Journal of Applied and Natural Science
13(4), 1172 - 1179 (2021)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

Phytostimulation and growth promotion activities of *Trichoderma* spp. on groundnut (*Arachis hypogaea* L.) crop

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Article Info
https://doi.org/10.31018/jans.v13i4.2936
Received: August 21, 2021
Revised: October 17, 2021
Accepted: October 22, 2021

How to Cite
Ayyandurai, M. *et al.* (2021). Phytostimulation and growth promotion activities of *Trichoderma* spp. on groundnut (*Arachis hypogaea* L.) crop. *Journal of Applied and Natural Science*, 13(4), 1172 - 1179. https://doi.org/10.31018/jans.v13i4.2936

Abstract
Groundnut **(***Arachis hypogaea* L.) suffers from many soil borne pathogens that deteriorate the quality of the seeds and are responsible for high yield loss. Practically *Trichoderma* sp. is used for seed treatment, it minimizes the seed and soil borne pathogens and supports plant growth promotion activities. In the present study, five different isolates of *Trichoderma* spp. were isolated from groundnut (*A. hypogaea*) rhizosphere soil. All the five isolates were confirmed by morphological methods and using molecular tools through Polymerase Chain Reaction (PCR) amplification of Internal Transcribed Spacer (ITS) region of *Trichoderma* sp. and DNA gets amplified in 650 bp to 700 bp. *Trichoderma* spp. were molecularly identified as T(SP)-20 (*Trichoderma longibrachiatum*), T(AR)-10 (*T. asperellum*), T(VT)-3 (*T. hamatum*), T(BI)-16 (*T. longibrachiatum*), T(TK)-23 (*T. citrinoviride*). Phytostimulation activities of all the six isolates *viz.,* phosphate solubilization, Ammonia production, IAA production, and Siderophore production, were evaluated. Among the six isolates, T(SP)-20, T(AR)-10, and TNAU-TA showed higher phytostimulation activities. The growth promotion of *Trichoderma* spp. on groundnut was assessed through the roll towel method. The isolate T (SP)-20 (*T. longibrachiatum*) produced the highest germination percentage of 93.33 and vigor index of 2246.2. This work developed a new isolate of *T. longibrachiatum* (T(SP)-20) which is a native isolate having significant phytostimulation and growth promotion activities and it could be exploited for other soil borne disease managing successfully.

Keywords: Groundnut, growth promotion, *Sclerotium rolfsii*, Soil-borne pathogens, *Trichoderma* spp.

INTRODUCTION

Groundnut is an important oilseed crop that belongs to the family of Leguminosae. It is grown widely throughout the world under various agroclimatic conditions. It is a valuable source of all essential nutrients. In India, Gujarat ranks first in groundnut area (16 lakh ha) with the production of 46 lakh tonnes. The highest productivity of 2980 kg/ha was recorded in the State of Tamil Nadu, while the productivity was only about 2751 kg/ha in Gujarat (www.indiastat.com). Groundnut is infected with a wide range of seed and soil borne pathogens like *Aspergillus niger, Sclerotium rolfsii, Macrophomina phaseolina*. The infection by these pathogens caused the qualitative and quantitative losses of the crop. The extensive use of chemical fungicides and fertilizer resulted in the emergence of fungicide-resistant pathogens and concerns have been raised over the residual

effects on the environment and human health, so biocontrol agents (BCAs) are an alternative to fungicides (Ons *et al*., 2020). It is important to highlight that pre-treating seeds with biocontrol agents is an alternative for controlling severe diseases that reduces yields more than 30%, making peanut production economically non-viable (Paredes *et al.,* 2017). *Trichoderma* spp. widely distributed soilborne antagonistic fungi used for seed treatment and significantly suppresses the growth of plant pathogenic microorganisms and enhance the rate of plant growth of groundnut Siddiquee *et al*., 2017). The seed and soil borne pathogen infection is reduced by seeds are treated with *Trichoderma* spp., which adhere the seed coat region to successfully colonize the harmful pathogens and also induce the growth of the plants (Prasad *et al* 2020). Halifu *et al*., (2019) reported that secretion of cell wall degrading enzymes such as cellulase, xylanase and glucanases by *Trichoderma* species impairs the microbial cell functions such as nutrient absorption in the rhizosphere and also strengthen the immune system of host plant. *Trichoderma* spp. enhanced plant growth by releasing hormone-like compounds that boost up root development and plant growth. The rapid growth of plant induces microbial populations through the secretion of significant amounts of root exudates, which in turn increases the availability of nutrients for microbial consumption (Carvalhais *et al*., 2015). Hence, *Trichoderma* spp. represents the most widely employed biocontrol agents, playing a useful role as plant growth promoters and valuable sources of secondary metabolites. The present study focuses on the selection of efficient *Trichoderma* sp. based on the biochemical activities and growth promotion on groundnut (*A. hypogaea* L.)

