Potential of *Gliocladium roseum* for Biocontrol of *Verticillium dahliae*

A. P. Keinath, D. R. Fravel, and G. C. Papavizas

U. S. Agricultural Research Service, Plant Sciences Institute, Biocontrol of Plant Diseases Laboratory, Beltsville, MD 20705.

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ABSTRACT

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A bioassay system was developed to evaluate fungi for biocontrol potential against *Verticillium dahliae*. Microsclerotia of *V. dahliae* were embedded in squares of nylon mesh. The squares were buried in nonsterile soil amended with preparations of the test fungi grown on vermiculite/wheat bran (3:1, w/w). After recovery from soil, viability of the microsclerotia was assessed by observing growth of *V. dahliae* on agar medium. *Gliocladium roseum* was the most effective species among 11 fungi tested. No viable microsclerotia were recovered from soils amended with *G. roseum* at a rate of 1.0% (w/w). The isolate of *G. roseum* used, the ratio of vermiculite to bran in the preparation, and the rate of antagonist preparation added to soil all significantly affected the viability of microsclerotia. The most effective isolate, *G. roseum* 632, significantly reduced the viability of microsclerotia by ≥36% when grown on vermiculite/bran and added to soil at a rate ≥0.01% (w/w). Three isolates of *G. roseum*, 632, W14, and Std, were effective in three different soils at −10 and −100 kPa soil matric potential. *G. roseum* has potential as a biocontrol agent of *V. dahliae*.

Additional keywords: *Gliocladium catenulatum, Gliocladium virens, Minimedusa sp., Penicillium* sp., potato early dying, *Talaromyces flavus, Trichoderma* spp.

Potato (*Solanum tuberosum* L.) early dying disease occurs in all potato-producing areas of the United States, resulting in substantial yield losses (15). *Verticillium dahliae* Kleb., which incites Verticillium wilt of potato and other crops, often is the major pathogen involved in this disease complex (9,15). This pathogen can persist up to 10 yr in the absence of potato. Current control measures are limited to the use of potato cultivars with varied degrees of tolerance to *V. dahliae* and fumigation with broad-spectrum biocides, often at rates up to 900 L/ha. Because of concern about potential adverse environmental effects of fumi-

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gation and the lack of other alternatives, biocontrol may be useful for management of potato early dying disease.

Selection of a biocontrol agent is one of the most critical steps in developing biocontrol (2,8). To devise a rational method for screening potential antagonists, it is helpful to consider the epidemiology of the disease to be controlled. Verticillium wilt of potato is a monocyclic disease. Microsclerotia of *V. dahliae* initially infect potato root tips (14), and infection can occur at any time throughout the growing season. Because of the large fibrous potato root system, the difficulty of protecting moving infection courts, and the long period during which root tips would have to be protected from infection, we focused this study on selecting antagonists that could inactivate microsclerotia before infection.

The objectives of this research were to screen potential fungal antagonists for control of *V. dahliae* in nonsterile soil and to determine the range of conditions under which the most promising biocontrol agent, *Gliocladium roseum* Bain. (teleomorph *Nectria ochraleuca* (Schw.) Berk.), could function.

MATERIALS AND METHODS

Bioassay system. The following procedure was used in all experiments, except where noted. *V. dahliae*, originally isolated from eggplant, was grown for ≥4 wk at 21 C on Czapek solution agar (Difco Laboratories, Detroit, MI). Microsclerotia were removed from the top 2 mm of agar, comminuted in a blender in sterile distilled water, and vacuum-infiltrated into 0.5-cm squares of 48-μm-mesh polyamide nylon (Tekto Inc., Briarcliff, NY) (10) to yield 15–20 microsclerotia per mesh square.

Potential biocontrol fungi were grown on a 3:1 (w/w) mixture of vermiculite (grade 4, W. R. Grace Company, Cambridge, MA) and wheat bran (ground to pass through a 2-mm sieve). The mixture was moistened with distilled water (35 ml of water per 12 g) and autoclaved 30 min on each of 2 consecutive days. Spore and mycelial suspensions were prepared from 2-wk-old potato-dextrose agar (PDA) (Difco) slant cultures. Vermiculite/bran was infested with these suspensions and incubated for 1 wk at 21 C.

