Calcium Interference with Zoospore Biology and Infectivity of *Phytophthora parasitica* in Nutrient Irrigation Solutions

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ABSTRACT

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Calcium, applied as either CaCl_2 or $\text{Ca}(\text{NO}_3)_2$ to water or calcium-free soluble fertilizer solution (Peters 20-10-20 Peat Lite Special), affected several important stages of *Phytophthora parasitica* zoospore behavior relevant to infection and disease spread. Release of zoospores from sporangia was suppressed by Ca^{2+} concentrations in the range of 10 to 50 meq. These concentrations also curtailed zoospore motility; 20 meq of Ca^{2+} in fertilizer solution caused all zoospores to encyst within 4 h, whereas 94% of zoospores remained motile in unamended solution. In addition, Ca^{2+} in the range of 10 to 30 meq stimulated zoospore cysts to germinate in the absence of an organic nutrient trigger, while suppressing

the release of a single zoospore (diplanetism) from cysts that did not germinate. In growth chamber experiments, the amendment of the fertilizer solution with 10 or 20 mM $\text{Ca}(\text{NO}_3)_2$ greatly suppressed infection of flood-irrigated, containerized vinca seedlings in a peat-based mix by motile or encysted zoospores of *P. parasitica*. These results demonstrate that Ca^{2+} amendments interfere with *P. parasitica* zoospore biology at multiple stages, with compounding effects on epidemiology, and suggest that manipulation of Ca^{2+} levels in irrigation water or fertilizer solutions could contribute to management of *Phytophthora* in recirculating irrigation systems.

Additional keywords: disease management, flood irrigation, greenhouse recirculating systems, oomycota.

Phytophthora, *Pythium*, and *Aphanomyces* species (Oomycota) are among the most damaging pathogens of plants in wet soils and irrigated cropping systems. These polycyclic pathogens can destroy plant tissues within a few days and then produce motile zoospores to initiate further cycles of disease. Although direct evidence for a role of zoospores in the infection process has been difficult to obtain in soil, there is increasing recognition that these propagules are important for epidemic development of *Phytophthora* root rots in crop production (10,23,29), and zoospores have been demonstrated to be the major agents for spread of both *Pythium aphanidermatum* and *Phytophthora capsici* in recirculating nutrient systems in greenhouses (27,28). Zoospores locate the most susceptible sites on roots by chemotaxis (6,18,35) and other mechanisms (22,30). This maximizes the chance of infection from individual spores and increases the inoculum potential when zoospores aggregate at infection sites (26). Zoospores also are transported more readily by bulk water flow through soil and other particulate materials than are nonmotile cells (33), and they can penetrate root mats in nutrient films (16). Thus, zoospores also facilitate spread of infection from primary disease foci.

Current control methods for the zoosporic oomycota center mainly on the use of fungicides, but pathogens have developed tolerance to some of these compounds and few fungicides are registered for use in irrigated greenhouse systems. An alternative and more durable approach to disease management would be to interfere with zoospore production or zoospore behavior. This has been achieved experimentally by manipulating the electrical con-

ductivity of nutrient irrigation solutions (29), using surfactants that lyse zoospores (27,28), and applying biocontrol bacteria that reduce zoospore taxis to roots (36) or that alter the ionic composition of media, causing zoospore lysis (11). We have explored a different approach based on the central role of intracellular and extracellular calcium in many aspects of growth and differentiation of the Oomycota (6).

At least a minimum level of extracellular calcium is necessary for production of zoosporangia by *Phytophthora* spp. (14), for normal motility of *Pythium* zoospores (9), for adhesion of *Pythium* and *Phytophthora* zoospore cysts to host or other surfaces (8,13), and for germination of *Pythium* (8), *Phytophthora* (32), and *Aphanomyces* (J. W. Deacon, unpublished data) zoospore cysts. These effects were demonstrated by direct manipulation of calcium levels, using calcium chelators, or using pharmacological agents known to interfere with calcium-mediated processes in other organisms (9). However, high levels of extracellular calcium can rapidly immobilize *Phytophthora* zoospores (2), induce zoospore cysts to germinate without a host or nutrient trigger (2,6), and suppress the production of a further zoospore (diplanetism) from a zoospore that encysts when it fails to locate a host (32). High soil calcium levels have also been associated with suppression of disease by several zoospore-forming pathogens (1,17,19,20,25), although the mechanisms of suppression are poorly characterized. They are reported to involve, inter alia, germination of sporangia of *Pythium splendens* (19), enhanced soil microbial numbers and soil fungistasis (1,19), and enhancement of host plant resistance to several pathogens (19,20).

