Axenic Culture of Cronartium fusiforme

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ABSTRACT

Cronartium fusiforme, the causal fungus of fusiform rust in southern pines, was isolated from slash pine and has been growing in the absence of host tissue for over 6 months on a chemically defined medium. Typical aeciospores formed in axenic culture on a yeast extract-peptone medium, and when inoculated onto oak leaves resulted in the production of uredia and telia. The method of establishing these axenic cultures is described, and the importance of this technique to fusiform rust research is discussed.

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Fusiform rust, caused by *Cronartium fusiforme* Hedgc. & Hunt ex Cumm., is the most important disease of slash pine (*Pinus elliottii* Engelm. var. *elliottii*) in the southeastern United States. Certain aspects of research on this disease are hindered by the inability to grow this obligate parasite apart from its pine and oak hosts. Studies of *C. fusiforme* nutrition and metabolism would be facilitated if in vitro experiments were possible. This report presents the procedures and results of the first successful establishment of *C. fusiforme* in axenic culture on a chemically defined medium.

Methods for the culture of rust fungi have been described (2, 8, 9, 11) as have procedures for culturing and maintaining *C. ribicola* on callus of western white pine (*Pinus monticola* Dougl.) (3) and *C. fusiforme* on callus of slash pine (10). By using the latter procedures, we established axenic cultures of *C. fusiforme* from callus cultures of infected host tissue.

One-year-old branch galls from field-grown slash pine were collected in April and used as the source of infected host tissue. Branches with galls 5 cm long were cut from the infected host and surface sterilized in a 15% solution of 5.25% NaOCl (Clorox, Clorox Co., Oakland, Calif.) for 1 hr. The periderm was removed aseptically, and 0.5 cm^3 sections of the young secondary phloem, cambium, and xylem were placed on nutrient agar medium (1). These excised sections were approximately one-third gall tissue and two-thirds "healthy" tissue. Callusing of the explants was visible within 10 days, and contaminated cultures could be identified and discarded within 30 days. Over 200 slash pine branch gall segments were cultured. Approximately one-third of these cultures became contaminated. Five noncontaminated cultures produced similar mycelial growths from the pine tissue in about 6 weeks. Of these, one was successfully subcultured.

The uncallused portion of one noncontaminated explant became necrotic after 6 weeks. A cream-colored, gelatinous mass of mycelium grew from the cambial region of the necrotic portion near the base of the callus (Fig. 1-A). This mycelial mass, at age 12 weeks, was transferred to healthy slash pine callus, where the fungus continued to proliferate on the surface of the medium. The mycelium was divided into six pieces, and each piece subsequently was placed 1 cm from healthy pine callus which had grown for 2 weeks on a medium (yeast extract-peptone) containing: inorganic salts (1); glucose, 30.0 g; yeast extract, 1.0 g; Difco peptone, 1.0 g; oleic acid, 1.0 mg; kinetin, 0.3 mg; ascorbic acid, 0.1 mg; 2,4-D 1.0 mg; agar, 10.0 g; and distilled H_2O to make 1.0 liter. We adjusted the pH of the medium to 6.2-6.4 with 1 N NaOH prior to autoclaving at 115 C for 15 min. The fungus was subcultured twice at 4-week intervals because the pine callus died within 6 weeks on the latter medium, whereas the fungus continued to grow. A third subculture onto this medium in the absence of pine callus was established and the fungus grew from ca. 1.0 cm^3 to ca. 2.0 cm^3 in 4 weeks. A total of 18 axenic cultures was obtained.

Suspecting that metabolites diffusing from the callus were supporting the growth of the fungus (4, 6) and that such components could be supplied by an artificial medium (5, 7), we prepared a chemically defined medium. This medium consisted of: inorganic salts (1); L-lysine, 0.3 mg; L-histidine, 0.4 mg; L-arginine, 8.5 mg; γ-L-aminobutyric acid, 75.0 mg; ornithine, 0.3 mg; L-aspartic acid, 4.0 mg; L-threonine, 0.9 mg; L-serine, 11.5 mg; L-asparagine, 100.0 mg; L-proline, 200.0 mg; L-glutamic acid, 2.6 mg; L-glycine, 0.2 mg; L-alanine, 17.0 mg; L-valine, 1.7 mg; L-methionine, 0.1 mg; L-isoleucine, 1.6 mg; L-leucine, 0.8 mg; L-tyrosine, 0.3 mg; L-phenylalanine, 0.5 mg; β-alanine, 0.8 mg; L-tryptophan, 0.1 mg; L-cysteine, 100.0 mg; L-glutamine, 100.0 mg; glutathione, 1.0 mg; folic acid, 0.1 mg; biotin, 0.1 mg; ascorbic acid, 0.3 mg; nicotinic acid, 0.5 mg; pyridoxin-HCl, 0.1 mg; thiamine-HCl, 0.1 mg; myo-inositol, 100.0 mg;

