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The histopathology of *Phaeocryptopus gaeumannii* on Douglas-fir needles

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Abstract: Germinating ascospores of *Phaeocryptopus gaeumannii* produce suprastomatal appressoria from which penetration pegs enter needles. Initial infection occurs between late May and early Jul and coincides with budbreak and shoot elongation. Colonization within needles is exclusively intercellular and increases continuously during Jul–May. No intracellular hyphae or haustoria were observed, but hyphae closely appressed to mesophyll and palisade cell walls are abundant by 3–5 mo after initial infection. Pseudothecial primordia begin to form in epistomatal chambers Oct–Apr, 4–9 mo after initial infection. Pseudothecial primordia developing in the epistomatal chamber are connected to the endophytic thallus by specialized cells in the substomatal chamber that have thickened apical walls and resemble phialides but are not involved in asexual reproduction. The apical wall thickenings instead appear to function as reinforcement against the turgor pressure of the guard cells, allowing cytoplasmic continuity to be maintained between the developing pseudothecium and vegetative hyphae within the needle. Concurrent with the formation of pseudothecial primorida, epiphytic hyphae emerge from the periphery of developing pseudothecia, grow across the needle surface, form numerous anastomoses and reenter the needle by producing appressoria above unoccupied stomata. Epiphytic hyphae and their associated appressoria gradually become more abundant during Oct–Jan.

Key words: ascocarp development, capnodiales, foliage pathogen, Swiss needle cast

INTRODUCTION

Phaeocryptopus gaeumannii (T. Rohde) Petr. first attracted notice in Switzerland in 1925 (Gäumann 1930) because of its association with severe defoliation in Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) plantations. This disease was called Swiss needle cast to distinguish it from Rhabdocline needle cast of Douglas-fir. The fungus subsequently was found to be widespread in western North America, where it apparently had escaped notice due to its inconspicuous habit and negligible effect on native Douglas-fir. The earliest confirmed records of *P. gaeumannii* in western North America are herbarium specimens collected by J.S. Boyce from Oregon and California in 1916 (Boyce 1940). Subsequent surveys have shown the fungus to occur on both coastal (*P. menziesii* [Mirb.] Franco var. *menziesii*) and interior (*P. menziesii* var. *glauca* [Beissn.] Franco) forms of Douglas-fir throughout the natural range of the host (Boyce 1940, Hood 1982). The fungus also has been reported from *Pseudotsuga macrocarpa* (Vasey) Mayr in New Zealand (Gadgil 2005), although it is not known to occur naturally on this host in North America.

Citing records of *P. gaeumannii*'s widespread distribution on native Douglas-fir, which preceded the Swiss needle cast outbreak in Europe, Boyce (1940) expressed the opinion that it is probably indigenous to the Pacific Northwest, where the fungus is prevalent but apparently harmless. Until recently Boyce's assessment of *P. gaeumannii* as a pathogen of negligible importance in western North American forests has been widely accepted. A survey of forest trees in southern British Columbia and northwestern Washington found the fungus widespread but with little associated loss of foliage (Hood 1982). However, as commercial Douglas-fir cultivation has expanded in the western Oregon Coast Range, where climate conditions are favorable for growth of the pathogen, Swiss needle cast has become a much more significant forest health problem than earlier in the past century (Hansen et al 2000, Manter et al 2005). Diseased trees are characterized as having chlorotic foliage, premature needle abscission and reduced height and diameter growth. In severely diseased stands trees may lose all but current-year foliage (Hansen et al 2000) and tree volume growth may be reduced 23–50% or more (Maguire et al 2002).

The careful observations of Rohde (1937) and Steiner (1937) first provided the essential details of the infection cycle and established that *P. gaeumannii* was a pathogen distinct from the similar conifer needle fungi *Asterina nuda* Peck (≡ *Phaeocryptopus nudus* [Peck] Petrak) and *Dimerosporium balsamicola*

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(Peck) Ell. & Everh. Subsequent reports published on the epidemiology and life history of *P. gaeumannii* have added details that generally reinforce the conclusions reached by Rohde and Steiner (Boyce 1940, Peace 1962, Ford and Morton 1971, Chen 1972, Hood and Kershaw 1975, Chastagner and Byther 1983, Michaels and Chastagner 1984, Hansen et al 2000). Asci mature during Apr–Jun in western Oregon and Washington, and ascospores are produced and infect needles mid-May–Jul (Rohde 1937, Chastagner and Byther 1983, Michaels and Chastagner 1984, Stone et al unpubl). Newly emerged needles are considered the primary substrate for new infection by ascospores (Rohde 1937, Chastagner and Byther 1983), although Rohde (1937) considered infection of older needles significant in disease and Hood and Kershaw (1975) reported sparse infection of previously unexposed 1 y old needles. Colonization of needles occurs in fall and winter, culminating in the emergence of pseudothecia from stomata in late winter and spring (Steiner 1937, Boyce 1940).

Pseudothecia form in the epistomatal chamber and are visible as minute black spheres aligned with rows of stomata (Boyce 1940, Stone and Carroll 1985). Pseudothecial primordia can begin to develop as early as 4 mo after initial infection of newly emerged needles (Hood and Kershaw 1975, Michaels and Chastagner 1984, Stone et al unpubl) but often are not visible until 9 mo after initial infection (early spring) or later (Boyce 1940). Pseudothecia are produced successively on foliage each year after infection, the number of pseudothecia increasing annually until needles are shed (Boyce 1940, Hansen et al 2000).

Entry through stomata has been suggested as the mode of infection for *P. gaeumannii* (Chen 1972, Fatuga 1978). Fatuga (1978) observed fungal cells in the stomata of foliage collected in winter months and concluded that these cells were germinating ascospores infecting through the stomata, although ascospore maturation has been reported only in late spring (Michaels and Chastagner 1984). Chen (1972) also observed hyphae on needle surfaces contiguous with hyphae in stomatal chambers and inferred that *P. gaeumannii* entered needles through stomata. However because few histological details were reported it is unclear whether the hyphae observed in either study actually originated from ascospores or whether the hyphae were entering and not emerging from stomata. Epiphytic vegetative hyphae of *P. gaeumannii* emerging from stomata during early development of pseudothecia were observed by Stone and Carroll (1985) and might account for the observations of Chen (1972) and Fatuga (1978). The present study was undertaken to further investigate the infection cycle of *P. gaeumannii*, specifically to examine the details of penetration, internal colonization, proliferation of epiphytic hyphae, and pseudothecial development.

MATERIALS AND METHODS

Ascospore germination and needle penetration.—One-year-old Douglas-fir seedlings, grown in an indoor container nursery (Pelton Reforestation Services, Maple Ridge, British Columbia) and assumed not to have been exposed to *P. gaeumannii* inoculum, were used for artificial inoculation and subsequent histological examination. Seedlings were transplanted into potting media in 8 L plastic pots and maintained at the Oregon State University Botany Field Lab. A single coastal Douglas-fir seed source from the Tillamook, Oregon, area was used for all studies.

Because *P. gaeumannii* does not sporulate in culture, ascospore germination and early stages of infection were observed on needles detached from potted seedlings and immediately exposed to *P. gaeumannii* ascospores. Inoculation of detached needles was performed by suspending field-collected infected needles with mature pseudothecia above uninfected, newly emerged detached shoots. Infected needles were affixed to Petri dish lids with petroleum jelly and suspended above the detached shoots that had been placed on moistened filter paper in the base of the Petri dish. Petri dishes were kept in an incubator in the dark at 18 C for 1 h to allow ascospore discharge onto the needles after which the lids were replaced with clean ones. Inoculated needles were maintained at 18 C in darkness and at various intervals were sampled and fixed for microscopic examination, described below, of the infection and colonization processes.

