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In vitro Evaluation of Groundnut (*Arachis hypogea* L.) Rhizospheric *Bradyrhizobium* Traits for Dinitrogen Fixation

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Authors' contributions

This work was carried out in collaboration among all authors. Authors Aravinthkumar A, Aravindh A and AN performed all the formal and statistical analysis, wrote the protocol and first draft of the manuscript. Author SD designed the study, formulated idea and reviewed the draft of the manuscript. All authors read and approved the final manuscript.

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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: Six random locations in the Cuddalore district of Tamil Nadu (India) were selected and groundnut rhizospheric soil samples were collected during Feb 2019. Traits of *Bradyrhizobium*

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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: The highest count of *Bradyrhizobium* cells, reaching, 5.87 log_{10} CFU g-1 of dry soil was recorded from the samples of rhizosphere soil, collected at Thiruvamur location (Cuddalore taluk). Whereas the lowest population was recorded in samples collected from Ramapuram location of Cuddalore district. In terms of nitrogen fixation efficiency per gram of mannitol, the strains BM-1 and BM-5 fell into the first classification, while the second category included BM-3 and BM-6, followed by BM-2 and BM-4 to the third category based on their performance.

Conclusion: This study, clearly deciphers the Nitrogen fixing potential of *Bradyrhizobium* traits of groundnut crop and the species holds the promise of using them as the efficient nitrogen fixers in legume crops in future.

Keywords: Groundnut; rhizosphere; Bradyrhizobium; dinitrogen fixation.

1. INTRODUCTION

Peanut, scientifically known as *Arachis hypogaea* L., holds a significant position as a primary oilseed crop cultivated across tropical and subtropical regions globally. In this context, India stands out as the largest groundnut producer, with its cultivation predominantly occurring under irrigated conditions. This annual legume serves as a premier oilseed crop, boasting the capacity to yield substantial quantities of oil and easily digestible protein within its seeds. However, the crop's productivity has shown fluctuations within irrigated settings, influenced by a plethora of both living (biotic) and non-living (abiotic) factors. Among these factors, inadequate nitrogen (N) nutrition and the prevalence of diseases emerge as the foremost challenges, detrimentally impacting groundnut yield. These constraints stem from various sources, including pests, pathogens, and environmental stressors. Notably, the application of synthetic chemical fertilizers and pesticides has been a common but unsustainable practice, particularly due to its high costs and associated ecological risks. This approach is especially impractical for farmers lacking sufficient resources. Consequently, the need for a more balanced and sustainable approach to groundnut cultivation is evident. Addressing nutrient deficiencies, adopting disease-resistant varieties, and implementing environmentally friendly farming practices are critical steps towards enhancing groundnut productivity without compromising long-term viability and ecological health. In addition, Nitrogen stands out as the most crucial nutrient that both plants and microorganisms require. It plays a central role in the growth of groundnut crops, particularly under irrigated conditions. The element 'N' serves as a pivotal nutrient, but its availability often becomes limited and costly, leading to constraints in groundnut production. Unfortunately, nitrogen is susceptible to various forms of loss, including denitrification, ammonia volatilization, leaching, and run-off. Of concern is the significant denitrification process, which can contribute to the emission of nitrous oxide—a greenhouse gas associated with global warming and the depletion of the ozone layer. This process is a direct consequence of excess nitrogen in the soil. Addressing the challenges posed by nitrogen management is essential not only for optimizing groundnut production but also for mitigating environmental impacts. In current practices, substantial amounts of synthetic chemical fertilizers and pesticides are commonly applied to supplement nitrogen levels in the soil. However, this approach comes with substantial downsides, including elevated production costs and threats to biodiversity. As a result, finding sustainable and effective strategies to manage nitrogen inputs is crucial for maintaining productivity while minimizing environmental harm. The escalating impact of continuous synthetic chemical utilization necessitates innovative strategies to sustain groundnut cultivation. This study explores the potential of *Bradyrhizobium*, a prominent inhabitant of groundnut cultivation regions, as a biological approach to counteract the detrimental consequences of synthetic chemical usage. *Bradyrhizobium* intrinsic traits, particularly tailored for the groundnut ecosystem, hold promise for enhancing crop productivity under irrigation conditions. In this investigation, we delve into the specific advantages conferred by *Bradyrhizobium* and its profound impact on groundnut growth. By capitalizing on its trait specialization, we propose a novel avenue for augmenting plant growth. This holistic approach offers a viable solution to circumvent both biological and environmental hazards stemming

