Direct Inoculation of Five-Needle Pines with *Cronartium ribicola* in Axenic Culture

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ABSTRACT

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Vegetative hyphae of *Cronartium ribicola* grown in axenic culture frequently penetrated the thin cuticle of hypocotyls of cultured *Pinus lambertiana* embryos without formation of appressoria. Hyphae penetrated the epidermal cells directly or entered between anticlinal walls and branched in the outermost cortical layer. In a few cases, vegetative hyphae entered stomates either with or without formation of an appressoriumlike structure. Vesicle formation in the substomatal cavity, although typical of natural penetration of the host in the field, was not observed in our materials. Thus, the typical, natural entry through stomates may represent the only penetration pathway available to germinal hyphae on needles otherwise protected by a thick cuticle.

Additional key words: blister rust, sugar pine, western white pine, white pine.

The germinal hyphae of the various plant pathogenic fungi and different spore stages of the same species exhibit different and characteristic modes of penetration during inoculation of their respective hosts. The germ tube may, without prior formation of an appressorium, penetrate the epidermal cell directly (8), grow through the middle lamella between epidermal cells and infect the cortex (18), or enter the host through a stomate (3,17). Conversely, appressorium formation may precede direct penetration of epidermal cells (8,19), intercellular penetration of the epidermis (20), or stomatal penetration (23). In some cases, infection is favored by hyphal contact with the surface of specific cells of the host (24).

In blister rust disease of five-needle pines such as western white pine (*Pinus monticola* Dougl.) and sugar pine (*Pinus lambertiana* Dougl.), germinal hyphae from basidiospores of *Cronartium ribicola* J. C. Fisch. ex. Rabenh. penetrate the needle through stomates and form characteristic substomatal vesicles from which infection hyphae ramify through the host tissue (17).

Direct penetration of the cuticle and of epidermal cells has been reported (2,12); it was, however, incompletely documented and may have involved artifacts (17). The extreme susceptibility of young and succulent stem tissue to inoculation by basidiospores (22) has thus remained unexplained.

We present evidence of both penetration of stomates and the more frequent direct penetration of the hypocotyl cuticle and epidermis by vegetative hyphae of the blister rust fungus grown in axenic culture. Further growth of the fungus with formation of

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intracellular haustoria in western white and sugar pines occurs without the development of specialized subepidermal infection structures.

MATERIALS AND METHODS

Preparation of fungal inocula. Leaves of *Ribes hudsonianum* var. *petiolare* Dougl. were inoculated with *C. ribicola* aeciospores collected from blister rust-infected *P. monticola* at sites near Moscow, ID. Basidiospores from telia produced on the inoculated leaves were shed onto a described medium supplemented with yeast extract, Bacto-peptone, and bovine serum albumin (11). Axenic cultures were generated as previously described (6) and maintained undisturbed for 16 mo. Subcultures were initiated by transferring 1.0 mm^2 sections from these stock cultures to fresh medium where new hyphal growth was then stimulated during 2 wk of incubation at 15 C. These subcultures, ~2 mm in diameter, were used as inocula for pine tissues.

Preparation and inoculation of host tissues. To stimulate germination, 30 seeds of *P. monticola* and 10 of *P. lambertiana* were nicked at the micropylar end and incubated 8 days at 29 C in aqueous 1% hydrogen peroxide, which was changed daily. Seed coats were removed from the germinated seeds, and gametophytes were surface sterilized for 15 min in aqueous 0.8% sodium hypochlorite solution (w/v) and rinsed with five volumes of sterile, distilled water. Embryos were aseptically excised and their radicles inserted into a 1%-agar-solidified half-strength GD medium (16). Hypocotyls of these 8-day-old embryos were immediately surface inoculated with 2-mm-diameter subcultures of *C. ribicola*. One subculture was applied to each embryo with the aerial hyphae in contact with the hypocotyl. Petri plates containing inoculated embryos were covered, sealed with Parafilm, and incubated 14 days at 20 C under 2,000 lux constant illumination (cool-white fluorescent).

Microscopic examination of inoculated tissues. For light microscopy, embryos were fixed in formalin-acetic acid-ethyl alcohol, dehydrated in an alcohol series, embedded in paraffin, sectioned, and stained with orseillin BB and aniline blue (14). For scanning electron microscopy, glutaraldehyde-fixed embryos were dehydrated in an alcohol series, exchanged by using freon, and critical point-dried with a Bowmar SP-50 EX critical point drier. They were then coated with gold to approximately 0.05 μm thickness in a Poloron Sputter Coater and examined in the ETEC Autoscan Scanning Electron Microscope at 20 kV.

