

**Effect of Extracellular Metabolites from *Gliocladium virens*
on Germination of Sporangia and Mycelial Growth of *Pythium ultimum***

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ABSTRACT

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Culture supernatants from *Gliocladium virens* grown in 5% bran extract or in minimal medium supplemented with autoclaved hyphae of *Pythium ultimum* inhibited sporangial germination and mycelial growth of *P. ultimum*. Culture supernatants from *G. virens* grown in 5% bran extract contained gliotoxin and laminarinase, amylase, carboxymethylcellulase, chitinase, and protease activities. Bran culture supernatants remained inhibitory after the above enzyme activities were inactivated by heating

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or removed by size-fractionation. Culture supernatants from *G. virens* grown in the supplemented minimal medium, which did not contain these enzyme activities, strongly inhibited sporangial germination and mycelial growth. The metabolite gliotoxin was the only substance that was present in the size-fractionated preparation that was inhibitory to sporangial germination and mycelial growth of *P. ultimum*.

Gliocladium virens Miller, Giddens, & Foster is an important potential biocontrol organism for use against several soilborne plant pathogens (24). It is an antagonist of *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, and *Pythium ultimum* Trow (13,21,25,34). Proposed mechanisms of antagonism resulting in biocontrol are antibiosis, lysis, mycoparasitism, and competition (1,9,26). However, none of these mechanisms has been proved definitively for *G. virens* (24).

Howell (13) reported the inhibition of growth, coagulation of protoplasm, and disintegration of hyphae of *P. ultimum* as a result of interaction with *G. virens*. These phenomena occurred in the absence of hyphal envelopment, hyphal coiling, and hyphal

penetration, suggesting the involvement of extracellular diffusible molecules, such as antibiotics and/or enzymes. *G. virens* produces several antibiotics including gliovirin (14), viridin (5), and gliotoxin (32). Gliovirin is extremely toxic to *P. ultimum* and is capable of coagulating protoplasm and disintegrating hyphae (14). A mutant of *G. virens* deficient in the production of gliovirin also was deficient in antagonistic ability against *P. ultimum*. Although this evidence suggests a major role for gliovirin in antagonism, definitive evidence is lacking. Production of gliovirin by *G. virens* has not been detected in soil (14). Evidence suggesting that gliotoxin and viridin are involved in antagonism is available (17,18), but also not definitive.

Extracellular enzymes produced by *G. virens* may play a role in antagonism of *P. ultimum* by degrading hyphal and sporangial cell walls. Circumstantial evidence for lysis of fungi by extracellular enzymes produced by closely related *Trichoderma* spp. and *G. roseum* has been reported (7,8,10,11,15,23,33). The hydro-

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lytic enzymes, chitinase and laminarinase, which are capable of catalyzing the degradation of fungal cell wall components, were detected in culture supernatants from the above fungi (7,8,10, 15,23,33) and in sterile soil (11). Culture supernatants containing laminarinase and/or chitinase lysed hyphae (8,33), and physical evidence for enzyme degradation of hyphal walls has been presented (23).

Xylanase, β 1,4-endoglucanase, β 1,4-exoglucanase, and β -glucosidase activities have been detected in supernatants of *G. virens* (12,31). However, nothing is known about the possible degradation of hyphae of *P. ultimum* by extracellular enzymes from *G. virens* or the overall role of these extracellular enzymes in biocontrol. The purpose of this research was to identify factors involved in inhibition of sporangial germination and mycelial growth of *P. ultimum* and to further characterize the extracellular proteins of *G. virens*. A preliminary report was published (28).

MATERIALS AND METHODS

Fungal isolates and preparation of mycelium. *G. virens* isolates G5, G10, G13, and G20 (previously designated MTD 356-15, GVM T, MTD2908-18, and GL21, respectively); and *P. ultimum* isolates PuZs1 and PuMxL1 were from the Biocontrol of Plant Diseases Laboratory culture collection. Experiments were performed with isolate G20 unless otherwise indicated. *P. ultimum*, isolate PuMxL1, was grown in still culture in potato-dextrose broth (PDB; Difco, Detroit, MI) at room temperature for 7 days. Mycelia were pelleted by centrifugation at 16,000 *g* at 4 C for 30 min, washed three times with distilled H₂O, dried overnight at 90 C, and ground in a Wiley mill to pass through a 425- μ m mesh screen.

