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Plant Pathology & Nematology

Reduction of Verticillium Wilt Symptoms in Cotton Following Seed Treatment with *Trichoderma virens*

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INTERPRETIVE SUMMARY

Verticillium wilt, caused by *Verticillium dahliae,* is a widespread disease that occurs in most cotton-producing areas. *V. dahliae* is a soil-borne pathogen that infects plants through the roots. Symptoms of infected cotton plants include stunting and wilting by some strains of *V. dahliae* and defoliation by other strains. The purpose of this research was to determine the potential of cotton seed treatment with biological control strains of *Trichoderma virens* to induce resistance to the fungal wilt pathogen, *Verticillium dahliae,* injected into the stem of cotton plants. *Trichoderma virens* is an effective biocontrol agent against soil-borne seedling diseases of cotton, but its ability to protect cotton against other diseases is unknown. Because another *Trichoderma* species, *T. harzianum*, is reported to give systemic protection to the foliar disease gray mold in bean, we hypothesized that *T. virens* also might provide systemic protection of cotton against wilt diseases.

Cotton plants grown from seed treated with two different strains of *T. virens* had significantly less severe symptoms after stem inoculation with *V. dahliae* than did controls not treated with *T. virens*. The introduced *Trichoderma virens* colonized the cotton roots and was not isolated from the stems in aboveground portions of the plants. *V. dahliae*, which was inoculated into the stems to avoid direct contact with the *T. virens* introduced on the seed, was readily isolated from stems following inoculation. This spatial separation between the two fungi indicates that direct antagonism between the fungi is not responsible for the reduction in disease following seed treatment with *T. virens*. This reduction in symptoms indicates that *T. virens* induces resistance in the host at a distance from where the fungus is established (the germinating seed) and that seed treatment with *T. virens* can provide some long-term protection of cotton plants from Verticillium wilt. However, although induction of defense responses, such as production of terpenoid phytoalexins in cotton, has been correlated with disease resistance, no evidence for increased induction of gossypol or related terpenoids, the best-known phytoalexins in cotton, was found in plants grown from seed treated with *T. virens*.

ABSTRACT

Strains of *Trichoderma virens* that control damping-off of cotton seedlings caused by either *Pythium ultimum* or *Rhizoctonia solani* were tested for their ability to induce resistance to Verticillium wilt. Cotton seeds were treated with dried preparations of *T. virens* and planted in field soil. Plants with six true leaves were inoculated with *Verticillium dahliae* by stem puncture. After 10 d, plants were rated for Verticillium wilt symptoms and plant heights measured. Two strains of *T. virens* significantly reduced ($\alpha = 0.05$) the disease-severity ratings in *V. dahliae*-inoculated plants of two cotton cultivars, Rowden and Deltapine 50. This result indicated that *T. virens* may induce a systemic resistance response in cotton, but concentrations of terpenoid phytoalexins in stele extracts were not significantly different in *V. dahliae*-inoculated plants that had been treated with the *T. virens* when compared with plants treated with the carrier alone. In the absence of *Verticillium*, plants treated with the G-4 isolate of *T. virens* were significantly taller than control plants treated without *T. virens*. This result indicates that some strains of *T. virens* may have growth-promoting activity.

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Abbreviations: HPLC = high-performance liquid chromatography.

Strains of *Trichoderma* (*Gliocladium*) *virens* (Miller, Giddens, and Foster) Arx. are effective biological control agents against Fusarium wilt (Zhang et al., 1996) and seedling diseases of cotton (*Gossypium hirsutum* L.) caused by *Pythium ultimum* Trow. and *Rhizoctonia solani* Kuhn (Howell, 1982). The mechanisms by which *T. virens* provides biological control are not fully understood. Although biocontrol of *Pythium ultimum* has been correlated with antibiotic production (Howell and Stipanovic, 1983; Wilhite et al., 1994), mutants that lack antibiotic production are still effective biocontrol agents against *Rhizoctonia solani* (Howell and Stipanovic, 1995). Also, mutants that are deficient for mycoparasitism are still effective in controlling damping-off caused by *R. solani* (Howell, 1987). Treatment with *T. virens* reduces the severity of Fusarium wilt in cotton (Zhang et al., 1996), but the mechanism has not been investigated. Strains of *T. virens* that are effective biocontrol agents against *R. solani* induce production of resistance-related compounds, such as terpenoids, and can lead to increased peroxidase activity in cotton roots (Howell et al., 2000).

