Microscopy of Cultured Loblolly Pine Seedlings and Callus Inoculated with *Cronartium fusiforme*

W. R. Jacobi, H. V. Amerson, and R. L. Mott

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

Portion of a Ph.D. thesis by senior author. North Carolina State University.

Journal Series Paper 6438 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 USDA, Forest Service, Asheville, for supplying plant and fungal material.

Accepted for publication 26 March 1981.

ABSTRACT

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.

Loblolly pine seedlings grown in axenic culture were readily penetrated and colonized in the hypocotyl region by *Cronartium fusiforme*. Germinating basidiospores with variable-length germ tubes frequently produced appressoria of various shapes and degrees of swelling. Penetration occurred directly through epidermal cells and occasionally through the guard cells or subsidiary cells of stomata. Loblolly pine callus was resistant both to cell penetration and to intercellular growth by *C. fusiforme* under the culture conditions utilized. Several culture media were used in efforts to alter the growth, form, and susceptibility of the callus to infection; none of these were successful. Basidiospores germinated and germ tubes grew normally on all callus tissues tested, but neither appressoria nor haustoria were observed. Two types of extracellular substances were detected primarily in senescing callus cells.

Additional key words: *Pinus taeda*, fusiform rust, scanning electron microscopy, stress metabolites.

A tissue culture system for the study of host-pathogen interactions has the advantage of allowing deliberate manipulations of the physical and chemical environment in which the pathogen interacts with the host. Success in the development of in vitro systems for disease study has been mixed. Major gene resistance has been detected in cultured callus of potato and tobacco against *Phytophthora infestans* (Mont.) DBy. and *Phytophthora parasitica*, var. *nicotianae* (Breda de Haan) Tucker, respectively (11,14). Although many attempts to obtain infection of cultured cells by rusts were unsuccessful (19), two cases involving conifer hosts were successful (6,9,13). Hotson and Cutter (13) and

0031-949X/82/01013806/$03.00/0

Cutter (6) were able to infect *Juniperus virginiana* L. with *Gymnosporangium juniperi-virginianae* Schw. and Harvey, and Grasham (9) achieved successful infection of white pine (*Pinus monticola* Dougl.) callus with spores of the blister rust fungus, *Cronartium ribicola* J. C. Fish. ex Rabenh. In contrast, Walkinshaw et al (23) maintained *Cronartium fusiforme* Hedgc. & Hunt ex Cumm. (*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Cumm.) Burds. et Snow.) in cultures of infected slash pine (*Pinus elliottii* Engelm. var. *elliottii*) gall tissue, but infection was not present in adjacent newly formed callus. Hollis et al (12) succeeded in isolating *C. fusiforme* from cultured slash pine gall tissue and Hare (8) reported intercellular colonization of slash pine callus by hyphal colonies, but again there were no haustoria. The compatibility of loblolly pine (*Pinus taeda* L.) callus to *C. fusiforme* has not been reported.

Incompatibility of host callus cultures with certain pathogens may occur for many reasons. The pathogen may be affected by unique structural or physiological features of callus cells, and by the lack of proper recognition features or stimuli offered by an epidermis. The production of antimicrobial substances by the callus cells may also affect the host's compatibility (15,19,22).

The purpose of this investigation was to compare the susceptibility of cultured loblolly pine seedlings and callus to in vitro infection by *C. fusiforme*. Several nutritive media were tested to attain infection of loblolly callus. The patterns of surface interaction between *C. fusiforme* and the seedlings and callus were compared.

MATERIALS AND METHODS

Media. Three media were used for the culture of loblolly pine callus and seedlings. A modified Gresshoff and Doy medium 1 (GD-1) (1) was further modified to provide better conditions for the growth of callus. Brown and Lawrence (B&L) medium (4), used primarily for callus culture, was modified as previously reported (16). A medium containing yeast extract and peptone (12) was used to grow callus in one experiment.