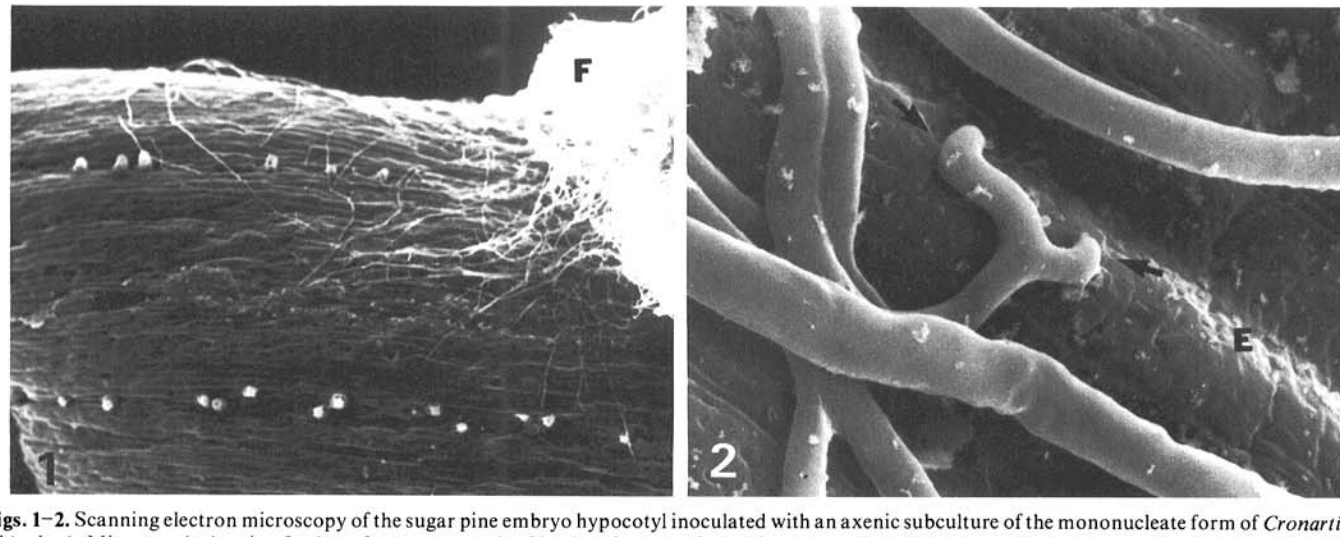
RESULTS AND DISCUSSION

Penetration and infection in the hypocotyl area of embryos of both pine species occurred within the 14-day experimental period in all cases. Fungal growth could be seen easily on the embryo surface, although no symptoms of infection were visible. The hypocotyl surface of *P. lambertiana* remained intact as the embryos grew from an initial length of about 15 mm to about 35 mm during the 14-day period. The smaller (5 mm) 8-day-old *P. monticola* embryos grew a proportional amount but tended to generate callus that distorted and ruptured the hypocotyl epidermis within 10 days.

