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Research Article

Isolation, Characterization and Molecular Identification of *Rhizobium* spp. and *Trichoderma* spp. from Nodule and Rhizospheric Soil of *Arachis hypogaea*

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Abstract

In practically all types of temperate and tropical soils, the fungus of the genus *Trichoderma* can be found. Additionally, they are found on decaying wood, bark, and other organic plant components. In this investigation, four distinct *Trichoderma* species were isolated from peanut rhizosphere soils (*Arachis hypogaea*). Isolates were taxonomically identified up to the species level using macro and micromorphological features. To identify the species of Trichoderma, morphological investigations such as pigmentation, colony growth, and anatomical studies such as conidiation appearances, size of conidia, conidiophores branching pattern, forms of phialides, and the absence or presence of chlamydospores were carried out. Based on macro, micro-morphological and 18s RNA sequencing analysis, Trichoderma isolates were classified into four species *T. viride* (GFT1), *T. harzianum* (GFT2), *T. longibrachiatum* (GFT3), and T. reesei (GFT4). Trichoderma harzianum species were prevalent in soil, with Trichoderma viren ranking second. A symbiont, the Rhizobium bacteria develops root nodules in association with the roots of leguminous plants. From the root nodules of *Arachis hypogaea* L., two bacterial strains were discovered. Three distinct confirmatory tests were carried out on the isolates as part of an investigation into their morphological and microscopic features. The biochemical features of the isolates were also examined. 16sRNA sequencing was used to provide taxonomy identification. Rhizobium leguminosarum PR7 and Rhizobium pusense PR4 were the two strains we obtained based on all the data. In the future, all isolated strains will be used for additional research on Trichoderma strains, including biocontrol and bioagumentor, and Rhizobium strains will be used to determine the effects of Trichoderma on Rhizobium under soil.

Keywords: *Trichoderma spp; Rhizobium spp;* Nodules; Confirmatory Test; Molecular Identification

Introduction

Peanut (*Arachis hypogaea*) plant produced over the world as they demand is day to day increasing, and it is also ancient crop of the new world which is widely grown in India. Ground nut or peanut is cultivated in all kinds and around the world of temperature like tropical, sub -tropical and warm climates also. Nowadays, India and China are the hugest producers of the peanut crop .Yield of plant on a global scale is increases slightly from 2 t/ha in the 2000 to 3 t/ha in the 2010 and 4 t/ha in the 2020.However, production of peanut more than 6t/ha can be obtained, when diseases are controlled, and good management practices are applied in agronomy field [25]. More production of ground nut due to their seed has 40% to 55% of oil content and used as cooking oil and aspect also. Other uses of peanut in food market as peanut butter, edible seed, and cooking oil and it has good protein and lipid value human is consumed directly that seed [2]. In addition to seeds, the foliage is an important fodder in regions where animals are used extensively on the farm, and the meal remaining after oil extraction is also an important source of animal feed. Moreover, According to reports, peanut receives between 70 and 90 percent of its nitrogen needs from symbiosis and contributes between 100 and 190 kg of nitrogen per hectare to the cropping system. In India, groundnuts can fix up to 101 kg of nitrogen per cropping season and receive 33-67% of their N nutrition from fixation [28].

Rhizobium is nodulating bacteria, an aerobic, gram-negative, motile, non-spore-forming soil bacterium called *Rhizobium* can colonise the rhizosphere of leguminous plants which can fix the environmental nitrogen into leguminous plant [7]. Since this genus has several strains, it is very diverse.In particular, *Rhizobium*

leguminosarum species has 64 mol% G+C content and *Rhizobium pusense* species has 59 mol% G+C, with the DNA base ratios of the *Rhizobium* genus ranging from 30 to 61% G + C [42]. A significant number of nitrogen fixing species has been found in leguminous plant such as *R. leguminosarum, R. alamii, R.lentis, R.japonicum, R.metallidurans, R.smilacinae, R.phaseoli, R.trifolii* and *R.pusense* [48]. Whereas another kind of rhizobia are present in soil such as *BradyRhizobium, SinoRhizobium*, *MesoRhizobium*, *AzoRhizobium*, and *AlloRhizobium* . Besides, many different types of organisms are capable of fixing nitrogen. Only a very small number of species, or about 87, have been identified as diazotrophs, or organisms that can fix nitrogen. These organisms come from 02 genera of archaea, 38 genera of bacteria, and 20 genera of cyanobacteria. In temperate and tropical forests, wetlands, fields, and disturbed sites, actinorhizal interactions (Frankia-nonlegume symbioses) are important sources of nitrogen inputs [38].