The modifications to GD-1 medium consisted of changes in the amounts and forms of nitrogen, the types of growth regulators, and the addition of yeast extract and peptone. Several combinations of the nitrogen forms NH_4^+ (from NH_4NO_3) and NO_3^- (from KNO_3) were examined along with glutamine as nitrogen sources in GD-1 medium. Among these were NH_4/NO_3 ratios of 3/10 mM (GD-1, 3/10), 20/40 mM (GD-1, 20/40), 5/35 mM (GD-1, 5/35), and 10 mM glutamine with 10 mM KCl. One growth regulator regime consisted of 0.23 μM 6-furfurylamino purine (kinetin) and 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The other regime consisted of 2.2 μM 6-benzylaminopurine (BAP) and 2.7 μM α-naphthaleneacetic acid (NAA). The 80% ethanol soluble fraction of yeast extract (YE[S]) was added in some experimental treatments at 0.05–0.1% (w/v) along with 0.05–0.1% (w/v) of peptone (P) (16).

Plant material. Susceptible loblolly seedlings from a known mother tree seed source were grown in axenic culture on GD-1 medium. Seeds were scarified at the micopyle end and soaked in 1% peroxide (which was changed daily) for 7 days. After emergence of the radicle, seed coats were removed and the seeds were surface sterilized in 0.8% sodium hypochlorite for 5 min. After being rinsed three times in sterile distilled water, the seedlings were removed from the gametophyte and placed on the medium.

Loblolly pine callus cultures were obtained from greenhouse-grown seedlings by methods described previously (16). Succulent stem tissue from 2- or 3-yr-old seedlings grown from seeds of field trees in Livingston Parish, LA, was used for original explants. The tissue was initiated and maintained on modified B&L medium in darkness at 22 C (16).

Inoculations. Basidiospores of *C. fusiforme* were cast directly from excised telial columns onto the pine seedlings or callus for 10–24 hr in complete darkness because the fungus is inhibited by light (H. V. Amerson, *unpublished*) (1,16). Seedlings, cultured 7–12 days, were inoculated on the hypocotyl just below the cotyledons. Callus pieces, subcultured every 3–4 wk, were inoculated on the top where active growth was occurring. Both seedlings and callus were placed in the dark on various media 5–8 days before inoculation. In one experiment, callus grown on B&L medium was washed three times in sterile 0.05 M phosphate buffer (KH_2PO_4 and $NaHPO_4$) at pH 7 for 3 hr immediately before inoculation in an attempt to remove any fungal inhibitors. In some experiments, callus was inoculated with hyphae of axenic cultures of basidiospore origin (1).

Tissue preparation for microscopy. At 10, 14, or 20 days after inoculation, seedlings and callus were prepared for light microscopy. Scanning electron microscope (SEM) examinations of seedlings and callus, grown for 2–5 days on the medium GD-1, 5/35, BAP, NAA, 0.1% YE(S) & P, were made at 24, 48, 60, 96, and 120 hr after inoculation. Tissue for histological observations was fixed in FAA, dehydrated, and infiltrated using *t*-butyl alcohol and paraffin (18). Sections 12 μm thick were cut and stained in orseillin BB and aniline blue (17). Seedlings and callus tissues were also prepared for epoxy embedding by fixation in a modified Karnovsky's fixative (2) buffered to pH 7.0 with 0.075 M sodium cacodylate buffer. Tissues were fixed for 12–24 hr and then washed three times with 0.075 M buffer for 12–24 hr, postfixed for 2 hr with buffered 2% osmium tetroxide, washed once in buffer and dehydrated in a graded series to 100% ethanol. Fixed tissues were embedded in Spurr's low-viscosity medium (Polysciences Inc., Paul Valley Industrial Park, Warrington, PA 18976). Sections were cut at a thickness of 1.0 or 2.5 μm and stained in 0.05% water soluble toluidine blue O (pH 5–6) at 70 C for 30–60 sec.

Tissue for SEM was prepared as described for material that was embedded in epoxy except the osmium tetroxide postfixation was eliminated. Dehydration in a graded ethanol series was followed by solvent exchange in increasing concentrations of Freon 113. The material was then critical-point dried in Freon 13, mounted on stubs, and gold coated with a sputter coater. Observations and photographs were made with an Etec Autoscan scanning electron microscope.

