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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

solution was centrifuged at 10,000 rpm for 5 min and supernatant aliquots were assayed for their absorbance at 500 nm. The amount of reducing sugars released was calculated from a glucose standard curve. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that catalyzed the release of 1.0 μmol of glucose equivalents per min during the hydrolysis reaction.

Cellulase assay

Cellulase activity was assayed by measuring the release of reducing sugar with DNS.[6] The assay mixture contained 1 mL of 0.5% cellulose (Sigma Co.) suspended in 50 mM citrate phosphate buffer (pH 4.8) and 1 mL of culture filtrates of different *Trichoderma* strains in 15-mL test tubes. The reaction mixture was incubated for 30 min at 50 °C and then centrifuged at 12,000 rpm for 5 min at 4 °C. The reaction was arrested by adding 3 mL of 1% DNS (dinitrosalicylate) reagent in 1 M NaOH and followed by heating for 10 min at 100 °C to develop a red-brown color. While it was hot, 1 mL of 40% Rochelle salt (potassium sodium tartrate) was added to stabilize the color. Blanks were generated the same way using distilled water in place of culture filtrate. After cooling the mixture to room temperature in a water bath, the absorbance was measured with a spectrophotometer (Systronics spectrophotometer) at 540 nm. The glucose content was obtained using a glucose standard prepared by the same procedure. One unit of cellulase activity was defined as the amount of enzyme in 1 mL of the reaction mixture that released 1 μmol of reducing sugar under assay conditions.

Competitive saprophytic ability (CSA) of the selected Trichoderma strains

This experiment was performed following the Cambridge method,[7] later modified by Ahmad and Baker.[8] A pure inoculum of *S. rolfsii* was prepared in sterilized 500-mL conical flasks each containing 200 g of acid-washed sand + 3% maize meal. Each flask was inoculated with three blocks of *S. rolfsii* culture (5-mm diameter). The moisture level of the sand-maize meal was maintained at 25%, and the pH was adjusted to 6.5. The flasks were incubated at 25 ± 2 °C for 10 days. The parent and mutant strains of *Trichoderma* strains were grown on PDA. The plates were incubated at 25 ± 2 °C for 5 days. The plates were then flooded with sterile distilled water, and the conidia were gently freed from the culture plate with the help of brush. The suspension was sieved through four layers of cheese cloth and then centrifuged at 2500 × *g* for 15 min. The number of conidia in the suspension was counted with a hemocytometer, and their numbers were then adjusted to the desired level. The *S. rolfsii* inoculum-soil mixture was mixed with freshly harvested conidia of parent and mutant strains of *Trichoderma* at rates of 10^1, 10^2, 10^3 and 10^4 conidial suspension in 0.1 mL per gram of soil. No conidia were added to the controls. The soil was mixed thoroughly by hand and was filled in 500 mL flasks. Twenty pieces (2 cm in length and 0.2–0.5 cm in diameter) of sterilized chickpea roots were buried in 500-mL conical flasks containing 250 g of inoculum mixture of antagonist and pathogen for each dilution. The flasks were incubated at 25 ± 2 °C for four weeks, and then, root bits were taken out and washed 10–12 times with sterilized distilled water. The washed root pieces were then surface-sterilized with 0.1% sodium hypochlorite solution and 5% ethanol for 5 min and again washed thoroughly with sterilized distilled water. The root pieces were then soaked between the folds of sterilized blotting papers and transferred to Petri dishes containing 20 mL TSM (*Trichoderma* selective medium). Five root pieces were placed onto the medium in each Petri dish. The experiments were set in three replicates for each dilution. All of the Petri dishes were incubated at 22 ± 2 °C for 6 days, and the percent colonization of root pieces by the test antagonist was recorded.

Biological control of collar rot of chickpea in the field

The parent strains of *T. harzianum*-1432 and *T. atroviride* and their most potent mutants, *Th-m1* and *T. atroviride* m_1, were used for field experiments. The plot size (1.5 m × 1.5 m) was prepared and used for each combination in triplicate. All of the plots were infected with the pathogen by amending 150 g of pure inoculum prepared on barley grains, and the plots were left for 20 days to allow the pathogen to establish itself in the soil. Then, 450 g of antagonists prepared on barley grains were also amended in field soil and left for 10 days. Surface-sterilized seeds of the susceptible variety of chickpea were sown at a rate of 100 seeds per plot. The development of disease was monitored regularly, and complete disease appearance was noted after 14 days of sowing. The percent mortality and percent disease control were calculated using the following formulae:

$$\text{Mortality (\%)} = \frac{\text{No. of seedlings in un-infested pot soil} - \text{No. of seedlings in infested pot}}{\text{No. of seedlings in un-infested pot soil}} \times 100$$

$$\text{Disease control (\%)} = \frac{\text{Mortality (\%) in control} - \text{Mortality (\%) in treatment}}{\text{Mortality (\%) in control}} \times 100$$

