hibit tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, or bind to dopaminergic receptors.

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Cultured Cells of White Pine Show Genetic Resistance to Axenic Blister Rust Hyphae

Abstract. *Hypersensitive resistance to axenically cultured* Cronartium ribicola *was displayed by subcultured callus of* Pinus lambertiana. *Cellular resistance to a destructive rust disease can now be studied at the macromolecular level through use of cloned cells of both host and pathogen in a system amenable to emerging recombinant DNA technology*

White pine blister rust, caused by *Cronartium ribicola* J. C. Fisch ex Rabenh, is responsible for annual losses of millions of cubic feet of timber from the several five-needle pine species in the United States alone. Selective breeding for genetic resistance is the "most feasible and promising approach" (*1*) to initiate recovery of five-needle pines from this pandemic. A single dominant gene in sugar pine (*Pinus lambertiana* Dougl.) affects the only qualitative and well-defined genetic resistance to this disease (*2*). Macroscopic expression of this resistance mechanism is the appearance of a brown fleck on the needle at the site of rust basidiospore inoculation, indicative of a hypersensitive reaction in the cells beneath.

Traditional field breeding programs for disease resistance, both in forest trees and agronomic crops, are labor-intensive, long-term, and usually dictated by generation times of both host and pathogen. Greenhouse and laboratory whole-plant assays for resistance are supportive but commonly involve natural modes of spore inoculation followed in weeks or months by appraisals of relative resistance in some organ-specific reaction. We now report the development of a rapid in vitro assay for a characteristic rust-resistance reaction displayed at the cellular level; the assay employed monocultures of both host and pathogen to challenge cell cultures (callus) with the vegetative pathogen.

Many blister rust–resistance mechanisms have been described in various host species (*3*) and characterized for breeding programs (*4*). In most cases, modes of inheritance of these resistance mechanisms (typically expressed only after secondary needle development in the 3-year seedling) have remained unresolved. However, the hypersensitive rust-resistance mechanism reported in sugar pine is expressed not only in spore-inoculated cotyledons and primary and secondary needles (*5*) but also in young embryos inoculated with vegetative hyphae of the rust fungus grown in axenic culture (*6*). Characteristic resistance expressed within 2 weeks by these embryos was similar histologically to that seen in the spore-inoculated needle. This suggested the possibility that resistance was being expressed independent of the degree of host tissue organization and thus on the cellular level.

Six callus lines were established from minced germinated embryos of sugar pine seeds (*7*) to test this hypothesis. Three seeds were heterozygous for hypersensitive resistance to blister rust; three were homozygous recessive for resistance and thus susceptible to rust. Callus cultures were maintained on a modified Brown and Lawrence agar-solidified medium (*8*) containing 2.2 μM benzylaminopurine (BAP) and 2.7 μM naphthalene acetic acid in petri plates at 20° ± 1°C under continuous cool white fluorescent light (2000 lux). Cultures were subcultured to fresh medium biweekly for 10 months before use. Axenic rust cultures were established (*8*) from basidiospores produced on the alternate host *Ribes hudsonianum* var. *petiolare* that had been inoculated with rust aeciospores collected in Idaho. Axenic cultures were maintained by subculture of segments excised from established colonies onto freshly prepared medium of the same composition (*8*).

For the infection assay, callus from each line was arranged in three pads (1 cm in diameter, 1.5 mm thick) on fresh pine callus medium. After 6 days of incubation, a smooth, fresh callus surface had formed. At this time two of the pads were inoculated at three sites each with axenic rust hyphae, and the third pad served as an uninoculated control.

Rust inocula consisted of colonies (2 mm in diameter) grown for 14 days in subculture from segments (1 mm^2) of older colonies that had been maintained in petri plates at 14°C in the dark. Inocula were applied inverted onto the callus surface such that aerial hyphae were in contact with host tissue. Inoculated calli in sealed plates were incubated for 2 weeks under the conditions described above for callus maintenance.

Hyphae grew radially 1 to 2 mm from the inoculum and over the callus within 4 days. After 2 weeks, radial overgrowth of hyphae on callus surfaces was measured. Depths of hyphal penetration into the host tissue were determined microscopically in callus sections stained with orseillin BB and aniline blue (*9*). Hyphal overgrowth and penetration were restricted in the three resistant lines compared to the susceptible lines (Table 1). Fungal growth, either on or within the resistant callus lines, was approximately 25 percent of that shown for susceptible callus lines.

