Etiology

# Germination of Oospores of *Pythium ultimum* in the Cotton Rhizosphere

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## ABSTRACT

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Microscope slides were dipped in agar suspensions of oospores of *Pythium ultimum*. Slides with hardened agar films with oospores were placed in soil in small plastic pots. Cotton seeds were planted adjacent to the slides, which were positioned so that the roots would grow down and across the agar film. After various incubation periods, soil was removed from the slides and those with small root segments adhering to the agar film were examined microscopically. Decrease in oospore wall thickness was observed after 12 hr in both rhizosphere and nonrhizosphere soil. After 48 hr, 30% of the oospores had thin walls, and after 45 days 85% had thin walls. Rate of conversion to thin walls was similar in sterilized and nonsterilized soil. Walls of 11- or 13-wk-old oospores were converted to thin ones faster than 5- or 9-wk-old oospores. Oospores germinated in the rhizosphere of both primary and secondary roots. Germinated oospores were not found at distances greater than 1.5 mm from the root surface. All germ tubes observed were oriented toward the root. Germination occurred mostly in the root hair zone, but occasionally oospores germinated near root tips. Germ tubes normally made contact with the root epidermis between root hairs or at the bases of root hairs. Rarely were contact and penetration of root hairs observed. Abnormal growth of some germ tubes prior to emergence through the oogonial wall was observed.

*Additional key words*: cotton seedling disease, root exudates.

Species of *Pythium* that attack cotton seedlings include *P. ultimum* Trow, *P. sylvaticum* Campbell and Hendrix, *P. heterothallicum* Campbell and Hendrix, and *P. irregulare* Buisman. Of these, *P. ultimum* is apparently the most prevalent in soils at the northern edge of the U.S. cotton belt and in California cotton soils (5,9). Cotton seedling disease caused by *Pythium* spp. is often severe when postplanting temperatures are low and soil moisture content is high, and results in stand failures and reduced yields (10).

Oospores of *P. ultimum* are thought to be primary survival propagules in soil. The fate of laboratory grown oospores of *P. ultimum* added to soil has been studied (2,7,16), but factors affecting oospore ripening (conversion from endogenously to exogenously dormant spores) and germination are not completely understood. A limiting factor to studying the fate of fungal spores or other propagules in soil is the dearth of techniques for examining them in situ. However, several methods have been described for recovering propagules added to soil. Spores were placed on or between layers of fiberglass tape (14), cellophane film (6), nylon mesh (15,17), membrane filters (1,21), and in agar on microscope slides (4) buried in soil and later recovered for study. Spores were added to soil in extremely high concentrations to facilitate later observation of the spores in smears of the soil on microscope slides (16,18,23).

Information on pathogen:root interactions has been obtained indirectly because of the opacity of the soil and lack of suitable techniques. Techniques utilizing cellophane bags (12), microscope slides (20,24), and root observation boxes (11) were described, but they lack the necessary precision for demonstrating details of spore germination and root infection by pathogens. There are few, if any, photographs in the literature of germinating spores in the rhizosphere with germ tubes growing toward and making contact

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with root hairs or root surfaces.

The following is a description of a technique for studying spores in soil and use of the method for observing maturation and germination of oospores of *P. ultimum* in the rhizosphere of cotton. A brief report of this study has been presented (8).

## MATERIALS AND METHODS

An isolate of *P. ultimum* designated B6-1, from a diseased cotton seedling grown in a field at the West Tennessee Experiment Station, Jackson, TN, was used in all experiments. Some experiments were repeated with other isolates to verify species reaction. All isolates used were highly virulent to cotton seedlings in greenhouse tests (9).

