Journal of Food Legumes
36(4): 278-287, 2023
DOI: 10.59797/jfl.v36.i4.164

Research paper

Evaluation of *Trichoderma* spp. as a plant growth promoter and antagonist of major pulse pathogens

Mantasha Arif[1], Vipin Verma[2], Aishwarya Priyadarshini[1], Lovkush Satnami[2], Aalok Mishra[2], Mariya Ansari[2], Anirudha Chattopadhyay[3], Dawa Dolma Bhutia[2] and Ankita Sarkar[2]*

[1]*Department of Botany, Institute of Science, Banaras Hindu University, Varanasi, Uttar Pradesh, India*
[2]*Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India*
[3]*Pulses Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat, India*

***E-mail:** ankitasarkar@bhu.ac.in*

***Received:** 25 August 2023*
***Accepted:** 9 February 2024*

Handling Editor:
Dr. RK Mishra, ICAR-Indian Institute of Pulses Research, Kanpur, India

ABSTRACT

***Trichoderma* spp. is mostly used for the management of soil-borne diseases and some foliage and fruit diseases in a variety of crop plants. It can help the environment by reducing agrochemical pollution, promoting plant growth, and enhancing plant resistance in addition to preventing plant diseases. *Trichoderma* spp. also functions as a secure, affordable, efficient, and environmentally friendly biocontrol agent for several crop species. In the present study, we obtained different *Trichoderma* isolates from rhizospheric soil samples of different locations and tested them for their antagonistic activity against major pulse pathogens. Among seven isolates, three isolates, viz., Pipal TH-2, ATH-Kashipur, and Mz/AP-2 were found to be highly effective by inhibiting the growth of *Fusarium udum* (64.04 to 78.65%), *Fusarium ciceris* (77.77 to 82.12%), *Sclerotium rolfsii* (59.09 to 69.30%), *Macrophomina phaseolina* (52.42 to 62.72%) and *Alternaria alternata* (80.12 to 83.22%). These isolates were also tested for growth-promoting traits (PGPR) in the present study and isolates having both plant growth-promoting ability and biocontrol potentiality were selected and preserved for further studies. These isolates of *Trichoderma* spp. would be a crucial partner for achieving the Green Earth goal due to their contribution to the sustainable growth of agriculture.**

***Key words*:** Trichoderma, Biocontrol, Pulse pathogen, Plant Growth Promoters

INTRODUCTION

Biocontrol agents are beneficial microbes that inhibit the growth of plant pathogens and enhance plant growth. They also increase the nutrient use efficiency and photosynthesis efficiency of plants and lower the effect of abiotic stresses (Tilocca *et al.*, 2020; Fontana *et al.*, 2021). Therefore, the use of fungal biocontrol agents is a vital, economically viable, environmentally benign, and long-term method for managing plant diseases (Ajilogba *et al.* 2013; Olowe *et al.*, 2020; Rojas *et al.*, 2020; Woo *et al.*, 2023)

Trichoderma species are soil-borne filamentous saprophytic fungal species and the sexual stage of this fungus includes Fungi, Ascomycota, Sordarimycetes, Hypocreales, Hypocreaceae, and *Trichoderma* spp. (Sun *et al.*, 2012). *Trichoderma* is used as an excellent biocontrol agent for the management of different soil-borne pathogens in a wide range of environments (Haggag and Abd – El Latif, 2001; Shali *et al.*, 2010; Yao *et al.*, 2023) due to their variety of plant disease prevention strategies, which include mycoparasitism, nutrient competition, and hydrolytic enzyme antibiosis (Filizola *et al.*, 2019; Mishra *et al.*, 2023) and plant growth promotion (Harman *et al.*, 2010; Ainhoa Martinez-Medina *et al.*, 2014; Druzhinina *et al.*, 2011; Tyskiewicz *et al.*, 2022). *Trichoderma* species have antibiosis effects on plant pathogenic fungi via secretion of antagonistic secondary metabolites (Kottb *et al.*, 2015; Izquierdo-García *et al.*, 2020; Morán-Diez *et al.*, 2020; Shobha *et al.*, 2020; El-Hasan *et al.*, 2022) including trichomycin, gelatinomycin, chlorotrichomycin, and antibacterial peptides (Maruyama *et al.*, 2020). Therefore, the present investigation aimed to isolate different *Trichoderma* spp. from various legume rhizosphere and to evaluate them against different legume pathogens. Thus primary objective of this study is to identify the new isolates of *Trichoderma* spp. having good biocontrol potentiality and plant growth-promoting ability.