Ascospore germination and needle penetration also were observed on attached needles of potted seedlings exposed to natural inoculum in a Douglas-fir plantation. Potted Douglas-fir seedlings were exposed to natural inoculum at the Salal progeny field site (see Hansen et al 2000) for 1 wk in early Jun, coinciding with shoot emergence and ascospore release. The exposed seedlings were moved to the Oregon State University Botany Field Lab and kept 12 mo. Foliage was collected at weekly intervals Jun–Aug 1997 and monthly thereafter for microscopic examination.

Foliage inoculation with macerated mycelium.—To compare infection in seedlings inoculated with ascospores from field-collected needles, described above, to infection with a known source of *P. gaeumannii*, seedlings were inoculated with macerated mycelium generated from single spore isolates grown in stationary liquid culture. To obtain single-spore isolates, Douglas-fir needles from the Juno Hill site (see Hansen et al 2000) bearing mature pseudothecia were affixed to Petri dish lids and suspended above the surface of 2% water agar to allow ascospore discharge for approximately 1 h. Individual spores were removed from the agar surface and transferred to potato dextrose malt yeast extract agar (PDMYA: 39 g potato dextrose agar, 1 g yeast extract, 1 g malt extract, [Difco, Becton Dickinson Diagnostics, Sparks, Maryland] and 1 L dH_20). After 1 mo on solid agar

a small piece of the *P. gaeumannii* agar culture was transferred to 500 mL malt broth (Difco, 1.5% w/v) and incubated 2 mo at 19 C.

For preparation of inoculum, fungal cultures were filtered and rinsed with sterile distilled water. The resulting mycelia were suspended in sterile distilled water (20 g fw of *P. gaeumannii* mycelium to 1 L dH_2O) and macerated 60 s in a tissue homogenizer (Brinkmann Instruments, Westbury, New York). Final concentration of the inoculum was adjusted to 1×10^5 fragments per mL with the aid of a haemocytometer. A portion of the macerated inoculum was diluted serially in sterile water and plated on malt agar to estimate the concentration of viable fragments. The macerated mycelium suspension was sprayed onto the foliage of potted seedlings to saturation by means of an airbrush aerosol spray apparatus. Seedlings were kept in a mist chamber 24 h after inoculation, then moved to a shade house at the Oregon State University Botany Field Lab.

Observations on field-collected foliage.—Infection and colonization were observed on Douglas-fir needles collected from naturally infected trees from six forest plantations in northern Oregon Coast Range (Hansen et al 2000). A single 10–15 y old tree was selected for foliage sampling from each site. Current-year needles were sampled from the lower crown, at approximately 1.8 m, from a secondary lateral branch from the north side of each tree at each site. Current-year needles were collected in Jun, Jul, Nov and Dec 1996, Jan and Feb 1997, as well as Sep 1997 and Mar 1998, to supplement observations from the first year of sampling.

Needle surface impressions.—Plastic impressions of needle surfaces were used to observe several stages of fungal development on needle surfaces from naturally infected trees in field plantations as well as on detached needles from potted seedlings inoculated in the laboratory. Abaxial needle surfaces were painted with clear fingernail polish and allowed to dry 1 h. Impressions were peeled from the needle surface and mounted on a microscope slide in a drop of 0.05% trypan blue in lactoglycerol, covered with a cover slip and sealed with fingernail polish. Plastic impressions from approximately 450 needles were used to examine fungal development.

Plastic impressions also were used for measurements of the abundance of *P. gaeumannii* hyphae on needle surfaces and the rates of epiphytic hyphal growth over time. Foliage was collected monthly Jun–Feb from six forest plantations in northern Oregon Coast Range chosen to represent a range of elevations and climate conditions typical for Douglas-fir management (Hansen et al 2000). For each collection time at each site, foliage was collected from a secondary lateral branch from one arbitrarily selected tree. The presence or absence of hyphae on needle surfaces was estimated from 10 randomly chosen fields of view at 400× with standard bright field microscopy for five current-year needles.

The abundance of epiphytic 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. Abundance of appressoria from epiphytic hyphae also was compared between one site characterized as having severe Swiss needle cast symptoms (Juno Hill) and a site with moderate disease (Upper Stone). Needle impressions were examined as described above and numbers of appressoria above stomata in each field of view were recorded. ANOVA (Statgraphics v. 5.1, Manugistics Inc., Rockville, Maryland) was used to compare abundance of appressoria from epiphytic hyphae between the two sites and at different sampling times.

Plastic embedded tissue.—Anatomical details of initial penetration and development of pseudothecia in stomata were examined in sections of plastic-embedded tissue by light microscopy. Needles were cut into approximately 5 mm segments and fixed in 3% glutaraldehyde in 0.1 M, pH 7.4 phosphate buffer under vacuum overnight. Segments were rinsed in two changes of buffer and dehydrated in a graded ethanol series through 95%, followed by infiltration with Historesin (Leica Microsystems, Bannockburn, Illinois). Embedded needle segments were cut into cross sections (perpendicular to needle long axis), approximately 15 μm thick, with a rotary microtome and steel knife. Sections were stained 5 min in acid-fuschin malachite green (Alexander 1969), rinsed with dH_20, and mounted on glass slides in Permount (Fisher Scientific, Fairlawn, New Jersey). Sections were examined under a Zeiss Axioskop 2 with standard bright field optics.

Cleared needles.—Cleared whole needles provided an additional means of observing internal foliage colonization and development of pseudothecia and epiphytic hyphae by means of light microscopy. Douglas-fir needles used for clearing were collected from naturally infected foliage from forest plantation sites and from potted seedlings exposed to natural inoculum at the Salal field site for 1 wk in Jun 1997 and maintained at the Oregon State University Botany Field Lab for 12 mo. Needles were collected at various intervals, placed in 10% KOH in 20 mL vials at 60 C and the solution was changed as needed until the needles became uniformly pale, approximately 7 d. The cleared needles were rinsed twice with dH_2O; the solution acidified with a few drops of 1N HCl followed by several drops of 30% H_2O_2. After 1 h the needles were rinsed twice in dH_2O, the excess water drawn off and then stained by gently heating for several minutes in aqueous 0.05% trypan blue. The excess stain was drawn off and needles were dehydrated in a graded ethanol series through 100% ethanol, 1:1 ethanol/xylene, then 100% xylene. Needles were mounted on microscope slides in Permount (Fisher Scientific), covered with 22×50 mm cover slips secured with small lead weights and allowed to cure 72 h. Slides were examined under a Zeiss Axioskop 2 with standard bright field optics.

Scanning electron microscopy.—Scanning electron microscopy (SEM) was used to observe internal colonization and the development of pseudothecia. SEM observations were made on needles from potted seedlings exposed to natural inoculum at the Salal field site and maintained at the Oregon State University Botany Field Lab. Additional SEM

observations were made on needles collected from field sites in Dec 1996, Jul 1997, Nov 1997 and May 1998 and mycelium-inoculated potted seedlings as described above. Needles were cut into 5 mm long segments, cut into longitudinal sections or cross sections to expose the needle interior. Segments were fixed in 3% glutaraldehyde in 0.1 M, pH 7.4 phosphate buffer in a vacuum chamber overnight, dehydrated in a graded ethanol series through 100% and dried by exchange with liquid CO_2 in a critical point drying apparatus (Balzers AG, Balzers, Switzerland). Dried needle segments were attached to aluminum SEM mounts, sputter coated with gold palladium and examined with an AMRAY 3300FE scanning electron microscope.

Culture sampling.—Internal fungi were cultured from mycelium-inoculated potted seedlings to verify the presence of *P. gaeumannii*. For culture sampling, needles first were surface sterilized by immersion in 95% ethanol for 1 min, 50% chlorine bleach (2% NaOCl) 10 min and a final rinse in 95% ethanol 1 min. Needles were cut aseptically into 5 mm long segments and placed on Petri dishes containing PDMYA and incubated 4 wk. Cultured needle segments were checked daily and scored for the presence of *P. gaeumannii*, which was recognized by dense growths of darkly pigmented, thick walled, slow growing sterile mycelium.

