Influence of *Gliocladium virens* on Germination and Infectivity of Sclerotia of *Sclerotium rolfsii*

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ABSTRACT

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A system was developed for: 1) testing the ability of sclerotia of *Sclerotium rolfsii* to infect snap bean plants or hypocotyls after their incubation in soil amended with *Gliocladium virens* (strain Gl-3); 2) relating infectivity to colonization of sclerotia by the antagonist; and 3) evaluating germination of antagonist-infected sclerotia on agar. Sclerotia were recovered from soil and plated (with or without surface treatment with 1% sodium hypochlorite) on a medium selective for *Sclerotium* to test for germination and on modified TME medium to determine the extent of sclerotial colonization by *G. virens*. Sclerotia recovered from soil were placed on 9-day-old snap bean plant hypocotyls cut into 9-cm sections. Hypocotyls were incubated at 26 C for 3 days and examined for lesions developing from germinating sclerotia. Infectivity of sclerotia also was assayed by placing two sclerotia 2 cm below the soil surface in contact with hypocotyls of snap bean plants growing in nonsterile field soil in the greenhouse. Positive correlation was found between the percentage of colonization of sclerotia by *G. virens* in soil and reduction of infectivity and germination when strain Sr-1 of *S. rolfsii* (small sclerotia, about 1.0 mm in diameter) was used. Rapid loss of viability and infectivity of sclerotia of strain Sr-1 (within 3 days in soil) was observed even at a low concentration of strain Gl-3 (3×10^3 colony-forming units [cfu] per gram of soil). In contrast, sclerotia of strain Sr-109 (sclerotia 1.5–2.0 mm in diameter) recovered from soil amended with 3×10^5 cfu of Gl-3 per gram of soil were about 60% infective at 32 days. Although 100% of sclerotia of strain Sr-3 of *S. rolfsii* (sclerotia >2.5 mm in diameter) from soil amended with Gl-3 were colonized by the antagonist at all times, their germinability was not reduced after 24 days and only slightly reduced after 32 days. Although all sclerotia of Sr-3 were germinable, only 73 and 42% were infective after 24 days at 6×10^3 and 9×10^3 cfu of Gl-3 per gram of soil, respectively. Sclerotia of the three strains of *S. rolfsii* tested from control soil were 100% viable and from 80–100% infective, depending on the strain.

Additional keywords: Phaseolus vulgaris.

Sclerotium rolfsii Sacc. causes extensive damage to plants in more than 100 families in warm regions of the world (1,15). Although progress has been made on experimental biological control of this important pathogen (3,10) very little is known about the mechanism of action of biocontrol agents such as *Gliocladium* spp. and *Trichoderma* spp. on this pathogen.

Mycoparasitism of sclerotia of *S. rolfsii* and other sclerotial fungi by soil antagonists, and the subsequent fate of sclerotia, has been studied by several investigators (4–7,9,12,14). Most studies on mycoparasitism of this pathogen, however, were performed in vitro. Elad et al (5), for instance, found that *Trichoderma harzianum* Rifai excreted β-1,3-glucanase and chitinase in a medium containing laminarin and chitin, respectively. Mutto et al (9) observed that hyphae of *T. harzianum* developed in the medulla and in the cell lumen of the sclerotia in vitro. Henis et al (6) also found that hyphae of *Trichoderma* penetrated the rind and cortex of sclerotia of *S. rolfsii* in vitro, lysed the medullar tissue, and produced chlamydospores inside the sclerotia. Davet (4) studied the ability of several strains of *Trichoderma* to parasitize and destroy sclerotia of *S. rolfsii* in nonsterile soil. He found that strain behavior in nonsterile soil varied from strain to strain and appeared to be associated with their competitive saprophytic ability, which could be used as a first criterion to screen strains of *Trichoderma* for biocontrol potential.

