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Calcium-regulated appressorium formation of the entomopathogenic fungus *Zoophthora radicans*

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Summary. The fungus *Zoophthora radicans* (Zygomycetes: Entomophthorales) requires external Ca^{2+} for appressorium formation but not for conidial germination. The number of appressoria formed depends on the Ca^{2+} concentration of the medium. At low $[Ca^{2+}]$ (100 pM) nuclear division and germ tube growth are significantly reduced compared to higher Ca^{2+} concentrations (10 and 1,000 μM). By contrast, neither external K^+ nor external Cl^- is needed for germination or appressorium formation.

Treatment of conidia with a Ca^{2+}-antagonist, Nd^{3+}, and a Ca^{2+} channel blocker, nifedipine, inhibits appressorium formation, showing that a Ca^{2+} influx is required for appressorium formation. Furthermore, the partial yet saturating inhibition by nifedipine and complete inhibition by Nd^{3+} indicates that at least two kinds of Ca^{2+} channels are involved in appressorium formation. A contribution of intracellular Ca^{2+} to the signal transduction chain for the formation of appressoria is demonstrated by the inhibitory effect of the intracellular Ca^{2+} antagonist TMB-8.

The calmodulin antagonists R24571, TFP, W-7, and W-5 inhibit appressorium formation at concentrations which have no effect on germination. The data presented in this paper are consistent with the hypothesis that a Ca^{2+}/calmodulin system is involved in regulating appressorium formation. However, since the direct effects of the drugs were not specifically tested on their proposed binding sites, we leave room for alternative hypotheses that have yet to be formulated.

Keywords: Calcium; Calmodulin; Appressorium formation; *Zoophthora radicans*; Entomopathogen.

Abbreviations: A-9-C 9-anthracenecarboxylic acid; DAPI 4′,6 diamino-2-phenylindole; EGTA ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid; HEPES N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; H-7 N-(2-methylamino)ethyl-5-isoquinolinesulphonamide dihydrochloride; IC_{50} concentration of inhibitor that causes 50% inhibiton; R24571 (calmidazolium) 1-[bis-(4-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl]-imidazolium chloride; TEA tetraethylammonium; TFP (trifluoperazine) 10-[3-(4-methylpiperazine-1-yl)-propyl]-2-trifluomethylphenothiazine; TMB-8 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride; W-5 N-(6-aminohexyl)-1-naphthalene-sulfonamide; W-7 N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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Introduction

The entomopathogenic fungus *Zoophthora radicans* (Zygomycetes: Entomophthorales) is a pathogen of various insect pests and has been considered to be an effective biological control agent (Milner and Mahon 1985, Roberts 1989). Its infective capability depends on cellular differentiation that leads to the production of a series of structures including germ tubes, appressoria, and penetration hyphae. The appressorium is a specialized infection structure produced by some plant and insect pathogenic fungi to assist in host penetration (e.g., Staples and Macko 1980, Charnley 1984). Appressorium formation is an important step in the infection of the potato leafhopper, *Empoasca fabae* (Homoptera: Cicadellidae), by this fungus (Wraight et al. 1990). The ability to produce these structures on demand in vitro has facilitated the study of the *Z. radicans* differentiation process (Magalhães et al. 1990 a).

The contribution of Ca^{2+} to fungal growth and development is similar to its action in animal and plant cells (Jaffe 1980, Hepler and Wayne 1985, Schmid and Harold 1988, St. Leger et al. 1989). Ca^{2+} is required

for growth and development in a wide variety of filamentous fungi, including the encystment of *Phytophthora cinnamomi* (Irving and Grant 1984, Grant et al. 1986), the induction of zoosporic germination in *Blastocladiella emersonii* (Van Brunt and Harold 1980) and *Aphanomyces astaci* (Cerenius and Södehäll 1984, Persson and Södehäll 1986), the growth of *Achyla* (Cameron and Lejohn 1972) and *Saprolegnia ferax* (Jackson and Heath 1989), the branching signal in *Neurospora crassa* hyphae (Reissig and Kinney 1983, Schmid and Harold 1988), and, under certain conditions, appressorium formation in *Uromyces appendiculatus* (Hoch et al. 1987). Ca^{2+} also regulates sexual reproduction in several oomycetes including *Saprolegnia diclina* (Fletcher 1988).

Other chemical signals such as those produced by the cation K^+ (Staples et al. 1983, Hoch et al. 1987) as well as by sugars such as sucrose, maltose, or glucose (Kaminskyj and Day 1984, Hoch et al. 1987, Magalhães et al. 1990 b) are known to promote appressorium formation. It is possible that Ca^{2+} also acts as a second messenger under these conditions. In this study, we show that differentiation of *Z. radicans* appressoria requires external Ca^{2+} whereas germ tube emergence and extension requires little or none.

Materials and methods

Fungal culture and inoculation

Zoophthora radicans, isolate ARSEF #1590 (USDA-ARS Collection of Entomopathogenic Fungi, Ithaca, N.Y. 14853), was removed from liquid nitrogen and maintained on Sabouraud dextrose agar (SDA) (Difco, Detroit, MI, U.S.A.) supplemented with 1% yeast extract (Difco) at 20 °C, and transferred weekly to fresh plates. Conidia were showered (= inoculated) onto 3% water-agar in 35 mm diameter Ca^{2+}-free polystyrene Petri plates (Falcon, Lincoln Park, ND, U.S.A.) as described in Magalhães et al. (1990 b), and then flooded with 3.5 ml of 1% yeast extract (YE) liquid medium or 0.3% Bacto-Soytone (Difco) plus 1% maltose (Fisher Scientific Co., Springfield, NJ, U.S.A.) liquid medium containing various agents. All media were autoclaved for 15 min at 120 °C and 1 bar and cooled to 50 °C before adding inhibitory drugs. Each plate was inoculated with 80–105 conidia/mm² and maintained at 25 °C in the dark. The noble agar (Fisher), yeast extract, and Bacto-Soytone contained 885, 1168, and 237 ppm Ca^{2+}, respectively (Table 1).