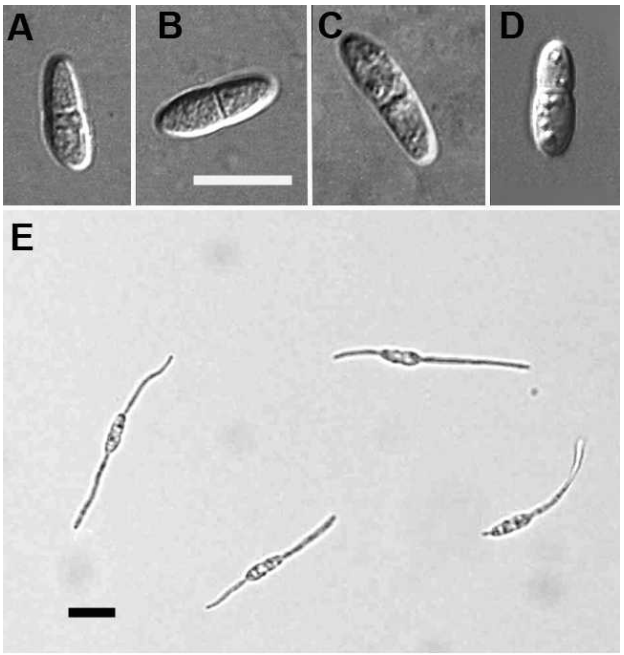

FIG. 1. Ascospores of *P. gaeumannii*. A–D. Typical ascospores. E. Ascospore germination on water agar. Bars = 10 μm.

RESULTS

Ascospore germination and hyphal growth on needle surfaces.—Ascospore germination was observed on plastic impressions of needle surfaces from seedlings exposed to natural inoculum at field sites and on detached needles inoculated in the laboratory. Ascospores are hyaline, ellipsoidal with obtuse ends, approximately 5.5 × 13 μm, two-celled with a median to slightly submedian septum, slightly constricted at the septum, the basal cell slightly narrower and tapering toward its base (FIG. 1). Germinating ascospores developed germ hyphae from polar ends of both cells (FIGS. 1E, 2A, C–F). Ascospores having a lateral germ hypha were infrequent. Germ hyphae of *P. gaeumannii* initially were hyaline, becoming pale olive-brown when up to 20 μm long, then becoming dark brown to black. Profuse growth of germ hyphae on needle surfaces, with irregular branching and frequent anastomoses was common on field-collected foliage (FIG. 2). Germ hyphae appeared to grow in random directions on needle surfaces. Hyphae were observed growing in the depressions between epidermal cells as well as across epidermal cells at various angles (FIG. 2). There was no indication that needle topography consistently influenced hyphal orientation.

Identification of *P. gaeumannii* hyphae on surfaces of needles collected from the field was based on several characters, taken in part from plastic impressions and also from observations of germinating ascospores on agar. Often identification of *P. gaeumannii* could be made based on the association of hyphae with ascospores or pseudothecia, and other foliar fungi could be identified based on hyphae associated with spores or fruiting structures different from *P. gaeumannii*. Hyphae of other species were typically narrower, hyaline, less convoluted in growth form and usually absorbed trypan blue stain more readily than hyphae of *P. gaeumannii*. However two common epiphytic fungi found on Douglas-fir needles, *Stomiopeltis* sp. and *Rasutoria pseudotsugae* (V.A.M. Mill. & Bonar) M.E. Barr, have dark-pigmented hyphae similar to that of *P. gaeumannii*. Hyphae of these two species were distinguished from *P. gaeumannii* by their dense, interwoven growth and roughly circular thalli on the needle surface (*Stomiopeltis* sp.) or their relatively larger, setose fruiting bodies that are produced superficially (*R. pseudotsugae*) instead of in stomata (*P. gaeumannii*).

Needle penetration.—Upon contacting a stoma, germ hyphae differentiated into thick-walled appressoria. Appressoria frequently were observed forming on short lateral branches from main germ hyphae, as well as forming laterally from germinating ascospores deposited next to, or directly above, stomata (FIGS. 2, 3). Single germ hyphae frequently produced several appressoria on lateral branches in succession. The subtending hyphae continued to elongate, some

