AN ABSTRACT OF THE THESIS OF

Bryan R. Capitano for the degree of Master of Science in Botany and Plant Pathology presented on May 25, 1999. Title: The Infection and Colonization of Douglas-fir Needles by the Swiss Needle Cast Pathogen, *Phaeocryptopus gaeumannii* (Rhode) Petrak.

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Abstract approved: _____
Everett M. Hansen

Phaeocryptopus gaeumannii is a pathogenic fungus on Douglas-fir needles which has become a serious threat to timber production in the last decade along the Oregon coast. This research was undertaken to describe the general biology and pathology of *P. gaeumannii* on Douglas-fir needles and how environmental variables affect fungal development. The biology of *P. gaeumannii* was described using histopathology methods such as scanning electron microscopy, thin section light microscopy, and plastic impressions of needle surfaces. Needle penetration occurred through stomata as ascospore germ tubes differentiated into appressoria in the outer stomatal chamber. Penetration pegs grew between the guard cells and into the needle mesophyll. Internal hyphae were observed only in intercellular spaces but were often well attached to host cells. Epiphytic growth of *P. gaeumannii* on needles was extensive and persisted throughout summer, fall, winter, and spring months. These hyphae continued to produce appressoria and penetrate stomata over time. Hyphal incidence on needle surfaces and inside needles was greater at high disease sites compared to low disease sites. No asexual stage was observed, however, new strands of hyphae grew from pseudothecial initials as these emerged from stomata in fall and winter months. These hyphae also persisted on needle surfaces.

Using temperature gradient plates, ascospores germinated and grew over a range of temperatures from 14°C to 30°C. Optimum germination and growth occurred at 18°C and 22°C respectively. Dry conditions for more than 24 hours significantly reduced both germination and growth of ascospores. Using inoculated seedlings, successful infection of needles occurred after 24 hours of exposure to moisture in a greenhouse mist chamber. As moisture periods increased, the overall colonization of needles by *P. gaeumannii* hyphae also increased.

The Infection and Colonization of Douglas-fir Needles by the Swiss Needle Cast Pathogen, *Phaeocryptopus gaeumannii* (Rhode) Petrak

by

Bryan R. Capitano

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented May 25, 1999
Commencement June 2000

Master of Science thesis of Bryan R. Capitano presented on May 25, 1999.

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ACKNOWLEDGEMENTS

Several individuals deserve a heartfelt thanks for their positive impact and guidance during my M.S. research. First, and foremost, I thank my major professor Everett Hansen for his patience, expert guidance, and gracious support. His leadership and training became the building blocks which helped me understand the foundations of scientific research. Additionally, I thank Dr. Jeff Stone for his encouragement, enthusiasm, active involvement, and support during my program. I must have worn him out with endless questions to which he was usually eager to answer. Thanks also to Everett and Jeff for reviewing the many drafts of this manuscript. Without their help, constructive criticisms and insights, I might still be working on it! Dr. George Carroll also deserves a warm thank you for his support during my initial steps in fungal-plant biology. His laboratory was the turnkey which crystallized my interest.

Several individuals have provided important technical assistance, advice, and training. Thanks especially to Cathy Cook, Al Soldner, and Fred Rickson for the time spent helping me become proficient at various histological techniques.

Many of my co-workers deserve a great deal of thanks for their insights, discussions, general help, and laughter. Particular thanks to Lori Winton who has been the most helpful and generous person with her time, even when she had none to spare. Thanks, too, for all those trips to Allan Brothers! Much thanks to Pablo Rosso for the thought provoking discussions and brainstorming sessions while driving back and forth from Tillamook this last three years. Thanks also to Wendy Sutton, Dan Manter, Alan Kanaskie, and Mike McWilliams. Most importantly, thanks to my wife Jessica for sharing in house disaster duty, helping to keep me sane, and believing it could be done.

This work is dedicated to my grandmother, Sorena Thompson whose encouragement that I stay in school has obviously paid off.

CONTRIBUTION OF AUTHORS

Dr. Everett Hansen was involved in the initial stages of project design and was instrumental in obtaining field study plots and other equipment and resources. Both Dr. Hansen and Dr. Jeff Stone assisted in the interpretation of results and in editing several drafts of these manuscripts.

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Chapter 1. Introduction and Literature Review

INTRODUCTION

The infection and colonization of Douglas-fir needles by the ascomycete *Phaeocryptopus gaeumannii* (Rhode) Petrak results in a foliar disease known as Swiss needle cast. Diseased trees are characterized by having chlorotic needles, poor needle retention, and a loss in tree height and diameter growth. In severely affected plantations, trees may lose all but current year foliage, and tree height and diameter growth may be reduced by fifty percent or more. In the last 10-15 years, this disease has become a serious threat to Douglas-fir plantations along the Oregon coast. However, very little is known about the histopathology of *P. gaeumannii* on and within Douglas-fir needles or how environmental factors affect disease development. This research was undertaken to address these issues. Such information would be useful primarily for understanding the general biology and pathology of *P. gaeumannii*, and to strengthen our understanding of the relationship between host, pathogen, and environment. This knowledge may help explain the current epidemic along the Oregon coast, and may illuminate possible control tactics for Swiss needle cast. More generally, this research may find broader application in our general understanding of foliar pathogens on conifer trees.

Swiss needle cast was first discovered around 1925 in Switzerland in 20-year-old Douglas-fir plantations. From Switzerland, the disease spread over a 15-year period throughout Germany, Denmark, Great Britain, and Ireland (Boyce, 1940; Liese, 1938; Rhode, 1937; Wilson and Waldie, 1928). As a result of severe defoliation and growth losses, Douglas-fir planting efforts had nearly stopped in Europe, except in mixed stands in a few less severely affected areas (Boyce, 1940). By 1940, Swiss needle cast had been discovered in six states in the eastern U.S., including Connecticut, Rhode Island, Massachusetts, Vermont, New Hampshire, and Maine (Boyce, 1940). Surveys of western North America in 1938 found that *P.*

gaeumannii was widely distributed throughout British Columbia, Washington, Oregon, and California, however, it appeared harmless compared to its effects in Europe and the eastern U.S. (Meinecke, 1939). Later examination of herbarium specimens by Boyce (1940) showed that *P. gaeumannii* was present in Oregon at least as early as 1916. Because of the wide distribution of *P. gaeumannii* in the natural range of Douglas-fir, and its relatively harmless effects, it was considered native to the Pacific Northwest.

Other locations where Douglas-fir has been planted as an exotic have also experienced injury related to Swiss needle cast. In 1959, Swiss needle cast was discovered on the North Island of New Zealand, and after 14 years had spread throughout most Douglas-fir plantations on both the North and South Islands, with moderate to severe symptoms showing in several infected areas (Hood and Kershaw, 1975). Swiss needle cast was also reported on Christmas trees in Michigan and Wisconsin in 1970 (Ford and Morton, 1971; Morton and Patton, 1970). In the mid 1970's, Christmas tree growers in the Pacific Northwest also reported damage due to *P. gaeumannii* (Hadfield and Douglass, 1982; Michaels and Chastagner, 1984)

In the last 15-20 years, investigators began noticing forest plantations affected by Swiss needle cast along the Oregon coast, especially near Tillamook. In the early 1980's, only a few plantations appeared unhealthy, however, the distribution and severity of the disease appears to have increased in the last 10 years (Winton, Capitano, Rosso, Sutton, Stone, and Hansen, 1998). In 1996, the Oregon Department of Forestry mapped approximately 130,000 acres (see Figure 2.1) where the disease was present and causing growth losses estimated at about $6 million per year (Kanaskie, pers. comm.). The current distribution of severe disease extends between Cloverdale and Nehalem, approximately 15 miles inland, and at elevations up to about 1500 feet. The heaviest concentrations of disease are still located in forest plantations near Tillamook, Oregon. This coastal strip region was historically dominated by Sitka spruce, western hemlock, and red alder, with Douglas-fir as a minor component. Since intensive logging operations began along the coast in the early to middle 1900's, this original mixture of species has gradually been replaced with young Douglas-fir plantations, due to superior growth and high market value. The area of severe disease corresponds roughly to what is considered the fog belt along the Oregon coast, and is characterized by a moderate climate with mild winters and relatively cool summers. Frequent

precipitation such as rainfall, fog, and heavy dew provide conditions favorable for infection and colonization of needles. These two factors—both the increase in Douglas-fir within the coastal fog belt, and a potentially favorable environment for disease—may explain the recent epidemic. Several other hypotheses have been suggested, including: improper or poorly adapted seed sources for replanting, poor soil fertility, and a new more virulent strain of *P. gaeumannii*. Research is underway to examine these possibilities.

Very little is known about the infection process of *P. gaeumannii* on and within Douglas-fir needles, or how the environment affects fungal development. This information may be valuable for understanding the general biology and pathology of *P. gaeumannii*, and may help explain the current Swiss needle cast epidemic. The objectives of this research were to describe the infection biology of *P. gaeumannii* and to evaluate whether environmental factors may, in part, account for the recent Swiss needle cast epidemic. Following this introduction is a general literature review describing the infection processes of several common pathogens on conifer needles as well as environmental variables which affect foliar pathogens. These examples will serve as a model for comparison to *P. gaeumannii*. The current state of knowledge about the infection biology of *P. gaeumannii* is also reviewed. The second two chapters are written in manuscript format each with their own introduction, discussion, and literature cited. Chapter two describes the processes of ascospore germination on needle surfaces, the mode of infection, the development of hyphae on and within needles, and the reproductive stages of *P. gaeumannii*. Standard histological methods were used to explore fungal development on needles including thin section light microscopy, scanning electron microscopy, and plastic impressions of needle surfaces. Chapter three describes the effects of temperature, moisture, and drying periods on *P. gaeumannii* ascospores, as well as the moisture requirements for successful infection of needles. The final chapter summarizes the overall thesis conclusions and is followed by a comprehensive literature cited.

LITERATURE REVIEW

Infection Processes of Other Conifer Needle Pathogens

Studies of the infection of host leaf tissues by other pathogenic foliar fungi show that discreet stages define the period between spore deposition and eventual colonization of host tissues by fungal hyphae (Emmett and Parbery, 1975; Howard, 1997; Mendgen and Deising, 1993; Mendgen, Hahn, and Deising, 1996). After impacting leaf surfaces, spores usually adhere strongly to host surfaces with mucilaginous substances (Epstein and Nicholson, 1997). Typically germination is either by direct formation of an appressorium or by means of a germ tube which differentiates into an appressorium at the site of infection (Mendgen, Hahn, and Deising, 1996). Germ tube growth toward infection sites, and appressorium formation, are often triggered by physical or chemical cues (Staples and Hoch, 1997). Appressoria, as defined by Howard (1997), are penetration peg bearing structures differentiating directly from a germ tube and preceding penetration into host tissue. Penetration pegs can penetrate host tissue either directly through the cuticle and epidermal cells, or between the guard cells of a stoma. Internal colonization may be inter- and intra-cellular with fungal hyphae in epidermal, hypodermal, parenchyma, vascular, and resin canal cells.

Patton and colleagues (1984) described the infection process of *Gremmeniella abietina* on Red and Scots pines. In artificial inoculation experiments, they showed that conidia germinated on needle bracts and entered the stomatal chambers on these bracts. The germ tubes penetrated between the guard cells without prior differentiation of an appressorium and penetration peg. Germ tubes then grew into the sub-stomatal cavity and immediately began colonizing the internal parenchyma tissue. Fungal hyphae were observed inside parenchyma and phloem cells. Epiphytic growth of the fungus on bract surfaces was reported to occur, however, the extent or importance of epiphytic growth was not described.

Cronartium ribicola, the cause of white pine blister rust, also penetrates needle stomata in five-needle pines. Patton and Johnson (1970) showed that basidiospores germinated and grew toward stomata. Germ tubes grew into the outer stomatal chamber, passed between the guard cells, and formed a vesicle in the sub-stomatal chamber. No appressoria or penetration pegs differentiated from germ tubes before

penetration of the stoma. Fungal hyphae were observed connecting vesicles in the sub-stomatal chamber to extensive intercellular mycelium. Haustoria were formed inside mesophyll cells and after tissue began to break down, vegetative hyphae began to invade the cells.

Dothistroma pini, red-band needle blight of various pines, also penetrates through the stoma. Several workers have observed that germ tubes from *D. pini* conidia are directed toward the stoma (Peterson, 1969; Ivory, 1972; Peterson and Walla, 1978). Germ tubes were capable of changing direction and curving toward the stoma. Additionally, germ tubes often developed on the side of the conidium nearest a stoma. Germ tubes did not always penetrate stomata but sometimes grew over the top of the stomatal pit (Ivory, 1972). These observations suggested that chemical signals from stomata influenced germ tube growth (Ivory, 1972). Multiple entries into the same stoma by a single conidium were common (Peterson and Walla, 1978). Differentiation of penetration structures in the outer stomatal chamber was variable. Occasionally the germ tube entered the stomatal pit and passed between the guard cells without differentiating into any penetration structure (Peterson and Walla, 1978). Sometimes germ tubes differentiated into appressoria (Gadgil, 1967), but more often germ tubes were swollen or coiled inside the stomatal pit (Ivory, 1972; Peterson and Walla, 1978). Penetration pegs were observed beneath appressoria and grew between the guard cells into the sub-stomatal cavity. These subsequently branched into the mesophyll as intra- and inter-cellular hyphae. Gadgil (1967) noted that mycelial growth did not take place on needle surfaces for long periods, and that surface growth disappeared soon after formation of appressoria.

Brown spot disease, caused by *Scirrhia acicola*, a close relative of *D. pini*, also infects pine needles through stomata. Work by Patton and Spear (1978) showed that germ tubes from *S. acicola* grew closely appressed to host epidermal cells. Often germ tubes displayed an obvious attraction to the stoma (Parris and Killebrew, 1969; Setliff and Patton, 1974; Patton and Spear, 1978). Germ tubes in the outer stomatal chamber sometimes differentiated into appressoria similar to *D. pini*. These appeared knobby, convoluted, melanized, and thick walled. Equally often, germ tubes entered the stomatal chamber and passed between guard cells without differentiation into appressoria. Germ tubes as long as 280 μm were recorded on needle surfaces, however, most were less than 100 μm (Patton and Spear, 1978). Penetration

pegs were observed between the guard cells, allowing penetration hyphae into the sub-stomatal chamber. Hyphae colonized both inter- and intra-cellular regions of the mesophyll (Setliff and Patton, 1974).

Direct penetration of needle epidermal cells has been documented for a variety of conifer pathogens. Basidiospores of *Cronartium quercuum* f. sp. *fusiforme* were observed penetrating epidermal cells on needles of Slash pine (Miller, Patton, and Powers, 1980). Germ tubes from basidiospores grew a short distance and terminated in an appressorium. Penetration pegs were formed beneath the appressorium and penetrated the underlying cuticle and epidermal cell, producing an oblong vesicle inside the infected cells. From the vesicle, a hypha penetrated the cell wall and developed both intercellular mycelium in the needle mesophyll as well as haustoria in surrounding cells.

Several species of *Lophodermium* are known for their ability to penetrate pine needles directly. *L. pinastri, L. conigenum,* and *L. seditiosum* infection processes on *Pinus sylvestris* were reviewed by Diwani and Millar (1986). Ascospore germ tubes sometimes grew a short distance before terminating in an appressorium. Spores could also germinate by direct formation of an appressorium. Usually the appressorium wall began to thicken and become melanized after about one week. By four weeks, all three *Lophodermium* species penetrated the cuticle and epidermal cells directly via a fine penetration peg produced beneath the appressorium. *L. seditiosum* colonized host tissue most abundantly, producing hyphae in epidermal cells surrounding the initial infection. After several weeks large numbers of epidermal, hypodermal, and mesophyll cells were colonized.

Rhabdocline diseases on Douglas-fir also penetrate needles directly. A single work by Farris (1967) showed that *R. pseudotsugae* conidia germinated directly into an appressorium on Douglas-fir needles. A fine penetration peg from the appressorium penetrated the cuticle and the underlying epidermal cell. Research by Stone (1988) revealed similar processes with the endophyte *Rhabdocline parkeri* on Douglas-fir needles. After deposition, conidia had attached to the needle surface with a mucilaginous substance. Conidia became two celled after germination and one cell developed a lateral appressorium. A fine penetration hypha was inserted into the host epidermal cell and the invading hypha enlarged to fill this cell. No further colonization of adjacent host tissue took place until natural needle senescence.

Environmental Variables Affecting Foliar Pathogens

Numerous reports have been published describing environmental conditions which favor fungal germination and growth on plant surfaces (Dickinson, 1976; Dickinson, 1986; Diem, 1971; Dix and Webster, 1995; Hudson, 1971; Park, 1982). Leaf surfaces, in particular, are very inhospitable environments due to rapid fluctuations in environmental conditions. Factors such as competition and antagonism, nutrient availability, solar radiation, wind, temperature, humidity, and leaf surface moisture all play a critical role in the ability of fungi to colonize and infect host leaves (Agrios, 1988). Of these factors, temperature and leaf moisture have been described as most important during initial colonization (Dix and Webster, 1995). Moisture and temperature regulate several processes of fungal development including spore formation, release, longevity, and particularly germination on and penetration into host tissues (Agrios, 1988).

Temperature and moisture requirements have been described for several important conifer needle pathogens. In studies on *Dothistroma pini*, for example, conidia were capable of withstanding 24 hours of desiccation and still germinating (Peterson and Walla, 1978). After germination, however, four hours of desiccation was sufficient to stop further development of germ tubes. In this same study, conidial germination and germ tube elongation were greatest at 24°C. In an earlier study, Peterson (1966) found that conidia could germinate and grow at temperatures ranging between 12-28°C. Peterson (1969) observed a correlation between cloudy, high-humidity weather and abundant conidial germination of *D. pini* on Ponderosa pine needles.

Van Arsdel (1967) determined that successful infection of pine seedlings by *Cronartium ribicola* required at least 48 hours of needle surface moisture and that infection occurred best at 16°C. Hansen and Patton (1977) also showed that the amount and distribution of water on needle surfaces was critical to infection. Dew formation on needles allowed maximum germ tube growth, however spores were often washed from needle surfaces when dew formation was too great. Moderate levels of dew formation promoted more uniform deposition of spores, more normal germ tube development, and higher levels of infection. Hansen and Patton (1977) also showed that temperature fluctuations reduced infection levels

while temperature shocks (short periods of temperature increases) did not significantly affect infection success but did stimulate the differentiation of vesicles.

Many other conifer needle diseases such as *Rhabdocline pseudotsugae*, *Scirrhia acicola*, *Cyclaneusma minus*, and *Lophodermium* spp. are similarly affected by needle wetness and moderate temperatures (Chastagner, Byther, and Riley, 1990; Kais, 1975; Wenner and Merrill, 1990; Merrill and Kistler, 1976)

Swiss Needle Cast Biology and Epidemiology

Several reports have been published on the epidemiology and basic life history of Swiss needle cast (Anonymous, 1939; Boyce, 1940; Chastagner and Byther, 1983; Chen, 1972; Michaels and Chastagner, 1984; Ford and Morton, 1971; Hood, 1983; Hood and Kershaw, 1975; Rhode, 1937). Swiss needle cast is a monocyclic disease which produces ascospores and infects needles in spring and summer months. Asci mature during March in western Oregon and Washington. Sporulation begins in late March, coinciding with host bud break and needle elongation, and continues throughout summer months. The greatest period of susceptibility of host tissue occurs during needle elongation. Most reports agree that current season needles are most susceptible, however, second year needles can also become infected. In heavily infected trees, current year needles may be cast during the first year of growth. However, less severely infected trees may retain several years of needles, and the shedding of any given year's leaves may extend over several seasons.

Entry through stomata has been suggested as the mode of infection for *P. gaeumannii* (Chen, 1972; Fatuga, 1978). Using paraffin embedding and sectioning, Fatuga (1978) observed fungal cells in stomata in winter months and concluded that these cells were ascospores infecting through the stoma. With similar techniques, Chen (1972) observed hyphae on needle surfaces connected to hyphae in stomatal chambers and concluded that *P. gaeumannii* infected needles through stomata. Because very few histological details were reported, it was unclear whether the fungal hyphae observed were entering or emerging from stomata. No detailed observations were reported of ascospore germination on needles, germ tube growth, appressorium differentiation, and penetration into needles.

Hyphae of *P. gaeumannii* are known to colonize the mesophyll of Douglas-fir needles. Using culture sampling, Rhode (1937) isolated *P. gaeumannii* from the mesophyll of Douglas-fir needles and found that older needles were colonized more heavily by *P. gaeumannii* hyphae. Using thin section light microscopy, Steiner (1937) documented the presence of hyphae in intercellular mesophyll spaces. However, no research has fully documented the distribution of hyphae in the mesophyll, palisade layer, or vascular cells, or whether hyphae penetrate cells inside Douglas-fir needles and produce absorptive structures similar to haustoria.

After a period of incubation inside needles, pseudothecia emerge from stomata in fall and winter months (Boyce, 1940; Stone and Carroll, 1986). These produce approximately 15 asci, each with 8 two-celled ascospores. Using spore trapping methods in Christmas tree plantations, Fatuga (1978) reported a second stage of spore release during fall months and hypothesized this to be a conidial stage for *P. gaeumannii.* Ultrastructural observations of developing pseudothecia by Stone and Carroll (1986) also suggested the possibility of an asexual stage. Stone and Carroll observed phialide-like cells in the substomatal chamber preceding the formation of pseudothecia. These cells showed laminar wall depositions characteristic of cells that successively produce and release conidia. To date, however, no anamorph has been described for *P. gaeumannii*. Stone and Carroll (1986) also noted vegetative hyphae of *P. gaeumannii* emerging from stomata prior to full development of pseudothecia. Their role in needle colonization and pathology remains undescribed.

Several reports have shown that spore release and infection are correlated with rainfall, high humidity, and needle wetness. In-vitro experiments on sporulation, for example, have demonstrated a direct relationship between needle wetness and release of ascospores from pseudothecia (Michaels and Chastagner, 1984). Surveys in southern British Columbia and the Olympic peninsula demonstrated positive correlations between regional infection levels and mean rainfall during May – July, and infection was higher in the coastal and interior rainbelts and comparatively less in the drier intermountain region east of the coast range (Hood, 1982). Ford and Morton (1971) also found a positive relationship between rainfall and spore release during May-July in Michigan plantations.

Chapter 2. The Histopathology of *Phaeocryptopus gaeumannii* On and Within Douglas-fir Needles

INTRODUCTION

The infection and colonization of Douglas-fir needles by *Phaeocryptopus gaeumannii* (Rhode) Petrak causes a disease known as Swiss needle cast. Within the last decade, this disease has become a serious problem in Douglas-fir plantations along the Oregon coast. The greatest concentrations of disease are around Tillamook, Oregon, however severely diseased plantations can also be found between Cloverdale and Nehalem (Figure 2.1). In severely affected plantations, trees may lose all but current year foliage, with consequent reductions in tree height and diameter growth.

