In vitro evaluation of groundnut (*Arachis hypogea* L.) rhizospheric *Bradyrhizobium* traits for dinitrogen fixation

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ABSTRACT

Aims: To decipher the nitrogen fixing potential of *Bradyrhizobium* sp.
Study design: Random sampling method was followed for the representative soil sample collection and Completely Randomized Design was used for the *in vitro* experiments.
Methodology: Random six locations of Cuddalore district (Tamil Nadu, India) was selected and groundnut rhizospheric soil samples were collected during Feb 2019. Traits of *Bradyrhizobium* sp. were isolated by serial dilution method. Followed by the purification of isolates and the purified isolates were subjected for biochemical characterization studies. After confirming, the traits were evaluated and graded for their nitrogen fixing ability by Microkjeldahl assay.
Results: A maximum number of *Bradyrhizobium* cells, 5.87 $\log_{10}$ CFU g-1 of dry soil was recorded from the rhizosphere samples, respectively, collected at Thiruvamur location of Cuddalore taluk and the lowest population was recorded in samples collected from Ramapuram location of Cuddalore district. Based on the amount of N fixed /g of mannitol, BM-1 and BM-5 constituted the first category, BM-3 and BM-6 constituted second category and BM-2 and BM-4 were ranked in the third category.
Conclusion: This study, clearly deciphers the Nitrogen fixing potential of *Bradyrhizobium* traits of groundnut crop and the species holds the promise of using the *Bradyrhizobium traits* as the efficient nitrogen fixers in legume crops in future.

Keywords: Groundnut, rhizosphere, Bradyrhizobium, dinitrogen fixation

1. INTRODUCTION

Groundnut or peanut (*Arachis hypogaea* L.) is a major oilseed crop widely grown in tropical and subtropical regions of the world. It, as an annual legume and premier oil seed crop, having the potential to produce high oil and easily digestible protein in the seeds. Poor 'N' nutrition and incidence of diseases are the major biotic and abiotic constraints which limit the productivity of groundnut. The injudicious, consistent use of synthetic chemical fertilizers and pesticides are too expensive for the resource poor farmers and lead to biological and environmental hazards. Hence, the use of PGPR for the enhancement of plant growth stimulation and biocontrol against phytopathogens might be a suitable biological approach to overcome the biological and environmental hazards. In the present study, it was observed that the population of *Bradyrhizobium* ranged between 0.77 and 1.17 per cent to the total bacterial community recorded in the population survey at Cuddalore district, Tamil Nadu, India. Six isolates of *Bradyrhizobium* were isolated and designated and the PGPR characteristics were analyzed. Two *Bradyrhizobium* isolates were ranked in I category. Finally, the *Bradyrhizobium* BM-5 was selected as the most efficient isolate and their speciation revealed that they were belonging to *Bradyrhizobium arachidis*. Considering the trait specialization of *Bradyrhizobium*, the study was carried out with the following objectives,

- Occurrence and community of *Bradyrhizobium*.
- Selection and evaluation of dinitrogen fixation.

2. MATERIAL AND METHODS

2.1.1 Glass Ware

Glassware was soaked in cleaning solution (100 g of potassium dichromate dissolved in 100 mL distilled water followed by addition of 500 mL of concentrated sulphuric acid) for 12 h, boiled in soap water for few minutes and washed in tap water. They were thoroughly rinsed in distilled water and dried. Glassware used for culture work was sterilized in a hot air oven at 180 °C for 4 h.

2.1.2 Chemicals

All the chemicals were of reagent grade quality unless otherwise stated and glass distilled water was used throughout the study.

2.1.3 Maintenance of *Bradyrhizobium* reference strains

The isolated *Bradyrhizobium* strains were maintained in yeast extract mannitol agar (YEMA) slants, at 30 ± 2^{o}C with monthly transfer and used throughout the study.

