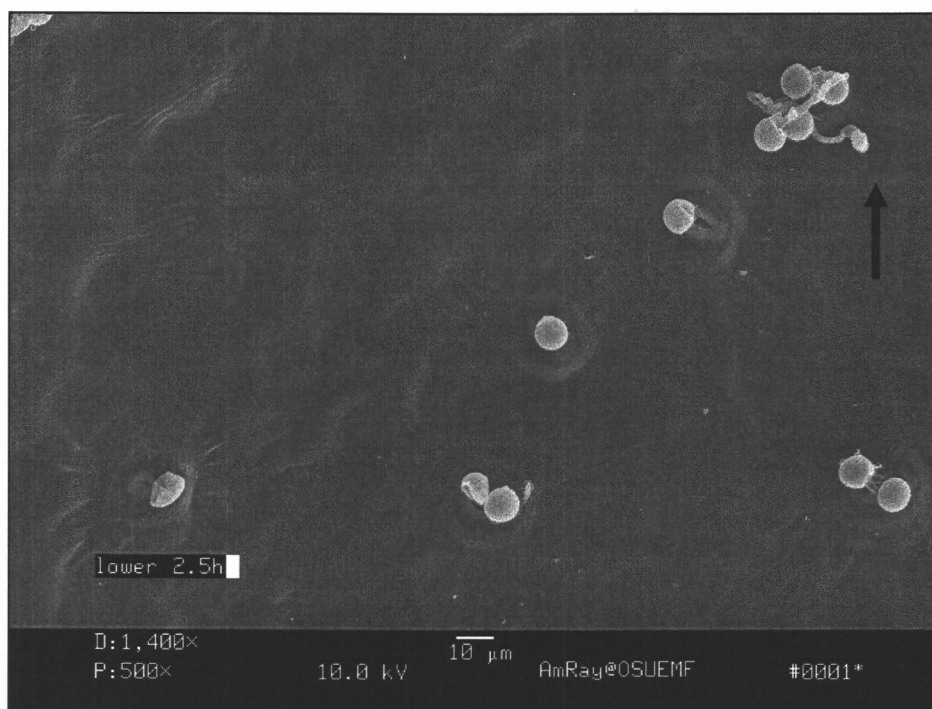


Fig. 20. Lower surface of tissue culture leaf 2.5 h post inoculation. All visible cysts are associated with stomata, some germ tube tips have “fuzzy structures” (arrow).

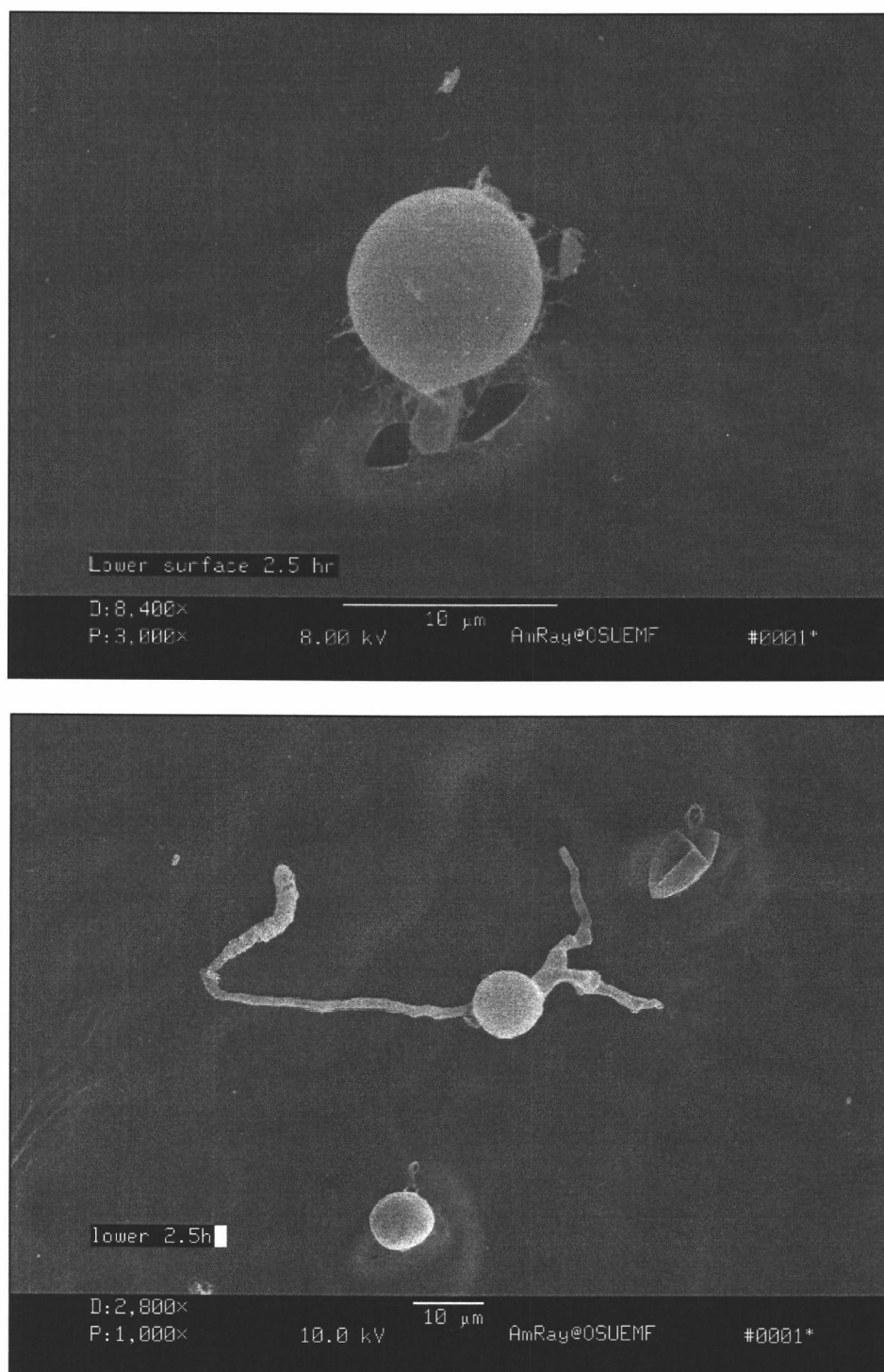


Fig. 21. Lower surface of tissue culture plantlet leaf 2.5 h post inoculation. Emerging tube visible penetrating stomata (top), germ tube producing "fuzzy" structure (bottom).



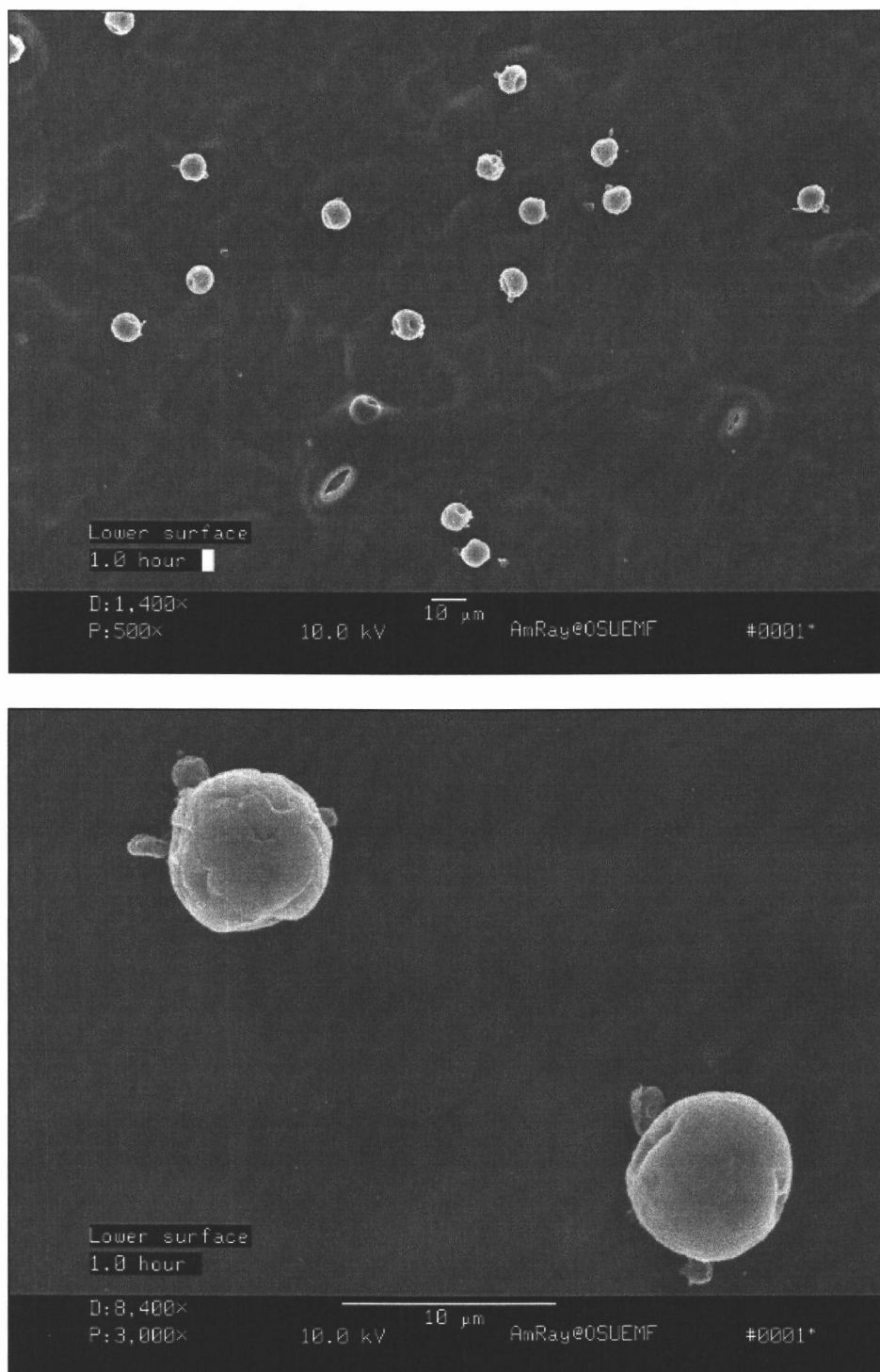


Fig. 22 Lower surface of tissue culture leaves 1.0 h post incubation. Emerging germ tubes 1-3  $\mu$ m in length are visible.



