Effects of Atmospheric Gases and Light on Changes in Thickness of Oospore Walls and on Germinability of Oospores of *Pythium ultimum*

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ABSTRACT

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Water suspensions of thick-walled oospores of *Pythium ultimum* were exposed to atmospheres of air, CO_2, O_2, N_2, or mixtures of air and CO_2. Aeration or O_2 was required for conversion to thin-walled oospores, but an atmosphere of 100% O_2 was not more effective than air. Oospores in water suspensions did not convert when exposed to atmospheres of 100% CO_2, and conversion was significantly inhibited in suspensions exposed to atmospheres containing 2.9% CO_2. Germinability of oospores (determined by plating on a nutrient medium) previously exposed to atmospheres of 100% CO_2 for 8 days was not appreciably affected. Conversion was inhibited in water suspensions adjusted to pH 6.0 or lower. Exposure to CO_2 atmospheres lowered the pH of the suspensions, but increased CO_2 atmospheric concentrations progressively inhibited conversion even in buffered solutions (pH 6.2–6.8). Exposure to light was required for conversion. At low light levels, linear increases in percentages of oospores converted were related to logarithmic increases in light quantity. Germination of thin-walled oospores on a nutrient medium was not affected by light. Evidence is presented that both thick- and thin-walled oospores are exogenously dormant.

Oospores of *Pythium ultimum* Trow are produced in hypocotyls and roots of plants previously invaded by the fungus (13) and in plant tissue introduced into soil (6). Because of their dormancy, thick walls, and resistance to dessication, they are considered to be primary survival structures (8,11,13). Thick-walled oospores of *P. ultimum* convert slowly to germinable thin-walled oospores when placed in nonsterile soil extract or in agar films on glass slides placed in soil (7,9,11,17). After 4 wk of incubation in nonsterile soil extract, 25% change to thin-walled oospores (11), and 60–80% change to thin-walled oospores in agar films in soil after 7 wk (9). Thin-walled spores remain dormant in soil extract but germinate when placed on nutrient media (11). Ultrastructural changes during conversion and germination have been reported (7).

Environmental factors affecting rates of conversion have not been clearly defined. In soil samples under laboratory conditions, Lumsden and Ayers (11) obtained maximum conversion at pH 7.0 in soil saturated with water at 25 C. Recently, Qian and Johnson (17) found that conversion was significantly faster in some soils than in others, but conversion rates were not affected by differences in soil pH, percent organic matter, texture, or concentrations of major and minor plant nutrient elements. Lysis of thin-walled oospores (or germination with subsequent lysis), however, was correlated positively with soil pH and concentrations of nitrogen and available phosphorus. Because changes in oospore wall thickness in *P. ultimum* are directly related to dormancy and survival of the fungus in soil, it is desirable to learn more about environmental conditions affecting these changes. The objective of this research was to determine the influence of atmospheric gases and light on oospore wall conversion.

MATERIALS AND METHODS

A culture of *P. ultimum* designated B6-1 (ATCC 56081) and isolated from a diseased cotton seedling from a field in western Tennessee was used in this study. Pathogenicity, oospore production in liquid media, and rates of conversion of oospores from thick to thin walls were similar to those of other isolates of *P. ultimum* from cotton seedlings grown at different locations (9).

Preparation of water suspensions of oospores. 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 through cheesecloth before autoclaving. The medium was dispensed in petri dishes (approximately 20 ml per dish), seeded with mycelial plugs of *P. ultimum* on cornmeal agar, and incubated at 17 C in darkness for 4 wk or more. Oospores were separated from hyphae by mixing the contents of five petri dishes with 100 ml of sterile, distilled water in a Waring blender. The material was blended intermittently at high speed 15 times for 1 or 2 sec each. Oospores in the blended material were washed through a sieve with a 150-μm (100-mesh) pore size and centrifuged at 5,000 g for 15 min. The supernatant was discarded, and the pellet was resuspended in sterile, distilled water, centrifuged a second time, and the pellet was taken up in 50 ml of water. Number of oospores was determined with a hemacytometer. This procedure yielded approximately 300,000 oospores per milliliter with very few hyphal fragments.