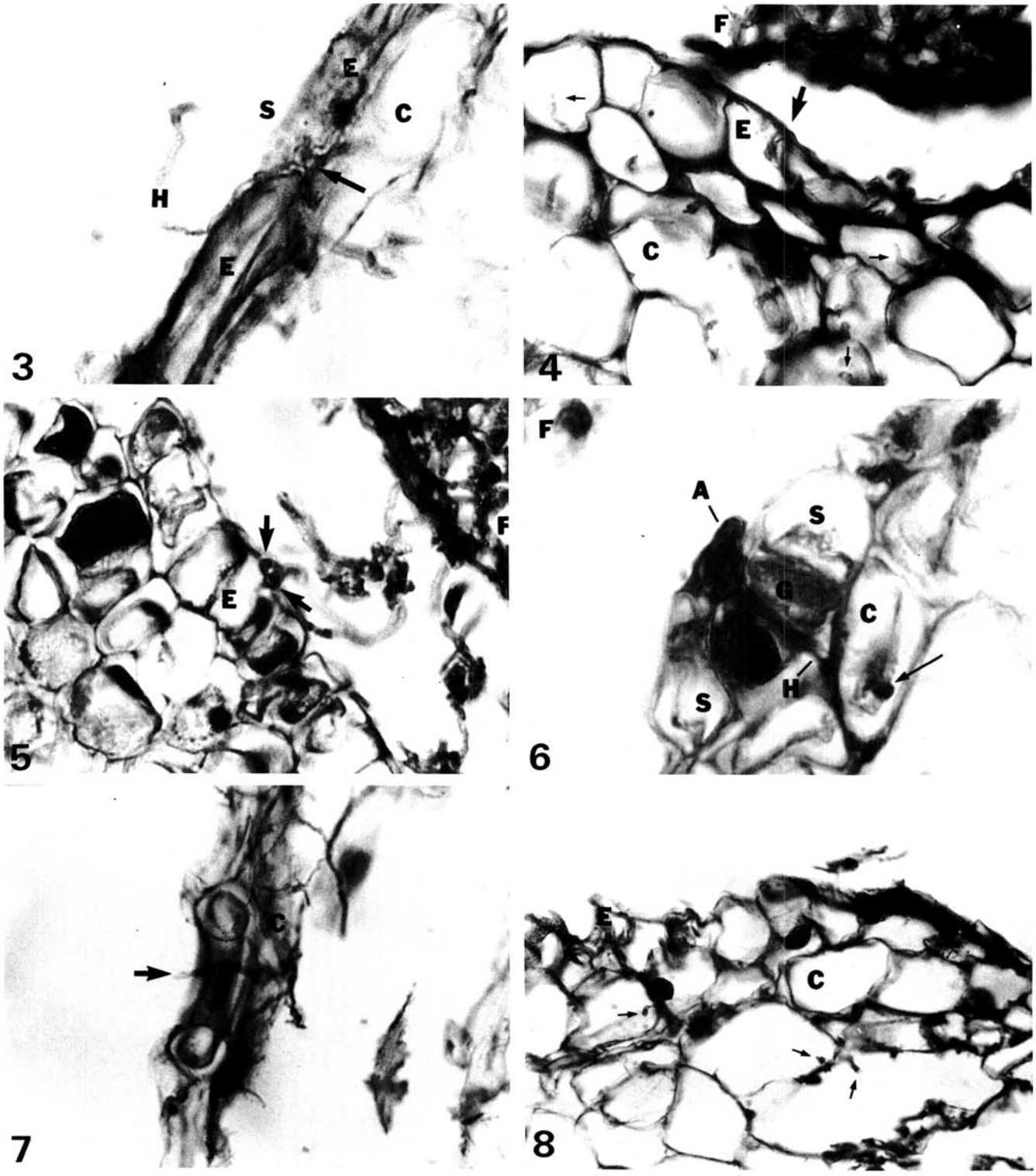
Hyphae grew from the inoculum onto the surface of the host hypocotyl within 4 days after application of the fungal subculture. This surface growth progressed radially from the inoculum through approximately 7 mm during the 14 days (Fig. 1), although a few hyphae grew to lengths >1 cm. Hyphal growth followed the host epidermal surface; aerial hyphae were isolated and rare. Such a substrate-proximal growth habit, reported also for germ tubes from *C. ribicola* basidiospores (9), suggests the presence of nutrients or growth stimulants associated with the host surface (7,10,13) and likely serves to enhance host penetration and infection. Hyphal tips were appressed against the epidermal surface (Fig. 2).

Hyphal entry was most easily followed in sugar pine where hyphae from the vegetative inocula commonly grew inward through the cuticle between epidermal cells and branched in the outermost layer of cortical cells (Fig. 3). Haustorial development was frequent and occurred after contact with the first few host cells (Fig. 4). Vegetative hyphae from the inoculum also penetrated epidermal cells directly through periclinal walls (Fig. 4). Rarely, appressoriumlike structures were observed either directly on the epidermis (Fig. 5) or over a stomate between guard cells (Fig. 6) in a fashion similar to that reported for infection of *Ribes* by *C. ribicola* aeciospore germ tubes (23). In our materials, a single hypha extended from the "appressorium" through the stomatal aperture and directly penetrated a cortical cell (Fig. 6). In another field of view of the same material (Fig. 7), a vegetative hypha penetrated a stomate without formation of an appressorium. Again, the single penetrating hypha branched within the cortex. No substomatal infection vesicle was produced.

