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RESEARCH ARTICLE

Morpho-cultural and biochemical characterization of *Trichoderma* isolates and their efficacy in controlling cotton root rot

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Abstract

Trichoderma species are essential for crop productivity, nutrient uptake, abiotic stress resistance and root growth and development. This study was conducted to evaluate the morpho-cultural, biochemical and biocontrol activities of different *Trichoderma* isolates against cotton root rot. It was observed that maximum mycelial growth (53.67 mm) was recorded by HST2, whereas the least (36.33 mm) was shown by HMT2 after 6 DAI (days after incubation). All the isolates had colonies that ranged in colour from light green to pale green to dark green. The native *Trichoderma* isolates showed reverse colony colours from creamish white and colourless to yellowish, accompanied by wavy to smooth colonyedges. Conidia ranged in length from 2.69 to 4.66 µm. Among the isolates, HFtT produced the longest conidia, with an average length of 4.66 µm. The conidial width of the isolates ranged from 2.31 to 3.65 µm, with the maximum width (3.65 µm) recorded in HFtT. The native HST2 isolateshowed positive results for all the biocontrol activities. The antagonistic potential of different native *Trichoderma* isolates against cotton root rot causing pathogens RB5 and RS2 was evaluated under both *in vitro* and *in vivo* conditions. *In vitro* dual-culture results revealed that HST2 exhibited the highest mycelial growth inhibition (72.78 % in RB5 and 72.50 % in RS2), followed by HMT2. Under *in vivo* conditions, minimum disease incidence and maximum disease control were shown by HST2 (10 % and 10 %) and (57.13 % and 57.13 %) respectively, in both Desi and American cotton.

Keywords: antagonistic; biocontrol; colony; enzyme; essential; mycoparasitic, *Trichoderma*

Introduction

Trichoderma has been recognized for its biocontrol properties since the 1920s, with various species shown to promote plant growth and effectively reduce disease occurrence. Rhizospheric competence is the capacity of *Trichoderma* species to colonize and flourish next to plant roots. The ability of *Trichoderma* species to biocontrol a wide variety of soil-borne illnesses, such as *Macrophomina*, *Pythium* and *Fusarium*, is well documented. *Trichoderma* species are well known for their ability to enhance plant systemic resistance, thereby influencing plant development and reducing susceptibility to disease. *Trichoderma* uses a range of tactics to fight phytopathogens, including resource competition, mycoparasitism, antibiotic synthesis, secondary metabolite production, etc. Mycoparasitism is the main biocontrol technique that *Trichoderma* employs to fight phytopathogens. One of the main theories put forth to explain *Trichoderma* spp.'s antagonistic activity against soil-borne plant-pathogenic fungi is their direct mycoparasitic activity (1 –4). Many enzymes, including chitinase, glucanase, cellulase, xylanase and lipase are produced by the fungus and play a major role in antagonism. These enzymes also aid in reducing plant infections. The polysaccharide, chitin and β-glucan chains that provide the cell wall its stiffness are attacked and broken down by these enzymes (5 –8).

Numerous studies have been conducted to gain a better understanding of the chitinase, cellulase, xylanase and glucanase systems of *Trichoderma* spp. during growth on different carbon sources. Understanding the mechanisms underlying the regulation of these enzymes are essential in selecting the most effective strain of *Trichoderma* for biocontrol. *Trichoderma* spp. is commonly employed as biocontrol agents in agriculture because of their ability to reduce the prevalence of diseases brought on by plant pathogenic fungi, particularly a number of prevalent soil-borne diseases (9, 10). It has been proposed that the level of biological control of *Sclerotium rolfsii*, *Rhizoctonia solani* and *Pythium aphanidermatum in vivo* can be linked to the lytic activity of multiple *T. harzianum* strains on their cell walls (3). *Trichoderma* undergoes morphological changes during mycoparasitism, such as coiling and the development of appressorium-like structures that facilitate the parasite's entry into the host (11). The current

study examined the capacity of *Trichoderma* species to produce cell wall-degrading enzymes.

Tubeuf and Smith coined the terms "biocontrol" and "biological control" in 1914 and 1919 respectively, with reference to plant pathogens and insects. As a component of integrated disease management, biocontrol is the process by which naturally occurring organisms reduce the population of plant pests. An antagonist, a biocontrol agent against plant infections, stimulates research and development across a wide range of domains to manage the pest and satisfy the demands of the growing human population. While plant pests include weeds, insects and plant pathogens, these hostile microorganisms are members of different bacterial and fungal groups.

Since biocontrol agents (BCAs), often referred to as bio-fungicides, are affordable, environmentally safe and capable of providing long-term control of pathogen growth, their isolation and application are necessary for sustainable disease management. Nearly all plant diseases and infections can be treated by biological control, or naturally existing microorganisms that are already present in the soil and the environment and have antagonistic effects. Only between 1–10 % of microbes show the ability to inhibit pathogen growth *in vitro* when fungal and bacterial isolates are tested for biocontrol activities. Certain agents are effective in suppressing plant pathogens under suitable in vivo conditions, whereas a few possess broad-spectrum activity against a wide range of pathogen taxa. *Agrobacterium*, *Ampelomyces candida*, *Bacillus*, *Coniothyrium*, *Pseudomonas*, *Streptomyces* and *Trichoderma* are some significant microbes from various genera that are presently being marketed globally to encourage farmers to adopt organic farming practices by lowering their use of chemical pesticides (12). These BCAs act against pathogens using a range of mechanisms, including hyperparasitism, competition, activation of host resistance, antibiotic synthesis (e.g., phenazines, 2,4-diacetylphloroglucinol), production of lytic enzymes (chitinases, proteases, glucanases), emission of metabolites (hydrogen cyanide, ammonia, carbon dioxide) and physical or chemical interference, such as blocking soil pores (13).

