Germination In Vitro of Pythium aphanidermatum Oospores

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Supported in part by ARS, USDA Grant No. 12-14-100-9919(34), administered by the Plant Science Research Division, Beltsville, Maryland.

Arizona Agricultural Experiment Journal Paper No. 1934

Accepted for publication 1 August 1972.

ABSTRACT

Oospores of *Pythium aphanidermatum* germinated in 1.5 hr, but maximum germination (20%) occurred after 12-hr incubation at 35 C on cornmeal agar. Germination was increased to 94% by oospore passage through live water snails. Cardinal temperatures for oospore germination, 15-35-45 C, coincided with those for linear growth. Germination occurred over a pH range of 5.2 to 8.6, with an optimum between 6.0 and 8.0. Oospore germination consisted of two distinct stages: (i) absorption of the endospore wall which was dependent upon an exogenous source of calcium; and (ii) production of a germ tube which was dependent upon an exogenous carbohydrate source.

Phytopathology 63:133-137

Additional key words: germination requirements, snail enzyme.

Thick-walled oospores are the major structures of *Pythium aphanidermatum* (Edson) Fitzp. capable of long term survival in soil (10, 13, 14). Few studies have been reported concerning factors affecting oospore germination (1, 4, 13). Studies on oospore germination are complicated by a reported dormancy period (4, 13), and the technical difficulty of obtaining oospores free of mycelium.

This investigation was undertaken to determine the effects in vitro of temperature, exogenous nutrients, and an enzyme from snails on oospore germination of *P. aphanidermatum*. A preliminary report has been published (11).

MATERIALS AND METHODS.—Four virulent *P. aphanidermatum* isolates, obtained from diseased sugar beet (P 1), sweet potato (P 19), potato (P 10), and watermelon (P 22) plants, were used. Single oospore isolates were maintained in culture on 10% V-8 juice agar contained in 9-cm-diam petri dishes incubated at 24 C.

Oospores obtained from agar cultures 3 weeks to 5 months old were used in germination studies. Ten ml sterile distilled water (SDW) were added to each culture, and the surface mycelial mat was scraped off with a rubber policeman. The water, containing numerous oospores and hyphal fragments, was strained through two layers of cheesecloth. The filtrate consisted of oospores nearly free of viable hyphal fragments. Oospores thus obtained will be referred to hereafter as fresh oospores.

The mycelial mat, containing numerous oospores attached to hyphae, was rinsed 3 times in SDW and placed in a beaker containing 40 ml SDW and five live water snails (*Planorbis* sp.) (9). The snails consumed the mycelial mat in 2 to 3 days and produced feces consisting of oospores free of mycelium (Fig. 1). Feces were collected with a pipet and the oospores suspended in SDW by agitation. Oospores thus obtained will be referred to hereafter as snail-ingested oospores.

Oospores suspensions were centrifuged at 7,000 *g* for 2 min. The supernatant solution was decanted and the oospores were centrifuged and rinsed 3 consecutive times in SDW containing 200 μg/ml vancomycin to eliminate bacterial contamination which occurred during snail ingestion. Preliminary studies showed that vancomycin had no effect on oospore germination. The oospores finally were suspended in SDW and stored at 24 C in darkness.

Germination studies.—In one method, oospore suspensions, adjusted to ca. 1,000 spores/ml, were spread on microscope slides coated with cornmeal agar (CMA) and incubated at various temperatures (± 1 C). CMA was adjusted to various pH values of 5.2 to 8.6 with increments of 0.5 units with 0.05 M tris maleate buffer. The pH, which remained stable, was measured before and after autoclaving and at the termination of the germination studies.

In a second method, 1 ml of an oospore suspension containing 300-500 spores was dispensed into 5-cm-diam petri dishes containing 7 ml of either (i) SDW; (ii) SDW containing a 1% carbon source (w/v); (iii) sterile tap water (STW); or (iv) STW containing a 1% carbon source. Final depth and pH of the solutions in the petri dishes were 2 mm and 6.5, respectively. Dishes were incubated at 35 C both in light and in darkness.

