Mechanisms of Survival of Zoospores of Phytophthora parasitica in Irrigation Water

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ABSTRACT

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Zoospores of *Phytophthora parasitica* were present in water placed on field soils from foot-rot infested citrus groves within 10 minutes, and citrus leaves in the water were infected within the same time. Zoospores retained motility in irrigation water up to 20 hours at 20 C but at higher temperatures the period of motility was shorter. Zoospores encysted rapidly after the addition of nutrients, orange peel, or citrus leaves. Zoospore cysts germinated in sterile distilled water (SDW) and sterile and nonsterile irrigation waste water. When growth ceased, the protoplasm contracted within the hyphae. Empty cysts or hyphae often lysed, but intact hyphal fragments remained viable for 40 days at 25 C in untreated waste water and resumed growth after the addition of supplemental nutrients. At concentrations of 10-1,000 mg glucose/liter of SDW plus 100 mg $CaCl_2$ or in the presence of orange peel or citrus leaves, hyphal tips in contact with container surfaces often produced appressoriumlike structures which usually produced microsporangia when nutrients were replaced with autoclaved or nonsterile irrigation waste water. Microsporangia could persist in nonsterile irrigation waste water at 24 C for 60 days, but most germinated sooner, releasing only one zoospore. Since mycelial inoculum from these zoospores was pathogenic to roots of citrus seedlings, zoospores of *P. parasitica* or structures produced by them probably play a significant role as survival or dispersal units in recycled water.

Additional key words: citrus, control.

Foot rot of citrus, which is common wherever citrus is grown, is caused by several species of *Phytophthora*, including *P. parasitica* Dast. This fungus usually infects small feeder roots and under certain conditions can invade buttress roots, girdle trunks, and kill trees. The disease is a limiting factor for production of citrus in Arizona, because some 20% of the 68,000 acres of citrus are affected (Allen, *unpublished*).

Another factor important to citriculture in Arizona, as well as other arid areas, is the shortage and expense of irrigation water. To offset this deficiency many growers collect excess irrigation water runoff from fields in large ponds or sumps. This water subsequently is recycled into the same groves. Since propagules of *Phytophthora* can be present in irrigation water (11, 12, 13, 17, 18, 25), use of recycled water for citrus may increase the incidence and severity of foot rot.

Activities of zoospores in soils and soil extracts have been described in several studies. Although zoospores of *P. parasitica* were observed swimming in soil extracts, encystment usually occurred within 12 hours (19). Zan (26) suggested that zoospores of *P. infestans* probably survive in soil as germ tubes rather than as ungerminated spores. Appressoriumlike structures (21), microsporangia (5, 19, 20, 21), and chlamydospores (5) also are produced by germinating zoospores. Zoospores seemed to survive longer in relatively dry soil (2 bars soil water suction) than in wet soil (0.1 bar soil water suction) (14).

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Zoospores of *Phytophthora* spp. have been recovered from recycled irrigation water in Arizona (17, 18). Although the motility of zoospores of some *Phytophthora* spp. has been studied with respect to temperature (3, 9, 10), pH, concentrations of carbohydrates, and physical contact (2, 3, 6, 7), their effect on the dissemination and survival of zoospores in irrigation water has not been described.

We present information on the survival of propagules of *P. parasitica* in irrigation water with special emphasis on zoospores.

MATERIALS AND METHODS

An isolate of *Phytophthora parasitica* recovered from irrigation drainage water near Phoenix, Arizona, was maintained on V-8 agar (19) and transferred monthly. A selective antibiotic medium, $P_{10}VP$ (22) was used for isolation and other laboratory cultural procedures.

Zoospores were obtained from *Phytophthora* cultures grown on V-8 agar. After 2-weeks' growth, four pieces (2-cm square) of the colonized V-8 agar were transferred to petri plates containing approximately 30 ml sterile distilled water (SDW) at 25 C. Cultures were incubated for 2-3 days, chilled for 15 minutes at 0 C, and then warmed to room temperature; 15 minutes later zoospores were released. Sporangia and mycelia were eliminated by passing the suspensions through a standard metal sieve with a pore size of 37 μm (W. S. Tyler Co., Mentor, Ohio). Zoospore numbers were determined with a Spencer Bright-Line hemacytometer (A. O. Instrument Co., Buffalo, N. Y.); and concentrations were adjusted by

dilution with SDW. Specific procedures involving zoospores are presented with results below.

