Insensitivity of Thick-Walled Oospores of *Pythium ultimum* to Fungicides, Methyl Bromide, and Heat

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ABSTRACT

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Thick-walled oospores, thin-walled, quiescent oospores, and quiescent sporangia of *Pythium ultimum* were treated for 24 hr with up to 1,000 mg of a.i./L of the fungicides captan, etridiazol, fenaminosulf, maneb, and thiram. Propagules also were treated with heat at 50 and 70 C for 30 min, and with methyl bromide gas at a concentration of 60 mg/L for up to 6 hr. Thick-walled oospores were killed only by heat at 70 C; their viability was reduced by heat at 50 C and by high levels of etridiazol, but was not affected by captan, fenaminosulf, maneb, methyl bromide, or thiram. In contrast, thin-walled oospores and sporangia, when treated while quiescent, were killed by heat at 50 or 70 C, by captan, thiram, or etridiazol at 100–500 mg a.i./L, by maneb at 1,000 mg of a.i./L, and by methyl bromide at 60 mg/L for 6 hr. Surprisingly, quiescent thin-walled oospores and sporangia were not greatly affected by fenaminosulf at up to 1,000 mg of a.i./L. However, when treated during germination, oospores and sporangia were killed by low levels of all toxicants tested, including fenaminosulf. Insensitivity of thick-walled oospores to fungicides and heat may result in the reappearance of germinable propagules in treated soils due to conversion of these survival structures to the thin-walled condition.

Additional keywords: constitutive dormancy, endogenous dormancy, fungal spore dormancy, pathogen eradication, soil sterilization.

Fungicides applied as soil drenches, fumigants, and heat treatments are commonly used to reduce or eliminate pathogen populations from soils and planting mixes. Control of *Pythium* spp. typically is a major goal of these treatments, because these fungi are present in most soils and soil mixes and cause seed, seedling, and root diseases of many plants. *Pythium* spp. generally are considered more sensitive than other pathogens and general soil microflora to eradicative treatments such as heat (4) and methyl bromide (6), and often are controlled by application of fungicides as soil drenches.

Pythium ultimum survives in soil as oospores and sporangia (8). Oospores initially are thick-walled and endogenously (constitutively) dormant (3,5), tolerant of adverse conditions, and capable of long-term survival. Thick-walled oospores do not germinate, do not initiate disease, and are not detected by soil-plating assays (3). Upon prolonged incubation in soil, thick-walled oospores become thin-walled and are then capable of germinating rapidly in response to available nutrients (3,5,8). *P. ultimum* also produces sporangia, which are similar in appearance and function to thin-walled oospores (8). Thus, thin-walled oospores and sporangia function as infective inoculum, whereas thick-walled oospores provide a reservoir of resistant propagules. Soil and soil-mix treatments that kill thin-walled oospores and sporangia, but not thick-walled oospores, may be only temporarily effective in controlling diseases caused by *P. ultimum*. Surviving thick-walled oospores can develop into thin-walled oospores and thus replenish the supply of germinable propagules.

The purpose of this study was to determine the sensitivity of thick-walled oospores, thin-walled oospores, and sporangia of *P. ultimum* to eradicative soil treatments, including the soil fumigant methyl bromide, several fungicides, and heat.

MATERIALS AND METHODS

P. ultimum Trow, American Type Culture Collection strain 32231, was used to produce oospores. *P. ultimum* strain P4, obtained from George Abawi, Geneva, NY, was used to produce sporangia. Stock cultures were periodically grown on cornmeal agar amended with 5 mg/L of pimaricin, 250 mg/L of sodium ampicillin, and 10 mg/L of rifampicin to prevent the appearance of bacteria in the cultures.

For spore production, the isolates of *P. ultimum* were grown in about 12 ml of V8-cholesterol broth in petri dishes in the dark at room temperature. After 6 days, the broth was replaced with sterile water, and the cultures were incubated for an additional 1–2 wk for spore production and maturation (1). Under these conditions, strains P4 and 32231 produced predominantly sporangia and thick-walled oospores, respectively, on mycelial mats.

Thin-walled oospores were produced by incubating oospore-bearing mycelium in nonsterile soil extract (1). The latter was prepared by mixing 10 g of freshly dug topsoil per liter of water, leaving the mixture to stand for 3 days, and filtering out the sediment with Whatman #1 filter paper. After about 4 wk, the mycelium was completely lysed, and the oospores formed brown aggregates. These aggregates included thin-walled and thick-walled oospores, but not oospores with walls of intermediate thickness, as determined by microscopic observation (Fig. 1).