MATERIALS AND METHODS

Isolation of *Trichoderma* spp. from the soil

The sandy, loamy soil samples were collected from five different groundnut-grown regions such as Alanganallur, Servarayanpatti, Vovval thotam, Bodi, and Thirupparankundram, of Tamil Nadu, in India. *Trichoderma* spp. were isolated through soil dilution plating technique in *Trichoderma* selective medium (TSM) (Elad and Chet, 1983). The isolates were further purified through a single hyphal tip method and sub cultured on PDA medium and incubated at 28±2°C for five days (Kumar *et al.,* 2014).

Morphological characters and molecular confirmation of *Trichoderma* spp.

The isolated five different *Trichoderma* spp. *viz.,* T(SP)-20 (*Trichoderma longibrachiatum* Acc. No - MZ277378), T(AR)-10 (*T. asperellum* Acc. No MZ277326), T(VT)-3 (*T. hamatum* Acc. No MZ675442), T(BI)-16 (*T. longibrachiatum* Acc. No MZ802988), T(TK)-23 (*T. citrinoviride*) were confirmed by morphological characters such as mycelia, conidia, phialides, colony growth pattern, and color (Rifai, 1969). The genomic DNA of *Trichoderma* spp. was isolated by Cetyl trimethylammonium bromide(CTAB) method (Narayanasamy and Saravana, 2009). The reaction mixture for Polymerase Chain Reaction (PCR) amplification of the DNA contained the volume of 10 µl in which the master mix was of 5 µl, 1 µl of forward and reverse primer individually, 2 µl of DNA, and 1 µl of sterile distilled water. Mastermix had 0.25 mM dNTP, 1.5 mM $MgCl_2$, Taq polymerase and buffer. The sequence of genomic DNA was identified using the Forward primer: ITS 1(5'- TCCGTAGGTGAACCTGCGG-3') and Reverse primer: ITS 4 (5'- TCCTCCGC TTATTGA TATGC-3'). PCR was done with a master cycler with an inbuilt program for initial denaturation at 94 ºC for 5 minutes, continued by 35 cycles composed of denaturing at 94 ºC for 1 minute, annealing at 46 ºC for 1 minute, extension at 72 ºC for 1 minute ending with a final extension at 72 ºC for 10 minutes (White *et al.*, 1990). The PCR products were subjected to analysis by gel electrophoresis on 1.5 per cent agarose gel, visualized and documented under UV light.

Phytostimulation activities

In the present study, all six *Trichoderma* spp. *viz*., [T (SP)-20, T(AR)-10, T(VT)-3, T(BI)-16, T(TK)-23, TNAU-TA] were assessed for the biochemical characteristics such as siderophore production, IAA production, phosphate solubilization, and ammonia production.

a. Siderophore production

Chrom Azurol S blue agar medium (CAS) medium was used to detect siderophore production of *Trichoderma* sp. ((Khamna *et al.*, 2009). The 8mm plug of *Trichoderma* sp was placed on the CAS medium containing Petri plate, which was incubated at 28°C for five days. After the incubation, color changes from yellow to orange zone produced around the colony indicated the production of siderophore by *Trichoderma* spp. (Schwyn and Neilands, 1987).

