JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Ultralow Calcium Requirements of Fungi Facilitate Use of Calcium Regulating Agents to Suppress Host Calcium-Dependent Defenses, Synergizing Infection by a Mycoherbicide

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Infection by many fungi activates a variety of calcium dependent defenses in the hosts, slowing or suppressing the attacker and limiting the efficacy of mycoherbicides. The calcium requirement for fungal growth is so low that it could only be implied based on fungi containing calcium-dependent signaling enzymes. Analytical grade media contain <2 μM calcium, and the addition of specific chelators does not affect fungal growth. Hydrophobic derivatives of the calcium-specific chelator BAPTA designed to traverse plant cuticles were synthesized in order to chelate calcium internally during fungal attack. Some chelators as well as calcium precipitating oxalate and channel blocker verapamil were applied with a weakly mycoherbicidal *Colletotrichum coccodes* to cotyledons of compatible *Abutilon threophrasti.* They suppressed calcium dependent callose biosynthesis in the weed and increased virulence but may have affected other calcium-dependent processes that facilitate virulence. The low calcium requirement of fungi, and their high affinity for calcium, allows the application of calcium-regulating agents as synergists for mycoherbicides where the weed uses calcium-dependent defenses.

KEYWORDS: ***Abutilon theophrasti*; BAPTA; *Colletotrichum coccodes,* calcium chelators; calcium requirements; callose; EGTA; infection; mycoherbicides; oxalate; synergists; virulence**

INTRODUCTION

The inundative use of compatible mycoherbicidal pathogens to control specific weeds in intensive row crops has been, in general, an abject failure because of insufficient virulence. This "inundative" approach to biocontrol, with a deluge of mycoherbicide inoculum, is designed to generate a homeostasis between the pathogen and the weed at a much lower weed density than natural infestations with the same organism. Even then, the inundative levels of inoculum often applied do not decimate all stages of weed growth. From an evolutionary standpoint, the lack of full control is understandable; if an organism is hyperpathogenic at normal inoculum levels, both it and the weed host would become extinct, or the weed would be diseased enough to render it to be just another wild but not weedy species (*1*). Intensive row crop agriculture demands that weeds be controlled young and relatively completely, early in the season when their damage potential is the greatest. Inundative biocontrol agents that cannot do this will not replace or even augment herbicides or mechanical cultivation.

Assuming that, for immediate control of a juvenile weed, a foliar or stem pathogen should create a series of contiguous 1 cm^2 lesions, ideally with one propagule per infection per lesion for 100% efficiency. Inundative biocontrol, inundates with 4−5 orders of magnitude more inoculum than the theoretical level for 100% efficiency at a near threshold requirement of 10^4−10^5 propagules applied per cm^2 (*1*). At levels below this, there is no infection at all, at best a "hypersensitive response", a small, constricted lesion of suicidal dead plant cells, that in their dying, kill or totally restrict the growth of the pathogen.

Mycoherbicidal pathogens are typically specific to single host species or to a few related hosts, which should allow the necessary selectivity between crop and weed host. Many *Colletotrichum* species have been proposed as biocontrol agents of weeds, as recently reviewed by Watson et al. (*2*). In this respect, the strain of *Colletotrichum coccodes* that specifically controls *Abutilon theophrasti* (*2*−*5*) is an ideal biocontrol agent, meeting a criterion that is not achieved by chemical herbicides (except in transgenic herbicide-resistant crops), the ability to selectively control *Abutilon* in closely related cotton. In an effort to find secondary hosts, L. A.Wymore, A. K. Watson, and C. Poirier, (McGill University, unpublished report) tried to infect

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10.1021/jf0201780 CCC: $22.00
Published on Web 09/18/2002

scores of other crop and weed species with this strain, including fellow Malvaceae such as cotton, yet the organism killed only *Abutilon theophrasti*, while causing at worst minor transient damage to other species. *Abutilon* has become a hard to control weed in many crops, often requiring the highest registered rates of the herbicides for its control. If it could be controlled by a mycoherbicide, the rates of such herbicides could be lowered to rates sufficient to control other, less pernicious weeds. Unfortunately, the *Colletotrichum* strain was never sufficiently virulent to be commercialized. Thus, ways must be found to circumvent the need for heavy inoculation as well as its hypovirulence, or else biocontrol with *Colletotrichum* cannot be economically sound. We view the *Colletotrichum/Abutilon* situation as a paradigm for the problems of most inundative mycoherbicides in row crops. Most industrial biotechnology efforts have been toward less-expensive production and formulation of the spores used as inocula, instead of finding ways to use less spores or to render the organism more efficient.

There are two major types of reasons for the need of inundations of fungal material: (a) There can be inherent problems in getting the organism to the right place and keeping it alive until an adequate infection is established, i.e., inadequate formulation, and (b) despite a pathogen being "compatible" with the weed, it does have to overcome the inherent defense mechanisms of the weed. This is a "quantitative" battle, i.e., a function of the level of inoculum used. In the past, we have found it fruitful to look at both approaches and have shown that superior formulations can enhance the utility of such agents, by both keeping water present around a spore during germination and establishment (*6*) and creating the ability to formulate mycelia where spores are hard to produce or preserve or where quick establishment is needed (*7*). In the first case, a formulant can be too potent, abolishing selectivity by causing lesions (*8*). We have also demonstrated that ascertaining the particular defenses used (such as phytoalexins, lignin, callose, and pathogenesis-related proteins) by a target weed (*9*) can allow the design of tactics to overcome those defenses (*10*).

In the case of *Colletotrichum* and *Abutilon*, we decided to use a "first principles" approach of first ascertaining then overcoming host defenses. There was reason to target our efforts toward defenses based on physical barriers composed of polymers of callose. A rapid increase in callose biosynthesis is typically seen in compatible responses to *C. coccodes* infection and in infection by many other *Colletotrichum* spp. (*11*). Mutant host plants that lack the ability to synthesize callose are hypersusceptible to fungal attack (*12*). Lignin-like material later becomes embedded in the callose, sterically preventing callose degradation by glucanases and thus inhibiting the progression of infective fungi (*13*). Traces of lignification can severely suppress degradation of carbohydrate polymers such as callose and cellulose by fungal enzymes (*14*). We reasoned that, if the production of callose could be blocked, the virulence of *Colletotrichum* would be enhanced. Callose synthase can also be inhibited by deoxyglucose, enhancing the virulence of a pathogen (*15*). Alas, deoxyglucose is only effective when applied through cut surfaces, because the antimetabolite does not penetrate plant cuticles. Deoxyglucose is not specific to callose synthase, and it is both phosphorylated as well as metabolized to a deoxysucrose derivative (*16*); both are considered to be generally toxic to plant cell metabolism (*17*).