Trichoderma species have been reported to induce systemic resistance in crops other than cotton. Application of *Trichoderma harzianum* Rifai to bean roots reduced the severity of the foliar disease, gray mold, caused by *Botrytis cinerea* Pers. ex Fr. (De Meyer et al., 1998). In that study, there was no examination of any of the resistance-related pathways in the host. An elicitor from *T. viride* Per. ex Fr. induced resistance-related responses in tobacco cells (Dean et al., 1989) and grapevine cells (Calderon et al., 1993). This elicitor induces ethylene biosynthesis in tobacco cells (Dean et al., 1989) and production of the phytoalexin, resveratrol, in grapevine cells (Calderon et al., 1993).

Biological control of *Verticillium dahliae* Kleb. in cotton with a mixture of lignin and *Trichoderma viride* (Azimkhodzbayeva and Ramasanova, 1990) and with *Gliocladium* species (Keinath et al., 1990) has been reported. This biocontrol of *Gliocladium* has been attributed to antagonism with the pathogen (Keinath et al., 1990).

Howell et al. (2000) reported that terpenoids and peroxidase activity were not stimulated in the shoots of cotton seedlings treated with *T. virens* that were compared with carrier-treated control seedlings. *Trichoderma virens* treatment might induce the host to respond more quickly to a pathogen attack than an untreated plant would. An increased accumulation of phytoalexins following pathogen attack, without any phytoalexin production in the absence of the pathogen, has been reported in carnation with biological control strains of *Pseudomonas* (van Peer et al., 1991). In addition, a more rapid host response, as indicated by terpenoid phytoalexin production and accumulation, has been correlated with *Verticillium* resistance in cotton (Bell, 1969; Joost et al., 1995). *Trichoderma* might increase the response of cotton to *Verticillium* infection even though no direct induction of defense-related compounds was observed.

The objective of this study was to determine whether seed treatment with *Trichoderma virens* would induce systemic disease resistance in cotton and, if systemic resistance was found, determine whether there was an increased rate of terpenoiod accumulation by the host to the pathogen. To determine whether systemic resistance occurred, we examined the effects of a *T. virens* seed treatment on the severity of Verticillium wilt symptoms in cotton. Although *Verticillium dahliae* naturally infects cotton through the roots, in these experiments it was injected into the stem and thus was used as a model for examining a systemic response by *T. virens*. To examine the effect of the *T. virens* treatment on the rate of response to the pathogen, we determined the terpenoid phytoalexin accumulation over time in the cotton plants.

MATERIALS AND METHODS

Preparation of Inoculum and Seed Treatment

Trichoderma virens isolates used in this study were G-4, a "P" strain that produces the antibiotic gliovirin and G-6, a "Q" strain that produces the antibiotic gliotoxin (Howell et al., 1993). Conidia of *T. virens* from potato dextrose agar cultures were used to inoculate 100 mL of deionized water containing 5% ground wheat bran (w/v) and 1% ground peat moss (w/v) adjusted to pH 4.0 with dilute HCl. Final concentration was approximately 10^5 conidia ml^{-1}, determined with a hemacytometer. The cultures were shaken (150 rpm) at 27 °C for 7 d, at which point the fungal growth was primarily in the

form of chlamydospores. Cultures then were centrifuged at 3000 × g for 10 min; supernatants were discarded, and the solid pellets were air-dried under a laminar flow hood for 24 h. The air-dried material was ground to a powder to provide inoculum (Howell et al., 1997).