2.1.4 Composition of media

Composition of Yeast extract mannitol Agar Medium (Allen, 1953), Mannitol - 10.0 g, Calcium carbonate - 3.0 g, Dipotassium hydrogen phosphate - 0.5 g, Magnesium sulphate - 0.2g, Sodium chloride - 0.1 g, Yeast extract - 0.5 g, Agar - 20.0g, Distilled water - 1000 mL, pH - 7.0.

2.2 Survey for *Bradyrhizobium* occurrence from the rhizosphere of irrigated groundnut in Cuddalore district, Tamil Nadu, India

2.2.1.1 Survey for *Bradyrhizobium* occurrence from groundnut rhizosphere

The survey was conducted at six villages in Cuddalore district, Tamil Nadu, India where groundnut is a predominant oil food crop grown under irrigated condition. Random selection of locations was made so that each and every sector of the experimental area would get a representation in the survey.

2.2.1.2 Details of Locations

The name of six locations selected for the survey of *Bradyrhizobium o*ccurrence from the rhizosphere of irrigated groundnut are given in Table 1.

2.2.1.3 Collection of rhizosphere soil sample from each location

A total number of five groundnut plants were selected randomly at various places in the irrigated groundnut field and the samples were considered as representative irrigated groundnut plant sample of that location. The selected groundnut plants were uprooted with entire root system together with the soil adhered to the roots. Then, they were aseptically packed up in polythene bags and transferred to the laboratory on the same day of collection for further analysis.

2.3 Isolation and Enumeration of *Bradyrhizobium* population from the rhizosphere of irrigated groundnut

2.3.1 Enumeration of *Bradyrhizobium* population from the rhizosphere of irrigated groundnut

The adhered soil of groundnut roots, collected from all the 5 groundnut plants of a particular locations, were pooled and 10 g of soil sample was transferred to 100 ml of sterile distilled water in a 250 ml Erlenmeyer flask and incubated on a rotary shaker (100 rpm) for 30 min at ambient temperature. The well mixed suspension of each soil sample was

subjected to tenfold serial dilution, ranging from 10^{-1} to 10^{-9}. One ml suspension from each dilution was transferred aseptically to Petri plates and melted YEMA medium was poured in each Petri plates. Then, they were rotated in clockwise and anticlockwise direction for uniform distribution and incubated at 37^{o} C. After incubation period, the *Bradyhizobium* colonies developed in each Petri plates were counted using Arnold colony counter. Three replications were maintained for each sample.

2.3.2 Enumeration of total heterotrophic population from the rhizosphere of irrigated groundnut

The enumeration of total heterotrophic population from the rhizosphere of groundnut was carried out on nutrient agar medium as described by Malik *et al.* (1997).

2.4. Authentication of *Bradyrhizobium* isolates

2.4.1.1 Gram staining

Smears were prepared from 24 h old culture of the isolates, dried and heat fixed. The smears were flooded for 1 min with Hucker's ammonium oxalate crystal violet. Excess stain was poured off and the slide washed in a gentle stream water. Lugol's iodine solution was applied and allowed to remain for one minute. Eighty per cent acetone alcohol was added drop by drop until the violet colour ceased to flow. The smear was washed in gentle stream of water and counter stained with saffranin for 30 to 60 sec and washed, dried and observed under oil immersion lens.

2.4.1.2 Congo – red agar medium

A loopful of each isolate was streaked on this medium and the plates were incubated for a week. Rhizobial colonies stand out as white, translucent, glistening, elevated, small ones with entire margin without absorbing red colour in contrast to red stained colonies of *Agrobacterium* species which is an allied contaminant of *Rhizobium*.

2.4.1.2.1 Composition of Congo Red YEMA Agar Medium (Allen, 1953)

Mannitol - 10.0 g, Calcium carbonate - 3.0 g, Dipotassium hydrogen phosphate - 0.5 g, Magnesium sulphate - 0.2 g, Sodium chloride - 0.1g, Yeast extract - 0.5 g, Agar - 20.0 g, Distilled water - 1000ml, pH - 7.0. To prepare this medium, 2.5 ml of 1% congo red solution was sterilized, separately and then added to sterilize YEMA medium.