A cornmeal medium (CMM) was prepared by heating 1 L of distilled water containing 10 g of yellow cornmeal at 60 C for 1 hr and filtering it through cheesecloth prior to autoclaving. To obtain oospores for maturation or germination studies, CMM in petri dishes was seeded with mycelial plugs of *P. ultimum* on potato-dextrose agar. Cultures were grown in darkness at 17 C for at least 4 wk. Five cultures were homogenized in a Waring blender (15 times for 3 sec each at high speed) with 100 ml of distilled water. The blended material was washed through a 150-$\mu$m (100-mesh) pore size sieve and centrifuged at 5,000 *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in either 20 ml of water which was mixed with 50 ml of 2.5% agar held at 42 C, or resuspended in 70 ml of 3% methyl cellulose at room temperature. Microscope slides were immediately dipped into a suspension of oospores and one side of each slide was wiped free of suspension with a dry cloth. Agar slides were placed in moist chambers to prevent drying, and slides dipped in methyl cellulose were dried at room temperature. Slides coated with agar films or with dried methyl cellulose were placed vertically, but at a slight angle, against the inside walls of 7.5-cm-diameter plastic pots. Soil (Sequatchie loam, pH 5.5) was added to the pots and packed carefully against the film surfaces. Pots with buried slides were incubated at 25 C, and the soil was watered once or twice per day to saturation as needed to prevent drying. To determine changes in oospore wall thickness and oospore germination, slides were removed periodically and soil was washed from the films with running tap water. Films were air-dried, stained with 0.03% acid fuchsin in 85% lactic acid, and examined microscopically.

To observe the fate of oospores in the rhizosphere, an acid delinted, nonchemically treated cotton seed (cultivar Stoneville 213) was planted in the soil in contact with the upper end of each slide which extended about 1 cm above the soil. Since the slides were placed at an angle in soil, roots from emerging seeds grew down across the agar film. The pots were placed in plant growth chambers with continuous light at 25 C. After various periods of incubation (4–10 days) aerial portions of the plants were cut and discarded, and slides with a quantity of soil and roots were removed from the pots and air-dried. Dry soil was brushed carefully from the slides with a small, soft-bristle brush, and roots not adhering to the film were removed with a scalpel. Soil was removed from adhering roots and root tips with careful brushing and/or gently flowing tap water. Films with adhering roots were air-dried, stained with acid fuchsin, and examined microscopically.

## RESULTS

Large numbers of oospores were produced in CMM. After 30 days of incubation at 17 C, each petri dish contained an average of $2.6 \times 10^6$ oospores. Over 99% of the oospores had thick walls which did not change in thickness while in the culture medium (Fig. 2). In one experiment, oospores retained their thick walls in CMM throughout an extended incubation period of 6 mo. Very few sporangia were produced in CMM. Both sporangia and oospores were produced, however, by the B6-1 isolate in water or on water agar culture.

Agar films containing oospores on slides buried in soil were superior to methyl cellulose films. When soil was washed from the slides, agar films adhered more tenaciously to the glass than did methyl cellulose films.

**Change in wall thickness of oospores in soil.** When agar films containing oospores on slides were buried in soil, many of the thick oospore walls began to change to thin ones (Fig. 3). After 48 hr of incubation about 30% of the oospores had thin walls. Thereafter, conversion to thin walls was less rapid with about 40% converted after 7 days and 85% after 45 days (Fig. 1A and B). Three additional cultures (P-7, C6-5, and VP-13) of *P. ultimum* were compared to isolate B6-1. All were highly pathogenic to cotton seedlings in greenhouse tests but were isolated from diseased cotton seedlings grown in different locations. Conversion of thick walls to thin walls of all three isolates was similar to that of B6-1. After 3 wk of incubation in agar film on slides buried in soil, over 70% of the oospores of all four isolates had thin walls.

Conversion was initially slightly faster in unsterilized soil than in sterilized soil, but this difference was not statistically significant and not apparent after 2 wk of incubation (Fig. 1A). A comparison was made of oospores taken from CMM cultures of different ages (Fig. 1B). At 7 days of incubation and thereafter, a higher percentage of 11- and 13-wk-old oospores had thin walls than did "younger" oospores. As an average of all days of incubation 5-, 9-, 11-, and 13-wk-old oospores had 37a, 40a, 51ab, and 60b% thin walls, respectively. Numbers followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

**Germination of oospores in the cotton rhizosphere.** On agar slides in soil, germinated oospores were observed in the vicinity of both primary and secondary roots (Figs. 4–10). Germination