Preparation of culture supernatants. *G. virens* was grown in 5% aqueous bran extract (prepared by boiling 50 g of wheat bran in 1 L of H₂O for 10 min followed by straining through coarse muslin), Weindling's synthetic liquid minimal medium as modified by Jones and Hancock (16) (per liter: 0.5% glycerol [w/v], 2.0 g of [NH₄]₂SO₄, 1.0 g of MgSO₄·7H₂O, 0.5 g of KH₂PO₄, and 3 ml of 1 mM Na-Fe EDTA), or in minimal medium plus 0.5% (w/v) autoclaved mycelium of *P. ultimum* for 5 days at room temperature and 150 rpm. Cultures were centrifuged at 16,000 *g* at 4 C for 30 min and the supernatants frozen until used. To compare enzyme activities in culture supernatants from *G. virens* isolates G5, G10, G13, and G20, these isolates were grown in 5% bran extract for 5 days at 90 rpm at room temperature. Culture supernatants were prepared as described above.

Enzyme assays. Culture supernatant was assayed for carboxymethylcellulase (CMCase), amylase, laminarinase, chitinase, and protease activities. For CMCase and amylase activities, 50 mM sodium acetate buffer, pH 4.2, plus 0.2% (w/v) carboxymethylcellulose or potato starch, respectively, were mixed with culture supernatant and incubated at 50 C. Laminarinase activity was determined by incubating culture supernatant with 50 mM sodium acetate buffer, pH 5.0, plus 0.2% (w/v) laminarin at 50 C. Reducing sugars liberated due to amylase, CMCase, and laminarinase activity were determined by the method of Nelson (22) with a glucose standard. Chitinase activity was determined by incubation of culture supernatant with 50 mM sodium acetate buffer, pH 4.2, plus 0.2% (w/v) chitin at 40 C. Liberated *N*-acetylglucosamine equivalents were determined by the method of Reising et al (27). Protease activity was determined by incubation of culture supernatant with 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5.0, plus 0.4% (w/v) azocoll at 50 C (6).

One unit of CMCase, amylase, or laminarinase activity was defined as the amount of enzyme that released 1 μ mole glucose reducing equivalent/min/mg of protein. One unit of chitinase activity was the amount of enzyme that released 1 μ mole *N*-acetylglucosamine equivalent/min/mg of protein. One unit of protease activity was the amount of enzyme that increased absorbance at 520 nm 1 unit/hr/mg of protein. Protein concentrations were determined by the method of Bradford (4) with bovine serum albumen as a standard.

Thin-layer chromatography of culture supernatants. Culture supernatant from *G. virens* that had been grown in 5% aqueous bran extract was prepared as described above and size-fractionated through an Amicon YM10 membrane (molecular weight cutoff = 10 kDa). Three milliliters of filtrate (<10 kDa fraction) was added to 5 ml of chloroform and extracted by vortexing three times for 30 sec. The aqueous phase was transferred and reextracted three times with chloroform as described above. The chloroform extracts were combined and dried under nitrogen to a final volume of approximately 20 μ l. Extracts were spotted onto Whatman 60A silica gel thin-layer chromatography (TLC) plates (Whatman, Hillsboro, OR) and developed with chloroform/acetone (7:3) or methylene chloride/acetonitrile (6:4). Compounds were detected as quenched spots against a fluorescent background under ultraviolet light.

