AN ABSTRACT OF THE DISSERTATION OF

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Breeding Port-Orford-cedar for resistance to *Phytophthora lateralis*, a causal agent of root disease, begins by screening, through artificial inoculation, phenotypically resistant trees selected from natural stands. The successful program selected tolerant or resistant POC parent trees for the purpose of disease management. Candidate resistant POCs were used in my dissertation to: 1. validate screening methods such as stem- and root-dip inoculation; 2. test for increased virulence of *P. lateralis*; and 3. evaluate detection techniques. The results showed that the established screening methods were appropriate, and no evidence of changed virulence was found. A PCR technique was more reliable than other techniques for detection of *P. lateralis* in seedlings. An additional test for foliar infection showed that initial penetration through wounds and natural openings was possible.

POC seedlings and rooted cuttings from resistant and susceptible families were used to demonstrate resistance mechanisms. In order to explain the mechanisms at the cellular level, the susceptible response of POC seedlings to *P. lateralis* was first observed with light microscopy. Zoospores encysted on lateral roots, germinated, and penetrated by means of appressoria. Direct penetration between epidermal cells was common but penetration through epidermal cell walls was also observed. The hyphae

colonized the root cortex inter- and intracellularly. Wound inoculation on stems resulted in inter- and intra cellular hyphal growth in cambial, sieve, and parenchyma cells in the secondary phloem.

Several resistance mechanisms were observed: 1) there was a difference in zoospore attraction between susceptible and certain resistant POCs revealed by microscopic observation, direct count of encysted zoospores, and quantitative real-time PCR; 2) the frequency of encystment, penetration, and colonization of resistant seedlings was much lower than susceptible seedlings, but no differences in infection pathway were observed by means of light or electron microscopy; 3) collapsed cell walls were present in resistant POCs showing increased cell wall thickness, wall appositions, and electron dense materials.

Resistance Mechanisms of Port-Orford-Cedar to *Phytophthora lateralis*

by

Eunsung Oh

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

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Eunsung Oh, Author

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Resistance Mechanisms of Port-Orford-Cedar to *Phytophthora lateralis*

Chapter 1

Introduction

Port-Orford-cedar (*Chamaecyparis lawsoniana* (Murr.) Parl., POC) is threatened by the Oomycota pathogen *Phytophthora lateralis* Tucker and Milbrath. Selection and breeding for resistance is a key management strategy. This research was designed to support the resistance breeding program. Objectives in my dissertation are to validate resistance screening methods, to evaluate zoospore attraction responses on susceptible and resistant POC, to characterize infection and colonization on susceptible hosts, and finally to investigate the bases for resistant reactions by means of histological methods.

Although its distribution is limited, POC is an ecologically and economically important tree in the forests where it naturally occurs. It is also planted widely as an ornamental. The natural range of POC is restricted to Southwestern Oregon and Northern California, with a disjunct population near Mt. Shasta in California (Trione 1959). POC typically grows in mixtures with other conifers. On serpentine soils it may grow in association with rare plants. Also, it grows along side streams so that it contributes to stream channel stabilization and stream shading for fish habitat. Because of its thick and fibrous bark, POC is fire resistant and one of the longest lasting standing trees. The wood is decay resistant, so snags in forests are important

for wildlife habitat. Until about 10 years ago, POC logs were exported to Asian countries where its price was about 10 times higher than Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco) for furniture and building construction (Betlejewski et al. 2003, Hansen et al. 2000).

In 1923 POC first received attention from pathologists, nursery managers, and foresters in the Pacific Northwest region because of the presence of root rot disease in nurseries near Seattle, Washington. C. M. Tucker at the University of Missouri studied cultures of the causal agent and considered it to be a new species of *Phytophthora* (Milbrath 1940). Later, Tucker and Milbrath (1942) first described the symptoms, development, and spread of the disease, and formally described the new species *Phytophthora lateralis* Tucker and Milbrath. In 1938 presence of the pathogen in Oregon was reported by Milbrath and McWhorter (Trione 1959).

P. lateralis is believed to be an introduced pathogen and causes severe root rot disease in POC. The approximately 80 known species of *Phytophthora* are all destructive plant pathogens, causing rots of roots, crowns, stems, leaves, and fruits of a huge range of agriculturally and ornamentally important plants. Some species such as *P. cinnamomi*, *P. parasitica*, *P. cactorum*, and *P. ramorum* each attack numerous different plant species. Others, such as *P. sojae* and *P. infestans*, have narrower host ranges, infecting just a few host plant species (Tyler 2002). *P. lateralis* is only known to infect POC and Pacific yew (*Taxus brevifolia*). Pacific yew was reported as an additional host in 1991 (DeNitto 1991, Murray 1995, Murray and Hansen 1997), however, symptoms on Pacific yew have only been reported from trees growing within an infested area of POCs.

Many POCs along roads and streams in either forests or nurseries have been killed by *P. lateralis*. The disease affects seedlings, rooted cuttings, and small trees, as well as old established trees with trunks many centimeters in diameter (Roth et al. 1957, Torgeson et al. 1954, Trione 1959, Tucker et al. 1942). The typical symptom is reddish brown necrotic phloem, with a distinct margin between healthy tissue and infected areas on roots and stems (Erwin and Ribeiro 1996, Hansen et al. 2000, Roth et al. 1957, Torgeson et al. 1954). The pathogen rapidly colonizes the tree and causes mortality to POC of all ages within 1 year after infection (Hansen and Lewis 1997). Because of serious damage to POC, much research has been carried out in various fields like pathogen biology and ecology to develop methods to control and manage the disease. The use of genetically improved resistant planting stock offers the most promising potential for long term management of the disease.

In early reports it was noted that occasional Port-Orford-cedars escaped mortality by *P. lateralis* in infected natural stands. Cuttings from such survivors were rooted and exposed to inoculum from pure cultures by L. F. Roth at Oregon State University to evaluate the response of POC to *P. lateralis* (unpublished). However, the results were equivocal because of either very limited resistance of the tested POCs or use of inoculum doses of *P. lateralis* that were too great. Despite initial failures, POC screening has been continuously pursued to identify potentially resistant parents and to better understand the genetic variation and genetic resistance within POC.

Hansen et al. (1989) tested POC using stem wound and root-and stem-dip inoculation techniques on cuttings, seedlings, and branches in the greenhouse to demonstrate variation in susceptibility to *P. lateralis*. Since the late 1980's, the US

Forest Service (USFS) and Bureau of Land Management (BLM) in cooperation with Oregon State University have conducted an intensive program to identify and test resistant trees from the field, and propagate them in a seed orchard with the goal of providing resistant seedlings for regeneration.

The resistance screening tests began with recognition of phenotypically resistant trees that escaped mortality in infested areas. Then branches from the resistant trees were collected and tested by stem dip inoculations at the OSU greenhouse. For the stem dip inoculation, lateral foliage was trimmed from the base of the branch and a fresh cut was made on the stem. Then the branches were immersed in a *P. lateralis* zoospore suspension for 24 hours and incubated for 3 weeks, after which the length of the necrotic lesion was measured. The top 10 % of trees with consistently smaller lesions were propagated by rooting at the USFS Dorena Genetic Resource Center (DGRC), Cottage Grove, Oregon. The rooted candidate resistant trees were then sent to OSU and tested by root dip inoculation. For the root dip inoculation, roots of the rooted cuttings were trimmed to standard length by cutting extra roots at the bottom of super cell containers and then the lower 1 cm of the root system was immersed in a *P. lateralis* zoospore suspension for 24 hours. The inoculated rooted cuttings were grown for 12 months, after which mortality was recorded. Uninoculated ramets from resistant clones were planted in a containerized seed orchard at DGRC. These trees were treated with hormones to induce flowering and open pollination as well as controlled crosses were performed, and seeds were collected from parent trees with the best performance in inoculation trials. Finally, the seedling or clonal families from the seeds were raised and

subjected to field validation trials in outdoor raised beds infested with *P. lateralis* at OSU and in infested outplanting sites in forested areas. Seedlings and rooted cuttings of resistant and susceptible parents were also sent to OSU for investigations on resistance mechanisms.

POC survivors from the root dip inoculation tests were evaluated in an outdoor raised bed at OSU and in field sites under natural conditions to validate screening methods and to examine the durability and variation in types of resistance (Betlejewski et al. 2003, Hansen et al. 2000, Linn et al 2003, Sniezko and Hansen 2000). In the resistance breeding program, over 10,000 field selections have been screened by means of a combination of stem and root dip inoculation techniques, and over 1100 trees have been chosen for further evaluation. Moreover, cones yielding over 1.5 million seeds were collected from resistant trees and raised at DGRC in 2002 and distributed to the USFS and BLM (Linn et al. 2003) for use in regeneration.
In the resistance screening tests, a few parent trees, such as PO-OSU-CF1 (from OSU), 510015 (from Gasquet Ranger District on the Six Rivers National Forest, California), and 117490 (from Gold Beach Ranger District, Siskiyou National Forest, Oregon), have consistently shown milder symptoms and reduced mortality (Betlejewski et al. 2003, Hansen et al. 2000, Linn et al 2003, Sniezko and Hansen 2000). Additionally, rooted cuttings or open-pollinated seedlings from these parents showing high resistance to *P. lateralis* have much lower mortality than those of parents rated low for resistance. These results suggest that the resistance to *P. lateralis* is heritable. A few parent trees appear to have simply inherited resistance, with resistance determined by a single dominant gene (Table 1.1). Parent trees 118569, 510044, and 117499 are

hypothesized to be homozygous recessives (susceptible) since progeny of these parents had almost 100 % mortality. In contrast, progeny from crosses with 510015, 117502, and CF1 showed low mortality and therefore the parent trees may be heterozygous for the resistance gene; progeny from crosses with tree 117490 showed almost zero mortality. Therefore, this parent tree appears to be homozygous dominant (resistant).

Table 1.1. Mortality and hypothesized resistance genotype of full-sib and half-sib Port-Orford-cedars tested by root dip inoculation with *P. lateralis* (Sniezko et al. 2003a, b).

Family	Parent		No. of seedlings	Test Mortality	Expected Mortality	Hypothesized Genotypes of Progeny
	Female	Male				
118569	118569	510042	18	1.00	1.0	rr
	510041	118569	24	0.96	1.0	rr
	118569	510044	18	0.94	1.0	rr
510044	118569	510044	20	1.00	1.0	rr
117499	117499	117499	11	0.91	1.0	rr
117490	117490	117490	12	0.00	0.0	RR
	117490	117499	24	0.00	0.0	Rr
	117490	117502	18	0.06	0.0	R_
	117335	117490	24	0.04	0.0	Rr
	117490	117344	24	0.00	0.0	R_
	117490	117490	48	0.02	0.0	RR
	117490	117499	24	0.00	0.0	Rr
	117490	118569	24	0.04	0.0	Rr
	117490	510015	24	0.00	0.0	R_
	117490	CF1	48	0.00	0.0	R_
	117490	Con1	24	0.21	0.0	Rr
50015	510015	70020	24	0.29	3:1	R_, rr
	510015	510015	18	0.28	3:1	R_, rr
	510015	CF1	24	0.19	3:1	R_, rr
CF1	CF1	CF1	24	0.04	3:1	R_, rr
	CF1	CF1	24	0.13	3:1	R_, rr
	510044	CF1	12	0.42	1:1	R_, rr
117502	117502	117499	24	0.46	1:1	Rr, rr
	117502	117502	24	0.46	3:1	R_, rr

The hypothesized genotypes of seedlings from crosses with 510015 and CF1 parent trees were predicted to be either R_ or rr regardless of which parent they were crossed with. Susceptible and resistant offspring should be produced in equal proportions when either of these parents is crossed with a susceptible tree. Parent tree 117490 generates only resistant offspring. Consequently, a dominant resistance gene at a single locus is suggested by mendelian segregation ratios to explain the high survival in these families (Sneizko et al. 2003a, b). Alternative genetic hypotheses are 1) other genes, not R gene, are dominant in some trees showing other resistance mechanisms, 2) the resistance is not controlled by a single locus in some trees but is multigenic.

In this thesis, I describe the cellular response of POC to infection by *P. lateralis*, and compare the reactions of susceptible and resistant seedlings to inoculation. Seedlings of several Port-Orford-cedar families and clones were raised at DGRC for this work. Sixteen full-sib and half-sib seedling families were mainly used (Table 1.2). Later, rooted cuttings of two susceptible and three resistant POC parent trees were available for histopathology studies (Table 1.3). These seedling and clonal families had been tested for resistance in routine screening trials from 2001 to 2003 (unpublished data) at OSU. Results from representative trials are presented in Tables 1.2 and 1.3, together with the responses we expected to observe in the histopathology work.

In summary, resistance screening tests have been used to identify resistant families with high rates of survival, susceptible families that suffer high rates of mortality, and other families with intermediate rates of mortality, perhaps resulting

Table 1.2. Observed reaction of full-sib and half-sib Port-Orford-cedar seedling families used in this thesis to root dip inoculation with *P. lateralis*.

Cross	Observed Mortality [1]	Expected Reaction [3]
117499 × OP	1.00	S
118051 × OP	0.93	S
CF1 × OP	0.55	S/R [2]
CF2 × CF2	0.65	S/R
CF3 × CF3	0.60	S/R
CF1 × CF1	0.05	S/R
510015 × OP	0.42	S/R
510015 × CF1	0.25	S/R
510015 × 510015	0.21	S/R
117490 × OP	0.00	R
117490 × 118054	0.07	R
117490 × 117650	0.00	R
117490 × 117505	0.00	R
117490 × 117490	0.00	R
117490 × 117344	0.00	R
117490 × 117335	0.05	R

[1] The mortality was recorded from 2001 to 2003 by root dip inoculation test.
[2] Segregating for susceptibility and resistance.
[3] S = Susceptible, R = Resistant.

Table 1.3. Observed reaction of rooted cuttings (RT) from Port-Orford-cedar trees used in this thesis to root dip inoculation with *P. lateralis*.

Clone	Observed Mortality	Expected Reaction
Con 1 RT	1.00	S
70037 RT	0.95	S
CF1 RT	0.10	R
CF2 RT	0.24	R
117490 RT	0.00	R

from segregation of an allele for resistance, or perhaps representing other resistance mechanisms. There has been no histological description of either susceptible or resistant reactions to *P. lateralis* in POC. Therefore, this dissertation investigates the bases for resistance mechanisms in POC, following the four main objectives outlined below:

• Resistance screening: Here we report 4 experiments done to support and validate the resistance screening program. Initial selection of trees was made by a stem dip inoculation method to test susceptibility of the branches collected from phenotypically resistant trees in infested areas. The top 10 % based on lesion length were then selected for root dip inoculation tests. In 1996, preliminary testing showed that there was a low positive correlation between the root and stem methods (Betlejewski et al. 2003). However, further validation of the stem and root dip methods has been needed. In this study, we compared results from the root and stem dip inoculation methods to determine whether the two tests are correlated.

Occasionally seedlings from resistant families die in raised bed and field validation plantings. Several ideas have been put forth to explain this unexpected mortality of resistant progeny such as 117490. The possible presence of a new race of *P. lateralis* with changed virulence was tested by isolations from symptomatic resistant seedlings. In most validation plantings, actual cause of death is not determined. Infected POCs were collected to compare techniques for detecting *P. lateralis*. Finally, the possibility that initial infection through the foliage might bypass resistance expressed in the roots was evaluated to understand alternative infection pathways.

• Zoospore attraction response: *P. lateralis* is a soil borne pathogen. Initial infection is caused by motile zoospores. It has been reported that the zoospores of *Phytophthora* species have the ability to swim toward hosts by chemo-, electro-, rheo-, and geotaxis (Morris et al. 1998). Therefore, we hypothesized that there are different zoospore attraction responses toward susceptible and resistant POC. Also, we were

interested in whether different parts of the root are more attractive to zoospores and whether this differed between susceptible and resistant trees. We compared zoospore attraction on roots by means of light and scanning electron microscopy, counting zoospores, and by real-time quantitative PCR to determine whether susceptible and resistant trees are differently attractive to zoospores.

• Initial infection and colonization: Although Tucker and Milbrath (1942) reported the microscopic morphological characteristics of asexual and sexual structures of *Phytophthora lateralis,* and Trione (1959 and 1974) made additional observations of asexual and sexual stages, there have been no microscopic observations to understand how *P. lateralis* encysts, germinates, penetrates, and colonizes in roots and stems at the cellular level. The differences between resistant and susceptible families of Port-Orford-cedar in terms of initial infection and colonization using histocytological methods have not been previously observed.

• Defense-related resistance response: In general, the main general resistance mechanisms are reduced infection, slowed growth, and reduced sporulation on the host plants (Wilson and Coffey 1980). There have been observations of different zoospore attraction and necrotic lesion lengths on susceptible and resistant POC seedlings. In addition, most resistant POC seedlings infected with *P. lateralis* showed an oozing response which is considered to be a resistant reaction from the host plants. This suggested that some seedlings may exhibit a general resistance response. Transmission electron microscopy was used to observe cytological and structural changes following exposure to inoculum to identify differences between susceptible and resistant POC seedlings at the subcellular level.

In this thesis, Chapter 2 addresses validation of resistance screening methods for POC selection, then Chapter 3 describes the zoospore attraction. Chapters 4 and 5 report microscopic observations at the cellular level. Finally, Chapter 6 provides a general discussion including the main conclusions of each chapter.

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Chapter 2

Validation of Methods for Screening Port-Orford-Cedar for Resistance to *Phytophthora lateralis*

Eunsung Oh

2.1 ABSTRACT

Phytophthora lateralis has been known as a destructive exotic pathogen of Port-Orford-cedar since the 1920s. Various disease management strategies have been applied to forests and horticultural nurseries because of the economic and ecological value of POC. One strategy, selection and breeding for resistance, was established in the 1980s and the program has been successful. Here I report four experiments to support and validate the POC resistance program.

For validation of screening methods, susceptible and resistant POC tree families were compared via stem- and root-dip inoculation. Differences in necrotic lesion length between susceptible and resistant tree families resulted from both inoculation methods. However, correlations between the root- and stem-dip methods for individual seedlings were not significant. To help explain unexpected mortality of resistant seedlings in the OSU raised bed validation tests, *P. lateralis* was reisolated from infected POC seedlings and tested for increased aggressiveness or changed virulence by means of the stem dip inoculation on resistant and susceptible POC trees. No evidence was found for changed virulence of *P. lateralis* since resistance testing began.

To evaluate detection techniques for *P. lateralis*, infected POC seedlings were collected from the 2002 OSU greenhouse and raised bed tests. Three detection techniques, cultural isolation, PCR, and ELISA were compared. The detection of *P. lateralis* by plating on the selective medium was successful but the proportion of successful detections by the method was very low, especially in resistant seedlings. Proportion of successful detections by PCR was greater than plating. Proportion of

successful detections by ELISA was the same as by PCR but ELISA was not specific for *P. lateralis*. Naturally infected POCs subjected to periodic flooding suggested the possibility of foliar infection. Inoculations of susceptible and resistant POCs by wound inoculation confirmed that *P. lateralis* was able to infect through both the wounds and natural openings in POC foliage.

2.2 INTRODUCTION

Port-Orford-cedar (POC, *Chamaecyparis lawsoniana* [Murr.]Parl.) is an ecologically and economically important species of coniferous forests in southwestern Oregon and northwestern California. Compared to other conifer species, POC is very fire and decay resistant and is an important stream-side species contributing to riparian structure. Due to its resistance to decay, POC snags provide habitat for wildlife because the snags also persist (Betlejewski et al. 2003, Hansen et al. 2000). POC is also valued as an ornamental tree in landscape plantings. With more than 200 named cultivars, POC has become one of the most widely used ornamental conifers. Port-Orford-cedar root disease was first noticed on ornamental nursery stock in the late 1930s. The disease spread to native POC in southwestern Oregon in 1952. The causal agent was identified as *Phytophthora lateralis* Tucker and Milbrath. Since then, *P. lateralis* has caused increasing mortality of POC in forests throughout its range as well as in horticultural nurseries (Torgeson et al. 1954, Trione 1959). In addition, Pacific Yew (*Taxus brevifolia* Nutt.) was found to be susceptible to infection by *P. lateralis* in the Six Rivers National Forest, northwestern California in 1991. However, root

disease on Pacific Yew was observed only when yew was growing in the understory of infested areas of POC (DeNitto 1991, Murray 1995, Murray and Hansen 1997).

P. lateralis is presumed to be an exotic pathogen although its origin remains unknown. It produces asexual, swimming zoospores in sporangia on the surfaces of infected tissues, and thick-walled chlamydospores, a resting stage, within infected tissue, as well as sexual oospores. Initial infection is caused by zoospores that are disseminated in streams and water films in saturated soil. Therefore, water-related management is important to prevent the spread of the disease. However, the limitations to controlling water in forests and nurseries are a challenge to disease management. Alternatively, the POC resistance breeding program was established in the mid 1980s by the USDA Forest Service and USDI Bureau of Land Management (BLM). Propagation and controlled crosses of candidate resistant parents have been conducted at the USFS Dorena Genetic Resource Center (DGRC). Oregon State University has been cooperating in the resistance breeding program by developing methods for screening POC trees for resistance to this root disease.

Over 10,000 field selections of healthy POC trees growing in diseased sites have been screened for resistance using a combination of stem and root dip inoculation techniques in the greenhouse (Hansen et al. 1989, McWilliams 1999 and 2001, Murray 1995, Murray et al. 1997). Over 1100 parent trees were selected for further evaluation. The results of inoculation tests showed that 50-75 % or more of the seedlings from resistant parents survived while only ~5 % from the most susceptible parents escaped mortality based on the short-term greenhouse tests. On the basis of inoculation tests, selected POC families were outplanted for further mortality tests

under field conditions. Resistant POC seed is now available from the program. Cones with over 1.5 million seeds were collected at DGRC in 2002 and distributed to the Forest Service and BLM (Linn et al. 2003). The first resistant seed crop, 2-year-old seedlings produced at DGRC's operational seed orchards, was used as a part of reforestation efforts of the Biscuit fire in southwest Oregon in 2004 (Sniezko and Elliott 2004). The resistance breeding program has been successful in identifying and propagating resistant POC, but several questions have arisen in the course of operational resistance screening.

1) Since the 1980s stem and root dip inoculation techniques have been continuously performed for screening POC for resistance at Oregon State University. However, the correlation between stem and root inoculations has been questioned.

2) One of the field validation sites is in the cold frame area outdoors next to greenhouse 3 on the OSU campus. For about 6 years, test seedlings have been repeatedly planted in infested soil in the raised beds to evaluate resistance (Figure 2.1). Mortality levels in the 2002 planting were higher than expected, raising the possibility that repeated plantings of resistant trees may have inadvertently selected for a more virulent strain of the pathogen.

3) Mortality is routinely tallied once or twice a year at field test sites. The specific symptoms of *P. lateralis* on POC seedlings are only visible for about one month after seedling death so actual cause of death is seldom confirmed. Better methods are needed to differentiate between mortality due to *P. lateralis* and other causes in field plantings.

Figure 2.1. Infected Port-Orford-cedars in soil inoculated with *P. lateralis* zoospores in 2002 OSU raised bed trial, Corvallis, Oregon.

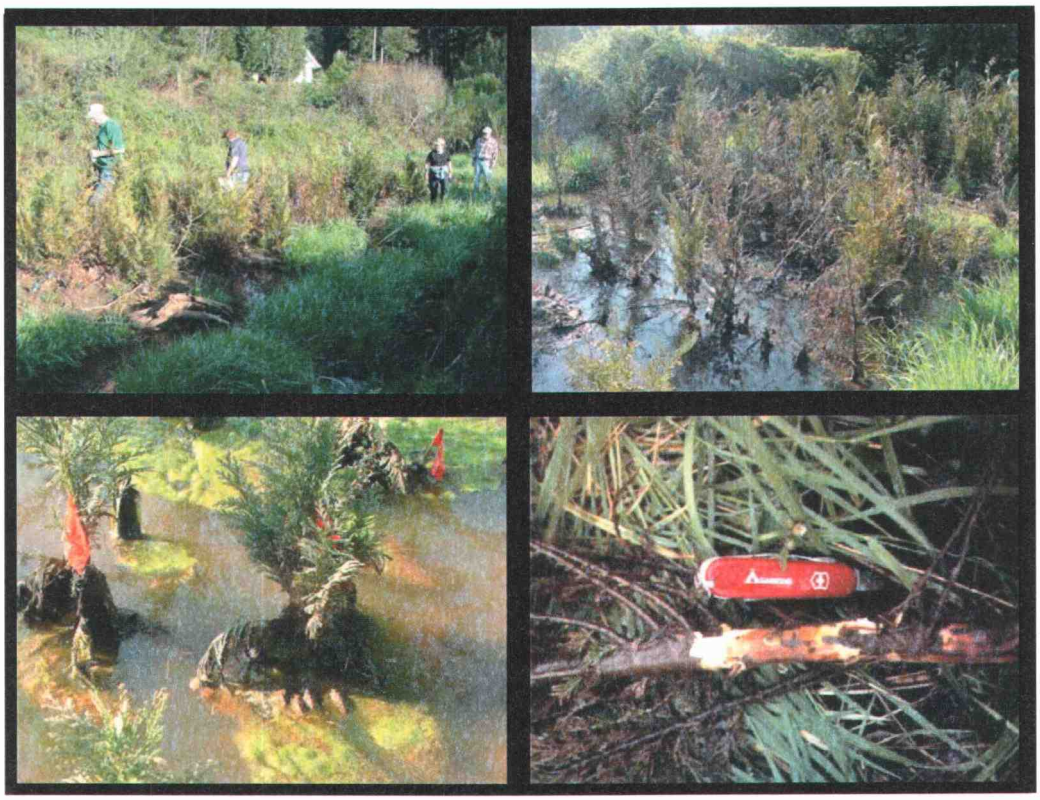

Figure 2.2. High mortality of resistant Port-Orford-cedars in 2000 and 2003 plantings at outplanting site at Hiouchi, California.

4) Mortality of 2000 and 2003 plantings of resistant trees at one field test site at Hiouchi, California, were greater than at other field test sites. The planting site at Hiouchi was relatively dry for the first two years, then in the winters of 2002 and 2003 much of the area had standing water. Soils were visually water-logged, with organic matter and soil deposited on foliage of POC. When the dead seedlings were examined, the typical symptom was high on the stem, not near the base or roots. This suggested the possibility of infection via water-soaked foliage during flood conditions (Figure 2.2).

Therefore, the primary objectives of this research were 1) to evaluate the correlation between stem and root dip inoculation techniques; 2) to test whether the virulence of the *P. lateralis* in the raised beds had changed; 3) to compare techniques for detecting *P. lateralis* on dying susceptible and resistant POCs from outplanting sites; and 4) to determine whether infection can occur through foliage.

In order to compare stem dip and root dip inoculation techniques, the upper 20 cm was clipped from individual seedlings and inoculated by the stem dip method. The basal portion of the seedling bearing the roots was inoculated by the root dip method, and the results of the two methods compared by measuring necrotic lesions on seedlings from each method. For the virulence test, *P. lateralis* was isolated from infected seedlings in the 2002 OSU raised bed trial. Virulence of the new isolates was compared with the original isolates by stem wound inoculations of susceptible and resistant seedlings. Cultural, molecular, and serological methods were compared for detection *Phytophthora* from the dying and dead POCs. A selective medium and morphological characteristics of *P. lateralis* in culture were used for cultural diagnosis (Cultural isolation). The molecular diagnostic technique employed simple Polymerase Chain Reaction (PCR with specific primers). PCR is a rapid and reliable detection and quantification technique for propagules and infection intensity on diseased plant materials by amplification of specific DNA sequence of the internal transcribed spacer (ITS) regions of ribosomal RNA genes. This technique is commonly used to detect *Phytophthor*a species for disease diagnosis (Grote et al. 2002, Judelson and Tooley 2000, Kong et al. 2003, Lacourt and Duncan 1997, Martin et al. 2004, Schubert et al. 1999, Tooley et al. 1997). Fortunately, primers for *P. lateralis* were developed by

Winton in 2001 even though the primers detect *P. ramorum* as well as *P. lateralis*. A serological method based on the dual-antibody system (=Double-antibody sandwich (DAS)-ELISA) (Ali-Shtayeh 1991, Benson 1991, MacDonald 1990, Miller et al. 1997, Murray 1995, Murray et al. 1997, Yuen 1998) also was compared with the other methods for detection of *P. lateralis*. Finally, foliage of susceptible and resistant POC seedlings was inoculated with *P. lateralis* to investigate the possibility of infection through foliage.

2.3 MATERIALS AND METHODS

2.3.1 Cultures

Three isolates of *P. lateralis* obtained from Oregon and California were used for inoculation of POC seedlings (Table 2.1). Cultures were grown on β-Corn Meal Agar (CMA, Difco) amended with 20 ppm β-sitosterol (Acros Organics) to stimulate zoospore production and stored in liquid nitrogen or water after they were originally isolated from infected POC. For zoospore production, cultures were incubated for 7 days at room temperature, then three agar disks with pure mycelium were transferred to pea broth (150 g split peas in 1 L dH_2O autoclaved for 4 minutes, 20 ppm β-sitosterol added to filtered pea broth, and the medium autoclaved for 25 minutes). The pea broth cultures were incubated for 7 days at 17 °C in Petri dishes, then pea broth was poured off, the colonies washed with distilled water, then flooded with 25 ml stream water from Oak Creek, Benton County, Oregon, and incubated for 2 days at 17 °C to induce sporangia.

Table 2.1. Host and origin of the isolates of *P. lateralis*.

Isolate	Host	Origin	Year Isolated
366	POC	Gasquet Ranger District, N. California	1986
368	POC	Gasquet Ranger District, N. California	1986
T_4P_3	POC	Galice District, Oregon	2000

2.3.2 Stem and root dip inoculation

Twelve container-grown seedlings from each of twelve tree families of POC were used to compare stem dip and root dip inoculation tests (Table 2.2). The seedlings in "super cell" containers were placed in a tray of water with the lower 3 cm of the cell submersed for 2-3 days. For inoculations with isolate T_4P_3, stems of each seedling were labeled and cut about 10 cm above the root crown. The top portions of the seedlings were inoculated by stem dip, and the lower portions with roots were inoculated by root dip. For stem dip inoculation, the upper stems from the seedlings of each family were trimmed to remove lower branches and the cut ends were immersed

Table 2.2. Tree families of cross and open-pollinated (OP) Port-Orford-cedar that were used in this experiment.

#	SN [1]	ID # [1]	Tree family
1	79	9430220	JG19 × OP
2	50	9430292	510015 × OP
3	48	9430196	117499 × OP
4	80	9430227	BP25 × OP
5	52	9430217	70045 × OP
6	84	9430252	70262 × OP
7	83	9430280	70465 × 70405
8	81	9430265	JQ23 × LO17
9	78	9430232	BV25 × OP
10	85	9430294	AS23 × OP
11	53	9430033	CF1 × OP
12	49	9430203	118051 × OP

[1] Sow number and ID number are Dorena codes.

overnight in 50 ml of zoospore suspension in paper cups. The stem ends were then inserted into wet vermiculite in super cells for incubation. For root inoculations, all exterior roots outside the cells were cut with a sharp razor blade for consistency. Seedlings were then inoculated by immersing the lower 1 cm of the super cell in 50ml zoospore suspension. Seedlings were exposed to zoospores overnight and then transferred to racks in the greenhouse. All stems and roots were carefully observed daily for visible symptoms in the green house. After 4 weeks the length of necrotic stem lesion (stem dip test), or length of the necrotic roots (root dip test) was measured and recorded. The data were log transformed to standardize variance and analyzed by Proc Mixed-generalized linear model (ANOVA), the SAS System version 8 (SAS Inc.). The ANOVA *F*-test provided P-values for main and interaction factors, and back-transformed least squares means were plotted for the comparisons.

2.3.3 Virulence test of *P. lateralis*

Twenty-four seedlings (Table 2.3) showing necrotic or chlorotic symptoms were collected from the 2002 OSU raised bed test (Figure 2.1), and isolations from those seedlings were performed. Pieces 2-3 mm square were cut from the necrotic margin on each seedling and transferred to selective medium (CMA with 20 mg pimaricin (Hansen et al. 1979), 200 mg ampicillin, and 10 mg rifampicin in methanol per liter (CARP)). *Phytophthora* colonies were evident after 2-4 days of dark incubation at room temperature. Each isolate was transferred to 3 plates of β-CMA for comparisons of growth rate and zoospore production. To compare growth rates, three plates of β-CMA per isolate were incubated for 3 days at room temperature in the dark,

then radial growth was measured in four directions. Growth increment was then measured daily for another 10 days. Each isolate was transferred to 3 plates of pea broth and then flooded with 25 ml stream water for 2 days for zoospore production. Then the stream water containing zoospores was collected in a beaker. One ml of stream water from the beaker was placed in a 1.7 ml eppendorf tube and vortexed for 2 minutes to cause the zoospores to encyst. Ten µl stream water from the vortexed tube was inserted in two cells on a hemacytometer and the number of zoospore cysts was counted. This procedure was repeated three times for each isolate.

In order to test virulence of *P. lateralis* isolates, 3 of 9 new isolates and 3 old isolates were selected as inocula for stem inoculations in August, October, and November 2002 (Table 2.4). Each of the three new isolates came from a dying seedling of a resistant family. Two susceptible and five resistant POC families and clones were chosen as host materials from plantings (Table 2.5) at the OSU Botany Farm (Figure 2.3A) and greenhouse at OSU.

Six branches, 20-30 cm long, were cut from each of eight trees from each family or clone except ESOC that was the only survivor of the family from the OSU Botany farm test, and Con1, a susceptible control tree growing on the OSU campus. Con1 and ESOC (3.3 % survival) were selected as susceptible POCs and the other 5 tree families and clones were selected as resistant POCs according to their high percentage of survival in earlier tests (Table 2.5). Branches were trimmed and bases freshly cut, then immersed 1 cm deep in 50 ml zoospore suspension (described above) and incubated overnight at root temperature in the dark (Figure 2.3B). One branch from each tree was exposed to each of the 6 isolates. After incubation overnight, the

Table 2.3. Tree families of Port-Orford-cedars from the 2002 OSU raised bed trial that were used for isolation.

#	SN[1]	ID #[1]	Tree family	Isolation of *P. lateralis*	Name
1	27	9430041	117509 × 117490	-	
2	56	9430123	510049 × 117490	-	
3	31	9430048	117852 × 510049	-	
4	15	9430021	117490 × 117502	-	
5	133	9430235	PO-OSU-CF1 × OP	-	
6	67	9430189	117341 × OP	+	Isolate 1
7	111	9430233	CF1× OP	+	Isolate 2
8	47	9430099	510008 × 510042	-	
9	84	9430206	118562 × OP	-	
10	112	9430234	CF1 × OP	+	Isolate 3
11	56	9430123	510049 × 117490	+	Isolate 4
12	71	9430193	117490 × OP	-	
13	42	9430079	118573 × 118567	+	Isolate 5
14	-	-	CF1 × OP	+	Isolate 6
15	67	9430189	117341 × OP	+	Isolate 7
16	29	9430044	117509 ×117502	-	
17	-	9430198	117502 × OP	-	
18	20	9430030	117500 × 117505	+	Isolate 8
19	56	9430123	510049 × 117490	-	
20	87	9430209	118839 × OP	-	
21	77	9430199	117503 × OP	-	
22	6	9430011	117344 × 117341	-	
23	111	9430233	CF1 × OP	-	
24	26	9430193	117490 × OP	+	Isolate 9

[1] Sow number and ID number are Dorena codes for sow year 1999.

Table 2.4. Isolates of *P. lateralis* that were used for stem inoculation.

Type	Name	Isolated date	Origin
Old	366	April 23, 1986	Gasquet Ranger District, N. California
	368	April 23, 1986	Gasquet Ranger District, N. California
	T_4P_3	? , 2000	Galice District, Oregon
New	Isolate 2	March 12, 2002	PO-OSU-CF1 × OP
	Isolate 4	March 12, 2002	510049 ×117490
	Isolate 9	May 13, 2002	117490 ×OP

Figure 2.3. Stem dip inoculation of Port-Orford-cedar with zoospores of *P. lateralis* for 3 weeks. **A** Collecting branches from survivors of resistant Port-Orford-cedar from OSU Botany farm test site, Corvallis, Oregon. **B** One branch from each tree of each family was placed in an inoculation cup that contained zoospores of one isolate. **C and D** Stems were incubated in vermiculite in a greenhouse.

Table 2.5. Tree family / clone of Port-Orford-cedar for stem dip inoculation.

Family / Clone	Expected reaction [1]	Tag #	No. of branches	Year Planted	% Survival	Location
Control 1 (Con1)	S	Con1	6	1960	-	OSU Campus
ESOC	S	10072	6	1990	3.3	Botany Farm
					2000 [2]	
CF1	R	10003	6	1989	48	Botany Farm
Rooted cutting		10011	6		2000	
		10013	6			
		10030	6			
		10032	6			
		10061	6			
		10071	6			
		10075	6			
CF2	R	10009	6	1989	80	Botany Farm
Rooted cutting		10014	6		2000	
		10040	6			
		10043	6			
		10053	6			
		10054	6			
		10056	6			
		10058	6			
510015 × OP	R	10078	6	1995	72.7	Botany Farm
		10084	6		2000	
		10092	6			
		10107	6			
		10109	6			
		10119	6			
		10121	6			
		10124	6			
117490 × 117490	R	785	6	1991	67	Greenhouse
		786	6		2003	
		787	6			
		788	6			
		789	6			
		790	6			
		791	6			
		792	6			
117490 × 117344	R	793	6	1991	90	Greenhouse
		794	6		2003	
		795	6			
		796	6			
		797	6			
		798	6			
		799	6			
		800	6			

[1] S = Susceptible and R = Resistant.

[2] Date of last assessment.

stems were inserted in vermiculite in super cells and incubated in a green house for 3 weeks (Figure 2.3C and D). The inoculations were repeated three times (August, October, and November 2002). The lengths of necrotic lesions were measured and recorded in each repeat. The data were log transformed to standardize variance and analyzed by Proc Mixed-generalized linear model (ANOVA) for randomized block design (trees were treated as blocks), the SAS System version 8 (SAS Inc.). The ANOVA *F*-test provided P-values for main and interaction factors, and back-transformed least squares means were plotted for the comparisons.

2.3.4 Detection of *P. lateralis* from symptomatic POC

In 2002 seedlings from resistant and susceptible POC families, initially selected by the resistance screening tests, were selected from two different trials to compare difference in detection rate between susceptible and resistant families and detection methods.

Test 1: Dead and dying seedlings from a root dip inoculation trial in the OSU greenhouse were selected. A total of 58 dying seedlings, 20 seedlings from 15 susceptible tree families and 38 seedlings from 12 resistant tree families (Table 2.6), were sampled for identification of the causal agent.

Test 2: Sixty-three of 77 tree families and clones tested from the 2002 raised bed test at OSU showed more than 50 % mortality. Thirty-eight seedlings from 15 susceptible families and 19 seedlings from 12 resistant families were selected (Table 2.7). All selected seedlings had clean necrotic margins typical of *Phytophthora* infection.

Table 2.6. Port-Orford-cedar seedlings infected by root dip inoculation in the 2002 OSU greenhouse trial and used to compare detection methods.

Sow #	ID #	Seedling #	Comments	Expected reaction
2	117490 × 117344	1	Two infection areas	R
3	117490 × 117499	1		R
3	117490 × 117499	2	All dead	R
4	117490 × 118569	1		R
5	117490 × 510015	1		R
6	117490 × 70231	1	All dead	R
6	117490 × 70231	2		R
7	117490 × con1	1		R
10	117490 × CF1	1		R
10	117490 × CF1	2	All dead	R
10	117490 × CF1	3	All dead	R
10	117490 × CF1	4		R
10	117490 × CF1	5	Gall?	R
10	117490 ×CF1	6	All dead	R
12	510015 × OP	1		R
13	CF1 × OP	1		S / R
13	CF1 × OP	2		S / R
13	CF1 × OP	3		S / R
15	117490 × 117490	1	Insect hole?	R
15	117490 × 117490	2		R

Table 2.6. Continued.

Sow #	ID #	Seedling #	Comments	Expected reaction
15	117490 × 117490	3		R
15	117490 × 117490	4	All dead, Gall?	R
15	117490 × 117490	5		R
15	117490 × 117490	6		R
15	117490 × 117490	7		R
16	117490 × OP	1		R
16	117490 × OP	2		R
16	117490 × OP	3		R
16	117490 × OP	4		R
16	117490 × OP	5	Insect hole?	R
16	117490 × OP	6		R
17	117490 × OP	1	All dead	R
17	117490 × OP	2		R
17	117490 × OP	3	All dead	R
17	117490 × OP	4		R
17	117490 × OP	5		R
17	117490 × OP	6		R
4	70112 × 70154	1	Gall?, Insect hole?	R
13	CF1 × OP	4		R
72	117344 × CF3	1	Insect hole?	S
88	70043 × 70015	1		S

Table 2.6. Continued.

Sow #	ID #	Seedling #	Comments	Expected reaction
81	70024 × 70137	1		S
86	70040 × 70037	1	All dead	S
55	70080 × 70092	1	All dead	S
52	70065 × 70103	1	All dead	S
85	70040 × 70015	1		S
76	17502 × 118574	1	Only one side-all dead	S
91	70047 × 70021	1		S
76	17502 × 118574	2		S
46	118463 × OP	1		S
42	70103 × OP	1	All dead	S
85	70040 × 70015	2		S
76	17502 × 118574	3		S
74	17500 × 117341	1		S
91	70047 × 70021	2		S
68	70256 × 70092	1		S
41	70024 × 70028	1	Two infection areas	S
55	70080 × 70092	2	All dead	S
71	117334 × CF1	1		S

Table 2.7. Mortality of Port-Orford-cedar in 2002 OSU raised bed test and number of seedlings that were tested for detection of *P. lateralis*.

Sow#	Seed ID	ID #	No. of seedlings	Comments	% mort	Total tested seedlings	Expected reaction
1	9430012	117344 × 117344	20		95		S
2	9430018	117490 × 117344	20		10	1	R
3	9430020	117490 × 117499	24		38	2	R
4	9430024	117490 × 118569	16		63	1	R
5	9430309	117490 × 510015	16		25	1	R
6	9430310	117490 × 70231	24		38	2	R
7	9420311	117490 × CON1	20		50	1	R
8	9430196	117499 × OP	16		100		S
9	9430152	CF1 × CF1	12		33		R
10	9430312	117490 × CF1	48	Control	19	6	R
11	9430027	117499 × 117499	36	Control	97		S
12	9430212	510015 × OP	48	Control	79	1	S
13	9430235	CF1 × OP	48	Control	58	4	R
14	9430191	117344 × OP	48		67		S
15	9430019	117490 × 117490	48	Control	33	7	R
16	9430193	117490 × OP	48	Control	44	6	R
17	9430194	117490 × OP	48	Control	38	6	R
18	9430004	117335 × 70020	24		54		R

Table 2.7. Continued

Sow#	Seed ID	ID #	No. of seedlings	Comments	% mort	Total tested seedlings	Expected reaction
19	9430254	117335 × 70057	16		44		R
20	9430016	117344 × 70020	20		75		S
21	9430313	117344 × 70057	16		56		S
22	9430314	117344 × 70276	24		58		S
23	9430072	118568 × 70020	16		75		S
24	9430315	118568 × 70057	12		75		S
25	9430316	118568 × 70276	16		56		S
26	9430134	70057 × 70020	12		83		S
27	9430138	70276 × 117335	6		83		S
28	9430140	70276 × 70020	16		63		S
29	9430317	70276 × -70276	12		58		S
30	9430188	117335 × OP	24		42		R
31	9430060	118558 × 118569	16		100		S
32	9430136	70138 × 70016	12		50		R
33	9430075	118569 × 510044	16		100		S
34	9430189	117341 × OP	48		63		S
39	9430213	510041 × OP	48		75		S
41	9430131	70024 × 70028	48		88	1	S
42	9430227	70103 × OP	24		92	1	S
46	9430205	118463 × OP	48	Control	98	1	S

Table 2.7. Continued.

Sow#	Seed ID	ID #	No. of seedlings	Comments	% mort	Total tested seedlings	Expected reaction
47	9430318	70055 × 70024	12		67		S
51	9430322	70065 × 70065	12		83		S
52	9430323	70065 × 70103	24		83	1	S
53	9430324	70065 × 70207	16		100		S
55	9430326	70080 × 70092	24		63	2	S
57	9430328	70119 × 70119	12		100		S
58	9430329	70149 × 70068	20		65		S
61	9430332	70178 × 70068	16		88		S
63	9430334	70207 × 70112	12		100		S
65	9430336	70221 × 70178	16		94		S
67	9430338	70252 × 70252	16		81		S
68	9430339	70256 × 70092	12		83	1	S
69	9430340	70274 × 70047	16		100		S
70	9430341	70274 × 70065	12		100		S
71	9430342	117334 × CF1	16		31	1	R
72	9430343	117334 × CF3	24		71	1	S
73	9430344	117334 × CF8	24		79		S
74	9430345	117500 × 117341	16		69	1	S
75	9430346	117502 × 118054	16		44		R
76	9430347	117502 × 118574	24		83	3	S

Table 2.7. Continued.