Irrigation waste water from several citrus orchards or recycling sumps near Phoenix, Arizona, was collected in sterile glass bottles, transported in an ice chest, and stored at 18 C. Samples ranged in pH from 7.2 to 8.6 and had microbial counts of 10^2-10^7 bacteria/ml and 10-10^4 viable propagules of fungi/ml. Samples of soil from the top 30 cm of empty irrigation ditches in citrus orchards with a high incidence of foot rot, were collected monthly in plastic bags and stored at 18 C.

A leaf-bait technique (8) was used in some instances to determine the presence of zoospores. Soils (5 g) to be tested were placed in a 125-ml beaker and SDW was added to provide approximately 2 cm of water above the soil surface. Pieces (3- to 5-mm square) of Rough lemon leaf (*Citrus jambhiri* Lush.) from upper branches of noninfected seedlings were floated on the irrigation water, removed at specific time intervals and floated on SDW (25 C). Controls consisted of leaf pieces floated on SDW only. Preliminary studies had indicated that chlamydospores, sporangia, cysts, and germlings sank and did not infect the leaf baits within a 10-hour period. However, when zoospores were used as inoculum, cysts were observed on the cut edge of leaf pieces within 10 minutes. Hyphae of zoospore germlings penetrated the floating leaf tissue and produced sporangia on the cut edges within 36 hours at 24 C.

A sieve-filter system was devised to determine which kinds of propagules of *Phytophthora* were present in water standing over infested soil. Sieves of monofilament nylon cloth (Tobler, Ernst, and Traber, Inc., New York) with pores of either 48, 31, or 10 μm were cut to fit the Sterifil Millipore filter apparatus (Millipore Corp., Bedford, Mass.). Millipore filters of 8-μm pore size were used for smaller pore size. Water samples were passed through sieves and filters of consecutively smaller pore sizes. Chlamydospores, sporangia, and other particles in the same size range were retained on the 48- or 31-μm sieves. Motile and encysted zoospores passed through all nylon sieves, but the 8-μm Millipore filter trapped these and other structures of similar size. Filters bearing zoospores were removed from the apparatus, inverted and placed on plates of $P_{10}VP$ medium for incubation. After 48 hours at 24 C, filters were removed and colonies were examined and counted.

TABLE 1. Percentages of citrus leaf pieces infected with *Phytophthora parasitica* after exposure to sterile distilled water in contact with untreated field soil at 24 C

Time interval	Leaves infected (%)[a]
0 minutes	0
5 minutes	0
10 minutes	35
15 minutes	51
30 minutes	76
1 hour	95
2 hours	97
3 hours	97
4 hours	98
5 hours	100

[a]Data are averages of 20 single-leaf replicates per time interval. Tests were repeated four times.

RESULTS

Propagules released into water from infested soil.—The leaf bait technique was used to determine when zoospores were present in water applied to infested soils from irrigation ditches. Twenty citrus leaf pieces were immediately floated on water after it was added to infested soil and, after removal and incubation, the numbers of infected pieces were noted at each time interval. The tests were repeated four times with each soil from four different orchards.

After incubation, 35% and 95% of the leaf baits, respectively, in contact with infested water for 10 minutes and 1 hour were infected with *Phytophthora* (Table 1). Leaf pieces exposed for a shorter period, those removed immediately and after 5 minutes of exposure, as well as those floated on SDW, were not infected with *Phytophthora*. Results were similar with soils from each of the four orchards tested. Leaf pieces washed for 1 minute in swiftly running tap water immediately following exposure to contaminated water and then floated on SDW were also infected at the same time intervals.

The sieve-filter system was used to process water standing over infested soil to determine when a propagule of a particular size was present. Twenty g of an infested soil from irrigation ditches (about 15% moisture) were placed in each of thirty 125-ml beakers and 50 ml of SDW were added to the soil. At 5-, 10-, 15-, 30-, and 60-minute intervals, 30 ml of water standing above the infested soils were removed with a wide-mouth pipette and passed successively through the sieve-filter series. Infested water from six beakers were sampled at each time interval. (Water from different beakers was sampled each time because the removal of 30 ml of water depleted the supply in the beaker). Sieves then were inverted on $P_{10}VP$ medium, and incubated for 48 hours at 24 C, after which counts were made of the *Phytophthora* colonies. These experiments were replicated six times with four different soil samples.