External calcium

Free Ca^{2+} as well as the free Mg^{2+} and H^+ concentrations were controlled in each experiment using a Ca^{2+}/EGTA buffer system (Wayne 1985). A computer program was used to calculate the apparent association constants (Wayne 1985). Solutions including 10 mM EGTA (titrated to pH 7.2 with NaOH), 10 mM HEPES (titrated to pH 7.2 with NaOH), 10 mM KCl (Sigma), 1 mM free Mg^{2+} added as $MgCl_2$, 1% YE, and different concentrations of $CaCl_2$ were prepared in Ca^{2+}-free polypropylene containers. Deionized H_2O (less than 20 nM total Ca^{2+}, as measured with inductively coupled argon plasma atomic emission spectroscopy), was used to mix solutions. The YE powder contained 0.1% Ca^{2+} (on a dry weight basis) (Table 1). Therefore the Ca^{2+} was removed from the 10% YE stock by passing it through a Chelex-100 ion exchange membrane (Bio-Rad Laboratories, Richmond, CA, U.S.A.) at a rate of 7.89 ml/min before addition to the Ca^{2+}/EGTA buffers. The Ca^{2+}/EGTA buffers were titrated to pH 7.2 with NaOH after all the components were added.

Ca^{2+}-free water was prepared by passing centrally available reverse osmosis water sequentially through a Barnstead D0803 high capacity, low affinity cation exchange column and a Barnstead D0809 low capacity, high affinity cation exchange column.

Table 1. Calcium content[a] of water, media components, conidia, and 5th instar potato leafhopper nymphs

Material	Ca^{2+} (ppm)
H_2O	0.0001
Noble agar	885
Yeast extract	1,168
Bacto-Soytone	237
Conidia	18.1
Empoasca fabae 5th instar nymphs	1,662

[a] Determination by inductively coupled argon plasma atomic emission spectroscopy

Calcium influx antagonists

Neodymium, an inorganic Ca^{2+}-channel blocker (Wayne 1985), was added as $NdCl_3$ (Aldrich Chemical Company Inc., Milwaukee, WI, U.S.A.) to the same buffer system (pCa 5) described above except that we substituted 0.3% Bacto-Soytone plus 1% maltose for YE as the organic nutrient source, since Nd^{3+} precipitates in the presence of YE but not in the presence of Bacto-Soytone plus maltose (as long as the [Nd^{3+}] is less than 1 mM). Bacto-Soytone plus maltose are almost as effective as YE in supporting germination and appressorium formation in *Z. radicans* (Magalhães et al. 1990 b). Ca^{2+} was removed from the 3% Bacto-Soytone + 10% maltose stock solution by passing it through the Chelex-100 ion exchange membrane. A computer program was used to calculate the apparent association constants of Nd^{3+}, Ca^{2+}, Mg^{2+}, and H^+ (Reiss and Wayne 1989). In all experiments described below Bacto-Soytone and maltose were used instead of YE as the nutrient source.

Nifedipine (Sigma Chemical Co., St. Louis, MO, U.S.A.), a voltage-dependent Ca^{2+}-channel blocker (Samuels et al. 1988), was dissolved in DMSO (Sigma) to make a 20 mM stock solution. DMSO was added to each solution to bring it up to 0.25% (35 mM) DMSO. This concentration of DMSO does not affect germ tube or appressorium formation.

Intracellular-calcium modulators

TMB-8 (Sigma), an inhibitor of intracellular Ca^{2+} release (Malogodi and Chiou 1974, Saunders and Hepler 1983, Saunders and Jones 1988), was dissolved in DMSO. All solutions contained 0.075% (10.5 mM) DMSO, which alone has no effect on appressorium formation. It has also been suggested that TMB-8 may affect various

other signaling systems, including inhibiton of the protein kinase C system (Simpson et al. 1984), which is present in fungi (Favre and Turian 1987). In order to test this hypothesis, we tested the effect of H-7, an inhibitor of protein kinase C (Hidaka and Tanaka 1987), on germination and appressorium formation. H-7 (Seikagaku America Inc., St. Petersburg, FL, U.S.A.) was dissolved in Ca^{2+}-free water.
Caffeine (Sigma), an agent that is thought to deplete intracellular Ca^{2+} stores (Paul and Goff 1973, Endo 1977, Bonsignore and Hepler 1985), was dissolved directly in the medium (pCa 5 and pCa 10).

Calmodulin antagonists

The calmodulin antagonists R24571 (Janssen Pharmaceutica, Belgium), W-7 (Sigma), and W-5 (Sigma) (Asano et al. 1985) were dissolved in DMSO to give 29, 20, and 20 mM stock solutions respectively. All solutions contained 0.25% (35 mM) DMSO. Trifluoperazine (TFP) (Sigma), another calmodulin antagonist (Asano et al. 1985), was dissolved in Ca^{2+}-free water (10 mM stock).

Potassium and chloride

A possible involvement of K^+ in germination and appressorium formation was checked by omitting KCl from the basal medium. In addition, we tested TEA, a K^+-channel blocker (Moczydlowski et al. 1988) (20 mM stock in Ca^{2+}-free water) as well as the Ca^{2+}-activated K^+-channel blockers apamin (Sigma) (0.2 mM stock in Ca^{2+}-free water) and charibdotoxin (Alomone Laboratories, Israel) (1 μM stock in Ca^{2+}-free water).
The effect of external chloride was investigated by omitting KCl from the medium, and by substituting $MgSO_4$ for $MgCl_2$ and $Ca(NO_3)_2$ for $CaCl_2$. The contribution of Cl^- fluxes were tested by treating cells with A-9-C (Aldrich) (1 mM), an inhibitor of Cl^- channels (Shiina and Tazawa 1988). A-9-C was dissolved in 0.5 M NaOH to give a stock solution of 500 mM.

Analysis of total calcium

Conidia of *Z. radicans*, 5th instar *E. fabae* nymphs, noble agar, and yeast extract were wet-ashed with redistilled HNO_3 + $HClO_4$ for total Ca^{2+} determination by inductively coupled argon plasma atomic emission spectroscopy (Jarrel-Ash Model ICAP 61, Thermo Jarrel Ash Corp, Franklin, MA, U.S.A.). Conidia were collected in a Ca^{2+}-free killing solution (0.1 M maleic acid + 1% Triton X-100). The molar concentration of Ca^{2+} was estimated by assuming the densities of the conidia are 0.1689 g dry weight/ml and 1.1570 g fresh weight/ml. Two hundred 5th-instar potato leafhopper nymphs were collected in Ca^{2+}-free plastic containers, frozen, and dried before analysis; the whole body Ca^{2+} concentration was determined. The molar concentration of Ca^{2+} was estimated by assuming the densities of the insects are 0.2261 g dry weight/ml and 0.9990 g fresh weight/ml. The Ca^{2+} content of the Ca^{2+}-free water used throughout this study was also determined.
Density (in terms of fresh weight) was measured by determining the fresh weight of conidia plus nymphs with an Automatic Electrobalance (Cahn 26, Cahn Instruments Inc., Cerritos, CA, U.S.A.) and measuring the packed volume in hematocrit tubes. Density, in terms of dry weight, was measured the same way after the specimens were dried at 23 °C for 24 h in a desiccator containing phosphorous pentoxide.

