Cellular anatomy of penetration and infection of containerized western larch seedlings by Botrytis cinerea

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The Cellular Anatomy of Penetration and Infection of Containerized Western Larch Seedlings by *Botrytis cinerea*

by

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B.S. University of Washington 1970

Submitted in partial fulfillment of the requirements for the degree of Master of Science University of Montana 1988

approved by

Chairman, Board of Examiners

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Abstract - *Botrytis cinerea* Pers.(ex Fr.) is a facultatively parasitic fungus which is a chronic problem in the production of containerized conifer seedlings. The pathological cellular anatomy of the infection of needles of containerized western larch (*Larix occidentalis* Nutt.) was investigated from whole mounts and serial sections with the light microscope. Both stomatal and cuticular penetration were evident. Direct penetration occurred most often on succulent needles and was more frequent with a more virulent fungal isolate. Germination, hyphal growth and stomatal entry were especially enhanced on senescent needles. Hyphae occasionally displayed taxis toward stomata on senescent or infected needles. Infective structures such as infection pegs, primary and secondary appressoria, and infection cushions, described in the literature on agricultural plants, were all present on western larch. Internally, growth of hyphae was parallel to and in contact with the inner periclinal walls of epidermal cells. Hyphal growth in the mesophyll was more random. The endodermis and enclosed vascular tissues, and the resin canals, were more resistant to invasion than the mesophyll. Mesophyll tissues generally became disorganized in advance of contact with hyphae. Vegetative hyphae and conidiophores exited via stomata or directly through the cuticle. Treatment with 1/10 or 1/100 the normal application rate of benomyl induced occasional alteration of fungal morphology on needle surfaces, but similar treatments with captan and dicloran did not. The effects induced by benomyl consisted of curling and twisting of germ tubes and hyphae.
Preface and Acknowledgements

Descriptions of the penetration and infection of western larch seedlings by Botrytis cinerea are arranged to give the reader two options. The mycologist and plant pathologist can proceed directly through the text unhindered by the terminology. Readers from other disciplines may wish to first examine the illustrations and read a few captions in order to acquaint themselves with the general appearance of the needle surface and interior, and to acquire a mental image for such terms as "germ tube" and "appressorium." The figures are for the most part arranged to present the usual sequence of events from contact between the conidia and the host cuticle to infection and renewed sporulation. Discussion of events in the text occasionally departs from this sequence when context demands it.

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Introduction

Botrytis cinerea Pers. (ex. Fr.) is a parasitic and saprophytic fungus which has become a chronic problem in the production of conifer seedlings. This damaging mold has long been a pest on stored bare-root seedlings, a concentrated product of high value on which Botrytis and other molds have been controlled through careful manipulation of temperature and humidity (Hopkins 1975, Hocking and Nyland 1971). Although Botrytis is an occasional pest in bare-root nurseries, its impact in forestry has escalated greatly with the advent of production of containerized conifer seedlings. "Grey mold [Botrytis] has caused greater losses in CTS [containerized tree seedling] facilities than any other pathogen," (Tinus and McDonald 1979). Botrytis cinerea is one of the most important chronic problems on containerized stock in western North America (James, personal communication). Careful adherence to cultural procedures, strict sanitation, and extended use and rotation of fungicides can diminish losses from Botrytis, but the fungus remains a recurrent problem because of its ability to rapidly evolve a tolerance to fungicides and because conditions in CTS facilities are so conducive to infection by and spread of the pathogen (James 1984, Sutherland and Van Eerden 1980). Repeated testing must be conducted to assess tolerance of B. cinerea to fungicides and to assess the degree of

*Botrytis cinerea* is also an important pathogen on a large number of agricultural and ornamental plants. An understanding of the biology of the infection process has enabled more efficient control of *Botrytis* in several agricultural crops (Maude 1980), and the histopathology of the infection process has been described for a number of agricultural host plants as briefly reviewed below. Unfortunately, comparable research regarding the infection process in conifers has not been available. A crucial step in understanding infection biology is a description of the process of penetration and infection at the cellular level. This study was intended to furnish a qualitative description of the cellular anatomy of penetration and infection of a suitable conifer seedling host. Western larch (*Larix occidentalis* Nutt.) was chosen as the host species because of its susceptibility to infection and because it is an important regeneration species in the Inland Northwest.
Objectives

The central objective of the study was the description of the cellular anatomy of the penetration and infection of needles of containerized western larch (Larix occidentalis) seedlings by B. cinerea. An additional objective was to discover whether that anatomy is altered in the presence of residual fungicides. Descriptions of interacting pathogen and host tissues were recorded for the following cases:

(1) Infections occurring via conidia on succulent, mature and senescent needles of seedlings in the absence of fungicides.

(2) Infections occurring via conidia on succulent, mature and senescent needles on seedlings which were treated with fungicides commonly used in forest nursery greenhouses.

Uninfected needles were observed to ascertain the normal appearance of seedling needle tissues. Qualitative differences in the infection process between needles of different ages and/or different fungicide treatments were described. These differences were quantified in relative terms whenever possible.

The Infection Process on Non-coniferous Hosts

Information on the penetration and infection process exists for a number of agricultural and ornamental plants on which Botrytis species, including B. cinerea, are
important pathogens. Although the complete life cycle of *B. cinerea* involves production of sclerotia, apothecia, ascospores and occasional microconidia, it is the conidia and the hyphae which arise from them that are the focus of most pathological and epidemiological investigations. (For an appreciation of the complexities of the complete life cycle of *B. cinerea* and its relation to the apothecial form, *Botryotinia fuckeliana*, see Jarvis 1980a.)

Verhoeff's 1980 review specified the important structures of penetration as conidial germ tubes, mycelium growing on dead parts of the plant or mycelium established in extraneous dead material. The ability to use moribund or senescent tissue as a basis for production of infection hyphae or conidia is noted by other authorities (van den Heuvel 1981, Jarvis 1980b, 1977, Mansfield 1980). Once in contact with the host, penetration may occur directly through the cuticle, through the stomata, or through wounds, punctures, or minute cracks in the cuticle (Blakeman 1980, Jarvis 1977, Verhoeff 1980). Apparently, penetration via stomata is the exception rather than the rule for *Botrytis* species, and when it does occur there is no evidence of attraction of the germ tubes toward stomata (Verhoeff 1980, Clark and Lorbeer 1976).

The structures of penetration are variable, but exhibit a few key features. Upon germination, penetration through the host cuticle may occur at the slightly swollen apices
of germ tubes, or at the more swollen, oval appressoria at
the ends of longer germ tubes or hyphae (Garcia-Arenal and
Sagasta 1980, van den Heuvel and Waterreus 1983). Occasional penetration may occur via larger,
dichotomously branched, compound appressoria, or through
even more branched, multi-digitate infection cushions
(Backhouse and Willets 1987, van den Heuvel and Waterreus
1983, Jarvis 1977). The hyphae, or at least the
appressoria and cushions, usually possess a mucilagenous
coat which probably serves to affix the fungus to the host.
The actual rupture of host cuticle and epidermal cell wall
is accomplished by a thin hypha termed a penetration or
infection peg (Blakeman 1980, van den Heuvel and Waterreus
exists as to whether the mechanism by which the infection
peg ruptures the host cuticle and wall is chemical
(enzymatic) or mechanical (concerted pressure.)
Considerable evidence exists for both views, and both
processes may occur simultaneously. The controversy was
reviewed by Verhoeff (1980), who regarded the question as
unresolved.

