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Effects of Calcium and Calmodulin on Spore Germination and Appressorium Development in *Colletotrichum trifolii*†

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Spore germination and appressorium formation are important steps in the process of fungal development and pathogenesis. These prepenetration events, which begin with spore attachment and culminate with appressorium maturation, a common scheme for many pathogenic fungi, are prerequisites for penetration of host external barriers and subsequent colonization. Conditions for in vitro spore germination and appressorium development in *Colletotrichum trifolii* are described. In addition, effects of Ca^{2+} and calmodulin on these processes have been examined. Results indicate that, as for other pathogenic fungi, appressorium development is induced on a hard surface. The data suggest that disturbance of calcium homeostasis, by ethylene-bis(oxyethylenenitrolo)tetraacetic acid (EGTA) or calcium channel blockers, impairs appressorium development. Moreover, calmodulin inhibitors affect both germination and differentiation, implying that the Ca^{2+}/calmodulin signal transduction pathway is important in the early development of *C. trifolii* on the plant host surface.

Colletotrichum trifolii Bain et Essary is the causal agent of alfalfa anthracnose. This plant-fungus interaction exhibits race and cultivar specificity, a common but poorly understood phenomenon in host-parasite relationships. Presently there are two established races of this fungus that are physiologically similar except for their pathogenic behavior on particular cultivars of alfalfa (6). Invasion by the fungus requires cellular differentiation, initiated by spore attachment, germination, germ tube elongation, appressorial initiation, and maturation, and subsequent cuticular penetration.

Appressoria are specialized structures which adhere to plant surfaces and are produced by a wide range of pathogenic fungi (10, 28). While not completely understood, appressoria are considered to function in breaching the cuticular barrier of the plant. In many cases, appressorium formation has been shown to be essential for fungal colonization (17).

There is little information concerning the mechanism(s) which triggers appressorial differentiation. Both physical and chemical stimuli can induce differentiation in vitro. Conditions including heat shock, salts, and cyclic AMP can induce appressorium development in vitro (13, 18, 28, 29). Of particular note was the demonstration that physical host topography induced appressorium formation in the bean rust fungus *Uromyces appendiculatus*. This thigmotropic signal response is quite specific, and thus appressorium development required a specific plant surface architecture (14, 40).

In order to study these early prepenetration events in greater detail in *C. trifolii*, conditions have been established for in vitro sporulation, germination, and appressorium formation. In this fungus, appressorium development is more generalized than in *U. appendiculatus* (28); virtually all germinated spores develop one or two appressoria if exposed to a hard surface such as glass or plastic. In addition, the conditions for nearly synchronous, abundant spore germination and appressorium formation have been determined, an essential requirement for these studies.

In preliminary studies, we found that by chelating Ca^{2+} with ethylene-bis(oxy-ethylenenitrolo)tetraacetic acid (EGTA), differentiation (appressorium development) was prevented, with no other discernible effect on the fungus. Consequently, this study focused on the relative contribution of calcium (Ca^{2+}) and the primary calcium-binding protein, calmodulin (CaM), in the regulation of these processes.

While calcium and calmodulin occupy central positions as intracellular regulators in animal cells (3) and evidence is mounting for a similar role in plants (2), the situation in fungi is not as well established. However, the involvement of Ca^{2+} in fungal differentiation has been known since the pioneering work of Griffin (11), in which the role of Ca^{2+} in hyphal branching was established. Calcium mediates homing behavior, encystment, and zoospore germination in both *Phytophthora* (15) and *Pythium* (7, 9) spp. Calcium is also important for appressorium formation in the entomopathogens *Zoophthora radicans* (20) and *Metarrhizium anisopliae* (32) and serves as a branching signal in *Fusarium graminearum* and *Neurospora crassa* (25, 26). Calcium is also involved in mycelial dimorphism in the ascomycete *Ceratocystis ulmi* (21) and photomorphogenesis in *Penicillium isariaeforme* (22).