Evaluation and statistics

The relative frequencies of germ tube initiation and appressorium formation in all experiments were determined by scoring approximately 100 randomly selected conidia in each replicate 24 h after inoculation, using an Olympus BH-2 microscope equipped with a phase contrast × 10 objective (A10PL, NA = 0.25) and a phase contrast condenser (NA = 1.25). Fungal development was also observed with Nomarski differential interference contrast optics including × 20 (SPLAN20 NA = 0.46) and × 40 objectives (SPLAN40, NA = 0.70) and a condenser (NA = 1.4). Photographs were taken with either Plus X or TMAX 400 film.
Germination is defined as the emergence of a germ tube from a conidium. All conidia with germ tubes are considered to have germinated regardless of the length of the germ tube. Percent germination was calculated based on the total number of germlings (germ tubes alone and germ tubes ending in appressoria) and non-germinated conidia. Appressorium formation is defined as a swollen structure on the tip of a germ tube as described in Magalhães et al. (1990 a). Percent appressoria was calculated only from the total sample of germlings. The maximal percentage of cells producing appressoria in vitro is approximately 50–60% because not all germlings form appressoria.
Nuclear division was determined by scoring approximately 100 germlings in each plate. Nuclei were examined using DAPI (Sigma), a fluorescent DNA-specific stain, as described in Magalhães et al. (1990 c). Since *Z. radicans* conidia are uninucleate, a germling was considered to have undergone nuclear division if two nuclei were present.
Each experiment was repeated three times, with three replicates per experiment unless stated otherwise. All experiments were conducted at 25 °C in the dark in plastic trays covered with aluminium foil.
The mean ± one standard error of the mean (SE) is indicated in the figures. Statistics were calculated with the aid of Minitab (Minitab, Inc., State College, PA, U.S.A.) and data were plotted with the aid of Sigma Plot (Jandel Scientific, Corte Madera, CA, U.S.A.). Orthogonal comparisons or pairwise-treatment differences were tested using the Student's t statistic when ANOVA indicated significant differences between treatments. Percent data were transformed to arcsin (square root of %) for mean comparisons to ensure equal variances in different treatments.

Results

The effect of external calcium on development of *Z. radicans*

The formation of appressoria in *Z. radicans* follows an orderly progression of developmental events. The oval conidium (Fig. 1 A) germinates approximately 6–8 h following inoculation. Germination results in the formation of a germ tube (Fig. 1 B). The germ tube elongates by tip growth (Fig. 1 C) and after 16–18 h the germ tube swells to form an appressorium, a structure that facilitates the penetrations of the fungus by the host.
External Ca^{2+} is required by *Z. radicans* for appressorium formation. The most effective concentration that stimulates appressorium formation is 10 μM (pCa

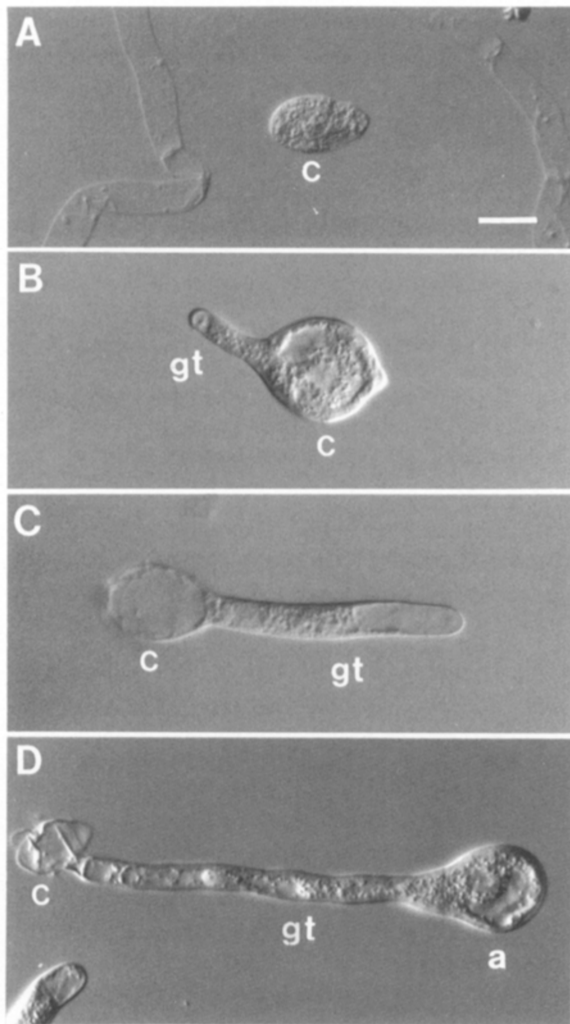


Fig. 1. Nomarski D.I.C. micrographs of various developmental stages of *Zoophthora radicans*. **A** Conidium. **B** Germ tube emergence. **C** Germ tube growth. **D** Appressorium. *c* Conidium, *gt* germ tube, *a* appressorium. Bar (A–D): 20 µm

5). In addition, there is a threshold for appressorium formation at 0.1 µM. Approximately 0.9 µM supports a half-maximal response (Fig. 2). There is an optimum external Ca^{2+} concentration since Ca^{2+} concentrations greater than 10 µM depress appressorium formation. By contrast, external Ca^{2+} is not required for germ tube formation although there is a slight stimulation in this process when the Ca^{2+} concentration is greater than 0.1 µM (Fig. 2). In order to determine whether or not wall-bound Ca^{2+} is rapidly utilized for germination before the buffer has a chance to equilibrate, conidia were washed with 10 mM EGTA (pH 7.2) at 0 °C for 10 min to remove the wall-bound Ca^{2+}. The EGTA-washed conidia germinated in Ca^{2+}-free water (mean (%) ± SE = 78.7 ± 3.3; n = 300) substantiating that germination is independent of external Ca^{2+}; however, no appressoria were formed. The EGTA-washed conidia germinated (mean (%) ± SE = 86.9 ± 2.7; n = 300) and differentiated into appressoria (mean (%) ± SE = 54.7 ± 5.4; n = 300) only when subsequently flooded with 1% YE nutrient medium (pCa 5).