In a recent paper, Papavizas and Lewis (12) reported that *Gliocladium virens* Miller, Giddens, & Foster was more effective than *T. harzianum* and *T. viride* Pers.: ex Fr. in suppressing damping-off and blight on snap bean caused by *S. rolfsii*. They observed that although 100% of sclerotia of some strains of *S. rolfsii* were colonized by *G. virens*, their germination was not always reduced, even when exposed to *G. virens* for 28 days in nonsterile soil. They postulated that the ability of *G. virens* to destroy sclerotia depended on the size and melanin content of sclerotia. The larger and darker the sclerotia, the less reduction in germination was observed even when the sclerotia were exposed to the antagonist in soil for 28 days. Tan sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary were much more readily invaded by various mycoparasites than black sclerotia of the same pathogen (8).

Colonization of sclerotia by *Trichoderma* spp. may not be entirely related to their germination on agar. Penetration of sclerotia of *S. rolfsii* by *Trichoderma* spp. alone did not always lead to sclerotial degradation (6,7). In the same context, colonization of sclerotia by *G. virens* or *Trichoderma* spp. and their germinability may not always be related to their infectivity. The objective of the present study was to develop a system to test infectivity of sclerotia of *S. rolfsii* and compare infectivity to colonization and ability to germinate after incubation in nonsterile soil amended with *G. virens*.

MATERIALS AND METHODS

Fungal strains and inoculum preparation. Strain Sr-1 of *S. rolfsii*, isolated from a diseased snap bean (*Phaseolus vulgaris* L.) plant at Beltsville, was used in most studies. We also used strains Sr-3 and Sr-109 from peanuts provided by R. Rodriguez-Kabana, Auburn University, Auburn, AL, and M. D. Porter, ARS Tidewater Research Center, Suffolk, VA, respectively. Sclerotia of Sr-1 are tan and about 1.0 mm in diameter. Sclerotia of Sr-109 and Sr-3 are dark (almost black) and 1.5–2.0 mm and 2.5–4.0 mm in diameter, respectively. All cultures of *S. rolfsii*

were maintained on Difco potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI). Sclerotia, produced on PDA in petri plates, were removed from the agar surface after 30 days of growth with a soft brush and used within 1–3 hr.

Strain Gl-3 of *G. virens*, from a sclerotium of *S. rolfsii* parasitized in a Beltsville soil also was maintained on PDA. Fungal biomass of Gl-3 was produced by growing it in 16-L fermenters as described previously (11). After 6 days of growth, the biomass was separated from the broth by filtration through a cotton muslin filter on an 18-cm-diameter Büchner funnel. Mats were air dried at room temperature for 2 days and ground in a Wiley mill to pass through a 425-μm-mesh screen. Colony-forming units in the dry preparation were determined by the dilution-plate method on modified TME medium described previously (13).

Soil infestation with *S. rolfsii* and *G. virens*. A loamy sand of pH 6.6 and 0.6% organic matter was used in all experiments. Soil was collected from a fallow plot 1 mo before the experiments were initiated. The untreated soil was kept moist on a greenhouse bench and passed through a 2.0-mm-mesh screen before use.

Portions (150 g) of moist (−30 kPa) soil were placed in 400-ml beakers and infested with sclerotia and fermenter biomass of Gl-3. Sclerotia were added at a rate of 200 sclerotia per 10 g of dry soil in all experiments. Strain Gl-3 was added to soil concomitantly with sclerotia as dry biomass mixed with Pyrax (a pyrophyllite, hydrous aluminum silicate, pH 7.0, R. T. Vanderbilt Co., Norwalk, CT). Pyrax does not provide any known nutrients. The Pyrax-fermenter biomass was added to soil at 3 mg/g of soil. The biomass was adjusted before addition to soil to provide various concentrations of the antagonist specified in each experiment.

After thorough mixing, soils containing sclerotia and Gl-3 biomass were transferred to 90- × 25-mm petri plates. The plates were placed in large polypropylene trays, covered with aluminum foil to maintain soil moisture and exclude light, and incubated at room temperature.

