Soil Matric Potential Effects on Changes in Wall Morphology, Germination, and Lysis of Oospores of *Pythium ultimum*

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ABSTRACT

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Thick-walled oospores of *Pythium ultimum* in agar films on glass slides were incubated in soil for 84 days. In soil with matric potentials of −0.03 to −0.30 MPa, almost all of the thick-walled oospores converted to thin-walled oospores, and 91% of these lysed. In soil near saturation (0 MPa) and in much dryer soil (−1.50 MPa), 82 and 62% of the oospores remained thick-walled, respectively. Occasionally, oospores with attached germ tubes were observed but not in soil with matric potentials of 0, −0.7, or −1.5 MPa. Oospores were germinated in corn meal agar films on glass slides and then were killed by fumigation with propylene oxide, aerated, and placed in soil. After 16 days of incubation, 98% of the germ tubes had disintegrated and were no longer visible. Based on rate of visual disappearance in soil of germ tubes killed with propylene oxide, calculations were made for the quantity of oospores buried in soil at different moisture levels that lysed directly and for those that germinated before lysis. As an average for soil with moisture levels of −0.03 to −0.30 MPa and soil with fluctuating moisture levels, it was calculated that 89.5% of the oospores lysed directly and 1.5% germinated.

Oospores of *Pythium ultimum* Trow are produced in hypocotyls and roots of plants previously invaded by the fungus (14) and in plant tissue introduced into soil (4). Initially, oospores are thick-walled, are resistant to desiccation, and survive for long periods in air-dry soil (5). In moist soils, thick-walled spores change to thin-walled, germinable oospores (7,12), many of which lyse (15). Rates of conversion from thick walls to thin walls are not related to concentrations of plant nutrients in soil, pH, or organic matter or to soil texture. Rates of lysis, however, are correlated positively with soil pH, C:N ratio, organic matter, and available phosphorus and negatively with clay content (15).

Germination of oospores was not observed by Lumsden and Ayers (12) in soil or in nonsterile soil extract. Only when oospores were removed from soil or soil extract and exposed to nutrients did they germinate. Qian and Johnson (15) occasionally observed germinated oospores (with attached germ tubes) on glass slides buried in soil, but in that study, data on oospore morphology were taken at 21-day intervals, and it was difficult to determine if many of the lysed spores observed had germinated before lysis. Germ tubes could have disintegrated near the spores and thus would not have been visible several days later when data were taken. This disintegration of germ tubes might have resulted in estimates of percent germination being far too low. Additional studies on visibility of germ tubes are needed to determine whether or not most thin-walled oospores germinate before lysis.

The objectives of the present study were to identify soil moisture tension effects on conversion of oospores from thick to thin walls, and to determine if a significant number of oospores germinate in soil before lysis.

MATERIALS AND METHODS

Incubation of oospores in soil. Composites of soil surface samples of a Dexter silt loam from a field in western Tennessee were used in this study. A culture of *P. ultimum* (ATCC 56081) was used that was originally isolated from a diseased cotton seedling from a field in western Tennessee. Pathogenicity, oospore production, and rates of conversion of oospores from thick to thin walls of this isolate were similar to other isolates of *P. ultimum* from cotton seedlings grown at different locations (7). Procedures for separating oospores from hyphae and incubating oospores in soil have been described (7). Briefly, oospores in liquid culture were separated from hyphae through blending, sieving, and centrifugation and then suspended in 2% water agar at 42 C. Microscope slides previously dipped into the suspension and coated with water agar films of oospores were buried in soil.

In experiments to determine the effects of moisture tension, 150-g portions of soil were equilibrated at 0, −0.03, −0.1, −0.3, −0.7, or −1.5 MPa with a pressure membrane apparatus (Soil Moisture Equipment Co., Santa Barbara, CA) at room temperature for 24 hr. Percent moisture determinations were made for each moisture tension level by weighing samples before oven drying. Samples of soil dried to different moisture tensions were placed in 473-ml Mason jars to a depth of 3.5 cm, with four replicates for each moisture level. Four slides with agar films of oospores were inserted into the soil in each jar. Lids were applied tightly, and jars with contents were weighed and incubated in a plant growth chamber with alternate 12-hr periods of 24 C and 18 C. At 7-day intervals during 84 days of incubation, lids were removed briefly and jars were rotated gently for air exchange. Jars with contents were weighed every 14 days to determine water loss due to evaporation. Mean water loss during the 84 days of incubation was negligible (0.16% per jar).

