Isolation and Localization of the Antibiotic Gliotoxin Produced by *Gliocladium virens* from Alginate Prill in Soil and Soilless Media

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ABSTRACT

Lumsden, R. D., Locke, J. C., Adkins, S. T., Walter, J. F., and Ridout, C. J. 1992. Isolation and localization of the antibiotic gliotoxin produced by *Gliocladium virens* from alginate prill in soil and soilless media. Phytopathology 82:230-235.

Gliocladium virens produced detectable levels of gliotoxin when grown with an alginate-wheat bran food base delivery system in peat moss-vermiculite soilless medium (PV medium). The fungus also effectively controlled damping-off of zinnia seedlings caused by *Pythium ultimum* and *Rhizoctonia solani* in this same system. Gliotoxin was not detectable, and biocontrol effectiveness was greatly reduced when the fungus was grown in a medium containing bark ash (charcoal). Aqueous extracts obtained from PV medium amended with *G. virens* when drenched onto planted flats were as effective for control of damping-off of zinnias as the intact *G. virens*-amended PV medium. Gliotoxin was detected in PV medium (0.42 $\mu g/cm^3$), composted mineral soil (0.36 $\mu g/cm^3$), clay soil (0.20 $\mu g/cm^3$), and sandy soil (0.02 $\mu g/cm^3$), all with natural microbiota but amended with 0.1% alginate prill containing *G. virens* (w/v). At a rate of 0.4% amendment, the amount of gliotoxin detected was quadrupled when compared to the 0.1% rate. Damping-off caused by *P. ultimum* was significantly suppressed by prill of *G. virens* in PV medium, composted mineral soil, and clay soil, but not in sandy soil. Gliotoxin was detected 4–5 cm away from the point source of *G. virens* growing from a single bran-alginate prill in PV medium and appeared to be associated with the mycelium of the advancing margins of the colony. In a concentrated mixture of soilless mix and nonsterile *G. virens* prill (1:1, v/v), gliotoxin was detected at high levels (up to 0.65 mg/g of prill) after 1 day of incubation but not at zero time. The amount of gliotoxin diminished with time but was still detectable after 18 days of incubation (<0.1 mg/g of prill). Gliotoxin was detectable over a wide range of incubation temperatures (15–30 C) in PV medium amended with prill of *G. virens*. After 3 days of incubation, detectable amounts were present at 15 C, but maximum production occurred at 30 C. These results show that gliotoxin can be detected in soils and soilless media and is correlated with disease suppressive activity toward *P. ultimum* and *R. solani* in nonsterile growing media. Factors such as time and temperature, and possibly organic matter, nutrient status, and other chemical and physical factors affect the ability and capacity of *G. virens* to produce gliotoxin.

Gliocladium virens J. H. Miller, J. E. Giddens & A. A. Foster has been effectively used for control of several soilborne plant pathogens (23), especially damping-off diseases caused by *Pythium* spp. and *Rhizoctonia solani* Kühn. Damping-off caused by *Pythium ultimum* Trow has been controlled by this fungus on several crops, including white mustard (36), cotton (11), and zinnia (20,21). Control of damping-off caused by *R. solani*, as well as control of potato scurf (1,3), also has been reported with this fungus on citrus seedlings (32), sugar beet (18), cotton (11), and zinnia and other bedding plants (20,21). A formulation of *G. virens* (isolate GL-21) was recently registered by the U.S. Environmental Protection Agency (EPA) for use against damping-off of greenhouse-grown vegetable and ornamental bedding plants (EPA Registration No. 11699-4 for product WRC-GL-21 and 11699-3 for WRC-AP-1). Although *G. virens* is well established as a potential biological control agent, improved performance and consistency of action of products can only be achieved through understanding the mechanisms of action.