RESULTS

Media effects on seedlings and callus. Seedlings grown on all media tested were infected by *C. fusiforme*. Extensive colonization occurred in all seedlings except those grown on media with high concentrations of nitrogen (20 mM NH_4^+ and 40 mM NO_3^-, Table 1). Callus cultures, even when given a preliminary wash in buffer, were immune to infection and did not support intercellular or surface colonization. Marked differences were observed in the rate of growth (volume increases), sizes of cells, degree of browning, and rate of senescence of the callus on the various media tested. Intercellular and extracellular hyphal growth occurred in callus cultures that became contaminated by fungi commonly present in the laboratory.

TABLE 1. Amount of colonization in tissue of susceptible loblolly pine seedlings and callus on various media

Media[a]	Colonization[b]	
	Seedlings	Callus
1. GD-1, 3/10	++	...[c]
2. GD-1, 3/10, K, 2,4-D	++	0
3. GD-1, 20/40, K, 2,4-D	+	0
4. B&L, 20/40, K, 2,4-D (washed)	+	0
5. Hollis, 20/40, YE & P	...[c]	0
6. GD-1, 5/35, BAP, NAA, 0.05% YE(S) & P	...[c]	0
7. GD-1, 5/35, BAP, NAA, 0.1% YE(S) & P	++	0
8. GD-1, Glut 10, KCl 10, BAP, NAA	...[c]	0

[a] Abbreviations used in media types: GD-1 = Gresshoff and Doy medium 1; B&L = Brown and Lawrence; Hollis YE & P = Hollis et al (12); 3/10, 5/35, 20/40 = the ratio of NH_4 to NO_3 in mM; BAP = 2.2 μM BAP; NAA = 2.7 μM NAA; K = 0.23 μM kinetin, 2,4-D = 4.5 μM 2,4-D; 0.05 or 0.1% YE(S) & P = the 80% ethanol soluble fraction of yeast extract and total peptone; and YE = whole yeast extract.

[b] Colonization of tissues was rated from 0 (none), to + (slight), to ++ (heavy).

[c] This combination was not tested.