Several reports have been published on the epidemiology of *P. gaeumannii* (Anonymous, 1939; Boyce, 1940; Chastagner and Byther, 1983; Michaels and Chastagner, 1984; Ford and Morton, 1971; Hood, 1983; Hood and Kershaw, 1975; Rhode, 1937). Very little is known, however, about the germination of ascospores on the needle surface, the mode of penetration, the development of hyphae on and within needles, and the reproductive stages of *P. gaeumannii.* This research was undertaken to address these issues. Such information would be useful not only for understanding the general biology and pathology of *P. gaeumannii*, but also to help explain the current epidemic along the Oregon coast. Understanding the biology may also illuminate possible control tactics for Swiss needle cast. More generally, this research may find broader application in our general understanding of foliar pathogens on conifer trees.

Entry through stomata has been suggested as the mode of infection for *P. gaeumannii* (Chen, 1972; Fatuga, 1978), however, no significant histological or microscopic observations have been published. Both Chen (1972) and Fatuga (1978) observed fungal cells in stomata in winter months and suggested that these cells were ascospores infecting through the stoma. However, because very few details were reported, it was unclear whether the fungal cells in stomata were infection related structures or immature pseudothecia emerging from stomata.

Using culture sampling, Rhode (1937) isolated *P. gaeumannii* from the mesophyll of Douglas-fir needles and showed that older needles were colonized more heavily by *P. gaeumannii* hyphae. Using thin section light microscopy, Steiner (1937) observed *P. gaeumannii* occupying intercellular portions of the needle mesophyll. Except for these few observations, no research has fully documented the distribution of hyphae in the mesophyll, palisade layer, or vascular cells, or whether hyphae penetrate cells inside Douglas-fir needles. Additionally, the presence and importance of vegetative hyphae on the surface of needles has received very little attention (see Stone and Carroll, 1986). Their role in needle colonization and pathology has not been fully described.

Scant evidence of an asexual stage was obtained in spore trapping experiments in Christmas tree farms (Fatuga, 1978). These studies noted a second stage of spore release during fall months and hypothesized this to be a conidial stage for *P. gaeumannii*. This was the only observation of a fall spore release, however, and no recent observations have substantiated its existence. Ultrastructural observations of developing pseudothecia by Stone and Carroll (1986) also suggested the possibility of an asexual stage. In this work, Stone and Carroll observed phialide-like cells preceding the formation of pseudothecia in the substomatal chamber. These cells showed laminar wall depositions characteristic of cells that successively produce and release conidia. To date, however, no anamorph has been described for *P. gaeumannii*.

The objectives of this research were to determine the general behavior and colonization patterns of *P. gaeumannii* on and within needles, to explore the possibility of an asexual stage, and to begin answering the question of how *P. gaeumannii* obtains nutrition from needles. Ascospore germination and the growth of hyphae on needle surfaces were studied using plastic impressions of needles. One question of interest concerned how surface colonization contributed to the overall pathology of needles. The mode of penetration and the anatomical details of penetration were studied with thin section light microscopy. Internal colonization of needles was studied using scanning electron microscopy to determine the extent of colonization over time, the distribution of hyphae within needles, and whether general growth habits suggested how *P. gaeumannii* obtains nutrition from needles. Pseudothecium development was monitored with both plastic impressions and scanning electron microcsopy to determine whether *P. gaeumannii* produces asexual propagules as precursors to pseudothecium formation. In addition to these

developmental studies, the amount of hyphae on and within needles was compared between high and low disease sites. These measurements were used to determine how the amount of hyphal colonization relates to disease severity along the Oregon coast.

MATERIALS & METHODS

Observations of the infection and colonization of Douglas-fir needles by *P. gaeumannii* were made on both naturally infected trees in field plantations and on artificially inoculated potted seedlings. Several histological methods were used. Plastic surface impressions of needles were obtained with nail polish for examination of ascospores, hyphae, and pseudothecia on the needle surface. Plastic embedding and sectioning for the light microscope were used to verify the mode of penetration into the needle. Scanning electron microscopy was used to observe the growth patterns of *P. gaeumannii* inside needle tissue.

Plant and Fungal Materials

Douglas-fir needles from naturally infected trees were collected from field plantations between Cloverdale and the Nehalem River along the Oregon coast (Figure 2.1). Six collection sites were used in the study: Juno Hill, Upper Stone Rd., Salal, Limestone, North Fork, and Acey Creek (Table 2.1). Throughout this report sites will be abbreviated as follows: Juno, Upper, Salal, Lime, N.Fork, and Acey. Sites were paired based on location and disease severity. Salal and Lime comprise the southernmost pair of moderate and low disease sites, near Cloverdale. Juno and Upper, near Tillamook, comprise the middle pair of high and low disease sites. N.Fork and Acey comprise the third pair of high and low disease sites near Nehalem. As shown in Figure 2.1, plot disease levels were in rough congruence with the aerial survey results from the Oregon Department of Forestry's 1996 survey. Estimates of disease severity were based on several factors including: foliage color, needle retention, crown transparency and density, height and diameter growth, and counts of pseudothecia.

Figure 2.1 Location of field collection sites in Tillamook County, Oregon. Plot locations are superimposed on a Swiss needle cast aerial survey from Oregon Department of Forestry, 1997. Colored squares represent plot locations. Red square = high disease. Yellow square = medium disease. Green square = low disease. Light yellow aerial survey polygons = high-moderate disease. Light green polygons =low disease. HWY 101 ( ); Towns (*); Bar = 4 miles.

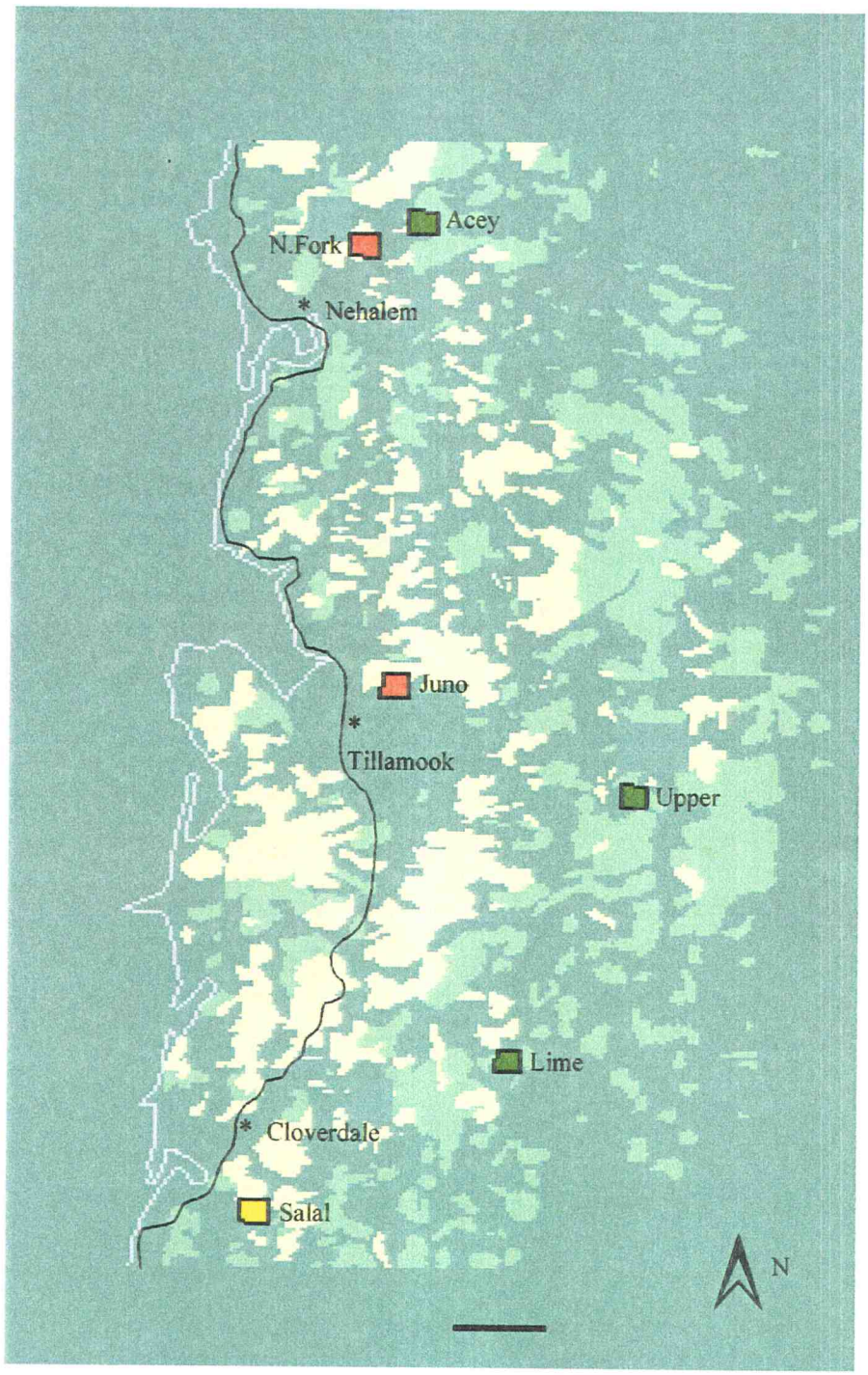


Table 2.1 Location and characteristics of Douglas-fir field plantations in Tillamook County, Oregon, where needles were collected.

SITE	DISEASE SEVERITY	TOWNSHIP/RANGE	ELEVATION (FT)	MILES TO OCEAN	AGE	ASPECT
JUNO	Severe	T1S R9W Sec17	380	2.25	14	NE
UPPER	Healthy	T1S R8W Sec35	1700	14.5	14	N
N. FORK	Severe	T3N R10W Sec12	160	4.75	10	SW
ACEY	Healthy	T3N R9W Sec5	670	8	10	E
SALAL	Moderate	T5S R10W Sec10	370	4	9	NW
LIME	Healthy	T4S R8W Sec6	890	12.25	9	N

At each site, a permanent weather station tree was designated from which all subsequent collections were obtained. From the north side of each tree at each site, current season needles were sampled at head height (approximately 5.5ft.) from a secondary lateral branch. Current season 1996 needles were collected in June 1996, July 1996, November 1996, December 1996, January 1997, and February 1997. Additionally, current season 1997 needles were collected in September 1997 and March 1998 to supplement observations from the first year. After using all needles from one lateral branch at a field site, a new lateral was chosen from the same main branch.

Potted Seedlings and Inoculation Techniques

Two-year-old potted Douglas-fir seedlings were used for artificial inoculation and subsequent histological examination. These seedlings were obtained as bareroot stock from DL Phipps nursery in Elkton, OR, an Oregon Department of Forestry nursery. The seed source was from Tillamook.

Both attached and detached needles from potted seedlings were inoculated with *P. gaeumannii* ascospores. Spore inoculum for attached needle inoculations was obtained by incubating diseased branchlets, approximately 4 inches in length, inside closed glass test tubes with 10 ml dH_2O. Test tubes were incubated overnight and then agitated vigorously for 60 seconds to release spores into the water suspension. Spore concentrations of 1×10^5 spores / ml were sprayed onto seedling foliage using an aerosol spray gun. Foliage was then enclosed in polyethylene bags for approximately 24 hours.

Afterwards, seedlings were kept in a mist chamber to insure free moisture on leaf surfaces during the course of monitoring.

Inoculation of detached needles was performed by suspending diseased needles with mature pseudothecia above healthy needles inside a petri plate. Needles with mature pseudothecia were attached to the petri plate lid with Vaseline. The petri plate enclosure was lined with moist filter paper to ensure high humidity. Petri plates were then incubated inside an enclosed humidity chamber for one hour.

Seedlings were also inoculated with macerated fungal mycelium. *P. gaeumannii* mycelium was obtained from single spore isolates from Juno needles. This procedure involved suspending needles with mature pseudothecia over an agar surface for approximately one hour. Individual spores were then removed from the agar surface and transferred to potato dextrose malt yeast extract agar (PDMYA: 39grams potato dextrose agar, 1g yeast extract, 1g malt extract, and 1L dH_20). After one month of growth on solid agar, a 5mm square section of *P. gaeumannii* and agar was transferred to 500ml liquid culture broth in a 1L flask. Fungal cultures were grown in liquid broth for two months at 19°C.

For preparation of inoculum, fungal cultures were filtered and rinsed using a vacuum filtration device. The resulting mycelia were then suspended in distilled water at a ratio of 20 grams of *P. gaeumannii* mycelium to 1L dH_2O. This mixture was macerated for 60 seconds with a tissue homogenizer. The final macerated mycelium suspension was sprayed onto the foliage of potted seedlings until runoff using an aerosol spray gun. (For similar mycelium inoculation techniques, see Hood, 1977.)

Plastic Impressions

Plastic impressions of needles were used to observe several stages of fungal development on needle surfaces. These included ascospore germination, germ tube development and differentiation, appressorium formation, and pseudothecium development. These processes were observed on both naturally infected trees collected from field plantations as well as on inoculated needles from potted seedlings.

For surface impressions, abaxial needle surfaces were painted with clear nail polish and allowed to dry for at least 1 hour. Afterward, plastic impressions were peeled from the needle surface. With practice,

suitable peels were obtained for microscopic examination. Following removal, ascospores and fungi on the undersurface of the impressions were stained by immersing the impression in several drops of trypan blue (Sigma) in lactoglycerol on a microscope slide (0.05% trypan blue and equal parts: lactic acid, dH_2O, and glycerol). Stained impressions were covered with a coverslip and sealed with clear nail polish. Bright field light microscopy was used to search for ascospores.

Plastic impressions were also used to monitor the frequency of hyphae on needle surfaces and the amount of vegetative hyphal growth over time. These measurements were used to compare surface colonization between high disease and low disease sites. From each collection time at each site, the presence or absence of hyphae on needle surfaces was estimated from 50 fields of view at 400X with standard bright field microscopy. These 50 observations were selected from 5 needles with 10 randomly chosen observations per needle. This frequency of occurrence of hyphae was expressed as a percent incidence from 50 fields of view. In each field of view where hyphae were present, mean lengths of hyphae were measured with a calibrated ocular micrometer and expressed as μm hyphae / mm^2 needle area. Data from Juno and Upper were used to compare the amount of hyphae between a high disease site and a low disease site. Questions for statistical analysis included: (1) whether the percent incidence of surface mycelium changed over time; and, (2) whether the mean lengths of hyphae, in fields of view where hyphae did occur, changed over time. Multiple regression routines were used to analyze changes over time and to compare slopes between sites.

Thin-Section Light Microscopy

While plastic impressions were used to examine germ tube development and appressorium formation, thin-section light microscopy (TLM) was used to study the structural details of penetration. TLM was also used to monitor the development of pseudothecia in stomata in fall months. Needles for TLM were cut into approximately 5mm segments and fixed in 3% glutaraldehyde in pH 7.4 phosphate buffer under vacuum overnight. Segments were then dehydrated in a graded ethanol series through 95% followed by infiltration with Historesin (Leica). Cross sections were cut from the embedded needles at a 15 μm thickness with a rotary microtome and steel knife. Sections were stained for 5 minutes in acid-

fuschin malachite green (Alexander, 1969), rinsed with dH_20, and mounted permanently in Permount (Fisher Scientific). Standard bright field light microscopy was used to search for examples of penetration and infection of the needle.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to study both internal colonization and the development of pseudothecia. Needles used for SEM observations were collected at Juno and Upper on December 1996, July 1997, November 1997, and May 1998. Additionally, SEM observations were made on needles from mycelium-inoculated potted seedlings. In preparation for SEM, needles were first cut into 5mm long segments. These were then cut into longitudinal sections or cross sections to expose the interior of the needle for viewing under the microscope. Segments were fixed in 3% glutaraldehyde in a vacuum chamber overnight, dehydrated in a graded ethanol series through 100%, and then critical point dried with a CPD 020 critical point drying machine. Dried needle segments were attached to aluminum SEM mounts with Duco household cement (ITW Brands). Samples were sputter coated with a mixture of argon gas and gold palladium. An AMRAY scanning electron microscope was used to search for examples of *P. gaeumannii*.

Culture Sampling

Internal fungi were cultured from mycelium-inoculated potted seedlings to verify the presence of *P. gaeumannii*. For culture sampling, needles were first surface sterilized with 95% ethanol for 1 minute, 50% Chlorox for 10 minutes, and a final rinse in 95% ethanol for 1 minute. Needles were then cut into 5mm long segments and plated onto potato dextrose malt yeast extract medium (PDMYA: 39 grams potato dextrose agar, 1.0g yeast extract, 1.0g malt extract, 1.0L dH_2O). Fungi were allowed to grow from the needles for 4 weeks. Afterwards, needle segments were scored for the presence of *P. gaeumannii* which was recognized by dense growths of darkly pigmented (charcoal colored), thick walled, frequently branched, slow growing mycelium.

RESULTS

Development of Ascospores and Hyphae on Needle Surfaces

Identification of *P. gaeumannii* on needle surfaces was based on several characters, taken in part from plastic impression observations, and also through observations of germinating ascospores on agar. Ascospores were hyaline, or sometimes lightly pigmented, and were approximately 5.5 x 13 µm (Figure 2.2). They were typical two-celled *Venturia*-like ascospores, with one cell slightly smaller in width and narrowing toward the apex. Hyphae were pigmented charcoal, or olive-brown. Their growth patterns were irregular, with frequent branching, curvatures, and enlarged regions. Hyphae on needle surfaces were often associated with ascospores or pseudothecia, thereby aiding identification. Hyphae of other fungi were likewise associated with spores or fruiting structures different from *P. gaeumannii*. Other hyphae were almost always more narrow, hyaline, less convoluted in growth form, and usually absorbed Trypan blue stain, thus appearing blue in color. Two common epiphytic fungi had similar dark pigmented hyphae. These included *Stomiopeltis*, and *Rasutoria*. These were distinguished from *P. gaeumannii* by their dense, interwoven growth which occupied roughly circular regions on the needle surface (*Stomiopeltis*), and their relatively larger fruiting bodies which were produced directly on needle surfaces instead of in stomata (*Rasutoria*).

Plastic impressions from over 450 needles were used to examine several stages of fungal development: ascospore germination, germ tube development, and development of vegetative hyphae. Ascospores were visible in plastic impressions from field collected needles between June 1996 and November 1996 (Table 2.2). In June 1996, needles from Upper, Lime, and Acey were too immature for plastic impressions. However, ascospore concentrations were highest in June for Juno, Salal, and N.Fork. Ascospore numbers decreased over time and no ascospores were observed on needles after November. With the exception of N.Fork and Acey, ascospore concentrations were greater on needles from high disease sites.

Figure 2.2 Ascospores (AS) of *P. gaeumannii* clumped together on abaxial surface of Douglas-fir needle. Stomata (S) are covered with wax and appear as depressions. SEM micrograph. Bar = 10 μm.

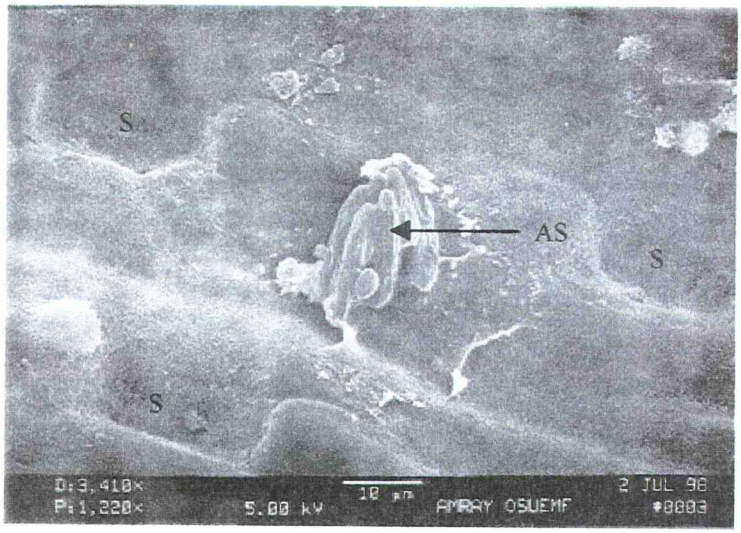


Figure 2.3 Ascospores (AS) and germ tubes (G) of *P. gaeumannii* on ascospore inoculated Douglas-fir needle. Note that one germ tube is usually longer than the other. S = stomata. Plastic impression. Bar = 20 μm.

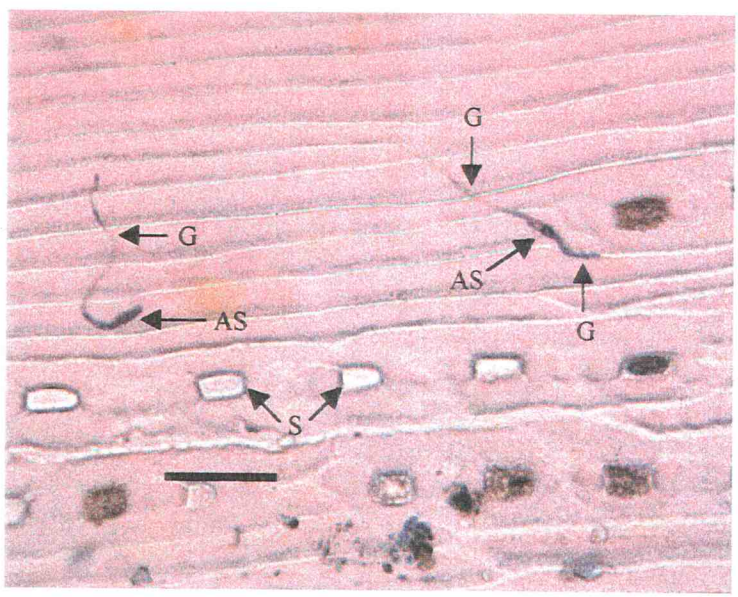


Table 2.2 Mean ascospore density on abaxial needle surfaces from six field sites in Tillamook County, OR. Sites paired based on location and disease severity.

Site:	26-June 96	28-July 96	6-Nov 96	12-Dec 96	6-Jan 97	5-Feb 97
Juno	0.65 ± 2.25*	0.53 ± 1.29	0 ± 0	ND	0 ± 0	0 ± 0
Upper	ND**	0.29 ± 0.89	0.12 ± 0.58	0 ± 0	ND	0 ± 0
Salal	0.24 ± 1.00	0.18 ± 1.25	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Lime	ND	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
N. Fork	0.12 ± 0.83	0.12 ± 0.58	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Acey	ND	0.24 ± 0.81	0 ± 0	0 ± 0	ND	0 ± 0

* Ascospores/ mm^2 ± standard deviation
**ND = Not determined due to immature needles or samples lost / destroyed.

No ascospores were found in over 40 plastic impressions of needles from spore-inoculated seedlings. Only one SEM micrograph from these seedlings showed ascospores on the needle surface. Shown in Figure 2.2, a group of six spores were clumped together on the abaxial surface of a needle. Because so few ascospores, or hyphae, were found on spore-inoculated seedlings, these seedlings were not used for further observations of fungal development on needle surfaces.