Intercellular hyphae and intracellular haustoria were apparent in all inoculated calli after 2 weeks (Figs. 1 and 2). Only the resistant lines showed brown-stained surface cell layers beneath and peripheral to the inocula when first examined at that time (Fig. 2). The depth of this stained region in all resistant lines exceeded that of hyphal penetration by several cell layers. Such a reaction front is typical of the hypersensitive response seen in naturally inoculated host trees (*10*) and in cultured excised embryos (*6*). Some less dense staining was attendant with all inoculations (Fig. 1), although it was distinguished easily from the darkly stained tissue and the more extensive hypersensitive reaction which was evident microscopically at 2 weeks. Gross macroscopic hypersensitivity was peculiar to inoculated resistant callus lines by 3 weeks and was characterized by callus browning and necrosis.

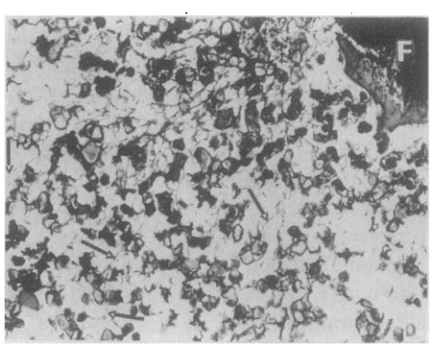
Table 1. Radial growth of vegetative hyphae of *Cronartium ribicola* on calli generated from rust-resistant and rust-susceptible sugar pine embryos. Measurements of surface radial hyphal overgrowth were made from the periphery of the applied fungal inoculum to host callus surface sites showing greatest distal hyphal growth. Microscopic measurements of hyphal penetration into the calli were made with the use of stained callus sections. Values are mean ± standard deviation (N = 6).

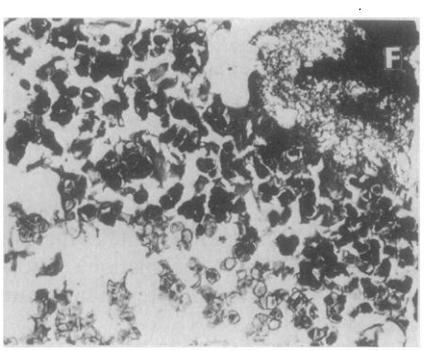
Callus clone	Surface radial growth (mm)	Subsurface radial growth (×120μm)
	Rust-resistant	
1	1.5 ± 0.6	2.1 ± 0.7
2	2.2 ± 0.9	2.5 ± 0.9
3	1.6 ± 0.6	1.9 ± 0.5
$\bar{x}$	1.8	2.2
	Rust-susceptible	
1	7.3 ± 1.9	9.8 ± 2.6
2	7.9 ± 2.3	8.2 ± 2.4
3	5.0 ± 1.6	6.6 ± 2.1
$\bar{x}$	6.7	8.2

Macroscopic expression of needle symptoms in natural infection or resistance in trees appears (*5, 11*) to depend on the threshold degree of colonization by the rust. This threshold in sugar pine callus is apparently reached 3 weeks but not 2 weeks after inoculation.

Cytokinin concentration in the range of 10 to 50 μM has been reported to suppress the hypersensitive reaction in plants to other fungal (*12*) and nonfungal (*13*) plant pathogens. In our case, cytokinin (BAP) at concentrations up to 88 μM did not depress the hypersensitive response. A higher concentration (110 μM) of medium cytokinin induced callus browning and necrosis in both control (uninoculated) and inoculated calli.

Hypersensitive resistance can be detected by both depressed fungal growth and host cell reaction in callus cultures. That both host and pathogen can be propagated separately and indefinitely in culture permits their continuous availability for examination in a variety of research perspectives. Interactions of selected genotypes of the resistant white pine hosts (*5, 14*) with races of the rust fungus (*2, 15, 16*) may be examined when studies of this and other modes of resistance are extended to the molecular level. Resistance expression at the cell level will help to identify mechanisms at work in mature trees, since callus can be readily generated from various parts of trees. Selection for resistance in natural or engineered cell populations can be a basis for propagation of superior trees when regeneration from cell cultures of white pines becomes possible.





Figs. 1 and 2. Light microscopy of sections of subcultured calli derived from blister rust–susceptible and rust–resistant sugar pine embryos. Calli were inoculated for 2 weeks with vegetative *Cronartium ribicola* grown in axenic culture. Fig. 1 (left). Inoculated rust-susceptible callus showing the fungal inoculum (*F*) from which intercellular hyphae (arrows) have grown to sites throughout and beyond the field of view. Abundant intracellular haustoria are resolved under higher magnification. Note the low frequency of dark, necrotic host callus cells beneath the inoculum (×30). Fig. 2 (right). Inoculated rust-resistant callus showing necrosis in the callus cell layer immediately beneath the fungal inoculum (*F*). A few intercellular hyphae, resolved at higher magnification, occupy sites within this layer; none are apparent beneath the necrotic zone (×30).

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