Percentage germination, measured by determination of the number of oospores with germ tubes, was expressed as the average of three counts, 50 spores /count for each treatment. All treatments were replicated 3 times, and the experiment was repeated twice.

RESULTS.—The effects of temperature and snail

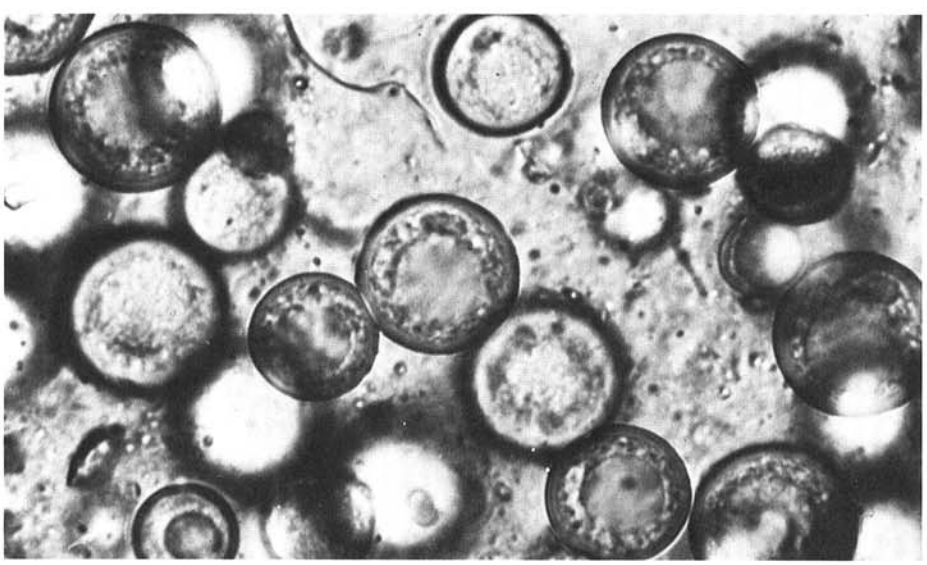

Fig. 1. Photomicrograph of mycelium-free oospores of *Pythium aphanidermatum* after passage through live water snails (× 1,000).

ingestion on rate and percentage germination of *P. aphanidermatum* oospores on cornmeal agar are presented in Fig. 2 and 3. Percentage germination increased after passage through live water snails (Fig. 2). Germination was initiated within 1.5 hr, and reached a maximum of 94 ± 2% after 12- to 14-hr incubation at 35 C. Maximum germination (94%) occurred over a pH range of 6.0 to 8.0. Fifty-four and 22% germination occurred at pH 5.2 and 8.6, respectively.

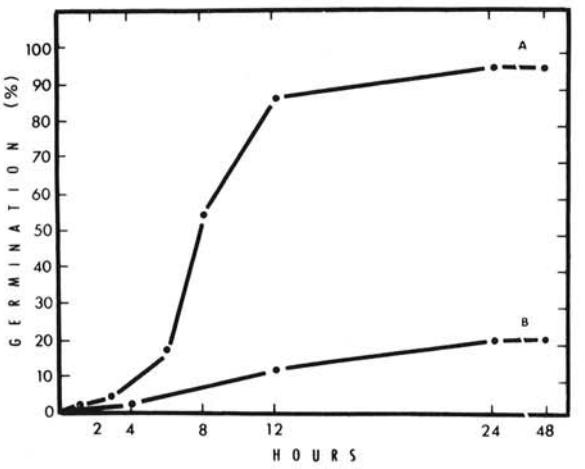

Fig. 2. Rate and percentage germination of *Pythium aphanidermatum* oospores incubated on cornmeal agar at 35 C. A = snail-ingested oospores. B = fresh oospores.