Rhizobium requires plant host for fixation of nitrogen and that live in symbiotic association with leguminous plant root nodules, since fixation of N_3 cannot be done independently. Although N_2 is present in the atmosphere, plants can only easily access it when it is in the form of soil nitrate. In agronomy field, *Rhizobium* is an essential source of nitrogen, for plant growth and metabolisms [14]. Nitrogen is an essential for all living organisms for the synthesis of biomolecules Viz., nucleic acid, proteins and other nitrogen containing compound. However, almost all soils have nitrogen deficiencies, making nitrogen a limiting element [19]. The increased utilization of chemical fertilizers as a source of nitrogen for crops results in increased emissions of nitrogen oxides, soil acidification and water pollution. Therefore, legume *Rhizobium* symbiosis mechanisms an essential role for in soil sustaining for healthy plant production. Geographical location, soil type, host-plant genotypes, as well as the rhizobia symbiont itself, all have an impact on this process. The growth and activity of the N_2-fixing plants are constrained by a number of environmental factors .The physiological state of the host plant has a strong bearing on the *Rhizobium*-legume symbiosis, which is a process of N_2 fixation [29]. As a result, a competitive and persistent rhizobia strain is not anticipated to express its full capacity for nitrogen fixation if the host legume's vigour is restricted by factors such as salinity, an unfavourable soil pH, nutrient deficiency, mineral toxicity, extremes in temperature, an excessive or inadequate amount of soil moisture, insufficient photosynthesis, plant diseases, and grazing [41].

All soils frequently contain the diverse group of free-living fungi known as *Trichoderma*, which belongs to the family Hypocreaceae. These parasitic ascomycetes fungi live in root ecosystems as cunning, non-pathogenic plant symbionts. They also prey on other fungi species. They multiply best at mesophilic temperatures (28-32°C) and a wide range of pH (6-6.5) levels and produce chlamydospores and ascospores for propagation [33]. There are numerous ways to isolate *Trichoderma*, but serial dilution of samples is one of the techniques that has been most frequently mentioned in the literature. Large samples can be handled using this method because it is straightforward, affordable, and efficient. Traditional techniques for identifying *Trichoderma* spp [34]. Prior attempts have been made using morphological and cultural approaches. Along with linear growth, colony colour, growth pattern, and hyphal pigmentation, these include the arrangement of conidiophores, phialides, and conidia. Initially growth of colony appearance different at grown on various medium [15]. Although more molecular methods are now being used, traditional taxonomy was based on differences in morphology, primarily of the asexual sporulation apparatus. The taxa have increased recently from nine to at least thirty-three species as a result. Some species have numerous nuclei or vegetative cell have more than 100, which is possible due to various genetic factors, including parasexual recombination, mutation, and other processes. As a result, Wild strains' genotype and phenotype exhibit a wide range of variation [45].

Trichoderma sp. It is a helpful fungus that primarily dwells in the soil or in the tissues of plants and are biological effectiveness against pathogenic fungi and capacity to promote plant growth have been the subject of extensive research on a global scale [40]. Through their highly effective antagonistic and microparasitic activity, Trichoderma spp. can lessen the severity of plant diseases. *Trichoderma* is a free-living and cosmopolitan fungi which are present in most of the rhizospheric soils as well as in other diverse environments [21]. In the rhizosphere soil there are many kinds of *Trichoderma* stains are found Viz., *Trichoderma harzianum*, *T. viride*, *T. longibrachiatum*, *T. Pseudoharzianum, T. afroharzianum, T. asperelloides, T. velutinum, T. virens, T. viridescens, T. atroviride, T. hamatum, T. asperelloides, T. brevicompactum* and *T. reesei* are a few strains of *Trichoderma* that have potent biocontrol activity, producers of secondary metabolites with significant medical value, and bioremediation characters, according to most of studies [30]. Some strains of the common soil-borne filamentous fungus *Trichoderma* species can form advantageous relationships with plants. For more than 70 years, *Trichoderma* Sp. has been used to biologically control plant diseases [4]. In the current framework. Utilizing a variety of techniques, we isolate the nodulating bacteria and biocontrol agent from peanut nodules and the rhizosphere soil, respectively.

