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EVALUATION OF *Trichoderma* spp. FOR THE COMPOUNDS RESPONSIBLE FORANTIBIOSIS AND MYCOPARASITISM ACTIVITY *IN VITRO*

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ABSTRACT

Biological control has become an important part of plant disease management, and it is a cost-effective and safe method in a variety of crops. *Trichoderma* spp. has attracted scientific attention due to its biocontrol effectiveness against a variety of economically important aerial, root and soil born diseases. In our current study, twenty strains were collected from different agro-climatic zone of Andhra Pradesh out of which, five strains of *Trichoderma* isolates (AT-1, AT-6, NT-3, KT-1 and KT-3) were screened for their biochemical activity. During the process of mycoparasitism phytopathogen cell walls are degraded by cell wall-degrading enzymes produced from *Trichoderma* were evaluated *in-vitro* through the production of cellulase, Protease, amylase, iron chelators (siderophores), cyanhydric acid (HCN) and ammonia (NH_3). *Trichoderma* isolate AT-6, NT-3 and KT-1 showed positive results for cellulase test. Isolates KT-1 showed positive for protease test. For amylase test KT-3, NT-3 and AT-1 showed positive with halo zone formation and for siderophore assay all the isolates showed positive results and for HCN assay the *Trichoderma* isolate KT-1, AT-6 and NT-3 showed positive results. For NH_3 assay KT-1, NT-3, AT-6 and KT-3 showed positive.

KEYWORDS: *Trichoderma* spp. lytic enzymes, cellulase, amylase, protease, siderophore, NH_3 and HCN.

INTRODUCTION

Disease management in economically significant crops is important for maintaining product quality and quantity. Fungicide treatment to the soil is costly and harmful to non-target microorganisms. *Trichoderma* spp. found all over the world and are commonly associated with soil surrounding plant roots and debris and has also been identified as promising biological agents for controlling plant diseases (Schuster and Schmoll, 2010). *Trichoderma* species are fast-growing free-living or entophytic fungi that thrive in soil and plant root habitats. They have attracted attention as cost-effective and secure biocontrol agents for various plant diseases and as boosters of plant defence mechanisms. There are several mechanisms involved in *Trichoderma* antagonism namely antibiosis; competition for nutrients; and mycoparasitism whereby *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as cellulase, chitinase, β-1, 3 glucanase, amylase and protease (Chet, 1987). In addition, Production of antifungal substances by *Trichoderma* spp. such as iron chelators (siderophore) and hydrogen cyanide may also promote plant growth (Samuels *et al*., 2002 and Ushamailini *et al*., 2008); these metabolites can protect plants against phytopathogens (Benitez *et al*., 2000 and Whipps 2001). Secondary metabolite production by fungi showing bio-control activity has been most commonly reported from isolates of *Trichoderma* spp. There are several large numbers of antibacterial and antifungal metabolites that have proven relevance for the management of diverse fungal infections. The aim of this study was to evaluate *In-vitro* biochemical properties of *Trichoderma* spp. Twenty isolates were collected from different agro-climatic zones of Andhra Pradesh. Moreover, investigating their enzymatic activity in order to select the promising *Trichoderma* species and these spsecies could be utilised as a potential biofertilizer.

MATERIAL AND METHODS

Trichoderma isolates *viz.,* Anakapalle *Trichoderma isolate* (AT-1 and AT-6) Nandyal *Trichoderma isolate* (NT-3) Kadiri *Trichoderma isolate* (KT-1 and KT-3) were collected from different agro climatic zones of Andhra Pradesh and these *Trichoderma* isolates were designated as the initial letter of the region from where it was collected. The plates were incubated at 26 ± 2°C for 5 days. *Trichoderma* colonies appeared in the plates were noted and maintained regularly by sub culturing. They were purified by single spore isolation method and maintained on potato dextrose agar (PDA) slants for future use.

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Qualitative assay of extracellular enzymes

Enzymatic assay of *Trichoderma* isolates were carried out by plate assay and test tube method on the respective media to study for extracellular enzymes. Assay were based on the formation of clear zones, change of colour and its intensity around the fungal colonies for production of cellulase, amylase, protease, siderophore, NH_3 and HCN enzymes. The independent experiments were performed with three replicates for each isolate.