Colonization of sclerotia by *G. virens* and their germination. After various time intervals specified for each experiment, the soil from each replicate plate was washed with running tap water on a 850-μm-mesh screen. Three hundred sclerotia were recovered individually with fine forceps from the residue that remained on the screen, and these were divided into two groups of 150 sclerotia each. One group from each replicate was surface-disinfested with 1.0% sodium hypochlorite for 1 min and washed with sterile distilled water. Sclerotia of the second group were not treated.

Fifty sclerotia (untreated and surface-disinfested) from each of five replicates were plated on five plates (10 sclerotia per plate) of modified TME medium to assess colonization of sclerotia by *G. virens*. Modified TME contained one-third the usual concentrations of chloroneb and nystatin of the TME medium described by Papavizas and Lumsden (13). *G. virens* is more sensitive to chloroneb and nystatin than *Trichoderma* spp. (Papavizas, *unpublished*). The TME plates were incubated under continuous fluorescent light and colonization was assessed visually after 8 days of incubation by counting the number of sclerotia supporting growth of Gl-3.

Sclerotial germination was determined by plating out 50 untreated and surface-treated sclerotia per replicate (10 sclerotia per plate) on PDA containing benomyl at 5.0 mg a.i./L to prevent growth of Gl-3 on the sclerotia and four antibacterial antibiotics (as in the original TME medium) to prevent bacterial growth around germinating sclerotia. Plates were incubated at room temperature and germination of sclerotia was assessed visually after 5 or 6 days of incubation.

Sclerotial infectivity. Snap bean seed (cultivar Bush Blue Lake 274) were treated with metalaxyl, 2E at 0.4 g a.i./kg of seed, to prevent Pythium damping-off. Metalaxyl does not affect *S. rolfsii* and *G. virens* (Papavizas, *unpublished*). Plastic pots containing 900 g each of nonsterile soil were planted with 10 seeds each and incubated at 28 C under a 12-hr artificial light regimen (approximately 700 $\mu E\ m^{-2}\ sec^{-1}$).

Two systems were developed for testing the infectivity of untreated and surface-disinfested sclerotia of *S. rolfsii* after incubation in soil with the antagonist. In the first system, sclerotia were placed 2 cm below the soil surface in contact with the hypocotyl of 9-day-old snap bean plants (two sclerotia per plant) and covered with soil. Plants were incubated as above for an additional 15 days, and blight caused by *S. rolfsii* was assessed by counting the total healthy stand (plants were considered healthy if there was no visible evidence of the pathogen on the roots or hypocotyl).

In the second system, three 9-day-old snap bean hypocotyls were cut into 9-cm sections, lightly rinsed with distilled water, and placed on two sterile, moist sheets of filter paper in 150- × 25-mm petri plates. Ten sclerotia were placed on the hypocotyls in each plate at a distance of 2.5–3 cm from each other (10 sclerotia per plate, five plates per replicate). Plates were placed in large

TABLE 1. Colonization of sclerotia of *Sclerotium rolfsii* (Sr-1) by *Gliocladium virens* (Gl-3)[v] after burial for 7 days in soil, their germination, and their ability to cause blight on snap bean

Treatment of sclerotia[w]	Colonization[x] of sclerotia by *G. virens* (%)	Germination[y] of sclerotia (%)	Healthy plant stand at 15 days (%)
Sclerotia from soil with Gl-3			
Untreated	88 a[z]	4 b	100 a
Surface-disinfested	50 b	9 b	95 a
Sclerotia from soil without Gl-3			
Untreated	0 c	100 a	28 bc
Surface-disinfested	0 c	100 a	45 b
Sclerotia from PDA		100 a	6 c
Control (no sclerotia)			100 a

[v] Dry, ground fermenter biomass was mixed with Pyrax (Pyrax 9 g/1 g fermenter biomass) and added to soil together with the sclerotia to provide 6×10^3 colony-forming units per gram of soil.
[w] Treatment of sclerotia in soil, or after being recovered from soil, before placement (two sclerotia per plant) 2 cm below the soil surface in contact with hypocotyls of beans growing in soil in 10-cm-diameter pots.
[x] Colonization of untreated and surface-treated (1% sodium hypochlorite for 1 min) sclerotia was assayed on modified TME medium.
[y] Germination of sclerotia was assayed on PDA containing antibiotics and benomyl.
[z] Values in each column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