Materials and Methods

Sample collection of nodulated roots of *Arachis hypogaea L.*

A sterile clean spade was used to dig for approximately 15 cm sideways and up to a depth of about 20 cm [44]. The clump of soil and the five healthy Nodulated plant of (Peanut) *Arachis hypogaea* (S20) were uplifted carefully from grown in agriculture field, Mavajinva, Amreli, Gujarat, India 21° 33' 13.9212'' N and 70° 54'

41.6988'' E, that placed in sterile aluminium foil where the those plants posing pink colour and heathy round shape nodules were detached from the roots and kept in screw-capped vials containing silica gel (GRM151Hi-Media) to prevent from desiccation.Transport immediately to the department of Microbiology and Biotechnology laboratory at Gujarat University, for isolation of *Rhizobium* [9].

Isolation of *Rhizobium* from root nodules

In this, healthy, unbroken, firm, and pink nodules were selected for the isolation. They were washed under tap water to remove adhering mud and soil particles. Further, nodules were washed with sterile distilled water (SDW) and hydrated with SDW overnight. Surface sterilization of the nodules was carried out by placing them in 1% $HgCl_2$ for 30 s and then washing them with SDW 5 times. The nodules were then treated with 1% sodium hypochlorite for 3 min, followed by 10 washes with SDW for free of sterilant. Each nodule was crushed in 100 µl of 15% glycerol, and then crushed with a sterilized crucible and prepared serial dilution up to 10^{-1} to 10^{-10} take 100 µl of the mixture from each series of dilution and was spread with help of a sterile micropipette on to Yeast mannitol extract agar medium (Hi-mediaM721)with itraconazole (Zydus.pvt.Lmt) 200 mg/l for reduced the growth of fungus and incubated at 28 ± 2°C for two days [18]. After two days, single colonies were picked -up and were re-streaked on fresh Yeast Mannitol agar medium for obtaining pure culture.

Morphological Identification of pure culture

Using the standard microbiological techniques, all the isolates were characterized for selected morphological parameters such as colony size, shape, border, elevation, colour, mucosity, transparency, and capacity to produce the exopolysaccharide gum other tests that were carried out included Gram staining where active isolates cultured on YEMA were smeared on clean microscope slides [20]. Three different confirmatory tests were performed to confirm the isolate as Rhizobia and to differentiate them from other contaminating microbes.

Biochemical test for *Rhizobium*

The Gram-negative isolates were further subjected to biochemical tests including catalase, nitrate reduction test, starch hydrolysis test, urease test, citrate utilization test, gelatine liquefaction test, oxidase test, voges-Proskauer, and indole test following standard procedure for confirmation of *Rhizobium* [3].

Confirmatory Test for *Rhizobium* growth on YEMA with congo red

In general, Rhizobia produce white colonies, whereas many other bacteria take up the dye strongly. YEMA-Cr media was prepared, and bacterial isolates were streaked on plates. The plates were then incubated dark condition at 28 ± 2°C for 48 hours [36].

Growth on glucose peptone agar (GPA) medium

Rhizobia show very heavy growth on glucose peptone agar medium and cause very little change in p^H when incubated at 28 ± 2°C for 2 days. The plates are then incubated at 28 ± °C for 48 hours [22].

Keto-lactose test

Keto-lactose agar medium was prepared, and bacterial isolates were streaked on plates. Inoculated plates were then incubated at 28 ± 2°C for 48 hours. Plates were then flooded with Benedict's Reagent, incubated dark condition at 28 ± 2°C and results were observed after 1 hour [37]. The yellow zone was observed which indicate that *Rhizobium* species.

Molecular characterization of *Rhizobium*

Molecular markers for this isolated *Rhizobium* included 16S rRNA was studied at Dravin Enterprise pvt. Lmt. 16s rRNA gene sequence analysis of strains PR4 and PR7 were performed to identify the organism. Universal Forward Primer (FP): 5'-AGAGTTTGATCCTGGCTCAG-3' and Reverse Primer (RP): 5'-AAGGAGGTGATCCAGCCGCA-3' were used to amplify 16s rRNA gene from the genomic DNA by the method described by Goswami., *et al*. 2013 [12]. Briefly, 1ml culture was withdrawn and centrifuged at 2700 x g for 10 minutes. Supernatant was discarded and 600µl of lysis solution (200mM Tris-HCL pH 8.5, 25mM EDTA in 1% Sodium dodecyl sulphate in water) was added. Tubes were kept at 80°C for 5-10 minutes followed by centrifugation at 2,700 x g for 10 min. Supernatant was collected in other tube and 200µl of 5 M ammonium acetate was added and centrifuged at 5,700 x g for 10 minutes. Again, supernatant was collected in other tube. 600µl of ice-cold isopropanol was added and tubes were inverted 30 to 40 times. Tubes were centrifuged at 5,700 x g for 10 min and supernatant was discarded. Pellet was washed with 600µl of 70% ethanol and then dissolved in 25 µl of TE (1mM EDTA, 10mM Tris-HCl pH 8.5) buffer.Above mentioned primers were used to amplify 16s rRNA gene from genomic DNA. Thereaction was carried out in a 50 µl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP,25 pmoles of FP and RP, 50 ng DNA template and 5 U Taq DNA polymerase. Amplification cycle parameters were set as: 94°C for 45 sec, 58°C for 45 sec, and 72°Cfor 105 sec for 34 cycles followed by a final extension of 10 min at 72°C. The reaction was carried out in a thermocycler. Amplified gene product (1.6 kb in size) was sequenced at 1st BASE (Dravin enterprise India Pvt. Ltd.). Gene sequence homology was determined using BLASTn search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Gene sequence obtained was aligned by Clus-

