New Forests **12:** 113–124, 1996.

Gliocladium virens in an alginate prill ineffective as a biological control of Fusarium root disease in container-grown Douglas-fir

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Received 6 December 1995; accepted in revised form 15 December 1995

Key words: *Fusarium oxysporum*, *Fusarium proliferatum*, seedling growth, seedling infection, germinants, virulence

Application. *Gliocladium virens*, commercially available as GL-21 microbial fungicide, was ineffective at controlling Fusarium root disease when applied as a top-dressing or incorporated into soilless growing medium at planting.

Abstract. An alginate prill formulation of *Gliocladium virens* (GL-21) was added as a top-dressing (54 g per m^2) or incorporated into medium (1.2 kg per m^3) used to grow Douglas-fir seedlings in styrofoam containers. Seedlings in the top-dress treatment were similar to control seedlings; infection and colonization by naturally-occurring *Fusarium* was unaffected by treatment. Incorporated *G. virens* reduced seedling growth and increased occurrence and colonization intensity of *Fusarium*. In a laboratory experiment, inoculating Douglas-fir seedlings with *G. virens* (10% w/w) prior to inoculation with *Fusarium* increased survival time when compared to concurrent inoculations of fungi.

Introduction

Fusarium spp. cause serious diseases of container-grown seedlings, especially Douglas-fir (*Pseudotsuga menziesii* var. *glauca* [Beissn.] Franco). Attempts to control these diseases by applying fungicidal drenches (Landis and others 1989) are usually unsuccessful (James and others 1987, 1988b; Williams 1989) probably because applied chemicals at sufficient concentrations fail to reach infection sites or root cortical cells where pathogens reside. Incorporating a slow-release, contact/systemic fungicide into soilless medium during planting was also ineffective at controlling Fusarium root disease (Dumroese and others 1990). Other fungicide problems include concern over pathogens developing resistance (Dekker 1976; Delp 1980), deteriorating water quality (Dumroese and others 1991), stricter environmental laws (Wilkinson 1989), and public pressure to reduce pesticide usage (Witt 1988).

Biological agents to control soilborne fungal pathogens have proven effective in agricultural crops (Papavizas 1985; Campbell 1989). Two important fungal genera used as biological control agents are *Trichoderma* and *Gliocladium* (Papavizas 1985). Selected strains of *Gliocladium virens* Miller, Giddens & Foster are effective biocontrol agents against Pythium damping-off on cotton (Howell 1991), cabbage and zinnia (Lumsden and Locke 1989), Rhizoctonia damping-off on white beans (Tu and Vaartaja 1981), and Phytophthora root rot on apples (Smith and others 1990). *Gliocladium virens* has been successfully introduced into soil (Lewis and Papavizas 1987) and soilless medium (Lumsden and Locke 1989) using alginate pellets with a bran substrate.

Gliocladium virens within an alginate pellet carrier (prill) has been commercially released as GL-21 microbial fungicide for control of *Pythium* and *Rhizoctonia* damping-off and root disease (Grace/Sierra, Fogelsville, PA). This *G. virens* formulation was evaluated as a possible biological control of Fusarium root disease in container-grown Douglas-fir seedlings. Our study objective was to determine effects of *G. virens* on seedling emergence and growth, and disease and root colonization by *Fusarium* spp.

Materials and methods

Greenhouse experiment

The greenhouse experiment was conducted at the University of Idaho Research Nursery, Moscow, Idaho (46.5°N latitude 117°W longitude). Two Douglas-fir seedlots, Bovill 3 and 8002, were surfaced sterilized in a solution of 2 parts bleach (5.25% sodium hypochlorite) with 3 parts tap water for 10 min (Wenny and Dumroese 1987), rinsed 48 h in running tap water and placed into cold-moist stratification at 3 °C for 28 days. After stratification, seeds were rinsed 24 h in running tap water prior to sowing.

