

# Assessing disease control, growth promotion, and root nodule development as affected by *Trichoderma longibrachiatum* GFT2 in *Arachis hypogaea*

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## ABSTRACT

In the ancient agriculture practices to present day farmers are using leguminous plants in field to enhance of soil fertility, which possess *Rhizobium* bacteria that has mechanism to acquire nitrogen from air to soluble form. In addition, in this research isolated strain *Trichoderma longibrachiatum* GFT2 act as biofertilizer and biocontrol agent which has been characterized by performing chitinase (18.56 U/ml), glucanase (13.82U/ml) and IAA production (7.3000µg/ml), PO<sub>4</sub> solubilization (10.7142 µg/ml), siderophore production (77.5%). Root colonization assay performed for detection of *Trichoderma longibrachiatum* GFT2 which colonized over the root or not, further compability test isolated *Rhizobium pusense* PR4 and *Trichoderma longibrachiatum* GFT2 for capable to each other on same environment or not. Moreover, Application of *Trichoderma longibrachiatum* GFT2 and *Rhizobium pusense* PR4 on peanut plant (*Arachis hypogaea*) seed germination and nodule formation. Maximum seed germination rate was shown in seed treated with PR4+GFT2 (39%), whereas least number (01) of nodules formed in plant treated with dual culture *Trichoderma longibrachiatum* GFT2 and *Rhizobium pusense* PR4. Maximum Sixteen nodules formation in plant treated with in *Rhizobium pusense* PR4 was observed. In this paper, we analyzed the how *Trichoderma longibrachiatum* GFT2 effected on peanut plant (*Arachis hypogaea*) nodulation process when applying in soil and reduced the number of nodules by indirectly inhibit or decline the growth of nodulation bacteria. Plant growth is facilitated by *Trichoderma* strain, but nitrogen fixation is insufficient in leguminous plants. We can determine the precise interaction between *Trichoderma longibrachiatum* GFT2 and whether their alteration of soil profiles is due to them by analysing soil samples for meta transcriptomics.

**Keywords:** Plant Growth Promoting traits; Compatibility; Peanut; *Rhizobium pusense*; *Trichoderma longibrachiatum*; Seed germination

## INTRODUCTION

Peanut (*Arachis hypogaea*) plants are grown all over the world as demand grows, and it is also an ancient New World crop that is widely grown in India (Hancock, 2022). Groundnuts and peanuts are grown in a variety of climates and locations around the world, including tropical, subtropical, and warm climates (Pramanik *et al.*, 2019). Nowadays, India and China are the biggest producers of the peanut crop (Mansurov-Dsc *et al.*, 2021). Plant yield increased slightly globally, from 2 t/ha in 2000 to 3 t/ha in 2010 and 4 m/ha in 2020 (Kamir *et al.*, 2020). In recent data, China has broadened its production of peanuts around the world. However, production of peanuts greater than 4 m/ha can be obtained when diseases are controlled and good management practises are applied in the agronomy field (Mahajan *et al.*, 2017). More groundnut production is required because their seed contains 40%-55% oil and is used as cooking oil, among other things (Maestri *et al.*, 2020). Other uses of peanut in the food market include peanut butter, edible seed, and cooking oil, and humans consume it directly due to its high protein and lipid content (Yanti *et al.*, 2022). In addition to seeds, the foliage is an important source of fodder in regions where animals are extensively used on the farm, and the meal remaining after oil extraction is also an important source of animal feed (Sagona *et al.*, 2020).

For many years, *Trichoderma species* have been used as biocontrol agents and biofertilizers in agricultural fields. *Rhizospheric soil, decaying tissue, and root ecosystems* (Kumar and Khurana, 2021). It has the ability to produce a variety of powerful antibiotic compounds, which allows it to inhibit the growth of pathogenic microbes (Górniak *et al.*, 2019). *Trichoderma longibrachiatum* is an opportunistic invader that produces antibiotics to act as an antagonistic mechanism (Kumar and Khurana, 2021). *Trichoderma longibrachiatum* is a free-living, cosmopolitan fungus that is present in most of the rhizospheric soils as well as in other diverse environments (Ghazanfar *et al.*, 2018). *T. longibrachiatum* acts as a biocontrol agent by producing hydrolytic enzymes such as proteases, chitinases, amylases, xylanases, and glucanases, which help to achieve biocontrol activity (Mishra *et al.*, 2020). They also help boost plant growth by producing plant growth regulators like siderophores, antibiotics, phosphate solubilization, HCN production, and ammonium production (Syed Ab Rahman *et al.*, 2018). *Rhizobium* is a nodulating bacterium that can fix nitrogen from the environment in leguminous plants. *Rhizobium* requires a plant host for nitrogen fixation and lives in symbiotic association with leguminous plant root nodules because N<sub>3</sub> fixation cannot be accomplished independently (Alam *et al.*, 2015). In the agronomy field, *Rhizobium* is an essential source of nitrogen for plant growth and metabolism (Checcucci *et al.*, 2017). Nitrogen is essential for all living organisms for the synthesis of biomolecules, such as nucleic acids, proteins, and other nitrogen-containing compounds (Sindhu *et al.*, 2019). The increased utilisation of chemical fertilisers as a source of nitrogen for crops results in increased emissions of nitrogen oxides, soil acidification, and water pollution (Tian *et al.*, 2020).

In this research, we must know the impact of *Trichoderma longibrachiatum* GFT2 on plant nodules of *Arachis hypogaea*. In view of the fact that, for the last two decades and today, farmers are using *Trichoderma* as a biofertilizer and biocontrol agent, what is the benefit of all microbes that interact with the plant biosphere, and are they rhizosphere microbes as well? Therefore, a series of experiments like the root colonisation assay for determining the growth of *Trichoderma* over the root act as biocontrol and biofertilizers to enhance plant growth. On the other hand, a compatibility test is conducted for their survival in the same surroundings. Aside from that seed germination assay for peanut seed germination percentage, finally, we analysed the number of nodules produced on the root of plants treated with *Trichoderma longibrachiatum* GFT2. At the end of the experiment, we found that *Trichoderma longibrachiatum* GFT2 reduced the number of nodules on the root and indirectly inhibited the growth of *Rhizobium pusense* PR4.