The antagonist preparation was added to a nonsterile Galestown gravely loamy sand (pH 5.8 [H_2O], 77.8% sand, 9.6% clay, 12.6% silt, 1.1% organic matter) collected in Beltsville, MD, and adjusted to a soil matric potential of −30 kPa. Uninoculated vermiculite/bran and unamended soil were used as controls. A randomized complete block design with four replicates was used. Each replicate consisted of five mesh squares buried in 31- × 10-mm plastic petri dishes with approximately 10 g of soil per plate. The dishes were covered with moist paper towels, placed in a plastic bag, and held at 21 C for 2–6 wk. The mesh squares were retrieved, washed in sterile distilled water, blotted dry, and placed in petri dishes on polygalacturonic acid-soil extract medium (6), containing 25 ml of soil extract, 975 ml of distilled water, 4.0 g of K_2HPO_4, 1.5 g of KH_2PO_4, 15 g of agar, 2.0 g of polygalacturonic acid, 0.05 g of streptomycin sulfate, 0.05 g of chlorotetracycline, and 0.05 g of chloramphenicol. The dishes were incubated at 21 C for 2 wk, when the mesh squares were examined microscopically for growth of *Verticillium* from viable microsclerotia. Viability was assessed as the production of new microsclerotia in the agar. Mycelial growth of *V. dahliae* on this low-nutrient medium was minimal and did not interfere with detection of microsclerotia. The number of mesh squares per dish with viable microsclerotia was recorded.

Screening fungal antagonists. Mesh squares with microsclerotia of *V. dahliae* were buried in soil amended with 10^5 colony-forming units (cfu) per gram dry soil of antagonist preparation (0.16–1.4%, w/w preparation, depending on the isolate) prepared as described previously; dishes were held at 21 C for 7 wk. The experiment was repeated with 1.0% (w/w; based on oven-dry soil) antagonist preparation. Selected antagonists included *Talaromyces flavus* (Klöcker) Stolk & Samson, *Penicillium* sp., four isolates of *G. roseum*, two isolates of *Minimedusa* sp., and two unidentified fungi isolated from a potato stem and from soil, respectively.

Another screening was performed twice with 1% (w/w) antagonist preparation and was incubated for 2 wk. This screening included six isolates of *Trichoderma* spp., two isolates of *G. virens* Miller, Giddens, & Foster, three isolates of *G. roseum*, one isolate of *G. catenulatum* Gilm. & Abbott, and two additional unidentified fungi isolated from a potato stem and a potato root, respectively.

Effect of rates and ratio of bran medium on antagonism. Three different ratios of vermiculite/bran were compared for growth and biocontrol effectiveness of *G. roseum*. Ratios of 3:1, 11:1, and 35:1 (w/w) were infested with *G. roseum* and incubated for 1 wk. The number of colony-forming units of *G. roseum* was determined by dilution plating 0.1 g of vermiculite/bran onto quarter-strength PDA, with two replicate flasks and three dishes at the 10^{-5} dilution. Antagonist preparations were added to soil at rates of 1.0 and 0.1% (w/w), and mesh squares with microsclerotia of *V. dahliae* were incubated in the soil for 2 wk. A randomized complete block design with four replicates was used. The experiment was repeated.

Different soil amendment rates of antagonist preparation were compared at different incubation times. Vermiculite/bran, 3:1, was added to soil at 1.0, 0.1, or 0.01% (w/w), and mesh squares with microsclerotia of *V. dahliae* were incubated in the soil for 2, 4, or 6 wk. In another experiment, vermiculite/bran at 11:1 (w/w) was added to soil at 0.5, 0.2, 0.1, 0.05, or 0.01% (w/w), and mesh squares were incubated in soil for 2 or 6 wk. A randomized complete block design with four replicates was used. Both experiments were performed twice.

In the first two experiments, three isolates of *G. roseum* were used, whereas two of these isolates were used in the latter experiment. Isolate Std originated from soil planted to alfalfa in Tompkins County, NY (11); isolate W14 was obtained from the surface of a butternut squash fruit grown in NY; and isolate 632 was isolated from soil planted to potatoes at the Beltsville Agricultural Research Center.