To treat spores with fungicides, brown aggregates containing thin-walled oospores, sporangium-bearing mycelium, or oospore-bearing mycelium were placed in test tubes. Aliquots of fungicides in water were added to achieve the desired final concentrations. After 24 hr, the mycelia were removed and rinsed in several changes of water. The brown aggregates were rinsed by gently suspending them in the test tube and leaving them to settle for a few minutes. Most of the fungicide remained in suspension and could be removed by aspiration. This process was repeated several times.

Spores were treated with captan 50WP, etridiazol 30WP, fenaminosulf 70WP, maneb 80WP, and thiram 75WP at 0, 1, 10, 100, 500, and 1,000 mg of a.i./L.

For heat treatment, spores were placed in 5 ml water in test tubes, immersed in a water bath for 30 min at 25, 50, or 70 C, and cooled to room temperature. For treatment with methyl bromide, spores were placed on moist filter paper. The filter paper, with one edge in water in a beaker to maintain moisture, was placed in a large side-arm flask. Methyl bromide and air from gas cylinders were mixed in a separate side-arm flask equipped with a large stir bar, and the mixture was diverted into the treatment flask. Gas samples were removed from the treatment flask at 15-min intervals throughout each experiment, and the methyl bromide content was determined by gas chromatography to maintain 60 mg of methyl bromide per liter. After 0, 2, 4, or 6 hr, the treatment flask was

purged with air and the spores transferred to water for testing.

The viability of sporangia and thin-walled oospores was determined by inducing germination. Treated or untreated spores were dispersed in 0.2% potato-dextrose-agar (PDA) syrup, incubated for 2 hr, and fixed by the addition of an equal volume of formalin-acetic acid-alcohol (2). Percent germination was determined by microscopic observation of at least 100 spores per sample for the presence of visible germ tubes.

Viability of thick-walled oospores was determined by first inducing conversion to the thin-walled condition (Fig. 1) by immersion in unsterile soil extract for 4 wk and then inducing germination as outlined above. In separate experiments, sporangia and thin-walled oospores were induced to germinate in the presence of fungicides rather than after exposure to fungicides while quiescent. For this, spores were incubated for 2 hr in PDA syrup containing 0–1,000 mg of a.i. fungicide per liter, fixed in formalin-acetic acid-alcohol, and observed microscopically for the presence of visible germ tubes.

Within an experiment, each dose-spore type combination was sampled three times, and at least 100 spores were observed microscopically for each sample. Each experiment was replicated three times, and data points presented are the means of the nine resultant determinations. Statistical analyses were conducted with the Minitab program (7). The effect of treatments on the ability of thick-walled oospores to become thin-walled in unsterile soil extract was tested by linear regression of the percent of oospores that became thin-walled as a function of fungicide concentration.

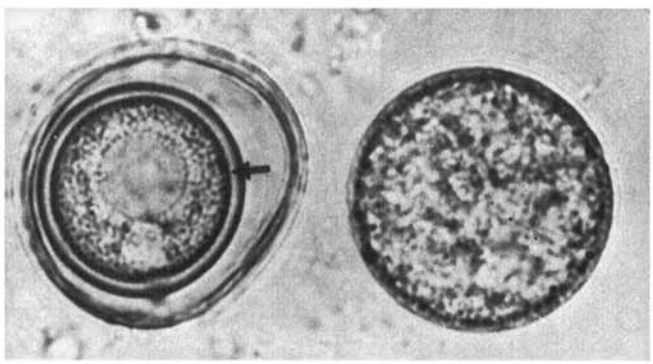

Fig. 1. Oospores of *Pythium ultimum* with a thick oospore wall (arrow) and a thin oospore wall.

The effect of treatments on spore germinability was determined by linear regression of the percent spore germination as a function of the logarithm of the sum of the treatment dose plus one. Treatment dose for fungicides was expressed as milligrams active ingredient and for methyl bromide as minutes of exposure to 60 mg/L. Goodness of fit of regressions was assessed by inspection of plotted data for linearity and of plotted residuals for random variability.

RESULTS

Exposure of thick-walled oospores to captan, etridiazol, fenaminosulf, maneb, or thiram at up to 1,000 mg of a.i./L for 24 hr, to methyl bromide gas at 60 mg/L for up to 6 hr, and to heat at 50 or 70 C for 30 min did not affect their ability to become thin-walled (Fig. 1) during subsequent incubation in unsterile soil extract (Fig. 2). For all treatments, slope values for regressions did not differ significantly ($P = 0.01$) from zero, and r^2 (coefficient of determination) values were below 0.420 (Fig. 2).

