

Selective Isolation and Enumeration of *Gliocladium virens* and *G. roseum* from Soil

YONG-HA PARK, Former Graduate Research Assistant, JAMES P. STACK, Former Assistant Professor, and CHARLES M. KENERLEY, Associate Professor, Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843-2132

ABSTRACT

Park, Y.-H., Stack, J. P., and Kenerley, C. M. 1992. Selective isolation and enumeration of *Gliocladium virens* and *G. roseum* from soil. *Plant Dis.* 76:230-235.

Selective media were developed for the isolation and enumeration of *Gliocladium virens* and *G. roseum* from soil. The basic antibiotic medium consisted of 3.0 g of glucose, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of K_2HPO_4 , 0.5 g of KCl, 1.0 g of $NaNO_3$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 50 mg of chloramphenicol, 50 mg of rose bengal, 50 mg of streptomycin sulfate, 500 μ g of benomyl (50WP), 500 mg of sodium propionate, and 20 g of Bacto agar in 1 L of distilled water. The medium was adjusted to pH 6.0 with 25% phosphoric acid before autoclaving. By the dilution plate method, selective isolation of *G. virens* or *G. roseum* was achieved from natural soil infested with *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *vasinfectum*, and *Pythium ultimum* either by adding 20 mg of gliotoxin (a secondary metabolite of *G. virens*) or 1.0 mg of gliotoxin, 20 mg of pentachloronitrobenzene (75WP), and 60 mg of acriflavine to 1 L of basic antibiotic medium, respectively. At 3 days after plating, selective isolation and quantification of *G. virens* was achieved on the selective medium for *G. virens* (GVSM). From nonsterile soil infested with the strains of *G. virens*, recovery of four gliotoxin-producing strains of *G. virens* on GVSM was significantly greater than or equal to recovery on three other media that have been used to isolate *G. virens*. However, these previously developed media did not discriminate *G. virens* from other fungi, including *Trichoderma* spp. Recovery of two non-gliotoxin-producing strains of *G. virens* was lower on GVSM than on other media tested. Total colony-forming units of *G. virens* from three natural soils was significantly higher on GVSM than on other media tested. At 4 days after dilution plating, selective isolation of *G. roseum* was achieved on the selective medium for *G. roseum* (GRSM). Recovery of five strains of *G. roseum* on GRSM was higher than or equal to the recovery on another medium that has been used to isolate *G. roseum*.

Strategies for development of selective media include selective inhibition of undesired microorganisms, selective enhancement of a target organism by providing a specific energy source, selective exclusion by use of environmental conditions preferentially favorable for the target organisms, and selective differentiation, e.g., pigmentation of the fungal colony through the use of stains or color indicators in the medium (21). Selective inhibition often is based on differential sensitivity of fungi to antibiotics, fungicides, and other chemicals (2-4,7-9,12,14,17). Although many useful and effective selective media are available for fungal isolation, there is no selective medium that uses differential sensitivity to secondary metabolites produced by the target organism.

Strategies during the past decade have demonstrated the efficacy of *Gliocladium* spp. as biocontrol agents in reducing the incidence of diseases (6,11,12). The population dynamics of these fungi, i.e.,

survival of conidia, mycelia, and chlamydospores, also have been examined (10,13,14). To detect the population densities of *Gliocladium* spp., several media have been developed for isolation and enumeration (3,12,14,16). However, there is no report of a medium that can exclusively differentiate *Gliocladium* spp. from *Trichoderma* spp. in soil dilution plates.

Gliotoxin, an antimicrobial metabolite produced by species of fungi, including *G. virens* J. H. Miller, J. E. Giddens, & A. A. Foster (1,19), was originally isolated from the culture filtrate of *T. lignorum* Tode (22). Its chemistry and toxicity have been investigated (19). Acriflavine, an antimicrobial agent which changes characteristics of nucleic acids by intercalation, has been used as a mutagen (5,23). Recently, selective media containing acriflavine for isolation of *Listeria monocytogenes* (Murray et al) Pirie from food products were developed (20). Strains of *G. roseum* Bainier have been found to be relatively insensitive to this antibiotic (C. M. Kenerley, unpublished). This study describes the development of selective media using differential sensitivity to gliotoxin and acriflavine as the basis of selectivity for *G. virens* and *G. roseum*, respectively, which permit the isolation of these fungi

from nonsterile soils. Also, the newly developed selective media are compared with other media (3,4,12) for recovery of *Gliocladium* spp. from soil.

MATERIALS AND METHODS

Strains. Strains of *G. virens*, *G. roseum*, *Nectria ochroleuca* (Schwein.) Berk, *Rhizoctonia solani* Kühn, *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *vasinfectum* (Atk.) W. C. Snyder & H. N. Hans., and *Pythium ultimum* Trow were used in this study (Table 1). All strains of these fungi were maintained on potato-dextrose agar (PDA) at 27 C in darkness.

Soil. A sandy loam soil (College Station, TX) and a Houston Black clay soil (Temple, TX) collected from cotton fields were infested with propagules of *G. virens* and *G. roseum*, respectively. In addition, sandy loam soils from a prairie (Bryan, TX) and another cotton field (College Station, TX) were used as sources of indigenous isolates of *G. virens*. All sandy loam soils were naturally infested with indigenous propagules of *G. virens* between 10 and 10² cfu/g of soil. The Houston Black clay soil was naturally infested with indigenous propagules of *G. roseum* between 10 and 10³ cfu/g of soil. All soils were passed through a 10-mesh sieve (1.68 mm diameter) before use.