Acid delinted and neutralized cottonseed coated with approximately 5 µL of latex sticker per seed (Rhoplex B 15J, Rohm and Haas, Philadelphia, PA) were mixed with 0.1 g of air-dried *T. virens* inoculum per gram of seed to coat the seed (Howell et al., 1997). Control seed was treated with latex sticker and a sterile wheat bran + peat moss preparation. The cultivars used were Rowden, a wilt-susceptible cultivar (Bell, 1969), and Deltapine 50, a cultivar with a moderate level of wilt resistance (Bell, 1992).

Seeds were planted in field soil (Lufkin fine sandy loam mixed 3:2 with sand) in 473-mL cups, two seeds per cup, with 1.23 cm^3 gypsum sprinkled over the soil surface after planting. Cups were incubated at 27°C with a 14-h photoperiod and watered daily. After 4 d, seedlings were thinned to one per cup. Cups were fertilized weekly with 50 mL of 3 g L^{-1} Peters 15-16-17 fertilizer in deionized water.

Plant Inoculation and Disease Evaluation

At six true leaves, the cotton plants were stem-inoculated with *V. dahliae* using the hanging drop method of Bugbee and Presley (1967). The *V. dahliae* strain used was strain PH, which belongs to the vegetative compatibility group P2 (Mace et al., 1990), obtained from Dr. A.A. Bell from the *V. dahliae* collection of the Cotton Pathology Research Unit, USDA, ARS, College Station, TX, and maintained on potato dextrose agar plates at 26°C. For inoculum preparation, plates were flooded with a conidial suspension, allowed to grow for 3 d, then washed with sterile water to remove conidia by the method of Joost et al. (1995). Conidia were washed once with sterile water and diluted to a concentration of 2 to 5×10^7 conidia mL^{-1}. The conidia suspension was drawn into a 3-mL syringe and, with a 22-gauge needle, a single drop was applied to the plant surface on the first internode above the soil line. The needle then was used to stab through the drop into the cotton stele. Plants were inoculated twice, once just below the cotyledonary node, and a second time on the opposite side of the stem halfway between the soil and the cotyledonary node. Controls were inoculated with sterile water. Each treatment consisted of 12 plants. Following inoculation, plants were incubated at 27°C with light and watering conditions described above.

Ten days after inoculation, plants were rated for leaf disease severity using a rating scale for each leaf: 100 = defoliated; 80 = both sides of the midrib of leaf showing chlorosis, necrosis, and wilting or epinasty; 50 = symptoms on only one side of the midrib of the leaf; and 0 = no visible chlorosis, necrosis, or epinasty. Results were combined for all leaves to determine a severity rating for the plant. Two people independently rated disease severity and the ratings for each plant were averaged. A leaf rating was used because yield losses are reported to correlate closely with leaf damage (Bell, 1992). Plant height from the soil line to the apical bud was measured 10 d after inoculation.

Tissue from the stele (the two internodes above the cotyledonary node) of each treatment were cut into 2-cm sections and plated on potato dextrose agar with 25 mg L^{-1} streptomycin sulfate, 25 mg L^{-1} penicillin G, and 50 mg L^{-1} rifampicin. Plates were incubated at 24°C for 14 d and examined every 2 d for growth of fungi from the tissues.

Induction of Defense-Related Terpenoids

Plants were grown from seed treated with G-6 or carrier alone, as described above, and harvested at 48, 72, or 168 h after inoculation with either *V. dahliae* or water and tested for the induction of terpenoids. The first two internodes above the cotyledonary node were collected and kept on ice. The epidermal layer was stripped off and the stele was weighed, cut into ca. 5-mm sections, then soaked for 24 h at 7°C in a volume of 90% acetone, 9.9% water, and 0.1% ascorbic acid equivalent to 3 mL g^{-1} of tissue. The acetone extract was drawn off with a pipette and placed at –20°C to allow particulate matter to settle out. The portion above the particulate matter was subjected to HPLC analysis as previously described (Zhang et al., 1993). HPLC results were compared to standard curves for the cotton terpenoids, desoxyhemigossypol, hemigossypol, desoxyhemigossypol 6-methyl ether,

hemigossypol 6-methyl ether, *p*-hemigossypolone, and gossypol.