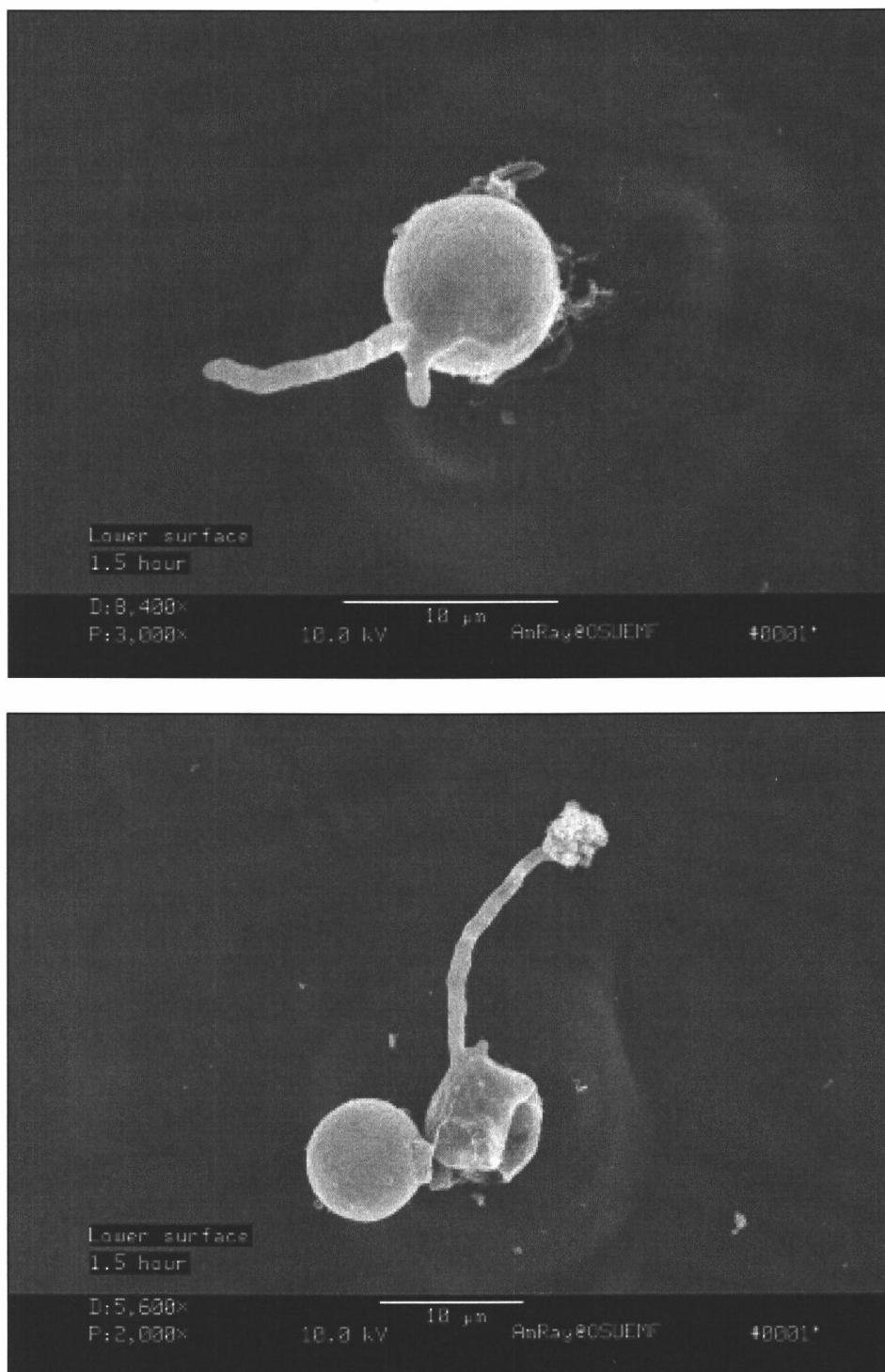


Fig. 23. Lower surface of tissue culture leaves 1.5 h post inoculation. Germinating cysts are associated with stomata, germ tube length has increased, and “fuzzy” structure is apparent (bottom).

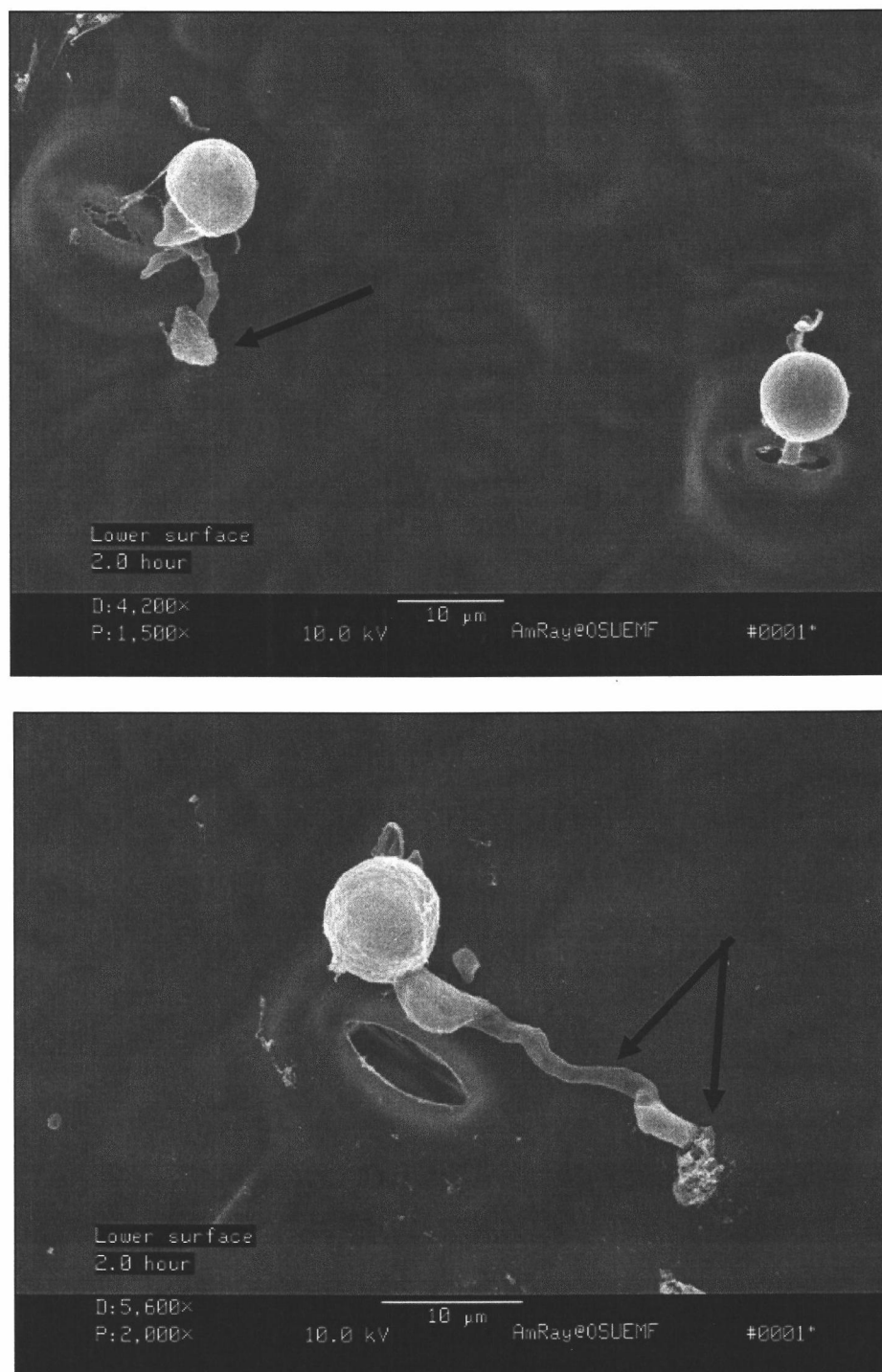


Fig. 24. Lower surface of tissue culture leaf 2.0 h post inoculation. Cysts adjacent to stomata are not penetrating the epidermis, germ tubes have increased in width, and the size of “fuzzy” structures have increased (arrows).

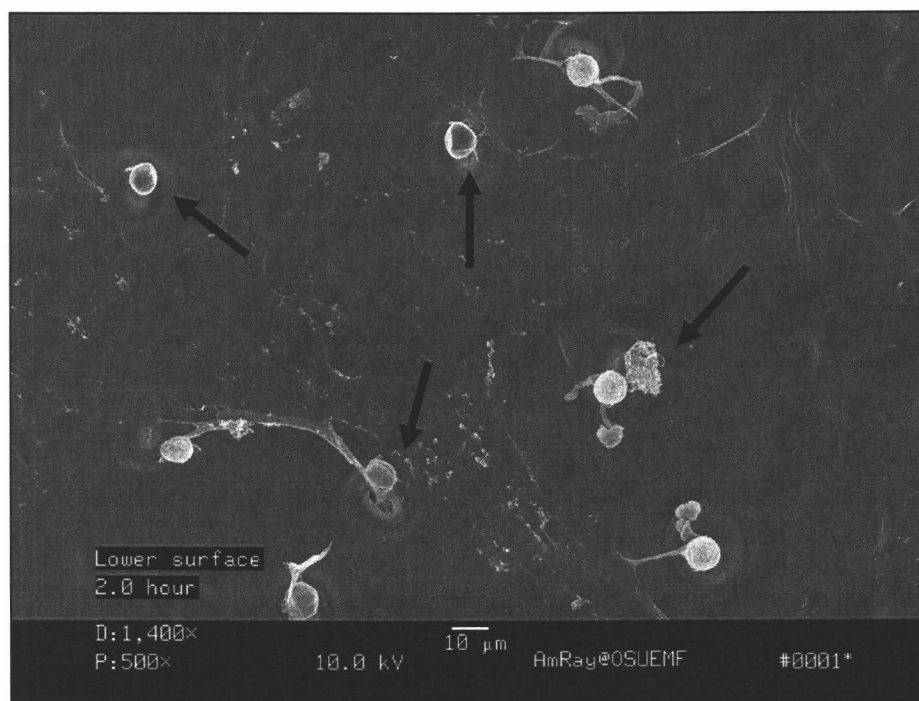


Fig. 25. Lower surface of tissue culture leaf 2.0 h post inoculation. Collapsed and degrading cysts (arrows) are visible.

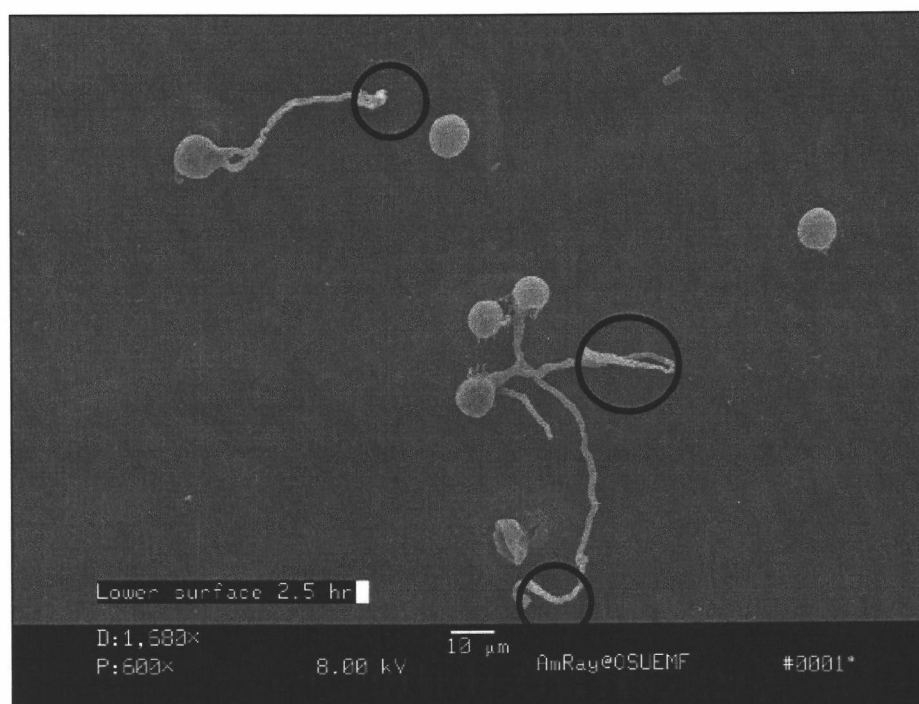


Fig. 26. Lower surface of tissue culture leaf 2.5 h post inoculation. Germ tubes have increased in length and "fuzzy" structures appear longer and thinner (circle).

### 3.3.2 Spread of Infection within Whole Plants from Foliar Inoculation

The experiment was first conducted in September, and repeated in February and March. Only the first experiment showed rapid disease development in less than 2 weeks. Of the inoculated plants from the first experiment, over half of all the inoculated leaves had senesced by harvest date, while in the subsequent repeats almost all the inoculated leaves had senesced by harvest date.

In the first trial, initial symptoms of infection on inoculated leaves were observed within 2 days. Disease development was more rapid on inoculated young leaves than on mature leaves (Fig. 28). The size and shape of the lesions did vary according to inoculation site, as lesions advanced farther on mid-rib inoculations as compared to mid-blade inoculations on all leaves regardless of age (Fig. 29). After 10 days plants were harvested, photographed, and samples removed for isolation and microscopy. Some of the leaves had abscised, while some remained attached. The lesion size or extent of spread did not appear to differ according to whether the leaf was still attached to the plant.

Lesions initiated from inoculation sites on young leaves were observed spreading into stems. Results from the first trial indicated that lesions expanded on young leaves through petioles into stems almost 100% of time (Fig. 30), while lesions appeared to remain within the inoculated mature leaves without spreading into the stems. The lesions in the upper stem appeared to spread in a vertical pattern before spreading around the stem and advancing into adjacent petioles above and below the inoculated leaf (Fig. 31). Removal of a leaf from the leading edge of the stem lesion exposed discoloration of the vascular bundles (Fig. 32).