## MATERIALS AND METHODS

### Isolation of *Rhizobium pusense* PR4 from Root Nodules:

A sterile, clean spade was used to dig approximately 15 cm sideways and up to a depth of about 20 cm. The clump of soil and the roots were carefully uplifted, placed in sterile aluminium foil where the nodules were detached from the roots, and kept in screw-capped vials containing silica gel to prevent desiccation (Etesami, 2022). The extraction was done a day after the nodules were harvested. Rhizobia was isolated from *Arachis hypogaea* (S20) fresh nodules grown in Mavajinva, Amreli, Gujarat, India. Fresh nodules were washed with sterile distilled water (SDW) and hydrated with SDW overnight (Prasanna Kumar *et al.*, 2022). Surface sterilisation of the nodules was carried out by placing them in 1% HgCl<sub>2</sub> for 30 s and then washing them with SDW five times (Mekonnen *et al.*, 2022). The nodules were then treated with 1% sodium hypochlorite for 3 minutes, followed by 10 washes with SDW. Each nodule was crushed in 100 l of 15% glycerol and then crushed with a sterilised glass rod, and 100 l of the mixture was spread on yeast mannitol extract agar with the help of a sterile micropipette and incubated at 28 ± 2 °C for two days (County-Kenya, 2021). The morphological and microscopic characteristics of *Rhizobium* were investigated. Individual colonies were characterised based on their size, color, shape, and elevation after 3-7 days of incubation at 28 ± 2 °C on yeast extract mannitol agar plates. Microscopic features of the isolates were studied by the gramme staining technique (Nagalingam *et al.*, 2020).

### Isolation of *Trichoderma longibrachiatum* GFT2 from rhizospheric soil:

A rhizospheric soil sample was collected from *Arachis hypogaea* (S20) at Mavajinva, Amreli, Gujarat, India. First, peanut plant uprooted, and soil was collected nearly 0.5 kg in airtight plastic bag and after that moist soil were dry and then use serial dilution and plate technique according to protocol (Nagalingam *et al.*, 2020). Make the serial dilution up to 10<sup>-1</sup> to 10<sup>-10</sup> and take 0.1 ml of each tube and spread over the PDA (HI-MEDIA, Pvt. Ltd., Mumbai, India) plate for isolation. Observed the growth of *Trichoderma* fungi on a daily basis in a BOD incubator at 28 ± 2 °C (Liu *et al.*, 2020). Elementary screening of fungi was done using macroscopic and microscopic features. The growth rate

and colour of colonies were revealed by macroscopic screening (Nagalingam et al., 2020). Each plate was prepared for microscopic screening slides, and fungi growth was mounted under 40 X magnification with 1% methylene blue, noting the conidial shape and filament length (Usman et al., 2021).

#### **Confirmatory test for *Rhizobium*:**

Three sorts of confirmatory tests were performed to confirm the rhizobium and distinguish it from other contaminating microbes. Tests were YEMA with Congo red, glucose peptone agar, and the ketolactose test (Patra et al., 2020).

#### **Peanut seeds and pot mixture:**

Peanut (*Arachis hypogaea*) seeds of the G-20 variety were obtained from Sarthi Agro Pvt. Ltd., Jamalpur, Ahmedabad, and Gujarat. The potting mixture was prepared using topsoil: perlite: soil in a ratio of 3:2:1 (Sayyed et al., 2019). To ensure complete elimination of soil-borne pathogens, the potting mixture was incubated in an oven at 90 °C for 24 hrs (Devarajan et al., 2021). After seed germination, an assay treatment with *Rhizobium* and *Trichoderma* inoculum was prepared and performed. The plants were grown in a greenhouse at 25±2 °C (Dörsch et al., 2002).

#### **Detection of PGP traits of fungal isolates under in vitro conditions**

##### **Assessment of IAA production:**

The spectrophotometric estimation of IAA was performed as per the method developed by Bric et al. (1991) with modifications adapted by Goswami et al. (2015). *Trichoderma longibrachiatum* GFT2 was incubated at 28± 2 °C in a potato dextrose broth medium supplemented with L-tryptophan (200 g/ml). The development of pink or brown colour in the assay system indicates the production of Indole. The optical density was recorded at 530 nm. The concentration of IAA produced by cultures was estimated against the standard curve of Indole 3-acetic acid (Hi-media-PCT0804) in the range of 10–100 g/ml.

##### **Phosphate solubilization:**

Goswami et al. (2015) described quantitative estimation of phosphate solubilization in 250-ml Erlenmeyer flasks containing 100 ml of Pikovskaya's broth medium and 1 ml of *T. longibrachiatum* GFT2 bioformulation containing 10<sup>6</sup> spores/ml. All tests were carried out in triplicate and the flasks were incubated at 28 ± 2 °C on a shaker (Nova shaking incubator, India) at 150 rpm for 10 days. The concentration of the soluble phosphate was estimated every 24 hours from the supernatant by using the stannous chloride method.

##### **Quantitative estimation of siderophore:**

Siderophores produced by *T. longibrachiatum* GFT2 were quantified using the CAS-shuttle assay. Cultures were grown in potato dextrose broth at 28±2 °C. Every 24 hours, 10 ml of sample was withdrawn and centrifuged at 2700 x g for 15 minutes. The CAS assay solution was added to the culture supernatant in equal parts, mixed, and left to stand in the dark for 20 minutes. The colour reaction was recorded at 630 nm, using potato dextrose broth as a blank, and % siderophore units were calculated by using the following formula: [(Ar–As)/Ar] 100 = % siderophore units. Where Ar = reference absorbance (minimal media + CAS assay solution) and As = sample absorbance (Patel et al., 2021a).

