# Relevance of Mycoparasitism in the Biological Control of *Rhizoctonia solani* by *Gliocladium virens*

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## ABSTRACT

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Conidia of two strains of *Gliocladium virens* that were strongly parasitic to *Rhizoctonia solani* were irradiated with ultraviolet light and screened for mycoparasitism in dual culture with *R. solani*. Three mutants that showed no mycoparasitic activity were isolated from each strain. The selected mutants retained the same antibiotic complement as the parent strains.

Peat moss-Czapek's broth cultures of parent and mutant strains had similar efficacy as biocontrol agents of cotton seedling disease induced by *R. solani* and as antagonists of sclerotia of *R. solani* in soil. These results indicate that mycoparasitism is not a major mechanism in the biological control of *R. solani*-incited seedling disease by *G. virens*.

Additional key words: cotton, mutation, seedling disease.

Biological control of soilborne pathogens with fungal mycoparasites has been demonstrated in a number of instances (7,8,10,12,15). In some cases, the biocontrol agent has been isolated directly from propagules or mycelium of the pathogen (8,11,15), and it has been assumed that mycoparasitism is a major mechanism in the biocontrol process. With some of the *Trichoderma* spp., e.g., *T. harzianum* Rifai, this may well be the case, because substantial production of antifungal compounds by these fungi has not been demonstrated (4,13,16). Also, Elad et al (5) found that excretion of different concentrations of $\beta$-1-3-glucanase, chitinase, and cellulase by strains of *T. harzianum* correlated with the ability of the strains to parasitize different pathogens and control various diseases. Other mycoparasites have been shown to produce antifungal antibiotics in addition to their parasitic activity (1,6,8,11,17). *Gliocladium virens* Miller et al, for instance, produces the antibiotics viridin (2) and gliotoxin (18), which are active against some of the Hyphomycetes, and gliovirin (9), which is inhibitory to *Pythium ultimum* Trow and a *Phytophthora* sp. (9). It has also been demonstrated that the antibiotics of *G. virens* can kill the sclerotia of *Rhizoctonia solani* Kühn (1). Therefore, with *G. virens*, the role of mycoparasitism in the antagonistic process is uncertain.

The purpose of the present study was to ascertain the relevance of mycoparasitism in the biological control by *G. virens*, of cotton seedling disease induced by *R. solani*, and in the antagonism of *G. virens* to sclerotia of *R. solani* in soil.

## MATERIALS AND METHODS

**Production and characterization of mycoparasitic-deficient mutants.** Conidia washed from potato-dextrose agar (PDA) plates of *G. virens* strains G-1 and G-6 were suspended in sterile water (1 $\times$ 10$^6$ conidia per milliliter), and 10-ml volumes of suspension in glass petri dishes containing a stirring bar were irradiated while stirring, with 6 W · m$^{-2}$ of ultraviolet light (254 nm) for 55 sec. Serial dilutions to 5 $\times$ 10$^3$ conidia per milliliter were made, and 0.1-ml aliquots were spread over PDA plates containing 0.4 $\mu$g/ml of benomyl (BPDA) to restrict colony growth. Plates were incubated in the dark for 96 hr at 25 C, then colonies were transferred at

random to the periphery of soil extract agar plates. Soil extract agar (SEA) consisted of an aqueous extract of an equal volume of soil with 0.1% glucose and 2% agar added. These plates were seeded in the center with PDA plugs containing *R. solani* and incubated for 48 hr at 25 C. The plates were then examined microscopically in areas where the hyphae of *R. solani* and those of the putative mutants had grown together for evidence of mycoparasitic activity. Those mutants showing no mycoparasitic activity were retained, and their growth rates were compared with those of the parent strains. SEA plugs containing parent and mutant strains were placed on SEA plates, and radial growth was measured after 24 and 48 hr of incubation at 25 C.

**Antibiotic production by parent and mutant strains.** Parent strains and mycoparasitic-deficient mutants of *G. virens* were assayed for antibiotic production by placing mycelial plugs on BPDA plates, incubating the cultures for 36 hr, then sprinkling the agar surface evenly with air-dried and ground wheat bran culture of *R. solani* (wheat bran-water, 1:1, by weight). After 24 and 48 hr the cultures were examined for evidence of mycelial free zones around the plugs of *G. virens*.