In vitro antagonism bioassay. Before the assay, isolates of *G. virens* were grown on 5% bran agar and *P. ultimum*, isolate PuZS1, was grown on cornmeal agar (BBL, Cockeysville, MD). Soil extract was prepared by mixing 100 g of soil in 1 L of water and filtering to remove the soil (2). The extract was autoclaved with 1% agar and 0.1% glucose. The resulting soil extract agar was covered with cellophane and seeded with one 5-mm-diameter plug each of *G. virens* and *P. ultimum*. Plugs were positioned 5 cm apart. After 24 hr the plates were scored for 1) inhibition of growth of *P. ultimum* by *G. virens* in advance of colony margins, 2) contact of *G. virens* with hyphal tips of *P. ultimum*, and 3) coiling of hyphae of *G. virens* around older portions of hyphae of *P. ultimum* (3–4 cm into the colony of *P. ultimum*). The percentage of growth inhibition of *P. ultimum* by *G. virens* was calculated with the formula, $1 - [(P + G)/P] \times 100$, where P equaled the increase in radius of colonies of *P. ultimum* on plates containing *P. ultimum* only and P + G equaled the increase in radius of colonies of *P. ultimum* on plates paired with *P. ultimum* and *G. virens*.

Sporangial germination inhibition bioassays. Sporangia of *P. ultimum*, isolate PuMxL1, were prepared by growing the pathogen on cornmeal agar at 25 C for 3 days. These plates were flooded with sterile 10% soil extract prepared as described previously (2) and incubated at 25 C for 7–10 days. Sporangia from these plates were rinsed into a test tube with sterile distilled H₂O, blended for 30 sec, filtered through a 100- μ m nylon mesh, and retained on a 15- μ m nylon mesh (Tetko Inc., Elmsford, NY 10523). The sporangia were washed four times with sterile distilled H₂O and suspended in 50 mM MES buffer, pH 5.0, at a concentration of 50,000–75,000 per milliliter.

In the bioassay to determine the effect of heat-treated culture supernatant on sporangial germination, culture supernatants from *G. virens* grown in 5% bran extract or minimal medium supplemented with hyphae of *P. ultimum* were adjusted to pH 6.7–7.0 with 1 N HCl and 1) not treated, 2) incubated at 80 C for 20 min (heat inactivated), or 3) autoclaved at 121 C and 15 psi for 30 min. Untreated culture supernatant (75 μ l), 75 μ l of heat inactivated culture supernatant, 75 μ l of autoclaved culture supernatant, or 75 μ l of buffer (50 mM MES, pH 5.0) were mixed with 75 μ l of sporangial suspension. Aliquots (25 μ l) of this mixture were mixed with 25 μ l of Difco nutrient broth and incubated at 30 C for 2 hr in sterile plastic petri dishes in a moist container. Sporangia were stained and killed with 0.08% lactofuchsin plus 0.1% Triton X-100 before determining the germination percentage.

In the bioassay to determine the effect of size-fractionated culture supernatants on germination of sporangia, 30 ml of culture supernatant from *G. virens* that had been grown in 5% bran extract was centrifuged at 14,400 *g* for 30 min at 4 C. Five milliliters of supernatant was refrigerated until used (nonfractionated preparation). The remaining 25 ml of supernatant were concentrated to approximately 15 ml by ultrafiltration through an Amicon YM10 membrane. The 10 ml of filtrate (<10 kDa fraction) was refrigerated until used. Ice-cold 50 mM MES buffer, pH 5.0 (200 ml), was combined with the retentate (>10 kDa) and concentrated through the YM10 membrane until 15 ml of retentate remained. The retentate (>10 kDa fraction) was refrigerated until used. All

fractions were used within 1 wk. The sporangial preparation (75 μ l) was combined with 75 μ l of the nonfractionated preparation; 75 μ l of the >10 kDa fraction; 75 μ l of the <10 kDa fraction; or 75 μ l of the 50 mM MES buffer, pH 5.0; mixed; and incubated at 25 C for 0, 2, or 4 hr. An aliquot of each mixture (25 μ l) was combined with 25 μ l of nutrient broth and incubated at 30 C for 2 hr. Sporangia were then stained, killed, and counted as described above.

