Use of Fluorescent Antibodies to Study the Survival of *Phytophthora megasperma* and *P. cinnamomi* Zoospores in Soil

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ABSTRACT

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Zoospore cysts of *Phytophthora megasperma* and *P. cinnamomi* were added to nonsterile soil and its matric potential was adjusted to predetermined values. The infested soils were held under laboratory and field conditions and periodically sampled to determine cyst viability and soil water potential. During sampling, soil containing cysts was suspended in water and aliquots were placed on a selective agar medium to allow germination of viable cysts. After germination, the cysts were stained by an immunofluorescence detection technique and counted. Under laboratory conditions, zoospore cysts of *P. megasperma* survived no longer than 3 wk at water potentials ranging from 0 to −15 bars, and under field conditions they survived no longer than 2 wk. Zoospore cysts of *P. cinnamomi* survived no more than 3 wk in soil at water potentials of 0 to −1 bar under either field or laboratory conditions. However, at water potentials of −5 or −15 bars, zoospore cysts of *P. cinnamomi* survived up to 6 wk in the laboratory. In a nonirrigated field plot, in which soil water potentials ranged from −7 to less than −100 bars, cysts of *P. cinnamomi* survived for 8–10 wk. Under more controlled humidity conditions, zoospore cysts of *P. cinnamomi* proved relatively tolerant of desiccation; many survived for 1 wk in soils that dried to water potentials as low as −75 bars. Compared to *P. cinnamomi*, zoospore cysts of *P. megasperma* were much more sensitive to desiccation; most were killed within 1 wk in soil dried to water potentials of −20 bars or less.

Motile zoospores of plant pathogenic *Phytophthora* spp. are important in dissemination and infection processes in both surface water (14,19), and in soil (4,5,6,9,16). However, zoospore movement through soil and subsequent infection of a host can be restricted by soil pore size, matric potential (ψ_m), and distance to host tissue (4,18). The inability of zoospores to move effectively through fine-textured soils may be due, in part, to a rapid encystment resulting from more frequent contact with soil particles (12). Conceivably, many of the zoospores released in soil, or which

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enter soil from surface water, may encyst without reaching a host plant. Encysted zoospores may survive in the soil to function as inoculum, but it is unclear from the literature just how long and under what conditions viability is maintained in field soils.

Hwang and Ko (8) reported that viable zoospores of *P. cinnamomi* could not be recovered from saturated or moist soils after 3 wk. Similarly, Mehrotra (17) found that zoospore cysts of *P. drechsleri* in a soil perfusion apparatus survived little more than 15 days in moist soil. Zoospore cysts of *P. megasperma* var. *sojae* appear to be relatively ephemeral, with infectivity disappearing from infested soils in less than 24 hr (17). In contrast to these reports, Turner (22) found that zoospores of *P. palmivora* retained infectivity for 6–18 mo when stored in soil maintained at 50% moisture-holding capacity. Infectivity was lost more quickly (10 to 32 days) in saturated or air-dried soils.

Most of the above studies utilized soil-baiting techniques in which the survival of zoospore cysts was evaluated by the successful infection of host tissues. Although such methods directly answer the question of infectivity of the surviving propagules, they do not provide quantitative information on the behavior of the zoospore cyst populations in soil. It is not known whether zoospore cysts were the surviving units, or if they germinated in the soil to form other structures which enhanced or reduced survival.

A notable departure from the soil-baiting method was used by McIntosh (15), who quantitatively measured the survival of *P. cactorum* zoospores in soil by dilution-plating techniques. He found that survival was favored in dry soils (−2 and −5 bars ψ_m) relative to wet soils (−0.1 and −0.2 bars ψ_m) and also confirmed that *P. cactorum* zoospores persisted only a few weeks in soil.

In each of the studies reported to date (8,15,17,22) zoospore survival was measured in soil maintained under controlled, laboratory conditions. In the present investigation, the survival potential of zoospore cysts in soil is examined under both field and laboratory conditions by quantitative methods of population assay. An immunofluorescent staining technique was developed to augment dilution-plate assays on a selective agar medium. The immunofluorescent stain aided the recognition of *Phytophthora* propagules among those of other fungi which also could germinate on the medium.