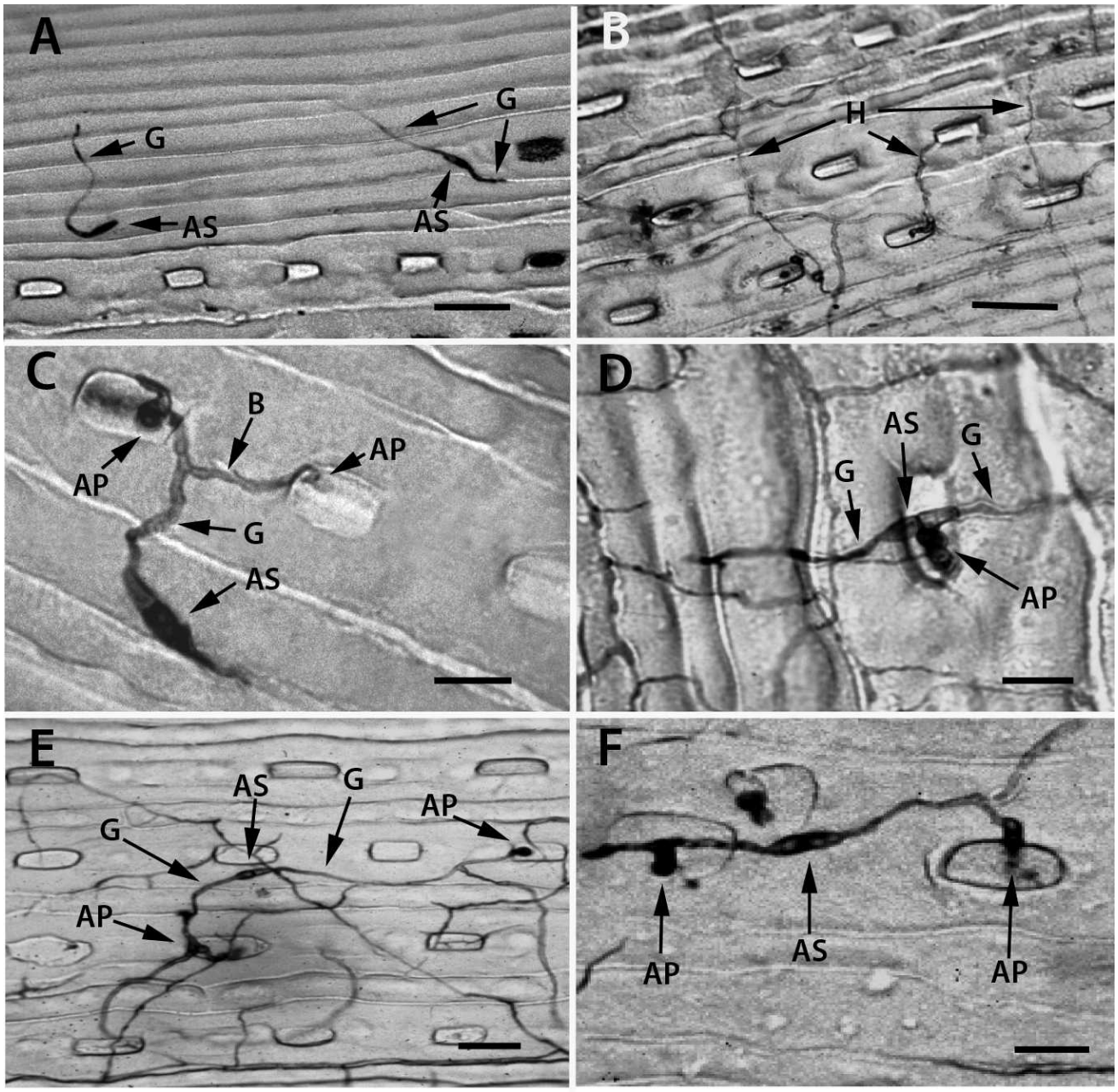

FIG. 2. Ascospore germination on needle surfaces from plastic impressions. A. Ascospore (AS) germination at approximately 24 h with polar germ hyphae (G). B. Germination at approximately 48 h with extensive hyphal growth (H) growth across and along epidermal cells. C. Ascospore germination at 48 h showing ascospore (AS), branched germ hypha (G). Appressorium (AP) has formed above the stoma on one branch of the hypha, and a second appressorium is being formed at the end of the other branch (B). D. Appressorium (AP) has formed directly from the side of an ascospore (AS) that was deposited on a stoma. Germ hyphae (G) also grew from the poles of the ascospore. E. Ascospore germination at 48 h. Germ hyphae (G) from the same ascospore (AS) formed two lateral appressoria (AP) above separate stomata. F. Two appressoria (AP) formed on each of the polar germ hyphae arising from a single ascospore (AS) above separate stomata. Bars: A, B = 20 μm; C–F = 10 μm.

more than 200 μm, contacting multiple stomata (FIGS. 2, 3). Several individual germ hyphae were observed with five or more successive lateral appressoria above multiple stomata. Two appressoria occasionally were observed above a single stoma (FIG. 3D).

Details of the penetration process were inferred from observations of 24 cross sections in which appressoria and penetration hyphae were present. In each instance an appressorium was produced above the epistomatal chamber. From this appresso-

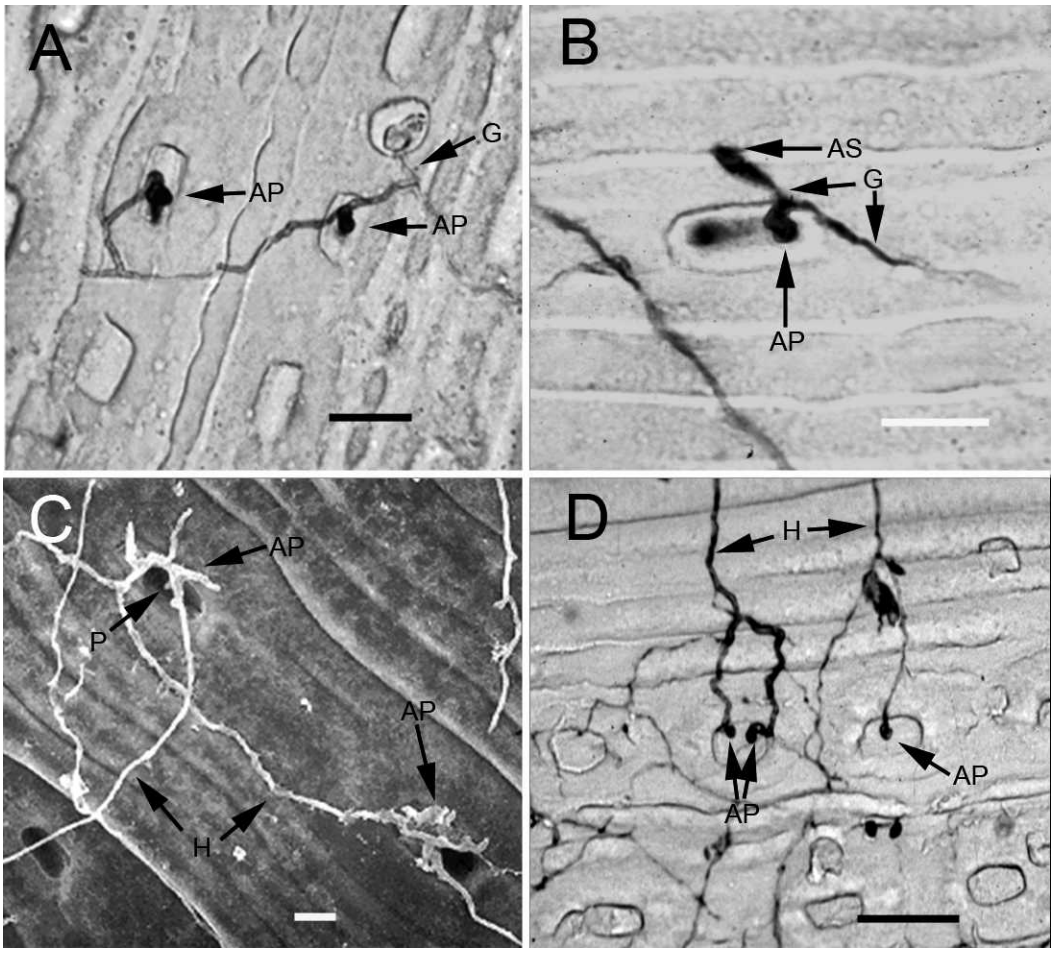

FIG. 3. Ascospore germination and penetration via stomata. A, B, D, plastic surface impressions. A. Two lateral appressoria (AP) arising successively from the same germ hypha (G). B. A germinated ascospore (AS) with appressorium (AP) above a stoma, the germ hypha (G) has continued to elongate. C. Scanning electron micrograph showing germ hyphae (G) forming appressoria (AP) above stomata. A penetration peg (P) is visible in the upper stoma. D. Branched germ hypha (G) with terminal appressoria (AP) above stomata. Note two appressoria on the same stoma. Bars: A, D = 20 μm; B, C = 10 μm.

rium an unbranched infection hypha grew through the stoma between the guard cells and into the substomatal chamber (FIG. 4), beyond which sparsely branching hyphae proceeded to colonize the intercellular mesophyll region of the needle (FIG. 4). Penetration pegs were approximately 2 μm wide and not distinctly different in morphology from vegetative hyphae observed within the mesophyll region of Douglas-fir needles.

Internal colonization.—Internal colonization increased continuously for 11 mo (Jul 1997–May 1998), as observed in SEM micrographs, in naturally infected current-year needles from field sites. Overall internal hyphae were more abundant in needles from sites having more severe disease (e.g. Juno Hill) (FIG. 5). Only intercellular colonization was seen in SEM preparations and cleared needles collected throughout the year. Hyphae were observed appressed to cell walls in both the palisade parenchyma layer and the spongy mesophyll (FIG. 5). Hyphal growth between the palisade cells however appeared generally less abundant than within the spongy mesophyll. In cross sections hyphae frequently were seen appressed to the endodermis cells, as well as mesophyll and palisade parenchyma cells (FIG. 5). Hyphae were never observed within the vascular region bounded by the endodermis, which appeared to effectively exclude growth of the fungus from the vascular bundle (FIG. 5E). Adhesive substances, apparently of fungal origin, frequently were observed attaching *P. gaeumannii* hyphae to the outer walls of host cells (FIG. 5F, G). No evidence of penetration of host cells (e.g. haustoria, intracellular colonization) was observed.