Among known regulators, calmodulin is probably the most important Ca^{2+} intracellular receptor. This small, acidic, remarkably conserved calcium-binding protein is found in all eukaryotes that have been examined (5). Calmodulin is the molecular link between free Ca^{2+} in the cell and regulation of numerous enzymes (35). Ca^{2+} binding induces conformational changes in calmodulin, resulting in activation of many enzymes, including the multifunctional, dedicated calmodulin-dependent protein kinases and protein phosphatases (39).

Chelation of calcium by EGTA or inhibition of calmodulin by trifluoperazine at physiological concentrations prevents spore development of *Penicillium notatum* (23). The data imply association of the Ca^{2+}-calmodulin (Ca^{2+}/CaM) system in spore development and suggest that a Ca^{2+}/CaM protein kinase may stimulate morphogenesis, but again the mecha-

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nism(s) awaits elucidation. Recent work by St. Leger and colleagues (31–33) suggests that a calcium-based second-messenger signaling system may function in appressorium development of the entomopathogen *M. anisopliae*. A parallel may exist between *C. trifolii* and *M. anisopliae*, as both fungi form appressoria as a requisite for disease development.

In this report, we evaluate the involvement of the Ca^{2+}/CaM system in the *C. trifolii* developmental pathway.

MATERIALS AND METHODS

Organisms and culture conditions. *C. trifolii* race 1 (ATCC 66954) (8) was used throughout these experiments and was isolated from single-spore cultures. The fungus was grown on solid YPSS medium (34) at 22°C under constant fluorescent light. Spores were obtained by inoculating liquid YPSS with a 1-cm^2 agar plug from colonies and incubated with vigorous agitation (150 rpm) at room temperature. Long-term storage involved placing spores and mycelia on dry, sterile filter paper at −20°C.

Spore germination and differentiation. To induce germination or differentiation, spores from liquid culture, 3 to 5 days old, were collected by centrifugation (10,000 × *g*, 5 min), resuspended in sterile distilled water, and washed twice. A final concentration of 10^4 spores ml^{-1} was pipetted onto various surfaces for spore germination and examination of appressorium development. Spores were considered to have germinated when germ tube length was equal to or greater than spore diameter. An appressorium was considered mature when melanization was evident. The evaluation of Ca^{2+}/CaM effectors was performed in polystyrene petri dishes. Growth was observed on colonies in petri dishes containing YPSS medium. All studies were conducted under constant fluorescent light at 22°C and monitored by phase-contrast light microscopy.

Chemicals. All chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise indicated. To determine the effect of Ca^{2+}/CaM on *C. trifolii* growth, chemicals were added to molten YPSS solid medium for radial growth, molten 0.7% agarose for germination, or sterile distilled water for germination and appressorium development. All aqueous solutions were sterilized by filtration.

The calcium ionophore A23187 was dissolved in ethanol and added from a 10 μM stock solution. EGTA was dissolved in distilled water and titrated to pH 8.0 with a solution of 5 N NaOH. Neodymium (Aldrich, Milwaukee, Wis.), an inorganic external Ca^{2+} blocker (38), was added as a chloride salt ($NdCl_3$) from an aqueous stock solution of 100 μM. Nifedipine, a voltage-dependent Ca^{2+} channel blocker, was dissolved in dimethyl sulfoxide (DMSO) and added from a 50 mM stock solution. Verapamil, also a Ca^{2+} channel blocker, was dissolved in DMSO and added from a 100 μM stock solution. TMB-8 [3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester], an inhibitor of intracellular calcium release, was dissolved in DMSO to give a 2.31 mM stock solution. The following calmodulin antagonists (1) were used: trifluoperazine dihydrochloride (TFP) was dissolved in water and added from a 10 mM stock solution; compound R 24571 [(1-[bis(4-chlorophenyl)-methyl]-3-[2-(2,4-dichlorophenyl)-methoxy]ethyl]-1H-imidazolium chloride] (calmidazolium) was dissolved in DMSO to give a stock solution of 1.45 mM; compound 48-80 (condensation product of *N*-methyl-*p*-methoxy-phenethylamine with formaldehyde) was dissolved in water to give a final concentration of 1 mg ml^{-1}; and W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] was dissolved in DMSO to give a 20 mM stock solution.

