Biological Control of Pythium Damping-Off of Cotton with Seed-Coating Preparations of *Gliocladium virens*

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ABSTRACT

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Air-dried and ground seed-coating preparations of *Gliocladium virens* were variable in their efficacy for biocontrol of cotton seedling disease induced by *Pythium ultimum*. Disease suppression was dependent on the proper strain-substrate combination and on the amount of material used. Treatment in excess of that required to coat the seed resulted in phytotoxicity. The loss of disease suppression with time in open storage in the lab was associated with a diminution in the amount of antifungal gliovirin in the preparation and a lengthening of time for propagule germination of the biocontrol agent. Combination treatments with reduced levels of metalaxyl fungicide and the biocontrol preparation resulted in a synergistic effect that gave disease suppression equal to that of full-strength fungicide treatment. Seed coat preparations of *G. virens* may be used to control Pythium damping-off of cotton seedlings, and in combination with fungicide, the amount of fungicide necessary to effect control can be reduced.

Additional keywords: antibiotics, phytotoxin, carboxin, pentachloronitrobenzene, *Gossypium hirsutum*.

Gliocladium virens J. H. Miller, J. E. Giddens, & A. A. Foster, a mycoparasite or antibiotic-producing antagonist of fungal plant pathogens, is an effective biocontrol agent of several soilborne root or seedling diseases (1,5,6,9,12,13,14). Recently, *G. virens* was effective in the biocontrol of damping-off of zinnias, where the biocontrol inoculum was added to unsterile soilless mix at the rate of 1% (v/v) on a dry-weight basis (9), and in the biocontrol of Phytophthora root rot of apples, when it was incorporated into potting mix at the rate of 0.6% (v/v) (12). Biocontrol inoculum at this rate is physically and economically feasible in a greenhouse operation. With field crops, however, this rate of inoculum would be economically prohibitive and would not be acceptable in a normal farming operation. The ideal delivery method of a biocontrol agent for field crops would be via seed treatment.

G. virens produces antifungal, antibacterial, and herbicidal compounds (3,4,6,7,11,15,16), and the kinds and amounts of these materials produced is influenced by the substrate on which it is grown (7,8). Our objective in this study was to ascertain which strain-substrate combinations of *G. virens* would best provide the necessary mix of antibiotic production, low herbicidal activity, and viability to enable the fungus to function as an effective biocontrol agent when coated onto cottonseed.

MATERIALS AND METHODS

Preparation and culture of *G. virens* strain-substrate combinations. Substrates of millet, wheat, sorghum, soybean, cotton, wheat bran, rice, rice hulls, or oats were wetted with water (w/w) and autoclaved twice on subsequent days at 121 C for 20 min. Peat moss was wetted with twice its weight of Raulin-Thom (10), Czapek-Dox (5), or fermentation (8) liquid media, respectively, and sterilized as above. Conidia of the various strains of *G. virens* were transferred to the media in 500-ml flasks and still-cultured in the dark at 25 C. After 6 days, the cultures were harvested, air-dried for 2 days, ground in a Wiley mill, and sieved to obtain particle sizes of $\leq$500 μm. The strain-substrate preparations were coated onto cottonseed (*Gossypium hirsutum* L. 'Stoneville 213') at the rate of 0.5–1.0 g/100 seeds, depending on the density of the material, with Rhoplex B-15 latex sticker (Rohm and Haas, Philadelphia, PA).

Pathogen inoculum and disease assay methods. *Pythium ultimum* Trow, strain P-1, was grown for 2 wk in V8-cholesterol broth (2) in still culture, and the mycelial mats were comminuted in sterile water with a blender. The population density of oospores was determined with the aid of a hemocytometer, and a suspension of the propagules was mixed into unsterile field soil at the rate of 2,000 oospores per gram of soil. The infested soil was incubated at 25 C for 6 wk before use to avoid dormancy problems associated with fresh oospores (2).

Treated cottonseed were planted in 5-g lots of unsterile cotton field soil (Lufkin fine sandy loam) infested with *P. ultimum*; the soil was contained in 18- $\times$ 150-mm test tubes. Before planting, the soil moisture was adjusted to 20% by weight, and one seed was planted in each tube. The tubes were incubated in the dark at 15 C for 7 days, then transferred to a growth chamber at 25 C, with a 12-h photoperiod. The numbers of emerged and surviving seedlings in the tubes were counted after 6 days, and the seedlings were examined for lesions.