External Ca^{2+} also influences other aspects of germ tube growth and development. At low Ca^{2+} concentrations (100 pM), nuclear division and germ tube length, width, and volume are significantly reduced ($P \leqslant 0.05$) compared to higher Ca^{2+} concentrations (10 and 1,000 µM) (Table 2).

The effect of calcium channel blockers on development

In order for appressorium formation to begin, external Ca^{2+} must enter the cell. This was demonstrated by treating the conidia with the inorganic Ca^{2+}-antagonist, Nd^{3+}, in the presence of 10 µM Ca^{2+} (pCa 5). The threshold concentration for the inhibition of appressoria is subnanomolar; the inhibitory concentration (IC_{50}) is 30 pM. Appressorium formation is almost totally inhibited at 1 nM but Nd^{3+} has no effect on conidial germination (Fig. 3). A particularly interesting and novel observation is that higher concentrations of

Table 2. The effect of Ca^{2+} on nuclear division and hyphal length, width[a] and volume of *Z. radicans* 24 h after inoculation

[Ca^{2+}]	Nuclear division (%)[b]	Hyphal length ($m \times 10^{-6}$)	Hyphal width ($m \times 10^{-6}$)	Hyphal volume[c] ($m^3 \times 10^{-18}$)
pCa 10 (1 pM)	0.5 ± 0.5 a	101.71 ± 2.38 a	5.83 ± 0.11 a	2,710
pCa 5 (10 µM)	34.9 ± 2.4 b	239.71 ± 8.75 b	6.93 ± 0.15 a	9,040[d]
pCa 3 (1,000 µM)	10.4 ± 1.5 c	230.36 ± 8.88 b	6.20 ± 0.13 a	6,950[d]

[a] Numbers indicate mean ± standard error of the mean; means within columns followed by the same letter are not significantly different by orthogonal comparisons ($\alpha = 0.05$, n = 300)

[b] Percent data transformed to arcsin (square root %) for analysis and retranslated

[c] Assuming hyphae are cylindrical

[d] Due to the spatulate form of the appressoria these values are underestimates

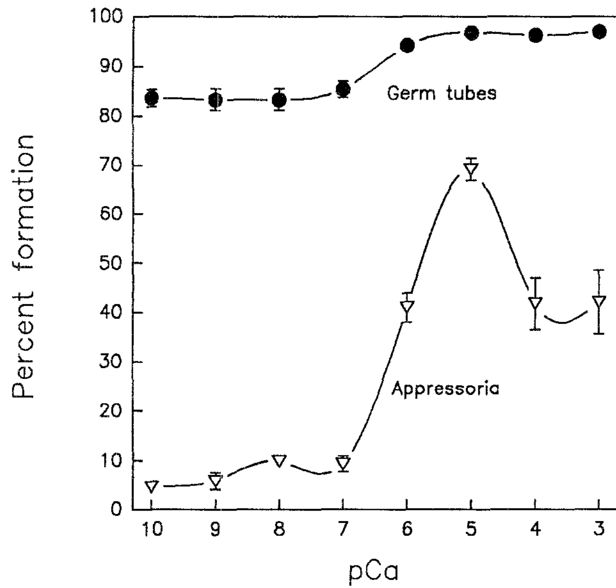


Fig. 2. The effect of external Ca^{2+} concentration on germination and appressorium formation. Cells were maintained in each concentration for 24 h in the dark. pCa = -log[Ca^{2+}]

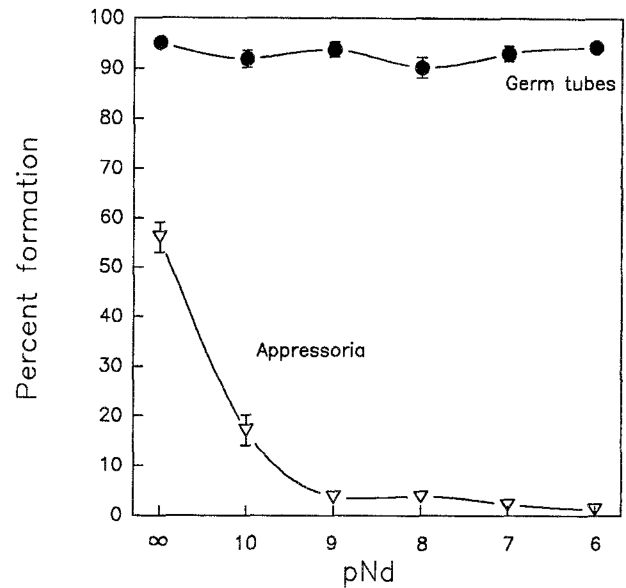


Fig. 4. The effect of $NdCl_3$ on the number of germ tubes emerging from each conidium determined 24 h after inoculation. Multipolarity means the production of more than one germ tube per conidium

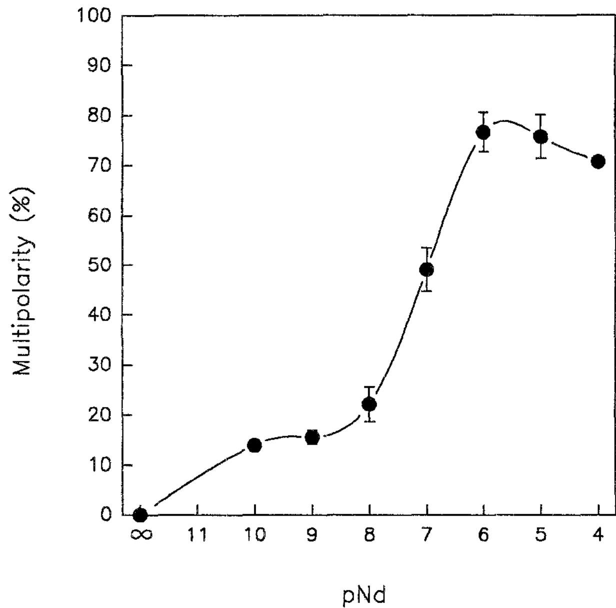


Fig. 3. The effect of external $NdCl_3$ concentration on germination and appressorium formation. Cells were maintained in each concentration for 24 h in the dark. pNd = -log[Nd^{3+}]