Media development. A minimal salts medium with 0.3% glucose was used as a basal medium throughout the experiment. The medium contained 3.0 g of glucose, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of K_2HPO_4 , 0.5 g of KCl, 1.0 g of $NaNO_3$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 20 g of Bacto agar in 1 L of distilled water. The medium was adjusted to pH 6.0 with 25% phosphoric acid before autoclaving.

Amendments with various concentrations and combinations of antibiotics, including gliotoxin, were added to the medium at 60 C after autoclaving. Antibiotics and fungicides included rose bengal, chloramphenicol, acriflavine, streptomycin sulfate, pentachloronitrobenzene (PCNB), benomyl (Benlate 50WP), propiconazole (Tilt 3.6 E), sodium propionate (propionic acid), and gliotoxin. Each antibiotic was dissolved in sterile distilled water except gliotoxin, which was dissolved in absolute ethanol. All antibiotics were kept at 4 C in darkness before mixing into the basal medium.

Present address of second author: EcoScience Laboratories, Inc., Amherst, MA 01002.

Accepted for publication 8 September 1991 (submitted for electronic processing).

Growth of the fungi tested was determined by placing a disk (5 mm diameter) from the margin of a colony on PDA onto the amended media (approximately 10 ml in a petri dish), incubating cultures at 27 C in darkness, and measuring colony radius from the disk at specific time intervals.

Efficacy of selective isolation and recovery of conidia and chlamyospores of *Gliocladium* spp. To increase the propagule density of fungi other than *Gliocladium* spp., the soil was infested with *F. o. vasinfectum*, *P. ultimum*, and *R. solani* 1 hr before adding *Gliocladium* spp. Propagule suspensions for each of the fungi were prepared by adding 10 ml of sterile water of 10-day-old PDA cultures of each fungus, scraping the mycelial mats with a flat spatula, macerating the mycelial mats with a razor blade, and filtering through two layers of sterile cheesecloth. A mixture of the three fungi in aqueous suspension was used for soil infestation. The propagule densities of *F. o. vasinfectum*, *P. ultimum*, and *R. solani* were $7.9 \pm 0.4 \times 10^5$, $5.48 \pm 1.1 \times 10^6$, and $4.0 \pm 0.8 \times 10^4$ cfu/g of soil, respectively. The colony-forming units of these fungi were determined after dilution plating on selective media that have been used to isolate these fungi (2,7,8).

Efficacy of selective isolation and recovery of conidia of *Gliocladium* spp. from soil was measured. Conidial suspensions were prepared by adding 10 ml of sterile water to 10-day-old PDA cultures of each fungus in the same manner as described above without macerating the mycelial mats. Ten milliliters of the suspension from each strain of *G. virens* (Gv-wt) ($3.8 \pm 0.3 \times 10^5$ conidia per milliliter) or *G. roseum* (Gr-1620) ($3.8 \pm 0.2 \times 10^5$ conidia per milliliter) was added to different 250-ml Erlenmeyer flasks containing 90 g of nonsterile soil. The number of conidia in the suspension was determined with a hemacytometer.

To measure the efficacy of isolation and recovery of chlamyospores of *Gliocladium* spp. from soil, 10 ml of a chlamyospore suspension from each strain of *G. virens* (Gv-wt) ($1.0 \pm 0.1 \times 10^5$ chlamyospores per milliliter) or *G. roseum* (Gr-1620) ($1.1 \pm 0.1 \times 10^5$ chlamyospores per milliliter) were added to a different 250-ml Erlenmeyer flask containing 90 g of nonsterile soil. Chlamyospore suspensions were prepared from biomass produced by growing the fungi in a molasses medium (13). The medium contained 25 ml of molasses and 5 g of yeast extract in 1 L of distilled water. One hundred milliliters of the medium was added to a 250-ml Erlenmeyer flask and autoclaved for 1 hr. One milliliter of a conidial suspension ($1.0 \pm 0.2 \times 10^7$ conidia per milliliter), as described above, was added to each flask. Flasks were held at room temperature (23 ± 2 C) with agitation (125 rpm) for

20 days. The mycelial mats were removed from the culture medium by filtration (grade 613 paper) on a 9-cm-diameter Büchner funnel, air-dried for 3 days, and ground through a 425- μ m (40-mesh) screen. The resultant powder was suspended in distilled water. The number of chlamyospores of *Gliocladium* spp. in the suspension was determined with a hemacytometer.

Selective recovery of *G. virens* and *G. roseum* from soils was determined 30 min after the fungal propagules were distributed throughout the soil. One gram of the soil in the flask was diluted serially in sterile water, and soil-water suspensions were spread on the surface of three replicate plates. Percent recovery of *Gliocladium* spp. from nonsterile soil containing *F. o. vasinfectum*, *P. ultimum*, and *R. solani* was compared to recovery

of *Gliocladium* spp. from sterile soil.