2.4.1.3 Hofer's alkaline medium

This medium is used to confirm whether the isolates are rhizobia or agrobacteria. Agrobacteria can withstand higher pH levels while rhizobia cannot. A loopful of the isolates can be streaked in this medium to find its growth.

2.4.1.3.1 Composition of Hofer's alkaline medium (Hofer, 1935)

Mannitol - 10.0 g, Calcium carbonate - 3.0 g, Dipotassium hydrogen phosphate - 0.5 g, Magnesium sulphate - 0.2 g, Sodium chloride - 0.1 g, Yeast extract - 0.5 g, Thymol blue 1.6% - 1.0 mL, Distilled water - 1000 mL, pH - 11.0. pH was adjusted to 11.0 by adding 28 mL of 1 N NaOH.

2.4.1.4 Glucose peptone agar

Agrobacterium readily utilize the glucose of this medium, grow and change its pH to yellow colour. On the other hand, Rhizobia grow poorly in this medium.

2.4.1.4.1 Composition of glucose peptone agar (Skinner and Lovelock 1979)

Glucose - 5.0 g, Peptone - 10.0 g, Bromo cresol purple - 10.0 mL (1% alcoholic solution), Agar - 15.0 g, Distilled water - 1000 mL, pH - 6.0

2.4.1.5 Keto lactose agar

This is prepared by replacing mannitol with lactose in YEMA medium. The rhizobial isolates were streaked on this medium and incubated. After incubation, the plates were flooded with Benedict's solution and yellow colour formation of colonies after one hour incubation indicates *Agrobacterium* contaminants.

2.4.1.5.1 Composition of Benedict's solution

Solution – A: Sodium citrate - 173.0 g, Anhydrous sodium carbonate - 100.0 g, Distilled water - 600.0 Ml

Solution –B: Copper sulphate - 17.3 g, Distilled water - 100.0 mL

Solution A and B were prepared separately. Solution B was added to solution A filtered and the resultant transparent blue solution stored.

2.5. *Bradyrhizobium* - Genus characterization

The genus *Bradyrhizobium* are Gram-negative, aerobic, non-spore-forming, motile rods of 0.46 to 0.53 × 1.30 to 1.97 µm in diameter. Colonies on YEMA medium is circular, convex and translucent, and have a diameter of 1 mm within 7 to 10 days incubation at 28 ºC. The generation time is 8.8 h in YEM broth. The pH range for growth is 6 to 8, with optimum growth at pH 7.0. Growth occurs between 20 and 30 ºC, with optimum growth at 28 ºC. *Bradyrhizobium* species are slow growing on bromothymol blue agar (Gachande and Khansole, 2011).

2.6 Determination of the dinitrogen fixing efficiency of *Bradyrhizobium* isolates under free living condition (*In vitro)*

2.6.1 Microkjeldahl assay (Bremner, 1960)

Hundred ml of the Yeast extract mannitol agar (YEMA) was taken into 250 mL Erlenmeyer flasks and sterilized by autoclaving. The flasks were separately inoculated with 1 mL of (1×10^7 CFU mL^{-1}) 48 h old cultures of *Bradyrhizobium, viz.,* BM-1 to BM-10, aseptically. The flasks were incubated at 30 ± 2 °C for one week under stationary condition. After the incubation period, 1 mL of the broth was transferred to 50 mL pyrex microkjeldhal flask. A quarter teaspoonful of the digestion mixture (10 g of reagent grade potassium sulphate, 1 g of cupric sulphate and 0.1 g of selenium metal powder) and 4 mL salicylic-sulphuric acid mixture (0.1 g of salicylic acid, 1.0 g of sodium thiosulphate and 30 mL of concentrated sulphuric acid) were introduced into it. The contents were slowly heated till frothing ceased and then heated strongly. Completion of the digestion was indicated by the solution turning into bluish green. After cooling, about 15 mL of distilled water was added to the flask, swirled and cooled. The contents were transferred into the distillation unit and 25 ml of 40 per cent sodium hydroxide was added. The ammonia was steam distilled for 15 min into an excess of 0.1 N sulphuric acid (10 mL) containing 2 drops of methyl red. The contents were back titrated with 0.1 N potassium hydroxide till the appearance of golden yellow colour. The nitrogen content of the sample was calculated using the factor, 1 mL of 0.1 N H_2SO_4 = 0.0014 g of N.