Effect of soil type and soil matric potential on antagonism by *G. roseum*. Antagonism by three isolates of *G. roseum* was tested in three different soils held at soil matric potentials of −10 or −100 kPa. The three soils were a Hammonton loamy sand from Haleyville, NJ (pH 5.1 [H_2O], 89.5% sand, 4.1% clay, 6.4% silt, 0.4% organic matter), the Galestown gravely loamy sand used in all other experiments, and a Hatboro loamy sand (pH 4.5 [H_2O], 74.3% sand, 8.6% clay, 17.1% silt, 0.3% organic matter) also from Beltsville. Soil moisture contents at −10 and −100 kPa were determined with a soil pressure plate (Soilmoisture Equipment Co., Santa Barbara, CA), and soil moisture content was adjusted gravimetrically to these two soil matric potentials. Vermiculite/bran (11:1, w/w) colonized by *G. roseum* was added to the soils at a rate of 0.2% (w/w), and mesh squares with microsclerotia of *V. dahliae* were incubated in the soils for 2 or 6 wk. Petri dishes were sealed with Parafilm M to reduce evaporation. Soil moistures were redetermined at the conclusion

TABLE 1. Reduction in viability of microsclerotia of *Verticillium dahliae* in soil with three rates of vermiculite/bran preparation of three isolates of *Gliocladium roseum*

Treatment	Viable *Verticillium*[a]		
	0.01%[b]	0.1%	1.0%
Control[c]	4.71 ± 0.11[d]	4.83 ± 0.10	4.83 ± 0.08
G. roseum Std	4.04 ± 0.29	2.04 ± 0.24	0.00 ± 0.00
G. roseum W14	4.21 ± 0.23	1.92 ± 0.32	0.04 ± 0.00
G. roseum 632	3.00 ± 0.32	1.00 ± 0.24	0.00 ± 0.00
MSD[e]	0.67	0.60	0.11

[a] Each value is the mean of 24 replicates and is the number of mesh squares with viable microsclerotia of *V. dahliae* (five mesh squares per experimental unit). Data from the 2, 4, and 6 wk incubation periods were averaged, as there was no significant effect of time ($P = 0.5$).

[b] Vermiculite/bran (3:1, w/w) colonized by *G. roseum* was added to soil on a w/w basis.

[c] Control was vermiculite/bran not colonized by *G. roseum*.

[d] Standard error of the mean.

[e] Minimum significant difference, Waller-Duncan k-ratio t test ($k = 100$).

of the experiment. A randomized complete block design with four replicates was used. The experiment was repeated.

The effect of a range of soil matric potentials on antagonism was determined with the Galestown gravely loamy sand and two isolates of *G. roseum*. Two series of matric potentials were tested: −30, −50, −100, −300, and −500 kPa; and −30, −170, −370, and −670 kPa. Antagonist preparation (vermiculite/bran, 11:1) was added to soil at 0.1%, and mesh squares with microsclerotia were incubated for 2 wk. Petri dishes were sealed with Parafilm M to reduce evaporation. A randomized complete block design with four replicates was used.

Data analysis. Data were expressed as the number of mesh squares with viable *V. dahliae* per petri dish (experimental unit). When variances were homogeneous between repeated experiments (two-tailed F test, $P < 0.05$), data were pooled for analysis. Analysis of variance, orthogonal contrasts ($P < 0.05$), and the Waller-Duncan k-ratio t test ($k = 100$) were used to determine significant effects of treatments (SAS Institute Inc., Cary, NC).

RESULTS

Comparison of fungi for antagonism against *V. dahliae*. Five isolates of *G. roseum* and one isolate of *G. catenulatum* eliminated all viable microsclerotia on all mesh squares when tested in the bioassay system. In this system, two isolates of *G. roseum* were ineffective, with 54 and 90% of the mesh squares with viable *V. dahliae*. Percent survival of *V. dahliae* ranged from 50 to 100% for *T. flavus*, *Penicillium* sp., *Minimedusa* sp., *Trichoderma* spp., *G. virens*, and four unidentified fungi.