In the following two trials performed on older, larger plants, spread of infection was slower and not as extensive. Incubation times were increased to 15 and 20 days prior to harvest. All lesions remained within the leaves and did not appear to advance through the petioles, except for one plant which did exhibit visible stem necrosis initiated from a mature leaf inoculation site. Inoculated leaves were

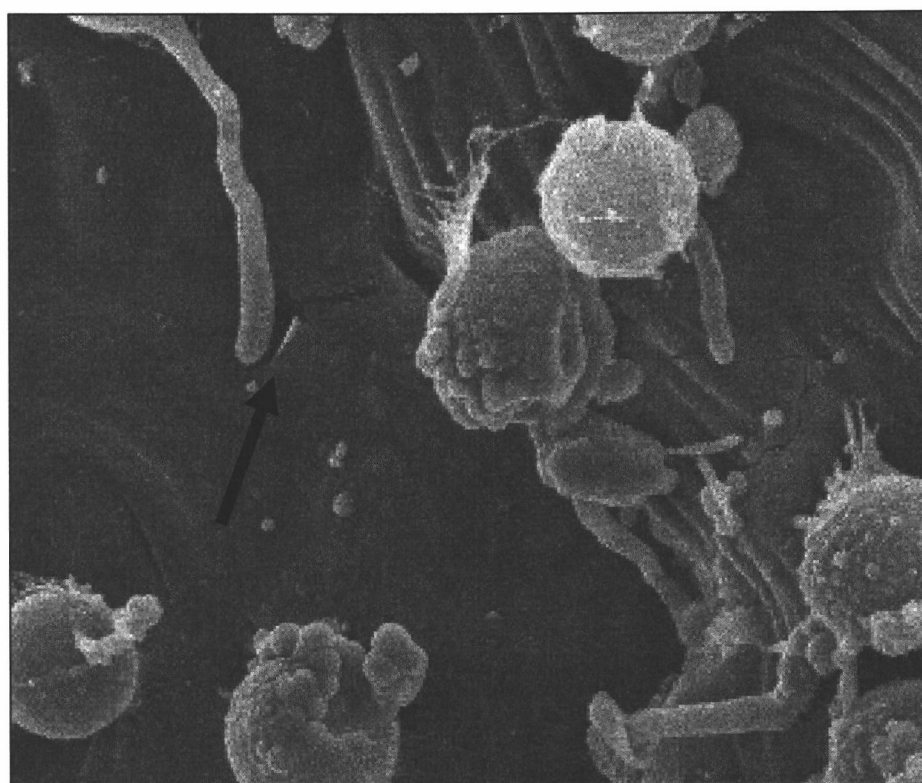
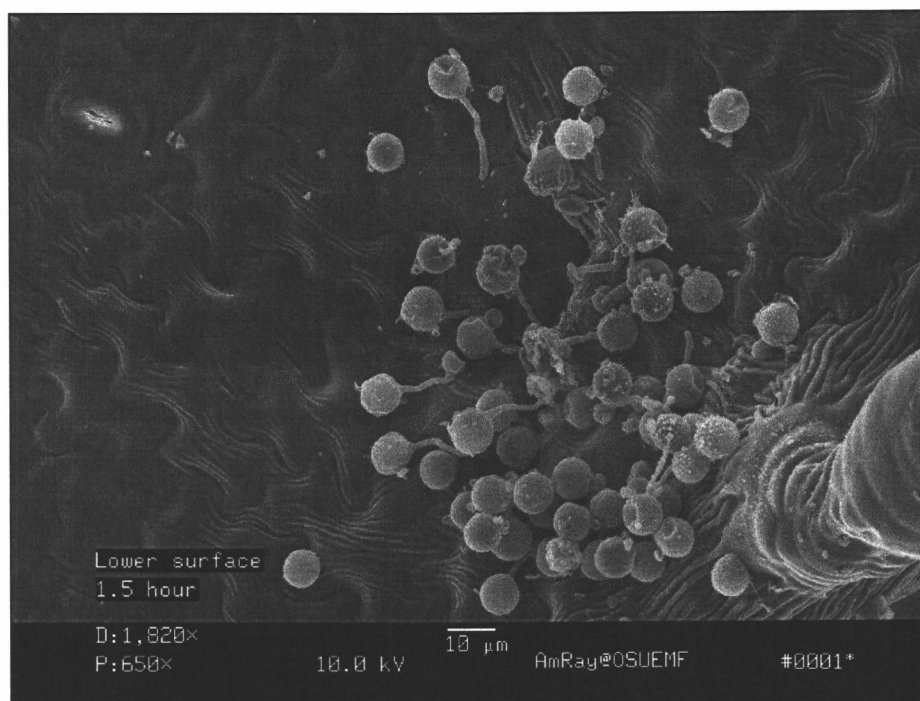


Fig. 27. Lower surface of tissue culture leaf 1.5 h post inoculation. Germ tubes of germinating cysts are oriented towards a crack (arrows) in cuticle (top), germ tube is penetrating into crack (enlarged view, bottom).



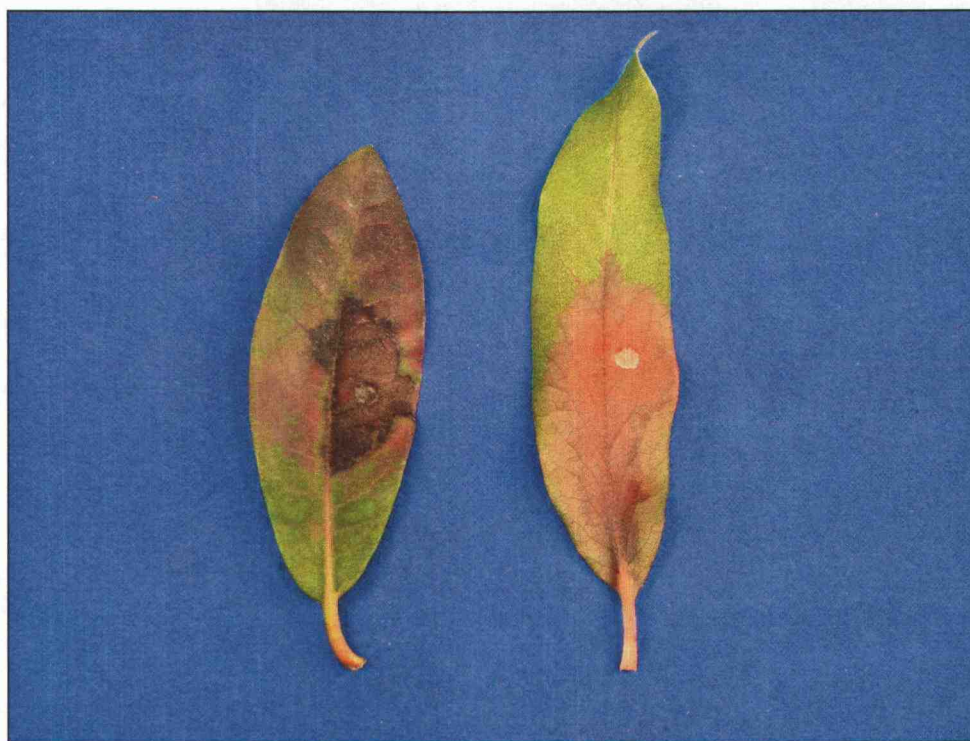


Fig. 28. Inoculated leaves from one plant. Lesions were larger and spread faster on young leaves (right leaf) compared to mature leaves (left leaf).



Fig. 29. Inoculated rhododendron leaves from two plants. Necrosis initiated from mid-rib inoculations (left) spread more rapidly than mid-blade inoculations (right).



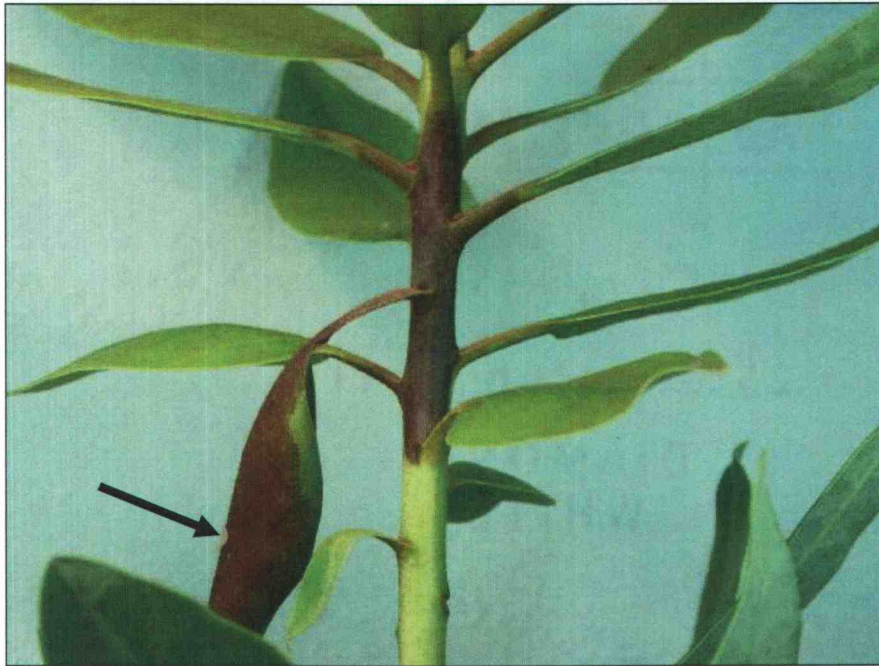


Fig. 30. Inoculated young leaf (arrow) and stem lesion. Infection spread from inoculated leaf through the petiole into the stem.

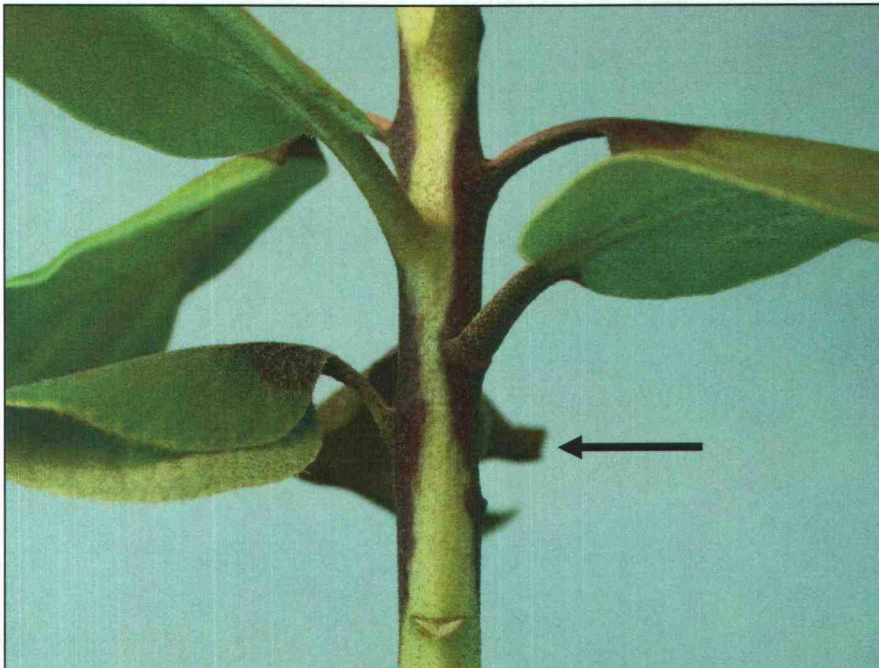


Fig. 31. Vertical lesion spread on stem of inoculated plant. Inoculated leaves indicated by arrows. Lesion from inoculated leaf (arrow) spread up and down stem into adjacent petioles.