MATERIALS AND METHODS

An isolate of *Phytophthora megasperma* Drechs. pathogenic to alfalfa, and an A_1 isolate of *P. cinnamomi* Rands, originally isolated from camellia, were studied. Both isolates were obtained from S. M. Mircetich, USDA-SEA/AR, Department of Plant Pathology, University of California, Davis, and both formed abundant aerial mycelium when grown on pea-dextrose agar (11). To obtain sporangia, disks of aerial mycelium were cut from 7- to 10-day-old cultures with a 7-mm-diameter cork borer, and were placed in petri dishes containing nonsterile soil extract to a depth of 2–4 mm. The soil extract was prepared by suspending 20 g of air-dried Yolo clay loam in 1,000 ml of water for 3 days followed by centrifugation for 30 min at 4,500 *g* to obtain the supernatant. Mycelial disks formed numerous sporangia in the extract within 5–8 days at 22–24 C. To stimulate zoospore release, mycelial disks bearing sporangia were transferred to petri dishes containing distilled water and left at 16 C for 1 hr. The water was warmed to 22–24 C for 1 hr and large numbers of zoospores were released. Zoospores were separated from the mycelium and sporangia by filtration through four layers of cheesecloth. Motile zoospores were stimulated to encyst by vigorous agitation with a magnetic stirring bar for 15 min.

Soil preparation. A Yolo clay loam was collected from a field plot near Davis, CA. The soil was sieved (1.4 mm openings) and used in experiments within a few days after collection without sterilization or extensive drying. Sieved soil was packed lightly into plastic rings having an inside diameter of 13 mm and a height of 8 mm. The rings were open at the top and had a plastic screen support at the bottom. Rings containing soil were placed on a 5-mm layer of moist soil on tension plates (2) or ceramic pressure plates (Soil Moisture Equipment Co., Santa Barbara, CA 93105). The soil in each ring was infested with zoospore cysts by pipetting the desired volume of spore suspension into the soil. The soils were wetted to saturation and the ψ_m was adjusted to different levels with the tension and pressure plates. Soils were allowed to drain water under the selected tensions or pressures for 24 hr, which was sufficient for nearly complete equilibration of soil ψ_m. After equilibration, four plastic rings of soil from each ψ_m value were used to determine initial populations of viable zoospore cysts by the dilution plate and immunofluorescence methods described below. The remaining plastic rings of infested soil were placed under the conditions used to examine zoospore survival, and populations were periodically determined by the same methods. Zoospore survival under the various treatments was expressed as a percentage of the initial population, and the percentages were transformed by an arc sine function for statistical analysis according to Duncan's multiple range test at $P = 0.05$.

Immunofluorescence and dilution plant techniques. Counts of viable zoospore cysts were made by plating a dilute soil suspension on a selective agar medium (2) which stimulated cyst germination, and staining the germinated cysts with fluorescent antibodies. Antisera were prepared against both species of *Phytophthora* by using the methods described by Malajczuk et al (13), with only the following modifications in antigen preparation and injection into rabbits. Because *P. megasperma* did not grow well on the medium of Burrel et al (1), Hohl's (7) P-3 medium was substituted for growth of that species. Only the washed cell-wall fraction of mycelial extracts was used as antigen; the soluble fraction was discarded. At the end of the standard injection series (13), each rabbit was given two interperitoneal (rather than subcutaneous) booster injections at 30-day intervals (13). All fluorescent staining was done indirectly with a commercially prepared fluorescence-labeled, goat antirabbit gamma globulin (Antibodies Inc., P.O. Box 442, Davis, CA 95616).

The success of the immunofluorescence technique required germination, staining, and examination of spores on very thin agar films so that UV transmission and antibody diffusion through the agar would occur at acceptable levels. Films of selective agar medium were prepared by layering 15 × 25-mm pieces of 10-μm mesh Nitex® (Tetco Inc., Elmsford, NY 10523) nylon screen onto the surface of solidified medium in petri dishes. A small quantity of melted medium was painted over the surface of the nylon mesh with a brush and, when solidified, it formed a continuum through the mesh to the medium in the dish. After preparation, the dishes containing the agar films were stored at 4 C until needed.