Each treatment consisted of 10 tube subsamples, and treatments were replicated three times. Replicates were completely randomized.

Untreated cottonseed and cottonseed coated with millet preparation of *G. virens* (G-9) were planted in soil tubes of uninfested, unsterile field soil as previously described. A third treatment consisted of cottonseed planted in tubes of uninfested soil, along with 0.1-g lots of millet preparation of *G. virens* per seed. The tubes were incubated for 6 days in a growth chamber at 25 C, with a 12-h photoperiod; the contents were then washed from the tubes and examined. Each treatment consisted of 10 replicate tubes arranged in a completely randomized design.

Solvent extraction and bioassay of preparations. Air-dried strain-substrate preparations (5 g) of *G. virens* were extracted with 100 ml of 80% aqueous acetone, and the acetone was removed in vacuo. Aqueous residue was extracted with an equal volume of chloroform; the chloroform was removed in vacuo, and the residue was dissolved in 2 ml of methanol. Samples were spotted (30 μl) on thin-layer chromatography plates of silica gel 4 GF and developed two-dimensionally in chloroform/acetone (70:30) and ethyl acetate. Developed plates were observed under 366- and 254-nm ultraviolet light. Purified gliotoxin (R_f 0.49 $\times$ 0.55), viridin (R_f 0.55 $\times$ 0.55), gliovirin (R_f 0.29 $\times$ 0.48), and viridiol (R_f 0.29 $\times$ 0.27) were used as standards. The extracts were assayed for antifungal activity by mixing 40 μl of extract with an equal volume of sterile H_2O and placing the mixtures in wells cut into the peripheries of agar in petri dishes. Potato-dextrose agar (PDA) plugs from 7-day-old cultures of the pathogen were placed in the center of the dishes. After 24 and 48 h, the dishes were examined for the presence of clear zones around the wells. Each dish contained three replicate extracts, and the dishes were arranged in a completely randomized design.

The antibiotics, viridin, gliotoxin, and gliovirin, and the herbicide, viridiol, were assayed for activity against *P. ultimum*. Aliquots of acetone (30 μl) containing 30, 20, 10, 5, 1, 0.5, or 0.1 μg of the respective antibiotics were mixed with 50 μl of sterile H_2O and placed in wells cut in the peripheries of agar in petri dishes and assayed as previously described.

Changes in biocontrol activity and propagule germination during storage. At 2-wk intervals during the 5-mo storage period, samples (0.3 g) of millet preparations of strains G-4 and G-9 of *G. virens* were coated onto seed and assayed for biocontrol activity as previously described; propagule germination and growth rate were determined by placing preparation particles on petri dishes of PDA and incubating them at 25 C for 15 h. Cultures were then observed microscopically and at intervals thereafter to ascertain germination and growth rate.

Fresh air-dried, biocontrol-active samples and aged (4 mo), inactive samples were extracted with solvent, and the extracts were assayed in vitro for antimicrobial activity against the pathogen as previously described.

Millet preparations of *G. virens*, freshly prepared and aged so that they were no longer active in biocontrol (2–3 mo) or no longer viable (5 mo), were coated onto cottonseed as previously described. The seed lots were divided, with one half incubated in a dry container and the other half incubated at 100% relative humidity for 24 h before planting. Coated seed then were assayed for biocontrol activity against *P. ultimum* as previously described.

Disease control efficacy of combined treatments of *G. virens* and reduced fungicide levels. Cottonseeds were treated with dilutions of the fungicide metalaxyl from 100 to 6.25% of the recommended rate, air-dried, and coated with millet preparation of *G. virens*. Seed treated with fungicide alone or in combination with the biocontrol agent were planted in soil infested with *P. ultimum* and assayed for disease suppression as previously described.

All experiments were repeated one or more times with similar results. The last data sets are presented here. The treatments within each experiment were replicated a minimum of three times. Data were analyzed with Student's *t*-test.

RESULTS

Biocontrol assay of strain-substrate combinations. Most of the strains of *G. virens* grown on millet were ineffective as biocontrol agents of cotton seedling disease (Table 1). However, strains G-4, G-9, and G-12 substantially inhibited damping-off when grown on this medium. Conversely, when strains G-9 and G-4 were grown on other substrates (Table 2), their efficacy in disease suppression varied with the substrate on which they were grown, and in most cases they were ineffective. Strains grown on millet, rice hulls, wheat bran, or the Raulin-Thom and peat moss mixture, however, gave good disease suppression. All strain-substrate preparations observed under the microscope contained conidia and chlamydospores.