TABLE 2. Effect of vermiculite/bran ratio on antagonism of microsclerotia of *Verticillium dahliae* by three isolates of *Gliocladium roseum*[a]

Treatment	Viable *Verticillium*[b] 3:1[c]	11:1	35:1
Control[d]	4.50 ± 0.29[e]	4.25 ± 0.25	3.50 ± 0.65
G. roseum Std.	1.75 ± 0.48	2.50 ± 0.87	3.00 ± 0.41
G. roseum W14	0.50 ± 0.50	1.75 ± 0.63	3.50 ± 0.50
G. roseum 632	0.25 ± 0.25	0.25 ± 0.25	3.25 ± 0.48
MSD[f]	1.28	2.14	NS[g]

[a] Antagonist preparation was added to soil at 0.1% (w/w), and mesh squares with microsclerotia were incubated in soil for 2 wk.
[b] Values are mean number of mesh squares with viable microsclerotia of *V. dahliae* based on four replicates (five mesh squares per experimental unit).
[c] Ratio of vermiculite to bran in the antagonist preparation.
[d] Control was vermiculite/bran not colonized by *G. roseum*.
[e] Standard error of the mean.
[f] Minimum significant difference, Waller-Duncan k-ratio t test ($k = 100$).
[g] Not significantly different at $P < 0.05$.

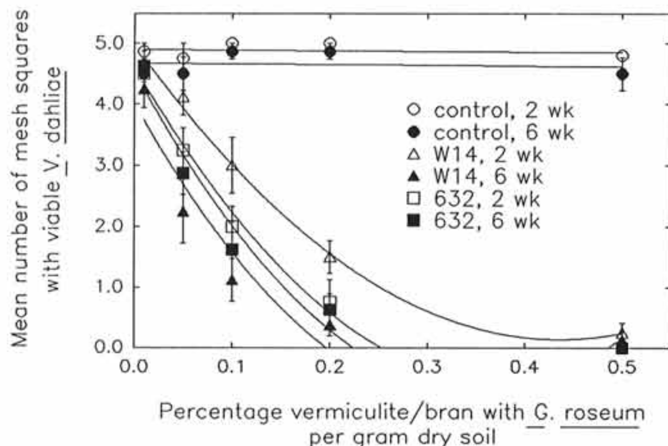

Fig. 1. Reduction in viability of microsclerotia of *V. dahliae* after 2 or 6 wk of incubation in nonsterile soil amended with different rates of *G. roseum* grown on vermiculite/bran (11:1 w/w). Each data point is the mean of eight replicates. Vertical bars give one standard error of the mean.

Effect of rates and ratio of bran medium on antagonism. *G. roseum* significantly reduced the viability of microsclerotia of *V. dahliae* when added to soil in vermiculite/bran medium (Table 1). The rate of preparation added to soil influenced the survival of microsclerotia of *V. dahliae*. At 1%, w/w, all three isolates of *G. roseum* used were equally effective. At 0.1%, all three isolates were still effective compared with vermiculite/bran without *G. roseum*, but isolate 632 was more effective (Waller-Duncan k-ratio t test, $k = 100$) than the other two isolates. At 0.01%, only isolate 632 significantly reduced the viability of microsclerotia. There was no significant effect of time of incubation (2, 4, or 6 wk) on reduction of viability (data not shown).

The ratio of vermiculite to bran in the antagonist preparation significantly ($P < 0.001$) influenced the effectiveness of *G. roseum*. The rate of application of antagonist preparation (1.0 or 0.1%) and the isolate also significantly affected biocontrol, as shown by significant interactions among ratio, rate, and treatment ($P < 0.01$). At a rate of 1% antagonist preparation added to soil, all three isolates significantly reduced the viability of the microsclerotia at all three ratios of vermiculite to bran (data not shown). At a rate of 0.1% (Table 2), there was no significant reduction in viability of the microsclerotia with antagonist preparation of vermiculite/bran at 35:1, w/w. At ratios of 11:1 and 3:1, all isolates significantly reduced the viability of the microsclerotia ($P < 0.01$) compared with noncolonized vermiculite/bran. With both ratios, isolate 632 was more effective than isolate Std ($P < 0.05$).