In certain experiments, microscope slides were buried in soil in plastic, 10.2-cm-diameter pots and incubated in growth chambers at a constant 24 C. Soil in these pots was kept moist by watering to saturation whenever the soil surfaces appeared dry. This soil is referred to as "moist" soil.

Determination of oospore morphology. At 21-day intervals during incubation, four replicate slides were removed from jars of soil at each moisture level, and the slide surfaces containing oospores were washed to remove soil particles. Oospores on the slides were stained with 0.03% acid fuchsin in 85% lactic acid and examined microscopically. Two hundred oospores chosen at random on each slide were classified as being thick-walled, thin-walled, germinated (with attached germ tubes), or lysed.

Fumigation of germ tubes with propylene oxide. To study disintegration of germ tubes in soil, the rate of germ tube disap-

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pearance was determined for oospores with attached germ tubes that were fumigated with propylene oxide and then placed in soil. Oospores with germ tubes were obtained by incubating thick-walled oospores in sterile-distilled water for 1 wk, after which 60–70% had converted to thin-walled oospores. After concentration by centrifugation, the oospores were incorporated in melted cornmeal agar (CMA) at 42 C. The CMA-oospore preparation was coated onto microscope slides as described and incubated in 1-L-capacity moist chambers at 20 C to promote germination. After 4.5 hr, 52% of the oospores had germinated. One milliliter of propylene oxide was added to the moist filter paper in the bottom of each chamber, which was sealed for 18 hr and then vented for 24 hr. Mortality of oospores after treatment with propylene oxide was determined by placing the agar surfaces of sample slides on a selective medium (6) in petri dishes. Complete mortality was indicated if growth did not occur on the medium within 5 days. Slides containing the fumigated oospores were buried in soil in plastic pots in growth chambers held at 24 C. A "moist" soil condition was maintained as described. Four slides were removed after 1, 2, 3, 4, 7, 11, and 16 days, washed, stained, and examined microscopically. Data were taken on numbers of thick-walled, thin-walled, germinated (with attached intact or identifiable-lysed germ tubes), and lysed oospores (without identifiable germ tubes). Four hundred oospores (100 selected at random on each slide) were classified at each time interval.

Statistical analyses. Effects of treatments on percentages of oospores were evaluated with analysis of variance procedures. A simple compartment model was used to estimate the quantities of thin-walled oospores that lysed directly and those that germinated before lysis. In the model, rates of conversion between categories of oospores are designated by differentials that identify the source category and resultant category (Fig. 1). The total rate of change in an oospore category is the sum of the rates of increase and decrease. For the four categories of oospores, these overall changes can be written as follows:

$$d(Thick)/dt = d(Thick \rightarrow Thin)/dt \quad (1)$$

$$d(Thin)/dt = -d(Thick \rightarrow (Thin/dt + d(Thin \rightarrow Germ)/dt + d(Thin \rightarrow Lysed)/dt \quad (2)$$

$$d(Germ)/dt = -d(Thin \rightarrow Germ)/dt + d\,(Germ \rightarrow Lysed)/dt \quad (3)$$

$$d(Lysed)/dt = -d(Thin \rightarrow Lysed)/dt - d(Germ \rightarrow Lysed)/dt \quad (4)$$

Where $d(Thick)/dt$, $d(Thin)/dt$, $d(Germ)/dt$, and $d(Lysed)/dt$ represent the overall rates of change in the numbers of thick-walled, thin-walled, germinated, and lysed oospores, respectively; and differentials containing two oospore class names represent the rates of conversion from the first to the second category (e.g. $d(Thick \rightarrow Thin)/dt$ represents the rate of conversion from thick-walled to thin-walled oospores).