TABLE 2. Colonization of sclerotia of *Sclerotium rolfsii* (Sr-1) by *Gliocladium virens* (Gl-3) after burial for 5 days in soil, their germinability, and their ability to cause lesions on excised snap bean hypocotyls

Concentration of Gl-3 in soil in which sclerotia were incubated (cfu/g soil)[v]	Colonization[w] of sclerotia by *G. virens* (%)	Germination[x] of sclerotia after burial in soil for 5 days (%)	Infective sclerotia[y] (%)
Control (no Gl-3)			
Sclerotia untreated	1 c[z]	100 a	100 a
Sclerotia surface-disinfested	1 c	100 a	100 a
3×10^3			
Sclerotia untreated	89 ab	29 b	0 b
Sclerotia surface-disinfested	73 b	27 b	0 b
6×10^3			
Sclerotia untreated	100 a	5 c	0 b
Sclerotia surface-disinfested	86 ab	4 cd	0 b
9×10^3			
Sclerotia untreated	100 a	0 d	0 b
Sclerotia surface-disinfested	97 a	0 d	0 b

[v] Dry, ground fermenter biomass of Gl-3 was mixed with Pyrax (Pyrax 9 g/1 g fermentor biomass) and added to soil together with sclerotia to provide the indicated concentrations (colony-forming units per gram soil, cfu/g).
[w] Colonization of untreated and surface-treated (1% NaClO for 1 min) sclerotia was assayed on modified TME medium.
[x] Germination of sclerotia was assayed on potato-dextrose agar containing antibiotics and benomyl.
[y] Infectivity was determined by placing sclerotia on 9-cm sections of 9-day-old bean hypocotyls in 150- × 25-mm petri plates.
[z] Values in each column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

polypropylene trays, covered with aluminum foil, and incubated in the dark at 26 C. After 3 days of incubation, the excised hypocotyls were examined for infection by the attached sclerotia. Infectivity was assessed as percentage of sclerotia that germinated and their germ tubes caused visible lesions.

Statistical analyses. All tests, performed twice with five replicates, were arranged in randomized complete block designs and contained appropriate controls. Mean separations were performed with Duncan's multiple range test with the Statistical Analysis System computer program (SAS Institute, Inc., Cary, NC). Since, in all cases, the second test for each experiment was identical with the first, only one test (five replicates) was analyzed statistically.

RESULTS

Sclerotial infectivity tested with whole plants. In the sclerotial infectivity assay with whole snap bean plants, there was a significant effect ($P \leq 0.05$) of colonization by *G. virens* of sclerotia in soil on their infectivity as demonstrated by the high healthy plant stand when colonized sclerotia were placed into contact with bean hypocotyls (Table 1). With strain Sr-1, which has small, tan sclerotia, there was a significant negative correlation ($P \leq 0.05$) between colonization by strain Gl-3 and germination ($r \leq 0.91$) and infection ($r \leq 0.95$). More than 90% of sclerotia recovered from soil amended with Gl-3, regardless of surface treatment, failed to germinate on the medium selective for *Sclerotium*. In contrast, 100% of the sclerotia from the soil without Gl-3 and from PDA germinated on agar.

Sclerotial infectivity tested with excised hypocotyls. The effect of various concentrations of fermenter biomass of strain Gl-3 on sclerotial germinability and infectivity with excised hypocotyls was studied by adding to soil Pyrax-fermenter biomass mixtures to achieve 0, 3×10^3, 6×10^3, and 9×10^3 cfu/g of soil and exposing sclerotia of strain Sr-1 to the antagonist in soil for 5 days only (Table 2). Only 1% of sclerotia from control soil, surface disinfested or not disinfested, were colonized by *G. virens*. Sclerotia from this treatment were 100% germinable and infective. However, sclerotia from soil amended with the lowest Gl-3 concentration, including those that were surface-disinfested, completely lost their infectivity. Although approximately 25% of the sclerotia from the low Gl-3 concentration (3×10^3) germinated, there was no infection on the hypocotyls. Almost all sclerotia from soil amended with the two high Gl-3 concentrations were colonized by Gl-3 and lost their ability to germinate.