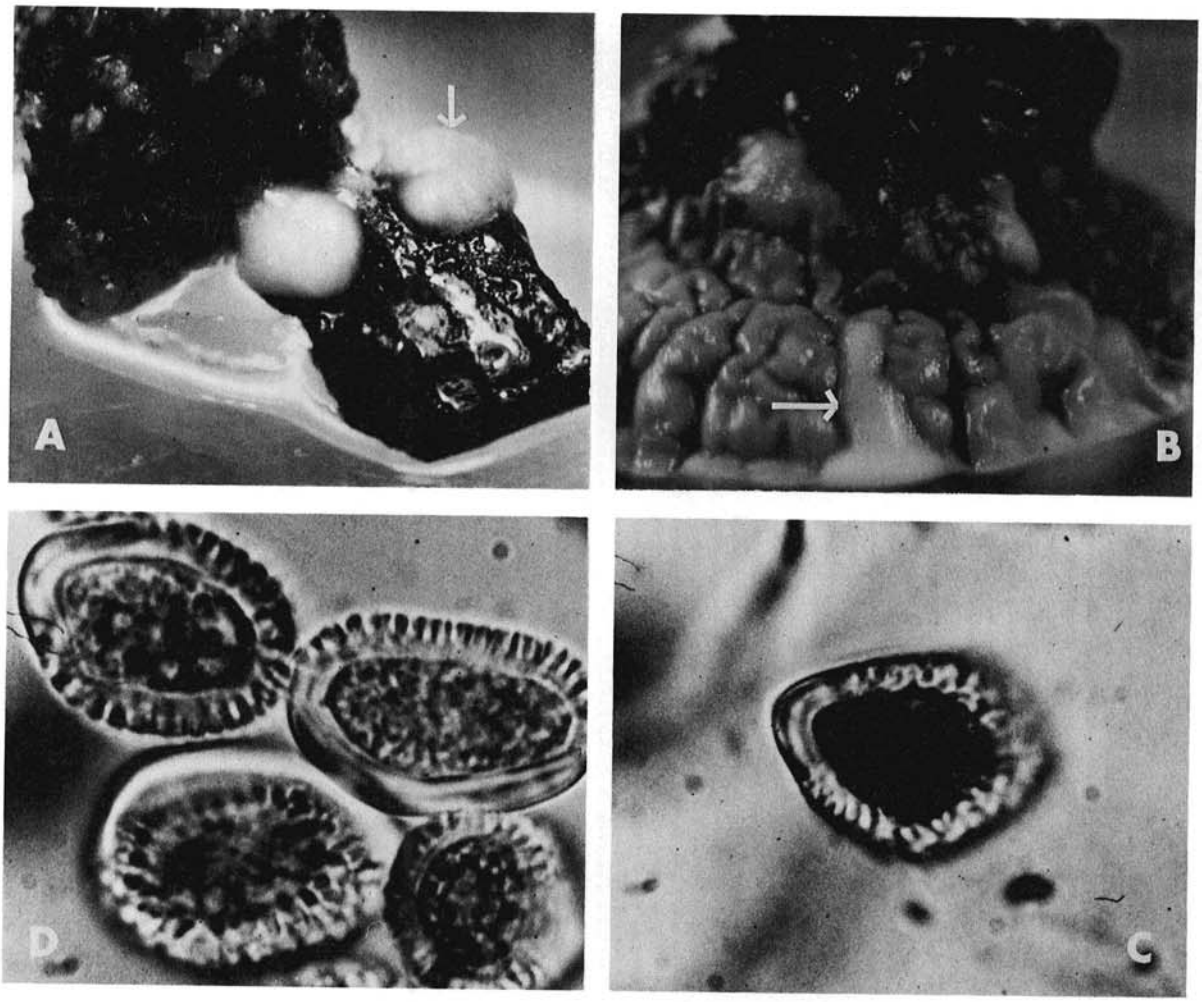

Fig. 1. Cultures and aeciospores of *Cronartium fusiforme*. **A)** A callused explant of a rust gall from slash pine showing mycelial mass (arrow) (× 4). **B)** Axenic culture of the fungus showing the position (arrow) of aecial sorus (× 2). **C)** Mature aeciospore obtained from aecial sorus shown in B (× 230). **D)** Mature aeciospores obtained from galls of field-grown slash pine (× 230).

kinetin, 0.3 mg; 2,4-D, 3.0 mg; oleic acid, 0.1 mg; citric acid, 3.0 g; glucose, 30.0 g; agar, 10.0 g; and distilled water to make 1.0 liter. The pH of the medium was adjusted to 6.2-6.4 with 1 N NaOH before autoclaving at 115 C for 15 min. The amino acids added and the concentrations used were based on analysis of susceptible slash pine leaders.

Pieces of mycelium from the yeast extract-peptone cultures without pine callus were placed on this chemically defined medium. The fungal masses grew to twice their original size in 4 to 6 weeks, and have been maintained for over 6 months through four subsequent subcultures. A total of 40 such cultures has been established. Throughout these studies, all cultures were maintained at 20 C with 1,000 ft-c of light on a 14-hr day.

One mycelial culture that remained on the yeast extract-peptone medium in the absence of pine callus produced a yellow structure resembling the aecial sorus of *C. fusiforme* on pine (Fig. 1-B). Freezing microtome sections and whole mounts of this tissue revealed mature aeciospores (Fig. 1-C) of *C. fusiforme* similar to those found in nature (Fig. 1-D). Subsequent inoculation of the primary host, *Quercus nigra* L., with aeciospores produced in axenic culture resulted in infection and subsequent formation of uredia and telia typical of *C. fusiforme*. No aeciospores were produced by the fungus growing on the chemically defined medium.

The establishment of *C. fusiforme* in axenic culture makes possible studies on fungal nutrition and genetics. In addition, this technique may be utilized to study the effects of metabolites from susceptible and resistant host tissues on growth of the fungus in vitro. Information gained from such studies could be used to develop procedures for indirect selection of resistant pines, and to provide insight into the nutritional relationships between the host and this obligate parasite.

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