from intensive synthetic chemical reliance. Our study underscores the significance of *Bradyrhizobium-based* plant growth promotion and sheds light on a sustainable path forward for irrigated groundnut cultivation.

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

2. MATERIALS AND METHODS

2.1 Glass Ware

Glass items were immersed in a cleaning solution consisting of 100 g of potassium dichromate dissolved in 100 mL of distilled water. To this mixture, 500 mL of concentrated sulfuric acid was added. The glassware remained in this solution for a duration of 12 hours. Then the glass items were subjected to a brief boiling in soapy water and then rinsed with tap water and allowed to dry. For glassware, sterilization was achieved by placing them in a hot air oven set at 180°C for a period of 4 hours.

2.1.1 Chemicals

All the chemicals were of reagent-grade quality unless specified and the study utilized distilled water exclusively.

2.1.2 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.

2.1.3 Composition of media

The Yeast Extract Mannitol Agar Medium is formulated with the following components per liter of medium [1]: Mannitol (10.0 g), Calcium carbonate (3.0 g), Dipotassium hydrogen phosphate (0.5 g), Magnesium sulfate (0.2 g), Sodium chloride (0.1 g), Yeast extract (0.5 g), Agar (20.0 g), and Distilled water (1000 mL). The pH of the medium is adjusted to 7.0.

2.2 Survey for *Bradyrhizobium* Occurrence from the Rhizosphere of Irrigated Groundnut in Cuddalore District, Tamil Nadu, India

2.2.1 Survey for *Bradyrhizobium* occurrence from groundnut rhizosphere

A survey was undertaken in the Cuddalore district of Tamil Nadu, India, encompassing six villages. These villages are known for cultivating groundnut as a major oil crop, primarily under irrigated conditions. The selection of survey locations followed a random approach, ensuring that every sector within the experimental area was adequately represented in the study.

2.2.2 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.

2.2.3 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.

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* (BM)**
Chidambaram	Vallampadugai	BM-1
	Mutlur	BM-2
Cuddalore	Karamanikuppam	BM-3
	Ramapuram	BM-4
Panruti	Thiruvamur	BM-5
	Vallam	BM-6

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 soil adhering to the roots of groundnut plants was gathered from a specific location is combined to form five individual samples. A portion of this soil is weighed to 10 grams, was placed into a 250 ml Erlenmeyer flask containing 100 ml of sterile distilled water. The mixture was then placed on a rotary shaker at 100 rpm and left to incubate at room temperature for 30 minutes. This process ensured proper suspension of the soil in the water.

Subsequently, the well-mixed suspension from each soil sample underwent a series of dilutions, spanning from 10^-1 to 10^-9. A volume of 1 ml from each dilution was carefully transferred to separate Petri plates. These plates were poured with molten YEMA medium. And the plates were gently rotated in both clockwise and anticlockwise directions to ensure uniform distribution. Following this, the Petri plates were placed in an incubator set at 37°C and the colonies of *Bradyhizobium* bacteria developed on each Petri plate were enumerated using an Arnold colony counter. To ensure accuracy, the sample were triplicated.

2.3.2 Enumeration of total heterotrophic population from the groundnut rhizosphere sample

The total heterotrophic population of the soil sample obtained from the groundnut rhizosphere was enumerated on nutrient agar medium [2].