The final concentrations of the solvents used had no discernible effects on fungal growth and development compared with water controls. All experiments were replicated at least three times. In spore and appressorium studies, a minimum of 100 independent structures were assessed for each replicate.

RESULTS

Germination and differentiation. Spores of *C. trifolii* do not germinate under agitation in liquid culture. Spores germinate only if surface contact has been made. Following germination, when the germ tube encountered a hard surface such as the plant cuticle, glass, plastic, or nitrocellulose, differentiation occurred and a mature appressorium developed. As found for other pathogenic fungi (18, 31), all hard, hydrophobic surfaces that were tested induced appressorium development, while with some hydrophilic surfaces, the fungus differentiated but at a reduced frequency, if at all (Table 1). For example, the hydrophilic face of GelBond (FMC Corp., Rockland, Maine) was less efficient in inducing differentiation than the hydrophobic face. A hydrophobic soft surface such as mineral oil failed to induce differentiation. Thus, it appears that the hardness of the surface and possibly the availability of calcium (see below) were the determining factors for appressorium induction. After inoculation in a plastic petri dish with water, spores began to develop germ tubes after 120 to 150 min. Half of the spores produced appressoria between 390 and 420 min, and nearly 100% of the spores produced appressoria by 450 min. Less than 0.3% of the spores which germinated failed to produce appressoria. Figure 1A shows mature appressoria of *C. trifolii.*

TABLE 1. Appressorium formation by *C. trifolii* on different substrates[a]

Surface	Mean % appressorium development ± SEM
Polystyrene	93.9 ± 5.9
Glass	82.2 ± 10.4
GelBond (hydrophobic face)	83.7 ± 11.4
GelBond (hydrophilic face)	23.4 ± 5.9
Parafilm	95.4 ± 3.1
Nitrocellulose	89.0 ± 7.9
Cellophane	81.6 ± 7.5
Agarose (0.7%)	0.0

[a] Spores of *C. trifolii* were obtained from growth in liquid culture in YPSS medium. Spores were harvested, washed in distilled water, added to polystyrene dishes to a final concentration of 2×10^{-4} spores ml^{-1}, and added to the appropriate surface. Appressorium development was observed after 12 h of incubation and is shown as the mean for three replicates of 300 spores each.

Effect of ionophore and EGTA on germination and differentiation. Disturbance of Ca^{2+} homeostasis may influence normal fungal development. Treatment of *C. trifolii* spores with EGTA (10 mM) under inducing conditions resulted in germination and germ tube elongation, but differentiation was inhibited (Fig. 1B). The involvement of external and internal calcium was evaluated with EGTA and the calcium ionophore A23187, respectively, and is shown in Table 2. Ionophore concentrations between 20 and 200 nM had significant effects on germination and differentiation, while levels above 200 nM seriously impaired germination and differentiation as well as colony growth in *C. trifolii*. Disruption of internal calcium homeostasis with EGTA plus ionophore had a deleterious effect on both germination and differentiation (Table 2). Impairment of internal calcium homeostasis was also deleterious for germination. Addition of Ca^{2+} (1 mM) partially restored the ability of spores to differentiate to mature appressoria (data not shown).

Effect of calcium channel blockers on germination and appressorium development. To further examine the role of external Ca^{2+} ions, the role of ion channels was investigated with several distinct external Ca^{2+} blockers. Table 3 summarizes the effects of selected Ca^{2+} channel blockers. These external blockers had little or no effect on germination (except at relatively high concentrations) but inhibited differentiation. Neodymium, an inorganic Ca^{2+} channel blocker, severely affected differentiation of germlings at 1 μM. Further support for the role of Ca^{2+} influx in differentiation was shown when nifedipine (a voltage-dependent Ca^{2+} channel blocker) was used. Nifedipine only partially reduced appressorium formation. In contrast to neodymium, nifedipine saturates at about 50% inhibition, suggesting that at least two types of Ca^{2+} channels may be involved in appressorium formation. However, verapamil, also a voltage-dependent Ca^{2+} channel antagonist, had negligible effects on *C. trifolii* (data not shown).

To examine the role of internal Ca^{2+}, we tested TMB-8, an inhibitor of intracellular Ca^{2+} release (Table 3). At 5 μM TMB-8, spore germination was unaffected, but few germinated spores matured into melanized appressoria. Thus, hyaline appressoria developed (96%) but did not become melanized (Fig.