Sensitivity of strains of *Gliocladium* spp. in vitro. Efficacy of recovery of 15 strains each of *G. virens* and *G. roseum* from the newly developed media was measured in vitro. Serial dilutions of conidial suspensions were spread on the surface of plates of the various media tested. The initial numbers of conidia in the suspensions were determined with a hemacytometer.

Comparison with other media for recovery of *Gliocladium* spp. from infested soil. Efficacy of selective isolation and recovery of *Gliocladium* spp. from soil infested with propagules of *Gliocladium* spp. and from noninfested natural soil was compared on several media. Extracted gliotoxin from a culture filtrate of *G. virens* (Gv-wt) was used as an ingredient of the newly developed media.

Table 1. Species and source of fungi used in the development of selective media for *Gliocladium virens* and *G. roseum*

Strain	Collector or source	Source identification
<i>G. virens</i>		
Gv-wt	C. Howell ^o	...
Gv-1 ^p	C. Howell	...
Gv-3	CVS ^q	126.65
Gv-5	CVS	344.47
Gv-6	BRC ^r	169262
Gv-7	G. Harman ^s	G-1 H
Gv-8	G. Papavizas ^t	GI 9
Gv-9	G. Papavizas	GI 12
G-3 ^p	C. Howell	G-3
Gv-6H	C. Howell	GV 6
GI-9	C. Howell	GI-9
GI-10	C. Howell	GI-10
GI-19	C. Howell	GI-19
GI-25	C. Howell	GI-25
GVP ^p	C. Howell	GVP
<i>G. roseum</i>		
Gr-86-283	C. Kenerley ^u	Gr-86-283
Gr-790	C. Kenerley	Gr-790
Gr-1064-2	C. Kenerley	Gr-1064-2
Gr-1320	C. Kenerley	Gr-1320
Gr-1398	C. Kenerley	Gr-1398
Gr-1498-2	C. Kenerley	Gr-1498-2
Gr-1536	C. Kenerley	Gr-1536
Gr-1577	C. Kenerley	Gr-1577
Gr-1620	C. Kenerley	Gr-1260
Gr-2722	C. Kenerley	Gr-2722
Gr-2827	C. Kenerley	Gr-2827
CKR-1	CVS	178.28
CKR-2	G. Jager ^v	M 16
CKR-3	CMI ^w	R 205
<i>Nectria ochroleuca</i> ^x	CVS	194.57
<i>Fusarium oxysporum</i> f. sp. <i>vasinfectum</i>		
	J. DeVay ^y	...
<i>Rhizoctonia solani</i> ^z	C. Kenerley	Rs-4
<i>Pythium ultimum</i>	C. Kenerley	P-18

^o United States Department of Agriculture, Agricultural Research Service, College Station, TX.

^p Nongliotoxin-producing strains.

^q Centralbureau Voor Schimmelcultures, Ag Baarn, The Netherlands.

^r Biosystematics Research Center, Ottawa, Ontario, Canada.

^s New York State Agricultural Experimental Station, Geneva, NY.

^t Biocontrol Plant Diseases Laboratories and Florist and Nursery Crops Laboratories, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD.

^u Texas A & M University, College Station, TX.

^v Instituut Voor Bodemvruchtbaarheid, Haren (Gr.), The Netherlands.

^w Commonwealth Mycological Institute, Richmond, England.

^x Teleomorph of *G. roseum*.

^y University of California at Davis, Davis, CA.

^z Anastomosis group 4.

Table 2. Colony radius^x of *Gliocladium virens*, *G. roseum*, *Rhizoctonia solani*, *Pythium ultimum*, and *Fusarium oxysporum* f. sp. *vasinfectum* after 24, 48, or 72 hr of incubation in darkness at 27 C on a basal medium amended with antibiotics

Treatment ^y	<i>G. virens</i> ^z		<i>G. roseum</i>		<i>R. solani</i>		<i>P. ultimum</i>		<i>F. o. vasinfectum</i>	
	48	72	48	72	24	48	24	48	48	72
Control	23	33	5	8	23	*	23	*	9	17
Rose bengal (50 mg)	21	35	4	6	18	*	10	20	10	16
Chloramphenicol (50 mg)	20	35	5	8	18	*	11	28	10	17
Streptomycin (50 mg)	23	35	4	7	18	*	10	27	9	15
Benomyl (500 µg)	23	35	3	9	22	*	23	*	10	17
Benomyl (2 mg)	0	0	0	2	0	0	0	0	0	3
Sodium propionate (500 mg)	30	*	5	8	16	30	4	16	8	16
Acriflavine (30 mg)	3	3	2	5	15	25	7	10	7	13
Acriflavine (300 mg)	2	2	2	4	20	28	6	10	6	9
PCNB (20 mg)	5	9	5	8	5	8	7	13	3	5
Propiconazole (4.3 ml)	10	16	2	3	13	27	23	*	2	2
Propiconazole (43 ml)	1	2	0	0	7	7	1	1	0	0

^x Each measurement is the average radius (mm) of colonies from agar disks in petri dishes from two trials, with four replicates per trial.

* = Colony reached edge of petri dish. Coefficient of variation for all measurements was < 8%.

^y Control was the basal medium described in the text. Unit indicates amount of antibiotic per liter of medium.

