Temperature and Water Stress Effects on Sporangium Viability and Zoospore Discharge in Phytophthora cryptogea and P. megasperma

J. D. MacDonald and J. M. Duniway

Assistant and Associate Professor, respectively, Department of Plant Pathology, University of California, Davis, CA 95616.

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ABSTRACT

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Mycelial disks from agar plate cultures of *Phytophthora cryptogea* and *P. megasperma* were incubated in soil at −150 millibars (mb) matric potential (ψ_m) on tension plates where they formed abundant sporangia in 2-4 days. When sporangia in soil were exposed to saturation ($\psi_m = 0$) conditions for various lengths of time ranging from 10-60 min to initiate zoospore release, and then dried again to −150 mb ψ_m, zoospore release was stopped short of completion. Such interruptions of the discharge process, which lasted 4 or 24 hr, did not affect sporangia adversely; when wetted again to saturation, they completed the release of zoospores and yielded a cumulative release similar to sporangia which were exposed to noninterrupted periods at saturation. Mycelial disks bearing sporangia also were removed from soil at −150 mb ψ_m and dried to various extents by vapor exchange with salt solutions. After drying to −40 bars water potential (ψ), many sporangia of *P. cryptogea* were still capable of releasing zoospores when placed in water and of germinating directly when plated on a selective agar medium. However, after drying to −70 bars ψ or less, most sporangia were no longer capable of either direct or indirect germination. Sporangia of *P. megasperma* appeared to be more sensitive to drying and most were killed by drying to −50 bars ψ or less. Sporangia of both species were capable of releasing zoospores in water over a wide range of temperatures, with the upper limits for release being 30 and 33 C for *P. megasperma* and *P. cryptogea*, respectively. Sporangia of *P. cryptogea* underwent a steady decline in capacity for indirect germination on prolonged exposure to temperatures of 33 or 36 C.

Additional key words: water potential, Phytophthora root rot.

Soilborne *Phytophthora* spp. have rather precise water requirements for sporangium formation in soil. Some species apparently require saturated soil for significant sporangium production (6, 14, 18), whereas other species or isolates require slightly dryer soils, at matric potentials (ψ_m) between −0.025 and −0.3 bar, for optimum sporangium production (3, 4, 11, 13, 15, 16). The lower ψ_m limit for sporangium formation by all species examined appears to be −3 to −5 bars (6, 15, 16). Once formed, the sporangia of *P. megasperma* and *P. cryptogea* have a very precise ψ_m requirement for zoospore release in soil, needing almost complete saturation ($\psi_m \geq -0.001$ bar) for optimum discharge (12). These narrow ranges of soil water status favoring sporangium formation and zoospore discharge suggest that sporangia may be sensitive to, and greatly affected by, water stresses in the soil. However, despite the epidemiological significance attached to zoospores (8), there is little precise data describing the effects of soil water on the survival of *Phytophthora* sporangia, and their subsequent ability to release zoospores.

The importance of moisture to the epidemiology of aerial members of the Peronosporales has long been recognized. However, even in the well-studied example of *Phytophthora infestans*, the influence of ambient humidity on sporangium viability is by no means clear, and experiments on survival can be influenced by the presence or absence of water films around the sporangia (19). Considerably less information is available concerning the effects of desiccation on sporangia of soilborne members of the Peronosporales. Sporangia of *Pythium ultimum*, which always germinate directly, can survive for 7 mo in air-dried soil (17) whereas those of *Phytophthora cactorum* appear much less tolerant of desiccation. Sporangia of *P. cactorum* can remain viable for 1-3 wk in moist soil (6, 13, 16), but they are quickly killed if the soil is allowed to air-dry (16). Meyer and Schönbeck (13) found that sporangia of *P. cactorum* were killed in soil when the soil was dried from 25% to 10% moisture holding capacity. However, the precise levels of soil moisture which are lethal to *Phytophthora* sporangia, as well as the effects of sublethal stresses on their capacity for direct or indirect germination have not been characterized adequately.

