Role of calcium in adhesion and germination of zoospore cysts of *Pythium*: a model to explain infection of host plants

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Newly-formed zoospore cysts of *Pythium aphanidermatum* adhered to glass and germinated without a further trigger, but both adhesion and germination were suppressed by EGTA. Older (10 min) cysts adhered and germinated poorly unless supplied with Ca^{2+} . Adhesion and germination were linked but separable, because young cysts germinated when dislodged from glass after 10 min, and older cysts kept in suspension germinated in response to high (7–10 mM) concentrations of Ca^{2+} , Mg^{2+} or Sr^{2+} . Other cations (Li^+ , Na^+ , K^+ , Ba^{2+} , Mn^{2+} , Fe^{3+} , Cu^{3+}) had no effect up to 25 mM concentration or were toxic. The naturally low germination of suspended cysts was suppressed by EGTA (chelator), La^{3+} or verapamil (Ca^{2+} entry blockers), amiloride (Ca^{2+} flux inhibitor), trifluoperazine or dibucaine (calmodulin antagonists), TMB-8 (intracellular Ca^{2+} antagonist) or A23187 (Ca^{2+} ionophore). Suppression by TMB-8 or A23187 was not relieved by any post-treatment. Suppression by EGTA was relieved only by divalent cations. Suppression by all other compounds was relieved only by specific L-amino acids that stimulated germination of control cysts (asparagine, aspartic acid and glutamic acid but not lysine or glutamine, where tested). Alanine relieved suppression by verapamil or dibucaine in *Pythium dissotocum* but not *P. aphanidermatum*. The findings indicated a central role of Ca^{2+} (presumably released during encystment) in adhesion and germination, such that zoospores would germinate autonomously by a Ca^{2+} -mediated cascade when they dock and encyst on roots with the fixed point of germination adjacent to the host. Amino acids are not essential but apparently synergize this process by species-specific receptor-binding which facilitates Ca^{2+} uptake or Ca^{2+} signalling.

Introduction

Zoospores of soil-borne Oomycetes (*Pythium*, *Phytophthora*, *Aphanomyces* spp.) are attracted to roots by chemotaxis and then undergo a developmental sequence leading to infection (Deacon, 1988). They search the root surface for a suitable site, then dock, encyst and adhere with precise orientation, so that the germ-tube emerges from a pre-determined point next to the host and immediately initiates penetration (Mitchell & Deacon 1986a; Hardham & Gubler, 1990; Jones *et al.*, 1991). Several stages of this sequence can be simulated *in vitro* and some of the potential host-derived triggers or elicitors have been identified (e.g. Hinch & Clarke, 1980; Mitchell & Deacon, 1986b, c; Longman & Callow, 1987; Jones *et al.*, 1991). However, it is unclear whether the

stages of the sequence are necessarily under separate control or are parts of a cascade. This is important because the success of infection depends on completion of the sequence.

Early in encystment, zoospores release glycoprotein by exocytosis of vesicles (Sing & Bartnicki-Garcia, 1975; Gubler & Hardham, 1988, 1991; Estrada-Garcia *et al.*, 1990). The glycoprotein forms the cyst coat beneath which the cyst wall is formed. It also serves as a non-specific adhesive, attaching the newly formed cyst to the host or other types of surface. In *Phytophthora cinnamomi* the glycoprotein released over the germination site is immunologically distinct from that over the rest of the cyst (Hardham & Gubler, 1990). An immunological difference was not found in *Pythium aphanidermatum* (Estrada-Garcia *et al.*, 1990). Nevertheless, both fungi germinate from the same region, corresponding to the former ventral groove of the zoospore (Paktitis *et al.*, 1986; Jones *et al.*, 1991). For both, therefore, the functional adhesive *in vivo* is that which is deposited over the germination site.

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Abbreviations: SDW, sterile distilled water; TFP, trifluoperazine dihydrochloride; TMB-8, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride.

Calcium has been implicated in adhesion of *Ph. cinnamomi* (Gubler *et al.*, 1989). Newly-formed cysts were naturally adhesive, but this property was lost on addition of EGTA, which chelates Ca^{2+} . Conversely, cysts lost their natural adhesiveness if they did not contact a surface within a few minutes, but this could be restored by addition of Ca^{2+} . It was proposed that the glycoprotein becomes adhesive when it interacts with Ca^{2+} (Gubler *et al.*, 1989), which was shown to be released in the early stages of encystment of *Ph. palmivora* (Irving *et al.*, 1984). The loss of adhesiveness in the absence of a surface was ascribed to dispersion of this released Ca^{2+} or, perhaps, its reabsorption. High levels of Ca^{2+} also induced encystment followed by 'spontaneous' germination of *Phytophthora* spp. *in vitro*, whereas most other ions induced encystment without germination (Byrt *et al.*, 1982; Irving & Grant, 1984; Grant *et al.*, 1986). These findings suggest that Ca^{2+} could provide the link in a possible cascade initiated by docking. In order to establish this, it is necessary to show a direct relationship between calcium, adhesion and germination, and also, ideally, to account for the roles of other factors such as root-derived amino acids which influence the infection sequence (Jones *et al.*, 1991). We have now done this for *Pythium* spp. *in vitro*, and the results suggest a simple model of Ca^{2+} -mediated autoregulation.

Methods

Fungal isolates and zoospore production. *Pythium aphanidermatum* (Edson) Fitzp. (isolate CBS 634.70) was used for most experiments, as in earlier work (Mitchell & Deacon, 1986a, b, c; Jones *et al.*, 1991). The cysts germinate in response to L-asparagine, L-glutamic acid or L-aspartic acid but not other amino acids. *Pythium dissotocum* Drechs. and *P. catenulatum* Matthews were also used; the cultures were supplied by M. E. Stanghellini (see Goldberg *et al.*, 1989). The cysts germinate in response to L-asparagine, L-glutamic acid, L-aspartic acid, L-alanine and (*P. catenulatum* only) L-glutamine. All fungi were grown at 25 °C on plates of V8 agar comprising Campbells V8 juice (100 ml), distilled water (900 ml) and Oxoid no. 3 agar (20 g). For production of zoospores, agar blocks (15 cm × 15 cm) were cut from colonies respectively 3, 5 and 7 d old and placed, six per dish, in 9 cm Petri dishes containing 20 ml sterile distilled water (SDW). Zoospores were released when cultures were incubated on a laboratory bench (16–20 °C) and used after about 6 h, when there were at least 10^5 zoospores ml^{-1} . Unless otherwise stated, the suspensions were standardized by dilution with SDW to 2×10^4 zoospores ml^{-1} .