In these equations, the value of a differential is considered to be negative if it represents a reduction in a category of oospores. Thus, $d(Thick)/dt$ is always negative because the percentage of thick-walled oospores can only decrease, whereas $d(Thin)/dt$ can be positive or negative. The question of whether most oospores germinate before lysis is equivalent to asking whether $d(Thin \rightarrow Germ)/dt$ is larger than $d(Thin \rightarrow Lysed)/dt$ (Fig. 1). Equation 3 can be rearranged to make $d(Thin \rightarrow Germ)/dt$ the dependent variable:

$$d(Thin \rightarrow Germ)/dt = -d(Germ)/dt + d(Germ \rightarrow Lysed)/dt \quad (5)$$

Similarly, equation 4 can be rearranged to make $d(Thin \rightarrow Lysed)/dt$ the dependent variable:

$$d(Thin \rightarrow Lysed)/dt = -d\,(Lysed)/dt - d(Germ \rightarrow Lysed)/dt \quad (6)$$

According to equations 5 and 6, $d(Thin \rightarrow Germ)/dt$ and $d(Thin \rightarrow Lysed)/dt$ can be estimated from values of $d(Germ)/dt$,

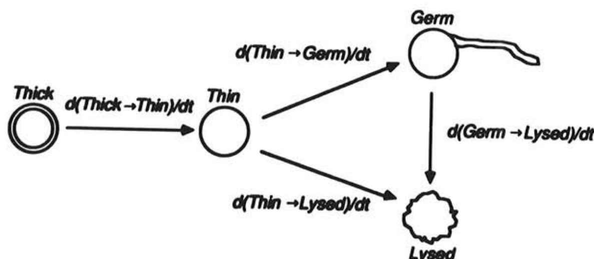

Fig. 1. Diagram of the compartment model used to evaluate relative rates of germination and lysis of oospores of *Pythium ultimum*. The differentials represent the rates of conversion between oospore states (e.g. $d(Thick \rightarrow Thin)dt$ is the rate at which thick-walled oospores convert to thin-walled oospores).

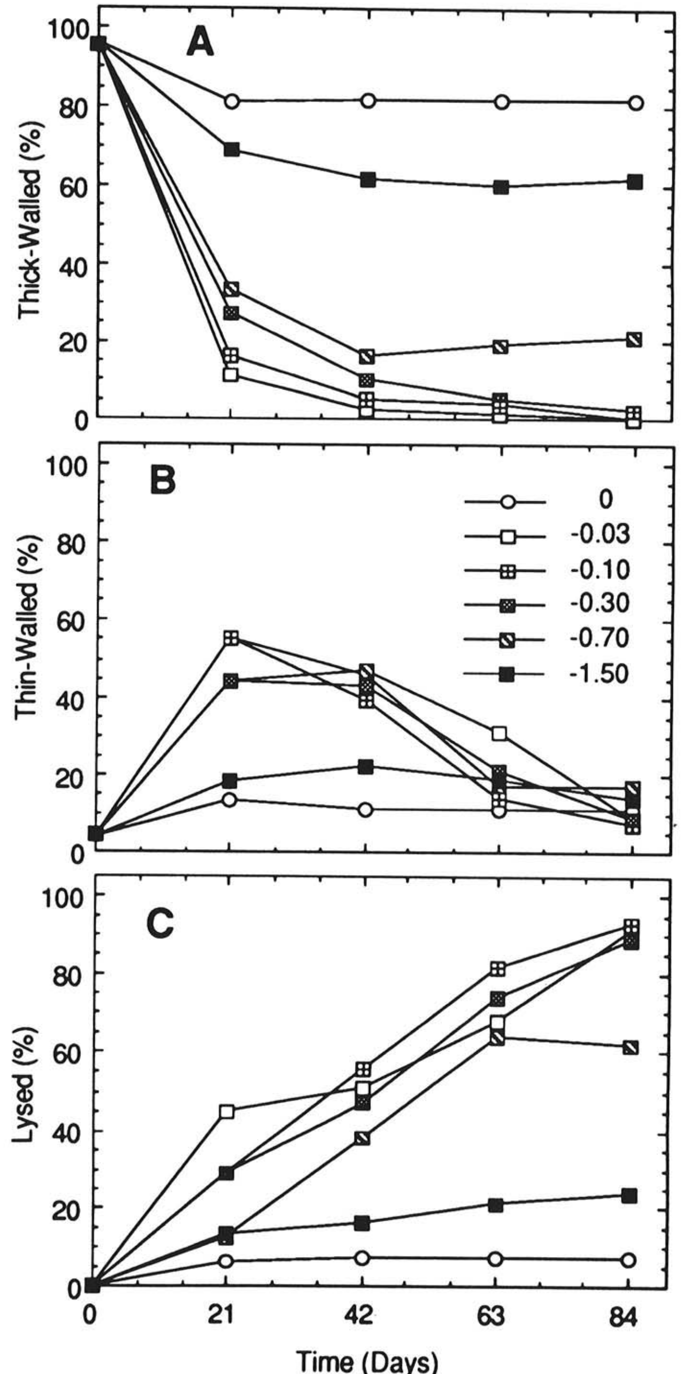

Fig. 2. Effect of soil moisture tension (0 to −1.5 MPa) on changes in morphology of oospores of *Pythium ultimum* in water agar films on glass slides buried in soil. **A**, Thick-walled oospores. **B**, Thin-walled oospores. **C**, Lysed oospores. Values are means of two experiments.