As part of our goal to understand the role of calcium in zoospore biology and exploit it for disease management, we have studied the effects of calcium amendments on *Phytophthora parasitica* Dastur in aqueous solutions and in Peters 20-10-20 Peat Lite Special soluble fertilizer solution (Scotts-Sierra Horticultural Prod-

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ucts Company, Marysville, OH), which is commonly used in irrigated greenhouse production units in the United States. Here we show that calcium amendments interfere with zoospore biology at multiple stages, which should have compounding effects in epidemiology, and that calcium amendment of irrigation solutions can suppress infection by zoospores significantly.

MATERIALS AND METHODS

Fungal culture and sporangium production. The isolate of *Phytophthora parasitica* (= *P. nicotianae* Breda de Haan) used in these studies originated from diseased vinca (*Catharanthus roseus* (L.) G. Don) in a commercial greenhouse in Oklahoma. The methods for culture and production of sporangia were as described previously (32), but with the following modification. Small (60 mm) petri dishes containing 3 ml of clarified V8 broth were inoculated with three agar inoculum blocks and incubated for 24 h at 27°C to produce mycelial mats. The mats were then rinsed thoroughly with mineral salts solution and reincubated in 3 ml of mineral salts solution for 4 to 5 days beneath fluorescent lights to induce formation of sporangia.

Effects of calcium and fertilizer solutions on zoospore release from sporangia. Sporangium-bearing mycelial mats were rinsed thoroughly with sterile distilled water (SDW), and then 4 ml of different experimental “release solutions” were added to each dish. Synchronous cleavage of the sporangium contents into zoospores was induced by chilling at 12°C for 30 min; the dishes were then removed to room temperature (20 to 21°C) and zoospores were released within 1 to 2 h. The release solutions in different experiments included SDW, aqueous 10 to 50 mM CaCl₂ or Ca(NO₃)₂ solutions, Peters 20-10-20 Peat Lite Special soluble fertilizer (Peters PLS) at 100 to 450 ppm of N, and Peters PLS at 150 ppm of N amended with 10 to 50 mM CaCl₂ or Ca(NO₃)₂. Peters PLS contains N, P, and K plus essential micronutrients, but no calcium; solutions of 150, 300, and 450 ppm of N are equivalent to electrical conductivities (ECs) of 1, 2, and 3 mS/cm. Addition of calcium to Peters PLS in the ranges tested did not measurably affect pH or EC.

The sporangia on the mycelial mats were examined in situ by mounting a petri dish on a Nikon Labophot research microscope

with a 10× objective (Nikon, Inc., Melville, NY). The dishes were scanned using a Sony DXC-151A PAC video camera connected to a Sony SVO 1610 VHS video recorder and a Sony PVM2030 Triniton monitor (Sony Corp. of America, Park Ridge, NJ). The number of sporangia out of 100 that had discharged their contents or that contained cleaved cytoplasm but were not discharged were determined from videotapes of random microscope fields recorded 1 to 3 h after the chilling treatment. Numbers of zoospores released into solutions at 1 to 2 h after chilling were determined by transferring 1-ml aliquots of release solution to Eppendorf tubes, encysting the zoospores by vortexing (70 s), and then counting them with a hemacytometer.

Effects of calcium in fertilizer solutions on zoospore motility. Zoospores were released as described above into Peters PLS solution (150 ppm of N) amended with 0, 10, 20, 30, or 40 mM Ca(NO₃)₂. The dishes were examined using the videotaping system at 2.5 to 3.5 h after chill induction of zoospore release. Both spores at the upper surface of release solutions and spores near the bottoms of dishes were recorded in three random microscope fields per dish. For assessing the time course of motility, the mycelial mats were removed from dishes at 1 h after chill induction to eliminate any additional release of zoospores. The spores near the upper surfaces of release solutions in three microscope fields per dish were recorded at 1, 2, 3, and 4 h after induction. Motility was assessed from videotape recordings by circling images of cells on plastic wrap overlaying the monitor while the tape was paused and then advancing the tape by frames to determine which cells were motile. Numbers of motile cells and total cells were counted for each field.