Encystment, adhesion and germination. Encystment was induced when an Eppendorf tube of zoospore suspension was held against a vortex mixer for 70 s (Tokunaga & Bartnicki-Garcia, 1971). The suspension was then incubated at room temperature in Eppendorf tubes or after transfer at different times to the wells of glass depression slides. Treatments were applied as detailed below; all supplements were adjusted to pH 7 with NaOH before use. Germination was assessed after 1.5 h, using a Leitz Orthoplan microscope with a video camera attachment (Jones *et al.*, 1991). Cells were scored as germinated when the germ-tube length at least equalled the cyst diameter (approx. 10 µm). Adhesion was assessed by comparing numbers of cells on a

slide before and after rinsing, described below. Then the slides were usually re-incubated in a humid chamber and re-assessed after 16 h.

Statistical methods. All treatments were replicated at least five times and, unless otherwise stated, 100 cysts were assessed for each replicate. Percentage adhesion or germination was recorded as mean $\pm$ standard error of the mean (SEM). Statistical comparisons were made by paired *t*-tests (Snedecor & Cochran, 1980) which are more sensitive than conventional *t*-tests. Pairing was necessary because the time constraints for assessment of germination meant that each experiment had to be done five or more times with a single replicate each time.

Relationship between adhesion and germination. Aliquots (80 µl) of cyst suspension were transferred to well slides immediately (t_0) or 10 min (t_{10}) after vortexing to induce encystment, from the same position in an Eppendorf tube. In each case, control slides immediately received 80 µl SDW; treatment slides immediately received 80 µl CaCl_2 solution or 80 µl EGTA solution or (t_0 samples only) 80 µl EGTA after 10 min delay. Each supplement was used at 7 mM final concentration. The number of cells on each slide and the number that had germinated were scored after 1.5 h. Then the liquid with non-adherent cells was removed by pipette, 160 µl SDW was added and similarly removed, this was repeated, and 160 µl SDW was finally added. Slides were then reassessed for the numbers of remaining cells and the numbers of these that had germinated.

To determine the time at which cysts lost their adhesiveness, aliquots of cyst suspension were transferred to slides immediately (t_0) or at 1 min intervals up to 10 min. Either EGTA solution (final concn 7 mM) or SDW was added, and germination was scored after 2 h.

The effect of dislodgement of cysts on germination was tested with cyst suspension (160 µl, 2×10^5 cysts ml^{-1}) that was added immediately to slides and rinsed with SDW after 10 min to remove non-adherent cysts. Then SDW (160 µl) was added and the wells were scraped with a sharp scalpel to dislodge some cysts. Eighty microlitres of water containing dislodged cysts was added to a fresh slide. The remaining water was removed from each original slide, leaving only attached cysts, and replaced with 80 µl SDW. Both sets of slides (representing detached and attached cells, respectively) were scored for germination after 1.5 h, then rinsed and scored for residual adhesion, using a video camera to locate the same microscope fields. The same experiment was done with cysts added to slides 10 min after vortexing but immediately rinsed to remove non-adherent cysts (the majority).

Effects of ions and EGTA on germination of suspended cysts. Immediately after vortexing, cyst suspensions (150 µl) of three *Pythium* spp. in Eppendorf tubes received an equal volume of SDW (control) or solutions of CaCl_2 , MgCl_2 , SrCl_2 , MnCl_2 , BaCl_2 , KCl , NaCl , LiCl , FeCl_3 or CrCl_3 , each at 1, 5, 7, 10 and 25 mM final concentration. The contents were mixed by inversion three times, and germination was scored after 1.5 h by transferring the 150 µl samples to a well slide. The slides were reassessed after 16 h in a humid chamber. In a similar experiment, fresh cysts in Eppendorf tubes received one of the following (7 mM final concentration): EGTA, EGTA + CaCl_2 , EGTA + MgCl_2 , EGTA + SrCl_2 , EGTA + KCl , EGTA + NaCl , SDW (control). The salts and EGTA had been mixed and incubated in solution for 24 h before use. The same treatments were also applied to motile zoospore suspension in well slides; both percentage encystment and percentage germination were assessed after 1.5 h and 16 h.

Effect of lanthanum on germination. Cysts of *P. aphanidermatum* (0.5 ml) were incubated in Eppendorf tubes for 10 min. Then 0.5 ml SDW (control) or 0.5 ml LaCl_3 solution (1.5 mM final concentration) was added and mixed by inversion. After a further 10 min (t_{20}) 80 µl aliquots were then transferred to further tubes which received the following (final concentrations): CaCl_2 (10 mM), SrCl_2 (10 mM), MgCl_2 (10 mM), CaCl_2 (10 mM) + LaCl_3 (1.5 mM), LaCl_3 (1.5 mM), MEP (3% malt extract and 1% peptone), SDW (control). Each treatment (SDW

Table 1. Relationship between adhesion and germination of cysts of *Pythium aphanidermatum*

Cysts produced by agitation of zoospores were transferred to glass slides immediately (t_0) or after 10 min (t_{10}). CaCl_2 or EGTA (7 mM final concn) was added to the slides immediately or after a 10 min delay. Percentage germination and adhesion after 1.5 h are shown as mean $\pm$ SEM for seven replicates.

