Effect of Sterol Biosynthesis Inhibitors on Phytotoxin (Viridiol) Production by *Gliocladium virens* in Culture

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ABSTRACT

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The steroid phytotoxin viridiol produced by *Gliocladium virens* is a major limiting factor in the amount of *G. virens*–millet preparation that can be used on cottonseed to control damping-off incited by *Rhizoctonia solani* and *Pythium ultimum*. Treatment of developing *G. virens* cultures with 1 μg ml^{-1} of the sterol production inhibitors propiconazole or flusilazole and 1–4 μg ml^{-1} of myclobutanil or triadimenol suppressed viridiol production without significant adverse effect on the growth of *G. virens* or on the biosynthesis of the nonsteroid antibiotics gliotoxin and glovirin. Greater amounts of the antibiotic-producing biocontrol preparation can thus be used to treat seed without concomitant phytotoxic effects on the emerging seedling radicle.

Gliocladium virens J.H. Miller, J.E. Giddens, & A.A. Foster is a mycoparasite and/or antibiotic-producing antagonist of plant pathogens, and it is an effective biocontrol agent of several soilborne root or seedling diseases (1–6,8,9,11,14,15). *G. virens* was effective in the biocontrol of Pythium damping-off of cotton seedlings when preparations of the fungus were coated onto cottonseed with a latex sticker (5). However, the amount of biocontrol preparation that could be used was limited by the concentration of the phytotoxin viridiol (5,7) in the strain–substrate preparation.

The most effective antifungal compounds produced by strains of *G. virens* are gliotoxin and glovirin (9), neither of which is a steroid. However, the antibiotic viridin and the phytotoxin viridiol are steroids, and the former is converted to the latter in vitro (10). There are several classes of compounds, some of which are used commercially as fungicides (13), that inhibit sterol biosynthesis in fungi. The imidazole and triazole derivatives used as fungicides inhibit cytochrome P-450 dependent monooxygenase that catalyzes the C_{14}-demethylation of 2,4-methylenedihydrolanosterol (12). Our objective in this study was to determine if the addition of some of these sterol inhibitors at sublethal concentrations to cultures of *G. virens* would block production of the phytotoxin viridiol in the biocontrol preparation without significant adverse effect on growth rate or nonsteroid antibiotic production by the fungus. If so, increased amounts of the biocontrol preparation could be used to treat seed without concomitant phytotoxic effects on the emerging cotton radicle.

MATERIALS AND METHODS

Preparation and culture of *G. virens* in media containing sterol inhibitors. Erlenmeyer flasks (250 ml) containing 5 g of ground millet (≤1 mm particle size) and 95 ml of H_2O were autoclaved twice on succeeding days. Sterol inhibitors dissolved in acetone (100 μg ml^{-1}) were then added to the flasks in 1-ml aliquants to make 1 μg ml^{-1} final concentrations in the medium. The sterol inhibitors used were propiconazole, flusilazole, myclobutanil, and triadimenol (13). Controls received 1-ml aliquants of acetone. Potato-dextrose agar (PDA) plugs (0.7 cm) from actively growing and sporulating agar cultures of *G. virens* strains were transferred to the liquid cultures, and the cultures were shake incubated (150 rpm) at 25 C for 6 days. Each treatment consisted of three replicate cultures.

Erlenmeyer flasks (250 ml) containing 100 ml of 5% ground millet were inoculated with *G. virens,* treated with 1 μg ml^{-1} of sterol inhibitors, and shake incubated as described above. Aliquants (1 ml) were removed from the cultures, suspended in 25 ml of sterile water, and macerated in a Waring blender for 2 min. Chlamydospores in the macerates were counted with a hemacytometer.

Harvest and extraction procedures. The culture contents of each of three replicates were centrifuged at 16,000 *g* for 10 min, and the supernatant fluids were separated from the pellets. Each pellet was extracted with 75 ml of 80% acetone, the solids were removed by centrifugation, and the acetone was removed in vacuo. The aqueous residue remaining was combined with the original supernate, and the entire mixture was extracted with 100 ml of chloroform. The chloroform was removed in vacuo, and the residue was dissolved in 2 ml of methanol. The contents of millet cultures treated and incubated as described above were also centrifuged, and the supernatant fluids were discarded. The pellets were spread on sterile petri dishes, and the contents were allowed to air-dry for 2 days. The air-dry preparations were ground in a Wiley mill, sieved to obtain particle sizes ≤500 μm, and stored at 5 C for later seed-treatment tests.

