Technique for Axenic Production and Application of Cronartium fusiforme Basidiospores

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ABSTRACT

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A technique, which utilized excised nondisinfested telial columns of *Cronartium fusiforme,* was developed to deliver contaminant-free basidiospores to areas approximately 1.4 mm in diameter. The spores germinated and occasionally produced mycelia on modified Gresshoff and Doy medium 1. *Pinus taeda* seedlings also were infected with basidiospores in vitro.

Additional key words: fusiform rust, loblolly pine.

Fusiform rust, which is incited by the pathogen *Cronartium fusiforme* Hedgc. & Hunt ex Cumm., is an important disease of pines in southern forests, and stumpage loss from this disease in 1972 was 28 million dollars for loblolly pine (*Pinus taeda* L.) and slash pine (*Pinus elliottii* var. *elliottii* Englem.) (7). Foresters use resistant planting stock in attempts to control fusiform rust in the field, but the continued increase of fusiform rust indicates the need for further study (Griggs and Schmidt, 1976, in Fusiform Rust Symposium Proceedings, Gainesville, FL).

Pine breeding and seedling assay programs for fusiform rust resistance have fostered the production of resistant planting stock. Recent tissue culture developments allow the production of cloned pine propagules (5) and offer the possibility of resistance studies under more controlled conditions, with rapid evaluation of resistance in vitro. Axenic cultures of *C. fusiforme* were obtained (1, and R. C. Hare, *unpublished*) which can yield hyphal inocula for the study of infection and resistance of pine tissues in vitro, but direct challenge with basidiospores is more desirable since this is the inoculum that pines encounter in the field.

In vitro challenge of pine propagules with *C. fusiforme* basidiospores requires that the basidiospore inoculum be free of other contaminating microorganisms, or other *C. fusiforme* spore stages suited to the alternate host, and that the basidiospore cast be directed to specific sites. The techniques presently used to inoculate pines with *C. fusiforme* basidiospores are not suitable for in vitro study since they do not produce contaminant-free inocula (2, 3, 4, 6). A recently developed technique using basidiospore casts from entire surface-sterilized oak leaves (R. C. Hare, *unpublished*; C. A. Hollis, *personal communication*) has been used to establish axenic cultures of *C. fusiforme,* but the basidiospore casts may contain uredospores or persistent aeciospore inocula, and the technique does not permit precise placement of the basidiospores. Thus, no presently existing technique for the casting of *C. fusiforme* basidiospores is both precise and capable of producing clean basidiospores, as required for efficient in vitro studies.

This paper reports a new technique for delivering contaminant-free *C. fusiforme* basidiospores directly from excised telial columns to specific target sites; thus meeting the requirements necessary for in vitro inoculation of cultured materials.

MATERIALS AND METHODS

Telial columns of *C. fusiforme* were produced on oak leaves, excised, and used for in vitro spore casts according to the following procedures. Aeciospore inoculations, 120 mg of spores/100 ml of tap water, were made with a chromatography sprayer on the undersides of 8- to 12-day-old leaves of young red oak (*Quercus rubra* L.) seedlings. Inoculation was followed by a 24- to 48-hr incubation period in a mist chamber, and inoculated plants were placed in a greenhouse following incubation. Three to 9 days after telial columns appeared (approximately 7-10 days after inoculation), the leaf was harvested, without surface sterilization, and immediately placed with the telial columns pointing upward in a 100-mm diameter petri dish containing moist filter paper, which served to anchor the leaf. Then the columns on the leaf were inspected in a sterile chamber with a dissecting microscope for the presence of contaminating microorganisms or uredospores. Uredospores adhering to the columns were easily seen (see Fig. 1 for size comparison of basidiospores and uredospores).