	Cyst age (min) at transfer, and treatment:						
	t_0	t_{10}	$t_0 + \text{Ca}^{2+}$	$t_{10} + \text{Ca}^{2+}$	$t_0 + \text{EGTA}$	$t_{10} + \text{EGTA}$	$t_0 + \text{EGTA}$ delayed
No of spores	188 $\pm$ 30	171 $\pm$ 29	185 $\pm$ 16	150 $\pm$ 19	143 $\pm$ 13	147 $\pm$ 15	162 $\pm$ 18
Germination (%)	59 $\pm$ 7	25 $\pm$ 5	59 $\pm$ 6	54 $\pm$ 5	10 $\pm$ 3	20 $\pm$ 4	57 $\pm$ 5
Adhesion (%)	59 $\pm$ 7	30 $\pm$ 7	61 $\pm$ 5	62 $\pm$ 5	21 $\pm$ 4	21 $\pm$ 3	53 $\pm$ 5
Germination (%) after washing	69 $\pm$ 5	51 $\pm$ 5	65 $\pm$ 7	63 $\pm$ 4	48 $\pm$ 8	51 $\pm$ 7	70 $\pm$ 5
Germinated cysts retained after washing (%)	71 $\pm$ 5	62 $\pm$ 8	67 $\pm$ 6	75 $\pm$ 5	75 $\pm$ 6	61 $\pm$ 6	68 $\pm$ 7
Non-germinated cysts retained after washing (%)	47 $\pm$ 7	23 $\pm$ 8	47 $\pm$ 3	45 $\pm$ 8	11 $\pm$ 3	12 $\pm$ 2	38 $\pm$ 6

or LaCl_3) thus had seven post-treatments. Germination was scored after 1.5 h by transferring the tube contents to wetted slides, and reassessed after 16 h on the slides. In a similar experiment, cysts were centrifuged at t_{20} (3 min in a microfuge at 15000 r.p.m.), the pellet was rinsed twice with SDW, then resuspended in SDW before post-treatment.

In identical experiments, t_{10} cysts in Eppendorf tubes were treated with LaCl_3 (1.5 mM final concn), EGTA (7 mM final concn) or SDW. At t_{20} , aliquots of these suspensions were post-treated with (mM final concn): CaCl_2 (10), L-asparagine (10), L-aspartic acid (10), L-glutamic acid (10), L-glutamine (10), L-lysine (10), LaCl_3 (1.5), EGTA (7) or SDW.

Effects of calcium modulators. t_{10} cysts in Eppendorf tubes were treated with SDW (control) or calcium modulators, then (t_{20}) post-treated with 10 mM- CaCl_2 , MgCl_2 or SrCl_2 or with individual L-amino acids (10 mM) known to stimulate or not to stimulate germination. The calcium modulators (Sigma; final concentration in parentheses) were verapamil hydrochloride (30 μM), amiloride hydrochloride (20 μM), dibucaine hydrochloride (50 μM), TFP (5 μM), TMB-8 (30 μM) and A23187 (5 μM). All except A23187 were dissolved in water and adjusted to pH 7 with NaOH. A23187 was initially dissolved in dimethylsulphoxide (DMSO) and then diluted to 0.05% (v/v) DMSO in water. DMSO alone at this concentration had no effect on germination.

Results

Cysts were easily pipetted from Eppendorf tubes, and direct comparisons of samples at 0 and 10 min after encystment showed no significant difference in retrieval (Table 1). Cysts incubated in the tubes were therefore considered to be suspended (non-adhered).

Relationship between adhesion and germination

Cysts transferred to slides immediately after encystment (t_0) germinated and adhered significantly better than did cysts (t_{10}) transferred after 10 min (Table 1). Addition of EGTA to t_0 cysts immediately after transfer reduced their germination and adhesion, but EGTA had no effect on t_0 cysts when added 10 min later. Ca_2^{+} had no effect

on t_0 cysts, but it markedly increased both germination and adhesion of t_{10} cysts. There was thus a consistent association between adhesion and germination, and Ca_2^{+} or other divalent cations seemed to mediate both processes.

A delay of up to 4 min in transfer of cysts to slides had no adverse effect on germination (Table 2); but a delay of 7 min or more significantly reduced germination. These times also marked a change in sensitivity to EGTA, which suppressed germination of cysts transferred up to 4 min but had no effect at 6 min or later. Consistent with these findings, cysts (t_0) that had been allowed to adhere to slides for 10 min could be physically dislodged without loss of germination, even though they did not readhere in large numbers (Table 3). The experiments indicated that competence to germinate was influenced by events in the first few minutes after encystment, was sensitive to interference by EGTA in this time and was associated with adhesion to a surface.

Effects of ions and EGTA on germination of suspended cysts

Cysts of three *Pythium* spp. held in suspension in Eppendorf tubes showed a significant increase in germination in response to Ca^{2+} , Mg^{2+} or Sr^{2+} at 7–10 mM but not to K^{+} or Na^{+} at even 25 mM (Table 4). Of the three divalent cations, Sr^{2+} was generally least effective in stimulating germination, especially at intermediate concentrations. In identical tests (not shown) Li^{+} and Mn^{2+} caused no significant response; Ba^{2+} , Fe^{3+} and Cr^{3+} caused many cysts to lyse and, even at 1 mM, significantly reduced germination of the intact cysts.

EGTA significantly suppressed the germination of suspended cysts compared with that in water controls (Table 5). This suppression was relieved by simultaneous addition of divalent cations but not by K^{+} or Na^{+} . When

Table 2. Effect of EGTA on germination of zoospore cysts of *Pythium aphanidermatum*

Cysts produced by agitation of zoospores were transferred to glass slides at different times and incubated for 2 h in distilled water or with EGTA (final concentration 7 mM)

Time of transfer (min)	Germination (%)†		Significance of difference‡
	Distilled water	EGTA	
0	69 ± 5.8	14 ± 2.7	***
1	71 ± 5.3	14 ± 2.9	***
2	71 ± 3.9	18 ± 3.6	***
3	70 ± 3.0	18 ± 2.8	***
4	70 ± 3.0	25 ± 3.2	***
5	50 ± 5.9	33 ± 4.1	*
6	37 ± 5.8	35 ± 5.7	NS
7	25 ± 2.4	26 ± 4.8	NS
8	30 ± 2.7	31 ± 3.8	NS
9	26 ± 2.1	26 ± 2.8	NS
10	28 ± 1.3	31 ± 3.7	NS

† Mean ± SEM for nine replicate slides; 100 cysts were assessed for each replicate.

