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Studies on *Rhizobium* from rhizosphere soil and its used as a Bio-fertilizer in *Cicer arietinum* (L)

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Abstract
Nutrient deficiency in the soil poses a big challenge to food production globally. The use of artificial nitrogen fertilizer to aid crop yield is a common farming practice, despite its undesirable effects and hazard to the environment and human population. This research work aimed at isolation, identification & characterization of *Rhizobium* species, chickpea rhizospheric soil samples collected from Samalpatti, Jogipatti and Valipatti Krishnagiri- Dist, Tamilnadu, India. Isolation of *Rhizobium* species was culture on Yeast Extract Mannitol Agar (YEMA) medium incubated 3 days at 320C. A total of 10 *Rhizobium* species isolates were isolated from rhizospheric soil samples. They are also found to be gram-negative, rod-shaped morphology, fast grower, indole producers and positive for catalase test. All isolates were found with bare absorption of Congo red dye & no growth on YEMA with 2% NaCl. 10 isolates were identified as *Rhizobium* species on the basis of the authentication test (nodulation check with (*Cicer arietinum*). The plant treated with higher concentration of Rhizobial culture at 5 days of time interval improved the plant growth than the other treatments. These three rhizobial isolates may be useful to increase the symbiotic biological nitrogen fixation in legume plant chickpea (*Cicer arietinum*) and can be used as potential biofertilizer owing to their plant growth-promoting characters.

Keywords: *Cicer arietinum***,** biofertilizer, *Rhizobium*, VBRS, rhizosphere

Introduction

Nitrogen the essential component which serves as the building blocks of proteins and nucleic acids is abundant in the earth's atmosphere but it cannot be utilized by plants because of the inert nature of the nature due to the presence of triple bonds between the nitrogen atoms. So for the nitrogen to be used by plants it must be fixed or converted to the form of ammonium or nitrite ions (Deshwal *et al*., 2013) [4]. There are certain microorganisms which are capable of converting the atmospheric nitrogen into ammonia or nitrite ions by a process known as nitrogen fixation (Roychowdhury *et al*., 2013) [12]. These nitrogen fixation can be done by certain soil microbes both symbiotically and as well as non-symbiotically. So among the symbiotic nitrogen fixing bacteria *Rhizobium* has been found to be having a greatest activity in fixing these atmospheric nitrogen for plants and also increasing the soil fertility (Rajeswari *et al*., 2017*:* Brockwell *et al*., 1995) [11, 2]. In this way the *Rhizobium* bacterial strains can be utilized for production of biofertilizers (Talukder *et al*., 2008*:* Atkins, 2004) [15, 1]. Biofertilizers are actually the natural mini- fertilizers which are responsible for providing safer plant nutrition and increasing the soil fertility through natural processes. These microorganisms actually can colonize the roots of different plants and the *Rhizobium* can form root nodules in leguminous plants (Zahrul islam *et al*, 2012) [16]. These rhizobial inoculants after forming the root nodules fixes the atmospheric nitrogen to ammonia which can be utilized by plants. Biofertilizers are the preparations of living cells of different strains of microorganisms that helps in enhancing the nutrient quality of soil by their interaction with the rhizosphere roots of plants when they are applied both on top soil and seed treatment (Siddiqui, 2006) [13]. Isolation of azotobacter and cost effective production of biofertilizer by Gauri Ashok kumar singh *et al*., (2011) [5]. Rhizobia bacteria that fix N2 (diazotroph) after becoming established inside root nodules of legumes (Fabaceae). The different genera of rhizobia, all of them belong to the Rhizobial, a probably monophyletic group of proteobacteria and they are soil bacteria characterized by their unique ability to infect root hairs of legumes and induce effective N2-fixing nodules to form on the roots (Hubbell & Kidder, 2003) [6]. They are rod shaped living plants which exist only in the vegetative stage. Unlike many other soil microorganisms, rhizobia produce no spores and they are aerobic and motile (Matiru and Dakora, 2004) [9].

The Rhizobial inoculants colonizes the roots of specific legumes to form tumor like growths called root nodules, which acts as factories of ammonia production. *Rhizobium* has ability to fix atmospheric nitrogen in symbiotic association with legumes and certain nonlegumes like Parasponia. Artificial seed inoculation is often needed to restore the population of effective strains of the *Rhizobium* near the rhizosphere to hasten N-fixation. Each legume requires a specific species of *Rhizobium* to form effective nodules (Mishra *et al*, 2012) [10]. In this research paper the main focus is in the isolation of Bacteria (*Rhizobium*) from chick pea and then its characterization followed by the identification through different biochemical tests for the identification, its pure culture growth in selective and optimized media and then the mass production of the bacterial strain to be utilized as a biofertilizer.

