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Suppressed germination and early death of *Phytophthora infestans* sporangia caused by pectin, inorganic phosphate, ion chelators and calcium-modulating treatments

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Abstract

In laboratory experiments, chemical treatments were applied to sporangia of *Phytophthora infestans* incubated at 12 °C, conducive to cytoplasmic cleavage and release of zoospores (indirect germination), and at 20 °C, conducive to germination by hyphal outgrowth (direct germination). Both types of germination were suppressed by applying increasing concentrations (1–5 mM) of $CaCl_2$ or $MgCl_2$, or by low concentrations of pectin, inorganic phosphate, chelators (EGTA, BAPTA), calcium channel-blockers (lanthanum, gadolinium, verapamil) or compounds that interfere with intracellular calcium-mediated processes (trifluoperazine, caffeine). The suppression by some treatments was partly overcome by adding Ca^{2+} or Mg^{2+} in the early stages of incubation, but Ca^{2+} was usually more effective than Mg^{2+}, and suppression at 20 °C was more easily overcome than at 12 °C. Pectin (0.1%) or BAPTA (5 mM) caused rapid death of sporangia at both 12 ° and 20 °, whereas EGTA (5 mM) or Na_2HPO_4 (5 mM) caused rapid death only at 12 °. The findings indicate that germinability and viability of *P. infestans* sporangia are strongly affected by the external availability of Ca^{2+} or other divalent cations, especially during zoosporogenesis.

Introduction

Phytophthora infestans (Mont.) de Bary, the cause of potato late blight, is one of the most important pathogens in agricultural history (Lucas et al., 1991). The disease swept through eastern USA in 1843-5 and through Europe in 1845, devastating potato crops and precipitating the great Irish Famine of 1845-6. More recently, potato blight has been controlled by a combination of plant breeding, disease forecasting and fungicide applications. But, coinciding with the appearance of the A2 mating type of *P. infestans* in Europe (Shattock et al., 1990), giving the potential for sexual recombination, the pathogen population has developed widespread resistance to the phenylamide fungicides that controlled it effectively in the 1980s (Davidse et al., 1991). There is urgent need to develop alternative and more durable strategies for control of this disease. As one approach to this, we have focused on the pre-infection behaviour of *P. infestans*, following our studies on pre-infection behaviour of other *Phytophthora*, *Pythium* and *Aphanomyces* spp. (Donaldson and Deacon, 1992; Deacon and Donaldson, 1993; von Broembsen and Deacon, 1996, 1997; Deacon and Saxena, 1998). Biflagellate zoospores are thought to have a major role as infective propagules of many of these fungi, including *P. infestans* (Pristou and Gallegly, 1954; Wilson and Coffey, 1980; Gees and Hohl, 1988). The zoospores are released after cytoplasmic cleavage in sporangia, accumulate at infection sites by chemotaxis or other mechanisms, then encyst and adhere before the cysts germinate with precise orientation to initiate infection (Deacon and Donaldson, 1993).

Studies on several *Pythium* and *Phytophthora* spp. indicate that Ca^{2+} has an important role in all stages of the pre-infection sequence (Irving and Grant, 1984; Irving, Griffith and Grant, 1984; Grant, Griffith and Irving, 1986; Iser et al., 1989; Donaldson and Deacon, 1992, 1993; von Broembsen and Deacon, 1996; Jackson and Hardham, 1996). The practical significance

of these findings was demonstrated recently by Ca^{2+} interference of infection by *Phytophthora parasitica* in nutrient irrigation systems (von Broembsen and Deacon, 1997). However, there is no equivalent information for *P. infestans* or the related downy mildew fungi that are dispersed as wind- or water-borne sporangia and that initiate infection either by hyphal outgrowth from the sporangia (direct germination) or by cytoplasmic cleavage in the sporangium to release zoospores (indirect germination).

In work reported here, we exploited the temperature-dependent differentiation of *P. infestans* sporangia, which germinate by hyphal outgrowth at warmer temperatures (20-25 °C) but release zoospores at 10–13 °C, simulating the cool, moist conditions which favour infection of potato leaves in field conditions (Harrison, 1992). For the two modes of germination in laboratory experiments, we compared the effects of treatments that might interfere with calcium-mediated processes. We show that the fungus is extremely sensitive to disruption by environmentally safe chemicals, including treatments that can raise or lower the external concentration of calcium or some other divalent cations.

Materials and methods

Fungal culture and production of sporangia

A laboratory stock culture (EU-PI_1) of *P. infestans* from a diseased potato tuber was maintained at 16-17 °C on rye grain infusion agar (Caten and Jinks, 1968). Sporangia were harvested by flooding 10–14-day-old cultures with sterile distilled water (SDW) and scraping the hyphal mat to dislodge the sporangia. The sporangial suspension was filtered through fine nylon mesh to remove large hyphal aggregates, then washed twice with SDW, using Whatman No. 3 filter paper to retain the sporangia. They were finally resuspended in SDW at approximately 10^4 sporangia ml^{-1}.