##### **Chitinase production and Endo β-1,4-glucanase production:**

Quantitative chitinase enzyme activity and endo1,4-β glucanase production estimation were carried out using the established dinitrosalicylic acid method. The developed colour was measured spectrophotometrically (EI) at 540 nm. Chitinase activity was determined by estimating reducing sugar concentrations as evidence for the quantity of released N-acetyl -D-glucosamine (NAGA), whereas endo β -1,4-glucanase activity was determined by estimating reducing saccharide concentrations as evidence for the quantity of released glucose (Rajninec et al., 2021). The concentration was calculated against the standard glucose curve (10–1000 g/mL) for endo β 1,4-glucanase activity. The concentration was calculated against the standard curve of NAGA (10–1000 g/mL) for chitinase enzyme activity. Senthilkumar et al. (2002) expressed enzyme activity in mole/ml/hr units.

##### **In vitro root colonization assay:**

An assay was performed as described by Yuan et al. (2015). *T. longibrachiatum* GFT2 suspensions of 20 ml (10<sup>6</sup> spores/ml) were inoculated in a sugar tube containing 20 g of sterile soil. Peanut seeds were planted up to 3 mm

deep in length in each tube, and the mouth of the sugar tubes was sealed with parafilm. Plants were then uprooted after 7 days with sterile forceps, and roots were properly excised. Before being cut into 1 cm pieces and placed on potato dextrose medium and yeast mannitol agar medium, plant roots were washed with sterile distilled water to remove any soil particles. Plates were incubated for 7 days at room temperature (28–30 °C), and the result was observed every day (Kaur et al., 2018).

#### **Compatibility assessment of *T. longibrachiatum* GFT2 with *Rhizobium pusense* PR4:**

The dual culture method was performed to evaluate the interaction between *T. longibrachiatum* GFT2 and *Rhizobium pusense* PR4 by inoculating them at the end of the plate and other periphery-streaked *Rhizobium* culture on the Petri plate. The plates were incubated at  $28 \pm 2^\circ\text{C}$  for 5 days. The percentage of inhibition was observed and calculated as per Qi et al. (2018).

#### **Seed germination assay:**

For seed germination, the pea plant seeds were surface sterilized for 15 minutes in a 1 percent  $\text{HgCl}_2$  solution before being rinsed six times with sterile distilled water. After sterilization, 100 seeds were transferred into 14-cm sterile petri dishes on sterile cotton and then wetted with 20 ml of sterile distilled water. Furthermore, one set of pea plant seed was kept as a control, and the others were treated with single *T. longibrachiatum* GFT2 and *Rhizobium pusense* PR4, also known as dual culture (GFT2+PR4), and sprayed with 10 ml of *Rhizobium pusense* PR4 ( $10^6$  cells/ml) and *T. longibrachiatum* GFT2 ( $10^6$  cells/ml) bioformulations. Moreover, sterile distilled water was sprinkled on both plates at regular intervals to maintain optimum moisture. To prevent infection, all the plates were closed with the lid. All the plates were marked and incubated in a germination chamber at  $25^\circ\text{C}$ – $28^\circ\text{C}$ , and the calculation of germination seeds was done daily until the end of the ten days. The germination percentage computation Germination percentage (%) =  $n/N$ , where  $n$  is the number of germinated seeds and  $N$  is the number of seeds (Kunova et al., 2016).

#### **Root development study:**

From June to September 2022, the experiment was carried out in a greenhouse to assess the effect of temperature and humidity on peanut plant nodule formation at Gujarat University in Gujarat, India. All pots (width and height: 21 cm) were used in triplicate, and four different treatments were given to them as controls: single *Rhizobium pusense* (PR4), *T. longibrachiatum* GFT2, and one combination treatment (GFT2+PR4). The pots were filled with 5 kg of soil after grinding and screening through a 2 mm sieve. Each pot was sown with 5 seeds of the G-20 variety of peanut (*Arachis hypogaea* L.) and allowed to grow for 3 months. Regular irrigation was carried out with distilled water after sowing. The number of nodules formed was calculated after 30, 35, 40, and 45 days (Sallam et al., 2019).

#### **Statistics Analysis:**

All biochemical assays (IAA, phosphate solubilization, siderophore production, and -glucanase and chitinase enzyme activity) were done in triplicate, and the average value was calculated along with the amount of variation, i.e., the standard deviation, and an ANOVA with one factor was calculated for significance. The formation of nodules on early root development and seed germination were also performed in triplicate, and data analysis was carried out (Patel et al., 2021b).

## **RESULTS**

A total of four fungal isolates were found in peanut rhizospheric soil samples in the current study, but only one, GFT2, is *Trichoderma longibrachiatum*. Based on the best growth and morphological characteristics obtained by growing on a variety of mediums, including WAM, BAM, CZPA, RBA, and PDA plates (Fig. 1), colonies of *Trichoderma* sp. initially appear whitish-woolly and become compact as they grow. During conidia formation, they turn a blue-green or yellow-green color. *Trichoderma* sp. grows in the form of concentric rings (Table 1). Further, the macromorphology features like phialides, conidial arrangement, and conidial morphology were determined (Fig. 1). The identity of the isolate was further confirmed by analysing the ITS sequence for 18sRNA characterization. Collectively, the isolate was identified as *T. longibrachiatum* based on the ITS sequence of *Trichoderma* assays (IAA, phosphate solubilization, siderophore production, and -glucanase and chitinase enzyme activity), which were done in triplicate, and the average value was calculated along with the amount of variation, i.e., the standard deviation, and an ANOVA with one factor was calculated for significance. The formation of nodules on early root development

and seed germination were also performed in triplicate, and data analysis was carried out (Patel *et al.*, 2021b). *longibrachiatum*. GFT2 was submitted to the National Center for Biotechnology Information database, and an accession number was obtained. Further, create the phylogenetic tree using MEGA 11 software (Fig. 2).

