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A study of the characteristics of "P" and "Q" strains of *Trichoderma virens* to account for differences in biological control efficacy against cotton seedling diseases

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Abstract

Strains of *Trichoderma virens* belonging to the "P" group are ineffective as biocontrol agents of seedling disease in cotton, and they are pathogenic to susceptible seed lots. Those strains belonging to the "Q" group are effective biocontrol agents of cotton seedling disease, and they are not pathogenic to cotton. To account for these behavioral differences, comparative assays were made of "P" and "Q" strains for production of phytotoxin, for cellulase, polygalacturonase and protease activity, for induction of phytoalexin synthesis in cotton roots, and for metabolism of pathogen germination stimulants. The results showed little difference in phyotoxin production or enzyme activity between the two groups, and that "P" strain mutants deficient for viridiol production were still pathogenic to cotton. There was also no difference between strains in their ability to metabolize pathogen germination stimulants. HPLC analyses of extracts from roots treated with "P" or "Q" strains, however, showed that "Q" strains induced high levels of phytoalexin synthesis, while "P" strains did not. Treatment of seeds or seedling radicles with combination "P" + "Q" seed coat preparations or cultures filtrates, respectively, ameliorated seedling kill, and increased phytoalexin production in treated roots. These results indicate that an inability by "P" strains to induce high levels of phytoalexins in cotton, not only makes them ineffective as biocontrol agents, but renders them pathogenic to susceptible cultivar seed lots. Induction by "Q" strains of high levels of phytoalexin synthesis in cotton makes them effective biocontrol agents, and it inhibits their development in cotton roots which might lead to pathogenesis.
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Keywords: *Trichoderma virens*; Germination stimulants; Cellulase; Polygalacturonase; Protease; Phytotoxin; Phytoalexins; Pathogenicity

1. Introduction

The imperfect fungus, *Trichoderma virens* (Miller, Giddens and Foster) von Arx, Beih., has been demonstrated on many occasions to be an effective biocontrol agent of seedling and root diseases on a number of crops (Beagle-Ristaino and Papavizas, 1985; Howell, 1982, 1991; Howell et al., 1997; Lumsden and Locke, 1989; Tu and Vaartaja, 1981). Strains of the biocontrol fungus *T. virens* can be separated into two distinct groups on the basis of their antibiotic production (Howell et al., 1993). The "Q" strains of *T. virens* produce the wide spectrum diketopiperazine antibiotic gliotoxin. The "P" strains do not produce gliotoxin, but they do produce a closely related compound, gliovirin, whose activity spectrum is confined to the Oomycetes (Howell and Stipanovic, 1983). Strains of *T. virens* also synthesize the steroid viridiol on low nitrogen containing substrates (Howell and Stipanovic, 1984; Jones and Hancock, 1987). Viridiol is a potent phytotoxin when applied to roots or seeds, and seed treatment with *T. virens* preparations containing high levels of the compound results in dead plants. Later studies on the mechanisms employed by *T. virens* to effect biological control have indicated that antibiotic production by this fungus has little or nothing to do with

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effective biocontrol (Howell and Stipanovic, 1995). The most important mechanisms in the biocontrol of cotton seedling diseases by this fungus appear to be plant phytoalexin induction and metabolism of pathogen germination stimulants produced by germinating cotton seedlings (Howell et al., 2000; Howell, 2002). Preliminary comparative studies on the efficacy of "P" and "Q" strains of *T. virens* as biocontrol agents of cotton seedling disease have indicated that "Q" strains give very effective disease control, while "P" strains do not. The purpose of this research was to determine what characteristics of "P" and "Q" strains of *T. virens* could account for the difference in their biocontrol efficacies. Answers to these questions should help to further elucidate what mechanisms are most important in the biological control process, and they may help to determine what characteristics might best be enhanced to optimize disease control by this fungus.