Trichoderma as a biological control agent

Trichoderma was first identified as a biocontrol agent in the early 1930s and species of the genus are cosmopolitan, free-living fungi that decompose organic and vegetable matter in soils (14, 15). *Trichoderma* species are abundant in almost all soil types and serve as effective antagonists with strong biocontrol potential against economically important soil-borne plant pathogens (16–18). Biocontrol antagonists are essential for managing plant diseases and parasitic microorganisms (19–21). *Trichoderma* combats other plant-pathogenic fungi while promoting the growth of plants and roots. It uses a range of tactics, including host cell resistance, mycoparasitism, competition for resources and space and antibiosis, to control plant pathogenic illnesses. *Trichoderma* species can control and antagonise a wide range of economically important postharvest phytopathogenic fungi, as well as certain bacteria and viruses (22, 23). Although there is a wealth of information on *Trichoderma* nutrition in the literature, little is known about the effects of particular carbon and nitrogen nutrients on the large-scale synthesis of *Trichoderma* antagonists (24). It is often regarded as an aggressive competitor that quickly colonizes the pathogen, particularly soil-borne pathogens like *Rhizoctonia*, *Phytophthora* and *Fusarium* species.

Materials and Methods

Isolation, purification and multiplication of native *Trichoderma* isolates

Collection of samples

Four cardinal sites inside the rhizosphere of cotton plants were used to gather soil samples from various cotton-growing regions in Haryana. Soil samples were taken from a depth of 10 to 15 cm at horizontal intervals of 100 to 120 cm using a soil auger that was driven into the ground to a depth of about 10 to 15 cm and then removed. A steel handle was used to push the dirt core out of the tubular auger and a sterile knife or blade was used to remove it. Approximately 500 g of the soil core sample were taken out, placed in polyethylene bags, covered with wire mesh or lids and labelled with the date and location of the sample collection sites. As quickly as feasible, collected soil samples were transferred to the laboratory and kept at 4 ℃ until needed. The soil samples were properly mixed and dried. For the purpose of isolating *Trichoderma* from soil, 1 g of the soil was obtained and serially diluted.

Isolation of native *Trichoderma* from soil samples

The serial dilution method was used to extract the isolated *Trichoderma* from the collected soil samples. The soil samples were collected from different cotton-growing districts in Haryana viz., Bhiwani, Charkhi Dadri, Faridabad, Fatehabad, Gurugram, Jhajjar, Mahendergarh, Rewari, Rohtak and Sirsa (Table 1).

The collected samples were finely crushed, mixed thoroughly and allowed to dry in the shade before being serially diluted. Test tubes were labelled 10^{-1}, 10^{-2}, 10^{-3} and up to 10^{-8} using glass marking pencils. The first test tube received 10 mL of distilled water, whereas the other test tubes received 9 mL. The test tubes capped with non-absorbent cotton plugs were autoclaved for 20 min at 121.6 ˚C and 15 psi of pressure. After

Table 1. Details of native *Trichoderma* isolates collected from different districts of Haryana

Sl. No.	District	Latitude	Longitude	Nomenclature of isolate
1.	Bhiwani	28.79	76.13	HBhT
2.	Charkhi Dadri	28.59	76.26	HCdT
3.	Faridabad	28.39	77.31	CFrT
4.	Fatehabad	29.51	75.45	HFtT
5	Gurugram	28.45	77.02	HGT
6.	Jhajjar	28.60	76.65	HJT
7.	Mahendergarh	28.26	76.15	HMT1
8.	Mahendergarh	28.27	76.15	HMT2
9.	Rewari	28.18	76.61	HRT
10.	Rohtak	28.89	76.60	HRkT
11.	Sirsa	29.55	75.00	HST1
12.	Sirsa	29.53	75.02	HST2

cooling, the first test tube, which held 10 mL of sterilized water, was filled with 1 g of representative soil to produce the initial dilution in test tube 10^{-1}. To ensure complete mixing, the test tubes were rolled back and forth between the palms for 5 min, resulting in a uniform dispersion of the soil sample. Using a fresh, sterile pipette, 1 mL of the suspension from the first dilution was removed while the object was in motion and placed into the test tube labelled 10^{-2}. Additionally, 9 mL of sterile water were added. The same procedure was carried out until the initial sample was diluted to a 10^{-8} ratio.

Every time a pipette was used, it was sterilized. One mL of the suspension from each dilution was added to the centre of the sterile water blanks using a sterile pipette. Following the final step, 1 cc of the dilution suspension was added to each petri plate containing potato dextrose agar (PDA). Glass marking pencils were used to mark the plates. A sterile, laminar air-flow environment was used for the entire process. For five to seven days, these plates were kept in a BOD incubator at 26±1 ˚C. Every day, the plates were inspected to look for *Trichoderma* spp. growth.

The macroscopic and microscopic features of the fungal colonies were analysed in order to conduct an initial screening for *Trichoderma* species. Slides were prepared for microscopic screening and the colonies' growth rate and colours were assessed for macroscopic screening. For microscopic examination, the mycelium of each isolate was removed from the PDA plate, spread onto a sterilized slide, mounted with a drop of water, covered with a coverslip and viewed under a light microscope at 10x and 40x.

Purification of *Trichoderma* isolates

For purification, a bit of mycelium was cut with the help of a sterilized cork borer and transferred to sterile PDA plates. The plates were incubated at 25˚C for 5–7 days in a BOD incubator. Later, the bit of mycelium was transferred onto PDA slants and a pure culture was obtained by the hyphal tip method (25). Within 5 –7 days pure culture was obtained and it was stored at 4 ˚C for further studies.

Cultural and morphological characterization of native *Trichoderma* isolates

The purpose of this experiment was to investigate potential morphological differences between various native *Trichoderma* isolates. To do this, 15 mL of sterilized PDA were added to petri plates, which were then inoculated with a 5 mm mycelial disc that had been cut off from the margin of the actively growing colony using a cork borer. The plates were then stored for five days at 25 ˚C in a BOD incubator. The colony characters were noted. For the mycelial, conidial and phialid characteristics, a loopful of *Trichoderma* isolates was taken from a five-day-old culture and put on glass slides that had been mounted with a drop of water and covered with a coverslip. The slides were examined under a compound fluorescent microscope with magnifications of 10x and 40x and notes were made on the observations.

Biochemical characterization of native *Trichoderma* isolates

Hydrogen cyanide (HCN) production assay

The Bakker and Schippers approach was used to measure the production of HCN (26). Glycine was subjected to cyanogenesis by microorganisms, which produced HCN. When HCN reacted with picric acid in the presence of Na_2CO_3, its colour shifted from deep yellow to orange to dark brown due to its volatile nature. Colour changes occurred when filter paper was dipped in a 0.5 % picric acid solution and 0.2 % (w/v) Na_2CO_3. After that, the filter paper was put in test tubes and allowed to incubate at 28 ˚C for 72 hr. A test was deemed positive when the strips' colour changed from deep yellow to brown; a test with no colour shift was deemed negative.