b. IAA production

Salkowski reagent was used for the detection and quantification of IAA (Hartmann *et al.*, 1983). A 20 ml of PDA broth containing conical flask was inoculated with all the six isolates of *Trichoderma* spp. Individually, and each flask was amended with 0.1% tryptophan served as treatment, a precursor for IAA. The broth without adding 0.1% tryptophan was maintained as a control (Gordon and Paleg, 1957). The broths were incubated at 28±2 °C for 5days. After that broth was centrifuged at 10000 rpm for 10 minutes. One ml of supernatant was added in 2ml Salkowski's reagent (1ml 0.5 M $FeCl_2$ in 50ml 35% perchloric acid) and kept

in dark for 30 minutes. The development of pink color indicates the production of IAA.

c. Phosphate solubilization

The Pikovskayc's broth was prepared in a sterile conical flask and added with 5g/l of tricalcium phosphate. The broth was inoculated with 7 days old culture *Trichoderma* sp and incubated at 28±2°C for four days. *Trichoderma* filtrate (50µl) was added with (5µl) of ammonium molybdate and shaken after adding chlorostannous acid 13µl, and distilled water (2.5ml) was added. Then K_2HPO_4 was added to make different concentrations of (0.2ppm, 0.4 ppm,0.6 ppm, 0.8 ppm, and 1 ppm standard solution. The development of blue color indicated phosphate solubilization. The blue color intensity was read with 600nm in a spectrophotometer (King, 1932).

d. Ammonia production

Trichoderma spp. culture was inoculated into the peptone water amended boiling tube. After 72hours, the addition of Nessler's reagent resulted in turning the brown color from yellow indicated a positive result of ammonia production (Dye, 1962).

Roll towel method

Efficacy of effective *Trichoderma* spp. on seed germination and growth promotion activity was assessed through the standard roll towel method (International Seed Testing Association, 1993). All the six isolates of *Trichoderma* spp. were grown in PDA broth at 28±2° C for five days. After that, centrifuged at 10000 rpm for 5 min; finally, the supernatant was discarded, and the pellet should be dissolved in sterile water. *Trichoderma* sp. suspension at 10^8 cfu /ml containing 100 mg of Carboxy Methyl Cellulose (CMC) was prepared. Likewise, the suspension of all the six isolates of *Trichoderma* spp. were prepared. The groundnut seeds were treated with 0.1% mercuric chloride for 5min and rinsed with sterile distilled water for surface sterilisation. The surface sterilized seed was soaked in *Trichoderma* sp. suspension for 12 hrs. In the case of control, the seed was soaked in sterile water. The soaked seeds of both treatment and control were arranged in wet blotter paper, rolled carefully, and then incubated for ten days; after the incubation, the germination percentage, root length, shoot length and vigor index were calculated (Agrawal and Agrawal, 2013).

Vigor Index = Germination % × mean total length of the seedling (root length + shoot length) …... .Eq.1

Statistical analysis

Mean differences of the treatment were evaluated with ANOVA at a significant level ($P< 0.05$), and means were compared by Duncan's Multiple Range Test (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Trichoderma spp. were isolated from five different groundnut grown regions in Tamil Nadu such as Alanganallur, Servarayanpatti, Vovval thotam, Thiruparankundram, and Bodi and designated with the isolated code like T(SP)-20 (*T. longibrachiatum*), T(SP)-10 (*T. asperellum*), T(VT)-3 (*T. hamatum*), T(TK)-23 (*T. citrinoviride*), T(BI)-16 (*T. longibrachiatum*) are shown in Table 1

Morphology of *Trichoderma* spp.

Cultural characteristics comprising growth rate, color, and colony appearance were regarded as taxonomically useful characteristics for *Trichoderma* spp. (Samuels *et al.*, 2002).The cultural growth of fungal antagonist *Trichoderma* spp. differed from dull to dark green color in ring-like zones. The antagonists identified morphologically with their colony characters showed that T(SP)-20 was greenish in color with a yellowish inner ring, and T(AR)-10 was dark green with sparse ring-like growth of culture (Table 2; Fig.1; Fig.2). Similarly, Sharma and Singh (2014) reported that *Trichoderma* sp initially produced flat pustules in concentric rings. The pustules appeared powdery due to dense conidiation. *T. virens* isolate showed predominant effuse conidiation without formation of any pustule.

Molecular characteristics of *Trichoderma* spp.