General experimental procedures

Aliquots (100 or 200 μl) of freshly harvested sporangial suspensions, prepared with SDW at 20 ° or SDW pre-chilled to 12 °, were incubated in 1.5 ml Eppendorf tubes at these temperatures to induce direct germination by hyphal outgrowth (20 °) or indirect germination by cytoplasmic cleavage and release of zoospores (12 °). Assessments of germination were made usually after overnight incubation (16 h), although indirect germination was initiated within 2 h, and direct germination within 4 h. For sampling, the tubes were inverted several times to mix the contents, and aliquots were examined microscopically at × 100 magnification. Indirect germination was assessed as the number of empty sporangia with a dissolved discharge papillum; direct germination as the number of sporangia with a hyphal outgrowth from the region of the papillum, at least equal to the sporangium length. All experiments involved at least three replicate tubes, and all counts were based on at least 100 sporangia (often > 200) in each replicate. The counts did not include the low percentage ($< 10\%$) of visibly damaged or abnormally shaped sporangia, nor the few sporangia (typically $< 2\%$) that germinated abnormally by indirect germination at 20 ° or by direct germination at 12 °. In some experiments the reduction of sporangial viability in response to treatments was assessed by the number of sporangia with coagulated or disrupted contents, in contrast to healthy sporangia which exhibited normal protoplasmic fluidity. These assessments were made at × 700 magnification, using a video camera attachment to the microscope (Jones, Donaldson and Deacon, 1991) and using slides with strips of tape to prevent the coverslip from crushing the sporangia.

Treatments

Eppendorf tubes containing 100 or 200 μl sporangial suspension received an equivalent volume of sterile distilled water (controls) or treatment solution adjusted to pH 7 by addition of NaOH. Treatments for various experiments included ethyleneglycol-bis(β-aminoethyl)N,N,N',N'-tetraacetic acid (EGTA, Sigma), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA, Sigma), citrus pectin (Sigma), sodium alginate (BDH Chemicals), D-galacturonic acid (Sigma), Na_2HPO_4, $CaCl_2$, $MgCl_2$, $BaCl_2$, NaCl, $LaCl_3$, $GdCl_3$ (all BDH Chemicals), trifluoperazine, verapamil and caffeine (all ICN Biomedicals), at final concentrations stated in the text. For attempted rescue from suppression of germination, 100 μl $CaCl_2$ or $MgCl_2$ solution or 100 μl water (control) was added at different times to tubes containing 100 μl sporangia suspension and 100 μl treatment solution.

Statistical analysis

Results for most experiments are presented as means with SE of the mean, because analysis of variance

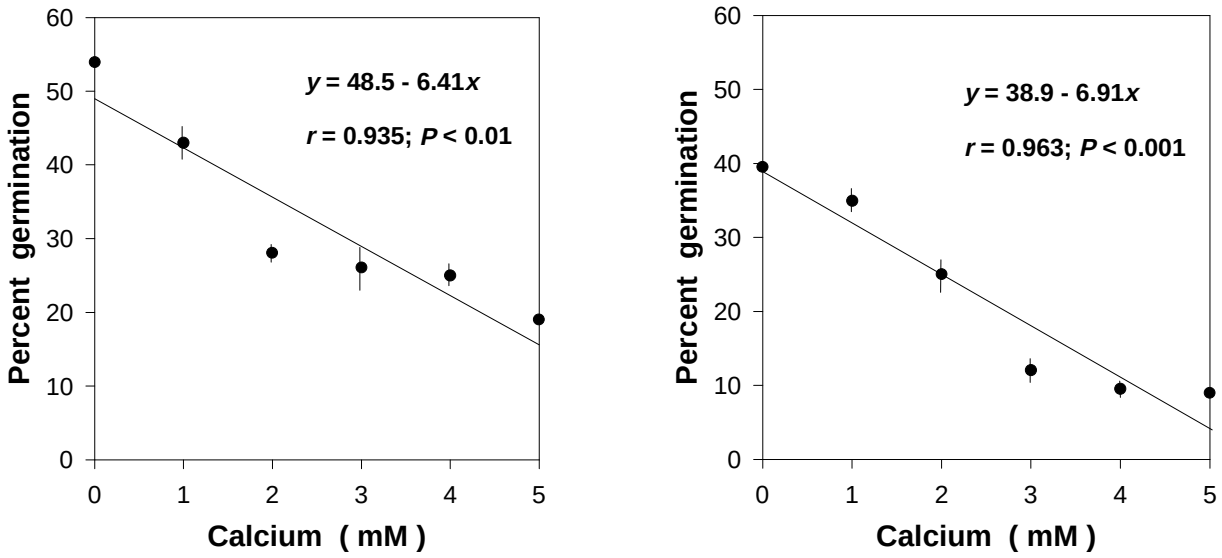

Figure 1. Effect of Ca^{2+} concentration on sporangial germination by hyphal outgrowth (left, 20 °C) or zoospore release (right, 12 °C); data points are means ± SE of 3 replicates, based on counts of 100 sporangia in each replicate *.
* Mg^{2+} supplements had similar effects, the regression equations being y = 48.8- 7.85x (r = 0.970, P <0.001) for germination by hyphal outgrowth and y = 36.4 - 6.06x (r = 0.967, P < 0.001) for germination by zoospore release.

would be biased by the inclusion of 'zero' data. Data from dosage response experiments were analysed by regression. Sigmoid curves for time course of sporangial death were analysed by regression after transforming percentage death to probits, and time (min) to $\log_{10}$ (min + 1).