The effect on germination of sporangia by compounds separated by TLC was determined by bioassay. Silica gel from lanes on plates developed with chloroform/acetone (7:3) was scraped into sterile test tubes in 2-cm portions or as individual quenched spots. The gliotoxin standard (Sigma, St. Louis, MO) and a portion of a lane that had no preparation added also were scraped into sterile test tubes as controls. One milliliter of sterile distilled water and 1 ml of PDB containing 5,000 sporangia per milliliter were added to each tube, the contents were mixed and incubated at 25 C. After 12–24 hr the tubes were examined and scored for growth.

Mycelial growth inhibition bioassays. A 50- μ l sporangial suspension, prepared as described above, was mixed with 200 μ l of nutrient broth in a 48-well tissue culture plate and incubated for 2 hr at 30 C until greater than 95% of the sporangia germinated. Nontreated, heat inactivated, and autoclaved preparations were prepared as described above from culture supernatants of *G. virens*. These preparations were filter-sterilized through a 0.22- μ m filter before use. Nontreated culture supernatant, heat-inactivated culture supernatant, autoclaved culture supernatant, or 50 mM MES, pH 5.0, were combined (250 μ l each) with the germinated sporangia-nutrient broth mixture and incubated at 30 C.

To determine the effect of size-fractionated culture supernatants on mycelial growth, 250 μ l of filter-sterilized (0.22- μ m filter) aliquots of the >10 kDa, <10 kDa, nonfractionated, and buffer (50 mM MES, pH 5.0) preparations (prepared as described above) were combined with the germinated sporangia-nutrient broth mixture and incubated at 30 C. Treatments in both mycelial growth inhibition bioassays were analyzed after 18 hr for mycelial growth inhibition by assessment of growth relative to the nutrient broth control. For the heat inactivation experiments, + equaled growth inhibition and – equaled no growth inhibition. For the size fractionation experiments growth inhibition was scored on a scale of 0–5 where 0 equaled no growth inhibition and 5 equaled complete growth inhibition.

To determine the effect of compounds separated by TLC on mycelial growth, size-fractionated culture supernatant was chromatographed and bioassays performed essentially as for the sporangial germination inhibition bioassay. However, sporangia in PDB were germinated to form hyphal filaments at 25 C for 2 hr before mixing with the TLC plate scrapings.

RESULTS

In vitro antagonism bioassay. In two experiments there was never physical coiling of hyphae of *G. virens* isolates G5, G10, or G13 around hyphae of *P. ultimum*. Hyphae of *G. virens*, isolate G20, occasionally coiled around older hyphae (older than 1 day) of *P. ultimum* but never around hyphal tips. When an additional 16 isolates of *G. virens* were observed for interaction with hyphae of *P. ultimum* there was never physical coiling of hyphae of *G. virens* around hyphal tips of *P. ultimum* and there was only occasional coiling of hyphae of *G. virens* around older portions of hyphae of *P. ultimum*. However, there was inhibition of growth of *P. ultimum* by all four isolates of *G. virens* in advance of colony margins of *G. virens*. Growth of *P. ultimum* was inhibited 68, 68, 64, and 50% by *G. virens* isolates G5, G10, G13, and G20, respectively.

Analysis of extracellular enzymes. Culture supernatants from *G. virens* grown in 5% bran extract contained, on average, 3.1 \pm 0.7 units of laminarinase, 2.0 \pm 0.4 units of amylase, and 51 \pm 6.8 units of protease and low levels of CMCase (0.1 \pm 0.5 units) and chitinase (0.006 \pm 0.002 units). Amylase, chitinase, and CMCase activities were not detected in culture supernatants

from *G. virens* grown in minimal medium, and only trace levels of laminarinase activity was detected in one of eight replicates. Protease, chitinase, and CMCase activities were not detected in culture supernatants from *G. virens* grown in minimal medium supplemented with hyphae of *P. ultimum*. Laminarinase and amylase activities were detected in only three of 13 replicates. Laminarinase and amylase activities were equivalent to those found in bran cultures in these three replicates. The reason for variation in some enzyme activities in minimal medium and minimal medium supplemented with hyphae of culture supernatants of *P. ultimum* is not known.