‡ Significance of difference between columns (paired *t*-test): ***, 0.001; *, 0.02; NS, not significant.

Table 3. Germination of adherent spores of *Pythium aphanidermatum* after detachment from glass slides

Spores encysted by agitation were added immediately (t_0) or after 10 min (t_{10}) to glass slides. Some of the adherent cells were scraped from the slides and reincubated on fresh slides. Germination and adhesion were assessed after 1.5 h (mean ± SEM for seven replicates, based on 100 cysts each).

Spore age and treatment	Germination (%)	Adhesion (%)
t_0		
Undisturbed	62 ± 7.0	65 ± 6.2
Detached, transferred to new slides	57 ± 5.2	27 ± 3.2
t_{10}		
Undisturbed	25 ± 4.9	29 ± 3.1
Detached, transferred to new slides	23 ± 3.2	29 ± 4.2

the same treatments were applied to motile spores on slides, EGTA alone or with any salt caused encystment of spores by 1.5 h whereas the spores remained motile in water (not shown). All spores had encysted by 16 h, when EGTA, EGTA + Na⁺ or EGTA + K⁺ had significantly suppressed germination, but EGTA + Ca²⁺ significantly enhanced germination over that in water controls. EGTA + Mg²⁺ significantly enhanced germination only by *P. aphanidermatum* and *P. dissotocum*, and EGTA + Sr²⁺ only by *P. aphanidermatum*.

These experiments showed that adhesion was not necessary for germination if an appropriate divalent cation was supplied at high concentration, and Mg²⁺ or Sr²⁺ often substituted for Ca²⁺ in this respect.

Effect of lanthanum on germination

La³⁺ displaces Ca²⁺ from superficial binding sites and also blocks transmembrane fluxes of Ca²⁺ (Godfraind *et al.*, 1986). When added to suspended cysts after 10 or 20 min, La³⁺ significantly suppressed germination to below the level in water controls (Table 6). This suppression was not relieved by post-treatment with Ca²⁺ or Sr²⁺. It was relieved by Mg²⁺, but only to the level in water controls, whereas Mg²⁺ significantly stimulated germination in the absence of La³⁺ (Table 6). La³⁺-suppression was relieved by a mixture of malt extract and peptone, which stimulated germination to the same level as it did in the absence of La³⁺. Identical results (not shown) were found when the cysts were centrifuged, and the pellet was rinsed and resuspended before the post-treatments were applied. La³⁺ should be removable by washing, but the rinses may not have been sufficient for this.

In further experiments (Table 7) suspended cysts were pretreated with La³⁺ or EGTA and then post-treated with L-amino acids known to stimulate germination (asparagine, aspartic acid and glutamic acid) or not to stimulate germination (lysine, glutamine) of *P. aphanidermatum* (Jones *et al.*, 1991). Other post-treatments were included for comparison. The results were identical whether or not the cells were rinsed before post-treatment. Suppression by EGTA was reversed only by Ca²⁺ (the only ion tested), whereas suppression by La³⁺ was reversed only by the 'stimulatory' amino acids, not by Ca²⁺.

Effects of calcium modulators

Germination of suspended cysts of *P. aphanidermatum* was significantly suppressed (relative to water controls) by addition of verapamil, dibucaine, TFP, amiloride, TMB-8 or A23187 at t_{10} or t_{20} (Table 8). In every case the suppression was not relieved by post-treatment with Ca²⁺, Mg²⁺ or Sr²⁺, although these ions significantly enhanced germination when added to controls. Lysine (non-stimulatory) did not relieve the suppression, but asparagine (stimulatory) reversed the effects of verapamil, dibucaine, TFP and amiloride. In a further experiment, a combination of verapamil (30 µM), dibucaine (50 µM), amiloride (20 µM), TFP (5 µM) and La³⁺ (0.75 mM) significantly reduced percentage germination from 24 ± 2.2 (controls) to 5.0 ± 1.4. Asparagine reversed this suppression (27.8 ± 3.6 per cent) but did not stimulate germination as it did with control cysts (65.0 ± 5.5). No post-treatment reversed the inhibition caused by TMB-8 or A23187 (Table 8).

Species-specificity of rescue by amino acids was found in a comparison of *P. aphanidermatum* with *P. dissotocum*

Table 4. *Effects of cations on germination of cysts held in suspension*

Zoospores of three *Pythium* spp. were encysted by agitation, dispensed to Eppendorf tubes and treated with water (control) or CaCl_2 , MgCl_2 , SrCl_2 , NaCl or KCl at 1, 5, 7, 10 or 25 mM final concentration. Percentage germination after 1.5 h and 16 h is shown as mean $\pm$ SEM for seven replicates, 100 cysts each.