Colony-forming units of *G. roseum* grown on vermiculite/bran ranged from 1.9×10^7/g for isolate Std grown on 35:1 to 3.8×10^8/g for isolate 632 grown on 3:1. The ratio 35:1 supported a significantly lower number of colony-forming units for all isolates than the two higher ratios, and isolate 632 produced significantly higher numbers of colony-forming units than the other two isolates on all vermiculite/bran ratios (Waller-Duncan k-ratio t test, $k = 100$).

G. roseum grown on vermiculite/bran (11:1, w/w) significantly reduced the viability of microsclerotia of *V. dahliae* when added to soil at rates from 0.5 to 0.01% (Fig. 1). With both isolates, viability decreased nonlinearly with the square of the rate. For isolate 632, there were no differences in intercept, slope, or second-order regression coefficient between 2 and 6 wk of incubation.

TABLE 3. Reduction in viability of microsclerotia of *Verticillium dahliae* by three isolates of *Gliocladium roseum* in three soils[a]

Treatment	Viable *Verticillium*: Loamy sand[b] Hammonton	Loamy sand[b] Hatboro	Gravely loamy sand[c] Ψ_m (kPa) −10	Gravely loamy sand[c] Ψ_m (kPa) −100
Unamended	4.73 ± 0.17	4.59 ± 0.19	4.92 ± 0.08	4.85 ± 0.08
Control[d]	4.81 ± 0.16	4.78 ± 0.09	4.60 ± 0.15	4.58 ± 0.19
G. roseum Std	0.31 ± 0.11	2.41 ± 0.26	0.86 ± 0.31	2.06 ± 0.32
G. roseum W14	0.06 ± 0.06	0.41 ± 0.11	0.63 ± 0.15	0.38 ± 0.18
G. roseum 632	0.03 ± 0.03	0.41 ± 0.13	0.87 ± 0.26	0.56 ± 0.22
MSD[e]	0.30	0.43	0.51	0.53

[a] Antagonist preparation (vermiculite/bran, 11:1) was added to soil at 0.2% (w/w), and mesh squares with microsclerotia were incubated in soil for 2 or 6 wk.
[b] Values are mean number of mesh squares with viable microsclerotia of *V. dahliae* ± standard errors. Values are means of 32 replicates, summed over matric potentials (no significant effect) and time (no interaction with treatment).
[c] Values are mean number of mesh squares with viable microsclerotia of *V. dahliae* ± standard errors. Values are means of 16 replicates, summed over time (no interaction with treatment).
[d] Control was vermiculite/bran not colonized by *G. roseum*.
[e] Minimum significant difference, Waller-Duncan k-ratio t test ($k = 100$).

However with isolate W14, all three values were significantly lower (t test, $P < 0.001$) for 6 wk than for 2 wk.

Effect of soil type and matric potential on antagonism. *G. roseum* was effective at reducing viability of microsclerotia in three different soils at two matric potentials (Table 3). In general, there were few differences among soils or between matric potentials of −10 and −100 kPa. The only significant difference between the two matric potentials was observed in the Galestown gravely loamy sand with isolate Std. Although this isolate significantly reduced viability of microsclerotia compared with the uninoculated preparation, it also was significantly less effective ($P < 0.01$) than isolates W14 and 632 at −100 kPa. This intermediate level of biocontrol also was observed with isolate Std in the Hatboro loamy sand.

Reduction of viability of microsclerotia by two isolates of *G. roseum*, W14 and 632, was examined over a series of matric potentials, ranging from −30 to −500 kPa. Within this range, *G. roseum* significantly reduced viability compared with uninoculated vermiculite/bran and with unamended soil (Waller-Duncan k-ratio t test, $k = 100$) at all matric potentials. Although there were differences in the level of biocontrol observed at different matric potentials, *G. roseum* was effective over the entire range of soil moistures.

DISCUSSION

G. roseum has potential for use as a biocontrol agent against *V. dahliae*. Three different isolates of *G. roseum* reduced the viability of microsclerotia of *V. dahliae* by as much as 100% when added to nonsterile soil. All isolates of *G. roseum* were effective in three soils representing different textural classes, including gravely loamy sand and loamy sand, soil types in which potatoes are grown in the United States. Where potatoes are grown with irrigation, the possibility of managing water to favor a biocontrol agent exists. However, *G. roseum* was able to control *V. dahliae* over a range of matric potentials, so matric potential does not appear to be a critical edaphic factor to consider in this biocontrol system.