Sow#	Seed ID	ID #	No. of seedlings	Comments	% mort	Total tested seedlings	Expected reaction
77	9430348	117852 × CF6	16		56		S
78	9430349	70015 × 70018	12		67		S
79	9430350	70015 × 70024	16		88		S
80	9430351	70015 × 70037	24		67		S
81	9430353	70024 × 70137	24		96	1	S
82	9430354	70024 × 70167	16		100		S
83	9430355	70030 × 70033	16		94		S
84	9430356	70033 × 70266	16		88		S
85	9430357	70040 × 70015	16		69	2	S
86	9430358	70040 × 70037	16		88		S
87	9430359	70040 × 70187	24		79		S
88	9430360	70043 × 70015	16		63	1	S
89	9430361	70043 × 70248	16		94		S
90	9430362	70047 × 70018	16		94		S
91	9430363	70047 × 70021	16		63	2	S
92	9430364	70248 × 70018	24		96		S
93	9430365	70248 × 70030	24		100		S
94	9430366	70266 × 70147	24		83		S
CF1	RC	PO-OSU-CF1	48	Control	2		R

Seedlings for Tests 1 and 2 were used to detect and diagnose *P. lateralis*. In order to verify the causal agent, three detection techniques, cultural isolation, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA), were performed and their efficacies compared in this study. Pieces of necrotic margins from infected stems were excised, and then divided into equal pieces (5 mm × 5 mm) for isolation, PCR, and ELISA simultaneously.

1) Cultural isolation technique

All procedures for isolation were the same as described above.

2) PCR technique

The tissue pieces were placed into 2 ml microfuge tubes with two 3 mm glass beads, frozen in liquid nitrogen, and pulverized in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) for 30 seconds at 4200 rpm for DNA extraction. After pulverization, samples were incubated in 1.5 ml CTAB extraction buffer (2 % (w/v) CTAB (Cetyltrimethylammonium bromide), 100 mM Tris, pH 8.0, 20 mM NaEDTA, 1.4 M NaCl, 1 % (w/v) polyvinylpoly-pyrrolidone, 0.1 % (v/v) 2-mercapto-ethanol) at 65 °C for 2 hours. The DNA was purified in 24 parts of chloroform and 1 part of isoamyl alcohol. Then, AL (Lysis buffer) from Qiagen Tissue Dneasy Extraction kit (Qiagen Inc., Valencia, CA) was added and incubated at 70 °C for 10 minutes followed by discarding the chloroform. Then, 100 % ethanol was added to the extracted DNA. Then, DNA was placed on Qiagen columns and wash buffers from the kit were added and pulled through by means of vacuum to clean the DNA. Elution

buffer AE warmed at 70 °C was added and the columns were centrifuged. The purified DNA was transferred to new tubes. PCR was performed in 15 µl multiplex reactions containing 1 × enzyme buffer, 200 µM dNTPs, 0.4 µM *P. lateralis* primers (Platf and Platr, Winton and Hansen 2001), 0.1 µM universal internal control primers (NS1 and NS2, White et al. 1990), 0.8 U RedTaq polymerase, and 1 µl template DNA. The reaction conditions were 2 min at 94 °C denaturing, 44 cycles of 30 s at 94 °C, 30 s at 50 °C annealing, and 1 min at 72 °C extension. Then the reaction was held for 4 min at 72 °C and 1 min at 24 °C. The PCR product was electrophoresed on 2 % (w/v) agarose gels in Tris-borate-EDTA buffer (pH 8.6) and visualized with ethidium bromide under UV light. The fragment sizes were estimated by comparison with a 100 bp DNA ladder (GeneRuler™, Hanover, MD). During the test, it was found that sometimes *P. lateralis* was positively identified by cultural isolation but the PCR test was negative. In these cases, new samples from the same seedlings were excised and the PCR was run again.

3) Serological technique

The reagent set for *Phytophthora,* DAS ELISA (Agdia Inc., Elkhart, IN), was used for a serological test. To determine optimum conditions for ELISA, pure mycelium of *P. lateralis* was tested as positive control at 4-fold concentrations (up to 10^{-4}). Polystyrene plates (Agdia Inc., Elkhart, IN) were coated for 4 h at room temperature or overnight at 4 °C with 100 µl per well of 50 µl *Phytophthora* antibody in 10 ml 1 × coating buffer. The plates were then washed three times with 1 × phosphate-buffered saline-Tween (PBST: 3.33 g buffer powder, 2 ml Tween-20, and

100 ml ddH_2O). Sample tissue pieces were placed into 2 ml microfuge tubes with two 3 mm glass beads, frozen in liquid nitrogen, and pulverized in a Mini-Beadbeater (Biospec Products) for 30 sec at 4200 rpm. Extraction buffer (600 µl) was added to the tubes, and vortexed thoroughly. 100 µl of samples from tubes were added to each well. Each sample was added to two replicate wells, and positive control (DNA of *P. lateralis*) and negative control (buffer) were also included in each assay. The plates were incubated in a moisture chamber for 2 h at room temperature or overnight at 4 °C. The plates were then washed and incubated for 2 h at room temperature with 100 µl of conjugate (50 µl concentrated enzyme conjugate in 10 ml 1 × ECI) per well and then washed again. 100 µl of substrate solution (0.1 % *p*-nitrophenyl phosphate in 10 mM diethanolamine, pH 9.8) was added and incubated at room temperature. The color reaction was measured after 15-30 min at 405 nm on a kinetic microplate reader, V max (Molecular devices Co., Sunnyvale, CA). The optical density (OD) values of the blank well were subtracted from the OD values of wells that contained samples, and the OD values were reported automatically. A sample was considered to have a positive reaction in the test if its mean OD value exceeded the 0.3 OD threshold established for the 10^{-2} positive control. For consistent conditions, all samples were tested simultaneously and repeated once with newly excised tissue pieces from the same seedlings.

For the final report, successful detection of *P. lateralis* from the susceptible and resistant families in Test 1 was combined with the detection from susceptible and resistant families in Test 2. The detection percentages of each of the three detection methods were log transformed to homogenize variance and analyzed by Proc Mixed-

generalized linear model (ANOVA), the SAS System version 8 (SAS Inc.). The ANOVA *F*-test provided P-values for main and interaction factors, and back-transformed least square means were plotted for the comparisons.

2.3.5 Infection through foliage

In order to test the possibility of infection by *P. lateralis* through foliage, 5 seedling families or clones were selected (Table 2.8). Branch tips from one seedling per family or clone were immersed in a zoospore suspension (1.3×10^4 / ml) of isolate 368 of *P. lateralis*. Treatments included wounded (5-6 punctures with a needle each side per branch tip) or unwounded, and exposure time (5, 24, or 48 hours). There were 3 replicate branch tips per treatment. Uninoculated foliage was also included as a control. Branch tips were rinsed with sterile ddH_2O, and incubated for 6 days at room temperature in a crisper box lined with wet paper towels. The test was repeated 3 times (Figure 2.4). In addition, a forth trial was performed with a 5 hour exposure period only. After incubation, inoculated foliage was carefully observed, and the

Table 2.8. Open-pollinated tree families and rooted cut tips of Port-Orford-cedar and that were used in this study.

Sow Year	ID #	Family / Clone	Expected Reaction [1]
About 1960	Con 1	Control 1	S
2001	RT1204	70037 RT	S
2001	RT1317	CF1 RT	R
2000	9430292	510015 × OP	R
2001	-	117490 RT	R

[1] S = Susceptible and R = Resistant.

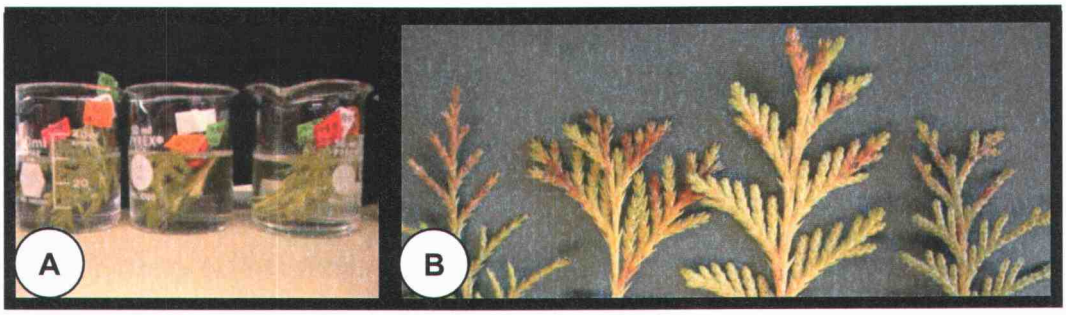

Figure 2.4. Inoculation of branches of Port-Orford-cedar. **A** POC branch tips were immersed in 25 ml zoospore suspension for 5, 24, and 48 hours. **B** Necrotic lesion lengths were measured after 6 days.

lengths of necrotic lesions were measured and recorded. The data were log transformed to homogenize variance and analyzed by Proc Mixed-generalized linear model (ANOVA), the SAS System version 8 (SAS Inc.). The ANOVA *F*-test provided P-values for main and interaction factors, and back-transformed least square means were plotted for the comparisons.

2.4 RESULTS

2.4.1 Stem and root dip inoculation

The results of the stem and root dip inoculation for POC seedlings indicated significantly different susceptibility among the seedlings (Table 2.9). There were significant differences among families and between tissue types (stem and root) ($P < 0.0001$). Interaction between family and tissue was not significant ($P = 0.4793$). In the stem dip inoculation, 510015 × OP and CF1 × OP showed significantly less root necrosis from *P. lateralis* than other families (Figure 2.5A). The remaining families gave variable results, but generally were not significantly different from each other.

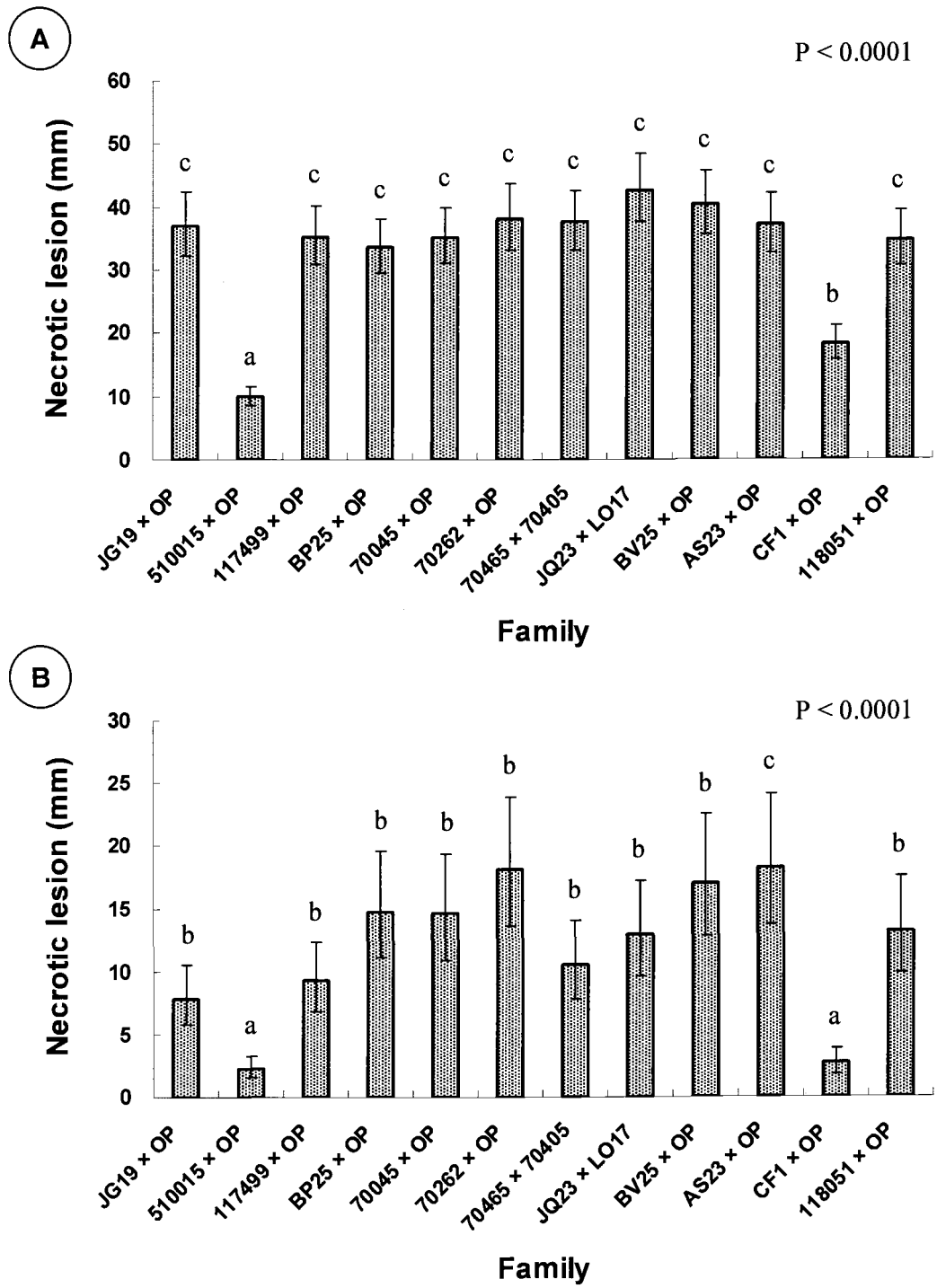

Figure 2.5. Least squares means of necrotic lesions on root and stem of POC seedlings by the stem dip (A) and the root dip (B) inoculation with zoospores of *P. lateralis* and incubation for 4 weeks. Bars with the same letter are not significantly different (P > 0.05).

Table 2.9. ANOVA analysis of necrosis measurements for the interaction between family and tissue after inoculation by *P. lateralis* zoospores overnight and incubated for 4 weeks.

Effect	Num. DF	Den. DF	F-value	Pr > F
Family	11	252	10.00	< 0.0001
Tissue (root / stem)	1	252	141.28	< 0.0001
Family*Tissue	11	252	0.96	0.4793

Table 2.10. Determination of susceptibility of Port-Orford-cedars by root and stem dip inoculation.

#	SN	ID #	Family	Susceptibility [1]
1	79	9430220	JG19 × OP	S
2	50	9430292	510015 × OP	R
3	48	9430196	117499 × OP	S
4	80	9430227	BP25 × OP	S
5	52	9430217	70045 × OP	S
6	84	9430252	70262 × OP	S
7	83	9430280	70465 × 70405	S
8	81	9430265	JQ23 × LO17	S
9	78	9430232	BV25 × OP	S
10	85	9430294	AS23 × OP	S
11	53	9430033	CF1 × OP	R
12	49	9430203	118051 × OP	S

[1] S=Susceptible and R=Resistant.

The CF1 and 510015 families also had shorter lesions than the others by root dip (Figure 2.5B). The remaining families showed little variation. JG19 × OP was the next resistant tree family by root dip inoculation but it showed the same susceptibility as the other families by stem dip inoculation. Therefore, 510015 × OP and CF1 × OP were characterized as resistant families by both tests, but the remaining families were not consistently differentiated (Table 2.10). In addition, there was no correlation in susceptibility between the root and the stem dip inoculation methods for most groups, but resistant 510015 × OP and CF1 × OP had significant correlations between the root and the stem dip inoculation methods (Figure 2.6).

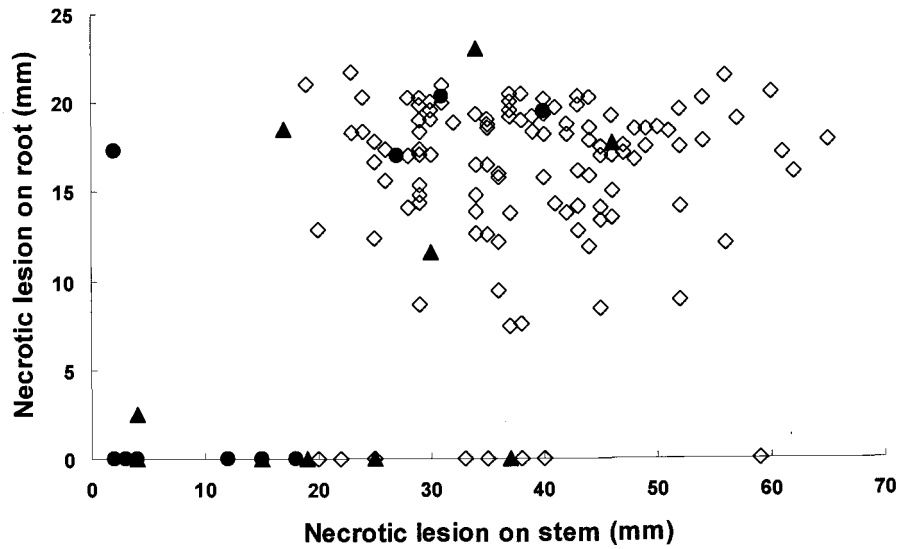

Figure 2.6. Correlation between the stem-and root-dip inoculation methods by necrotic lesion length. ● = CF1 × OP seedlings; ▲= 510015 × OP seedlings; ◊ = All seedlings in Table 2.12 except CF1 and 510015 × OP .

2.4.2 Virulence test of *P. lateralis*

P. lateralis was successfully isolated from the necrotic lesion in 9 of 24 dying POC seedlings in the raised bed (ca. 40 % isolation detection). When growth of these new isolates was compared with the standard isolates, no significant differences in growth rate or zoospore production among isolates were found (Table 2.11). However, standard isolates produced slightly more zoospores (average 2×10^5) than new isolates (average 10^5). Isolates 368 and T_4P_3 especially produced many active zoospores.

Standard isolates and three new isolates were selected for stem inoculations on the Port-Orford-cedars from the resistance test at the OSU Botany farm. The interaction between family / clone and isolate was not significant (P = 0.6624, Table 2.12). However, there were significant differences between families / clones and

between the isolates, respectively ($P < 0.001$ and $= 0.0027$, ANOVA *F*-test) (Figure 2.7, Table 2.12). Con 1 from the OSU campus had a 33 mm average length of necrotic

Table 2.11. Comparison of growth rate and zoospore production among old and new isolates of *P. lateralis*.

Type	Name of Isolate	Isolated from	Date of Isolation	mm [1]	mm [2]	10^5 *
Old	366	POC	1986	9.9	1.3	1.5
	368	POC	1986	10.8	1.3	2.0
	T4P3	POC	2000	9.9	0.9	1.9
Newly isolated	Isolate 1	117341 × OP	2002	10.2	1.4	1.3
	Isolate 2	PO-OSU-CF1 × OP	2002	10.1	1.4	1.3
	Isolate 3	CF1 × OP	2002	10.1	1.5	1.3
	Isolate 4	510049 ×117490	2002	9.2	1.3	1.4
	Isolate 5	118573 × 118567	2002	11.0	1.4	1.1
	Isolate 6	CF1 × OP	2002	9.4	1.4	0.9
	Isolate 7	117341 × OP	2002	10.5	1.4	0.7
	Isolate 8	117500 × 117505	2002	8.9	1.2	0.9
	Isolate 9	117490 × OP	2002	-	-	-

[1] Mean 3 day growth rate along 4 radii from three plates per isolate.
[2] Mean daily growth rate of radii from 4th to 13th per day from three plates per isolate.
*Mean of three measurements from zoospore suspension by means of a hemacytometer.

Table 2.12. ANOVA analysis of necrosis measurements for the interaction between family and isolate after inoculation by *P. lateralis* zoospores overnight and incubated for 4 weeks.

Effect	Num. DF	Den. DF	F-value	Pr > F
Family	6	962	31.29	< 0.0001
Isolate	5	962	3.66	0.0027
Family*Isolate	30	962	0.87	0.6624

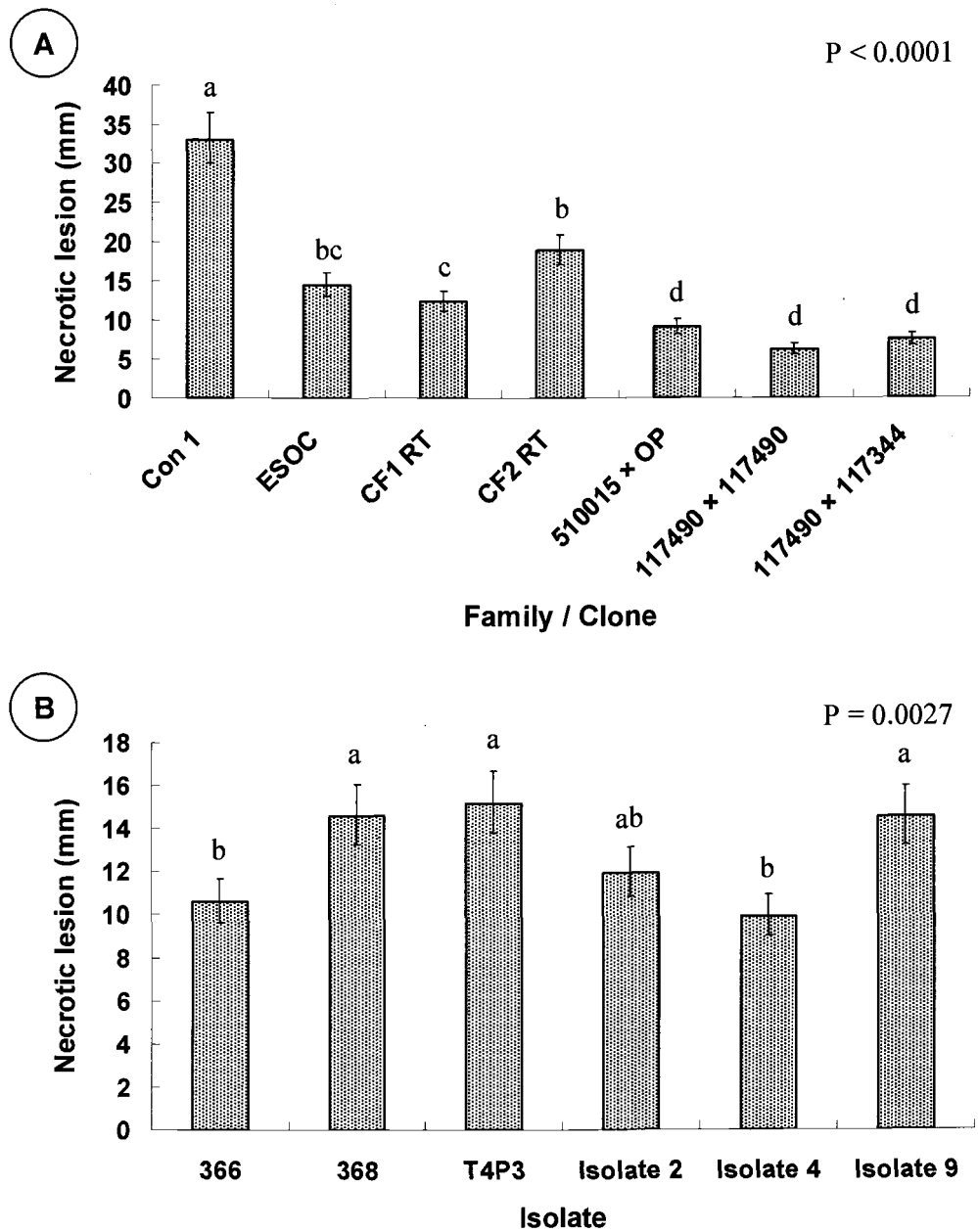

Figure 2.7. Least squares means of necrotic lesion lengths on stems of Port-Orford-cedar following inoculation with zoospores of *P. lateralis* and incubation for 4 weeks. **A** Difference by family / clone. **B** Difference by isolate. Bars with the same letter are not significantly different ($P > 0.05$).

lesion that was greater than other trees, as expected. Interestingly, the length of necrotic lesion from one surviving ESOC tree was 14 mm which was not significantly different than lengths from the CF 1 (12 mm) and CF 2 (19 mm) clones (Figure 2.7A). 510015 × OP, 117490 × 117490, and 117490 × 117344 had smaller lesions (9, 6, and 8 mm, respectively) than others that were very resistant to the pathogen. They were significantly different than other tree families and clones but not different among themselves. Because the lengths of necrosis from ESOC, CF1 RT, and CF2 RT were neither greater than Con 1 nor smaller than 510015 × OP, 117490 × 117490, and 117490 × 117344, those trees were intermediate in susceptibility to *P. lateralis* in this test. Isolates 368, T_4P_3, Isolate2, and Isolate 9 did not differ in lesion length from each other but 366 and Isolate 4 produced shorter lesions (Figure 2.7B).

2.4.3 Detection of *P. lateralis* from symptomatic POC

Test 1: A total of 58 seedlings from POC families tested in 2002 from the OSU greenhouse were used to compare detection techniques. Isolation success from seedlings from susceptible families was 30 % (6 / 20 seedlings) while the detection from seedlings from resistant families was 5 % (2 / 38 seedlings), about 6 times less detection than susceptible POC (Table 2.13). Detection by PCR was a little higher than by cultural isolation. Figure 2.8A shows an example of PCR detection. A 738 bp amplication product was detected for *P. lateralis*, and a 550 bp product, the internal control, indicated successful PCR amplication. Figure 2.8B shows the comparison between direct PCR of DNA extracted from wood pieces and PCR of DNA extracted from mycelium from isolation plates. Sample 41 was culture positive but *P. lateralis*

was not detected from the wood pieces in the first PCR. The culture DNA extract was PCR positive and a repeated PCR of DNA extracted from the lesion from the seedling was positive. All samples were characterized as detection or failure by reconfirmatory test as needed as described in the Methods. Detection by PCR from susceptible seedlings was 35 % (7 / 20 seedlings) and from resistant seedlings it was 21 % (8 / 38 seedlings) (Table 2.13).

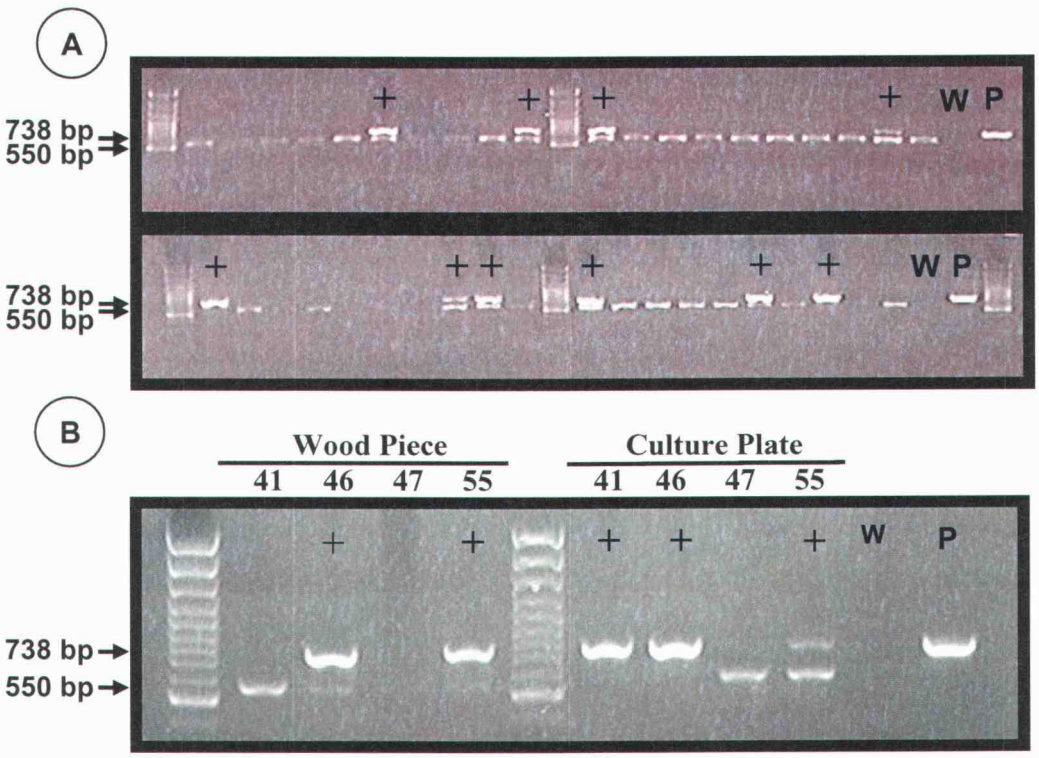

Figure 2.8. Gel electrophoresis of amplification products after PCR with DNA extracted from the infected POC seedlings at the OSU greenhouse (Test 1). **A** An example of successful detection for 10 out of 40 samples. **B** Comparison of PCR results from wood samples and culture plates. W = Water (ddH_2O); P = Positive control.

Table 2.13. Detection of *P. lateralis* from dying Port-Orford-cedars by root dip inoculation in the 2002 OSU greenhouse (Test 1) and raised bed (Test 2).

#	Sow #	ID #	Seedling #	Expected reaction	Greenhouse			Raised Bed		
					Plating	PCR	ELISA	Plating	PCR	ELISA
1	2	117490 × 117344	1	R	-	-	-	-	-	-
2	3	117490 × 117499	1	R	-	+	+	-	+	+
3	3	117490 × 117499	2	R	-	-	-	-	-	-
4	4	117490 × 118569	1	R	-	-	-	-	-	-
5	5	117490 × 510015	1	R	-	-	-	-	-	-
6	6	117490 × 70231	1	R	-	-	-	-	-	-
7	6	117490 × 70231	2	R	-	+	+	-	+	+
8	7	117490 × con1	1	R	-	-	+	-	-	+
9	10	117490 × CF1	1	R	-	-	-	-	-	-
10	10	117490 × CF1	2	R	-	-	-	-	-	-
11	10	117490 × CF1	3	R	-	-	-	-	-	-
12	10	117490 × CF1	4	R	-	-	-	-	-	-
13	10	117490 × CF1	5	R	-	-	+	-	-	+
14	10	117490 ×CF1	6	R	-	-	-	-	-	-
15	12	510015 × OP	1	R	-	-	+	-	-	+
16	13	CF1 × OP	1	R	-	-	-	-	-	-
17	13	CF1 × OP	2	R	-	-	-	-	+	-
18	13	CF1 × OP	3	R	-	+	+	-	+	+

Table 2.13. Continued.

#	Sow #	ID #	Seedling #	Expected reaction	Greenhouse			Raised Bed		
					Plating	PCR	ELISA	Plating	PCR	ELISA
19	13	CF1 × OP	4	R	-	+	-	-	-	-
20	15	117490 × 117490	1	R	-	+	+	-	+	+
21	15	117490 × 117490	2	R	-	-	-	-	-	+
22	15	117490 × 117490	3	R	-	-	-	-	-	-
23	15	117490 × 117490	4	R	-	-	-	-	-	-
24	15	117490 × 117490	5	R	-	-	-	-	-	-
25	15	117490 × 117490	6	R	-	-	-	-	-	-
26	15	117490 × 117490	7	R	-	-	+	-	-	-
27	**16**	**117490 × OP**	**1**	R	+	+	+	+	+	+
28	16	117490 × OP	2	R	-	-	-	-	+	-
29	16	117490 × OP	3	R	-	-	-	+	+	+
30	16	117490 × OP	4	R	-	-	-	-	-	-
31	16	117490 × OP	5	R	-	+	-	-	-	-
32	**16**	**117490 × OP**	**6**	R	+	+	+	-	-	-
33	17	117490 × OP	1	R	-	-	-	-	-	+
34	17	117490 × OP	2	R	-	-	-	-	-	-
35	17	117490 × OP	3	R	-	-	+	-	-	-
36	17	117490 × OP	4	R	-	-	-	-	-	-
37	17	117490 × OP	5	R	-	-	-	-	-	-

Table 2.13. Continued.

#	Sow #	ID #	Seedling #	Expected reaction	Greenhouse			Raised Bed		
					Plating	PCR	ELISA	Plating	PCR	ELISA
38	17	117490 × OP	6	R	-	-	-	-	-	-
39	-	70112 × 70154	1	S	-	-	-			
40	**41**	**70024 × 70028**	**1**	**S**	+	+	+	+	+	+
41	42	70103 × OP	1	S	-	-	-	-	-	-
42	**46**	**118463 × OP**	**1**	**S**	+	+	+	+	+	+
43	52	70065 × 70103	1	S	-	-	-	-	-	-
44	55	70080 × 70092	1	S	-	-	-	-	-	-
45	55	70080 × 70092	2	S				-	-	-
46	68	70256 × 70092	1	S	-	-	-	-	-	-
47	71	117334 × CF1	1	S				-	-	-
48	**72**	**117344 × CF3**	**1**	**S**	+	+	+	+	+	+
49	72	117344 × CF3	1	S	-	-	-			
50	74	117500 × 117341	1	S	-	-	-			
51	76	17502 × 118574	1	S	-	-	-	-	-	-
52	76	17502 × 118574	2	S	-	-	-	-	-	-
53	76	17502 × 118574	3	S	-	-	-	-	-	-
54	81	70024 × 70137	1	S	-	-	-	-	-	-
55	**85**	**70040 × 70015**	**1**	**S**	+	+	+	+	+	+
56	85	70040 × 70015	2	S	-	-	+	-	-	+

Table 2.13. Continued.

#	Sow #	ID #	Seedling #	Expected reaction	Greenhouse			Raised Bed		
					Plating	PCR	ELISA	Plating	PCR	ELISA
57	86	70040 × 70037	1	S	-	-	-			
58	88	70043 × 70015	1	S	+	+	-	+	+	-
59	**91**	**70047 × 70021**	**1**	S	+	+	+	+	+	+
60	91	70047 × 70021	2	S	-	+	+	-	+	+
Total result		Susceptible	# detection / # total tested (% detection)		6 / 20 (30)	7 / 20 (35)	7 / 20 (35)	6 / 19 (32)	7 / 19 (37)	7 / 19 (37)
		Resistant	# detection / # total tested (% detection)		2 / 38 (5)	8 / 38 (21)	11 / 38 (29)	2 / 38 (5)	8 / 38 (21)	11 / 38 (29)

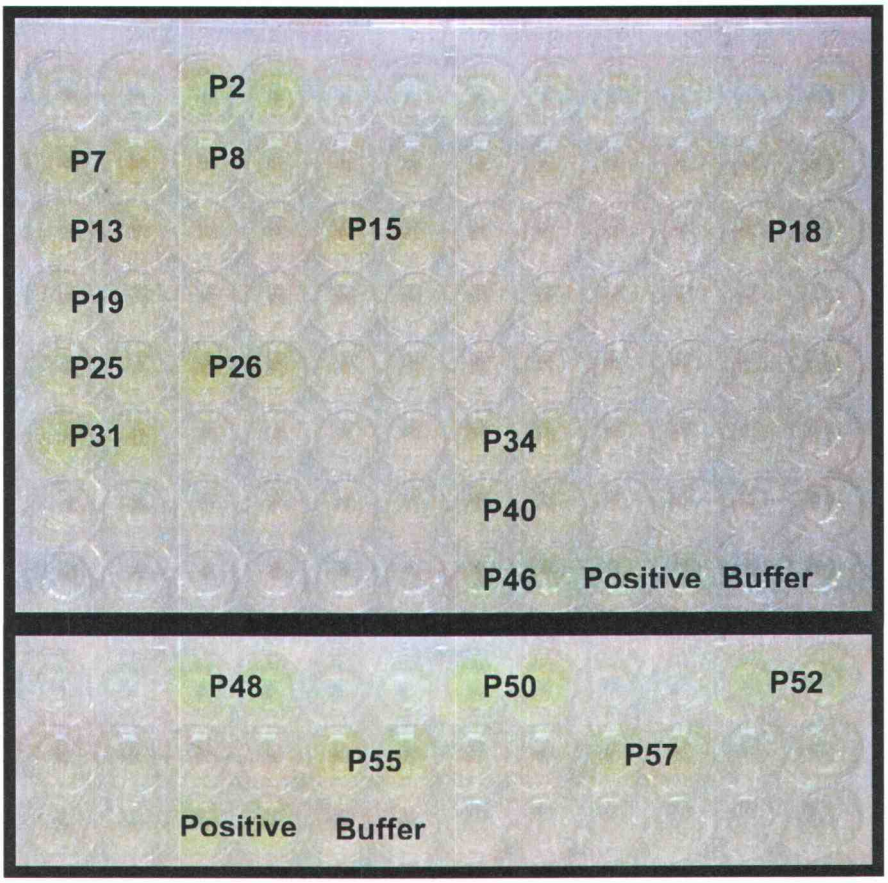

Figure 2.9. ELISA reactions for *Phytophthora lateralis* extracted from infected POC seedlings in the 2002 OSU greenhouse (Test 1). The numbers indicated the samples.

ELISA test results were based on absorbance (Optical density = OD) values proportional to the amount of bound antigen. The detection threshold for ELISA was set at 0.3, the mean value for a 4-fold dilution of the positive control standard. Only 18 positive reactions were detected from the 58 dying seedlings (Figure 2.9). The positive detection rate for susceptible seedlings was 35 % (7 / 20 seedlings) and 29 % (11 / 38 seedlings) for resistant. The rate of detection by ELISA was greater than detection by cultural isolation and PCR. Only 5 susceptible and 1 resistant families showed positive reactions for all three detection techniques.

Test 2: A total of 57 seedlings from susceptible and resistant POC families in the OSU raised bed trial were used for a second test of detection methods. The procedure for the three detection techniques was the same as that used for Test 1. *P. lateralis* was successfully isolated from 6 of 19 seedlings from susceptible families (32 % detection), while it was isolated from only 2 of 38 seedlings of resistant families (5%) (Table 2.13). The rate of isolation detection for resistant POCs was about 8 times less than the susceptible POCs. The rate of PCR detection result for susceptible POC families was 37 % (7 / 19 seedlings) but the rate of detection for resistant POCs was only 21 % (8 / 38 seedlings). The detection of *P. lateralis* by PCR was higher than by cultural isolation. Again, detection by ELISA was even higher than the PCR result; 37 % (7 / 19 seedlings) for susceptible seedlings and 29 % (11 / 38) for resistant seedlings. Interestingly, only 5 seedlings from susceptible families and 2 resistant clones were positive for all three detection methods.

Combined results from Test 1 and Test 2 (Figure 2.10) indicated that there were interactions between susceptibility and detection technique ($P < 0.0001$, Table 2.14). There was a distinct difference in detection rate between susceptible and resistant seedlings for all techniques. Rate of detection by cultural isolation was less than that for both PCR and ELISA for both susceptible and resistant seedlings.

Table 2.14. ANOVA analysis of three methods for detecting *P. lateralis* on infected susceptible and resistant Port-Orford-cedar seedlings from Tests 1 and 2.

Effect	Num. DF	Den. DF	F-value	Pr > F
Detection method	2	6	1078.83	< 0.0001
Reaction (sus. / res.)	1	6	2366.42	< 0.0001
Detection method*Reaction	2	6	732.22	< 0.0001

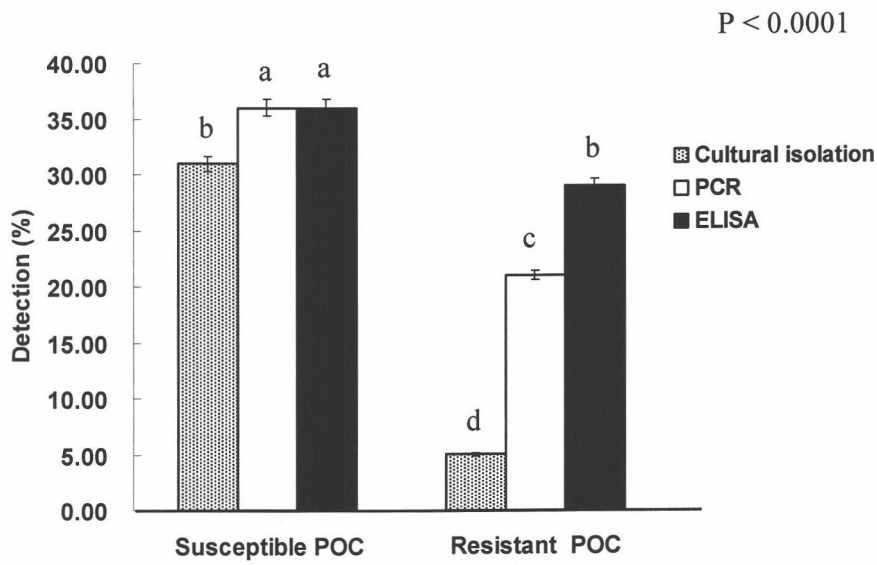

Figure 2.10. Least squares means of detection percentages by three techniques for *P. lateralis* detection from infected Port-Orford-cedars for Tests 1 and 2. Bars with the same letter are not significantly different (P > 0.05).

2.4.4 Infection through foliage

High levels of mortality in resistant POC seedlings from the 2000 and 2003 outplantings at Hiouchi, California suggested the possibility that infection by *P. lateralis* may have occurred through foliage under standing water conditions, thereby bypassing resistance expressed in the roots. To test this possibility, wounded and unwounded foliage of resistant and susceptible trees was immersed in a zoospore suspension. The results indicated a significant interaction between tree family, wound treatment, and time of exposure to zoospores (P = 0.0071, ANOVA *F*-test) (Table 2.15). For wounded foliage, the necrotic lesion length for the 5 hr exposure period was not different than for the 48 hr exposure period, and the 27 hr exposure period showed

Table 2.15. ANOVA analysis of infection of foliage after dipping in *P. lateralis* zoospore suspension for 5, 24, or 48 hours with and without wounds.

Effect	Num. DF	Den. DF	F-value	Pr > F
Family	4	12.9	16.21	< 0.0001
Wound	1	23.4	1101.08	< 0.0001
Time	2	16	0.77	0.4775
Family*Wound	4	12.9	33.05	< 0.0001
Family*Time	8	12.6	2.12	0.1123
Wound*Time	2	16	0.03	0.9661
Family*Wound*Time	8	12.6	4.75	0.0071

Table 2.16. ANOVA analysis of infection of foliage after dipping in *P. lateralis* zoospore suspension for 5 hours with and without wounds.

Effect	Num. DF	Den. DF	F-value	Pr > F
Family	4	52.5	1.60	0.1872
Wound	1	52.5	23.46	< 0.0001
Family*Wound	4	52.5	0.99	0.4237

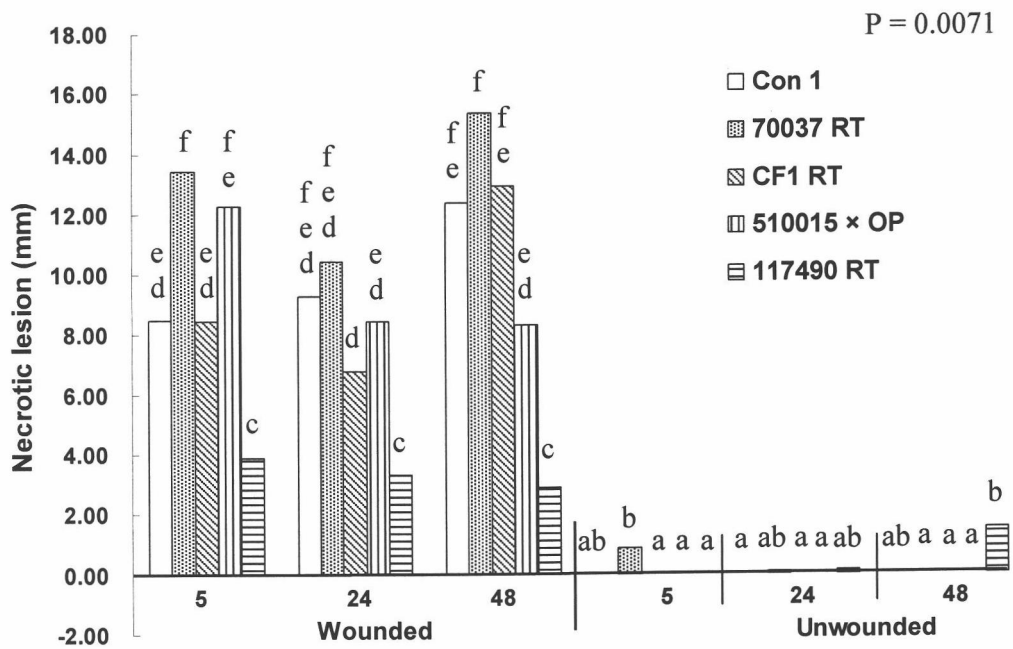

Figure 2.11. Least squares means of foliage infected by *P. lateralis* under condition of unwounded and wounded for 5, 24, or 48 hours. Bars with the same letter are not significantly different (P > 0.05).

less infection than 5 and 48 hr exposure periods for all tree families, except for 117490 RT. 117490 RT showed no difference between exposure times and also had significantly shorter necrotic lesions than other families (Figure 2.11). For the unwounded foliage, necrotic lesions were much smaller. However, some necrotic lesions were observed regardless of tree family and wound treatment. Since there was no main effect of exposure time (Table 2.15), the experiment was repeated with only a 5 hour exposure period. The result indicated that there was no interaction between family and wound treatment (Table 2.16). Instead, there was a significant difference between wounded and unwounded ($P < 0.0001$, ANOVA *F*-test) (Figure 2.12A). Although families did not differ significantly, 117490 RT had fewer lesions and those lesions were smaller than the other families (Figure 2.12B).