The enzyme callose synthase uses calcium as an obligate cofactor, and the activity of this enzyme in vitro can be blocked by chelators and other regulators (**Figure 1**) that render calcium unavailable (*2, 11, 18*). Calcium is immediately released from

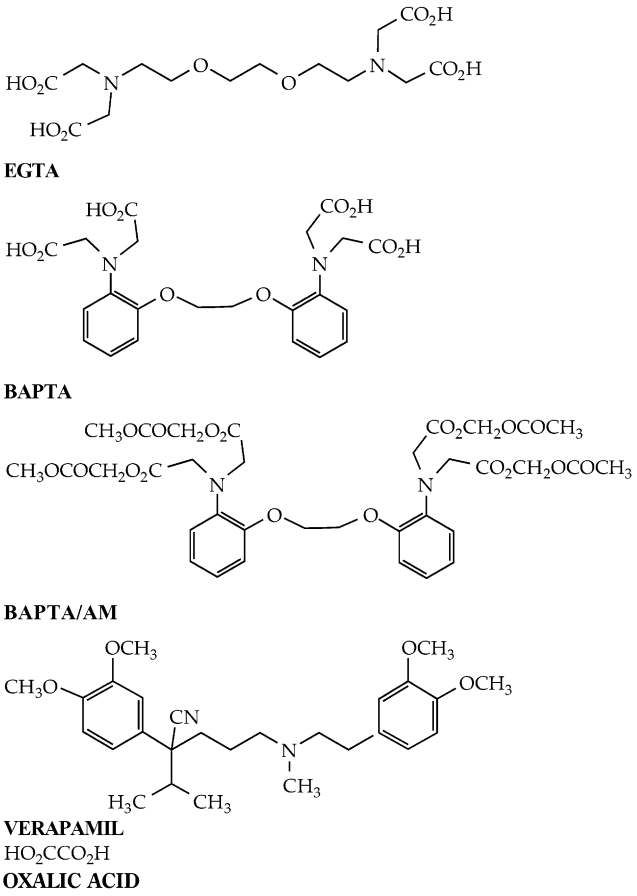

Figure 1. Structures of the available calcium regulators used in this study.

cells into the cell wall matrix and intracellular spaces immediately after fungal attack is sensed by the plant (*19*). Some fungi already seem to use this tactic of rendering calcium unavailable or have been engineered to use it; *Sclerotinia* sp. is a heavy excreter of oxalate, which immediately forms highly insoluble calcium oxalate crystals. This process is an important (*20*) but not sole determinant of virulence (*21*). Conversely, mutants of oxalate producing organisms that lose the ability to secrete oxalate lose virulence (*22*). A gene for overexpression of an oxalate degrading decarboxylase has been engineered into crops, conferring resistance to *Sclerotinia* (*23*). Plants engineered to overproduce oxalate oxidase were also resistant to fungal attack (*24*).

Fungi have very low requirements for calcium that can only be inferred, as there are no reports demonstrating an ability to deplete the fungi or their growth media of calcium to a point limiting growth (*25*). Low calcium in media sometimes has morphological consequences (*25*), but an absolute calcium requirement for fungal viability has yet to be demonstrated. *Colletotrichum* and many other fungi must be able to grow on the traces of calcium in reagent grade chemicals, as many documented media do not contain calcium (*26*). Conversely, there is a large literature in plant pathology showing that augmenting the calcium nutrition of crop plants diminishes pathogen attack, e.g., (*27*), including infection by *Colletotrichum* (*28*).

We wished to test the hypothesis that the virulence of *Colletotrichum* and other fungi that must overcome the formation of callose-containing papillae could be synergized by adding inhibitors of callose synthase. The calcium chelator EGTA and its fluorescent derivative BAPTA have a 10^5 greater affinity for calcium than magnesium (*29, 30*). As most calcium chelators