Indole acetic acid production assay

The production of IAA was measured using a modified version of the Brick technique (27). Fungal cultures of native *Trichoderma* isolates were grown on potato dextrose broth (PDB) with 1 g/L of L -tryptophan as a precursor for 72 hr at 25 ˚C. The native *Trichoderma* isolate cultures were incubated and the supernatant was collected by centrifuging them for 30 min at 3000 rpm. Two drops of orthophosphoric acid were added to 2 mL of supernatant, followed by 4 mL of Salkowski reagent (50 mL 35 % perchloric acid, 1 mL 0.5 M FeCl3). The development of a pink tint in the solution indicated the synthesis of IAA.

Siderophore production

Siderophore production was detected by CAS (Chrome azurol S) assay (28).

Solutions

Chrome azurol S (CAS) agar medium

Dye solution (Solution A)

A dye solution (50 mL) containing 60.5 mg of CAS in distilled water was mixed with 10 mL of an iron(III) solution prepared by dissolving $FeCl_3 \cdot 6H_2O$ to obtain a 1 mM iron (III) solution in 10 mM HCl. The mixture was progressively added to a solution that contained 72.9 mg of hexadecyltrimethyl-ammoniumbromide (HDTMAC) in 40 mL of distilled water. A 100 mL autoclave was filled with 10 mL of the resultant dark blue liquid and it was operated for 10 min at 15 psi.

Solution B

PIPES buffer of 30.42 g, 15 g of agar-agar, 2 % K_2HPO4 (10 mL), 10 % NaCl (2 mL), 5 % Na_2MoO_4 (0.1 mL), deferrated 1 M sucrose (3 mL), deferred 1 M $CaCl_2$ (0.4 mL) and deferrated 1 M $MgSO_4.7H_2O$ (0.8 mL) were combined with 800 mL of distilled water to create a base medium. After adding 50 % (w/w) NaOH to bring the medium's pH to 6.8, it was autoclaved for 20 min at 15 psi.

Once the mixture had cooled to 50 ˚C, 30 mL of a 10 % mannitol solution were added as a carbon source. After that, the dye solution (solution A) was added and mixed thoroughly to prevent air bubbles from forming. Sterilized petri plates were poured with the liquefied medium. After inoculating 5 µL of each log - phase-grown culture onto siderophore plates, the plates were incubated for three to four days at 28 ± 2 ºC. The decolorization of the blue ferric dye complex and the development of yellow halo zones around the cultures indicated the presence of iron chelators, also known as siderophores.

Cellulase production

Carboxymethylcellulose (CMC) agar plates were used to measure the cellulolytic activity of native *Trichoderma* isolates. Freshly growing fungal cultures were spot inoculated onto CMC agar plates and left to grow for 48 hr at 28 ± 2 ºC. Following 15–20 min of flooding the plates with a 0.1 % aqueous solution of Congo red, 1 M NaCl was used to destain the surface. The production of cellulase was observed as a clear zone surrounding the colony.

Pectolytic activity

The Pectinase Screening Agar Medium (PSAM) had the following ingredients (g/L): 20 g of agar, 10 g of pectin, 0.5 g of KCl, 0.5 g of $MgSO_4$, 1 g of K_2HPO_4, 0.5 g of trypton and 2 g of $NaNO_3$. The medium mentioned above was used to inoculate petri dishes with PSAM. Pectolytic activity was indicated by a clear zone surrounding the inoculated culture.

Zinc solubilization

All isolates were evaluated for their ability to solubilize zinc using a modified Bunt and Rovira medium (glucose 10 g, $(NH_4)_2SO_4$ 1.0 g, KCl 0.2 g, K_2HPO_4 0.1 g, $MgSO_4.7H_2O$ 0.2 g, ZnO 1 g and agar 15 g) supplemented with 0.1 % zinc oxide. About 3 µL of log-phase-grown isolate cultures were spotted on medium plates containing zinc oxide and the plates were then incubated for 6 days at 28 °C. A measure of zinc solubilization activity was the formation of the solubilization zone.

Phosphate solubilization

For each of the native *Trichoderma* isolates, the solubility of phosphate on Pikovskaya agar (10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.5 g yeast extract, 25 mg $MnSO_4$, 25 mg $FeSO_4$ and 20 g agar-agar in 1 L, pH -7) was examined separately. Cultures that generated a translucent halo zone surrounding each colony after being cultured at the appropriate temperature are identified as phosphate-solubilizing microorganisms (PSM).

Ammonia production

It was examined whether native *Trichoderma* isolates could generate ammonia in peptone water. Freshly formed cultures were inoculated with 10 mL of peptone water in each tube and they were thereafter incubated at 28 °C for 48 hr. Nessler's reagent (0.5 mL) was added to each tube. The formation of a brown to yellow tint indicated a positive test result for ammonia production.

In vitro evaluation of antagonistic activity of native *Trichoderma* isolates

Using the dual culture method, all native *Trichoderma* isolates collected from several districts of Haryana were assessed for their antagonistic capability against *Rhizoctonia solani* and *Rhizoctonia bataticola in vitro* (29). A sterile petri plate was filled with 15 mL of melted PDA medium, which was then left to harden.

The antagonistic fungus *Trichoderma* was then placed on the other side of the PDA plate just opposite to the first disc, i.e., at an angle of 180°. A 5 mm mycelial disc was then cut from the margin of the actively growing colony from 7 day old culture using a sterilized cork borer and placed near the periphery on one side of the PDA. When the control petri plates were fully filled with the growth of test pathogen, split growth of the pathogen was observed. The petri plates were incubated at 25 ° C.

In CRD, every treatment was repeated three times and relevant controls were kept up to date. A well-established formula was used to compute the percentage growth inhibition (30).

$$\text{Mycelial growth inhibition, I (\%)} = \frac{C—T}{C} \times 100$$

Where;

I = Percent inhibition of mycelial growth

C = Radial mycelial growth of *R. bataticola* and *R. solani* in control

T = Radial mycelial growth of *R. bataticola* and *R. solani* in treatment

Evaluation of bio agents under *in vivo* conditions

Among the different native *Trichoderma* isolates which were tested against *R. bataticola* and *R. solani* (RB5 and RS2) under *in vitro* conditions, the first four isolates which showed maximum mycelial growth inhibition of *Rhizoctonia* were further tested under *in vivo* conditions. *In vivo* experiments on American and Desi cotton were performed, with disease incidence and disease control measured. Every treatment was repeated 3 times and relevant controls were maintained.