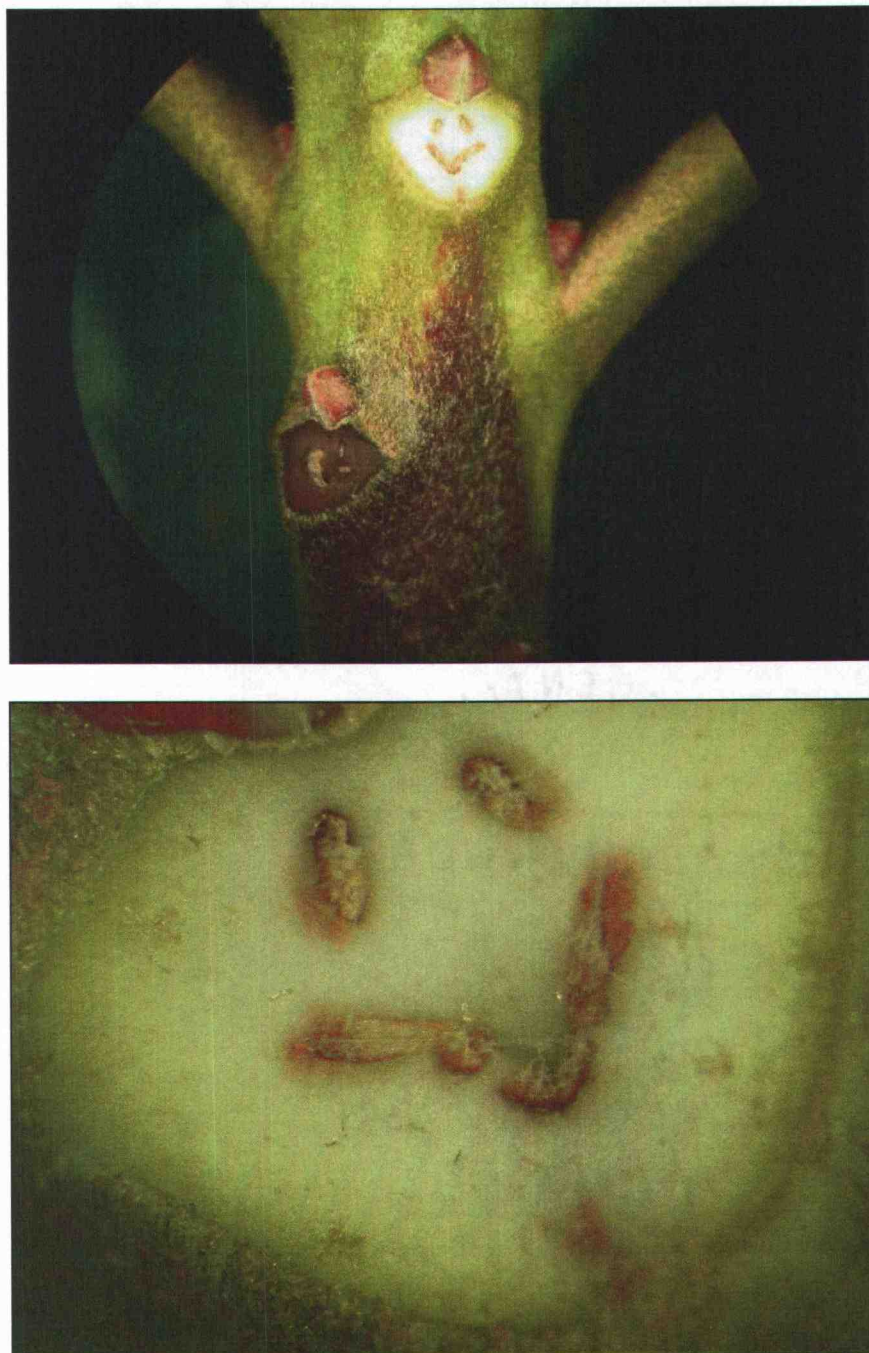


Fig. 32. Stem from inoculated plant with leaf removed just above the leading edge of infection (top). Leaf scar with discolored vascular bundles (bottom).

discolored showing dark green, brown and reddish areas (Fig. 33) when compared to the healthy green of the non-inoculated leaves (Fig. 14).

Isolation results from all three trials varied regardless of age, lesion size or pattern of spread, as there were often gaps in recovery of *P. ramorum* from symptomatic and non-symptomatic tissue. Often there was no recovery in tissue near the point of inoculation, but the pattern of recovery was not consistent. *P. ramorum* was recovered from brown necrotic tissues in addition to what appeared to be healthy green tissue (Fig. 34). Recovery frequency was greater in tissues between the inoculation site and the plant stem than it was between the inoculation site and the leaf tip. *P. ramorum* was only rarely recovered from the leaf tips.

In the first trial isolations were made from stem tissues from the leaf scar of an inoculated leaf, and just above and below the leading edge of the stem lesion. *P. ramorum* was recovered up to 1 cm from a visible lesion (Fig. 35). Isolations of stem tissues at the leaf scar of an inoculated leaf only yielded *P. ramorum* if the leaf was attached at the time of harvest. Isolation results were negative if the leaf had abscised.

Tissues from the first trial were collected for microscopy also from leading edges of lesions from both stems and petioles. Slides were stained with Calcofluor White M2R and viewed with epi-fluorescence. Evaluations of thin sections did not reveal any clear hyphal structures.

Additional stem and petiole samples were collected from the third trial performed in March to be used for microscopy and isolation evaluations. Plants were harvested 20 days after inoculation. Leaves of three of the *P. ramorum*-inoculated plants had abscised while the fourth *P. ramorum*-inoculated plant and the control plants retained all treated leaves.

Samples for isolation were collected from 4 *P. ramorum*-inoculated plants (2 with mid-blade, and 2 with mid-vein inoculation sites) while 2 control plants included





Fig. 33. Mid-blade inoculated leaves. Necrosis did not advance into petioles of mature leaves (left leaf), but necrosis advanced through petioles into stems on young leaves (right leaf).

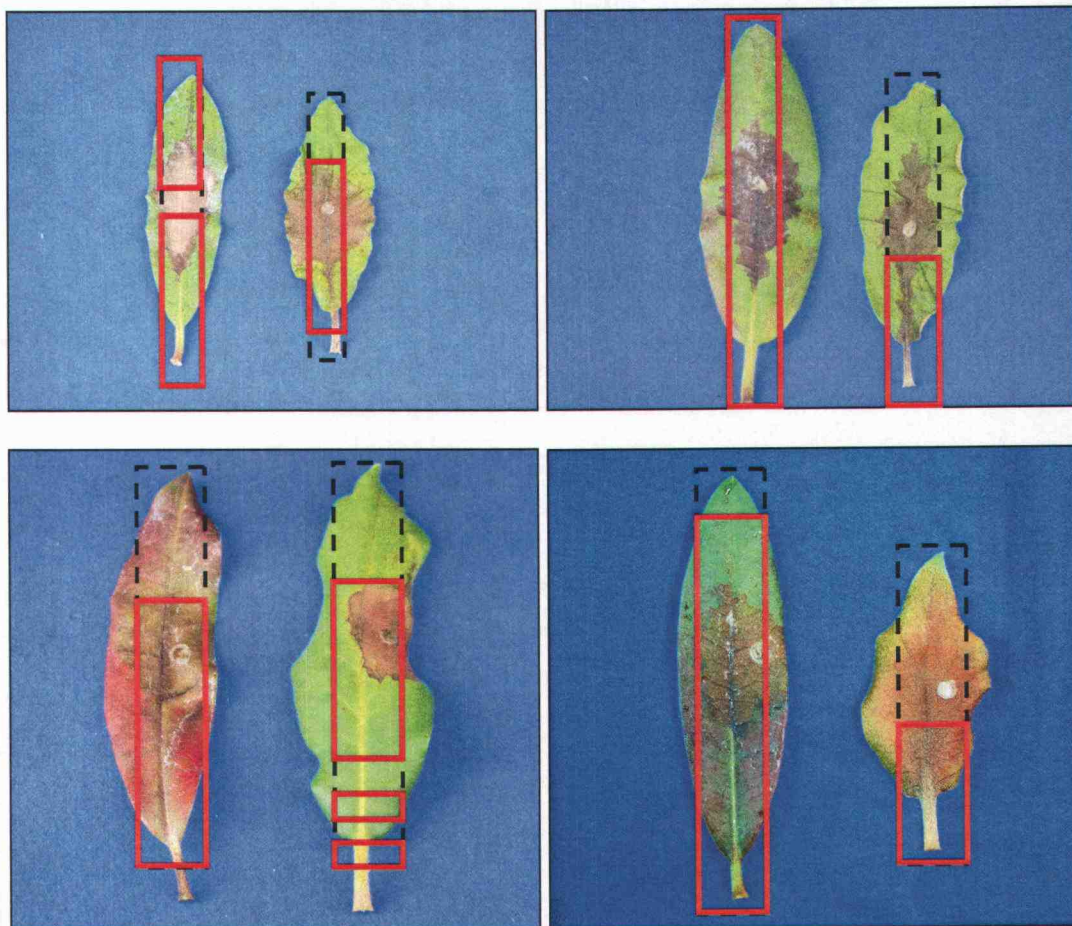


Fig. 34. Inoculated leaves from four plants. Leaves were trimmed for isolation (black dashed line). *P. ramorum* was recovered from areas surrounded by the red solid line. For 5 of the 8 leaves, recovery was greater between the inoculation site and petiole than for the area between the inoculation site and the leaf tip.



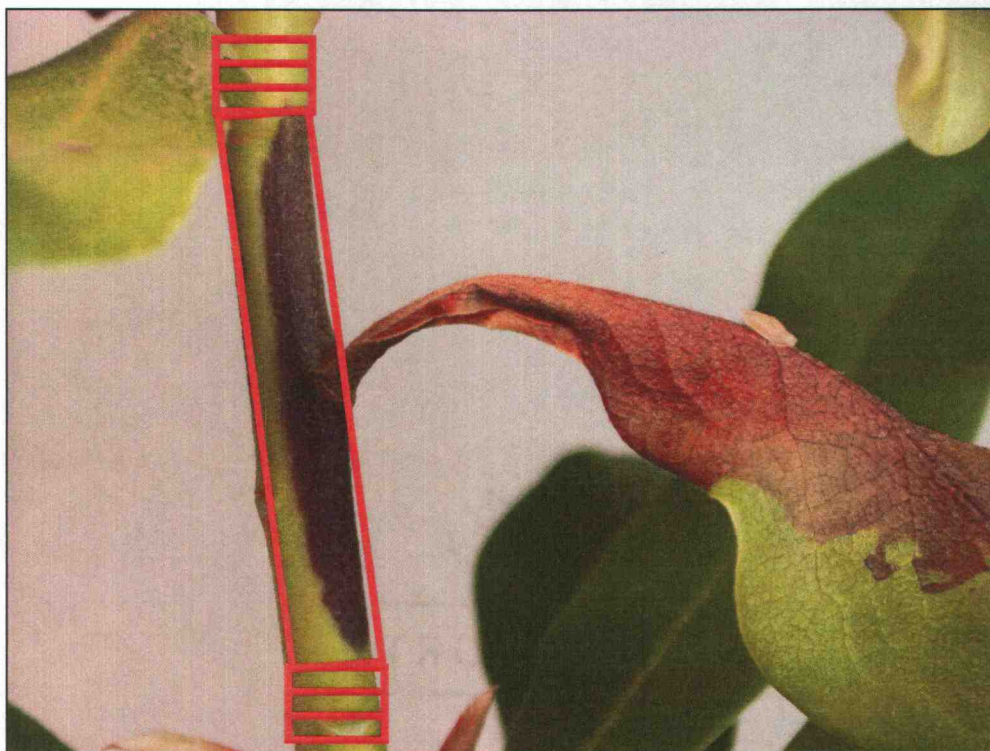


Fig. 35. Stem tissue sampled for isolation of *P. ramorum*. Isolation results yielded *P. ramorum* from entire lesion area and up to 1 cm beyond lesion (red lines).

one of each wounding method. Results showed that *P. ramorum* had spread from the inoculated leaf through the petiole and into the stems. The sampled stem tissues include 1 cm above and below the leaf scar of the inoculated leaf (Fig. 36).

*P. ramorum* was not recovered from any tissues collected from control plants. Stem tissues from the lower mature leaf sites from inoculated plants were positive for *P. ramorum* for 4 of 4 plants, even though only one plant had retained its inoculated leaf. Stem tissues from the young leaf sites were positive 1 of 4 times, but not from the only plant that had retained its inoculated leaf (Fig. 36). Stem tissue collected from the young leaf scar of the only plant that had retained its leaf, was negative in isolation even though it exhibited vascular discoloration (Fig. 37). The lower stem tissues were positive at the leaf scar, above and below the leaf scar, but the tissues opposite the leaf scar were negative. Lower stem tissue from the plant that had retained its leaf was sampled extensively. Isolations from those samples that revealed tissue from below the leaf scar was positive for *P. ramorum*, petioles adjacent to the inoculation site were positive, but stem and petioles opposite the inoculation site were negative (Fig. 38 and Fig. 39).