High-pressure liquid chromatography fractionation of culture extracts. Compounds in the chloroform extracts were separated by using a Hewlett Packard 1090 liquid chromatograph equipped with a diode array detector. The effluent was monitored at 254 nm, and the column (Scientific Glass Engineering C-18, 250 × 4.6 mm, 5-μm packing) was held at 40 C. The mobile phase (1.25 ml/min) consisted of dilute acid (0.07% H_3PO_4) and acetonitrile. Development of the chromatogram was isocratic (80% acid:20% acetonitrile) for 5 min, followed by a linear gradient to 72% acid:28% acetonitrile over 1 min, and held at this ratio for an additional 12 min. The instrument was calibrated by injecting known quantities of authentic gliotoxin, glovirin, viridin, and viridiol.

Phytotoxicity and disease assay methods. Cottonseed treated with 0.05-g lots per seed (approximately 10 times the normal seed treatment) of air-dried *G. virens*–millet preparations, with and without sterol inhibitor added (propiconazole for strain G-4, flusilazole for strain G-6), were planted in 5 g each of unsterile cotton field soil (Lufkin fine sandy loam). The soil was contained in 18 × 50 mm test tubes. Soil moisture was 20% by weight (matric potential, 0.07 MPa), and one seed was planted in each tube. The tubes were incubated for 6 days at 25 C and a 12-h photoperiod; the contents were then washed from the tubes, and the seedling roots were examined for evidence of phytotoxicity. Each treatment consisted of 10 replicate tubes arranged in a completely randomized design. Controls consisted of untreated seed or seed treated with sterol inhibitor alone (1 $\mu g\ g^{-1}$ in ground vermiculite). The same procedure was carried out in soil infested with *Pythium ultimum* Trow at the rate of 2,000 oospores per gram of soil, except that the treatments (0.05 g) were coated onto 10 seeds with a latex sticker (Rhoplex B 15J, Rohm & Haas Co.), and the tubes were incubated in the dark at 18 C for 1 wk before transfer to 25 C and a 12-h photoperiod. After 4 days of incubation, the numbers of surviving seedlings were counted. Treatments consisted of P strains (gliovirin producers) (9) with and without propiconazole (1 $\mu g\ ml^{-1}$) added to the growth medium. The control was untreated. The treatments were each replicated three times and arranged in a completely randomized design.

Q strains (gliotoxin producers) (9) with and without triadimenol (4 $\mu g\ ml^{-1}$) added were assayed for efficacy against *Rhizoctonia solani* Kühn as previously described (9). The biocontrol activity of P strains was assayed against *P. ultimum,* and that of Q strains against *R. solani,* because previous work (9) has shown these strain–pathogen combinations to be the most effective.

Fungitoxicity of sterol biosynthesis inhibitors. The sterol biosynthesis inhibitors propiconazole, flusilazole, myclobutanil, and triadimenol were assayed for fungitoxicity to *G. virens* by adding the compounds, dissolved in acetone, to PDA to achieve concentrations of 2, 4, 6, 8, or 10 $\mu g\ ml^{-1}$. The sterol inhibitor-containing agar media were then spotted with 5 μl aliquants of *G. virens* conidia suspension. After 48 h of incubation at 25 C, the resulting colony diameters were measured. The control contained no sterol inhibitor, and each treatment was replicated three times.

All experiments were repeated one or more times with similar results. Data were analyzed by regression analysis in the AXUM 3.0 computer program or by Student's *t* test.

RESULTS

Chlamydospore counts from control and flusilazole-treated Q strain cultures were not significantly different ($P \leq 0.05$) (Table 1). The propagule numbers in P strains G-4 and G-8 were significantly reduced by propiconazole treatment, but only by 17 and 24%, respectively; and chlamydospore numbers in strain G-9 were actually increased 27% by propiconazole treatment. Microscopic examination of the culture solids from control and treated cultures did indicate some suppression of mycelial growth in the treated cultures. Air-dried pellet granules from treated and control cultures sprinkled on PDA showed equivalent germination and growth after 24 h.