Figure 2.13 illustrated unwounded and wounded POC foliage. The difference between unwounded and wounded treatments was obvious in Figure 2.13A. Control (a) and unwounded (b) foliage did not show infection, whereas wounded (c) foliage of resistant 117490 RT developed small necrotic lesions around the wound site after 24 hr exposure. In some cases, different exposure periods did not affect lesion size on wounded foliage (Figure 2.13B). In addition, there was a difference between susceptible (a and b) and resistant (c) foliage (Figure 2.13C). The necrotic lesions on 117490 RT resistant foliage were limited to the wounded site (b) compared to broad necrotic lesions on 70037 RT (susceptible) wounded foliage (a) (Figure 2.13D). Among wounded foliage, the differences between families in necrotic lesion size were very obvious (Figure 2.13E). Sometimes, unwounded foliage of 70037 RT and CF1 RT (b) was infected on the upper surface of the scale (Figure 2.13F and G). More

often a necrotic lesion on unwounded 117490 RT foliage developed on the upper surface of the scale (Figure 2.13H). In summary, infection in unwounded foliage developed with all incubation times and in all POC families. However, the lesion was much smaller in unwounded than in wounded foliage. Interestingly, all wounded foliage of resistant POCs showed an oozing response around wound sites irrespective of exposure time. First the ooze was clear and hyaline, later it was viscous and brown to black colored (Figure 2.13 I and J). This response was absent in susceptible POCs.

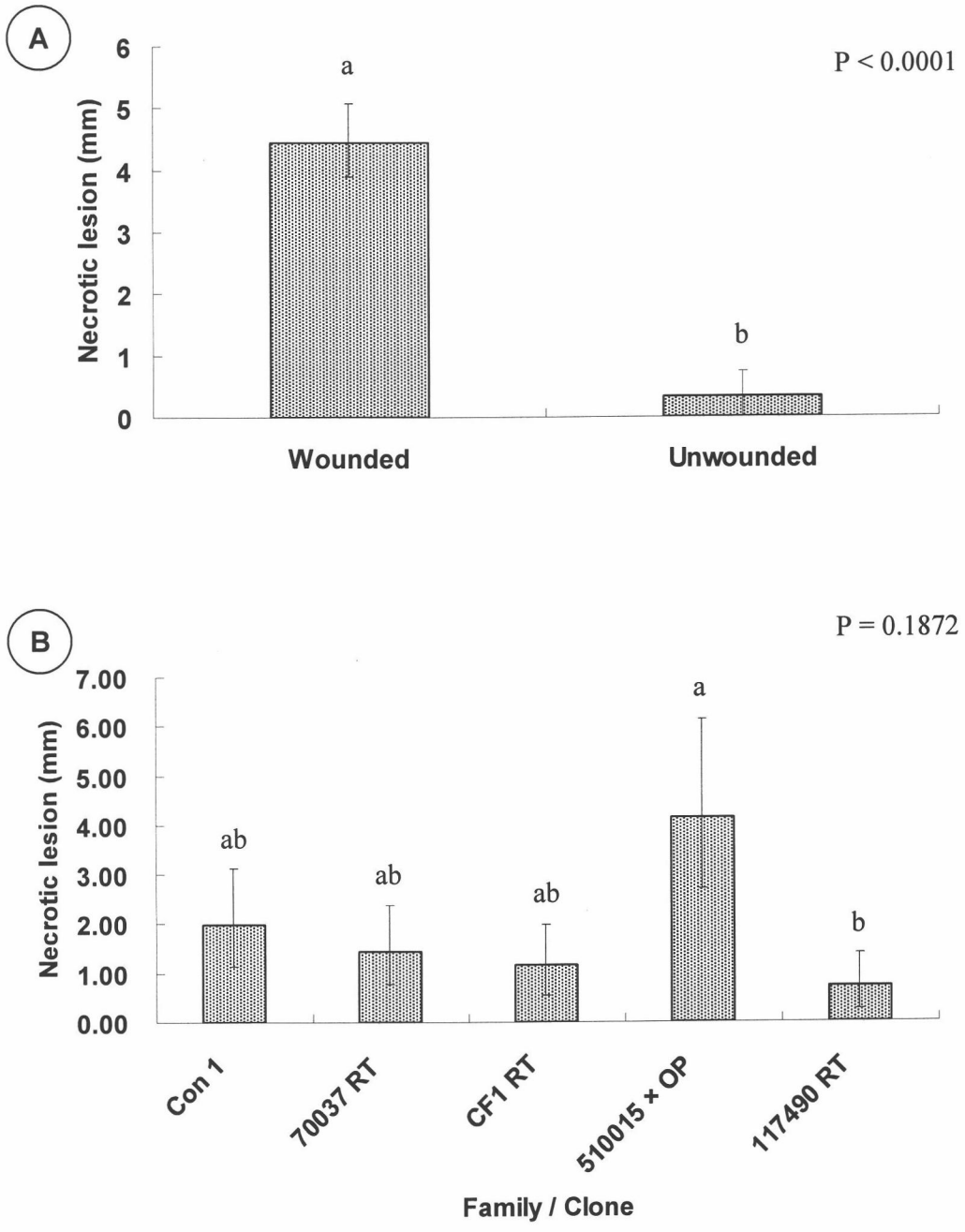

Figure 2.12. Least squares means of infected foliages by *P. lateralis* under condition of unwounded and wounded for only 5 hours. **A** Difference between wounded and unwounded. **B** No difference among family. Bars with the same letter are not significantly different ($P > 0.05$).

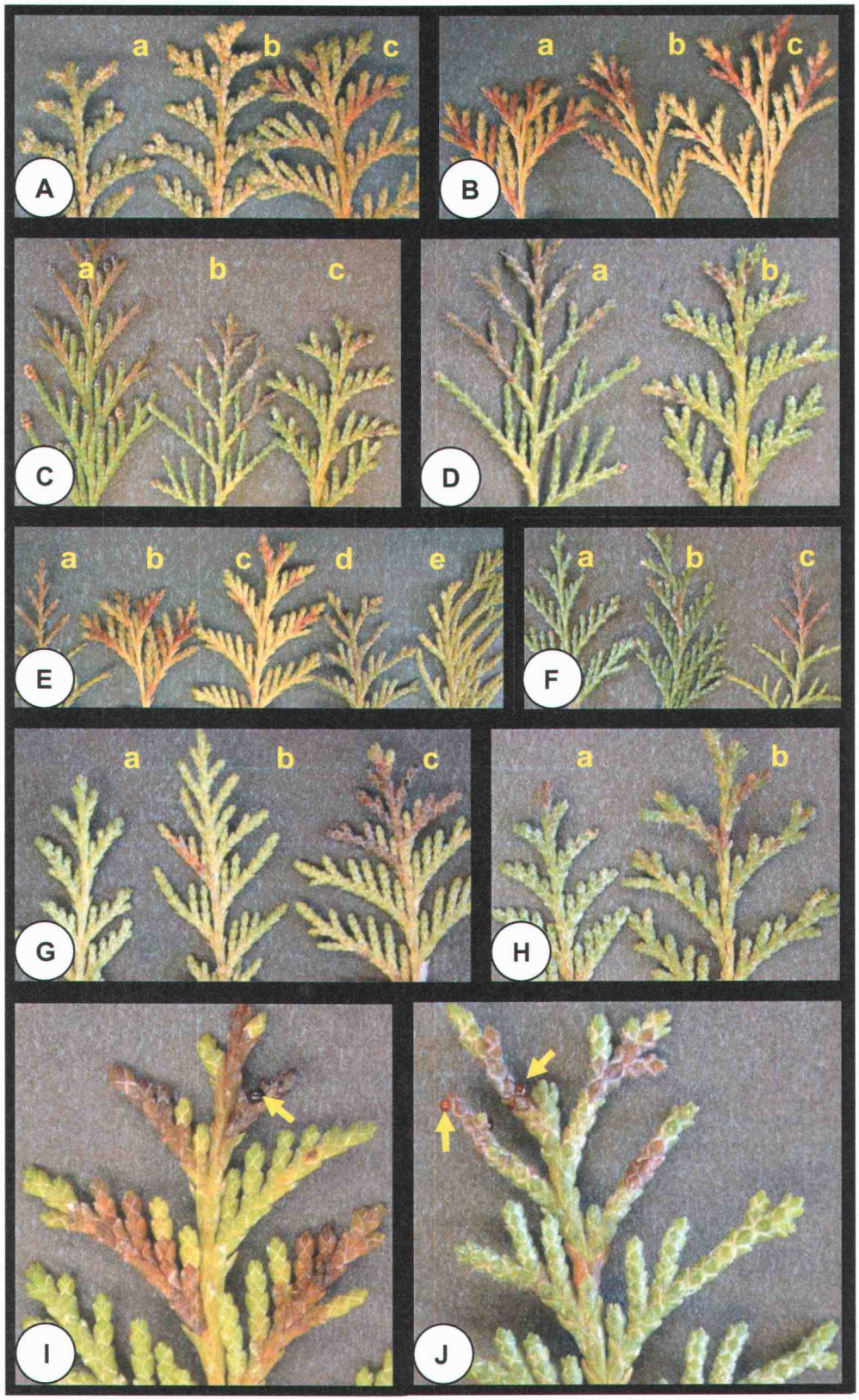
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Figure 2.13. Port-Orford-cedar foliage wounded and unwounded, then infected with *P. lateralis* zoospores. **A** Control (a), unwounded (b), and wounded (c) foliage of 117490 RT for 24 hr. **B** wounded 510015 × OP foliage for 5 (a), 24 (b), and 48 (c) hr incubation periods. **C** Wounded Con1 (a), 70037 RT (b), and 117490 RT (c) for 5 hr. **D** wounded 70037 RT (a) and 11740 RT (b) for 24 hr. **E** wounded foliage of 70037 RT (a), 510015 × OP (b), CF1 RT (c), 117490 RT, and control (e) for 5 hr. **F** Clean control (a) and necrotic lesions on unwounded (b), and wounded (c) of 70037RT for 5 hr. **G** Clean control (a) and necrotic lesions on unwounded (b), and wounded (c) of CF1 RT for 48 hr. **H** necrotic lesions on unwounded (a) and wounded 117490 RT for 48 hr. **I** and **J** Oozing response (arrows) on both CF1 RT for 24 hr and 117490 RT for 5 hr, respectively.

2.5 DISCUSSION

Both root and stem dip inoculations are useful techniques and routinely used for classifying POC families by mortality level (Hansen et al. 1989). In order to test the correlation between the root and stem dip inoculation techniques, roots and stems from the same individual seedlings were inoculated with *P. lateralis* and results compared by tree family. Unlike a previous trial result (Betlejewski et al. 2003), there was no significant correlation found between the root- and stem-dip inoculation methods. However, CF1 × OP and 510015 × OP had smaller necrotic lesions than the other families with both techniques. The concern has been expressed that using the stem dip test for the first screening of resistant families results in discarding some trees from the program that might prove resistant in root inoculation tests. While there was overall a poor correlation between the root- and stem-dip tests, at least both tests identified the same trees as most resistant.

Comparison between isolates of *P. lateralis* obtained up to 16 years ago with isolates obtained more recently from the raised beds at OSU did not reveal any differences in growth rate and zoospore production. That suggests that *P. lateralis* has not been selected for aggressiveness by continual challenge with resistant seedlings. Inoculation of resistant and susceptible stems revealed no differences between new and standard isolates, and no significant interaction between age of isolate and family. Thus there was no evidence for altered virulence in *P. lateralis* used for the screening. In stem inoculation, interestingly CF1 RT was intermediate in susceptibility with new isolates as well as standard isolates. In addition, CF1 RT showed similar necrosis development as the susceptible 70037 RT for the foliage test.

Isolation, of *P. lateralis* from soil by baiting (Hamm and Hansen 1984, Hansen et al. 1979, Ostrofsky et al. 1977), and direct plating from root and stem tissue have been used for *Phytophthora lateralis* detection (Linderman et al. 1977, Hansen et al. 1979, Hansen and Hamm 1996). Phytophthoras in general, and the slow-growing *P. lateralis* in particular, are considered difficult to isolate directly from diseased plants, especially after the plants are dead. Three methods, direct plating in culture, PCR, and ELISA, were compared for detecting *P. lateralis*. Rate of successful isolation of *P. lateralis* from symptomatic seedlings was low by direct plating. The rate of detection was very low for both susceptible and resistant POCs but the rate of detection from resistant POCs was lower than that from susceptible POCs. *P. lateralis* was the only *Phytophthora* species isolated from these seedlings. PCR detection was more successful than cultural isolation. All seedlings that were positive for *P. lateralis* by culture were also positive by PCR (and ELISA). In addition, PCR amplified a product of *P. lateralis* from the sample in a number of cases where the pathogen was not recovered by direct plating. Because the PCR amplification for the detection was made by the primers for only *P. lateralis* and *P. ramorum*, and ELISA might detect other *Phytophthora* or even some *Pythium* species, the PCR technique was more precise and believable than ELISA. *P. lateralis* was detected more frequently on both susceptible and resistant POCs by ELISA than by culture and PCR. Even though detection of *P. lateralis* by three methods was lower than expected, there was no difference in detection frequency between greenhouse inoculated and raised bed seedlings. In other words, both greenhouse and raised bed trials showed consistent low detection frequency for resistant tree families. Detection of *P. lateralis* by all methods was 25 %

for susceptible and 2.6 % for resistant POCs from the 2002 OSU greenhouse trial. The detection was 26 % for susceptible and 5 % for resistant POCs at the OSU raised bed, one of the field validation sites. Possible explanations for low frequency of detection by plating are 1) less hyphae present in resistant families than in susceptible families, 2) the hyphae were already dead at the margin due to host reactions, 3) the oozing response observed only in resistant families may be an inhibitor for isolation.

Although the ELISA test produced more positive reactions than other techniques, it must be remembered that the ELISA kit will detect other *Phytophthora* species and some *Pythium* species. Also, sampling error from the extract during the assay is possible. Therefore, false positives could have existed and increased the apparent detection percentage. MacDonald (1990) also compared culture plate and ELISA results for *Phytophthora*, *Pythium*, and *Rhizoctonia* in ornamental plants. He mentioned the false positives on the ELISA test and suggested that the cause might be an unmeaningful threshold of 0.3, which was the same as we had.

Dying resistant POCs were not predicted at the Hiouchi, California test site. Also, the typical symptom, a water soaked brown lesion, was found on seedling stems, but roots were sometimes symptom free. Because of extreme environmental conditions, standing water, initial infection through submerged foliage was suggested. The possibility of foliar infection was tested by inoculation of foliage. The results indicated that development of infection among tree families / clones was different on wounded foliage. Interestingly, unwounded foliage of both susceptible and resistant families developed necrotic lesions, and the lesions were usually located on the upper surface of scales. This means that the shoot, with extending scales, may have natural

openings. After all, *P. lateralis* has the ability to enter through physical wounds and natural openings and cause initial infection. However, the necrotic length on unwounded foliage was much shorter than in wounded. The unwounded foliage may need more time to infect and colonize than wounded foliage. Much longer incubation periods than 48 hours may lead to larger necrotic lengths leading to killing young POC seedlings in nature. In addition, there was no difference in necrotic length between CF1 RT and susceptible families, such as Con 1 and 70037 RT. Also, 510015 × OP was very susceptible by wound inoculation on foliage. However, 117490 RT was more resistant than other tree families.

In summary, the length of necrotic lesions on susceptible and resistant tree families are a useful indicator of susceptibility to *P. lateralis*, whether seedlings are inoculated on roots, stems, or foliage. However, as we mentioned in chapter 1, the CF1 parent tree segregates susceptible and resistant progeny at either 3:1 or 1:1 ratios depending on the other parent. Samples of CF1 seedlings for each test gave inconsistent results for the susceptible level, and even rooted cuttings were intermediate in susceptibility. This may be due to acquired susceptibility somehow or possibly the result of a different resistance mechanism than other resistant tree families.

The results in this study support the root and stem dip inoculation and raised bed screening methods for selection of resistance. Also, the comparison of the three detection methods in this chapter was the first attempt for *P. lateralis* to validate detection methods for efficacy. Indeed, there were differences in susceptibility among POC tree families, and the POC resistance breeding program has successfully selected

resistant families. In succeeding chapters, some of the selected resistant tree families will be used with susceptible tree families in order to understand the differences between susceptible and resistant families in expression of the resistance mechanisms at the cellular level.

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Chapter 3

Attraction of *Phytophthora lateralis* Zoospores to Roots of Port-Orford-Cedar

Eunsung Oh

3.1 ABSTRACT

Phytophthora lateralis is a destructive root pathogen on Port-Orford-cedar (*Chamaecyparis lawsoniana*) in ornamental plantings and natural forests in northern California and southern Oregon. Swimming zoospores of *P. lateralis* play an important role in infection. In general, zoospores are attracted to natural wounds and growing root tips that stimulate chemotaxis (Tyler et al. 1996, Zentmyer 1961). Zoospore accumulation on POC roots was compared between susceptible and resistant seedlings. Zoospore attraction to susceptible and resistant roots was quantified by light microscope estimates of numbers of zoospores and quantitative real-time PCR, and scanning electron microscopy was used to show visual differences. Zoospores were attracted most abundantly to the zones of root cell division and elongation, between 2 and 6 mm back from the root tip. Cross pollinated seedlings of POC tree 117490 showed significantly fewer zoospores attracted than seedlings of susceptible family 118051× OP. Otherwise, there were no other significant differences in zoospore attraction between families /clones. More zoospores of isolate T_4P_3 were attracted to roots than of isolates 366 and 368. POC specific primers, a new DNA extraction method, and SYBR green real-time QPCR were used to quantify pg *P. lateralis* DNA per ng POC DNA. In these QPCR trials, there were no significant differences between susceptible and resistant POC seedlings but differences in zoospore attraction by position along the root were significant. The results gave further support for the attraction of zoospores to the zones of root cell division (2-4 mm) and root elongation (4-6 mm).

3.2 INTRODUCTION

Oomycetous fungi are dispersed primarily by asexual, flagellated zoospores. Among the Oomycetes, *Phytophthora* and *Pythium* species mostly cause severe diseases in important crops, ornamental shrubs, and trees in forests. Zoospores play an important role in initiating infection. Movement of soil-borne zoosporic fungi depends on water saturation of soil. Zoospores have a limited time (less than 7 hours) and distance (a few millimeters to centimeters) in which to contact a viable host. However, different factors in the environment and *in vitro* influence the duration of activity and their motility. Evolution of tactic responses by zoospores to host roots has conferred selective advantages to Oomycetous fungi; tactic responses include directed movements in chemical gradients (chemotaxis), in electrical fields (electrotaxis), in water currents (rheotaxis), and due to gravity (geotaxis) (Morris and Gow 1993). Deacon and Donaldson (1993a, b) listed some factors that influence zoospore aggregation on roots (Table 3. 1). These factors also influence encystment, cyst germination, and hyphal chemotropism. Also, Carlile (1983) discussed the epidemiological and ecological significance of motility, taxis, and tropism in *Phytophthora* species.

Deacon and Donaldson (1993a, b) summarized host-specific factors such as isoflavones, and host-nonspecific factors such as amino acids, calcium, and electrical fields that have been analyzed for their influence on host-plant recognition phenomena (Table 3.1). Chemotaxis enables zoospores to target plant roots by swimming toward regions of nutrient exudation, such as the root apex and wound sites (Morris et al. 1998, Van West et al. 2002, Zentmyer 1961). Electrotaxis enables zoospores to be

attracted by external electrical currents and fields induced by the flow of protons and other ions into and out of growing and wounded regions (Morris and Gow 1993, Van West et al. 2002). Rheotaxis and geotaxis are advantageous because the zoospore population stays in the aerobic surface layers of soil.

Table 3.1. Factors that influence zoospore aggregation on roots (Deacon and Donaldson 1993a).

Types	Zoospore taxis
Factors involved	Chemical diffusates: amino acids, sugars, aldehydes, alcohols, isoflavones Electrical fields Auto-aggregation? Can be host-specific
Synergism/antagonism	Synergism and antagonism of diffusate mixtures (fungus-specific)
Role of calcium	Mediates motility pattern

There is suggestive evidence that chemotaxis and electrotaxis are important for zoosporic fungi. Chemotaxis has been studied in various *Phytophthora* and *Pythium* species such as *Pythium aphanidermatum* (Deacon and Donaldson 1993b, Royle and Hickman 1994a), *Phytophthora sojae* (Ho and Hickman 1967, Morris et al. 1998), *Ph. cinnamomi* (Allen and Newhook 1973), *Ph. drechsleri* (Barash et al. 1965), *Ph. palmivora* (Iser et al. 1989), and *Ph. megasperma* (Chi and Sabo 1978). Especially, Zentmyer (1961) tested zoospore attraction in different chemoattractants with *P. cinnamomi* by counting zoospores on fine lateral roots (about 1-2 cm from root tip) of avocados under a dissecting microscope. The result showed that there were different chemotatic and chemotropic activities of zoospores. Electrotaxis was demonstrated because in a few examples chemo-attraction failed to provide a clear explanation of zoospore aggregation. It is not known whether the test compounds enhance

metabolism of zoospores or if they only attract zoospores around roots. Also, it has been suggested that all plant roots exude nutrients from their roots. Van West et al. (2002) observed that there were different electrical fields among plant species and even between *Pythium* species and *Phytophthora* species. Nevertheless, there is no clear explanation of zoospore aggregation or its mechanisms on roots.

Murray and Hansen (1995, 1997) studied zoospore attraction in *Phytophthora lateralis*. In her work, Murray compared differences in *P. lateralis* zoospore attraction to Pacific yew (*Taxus brevifolia* Nutt.) and Port-Orford-cedar rootlets and found that the number of zoospores on POC rootlets was significantly greater than on Pacific yew rootlets, and that encystment of zoospores on POC occurred faster than on Pacific yew. Importantly, the zoospores were mostly aggregated and encysted on the zone of elongation of roots of POC and in specific areas along the region of maturation, in layers up to 10 deep. In contrast, the zoospores were attracted and commonly encysted in clumps on root hairs of Pacific yew. In addition, zoospores did not aggregate or swarm around the Douglas-fir control rootlets but did encyst on rootlets by chance. However, she described the relative attraction of zoospores, not absolute numbers; there has been no further work on zoospore attraction to POC, and no research on the differences in zoospore attraction between susceptible and resistant tree families of POC.

Simple and nested Polymerase Chain Reaction (PCR) methods have been developed for rapid and reliable detection and quantification of propagules and infection intensity on diseased plant materials. The methods rely on amplification of a specific sequence of the internal transcribed spacer (ITS) regions of ribosomal RNA

genes, at a high level of quantitative sensitivity. PCR primers with specificity to *P. infestans* (Aylor et al. 2001, Judelson and Tooley 2000, Tooley et al. 1997), *P. nicotianae* (Grote et al. 2002, Kong et al. 2003, Lacourt and Duncan 1997), *P. ramorum, P. nemorosa,* and *P. pseudosyringae* (Martin et al. 2004), and other *Phytophthora* species (Baily et al. 2002, Schubert et al. 1999) have been used to discriminate *Phytophthora* species for diagnostic purposes. Also, primers for *P. lateralis*, which also detect *P. ramorum*, were developed by Winton and Hansen in 2001.

Quantitative real-time polymerase chain reaction (QPCR) is an innovative and reliable technique for quantitative analysis of gene expression, mutation detection, allelic discrimination, and single nucleotide polymorphism (SNP) genotyping. QPCR was developed to overcome the basic weakness of classical PCR technology which can not directly quantify the amount of amplicon and measure low amounts of DNA. QPCR quantifies the amount of amplified product through the detection and quantitation of a fluorescent dye during the exponential phase of the amplification cycle. Most studies on QPCR have used a species specific TaqMan (dual-labeled) probe in true fungi (Böhm et al. 1999, Winton et al. 2002), as well as the Oomycetous fungi *P. infestans* and *P. citricola* (Böhm et al. 1999), and *P. ramorum* (Ivors and Garbelotto 2002). However, design of specific TaqMan probes can be difficult, time-consuming, and expensive. Alternatively, SYBR® green fluorescent dye is an inexpensive but non-specific dye that can be used instead of TaqMan probes. SYBR green has a high affinity for double-stranded DNA (dsDNA), and emits the fluorescence when it binds to dsDNA during the PCR annealing step. The rate of

increase in fluorescence signal intensity depends on the initial concentration of target present in the PCR reaction. There are a few published studies on quantification of fungal DNA by means of SYBR green real-time PCR (Alkan et al. 2004, Hayden et al. 2004, Vettraino et al. 2004).

The primary objective of this study was to compare the differences in *P. lateralis* zoospore attraction to susceptible and resistant Port-Orford-cedar in order to test two hypotheses. 1) *P. lateralis* zoospores are not uniformly attracted to the entire root and certain regions of the root are more attractive than others; and 2) zoospore aggregation and encystment differs between roots of susceptible and resistant POC. These hypotheses were investigated by means of scanning electron microscopy to visualize zoospore aggregation on roots of POC, direct counts of zoospore abundance under a light microscope, and finally, development of SYBR green quantitative real-time PCR to quantify both *P. lateralis* and POC DNA in infected roots and derive a normalized measurement of zoospore aggregation on susceptible and resistant POC.

3.3 MATERIALS AND METHODS

3.3.1 Cultures and POC seedlings

Three isolates of *P. lateralis* obtained from Oregon and California were used for inoculation of POC seedlings (Table 3.2). Cultures were grown on Corn Meal Agar (CMA, Difco) amended with 20 ppm β-sitosterol (Acros Organics) (βCMA) to stimulate zoospore production and stored in liquid nitrogen or water after they were originally isolated from infected POC. For zoospore production, cultures were incubated for 7 days at room temperature, then three agar disks with pure mycelium

were transferred to pea broth (150 g split peas in 1 L dH_2O autoclaved for 4 minutes, 20 ppm β-sitosterol added to filtered pea broth, and the medium autoclaved for 25 minutes). The pea broth cultures were incubated for 7 days at 17 °C in Petri dishes, then pea broth was poured off, the colonies washed with distilled water, then flooded with 25 ml stream water from Oak Creek, Benton County, Oregon and incubated for 2 days at 17 °C to induce sporangia.

Two-year-old seedlings or rooted cuttings of susceptible and resistant POCs were provided by Dorena Genetic Resource Center (DGRC), USDA Forest Service in Cottage Grove, Oregon. The seedlings were held in an enclosed greenhouse before they were used for inoculation (Table 3.3). The seedlings included open, self, and cross-pollinated families, and clones produced by rooting cuttings. The seedling families and clones had been previously tested for their susceptibility to *P. lateralis* by stem and root dip inoculations (Chapter 2).

Table 3.2. Host and origin of the isolates of *P. lateralis* used in zoospore attraction experiments.

Isolate	Host	Origin	Year Isolated
366	POC	Gasquet Ranger District, N. California	1986
368	POC	Gasquet Ranger District, N. California	1986
T_4P_3	POC	Galice District, Oregon	2000

3.3.2 Scanning electron microscopy

Fixation methods developed by Beagle-Ristaino and Rissler (1983) were modified for this study. The tip 2 cm of lateral roots of susceptible and resistant POCs was excised. Then the roots were carefully washed with distilled water, and placed in

30 ml zoospore suspension (approximately 1.8×10^4 zoospores per milliliter) of isolate T_4P_3 for 24 hours. Root pieces were transferred to 2.5 % glutar-paraformaldehyde in 0.2 M sodium phosphate buffer (0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 with pH 6.8) at 4 °C and fixed overnight. The fixed roots were rinsed three times with cold 0.2 M sodium phosphate buffer (pH 6.8) for 10 minutes. Then dehydration of the infected

Table 3.3. Port-Orford-cedar families and clones that were used in this chapter.

Experiment	Expected Reaction [1]		Family/Clone [2]
Scanning electron microscopy	S		118051 × OP
(2 roots from 1 seedling)	S/R		PO-OSU-CF1 [3] × OP
	Group 1	S	118051 × OP
		R	117490 ×117650
		R	117490 ×117335
		R	117490 × 118054
		R	117490 × 117505
	Group 2	S	118051 × OP
		S/R	CF1 × OP [3]
		R	CF1 RT
Count zoospores		R	CF1 × CF1 [3]
(3 roots from 3 different		R	CF2 × CF2
seedlings of each family)		R	CF3 × CF3
	Group 3	S	118051 × OP
		R	510015 × 510015 [3]
		R	510015 × CF1[3]
		R	510015 × OP [3]
	Group 4	S	118051 × OP
		S	117499 × OP
		R	510015 × OP [3]
		S/R	CF1 × OP[3]
	S		70037 RT
Quantitative real-time PCR	R		CF1 RT
(Total 20 roots from 2-3	R		510015 × OP [3]
different seedlings each assay)	R		117490 RT
	R		117490 × OP

[1] S = Susceptible and R = Resistant.
[2] Family by cross, open (OP), and self pollination and clone by rooted cutting (RT).
[3] Hypothesized to segregate susceptible and resistant offspring at 3:1 or 1:1 ratios (Chapter 1).

roots was carried out in a graded series of ethanol (30 %, 50 %, 75 %, and 95 % or 100 %) for 10 minutes each. The last step was performed for 20 minutes. The roots were mounted on aluminum stubs, sputter coated with 60 / 40 % by weight of Au / Pd alloy and scanned on an Am Ray 3300 FE model scanning electron microscope (FESEM). The electron microscopic images were recorded on Polaroid film.

3.3.3 Direct counts of zoospores attracted to roots

Resistant and susceptible families and clones were divided into 4 groups for measuring zoospore attraction (Table 3.3). This study was designed as repeated measures containing between-subject factors: resistance, family, position, and isolates. Each test included three randomly selected roots from each of three seedlings for each isolate. For Groups 1 and 4 (Table 3.3), a total of 81 roots (9 replications × 3 isolates × 3 repeated trials) of each POC seedling were used and for Groups 2 and 3, 27 roots (9 replications × 3 isolates × 1 trial) of each POC seedling were collected. One seedling per family was used for all four groups.

Zoospore production differed among isolates. Zoospores of T_4P_3 were abundant and very active while other isolates produced low numbers of relatively inactive zoospores (Chapter 2). In order to standardize experimental conditions, the zoospore concentration of each isolate was determined with the aid of a hemacytometer and adjusted to the same concentration (ca. 2×10^4 zoospores / ml) for each trial. The roots from each seedling were immersed in zoospore suspension in a Petri dish simultaneously.

After 24 hours, the roots were fixed overnight in 2.5 % glutar-paraformaldehyde in 0.2 M sodium phosphate buffer at 4 °C. Then the roots were placed on slides in a drop of 0.001 % aqueous calcofluor White M2R (fluorescent brightener, Sigma chemical Co. St. Louis) (Tsao 1970) and covered with a cover slip. The cover slip was marked every 2 mm from root tip to 10 mm (Figure 3.1). The slides were examined under a fluorescent- light microscope (Zeiss, Axioskop 2) with 100-200 × magnification at excitation λ 450-490 and emission λ 515 and encysted zoospores in each 2 mm root segment were counted in a single focal plane. The number of encysted zoospores on each 2 mm segment of a root was counted and compared (Figure 3.1). Data for each test were recorded and the number of zoospores were log transformed to standardize variance and analyzed by Proc Mixed-generalized linear model (ANOVA), The SAS System version 8 (SAS Inc.). The ANOVA *F*-test provided P-values for main and interaction factors, and back-transformed least squares means were plotted for comparisons.

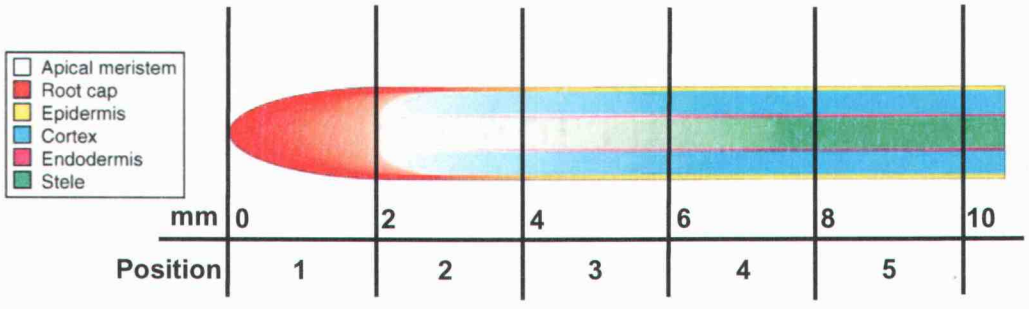

Figure 3.1. A diagram of a root tip showing tissue types and the 2 mm segments that were used for quantification. Each segment was considered as position 1 (0-2 mm) = Root cap, 2 (2-4 mm) = Root cell division, 3 (4-6 mm), = Root elongation, 4 (6-8 mm) and 5 (8-10 mm) = Root maturation.

3.3.4 Quantification of POC and *P. lateralis* DNA with Real-time PCR

1) POC DNA extraction for sequencing

Total genomic DNA was extracted using a modification of the Winton et al. (2002) protocol. A *Phytophthora*-free root of Port-Orford-cedar was placed into a 2 ml microfuge tube with two 3 mm glass beads, frozen in liquid nitrogen, and pulverized in a Mini-Beadbeater for 30 seconds at 4200 rpm. After pulverization, samples were incubated in 1.5 ml CTAB extraction buffer (2 % (w/v) CTAB (cetyltrimethylammonium bromide), 100 mM Tris, 20 mM NaEDTA pH 8.0, 1.4 M NaCl, 1 % (w/v) polyvinylpolypyrrolidone, 0.1 % (v/v) 2-mercaptoethanol) at 65 °C for 2 hours. The DNA was purified in chloroform / isoamyl alcohol (24:1) and precipitated from the aqueous phase by the addition of isopropanol after adding 5 µl ribonuclease A for 10 minutes at room temperature. After centrifugation, the DNA pellet was washed in ice-cold 70 % ethanol and resuspended in TE (5 mM Tris pH 8.0, 0.5 mM Na_2EDTA). Purified POC DNA was electrophoresed on 2 % (w/v) agarose gels in Tris-Borate-EDTA (TBE) buffer (pH 8.6) and visualized with ethidium bromide under UV light. The fragment sizes were estimated by comparison with a low mass ladder (Invitrogen, Carlsbad, CA). The extracted DNA was purified with a Gene Clean kit (Q·Biogene, Inc. Carlsbad, CA) and then electrophoresed on a 2 % agarose gel with the low mass ladder again to get purified and concentrated DNA.

2) PCR amplication for sequence

In order to get ITS region sequences from total genomic DNA, the purified POC DNA was amplified with various forward (ITS1, ITS3, ITS5, ITS5*, 5.8SR, and

5.8S-79F) and reverse (ITS2, 26S-25R, and 5.8GYM) primer combinations (Table 3.4). None of the primer combinations yielded an amplification product except for the set of ITS1-26S-25R and ITS5*-26S-25R primers.

For sequencing, amplification reactions were performed in 50-µl volumes containing 1× enzyme buffer, 200 µM dNTP, 0.05 U/µl RedTaq DNA polymerase (Sigma, St. Louis, MO), 0.4 µM primer ITS sets (ITS1, 26S-25R, 5.8 GYM, and 5.8S-79F) and 1 µl genomic DNA. The thermal cycler was programmed for 1 cycle of 60 s at 95 °C, and 39 cycles of 60 s at 94 °C denaturing, then 60 s at 52 °C annealing, and 60 s at 72 °C extension, and 7 min at 72 °C and finally 5 min at 10°C for hold. PCR products were electrophoresed on 2 % (w/v) agarose gels in TBE buffer (pH 8.6) and visualized with ethidium bromide under UV light. The fragment sizes were estimated by comparison with a 100 bp DNA ladder (GeneRuler ™ , Hanover, MD) and a low mass ladder. The PCR products contained 15 ng DNA / µl. The products were incubated overnight at room temperature after adding 0.5 µl ExoSAP-

Table 3.4. Forward and reverse primers tested for the amplification of ribosomal DNA from Port-Orford-cedar.

Primer Name	Sequences (5'-3')	Reference
ITS1 (F)[1]	TCCGTAGGTGAACCTGCGG	White et al. 1990
ITS2 (R)	GCTGCGTTCTTCATCGATGC	White et al. 1990
ITS3 (F)	GCATCGATGAAGAACGCAGC	White et al. 1990
ITS5 (F)	GGAAGTAAAAGTCGTAACAAGG	White et al. 1990
ITS5* (F)	GGAAGGAGAAGTCGTAACAAGG	Liston et al. 1996
5.8SR (F)	TCGATGAAGAACGCAGCG	Vilgalys 1990 &1994
5.8GYM (R)	CAGAATCCCGTGAATCATC	Liston et al. 1996
5.8S-79F (F)	GCAGAATCCCGTGAACCATC	Nickrent (?)
26S-25R (R)	TATGCTTAAACTCAGCGGGT	Liston et al. 1996

[1] Direction of the primers; F = Forward and R = Reverse.

IT (USB, Cleveland, OH). The ExoSAP-IT removed any unconsumed dNTPs and primers remaining in the PCR product mixture to prevent interference with sequencing reactions. 12 µl volumes of 150 ng DNA for sequencing included 1.2 µl primers, 10 µl PCR product, and 0.8 µl ddH_2O. Cycle sequencing was performed using dye-terminator chemistry on an ABI model 377 fluorescent sequencer (PE Applied Biosystems, Inc., Foster City, CA). Contigs were assembled and the overlapping sequences edited using the Staden package (Staden 1996). Because sequence signals of the PCR product itself were very low, an alternative protocol, cloning by bacterial transformation, was necessary for sequencing.

3) Cloning PCR amplification product for sequence

PCR products from primers ITS5*-26S-25R and ITS1-26S-25R were cloned with TOPO-TA for sequencing cloning kit (Invitrogen) according to manufacturer's instructions. 1.1 kb PCR amplification products were ligated into the TOPO vector (2 µl PCR product, 0.5 µl salt solution, and 0.5 µl pCR®4-TOPO (vector) for 30 minutes at room temperature and placed on ice. For chemical transformation, competent cells were thawed on ice and 25 µl removed to a new tube. 1 µl ligated TOPO vector was added and incubated on ice for 6 minutes. The tube was placed in a water bath at 42 °C for 30-second heatshock and then placed on ice. 125 µl SOC from the kit was added to the tube and put in a rotating incubator at 37 °C for 60 minutes. 25 µl cloning liquid was spread in pre-prepared LB+kan plates (autoclaved 10 g LB Agar, 250 ml dH_2O at 50 °C + 250 µl kanamycin sulfate), and the plates were incubated for 12 hours at 37 °C. Positive amplified bacterial colonies were picked with sterile

toothpicks and twirled in 50 µl ddH_2O. For each colony, 2 µl was transferred to one well of a 96 well PCR plate followed by sealing with adhesive foil and the plate was stored frozen until used for PCR. Positive clones were amplified by PCR using 2.1 µl 10 × buffer, 1.68 µl 2.5 mM dNTPs, 1.05 µl 1 U RedTaq, 0.84 µl 0.4 µM forward TOPOM13 primer, 0.84 µl 0.04 µM reverse TOPOM13 primer, and 2 µl sample template. The reaction conditions were 1 cycle of 3 min at 95 °C denaturing, 34 cycles of 20 s at 95 °C, 20 s at 50 °C annealing, and 1.5 min at 72 °C extension, and finally hold at 15 °C. The amplified cloned PCR-products were electrophoresed on 2 % (w/v) agarose gels in TBE buffer (pH 8.6) and visualized with ethidium bromide under UV light. The fragment sizes were estimated by comparison with a 100 bp DNA ladder and a low mass ladder. The amplified cloned PCR products contained 100 ng DNA / µl. The PCR products were incubated overnight at room temperature after adding 0.5 µl ExoSAP-IT and cleaned by running a thermal cycler as described above. 12 µl volumes of 150 ng DNA for sequencing included 1.2 µl primers, 1.5 µl PCR product, and 9.3 µl ddH_2O. Cycle sequencing was performed using dye-terminator chemistry on an ABI model 377 fluorescent sequencer (PE Applied Biosystems, Inc., Foster City, CA, USA). Contigs were assembled and the overlapping sequences edited using the Staden package (Staden 1996). Ribosomal DNA alignment of ITS1, 5.8S, and ITS2 of Port-Orford-cedar was conducted to identify candidate primers.

4) POC primer design

Three possible forward and reverse primers, Pocits678F-1122R, Pocits331F-896R, and Pocits13F-697R, were identified with the aid of 'primer express' software v2.0 (PE Applied Biosystems). Additionally, one candidate primer set was modified with existing ITS primers by comparing nucleotides with POC ITS region sequenced previously by ITS1F and 26S-25R and ITS5* and 26S-25R. The two primer sets, Pocits678F-1122R and Pocits13F-697R, from the software and one modified primer set were synthesized (Integrated DNA Technologies, Inc.) (Table 3.5).

Table 3.5. Primers of Port-Orford-cedar designed for the quantitative real-time polymerase chain reaction in the present study.

Primer name	Direction [1]	Sequence (5'-3')	Position	Fragment length (bp)
Pocits342	F	GGG AAG ATA TGA GCC TTG TC	342	-
Pocits842	R	TTC GCT ACA TTC TTC ATC GT	842	
Pocits678	F	ATG TGT CAA CAC CAA CAC AC	678	445
Plat1122	R	GCC AAT TTA AAG TTC CAC AC	1122	
Pocits13	F	CGT AAC AAG GTT ACC GTA GG	13	685
Pocits697	R	GTG TGT TGG TGT TGA CAC AT	697	

[1] F = Forward and R = Reverse.

Extracted DNA from roots and stems of POC, as well as negative controls such as *P. lateralis* and *P. ramorum* DNA, Douglas-fir DNA, and ddH_2O were included in PCR reactions with the three sets of primers. The products were electrophoresed on 2 % (w/v) agarose gels in TBE buffer (pH 8.6) and visualized with ethidium bromide under UV light. The fragment sizes were estimated by comparison with a 100 bp DNA ladder. Only forward Pocits13f: 5'-CGT AAC AAG GTT ACC GTA GG-3' and

reverse Poctis697r: 5'-GTG TGT TGG TGT TGA CAC AT-3' clearly amplified a 685 bp product of nr ITS DNA of POC.

5) Optimization

The ITS primer set for *P. lateralis* was developed by Winton and Hansen (2001). The optimum concentrations of forward (Plat87f: 5'-TTA GTT GGG GGC TTC TGT TC-3') and reverse (Plat786r: 5'-AGC TGC CAA CAC AAA TTT C-3') primers for *P. lateralis* and forward (Pocits13f: 5'-CGT AAC AAG GTT ACC GTA GG-3') and reverse (Poctis697r: 5'-GTG TGT TGG TGT TGA CAC AT-3') primers for POC were determined by comparing the quality of the PCR products amplified at different final primer concentrations (50, 75, 100, and 150 nM) in reagent mix. 75 nM concentration for primers was selected for amplification of infected root DNA.

6) Standards

Assay standards, containing both *P. lateralis* and POC DNA in proportions representative of inoculated roots were prepared from genomic DNA extracted from mycelium in pea broth and uninfected POC foliage. POC and *P. lateralis* DNA were extracted as described above except that ribonuclease A was excluded in *P. lateralis* DNA (Sambrook 1989). Extracted plant and fungal DNA was electrophoresed on 2 % (w/v) agarose gels in TBE buffer (pH 8.6) and visualized with ethidium bromide under UV light. The fragment sizes were estimated by comparison with a low mass ladder. Then, extracted DNA was cleaned with a Gene Clean kit and electrophoresed on a 2 % agarose gel with the low mass ladder again to get purified and concentrated DNA. The

amount of the gene cleaned fungal and plant DNA was accurately measured by spectrophotometer. DNA concentrations in extracts from *P. lateralis* and POC samples were determined by measuring optical density at 260 nm with the aid of a spectrophotometer (Amersham Pharmacia Biotech, Buckinghamshire, UK), then diluted as needed and combined so that the ratio of POC: *P. lateralis* in the DNA standards was comparable to that of infected roots. Serial dilutions for *P. lateralis* and POC were prepared for use in calibration experiments. *P. lateralis* standards ranged from 0.0005 to 50 pg and POC standards ranged from 0.05 to 50 ng.

7) Root inoculation and DNA extraction

Twenty lateral root tips, 2 cm long, were excised from 2 or 3 seedlings of each family (Table 3.3) The roots of each seedling were carefully washed twice with distilled water to remove extra soil and were inoculated in 30 ml zoospore suspension of isolate 368 (Ca. 1.5×10^4 zoospores / ml) for 5 hours. A set of CF1 RT roots was incubated in 30 ml ddH_2O without zoospores as control. Roots were carefully removed from the zoospore suspension and cut into 5, 2 mm long segments (positions 1 - 5) starting from the root tip (Figure 3.1). Segments from each position (one from each root) were collected in a tube and DNA was extracted for quantitative PCR.

DNA extraction was performed with a new technology, Quick Pick ™ plant DNA purification kit (Q·Biogene, Inc., Carlsbad, CA) according to manufacturer's instruction, to prevent loss of DNA through the many steps of the standard CTAB procedure. The extraction procedure was the same as described above until pulverization. Ground samples (5 mg) were incubated in 10 µl lysis buffer and 1 µl

proteinase K solution for 30 minutes at 65 °C and vortexed. 1 µl Magazorbe (magnetic particles) and 25 µl binding buffer was added to the supernatant and the mixture was incubated for 10 minutes. The magnetic particles were transferred with a PickPen (Q·Biogene, Inc. Carlsbad, CA) for each step. The particles were rinsed twice in 40 µl wash buffer. The particles were discarded after 5-10 minutes in 30 µl elution buffer. The elution buffer then contained both *P. lateralis* and POC DNA.

