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RESEARCH ARTICLE

Isolation and Characterization of Nodule Endophytes from Bunching and Semi-spreading Groundnut Genotypes

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ABSTRACT

Bacterial endophytes prevalently colonize the internal tissues of plants and are shown to positively influence plant growth. They play a significant role in maximizing crop productivity while reducing the environmental impacts of agriculture. The aim of the present work was to isolate and characterize root nodule endophytes from two groundnut genotypes (Bunching & Semi-spreading). A total of 20 bacterial isolates were recovered from groundnut nodules using the yeast extract mannitol agar medium supplemented with congo red. The isolates were characterized morphologically, biochemically and compared with the reference strains *Rhizobium* sp. TNAU 14, *Rhizobium* sp. COS 1 and *Bradyrhizobium* sp. The isolates showed close similarity to the reference strains in their biochemical and morphological characteristics. Based on these results, it is concluded that the isolates YBB1, YBB6, YBB8, YBB14A, S27 from the bunching type and YSB 3, YSB4 and YSB5 from the semi-spreading genotype tentatively represent *Rhizobium*.

Keywords: *Groundnut, Isolation, Rhizobial endophytes, Rhizobium.*

INTRODUCTION

Endophytes are micro-organisms (bacteria and fungi) that live inside the plant tissues for at least part of their life without causing any apparent disease symptoms in the host. Endophytic bacteria colonize the internal tissue of the plants but do not show any external sign of infection or adverse effect on infected host plants and avail the benefit of the host (Castro *et al*., 2007). Symbiotic and non-symbiotic bacteria have been isolated from the root nodules of a wide range of legumes (Sturz *et al*., 1997; Zakhia *et al*., 2006) such as alfalfa (Gagne *et al*., 1987), clover (Sturz *et al*., 1997) and soybean (Oehrle *et al*., 2000). Endophytic bacteria exert several beneficial effects on host plants, such as induction of resistance to plant pathogens (Chen *et al*., 1995), nitrogen fixation (Kirchhof *et al*., 1997) and stimulation of plant growth (Sturz *et al*., 1997). Such plant beneficial microorganisms are significant in the field of agriculture either as biofertilizers, pesticides or for phytoremediation applications (Zakhia *et al*., 2006).

Groundnut is an important oilseed crop suitable for cultivation in tropical areas of the world. It belongs to *Leguminosae* family, subfamily *Papilionidae* and tribe *Aeschynomeneae*. The genus *Arachis* comprises of 22 species out of which 9 are reported to be nodulated. Poiteau first noted groundnut nodules as early as 1853. Groundnut possesses a high symbiotic nitrogen-fixing capacity and the amount of nitrogen accumulated by groundnut is high compared to other tropical legumes.

Bacteria belonging to the family *Rhizobiaceae* (*Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*) are dominant symbiotic endophyte bacteria that infect plants of the *Leguminosae* family by forming nodules in their roots and thereby aiding in biological nitrogen fixation, apart from other direct and indirect benefits to the plant host. The symbiosis between legume plants and rhizobia in the soil is widely studied and is highly significant in agriculture. Nodules, especially those collected from the field, are not always occupied by a single rhizobial isolate or by a single micro-organism. Nodules of pea and lupin, have been described to contain both the nitrogen-fixing symbiont and associative organisms such as *Micromonospora* (Trujilo *et al*., 2010). Recognition of rhizobia by observing the morphological characters when growing on a solid medium is important. Understanding the nodule endophytic bacterial diversity between two different groundnut genotypes bunching and semi-spreading is the need of the hour. Hence, the present study was aimed to isolate and characterize the nodule endophytes from bunching and semi-spreading groundnut genotypes.

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MATERIAL AND METHODS

Sample collection

The groundnut nodule samples were collected from TamilNadu Agricultural University, Regional Research Station, Vridachalam, TamilNadu, India (11°30′32.43″N and 79°20′14.68″E). Two groundnut genotypes namely VRI – 2 (Bunching type) and VRI – 7 (Semi-spreading type) were chosen. Plants are in the vegetative stage (25-30 days old) were selected, uprooted gently by wetting the root zone. The root portion was gently washed with running tap water to remove adhering soil particles without damaging the nodules. The plant samples were packed in air-tight pouches and brought to the laboratory.