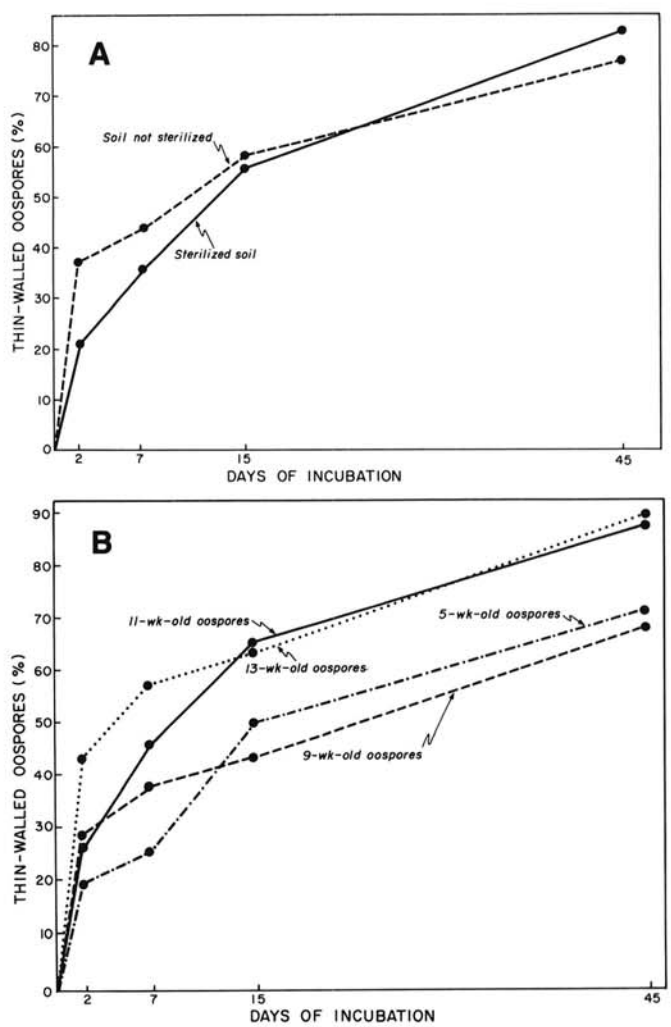
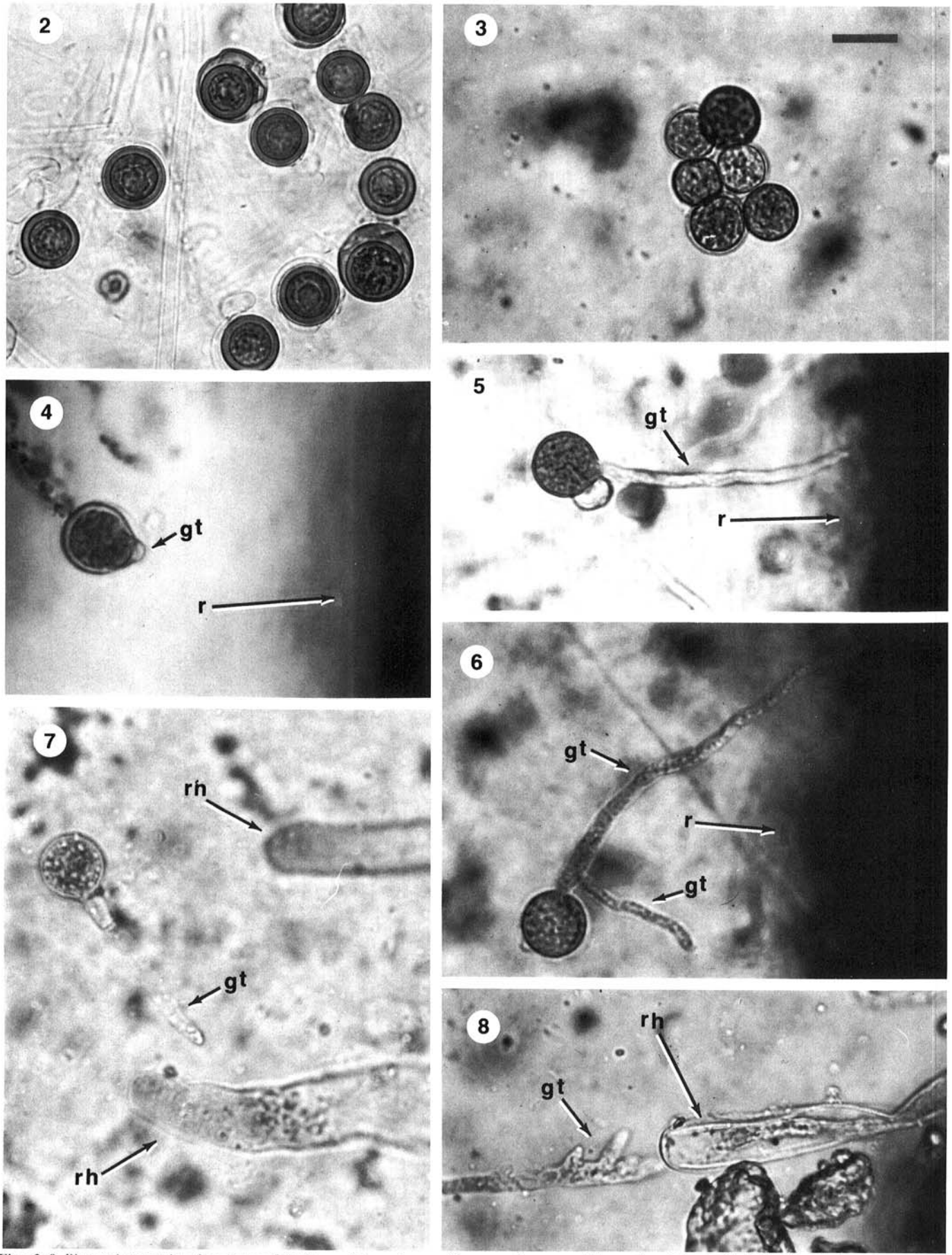