Results

Effect of temperature on sporangial germination

Initial experiments with a range of temperatures (4, 12, 16, 20, 27 °C) established that 12 °C was near optimal for indirect germination (typically > 60%, with < 2% direct germination) and 20 °C for direct germination (c. 40%, with < 2% indirect germination). Indirect germination reached a maximum within 2-3 h, whereas direct germination typically required 4 h or more. However, 30-40% of sporangia failed to germinate at either temperature.

Effect of divalent cations on sporangial germination

Calcium and magnesium at final concentrations as low as 5 mM significantly reduced both direct and indirect germination (Figure 1). Barium (not shown) similarly reduced direct germination but was not tested for indirect germination. In contrast, sodium was much less active, with no effect on direct germination at 10 mM and it only reduced indirect germination by 50% at 10 or 15 mM (not shown).

Table 1. Inhibition of direct (at 20 °) and indirect (at 12 °) germination of *P. infestans* sporangia by calcium-modulating treatments. Means ± SEM of three replicates, assessed after 16h, based on counts of at least 100 sporangia in each replicate

Treatment	Percent germination	
	Direct (hyphal outgrowth)	Indirect (zoospore discharge)
Water (control)	54.3 ± 0.9	19.1 ± 0.4
Lanthanum, 1 mM	1.0 ± 0.6	0.6 ± 0.6
Verapamil, 30 μM	4.3 ± 0.8	12.6 ± 2.0
Trifluoperazine, 5 μM	1.5 ± 0.2	0

Effects of potential calcium-modulating treatments

Germination of sporangia at either 12 ° or 20 ° was reduced to less than 5% by low concentrations of the chelator BAPTA (2 mM or less), by the calcium channel blockers lanthanum (50 μM or less) and gadolinium

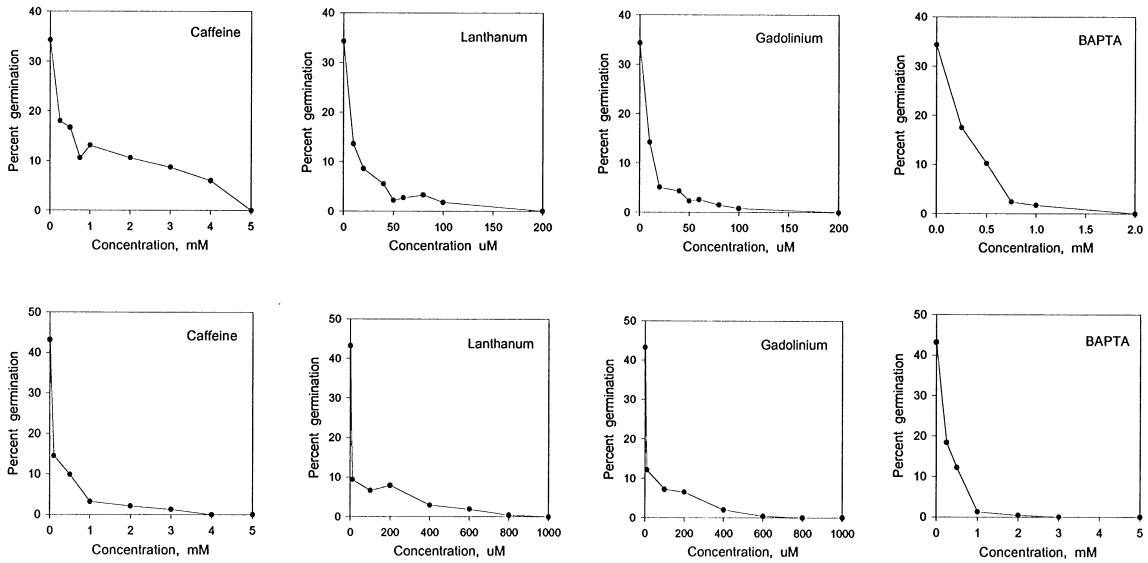

Figure 2. Effects of the calcium modulators, caffeine, lanthanum, gadolinium and BAPTA, on sporangial germination of *P. infestans*. Top row, direct germination (20 °C); bottom row, indirect germination (12 °C). Data points are means of three replicates, based on counts of at least 100 sporangia in each replicate; standard errors (not shown) were always $< \pm$ 5%.

(100 μM or less), and by caffeine (3 mM or less) which reportedly depletes the calcium in intracellular stores (Figure 2). The chelator EGTA at 2 mM concentration (not shown) reduced both direct and indirect germination from 40–45% (controls) to 4.5% but was not tested at lower concentrations. In separate tests, La^{3+} and verapamil (Ca^{2+} channel blockers) and trifluoperazine (TFP, a calmodulin antagonist) also severely reduced sporangial germination (Table 1). In all these assays a proportion of the sporangia were seen to be damaged irreversibly by the treatments: either the entire sporangial content was coagulated, with no cytoplasmic movement, or regions of the contents exhibited Brownian motion. This contrasted with the normal protoplasmic fluidity of untreated sporangia. To quantify this, sporangia incubated at either 12 ° or 20 ° with La^{3+} (1 mM), verapamil (30 μM) or TFP (5 μM) were sampled periodically for microscopic assessment (Figure 3). The data of Figure 3, when transformed to probits of cell death against logarithm (time + 1 min), gave highly significant straight-line relationships, with estimated times for 50% sporangial death ranging from 20–30 min (TFP) to an extrapolated value of more than 3 h for La^{3+} at 20 °C (Table 2).