Isolation

The nodules were separated from the roots and surface disinfected with 95% alcohol for the 30s and with 0.1% (w/v) mercuric chloride for 2 min, rinsed 6-8 times with sterile distilled water. The surface sterilized nodules were crushed and streaked on yeast extract mannitol agar supplemented with 0.025% congo red indicator (Composition per litre : Yeast extract 1g, Mannitol 10g, Dipotassium hydrogen phosphate 0.5g, Magnesium sulphate 0.2g, Sodium chloride 0.1g, Congo red 0.025g, Agar 20.0g, pH 6.8±0.2). The plates were incubated at 28°C and the single colonies obtained were further purified by repeatedly streaking on the same medium. Pure cultures were stored in 50% glycerol stocks at −80°C for further experiments.

Confirmatory tests

Confirmatory tests were conducted to tentatively confirm the *Rhizobium* isolates. Standard strains *viz., Rhizobium* TNAU 14, *Bradyrhizobium* sp. and *Rhizobium* COS 1 were obtained from Biofertilizer production unit, Department of Agricultural Microbiology, TNAU and were used as standard strains in characterization experiments.

Growth on glucose peptone agar

The nodule endophytic isolates were streaked on GPA plates and incubated at 28 ±2 °C overnight.. *Rhizobium* shows poor growth or no growth when it is streaked on glucose peptone agar. Heavy growth is indicative of other non-rhizobial endophytes (Vincent, 1970).

Growth on lactose agar

The bacterial isolates were streaked on lactose agar plates at 28 ±2 °C for 48 hours. Freshly prepared Benedict reagent was flooded on the plates and left for 1-2 hrs. Bacteria other than *Rhizobium* utilizes lactose in the medium and transforms it to alpha keto glutarate when Benedict's reagent is added, it is indicated by a change in colour from blue to yellow. *Rhizobium* utilizes lactose hence no change of colour is found (Sadowsky *et al*., 1983)

Growth on YEMA with BTB

Yeast extract mannitol agar medium was prepared with 0.002 g/l bromothymol blue indicator. The bacterial isolates were streaked and incubated at 28 ±2 °C overnight. *Rhizobium* colonies while growing changes the pH of the medium which will be indicated by a change of the colour from green to yellow.

Growth on Luria Bertani agar

The nodule endophytic bacterial isolates were streaked onto Luria Bertani medium and incubated at 28 ±2 °C overnight. *Rhizobium* colonies show poor/no growth in Luria Bertani medium.

Growth on alkaline Hoffers broth

Alkaline Hoffers broth with BTB indicator medium was prepared and the pH of the broth was adjusted to 11 using 0.1 N NaOH. The bacterial isolates were streaked and incubated at 28 ±2 °C overnight. *Rhizobium* does not grow at a higher pH.

Characterization of isolates

Gram's staining

Twenty-four hours old bacterial suspension smear was taken on a clean glass slide, air dried and fixed by gentle heating. Crystal violet dye was spread over the smear and air dried for 60 seconds and then washed with water. The smear was then flooded with Gram's iodine solution for a minute and rinsed in tap water and decolorized with 95% of ethanol until colour runoff, washed with water and treated with counter stain safranin for about 10 seconds, washed with water, air dried and observed under microscope at 40X (Schaad *et al*., 1980).

Indole production test

The bacterial isolates were inoculated in peptone broth and incubated at 28±2 °C for 48-96 h. After the incubation period, 0.5 ml of Kovac's reagent was added and shaken gently. Development of pink or red colour indicated a positive reaction (Gillus, 1956).