The host plants examined by the aforementioned reviewers
and investigators include tomato fruits, onions, and a
number of bean species. Artificial surfaces of various
sorts have also been used. Although the main features of
penetration and infection are similar throughout, the exact
mode of entry may vary. For example, Rijkenberg et al. (1980), working with tomato fruits, noted that the centers of epidermal cells, where the cuticle and cell wall are thinnest, were the optimal sites for rapid penetration by germinating conidia. Clark and Lorbeer (1976) however, noted that direct penetration of onion cuticle usually took place at the anticlinal walls of epidermal cells. Such cell junctions were also the usual sites of penetration by B. cinerea of broad bean (Vicia faba) leaves (Mansfield and Richardson 1981). Another example of variability is the type of appressorium from which penetration ensues. Although several authorities cite instances of penetration from the ends of short germ tubes or from terminal appressoria on the ends of germ tubes, Akutsu, Kobayashi et al. (1981) noted specifically that further hyphal growth and the formation of secondary, multicellular appressoria were necessary for infection of cucumber leaves. Alternatively, if conidia were able to fuse with each other and form a network of hyphae, the resultant simple (unicellular) appressoria were also able to achieve penetration of the same host (Akutsu, Ko, and Misato 1981). Other obvious differences in mode of entry concern the age and type of tissues invaded. The role of senescent tissue has been mentioned, and it should be noted that young, succulent tissue is susceptible as well. Floral parts offer opportunities for infection in a number of hosts, but
the variabilities inherent in these infections will be ignored here, since conifer seedlings have no such structures. Jarvis (1977) and Verhoeff (1980) adequately reviewed the types of tissues attacked.

The initial reaction of penetrated host cells determines the success or failure of establishment of *B. cinerea*, and has therefore been described in detail for several host plants. The deposition of granular reaction material at sites of attempted penetration is a common feature. According to Rijkenberg et al. (1980), if penetration of tomato fruits is successful there is little discoloration at the site, but sites at which growth is arrested show papillae of granular material opposite penetration tubes. Mansfield and Richardson (1981) noted similar events in broad bean leaves. *B. cinerea* hyphae penetrated epidermal cells, but were confined to cell walls and eventually the hyphal cytoplasm became disorganized and died. Here too the failure to establish infection was associated with dense granular deposits. Successful establishment was associated with rapid necrosis of invaded cells. Failure of infection was associated with little or no cell death and with formation of reaction material in similar experiments on broad bean and tulip leaves (Mansfield and Huston 1980, Mansfield 1980). Events on onion leaves and bulb scales, and on narcissus bulbs also displayed the formation of granular papillae in resistant reactions
(O'Neill and Mansfield 1982, Stewart, Alison and Mansfield 1984). Compounds associated with reaction material or with walls of invaded cells included callose and suberin (De Leeuw 1985), lignin and phenol (Mansfield and Hutson 1980), and lignin, callose and phytoalexins (O'Neill and Mansfield 1982). If B. cinerea does succeed in establishing itself in host tissues it may remain latent or dormant, but usually growth proceeds to an entirely necrotrophic phase in which hyphae emit toxins which kill host tissues in advance of hyphal growth. The nature of these toxins is essentially unknown (Jarvis 1977, Verhoeff 1980).

Since both the mode of infection and the responses of host tissues are variable, several investigators have attempted to assess the role of the host cuticle in accounting for this variability. This has been done chiefly by comparing responses on unmodified cuticle with those on cuticle modified by some form of pretreatment, or by modifying cuticle surfaces by adding nutrients to the inoculum. Sometimes artificial substrates are used in place of host cuticles. The effects of the modifications may then be assessed in terms of germination, morphology of resultant hyphae, and infectivity. Germination of some isolates may proceed without exogenous nutrients, whereas other isolates require a supply of nutrients (Verhoeff 1980). Some results suggested that growth may occasionally proceed as far as formation of germ tubes and primary
appressoria in spite of a lack of exogenous nutrients (Shirane and Watanabe 1985). There is general agreement that a supply of nutrients such as glucose or sucrose enhances germination (Akutsu, Ko, and Misato 1981, Akutsu, Koobayashi et al. 1981, Blakeman 1980). Schütt (1973) wrote that volatile exudates of leaves and shoots of various conifers inhibited or stimulated spore germination and growth of mycelium. The effects were temperature and light dependent, but the overall effects were congruent with differences in susceptibility between the conifer test species. Hill et al. (1980) wrote that host cuticular waxes may contain substances inhibitory to germination.

Investigators also noted changes in the manner of infection and the cellular infective structures with respect to presence or absence of surface nutrients. The presence of nutrients in the inoculum can enhance entry via the stomata (Clark and Lorbeer 1976). The formation of appressoria is stimulated by a supply of nutrients in the inoculum, as well as by other features of the cuticle surface. This stimulation of appressorial formation by nutrients is not unique to B. cinerea, but common to a wide variety of fungi (Emmett and Parbery 1975). Such stimulation by glucose aids formation of simple unicellular (primary) appressoria, and a supply of purine-related compounds furthers production of multicellular (secondary) appressoria (Akutsu et al. 1987, 1983, Akutsu, Ko, and
Misato 1981, Akutsu, Kobayashi et al. 1981, 1982, Shirane and Watanabe 1985). Similarly Garcia-Arenal and Sagasta (1980) reported enhanced formation of appressoria and infection cushions in media amended with Czapek broth. Nutrients, however, may also promote hyphal extension instead of appressorial formation (Emmett and Parbery 1975) or appressoria may be stimulated to germinate and continue hyphal growth (Blakeman 1980).

Aside from nutrient levels, other cuticular properties may influence the formation of structures. Hill et al. (1980) stated that appressorial formation proceeds more readily on more hydrophobic surfaces. Presence of free water on the surface is required for germ tube growth (Carre and Coyier 1984). If *Gladiolus* leaves were thoroughly wet, direct penetration of the cuticle predominated, but if moisture consisted of water droplets in the stomata, tubes entered leaves via the stomata (Blakeman 1980).

If cuticular surface properties influence germination and the formation of fungal structures, then such cuticular properties as nutrient levels may also influence infectivity. This is indeed the view of most authorities. For example, Jarvis (1977) stated that the addition of nutrients to inoculum may induce infection on plants on which *B. cinerea* is not normally parasitic. Mansfield (1980) found that presence of pollen grains on plant
surfaces, or the addition of a source of sugars such as orange juice, can transform \textit{B. cinerea} into an aggressive pathogen. The above series of experiments performed by Akutsu and co-workers demonstrated considerable increases in penetration and infection when appropriate nutrients were present in the inoculum. Such additions of nutrients do not always increase infection however. Van den Heuvel and Waterreus (1983) observed that penetration from germ tube tips and simple and multicellular appressoria was not markedly increased by the addition of glucose. Akutsu's work indicates that actual penetration and infection may depend on the additional presence of other compounds, such as purine-related substances. Finally, substances which themselves are not nutrients in the ordinary sense may promote leaching of nutrients from plant surfaces and thereby alter fungal behavior. Verhoeff (1980) mentioned a case in which the fungicide zineb stimulated mycelial growth on tomato leaves by this process, and also stimulated growth of \textit{B. cinerea} hyphae \textit{in vitro}. Ammonium nitrogen had a similar effect on leachates of \textit{Vicia faba} leaves.