Treatment	Concn* (mM)	Germination (%)					
		<i>P. aphanidermatum</i>		<i>P. catenulatum</i>		<i>P. dissotocum</i>	
		1.5 h	16 h	1.5 h	16 h	1.5 h	16 h
Ca^{2+}	0	38 $\pm$ 5.2	44 $\pm$ 4.1	18 $\pm$ 1.6	24 $\pm$ 2.1	23 $\pm$ 2.3	37 $\pm$ 2.9
	1	36 $\pm$ 4.1	42 $\pm$ 5.0	16 $\pm$ 2.6	23 $\pm$ 3.9	28 $\pm$ 3.9	35 $\pm$ 3.3
	5	47 $\pm$ 4.9	51 $\pm$ 6.1	20 $\pm$ 1.6	30 $\pm$ 3.1	25 $\pm$ 2.8	33 $\pm$ 2.1
	7	72 $\pm$ 5.0	74 $\pm$ 5.0	29 $\pm$ 3.9	40 $\pm$ 5.5	35 $\pm$ 3.2	45 $\pm$ 3.8
	10	72 $\pm$ 6.1	77 $\pm$ 4.8	51 $\pm$ 6.9	57 $\pm$ 5.9	72 $\pm$ 4.2	76 $\pm$ 3.6
	25	71 $\pm$ 4.3	74 $\pm$ 3.8	53 $\pm$ 4.2	59 $\pm$ 4.1	63 $\pm$ 3.3	70 $\pm$ 3.0
Mg^{2+}	0	30 $\pm$ 3.4	36 $\pm$ 3.1	20 $\pm$ 2.6	28 $\pm$ 2.4	28 $\pm$ 2.5	32 $\pm$ 3.0
	1	29 $\pm$ 3.4	34 $\pm$ 3.2	21 $\pm$ 2.6	28 $\pm$ 2.4	30 $\pm$ 3.5	38 $\pm$ 3.8
	5	36 $\pm$ 5.8	40 $\pm$ 5.7	20 $\pm$ 2.5	27 $\pm$ 2.4	28 $\pm$ 2.4	34 $\pm$ 2.0
	7	48 $\pm$ 5.8	56 $\pm$ 4.9	30 $\pm$ 2.8	43 $\pm$ 4.4	37 $\pm$ 3.6	46 $\pm$ 2.8
	10	67 $\pm$ 7.6	70 $\pm$ 6.7	49 $\pm$ 6.2	57 $\pm$ 5.4	57 $\pm$ 4.6	66 $\pm$ 4.9
	25	71 $\pm$ 3.8	76 $\pm$ 2.4	59 $\pm$ 6.3	65 $\pm$ 4.6	57 $\pm$ 3.5	64 $\pm$ 4.0
Sr^{2+}	0	39 $\pm$ 3.7	43 $\pm$ 5.0	22 $\pm$ 1.9	30 $\pm$ 3.2	23 $\pm$ 3.0	29 $\pm$ 3.0
	1	38 $\pm$ 2.8	42 $\pm$ 3.2	22 $\pm$ 3.1	32 $\pm$ 2.1	21 $\pm$ 3.6	28 $\pm$ 3.4
	5	46 $\pm$ 4.5	52 $\pm$ 3.6	19 $\pm$ 2.4	25 $\pm$ 2.1	22 $\pm$ 2.7	27 $\pm$ 2.4
	7	50 $\pm$ 5.3	58 $\pm$ 5.4	19 $\pm$ 2.3	29 $\pm$ 2.8	27 $\pm$ 2.5	32 $\pm$ 2.7
	10	60 $\pm$ 6.7	65 $\pm$ 5.7	34 $\pm$ 4.5	44 $\pm$ 4.2	53 $\pm$ 4.4	61 $\pm$ 4.5
	25	72 $\pm$ 3.3	78 $\pm$ 2.8	54 $\pm$ 4.5	60 $\pm$ 4.6	49 $\pm$ 6.0	56 $\pm$ 5.8
$\text{Na}^+/\text{K}^{\dagger}$	0	38 $\pm$ 3.1	43 $\pm$ 3.8	22 $\pm$ 3.0	33 $\pm$ 3.3	23 $\pm$ 2.2	29 $\pm$ 2.8
	1	36 $\pm$ 6.8	41 $\pm$ 4.6	26 $\pm$ 3.7	32 $\pm$ 3.2	23 $\pm$ 2.5	31 $\pm$ 1.6
	5	43 $\pm$ 6.1	47 $\pm$ 5.9	21 $\pm$ 3.9	30 $\pm$ 3.7	23 $\pm$ 3.3	31 $\pm$ 2.1
	7	41 $\pm$ 3.1	48 $\pm$ 1.7	26 $\pm$ 3.2	33 $\pm$ 3.2	23 $\pm$ 1.9	30 $\pm$ 2.3
	10	40 $\pm$ 6.8	43 $\pm$ 6.2	22 $\pm$ 3.6	30 $\pm$ 4.0	22 $\pm$ 3.1	28 $\pm$ 2.3
	25	40 $\pm$ 7.4	46 $\pm$ 6.8	26 $\pm$ 3.5	31 $\pm$ 3.3	23 $\pm$ 2.1	28 $\pm$ 1.8

* Each test with a cation had a designated water control.

$\dagger$ Combined data: the highest data point for Na^+ or K^+ is shown.

Table 5. *Effects of EGTA with or without ions on cyst germination by three Pythium species*

Cysts produced from zoospores by agitation were treated with EGTA, with or without cations (all at 10 mM final concentration) in Eppendorf tubes. Percentage germination after 1.5 h and 16 h is shown as mean $\pm$ SEM for nine replicates, 100 cysts each.

Treatment	Germination (%)					
	<i>P. aphanidermatum</i>		<i>P. dissotocum</i>		<i>P. catenulatum</i>	
	1.5 h	16 h	1.5 h	16 h	1.5 h	16 h
None (water)	35 $\pm$ 6.7	38 $\pm$ 6.5	20 $\pm$ 2.7	29 $\pm$ 2.3	24 $\pm$ 1.7	30 $\pm$ 2.4
EGTA*	3 $\pm$ 0.8	15 $\pm$ 2.0	5 $\pm$ 1.1	11 $\pm$ 2.0	4 $\pm$ 1.6	15 $\pm$ 1.7
$\text{Na}^+/\text{EGTA}^*$	4 $\pm$ 1.0	13 $\pm$ 2.2	6 $\pm$ 1.3	11 $\pm$ 2.0	5 $\pm$ 1.1	16 $\pm$ 2.0
K^+/EGTA^*	4 $\pm$ 1.3	16 $\pm$ 3.7	6 $\pm$ 1.1	13 $\pm$ 2.4	7 $\pm$ 1.4	16 $\pm$ 3.7
$\text{Ca}^{2+}/\text{EGTA}$	48 $\pm$ 6.5	67 $\pm$ 4.4	37 $\pm$ 4.3	54 $\pm$ 7.1	37 $\pm$ 5.4	55 $\pm$ 5.9
$\text{Mg}^{2+}/\text{EGTA}$	55 $\pm$ 6.8	67 $\pm$ 5.3	28 $\pm$ 4.6	43 $\pm$ 4.2	26 $\pm$ 2.5	45 $\pm$ 5.6
$\text{Sr}^{2+}/\text{EGTA}$	40 $\pm$ 4.8	54 $\pm$ 4.8	25 $\pm$ 2.8	42 $\pm$ 5.0	22 $\pm$ 2.5	41 $\pm$ 3.5

* Significantly different from water controls in all cases.