G. virens is closely related to species of *Trichoderma* and has been referred to recently as *Trichoderma virens* (Miller et al) Von Arx (22), earlier as *Trichoderma lignorum* (Tode) Harz (31), amended to *Gliocladium fimbriatum* Gilman et Abbott (30), and as *Trichoderma viride* Persoon ex Fries (4,5,34–38). It is likely that these various species' names represent the same organism as *G. virens*. The name *G. virens*, which is well established in the literature (23), is used here to avoid further confusion. *G. virens* and these closely related species produce secondary metabolites that have biological activity in vitro against plant pathogens (5,6,13–15,25,29–31, 34,35) and that are likely involved to some degree in the mechanism of action in vivo. Additionally, a mycoparasitic mode of action has been suggested (3,11). *T. lignorum* was originally described as parasitic on mycelium of *R. solani* but the same fungus, renamed *G. fimbriatum*, was also reported to produce an antibiotic, gliotoxin (30). Other attempts to attribute primary mycoparasitic action toward *Rhizoctonia* (12) or *Pythium* (25) as a mode of action were negative.

Antibiosis is potentially a principal component of the mechanism of biocontrol by *G. virens*, but understanding the mechanism is complicated by the possible presence of several metabolites produced by *G. virens*. The fungus produces an array of metabolites including gliotoxin, gliovirin, gliocladic acid, heptelidic acid (avocetin), viridin, viridiol, and valinotricin (27). Although gliovirin and viridin may be involved to some extent (13,36), gliotoxin specifically has been implicated in biocontrol mechanisms by Weindling (30) and Wright (35). Other antibiotics also have been implicated, especially gliovirin against *P. ultimum* (13), and viridin against *R. solani* (36,38).

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The objectives of this research were to investigate gliotoxin production under a range of conditions, to determine its importance in the mechanism of biological control, and to establish whether this biologically active metabolite could be isolated from mineral soils or organic potting media. The ultimate aim of this research is to understand the mechanism of action of *G. virens* and use this knowledge to improve performance against *Pythium*, *Rhizoctonia*, and other soilborne plant pathogens.

MATERIALS AND METHODS

Fungal isolates and preparation of inoculants. Subisolate G-20 of *G. virens* (GL-21) (20) was used in all experiments. An alginate prill formulation containing *G. virens*, which was used previously (20), was prepared and provided by W. R. Grace & Co.-Conn., Columbia, MD.

Sporangia of *P. ultimum* (PuZS1), prepared as previously described (2), were drenched onto planted containers (10 × 12 × 6 cm) at a rate of 50 sporangia per cm^2 of surface area to provide about 50% damping-off. *R. solani* (RSSF1, AG4) was grown for 2 wk in sterile soilless medium amended with 0.1% cornmeal (w/w) (autoclaved twice on two consecutive days before infestation), dried, and added to the potting media at a level sufficient to induce about 50% damping-off (0.25 g dry wt/L of medium). Inoculum of *R. solani* was added at the same time with prills of *G. virens*, usually 3 days before planting zinnia seeds.

Test media. Unless noted, experiments were done in soilless medium (Redi-earth, W. R. Grace & Co.-Conn., Cambridge, MA), which is composed of Canadian peat moss, vermiculite, and a nutrient charge (PV medium). Other soilless media used were Metro Mix 350 (PSV medium), Metro Mix 500 (PVC medium) (W. R. Grace & Co.-Conn.), and Ball Growing Mix II (PP medium) (Ball Seed Co., West Chicago, IL). The pH in water of all media was between 6.1 and 6.5. A composted potting soil with a pH of 6.4 consisted of composted loamy soil mixed with peat moss and perlite (3:3:1, v/v). A Beltsville Galestown gravely loamy sand with 78% sand, 12% silt, and 10% clay, (pH 5.8–6.2) and a Beltsville silty clay loam with 18% sand, 50% silt, and 32% clay with 1.4% organic matter (pH 6.3) also were used.

Biocontrol assay. A standard biocontrol assay (20) consisting of potting medium amended with alginate prill of *G. virens* at the standard rate of 1.0% (w/w or 0.1% w/v) was used throughout the study. The amended media were moistened with a volume of 20-10-20 fertilizer solution (Peter's, W. R. Grace & Co.-Conn.) equal to the weight of the medium and incubated for 3 days at 20–25 C before planting 10 zinnia seeds (*Zinnia elegans* Jacq. cv. 'State Fair') in each of four rows in 10- × 16- × 6-cm market pack flats. Flats inoculated with *P. ultimum* were incubated in a growth room at 15–20 C. Flats inoculated with *R. solani* were incubated in a growth chamber at 25–30 C. Seedling stand was determined after 1 wk for *P. ultimum* and after 2 wk for *R. solani*. All tests were repeated at least twice and had four replicated flats per treatment. The number of colony-forming units (cfu) of *G. virens* was assessed with a semi-selective medium (TME) (24) at the time of planting as previously described (20).