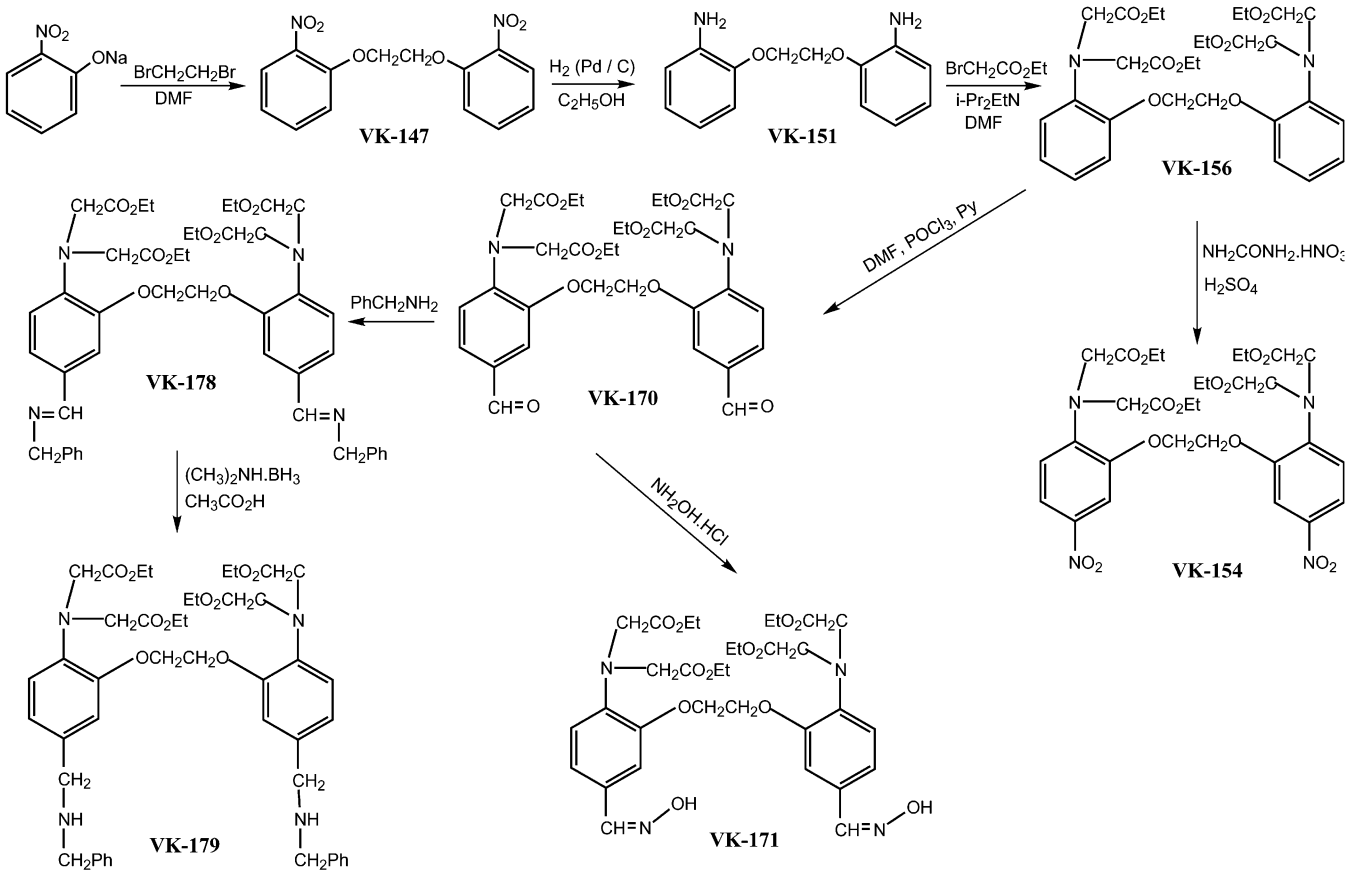

Figure 2. Scheme of synthesis BAPTA esters especially prepared for this study. The precise conditions are described in the text.

are too hydrophilic to penetrate plant cuticles, BAPTA derivatives were synthesized with hydrophobic tails for this study (**Figure 2**) and used with oxalate, a natural complexing agent, as well as verapamil (**Figure 1**), a blocker of calcium transport channels (*31*). We demonstrate that less callose appears when such compounds are used, and the virulence of *Colletotrichum coccodes* on *Abutilon theophrasti* is enhanced.

MATERIALS AND METHODS

Organisms. The *Colletotrichum coccodes* (Wallr.) Hughes strain AG-90 specific to *Abutilon theophrasti* used in many previous studies (*2*−*5*) was kindly provided by Prof. Alan Watson, McGill University. It has been deposited as accession DAOM 183088 in the National Mycological Herbarium, Department of Agriculture, Ottawa, Canada. The inoculum used was always within three transfers of passing through an infected *Abutilon* plant to sustain pathogenicity. The original stocks were maintained under 15% sterile glycerol and stored at −80 °C.

Standard culture was on Difco potato glucose agar (PDA) or broth (PDB). For studies on the need for/effect of calcium, small amounts of mycelia were transferred from PDA culture to a liquid "minimal" medium 7 mM potassium phosphate buffer (pH 7.0), 4 mM $MgSO_4$, 0.5 g L^{-1} l-alanine, and 20 g L^{-1} glucose as well as 0.1 mL L^{-1} Vogel microelement solution (both in (*32*). All reagents used to prepare the latter minimal medium were of the highest analytic purity available to minimize inclusion of calcium. Deionized and then glass-distilled water was used to prepare media. All glassware was soaked in 1.5 N HC1 and twice rinsed with distilled with water, for the same reason.

Abutilon theophrasti Medic. seeds were collected from cotton fields south of Rehovot, Israel. The germination of the seeds was enhanced by bleaching for 1 h in 2% sodium hypochlorite on a shaker, followed by extensive rinsing and then imbibition in water at 37 °C overnight. Seeds were sown in a peat vermiculite mixture in small trays and cultivated in a 25−30 °C day/15−25 °C night greenhouse, 13.5 h average day length.

Sources of Chelators. When this study was begun, BAPTA-AM (**Figure 1**) was the only lipophilic BAPTA analogue commercially available and was purchased from Calbiochem. The calcium channel blocker verapamil (**Figure 1**) was purchased from Sigma.

The BAPTA analogues were synthesized basically according to the scheme in **Figure 2**, as follows:

VK-156 BAPTA-tetraethyl Ester. It was synthesized according to the procedure of Tsien (*29*).

VK-154 5,5′-Dinitro-BAPTA-tetraethyl Ester. 5,5′-Dinitro BAPTA was synthesized according to the procedure of Pethig et al. (*33*).

VK-170 5,5′-Diformyl-BAPTA-tetraethyl Ester. 5,5′-Diformyl BAPTA was synthesized based on the procedure for 5-formyl BAPTA of Grynkuwicz et al. (*34*). Phosphorus oxychloride (15.8 mL, 0.170 mol) was added dropwise to a solution of BAPTA (6.08 g, 0.010 mol) and pyridine (21 mL, 0.260 mol) in DMF (30 mL) at 0 °C. The reaction mixture was stirred for 0.5 h at room temperature, followed for 1 h at 70 °C and overnight at room temperature. Chloroform (300 mL) was added, and the obtained solution was extracted with a solution of NaOH (23 g) in water and ice, then washed with H_2O to pH = 7, $NaHCO_3$ (aq), and brine, then dried over Na_2SO_4, and evaporated under vacuum. The residue was crystallized from ethyl acetate to yield the product (3.61 g, 54%).

1H NMR: δ ($CDCl_3$) = 9.80 (2H, s, 2x **CH**O); 7.39 (2H, dd, J_1 = 1.6 Hz, 2x aromatic ring (H_2)); 7.37 (2H, d, 2x aromatic ring (H_3)); 6.76 (2H, d, J = 8.3 Hz, 2x aromatic ring (H_1)); 4.31 (4H, s, O**CH$_2$CH$_2$**O); 4.22 (8H, s, 4x N**CH$_2$**); 4.06 (8H, q, J = 7.1 Hz, 4x **CH$_2$**CH_3); 1.14 (12H, t, J = 7.1 Hz, 4x CH_2**CH$_3$**).