In addition to desiccation, the viability of sporangia may be adversely affected by cycles in water status. Cohen et al (2) found that when sporangia of aerial members of the Peronosporales were wetted for intervals too short to allow zoospore release and then dried to low humidities, their viability on subsequent wetting, as measured by zoospore release and plant infection, was greatly reduced relative to sporangia that were not wetted

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before drying. Sporangia subjected to intervals of drying underwent cytological and physiological alterations which Cohen et al (2) attributed to the abrupt stoppage of zoosporogenesis. Other aerial plant pathogens not in the Peronosporales appeared to be much less affected by similar moisture cycles (7). Although sporangia in a soil environment would never dry as rapidly, or to the same extent after wetting, as those in an aerial environment, zoospore discharge in soil is extremely sensitive to small ψ_m deficits (12). Conceivably, a well-drained soil could drain from saturation to a ψ_m value limiting to zoospore release before the discharge process was completed. However, the effects of such cycles of soil moisture on zoospore discharge by sporangia in soil are unknown. It has not been determined whether the small ψ_m tensions which normally limit zoospore release (12) could effectively stop the discharge process once initiated, or result in an impairment of the discharge process during subsequent wet periods, similar to the impairment observed by Cohen et al (2) for aerial members of the Peronosporales.

The present study examines the effects of cycles of soil moisture on the indirect germination of sporangia of two *Phytophthora* spp. and the extent to which the sporangia can tolerate desiccation in soil. Some effects of temperature on the indirect germination of sporangia also were investigated.

MATERIALS AND METHODS

Two species of *Phytophthora* were used in these studies: an isolate of *P. cryptogea* Pethyb. and Laff. pathogenic to safflower (5), and an isolate of *P. megasperma* Drechs. pathogenic to alfalfa (12). Both isolates formed abundant aerial mycelium when cultured in plates containing pea-dextrose agar (12). Disks of aerial mycelium were cut from 7- to 10-day-old cultures with a 7-mm-diameter cork borer and were buried 2-4 mm deep in soil on Büchner funnel tension plates (3, 5). The soil used in these experiments was a coarse sand fraction ($>$ 250 μm) of Yolo fine sandy loam obtained by wet-sieving autoclaved soil. The tension plates were set to maintain the soil at -150 millibars (mb, 1,000 mb = 1 bar) ψ_m where abundant sporangia formed on the disks in 2-4 days. After their formation, sporangia on mycelial disks were exposed to various experimental conditions, and their subsequent ability to release zoospores was evaluated by the methods described below. Unless stated otherwise, temperatures were maintained constant at 22-24 C.

Zoospore release during cycles of soil moisture.—In experiments on the effects of soil moisture cycles on zoospore release, sporangia were formed on mycelial disks which were buried individually in soil-filled rings of plastic pipe having an inside diameter of 13 mm and a height of 8 mm. The rings stood on tension plates and were open at the top with a nylon mesh support (2-mm openings) at the bottom. They served to confine the zoospores in a small volume of soil after their release and thus maximized their recovery from soil. The numbers of zoospores released under various experimental conditions were estimated by suspending the entire contents of each plastic ring in 10 ml of distilled water. The resulting soil suspension was thoroughly mixed, and 20-μliter drops were pipetted onto the surface of a selective agar medium (3). The plates were incubated at 22-24 C for 18 hr, after which the germinated zoospore cysts were stained with acid fuchsin and counted at $\times$100 magnification (12).

After sporangia had formed at -150 mb ψ_m, soil moisture cycles were initiated by adjusting tension plates to $\psi_m = 0$ and wetting the soil surface with distilled water to immediately bring it to saturation. This provided optimum conditions for zoospore release (12) and sporangia were held under these conditions for various lengths of time ranging from 10-60 min. At the end of these initial periods at $\psi_m = 0$, assays were made to determine numbers of zoospores released and the tension plates were reset to drain the soil again to -150 mb ψ_m, a ψ_m value too dry for zoospore discharge (12). After 4 hr at -150 mb ψ_m, an assay for zoospores was made again to determine if additional release had occurred at the lower ψ_m value. Finally, the tension plates and soil were returned to saturation conditions for 4 hr to allow complete release (12) and a final assay was made to determine the numbers of zoospores released.