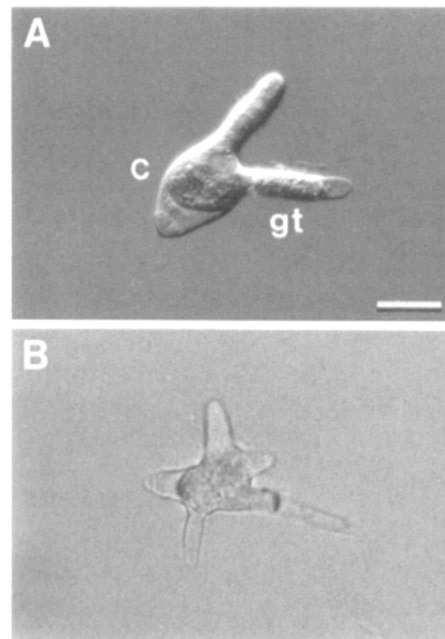


Fig. 5 A, B. Micrographs of germlings treated with $NdCl_3$ showing multipolar germination. **A** Nomarski D.I.C. micrograph. **B** Phase-contrast micrograph. Bar (A, B): 20 µm

Nd^{3+} (> pNd 8) cause *Z. radicans* to form two or more (up to five) germ tubes from each conidium (Figs. 4 and 5 A, B) as opposed to only one germ tube and appressorium formed in normal conditions (Fig. 1 B and C).

Further support that a Ca^{2+}-influx is required for appressorium formation comes from the observation that the voltage-dependent Ca^{2+} channel blocker nifedipine reduces appressorium formation. In contrast to the complete inhibition by Nd^{3+}, the inhibition of appressorium formation by nifedipine saturates at approximately 50% inhibition (about 22% appressoria) (Fig. 6), indicating that at least two kinds of Ca^{2+}

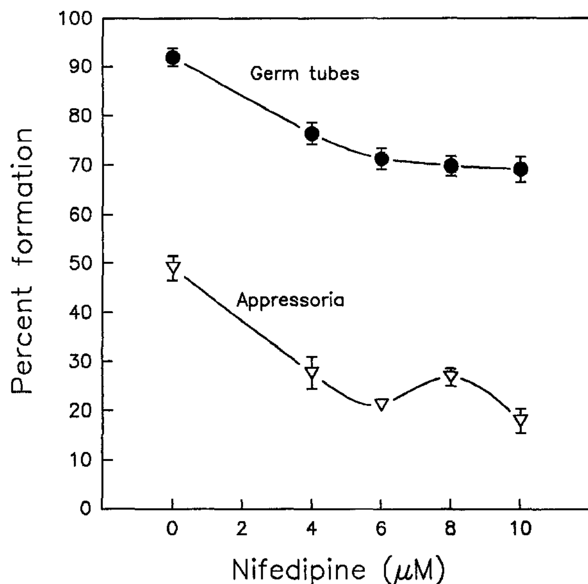


Fig. 6. The effect of nifedipine on germ tube and appressorium formation determined 24 h after inoculation

channels may be involved in appressorium formation. Nifedipine also slightly (but significantly) inhibits germination, although it is more than twice as effective in inhibiting appressorium formation. Ten μM nifedipine depresses germination by ca. 20%, while inhibiting appressorium formation by ca. 50% (Fig. 6). Higher nifedipine concentrations (up to 50 μM) do not increase inhibition of either germination nor appressorium formation. The inhibition of germination may be nonspecific since this process is independent of external Ca^{2+}

Time course for external calcium requirement

In order to determine when external Ca^{2+} is needed for appressorium formation, Nd^{3+} was added at various times following inoculation. While Nd^{3+} completely inhibits appressorium formation when given at zero time, it is ineffective when added 8 h after inoculation; this suggests that the influx of Ca^{2+} occurs within the first 8 h of development (Fig. 7 A). The reversibility of the inhibition of appressorium formation by Nd^{3+} was examined by removing the Nd^{3+} at various times following inoculation and scoring germination and appressorium formation 24 h after inoculation and Nd^{3+} removal. The presence of Nd^{3+} for up to 4 h postinoculation does not repress appressorium formation (Fig. 7 B).

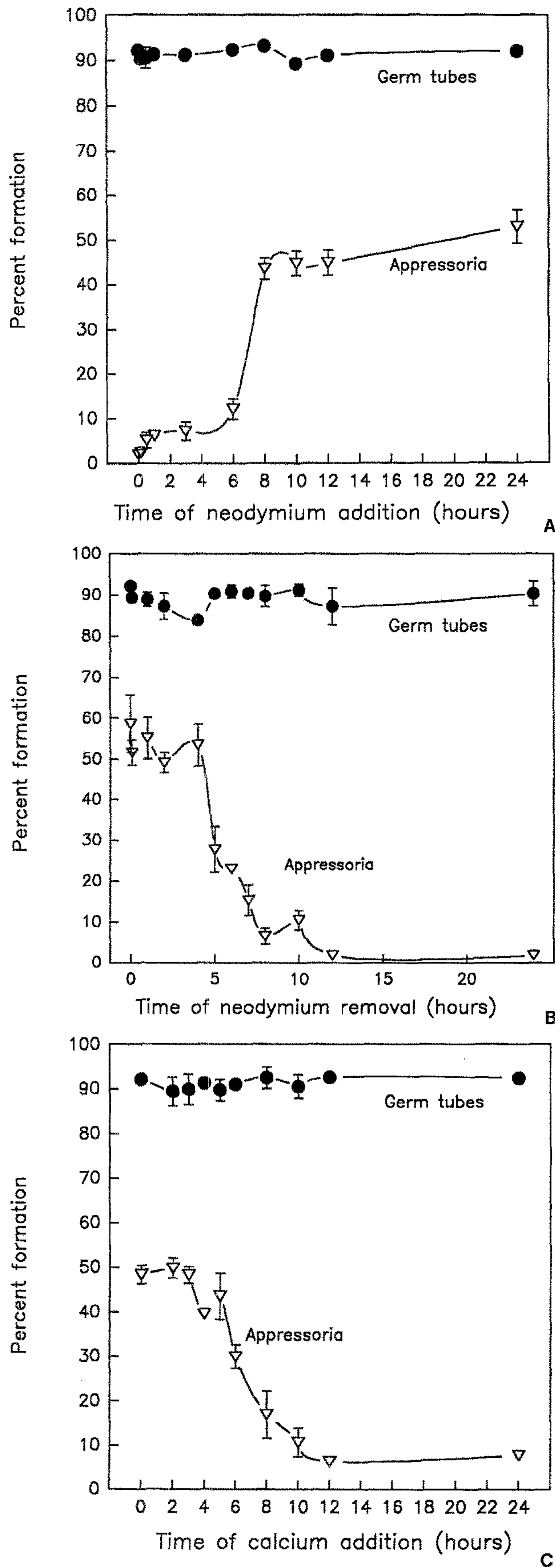
The effect of Ca^{2+} on signal transduction was also evaluated by adding Ca^{2+} (pCa 5) at various times postinoculation. Appressorium formation is maximal when the conidia/germlings are transferred from a low Ca^{2+} medium (pCa 10) to a high Ca^{2+} medium (pCa 5) between 0–5 h postinoculation, and still occurs even when the conidia are transferred up to 10 h postinoculation, indicating that the cell has a signaling system with a half-life of 6–8 h which is poised to accept and respond to Ca^{2+} (Fig. 7 C).