Effects of calcium and fertilizer solutions on cyst germination. Zoospores were released as described above into aqueous solutions of 0, 10, or 20 mM CaCl₂ or into Peters PLS (150 ppm of N) amended with 0, 10, or 20 mM Ca(NO₃)₂ or CaCl₂. At 1.5 to 2 h after the chilling of sporangia, zoospores in 1-ml aliquots of release solutions were transferred to Eppendorf tubes, vortex-encysted as described above, and incubated at room temperature for 2 h. The Eppendorf tubes were then vortexed for 10 s to resuspend cells, and 80-μl aliquots were transferred to slides. Germination was assessed by scoring 100 cysts on each slide. Cysts were considered to have germinated if the length of the germ tube was greater than the diameter of the cyst. The remaining spores in the Eppendorf tubes were assessed after 8 h; 100 cysts were scored for germination by germ tubes and for release of a further single zoospore. Empty cyst cases (ghosts) were counted as cysts that had released a zoospore.

Effects of calcium in fertilizer solutions on infection of vinca seedlings. Three-week-old vinca seedlings, cultivar Grape Cooler, were produced from seed sown in Redi-Earth Peat-Lite Mix (Scotts-Sierra Horticultural Products Company) in 288-plug plastic seedling trays. The trays were incubated in a growth chamber with a 12-h light (28°C)/12-h dark (22°C) cycle and irrigated daily with tap water. Blocks of 12 or 16 seedlings, arranged three by four or four by four, were cut from the plastic seedling trays and

TABLE 1. Effects of aqueous solutions of CaCl₂ on cytoplasmic cleavage in sporangia and on discharge of motile cells from the cleaved sporangia^a

Sporangia	Calcium concentration (mM)					5% LSD	P (ANOVA)
	0	10	20	35	50		
Cleaved							
Discharged	73	68.3	50	35.7	33	12.94	9.5 × 10 ⁻⁵
Not discharged	0	8	26	45.7	63	11.78	1.6 × 10 ⁻⁶
Uncleaved	27	23.7	24	18.7	4	9.16	1.7 × 10 ⁻³

^a Numbers of sporangia (out of 100 assessed); means of counts in three replicate dishes at 2 h after a 30-min chill induction of sporangial cleavage.

TABLE 2. Effects of different concentrations of soluble fertilizer solution (Peters 20-10-20 Peat Lite Special) on release of zoospores from sporangia^a

	Peters concentration (ppm of nitrogen)						5% LSD	P (ANOVA)
	0	100	150	200	300	400		
Discharged sporangia ^b								
Experiment 1	38	59.7	...	57.7	44.7	38.3	10.5	1.2 × 10 ⁻³
Experiment 2	49	...	74.3	...	58.7	...	49	5.8 × 10 ⁻³
Discharged cell count ^c								
Experiment 1	94.8	139	...	139.7	77.7	57	39.2	4.0 × 10 ⁻⁴
Experiment 2	93.2	...	181.3	...	51.2	...	22.2	1.4 × 10 ⁻⁴

^a Data from two experiments.

^b Numbers of sporangia with discharged contents (means of 100 assessed in each of three replicate dishes).

^c Numbers of spores in the bathing medium (means of hemacytometer counts of vortex-encysted spores in aliquots taken near the top of suspensions in three replicate dishes).

placed in the bottoms of 100-mm-square petri dishes 24 h prior to inoculation. Seedling blocks were, thereafter, irrigated by adding nutrient solutions to individual dishes to simulate flood irrigation. Inoculum was prepared from zoospores released into 150 ppm of N Peters PLS as previously described. The suspensions were used as either motile zoospores or vortex-encysted (70 s) spores and were diluted to approximately 10^2 cells/ml by addition of 150 ppm of N Peters PLS or 150 ppm of N Peters PLS amended with 10 or 20 mM $\text{Ca}(\text{NO}_3)_2$. In a preliminary experiment, suspensions of 10^2 , 10^3 , and 10^4 motile cells/ml were used to determine that 10^2 cells/ml was usually sufficient to give >95% infection of inoculated plants.

Seedling blocks were flood-irrigated on the morning of the day of inoculation with 25 ml of 150 ppm of N Peters PLS with or without $\text{Ca}(\text{NO}_3)_2$ to ensure that the peat mix was saturated. Later that day, the seedling blocks were flooded again with 20 ml of the corresponding solution with or without (controls) inoculum. Thereafter, seedlings were flood-irrigated daily with 25 ml of the appropriate irrigation solution that did not contain inoculum. Symptoms and plant deaths were recorded daily. After 10 days, all seedlings were removed, washed free of peat mix, and placed on a *Phytophthora*-selective medium (31) to detect infection by *P. parasitica*. In some experiments, additional blocks of uninoculated plants were included to test the effects of the irrigation solutions on plant growth as measured by total dry weight. These plants were removed, washed, placed in small paper bags, oven-dried (72 h at 180°C), and weighed.