$d(Lysed/dt$, and $d(Germ \rightarrow Lysed)dt$. Values of $d(Germ)/dt$ and $d(Lysed)/dt$ were estimated as the rates of change in Germ and Lysed during each period between assays. Values of $d(Germ \rightarrow Lysed)/dt$ were estimated from the propylene oxide experiment. This was done by fitting an exponential equation to the data for the observed number of germinated oospores, and then using one of the fitted regression variables to estimate $d(Germ \rightarrow Lysed)/dt$. The equation fitted to the treated spore data was:

$$Visible = K_1 * \exp(k_2 * Time) \quad (7)$$

where *Visible* is the percentage of oospores with visible germ tubes, and *Time* is days of incubation. Differentiating equation 7 with respect to *Visible* gives:

$$d(Visible)/dt = k_2 * Visible \quad (8)$$

In order to calculate $d(Germ \rightarrow Lysed)/dt$, it was assumed that the rate of disintegration and disappearance of germ tubes in the oospore conversion experiments was similar to the rate of disappearance of germ tubes killed by exposure to propylene oxide. Equation 7 was fitted to data from the propylene oxide experiment by nonlinear regression. Initial parameter estimates used in the nonlinear regression were derived from linear regression with the linearized form of equation 7 ($\ln Visible = \ln [k_1] + [k_2 * Time]$). The percentage of germinated oospores for each period between assays was calculated as the average of the observed percentages at the two assays, and this value was multiplied by k_2 to give an estimate of $d(Germ \rightarrow Lysed)/dt$. All experiments were repeated.

RESULTS

Effects of moisture tension on changes in oospore morphology. At the beginning of the experiment, 96% of the oospores were thick-walled, and 4% were thin-walled (Fig. 2). During incubation in soil near saturation (0 MPa), oospore wall conversion was low. At this moisture level, all of the significant changes ($P < 0.05$) in wall thickness and lysis that occurred did so during the first 21 days. In soil with moisture tension near the permanent wilting point (−1.5 MPa), 62% of the oospores remained thick-walled during the 84 days, and all of the significant changes ($P < 0.05$) in wall thickness and lysis occurred during the first 42 days. At soil moisture levels more suitable for plant growth (−0.03 to −0.3 MPa), almost all of the thick-walled oospores converted, and 91% lysed during 84 days of incubation. Rates of increase in lysed oospores were relatively constant during incubation, e.g., as means of the −0.03, −0.1, and −0.3 MPa moisture tensions, 34, 51, 75, and 91% of the oospores were lysed at 21, 42, 63, and 84 days of incubation, respectively.

Occasionally, evidence of germination of oospores was observed. In soil with moisture tensions of −0.03, −0.10, and −0.30 MPa, an average of 0.04, 0.04, 0.02, and 0.11% of the oospores had attached germ tubes at 21, 42, 63, and 84 days of incubation, respectively. Oospores with germ tubes were not observed on slides taken from soil with moisture tensions of 0, −0.7, or −1.5 MPa.

To obtain more precise data on lysis and germination, oospores on slides were incubated in pots of soil with fluctuating soil moisture for 120 days, and data on oospore morphology were taken at 7-day intervals. Changes in morphology (Fig. 3) were similar to those that occurred at moisture levels of −0.03 to −0.3 MPa (Fig. 2). A total of five oospores with attached germ tubes were observed during incubation: one spore each on days 7, 14, 49, 63, and 91.