Desiccation tolerance of sporangia.—To determine the levels of water stress that reduce sporangium viability, mycelial disks bearing sporangia were gently removed from soil held at -150 mb ψ_m and shaken free of excess soil. Seven disks were suspended 15-20 mm above distilled water or NaCl solutions with solute potentials (ψ_s) ranging from -10 to -80 bars in each of several closed humidity chambers. Additional disks bearing sporangia were held in soil on tension plates at constant $\psi_m = -20$ mb, the wettest ψ_m value that prevents zoospore discharge (12). After 48 hr in the humidity chambers or at $\psi_m = -20$ mb, the total water potential (ψ) of four disks from each treatment was determined in a thermocouple psychrometer (4). The remaining three disks from each treatment were placed individually in 10 ml of distilled water and the numbers of zoospores discharged in water were determined by plating on selective medium after 2-, 4-, and 6-hr intervals.

After measuring their ψ value, the four mycelial disks from each treatment were removed from the psychrometer. Two disks were comminuted in 10 ml of distilled water in a blender for 60 sec at low speed, pelleted in a clinical centrifuge, and resuspended in 5 ml of distilled water. Several 20-μliter aliquots of the resulting suspension were pipetted onto the surface of a selective agar medium to determine the ability of sporangia to germinate directly. The remaining two disks from the psychrometer were used to count total numbers of sporangia present without regard to their viability, by methods described previously (3).

Effect of tempeature on zoospore release.—Mycelial disks bearing sporangia were removed from soil held at -150 mb ψ_m and 22-24 C, and were individually placed in 10 ml of distilled water to stimulate zoospore release. The water had equilibrated to various temperatures ranging from 6-36 C before immersion of the disks, and unless stated otherwise, was maintained at the same constant temperatures throughout the experiment. Numbers of zoospores released were determined by pipetting 20-μliter aliquots onto selective agar medium after 2-, 4-, and 6-hr intervals in water.

RESULTS

Zoospore release during cycles in soil moisture.—Both species responded similarly to cycles of soil moisture between zero and −150 mb ψ_m, and only the results for *P. cryptogea* are presented (Fig. 1 and 2). When sporangia which had formed at −150 mb were exposed to saturated soil conditions ($\psi_m = 0$) for periods of 10 to 20 min, there was no zoospore release during either the initial 10- or 20-min period at saturation, or the following 4-hr period when the tension plates were adjusted to return the soil to −150 mb ψ_m (Fig. 1). All discharge that was observed occurred during the final 4-hr period at $\psi_m = 0$, and resembled discharge from sporangia which were exposed to only a single, noninterrupted 4-hr period at saturation (Fig. 1). When the initial period of saturation to which sporangia were exposed was extended to 30, 40, or 50 min, there was again no zoospore release during the initial period of saturation ($\psi_m = 0$), but some release did occur during the 4-hr interval after the tension plates were adjusted to return the soil to −150 mb ψ_m (Fig. 1). The relatively small amount of release that occurred during this drier period probably occurred during the 20-30 min required for the soil to drain from saturation to a ψ_m limiting for zoospore release (12). It was evident, however, that drying the soil from saturation to −150 mb ψ_m stopped zoospore release short of completion, because it did not continue until the final 4-hr saturation period. If soils were saturated initially for 60 min, some discharge occurred during that initial 60 min, but it also was stopped short of completion by the intervening 4-hr period at −150 mb ψ_m. Again, discharge was not completed until the final 4-hr period at saturation. The final cumulative numbers of zoospores released during all sequences of soil moisture tested (Fig. 1) were not significantly different from the numbers released by sporangia exposed to a single 4-hr period at saturation.

Additional experiments were done in which sporangia were initially exposed to saturated soil conditions ($\psi_m = 0$) for 30 or 60 min, followed by either 4- or 24-hr intervals at −150 mb ψ_m before the final 4-hr period at saturation. A comparison of the cumulative numbers of zoospores released under these conditions with the numbers released from sporangia exposed to a continuous period at saturation (Fig. 2), showed that interruptions of the discharge process lasting 4 or 24 hr did not significantly affect the capacity of sporangia to release zoospores.