A further consideration for assessing the germination, growth and infectivity of \textit{B. cinerea} on host surfaces is the concentration of conidia in the inoculum. Naturally, inoculum density must be sufficiently high to enable observation of fungal behavior and host response, but if
densities are too high, conidial germination is inhibited. Blakeman (1980) reviewed the effects of inoculum density on a number of agricultural hosts. Most investigators have used densities from $10^3$ to $10^5$ conidia per ml. In general, increasing conidial concentrations seems to increase infection until the spores appear to mutually inhibit germination (Blakeman 1980, van den Heuvel 1981, Mansfield 1980). Akutsu, Ko, and Misato (1981) observed that at high concentrations conidia were able to fuse and form a network of hyphae from which infection readily occurred, but infection in the absence of nutrients was lacking at lower conidial concentrations. Other workers have also noted the effect of conidial concentrations on fungal structures. Stewart and Mansfield (1981) found that development of long germ tubes on onion surfaces increased, and that of short tubes decreased, as inoculum density declined. Van den Heuvel and Waterreus (1983) discovered that greater concentrations of spores on french bean leaves promoted penetration from germ tube apices, whereas lower concentrations enhanced penetration by hyphal appressoria or infection cushions.

Thus the basic elements of germination, growth and infection can be altered in a multiplicity of ways depending on conditions at the host cuticle surface. The extent to which the results of any single investigation can be applied to other hosts or conditions must be viewed with
caution. Variability probably increases when the genetic variations of host and pathogen are considered, but the literature provides a context for the behavior of *B. cinerea* on conifer seedlings and for seedling tissue responses. Additional contextual material for cellular level behavior of pathogenic fungi and plant response can be found in Aist (1976) and Emmett and Parbery (1975). Beckett et al. (1974) and Gull and Trinci (1971) can be consulted for a general view of the ultrastructure of *B. cinerea* spores and hyphae.

**Materials and Methods**

**General Design:**

Fungicide-treated and untreated containerized western larch seedlings were infected with *Botrytis cinerea* by inoculation with suspensions of conidia. Needles from these seedlings were made into whole mounts by clearing and staining. Whole mounts enabled observation of the behavior of germinating conidia and the resultant hyphae on the needle surfaces. Internal patterns of infection were observed by means of serial sections of infected needles. Noninfected needles were similarly treated and observed so that normal host tissues could be compared to infected host tissues. Results were recorded photographically. Details of these procedures follow by sections.
Production of conidia and inoculation:

Needles of infected seedlings from forest nursery greenhouses were placed onto water agar. Hyphal tips were taken from the resultant fungal growth and aseptically transferred onto potato dextrose agar. Incubation at 20 degrees C and exposure to cycles of 12 hours black light (General Electric 15 watts) alternating with 12 hours of darkness induced sporulation on the agar plates (Dhingra and Sinclair 1985, Epton and Richmond 1980, with modifications). Spores were floated off agar with distilled water and Tween® solution (1% vol./vol.) and the solution filtered through cheesecloth to remove hyphae. The density of the resultant inoculum was computed with a Neubauer hemacytometer and adjusted with distilled water to 5000 conidia per ml. The inoculum was then sprayed with a spray bottle onto the seedlings until run-off, and groups of four to six seedlings were placed into incubation chambers. The incubation chambers consisted of gallon jars with clear plastic lids. Each had a smaller jar inside to hold the seedlings upright. The insides of the incubation chambers were maintained as close to 100% relative humidity as possible by a reservoir of water at the bottom of each chamber, and by a daily light spray of distilled water onto the seedlings. After inoculation, the groups of seedlings in their incubation chambers were placed into a growth chamber and maintained with alternating cycles of 12 hours
fluorescent light at 20-21 degrees C and 12 hours of darkness at 15-16 degrees C.

Three groups of four to six untreated seedlings were inoculated on different dates in spring of 1987. With each group an additional group of seedlings was sprayed with distilled water and Tween® only, (i.e., no spores,) in order to affirm the effectiveness of inducing infection. In the fall of 1987, these procedures were repeated for two more inoculations. Each of these latter two inoculations contained a group of four to six untreated seedlings and three similar groups each treated with one of three fungicides; dicloran (Botran®), benomyl (Benlate®), or captan. As before, a group of seedlings sprayed only with distilled water and Tween® (i.e., no conidia,) was present to test the effectiveness of the inoculum in inducing infection. The three spring inoculations and the first fall inoculation all utilized spores cultured from B. cinerea from western larch grown at the Montana State Nursery greenhouse at Missoula, Montana. This isolate was maintained on infected seedlings in the growth chamber for use in producing inoculum. The fourth inoculation was conducted with spores of an isolate of B. cinerea obtained from stored lodgepole pine container stock from the Western Forest Systems Nursery, Lewiston, Idaho.
Treatment with fungicides:

The levels of fungicides applied to seedlings in these tests were to be lower than levels known to exert effective control, but within levels likely to be encountered by Botrytis between fungicide applications in greenhouses. Growth of B. cinerea on fungicide-amended potato dextrose agar was observed in order to find suitable levels of fungicide for treatment of the seedlings. Procedures for amending agar with fungicides followed James and Gilligan (1983). Ideally, there would be sufficient fungicide on the seedlings to affect growth, but not so much as to totally inhibit infection. Levels tested on the agar plates therefore ranged from one in the vicinity of a full application rate, (to check for possible fungicide tolerance,) to one tenth and one hundredth of that rate for each fungicide. Plates were sprayed with conidia at the same concentration at which seedlings were inoculated, 5000 spores per ml. Two such trials with the amended agar revealed that the 1/100 rates were potentially suitable for treatment of the seedlings. Therefore 1/100 normal application rates were used for the first fall inoculation. The rates were increased to 1/10 of normal application rates for the last fall inoculation. Although application rates vary somewhat, full field application rates for dicloran and benomyl are generally about one pound of 50% active ingredient wettable powder per 100 gallons of water.
This is equivalent to nearly 1.195 grams 50% wettable powder per liter of water or 1195 ppm, (= 597.5 ug/ml of active ingredient.) One tenth and one hundredth of normal rates were therefore administered as approximately 0.12 g 50% wettable powder/liter and 0.012 g 50% wettable powder/liter respectively. Application rates for captan are somewhat higher, generally 1.5 to 2 times that for the other two fungicides, hence the captan in these experiments was applied at 0.24 and 0.024 grams of 50% wettable powder per liter of water.

All fungicides were weighed and added as 50% wettable powder to distilled water and mixed for one to three hours until dissolved. The resultant solutions were sprayed onto seedling groups until run-off, and the seedlings allowed to dry before inoculation with conidia.

Production of whole mounts:

Procedures follow Dhingra and Sinclair (1987) and Kelman (1967) with slight modifications.

Needles were harvested from seedlings at intervals of 24, 48, and 72 hours after inoculation and placed into vials of Carnoy's solution for fixation and clearing. Vials were labeled as to date of inoculation, date of harvest, type of fungicide (if any), and relative age of needle tissue, (succulent, mature or senescent.) Clearing in Carnoy's solution required five to seven days. Subsequent to clearing, whole needles were placed into labeled vials of
0.1% acid-fuchsin in lactophenol or 0.1% cotton blue in lactophenol and left one to several hours for staining of spores and hyphae. Needles could then be placed on glass slides with a few drops of stain and examined with a microscope. Because of the appreciable thickness of whole needles, it was necessary to place pieces of coverslips at each end of the needles and a whole coverslip over the top, so that the mounting medium, the stain, would be well dispersed over the specimens. Pertinent features were recorded photographically and a record kept in a log book.