Treatment of *G. virens*–millet cultures with sterol biosynthesis inhibitors completely suppressed the production of the steroids viridin and viridiol in the Q strains; while in the P strains, biosynthesis of the pathway intermediate viridin (10) was not suppressed and was significantly elevated by the treatment in strain G-9 (Table 2). Viridiol synthesis was suppressed in all P strains. Synthesis of the nonsteroid metabolites, gliotoxin and gliovirin, was not suppressed by the sterol inhibitor treatments (Table 2). The efficacy of a given inhibitor was dependent on the group of *G. virens* strains on which it was used (Table 3). In general, propiconazole was more effective as a viridiol suppressant with strains belonging to the P group (gliovirin producers) than with strains of the Q group (gliotoxin producers). The others—triadimenol, myclobutanil, and flusilazole—were more effective with Q group strains.

The radicles of cottonseed treated with *G. virens* preparations to which sterol inhibitor had not been added were stunted, and the apices were necrotic (Fig. 1). Cottonseed treated with *G. virens* preparations containing sterol inhibitor produced healthy radicles, although total seedling development was slightly retarded. Seedlings treated with sterol inhibitor alone were not retarded. Cottonseed planted in soil infested with *P. ultimum* and coated with *G. virens* (P strain) preparations, with and without sterol inhibitor

TABLE 1. Effect of sterol inhibitors on chlamydospore production by *Gliocladium virens* strains in culture

Strains	Chlamydospore production ($\times 10^7\ ml^{-1}$)		
	Control	PC[a]	FL
P group			
G-4	1.62 ± 0.02	1.34 ± 0.04*[b]	...
G-8	0.79 ± 0.04	0.60 ± 0.02*	...
G-9	0.44 ± 0.02	0.60 ± 0.01*	...
Q group			
G-6	2.02 ± 0.06	...	1.98 ± 0.04 NS[c]
G-11	3.23 ± 0.04	...	3.23 ± 0.05 NS
G-20	2.04 ± 0.05	...	2.20 ± 0.06 NS

[a]PC = propiconazole; FL = flusilazole.
[b]* = Significant difference from control as assessed by Student's *t* test at $P \leq 0.05$.
[c]NS = no significant difference from control.

TABLE 2. Effect of sterol biosynthesis inhibitors on production of the phytotoxin viridiol and other secondary metabolites by *Gliocladium virens* in culture

Strain/inhib.[a]	Phytotoxin and antibiotics ($\mu g\ ml^{-1}$)			
	Viridiol	Viridin	Gliotoxin	Gliovirin
P group				
G-4 + UT	1,510 ± 100*[b]	110 ± 30	NP	1,560 ± 100
G-4 + PC	0	47 ± 12	NP	1,430 ± 60
G-8 + UT	1,460 ± 100*	37 ± 6	NP	1,680 ± 50
G-8 + PC	0	56 ± 6	NP	1,460 ± 100
G-9 + UT	1,480 ± 90*	37 ± 6*	NP	1,730 ± 100
G-9 + PC	0	170 ± 50	NP	1,560 ± 130
Q group				
G-6 + UT	2,340 ± 90*	30 ± 17*	650 ± 100	NP
G-6 + FL	0	0	890 ± 150	NP
G-11 + UT	2,460 ± 20*	37 ± 15*	700 ± 80	NP
G-11 + FL	0	0	950 ± 200	NP
G-20 + UT	2,350 ± 30*	30 ± 10*	700 ± 100	NP
G-20 + FL	0	0	800 ± 70	NP

[a]UT = untreated control; PC = propiconazole; FL = flusilazole; NP = not produced. Sterol inhibitor concentrations = 1 $\mu g\ ml^{-1}$.
[b]Numbers followed by * within a strain are significantly different ($P \leq 0.05$) according to Student's *t* test.