2.6.2 Grading *Bradyrhizobium* sp. on the basis of the dinitrogen fixing efficiency

All the six isolates of *Bradyrhizobium* were graded into three categories on the basis of their dinitrogen fixing efficiency determined by Microkjeldahl assay (Bremner, 1960). I category - > 15 mg 'N' fixed g^{-1} of mannitol, II category - 10 to 14.99 mg 'N' fixed g^{-1} of mannitol, III category - below 10.0 mg 'N' fixed g^{-1} of mannitol.

3. RESULTS AND DISCUSSION

3.1 survey, occurrence and population of *Bradyrhizobium*

Three major groundnut growing taluks of Cuddalore district in Tamil Nadu state, India, were surveyed for the occurrence of *Bradyrhizobium* in the rhizosphere region of plants (Feb 2019). Six different villages of three different taluk of Cuddalore district were selected in a random manner so that each sector of the experimental area would get a representation in the survey. Groundnut is grown continuously as monoculture in all the six locations of the surveyed area under irrigated condition. The details of locations and their respective taluk is given in table 1.

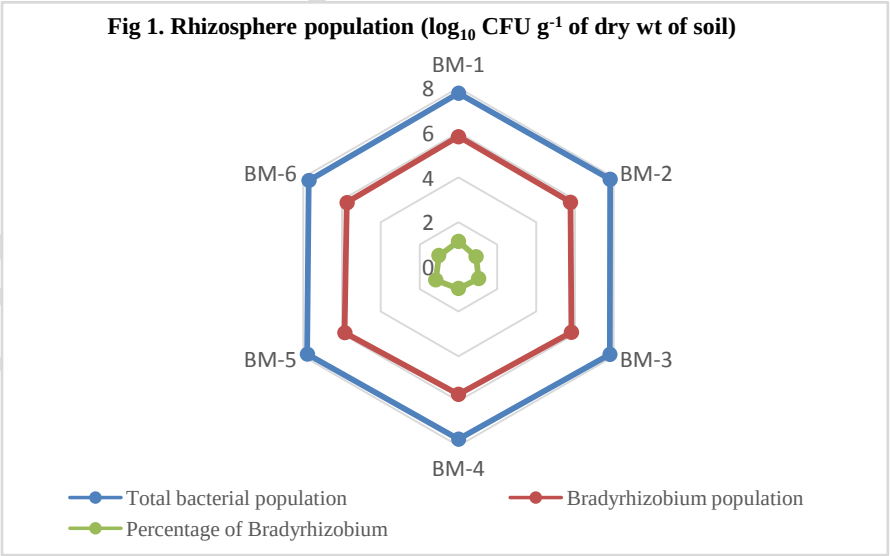
Table 1. Designation of *Bradyrhizobium* isolates, obtained from the rhizosphere of irrigated groundnut, grown at different location of Cuddalore district, Tamil Nadu

Name of Taluk	Location	Isolate designation
		Bradyrhizobium
Chidambaram	Vallampadugai Mutlur	*BM-1 BM-2
Cuddalore	Karamanikuppam Ramapuram	BM-3 BM-4
Panruti	Thiruvamur Vallam	BM-5 BM-6

*BM - *Bradyrhizobium*

A total number of six PGPR isolates, viz., *Bradyrhizobium*, were obtained from rhizosphere samples of groundnut, collected from different locations of Cuddlore district. The *Bradyrhizobium* isolates were designated as 'BM' series and numbered randomly. The high incidence of *Bradyrhizobium* in tropical soils may be attributed to the low level of available nitrogen or to higher temperature requirements of these organisms (Kaluza et al., 1985). The occurrence of Rhizobium in legume soils was first reported by Beijernck (1888). Itakura et al. (2002) suggested the occurrence of *Bradyrhizobium* population within the range of 0.001-1.0% of total bacterial population.

