

Inhibition of *Cronartium fusiforme* by Loblolly Pine Callus

W. R. Jacobi

Graduate research assistant, Department of Plant Pathology, North Carolina State University, Raleigh 27650. Current address of author, Department of Botany and Plant Pathology, Colorado State University, Fort Collins 80523.

Portion of a Ph.D. thesis submitted by the author to North Carolina State University.

Journal Series Paper 6522 of the North Carolina Agricultural Research Service, Raleigh 27650.

Mention of trade or company names does not imply endorsement by the North Carolina Agricultural Research Service of the products named, or criticism of similar ones not mentioned.

Appreciation is given to the Tree Improvement Cooperative, School of Forest Resources, North Carolina State University and the Resistance Screening Center of the U.S. Forest Service, Asheville, NC, for supplying plant and fungal material.

Accepted for publication 1 April 1981.

ABSTRACT

Jacobi, W. R. 1982. Inhibition of *Cronartium fusiforme* by loblolly pine callus. *Phytopathology* 72:143-146.

Loblolly pine callus grown in vitro inhibited mycelial growth of *Cronartium fusiforme* derived from both basidiospores and urediospores. Phosphate buffer washes of loblolly and slash pine callus inhibited growth of *C. fusiforme* in culture. Colonization of susceptible loblolly seedlings by

C. fusiforme was restricted by growing seedlings in the callus washes, or on existing callus, and by inducing callus formation in the seedlings. The inhibitory factor from pine callus was heat labile, of high molecular weight, and water soluble.

Additional key words: basidiospores, slash pine.

When loblolly pine (*Pinus taeda* L.) callus is grown on a variety of media and growth factors it is resistant to intracellular and intercellular colonization by *Cronartium fusiforme* Hedgc. and Hunt ex Cumm. (*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Cumm.) Burds. et Snow). In fact, the fungus does not survive on the surface of loblolly pine callus (11). Resistance of callus tissues to fungal pathogens, especially rust fungi (15), is not uncommon, although some authors have reported successful infection (5,7). Harvey and Grasham (7) routinely succeeded in infecting callus of western white pine (*Pinus monticola* Dougl.) with *Cronartium ribicola* J. C. Fisch. ex Rabenh.

Resistance of plant callus to infection has been attributed to the lack of the stimulus afforded by an epidermis and/or cuticle (19), or to antimicrobial metabolites exuded by the host tissue (4,15). A variety of toxic substances has been isolated from callus or media supporting callus (13,16). Phenolic substances are produced by potato callus cells and stationary-phase cells of *Acer* sp. (18,21). Phytoalexins are produced in callus cultures challenged by compatible pathogens (6,8).

The purpose of this investigation was to determine whether toxic substances are produced by loblolly and slash pine callus and, if so, the role they may play in the resistance of intact plants.

MATERIALS AND METHODS

Two approaches were used in these studies: first, callus washes were assayed with cultures of *C. fusiforme*, and second, susceptible loblolly seedlings that contained callused cells or had been grown

on callus or callus washes were inoculated.

Plant materials. Callus tissues of loblolly and slash pine (*Pinus elliotii* Engelm. var. *elliotii*) were obtained by routine tissue culture methods (3). Loblolly pine callus was initiated from succulent stems of 3-yr-old seedlings of resistant and susceptible plants used at the Resistance Screening Center, U.S. Forest Service, Asheville, NC. Slash pine callus also was initiated from susceptible plants used at the Resistance Screening Center. Pine callus was initiated and maintained on a modified Brown and Lawrence (B & L) medium (3,10). Callus used for interactive studies with the fungus and seedlings was grown on a modified Gresshoff and Doy medium 1 (20) with 5 mM NH₄⁺, 35 mM NO₃⁻, 2.2 μM 6-benzylaminopurine (BAP), 2.7 μM α-naphthalenacetic acid (NAA), (hereafter designated GD-1) with or without 0.05% of an 80% ethanol-soluble fraction of yeast extract and peptone (YE & P) to allow fungal growth (10). This medium previously was shown to be suitable for both the host and pathogen (10).

Loblolly seedlings used in studies to assess the effect of callus on colonization were cultured in vitro from a susceptible half-sib family line. Pine seedlings were cultured axenically on a modified GD-1 medium (20).