Untreated cottonseed or seed coated with millet preparations of *G. virens* and planted in uninfested soil emerged and produced healthy radicles without lesions. Those seed treated with 0.1-g lots of millet preparation of *G. virens* per seed did not emerge, and the radicle tips were necrotic. The remainder of the seedling was healthy.

Thin-layer chromatography of strain-substrate extracts. Strains G-1, G-3, G-4, G-8, G-9, and G-12 produced viridin, viridiol, and gliovirin, whereas strains G-2, G-5, G-6, G-10, G-11, G-13, G-15 through G-18, and G-20 produced viridin, viridiol, and gliotoxin, but not gliovirin (data not shown). All strain-substrate combinations with efficacy as biocontrol agents of Pythium damping-off were capable of producing the antibiotic gliovirin. None of those combinations that failed to produce any detectable gliovirin strongly suppressed the disease, although strains G-16

and G-17 suppressed the disease to a moderate extent.

Strain-substrate extracts of biocontrol-effective strains strongly inhibited the growth of *P. ultimum* in vitro (G-4, G-9, and G-12 gave 18- to 20-mm clear zones), whereas those strains that produced smaller zones of inhibition (G-16 and G-17 gave 6-mm clear zones) were only partially effective. Those that did not inhibit the pathogen at all (G-2, G-5, G-6, G-10, G-11, G-13, G-15, G-18, and G-20) were not effective in disease suppression.

Viridin and viridiol were not inhibitory to the pathogen in vitro, and gliotoxin gave only a 2.5-mm clear zone at a concentration of 30 μg. Gliovirin, however, was very active against *P. ultimum*, giving a 12-mm clear zone at a concentration of 0.5 μg.

Effect of storage period on biocontrol efficacy and propagule germination of *G. virens*. Strains G-4 and G-9 on millet that were stored at room temperature (25 C) and monitored at 2-wk intervals for biocontrol efficacy showed a loss of disease suppression (80 and 70% damping-off, respectively) after 2 mo. This was accompanied by an increase in the time necessary for propagule germination of the biocontrol strains. The fungus in fresh strain-millet preparations germinated within 16 h after plating on PDA. Preparations held in storage for 2 mo required 24 h before germination, and preparations held for 3 mo required between 24 and 36 h.

Disease control efficacy of combined biocontrol and reduced fungicide treatments. Strains of *G. virens* were not inhibited by the metalaxyl concentration commonly used to control seedling disease in soil (Table 3). The treatment of seeds with progressive dilutions of metalaxyl before planting in soil infested with *P. ultimum* resulted in increased damping-off. Combined treatment with the biocontrol agent and any dilution of fungicide restored disease suppression to that obtained with undiluted fungicide.

DISCUSSION

A comparison of the biocontrol efficacies of many strains on a single substrate and two strains on many substrates indicates that the right combination of strain and substrate must be used to obtain disease suppression. Disease suppression can be effected by coating a small amount of an effective strain-substrate preparation directly on seed, rather than adding material to the furrow or incorporating it into the soil. Because *G. virens* produces the herbicide viridiol (7), treatment with preparations in excess of that which could be coated on seed with sticker resulted in phytotoxicity to the host plant. Disease-suppression efficacy of the strain-substrate preparations against damping-off induced by *P. ultimum* was associated with the production of gliovirin by the biocontrol agent. Although viridin is produced by effective biocontrol strains, it also is produced by ineffective strains, and it does not inhibit the growth of *P. ultimum*. Gliotoxin is inhibitory to *P. ultimum* at high concentrations, but it was not produced by the effective biocontrol strains. Those strains not able to produce gliovirin were ineffective or only partially effective (G-16 and G-17) against *P. ultimum* when the seed-coating technique was used.