Fig 1. Rhizosphere population (log_{10} CFU g^{-1} of dry wt of soil)



A maximum number of *Bradyrhizobium* cells, 5.87 log_{10} CFU g-1 of dry soil was recorded from the rhizosphere samples, respectively, collected at Thiruvamur location of Cuddalore taluk (Fig 1). The lowest population of *Bradyrhizobium* (5.69 log_{10} CFU g-1 of dry

soil) was recorded with rhizosphere sample collected at Ramapuram location of Cuddalore district. The rhizosphere samples collected from all other location of Cuddalore taluk recorded the *Bradyrhizobium* population as intermediary to the above two levels. In general, rhizosphere samples collected from six different locations of Cuddalore district recorded a population of *Bradyrhizobium* in a range of 5.67 to 5.87 $\log_{10}$ CFU g-1 of dry soil is presented (Fig 2). The occurrence of *Bradyrhizobium* in different niches, such as, soils, roots, and rhizosphere of various crop plants including groundnut has been reported by many authors (Agah et al.,2013; Oscar yedeou Didagde et al., 2014 and Patil 2014). Oscar Yedeou Didagbe (2014) and Sadaf et al., (2016) reported the ubiquitous occurrence of Bradyrhizobium in groundnut rhizosphere. The size of the *Bradyrhizobium* population, expressed as per cent of total bacterial population of soil, has been reported as 1 to 10% by Kalaiarasi (2015). Valeria et al., (2015) and Sadaf et al., (2016) reported the occurrence of *Bradyrhizobium*, as PGPR, in the rhizosphere and nodules of groundnut. The total microbial population can also be estimated using latest high throughput analysis involving Omics sciences (Aravinthkumar et. al., 2022).

3.2 Authentication of the isolates

The isolated strains were authenticated as *Bradyrhizobium* by conducting several confirmative tests viz., infectivity test, gram staining, growth on congo red agar medium, growth on Hofer's alkaline medium, growth on yeast extract mannitol agar and growth on glucose peptone agar medium. The results are presented in the table 2. The isolated are white, translucent, mucoid colonies on congo red agar and YEMA. The results confirmed the six isolates as groundnut *Bradyrhizobium* sp. The isolates are gram negative, did not absorb colour in congo red agar and with no growth on glucose peptone agar and no yellow colouration in ketolactose agar. These organisms exert PGPR characteristics in the rhizosphere and promote the growth of groundnut plant (Gayathri Krishnaswamy 2017).

Isolates	Infectivity test	Gram staining	Growth on			
			Congo red agar	Hofer's alkaline medium	Glucose peptone agar	Keto-lactose agar
BM-1	*	-	NA	NG	NG	NC
BM-2	*	-	NA	NG	NG	NC
BM-3	*	-	NA	NG	NG	NC
BM-4	*	-	NA	NG	NG	NC
BM-5	*	-	NA	NG	NG	NC
BM-6	*	-	NA	NG	NG	NC

Table 2. Authentication of the isolates

* - Infection occurred
\- - Gram negative
NA - No adsorption of red colour
NG - No growth
NC - No yellow coloration

All the six isolates of *Bradyrhizobium*, designated in BM series, were subjected to genus characterization namely, cell size, cell shape and motility, responded positively for the above tests. The results are presented in Table 3 colony morphology, growth on BTB agar and gram reaction.