A second test was performed to study the effect of length of incubation of Sr-1 sclerotia in soil amended with two Gl-3 concentrations (0, 3×10^3, 6×10^3). Sclerotia were recovered as before after 3, 6, 12, and 18 days, and untreated sclerotia were used to assay colonization and germination on agar and infectivity on excised hypocotyls. After only 3 days in the soil at the low Gl-3 concentration, 60% of the sclerotia lost their ability to cause lesions on hypocotyls despite the fact that 90% of them were germinable (Fig. 1). After 6 days, only 3 and 0% of the sclerotia from the mid and high Gl-3 concentration, respectively, were infective. Intact sclerotia could not be recovered after 12 and 18 days. After 3 days, more than 90% of the sclerotia were colonized by Gl-3 at both concentrations. No colonization by *Gliocladium* or *Trichoderma* was observed on sclerotia from the control soil.

To determine whether strain Gl-3 of *G. virens* was effective on other strains of the pathogen, strain Sr-109 of *S. rolfsii* (medium size sclerotia) and strain Sr-3 (large sclerotia), both from peanuts, were incubated separately for 8, 16, 24, and 32 days in soil amended with Pyrax-fermenter biomass of Gl-3 at 0, 3×10^3, and 6×10^3 cfu/g for Sr-109, and at 0, 6×10^3, and 9×10^3 for Sr-3.

Ability to cause lesions on excised hypocotyls was the only criterion used with Sr-109 (Fig. 2). Sixty percent of the sclerotia recovered from soil amended with 3×10^3 cfu of Gl-3 per gram of soil and assayed without surface treatment were infective even after 32 days of exposure, at which time the test was terminated. About 39, 28, 22, and 13% of the sclerotia from soil amended with 6×10^3 cfu were infective after 8, 16, 24, and 32 days in soil, respectively. More than 80% of the sclerotia from control soil were infective at all times.

Although 100% of sclerotia of strain Sr-3 from soil amended with both concentrations of Gl-3 (6×10^3 and 9×10^3) were colonized by the antagonist at all times, their germinability was not reduced at all after 24 days and only slightly after 32 days in soil (Fig. 3). Only the 24- and 32-day assays are shown in Figure 3. Although all sclerotia were germinable, 73 and 42% were infective after 24 days at antagonistic inoculum densities of 6×10^3 and 9×10^3 cfu/g, respectively. No further reduction in ability to cause lesions on hypocotyls was observed at the 32-day assay, at which time the test was terminated. Sclerotia

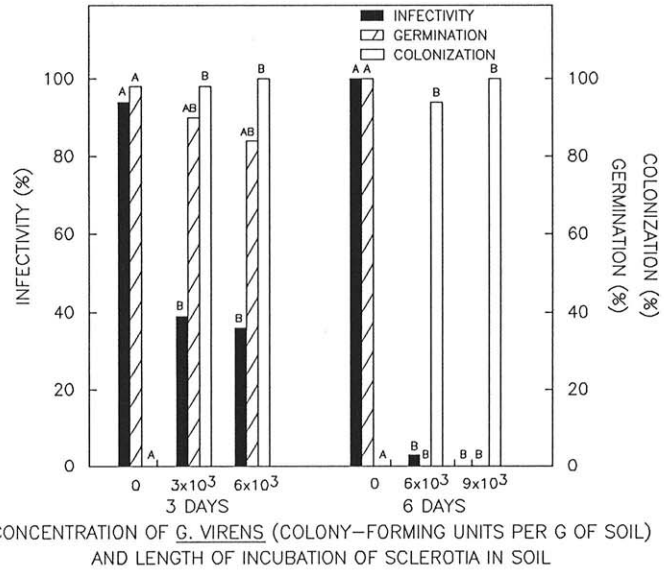

Fig. 1. Colonization of sclerotia of *Sclerotium rolfsii* (strain Sr-1) by *Gliocladium virens* (strain Gl-3), their germinability on agar, and their ability to cause lesions on excised snap bean plant hypocotyls after burial of sclerotia for 3 and 6 days in soil amended with the biocontrol agent at two concentrations. Within a given parameter and incubation period, bars with the same letters do not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test.