MATERIALS AND METHODS

The present investigation was conducted in the Mycology Research Laboratory, Department of Plant Pathology, Institute of Agricultural Sciences, BHU, Varanasi, Uttar Pradesh.

Maintenance of biocontrol agents and pathogens

Isolates of seven *Trichoderma* spp. obtained from various locations were used in this study. (Table 1) and antagonistic microbes *Fusarium udum, Sclerotium rolfsi and Alternaria alternata* were obtained from Mycology Research Laboratory, Department of Mycology and Plant Pathology, IAS, BHU. The pure culture of biocontrol agents and pathogens were isolated, purified, and maintained on Potato Dextrose Agar (PDA) by periodic subculturing throughout the investigation.

Catalase assay

Catalase test of *Trichoderma* isolates was done by the method described by Clarke and Cowan (1952). In this test, fresh culture was taken on a glass slide and a few drops of H_2O_2 (30%) were put into it. Effervescence on the slide indicates a positive result.

Cellulase test

Cellulase test of *Trichoderma* isolates was performed following the method described by Halliwell and Griffin (1973). Cellulose media was poured on a petri plate and isolates inoculated after solidification. After 48 hours of incubation period, 2% Congo red solution was flooded onto the culture plates, and incubated for 15 minutes. After 15 minutes, the solution was decanted and de-stained with 0.5 M NaCl solution. Subsequently, the NaCl solution was decanted after 15 minutes, and culture plates were checked for clear zones around the colony indicating positive results.

HCN assay

HCN production test of *Trichoderma* isolates was performed following the method described by Bakker and Schippers (1987). Screening fungus for HCN production was done in PDA media. The spot inoculation of *Trichoderma* isolates was done on a poured media, Whatman No.1 filter paper was cut according to the size of Petri plates and dipped in the solution of 0.5% picric acid and 2% Na_2CO_3. Then filter paper was attached to the lid of Petri plates containing spot inoculant and sealed with paraffin to prevent the leakage of HCN gas produced. Similarly, control plate was maintained without the inoculation of the fungal strains. The petri plates were incubated at 28 ± 2 °C for 3-4 days in a BOD incubator. The positive result was indicated by the filter paper's transformation from yellow to orange whereas the negative result was determined by the unchanged color of the filter paper similar to control plates.

Protease test

Protease media was prepared, autoclaved, and poured into the petri plates. After solidification, spot inoculation of *Trichoderma* isolates was done and incubated in a BOD incubator. After 48 hours, a clear zone was visualized around the colony indicating a positive result.

Phosphate solubilization assay

The phosphate test was performed following the method described by Mehta and Nautiyal (2001). The Petri plates containing NBRI media were spot inoculated with 2 mm mycelium bits of *Trichoderma* spp. All the plates were incubated at 28 ± 2 °C for 2-3 days in a BOD incubator. The emergence of a clean zone around the spot of inoculated colonies indicated a positive result.

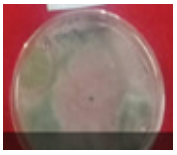
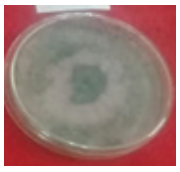
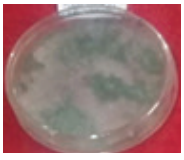
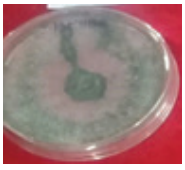
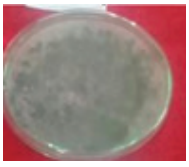
Chitin production assay

The chitin production test of *Trichoderma* isolates was done following the method described by Hsu and Lockwood (1975). Chitin agar media was prepared, autoclaved (121 °C, 15 psi for 15 minutes), and poured on petri plates. The spot inoculation of the bit of *Trichoderma* strain was done and incubated at 28 ± 1 °C for 3-4 days in a BOD incubator. After 3-4 days staining was done with Lugol's iodine and the production of a halo zone indicates a positive result.

Amylase test

Amylase assay of *Trichoderma* isolates was done by the method described by Chavez *et al.* (2004). This was also known as the starch hydrolyzing test. Starch agar media was prepared, autoclaved (121 °C, 15 psi for 15 minutes), and poured into a petri plate after a few minutes when the media was solidified then spot inoculation with bits of *Trichoderma* isolates was done. Plates were incubated in a BOD incubator for 48 hours. After 48 hours culture plate was stained with iodine solution and a clear area around colonies was visualized which indicates a positive result.