All the six *Trichoderma* spp. were characterized molecularly by PCR using a universal primer of ITS1 and ITS4 and produced the amplicon size in the range of 650 bp to 700 bp (Fig.3). The PCR amplified *Trichoderma* spp. product was sent to the Eurofins Genomics Bangalore, India for DNA sequencing, and the sequenced product was submitted to the NCBI database. The accession numbers received T(SP)-20 (*Trichoderma longibrachiatum* Acc. No MZ277378), T(SP)-10 (*T. asperellum* MZ277326), T(VT)-3 (*T. hamatum* Acc. NoMZ675442), T(TK)-23 (*T. citrinoviride*) are (Table 2). Similarly, Castle *et al.* (1998) reported that PCR of *T. koningii* and *T. longibrachiatum*. *T. citrinoviride, T. koningii*, *T. atroviride* isolates using universal primers (ITS1, and ITS4) were amplified in 700 bp. Also, Shahid (2013) reported universal primers (ITS-1& ITS-4) used for the amplification of 28S rRNA gene fragment of *T. longibrachiatum* that produced a sharp band of about 654-700 bp on the gel.

Phytostimulation activities of *Trichoderma* spp.

The isolates, namely T(SP)-20. T(AR)-10, T(VT)-3, T(TK)-23, TNAU-TA, showed a positive result in phosphate solubilization by observing the blue color appearance. T(SP)-20, T(AR)-10 produced the high blue color intensity among the six isolates. The isolate T(BI)-16

Table 1. *Trichoderma* spp. isolated from rhizosphere region of groundnut plant

S.No	Place of collection	Districts	*Trichoderma* sp.	Isolate code	Geo-coordinates	
					Latitude	Longitude
1	Servarayanpatti	Sivagangai	*T. longibrachiatum*	T(SP)-20	9.86670°N	78.4832°E
2	Alanganallur	Madurai	*T. asperellum*	T(AR)-10	10.0474°N	78.0904°E
3	Vavval Thottam	Madurai	*T. hamatum*	T(VT)-3	9.9699°N	78.2040° E
4	Bodi	Theni	*T. longibrachiatum*	T(BI)-16	10.0106°N	77.3497°E
5	Thirupparan kundram	Madurai	*T. citrinoviride*	T(TK)-23	9.8823° N	78.0720°E
6	Culture collection of	Coimbatore	*T. asperellum*	TNAU-TA	11.0122°N	76.9354°E

Table 2. Morphological characterization and molecular confirmation of *Trichoderma* spp.

Isolate code	Colony character	Accession number
T(SP)-20	Greenish towards outside with yellowish inner ring	*Trichoderma longibrachiatum (*MZ277378)
T(AR)-10	Dark green with sparse ring-like growth	*Trichoderma asperellum* (MZ277326)
T(VT)-3	Dull green culture with ring-like pattern	*Trichoderma hamatum* (MZ675442)
T(BI)-16	Light green to yellowish culture with whitish mycelial growth	*Trichoderma longibrachiatum* (MZ802988)
T(TK)-23	Complete dull green tufts or pustules	*Trichoderma citrinoviride*

Table 3. Phytostimulation activities of *Trichoderma* sp.

S.No.	ISOLATE	Phosphate Solubilization	Ammonia production	IAA Production	Siderophore production
1	T(SP)-20	++	+	+++	+
2	T(AR)-10	+	+	++	+
3	T(VT)-3	+	-	+	++
4	T(BI)-16	+	-	+	-
5	T(TK)-23	-	+	+	+
6	TNAU-TA	++	++	+++	++
7	CONTROL	-	-	-	-

"+++", good producers; "++", medium producers; "+", low producers; "-", negative reaction

did not show any phosphate solubilization reaction (Table 3; Fig.4). Our results were in accordance with the findings of Azarmi *et al.* (2011), who reported that *T. harzianum* boosted the phosphorus and other nutrient content in tomato seedlings, as well as stem height and diameter, and fresh and dry weights.

All the *Trichoderma* spp. showed deep yellowish to brown color during the test of ammonia production except control. The dark brown color was observed in *T. longibrachiatum* (T(SP)-20), *T. asperellum* (T(AR)-10), *T. asperellum* (TNAU-TA) which indicated that production of ammonia. (Table 3, Fig.4). Similarly, Prasad *et al.* (2017) reported that ten *Trichoderma* spp. used for the biochemical test of ammonia production, exhibited positive reaction by the appearance of brown color (positive result) except *T. harzianum*-6.