Attempted rescue of germination-suppression caused by chelators

The reduction of sporangial germination by either Ca^{2+} supplements or chelating compounds indicated that sporangia were highly sensitive to the level of Ca^{2+} or other divalent cations in the external environment. To test this, sporangia were incubated with either EGTA or BAPTA (5 mM), and 5 mM $CaCl_2$ or $MgCl_2$ was added at different times to try to reverse the suppression of germination (Table 3). Although the cations were partly suppressive when used alone, they also partly relieved the suppression caused by EGTA. Ca^{2+} also substantially relieved the suppression of direct germination (at 20 °) or indirect germination (at 12 °) caused by BAPTA, but Mg^{2+} was only effective for direct germination. Both cations were most effective in relieving the suppression when added simultaneously with the chelators, or within the first 10–20 min; they had little effect when added after 45 min (Table 3).

Effects of uronates and phosphate

Inorganic phosphate and various uronates were tested, in the expectation that they could influence the availability of Ca^{2+} in the sporangial environment. In initial tests at 20 °C (Figure 4) pectin at 0.05% (w/v) significantly reduced germination and it completely

Table 2. Regressions of straight-line plots of probit (percent death of sporangia) against log (min + 1) for the curves in Figure 3, with estimated times (min + 1) for 50% death (probit value 5) of sporangia caused by calcium-modulators

	Correlation coefficient	Probability of regression (P)	Regression equation	Estimated time (min + 1) for 50% death
Direct germination				
Lanthanum	0.955	6.25×10^{-5}	y = 0.760x + 3.276	185.5*
Verapamil	0.955	6.31×10^{-5}	y = 1.140x + 3.084	47.9
Trifluoperazine	0.951	7.95×10^{-5}	y = 1.219x + 3.167	31.9
Indirect germination				
Lanthanum	0.942	1.46×10^{-4}	y = 1.341x + 2.318	109.9
Verapamil	0.947	1.08×10^{-4}	y = 1.090x + 2.798	104.8
Trifluoperazine	0.986	1.07×10^{-6}	y = 1.376x + 3.160	21.7

* extrapolated

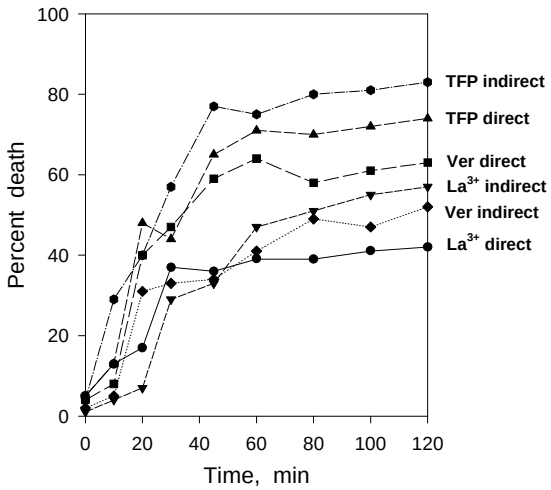

Figure 3. Time course of sporangial death caused by the Ca^{2+} modulators, trifluoperazine (TFP, 5 μm), verapamil (Ver, 30 μm) and lanthanum (1 mM), when sporangia were incubated to induce direct germination (20 °C) or indirect germination (12 °C). Data points are assessments of 100 sporangia at each time.

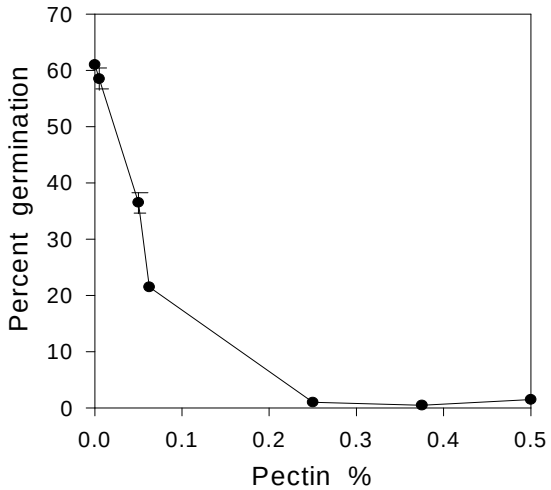

Figure 4. Effect of pectin concentration on direct germination of *P. infestans* sporangia (20 °C), means ± SEM of 2 replicates.

suppressed germination at 0.25%. So, pectin was used at a standard 0.1% concentration for comparison with other uronates (0.1%) or 5 mM (0.07%) Na_2HPO_4 (Table 4). Pectin strongly suppressed both direct and indirect germination, but sodium alginate was much less suppressive, and D-galacturonic acid (not shown) had no effect on indirect germination and stimulated direct germination. Na_2HPO_4 (5 mM) strongly suppressed indirect germination, but only partly suppressed direct germination. When Ca^{2+} or Mg^{2+} (5 mM) was added simultaneously with the treatments the cations partly reduced the suppression by pectin, alginate or Na_2HPO_4. Overall, Ca^{2+} was more effective than Mg^{2+}, and suppression of direct germination was overcome more than was suppression of indirect germination (Table 4). A delay of more than 10 min in applying the cations caused a reduction in their effectiveness, especially for overcoming the suppression of indirect germination.