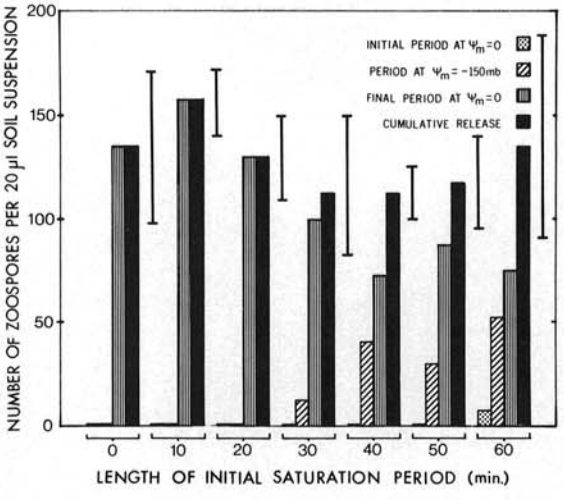

Fig. 1. Influence of cycles in soil matric potential (ψ_m) on zoospore release by sporangia of *Phytophthora cryptogea*. Sporangia were exposed to initial periods at saturation ($\psi_m = 0$) of 10 to 60 min followed by 4 hr at −150 mb ψ_m, and a final 4-hr period at saturation. Bars are grouped on the horizontal axis by the length of the initial period at saturation. Bar heights represent the increments of zoospore release during each sequential treatment (obtained by difference) and the cumulative numbers of zoospores released during all treatments (final count). Vertical lines show total variation in cumulative numbers of zoospores.

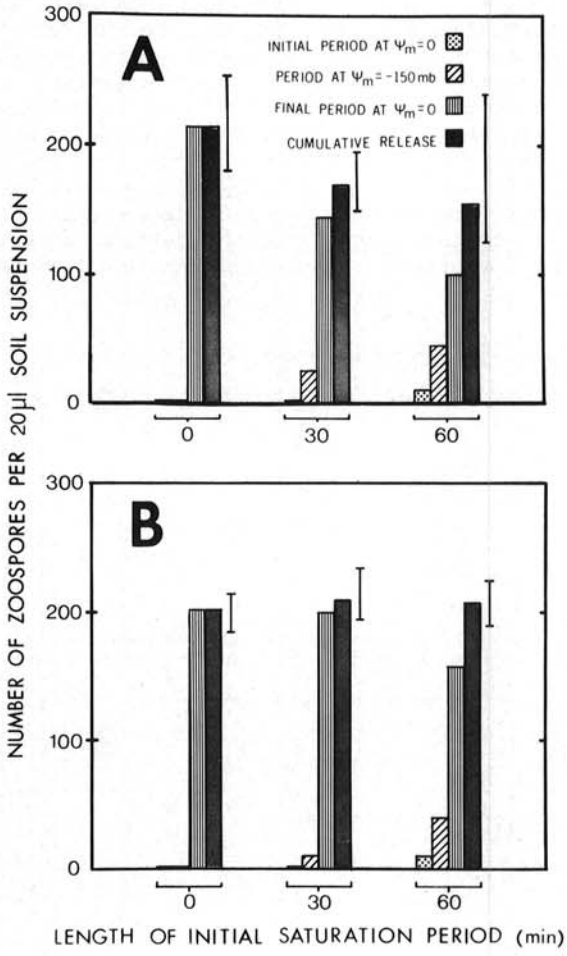

Fig. 2-(A, B). Influence of cycles in soil matric potential (ψ_m) on zoospore release by sporangia of *Phytophthora cryptogea*. Sporangia were exposed to initial periods at saturation ($\psi_m = 0$) for 0, 30, or 60 min and then dried to −150 mb ψ_m for **A)** 4 hr, or **B)** 24 hr before a final 4-hr at saturation. Bars are grouped on the horizontal axis by the length of the initial period at saturation. Bar heights represent the increments of zoospore release during each sequential treatment (obtained by difference) and the cumulative numbers of zoospores released during all treatments (final count). Vertical lines show total variation in cumulative numbers of zoospores.