8) SYBR PCR conditions and analysis

Reactions were performed in 25 µl-volumes containing 2 × Brilliant SYBR Green® Master Mix (Stratagene, La Jolla, CA), 75nM forward and reverse primers for both organisms, 1 mM reference dye, and 0.5 µl template (various concentrations). Real-time quantitative PCR was performed with an automated ABI Prism 7000 SDS (Applied Biosystems, Foster City, CA) in MicroAmp optical 96-well plates or single tubes (Applied Biosystems, Foster City, CA). Thermal cycling was completed in 3 hours 32 minutes and conditions consisted of 10 min at 95 °C and then 40 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min 30 s and finally 72 °C for 3 min. The PCR products generated during the PCR amplification were detected by 7000 SDS software (PE Applied Biosystems). The threshold cycle (Ct) was calculated to indicate significant fluorescence signals rising above background during the early cycles of the exponential amplification phase of the PCR amplification process. The PCR products were analyzed on 2 % (w/v) agarose gels in TBE buffer (pH 8.6)

without ethidium bromide and visualized under UV light with the SYBR Green filter in order to verify that the products of both *P. lateralis* and POC were being generated.

No template (negative) controls and the genomic DNA standards (positive) for both *P. lateralis* and POC were included as PCR controls in each assay. Unknown samples of infected roots and standards were included in triplicate in each plate, and the assay was repeated three times and data averaged. Concentration gradients of *P. lateralis* and POC DNA were used for generating the calibration curve. The concentration of input DNA was plotted against threshold cycle (Ct) to obtain a standard quantification curve by the SDS software and a regression equation was calculated. The amount of DNA in unknown samples was calculated by interpolation using measured CT-values and the regression equation. Normalized estimates of attracted *P. lateralis* zoospores on POC roots were obtained by dividing *P. lateralis* DNA estimates by POC DNA estimates for individual samples. The normalized estimates were recorded and were log transformed to standardize variance and analyzed by Proc Mixed-generalized linear model (ANOVA), The SAS System version 8 (SAS Inc.). The ANOVA *F*-test provided P-values for main and interaction factors, and back-transformed least squares means were plotted for comparisons.

3.4 RESULTS

3.4.1 Scanning electron microscopy

After 24 hour incubation with zoospores, the zoospores accumulated on both susceptible (118051 × OP) and resistant (PO-OSU-CF1 × OP) rootlets of POC. Zoospores encysted on the surface germinated, and hyphae grew to the epidermis.

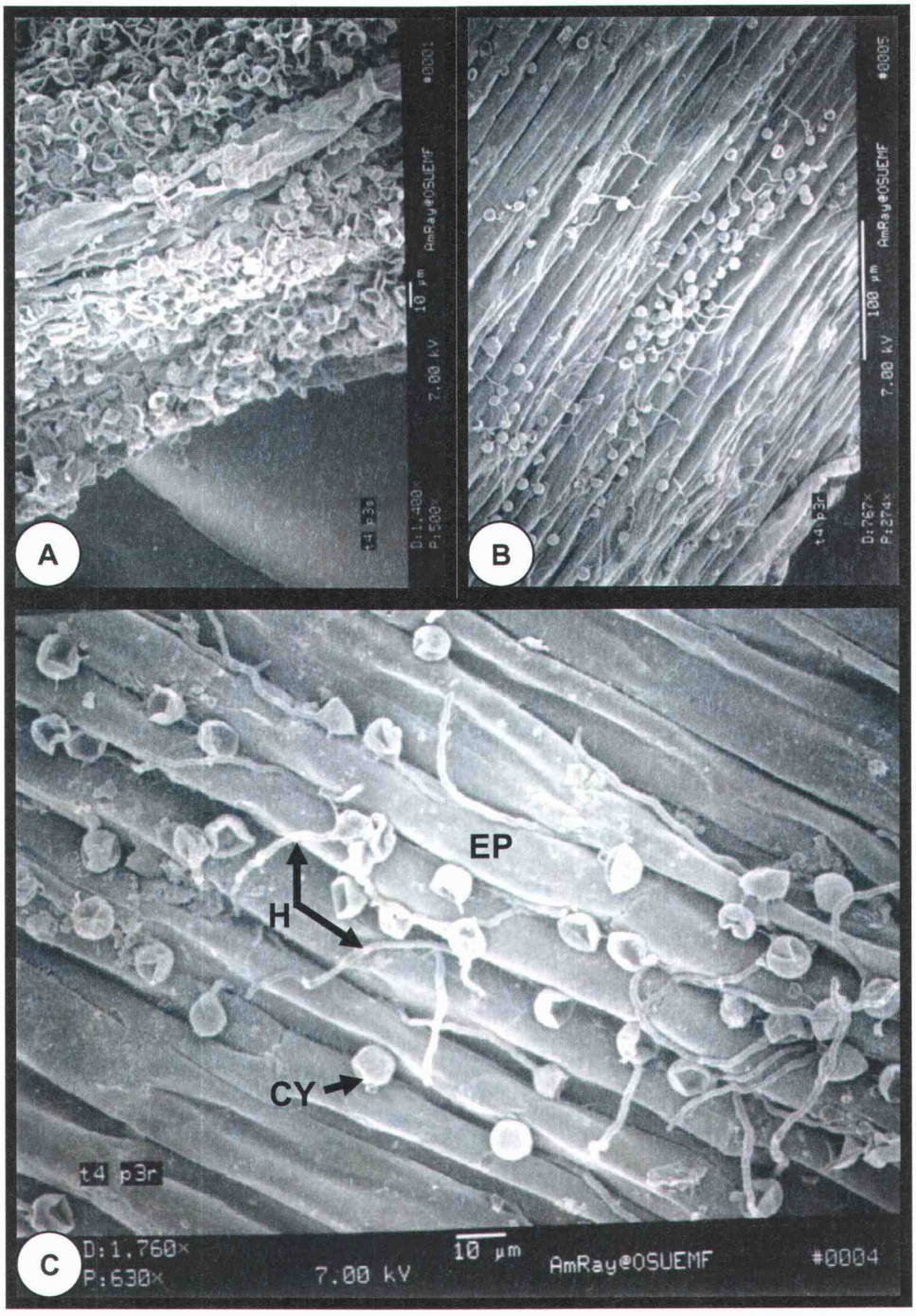

Figure 3.2. Scanning electron micrographs of the zoospore attraction on susceptible and resistant roots of POC infected by *Phytophthora lateralis* zoospores for 24 hours. **A** Many zoospores on susceptible 118051 × OP root. **B and C** Fewer zoospores on resistant CF1 × OP root. CY = Cyst; EP = Epidermal cell; H = Hypha.

Hyphae were observed in the process of penetrating the epidermis on both resistant and susceptible roots. However, there were apparent differences in zoospore aggregation on root tips of susceptible and resistant rootlets of POC (Figure 3.2). Zoospores accumulated abundantly on the surfaces of susceptible roots (Figure 3.2.A); many fewer zoospores were visible on roots of family CF1 × OP (Figure 3.2.B). Close observation of the resistant root showed that the zoospores germinated, and hyphae penetrated between epidermal cells (Figure 3.2.C).

3.4.2 Direct counts of zoospores on susceptible and resistant roots

Zoospore attraction of *P. lateralis* to POC was compared by family, root position, and isolate. In Group 1 families, there was a significant difference between susceptible and resistant tree families ($P < 0.0001$, ANOVA *F*-test, Table 3.6). The four cross pollinated 117490 POC families exhibited similar zoospore attraction. Position 1 (tip 2 mm) attracted the fewest zoospores, and positions 2 and 3 the most (Figure 3.3.A). Positions 4 and 5 showed fewer zoospores than position 2 and 3 but more than position 1 ($P < 0.0001$, ANOVA *F*-test, Figure 3.3.B). In other words, the zones of root cell division (2-4 mm) and elongation (4-6 mm) attracted more zoospores than other regions. Zoospores of isolate T_4P_3 aggregated on roots more abundantly than isolates 366 and 368 ($P = 0.003$, ANOVA *F*-test, Figure 3.3.C).

In Group 2 families, there were no significant differences among families, positions, or isolates (Table 3.7). The mean estimate of zoospores attracted to susceptible 118051 × OP was not different from the estimate of zoospores on the resistant CF series ($P = 0.3558$, ANOVA *F*-test, Figure 3.4.A). In addition, there were

Table 3.6. ANOVA analysis of Group 1 for zoospore counts on roots after 24 hour inoculation.

Effect	Num. DF	Den. DF	F-value	Pr > F
Family	4	28	16.24	< 0.0001
Isolate	2	28.1	7.19	0.0030
Family*Isolate	8	28	0.43	0.8946
Position	4	1056	73.16	< 0.0001
Family*Position	16	1054	1.31	0.1839
Isolate*Position	8	1056	0.84	0.5703
Family*Isolate*Position	32	1054	0.68	0.9105

Table 3.7. ANOVA analysis of Group 2.

Effect	Num. DF	Den. DF	F-value	Pr > F
Family	5	1	4.42	0.3458
Isolate	2	1	74.57	0.0816
Family*Isolate	10	1	1.14	0.6295
Position	4	1	14.82	0.1921
Family*Position	20	1	0.70	0.7543
Isolate*Position	8	1	6.08	0.3043
Family*Isolate*Position	40	1	0.52	0.8258

Table 3.8. ANOVA analysis of Group 3.

Effect	Num. DF	Den. DF	F-value	Pr > F
Family	3	22	1.91	0.1580
Isolate	2	22	68.97	< 0.0001
Family*Isolate	6	22	0.57	0.7477
Position	4	187	9.41	< 0.0001
Family*Position	12	187	3.95	< 0.0001
Isolate*Position	8	187	1.67	0.1090
Family*Isolate*Position	24	187	2.00	0.0057

Table 3.9. ANOVA analysis of Group 4.

Effect	Num. DF	Den. DF	F-value	Pr>F
Family	3	149	0.34	0.7988
Isolate	2	149	8.01	0.0005
Family*Isolate	6	149	0.49	0.8144
Position	4	757	4.45	0.0015
Family*Position	12	757	0.44	0.9472
Isolate*Position	8	757	1.52	0.1475
Family*Isolate*Position	24	757	0.42	0.9935

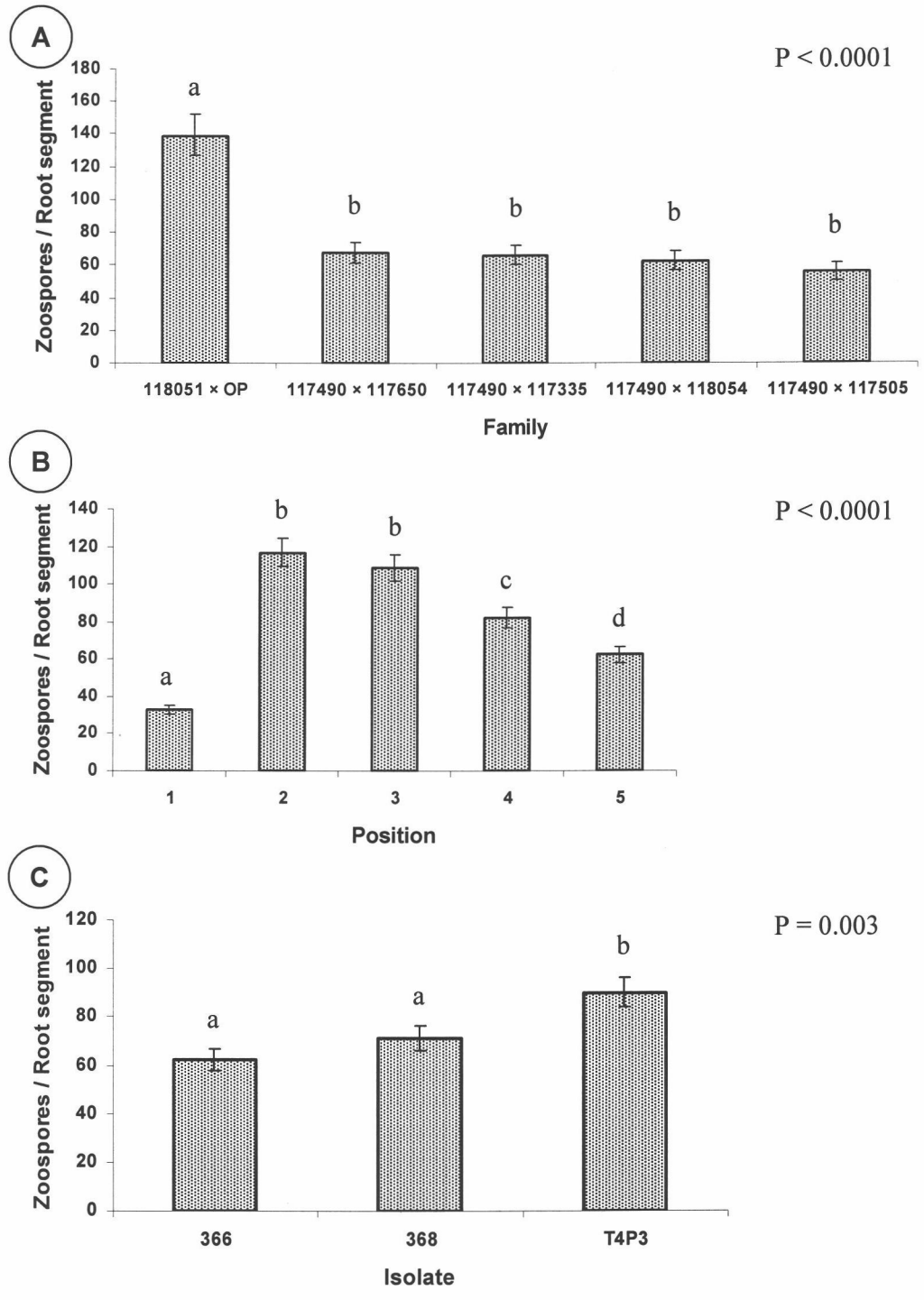

Figure 3.3. Least squares means of counted zoospores on roots of Group 1 families, including susceptible (118051 × OP) and resistant (Cross pollinated with 117490) Port-Orford-cedars exposed to *P. lateralis* for 24 hours. **A** Zoospore count by family. **B** Zoospore count by root position. **C** Zoospore count by *P. lateralis* isolate. Position 1 = 0-2 mm; 2 = 2-4 mm; 3 = 4-6 mm; 4 = 6-8 mm; 5 = 8-10 mm. Bars with the same letter are not significantly different (P > 0.05).

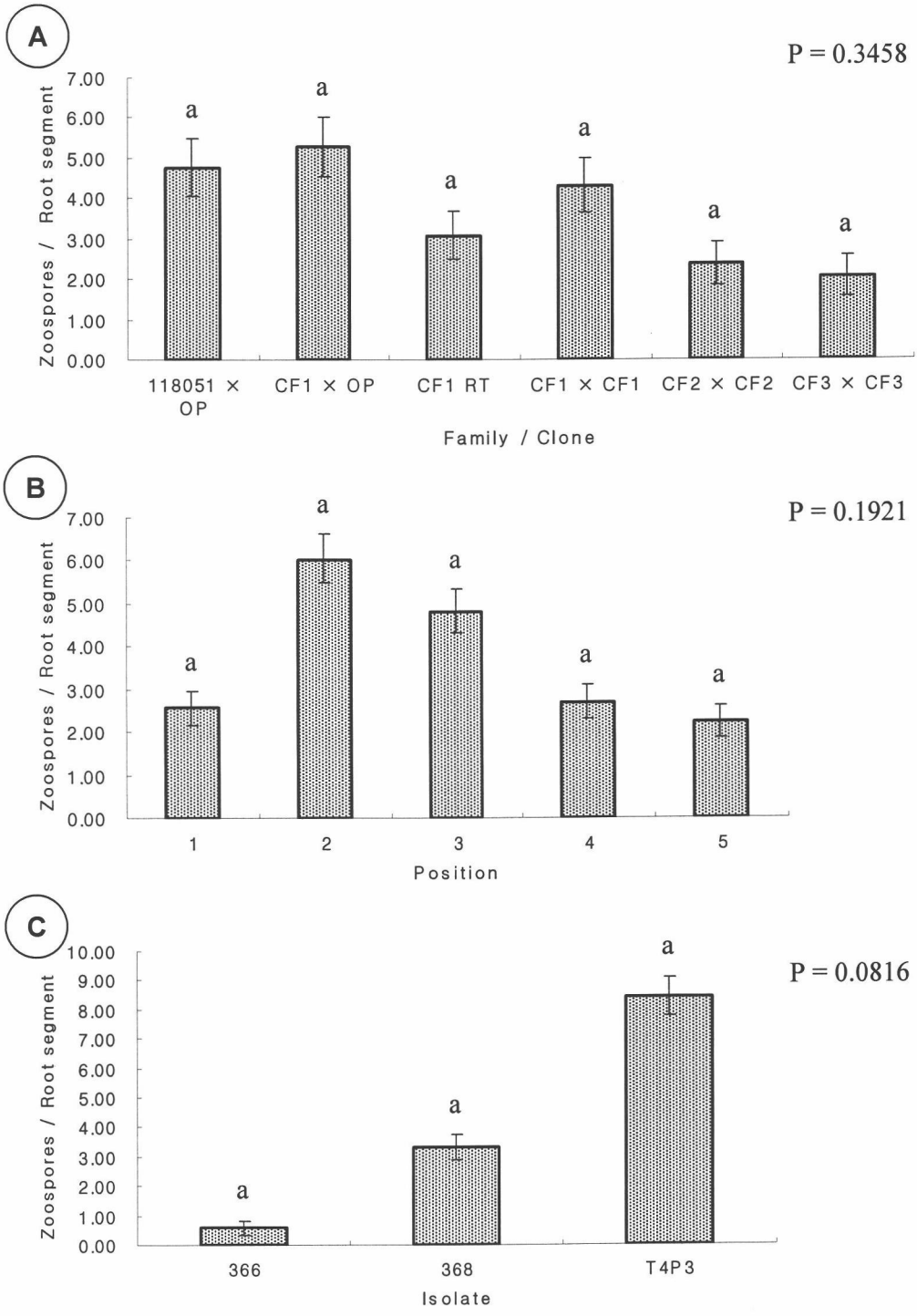

Figure 3.4. Least squares means of counted zoospores on roots of Group 2 families, including susceptible (118051 × OP) and resistant (Self and open pollinated CF series) Port-Orford-cedars exposed to *P. lateralis* for 24 hours. **A** Zoospores count by family. **B** Zoospores count by root position. **C** Zoospores count by *P. lateralis* isolate. Position 1 = 0-2 mm; 2 = 2-4 mm; 3 = 4-6 mm; 4 = 6-8 mm; 5 = 8-10 mm. Bars with the same letter are not significantly different ($P > 0.05$).

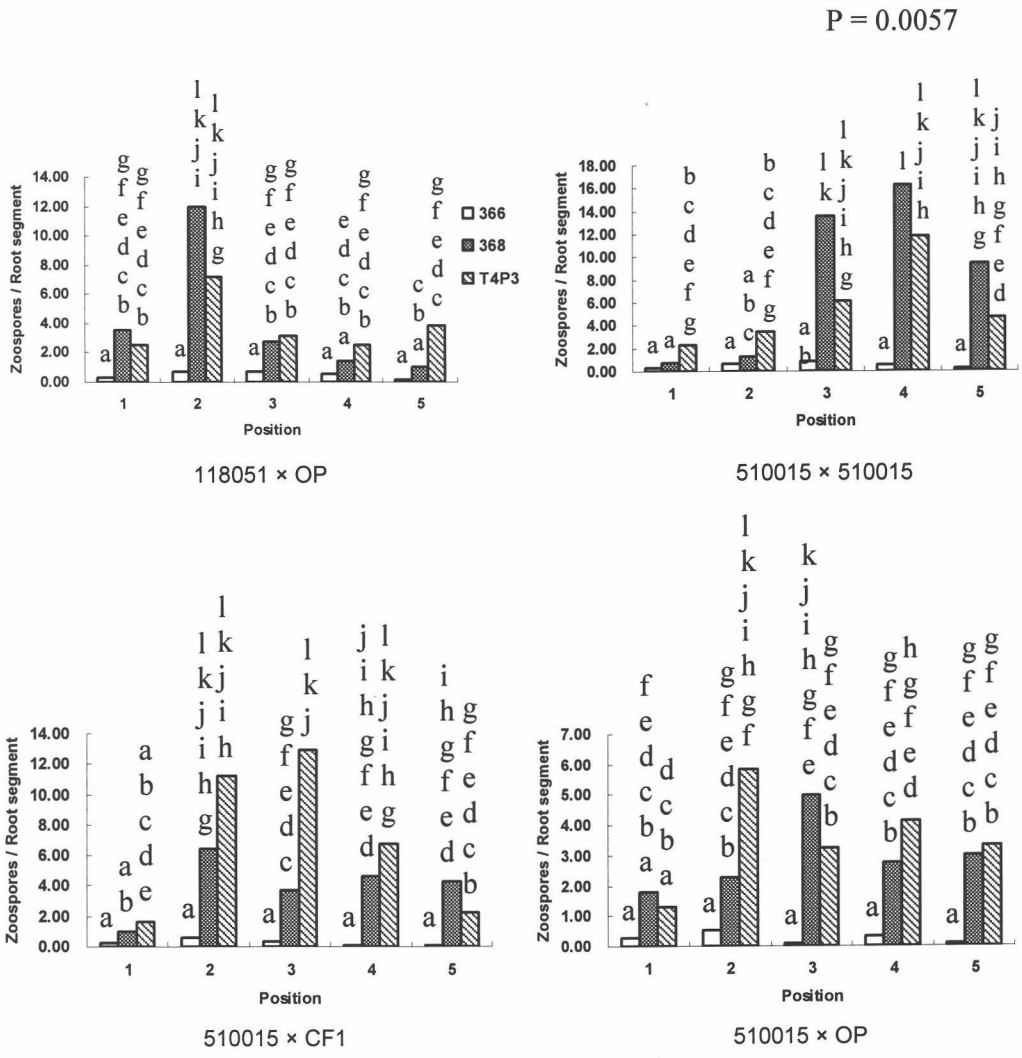

Figure 3.5. Least squares means of attracted zoospores on roots of Group 3 families, including susceptible (118051 × OP) and resistant (Cross, self and open pollinated with 510015) Port-Orford-cedars exposed to *P. lateralis* for 24 hours. Bars with the same letter are not significantly different (P > 0.05). Position 1 = 0-2 mm; 2 = 2-4 mm; 3 = 4-6 mm; 4 = 6-8 mm; 5 = 8-10 mm. Bars with the same letter are not significantly different (P > 0.05).

no significant differences in zoospore attraction among positions and isolates (P = 0.1921 and 0.0816, respectively, ANOVA *F*-test, Figure 3.4.B and C), although there were apparent differences.

There was a significant interaction in zoospore attraction between family, position and isolate in Group 3 families (P = 0.0057, ANOVA *F*-test, Table 3.8). There were no differences in zoospore attraction among positions and families with isolate 366. Root position 1 consistently had the fewest zoospores, but zoospore counts for the other positions varied by family and isolate (Figure 3.5). Overall, there were no consistent differences between isolates 368 and T_4P_3.

For Group 4 (Table 3.9), there were no significant differences between two open pollinated susceptible POC families and two open pollinated resistant POC families (P = 0.7988, ANOVA *F*-test, Figure 3.6.A). However, differences among root position and isolate were evident (P = 0.0015 and 0.0005, respectively, ANOVA *F*-test, Figure 3.6.B and C). More zoospores were counted for position 2, the root cell division zone than any other position. Also, zoospores of T_4P_3 accumulated more abundantly on roots than isolates 366 and 368.

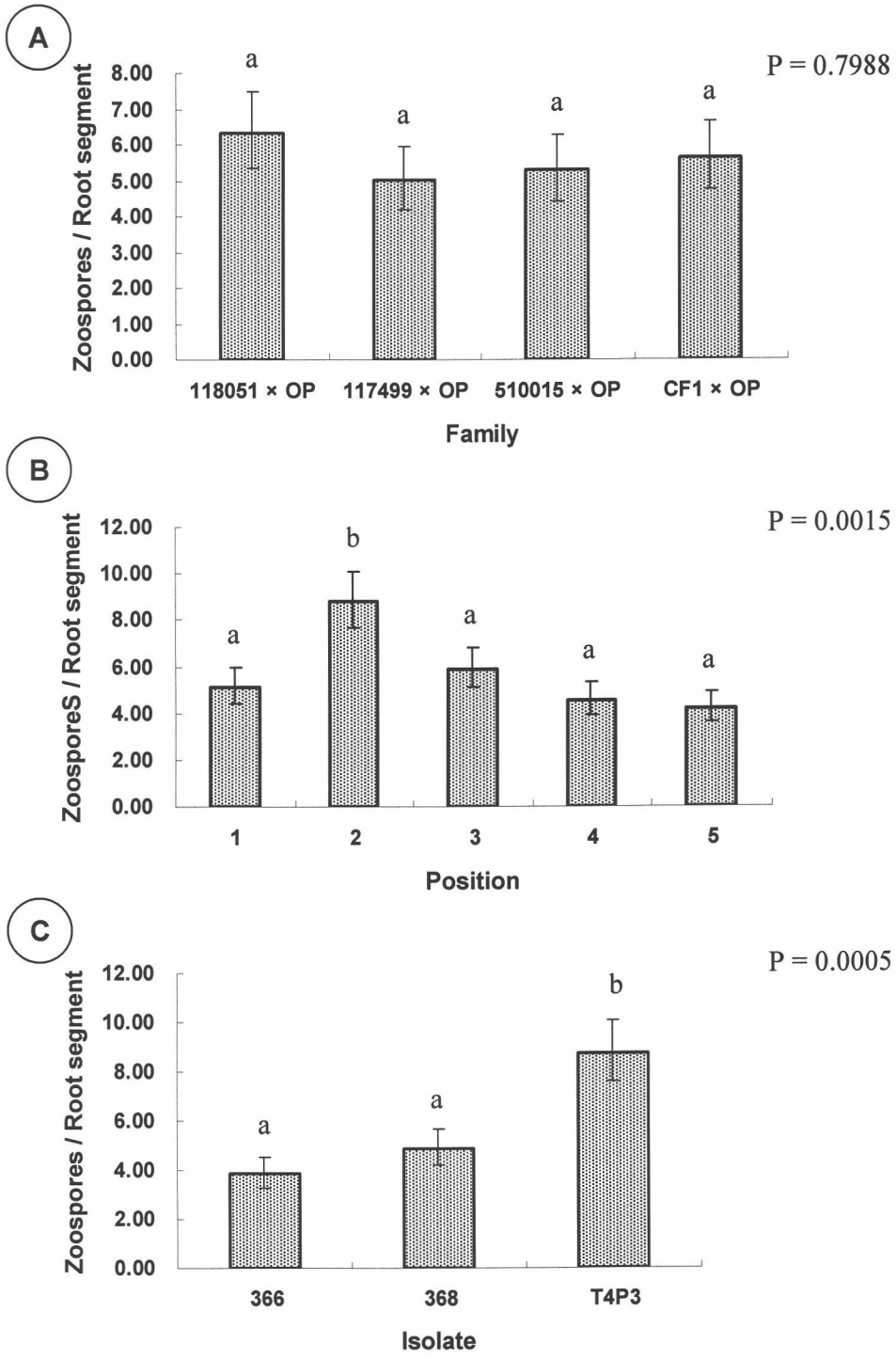

Figure 3.6. Least squares means of attracted zoospores on roots of Group 4 families, including susceptible (Open pollinated 118051 and 117499) and resistant (Open pollinated 510015 and CF1) Port-Orford-cedars exposed to *P. lateralis* for 24 hours. **A** Zoospores count by family. **B** Zoospores count by root position. **C** Zoospores count by *P. lateralis* isolate. Position 1 = 0-2 mm; 2 = 2-4 mm; 3 = 4-6 mm; 4 = 6-8 mm; 5 = 8-10 mm. Bars with the same letter are not significantly different (P > 0.05).

3.4.3 Quantification of POC and *P. lateralis* DNA with Real-time PCR

After successful cloning of Port-Orford-cedar DNA, ribosomal DNA alignment of ITS1, 5.8S, and ITS2 were completed to find primers. The sequences were compared against the GENBANK database using the program BLAST in the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Ribosomal DNA alignments of *Cryptomeria japonica* (Cupressaceae) and *Glyptostrobus pensilis* (Taxodiaceae), were the most similar sequences to POC. The three primers for POC were designed for quantitative PCR based on the ribosomal DNA alignment. In Figure 3.4, group Ⅰ, forward (Pocits342) and reverse (Pocits842) primers, showed no amplification reaction. In contrast, group Ⅱ, forward (Pocits678) and reverse (Pocits1122), amplified a 445 bp segment of POC DNA but it also amplified products in Douglas-fir needles (Lane 3) and *P. lateralis*

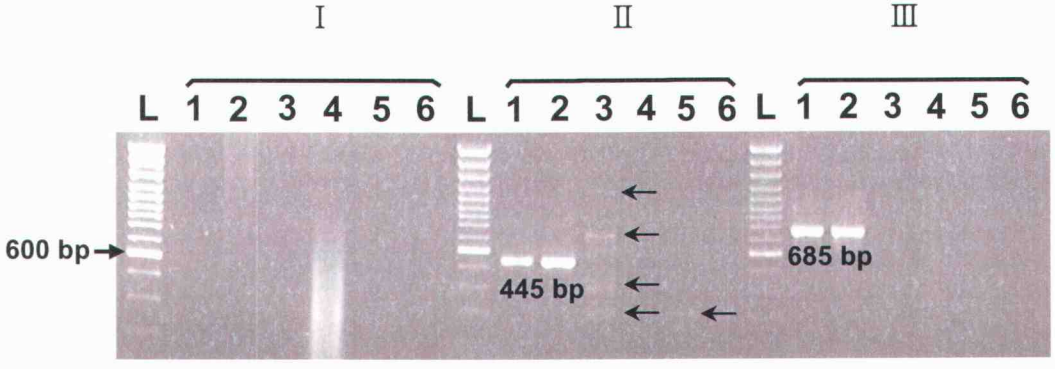

Figure 3.7. Amplification of Port-Orford-cedar target sequences with specific primers designed for quantitative real-time PCR application. Group Ⅰ - Ⅲ=Sets of forward and reverse primers; Ⅰ = Pocits342 and Pocits842, Ⅱ = Pocits678 and Pocits1122, and Ⅲ = Pocits13 and Pocits697. Group I; Lane 1 = POC root, 2 = POC foliage, 3 = Douglas-fir needles, 4 = Mycelium of *P. lateralis*, 5 = Mycelium of *P. ramorum*, and 6 = Water control. Group Ⅱ and Ⅲ; Lane 1 = POC root, 2 = POC foliage, 3 = Douglas-fir needles, 4 = Mycelium of *P. ramorum*, 5 = Mycelium of *P. lateralis*, 6 = Water control. L = 100bp DNA ladder. Arrows indicating amplification of unexpected products.

(Lane 5). Group III, forward (Pocits13) and reverse (Pocits697) amplified the expected 685 bp band and nothing else. The development of POC primers was successful to amplify a target DNA from POC roots and foliage.

The POC and *P. lateralis* primer sets specifically amplified the target DNA for POC and *P. lateralis* from root samples using SYBR green real-time QPCR. For standards, a linear relationship was obtained between threshold cycle (Ct) of the SYBR green real-time QPCR amplification reaction and DNA concentration on a logarithmic scale, and the regression coefficient value (R^2) was high for both *P. lateralis* (0.986) and POC (0.995) (Figure 3.8. A and B). *P. lateralis* and POC DNA was quantifiable over a range from 0.0005 to 50 pg and from 0.005 to 50 ng respectively. By interpolating values for unknown root samples on the linear regression, the graphs showed the quantities of DNA for both *P. lateralis* and POC. A low amount of DNA (pg) of *P. lateralis* was obtained from 20 2mm-long roots but it differed among tree families and positions while the amount of POC DNA (ng) of roots was very similar, as expected.

By ANOVA analysis of normalized DNA estimates from SYBR green real-time PCR, there were significant differences in zoospore attraction by root position and family ($P < 0.0001$, ANOVA *F*-test, Table 3.10). Positions 2 and 5 of 70037 RT had higher *P. lateralis* DNA content than other positions. Position 2 of CF1 RT contained higher *P. lateralis* DNA than other positions. Positions 2 and 3 of 510015 × OP were different than others. Positions 1, 2, and 3 of 117490 RT attracted more zoospores than other positions. Position 3 of 117490 × OP was the lowest. Overall, positions 2 and 3, zones of root cell division (2-4 mm) and elongation (4-6 mm),

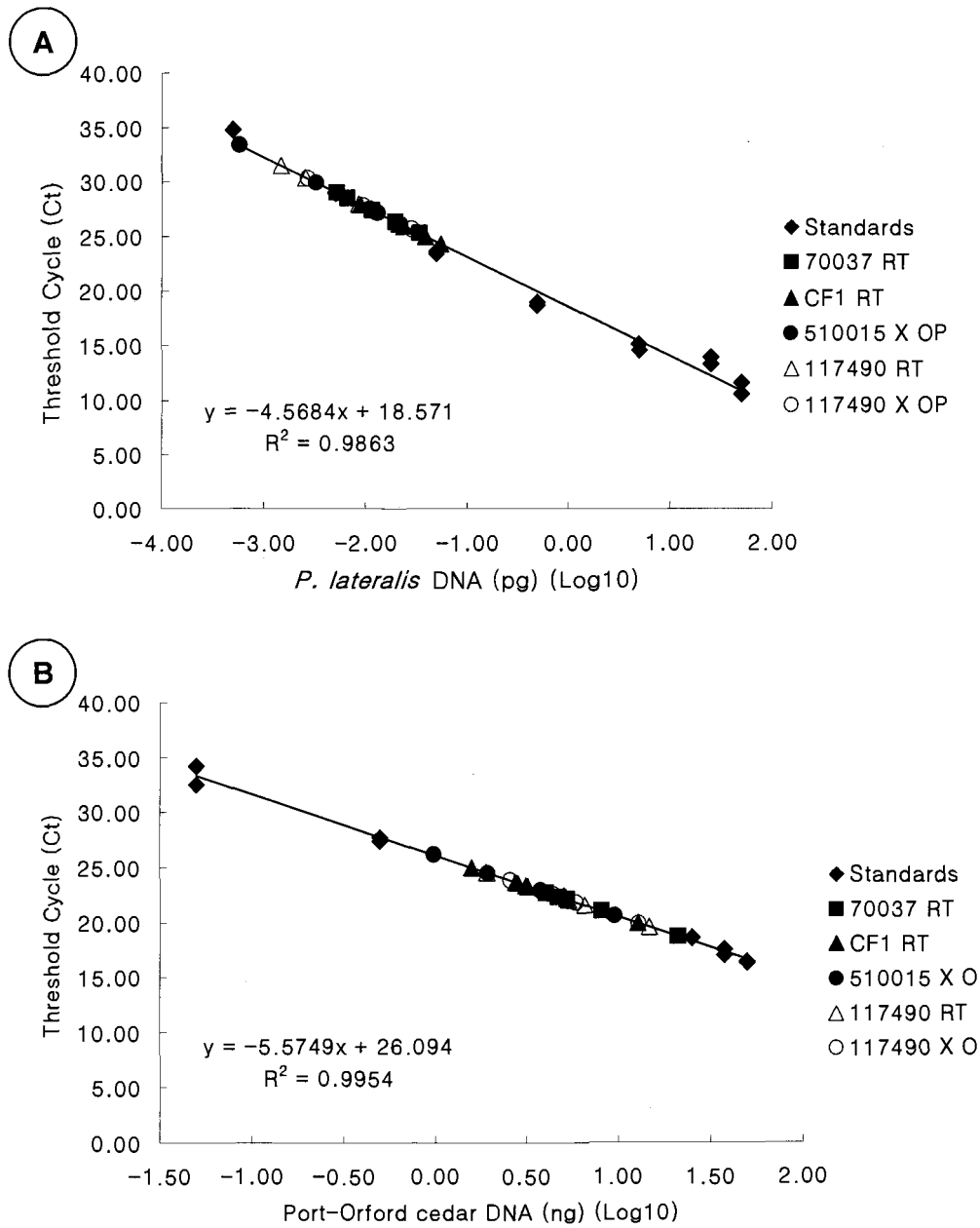

Figure 3.8. Standard curves and DNA values from infected roots demonstrating the quantification of *P. lateralis* (A) and Port-Orford-cedar (B) DNA present in root samples using SYBR green real-time PCR. Cycle thresholds (Ct) were plotted against the logarithmic scale of genomic DNA standards of known concentration and linear regression equations were calculated for the quantification of unknown root samples by interpolation.

Table 3.10. ANOVA analysis of *P. lateralis* DNA / POC DNA on Port-Orford-cedar roots.

Effect	Num. DF	Den. DF	F-value	Pr > F
Family	4	36.4	39.49	< 0.0001
Position	4	147	11.09	< 0.0001
Family*Position	16	147	11.16	< 0.0001

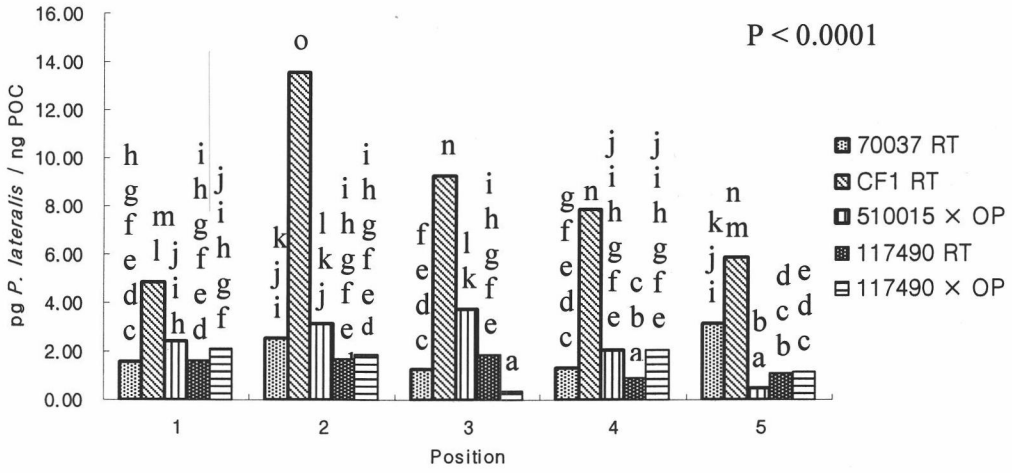

Figure 3.9. Least square means of normalized estimates of attracted zoospores of *P. lateralis* on Port-Orford-cedar for 5 hour inoculation (pg *P. lateralis* DNA / ng POC DNA). Bars with the same letter are not significantly different (P > 0.05). Position 1 = 0-2 mm; 2 = 2-4 mm; 3 = 4-6 mm; 4 = 6-8 mm; 5 = 8-10 mm.

generally aggregated more zoospores than other positions. Also, all positions on CF1 RT had significantly more *P. lateralis* DNA than other families, even the susceptible 70037 RT.

3.5 DISCUSSION

The initial infection of Port-Orford-cedar by *Phytophthora lateralis* is known to be caused by zoospores. Therefore, study of zoospore attraction to POC roots is central to an understanding of zoospore behavior and its importance to POC root disease management. Although zoospores are attracted to natural wounds on both resistant and susceptible roots (Widmer et al. 1998), there have been reports that showed that *Phytophthora* zoospores are attracted to chemical stimuli from undamaged plant roots. Attraction of zoospores of *Phytophthora* and *Pythium* species to capillary tubes filled with chemo-attractants has been demonstrated (Morris et al. 1998, Tyler et al. 1996). Encystment, cyst adhesion, germination, and germ tube tropism are additional components of the infection process affected by chemical signals (Deacon and Donaldson 1993).

In this study, we tested the hypothesis that roots of resistant and susceptible POC differed in attractiveness to zoospores of *P. lateralis.* It was initially visually evident that there were more encysted zoospores on a susceptible root than on a resistant root in SEM micrographs of susceptible and resistant roots. The initial inoculum (number of zoospores) is critical in the starting point of infection and different inoculum levels could lead to large differences in colonization and infection severity on roots later on. This preliminary result encouraged us to measure zoospore accumulation in other susceptible and resistant families. Zoospores were counted directly under the light microscope for comparisons between resistant and susceptible families. Murray and Hansen (1995, 1997) also studied differences in zoospore aggregation between POC and Pacific yew but did not compare susceptible and

resistant POC. Direct counts of encysted zoospores however, proved to be an inefficient, time consuming, inaccurate, and slow procedure.

Differences in zoospore attraction were found to vary by family, position, and isolate. Overall, the results by family indicated that there were few consistent differences in zoospore aggregation between susceptible and resistant family / clones. Only families that included tree 117490 as one parent had consistently fewer zoospores than susceptible families. There are several possible explanations for this: 1) extraneous factors, such as inadvertent root wounding, may have masked inherent differences in zoospore attraction; 2) because cross pollinated seedling families segregate for resistance, any single seedling from even an overall resistant family might be susceptible; 3) there may be more than one mechanism of resistance expressed in different POC families; 4) although individual trees and families may vary with respect to their attractions to *P. lateralis* zoospores, these differences may not be actually related to resistance to infection or disease. Reduced zoospore attraction may play a role in only some resistant families.

In general, zoospores accumulated most abundantly in positions 2 (2-4 mm behind the root tip) and 3 (4-6 mm), corresponding roughly to the zone of root cell division and the elongation zone. Murray and Hansen (1995, 1997) observed a similar concentration of zoospores in the elongation zone of POC roots. Possibly more chemical stimuli are generated from the actively growing elongation zone and nearby areas, and this resulted in more zoospore aggregation on these zones. Differences in zoospore accumulation between isolates were expected; more zoospores of T_4P_3 encysted on roots because sporangia and zoospores of this isolate formed more

abundantly and more quickly than the other isolates. Even though the same concentration of zoospores was used for all isolates in the tests, it took longer for isolate 366 in particular to reach the desired sporulation levels. Therefore, although the same concentration of inoculum was used, zoospores from each isolate may have varied in age.

Quantitative PCR was adapted for use with *P. lateralis* as an independent way to compare zoospore attraction to susceptible and resistant roots. Development of PCR assays gave us a new way to detect and quantify propagules of organisms in irrigation water, soil, crops, and woody plants (Aylor et al. 2001, Judelson and Tooley 2000, Kong et al. 2003, Nechwatal et al. 2001, Schubert et al. 1999, Tooley et al. 1997). Moreover, real-time QPCR was created for reliable and direct quantification of DNA. The TaqMan probe is commonly used to target specific DNA in QPCR but we chose to use SYBR green real-time QPCR in this study because specific primers for *P. lateralis* had been developed by Winton and Hansen (2001). The specific primers for Port-Orford-cedar designed in this study gave us the possibility to normalize the QPCR measurement of *P. lateralis* DNA and enabled comparisons of root infection independently of root size. For QPCR, roots were exposed to zoospores for only 5 hours. Therefore, it was assumed that *P. lateralis* DNA came from encysted zoospores and their germ tubes and appressoria but not internal hyphae. Because only a very small amount of roots was used (5 mg for 20, 2 mm-long root segments), DNA extraction was carefully carried out by the purification of DNA with a PickPen. This method allowed as to obtain both *P. lateralis* and POC DNA from small samples.

The results from QPCR were similar to the results from direct counts of zoospores. Overall, more *P. lateralis* DNA accumulated on positions 2 and 3 than elsewhere on the roots. Family CF1 RT accumulated more zoospores at all positions than the other families. A similar result was obtained with open pollinated seedlings of CF1 by direct counts.

Even though there were no consistent differences in zoospore attraction between susceptible and resistant POCs, this chapter does confirm zoospore attraction to the root cell division zone (2-4 mm) and root elongation zone (4-6 mm), as suggested by Murray and Hansen (1995, 1997). Further comparisons of zoospore attraction to susceptible and resistant POC will require seedlings of known resistance genotype, and careful growing and harvesting of roots under standard conditions. Also, the development of POC primers and use of the new DNA purification method will be valuable for future studies with *P. lateralis*. Finally the development of SYBR green real-time QPCR for quantification of both *P. lateralis* and POC DNA provides a model for further work with other species.

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Chapter 4

Cytological Comparison of Initial Infection and Colonization of Susceptible and Resistant Port-Orford-Cedar Seedlings by *Phytophthora lateralis*

Eunsung Oh

4.1 ABSTRACT

Encystment of *Phytophthora lateralis* zoospores on roots, the penetration and colonization of roots, and colonization of stems of seedlings, as well as host reactions to infection were examined histologically. Young lateral roots and woody stems of POC seedlings were exposed to motile zoospores or inoculated with pure mycelium. Two groups of POC were compared: 70037 RT, 117499 × OP, and 118051 × OP which appeared to be highly susceptible, and 117490 RT, CF1 RT, and 510015 × OP, which were resistant. The frequency of encystment, penetration, and colonization of susceptible seedlings was much greater than that of resistant seedlings. However, no differences in infection pathway were observed between the two groups. *P. lateralis* penetrated root epidermal cells both directly and indirectly. Hyphal colonization occurred both inter- and intracellularly in both roots and stems. Hyphae grew more slowly in cortical cells of resistant roots. Hyphae were abundant in stem phloem tissues of susceptible seedlings, but resistant stems had only a few intracellular hyphae, found in two or three layers of parenchyma cells in the secondary phloem. These results suggest that slowed growth of the pathogen may be due to an active resistance mechanism. Therefore, a combination of reduced zoospore attraction and retardation of hyphal growth may lead to resistance.