Ascospores typically developed germ tubes from both cells (Figure 2.3), however one germ tube was usually longer. Rarely, a third germ tube developed from the side of an ascospore. Germ tubes of between 10 to 20 μm were sometimes hyaline or only slightly pigmented, but could also be pigmented dark brown. Germ tube growth was often extensive and germ tubes longer than 20 μm were always darkly pigmented. Germ tubes and hyphae appeared to grow in random directions on the needle surface (Figures 2.4 and 2.5). There was no indication that needle topography, such as ridges or depressions, consistently influenced hyphal orientation. Hyphae were observed growing in the depressions between epidermal cells as well as across epidermal cells at various angles. In general, hyphae were closely appressed to host epidermal cells (Figure 2.5).

Figure 2.4 Hyphae (H) of *P. gaeumannii* growing on abaxial surface of Douglas-fir needle. S = stomata. Plastic impression. Bar = 20μm

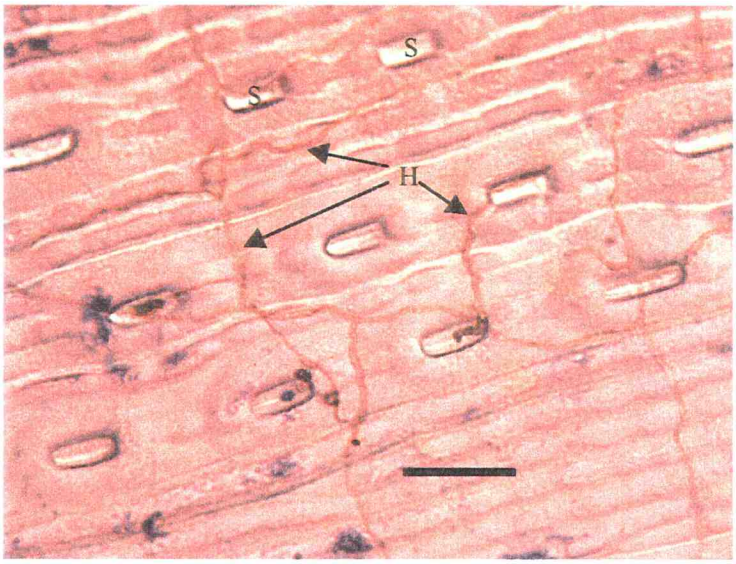
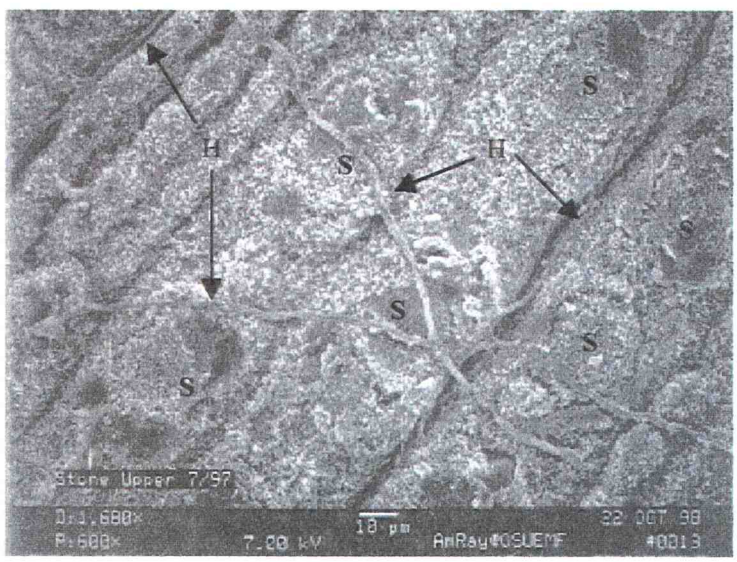


Figure 2.5 Hyphae (H) of *P. gaeumannii* growing over and around several wax covered stomata (S) on Douglas-fir needle. Note hyphae are closely appressed to epidermal cells. SEM micrograph. Bar = 10μm.



Epiphytic Growth

Following germination from ascospores, hyphae continued growing and persisted on needle surfaces over time (Tables 2.3-2.6). Plastic impressions were used to measure hyphal incidence and abundance, and to describe the characteristics of hyphal colonization over time. Because hyphae persisted on needles, the amounts reported for each collection time were cumulative and not measures of new growth occurring between collection times.

As shown in Table 2.3, the incidence of hyphae on current year needles increased through November or January during 1996-1997 at most sites. Surface hyphae were clumped early in the summer. In July 1996, when needles were approximately 2-3 months old, only 18% of fields of view contained hyphae (averaging values from all sites). By February 1997, the incidence of hyphae increased to an average of 64% from all sites. Hyphae generally appeared more evenly distributed on needle surfaces by February. Although most sites showed a greater incidence of hyphae in February compared to July, hyphal incidence did not always increase steadily, but showed some fluctuations over time.

With the exception of N.Fork and Acey, the incidence of surface hyphae was greater at high disease sites. For comparison, the incidence of hyphae at Juno and Upper are displayed in Figure 2.6. From regression analysis, Juno showed a significant increase over time ($p = .0038$). Although the increase in incidence at Juno appeared to level off near 100%, the data did not support an x^2 term for curvature ($p = .28$). In contrast to the increase at Juno, Upper failed to show an appreciable increase with time ($p = .79$). Using multiple regression to compare sites, Juno showed an estimated 37.8% greater incidence of hyphae over time compared to Upper ($p = .02$).

Within areas of the needle surface where hyphae occurred, the abundance of hyphae also increased over time (Table 2.4). (Because of frequent branching and extensive growth, the total lengths of surface hyphae were often not easily determined. Measurements of hyphae, therefore, were confined to fields of view at 400X and expressed as $\mu m / mm^2$). The length of hyphae in July averaged 414.4$\mu m / mm^2$ (average from all sites). Because hyphal growth was not very dense or overlapping in July, single fungal individuals could usually be identified. Hyphae could often be traced to ascospores in July, or to the needle's edge indicating the ascospore's origin on the upper surface of the needle. Growth from a

Table 2.3 Percent incidence of *P. gaeumannii* hyphae on current year 1996 needles from six field sites in Tillamook County, OR.

	26-Jun-96	28-Jul-96	6-Nov-96	12-Dec-96	6-Jan-97	5-Feb-97
Juno	14*	28	84	ND	88	90
Upper	ND**	20	28	32	ND	20
Salal	10	26	86	66	74	84
Lime	ND	0	24	42	58	38
N.Fork	0	10	42	30	42	76
Acey	ND	24	86	86	ND	78

*Percentage of microscope fields of view (400X) containing hyphae.
**ND = Not determined due to immature needles or samples lost / destroyed.

Table 2.4 Mean abundance of *P. gaeumannii* hyphae on current year 1996 needles from six field sites in Tillamook County, OR.

	26-Jun-96	28-Jul-96	6-Nov-96	12-Dec-96	6-Jan-97	5-Feb-97
Juno	272.1 ± 138.7*	336.2 ± 271.8	798.0 ± 662.4	ND	818.7 ± 613.5	1180.7 ± 1071.1
Upper	ND**	307.4 ± 223.3	497.5 ± 512.2	1783.7 ± 1325.9	ND	1656.8 ± 1741.1
Salal	261.2 ± 193.9	719.91 ± 402.2	1919.0 ± 1671.0	631.5 ± 645.1	1303.7 ± 798.9	1225.6 ± 901.5
Lime	ND	0.0 ± 0.0	725.5 ± 552.6	1252.8 ± 977.1	1086.4 ± 959.0	1058.7 ± 910.1
N.Fork	0 ± 0	587.7 ± 375.8	1256.7 ± 1011.8	954.0 ± 806.5	583.0 ± 594.1	620.0 ± 571.9
Acey	ND	535.1 ± 231.8	864.3 ± 940.5	2240.4 ± 1287.6	ND	1081.3 ± 769.8

*Hyphal abundance (um/mm2) ± standard deviation. Abundance measurements are averages only of areas on needle surface where hyphae were present; areas lacking hyphae were not included in mean amounts.
**ND = Not determined due to immature needles or samples lost / destroyed.

Table 2.5 Percent incidence of *P. gaeumannii* hyphae on current year 1997 needles from six field sites in Tillamook County, OR.

	1-Sep-97	25-Mar-98
Juno	44*	92
Upper	22	78
Salal	14	60
Lime	82	78
N.Fork	12	50
Acey	76	70

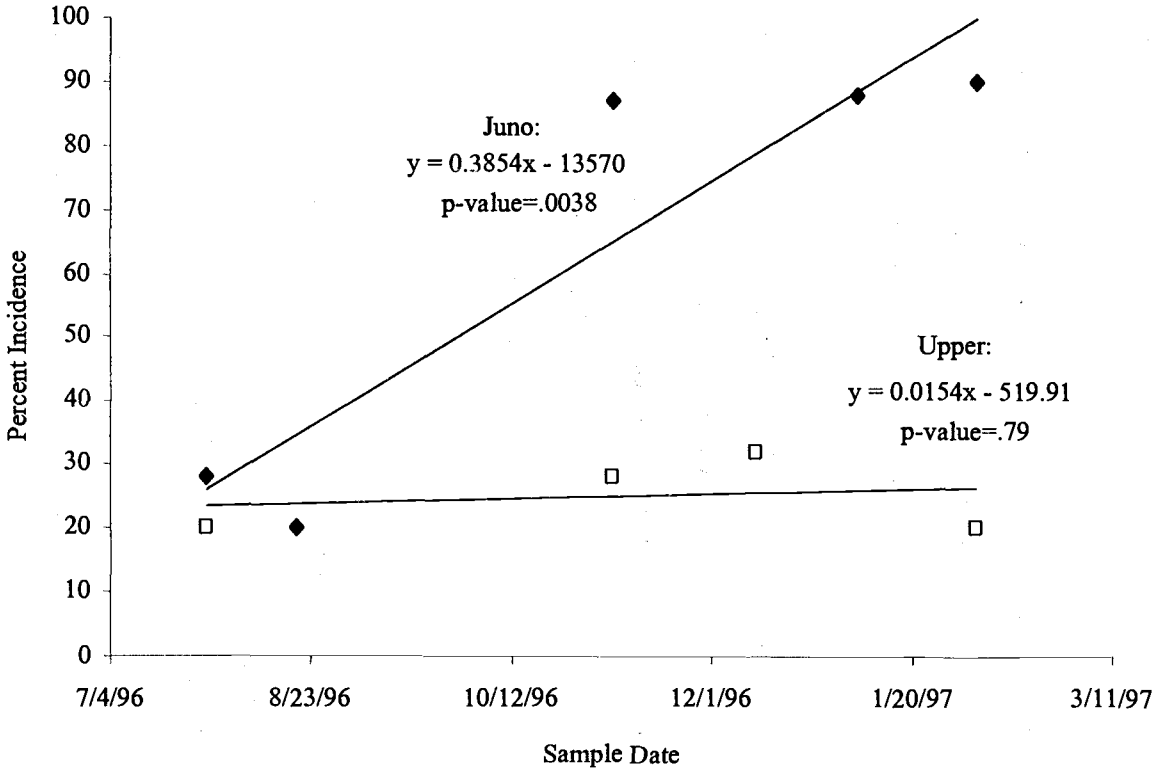
*Percentage of microscope fields of view (400X) containing hyphae.

Table 2.6 Mean abundance of *P. gaeumannii* hyphae on current year 1997 needles from six field sites in Tillamook County, OR.

	1-Sep-97	25-Mar-98
Juno	681.4 ± 467.1*	1163.4 ± 606.7
Upper	1728.8 ± 1415.3	968.3 ± 866.2
Salal	668.5 ± 468.8	1097.5 ± 940.1
Lime	1424.0 ± 993.8	2737.3 ± 1787.6
N.Fork	625.7 ± 573.5	624.7 ± 683.8
Acey	1267.2 ± 668.2	595.4 ± 560.0

*Hyphal abundance (um/mm2) ± standard deviation. Abundance measurements are averages only of areas on needle surface where hyphae were present; areas lacking hyphae were not included in mean amounts.

Figure 2.6 Incidence of epiphytic hyphae on current year Douglas-fir needles from July 1996 through February 1997 at Juno (◆) and Upper (□). Incidence measured as the percentage of microscope fields of view (400X) containing hyphae.



single ascospore often spread across several microscopic fields of view. Hyphal lengths of 3mm were observed in July, although accurate measures of total length were often difficult to determine because of frequent branching and curved growth. By February, the length of hyphae averaged 1137.2μm / mm^2 (average from all sites). Because of dense and often overlapping growth in winter months, single individuals were not possible to identify. However, individual hyphal strands could often be traced through several fields of view, and sometimes over large portions of the whole needle. Abundance of hyphae also fluctuated over time, similar to hyphal incidence.

While most sites showed an increase in abundance over time, the amounts of hyphae (in areas where hyphae were found) were not consistently different between high disease and low disease sites. For comparison (Figure 2.7), both Juno and Upper showed similar increases in abundance over time ($p = .0001$ and .0002 respectively). There were no significant differences, however, between the overall abundance of hyphae at Juno compared to Upper ($p = .44$).

Hyphae also persisted over time on current year needles collected during 1997-1998 (Tables 2.5 and 2.6). However, hyphae did not show a consistent increase between the sample collection dates. The percent incidence of hyphae increased over time for four out of six sites (Table 2.5). The length of hyphae increased between the two sample collection times for only three out of six sites. No consistent differences in percent incidence or abundance were present between high disease and low disease sites.

Needle Penetration.

Needle penetration occurred via stomata as germ tubes grew over a stoma and differentiated into an appressorium. Using plastic impressions of needles from field sites, over 1,050 appressoria were observed in stomata from needles collected in 1996-1997 and 1997-1998. On current year 1996 needles, the mean percentage of stomata with appressoria increased over time for all sites (Table 2.7), suggesting that penetration events also increased over time. (Because plastic impressions were impressions of surface features only, they were not capable of verifying actual penetration into needles.) Because appressoria persisted on needles over time, the amounts reported for each collection time were cumulative and not measures of new appressoria formed between collection times.

Figure 2.7 Mean abundance of epiphytic hyphae on current year Douglas-fir needles from July 1996 to February 1997 at Juno (Graph A) and Upper (Graph B). Abundance measurements are averages only of areas on needle surface where hyphae were present; areas lacking hyphae were not included in mean amounts. Abundance measures are expressed as $\mu m/mm^2$ and were transformed to the Log scale to correct for non-constant variance.

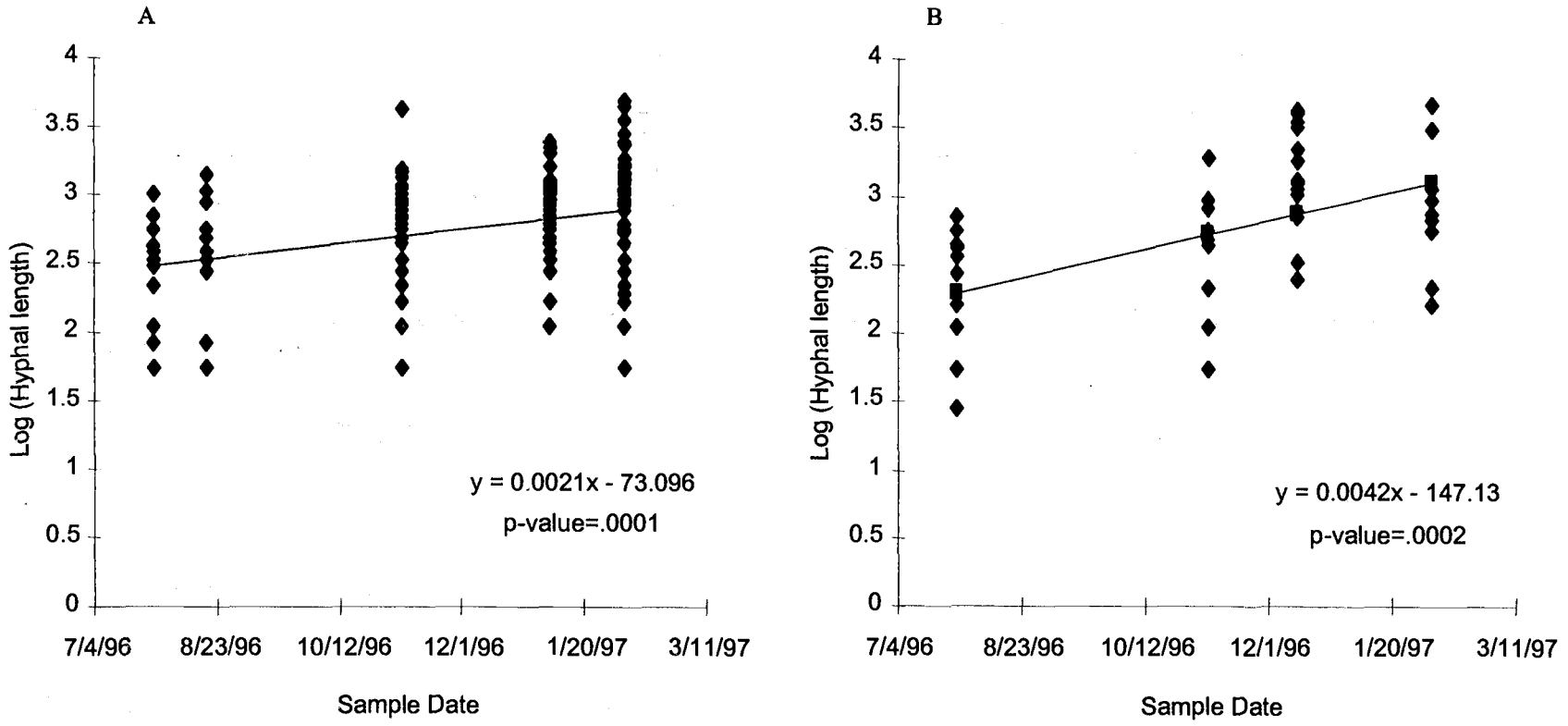


Table 2.7 Mean percentage of Douglas-fir stomata occupied by *P. gaeumannii* appressoria on current year 1996 needles. Needles were collected from six field sites in Tillamook County, OR.

	6/26/96	7/28/96	8/19/96	11/6/96	12/12/96	1/6/97	2/5/97
Juno	0.00 ± 0.00*	1.59 ± 3.07	0.32 ± 1.66	3.17 ± 4.65	ND	5.83 ± 8.20	8.28 ± 9.29
Upper	ND**	0.76 ± 2.09	ND	0.74 ± 2.80	2.71 ± 6.10	ND	1.62 ± 4.85
Salal	0.39 ± 1.58	2.08 ± 4.47	0.31 ± 1.62	7.42 ± 9.27	1.42 ± 3.08	3.08 ± 5.02	4.65 ± 6.99
Lime	ND	0.00 ± 0.00	ND	1.22 ± 3.11	2.79 ± 5.62	3.42 ± 7.56	1.31 ± 3.82
N.Fork	0.00 ± 0.00	0.71 ± 2.36	ND	0.26 ± 1.30	0.72 ± 2.20	0.42 ± 1.74	1.84 ± 3.05
Acey	ND	1.15 ± 2.90	ND	2.57 ± 4.08	11.68 ± 13.97	ND	4.63 ± 8.21

*Percentage of stomata occupied by appressoria ± standard deviation.
**ND = Not determined due to immature needles or samples lost / destroyed.

Table 2.8 Mean percentage of Douglas-fir stomata occupied by *P. gaeumannii* appressoria on current year 1997 needles. Needles were collected from six field sites in Tillamook County, OR.

	9/1/97	3/25/98
Juno	4.35 ± 7.55*	6.59 ± 15.47
Upper	6.82 ± 8.67	1.77 ± 5.04
Salal	0.70 ± 2.32	2.58 ± 7.54
Lime	5.14 ± 9.95	3.49 ± 6.29
N.Fork	0.91 ± 2.82	0.84 ± 2.94
Acey	5.03 ± 6.03	2.90 ± 4.95

*Percentage of stomata occupied by appressoria ± standard deviation.

Needles from Juno and Upper were used to compare the increase in appressoria produced over time between a high disease and low disease site (Figure 2.8). From regression analyses, the percentage of needle stomata occupied by appressoria at Juno increased significantly with time ($p < .0001$). In contrast, the percentage of stomata with appressoria at Upper did increase slightly with time; however, this increase was not statistically significant ($p = .13$). From multiple regression analyses, there was strong evidence that the increase at Juno was greater than the increase at Upper ($p < .0001$). By March, Juno had 6.7% more stomata with appressoria than Upper.

Appressoria also persisted in stomata over time on current year 1997 needles (Table 2.8). However, the percentage of stomata with appressoria did not consistently increase between the two sample collection dates. Additionally, there were no consistent differences between the amount of appressoria at high disease sites compared to low disease sites.

In addition to estimating the amount of penetration over time, plastic impressions were used to describe the process of penetration. Needle penetration began as germ tubes grew towards stomata. Some observations suggested that germ tubes were attracted to stomata. In figure 2.9a, for example, a germ tube has grown toward a stoma on the left and produced an appressorium above the stoma. Growth toward this stoma was not very direct; however, a single branch was also produced from this germ tube which grew more directly toward the stoma on the right. Germ tubes also developed from the side of an ascospore nearest a stoma and grew directly into the stomatal pit. In figure 2.9b, for example, an ascospore was visible directly above a stoma. Two germ tubes have grown away from the stoma, while a third germ tube has differentiated into an appressorium directly above the stoma. Sometimes germ tubes displayed changes in direction and grew in curved paths towards stomata (Figure 2.9c). Germ tubes from each cell of an ascospore could also grow toward the stoma nearest each germ tube (Figure 2.9d). Directed growth toward stomata was not always evident, however. As shown in Figure 2.5, germ tubes sometimes grew near, or around, or over stomata without differentiating into appressoria.

Appressoria were usually produced on a short lateral projection from a hypha (Figure 2.9d) but were sometimes produced directly after germination if an ascospore was next to, or directly above, a stoma (Figure 2.9b). Single germ tubes were capable of producing several appressoria, either as a result of

Figure 2.8 Percentage of Douglas-fir stomata occupied with *P. gaeumannii* appressoria on current year needles from July 1996 to February 1997at Juno (◆) compared to Upper (□).