talW using MEGA 11 software and a neighbor-joining (NJ) tree with bootstrap value 1000 was constructed.

Rhizospheric soil sample collection

Rhizospheric soil (500 gm) sample was collected from grown *Arachis hypogaea* in agriculture field, Mavajinva, Amreli, Gujarat, India 21° 33' 13.9212'' N and 70° 54' 41.6988'' E for isolation of biocontrol fungi as *Trichoderma* spp. Soil sample collection was done by removed 10 cm of surface soil with the help of spade and sample were taken 20 cm depth and were transferred to sterile airtight zip bag and immediately carried to the laboratory. Firstly, soil sample were sieved through a mesh of 2mm and maintain humidity 45%. Prepared serial dilutions up to 10^{-1} to 10^{-10} and from each dilution, 100 µl were inoculated by spread plate method on Potato dextrose agar medium (Hi-media MH096) amended with streptomycin a broad-spectrum antibiotic at concentration of (100 mg/l) (Hi-mediaSD031) for inhibiting native bacterial growth. Incubated at 28 ± 2°C for 7 days [50].

Morphological characteristics of *Trichoderma* Spp.

Elementary screening of fungi was done by macroscopic and microscopic features. Macroscopic screening of *Trichoderma* Strains were identified based on their phenotypic nature such as surface colour, texture, margin, pigmentation and colony appearance and sporulation pattern were examined from cultures grown on six media: Potato dextrose agar medium (PDA), Rose Bengal agar medium (RBA) (Hi-mediaM842), Czapek's dox agar medium (CZDA) (Hi-media,M075),Wheat flour agar medium (WAM) (1gm/100ml ; wheat flour, 1% sugar and 3% agar -agra powder) , Soyabean agar medium (SAM) (1gm/100ml; Soyabean flour, 1% sugar and 3% agar -agra powder) and Bengal gram flour medium (BAM) (1gm/100ml ; Bengal gram flour, 1% sugar and 3% agar -agra powder) at 28 ± 2°C for 4 and 7 days. For observing colony characteristics and growth rate, inoculum was taken from the actively growing margin of 4 days culture, grown on Potato dextrose agar medium. A 7 mm mycelia disc was placed at centre of all Petri dishes. The dishes were kept dark condition for incubation at 28 ± 2°C for 7 days. Radial growths were measured at 24 h intervals until colony covers the whole Petri dish. In addition, all micro morphological data were examined on cultures grown on different media and incubated for four days at 28 ± 2°C. The microscopic characteristics were (Compound microscope MICROS Austria) examined and measurements of conidiophores, shape of conidia and phialide, filament length were made from slide preparations stained with lactophenol-cotton blue /1% Methylene blue were observed at 40X magnification of objective lens and photographed. Fungal strains were sub-cultured and grown on PDA for routine experiments and the long-term storage, fungal suspension was prepared in cryovials overlaid with 80% glycerol and stored at 4°C [24].

Molecular identification of fungal isolates

Amplification of 18s rRNA gene from the purified genomic DNA was carried out using universal primer set for fungal 18s rRNA gene amplification (forward primer (FP) 5'-GGAAGTAAAAGTCGTAACAAGG-3' and reverse primer 5'TCCTCCGCTTATTGATATGC-3'. The method describe by Goswami., *et al*. 2015 [13]) as followed. The reaction was carried out in a 50 µl reaction mixture containing 1.5 mM MgCl2, 0.2 mM each dNTP, 25 pmoles of FP and RP, 50 ng DNA template and 5 U Taq DNA polymerase.Amplification cycle parameters were set as: 94°C for 1 min, 55°C for 1 min, and 72°C for 105 sec for 34 cycles followed by a final extension of 10 min at 72°C. The reaction was carried out in a thermocycler. Amplified gene product was sequenced at 1st BASE (Dravin enterprise India Pvt. Ltd). Gene sequence homology was determined using BLASTn search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Gene sequence obtained was aligned by ClustalW using MEGA 11 software NJ tree with bootstrap value 1000 was constructed [46].