Table 1. *Trichoderma* isolates and their morphological characteristics

S. No	Isolate name	Region of isolation	Location	Pattern of spore formation	Pure culture plates
1	ATH Kashipur	Chickpea rhizosphere	Kashipur, Varanasi	Margin	
2	CTH Kashipur	Chickpea rhizosphere	Kashipur, Varanasi	Margin and centre	
3	Kashipur B	Chickpea rhizosphere	Kashipur, Varanasi	Patches	
4	Pipal TH -1	Rhizosphere *of Ficus religiosa*	AICRP-Pigeon pea, Plant Pathology field IAS, BHU Varanasi	Margin and centre	
5	Pipal TH- 2	Rhizosphere *of Ficus religiosa*	AICRP-Pigeon pea, Plant Pathology field IAS, BHU Varanasi	Centre	
6	Mz/Ap-1	Pigeopea rhizosphere	Andhra Pradesh	Scattered	
7	Mz/Ap-2	Pigeopea rhizosphere	Andhra Pradesh	Full plate	

Zinc solubilization test

This test was done following the method described by Saravanan *et al*. (2004). A zinc solubilization test was carried out by preparing the Zinc media. The isolates of *Trichoderma* spp. were embedded in media by cutting the mycelium bits and incubated for 48 hours. The production of the hollow zone around the growth of isolates indicates a positive result.

Siderophore assay

This test was performed following the method described by Milagres (1999). Siderophore assay was done in Chrome Azurol S (CAS) (Schwyn and Neilands, 1987) and PDA media. 0.5 mg CAS was dissolved in 50 mL of distilled water and mixed thoroughly with 10 mL of 1 mM $FeCl_3.6H_2O$. Hexadecyltrimethylammonium bromide (HDTMA) solution (72.9 mg of HDTMA is dissolved in 40 mL of D.W.) is added slowly by stirring which is used as an indicator. The resultant solution is autoclaved at 121 ºC for 15 min. Petri plates were poured with an appropriate quantity of PDA, after solidification, media was cut into halves, and one half was replaced with CAS agar. The half containing PDA was inoculated with *Trichoderma* spp. The plates were incubated for 1 week in a BOD incubator. The change in color of CAS medium from blue to reddish-orange or purplish-red indicated a positive result.

Ammonia production test

Ammonia production of *Trichoderma* isolates was done following the method described by Dye, 1962. Peptone water was prepared, 10 mL poured into a 21 mL test tube, and autoclaved (121 ºC, 15 psi for 15 minutes). After allowing them to cool at room temperature, the tubes were inoculated with fungal spores and allowed to grow at 30 °C for 4 days. Four days after inoculation, 1mL of Nessler's reagent was added to the tubes. The formation of a faint yellow color signaled the creation of tiny amounts of ammonia, while a deep yellow to brown color signified the highest synthesis of ammonia.

Antagonistic test

The antagonistic potential of *Trichoderma* isolates was tested against pathogens *Fusarium udum* (ON797461.1), *Sclerotium rolfsii*, *Alternaria alternata, Fusarium oxysporum* f.sp. *ciceris* and *Macrophomina phaseolina* based on a method performed by Broadbent *et al*. (1971). The molten media was poured on sterile petri plates and allowed for solidification under laminar airflow. The two mm mycelium disc isolates of *Trichoderma* spp. and pathogen were inoculated on PDA at opposite edges. Both the mycelium disc was two cm away from the periphery and 4.5 cm apart. The control plates were maintained by inoculating the pathogen on petri plates. All the plates were incubated at 28 ± 2 °C in a BOD incubator. After 6 days, colony diameter was measured and percent inhibition of each isolate against pathogen was calculated by the following the formula:

$$I\ \% = (C - T)/C \times 100$$

Where, I%= % inhibition of mycelium growth; C= growth of the pathogen in control petri plates; T= growth of the pathogen in dual petri plates.

RESULTS AND DISCUSSION

Catalase test

In the catalase test, all seven strains of *Trichoderma* were positive (Fig 6a). *Trichoderma koningiopsis* UFSMQ40 using solid-state fermentation produces the catalase enzymes (Yao *et al*., 2023). Aman (2004) reported catalase production activity in *Trichoderma reesei* by cultivating it in a medium containing ground wheat and barley straw.