Production of serial sections:

   Fixation, dehydration, embedding, sectioning and staining; procedures follow Feder and O'Brien (1968) and Johansen (1940) with slight modifications.

   Fixation:

   Needles were selected for fixation as soon as infection became visible with a dissecting microscope or a hand lens. Suitable pieces of needle about 3 to 4 mm in length were excised with a sharp razor blade. Each piece ideally included tissue not yet penetrated by hyphae, as well as thoroughly infected tissue. Tissue pieces were placed into vials of buffered glutaraldehyde (5.1 ml of 0.2M KH₂PO₄, 4.9 ml of 0.2M K₂HPO₄, 25.0 ml distilled water, and 5.0 ml practical grade glutaraldehyde,) and subjected to a vacuum to remove air. Addition of one ml of Tween® solution to 100 ml of buffered glutaraldehyde greatly assisted
reduction of surface tension and removal of air. Tissue was left in the vacuum overnight and subjected to one or more vacuum conditions the next day.

Dehydration:

Two dehydration series were employed; one preparatory to embedding with glycol methacrylate and the other preparatory to embedding with paraffin.

Dehydration for methacrylate embedding was through a graded series of ethanol and water, (% ethanol: 10, 20, 30, 50, 70, 95, 100, 100.) Transfer of tissue to monomer embedding fluid was preceded by a graded series of n-butanol in ethanol, (% n-butanol: 50, 75, 100, 100.) Changes of liquid were made throughout at one hour intervals.

Dehydration for paraffin embedding was through a graded series of ethanol, water, and tertiary butyl alcohol, (Table 1.)

Table 1. Dehydration series for paraffin embedding (%)

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Changes of liquid were made throughout at one hour intervals.

Embedding:

Two embedding media were used. Initially sections were embedded in glycol methacrylate, but too frequently tissue separated from the plastic during sectioning. Failure of plastic to adequately adhere to the waxy cuticle was the apparent cause. Since the possibility of discovering significant events on the cuticle surface was evident, cuticles were not removed with xylene as is commonly done in such cases. Instead, paraffin embedding was employed, since the latter material adhered more strongly to the cuticle. Glycol methacrylate did prove adequate for embedding young succulent needles, assumedly because the cuticle was less developed, and senescent tissue, assumedly because the cuticle was eroded.

Glycol methacrylate embedding:

Subsequent to the graded series described above, tissue was placed into glycol methacrylate monomer for 12 hours, then into an additional change of monomer for 12 more hours of infiltration. The tissue was then placed with monomer and small paper labels into gelatin capsules and placed in an oven. After incubation at 30 degrees C for 3 to 5 days, followed by incubation at 40-45 degrees C for an additional 5 days or longer, the capsules were removed from the oven.
The polymerized plastic could then be trimmed with a razor blade prior to sectioning.

**Paraffin embedding:**

After dehydration, the pieces of needles were transferred from TBA to a 1:1 mixture of TBA and paraffin oil in which they remained from one to several hours. During this time, 52 degree paraffin was poured into the bottoms of small vials and allowed to solidify. The tissue in the 1:1 mix was placed on top of the solidified paraffin at about 1/3 the volume of the paraffin, then the vials were placed in a 60 degree C oven overnight. The next morning the fluids in the vials were replaced with two changes of 52 degree paraffin at 2 hour intervals, then with two changes of 56 degree paraffin at equally long or longer intervals. The paraffin and tissues were then poured into labeled paper boats. The tissues were arranged, and the boats cooled quickly by placement in an ice box. Small blocks of paraffin containing the tissue were cut from the boats and mounted on wooden blocks for sectioning.

**Sectioning:**

All sectioning was done on a Spencer 820 rotary microtome. Plastic sections were cut individually in thicknesses ranging from 5 to 13 micrometers. The sections were placed onto drops of water on a glass slide, and the slide heated to 150 degrees C for 15 minutes to fix sections to the glass prior to staining.  Paraffin
sections were cut as ribbons at 15 or 18 micrometers and fixed to the slide with Haupt's adhesive. 4% formalin was used to enable ribbons to expand on the slide, i.e., to remove any compression from sectioning. Slides with ribbons were put on a slide warmer at 40 degrees C, excess formalin drained, and left overnight.

Staining:

Tissue sectioned in paraffin must be de-paraffinized prior to staining. This was accomplished with the following graded series: 100% xylene, xylene and absolute ethanol 1:1, 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, and distilled water. Sections remained in xylene for 10 minutes and in the other solutions for 5 minutes. Dehydration after staining and prior to mounting was done by using the series in reverse.

Both the methacrylate plastic sections and hydrated (de-paraffinized) sections were stained with periodic acid-Schiff reagent with aniline blue-black as a counterstain. The schedule was as follows:

1. Place slides in freshly prepared, saturated (0.5 g per 100 ml water) dimedone overnight.
2. Rinse in running tap water 1 hour.
3. Place slides in 1% periodic acid for 5-10 minutes.
4. Rinse in running tap water 5 minutes.
5. Place in Schiff reagent for 4-5 minutes.
6. Place slides into two changes of fresh metabisulfite rinse, allowing 2 minutes for each change. (Metabisulfite rinse: 5 ml of 10% potassium metabisulfite, 5 ml 1.0 N HCl, 90 ml distilled water.)
7. Rinse in running tap water 5 minutes.
8. Dip slides in a 1:1 solution of 1% aniline blue-black (1% stain in ethanol) and 7% acetic acid.
9. Dip slides into two changes of 7% acetic acid and one change of distilled water.

At this point plastic sections were dried and saved for mounting with Permount® and coverslips. Paraffin sections were dehydrated with the previously described graded series and immediately mounted. Mounted slides were placed on slide warmers for 5-10 days with their coverslips firmly pressed to the slides by lead plugs. After removal from the warmers, slides were cleaned of excess mounting medium with razor blades and xylene.

Microphotography:

A 35 mm Bell and Howell/Canon camera was mounted on a Zetopan Reichart microscope. A Zeiss T-mount adapter with a horizontal viewing tube facilitated focusing. Photographs of selected events on whole mounts and serial sections were taken at one or more powers of magnification, ranging from 80X to 1000X, with Ectachrome 160 film. A written log was kept of all photographed events.
Results:

Examination of whole mounts and serial sections revealed two essential features of *Botrytis* infection of the western larch seedling needles. First, nearly all the fungal structures described as playing a role in the infection of other host plants were present on the needles. The exception was the absence of an obvious mucilage sheath around appressoria and infection hyphae. Second, there seemed to be no consistent effects on fungal morphology on the needle surfaces from treatment with fungicides at levels of 1/100 or 1/10 normal application rates. Effects were noted in rare instances on benomyl-treated needles. The following sections describe the results in detail.