**Fig. 1.** Effects of **A,** soil sterilization and **B,** age of oospores on changes in wall thickness of oospores of *Pythium ultimum* in agar films on microscope slides placed in soil.




**Figs. 2–8.** Photomicrographs of oospores of *Pythium ultimum* in agar films on microscope slides retrieved from soil. **2,** Thick-walled oospores prior to incubation in soil. **3,** Thin-walled oospores incubated in soil for 15 days. **4,** Initiation of germ tube (gt) near out-of-focus root (r). **5,** Germ tube (gt) making contact with out-of-focus root (r). **6,** Branched germ tube (gt) making contact with out-of-focus root (r). **7,** Germ tube (gt) oriented toward root hair (rh). **8,** Branched germ tube (gt) making contact with root hair (rh). Scale bar in Fig. 3 applies to all figures in this composite and represents 20 $\mu$m.

occurred near roots in both natural and in previously sterilized soil. More oospores adjacent to and in contact with root surfaces germinated than did oospores located at a distance from the roots. This was evidenced by the large number of germinated spores found on the film previously in direct contact with the roots. Germ tubes from oospores in contact with roots were not oriented in any particular direction. Germ tubes from oospores not in direct contact with a root were always oriented toward the root. Occasionally, branched germ tubes were observed; both branches always were oriented toward the root (Fig. 6). Germinated oospores were not observed at distances greater than 1.5 mm from the root surface. Germination occurred mostly in the root hair zone, and only occasionally near root tips (Fig. 10). Germ tubes normally made contact with the root epidermis between root hairs or at the bases of root hairs. Only rarely was contact and penetration of root hairs observed. In the few cases where contact was observed between root hairs and germ tubes, the germ tubes had branched prior to contact (Figs. 7 and 8).