The effect of intracellular-calcium modulators on development

While external Ca^{2+} is required for appressorium formation it is possible that intracellular Ca^{2+} also contributes to the signal transduction chain either by being released directly by the primary developmental signal or through Ca^{2+}-induced Ca^{2+} release (Endo 1977). Therefore, we tested the effect of TMB-8, an inhibitor of intracellular Ca^{2+} release, on germ tube and appressorium formation. TMB-8 depresses the formation of both germ tubes and appressoria but is more effective in inhibiting appressorium formation than germination (Fig. 8). At 1 μM, there is approximately 20% inhibition of appressorium formation as opposed to less than 5% inhibition of germination. Caffeine, an agent that is thought to reduce the intracellular Ca^{2+} content, drastically reduces germination (%, mean ± SE = 14.1 ± 0.7, n = 600) and appressorium formation (%, mean ± SE = 5.5 ± 2.87, n = 600) when it is included in pCa 5 medium. Caffeine also depresses germination (%, mean ± SE = 16.6 ± 1.5, n = 600) and completely inhibits appressorium formation when it is included in the pCa 10 medium.

The effect of calmodulin antagonists on development

Once Ca^{2+} enters the cytosol, it may bind to the Ca^{2+} binding protein calmodulin (Asano and Stull 1985). In order to test this hypothesis, cells were challenged with a series of calmodulin antagonists: R24571, TFP, W-7, and W-5. The calmodulin antagonists inhibit both germ tube and appressorium formation. Appressorium formation is depressed by some of these antagonists at concentrations that have little or no effect on germination (Fig. 9). The inhibition of germination may be nonspecific since there is little relationship between the inhibition of germ tube formation and the binding affinity to calmodulin. The order of effectiveness of the CaM antagonists tested in inhibiting appressorium formation is R24571 > TFP > W-7 > > > W-5 (Table 3).



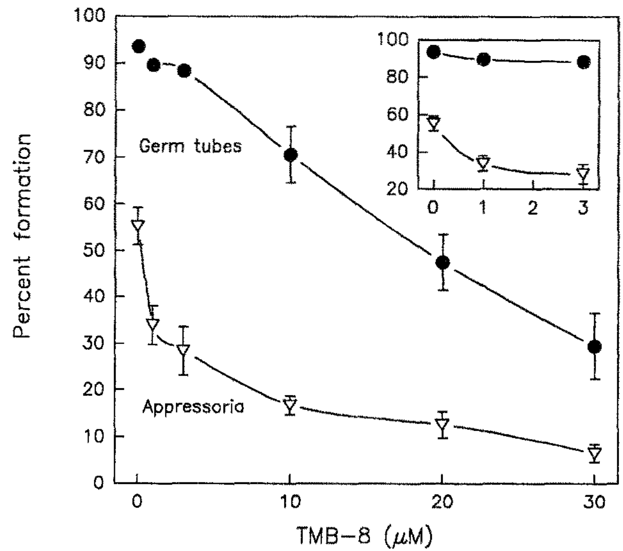


Fig. 8. The effect of TMB-8 on germination and appressorium formation 24 h postinoculation. Note different scales (from 0 to 3 μM) in the inset plot

The contribution of potassium and chloride to formation of appressoria

Since appressorium formation requires a swelling of the germ tube, it was postulated that K^+ may be involved in the response and that Ca^{2+} may function to activate K^+ channels. These activated channels may allow an influx of K^+ that would increase the cellular osmotic pressure and consequently the turgor pressure that may lead to swelling. However, the Ca^{2+}-activated K^+-channels blockers apamin (0.001–1 μM) and charibdotoxin (0.001–0.9 μM) have no effect on germ tube and appressorium formation (Table 4). Furthermore, external application of the K^+-channel blocker TEA (Aldrich) (0.1–10 mM) also has no effect on germ tube

Fig. 7. The kinetics of the Ca^{2+} signaling system. **A** The effect of time of addition of neodymium on germination and appressorium formation. Nutrient medium containing a high concentration of $NdCl_3$ (pNd 6) was added at different times starting at time zero. Appressorium formation was determined 24 h postinoculation. **B** The time course of the effect of Nd^{3+} removal on germination and appressorium formation. At the indicated times pNd 6 medium was replaced with pCa 5 medium. Appressorium formation was determined 24 h after inoculation. Similar results are obtained when appressorium formation is scored 24 h after Nd^{3+} removal. **C** The time course of the effect of initial Ca^{2+} deprivation. Nutrient medium containing a high Ca^{2+} concentration (pCa 5) was added at different times starting at time zero. Appressorium formation was determined 24 h postinoculation