^z Fungal strains: *G. virens* (Gv-wt), *G. roseum* (Gr-1620), *R. solani* (Rs-4), *P. ultimum* (P-18), and *F. o. vasinfectum* (J. DeVay).

Table 3. Colony radius^x of *Gliocladium virens*, *G. roseum*, *Rhizoctonia solani*, *Pythium ultimum*, and *Fusarium oxysporum* f. sp. *vasinfectum* at 48 hr after seeding onto a basic antibiotic medium amended with antibiotics

Treatment ^y	<i>G. virens</i> ^z	<i>G. roseum</i>	<i>R. solani</i>	<i>P. ultimum</i>	<i>F. o. vasinfectum</i>
Control	24	5	*	8	10
Basic antibiotic medium (BAM)	19	5	25	8	10
BAM + propaconazole (4.3 ml)	10	2	15	6	2
BAM + gliotoxin (10 mg)	14	3	12	0	6
BAM + gliotoxin (20 mg) (GVSM)	12	1	3	0	2
BAM + propaconazole (4.3 ml) + gliotoxin (10 mg)	9	3	12	0	1
BAM + acriflavine (60 mg)	2	4	16	5	9
BAM + gliotoxin (10 mg) + PCNB (20 mg) (BAGP)	3	4	8	4	3
BAGP + acriflavine (60 mg)	0	3	3	0	1

^x Each measurement is the average radius (mm) of colonies from agar disks in petri dishes from two trials, with four replicates per trial. * = Colony reached edge of petri dish. Coefficient of variation of all measurements was < 8%.

^y Control was the basal medium described in the text. Basic antibiotic medium (BAM) consisted of 50 mg of chloramphenicol, 10 mg of rose bengal, 50 mg of streptomycin sulfate, 500 µg of benomyl, and 500 mg of sodium propionate in 1 L of basic antibiotic medium. Unit indicates amount of antibiotic per liter of medium.

^z Fungal strains: *G. virens* (Gv-wt), *G. roseum* (Gr-1620), *R. solani* (Rs-4), *P. ultimum* (P-18), and *F. o. vasinfectum* (J. DeVay).

Ten milliliters of a conidial suspension (10⁴ to 10⁵ conidia per milliliter, prepared as described above) from each of five strains of *G. virens* and *G. roseum* were added to 250-ml Erlenmeyer flasks containing 90 g of nonsterile soil. Efficacy of recovery of propagules from soil infested with *G. virens* by dilution plating was compared using Trichoderma medium E (TMESA) (12), Trichoderma selective medium (TSM) (4), Davet's medium (3), and the newly developed selective medium for *G. virens*. Comparison of recovery of propagules of *G. roseum* from infested soil by dilution plating was made between the newly developed selective medium for *G. roseum* and Davet's medium (3).

Comparison with other media for recovery of indigenous isolates of *G. virens*

from natural soil. Selective isolation of indigenous propagules of *G. virens* by dilution plating was compared on four media. Serial dilutions were made for each of the soils described previously. The media tested were TMESA (12), TSM (4), Davet's medium (3), and the newly developed selective medium for *G. virens*.

All inoculated media were incubated at 27 C in darkness, except those containing TMESA, which were incubated at room temperature under fluorescent light (14). Colony-forming units were recorded daily for 14 days. Colony identity was verified by examination with dissecting and compound microscopes.

Statistics. Experiments were conducted at least twice with at least three

replicates per treatment. Means of two and more than two treatments were separated by *t* test and Duncan's multiple range test, respectively. Statistical values were obtained using the *t* test and the analysis of variance procedure of SAS (SAS Institute, Cary, NC) (15).

RESULTS AND DISCUSSION

Inhibition of radial growth in vitro of *G. virens* (Gv-wt), *G. roseum* (Gr-1620), *F. o. vasinfectum*, *P. ultimum*, and *R. solani* varied with the antibiotic and antibiotic concentration (Table 2). Rose bengal at 50 mg/L, chloramphenicol at 50 mg/L, and streptomycin sulfate at 50 mg/L did not suppress growth of *G. virens* (Gv-wt), *G. roseum* (Gr-1620), or *F. o. vasinfectum* but inhibited growth of *P. ultimum* (52–57%) and *R. solani* (22%) at 24 hr of incubation. Benomyl (50WP) at 2 mg/L inhibited growth of all fungi tested (75–100%). Benomyl at 500 µg/L did not inhibit growth of the five species assayed but did restrict growth of some background fungi on nonsterile soil dilution plates (*data not presented*). Sodium propionate decreased colony size of *R. solani* (30%), *P. ultimum* (83%), and *F. o. vasinfectum* (11%), enhanced colony size of *G. virens* (Gv-wt) (130%), and had no effect on *G. roseum* (Gr-1620). Acriflavine at 30 and 300 mg/L suppressed the growth of all fungi tested (13–91%). Moreover, hyphae of *G. virens* (Gv-wt), *R. solani*, *P. ultimum*, and *F. o. vasinfectum* that grew from 5-mm-diameter mycelial disks on the basal medium amended with 30 mg/L acriflavine were very sparse, whereas hyphal density of *G. roseum* (Gr-1620) was not different from the control. PCNB (75WP) at 20 mg/L reduced the colony size of all test fungi (67–88%) except *G. roseum* (Gr-1620). Except for *P. ultimum*, propiconazole at 4.3 ml/L suppressed growth of all test fungi (47–89%), especially *F. o. vasin-*

fectum (89%).