The effects of uronates, Na_2HPO_4, EGTA and BAPTA on sporangial death were compared in a repeat experiment by periodic sampling of sporangia incubated at 12 ° and 20 ° (Figure 5). Control sporangia incubated in water showed consistently low death (usually $< 10\%$), whereas all the treated sporangia exhibited at least 37% death after 120 min. However, there

Table 3. Inhibition of direct (at 20 °) and indirect (at 12 °) germination of *P. infestans* sporangia by the calcium-chelators, EGTA or BAPTA (5 mM), and by 5 mM $CaCl_2$ or $MgCl_2$ added alone or at different times after the chelators. Means ± SEM of three replicates, assessed after 16 h, based on counts of at least 100 sporangia in each replicate. Composite table for 4 experiments, with their respective controls

Treatment	Percent germination	
	Direct (20 °)	Indirect (12 °)
Water (control)	38.6 ± 1.7	21.3 ± 1.1
Ca^{2+} 5 mM	12.2 ± 3.0	10.6 ± 1.7
EGTA 5 mM	0.5 ± 0.3	1.3 ± 0.2
EGTA + Ca^{2+} 0 min	12.9 ± 1.3	15.4 ± 0.3
+ Ca^{2+} 10 min	10.8 ± 1.0	8.2 ± 1.5
+ Ca^{2+} 20 min	10.9 ± 1.7	2.3 ± 0.5
+ Ca^{2+} 45 min	5.8 ± 2.2	1.0 ± 0.6
Water (control)	48.8 ± 2.5	53.1 ± 4.5
Mg^{2+} 5 mM	28.9 ± 0.2	11.9 ± 1.8
EGTA 5 mM	0.5 ± 0.2	1.6 ± 0.3
EGTA + Mg^{2+} 0 min	12.4 ± 2.0	10.7 ± 2.3
+ Mg^{2+} 10 min	17.0 ± 4.0	10.8 ± 1.7
+ Mg^{2+} 20 min	9.0 ± 1.2	4.5 ± 1.2
+ Mg^{2+} 45 min	6.4 ± 0.7	1.3 ± 0.5
Water (control)	60.3 ± 2.2	47.2 ± 5.5
BAPTA	0.4 ± 0.4	0
BAPTA + Ca^{2+} 0 min	51.1 ± 3.2	27.3 ± 3.1
+ Ca^{2+} 10 min	49.2 ± 4.3	24.2 ± 2.4
+ Ca^{2+} 20 min	31.2 ± 3.0	16.3 ± 0.7
+ Ca^{2+} 45 min	14.6 ± 7.2	2.3 ± 0.5
Water (control)	63.1 ± 5.4	51.1 ± 3.1
BAPTA	0.3 ± 0.3	0
BAPTA + Mg^{2+} 0 min	40.4 ± 2.6	3.8 ± 0.8
+ Mg^{2+} 10 min	41.9 ± 2.7	0.7 ± 0.5
+ Mg^{2+} 20 min	29.8 ± 8.0	0.7 ± 0.4
+ Mg^{2+} 45 min	7.8 ± 2.3	0

Table 4. Inhibition of direct (at 20 °) and indirect (at 12 °) germination of *P. infestans* sporangia by pectin, sodium alginate or Na_2HPO_4, and effects of 5 mM $CaCl_2$ or $MgCl_2$ added to the treatments at different times. Means ± SEM of three replicates, assessed after 16 h, based on counts of at least 100 sporangia in each replicate. Composite table for 2 experiments, with their respective controls