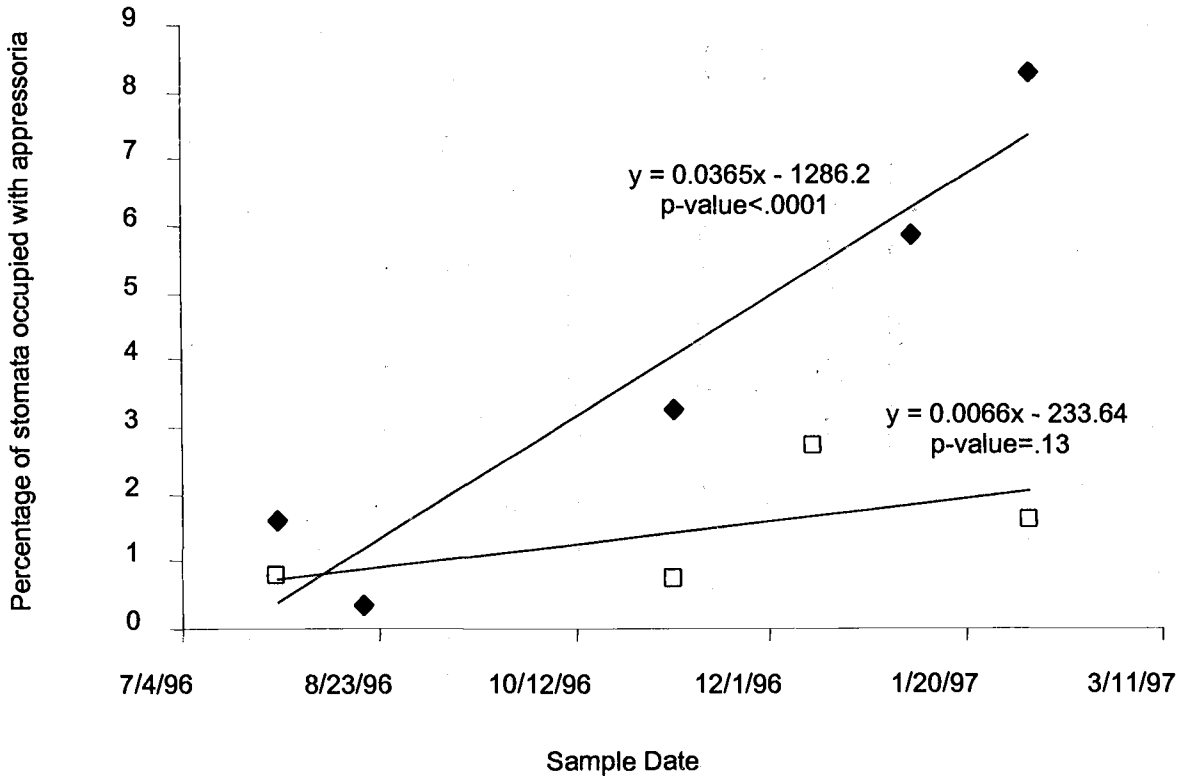
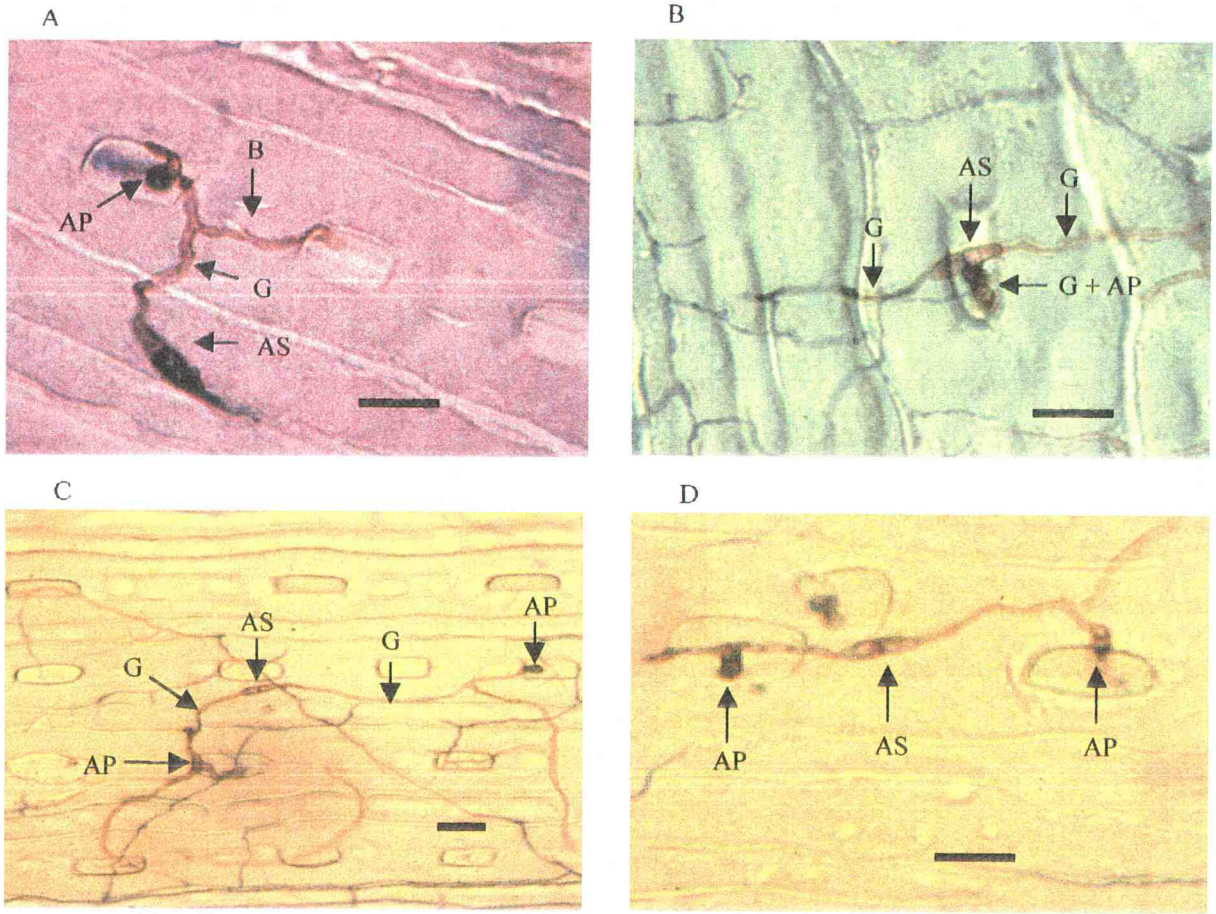


Figure 2.9 Development of *P. gaeumannii* ascospores (AS), germ tubes (G). and appressoria (AP) on Douglas-fir needles. A. Germ tube from ascospore produced an appressorium in the outer chamber of stoma on left A single germ tube branch (B) also grew toward stoma on right, however no appressorium is visible at the end of this germ tube. B. Germ tube from lateral side of ascospore differentiated directly into appressorium above stoma (G + AP) while other germ tubes grew away from stoma. C. Germ tubes grew in curved paths toward separate stomata. D. Both germ tubes from same ascospore grew toward separate stomata Appressoria produced on short lateral branches from germ tube. Germ tube on right continued growing after production of appressorium. Plastic impressions. Bars = 10 μm.



multiple branches or in succession (Figures 2.10 and 2.11). In Figure 2.10, a single germ tube has produced two appressoria over adjacent stomata. In this example, the ascospore is out of the field of view, to the right. (The nature of the structure at the end of one of the germ tube branches is unclear; however, it is not pigmented like an appressorium and is likely an artifact resulting from an air pocket beneath the plastic impression.) Single germ tubes commonly produced 5 or more appressoria through branching and further growth.

The morphology of appressoria was variable. Appressoria usually appeared spherical, thick walled, and darkly pigmented (Figure 2.9). However, germ tubes did not always differentiate into spherical structures but could appear as a swollen hypha above a stoma (Figure 2.11). Usually one appressorium occupied a stoma; however, on rare occasions two appressoria occupied a single stoma (Figure 2.12).

Following appressorium development, a single penetration peg was produced beneath the appressorium, growing into the stoma and between the guard cells. In plastic impressions, penetration pegs were often visible as a small refractive circle in the center of the appressorium. Alternatively, the penetration peg grew off-center so that it became visible to the side of the appressorium just before passing between the guard cells (Figure 2.13).

Of approximately 400 cross sections of needles from thin section light microscopy, 24 examples of appressoria and penetration pegs were observed. In each instance, an appressorium was produced in the outer stomatal chamber (Figure 2.14). From this appressorium a penetration peg grew between the guard cells, into the substomatal chamber, and began to colonize the intercellular mesophyll region of the needle (Figure 2.15). Penetration pegs were approximately 2μm in width, and not significantly different from hyphae which grew into the mesophyll region of Douglas-fir needles. No obvious morphological features distinguished penetration pegs from internal vegetative hyphae.

Figure 2.10 Two appressoria (AP) of *P. gaeumannii* are produced in succession over adjacent stomata from a single germ tube (G). Appressoria are produced on short lateral projections from germ tube. Ascospore is out of picture frame, to the right U= undefined structure. Plastic impression. Bar = 20 μm.

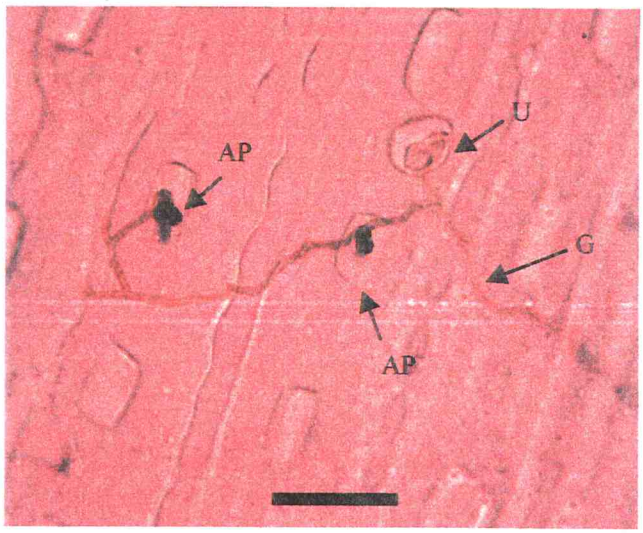


Figure 2.11 *P. gaeumannii* hyphae (H) growing across needle epidermis and producing appressoria (AP) above stomata. Penetration peg (P) visible beneath appressorium, descending into stomatal cavity. SEM micrograph. Bar = 10μm.

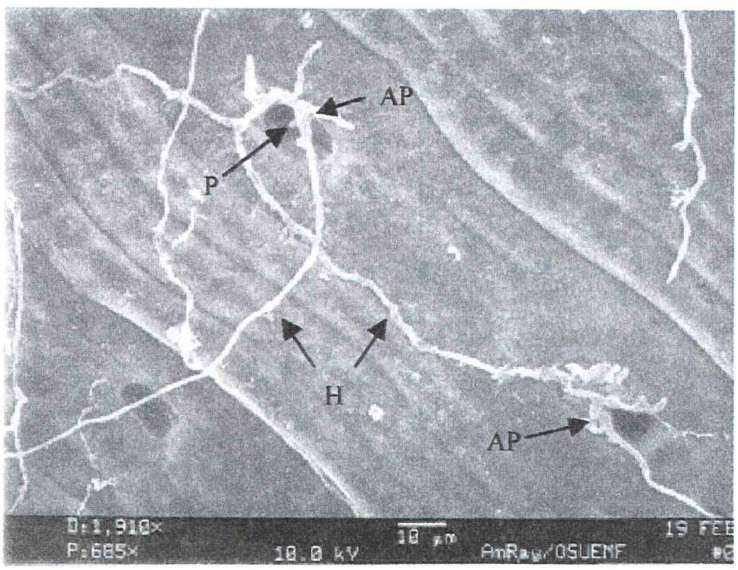


Figure 2.12 *P. gaeumannii* hyphae (H) growing across needle epidermis and producing appressoria (AP) above stomata. Stoma on left has two appressoria. Plastic impression. Bar = 20 μm.

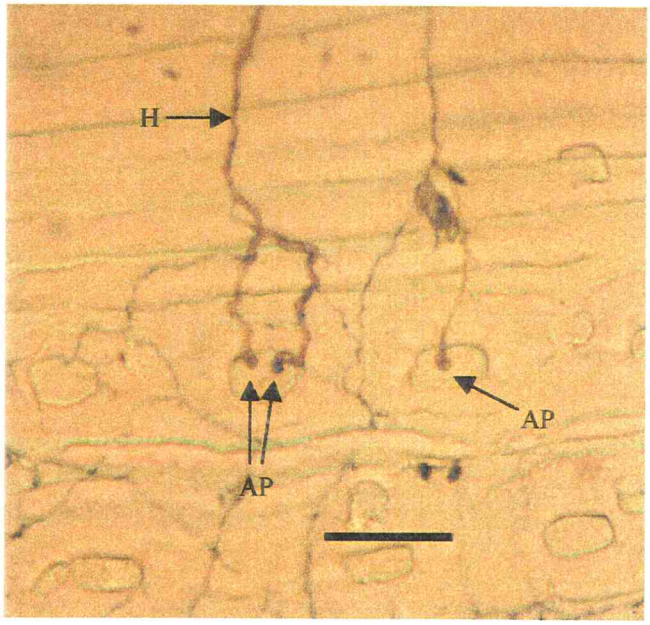


Figure 2 13 Ascospore (AS) of *P. gaeumannii* producing infection structures in nearby stoma. Germ tube (G) extending from ascospore produces lateral appressorium (AP) and penetration peg (P). Penetration peg is descending into stomatal cavity, however, this could not be focused sharply. Plastic impression Bar = 10 μm.

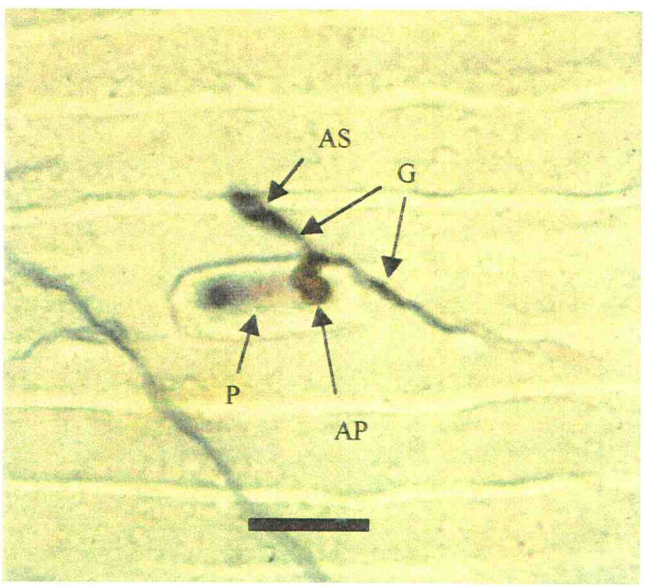


Figure 2.14 *P. gaeumannii* appressorium (AP) and penetration peg (P) in Douglas-fir stoma. Note: appressoria and penetration peg appear separated from stoma due to sample shrinkage. Thin section light micrograph. Bar = 10 μm.

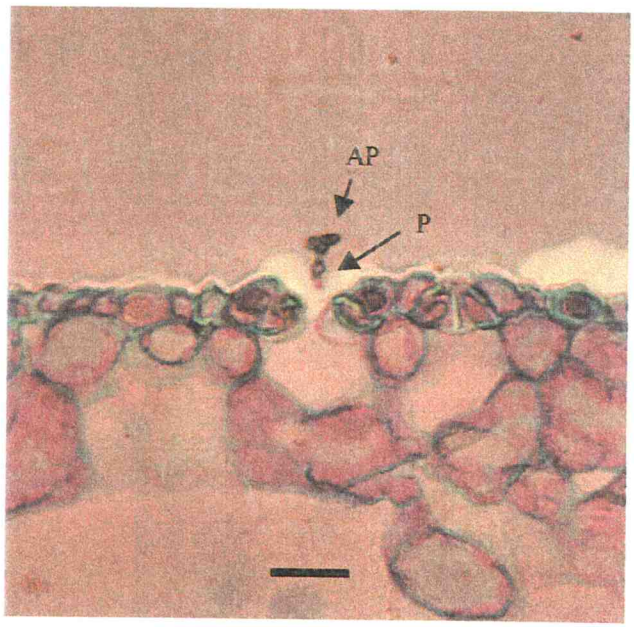
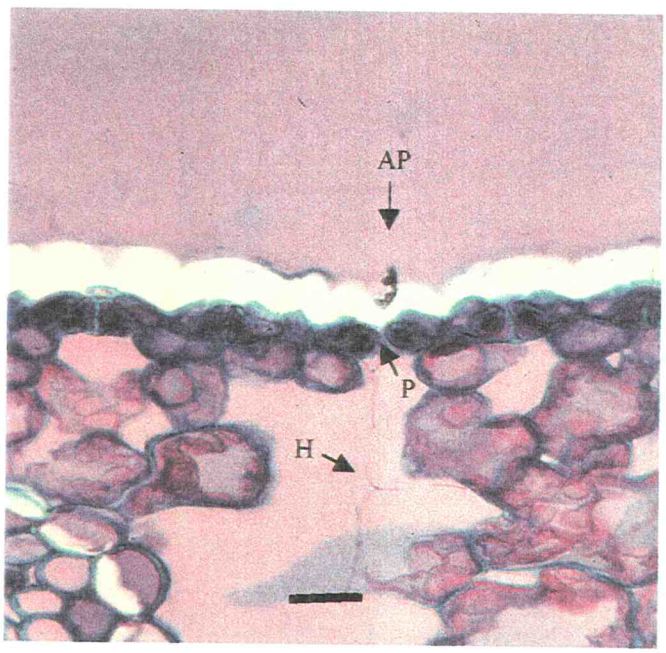


Figure 2.15 Internal hyphae (H) of *P. gaeumannii* extending into Douglas-fir mesophyll from appressorium (AP) and penetration peg (P). Note: embedding plastic has separated from needle surface giving impression that appressorium is above the stoma. Thin section light micrograph. Bar = 10 μm.



Internal Colonization

Observations of SEM micrographs revealed that internal hyphae increased substantially over an eleven month period (July 1997 to May 1998) in current year needles from Juno and Upper. Overall, more internal hyphae were observed in Juno needles (Figure 2.16).

In July 1997, hyphae were absent among parenchyma cells in needles from Upper (Figure 2.16a). In contrast, every needle segment from Juno contained intercellular hyphae in the spongy mesophyll and palisade layer (Figure 2.16b). Although present in every Juno segment, the frequency of hyphae was low. About one in five fields of view at 300X magnification contained strands of *P. gaeumannii* hyphae.

In November 1997, examples of internal colonization were noticeably more frequent. Upper contained long strands of intercellular hyphae in every needle segment, although their presence was infrequent (Figure 2.16c). In contrast, intercellular hyphae were observed in every field of view in Juno needles (Figure 2.16d). Hyphae were both appressed to mesophyll cell walls and growing in long, infrequently branched strands between mesophyll cells.

In May 1998, both Juno and Upper needles contained considerably greater levels of hyphal colonization than previous months. However, Upper needles still contained less hyphae than Juno (Figures 2.16e, f). The amount of hyphae in Upper needles from May 1998 appeared roughly equivalent to Juno needles from November 1997.

Intercellular growth patterns were consistent throughout the year. Hyphae were distributed between both the mesophyll (Figure 2.16) and the palisade parenchyma layer (Figure 2.17). Growth between the palisade cells, however, was generally less abundant than between the spongy mesophyll. In cross sections, hyphae were frequently appressed to the outer bundle sheath cells, as well as mesophyll and palisade parenchyma cells (Figure 2.18). No examples were found, however, of hyphae growing inside xylem or phloem tubes, or any other cells observed in cross sections. Adhesive substances were frequently observed connecting *P. gaeumannii* to the outer wall of host cells (Figure 2.19). In this example, the adhesive substances between the hypha and the wall have partially detached, most likely during sample preparation.

Figure 2.16 Comparison of *P. gaeumannii* internal colonization in current year needles from Upper and Juno. A. Upper, 7/97; no internal hyphae. B. Juno, 7/97; one strand of intercellular hyphae visible. C. Upper, 11/97; intercellular hyphae present in every needle, although infrequent. D. Juno, 11/97; several strands of hyphae present in every needle segment. E. Upper, 5/98; several strands of hyphae present in every needle segment. F. Juno, 5/98; abundant levels of hyphae present in every needle segment. MW = mesophyll cell wall. H = hyphae. SEM micrographs. Bars = 100μm.

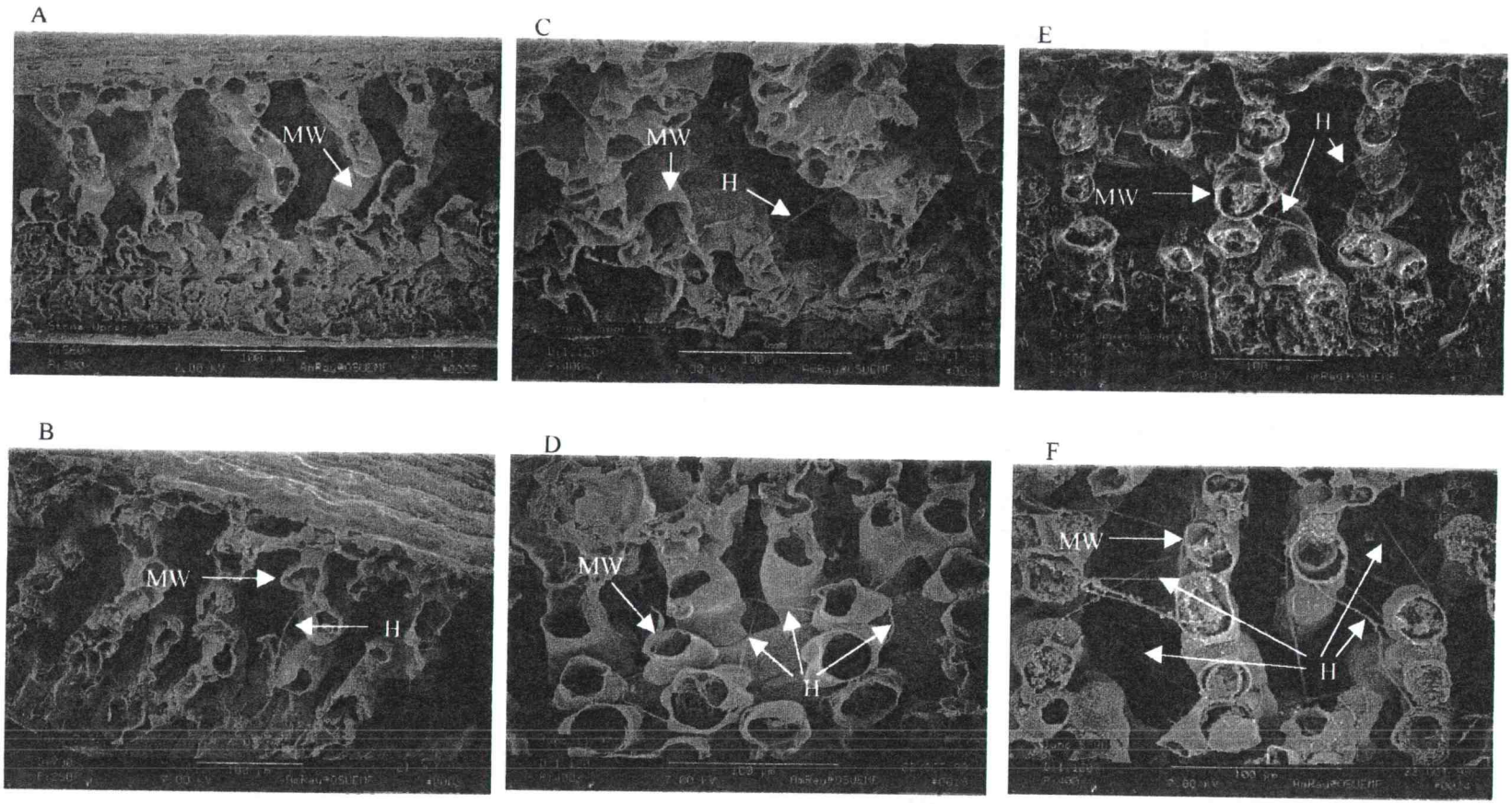


Figure 2.17 Hyphae (H) of *P. gaeumannii* growing between Douglas-fir palisade parenchyma cells (PP). SEM micrograph. Bar = 10 μm.