Phytophthora propagules were recovered only from the 8-μm filter. We believe these were zoospores because the 48- and 31-μm sieves in previous tests removed all propagules with the dimensions of culturally-produced sporangia and chlamydospores. Propagules could not be recovered from water sieved immediately following its addition to *Phytophthora*-infested soil. However, samples that had stood for 10 minutes contained an average of 33 propagules in 30 ml of water as shown by colony counts on agar. The number of recovered propagules increased with each time increment up to 107 propagules/30 ml at 60 minutes.

Motility and survival of zoospores.—In preliminary studies, we noted that infective propagules of *P. parasitica* were present in irrigation water that had remained 46 days above infested soil in the laboratory (Thomson and Allen, *unpublished*). Therefore, we studied the influence of irrigation water on the motility and survival of zoospores, and structures produced by them.

For motility studies, 20 ml of a suspension of 1×10^4 zoospores/ml of irrigation waste water were placed in plastic petri dishes (60×15 mm) and incubated at 10, 20, 25, 30, and 35 C. At specific times throughout a 20-hour

period the numbers of motile zoospores observed during 1-minute intervals per ×100 microscope field were counted. This experiment was performed three times with three replicates at each temperature.

Some zoospores were motile for 20 hours at 20 C but none was observed at 4 hours at temperatures of 25 C and above. The numbers of zoospores retaining motility declined rapidly at each temperature and less than 10% of the initial number were motile at the longest time intervals. For example, at 10 C only 5% of the zoospores were motile after 5 hours and none was motile after 10 hours.

For survival studies, zoospore suspensions of 1×10^4/ml irrigation water were incubated in the dark in covered petri dishes at 10, 25, and 35 C for 40 days. Survival was determined by removing and mixing 1 ml of a suspension with melted $P_{10}VP$ medium (45 C). Plates were incubated for 48 hours, then the numbers of *Phytophthora* spp. colonies were counted. For controls, duplicate suspensions of zoospores were incubated in SDW. This experiment was performed twice and included three replicates at each temperature.

Survival time of zoospores in irrigation water was shortened considerably at higher incubation temperatures. Five percent of the original number of colony-forming units (CFU) survived 40 days at 10 C, but only 2% survived 15 days and 15% survived 10 days at 25 and 35 C, respectively. However, 90%, 81%, and 95% of the CFU in SDW were viable after 40 days at 10, 25, and 35 C, respectively.

Structures produced by zoospores.—Twenty milliliters of the following liquids were placed in separate plastic petri plates: autoclaved or nonsterile sump water; SDW; SDW amended with glucose at 10, 100, 250, and 1,000 mg/liter; and asparagine at 250 mg/liter. Glucose and asparagine solutions were amended with $CaCl_2$ at 100 mg/liter since preliminary studies had shown that the percentage of zoospore germination was increased (Thomson, *unpublished*). Conversely, $CaCl_2$ without glucose did not induce a high percentage of zoospore germination. Sporangia still attached to mycelia were placed in each dish. Indirect germination by means of zoospores occurred within 5 minutes after sporangia were placed in any of the solutions.

We incubated the spore suspensions at 24 C for 36 hours, then removed the liquid, washed the plates thoroughly with running distilled water (all sporangia, mycelium, and any zoospores not adhering to the container were therefore removed), and added 20 ml of sterile or nonsterile irrigation water. Subsequently, cultures were incubated for an additional 60 days at 24 C. At appropriate intervals the plates were examined microscopically for the presence of structures produced by zoospores. This experiment was performed three times with three replicates of each suspending medium.

Zoospores were nonmotile within 0.5-2.0 hours in all of the glucose- and asparagine-amended solutions, but were still motile in the $CaCl_2$ solution, SDW, and autoclaved and nonsterile sump water after 10 hours (Table 2). Rapid encystment occurred in the higher glucose concentrations. For example, there were no motile zoospores in solutions containing 1,000 mg/liter glucose plus $CaCl_2$ after 0.5 hours, whereas zoospores still were motile after 2 hours in solutions containing 10 mg/liter glucose plus $CaCl_2$.