Disintegration and decrease in visibility in soil of germ tubes previously treated with propylene oxide. After 4.5 hr in CMA films on glass slides, 52% of the oospores had attached germ tubes. Length of germ tubes varied to up to 10 times the oospore diameter. Mortality of germinated and ungerminated oospores was 100% after treatment with propylene oxide as determined on a *Pythium*-selective medium. During the 16 days of incubation in soil, quantities of dead thick-walled or dead thin-walled ungerminated oospores did not change significantly ($P = 0.05$). Germ tubes began to disintegrate, and 19% became invisible within 24 hr. Numbers of oospores with attached visible germ tubes decreased progressively during the 16 days of incubation. Decreased visibility over time fit well with equation 7 ($Visible = k_1 * \exp [k_2\ Time]$) with a value of $k_2 = -0.2193 \pm SE = 0.0138$ (Fig. 4).

Rates of oospore germination and lysis. To evaluate the significance of the difference between rates of germination and lysis, values of $d(Thin \rightarrow Germ)/dt$ and $d(Thin \rightarrow Lysed)/dt$ were calculated for each of 17 observation periods during the moist soil experiment lasting 126 days (Fig. 3). The average calculated rate of germination of thin-walled oospores ($d[Thin \rightarrow Germ]/dt$) was −0.0172 oospores per day, and the average rate of lysis ($d[Thin \rightarrow Lysed\ /dt]$) was −0.717 oospores per day. Wilcoxon's signed rank test (16) indicated a significant difference ($P < 0.01$) between the two rates. Multiplication of the calculated mean values of $d(Thin \rightarrow Germ)/dt$ and $d(Thin \rightarrow Lysed)/dt$ by the duration of the moist soil experiment indicated that 88.9% of

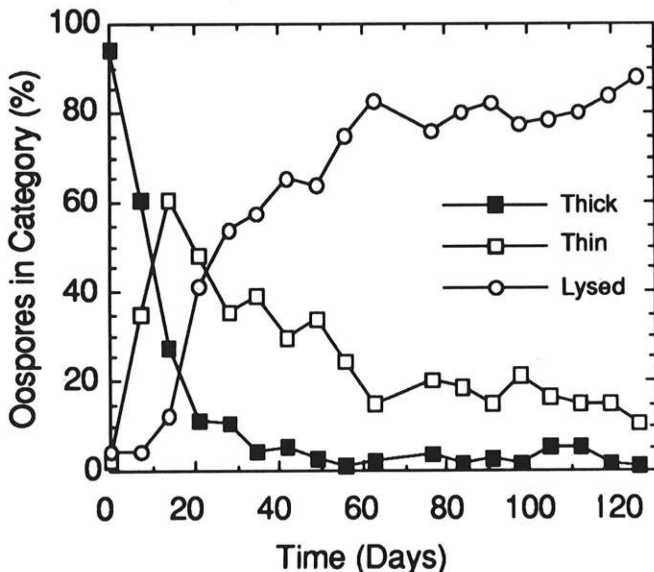

Fig. 3. Changes in morphology of oospores of *Pythium ultimum* in soil with fluctuating moisture tension. Oospores were in water agar films on glass slides buried in soil.

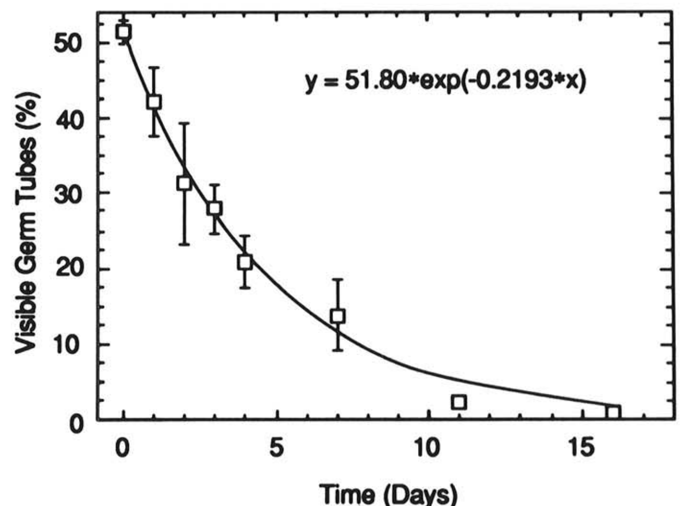

Fig. 4. Percentage of propylene oxide-fumigated oospores of *Pythium ultimum* (in agar films on glass slides) with visible germ tubes attached plotted against time of incubation in soil. Values are means of three experiments. Bars = ± standard error.