Potato-dextrose broth cultures (100 ml) of parent and mutant strains of *G. virens* were shake-incubated at 25 C for 4 days, and then the culture filtrates were adjusted to pH 3.5 and extracted with equal volumes of chloroform. The extracts were concentrated to 1 ml in vacuo, and 25-$\mu$l volumes were spotted along with authentic samples of gliotoxin and viridin on silica gel GF plates (J. T. Baker Chemical Co., Phillipsburg, NJ) buffered with 0.05 M oxalic acid-NaOH to pH 4.0. The plates were developed two dimensionally in chloroform-acetone-formic acid (70:28:2) and ethyl acetate-formic acid (98:2). Chromatograms were observed under UV light (254 nm) for spots with $R_f$ values corresponding to those obtained with authentic samples.

**Efficacy of mycoparasitic-deficient mutants as suppressants of cotton seedling disease.** Parent and mutant strains of *G. virens* were cultured 1 wk on a peat moss-Czapek's broth medium (PMCZB), air dried, and ground to pass through a 20-mesh sieve (8). The cultures were stored at 5 C until used. Comparison of the parent and mycoparasitic-deficient strains as suppressants of cotton seedling disease, induced by *R. solani*, was made in cotton field soil infested with 0.7 g of air-dried wheat bran culture of *R. solani* per 45 kg of soil. Seeds of cotton (*Gossypium hirsutum* L. 'Stoneville 213') were planted in the infested soil or in uninfested soil, and parent or mutant inoculum of *G. virens* was added as an

in-furrow treatment at the rate of 8 g/m of row. The tests were carried out in soil flats in growth chambers at 26 C day and 18 C night temperatures and a 14-hr photoperiod. After 2 wk, the numbers of damped-off cotton seedlings were counted. Each treatment was replicated four times, and the experiment was done twice with similar results.

**Antagonism of mycoparasitic-deficient mutants to sclerotia of *R. solani* in natural soil.** Sclerotia of *R. solani* grown in sand-cornmeal culture (14) were incorporated into natural field soil at the rate of 1% by weight. Air-dried PMCZB cultures of the parent or mutant isolates were added to 100-g lots of infested soil, with mixing, at the rate of 1% by weight and placed in covered glass dishes. Controls were infested with sterile peat moss. The moisture level of each sample was adjusted to 15% by weight (corresponding to −0.15 bar), and the mixtures were incubated at 25 C for 3 wk. At the end of this period, a 25-g lot from each sample was washed over 1-mm-mesh and 500-μm-mesh sieves and the retentate from the 500-μm-mesh sieve was transferred to 100 ml of molten (45–50 C) 2% water agar. The agar was dispensed in 10-ml aliquots into petri dishes. The plates were incubated at 25 C, and after 24 and 48 hr, counts of the number of viable propagules of *R. solani* were made. Each treatment was replicated three times, and the experiment was done twice with similar results.

## RESULTS

Ultraviolet irradiation and in vitro screening of surviving colonies of *G. virens* for mycoparasitic activity resulted in the isolation of three mycoparasitic-deficient mutants from strain G-1, and three from strain G-6. All six mutants were characterized by the capacity of their hyphae to grow among and in close proximity to the hyphae of *R. solani* without the characteristic coiling around and penetration of host hyphae exhibited by the parent strains (Fig. 1). However, those host hyphae that grew into the older areas of the mutant colony were killed, and the hyphae were devoid of contents.

Comparison of the radial growth rates of the parent and mutant strains of *G. virens* showed that in the case of strain G-1, mutation resulted in radial growth rate reductions of 21, 36, and 52% for G-1-62, G-1-10, and G-1-42, respectively. Mutation of strain G-6 resulted in a radial growth rate reduction of 64% for strain G-6-57, whereas the growth rates of both G-6-15 and G-6-2 exceeded that of the parent strain by 10 and 13%, respectively.