From this survey, a basic antibiotic medium (BAM) that suppressed growth of soilborne fungi and bacteria and least inhibited the growth of *Gliocladium* spp. was formulated. The selected antibiotics and effective concentrations for BAM

included 10 mg of rose bengal, 50 mg of chloramphenicol, 50 mg of streptomycin sulfate, 500 µg of benomyl (50WP), and 500 mg of sodium propionate in 1 L of distilled water. The medium was adjusted to pH 6.0 with 25% phosphoric acid, which resulted in opti-

mal growth of *G. virens* (Gv-wt) and *G. roseum* (Gr-1620) (data not presented). Rose bengal at 50 mg/L, chloramphenicol at 50 mg/L, and streptomycin sulfate at 50 mg/L were selected for suppressing growth of bacteria that appeared on soil dilution plates. Benomyl at 2–20 mg/L has been used to isolate *Trichoderma* spp. from soil (4,14). Because benomyl (50WP) at 2 mg/L inhibited growth of *Gliocladium* spp., the concentration selected was 500 µg/L. Although benomyl at this concentration did not inhibit growth of the fungi tested, this concentration of benomyl was effective for suppressing growth of other fungi that appeared on soil dilution plates. Sodium propionate at 500 mg/L was selected for suppressing the growth of rapidly growing fungi (e.g., *Rhizopus* spp.) (14).

Gliotoxin and other concentrations of the previously tested antibiotics were added to BAM in an attempt to further distinguish *G. virens* (Gv-wt) and *G. roseum* (Gr-1620) from other fungi tested (Table 3). BAM suppressed the growth rates of *R. solani* (>44%), *P. ultimum* (>82%), and *G. virens* (Gv-wt) (21%) but not those of *G. roseum* (Gr-1620) or *F. o. vasinfectum*. Although the combination of antibiotics in BAM was not effective for distinguishing *Gliocladium* spp. from other fungi, the combination was useful for suppressing growth of fungi and bacteria that would usually appear on soil dilution plates inoculated with soil suspensions. BAM plus gliotoxin at 20 mg/L, hereafter referred to as a selective medium for *G. virens* (GVSM), was effective in suppressing growth of *R. solani* (>93%), *P. ultimum* (100%), *F. o. vasinfectum* (80%), and *G. roseum* (Gr-1620) (80%). Growth of *G. virens* (Gv-wt) also was reduced on this medium (50%); however, colonies of *G. virens* (Gv-wt) were easily distinguishable after 48 hr by their greater colony size and the sparse mycelia of other fungi. Propiconazole (4.3 ml/L) added to BAM inhibited *F. o. vasinfectum* more than other fungi listed (Table 3). This suggests another compound that may be used to differentiate *G. virens* from *F. o. vasinfectum* in soil. A combination of BAM, 10 mg of gliotoxin, 20 mg of PCNB (75WP), and 60 mg of acriflavine effectively inhibited fungi other than *G. roseum* (Gr-1620) and *R. solani*. Although colonies of *R. solani* developed on this medium, only sparse hyphae grew from a 5-mm-mycelial disk which distinguished colonies of *R. solani* from *G. roseum* (Gr-1620).

However, recovery of propagules of *G. roseum* (Gr-1620) on the combination of BAM, 10 mg of gliotoxin, 20 mg of PCNB (75WP), and 60 mg of acriflavine as described above from soil was approximately 35% of the initial number of conidia of *G. roseum* (Gr-1620) added to the soil (Table 4). By reducing the concentration of gliotoxin to 1 mg/L,

Table 4. Recovery of *Gliocladium roseum* (Gr-1260) on basic antibiotic media amended with acriflavine and gliotoxin from nonsterile soil

Medium ¹	<i>G. roseum</i> (× 10 ⁴) ²
BAM	6.8 a
BAM + acriflavine	6.9 a
BAM + acriflavine + gliotoxin (1 mg) (GRSM)	6.7 a
BAM + acriflavine + gliotoxin (10 mg)	2.7 b
BAM + acriflavine + gliotoxin (20 mg)	... c

¹ BAM = basic antibiotic medium. Unit indicates amount of antibiotic per liter of medium. In all cases, the amount of acriflavine was 60 mg.

² Each measurement is the average number of colony-forming units per gram of soil by the dilution plate method from two trials, with three replicates per trial. Means with the same letter in each column are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. Nonsterile soil was infested with 7×10^4 conidia per gram of soil of *G. roseum* (Gr-1620).