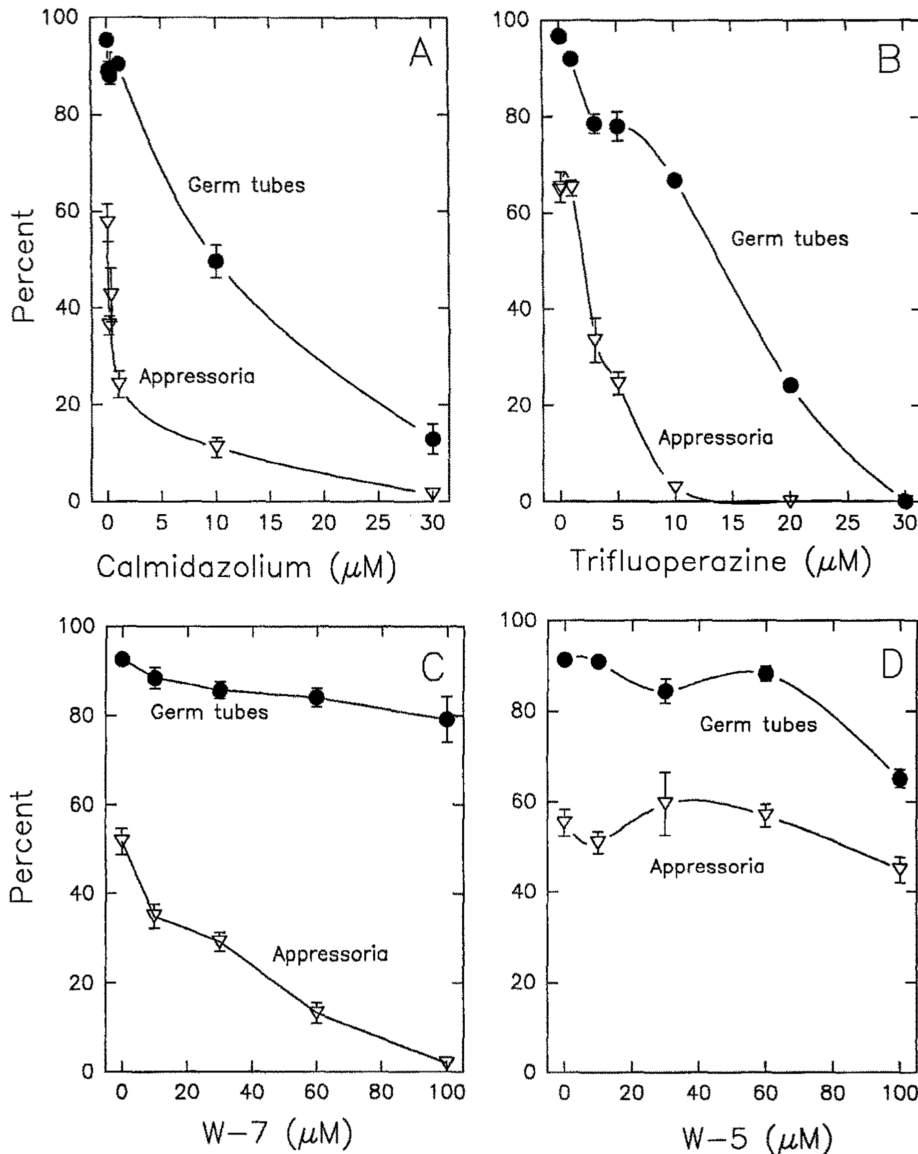


Fig. 9. The effect of inhibitors of the Ca^{2+}/calmodulin complex on germ tube and appressorium formation 24 h postinoculation. **A** R24571. **B** Trifluoperazine. **C** W-7. **D** W-5. The experiment with W-5 was repeated two times

and appressorium formation. In fact, germ tube and appressorium formation occurs even when K^+ is omitted from the medium, indicating that K^+ is not required for germination or appressorium formation (Table 4). Thus it is likely that hyphal cells enlarge because of site re-directed deposition of apical vesicles.

Chloride deprivation by omitting KCl and substituting $Ca(NO_3)_2$ for $CaCl_2$ and $MgSO_4$ for $MgCl_2$ does not affect germination and appressorium formation (Table 4), indicating that external Cl^- is not required for germination nor appressorium formation. Furthermore, experiments that substitute $Ca(NO_3)_2$ for $CaCl_2$ show that it is the cation Ca^{2+}, not the anion Cl^- that is required for appressorium formation. However, a Cl^- efflux may be required for germination since A-9-C, a chloride channel blocker. completely inhibits germ tube formation.

Calcium content analysis

In order to test whether or not sufficient Ca^{2+} is available to induce appressorium formation in vivo, that is, on *Empoasca fabae*, the natural host, we determined the calcium content of *E. fabae* nymphs and the conidia of *Z. radicans*. Fifth instar nymphs are composed of 1662 ppm total calcium which, if it were evenly distributed, is equivalent to 9.4 mM calcium. Conidia contain 18.1 ppm total calcium which is equivalent to only 76.5 μM, indicating that sufficient calcium is available to regulate appressorium formation when the conidium

Table 3. The inhibitory concentrations (IC_{50}) of germ tube and appressorium formation, and affinity constants (K_a) of four calmodulin antagonists

Antagonists	IC_{50} (μM)		K_a (μM)[a]
	Germ tube formation	Appressorium formation	
R 24571	10	0.55	0.3
Trifluoperazine	15	3	1
W-7	>100	37	11
W-5[b]	>100	>100	90

[a] Data from Asano and Stull (1985)
[b] Experiment repeated two times

Table 4. The effect of K^+ and Cl^- channel blockers and elimination of K^+ and Cl^- on germination and appressorium formation of *Z. radicans*

Medium[a]	Germ tubes[b]	Appressoria[b]
Control	93.6±0.9	43.9±5.1
K^+ experiments		
$-K^+$	94.6±1.5	43.0±0.9
+TEA (1 mM)	93.2±1.6	52.7±2.4
+apamin (1 μM)	90.3±3.5	45.9±4.9
+charibdotoxin (0.9 μM)	92.8±0.9	36.1±2.9
Cl^- experiments		
$-Cl^-$	93.2±0.7	47.4±2.9
+A-9-C (1 mM)	0.24±0.03[c]	0.0[d]

[a] In a basal solution containing 10 mM EGTA, 10 mM HEPES (pH 7.2, titrated with NaOH), 10 mM KCl, 1 mM KCl, 10 μM $CaCl_2$, 0.3% Bacto Soytone and 1% maltose (pH 7.2)
[b] From 3 replicates of 100 conidia or germlings each; numbers indicate percent mean ± SE
[c] Significantly different from control ($P<0.01$, $t=23.02$, $df=5$); there were no significant differences between the means of the remaining treatments as indicated by ANOVA ($P>0.05$, $F=1.65$, $df=6, 14$), using the transformation arcsin (sqare root of %) for analysis
[d] The few conidia that germinated did not form any appressoria

lands on the host in the natural environment (Table 1).

Discussion

The present observations demonstrate that in *Z. radicans*, appressorium formation, in contrast to germ tube initiation, is strongly dependent on external Ca^{2+}. At low Ca^{2+} concentrations conidial germination is slightly inhibited whereas germ tube growth is significantly reduced. Similarly, a Ca^{2+} influx is not required for conidial germination of the entomopathogenic hyphomycete *Metarhizium anisopliae*. However, germ tube length in *M. anisopliae* is limited to 85–130 μm in the absence of Ca^{2+} as opposed to 400 μm in YE liquid medium 30 h after inoculation (St. Leger et al. 1989). Hoch et al. (1987) report that Ca^{2+} may also be necessary for the induction of infection structures in the plant pathogen *Uromyces appendiculatus*.