When samples were collected from the various treatments to determine the number of viable cysts, the soil from each plastic ring was suspended in 5 ml of distilled water, thoroughly mixed, and 50 μl was pipetted onto the surface of a single agar film. These spore suspensions were incubated on the films of selective medium for 18 hr at 22–24 C to allow germination of viable spores. After incubation, the surface of the agar films was gently rinsed with water to remove excess soil. Germinated cysts were not affected by washing, because they were securely attached to the agar by their germ tubes. After washing, the agar films were lifted from the surface of the medium by means of the nylon mesh which served to support them during handling for the staining procedure. The films were immersed for 45 min at 22–24 C in a 1/16 dilution of whole rabbit serum containing *Phytophthora*-specific antibodies. Films subsequently were immersed twice in 0.1 M phosphate-buffered saline for 15 min, once in a 1/64 dilution (0.31 mg protein/ml) of fluoresence-labeled goat antirabbit gamma globulin for 45 min at 22–24 C, and then twice more in phosphate-buffered saline for 15 min. Finally, they were laid agar surface downward on clean 22 × 40-mm glass coverslips and allowed to air dry for 2 hr, after which the mesh screens could be removed with forceps. The dried agar films adhered to the glass coverslips, which were then inverted and mounted with water on glass microscope slides. The agar films were observed through a Leitz microscope fitted with a mercury vapor lamp, BG12 and BG38 excitation filters, a K530 barrier filter, and a dark-field condenser. The total number of germinated, fluorescent spores in each 50 μl spot was counted.

Zoospore survival. Zoospore survival under laboratory conditions was evaluated in soils adjusted to ψ_m values of 0, −0.3, −1, −5, and −15 bars. The infested soil samples in plastic rings were removed from the tension and pressure plates and, along with additional soil equilibrated to the same ψ_m values, were placed in 400-ml jars. The additional soil at the same ψ_m was packed around the samples in the jars to buffer the infested soil against changes in moisture, and the jars were sealed with rubber stoppers. The jars were stored at 22–24 C and periodically samples were removed and examined for viable cysts. Five sample rings were removed from each treatment at the various assay intervals. Four were used in assays for viable cysts using the methods described above, and one was placed in a thermocouple psychrometer (3) to measure water potential (Ψ).

To study survival under field conditions, soil samples and additional volumes of soil were adjusted to ψ_m values of −0.3 and −5 bars. Five samples were packed in soil in each of several plastic cylinders (2.5 cm in diameter × 10 cm long) which were perforated by numerous holes to facilitate vapor exchange between the samples and the field soil in which they were buried. The cylinders were buried at depths of 10 or 40 cm during May 1977, in the same field plots from which the soil originally had been collected. Samples initially adjusted to −0.3 bars ψ_m were buried in a plot that was kept wet with frequent irrigations, as well as a plot that was allowed to dry slowly after receiving a single irrigation at the time of sample placement. A safflower crop was grown in both of these plots throughout the course of the experiment. The remaining samples, initially adjusted to −5 bars ψ_m, were buried in a fallow plot which received no irrigation. A single cylinder was removed from each of the treatments at various intervals for determinations of cyst viability, and the five sample rings in each cylinder were handled as described above. Soil temperatures in the field plots ranged 20–28 C during the experiment.

RESULTS

As found by Malajczuk et al (13), antisera prepared for individual *Phytophthora* spp. were not species specific. The antiserum produced against *P. megasperma* reacted equally well with *P. cinnamomi* and vice versa. While the lack of specificity simplified the staining process by allowing use of a single antiserum (*P. megasperma*) in assays for both species, it raised concern over the potential for nonspecific binding with other fungi. However, the levels of nonspecific binding and autofluorescence, as determined with soil samples that were not artificially infested with zoospore cysts, were invariably low. Individual germinating zoospore cysts of *Phytophthora* were readily distinguished from germinating propagules of other fungi on selective agar medium by the immunofluorescence technique (Fig. 1), and the only structures growing on the agar films, identified as *Phytophthora* by the specific stain, were germinated zoospore cysts.