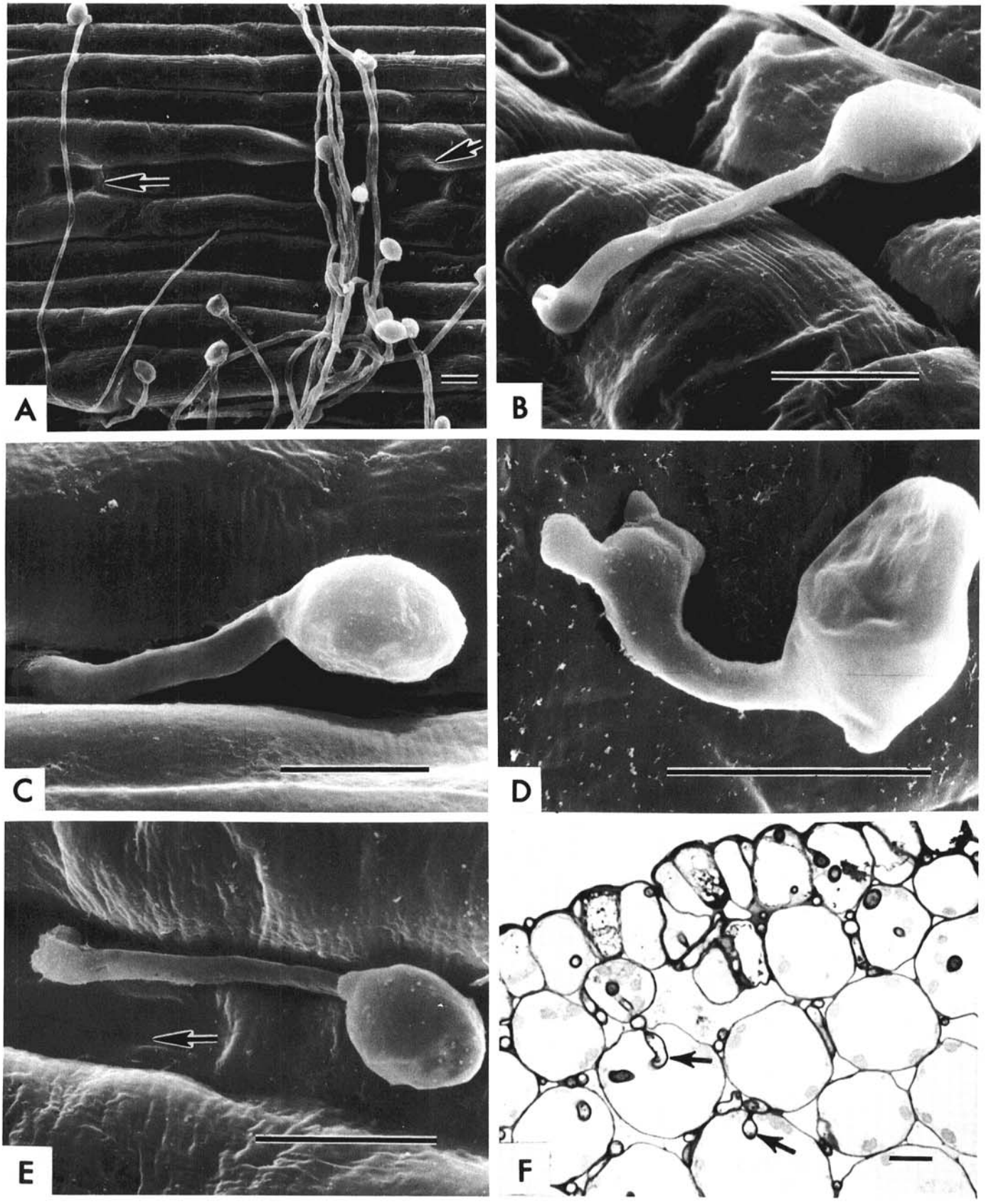

Fig. 1. Germinated basidiospores of *Cronartium fusiforme* on the hypocotyls of loblolly pine seedlings after 96–120 hr. **A,** Basidiospores with long germ tubes on a hypocotyl with stomata (arrows). **B,** Germinated basidiospore with an appressorium. **C,** Direct penetration in the valley area between two epidermal cells. **D,** Direct penetration by an appressorium with appendages or new germ tube growth. **E,** Direct penetration of a guard cell or subsidiary cell near a stomate (arrow). **F,** Transverse section of a seedling hypocotyl with numerous intercellular hyphae and haustoria (arrows). Bars = 10 μm.

Seedling inoculation. Figure 1A shows the typical surface topography of seedling hypocotyls. The cells of the epidermal layer on 1- to 2-wk-old seedlings formed a series of parallel ridges and valleys (Fig. 1A and B). Sunken stomata were present about every sixth to eighth ridge (Fig. 1A).

Basidiospores germinated well on the loblolly seedlings, forming a mass of germ tubes of varied lengths over the surface of the hypocotyls. The germ tubes frequently grew perpendicularly to the ridges of cells until a suitable infection site was located (Fig. 1A and B). As indicated by the presence of appressoria, infection usually was achieved by direct penetration of epidermal cells (Fig. 1B and D). Additional penetrations occurred through guard cells or subsidiary cells (Fig. 1E). Germ tubes showed no evidence of attraction to the nearest stomata since many were passed over by the elongating hyphae (Fig. 1A). In most instances of direct penetration, the germ tubes swelled and in many cases produced distinct appressoria (Fig. 1B), some with appendages (Fig. 1D).

Intercellular hyphae with haustoria penetrating into infected cells were commonly observed in the seedlings 14 days after inoculation (Fig. 1F). The fungus usually colonized the cortex, occasionally penetrated the endodermis, and then entered the central tissue of the hypocotyl. In many cases, one half to three

