

## In Vitro Inoculation of Western White Pine Tissue Culture Propagules with Vegetative Hyphae of *Cronartium ribicola*

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### ABSTRACT

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Propagules of western white pine were inoculated in vitro through intact needles and shoot apex wounds with vegetative hyphae of the blister rust fungus grown from basidiospores in axenic culture. Successfully inoculated propagules showed intracellular haustoria, characteristic of natural

inoculation by basidiospores. The opportunity to challenge different tissues of selected host genotypes with cloned replicates of specific rust isolates in vitro may be useful in studying basic processes of resistance and pathogenesis.

Breeding for resistance to blister rust in white pines is a long-term prospect complicated by pathogen genetic variation (11,14), short seasonal availability of basidiospores for inoculation (1), and the several years required to evaluate resistance (2).

Recent in vitro studies of white pine blister rust demonstrated that vegetative hyphae of the mononucleate stage of the blister rust fungus (*Cronartium ribicola* J. C. Fisch.: Rabenh.) grown from basidiospores in axenic culture were virulent in cell cultures (8) and intact young embryos (7) of western white pine (*Pinus monticola* Dougl.) and sugar pine (*Pinus lambertiana* Dougl.). Genetic resistance was phenotypically differentiable both in cell cultures (8) and embryos (5) of sugar pine within two weeks following inoculation.

Some rust resistance mechanisms reported for *P. monticola* are expressed only by specific mature tissues or organs (13) such as the short shoot (9). Where tissue or organ specificity is critical, the use of white pine cell cultures, embryos, or young seedlings in assays for resistance may be inappropriate. For example, cell cultures of *P. monticola* generated from cambial explants of trees which had shown "bark resistance reaction" (13) in the field, did not display resistance when inoculated with vegetative rust hyphae in vitro (*unpublished*).

This paper describes procedures for in vitro inoculation of stems and intact needles of *P. monticola* using vegetative hyphae of the blister rust fungus.

### MATERIALS AND METHODS

Primary colonies of *C. ribicola* were grown from basidiospores in axenic culture (4), during 40 days incubation on an agar-solidified medium in the dark at 15 C. These staled (viable but nongrowing) whole colony inocula were removed from their agar substrate and applied inverted to the adaxial surface of primary needles of tissue culture-generated propagules of *P. monticola*. Alternately, 1-mm segments cut from the periphery of these staled axenic cultures were applied to needles either immediately (fresh-

cut inocula) or after a preliminary 14-day incubation of fresh-cut inocula segments on nutrient medium, during which time they had grown to 2-mm diameter (14-day subcultures).

Cloned shoots of *P. monticola* were grown in vitro from adventitious buds developed on excised cotyledons (15). Seed used were from a general collection of undescribed genotypes. Shoots 1.0-1.5 cm in length and eight to 12 wk old were selected for inoculation. The three types of inocula (whole/staled, fresh-cut, and 14-day subcultures from whole/staled colonies) were each applied to green primary needles in situ on the upper half of the shoot. The cut surface of each freshly cut subcultured colony segment was placed in contact with the needle, because new hyphal growth is commonly initiated only from freshly cut surfaces of staled colonies of *C. ribicola* (*unpublished*).

For stem inoculations, plants were wounded either at the apex or at the mid-part on the stem. Apical wounds were made by removing the surrounding needles and excising approximately 1 mm from the stem apex. Other stem inoculations were attempted through a wedge-shaped wound, approximately one-third the stem thickness, at a point midway along the length. Immediately after wounding, 14-day subcultures of primary rust colonies were applied to cut apices or mid-stem wounds with the aerial hyphae in contact with host tissue.

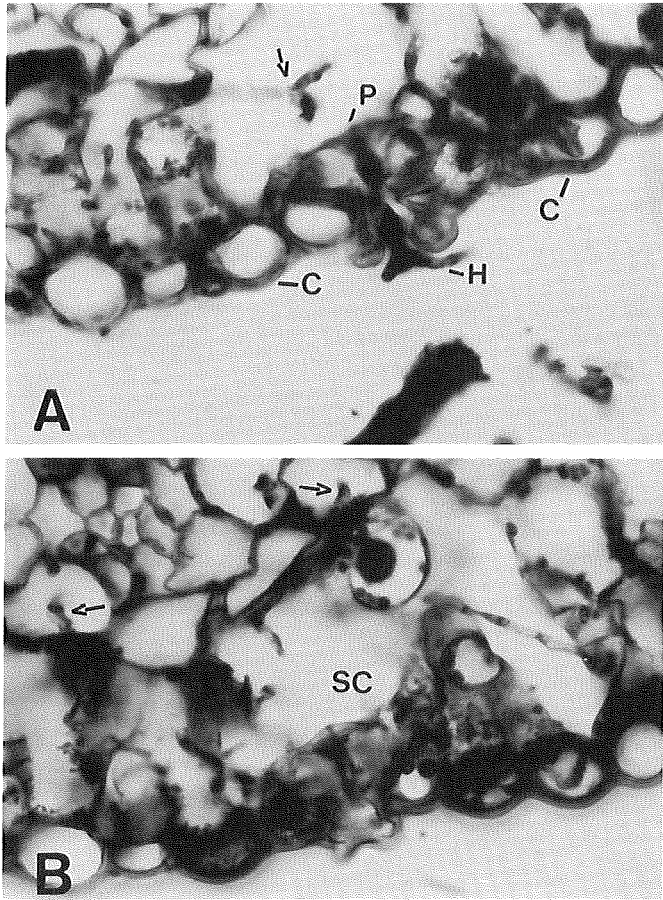
All inoculated materials were incubated at 20 C under constant 2,000 lux cool-white fluorescent illumination. Cultures were examined during a 4-wk period for hyphal overgrowth on the host surface, infection of host tissues, and evidence of host response.