IR: asymmetric CC(═O)─OC, CC(═O)O─C, C─O and O─C(.../)C stretch 1166, 1179, 1201, 1249 cm^{-1}; C═O stretch (aromatic aldehyde) 1683 cm^{-1}; C═O stretch (aliphatic ester) 1746 cm^{-1}. TLC: Rf (EtOAc/Hex 1:1) = 0.30. mp = 147−8 °C.

VK-171 5,5′-Diformyldioxime-BAPTA-tetraethyl Ester. A mixture of diformyl−BAPTA (1.05 g, 1.6 mmol), hydroxylamine hydrochloride (1.13 g, 16.3 mmol), and $NaHCO_3$ (1.37 g, 16.3 mmol) was stirred in MeOH/H_2O (22 mL, 10/1) at 70 °C for 20 h. After cooling, the mixture

was extracted with $CHCl_3$ (150 mL), and the obtained organic phase was washed with H_2O, 1 M H_2SO_4, H_2O, and 1 M $NaHCO_3$; dried over Na_2SO_4; and evaporated to yield the dioxime product (0.91 g, 83%), mp = 70 °C.

1H NMR: δ ($CDCl_3$) = 8.39 (2H, broad s, N−**OH**), 8.01 (2H, s, 2x **CH**═N); 7.18 (2H, s, 2x aromatic ring (H_3)); 6.95 (2H, d, J = 8.2 Hz, 2x aromatic ring (H_2)); 6.71 (2H, d, J = 8.2 Hz, 2x aromatic ring (H_1)); 4.23 (4H, s, O**CH$_2$CH$_2$**O); 4.16 (8H, s, 4x N**CH$_2$**); 4.04 (8H, q, J = 7.1 Hz, 4x **CH$_2$**CH_3); 1.14 (12H, t, J = 7.1 Hz, 4x CH_2**CH$_3$**). TLC: Rf (1% MeOH/$CHCL_3$) = 0.70.

VK-178 5,5′-Di(benzyliminomethyl)-BAPTA-tetraethyl Ester. A mixture of diformyl-BAPTA (0.45 g, 0.7 mmol) and benzylamine (0.17 mL, 1.56 mmol) in toluene (40 mL) was stirred at 100 °C for 1 h. The toluene was then distilled and the residue dried under vacuum and crystallized from EtOAc/Hex to yield the product (0.283 g, 50%).

1H NMR: δ ($CDCl_3$) = 8.25 (2H, s, 2x **CH**═N); 7.40 (2H, d, J = 1.7 Hz, 2x aromatic ring (H_3)); 7.32 (10H, m centered at 7.32, benzyl ring); 7.16 (2H, dd, J_1= 1.6 Hz, J_2 = 8.2 Hz, 2x aromatic ring (H_2)); 6.76 (2H, d, J = 8.2 Hz, 2x aromatic ring (H_1)); 4.78 (4H, s, 2x **CH$_2$**-Ph); 4.32 (4H, s, O**CH$_2$CH$_2$**O); 4.18 (8H, s, 4x **CH$_2$**N); 4.04 (8H, q, J = 7.1 Hz, 4x **CH$_2$**CH_3); 1.11 (12H, t, J = 7.1 Hz, 4x CH_2**CH$_3$**).

VK-179 5,5′-Di(benzylaminomethyl)-BAPTA-tetraethyl Ester. A mixture of 4,4′-benzyliminomethyl-BAPTA tetraethyl ester (0.18 g, 0.22 mmol) and dimethylaminoborane (0.045 g, 0.76 mmol) in acetic acid (3 mL) was stirred at 120 °C for 2 h. After cooling, chloroform was added, and the solution was washed with $NaHCO_{3(aq)}$, distilled water, and brine; dried over Na_2SO_4; and evaporated under vacuum. The residue (182 mg) was purified by flash chromatography on silica using 2% MeOH in $CHCl_3$ as the eluent. Yield 62 mg, 34%.

1H NMR: δ ($CDCl_3$) = 7.37−7.30 (8H, m, 2x benzyl group); 7.30−7.24 (2H, m, 2x benzyl group); 6.91 (2H,d, J = 1.5 Hz, 2x aromatic ring (H_3)); 6.83 (2H, dd, J_1= 1.4 Hz, J_2= 8.1 Hz, 2x aromatic ring (H_2)); 6.77 (2H, d, J = 8.1 Hz, 2x aromatic ring(H_1)); 4.32 (4H, s, O**CH$_2$CH$_2$**O); 4.14 (8H, s, 4x O_2C**CH$_2$**N); 4.05 (8H, q, J = 7.1 Hz, 4x **CH$_2$**CH_3); 3.79 (4H,s, 2x **CH$_2$**NH); 3.71 (4H, s, 2x **CH$_2$**NH); 1.26 (2H, broad s, 2x **NH**); 1.15 (12H, t, J = 7.1 Hz, 4xCH_2**CH$_3$**).

TLC: Rf (10% MeOH in $CHCl_3$) = 0.35. IR: asymmetric CC(═O)−O−C, C−O−C(.../) stretch 1176, 1255 cm^{-1}; C═O stretch (aliphatic ester) 1746 cm^{-1}.

Measurements of Calcium. As neither a Varian model 1-1000 nor a Perkin-Elmer 5100 PC atomic absorption spectrometer (sensitive above a few μM Ca^{+2}) could detect calcium in the media, a Spectroflame Specto Inductive Coupled Plasma (ICP) atomic emission spectrometer (ca. 20 nM Ca^{+2} sensitivity) was used. Spent liquid culture media were separated from mycelia by centrifugation at 5000 *g* for 1 h at 4 C, and supernatant (media) were fed into the instrument. The precipitated fungal mycelia were extracted by boiling 10 min in 5 mL of 1.5N analytical grade HCl, and the acid solutions were fed into the instrument.