2.4 Authentication of *Bradyrhizobium* Isolates

2.4.1 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 specimen on the slide was rinsed with a gentle stream of water, followed by counterstaining with saffranin for a duration of 30 to 60 seconds. Afterward, it was washed, allowed to dry, and then observed using an oil immersion lens.

2.4.2 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*.

2.4.3 Composition of Congo Red YEMA Agar Medium [1]

The composition of the medium consists of the following components: Mannitol (10.0 g), Calcium carbonate (3.0 g), Dipotassium hydrogen phosphate (0.5 g), Magnesium sulfate (0.2 g), Sodium chloride (0.1 g), Yeast extract (0.5 g), Agar (20.0 g), and Distilled water (1000 ml). The pH of the medium is adjusted to 7.0. To prepare the medium, a separate sterilization of 2.5 ml of a 1% solution of congo red is conducted. This sterilized congo red solution is then added to the sterilized YEMA medium.

2.4.4 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.

2.4.5 Composition of Hofer's alkaline medium [3]

The composition of the solution includes 10.0 grams of Mannitol, 3.0 grams of Calcium Carbonate, 0.5 grams of Dipotassium Hydrogen Phosphate, 0.2 grams of Magnesium Sulphate, 0.1 grams of Sodium Chloride, 0.5 grams of Yeast Extract, and 1.0 mL of Thymol Blue 1.6%. These ingredients are dissolved in 1000 mL of Distilled Water. To achieve a pH of 11.0, 28 mL of 1 N Sodium Hydroxide (NaOH) was added and adjusted.

2.4.6 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.

2.4.7 Composition of glucose peptone agar [4]

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.

2.4.8 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.

2.4.9 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. These species are slow growing on bromothymol blue agar [5].

2.6 Determination of the Dinitrogen Fixing Efficiency of *Bradyrhizobium* Isolates under Free Living Condition (*In vitro)*

2.6.1 Microkjeldahl assay [6]

A volume of 100 ml of Yeast Extract Mannitol Agar (YEMA) was placed into 250 mL Erlenmeyer flasks and then subjected to sterilization through autoclaving. Each flask was individually inoculated with 1 mL of a 48-hour-old culture of *Bradyrhizobium*, specifically strains BM-1 to BM-10, maintaining aseptic conditions. These flasks were then incubated at a temperature of 30 ± 2°C for a period of one week, with no agitation. Following the incubation period, 1 mL of the culture broth was transferred to a Pyrex micro-Kjeldahl flask with a capacity of 50 mL. To this flask, a quarter teaspoon of a digestion mixture consisting of 10 g of high-purity potassium sulfate, 1 g of cupric sulfate, and 0.1 g of selenium metal powder was added. Additionally, 4 mL of a salicylic-sulfuric acid mixture, composed of 0.1 g of salicylic acid, 1.0 g of sodium thiosulfate, and 30 mL of concentrated sulfuric acid, was introduced into the flask. The contents of the flask were gradually heated until frothing subsided, and then vigorous heating was applied. The completion of digestion was indicated by the transformation of the solution color to bluish-green. Once cooled, around 15 mL of distilled water was incorporated into the flask, mixed gently, and allowed to cool further. Subsequently, the flask's contents were transferred to a distillation apparatus, and 25 ml of a 40% sodium hydroxide solution was added. Ammonia was then subjected to steam distillation for a duration of 15 minutes, with the distillate being collected in an excess of 0.1 N sulfuric acid solution (10 mL) containing 2 drops of methyl red as an indicator. The collected distillate was back-titrated using 0.1 N potassium hydroxide until a distinct golden-yellow color emerged. The nitrogen content of the original sample was determined using the conversion factor, where 1 mL of 0.1 N H_2SO_4 corresponds to 0.0014 g of nitrogen.