Treatment of oospores with gases. Medical grade carbon dioxide, oxygen, nitrogen, air, and mixtures of CO_2 and air were supplied by Burdett Gas Products, Knoxville, TN. Gases from supply tanks were passed through latex tubing attached to glass tubing immersed in 60-ml water suspensions of oospores in 70-ml test tubes. Gases were bubbled through the suspensions at the rate of 10 bubbles per minute (0.7 ml/min). In certain experiments, gases were passed over the surfaces of 20-ml water suspensions or buffered solutions containing oospores in 250-ml Erlenmeyer flasks. Rubber-stoppered flasks containing water suspensions of oospores were connected to gas supply valves with glass tubing that extended through the stopper and ended approximately 1 cm above the suspension surface. An outlet glass tube through the stopper was attached to general purpose latex tubing, which ended in a flask of distilled water. In some experiments, three or four flasks were connected in series, each with glass tubing connections as described. Such systems were purged for 10 min with the gas, then flow rates were reduced, measured with a manometer, and adjusted to 2 ml/min. Buffers were prepared according to Gomori (4). Acidity of the suspensions was measured with an electronic pH meter.

Oospore condition was determined at 2- to 4-day intervals by stirring a suspension with the tip of a 1-ml pipette and removing approximately 0.3 ml. Two drops were mixed with one drop of 0.03% acid fuchsin in 85% lactic acid on a microscope slide. A cover glass was applied and oospores were examined at 400× with a microscope. Two hundred oospores selected at random on each slide were classified as thick-walled, thin-walled, germinated, or lysed.

Germinability of oospores was determined by applying 1 ml of

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an oospore suspension (diluted 1:100 with sterile water) on the surface of a selective cornmeal agar medium (SCMA) in a petri dish. Cornmeal broth was prepared as for CMM, and 15 g of agar per liter was added. After the medium was autoclaved and had cooled to 42–46 C, the following materials per liter were added: penicillin-G, 80,000 units; pimaricin, 20 mg; PCNB (75%), 20 mg; and streptomycin sulfate, 50 mg. After 24 hr of incubation at 20 C, percentages of oospores with attached germ tubes were recorded.

Exposure of oospores to light. Cultures of *P. ultimum* in CMM containing oospores that developed in darkness at 17 C were removed from the incubation chamber under subdued light to a photographic darkroom. There, cultures were blended as previously described, but in darkness. Blender contents (oospores and mycelial fragments) were bulked, and 35 ml was transferred to each of 25 250-ml Erlenmeyer flasks. During this procedure, a safelight equipped with a Kodak OC filter and 15 W bulb was used momentarily when necessary for measurements. Five incubation boxes, each 20 × 22 × 20 cm high, were fashioned from pieces of three-ply corrugated cardboard. The inside of each box was lined with black construction paper and vented on two sides with 30-cm-length coils of black latex tubing. Five flasks containing blended material were placed in each box. The top of one box was sealed "light-tight," another was left with an open top, and one, three, or six sheets of mimeo bond typing paper (75 g/m^2) placed on tops of the remaining boxes served as light filters. All boxes with flasks were placed in a plant growth chamber with a combination of incandescent and fluorescent light and held at a constant 25 C with alternating 12-hr periods of light and darkness. Light quantity received in each box was measured with a Quantum/Radiometer/Photometer (Li-Cor, Inc., model LI-185A). At intervals of 2 or 3 days, samples were withdrawn from the flasks in the darkroom with safelight, and oospore conditions were determined as previously described.

For verification of results, all experiments were repeated. Data were analyzed by analysis of variance procedures, and treatment means were separated with Duncan's new multiple range test.

RESULTS

Water suspensions of oospores prepared from 4-wk-old CMM cultures of *P. ultimum* contained an average of 95% thick-walled oospores, 2% thin-walled oospores, and 3% lysed oospores. Less than 0.3% of the oospores had attached germ tubes. Germination was not increased by any of the treatments in this study, but lysis without prior germination increased up to 10% after 3 wk. Because none of the gases or light was correlated with increased lysis during the 2-3 wk of incubation, data on germination and lysis are not included in the tables or graphs presented.

Effect of aeration. Mean rates of increase in percentages of thin-walled oospores were greater in stationary suspensions in 125-ml flasks than in similar volumes of stationary suspensions in 15-ml test tubes (Fig. 1). Rates of increase in percentages of thin-walled oospores in tubes were inversely related to the concentrations of oospores in the suspensions ($P < 0.05$). Aeration of water suspensions of oospores in tubes caused increases in rates of conversion (Fig. 2). Conversion was slower in nonsterile soil extract than in water but was increased significantly ($P < 0.01$) by aerating the soil extract.