Cellulase assay

Carboxymethyl cellulose (CMC) agar plate (Hankin and Anagnostakis, 1975) was prepared to screen for cellulase production. The medium composition (per litre): Cellulose 0.5 g, K_2HPO_4 0.099 g, Magnesium sulphate 0.049 g, Yeast extract 0.05 g, Congo red 0.05 g, Agar 20 g, distilled water 1 litre. The medium was aseptically transferred to petri dishes and inoculated with a 6 mm agar disc cut from 5-day old *Trichoderma* culture of each strain separately and incubated at 26 ± 2°C in darkness for 3 to 5 days. Halo zone formation around the fungal colonies indicates the cellulase enzyme production.

Amylase assay

Amylase activity (Hankin and Anagnostakis, 1975) was assessed by growing the *Trichoderma* strains on Starch Agar Medium (Starch 20.00 g, Beef extract 3.00 g, Peptone 5.00 g, Agar 16.00 g, Distilled water 1000 ml). The medium was aseptically transferred to petri dishes and inoculated with a 6 mm agar disc cut from 5-days old culture of each strain separately and incubated at 26 ± 2ºC in darkness for 3 to 5 days. Then the plates were flooded with 1% iodine in 2% potassium iodide. The clear zone formed surrounding the colony was considered as positive for amylase activity

Protease assay

Protease activity of *Trichoderma* isolate was determined according to the modified method of Berg *et al.* (2002). Skim milk agar medium (51.5 g/litre) was used for detection of protease activity. Culture disc from 5-6 days old *Trichoderma* cultures were inoculated on skim milk agar medium and incubated at 28°C ± 2ºC for three to four days. Positive *Trichoderma* spp. strain gave a clear zone indicating the production of protease enzyme.

Siderophores production

The fungal isolates were screened for siderophore production by the universal chrome Azurol S assay as described by Schwyn and Neilands (1987). The culture was inoculated into the autoclaved Kings' B media broth (**Kings' B medium g/litre** Peptone 5.0 g, K_2HPO_4 1.2 g, Magnesium sulphate 1.5 g, Glycerol 2 ml**,** 1 L distilled water**,** pH 7.2) and incubated for 2-3 days at room temperature. The incubated cultures were centrifuged for 12 min at 5000 g. CAS solution was added to the culture supernatant and incubated for 30 min in the dark. Blue to orange pinkish colour change indicates the presence of siderophores.

Ammonia production

Ammonia production was determined by the method given by Dye (1962) growing the different *Trichoderma* cultures in peptone water broth. The tubes were incubated at 30°C for 4 days, after which 1 ml of Nessler's reagent was added to each tube. Observations were recorded in terms of a faint yellow colour to deep yellow colour.

Production of HCN

The fungal isolates were cultured on Potato dextrose agar plates amended with glycine (4.4 g/L). Whatman No. 1 filter paper was soaked in 1% picric acid and sprayed with 1ml of 10% Na_2CO_3 and placed under the Petri dish lids. The plates were carefully sealed with parafilm to prevent the leakage and were kept for incubation for 5 days at 28±2°C. Color change from yellow to reddish brown indicates the production of HCN. Bakker and Schippers (1987) had reported in their study that a change in colour of the filter paper from yellow to light brown or reddish brown indicated the production of HCN.

RESULTS AND DISCUSSION

When twenty isolates were screened *in vitro* against three pathogens *viz., Sclerotium rolfsii, Aspergillus niger* and *Rhizcotonia batatticola* in dual culture, five isolataes (AT-1, AT-6, NT-3, KT-1 and KT-3) were selected as potential strains based on per cent inhibition.

Trichoderma isolates *viz.,* AT-6, NT-3 and KT-1 showed positive for the cellulase activity (Table 1, Plate 1A). Strong evidence for the production of cellulase enzymes was provided by the clear zone that appeared around the colony. Cellulases are the enzymes responsible for the cleavage of the β–1, 4–glycosidic linkages in cellulose. The two enzymes that are crucial in the enzymatic breakdown of the cell walls of phytopathogenic fungi are cellulase and 1, 3-glucanase during mycoparasitic interaction (Kamala and Indira, 2014). Benhamou and Chet (1997) reported that when *Trichoderma* attempts to penetrate the host cell walls result in the production of significant amounts of cellulytic enzymes, which are crucial in breaching the host cell walls.