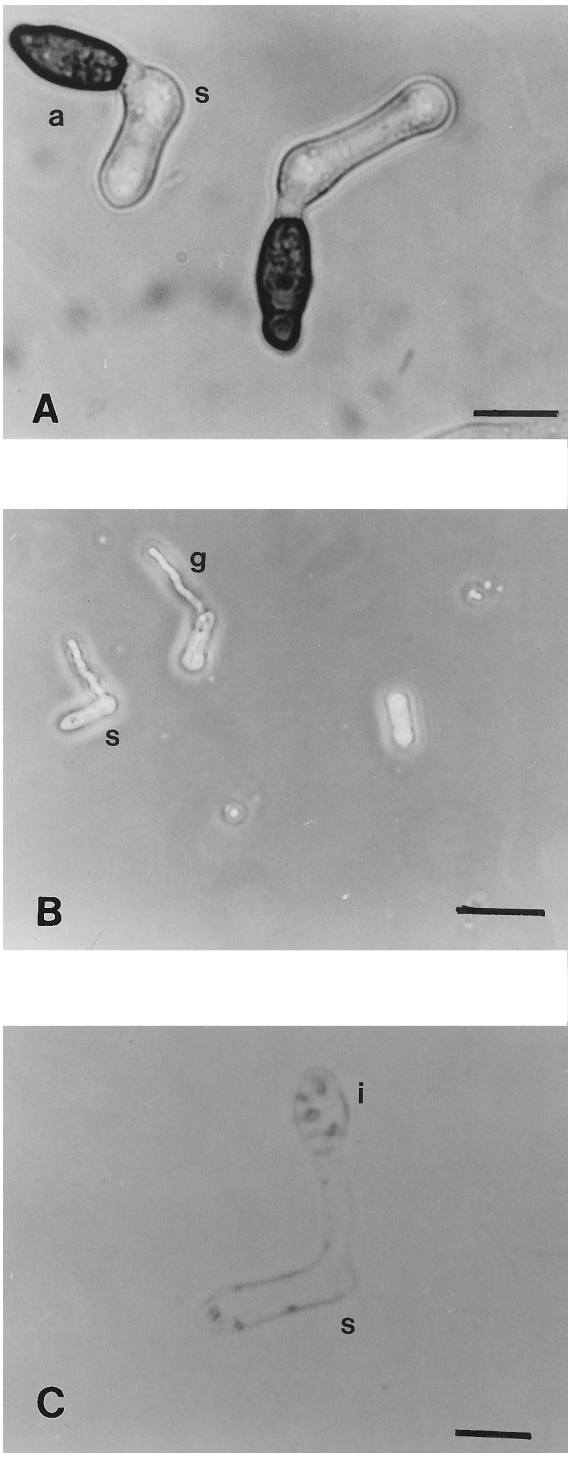

FIG. 1. (A) Appressorium of *C. trifolii* race 1. (B and C) Effect of EGTA (10 mM) (B) and of TMB-8 (5 μM) (C) on appressorium development. Spores of *C. trifolii* obtained by liquid culture in YPSS medium were harvested, washed in distilled water, added to polystyrene dishes to a final concentration of 2×10^{-4} spores ml^{-1}, and incubated for 12 h. Structures: a, appressorium; g, germ tubes; i, immature appressorium; s, spores. Bars: 10 μm (A and C); 5 μm (B).

1C). At higher concentrations (30 μM), appressoria failed to initiate, spores germinated poorly, and colony growth was reduced. These data suggest that proper appressorium development and maturation, but not initiation, are dependent on mobilization of internal Ca^{2+}.