A loss of biocontrol activity with time in storage by air-dried preparations of *G. virens* was associated with a loss of gliovirin in the material and with an increase in the time required for germination of the biocontrol agent propagules. Biocontrol activity in a 10- to 12-wk-old preparation could be restored by incubating the material in an atmosphere of 100% relative humidity for 24 h before it was used to coat seed; approximately 30 days after the initial loss in biocontrol activity, however, this restorative technique was no longer effective (data not shown). A constitutive amount of gliovirin in the biocontrol preparation may be required to suppress the pathogen until the biocontrol

TABLE 1. Gliovirin production and biocontrol efficacy of strains of *Gliocladium virens* grown on millet against cotton seedling disease induced by *Pythium ultimum*

Strain	% Damping-Off[a]			Gliovirin production
	Preemergence	Postemergence	Total	
G-1	70	10	80	+
G-2	100	0	100	−
G-3	80	20	100	+
G-4	10	10	20	+
G-5	90	0	90	−
G-6	90	10	100	−
G-8	60	20	80	+
G-9	10	0	10	+
G-10	100	0	100	−
G-11	40	10	50	−
G-12	10	0	10	+
G-13	60	10	70	−
G-15	80	20	100	−
G-16	40	0	40	−
G-17	40	0	40	−
G-18	90	0	90	−
G-20	60	10	70	−
Control	100	0	100	

[a]Data are the means of three replicates. Standard deviations for all treatments were ≤10%.

TABLE 2. Effect of substrate on the biocontrol efficacy of strains G-4 and G-9 of *Gliocladium virens* against cotton seedling disease induced by *Pythium ultimum*

Substrate[a]	% Damping-off[b]					
	Preemergence		Postemergence		Total	
	G-4	G-9	G-4	G-9	G-4	G-9
Control	100	100	0	0	100	100
PMCZB	90	80	10	0	100	80
Wheat	100	100	0	0	100	100
Soybean	100	100	0	0	100	100
Sorghum	100	100	0	0	100	100
Oats	100	100	0	0	100	100
Rice	100	100	0	0	100	100
FPM	100	100	0	0	100	100
Rice hulls	40	10	0	0	40	10
Millet	10	10	10	0	20	10
Wheat bran	10	20	10	10	20	30
RTPM	10	20	20	20	30	40

[a]PMCZB = peat moss + Czapek-Dox broth; FPM = fermentation medium + peat moss; RTPM = Raulin-Thom medium (10) + peat moss.

[b]Data are the means of three replicates. Standard deviations for all treatments were ≤10%.

TABLE 3. Synergistic effect of metalaxyl and biological treatments on cotton seedling disease induced by *Pythium ultimum*

Chem/Bio mix[a]	% Damping-Off[b]		
	Preemergence	Postemergence	Total
Control	80	20	100
G-9/millet	30	0	30
Metalaxyl			
100%	10	10	20
100% + G-9	10	0	10
50%	0	10	10
50% + G-9	10	0	10
25%	10	20	30
25% + G-9	10	10	20
12.5%	20	0	20
12.5% + G-9	10	0	10
6.25%	40	0	40
6.25% + G-9	10	0	10

[a]Strain G-9 of *Gliocladium virens* grown on millet seed was air-dried, comminuted to ≤500 particle size, and coated onto cottonseed after the fungicide. Metalaxyl dilutions represent percentages of the recommended rate for cottonseed treatment.

[b]Data are the means of three replicates. Standard deviations were ≤10% for all treatments.

agent starts to grow and produce more of the antibiotic. The speed of germination and growth by the biocontrol agent also may be an important factor in successful disease suppression, particularly with a pathogen like *P. ultimum*, which tends to attack early in the seed germination process. This may explain the success of the 24-h restorative technique.

Strains of *G. virens* apparently are not inhibited by the fungicides carboxin, pentachloronitrobenzene, or metalaxyl, which are commonly used as cottonseed treatments to control seedling diseases. When reduced concentrations of metalaxyl were used in combination with the biocontrol agent as a seed coating, a synergistic effect was observed, and disease suppression was restored to the level usually observed with full-strength fungicide. Again, this may be a phenomenon where the initial burden of pathogen inhibition is carried by the fungicide until the biocontrol system becomes operational.

From this study, it is concluded that the proper combination of strain of *G. virens* and substrate must be used for coating cottonseed to suppress damping-off induced by *P. ultimum*.

Combination treatments of fungicide and biocontrol preparation exhibited a synergism that may allow a significant reduction in the amount of fungicide needed to effect disease suppression. The use of small amounts of fungicide may be necessary during the interim period before biocontrol agents are developed that are better tailored to the requirements of agriculture than those that are currently isolated from nature.

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