Fungal infection and host tissue responses:

Conidia sprayed onto needle surfaces were seldom observed to be uniformly distributed on those surfaces and often accumulated in dense aggregations alternating with smaller groups. Conidia often lodged in open stomata (Figure 1). Tubes from germinated conidia appeared to grow at random, although sometimes tubes would grow along the anticlinal divisions between the epidermal cells. The vast majority of tubes from germinated conidia failed to penetrate host tissues, although germ tube tips or blunt appressoria often adhered to the cuticle. Both stomatal penetration and direct penetration did occur however. Stomatal penetration
occurred via short, single germ tubes (Figure 2 on the right, and Figure 3), or more rarely via longer tubes which could be traced to germinated spores (Figure 6). Where more dense spore aggregations occurred it was possible to see tubes from several conidia entering a single stomatum (Figures 4 and 5, and Figure 2 on the left). Very occasionally, a conidium would give rise to tubes, one of which penetrated via a stomatum, the other directly (Figure 7). Direct penetration most frequently happened at the ends of short tubes with blunt tips or small, rounded appressoria. Figures 8, 9 and 22 illustrate typical events. The anticlinal wall between epidermal cells was the most common site for direct penetration, and sometimes the hyphal growth subsequent to penetration seemed to follow this wall (Figure 9). The centers of epidermal cells could also be penetrated, as could guard cells. Direct penetration was much more frequent with the use of the more virulent Western Forest Systems isolate. Stomatal penetration greatly predominated with the Montana State Nursery isolate. Direct penetration was most frequently seen on the more succulent needles.

The mechanics of initiation of penetration are for the most part below the resolution of the light microscope. Clark and Lorbeer (1976) stated that the penetration pegs of *B. cinerea* are not seen with the light microscope. However several peg-like structures were observed in these
studies. These may have been rendered visible by expansion from a much smaller diameter. Such "pegs" were observed as a narrow spike protruding into host tissue from the swollen apices of short germ tubes or from a swollen hyphal tip (Figure 10). Shorter, more blunt, more deeply stained "pegs" could be seen entering host tissues from the swollen apices of short germ tubes (Figures 11 and 12). In other instances it was difficult to tell if such blunt "pegs" were associated with penetration or merely attachment (Figure 23). Likewise the initial reactions of host tissues were difficult to discern and interpret in these mounts. A clouding and discoloration of host tissue may be seen at the site of actual or attempted penetration as shown in Figures 10 and 14. Other instances, such as Figure 13 may also show host reactions, although it was difficult to separate possible host reactions from partially stained or unstained subsurface fungal plasm in some whole mounts. Figures 15 and 16 seem to show either an initial host reaction or erosion of the cuticle near a hyphal tip.

As mentioned above, direct penetration of host tissues occurred more frequently on succulent needles. The more senescent tissues supported the most extensive hyphal growth on the needle surfaces, although succulent tissue also occasionally supported such growth. Spores germinated readily on senescent tissue, and the resultant hyphae
frequently formed an anastomosing network over the surface (Figures 17 and 18). Although senescent needles frequently did not clear well, making it difficult to find instances of direct penetration, stomatal penetrations were commonly observed (Figure 19). Mature needles yielded fewer instances of direct penetration than did succulent needles, and fewer instances of stomatal penetration than did senescent needles.

The appressoria and other attachment structures which are described in the literature were apparent in these experiments. Penetration from germ tube apices has been mentioned above and is illustrated in Figure 22. Figures 20 and 21 display attachment of the fungus to the cuticle by means of tube apices; one instance shows attachment by a pair of blunt tubes, the other by a small appressoria-like swelling. More developed, larger appressoria appeared at the ends of long tubes or in association with a hyphal network. Such appressoria are often oval swellings on the ends of tubes or hyphae, but sometimes are comprised of a connected pair of swellings. These simple, unicellular appressoria and the consequent penetrations are illustrated with Figure 24 and in the upper right of Figure 25. Simple appressoria grade into multicellular, compound (secondary) appressoria. The multi-digitate compound appressoria were observed less frequently than simple appressoria and were usually associated with heavily infected tissue (Figures 26
and 27). Even more rarely seen were the yet larger infection cushions (Figure 28). Some larger structures were the size of infection cushions, but lacked conspicuous appressoria and may have been sclerotal initials similar to those recorded by Akutsu et al. (1987). The mucilage around tubes and appressoria which was mentioned in the literature was seldom evident however. Techniques of fixation and clearing may have dissolved much of the mucilage.

Other branching, appressoria-like structures were associated with entry via stomata. These structures intergraded with structures which formed coils around guard cells prior to stomatal penetration. Figure 29 displays a multi-digitate structure with coils all pointing into a stomatal aperture. A bi-lobed simple appressorium is immediately adjacent. A much simpler coil is shown in Figure 30. A series of such structures would sometimes form over stomata as a hypha grew along the needle surface (Figures 31 and 32). Figure 32 demonstrates that on at least two stomata penetration has not yet occurred. Figure 33 shows another set of coils, also prior to penetration, while figures 34 and 35 are of coils over penetrated stomata. These events strongly imply a taxis of hypal tips toward stomata. In many other instances however, hyphae were seen to grow nearby to or even directly over stomata
without entering. Sometimes conidia germinated within stomata only to have tubes grow away from the aperture.

As with the external features of infection, the internal events were similar to descriptions from other hosts. Upon entry, hyphae seemed to disorganize and kill tissue well in advance of physical contact (Figure 48). Figure 36 depicts normal tissue and Figure 37 infected tissue. The appearances of normal tissue, necrosis in advance of hyphae, and tissue in contact with hyphae are recorded in a series of photographs from neighboring sections (Figures 49, 50 and 51). A longitudinal section of tissue not yet disorganized, but with chloroplasts characteristically disintegrated, or at least unstained, is shown in Figure 47. This disappearance of the chloroplasts, followed by disorganization of other cellular components, and ultimately, maceration, seems to be the most common sequence, but occasionally the chloroplasts were still well intact, or at least readily absorbing stain, in tissues in close proximity to hyphae (Figures 46 and 55).

The spatial arrangement of hyphae in infected needles varied with the type of tissue. Hyphae grew just below the epidermis and possibly sometimes within the anticlinal and lower periclinal walls of epidermal cells. Growth of such hyphae was in a direction more or less parallel to the long axis of the epidermal cells, i.e., longitudinal to the axis of the whole needle (Figures 38, 39, 40 and 41). Figure 42
shows the more rare growth within an epidermal cell. Hyphal growth within the mesophyll tissues was much less regular, with most hyphae seemingly growing in random directions. Occasional whole mounts were sufficiently cleared and penetrated by stain to render the internal hyphal patterns visible (Figure 43), but the patterns are more readily appreciated in transverse and longitudinal sections (Figures 44, 45 and 51.) When less random patterns were apparent the general orientation of hyphae was somewhat longitudinal (Figure 46), especially in mesophyll tissues adjacent to the endodermis (Figures 54 and 55). In general, mesophyll tissues were more readily penetrated and disorganized than epidermal tissues, resin ducts or the tissues of the vascular bundle. The endodermis often remained intact and the vascular tissues, including the vascular parenchyma, unpenetrated even when the mesophyll was permeated with hyphae (Figures 54 and 55). The beginnings of collapse of some endodermal cells and the penetration of some vascular tissues can be seen in Figures 37 and 44. Figure 52 illustrates intense infection in and around a resin duct and the consequent collapse of some cells lining the duct. In some instances guard cells and/or immediately adjacent epidermal cells seemed prone to invasion (Figure 53).