The disease incidence was calculated by the following formula:

$$\text{Disease incidence} = \frac{\text{No. of infected plants}}{\text{Total no. of plants assessed}} \times 100$$

$$\text{Disease reduction (\%)} = \frac{\text{PDI in control—PDI in treatment}}{\text{PDI in control}} \times 100$$

Results and Discussion

Mycelial growth of native *Trichoderma* isolates

Soil samples from Bhiwani, Charkhi Dadri, Faridabad,

Table 2. Radial growth of native *Trichoderma* isolates

Sl. No.	Name of Isolates	6 DAI (mm)
1.	HBhT	37.00* (37.43)**
2.	HCdT	42.67 (40.75)
3.	HFrT	40.00(39.20)
4.	HFtT	36.67 (37.24)
5.	HGT	42.00 (40.37)
6.	HJT	39.33 (38.82)
7.	HMT1	38.33 (38.22)
8.	HMT2	36.33 (37.02)
9.	HRT	41.33 (39.98)
10.	HRkT	39.33 (38.82)
11.	HST1	40.33 (39.40)
12.	HST2	53.67 (47.09)
13.	T13	40.33 (39.40)
CD (p=0.05)		5.81
SE(m) ±		1.98
SD		4.41

*Mean of 4 replications ** Values in parenthesis are angularly transformed.