Experimental design and statistical analysis. All treatments were replicated three times, and all treatment effects were repeated at least twice. Results were analyzed by analysis of variance (ANOVA) and, in some cases, also by regression using Microsoft Excel version 5 (Microsoft Corp., Redmond, WA). Numbers of infected plants or plant deaths were analyzed by chi-squared, using Yates' correction when there was only one degree of freedom. Percentage data were converted to arcsine values for analysis.

RESULTS

Effects of calcium in aqueous solution on zoospore release from sporangia. Ca^{2+} treatments affected the production of zoospores from sporangia at two different stages: during cleavage of the sporangium protoplasm into uninucleate cells and at the stage when zoospores were released by dissolution of the sporangium papillum. CaCl_2 had a concentration-dependent effect on both of these processes (Table 1). The number of sporangia with cleaved contents was positively correlated with CaCl_2 concentration in the range of 0 to 50 mM ($r = 0.972$, $P = 1.55 \times 10^{-9}$) and was significantly enhanced by 50 mM CaCl_2 compared with the water controls (ANOVA). But discharge of zoospores from the cleaved sporangia was negatively correlated ($r = 0.91$, $P = 3.3 \times 10^{-6}$) with CaCl_2 concentration over the range 0 to 50 mM and was significantly reduced by even 20 mM CaCl_2 (ANOVA). Therefore, the overall effect of CaCl_2 was to reduce significantly the release of

TABLE 3. Effects of CaCl_2 and $\text{Ca}(\text{NO}_3)_2$ amendments in Peters Peat Lite Special (PLS) soluble fertilizer solution (150 ppm of N) on release of zoospores from sporangia^a

Exp.	Assessment	Water control	Peters PLS 150 ppm of N + calcium as mM CaCl_2 or $\text{Ca}(\text{NO}_3)_2$								5% LSD	P (ANOVA)
			0 mM	10 mM Cl	10 mM NO_3	20 mM Cl	20 mM NO_3	30 mM Cl	30 mM NO_3	40 mM NO_3		
1	Discharged sporangia ^b	...	61.7	51.7	50	37.3	35	23.3	17.7	...	13.7	7.0×10^{-5}
2	Discharged sporangia	49	51	...	31.3	...	9.7	...	3.7	3	9.9	1.6×10^{-7}
	Spore count ^c	135.7	113.2	...	104	...	26.5	...	10.8	5	27.9	8.2×10^{-12}
3	Discharged sporangia	47	81	...	65	...	40	...	15	6.3	16.4	8.6×10^{-7}
	Spore count	212.7	143.7	...	127	...	127	...	15	4	55.4	1.9×10^{-5}

^a Data from three experiments.

^b Numbers of sporangia (means of 100 assessed in each of three replicate dishes) that had discharged their contents as zoospores.

^c Numbers of spores in the bathing medium (means of hemacytometer counts of vortex-encysted spores in aliquots taken near the top of suspensions in three replicate dishes).

TABLE 4. Effects of $\text{Ca}(\text{NO}_3)_2$ amendments in Peters Peat Lite Special (PLS) soluble fertilizer solution (150 ppm of N) on zoospore motility

	Water control	Ca level (meq) in Peters PLS solution					5% LSD	P (ANOVA)
		0	10	20	30	40		
Motile spore count ^a	194.3	205.7	59.7	9.3	0	0	84.3	3.4×10^{-2}
Motile as percent of total ^b	89.4	84.0	27.8	1.4	0	0		

^a Means of three replicate dishes, based on in situ counts of spores 2 h after chill induction of zoospore release; three random microscope fields were used for each dish, and the data were recorded as the sum of spores near the top and at the bottom of the suspension in each microscope field.

^b Calculated from the grand means (of three dishes) of motile and total cells; 86% of all motile spores were found at the top of the spore suspensions.