Biological activity of PV aqueous extracts. Aqueous extracts of PV medium with or without *G. virens* were obtained by treatment of the medium with sterile distilled water (1:1, w/w) separated from the medium with a Carver hydraulic laboratory press (Menomonee Falls, WI). Sporangia of *P. ultimum* were prepared and added to 100 ml of extract obtained from 1 L of PV medium. The extract with the pathogen was poured onto containers planted with zinnia seed in PV medium as in the standard biocontrol assay.

The aqueous extracts of PV medium were further tested for biological activity after filter sterilization with 0.2-μm syringe filters (MG Scientific, Bufflaoe Grove, IL). Aliquots (0.5 ml) were mixed with 0.5 ml of potato-dextrose yeast extract (PDB-YE) broth (1.0% PDB-0.1% YE) containing 5,000 sporangia of *P. ultimum* in 10- × 75-mm test tubes. After 24 h of incubation at 25 C, growth of mycelial mats was evaluated on a visual scale of 0–3 (0 = no growth, 1 = slight growth, 2 = moderate growth, and 3 = dense growth).

Extraction of gliotoxin from media and soils. A preliminary study was done to determine the efficiency of a chloroform extraction procedure for extraction of gliotoxin from PV medium. PV medium (10 g dry wt) was amended with 1, 5, 10, or 50 μg of standard gliotoxin (Sigma Chemical Co., St. Louis, MO) and extracted with 10 ml of high pressure liquid chromatography (HPLC)-grade chloroform. Chloroform (8 ml) was recovered from the medium with a No. 18 cannula (Luer-loc, BECTON, Dickinson

TABLE 1. Effect of soilless media with different components on biocontrol ability of *Gliocladium virens* against damping-off of zinnia caused by *Pythium ultimum* and *Rhizoctonia solani*

Pathogen and medium	Percentage plant stand		
	Nontreated control	*G. virens*[x] treatment	Pathogen[y] control
P. ultimum			
Peat-vermiculite	89.7 a[z]	83.4 a	63.9 b
Peat-sand-vermiculite	83.9 a	71.3 b	55.5 c
Peat-perlite	79.6 a	64.7 b	48.4 c
Peat-vermiculite-charcoal	78.8 a	59.4 b	43.9 d
R. solani			
Peat-vermiculite	88.1 a	68.9 b	49.0 c
Peat-sand-vermiculite	91.3 a	83.0 a	67.0 b
Peat-perlite	86.9 a	76.9 a	50.3 b
Peat-vermiculite-charcoal	83.8 a	75.7 b	75.0 b

[x] *Gliocladium virens* was added in wheat-bran alginate prill at the rate of 0.1% (w/v).
[y] Pathogen inoculum was added at a rate to provide about 50% disease level. Sporangia (50/cm^2 of pot surface) were added for *P. ultimum*, and dried cultures (0.1%) were added for *R. solani*, grown in sterile soilless medium (peat-vermiculite) with 0.5% cornmeal.
[z] Values ($P \leq 0.05$) in each row for each pathogen followed by the same letter are not significantly different according to an analysis of variance and Duncan's multiple range test. Data were subjected to the arcsine-square root transformation for statistical analysis; tabulated numbers represent back-transformed data. Data are the composite results of three experiments with similar results.

TABLE 2. Effect against damping-off of zinnia caused by *Pythium ultimum* of aqueous extracts from peatmoss-vermiculite soilless medium compared to nonextracted medium, both amended with bran alginate prill of *Gliocladium virens*

Treatment[x]	Percentage of healthy plants	Growth of *P. ultimum*[y]
Healthy check		
Complete	91.8 a[z]	NT
Drench	91.4 a	+++
Bran prill, 4× rate		
Complete	89.4 a	NT
Drench	74.9 a	0
Bran prill, 1× rate		
Complete	73.3 b	NT
Drench	71.4 b	+
Pythium check		
Complete	53.4 c	NT
Drench	54.5 c	+++