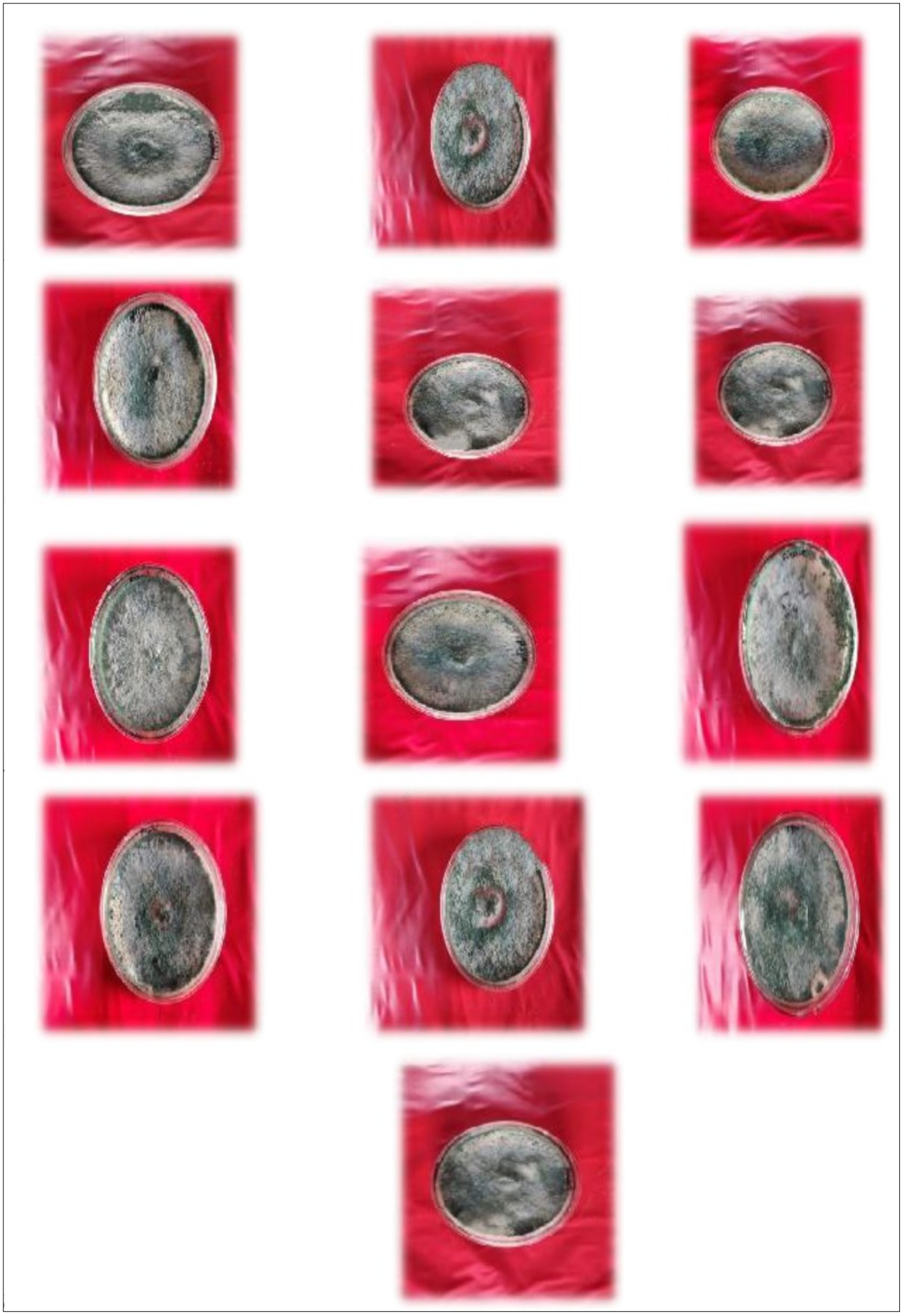

Fig. 1. Pure Cultures of native isolates of *Trichoderma.*

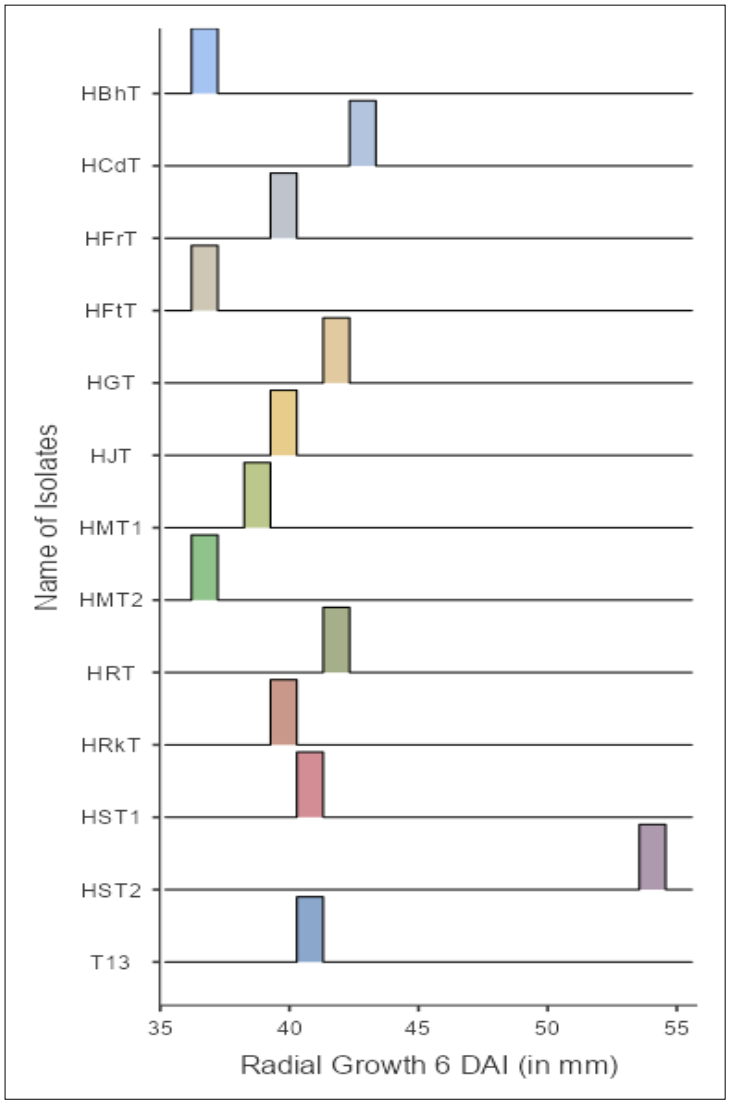

Fig. 2. Radial growth (mm) of native *Trichoderma* isolates after 6 days of incubation.

Fatehabad, Gurugram, Jhajjar, Mahendergarh, Rewari and Sirsa yielded a total of twelve *Trichoderma* isolates. A unique code, such as HBhT, HCdT, HFrT, HFtT, HGT, HJT, HMT, HRT or HST, was assigned to each isolate (Fig. 1) After six days of incubation, HST2 exhibited the highest mycelial growth (53.67 mm), which was statistically significantly greater than that of the other native *Trichoderma* isolates. However, HMT2 showed the least amount of growth (36.33 mm), as seen in Table 2 and Fig. 2.

Morphology and measurement of mycelial and conidia characters of *Trichoderma* isolates

The most important characteristics of a fungus are its morphological characteristics, which facilitate the differentiation of various native isolates of *Trichoderma.* It is evident from Table 3 that the colonies' colours varied, ranging from light green to pale green to dark green. HST1, HRkT, HFrT and HMT2 isolates displayed a pale/whitish green colony colour, while HCdT, HFtT and HJT isolates displayed a light green colony colour. HRT, HGT, HST2, HBhT, HMT1 and T13 isolates displayed a dark green colony color. The reverse colony color of native *Trichoderma* isolates varied from creamish white to colorless to yellowish. The isolates of HRT, HGT, T13, HST2 and HBhT were seen to have a cream-colored, whitish look of the reverse colony. The color of the reverse colony was observed to be colorless in isolates of HST1, HCdT, HFtT, HJT and HMT2 and colorless to yellowish in isolates of HRkT, HFrT and HMT1 (Table 3 and Fig. 1). The colony's edge was observed to be either wavy or smooth. The isolates of HBhT, HCdT,

Table 3. Morphological characters of native isolates of *Trichoderma* collected from different districts of Haryana

Isolate	Colony color	Reverse colony colour	Colony edge	Mycelia colour
HBhT	Dark Green	Creamish white	Wavy	Watery white
HCdT	Light Green	Colourless	Wavy	Watery white
HFrT	Whitish Green	Colourless to Yellowish	Smooth	Watery white
HFtT	Light Green	Colourless	Smooth	Watery white
HGT	Dark Green	Creamish White	Wavy	Watery white
HJT	Light Green	Colouless	Wavy	Watery white
HMT1	Dark Green	Colourless to yellowish	Wavy	Watery white
HMT2	Whitish Green	Colourless	Smooth	Watery white
HRT	Dark Green	Creamish white	Smooth	Watery white
HRkT	Whitish Green	Colourless to yellowish	Smooth	Watery white
HST1	Whitish Green	Colourless	Smooth	Watery white
HST2	Dark Green	Creamish white	Wavy	Watery white
T13	Dark Green	Creamish white	Smooth	Watery white

Table 4. Conidial and mycelial characters of native isolates of *Trichoderma*

Name of isolate	Mycelial width	Width of conidia	Length of conidia
HBhT	3.32* (10.49)**	2.89 (9.78)	3.36 (10.56)
HCdT	3.87 (11.34)	3.02 (9.99)	4.12 (11.70)
HFrT	4.21 (11.84)	3.1 (10.12)	4.54 (12.30)
HFtT	4.23 (11.86)	3.65 (10.96)	4.66 (12.46)
HGT	3.22 (10.32)	3.07 (10.08)	3.46 (10.72)
HJT	2.85 (9.72)	2.42 (8.93)	3.08 (10.11)
HMT1	2.56 (9.21)	2.31 (8.73)	2.85 (9.71)
HMT2	4.15 (11.75)	2.67 (9.41)	4.23 (11.86)
HRT	2.72 (9.47)	2.0 (8.12)	3.05 (10.05)
HRkT	3.08 (10.10)	2.58 (9.24)	3.26 (10.40)
HST1	3.86 (11.32)	3.08 (10.11)	4.37 (12.07)
HST2	4.22 (11.84)	2.88 (9.77)	4.46 (12.19)
T13	2.46 (9.01)	2.35 (8.77)	2.69 (9.44)
CD (p=0.05)	0.35	0.58	0.27
SE (m) ±	0.12	0.20	0.09
SD	0.67	0.43	0.71

*Mean of 3 replications ** Values in parenthesis are angularly transformed.