The percentages of damped-off cotton seedlings from seed planted in soil infested with *R. solani* and treated in-furrow with parent or mutant strains of *G. virens* are shown in Table 1. Treatment of seed with either parent strains or mycoparasitic-deficient mutants significantly reduced the number of damped-off seedlings when compared with the control. There were no significant differences in the number of damped-off seedlings between parent and mutant isolate treatments in all but one case. In-furrow treatment of cotton seed with parent or mutant isolates in uninfested soil did not adversely affect emergence, survival, or development of the seedlings.

Examination of BPDA plates seeded first with PDA plugs of parent and mutant strains and subsequently with air-dried inoculum of *R. solani* showed clear zones of equivalent size in the lawn of *R. solani* around both parent and mutant colonies. On thin-layer chromatography plates developed with chloroform-acetone-formic acid and ethyl acetate-formic acid, spots corresponding to viridin were found at $R_f$ 0.58 and $R_f$ 0.65, respectively, in extracts from either parent or mutant strains of isolate G-1. Extracts from parent and mutant strains of isolate G-6 gave spots at $R_f$ 0.52 and $R_f$ 0.61, corresponding to authentic gliotoxin. These results indicate that loss of mycoparasitic activity by the mutants was not accompanied by a loss in the antifungal activity characteristic of these fungal strains.

The numbers of viable sclerotia of *R. solani* recovered from unsterile soil after 3 wk of incubation with propagules of parent and mutant isolates of *G. virens* are shown in Table 2. Treatment of sclerotia-infested soil with parent and mutant isolates of *G. virens* reduced the number of viable sclerotia by an average of 85% when compared with the nontreated control. Differences in viability among sclerotia treated with parent or mutant strains of *G. virens* were small and inconsistent.
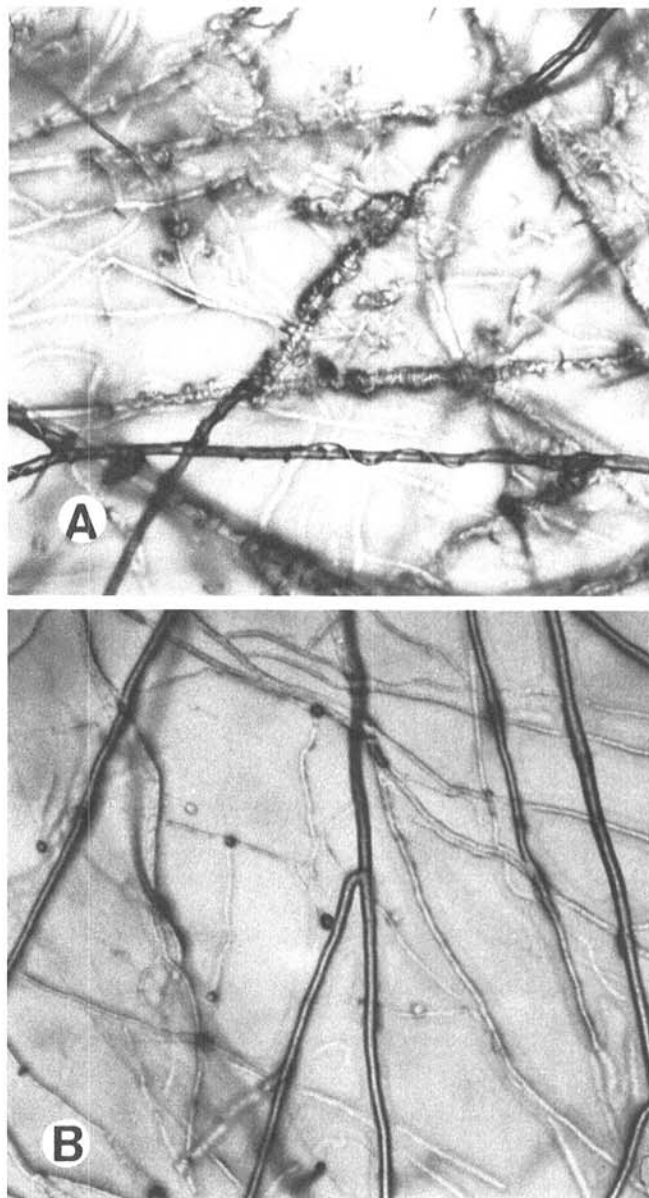


**Fig. 1. A,** Hyphae of a parent strain of the mycoparasite *Gliocladium virens* tightly coiled around the hyphae of its host *Rhizoctonia solani*. **B,** Hyphae of *R. solani* and a mycoparasitic-deficient mutant of *G. virens* growing in close proximity. Note the lack of contact and subsequent coiling that were observed in **A** (× 100).