Cellulase test

A cellulase test was carried out for all the isolates of *Trichoderma spp*. and all isolates gave positive results except Mz/Ap-1 (Fig 6b). Saravanakumar *et al*. (2018) reported the biocontrol potential of cellulase enzyme produced by *T. harzianum* strain Th22 in stimulating DIMBOA and defense-related genes against *F. graminearum* in maize.

HCN production assay

Secondary metabolite Hydrogen cyanide (HCN) is produced by PGPF and PGPR which are volatile (Schippers *et al*., 1990). The metabolite possesses broad spectrum characteristics for biocontrol (Ramette *et al*., 2003). Positive *Trichoderma* isolates changed the color of Whatman no.1 filter paper dipped in 0.5% picric acid and 2% Na_2CO_3 from yellow to light brown due to HCN gas produced by *Trichoderma* spp. and formed the picrate solution as reported earlier by Williams and Edwards (1980). Thus Pipal TH-2, Kashipur -B, Mz/Ap-1, and Mz/Ap-2 isolates gave positive results in this HCN production test (Fig 6c).

Chitinase assay

Trichoderma spp. produce chitinase which significantly hydrolyzes pathogenic fungal cell walls. Thus, this application shows promising effects for disease control mainly of fungal origin since plant tissue does not contain chitin, so chitinase shows excellent potential for controlling disease (Neeraja, 2010). In the chitinase test, only ATH- Kashipur isolate was found positive (Fig 6d). Monteiro *et al*. (2010) and Naher *et al*. (2014) reported that chitinase, glucanase, and protease enzymes produced by *Trichoderma* spp. are the main administrators of phytopathogen cell wall degradation.

Protease test

In the protease test, the *Trichoderma* isolates Pipal TH-2, ATH-Kashipur, and Kashipur-B gave positive results (Fig 6e). Rai *et al*. (2019) reported the differential potential of protease enzymes produced by *Trichoderma* spp. in destructing host cell walls.

Phosphate solubilization assay

Among all the isolates tested only two isolates Mz/Ap-2 and Pipal TH-2 had a positive response to phosphate solubilization (Fig 6f). The production of the halo zone in the NBRI medium was due to the decolorization of Bromophenol blue, which showed a blue color as a result of the production of organic acid (Onyia and Anyanwu, 2013). In the natural environment, numerous microorganisms and rhizospheric bacteria exist, which can solubilize mineralized phosphorus and make them available to plants (Bhattacharyya and Jha, 2012).

Amylase test

According to earlier research, *Trichoderma* spp. may produce significant amounts of extracellular amylase and glucoamylase from soluble potato starch. In a similar study, α-amylase was purified and characterized from *Trichoderma pseudokoningii* grown on orange peel under solid-state fermentation (Abdulaal, 2018). In the amylase test, all the isolates gave positive results except Pipal TH-2, Mz/Ap-2 isolates (Fig 6g).

Ammonia production assay

After the addition of Nessler's reagents, only Mz/Ap -2 isolate of *Trichoderma* spp. was able to change its color from light yellow to dark yellow (Fig 6h). The intensity of color change indicated the amount of production of ammonia. In an earlier study, Mohiddin *et al*. (2017) observed that out of 20 isolates of *Trichoderma* spp. 13 gave positive results for the ammonia production test.

Zn solubilization test

In the zinc solubilization test, all the *Trichoderma* isolates indicated a positive result except Mz/Ap-1, pipal TH-1, and ATH- Kashipur (Fig 6i). Altomare *et al*. (1998) observed the media becoming more acidic, chelating metabolites being produced, and an increase in redox activity as *T. harzianum* solubilized metallic zinc.

Siderophore Production test

CTH-Kashipur, Kashipur-B, and Pipal TH-1 isolates gave positive results in siderophore production test (Fig 6j). In this assay, a siderophore-producing microorganism scavenges iron from the Fe-CAS-HDTMA complex and subsequently releases the CAS dye (Schwyn and Neilands, 1987). The release of CAS dye resulted in a change in color from blue to orange (Louden *et al.,* 2011; Qi and Zhao, 2013) detected a positive result for siderophore activity by *Trichoderma asperellum*. *Arabidopsis thaliana* was able to grow in iron-deficient or insoluble iron-containing media when treated by *Trichoderma asperellum* producing a positive result for siderophore activity (Zhao *et al*., 2020). *Trichoderma atroviride* LBM 112 was able to show positive results for siderophore production activity (López *et al*., 2019). These all results support our experimental findings.