Table 5. Recovery¹ of *Gliocladium virens* (Gv-wt) or *G. roseum* (Gr-1620) on media after infestation of sterile or nonsterile soil with conidia

Soil	Medium ²	Colony-forming units (× 10 ⁴) per gram of soil	
		<i>G. virens</i>	<i>G. roseum</i>
Sterile soil	B	3.4 a	4.0 a
	BAM	3.5 a	4.1 a
	GVSM	3.8 a	ND b
	GRSM	ND b	4.0 a
Nonsterile soil	GVSM	3.9 a	ND b
	GRSM	ND b	4.0 a

¹ Each measurement is the average number of colony-forming units per gram of soil by the dilution plate method from two trials, with three replicates per trial. Means with the same letter in each column for each soil condition are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. The numbers of conidia per gram of soil introduced to both sterile and nonsterile soils were 3.8×10^4 for *G. virens* (Gv-wt) and 3.8×10^4 for *G. roseum* (Gr-1620). Nonsterile soil was infested with 4×10^4 , 7.9×10^5 , and 5.4×10^6 cfu/g of *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *vasinfectum*, and *Pythium ultimum*, respectively. ND = no colonies detected from any dilution.

² B = basal medium, BAM = basic and biotic medium, GVSM = selective medium for *G. virens*, and GRSM = selective medium for *G. roseum*.

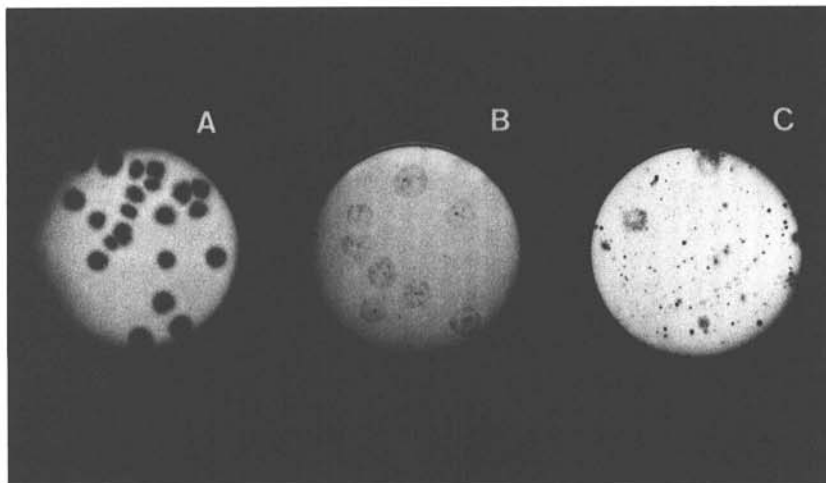


Fig. 1. Selective isolation of *Gliocladium virens* and *G. roseum* on selective media from natural soil infested with *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *vasinfectum*, and *Pythium ultimum* by the dilution plate method. (A) Selective medium for *G. roseum* (GRSM); (B) selective medium for *G. virens* (GVSM); and (C) basic antibiotic medium (BAM).

Table 6. Recovery^y of strains of *Gliocladium virens* and *G. roseum* from nonsterile soil

Medium ^z	Strain of <i>G. virens</i> (cfu × 10 ⁴ /g of soil)						Strain of <i>G. roseum</i> (cfu × 10 ⁴ /g of soil)				
	Gv-wt	Gv-3	Gv-7	Gv-9	GVP	G-3	CKR-1	CKR-2	Gr-1398	Gr-1620	Gr-2827
GVSM	7.0 a	4.0 a	7.7 a	2.2 a	2.3 b	2.8 b					
TMESA	5.3 a	2.2 a	4.7 ab	1.7 ab	3.7 b	10.2 b					
TSM	5.0 a	3.6 a	4.3 b	1.4 ab	41.0 a	9.1 b					
Davet	4.0 a	4.0 a	2.3 b	1.2 b	0.7 b	19.2 a	1.8 b	9.4 a	2.1 b	2.1 a	2.4 a
GRSM							6.6 a	5.9 a	4.0 a	1.6 a	2.5 a

^y Each measurement is the average number of colony-forming units per gram of soil by the dilution plate method from two trials, with three replicates per trial. The soil was infested with each strain of *G. virens* or *G. roseum*. Means with the same letter in each column are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

^z GVSM = selective medium for *G. virens*, TMESA = Trichoderma medium E by Papavizas and Collins (12), TSM = Trichoderma selective medium by Elad and Chet (5), Davet = Davet's medium (4), and GRSM = selective medium for *G. roseum*.

Table 7. Recovery of indigenous *Gliocladium virens* from nonsterile soil

Medium ^x	Colony-forming units (× 10 ⁴) per gram of soil ^y of <i>G. virens</i>		
	Soil 1 ^z	Soil 2	Soil 3
GVSM	64 a	138 a	31 a
TMESA	2 b	15 b	2 b
TSM	2 b	13 b	1 b
Davet	NS	NS	NS

^x GVSM, TMESA, TSM, and Davet = selective medium for *G. virens*, Trichoderma medium E by Papavizas & Collins (12), Trichoderma selective medium by Elad & Chet (5), and Davet's medium (4), respectively.

^y Each measurement is the average number of colony-forming units per gram of soil by the dilution plate method of two trials, with three replicates per trial. NS = nonselective for *G. virens* recovery from soil. Means with the same letter in each column are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

^z Soil 1 and soil 2 were collected from different cotton fields in College Station, TX. Soil 3 was from a prairie in Bryan, TX.

recovery of propagules of *G. roseum* (Gr-1620) from the soil was not significantly different from the initial number of conidia of the fungus ($P = 0.67$). Inhibition of *G. roseum* on this combination was attributed to the sensitivity of *G. roseum* to the gliotoxin in the medium. The selective medium for *G. roseum* (GRSM) contained 60 mg of acriflavine, 20 mg of PCNB, and 1 mg of gliotoxin in 1 L of BAM.