Under sterile conditions, fine-tipped forceps were used

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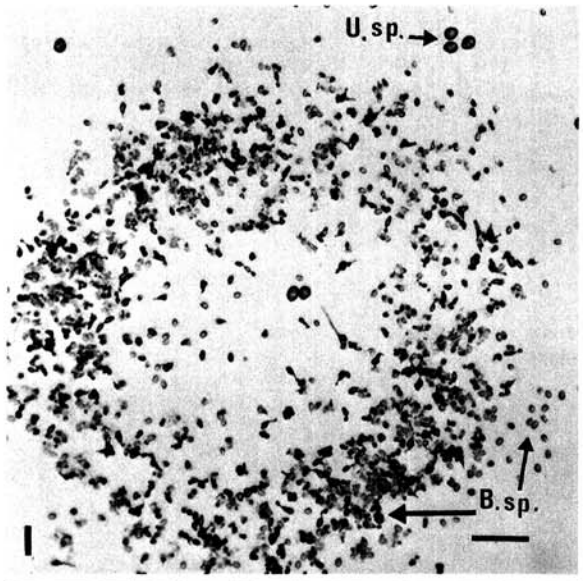

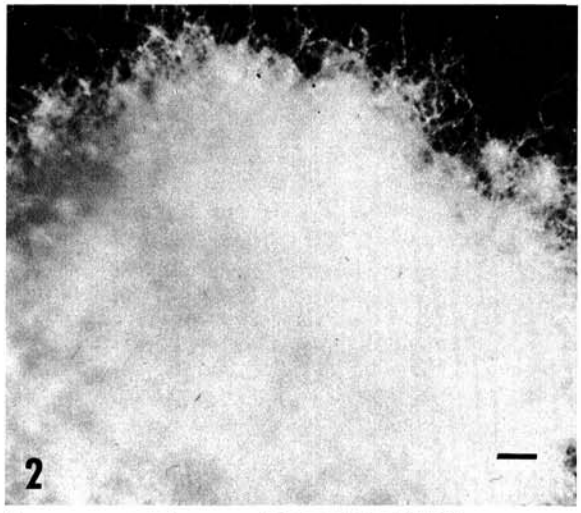

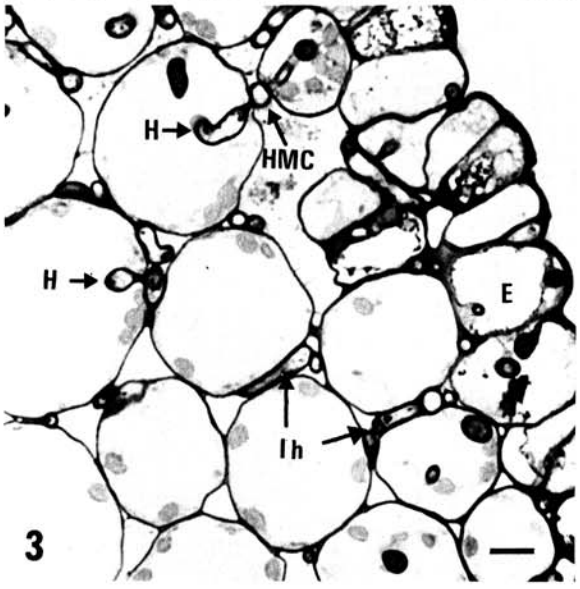

to remove well-formed telial columns with few or no uredospores. The excised telial columns were placed on 1.5% (w/v) water-agar, and with the aid of a dissecting microscope any adhering particles or uredospores were removed with a needle or pointed scalpel. The telial columns were turned to check all areas, and especially to note the column bases which sometimes must be removed owing to high numbers of uredospores. Next, the apex of the telial column was inserted into the water-agar, and the column was pushed from the base, propelling it through the agar for a final cleaning. The column was immediately removed from the agar and inspected for persistent uredospores. If any were present, the column was discarded.