Data Analysis

Data were subjected to analysis of variance with the general linear models procedure of SAS (version 6, SAS Institute, Cary, NC) using seed treatment and *Verticillium* inoculation as variables. Data for the different cultivars were analyzed separately. Mean separations were determined by Fisher's protected least-significant-difference (FLSD) test. T-tests were performed using Excel (Microsoft, Bothell, WA). All experiments were performed at least twice.

RESULTS AND DISCUSSION

Wilt symptoms developed on *Verticillium*-inoculated plants of both cultivars, but were not observed on any of the water-inoculated plants. For both cultivars, Rowden and Deltapine 50, plants grown from seed treated with either *T. virens* G-6 or G-4 had significantly lower leaf disease ratings than plants grown from seeds treated with the carrier alone (Fig. 1). There was very little defoliation on Deltapine 50, but the severity ratings were indicative of the level of defoliation on the susceptible cultivar Rowden (26% for control, 18% for G-4, and 16% for G-6). This outcome indicates that seed treatment with *T. virens* offers some amelioration of disease symptoms that extends beyond the seedling stage and affects the aboveground portions of the plant. No significant interactions between seed treatment and *Verticillium* inoculation were found in these tests.

Verticillium dahliae was isolated from the stele tissue of plants with or without *T. virens* treatment following inoculation with the pathogen. The isolation of *V. dahliae* from all inoculated plants tested indicated that the seed treatment did not prevent infection and that the reduction in disease severity was not due to prevention of *V. dahliae* infection following stem inoculation. During natural infection, *V. dahliae* penetrates through the roots; thus *T. virens* would be expected to influence the infection of *V. dahliae* penetrating the roots, similar to the effect seen for *Fusarium oxysporum* infection

