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DISEASE

Field Control of Cotton Seedling Diseases with *Trichoderma virens* in Combination with Fungicide Seed Treatments

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INTERPRETIVE SUMMARY

The purpose of this research was to determine if biological and fungicide seed treatment combinations could effectively control cotton seedling diseases on different cotton varieties and under diverse environmental conditions. There are several reports indicating that *Trichoderma virens* is an effective biological control agent for some cotton seedling diseases, but these tests were all conducted under controlled conditions and only indicate the potential for control in the field. A biological control agent that can function in combination with fungicide seed treatments to suppress seedling disease in the field might have value as a commercial product for seedling disease control.

Two years of field tests in California and 1 year in Arkansas, Indiana, Louisiana, Mississippi, and Oklahoma of combined biological and fungicide cotton seed treatments provided control of cotton seedling diseases in areas of moderate to heavy disease pressure. The biological component is the fungus *T. virens*, cultured on millet or wheat bran + peat moss, air-dried, and ground into fine granules. The chemical component is the fungicide, metalaxyl. The seed was treated first with the fungicide, followed by a latex sticker and the fungus granules. Cotton seed treated with the *T. virens* and metalaxyl combination and planted in soil infested with seedling disease pathogens, in some instances, produced stands equal to those obtained with standard fungicide treatments and, more often, significantly better than those from obtained untreated seed. Biological and fungicide seed treatments may also provide longer term protection than that provided by fungicides alone, through colonization and protection of the developing root system and reduction of pathogen inoculum by parasitism of pathogen propagules in the soil.

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ABSTRACT

The purpose of this study was to assess the biocontrol efficacy of the fungus *Trichoderma virens* in combination with fungicides against cotton (*Gossypium hirsutum*) seedling disease pathogens in the field under different soil and ambient environmental conditions. Cotton seed treated with fungicides and/or coated with a latex sticker and air-dried granules of *T. virens* was planted in field plots with a history of seedling disease in California (CA), Oklahoma (OK), Arkansas (AR), Louisiana (LA), Mississippi (MS) and Indiana (IN), and surviving seedlings counted. Seedling stands in CA from *T. virens* plus metalaxyl-treated seed plots were greater than the untreated control and most of the plots in which seed was treated only with fungicide. In AR and MS, *T. virens* plus metalaxyl treatments produced significantly better stands than the untreated controls. In some cases, this combination produced stands equal to those in fungicide-treated controls. In LA, OK, and IN, *T. virens* plus metalaxyl-treated seed resulted in stands equal to those in untreated controls, but less than those in the fungicide-treated controls. The addition of triademenol or fludioxonil to the seed treatment combinations did not improve stands above *T. virens* plus metalaxyl alone. The treatment of cotton seed with *T. virens* plus metalaxyl generally resulted in greater seedling stands than those in untreated controls and equal to those of the fungicide control, except where disease pressure is very heavy (IN) or light (LA and OK).

Abbreviations: FLSD, Fisher's least significant difference.

The hyphomycetous fungus, *T. virens* = (*Gliocladium virens)* (J. H. Miller, Giddens & A. A. Foster) Arx, is reported to be a mycoparasite of the cotton seedling pathogen, *Rhizoctonia solani* Kühn (Weindling, 1932). Strains of *T. virens* ("Q") also produce the antibiotic, gliotoxin, that is active against *R. solani*, but less active against *Pythium ultimum* Trow (Howell et al., 1993; Weindling and Emerson, 1936). Other strains ("P") of the fungus produce the antibiotic, gliovirin, that is highly active against *P. ultimum* and other oomycetes, but has no effect on hyphomycetes such as *R. solani* (Howell and Stipanovic, 1983). All strains of *T. virens* are capable of producing the steroid phytotoxin, viridiol, on substrates with high C/N ratios (Howell and Stipanovic, 1984; Jones and Hancock, 1987). Preparations containing viridiol, when placed in close proximity to the seed, can have devastating effects on the emerging cotton radicle. This problem can be eliminated by adding low levels of a steroid-inhibiting fungicide to the growth medium prior to culture of the biocontrol agent eliminates this problem (Howell and Stipanovic, 1994). Removal of viridiol production through deletion mutation of *T. virens* strains does not appear to enhance biocontrol activity, at least not at the concentration coated on seed (Howell et al., 1997).