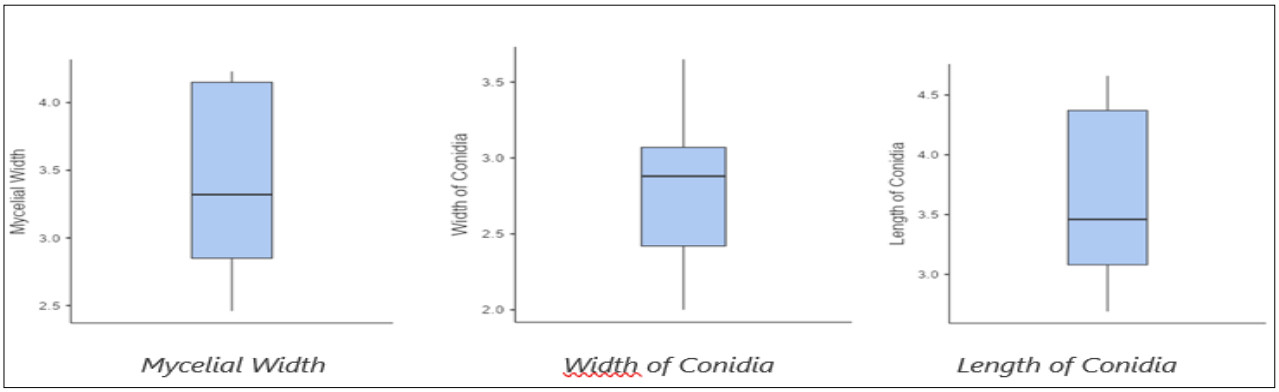

Fig. 3. Mycelial width and the width and length of conidia of different native *Trichoderma* isolates.

Fig. 4. Microscopic view of (a)mycelia; (b)phialides of *Trichoderma* spp.

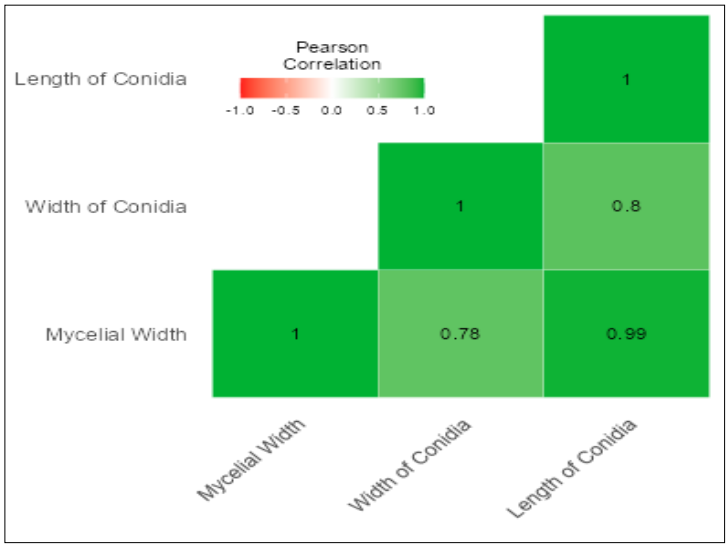

Fig. 5. Correlation between mycelial width and the width and length of condia of different native *Trichoderma* isolates.

HGT, HJT, HMT1 and HST2 had wavy colony edges, whereas isolates of HFrT, HFtT, HMT2, HRT, HRkT, HST1 and T13 have smooth edges (Table 3). The mycelial width of native isolates of *Trichoderma* ranged from 2.46 to 4.23 µm, according to the findings in Table 4 and Fig. 3.

The maximum width was recorded in HFtT (4.23 µm) followed by HFrT (4.21 µm) whereas, the minimum width of 2.46 µm was recorded in T13. A correlation heat map showing the relationship between mycelial width and the width and length of conidia of different native *Trichoderma* isolates is presented in Fig. 4 & 5.

Bio control activities of native *Trichoderma* isolates

Potency of native *Trichoderma* isolates for production of HCN and IAA

According to the data presented in Table 5, the isolates HGT, HCdT, HST2 and HRkT showed positive results for HCN production, while the other isolates showed negative results. IAA production was positive for all isolates, although isolate HST2 produced the greatest IAA (21.78 µg/mL), followed by isolates HST1 (21.48 µg/mL) and HBhT (16.57 µg/mL). IAA production was lowest in T13 (11.48 µg/mL). Among the isolates HGT, HST2, HFtT, HMT2 and T13 produced positive cellulase results, while the remaining isolates showed negative cellulase activity. Among the isolates HST2, HFtT, HMT2 and T13 showed positive pectolytic activity results, whereas the other isolates showed negative results. Ammonia production was positive only for isolates HRT, HGT, HST2, HMT2 and T13.

Siderophoe production was observed for isolates, such as HRT, HGT, HST1, HST2 and HFtT, while the remaining isolates showed negative results. Isolates HRT, HST2, HFtT and T13 showed positive zinc and phosphate solubilization activity, but the remaining isolates showed negative results from both activities (Table 5 and Fig. 6).

Antagonistic activity of different *Trichoderma* isolates against *Rhizoctonia* spp.

A pure culture of *Rhizoctonia* was maintained to study the bio-efficacy of thirteen isolates, including twelve native isolates and one commercial formulation of *T. viride*. Various *Trichoderma* isolates demonstrated broad-spectrum antagonistic activity against *R.*

Table 5. Bio control activities of native *Trichoderma* isolates

Isolate	HCN production	IAA production	Cellulase activity	Pectolytic activity	Ammonia production	Siderophore production	Zn solubilisation	Phosphate solubilisation
HBhT	–	(16.57)	–	–	–	–	–	–
HCdT	+	(16.13)	–	–	–	–	–	–
HFrT	–	(15.35)	–	–	–	–	–	–
HFtT	–	(17.22)	+	+	–	+	+	+
HGT	+	(12.74)	+	-–	+	+	–	–
HJT	–	(12.35)	–	–	–	–	–	–
HMT1	–	(12.35)	–	–	–	–	–	–
HMT2	–	(16.26)	+	+	+	–	–	–
HRT	–	(13.78)	–	–	+	+	+	+
HRkT	+	(11.57)	–	–	–	–	–	–
HST1	–	(21.48)	–	–	–	+	–	–
HST2	+	(21.78)	+	+	+	+	+	+
T13	–	(11.48)	+	+	+	–	+	+

Table 6. Mycelial growth inhibition of RB5 and RS2 isolate by native isolates of *Trichoderma*