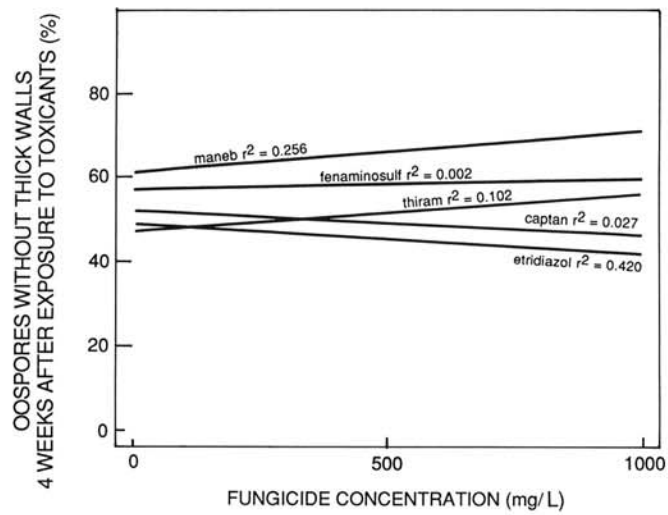

Fig. 2. Effect of exposure of thick-walled oospores to various treatments on their subsequent ability to become thin-walled during incubation in unsterile soil extract. Fitted linear regressions and r^2 values are shown for five fungicides. For all treatments, the slope estimates for the regressions did not differ significantly ($P = 0.01$) from zero.

TABLE 1. Results of regression of percent germination of oospores and sporangia of *Pythium ultimum* as a function of the logarithm of the sum of toxicant dose plus one for each propagule type and treatment

Spore type and treatment	df error	r^2	Slope			
			Slope	SD[a]	*P*	ED50
Thick-walled oospores						
Captan	19	.128	−4.7	2.8	>.10	>1,000 mg/L
Etridiazol	19	.572	≤−17.2	3.4	<.001	569 mg/L
Fenaminosulf	19	.163	−6.1	3.2	>.05	>1,000 mg/L
Maneb	19	.230	−5.8	2.4	>.05	>1,000 mg/L
Thiram	19	.045	−3.8	4.1	>.10	>1,000 mg/L
Methyl bromide	19	.118	−0.6	4.8	>.10	>6.0 hr
Thin-walled oospores						
Captan	10	.776	−32.1	5.5	<.001	1 mg/L
Etridiazol	13	.653	−28.6	8.9	<.001	14 mg/L
Fenaminosulf	19	.491	−14.7	3.4	<.01	158 mg/L
Maneb	19	.697	−24.8	3.8	<.001	12 mg/L
Thiram	10	.572	−34.4	9.4	<.001	5 mg/L
Methyl bromide	10	.818	−21.9	3.3	<.001	0.1 hr
Quiescent sporangia						
Captan	10	.756	−34.7	6.2	<.001	2 mg/L
Etridiazol	13	.696	−30.1	5.5	<.001	4 mg/L
Fenaminosulf	19	.730	−29.4	4.1	<.001	28 mg/L
Maneb	19	.730	−29.4	4.1	<.001	28 mg/L
Thiram	13	.843	−34.7	4.2	<.001	19 mg/L
Methyl bromide	10	.966	−31.9	1.9	<.001	0.2 hr

[a] Standard deviation.

In most cases, treatment of thick-walled oospores did not affect their ability to germinate after becoming thin-walled (Table 1, Fig. 3). Slopes of regressions for captan, fenaminosulf, maneb, methyl bromide, and thiram did not differ significantly from 0; *P* values, as determined by t-rations for slope estimates, were greater than 0.05. Also, r^2 values were low, indicating lack of correlation, and the ED50 values calculated from the fitted regressions were high, indicating lack of effect of treatment on spore germinability (Table 1). Etridiazol significantly reduced the germinability of oospores treated while thick-walled (Table 1). Exposure of thick-walled oospores to 50 C for 30 min reduced their germinability by 59% as compared with oospores held at 25 C. Thick-walled oospores were killed by treatment at 70 C; treated oospores lost the thick oospore wall during immersion in unsterile soil extract but failed to germinate when subsequently exposed to nutrients.