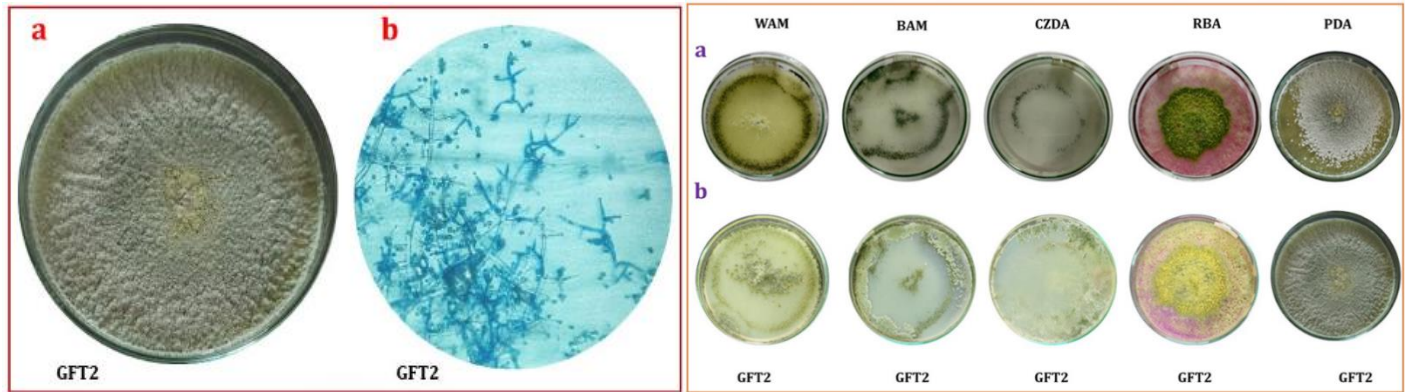


Fig. 1. Colony Morphology of *Trichoderma longibrachiatum* GFT2 grown on various medium WAM, BAM, CZDA, RBA and PDA for (a) is 4 days and (b) is 7 days at 28 ± 2 °C

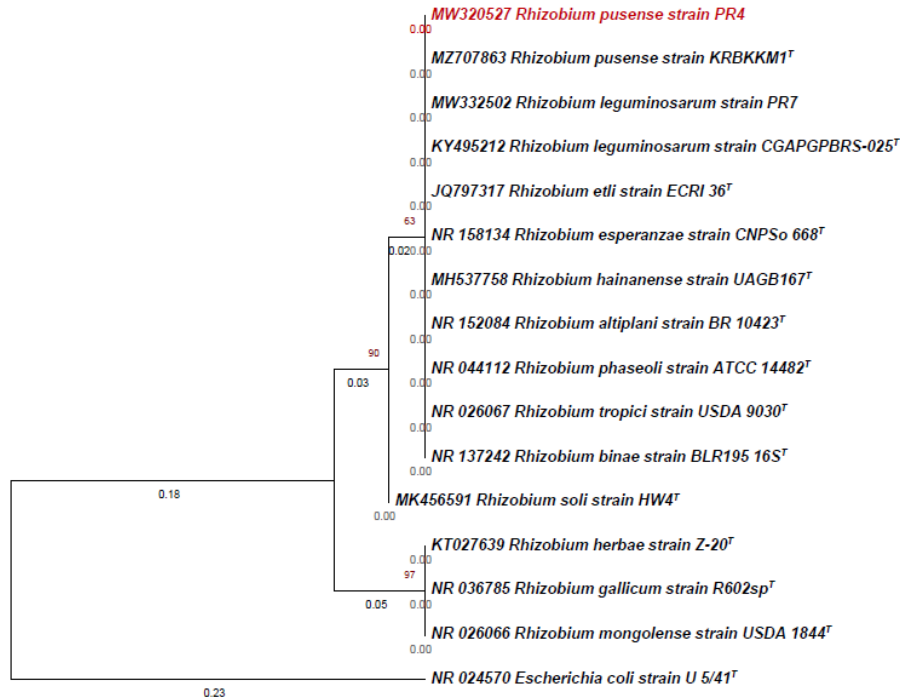
Table 1. Microscopic and culture morphological characteristic of *Trichoderma longibrachiatum* GFT2.

Culture Name	Growth Media Name	Colony growth (mm) after 2 days (28 °C)	Culture Colour	Conidia Shape	Pigmentation and Concentric ring	Sporulation initiate After (hrs)	Margin	Texture	Philiade Shape	Conidia Color
GFT2	CZDA	52	Green	OVAL	1	98	Filamentous	Loose	Subglobose	Green
	BAM	78.8	Yellow Green		1	98		Compact		Green
	PDA	79.8	White green		2 /Yellow Pigm	98		Loose Puffy		Green
	RBA	50.2	Yellow		1	48		Loose		Green
	WAM	64.2	Green		2	98		Loose Puffy		Green
	SAM	52	White green		1	98		Loose		

