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K e y w o r d s : Biocontrol, wood decay, *Trichoderma, Gliocladium*

1. Introduction

Trichoderma *(Gliocladium) virens* is one of the most widely studied species of fungal biocontrol agents. It has proven effective as a suppressant of several soil-borne pathogens (G. L. PAPAVIZAS, 1985), but. *T. virens* has received little attention as a biocontrol agent for wood attacking fungi. However, T. L. HIGHLEY and L. FERGE (1995) found that a commercially formulated *T. virens* biofungicide, Gliogard-GL-21 (W. R. Grace and Co., CT)[3], was an effective pretreatment in the protection of wood against decay by all the white-rot and brown-rot fungi examined in laboratory tests. Although GL-21 produced metabolites in an agar medium that inhibited growth of the decay fungi, wood treated with the metabolites was not decay resistant nor was wood treated with GL-21 resistant to decay after death of GL-21.

Production of the antifungal antibiotics, gliotoxin and gliovirin, by *T. virens* has been associated with its efficacy as a biocontrol agent of most

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soil-borne diseases, but its importance to biocontrol of wood-attacking fungi is unknown. C. R. HOWELL and R. D. STIPANOVIC (1995) produced gliotoxin-deficient [GLT-] mutants of *T. virens* to help ascertain the role of gliotoxin production in the *T. virens/Rhizoctonia solani* biocontrol phenomenon. They found the biocontrol efficacy of the GLT- mutants to be equal to that of the parent strains, indicating that gliotoxin is not necessary for control of the disease.

The objective of this study was to compare the biocontrol efficacy of GLT-mutants of *T. virens* with that of parent strains in the prevention of fungal wood deterioration. This would help in understanding the role of fungitoxic metabolites produced by *T. virens* in the control of wood deterioration.

2. Methods

2.1 Fungi

The following Basidiomycete decay fungi were used: Brown-rot-*Postia* (= *Poria*) *placenta* (Fr.) M. Lars. et. Lomb. [MAD-698], *Neolentinus* (= *Lentinus*) *lepideus* (Fr.:Fr:) Redhead and Ginns [MAD-534] and *Gloeophyllum trabeum* (Pers.:Fr.) Murr. [MAD-617]; white-rot-*Phlebia brevispora* Nakas. in Nakasone et Eslyn [HBB-7030-sp], *Irpex lacteus* (Fr:Fr.) Fr. [HBB-7328-sp.], *Trametes* (= *Coriolus*) *versicolor* L. ex Fr: Pilate [MAD-697], and *Phanerochaete chrysosporium* Burds. [ME-461].

The following mold and stain fungi were used: *Trichoderma* sp., *Aspergillus niger* van Tieghem, *Penicillium* sp., *Ceratocystis pilifera* (Fries) Moreau, *Ceratocystis minor* (Hedgcock) Hunt, and *Aureobasidium pullulans* (de Bary) Arnaud.

Trichoderma (Gliocladium) virens (Miller *et al.*) [GL-21] was donated by W. R. Grace and Co., CT, in the form of dispersible granules. *T.* virens strains G-11 and the UV-induced mutants G-11-4 and Gil-13, deficient for gliotoxin production, were provided by C. R. HOWELL, Southern Crops Research Laboratory, USDA/ARS, College Station, TX.

2.2 Tests for antagonistic effectiveness of *T. virens* strains in agar medium

Dual culture on malt-agar medium. A mycelial plug of the target fungus was placed on one edge of a Petri plate containing 2% malt extract in agar and a *T. virens* strain on the opposite edge. Plates inoculated with mold or stain fungi were inoculated immediately on the opposite edge of the Petri plate with a *T. virens* strain. If a mold or stain fungus was the target fungus, plates were inoculated at the same time with *T. virens.* If a Basidiomycete was the target fungus, plates were inoculated with *T. virens* 2 days following inoculation with the Basidiomycete.

Filtrate inhibition. GL-21 was grown on a basal salts medium (T. L. HIGHLEY, 1973) with ammonium sulfate (0.2%) as the nitrogen source and glucose (1%) as

the carbon source. The pH was adjusted to 3.5. Cultures were incubated for 7 days at 27 °C and 70% relative humidity. Mycelial mats were removed over a Buchner funnel, and the filtrate was sterilized through a 0.20 µm sterile Millipore filter. Ten ml of the filtrate were placed in Petri dishes (90 mm), and 10 ml of the nutrient agar (2X) were added and mixed with the filtrate. Plates were inoculated with agar plugs of the decay fungi. Nutrient agar plates without the filtrate were inoculated with decay fungi to serve as controls. Cultures were incubated at 27 °C, and colony diameter was periodically measured until the control fungi completely covered the plate.