Germinability of thin-walled oospores and sporangia was significantly reduced by fungicides applied to quiescent spores, except with fenaminosulf (Table 1, Fig. 3). Average percent germination of untreated thin-walled oospores and sporangia for all experiments was 74 ± 10 and 81 ± 10, respectively. Thin-walled

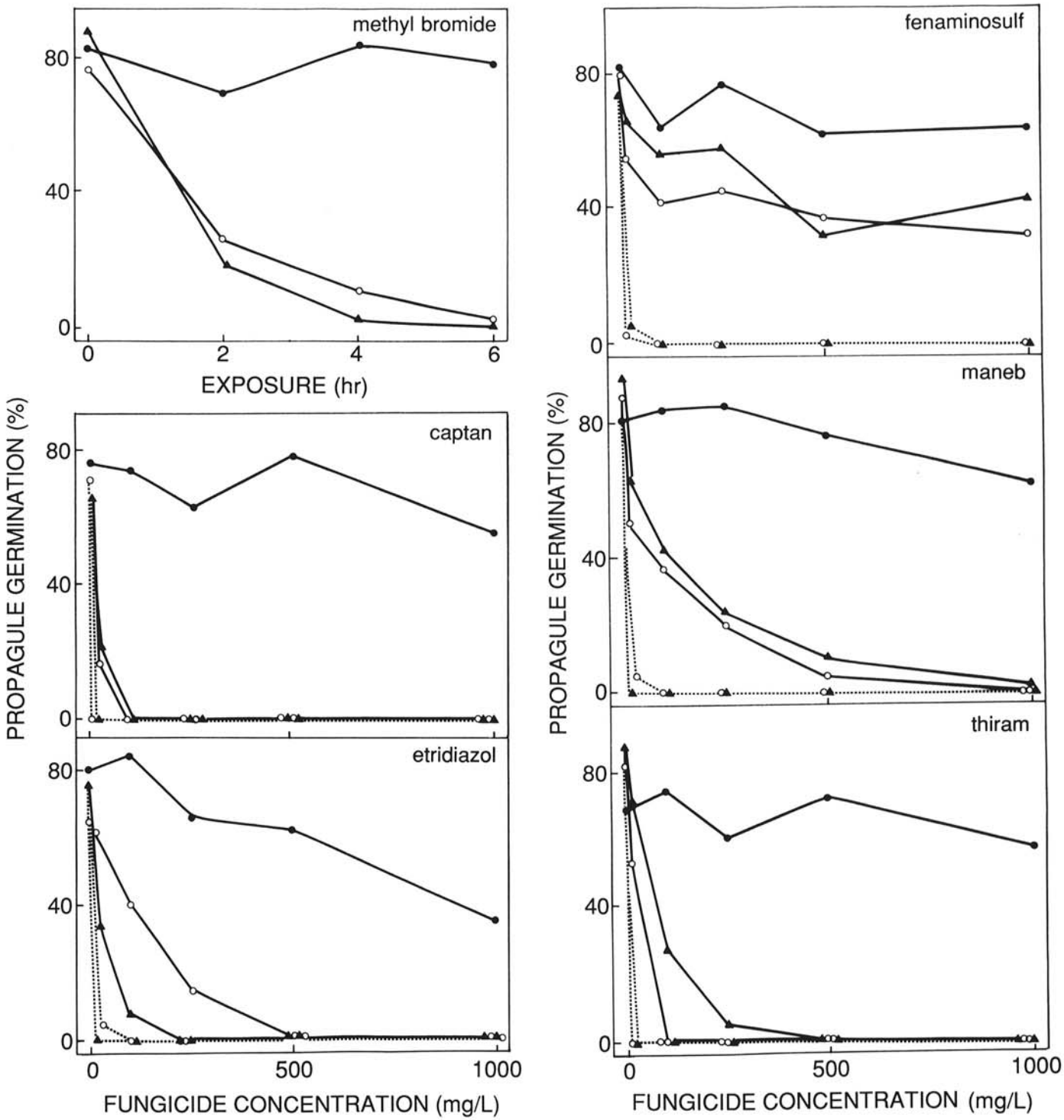

Fig. 3. Effect of fungicides and methyl bromide on germinability of oospores and sporangia of *Pythium ultimum*. Thin-walled oospores (open circles) or sporangia (triangles) were treated, removed from the toxicant, and incubated in 0.2% potato-dextrose agar (PDA) to induce germination. Thick-walled oospores (closed circles) were incubated in unsterile soil extract, following removal of the toxicants, to induce loss of the thick walls, and then induced to germinate in PDA. Quiescent propagules (solid lines) were exposed to fungicides for 24 hr or to methyl bromide at 0.06 gm/L. Germinating propagules (dashed lines) were treated with fungicides in 0.2% PDA for 2 hr.