Results

Isolating nodulating bacteria were growing on YEMA medium after incubated 28 ± 2 °C for 2 days. Total 6 isolates were found, and all were fast-growing organisms, produced creamy margin, smooth, and round with diameter range 2-5 mm and some of the colonies were milky and translucent or yellow with diameter range 2-3 mm. The shapes of the colonies in most isolates were round except a few isolates which had rhizoid, and some were irregular. The colonies were having sticky appearance showing the production of mucous. The margins were smooth in many isolates or undulated in a few isolates (Table 1). Microscopic examination revealed that the isolates were gram negative and elongated rod in shape, possess flagella on their polar end. Microscopic observation gives little additional information as to the genus and species of a particular bacterium.

Performed biochemical reaction allow to organisms grow and identifying a suspected organism. In biochemical reaction all the isolates showed positive result for Urease, Oxidase, Nitrate reduction, whereas PR6 isolate showed negative test. Moreover, PR1, R2 and PR3 showed positive test of Catalase, Methyl-Red (MR) test, Vogus- Proskauer (VP) test, Citrate utilization, Starch hydrolysis, Gelatine test, and other side indole production test showed negative. Also, in PR4 and PR7 isolates observed positive test of Catalase, Indole production, Methyl-Red (MR) test, whereas negative tests were examined of Vogus- Proskauer (VP) test, Citrate utilization, Starch hydrolysis, Gelatine test (Table 2). Confirmatory test was performed with all the 06 bacterial isolates grown on various medium. In GPA test 03 isolates showed no growth on GPA media and 2 isolates, PR4 and PR7 showed yellow colour growth on GPA

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

Table 1: Phenotypic characteristics of isolates.

Tests	PR1	PR2	PR3	PR4	PR7	PR6
Urease	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	-
Indole production	-	-	-	+	+	-
Methyl-Red (MR) test	+	+	+	+	+	-
Vogus- Proskauer (VP) test	+	+	+	-	-	+
Nitrate reduction	+	+	+	+	+	+
Citrate utilization	+	+	+	-	-	-
Starch hydrolysis	+	+	+	-	-	+
Gelatine test	+	+	+	-	-	+

Table 2: Biochemical analysis of isolates.

Note: Where '+' indicates positive trait and '-' indicates negative trait

media, also PR3 showed minor white colour growth on GPA medium. In the YEMA+Cr medium results revealed that 01 isolate (PR3) showed growth on YEMA-CR media without absorbing the Congo red dye and 04 (PR1, PR2, PR4, PR7) isolates also showed growth on YEMA-CR media absorbing little amount of dye whereas PR6 isolates showed growth on YEMA-CR media by extremely absorbing the Congo red dye. In keto-lactose test all the isolates showed no yellow colour formation on keto-lactose media after adding Benedict's reagent except 02 (PR4, PR7) isolates, showed yellow colour formation (Table 3).

Phylogenetic analysis of *Rhizobium leguminosarum* (PR7) and pusense (PR4) based on 16srRNA gene sequences obtained and was compared to other sequences with the help of BLASTn database. Similar sequences with highest sequence homology and query coverage with lowest E values were used to develop NJ tree using MEGA 11 software which uses ClustalW algorithm for multiple sequence alignment. Phylogenetic analysis revealed that *Rhizobium leguminosarum* PR7 (MW332502) have maximum similarity *Rhizobium leguminosarum* strain CGAPGPBRS-025 (KY495212) and *Rhizobium pusense* PR4 (MW320527) have maximum similarity with the *Rhizobium pusense strain* KRBKKM1 (MZ707863). There was a total of 52 positions in the final dataset. Evolutionary analyses were constructed in MEGA11software (Figure).

In the present study total 10 fungal isolates were examined from the rhizospheric soil of leguminous plant peanuts. Isolated fungal Cultural characteristics comprising Colony growth rate, colour, Conidia Shape, pigmentation, Margin, Texture, Phialide Shape, Conidia colon and colony appearance were examined. These characteristics were regarded as taxonomically useful characteristics for typical *Trichoderma*. All fungal isolates grown on various kinds of media like PDA, CZDA, BAM, RBA, WAM, SAM. In addition, on PDA medium growth of GFT1 fungal isolates produced a concentric ring with green conidial and white filamentous mat over the growth

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

Table 3: Confirmatory test of isolates.