[x] Extract from soilless medium (amended with 0.1% or 0.4% (w/v) of *G. virens* and incubated at 25 C for 3 days) forced from the mix with hydraulic pressure was drenched onto nonamended medium after planting. Complete medium was amended with *G. virens* but not extracted.
[y] Filter-sterilized (0.2-μm syringe filter) aqueous extract (1.0 ml) incubated with 5,000 sporangia of *P. ultimum* in 1.0 ml of potato-dextrose broth and incubated 24 h at 25 C. 0 = no growth, + = slight growth, ++ = moderate growth, +++ = dense growth, and NT = not tested.
[z] Values followed by a common letter are not significantly different at $P \leq 0.05$ according to an analysis of variance and Duncan's multiple range test. Data were subjected to the arcsine-square root transformation for statistical analysis. Tabulated numbers represent back-transformed data. Data are the composite results of three experiments with similar results.

& Co., Rutherford, NJ) and glass syringe. The chloroform extract was reduced to dryness, the residue was resuspended in HPLC-grade chloroform (100 μl) and evaluated by HPLC. Samples were injected directly into a Beckman Ultrasphere ODS 5μ 4.6-mm × 25-cm reverse-phase column with a Whatman Universal guard cartridge precolumn. The eluent (1 ml/min flow rate) was 65% double-distilled water, 20% HPLC-grade acetonitrile, and 15% HPLC-grade methanol, adjusted to pH 4.0 with acetic acid. The detector was set at 254 nm. Elution time for gliotoxin was 15 ± 1 min. The recovery of gliotoxin for each of the above samples was 12.2, 16.6, 18.9, and 10.4%, respectively. A subsequent preliminary experiment was intended to determine the effect of repeated extraction on recovery of gliotoxin. Serial extractions of 5.0 g of PV medium amended with 100 μg of gliotoxin yielded 15.1, 7.5, 3.6, and 1.6 μg of gliotoxin with each 10-ml vol of chloroform, for a total recovery of 27.7 μg. Thus, 22.6% of the gliotoxin was recovered in the first two extractions. Therefore, chloroform extracts were made from soilless media and soils amended with prill of *G. virens* by adding two sequential aliqots of 5 ml of chloroform to 5 cm^3 of compacted medium. Each sample was mixed thoroughly, and the chloroform was withdrawn with the No. 18 cannula and glass syringe. The extracts were filtered (0.45-μm nylon syringe filter) and pooled. Extracts were dispensed in decreasing volumes (1.0, 0.5, 0.25 ml) into 10- × 75-mm test tubes or 4-ml vol into capped vials; all samples were evaporated to dryness using either a stream of N_2 gas or a vacuum. The sample tubes containing decreasing amounts of dried extract were filled with 1.0 ml of PDB-YE containing 5,000 sporangia of *P. ultimum* or diluted *R. solani* mycelial fragments and incubated 24 h at 25 C. Growth was assessed as described above. The 4.0-ml samples were reconstituted in HPLC-grade chloroform (100 μl) for analysis by thin-layer chromatography (TLC).

TABLE 3. Detection of gliotoxin and biological activity of chloroform extracts of soils and soilless medium with biocontrol activity induced by *Gliocladium virens* againts Pythium damping-off of zinnia

Medium/ treatment[w]	Percentage of healthy plant stand[x]	Growth of Pythium[y]	Gliotoxin μg/cm[3z]
Nontreated soilless medium	97.0 a	+++	0.0
Pythium check	22.7 d	NT	NT
G. virens, 0.1%	61.4 c	+	0.418 ± 0.021
G. virens, 0.4%	81.2 b	0	1.320 ± 0.047
Nontreated compost soil	72.6 a	+++	0.0
Pythium check	28.3 c	NT	NT
G. virens, 0.1%	51.3 b	++	0.355 ± 0.003
G. virens, 0.4%	65.9 a	+	1.110 ± 0.004
Nontreated clay soil	34.1 b	+++	0.0
Pythium check	28.1 b	NT	NT
G. virens, 0.1%	42.5 b	++	0.20 ± 0.001
G. virens, 0.4%	57.7 a	+	0.90 ± 0.004
Nontreated sandy soil	40.5 a	++	0.0
Pythium check	46.1 a	NT	NT
G. virens, 0.1%	52.7 a	++	0.016 ± 0.001
G. virens, 0.4%	49.0 a	+	0.145 ± 0.002

[w]Growing media amended with 0.1% or 0.4% alginate-wheat bran prill of *G. virens* (G-20), added 3 days before planting and infested with *Pythium ultimum*.