TABLE 2. Effect of EGTA and calcium ionophore on appressorium development of *C. trifolii*[a]

Treatment	Concn	Mean % appressorium development ± SEM
None (control)		86.2 ± 3.2
EGTA	1 μM	63.9 ± 4.0
	10 μM	49.4 ± 12.4
	1 mM	41.2 ± 5.2
	10 mM	19.6 ± 3.2
A23187	2 nM	84.5 ± 8.3
	20 nM	76.1 ± 8.1
	200 nM	35.2 ± 9.0
A23187 + EGTA	20 nM + 10 μM	50.6 ± 2.6
	20 nM + 100 μM	3.6 ± 0.9
	20 nM + 1 mM	0.0
	200 nM + 10 μM	38.9 ± 13.9
	200 nM + 100 μM	8.9 ± 2.6

[a] Spores of *C. trifolii* obtained by liquid culture in YPSS medium were harvested, washed in distilled water, and added to polystyrene dishes to a final concentration of 2×10^{-4} spores ml^{-1} with the treatments shown. Appressorium development after 12 h is shown as the mean for three replicates, 100 spores each.

Effect of calmodulin inhibitors on germination and appressorium development. Having established the involvement of Ca^{2+} in germination and appressorium development, the effect of calmodulin was examined by use of pharmacological antagonists (Table 4). TFP, a phenothiazine, inhibited germination by nearly 100% at 15 μM. However, at this concentration, germ tube length and colony growth were also affected. Since TFP interacts with proteins in addition to calmodulin (37), the specific basis for these results is not clear.

W-7, R24571, and compound 48-80, which are more specific and potent calmodulin inhibitors (1), were also tested. R24571 completely inhibited differentiation on induction surfaces at 1.46 μM (1 μg ml^{-1}) and spore germination at concentrations above 7.27 μM (5 μg ml^{-1}). For all differentiation studies, germinated spores were obtained prior to treatment with the antagonist. Similar results were observed for compound 48-80 (Table 4); at 1 μg ml^{-1}, germinated spores failed to differentiate into mature appressoria. The concentrations which inhibited spore germination and appressorium development had no effect on colony growth.

DISCUSSION

The experiments described in this report were designed to examine whether calcium and/or calmodulin regulates the prepenetration events of spore germination and appressorium development in *C. trifolii*. Our approach exploited pharmacological agents that affect calcium and/or calmodulin activities. The results demonstrated that *C. trifolii* appressorium formation, in contrast to spore germination and germ tube formation, strongly depends on external Ca^{2+}. The calcium channel blockers neodymium and nifedipine had negligible effects on spore germination and germ tube growth but markedly reduced differentiation (Table 3). Similarly, Ca^{2+} influx was not required for spore germination in the entomopathogenic fungi *Z. radicans* and *M. anisopliae*, although the latter organism exhibited limited germ tube length in the absence of Ca^{2+} (20, 31). It was also reported that Ca^{2+} may be necessary for the induction of infection structures in the plant pathogen *U. appendiculatus* (13). In addition to calcium independence, *C. trifolii* spore germination did not require de novo protein syn-

TABLE 3. Effect of calcium channel blockers on germination, differentiation, and colony growth of *C. trifolii*[a]

Compound	Concn	Mean % germination (noninductive surface) ± SEM	Mean % differentiation at 12 h (inductive surface) ± SEM	Mean colony diam (cm) ± SEM
Nifedipine	0 μM	94.0 ± 3.6	94.3 ± 4.9	7.7 ± 0.1
	2 μM	93.7 ± 3.1	67.4 ± 4.2	7.3 ± 0.4
	4 μM	94.0 ± 1.0	67.3 ± 5.6	6.8 ± 0.1
	6 μM	88.7 ± 1.5	63.0 ± 4.4	6.7 ± 0.2
	8 μM	88.7 ± 3.2	59.7 ± 7.5	7.4 ± 0.5
	10 μM	90.7 ± 2.1	67.3 ± 3.1	6.7 ± 0.6
Neodymium	0 M	97.7 ± 2.5	67.3 ± 9.5	7.1 ± 0.4
	10^{-8} M	97.0 ± 1.7	64.7 ± 4.5	6.4 ± 0.1
	10^{-7} M	93.3 ± 6.0	71.3 ± 12.8	6.5 ± 0.2
	10^{-6} M	91.0 ± 0.0	34.3 ± 1.2	6.4 ± 0.3
	10^{-5} M	88.0 ± 5.3	26.0 ± 4.0	6.8 ± 0.3
	10^{-4} M	90.3 ± 6.4	11.3 ± 2.1	6.8 ± 0.5
	10^{-3} M	79.7 ± 9.3	0.0	6.7 ± 0.1
	10^{-2} M	52.0 ± 12.1	0.0	6.5 ± 0.5
TMB-8	0 μM	79.1 ± 4.9	99.1 ± 0.7	6.4 ± 0.5
	5 μM	82.0 ± 4.8	86.5 ± 18.6[b]	6.5 ± 0.1
	10 μM	78.8 ± 4.0	87.2 ± 3.4[b]	4.7 ± 0.5
	20 μM	73.6 ± 3.3	3.6 ± 2.7[b]	4.5 ± 0.5
	30 μM	17.6 ± 4.1	0.0	3.3 ± 0.1