On the other hand, six bacterial samples were isolated from the root nodule of the peanut (*Arachis hypogaea L.*). Most of the isolates showed similar colony morphology, produced white or creamy white colonies, and had raised edges when grown on YEMA plates. All of the isolates grew quickly, with colonies reaching 1-3 mm in diameter after 2-3 days of incubation at 28 ± 2°C. The colonies had a sticky appearance, showing the production of mucous. Microscopic examination revealed that the isolates were gram-negative and rod-shape. Isolate PR4 was *Rhizobium pusense* based on confirmatory tests (Table 2), morphological and microscopic colony characteristics (Table 3), and molecular identification using a universal primer, retrieving FASTA sequencing, uploading to the NCBI, and getting the accession number. A further phylogenetic tree was generated using MEGA 11 software (Fig. 3). Quantitative analysis of IAA showed that *Trichoderma longibrachiatum* GFT2 was able to produce a good amount of IAA, i.e., 12.25 ± 1.08 µg/ml (P = 0.0622, ANOVA) after 120 hrs of incubation (Fig. 4). Quantitatively, maximum phosphate solubilization was observed in *Trichoderma longibrachiatum* GFT2 (10.71 ± 2.75 µg/ml, P = 0.0419, ANOVA) after 10 days of incubation. In addition, the pH value fluctuated (Fig. 6). The quantitative production of siderophore by *Trichoderma longibrachiatum* GFT2 was assessed by CAS-shuttle assay. GFT2 was able to produce a



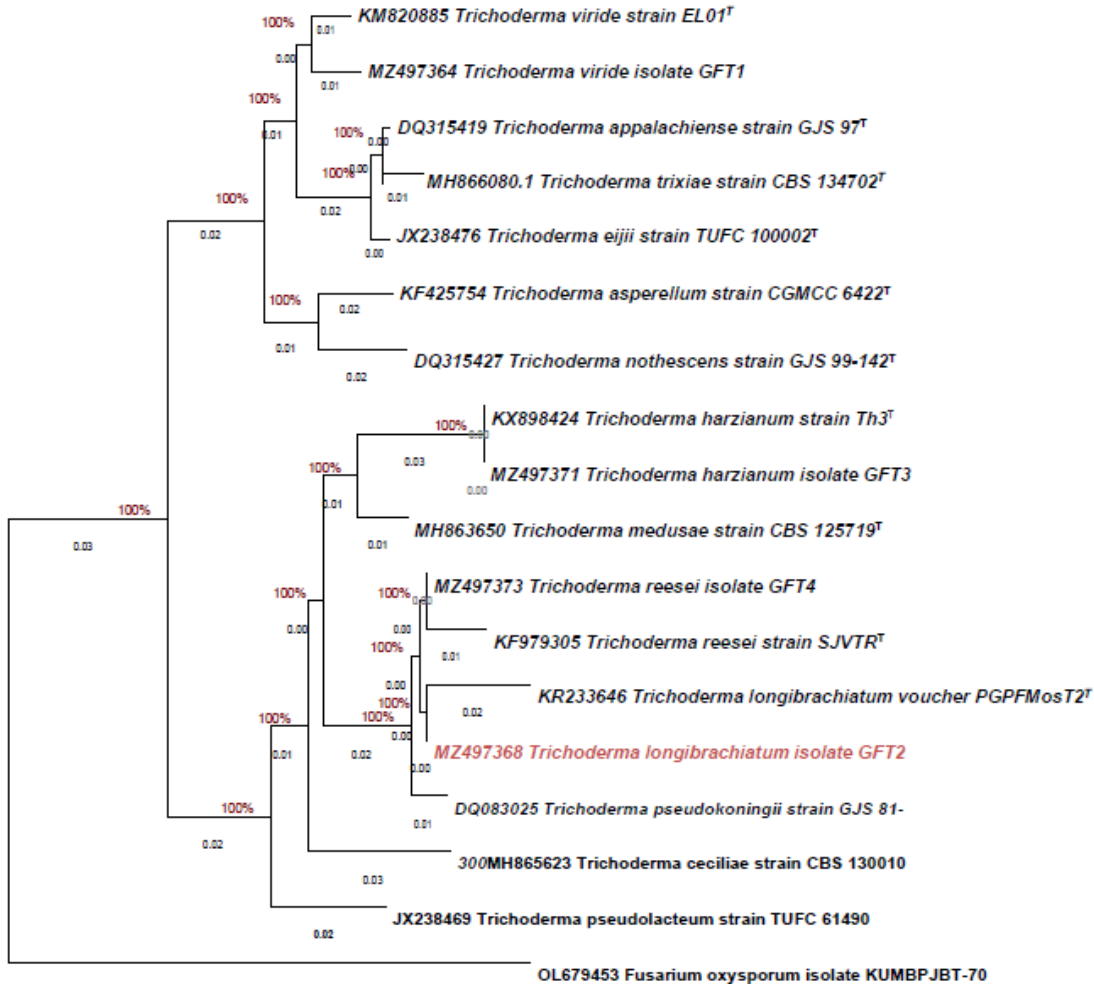
siderophore in a minimal medium, which was indicated by changing the CAS dye colour from cyan blue into purple and pink. It was observed that after 4 days of incubation, the blue colour was converted into purple, and after 7 days of incubation, the blue colour disappeared, leaving a pink colour. The formation of siderophores in *Trichoderma longibrachiatum* GFT2 was observed after 7 days of incubation (77.5% 3.54 P = 0.0448, ANOVA) (Fig. 4).



**Fig. 2.** Phylogenetic analysis of *Trichoderma longibrachiatum* GFT2 on 18srRNA gene sequences available from NCBI library constructed after multiple alignments of data ClustalW. Distances and clustering with the neighbor-joining method were performed using MEGA 11 software.

**Table 3.** Morphological, microscopic and colony characteristics of *Rhizobium* isolates.

Colony Characterize	PR1	PR2	PR3	PR4	PR7	PR6
Appearance	Dry	Dry	Dry	Gummy	Gummy	Chalky
Size	2mm	2mm	2mm	3-4mm	3-4mm	1-3mm
Shape	Spherical	Spherical	Spherical	Round	Round	Irregular
Arrangement	Single	Strepto-bacillus	Strepto bacillus	Single	Single	Cluster
Sides	Parallel	Parallel	Parallel	Parallel	Parallel	Parallel
Ends	Round	Round	Round	Round	Round	Truncate
Flagella	-	-	-	-	-	-
Capsule	+	+	+	+	+	+
Endospore	+	+	+	-	-	+
Gram Staining Reaction	+	+	+	-	-	+
Color	White	White	White	White	White	Yellow
Negative staining	Rod shape	Rod shape	Rod shape	Short rod	Short rod	Rod shape



**Fig. 3.** Phylogenetic analysis of *Rhizobium pusense* (PR4) based on 16srRNA gene sequences available from NCBI library constructed after multiple alignments of data ClustalW. Distances and clustering with the neighbor-joining method were performed using MEGA 11 software.