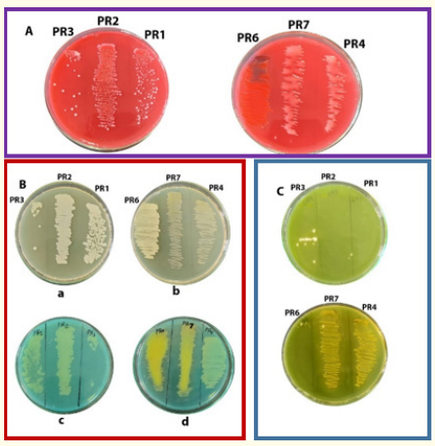

Figure 1: Confirmatory test of *Rhizobium*. The different isolates were grown on various medium. A. YEMA+Cr Rhizobia produce white colonies, whereas many other bacteria take up the dye strongly B. Keto-lactose Test. The yellow zone was observed which indicate that *Rhizobium* species C. Growth on Glucose Peptone Agar (GPA) medium, *Rhizobium* show growth on glucose peptone agar medium and produce yellow colour.

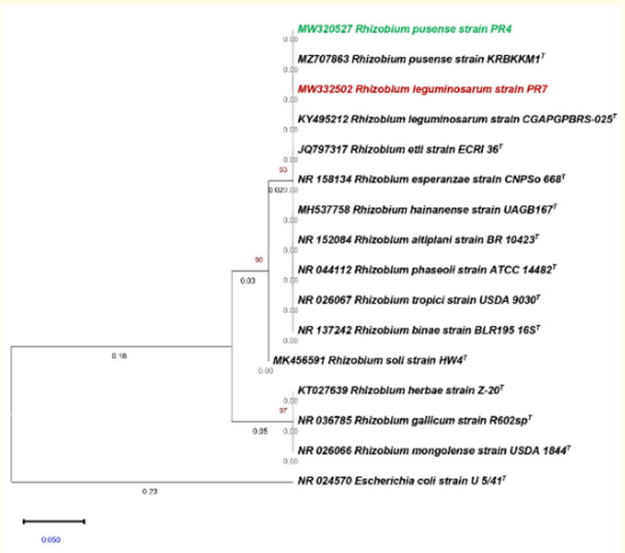

Figure 2: Phylogenetic analysis of *Rhizobium leguminosarum* (PR7) and *Rhizobium pusense* (PR4) based on 16srRNA gene sequences available from NCBI library constructed after multiple alignments of data ClustalW. Distances and clustering with the neighbor-joining method were performed using MEGA 11 software.

after 4 days incubation at 28 ± 2°C. The conidia production was denser in centre then towards the margins. GFT2 isolate appears to be a bit granular on PDA, with green conidia distributed throughout. An irregular yellow zone without conidia was present around the inoculum. All plate filled with the white filaments over the mat and in the centre produced green conidia with two concentric rings in GFT3 isolate. In the GFT4 isolate formed many concentric rings gradually growing and cover all area in plate and produced green conidia in centre with one yellow concentric ring, also produced in centre pustule yellow colony all morphology characteristics observed after fungal isolates incubated for 4 days at 28 ± 2°C (Figure 3). Moreover, rest of the morphology of fungal isolates grown on various media data mention in Table.

Microscopic identification of (GFT1, GFT2, GFT3 and GFT4) isolates were carried out using of staining method. The microscopic morphological details revealed by 1% Methylene blue staining are summarized in *Trichoderma* ITS sequences were used to create a phylogenetic tree along with other *Trichoderma* sequences downloaded from the GenBank database at the NCBI to study the relationships between the *Trichoderma* isolates (identified in the text by their accession numbers). Phylogenetic analysis of *T. viride* (GFT1), *T. harzianum* (GFT2), *T. longibrachiatum* (GFT3), and *T. reesei* (GFT4) based on ITS sequences obtained and was compared to other sequences with the help of BLASTn database. Similar sequences with highest sequence homology and query coverage with lowest E values were used to develop NJ tree using MEGA 11 software which uses ClustalW algorithm for multiple sequence alignment. Phylogenetic analysis revealed that *T. viride* (GFT1) (MZ497364) have maximum similarity *Trichoderma viride* strain EL01 (KM820885) similarly *Trichoderma harzianum* GFT3 (MZ497371) have maximum similarity with the *Trichoderma harzianum* strain Th3 (KX898424), *Trichoderma longibrachiatum* GFT2 (MZ497368) have maximum similarity with the *Trichoderma*