Eighteen non-sterilized styroblock containers (each containing 160 individual 66 cm^3 cells) in which Douglas-fir seedlings were previously grown, were used in this experiment. Background inoculum levels of *Fusarium* on container surfaces were assayed. Two pieces of styroblock, each about 0.5 cm^3, were removed from the bottom drainage hole of 10 randomly selected cells per container, placed on a selective medium for *Fusarium* (Komada 1975), and incubated as described below. All containers were filled with 50:50 peat:vermiculite growing medium (Grace/Sierra, Portland, OR). In six containers the medium was top-dressed with the alginate prill formulation of *G. virens* (GL-21) at a rate of 54 g per m^2. The alginate prill consisted of sodium alginate, ground bran, and fungal chlamydospores. A mixture of

1.2 kg alginate prill per m^3 of the growing medium was added to another six containers. Prill application rates were recommended by the manufacturer. The remaining containers were kept as controls. Three seeds per cell were sown into the containers (3 styroblocks/treatment/seedlot) and covered with 0.5 cm silica grit. Filled styroblocks were randomly placed on greenhouse benches. Seedlings were grown using the standard growing regime of Wenny and Dumroese (1992). No fungicide applications were made.

Seedling emergence was determined 28 days after sowing. Seedlings that died throughout the experiment were assayed for *Fusarium*, as was a subsample of 6 seedlings lacking above ground disease symptoms (non-diseased) from each styroblock/treatment/seedlot combination collected seven months after sowing. Ten root tips from each non-diseased seedling were randomly removed and the entire root system of diseased seedlings was used. Roots were washed, surface sterilized in a 1:10 bleach:water solution for 1 min, rinsed in sterile water, and aseptically placed on a selective medium for *Fusarium* (Komada 1975). These were incubated under cool fluorescent, diurnal light (12 h photoperiod) at 22–24 °C for 7 days. Samples were assayed for infection; percentage root colonization of infected seedlings was calculated by counting root pieces colonized.

Seven months after sowing, 25 non-diseased seedlings from each styroblock/treatment/seedlot combination were measured for root collar diameter (RCD) and height from groundline to the tip of the terminal bud. Oven-dry weights were determined on 6 seedlings from each styroblock/treatment/seedlot combination after drying 48 h at 60 °C.

Laboratory experiment

A laboratory experiment was used to test potential biocontrol of *G. virens* (GL-21) against selected isolates of *Fusarium oxysporum* Schlecht. and *F. proliferatum* (Matsushima) Nirenberg. These isolates, all collected from container-grown Douglas-fir, were used because of their different ranges of pathogenicity, as determined by laboratory assays described by James (1996). There were five treatments including four combinations of *G. virens* with each of five *Fusarium* isolates: 1. *G. virens* inoculated alone, 2. *G. virens* and *Fusarium* isolates inoculated concurrently, 3. *Fusarium* isolates inoculated four days after inoculation with *G. virens*, 4. *Fusarium* isolates inoculated alone, and 5. control with neither organism.

Glass vials (23 ml) were filled to two-thirds capacity with sterile, soilless, 50:50 peat:vermiculite growing medium. *G. virens* alginate prill (0.22 g/vial) was mixed thoroughly at a rate of 10% w/w. The *Fusarium* inoculum was prepared using the techniques of Miles and Wilcoxon (1984), a method effective in previous pathogenicity tests on conifer seedlings (James and Gilligan

1984; James and others 1989). Perlite, an inert, inorganic, siliceous rock of volcanic origin, commonly used in potting mixtures, was the matrix for fungal growth. Moistened cornmeal and 1% potato dextrose agar (PDA) were added to perlite to provide nutrients for fungal growth. Perlite/cornmeal/PDA mixtures were inoculated with spore suspensions of selected *Fusarium* isolates and incubated in the dark at ≈24 °C for at least 24 days. Following incubation, inoculum was air dried and ground to a powder with mortar and pestle. Inoculum powder was added to vials and mixed thoroughly at a rate of 0.05 g (2.2% w/w). For test 5, non-inoculated perlite powder was added to vials at the same rate as *Fusarium* inoculum.