When the biocontrol preparation was added in-furrow with the seed in 1982, *T. virens* was reported to be an effective biocontrol agent of *R. solani*-incited cotton seedling damping-off (Howell, 1982). It was speculated that mycoparasitism and antibiotic production might be the mechanisms involved in the biocontrol process. However, subsequent research using mutants of *T. virens* deficient for gliotoxin production or mycoparasitic activity has demonstrated that neither mechanism is necessary for disease control (Howell, 1987; Howell and Stipanovic, 1995). The operative mechanisms involved in this biocontrol phenomenon have yet to be determined. Regardless of the mechanisms involved, the substrate on which the agent is grown has a profound effect on its biocontrol efficacy (Howell, 1991).

The biocontrol efficacy of *T. virens* in suppressing cotton seedling damping-off by *R. solani* has been demonstrated repeatedly in the growth chamber (Howell, 1982; 1987; Howell et al., 1993; Howell and Stipanovic, 1995). However, before commercial applications will be developed, its biocontrol activity must be demonstrated in the field under diverse environmental conditions and in the presence of other seedling disease pathogens. Field comparisons of fungal biocontrol preparations in California in 1995 demonstrated that *T. virens* could effectively control cotton seedling diseases (DeVay et al., 1996). However, the results of metalaxyl (Apron FL) treatment indicated that *Pythium* spp. was not a problem in California that year. Preparations of *T. virens* with and without metalaxyl significantly increased seedling stands over the untreated control, the fungicide-treated control, and seed treated with the biological control agent, Binab T, a mixture of *T. polysporum* (Link ex Pers.) Rifai and *T. harzianum* Rifai produced by Binab Bioinnovation AB, Algaras, Sweden.

The purpose of the current study was to determine whether air-dried preparations of *T. virens* alone, or in combination with metalaxyl and/or other fungicides and coated on cotton seed, could effectively and consistently prevent cotton seedling diseases in the field.

MATERIALS AND METHODS

Culture and Handling of Biological Control Preparations

Inoculum of several *T. virens* "Q" (gliotoxin-producing) strains, isolated from diverse geographic areas, was produced by inoculating liters of medium consisting of 5% ground millet or wheat bran and 1% ground peat moss in deionized water with conidia. The medium was adjusted to pH 4.0 with dilute HCl. The cultures were shaken (150 revolutions per min) at 27°C for 12 d, then the contents were centrifuged at 3000 x *g* for 10 min. The supernatant fluids were discarded and the solids were spread to air-dry under a positive pressure hood for 48 h. The air-dried materials were then ground to a 500 μm particle size and stored in plastic bags at 5°C until used.

Seed Treatment Technique

Acid delinted seed of cotton (*Gossypium hirsutum* L.) 'Acala Maxxa' was treated with metalaxyl (Apron FL, 22.1 mL/45.4 kg), metalaxyl + triadimenol (Baytan,29.1 mL/45.4 kg) or metalaxyl + fludioxonil (Maxim, 2.33 mL/45.4 kg) by diluting the fungicides in sufficient water to get coverage of the seed and applying the dilutions to

Table 1. Effect of seed treatments with *Trichoderma virens* on final stands of cotton in Arkansas (AR), Indiana (IN), Louisiana (LA), Mississippi (MS) and Oklahoma (OK), 1996.