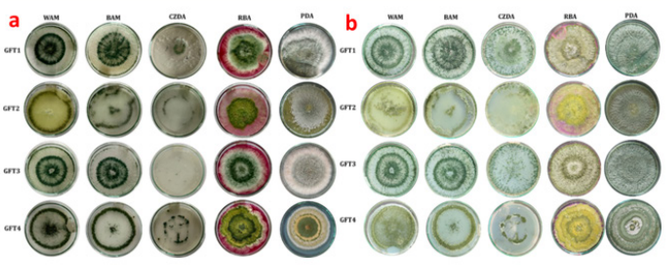

Figure 3: Colony Morphology of four different Strains of *Trichoderma* grown display of(a) for 4 days and (b) 7 days at 28 ± 2°C on, WAM, BAM, CZDA, RBA and PDA.GFT1, GFT2, GFT3, and GFT4.

longibrachiatum voucher PGPFMos-T2(KR233646), also *Trichoderma reesei* GFT4(MZ497373) have maximum similarity with the *Trichoderma reesei* strain SJVTR(KF979305).Evolutionary analyses were conducted in MEGA11 (Figure 4).

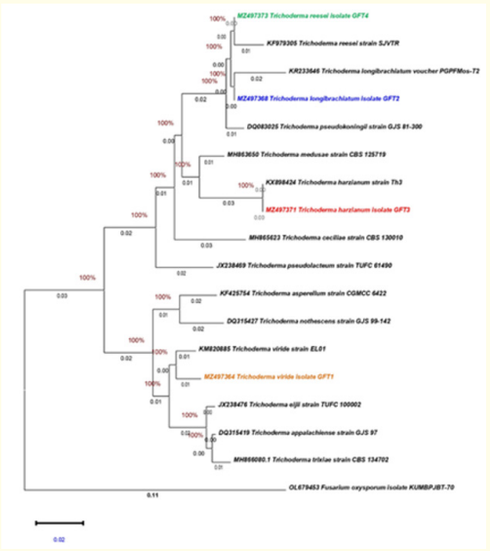

Figure 4: Phylogenetic analysis of *Rhizobium leguminosarum* (PR7) and *Rhizobium pusense* (PR4) based on 16srRNA gene sequences available from NCBI library constructed after multiple alignments of data ClustalW. Distances and clustering with the neighbor-joining method were performed using MEGA 11 software.

Discussion

In the current study, a total aof 06 bacterial samples were isolated from root nodules of *Arachis hypogaea* growing in Saurashtra, Gujarat and characterised using morphological, biochemical, and phylogenetic analysis. Several studies have been published that describe the morphological, biochemical characteristics and phylogenetic analysis of rhizobia. Singha., *et al*. (2013) isolated *Rhizobia* from root nodules of Soybean (*Glycine max L*.) and identified them as *Rhizobium joponicum* and *BradyRhizobium japonicum* based on morphological, cultural, and biochemical characteristics [43]. In addition, morphological, biochemical, and phylogenetic analysis were used to isolate *Rhizobium* species from *Crotolaria junceae L* plant and isolated strain were *Rhizobium leguminoserum* and *MesoRhizobium thiogangeticum.*According to Agah, Jain., *et al*. (2020), the *Rhizobium* species have also been distinguished basis of morphological attributes and molecular analysis of *Rhizobium* from Root Nodules of *Arachis hypogaea L*. and *Telfairia occidentalis* in South-East, Nigeria [17].