Character*	**Response of *Bradyrhizobium* sp.**
Gram's nature	Gram negative
Cell shape	Rod shaped
Spore formation	Non-spore forming
Oxygen demand	Aerobic
Motility	Motile
Shape of colony	Circular
Size of colony	3.1 mm dia
Opacity	Opaque
Elevation	Convex
Margin	Regular/Entire
Colour/pigmentation	Whitish pink and glistening
Colony on BTB agar	Forms blue colony due to alkaline reaction
Growth on BTB agar	Slow growing

Table 3. General characteristics of *Bradyrhizobium* species

* - According to Gachande and khansole (2011)

3.3. Nitrogen fixing efficiency of *Bradyrhizobium*

The dinitrogen fixing efficiency of *Bradyrhizobium* isolates, namely, BM-1 to BM-6 was assayed by Microkjeldahl assay under free living condition and the results presented in Table 4. It was found that all the *Bradyrhizobium* isolates fixed atmospheric dinitrogen under free living condition, but with variation in their dinitrogen fixing efficiency. The *Bradyrhizobium* strain, BM-5, isolated from the rhizosphere soil sample of Karamanikuppam recorded the lowest 'N' fixation, viz., 9.36 mg g1 of mannitol. On the other hand, the strain BM-5, isolated from the rhizosphere soil sample of Thiruvamur recorded the highest 'N' fixation, viz., 15.75 mg g-1 of mannitol under free living condition. The 'N' fixation recorded with the remaining isolates was intermediary to these two levels. In our laboratory, Srinivasan (2013) reported that the efficiency of Rhizobium to fix 'N' per g of mannitol was 0.9 to 12.5 mg. Hence, the development and deployment of these organisms, as an agricultural bioinoculant, will be the suitable biological approach for the maximization of growth and yield in groundnut plant (Kumar Naik et al.,2017 and Tounsi Hammami Soumaya 2016).

Table 4. Screening the *Bradyrhizobium* isolates for their dinitrogen fixing efficiency under *invitro* condition (Microkjedahl assay)

Isolate Number**	Nitrogen fixing efficiency * [a]
BM-1	15.21 ±0.41
BM-2	13.41 ± 0.14
BM-3	09.36 ± 0.17
BM-4	14.10 ±0.19
BM-5	15.75 ± 0.09
BM-6	09.55 ± 0.11

** - Inoculum level at 1 x 10^7 cfuml^{-1}

* - Amount of 'n' fixed by *Bradyrhizobium* (mg g^{-1} of mannitol)

a - Values are average of three replications ± SD

4. SUMMARY AND CONCLUSIONS

Groundnut (*Arachis hypogaea* L.) is the most important premier oil seed crop and India is the largest producer of groundnut where the same is generally grown under irrigated condition. However, the productivity of groundnut has been volatile under irrigation condition and the yield of the same is affected by a lot of biotic and abiotic factors, including, 'N' nutrition, temperature and moisture stress, pest and disease incidence. Nitrogen is the most essential nutrient required by both plants and microorganisms. 'N' are the key essential nutrients and expensive inputs that most frequently limit the groundnut production under irrigated condition. Nitrogen is one of the costly nutrient inputs to groundnut crop and often lost through denitrification, ammonia volatilization, leaching and run-off because of groundnut is grown in an environment conducive to 'N' losses. In addition, large denitrification process may represent an important source of nitrous oxide, a gas linked with greenhouse effect and the destruction of stratospheric ozone layer. Generally, large quantities of synthetic chemical fertilizers and pesticides are used to replenish the same, resulting in high productivity costs and severe biological and environmental hazards. Recently, a biological approach of using "Plant Growth Promoting Rhizobacteria (PGPR)" was attempted to reduce the drastic effects caused by the consistent use of synthetic chemical fertilizers and pesticides and to improve the crop productivity of groundnut under irrigation condition.