	Seeding survival,%‡				
Treatment†	**AR**	**IN**	**LA**	**MS**	**OK**
TV-111/Met	**70.7a**	**19.5abcde**	**62.8bcd**	**63.0abc**	**71.7cd**
TV-110/Met	**60.8bcd**	**14.7cdefgh**	**62.8bcd**	**58.5abcde**	**60.3f**
TV-109/Met	**66.8ab**	**18.2bcdef**	**68.0abc**	**54.5bcdefg**	**74.7cd**
TV-108/Met	**61.0bcd**	**15.8cdefg**	**66.8bc**	**61.2abcd**	**62.0f**
TV-G-4/Met	**64.7bc**	**22.5abc**	**60.7cd**	**56.5abcdef**	**71.8cd**
Carb-PCNB/Met	**79.5a**	**31.0a**	**73.5ab**	**67.7ab**	**84.2ab**
Metalaxyl	**68.7ab**	**28.3ab**	**61.0cd**	**44.2efgh**	**70.5de**
Carb-PCNB	**52.2cde**	**13.5cdefgh**	**77.7a**	**73.1a**	**83.0ab**
Untreated	**43.8efgh**	**7.7efgh**	**60.6cd**	**39.7gh**	**71.0cd**

†TV = *T. virens* strain coated on cotton seed with latex sticker (Rhoplex B 15J). Carb-PCNB = Vitavax-Pentachloronitrobenzene (174.6 mL/45.4 kg); Met = Metalaxyl (22.1 mL/45.4 kg).

‡Percent of 'Deltapine 50' cotton plants surviving per plot, mean of six replications. Based on 100 seeds/plot. Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD test at $P < 0.05$.

the seed with stirring. The treated seed was then air-dried. Seed of cotton 'Deltapine 50' was treated with metalaxyl and/or carboxin-pentachloronitrobenzene (Vitavax-PCNB, 174.6 mL/45.4 kg) in the same manner. The seed to be treated with the *T. virens* was then coated with a latex sticker (Rhoplex B 15J, Rohm and Haas) by mixing 39 mL with each pound of seed and shaking with the air-dried, ground fungal preparation described above (7% w/w). The 'Acala Maxxa' treated seed was packaged and sent to cooperators in California, while 'Deltapine 50' treated seed was sent to Arkansas, Indiana, Louisiana, Mississippi, and Oklahoma for planting and evaluation of control of seedling diseases.

Experimental Plots

Cotton fields with a history of seedling disease were selected for planting. Plots in the Southern Cotton Belt states (Arkansas, Louisiana, Mississippi, and Oklahoma) and Indiana were 12.2 m in length and planted with 100 seeds. Each treatment was replicated six times. In California the plots were 9.2 m in length and planted with 120 seeds. Each treatment was replicated eight times. A randomized complete block design was used in all experiments. Biological treatments in California were compared with untreated and metalaxyl-treated seed in 1996, and with untreated seed, metalaxyl plus tiradimenol and metalaxyl plus fludioxonil treated seed in 1997. In the Southern Cotton Belt states and in Indiana, the results of the biological treatments were compared with untreated seed, and seed treated with metalaxyl, carboxin-PCNB, and carboxin-PCNB plus metalaxyl.

The California plantings in 1996 and 1997 were made on 29 and 28 March at Shafter I, on 8 and 2 April at Shafter II, and on 24 and 17 April at Dos Palos, respectively. The soil type was a Wasco sandy loam (coarse-loamy, mixed, nonacid, thermic Typic Torriorthents) at Shafter I and II, and a silty clay loam at Dos Palos. The planting dates and soil types for the other locations were as follows: Arkansas on 4 May, in a Dubbs-Dundee silt loam soil (fine-silty, mixed, thermic Typic Hapludalfs); Indiana on 5 June, in Chalmers silt loam (fine-silty, mixed, mesic Typic Haplaquolls); Louisiana on 16 May, in Olivier silt loam (fine-silty, mixed, thermic Aquic Fragiudalfs); Mississippi of 26 April, in Marietta fine sandy loam (fine-loamy, siliceous, thermic Fluvaquentic Eutrochrepts); and Oklahoma on 17 May, in Norge loam (fine-silty, mixed, thermic Aeric Ochraqualfs).