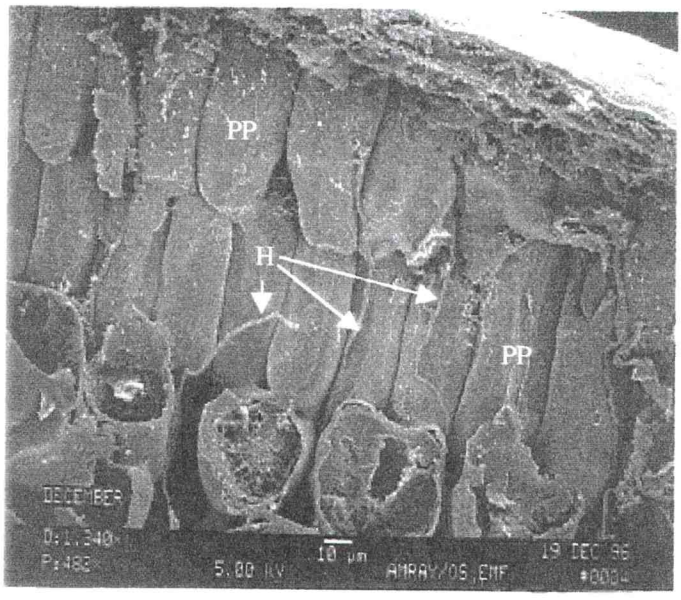


Figure 2.18 Hyphae (H) of *P. gaeumannii* growing around perimeter of bundle sheath cells (BS) and in surrounding parenchyma of Douglas-fir needle. SEM micrograph of needle cross-section. Bar = 100 μm.

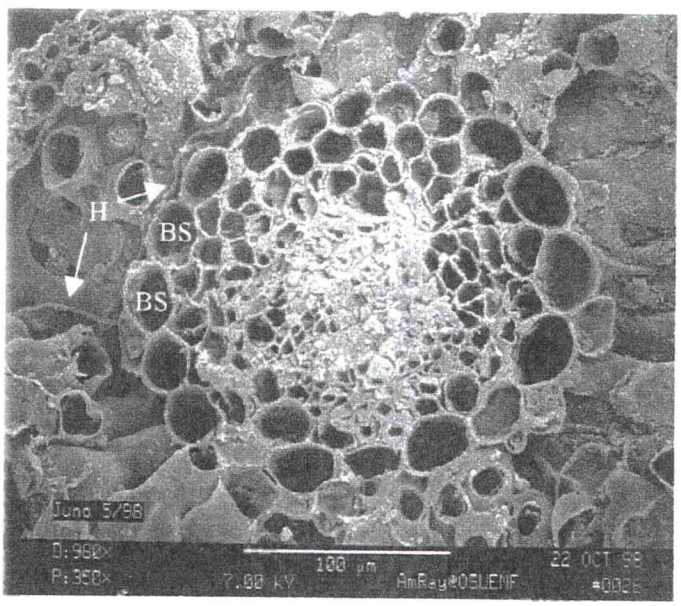


Figure 2.19 *P. gaeumannii* hyphae (H) attached to outer mesophyll cell wall (MW) inside Douglas-fir needle. SEM micrograph. Bar = 10 μm.

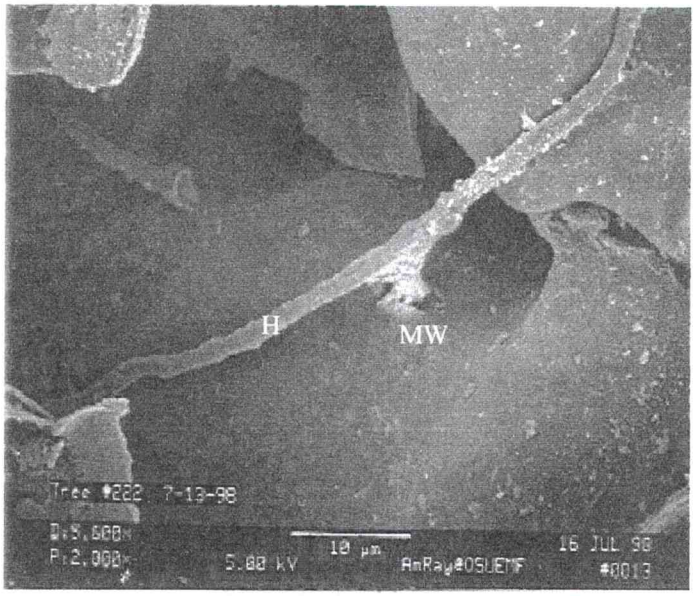
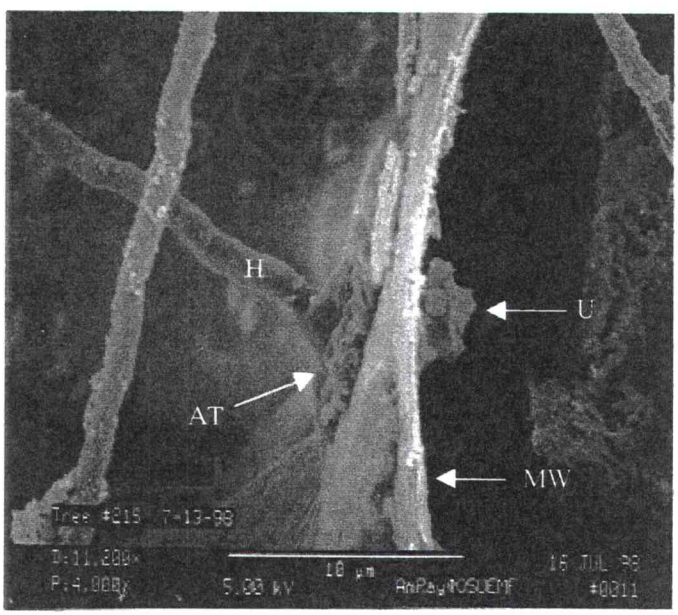


Figure 2.20 Possible penetration of mesophyll cell inside Douglas-fir needle by *P. gaeumannii*. A single hypha (H) has attached (AT) to the outside of a mesophyll cell wall (MW). Structure inside mesophyll wall (U) may be callose, haustorial development, or an article of debris. SEM micrograph. Bar = 10 μm.



One observation from an artificially inoculated seedling suggested that *P. gaeumannii* may penetrate host cell walls inside needles. Shown in Figure 2.20, a single hypha has come into contact with a mesophyll cell wall. The nature of the depositions on the outer cell wall are unclear but could be adhesive substances or a structure similar to an appressorium. Opposite the fungal attachment, on the inner side of the host mesophyll cell wall, there is another unidentified structure. The nature of this structure is unclear. It may be a host cell defense reaction and subsequent deposition of callose or other protective substances. It is possible that the structure is an article of debris or an artifact of sample preparation. It is also possible that the hyphal strand on the outer mesophyll wall has successfully penetrated through the mesophyll wall and the inner structure is a precursor of new hyphal growth, or an absorptive cell like a haustorium.

In potted seedlings which had been inoculated with *P. gaeumannii* macerated mycelium, hyphal growth appeared consistent with growth patterns observed from field specimens. Hyphae were approximately 2-3μm in width and were observed in long, mostly unbranched strands growing between and attached to parenchyma cells. Culture samples from mycelium inoculated seedlings were used to verify the presence of *P. gaeumannii* inside inoculated needles. *P. gaeumannii* was positively identified in 40 needles from inoculated seedlings.

Development of Pseudothecia

Observations of thin sections, plastic impressions, and SEM micrographs showed pseudothecia forming in stomata from early fall months through early spring on current season needles. Formation of pseudothecia began as intercellular hyphae in the mesophyll grew through the substomatal chamber, and passed between the guard cells into the outer stomatal chamber. These hyphae differentiated into a condensed mass of darkly pigmented cells in the outer stomatal chamber (Figures 2.21 and 2.22). This aggregation of cells enlarged to fill the entire stomatal cavity, and emerged from the stoma often covered by remnants of cuticular wax. The cells appeared roughly spherical at first, but slowly increased in size and grew into new strands of hyphae on the needle surface (Figure 2.21).

Figure 2.21 Early development of *P. gaeumannii* pseudothecium in a Douglas-fir stoma. Condensed mass of spherical (SP) cells emerge from stoma. Pseudothecial initial is covered with remnants of needle wax (W). SEM micrograph. Bar = 10 μm.

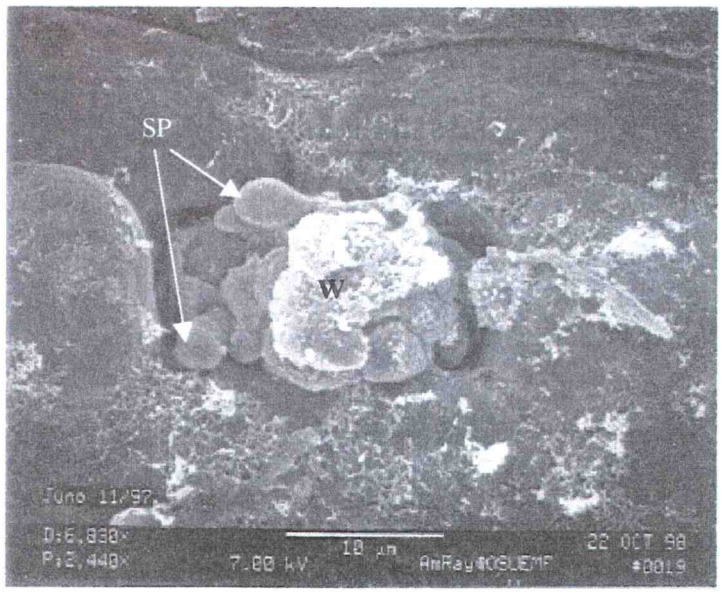
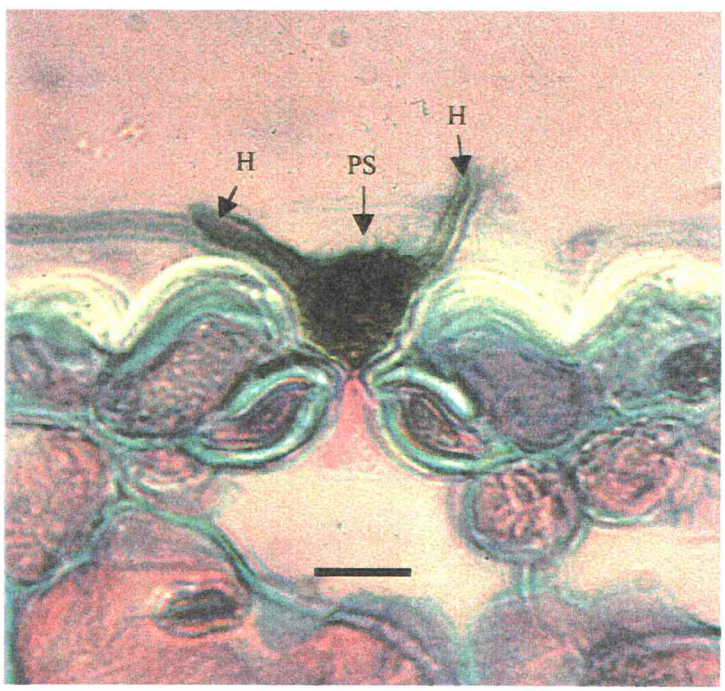


Figure 2 22 Later development of *P. gaeumannii* pseudothecium (PS) in a Douglas-fir stoma. Hyphae (H) have emerged from pseudothecial initial in multiple locations. SEM micrograph. Bar = 10 μm



In plastic impressions, these spherical cells appeared first around the perimeter of the stoma (Figure 2.23a). (Presumably, the plastic impressions did not show immature pseudothecia because they were still covered with needle wax.) Hyphae that grew from these spherical cells were indistinguishable from ascospore germ tubes or other somatic hyphae, both in size and pigmentation, and in their irregular patterns of growth (Figures 2.23b-d). In SEM micrographs this process of spherical cell development and emergence of new hyphae was also observed (Figures 2.23c, d). In all samples examined, no examples of asexual cells, such as conidia or spermatia, were found during the course of pseudothecial development. At the same time that new hyphae emerged, pseudothecia began to enlarge. By early to mid winter, the spherical cells making up the pseudothecial initial became gradually less distinct and formed the outer layer of the pseudothecium (Figure 2.24). General observations revealed that pseudothecia in different stomata developed at different times and not always simultaneously.

During the course of pseudothecial development, hyphae from the original spherical cells persisted on the needle surface and increased over time. Because these hyphae were indistinguishable from ascospore related hyphae, their incidence and abundance on needle surfaces were included in the measurements of hyphal growth over time reported in Tables 2.3-2.6.

Hyphae from pseudothecia also produced appressoria above stomata (Figure 2.25). In plastic impressions, penetration pegs were sometimes visible extending into the stoma (Figure 2.26). No examples were found in thin section light microscopy, however, of penetration pegs entering through the stomata from these hyphae. The amount of appressoria produced from these emergent hyphae was also included in the mean percentage of stomata occupied by appressoria over time in Tables 2.7 and 2.8.

In addition to producing appressoria, hyphae from pseudothecial initials were seen connecting pseudothecia. Shown in Figure 2.27, hyphae from pseudothecial initials were frequently observed connecting several adjacent pseudothecia.

Between January and March, individual strands of hyphae began to deteriorate. Disintegrating hyphal cell walls appeared less melanized and became less distinct.

Figure 2.23 Development of *P. gaeumannu* hyphae emerging from pseudothecial initials. A. Emergence of spherical cells (SP) of *P. gaeumannii* around perimeter of stoma prior to emergence of pseudothecial initial. Plastic impression. Bar = 10 μm. B. Extensive hyphal growth (H) from pseudothecial initial (PS). Plastic impression. Bar = 10 μm. C. Spherical cells (SP) comprising the pseudothecial initial and hyphae (H) which arise from these cells. SEM micrograph Bar = 10 μm. D. Several developing pseudothecia (PS), and extensive hyphal growth (H) on needle surface resulting from pseudothecial initials. SEM micrograph. Bar = 100 μm.

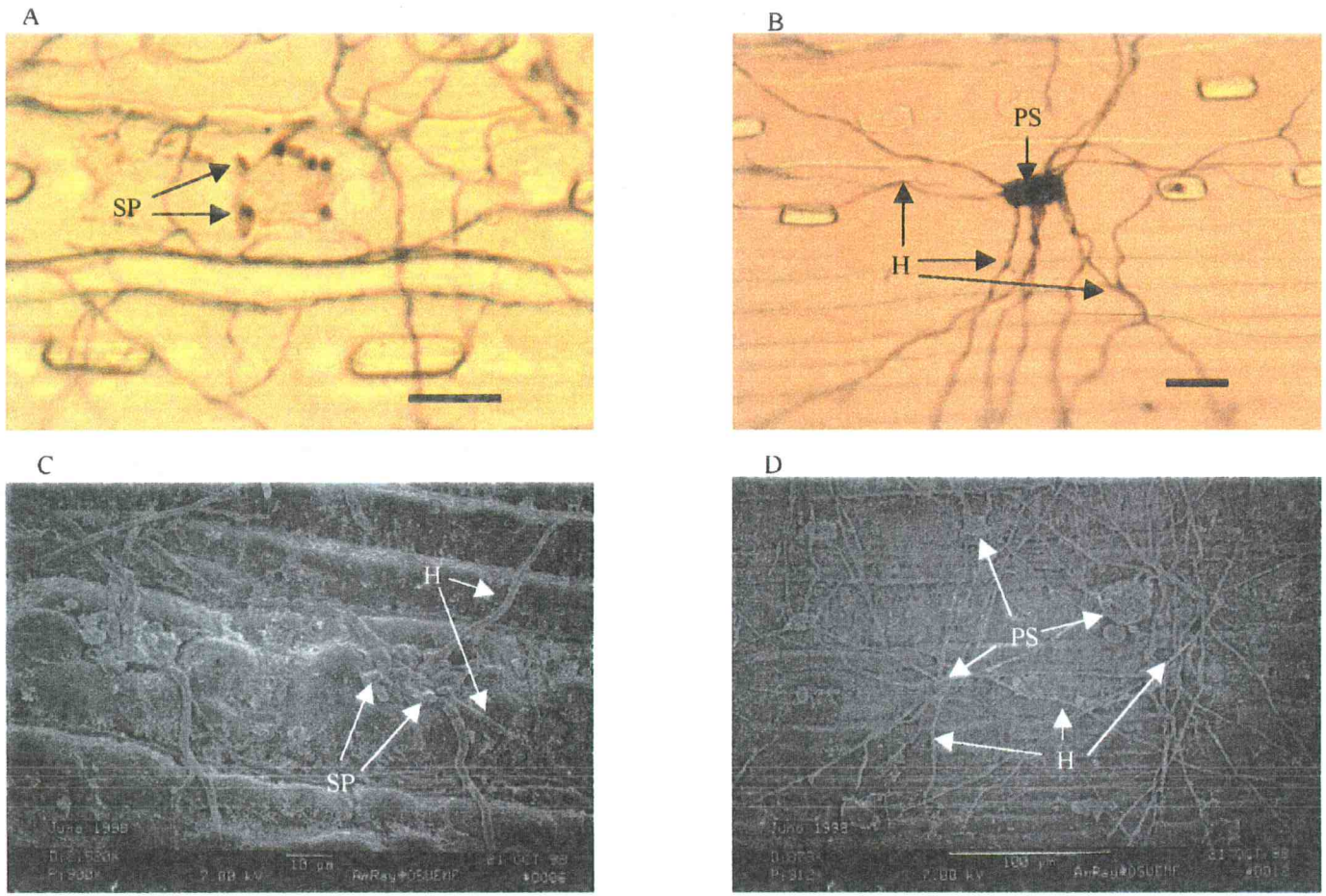


Figure 2.24 Development of *P. gaeumannii* pseudothecia (PS) in several stomata. Hyphae (H) from pseudothecial initials covers extensive portions of needle surface. SEM micrograph. Bar = 100 μm.

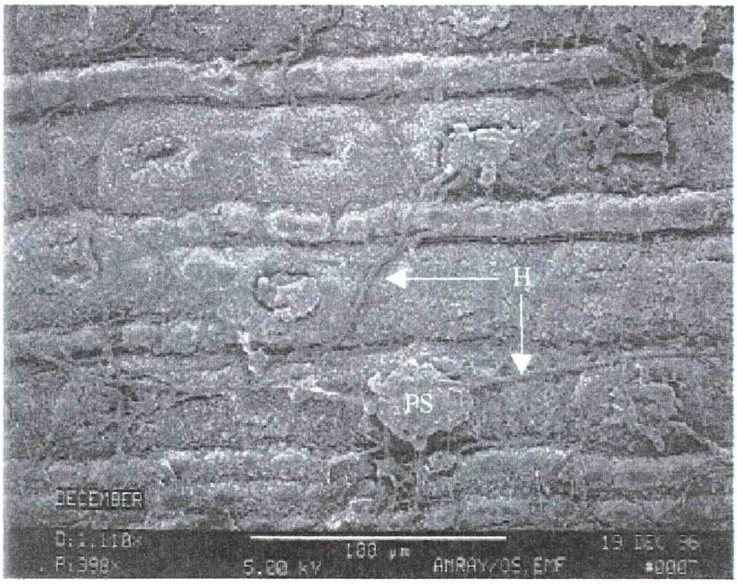


Figure 2.25 *P. gaeumannii* hyphae (H) emerging from pseudothecial initial, growing across needle surface, and producing appressorium (AP) in unoccupied stoma. Plastic impression. Bar = 10 μm.

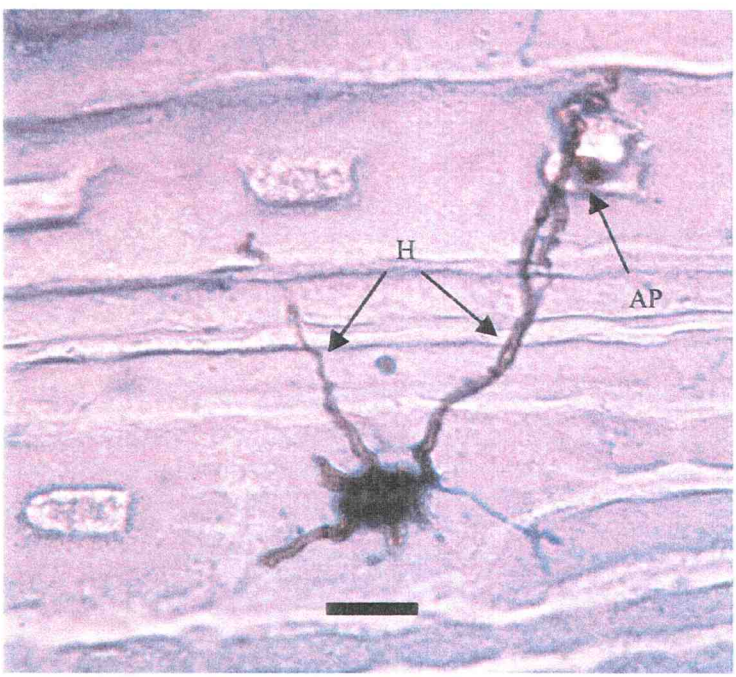


Figure 2 26 *P gaeumannii* appressorium (AP) and penetration peg (P) produced in stoma as a result of hyphae (H) from pseudothecial initial. Pseudothecial initial is out of field of view, below. Pseudothecium (PS) to the right is unassociated with hyphae and appressorium. Plastic impression. Bar = 10 μm.