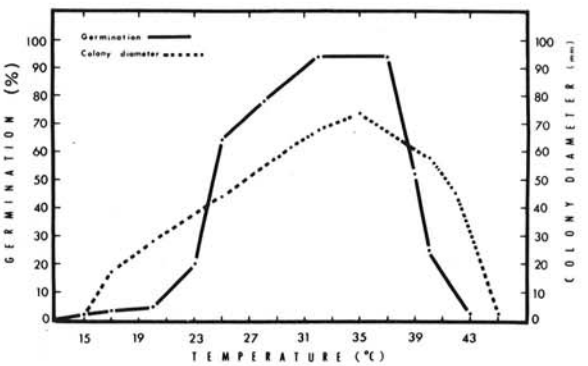

Fig. 3. Effect of temperature on oospore germination and linear growth of *Pythium aphanidermatum* on cornmeal agar.

Optimum temperatures for oospore germination ranged between 32 and 37 C, and coincided with the optimum temperatures for mycelial growth (Fig. 3). Mycelial growth was determined by measuring the diameter of colonies after 24-hr incubation on CMA contained in 9-cm-diam petri dishes. Fungal colonies originated from 5-mm-diam plugs cut from the advancing margin of a 12-hr-old culture of *P. aphanidermatum*.

Sequential development stages during the germination of a single mature oospore are presented in Fig. 4. The cytoplasm of mature oospores appeared finely textured, and contained a well-developed central to subcentral reserve globule and a crescent- to oblong-shaped refringent body. Mature oospore walls, averaging 1.8 ± 0.19 μ in diam, consisted of a thin outer (exospore) wall and a thick inner (endospore) wall. Pregermination stages (A-G) included absorption of the endospore wall and a reduction in size of the central reserve globule. Ninety percent of the oospores germinated with one germ tube, the remainder with two. Lateral branching of the germ tube frequently occurred immediately after its emergence.

Microscopic examination of 100 mature oospores before and after snail ingestion was made to determine the presence of any mechanical abrasion of the oospore wall due to rasping mouth parts of the snail. No changes were observed in either wall thickness or morphology. We noted, however, that small snails (less than 4-mm in diam) tended to remove the oogonial wall, whereas larger snails did not. Presence or absence of the oogonial wall after snail ingestion did not affect subsequent oospore germination.

Fresh oospores were incubated in a 5% aqueous solution of commercial snail enzyme obtained from *Helix pomatia* (L'Industrie Biologique Francaise, Genevilliers, France), to determine whether the increase in percentage germination after snail ingestion was enzymatically induced. After a 24-hr incubation period, oospores were washed in SDW and incubated on CMA at 35 C. Nontreated fresh oospores served as controls. Seventy-five percent of the enzyme-treated oospores germinated, whereas only 15% of the nontreated oospores germinated.

Effects of various carbohydrates on germination of fresh and snail-ingested oospores are presented in Table 1. No germination of either fresh or snail-ingested oospores occurred in SDW or SDW containing various 1% carbohydrate sources. In STW, the thick endospore wall was absorbed in 84% of the snail-ingested oospores, but only 6% produced germ tubes. Germ tubes seldom exceeded 100 μ in length. Absorption of the endospore wall of nongerminated fresh oospores was not observed in STW. High percentage germination of snail-ingested oospores occurred only in STW containing certain carbohydrate sources.

The specific effect of STW was studied by placement of snail-ingested oospores in saline solutions (NaCl) of various osmotic values and in Hoagland's solution (7). Absorption of the endospore wall occurred only in Hoagland's solution. No germ tubes were produced. Upon addition of sucrose, 94% of the oospores germinated within 6-10 hr. Calcium, supplied either as $CaCl_2$ or $Ca(NO_3)_2$ at concentrations ranging from 10-200 μg/ml, was subsequently shown to be the active ingredient in Hoagland's solution capable of inducing endospore wall absorption.