TABLE 5. Numbers of motile zoospores 1 to 4 h after their release into Peters Peat Lite Special fertilizer solution (150 ppm of N) with or without 20 mM $\text{Ca}(\text{NO}_3)_2$

	1 h	2 h	3 h	4 h	5% LSD	P (ANOVA)
Peters solution alone						
Motile zoospore count ^a	240.3	222.3	176.3	180	ns	6.3×10^{-1}
Motile cells as percent of all cells		98.8	97.0	95.3	94.4	
Peters solution + 20 mM $\text{Ca}(\text{NO}_3)_2$						
Motile zoospore count ^a	149.7	66.3	4.3	0	31.8	1.4×10^{-5}
Motile cells as percent of all cells		78.4 ^b	33.3 ^b	1.7 ^b	0 ^b	

^a Means of three replicate dishes, based on counts in three random microscope fields per dish. Two-way analysis of variance showed significant effects of presence/absence of Ca^{2+} ($P = 2.27 \times 10^{-6}$) and time ($P = 7.0 \times 10^{-3}$), but no significant Ca^{2+} /time interaction ($P = 0.44$).

^b Significantly less than in Peters solution alone ($P < 0.0032$), by *t* test of arcsine-transformed percentages.

infective propagules, even though CaCl_2 enhanced differentiation in the sporangia.

Effects of Peters PLS solution on zoospore release from sporangia. In the absence of calcium, Peters PLS solution at 100 to 200 ppm of N enhanced the number of discharged sporangia and also the number of spores assessed in the bathing solutions compared with water controls (Table 2). Higher concentrations of Peters PLS solution (300 to 450 ppm of N) neither enhanced nor suppressed sporangium discharge compared with the water controls. However, the number of spores assessed in aliquots of the bathing solutions was reduced by high concentrations of Peters PLS (Table 2). This effect was explained by assessment of zoospore motility from videotapes in the second experiment in Table 2. Based on counts of at least 150 cells in each dish after 2 h, the percentage of motile spores was 59, 91, 81, and 59 for the 0, 150, 300, and 450 ppm of N treatments, respectively. All the motile cells in the 450-ppm treatment were near the bottom of the dishes and were swimming slowly and erratically. In the other solutions, the motile spores were swimming normally, near the top of the suspensions where samples had been taken for the tabulated cell counts.

Effects of calcium in Peters PLS solution on zoospore release from sporangia. In each of three experiments (Table 3), amendment of Peters PLS solution (150 ppm of N) with calcium reduced sporangial discharge progressively over the range of 0 to 40 meq of Ca^{2+} . This was reflected in both the numbers of discharged sporangia and the numbers of spores assessed in the release solutions. CaCl_2 and $\text{Ca}(\text{NO}_3)_2$ were equally effective, indicating that the effect was caused by the Ca^{2+} ion. The concentrations of 30 and 40 meq of Ca^{2+} were highly suppressive, but significant reductions compared with Peters PLS solution alone were obtained at even 10 or 20 meq of Ca^{2+} .

Effects of calcium on zoospore motility in fertilizer solution. Motility was assessed for spores released from sporangia into solutions of different calcium content. In the absence of Ca^{2+} , most zoospores remained motile for at least 3 h in Peters PLS solution (150 ppm of N). Only 16% of the zoospores had encysted at this time (Table 4), and the cysts had deposited to the bottom of the suspensions, whereas most of the motile spores remained near

the liquid surface. These findings were similar to those for spores in the water controls. The presence of even 10 mM $\text{Ca}(\text{NO}_3)_2$ in Peters PLS solution (150 ppm of N) significantly reduced the number of motile zoospores after 3 h; most of the spores had encysted. At 20 mM Ca^{2+} , there were few motile spores and, at the higher Ca^{2+} concentrations, there were few spores to assess (and none motile), because the release of spores from sporangia had been strongly suppressed.

In a further experiment (Table 5), the sporangia were removed from the spore suspensions after 1 h so that no further zoospores would be released, and motility of the spores was assessed periodically. In Peters PLS solution alone (150 ppm of N), there was no significant reduction in motility during the first 4 h. Amendment of Peters PLS solution with 20 mM $\text{Ca}(\text{NO}_3)_2$ caused a small, significant reduction in motility during the first hour and major reductions after 2 and 3 h. Again, the motile spores were found mainly near the surface of the suspensions, whereas most cysts were found at the bottom of the dishes.

Effects of soluble fertilizer solutions and calcium on cyst germination. Zoospores that had been encysted by vortex treatment after release into the different solutions either germinated within 2 h by producing a germ tube, or the cysts remained quiescent for 5 to 6 h and then released a further single zoospore from a short protuberance. The numbers of cells that responded in these ways were assessed after 7 to 8 h, because the data for germ tube production at that time interval did not differ significantly from the data at 2 h in initial experiments.