Effect of oxygen, nitrogen, and carbon dioxide. Conversion of thick-walled oospores was rapid in suspensions in tubes into which O_2 or air was bubbled, but little conversion occurred in control tubes (without bubbles) or in tubes in which N_2 was bubbled (Fig. 3). Conversion did not occur in suspensions in which pure CO_2 was bubbled, or in stationary suspensions in Erlenmeyer flasks exposed to 100% CO_2 atmospheres (Table 1). Germinability of oospores removed after 8 days from suspensions exposed to atmospheres of 100% CO_2 was determined by plating on SCMM. Germination after 24 hr from air-treated, CO_2-treated, and control suspensions (no atmosphere changes) was 83, 75, and 78%, respectively.

Conversion of oospores was inhihited in water suspensions of oospores with atmospheres containing mixtures of air and CO_2 (Table 1). Mean percentages of oospores with thin walls after 2, 4, 7, and 10 days of incubation in suspensions with atmospheres containing 2.9% CO_2 were significantly less ($P < 0.01$) than corresponding percentages of thin-walled oospores with ambient air atmospheres. Acidity of the suspensions was increased by CO_2 treatments. In a separate experiment, distilled water was adjusted to six hydrogen ion concentrations from pH 4.0 to 8.0 with 0.01 N NAOH or 0.1 N HCl. Conversion was reduced in such solutions at pH 6.0 or lower and completely inhibited at pH 4.0. Buffer chemicals, in solutions buffered for pH stability, inhibited

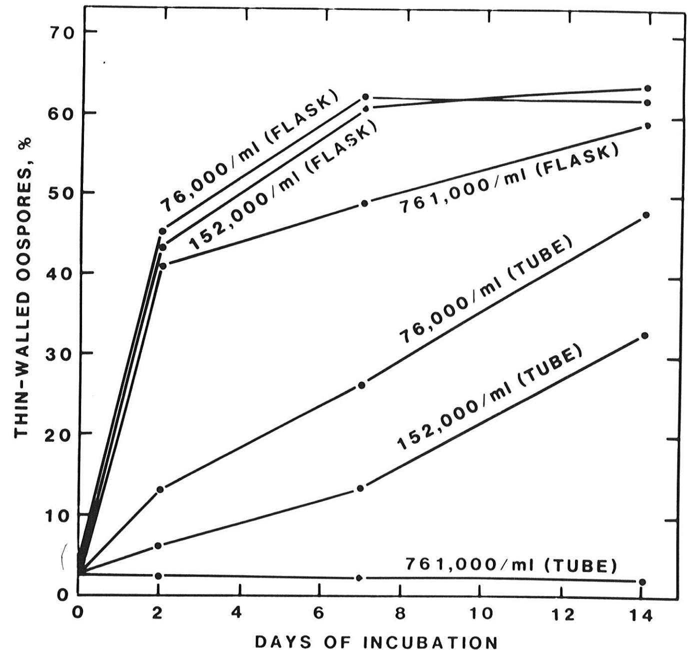

Fig. 1. Effect of oospore concentration on conversion of thick-walled oospores of *Pythium ultimum* in 12-ml water suspensions in 125-ml Erlenmeyer flasks and on conversion in similar volumes in 15-ml test tubes. $LSD_{0.05}$ among means of container-concentrations = 11%.