In very extensively infected needles hyphae were readily observed exiting the interior tissues and growing back out
onto the surface. Some of these hyphae grew in a procumbent fashion, while others, much greater in diameter, grew away from the host tissues and formed conidiophores. Hyphae could occasionally exit directly through the epidermis and cuticle, but more commonly exited via the stomata (Figure 56). A pair of thicker hyphae are seen in Figure 57. These are of the type which seemed to eventually form conidiophores (Figure 60). Such thick hyphae also erupted directly through the epidermis (Figure 58). Exit via a stoma and the resultant procumbent, branching pattern of surface growth is displayed in Figure 59.

The rates at which infection progressed were somewhat variable, even within an age class of needles. In most cases infection was noticeable with a hand lens in 24 to 48 hours. Interior tissues became disorganized in about three days, and maceration was quite evident at seven days. The exit of hyphae from infected needles could occur within two days, but was more common after three days.

Effects of fungicide treatments on fungal morphology:

The lowest concentrations of benomyl and dicloran, (1/10 and 1/100 of normal field rates,) when present in potato dextrose agar plates, readily inhibited growth and induced characteristic changes in fungal morphology. Benomyl induced curling and lysis of germ tubes (Figure 63), while dicloran elicited an abnormally frequent dichotomous
branching habit (Figure 62). These effects may be compared to the growth habit on unamended potato dextrose agar (Figure 61). The only discernable effect of captan was to inhibit or delay germination of conidia. However, fungicides on seedling needles did not affect fungal morphology. An exception was benomyl, which resulted in very isolated instances of distorted germ tubes (Figures 64 and 65). There were in addition two isolated instances in which conspicuously abnormal hyphal growth was present on the benomyl-treated needles. The abnormality consisted of regions of corkscrew twists alternating with slender stringy growth (Figure 66).

Discussion

Although results indicated that the course of infection involves the same basic features encountered in other hosts, some aspects invite closer scrutiny. These aspects are the pattern of hyphal growth on senescent tissues, the differences in frequency of stomatal versus direct penetration between tissue types and fungal isolates, and the occasional evidence for taxis of hyphal tips toward stomata. The conspicuous lack of fungicide-induced morphological effects on needle surfaces compared to the consistent production of such effects on amended agar also requires explanation. A more minor, but still interesting result, is the occasional persistence of chloroplasts in
heavily infected tissue, an event which represents a departure from the usual disappearance of chloroplasts, or at least their failure to stain, in tissues in close proximity to hyphae.

The patterns of hyphal growth on senescent tissue are of importance not because of any departure from other studies, but because of conformity to the general pattern. The ability of the fungus to readily colonize senescent tissue and utilize it as a base from which infection spreads to the remainder of the seedling and adjacent seedlings has often been observed by growers of conifer stock. Typically, senescent needles are common in the lower canopy of containerized larch seedlings. The difficulty of targeting the lower seedling canopy with fungicide concentrations sufficient to inhibit *Botrytis* in an environment so favorable to its growth is part of the problem of disease containment. Fungicides are periodically applied mixed with water from the overhead sprinkler system and must contact succulent seedling tops which can be damaged by strong concentrations of fungicides (Bodmer, personal communication, James, personal communication). The problem can be compounded by the development of resistance to fungicides by *Botrytis*, as mentioned in the introduction. Hence it is possible that better containment may be achieved by adding some form of biological control, most probably in the form of organisms
which singly or in combination can more quickly utilize the
nutrient base provided by senescent tissues. Such
organisms should be fungicide tolerant without being
pathogenic to plants, so they may be used in conjunction
with fungicides if necessary. Removal of competing
organisms by fungicide application has been shown to
actually increase damage from fungicide resistant strains
of B. cinerea (Cook and Baker 1983). The goal of a
fungicide tolerant control agent may well be achievable.
Some strains of Trichoderma harzianum Rifai, for instance,
are tolerant of fungicides commonly used in conifer
greenhouses and are antagonistic to many fungi, including a
close relative of Botrytis. Other organisms, bacterial and
fungal, of potential use in control of B. cinerea are
catalogued by Cook and Baker (1983).

An equally intriguing result was the variation in mode of
penetration with host tissue age and fungal isolate.
Unfortunately, the procedures employed in the experiments
did not always work well enough to generate reliable
quantitative results. The chief difficulties in this
regard were, first, the failure of some needles to clear
sufficiently to enable differentiation between penetration
and mere attachment, and second, in some cases of
penetration, fungi did not absorb stain, probably because
the fungal cytoplasm had already moved out of the conidium
and germ tube and into subcuticular or subepidermal hyphae.
Instances of the latter event could occasionally clearly be seen, but in the absence of consistent and thorough staining of fungi on the surface there was no way to effectively spot such events when scanning needle surfaces at low power. These and other more minor difficulties would together render formal statistical analysis somewhat spurious. Nevertheless, as stated in the results, direct penetration of tissues occurred only rarely when inoculations were performed using the isolate from the Montana State Nursery greenhouse. Nearly all the observed penetrations were stomatal. The Western Forest Systems isolate produced far more intense infection on the seedlings, even though these seedlings received a higher dose of fungicide. Direct penetrations were very frequently observed with the Western Forest Systems isolate, especially on more succulent needles. This implies that greater pathogenicity is correlated with enhanced ability to achieve direct penetration, an entirely reasonable interpretation and one well documented for Botrytis species on agricultural crops (e.g., Clark and Lorbeer 1976, Mansfield and Hutson 1980, Mansfield and Richardson 1981, Stewart and Mansfield 1984). Whether direct penetration occurred frequently on senescent tissue was unresolved due to the difficulties described above. Nonetheless, stomatal penetration was abundant and more frequent on senescent needles than on mature or succulent
needles, presumably because the fungus was more able to enzymatically extract nutrients from senescent tissue or because it found them already present on the surface. Such nutrient availability would stimulate growth and enhance the possibility of random contact between hyphae and stomatal apertures.

Growth on senescent needles may not always be random however. Although many growth patterns, including many involving contact and entry of stomata, seemed random, some observations are best interpreted as evidence for taxis. Figures 29-35 taken together imply that under some circumstances hyphae grow in a directed manner, such as a diminishing spiral, toward the stomatal aperture. That this taxis was observed in instances in which the needle was senescent or the interior already infected would be consistent with the idea that substances inducing taxis could leak through the stomata from the damaged tissues within the needle. Taxis may also be involved in the favoring of the anticlinal epidermal cell wall as a site for direct penetration, but there is little evidence for that here, except for the occasional observation that surface hyphae sometimes grew for a distance along such anticlinal divisions after contact.

The degree of taxis aside, that *B. cinerea* grew so successfully on fungicide-treated needles was surprising. Growth at the same levels of fungicide on the agar plates
was not only considerably inhibited, but also strikingly affected morphologically with benomyl and dicloran. The most reasonable explanation for the successful germination and growth of the fungus on needles may lie with the water repellent properties of cuticular waxes. These same properties are no doubt responsible for the lack of uniform distribution of the conidia, which like the fungicides had been applied as a water spray solution. Nevertheless, even though water, and the dissolved fungicides, may have been shed by much of the cuticle surface, much of the needle surfaces remained obviously wet after spraying, and evaporation should have deposited the dissolved fungicide onto the needle surface and perhaps even concentrated it in places as it did the conidia. Allowing for further dilution when the seedlings were again sprayed with spores would also explain part of the results, but probably would not serve to dilute the 1/10 application rate treatment to a concentration below the 1/100 application rate treatment. The 1/100 rate treatments of dicloran and benomyl on agar had obvious and consistent effects on fungal morphology. The differences between the results of the amended agar pilot tests and the results on the seedlings are therefore still largely unexplained. That the only abnormalities observed were those that correspond to deformities induced by benomyl may be because of the partially systemic properties of that fungicide. It's also plausible that
minute undissolved particles of benomyl caused high local concentrations. Benomyl was the least soluble of the three wettable powders.