Antagonistic test

Antagonistic test of Mz/Ap-1, Pipal TH-1, and Kashipur -B isolates of *Trichoderma spp.* against the *Fusarium udum, Sclerotium rolfsii* and *Alternaria alternata* were observed by dual culture method. All the isolates of *Trichoderma* spp. used in antagonistic tests against pathogens inhibited the growth of pathogens through the restriction zone formation. A 77-88% mycellial growth inhibition of *Alternaria* sp. by different strains of *Trichoderma* was reported following the dual culture method (Bhattacharya and Chakraborty, 2020). *In-vitro* antagonistic test showed a 74-79% reduction in mycelial growth of *F. oxysporum* f.sp. *ciceri* by Trichoderma strains (Katyayani *et al*., 2020). *T. asperellum* showed the highest percent inhibition against chickpea pathogen *R. solani* and *M. phaseolina* under *in-vitro* evaluation (Jaisani and Gohel, 2021).

Table 2. Mycelium growth inhibition of *F. udum, S. rolfsii and A. alternata* by isolates of *T. spp.* (Mz/Ap-1, Pipal TH-1 and Kashipur-B)

		Inhibition percentage (%)				
S.NO.	**Strain name**	***Fusarium udum***	***Sclerotium rolfsii***	***Alternaria alternata***	***Macrophomina phaseolina***	***Fusarium ciceris***
1	Mz/AP-2	64.04	59.09	80.42	52.42	78.32
2	ATH-Kashipur	78.65	69.30	83.22	60.33	77.77
3	Pipal TH-2	75.28	68.18	81.12	62.72	82.12

Table 3. Performance of Trichoderma isolates against different biochemical tests

S. No.	**Strain**	**Catalase**	**Phosphate solubilization**	**Chitinase**	**HCN production**	**Zinc solubilization**	**Protease**	**Cellulase**	**Siderophore production test**	**Amylase**	**Ammonia production**
1.	Pipal TH-1	+	-	-	-	+	-	+	+	+	-
2.	Pipal TH-2	+	+	-	+	-	+	+	-	-	-
3.	Mz/Ap-1	+	-	-	+	-	-	-	-	-	-
4.	Mz/Ap-2	+	+	-	+	-	-	+	-	-	+
5.	CTH-Kashipur	+	-	-	-	+	-	+	+	+	-
6.	ATH-Kashipur	+	-	+	+	+	+	+	-	+	-
7.	Kashipur-B	+	-	-	-	-	+	+	+	+	-

