## Aseptic Zoospore Production by Phytophthora fragariae

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ABSTRACT

*Phytophthora fragariae* consistently generated large numbers of zoospores when cultured on a medium containing blended lima beans. Requirements for formation of zoospores included flooding the cultures and low temperature incubation. Changing the flooding liquid during incubation increased yields, and the use of aseptic conditions gave optimum zoospore production.

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Problems in the consistent production of high titers of infective zoospores from *Phytophthora fragariae* Hickman have limited investigations of red stele of strawberries (9, 10, 11). Although there is general agreement that low-temperature flooding of cultures of *P. fragariae* is essential for stimulating zoospore formation, the media and flooding liquids suggested have been esoteric, and in many cases unavailable (1, 2, 5, 6, 7). We have investigated the conditions required for zoospore formation in order to develop a procedure that would utilize materials that are generally available, be simple to carry out, and result in uniformly high yields of zoospores.

MATERIALS AND METHODS.—Media included in this study were: red kidney bean agar (KBA) as described by Wynn (11), modified Davies medium (4, 9), V-8 juice agar (8), and commercially available lima bean and potato-dextrose agars prepared according to manufacturer's directions (Difco). In addition, frozen lima bean agar (FLA) was prepared by blending 140 g frozen lima beans in 500 ml water, then making the medium up to 1 liter at 1.5% agar; and wheat germ agar was prepared using 20 g wheat germ and 15 g agar per liter.

Races A2 and A4 of *P. fragariae* (3) were used throughout these studies, and each experiment was replicated three times with each race. Initial zoospore suspensions were produced from cultures grown on KBA (11), while subsequent suspensions were produced from cultures on FLA as described below. Zoospore inoculations were carried out by applying 1 ml of water containing 10,000 to 15,000 zoospores to the agar surface and spreading the liquid evenly across the plate. Vegetative inocula were 3-mm disks of mycelium from the edges of 12- to 15-day-old cultures grown on FLA. Disks were placed mycelium-down in the center of the plates. Except where indicated, sterile distilled deionized water was used for all media preparation and flooding. Conditions for vegetative growth of *P. fragariae* were as described previously (9, 10).

After various periods of vegetative growth, zoospore production was initiated by sectioning the cultures into quarters, transferring each section to a sterile 100 × 20 mm petri dish, and flooding the sections with 50 ml of sterile liquid. Flooded sections were incubated sequentially at 10 C for 48 hr, 20 C for 1 hr, 4 C for 1 hr, then returned to 20 C for the release of zoospores (11). In some experiments, the flooding liquid was changed after 24 hr. In all experiments the flooding liquid was changed prior to the first exposure to 20 C. Sporangial counts were made 48 hr after flooding, using a dissecting microscope at ×30. Counts of sporangia and total number of hyphal tips were made on eight fields around the perimeter of mycelial growth, and sporangial production was calculated as the percentage of the total number of observed hyphal tips which produced sporangia. Motile zoospores were counted with a hemocytometer 3-4 hr after the cultures had been returned to 20 C. Vegetative growth on these media was compared after 14 days' growth. Because the amount of aerial mycelial growth varied among the media tested, growth was estimated

TABLE 1. Mycelial growth, sporangia production, and zoospore formation by *Phytophthora fragariae* (Race A4) on various solid media

| Medium | Vegetative growth[a] | % Hyphal tips forming sporangia | Optimal culture age for zoospore formation (days) | | Yield (zoospores/ml) | |
|---|---|---|---|---|---|---|
| | | | Vegetative inoculation | Zoospore inoculation | Maximum observed | Average |
| Red kidney bean | 3 | 60 | 18 | 9 | 100,000 | 20,000 |
| Modified Davies medium | 4 | 35 | 10 | 5 | 60,000 | 10,000 |
| Wheat germ agar | 5 | 0 | | | 0 | 0 |
| V-8 juice agar | 3 | 20 | 8 | | 75,000 | 15,000 |
| Frozen lima bean agar | 3 | 95 | 7 | 5 | 250,000 | 40,000 |
| Lima bean agar (Difco) | 2 | 0 | | | 0 | 0 |
| Potato-dextrose agar (Difco) | 1 | 0 | | | 0 | 0 |

[a] Relative scale: 0 = no growth, 5 = maximum growth.