The presence of 10 or 20 mM CaCl_2 in aqueous solution significantly increased the germination by germ tubes, but markedly suppressed the release of further zoospores from the same population of cysts (Table 6). In the absence of Ca^{2+} , Peters PLS fertilizer solution had a significant concentration-dependent effect on germination in each of two experiments (Table 7). Germination by germ tubes was enhanced by increasing concentrations of Peters PLS solution, but the lowest concentration (100 ppm of N) had no significant effect compared with the water controls. Conversely, Peters PLS solution caused a concentration-dependent suppression of zoospore release from ungerminated cysts, and even the low concentrations (100 or 200 ppm of N) significantly suppressed the release of further zoospores. The same patterns of response to treatments were seen in two experiments, even though the overall level of germination by germ tubes differed between these experiments (Table 7). The presence of either CaCl_2 or $\text{Ca}(\text{NO}_3)_2$ in Peters PLS solution (150 ppm of N) enhanced germination by germ tubes (significant for 20 meq of Ca^{2+}) and suppressed the release of a further zoospore from cysts (significant for both 10 and 20 meq of Ca^{2+}) (Table 8).

Effects of calcium on infection of vinca seedlings. In an initial experiment with blocks of 12 seedlings, flood inoculation of vinca with 10^2 , 10^3 , or 10^4 zoospores/ml of Peters PLS solution (150 ppm of N) caused infection of all (36) or nearly all (35 of a

TABLE 6. Effects of aqueous solutions of CaCl_2 on percentage of germination of zoospore cysts by germ tubes and on release of a further zoospore from nongerminated cysts^a

Mode of germination	CaCl ₂ concentration (mM)			5% LSD	P (ANOVA)
	0	10	20		
Germ tube	18.3	39.7	51.7	16.6	7.4×10^{-3}
Zoospore release	42.7	7.3	7	12.3	5.5×10^{-4}
Total	61	47	58.7	ns	7.2×10^{-2}

^a Means of assessment of 100 cysts in each of three replicates assessed 7 to 8 h after vortex-encystment of zoospores released into the different solutions.

TABLE 7. Effects of concentration of Peters Peat Lite Special (PLS) fertilizer solution on percentage of germination of zoospore cysts by germ tubes and on release of a further zoospore from nongerminated cysts^a

Experiment and mode of germination	Peters PLS concentration (ppm of N)					5% LSD	P (ANOVA)
	0	100	200	300	400		
Experiment 1							
Germ tube	34	35	...	83.7	...	20.2	1.4×10^{-3}
Zoospore	46.7	18.3	...	1.3	...	15.8	1.2×10^{-3}
Total	80.7	53.3	...	85	...	11.8	1.2×10^{-3}
Experiment 2							
Germ tube	11.3	10.3	22	30.7	32	ns ^b	6.7×10^{-1}
Zoospore	62.5	40	14.5	24.5	21.5	29.44	4.4×10^{-2}

^a Data from two experiments. Means of assessment of 100 cysts in each of three replicates assessed 7 to 8 h after vortex-encystment of zoospores released into the different solutions.

^b No significant difference by analysis of variance, but regression analysis ($r = 0.65$, $P = 8.2 \times 10^{-3}$) showed concentration-dependent suppression of zoospore release.

total 36) seedlings assessed by reisolation of the pathogen from the roots. The cumulative plant deaths over 10 days were 13, 23, and 23, respectively, for the three inoculum levels. As an additional treatment, 20 mM Ca(NO₃)₂ was used in Peters PLS solution containing 10² zoospores/ml. Only two of a total 36 seedlings were infected, and none of them died. The effect of Ca²⁺ in suppressing infection from 10² zoospores/ml was highly significant ($\chi^2 = 28.66$, $P < 0.001$).

A second experiment with blocks of 16 seedlings and 10² zoospores/ml (not shown) confirmed the suppressive effect of calcium: 22 of a total 48 seedlings were infected (12 deaths) when flood-irrigated with Peters PLS solution (150 ppm of N), but only four of a total 48 seedlings were infected (two deaths) when the Peters PLS solution was amended with 20 mM Ca(NO₃)₂. The suppression of infection by calcium was highly significant ($\chi^2 = 11.11$, $P < 0.001$).

In two further experiments (Table 9), the zoospores (10²/ml) were either motile or pre-encysted by vortex treatment. In Peters PLS solution (150 ppm of N) alone, the seedlings were infected from both motile and encysted spores, but amendment of Peters PLS solution with 20 mM Ca(NO₃)₂ almost completely suppressed infection from motile spores ($P < 0.001$ by χ^2 in each experiment) and reduced infection from pre-encysted spores. Even 10 mM Ca(NO₃)₂ was suppressive in the one experiment in which it was used. None of the 324 uninoculated control seedlings in the four experiments became infected. There was no significant difference in plant dry weight between uninoculated controls irrigated with Peters PLS solution (150 ppm of N) alone or containing 20 mM Ca(NO₃)₂ (mean dry weights, 43.1 and 42.0 mg, respectively; $P = 0.71$).