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 [6]. 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*

A survey was conducted in February 2019 in the Cuddalore district of Tamil Nadu, India, focusing

on the presence of *Bradyrhizobium* in the rhizosphere of groundnut plants. The survey targeted three primary groundnut cultivating taluks in the district. To ensure comprehensive coverage, a random selection process was employed to choose six villages from these three taluks. This approach aimed to include representation from every sector of the experimental area. The cultivation of groundnut in the surveyed region followed a continuous monoculture practice and was carried out under irrigated conditions. The locations and corresponding taluks are detailed in Table 1.

Six PGPR isolates were derived from rhizosphere samples of groundnut taken from various locations in the Cuddlore district. These isolates belonged to the *Bradyrhizobium* group and were labeled as the 'BM' series, with random numbering. The prevalence of these *Bradyrhizobium* isolates in tropical soils could be linked to either the limited availability of nitrogen or the organisms' preference for higher temperatures [7]. The existence of Rhizobium in legume soils was initially documented by Deshwal et al. [8]. Idriss et al. [9] proposed that their population makes up about 0.001-1.0% of the overall bacterial population range.

The Thiruvamur location in Cuddalore taluk exhibited the highest recorded population of *Bradyrhizobium* cells, with 5.87 log10 CFU g-1 of dry soil in the collected rhizosphere samples. On the other hand, the lowest *Bradyrhizobium* population (5.69 log10 CFU g-1 of dry soil) was found in rhizosphere samples from the Ramapuram location in the Cuddalore district. The rhizosphere samples from the remaining locations within Cuddalore taluk displayed intermediate *Bradyrhizobium* population levels compared to the aforementioned extremes. In a broader *overview*, the *Bradyrhizobium* populations in rhizosphere samples from six distinct locations across Cuddalore district ranged from 5.67 to 5.87 log10 CFU g-1 of dry soil, as depicted in Fig. 1. Numerous researchers, including Agah et al. [10] and Didagde et al. [11] have documented the presence of *Bradyrhizobium* in various niches like soils, roots, and the rhizosphere of diverse crops, including groundnut. Groundnut rhizosphere's ubiquity as a habitat for *Bradyrhizobium* has been reported by Didagbe [11] and Sadaf et al. [12]. The relative size of this population, as a percentage of the total soil bacterial population, has been quantified as 1 to 10% according to Kalaiarasi and Dinakar [13]. Additionally, Silva et al. [14] and Sadaf et al. [12] have affirmed the presence of *Bradyrhizobium* as a plant growth-promoting rhizobacterium (PGPR) in both groundnut rhizosphere and nodules. Modern high-throughput techniques encompassing Omics sciences have also enabled the estimation of overall microbial populations, as noted by Aravinthkumar et al. [15].