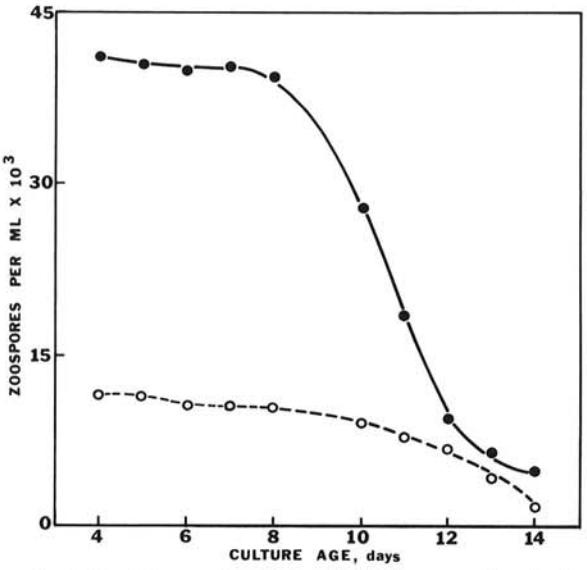


**Fig. 1.** The influence of culture age on zoospore production by *Phytophthora fragariae* (Race A4) when grown on frozen lima bean agar. Cultures were flooded with sterile distilled deionized water which was maintained for 48 hr (open circles) or drained and replaced after 24 hr (closed circles).

subjectively on a relative scale of 0 for no growth to 5 for the maximum growth observed.

RESULTS AND DISCUSSION.—*P. fragariae* produced zoospores when cultured on four of the seven media tested (Table 1) with maximum yields obtained using FLA. Results obtained with both races of the fungus were similar for all media tested. Maximum zoospore production was obtained with FLA when the flooding liquid was changed after 24 hr (Fig. 1). Using a zoospore inoculum and FLA, we observed that changing the flooding liquid not only increased yields of zoospores, but also increased the range of colony ages over which maximum yields could be obtained (Fig. 1).

Colonies which had been initiated with zoospore inoculum produced copious quantities of zoospores when cultured on FLA or KBA (Table 1) and sporangia were differentiated throughout the mycelial mat. On the other hand, colonies which had developed from vegetative inoculum produced fewer zoospores, and usually differentiated sporangia only around the periphery of the mycelium.

With the above techniques, we have determined that flooding with sterile distilled deionized water results in maximal yields of zoospores. In our experiments, non-sterile distilled deionized water produced yields ranging from 55 to 75% of those obtained under sterile conditions, while local tap water resulted in yields of 25 to 40% of those generated under optimum conditions. Flooding cultures with 2 mM solutions of sodium nitrate, potassium nitrate, magnesium nitrate, or calcium nitrate did not increase yields over those obtained in sterile water.

We have tested eight brands of frozen lima beans, and have found no variation between brands in their ability to support growth and zoospore formation by *P. fragariae*. Difco lima bean agar, on the other hand, supported very poor growth of the fungus, and we have not been able to trigger zoospore production on this medium.

With the methods described here, 25 cultures of the fungus consistently yielded 10 liters of zoospore suspension containing 20,000 spores per ml. These methods eliminate the requirements for unusual media, exotic flooding liquids (1, 2, 5, 6, 7), and precise timing during zoospore production. The culture age over which *P. fragariae* will produce maximum yields of zoospores is also considerably lengthened. These techniques have proven very useful in a program of screening strawberry seedlings for resistance to *P.* fragariae (10).

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