Methyl red test

The bacterial isolates were inoculated in 5 ml MRVP broth and incubated at 28±2°C for 48h. After the incubation period, five drops of 0.04% solution of alcoholic methyl red was added. Development of bright red colour indicated a positive result whereas a negative result is indicated by the development of yellow colour (Olutiola *et al*., 2008)

Voges- Proskauer test

The bacterial isolates were inoculated in 5 ml

MRVP broth and incubated at 28±2˚C for 48 h. After the incubation period, 1ml of potassium hydroxide containing 0.3% creatine and 3ml of the α-napthol solution was added. A positive reaction is indicated by the development of pink colour within 2-5 min (Olutiola *et al*., 2008)

Citrate utilization test

Citrate utilization test was performed by streaking the bacterial isolates on the Simmon's citrate agar slants. The slants were incubated at 28±2˚C for 48 h. Colour change in medium from green to blue indicated the positive result (Simmons, 1976).

Catalase test

The bacterial isolates were streaked on YEMA plates, incubated at 28±2˚C for 48 h. A drop of 3% H_2O_2 was placed on colonies. The appearance of effervescence indicates positive reaction (Smibert and Kreig, 1981).

Urease test

The urease test is used to determine the ability of an organism to split urea, through the production of the enzyme urease. Hydrolysis of urea produces ammonia and CO_2. The formation

Table 1. Morphological characterization of nodule endophytes from bunching and semi-spreading groundnut genotypes cultured on yeast extract mannitol agar medium supplemented with congo red.

Isolate	Growth rate	Colony colour	Colony shape	Colony diameter (mm)	Gram's reaction
VRI 2 (Bunching type)					
YBB1	++	Translucent white	Circle	1-2	Gram negative
YBB2	+++	Creamy yellow	Irregular	2-3	Gram positive
YBB3	+++	Slimy pale white	Circle	2-3	Gram negative
YBB5	++	Creamy yellow	Irregular	1-2	Gram positive
YBB6	+	Pale yellow	Circle	1-2	Gram negative
YBB7	+	Light pink	Circle	1-2	Gram negative
YBB8	++	Translucent white	Circle	2-3	Gram negative
YBB9	++	Light pink	Circle	2-3	Gram positive
YBB10	+++	Translucent white	Circle	2-3	Gram negative
YBB11	+	Pale yellow	Circle	1-2	Gram positive
YBB12	+	Light pink	Irregular	1-2	Gram positive
YBB14A	+++	Translucent white	Circle	2-3	Gram negative
S27	+	Slimy pale white	Circle	1-2	Gram-negative
YBB19B	+	Light pink	Circle	1-2	Gram positive
S10	+	Light pink	Circle	1-2	Gram positive
S11	+	Slimy pale white	Circle	1-2	Gram-negative
VRI 7 (Semi-spreading type)					
S19	+	Pale white	Circle	1-2	Gram-negative
YSB1B	++	Light pink	Irregular	2-3	Gram positive
YSB3	++	Translucent white	Circle	2-3	Gram negative
YSB5	+	Pale yellow	Circle	1-2	Gram negative
Reference Strains					
**Rhizobium* TNAU 14	+++	Translucent white	Circle	2-3	Gram negative
**Rhizobium* COS 1	++	Translucent white	Circle	2-3	Gram negative
**Bradyrhizobium* sp.	+	Translucent white	Circle	1-2	Gram-negative

(+++) Very fast colonies appeared after 24 hours of incubation. (++) Fast colonies appeared after 2-3 day incubation. (+) Slow colonies appeared after 4-5 days of incubation. The morphological characteristics are compared with three reference strains (*)

of ammonia alkalinizes the medium, and the pH shift is detected by the colour change of phenol red from light orange at pH 6.8 to magenta (pink) at pH 8.1. Rapid urease-positive organisms turn the entire medium pink within 24 hours (Christensen, 1946).

Starch hydrolysis

Bacteria capable of hydrolyzing starch to maltose possess the enzyme amylase. The presence or absence of amylase can be detected in the test. Bacterial isolates were streaked on starch agar plates and incubated at 28±2 ºC for 48-72 hrs. After

incubation, the iodine solution was flooded to the plates. Development of the blue colour indicated that starch had not been hydrolyzed. Complete hydrolysis and partial hydrolysis of starch were indicated by the development of clear white or brownish white colour respectively (Clarke, 1952)

Gelatin test

The ability of bacteria to produce gelatinase enzyme by utilizing gelatin as a source was tested. Degradation of gelatin indicates the presence of gelatinase enzyme. The actively grown cultures were inoculated in nutrient gelatin medium and grown for 48 hrs, when the growing culture is exposed at 4°C for 30 min, the cultures which produce gelatinase remain liquefied while others due to the presence of gelatin remains solid (Aneja, 2003).