4.2 INTRODUCTION

Since Port-Orford-cedar (*Chamaecyparis lawsoniana*, POC) root disease was first reported in nurseries in the Seattle area in the 1920s, and in natural coniferous forests in Oregon in the 1950s, the distinctive symptoms of infection on roots and

stems of POC have been described many times (Erwin and Ribeiro 1996, Hansen et al. 2000, Roth et al. 1957, Torgeson et al. 1954, Trione et al. 1957, 1959). The diagnostic symptom, a red-brown necrotic lesion in the inner bark (phloem), is visible in recently killed trees after outer bark is peeled off in the field or in the laboratory. Most *Phytophthora* species are soil-borne pathogens, and the root-soil interface is the place of original infection. The infection on fine lateral roots of POC is usually initiated by motile zoospores of *Phytophthora lateralis* under favorable environmental conditions, and the rootlet is colonized by hyphae (Hansen et al. 2000). Colonizing the live inner bark, the hyphae then move up the root into the stem. Young and small seedlings are killed within one year after infection, and old-growth POC trees are dead within a few years (Hansen et al. 1998, Trione 1959). While the general course of infection is known, and the resulting necrotic symptoms are familiar, there has been no description of infection and pathogenesis at the histological level.

Flagellated zoospores are produced in Plasmodiophoromycota, of the Kingdom Protozoa, Oomycota of Kingdom Chromista, and Chytridiomycota of Kingdom Fungi. All zoospores in these kingdoms are motile for a limited time and act as important asexual structures for infection, because of the zoospore's ability to locate a viable host using homing responses to host cues. It has been reported that directed zoospore movement results from chemotaxis, electrotaxis, rheotaxis, and geotaxis (Cameron and Carlile 1977, Morris et al.1998, Morris and Gow 1993, Tyler et al. 1996, Van West et al. 2002, Zentmyer 1961). Chemotaxis and electrotaxis have been intensively studied in oomycetous fungi. There is strong evidence of differences in zoospore attraction in several *Phytophthora* species between different species of host plants and

within the same host species. Simply, different cultivars or genotypes of a host may react differently to pathogens (Blaker and Hewitt 1987, Tyler et al. 1996). In *Phytophthora lateralis*, Murray and Hansen (1995, 1997) demonstrated differences in zoospore attraction to roots of two host species, Port-Orford-cedar and Pacific yew.

Light microscopy is one of the oldest scientific techniques and also one of the newest. The power behind light microscopy is the visualization of intact cytological structures. In *Phytophthora* species, histological and cytological studies of Oomycetes have been limited to a few species, including *P. nicotianae* (syn. *parasitica*), *P. palmivora*, *P. infestans*, *P. sojae*, *P. cinnamomi*, and *P. ramorum.* There have been histological comparisons of *Phytophthora* infection in resistant and susceptible cultivars of important crops. Infection by *P. nicotianae* and *P. palmivora* on fibrous roots of citrus species that have different host responses to infection were compared by means of light and electron microscopy. No differences were observed in mode of penetration of the hypodermis or in the hosts' response to infection. However, *P. palmivora* had significantly higher colonization of cortical cells in susceptible sour orange (*Citrus aurantium*) than in disease tolerant trifoliate orange (*Poncirus trifoliata*), and intracellular hyphae of both *Phytophthora* species were observed in the cortex of both hosts. Trifoliate orange colonized by *P. nicotianae* hyphae near cortical cells showed hypersensitivity of host tissue and necrosis of fungal tissue during the infection process. In addition, cortical cells in trifoliate orange had less fungal ingress and cell disruption than in sour orange. In contrast, intercellular hyphae of *P. nicotianae* did not affect the cell structure at all (Widmer et al. 1998). Histopathological study of soybean has demonstrated the difference between resistant

and susceptible soybean hypocotyls infected with *P. sojae*. In this study, intercellular and intracellular hyphae of *P. sojae* were present in all tissues of the susceptible hypocotyls. In contrast, the resistant hypocotyls were intact and showed resistant responses: the hyphae were restricted to small infected areas, and host cells close to the infected areas were filled with granular, dark-staining cytoplasm which functioned as a barrier to prevent further infection of adjacent intact cells (Klarman and Corbett 1974).

Similar cytological events have been observed in potato tuber and leaf tissues colonized by *P. infestans*. When zoospores of *P. infestans* encyst and germinate, the cysts form appressoria on the surface of leaves of potato. Commonly, appressorial hyphae directly penetrate the periclinal wall of an epidermal cell. It has been sometimes observed that the hyphae penetrate through stomata. However, the anatomy of the host tissue in part determines the invasion strategy of *Phytophthora* species (Coffey and Wilson 1983). In addition, histological changes in susceptible and resistant plant species inoculated with *P. cinnamomi* were examined (Cahill et al. 1989). The result showed resistance responses such as lignification of cell walls, deposition of phenolics, callosic papillae, and cell wall distortion and disruption in resistant plant species. Tainter et al. (1999) found that *P. cinnamomi* hyphae were most abundant in the secondary phloem of the *Quercus* species studied and that within the secondary phloem, some parenchyma cells showed resistance to the invasion of *P. cinnamomi*. Recently, a histological study of *P. ramorum*, the causal agent of sudden oak death, was conducted by Pogoda and Werres (2004) in Germany in order to understand how *P. ramorum* colonizes the tissue in *Rhododendron*. They reported

hyphae of *P. ramorum* present in brown tissue of necrotic twigs as well as in healthy-appearing tissues beyond the necrotic areas. Also, chlamydospores were observed only in the old necrotic twigs but not in transition areas between healthy and unhealthy tissues on twigs. Old necrotic twigs contained collapsed parenchyma and cambial cells.

Port-Orford-cedars resistant to POC root disease have been identified, and a resistance breeding program is central to POC root disease management on public and private lands. The program has been established since the mid 1980s by the USDA Forest Service and USDI Bureau of Land Management (BLM), working cooperatively with Oregon State University. Over 10,000 field selections of POC have been screened using a combination of stem and root dip inoculation techniques in the greenhouse (Hansen et al. 1989, McWilliams 1999 and 2001, Murray 1995, Murray and Hansen 1997), and over 1100 parent trees were selected for further evaluation. The results showed that 50-75 % of the seedlings from resistant parents survived while only ~5 % from the most susceptible parents escaped mortality, in the short-term green house tests. Also, selected POCs were outplanted for further testing under more realistic conditions. Cones with over 1.5 million seeds were collected from resistant trees at Dorena in 2002 and distributed to the Forest Service and BLM (Linn et al. 2003). Resistant POC seedlings are now available from the program (Chapters 1 and 2).

Tucker and Milbrath (1942) first described the symptoms and development of POC root disease caused by *Phytophthora lateralis.* They also reported the microscopic morphological characteristics of the asexual and sexual structures of *Phytophthora lateralis*. Trione (1957) made additional light microscope observations

of asexual and sexual stages of *P. lateralis*. However, there were no further observations of the cytological histology of *Phytophthora lateralis* on Port-Orford-cedar. Therefore, this study was undertaken to follow the processes of how *P. lateralis* encysts, germinates, penetrates, and colonizes in roots and stems. Colonization patterns in resistant and susceptible families of Port-Orford-cedar were compared to determine whether histocytological observations reveal any basis for the mechanisms of resistance.

4.3 MATERIALS AND METHODS

4.3.1 Cultures and POC seedlings

Three isolates of *P. lateralis* obtained from Oregon and California were used for inoculation of POC seedlings (Table 4.1). Cultures were grown on β-Corn Meal Agar (CMA, Difco amended with 20 ppm β-sitosterol (Acros Organics)) to stimulate zoospore production, and stored in liquid nitrogen or water after they were originally isolated from infected POC. For zoospore production, cultures were incubated for 7 days at room temperature, then three agar disks with pure mycelium were transferred to pea broth (150 g split peas in 1 L dH_2O autoclaved for 4 minutes, 20 ppm β-sitosterol added to filtered pea broth, and the medium autoclaved for 25 minutes). The pea broth cultures were incubated for 7 days at 17 °C in Petri dishes, then pea broth was poured off, the colonies washed with distilled water, then flooded with 25 ml stream water from Oak Creek, Benton County, Oregon and incubated for 2 days at 17 °C to induce sporangia.

Table 4.1. Host and origin of the isolates of *P. lateralis*.

Isolate	Host	Origin	Year Isolated
366	POC	Gasquet Ranger District, Six Rivers NF, CA	1986
368	POC	Gasquet Ranger District, Six Rivers NF, CA	1986
T_4P_3	POC	Galice District, Siskiyou NF, OR	2000

Two year-old seedlings or rooted cuttings of susceptible and resistant POCs were provided by Dorena Genetic Resource Center (DGRC), USDA Forest Service in Cottage Grove, Oregon. The trees were placed in the green house before they were used for inoculation (Table 4.2). The trees included seedlings from open, self, and cross-pollinated families as well as rooted cuttings from selected parent trees. The seedling families and parent trees had been previously tested for their susceptibility to *P. lateralis* (Chapter 2).

4.3.2 Inoculation of roots and stems

Three lateral roots of each seedling or rooted cutting were collected for inoculation, and the inoculation was repeated three times for fluorescent-light microscopy (Table 4.2). Two-cm-long root tips were excised and immersed in 30 ml of a zoospore suspension of isolates 368 and T_4P_3 for 24 hours (Figure 4.1A). Zoospore densities were ca. 3.6×10^5 zoospores per milliliter. After inoculation, the terminal 1 cm was removed from the root piece and embedded in plastic. Most thin sections were cut between 2 mm to 6 mm from the root tip. Control roots were immersed in distilled water at the same time and prepared for microscopy similarly. Stem inoculations were made at two sites on each of two trees of each family or clone.

Table 4.2. Reaction in resistance screening tests and origin of POC seedlings.

Sow [1]	Family / Clone [2]	Expected Reaction [3]	Part Inoculated	Origin
48	117499 × OP	S	Root and Stem	Gold Beach District, Siskiyou NF, OR
-	118051 × OP	S	Root	Illinois Valley District, Siskiyou NF, OR
50	510015 × OP	S/R [4]	Root and Stem	Gasquet District, Six Rivers NF, CA
53	CF1 × OP	S / R [4]	Stem	Coos Bay, OR
16	117490 × OP	R	Stem	Gold Beach District, Siskiyou NF, OR
RT1204	70037 RT	S	Stem	
RT1317	CF1 RT	R	Stem	Coos Bay, OR
-	117490 RT	R	Root and Stem	Gold Beach District, Siskiyou NF, OR

[1] Sow number is Dorena code.
[2] Family by open pollination (OP) and clone by rooted cutting (RT).
[3] S = Susceptible and R = Resistant based on root dip inoculation (Chapter 1).
[4] Hypothesized to segregate susceptible and resistant offspring at 3:1 or 1:1 ratios (Chapter 1).

Inoculations were on the lower stem (diameter 6 mm) and on the upper stem (diameter 4 mm). Stem inoculations were repeated three times. Stems of resistant and susceptible POC seedlings and rooted cuttings were sliced transversely to produce a 1 cm bark flap, and about 30 mg mycelium from liquid cultures of two isolates, 368 and T_4P_3, was inserted under the flap to contact the cambium. The infection sites then were wrapped with paraffin film, and the seedlings were incubated in a greenhouse for 4 weeks (Figure 4.1B). The lengths of necrotic lesions were recorded after bark was peeled off on one side of the stem, and then two, 2 mm long pieces were removed from the opposite side of the stem for sectioning. These pieces were removed at the necrotic margin (transition between green and necrotic tissues) and about 5 mm beyond the margin, in healthy-appearing tissue.

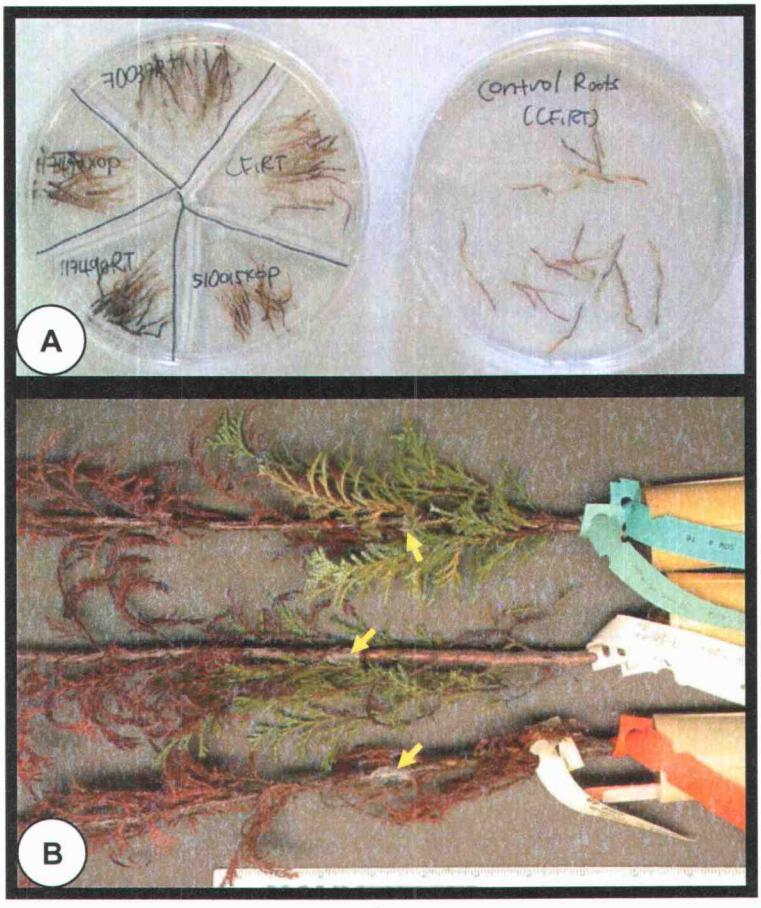

Figure 4.1. Inoculation of roots (A) and stems (B) with motile zoospores and mycelium of *P. lateralis*, respectively. Arrows indicate inoculation sites.

4.3.3 Preparation for light microscopy

The samples of roots and stems were individually fixed in 2.5 % glutaraldehyde in 0.2 M sodium phosphate buffer (pH 6.8) for 1 hour under vacuum and then stored at 4 °C overnight. The samples were washed three times with 0.2 M phosphate buffer for 15 minutes each time. Dehydration was performed with a graded ethanol series (30 %, 50 %, 75 %, and 95 % or 100 %). The duration of each step was 1 hour. The last step was repeated once. After dehydration, the samples were

infiltrated in four steps with 30 %, 50 %, 75 %, and 100 % infiltration solution in ethanol (100 ml Technovit 7100-HEMA containing co-catalyst XCL and 1g hardener I-dibensoyl peroxide, H_2O content 20 %) from a Technovit 7100 embedding kit (EBScience Co., Agawam, MA). The duration of the first three steps was 1 hour under vacuum, then 100 % infiltration solution for 2 hours in vacuum. The samples were put in 580 µl embedding solution (15 parts infiltration solution and 1 part hardener Π-barbituric acid derivative) in embedding capsules (Conical tip, Polysciences, Inc., Niles, IL) overnight. The embedded samples were sectioned to 4 µm thick using a microtome (Spencer Lens Co. Buffalo, N.Y. U.S.A). Then the sections were transferred to slides in a drop of ddH_2O, and the slides were placed on a slide warmer at 57 °C for 15-30 minutes. After all staining (see below), slides were dried at room temperature for 30 minutes and then mounted with Polymount (Polysciences, Inc.). The mounted slides were pressed with lead weights in a 50 °C oven for at least 2 hours, or overnight. The slides were observed under a fluorescent-light microscope (Zeiss, Axioskop 2) at various magnifications.

Several stains were evaluated to distinguish between host tissues and hyphae, and to visualize various chemical constituents of healthy and diseased cells. Three stains were tested to differentiate hyphae of *P. lateralis* from host cells in stems:

1) Calcofluor (Ruzin 1999, Tsao 1970): sectioned roots and stems were stained with drops of 0.001 % Calcofluor White M2R (Fluorescent brightener (Sigma Co.) in 0.05 M Tris-HCl (pH 8.0) or in ddH_2O for 10 minutes, then rinsed with ddH_2O for a few seconds. Calcofluor was the only stain used with roots. Stained cell walls fluoresce blue. Samples were exposed to UV light for viewing (excitation filter 450 – 490 and emission filter 515).
2) Malachite green and acridine orange (Schans et al. 1982): sectioned stems were stained with 0.5 % aqueous malachite green (Sigma Co.) for 5 minutes, rinsed with ddH_2O, and air dried. The sections then were stained with 0.001 % acridine orange (Sigma Co.) in boric acid-borax buffer (pH 8.6) for 10 minutes,

rinsed, and dried. Malachite green stains lignified cells and chromatin and fluoresces green, and acridine orange stains nuclei and fluoresces flame red when exposed to light (excitation filter 550 and emission filter 526).

3) Johansen's Safranin O and fast green method (Clark 1983, Johansen 1940): sectioned stems were stained with Safranin (Sigma Co.) for 2 minutes, rinsed with dH_2O, and air dried. Then, the sections were stained with fast green (Sigma Co.) for 5 minutes, rinsed, and dried. Safranin stains chromosomes, nuclei, and lignified walls bright red and fast green stains cytoplasm and cellulose walls green.

Staining protocols for specific targeted compounds included:

1) For starch, two techniques were used. I (Gahan 1984): Sections were stained with 0.5 % iodine (Sigma Co.) in 5 % aqueous potassium iodide (Sigma Co.) for 2 minutes and rinsed with ddH_2O. Short chain starch grains are red brown, and long chain starch grains are deep blue.
2) For starch, II: Slides were immersed for 2 minutes in 0.5 % iodine in 5 % aqueous potassium iodide after staining with ferric chloride after chlorine-sulfate (see 6 below).
3) For lignin, Johansen's Safranin O and fast green method (Clark 1983). Lignified cell walls are bright red or red, and unlignified cell walls are green.
4) For callose, Ruzin's method (1999) was modified: Sections were stained with 0.1 % aniline blue (0.01 g aniline blue in 100 ml 0.2 M Phosphate buffer, pH6.8) for 10 minutes and washed with dH_2O. Callose stains yellow to reddish brown, normal plant cells are blue-green.
5) For polyphenolics, two techniques were used. I (Gahan 1984): Ferric chloride. Sections were stained with 2 % ferric chloride in 95 % EtOH for 10 minutes and washed with 95 % EtOH. Polyphenols are green.
6) For polyphenolics, II (Campbell et al. 1937, Ride 1975): Ferric chloride after chlorine-sulfate. Slides were put in boiling 75 % EtOH for 30 minutes and washed with dH_2O, then immersed in 1 part of 1% $KMnO_4$ and 1 part of conc. HCL for 4 hours, then rinsed with dH_2O. Slides were then immersed in 20 % sodium-sulfate. Finally, 2 % ferric chloride was applied to the sections.
7) For nuclei and lignified cells, I (Gahan 1984): Toluidine blue O. Sections were processed with 1 % aqueous toluidine blue (Sigma Co.) for 30 minutes followed by a rinse of absolute n-butanol (Sigma Co.) for 2 minutes. Nuclei are blue, and lignified cells are blue-green.
8) For nuclei and lignified cells, II: Toluidine blue O after chloride-chlorine sulfate. 0.05 % toluidine blue O (0.0025 g toluidine blue O in 50 ml 0.2 M phosphate buffer, pH 6..8) was used as a counterstain after ferric chloride-chlorine sulfate treatment (See 6 above).

4.3.4 Analysis

In the comparison of resistant vs. susceptible host inoculations by stem wound inoculation, necrotic lesion lengths from inoculated susceptible and resistant seedlings were analyzed by Proc Mixed-generalized linear model (ANOVA), The SAS System version 8 (SAS Inc.). The ANOVA *F*-test provided P-values for the differences among families.

4.4 RESULTS

4.4.1 Susceptibility of seedlings and rooted cuttings used for histology

When the outer bark was scraped off, the infected sites on the inoculated stems were obvious to the naked eye (Figure 4.2). There was a distinct brown margin between healthy and unhealthy tissues (Figure 4.2A and B). The typical symptom, water-soaked necrosis, appeared both in susceptible and resistant seedlings, but the extent of necrosis was different; resistant seedlings had shorter lesion lengths than susceptible seedlings. In addition, the resistant seedlings showed an oozing resin response near necrotic areas (Figure 4.2C). Statistical analysis showed that there were significant differences in necrotic lesion length among families (P = 0.0003, ANOVA *F*-test). Seedlings of families 117499 × OP and CF1 × OP and rooted cuttings of 70037 RT had longer average necrotic lesions (family average) than other families or clones and were considered susceptible. Seedlings of family 510015 × OP were intermediate in average lesion length. 117490 × OP seedlings did not differ from rooted cuttings of CF1 RT and 117490 RT. The latter had the shortest necrotic lesions of the trees tested (Figure 4.3).

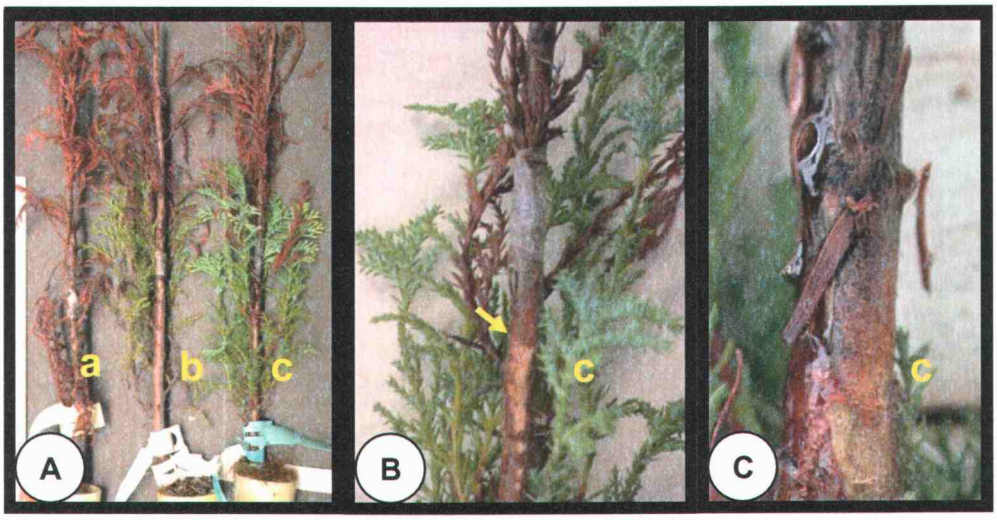

Figure 4.2. Stem inoculation on seedlings of Port-Orford-cedar infected with mycelium of *P. lateralis* for 4 weeks. **A** Susceptible 117499 × OP (a) and resistant 510015 × OP (b) and 117490 × OP (c). **B** Distinct necrotic margin on 117490 × OP (arrow). **C** An oozing response of 117490 × OP.

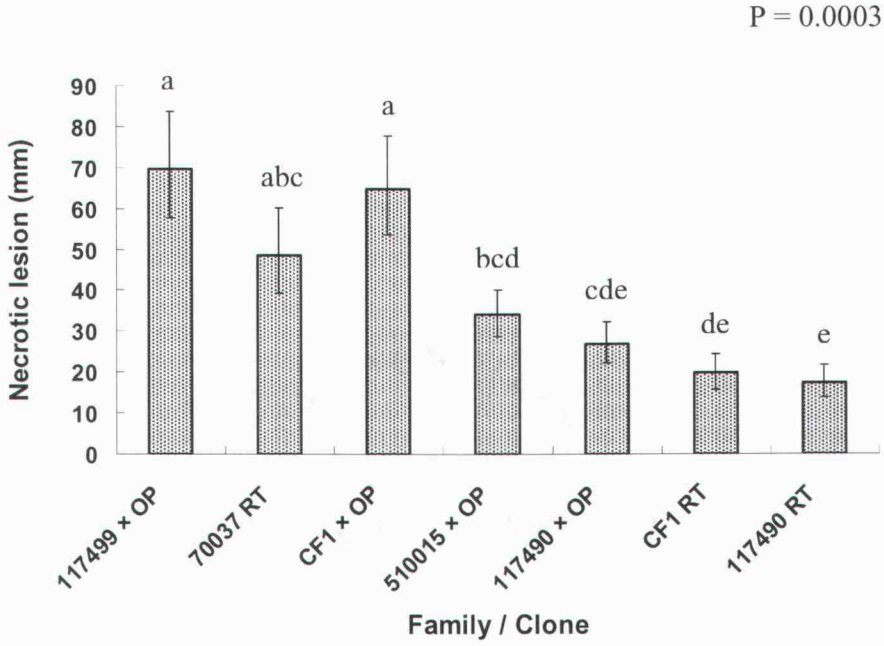

Figure 4.3. Least squares means of necrotic lesions on stems of POC seedlings inoculated with *P. lateralis* mycelium and incubated for 4 weeks. Bars with the same letter are not significantly different (P > 0.05).

4.4.2 Histological observations of healthy and infected roots

1) Anatomy of healthy roots

Healthy, uninfected roots were sectioned and examined to interpret root structure of Port-Orford-cedar. Transverse sections showed that the young roots are composed of layers of epidermal cells and cortical cells surrounding the vascular system. The outer layer of epidermis had irregular cell shape, size, and arrangement, especially near the root cap. Also, the outer layers sloughed off easily (Figure 4.4A). Figure 4.4.B is a cross-section through the apical meristem just behind the root tip. The vascular cylinder was composed of xylem and phloem surrounded by a layer of pericycle and endodermis (Figure 4.4C). Cells in the cortex were uniform in shape and size (Figure 4.4D and E). Cell walls of epidermal and some exodermal cells fluoresced more brightly than cortical cells after staining with calcofluor (Figure 4.4F). Longitudinal sections showed the primary tissues of lateral roots (Figure 4.4G and H). No structures resembling zoospores, cysts, or hyphae were observed in or on uninoculated roots.

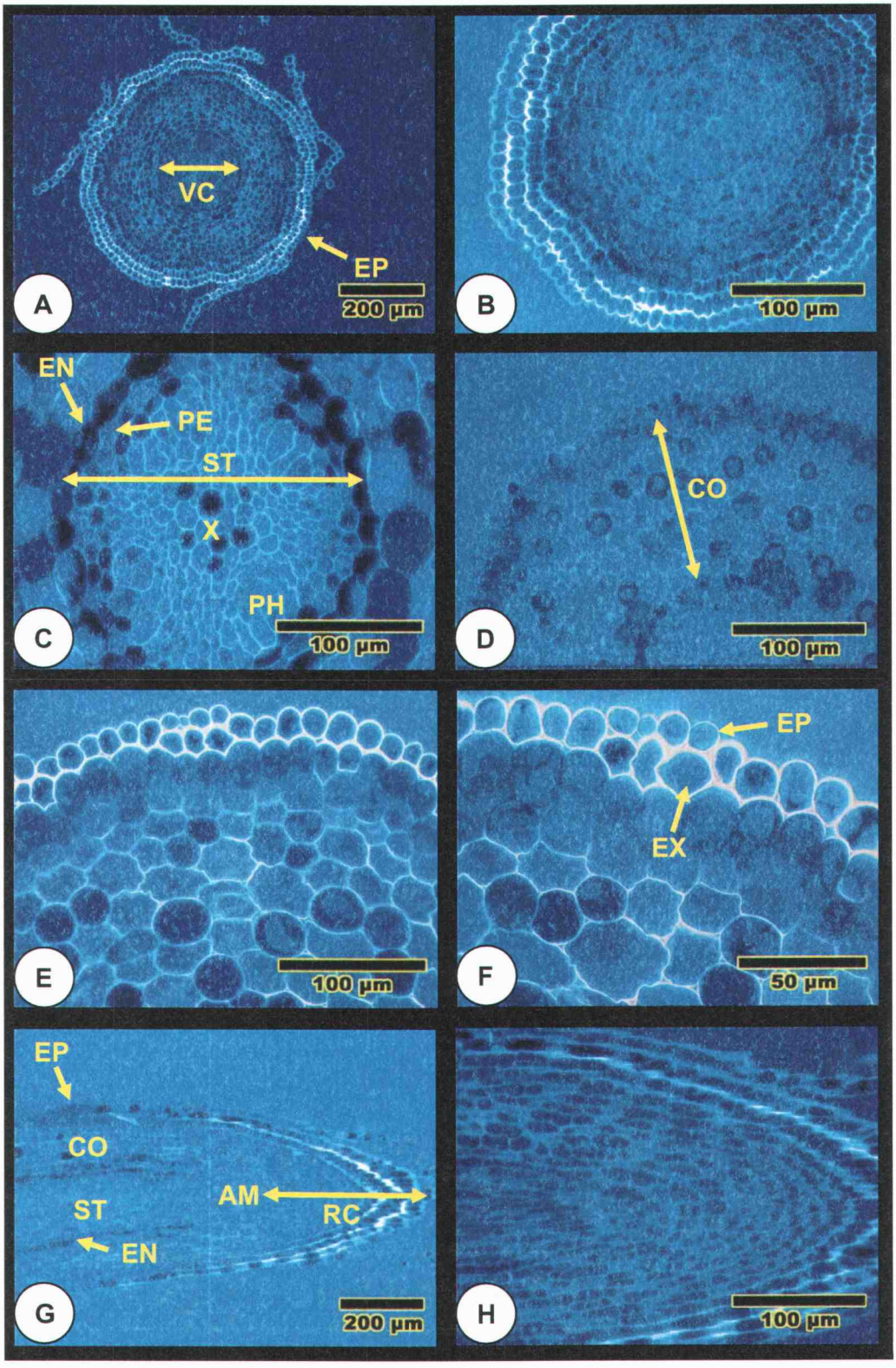
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Figure 4.4. Transverse and longitudinal sections of uninoculated primary roots of susceptible and resistant Port-Orford-cedar stained with 0.001 % calcofluor. **A** Irregular outer layers of cells. 117499 × OP. **B** Apical meristem of 118051 × OP. **C** Vascular cylinder (stele) of 510015 × OP. **D** Cortex of 117499 × OP. **E** Cortex of 118051 × OP. **F** Layers of epidermal and exodermal cells of 118051 × OP. **G** A primary lateral root of 510015 × OP. **H** Apical meristem of 510015 × OP. AM = Apical meristem; CO = Cortex; EN = Endodermis; EP = Epidermis; EX = Exodermal cell; PH = Phloem; PE = Pericycle; RC = Root cap; ST = Stele (vascular cylinder); X = Xylem.

2) *P. lateralis* in susceptible roots

• Germination and penetration: Excised, non-suberized root tips were inoculated with zoospores of *P. lateralis* for 24 hours, stained with calcofluor, and observed directly under a fluorescence microscope. Zoospores aggregated on the root surface and encysted, losing the two flagellae (Figure 4.5A). Most cysts were attached to the epidermis. Cysts germinated and formed germ tubes and appressoria on the epidermal cells (Figure 4.5B). These direct observations of the root surface were confirmed by longitudinal sections of roots. The germ tubes were sometimes divided by a septum and formed an appressorium for penetration of the root (Figure 4.5C). Initial penetration usually occurred between the anticlinal walls of the epidermal cells (Figure 4.5D, surface view; and Figure 4.5E-G, cross section). After penetration between epidermal cells, the hyphae were delimited by septa and continuously elongated until cortical cells were met (Figure 4.5E and F). Also, unattached cysts near the surface of the root germinated and extended their hyphae to the root surface. The hyphae penetrated between epidermal cells (Figure 4.5G). Direct penetration through an epidermal cell wall was also observed (Figure 4.5H).

Observations were repeated on additional seedlings (Figure 4.6). Zoospores aggregated on the surface of a root and encysted (Figure 4.6A). The cysts germinated, and germination hyphae penetrated the epidermal cells (Figure 4.6B and C). After penetration, the hyphae in a layer of epidermal cells were delimited by septa (Figure 4.6D). The cortex was occupied by both intercellular and intracellular hyphae (Figure 4.6E and F).

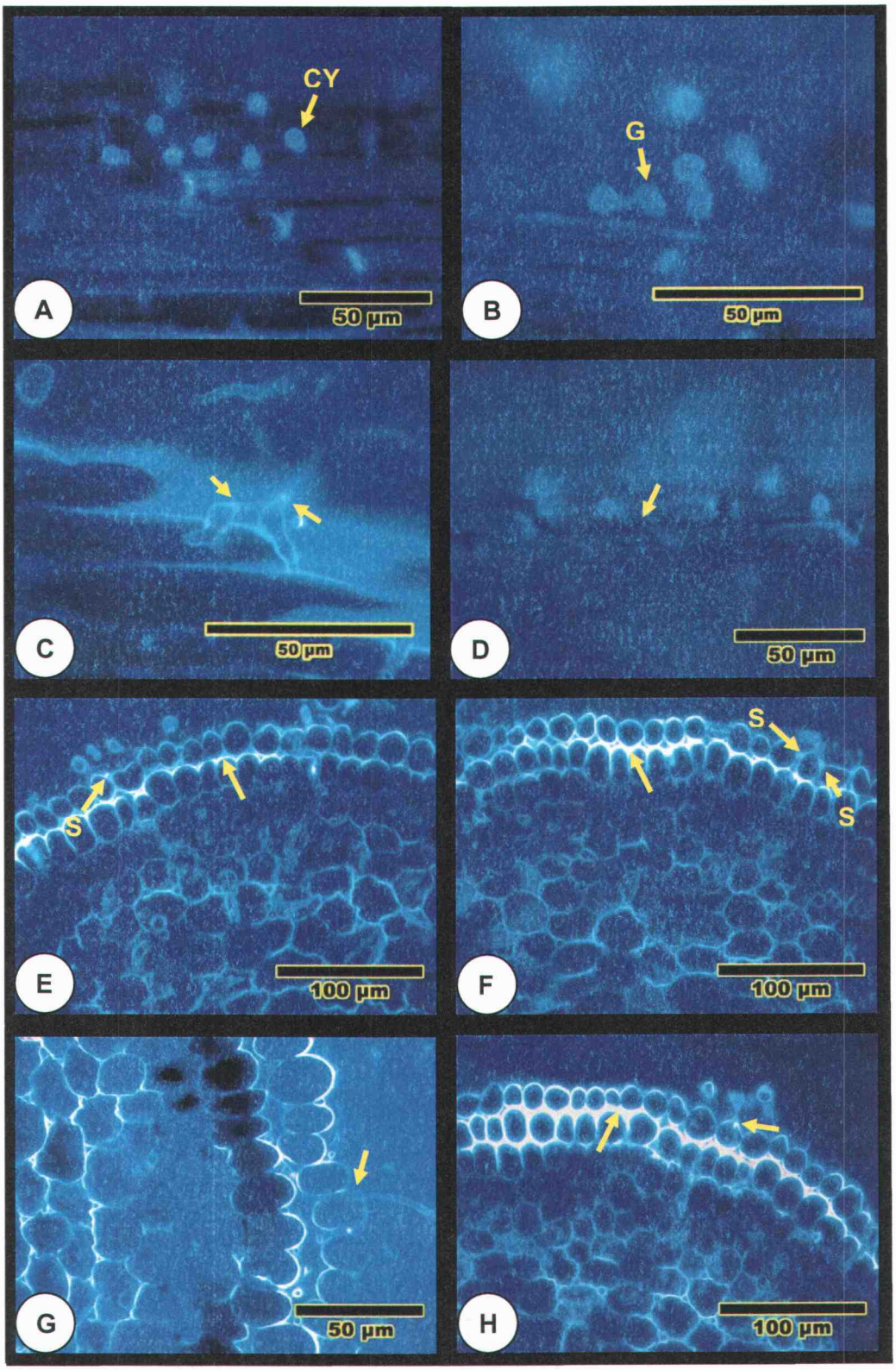
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Figure 4.5. Fluorescent micrographs of susceptible roots exposed to zoospores of *P. lateralis* for 24 hours and stained with 0.001 % calcofluor. **A and B** Surface view of intact roots showing cysts and germination on 118051 × OP roots. **C** Longitudinal sectioned roots of 118051 × OP showing appressoria delimited by septa (arrows). **D** Penetration of a hypha through the epidermis of 118051 ×OP (arrow, longitudinal section). **E, F, and H** Transverse sectioned roots of 118051 × OP showing penetrating hyphae divided by septa (arrow). **G** Transverse sectioned root of 117499 × OP showing penetrating hypha (arrow). CY = Cyst; G = Germ tube; S = Septum.

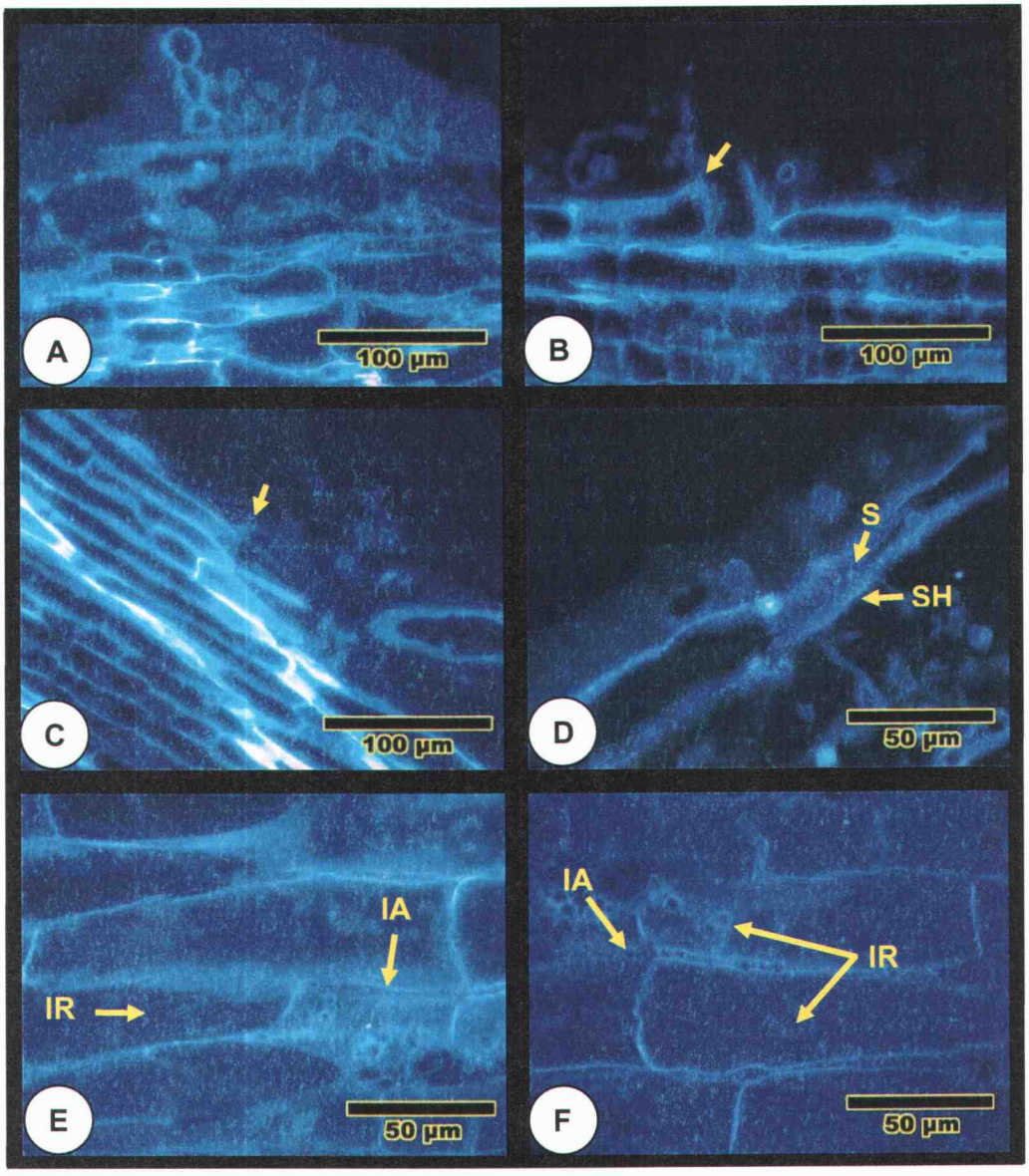

Figure 4.6. Fluorescent micrographs of longitudinal sections of 118051 × OP susceptible roots exposed to zoospores of *P. lateralis* for 24 hours and stained with 0.001 % calcofluor. **A** Zoospore aggregation and germination. **B, C, and D** Penetration process. **E and F** Colonization with intercellular (IR) and intracellular (IA) hyphae. S = Septum; SH = Swollen hypha.

• Colonization: The main penetrating hyphae became swollen (Figure 4.7A) and extended their branches to neighboring intact cortical cells (Figure 4.7B). Many-branched hyphae elongated and colonized the parenchyma cells of the cortex. The branched hyphae grew intercellularly and intracellularly in the cortex (Figure 4.7C, D, and E).

Fewer hyphae were observed in 117499 × OP (Figure 4.7F) than in 118051 × OP (Figure 4.7E), but there were no apparent differences in the pattern of germination, penetration, and colonization. Cysts germinated and hyphae grew through the epidermis to the open space between the separated outer layer of epidermis and the first layer (hypodermis) of cortex (Figure 4.7G). In addition, there was often a layer between the outer layers of epidermis and the first layer of the cortex that fluoresced brightly (Figure 4.7H).

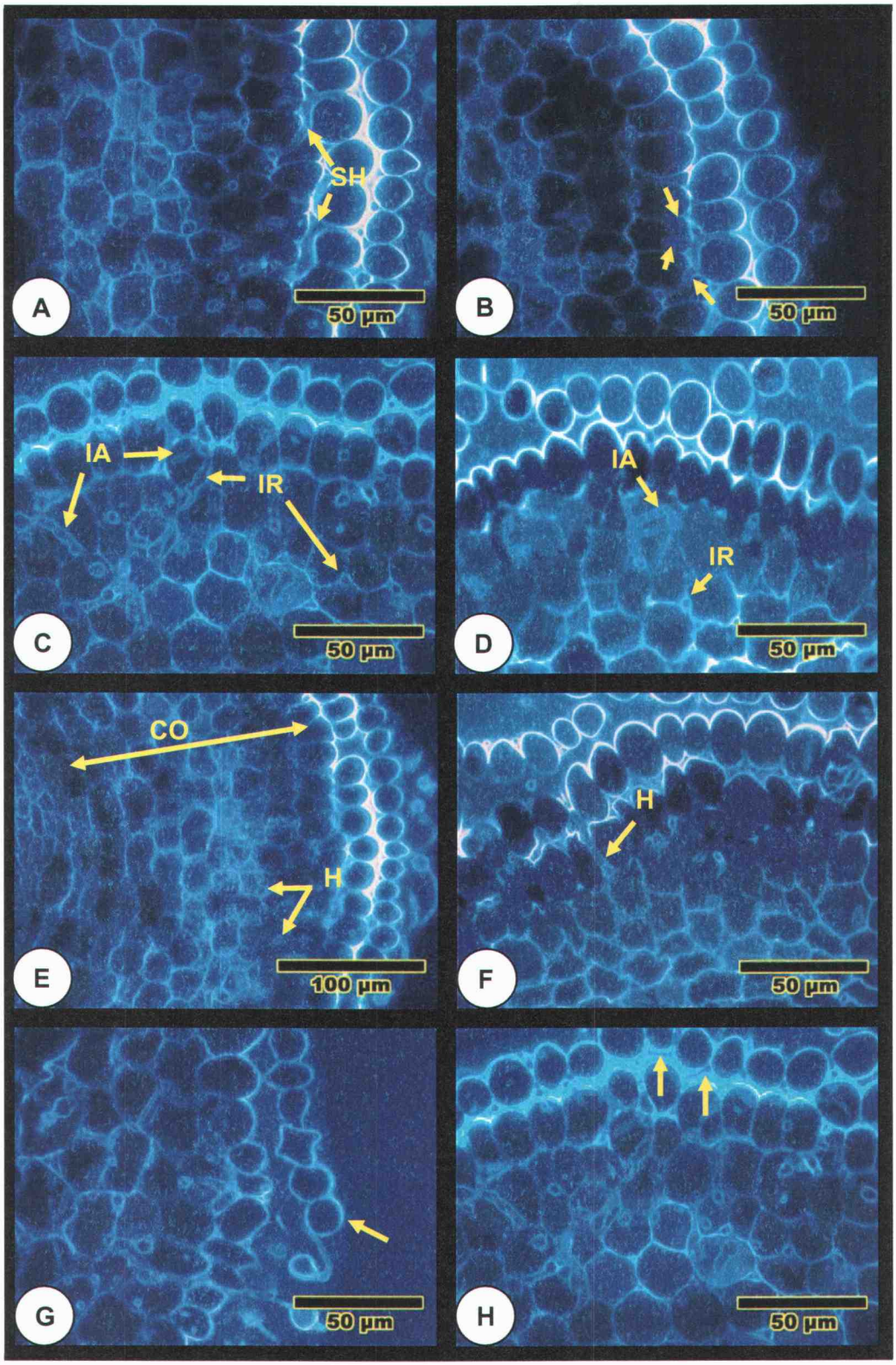
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Figure 4.7. Fluorescent micrographs of transverse sections of susceptible roots exposed to *P. lateralis* for 24 hours and stained with 0.001 % calcofluor. **A** Swollen hyphal structures on 118051 × OP (arrows). **B** Branching hypha at epidermis-cortex interface (arrow). **C and D** Intercellular (IR) and intracellular (IA) penetration on 118051 × OP and 117499 × OP. **E** Colonization in cortex of 118051 × OP. **F** Fewer hyphae on 117499 × OP. **G** A separated outer layer of epidermis of 118051 × OP (arrow). **H** Penetration to interface space between epidermis and cortex of 118051 × OP (arrows). CO = Cortex; H = Hypha; SH = Swollen hypha.