The Ψ values of the soil samples which were stored in sealed jars remained fairly constant throughout the experiment (± 10% of initial Ψ). When stored in sealed jars, all the zoospore cysts of both species in saturated soil ($\psi_m = 0$) died within 1 wk (Fig. 2). Cysts of *P. megasperma* died within 2–4 wk in all remaining treatments (Fig. 2A), as did those of *P. cinnamomi* maintained at −0.3 or −1.0 bar ψ_m (Fig. 2B). However, a considerable percentage of *P. cinnamomi* cysts survived for 4 wk in soil at ψ_m values of −5 and −15 bars, although none survived for 6 wk (Fig. 2B).

Storage in sealed jars reduced water loss from the soil, but the lack of ventilation resulted in a 60- to 100-fold increase in the CO_2 levels (as determined by gas chromatography) in the soil after 2–4 wk. Because it was not known whether CO_2 influenced cyst viability either directly or indirectly, an additional experiment was done in which jars of soil were closed with polyethylene bags rather than rubber stoppers. Even though this method of closing the jars allowed some escape of CO_2 (internal concentration = 1.5 to 2.5 times atmospheric levels) and the samples in the jars gradually dried (25 to 50% lower Ψ after 4 wk), the results obtained were virtually identical to those of the previous experiment (Fig. 2).

In the field, zoospore cysts of *P. megasperma* lost viability within 1–2 wk in all treatments, but the loss of viability was most rapid in the nonirrigated plots (Fig. 3A). Cysts of *P. cinnamomi* also died within 2–4 wk in the irrigated field plots, but 55–70% of the initial population was still viable after 4 wk in the nonirrigated plot (Fig. 3B) where they survived for 8–10 wk. The survival of *P. cinnamomi* cysts at depths of 10 and 40 cm in the nonirrigated plot was very similar even though the soil Ψ values differed at the two depths. The samples buried at 40 cm remained between −7 and −10 bars Ψ, whereas those buried at 10 cm dried to Ψ values less than −100 bars (Fig. 3C). The results obtained in the nonirrigated plot (Fig. 3) suggested that the zoospore cysts of *P.*