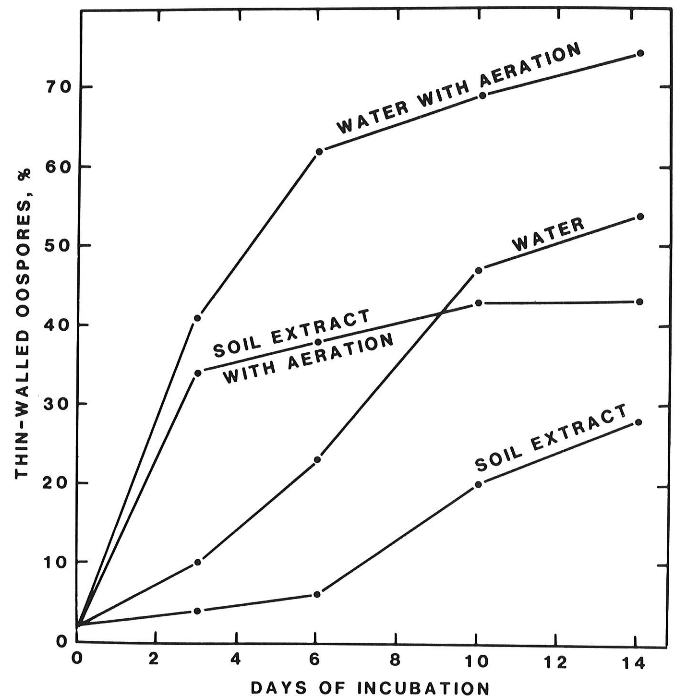

Fig. 2. Effect of aeration on conversion of thick-walled oospores of *Pythium ultimum* in water or soil extract suspensions in test tubes. Oospore concentration = 180,000 per milliliter. $LSD_{0.05}$ among treatment means = 7.9%.

conversion. Conversion was completely inhibited during an incubation period of 10 days in 0.1 M citrate-sodium phosphate buffer at pH 7.0, in 0.2 M sodium phosphate buffer at pH 6.7, or in 0.2 M Tris buffer at pH 6.6. Conversion rates were reduced also in both Tris and sodium phosphate buffers diluted to 0.0125 M concentrations (Table 2). Tris was less inhibitory to conversion than was sodium phosphate at low molarities. Suspensions of oospores in 0.0125 M Tris were exposed to atmospheres of mixtures of CO_2 and air. Conversion was reduced significantly ($P < 0.05$) in atmospheres of 2.9 or 4.8% CO_2 and completely inhibited in an atmosphere of 9.5% CO_2 (Fig. 4). All solutions were held at pH 6.2 or higher.

Effect of light. In preliminary experiments, no appreciable conversion in water suspensions occurred in darkness during 15 days of incubation. In a replicated experiment, light quantity affected rate of conversion (Fig. 5). Very little conversion occurred in darkness and progressively higher percentages of oospores converted during 12 days of incubation with exposure to increased amounts of light. Linear increase in percentage of oospore conversion was related to a logarithmic increase in light quantity (Fig. 6). For each 2.75-fold increase in light quantity (w/m^2) there was a corresponding 1% increase per day in formation of thin-walled oospores.

To determine if light affects germination of thin-walled oospores, Erlenmeyer flasks containing thick-walled oospores in sterile, distilled water were placed in growth chambers held at 25 C in continuous light. After 6 days of incubation, 7.3% were thick-walled, 82.3% were thin-walled, and 10.4% were lysed. None of the spores had attached germ tubes. One-milliliter samples of this material were spread over SCMA in petri dishes, nine of which were exposed to continuous light, and nine placed in light-tight boxes. After 24 hr, 78% of the spores in darkness and 79% exposed to light had attached germ tubes. This experiment was repeated twice with similar results.

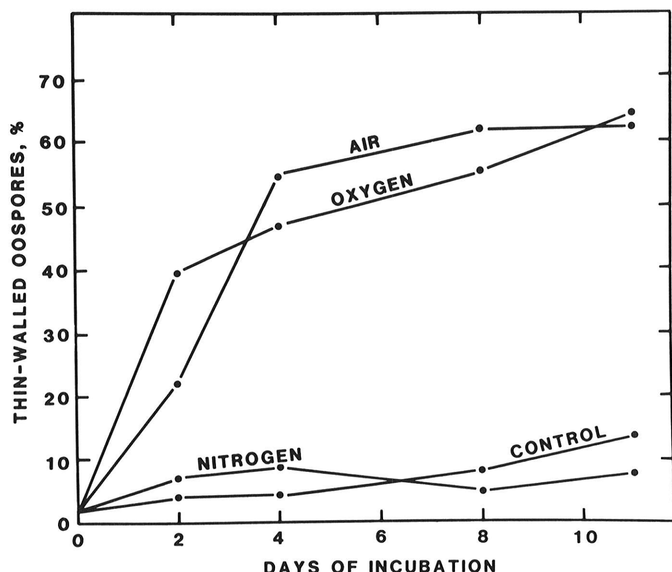

Fig. 3. Effect of air, 100% CO_2 or 100% N_2 bubbled through suspensions on conversion of thick-walled oospores in water suspensions in test tubes. Oospore concentration = 91,000 per milliliter. $LSD_{0.05}$ among treatment means = 12.7%.