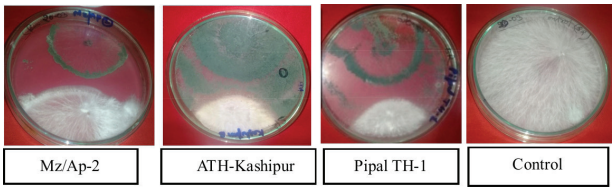


Fig 1. Antagonistic test of Trichoderma isolates against *Sclerotium rolfsii*

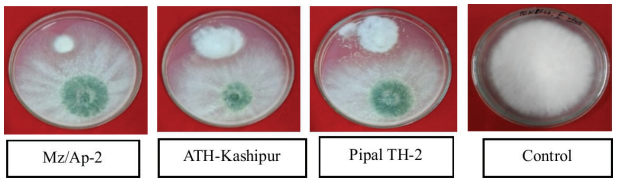


Fig 2. Antagonistic test of Trichoderma isolates against *Fusarium udum*

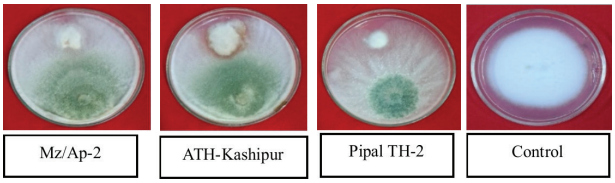


Fig 3. Antagonistic test of Trichoderma isolates against *Fusarium ciceris*

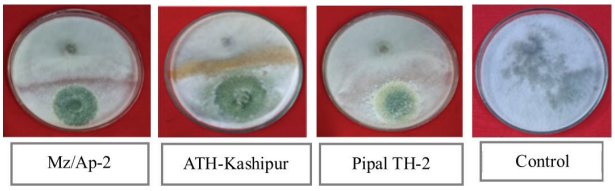


Fig 4. Antagonistic test of Trichoderma isolates against *Macrophomina sp.*

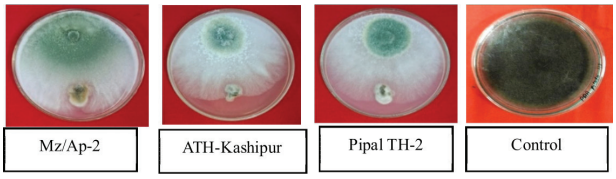


Fig 5. Antagonistic test of Trichoderma isolates against *Alternaria alternate*

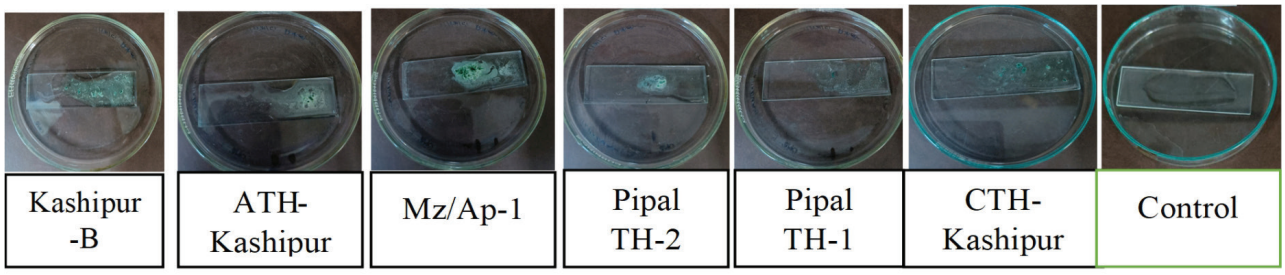


Fig 6a. Catalase test

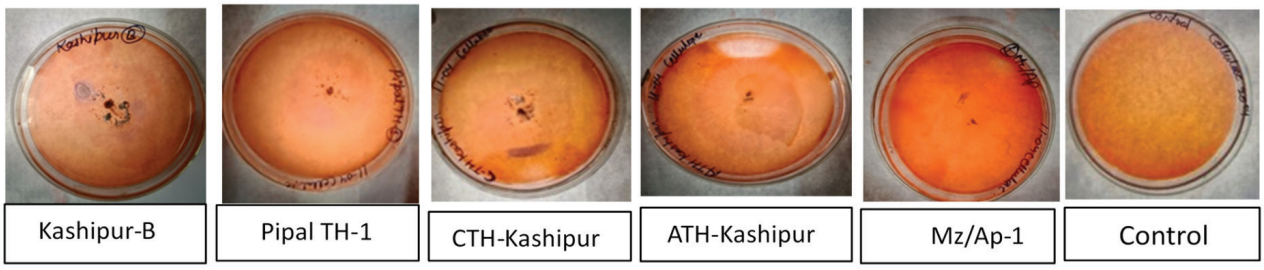


Fig 6b. Cellulase test

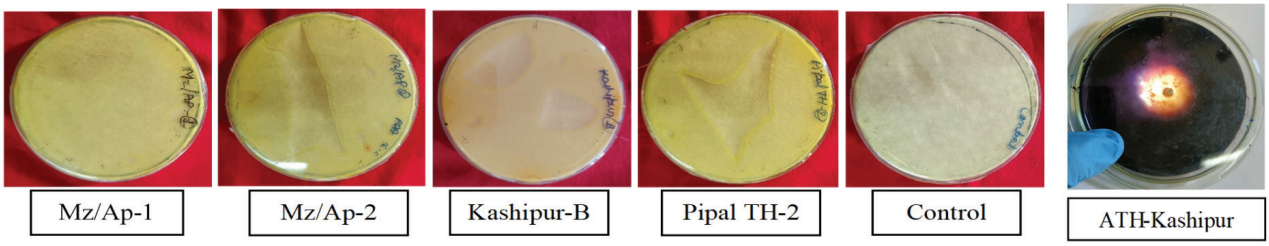


Fig 6c. HCN test

Fig 6d: Chitinase test

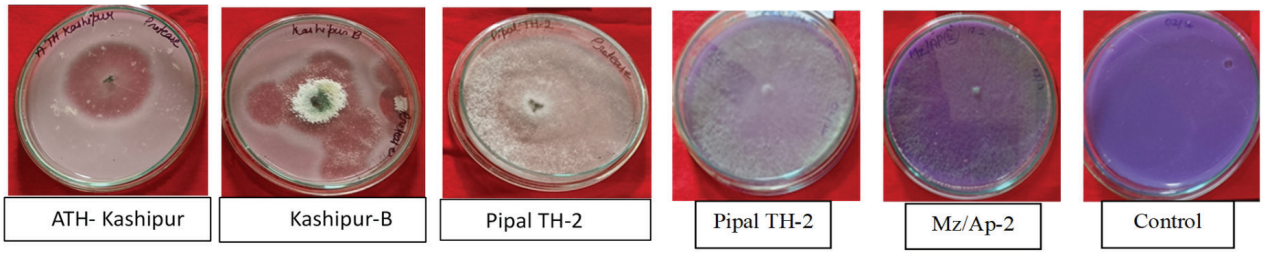


Fig 6e. Protease test

Fig 6f. Phosphate solubalization

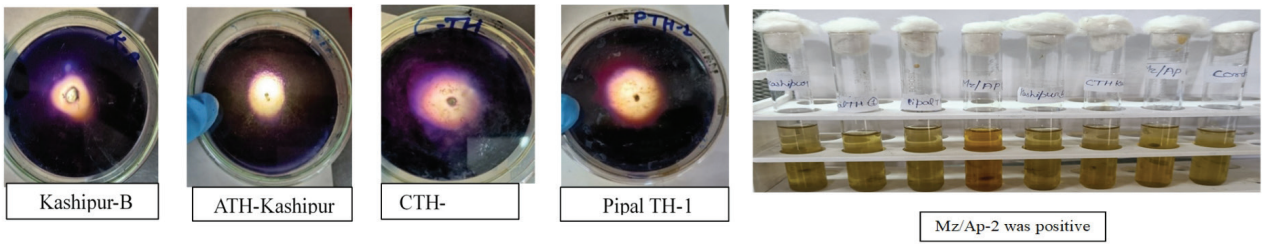


Fig 6g. Amylase test

Fig 6h. Ammonia production

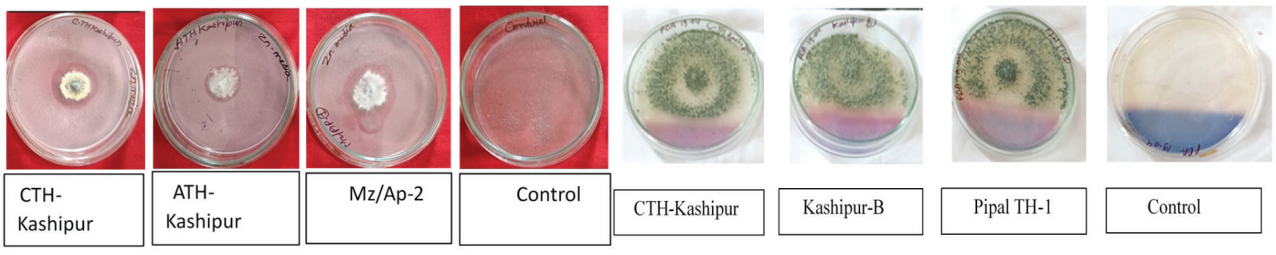


Fig 6i. Zn solubilization

Fig 6j. Siderophore production

Fig 6. *In vitro* assay of plant-promoting traits and extracellular enzymes secreted by the *Trichoderma* isolates

CONCLUSION

In conclusion, this study evaluated the biocontrol potential and beneficial attributes of *Trichoderma* isolates. The isolates exhibited diverse enzymatic activities, including catalase, chitinase, and amylase production. Positive responses were seen in assays for phosphate solubilization, HCN production, and zinc solubilization with strains Mz/Ap-2 and ATH-Kashipur showing the best results in the majority of the tests performed during the investigation. Notably, the isolates displayed significant antagonistic effects against key pathogens of pulse crops, emphasizing their potential as biocontrol agents. The findings underscore the multifaceted role of *Trichoderma* sp. in promoting plant health and disease management, highlighting its value as an eco-friendly and sustainable strategy for agricultural applications. Further studies are required to evaluate the performance of these isolates in on-field assays and thereafter development of dry and liquid formulations of the best *Trichoderma* spp. isolate as a product for the betterment of the farming community.

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