Treatment	Percent germination	
	Direct (20 °)	Indirect (12 °)
Water (control)	37.0 ± 3.4	31.2 ± 1.1
Pectin 0.1%	0	0
+ Ca^{2+} 5 mM, 0 min	14.0 ± 8.5	4.6 ± 1.2
+ Ca^{2+} 5 mM, 20 min	5.4 ± 2.5	3.4 ± 0.6
+ Ca^{2+} 5 mM, 45 min	0	2.3 ± 0.4
Water (control)	51.0 ± 1.7	25.1 ± 0.8
Pectin 0.1%	0	0
+ Mg^{2+} 5 mM, 0 min	18.6 ± 2.1	4.8 ± 1.3
+ Mg^{2+} 5 mM, 20 min	8.8 ± 1.1	7.7 ± 0.8
+ Mg^{2+} 5 mM, 45 min	7.1 ± 0.5	1.1 + 0.6
Water (control)	37.0 ± 3.4	31.2 ± 1.1
Alginate 0.1%	22.3 ± 2.2	16.3 ± 5.2
+ Ca^{2+} 5 mM, 0 min	37.4 ± 4.2	14.6 ± 2.3
+ Ca^{2+} 5 mM, 20 min	47.0 ± 1.9	14.9 ± 1.8
+ Ca^{2+} 5 mM, 45 min	44.6 ± 2.4	5.8 ± 1.4
+ Mg^{2+} 5 mM, 0 min	30.0 ± 2.6	13.7 ± 1.4
+ Mg^{2+} 5 mM, 20 min	28.7 ± 3.2	4.1 ± 1.5
+ Mg^{2+} 5 mM, 45 min	13.2 ± 1.4	6.1 ± 0.7
Water (control)	37.0 ± 3.4	31.2 ± 1.1
Na_2HPO_4 5 mM	20.3 ± 3.1	0
+ Ca^{2+} 5 mM, 0 min	27.8 ± 1.8	14.1 ± 2.6
+ Ca^{2+} 5 mM, 20 min	36.8 ± 3.0	9.8 ± 0.6
+ Ca^{2+} 5 mM, 45 min	25.0 ± 7.4	12.1 ± 3.4
+ Mg^{2+} 5 mM, 0 min	19.7 ± 0.1	2.5 ± 0.7
+ Mg^{2+} 5 mM, 20 min	28.3 ± 2.0	2.2 ± 0.6
+ Mg^{2+} 5 mM, 45 min	17.5 ± 1.0	2.2 ± 0.8

were major differences between the treatments, and there was evidence of interaction between treatments and temperature of incubation. At 20 ° (conducive to direct germination) pectin (0.1%) and BAPTA (5 mM) caused rapid onset of cell death, whereas EGTA (5 mM), Na_2HPO_4 (5 mM), D-galacturonic acid (5 mM) and sodium alginate (0.1%) caused less death. At 12 °C (conducive to indirect germination) pectin, BAPTA, EGTA and Na_2HPO_4 all caused rapid cell death, but D-galacturonic acid and sodium alginate caused less death. The estimated times for 50% sporangial death derived from log (time + 1 min)/ probit plots (Table 6) show that, except for pectin and BAPTA which were the most toxic treatments, sporangia were more sensitive to disruption by treatments at 12 ° than at 20 °. In other words, sporangia were more sensitive to disruption when induced to undergo cytoplasmic cleavage than to germinate by hyphal outgrowth.

Discussion

The major finding in this work is that, in laboratory conditions, sporangia of *P. infestans* are highly sensitive to mild chemical treatments that either raise or lower the external concentration of Ca^{2+} or other divalent cations. The treatments not only suppressed sporangial

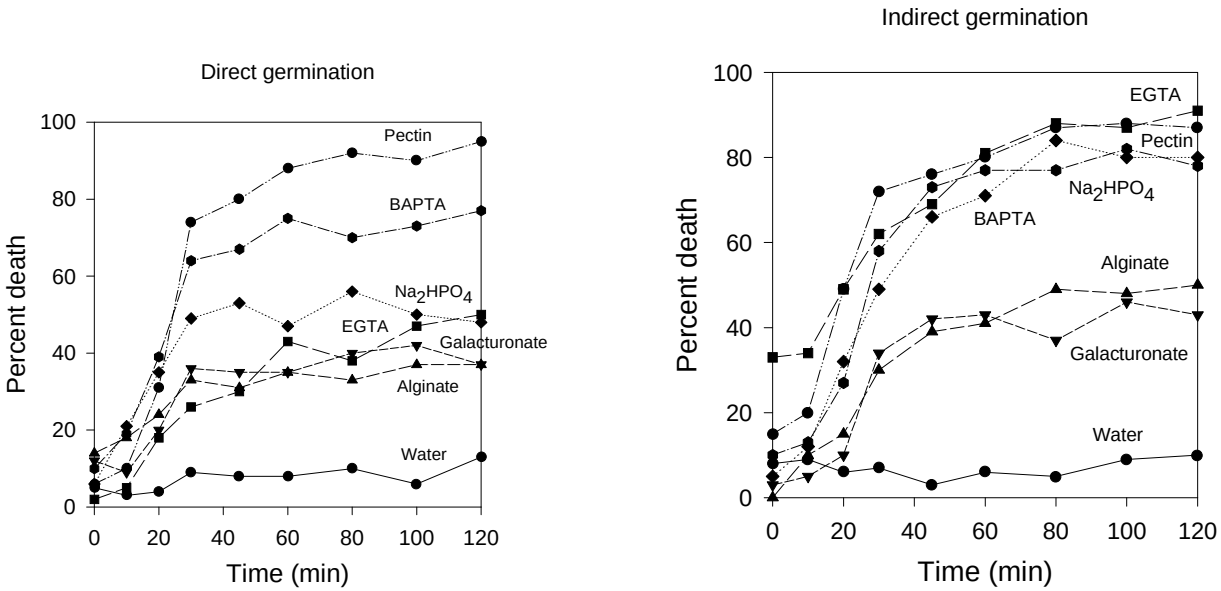

Figure 5. Time course of sporangial death caused by pectin (0.1%), sodium alginate (0.1%), D-galacturonic acid (0.1%), BAPTA (5 mM), EGTA (5 mM) or Na_2HPO_4 (5 mM). Sporangia were incubated to induce direct germination (20 °C) or indirect germination (12 °C). Data points are assessments of 100 sporangia at each time.