Sl. No.	Name of isolate	Growth inhibition (%)	
		RB5	RS2
1.	HBhT	63.05* (52.54)**	62.50 (52.21)
2.	HCdT	68.33 (55.74)	70.00 (56.76)
3.	HFrT	62.22 (52.06)	61.66 (51.73)
4.	HFtT	63.61 (52.88)	62.78 (52.38)
5.	HGT	70.00 (56.77)	69.44 (56.42)
6.	HJT	63.33 (52.71)	63.61 (52.87)
7.	HMT1	61.66 (51.72)	62.22 (52.05)
8.	HMT2	70.83 (57.29)	71.11 (57.46)
9.	HRT	60.28 (50.90)	56.94 (48.96)
10.	HRkT	60.28 (50.91)	59.44 (50.42)
11.	HST1	64.72 (53.54)	65.83 (54.20)
12.	HST2	72.78 (58.53)	72.50 (58.35)
14.	T13	54.44 (47.52)	54.72 (47.68)
CD (p=0.05)		2.76	2.23
SE(m) ±		0.96	0.77
SD		5.06	5.48

*Mean of 4 replications ** Values in parenthesis are angularly transformed.

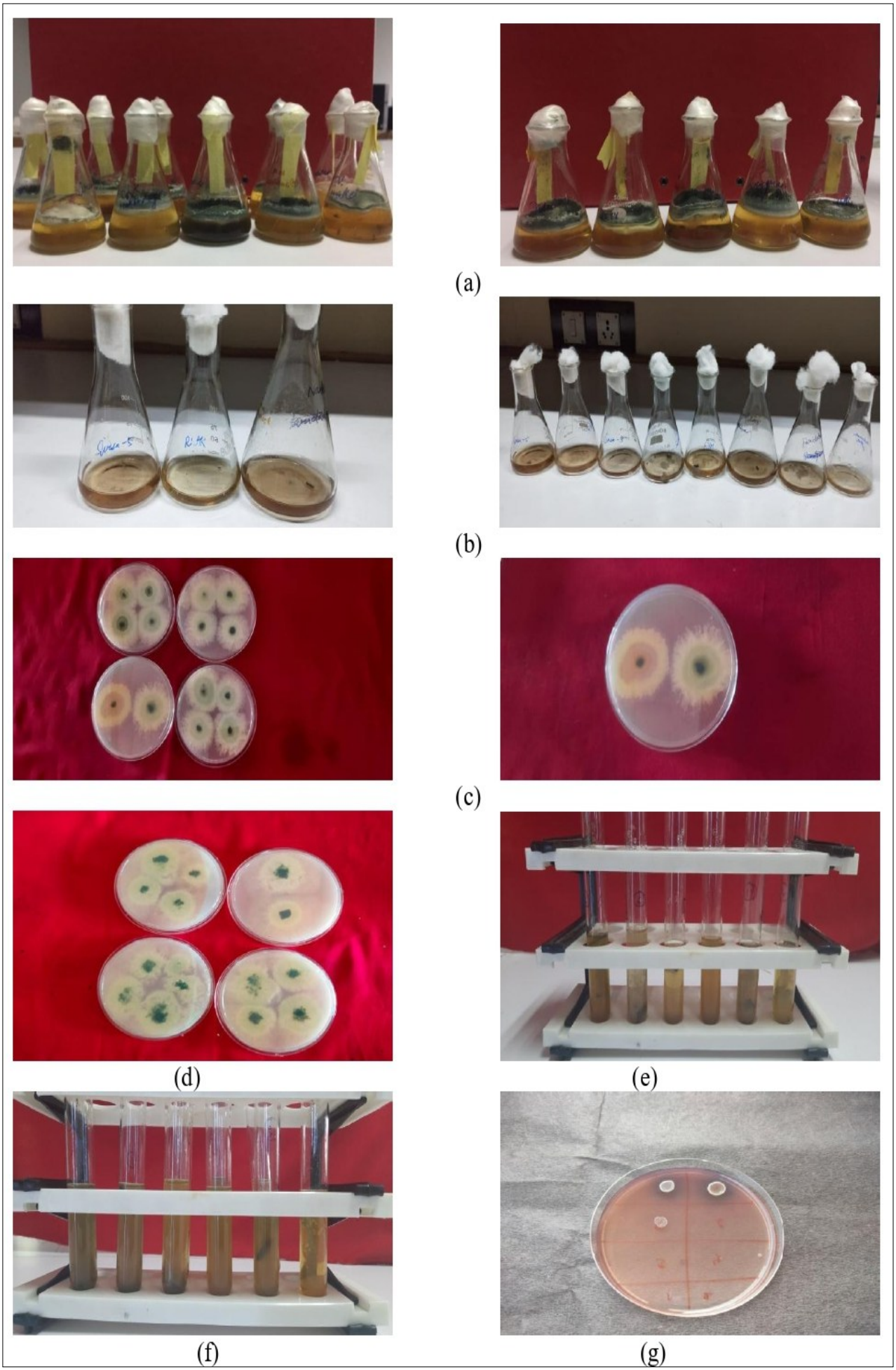

Fig. 6. Biocontrol activities of native isolates of *Trichoderma* (a) HCN Production (b) IAA Production (c) Cellulase Production (d) Pectolytic Activity (e) Ammonia Production (f) Zinc and Phosphate solubilisation (g) Siderophore Production.

Fig. 7. Efficacy of native *Trichoderma* isolates against RB5 and RS2 under *in vitro* conditions.