TABLE 3. Viridiol production by P and Q strains of *Gliocladium virens* in the presence of sterol inhibitors

Strain	Viridiol production			
	PC[a]	FL	MB	TD
P strains				
G-4	–[b]	+	+	+
G-8	–	–	+	+
G-9	–	+	+	+
GLA-2	–	–	+	+
Q strains				
G-6	+	–	–	–
G-12	+	–	–	–
G-11	+	–	–	–
G-20	+	–	–	–

[a]PC = propiconazole; FL = flusilazole; MB = myclobutanil; TD = triadimenol. Sterol inhibitor concentrations = 1 $\mu g\ ml^{-1}$.
[b]+ = Viridiol production; – = no viridiol production with sterol inhibitor present.

added, produced 10–30% damped-off seedlings; whereas 100% of the nontreated control was damped-off (Table 4). The biocontrol efficacy of preparations treated with sterol inhibitor was not significantly different from those not treated.

Seed treated with *G. virens* (Q strain) preparations, with and without sterol inhibitor added, and planted in soil infested with *R. solani* showed 37–50% damping-off, while none of the nontreated controls survived (Table 4). Treatment with triadimenol had no significant adverse effect on the biocontrol efficacy of the *G. virens* preparations.

Fungitoxicity to *G. virens* varied substantially among the sterol inhibitors tested (Fig. 2). Propiconazole and flusilazole completely inhibited growth of the fungus at a concentration of 2 μg ml^{-1}. Conversely, myclobutanil and triadimenol only inhibited the growth of *G. virens* 49 and 30%, respectively, at a concentration of 4 μg ml^{-1}. Growth was not completely inhibited by either myclobutanil or triadimenol at 10 μg ml^{-1}.

DISCUSSION

The production of viridiol by both P and Q strains of *G. virens* is a major limiting factor in the amount of biocontrol preparation that can be used as a treatment for seedling disease control (5,8,9). The addition of sterol inhibitors to the culture medium after autoclaving and prior to inoculation with *G. virens* results in complete suppression of viridiol production in the cultures. It also results in complete suppression of the sterol pathway intermediate viridin in the Q strains, while in the P strains viridin levels remain constant or are elevated. This may indicate that the inhibitors act at different points in the pathway. Production of antifungal antibiotics gliotoxin and gliovirin are unaffected by the treatments. The final air-dried preparations of *G. virens* therefore contain no detectable phytotoxin and high levels of the antibiotics. Thus, more *G. virens* preparation can be used as a treatment for seedling disease control without phytotoxic effects than was possible with preparations that were not treated with sterol inhibitor. Sterol inhibitor treatment of the culture medium during the growth process does not preclude production of viridiol by the fungus after seed treatment and planting in the soil, and the slight reduction in growth rate observed with treated seedlings may be attributable to subsequent production of viridiol. However, since no phytotoxicity was observed, viridiol production in that environment may be below the threshold required to induce symptoms of phytotoxicity.

Treatment of *G. virens* cultures with sterol inhibitors does not adversely affect the propagule numbers or efficacy of the air-dried fungus–millet preparations as biocontrol agents of cotton seedling diseases incited by *P. ultimum* or *R. solani*. In cases where the use of higher concentrations of sterol inhibitor is possible, it may even be beneficial. These fungicidal compounds may act synergistically with the biocontrol agent to enhance or broaden its antifungal activity spectrum.

Sterol inhibiting fungicides vary considerably in their toxicity to the biocontrol agent *G. virens,* and they are effective inhibitors of viridiol production at low concentration. Therefore, it is possible to achieve viridiol suppression with low concentrations of the more toxic inhibitors, but they will be useful for little else. Treatment with less toxic sterol inhibitors at higher concentration will achieve the same effect and may act to ward off other plant pathogens and fungal competitors of the biocontrol agent.

