Mechanisms in the Biocontrol of *Rhizoctonia solani*-Induced Cotton Seedling Disease by *Gliocladium virens*: Antibiosis

C. R. Howell and R. D. Stipanovic

First author, research plant pathologist, and second author, supervisory research chemist, Southern Crops Research Laboratory, USDA/ARS, 2765F&B Road, College Station, TX 77845.

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ABSTRACT

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Production of the antifungal antibiotics gliotoxin and gliovirin by the biocontrol fungus *Gliocladium virens* has been associated with its efficacy as a biocontrol agent of seedling diseases incited by *Rhizoctonia solani* and *Pythium ultimum*. The association of gliotoxin and gliovirin with control of *P. ultimum*-incited disease has been confirmed by mutational analysis, but this is not true of gliotoxin and *R. solani*-incited disease. Gliotoxin-deficient (GLT−) mutants of *G. virens* were produced with UV and detected with *Bacillus subtilis*. Gliotoxin loss in the mutants was confirmed by high-pressure liquid chromatography analysis. Gliotoxin deficiency also results in loss of resistance to gliotoxin by GLT− strains. Assay of the GLT− mutants for suppression of *R. solani*-incited cotton seedling disease showed their biocontrol efficacy to be equal to that of the parent strains, indicating that gliotoxin is not necessary for protection of the infection court. These results and prior research put into question the importance of mycoparasitism and antibiosis as mechanisms in control of this disease, and suggest that the role of competition should be investigated more closely.

Additional keyword: UV mutation.

The physiology and biocontrol efficacy of the Deuteromycete fungus *Gliocladium virens* J. H. Miller, J. E. Giddens, & A. A. Foster has been much studied in recent years (4,7,10–12,16–18, 20–23). *Gliocladium virens* has proven effective as a suppressant of damping-off diseases incited by *Pythium ultimum* Trow and *Rhizoctonia solani* Kühn (1,5,7,11,15,21). The mechanisms of biocontrol of plant diseases fall into five general categories: parasitism, antibiosis, competition, induced resistance, and lysis (2). Research on mycoparasitism as a mechanism in the biocontrol of plant pathogens by *G. virens* has shown that although it may play an important role in pathogen inoculum reduction by attacking resting structures in the soil (5), it does not appear to be necessary in protection of the infection court by the antagonist (6). Antibiosis, however, is very much involved in protection of the infection court from *P. ultimum* by *G. virens*. The production of gliovirin by "P" strains and of gliotoxin by "Q" strains of *G. virens* (10) appears to play a vital role in this particular antagonist/pathogen interaction, and disease suppression is dependent on their synthesis (8,23). The importance of gliotoxin in the suppression of *P. ultimum*-induced seedling disease has recently been confirmed by mutational analysis (23).

The role of gliotoxin in the biocontrol of *R. solani*-incited seedling damping-off by *G. virens* is less certain, and gliovirin and viridin are not active against this pathogen (10). Although gliotoxin-producing strains are the most effective suppressors of Rhizoctonia damping-off, and gliotoxin is strongly inhibitory to *R. solani* AG-4, the importance of this antibiotic to the biocontrol process has not been comfirmed by mutational analysis.

The objectives of this study were to produce UV-induced mutants of *G. virens* strains that were deficient for gliotoxin production, and compare their biocontrol efficacies against *R. solani* AG-4 with that of parent strains. This would help to ascertain the role of gliotoxin production in the *G. virens*/*R. solani* biocontrol phenomenon, and allow us to focus on more efficient means of screening for effective biocontrol strains.