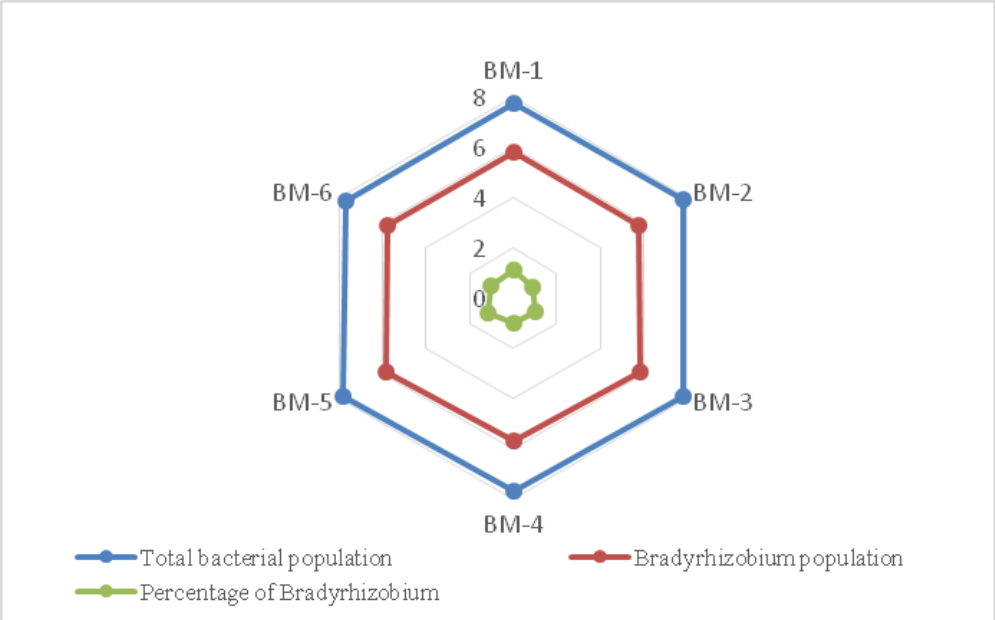

Fig. 1. Rhizosphere population (log_{10} CFU g^{-1} of dry wt of soil)

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 [16].

The six *Bradyrhizobium* isolates, identified under the BM series nomenclature, underwent a comprehensive genus characterization, encompassing attributes such as cell size, cell shape, and motility. Notably, all isolates exhibited affirmative responses in these evaluations. The outcomes have been tabulated in Table 3, encompassing details regarding colony morphology, growth patterns on BTB agar, and gram reactions.

3.3 Nitrogen Fixing Efficiency of *Bradyrhizobium*

The efficiency of dinitrogen fixation was assessed for various *Bradyrhizobium* isolates, designated as BM-1 to BM-6, through the Microkjeldahl assay under conditions of independent growth. The results, presented in Table 4, indicated that all these *Bradyrhizobium* isolates were capable of fixing atmospheric dinitrogen while existing in a free-living state. However, there were notable variations in their respective abilities to fix dinitrogen efficiently. Among the isolates, BM-5 strain, obtained from the rhizosphere soil sample of Karamanikuppam, exhibited the lowest recorded nitrogen (N) fixation rate at 9.36 mg per gram of mannitol.

Table 2. Authentication of the isolates

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

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

Table 3. General characteristics of *Bradyrhizobium* species

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

** - According to Gachande and khansole [5]*

Table 4. Screening the *Bradyrhizobium* isolates for their dinitrogen fixing efficiency under *in vitro* 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

Conversely, the BM-5 strain originating from the rhizosphere soil sample of Thiruvamur demonstrated the highest nitrogen fixation efficiency, registering a rate of 15.75 mg per gram of mannitol under free-living conditions. The remaining isolates displayed levels of nitrogen fixation that fell between these two extremes. It is pertinent to note that previous work conducted by Srinivasan in our laboratory in 2013 documented the nitrogen fixation efficiency of Rhizobium to range between 0.9 and 12.5 mg per gram of mannitol. Consequently, leveraging these organisms for agricultural bioinoculation holds promise as a viable biological strategy to enhance growth and yield in groundnut plants, as highlighted by Kumar et al. [17] and Soumaya et al. [18].