(Table 9). Asparagine rescued cysts of both fungi from suppression by verapamil or dibucaine, but alanine rescued only *P. dissotocum*, consistent with its ability to stimulate germination of control cysts of this fungus.

Discussion

Pythium and *Phytophthora* are closely related genera, with a fundamentally similar infection sequence from

Table 6. *Effects of lanthanum and post-treatment with other substances on cyst germination by Pythium aphanidermatum*

Zoospores encysted by agitation and held in suspension were treated after 10 min with La^{3+} and post-treated (at 20 min) as shown. Percentage germination after 1.5 h and 16 h is shown as mean $\pm$ SEM for seven replicates, 100 cysts each.

Treatment (1.5 mM)	Post-treatment (10 mM unless stated)	Germination (%)	
		1.5 h	16 h
None	None	19 $\pm$ 2.0	22 $\pm$ 2.9
	Ca^{2+}	47 $\pm$ 4.6	51 $\pm$ 3.6
	Sr^{2+}	36 $\pm$ 3.0	40 $\pm$ 2.7
	Mg^{2+}	39 $\pm$ 3.7	42 $\pm$ 3.5
	La^{3+} (1.5 mM)	4 $\pm$ 1.1	5 $\pm$ 1.3
	La^{3+} (1.5 mM) + Ca^{2+}	7 $\pm$ 1.1	9 $\pm$ 0.9
	MEP*	47 $\pm$ 4.6	47 $\pm$ 2.2
La^{3+}	None	8 $\pm$ 1.3	11 $\pm$ 1.6
	Ca^{2+}	12 $\pm$ 2.4	14 $\pm$ 2.2
	Sr^{2+}	12 $\pm$ 1.3	14 $\pm$ 1.8
	Mg^{2+}	23 $\pm$ 3.3	27 $\pm$ 3.3
	La^{3+} (1.5 mM)	5 $\pm$ 1.2	8 $\pm$ 1.3
	La^{3+} (1.5 mM) + Ca^{2+}	8 $\pm$ 1.0	12 $\pm$ 0.9
	MEP*	40 $\pm$ 7.3	46 $\pm$ 2.2

* MEP, malt extract (3%) + peptone (1%).

zoospores (e.g. Deacon, 1988). Here, *P. aphanidermatum* was found to behave like *Ph. cinnamomi* (Gubler *et al.*, 1989) in adhesion. New cysts naturally adhered to a surface, but this was prevented by EGTA; older cysts lost their adhesiveness in the absence of a surface, but it was restored by addition of Ca^{2+} . In explanation of this, Gubler *et al.* (1989) proposed that Ca^{2+} interacts with cyst coat glycoprotein for adhesion to surfaces. The Ca^{2+} was assumed to be released early in encystment, as Irving *et al.* (1984) showed for *Ph. palmivora*, and loss of adhesiveness with age of non-attached cells probably occurred by dispersion of the released Ca^{2+} .

Ca^{2+} and other divalent cations promote germination by *Phytophthora* spp., as now confirmed for *Pythium*. Here, cysts that had adhered needed no further trigger for germination, but even young (t_0) non-attached cysts required an exogenous factor such as a divalent cation (7–10 mM) or specific amino acids (Tables 4–7). Self-sufficiency for germination was retained during 4–5 min delay in contacting a surface but lost after 7 min delay (Table 2); adhesion for the initial 10 min was sufficient to

Table 7. *Rescue of lanthanum- or EGTA-suppressed germination of cysts of P. aphanidermatum by calcium and amino acids*

Zoospores encysted by agitation were treated 10 min later with La^{3+} or EGTA then post-treated at 20 min as shown. Percentage germination after 1.5 h or 16 h is shown as mean $\pm$ SEM for seven replicates, 100 cysts each, in two separate experiments.