In potted seedlings inoculated with *P. gaeumannii* macerated mycelium, hyphal growth within needles was identical to colonization patterns observed in field collected samples. Internal hyphae were approx-

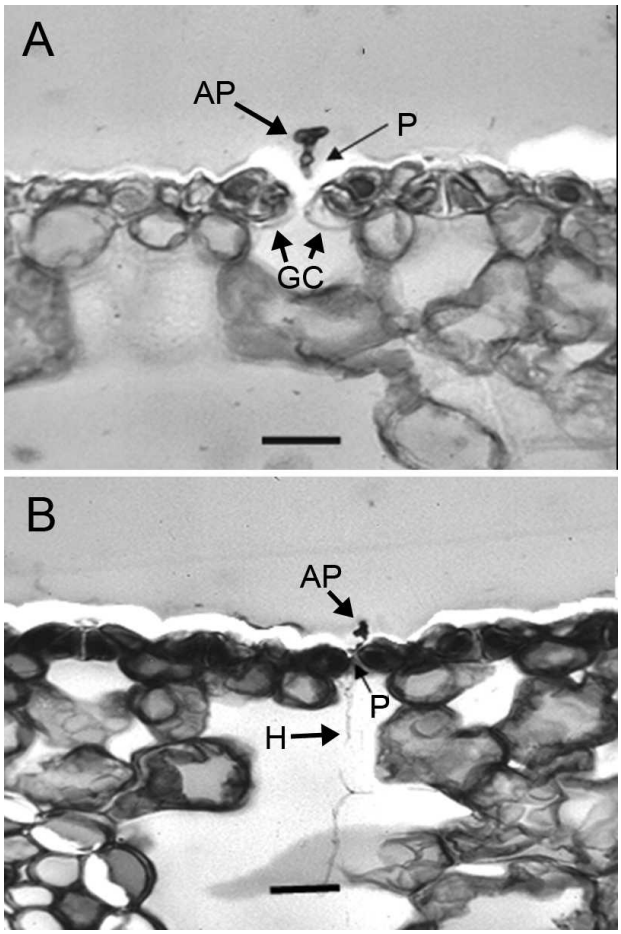

FIG. 4. Cross sections through stomata. A. Appressorium (AP) with short penetration peg (P) growing between guard cells (GC). B. Appressorium (AP) with penetration peg (P) contiguous with a branched intercellular hypha (H). The embedding resin has separated at the needle surface, making the appressorium appear above the needle surface. Bars = 10 μm.

imately 2–3 μm wide and were observed in long, mostly unbranched strands growing between and attached to mesophyll cells. Identity of the hyphae as *P. gaeumannii* was confirmed by re-isolation of the fungus in culture and by observations of development of pseudothecia on attached needles after 12 mo incubation following inoculation. Presence of *P. gaeumannii* also was confirmed by re-isolation in 40/40 needles from inoculated seedlings.

Development of pseudothecia.—Pseudothecial primordia formed in stomata Sep–Oct (early fall months) through Mar–Apr (early spring) on current-year needles, as observed in cleared whole needles, sectioned embedded tissue, plastic surface impressions and SEM micrographs. Formation of pseudothecial primordia begins as intercellular hyphae in the mesophyll differentiate into a cluster of specialized cells in the substomatal chamber immediately beneath the guard cells. The cluster of 4–6 cells arises as a whorl from a single branch point of the parent hypha and is aligned along the elliptical stomatal pore with the cell apices in line with the guard cells (FIG. 6A–C). The apices of these cells have thickened cell walls that resemble the periclinal thickening of phialides (Stone and Carroll 1985). The thickened cell apices are oriented transversely to the long axis of the stoma and stain strongly by trypan blue in cleared needle preparations, making them readily visible within the stomata. A narrow channel maintaining cytoplasmic continuity between the developing pseudothecium and the internal thallus is visible between the thickened hyphal apices (FIG. 6B–C).

Pseudothecial primordia first were visible on the needle surface as a cluster of spherical cells in the epistomatal chamber, contiguous with the cluster of cells in the substomatal chamber described above (FIG. 6B–E). These cells form beneath the stomatal wax plug, and as they divide the stomatal wax plug is forced outward, the remnants of which often remain as a small cap atop the developing pseudothecium (FIG. 6G). The pseudothecial primordia enlarge as cells continue to divide to form a spherical mass of darkly pigmented cells filling the entire epistomatal chamber and eventually emerging above the needle epidermis (FIG. 6D–G). Primordia began to form as early as Oct, approximately 4 mo after initial infection but more abundantly at 6–9 mo. These continued to enlarge Nov–May. By Dec–Jan the spherical cells forming the pseudothecial primordia gradually differentiated into a layer of sclerenchyma that formed the outer layer of the pseudothecia. General observations revealed that pseudothecia matured at different rates and not always simultaneously; at all times of the year infected needles had a population of pseudothecia in varying stages of maturity. Immature asci initially were present as early as mid-Apr and increased in abundance through mid-May (FIG. 6H).

Epiphytic growth.—Coinciding with the formation of pseudothecial primordia in the epistomatal chamber, cells around the periphery of the developing pseudothecium give rise to dark-pigmented hyphae that radiate from the epistomatal chamber and grow across the needle surface. These sparsely branched, septate, stolon-like hyphae began to emerge from stomata Aug–Sep (late summer) and continued to proliferate on needle surfaces throughout Oct–Jan (fall and winter) (FIGS. 6F, 7; TABLE I). The abundance of these epiphytic hyphae varied among field sites. Profuse epiphytic growth, occasionally covering the needle with a loose superficial mass, was observed on foliage at some sites (FIG. 7).