Western white pine embryos in vitro tended to form callus (Fig. 8), which resulted in distorted epidermal and cortical tissues and generally obscured resolution of exact pathways of intercellular hyphal growth. However, haustoria were visible at similar depths (12–15 cells) in stained sections of the cortex of each of the sugar pine and western white pine embryos after the 14-day incubation period. Therefore, both host species were probably penetrated within a brief period after application of the fungal inoculum and prior to callus production by the western white pine embryos. Thus, the processes of infection were considered to be similar in both host species.



Figs. 1–2. Scanning electron microscopy of the sugar pine embryo hypocotyl inoculated with an axenic subculture of the mononucleate form of *Cronartium ribicola*. **1,** Micrograph showing 2-wk surface overgrowth of hyphae from the fungal inoculum (F) (×90). **2,** An enlarged view of the hypocotyl showing surface-associated fungal growth, with hyphal tips (arrows) appressed to the epidermis (E) (×3,100).



Figs. 3–8. Light microscopy of inoculated pine embryos. **3,** A vegetative hypha is seen as having grown from the outer surface (S) of the sugar pine embryo, between anticlinal walls of two epidermal cells (E) (shown in longitudinal section), then branching (arrow) at the outer cortex (C) (×700). **4,** Inoculated sugar pine hypocotyl in partial cross section showing direct penetration of an epidermal cell (E) by a vegetative hypha (large arrow) grown from the axenic fungal inoculum (F). Numerous intracellular haustoria (small arrows) are shown in the epidermis and cortex (C) (×500). **5,** Appressoriumlike structures (arrows) at tips of short, vegetative hyphal branches of *C. ribicola* grown from an axenic fungal inoculum (F), and appressed against the epidermis (E) of the sugar pine embryo hypocotyl (×575). **6,** Penetration through the stomate of a sugar pine hypocotyl by a single vegetative hypha (H) of *C. ribicola*, grown from an appressoriumlike structure (A). Guard (G) and subsidiary (S) cells of the stomate are shown in cross section. A haustorium (arrow) is seen within that cortical cell (C) first encountered by the hypha. A portion of the axenic fungal inoculum (F) is visible at the upper left of the field of view (×950). **7,** Penetration of a sugar pine hypocotyl stomate (shown in longitudinal section) by a single vegetative hypha (arrow) of *C. ribicola*, which then branches in the cortex (C) (×425). **8,** Western white pine hypocotyl (in partial cross section) two weeks after inoculation with an axenic culture of *C. ribicola* (not shown in this field of view). The epidermis (E) and cortex (C) are distorted by the in vitro tendency to callus by these embryos. Haustoria (arrows) are visible within cortical cells (×220).

In nature, penetration of the needle by stomatal entry of the germinal hyphae may be a consequence of the thick needle cuticle. The mature cuticle has wax esters, hydrocarbons, and long-chain fatty alcohols (15) that are a thick (3–5 μm) barrier to penetration (4). Thus, the needle cuticle may prevent direct penetration with the result that stomates provide the only sites available for hyphal entry.

The hypocotyl cuticle is thin (0.5 μm) on the cultured embryos we used. A thin cuticle is typical of very young plants and usually consists mainly of C_2–C_8 fatty acid monomers (15). Thus, on our cultured embryos the cuticle would offer little resistance to the diffusion of nutrients outward to sustain overgrowth of hyphae or even opportunistic hyphal penetration, similar to penetration of exposed parenchyma of flax (*Linum usitatissimum* L.) cotyledons by vegetative hyphae of axenic *Melampsora lini* (21).

No substomatal or other infection "vesicles" were observed in our materials. Substomatal vesicles are typical in needles infected in the field. In the needles, such vesicles may be characteristic penetration structures produced only by germ tubes. Germinal hyphae of *C. ribicola* differ from vegetative hyphae in their response to light and gravity (3). Other differences between germinal and vegetative hyphae may also exist, as they do between the other rust fungi (1). Vegetative hyphae from axenic cultures of *C. ribicola* show infrequent stomatal penetration of intact primary needles of *P. monticola* (Diner and Mott, *unpublished*). We used a dense inoculum similar to that reported here for use with embryos, but have not observed penetration through the cuticle and intact epidermis at other sites on the needle.

Characteristic disease pathology and a genetic resistance were expressed by the intact host embryo inoculated with vegetative hyphae from axenic culture (5). We consider that inoculation of pine embryos with vegetative hyphae of *C. ribicola* in axenic culture is an opportunistic event made possible by the thin permissive nature of the embryo cuticle. The modes of penetration we have described may also be reflected in the disproportionate vulnerability of succulent white pine stems in the field (22).

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