The remaining aspect, that of the occasional persistence of chloroplasts in tissue permeated by hyphae, is also puzzling. It may be that hyphal extension and production of toxins are not in a consistent equilibrium, so that sometimes hyphae may grow into tissues without producing the usual disruptions. As noted in the discussion of the literature, the nature of toxin production by B. cinerea is relatively unknown.

Summary and Conclusions

Infection of needles of young western larch seedlings by conidia of B. cinerea followed essentially the same pattern as that described for other hosts. Conidial germ tubes and hyphae penetrated via stomata or directly through the cuticle. Senescent tissue was readily colonized and provided a base for extensive hyphal growth and production of conidia. Succulent tissues were more readily penetrated directly through the cuticle than are mature tissues. The simple appressoria, compound appressoria and infection cushions present on other hosts were seen on western larch. Strong evidence existed for occasional taxis of hyphal tips toward stomata on senescent or damaged needles. Once entry was gained, hyphae grow longitudinally in or just below the
lower periclinal wall of the epidermal cells, and more or less randomly in the mesophyll. The endodermis and enclosed vascular tissues, and the resin canals, were usually invaded only after disorganization of the mesophyll. The fungus tended to kill tissues in advance of hyphal growth. Hyphae exited thoroughly infected tissue either directly through the cuticle or through stomata. Once back on the cuticle surface, hyphae grew procumbently on the surface or formed conidiophores. Application of captan, benomyl, or dicloran at 1/10 or 1/100 normal application rates failed to consistently alter fungal morphology on the needle surfaces.

The results strongly imply that senescent tissues in the lower canopy may enhance the rate of infection by furthering both hyphal growth and production of conidia. The augmentation of chemical controls with fungicide tolerant microbial saprophytes might help control Botrytis populations in senescent material under the seedling canopy. This may be all the more desirable since residual fungicides seem to little affect Botrytis growth patterns. Experimentation with Botrytis and its potential microbial competitors on needle surfaces is desirable, especially if conducted in the presence of varying levels of fungicides.
Illustrations

The following figures are photographs of whole mounts and sections as described in procedures. Sections are labeled as transverse or longitudinal; other photographs are of whole mounts. Fungal structures in whole mounts are stained magenta or blue, while host tissue is pale green-grey to tan. Fungal structures are lavender to purple in the sections, while host structures are pink to magenta. Figures are labeled as to age of needle, (succulent, mature, or senescent,) and fungicidal treatment, if any. The photographs were chosen on the basis of the ability to display the events under discussion in the text, and not all needle ages or fungicide treatments are equally represented.
Figure 1. Conidium in a stomatal aperture on a mature needle. x3200.

Figure 2. Stomatal penetration via a short germ tube on a succulent needle. Tubes from several spores enter a stoma on the left. x800.
Figure 3. Stomatal penetration by a short germ tube on a succulent needle. x3200.

Figure 4. Tubes from two spores penetrate a stoma on a succulent needle. x3200.
Figure 5. Tubes from spores enter a stoma on a succulent needle. x3200.

Figure 6. Hypha traced to a germinated conidium enters a stoma on a succulent needle. x3200.
Figure 7. A hypha traced to a conidium bifurcates; one tube penetrates directly, the other enters a stoma. x1800.

Figure 8. Penetration from the apex of a short germ tube. Succulent needle. x3200.
Figure 9. Penetration at anticlinal wall of epidermal cells; subsequent hyphal growth seems to follow this wall. Captan at 1/100 usual rate, succulent needle. x3200.

Figure 10. A narrow spike from the end of a hypha traced to a conidium; the spike penetrates host tissue and is probably an infection peg expanded from an originally smaller diameter. Discoloration of host tissue at the site is evident. Succulent needle x3200.
Figure 11. A shorter "peg" enters host tissue from the swollen apex of a short germ tube. Succulent needle. x3200.

Figure 12. Another "peg" enters host tissue from the end of a short tube. Succulent needle. x3200.
Figure 13. Direct penetration from a short, blunt tube branching off a hypha. There are two unstained spores just above and in contact with the hypha. Partially stained fungal cytoplasm is visible in the host. Benomyl at 1/100 usual rate on a succulent needle. x3200.

Figure 14. Discoloration of host tissue at site of initiation of penetration. Benomyl at 1/10 usual rate on a succulent needle. x4000.
Figure 15. Reaction of host tissue or erosion of cuticle near a hyphal tip. Mature needle. x3200.

Figure 16. Deeper focus of event in Figure 15.
Figure 17. Germinated conidia on a senescent needle. x800.

Figure 18. Anastomosing hyphae from germinated conidia on a senescent needle. Benomyl at 1/10 usual rate. x800.
Figure 19. A hypha from an anastomosed mass of hyphae enters a stoma. Senescent needle with captan at 1/100 normal rate. x1440.

Figure 20. A conidium attached to the cuticle by two blunt tubes. Mature needle. x3200.
Figure 21. A small appressorium-like swelling at the apex of a short germ tube. No penetration is evident. Succulent needle. x3200.

Figure 22. Direct penetration from the blunt apex of a short germ tube. Succulent needle with captan at 1/100 normal rate. x3200.
Figure 23. A small papilla-like projection off a short germ tube makes contact with the cuticle. In some instances such projections were clearly associated with penetration, (Figures 11 and 12,) while in other instances such as this and Figure 27 the papillae-projections merely seem to contact the cuticle. Succulent needle. x3200.

Figure 24. Direct penetration by a simple bi-lobed appressorium; another simple appressorium is seen to the right. Succulent needle. x1000.
Figure 25. Direct penetration by a simple appressorium, (upper right.) Several hyphae enter a stoma on the left. Succulent needle treated with benomyl at 1/10 normal rate. x1000.

Figure 26. A multi-digitate, compound, (secondary,) appressorium. No penetration is visible. Succulent needle with benomyl at 1/10 normal rate. x1800.
Figure 27. Lobes of a compound appressorium; one lobe has a projected papilla which contacts the anticlinal junction between epidermal cells. Succulent needle treated with benomyl at 1/10 usual rate. x4000.

Figure 28. An infection cushion; no penetration is evident. Benomyl applied at 1/10 the usual rate to a succulent needle. x1800.
Figure 29. Multi-digitate structure with coiling hyphae all pointing toward stomatal aperture. Succulent needle treated with benomyl at 1/10 usual rate. x1800.

Figure 30. A relatively simple hyphal coil has its tip pointed toward the stomatal aperture. Succulent needle. x4000.
Figure 31. A hypha traverses the needle surface, forming coiled structures over the stomata. Figure 31 focuses on the coils, and Figure 32 on the stomata. The lower focus demonstrates that penetration of the stomata has not yet occurred. Succulent needle. x1000.