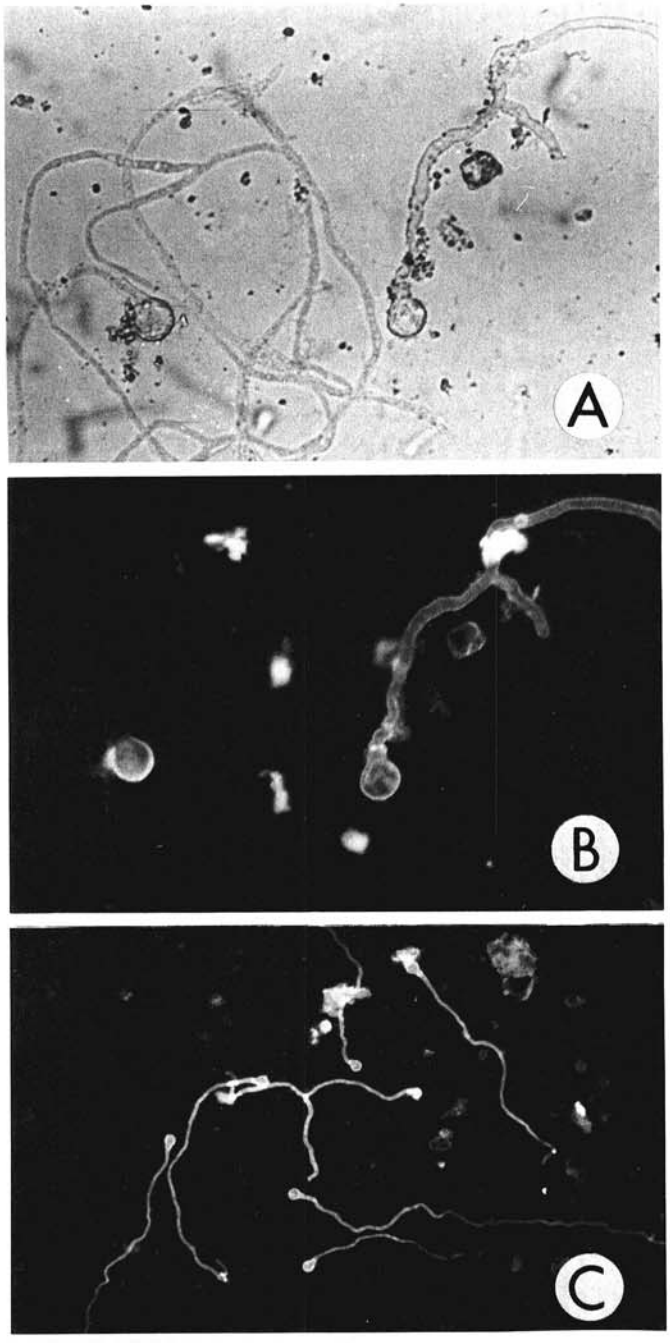

Fig. 1. Immunofluorescent stains of germinating zoospore cysts of *Phytophthora cinnamomi*. **A)** Germinating propagules in a soil assay at ×450 magnification illuminated with white light. **B)** The same propagules when illuminated with UV light showing the fluorescent cyst and germ tube of *P. cinnamomi* on the right. The spore on the left was not *P. cinnamomi* and exhibited a reddish autofluorescence which was readily distinguished from the bright green fluorescence of the specific antibody stain. **C)** Germinated zoospore cysts at ×100 magnification showing ease of recognition in soil assays.

cinnamomi, unlike those of *P. megasperma*, can tolerate considerable desiccation in the soil.

The influence of low Ψ values on the viability of zoospore cysts was examined further by inoculating soil in plastic rings with cysts and drying the soil to −5 bars ψ_m on the pressure plate for 24 hr. The rings containing infested soil then were suspended for 1 wk in sealed humidity chambers over NaCl solutions with solute potentials (ψ_s) ranging from −5 to −100 bars. The humidity chambers were equipped with capillary tubes for adequate gas exchange and were immersed in a large water bath to moderate changes in laboratory temperature. The actual Ψ values to which infested soils had finally dried were measured in thermocouple psychrometers (3) before the numbers of viable cysts were determined. Zoospore cysts of *P. cinnamomi* tolerated drying to Ψ values as low as −43 bars (Fig. 4), and 50% tolerated drying to −75 bars Ψ. However, most *P. megasperma* cysts were killed by drying soil to −21 bars Ψ (Fig. 4).

DISCUSSION

The immunofluorescent staining technique proved to be a simple and reliable method for identifying the germinating zoospore cysts of *Phytophthora* species on selective agar medium. There are few precedents in the literature for the successful use of immunofluorescent methods in studies of fungi in natural soil. One difficulty has