Results

The pure culture of the pathogen isolated from the infected field of chickpea suffering from collar rot disease and the different strains of *Trichoderma* were maintained on PDA medium at 25 ± 2 °C. Fig. 1 shows the effect of different concentrations of culture filtrates of potent *Trichoderma* species on the percent inhibition of *S. rolfsii* radial growth. Three concentrations of culture filtrates (10%, 20%, and 40%) were used in this study. The inhibition rates of *S. rolfsii* radial growth varied at every concentration of culture filtrate for all of the *Trichoderma* used. Fig. 1 shows that the maximum inhibition of radial growth of *S. rolfsii* by all strains was found at the 40% concentration of culture filtrate, compared to the 10% and 20% culture filtrate concentrations. It is evident that the maximum percent inhibition of *S. rolfsii* was found at 40% *T. harzianum*-1432 (42.2%) followed by *T. atroviride* (40.3%).

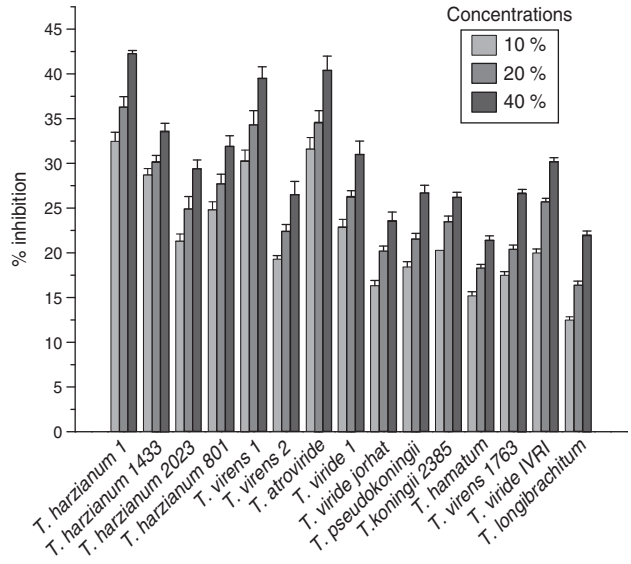

Fig. 1 – The effect of different concentrations of *Trichoderma* species culture filtrate on *Sclerotium rolfsii* radial growth.

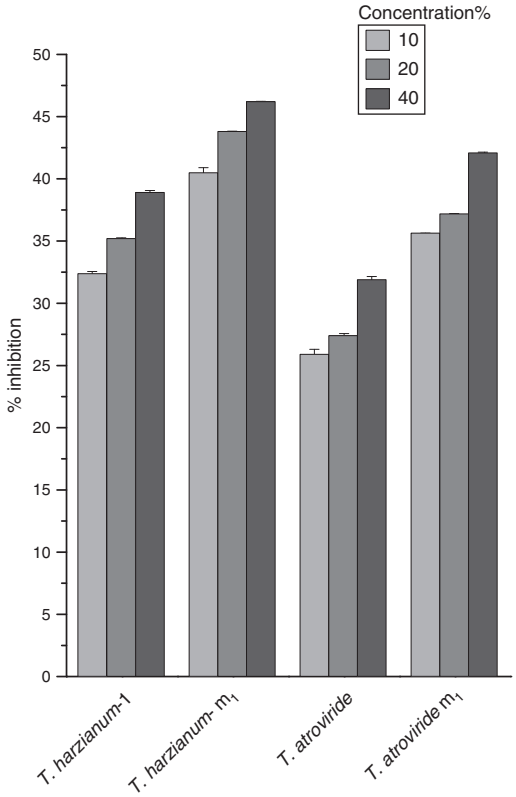

Fig. 2 – Effect of culture filtrates of *Trichoderma* parent and mutant strains on the percent inhibition of *S. rolfsii* radial growth.