2. Materials and methods

2.1. Maintenance and culture of microbial isolates

Trichoderma virens "Q" strains G-6, Ga6-3, Fl7-6 and Al5-4 and "P" strains G-4, G-9, Ga6-4, and NC16-1 were maintained on PDA plates containing 50 μg ml^{-1} rifampicin until used. Biocontrol preparations of each strain were produced as shake cultures in a medium consisting of 5 g wheat bran, 1 g peat moss and 100 ml water, adjusted to pH 4.0 with HCl. The cultures were incubated at 27 °C and 150 rpm for 6 days before harvest, and then they were centrifuged at 5000 rpm for 10 min. The supernates were decanted and stored in the freezer, and the solids were spread in sterile petri dishes under a positive pressure hood to dry overnight. The air-dried solids were then placed in Ziploc bags and stored in the refrigerator at 5 °C until used.

2.2. Bioassay of "P" and "Q" strain preparations for pathogenicity to cotton seedlings

Susceptible seed lots of the cotton cultivar Deltapine 451 B/RR were coated with fine granules of the air-dried culture solids of *T. virens* "P" and "Q" strains, described in Section 2.1, after coating the seed with a latex sticker. The seeds were then planted in test tubes containing 10 g moist soil that was not infested with cotton seedling disease pathogens. This soil was the same Lufkin fine sandy loam found in the seedling disease plots, except that it had never been planted with crops. Each treatment, consisting of five tubes, was replicated three times, and the experiment was repeated twice. After 7 days incubation in a growth chamber at 25 °C and with a 14 h photoperiod, the numbers of surviving seedlings were counted. Seeds and seedlings that did not emerge in non-infested soil were harvested, surface sterilized with 1% sodium hypochlorite and 70% ethanol, then washed in sterile water and the embryos plated on PDA containing 50 μg ml^{-1} rifampicin to observe for fungal growth.

2.3. Comparison of "P" and "Q" strains for viridiol production and cellulase, polygalacturonase, and protease activities

2.3.1. Viridiol

The "Q" strain G-6, "P" strain G-4 and viridiol-deficient mutants G4-10V and G4-21V were shake incubated in 100 ml cultures containing 5% ground millet. Cultures were incubated for 6 days at 25 °C, and then the supernates were each extracted twice with 50 ml volumes of chloroform. The chloroform extracts were taken to dryness and dissolved in 2 ml volumes of methanol. Aliquants of the extracts were subjected to HPLC analysis as described previously (Howell et al., 1993), and the viridiol concentrations in the "Q," "P" and viridiol-deficient strains were compared. The pathogenicities of "P" strains G-4, G4-10V, and G4-21V to the cotton cultivar DP451B/RR were also assayed as described in Section 2.2.

2.3.2. Cellulase assay

An agar gel containing 1.7% agar and 0.5% carboxymethylcellulose was prepared, autoclaved, and poured in 15 ml lots into petri dishes. Three 7 mm diameter wells were cut into each plate with a cork borer, and each well was filled with 80 μl of supernates from 6 day shake cultures (5% wheat bran + 1% peat moss) of "Q" strains G-6, Fl7-6 and Al5-4, and "P" strains G-4, G-9, and NC16-1. The wells in control plates were filled with heat killed culture filtrates. After 48 h at 30 °C, the plates were flooded with 2 ml congo red dye (1 mg ml^{-1}). The dye was decanted after 15 min, and the plates were washed with 1 M NaCl. The plates were then observed for the presence of clear zones around the wells. Three replicates were made for each treatment, and the experiment was repeated twice.

2.3.3. Polygalacturonase assay

An assay medium consisting of Raulin-Thom agar medium (Raper and Thom, 1949) containing 0.5% sodium polygalcturonate was prepared, adjusted to pH 5.0, and amended with 0.3 μg ml^{-1} benomyl. The medium was autoclaved and 15 ml aliquants were poured into petri dishes. The plates were inoculated with three 4 mm plugs each of "Q" strains G-6, Fl7-6 and Al5-4, and "P" strains G-4, G-9 and NC16-1. After 72 h incubation at 27 °C, the plates were flooded with 1% hexadecyltrimethylammonium bromide. After 10 min, the excess was decanted and the plates observed for clear zones around the colonies.