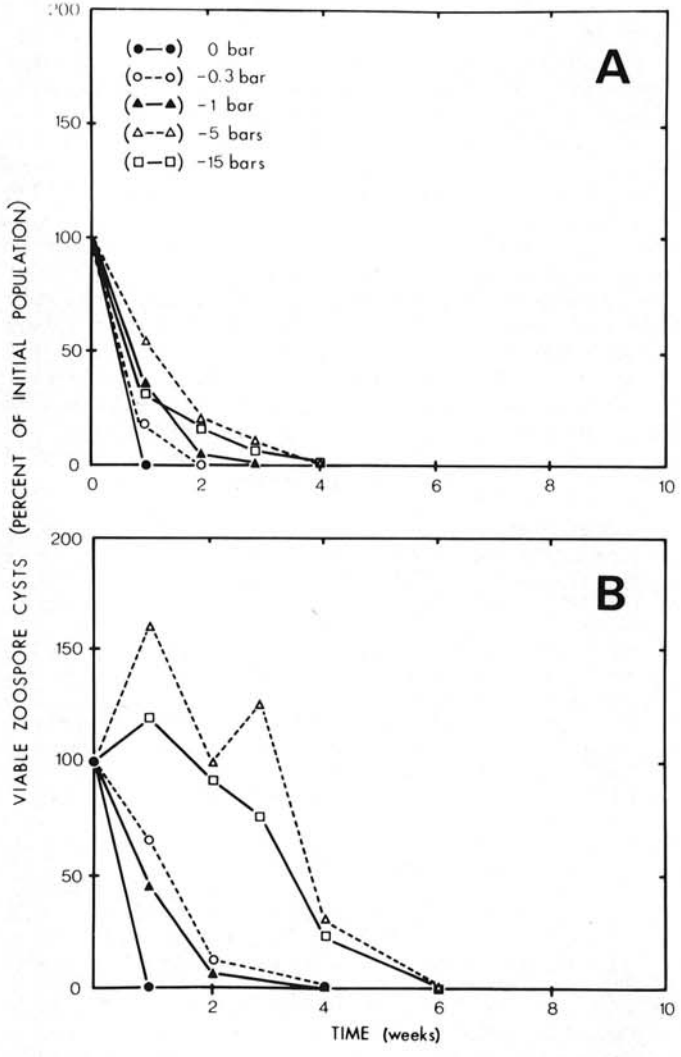

Fig. 2. Survival of zoospore cysts of **A)** *Phytophthora megasperma* and **B)** *P. cinnamomi* in field soil maintained at constant levels of moisture in the laboratory. Initial populations were determined after adjusting soil matric potentials to the values indicated, and subsequent populations of viable cysts are expressed as percentages of the initial population (LSD = 10.5 and 15.7% at $P = 0.05$ for *P. megasperma* and *P. cinnamomi*, respectively).