Test fungi. Axenic cultures were derived from two types of spores of *C. fusiforme*: basidiospores abjected from excised telial columns (1), and urediospores from nonruptured or freshly-ruptured urediospore pustules. Colonies derived from both spore types were initiated and maintained on a modified GD-1 medium with 0.1% whole yeast extract and peptone (GD-1, YE & P) (1).

Callus washes. Callus, transferred 2-3 wk earlier on B&L medium, was washed in 1.4 ml of 0.05 M phosphate buffer, (KH₂PO₄ and NaHPO₄ at pH 7) per gram fresh weight, for 1 hr at 4C on a wrist-action shaker. Callus and cellular debris were

removed by filtration through cheesecloth followed by centrifugation at 40,000 g for 20 min.

Bioassay of callus washes. Mycelial colonies derived from basidiospores of *C. fusiforme* were used to assay the washes for inhibitory substances. The washes and the buffer were filter-sterilized (0.45 μm Nalgene filter unit; Nalge/Sybron Corp., Rochester, NY 14625) and added at 50% of the liquid volume of GD-1, YE & P. Each of five replicated plates was seeded with two 6-mm plugs of mycelium. Diameter measurements were taken with the aid of a dissecting microscope every 5 days for 35 days.

Characterization of the inhibitory factor. The heat stability of the fungal inhibitory factor was determined by bioassaying the wash after heating for 20 min at either 50, 70, or 100 C in a water bath or after autoclaving the wash and media mixture. The approximate molecular weight of the inhibitor(s) was determined by eluting it through ultrafiltration membranes of 10,000, 50,000, and 200,000 nominal molecular weight exclusion limits (NMWL) (Amicon Corp., Lexington, MA 02173). Agglutination tests were

made with rabbit red blood cells by the procedures of Lis and Sharon (14).

Assessment of infection and colonization. Loblolly pine seeds were germinated (11) and placed on GD-1 medium. After 7–10 days, the germinated seedlings were used for one of three experimental treatments: seedlings with 2mm of the radicle end removed were grown in a liquid inhibitor + GD-1 medium mixture that was placed in wells cut in GD-1 agar plates; seedlings were grown on callus cultures of loblolly pine by placing the cut radicle end in the callus mass; and callus formation was induced in certain locations of the cortex of the seedlings by growing them on GD-1 medium supplemented with growth factors (2.2 μM BAP, 2.7 μM NAA).

After 4–5 days on the particular substrate, the upper hypocotyl of each of the seedlings were inoculated with basidiospores cast (for 10–24 hr) from excised telial columns (1). The inoculum concentrations were not determined specifically, and were similar only within a single experiment. All seedlings were collected after 14 days, fixed, dehydrated, embedded, and sectioned on a rotary microtome (12). The degree of colonization, which was determined by microscopic examination of transverse sections of the upper hypocotyl, was rated according to the amount of tissue containing hyphae: 0 = none, 1 = very slight (one to four cells), 2 = slight (one eighth of the transverse area), 3 = moderate (one half of the transverse area), 4 = heavy (one half to all of the transverse area). Seedlings under all experimental conditions produced callus in response to minor damage from handling and culturing. Thus, the condition of each seedling was rated according to the amount of damage: 0 = no damage, 1 = slight physical damage (no callus), 2 = slightly callused cortex, 3 = moderately callused cortex, 4 = heavily callused cortex.

TABLE 1. Growth of *Cronartium fusiforme* basidiospore- and urediospore-derived colonies placed next to loblolly callus and to loblolly seedlings with and without callus^a

Treatment	Colony growth ^b	
	Basidiospore ($\mu\text{m}/5$ days)	Urediospore ($\mu\text{m}/5$ days)
Experiment 1—Callus		
Control (no callus)	780 x	563 x
Callus 2 cm ^c	518 y	80 y
Callus 1 cm ^d	280 z	52 y
Experiment 2—Seedlings		
Control (no seedling)	1,000 x	350 x
Seedling 2 cm ^c	900 x	300 x
Seedling 1 cm ^d	840 x	360 x
Seedling with callus 1 cm	690 y	... ^e

^a Illustrated in Fig. 1.

^b Means of diameter growth over 35 days of 10 colonies started from plugs grown on GD-1 medium with the ethanol soluble fraction of yeast extract and peptone. Values in a column or experiment with a common letter were not significantly different based on Duncan's multiple range test, $P=0.05$.