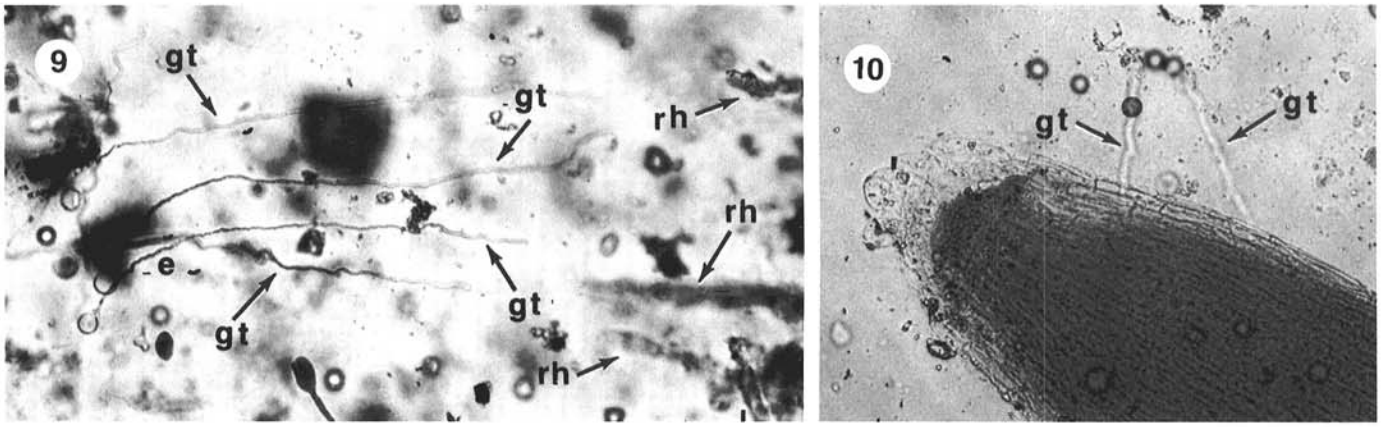
Most oospores germinated on agar slides near roots by forming an appressoriumlike structure that pushed directly through the oogonial wall. Occasionally, germ tubes would curve around the oospore for some distance inside the oogonial wall prior to emergence (Fig. 11).

## DISCUSSION

Lumsden and Ayers (16) studied the influence of the soil environment on germination of oospores of *P. ultimum*. Damping-off of snapbean seedlings occurred in soil infested with thin-walled, germinable oospores, but not in soil infested with thick-walled, dormant oospores, until enough time had elapsed for conversion to germinable oospores. After 1, 2, and 3 wk of incubation in soil, 0.1, 0.3, and 11.6%, respectively, of the oospores had converted to thin-walled oospores. In similar experiments, Hancock (7) obtained virtually no change in oospore wall thickness during the first 2 wk of incubation in soil. After 6 wk, approximately half the spores had thin walls. In the present study, conversion was much faster. After 2 wk in agar film on slides buried in soil, 40–60% of the oospores had thin walls. The reason conversion in spores on agar slides occurred much more rapidly than in spores not on slides is not readily apparent. In addition to differences in isolates of *Pythium*, soil type, and soil pH which could affect rate of conversion, there were considerable differences in the concentrations of oospores in the soils. In the present study, numbers of oospores per gram of soil were much smaller.

The rate of germination appeared to be greatest with oospores that were very close to roots. This was illustrated in certain slide preparations in which roots were removed from contact with the agar film, and the film area in contact with the root remained on the slide. In most cases when a root was removed from intimate contact with the agar film, the film adhered to the root segment. Only a small percentage of the oospores germinated when not in intimate contact with roots. Many were observed to have thin walls and were probably capable of germinating. Data were not taken on percentages of germinated oospores in the rhizosphere because there was no effective method for determining the distance between the root epidermis and the agar film, a factor which could affect diffusion of root exudates into the film.

Stanghellini and Hancock (23) described germination of sporangia of *P. ultimum* in the bean spermosphere. Sporangial germination occurred up to 10 mm from the seed surface after 12 hr of incubation in soil at 28% moisture (pF 1.7). In the present study where much longer incubation periods (5–10 days) were necessary, and where constant moisture levels would be difficult to maintain,




**Figs. 9-10.** Germinated oospores of *Pythium ultimum* near cotton roots as viewed with the low power (×10) microscope objective. **9,** Several oospores with germ tubes (gt) oriented toward root and out-of-focus root hairs (rh). **10,** Germinated oospores with germ tubes (gt) making contact with root tip.