2.3 Tests for antagonistic effectiveness of *G. virens* strains in wood

Southern Pine or sweetgum test blocks (25 mm × 25 mm × 9 mm, with the 9-mm dimension in the grain direction) were treated with the *T. virens* strains and tested for decay resistance by the soil-block method (ASTM, 1971). To determine if live *T. virens* was necessary to render blocks resistant to decay, sterile filtrates (concentrated and unconcentrated) of GL-21, grown as previously noted, were used to treat blocks. Green maple and Southern Pine blocks (3 mm × 28 mm × 42 mm, with the 3-mm dimension in the grain direction) were also dip treated with sterile filtrates of GL-21 and exposed to mold and stain fungi.

Blocks vacuum-impregnated or dip-treated with culture filtrate. GL-21 was grown in 500-ml flasks containing 100 ml of the basal salts medium containing ammonium sulfate and 1% glucose. Cultures were incubated for 7 days at 27 °C and 70 % relative humidity, blended for 10 seconds in a Waring blender, filtered through a Buchner funnel, and the filtrate sterilized through 0.45 and 0.20 µm sterile Millipore filters. The total volume of filtrate obtained was 2000 ml. The entire solution was lyophilized and reduced to 200 ml. This was again filtered through a 0.45 µm sterile Millipore, then further filtered through a 0.22 µm sterile filter. The solution was reduced to 1/10 the original volume and was taken as the high concentration. The 10X solution was combined with an equal volume of sterile water to obtain a concentration of 5X the original solution. This was taken as the low concentration but was certainly greater than the original filtrate solution. For decay tests, steam sterilized blocks were vacuum impregnated with the sterilized filtrates and dried down in absorbent lined pans for 3 hours prior to exposure to decay fungi. The soil blocks were incubated for 6 weeks. For mold and stain tests, blocks were steam sterilized, then dipped for 1 minute in the sterile filtrate preparations.

Blocks treated with living T. virens strains. The *T. virens* strains, G-11, G-11-4 and G11-13, were cultured as previously noted for 1 week, and the cultures were blended for 10 seconds in a Waring blender. Test blocks were vacuum impregnated with the blended culture and exposed to decay fungi in soil-block tests for 10 weeks.

2.4 Wood-block tests

Blocks were evaluated for decay resistance using the standard American Society for Testing and Materials (ASTM, 1971) soil-block test. Bottles were incubated at 27 °C and 70% relative humidity in the dark for 6 or 10 weeks. Percentage weight loss, the measure of decay, was calculated from the weight of decayed blocks after

equilibration at 70% relative humidity and 27 °C. Original weights had been recorded after similar equilibrations. Five replications were used for each treatment.

Blocks treated with sterile culture filtrate were evaluated for resistance to stain and mold attack by placement in sterile 140-mm-diameter Petri plates, lined with 3 layers of white paper towels saturated with water. Plates were inoculated with a spore suspension of the test fungi, placed in plastic bags, and incubated for 8 weeks at 27 °C and 70% relative humidity.

3. Results

The gliotoxin-producing *T. virens* [GLT+] strain quickly overgrew the decay fungi and completely inhibited their growth in dual culture on a malt-agar medium (Table 1). The gliotoxin-deficient [GLT-] mutants also completely inhibited the decay fungi, *P. placenta, N. lepideus, T. versicolor,* and *P. brevispora* in dual culture but caused lesser inhibition to *G. trabeum* and *I. lacteus.* Both the GLT+ and GLT- strains had no inhibitory effect on the growth of the mold fungi in dual culture malt-agar medium. The stain fungi were inhibited similarly by the GLT+ and GLT- strains, although inhibition was not 100%.

Table 1: Interaction of wood-attacking fungi with *Trichoderma virens* [GLT+] or gliotoxin-deficient [GLT-] mutants in agar medium.