Germination of the encysted zoospores occurred within 2 hours after encystment and ranged from 30-75% germination in the nonamended solutions to 80-95% in glucose-amended water. Germ-tube growth ceased after about 12 hours in SDW and in sterile and nonsterile irrigation water; the protoplasm of the germlings subsequently contracted several micrometers into the

TABLE 2. Duration of motility and percentage germination of zoospores of *Phytophthora parasitica* incubation for 36 hours in liquid media at 24 C and the occurrence of germination structures after a subsequent 24-hour incubation in sterile irrigation water

Suspending media	Zoospores		Germination structures	
	Motility period (hours)	Germination (%)	Appressoriumlike structures[a]	Microsporangia[a] (%)
Glucose (mg/liter) + $CaCl_2$ (100 mg/liter)				
10	2[b]	80[c]	+	40
100	1.5	95	+	88
250	1	95	+	90
1,000	0.5	95	+	75
Asparagine 250 mg/liter + $CaCl_2$ (100 mg/liter)	1	96	+	87
Glucose 250 mg/liter	1	94	−	0
$CaCl_2$ 100 mg/liter	10	30	−	0
SDW[d]	10	70	−	1
Sump water (sterile)	10	67	−	1
Sump water	10	75	−	1
SDW + orange peel	0.25	98	+	175[e]
SDW + citrus leaf pieces	0.5	75	+	25

[a]Germlings produced appressoriumlike structures and microsporangia 24 hours after the initial suspending media was replaced with sterile irrigation water.

[b]All results are the averages of three replicates per treatment in tests repeated three times.

[c]Percentage of germinated zoospores per 100 observed.

[d]SDW = sterile distilled water.

[e]Some germlings produced more than one microsporangium.

hyphae. Empty cysts or germ tubes often lysed within 4 days, but intact propagules containing protoplasm survived more than 40 days at 24 C.

Germlings in the amended solutions grew for 4-12 hours and each then formed a terminal hyphal swelling resembling an appressorium (Fig. 1). The appressoriumlike (AL) structures always were formed in glucose-amended water but formed only rarely in nonamended waters. The AL structures developed most frequently from germlings grown in 250 mg/liter glucose plus 100 mg/liter $CaCl_2$. The structures were reniform to spherical and averaged 24×11 μm (max 30×15 μm) (Fig. 1). Incubation in nutrient-amended solutions for 12-36 hours allowed continued growth of some germlings. As a consequence, as many as four AL structures were occasionally formed in succession by one germling. Structures were closely appressed to the bottoms of the petri dishes and were not easily washed off. Adherence to a surface apparently was necessary for formation of AL structures since floating germlings produced none.