3) *P. lateralis* in resistant roots

Few cysts (less than 5) were observed on the surface of 510015 × OP (resistant) roots (Figure 4.8A). Most cysts did not germinate (Figure 4.8B) but if they germinated, penetration occurred as in susceptible roots, with both direct invasion into the epidermal cells and penetration between epidermal cells (Figure 4.8C-E). Also, a septum formed in the penetration hypha as in susceptible roots (Figure 4.8F). There were no differences in the process of germination and penetration between resistant roots and susceptible roots, but the frequency of germination and penetration observed in resistant roots was less than in susceptible roots. Also, the germinated hyphae did not penetrate as deeply in resistant roots as in susceptible roots. Colonization of the cortex was not observed in resistant roots in the 24 hour inoculation period used in this study.

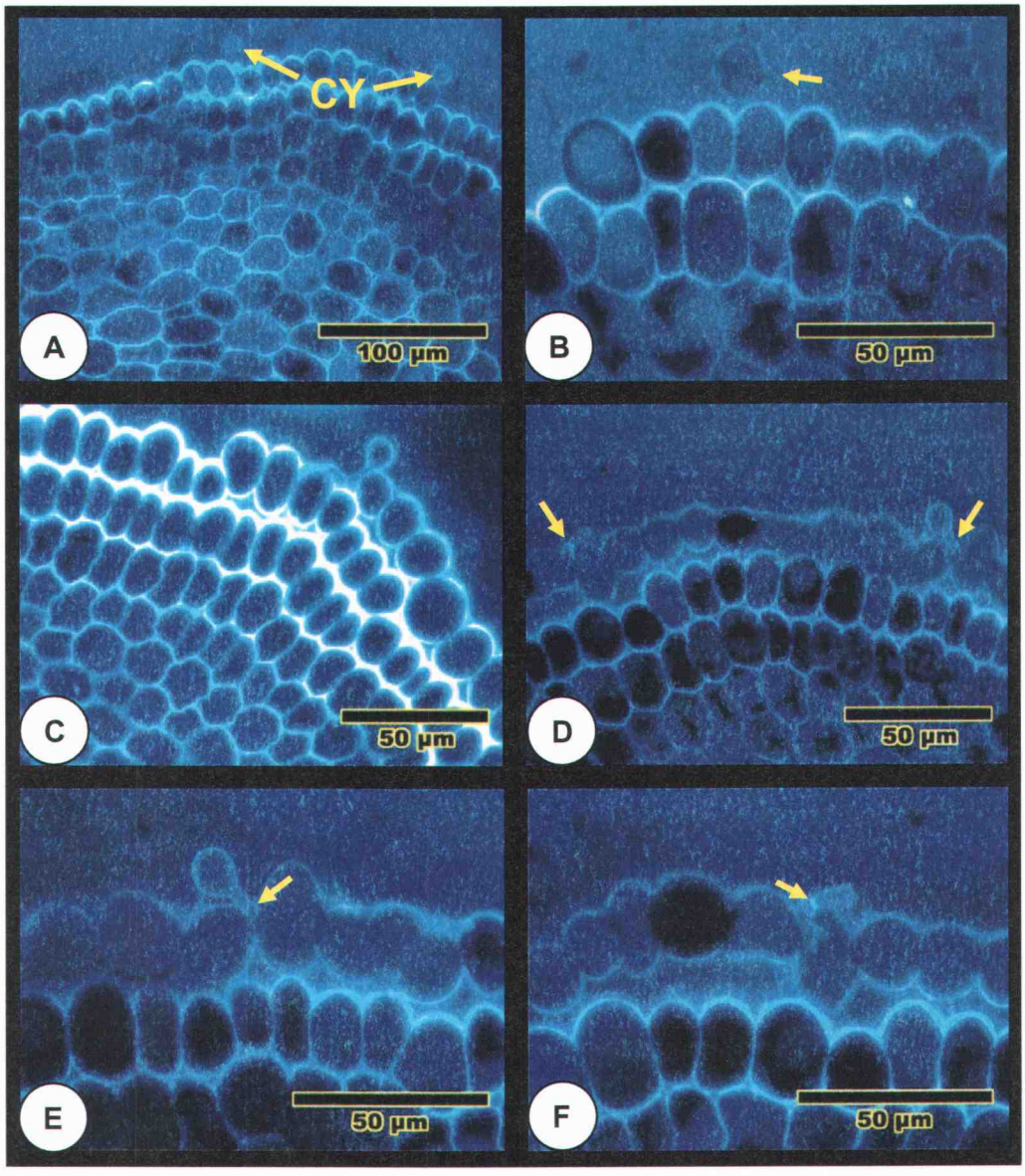

Figure 4.8. Fluorescent micrographs of transverse sections of 510015 × OP resistant fine roots exposed to zoospores of *Phytophthora lateralis* for 24 hours and stained with 0.001 % calcofluor. **A and B** Cysts on epidermis. **C and D** A germinated cyst. **E and F** Penetration of a hypha.

4.4.3 Histological observations in stems

1) Comparison of staining methods

Different stains were evaluated for best visualization of host and pathogen tissues on the sections from stems (Figure 4.9). Safranin and fast green differentiated fungal hyphae (green) from phloem cells (red) (Figure 4.9B). The bright red indicated lignified cells. In uninoculated stems, however, phloem cells were green and fiber cells red (Figure 4.9A). Toluidine blue stained hyphae as well as nuclei in phloem cells (Figure 4.9C and D). Malachite green and acridine orange separated bright orange hyphae from green phloem cells in inoculated stems while most phloem cells were green in uninoculated stems (Figure 4.9E and F). However, some nuclei in phloem cells and the cells themselves fluoresced bright orange, especially after long exposure to UV light. Therefore, there was always the possibility of misinterpreting results with these stains, although hyphae of *P. lateralis* and POC cells were distinguishable with trained eyes.

Calcofluor stained POC cell walls fluoresced blue in both uninoculated and inoculated stems, as did the cellulosic *Phytophthora* cell walls. The latter often fluoresced more brightly blue or white, however. Therefore, calcofluor was used for most examinations of colonization of *P. lateralis* in susceptible and resistant stems after inoculation.

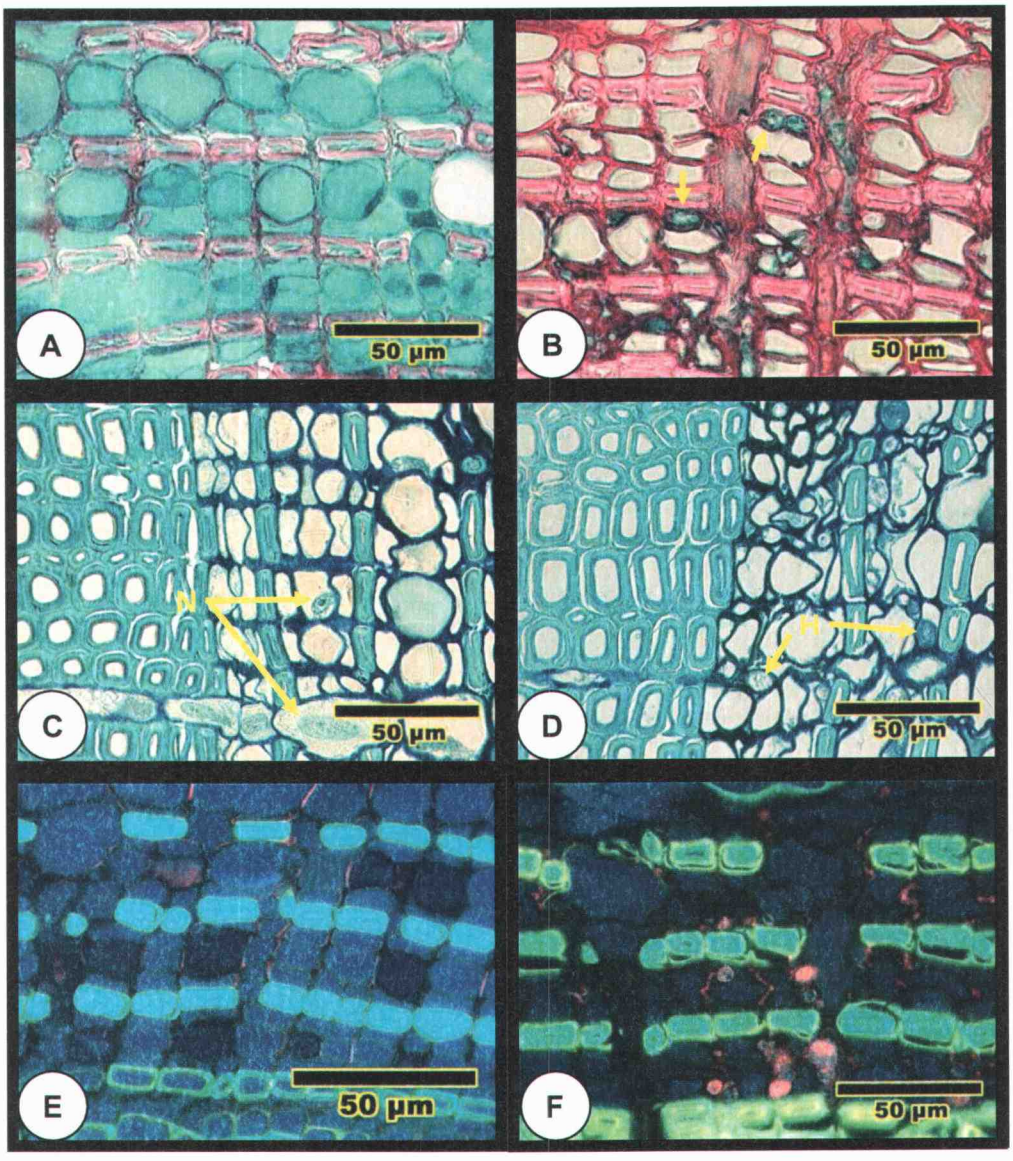

Figure 4.9. Light micrographs of transverse sections of susceptible stems four weeks after inoculation with *P. lateralis*. **A and B** stained with safranin and fast green. **A** Uninoculated stem of 118051 × OP. **B** Green hyphae (arrows) in red functional phloem of 118051 × OP stems. **C and D** stained with 1% toluidine blue. **C** Uninoculated stem of 118051 × OP. **D** Green hyphae in the inoculated stem of 118051 × OP. **E and F** stained with malachite green and acridine orange. **E** uninoculated stem of CF1 × OP. **F** Bright orange hyphae in green functional phloem of CF1× OP. H = Hypha; N = Nucleus.

2) Anatomy of healthy stems

Cross sections of uninoculated stems stained with calcofluor revealed pith, secondary xylem, vascular cambium, phloem, and a developing peridial tissue (Figure 4.10). The peridial tissue included primary phloem, cork cells, epidermis, and cuticle (Figure 4.10C and D). The secondary phloem was comprised of functional phloem, ray parenchyma, and the non-functional phloem cells, the cortex (Figure 4.10 D). The functional phloem included phloem mother cells, (Figure 4.10E) followed by fiber cells surrounding two layers of sieve cells, enclosing the parenchyma cells (Figure 4.10F). All cells in cross section of uninoculated stems were healthy, and no hyphae were observed in uninoculated stems.

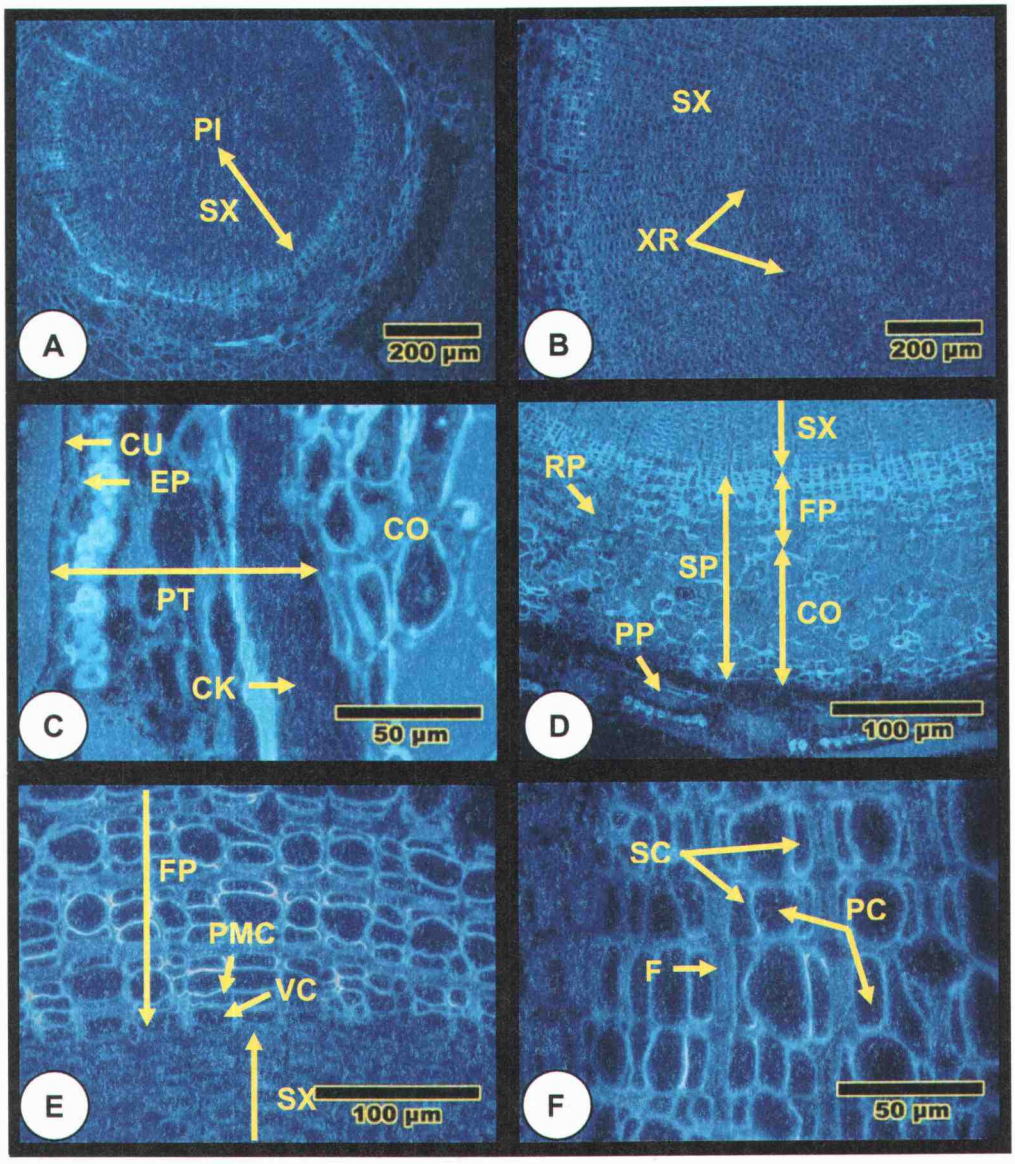

Figure 4.10. Fluorescent micrographs of transverse sections of uninoculated 70037 RT and 118051 × OP susceptible stem of POC stained with 0.001 % calcofluor. **A** Entire cross sectioned stem. **B** Secondary xylem and xylem ray. **C** Peridial tissues with developing cork layer. **D** Secondary phloem. **E** Functional phloem. **F** Cells in functional phloem. CO = Cortex (non-functional phloem); CU = Cuticle; EP = Epidermis; F = Fiber; FP = Functional phloem; PC = Parenchyma cell (tannin cell); PI = Pith; PMC = Phloem mother cell; PP = Primary phloem; PT = Peridial tissue (periderm); RP = Ray parenchyma (living); SC = Sieve cell; SP = Secondary phloem (Inner bark); SX = Secondary xylem; XR = Xylem ray; VC = Vascular cambium.

3) Susceptible and resistant stems inoculated with *P. lateralis*

Susceptible and resistant POC were sectioned through the margin of necrosis (M) on inoculated trees, and about 2 mm beyond the margin (AM). In susceptible trees, hyphae were seen in the cambium, in the sieve and parenchyma cells of the functional phloem, and in cortical cells (Figure 4.11). Hyphae were intercellular in the cambial layer of 117499 × OP (Figure 4.11A and B). Many intercellular and intracellular hyphae were observed in phloem sieve and parenchyma cells in 117499 × OP. Intracellular hyphae were especially abundant in phloem parenchyma cells of 117499 × OP, and fiber cells were intact (Figure 4.11C and D). Also, intercellular and intracellular hyphae were present in phloem sieve and parenchyma cells of 70037 RT but fewer hyphae were observed in 70037 RT than in 117499 × OP. A few hyphae were also present in cortical cells of 70037 RT (Figure 4.11F), however, some cells did not have any hyphae and did not fluoresce (Figure 4.11E).

There were different fluorescent responses between uninoculated and inoculated stems. The healthy control stems stained with calcofluor showed white or bright blue, especially the functional phloem (Figure 4.12A). In contrast, infected stems, sectioned at the margin and above the margin of susceptible as well as resistant POC were also brightly fluorescent blue, except in the functional phloem. Figure 4.12B and C were examples of the dark functional phloem and showed the dead cambial cells and the first layer of sieve and parenchyma cells.

All sections above the necrotic margins of 70037 RT susceptible POC had either no hyphae or only a few hyphae. Most cells were dead regardless of the presence of hyphae (Figure 4.13A). The necrotic margins of 70037 RT susceptible

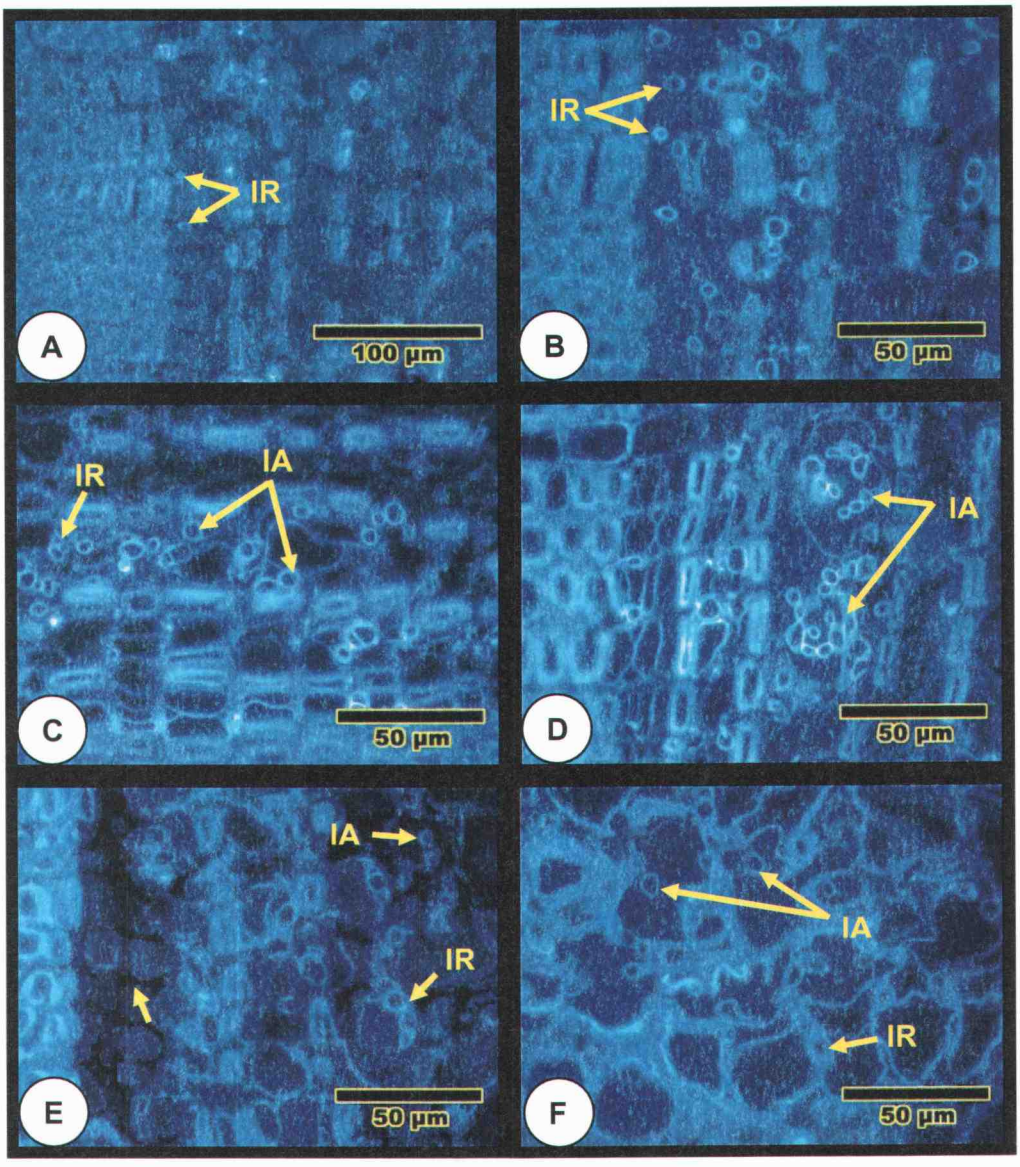

Figure 4.11. Fluorescent micrographs of transverse sections of susceptible stems four weeks after inoculation with *P. lateralis* and stained with 0.001 % calcofluor. **A and B** 117499 × OP, intercellular hyphae in the vascular cambium and in phloem mother cells. **C and D** 118051 × OP intercellular hyphae in phloem, intracellular hyphae in sieve and parenchyma cells. **E and F** 70037 RT, non-fluorescent vascular cambium, phloem mother, sieve, and parenchyma cells with no hyphae (arrow). Some hyphae in cortical parenchyma. IR = Intercellular; IA = Intracellular.

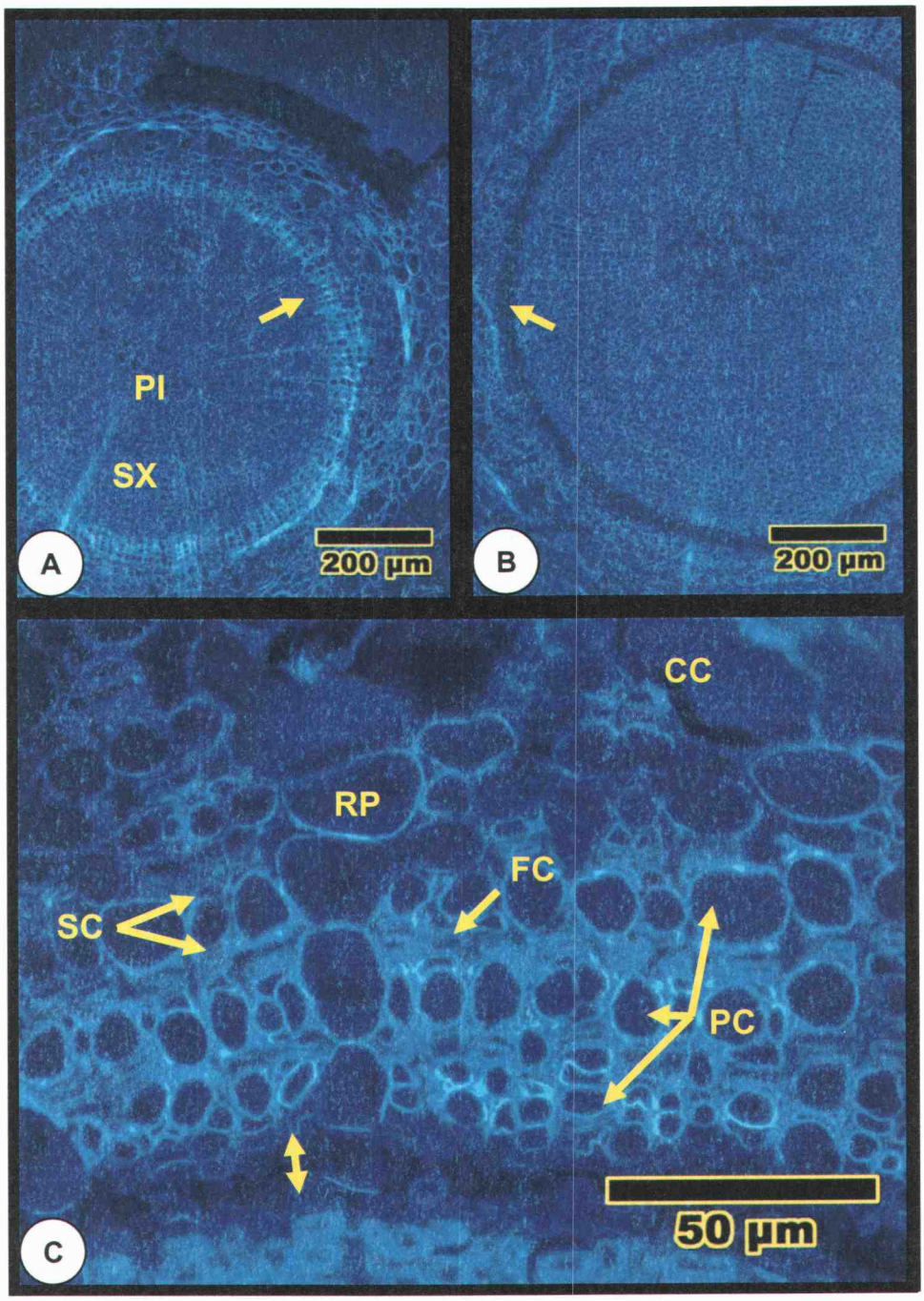

Figure 4.12. Fluorescent micrographs of 70037 RT (rooted cutting) stems stained with 0.001 % Calcofluor. **A** Uninoculated stem as a control. **B** and **C** Inoculated stems. Arrows indicating non-fluorescent cambial layer as well as the first layer of sieve and parenchyma cells. **C** A magnified area of the phloem of 70037 RT, sectioned above the margin. CC = Cortical Cell; FC = Fiber Cell, PC = Parenchyma Cell; PI = Pith; RP = Ray Parenchyma; SC = Sieve Cell; SX = Secondary Xylem.

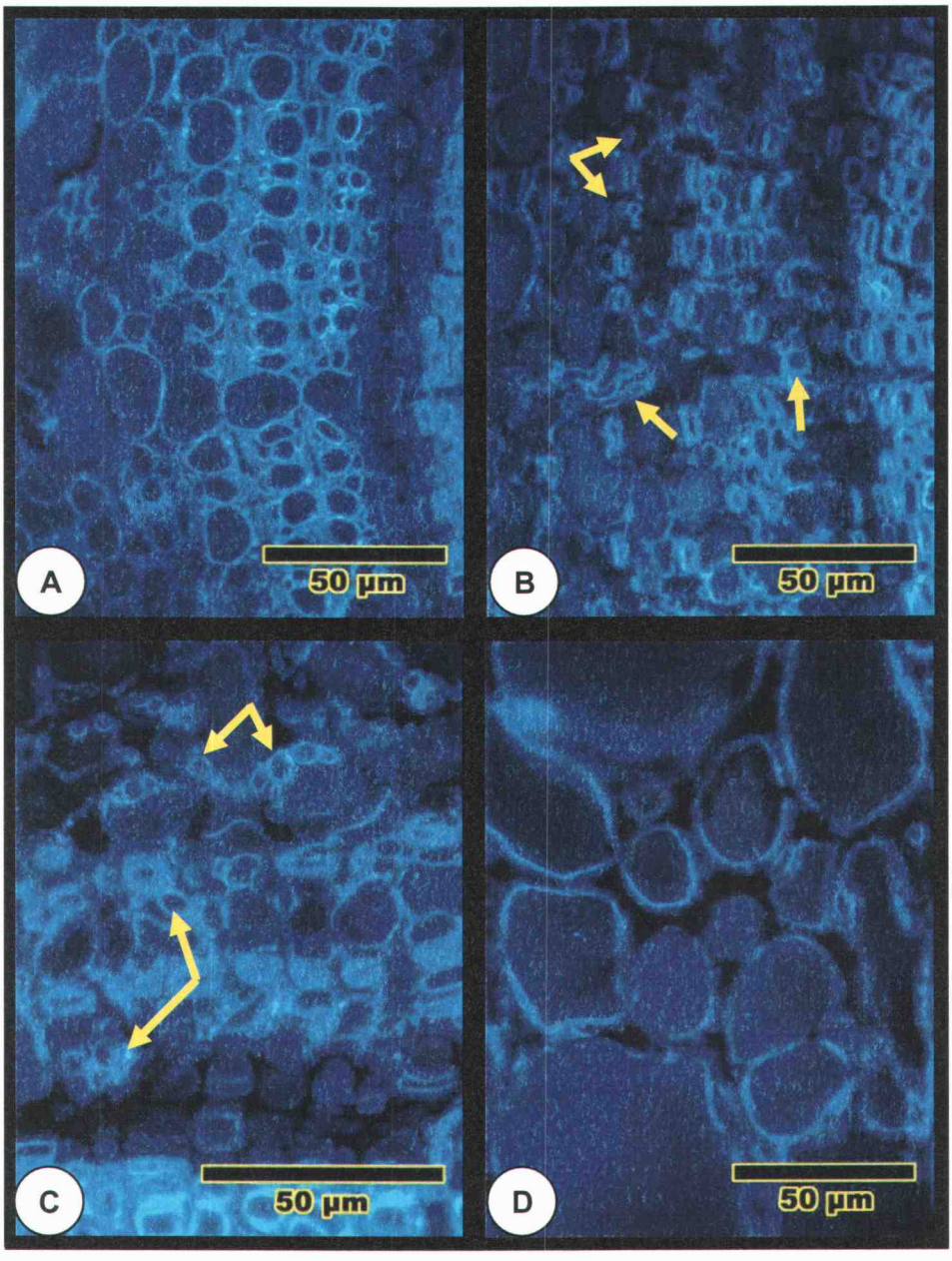

Figure 4.13. Fluorescent micrographs of cross sectioned 70037 RT susceptible stem four weeks after inoculation *P. lateralis*. The sections were stained with 0.001 % Calcofluor. **A** Non-fluorescent cells in one or two layers of phloem including cambial cells in sections from above the necrotic margin. **B** and **C** Inter- and intracellular hyphae (arrows) in functional phloem at the necrotic margin. **D** Dead cortical cells at the margin.

POC showed many inter-and intracellular hyphae in sieve and parenchyma cells (Figure 4.13B and C). Cortical cells outside the functional phloem did not fluoresce but no hyphae were observed in this study (Figure 4.13D).

In all resistant stems, colonization by the hyphae was highly restricted. Hyphae were only present intercellularly in a layer of cambium, phloem sieve and parenchyma cells (Figure 4.14A). Interestingly, a few hyphae were observed in xylem cells near the cambium, but only in CF1 × OP (Figure 4.14B). Most hyphae were observed in cambial cells and outer most layers of sieve and parenchyma cells (Figure 4.14C and D). Some cells were non-fluorescent without showing hyphae (Figure 4.14E), and usually most cells in the cambium and phloem were collapsed or disorganized (Figure 4.14F).

Very few hyphae were observed in sections from above the necrotic margin of 117490 RT. Only cambial cells appeared to be non-fluorescent (Figure 4.15A and B). No hyphae were observed in sections from the necrotic margin of 117490 RT, but the POC cell structure in the necrotic margin was completely different than in sections from above the necrotic margin, as well as from susceptible stems. Sieve and parenchyma cells were highly modified at the necrotic margin of resistant stems (Figure 4.15C and D) but fiber cells were intact. The modified cells were severely collapsed and irregularly shaped. This result was seen in all sections of the necrotic margin of 117490 RT.

A second fluorescent stain, malachite green and acridine orange, was used to observe the cytological changes in the necrotic margin of 117490 RT. Xylem and fiber cells of POC fluoresced green, and the sieve and parenchyma cells fluoresced bright

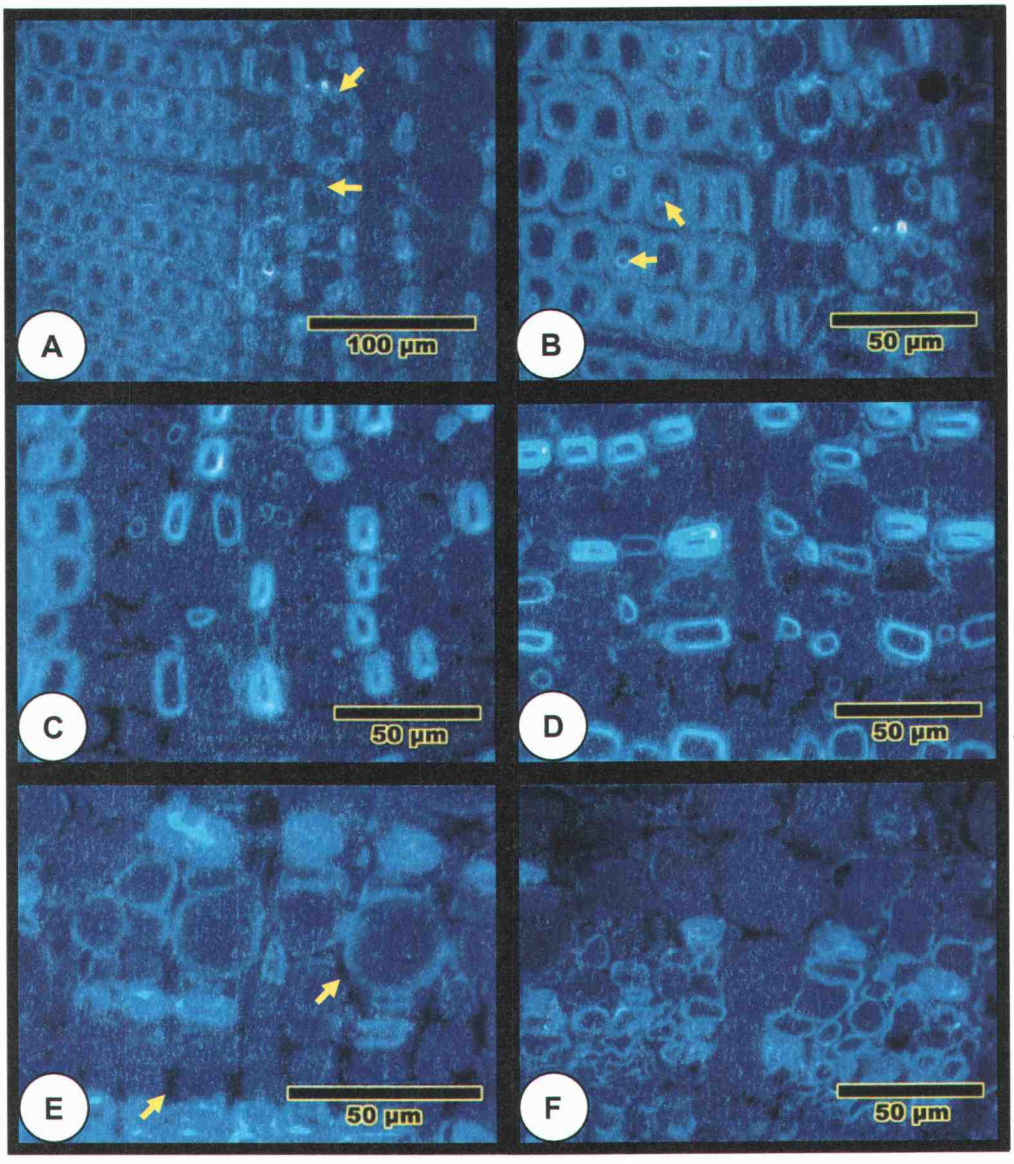

Figure 4.14. Fluorescent micrographs of transverse sections of resistant stems four weeks after inoculation with *P. lateralis* and stained with 0.001 % calcofluor. **A and B** CF1× OP, inter-and intracellular hyphae in cambial, sieve, and parenchyma cells of phloem as well as intracellular hyphae in xylem (arrows). **C and D** 117490 × OP, fewer hyphae in phloem. **E and F** 117490RT, non-fluorescent (arrows) and collapsed cells.

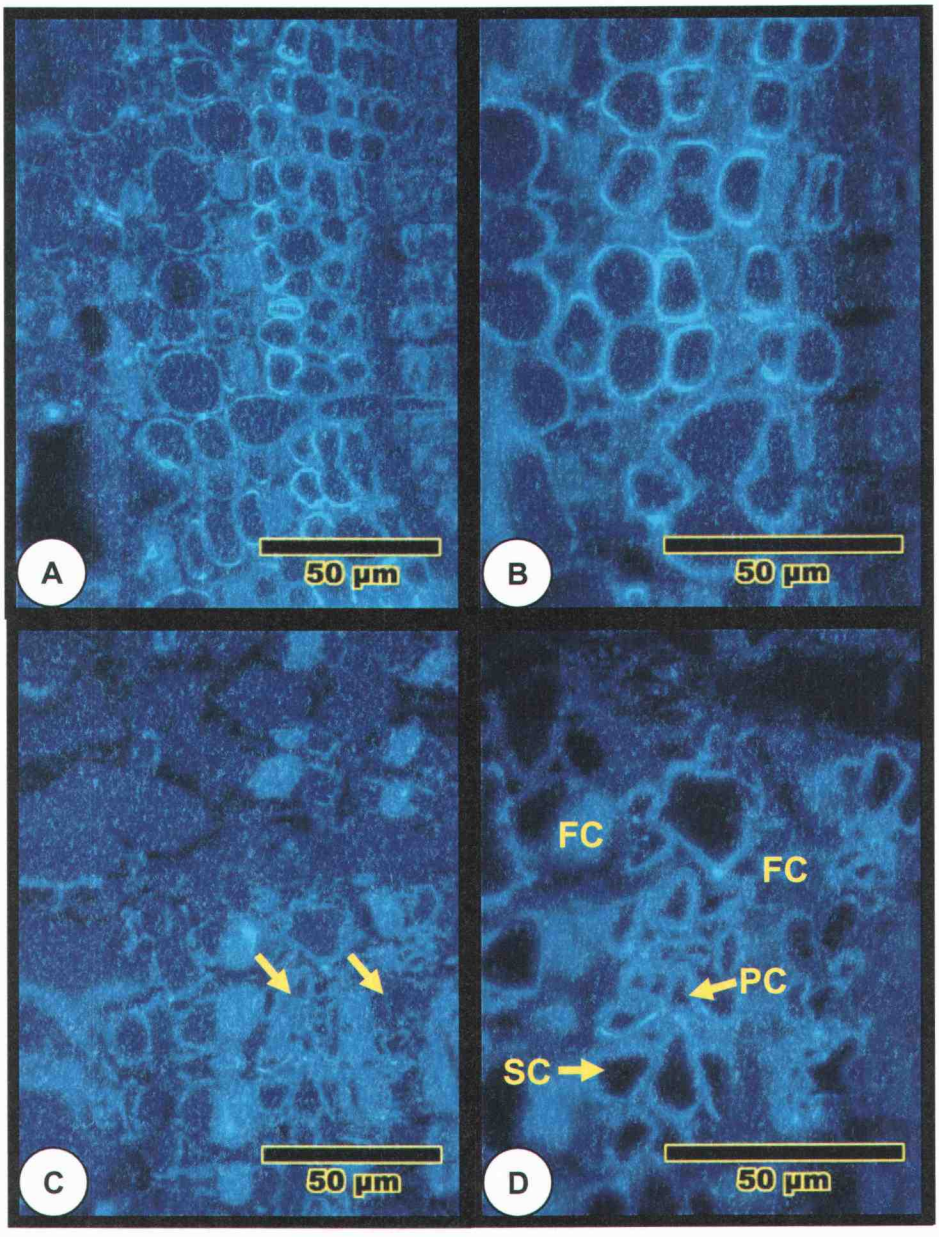

Figure 4.15. Fluorescent micrographs of cross sectioned 117490 RT resistant stem four weeks after inoculation *P. lateralis*. The sections were stained with 0.001 % Calcofluor. **A** and **B** no hyphae and dead cambial cells but intact cell shape above the necrotic margin, **C** and **D** Few or no hyphae at the necrotic margin, but severely collapsed cells (arrows). FC = Fiber Cell; PC = Parenchyma Cell; SC = Sieve Cell.

orange. The sieve and parenchyma cells of the control stem were either full of reddish orange fluorescing cytoplasm or were empty (Figure 4.16A and B). The cells in the necrotic margin of the resistant stem fluoresced green. Some cells showed the separation of membrane and wall with green fluorescing materials inside the host cells (Figure 4.16C and 4.16G). Also, a sieve plate that is a typical primary cross wall with plasmodesmata between two sieve elements was observed in Figure 4.16C and D. Some sections of the necrotic margin of 117490 RT showed red fluorescing intracellular hyphae in sieve and parenchyma cells (Figure 4.16E and F). Cytoplasmic changes, including red staining, dense materials, were observed in sieve and parenchyma cells (Figure 4.16H).

In summary differences were observed between susceptible and resistant POC stems. More inter- and intracellular hyphae of *P. lateralis* were seen in the cambial cells, phloem sieve cells and parenchyma cells in susceptible stems than in resistant stems. Sometimes dead cells were observed in the functional phloem of both susceptible and resistant stems without evident hyphae, but the frequency of dead cells in resistant stems was higher than in susceptible stems. Only resistant stems had collapsed cells in the phloem.

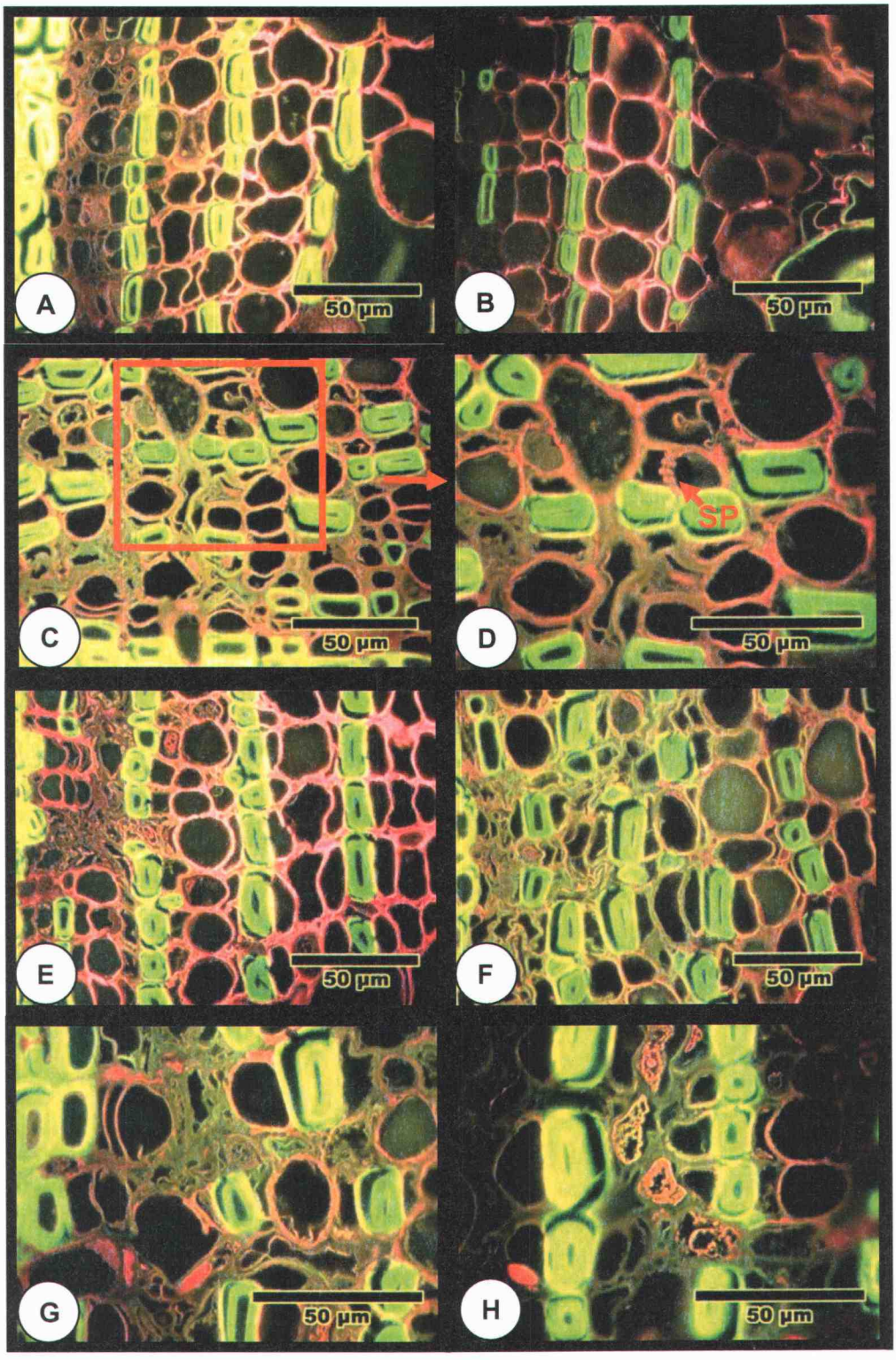
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Figure 4.16. Fluorescent micrographs of cross sectioned 117490 RT resistant stem. The sections were stained with 0.5 % aqueous malachite green following 0.001 % acridine orange. **A** and **B** Functional phloem of uninoculated stem. **C** and **D** Abnormal cells and sieve plate (SP) at the necrotic margin of tissue infected by *P. lateralis*. **E** and **F** Hyphae and disorganized cells at the necrotic margin. **G** and **H** Collapsed cells, separating cell walls, and wall thickening.