Figure 32. Focus on the stomata under the hyphal coils of Figure 31.
Figure 33. Hyphae coil in a diminishing spiral toward the stomatal aperture; penetration has not yet occurred. Succulent needle. x1800.

Figure 34. Hyphal coils over a stoma. A lower focus, (not shown,) confirms entry. Senescent needle with dicloran at 1/100 normal rate. x3200.
Figure 35. Branching hyphae coil in diminishing spirals into two stomata. Senescent needle with dicloran at 1/100 normal rate. x1800.

Figure 36. Transverse section of normal tissue in a succulent needle. 5 micrometers, glycol methacrylate. x1000.
Figure 37. Transverse section of infected tissue in a succulent needle. Some endodermal cells are beginning to collapse. 10 micrometers, glycol methacrylate. x1000.

Figure 38. Longitudinal section of an infected succulent needle. Hyphae grow subepidermally, parallel to the axis of the needle and the long axis of the epidermal cells. 18 micrometers, paraffin. x1440.
Figure 39. Transverse section showing clustering of hyphae under epidermis. Mature needle with 1/100 the usual dose of dicloran. 15 micrometers, paraffin. x1000.

Figure 40. Transverse section of hyphae under epidermis. Succulent needle, 10 micrometers, glycol methacrylate. x3200.
Figure 41. Transverse section of hyphae under epidermis. Mature needle with 1/100 usual dose of dicloran. 15 micrometers, paraffin. x4000.

Figure 42. Transverse section showing hyphae within an epidermal cell. Succulent needle with 1/100 full dose of captan. 15 micrometers, paraffin. x4000.
Figure 43. Pattern of hyphal growth inside a cleared mature needle. Treated with dicloran at 1/100 usual rate. x800.

Figure 44. Transverse section depicting pattern of hyphae in mesophyll. Some vascular tissues are affected. Mature needle with 1/100 usual rate of dicloran. 15 micrometers, paraffin. x1000.
Figure 45. Longitudinal section showing hyphal growth in the mesophyll. Mature needle with 1/100 normal rate of benomyl. 15 micrometers, paraffin. x1000.

Figure 46. Longitudinal section illustrating hyphal growth in the mesophyll. The normal appearance of the chloroplasts in cells in such close proximity to hyphae is unusual. See also Figure 55. Succulent needle, 18 micrometers, paraffin. x1440.
Figure 47. Longitudinal section showing the characteristic disappearance of chloroplasts in cells in advance of contact with hyphae. Succulent needle with 1/100 the normal rate for captan. 15 micrometers, paraffin. x1000.

Figure 48. Transverse section of highly disorganized tissues in advance of contact with hyphae. Succulent needle, 5 micrometers, glycol methacrylate. x800.
Figure 49. Transverse section of normal tissues far in advance of hyphal contact. Note normal appearance of chloroplasts. Succulent needle with 1/100 the usual rate for dicloran. 15 micrometers, paraffin. x1000.

Figure 50. Transverse section of disorganized mesophyll not yet penetrated by hyphae. This section is a few sections away from that of Figure 49. x1000.
Figure 51. Transverse section of tissue invaded by hyphae. This section is neighboring the area displayed in Figure 50. This sequence of figures 49-50 shows the usual transition in appearance from normal to invaded mesophyll tissues. x1000.

Figure 52. Transverse section of an infected resin canal on a mature needle. Collapse of some canal cells is evident. 18 micrometers, paraffin. x3200.
Figure 53. Transverse section showing infection around guard cells on a mature needle. 18 micrometers, paraffin. x3200.

Figure 54. Longitudinal section showing that the endodermis and enclosed tissues are still intact in spite of intense infection in the mesophyll. Mature needle with 1/100 full application rate for dicloran. 15 micrometers, paraffin. x1800.
Figure 55. Longitudinal section illustrates intact endodermis near intense infection in mesophyll. Succulent needle, 18 micrometers, paraffin. x800.

Figure 56. Hyphal tip exits an infected needle via a stoma. Succulent needle. x3200.
Figure 57. Two large hyphae exit via a stoma on a senescent needle. x3200.

Figure 58. A large hypha exits directly through the cuticle. Mature needle with captan at 1/100 normal rate. x3200.
Figure 59. A hypha exits via a stoma and subsequently displays the characteristic normal dichotomous branching. Compare to the abnormal dichotomous branching, (Figure 62.) Mature needle with 1/100 normal rate for dicloran. x320.

Figure 60. A conidiophore in the process of formation of conidia. The hypha giving rise to the conidiophore exited directly through the cuticle. Succulent needle. x1440.
Figure 61. Normal growth habit of *B. cinerea* on potato dextrose agar, (PDA). x800.

Figure 62. The pattern of frequent dichotomous branching induced by amending PDA with dicloran (botran). This pattern was evident at all concentrations of dicloran tested on PDA. x800.
Figure 63. Typical pattern of curling and lysis of germ tubes induced by benomyl in PDA. This pattern was evident at all concentrations of benomyl on PDA. x800.

Figure 64. Distorted germ tubes on a conidium subjected to the 1/10 rate treatment of benomyl on a succulent needle. This type of event was rarely observed on needle surfaces. x1800.
Figure 65. Distorted germ tubes on conidia subjected to the 1/100 rate treatment of benomyl on a senescent needle. This distortion was rarely observed on needle surfaces. x1440.

Figure 66. Corkscrew twists in hyphae from the 1/10 rate treatment with benomyl on a mature needle. This type of distortion was observed only twice on needle surfaces. x400.
Appendix

Production of seedlings:

Western larch were grown from Champion Timberlands seed, lot number 82/ 1 - 14 - 17. Seed was stratified for 30 days initially, but later tests indicated that unstratified seed germinated just as well. Seed was rinsed with 30% hydrogen peroxide for a few minutes to provide surface sterilization prior to sowing into six inch leach tube containers. Forestry potting mix (Terra-lite®) was autoclaved before being poured into leach tubes. The seedlings were watered twice daily until they were about an inch tall, then removed to the Botany greenhouse. Here they were watered twice weekly with a nutrient solution.

Some seedlings developed symptoms of Fusarium wilt, and a batch of six seedlings tested positive for *Fusarium oxysporum* when root tips where plated onto Komada agar. More frequent watering greatly reduced Fusarium wilt symptoms. Seedlings with such symptoms were discarded, but it can be assumed that some *Fusarium* was present on the experimental seedlings.

In addition to the seedlings raised in the greenhouse, 10 seedlings from the Coeur d'Alene USFS nursery were utilized in the last fall inoculation. This was because the remainder of the seedlings in that inoculation were mostly comprised of succulent needles, and it was desirable to
have more mature and senescent needles included in the experiment.

The formula for 2 liters of nutrient stock solution is as follows:

- \( \text{NH}_4\text{NO}_3 \) 5.14 g
- \( \text{NaH}_2\text{PO}_4\text{H}_2\text{O} \) 2.84 g
- \( \text{KNO}_3 \) 5.16 g
- \( \text{Ca(NO}_3\text{)}_2\text{H}_2\text{O} \) 2.37 g
- \( \text{MgSO}_4\text{H}_2\text{O} \) 3.94 g
- \( \text{NaCl} \) 0.23 g

2 liters distilled water

40 ml of Hoagland's micronutrient solution was added to each 2 liters of stock. The stock solution was diluted with 19 parts tap water per one part of stock.
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