The current study revealed that most isolates have very different morphological and microscopic features, with some having rod-shaped, short rod, and endospore-forming strains under the microscope. Morphological features, on the other hand, have varied in isolated strains' appearance. PR1, PR2, and PR3 isolates were dry, while PR6 isolates were chalky and PR4, PR7 isolates were gummy. Another distinguishing feature was the colour produced on YEMA medium, with all isolates producing white colour except isolate PR6. The biochemical analyses of the isolates revealed that majority of the tests for indole, urease, catalase, oxygenase, Voges-Proskauer, nitrate reduction, and Oxidase were positive. Voges-Proskauer tests on *Rhizobium* isolates had negative. Most of the isolates were oxidase, catalase, and urease positive, nitrate reduction positive, and unable to utilise citrate, according to biochemical characterization of the isolates, which supports Kumar., *et al*. (2020) [23]. The same tests were employed by Ali., *et al*. (2012) to establish the identity of similar isolated bacterial strains as *Rhizobium spp*. When isolating and describing *Rhizobium meliloti* [1]. These results closely align with those of Shahzad., *et al*. (2012), who previously identified the *Rhizobium* from soil and groundnut root nodules using the same favourable biochemical tests [39]. Additionally, it was noted that the isolates failed to produce gelatinase enzymes. Similar result Nohwar., *et al*. (2019) also discovered that *Rhizobium* had negative gelatinase activity [31]. Three confirmatory tests were carried out to establish that the isolates were rhizobia. On YEMA-Cr media, most of the isolates, apart from PR4 and PR7, showed no Congo red dye absorption, which is similar to the results of Verma., *et al*. (2020) who reported that rhizobia wouldn't absorb Congo red dye or absorb it only occasionally compared to other bacteria [49]. According to Jain., *et al*. (2020a) report on the growth in glucose peptone agar, rhizobia either showed no growth or grew very slowly on GPA media [16]. However, this result conflicted with the current finding, which showed that *Rhizobium* strains were growing on GPA media. The results of the ketolactose test showed that most of the isolates did not produce 3-ketolactose from lactose. The results of the two isolates, PR4 and PR7, however, were positive, which contrasts with earlier research done by MD., *et al*. 2012 [27]. Additionally, according to Singha., *et al*. (2013) the results of the *Rhizobium* ketolactose test were negative, which is a

contrary finding [43]. According to Di Lelio., *et al.* (2021), *Trichoderma* species were isolated using *Trichoderma* selective medium (TSM), and subsequently sub-cultured on potato dextrose agar [8]. Even so, India has been home to more than 200 different species of *Trichoderma*. Only four species were found in the rhizospheric soil of peanut during the current investigation [32]. According to Gajera., *et al*. (2016), there were fourteen different species of *Trichoderma*, of which *T. harzianum* and *T. viride* were determined to be the most prevalent in ground nut rhizospheric soil [11]. *Trichoderma* colony morphological characteristics have been documented by Bunbury-Blanchette and Walker (2019) utilising a variety of parameters, including growth pattern, growth rate, and colour [5]. *Trichoderma* isolates were species-level identified using the colour of the colony, the development of chlamydospores, conidiophores and phialides characteristics, and the shape of the conidia as the primary characters to identify the species [35]. Additionally, Tegene., *et al*. (2021) employed these traits as the main criteria for isolating *Trichoderma* colonies from soils [47]. In addition, Frisvad., *et al*. (2018) has been documented these features are taxonomically useful for *Trichoderma* [10]. According to Cai and Druzhinina (2021)*Trichoderma* spp. microscopic characteristics and colony morphology were used to determine the taxonomic classification of the *Trichoderma* [6]. Mazrou., *et al*. (2020) also described the morphological characters of *Trichoderma* spp grown on various nutrient media employed in the current study and examined the micro-morphological traits [26]. To confirm species identity, which was previously made only based on morphological criteria, genetic sequence 18s RNA and 16s RNA analysis of four isolates of *Trichoderma* spp. and two isolates of *Rhizobium* spp. was performed.The current study is based on a preliminary study that was done on isolates of biocontrol and biofertilizer agents from soil around peanut plants, as well as nodulation or nitrogen-fixing bacteria from the same peanut plant root nodules. According to morphological, biochemical, confirmatory testing, molecular identification based on completed 16s RNA sequence, and built phylogenetic tree, there were two strain isolates of *Rhizobium leguminosarum* PR7 and *Rhizobium pusense* PR4. The majority of fungi used for biofertilizer, and biocontrol, identified based on their morphological characteristics grown in various nutrient medium and microscopic observation, a total of four *Trichoderma* strains were obtained from the rhizosphere soil of the *Archis hypogaea L*. plant. The most powerful analysis was molecular identification by 18s RNA sequencing and the construction of phylogenetic trees. The strains that were ultimately identified were *T. viride* (GFT1), *T. harzianum* (GFT2), *T. longibrachiatum* (GFT3), and *T. reesei* (GFT4). The following strains of nitrogen-fixing fungus (PR4 and PR7) and as well as the biocontrol fungi GFT1, GFT2, GFT3, and GFT4 were processed for continued study.The upcoming research, only all *Trichoderma* strains will have their biocontrol and biofertilizer properties defined.

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Conflict of Interest

The authors declare no competing interests.

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