Samples for microscopy were only collected from 3 of the 6 plants: two plants inoculated on the mid-blade as well as the one control plant with the mid-blade wound and agar plug. Of the plants sampled for microscopy, they included the only plant that had retained its leaves. Microscopic examination of the petioles from the plant that had lost its leaves revealed that they were similar to the petioles of the control plants and showed no visible hyphal structures. On initial inspection of the plant that had retained its leaves, there were large necrotic areas in the lower mature leaf only, as the necrosis on the young leaf had not spread into the petiole (Fig. 40). Of all 4 *P. ramorum*-inoculated plants, this was the only one for which there was less necrosis on the younger leaf than on the mature leaf. Microscopic observation of thin sections did not reveal any hyphae in the petiole of the young leaf, while observations of the petiole from the mature leaf revealed hyphae of *P.*

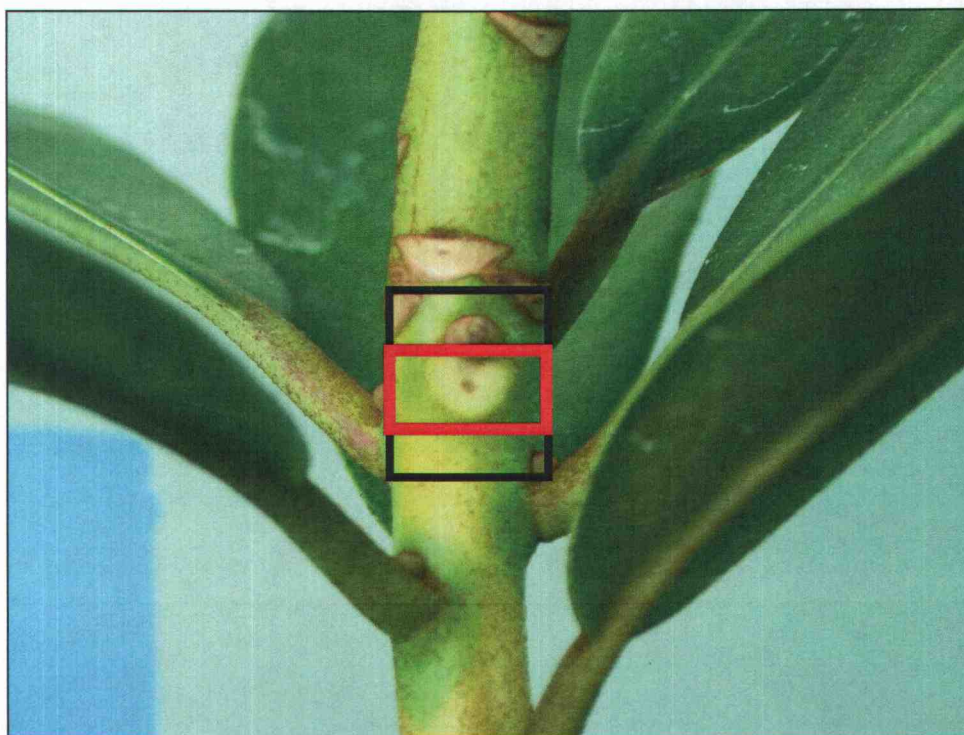


Fig. 36. Stem tissue around leaf scar collected for isolation from inoculated plant. Red rectangle shows the area near the leaf scar from which *P. ramorum* was recovered; *P. ramorum* was not recovered from the black rectangles above and below the leaf scar.



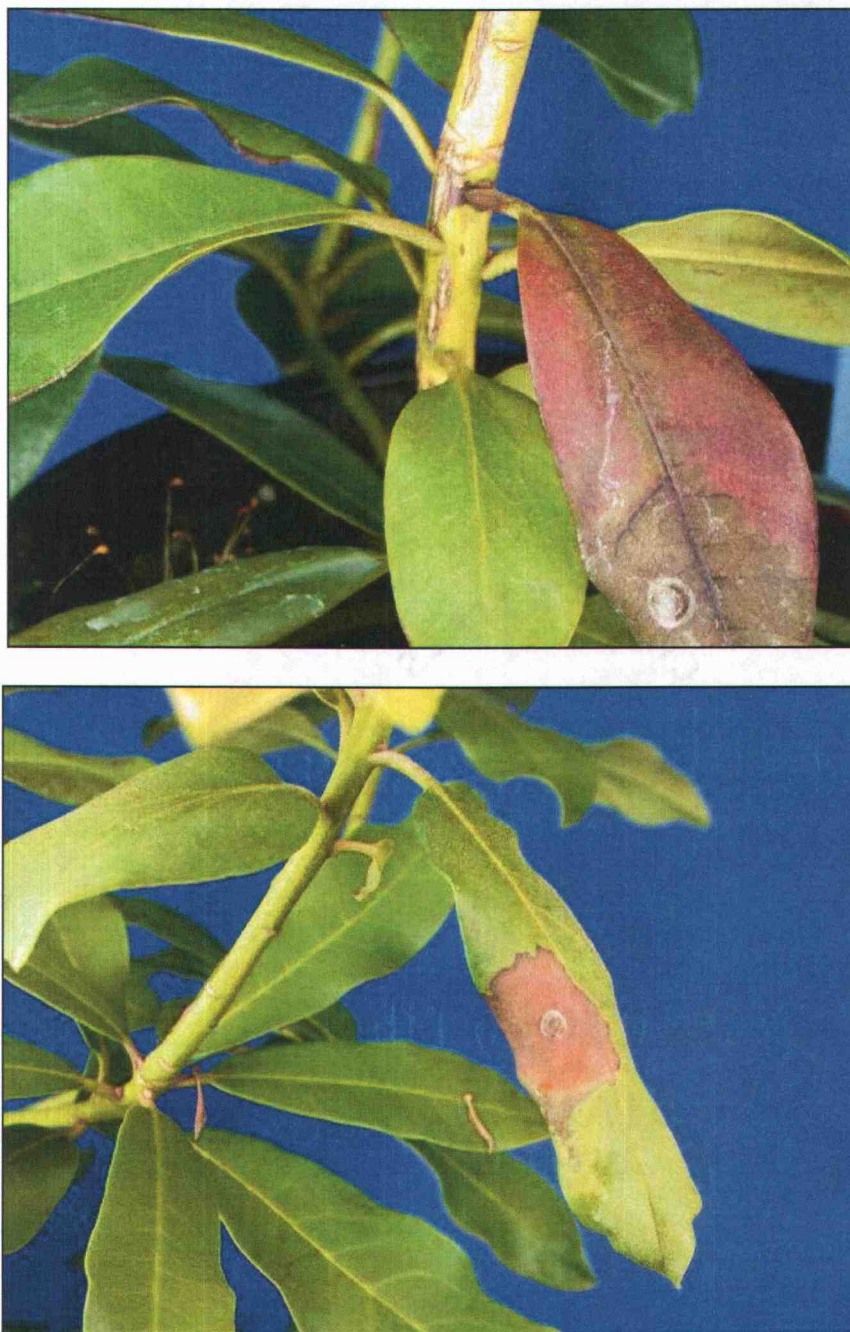


Fig. 37. Mature (top) and young (bottom) inoculated leaves. In only one of four plants, necrosis advanced through the mature leaf into the stem but spread was limited within the young leaf.



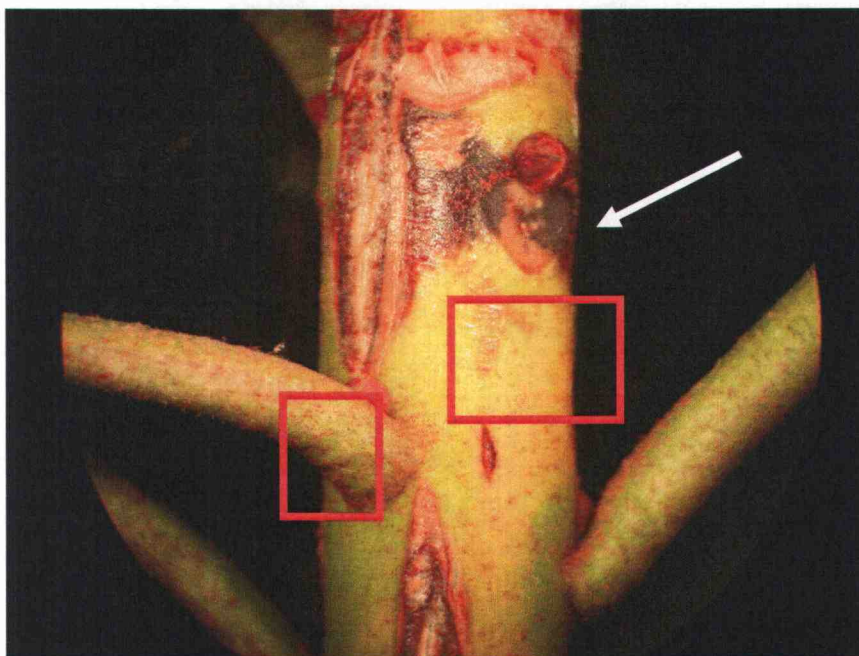


Fig. 38. Positive isolation results of inoculated lower stem. Discolored leaf scar (arrow) is where inoculated leaf was attached. Rectangles show areas of the stem below the leaf scar and adjacent petiole from which *P. ramorum* was recovered. Hyphae were visible in thin sections of petiole (Fig.40) and stem (Fig.41) from this plant.

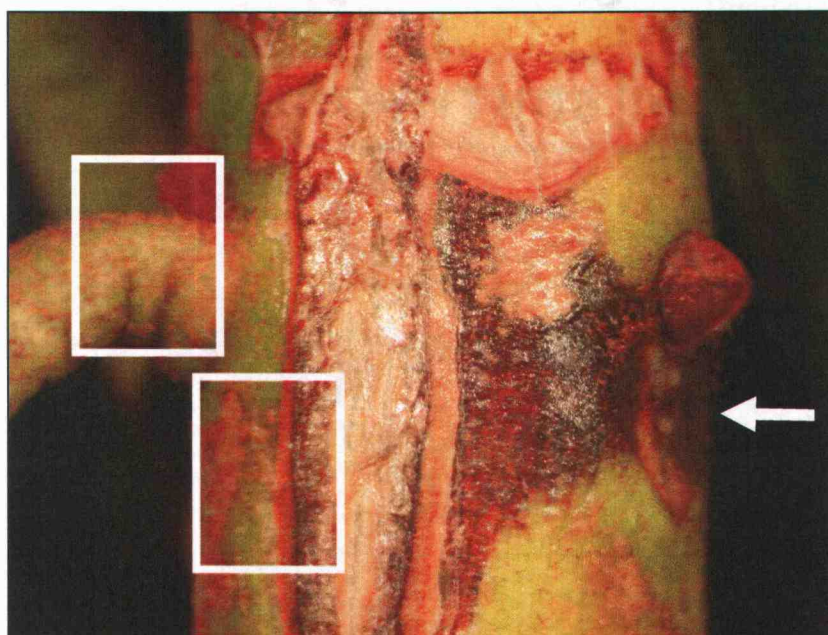


Fig 39. Negative isolation results of inoculated lower stem. The same stem from Fig. 38. *P. ramorum* was not recovered in isolation from stem or petiole opposite leaf scar (rectangles) where inoculated leaf was attached (arrow).

*ramorum* in the main vascular bundle and concentrated in the primary xylem tissues (Fig. 41).

Samples of stem tissues collected for microscopy included the entire leaf scar and the stem just below the scar (similar to Fig. 36). Evaluations of the thin sections revealed that hyphal structures were not visible in any of the tissues from the leaf scar itself even though adjacent tissues were positive in isolation. Hyphae were only visible in the primary xylem cells where the vascular tissue of the inoculated leaf had been connected to vascular tissue of the stem (Fig. 42, Fig.43). This juncture of vascular tissue is lower inside the stem than the leaf scar itself (Fig. 44).