Enzyme activities associated with isolates of *G. virens* that differ in biocontrol efficacy. Chitinase, CMCase, protease, and laminarinase activities associated with *G. virens* isolates G5, G10, G13, and G20 were compared to determine whether levels of these enzymes correlated with biocontrol efficacy. There were no significant differences in protease (on average, 59.3 \pm 19.6 units) or laminarinase (on average, 2.76 \pm 0.6 units) activities. All four isolates produced low levels of chitinase (on average, 0.006 \pm 0.004 units) and CMCase (on average, 0.08 \pm 0.006 units) activities.

Sporangial germination inhibition bioassays. Size fractionation of culture supernatants from *G. virens* grown in 5% bran extract by ultrafiltration removed laminarinase, amylase, protease, chitinase, and CMCase activities from the <10 kDa fraction. This <10 kDa fraction and the nonfractionated preparation both prevented germination of sporangia of *P. ultimum* (Table 1). When the <10 kDa fraction was further fractionated by TLC, only one region of the chromatogram inhibited germination of sporangia. This region represented a spot that comigrated with the gliotoxin standard. The gliotoxin standard from the chromatogram also inhibited germination of sporangia, whereas the silica gel control did not.

The inhibition of germination of sporangia with the <10 kDa fraction and the nonfractionated preparation was reversible. Sporangia that were not treated or that were treated with the <10 kDa fraction or the nonfractionated preparation had 99, 0, and 0% germination, respectively. However, when these sporangia were washed with 100 ml of sterile distilled water the sporangia treated with the <10 kDa fraction and the nonfractionated preparation had 88 and 89% germination, respectively.

The >10 kDa fraction, which contained the above enzyme activities, did not inhibit germination of sporangia of *P. ultimum*. Treatment of sporangia with this >10 kDa fraction resulted in a level of germination similar to that of sporangia that had been treated with buffer or nutrient broth (Table 1). There was no

TABLE 1. Effect of size-fractionated bran culture supernatant from *Gliocladium virens* on sporangial germination and mycelial growth of *Pythium ultimum*

Preparation ^a	Sporangial germination (%) ^b	Growth inhibition of mycelia ^c	Enzyme activity ^d
<10 kDa	0	4.5 \pm 0.58	–
>10 kDa	78.4 \pm 3.4	0	+
Nonfractionated	0	5.0 \pm 0	+
Buffer	72.2 \pm 7.9	0	–
Nutrient broth	67.0 \pm 5.1	0	–

^a Culture supernatants from *G. virens*, isolate G20, grown in 5% bran extract were size fractionated by ultrafiltration through an Amicon YM10 membrane. Retentate (>10 kDa), eluent (<10 kDa), nonfractionated supernatant, 50 mM MES, pH 5.0 (buffer), or nutrient broth were incubated with sporangia or hyphae of *P. ultimum*.

^b Data are representative of three experiments. Sporangia of *P. ultimum* were mixed with the preparations and then incubated with nutrient broth before counting.

^c The experiment was performed three times. Hyphae of *P. ultimum* were mixed with nutrient broth and preparations of *G. virens*. Mixtures were scored on a scale of 0–5 where 0 = no mycelial growth inhibition and 5 = complete mycelial growth inhibition.

^d Presence (+) or absence (–) of amylase, chitinase, CMCase, laminarinase, and protease activities.

decrease in sporangial germination when sporangia were incubated with the >10 kDa fraction for 4 hr. Sporangia that were incubated with the >10 kDa fraction for 4 hr before the addition of nutrient broth germinated at the same level (70.8% sporangial germination) as the sporangia that had been mixed with the >10 kDa fraction or buffer and immediately incubated with nutrient broth (Table 1).