No significant differences were observed either in rate, behavior, or percentage germination of fresh or snail-ingested oospores obtained from 3-week- to 5-month-old cultures of the four *P. aphanidermatum* isolates used throughout this study.

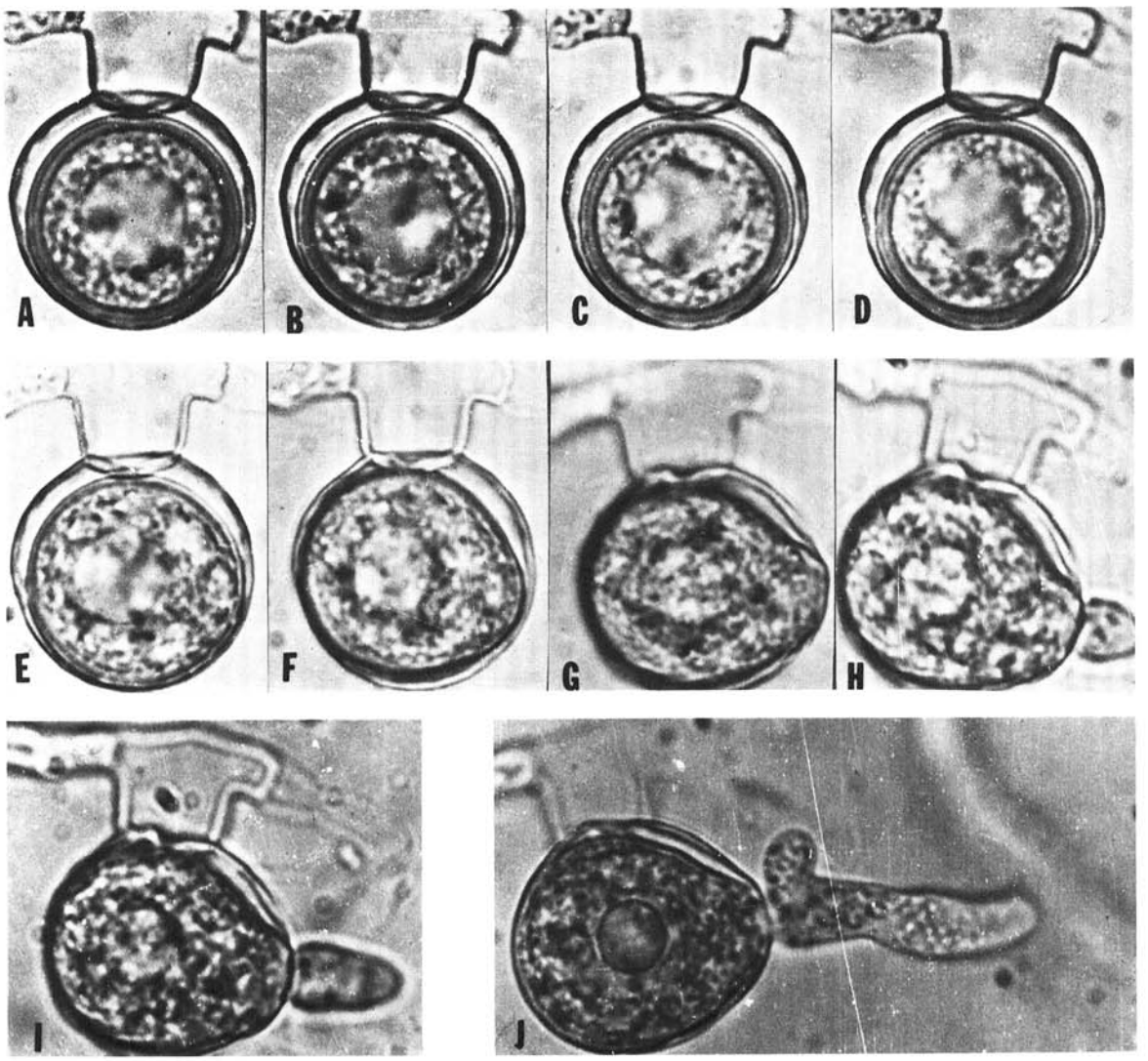

Fig. 4. Sequential stages during the germination of a single mature oospore of *Pythium aphanidermatum*. **A-E)** Taken at 30-min intervals, showing absorption of endospore wall and decrease in size of central reserve globule. **F-J)** Taken at 15-min intervals, showing germination.