### 3.4 Discussion

Foliar infection initiation sites were evaluated to determine if stomata were required for infection by *Phytophthora ramorum*. After inoculating with a zoospore suspension and comparing attachment sites and germination of cysts, differences were observed between mature leaves of greenhouse grown plants with leaves of tissue culture plantlets. During the first trial I did not observe germinating cysts of *Phytophthora ramorum* in association with stomata on mature leaves. In the second and third trials using tissue culture plantlet leaves, germinating cysts were commonly found either directly on or very near stomatal openings, many of them germinating directly into the stomate. One explanation for the difference is that the few, small areas examined with SEM were inadequate to randomly and thoroughly sample the leaf surfaces, leading to biased results. Another possible explanation is that tissue culture plantlets are physiologically or morphologically different from mature, greenhouse-grown plants. Leaf surfaces of mature leaves were colonized by other organisms not present on tissue culture leaves. Another explanation might be that those organisms could either act competitively with *P. ramorum*, or have initiated a host defense response prior to inoculation, neither of which would be occurring with sterile tissue culture leaves. Tissue culture plantlets are produced in agar that contains hormones used to control and stimulate their growth. It is also



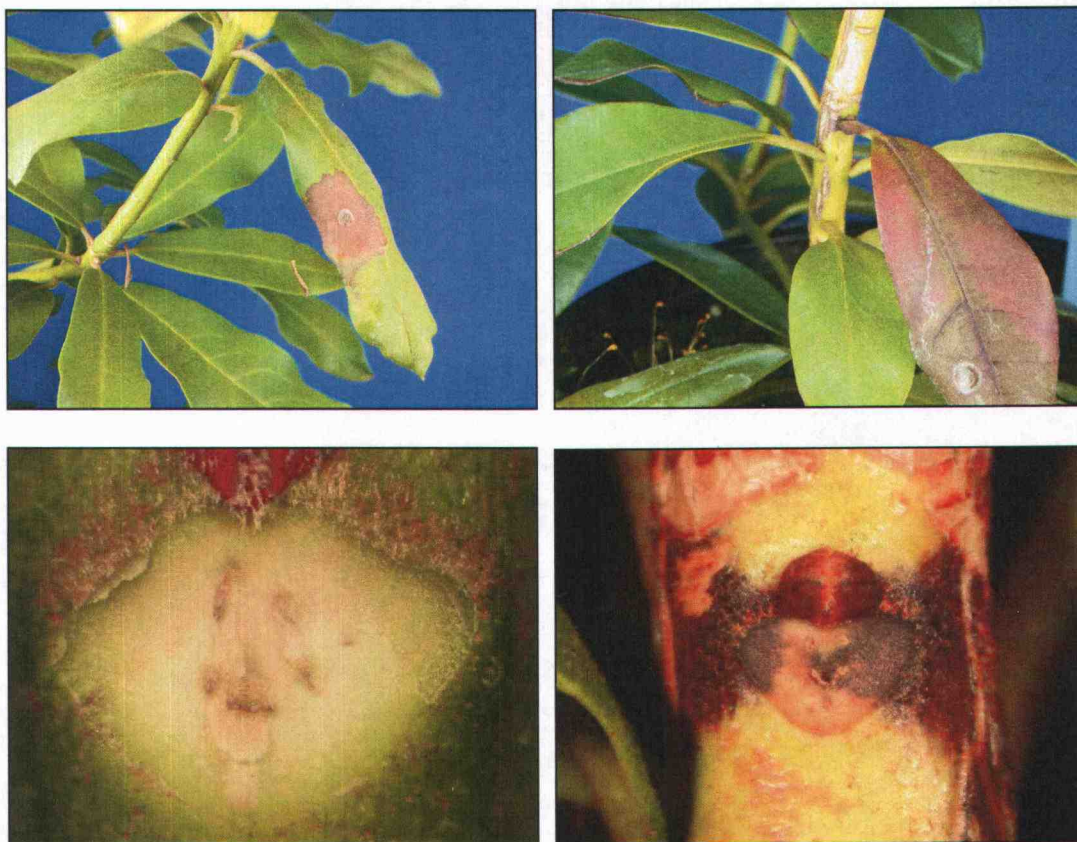


Fig. 40. Inoculated leaves and corresponding leaf scars. Young inoculated leaf (upper left) and leaf scar (bottom left): necrosis was limited to the leaf and *P. ramorum* was not recovered from the leaf scar. Mature inoculated leaf (upper right) and leaf scar (bottom right): necrosis spread from leaf through petiole into stem. *P. ramorum* hyphae were observed in xylem of petiole and the stem tissue.

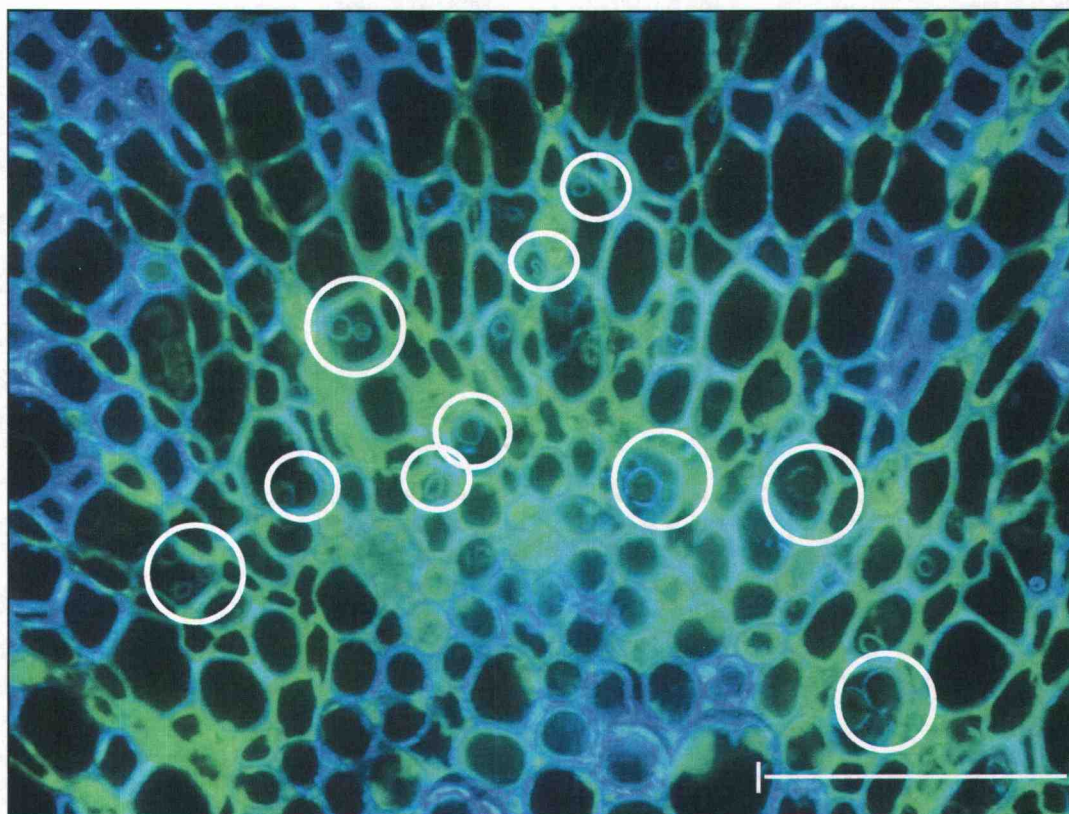


Fig. 41. Primary xylem of petiole from mature inoculated leaf. *P. ramorum* hyphae (circles) in cross section inside cells. bar = 50  $\mu$ m





Fig. 42. Cross section of inoculated stem. *P. ramorum* hyphae observed in primary xylem tissues of areas where vascular systems from leaf and stem connect (circles) (refer to Fig. 44). Bar = 200  $\mu$ m

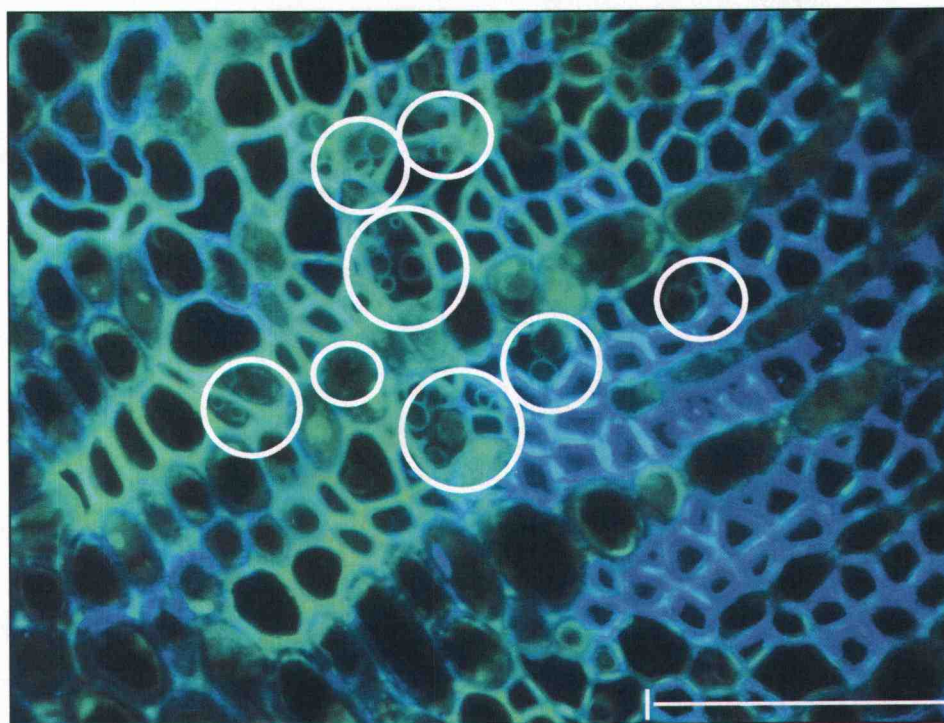


Fig. 43. Cross section of stem tissue to which the inoculated leaf was attached. Hyphae (circles) visible inside primary xylem cells. Bar = 50  $\mu$ m



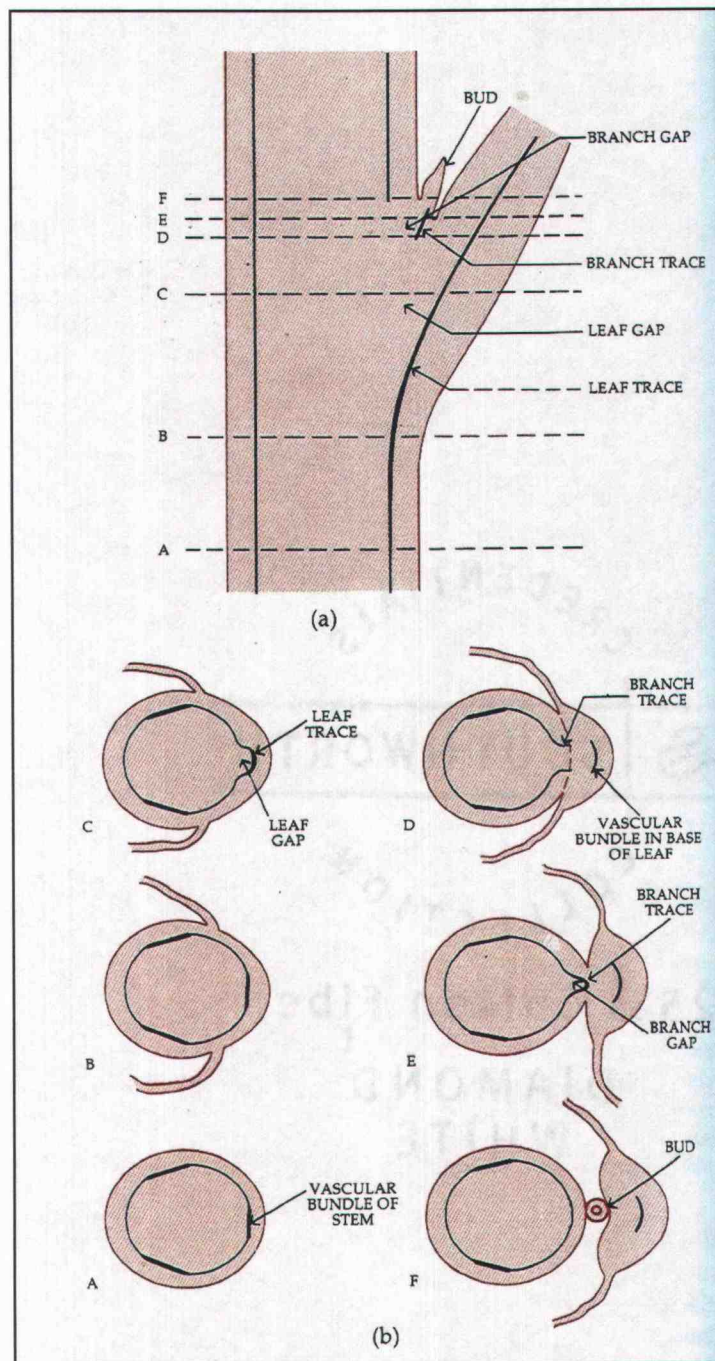


Fig. 44. Drawing of longitudinal section of stem (a) and corresponding cross-sections (b) (Raven et al. 1986). Hyphae were not visible in stems of inoculated plants when tissues were collected at leaf scar (C), while hyphae were visible in primary xylem when tissues were collected from below leaf scar (B) where vascular system of leaf and stem combine.

possible that the presence of these hormones in the tissues may act as an attractant, something that needs further investigation (S. Werres, personal communication). The fact that cysts were observed germinating and penetrating the cuticle directly suggests that stomata are not absolutely required for infection, although this might occur preferentially on some tissues.