Indole acetic acid (IAA) production of *Trichoderma* spp. was observed to be positive for all the six *Trichoderma* spp. production of reddish-pink color except for the control plate. Isolates of T(SP)-20, T(AR)-10, TNAU-TA were noticed with the highest IAA production. The IAA was highly noticed in the isolate T(SP)-20, which induced higher growth than the remaining isolates. The IAA was responsible for the growth promotion activity (Table 3; Fig.4). Guey *et al.* (2018) reported that out of 20 *Trichoderma* strains, only eight strains showed the maximum IAA production. Among the eight strains, TG 4 showed maximum (90 µg/ml) IAA production. In Arabidopsis, seedlings treated with *T. virens,* or *T. atroviride* produced auxin-related substances such as indole-3-acetic acid, indole-3-acetaldehyde, and indole-3-ethanol, which were responsible for increased biomass production (Contreras-Cornejo *et al.*, 2014).

All the *Trichoderma* spp. isolates showed siderophore production in the plate assay in which they showed a prominent yellow color zone. Among the six *Trichoder-*

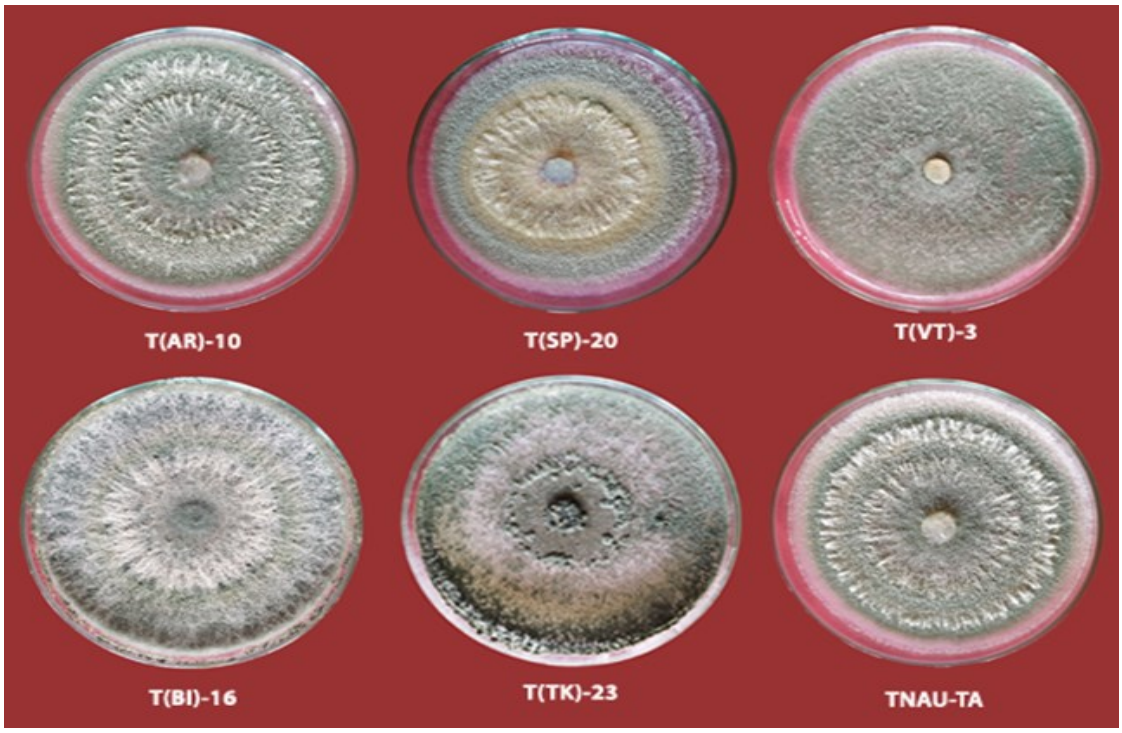

Fig.1. *Isolates of Trichoderma spp*.