Recovery of conidia of *G. virens* (Gv-wt) on GVSM and *G. roseum* (Gr-1620) on GRSM from nonsterile soil or sterile soil infested with these strains was not significantly different from the initial number of conidia added to the soil ($P \leq 0.05$) (Table 5). In the nonsterile soil, the colony-forming units on GVSM and GRSM are only for *G. virens* and *G. roseum*, respectively. There were no significant differences between recovery of chlamydospores of *G. virens* (Gv-wt) from sterile soil on BAM or from nonsterile soil on GVSM ($P = 0.89$). The number of colonies of *G. roseum* (Gr-1620) on BAM from sterile soil and on GRSM from nonsterile soil also were similar (*data not presented*).

Selective isolation of *G. virens* on GVSM from natural soil infested with *G. virens* (Gv-wt), *G. roseum* (Gr-1620), *R. solani*, *P. ultimum*, and *F. o. vas-infectum* was successful (Fig. 1). Of 232 colonies collected from 20–30 plates of GVSM 3 days after plating, only *G. virens* was detected; colony size of *G.*

virens was optimal for quantification. Although a few colonies of *G. roseum*, *R. solani*, and unidentified fungi began to grow at 5 or 6 days after incubation, the colony size and shape of *G. virens* on GVSM were easily distinguishable. At 4 days after plating, eight of 318 fungal colonies collected from 20–30 plates of GRSM were fungi other than *G. roseum*. Despite a few colonies of other fungi at 4 or 5 days after plating on GRSM, counting of colonies of *G. roseum* was feasible because of the distinct shape of colonies of *G. roseum* from other fungi. Even 7 days after plating, colonies of *G. roseum* could be distinguished from other fungi on GRSM based on colony shape. Similar differentiation in the presence of interfering fungi has been reported for other soilborne fungi (17).

Recovery of three (Gv-1, G-3, and GVP) of 15 strains of *G. virens* on GVSM was significantly less than the mean of recovery on BAM ($P \leq 0.05$) (*data not presented*). These three strains were nongliotoxin-producing strains of *G. virens*. Recovery of gliotoxin-producing strains of *G. virens* on GVSM from infested soil was greater than or equal to recovery on other media tested ($P \leq 0.05$) (Table 6). Only two of 295 colonies were not confirmed as isolates of *G. virens* after 3 days of incubation on GVSM. However, on TMESA and TSM, numbers of colonies of *G. virens* were overestimated by approximately 20–25% because of similarities of colo-

nies of *G. virens* and *Trichoderma* spp. We were able to differentiate colonies of *G. virens* unambiguously from *Trichoderma* spp. on these media only by observing sporulating colonies under a dissecting microscope after 7–15 days of incubation. Exclusive differentiation of *G. virens* from *Trichoderma* spp. on TMESA and TSM may require microscopic examination for estimating populations of *G. virens* from soil. On Davet's medium, selective isolation of *G. virens* was not achieved because of nondiscrimination of other fast-growing fungi and bacteria on the medium. Counting colonies of *G. virens* on this medium was possible only by marking each colony on the plate daily and later checking the marked colonies for sporulation under a dissecting microscope. A decrease in the recovery of nongliotoxin-producing strains of *G. virens* (GVP and G-3) on GVSM is probably attributable to their sensitivity to gliotoxin.

Recovery of propagules of 10 of 15 strains of *G. roseum* were equal to recovery on PDASA (PDA containing 50 µg/ml of streptomycin sulfate and 50 µg/ml of chloramphenicol) and 13 of 15 were similar to recovery on BAM ($P \leq 0.05$) (*data not presented*). Five strains of *G. roseum*, which had less recovery on GRSM than on PDASA, were CKR-1, Gr-1498-2, Gr-790, Gr-1064-2, and *N. ochroleuca*. Among the strains, two strains (Gr-790 and Gr-1064-2) were recovered less on GRSM than on BAM. With reducing the amount of acriflavine (25 mg/L) in GRSM, recovery of these two strains on the medium was equal to recovery on BAM. Acriflavine (60 mg/L) in GRSM probably is the ingredient that causes less recovery of these two strains on GRSM. We did not determine if one compound or a combination of compounds was responsible for the sensitivity of the other strains (CKR-1, Gr-1498-2, and *N. ochroleuca*).

Recovery of five strains of *G. roseum* on GRSM from infested soil was significantly higher or not different from that on Davet's medium ($P \leq 0.05$) (Table 6). No difficulty was encountered in detecting colonies of *G. roseum* on GRSM because of the selective exclusion of other contaminating fungi. However, recov-

ered propagules of *G. roseum* on Davet's medium could be determined only by examination of plates under a dissecting microscope.

Recovery of indigenous isolates of *G. virens* from nonsterile soil was greater on GVSM than the other media tested ($P \leq 0.05$) (Table 7). Only estimates of the number of colonies of *G. virens* could be made on TMESA and TSM because of the growth of colonies of *Trichoderma* spp. and other fungi on the media. Although there were fungi other than *G. virens* on GVSM, there was no difficulty in counting the number of colonies of *G. virens* because of the distinctive shape of colonies of *G. virens*.