Conflicting findings on the cytology and histology of *Phytophthora* spp. infection indicates that the need for stomatal penetration is not resolved, even for the well-studied *P. infestans*. I observed one occasion of penetration of a guard cell, which has been documented as a preferential infection site by *P. infestans* on potato (Hohl and Suter 1976) while other studies of *P. infestans* document stomata as preferred entry sites through the leaf. My observations of penetration of the cuticle through both the periclinal wall (cell wall parallel to surface) and anticlinal walls (cell wall perpendicular to surface) of the epidermis are supported by previous reports on *P. infestans* and *P. megasperma* var. *sojae* exhibiting the same infection methods (Stossel et al. 1980). A preliminary report indicated the apparent association of germinating cysts of *P. ramorum* with stomata on *Vaccinium ovatum* (Florance 2002). Subsequent work comparing *P. ramorum* germination patterns on rhododendron and other foliar hosts indicated *P. ramorum* can penetrate stomata (Oh et al. 2005). Host factors such as plant species or cultivar, leaf age, leaf position, thickness of the cuticle, and other factors may contribute to the inconsistency in the requirement for stomatal penetration by *Phytophthora* species. To fully resolve this question, trials comparing different hosts, in addition to the frequency of cyst association with and penetration of stomata, should be quantified on random samples of leaves inoculated with *P. ramorum* zoospores.

The “fuzzy” structures observed on hyphae of germinating cysts are of unknown function. Although they resemble appressoria, they appear different from appressoria of other *Phytophthora* species in several ways (H. Judelson, personal communication). Hyphal penetration directly underneath the “fuzzy” structures was not observed, and although the swollen structures initially appear to be robust,

as time progresses, they continue to elongate. It would be interesting to observe *P. ramorum* cyst germination and penetration on other species to see if “fuzzy” structures are formed on leaves of other hosts.

The progression of infection and disease spread I observed is similar to other laboratory experiments using artificial inoculation methods on plants from the Ericaceae family (Hansen et al. 2005). Inoculated plant leaves often responded to inoculation by senescing and abscising. This could be caused by increased ethylene production due to stress in addition to infection from the pathogen (Agrios 1979). Often *P. ramorum* could not be recovered from stem tissue if the leaf was no longer attached, possibly the result of a hypersensitive response to prevent advancement of infection. For plants that retained their leaves and for which tissue were young and succulent, infections that did advance into stems followed a vertical pattern, spreading both up and down the stems as previously described (Davidson et al. 2005; Tjosvold et al. 2004). Isolation results provide more evidence of a vertical pattern of colonization. In more mature plants the spread of infection was slower, but given longer incubation times, followed the same pattern. This pattern of spread indicates the possibility of colonization and advancement through vascular tissue. The discolored vascular bundles found in leaf scars at the leading edge of stem lesions are other indicators that the vascular system is being colonized by the pathogen. While *P. ramorum* is described as a strictly aerial pathogen infecting leaves, twigs, and stems or boles (Davidson et al. 2005; Rizzo 2003), no mention is made of the tissues actually colonized, or the mode of spread within the plant. My results (Chapters 2 and 3) suggest that it can spread in the vascular tissue and it may not be restricted to aerial plant parts (Chapter 2).

In addition to better understanding its biology, understanding what tissues are colonized by *P. ramorum* and how the pathogen spreads through the plant is critical in understanding how to control the pathogen. Knowledge of its infection biology will assist in establishing regulatory and mitigation guidelines, as well as developing control strategies that can be employed in the nursery setting. If plants



such as rhododendron can be infected with *P. ramorum* and appear asymptomatic, then the risk for spread of the pathogen is potentially very high. My work has shown that an infected leaf can abscise, leaving a stem with no sign of infection to the untrained eye, yet the pathogen can still be recovered from the plant. There is evidence to suggest that this may also occur in commercially propagated nursery stock infected naturally with *P. ramorum*. In January, 2005, a shipment of asymptomatic rhododendrons arrived at UC Davis from a local California nursery. Plants were placed in a lath house and did not express visible symptoms until one month later, when the pathogen was recovered from the stems, leaves, and roots in 40 out of 44 plants (Bienapfl et al. 2005). Modification of the current regulatory guidelines to include the sampling of asymptomatic tissues could decrease the likelihood that infected plants are shipped.

## Literature Cited

- Agrios, G.N. 1979. Plant Pathology. Academic Press. San Diego, CA.
- Bienapfl, J., Zanzot, J.W., Murphey, S.D., Garbelotto, M., and Rizzo, D.M. 2005. Isolation of a new lineage of *Phytophthora ramorum* from asymptomatic stems and roots of a commercial lot of rhododendron in California. *Phytopathology* 95:S20.
- Davidson, J.M., Wickland, A.C., Patterson, H.A., Falk, K., and Rizzo, D.M. 2005. Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. *Phytopathology* 95:587-596.
- Erwin, D.C., Bartnicki-Garcia, S., and Tsao, P.H. 1983. *Phytophthora: its Biology, Taxonomy, Ecology, and Pathology*. APS Press, St. Paul, MN.
- Erwin, D.C., and Ribeiro, O.K. 1996. *Phytophthora Diseases Worldwide*. APS Press, St. Paul, MN.
- Florance, E.R. 2002. Plant structures through which *Phytophthora ramorum* establish infections. Sudden Oak Death Science Symposium - State of Our Knowledge. Monterey, CA.
- Hansen, E.M., Parke, J.L., and Sutton, W. 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. *Plant Dis.* 89:63-70.
- Lewis, C., and Parke, J.L. 2005. Pathways of infection of *Phytophthora ramorum* in rhododendron. in: Proc. Sudden Oak Death Sci. Symp., 2nd. P.J. Shea and M. Haverty, (eds). Pacific Southwest Res. Stn., For. Serv., USDA. Albany, CA. (In press).
- Oh, E., Stone, J.K., and Hansen, E.M. 2005. Comparison of foliar infection by *Phytophthora ramorum* on different host. in: Proc. Sudden Oak Death Science Symposium Sci. Symp., 2nd. P.J. Shea and M. Haverty, (eds). Pacific Southwest Res. Stn., For. Serv., USDA..Albany, CA. (In press)
- Raven, P.H., Evert, R.F., and Eichorn, S.E. 1986. *Biology of Plants*. Fourth ed. Worth Publishers. New York, NY.
- Rizzo, D.M., Garbelotto, M., Davidson, J.M., Slaughter, G.W., and Koike, S.T. 2002. *Phytophthora ramorum* and sudden oak death in California: I. Host relationships. Pages 733-740 In: Proc. Fifth Symp. Oak Woodlands in Californias Changing Landscape, San Diego, CA. Gen. Tech. PSW-GTR-184 R. Standiford, D. McCreary and K.L. Purcell, (eds). Pacific Southwest Res. Stn. For. Serv. USDA. Albany, CA.

### Literature Cited (Continued)

- Rizzo, D.M. 2003. Sudden Oak Death: Host plants in forest ecosystems in California and Oregon. <http://sod.apsnet.org/> [Cited July 20, 2005].
- Stossel, P., Lazarovits, G., and Ward, E.W.B. 1980. Penetration and growth of compatible and incompatible races of *Phytophthora megasperma* var. *sojae* in soybean hypocotyl tissues differing in age. Can. J.Bot. 58:2594-2601.
- Tjosvold, S., Buermeyer, K.R., Blomquist, C., and Frankel, S. 2004. Nursery Guide for Diseases Caused by *Phytophthora ramorum* on Ornamentals: Diagnosis and Management. University of California Division of Agriculture and Natural Resources. Publ. 8156.

## Chapter 4

### Conclusion and Future Directions

The goal of this research was to help better understand the biology and histopathology of *Phytophthora ramorum*. *P. ramorum* is known primarily as a foliar pathogen, infecting its plant hosts through dispersal of infective propagules onto above-ground trunks, stems, and leaves. I discovered that *P. ramorum* can, at least under certain circumstances, infect plants and cause disease through below-ground inoculum. I also discovered that *P. ramorum* infects initially through vascular tissue (xylem) leading to subtle signs of infection that could possibly go undetected as the plants initially appear asymptomatic. These new aspects of *P. ramorum*'s biology are important for control and mitigation decisions because it increases the potential risk of long term transport of the pathogen via nursery crops. My work suggests that other sources of *P. ramorum* (roots, above and below-ground stems and potting media) should be considered when investigating potential sources of contamination in nurseries.

Rhododendrons, due to their popularity, will continue to be a major component of ornamental landscapes in residential and commercial settings for many years to come. Because they are easily cultivated and have many uses in ornamental landscapes, countless new varieties are added to the market every year. In the past introductions of these new varieties have been the results of accomplishments in improving existing lines breeding for shape, size, color, size of flower instead of developing varieties resistant to diseases or insects (Baker and Linderman 1979). Selection of rhododendron cultivars resistant to *Phytophthora* diseases has not been a high priority for the nursery industry despite their susceptibility and importance as vectors, but the threat of *P. ramorum* could change this. It is possible that the driving force might not come from the nurseries themselves, but from the public. It may take an educated consumer demanding resistant varieties when purchasing ornamental stock to encourage the production and availability of safer options.

The severity of disease on rhododendron cv. "Nova Zembla" was dramatic when inoculated with *P. ramorum* isolate 03-74-N11-A. The resulting infections were rapid with large amounts of necrosis. While this isolate did originate from an Oregon nursery, it is atypical of most U.S. nursery isolates in that it has the mating type (A1) and genotype characteristic of European isolates. Future work on rhododendrons could include the evaluation of infections caused by isolates with the A2 mating type and North American genotype. In general, isolates with the A2 mating type are reported to be less virulent than isolates with the A1 mating type (Brasier et al. 2002), but both A1 and A2 isolates have been shown to infect rhododendrons from artificially infested potting media (Parke et al. 2004).

*P. ramorum* has caused major economic and logistic problems for the nursery industry in the United States, especially on the west coast. It has also caused extensive tree mortality in coastal California regions changing the ecosystems of those forests for many years to come. These changes have occurred in an unprecedented short amount of time. With new hosts still being confirmed and new aspects of its biology being discovered, there is still much to be learned about the full potential of the pathogen. The seriousness with which *P. ramorum* can alter our native forests is the biggest concern driving scientific and regulatory efforts to understand and control this pathogen. Given the current practice of global transport of plant and plant products, and the vulnerability of our own eastern forests, the implications of this research are serious. Current guidelines restricting the use of *P. ramorum* required by quarantine regulations limits the flexibility of researchers. There is much work left to be done with regard to this pathogen, as there are still unanswered questions about *P. ramorum*'s survivability and infection potential. This is a long term goal which can only be reached with the commitment and cooperation of scientists and regulators as well as nursery growers.