[a] Spores of *C. trifolii* obtained by liquid culture in YPSS medium were harvested, washed in distilled water, and added to polystyrene dishes (10^4 spores ml^{-1}) (inducing conditions) or 0.7% agarose (10^4 spores) (noninducing conditions) for evaluation of differentiation and germination, respectively. Colony growth was assessed by inoculating an agar plug containing mycelia of *C. trifolii* in YPSS medium and was measured after incubation for 15 days at 22°C. Values are the means for three replicates, 100 spores each.

[b] Hyaline appressoria.

thesis. Cycloheximide at a physiologically relevant concentration (1 μM) had no effect on spore germination, but following germ tube initiation, fungal growth ceased (data not shown).

Making the membranes permeable to calcium with A23187 did not induce appressorium development, suggesting that differentiation is a product of a sustained rather than transient increase in the concentration of calcium ions in the cytosol. An increase in calcium influx was not sufficient for this cellular response. The data support the idea that rather than Ca^{2+} influx, calcium cycling across the membrane is required for differentiation. These observations are in agreement with the model proposed to explain the differentiation of appressoria in *M. anisopliae* (33). The data presented here are also in agreement with a report concerning *Z. radicans* (20), *U. appendiculatus* (13), and the homing responses of zoosporic fungi (9), for which Ca^{2+} flux is believed to be important.

In addition to external Ca^{2+} transit being important for differentiation, release of intracellular Ca^{2+} is also necessary, as indicated by the inhibition of appressorium maturation by the internal Ca^{2+} channel blocker TMB-8. TMB-8 may play a role in other signal pathway components, including protein kinase C (27).

A relationship between Ca^{2+} and calmodulin in germination and appressorium formation is shown by the effect of the calmodulin antagonists TFP, calmidazolium, W-7, and compound 48-80. The order of effectiveness of those antagonists on appressorium formation of *C. trifolii* parallels the binding affinities reported by Asano et al. (1), St. Leger et al. (31), and Magalhães et al. (20). The efficacy of these antagonists corresponds directly with their specificity towards calmodulin; that is, compound 48-80 is the most specific and potent calmodulin antagonist, while W-7 and TFP are the least specific. These different levels of specificity are reflected in a variety of nonspecific effects on *C. trifolii*, including lethality. However, at physiologically relevant concentrations, these compounds affected germination and appressorium formation in a manner related to their binding affinities for calmodulin. At the concentrations used, these compounds had no other discernible effect on the fungus. Calmodulin inhibitors are likely to be acting on the Ca^{2+}/CaM complex, affecting germination and infection structure formation.

The use of metabolic inhibitors or antagonists to block biochemical reactions or developmental processes must be interpreted with caution. Disturbance of Ca^{2+} homeostasis may influence normal fungal development. Any conclusion based on their use should address questions relating to the specificity of the inhibitors. In fact, the Ca^{2+}/CaM antagonists used in this study are known to have secondary effects. For example, TFP has been reported to inhibit CaM-insensitive Mg^{2+} ATPase in animal cells (36). These antagonists can also accumulate in membranes, which can disrupt general structure-function properties (4). Despite this caveat, Ca^{2+}/CaM antagonists have been used in a variety of plant, animal, and fungal systems to obtain initial evidence for Ca^{2+}/CaM-dependent processes (12). The demonstration that distinct classes of antagonists (phenothiazines and naphthalenesulfonamides [TFP and W-7, respectively]) had similar physiological effects on *C. trifolii* supports a common, specific mode of action. The antagonists did not interfere with other general physiological parameters such as growth and sporulation. The importance of appressoria for pathogenicity is illustrated by the fact that these antagonists prevent fungal infection of alfalfa (unpublished data). A more precise evaluation of the role of calmodulin in *C. trifolii* development can be done by cloning the calmodulin gene and inhibiting expression. This approach had been used for *Aspergillus nidulans* (24) as well as a *Dictyostelium* sp. (19). The unique calmodulin gene of *C. trifolii* has been cloned, and the construction of an antisense vector is in progress in order to address this issue more directly.