From the data reported here, we concluded that the addition of low concentrations of sterol inhibiting fungicides to developing cultures of the biocontrol agent *G. virens* is a feasible means of suppressing production of the phytotoxin viridiol in the biocontrol preparation. Other sterol inhibiting compounds are avail-

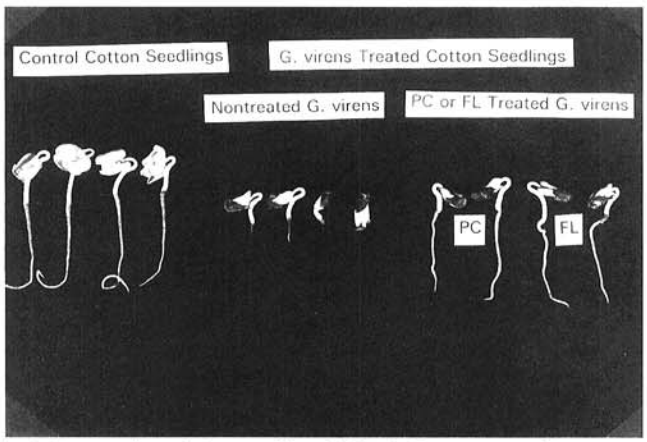

Fig. 1. Suppression of phytotoxicity of *Gliocladium virens*–millet preparations to the radicles of developing cotton seedlings by treatment of strain G-4 with propiconazole (PC) or strain G-6 with flusilazole (FL).

TABLE 4. Effect of sterol inhibitors on the biocontrol efficacy of *Gliocladium virens* P and Q strains against *Pythium ultimum*– and *Rhizoctonia solani*–incited cotton seedling disease, respectively

	Seedling damping-off (%)[b]	
Strain/inhib.[a]	*P. ultimum*	*R. solani*
P group		
G-4 UT	20 ± 5.8 NS[c]	NT
G-4 PC	10 ± 0.0 NS	NT
G-9 UT	20 ± 5.8 NS	NT
G-9 PC	30 ± 5.8 NS	NT
Q group		
G-6 UT	NT	50 ± 5.8 NS
G-6 TD	NT	43 ± 3.3 NS
G-11 UT	NT	50 ± 5.8 NS
G-11 TD	NT	37 ± 3.3 NS

[a] UT = untreated; PC = propiconazole; TD = triadimenol; NT = not tested.
[b] % Control, where controls produced 100% damping-off.
[c] NS = no significant difference between untreated and sterol inhibitor treated biocontrol preparations as assessed by Student's t test at $P \leq 0.05$.

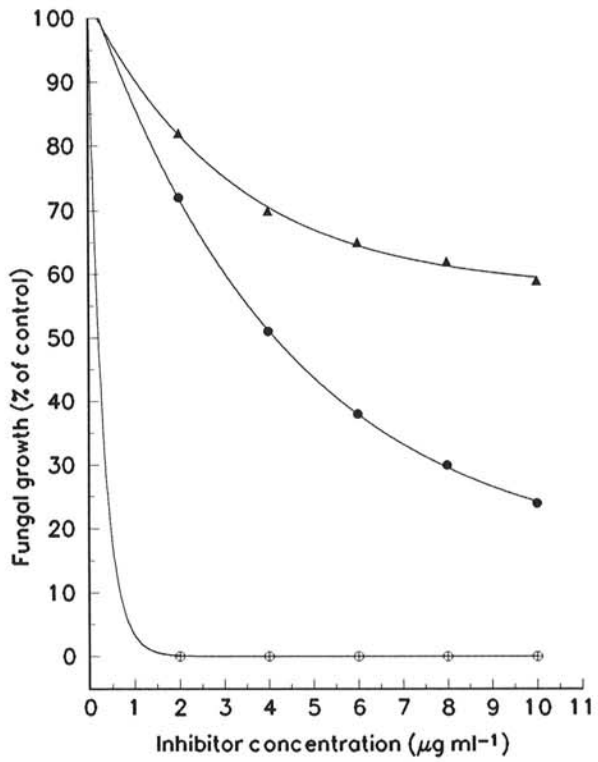

Fig. 2. Fungitoxicity of the sterol biosynthesis inhibitors (○) propiconazole, (+) flusilazole, (●) myclobutanil, and (▲) triadimenol to *Gliocladium virens* on potato-dextrose agar, as measured by reduction in colony diameters. Data are a composite of measurements from strains G-4 and G-6.

able, and a search is currently underway for those that are effective viridiol suppressants and are less toxic to *G. virens*. These compounds may allow the development of combination biocontrol agent–fungicide treatments that are harmless to the plant and that enhance disease control.

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