Table 5. Regressions of straight-line plots of probit (percent death of sporangia) against log (min + 1) for the curves in Figure 5, with estimated times (min + 1) for 50% death (probit value 5) of sporangia caused by treatments

	Correlation coefficient	Probability of regression (P)	Regression equation	Estimated time (min + 1) for 50% death
Direct germination				
EGTA	0.959	4.54×10^{-5}	y = 1.062x + 2.727	138*
Alginate	0.945	1.24×10^{-4}	y = 0.394x + 3.848	839*
Na_2HPO_4	0.951	7.95×10^{-5}	y = 0.815x + 3.500	69
BAPTA	0.942	1.44×10^{-4}	y = 1.076x + 3.518	24
Pectin	0.918	4.89×10^{-4}	y = 1.709x + 2.881	17
D-Galacturonate	0.838	4.77×10^{-3}	y = 0.548x + 3.604	353*
Indirect germination				
EGTA	0.882	1.64×10^{-4}	y = 0.935x + 4.100	9
Alginate	0.991	2.52×10^{-7}	y = 1.526x + 2.022	89
Na_2HPO_4	0.946	1.13×10^{-4}	y = 1.383x + 2.993	28
BAPTA	0.902	8.71×10^{-4}	y = 1.219x + 3.305	25
Pectin	0.929	2.92×10^{-4}	y = 1.217x + 3.607	14
D-Galacturonate	0.900	9.52×10^{-4}	y = 0.982x + 2.859	151*

* Extrapolated

germination but also, where tested, caused rapid loss of sporangial viability. The treatments were generally more effective in conditions conducive to zoospore release (12 °C) than to germination by hyphal outgrowth (20 °C). These findings suggest a possible new approach to control of *P. infestans* by the use of environmentally safe chemicals, compatible with recent reports on *Phytophthora parasitica* (von Broembsen and Deacon, 1996, 1997). However, we used only one isolate of *P. infestans*, so future work should explore a wider range of isolates and the effects of treatments

applied to leaf surfaces, where the nutritional conditions might be different from those *in vitro*.

Calcium has a central role in intracellular signalling processes of fungi and other eukaryotic cells. In previous work, the use of pharmacological drugs and other Ca^{2+}-modulating treatments suggested that normal zoospore motility and the germination of *Pythium* and *Phytophthora* zoospore cysts depend on the availability of external Ca^{2+}, the functioning of membrane Ca^{2+} channels and the release of Ca^{2+} from intracellular stores (Irving and Grant, 1984; Donaldson and Deacon, 1992, 1993; Reid, Morris and Gow, 1995). However, excessive Ca^{2+} levels in nutrient irrigation solutions can suppress zoospore release from sporangia, curtail zoospore motility, prevent further zoospore release (diplanetism) from zoospore cysts and significantly reduce root infection of containerized seedlings by *P. parasitica* (von Broembsen and Deacon, 1997). Ca^{2+} is known to have a role in flagellar function (Bloodgood, 1991) and cells have a complex homeostatic system which maintains a low intracellular free Ca^{2+} concentration (Miller, Vogg and Sanders, 1990) to prevent Ca^{2+} toxicity. A transient rise in the cytosolic Ca^{2+} level can act as a trigger for differentiation or gene expression, and this has been shown to be required for cytoplasmic cleavage to produce zoospores in sporangia of *Phytophthora cinnamomi* (Jackson and Hardham, 1996). However, excessively high external Ca^{2+} concentrations might disrupt the normal homeostasis, which is regulated by release of Ca^{2+} from intracellular stores and by uptake or release of Ca^{2+} through the cell membrane.

We have now shown that sporangia of *P. infestans* are sensitive to several Ca^{2+}-modulating treatments. The suppression of germination by caffeine (Figure 2) suggests a role for intracellular Ca^{2+} stores, because caffeine reportedly depletes these stores (Tsien and Tsien, 1990). Similarly, the suppression by La^{3+}, Gd^{3+} and verapamil (Figure 2; Table 1) suggests a role for Ca^{2+} channel activity (Godfraind, Miller and Wibo, 1986) and the inhibition by trifluoperazine (Table 1) indicates the involvement of calmodulin in sporangial germination (Bereza, Brewer and Mizukami, 1982). We also show that high external levels of Ca^{2+}, Mg^{2+} or Ba^{2+} partly suppress germination of *P. infestans* sporangia (Figure 1; Table 3, and text). The suppressive levels (2–5 mM) are much higher than normal ambient levels, and might have caused excessive Ca^{2+} uptake or Ca^{2+}-induced Ca^{2+} release from intracellular stores (Berridge and Irvine, 1989).