Inoculation of *Abutilon*. Conidia and sclerotia were scraped off mycelium growing on solid culture and resuspended in 2 mL of glass-distilled H_2O. The suspension was vortex mixed for 10 min with no. 3 solid glass balls (Scientific Glass Apparatus, NJ) and filtered as above. The filtered inoculum was washed according to Wymore et al. (*3*) and diluted based on hemocytometer counts. The hemocytometer spore count was consistently five times greater than plate counts suggesting about 20% vitality after inoculum application. Plants were always inoculated at the cotyledonary stage before true leaves appeared, for reasons of both greatest sensitivity and highest uniformity. Inoculum was applied in 0.02% Tween 80 as a single 2 μL droplet containing 10 viable spores to each cotyledon. The water insoluble chelators were dissolved in spectroscopic grade dimethyl sulfoxide (DMSO; Merck, Darmstadt), which had been found to be devoid of calcium (*35*) and verified in this study by ICP. A solution of 1% DMSO alone (the maximum used in the final solutions) served as a control.

After inoculation, the pots or trays were put in a covered plastic box with free water on the bottom to ensure near saturating humidity, in the dark at 23 °C for 24 h, and then opened to the light.

Callose Determination. Callose was determined histochemically, basically as outlined by Shipton and Brown (*36*), after a series of experiments testing other protocols for bleaching the small, fragile cotyledons, and then staining the callose. Variously infected and mock-infected cotyledons were placed in 25 mL vials with 15 mL of a prefiltered solution made from stocks with a final concentrations containing: 65% ethanol, 11.6% w/v phenol, 11.6% v/v glycerol, 11.6% v/v lactic acid, 11.6% water, and 0.67% w/v aniline blue. The sealed vials were placed in a boiling-water bath until the leaves were devoid of pigments (ca. 1 min). The vials were cooled to room temperature, the staining solution was removed and replaced with fresh solution and placed in the hot water bath for another 30 s. The staining solution was replaced first by water, to remove excess stain and then by 20% chloral hydrate for up to 2 days. Just before microscopy, the chloral hydrate was replaced by a fresh solution of chloral hydrate. The cotyledons were picked carefully onto a spatula, excess chloral hydrate was removed using a piece of filter paper, and each cotyledon was placed in a drop of 50% glycerol on a microscope slide and covered. Callose was visualized issuing a Zeiss fluorescence microscope with 365 nm excitation filter and 395 nm cut off filter. The callose fluoresced blue, as described by Kauss (*11*). Cleared leaves were photographed with a camera having an automatic exposure meter that ensured that each picture showed an equal level of fluorescence. Thus, the duration of exposure of each picture is important for analyzing results.

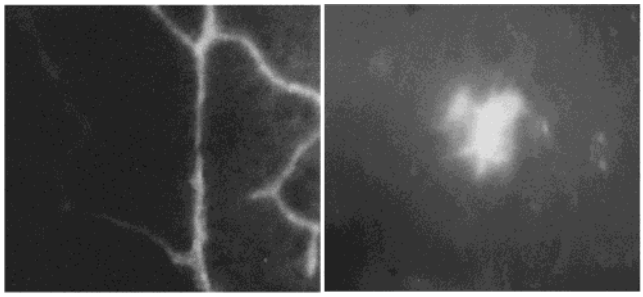

Figure 3. Infection of *Abutilon threophrasti* by a compatible strain of *Colletotrichum coccodes* induces callose biosynthesis. A. Uninfected leaf with aniline blue fluorescing material only in the vascular tissue. B. Callose appearing around an infection site. Leaves were cleared and stained with aniline blue and photographed with a fluorescence microscope.

Effect of Calcium Deprivation of the Fungus. In preliminary experiments, agar, noble agar, and ultrapure agarose were all found to contain a considerable amount of calcium. Thus, calcium deprivation was tested by transferring the fungus to liquid minimal medium for a number of generations in shake culture or by cultivating in a sterile petri plate as a stagnant, thin layer culture. In the latter case, a small piece of scraped mycelium was placed in the center of 5 cm diameter sterile plastic petri dishes in 7 mL of liquid minimal medium with or without calcium-specific regulators. Growth diameter was measured at appropriate intervals, and mycelial density was visually estimated until control cultures reached within a cm of the edge of the dishes. The experiments were then terminated, the mycelia were collected into glass-distilled water, centrifuged, and fresh and dry weight determined, and the mycelium was extracted for calcium analysis by ICP, as described above.

Measurements of Fungal Growth (and Virulence). A total of 11 days after single spot treatment of the cotyledons, virulence was visually scored, and a 9 mm diameter disk was punched from the leaf, and the amount of fungal protein in each leaf disk was determined immunologically. Our previous studies (*37*), as well as those of others have indicated that this is the best quantitative method of determining levels of virulence because of established fungus in leaf tissue.

An antigen was prepared as described by Sharon et al. (*37*) except that the present strain of *C. coccodes* was used. Antisera collected on the 93rd day after the first injection were ammonium sulfate precipitated and biotinylated according to Harlow and Lane (*38*).

Washed and liquid nitrogen frozen leaf tissue was ground for 90 s in an Ultra-Turrax (Jankle & Kunkel, Germany) homogenizer in 150 mM Na_2CO_3, 15 mM $Na_2S_2O_5$, and 25 g L^{-1} PVPP, pH 9.6. The suspension was centrifuged at 40 000 *g*, for 2 h at 4 °C, and the supernatant was used. After general protein determination (*39*), the