After 4 wk, tissues beneath and immediately adjacent (1-2 mm) to the inoculum were sampled, fixed, dehydrated through a graded ethanol series, embedded in paraffin, sectioned, and stained (10). Sections (average thickness 15  $\mu$ m; 100-450 sections per sample) were examined for sites of hyphal penetration and for presence and location of intercellular hyphae and intracellular haustoria.

### RESULTS

Intact needles displayed no surface overgrowth of hyphae from any of the three inoculum types. Infection occurred with the fresh-cut inocula but not with whole-colony or 14-day subculture inocula. In 42% of 36 trials, the needles inoculated with fresh-cut mycelia showed presumptive penetration at one or two sites where, in each case, the mesophyll was colonized by intercellular hyphae

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**Fig. 1.** Cross sections of western white pine propagule needles inoculated intact with vegetative blister rust hyphae. **A**, Penetration of a stomatal pore (P) by a hypha (H) grown from the applied inoculum. Another, similar hypha (arrow) is shown in the substomatal chamber ( $\times 700$ ). Epidermal cells with cuticle (C) can be seen adjacent to the stomate. **B**, Intracellular haustoria (arrows) in the mesophyll beneath the substomatal chamber (SC) ( $\times 700$ ).

that were progressively more thinly dispersed with increasing distance from stomata. Microscopic sections of three needles exhibited hyphae grown inward from the stomatal opening between subsidiary cells (Fig. 1A). Substomatal chambers beneath these penetrated stomata contained hyphae but lacked the substomatal infection vesicle typical of infection from basidiospores in the field. Numerous intracellular haustoria were visible in the substomatal mesophyll (Fig. 1B). Exhaustive examination provided no evidence of hyphal penetration through the intact epidermis.

In contrast to fresh-cut inocula, the 14-day subcultures failed to penetrate needles even once in 20 trials, although inoculum viability had been demonstrated by growth of hyphae during the 14-day preliminary incubation. These growing hyphae became bent as inocula were applied to the host surface. It is presumed that these bent hyphae, lacking both directed attraction to stomatal pores (7) and available nutrients for continued growth on the intact needle cuticle, were not prone to encounter and enter stomata. With fresh inocula, new hyphae were initiated from the cut surfaces appressed to the needle epidermis. It is likely that some hyphae grew straight to enter stomata directly and by chance during the early flush of randomly oriented growth from the fresh cut surface of the staled colony. These hyphae, in contrast to those showing no further growth on the non-nutritive surface of the needle cuticle, continued to grow and invade the needle mesophyll, undoubtedly supported nutritionally by internal host tissues. None of 13 trials with the whole colony inocula led to observable hyphal growth or infection.

When 14-day subcultures were applied to apical wounds, 8 of 10 apices were infected after 4 wk. Infected apices exhibited numerous intercellular hyphae and intracellular haustoria in the stem cortex

beneath the inoculated wounds. In contrast, lateral wounds made to stems of similar propagules immediately became soaked with fluid exuding from the wounds. Wounds continued to exude even after repeated removal of fluid by absorption into sterile filter paper. Inocula applied to these wet areas immediately became visibly soaked and showed no hyphal growth or infection at the wound sites during the 30-day incubation.

## DISCUSSION

Axenic, vegetative hyphal inocula of the blister rust fungus infected both needles and stem apices of tissue culture propagules of *P. monticola* in vitro. Penetration of needles depended upon inoculum age and mode of application to the host; stem infection depended upon the site of the wound. The failure of rust hyphae to overgrow the needle surface contrasted with their extensive surface growth on young intact embryos (7), where exudation of nutrients through the thin cuticle sustained inoculum growth and opportunistic penetration of the host.

Axenically grown hyphae of other rust fungi have not been shown to infect intact susceptibles, with one possible exception (3) involving young and succulent host tissues. Successful host inoculation was realized in some other cases by applying vegetative mycelia to exposed mesophyll (12,16). Our in vitro methods using vegetative inocula with cell cultures (8), young embryos (5), needles, and stems are unique. Because methods exist to clone both the white pine host (15) and blister rust pathogen (6), the genetic/biochemical regulation of infection and resistance may now be examined in culture.

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