The effects of chelators and other treatments on sporangia are more difficult to interpret. EGTA and BAPTA have a high binding affinity for Ca^{2+} but are not Ca^{2+}-specific; they also have a high affinity for Zn^{2+}, Mn^{2+} and Fe^{2+} (Youatt, 1993; Loukin and Kung, 1995). Thus, we cannot exclude the possibility that the chelators suppressed sporangial germination by removing ions other than Ca^{2+} from the bathing medium. Changes in the external ionic balance are reported to affect the germination of *Phytophthora* zoospore cysts (Grant, Griffith and Irving, 1986). In an attempt to overcome these difficulties, we used Mg^{2+} as well as Ca^{2+} to try to rescue sporangia from chelator-induced suppression. EGTA and BAPTA have very low affinity for Mg^{2+}, so this ion should not displace chelated Ca^{2+} or Zn^{2+} (Tsien, 1980). The finding that Mg^{2+} was sometimes as effective as Ca^{2+} in partly reversing the effects of chelators or other treatments (Tables 3, 4) is consistent with reports that Mg^{2+} can substitute for Ca^{2+} in some other cellular processes: to initiate trout sperm swimming (Boitano and Omoto, 1992), to trigger germination of pre-encysted zoospores of *Pythium* (Donaldson and Deacon, 1993) and to reverse the EGTA-imposed suppression of *Pythium* cyst germination (Donaldson and Deacon, 1993). However, Mg^{2+} was not always as effective as Ca^{2+} in overcoming suppression of sporangial germination; for example, it was not effective in reversing suppression by BAPTA or Na_2HPO_4 at 12 °C, although it was effective at 20 °C. These temperature-differential effects indicate complex interactions that require further study.

One of the most interesting findings was that sporangia of *P. infestans* lost viability rapidly in response to treatments (EGTA, BAPTA, pectin, Na_2HPO_4) that can sequester external Ca^{2+} or other polyvalent cations. For some treatments (EGTA, Na_2HPO_4) the effect was more drastic at 12 °C than at 20 °C, indicating that sporangia were particularly susceptible to disruption when induced to undergo cytoplasmic cleavage for zoospore production. The generally poorer ability of Ca^{2+} or Mg^{2+} to reverse suppression of sporangial germination at 12 °C than at 20 °C (Tables 3, 4) is further evidence of the extreme sensitivity of sporangia induced to undergo cytoplasmic cleavage.

Some of the treatments used here might have sequestered Ca^{2+} or other cations within the sporangial wall, leading to changes in structural stability or, indirectly, to changes in intracellular events. There is evidence for Ca^{2+}-binding sites on sporangia of the downy mildew pathogen, *Pseudoperonospo-*

ra humuli, because Griffin and Coley-Smith (1975) found that streptomycin, which behaves as a divalent cation in solution, was adsorbed by sporangia and could not be removed by water washes, but up to 95% of the antibiotic could be displaced by addition of Ca^{2+}. Other divalent metal cations also could displace the adsorbed streptomycin, but Mg^{2+} was much less effective than Ca^{2+}. Beakes and Gay (1980) found that sub-lethal concentrations of streptomycin inhibited zoospore cleavage and the release of zoospores from sporangia of *Saprolegnia* spp., similar to its effects on sporangia of *P. humuli* (Griffin and Coley-Smith, 1971). They interpreted this as interference with Ca^{2+} function. In our study the suppression of germination and rapid loss of sporangial viability caused by treatments such as pectin or BAPTA would be compatible with interference of Ca^{2+} or other cation function on or near the cell surface. The roles of Ca^{2+} in plant cell walls have been reviewed in depth (Sinclair and Trewavas, 1997) but the physiological roles of wall Ca^{2+} have received little study in fungi.

Pectin at even low concentrations ($< 0.1\%$) was remarkably suppressive to sporangial germination and also caused rapid loss of viability at both 12 ° and 20 °. Its effects were significantly, but only weakly, reversed by simultaneous addition of either Ca^{2+} or Mg^{2+}. At equivalent concentrations to pectin, D-galacturonic acid was non-inhibitory, and sodium alginate was only weakly suppressive, the effect on direct germination being strongly overcome by Ca^{2+} or Mg^{2+}. Pectin might have had a direct effect on sporangia, in addition to a role in sequestering Ca^{2+}. For example, pectin causes rapid encystment of motile zoospores of *Phytophthora* spp. (Grant, Irving and Radda, 1985; Zhang et al., 1990). Na_2HPO_4 was strongly suppressive to both direct and indirect germination, and was the only treatment for which Mg^{2+} was essentially ineffective in reversing the inhibition. Woloshuk et al. (1991) found that 25 meq HPO_4^- was required for complete inhibition of direct sporangial germination by *P. infestans* at pH 7, and 50 meq HPO_4^- at pH 6 in a nutritionally complex medium. Grant, Grant and Harris (1992) found almost complete inhibition of mycelial growth of two isolates of *P. infestans* at 1-5 mM phosphate in a nutritionally defined medium of pH 6.5. Other *Phytophthora* spp. were much more tolerant of high phosphate levels. Our germination assays were in nutrient-deprived conditions, which might explain why phosphate and other treatments caused dramatic reductions of germination. Experiments are in progress to assess the effects of these treatments on leaf surfaces.

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