Carbon utilization

All the 20 bacterial isolates were tested for their growth in different carbon sources (sucrose, mannitol, sorbitol, fructose, glucose, sodium citrate) in the MS minimal medium supplemented with 0.1% of respective carbon sources. Glucose was used as a control. The test tubes containing different carbon sources after inoculation were incubated at 28°C for a week and the growth was measured in a spectrophotometer at 600nm.

RESULTS AND DISCUSSION

Morphological characterization

Groundnut (*Arachis hypogaea L.*) is usually nodulated by rhizobia. Isolation and characterization of root nodulating endophytes in several legumes

Table 2. Confirmatory test for nodule endophytes isolated from bunching and semi-spreading groundnut genotypes.

Isolates	Glucose peptoneagar	Lactose agar	YEMA with BTB	LB agar	Alkaline Hoffers broth
VRI 2 (Bunching type)					
YBB1	-	No Yellow colour formation	Green to Yellow	-	-
YBB2	+	Yellow colour formation	Green	-	-
YBB3	-	No Yellow colour formation	Green to Yellow	+	-
YBB5	+	No Yellow colour formation	Green to Yellow	+	-
YBB6	-	No Yellow colour formation	Green to Yellow	+	-
YBB7	-	No Yellow colour formation	Green to Yellow	+	-
YBB8	-	No Yellow colour formation	Green to Yellow	+	-
YBB9	+	Yellow colour formation	Green	+	-
YBB10	-	No Yellow colour formation	Green to Yellow	-	-
YBB11	-	No Yellow colour formation	Green to Yellow	-	-
YBB12	+	No Yellow colour formation	Green to Yellow	-	-
YBB14A	-	No Yellow colour formation	Green to Yellow	-	-
S27	-	No Yellow colour formation	Green to Yellow	-	-
YBB19B	+	Yellow colour formation	Green	+	-
S10	+	Yellow colour formation	Green to Yellow	+	-
S11	-	No Yellow colour formation	Green to Yellow	+	-
VRI 7 (Semi-spreading type)					
S19	-	Yellow colour formation	Green	+	-
YSB1B	-	No Yellow colour formation	Green to Yellow	-	-
YSB3	+	No Yellow colour formation	Green to Yellow	-	-
YSB5	-	No Yellow colour formation	Green to Yellow	-	-
Reference strains					
**Rhizobium* TNAU 14	-	No Yellow colour formation	Green to Yellow	-	-
**Rhizobium* COS 1	-	No Yellow colour formation	Green to Yellow	-	-
**Bradyrhizobium* sp.	-	No Yellow colour formation	Green to Yellow	-	-

(+ Growth; - No growth; (*) Reference strains).

have already been investigated and the present study focuses on the diversity of nodule endophytes between two different groundnut genotypes. 20 endophytic bacteria were isolated from two groundnut genotypes' surface sterilized root nodules, to avoid any contamination from the nodule surface. Based on unique colony morphology, colonies were picked and purified by repeated streaking and stored. Table 1. represents the morphological characters of the isolated nodule endophytes. Most of the colonies appeared within 24-48 hrs whereas few appeared after 3-5 days. The colonies on YEMA plates supplemented with congo red were translucent white in colour, round in shape, fast