Furthermore, results similar to those in Fig. 2 were obtained when the soil was dried to −1 bar ψ_m on a pressure plate apparatus for 24 hr between saturation intervals.

Desiccation tolerance of sporangia.—After being dried to −40 bars ψ, many sporangia of *P. cryptogea* were still capable of releasing zoospores when placed in water (Fig. 3-A). Also, it was observed that drying sporangia of *P. cryptogea* to between −3 and −8 bars ψ consistently stimulated a more vigorous release of zoospores, as measured by numbers released (Fig. 3-A) and the rate at which discharge occurred (data not shown). The decline in the numbers of zoospores released upon the drying of sporangia to progressively lower ψ values was associated with a decline in the percentage of sporangia capable of direct germination (Fig. 3-A). Apparently all sporangia were killed when dried to ψ values of approximately −85 bars and light microscope observation of these sporangia revealed (Fig. 4) that the cytoplasm had become coarsely granular in appearance and had shrunken away from the sporangium wall. The sporangia of *P. megasperma*, on the other hand, appeared somewhat more sensitive to drying than those of *P. crytpogea*. Apparently all the sporangia of this species were killed by drying to a ψ value of −52 bars (Fig. 3-B), and a slight drying of sporangia never stimulated a more vigorous zoospore release. The sporangia of both species behaved in the manner shown in Fig. 3 when dried in humidity chambers either free from excess soil or while still buried in soil. Furthermore, sporangium counts showed that there were no significant differences in the total numbers of sporangia between treatments and differences in germination were probably due only to the effects of desiccation.

Effect of temperature on zoospore release.—Although both species were capable of releasing some zoospores in distilled water over a wide range of temperatures, there were distinct temperature optima for release by both species (Fig. 5). Observations at 2-, 4-, and 6-hr intervals showed that release proceeded much more slowly at 9 and 12 C (the lowest temperatures that permitted release) than at more optimum temperatures. Zoospore discharge decreased abruptly to zero when the temperature increased from 27 to 30 C for *P. megasperma* and from 30 to 33 C for *P. cryptogea*.

Additional experiments with *P. cryptogea* at 33 and 36 C showed that as long as sporangia were held in water at these temperatures they did not release zoospores, but after removal to lower temperatures, some zoospore release then occurred. However, prolonged exposure to such high temperatures resulted in a steady decline in the subsequent ability of sporangia to release zoospores (Fig. 6), so that sporangia exposed to 36 C for 24 hr were no

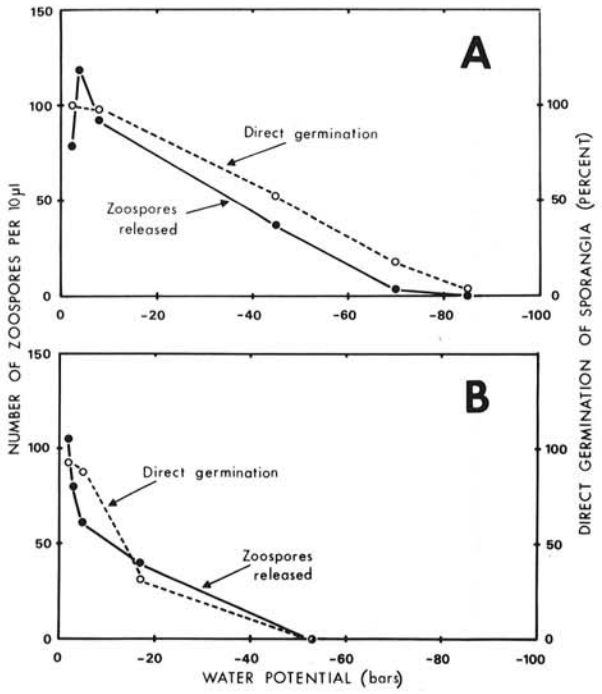

Fig. 3-(A, B). Zoospore release and direct germination by sporangia of **A)** *Phytophthora cryptogea* and **B)** *P. megasperma* after drying to various water potentials. Sporangia were formed on mycelial disks in soil (ψ_m =150 mb) and then dried during 48 hr in humidity chambers to the water potentials shown before placement in distilled water or on selective agar medium to evaluate zoospore release and direct germination, respectively.