MATERIALS AND METHODS

UV mutagenesis of *G. virens* strains, and detection and isolation of gliotoxin deficient mutants. Conidia of *G. virens* strains G-6, G-11, and G-20 were suspended in sterile water (2×10^6 conidia per milliliter), and 10-ml aliquants in glass petri dishes were stirred and irradiated with 600 W/cm^2 of 254 nm UV for 55 s. Serial dilutions of the suspensions were made to give 5×10^2 conidia per milliliter, and 0.1-ml aliquants were spread on potato-dextrose agar (PDA) plates containing 0.4 μg ml^{-1} benomyl to restrict colony growth. After incubating at 25 C in the dark for 96 h to allow colony development, the cultures were overlaid with Quantum 4000 (a dry *Bacillus subtilis* powder from Gustafson Inc., Plano, TX) by sifting 5 mg of the preparation through Miracloth (Chicopee Mills, Inc. NY) onto the agar surface. The cultures were further incubated for 24-48 h, then observed for the presence of fungal colonies that had produced no clear zones in the bacterial lawn. These putative antibiotic-deficient mutants were subtransferred to fresh PDA amended with benomyl, incubated for 48 h, and re-tested with the Quantum 4000 preparation to confirm the loss of antibiotic activity.

Production, extraction, and HPLC analysis of secondary metabolites synthesized by parent and mutant strains of *G. virens*. PDA plugs (0.7 cm diameter) from actively growing cultures of parent and putative mutants of *G. virens* strains were transferred to liquid cultures (50 ml) consisting of 5% ground millet, 1% ground peat moss (w/w), and water adjusted to pH 4.0. The cultures were shake incubated (150 rpm) for 4 days at 25 C. Each treatment consisted of three replicate cultures. The contents of the replicate cultures were then centrifuged at 16,000 *g* for 10 min, and the supernatant fluids were decanted from the pellets. The pellets were spread on sterile petri dishes and air dried under a positive pressure hood for 24 h, then they were ground to a

particle size ≤ 500 μm and stored at 5 C until used. One-gram samples of the air-dried pellets were extracted with 50 ml of 80% acetone and the acetone was removed in vacuo. The aqueous residues were then extracted with equal volumes of chloroform, the chloroform was removed in vacuo, and the residues were dissolved in 1-ml aliquants of methanol. The secondary metabolites in the extracts were separated with high-pressure liquid chromatography (HPLC), using a Hewlett Packard 1090 liquid chromatograph equipped with a diode array detector and a column and elution method previously described (10).

Antifungal activity of culture extracts from parent and mutant strains. The contents of chloroform extracts from air-dried pellets of parent and mutant strains, redissolved in acetone from methanol, were assayed for activity against an *R. solani* AG-4 strain from cotton as follows: the extracts were diluted 1:1 with sterile water, 80-μl aliquants were placed in 7-mm wells cut into PDA plates, and the plates were simultaneously inoculated with 5-mm PDA plugs of *R. solani,* 2 cm from the wells. Each treatment consisted of four replicates. After 24 h, the distance from the PDA plug to the hyphal front in line with the well was measured.

Growth, sporulation, mycoparasitic activity, and resistance to gliotoxin of parent and mutant strains. Conidial suspensions (1 ml of 1×10^{-6} conidia per milliliter) of parent and mutant strains were added to 50 ml of Raulin-Thom medium (19) in 125-ml flasks, and were shake incubated for 5 days. The cultures were then filtered through pre-weighed filter paper and dried for 24 h at 60 C. The mycelial dry weights of three replicates for each strain were calculated by subtracting the filter paper weight from the total. PDA plugs (5 mm) of parent and mutant strains were placed on PDA plates, and after 48 h hyphal growth from the plugs was measured. After colony growth covered the plate, conidia produced by 5-mm plugs from the colonies on PDA were counted with a hemacytometer. PDA plugs of parent and mutant strains were also placed on the periphery of soil extract agar plates (6), and a PDA plug of *R. solani* was placed in the center. After 72 h incubation, the plates were observed for evidence of mycoparasitism of the *R. solani* hyphae by the parent and mutant strains of *G. virens*.

Gliotoxin in acetone was added to molten PDA to give final concentrations of 10, 20, and 30 μg ml^{-1}, and the medium was dispensed into petri dishes. Aliquants (5 μl) of conidial suspensions containing 10^6 conidia per milliliter of parent and mutant strains were added to the surface of the gliotoxin agar. After 24 h the plates were examined for evidence of conidia germination, and after 40 h mycelial growth from the original inoculum was measured.