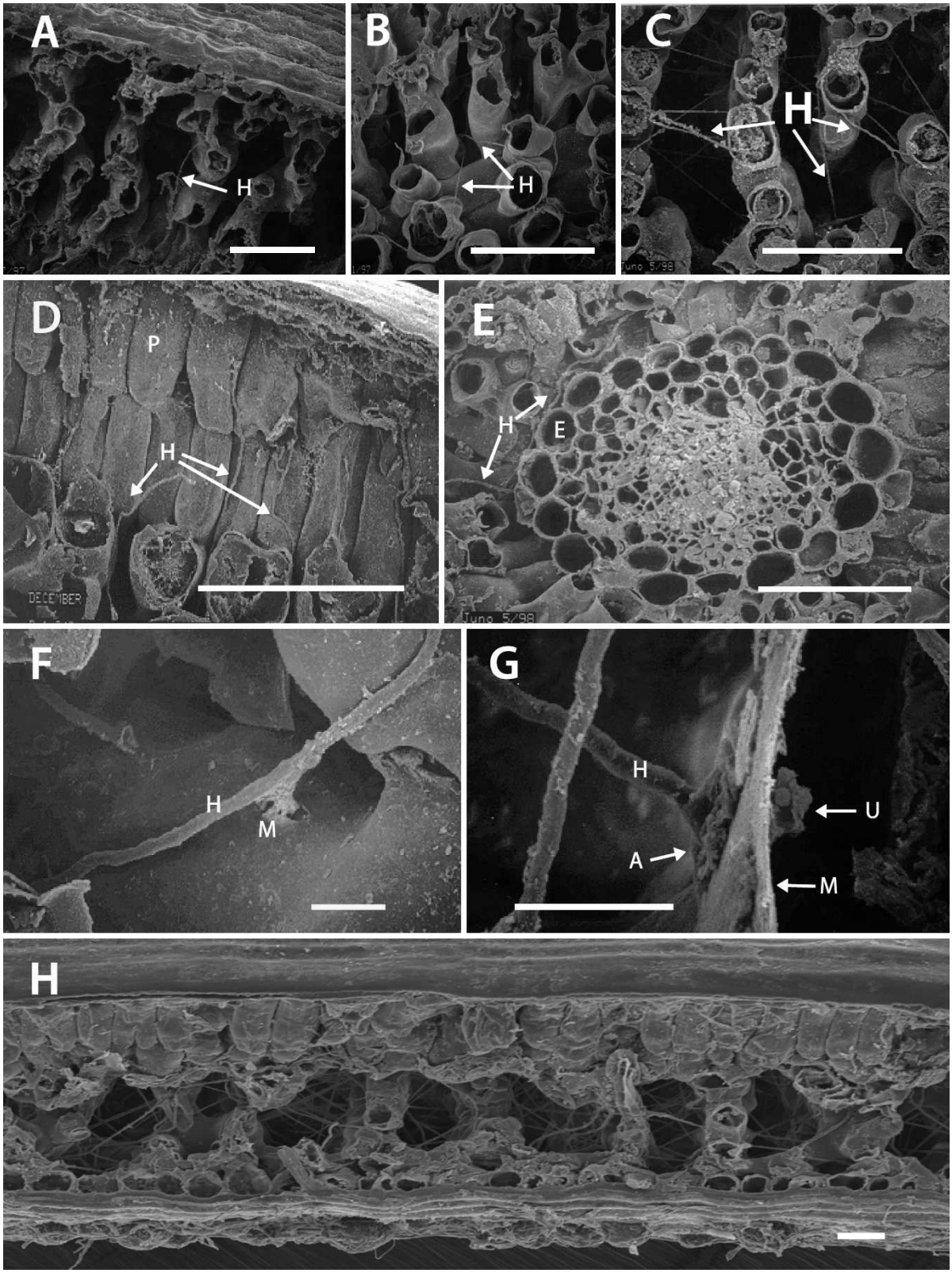

FIG. 5. Scanning electron micrographs of internal needle colonization. A–C. Progress of colonization 1–11 mo after exposure to inoculum. A. Colonization at 1 mo, few hyphae (H) are visible. B. Colonization at approximately 5 mo after inoculation. Internal hyphae (H) are more numerous. C. Colonization at 11 mo after inoculation. Internal hyphae (H) are abundant in the intercellular spaces. D. Hyphae (H) appressed to palisade mesophyll cells (P). E. Internal hyphae (H) outside

Observations on epiphytic hyphal growth made from plastic impressions, cleared whole needles and SEM were used to measure hyphal incidence and abundance and to describe the progression of epiphytic hyphal colonization over time. The length of hyphae on current-year needles increased Jul–Feb in 1996–1997 at most sites (TABLE I). For two sites examined in greater detail (Juno, Upper) distribution of epiphytic hyphae in early summer (Jun–Jul) was scattered. In Jul 1996, when current-year needles were approximately 2 mo old, only 18% of fields of view contained hyphae (averaging values from both sites). By Feb 1997 hyphae were distributed more evenly on needle surfaces. The incidence of epiphytic hyphae increased to an average of 64% for both sites.

Epiphytic hyphae growing across needle surfaces were observed to frequently anastomose, resulting in multiple interconnections between pseudothecial primordia within stomata (FIGS. 7, 8). Epiphytic hyphae also appeared to function as a means for expanding internal needle colonization. Upon contacting an unoccupied stoma, epiphytic hyphae were observed to form appressoria identical to those produced by germ hyphae (FIG. 8). The proportion of stomata having appressoria also increased during the fall–winter in parallel with the development of epiphytic hyphae (FIG. 9).

DISCUSSION

The infection and colonization of Douglas-fir needles begins as ascospore germ hyphae grow toward stomata and differentiate into appressoria in the outer stomatal chambers. Infection via stomata appears to be the exclusive penetration pathway of *P. gaeumannii*. Direct penetration of cell walls or penetration between adjacent epidermal cells was never observed. Single germ hyphae of *P. gaeumannii* are capable of producing appressoria above several stomata, either as a result of multiple branches or through successive growth. Multiple appressoria arising from the same hypha were relatively common in needle surface impressions and apparently represent multiple sites of needle penetration originating from the same ascospore.

Internal colonization by *P. gaeumannii* appears to be exclusively intercellular. No intracellular hyphae or haustoria were observed, although hyphae frequently were seen closely appressed to mesophyll cell walls and attached by mucilaginous material that appeared to be of fungal origin. Hyphae were visible in the intercellular spaces as early as 2 mo after exposure to inoculum and gradually increased in abundance 4–9 mo after infection. Hyphae in needles were confined to the mesophyll, never penetrating beyond the endodermis, and never were observed in vascular elements. *P. gaeumannii* therefore appears to be restricted to growth in foliage and unable to colonize shoots via vascular tissue.

Although *P. gaeumannii* readily grows on artificial culture media (Rohde 1937, Hood 1982), ecologically and functionally it behaves as an obligate parasite. It grows and reproduces only on living needles, does not persist saprotrophically in needle litter and has a narrow host range, species of *Pseudotsuga* being its only known hosts. However *P. gaeumannii* lacks intracellular hyphae or haustoria, the hallmarks of biotrophic nutrition. Apparently *P. gaeumannii* obtains carbon by direct absorption from the apoplast. Nutrient exchange between host and hyphal cells appears to occur via hyphae appressed to mesophyll cell walls but not penetrating host cells.

The principle symptom of Swiss needle cast is premature needle abscission. Pathogenicity of *P. gaeumannii* has been attributed to impaired CO_2 uptake, which is reduced proportionally with increasing occlusion of stomata by pseudothecia (Manter et al 2000). Occlusion of 20% of stomata was estimated to reduce stomatal conductance by approximately 20% and daily net CO_2 assimilation by 60%. On an annual basis net respiration exceeds assimilation if about 25% of stomata are occluded (Manter et al 2003a) and needles having greater than 50% of stomata occluded rarely remain attached (Hansen et al 2000). Abscission is thought to be triggered when needles switch from being a carbon source to a carbon sink (Cannell and Morgan 1990) and is strongly correlated with increasing abundance of pseudothecia on needles (Manter et al 2003b, 2005). The obstruction of stomata by pseudothecia therefore appears sufficient to account for premature needle loss caused by *P. gaeumannii*. Cellular disruption does not appear to be a factor in the pathogenicity of *P. gaeumannii*.

Development of *P. gaeumannii* pseudothecia is unusual compared to most other conifer foliage

←

the endodermis and bundle sheath cells (E). F. Hypha (H) on a mesophyll cell, the hypha appears to be adhered to the cell by mucilage (M). G. Hyphal attachment to a mesophyll cell by what appears to be mucilage (M). An unknown structure (U) is present inside the cell opposite the attachment point. H. An 11 mo old needle cut longitudinally to show extensive internal colonization. Bars: A–E = 100 μm; F, G = 10 μm; H = 10 μm.