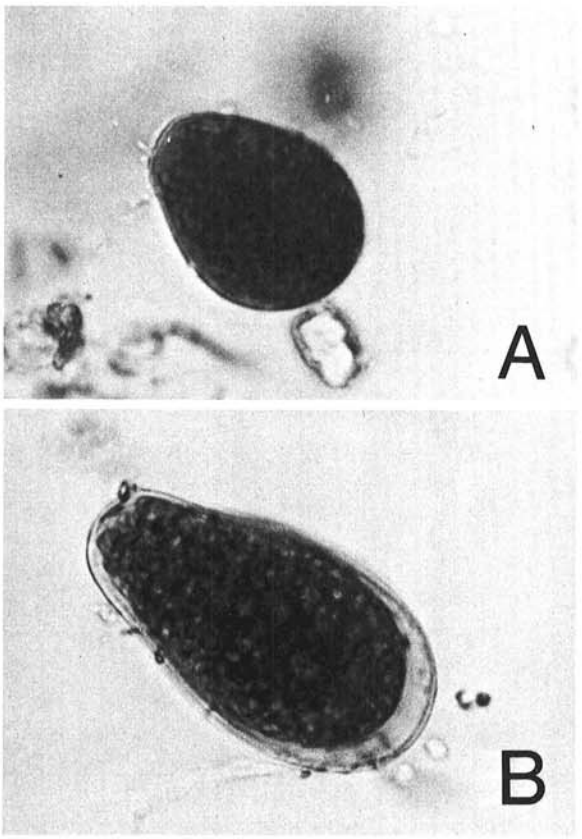

Fig. 4-(A, B). Sporangia of *Phytophthora cryptogea* (×830) stained with acid fuchsin and mounted in distilled water. **A)** Viable sporangium from a mycelial disk with a water potential > −10 bars. **B)** Nonviable sporangium from a mycelial disk dried to a water potential < −70 bars.

longer able to release zoospores when moved to more favorable temperatures. Microscopic examination of sporangia treated in this manner showed that the cytoplasm had become coarsely granular in appearance, similar to that of water-stressed sporangia (Fig. 4), but without shrinkage of the cytoplasm away from the sporangium wall.

DISCUSSION

Under the conditions used in the present study, a population of sporangia required 60 min of optimum ψ_m conditions ($\psi_m = 0$) before any zoospore release occurred (Fig. 1), and 4 hr of optimum conditions are needed for completion of release (12). It was shown previously (12) that zoospore release by *P. cryptogea* and *P. megasperma* is extremely sensitive to matric forces in the soil, being severely restricted at −10 mb ψ_m and fully prevented at −25 mb ψ_m. Such high ψ_m values represent near-saturation conditions in soil and, following rain or irrigation, a well-drained soil might reach these ψ_m values fairly rapidly. Therefore, it is conceivable that a well-drained soil could reach a ψ_m value limiting to zoospore release before a population of sporangia could complete the discharge process. Our results (Fig. 1 and 2) clearly show that if a saturated soil drains to a ψ_m value limiting for zoospore discharge, zoospore discharge by a population of sporangia can be arrested short of completion. However, in terms of the cumulative numbers of zoospores released during successive periods at saturation, zoospore discharge from the sporangia of *P. cryptogea* (Fig. 1 and 2) and *P. megasperma* (data not shown) was not adversely affected by interruptions lasting as long as 24 hr.

The ability of sporangia of *Phytophthora* spp. to remain unaffected by cycles of soil ψ_m between values favorable and limiting for zoospore release may be important in the epidemiology of diseases caused by these fungi, by assuring a persistent and continual supply of sporangia capable of releasing zoospores. Furthermore, if events in the discharge process are not reversed during periods of low soil ψ_m, then the cumulative effects of short periods of saturation could be as important in zoospore release as prolonged periods of saturation. However, in addition to zoospore release, the movement of zoospores in soil may also be affected by the length of the saturation interval (5).