TABLE 1. Changes in pH and thickness of walls of oospores of *Pythium ultimum* in Erlenmeyer flasks containing water suspensions of oospores exposed to atmospheres of mixtures of CO_2 and air[a]

	Day 2		Day 10		
Atmosphere	pH	Thin-walled oospores (%)	pH	Thin-walled oospores (%)	Mean[b]
Air	6.9	69	7.3	92	83
2.9% CO_2	5.9	48	7.2	63	57
9.5% CO_2	5.5	10	6.5	55	32
100% CO_2	4.6	2	5.7	2	2

[a] At the beginning of the experiment (day 0), 2% of the oospores had thin walls. Concentration of suspensions was 107,000 oospores per milliliter.
[b] Mean thin-walled oospores (%) for days 2, 4, 7, and 10. Differences among all means were significant ($P = 0.05$) according to Duncan's new multiple range test.

TABLE 2. Changes in thickness of walls of oospores of *Pythium ultimum* in water solutions of two buffers in Erlenmeyer flasks after 10 days of incubation[a]

	Sodium phosphate buffer[b]		Tris buffer[c]	
Molar concentration	Thin-walled oospores (%)	pH	Thin-walled oospores (%)	pH
0.2	2	6.6	2	6.5
0.1	2	6.7	6	6.6
0.05	2	6.7	36	6.8
0.025	2	6.8	61	7.0
0.0125	14	7.0	59	7.2
0	52	6.6	72	6.6

[a] The two buffers were tested at different times and two sources of oospores were used. At the beginning of each experiment, 2% of the oospores had thin walls.
[b] Concentration of oospores = 148,000 per milliliter.
[c] Concentration of oospores = 157,000 per milliliter.

DISCUSSION

In studies on the effects of CO_2 on growth and sporulation of species of *Pythium* and the related genus, *Phytophthora*, CO_2 concentrations above 15% reduced growth of *Pythium graminicola* (22), *Phytophthora parasitica* var. *nicotiana* (3), and other species of *Phytophthora* (14). Carbon dioxide concentrations above 1% reduced sporulation of *Phytophthora* spp. (15) and *Pythium aphanidermatum* (12). Few, if any, studies of the

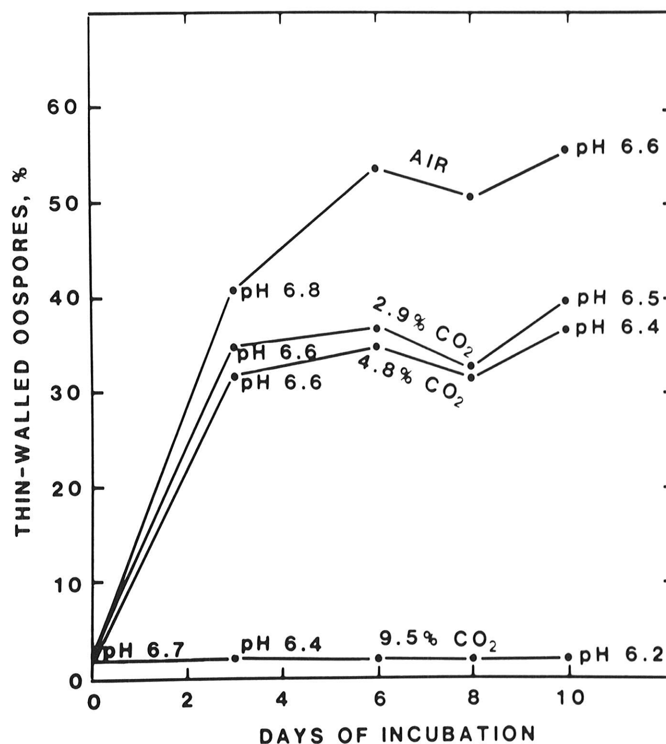

Fig. 4. Effect of air or mixtures of CO_2 and air on conversion of thick-walled oospores of *Pythium ultimum* in suspensions buffered with 0.0125 M Tris in Erlenmeyer flasks. Oospore concentration = 96,000 per milliliter. $LSD_{0.05}$ among treatment means = 5.0%.