After stand counts, diseased seedlings were removed from untreated control plots, washed throughly with tap water, surface sterilized with 0.5 % sodium hypochlorite for 1.5 min, and plated on water agar containing rifamycin (10 μg mL^{-1}) and amphicillin (250 μg mL^{-1}). The resulting colonies

Table 2. Field efficacy of seed treatments with *Trichoderma virens* for biological control of cotton seedling diseases at three locations in California, 1996.

	Seedling survival,%‡		
Treatments†	Shafter 1	Shafter 2	Dos Palos
TV-G-6	72.9a	75.5a	81.3a
TV-108	71.5a	63.0b	73.3b
TV-109	69.9ab	76.1a	81.6a
TV-110	67.7ab	69.4ab	77.5ab
TV-111	71.6a	73.8a	80.3a
TV-115	62.3b	73.0a	80.4a
Metalaxyl	61.9b	62.3b	63.4b
Untreated	59.2c	16.0c	29.8c

†All seed were treated with metalaxyl (Apron FL 22.1 mL/45.4 kg) except the untreated control. TV = *Trichoderma virens* strain applied after fungicide.

‡Percent of 'Acala Maxxa' cotton plants surviving per plot, mean of eight replications. Based on 120 seeds/plot. Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD test at $P < 0.05$.

were transferred to potato dextrose agar and identified under the microscope.

Data Collection and Analysis

Stand counts were made and percentage seedling survival calculated approximately 1 month after planting. The data were subjected to analysis of variance with the general linear models procedure of SAS. Mean separations were determined by Fisher's protected least significant difference (FLSD) test.

RESULTS AND DISCUSSION

Microscopic examination of the contents of 12-d-old shake cultures of *T. virens* strains grown on millet or wheat bran + peat moss medium revealed that the solids contained large numbers of well-developed thick-walled chlamydospores. The spores were mostly separate, with very little connecting mycelium present. Granules of the dried and ground preparation sprinkled on potato dextrose agar plates produced viable *T. virens* mycelium with no contaminants. Cotton seed coated with the latex sticker and the air-dried granular preparations was uniformly coated, and little of the biological preparation was lost from the seed during handling and packaging.

Cotton stands of seed treated with *T. virens* and metalaxyl were equal to stands provided by chemical seed treatments and greater than the untreated controls in Arkansas and Mississippi where disease pressure was moderate to heavy (Table 1). In Louisiana and Oklahoma where disease pressure was light, seed treatment with carboxin-PCNB and carboxin-PCNB plus metalaxyl generally resulted in better seedling stands than metalaxyl alone, *T. virens* plus metalaxyl combinations and the untreated controls (Table 1). In Indiana, where growing conditions for cotton are marginal and disease pressure was heavy, fungicide seed treatments generally provided greater stands than the biological plus metalaxyl combinations and the untreated seed. Isolations from diseased seedlings for the presence of seedling pathogens identified *R. solani* in Louisiana and Mississippi, *Pythium ultimum* in Arkansas and Oklahoma, and *T. basicola* Zoph in Arkansas.

Results from the 1996 field trials in California on soils heavily infested with seedling disease pathogens (Table 2) were similar to those obtained in the other states. *Trichoderma virens* plus metalaxyl

Table 3. Field performance of seed treatments with *Trichoderma virens* plus fungicide combinations for control of seedling diseases of cotton at three locations in California, 1997.