To determine whether oospores produced in vivo were functionally similar to those produced in vitro, infected oat roots containing numerous oospores (10) were removed from soil, washed in STW, and divided into two lots. One lot was fed to water snails which, after 4 days' feeding, produced feces containing oospores. The other lot of infected roots was macerated between two ground glass microscope slides, and the oospores were collected after sedimentation in a column of water. Oospores from both lots were washed as previously described and incubated on CMA at 35 C. Eighty-two percent of the snail-ingested oospores germinated, whereas only 21% of the oospores mechanically separated from infected host tissue germinated.

DISCUSSION.—The apparent dormancy of mature oospores of *P. aphanidermatum*, produced either in vivo or in vitro, was broken after passage through live water snails. Although several researchers have used snails to obtain mycelium-free oospores, no increase in percentage germination has been reported (2, 6, 9). Germination of *P. aphanidermatum* oospores was shown to be enzymatically induced. It is possible that enzymes present in the snail intestine increased oospore permeability which allowed the diffusion of nutrients required for subsequent germination. Induction of oospore germination of *Aphanomyces euteiches* with proteolytic enzymes has been reported (16).

Oospore germination had two distinct stages, each with a different exogenous nutrient requirement. Firstly, a pregermination stage which was dependent upon the presence of calcium and consisted of the absorption of the endospore wall and a reduction in

TABLE 1. Effect of various carbohydrates on germination of fresh and snail-ingested oospores of *Pythium aphanidermatum*

Substrate	% Oospores with germ tubes[a]	
	Fresh oospores	Snail-ingested oospores
Sterile distilled water	0	0
Sterile tap water	2	6
Arabinose[b]	4	75
Dextrin	2	94
Dextrose	3	74
Galactose	4	86
Lactose	2	61
Levulose	10	93
Maltose	8	95
Mannitol	3	6
Mannose	2	9
Raffinose	5	6
Rhamnose	4	5
Sorbitol	2	7
Starch	15	94
Sucrose	10	92

[a] Percentage germination recorded after 24-hr incubation at 35 C.

[b] All carbohydrates: 1% (w/v) in sterile tap water. No germination of either fresh or snail-ingested oospores occurred in sterile distilled water containing 1% carbon sources.

size of the central reserve globule. Apparently the endogenous reserves utilized during pregermination were not sufficient to support germination. Secondly, a germination stage that involved the production and growth of a germ tube which was dependent upon an exogenous carbohydrate source. Developmental stages in oospore germination were similar to those described for *Phytophthora megasperma* var. *sojae* (5) and *Phytophthora cactorum* (3).

The rapid rate of oospore germination, coupled with their high temperature optima and utilization of diverse carbohydrate sources, corresponds with the known ecology of *P. aphanidermatum* (1, 10, 13).

In addition to their usefulness as a tool in understanding factors affecting oospore germination, snails, and possibly other soil fauna, may play a role as passive vectors in nature. *Pythium aphanidermatum* was isolated from feces produced by water snails that were collected from agricultural irrigation ditches, and from casts produced by earthworms that were collected in an alfalfa field (M. E. Stanghellini, *unpublished data*). Browsing and feeding habits of these two organisms could disseminate and increase both the inoculum density and inoculum potential of soil-borne fungal plant pathogens. Although their importance in heavily cropped agricultural areas would probably be minor, they could account for reinfestation in plant nurseries and other enclosed agricultural operations. Both snail and earthworm transmissions of soil-borne fungi have been reported (8, 12, 15).

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