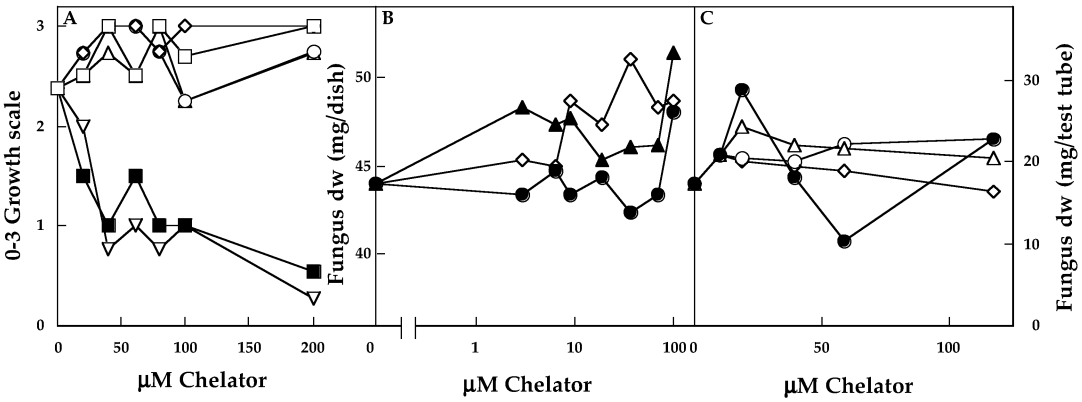

Figure 4. Effects of calcium chelators on fungal growth (results from representative experiments). A. One hundred washed spores were inoculated in each petri dish containing a thin layer of liquid minimal medium. Cultures were visually scored after 14 days. Fungal growth was measured visually on an arbitrary scale: (3) growth similar to untreated control; (0) no growth. B. Lack of effect of the presence of EGTA, BAPTA, and BAPTA ester VK-178 on fungal growth. C. Lack of effect of EGTA, BAPTA-AM, and BAPTA esters VK 170 and VK-178 on fungal growth. B and C. Fungal growth (dry weight) resulting from seeding 25 spores (to limit calcium input with inoculum) seeded in culture tubes after 15 days of incubation. The chelators were introduced as solutions containing 0.1% ethanol, which by itself was slightly inhibitory. The incubation was in darkness, as some BAPTA chelators are photosensitive. Symbols: ▲ BAPTA; □ BAPTA-AM; ● EGTA; ◇ BAPTA ester VK-178; ■ BAPTA ester VK-171; ▽ BAPTA ester VK-179; △ BAPTA ester VK-154; ○ BAPTA ester VK-170. There were no significant differences among any of the treatments and between the treatments and the unamended controls with all chelators other than VK-171 and VK-179 ($p > 0.05$), whereas VK-171 and VK-179 significantly inhibited fungal growth ($p < 0.01$).

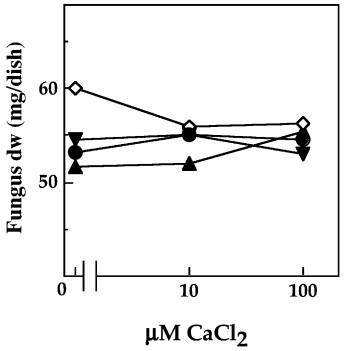

Figure 5. Lack of stimulatory effect of added calcium on fungal growth in the presence of chelators. The concentrations were chosen to give positive and negative molar ratios of Ca^{+2} to chelators. A total of 500 spores were added to each plate containing minimal medium enriched with various levels of $CaCl_2$ and incubated 16 days. Symbols: ● 25 μM EGTA; ◇ 25 μM BAPTA ester VK-178; ▲ 25 μM BAPTA; ▼ 100 μM BAPTA. EGTA, BAPTA BAPTA-AM, and BAPTA esters VK-178, VK-170, and VK-154. There were no significant differences in effect among themselves or with the unamended controls ($p > 0.05$).

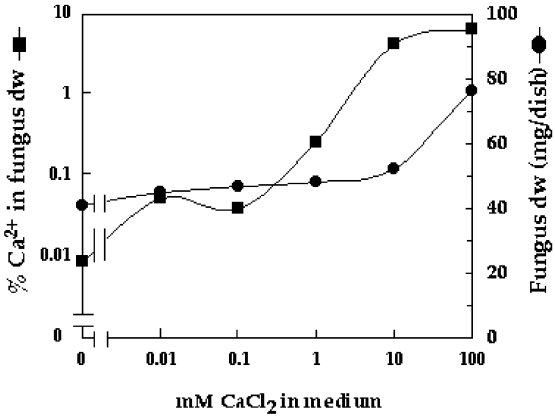

Figure 6. Effect of calcium in the medium on hyphal calcium concentration. A total of 50 spores were planted per dish and incubated with minimal medium containing the indicated amounts of Ca^{+2} (as $CaCl_2$). The zero calcium added (control) medium contained less than 2 μM Ca^{+2}. 1 M Ca^{+2} was toxic to the tissue (not shown). The level of Ca^{+2} in the tissue was measured by extracting the dry tissue and measuring Ca^{+2} in acid extracts by ICP.

amount of fungal protein in the tissue was determined by a sandwiched ELISA technique (*38*) as follows: An ascites cell monoclonal antibody against *C. coccodes* (kindly provided by Prof. I. Barker, Central Science Laboratory, MAFF, Hatching Green, Harpenden U.K.) was diluted 1/600 to 1/1000 depending on total protein, and 100 μL were added per well of F96 Maxisorb NUNC Immuno-Plates. The plates were incubated overnight at 4 °C, washed by rinsing three times in wash solution containing 0.05% Tween-20 (Sigma) in phosphate buffered saline (PBS) and washing one time in glass distilled water (*38*), and then incubated 2 h at room temp with 100 μL blocking solution. After washing, 100 μL of antigen (fungus) containing leaf extract was added and incubated overnight at 4 °C. Plates were washed, and 50 μL of 1/800 diluted biotinylated polyclonal antibody in blocking solution was incubated overnight at 4 °C. The blocking solution contained 1% bovine serum albumin in PBS (*38*). The blocking solution, rather than the biotinylated antibody, was used as a blank. Plates were washed, and 50 μL of 1/800 diluted alkaline phosphatase conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc., PA) in blocking solution was incubated 2 h at room temperature. Finally, the plates were washed with 100μL of substrate (one tablet of Sigma 104TM phosphatase substrate dissolved in 10 mL of 10 mM diethanolamine, 0.5 mM $MgCl_2$, pH = 9.5). The plates were incubated at room temperature until color developed. The color in the plates was quantified at 405 nm with a Dynatech MR5000 microplate reader. Serial dilutions were made of determined amounts of fungal protein on a fixed, identical background of homogenate from uninoculated leaves for calibration. These calibration extracts were incubated in parallel with the samples on the assay plates and experimental sample readings were interpolated from the calibration curve as fungal protein.