Treatment (mM)	Post-treatment (10 mM unless shown)	Germination (%)			
		Experiment 1		Experiment 2	
		1.5 h	16 h	1.5 h	16 h
None	None	21 $\pm$ 1.4	25 $\pm$ 1.1	20 $\pm$ 1.9	25 $\pm$ 1.7
	EGTA (7 mM)	8 $\pm$ 0.8	10 $\pm$ 0.8	9 $\pm$ 1.6	13 $\pm$ 2.2
	La^{3+} (1.5 mM)	9 $\pm$ 0.8	12 $\pm$ 1.0	9 $\pm$ 1.0	15 $\pm$ 1.3
	Ca^{2+}	61 $\pm$ 4.6	64 $\pm$ 5.1	57 $\pm$ 5.3	63 $\pm$ 3.9
	Asparagine	71 $\pm$ 4.8	75 $\pm$ 3.4	53 $\pm$ 5.3	61 $\pm$ 5.2
	Glutamine	23 $\pm$ 1.8	25 $\pm$ 1.3	—	—
	Glutamic acid	—	—	46 $\pm$ 4.2	53 $\pm$ 4.7
	Aspartic acid	—	—	48 $\pm$ 3.9	56 $\pm$ 4.7
	Lysine	—	—	18 $\pm$ 2.8	28 $\pm$ 1.5
	EGTA (7)	8 $\pm$ 1.0	11 $\pm$ 1.0	11 $\pm$ 0.9	15 $\pm$ 1.5
	EGTA (7 mM)	9 $\pm$ 0.8	12 $\pm$ 0.7	10 $\pm$ 0.9	14 $\pm$ 1.3
EGTA (7)	La^{3+} (1.5 mM)	7 $\pm$ 0.8	9 $\pm$ 0.6	7 $\pm$ 1.0	12 $\pm$ 1.3
	Ca^{2+}	56 $\pm$ 5.4	60 $\pm$ 4.5	38 $\pm$ 3.5	42 $\pm$ 3.7
	Asparagine	10 $\pm$ 1.0	13 $\pm$ 1.6	13 $\pm$ 2.1	19 $\pm$ 2.4
	Glutamine	9 $\pm$ 0.8	13 $\pm$ 1.8	—	—
	Glutamic acid	—	—	10 $\pm$ 1.4	16 $\pm$ 1.0
	Aspartic acid	—	—	14 $\pm$ 1.9	17 $\pm$ 2.2
	Lysine	—	—	8 $\pm$ 1.1	14 $\pm$ 1.9
	La^{3+} (1.5)	9 $\pm$ 0.9	12 $\pm$ 1.3	10 $\pm$ 1.0	14 $\pm$ 1.5
	EGTA (7 mM)	6 $\pm$ 0.5	8 $\pm$ 0.8	9 $\pm$ 0.8	14 $\pm$ 1.8
	La^{3+} (1.5 mM)	6 $\pm$ 1.1	9 $\pm$ 0.9	8 $\pm$ 1.0	13 $\pm$ 1.6
	Ca^{2+}	9 $\pm$ 1.6	11 $\pm$ 0.7	14 $\pm$ 2.3	17 $\pm$ 2.1
La^{3+} (1.5)	Asparagine	43 $\pm$ 4.3	48 $\pm$ 3.9	37 $\pm$ 4.6	42 $\pm$ 3.7
	Glutamine	10 $\pm$ 1.1	13 $\pm$ 0.8	—	—
	Glutamic acid	—	—	33 $\pm$ 2.4	38 $\pm$ 3.3
	Aspartic acid	—	—	40 $\pm$ 4.1	46 $\pm$ 5.2
	Lysine	—	—	9 $\pm$ 1.1	14 $\pm$ 1.3

Table 8. *Effects of calcium modulators on germination of cysts of Pythium aphanidermatum*

Zoospores encysted by agitation were treated 10 min later with calcium modulators then post-treated at 20 min as shown. Percentage germination after 16 h is shown as mean $\pm$ SEM for five replicates, 100 cysts each.

Post-treatment†	Germination (%)						
	Treatment (μ M)*:						
	None	Verapamil (30)	TFP (5)	Dibucaine (50)	A23187 (5)	Amiloride (20)	TMB-8 (30)
None (water)	24 $\pm$ 3.4	10 $\pm$ 1.6	9 $\pm$ 4.0	5 $\pm$ 1.4	5 $\pm$ 0.9	6 $\pm$ 1.1	11 $\pm$ 2.3
Ca ²⁺	55 $\pm$ 5.5	6 $\pm$ 1.6	10 $\pm$ 2.5	8 $\pm$ 1.4	9 $\pm$ 1.3	5 $\pm$ 1.5	9 $\pm$ 2.8
Mg ²⁺	39 $\pm$ 3.9	7 $\pm$ 1.5	12 $\pm$ 3.0	6 $\pm$ 1.4	6 $\pm$ 1.6	6 $\pm$ 2.4	9 $\pm$ 2.8
Sr ²⁺	46 $\pm$ 2.9	9 $\pm$ 1.8	15 $\pm$ 4.0	6 $\pm$ 1.0	3 $\pm$ 1.7	8 $\pm$ 3.6	7 $\pm$ 1.4
Asparagine	66 $\pm$ 5.7	53 $\pm$ 5.6	75 $\pm$ 6.5	61 $\pm$ 9.7	6 $\pm$ 1.4	69 $\pm$ 4.6	6 $\pm$ 1.9
Lysine	19 $\pm$ 1.4	10 $\pm$ 1.5	10 $\pm$ 2.6	8 $\pm$ 2.4	5 $\pm$ 1.1	7 $\pm$ 1.2	8 $\pm$ 1.1
Verapamil	8 $\pm$ 1.5	8 $\pm$ 1.0	—	—	—	—	—
TFP	12 $\pm$ 2.3	—	12 $\pm$ 2.0	—	—	—	—
Dibucaine	11 $\pm$ 2.1	—	—	5 $\pm$ 1.1	—	—	—
A23187	6 $\pm$ 1.2	—	—	—	4 $\pm$ 1.3	—	—
Amiloride	10 $\pm$ 2.3	—	—	—	—	6 $\pm$ 1.4	—
TMB-8	11 $\pm$ 2.8	—	—	—	—	—	6 $\pm$ 1.0

* In the absence of post-treatment, all treatment compounds significantly suppressed germination compared with water controls.

† Post-treatments were at 10 mM final concentration except for calcium modulators, which were used at the same concentrations as for treatments.

Table 9. *Reversal of suppression by verapamil and dibucaine in Pythium aphanidermatum and P. dissotocum*

Zoospores encysted by agitation were treated 10 min later with verapamil or dibucaine, then post-treated at 20 min as shown. Percentage germination after 16 h is shown as mean $\pm$ SEM for five replicates, 100 cysts each.

Post-treatment (10 mM)	Germination (%)					
	<i>P. aphanidermatum</i>			<i>P. dissotocum</i>		
	No treatment	Verapamil (30 μ M)	Dibucaine (50 μ M)	No treatment	Verapamil (30 μ M)	Dibucaine (50 μ M)
None (water)	20 $\pm$ 3.8	9 $\pm$ 0.9	6 $\pm$ 0.7	23 $\pm$ 2.6	7 $\pm$ 2.3	4 $\pm$ 1.0
Ca ²⁺	42 $\pm$ 5.4	7 $\pm$ 1.1	7 $\pm$ 1.4	54 $\pm$ 3.8	9 $\pm$ 2.5	4 $\pm$ 1.9
Asparagine	64 $\pm$ 9.2	63 $\pm$ 7.6	45 $\pm$ 6.1	66 $\pm$ 6.3	47 $\pm$ 6.1	52 $\pm$ 8.9
Alanine	18 $\pm$ 2.4	12 $\pm$ 2.6	8 $\pm$ 2.1	41 $\pm$ 5.0	26 $\pm$ 1.3	28 $\pm$ 4.4

