Inoculation of Slash Pine Seedlings with Stored Basidiospores of Cronartium fusiforme

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ABSTRACT

Precast basidiospores of *Cronartium fusiforme* were concentrated onto Millipore filters. Inoculation instruments made from glass capillary tubes were used to transfer basidiospores from filters to specific points on slash pine seedlings. Infections occurred on hypocotyls, cotyledons, stems, and secondary needles. Basidiospores stored on filters at 5 C have retained infectivity for 8 weeks. Phytopathology 60:1773-1774.

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Several techniques have been used to inoculate pine seedlings with basidiospores of *Cronartium fusiforme* Hedgc. & Hunt ex Cumm. These have included allowing basidiospores to be cast from telia suspended over the pines (2), placing telia-bearing oak leaves on the parts of the pines to be inoculated (1), inserting telial columns into slits cut in the stems of pines (1), and injecting a suspension of basidiospores into the pines with a syringe (4). All of these methods involve the use of freshly cast basidiospores, since it has been assumed that basidiospores of *C. fusiforme* are so fragile that storage and handling is difficult, if not impossible. None of these techniques is satisfactory both for restricting inoculum to a very specific point and, at the same time, avoiding injury to the host. This report describes a technique which can be used to inoculate different organs of pine seedlings at specific points, without injury, using precast basidiospores of *C. fusiforme*. A procedure is also presented for storing the basidiospores of this fungus for several weeks without a loss of their capacity to cause infections.

Basidiospores are collected by suspending oak leaves bearing telia of *C. fusiforme* over distilled water in petri dishes and incubating at 20 C. The basidiospores are concd from the water by vacuum filtration onto Millipore filters (SSWP, 47 mm, 3 μ pores) at 2-hr intervals. The basidiospores may be concd further by washing the spores from the different filters into a beaker of water and filtering again onto a single filter disc. The slight vacuum used for filtration is released when most of the liquid has passed through the filter, but while the basidiospores are still moist. The filter disc is transferred with forceps to a chilled petri dish lined with moistened filter paper in the top and bottom. The petri dish is wrapped with aluminum foil and stored at 5 C. Distilled water is added to the filter paper during prolonged periods of storage to prevent desiccation of the basidiospores.

The instruments used to transfer the basidiospores from the filter discs to the point of inoculation are constructed from capillary tubes 100 mm long by 0.8 mm in diam. A tube is heated near one end and pulled out to a diam of about 0.3 mm. The small end of the tube is heated again until a small bead of glass is formed at the end. The bead is then gently pressed against a piece of glass to produce a flattened surface of the desired diam.

Seedlings to be inoculated are first sprayed with a fine mist of distilled water. Inoculation is accomplished by touching the flattened surface of the instrument gently to the basidiospores on the filter disc and then to the specific point selected for inoculation of the plants. The relative quantity of basidiospores transferred may be controlled either by changing the size of the flattened end of the instrument or by altering the concn of basidiospores on the filter discs. The inoculated pines are immediately placed in a mist chamber at about 20 C and incubated for 54 hr. It is important that the seedlings be moved into the mist chamber as quickly as possible after inoculation to prevent desiccation of the basidiospores.

Spores labeled with a fluorescent brightener are used to inoculate organs such as secondary needles while they are still elongating. The point of inoculation can be located at the time specimens are collected for examination by removing the specimen from the plant and placing it in the beam from an ultraviolet light source. The basidiospores to be brightened are cast into a solution of calcofluor white ST [the disodium salt of 4,4′-bis-4-anilino-6-bis (2-hydroxyethyl) amino-*s*-triazin-2-ylamino-2,2′-stilbenedisulfonic acid] (American Cyanimid Co., Bound Brook, N. J.) (0.025 ml brightener to 50 ml distilled water) (3). Basidiospores can also be labeled by pouring the brightener solution over the spores on the filter, applying a vacuum to remove the excess liquid, and washing the brightened spores twice with distilled water.

This technique has been used to inoculate different organs of 380 slash pine (*Pinus elliottii* var. *elliottii* Engelm.) seedlings ranging in age from 17 days to 4.5 months. Infections were confirmed by histological examination or by the development of a spindle-shaped

stem gall which is a characteristic symptom of fusiform rust. Infections were produced on stems, cotyledons, and secondary needles but not on primary needles (Table 1). The highest per cent infection was obtained on the seedlings which were inoculated on the hypocotyl 1 cm below the cotyledons. The lowest per cent infection was on the seedlings which were inoculated on the stem in the area where fascicles of secondary needles were produced.

This method makes it possible to inoculate pines, with no observable injury, at very specific points with a relatively constant number of basidiospores from a uniform source. The localization of inoculum permits the evaluation of the susceptibility of different organs, and makes it possible to follow accurately the development of the fungus from a known point. One of the most promising uses of the localized sites of infection may be in investigations of mechanisms of resistance. Tissues can be analyzed for biochemical and physiological changes, starting with the very earliest stages of infection and colonization rather than waiting until symptoms develop before the sites of infection can be located.

An additional advantage of using inoculum concd on Millipore filters is that the basidiospores survive extended periods of storage without losing infectivity. This makes it possible to use a known, or the same, source of basidiospores for making inoculations over an extended period of time. One collection of basidiospores used for inoculations at weekly intervals, and stored at 5 C, retained the ability to infect slash pine seedlings for 8 weeks (Table 2). A second collection of basidiospores stored at 5 C has retained viability for 8 weeks and infectivity for at least 5 weeks (Table 2).

The inoculation technique and method of basidiospore storage described present an opportunity to overcome some of the problems previously associated with the inoculation of pines with the basidiospores of *C. fusiforme*. It is now possible to collect and store for relatively long periods of time a source of inoculum that will be uniform from plant to plant and from place to place on the same plant. The infection court can be selected and the inoculum applied in relatively constant quantities to the selected sites.

TABLE 1. Slash pine seedlings infected following inoculation of different organs with precast basidiospores of *Cronartium fusiforme*

Point of inoculation	Seedlings inoculated	Age of seedlings at inoculation	Infection
	no.	*days*	*%*
Hypocotyl, 1 cm below cotyledons	130	17	48[d]
Cotyledons, 1 cm from stem[a]	50	29	44[d]
Stem, 1 cm above cotyledons	50	51	44[d]
Primary needles, 1 cm from stem[b]	50	51	0[d]
Secondary needles at end of fascicle sheath[c]	50	135	38[e]
Stem between 2nd and 3rd fascicles of secondary needles	50	135	24[d]

[a] All cotyledons inoculated.
[b] Four needles inoculated/plant.
[c] Four fascicles of secondary needles: two needles per fascicle inoculated per plant.
[d] Per cent infection calculated from seedlings developing stem galls.
[e] Per cent infection calculated from inoculated fasicles with at least one secondary needle infected. Infection confirmed by histological examination.

TABLE 2. Viability and infectivity of basidiospores of *Cronartium fusiforme* stored at 5 C

Weeks of storage at 5 C	% Germination,[a] basidiospore collection		Infection of pine seedlings,[c] basidiospore collection	
	1	2	1	2
0	80	84	+	+
1	94	90	+	+
2	92	[b]	+	+
3	92	[b]	+	+
4	94	[b]	+	+
5	90	79	+	+
6	88		+	
7	90		+	
8	83		+	

[a] Direct germination of basidiospores determined on 1% water agar supplemented with an ether extract of slash pine seedlings.
[b] No germination counts made for weeks 2, 3, and 4.
[c] + = One or more of inoculated seedlings infected with *Cronartium fusiforme*.

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