RESULTS

*Colletotrichum coccode*s is a rather poor pathogen of *Abutilon*. It is most virulent on the cotyledons, with less virulence on later growth stages (*3*). Single point inoculations of up to 10^3 spores (in a 2 μL droplet) in preliminary experiments only resulted in about 30% of the leaves having a necrotic spot, many of which were similar to a hypersensitive response. This indicates that there was a considerable margin for improvement of virulence.

When *Abutilon* was inoculated with the specific strain of *Colletotrichum*, there was a massive appearance callose at the

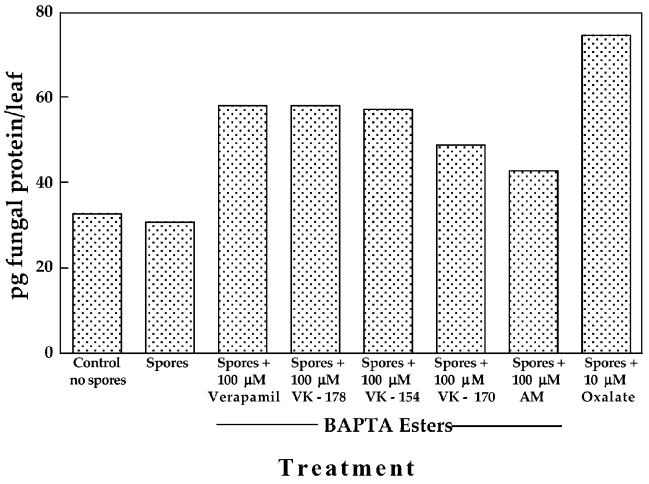

Figure 7. Treatment with calcium chelators and blocker enhances fungal growth in *Abutilon* cotyledons. Cotyledons were inoculated with 2 μL droplet carrying 10 viable spores, with and without chelators and 1% of DMSO carrier. Leaf disks containing the infection zone were excised 11 days later, and the magnitude of fungal infection was immunologically measured 11 days later. Controls with no spores but with oxalate or with VK178 gave similar results as the controls shown (data not shown).

sites of infection (**Figure 3**). This, and the literature discussed above, suggests that the suppression of callose biosynthesis could increase virulence of this mycoherbicide.

***Calcium and* Colletotrichum.** The first step in testing the hypothesis that disruption of calcium mediated effects such as suppressing callose biosynthesis could affect fungal establishment and growth in the host was to ascertain that the fungi could grow in the presence of calcium modulating agents.

The level of calcium in the minimal medium prepared from reagent grade materials was less than 2 μM (*40*). A series of chelators was added to the media, all with affinity constants such that the amounts were orders of magnitude greater than the amounts needed to complex the final residual calcium, as well as with any calcium in the inoculum. The fungi appeared to grow normally on the commercially available BAPTA-AM, as well as on the newly synthesized BAPTA-esters VK-178, VK-154, and VK-170 specially produced for this study (**Figure 4A**). Other experiments yielded similar results with EGTA, the most commonly used calcium chelator, oxalate, and verapamil (*40*). For unexplored reasons, chelators VK-171 and VK-179 inhibited fungal growth (**Figure 4A**) and were not used further. Quantitative studies, measuring dry weight, also indicated that EGTA, BAPTA BAPTA-AM, and BAPTA esters VK-178, VK-170, and VK-154 did not inhibit mycelial growth in the dark (**Figure 4B,C**) or on rich media (*40*).

Conversely, adding calcium to the minimal medium did not enhance fungal growth, without chelator and with different ratios of chelator to calcium (**Figure 5**). Added calcium was accumulated by the fungus when it was in the medium, and the calcium content reached 10% in fungal dry matter at 0.1 M Ca^{++} in the medium (**Figure 6**). This calcium accumulation probably accounts for the increased dry weight at high $CaCl_2$ concentrations. A higher concentration of 1 M $CaCl_2$ in the medium was toxic to the fungus, as might be expected (*40*) from both the osmoticum and from chloride toxicity.

These results suggest that calcium could be preloaded into inoculum during its preparation to ensure that there will be sufficient calcium for all cellular needs, when the fungus is to be later inoculated with a chelator. The above results suggest that either the fungi (a) do not need calcium; (b) have sufficient reserves to not need material from the medium (i.e., have a finite but ultralow calcium requirement); (c) have stronger chelators than those used and can abstract the calcium; and/or (d) degrade the chelators releasing bound calcium. Although a cannot be ruled out (see also ref *25*), the presence of calcium-regulated signaling pathways in fungi that are similar to those in plants and animals, suggests that it is unlikely that fungi are totally without a calcium requirement. It is more likely that *Colletotrichum* has a finite, albeit minuscule, calcium requirement, many orders of magnitude lower than plants and animals, which use calcium mostly for structural needs. The important aspect of these results is that *Colletotrichum* can grow in the presence of calcium chelators, either extracting minuscule amounts from the environment and /or living on stored reserves.

Calcium binding agents and verapamil, a calcium channel blocker, were applied simultaneously with a low, typically sublethal, dose of inoculum, and the level of fungal infection was discerned immunologically. Only the background (noise) level of fungal protein (i.e., not really fungal protein) was detected in the infected tissue without calcium regulators (**Figure 7**). Conversely, both the calcium channel blocker and the various calcium binders supported a massive growth of fungus (in comparison with the background levels of spores alone, which elicited a hypersensitive response and not an infection; **Figure 7**). The different levels of stimulation of hypervirulence among the calcium regulators shown were not significantly different from each other ($p > 0.05$) but are highly significantly different