Materials and Methods
Sample collection
Rhizospheric soil samples were collected from root zone of chickpea field at Samalpatti, Jogipatti and Valipatti Krishnagiri- Dist, Tamilnadu, South India. Samples were kept in clean sterile bottles sealed and transferred to the Research Department of Botany, Sri Moogambigai Arts and Science College (Women), Mallupatti, Palacode, Tamilnadu, India. stored at 4$0^{\circ}$C.

Preparation of sample
Sample kept at room temperature before analysis. There are different ways to carry out physical fermentation process: Ten grams Rhizospheric soil samples were absorb in 90 ml of normal saline solution (8.5 g NaCl/L), homogenized for 20 min, appropriately mixed in normal saline. From every sample, serial dilutions were formed by following the method of Islam (Islam *et al*., 2020).

Isolation of nitrogen fixing bacteria (*Rhizobium*)
The soil samples were suspended in water by forceful vortexing and serial dilutions were formed up to 10-6 in barren distilled water. After appropriate dilution were added to petriplate on YEM Agar plate with the right calibration of pH (6.8-7) and cover for 72 hrs at 320C. Bacterial culture was repeated for three times by single colony streaking on YEMA medium. The cultures were subsequently sub-cultured and used regularly.

Morphological and biochemical characterization gram staining
The colony feature (i.e. form, guise, colour, mound, margin of the bacterial colony and their growth rate) were destined by observing the colonies on YEMA plates of the overnight grown microorganisms at 32°C. Microscopic watching of the isolates was done using Gram staining fetch.

Catalase activity
Isolates of 48 hours old culture were flooded with hydrogen peroxide to look on the release of
bubbles of oxygen around the bacterial colonies.

Indole-3-acetic acid test
YEMA Culture media add tryptophan (0.1%) then all isolates inoculated with this media. Then it kept Shaking incubator (32ºC, 100 rpm) for 48 hours.

Characterization and plant nodulation check test growth on 2% NaCl
To the basal medium of YEMA, 2% NaCl was added to check the growth of isolates. As 2% NaCl is inhibitory for some rhizobial isolates it may can be able to serve as tools for identification of isolates.

Congo red test
The integrity of the rhizobial isolates was discovering by adding Congo red in YEMA media. Most rhizobia bury the dye only poorly whereas contaminants including Agrobacteria, will absorb highly.

Plant nodulation check test
The different isolates were tested for their capability to nodulate *Cicer arietinum* plants grown in mud pots. Seeds of *Cicer arietinum* were inoculated with 10 *Rhizobium* isolates by soaking seeds. Plants were gingerly uprooted after 15 days, 26 days and 40 days respectively and observed for nodulation.

Inoculum preparation
Rhizobium inoculant was prepared by using broth culture. Yeast mannitol broth was prepared on Erlenmeyer flask and was sterilizes at 1210C for 15 minutes. Then the liquid medium was kept for cooling. After cooling down, a small amount of *Rhizobium* species was transferred aseptically from the agar medium to liquid medium with the aid of a sterile inoculating needle. The flask containing broth and isolates was then placed on the shaker at 28^{0}C under 120 rpm for three days to accelerate the growth of *Rhizobium*. After three days, growth was observed on the flask and it was taken out from the shaker for inoculation process.

Seed preparation
Before placing, seeds were washed with tap water then it sterilized by 98% alcohol for 5 minutes afterward washed up with distilled water for 5 minutes and again with sterile distilled water for 2 minutes. Seeds were kept at laminar air flow for few minutes to become dry.

Inoculation process
Broth culture poured onto the few amount of three times autoclave sterile soils and mixed with hands by using sterile hand gloves. Sterile soils mixed with *Rhizobium* isolates were placed on one pot whereas another pot contained only sterile soil but no *Rhizobium* isolates. Seeds were then placed in the pots by maintaining a certain amount of distance between them. Every pot contained four seeds and was kept in a place full of sun light for proper growth.

Plant growth experiment on enhancement by *Rhizobium* bacteria
At that time, Leonard container experiment used the selected rhizobia culture for the plant development study (Sunil 2013) [14] The *Cicer arietinum* plant was selected for the plant development study because of its accessibility and healthy substance. was in different time periods (T0-0, T1-5ml, T2-5+5ml, T3-5+5+5ml, T4-5+5+5+5ml) at various time stretches (T0-0th day, T1-1st day& 6th day, T2 -1st, 6th, 11th &16th). The progress of the plant development was determined by the shoot - and root length, the number of framed humps and the evaluation of the chlorophyll content are determined. After 21 days of plant development, the results were noted.