It is assumed that throughout the first 60-min interval, while the soils were sufficiently wet for zoospore discharge, sporangia were undergoing the initial steps of zoosporogenesis (1, 20). Stopping zoospore discharge by draining soil at the frequent intervals used in these experiments (Fig. 1) probably interrupted zoosporogenesis at many intermediate steps. However, there is no evidence to indicate what happens to a given sporangium when zoospore release is interrupted by decreases in soil ψ_m. Whether the events in zoosporogenesis stop in place, proceed to some specific intermediate point and stop, or are actually reversed and subsequently restarted when conditions again become favorable is not known. It is evident, however, that exposure to limiting ψ_m conditions at all stages of the zoospore discharge process has no permanent injurious effects on sporangia like those observed by Cohen et al (2).

Even though sporangia of both *P. cryptogea* and *P. megasperma* may be more sensitive to desiccation than sporangia of aerial members of the Peronosporales (2, 19), nevertheless they appear capable of surviving the levels of dryness that are commonly encountered in cultivated soils. For example, half the sporangia of *P. cryptogea* survived drying to −40 bars ψ and still were capable of releasing zoospores when wetted, or germinating directly when placed on agar (Fig. 3). This is

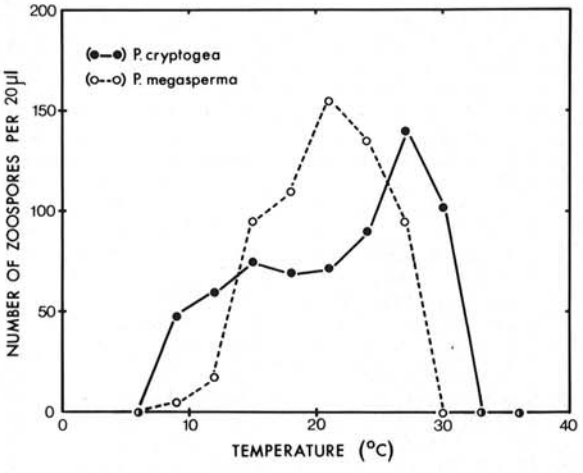

Fig. 5. Influence of temperature on zoospore release by sporangia of *Phytophthora cryptogea* and *P. megasperma*. Numbers of zoospores released were determined 6 hr after sporangia, which had formed in soil at −150 mb matric potential and 22-24 C, were placed in distilled water equilibrated to the indicated temperatures.

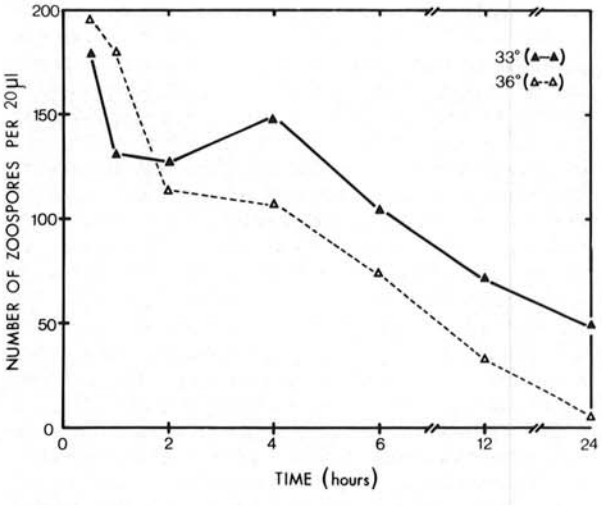

Fig. 6. Influence of the length of time which sporangia of *Phytophthora cryptogea* were held in water at 33 and 36 C on their subsequent ability to release zoospores. Sporangia, which had formed in soil at −150 mb matric potential and 22-24 C, were placed in water equilibrated to 33 or 36 C for the indicated lengths of time and then cooled to 24 C for 6 hr, after which zoospore release was determined.