The most effective antagonists tested, *T. harzianum*-1432 and *T. atroviride*, were selected for the generation of mutant strains to enhance the efficacy of anti-*S. rolfsii* antagonism. The radial growth of wild-type isolates was inhibited by approximately more than 90% at 50 ppm of carbandazim in the medium. The treatment of spores with NTG was much more stable than with UV irradiation and its resultant isolates. These mutagenized strains possessed significant levels of carbandazim tolerance and a higher growth rate than their parent strains. Ultimately, 2 isolates, one derived from *T. harzianum*-1432 and one derived from *T. atroviride*, were obtained after a series of 11-to-13 serial transfers on media with increasing concentrations of fungicide up to 100 ppm. Tolerant isolates were capable of growing at fungicide concentrations lethal to wild-type isolates and were stable following culture on unamended medium. Tolerance was also found to be unaltered when isolates were retrieved from long-term storage cultures in the absence of fungicide (PDA slants covered with paraffin oil). These mutants were named *Th*-m_1 and *T. atroviride* m_1. They showed different and characteristic morphological features as well as specific growth patterns, which differed from their parent strains.

The effect of different culture filtrate concentrations of *Trichoderma* parent and mutant strains on the percent inhibition of *S. rolfsii* radial growth is shown in Fig. 2. As the concentration of culture filtrates was increased (20–40%), the percent inhibition also increased. It is evident from Fig. 2 that *Th*-m_1 showed maximum percentage of *S. rolfsii* inhibition at all three concentrations tested in this study compared with other mutant and parent strains tested.

The production of β-1,3-glucanase by mutant strains compared to their respective parent after 2, 4 and 7 days of incubation has been presented in Fig. 3A. It is evident from this figure that the β-1,3-glucanase activity of each strain increased as the days passed, but after 4 days of incubation, they follow the same pattern as in cellulase activity. The maximum enzyme activity was shown by *Th*-m_1 (61.9 U/mg) followed by *T. atroviride* m_1 (46.5 U/mg) in 4 days of growth under the same growth conditions.

Fig. 3B shows the production of cellulase by *Trichoderma* parent and mutant strains at 2, 4 and 7 days. The maximum cellulase activity was recorded for all strains used in the study 4 days after the inoculation of spores. The rate at which the enzyme appeared in the culture medium was higher in the case of *Th*-m_1 (5.69 U/mL) followed by *T. atroviride* m_1 (4.11 U/mL).

The percent colonization of chickpea root segments by selected parent and mutant strains of *Trichoderma* is shown in Fig. 4. The data revealed that when root segments were removed after 2, 4 and 6 days of incubation from the soil infested with the conidia of *Trichoderma* strains, the two parent strains, *T. harzianum*-1432 and *T. atroviride*, were recovered from root segments less frequently than the mutant strains used in the study and were slow to colonize root segments at higher population densities. However, the mutant *T. atroviride* m_1 was isolated from the root pieces only at higher population densities. From Fig. 4, it is clear that *Th*-m_1 showed a significantly higher percent colonization than the other mutant strain at all population densities on all studied days.

Results presented in Fig. 5 show that *Th*-m_1 possessed the maximum percent of disease control (82.9%) and minimum percent mortality (10.6%). The two parent strains, *Th*-1432, *T. atroviride* and the mutant *T. atroviride* m_1, were found less significant to control the disease than *Th*-m_1.