[x]There was a significant treatment × soil interaction ($P \leq 0.0001$), therefore means were separated for each soil by Duncan's multiple range test. Data were arcsine-square root transformed. Tabulated numbers represent back-transformed data. Values for each soil followed by the same letter are not significantly different at $P \leq 0.0001$. Data are composite results of two experiments with similar results.

[y]Growth of *P. ultimum* in potato-dextrose broth in the presence or absence of dried samples of chloroform extracts of amended soil. 0 = no growth, + = slight growth, ++ = moderate growth, +++ = dense growth, and NT = not tested.

[z]Micrograms of gliotoxin per cubic centimeter of growing media (± standard error) extracted with 10 ml of chloroform and assayed by high pressure liquid chromatography (chloroform/methanol, 7:3).

Aliquots (20 μl) were spotted on channelled, 20- × 20-cm fluorescent indicator plates (Whatman LK6DF) and developed with chloroform/acetone (7:3, v/v) for 45 min. Gliotoxin standards were also applied, and the developed plates were visualized with shortwave UV light (254 nm). The R_f value for the standard gliotoxin was approximately 0.54. Areas on the TLC plates that contained visible spots of gliotoxin were scraped from the glass plates with a single-edged razor blade. The scrapings were transferred to sterile 10- × 75-mm test tubes and assayed for the effect on growth of *P. ultimum* and *R. solani* as described above.

Effect of time, temperature, and distance on gliotoxin production. Gliotoxin production by *G. virens* in vitro was measured by growing the fungus in 3- × 5-cm glass vials containing 1 g of sterile or nonsterile PV medium and 1 g of alginate prill containing *G. virens*. The components were mixed thoroughly after adding 2.0 ml of water and were incubated at 25 C for 1–18 days. The effect of temperature on gliotoxin production was studied by preincubation of 0.1% (w/v) prill of *G. virens* in PV medium at various temperatures. Extracts were made by mixing chloroform (5 ml) with 5-cm^3 samples of PV medium and recovering the chloroform extract as described above. The extraction procedure often required pressure to compact the medium and draw the chloroform into the syringe. Chloroform-extracted samples were handled for TLC and HPLC as described above.

The presence of gliotoxin at different distances from a single alginate prill containing *G. virens* was determined in moistened PV medium (an equal volume of water to weight of medium) in plastic petri dishes. After incubation for 4 days at 25 C, the medium was sampled at 1-cm intervals from the single prill previously placed in the center of the dish. The PV medium samples containing *G. virens* were extracted with a volume of chloroform equal to the weight of the medium (1 ml/1 g). HPLC was performed on samples as described above. Similar 1-cm interval sampling (total 6 cm) and analysis were conducted from samples taken from 3- × 10-cm glass vials in which 1.0 g of *G. virens* alginate prill (massed prill) was added to the top 1 cm of medium in the containers.

Statistical analyses. Experiments were repeated twice, unless otherwise indicated, with four replicates. Tabulated data were averaged and subjected to the arcsine-square root transformation, analysis of variance, and Duncan's multiple range test. Tabulated numbers represent back-transformed data. Graphic data was analyzed by a simple *t* test, and standard errors are indicated.

RESULTS

Disease control in soilless media. Damping-off diseases caused by *P. ultimum* and *R. solani* were suppressed by *G. virens* in wheat bran-alginate prill in PV medium (Table 1). Also, with two other commercial media containing peat moss as the primary ingredient (PSV and PP media), plant stand was significantly

TABLE 4. Localization of gliotoxin produced by *Gliocladium virens* growing from prill in soilless medium

Distance from source (cm)	Gliotoxin μg/g dry wt[y]	
	Massed prill[z]	Single prill[z]
0–1	1.311 ± 0.533	0.678 ± 0.026
1–2	0.190 ± 0.023	0.087 ± 0.005
2–3	0.089 ± 0.020	0.061 ± 0.021
3–4	0.109 ± 0.009	0.041 ± 0.046
4–5	0.024 ± 0.013	0.0
5–6	0.0	0.0

[y]Gliotoxin extracted with chloroform and quantified by high pressure liquid chromatography (chloroform/methanol, 7:3). Values are micrograms of gliotoxin per gram dry wt ± standard error.