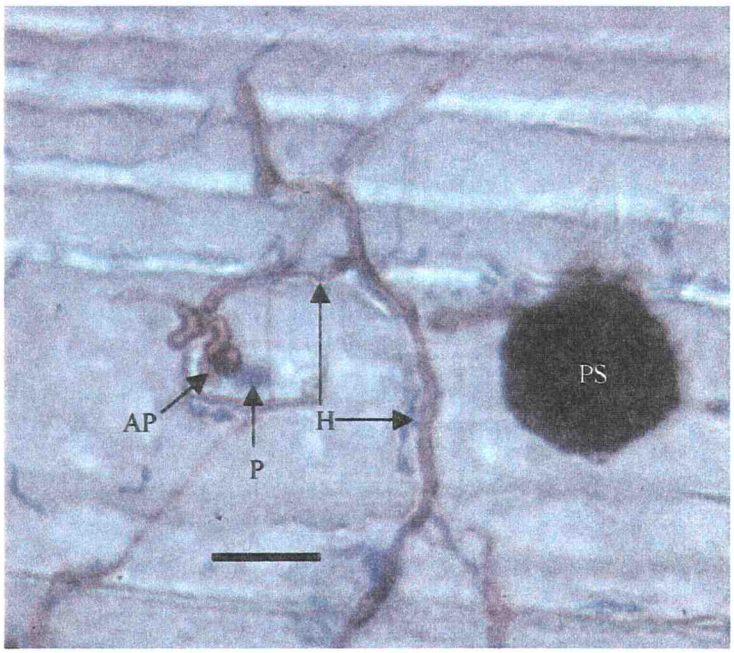
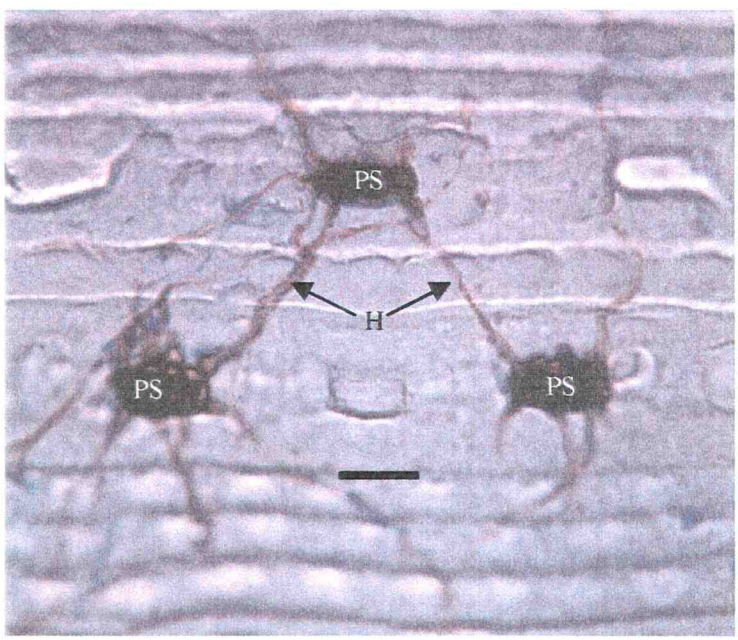


Figure 2.27 Three pseudothecia of *P. gaeumannii* (PS) connected by hyphae (H) Plastic impression. Bar = 10 μm. .



DISCUSSION

The infection and colonization of Douglas-fir needles began as ascospore germ tubes grew towards stomata and differentiated into appressoria in the outer stomatal chamber. Single germ tubes were capable of producing several appressoria, either as a result of multiple branches or in succession. Appressoria were produced from both germ tubes and somatic hyphae on needle surfaces, and these hyphae persisted and increased over time. As a result of epiphytic growth, hyphae continued to produce appressoria over time, suggesting that penetration events also increased. Needle penetration occurred through the stoma as penetration pegs grew between the guard cells and into the needle mesophyll. Internal hyphae were observed only in intercellular regions but were often well attached to host cell walls. As pseudothecia began to develop in stomata in the fall, new hyphae emerged from pseudothecial initials. These hyphae contributed to epiphytic colonization and produced new appressoria in adjacent stomata.

Several aspects of the biology of *P. gaeumannii* are unique compared to other fungal pathogens on conifer needles. For example, the fact that *P. gaeumannii* was capable of producing multiple appressoria from single germ tubes was unusual. Most research suggests that fungal spores germinate directly into appressoria or germ tubes grow only a short distance and terminate in a single appressorium (Diwani and Millar, 1986; Ferris, 1967; Gadgil, 1967; Ivory, 1972; Parris and Killebrew, 1969; Patton and Johnson, 1970; Miller, Patton and Powers, 1980; Patton and Spear, 1978; Patton et. al., 1984; Peterson, 1969; Peterson and Walla, 1978; Setliff and Patton, 1974; Stone, 1988). In addition, germ tubes of *P. gaeumannii* were capable of extensive growth and epiphytic hyphae were observed on needle surfaces for over 10 months. *Dothistroma pini* germ tubes have been known to persist on needle surfaces for up to 45 days, however, these disintegrated shortly after appressoria were formed (Gadgil, 1967). Most other conifer pathogens produce transient germ tubes whose function is to gain immediate ingress into needles. The production of hyphae from developing pseudothecia is also unique among conifer pathogens. These hyphae also contributed to further surface growth and needle penetration over time. Such extensive growth for *P. gaeumannii* indicates that germ tubes and vegetative hyphae may be capable of utilizing nutrients from the needle surface. Based on these observations, *P. gaeumannii* may be considered an internal fungus, with a strong secondary ability for epiphytic colonization.

While observations of surface hyphae suggested that epiphytic growth was extensive, especially at high disease sites, the amounts reported may actually have been underestimates. Because high disease sites have poor needle retention, the measurements of surface hyphae were only estimates of the amount of hyphae remaining on attached needles. Abscised needles were not taken into account. If prematurely abscised needles had shown larger amounts of colonization, the reported amounts of hyphae may have been underestimates of true epiphytic colonization. With the same line of reasoning, the number of appressoria observed in stomata may also have been underestimates, especially at sites with greater needle loss.

Although observations from 1996-1997 showed an increase in epiphytic colonization over time, these same results were not repeated during 1997-1998. In particular, more fluctuations in epiphytic colonization were observed during the second year. These fluctuations may represent natural senescence of epiphytic hyphae. They may also be a result of lowered needle retention, as described above, and a consequent misrepresentation of actual colonization. However, measurements were only taken on two occasions during 1997-1998, and without greater numbers of sample times it would be difficult to establish a trend for increase. In general, epiphytic growth persisted on needles throughout the year for both years sampled.

Several details of the histopathology of *P. gaeumannii* on Douglas-fir needles will require further research. For example, the observations reported in this research for directed germ tube growth towards stomata were suggestive but incomplete. Work on other fungal plant pathogens has shown that fungal germ tubes can be attracted to chemical signals from stomata, however, the precise chemical signals have rarely been defined (Staples and Hoch, 1997; Millar, 1981). The fact that some germ tubes grew over or around stomata without producing appressoria suggests that, if chemotropic signals are present, these signals may not always be produced or detected from every stoma. It is possible that stomatal wax may prevent detection or penetration of stomata (Millar, 1981), however very little evidence was found to support this hypothesis for *P. gaeumannii*. SEM micrographs revealed a heterogenous distribution of wax on needle surfaces and in stomata, however, this may have been an artifact of sample preparation. Because germ tubes and somatic hyphae on needles grew in random directions, there is very little to suggest that

thigmotropic signals direct the growth of *P. gaeumannii* on needle surfaces. Overall, the role of chemical signaling, stomatal wax, and other factors which influence infection by *P. gaeumannii* needs further study.

Details concerning the precise nutritional relationships between *P. gaeumannii* and Douglas-fir host cells are still unknown. In SEM observations, there was very little evidence that *P. gaeumannii* penetrated host cells directly; only one observation resembled cellular penetration. However, intercellular hyphae were often well attached to mesophyll and palisade parenchyma cells. Further, the amount of intercellular hyphae increased over time, especially at high disease sites like Juno. Previous transmission electron microscopy (TEM) observations of these points of contact between *P. gaeumannii* and host cells show cellular appositions in the host and indicated a host defense reaction (Stone, pers. comm.). Research regarding nutrient movement between hosts and parasites suggests that parasites can intercept solutes diffusing through the host apoplast (Hancock and Huisman, 1981). Additionally, some parasites are known to produce compounds that alter the permeability of host cell membranes (Wheeler, 1976). These current observations of host wall contact, combined with earlier TEM work, suggest that *P. gaeumannii* obtains nutrition primarily from the apoplast, and that membrane altering substances may be produced. Understanding the precise nutritional relationships between *P. gaeumannii* and Douglas-fir host cells will also require further study.

No examples of asexual cells such as conidia or spermatia were observed during the course of pseudothecium development, or from any other stage of fungal development on needle surfaces. While these findings do not dismiss the possibility of an anamorph, they also do not support the observations of a fall spore release by Fatuga (1978), which was hypothesized to be a conidial stage for *P. gaeumannii*. Instead of the production of conidia from developing pseudothecia, a unique stage of hyphal growth was observed from spherical cells produced during pseudothecial development. Such observations may support the work of Stone and Carroll (1986). In their ultrastructural observations of developing pseudothecia, phialide-like cells were found preceding the formation of pseudothecia. These cells showed laminar wall depositions characteristic of cells that successively produce and release conidia. While no conidia were observed in the present study, it seems possible that these phialide-like cells may be responsible for the hyphal growth observed from pseudothecial initials. In monitoring several life stages of pseudothecia, it

appeared that the spherical cells which emerged from stomata gave rise to multiple strands of hyphae as well as forming the outer cell layer of the pseudothecium. This continuous production of cells over time may explain the laminar depositions observed on phialide-like cells. Further histological studies will be needed to determine the exact relationship between the phialide-like cells, pseudothecia, and this unique stage of hyphal growth.

The results presented in this research may help explain the current Swiss needle cast epidemic in Tillamook County, Oregon. Particular locations around Tillamook are known to receive almost daily occurences of precipitation, even through summer months, in the form of rain, fog, or dew formation. Epidemiology studies of *P. gaeumannii* have shown a strong link between mean infection levels and high amounts of rainfall and other precipitation in Vancouver, B.C. and the northern Cascade mountains. These increased periods of leaf moisture may favor epiphytic colonization of *P. gaeumannii*. In turn, stomatal penetration and internal colonization of Douglas-fir needles would also increase. Hyphae which persist on needles throughout fall, winter, and spring months may, therefore, contribute to disease progress in a cumulative fashion over time, given optimal conditions. In comparing the amount of hyphal colonization at Juno and Upper, greater levels of both epiphytic and internal hyphae were found on Juno needles. Additional information on the relationship between environmental factors and fungal growth, therefore, may help improve our understanding of Swiss needle cast severity along the Oregon coast.

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Chapter 3. Temperature and Moisture Conditions Favoring Ascospore Germination, Germ tube Growth, and Fungal Colonization of Douglas-fir Needles.

INTRODUCTION

Infection of Douglas-fir needles by the ascomycete *Phaeocryptopus gaeumannii* has resulted in serious damage within the last decade to forest plantations along the Oregon coast, particularly near Tillamook. Since its initial discovery in Switzerland in the 1920's Swiss needle cast has resulted in severe foliage loss and growth reductions on Douglas-fir trees planted in exotic locations throughout Europe, the Midwest and eastern U.S., and in New Zealand. Until relatively recently, Swiss needle cast has not been a serious disease in coastal Oregon, where it occurs naturally. One explanation for the recent epidemic is that reforestation practices have favored replanting large acreage's along the coast to Douglas-fir where alder, hemlock and spruce once predominated. Environmental factors along the coast are more favorable to disease development. Specifically, the coastal strip is characterized by a moderate climate with warm winters and relatively cool summers. Frequent periods of precipitation such as rainfall, fog, and heavy dew provide conditions favorable for infection and colonization of needles.

Several reports have shown that spore release and infection are correlated with rainfall, high humidity, and needle wetness. In-vitro experiments on sporulation, for example, have demonstrated a direct relationship between needle wetness and release of ascospores from pseudothecia (Michaels and Chastagner, 1984). Surveys in southern British Columbia and the Olympic peninsula demonstrated positive correlations between regional infection levels and mean rainfall during May – July and infection was higher in the coastal and interior rainbelts and comparatively less in the drier intermountain region east of the coast range (Hood, 1982). Ford and Morton (1971) also found a relationship between rainfall and spore release during May-July in Michigan plantations.

While the dependence of infection on moisture has been established, more detailed knowledge of the effect of environment on specific aspects of *P. gaeumannii* biology is lacking. This research was undertaken to determine the effects of several environmental factors on *P. gaeumannii* ascospore germination and on subsequent colonization of needles. Spore germination and germ tube elongation were

observed across a range of temperatures. The effect of drying on ascospore germination and germ tube growth was also explored. Finally, the relationship between the duration of needle surface moisture and colonization of needles was examined on artificially inoculated seedlings. Such information may allow a better understanding of the relationship between host, pathogen, and environment, and may strengthen the hypothesis that the recent Tillamook epidemic is related to environmental factors. This knowledge may also prove valuable in risk assessments and in helping to choose low risk areas for Douglas-fir plantations.

MATERIALS & METHODS

Plant and Fungal Materials

Two-year-old potted Douglas-fir seedlings were used for artificial inoculations and subsequent histological examination. These were obtained from DL Phipps nursery in Elkton, OR, an Oregon Department of Forestry nursery, and were grown as standard 2-0 bareroot seedlings. The geographic origin of the seed source was from the Tillamook district, Oregon Department of Forestry. Samples of *P. gaeumannii* were obtained from diseased 15 year-old Douglas-fir trees at Juno Hill, an Oregon Department of Forestry plantation approximately 5 miles north of Tillamook, OR.

Temperature Studies

Ascospore germination and growth were examined across a range of temperatures, and on two different media, potato dextrose agar (PDA) and water agar (WA). Ascospores were first discharged onto agar plates by suspending field infected Douglas-fir needles with mature pseudothecia above the agar surface. Agar plates were incubated inside a humidity chamber for 4 hours during sporulation. Afterwards, one plate of each medium was incubated at 14, 18, 22, 26, 30, and 34°C on a temperature gradient plate. After 72 hours, ascospore germination and germ tube elongation were measured on each petri plate. Sixty ascospores were measured on each plate-by-temperature combination. This entire experiment was repeated for temperatures between 18°C and 25°C, at 1° intervals. At each temperature, ascospores were grown on both PDA and WA.

Chi^2 analyses were used to test for differences in germination at different temperatures and on different media. Two-way ANOVA was used to test for differences in germ tube lengths at different temperatures and on different media.

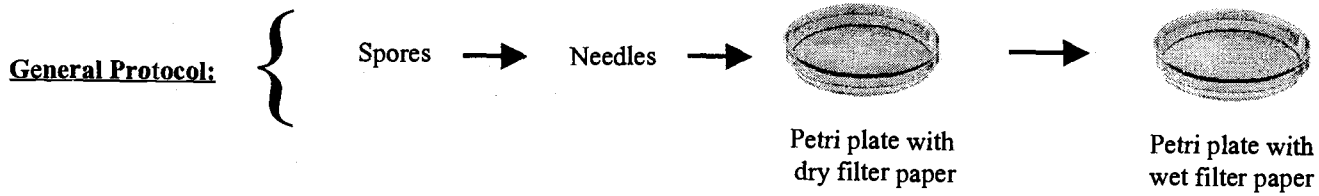
Effect of Drying on Germination and Germ Tube Growth.

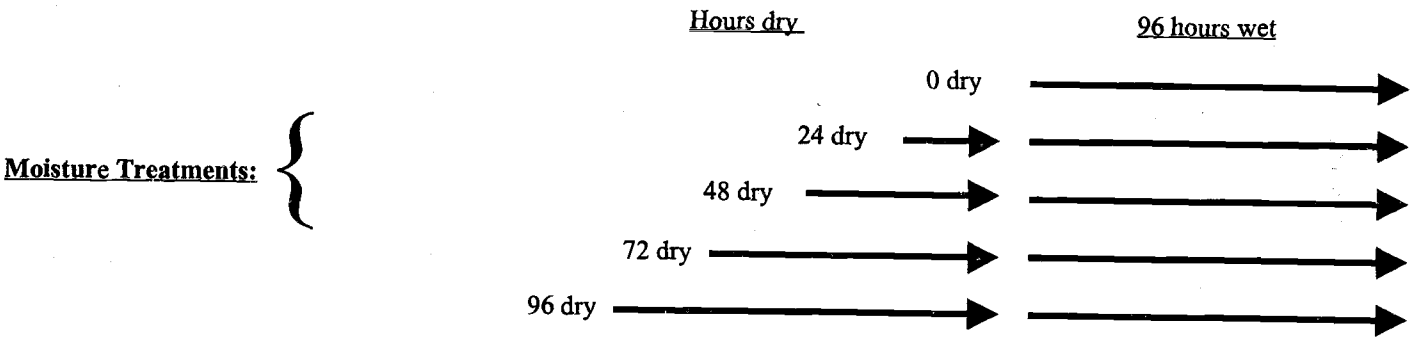
The effect of drying on ascospore germination and growth was examined after inoculating detached healthy needles with ascospores. Current year needles from healthy, potted seedlings were used. These were detached from the tree and then placed inside petri plates designed as mini-inoculation chambers. Diseased needles, collected from naturally infected trees at Juno Hill, were attached to the petri plate lid with Vaseline and suspended above healthy needles. All needles were misted lightly with distilled water and the plates were incubated inside an enclosed humidity chamber for 4 hours, until sporulation occurred.

Inoculated needles were transfered to new petri plates and then assigned to one of five drying treatments: zero hours, 24 hours, 48 hours, 72 hours, or 96 hours. Two needles underwent each drying treatment; 10 needles were used in all. (Needles received drying treatments prior to germination of ascospores.) Needles undergoing dry treatment were incubated in petri plates with dry filter paper for the specified length of time. Afterwards, filter papers were saturated with distilled water in order to create a humid environment inside the petri plate enclosure. (Visual inspection revealed that condensation was common on needles in moistened petri plates.) Each treatment received 96 hours of moist conditions following the specified drying treatment. Plates were incubated at 19°C during all treatments. Figure 3.1 illustrates the experimental design and treatment protocols.

After 96 hours, plastic impressions were made of needle surfaces to assess germination and germ tube growth rates. Abaxial needle surfaces were painted with a thin coat of clear fingernail varnish. After drying for 1 hour, the plastic impressions were peeled from the needle surface and stained in several drops of trypan blue (equal parts: lactic acid, dH_2O, glycerol, and 0.05% trypan blue (Sigma)). Stained peels

Figure 3.1 Treatment protocol for the effect of drying on ascospore germination and germ tube growth. Ascospores were first inoculated onto needles. Needles were then incubated inside petri plates with dry filter paper. All plates received one of five drying treatments. Needles were then incubated in petri plates with wet filter paper for 96 hours. Plastic impressions were made of needle surfaces to assess ascospore germination and germ tube growth on needle surfaces.





were covered with a coverslip and sealed with clear nail varnish. Germination and germ tube growth were assessed with standard light microscopy.

Germination percentages were calculated from approximately 20 ascospores per needle (two needles / treatment) and germ tube lengths were measured at 400X magnification using an ocular micrometer. Germination data were categorized into five levels, depending on the five levels of dry treatment, and analyzed using a one-way ANOVA. Multiple comparisons were performed using the Tukey-Kramer procedure. The questions of interest included whether germination varied depending upon length of drying, and if so, which treatment afforded optimal germination. Regression was used to assess whether a simple linear model described the change in germination over time. Differences in germ tube lengths were also analyzed with one-way ANOVA. Multiple comparisons were used to determine which treatments afforded optimal germ tube growth. Regression was used to analyze whether the data fit a simple linear model.

Effect of Moisture on Colonization of Needles.

The effect of moisture on the infection and colonization of Douglas-fir needles was examined after inoculating healthy seedlings with *P. gaeumannii* mycelium and exposing the seedlings to various moisture periods in a mist chamber. *P. gaeumannii* mycelium was obtained from ascospores from naturally infected needles collected at Juno Hill. Needles were suspended over water agar for approximately one hour, until sporulation occurred. Individual spores were then removed using a glass pipet tip and transferred to potato dextrose malt yeast extract (PDMYA: 39 grams potato dextrose agar, 1g yeast extract, 1g malt extract, and 1L dH_20.) After one month of growth on solid agar, a 5mm square section of *P. gaeumannii* and agar was transferred to 500 ml of liquid culture broth in a 1000ml flask. Fungal cultures were grown in liquid broth for two months at 19°C.

For preparation of inoculum, fungal cultures were filtered and rinsed using a vacuum filtration device. The resulting mycelia were then suspended in water at a ratio of 20g of *P. gaeumannii* mycelium to 1L dH_2O. This mixture was macerated for 60s with a tissue homogenizer. The final macerated

mycelium solution was sprayed onto the foliage of potted seedlings until runoff using an aerosol spray gun. (For similar mycelium inoculation techniques, see Hood, 1977.)

Following inoculation, seedlings were exposed to one of six moisture treatments in a mist chamber: zero-hours, 1 hour; 1 day, 3 days, 5 days, and 14 days. Mist chamber cycles were set to maintain continuous free moisture on the foliage. A total of 12 trees were inoculated with macerated mycelium. Two trees were assigned to each moisture treatment. After the specified treatment, seedlings were removed from the mist chamber and held in relatively dry atmospheric conditions in a greenhouse for 12 months. Colonization of needles by *P. gaeumannii* was then assessed through both culture sampling and scanning electron microscopy.

For culture sampling, 20 needles from each tree were surface sterilized with 95% EtOH for one minute, 50% Chlorox for 10 minutes, and a final rinse in 95% EtOH for one minute. Needles were then cut into six segments and placed onto PDMYA and allowed to incubate three weeks. All segments were scored for the presence or absence of *P. gaeumannii* hyphae. The proportion of needle segments colonized was calculated for each tree. One main question of interest was the minimum amount of moisture required for successful infection and colonization of needles. Regression was also used to determine if the ratio of colonized to uncolonized needle segments (logit transformation) increased as a function of moisture exposure.

Scanning electron microscopy (SEM) was used to assess the amount of internal colonization on a qualitative, visual level. Needles were first cut into 5mm longitudinal sections with a razor blade. Segments were then fixed in 3% glutaraldehyde in a vacuum chamber overnight and then dehydrated in a graded EtOH series up to 100%. Samples were critical point dried with a CPD 020 critical point drying machine. Dried needle segments were attached to aluminum SEM mounts with Duco household cement (ITW Brands) and sputtercoated with a mixture of argon gas and gold palladium. All viewing was performed with an AMRAY scanning electron microscope. Six needle segments were viewed from each treatment group.

RESULTS

Ascospore Germination

Ascospores germinated over a range of temperatures from 14°C to 30°C, but failed to germinate at 34°C (Table 3.1). Overall, there was significant evidence for a difference in germination between 14°C and 30°C ($p = 0.001$, Chi^2 test). Germination was optimal at both 18°C and 22°C for this first temperature experiment. In the second temperature experiment (Table 3.2), there was significant evidence that germination varied over the range of temperatures from 18°C to 25°C ($p = 0.001$). Optimal germination occured at 18°C. As temperatures increased, germination usually decreased.

Table 3.1 Germination responses of *P. gaeumannii* ascospores between 14°C and 34°C. A total of 120 ascospores were examined at each temperature. Data at each temperature reflect combined ascospore counts from both PDA and WA media.

	Germination		
Temp:	Yes	No	Percent
14C	119	1	99.2
18C	120	0	100
22C	120	0	100
26C	116	4	96.7
30C	53	67	44.2
34C	0	120	0

Table 3.2 Germination responses of *P. gaeumannii* ascospores between 18°C and 25°C. A total of 120 ascospores were examined at each temperature. Data at each temperature reflect combined ascospore counts from both PDA and WA media.

	Germination		
Temp:	Yes	No	Percent
18C	86	34	71.7
19C	78	42	65.0
20C	68	52	56.7
21C	62	58	51.7
22C	67	53	55.8
23C	60	60	50.0
24C	29	91	24.2
25C	26	94	21.7

Ascospore germination was not consistently affected by media type. In the first experiment (Table 3.3), ascospore germination was 7.4% greater on WA compared to PDA ($p = 0.006$, Chi^2 test). In the second experiment, however, (Table 3.4), ascospore germination was 9.6% greater on PDA compared to WA ($p = 0.003$, Chi^2 test).