Bovill 3 seed, treated as described above, were placed on moistened filter paper (Whatman No. 3, Whatman International Ltd., Maidstone, England) within petri dishes and incubated in the dark at ≈24 °C. Germinants, selected when their primary root was at least 5 mm long, were carefully placed with their primary root pointing downward, one per vial. Four ml sterile water were added to each vial just before germinant placement. Each treatment consisted of 24 vials with germinants, incubated under cool fluorescent, diurnal light (12 h photoperiod) at 22–24 °C. Germinants within inoculation vials were examined daily for disease symptoms (damping-off and hypocotyl rot). Two ml sterile water were added to germinants surviving 7 days. Tests concluded at 14 days, and all surviving germinants were removed and examined for disease symptoms. Reisolations were made from all inoculated germinants onto Komada's medium to determine if infection by inoculated isolates occurred.

A numerical rating system was developed to compare levels of *Fusarium* virulence and protection afforded by *G. virens* on inoculated germinants (James 1996). The rating system awarded points based on duration of germinant survival within inoculated vials, occurrence and type of disease, reisolation of inoculated fungal isolate, and primary root growth within the vial. Germinants received one point for each day of survival from day 3–14 (12 points maximum), another 1, 2 or 4 points depending on type of disease apparent (maximum points if non-diseased), an additional 1, 2 or 4 points based on fungal reisolation (maximum points if no organism reisolated), and 3 points more if the root grew to the bottom of the vial. The range of possible points was 3–23, with higher point values reflecting less aggression by the tested isolate against the germinant. To convert points to a score in which higher numbers represent greater virulence, we devised a reciprocal rating. Ratings ranged from 0 to 100; 0 indicating germinants were uninfected within 14 days and 100 indicating all germinants killed within 3 days by the inoculated isolate.

For both greenhouse and laboratory experiments, dependent variables were analyzed with a one-way analysis of variance (Snedecor and Cochran 1989) and means compared with Tukey's HSD at the 0.05 level of significance.

Results and discussion

Inoculum for *Fusarium* infection in the greenhouse was provided by natural levels on seed and non-sterile styroblock containers and by natural introduction of airborne spores. About 60% of the cells in each styroblock container were colonized with *Fusarium* spp., with three quarters of the isolates identified as *F. proliferatum*. *Fusarium* inoculum was almost certainly not uniformly distributed throughout all treatments at the beginning of the experiment. However, our experience indicates a large percentage of seedlings become infected with *Fusarium* from natural inoculum, although not necessarily resulting in disease, by the end of the growth cycle (James and others 1987, 1988b). Therefore, we believe valid comparisons can be made among treatments based on prevalence of natural inoculum.

Douglas-fir seedling emergence was unaffected by treatment (data not shown). However, for both seedlots, post-emergence damping-off, when compared with the control, was significantly greater when *G. virens* was incorporated (Table 1). For the Bovill seedlot, seedling mortality (less damping-off) was significantly higher in the prill incorporated treatment than the control. Isolations from dead seedlings revealed no significant differences in incidence of *Fusarium* infection among treatments even though disparity between average percent infection of control and treated seedlings appeared large. This lack of significance was likely due to the relatively low disease level encountered in the experiment. A large proportion of diseased seedlings were infected with either *F. oxysporum* or *F. proliferatum*, although no treatment effects on differential infection by these two fungi were found.

In general, *Fusarium* spp. caused significantly greater root infection of non-diseased seedlings in the *G. virens* incorporated treatment (Table 1). Data also indicate *F. proliferatum* and *F. oxysporum* made up most isolates colonizing roots on non-diseased seedlings by the end of the experiment. Seedlings grown with incorporated prill had, for the most part, significantly less height, RCD and oven-dry weight. *Gliocladium virens* prill added as a top dressing had little effect on either *Fusarium* root infection or seedling growth.

Ineffectiveness of *G. virens* incorporated into growing media may be attributable to carrier characteristics as well as the fungus. Alginate pellets are an efficient method for delivering biocontrol agents to soil (Favel and others 1985) and *G. virens* requires a food base of bran rather than kaolin

Table 1. Effects of *Gliocladium virens* on disease and non-diseased seedling infection and colonization by *Fusarium* and subsequent morphology of Douglas-fir seedlings.