[z]Alginate prill containing *G. virens* added to top 1 cm of container with soilless medium (1 g:1 g) (massed prill) or as a single prill. Medium sampled and extracted at various distances after 3 days.

increased by the *G. virens* amendment compared to the pathogen controls. In a medium containing peat moss, vermiculite, and a charcoal-like "bark ash" component (PVC medium), however, *G. virens* was not effective for control of either pathogen. In all media, the population densities of *G. virens* produced from the alginate prill were approximately equal, ranging from 1.3 to 3.7×10^7 cfu/g dry weight of medium. The nonamended media had background populations of 10^3–10^4 cfu of *G. virens* and *Trichoderma* spp. The pH values for all four media were similar (pH 6.0–6.3).

Detection and extraction of gliotoxin. Aqueous extracts, which were obtained from the PV medium amended with prill of *G. virens*, provided protection from damping-off caused by *P. ultimum* equal to the standard incorporated prill treatment when drenched along with sporangia of *P. ultimum* onto flats of zinnia (Table 2). The treatment with 4× the standard rate of prill (0.4% w/v) was statistically equal to the noninoculated healthy control (Table 2). Aqueous extracts subsequently re-extracted with chloroform (1:10, v/v) and applied to thin-layer chromatograms did not contain detectable levels of gliotoxin. However, aqueous extracts filter-sterilized and incubated in the presence of *P. ultimum* sporangia inhibited growth when tested in vitro in PDB. Extracts from nonamended controls did not inhibit growth (Table 2).

Gliotoxin was detected in chloroform extracts from PV medium, composted mineral soil, clay loam field soil, and sandy loam field soil that were amended with the same amount of *G. virens* (Table 3). Gliotoxin was never recovered from the soilless medium containing bark ash (PVC medium). In general, gliotoxin levels at approximately 1.0 $\mu g/cm^3$ of medium were associated with significantly improved levels of disease control compared to treatments in which less than 1.0 μg of gliotoxin was detected. Media amended with 4× the standard rate of prill (0.4%, w/v) yielded about 4× as much gliotoxin in chloroform extracts. Disease control also was enhanced by the increased level of *G. virens* amendment except in sandy soil. Zinnia seedling stand, however, was greatly reduced in both mineral soils even in the treatments without added pathogen inoculum when compared to media containing abundant organic matter. Chloroform extracts, from the PV medium concentrated 8×, strongly inhibited germination of sporangia and growth of *P. ultimum* especially with the 4× *G. virens* amendment. Population densities of *G. virens* were equal with both rates of amendment in PV medium (3×10^7), approximately equal in compost soil (0.5–1.3×10^7), and approximately equal but lower than the above in clay (1.7–3.0×10^6) and sandy soils (1.8–4.3×10^5).

Localization of gliotoxin. Gliotoxin was detected by HPLC in chloroform extracts of PV medium amended with a single alginate prill containing *G. virens* (Table 4). The gliotoxin was most abundant around the individual prill recovered from the medium. Appreciable gliotoxin was detected at 1-cm intervals as far as 3–4 cm from the individual prill. This distance was the same as the distance that *G. virens* had grown from the prill as detected by plating PV medium on TME. In fact, gliotoxin was detected in the entire sphere of adhering mycelium and medium surrounding the prill from which the *G. virens* grew (the "fungal aggregate"). When a quantity of massed prill was applied to the PV medium (1 g of prill/1 g of medium), the amount of gliotoxin detected usually doubled and was detected at a greater distance than with the single prill (Table 4).

Gliotoxin was detected 1–2 days after amendment with concentrated amounts of prill of *G. virens* in PV medium (1 g of prill/1 g of medium) (Fig. 1). The maximum amount of gliotoxin produced peaked between 1 and 4 days, then diminished to low levels between 9 and 18 days. No gliotoxin was detected in prill alone nor at time zero in soilless medium. The amount of gliotoxin was approximately the same whether produced under sterile (autoclaved PV medium and autoclaved alginate prill with conidia of *G. virens* added) or nonsterile conditions (PV medium with resident microflora and with nonsterile prill of *G. virens* incorporated) (data not shown). Extracts containing as little as 1.0 μg of gliotoxin per gram of prill inhibited germination or growth of both *P. ultimum* and *R. solani*. When chloroform extracts were chromatographed, the TLC band comigrating with the gliotoxin standard was always inhibitory to both pathogens when scraped off the TLC plate and bioassayed in PDB-YE broth. Growth of *R. solani* mycelium and germination and growth of *P. ultimum* sporangia were always inhibited when gliotoxin could be seen on the chromatogram (not shown).