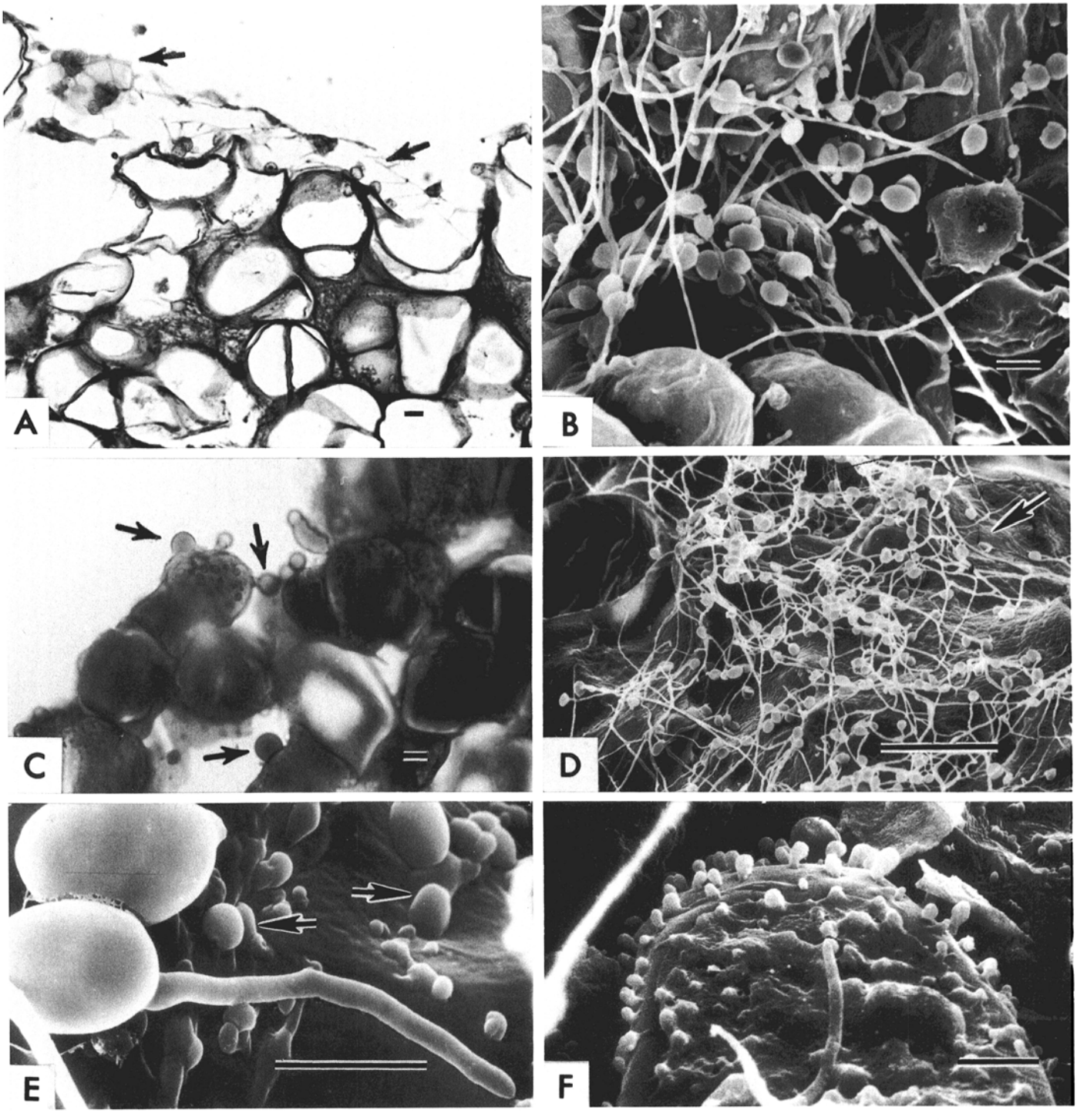

Fig. 2. Callus of loblolly pine inoculated with basidiospores of *Cronartium fusiforme*, and callus cells with exuded substances. **A,** Basidiospores and germ tubes on callus 3 wk after inoculation. **B,** Basidiospores and germ tubes on callus 24 hr after inoculation (arrows). **C,** Globular material on the surface of declining callus cells (arrows). **D,** Basidiospores and germ tubes on loblolly pine callus with cells covered by slime (arrow). **E,** Germinating basidiospores with no appressorium and globular substances (arrows). **F,** Large projections of exuded material on a cell of loblolly pine callus. Bars for A, B, C, E, F = 10 μm. Bar for D = 100 μm.

quarters of the seedling circumference was colonized. Hyphae did not move into regions of seedlings where callus was generated.

Callus inoculation. The irregular surface of loblolly pine callus was unlike the orderly surface of an intact seedling (Fig. 2B). The callus cells grew in an irregular pattern giving rise to a "lumpy" surface with large intercellular spaces. The texture of the callus was friable, especially when recently initiated or subcultured, with little cell-to-cell contact (Fig. 2A and B).