Fungus	Inhibition of growth (%) by *T. virens* isolates		
	G-11 [GLT+]	G-11-4 [GLT-]	G-11-13 [GLT-]
Brown rot			
Postia placenta	100	100	100
Neolentinus lepideus	100	100	100
Gloeophyllum trabeum	100	72	62
White rot			
Trametes versicolor	100	100	100
Phlebia brevispora	100	100	100
Irpex lacteus	100	62	65
Mold			
Trichoderma sp.	0	0	0
Aspergillus niger	0	0	0
Penicillium sp.	0	0	0
Stain			
Ceratocystis pilifera	76	66	60
Ceratocystis minor	56	50	54
Aureobasidium pullulans	85	85	71

T a b 1 e 2: Fungistatic ability of culture filtrate[a] from GL-21 in agar medium.

Fungus	Inhibition of growth (%)
Brown rot	
Postia placenta	80
Gloeophyllum trabeum	56
Neolentinus lepideus	87
White rot	
Trametes versicolor	79
Phlebia brevispora	24
Irpex lacteus	43
Mold and stain	
Trichoderma sp.	16
Aspergillus niger	0
Penicillium sp.	0
Ceratocystis pilifera	100
Aureobasidium pullulans	29

[a] Filtrate from GL-21 grown on a basal salts medium with 0.30% ammonium sulfate as nitrogen source and pH adjusted to 3.5.

Tab 1 e 3: Decay resistance of wood blocks treated with *Trichoderma virens* [GLT+] or gliotoxin-deficient [GLT-] mutants.

Wood decay fungus	Weight loss[a] produced by wood-rotting Basidiomycete (%)			
	Control	G-11 [GLT+]	G11-4 [GLT-]	G11-13 [GLT-]
Southern Pine				
Brown rot				
Postia placenta	53.0 ± 5.6	0	0	0
Neolentinus lepideus	30.3 ± 2.3	0	0	0
Gloeophyllum trabeum	36.3 ± 4.0	0	31.6 ± 0.9	0
Maple				
White rot				
Irpex lacteus	61.8 ± 6.3	0	32.5 ± 24.4	63.5 ± 7.5
Trametes versicolor	45.1 ± 1.7	0	40.4 ± 2.6	43.2 ± 1.7
Phlebia brevispora	30.2 ± 5.2	0	13.7 ± 2.3	18.2 ± 6.4

[a] Mean weight loss with standard deviation (10 weeks exposure).

Growth of the decay fungi on agar medium containing sterilized culture filtrate from GL-21 [GLT+] (prepared from a sulfur-containing medium, pH 3.5) was slowed but not completely inhibited (Table 2). The stain fungus, *C. pilifera*, was unable to grow on the culture filtrate containing medium, but the culture filtrate had little effect on the growth of the other mold and stain fungi.

Pretreatment of wood blocks by vacuum impregnation with a blended culture of the GLT+ *T. virens* strain prevented decay by all the brown-rot and white-rot fungi tested (Table 3). Decay by the brown-rot fungi, *N. lepideus* and *P. placenta*, was also prevented by the GLT- strains of *T. virens*. Decay by the brown rotter, *G. trabeum*, was prevented by one GLT- strain but not by the other. The GLT- strains did not stop decay by any of the white-rot fungi, but progression of decay was slowed in most cases.

Decay was reduced in blocks treated with a sterile filtrate from GL-21 cultured on a sulfur-containing medium, pH 3.5, to promote antibiotic production, but was not completely stopped (Table 4). Concentration of the filtrate further reduced the amount of decay in blocks but weight loss still occurred. Treatment of wood with the previously noted filtrates was ineffective in preventing growth of stain and mold fungi on wood.

Table 4: Decay resistance of blocks treated with sterilized GL-21 filtrates.

Wood decay fungus	Weight loss[a] produced by wood-rotting Basidiomycete (%)		
	Control (untreated)	Filtrate (low concentration)	Filtrate (high concentration)
Southern Pine			
Brown rot			
Postia placenta	26.5 ± 2.0	8 .9 ± 1.8	4.7 ± 2.2
Gloeophyllum trabeum	26.7 ± 2.4	14.9 ± 2.9	14.1 ± 2.4
Sweetgum			
White rot			
Phanerochaete chrysosporium	54.9 + 8.6	49.4 ± 6.5	15.6 ± 12.5
Trametes versicolor	40.3 ± 5.1	47.9 ± 4.5	18.1 ± 5.1

[a] Mean weight loss with standard deviation (6 weeks exposure)

4. Discussion

The development of successful strategies for the use of biocontrol agents to protect wood from fungal attack will require a detailed knowledge of the mechanisms employed by the biocontrol agents to effect control. *Trichoderma virens* produces a number of well-established antifungal agents, such as gliovirin and gliotoxin, to what the biological activity of the fungus is often ascribed. We report in this paper the contribution of gliotoxin to the control of wood-attacking fungi by using gliotoxin-deficient mutants of *T. virens* to study biological efficacy against wood-attacking fungi.