growing and produced polysaccharides (Figure 1). Most of the isolates showed no absorption of congo red dye on YEMA medium which is consistent with the results of Trinick *et al.* (1982) who reported that rhizobia do not absorb congo red dye or absorbed very weekly compared with other bacteria. Colonies of *Rhizobium*, isolated from a variety of legume hosts were white, rounded, had a diameter from 5 to 7 mm and produced mucous substances which are the characteristic feature of rhizobia. Similar morphological characters of rhizobia were earlier reported by several workers from different hosts like Berseem (Gauri *et al*., 2011), Faba bean (Anteneh, 2012), Mungbean (Shraddha *et al*., 2013 ; Amin, 2014) , Pea (Deshwal and Chaubey, 2014), Soybean (Pawar *et al*., 2014), Black gram (Satyanandam *et al*., 2014), Groundnut (Benson *et al*., 2015), and fenugreek (Tsegaye *et al*., 2015). There are several reports describing the characterization of rhizobia based on morphological and biochemical features. Gachande and Khansole (2011) isolated rhizobia from root nodules of Soy bean (*Glycine max* L.) and characterized them as *Rhizobium japanicum* and *Bradyrhizobium japanicum* based on morphological, cultural and biochemical characteristics. The present study revealed that the morphological features of most of the isolates were very much similar to that of the reference strains and the colonies tentatively represent rhizobial species. However, YBB2, YBB5, YBB9, YBB11, YBB12, YBB19B, S10 from the bunching type and YSB1B from semi-spreading type varied in colony morphology like colour, shape and did not resemble with the colony characteristics of reference strains and hence, these isolates may not be considered as probable rhizobial isolates.

Table 3. Biochemical characterization of nodule endophytes isolated from bunching and semi-spreading groundnut genotypes

Isolate	Biochemical tests								Carbon source utilization					
	Ind	MR	VP	Cit	Cat	Sta	Ure	Gel	Suc	Man	Glu	Fru	Sor	Sod cit
VRI 2 (Bunching type)														
YBB1	-	-	-	-	+	-	+	-	+	+	+	+	+	-
YBB2	-	-	+	-	+	+	+	+	+	+	+	+	+	-
YBB3	-	+	+	-	+	-	+	-	-	+	+	+	+	-
YBB5	-	-	+	-	+	-	+	-	-	+	+	+	+	+
YBB6	-	-	+	-	+	-	-	-	+	+	+	+	+	+
YBB7	-	-	+	-	+	-	+	-	+	+	+	+	+	-
YBB8	-	-	-	-	+	-	+	-	+	+	+	+	+	+
YBB9	-	-	+	+	+	+	-	-	+	+	+	+	+	-
YBB10	+	-	+	-	+	-	+	-	-	+	+	+	+	-
YBB11	+	-	+	-	+	-	+	-	+	+	+	+	+	+
YBB12	-	-	+	+	+	-	+	-	+	+	+	-	-	-
YBB14A	+	-	+	-	+	+	-	-	+	+	+	+	+	-
S27	-	-	-	-	+	-	+	-	+	+	+	-	-	-
YBB19B	+	+	+	-	+	-	-	-	-	+	+	+	+	-
S10	+	+	-	-	+	-	+	-	+	+	+	+	-	-
S11	-	-	-	-	+	-	+	-	-	+	+	-	+	-
VRI 7 (Semi-spreading type)														
S19	-	-	-	-	+	-	+	-	+	+	+	-	-	-
YSB1B	+	+	+	+	-	-	-	-	-	+	+	+	+	-
YSB3	-	+	+	-	+	-	+	-	+	+	+	+	-	-
YSB5	-	-	+	-	+	-	+	-	-	+	+	-	+	+
Reference strains														
**Rhizobium* TNAU 14	-	-	+	-	+	-	+	-	+	+	+	+	+	-
**Rhizobium* COS 1	-	-	+	-	+	-	+	-	+	+	+	+	+	-
**Bradyrhizobium* sp.	-	-	+	-	+	-	+	-	+	+	+	-	-	-

(Ind-Indole production test; MR-Methyl red test; VP- Voges- Proskauer test; Cit-Citrate utilization test; Cat-Catalase oxidation test; Sta-Starch hydrolysis test; Ure-Urease test; Gel-Gelatin hydrolysis test) Carbon utilization (Suc-sucrose; Man-mannitol; Gluc-glucose; Fruc-fructose; Sorb-sorbitol; Sod cit-sodium citrate) + Positive result; - Negative result; (*) Reference strains.