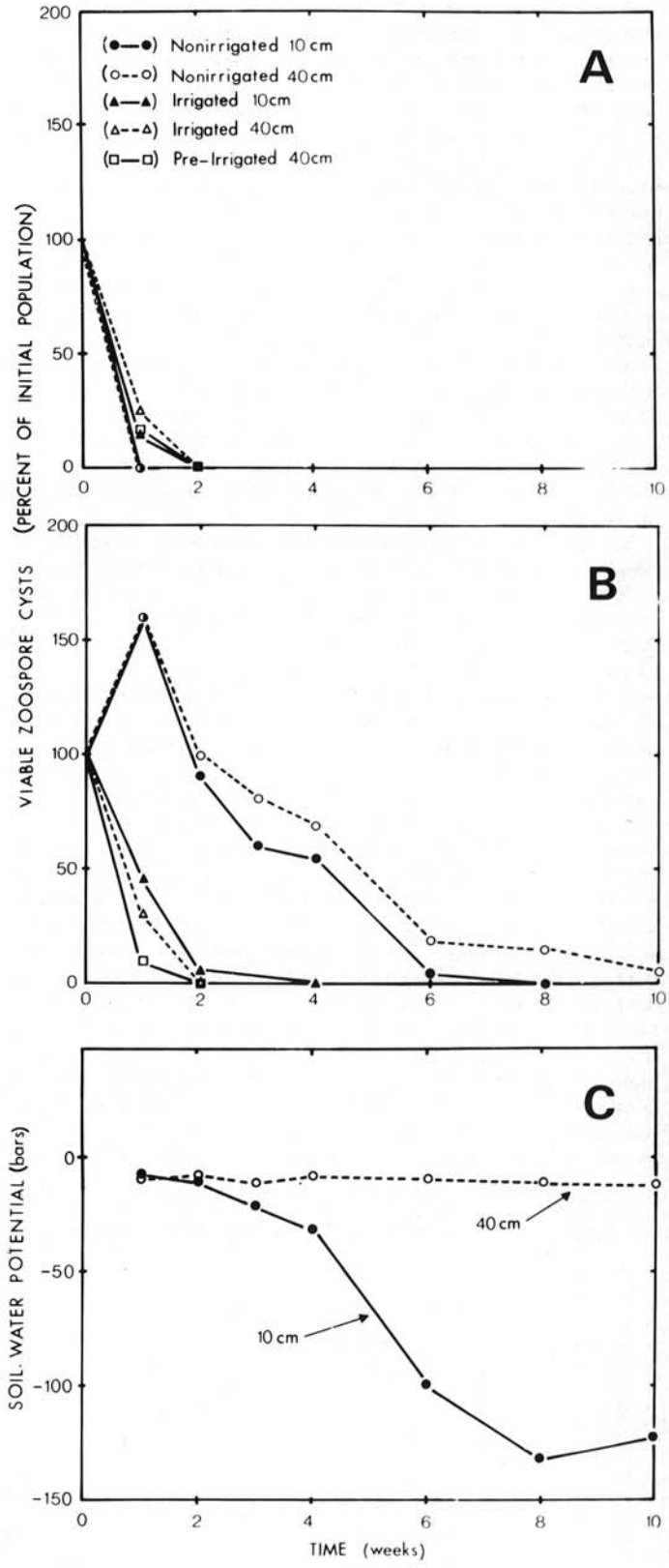

Fig. 3. Survival of zoospore cysts of **A)** *Phytophthora megasperma* and **B)** *P. cinnamomi* under field conditions. Soil containing zoospores was buried at depths of 10 and 40 cm and irrigated as indicated. Samples buried in irrigated and preirrigated plots were initially adjusted to −0.3 bar matric potential (ψ_m), and those in nonirrigated plots to −5 bars ψ_m. Initial zoospore populations were determined after adjusting the ψ_m of the samples, and subsequent populations of viable cysts are expressed as percentages of the initial population (LSD = 11.1 and 15.6% at $P = 0.05$ for *P. megasperma* and *P. cinnamomi*, respectively). **C)** Water potentials of soil samples from nonirrigated treatments infested with zoospore cysts of *P. cinnamomi*.

been background fluorescence caused by the adsorption of antibodies onto soil particles (13). This was not a significant problem in the present study because excess soil was washed from the surface of agar films prior to antibody application. Spores that germinated remained securely attached to the washed agar films by their germ tubes. Another difficulty with immunofluorescence methods for detection of specific fungi in natural soil is the presence of other fungi which may autofluoresce or bind fluorescent antibodies. This can greatly limit the usefulness of immunofluorescent methods in routine assays of natural soil populations, but in studies such as this one in which soils were artificially infested with large numbers of propagules, noninfested soil can serve as a control to determine the relative amount of nonspecific fluorescence. Furthermore, use of a selective medium reduced the numbers of other fungi which germinated in the soil assays. When combined with such techniques, immunofluorescent stains can be used effectively in soil assays for specific fungi. In long-term survival studies, they may prove more suitable than vital fluorescence techniques (21) because the propagules are not treated with chemical markers until after removal from the soil and germination on media.

By examining the selective medium so soon after germination, it always was possible to distinguish the propagules of *Phytophthora* which had given rise to germ tubes. On the basis of these observations, it is evident that zoospore cysts can survive in their original form in soil and do not need to convert to other structures to maintain viability. Tsao (20) reported that zoospore cysts are able to germinate in soil and form mycelium or microsporangia. These structures, however, were not observed by the methods used here and if formed, evidently did not contribute significantly to survival.

The reason for the increases in *P. cinnamomi* populations that sometimes occurred during the first 1–3 wk in the drier soil treatments is unknown. The increase was statistically significant in two experiments (Fig. 2 and 3), but was not statistically significant in a third experiment (Fig. 4). No increase over initial populations was ever observed with *P. megasperma*, or with *P. cinnamomi* at ψ_m values higher than −5 bars. Increases in zoospore populations have not been reported in previous soil-baiting studies (8,17,22), but were observed by McIntosh (15) who also used quantitative methods to assay populations of *P. cactorum* zoospores. However, the soils in which he observed population increases were maintained at −0.5 bars ψ_m and contained host tissues. Under such conditions, there may have been sporangial formation on colonized tissues. When increases in zoospore populations were observed in the present study (Fig. 2,3), host tissues were not present in the soil and the dry soil conditions would not have permitted sporangium formation or zoospore release (3,11). Rather than an actual population increase in these samples, we believe that there may be an unrecognized error in determining the initial populations of samples treated in the pressure plate apparatus. Although studies showed that the pressures used did not adversely affect zoospores, there might be a transient interaction between the pressure and the assay method used to determine initial populations.