In dual cultures on a malt agar medium, both GLT+ and GLT- strains generally had a similar inhibitory effect on the decay fungi and mold and stain fungi. Loss of gliotoxin had no effect on biocontrol performance against the brown-rot fungi in wood. With the white-rot fungi, the GLT- strains did not prevent decay, indicating that gliotoxin must play a very important part in biocontrol of these fungi. However, metabolites from GL-21 were not effective in preventing decay, indicating that the living mycelium produces other components essential to biocontrol of white-rot decay.

Previously, T. L. HIGHLEY and L. FERGE (1995) found that although treatment of wood blocks with living GL-21 prevented decay, treatment with sterilized culture filtrate (metabolites) was ineffective. Also, wood treated with GL-21 was not decay resistant after death of GL-21. Substrate and environment have a profound effect on secondary metabolite production by *T. virens* (C. R. HOWELL and R. D. STIPANOVIC, 1995). In this test, even though growth on sulfur-containing medium at low pH enhanced antibiotic production, treatment of blocks with sterilized culture filtrate from GL-21 reduced but did not eliminate decay, even when filtrates were concentrated. This is additional evidence that the mechanism employed by *T. virens* to prevent wood decay involves other factors in addition to production of fungitoxic metabolites. Competition may play an important role in the mechanism of decay prevention by *T. virens* similar to that suggested with the soil-borne disease *R. solani* (C. R. HOWELL and R. D. STIPANOVIC, 1995).

T. virens continues to show promise as a biocontrol treatment to prevent wood decay. However, the results thus far indicate that neither living *T. virens* nor its metabolites show promise as a preventive treatment for control of discoloring fungi that colonize on green wood.

5. Summary

Trichoderma (*Gliocladium*) *virens* has shown good antagonism against decay fungi in agar medium and in wood blocks. Gliotoxin produced by *T. virens* is associated with biocontrol of some plant diseases, but its importance to biocontrol of wood-attacking fungi is unknown. We investigated the ability of gliotoxin-producing [GLT+] isolates of *T. virens* and gliotoxin-deficient [GLT-] mutants of *T. virens* to inhibit growth of wood-attacking fungi in agar medium and to prevent decay in wood. The brown-rot fungi *Postia placenta* and *Neolentinus lepideus* and the white-rot fungi *Trametes versicolor* and *Phlebia brevispora* were completely inhibited by the GLT+ isolate and the GLT- mutants in agar medium. The GLT+ isolate also completely inhibited the growth of the brown-rot fungus *Gloeophyllum trabeum* and the white-rot fungus *Irpex lacteus,* but the GLT- mutants caused lesser inhibition. The GLT+ isolate and GLT- mutants were ineffective in preventing growth of mold fungi, but both inhibited stain fungi in dual agar culture. Pretreatment of wood blocks with the GLT+ isolate or GLT- mutant prevented decay by the brown-rot fungi. Although the GLT+ isolate prevented decay by the white-rot fungi, the GLT- mutants did not. *G. virens* [GL-21] was grown on a sulfur-containing medium at pH 3.5 to enhance antibiotic production in culture filtrates. However, inhibition of growth of decay fungi on agar medium containing culture filtrates was not enhanced. Decay was reduced in blocks treated with the culture filtrates but was not completely stopped. The filtrates were also ineffective in preventing growth of mold and stain fungi on wood.