4. SUMMARY AND CONCLUSIONS

4.1 Occurrence of *Bradyrhizobium* in Groundnut Rhizosphere

A survey was conducted in the Cuddalore district of Tamil Nadu, India, across six locations, to assess the presence of *Bradyrhizobium* in irrigated groundnut crops. The study involved isolating *Bradyrhizobium* cultures *from* all chosen sites and investigating their abilities in fixing dinitrogen and solubilizing phosphate. Detailed characterization of these isolates was also carried out. This research aimed to explore the population dynamics of *Bradyrhizobium* in the context of groundnut cultivation, specifically in areas with irrigation. The six chosen locations encompassed both monocrop groundnut fields and those under irrigation. The outcomes of the study highlighted that *Bradyrhizobium* was prevalent throughout the study area, but its distribution in terms of population varied considerably. Notably, the population was more pronounced in the rhizosphere of the groundnut plants. Quantitatively, the population of *Bradyrhizobium* ranged between 0.77% and 1.17% of the total bacterial community within the given population. Remarkably, the Thiruvamur block exhibited a notably higher population of *Bradyrhizobium*, surpassing 1.17% of the total bacterial community. Across the other locations, the *Bradyrhizobium* population fell within the range of 0.85% to 1.14% of the total bacterial community. Notably, the presence of *Bradyrhizobium* in the rhizosphere of groundnut plants was found to be comparatively lower in the Karamanikuppam taluk in contrast to the other isolates. In summary, this study conducted in the Cuddalore district, Tamil Nadu, shed light on the distribution of *Bradyrhizobium* across irrigated groundnut crops. The research demonstrated that while *Bradyrhizobium* was present widely, the population density varied between different locations. The most significant populations were identified in the Thiruvamur block, while the Karamanikuppam taluk exhibited relatively lower levels of *Bradyrhizobium* presence compared to other areas. Their predominance occurrence over in groundnut soils of Cuddalore district is also confirmed based on their population. the population of *Bradyrhizobium* observed in six selected locations ranged at 10^5 CFU g-1 of soil. The maximum population (5.87 $\log_{10}$ CFU g-1 of soil) was recorded at Thiruvamur location.

4.2 Isolation and Characterization of *Bradyrhizobium* from Groundnut rhizosphere Soil

Six distinct cultures of Bradyrhizobium (designated as BM-1 to BM-6) were obtained from separate locations within Cuddalore district, Tamil Nadu state, India. The method of identification encompassed an analysis of colony characteristics along with the scrutiny of individual cells utilizing a phase contrast microscope. Conspicuously, all six isolates (BM-1 to BM-6) exhibited hallmark traits of Bradyrhizobium. These traits encompassed

circular and intact colony formations, measuring 3.1 mm in diameter, displaying a whitish-pink hue on solid yeast extract mannitol agar plates. Moreover, under the classification of Gram staining, these isolates were determined to be Gram-negative, displaying motility and lacking spore-forming capabilities. This study conclusively illuminated the prevalence of these isolates within the rhizospheric soils of groundnut crops cultivated in the Tamil Nadu state of India [5].

4.3 Nitrogen Fixation by *Bradyrhizobium* Isolates

The dinitrogen-fixing efficiency of six *Bradyrhizobium* isolates was assessed using the Microkjeldahl method developed by Bremner in 1960. This evaluation took place under controlled *in vitro* conditions. The isolates were categorized based on the amount of nitrogen (N) they fixed per gram of mannitol, yielding three distinct groups: Above 15 mg 'N' Fixed per g of Mannitol: Among the six isolates, two isolates, namely BM-1 and BM-5, fell into this category. 10 - 14.99 mg 'N' Fixed per g of Mannitol: Two other isolates, BM-3 and BM-6, were placed within this range, constituting the second category. Below 10 mg 'N' Fixed per g of Mannitol: The remaining two isolates were grouped in the third category due to their lower nitrogen-fixing efficiency. The study's findings showcased that the *Bradyrhizobium* isolate BM-5, obtained from Thiruvamur, exhibited the highest atmospheric dinitrogen fixation, measuring at 15.75 mg 'N' fixed per g of mannitol. This result suggests that BM-5 has the capacity for significant nitrogen fixation when evaluated under in vitro conditions.

CONFERENCE DISCLAIMER

Some part of this manuscript was previously presented in the conference: 6th International Conference on Strategies and Challenges in Agricultural and Life Science for Food Security and Sustainable Environment (SCALFE-2023) on April 28-30, 2023 in Himachal Pradesh University, Summer Hill, Shimla, HP, India. Web Link of the proceeding: https://www.shobhituniversity.ac.in/pdf/Souvenir-Abstract%20Book-Shimla-HPU-SCALFE-2023.pdf

ETHICAL APPROVAL

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

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COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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