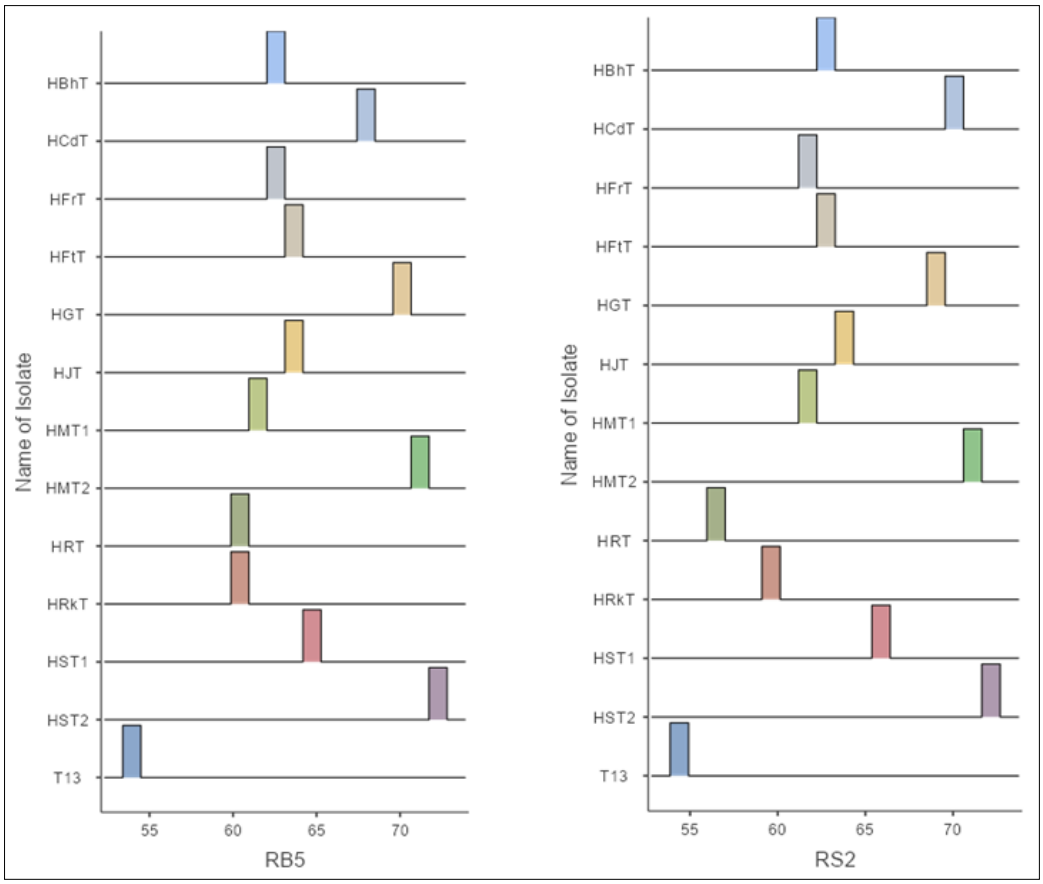

Fig. 8. *In vitro* evaluation of native *Trichoderma* isolates in per cent growth inhibition of RB5 and RS2.

bataticola and R. solani. Among the isolates, HST2 showed the highest mycelial growth inhibition (72.78 %), which was statistically significant compared to all other isolates except HMT2 and HGT, which were statistically at par. T13, on the other hand, displayed the least mycelial growth inhibition (54.44 %). HST2 measured the highest mycelial growth inhibition (72.50 %) in RS2 of *R. solani*, which was statistically significant compared to all other isolates with the exception of HCdT, HGT and HMT2, which were statistically at par with HST2. Conversely, T13 showed the lowest mycelial growth inhibition (54.72 %) (Table 6 & Fig. 7, 8).

When the selected native *Trichoderma* isolates were examined *in vivo*, their effectiveness was evaluated based on disease incidence and disease control percentage (Table 7). Native *Trichoderma* isolate HST2 from the Sirsa district recorded the lowest disease incidence (10 %) in both Desi and American cotton, which is statistically significant when compared to all other treatments. HST2 demonstrated 57.13 % disease control, while HMT2 demonstrated 42.46 % disease control, which were statistically significant when compared to the remaining treatments.

Molecular characterization of *Trichoderma* isolate

In the present study, universal primers (ITS 1 and ITS 4) were used to carry out the molecular characterization of *Trichoderma* isolate that was found to be the best performer under both *in vitro* and *in vivo* conditions.

DNA isolation of *Trichoderma* isolate and quality testing

The DNA was isolated using the CTAB method (31). Agarose gel electrophoresis (0.8 %) was used to check the quality and quantity of the DNA sample. On agarose gel electrophoresis, a single band of high molecular weight was observed and it was free of mechanical or enzymatic degradation or contamination with RNA.

Polymerase chain reaction (PCR) amplification

Standard polymerase chain reaction (PCR) conditions, including DNA, primers, Taq polymerase and $MgCl_2$ were used to amplify the ITS region. The PCR reaction was carried out in a 20 µL reaction mixture containing 2 µL of template DNA, 1 µL of primer, 2.5 µL of $MgCl_2$ (2.5 mM), 2.0 µL of PCR buffer, 0.25 µL of Taq polymerase and 0.4 µL of dNTP mix. The total volume was raised to 20 µl by adding nuclease-free water. The PCR amplification reaction was carried out under the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The sequencing of the PCR amplified product was done by Biokart India Pvt. Ltd., as the isolate was identified as *Trichoderma asperelloides* with GenBank accession number PP301378.

Conclusion

The relationship between *Trichoderma*, plants and pathogens is a complex and dynamic system. *Trichoderma* interactions with plants and pathogens constitute a complex system and understanding these relationships can improve its biocontrol effectiveness. To defend itself against biotic stressors like a wide range of pathogenic microorganisms (fungi, bacteria, insects and nematodes) and abiotic pressures like unfavorable environmental circumstances, *Trichoderma* employs a number of complex direct and indirect biocontrol mechanisms. Treatments that increase *Trichoderma's* competitiveness in the rhizosphere and rhizoplane are expected to be used in the upcoming years to improve the techniques for boosting the efficacy and dependability of *Trichoderma* preparations. This can be done by strong and consistent colonization of these areas and combining Tricoderma with microorganisms known as "supporting strains," which enhance its beneficial effects.

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Authors' contributions

PV, NKY designed the study and prepared the manuscript. PV and PP analyzed the data. NKY, VKM, P, PY, DK and S helped in the preparation of the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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Table 7. *In vivo* evaluation of native *Trichoderma* isolates against root rot of cotton

Sl. No.	Treatments	% Disease incidence (Desi cotton)	Disease control (%)	% Disease incidence (American cotton)	Disease control (%)
T_1	HST2	10* (18.42)**	57.13	10 (18.42)	57.13
T_2	HMT2	13.33(18.42)	42.46	13.33(18.42)	42.46
T_3	HGT	16.66 (24.83)	28.5	13.33 (24.83)	42.86
T_4	HCdT	20 (23.02)	14.2	20(19.42)	14.2
T_5	Control	23.33 (26.55)		23.33 (26.55)	
CD (p=0.05)		4.21		2.83	
SE (m) ±		1.32		0.88	

*Mean of 4 replications ** Values in parenthesis are angularly transformed.

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