## Literature Cited

- Baker, K.F., and Linderman, R.G. 1979. Unique features of the pathology of ornamental plants. *Ann. Rev. Phytopathol* 17:253-277.
- Brasier, C., Rose, J., Kirk, S.A., and Webber, J.F. 2002. Pathogenicity of *Phytophthora ramorum* isolates from North America and Europe to bark of European *Fagaceae*, American *Quercus rubra* and other forest trees. Sudden Oak Death Sci. Symp: The State of Our Knowledge. Monterey, CA.
- Parke, J.L., Linderman, R.G., Osterbauer, N.K., and A., G.J. 2004. Detection of *Phytophthora ramorum* blight in Oregon nurseries and completion of Koch's Postulates on *Pieris*, *Rhododendron*, *Viburnum*, and *Camellia*. *Plant Dis* 88:87.



## Bibliography

- Agrios, G.N. 1979. Plant Pathology. Academic Press. San Diego, CA.
- Baker, K.F., and Linderman, R.G. 1979. Unique features of the pathology of ornamental plants. *Ann. Rev. Phytopathol.* 17:253-277.
- Benson, D.M., and Hoitink, H.A.J. 1986. *Phytophthora dieback*. In: Compendium of Rhododendron and Azalea Diseases. Pages 12-15. D.L. Coyier and M.K. Roane eds. St. Paul, MN. APS Press.
- Bienapfl, J., Zanzot, J.W., Murphey, S.D., Garbelotto, M., and Rizzo, D.M. 2005. Isolation of a new lineage of *Phytophthora ramorum* from asymptomatic stems and roots of a commercial lot of rhododendron in California. *Phytopathology* 95:S20.
- Bonants, P., Verstappen, E., Weijacha, K., de Vries, I., and Ivors, K. 2005. Molecular identification and detection of *Phytophthora ramorum*. In: Proc. Sudden Oak Death Sci. Symp., 2nd, P.J. Shea and M. Haverty, (eds). Pacific Southwest Res. Stn., For. Serv., USDA. Albany, CA. (In press).
- Brasier, C., Rose, J., Kirk, S.A., and Webber, J.F. 2002. Pathogenicity of *Phytophthora ramorum* isolates from North America and Europe to bark of European *Fagaceae*, American *Quercus rubra* and other forest trees. Sudden Oak Death Sci. Symp: The State of Our Knowledge. Monterey, CA.
- Colburn, G.C., Sechler, K., and Shishkoff, N. 2005. Survivability and pathogenicity of *Phytophthora ramorum* chlamydospores in soil. *Phytopathology* 95:S20.
- COMTF. 2005. A disease chronology of *Phytophthora ramorum*, cause of sudden oak death and other foliar diseases. <http://www.suddenoakdeath.org/>.
- Davidson, J.M., Rizzo, D.M., Garbelotto, M., Tjosvold, S., and Slaughter, G.W. 2002. *Phytophthora ramorum* and sudden oak death in California: IV. Transmission and survival. Pages 741-749 In: Proc. Fifth Symp. Oak Woodlands: Oak Woodlands in California's Changing Landscape R.B. Standiford, D. McCreary and K.L. Purcell, (eds). Gen. Tech. Rep. PSW-GTR-184. Pacific Southwest Res. Stn., For. Serv., USDA, Albany, CA.
- Davidson, J.M., Werres, S., Garbelotto, M., Hansen, E.M., and Rizzo, D.M. 2003. Sudden oak death and associated diseases caused by *Phytophthora ramorum*. *Plant Health Progress*. doi:10.1094/PHP-2003-0707-01-DG.
- Davidson, J.M., Wickland, A.C., Patterson, H.A., Falk, K., and Rizzo, D.M. 2005. Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. *Phytopathology* 95:587-596.

## Bibliography (Continued)

- Erwin, D.C., Bartnicki-Garcia, S., and Tsao, P.H. 1983. *Phytophthora*: its Biology, Taxonomy, Ecology, and Pathology. APS Press, St. Paul, MN.
- Erwin, D.C., and Ribeiro, O.K. 1996. *Phytophthora* Diseases Worldwide. APS Press, St. Paul, MN.
- Florance, E.R. 2002. Plant structures through which *Phytophthora ramorum* establish infections. Sudden Oak Death Science Symposium - State of Our Knowledge. Monterey, CA.
- Fichtner, E.S., Lynch, S., and Rizzo, D.M. 2005. Summer survival of *Phytophthora ramorum* in forest soils. Phytopathology 95:S20.
- Fichtner, E.S., Lynch, S., and Rizzo, D.M. 2005. Seasonal survival of *Phytophthora ramorum* in soils. Phytopathology 95:S20.
- Garbelotto, M., Rizzo, D.M., and Marais, L. 2002. *Phytophthora ramorum* and sudden oak death in California: IV. Chemical control. Pages 811-818 In: Proc. Fifth Symp. Oak Woodlands in California's Changing Landscape R. Standiford, D. McCreary and K.L. Purcell, (eds). Gen. Tech. Rep. PSW-GTR-184. Pacific Southwest Research Station, Forest Service, USDA, Albany, CA.
- Goheen, E.M., Hansen, E.M., Kanaskie, A., McWilliams, M.G., Osterbauer, N., and Sutton, W. 2002. Eradication of sudden oak death in Oregon. Phytopathology 92:S30.
- Hansen, E.M., Sutton, W., Parke, J.L., and Lindermann, R.G. 2002. *Phytophthora ramorum* and Oregon forest trees - one pathogen, three diseases. Sudden Oak Death, a Science Symposium: The State of Our Knowledge, Monterey, CA.
- Hansen, E.M., Reeser, P., Sutton, W., and Winton, L.M. 2003. First report of A1 mating type of *Phytophthora ramorum* in North America. Plant Dis. 87:1267.
- Hansen, E.M., Parke, J.L., and Sutton, W. 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. Plant Dis. 89:63-70.
- Lewis, C., and Parke, J.L. 2005. Pathways of infection of *Phytophthora ramorum* in rhododendron. in: Proc. Sudden Oak Death Sci. Symp., 2nd. P.J. Shea and M. Haverty, (eds). Pacific Southwest Res. Stn., For. Serv., USDA. Albany, CA. (In press).

## Bibliography (Continued)

- Ivors, K., Hayden, K., Bonants, P., Rizzo, D.M., and Garbelotto, M. 2003. AFLP and phylogenetic analyses of North American and European populations of *Phytophthora ramorum*. *Mycol. Res.* 108:378-392.
- Jeffers, S. 2005. Recovery of *Phytophthora ramorum* from soilless mixes around container-grown ornamental plants. *Phytopathology* 95:S20.
- Kaminski, K.S., Wagner, S., Werres, S., Beltz, H., Seipp, D., and Brand, T. 2005. Infectivity and survival of *P. ramorum* in recirculating water of nurseries. In: Proc. Sudden Oak Death Sci. Symp. 2nd. P.J. Shea and M. Haverty, (eds). Pacific Southwest Res. Stn., For. Serv., USDA. Albany, CA. (In press).
- Kelly, M., and Meentemeyer, R.K. 2002. Landscape dynamics of the spread of sudden oak death. *Photogramm. Eng. Remote Sensing.* 68:1001-1009.
- Linderman, R.G., and Davis, A. 2005. Survival of *Phytophthora ramorum* in potting mix components or soil and eradication with aerated steam treatment. *Phytopathology* 95:S20.
- Matheron, M.E., and Mircetich, S.M. 1985. Influence of flooding duration on development of *Phytophthora* root and crown rot of *Juglans hindsii* and Paradox walnut rootstocks. *Phytopathology* 75:973-976.
- McLaughlin, I., Sutton, W., and Hansen, E.M. 2005. Survival of *Phytophthora ramorum* in tanoak and rhododendron leaves. *Phytopathology* 95:S20.
- ODA. 2003. Final report on *Phytophthora ramorum* in Clackamas County, Oregon. Oregon Department of Agriculture.  
<http://oda.state.or.us/plant/ppd/path/SOD/030624SODrpt.pdf>. [Cited on July 20, 2005]
- Oh, E., Stone, J.K., and Hansen, E.M. 2005. Comparison of foliar infection by *Phytophthora ramorum* on different host. in: Proc. Sudden Oak Death Science Symposium Sci. Symp., 2nd. P.J. Shea and M. Haverty, (eds). Pacific Southwest Res. Stn., For. Serv., USDA. Albany, CA. (In press)
- Parke, J.L., Roth, M., and Choquette, C. 2005. *Phytophthora ramorum* disease transmission from infested potting media. in: Proc. Sudden Oak Death Sci. Symp., 2nd. P.J. Shea and M. Haverty, (eds). Pacific Southwest Res. Stn., For. Serv., USDA. Albany, CA. (In press).

## Bibliography (Continued)

- Parke, J.L., Linderman, R.G., Osterbauer, N.K., and A., G.J. 2004. Detection of *Phytophthora ramorum* blight in Oregon nurseries and completion of Koch's Postulates on *Pieris*, *Rhododendron*, *Viburnum*, and *Camellia*. Plant Dis 88:87.
- Raven, P.H., Evert, R.F., and Eichorn, S.E. 1986. Biology of Plants. Fourth ed. Worth Publishers. New York, NY.
- Rizzo, D.M., Garbelotto, M., Davidson, J.M., Slaughter, G.W., and Koike, S.T. 2002. *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. Plant Dis. 86:205-214.
- Rizzo, D.M., Garbelotto, M., Davidson, J.M., Slaughter, G.W., and Koike, S.T. 2002. *Phytophthora ramorum* and sudden oak death in California: I. Host relationships. Pages 733-740 In: Proc. Fifth Symp. Oak Woodlands in Californias Changing Landscape, San Diego, CA. Gen. Tech. PSW-GTR-184 R. Standiford, D. McCreary and K.L. Purcell, (eds). Pacific Southwest Res. Stn. For. Serv. USDA. Albany, CA.
- Rizzo, D.M. 2003. Sudden Oak Death: Host plants in forest ecosystems in California and Oregon. <http://sod.apsnet.org/> [Cited July 20, 2005].
- Rizzo, D.M., and Garbelotto, M. 2003. Sudden oak death: endangering California and Oregon forest ecosystems. Front. Ecol. Environ. 1:197-204.
- Rizzo, D.M., Garbelotto, M., and Hansen, E.M. 2005. *Phytophthora ramorum*: integrative research and management of an emerging pathogen in California and Oregon forests. Annu. Rev. Phytophology 43:309-335.
- Shishkoff, N., and Tooley, P.W. 2004. Persistence of *Phytophthora ramorum* in nursery plants and soil. Phytopathology 94:S95.
- Stossel, P., Lazarovits, G., and Ward, E.W.B. 1980. Penetration and growth of compatible and incompatible races of *Phytophthora megasperma* var. *sojae* in soybean hypocotyl tissues differing in age. Can. J.Bot. 58:2594-2601.
- Tjosvold, S., Buermeyer, K.R., Blomquist, C., and Frankel, S. 2004. Nursery Guide for Diseases Caused by *Phytophthora ramorum* on Ornamentals: Diagnosis and Management. University of California Division of Agriculture and Natural Resources. Publ. 8156.

## Bibliography (Continued)

- Tjosvold, S.A., Chambers, D.L., Davidson, J.M., and Rizzo, D.M. 2002. Incidence of *Phytophthora ramorum* inoculum found in soil collected from a hiking trail and hikers' shoes in a California park. Sudden Oak Death Sci. Symp: The State of Knowledge. Monterey, CA.
- USDA-APHIS. 2005. List of hosts and plants associated with *Phytophthora ramorum*. [http://www.aphis.usda.gov/ppq/ispm/pramorum/pdf\\_files/usdaprlist.pdf](http://www.aphis.usda.gov/ppq/ispm/pramorum/pdf_files/usdaprlist.pdf). [Cited Aug 21,2005]
- Werres, S., Marwitz, R., Man in't Veld, W.A., Cock, W.A.M.d., Bonants, P.J.M., Weerdt, M., Themann, K., Ilieva, E., and Baayen, R.P. 2001. *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. Mycol. Res. 105:1155-1165.
- Werres, S., and De Merlier, D. 2003. First detection of *Phytophthora ramorum* mating type A2 in Europe. Plant Dis. 87:10.
- Winton, L.M., and Hansen, E.M. 2001. Molecular diagnosis of *Phytophthora lateralis* in trees, water, and foliage baits using multiplex polymerase chain reaction. For. Pathol. 31: 275-283.