Techniques

A Rapid Axenic Assay for Hypersensitive Resistance of *Pinus lambertiana* to *Cronartium ribicola*

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ABSTRACT

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Axenic cultures of *Cronartium ribicola* were used as inocula for young embryos of *Pinus lambertiana* from seed lots expressing either blister rust susceptibility or hypersensitive resistance to the disease in the seedling or mature tree. Light-microscopic examination of both groups 14 days after inoculation showed characteristic expression of progressive disease in the embryos of susceptible genotypes and tissue necrosis with limitation of the disease in embryos of the hypersensitive genotype. This rapid in vitro display of resistance characteristics is independent of both the "natural" mode of host penetration via stomates and the ontogenic state of the inoculum. The assay should prove useful in breeding for host resistance and in studies of host-parasite biology.

Resistance to the white pine blister rust fungus (*Cronartium ribicola* J. C. Fisch. ex. Rabenh.) is expressed by several five-needle pines (6). These natural hosts employ a variety of resistance responses (7,11,12). Breeding programs have improved genetic resistance to blister rust in *Pinus monticola* Dougl. (Western white pine) and *Pinus lambertiana* Dougl. (sugar pine) (9,12). Progeny selections were usually made from inoculated pine seedlings at least 2 yr of age (1,13).

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Recently, methods were developed to assay for a hypersensitive resistance response in sugar pine using seedlings inoculated at 6 wk and evaluated by macroscopic examination 2 mo later (10). Inoculation was by basidiospores shed from telia on leaves of *Ribes* suspended over the host seedlings. The resistance was controlled by a single dominant gene, and expression in seedlings was equivalent to that seen in inoculated, fully developed secondary needles of resistant individuals of similar genotypes (11).

C. ribicola was recently established in axenic culture from basidiospores (3). Axenic subcultures of these colonies were virulent on embryos of sugar pine (*unpublished*), and infection was established by direct penetration of the hypocotyl cuticle by the vegetative hyphae.

This study demonstrates that the genetically controlled,

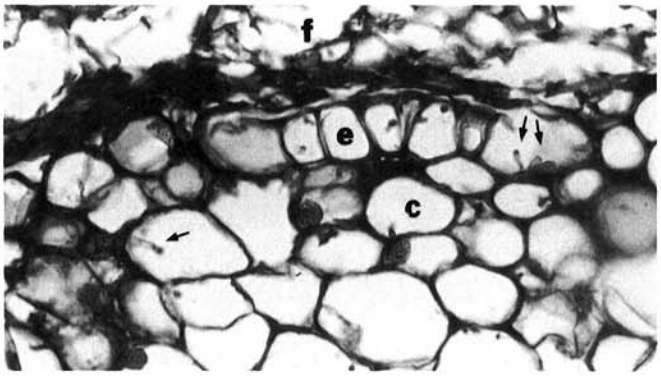

Fig. 1. Light micrograph showing fungal overgrowth (f) on the hypocotyl surface, and intracellular haustoria (arrows) in the epidermis (e) and cortex (c) of a blister-rust-susceptible sugar pine embryo, 14 days after inoculation with an axenic culture of *Cronartium ribicola* (×250).

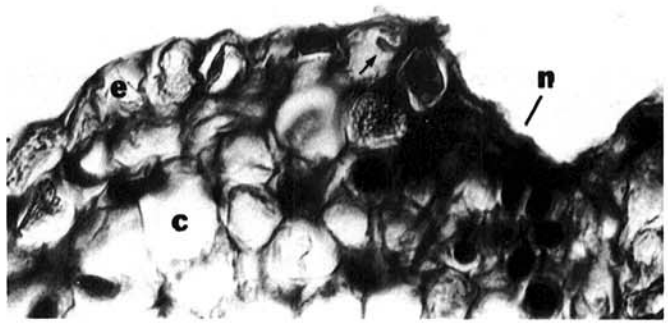

Fig. 2. Light micrograph showing necrosis of epidermis (e) and cortex (c) of blister-rust-resistant sugar pine embryo, 14 days after inoculation with an axenic fungal culture. A haustorium (arrow) is seen in one epidermal cell adjacent to the necrotic area (N). The fungal overgrowth often became partially detached during specimen preparation, and is not shown in this field of view (×250).

hypersensitive reaction in sugar pine (11) can be observed even in embryos cultured in vitro. The reaction is faithfully elicited within 2 wk after inoculation with vegetative hyphae from axenically cultured *C. ribicola*.

MATERIALS AND METHODS

Following preliminary experiments, six seeds from full-sib families of known genotype (three homozygous dominant for hypersensitive resistance to blister rust; three homozygous recessive for resistance) were each nicked at the micropylar end and germinated 8 days at 29 C in aqueous 1% hydrogen peroxide, which was changed daily (2). Embryos were then aseptically excised and placed in petri plates with their radicles inserted into 1% agar-solidified, half-strength medium #1 (14) lacking growth regulators. The intact cotyledonary node of each embryo was inoculated with a 14-day-old, 2-mm-diameter colony generated from subculture of the blister rust fungus. Fungal subcultures had been prepared by inoculating a described medium (4) with 1-mm sections of 6-mo-old axenic cultures of *C. ribicola* generated from basidiospores germinated on medium of the same composition. Once inoculated, embryos were incubated at 20 C under constant 2,000 lux illumination (cool-white, fluorescent).

After 14 days of incubation, the inoculated embryos were fixed in formalin-acetic acid-ethyl alcohol, dehydrated in ethanol series, and embedded in paraffin. Thin sections of the paraffin-embedded embryos were stained with orseillin BB and aniline blue (8).

RESULTS AND DISCUSSION

Hyphae grew from the inocula to the hypocotyl and cotyledonary surfaces within 4 days on all embryos whether susceptible or resistant. This surface growth of hyphae progressed radially from the inoculum. No differences in extent of surface growth could be discerned between that on susceptible and resistant embryos. The hyphae grew to a maximum 1.5 cm radius during the 14-day experimental period with no apparent symptoms of disease.

Infection of cotyledons and hypocotyls was observed by light microscopy in all embryos from both rust-resistant and susceptible seed lots. Intracellular haustoria and hyphae were plentiful in susceptible embryos (Fig. 1). No response to infection was apparent in these embryos examined by light microscopy. We have observed (*unpublished*) that vegetative hyphal penetration of sugar pine embryos occurs directly through the cuticle, a likely consequence of an opportunistic invasion through an epidermis coated by a very thin cuticle that is easily penetrated.

Sections of inoculated embryos with hypersensitive resistance showed numerous small areas of necrosis at infection sites beneath the inoculum (Fig. 2). A few intracellular haustoria could be seen in the epidermis and outermost cortex. Although surface hyphal growth external to the host was resolved in section, intercellular hyphae were not. This failure was due to necrotic collapse and distortion of tissue in the inoculated area as well as in cortical cell layers deeper than those in which haustoria could be resolved. Such a host reaction essentially precludes further fungal invasion, and typifies the hypersensitive response in sugar pine (11) as well as in other pine (7,15) and nonpine (5) species. To date, only spore inocula have been used in other studies of host resistance to infection of callus or organized plant tissues by parasitic fungi. Relatively few axenic cultures of these pathogens have been generated. The successful use of axenic cultures as inocula for in vitro expression of specific host resistance responses has not, until now, been reported.

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