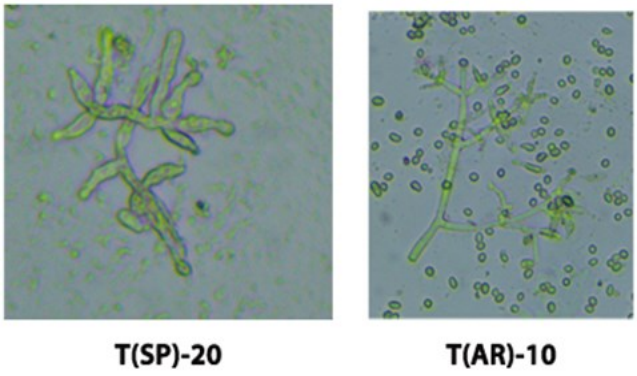

Fig. 2. *Showing conidia with phialides of Trichoderma spp.*

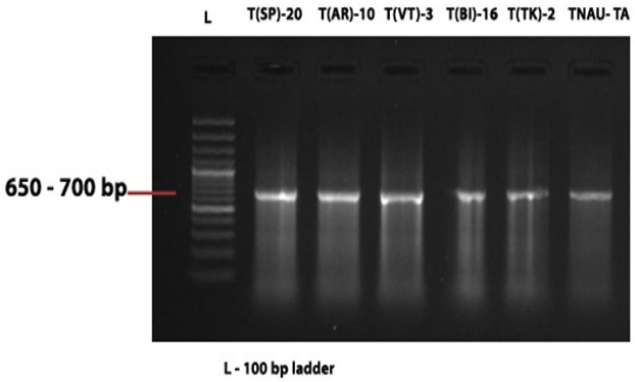

Fig. 3. *Showing PCR amplification of Trichoderma spp.*

Table 4. Growth promotion activities of *Trichoderma* spp.

S.No.	ISOLATE	Shoot length(cm)	Root length(cm)	Germination %	Vigor index
1	T(SP)-20	10.6^{a} (19.00)	13.4^{a} (21.47)	93.33^{a} (75.03)	2246.2^{a}
2	T(AR)-10	8.7^{b} (17.15)	11.6^{b} (19.91)	86.66^{b} (68.58)	1762.2^{b}
3	T(VT)-3	8.3^{c} (16.74)	8.4^{c} (16.84)	80^{c} (63.43)	1341.3^{c}
4	T(BI)-16	6.5^{e} (14.77)	8.5^{c} (16.95)	73.33^{d} (58.90)	1107.3^{de}
5	T(TK)-23	8.5^{bc} (16.95)	7.6^{d} (16.00)	66.66^{e} (54.73)	1075.5^{e}
6	TNAU-TA	7.4^{d} (15.78)	6.9^{e} (15.22)	80^{c} (63.43)	1150.13^{d}
7	CONTROL	2.8^{f} (9.63)	4.5^{f} (12.24)	46.66^{f} (43.08)	342.2^{f}
	CD (P = 0.05)	0.36	0.29	2.70	60.88

Values are the means of three replicates; means in a column followed by the same letters are not significantly different according to Dungun's multiple range test at P= 0.05. Values in parentheses are arcsine transformed values