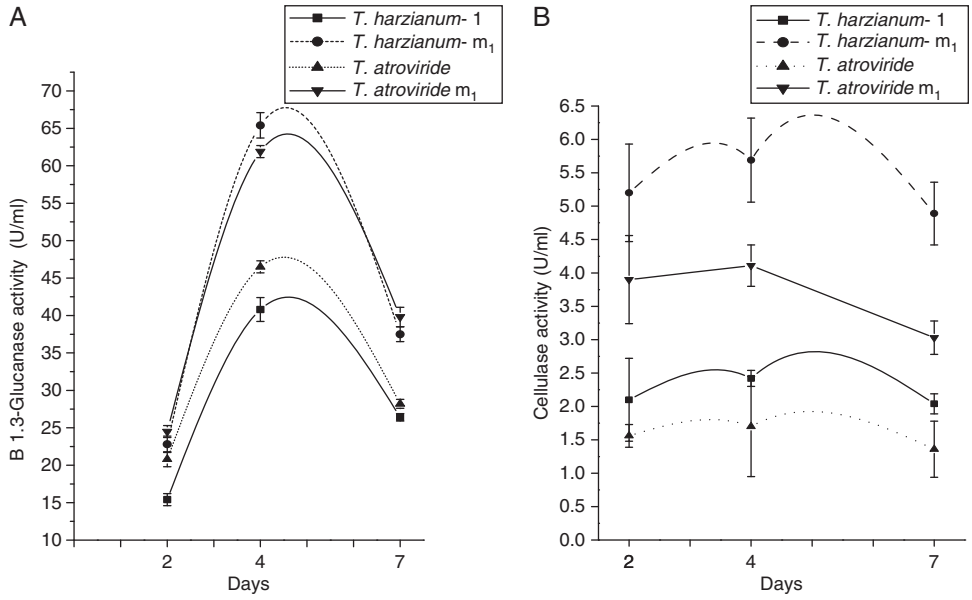

Fig. 3 – (A) The β-1,3 glucanase activity of selected *Trichoderma* species. (B) The cellulase activity of selected Trichoderma species.

Discussion

The pathogen *S. rolfsii*, which responsible for chickpea collar rot, is globally distributed and is more competitive for nutrients than most other soil microorganisms. The biocontrol of this pathogen through *Trichoderma* species has proven to be a good alternative because of their high reproductive capacity, ability to survive under unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defense mechanisms.

Culture filtrates have been used in the present study to demonstrate the possible presence and role of fungal metabolites in the process of *Trichoderma* species antagonist behavior.[9,10] Three concentrations of culture filtrates (10%, 20%, and 40%) were used in this study. Fifteen strains of *Trichoderma* species were screened against *S. rolfsii in vitro* and were found to produce varying degrees of inhibition on *S. rolfsii* radial colony growth. Only two strains were found effective (Fig. 1). The maximum percentage of *S. rolfsii* inhibition was found at the 40% concentration of *T. harzianum*-1432 (42.2%), which was followed by *T. atroviride* (40.3%) (Fig. 1). The inhibition of *S. rolfsii* radial growth by all strains was found to be higher at 40% concentration than at the 10% and 20% culture filtrate concentrations. It has been reported that the production of metabolites from different *Trichoderma* strains depends on ecological factors, so the strains showed varying effect on pathogens.[11] *T. harzianum*-1432 and *T. atroviride* appeared to be strong antagonists against the pathogen. The antagonism of *Trichoderma* species against several pathogens has been reported.[12–14]

It has been shown that not all *Trichoderma* species possess the mechanisms and characteristics deemed necessary for optimum biocontrol. Very often, those strains that have the capacity to produce enzymes and antibiotics that are associated with biocontrol are not the ones that have good storage qualities or function well at temperatures and moisture levels where pathogen flourish. Additionally, effective pathogen control may require 10^5–10^6 propagules to be present in one gram of soil in the case of *Trichoderma*. The presence of such high amounts of conidia is undesirable from an ecological, economic, and public health perspective. To solve this problem, one of the important aims of the present investigation was to develop a biocontrol strain that had improved biocontrol potential and increased tolerance to carbandazim.

Effective tools for strain improvement include mutagenesis.[15,16] Peterson and Nevalainen,[17] discussed progresses in the field of *Trichoderma* strain improvements. Mutation has been manipulated to improve production of antifungal metabolites and the antagonistic potential of biocontrol agents to control a broad spectrum of phytopathogens.[16,18]

To obtain a mutant strain that showed enhanced antagonistic capability against *S. rolfsii*, mutation was performed through NTG treatment. In this way, we obtained two mutants, namely *Th*-m1 and *T. atroviride* m_1 that can also tolerate high levels of fungicide. The development of *Trichoderma* mutants for the suppression of fungal plant pathogens is an important method of strain improvement, which yields effective and reliable strains for biological control. After the development of mutants, assessing the bio-efficacy through various techniques is equally important for the suppression of the pathogen.[19] The effect of different culture filtrate concentrations of the parent and mutant *Trichoderma* strains on the percent inhibition of *S. rolfsii* radial growth were also studied. It was evident from the observation that maximum percent inhibition of *S. rolfsii* was found at 40% for *Th*-m_1 (46.2%) Variations among the mutants have been due to silent mutations