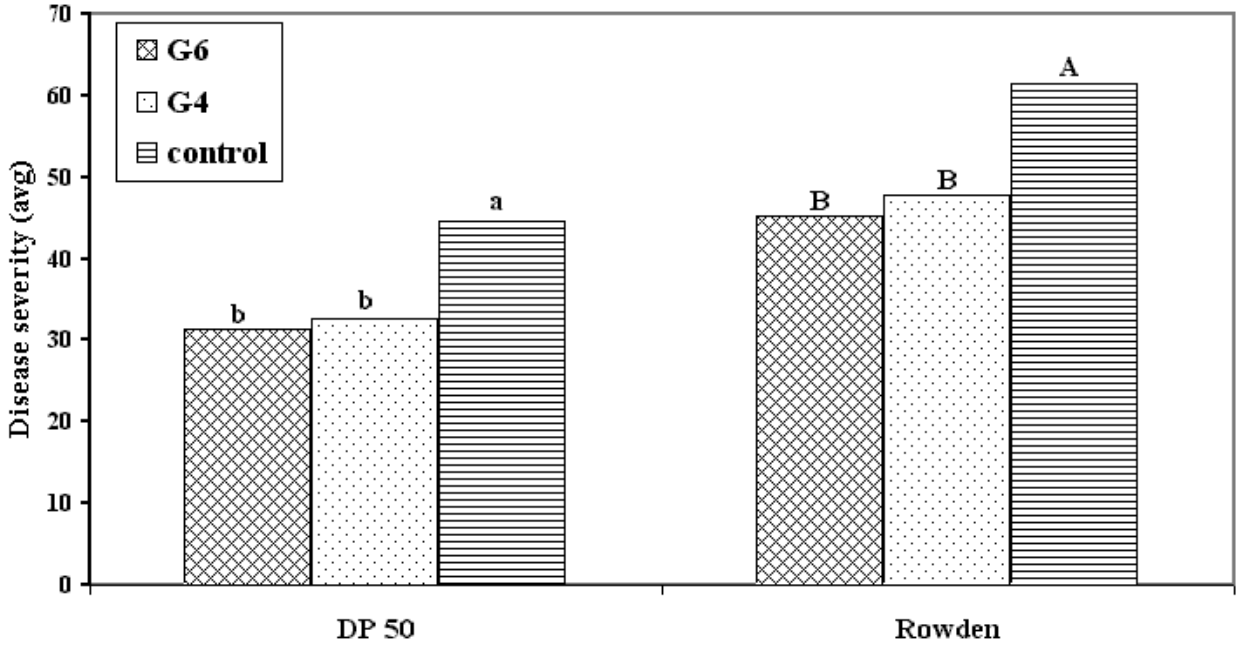

Fig. 1. Effect of seed treatments with *Trichoderma virens* on Verticillium wilt severity of cotton cultivars Rowden (wilt-susceptible) and Deltapine 50 (DP 50, moderately wilt-resistant). Seeds were treated with *T. virens* G-6 and G-4, "Q" and "P" strains, respectively. The control is seed treated with wheat bran + peat moss carrier without *T. virens*. Wilt severity was determined for each leaf and averaged over the entire plant using a scale of 100 = defoliated, 80 = both sides of the midrib of leaf showing chlorosis, necrosis, and wilting or epinasty, 50 = symptoms on only one side of the midrib of the leaf, and 0 = no visible chlorosis, necrosis, or epinasty. Bars within a cultivar topped by the same letter are not significantly different by FLSD (α = 0.05).

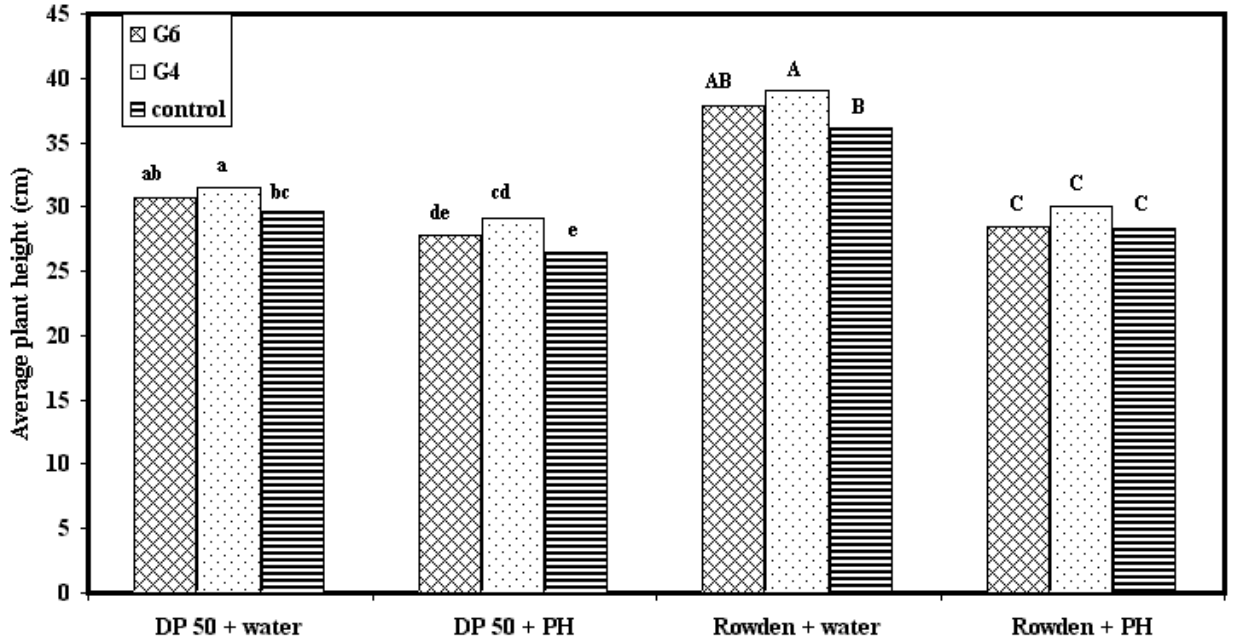

Fig. 2. Effect of seed treatments with *Trichoderma virens* and with and without stem inoculations with *Verticillium dahliae* (Vd) on the height of cotton plants of cultivars Rowden (wilt-susceptible) and Deltapine 50 (DP 50, moderately wilt-resistant). Seed was treated with *T. virens* G-6 and G-4, "Q" and "P" strains, respectively. The control is seed treated with wheat bran + peat moss carrier without *T. virens*. Bars within a cultivar with the same letter are not significantly different by FLSD (α = 0.05).