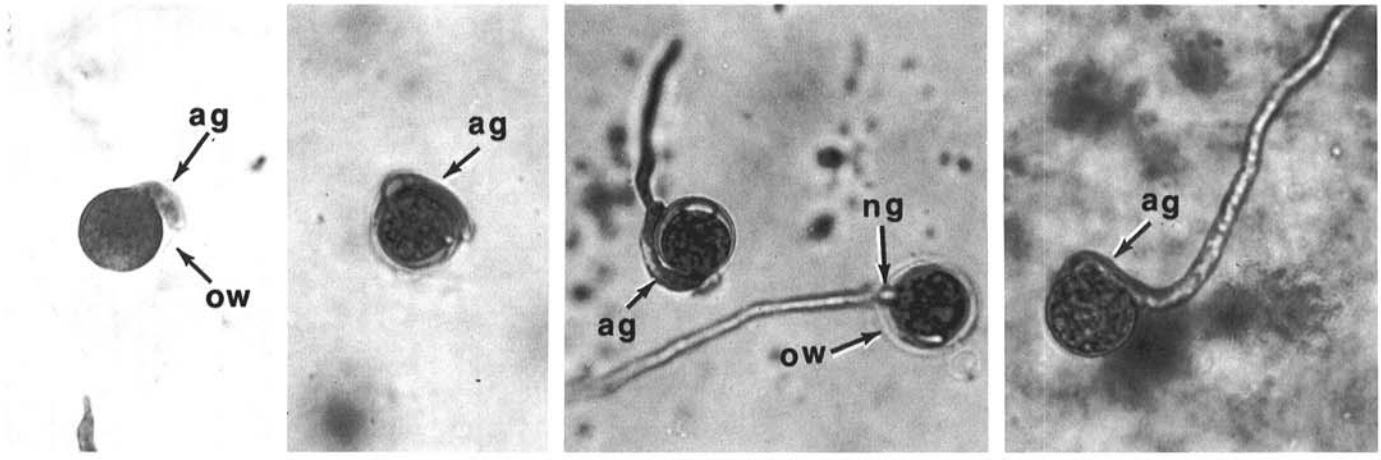



**Fig. 11.** Normal (ng) and abnormal growth (ag) of germ tubes of *Pythium ultimum* prior to penetration of oogonial walls (ow). Scale bar in Fig. 3 applies to this figure.

oospore germination was not detected further than 1.5 mm from the root surface. In addition to differences in moisture tension, different or greater quantities of stimulatory substances may be produced by germinating seed than are produced by roots. Also, sporangia may be more easily stimulated to germinate than are oospores. Compared to sporangia, oospore germination would be delayed because their thick walls must first be converted to thin walls before germination can take place. There was no evidence in the present study that root exudates affected the rate of conversion from thick to thin walls.

It is well known that root exudates stimulate germination of spores of many pathogens. Zoospores of several species of *Pythium* have been shown to be attracted to, swim toward, and collect on root surfaces (13,19,22). Germination of zoospores in contact with roots and penetration of root epidermis by zoospore germ tubes have been illustrated. There are few reports, however, on tropistic responses of germ tubes from germinating spores some distance from roots. When excised roots of peach were placed in water containing sporangia of *P. irregulare*, germ tubes from some nearby sporangia grew towards the excised roots (3). However, Spencer and Cooper (22) found no tropistic response of the mycelium of *P. ultimum* to roots of cotton seedlings grown in buried cellophane or dialysis bags that were adjacent to actively growing cultures of *Pythium*. In the present study, germ tubes from oospores of *P. ultimum* exhibiting positive tropism to roots were observed and photographed. Scores of germ tubes from oospores near roots were observed; all were oriented toward the roots.

The agar-slide method described herein is suitable and useful for studying the fate of fungal propagules in soil and in the plant rhizosphere. Even though cotton seedling primary roots are relatively large in diameter and there was some difficulty in preparing mounts suitable for observation under the microscope high power objective, many excellent specimens were obtained. Use of the method with plants having seedling roots smaller in diameter than those of cotton should facilitate the preparation of acceptable mounts for photomicrography.

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