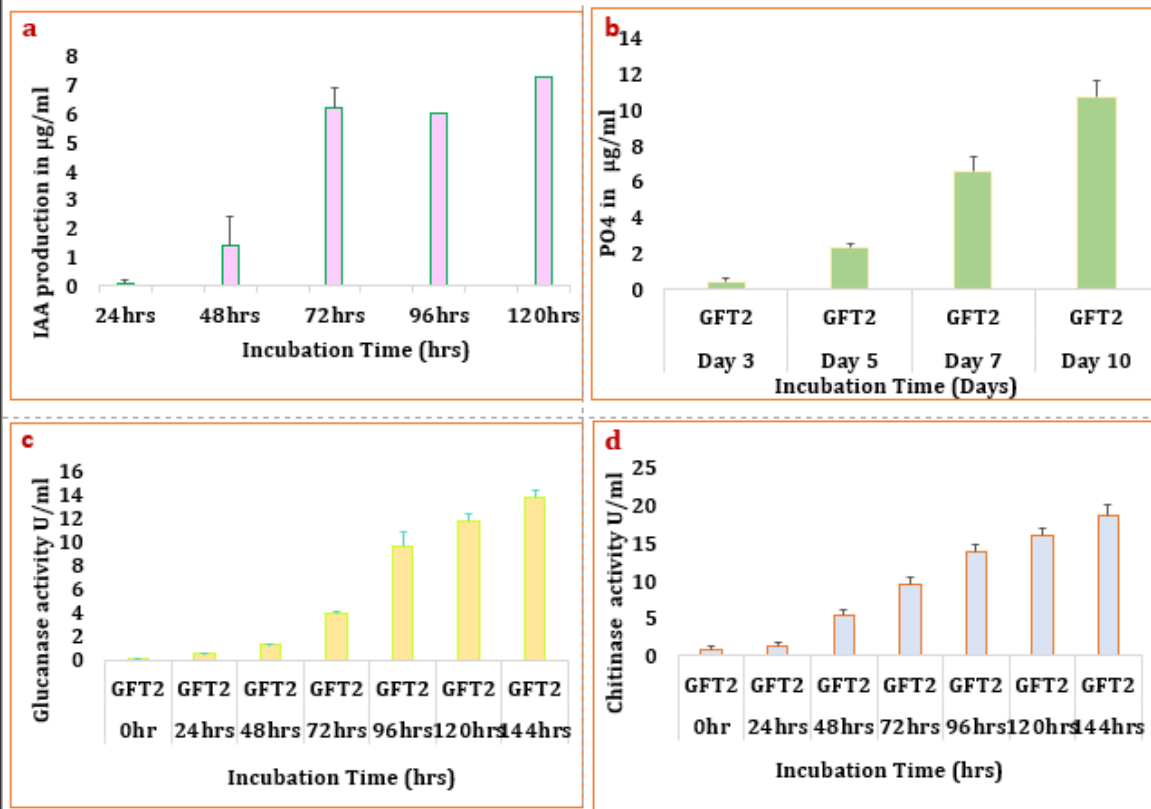
**Table 2.** Confirmatory test of *Rhizobium* isolates.

Isolates	YEMA + Congo Red		GPA		Keto -Lactose	
	24hr	48hr	24hr	48hr	24hr	48hr
PR1	<b>Reddish Growth</b>	<b>Reddish Growth</b>	<b>No Growth</b>	<b>No Growth</b>	-----	-----
PR2					-----	-----
PR3					-----	-----
PR4	<b>White Poor Growth</b>	<b>White Growth</b>	<b>Poor Growth</b>	<b>Poor Growth</b>	<b>Yellow Zone</b>	<b>Yellow Zone</b>
PR7						
PR6	<b>Yellowish Growth</b>	<b>Dark Red Growth</b>	<b>No Growth</b>	<b>No Growth</b>	-----	-----

*Trichoderma longibrachiatum* GFT2 was able to produce both enzymes chitinase as well as Endo-  $\beta$ -1,4- glucanase by sung DNSA method. After 144 hrs of incubation, chitinase enzyme activity was assessed activity observed in the GFT2 strain ( $18.56 \pm 18.49$  U/ml,  $P=0.0974$ , ANOVA) (**Fig.4**). In addition, GFT2 was also able to yield glucanase enzyme activity after 144 hrs of incubation GFT2 ( $13.82 \pm 0.64$  U/ml,  $P=0.0919$ , ANOVA) (**Fig.4**). The dual