At least two major types of extracellular substances were observed on the callus cultures: a brown extracellular slime and various-sized globules. Presumably these deposits were stress responses of the pine cells since they were observed on senescing or injured callus and occasionally under areas of heavy basidiospore inoculation. The homogenous extracellular slime was apparent, however, to a limited extent even in healthy callus cultures. It was commonly deposited intercellularly and on the callus surface (Fig. 2D). Some callus cells had a rough and pimpled surface (Fig. 2F). These projections, cell walls, and the extracellular slime all stained dark blue when exposed to aniline blue and orseillin BB.

The second type of extracellular substance consisted of globules (Fig. 2C and E) which stained a brilliant red in contrast with the blue stained projections. These substances retained their globular character and staining characteristics after they became detached from the cell wall. They coalesced occasionally in confined intercellular spaces. Small globules similar to those found outside the cell were commonly seen inside declining cells.

Basidiospores were cast abundantly over the surface of the callus cultures. The spores landed directly on callus cells (Fig. 2A and B) or on a matrix of extracellular deposits (Fig. 2D). Spores germinated well in either case. Germination of basidiospores occurred within 10 hr after inoculation unless there was a film of surface fluid that inhibited spore germination. The growth of germ tubes was considerable, but growth was not sustained past the germ tube stage. Penetration structures, appressoria, or swollen germ tube tips were not observed with either SEM (Fig. 2B and E) or light microscopy (Fig. 2A). Penetration was not observed even on vigorous callus where spores, germ tubes and callus cells were in close contact and no extracellular material was observed (Fig. 2A).

Hyphal colonies derived from basidiospores did not infect or proliferate on the callus nor did they grow when placed on the agar media near callus. These colonies were irreversibly injured; they failed to survive upon transfer to a fresh medium known to support growth.

DISCUSSION

The infection and colonization of loblolly pine seedlings by *C. fusiforme* in a tissue culture system allowed detailed studies of host-pathogen interactions. The epidermal surface of the hypocotyls appeared similar to that of needle tissue from 5-wk-old slash pines (3). Both the pathogen and the cultured seedlings appeared to interact at penetration and subsequent colonization as they would under natural conditions (3,21). Penetration of guard cells or subsidiary cells, formation of appressoria, end swellings of germ tubes, and other surface manifestations of direct penetration occurred randomly as other reports have indicated (3,21).

In contrast to the cultured seedlings, loblolly pine callus appeared resistant to both intercellular and intracellular colonization. Resistance of the callus to *C. fusiforme* was not altered by variations in the media even though callus growth and cell size were affected. These inoculation trials resembled the many unsuccessful attempts to infect callus tissues with urediospores, basidiospores, or mycelia of various rust fungi (19,22) including *C. fusiforme* (8,23). These trials contrast with the extensive surface colonization and successful infection of western white pine callus by *C. ribicola* (9) and the surface growth of *C. fusiforme* on slash pine callus (8). *C. ribicola* also penetrated, colonized, and formed haustoria in nonhost species, *Pseudotsuga menziesii* (Mirb.) Franco (10). The failure of *C. fusiforme* to exist saprophytically on the callus or near callus grown on media known to support axenic colonies may result from the presence of toxic substances similar to those reported in other callus cultures (19,20). The resistance of the callus was, however, partially specific since a variety of fungal contaminants grew on the callus. In some cases, cellular exudates seemed to prevent germ tube contact with the cell wall, but in other cases colonization and infection failed in the absence of exudates.

SEM and histological observations revealed at least two distinct types of cell exudates associated with stressed callus cells. Similar substances were reported in cultures of sycamore callus that were grown under less-than-optimum concentrations of sucrose or growth regulator substances (5). Carceller et al (5) suggested that the homogenous slime and the globules corresponded to lignin (a complex phenolic) or a lignin-hemicellulose complex. Other "polyphenols" and "tannins" have been reported in callus cells, including slash pine cells, and appear similar to the globules observed in loblolly pine callus (7).