Seedlots and treatments	Diseased seelings			Non-diseased seedling infection and root colonization						
				Seedling infection		Root colonization				
	Damped-off per 100 cells (n)	Number per 100 cells (n)	With *Fusarium* spp. (%)	Of those with *Fusarium*, percentage with *F. oxysporum* or *F. proliferatum*	With *Fusarium* spp. (%)	Of those with *Fusarium*, percentage with *F. oxysporum* or *F. proliferatum*	With *Fusarium* spp. (%)	Of those with *Fusarium*, percentage with *F. oxysporum* or *F. proliferatum*	Height (cm)	RCD (mm)	Oven-dry weight (g)
Bovill 3											
Top-dressed	6.0 ab[1]	8.1 ab	55 a	65 a	45 a	84 ab	38 a	36 a	13.8 a	2.28 a	1.23 a
Incorporated	7.4 a	9.7 a	40 a	71 a	88 b	100 a	54 a	52 a	12.2 b	2.02 b	0.98 b
Control	1.3 b	2.4 b	22 a	0 b	40 a	76 b	44 a	41 a	13.1 a	2.20 a	1.22 a
8002											
Top-dressed	4.0 ab	8.4 a	62 a	28 a	48 a	80 a	19 a	19 a	13.0 a	2.26 a	1.20 a
Incorporated	7.6 a	7.5 a	51 a	53 a	86 b	94 a	53 b	53 b	12.0 b	2.08 b	1.06 a
Control	1.6 b	3.5 a	29 a	50 a	43 a	89 a	21 a	21 a	13.3 a	2.20 a	1.15 a

[1] Values in each column for each seedlot followed with different letters are significantly different at $p \leq 0.05$.

clay as the carrier in the pellet (Lewis and Papavizas 1985). Unfortunately, the bran substrate may also serve as a food source for *Fusarium* (Mousseaux 1992), thus increasing pathogen populations.

The laboratory test indicated *G. virens* prill in combination with certain *Fusarium* isolates can have a deleterious effect on Douglas-fir germinants (Table 2). Three isolates of *F. proliferatum* were tested, two of which were quite virulent on germinants. Of the two *F. oxysporum* isolates, one was virulent and the other moderately so. Some protection of young germinants from the most virulent *Fusarium* isolates was afforded by inoculating first with *G. virens*. However, if both *G. virens* and *Fusarium* were inoculated simultaneously, no protection was evident. In fact, for most tested *Fusarium* isolates, *G. virens* significantly exacerbated disease severity when both fungi were added concurrently.

Gliocladium virens produces several antifungal enzymes (DiPietro and others 1993) and secondary metabolites (Park and others 1991) important in antagonism and mycoparasitism (Ridout and others 1992). One such chemical, gliotoxin, is produced in especially high levels by strain GL-21 (Lumsden and others 1992a). Beside gliotoxin, gliovirin (Howell and Stipanovic 1983; Phillips 1986) and viridin (Lumsden and others 1992b) are also known to exert antibiotic properties against other microorganisms. Viridiol, a reduced form of viridin with little or no anti-microbial activity (Jones and Hancock 1987; Lumsden and others 1992b), may induce phytotoxic effects if present at sufficient concentrations (Howell and Stipanovic 1984; Jones and others 1988; Lumsden and others 1992b). There was some evidence for phytotoxicity by *G. virens* on Douglas-fir in both greenhouse and laboratory experiments. Greenhouse seedlings grown with *G. virens* incorporated into their medium were consistently smaller than those grown without this fungus. In addition, our laboratory experiment showed *G. virens* had some pathogenic/phytotoxic tendencies toward Douglas-fir; average of control treatments rated 22, significantly lower than the 74 rating for *Gliocladium* alone (Table 3).