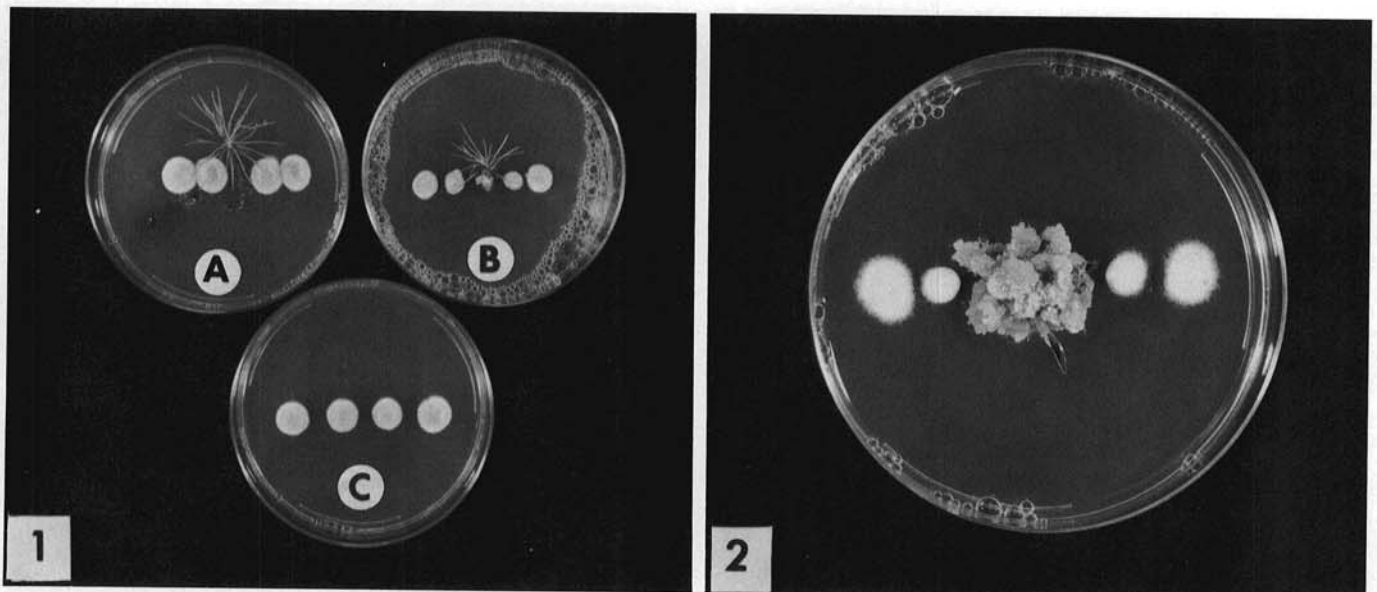
^c Fungus placed 2 cm from callus or seedling.

^d Fungus placed 1 cm from the callus or seedling.

^e No data available.

RESULTS AND DISCUSSION

Radial growth of colonies of *C. fusiforme* was inhibited when placed 1 or 2 cm from loblolly pine callus (Table 1, Fig. 1). Colonies of *C. fusiforme* derived from either basidiospores or urediospores grew well next to noncallused loblolly pine seedlings, but grew poorly near seedlings which had formed callus at the hypocotyl base (Table 1, Fig. 2). Urediospore colonies did not grow in the presence of loblolly pine callus (Table 1). The high sensitivity of urediospore colonies to an inhibitory factor from the callus was not surprising because oak is the normal host of this spore stage. In a second test, mycelium of basidiospores did not grow when placed



Figs. 1 and 2. Mycelial colonies derived from basidiospores of *C. fusiforme* at 1 and 2 cm for loblolly pine seedlings and callus at 30 days on GD-1, 5/35 with the ethanol soluble fraction of yeast extract and peptone: 1, Colonies growing near callus. 2, Colonies growing near (A) healthy seedling, (B) seedling with callus, (C) on medium alone ($\times 0.8$).

TABLE 2. Inhibition of *Cronartium fusiforme* colonies by phosphate buffer washes of loblolly and slash pine callus

Callus wash	Colony growth ^a ($\mu\text{m}/5$ days)
Experiment 1	
Buffer control	1,590 x
Loblolly (resistant)	155 y
Loblolly (susceptible)	290 y
Experiment 2	
Buffer control	650 x
Slash (susceptible)	330 y

^a Means of diameter growth over 35 days of 10 colonies started from plugs grown on GD-1 medium with yeast extract and peptone. Values in an experiment with a common letter were not significantly different based on Duncan's multiple range test, $P = 0.05$.

for 2 wk on loblolly pine callus grown on GD-1, with the ethanol-soluble fraction of yeast extract. Only 5% of the colonies survived upon transfer to GD-1, YE & P medium. Thus, loblolly pine callus appeared to produce inhibitory or toxic factors which diffused readily into the agar, restricting or killing the mycelium of *C. fusiforme*.