Effect of temperature on gliotoxin production. The temperature of incubation of prill of *G. virens* in PV medium affected the amount of gliotoxin produced after 3 days (Fig. 2). Gliotoxin production increased as the incubation temperature increased from 15 to 30 C.

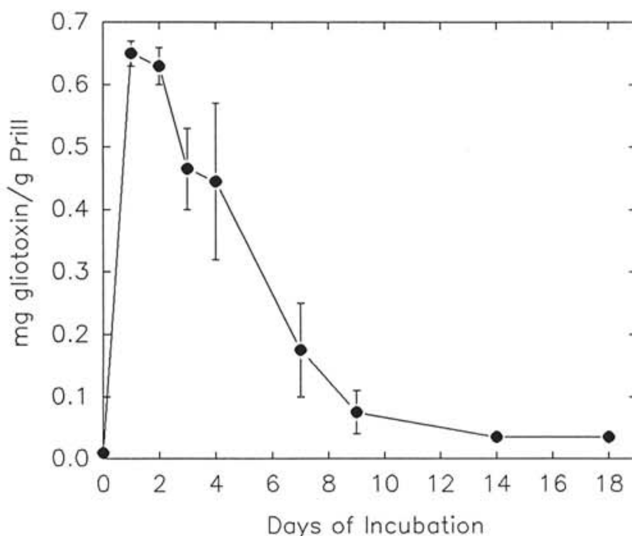

Fig. 1. Effect of time on the amount of gliotoxin extracted from peatmoss-vermiculite soilless medium containing *Gliocladium virens* in sodium alginate prill. Samples of medium were extracted with an equal volume to weight of chloroform and assayed by high pressure liquid chromatography. Values are means ± standard error and represent a typical experiment repeated three times.

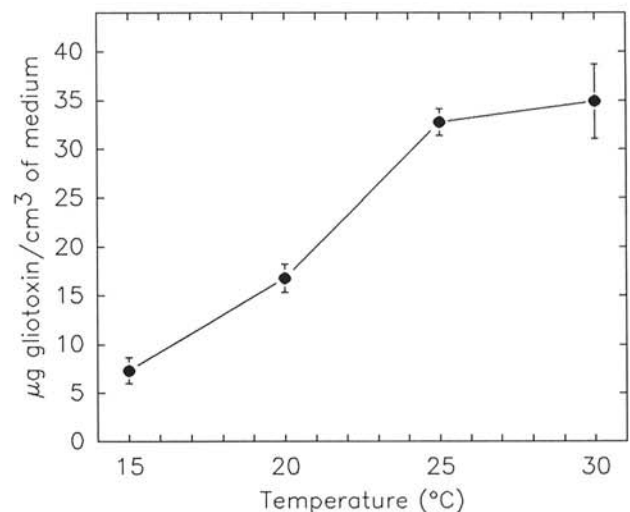

Fig. 2. Effect of temperature on production of gliotoxin in peatmoss-vermiculite soilless medium by *Gliocladium virens* in sodium alginate prill. Samples of medium were extracted with an equal volume to weight of chloroform and assayed by high pressure liquid chromatography after 3 days of incubation. Values are means ± standard error and represent a typical experiment repeated twice.

DISCUSSION

The biological significance of antibiotics produced by soil-inhabiting fungi, bacteria, and actinomycetes has been a controversial topic for several decades (8,33). The implication of antibiotics in biocontrol mechanisms has been clearly demonstrated in only limited cases in sterilized soil (26,35), in organic matter-amended soil (34), in wheat plant rhizospheres (28), and around rotting seed (38). Gliotoxin adversely affects membranes of *Pythium* and *Rhizoctonia* (16) and may be a factor in the leakage of metabolites from hyphae of *Rhizoctonia* treated with extracts from cultures of *G. virens* (19).