The bases of the cleaned telial columns were implanted in a patch of 1.5% (w/v) water-agar that was attached to the top of a 100-mm diameter petri dish. The dish was reassembled such that the desired target (agar or plant material) was directly beneath the implanted columns now hanging from the petri dish top. The dish was incubated at 20-22 C for 24-72 hr in darkness to allow for basidiospore formation and casting. After the spores were cast, the implanted telial columns were removed by replacing the petri dish top. Failure to remove the columns and/or agar often led to contamination.

Axenic hyphal colonies were initiated and maintained on a modified Gresshoff and Doy medium I (8) which the authors further altered by replacing 20 g/liter of sucrose with 20 g/liter of glucose, and by reducing the NAA concentration from 2 mg/liter to 0.1 mg/liter. Additionally, 6-benzyladenine was omitted and 1 g/liter of both yeast extract and peptone was added.

RESULTS

Each excised column produced a ring-like spore cast pattern on agar with few if any spores in the central region, which coresponds to the apex of the implanted column (Fig. 1). Cast patterns had a mean diameter of 1.4 mm (range of 0.5 to 2.25 mm). Column size, conformation, and the angle of implant affected the cast patterns. Large or curved columns produced large spore cast rings; columns implanted at angles varying greatly from vertical produced typically elongated rings with few spores in the central regions. The concentration of the spore cast could be roughly regulated by removing the telial columns at prescribed times. Thus far, 646 telial columns have been implanted over various nutritive media and living pine surfaces. Eighty-nine percent of the columns have produced spores free of any visible

Fig. 1-3. *Cronartium fusiforme* spores, hyphae, and infective structures. **1)** Ring-shaped basidiospore cast pattern. Note size difference between basidiospores (B. sp.) and uredospores (U. sp.), which were intentionally placed in this cast pattern. Bar equals 100 μm in Fig. 1-2. **2)** Axenic hyphal culture of *Cronartium fusiforme* derived from basidiospores. **3)** Section of a cultured *Pinus taeda* seedling infected in vitro with *Cronartium fusiforme* basidiospores. Note haustoria and intercellular hyphae within the hypocotyl. Legend: E, host epidermis; Ih, intercellular hyphae; H, haustoria; HMC, haustorial mother cell. Bar equals 10 μm.

contamination for periods up to 3 mo, at which time observation was discontinued.

Basidiospores cast onto various agar media germinated readily and occasionally produced axenic hyphal cultures of *C. fusiforme* (Fig. 2) on modified Gresshoff and Doy medium 1 (8). The early precautions taken in the cast technique to minimize uredospore production and assure their removal from telial columns, coupled with careful inspection of cast patterns, insured the absence of uredospores from patterns used to obtain axenic hyphal cultures. Thus, for the first time, axenic cultures of *C. fusiforme,* known to be strictly of basidiospore origin, were obtained (Fig. 2). The virulence of basidiospores produced from excised telial columns was established by the in vitro infection of *Pinus taeda* seedlings (Fig. 3). This represents the first documentation of in vitro infection by *C. fusiforme*.

DISCUSSION

Telial columns obtained from an oak leaf grown in a greenhouse or in the field are likely to be contaminated with bacteria and fungi. Seemingly this is true since failure to remove the telial columns and/or the water-agar implant material frequently resulted in contamination. Paradoxically however, the contaminated columns consistently (89% of the cases) produced basidiospores free from visible contamination. This may result from the speed with which teliospores germinate (produce promycelia) and also by the speed of basidiospore formation and cast from these promycelia. Presumably the rapid pace of germination, spore formation, and spore cast simply allowed *C. fusiforme* to outgrow contaminating organisms. Thus, a telial column scraped clean of existing spores and debris produced contaminant-free basidiospores for a period of ca. 24-72 hr. The technique is ideal for studying races of *Cronartium* since uncontaminated basidiospores can be obtained from specific telial columns produced in the greenhouse or in the field.

Although this simple technique for obtaining contaminant-free inocula and axenic cultures has been tested only for *C. fusiforme,* it seems applicable to many rust fungi.

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