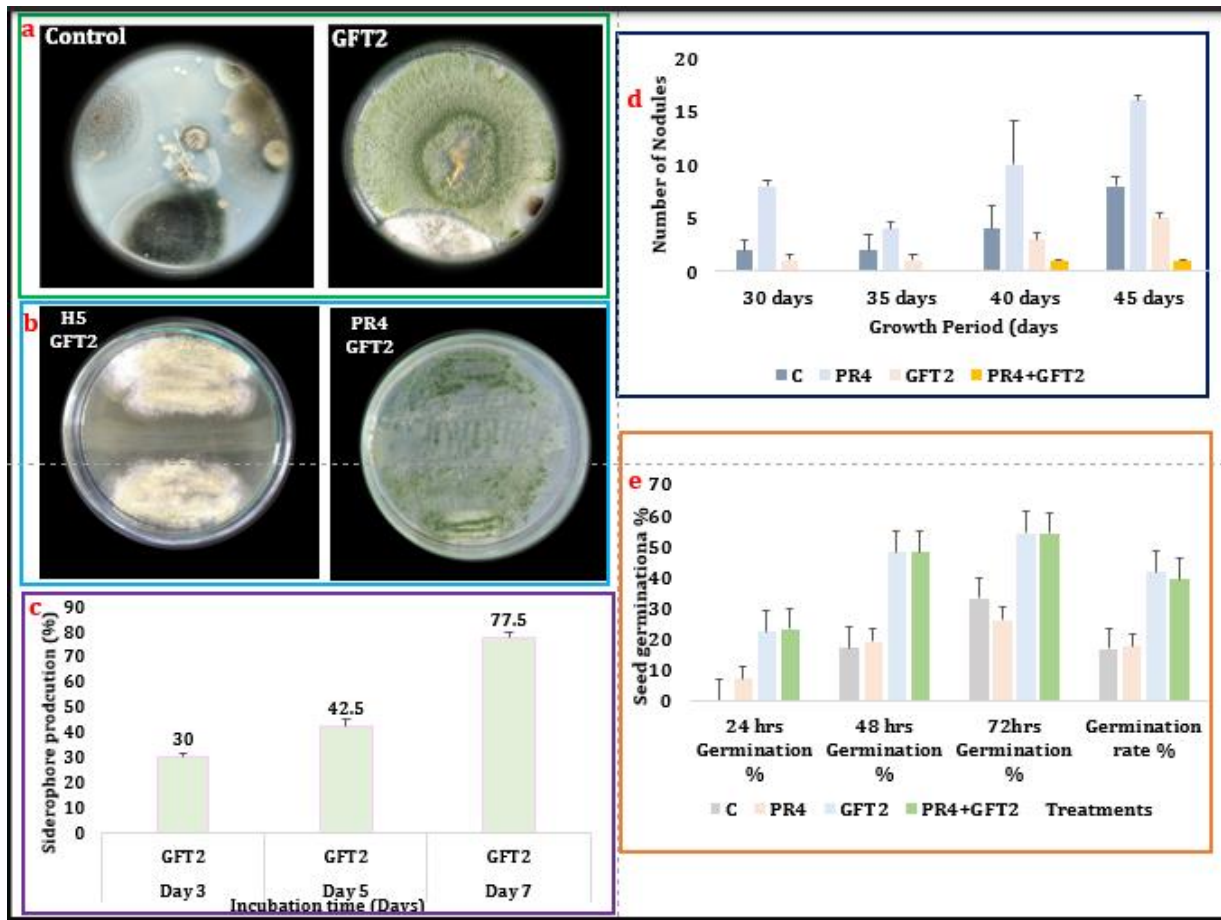
culture plating method was performed to analyse compatibility among *Trichoderma longibrachiatum* GFT2 and *Rhizobium pusense* PR4. *Trichoderma longibrachiatum* GFT2 was found to be dominating and had overgrown *Rhizobium pusense* PR4. Besides, *Pseudomonas aeruginosa* was taken as control and found that *Trichoderma longibrachiatum* GFT2 was not able to overgrow on *Pseudomonas* as this genus was resistant against *Trichoderma*, in contrast, *Rhizobium pusense* PR4 was sensitive (Fig. 5).

A close test tube assay was used to assess the colonising ability of *Trichoderma longibrachiatum* GFT2, and seeds were treated with *Trichoderma longibrachiatum* GFT2 while a control was kept for reference. After 7 days of incubation, it was observed that *Trichoderma longibrachiatum* GFT2 was grown on medium with other soil flora such as *Fusarium*, *Aspergillus*, and *Penicillin* spp. In the control plate, no *Trichoderma* strains were found, but other fungi like *Fusarium*, and *Aspergillus* were observed. Thus, the root colonisation assay determined that *Trichoderma longibrachiatum* GFT2 easily colonised on roots (Fig. 5). A seed germination assay was performed to find out the biofertilizer traits of *Trichoderma longibrachiatum* GFT2. Therefore, in the seed germination assay, seed was treated with different cultures as described in the method section; here, the maximum seed germination rate was found in PR4 + GFT2 (39%), compared to the other treatments of *Trichoderma longibrachiatum* GFT2 (41%), control (16.66%), and PR4 (17.33 %). Figure 6 depicts data. A greenhouse experiment was conducted for nodule estimation after co-inoculation of *Trichoderma longibrachiatum* GFT2 with *Rhizobium pusense* PR4 in the pot. Maximum nodule formation of  $16 \pm 0.47$  ( $P = 0.9780$ , ANOVA) nodules per plant was observed after 45 days in plants treated with *Rhizobium pusense* PR4. Minimum nodule formation was observed in the plants treated with *Trichoderma longibrachiatum* GFT2, i.e.,  $05 \pm 0.41$  ( $P = 0.8507$ , ANOVA). Plants treated with co-inoculation culture GFT2 + PR4 formed the fewest nodules after 45 days, namely one ( $P = 0.9801$ ; ANOVA). The untreated plant had one nodule per plant, i.e.,  $08 \pm 0.81$  ( $P = 0.9817$ , ANOVA), as compared to the plant treated with a single strain of *Trichoderma longibrachiatum* GFT2 and *Trichoderma* with *Rhizobium* (Fig. 5).



**Fig. 4.** Display of (a) IAA production in  $\mu\text{g/ml}$  by *Trichoderma longibrachiatum* GFT2 on successive days after inoculation. (b) phosphate solubilization by *Trichoderma longibrachiatum* GFT2 on successive days after incubation (c) glucanase production by U/ml by *Trichoderma longibrachiatum* GFT2 on successive days after incubation (d) chitinase production U/ml by *Trichoderma longibrachiatum* GFT2 on successive days after incubation.