Zusammenfassung

Bekämpfung der Holzfäule durch *Trichoderma (Gliocladium) virens*

II. Antibiotische Wirkung

Trichoderma (Gliocladium) virens hat gute antagonistische Wirkungen gegenüber holzzerstörenden Pilzen in Agar-Kulturen und in Holzklötzchen gezeigt. Das von *T. virens* produzierte Gliotoxin wird für die biologische Kontrolle einiger Pflanzenkrankheiten eingesetzt, nichts ist jedoch über seine Bedeutung im Hinblick auf die biologische Bekämpfung von holzzerstörenden Pilzen bekannt. Wir haben die Fähigkeit von Gliotoxin-produzierenden Isolierungen (GLT+) und von Mutanten von *T. virens* ohne Gliotoxinproduktion (GLT-) in bezug auf ihre wachstumshemmende Wirkung und ihre Fähigkeit Holzabbau durch holzzerstörende Pilzen in Agar-Kulturen zu verhindern, untersucht. Die Braunfäulepilze *Postia placenta* und *Neolentinus lepideus* und die Weißfäulepilze *Trametes versicolor* und *Phlebia brevispora* wurden in Agar-Kultur durch die Isolierungen GLT+ und die Mutanten GLT- vollständig gehemmt. Die Isolierungen GLT+ hemmten auch das Wachstum des Braunfäulepilzes *Gloeophyllum trabeum* und des Weißfäulepilzes *Irpex lacteus,* wobei die Mutanten GLT- eine geringere Hemmung bewirkten. Die Isolierungen GLT+ und die Mutanten GLT- konnten das Wachstum von Moderfäulepilzen nicht hemmen, aber beide hemmten das Wachstum holzverfärbender Pilze in Doppelkultur. Eine Vorbehandlung der Holzklötzchen mit der Isolierung GLT+ oder der Mutanten GLT- verhinderte eine Holzzerstörung durch Braunfäulepilze. Obwohl die Isolierung GLT+ Weißfäulepilze verhinderte, konnte dies die Mutante GLT- nicht. *G. virens* (GL-21) wurde auf einem schwefelhaltigem Kulturmedium bei einem pH-Wert von 3,5 gezüchtet, um die Produktion von Antiobiotika in den

Kulturfiltraten zu erhöhen. Die Hemmung des Wachstums der holzzerstörenden Pilze in einer Agar-Kultur mit diesen Filtraten wurde jedoch nicht verbessert. Holzfäule von Klötzchen, die mit diesem Filtraten behanelt worden waren, war zwar geringer, das Pilzwachstum konnte jedoch nicht gestoppt werden. Die Filtrate konnten auch das Wachstum von Moderfäule- und holzverfärbenden Pilzen nicht verhindern.

Résumé

Contrôle des pourritures du bois par ***Trichoderma (Gliocladium) virens***

II. L'effet antibiotique

Sur milieux gélosé et sur les échantillons du bois, *Trichoderma (Gliocladium) virens* a montré une activité antagoniste contre les champignons des pourritures. La gliotoxine produite par *T. virens* est associé au biocontrôle dans certaines maladies de plantes, mais son rôle dans le biocontrôle des champignons attaquant le bois est inconnu. Nous avons établis le rôle des souches produisant de la gliotoxine (GLT+) et des mutants déficient en glioxine (GLT-) de *T. virens,* dans le contrôle des champignons de pourriture sur milieux gélosés et dans le bois. Les pourritures brunes *Postia placenta* et *Neolentinus lepideus* et les pourritures blanches *Trametes versicolor* et *Phlebia brevispora* sont totalement inhibées en milieux gélosés par les souches GLT+ et les mutants GLT-. Les souches GLT+ inhibent totalement la croissance de la pourriture brune *Gloeophyllum trabeum* et de la pourriture blanche *Irpex lacteus,* les mutants GLT- sont moins inhibitrices. Sur milieux gélosés les souches GLT+ et les mutants GLT- sont actifs contres les champignons colorés, mais inactifs contre les moisissures. Le prétraitement des échantillons de bois avec les souches GLT+ et les mutants GLT- contrôle la deterioration par les champignons des pourritures brunes. Bien que les souches GLT+ controlent la deterioration par les pourritures blanches, les mutants GLT- sont sans effets. Lorsque *G. virens* (GL-21) est cultivé sur un milieu pH 3,5 contenant du soufre la production d'antibiotiques dans le milieu est augmentée. Cependant, l'inhibition de la croissance des champignons des pourritures, sur milieux gélosés contenant les filtrats de cultures, n'a pas augmentée. La pourriture a diminuée mais n'a pas été totalement arrêté dans les échantillons de bois traités avec les filtrats. Les filtrats ont été aussi inactifs sur la croissance des moisissures et des champignons colorant le bois.

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