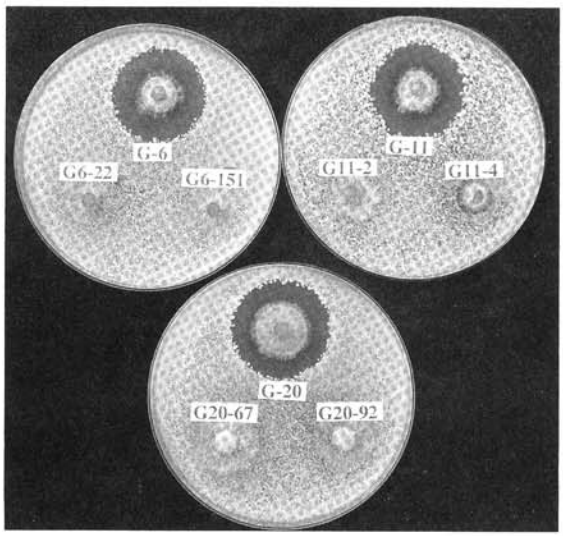

Fig. 1. Detection of gliotoxin-deficient mutants (no clear zones) among gliotoxin-producing parents (clear zones) of *Gliocladium virens* on media seeded with a lawn of *Bacillus subtilis,* after 48 h incubation.

Preparation of pathogen inoculum and biocontrol efficacy assay of parent and mutant strains of *G. virens.* *Rhizoctonia solani* was cultured on a medium consisting of 25 g of ground millet and 30 ml of distilled water for 3 wk, then the cultures were air dried and ground to a particle size ≤500 μm. The inoculum was mixed with ground vermiculite (1:3, w/w) just before use. Cottonseed, coated with latex sticker (Rhoplex B 15J, Rohm and Haas, Philadelphia, PA) and the air-dried preparations of parent and mutant strains of *G. virens* held previously at 5 C (0.01 g/seed), were placed in furrows of nonsterile cotton field soil in flats (50 × 35 cm). Inoculum of *R. solani* was sprinkled over seed and soil in the furrow at the rate of 0.1 g per 10 cm of furrow. The furrows were closed, each flat watered with distilled water, and the flats were incubated at 25 C with a 14 h photoperiod for 2 wk. Counts were then made of surviving seedlings in each treatment. Treatments were replicated four times and completely randomized.

Assay of gliotoxin production in the cotton spermosphere and surrounding soil. Air-dried and ground preparations of parent and mutant strains of *G. virens* (0.05-g samples) were mixed with 3-g samples of nonsterile field soil and placed in 18 × 50 mm test tubes containing 0.5 ml of water. One cotton seed was planted in each tube and the tubes were incubated at 25 C with a 12-h photoperiod. Each treatment consisted of 20 tubes, and treatments were arranged in a completely randomized design. After 4 days incubation, the contents of the tubes in each treatment were washed into a flask with 80% acetone, the seeds removed, and the remainder extracted with an additional 50 ml of the solution. The extracts were centrifuged, the pellets discarded, and acetone was removed from the supernatant fluids in vacuo. The aqueous residues were extracted with chloroform, the chloroform was removed in vacuo, and the residues were dissolved in 1-ml aliquants of methanol. The extracts were subjected to HPLC analysis as described previously.

All experiments were repeated at least once, with similar results, and the data were analyzed by Student's *t* test. Data from the second trial of each experiment are presented here.

RESULTS

Ultraviolet irradiation of conidia from *G. virens* strains G-6, G-11, and G-20 resulted in the isolation of two mutants from each strain that were deficient for production of antibiotics inhibitory to *B. subtilis* (Fig. 1). Approximately 750 mutated colonies were screened for each gliotoxin-deficient mutant obtained (0.13%).