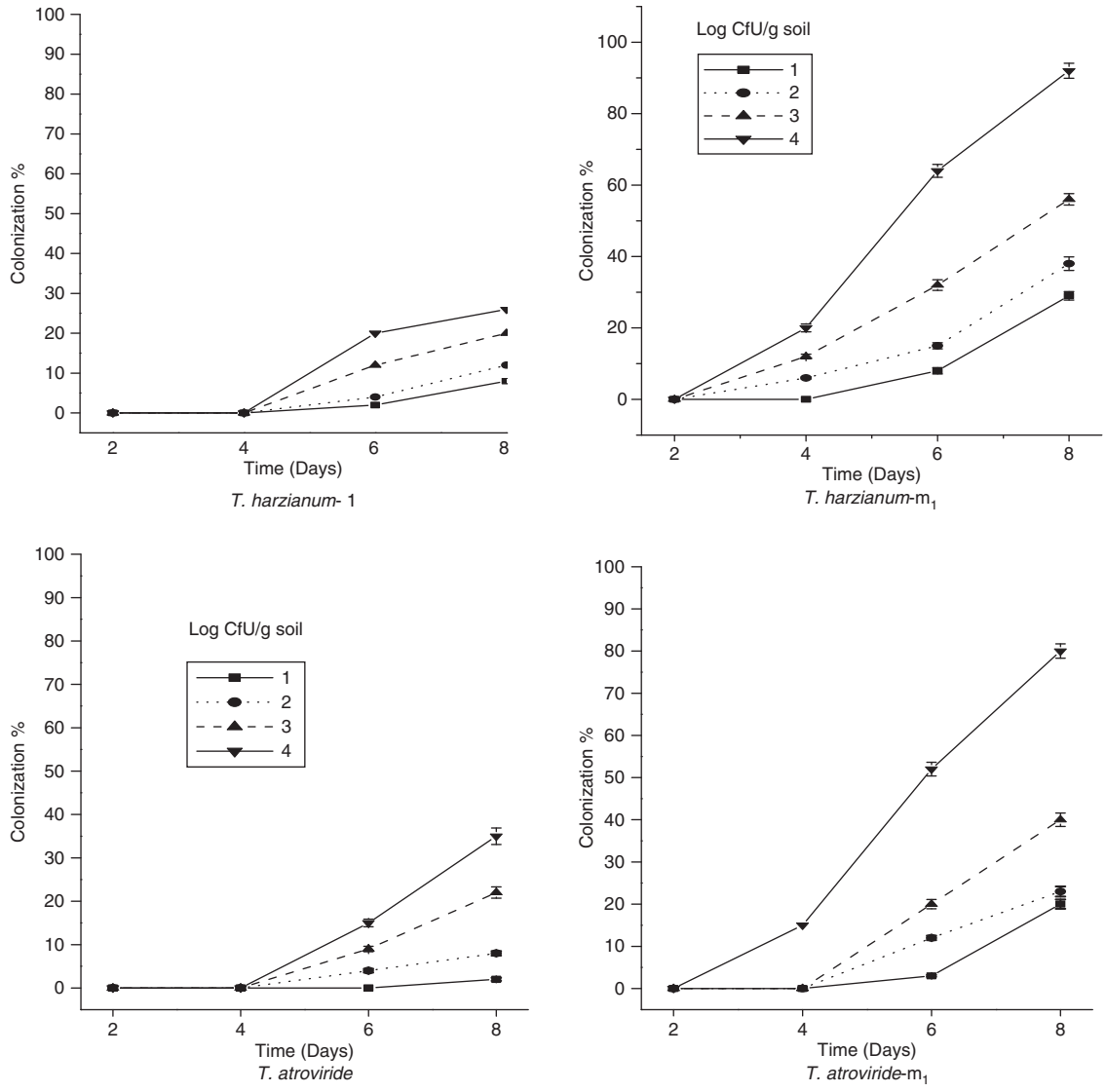

Fig. 4 – The percent colonization of *Trichoderma* strains on root fragments when 1, 2, 3 and 4 log cfu of these strains were added to the soil.

induced during mutagenesis of wild-type *Trichoderma* strains to produce different levels of hydrolytic enzymes and antifungal metabolites.

The isolation of mutants showing increases in the production of several extracellular enzymes is a promising approach to improve biocontrol agents.[20] Enzyme activities of the selected parent and mutant strains were therefore studied. Observations of enzyme activities were made after 2, 4 and 7 days. *Trichoderma* strains have been extensively studied for their ability to produce extracellular cellulolytic enzymes, namely endoglucanases, exoglucanases and cellobiase, which act synergistically to convert cellulose to glucose.[21] These strains have often been mutagenized and genetically modified to obtain improved strains capable of producing higher cellulase levels.[22,23]

The mutant *Th*-m_1 produced the maximum amount of cellulase in the medium when supplemented with cellulose as a substrate, whereas other *Trichoderma* strains released this enzyme in much lower amounts comparatively. Ahmad and Baker[8] reported that mutants with higher cellulase activity than that of wild-type strains can utilize cellulose substrates on or near the root more efficiently.

