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The improvement of competitive saprophytic capabilities of *Trichoderma* species through the use of chemical mutagens

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ABSTRACT

The antagonistic potential of *Trichoderma* strains was assayed by studying the effect of their culture filtrate on the radial growth of *Sclerotium rolfsii*, the causal agent of chickpea collar rot. *Trichoderma harzianum*-1432 (42.2%) and *Trichoderma atroviride* (40.3%) were found to be strong antagonists. To enhance their antagonistic potential, mutagenesis of these two selected strains was performed. Two mutants, *Th*-m_1 and *T. atroviride* m_1, were found to be more effective than their parent strains. The enzymatic activities of the selected parent and mutant strains were assayed, and although both mutants were found to have enhanced enzymatic activities compared to their respective parent strains, *Th*-m_1 possessed the maximum cellulase (5.69 U/mL) and β-1,3-glucanase activity (61.9 U/mL). *Th*-m_1 also showed high competitive saprophytic ability (CSA) among all of the selected parent and mutant strains, and during field experiments, *Th*-m_1 was found to successfully possess enhanced disease control (82.9%).

Introduction

Chickpea (*Cicer arietinum*) collar rot (*Sclerotium rolfsii* Sacc.) is one of the most devastating soil-borne diseases of fungal origin and results in a 10–30% yield loss annually, depending on disease severity. The chemical control of soil-borne pathogens provides a certain degree of control but can simultaneously have adverse effects on the environment affecting beneficial soil microorganisms. Therefore, the biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to existing chemical treatment methods.[1,2] *Trichoderma* spp. are free-living fungi that are common in soil and root ecosystems and have now been established to be opportunistic, avirulent plant symbionts and parasites of several soil-borne phytopathogens.[3] Depending on the strain, the use of *Trichoderma* in agriculture can provide numerous

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advantages: (1) the colonization of the rhizosphere (rhizosphere competence), allowing rapid establishment within stable microbial communities in the rhizosphere; (2) the control of pathogenic and competitive/deleterious microflora through a variety of mechanisms; and (3) improvements in plant health.

In the present study, attempts were made to develop an improved strain of *Trichoderma* for the better management of this disease.

Material and methods

Origin, isolation and maintenance of Trichoderma strains

The sampling sites selected for the isolation of *Trichoderma* from soil were in the areas where the disease caused by *S. rolfsii* was either very low or non-existent in the presence of susceptible hosts. The soil samples were transferred to the laboratory and air-dried at room temperature. *Trichoderma* species were isolated on a selective medium [(TSM) Elad, 1981]. Approximately 20 mL of TSM medium was poured into Petri dishes and allowed to solidify. The serial dilution method was employed to isolate *Trichoderma* from soil samples. One gram of dried soil was added to 9 mL of sterilized distilled water and was serially diluted to a dilution factor of 10^4. Thereafter 200-μL aliquots of soil suspension were spread on TSM. The plates were incubated at 25 ± 2 °C. The colonies appearing on the medium were transferred to PDA. The cultures of *Trichoderma* were also maintained on PDA slants at 4 °C for further study. The identification of *Trichoderma* isolates was made using the taxonomic keys of Rifai (1969), Bisset (1984, 1991a,b and 1992), Kubicek and Harman (1998) and Nagamani et al. (2002).

Effect of culture filtrates of the selected Trichoderma strains on S. rolfsii

The selected *Trichoderma* spp. and *S. rolfsii* were grown on PDA medium in Petri dishes at 25 ± 2 °C for 4 days. Two equal size blocks (5 mm each) of *Trichoderma* species, cut from the actively growing margins of 4-day-old cultures, were inoculated separately into 250-mL Erlenmeyer flasks each containing 100-mL sterilized potato dextrose broth in triplicate. After 10 days of incubation at 25 ± 2 °C, the static cultures were filtered through Whatman filter paper number 44 and then through a Seitz filter (G 4) attached to a vacuum pump to obtain cell-free culture filtrates. Three concentrations of the culture filtrates (10, 20 and 40%) were used for this study. Five-millimeter agar blocks of actively growing colonies from 5-day-old *S. rolfsii* cultures were cut from the colony margin and inoculated at the center of a Petri dish separately containing PDA medium and the culture filtrate. The control set was made by pouring 20 mL of PDA medium only in sterilized Petri dishes. The inoculated Petri dishes were incubated at 25 ± 2 °C and the radial colony growth was measured after 4 days of incubation. The percent inhibition in the radial growth of the colony was calculated using the following formula:

$$\text{Percent growth inhibition} = (C - T)/C \times 100,$$

where *C*, growth in control and *T*, growth in treatment.

Generation of mutant strains through N′-methyl-N′-nitro-N′-guanidine (NTG) treatment

The method of Chadegani and Ahmadjian[4] was followed for mutagenesis of *T. harziaunum*-1432 and *T. atroviride*, which exhibited maximum activity against the pathogen during screening, using N′-methyl-N′-nitro-N′-guanidine (NTG). Spore suspensions from a 10-day-old culture of the selected *Trichoderma* isolates were prepared in 5 mL of sterile 0.1 M sodium citrate buffer (pH 5.5), filtered through cheese cloth, centrifuged twice at 10,000 rpm and subsequently washed with the same buffer. After the second washing, the pellets were resuspended in 5 mL of sodium citrate buffer and the spore concentration was adjusted to 1×10^5 spores/mL using hemocytometer. A stock solution of NTG (1 mg/mL) was prepared in sodium citrate buffer immediately before the treatment and the final concentration used was 50 μg/mL of spore suspension. The NTG-treated spores were incubated at 37 °C in a shaking water bath for 45–90 min to achieve 5–10% viability. At selected intervals, mutagenesis was stopped by passing the entire 4-mL sample through a 0.45-μm Millipore filter, washing the spores twice with 0.1 M phosphate buffer, and finally resuspending the spores in the same buffer. The spores treated with NTG were inoculated on minimal medium for colony forming units. The sensitivity of wild-type isolates of *Trichoderma* to fungicide was tested by amending the culture medium with increasing concentrations of the fungicide.

Effect of culture filtrate of the parent and mutant strains of Trichoderma strains on S. rolfsii radial growth

The effect of culture filtrate of the parent and mutant *Trichoderma* strains on the radial growth of *S. rolfsii* was assayed using the method described in the "Material and methods" section.

Effect on the cellulase and β-1,3-glucanase activity of the selected Trichoderma strains

The selected parent and mutant strains of *Trichoderma* were cultured at 30 °C on a synthetic medium (SM medium). Flasks containing 50 mL of liquid SM medium were inoculated with 2 blocks (5 mm) of mycelia discs cut from the actively growing cultures of the selected mutant and parent strains of *Trichoderma*. The glucose in the medium was substituted with selected carbon sources (0.2%, v/v) and nitrogen with selected nitrogen sources (0.1%, v/v). Cultures were incubated at 30 °C in a rotary shaker at 120 rpm for 4 days and then centrifuged at 15,000 × *g* at 4 °C for 10 min. The supernatant was assayed for chitinase and β-1,3-glucanase activity by the method described as follows.

β-1,3-Glucanase assay

β-1,3-Glucanase was assayed by measuring the release of reducing sugar with DNS.[5] One milliliter of enzyme sample was incubated with 1 mL of 0.2% laminarin in 50 mM sodium acetate buffer (pH 4.8) at 50 °C for 1 h. Two milliliters of copper reagent was then added, and the reaction mixture was boiled for 10 min in a water bath. The tubes were cooled, and 2 mL of arsenomolybdate reagent was added and vertexed; the final volume was adjusted to 25 mL with distilled water. The