(Zhang et al., 1996). Because defense-related compounds are induced in cotton roots with *T. virens* seed treatment (Howell et al., 2000), there is a potential for reduction in initial infection that could provide an additional decrease in Verticillium wilt. Also, the presence of *T. virens* in the roots could lead to direct antibiosis by *T. virens* against *V. dahliae*. This possibility was bypassed by inoculating the stem of the plants. Because *Trichoderma* was never isolated from stele tissue of any of the plants, there is no evidence of an opportunity for direct antibiosis or competition between the biological control agent and the pathogen in the aerial portions of the cotton plant.

Inoculation with *V. dahliae* led to a reduction in height of the cotton plants for all of the seed treatments (Fig. 2). Treatment with isolate G-6 of *T. virens* did not have a significant effect on the height of the cotton plants of either Rowden or Deltapine 50. However, plants of both cultivars not inoculated with *V. dahliae* and grown from seed treated with the "P" strain, isolate G-4, were significantly taller than plants grown from seed treated with the carrier alone. Deltapine 50 plants treated with G-4 and inoculated with *V. dahliae* also were significantly taller than the plants grown from seed treated with the carrier alone, whereas the heights of Rowden plants inoculated with *V. dahliae* were not significantly different among seed treatments. This result indicates that some strains of *T. virens* have a growth-promoting effect on cotton. Growth promotion has been reported in other crops with strains of *T. harzianum* (Chang et al., 1986; Windham et al., 1986) and *T. koningii* (Windham et al., 1986), but growth promotion has not been demonstrated with *T. virens* on cotton. Seedling germination or growth during the first 10 d after planting was not significantly different between seeds with and without *T. virens* treatment. Thus, the growth promotion does not appear early in the growth of the plants and is not as pronounced as growth promotion reported on other crops caused by other *Trichoderma* species (Harman, 2000).

Terpenoids were not detected in the stele tissue from water-inoculated plants of either cultivar with any of the seed treatments at 48 or 72 h after inoculation (Table 1). In water-inoculated plants of Deltapine 50, the terpenoids, including desoxyhemigossypol, hemigossypol, desoxyhemigossypol 6-methyl ether, hemigossypol 6-

Table 1. Induction of terpenoids in cotton with and without *Trichoderma virens* seed treatment and inoculation with and without *Verticillium dahliae*.