Table 1. Qualitative assay of biochemicals produced by *Trichoderma* spp.

S. No.	Enzymatic activity	*Trichoderma* isolates				
		AT-1	AT-6	NT-3	KT-1	KT-3
1	Cellulase	-	+	+	+	-
2	Protease	-	-	-	+	-
3	Siderophore	+	+	+	+	+
4	Amylase	+	-	+	-	+
5	HCN	-	+	+	+	-
6	NH_3	-	+	+	+	+

+ indicates positive for enzymatic activity and – indicates the negative for enzymatic activity
(No enzyme activity was produced)

Trichoderma isolate AT-1, NT-3 and KT-3 exhibited amylase activity by forming halo zone (Table 1, Plate 1B). Amylase is the extracellular enzyme that randomly cleaves the 1,4 α -D-glucosidic linkages between adjacent glucose units in the linear amylose chain to produce glucose thus making nutrients available for the bioagent. The results are agreement with Abdenaceur *et al.* (2022) who showed that among the 15 *Trichoderma* isolates isolated from rhizosphere soil in Northern Algeria only five isolates T1, T6, T10, T12 and T15 showed positive for amylase activity by forming starch hydrolysing zone.

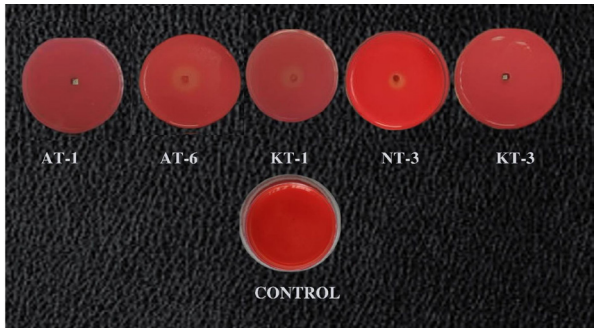
Only one *Trichoderma* isolate KT-1 showed positive for protease activity (Table 1, Plate 1C). It has been suggested that this protease is involved in the degradation of pathogen cell walls, membranes and even proteins released by the lysis of the pathogen, thus making nutrients available for the endophytes (Goldman *et al*., 1994). Fungal proteases play a significant role in cell wall lyses by catalysing the cleavage of peptide bonds in proteins (Mata *et al*., 2001).

All the five-isolate showed positive results for the siderophore by changing the colour from blue to orange and pinkish colour resulting from siderophore removal of Fe from the dye (Table 1, Plate 1D). Siderophores are among the strongest (highest affinity) Fe^{3+} binding agents known and compete with pathogen and can supress the growth of pathogen by depriving the necessary micronutrients. These results are similar with Singh *et al.* (2022) when conducted siderophore production test qualitatively by inoculation of *Trichoderma* isolates on chrome azurol sulfonate (CAS) agar medium. All 25 isolates showed positive results for siderophore production. Among the tested isolates, *Trichoderma* isolates (T3, T4, T5, T7, T8, T9, T10, T11, T14, T15, T18 and T21) exhibited strong siderophore production by pink and orange halo colour development.