	Seedling survival,%‡		
Seed treatments†	Shafter-1	Shafter-2	Dos Palos
Untreated	57.9b	59.0c	56.1e
TV-G-6	64.5b	58.5c	60.6de
TV-G-6 + Met	75.4a	79.7a	71.9a
TV-G-6 + Met + Tri/1	62.9b	73.9ab	67.5abc
TV-G-6 + Met + Tri/2	74.8a	72.2b	64.9bcd
TV-G-6 + Met + Flu	73.1a	72.2b	64.4cd
Met + Tri	60.1b	74.8ab	70.6ab
Met + Flu	75.4a	76.4ab	70.4ab

†Met = metalaxyl (Apron FL 22.1 mL/45.4 kg.); Tri/1 = triadimenol (Baytan 29.1 mL/45.4 kg); Tri/2 = triadimenol (Baytan 14.55 mL/45.4 kg); Flu = fludioxonil (Maxim 2.33 mL/45.4 kg TV = *T. virens* strain G-6 coated on seed with a latex sticker (Rhoplex B 15J) after fungicide treatment.

‡Percent of 'Acala Maxxa' cotton plants surviving per plot, mean of eight replilcations. Based of 120 seeds/plot. Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD test at $P < 0.05$.

combination seed treatments resulted in seedling stands that were significantly greater or equal to metalaxyl alone, and greater than the stands in the untreated control. Variations in disease control efficacy among the biological treatments may have been due to silent mutations induced during mutagenesis of *T. virens* to produce strains deficient for viridiol production (Howell et al., 1997). The improvement in plant stands associated with metalaxyl indicate that the seedling disease pathogen, *P. ultimum,* was a major contributor to seedling damping-off in these fields.

The 1997 field tests in California confirmed the data obtained in 1996 (Table 3). Seed treatment with the *T. virens* plus metalaxyl combination generally produced seedling stands equal to the chemical control and significantly better than the untreated control. The addition of triadimenol or fludioxonil to the biological plus metalaxyl combinations did not improve seedling survival. This was probably due to the growth inhibiting effects of these fungicides on *T. virens*.

Diseased seedlings from both the Shafter and Dos Palos sites were heavily infested with *R. solani*. The Shafter plots contained higher numbers of *P. ultimum* than those at Dos Palos. Diseased seedlings from all plots yielded *Fusarium spp.* of uncertain pathogenicity.

CONCLUSIONS

In seedling disease tests in California over 2 years and 1 year in Arkansas, Indiana, Louisiana, Mississippi, and Oklahoma, seed treatment of two cotton cultivars with *T. virens* strains in combination with metalaxyl provided effective seedling disease control in field soils naturally infested with seedling pathogens. Optimum disease control occurs in areas where disease pressure (inoculum concentration and environmental conditions) is moderate to heavy. Excessive disease pressure can undermine the effectiveness of biological seed treatments. *Trichoderma virens* plus metalaxyl seed treatments were most effective in controlling cotton seedling diseases incited by *R. solani* and *P. ultimum*. They may not be as effective against other seedling disease pathogens. *Trichoderma virens* applied as granular inoculum to cotton seed has been shown to colonize tap roots and secondary roots of developing cotton plants, reduce root colonization by *Fusarium* spp., and suppress *Fusarium* wilt of cotton incited by *F. oxysporum f. sp. vasinfectum* (Zhang et al., 1996). Therefore, seed treatment with *T. virens* preparations may provide longer term protection of the developing root system than that afforded by chemical seed treatments alone. Annual seed treatment with *T. virens* over time may also reduce the inoculum potential of *R. solani* through mycoparasitism of pathogen sclerotia (Howell, 1982).

The fact that these experimental results were obtained under the uncontrolled soil and ambient conditions that occur in the field during the planting season is significant. There are several reports indicating that *T. virens* is an effective biological control agent for some cotton seedling diseases (Howell, 1982, 1987, 1991; Howell and Stipanovic, 1983, 1995; Howell et al., 1993), but these tests were all conducted under controlled conditions and, as such, only indicate the potential for control in the field. A biological control agent that can function in combination with fungicide seed treatments to suppress seedling disease in the field might have value as a commercial product for seedling disease control.

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