Experiments described here demonstrated that *G. virens* produces the antibiotic gliotoxin early in the growth cycle rather than later, as is often the case with secondary metabolites (7). The compound is produced over a period of several days in a nonsterile soilless medium and in nonsterile agricultural soils. Gliotoxin is produced in association with the mycelium of the fungus in the medium away from the point of origin of growth and food base location. This evidence does not address the argument that microorganisms cannot produce antibiotics in soil without nutrient amendment but does show that sterilization of soil is not required and that gliotoxin is produced by the leading edge of the mycelial colony.

Nutrients are required for biological control activity of *G. virens* (17,18) and production of gliotoxin (7,8). The presence of organic matter in soil in addition to the food base may also be important. This is suggested by the strikingly greater production of gliotoxin in organic-based media versus mineral soils with low organic matter content. The organic matter may supplement nutrient requirements provided by the alginate-wheat bran food base, increase porosity, or reduce binding by clay, silt, or colloids that affect antibiotic activity in mineral soils (33). Organic matter may also have some protective effect against instability. Acidic conditions enhance chemical stability of gliotoxin, and rapid breakdown occurs above pH 7.0 (5,30,31). Increased calcium levels in organic soils may also enhance production of gliotoxin (38).

Alginate prill (9) provides a rich food base for growth and biocontrol activity of *G. virens* (17,18,20). This food base serves adequately as the nutrient source for gliotoxin production. Gliotoxin is produced abundantly from *G. virens* or other fungi grown on food bases such as wheat meal and clover (34,35), wheat straw (37), or seed coats (38). Although these natural food bases provide nutrients for gliotoxin production, alginate prill has the potential for superior performance, because selective ingredients can be added to the granule to enhance growth and antibiotic production.

The alginate prill formulation can enhance the successful competitiveness of *G. virens* in soil as defined by Garrett (10): rapid germination, rapid growth, enzyme production, and antibiotic production. Chlamydospores of *G. virens* in the alginate prill germinate and outcompete other soil microorganisms, because the fungus has possession of the substrate for satisfaction of Garrett's first two requirements. Furthermore, germinated chlamydospores of *G. virens* can produce necessary enzymes from the food base for supplying primary precursors for metabolism and for production of gliotoxin (25). The gliotoxin also may serve to inhibit soil microbial competitors, and in the process may affect germination and growth of *Pythium* and *Rhizoctonia* to bring about biological control.

The fact that gliotoxin was a major metabolite detected in this system and that it had biological activity does not discount involvement of other metabolites. Other metabolites of *G. virens* with antifungal activity include viridin (6,14,37,38) and gliovirin (13). These antibiotics may also be produced by *G. virens* (G-20) in small quantities or they may be less efficiently extracted than gliotoxin. Even gliotoxin was only partially recovered (10–20%).

Other evidence, however, indicates that gliotoxin is a major component of the biocontrol systems of *G. virens*. The biocontrol activity was transferrable in aqueous extracts and was reversible in a medium containing charcoal, which could have absorbed the gliotoxin. Furthermore, previously (36) gliotoxin-producing strains were most effective for biocontrol of Pythium damping-off of mustard, and a viridin-producing strain was intermediate in effectiveness. In contrast, gliotoxin production was not correlated with control of *R. solani*, but the variability was attributed to variable sensitivity of anastomosis groups of *R. solani* to gliotoxin (15). Groups AG1, AG4, and AG5 required much more gliotoxin for inhibition than other groups tested. Our AG4 isolate of *R. solani*, however, was sensitive to gliotoxin. Variation in sensitivity of pathogens to gliotoxin needs further examination.

Other mechanisms of biocontrol may play a minor, if any, role in this biocontrol system. Mycoparasitism has not been shown to be involved in the biocontrol of *Pythium* and is of minor significance in the biocontrol of *Rhizoctonia* (12). Antibiosis as a possible primary mechanism of biocontrol in this instance is one that can be readily manipulated for improvements in biological control ability. Immediate improvements may be implemented by selecting strains superior or exclusive for production of gliotoxin, selecting fermentation systems for *G. virens* that favor later production of gliotoxin, and incorporating formulation ingredients that enhance gliotoxin production in soil.

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