Confirmatory test

Five confirmatory tests were performed to confirm the isolates belonged to rhizobia (Table 2). In the glucose peptone agar, out of the 20 isolated bacteria, 6 showed profuse growth in the medium. This is in contradiction with the results reported by Vincent (1970), that rhizobia showed either no growth or grow very poorly on GPA medium. Result of

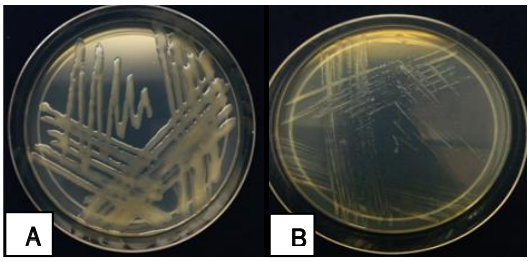

Figure 1. Colonies streaked on YEMA medium supplemented with congo red. Appearance of translucent white colonies with polysaccharide production was indicative of *Rhizobium* sp.

lactose agar test revealed that most of the isolates showed the negative result for the production of alpha keto glutarate from lactose which was in accordance with Sadowsky *et al*. (1983). In YEMA medium supplemented with BTB, 4 isolates showed negative results and in LB medium, 10 isolates showed good growth which is contradictory to rhizobial nature (Figure 2). None of the bacterial isolates exhibited growth in alkaline Hoffer's broth.

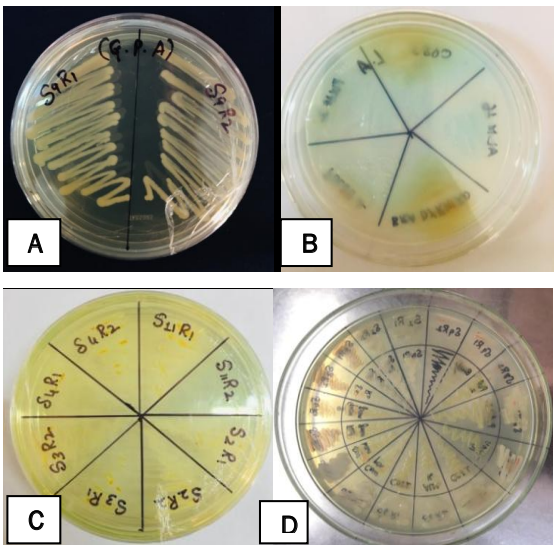

Figure 2. Confirmatory tests A). Glucose peptone agar- Colonies showed profuse growth indicating non-rhizobial endophytes; B). Lactose agar- Colour change from green to yellow indicated endophytes other than rhizobia; C).YEMA medium supplemented with BTB-colour changed from green to yellow indicated *Rhizobium*; D). LB agar- Bacterial isolates showed no/ poor growth in LB agar plates

Biochemical and Carbon source utilization

In regard to the biochemical tests, most of the isolates showed a positive result for catalase test, urease test which complemented with the results of Lupwayi and Hague (1994) and a negative result for starch hydrolysis, citrate utilization and gelatin hydrolysis test. Negative gelatinase activity is a characteristic feature of *Rhizobium*, as reported by Hunter *et al.* (2007). The results confirmed that the biochemical features of all the isolates except YBB2, YBB9, YBB12, S19 and S10 were similar to the biochemical features of reference strains. In carbon source utilization tests, glucose, mannitol, sorbitol and fructose were the most preferred sources whereas sodium citrate was the least utilized source (Table 3).

CONCLUSION

From the present study it can be concluded that most of the bacterial isolates recovered from two groundnut genotypes (bunching and semi-spreading) were closely related to the reference strains *Rhizobium* TNAU 14, *Bradyrhizobium* sp. and *Rhizobium* COS 1 based on their morphological, confirmatory and biochemical characteristics and tentatively represent rhizobial species. However, the ability of the isolates to nodulate the groundnut plants are yet to be ascertained. Further study is required to understand the nodule forming bacterial diversity in legumes, interactions between plant-nodule endophytic bacteria, nodulation process and presence of symbiotic genes. Suitable PCR based genotypic techniques can be employed for confirming the identity of the isolates and for predicting the phylogenetic relationship.

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