Callus and seedling cultures of loblolly pine represent opposite points on a continuum of disease resistance and susceptibility. Callus resists both penetration and intercellular growth by *C. fusiforme*, while intact seedlings allow normal infection and colonization. Tissue culture provides the controlled environment necessary for future elucidation of the factors responsible for resistance and susceptibility in both types of tissue.

LITERATURE CITED

1. Amerson, H. V., and Mott, R. L. 1978. Technique for axenic production and application of *Cronartium fusiforme* basidiospores. Phytopathology 68:673-675.
2. Amerson, H. V., and Van Dyke, C. G. 1978. The ontogeny of echinulation (spines) in uredospores of *Puccinia sparganioides*. Exp. Mycol. 2:41-50.
3. Ammon, V., Walkinshaw, C., and Seifers, D. 1976. Observations of tolerant and susceptible slash pine seedling needles inoculated with *Cronartium fusiforme* using scanning electron microscopy. (Abstr.) J. Miss. Acad. Sci. 21(Suppl):5.
4. Brown, C. L., and Lawrence, R. H. 1968. Culture of pine callus on a defined medium. For. Sci. 14:62-64.
5. Carceller, M., Davey, M. R., Fowler, M. W., and Street, H. E. 1971. The influence of sucrose, 2,4-D, and kinetin on growth, fine structure, and lignin content of cultured sycamore cells. Protoplasma 73:367-385.
6. 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.
7. Hall, R. H., Baur, B. S., and Walkinshaw, C. H. 1972. Variability in oxygen consumption and cell morphology in slash pine tissue cultures. For. Sci. 18:298-307.
8. Hare, R. C. 1978. Axenic culture of *Cronartium fusiforme* from three spore forms. Can. J. Bot. 56:2641-2647.
9. 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.
10. Harvey, A. E., and Grasham, J. L. 1971. Inoculation of a nonhost tissue culture with *Cronartium ribicola*. Can. J. Bot. 49:881-882.
11. Helgeson, J. P., Haberlack, G. T., and Upper, C. D. 1976. A dominant gene conferring disease resistance to tobacco plants is expressed in tissue cultures. Phytopathology 66:91-96,
12. Hollis, C. A., Schmidt, R. A., and Kimbrough, J. W. 1972. Axenic culture of *Cronartium fusiforme*. Phytopathology 62:1417-1419.
13. Hotson, N. H., and Cutter, V. M. 1951. The isolation and culture of *Gymnosporangium juniperi-virginianae* Schw. upon artificial media. Proc. Nat. Acad. Sci., USA 37:400-403.
14. Ingram, D. S. 1967. The expression of *R*-gene resistance to *Phytophthora infestans* in tissue cultures of *Solanum tuberosum*. J. Gen. Microbiol. 49:99-108.
15. Ingram, D. S. 1973. Growth of plant parasites in tissue culture. Pages 392–421 in: H. E. Street, ed. Plant Tissue and Cell Culture. University of California Press, Berkeley. 502 pp.
16. Jacobi, W. R. 1982. Growth medium for dual cultures of loblolly pine callus and *Cronartium fusiforme*. Phytopathology 72:136-138.
17. Jewell, F. F., True, R. P., and Mallett, S. L. 1962. Histology of *Cronartium fusiforme* in slash pine seedlings. Phytopathology 52:850-858.
18. Johansen, D. A. 1940. Plant Microtechnique. McGraw-Hill, New York. 523 pp.
19. Maheshwari, R., Hildebrant, A. C., and Allen, P. J. 1967. Factors affecting the growth of rust fungi on host tissue cultures. Bot. Gaz. 153-159.

20. Mathes, M. C. 1967. The secretion of antimicrobial materials by various isolated plant tissues. Lloydia 30:177-181.
21. Miller, T., and Cowling, E. B. 1977. Infection and colonization of different organs of slash pine seedlings by *Cronartium fusiforme*. Phytopathology 67:179-186.
22. Turel, F. L. M., and Ledingham, G. A. 1957. Production of aerial mycelium and uredospores by *Melampsora lini* (Pers.) Lev. on flax leaves in tissue culture. Can. J. Microbiol. 3:813-819.
23. Walkinshaw, C. H., Jewell, F. F., and Walter, N. M. 1965. Callus culture of fusiform rust-infected slash pine. Pine Dis. Rep. 49:616-618.