enable germination because cysts could then be dislodged without effect (although adhesion is effectively permanent in nature). The flux of Ca²⁺ across the cyst membrane, measured for *Ph. palmivora*, is sufficient to explain this: up to 30% of cell Ca²⁺ was released within 2 min of encystment (Irving *et al.*, 1984) and labelled Ca²⁺ was taken up within 2 min (Iser *et al.*, 1989). Both Sr²⁺ and Mg²⁺ were found to substitute for Ca²⁺ in promoting germination of *Pythium* (Table 4). However, the suppression of germination by EGTA was almost certainly caused by chelation of Ca²⁺, because the stability constant for binding of EGTA to Ca²⁺ is at least five orders of magnitude higher than for Mg²⁺, and nearly three orders of magnitude higher than for Sr²⁺ (Schmid

& Reilley, 1957). The effects of the Ca²⁺ entry blockers La³⁺ and verapamil (Godfraind *et al.*, 1986) supported a central role of Ca²⁺ uptake in germination. The low background germination of suspended cysts seemed to depend on uptake of Ca²⁺ over a prolonged time because EGTA suppressed germination even when added at 20 min (Table 7).

These findings can be related to behaviour *in vivo* by a simple model. Zoospores dock and encyst precisely on roots, such that the fixed point of germination is next to the host (Hardham & Gubler, 1990; Jones *et al.*, 1991) and the glycoprotein secreted over the germination site adheres the cyst in this orientation (Hardham & Gubler, 1990). As on slides, adhesion is likely to involve Ca²⁺,

released during encystment, and adhesion should then impede dispersion of Ca^{2+} from the germination site, perhaps by ionic bridging but at least by lengthening the diffusion path. Ca^{2+} could then be reabsorbed and trigger germination. Thus, docking would lead automatically to germination, independent of other triggers, as when cysts adhere to glass, and the orientation of germination would lead automatically to host-penetration. The zoospore is thus envisaged to have an in-built reporter system to signal that it has docked correctly, and infection follows by a Ca^{2+} -mediated cascade.

A corollary of this hypothesis is that the pre-determined site of germination is a control centre which receives the Ca^{2+} signal. This is not essential because, theoretically, adhesion and Ca^{2+} uptake might occur at any point on the cyst surface *in vitro*. But *in vivo* docking will ensure that it invariably involves the germination site. The possibility of a control centre might focus future work. Gubler *et al.* (1990) found localized intense antibody reaction for calmodulin on the knob-like base of the anterior flagellum of *Ph. cinnamomi*, which is near the point of germ-tube outgrowth of both *Phytophthora* (Paktitis *et al.*, 1986) and *Pythium* (Jones *et al.*, 1991). It is unknown whether the knob-like base is retained when flagella are shed and whether calmodulin is similarly localized in *Pythium*. Nevertheless, germination of *Pythium* was suppressed by dibucaine and TFP, which are calmodulin inhibitors in mammalian systems (Bereza *et al.*, 1982). The region of flagellar insertion is also rich in microtubules (Grove & Bracker, 1978) and associated proteins, which are widely implicated in signal transduction.

Specific amino acids triggered germination, but were not effective in the presence of EGTA (Table 7), suggesting that their actions were intimately linked with Ca^{2+} uptake or Ca^{2+} signalling at the cell surface. Consistent with this, the amino acids reversed the suppression caused by calcium entry blockers (La^{3+} , verapamil) or by amiloride, which inhibits $\text{Na}^+/\text{Ca}^{2+}$ flux across membranes of mammalian cells (Godfraind *et al.*, 1986) and can also block Ca^{2+} channels (Tytgat *et al.*, 1990). The same amino acids reversed the suppression caused by calmodulin inhibitors (dibucaine, TFP), but did not overcome suppression by TMB-8, an intracellular calcium antagonist in mammalian systems (Owen & Villereal, 1982). The failure of amino acids or even Ca^{2+} to reverse suppression by A23187 is not easily explained. A23187 is an ionophore (Pressman, 1976) and should cause equilibration of Ca^{2+} across the cell membrane. Irving *et al.* (1984) found that it induced encystment and germination of *Phytophthora* zoospores in the presence of 1 mM- Ca^{2+} , but not with 100 μM - Ca^{2+} , presumably because equilibration of Ca^{2+} at this concentration was too low to induce differentiation. In

our study, 10 mM- Ca^{2+} did not reverse suppression of *Pythium* by A23187 (Table 8), perhaps because equilibration of Ca^{2+} at this concentration was toxic or A23187 had inhibitory effects unrelated to Ca^{2+} fluxes.

Amino acids are unlikely to have been taken up by the cysts, because Penington *et al.* (1989) found no significant uptake of labelled glutamate by zoospores or cysts of *Ph. palmivora* until after germination, and we (unpublished) found no evidence of uptake of labelled glutamate by spores of *P. aphanidermatum*. This would preclude the uptake of Ca^{2+} by symport and indicates, instead, that amino acids acted through receptors on the cyst membrane to open Ca^{2+} channels. Ca^{2+} -channel-linked glutamate receptors are widely reported in other organisms (e.g. Gilbertson *et al.*, 1991; Hollmann *et al.*, 1991) and might be expected to occur in Oomycete zoospores. To our knowledge, Ca^{2+} channels linked to aspartate, asparagine or alanine receptors have not been reported before. In any case, a receptor-mediated role of amino acids would be consistent with their species-specific effects, as found for alanine in this work (Table 9).

The question remains as to how amino acids might influence germination on roots. They are not essential, because young adherent cysts needed no supplementary trigger *in vitro*. Nevertheless, if amino acids can open Ca^{2+} channels, then they could synergize Ca^{2+} uptake during the docking sequence on roots. This could explain the common observation (e.g. Jones *et al.*, 1991) that cysts germinate rapidly and with almost 100% efficiency on roots, in contrast to the maximum of approximately 70% germination in this study.

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