Action of the inhibitory factor. Phosphate buffer washes from callus of loblolly and slash pine inhibited radial growth whether the washes originated from resistant and susceptible loblolly, or from susceptible slash pine callus cells (Table 2). Susceptible seedlings of loblolly pine were used to further assess the effect of callus and callus washes on infection by *C. fusiforme*. The inhibitory factor washed from loblolly pine callus appeared to become systemic and to reduce the ability of the pathogen to grow intercellularly and intracellularly in the seedlings (Table 3). The volume of tissue colonized by *C. fusiforme* was significantly less in seedlings exposed to the inhibitory factor contained in the growth medium than in the control seedlings (Table 3). The fungus was not prevented from penetrating the seedlings, however, since 90% of the seedlings were infected in Experiment 1. The amount of damage or callus formation in seedlings did not differ between the heavily colonized control seedlings and the wash-treated seedlings (Table 3). Thus, there are no anatomical differences that would explain the restricted colonization.

Seedlings grown in direct contact with established callus resisted infection and colonization by *C. fusiforme* (Table 3). Only 32% of the seedlings were infected and only a few cells near the epidermis of the remaining seedlings were colonized. Again, there were no obvious anatomical differences between the controls and the seedlings grown on callus that could account for resistance (Table 3). Seedlings grown on a medium with callus-inducing growth regulators for 48 hr formed very little callus and were colonized well (Table 3). In other tests, seedlings grown for 4-5 days on growth factors had heavily callused cortices with little colonization (9).

Properties of the inhibitory factor. The physical properties of the inhibitory factor in the callus washes suggested that the factor was a protein, a substance bonded to a protein, or a glycoprotein. The inhibitory factor was water soluble and heat labile. Partial (75%) inactivation occurred at 70 C and complete inactivation at 121 C. The inhibitory factor was retained on the 10,000 NMWL filter; fractions retained on the 50,000 and 200,000 NMWL filters were increasingly more inhibitory. Red blood cells were agglutinated by the crude callus wash, large molecular weight (>10,000), and concentrated wash fractions. Neither the low-molecular-weight fraction, nor the buffer caused any agglutination. Agglutinating glycoproteins from other plants including soybeans, wheat germ, and peanuts inhibit the growth of various fungi (2,17), presumably because they inhibit the incorporation of cell wall precursors (2).

Conclusions. The inhibitory factor produced by loblolly pine callus restricts hyphal growth and may account for the lack of any intercellular or surface growth of *C. fusiforme* after germination of basidiospores on the callus. An inhibitory factor from the callus which became systemic in the seedlings prevented infection of

TABLE 3. Colonization of susceptible loblolly pine seedlings by *Cronartium fusiforme* after growth in liquid washes of loblolly pine callus, on callus, and with induced callus

Seedling substrate	Number of seedlings	Seedling damage index ^a	Colonization index ^b
Experiment 1			
Wash ^c	32	1.0 x	1.5 x
Buffer control ^d	32	1.1 x	2.4 y
Experiment 2			
Callus ^e	20	1.6 x	0.3 x
Callus inducing medium ^f (48 hr)	20	1.2 xy	2.5 y
Medium control	20	0.9 y	2.9 z

^a Degree of damage and callus formation in seedlings, rated 0-4 (none to heavy).

^b Colonization was rated 0-4 (none to heavy). Means of seedling damage index or colonization index, in an experiment with common letters, were not significantly different at $P = 0.05$ based on Duncan's multiple range test.

^c Callus wash and GD-1 medium mixture (1:1).

^d Buffer and GD-1 medium mixture (1:1).

^e Seedlings placed directly in callus growing on GD-1.

^f Seedlings placed on GD-1 for 48 hr then transferred to GD-1 minus growth factors.

seedlings and severely restricted colonization in infected seedlings. This same factor may be responsible for the lack of infection or intracellular colonization of callus. The inhibitory factor in the callus washes inhibited the fungus directly, but also may affect the physiology of host cells in either callus or seedling, resulting in an incompatible host.