HPLC analyses of extracts of air-dried preparations from parent and mutant strains for secondary metabolite production showed that mutants differed from parents only by their failure to produce gliotoxin and dimethylgliotoxin. Both parent and mutant strains produced viridiol, viridin, and heptelidic acid in similar amounts

TABLE 1. Production of secondary metabolites in cultures of parent (GLT+) and mutant (GLT−) strains of *Gliocladium virens*

Strain[x]		Metabolite concentration (μg/g)[y]				
		VDL[z]	GLT	dmGLT	VDN	HPA
G6	+	2,000 a	163 a	53 a	50 a	tr
G6-22	−	2,357 a	0 b	0 b	74 a	tr
G6-151	−	2,033 a	0 b	0 b	130 a	tr
G11	+	2,200 a	210 a	0 a	30 a	153 a
G11-2	−	2,183 a	0 b	0 a	120 a	tr
G11-4	−	2,133 a	0 b	0 a	83 a	197 a
G20	+	2,350 a	233 a	60 a	10 b	tr
G20-67	−	2,237 ab	0 b	0 b	43 a	tr
G20-92	−	1,770 b	0 b	0 b	47 a	tr

[x] + = GLT-producing parent strain; − = GLT minus mutant.

[y] Data within a parent/mutant group with the same letter designations are not significantly different as assessed by Student's *t* test at $P \leq 0.05$; tr = a trace of the compound.

[z] VDL = viridiol; GLT = gliotoxin; dmGLT = dimethylgliotoxin; VDN = viridin; HPA = heptelidic acid.

(Table 1). Mutant strains tended to produce more viridin than their parents, but production was variable and the differences were sometimes not significant.

Bioassay of extracts from parent and mutant strains of G-6, G-11, and G-20 for activity against *R. solani* showed that extracts from parent strains inhibited pathogen growth by 70%, while extracts from mutants inhibited pathogen growth by only 25–30%, compared with the control (Fig. 2).

A comparison of the growth characteristics and mycoparasitic activities of parent and mutant strains of *G. virens* showed that although mycelial dry weight, hyphal extension, and conidial production by mutant strains were sometimes significantly reduced, they were just as often equal to or significantly better than the parent strains (Table 2). There was no consistent pattern of reduction except for hyphal extension in the G-11 mutant strains, and this amounted to only 6%. Both GLT+ parents and GLT− mutants of all strains were vigorous mycoparasites of the hyphae of *R. solani* AG-4.

Bioassay of parent and mutant strains for resistance to gliotoxin showed that, after 24 h on media containing 10 and 20 $\mu g\ ml^{-1}$ gliotoxin, all parent strains and mutants G6-151, G11-2, and G11-4 had germinated. Strains G20-67 and G20-92 showed reduced germination, and G6-22 did not germinate. On 30 $\mu g\ ml^{-1}$ gliotoxin, all of the parent strains germinated, but none of the mutant strains did.

On gliotoxin-containing media, the radial growth of all strains was progressively inhibited by increasing concentrations of the antibiotic (Table 3). However, on media containing 30 $\mu g\ ml^{-1}$ the parent strains were only inhibited 27–33%, while the mutant strains were inhibited 77–83%. At all gliotoxin concentrations, G6-151 was significantly more resistant to the antibiotic than were the other mutants.

When air-dried preparations of parent and GLT− mutant strains of *G. virens* were used to coat cotton seed that was subsequently planted in soil infested with *R. solani,* the biocontrol efficacies of the mutant strains were not significantly different from that of the GLT+ parents (Table 4). In heavily infested soils in which 98% of the seedlings in the nontreated control were damped-off, disease incidence was reduced to 40–55% by treatment with either parent or mutant strains of the biocontrol agent.

HPLC profiles of extracts of soil tube cultures treated with air-dried preparations of parent and mutant strains showed the presence of dimethylgliotoxin from parent strains G-6 (240 $\mu g\ ml^{-1}$), G-11 (340 $\mu g\ ml^{-1}$), and G-20 (230 $\mu g\ ml^{-1}$), but there were only trace amounts of its precursor gliotoxin. Extracts of the mutant strains G6-22, G6-151, G11-2, G11-4, G20-67, and G20-92 were devoid of either gliotoxin or its dimethyl derivative.