the oospores lysed directly, and 2.1% germinated before lysis. Similar calculations were also performed for each of the four observations for the combined −0.03, −0.10, and −0.30 MPa treatments in the controlled moisture experiment. At these moisture tensions, it was calculated that 90.2% lysed directly, whereas 0.8% germinated. To evaluate the sensitivity of the analysis to values of k_2 and the counted numbers of germinated oospores, the analysis of the moist soil experiment data was repeated with a range of values for k_2 and for observed *Germ*. We found that a k_2 value of −4.98 (22.7 times the −.2193 estimate) resulted in equivalent values of $d(Thin \rightarrow Germ)dt$ and $d(Thin \rightarrow Lysed)/dt$, and values greater than 0.9 resulted in no significant difference according to Wilcoxon's signed rank test. Similarly, counted *Germ* percentages had to be 22.0 times larger to result in equivalence of $d(Thin \rightarrow Germ)/dt$ and $d(Thin \rightarrow Lysed)/dt$ and 3.9 times larger to result in no significant difference.

DISCUSSION

The agar-film technique is a method for placing spores (or other biological propagules) in soil and then later retrieving these same spores for microscopic examination. The technique has been labeled by critics as too artificial, and results may not reflect a true picture of the fate of spores in soil. However, the technique has been shown to be useful for determining spore germination of a number of fungi and actinomycetes in soil (2); the effects of antibiotics in soil on germination of spores and morphology of developing hyphae (17); the effects of soil organic amendments on germination of spores and lysis of hyphae of *Helminthosporium sativum* (2); the effect of soil organic amendments on hatching of nematode eggs (8), germination of oospores of *P. ultimum*, and penetration of roots in the cotton rhizosphere (7); and the effects of pH, temperature, water potential (12), and soil chemical characteristics on conversion and lysis of oospores of *P. ultimum* (15). None of the results of these studies appeared to be influenced by the presence of agar films.

Conversion of oospores of *P. ultimum* from thick to thin walls is a process necessary for germination of the spores (6,12). In the laboratory, conversion is rapid under optimum conditions of available water, O_2, and light and in the absence of inhibiting concentrations of CO_2 (6). Conversion is slower in soil because of the reduced amount of light, lower O_2 availability, and increased concentration of CO_2. In our study, conversion was reduced significantly in soil with a matric potential of −1.5 MPa. Apparently, sufficient water was not available for maximum conversion at this moisture tension. Conversion was almost completely inhibited in soil near saturation, a condition in which O_2 availability is limited and CO_2 concentrations may be high. At this moisture level, about 15% of the oospores converted during the first 21 days and numbers of thin-walled oospores remained relatively constant thereafter and did not lyse. If lysis of oospores in soil is related to microbial activities, then anaerobic microorganisms apparently are not involved.

In a previous study, Lumsden and Ayers (12) reported a high rate of conversion of oospores of *P. ultimum* in soil at O bar moisture tension. Differences in methodology could account for the apparent differences in our results. After establishing a moisture curve for the soil used, we dried soil samples on pressure-plate membranes to the desired water tensions. These were placed in sealed mason jars that were aerated briefly at 7-day intervals. Loss of water was minimal. Lumsden and Ayers added water to samples of soil, let the samples "equilibrate" for 2 days, and then placed them in petri dishes. Lids were applied, but no mention was made of any special precautions to prevent moisture loss from the dishes. It is probable that soil in the dishes, originally at saturation, was closer to −0.03 MPa moisture tension at the end of their 6-wk period of incubation. Thus, their results could be similar and comparable to ours, in which high rates of conversion occurred in soils at −0.03 and −0.01 MPa moisture tensions.

Percentages of thin-walled oospores increased to about 45% (Fig. 2) after 6 wk in soils with water potentials of −0.03 to −0.70 MPa. These percentages were lower than the 80% found by Lumsden and Ayers (12) to be thin-walled after 6 wk in soil at −0.1 MPa. In another study with a "soil drop" technique for determining quantities of germinable propagules associated with soil organic debris, Lifshitz and Hancock (9) found that less than 4% of the spores were germinable (and presumably thin-walled) at any time during 17 wk in soil at −0.04 MPa. The authors, however, gave no quantitative data on thick-walled oospores remaining in the soil, or on nonviable thin-walled oospores. The difference in results in this study and ours is considerable and could be due to differences in methodology, strains of *P. ultimum* used, or soil characteristics.