Incubation of culture supernatants from *G. virens* grown in 5% bran extract at 80 C for 20 min (heat inactivation) decreased laminarinase, amylase, protease, chitinase, and CMCase activities to nondetectable levels (<0.012 units for laminarinase and amylase, <0.0017 units for chitinase, <0.0097 units for CMCase, and <1.2 units for protease). However, heat-treated and nontreated bran culture supernatants had similar levels of inhibition of sporangial germination. In both preparations the dilution end point for inhibition of sporangial germination was between 10^{-1} and 10^{-2} (Table 2). Autoclaving the bran culture supernatant for 30 min inactivated the inhibitory factor(s) (Table 2).

Culture supernatant from *G. virens* grown in minimal medium containing hyphae of *P. ultimum* did not contain detectable levels of laminarinase, amylase, protease, CMCase, and chitinase activities, but it was still capable of inhibiting sporangial germination. In fact, there was more sporangial germination inhibitory factor in these preparations (dilution end point between 10^{-2} and 10^{-3}) than in nontreated bran preparations (dilution end point between 10^{-1} and 10^{-2}) (Table 2), even though the bran extract culture supernatants contained the above enzymes. The inhibitory factor(s) in preparations from media that contained hyphae of *P. ultimum* appeared to be slightly less active after incubation at 80 C for 20 min.

Mycelial growth inhibition bioassay. The <10 kDa fraction, which contained none of the above enzymes, prevented mycelial growth. Absence of mycelial growth was also associated with the nonfractionated bran culture supernatant from *G. virens* (Table 1). As with sporangial germination, when the <10 kDa fraction was further fractionated by TLC only a single compound that comigrated with the gliotoxin standard inhibited mycelial growth. Mycelial growth was not inhibited by the >10 kDa fraction. This fraction allowed profuse mycelial growth as did the buffer and nutrient broth controls (Table 1).

Nontreated and heat-treated bran preparations from *G. virens* inhibited mycelial growth similarly. In both preparations the dilution end point for mycelial growth inhibition was between 10^{-1} and 10^{-2} (Table 2) even though the heat-treated preparation contained no detectable laminarinase, amylase, protease, CMCase, or chitinase activities. As with inhibition of sporangial germination, the factor responsible for inhibiting mycelial growth was inactivated by autoclaving (Table 2).

Both heat-treated and nontreated preparations from media that contained hyphae of *P. ultimum* inhibited mycelial growth. As with sporangial germination, the mycelial growth inhibitory factor

in these preparations was slightly less active after incubation at 80 C for 20 min than in the nontreated preparations (Table 2). There appeared to be more inhibition of mycelial growth associated with these untreated preparations (dilution end point between 10^{-2} and 10^{-3}) than with nontreated bran preparations (Table 2) despite the absence of laminarinase, amylase, protease, chitinase, and CMCase activities.

Identification of the inhibitory factor. The compound that inhibited germination of sporangia and mycelial growth comigrated with a gliotoxin standard on TLC plates developed with chloroform/acetone (7:3). In addition, chloroform extracts of bran culture supernatants were mixed with gliotoxin standard and chromatographed using chloroform/acetone (7:3) and methylene chloride/acetonitrile (6:4) solvent systems. With both solvent systems a single defined spot was present in the mixed preparation that comigrated with the gliotoxin standard.

DISCUSSION

The work reported here and the work of others (14,17) indicate that *G. virens* produces a factor such as an enzyme or antibiotic that is released into culture medium and inhibits growth of *P. ultimum* in vitro. This factor appears to be one or more antibiotics, as suggested by Howell (13). *P. ultimum* is highly susceptible to antibiosis (30) and is highly subject to fungistasis (2,30). In our bioassays the antibiotic gliotoxin was the only metabolite that inhibited mycelial growth and sporangial germination. This antibiotic causes cellular stasis rather than cellular death (17), explaining the reversible nature of inhibition of sporangial germination. Gliovirin, which is toxic to *P. ultimum* (14), and other antibiotics are <10 kDa in size and have been isolated from *G. virens* (32). It is possible that these compounds, not produced under our growth conditions, also inhibit mycelial growth and germination of sporangia.