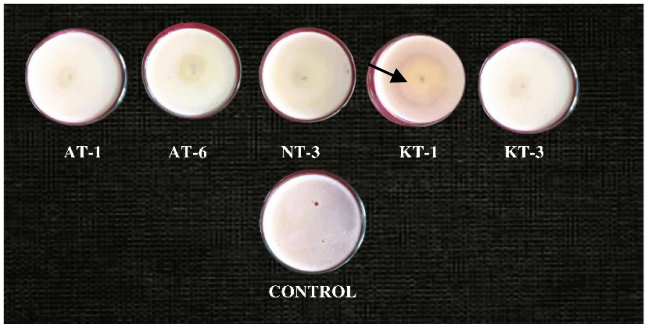
Trichoderma isolates such as KT-1, AT-6 and NT-3 showed positive for HCN assay by changing the filter paper colour from yellow to reddish brown on colour (Table 1, Plate 1E). HCN synthesizes some antibiotics or cell wall degrading enzymes (Ramette *et al*., 2006). HCN toxicity inhibits cytochrome c oxidase as well as other important metalloenzymes (Nandi *et al*., 2017). These results were in agreement with Mohiddin *et al*. (2017) stated that HCN production is an important trait found in many bioagents as it indirectly promote plant growth by controlling some soil borne pathogens, while screening for HCN among five isolates three *Trichoderma* isolate AT-3, AT-5 and AT-7 were found positive and rest were found negative.

Trichoderma spp. isolates AT-6, NT-3, KT-1 and KT-3 showed positive for ammonium production by changing colour from faint yellow to bright yellow colour (Table 1, Plate 1F). Abdenaceur *et al.,* (2022). Quantitative screening of NH_3 production revealed that isolates of *Trichoderma* isolate T2, T4, T6, T11, T12 had showed positive for NH_3 production (Table 1 and Fig. 1).

The results obtained showed that the qualitative methods are valid and important in selection of biocontrol agents. These methods in plates reveal feasibility for an initial selection of strains for screening large number of samples. These *Trichoderma* isolates can be applied as biocontrol agents in management of disease and increasing yield and to increase production in the



(1A)



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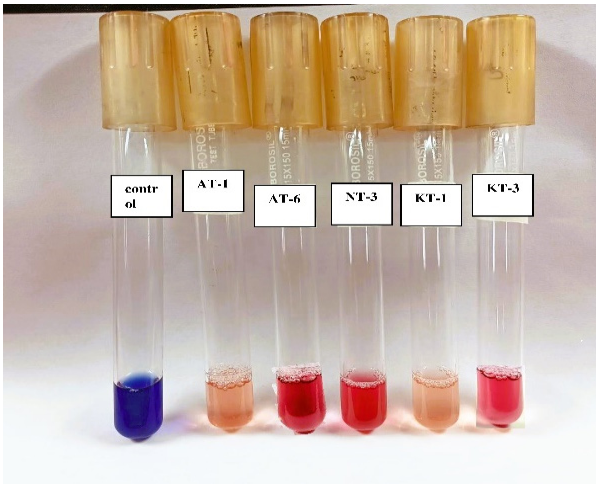
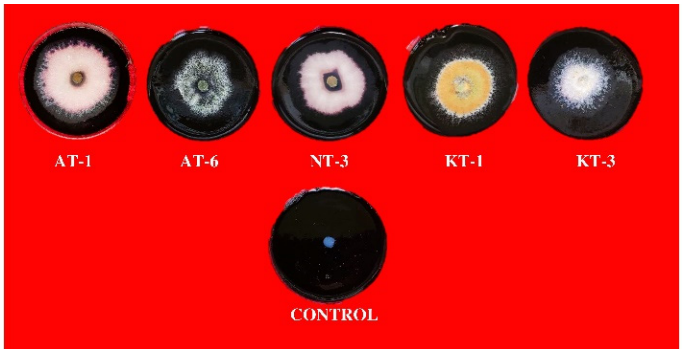


(1C)



(1D)

(1E)

(1F)

The figure shows the production of (1A) Cellulase; (1B) Protease; (1C) Siderophore; (1D) amylase; (1E) HCN; (1F) Ammonium by *Trichoderma* spp.

Fig. 1. Qualitative assay of biochemicals produced by *Trichoderma* spp.

agriculture. *Trichoderma* isolates KT-1 may have good biocontrol ability as it showed positive for five test out of six qualitative tests performed followed by NT-3 and KT-3. *Trichoderma* isolates AT-1 may be least effective as it shown positive for the two test only. However, knowledge of the types, amounts and characteristics of enzymes produced by *Trichoderma* cited above would be studied for selecting organisms best suited for biocontrol in agriculture and industrial requirements. Further research has to be done to quantify the lytic enzymes and *in vivo* experiments to be conducted against phytopathogens.

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