Pathogenesis in *C. trifolii* requires spore germination followed by infection structure development. The results pre-

TABLE 4. Effect of calmodulin antagonists on germination, differentiation, and colony growth of *C. trifolii*[a]

Compound	Concn	Mean % germination (noninductive surface) ± SEM	Mean % differentiation (inductive surface) ± SEM	Mean colony diam (cm) ± SEM
TFP	0 μM	85.8 ± 8.4	87.7 ± 8.4	6.5 ± 0.4
	5 μM	79.5 ± 10.6	35.3 ± 15.1	6.8 ± 1.8
	10 μM	46.3 ± 13.0	7.6 ± 12.7	6.7 ± 0.3
	15 μM	0.3 ± 0.6	3.0 ± 5.9	6.7 ± 0.1
	20 μM	0.0	2.2 ± 4.8	5.4 ± 0.1
	25 μM	0.0	2.5 ± 4.9	5.8 ± 0.1
	50 μM	0.0	0.7 ± 1.4	3.6 ± 0.3
W-7	0 μM	89.3 ± 7.0	77.0 ± 10.0	6.4 ± 0.0
	20 μM	78.7 ± 2.1	0.0	8.0 ± 0.6
	40 μM	74.3 ± 5.0	0.0	7.3 ± 0.6
	60 μM	75.7 ± 5.0	0.0	7.6 ± 0.4
	80 μM	87.0 ± 8.7	0.0	7.3 ± 0.3
	100 μM	75.3 ± 3.8	0.0	7.7 ± 0.2
R-24578	0 μM	95.0 ± 2.9	78.5 ± 9.0	6.7 ± 0.1
	1.5 μM	77.9 ± 14.9	0.0	7.6 ± 0.2
	3 μM	84.3 ± 4.4	0.0	7.3 ± 0.3
	6 μM	79.4 ± 2.1	0.0	7.3 ± 0.2
	12 μM	72.2 ± 9.1	0.0	6.2 ± 0.4
	24 μM	37.8 ± 3.9	0.0	6.9 ± 0.1
48-80	0 μg/ml	88.7 ± 3.3	83.45 ± 2.33	7.8 ± 0.6
	1 μg/ml	87.0 ± 1.3	0.0	7.0 ± 0.4
	5 μg/ml	90.2 ± 3.5	0.0	7.4 ± 0.4
	10 μg/ml	89.3 ± 2.0	0.0	7.3 ± 0.2
	15 μg/ml	83.2 ± 3.0	0.0	7.2 ± 0.0
48-80 + Ca^{2+}	5 μg/ml + 1 mM	0.0	10.20 ± 5.6	0.0
	5 μg/ml + 10 mM	0.0	9.60 ± 6.30.0	0.0

[a] Spores of *C. trifolii* were obtained from liquid culture growth in YPSS medium. Spores were harvested, washed in distilled water, and added to polystyrene dishes (10^4 spores ml^{-1}) (inducing conditions) or 0.7% agarose (10^4 spores) (noninducing conditions) for evaluation of differentiation and germination, respectively. Colony growth was assessed by inoculating an agar plug containing mycelia of *C. trifolii* in YPSS medium and was measured after incubation for 15 days at 22°C. The diameter is the mean for three replicates, 100 spores each. Percent germination or appressorium development after 12 h is shown as the mean for three replicates, 100 spores each.

sented here suggest that the Ca^{2+}/CaM system plays a role in the prepenetration development required for pathogenesis in *C. trifolii*.

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