well below the range of soil ψ suitable for plant growth (10), and suggests that significant numbers of sporangia might be capable of surviving, at least for a short time, in soil drier than that tolerated by cultivated plants. Sporangia of *P. megasperma* appeared somewhat more sensitive to drying than those of *P. cryptogea* (Fig. 3), although apparently they too can tolerate greater stresses than those tolerated by most crop plants. Though previous studies on persistence of sporangia in soil did not involve as wide a range of precise moisture levels as this one, the results do suggest that sporangia of *P. cactorum* (13) and *P. infestans* (21) also may survive drying to extents common in all but the topmost layers of cultivated soils. The stimulation of zoospore released by *P. cryptogea* caused by slight drying (−3 to −8 bars) of sporangia (Fig. 3-A) could be important in the epidemiology of this pathogen if it results in an unusually prompt and abundant release of zoospores when soil becomes saturated.

Portions of the data presented (Fig. 3) may actually under-represent the tolerance of sporangia to moderate desiccation. When mycelial disks bearing sporangia were incubated in humidity chambers over NaCl solutions at $\psi_s \leqslant -40$ bars, their ψ nearly equilibrated with the solutions within 48 hr. The ψ of disks incubated over solutions of higher ψ_s on the other hand, did not fully equilibrate after 48 or even 72 hr in the humidity chambers. However, microscopic observation of disks removed from soil showed that sporangia formed on the surface and periphery of the disks, and in those locations, they may have been exposed to drier conditions in the humidity chambers than ψ measurements of whole disks would suggest. Because it was not possible to measure ψ of the sporangia themselves, the measured ψ values may in fact underestimate the extent to which sporangia were actually dried.

Viability of sporangia after desiccation was determined by examining their ability to germinate both directly and indirectly (Fig. 3). This was done because reports in the literature (1, 9, 13, 22) suggest that these two modes of germination may be affected differentially by environmental stresses. Katsura (9) reported that sporangia of *P. capsici* were capable of direct germination in sucrose and glucose solutions with ψ_s values too low (ie, −12 to −15 bars) to permit zoospore release. Also, there are reports that specific concentrations of oxygen may favor one mode of germination over another (22), and that as sporangia age (1), or dry (13), they may lose the ability to release zoospores but still remain capable of direct germination. However, our experiments indicate that the reduced ability of sporangia to release zoospores following desiccation is not the result of a differential impairment of the discharge mechanism because they showed a similar reduction in direct germination (Fig. 3). In fact, sporangia dried to <-70 bars ψ appeared to have been severely injured by such treatment. The appearance of these sporangia with their shrunken cytoplasm (Fig. 4) is similar to those of *P. infestans* exposed for several minutes to 90% RH (about −150 bars ψ at 25 C) (19). Warren and Colhoun (19) were unsuccessful in attempts to induce germination of sporangia dried to such an extent and considered them to be dead.

It was shown previously (12) that zoospore release will not occur in soil unless a very high ψ_m (> -25 mb) is provided. If this requirement is not satisfied, zoospore release will not occur at any temperature, or respond to shifts in temperature (12). However, temperature can also exert a limiting influence on zoospore release. At high or low temperature extremes, zoospore release did not occur even though the ψ_m requirement was satisfied (Fig. 5). Furthermore, prolonged exposure to high temperatures had a deleterious effect on the capacity of *P. cryptogea* sporangia for subsequent zoospore discharge (Fig. 6).

The precise water requirements which have been demonstrated for sporangium formation (3, 4, 6, 11, 13, 14, 15, 16, 18) and zoospore release (12) by *Phytophthora* spp. in soil may give the impression that sporangia are sensitive to environmental stresses in the soil. However, our experiments demonstrate that sporangia can withstand significant interruptions of the discharge process with no apparent injury, they can withstand soil water stresses far in excess of those tolerated by most crop plants and retain the ability to release zoospores or germinate directly, and they are capable of releasing zoospores over a wide range of temperatures. In fact, sporangia appear more tolerant of environmental stresses than the previous literature suggests, and this ability to function under a variety of soil conditions attests to the significance of sporangia in the epidemiology of diseases caused by this important group of pathogens.

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