influence of CO_2 on spore germination in these two genera have been reported. In the present study, CO_2 at a concentration of 2.9% or higher in the atmosphere above solutions containing oospores of *P. ultimum* reduced the rate of conversion of these spores from thick to thin walls. The 2.9% concentration of CO_2 is not uncommon in natural soil. In the atmosphere above ground, CO_2 concentration is approximately 0.03% (23), but in the soil atmosphere it is much higher and depends largely on microbial activity. Normally, the CO_2 concentration in soil ranges from 2 to 4%, but after a rain or after addition of decomposible organic materials, the CO level can rise as high as 10% (2,10,16,21).

In solutions in test tubes containing high concentrations of oospores, conversion was reduced. When such solutions were aerated, conversion proceeded rapidly, but pure O_2 as an aerating gas was not more effective than air. Nitrogen concentration in air, an atmosphere suitable for conversion, is normally 78.1% (23). In an atmosphere of pure N_2, conversion was completely inhibited, presumably because of lack of O_2. The minimum concentration of O_2 required for conversion was not determined in this study, but formation of thin-walled oospores in solutions in tubes where oxygen was increasingly limited was reduced correspondingly. In waterlogged soils, or in soils where microbial respiratory activity is high, CO_2 levels tend to increase and O_2 levels decrease. Both of these conditions favor inhibition of oospore wall conversion.

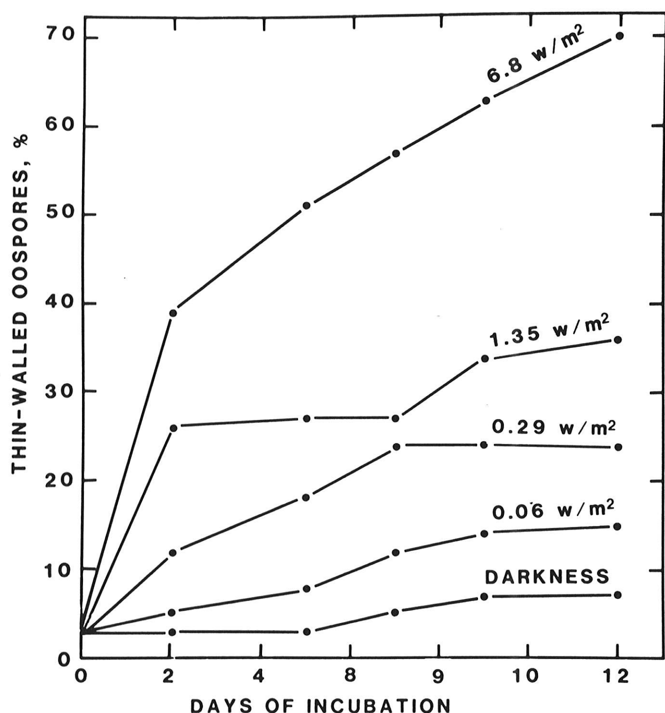

Fig. 5. Effect of quantity of light on conversion of thick-walled oospores of *Pythium ultimum* in water suspensions in Erlenmeyer flasks. Oospore concentration = 96,700 per milliliter. $LSD_{0.05}$ among treatment means = 6.8%.

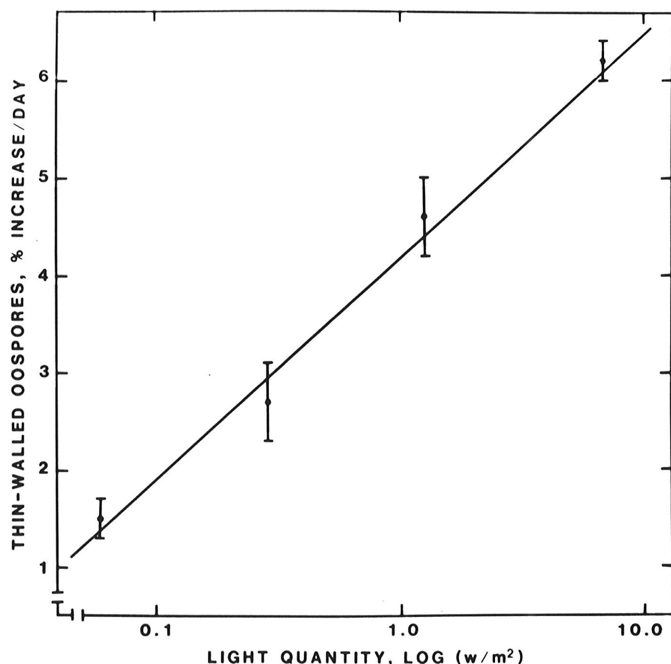

Fig. 6. Logarithmic relationship of light quantity on conversion of thick-walled oospores of *Pythium ultimum* in water suspensions in Erlenmeyer flasks. Vertical lines represent standard error of the mean.