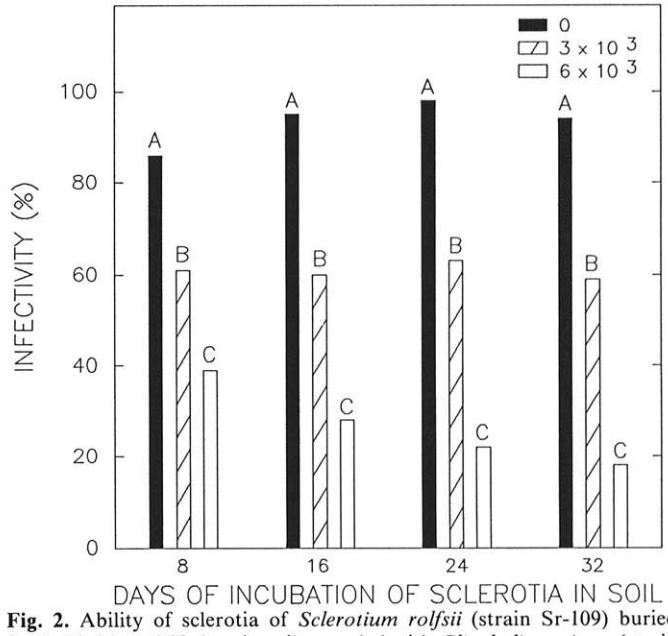

Fig. 2. Ability of sclerotia of *Sclerotium rolfsii* (strain Sr-109) buried for 8, 16, 24, and 32 days in soil amended with *Gliocladium virens* (strain Gl-3) at two concentrations to cause lesions on excised snapbean hypocotyls.

of Sr-3 from control soil were 100% germinable and more than 80% infective at all times.

DISCUSSION

The objective of this study was to develop a system to assay the infectivity of sclerotia of *S. rolfsii* that were exposed to a biocontrol agent (*G. virens*, strain Gl-3) in soil and to compare this characteristic with the colonization of sclerotia by the biocontrol agent and with sclerotial germinability. This was accomplished by recovering sclerotia from soil infested with the biocontrol agent and testing them for the three characteristics (infectivity, germinability, and colonization) rather than allowing plant roots to come into contact with sclerotia in soil where it is very difficult to observe the behavior of individual sclerotia. The infectivity assay on excised hypocotyls of snap bean plants takes only 2 or 3 days to complete after the sclerotia are recovered from soil. The system with excised hypocotyls can easily be used to screen strains of microorganisms for biocontrol capabilities against *S. rolfsii* and other sclerotial pathogens in the laboratory under more natural conditions than those prevailing in agar assays.

Fresh sclerotia of strain Sr-1 (small sclerotia) of *S. rolfsii* produced on PDA were rapidly degraded in soil by *G. virens* (Gl-3), which in previous studies (12) provided excellent control of *S. rolfsii* in field soil. This appears to be in contrast with the findings of Henis and Papavizas (7), who observed that sclerotia from PDA neither germinated nor were degraded by *T. harzianum* in field soil. Henis and Papavizas used sclerotia of strain Sr-3 of *S. rolfsii*, which, even in the present studies with a more effective biocontrol agent (Gl-3) than *T. harzianum*, were not degraded in soil even after 32 days of incubation, despite the fact that the Sr-3 sclerotia were colonized 100% by Gl-3. Resistance of sclerotia of this strain (large sclerotia) may be because the darker sclerotia of Sr-3 are up to 15–20 times larger in volume than those of strain Sr-1, and it may be more difficult to destroy the entire sclerotial contents. Sclerotia of Sr-3 appear to contain more melanin than those of Sr-1. The presence of melanin in the rind of Sr-3 sclerotia could account for their resistance to the degrading enzymes of *G. virens* (2,7).