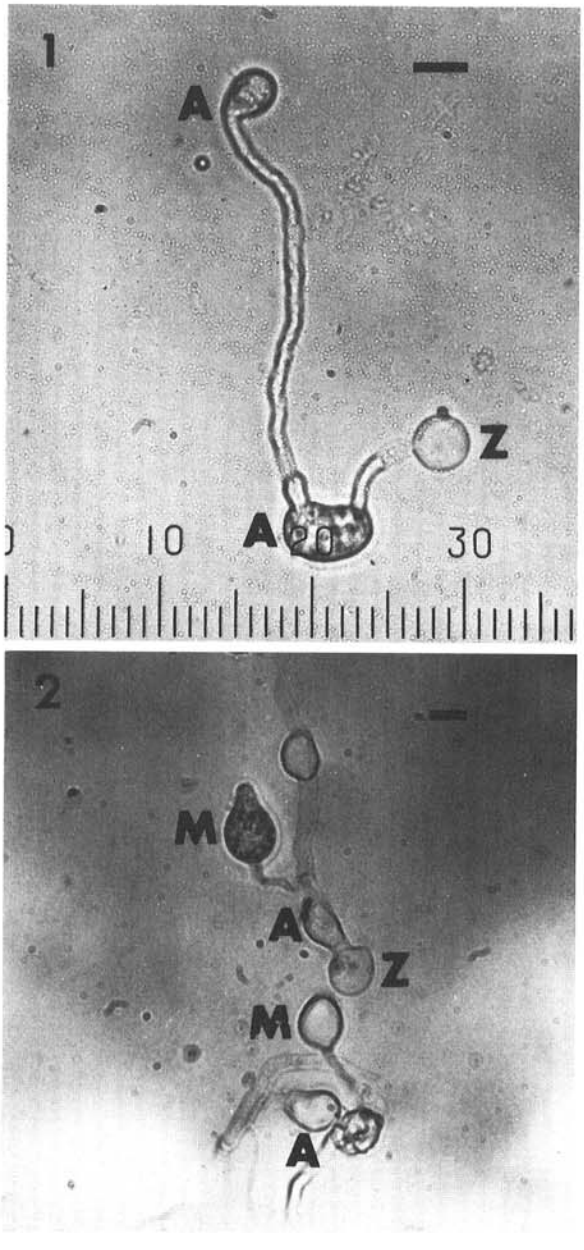

Fig. 1-2. Photomicrographs of germinated zoospore cysts (Z) of *Phytophthora parasitica* at 25 C. Scale bars represent 10 μm. **1)** Appressoriumlike structures (A) produced in water amended with glucose (250 mg/liter) and $CaCl_2$ (100 mg/liter) after 12 hours of incubation. **2)** Germinated and nongerminated microsporangia (M) 24 hours after replacement of glucose and $CaCl_2$ amendments with sterilized irrigation drainage water.

Microsporangia (avg. 23×13.8 μm) were produced 24 hours after the original nutrient solution was removed and replaced with sterile or nonsterile irrigation water (Fig. 2). Sporangiophores usually originated from or near the AL structures. Chilling at 4 C for 15 minutes induced the release of one zoospore per microsporangium. These zoospores formed normal colonies of *P. parasitica* which, when used as inoculum, were pathogenic to citrus seedlings. Some of the microsporangia and AL structures survived in nonsterile irrigation water for at least 60 days.

We also noted that a piece of orange peel (a 5-mm cube), small pieces of citrus leaves, and 250 mg/ml fructose, maltose, galactose, and sucrose, when substituted for the glucose amendment, also induced formation of AL structures and microsporangia.

DISCUSSION

Zoospores of *P. parasitica* were present within 10 minutes after water was placed on naturally infested soil. These zoospores became attached to floating citrus leaf tissue within the same time interval since swiftly running water did not remove them. This supports the observation that zoospores of *P. parasitica* are present in natural irrigation waste water (18). The ability of sporangia to germinate rapidly after inundation indicates that *P. parasitica* (and likely other *Phytophthora* spp.) is stimulated to release zoospores by short periods of flooding. If so, *P. parasitica* should be able to colonize a substrate more rapidly than many other microorganisms.

Other workers (23, 24) have indicated that flooding reduced or eradicated the propagules of some *Phytophthora* spp. However, we found that zoospores, or the structures produced by them, survived for 40-60 days in irrigation water. Our results indicate that zoospores remain motile up to 20 hours, which may be sufficient time for them to be recycled to susceptible crops. Agitation and physical contact, however, stimulate rapid encystment (3). Therefore, turbulent water associated with a pump-operated recycling system probably would cause encystment of zoospores.

Hyphal fragments produced by germinating cysts also may be a means for survival of *P. parasitica* in irrigation water. We noted that protoplasm of germlings contracted when insufficient nutrients were present to support continued vegetative growth. Some of the resulting fragments survived 40 days in untreated waste water and germinated upon addition of nutrients. This survival mechanism has also been reported for *Pythium ultimum* (16) and for germlings of other members of the Peronosporales, including *Phytophthora* spp. (1).

Survival may be facilitated by the formation of AL

structures (Fig. 1) which are produced when zoospores germinate in water amended with carbohydrates and with plant materials such as orange peels or citrus leaves which are common in sumps. The AL structures can survive for at least 60 days in irrigation water and conceivably they may be produced when zoospores germinate in the vicinity of diffused nutrients from citrus fruit or leaves. Appressoria, which are not uncommon in the genus *Phytophthora,* are produced by germinating zoospores of *P. infestans* in water in petri plates and during infection of potato leaves (15). Tsao (21) also has reported occasional production of AL structures in nonamended soil, but specific requirements for their formation were not defined.

Another method of survival involves the production of microsporangia. Microsporangia are produced after removal of a nutrient source from AL structures (Fig. 2), a process analogous to the production of regular sporangia on normal mycelia after nutrients are depleted (4). Some microsporangia survived in irrigation water for over 60 days; others germinated sooner, releasing only one zoospore. These zoospores could cause new infections if released in the proper environment. The method of induction of microsporangia is not unique to *P. parasitica* because such structures were observed in nonsterile soil infested with sporangia of *P. palmivora* (19).

Since *P. parasitica* survives in irrigation water for several weeks, water recycled from citrus should not be used on citrus orchards, and new orchards should not be planted where there is likelihood of exposure to contaminated irrigation water.

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