4.1 Occurrence of *Bradyrhizobium* in groundnut rhizosphere

In the present study, the occurrence of *Bradyrhizobium* in irrigated groundnut crop grown cuddalore district, Tamil Nadu state, India was surveyed in six selected locations. The *Bradyrhizobium* cultures were isolated from all the selected locations, their dinitrogen fixing and phosphate solubilizing efficiency of the same were studied and all the isolates were characterized. With selected *Bradyrhizobium* isolates, the IAA and EPS production efficiency, adsorption to groundnut roots were investigated. After determining the efficiency of the above-mentioned characters, one of the most efficient isolates from each genus, viz., *Bradyrhizobium* has been selected and used for the optimization of different factors that influencing the "intergeneric co-aggregation of PGPR cells" was studied. A pot culture study was also conducted to evaluate the effect of 'N' fixation cells on the enhancement of growth and yield parameters in groundnut crop under irrigated condition. In the present study, the community population of *Bradyrhizobium* in six selected locations of cuddalore district, Tamil Nadu, India was studied where groundnut is grown as mono crop and under irrigated condition. The results of the present study revealed the ubiquitous occurrence of *Bradyrhizobium* in irrigated groundnut soils of Cuddalore district, Tamil Nadu, but with variation in the community population level. The population of *Bradyrhizobium* was found to be more in groundnut rhizosphere. A range of 0.87 to 1.17 per cent of *Bradyrhizobium* to the total bacterial population was observed in the survey to the total bacterial population.

In the present study, one location, namely, Thiruvamur was found to have higher *Bradyrhizobium* population as above 1.17 per cent of the total bacterial population. In the remaining nine locations, the population was found to range from 0.85 to 1.14 per cent of the total bacterial population. In general, the occurrence of *Bradyrhizobium* in rhizosphere of groundnut was found to be less in Karamanikuppam taluk when compared to other isolates. The results of the present study revealed the predominance of *Bradyrhizobium* over in groundnut soils of Cuddalore district, Tamil Nadu, India. In the present study, the population of *Bradyrhizobium* observed in six selected locations ranged at 10^5 CFU g-1 of soil. The maximum *Bradyrhizobium* 5.87 $\log_{10}$ CFU g-1 of soil, respectively, recorded at Thiruvamur location of Cuddlore district, Tamil Nadu state, India.

4.2 Isolation and characterization of *Bradyrhizobium* from groundnut rhizosphere soil

In the present study, six cultures of *Bradyrhizobium* (BM-1 to BM-6) isolated from each of the six locations situated in Cuddalore district, Tamil Nadu state, India. The isolates were identified based upon colony characters and examination of individual cells under phase contrast microscope. All the six isolates (BM-1 to BM-6) exhibited the characters of *Bradyrhizobium* viz., circular, entire colonies, 3.1 mm in dia., whitish pink on solid yeast extract mannitol agar plates and gram negative, motile and non-spore former (Gachande and Khansole, 2011). The results of the present study also clearly revealed the occurrence of both *Bradyrhizobium* in the rhizosphere soils groundnut grown at Tamil Nadu state, India.

4.3 Nitrogen fixation by *Bradyrhizobium* isolates

All the six *Bradyrhizobium* isolates were evaluated for their dinitrogen fixing efficiency by Microkjeldahl method of Bremner (1960) under in vitro condition. Based on the amount of N fixed /g of mannitol, all the six isolates were made into 3 categories: 1) Above 15 mg 'N' fixed g-1 of mannitol, 2) 10 - 14.99 mg 'N' fixed g-1 of mannitol and 3) below 10 mg 'N' fixed g-1 of mannitol. In the present study, two isolates viz., BM-1 and BM-5 constituted the first category. Two isolates viz., BM-3 and BM-6 constituted second category and the remaining two isolates were ranked in the third category. The results of the present study revealed a maximum amount of atmospheric dinitrogen (15.75 mg 'N' fixed g-1 of mannitol) fixed by *Bradyrhizobium* isolate viz., BM-5, an isolate collected from Thiruvamur, Cuddlore of Tamil Nadu, aids in maximum 'N' fixation of 15.75 mg N per g of mannitol under *in vitro* assay conducted. The other isolates showed 'N' fixation in a range of 9.36 – 15.21 mg 'N' fixed g-1 of mannitol.

CONSENT

Not applicable for this research article.

ETHICAL APPROVAL

All the ethics of research and experiment have been taken into consideration and followed.

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