In the past, most Douglas-fir seedling root disease was attributed to *F. oxysporum* (Bloomberg 1973, 1976). However, this species, although present, was not the predominant *Fusarium* spp. isolated from either diseased or healthy seedlings. *F. oxysporum* was isolated more frequently during early stages of the growth cycle. This species commonly colonizes seed (James and others 1987, 1988b) and was likely introduced at some level on contaminated seed. In this study, other incitants of post-emergence damping-off and root disease included *F. proliferatum*, *F. acuminatum* Ell. & Ev., *F. sporotrichioides* Sherb. and *F. solani* (Mart,) Appel & Wollenw; all but *F. proliferatum* were uncommon colonizers of seedling roots by the end of the growth cycle. *Fusarium proliferatum* was uncommon on young damped-off seedlings but

Table 2. Effects of *Gliocladium virens* on virulence of selected *Fusarium* isolates.

Time of *G. virens* placement with *Fusarium* isolates	Ratings[1] *Fusarium* isolates				
	F. oxysporum 9066N	*F. proliferatum* 9201E	*F. proliferatum* 9112F	*F. oxysporum* 9051C	*F. proliferatum* 9202T
Absent (*Fusarium* only)	56 c[2]	68 c	89 a	83 b	91 a
Before	69 b	86 b	68 b	69 c	71 b
Concurrent	91 a	94 a	92 a	91 a	94 a

[1] Virulence increases with rating from 0 to 100, see text and James 1996.
[2] Values in each column followed with different letters are significantly different at $p \leq 0.05$.

Table 3. Laboratory ratings of *Gliocladium virens* and *Fusarium* spp.

	Average ratings[1]
All *Fusarium* isolates	81 b[2]
G. virens inoculated before *Fusarium*	69 c
G. virens and *Fusarium* added concurrently	92 a
G. virens alone	74 c
Control (neither organism)	22 d

[1] Virulence increases with rating from 0 to 100, see text and James 1996.
[2] Values in each column followed with different letters are significantly different at $p \leq 0.05$.

incidence increased with seedling age, and it was very common on roots of seven-month-old non-diseased seedlings. James (1993) found it to be an important cause of root disease in older container-grown seedlings. In our experience, *F. proliferatum* is an uncommon colonizer of seed, but readily colonizes seedling containers at high levels (James and others 1988a, b) as confirmed by this study. We suspect this species initially infected seedlings from inoculum carried on containers and later spread rapidly throughout the greenhouse due to its characteristic mass spore production.

Evidence from the laboratory experiment showed both *F. oxysporum* and *F. proliferatum* were slightly affected by *G. virens*. We did not test antagonistic properties of *G. virens* against the other *Fusarium* species recovered from Douglas-fir seedlings during our experiment (*F. acuminatum, F. sporotrichioides, F. solani*), but we would expect different *Fusarium* species and perhaps even different strains to respond differently to toxic metabolites produced by *G. virens*. Conversely, different *G. virens* strains may react differently to *Fusarium* spp. It is unknown if *G. virens* was hyperparasitic on *Fusarium* in our experiments, even though it has been parasitic on other plant pathogenic fungi (Howell 1987; Papavizas and Collins 1990; Ristaino and others 1991).

Our test results indicated the commercial alginate prill formulation of *G. virens* (GL-21) was ineffective as biological control of Fusarium root disease in Douglas-fir seedlings. Although some level of antagonism by *G. virens* may occur against Fusarium isolates (Park and others 1992), prevention of disease and/or root colonization by fusaria was not evident in our study.

It should not be too surprising *G. virens* (GL-21) was ineffective in controlling Fusarium root disease on Douglas-fir seedlings. The particular *G. virens* strain used in our experiments was developed primarily to control *Pythium ultimum* and *Rhizoctonia solani* on several different crops (Lawson and Dienelt 1989; Lumsden and Locke 1989; Roberts and Lumsden 1990; Lumsden and others 1992b). *Fusarium* is less sensitive to gliotoxin than either

P. ultimum or *R. solani* (Park and others 1992). Another type of *G. virens* carrier, different application rates, and/or perhaps other strains of *G. virens* are more suited to control diseases caused by *Fusarium* spp.

Acknowledgements

We thank James F. Knauss of Grace/Sierra for providing us with the alginate prill, James F. Walter of Grace Biopesticides for his comments, Kenneth E. Quick for assisting with the crop and data collection, and for manuscript reviews by Deborah S. Page-Dumroese and Guy R. Knudsen.

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