Many useful and effective selective media are available for isolation and population studies of fungi (21). However, because of low percentages of recoveries of the desired fungi, some selective media are inadequate for estimating the number of fungal propagules. The success of some selective media depends on accompanying techniques, e.g., controlling temperature and moisture (18). The performance of the semiselective media (GVSM and GRSM) developed in this study permits the direct isolation and enumeration of *G. virens* and *G. roseum* from natural soil by the dilution plate method. Gliotoxin is the basis of selectivity for *G. virens* on GVSM. To our knowledge, this is the first report of using the target organism's own secondary metabolite as a key ingredient for selective isolation of a fungus from natural soil. This may be a useful approach

for other soil fungi and microorganisms in other habitats. These semiselective media are currently being used to study the ecology of these two biocontrol fungi in the same ecosystem.

LITERATURE CITED

1. Brian, P. W., and Hemming, H. G. 1945. Gliotoxin, a fungistatic metabolic product of *Trichoderma viride*. Ann. Appl. Biol. 13:214-228.
2. Conway, K. E. 1985. Selective medium for isolation of *Pythium* spp. from soil. Plant Dis. 69:393-395.
3. Davet, P. 1979. Technique pour l'analyse des populations de *Trichoderma* et de *Gliocladium virens* dans le sol. Ann. Phytopathol. 11:529-533.
4. Elad, Y., and Chet, I. 1983. Improved selective media for isolation of *Trichoderma* spp. or *Fusarium* spp. Phytoparasitica 11:55-58.
5. Franklin, T. J., and Snow, G. A. 1981. Biochemistry of Antimicrobial Action. Chapman and Hall, London. 217 pp.
6. Howell, C. R. 1982. Effect of *Gliocladium virens* on *Pythium ultimum*, *Rhizoctonia solani*, and damping-off of cotton seedlings. Phytopathology 72:496-498.
7. Ko, W., and Hora, F. K. 1971. A selective medium for the quantitative determination of *Rhizoctonia solani* in soil. Phytopathology 61:707-710.
8. Komada, H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Rev. Plant Prot. Res. 8:114-125.
9. Mircetich, S. M., and Kraft, J. M. 1973. Efficiency of various selective media in determining *Pythium* populations in soil. Mycopathol. Mycol. Appl. 50:151-161.
10. Mueller, J. D., and Sinclair, J. B. 1986. Occurrence and role of *Gliocladium roseum* in field-grown soybeans in Illinois. Trans. Br. Mycol. Soc. 86:677-680.
11. Papavizas, G. C. 1985. *Trichoderma* and *Gliocladium*: Biology, ecology, and potential for biocontrol. Annu. Rev. Phytopathol. 23:23-54.
12. Papavizas, G. C., and Collins, D. J. 1990. Influence of *Gliocladium virens* on germination and infectivity of sclerotia of *Sclerotium rolfsii*. Phytopathology 80:627-630.
13. Papavizas, G. C., Dunn, M. T., Lewis, J. A., and Beagle-Ristaino, J. 1984. Liquid fermentation technology for experimental production of biocontrol fungi. Phytopathology 74:1171-1175.
14. Papavizas, G. C., and Lumsden, R. D. 1982. Improved medium for isolation of *Trichoderma* spp. from soil. Plant Dis. 66:1019-1020.
15. SAS Institute, Inc. 1982. SAS User's Guide: Statistics. SAS Institute, Inc., Cary, NC. 584 pp.
16. Smith, V. L., Wilcox, W. F., Harman, G. E. 1990. Potential for biological control of *Phytophthora* root rot and crown rots of apple by *Trichoderma* and *Gliocladium* spp. Phytopathology 80:880-885.
17. Stack, J. P., and Millar, R. L. 1985. Isolation and characterization of a metalaxyl-insensitive isolate of *Phytophthora megasperma* f. sp. *medicaginis*. Phytopathology 75:1387-1392.
18. Stack, J. P., and Millar, R. L. 1985. Relative survival potential of propagules of *Phytophthora megasperma* f. sp. *medicaginis*. Phytopathology 75:1398-1404.
19. Taylor, A. 1971. The toxicology of sporidesmins and other epipolythiodioxopiperazines. Pages 337-376 in: Microbial Toxin VII. S. Kadis, A. Ciegler, and S. J. Ajl, eds. Academic Press, New York.
20. Tiwari, N., and Aldenrath, S. G. 1990. Isolation of *Listeria monocytogenes* from food products on four selective plating media. J. Food. Prot. 53:382-385.
21. Tsao, P. H. 1970. Selective media for isolation of pathogenic fungi. Annu. Rev. Phytopathol. 8:157-186.
22. Weindling, R., and Emerson, O. H. 1936. The isolation of a toxic substance from the culture filtrate of *Trichoderma*. Phytopathology 26:1068-1070.
23. Wolfe, A. O. 1975. Quinacrine and other acridines. Pages 203-233 in: Antibiotics III (Mechanisms of Actions of Antimicrobial and Antitumor Agents). J. W. Corcoran and F. E. Hahn, eds. Springer-Verlag, New York.