**Fig. 5.** Display of (a) Root colonization by *Trichoderma longibrachiatum* GFT2 where the control plate has grown only contaminated fungi, the GFT2 plate grown *Trichoderma longibrachiatum* (b)compatibility test between *Pseudomonas aeruginosa* (H5) and, *Trichoderma longibrachiatum* between *Rhizobium pusense*(PR4) (c) siderophore production in percentage by *Trichoderma longibrachiatum* GFT2 on succeeding days after incubation(d) The impact of co-inoculation of *Trichoderma longibrachiatum* GFT2 and *Rhizobium pusense* PR4 on peanuts seed germination. (e) several nodules produced after the plants is treated with different microorganisms (PR4, GFT2, GFT2+PR4) on successive days of growth.

## DISCUSSION

A significant number of *Trichoderma* strains have been reported as biocontrol agents and bioaugmentors in the agriculture field (Eida *et al.*, 2020). *Trichoderma* strains boost soil fertility and crop development through their plant growth-promoting traits. In the present work, it was shown that *Trichoderma longibrachiatum* GFT2 enhanced the plant growth of certain areas of the plant and inhibited the growth of nodulating bacteria (Berbel *et al.*, 2020). Firstly, fungi isolate was determined and done their molecular identification based on its ITS sequencing, (El-Dawy *et al.*, 2021) has also reported the isolation of *T. longibrachiatum* (GFT2) from peanut rhizospheric soil. *Rhizobium pusense* PR4 was isolated from root nodules of the peanut plant by Singha *et al.* (2015). In the current study, *Trichoderma longibrachiatum* GFT2, has shown great efficiency in plant growth promotion as well as biocontrol activity. PGP traits like IAA production, solubilization of tricalcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ ), and biocontrol activity such as chitinase, glucanase, and siderophore production. Indole-3-acetic acid and its derivative compounds are one of the most abundant phytohormones found in nature and are essential to initial root and shoot development (Bunsangiam *et al.*, 2020.). Apart from root and shoot development, it also helps in cell division, cell expansion, fruit development, and apical dormancy (Mulani *et al.*, 2021). Saber *et al.* (2017) have also reported that *T. longibrachiatum* VKY<sub>1</sub> was able to yield 138.9  $\mu\text{g/ml}$  auxin when amended with 500  $\text{mg/ml}$  of auxin precursor in broth medium. The studies

confirmed that IAA production is enhanced when the concentration of the precursor is increased (Krishnan and Siril, 2018). Phosphate solubilization was also seen in *Trichoderma longibrachiatum* GFT2. Rudresh et al. (2011) reported from the study that the highest phosphate solubilization was observed in the *Trichoderma longibrachiatum* TV97 strain, i.e. 9.03 µg/ml among the other strains of *Trichoderma*, viz., *T. viride* PDBCTV32, and *T. viride* TVA7. Biocontrol fungi and rhizobacteria both can produce siderophores, but their chemical natures differ from each other (Mulani et al., 2021). Iron sequestration by microbes is classified into catecholates, hydroxamates, carboxylates, and phenolates. However, most of the fungi produced hydroxamate-type siderophores in the PDB medium. *Trichoderma* strains produced hydroxamate and carboxylate types of siderophore, according to Ghosh et al. (2017), with *Trichoderma longibrachiatum* GFT2 producing 42.42% of the siderophore. Fungi biocontrol activity is typically accomplished through the production of various hydrolytic enzymes such as cellulase, chitinase, pectinase, glucanase, and xylanase (Osman et al., 2019). Mukhammadiev et al. (2020) investigated that *Trichoderma longibrachiatum* GFT2 produced hydrolytic chitinase (23.8 U/ml) in production media which contained 0.2% colloidal chitin from shrimp shells as substrate. Root colonization where root is produced ample number of biochemical substances which is improve the growth and survival for soil microbiome (Riaz et al., 2021).

In the root colonisation experiment, *T. longibrachiatum* GFT2 has the potential to colonise the peanut plant root. Rashid et al. (2016) have also reported the colonisation assay of *T. longibrachiatum* JTL2 in peanut plant roots. a compatibility test between *Trichoderma* and *Rhizobium* to determine their viability in the same environment and whether there are any factors that inhibit their growth (Sutton, 2006). In present study revealed that *Trichoderma longibrachiatum* GFT2 has the capability to inhibit the growth of *Rhizobium pusense* PR4 in plate medium. Mahapatra et al. (2017) studied the *in vitro* compatibility test between *Trichoderma* and *Rhizobium* using the same approach, and the result displayed that *Trichoderma* inhibits the growth of *Rhizobium* in both solid media and broth media. When culture spraying is done over the seeds, both microbes help to increase the germination percentage, but no literature has been found to support germination by dual culture of *T. longibrachiatum* GFT2 and *Rhizobium pusense* PR4. For nodule estimation, the least number of nodules were produced in the plant treated with *T. longibrachiatum* GFT2 and *Rhizobium pusense* PR4. Similarly to seed germination, no literature was found to conduct a pot experiment with dual culture and nodule formation. We were unable to find the proper mechanism to inhibit the growth of nodulating bacteria as well as beneficial microbes in the soil, and we were also unable to work on all leguminous plants, their produced nodules, and the effect of *Trichoderma longibrachiatum* GFT2 on early root development in this study.

## CONCLUSION

In the present framework said that *T. longibrachiatum* GFT2 has capable to promoting plant growth by producing IAA, solubilization of tri-calcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ ), siderophore and biocontrol activity as chitinase, glucanase. On the other side, it was also easily colonized the root surface and reduced the growth of *Rhizobium*, therefore the number of root nodules was decreases and depriving the nitrogen to plant for their extinguish growth. Furthermore, clarification and how *T. longibrachiatum* GFT2 inhibitor decline the growth of Nodulating bacteria invade environment. Future perspective is doing meta- transcriptomics of soil for gaining more idea about this hypothesis.

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