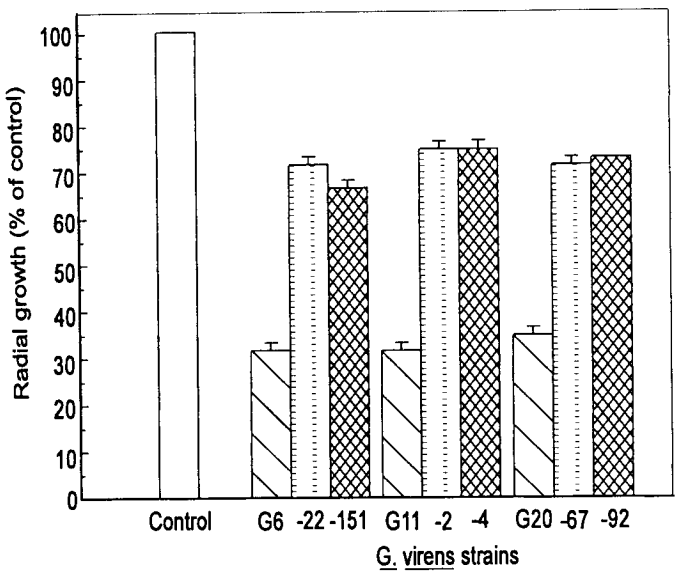

Fig. 2. Growth inhibition of *Rhizoctonia solani* AG-4 by extracts of gliotoxin-producing parent (G6, G11, G20) and gliotoxin-deficient mutant (6-22, 6-151, 11-2, 11-4, 20-67, 20-92) strains of *Gliocladium virens.* Extracts were from air-dried pellets of 4-day-old 5% millet and 1% peat moss shake cultures. Error bars represent the standard error of the mean.

DISCUSSION

Efficient means of screening for biocontrol agents, and the development of strategies for their use, require detailed knowledge of the mechanisms employed by the agents to effect disease control. Biocontrol activity has most often been ascribed to antibiosis, mycoparasitism, or competition (2), and, with *G. virens,* research has tended to focus on the first two (1,6,8,10,13,16). Both of

TABLE 2. Comparison of growth characteristics of GLT+ parent and GLT− mutant strains of *Gliocladium virens*

Strain[x]		Mycelial dry weight[y]	Number of conidia[z]	Hyphal extension
G6	+	162 a	5.4 b	28.3 a
G6-22	−	213 a	7.9 a	28.3 a
G6-151	−	205 a	2.5 c	26.3 b
G11	+	85 b	5.7 b	28.6 a
G11-2	−	183 a	4.0 c	27.0 a
G11-4	−	172 a	6.4 a	27.0 a
G20	+	137 b	5.0 b	27.3 a
G20-67	−	148 b	6.2 a	26.3 b
G20-92	−	217 a	6.4 a	28.0 a

[x] + = GLT-producing parent strain; − = GLT-minus mutant strain.
[y] Data within a parent/mutant group with the same letter designations are not significantly different as assessed by Student's *t* test at $P \leq 0.05$. Mycelial dry weight is expressed in mg.
[z] Conidia numbers are $\times 10^6\ ml^{-1}$. Hyphal extension is expressed in mm.

TABLE 3. Resistance of parent and gliotoxin-deficient mutant strains of *Gliocladium virens* to gliotoxin

Strain	GLT[y]	Gliotoxin concentration ($\mu g\ ml^{-1}$)[z] 10	20	30
G6	+	12 a	24 a	33 a
G6-22	−	48 c	79 c	82 c
G6-151	−	34 b	61 b	77 b
G11	+	0 a	14 a	29 a
G11-2	−	39 b	74 b	81 b
G11-4	−	46 c	77 b	81 b
G20	+	1 a	18 a	26 a
G20-67	−	43 b	76 c	81 b
G20-92	−	44 b	70 b	82 b

[y] GLT = gliotoxin (+) positive or (−) negative.
[z] Data are expressed as % inhibition of, compared with the nontreated control. Data within a parent/mutant group with the same letter designations are not significantly different as assessed by Student's *t* test at $P \leq 0.05$.