4) Histochemical staining of susceptible and resistant stems inoculated with *P. lateralis*

Histochemical staining for specific targeted compounds was used to investigate possible biochemical changes induced by infection. In transverse sections of uninoculated stems, starch was abundant in pith and xylem rays. Starch plastids appeared red-brown (Figure 4.17A and B). Combination stains for starch and nuclei stained only the target compounds in sections. Starch grains in pith and xylem ray cells were reddish brown and plant cell walls were greenish blue in the xylem (Figure 4.17C and D). Nuclei were found in phloem cells but not in the cortex. Few starch grains were observed in the phloem but many starch grains were found in cortex cells (Figure 4.17E and F).

In longitudinal sections of uninoculated seedlings, many starch grains were found in ray parenchyma as well as in the cortex. Some starch grains were observed in phloem (Figure 4.18A). Starch grains were also scattered in ray parenchyma cells, and some starch grains surrounded nuclei (Figure 4.18B). Starch grains in inoculated stems were found in xylem ray cells but usually fewer starch grains were observed in ray parenchyma and phloem than in uninoculated stems (Figure 4.18D and E).

Because structural changes had been observed in sections from the necrotic margin of 117490 RT resistant stems stained with calcofluor and malachite green-acridine orange, other staining methods were used to reveal substances that might be associated with the structural changes. The cross section of a control stem was stained with safranin and fast green to observe the normal plant cell walls (Figure 4.19A). In uninoculated resistant stems green-colored phloem cell walls were observed, but fiber

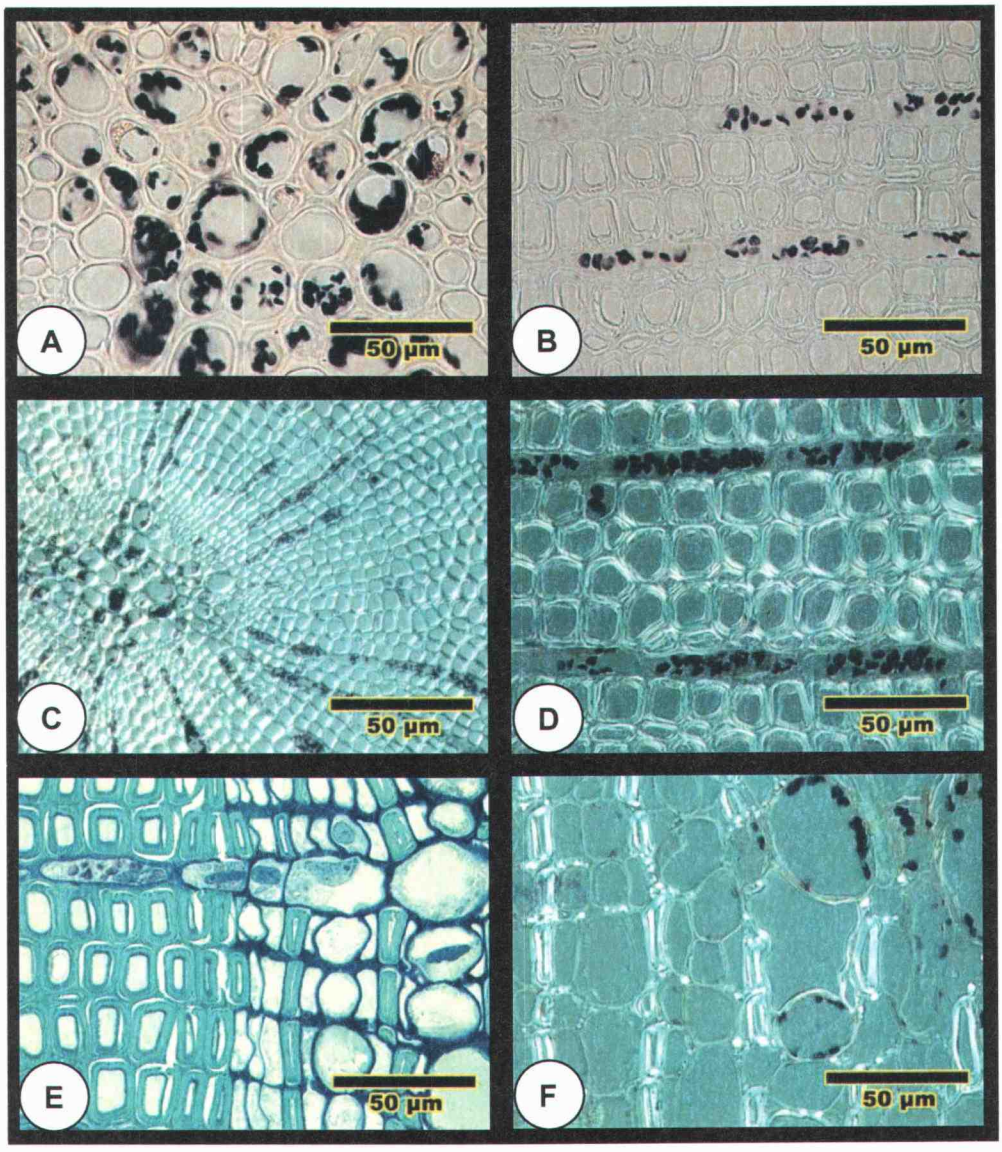

Figure 4.17. Light micrographs of transverse sections of uninoculated 118051 × OP stem stained with 0.5 % iodine in 5 % potassium iodide for starch and combination of iodine and 1 % toluidine blue for nuclei. **A** Many starch grains in pith. **B** Starch grains in xylem ray stained with only iodine. **C** Starch grains in pith stained with both iodine and toluidine blue. **D** Starch grains in xylem ray stained with both iodine and toluidine blue. **E** Starch grains and nuclei in xylem and phloem stained with iodine and toluidine blue. **F** Starch grains in cortex stained with both iodine and toluidine blue.

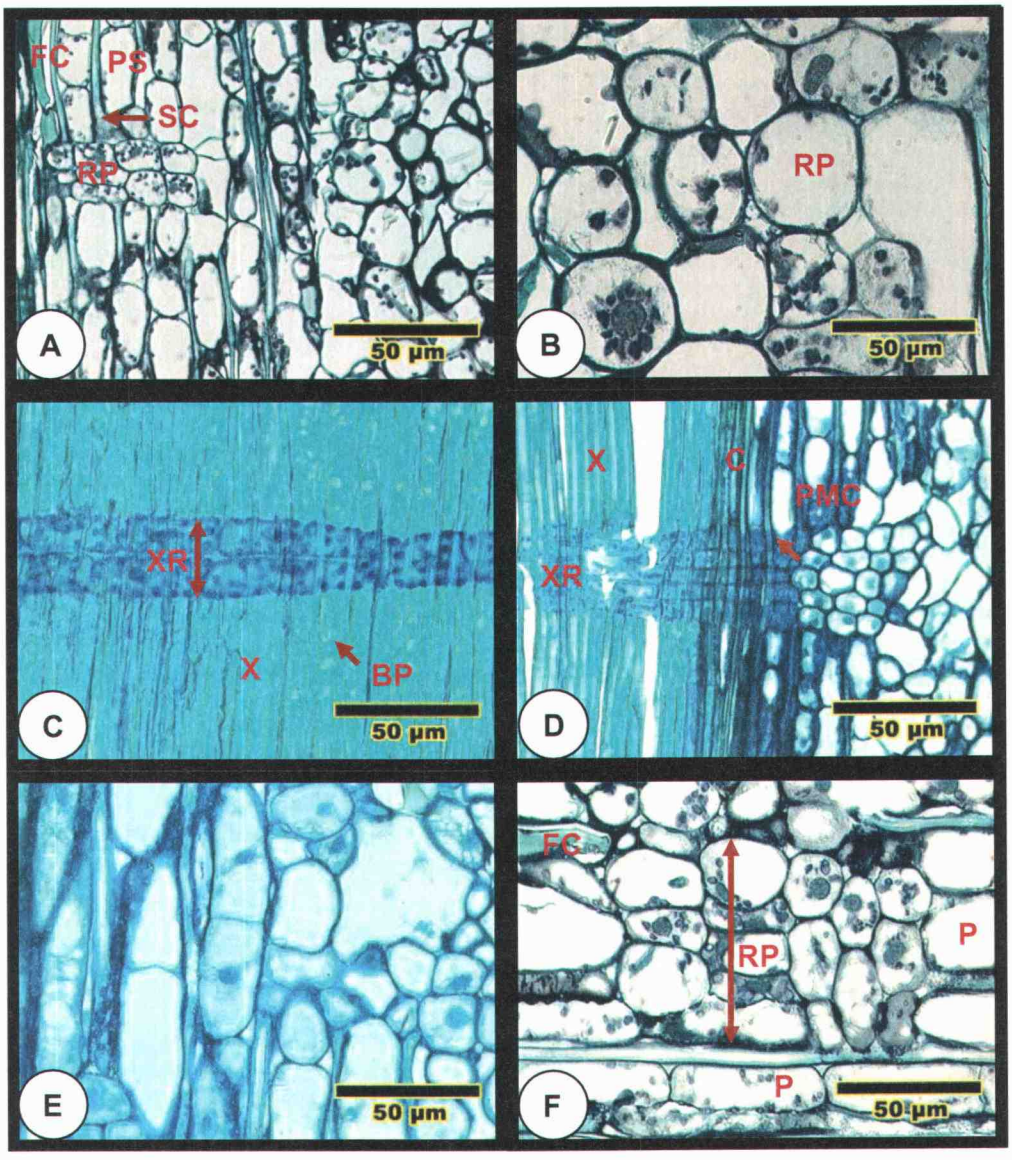

Figure 4.18. Light micrographs of longitudinal sections of susceptible stems stained with combination of 5 % potassium iodide for starch and 1 % toluidine blue for nuclei. **A and B** Starch grains and nuclei in phloem of uninoculated 118051 × OP stems. **C and D** Starch grains in secondary xylem of inoculated 117499 × OP stems. **E and F** Starch grains in inoculated 117499 × OP stems. BP-Bordered pit; FC-Fiber cell; PS-Parenchyma strand; RP-Ray parenchyma; SC-Sieve cells; X-Xylem; XR = Xylem ray.

cells were pinkish red (Figure 4.19A). In contrast, the resistant stem infected with *P. lateralis* showed bright red phloem cell walls, indicating lignification (Figure 4.19B). Also, the cross section of a control stem was stained with aniline blue to visualize the normal callose distribution in the functional phloem. The cambium was stained to white or faint yellow to reddish brown (Figure 4.20A and B). In cross section the necrotic margin of 70037 RT susceptible stem cells were stained white or reddish brown in a few layers of sieve and parenchyma cells including the cambium (Figure 4.20C and D). The necrotic margin of the inoculated resistant stem showed several layers of collapsed cells stained reddish brown (Figure 4.20E and F).

Uninoculated stem sections were stained with ferric acid after chlorine-sulfate to detect polyphenols. Polyphenols were expected to be stained green but only brownish colors were evident on the cross sectioned stems regardless of infection and susceptibility. The uninoculated stem had white or light brownish colored cells in the functional phloem (Figure 4.21A, B, and G). Compared to the control stem, cells at the necrotic margin of the inoculated susceptible stems were brown and dark brown (Figure 4.21C and D). There were no visual differences between susceptible and resistant stems, in sections from the necrotic margin or above the margin (Figure 4.21E and F). However, brownish materials were observed in the cortex of inoculated resistant stems only, and not in uninoculated or susceptible stems (Figure 4.21H). The combination treatment of ferric chloride and iodine for starch revealed abundant starch in cortical cells but not in phloem of uninoculated stems (Figure 4.22A and B). The phloem and cortical cells in the necrotic margin of susceptible stems were stained light brown to brown (Figure 4.22C and D) while the cortical cells in the necrotic margin

of resistant stems were stained dark brown (Figure 4.22E). Interestingly, no starch was evident in either the inoculated susceptible or resistant stems. Resistant stems contained unidentified spherical materials, but otherwise no visual differences in polyphenols between resistant and susceptible stems were detected with ferric chloride in this study. Toluidine blue stained all cells including xylem cells in uninoculated stems (Figure 4.23A and B). Sections from the necrotic margin of 70037 RT showed light brown colored intracellular hyphae in sieve and parenchyma cells which were light green (Figure 4.23C and D). The necrotic margin of 117490 RT contained dark green stained cambium cells and phloem mother cells. No hyphae were present in this case (Figure 4.23E). Unidentified materials were stained blue in cortical cells (Figure 4.23F).

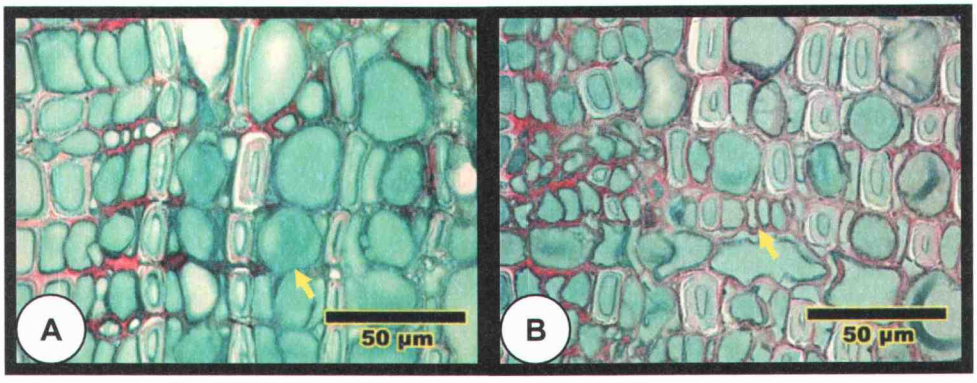

Figure 4.19. Light micrographs of transverse sections of 117490 × OP stained with safranin and fast green. **A** uninoculated stem indicating green phloem cells (arrow). **B** inoculated stem indicating red phloem cells (arrow) 4 weeks after inoculation with *P. lateralis*.

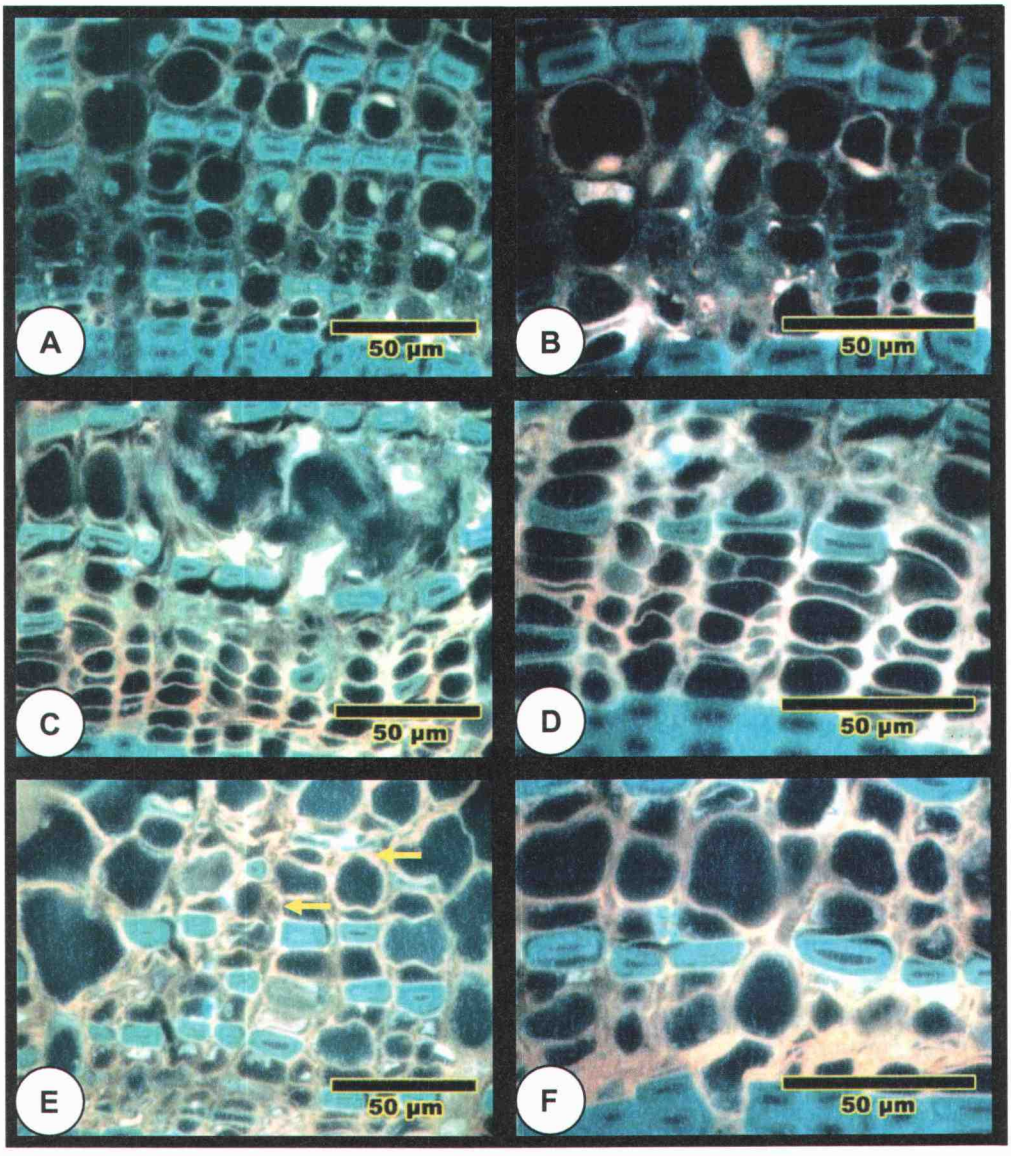

Figure 4.20. Fluorescent micrographs of cross sectioned 70037 RT susceptible and 117490RT resistant stems. All sections were stained with 0.1 % aniline blue (1000 ×) for callose. **A and B** Uninoculated stem of 117490 RT as a control to show localization of natural callose. **C and D** Yellow to reddish brown cambial and phloem mother cells at M of 70037 RT infected by *P. lateralis*. **E and F** Sieve and parenchyma cells (arrows) including cambial and phloem mother cells and disorganized cells beyond xylem at the M of 117490 RT infected by *P. lateralis*.

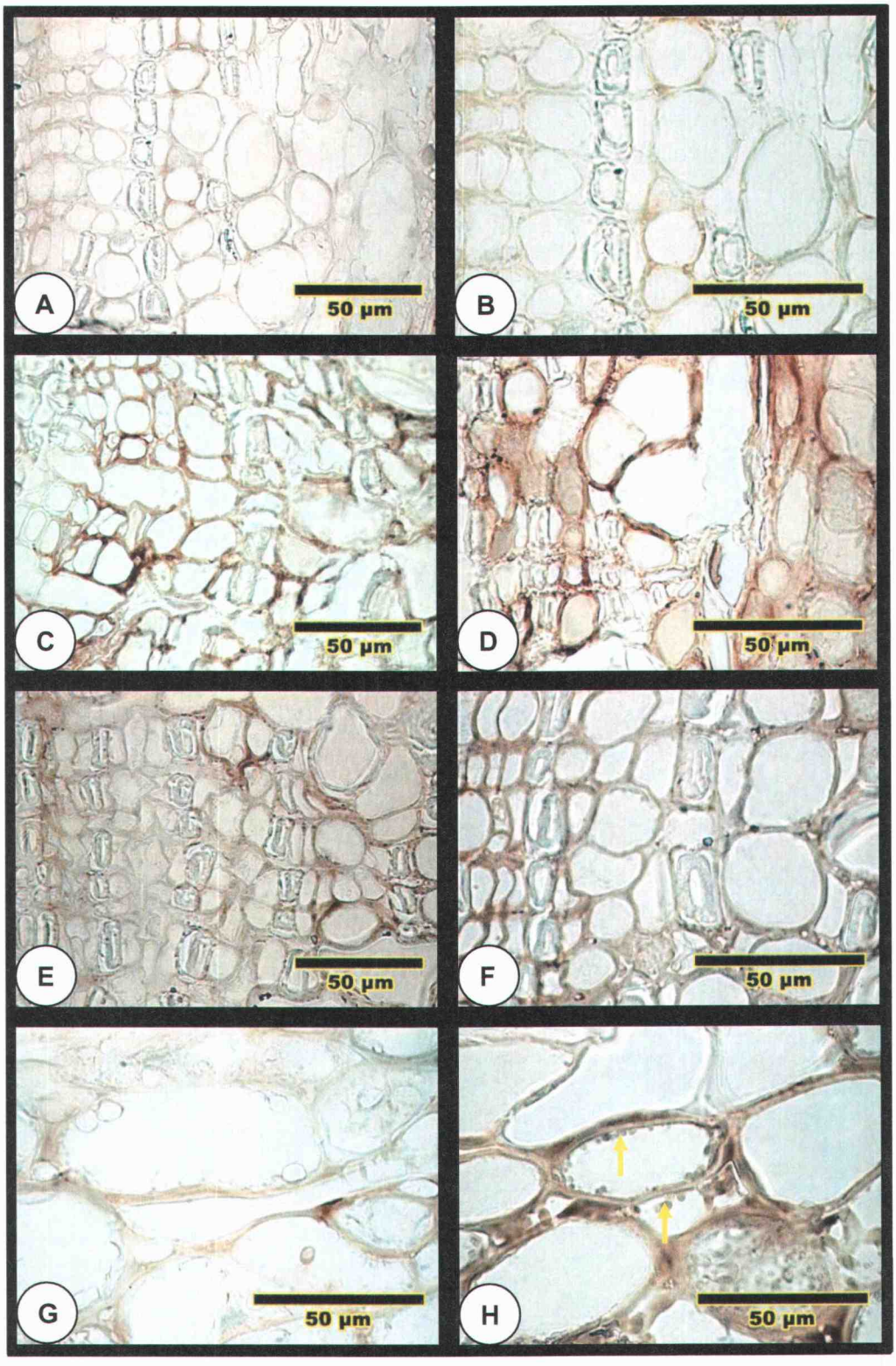
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Figure 4.21. Light micrographs of cross sectioned POC stems stained with 2 % ferric chloride after chlorine-sulfate treatment for polyphenolics including lignin. **A, B, and G** Uninoculated 117490RT stem. **C and D** brownish colored cells at the M of 70037 RT infected by *P. lateralis*. **E and F** unstained cells and dark brownish cambial cells (arrow) at the M and AM of 117490 RT. **H** Brown colored materials in cortex (arrow).

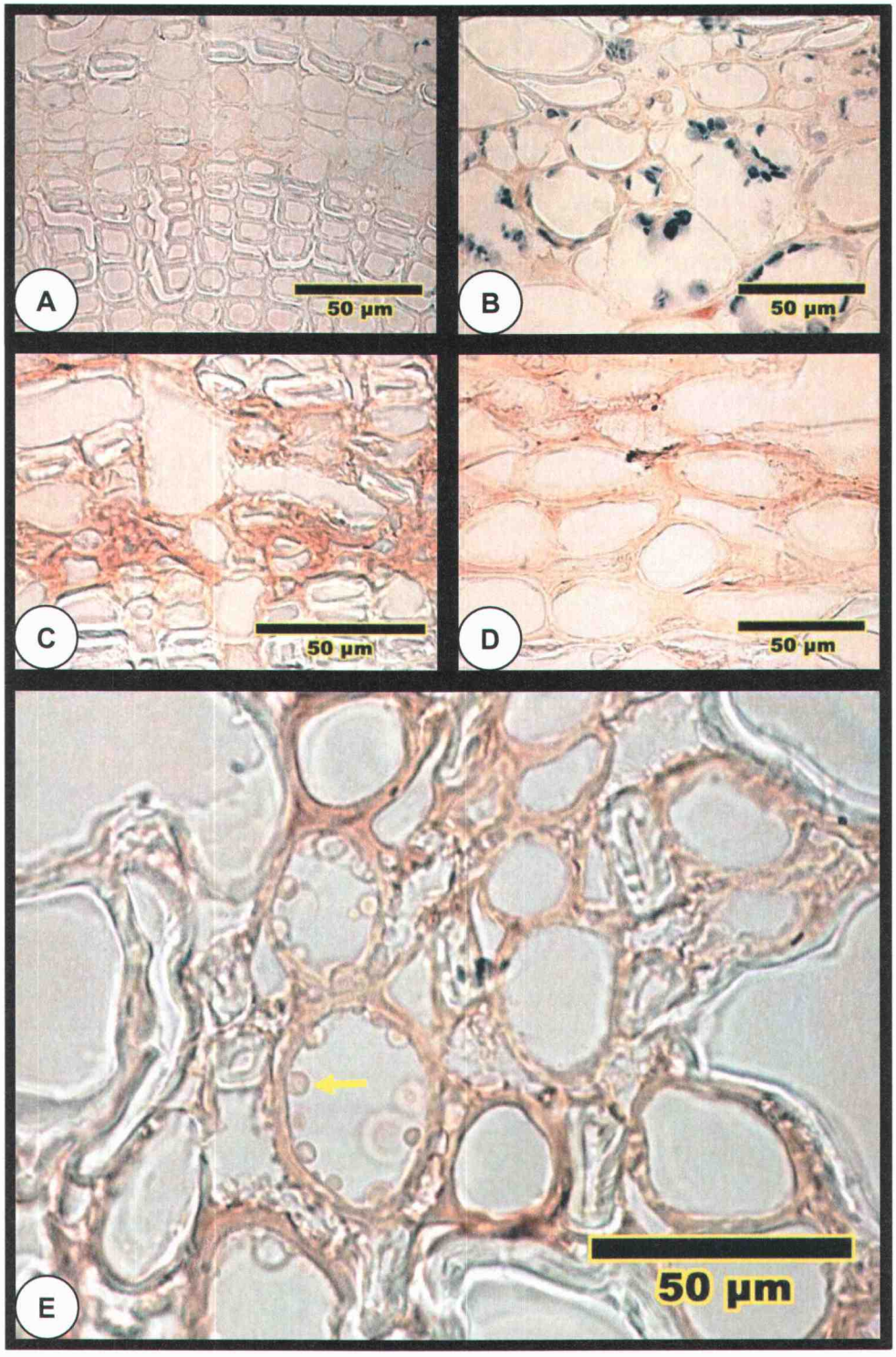
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Figure 4.22. Light micrographs of cross sectioned POC stems stained with 2 % ferric chloride followed by potassium iodide for starch. **A and B** No starches in phloem and light brownish cortical cells and many starch grains in uninoculated 117490 RT stem. **C and D** brownish phloem and brown cortical cells at the necrotic margin of 70037 RT infected by *P. lateralis*. **E** Dark brown cortical cells containing spherical materials (arrow) at the margin of 117490 RT. Note absence of starch in both **B** and **D**.

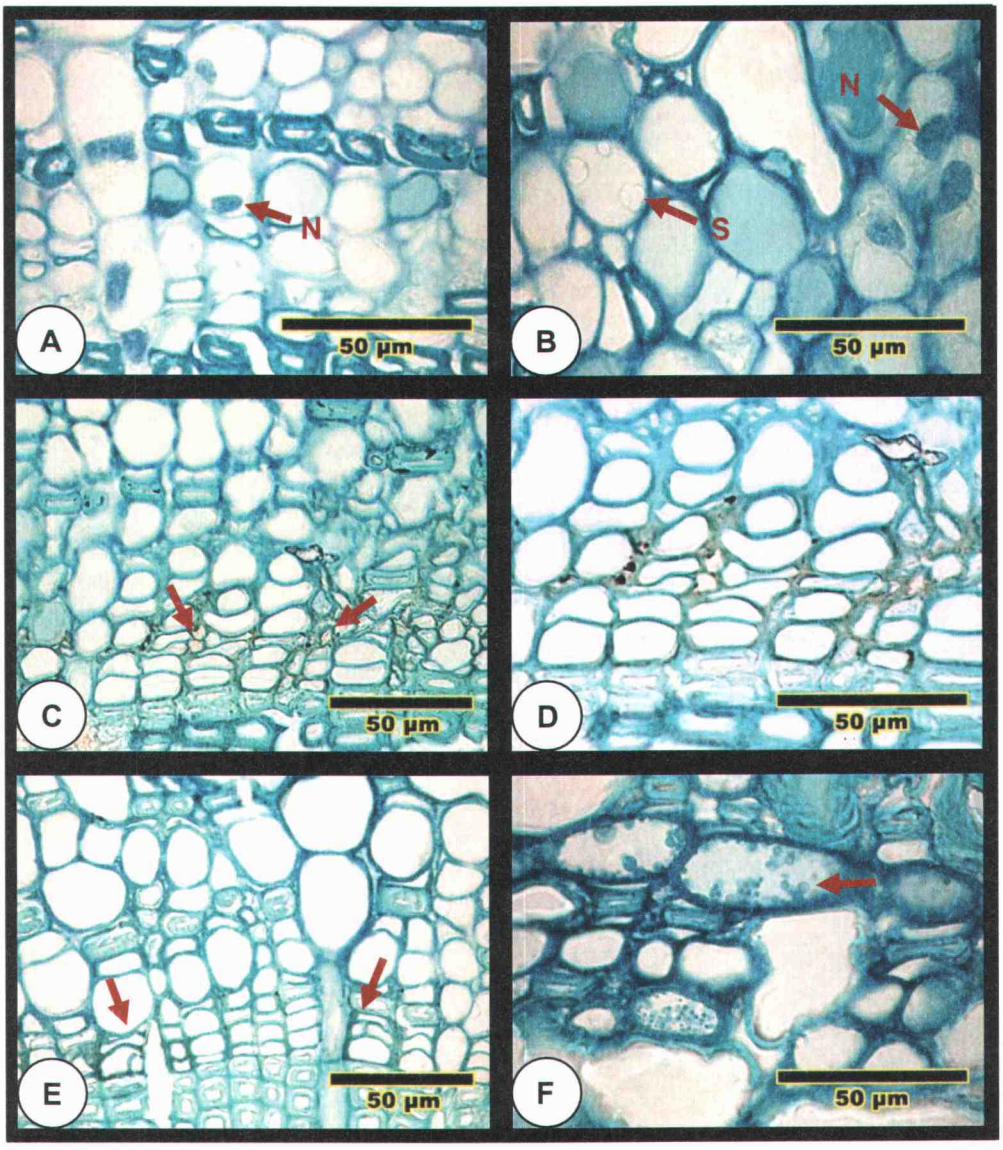

Figure 4.23. Light micrographs of cross sectioned POC stems. The sections were stained with toluidine blue after chlorine-sulfate treatment. **A and B** Faint blue stain in uninoculated 117490 RT. **C and D** Bright green parenchyma cells with intracellular hyphae (arrows) at the necrotic margin of 70037 RT. **E** Dark green cambial and phloem mother cells (arrow) at the margin of 117490RT. **F** Unidentified spherical materials (arrow) in cortical cells at the necrotic margin of 117490 RT. N = Nucleus; S = Starch.

In conclusion, there were no substantial histochemical differences between susceptible and resistant stems either at the necrotic margin or above the margin. Structural changes, including collapsed cells, separating cell walls, and wall thickening were evident in the necrotic margin of resistant stems.

4.5 DISCUSSION

Histocytological study in fungal biology provides insight to epidemiology as well as the interactions between microorganisms and host plants. Since the microscopic interactions between *P. lateralis* and POC have not been described previously, histological studies of fungal penetration and colonization were undertaken in order to better understand both initial infection and colonization of the host plant. In this chapter, transverse sections of uninoculated roots and stems provided fundamental information on anatomical features of Port-Orford-cedar. This provided a baseline for study of interactions with *Phytophthora lateralis*. Most *Phytophthora* species form a specialized structure called the appressorium during the infection process of host plants. Appressoria of *P. lateralis* were observed in POC roots inoculated with zoospores. The processes of zoospore encystment, germination and penetration by *P. lateralis* were found to be similar in susceptible and resistant seedlings, but the frequency of encystment and penetration was greater in susceptible seedlings. Penetration between anticlinal epidermal cell walls was typical, and it is a common penetration process in *Phytophthora* species on roots and hypocotyl tissues (Stössel et al. 1980). Some *Phytophthora* species, such as *P. infestans* and *P. nicotianae*, attack foliage or roots and penetrate directly through the periclinal walls of

an epidermal cell or through stomata (Coffey and Wilson 1983, Pristou and Gallegly 1954, Widmer et al. 1998). Occasionally, penetration of *P. lateralis* occurred directly into the outer walls of epidermal cells.

In general, the main resistance mechanisms are reduced infection frequency, slowed growth, and reduced sporulation on the host plants (Wilson and Coffey 1980). Beagle-Ristaino and Rissler (1983) investigated *P. sojae* on susceptible and resistant soybean roots. After inoculation of zoospores onto roots, reduced colonization after 72 hr exposure period and fewer oogonia and oospores after 8 days incubation were observed in resistant soybean roots. In this chapter, the apparent quantitative differences in encystment and penetration between susceptible and resistant seedlings included the initial number of cysts on the epidermal cells and the growth rate of hyphae after penetration. After 24 hours, susceptible seedlings were massively colonized by hyphae, while only short germinated hyphae from cysts were evident on resistant seedlings. Swollen germ tubes and septum-like structures separating an appressorium from the penetrating hypha have been shown in root sections of susceptible, as well as resistant POC. Similar features were present on hyphae of *P. sojae* penetrating soybean hypocotyls (Stössel et al. 1980). Presence of appressoria may indicate that penetration of young root cells by *P. lateralis* was facilitated by enzymatic action and mechanical pressure (Pristou and Gallegly 1954).

Colonization frequency of hyphae in the sectioned roots was consistent with observations of colonization in the sectioned stems. Several differences were observed between susceptible and resistant seedlings that suggested a general resistance response, in contrast to a more specialized hypersensitive reaction: 1) more zoospores

were attracted to susceptible than resistant roots. 2) frequency of encystment and germination in resistant roots was lower than in susceptible roots. 3) hyphae in resistant roots and stems did not colonize as extensively in susceptible roots and stems. 4) more non-fluorescent cells were present in phloem and cortex of resistant stems than in cells of susceptible stems. 5) while phloem cells were intact in susceptible stems, phloem cells in resistant stems were collapsed.

There were no asexual structures (sporangia or chlamydospores), or sexual structures (oogonia and oospores) observed in the root and stem inoculation study. In roots, because the tissues were fixed after 24 hours, there was not enough time for sporulation. In stems infected for 4 weeks, however, we might have expected to find chlamydospores. In contrast, in histological studies of *P. ramorum* in rhododendron twigs (Pogoda and Werres 2004), chlamydospores were observed in necrotic tissue beneath the inoculation point and in discolored tissues beyond that point, but not in the transition area between the discolored and healthy looking parts of the stem.

The microscopic observations were generally consistent with the results (lesion lengths) from stem wound inoculation: 117499 × OP seedlings were susceptible to stem inoculation, and microscopic observations indicated rapid colonization and extensive cell death in colonized tissues. The hypothesized reaction of CF1 × OP seedlings was either susceptible or resistant depending on the segregation of resistance genes in the progeny. The particular CF1 × OP seedlings used in this study were very susceptible to *P. lateralis* by stem wound inoculation, and histological observations were similar to the susceptible 117499 OP. The observed reaction (lesion length) of 70037 RT and 510015 × OP, and 117490 × OP suggested intermediate resistance. The

microscopic observations also suggested that 70037 RT was less susceptible than 117499 × OP and CF1 × OP seedlings. However, CF1 × OP seedlings showed the oozing response that was otherwise only seen among resistant POC seedlings.

Overall, calcofluor fluorescent dye worked better than malachite green and acridine orange to differentiate the cells of POC and *P. lateralis*, however calcofluor did not show the detail of the structural changes in the necrotic margin of resistant stems. The second fluorescent dye, malachite green and acridine orange enabled better visualization of structural changes than calcofluor.

The safranin and fast green staining method worked well to reveal differences between susceptible and resistant plants infected with *P. sojae* and *P. infestans* (Klarman and Corbett 1974, Pristou 1954). With POC, fiber cells on both susceptible and resistant plants were intact after infection, similar to observations on soybeans and oaks infected with *P. sojae* and *P. cinnamomi*, respectively (Klarman and Corbett 1974, Tainter et al. 1999). Results for lignification of cell walls after infection with *P. lateralis* were inconsistent, however.

Biochemical responses develop in the first few hours in plants after invasion of a pathogen. These responses are a key to differentiate susceptibility of plant species to pathogens (Ward et al. 1989). Histochemical staining for specific polyphenols was generally inconclusive. However, the staining for starches differentiated infected and healthy stems. Only healthy tissues contained starches, as reported on oak trees infected with *P. cinnamomi.* In addition, even though there were no differences in polyphenolics between susceptible and resistant POC stems, unidentified yellowish brown materials in parenchyma cells of resistant stem cortex suggested that the

content of parenchyma cells appeared to break down and became granular. The expected color of granular polyphenolics, however, was dark, blue-gray-green (Tainter et al. 1999). Also, there was suggestive evidence that yellow to reddish brown colored phloem cells indicated presence of callose in resistant stems.

Nevertheless, this study provided fundamental information on the anatomy of young roots and young secondary stems of Port-Orford-cedar. In addition, we reported the first study on initial infection, penetration, and colonization of *P. lateralis* on the roots of POC seedlings as well as colonization of the stems of POC seedlings. In this study we demonstrated cytological differences between resistant and susceptible seedlings of POC that suggest involvement of general resistance mechanisms in certain families.

Although this study included statistical data to show quantitative differences of *P. lateralis* on the stems of susceptible and resistant POC seedlings and associated cytological evidence for a general resistance response in some POC tree families resistant to *P. lateralis*, more work is needed. In particular, it will be important to work with rooted cuttings to avoid the uncertainties of open-pollinated seedlings with segregating resistance genes. It would also be useful to extend the observations of the extent of colonization of resistant and susceptible roots to longer incubation times and to include measures of subsequent sporulation from infected roots.

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Chapter 5

Ultrastructural Changes in Susceptible and Resistant Port-Orford-Cedar Seedlings Inoculated with *Phytophthora lateralis*

Eunsung Oh

5.1 ABSTRACT

Histological studies on the pathogenesis of *Phytophthora* species are essential to an understanding of the relationship between susceptible and resistant hosts (Chapter 4). In this chapter, transmission electron microscopy (TEM) is used to examine ultrastructural changes in infected roots and stems of susceptible and resistant POCs. Those changes included increased cell wall thickness, cell wall appositions, presence of electron osmiophilic granules and secretory bodies, and encasement of hyphae by electron dense opaque materials.

5.2 INTRODUCTION

The history of plant pathology began with potato late blight caused by *Phytophthora infestans* (Mont.) de Bary, and *P. infestans* is the best known and most researched species in the Oomycota. Studies of the histology and cytology of *P. infestans* on potato date to the 1950s. The light microscope gave scientists a new tool to understand anatomy of the host and parasite as well as their interfaces at the cellular level. However, the small size of fungal and plant structures, and the physical limitation of light to 1000 × magnification and a resolution of 0.2 μm limited the usefulness of light microscopy at the subcellular level. The introduction of the electron microscope (EM) in the 1930s provided added magnification and resolution power, but also has limitations. In 1942, the first observation on a solid specimen was performed, but EM was not widely used until the 1960s. The first ultrastructural studies of plant pathogens were made with powdery mildews and rusts examining host-parasite interactions in the late 1960s and 1970s (Bracker et al. 1973).

In Oomycota, reproductive structures in the vegetative stages of *Peronospora manshurica,* in the Family Peronosporaceae, the casual agent of downy mildew in soybeans, were observed with EM to observe details of organelles in fungal structures (Peyton and Bowen 1963). Meanwhile, Hawker and Abbott (1963) examined the hyphal structure of *Pythium debaryanum* in Family Pythiaceae, and Hendy made further observations on the structure of organelles in the same species in 1966. Fine structure of the growth zone in hyphal tips of *Pythium ultimum* was also observed by EM (Grove and Bracker 1968). In addition, EM was used to observe centrioles during formation of zoospores in *Albugo candida* in the Family Albuginaceae, causing white rust of crucifers (Berlin and Bowen 1964a, b). Among *Phytophthora* species, organelles in sporangia of *P. erythroseptica* were described to show structural changes during the developmental process (Chapman and Vujičić 1965, Vujičić et al 1965). Morphology of haustoria and hyphae of *P. parasitica* and *P. infestans* was observed with the aid of EM by Ehrlich et al. (1966). Hohl and Hamamoto (1967) described the fine structural changes all along the zoospore development of *P. parasitica.* Ultrastructural studies on oomyceteous fungi are continuing today, often emphasizing the interactions between host and parasite.

Cell wall modification was reported in tobacco roots infected with *Phytophthora parasitica* var. *nicotianae* (Hanchey and Wheeler 1971). In 1974, Klarman and Corbett used both light and electron microscopy to view cytological differences between susceptible (S = compatible) and resistant (R = incompatible) soybeans after the plants were infected by *Phytophthora sojae.* Cytological differences between susceptible and resistant hosts to *P. infestans* (Coffey and Wilson 1983, Hohl

and Stössel 1976, Hohl and Suter 1976, Wilson and Coffey 1980), *P. sojae* (Stössel et al. 1981, Ward and Cahill 1989) *P. nicotianae* and *P. palmivora* (Widmer et al. 1988), and *P. cinnamomi* (Cahill et al. 1989) were observed to follow the interaction between host and pathogen. From the 1980s until now, scientists have been focused on recognition events between the host and pathogen as being key elements of infection processes leading to a resistant or susceptible reaction (Blein et al. 2002, Boissy et al. 1999).

Differences in zoospore attraction between susceptible and resistant roots of POC, and cytological differences in susceptible and resistant stems of POC have been observed (Chapters 3 and 4). Therefore, the objective of this chapter is to extend this work to the ultrastructural level. TEM was employed to compare changes in roots and stems of susceptible and resistant POCs following infection by *P. lateralis.*

5.3 MATERIALS AND METHODS

5.3.1 Preparation of samples

Isolate 368 of *P. lateralis* was used for root inoculation of POC seedlings. The pathogen was stored, grown, and induced to produce zoospores as described previously (Chapter 3). Actively growing root tips from three rooted cuttings of clone 117490 RT (resistant) or 70037 RT (susceptible) were inoculated, as described previously (Chapter 4). Roots were inoculated with 25 ml zoospore suspension for 1, 3, 5, 7, 9, 12, and 24 hours. Concentration of zoospores was ca. 1.6×10^4 zoospores per ml. Stem inoculations were done on rooted cuttings of the same clones that were used for root inoculation, using methods described in Chapter 4. Sections for electron

microscopy were taken from the margin between necrotic and healthy tissue (Figure 5.1).

Figure 5.1. Inoculated stem, showing the necrotic lesion (bold arrow) and the margin between necrotic and healthy tissue (fine arrow).

5.3.2 Transmission electron microscopy

The inoculated roots and stems were fixed in 2.5 % glutaraldehyde in 0.05 M sodium cacodylate buffer and post-fixed with 2 % OsO_4 (Osmium tetroxide) for 2 hours at room temperature. After rinsing with buffer, the roots and stems were embedded in resin (Spurr 1969) as described in Chapter 4.

For root samples, a two mm section of the embedded block was cut from the root tip to uniformly obtain ultra-thin sections from the same region of the roots. The blocks were trimmed and these sections were cut with a diamond knife (Polysciences Inc. Niles, IL) using an ultra-microtome MT-2 (Servall Poster-Blum). The sections were 90 nm to 150 nm. The sections were mounted on formvar-coated copper grids.