LITERATURE CITED

- Amerson, H. V., and Mott, R. L. 1978. Technique for axenic production and application of *Cronartium fusiforme* basidiospores. *Phytopathology* 68:673-675.
- Barkai-Golan, R., Mirelman, D., and Sharon, N. 1978. Studies on growth inhibition by lectins of penicillia and aspergilli. *Arch. Microbiol.* 116:119-124.
- Brown, C. L., and Lawrence, R. H. 1968. Culture of pine callus on a defined medium. *For. Sci.* 14:62-64.
- Campbell, G., Chan, E. C. S., and Barker, W. G. 1965. Growth of lettuce and cauliflower tissues in vitro and their production of antimicrobial metabolites. *Can. J. Microbiol.* 11:785-789.
- Cutter, V. M., Jr. 1959. Studies on the isolation and growth of plant rusts in host tissue cultures and upon synthetic media. I. *Gymnosporangium*. *Mycologia* 51:248-295.
- Dixon, R. A., and Fuller, K. W. 1978. Effects of growth substances on non-induced and *Botrytis cinerea* culture filtrate-induced phaseollin production in *Phaseolus vulgaris* cell suspension cultures. *Physiol. Plant Pathol.* 12:279-288.
- Harvey, A. E., and Grasham, J. L. 1970. Inoculation of western white pine tissue cultures with basidiospores of *Cronartium ribicola*. *Can. J. Bot.* 48:1309-1311.
- Helgeson, J. P., Budde, A. D., and Haberslach, G. T. 1978. Capsidiol: a phytoalexin produced by tobacco callus tissues. (*Abstr.*) *Plant Physiol.* (Suppl.) 61:58.
- Jacobi, W. R. 1979. I. Interactions of cultured callus and seedlings of loblolly pine with *Cronartium fusiforme*. II. Improving forest disease and insect impact assessment. Ph.D. thesis, N. C. State University, Raleigh. 75 pp. (Diss. *Abstr.* 40:4053B).
- Jacobi, W. R. 1982. Growth medium for dual cultures of loblolly pine callus and *Cronartium fusiforme*. *Phytopathology* 72:136-138.
- Jacobi, W. R., Amerson, H. V., and Mott, R. L. 1982. Microscopy of cultured loblolly pine seedlings and callus inoculated with *Cronartium fusiforme*. *Phytopathology* 72:138-143.
- Jewell, F. F., True, R. P., and Mallett, S. L. 1962. Histology of *Cronartium fusiforme* in slash pine seedlings. *Phytopathology* 52:850-858.
- Khanna, P., Uddin, A., Sharma, G. L., Manot, S. K., and Rathore, A. K. 1976. Isolation and characterization of sapogenin and solasodine from in vitro tissue cultures of some solanaceous plants. *Indian J. Exp. Biol.* 14:694-696.
- Lis, H., and Sharon, N. 1972. Soybean (*Glycine max*) agglutinin. Pages 360-368 in: V. Ginsburg, ed. *Methods in Enzymology* 28B.

15. Maheshwari, R., Hildebrandt, A. C., and Allen, P. J. 1967. Factors affecting the growth of rust fungi on host tissue cultures. *Bot. Gaz.* 128:153-159.
16. Mathes, M. C. 1967. The secretion of antimicrobial materials by various isolated plant tissues. *Lloydia* 30:177-181.
17. Mirelman, D., Galun, E., Sharon, N., and Lotan, R. 1975. Inhibition of fungal growth by wheat germ agglutinin. *Nature* 256:414-416.
18. Robertson, N. F., Friends, J., Aveyard, M., Brown, J., Huffee, M., and Homans, A. L. 1968. The accumulation of phenolic acids in tissue culture pathogen combinations of *Solanum tuberosum* and *Phytophthora infestans*. *J. Gen. Microbiol.* 54:261-268.
19. Rossetti, V., and Morel, G. 1958. Le développement du *Puccinia antirrhina* sur tissu de muflier cultivés in vitro. *C. R. Hebd. Séances, Acad. Sci., Paris* 247:1893-1895.
20. Sommer, H. E., Brown, C. L., and Kormanik, P. P. 1975. Differentiation of plantlets in longleaf pine (*Pinus palustris* Mill.) tissue culture in vitro. *Bot. Gaz.* 136:196-200.
21. Westcott, R. J., and Henshaw, G. G. 1976. Phenolic synthesis and phenylalanine ammonia-lyase activity in suspension cultures of *Acer pseudoplatanus* L. *Planta (Berl.)* 131:67-73.