Table 3.3 Germination responses of *P. gaeumannii* ascospores on PDA and WA. Experiment #1. 300 ascospores were examined on each medium. Data for each medium reflects combined ascospore counts from all temperatures between 14°C and 30°C.

	Germination		
Media:	Yes	No	Percent
PDA	253	47	84.3
WA	275	25	91.7

Table 3.4 Germination responses of *P. gaeumannii* ascospores on PDA and WA. Experiment #2. 480 ascospores were examined on each medium. Data for each medium reflects combined ascospore counts from all temperatures between 18°C and 25°C.

	Germination		
Media:	Yes	No	Percent
PDA	261	219	54.4
WA	215	265	44.8

Germination was significantly affected by exposure to dry periods (p-value = 0.002, Table 3.5). Multiple comparisons of dry period treatments showed that germination was significantly reduced when exposure to dry conditions exceeded 24 hours (Figure 3.2). As dry periods increased up to 96 hours, germination decreased in a linear fashion (Figure 3.3).

Figure 3.2 Effect of drying on ascospore germination. Error bars represent standard error of estimate. Multiple comparisons identified two subsets (a and b) which were significant at the .05 level using Tukey's HSD.

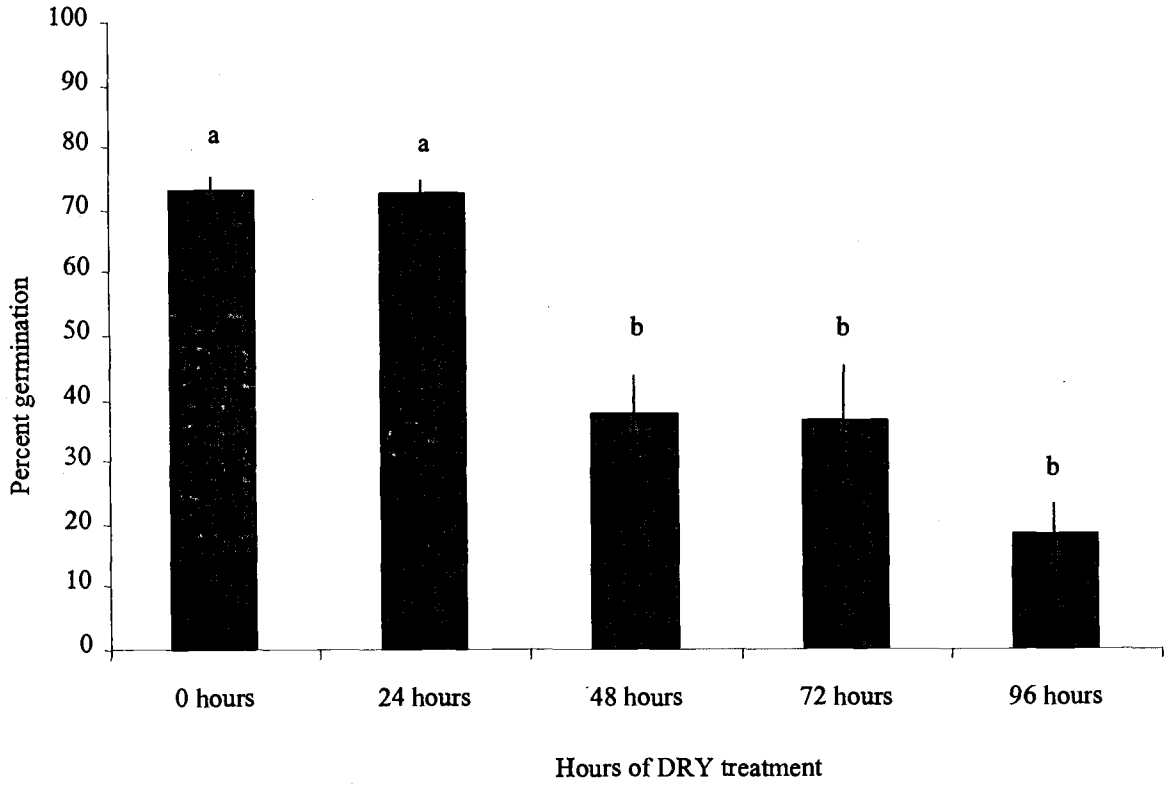


Figure 3.3 Relationship between *P. gaeumannii* ascospore germination and duration of dry period exposure. Data points represent summary of ascospore germination on a single needle (ca. 20 ascospores). Two needles were exposed to each dry period treatment.

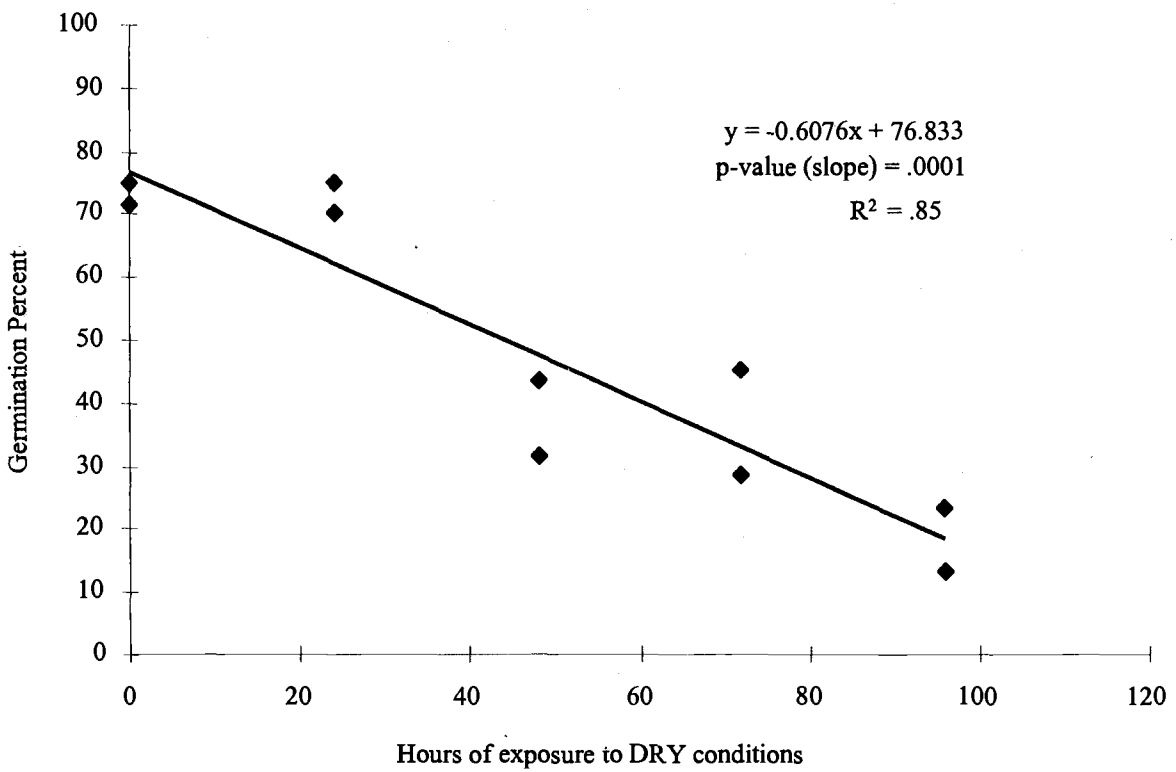


Table 3.5 One-way ANOVA testing for the effect of dry period exposure on germination of *P. gaeumannii* ascospores.

Source of Variation	SS	df	MS	F	P-value
Between Groups	4720.556	4	1180.139	21.45707	0.002391
Within Groups	275	5	55		
Total	4995.556	9			

Germ Tube Growth

Overall, there was a significant difference in germ tube lengths between temperatures ($p = 0.014$, Table 3.6). Germ tube growth was optimal at 22°C for the experiment using 4° temperature intervals (Figure 3.4). However germ tube lengths at 18°C, 22°C, and 26°C were not statistically different based on multiple comparisons. In the second temperature experiment (Figure 3.5), there was no significant difference between temperatures ($p = 0.15$, Table 3.7). The data showed a slight increase in growth between 20°C and 23°C, however, no single temperature stood out as the clear optimum.

Table 3.6 Two-way ANOVA without replication testing for differences in germ tube growth on different media and temperatures for temperatures between 14 and 30°C.

Source of Variation	SS	df	MS	F	P-value
Media	0.354184	1	0.354184	7.767539	0.049462
Temperature	2.472389	4	0.618097	13.55536	0.013512
Error	0.182392	4	0.045598		
Total	3.008965	9			

Table 3.7 Two-way ANOVA without replication testing for differences in germ tube growth on different media and temperatures for temperatures between 18 and 25°C.

Source of Variation	SS	df	MS	F	P-value
Media	0.687022	1	0.687022	50.83343	0.000189
Temperature	0.212931	7	0.030419	2.250714	0.153279
Error	0.094606	7	0.013515		
Total	0.994559	15			

Figure 3.4 Effect of media and temperature on germ tube growth for temperatures between 14 and 30C. Error bars represent the standard error of the estimate. PDA = ▨ WA = ■

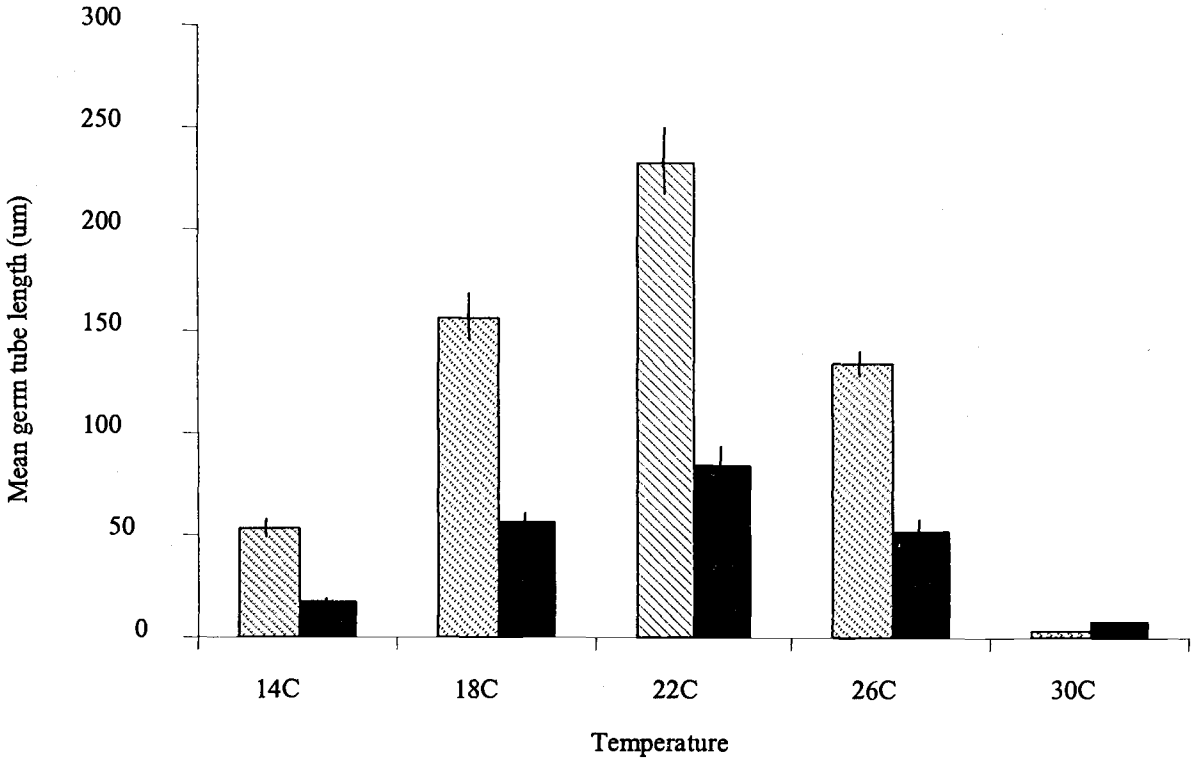
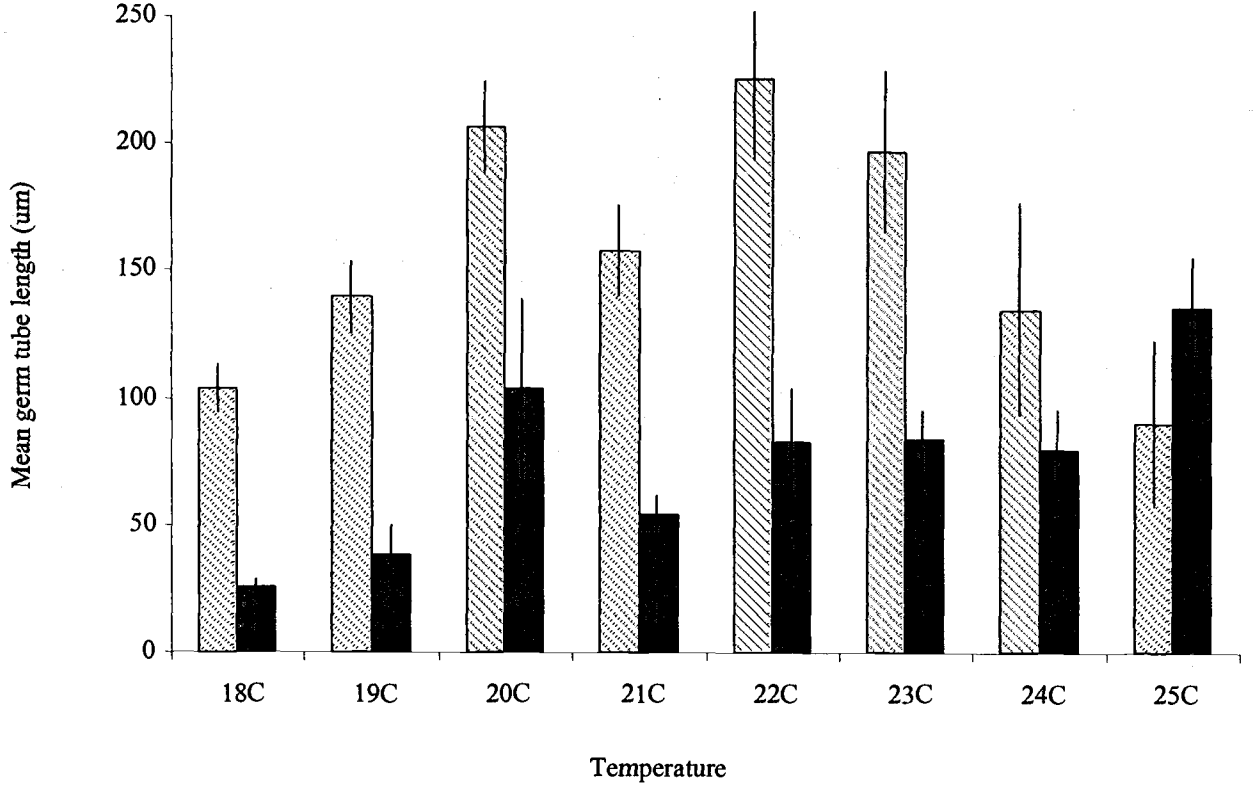


Figure 3.5 Effect of media and temperature on germ tube growth for temperatures between 18 and 25C. Error bars represent standard error of the estimate. PDA = ▨ WA = ■



There was evidence that media type affected germ tube growth (Tables 3.6 and 3.7). Germ tube growth was greater on PDA in the first temperature study (Figure 3.4), ($p = 0.049$). On average, germ tube elongation was 2.4 times greater on PDA than on WA. In the second temperature experiment (Figure 3.5), germ tubes grew 2.6 times more on PDA than on WA ($p = 0.0002$).

There was also evidence that exposure to dry conditions affected germ tube growth (Table 3.8, Figure 3.6). Multiple comparisons of dry period treatments showed that germ tube growth was significantly reduced after only 24 hours exposure to dry conditions. Another significant reduction in growth occurred after exposure to dry conditions for 72 hours. Overall, germ tubes decreased in length as exposure to dry periods increased (Figure 3.7a, b). Germ tubes became asymptotic near the x-axis ($p = 0.008$, testing an x^2 term for curvature), however, the data show an unusual increase in germ tube lengths at 96 hours.

Table 3.8 One-way ANOVA testing for the effect of exposure to dry conditions on growth of *P. gaeumannii* germ tubes.

Source of Variation	SS	df	MS	F	P-value
Between Groups	15842.194	4	3960.5485	213.69	0.0001
Within Groups	92.67	5	18.534		
Total	15934.864	9			

Effect of Moisture Durations on Needle Infection and Colonization

P. gaeumannii hyphae required a minimum of 24 hours of needle surface moisture to infect needles (Table 3.9). Overall, the proportion of needle segments colonized increased with increasing exposure to moisture ($p = 0.0001$, Figure 3.8). Considerable variation was found between trees, however.

Table 3.9 Proportion of needles segments colonized from six moisture exposure treatments.

Tree#	Control	1 hour	1day	3 days	5 days	14 days
1	0	0	0.18	0.60	0.68	0.81
2	0	0	0.37	0.03	0.1	0.81

Figure 3.6 Effect of drying on germ tube growth. Error bars represent standard errors of each estimate. Multiple comparisons identified three distinct subsets (a, b, c) significant at the .05 level using Tukey's HSD.

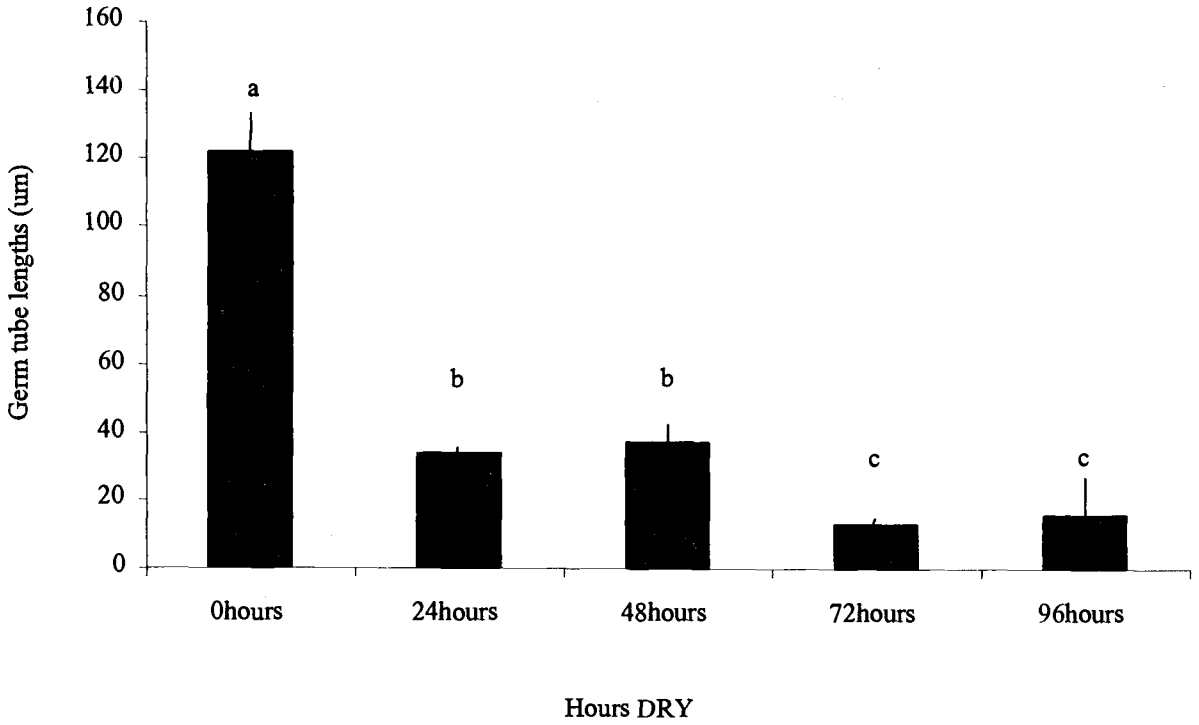


Figure 3.7 Relationship between germ tube lengths of *P. gaeumannii* and duration of exposure to dry periods. Graph A depicts simple linear relationship while graph B includes x^2 term for curvature.