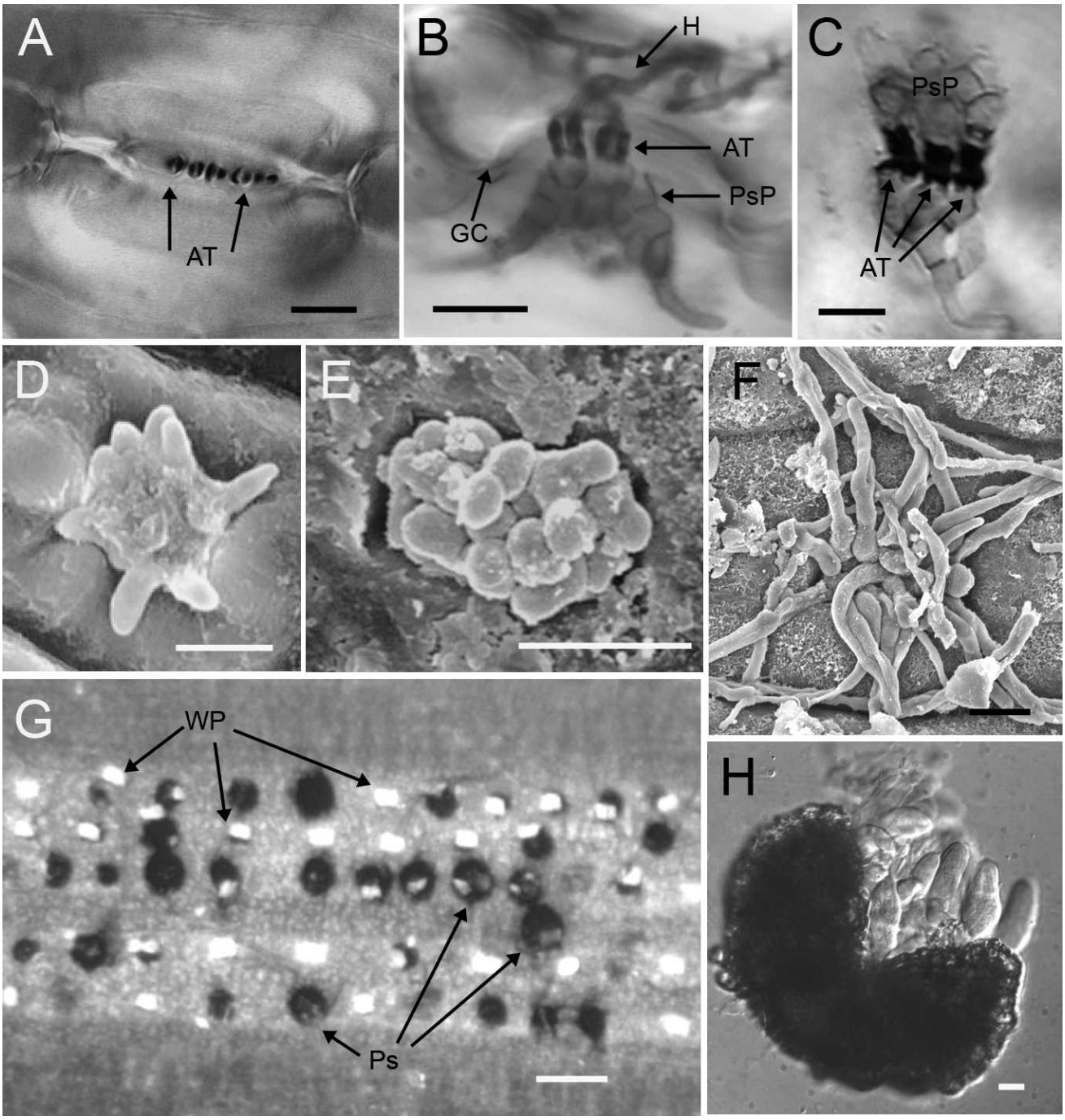

FIG. 6. Pseudothecium development. A–C. Pseudothecial primordia in cleared needle preparations. A. Outer view of stoma with stained apical thickenings (AT) of ''phialide-like'' cells in the substomatal chamber. B. Side view of developing pseudothecial primordium (PsP) emerging above the guard cell (GC), showing the apical thickenings at the midpoint of the guard cell and attached to internal hyphae (H). C. The hyphal apparatus that gives rise to the pseudothecial primordium (PsP), with stained apical thickenings (AT). D–F. Scanning electron micrographs of pseudothecial primordia. D. Pseudothecial primordium with peripheral cells beginning to elongate. E. A pseudthecial primordium composed of a cluster of spherical cells. F. Later stage of development with peripheral cells that have produced hyphae radiating from the stoma. G. Pseudothecia (Ps) at various stages of development on an 11 mo old needle, as seen under the dissecting microscope. Wax plugs (WP) are pushed up by the enlarging pseudothecia. H. A mature pseudothecium with asci. Bars: A–F, H = 10 μm; G = 50 μm.

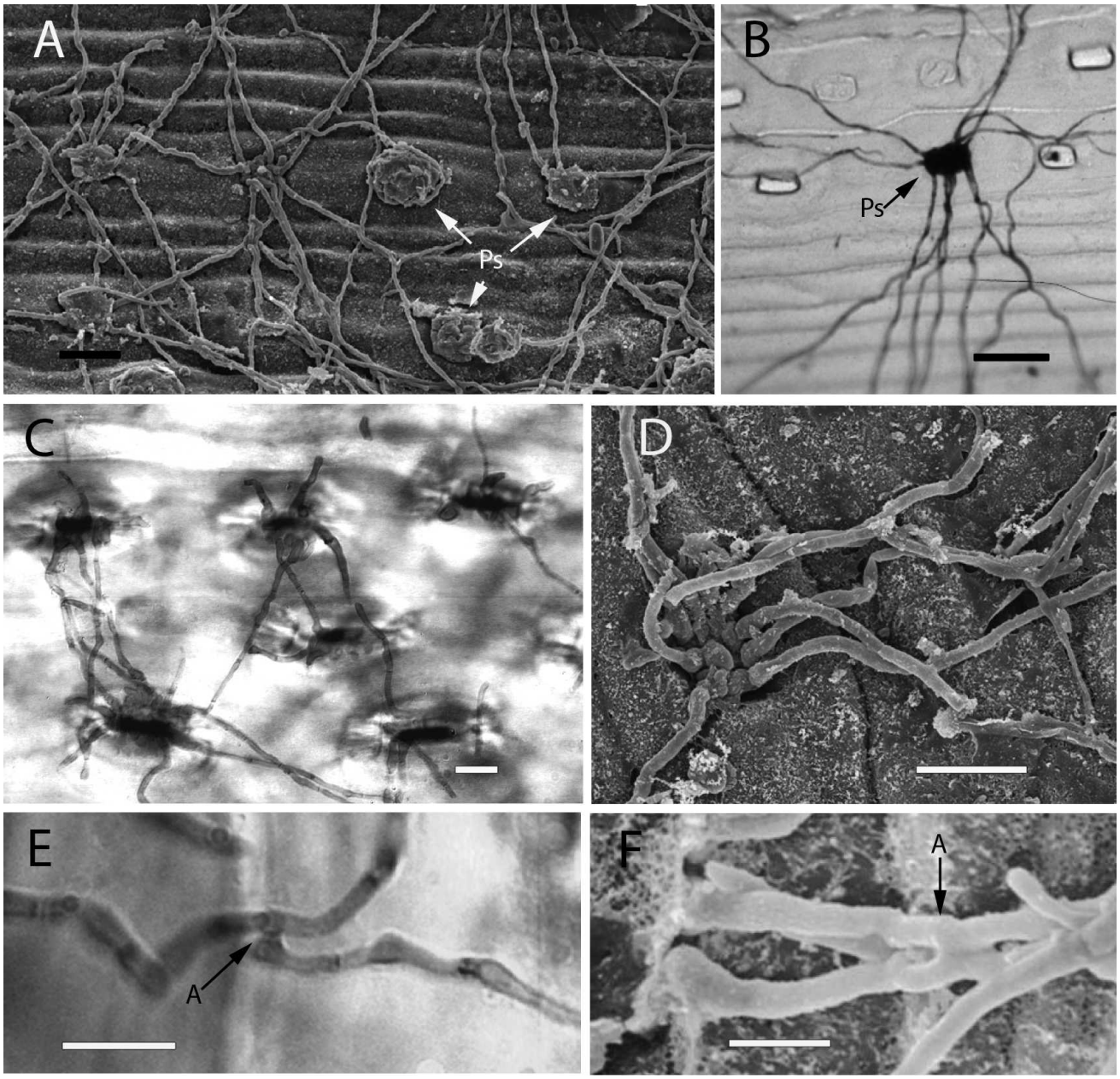

FIG. 7. Epiphytic hyphae arising from developing pseudothecia and anastomoses. A. Scanning electron micrograph of a 5 mo old needle with developing pseudothcia (Ps) and surface hyphae. B. Plastic impression of a developing pseudothecium (Ps), with radiating hyphae, in a stoma. C. Cleared needle preparation showing the surface hyphal interconnections among the developing pseudothecia in multiple stomata. D. Scanning electron micrograph showing hyphae arising from the peripheral cells of a developing pseudothecium. E. Anastomosis (A) as seen in cleared needle preparation. F. Scanning electron micrograph showing surface hyphal anastomosis (A). Bars: A, B = 20 um; D = 10 um; E = 5 um; F = 2.5 um.