TABLE 4. The biocontrol efficacies of GLT+ parent and GLT− mutant strains of *Gliocladium virens* against *Rhizoctonia solani*-induced cotton seedling disease

Strain	GLT[y]	Percentage of seedling damping-off[z]
None		98 a
G6	+	48 b
G6-22	−	53 b
G6-151	−	40 b
G11	+	50 bc
G11-2	−	40 c
G11-4	−	55 b
G20	+	55 b
G20-67	−	50 b
G20-92	−	48 b

[y] GLT = gliotoxin (+) positive or (−) negative.
[z] Data within a parent/mutant group with the same letter designations are not significantly different as assessed by Student's *t* test at $P \leq 0.05$.

these mechanisms in *G. virens* have been subjected to mutational analysis with respect to other diseases (6,23) and other antibiotics (8), but much remains to be done. We report here the production, isolation, and bioassay of mutants deficient for gliotoxin synthesis, and the effect of this mutation on biocontrol efficacy against *R. solani*–induced cotton seedling disease.

HPLC analysis of extracts from mutants of *G. virens* that failed to produce clear zones in lawns of *B. subtilis* showed that the mutants were consistently deficient for gliotoxin production. Since gliotoxin inhibits *B. subtilis (1),* and none of the other secondary metabolites known to be produced by *G. virens* have been demonstrated to have activity against it or other *Bacillus* spp. (1,3,8,9, data not shown), this system appears to be rather specific for detecting gliotoxin production or a deficiency for it in this fungus.

Bioassay of GLT+ parent and GLT− mutant strains for efficacy as biocontrol agents of cotton seedling damping-off in *R. solani*–infested flats of cotton field soil showed that loss of capacity to synthesize gliotoxin had no significant effect on biocontrol performance against *R. solani* in this system. These results indicate that gliotoxin activity is not a primary mechanism in disease control when *R. solani* is the pathogen.

A recent report dealing with the production by *G. virens* of chitinolytic enzymes (4) demonstrated a synergistic effect between endochitinase and gliotoxin in vitro. This could reasonably lead to speculation that gliotoxin synthesis is necessary for mycoparasitism. Our results with GLT− mutants, however, show that they are just as efficient as the parent strains at acting as mycoparasites of *R. solani.* This indicates that the absence of gliotoxin neither adds to nor detracts from the mycoparasitic process.

The results of a bioassay of conidia from parent and GLT− mutant strains for resistance to gliotoxin indicate that loss of gliotoxin biosynthesis is accompanied by susceptibility to the antibiotic. All mutants that no longer produced either gliotoxin or its derivative dimethylgliotoxin were subject to growth inhibition by gliotoxin.

Since previous experience has shown that substrate and environment have profound effects on secondary metabolite production by *G. virens* (data not shown), demonstration of the presence or absence of gliotoxin in the spermosphere of cotton seed treated with parent or mutant strains was considered necessary. The results of the experiment showed that although only trace amounts of the precursor gliotoxin could be found in the spermosphere of seed treated with parent strains, substantial amounts of its derivative dimethylgliotoxin were present. Because dimethylgliotoxin is a biosynthetic product of gliotoxin (14), gliotoxin must also have been produced in substantial amounts. In the spermospheres of seed treated with mutants, there was no evidence of either gliotoxin or its dimethyl derivative, indicating that the antibiotic was not produced in that environment. Previous work has demonstrated the production of gliotoxin by *G. virens* in a soilless mix (16). However, the pH of that medium is acid and the pH of the soil used in the work reported here is basic. Since the ratio of gliotoxin production to that of its dimethyl derivative is increased by culture in an acid medium and decreased in a basic one (data not shown), it is no surprise that only trace amounts of gliotoxin were found in the basic soil extracts.

The fact that mycoparasitism (6) and antibiosis do not appear to be critical mechanisms in the biocontrol of *R. solani* by *G. virens* does not mean that these mechanisms are not important to the overall process. Mycoparasitism and antibiosis may be involved in reduction of pathogen inoculum (6), and they may aid in the long-term survival of the biocontrol agent and in its ability to colonize the cotton root system (18). These two phenomena, however, do not appear to be critical to protection of the infection court from *R. solani,* and perhaps the mechanism of competition should be examined more closely. We may not be screening for the most effective mechanisms in biocontrol, and we may not have given competition the credit that it deserves.

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