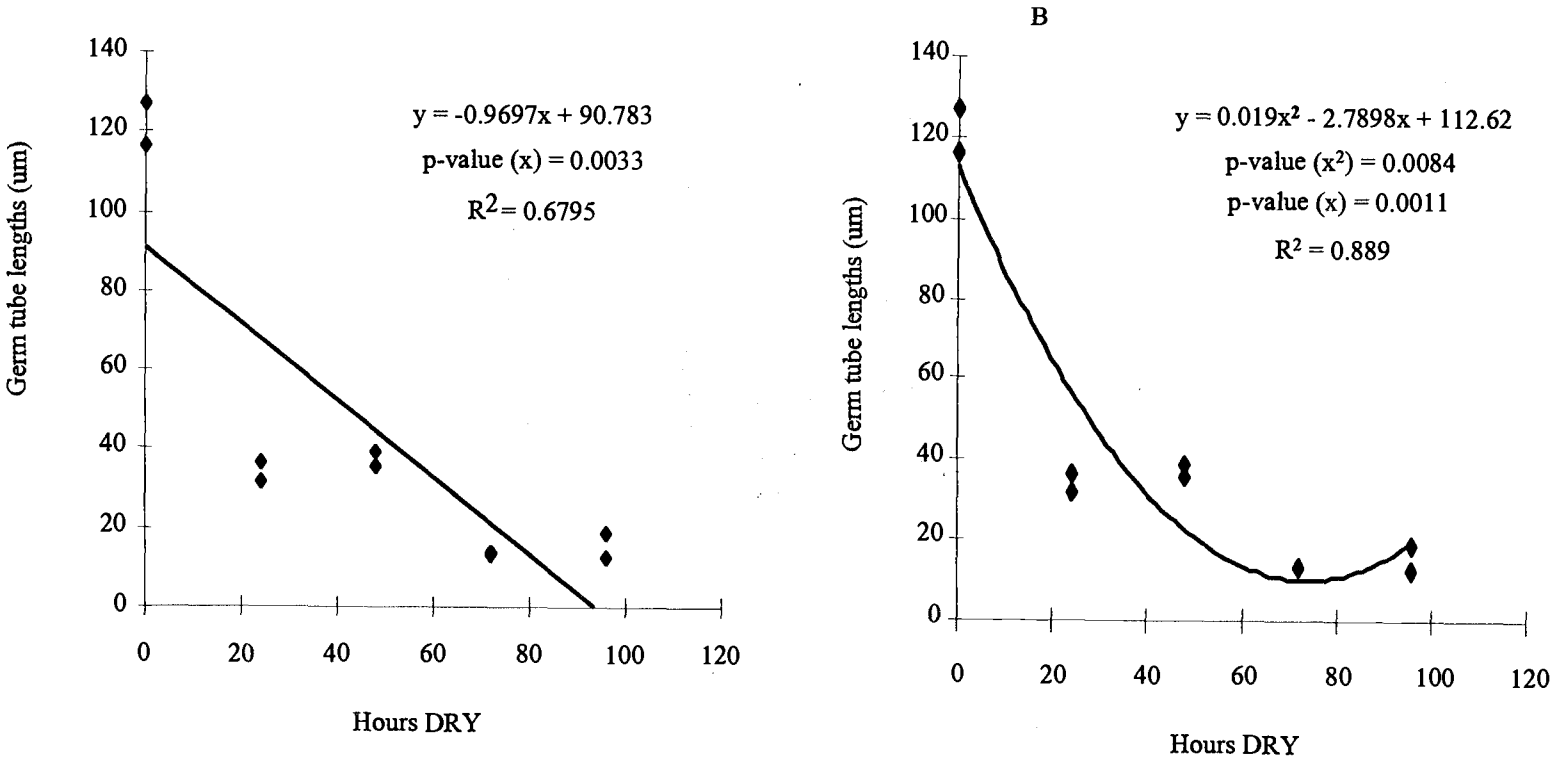
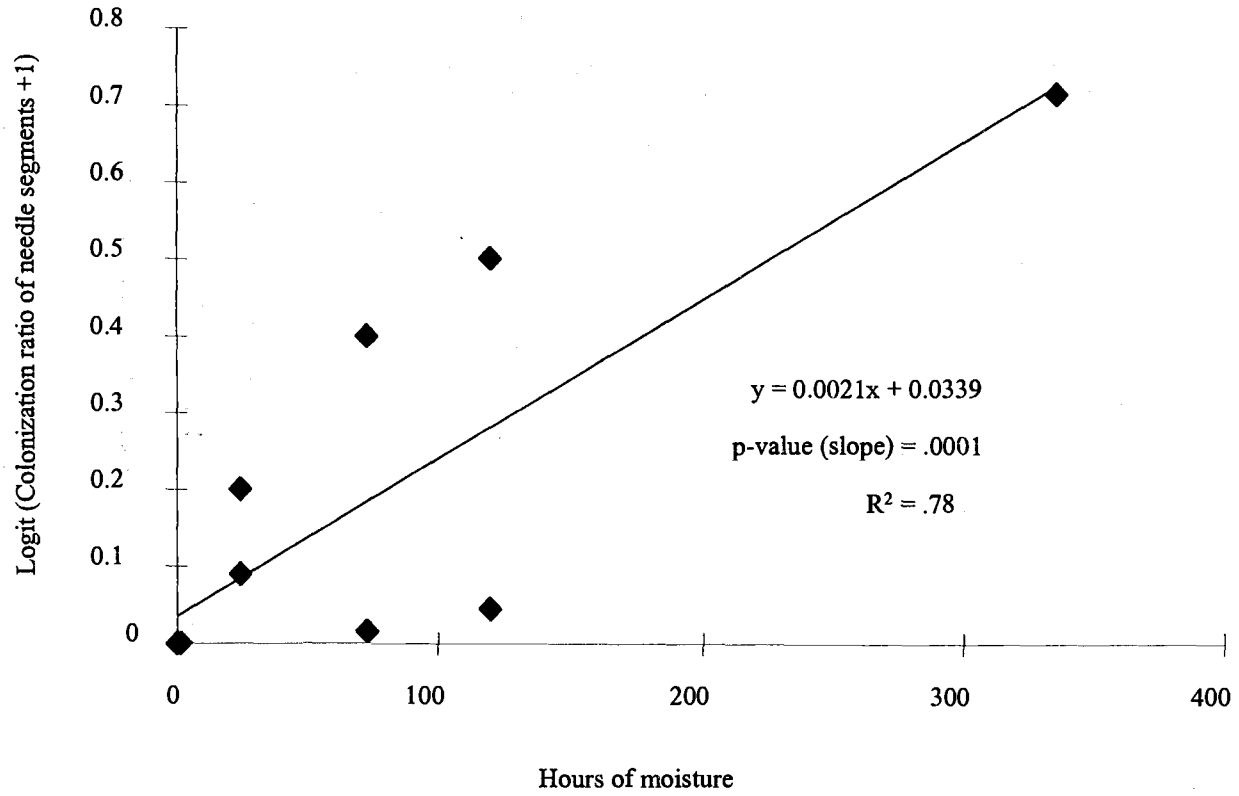


Figure 3.8 Relationship between the ratio of needle segments colonized by *P. gaeumannii* hyphae and the duration of exposure to moisture in a greenhouse mist chamber following inoculation of seedlings with *P. gaeumannii.* Each data point represents summary of colonization ratio from 120 needle segments (20 needles) from each tree. Two trees were used for each moisture class. Response values transformed to the logit scale (log of the ratio of colonized to uncolonized needle segments).



Internal Colonization

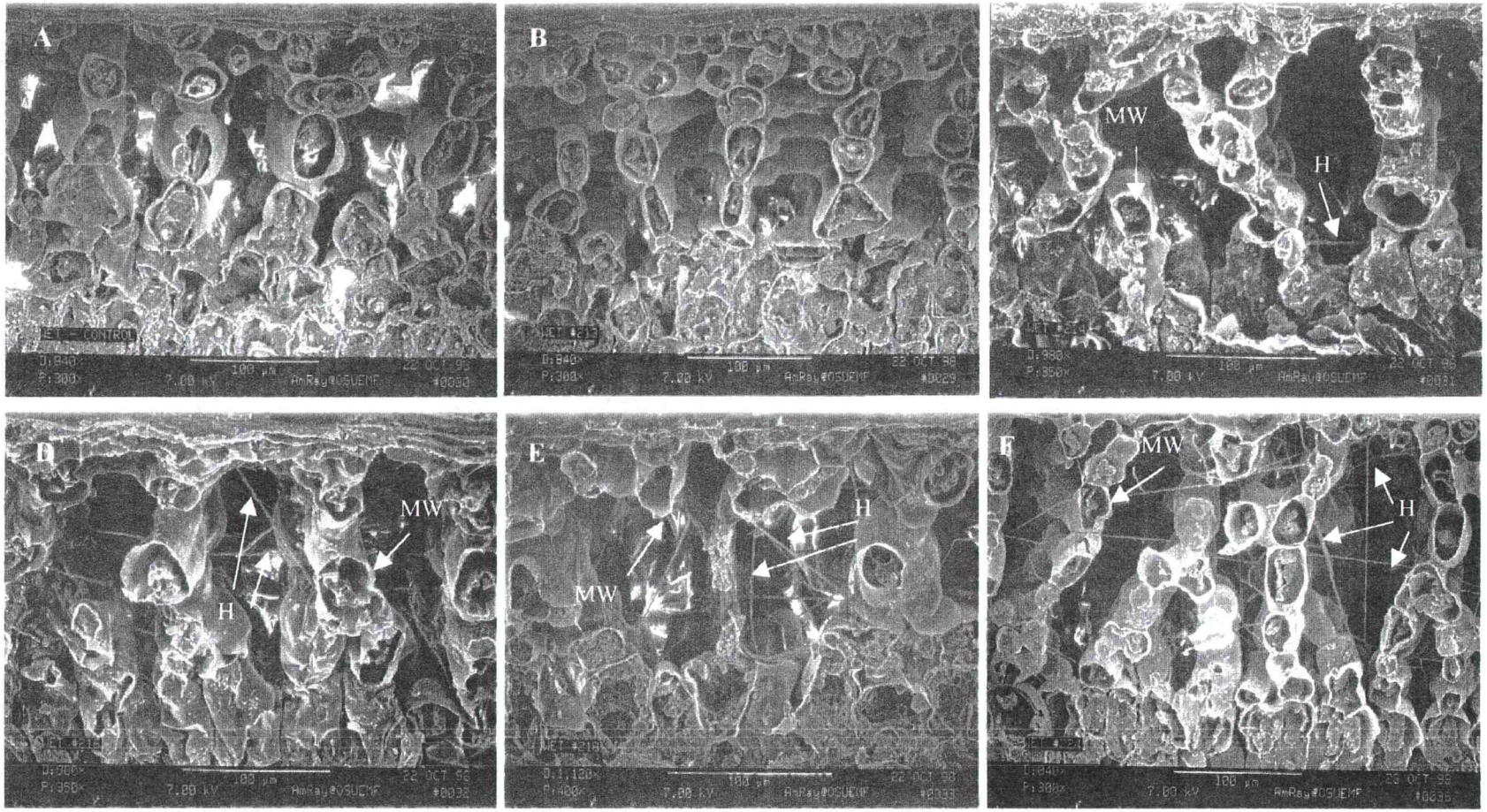
Observations of SEM micrographs revealed that the amount of internal colonization agreed with culture sampling results. Specifically, no hyphae were found in either the control or the one hour treatment (Figure 3.9a, b). However, hyphae were found in needles from the 1 day, 3 day, 5 day, and 14 day treatments (Figure 3.9c-f). In treatments where hyphae were present, the frequency of hyphae increased relative to the length of moisture treatments. In the 1 day treatment, hyphae were present in two out of six needle segments viewed with the scanning microscope. In these two segments, the frequency of hyphal strands was low. On average, one in five fields of view at 300X magnification contained *P. gaeumannii* hyphae. In the 3 day treatment, the frequency of hyphae increased to 4 out of 6 needle segments. In these segments, 3 of 5 fields of view at 300X magnification contained *P. gaeumannii.* In the 5 and 14 day treatments, all segments contained hyphae in all fields of view. More hyphae were present in any given field of view in the 14 day treatment compared to the 5 day treatment.

DISCUSSION

The effects of several environmental factors on *P. gaeumannii* ascospore germination and needle colonization were explored. Ascospore germination occurred at temperatures ranging between 14 and 30°C. No lower temperature limit was observed, however, ascospores failed to germinate at 34°C. Ascospore germination was optimal at 18°C, however there were some inconsistencies between experiments. Germ tube growth was optimal at approximately 22°C, although growth between 18°C and 26°C were not statistically different. No consistent optimum temperature was identified in the second experiment looking at a finer range of temperatures. Media did not significantly affect germination but germ tube growth was better on PDA compared to WA. Exposure to dry conditions significantly reduced germination and germ tube growth. Infection and colonization of needles occurred after only 24 hours when moist conditions were present on needle surfaces. Needle colonization increased with increasing moisture duration, however, there was considerable variation among trees.

While this research has begun to define the effects of temperature, dry periods, and moisture durations on *P. gaeumannii*, several experimental details need to be refined in future experiments. For

Figure 3.9 Colonization of Douglas-fir mesophyll by *P. gaeumannii* following artificial inoculation and exposure of seedlings to various moisture treatments in a greenhouse mist chamber. A. Zero-hour control. No Hyphae found. B. 1 hour treatment. No hyphae found. C. 1 day treatment with hyphae in 2 out of 6 needle segments. D. 3 days with hyphae in 4 of 6 needle segments E 5 days with hyphae in every needle segment. F. 14 days with extensive hyphae in every needle segment. MW = mesophyll cell wall. H = hyphae. SEM micrographs. Bars = 100μm.



example, no statistical differences were found in germ tube growth between 18°C and 26°C. These results indicate that larger sample sizes will be necessary to identify optimum growth temperatures more precisely. Results from the dry exposure experiment showed that germ tube lengths increased slightly at longer exposure times (Figure 3.7). This anomaly may have also resulted from small sample sizes and too few replicate observations. Finally, some inconsistencies in needle colonization were found between trees in the moisture exposure experiment. This may have been related to sampling error or poor inoculation success. In either case, larger sample sizes and more replicate trees would likely have remedied this problem as well.

Some difficulties with obtaining larger sample sizes were, in part, a result of unreliable inoculation techniques. In general, whole tree inoculations which rely on ascospore suspensions have not been successful (see Chapter 2). While ascospores have been collected and concentrated into a water suspension, ascospores either did not adhere to needle surfaces or were too easily washed off following inoculation of seedlings. Of the ascospores that remained on needles, no examples of germination were found. Some of these problems may be remedied by investigating the conditions that favor ascospore adhesion onto needle surfaces. Mycelium inoculations, on the other hand, were effective for ensuring successful infection of seedlings. However, a relatively large amount of time and space is required to grow enough *P. gaeumannii* cultures for large numbers of trees to be inoculated. Inoculation of detached healthy needles (by way of casting ascospores from diseased needles overhead) was somewhat successful, however, this method was unreliable. In particular, the diseased needles chosen as the source of inoculum did not always carry mature pseudothecia ready for sporulation. Additionally, sporulation often resulted in a very heterogeneous and unreliable distribution of ascospores on the target needles. Because of this lack of dependability, inoculation of large numbers of needles was often unsuccessful. In general, there is a need to develop inoculation techniques for *P. gaeumannii* which are more efficient and reliable than current methods.

Further characterization of the temperature and moisture requirements for ascospores and epiphytic hyphae will allow a better understanding of the requirements for disease development. For example, temperature fluctuations have been shown to reduce infection levels for *Cronartium ribicola*

(Hansen and Patton, 1977). Moisture fluctuations and wetting and drying cycles may also affect the viability of germ tubes and epiphytic hyphae (Park, 1982). Other research has shown that germ tube elongation is often more sensitive to desiccation than spore germination (Park, 1982; Peterson and Walla, 1978). In this study, only non-germinated ascospores were subjected to drying. Future studies will be needed to examine the effect of drying on germ tubes and actively growing mycelium. While the effect of temperature and moisture have been examined on ascospores in-vitro, the requirements for spore based infection of intact needles on seedling trees has not yet been investigated. This is largely due to difficulties with spore inoculation techniques. Results from mycelium inoculation experiments showed that infection takes place within 24 hours and that increased moisture duration allowed greater colonization needles. However, understanding the timing between ascospore deposition on needle surfaces and ultimate infection from ascospores is still a critical gap in our knowledge.

While further studies are needed, the results from this research strengthen our understanding of the relationship between environmental factors and disease severity for Swiss needle cast. As noted above, germination and germ tube elongation were significantly reduced when needles were held in dry conditions for longer than 24 hours. Additionally, successful infection of mycelium inoculated seedlings occurred after only 24 hours of available moisture. Most research confirms that fungi require high relative humidity or a film of water before germination or growth can take place and that spores are not capable of withstanding periods of desiccation for longer than 24 to 48 hours (Diem, 1971; Park, 1982). Indeed, most foliar pathogens reach epidemic levels during extended periods of rainfall, cloudcover, and moderate temperatures (Agrios, 1988). Particular locations around Tillamook County are known to receive almost daily occurences of precipitation, even through summer months, in the form of rain, fog, or dew formation. Previous epidemiological studies with Swiss needle cast have shown a strong link between mean infection levels and high amounts of rainfall and other precipitation in Vancouver, B.C. and the northern Cascade mountains (Hood, 1982; Ford and Morton, 1971). The results of this research suggest that such frequent periods of moisture will favor infection and colonization of needles by *P. gaeumannii*, as well as long term epiphytic growth. Given optimal conditions, hyphae which persist on needles throughout fall, winter, and spring months are likely to contribute to disease progress in a cumulative fashion over time.

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Chapter 4. Conclusions

The development of *P. gaeumannii* on Douglas-fir needles proceeded as ascospore germ tubes differentiated into appressoria in the outer stomatal chamber. Penetration pegs grew between the guard cells and into the needle mesophyll. Internal hyphae were observed only in intercellular spaces but were often well attached to host cells. Epiphytic growth of *P. gaeumannii* on needles was extensive and persisted on needle surfaces throughout summer, fall, winter, and spring months. These hyphae continued to produce appressoria and penetrate stomata over time. Hyphal incidence on needle surfaces and inside needles was generally greater at high disease sites compared to low disease sites. No asexual stage was observed, however, new strands of hyphae grew from pseudothecial initials as these emerged from stomata in fall and winter months. These hyphae also persisted on needle surfaces.

Ascospore germination and growth were relatively sensitive to temperature and moisture conditions. Ascospores germinated and grew over a range of temperatures from 14 to 30°C. Optimum germination and growth occurred at 18°C and 22°C respectively. Dry conditions for more than 24 hours significantly reduced both germination and germ tube elongation. Using inoculated seedlings, successful infection of needles occurred after 24 hours of exposure to moisture in a greenhouse mist chamber. As moisture periods increased, the overall colonization of needles by *P. gaeumannii* hyphae also increased.

This research may be instructive for understanding how *P. gaeumannii* compares to other foliar pathogens on conifers. Most other needle pathogens produce transient germ tubes whose function is to gain immediate ingress into needles (Millar, 1981). In addition, pathogens such as *Lophodermium*, *Dothistroma*, *Cronartium*, and *Rhabdocline* derive nutrition from their host by penetrating host cells directly via hyphae or, in the case of rust pathogens like *Cronartium*, producing haustoria which are specialized for nutrient absorption (Diwani and Millar, 1986; Ferris, 1967; Patton and Johnson, 1970; Peterson and Walla, 1978). In contrast, no obvious evidence was found to suggest than *P. gaeumannii* obtains nutrition from host needles through direct colonization of mesophyll cells. Instead, only intercellular hyphae were present, albeit many of these were well attached to the outside of mesophyll walls. These findings suggest that *P. gaeumannii* may obtain nutrition either from direct diffusion across the host apoplast, or by absorption of solutes in the intercellular region of needles. The large degree of

epiphytic growth also contrasts markedly with the overt pathogens mentioned. Such extensive and persistent growth suggests that *P. gaeumannii* is also capable of obtaining nutrition from needle surfaces. These observations also suggest some ecological similarity between *P. gaeumannii* and black mildews in the families Meliolaceae and Asterinaceae. Fungi such as *Rasutoria, Stomiopeltis,* and *Eupelte* also produce extensive mats of superficial hyphae on needle surfaces and can penetrate needles through stomata (Pirozynski and Shoemaker, 1970; Stone, 1997). Similar to *P. gaeumannii*, the surface mycelia of these fungi are characteristically large in diameter (2-4μm), melanized, frequently branched, and closely appressed to host epidermal cells. These patterns of growth offer ecological advantages for organisms in phylloplane habitats, including the ability to withstand moderate fluctuations in temperature, moisture, and solar radiation (Dickinson, 1976; Dickinson, 1986; Park, 1982). Although their nutritional modes are varied, members of the Meliolaceae and Asterinaceae do not aggressively colonize and injure host tissues. In some instances, however, they may cause chlorosis and premature abscission of older needles (Stone, 1997). Although the nutritional relationships between *P. gaeumannii* and host cells needs further characterization, there was very little evidence to suggest that *P. gaeumannii* is an aggressive pathogen similar to rust species like *Cronartium*, or needle pathogens such as *Dothistroma* and *Rhabdocline*.

The epiphytic colonization strategy of *P. gaeumannii* may help to explain the current Swiss needle cast epidemic along the Oregon coast. Until the last decade, Swiss needle cast was not considered injurious to forest plantations of Douglas-fir in its native range (Boyce, 1940; Meinecke, 1939). Over the last half-century, however, reforestation practices have been introducing greater levels of Douglas-fir in a region historically dominated by spruce, hemlock, alder, and only a small percentage of Douglas-fir. This area corresponds roughly to what is considered the fog zone, and is characterized by a moderate climate with warm winters and relatively cool summers. Frequent periods of precipitation such as rainfall, fog, and heavy dew are common along this coastal strip. As described by Agrios (1988), most foliar pathogens depend on the presence of free moisture on host surfaces only during germination of their spores. After penetration into host cells, they become reliant on host cells for nutrients and water. Moderate coastal climates with frequent periods of moisture will favor the initial stages of disease development including sporulation, germination, and infection. Because *P. gaeumannii* has an epiphytic colonization strategy, however, the cumulative amount of leaf moisture throughout the year will also affect the ability of *P.*

gaeumannii to colonize host needles year-round. As suggested by inoculation studies in this research, increased moisture on needle surfaces favored colonization by fungal hyphae. As epiphytic growth increases, stomatal penetration and internal colonization of Douglas-fir needles would also increase. Hyphae which persist on needles throughout fall, winter, and spring months may, therefore, contribute to disease progress in a cumulative fashion over time, given optimal conditions.

Some aspects of the biology of *P. gaeumannii* suggest potential control options for Swiss needle cast. For example, identifying sites where moisture events and mean temperatures are suitable for *P. gaeumannii* germination and growth may allow more strategic planning and management of Douglas-fir plantations. The use of environmental, or remote sensing technologies, may help in choosing low risk areas for Douglas-fir at the landscape level (Rosso, 1998). Second, where fungicide treatments are practical or desirable, control programs may wish to introduce additional applications of fungicides in fall months. Such treatments may control additional disease development due to epiphytic growth and colonization.

While the environment in coastal climates like Tillamook County may be advantageous for spore germination, infection, and increased epiphytic colonization, this explanation may not entirely account for the current epidemic. Factors such as poorly adapted Douglas-fir seed sources, and increases in inoculum level may also contribute to the current situation. As noted by several investigators, the few Douglas-fir plantations existing in Tillamook County 10 to 20 years ago appeared relatively green and healthy. Many of those same plantations are now severely diseased (Hansen, pers. comm.). These observations suggest that environment alone was not sufficient for severe disease in some of these originally healthy stands. One possible explanation for increased disease levels is that ascospore inoculum levels may have been increasing coincident with the rise in young Douglas-fir plantations. The historically small numbers of host species may have kept *P. gaeumannii* spore populations small. Such an explanation would account for the few healthy plantations seen 10 to 20 years ago. To date, these hypotheses have not been adequately addressed due to technical difficulties with obtaining ascospores for quantitative epidemiology studies. However, molecular detection methods are currently being developed which may allow quantitative estimates of ascospore inoculum in the field. The ability to perform quantitative epidemiology should aid in determining the effect of ascospore inoculum levels on disease severity.

Inappropriate seed sources has also been suggested as a contributing factor to disease severity. In replanting the Tillamook burns in the 1930's, seed was gathered from various geographical regions around Oregon. At present, many of the young plantations in Tillamook County which are severely diseased are planted with seed collected from these Tillamook burn sites. Because of poor records, the actual origin of much of this seed is still unknown (Hansen, pers. comm.). General observations as well as provenance trials have shown that coastal seed sources are more tolerant to Swiss needle cast (Boyce, 1940; Hood, 1971; Hood, 1982; McDermott and Robinson, 1989). In general these studies show that variation among provenances does exist, and that provenances from inland, higher elevation areas were more susceptible to Swiss needle cast, while low elevation coastal provenances were more tolerant. McDermott and Robinson (1989) also noted that this provenance variation was correlated with rainfall so that "provenances from locations with higher rainfall, and presumably higher selective pressure for disease resistance, expressed higher resistance to Swiss needle cast."

This research has shown that fungal development on and within needles is extensive and will likely be favored by conditions found along the Oregon coast. However, the current Swiss needle cast epidemic is likely the result of several weak points in the disease triangle. According to this model, disease outbreaks require a favorable environment, a pathogenic organism, and a susceptible host (Agrios, 1988). Sustainable control measures for the current epidemic, therefore, must rely on a thorough understanding of disease biology, careful selection of environments for Douglas-fir plantations, and the use of suitable host provenances.

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