Although all sclerotia of Sr-3 were colonized by *G. virens* after exposure to soil amended with strain Gl-3 for 24 and 32 days, 100% of colonized sclerotia germinated on the medium selective for *S. rolfsii* even when plated without surface treatment with sodium hypochlorite. Despite this, a considerable number of viable sclerotia did not cause lesions on excised hypocotyls (Fig. 3). This also was demonstrated in the tests with sclerotia of Sr-1 (Table 2, Fig. 1), suggesting three possibilities that are not necessarily mutually exclusive: 1) the antagonist on the surface of germinable sclerotia may serve as a physical barrier protecting the hypocotyls; 2) *G. virens* produces metabolites on the sclerotial-hypocotyl surface interface that prevent germination of sclerotia, infection of tissue, or both; and 3) the inoculum potential of the sclerotia is reduced because their vigor is impaired as a result of colonization by the antagonist.

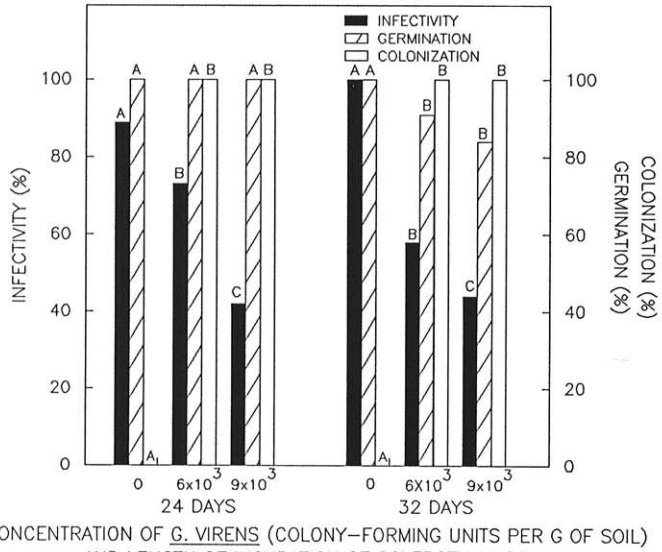

Fig 3. Colonization of sclerotia of *Sclerotium rolfsii* (strain Sr-3) by *Gliocladium virens* (strain Gl-3), their germinability on agar, and their ability to cause lesions on excised snap bean plant hypocotyls after burial of sclerotia for 24 and 32 days in soil amended with the biocontrol agent at two concentrations. Within a given parameter and incubation period, bars with the same letters do not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test.

We suggest, therefore, that there were at least two mechanisms of biological control preventing infection of excised hypocotyls or whole plants by sclerotia that had been exposed to *G. virens* in field soil. Sclerotia of *S. rolfsii*, at least those of Sr-1, and to a lesser degree those of Sr-3 and Sr-109, may be attacked and degraded by the mycoparasitic action of *G. virens* in soil in the absence of the host. We also suggest that, in addition to mycoparasitism, antibiosis may be involved in the degradation of sclerotia by *G. virens* in soil and in the prevention of infection of hypocotyls by germinable sclerotia. *Gliocladium* spp. are known to produce various toxic metabolites and antibiotics, as well as various enzymes, such as exo- and endoglucanases, cellobiase, and chitinase (10). However, antibiotic production by *G. virens* on sclerotia in soil has not been demonstrated.

Differences in susceptibility to degradation in soil and infectivity of sclerotia of various strains of *S. rolfsii* suggest that there is considerable specificity in biocontrol due to differences in susceptibility of strains of the same pathogen to a single biocontrol strain, in addition to specificity due to various strains of a biocontrol agent. Either way, specificity between a biocontrol agent and a target pathogen is an obstacle to biocontrol that should be considered when studying biocontrol in the laboratory, or attempting to use biocontrol agents in an applied setting.

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