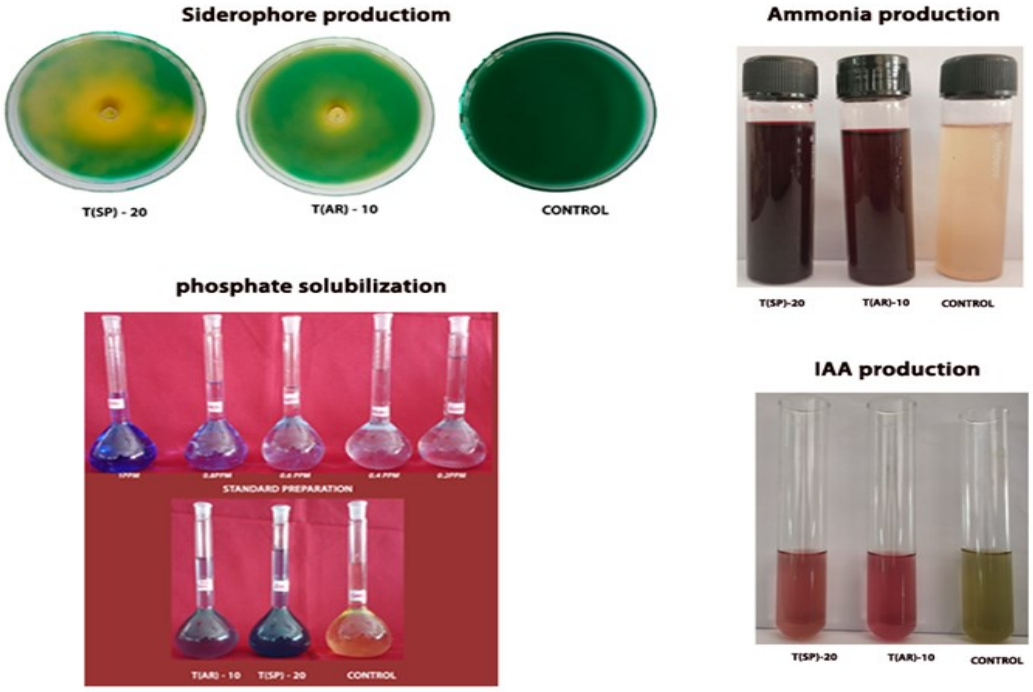

Fig. 4. *Showing phytostimulation activities of Trichoderma spp.*

Fig. 5. *Roll towel method:A. T(SP)-20 (T. longibrachiatum) and B. T(AR)-10 (T. asperellum) treated groundnut seeds showing high shoots, root length and germination percentage, C. Control (without Trichoderma sp. treatemet) showing poor shoots, root length and germination percentage*

ma spp. T(SP)-20, T(AR)-10 showed high production of siderophores than other isolates (Table 3, Fig.4). Comparable findings were reported by Zhao *et al*. (2014) with the siderophore-producing *T. asperellum* strain in cucumber. It boosted the available iron level in sterilized soil and increased the cucumber plants ability to absorb the nutrient elements. Our findings were in accordance with Prasad *et al.* (2017), who reported positive results with *Trichoderma* spp. isolates for ammonia production and IAA production, and siderophore production and those isolates effectively inhibited the mycelial growth of *S. rolfsii* (95.1%).

Growth promotion activities of *Trichoderma* spp.

Growth promotion activities of six *Trichoderma* spp. were assessed by roll towel method. The resulted revealed that T(SP)-20 produced a higher vigor index (2246.4) followed by T(SP)-10 (1762.2) and TNAU- TA (1341). The isolates T(SP)-20 and T(AR)-10 showed major growth-promoting activity as compared to the control. The *T. longibrachiatum* T(SP)-20 showed high germination percentage of 93.33%, with a high shoot length of 10.6cm, and root length of 13.4cm and this was followed by T(AR)-10(86.6) and TNAU- TA (80) as compared to the control in which germination percent-

age was only 46% and vigor index 342 (Table 4, Fig.5). Similarly, Rajaput and Rao (2019) reported that roll towel test with *T. harzianum* treated tomato seeds revealed that seed born pathogen infection was minimized to 15.5% and produced the high seed germination percentage (92.67%) and vigor index(2006.78) as compared to control in which the seed germination was 65% and vigor index was 1027.83. Similarly, Kumar *et al.* (2014) reported that *T. harzianum* treated chickpea seeds produced a high seedling length (16.50) and germination percentage (90%), and high vigor index (1485) as compared to control (germination percentage 58% and vigor index 705.86).

Conclusion

The current investigation provided strong evidence that *Trichoderma* spp. isolated from groundnut (*A. hypogea* L.) rhizosphere exhibits a great morphological and biochemical diversity. Screening of their ability to synthesize plant-growth-promoting and antifungal compounds revealed that the isolates were able to produce siderophore, synthesize IAA, and solubilize phosphate. Two *Trichoderma* isolates like T(SP)-20 (*T. longibrachiatum),* T(AR)-10 (*T. asperellum*) having higher plant growth-promoting activity were considered as the elite *Trichoderma* spp., which may be screened for the management of groundnut stem rot in both pot and field trials.

Conflict of interest

The authors declare that they have no conflict of interest.

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