In the laboratory, under optimum conditions and in the presence of available nutrients, conversion and germination of oospores of *P. ultimum* is rapid and, apparently, is a continuous process (6). In soil, where available nutrients are limited, thin-walled oospores accumulate and many lyse, but a few of the thin-walled spores apparently are resistant to lysis. In the present study, under optimum conditions of soil moisture for lysis (−0.03 to −0.3 MPa), 7–9% of the oospores were thin-walled after 84 days, and 89–93% had lysed. In a previous study (15), fewer oospores (20–75% in 84 days) lysed, perhaps because moisture conditions of the soils were not held constant. Fewer oospores may lyse in the field where moisture levels fluctuate from saturation to near dryness. Lysis appears to be related to soil biological activities. Qian and Johnson (15) showed that available soil P, pH, and C:N ratio individually were strongly correlated with lysis of oospores in agar films in soil. In stepwise regression analysis, additive effects of these soil variables were demonstrated. Amendments of $Ca(OH)_2$ necessary to bring an acid soil from pH 4.3 to 6.1 resulted in doubling the rate of lysis. Additions of $Ca(H_2PO_4)_2 \cdot H_2O$ did not raise the pH, yet lysis was increased considerably. In additional experiments (data not shown), oospores did not lyse significantly during 84 days of incubation in autoclaved soil.

The calculated rates at which thin-walled oospores germinated and lysed depended in part on the assumption that germ tubes killed with propylene oxide decayed and disappeared in soil at a rate similar to untreated germ tubes. Agnihotri and Vaartaja (1) reported that germ tubes from sporangia of *P. ultimum* lysed within 16 hr when germinated on natural soil. Lloyd and Lockwood (11) showed that hyphae of *Glomerella cingulata* and *Fusarium solani* f. sp. *phaseoli*, killed either by propylene oxide gas or by gamma radiation, lysed on soil at the same rate as living hyphae. Lysis, however, is just the first step in the decay process. It is possible that fumigation with propylene oxide induces chemical changes in the germ tubes that make them more resistant to decay. It is more likely, however, that living germ tubes decay at a slower rate, because fumigated germ tubes were older and were already lysed when placed in soil. If decay to invisibility is slower for germ tubes produced in soil than for those treated with propylene oxide and then placed in soil, then the values for rates of germination calculated in this study are too high, and, thus, a lower percentage of oospores actually germinate before lysis.

The significance of the low germination rate, and the high rate of lysis of oospores of *P. ultimum* on survival in nature is not clearly understood. Very high rates of lysis were found in this study in soil with constant moisture tensions. In experiments that were more comparable to field conditions with fluctuating moisture tensions, rates of lysis were not nearly as high (15). In one soil, only 20% of the oospores lysed after 84 days. In the present study, the calculated $d(Thin \rightarrow Lysed)/dt$ values tended to decrease with time through the entire experiment (Fig. 3). Through regression analysis, there was no significant ($P < 0.10$) correlation of numbers of thin-walled oospores with time during 63–84 days of incubation. Numbers of thin-walled oospores became essentially static. Our results, therefore, do not indicate that *P. ultimum* will not survive in soil, but only that the fungus may survive at low population densities. Field studies (3,13) have shown wide fluctuations in population densities in soil. High population densities were associated with high moisture (after

dryer periods where nutrients could be released by rewetting) (10) and with additions of organic matter (3). These population increases could be initiated by germination of sporangia, thin-walled oospores, or thick-walled oospores that converted and germinated rapidly (6). After decomposition of nutrients, it is likely that populations gradually decrease. Our results, where there was an absence of weed roots or falling leaves that could provide nutrients, could be comparable to field conditions where low population densities are found.

Our concepts of survival of oospores of *P. ultimum* are as follows: plant roots, hypocotyls, and organic residues in soil are invaded by hyphae, and oospores are produced within these tissues. Oospores probably remain thick-walled until the organic matter decomposes (although there is no data to confirm this directly). As organic matter decomposes, conditions favorable for conversion to thin-walled oospores become more prevalent, and the rate of conversion depends on pH of the substrate, available O_2 and light, and absence of inhibiting concentrations of CO_2. If nutrients are not present in quantities sufficient to induce germination, many of the spores will lyse. A few (perhaps 1–3%) may germinate under low nutrient conditions. A few of the thin-walled oospores may be resistant to lysis and survive for relatively longer periods.

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