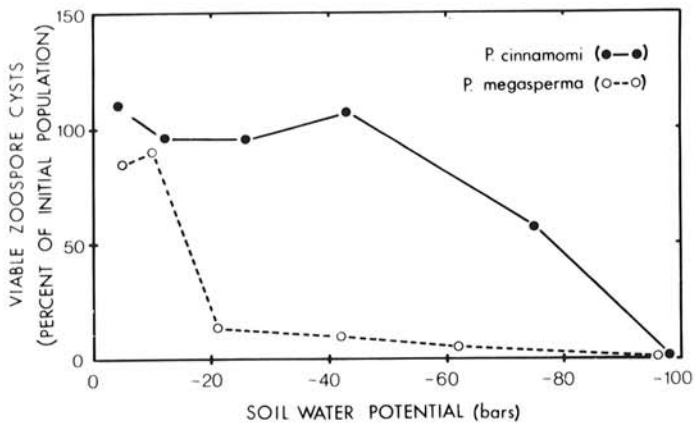

Fig. 4. Viability of *Phytophthora megasperma* and *P. cinnamomi* zoospore cysts plotted as a function of the total water potential to which they were dried in soil. All samples were initially adjusted to −5 bars matric potential before initial zoospore populations were determined. Samples then were placed in humidity chambers over NaCl solutions for 1 wk after which the total water potentials and zoospore populations were determined. Populations of viable zoospore cysts are expressed as percentages of the initial populations (LSD = 12.7% at $P = 0.05$).

Conditions of high soil moisture, especially saturation, do not favor long-term survival by cysts of either *P. megasperma* or *P. cinnamomi* (Fig. 2,3) Others (8,15,17,22) reported similar results with other species and the short survival has been attributed to germination and subsequent lysis of cysts under high soil moisture conditions (15). However, drier soil conditions did not greatly enhance the survival of *P. megasperma* cysts. In fact, zoospore cysts of *P. megasperma* appeared to be only slightly tolerant of desiccation in the soil (Fig. 4), as most cysts of this species lost viability at soil Ψ values near the permanent wilting percentage of the soil (10). In contrast to *P. megasperma*, zoospore cysts of *P. cinnamomi* survived much better in dry soil. Furthermore, they appeared to be relatively tolerant of desiccation in soil (Fig. 4), being able to survive, at least for the short term, water stresses more severe than those tolerated by most agronomic plants (10). The results obtained here with *P. cinnamomi*, however, may not represent the upper limit of zoospore tolerance to desiccation since Turner (22) reported that some soils infested with zoospores of *P. palmivora* retained infectivity for 10–21 days after air drying.

The survival of zoospore cysts in soil could be an important factor in the epidemiology of diseases caused by *Phytophthora* spp. Zoospores that encyst before reaching a susceptible host may survive to greatly increase the inoculum density in soil. If the soil is drained to water contents less than saturation the cysts of several *Phytophthora* spp. may survive for days or weeks (8,15,17, and Fig. 2,3). Under favorable circumstances, the cysts of *P. cinnamomi* may survive over 2 mo in soil (Fig. 3), and those of *P. palmivora* for over 6 mo (22). If roots of a host passed close enough to the cysts during this time, the cysts presumably could germinate and infect directly or, under suitable conditions, form germ sporangia and release zoospores (20). The data reported here indicate that zoospore cysts of *Phytophthora* species differ in abilities to persist in soil, or tolerate conditions of low soil Ψ, and that cysts of some species may be important survival structures in field soil.

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