Because β-1,3-glucanase is a structural component of fungal cell walls, the production of this enzyme has been reported to be an important enzymatic activity in the biocontrol of microorganisms.[3] It is evident from Fig. 3B that maximum β-1,3-glucanase activity was exhibited by *Th*-m_1 (61.9 U/mL). Direct evidence for the involvement of glucanases in mycoparasitism has been demonstrated by Lorito et al.[3] Many β-1,3-glucanases have been isolated, and their genes have been cloned.[24]

The data from the present study demonstrate that *Th*-m_1 produces higher amount of enzymes with higher cellulase and β-1,3-glucanase activity than that of the other selected strain. These results strongly support the contention that *Th*-m_1, as it released cellulase and β-1,3-glucanases in

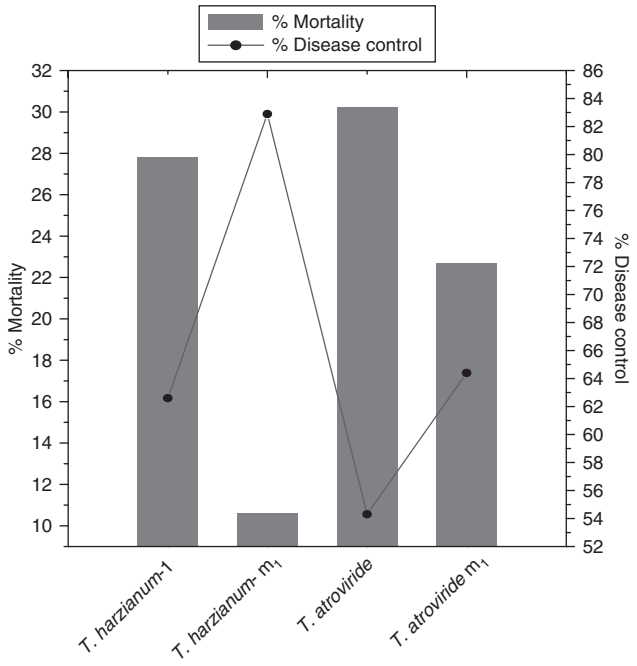

Fig. 5 – The effect of selected *Trichoderma* parent and mutant strains on the percent mortality due to chickpea collar rot and the percent reduction in disease over the control in sterilized potted soil.

significant amounts, possessed stronger mycoparasitic capabilities than the other selected parent and mutant *Trichoderma* strains. The coordinated activity of these enzymes released by *Th*-m_1 makes it capable of degrading different polymers, which leads to the effective breakdown of pathogen cell walls.

The extent to which an antagonist can survive in the presence of soil-borne pathogen depends on its competitive saprophytic ability, which may be assessed by the competitive saprophytic colonization of substrate units buried in graded series of inoculum dilutions of the pathogen.[8] The soil moisture content plays an important role in the activity and survival of *Trichoderma*.[25] Cumagun et al.[26] also found that the higher the amount of inoculum of *T. harzianum* strain No. 94-016, the higher the percent colonization of rice straw in the soil. The CSA of selected *Trichoderma* strains was studied in the present investigation. *Th*-m_1 possessed a significantly higher percent colonization than the other mutant strains at all population densities on all days of incubation, whereas *T. atroviride-*m_1 were recovered only after 4 days of incubation. The parent strains *Th*-m_1 and *T. atroviride* were recovered from root segments less frequently than the mutant strains and were very slow to colonize the root segments at higher population densities (Fig. 4). *Th*-1432 possesses higher CSA, which may be directly correlated with its higher cellulolytic activity.[8]

The parent and mutant strains of *T. harzianum*-1432 and *T. atroviride* were applied for field studies. When they were amended in the soil, it was observed that *Th*-m_1 significantly suppressed the pathogen population (Fig. 5). Under field conditions, *Th*-m_1 significantly decreased disease incidence.

The results obtained after field application of the antagonist revealed that the disease incidence was significantly reduced because of the high efficacy of the antagonist mutant strain against the pathogen by the enhanced production of extracellular enzymes.[27,28,29] The disease control potential of *Th*-m_1 may also be correlated with its high CSA. The results of biological control studies in pot and field conditions indicate that *Th*-m_1 has the potential to serve as a biocontrol agent of chickpea collar rot.

Conflicts of interest

The authors declare no conflicts of interest.

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