pathogens, whose fruiting bodies typically erupt through the epidermal tissue. The process begins with the formation of a branched structure in the substomatal chamber and the formation of 4–6 cells aligned in the stomatal opening (FIG. 6). These cells have thickened apices that in electron micrographs appear to comprise several successively deposited wall layers, much like the apical thickening of phialides (Stone and Carroll 1985). Because of the resemblance of these cells to phialides Stone and Carroll (1985) postulated the possible existence of an undiscovered anamorph of *P. gaeumannii*. In the present study no instances of asexual propagules were observed in numerous microscopic examinations of infected needles at any stage of development. Furthermore infection by ascospores alone accounts for the

TABLE I. Mean abundance of *P. gaeumannii* hyphae on 1996 needles from six field sites in Tillamook County, Oregon

Site	26 Jun 1996	28 Jul 1996	6 Nov 1996	12 Dec 1996	6 Jan 1997	5 Feb 1997
Juno	272.1 ± 138.7[1]	336.2 ± 271.8	798.0 ± 662.4	ND	818.7 ± 613.5	1180.7 ± 1071.1
Upper	ND[2]	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

[1] 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.
[2] ND = Not determined due to immature needles or samples lost / destroyed.

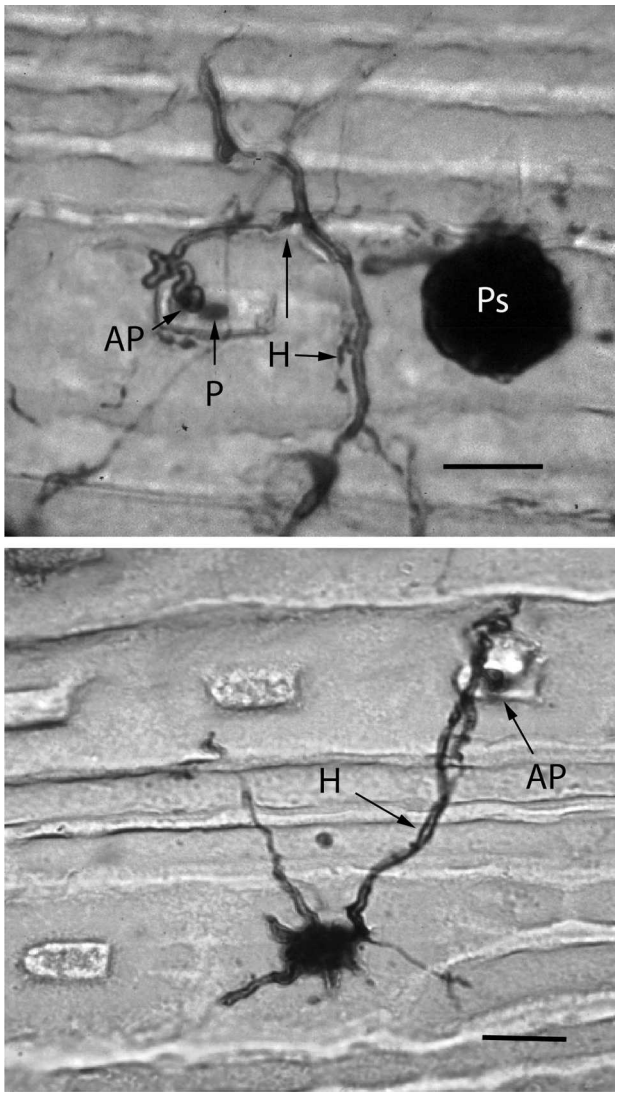

FIG. 8. Plastic needle impressions showing epiphytic hyphae forming appressoria above unoccupied stomata. A. An appressorium (AP) with penetration peg (P) has formed on a lateral branch of an epiphytic hypha (H). A pseudothecium (Ps) is also visible. B. A hypha (H) that arose from a developing pseudothecium grew toward an unoccupied stoma and formed an appressorium (AP). Bars = 10 um.

infection patterns observed in the field. While these negative findings do not eliminate the possibility of an anamorph, it appears unlikely that these cells, which are invariably present in infected needles, function in conidium formation.

Instead of representing an anamorph these phialide-like cells appear to be a preliminary stage in pseudothecium development, forming an attachment from which the pseudothecial primordium develops and from which the mature pseudothecium dehisces after ascospore release. The apical wall thickenings are oriented along the central midline of the stoma, in line with the guard cells, and might provide structural reinforcement to the hyphal wall, allowing it to resist being crushed by the turgor pressure of closing guard cells. Structural reinforcement at this point would help maintain cytoplasmic continuity between the developing pseudothecial primordium and the nutritional supply of the vegetative thallus within the needle.

Another unusual aspect of *P. gaeumannii* colonization is the emergence and growth of epiphytic hyphae coinciding with the initiation of pseudothecial primordia. These sparsely branched hyphae originate

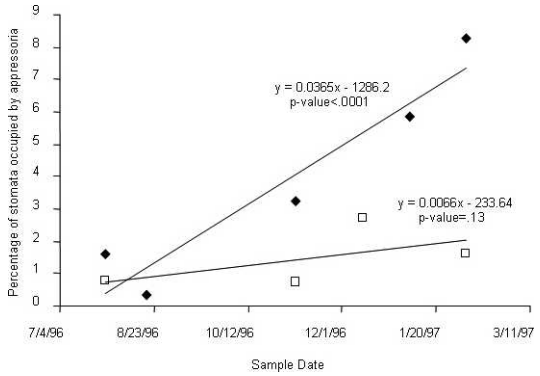

FIG. 9. Increase in numbers of appressoria associated with epiphytic hyphae over 3–10 mo after infection at a severely diseased site (Juno, ♦) and a moderately diseased site (Upper Stone □).

from the periphery of developing pseudothecia, grow across the needle surface and reenter the needle through nearby unoccupied stomata. Epiphytic hyphae were observed to frequently anastomose, resulting in a dense, interconnected hyphal network. Superficial hyphal growth has not been reported previously for *P. gaeumannii*. However recent phylogenetic analyses have placed *P. gaeumannii* in Capnodiales (Winton et al 2007), a group of fungi collectively known as the sooty molds because of their profuse, brown pigmented superficial mycelia (Reynolds 1998). Molecular phylogenetic studies have provided further evidence that *P. gaeumannii* is not congeneric with *P. nudus* (Peck) Petrak, the genus type, which instead is allied to members of Dothioraceae (Dothidiales). In fact *P. gaeumannii* groups within genus *Mycosphaerella* and is closely related to *Rasutoria pseudotsugae* (Winton et al 2007), which also produces profuse epiphytic hyphae but does not colonize the needle mesophyll.

The epiphytic hyphae appear to function in expanding colonization of the needle by producing appressoria above unoccupied stomata, through which reentry into the interior needle presumably occurs. Penetration pegs from these secondary appressoria could not be distinguished from other internal hyphae in cross sections or cleared needles because needles already are heavily colonized when the epiphytic hyphae begin to emerge. Secondary appressoria produced by the epiphytic hyphae appeared to be identical to primary appressoria formed from germ hyphae, and these continued to increase in abundance during the winter concurrently with internal needle colonization. This behavior is puzzling in light of the observation that older needles are much less susceptible to ascospore infection than young, newly emerged needles (Hood and Kershaw 1975, Stone et al unpubl). Assuming that the majority of these appressoria represent successful penetration points, the epiphytic hyphae and ascospore germ hyphae appear to differ in their ability to infect older needles. Possibly the greater melanization of the epiphytic hyphae adds sufficient rigidity to the cell wall (Henson et al 1999) to enable successful penetration between hardened guard cell walls that form a barrier to penetration from less melanized primary appressoria. The epiphytic colonization by *P. gaeumannii* is novel, and its role in the epidemiology of Swiss needle cast disease invites further study.

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