DISCUSSION

The 16 representative experiments reported here show that Ca²⁺ amendments in the range of 10 to 30 meq in either water or Peters PLS fertilizer solution significantly affect several stages of the production and behavior of *P. parasitica* zoospores. The effects of Ca²⁺ on the different stages were analyzed separately, but they were cumulative, so manipulation of Ca²⁺ has the potential to reduce markedly the availability of inoculum for primary infection and secondary spread of infection by *P. parasitica* in irrigation systems. This was confirmed by the dramatic reduction of infec-

tion of vinca seedlings when even low levels of Ca²⁺ were added to fertilizer solution in a simulated flood irrigation system. The effects of other cations were not tested in this study, but the findings for Ca²⁺ are important because they demonstrate that this ion, which is essential for normal zoospore behavior (6,8,9,13,14,32), can profoundly interfere with zoospore biology when supplied at supra-optimal levels for the fungus.

Calcium salts in the range of 10 to 40 mM had a concentration-dependent effect in reducing the release of zoospores from sporangia. Yet Ca²⁺ did not visibly suppress the cleavage of sporangial contents—a necessary prelude to zoospore release—and high Ca²⁺ concentrations even enhanced sporangial cleavage. Thus, high Ca²⁺ levels might have interfered with the later stages of zoospore differentiation in sporangia or with the process of zoospore release that occurs by dissolution of the sporangial papillum (15). In any case, this suppressive effect of Ca²⁺ on release from sporangia was paralleled by suppression of release of a further zoospore from zoospore cysts.

The higher concentrations of Peters PLS fertilizer solution (300, 400, and 450 ppm of N) also suppressed zoospore discharge from sporangia, but the fertilizer concentrations more commonly used for containerized ornamental crops (100 to 200 ppm of N) either had no effect on zoospore discharge or enhanced it compared with that of water controls. This stimulatory effect of low salt concentrations on release of zoospores from sporangia is commonly reported (12,14). It was negated by the addition of 10 or 20 meq of Ca²⁺ to Peters PLS solution (150 ppm of N), suggesting that calcium amendment of either irrigation water or commercial nutrient solution should reduce the production of secondary inoculum from primary infections of plants.

In addition to suppression of zoospore production, Ca²⁺ concentrations of 20 meq or more curtailed the motility of zoospores in a commercial fertilizer solution, causing them to encyst. This effect of Ca²⁺ on zoospore encystment is also reported for *P. cinnamomi* (2). Ca²⁺ amendment, therefore, should diminish the role of zoospores in locating the preferred infection sites on roots (6,35) and reduce the autonomous movement or passive transport of zoospores through particulate materials (24,33) or root mats (16). Furthermore, the curtailment of zoospore motility would influence the distribution of inoculum in irrigation systems, be-

TABLE 8. Effects of CaCl₂ or Ca(NO₃)₂ in Peters Peat Lite Special fertilizer solution (150 ppm of N) on percentage of germination of zoospore cysts by germ tubes and on release of a further zoospore from nongerminated cysts^a

Mode of germination	Concentration (mM) of CaCl ₂ or Ca(NO ₃) ₂					5% LSD	P (ANOVA)
	0	10 Cl	10 NO ₃	20 Cl	20 NO ₃		
Germ tube	18.0	30.0	32.67	41.33	62.67	19.11	4.6 × 10 ⁻³
Zoospore	28.0	7.67	6.67	3.0	1.67	5.09	2.6 × 10 ⁻⁶
Total	46.0	37.67	39.33	44.33	64.33	17.18	3.9 × 10 ⁻³

^a Means of assessment of 100 cysts in each of three replicates assessed 7 to 8 h after vortex-encystment of zoospores released into the different solutions.