Several fungi (among them *Phytophthora* spp.) require light for breaking of dormancy of spores (1,20). Only 12% of the oospores of *Pythium aphanidermatum* in intact culture mats germinated in darkness, whereas 94% germinated when previously exposed to 300 ft-c of white light for 36 hr (19). However, when separated from the hyphae on which they were formed, 50% of the oospores germinated in darkness. In the present study, initiation of germination (conversion to thin-walls) was completely or almost completely inhibited in darkness. Thin-walled oospores, obtained by exposure of thick-walled oospores to light, germinated equally well in darkness or light.

Increases in conversion of oospores responded arithmetically to logarithmic increases in light exposure. A measurable amount of conversion occurred in a very low light exposure of 0.06 w/m^2. This value is considerably less than 350 w/m^2, a value obtained in direct sunlight (hazy, but cloudless day at 1:20 p.m. on 25 September 1986 at Knoxville, TN). Wooley and Stroller (24) found that in a silty clay loam, about 5% of sunlight was transmitted through 0.5 mm and less than 2% through 1.1 mm of soil. Two percent, or 7 w/m^2, of the sunlight value at Knoxville, TN, was a light exposure permitting good conversion. It is assumed, therefore, that oospores occurring in the upper 1–2 mm of soil receive adequate light for oospore conversion. Spring soil preparation by discing or plowing and planting procedures exposes soil to light and probably increases oospore conversion. The effect of no-till agricultural practices on conversion should be investigated.

Sussman and Halvorson (20) described constitutive dormancy in spores as the delay of germination due to an innate property of the dormant stage such as a barrier to penetration of nutrients, a metabolic block, or the production of a self inhibitor. Exogenous dormancy was described as a condition in which development is delayed because of unfavorable chemical or physical conditions of the environment. Lumsden and Ayers (11) regarded thick-walled oospores of *P. ultimum* to be constitutively dormant, and the thin-walled oospores as exogenously dormant. Evidence that both thick- and the thin-walled oospores are exogenously dormant is presented in the present study. Thick-walled oospores separated from the hyphae changed to thin-walled oospores very rapidly (69% in 2 days, Table 1) when subjected to proper aeration, pH, and light conditions. Nutrients apparently are not required for this change. Thick-walled oospores prevented from converting in an atmosphere of 100% CO_2 were removed from the CO_2 substrate and placed on a selective agar medium containing nutrients (and exposure to light) favorable for germination. Within 24 hr, 75% had germinated, 7% were thin-walled, and 18% were thick-walled. There is no evidence for an innate property responsible for dormancy of thick-walled oospores. Because exposure to light, oxygen, and nutrients (in quantities commonly occurring in nature) results in conversion and germination, both of these spore types must be exogenously dormant. Conversion to thin-walled spores and germination is apparently a continuous process. Under the proper conditions of aeration, light, and nutrients, and in the absence of inhibiting amounts of CO_2, germination of thick-walled oospores proceeds rapidly without development of a dormant thin-walled stage.

One of the significant aspects of the present findings is the

possible relationship of seedling infection sites to these atmospheric conditions. The isolate of *P. ultimum* used in this study is highly pathogenic to a variety of plant taxa, including green beans, okra, cotton, and cucumber. Although roots are attacked, most of the plant damage occurs in the seedling stage on young hypocotyls at or just below the soil surface. This site appears to be the most favorable for maturation of oospores and subsequent hypocotyl invasion. At this location (as compared with root sites at greater soil depths), oxygen levels are high, CO_2 levels are low, and light conditions are favorable for oospore conversion. Nutrients in exudates from hypocotyls (5,18) could then induce germination of thin-walled oospores and chemotrophic responses of germ tubes toward the hypocotyl sources (9).

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