	Terpenoid concentration (µg compound/g tissue)‡					
Treatment (time)†	**dHG**	**HG**	**dMHG**	**MHG**	**HGQ**	**G**
Rowden, water-inoculated						
Control (72 h)	**n.d.§**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
G-6 (72 h)	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
Control (168 h)	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
G-6 (168 h)	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
Rowden with *Verticillium*						
Control (72 h)	**7.5 ± 3.4¶**	**28.4 ± 10.6**	**3.3 ± 1.5**	**0.05 ±0.08**	**6.7 ± 1.6**	**5.4 ± 1.3**
G-6 (72 h)	**7.2 ± 3.5**	**26.6 ± 11.2**	**2.5 ± 1.3**	**0.14 ±0.20**	**7.1 ± 3.2**	**4.9 ± 1.5**
Control (168 h)	**62.0 ± 38.5**	**137.8 ± 67.9**	**5.3 ± 3.2**	**1.5 ± 1.2**	**26.0 ±15.1**	**39.6±22.7**
G-6 (168 h)	**62.3 ± 26.9**	**130.8 ± 50.6**	**5.4 ± 2.5**	**1.3 ± 1.2**	**18.7 ±12.0**	**31.1±17.2**
DeltaPine 50, water-inoculated						
Control (72 h)	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
G-6 (72 h)	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
Control (168 h)	**45.8 ± 25.5**	**80.2 ± 45.5**	**4.2 ± 3.8**	**4.5 ± 3.8**	**7.1 ± 3.4**	**15.2 ± 9.0**
G-6 (168 h)	**44.7 ± 19.2**	**74.9 ± 31.9**	**3.1 ± 1.8**	**3.6 ± 2.8**	**6.5 ± 2.6**	**13.4 ± 5.8**
DeltaPine 50 with *Verticillium*						
Control (72 h)	**38.9 ± 3.6**	**77.1 ± 6.7**	**7.7 ± 1.8**	**2.6 ± 0.8**	**6.4 ± 1.4**	**7.7 ± 0.8**
G-6 (72 h)	**31.8 ± 7.2**	**67.4 ± 14.7**	**5.0 ± 1.6**	**2.0 ± 0.8**	**7.4 ± 2.0**	**5.6 ± 0.7**
Control (168 h)	**170.3 ± 53.5**	**435.3±102.6**	**23.4±6.0**	**12.9 ± 3.4**	**64.2 ±26.8**	**106.7 ± 23.3**
G-6 (168 h)	**159.7 ± 46.9**	**386.6 ± 99.8**	**21.6±8.4**	**13.3±4.9**	**54.0 ±23.9**	**90.1 ± 40.2**

† Control is seed coated with wheat bran + peat moss carrier without *T. virens*. G-6 is seed coated with isolate G-6 of *T. virens*. Time is the incubation period between inoculation and tissue extraction.

‡ dHG = desoxyhemigossypol, HG = hemigossypol, dMHG = desoxyhemigossypol 6-methyl ether, MHG = hemigossypol 6-methyl ether, HGQ = hemigossypolone, G = gossypol.

§ n.d. = not detected.

¶ Each number is the average from eight samples with standard deviation.

methyl ether, hemigossypolone, *p*-hemigossypolone and gossypol, were detected 168 h after inoculation. However, the levels were significantly lower than in the *V. dahliae*-inoculated plants 168 h after inoculation ($\alpha = 0.01$). Terpenoids were induced in both cultivars at all times tested after inoculation with *V. dahliae*. As expected, the levels of terpenoids were higher in the moderately resistant cultivar, Deltapine 50, than in the susceptible cultivar, Rowden, at all times tested. This result is consistent with previous reports on a correlation between a more rapid host response, leading to higher levels of terpenoids earlier in infection, and *Verticillium* resistance in cotton (Bell, 1969; Joost et al., 1995). However, there was no significant difference in the levels in control plants versus the *T. virens*-treated plants (Table 1).

These results suggest that the reduction in Verticillium wilt found in plants treated with *T. virens* was caused by a mechanism other than an earlier induction of defense responses, at least as far as terpenoid phytoalexins are concerned. This is in contrast to the increased phytoalexin accumulation following stem inoculation with *Fusarium* caused by bacterizing carnation roots with *Pseudomonas* (van Peer et al., 1991).

Further work is needed to determine whether other resistance pathways are induced. Katz et al. (1998) reported that a synthetic activator of acquired resistance in plants could directly induce genes for anionic peroxidase in parsley cells, while genes for Phe ammonia-lyase were potentiated, responding more rapidly to pathogen infection than genes from untreated plants did. Other defense-related systems in cotton might respond differently.

Our results show that seed treatment with *T. virens* can reduce symptoms of Verticillium wilt on cotton in the greenhouse. This reduction occurs even when the pathogen is inoculated directly into the cotton stems, and may be due to induction of resistance in cotton by *T. virens* that can continue beyond the seedling stage. Further research will be conducted with other pathogen systems to determine whether this is a true systemic resistance response, providing reductions in the severity of diseases that are strictly foliar and have no potential for a direct interaction with the *T. virens* colonizing the plant root. Also, treated plants will be tested in the field to determine whether seed treatment with *T. virens* might allow growers to use wilt-susceptible cotton cultivars in areas where *Verticillium* limits cultivar choice.

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