The fact that low concentrations of Ca^{2+} inhibit nuclear division and slightly inhibit germ tube growth does not imply that this is the mechanism of the inhibition of appressorium formation, since appressoria form in the absence of mitosis and on short germ tubes (Magalhães et al. 1990 c). The importance of Ca^{2+} in *Z. radicans* development is also highlighted by the initial loss of polarity caused by Nd^{3+}, which leads to multipolar germ tube formation. This may be caused by a loss of a hyphal "apical dominance" where the first outgrowth inhibits the growth of other potential outgrowths in a Ca^{2+} independent manner, or by the depletion of a Ca^{2+} gradient required for polarity (Kühtreiber and Jaffe 1990). Whatever the mechanism may be, any proposal must explain that loss of polarity occurs when Nd^{3+} is in the medium but not in low Ca^{2+} solutions.

The time course study shows that *Z. radicans* utilizes Ca^{2+} for maximal appressorium formation 0–6 h after inoculation, indicating that Ca^{2+} may act as a signal for appressorium differentiation. Initial Ca^{2+} deprivation (up to 6 h postinoculation) does not hamper appressorium formation. The primary developmental signal may activate Ca^{2+} channels such that they remain maximally open for 6 h and slowly decline thereafter. This is similar to the Ca^{2+} utilizing system in fern spores (Wayne and Hepler 1984, Scheuerlein et al. 1989). This contrasts with the effect of Ca^{2+} on zoospore emergence from isolated spore cysts of *Aphanomyces astaci* where the Ca^{2+} utilizing system has a half-life of 15 min (Cerenius and Södehäll 1984). The ability of *Z. radicans* to utilize Ca^{2+} after a long restriction period (6 h) can be particularly important if a similar phenomenon should occur in the natural environment where the germling may experience an initial lack of Ca^{2+}. In that case, it could recover its ability to cause infection by forming an appressorium as Ca^{2+} becomes available.

We assume that the putative primary developmental signal activates both voltage-dependent and voltage-independent Ca^{2+} channels, since nifedipine inhibition saturates at about 50% inhibition while Nd^{3+} treatment leads to 100% inhibition. This indicates that *Z. radicans* has two kinds of channels possibly homolo-

gous with nifedipine-sensitive L-type and nifedipine-resistent N-type channels in animal cells (Tsien et al. 1987). A similar phenomenon has been seen in algal cells (Wayne et al. 1990) and cucumber cells (Reiss and Wayne 1989). However, the direct affect of these drugs on Ca^{2+} channels in fungi has not yet been demonstrated and so these results must be interpreted cautiously.

Not only is a Ca^{2+} influx required, but the release of intracellular Ca^{2+} is also necessary for appressorium formation as indicated by the suppression of appressoria by TMB-8. TMB-8 may affect various other signaling systems, including inhibition of the protein kinase C system (Simpson et al. 1984). A possible inhibition of the protein kinase C system by TMB-8 seems unlikely in the case of appressorium formation by *Z. radicans* since H-7 (0.1–100 μM), an inhibitor of protein kinase C, has not effect on germination (mean ± SE = 92.2±2.6, n = 300, $P > 0.05$, t = 1.39) and appressorium formation (mean ± SE = 42.4±4.2, n = 300, $P > 0.05$, t = 2.98). In addition, the inhibition of germination by TMB-8 indicates that this process is actually dependent on internal Ca^{2+} previously stored in the conidium. The observation that caffeine also inhibits germination supports the conclusion that intracellular Ca^{2+} is required for germination, since caffeine inhibits the uptake of Ca^{2+} by enhancing the Ca^{2+}-induced Ca^{2+} release mechanism (Endo 1977) which may lead to a decrease in the intracellular Ca^{2+} content (Paul and Goff 1973). However, the caffeine results must be interpreted cautiously since caffeine also affects the cAMP signaling system. Note that the effectiveness of TMB-8 and caffeine in modulating intracellular Ca^{2+} concentrations has not yet been demonstrated in fungi, leaving room for other interpretations of these results.

A linkage between Ca^{2+} and calmodulin in appressorium formation is indicated by the effect of the calmodulin antagonists. R24571, TFP, W-7, and W-5 inhibit appressorium formation. The order of effectiveness of the calmodulin antagonists on appressorium formation by *Z. radicans* follows the same trend as the binding affinity reported by Asano et al. (1985), and is the same as that described by St. Leger et al. (1989) for conidial germination of *M. anisopliae*. While high concentrations of these inhibitors may have nonspecific effects in *Z. radicans*, it seems that the low concentrations needed to inhibit appressorium formation may be binding specifically to calmodulin or calmodulin-like proteins since the order of effectiveness of these agents in inhibiting appressorium formation is similar to their binding affinity for calmodulin. This indicates that the inhibitors may be acting on the Ca^{2+}/calmodulin complex. By contrast, our data do not support the hypothesis that the Ca^{2+}/calmodulin system is involved in germ tube formation. These conclusions are based on pharmacological studies whose interpretations are based, to a large degree, on studies done on animal and plant systems. While we have used a wide range of inhibitors that are thought to act on various points of the Ca^{2+}/calmodulin signal transduction chain to support the "Ca^{2+}/calmodulin hypothesis", the conclusions would be strengthened by testing the direct effects of these pharmacological agents on their proposed binding site in *Z. radicans*.

Originally, we thought that K^+ or Cl^- influx was involved in hyphal swelling of *Z. radicans*, but appressorium formation is not repressed when the fungus is deprived of K^+ or Cl^- indicating that neither K^+ nor Cl^- is required for appressorium formation and the redistribution of wall precursor-containing vesicles may be the mechanism of swelling. This redistribution may be under Ca^{2+} control. This hypothesis is supported by the fascinating observation that a redistribution of chlortetracycline staining occurs during appressorium formation (unpubl. data). In contrast, K^+ is required by uredospores of the bean rust, *Uromyces phaseoli*, to produce appressoria on water agar, and, at low levels, its effect is potentiated by Ca^{2+} (Staples et al. 1983). For *Z. radicans*, we do not know yet what the primary developmental signal is for appressorium formation, however, it seems that Ca^{2+} acts as a second messenger through the Ca^{2+}/calmodulin signaling system.

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