Our data indicate that the extracellular enzymes laminarinase, amylase, protease, and CMCase of *G. virens* are not primarily responsible for antagonism of *P. ultimum* in vitro. *G. virens* isolates G5, G10, G13, and G20 were studied for their efficacy in controlling damping-off of zinnia caused by *P. ultimum* (21). Of these, isolate G10 was the most effective, resulting in a plant stand that was equal to the control. *G. virens* G20 was intermediate in its biocontrol capability while isolates G5 and G13 were least effective (21). Yet, culture supernatants from these four isolates contained similar enzyme activities. More convincingly, experiments where these enzyme activities were removed from culture supernatants of *G. virens* by heat inactivation or size fractionation showed that these enzymes were not involved in inhibiting sporangial germination or hyphal growth of *P. ultimum* in vitro.

Some of these enzymes, however, may have a subtle role in fungal antagonism in situ. Jones and Hancock (17) present evidence that suggests that gliotoxin toxicity may be affected by

TABLE 2. Inhibition of sporangial germination and mycelial growth of *Pythium ultimum* by culture supernatants of *Gliocladium virens*

Preparation ^a	Sporangial germination (%) at indicated dilution ^b				Mycelial growth inhibition at indicated dilution			
	10^0	10^{-1}	10^{-2}	10^{-3}	10^0	10^{-1}	10^{-2}	10^{-3}
Bran extract								
not treated	0	0	93.8	>90	+	+	-	-
heat-treated	0	0	88.6	97.4	+	+	-	-
autoclaved	85.6	94.8	>90	>90	-	-	-	-
Minimal								
not treated	0	0	0	83.2	+	+	+	-
heat-treated	0	0	90.8	>90	+	+	-	-
autoclaved	91.2	91.4	>90	>90	-	-	-	-
50 mM MES, pH 5.0	83.4				-			
WM+Pu medium	81.4				-			
Bran extract medium	91.8				-			
Nutrient broth	92.4				-			

^a Culture supernatants were prepared by growing *G. virens*, isolate G20, in 5% bran extract or minimal medium containing hyphae of *Pythium ultimum*. Supernatants were incubated at 80 C for 20 min (heat-treated), autoclaved for 30 min, or not treated.

^b Data are representative of three experiments. The coefficient of variation for all treatments was <11%. In all experiments, incubation of sporangia with buffer resulted in approximately 75% sporangial germination. Culture supernatants were diluted with 50 mM MES, pH 5.0.

the chemical composition and architecture of the cell wall of the target organism. They suggested that enzyme degradation of the cell wall may facilitate the diffusion of gliotoxin to the gliotoxin binding site on the cytoplasmic membrane (17). The hyphal cell walls of Oomycetes (including *Pythium* spp.) contain 80–90% carbohydrate, 3–8% lipid, and 4% protein (29). The carbohydrate consists primarily of an insoluble glucan containing β 1,3- and β 1,6-linkages (3). The enzymes laminarinase, protease, and CMCase have the potential to degrade cell wall and membrane constituents of *P. ultimum*. Therefore it is possible that laminarinase, CMCase, and protease allow the diffusion of gliotoxin by degrading the cell wall of *P. ultimum* and exposing the metabolic site of action. Although our data indicate that these cell wall degrading enzymes are not important in the inhibition of sporangial germination and mycelial growth in vitro, their importance may be magnified under conditions where low concentrations of the metabolite are present due to low levels of metabolite production, metabolite inactivation, or binding of the metabolite. These conditions may be those associated with growth of *G. virens* in soil.

A more likely role for enzymes in biological control of *P. ultimum* is in the degradation of organic matter during saprophytic growth of *G. virens* in soil. It is well recognized that *G. virens* and related *Trichoderma* spp. require an organic food base to effectively control soilborne pathogens (18–21,24). The food base often contains wheat bran which must be degraded enzymatically for the fungus to produce secondary metabolites, grow rapidly in soil, and sporulate (19,20,24). It is possible that the enzymes detected in bran extract culture supernatants convert compounds in the bran food base into assimilable nutrients for *G. virens*. Clearly, further investigation of the production, location, and activity of these enzymes under agriculturally relevant conditions is necessary to define their role in biocontrol.

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