TABLE 9. Numbers of vinca seedlings infected (and plant deaths in parentheses) 10 days after blocks of 16 seedlings were flood-irrigated with Peters Peat Lite Special fertilizer solution (150 ppm of N), with or without Ca(NO₃)₂, and containing either motile zoospores or vortex-encysted zoospores (10² cells/ml)

Experiment and replicate	Zoospore inoculum			Cyst inoculum		
	Peters alone	Peters + 20 meq of Ca	Peters + 10 meq of Ca	Peters alone	Peters + 20 meq of Ca	Peters + 10 meq of Ca
Experiment 1						
1	16 (3)	0 (0)	...	16 (0)	2 (0)	...
2	16 (3)	0 (0)	...	16 (2)	3 (1)	...
3	16 (2)	1 (0)	...	15 (0)	4 (1)	...
χ^2	43.18 ($P < 0.001$)			24.44 ($P < 0.001$)		
Experiment 2						
1	5 (2)	0 (0)	0 (0)	6 (3)	1 (0)	3 (3)
2	6 (3)	1 (0)	0 (0)	2 (0)	2 (2)	1 (0)
3	9 (3)	1 (1)	2 (1)	2 (1)	0 (0)	0 (0)
χ^2	33.5 ($P < 0.001$)			na ^a		

^a Not applicable; insufficient counts for χ^2 analysis.

cause zoospores of *Phytophthora* spp. swim towards the surface of a liquid (3,4), whereas cysts settle out of suspension, as confirmed here for *P. parasitica*. This might be exploited in the design of recirculating systems.

High levels of Ca^{2+} applied soon after encystment can trigger the production of germ tubes from zoospore cysts of *P. cinnamomi* (2), *P. parasitica* (32), *Pythium* spp. (8), and *Aphanomyces* spp. (5). For *P. cinnamomi*, this effect was unique to Ca^{2+} among all the cations tested (2), but for *Pythium*, the effect of Ca^{2+} could be mimicked by Mg^{2+} or Sr^{2+} , which act as surrogates for Ca^{2+} in several physiological processes (8). Previously, we reported that Ca^{2+} applied post-encystment could markedly suppress the release of a further zoospore from cysts of *P. parasitica* that had not produced germ tubes (32). Here we show the same effects of Ca^{2+} on cysts derived from zoospores that were released initially into solutions of different calcium content. The production of a germ tube in the absence of a host or organic nutrients would lead to energy depletion of the propagules (21). The suppression of further zoospore release from ungerminated cysts would ensure that these propagules do not contribute to further infection.

As a test of the practical relevance of these findings, Ca^{2+} at 20 meq was shown significantly and markedly to reduce infection of containerized vinca seedlings from zoospores in a flood-irrigated system employing Peters PLS fertilizer solution. The effect was consistent in each of four experiments. Even 10 meq of Ca^{2+} was effective in the one experiment in which it was tested, and 20 meq of Ca^{2+} also significantly reduced infection from encysted zoospore inoculum. The design of these experiments ensured that relatively high levels of viable inoculum were introduced into the bases of plugs of peat containing susceptible seedlings, and the criterion for infection was reisolation of the pathogen from any part of a seedling root system. Thus, these were rigorous tests of the effectiveness of Ca^{2+} in suppressing infection in a simulated ebb and flow irrigation system. Moreover, the tests employed a standard inoculum concentration across treatments, so they did not take account of any additional suppressive effect of Ca^{2+} on zoospore release from sporangia.

Peters PLS contains no calcium so that it can be used in water from sources that differ in water quality. The calcium levels in water used for ornamentals are usually much lower than 20 meq; typically the Ca^{2+} level is less than 2 meq, although 7 meq is not uncommon for hydroponically grown vegetables in which $\text{Ca}(\text{NO}_3)_2$ is used as a nitrogen source. In short term experiments, we found no adverse effect of Ca^{2+} on growth of vinca seedlings at the Ca^{2+} levels effective for disease control. But high Ca^{2+} levels could affect plants if applied continuously, especially for plants grown in peat mixes, which accumulate salts with time (7,34). This accumulation, however, could be an advantage in reducing the total amount of Ca^{2+} applied, or the time over which it needs to be applied. Currently, no fungicides are registered for application through irrigation water; surfactants also are not registered for use in disease control. Fungicides can be applied as drenches to ornamentals in containerized media, but with the risk that pathogens could develop fungicide resistance during constant exposure to low doses.

Manipulation of calcium levels with multifactorial and cumulative effects on zoospore inoculum could be part of a disease management strategy for calcium-tolerant crops. It is relevant to infection in recirculating irrigation systems, in peat-based media, and probably also in soil. Indeed, some of the modes of action of Ca^{2+} on *P. parasitica*, demonstrated here, could help to explain the many reports of calcium-mediated suppression of disease caused by *Phytophthora* and other zoosporic fungi.

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