TABLE 1. Effect of in-furrow treatment with mycoparasitic parent (G-1 and G-6) and nonmycoparasitic mutant strains of *Gliocladium virens* on damping-off of cotton seedlings in soil infested with *Rhizoctonia solani* in a growth chamber

| Treatment | Damping-off (%)[a] |
|---|---|
| Control | 74 ± 1[b] |
| G-1 | 16 ± 4 |
| G-1-10 | 26 ± 9 |
| G-1-42 | 22 ± 5 |
| G-1-62 | 17 ± 0 |
| G-6 | 12 ± 2.5 |
| G-6-2 | 25 ± 0 |
| G-6-15 | 19 ± 6.5 |
| G-6-57 | 12 ± 4.1 |

[a] Values are the means of four replicate plantings of 40 seeds each.
[b] Values following the ± symbol are standard deviations.

TABLE 2. Viable propagules of *Rhizoctonia solani* in soil treated with mycoparasitic parent (G-1 and G-6) and nonmycoparasitic mutant cultures of *Gliocladium virens* and incubated for 3 wk in a growth chamber

| Treatment[a] | Propagules/25 g of soil[b] |
| --- | --- |
| Control | 75 ± 2.9[c] |
| G-1 | 3 ± 2.3 |
| G-1-10 | 10 ± 2.9 |
| G-1-42 | 3 ± 2.5 |
| G-1-62 | 19 ± 3.7 |
| G-6 | 16 ± 1.0 |
| G-6-2 | 5 ± 1.0 |
| G-6-15 | 19 ± 4.4 |
| G-6-57 | 17 ± 5.0 |

[a] Treatments consisted of air-dried peat moss-Czapek's broth cultures of parent and mutant strains and a sterile air-dried peat moss control added to natural field soil at a rate of 1% by weight.

[b] Values are the means of three replicate samples.

[c] Numbers following the ± symbols are standard deviations.

## DISCUSSION

The frequency with which mycoparasitic-deficient mutants were obtained by the rather laborious process described shows that they occurred in relatively high numbers. This probably indicates that the mycoparasitic process is a complex one, and that any one of a number of mutations may result in its failure. Although these mutations allow the hyphae of the host and mycoparasite to intermingle in close proximity without direct parasitic attack of the host colony, it is interesting that host hyphae growing into the area occupied by the mycoparasite are often dead and devoid of contents. This phenomenon gives the appearance of antibiosis, and this interpretation is supported by the results of the bioassay for antibiotic activity and chromatography of culture extracts. Both parent and mutant strains were equally capable of producing antifungal compounds.

The results of the in-furrow treatment of cottonseed with parent and mycoparasitic-deficient strains of *G. virens* in soil infested with *R. solani* indicate that mycoparasitism is not necessary to effect disease control. If this is so, then some other mechanism(s) must be acting in that capacity.

The death of sclerotia of *R. solani* in similar numbers in soil treated with either parent or mycoparasitic-deficient mutants of *G. virens* indicates that mycoparasitism is not necessary for the reduction of pathogen propagule numbers in soil. The supposition that the mechanism involved in sclerotial death is antibiosis by *G. virens* is supported by the work of Aluko and Hering (1) who showed that the death of sclerotia of *R. solani* on potato tubers treated with *G. virens* was due to antibiotics produced by the mycoparasite. Similarly, the present results support their contention that sclerotial death was not due to mycoparasitism.

The results obtained from this study indicate that, in contrast to the studies done with *T. harzianum* (5), mycoparasitism by *G. virens* does not appear to be the primary mechanism in the biocontrol process. It may, however, function in concert with antibiosis to effect control. The relative importance of antibiosis in this process is currently being investigated.

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