TABLE 2. Percent germination of thin-walled oospores and sporangia of *Pythium ultimum* treated with fungicides at 10 mg of a.i./L either for 24 hr while quiescent or for 2 hr while germinating

Treatment	Oospores		Sporangia	
	Quiescent	Germinating	Quiescent	Germinating
Captan	18 e[a]	0 f	23 e	0 f
Etridiazol	64 abc	6 f	36 de	1 f
Fenaminosulf	55 bcd	2 f	66 abc	5 f
Maneb	49 cd	10 f	62 abc	2 f
Thiram	53 bcd	0 f	74 ab	0 f
Untreated	74 ab	74 ab	81 a	81 a

[a]Values in any column followed by a common letter do not differ significantly at $P = 0.05$ according to Waller and Duncan's multiple-range test; means of three replicates with three samples per replicate.

oospores and sporangia were also sensitive to heat; none germinated after exposure to 50 or 70 C for 30 min. For each treatment, slope values for thin-walled oospores did not differ significantly ($P = 0.05$) from those for sporangia.

Germinating oospores and sporangia were highly sensitive to fungicides (Table 2, Fig. 3). Germination was very low (0–10%) when induced in the presence of as little as 10 mg of a.i. fungicide per liter (Table 2), and no spores germinated when induced in the presence of 100 mg/L (Fig. 3). In contrast, exposure of quiescent spores to 10 mg/L for 24 hr resulted in little to no decrease in subsequent germination for fenaminosulf, maneb, thiram, and etridiazol. Captan reduced the germinability of quiescent oospores and sporangia to 18 and 23%, respectively, as compared with 74 and 81% for untreated checks. Sensitivity of quiescent or germinating oospores was similar to that of comparable sporangia for all treatments (Table 2, Fig. 3).

DISCUSSION

Thick-walled oospores of *P. ultimum* were not killed by exposure to fungicides, methyl bromide, or moderate heat. For most treatments, oospores became thin-walled in unsterile soil extract and then germinated normally when supplied with nutrients. Of the treatments tested, only heat at 70 C killed thick-walled oospores, although heat at 50 C and etridiazol at high levels reduced their germinability.

In contrast, thin-walled oospores and sporangia were highly sensitive to some treatments when exposed while quiescent, including captan and heat (50 C), and were sensitive to moderate levels (100 to 500 mg/L) of thiram, etridiazol, or maneb, or 4- to 6-hr exposure to methyl bromide. Surprisingly, thin-walled oospores and sporangia were nearly insensitive to fenaminosulf, even at high levels, although this material is widely and effectively used for control of *Pythium* spp. However, when exposed while germinating instead of while quiescent, oospores and sporangia were highly sensitive to all fungicides tested, including fenaminosulf.

These experiments indicate that choice of treatments for control of *Pythium* spp. should include consideration of different sensitivity of propagules to various treatments and whether short-term or long-term control of the pathogen is required. Some fungicides, especially fenaminosulf, are effective only during or after spore germination and, thus, must be available in the soil solution when host tissue is available for infection. Other treatments, such as captan, thiram, etridiazol, methyl bromide, and 50 C heat can effectively kill thin-walled oospores and sporangia, which are the germinable propagules in soil, and provide short-term disease control. However, thick-walled oospores largely survive these treatments and can then become thin-walled during exposure to unsterile soil. Thus, a supply of germinable propagules can be replenished and a potential for disease reestablished.

The ability of thick-walled oospores to survive control treatments and replenish the supply of germinable propagules must also be considered when soil-plating assays are used to detect *Pythium* spp. Thus, no detectable propagules of *Pythium* spp. would be expected immediately after soil treatments are applied if thin-walled oospores and sporangia are killed. The subsequent reappearance of detectable propagules, however, could be due to surviving oospores and not necessarily to reinvasion of the soil by *Pythium*.

Long-term control of *Pythium* spp. may require eradication of thick-walled oospores, persistence of toxicants effective against germinable or germinating propagules, or effective pretreatments designed to induce loss of thick oospore walls. In the present study, only heat at 70 C for 30 min effectively killed thick-walled oospores of *P. ultimum*.

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