G. roseum is a biocontrol agent of several soilborne phytopathogenic fungi that produce melanized resting structures. For example, it was the fungus most frequently isolated from sclerotia of *Phymatotrichum omnivorum* buried in several different soils in Texas (7), and it was observed frequently on the sclerotia of *Botrytis aclada* in Britain (18). In Canada, the isolation of *G. roseum* from strawberry plants was negatively correlated with the recovery of *V. dahliae* from the same plants (5). *G. roseum* has been used as a biocontrol agent against *Phomopsis sclerotioides* on greenhouse-grown cucumbers in Switzerland (12).

Biocontrol by *G. roseum* operates by several different mechanisms. This antagonist is a mycoparasite of *P. omnivorum* (7). In vitro, *G. roseum* produced antibiotics against *B. aclada* (13). Further, *G. roseum* appeared to compete effectively against *V. dahliae* for infection sites in the crowns of strawberries (5). In our system, *G. roseum* reduced the viability of microsclerotia of *V. dahliae*. Although the mechanism of this antagonism is not known currently, some microsclerotia exposed to *G. roseum* appeared degraded (A. P. Keinath and D. R. Fravel, *unpublished*). However, it is possible that the microsclerotia remained viable, but germination was inhibited by *G. roseum*. The potential of *G. roseum* to function as a biocontrol agent with a variety of mechanisms makes this antagonist a possible candidate for biocontrol of Verticillium wilt of potato.

Rate of application of the antagonist is often not considered when evaluating antagonists, yet it is an important consideration for the adoption of biocontrol (1). *G. roseum* was more effective with higher rates of antagonist preparation added to soil, e.g., 1%, w/w, than with lower rates. However, the most effective isolate was able to reduce the viability of microsclerotia by 36% when added to soil at a rate of 0.01% (= 224 kg/ha for 15-cm plow layer) of the vermiculite/bran preparation, which was moist (75% moisture content) when used. This preparation was selected for convenience of preparation and handling, not because it would be the most effective way to apply *G. roseum* in the field. Therefore, with an improved formulation of *G. roseum* and a greater understanding of the ecology of this antagonist, it should be possible to obtain the same or greater levels of control with a lower application rate.

In the initial screening of fungi for biocontrol potential, five isolates of *G. roseum* and an isolate of *G. catenulatum*, a closely related species (4), were equally effective when added to soil at a rate of 1.0%. When three isolates of *G. roseum* were tested in a series of experiments at different rates, in different soils, and at different matric potentials, some differences among the isolates were apparent. In general, isolate Std was the least effective isolate, isolate 632 was the most effective, and isolate W14 was intermediate. The latter two isolates were highly effective under a variety of conditions, indicating that it is possible to find isolates that are more effective than others. *G. roseum* and *G. catenulatum* also were the most effective antagonists screened against *P. omnivorum* when incorporated into soil on an inert carrier, and differences among isolates also were apparent in this system (8).

The population density of *G. roseum* added to soil may be important in regulating the level of biocontrol that can be achieved. Different formulations of the vermiculite/bran preparation, which contained different numbers of colony-forming units, and different rates of the preparation added to soil resulted in different percentage reductions in viability of microsclerotia. Larger reductions occurred with higher initial population densities of *G. roseum*.

G. roseum is a root surface colonizer of older roots (3,16,17) and may not be able to protect potato roots from infection by *V. dahliae*, which infects through root tips. We believe that *G. roseum* acts through reduction of microsclerotia viability. Destruction of initial inoculum is a feasible control approach, because *V. dahliae* has a monocyclic disease cycle. *G. roseum* could be applied to the soil before or at planting, or it could be applied to potato residues at the end of the season to possibly reduce the formation of microsclerotia in infected stems. Until tested in the field, the degree of biocontrol of potato early dying disease obtainable cannot be known. *G. roseum* may not completely control the Verticillium wilt component of potato early dying disease. However, *G. roseum* may be useful in combination with other control measures, such as tolerant cultivars, which also offer only partial control.

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