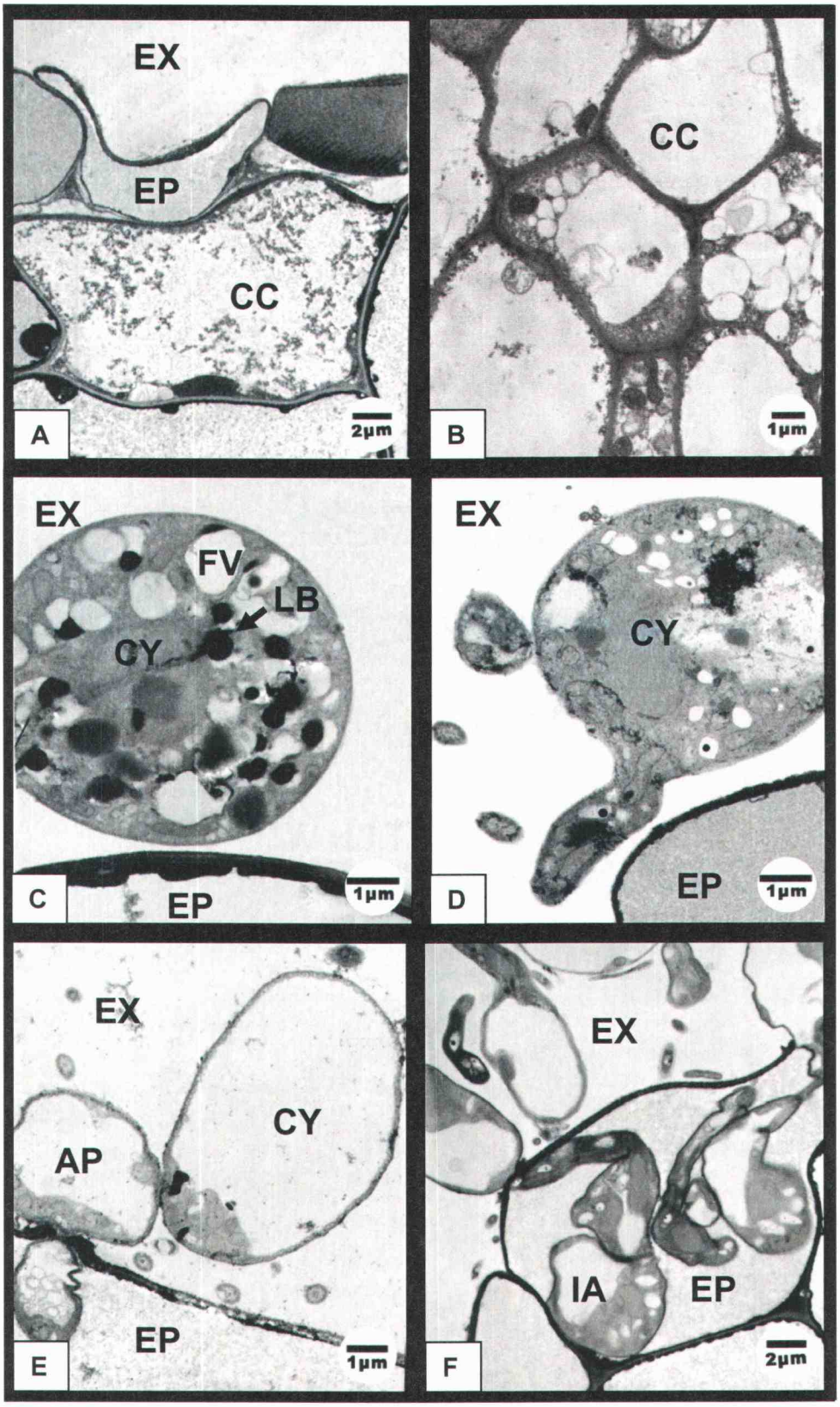
Ultra-thin sections were mounted and stained with lead citrate (Reynold's stain, Venable and Coggeshall 1965) using the double lead stain technique (Daddow 1986). After staining the grids were washed with ddH_2O. The grids were then stained in uranyl acetate (UA; 0.5 g UA in 15 ml ddH_2O) for 2 minutes. The grids then were placed directly into lead citrate again for 2 minutes. Finally, the grids were rinsed with ddH_2O and air dried. The sections on the grids were examined with a transmission electron microscope (Philips CM12) at 60 kV with 100 μm objective apertures at magnifications ranging from 3,800 × to 22,000 ×. Photographic images were recorded on a 3 1/4" × 4" plate camera, using Kodak 4489 film.

5.4 RESULTS

5.4.1 Transmission electron microscopy of roots

Ultra-thin sections of uninoculated roots were examined as a basis for comparison with infected roots, concentrating on epidermal and cortical cells near the surface of the root. Usually, the dead epidermal cells did not contain cytoplasm and were clean (Figure 5.2A). The cortical cells beneath epidermal cells were either empty or full of cytoplasmic materials including organelles (Figure 5.2B).

Cysts were observed on roots at 1 and 3 hour after inoculation, but cysts were more commonly present at 5 hour after inoculation (Figure 5.2C) and had germinated on epidermal cells (Figure 5.2D). After 9 hour inoculation, a germinated cyst with an appressorium and penetration hypha was observed (Figure 5.2E). Numerous intra-and intercellular hyphae were present in cortical cells as well as epidermal cells after 12 hour inoculation (Figure 5.2F and G). No or a few electron dense materials were near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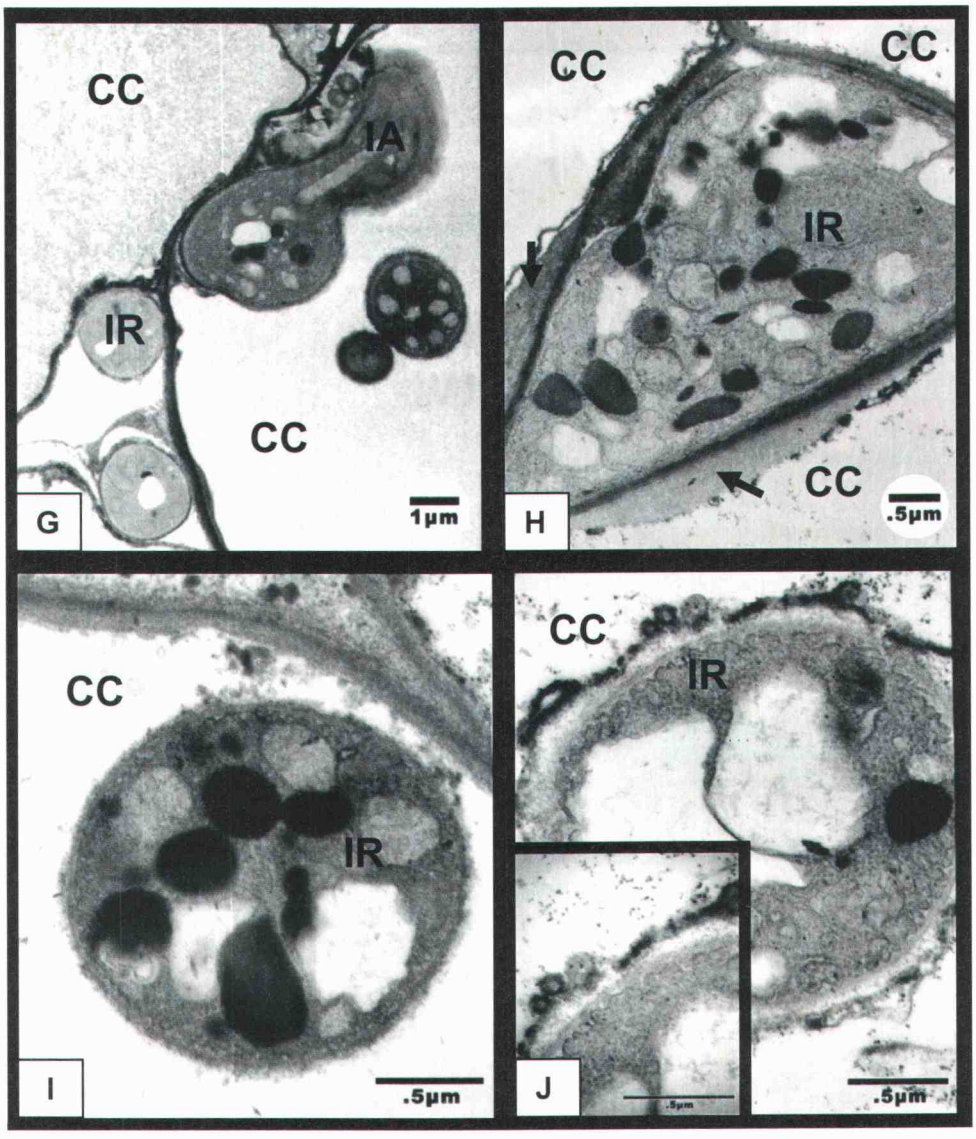
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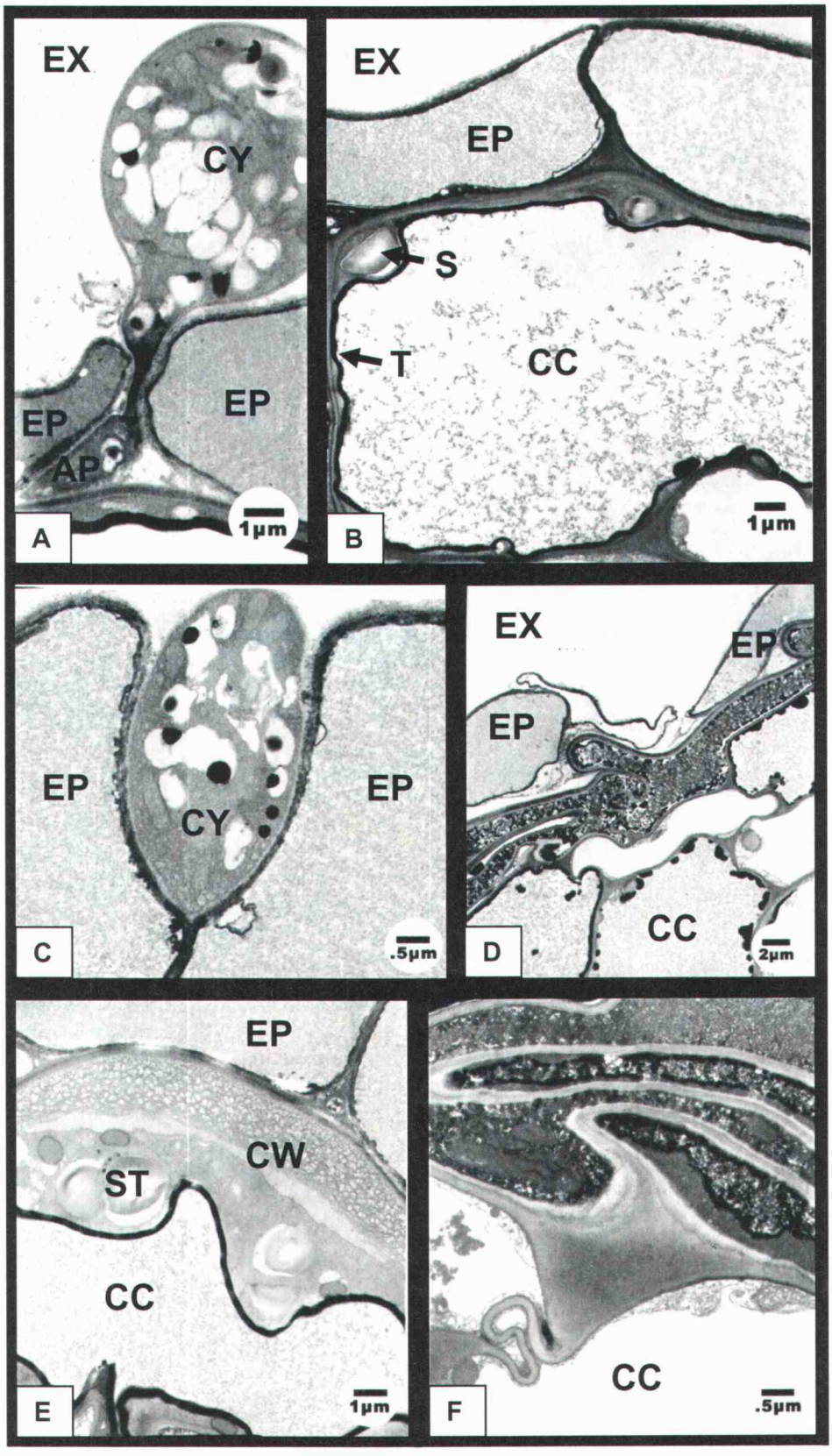
Figure 5.2. Electron micrographs of ultrathin sections of susceptible roots of POC inoculated by *P. lateralis*. **A and B** Control roots of 510015 × OP. **A** Epidermal cells (EP) and external zone (EX). **B** Cortical cells (CC). **C-J** 70037 RT inoculated with *P. lateralis*. **C** Cyst (CY) on an epidermal cell after 5 h inoculation. FV=Fingerprint Vacuoles; LB=Lipidlike Body. **D** Germinating cyst after 5 h inoculation. **E** Cyst with an appressorium (AP), and swollen structure penetrating into an epidermal cell after 9 h. **F** Intracellular (IA) hyphae in an epidermal cells as well as on surface of a root after 12 h. **G** Abundant intercellular (IR) and intracellular hyphae in cortical cells after 12 h. **H** Intercellular hypha and electron dense layers near cortical cell walls after 24 h (arrows). **I** Intercellular hypha after 24 h. **J** Intracellular hypha surrounded by electron dense materials.

hyphal cell walls after 24 hour inoculation (Figure 5.2H, I, and J). On roots of resistant POCs, cysts were present at 1 hour after inoculation as on susceptible POC, but fewer cysts were observed on the surface of roots. A germinated cyst produced an appressorium that penetrated between epidermal cells (Figure 5.3A). However, the appressorium appeared unhealthy or electron dense with cytoplasmic disruption near a thick cortical wall compared to the germinated cysts in the susceptible root. Wall thickening also was observed on cortical cells beneath the epidermal cells without presence of cysts at 3 hour after inoculation. The walls of their cortical cells were about 2.6 times thicker than walls in the control and susceptible roots (Figure 5.3B). The cell wall thickening occurred regardless of the presence of cysts. An ungerminated cyst was observed between epidermal cells (Figure 5.3C), and it had not germinated after 5 hour incubation.

Compared to susceptible roots that were inoculated at the same time, germinated cysts were infrequent on resistant roots, and when observed, they were abnormal. Cell modification of POC including increased cortical wall thickness was commonly observed in the resistant roots. After 5 hour incubation, wall appositions were seen in cortical cells beneath a layer of epidermal cells without attached cysts (Figure 5.3D). Cell walls with appositions were up to 14 times thicker than normal cell walls (Figure 5.3E). In addition, electron dense materials were observed in the cortical cells. Some cortical cells appeared collapsed (Figure 5.3F). After 7 hour incubation, intracellular hyphae were seen in cortical cells (Figure 5.3G). The hyphae were surrounded by electron osmiophilic materials. A collar-like wall apposition that included electron osmiophilic granules between cell wall and membrane was observed where a hypha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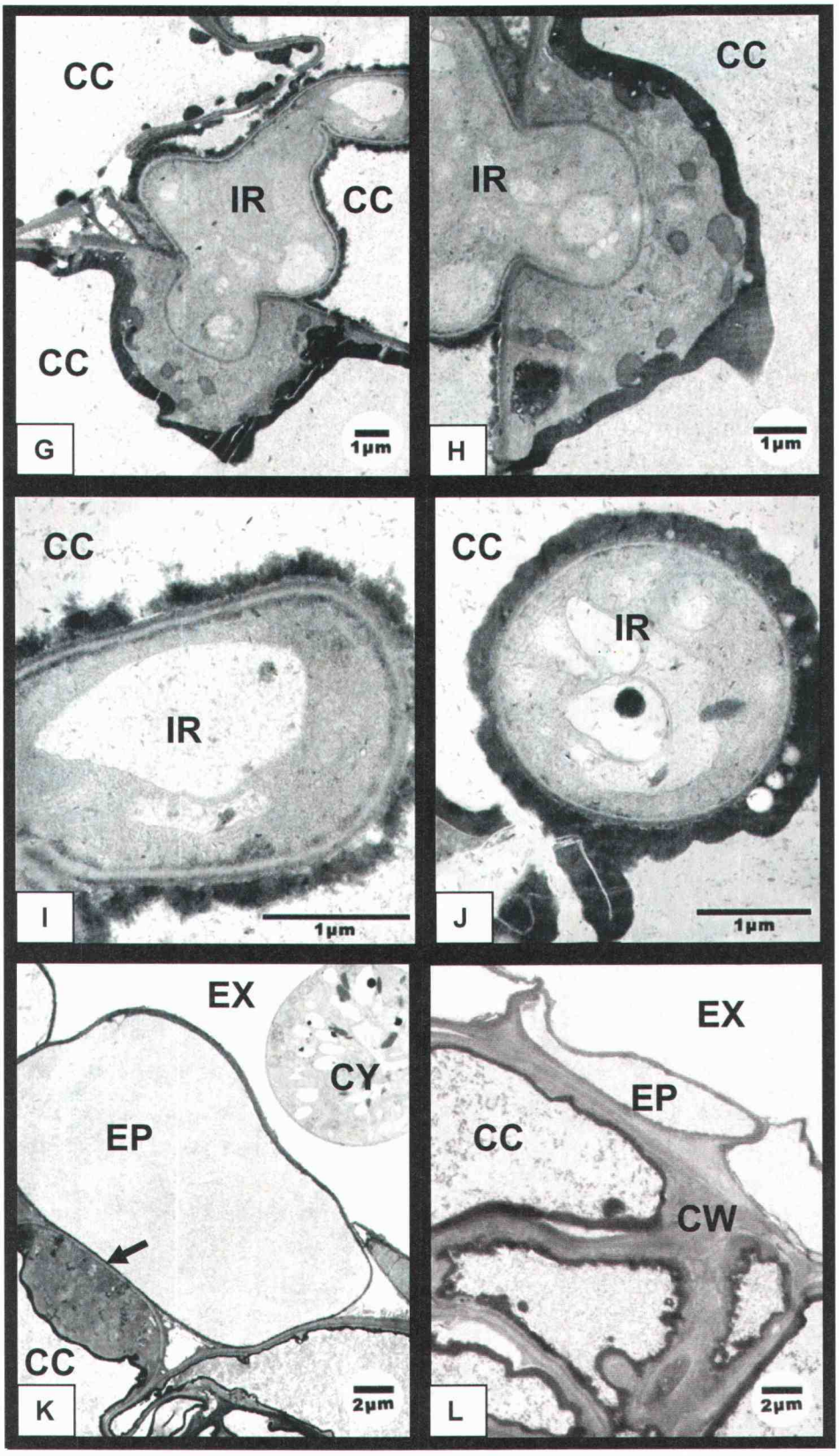
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Figure 5.3. Electron micrographs of ultrathin sections of 117490 RT resistant roots inoculated with *P. lateralis*. **A** Germinated cyst with an appressorium after 1 h. **B** Cell wall thickening after 3 h. **C** Abnormal ungerminated cyst after 5 hour. **D** Wall apposition beneath the epidermis after 5 hour. **E** Wall thickening after 5 h. **F** Wall apposition and collapsed cell walls. **G** Simultaneous wall modification and penetration of intracellular hypha in cortical cells after 7 h. **H** A closer look at **G** showing collar-like wall apposition containing lipidlike body. **I** A close look at **G** with hypha surrounded by electron dense osmiophilic granules. **J** Intracellular hypha encased by electron dense materials in cortical cell after 7 h. **K** Ungerminated cyst and wall apposition by electron opaque materials beneath epidermis after 9 h. **L** Cell wall (CW) thickening behind the epidermis. T = Tonoplast of host.

was penetrating a host cell wall (Figure 5.3H). The main part of the hypha was also surrounded by numerous dense osmiophilic granules (Figure 5.3I). Another intracellular hypha seen in a cortical cell was encased by electron dense material (Figure 5.3J). An ungerminated cyst was observed at 9 hour after inoculation, and wall appositions were observed in nearby cortical cells (Figure 5.3K). Wall thickenings, as well as electron osmiophilic materials, were also present along the walls (Figure 5.3L). Cysts and hyphae were not observed in resistant roots inoculated for 12 and 24 hours, but thickened walls and electron dense materials were noted. Furthermore, no inter- or intracellular hyphae were observed in resistant roots.

5.4.2 Transmission electron microscopy of stems

Ultra-thin sections of an uninoculated stem of 510015 were examined to establish normal structure of POC stem cells. Parenchyma cells contained nuclei, starch grains, and lipidlike bodies and appeared healthy (Figure 5.4A). In inoculated stems of susceptible 70037RT, intracellular hyphae were observed in sieve (Figure 5.4B) and parenchyma cells (not shown). Electron dense materials were seen near starch plasmids in parenchyma cells (Figure 5.4C) and electron dense materials were present along cell walls (Figure 5.4D).

In sections from the necrotic margin of resistant 117490RT stems, starches and lipidlike bodies in parenchyma cells looked different than in control and susceptible stems (Figure 5.5A). The starches were surrounded by osmiophilic granules (Figure 5.5B), and the lipidlike bodies were not solid but full of granular materials (Figure 5.5C). An intracellular hypha with a broken cell wall was observed in one parenchyma

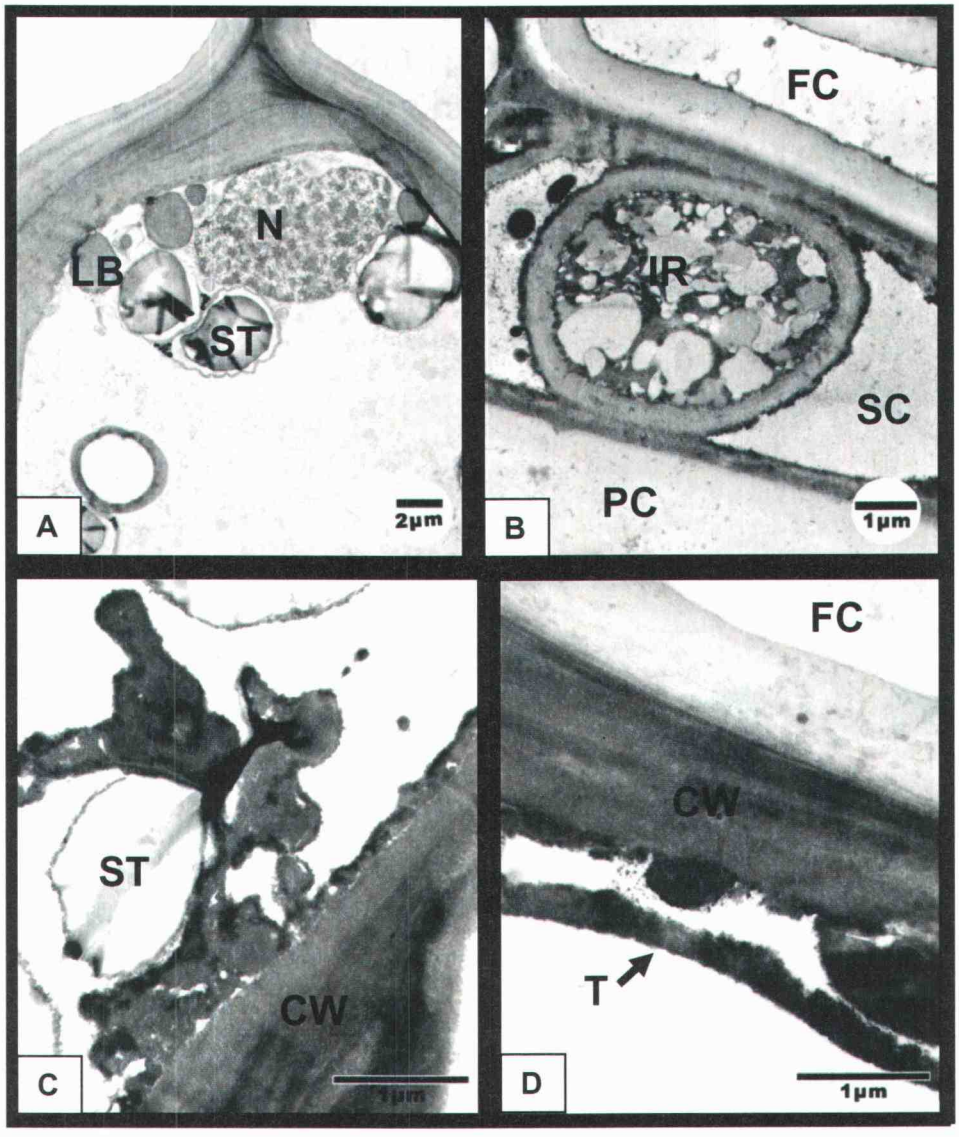
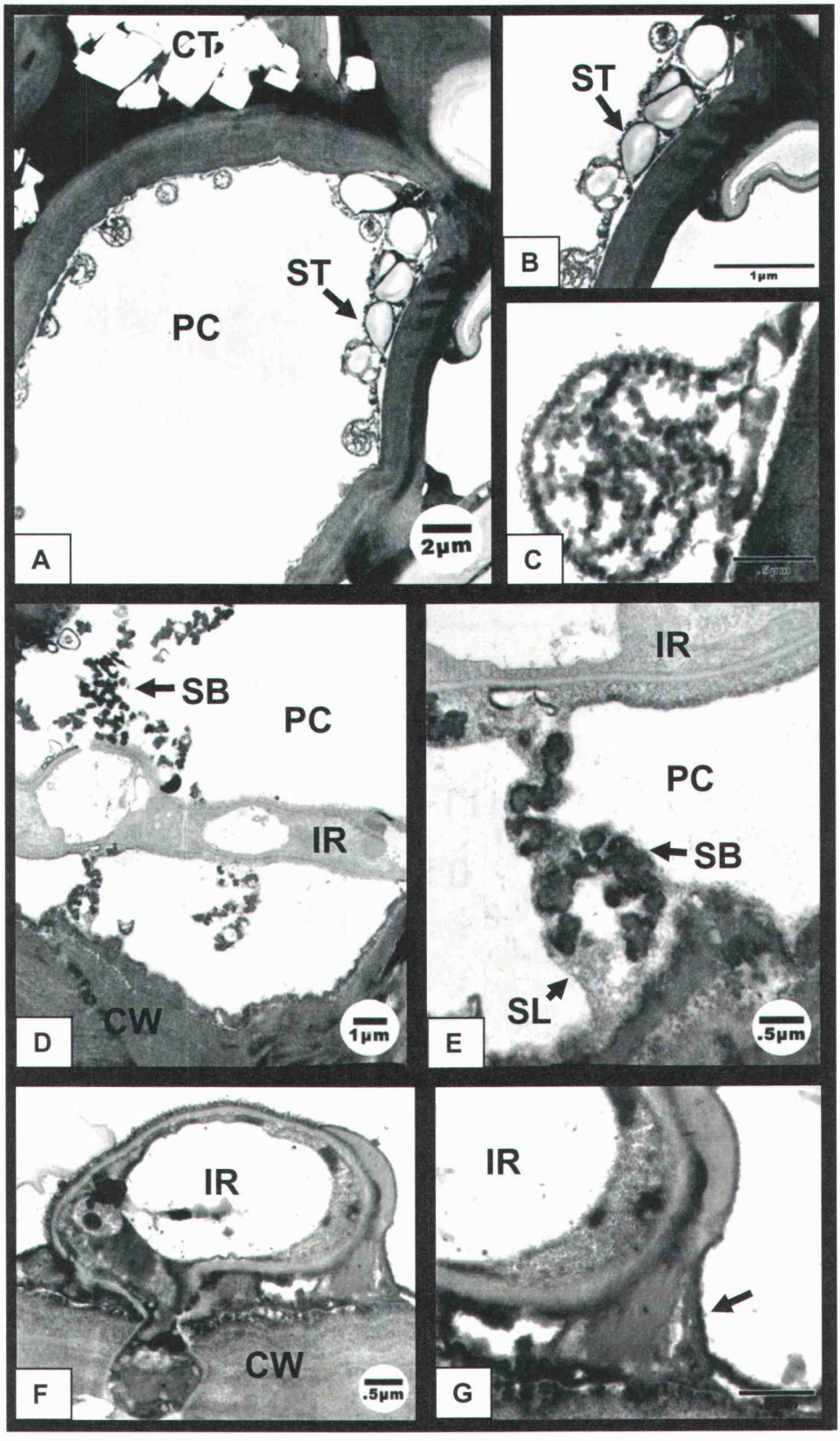


Figure 5.4. Electron micrograph of ultrathin sections of POC stems. **A** Parenchyma cell of uninoculated control stem of 510015 × OP with nucleus (N), starches (ST), and lipidlike bodies (LB). **B** 70037RT inoculated with *P. lateralis*. An intracellular hypha (IR) in a sieve cell. **C** Electron dense materials near starch grains. **D** Lipidlike bodies between cell wall and cell membrane. CW=Cell Wall of host; T=Tonoplast of host.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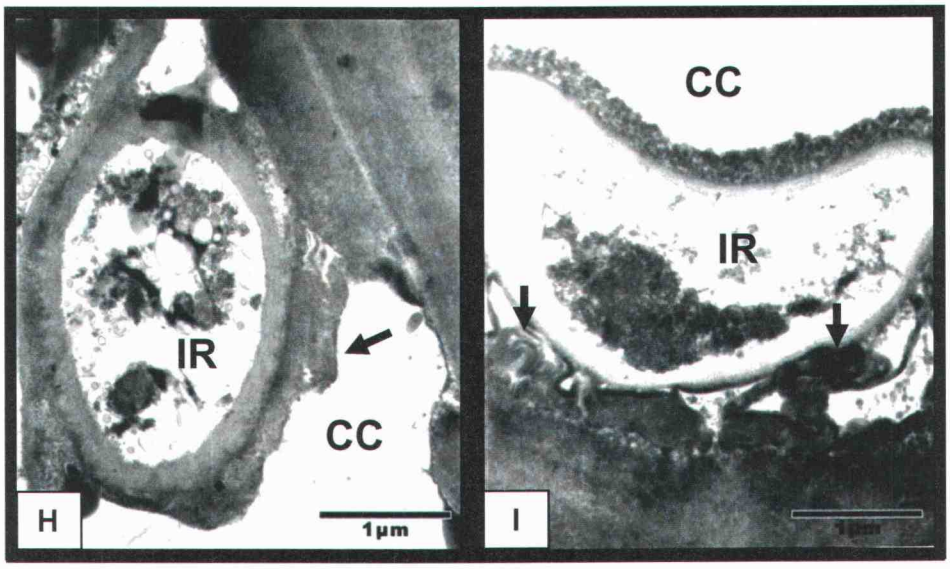

Figure 5.5. Electron micrograph of ultrathin sections of 117490 RT resistant stem inoculated by *P. lateralis*. **A** Intracellular crystals (CT) in a parenchyma cell containing starches and lipidlike bodies. **B** Starch grains surrounded by electron osmiophilic granules. **C** Numerous dense osmiophilic granules in lipidlike body. **D** Intracellular hypha in a parenchyma cell near numerous secretory bodies (SB) that are contiguous with the sheath-like layer (SL) from thickened wall. **E** Close look at **D**, secretory bodies derived from between invaginated host plasma membrane reaching the hypha. **F** Hypha in a parenchyma cell and its penetration into a wall (CW). **G** A close look at **F** showing encasement of the hypha. **H** and **I** Electron dense materials (arrows) near a hypha.

cell. The hypha was surrounded by numerous secretory bodies in the host cytoplasm, and the bodies were contiguous with the sheath-like layer. Cell walls were thicker than the walls in control and inoculated susceptible stem sections (Figure 5.5D). The secretory bodies were covered with a kind of sheath that was associated with the cell wall (Figure 5.5E). In addition, an intracellular hypha in a parenchyma cell was observed penetrating to the adjacent host cell. The cell wall of POC was thickened at the point where the hyphae penetrated (Figure 5.5F). Electron dense material was observed between the wall and the hypha, appearing to prevent attachment of the

hypha to the host cell wall. The hypha appeared to be dead (Figure 5.5G). In general, intracellular hyphae in resistant stems looked more abnormal than in the susceptible stem. Additional observations were made on intracellular hyphae in parenchyma cells (Figure 5.5H and I).

5.5 DISCUSSION

Transmission electron microscopy was used to observe physical, cytological, and structural changes at the cellular and subcellular level in roots and stems of susceptible and resistant Port-Orford-cedars infected with *P. lateralis*. The results provide insight to the mechanisms of resistance expressed by rooted cuttings of POC tree 117490.

Fewer *P. lateralis* zoospores encysted and germinated on resistant roots than on susceptible roots during 1-5 hour inoculation periods. After 9 and 12 hours incubation period, hyphae from cysts penetrated and colonized both inter- and intracellularly, in both susceptible and resistant roots. However, hyphae were observed much less frequently in resistant roots than in susceptible roots. This result was consistent with evidence provided by light microscopy in Chapter 4. Subcellular observations by EM showed cytological changes in resistant roots during the infection process by *P. lateralis*. Cell wall thickening was observed in resistant roots after only 1 hour exposure to zoospores, and after 3 hour incubation a resistant root cortical cell wall was 2.6 times thicker than cell walls in uninoculated and susceptible roots. Moreover, after 5 hour incubation some cortical cell walls in resistant roots were up to14 times thicker. This extraordinary wall thickness was observed in root sections

both with and without visible cysts or hyphae of *P. lateralis.* A similar resistant response to *P. sojae* was observed in some soybean cultivars (Stössel et al. 1981). Increased wall thickness, a type of induced structural change, is commonly present in the interaction of host and pathogen as a physical defense mechanism (Agrios 1997). This wall thickening in POC appears to be one of the general resistance reactions to *P. lateralis.*

In addition, cortical cell walls below the epidermis in inoculated resistant roots were crumpled and cells appeared to be abnormal, with electron dense materials. Intracellular hyphae encased by osmiophilic particles were observed, and host cell wall appositions at points of penetration were present. Wall apposition is also a general resistance response (wound) and has been observed in many other plant hosts in response to pathogenic fungal infection, including *Phytophthora* species. Interactions between *P. sojae* and resistant soybean cultivars and between *P. infestans* and resistant potato cultivars caused similar wall appositions that were attributed to a resistant response (Ward et al. 1989, Wilson and Coffey 1979). Osmiophilic granules were frequently seen on the surface of fungal hyphae adjacent to host cells. These may represent antifungal materials produced by the host in resistance to infection. Similar ultrastructural changes have been observed in other *Phytophthora* species. Invasion of *P. lateralis* on resistant POC changed stem cells biochemically and cytologically such as osmiophilic materials, cortical cell wall thickening, and wall appositions.

Cytological changes, including cell wall thickening, were also noted in resistant stems inoculated with *P. lateralis*. Interestingly, numerous secretory bodies, electron dense opaque materials in the host cytoplasm, were observed near hyphae,

and the bodies were continuous with the sheath-like layer surrounding hyphae and adjacent host cell walls. Both secretory bodies and sheath-like layer were related to the cell wall and presumably produced from the host cell wall. Eventually, the secretory bodies were possibly moved from the cell wall to the hypha resulting in slowed growth or death of the hypha (Bracker et al. 1973). It is evident that resistant POC cells were biochemically changed after inoculation as outcome of host and pathogen interactions (Coffey and Wilson 1983). Hyphae in resistant stems of 117490 were encased with electron dense materials (Figure 5.5F-I). Dense materials apparently derived from the POC cell wall were attached to the cell wall of the hypha. Perhaps this cell modification formed a barrier, preventing extension of hyphae and restricting colonization of host tissue as a role of barriers (Coffey and Wilson 1983, Klarman and Corbett 1974, Stössel et al. 1981).

In conclusion, the first ultrastructural study of responses of Port-Orford-cedar to infection by *P. lateralis* revealed distinct physical, biochemical, and cytological differences between susceptible and resistant POCs. The roots and stems of resistant POC seedlings exhibited general resistance responses, including increased cell wall thickness, wall appositions, osmiophilic granules, secretory bodies, and encasement of hyphae by electron dense opaque materials. Therefore, this study provided evidence for resistance mechanisms based on induced physical (barrier) and mechanical defense of POC to *P. lateralis*. Although no rapid cell death by inducing lignification and phenolic compounds as a hypersensitive response was documented, this study provides evidence for general resistance mechanisms in POC to *P. lateralis* and reveals the relationship between POC and *P. lateralis*. For future work, histological

methods for identification of biochemical materials (phenolics, callose) must be improved to better evaluate the cytochemical differences between susceptible and resistant POC. It will also be important to examine other resistance genotypes to determine if there are a variety of resistance mechanisms in POC.

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Chapter 6

Summary and Discussion

The Port-Orford-cedar resistance breeding program has been selecting and evaluating POC resistant to *Phytophthora lateralis* using artificial and natural inoculation tests at the OSU greenhouse, Botany farm, and various outplanting sites since the 1980s. This study was the first to examine resistance mechanisms of POC to *Phytophthora lateralis* at the cellular level. The following main conclusions summarize our results.

- There was no statistically significant correlation between the stem- and root-dip inoculations of susceptible families, although both inoculation techniques identified the most resistant families. After isolation of 9 new isolates from the raised bed inoculation site, the new isolates and standard isolates were compared. The result showed that there were no differences in growth rate and zoospore production. Additionally, there was no evidence of change of aggressiveness of the isolates by stem dip inoculation on resistant and susceptible trees from the greenhouse and Botany farm. There were significant differences among the POC families. Three techniques to detect *P. lateralis* in susceptible and resistant seedlings were compared. The rate of detection by direct isolation of *P. lateralis* in susceptible and resistant seedlings was lower than detection by PCR or ELISA, especially in resistant seedlings. The rate of detection by PCR was similar to ELISA. Inoculation tests showed that *P. lateralis* is capable of entering through wounded foliage as well as unwounded foliage to cause initial infection on POC seedlings.

- There were differences in zoospore attraction on roots of some families. In general, most zoospores were aggregated to the cell division and elongation zones of the roots. 117490 RT seedlings accumulated significantly fewer zoospores than other seedling of other families, as measured by scanning electron microscopy, direct counts, and quantitative PCR.

- Cytological observations indicated that encystment, penetration, and colonization of *P. lateralis* were the same on both susceptible and resistant POCs but their frequencies in resistant seedlings were lower than in susceptible seedlings. During initial infection on susceptible seedlings, *P.*

lateralis zoospores encysted, germinated, and produced an appressorium. The infection hypha from the appressorium penetrated directly between epidermal cells and a swollen hypha ramified and branched for further infection in the root. Hyphae from unattached cysts on the root indirectly penetrated between epidermal cells, as well. Inter- and intracellular hyphae were commonly present in infected stem phloem, especially in sieve and parenchyma cells. Fewer hyphae were observed in resistant stems than in susceptible stems. Dead cells were found in both types of seedlings, however collapsed cells were found in only resistant stems after infection.

• Transmission electron micrographs showed the ultrastructure of both POC and *P. lateralis*. There were fewer cysts and hyphae in resistant roots than in susceptible roots. Also, there were fewer inter- and intracellular hyphae in resistant stems than in susceptible stems. Both resistant roots and stems had unique cytological and structural changes, such as cell disruption and collapse following increases in wall thickness, wall appositions, presence of osmiophilic granules, and encasement of hyphae and host cell walls by electron dense materials.

OSU has been cooperating with the US Forest Service and Bureau of Land Management to test candidate POCs for resistance by means of stem- and root-dip tests in the greenhouse, longer term mortality tests in the raised beds infested with *P. lateralis*, and continuing survival / mortality tests at the Botany farm under natural conditions. Chapter 2 reported four experiments designed to validate the POC resistance screening tests. The concern has been expressed that using the stem dip test for the first screening of resistant trees results in discarding some trees from the program that might prove resistant in root inoculation tests. The result showed that while overall there was a poor correlation between the stem- and root-dip tests, at least both tests identified the same trees as most resistant. Since 2000 the raised beds adjacent to the OSU East Greenhouses have been in continual use to test POCs. The question was raised whether there has been selection for a more aggressive race or

strain of *P. lateralis* over that period. There was no evidence by direct isolation and virulence tests of change in aggressiveness or virulence.

Dying resistant as well as susceptible seedlings in the outplanting sites as well as the raised bed was very disappointing. When three techniques were compared for detecting *P. lateralis* from those seedlings, we confirmed that the causal agent was *P. lateralis* as expected. In addition, the detection rate of *P. lateralis* in resistant seedlings was lower than in susceptible seedlings by all three methods, especially direct isolation. The detection rate by PCR and ELISA was greater than by direct isolation. However, PCR positive in some seedlings was not matched with the ELISA positive from the same seedlings. PCR results were more convincing than ELISA because PCR was done with *P. lateralis* primers although the primers would amplify *P. ramorum* as well. There was no evidence of *P. ramorum* at the test sites. In contrast, the commercial ELISA kit will react with other *Phytophthora* species and some *Pythium* species. That gives the potential for a false positive for the tests. Also, the positive reactions indicated by the color-based precipitates were scored by optical density. Therefore, inappropriate threshold values as well as possible sampling errors may have led to misinterpretation of the results.

Hiouchi, California was an outplanting site where many resistant seedlings as well as susceptible seedlings died after prolonged winter flooding of the site. The question was raised how *P. lateralis* infected POC seedlings in the field, especially at Hiouchi where seedlings were immersed in flood water. Localized necrotic lesions on the stems but not on roots was the first indication of another possible pathway for the infection. Although *P. lateralis* is known as a soil-borne pathogen, there was no

evidence of infection through the roots. I inoculated foliage of resistant and susceptible seedlings to see if foliar infection might bypass resistance normally expressed in the roots. The result showed evidence of *P. lateralis* entering through wounds and natural openings on foliage. Resulting lesions were much larger on foliage from susceptible seedlings than from resistant seedlings, however.

In conclusion, the chapter 2 provides evidence that the resistant screening tests that are used at OSU are valid for future work. Then, the next question was raised " What makes some POC families very resistant to *Phytophthora lateralis*? " The chapters 3, 4, and 5 describe differences in the responses of resistant and susceptible POCs to *P. lateralis*. In chapter 3, the attraction of zoospores to host roots by taxis is explored (Carlile 1983, Deacon et al. 1993, Morris et al. 1993, Tyler et al. 1996, 2000). In addition, natural and physical wounds stimulate zoospore aggregation (Widmer et al. 1998). Attraction of *P. lateralis* zoospores to POC roots has been demonstrated (Murray 1995, 1997). Studies on zoospore attraction of other *Phytophthora* species and their subsequent colonization have been done, comparing susceptible and resistant hosts (Beagle-Ristaino et al. 1983, Cahll et al 1989, Klarman et al. 1974, Widmer et al. 1998). In the present work, similar results indicating a difference in zoospore attraction between susceptible and resistant POC roots of at least some families were obtained by direct observation, direct counts, and quantification of *P. lateralis.* Also, roots of some resistant POC families attracted fewer zoospores than susceptible roots. These results suggest that zoospore attraction may be a factor to reduce the initial inoculum and further to decrease infection level on seedlings, especially resistant seedlings. However, use of a very low concentration of *P. lateralis* zoospores led to

poor statistical support for differences in zoospore attraction. Therefore, the work should be repeated with higher concentrations of *P. lateralis* zoospores in order to see significant differences.

Development of POC primers and a new extraction method for real-time quantitative PCR using SYBR green was quite a challenge but successful. However, the result obtained was unexpected and may have been confounded by factors such as natural wounds on collected roots and random sampling errors. CF1 family / clone is resistant to *P. lateralis* in most tests, but QPCR indicated that more zoospores encysted on CF1 roots than on roots of susceptible families. Natural wounds are common on delicate fine roots (Widmer et al. 1998) and provide sites for zoospore attack on both susceptible and resistant roots by emitting root exudates. The most active areas of root cell division (2-4 mm from root tip) and elongation (4-6 mm) attracted more zoospores than other regions as measured by QPCR as well as direct counting, and that was the expected response. The actively growing root zones may produce more nutrients and exudates. This result supports Murray and Hansen's work (1995, 1997). In addition, differences in zoospore number were observed in transverse sections of susceptible and resistant roots.

In Chapters 4 and 5, differences in response of susceptible and resistant roots were explored using histocytological methods. Light and transmission electron microscopy showed fewer encysted zoospores and less subsequent colonization on sections from resistant roots than on sections from susceptible roots. Germination of cysts on resistant roots was lower and slower than on susceptible roots. We believe that reduced initial zoospore attraction and slower germination and colonization of *P.*

lateralis on resistant roots may be one of the resistance mechanisms exhibited by these trees.

Differences in the responses of POC cells to infection were evident between resistant and susceptible roots. Collapsed POC cells, increases in wall thickness, appositions, and encasement of host cell walls and invading hyphae by electron dense materials were evidence of a general resistance response of resistant POC roots and stems. Histochemical staining for phenolic compounds including lignin failed to show differences between susceptible and resistant POCs. Observations at both the cellular and subcellular levels indicated the possible induction of biochemical substances for resistance. Further biochemical study will enhance the cytological evidence here in future work.

In conclusion, chapters 3, 4, and 5 provide evidence that 1) zoospore attraction may an important factor to reduce initial inoculum in some resistant POC families; 2) slowed growth of the pathogen in resistant POC families is a general resistance response to *P. lateralis*; 3) resistance responses such as increased wall thickness, wall appositions, encasement of host and hyphal cell walls by electron dense materials were only found in some resistant POC families. Those three responses may combine, at different times, places, and levels to increase resistance in some POC families or may act simultaneously to make POC very resistant in trees like 117490.

Because parent tree CF1 segregates susceptible and resistant progeny at either a 3:1 or 1:1 ratio depending on the parent tree cross, CF1 resistance appears to be variable depending on the particular seedling chosen for testing. CF1 seedlings that

were used in stem- and root-dip inoculation tests, stem inoculations for the virulence of new isolates from the raised bed, and infection pathway on foliage indicated that CF1 was more susceptible than suggested by earlier standardized tests of resistance (Chapter 2). Also, CF1 seedling roots attracted more zoospores as measured by direct counts and QPCR (Chapter 3) although direct observation of root surfaces by means of SEM showed fewer zoospores on the roots than on susceptible roots. However, cytological evidence indicated that CF1 seedlings had general resistant responses at the cellular level to *P. lateralis* including fewer hyphae of *P. lateralis* and collapsed cells of POC. CF1 was the only family where hyphae of *P. lateralis* were found in xylem. This variable evidence may be the result of several factors. Use of open pollinated seedling families in some cases may have resulted in selection of susceptible individuals. Unexpected results were also obtained with rooted cuttings of CF1, however. It may be that this tree expresses different resistance mechanisms than other resistant parents such as 510015 and 117490. Our hypotheses could not consistently explain the observed resistance of CF1.

In spite of that, the research presented in this dissertation provided important information in support of the resistance screening protocols for POC. The comparison of three detection methods for *P. lateralis* will be useful for the evaluation of field plantings when visual symptoms are inadequate, and the reduced isolation success from seedlings from resistant families provides another indication of their resistance. In addition, this work contributed a fundamental study of differences in zoospore attraction on POC by direct observation, direct counts, development of POC primers, and real-time quantitative PCR with SYBR green. Finally, microscopic observations

documented histocytological and structural changes of POC that are associated with resistance.

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