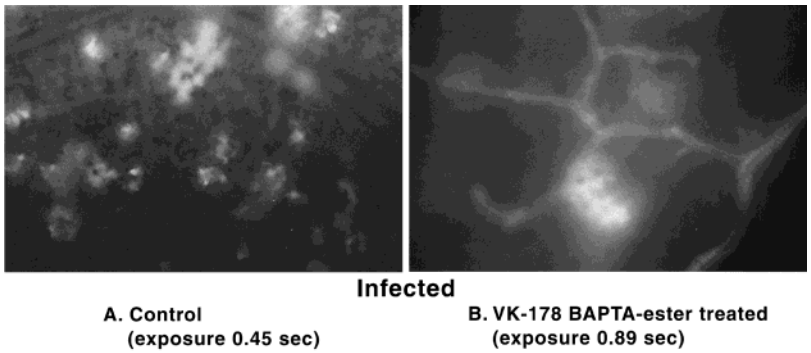

Figure 8. Treatment of cotyledons with calcium chelator BAPTA-ester VK-178 (with fungal inoculum) suppresses callose biosynthesis. Inoculation was by spraying a 5×10^4 mL^{-1} spore suspension (10^4 live spores mL^{-1}) with and without 1 mM BAPTA-ester VK-178, and the leaves were cleared and stained for callose 24 h after inoculation. A microscopic examination representative untreated and chelator-treated infection sites is shown. There was heavy callose deposition (exposure 0.45 s) beneath an infection site, whereas the BAPTA-ester VK-178 treated cotyledon shows faint callose spots and aniline blue staining of the vascular bundles that become visible with the longer (0.89 s) exposure. The magnifications are identical.

from the controls without chelator ($p < 0.01$) or from chelator alone controls without fungus (data not shown).

This supportive effect of free calcium deprivation of host tissue upon inoculation can be correlated with a lack of callose biosynthesis in the cotyledonary tissue. This was seen by clearing and staining tissue for callose, 24 h after inoculation. Bright fluorescence was visible from a multitude of infection sites when no chelator was present as evidenced on macroscopic analysis of whole cotyledons in ultraviolet light, whereas a faint fluorescence was visible from the veins of the chelator-treated cotyledons (not shown). A low magnification fluorescent microscope analysis revealed large callose deposits in many cells near each infection site of the leaves infected without chelator. A representative infection site is shown in **Figure 8A**. A doubled exposure time was typically required to achieve the same fluorescence, as measured by the automatic exposure system, when BAPTA ester VK-178 was used (**Figure 8B**). With the chelator, the fluorescence emanated mainly from polysaccharide in the vascular bundles.

As the data in **Figure 7** indicated far better establishment of the fungus in the presence of the calcium regulators, there is a three way correlation between virulence, deprivation of free calcium, and the suppression of callose biosynthesis. The lack of callose biosynthesis need not be, and is probably not, the only cause of hypervirulence brought about by the presence of calcium regulators, considering all known calcium modulating signaling processes. Callose biosynthesis is probably the process requiring the most calcium and, thus, the process most likely to be affected by calcium deprivation by the chelators.

DISCUSSION

The requirement for calcium is so low that *Colletotrichum coccodes* could be grown for generations on putatively calcium-free media containing EGTA, a very strong calcium specific chelator. The analytical grade reagents used contain enough calcium for normal growth, and calcium was still present in the mycelia (as assayed by ICP). *C. coccodes* could be cultivated on EGTA and other calcium-specific chelators without affecting growth, yet trace amounts of calcium were still routinely found in mycelia using the ultrahigh sensitivity ICP equipment.

As most calcium chelators are too hydrophilic to penetrate plant cuticles, BAPTA derivatives were synthesized with hydrophobic tails (**Figure 2**), and some were not inhibitory to the fungus (**Figure 4**). The calcium regulators were applied along with *Colletotrichum* to *Abutilon*. Immunochemical determination of *Colletotrichum* in the leaves showed greatly enhanced levels of mycelia when the calcium-regulating agents were used (**Figure 7**). Concomitantly, microscopic analysis showed that far less callose was present after such treatments (**Figure 8**). Thus, calcium deprivation increased infectivity while decreasing callose content. This correlation fits the hypothesis but does not prove it, as calcium deprivation can have many effects in plants. *Abutilon theophrasti* is known to contain unidentified allelochemicals that suppress the growth of other plants (*41*, *42*). It is not known whether such compounds also affect fungal pathogens or if they can be induced (i.e., serve as phytoalexins) and whether there is a calcium requirement for their biosynthesis.

Indeed, there is considerable evidence that oxalate is naturally used by fungi as part of their pathogenesis process (*43*). Oxalate can be synthesized in fungi (a) by cleavage of oxaloacetate from Kreb's cycle (TCA cycle) by the enzyme oxaloacetase (*44*); (b) by oxidation of glyoxylate by glyoxylate NADP-1 oxyreductase (glyoxylate dehydrogenase) (*45*); and (c) by oxidation of ascorbate analogues erythroascorbate and its galactoside, by ascorbate oxidase (*46*).

A database search suggests that the genes responsible for oxalate production in fungi have not been isolated. The enzyme glyoxalate dehydrogenase has been completely purified and is dauntingly large (331 kD) (*47*). The gene will probably not be large, as the enzyme is composed of six identical homopolymers.

It is valid to ask whether increasing hypervirulence through oxalate production by the biocontrol agent should be transgenic, based on two arguments: (a) Perhaps it is metabolically and economically "cheaper" to provide commercial oxalate in the formulation than produce it from metabolites in the biocontrol agent. Such production can be at the expense of growth; and (b) providing oxalate in the formulation confers transient hypervirulence. The spores later formed do not have greater virulence. Using exogenous oxalate can act as a failsafe mechanism and preclude the necessity to register a transgenic organism. Oxalate is especially inexpensive to provide.

There is always the possibility that a target weed will evolve resistance to oxalate overproducers or to added oxalate. The oxalate catabolic enzyme oxalate decarboxylase from *Collybia velutipes* (a basidiomycete) has significant sequence homology with germin-like proteins from *Arabidopsis*, tomato, and rice (*23*). Oxalic acid can also be oxidized in plants by oxalate oxidase. Engineering poplars to over-produce oxalate oxidase rendered the plants resistant to *Septoria* (*48*).

Weeds evolve resistance to herbicides, plants in general evolve resistance to fungal pathogens, including those producing oxalate, and weeds can then be expected to evolve resistance to synergized mycoherbicides. Such evolution takes time, but with good crop and technology rotations, the utility of a herbicide or mycoherbicide can be prolonged.

ACKNOWLEDGMENT

Ms. Nava Kahana kindly assisted in posthumously summarizing Prof. Warshawsky's contribution to this study.

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Received for review February 11, 2002. Revised manuscript received July 29, 2002. Accepted July 29, 2002.

JF0201780