Results and Discussion
In this Research paper Figure 1 shows the root nodules from the chick pea plants and Fifure2 shows the extraction of root nodules in the Phosphate buffer medium. All the isolates morphological characteristics and biochemical test result are given in (Table 1). *Rhizobium* isolate (Vimala Botany Research Scholar-*VBRS*-2) on YEMA media showed in (Figure 3). Microscopic observation of *Rhizobium* isolate (*VBRS*-5) showed in (Figure 2). Cultural growth on different media and nodulation check result of *Rhizobium* species isolates are given in (Table 2).Observation of nodulation check of *Rhizobium* isolate (*VBRS*-7) with *Cicer arietinum* plant after 26 days showed in (Figure 1). The plant treated with 20 ml (Different time interval) of *Rhizobium* culture (T2) showed better result (25th day). The VBRS8 strain

treated plants showed maximum shoot length is 39.8+0.020cm, the root length is 20.5+0.013 cm and the plant formed 23.2+0.013 nodules in its root. T2 plants have higher chlorophyll content compare to control and other tested plant. Total chlorophyll value is maximum in VBRS8 strain (T2=0.197+0.002). The Treatment-2 showed the better plant growth than other treatment (T0 and T1) and control.

Table 1: Morphological identification and Biochemical test

Name of the Isolates	Shape	Gram stain	Colour	Catalase test	Methyl Red	Vp test	Catalase test	Starch Hydrolysis	IAA test
VBRS-1	Small rod	Negative	White, Translucent	Positive	Positive	Negative	Positive	Negative	Negative
VBRS-2	Small rod	Negative	White,Translucent	Negative	Positive	Negative	Positive	Negative	Positive
VBRS-3	Small rod	Negative	White Translucent	Positive	Positive	Negative	Positive	Negative	Positive
VBRS-4	Medium rod	Negative	White,Translucent, Gummy	Positive	Positive	Negative	Positive	Negative	Negative
VBRS-5	Small rod	Negative	White, Translucent	Positive	Positive	Negative	Positive	Negative	Positive
VBRS-6	Small rod	Negative	White,Translucent	Negative	Positive	Negative	Positive	Negative	Positive
VBRS-7	Medium rod	Negative	White Translucent	Positive	Positive	Negative	Positive	Negative	Positive
VBRS-8	Medium rod	Negative	White,Translucent, Gummy	Positive	Positive	Negative	Positive	Negative	Positive
VBRS-9	Small rod	Negative	White, ranslucent	Positive	Positive	Negative	Positive	Negative	Positive
VBRS-10	Medium rod	Negative	White,Translucent Gummy	Positive	Positive	Negative	Positive	Negative	Positive

Table 2: Biochemical test

Name of the Isolates	Congo red dye	YEMA with 2% NaCl	Nodulation check with *Cicer arietinum* plant
VBRS-1	Positive	Absent growth	Absent
VBRS-2	Positive	Absent growth	Absent
VBRS-3	Positive	Absent growth	Nodule present
VBRS-4	Positive	Absent growth	Absent
VBRS-5	Positive	Absent growth	Absent
VBRS-6	Positive	Absent growth	Absent
VBRS-7	Positive	Absent growth	Nodule present
VBRS-8	Positive	Absent growth	Absent
VBRS-9	Positive	Absent growth	Nodule present
VBRS-10	Positive	Absent growth	Absent

Table 3: The plant growth enhancement experiment using by VBRS strains

S. No	Sample	Shoot length	Root length	No. of Root nodules	Chlorophyll a (mg/ml)	Chlorophyll b (mg/ml)	Total Chlorophyll (mg/ml)
VBRS-1	T0	13.1+0.012	3+0.010	2+0.013	0.015+0.010	0.025+0.03	0.081+0.021
	T1	20.3+0.011	4.5+0.021	10+0.012	0.02+0.010	0.032+0.03	0.096+0.012
	T2	23.2+0.014	6+0.011	18+0.012	0.022+0.010	0.037+0.03	0.102+0.011
VBRS-2	T0	14.5+0.013	3.5+0.013	5+0.013	0.013+0.010	0.022+0.03	0.071+0.011
	T1	17.7+0.011	7.2+0.013	10+0.011	0.014+0.010	0.031+0.03	0.098+0.015
	T2	26.3+0.012	13+0.023	13+0.013	0.017+0.010	0.035+0.03	0.101+0.017
VBRS-3	T0	10.2+0.013	4.5+0.03	3.5+0.012	0.011+0.010	0.015+0.03	0.067+0.009
	T1	14.1+0.021	6+0.015	11.0+0.013	0.023+0.010	0.022+0.03	0.086+0.012
	T2	17.4+0.017	10.5+0.012	11.8+0.013	0.035+0.010	0.027+0.03	0.993+0.014
VBRS-4	T0	12.4+0.015	5.1+0.013	1.5+0.011	0.014+0.010	0.014+0.03	0.061+0.021
	T1	19.6+0.020	9.8+0.011	5+0.021	0.019+0.010	0.021+0.03	0.083+0.009
	T2	22.5+0.018	14.5+0.013	10+0.013	0.026+0.010	0.027+0.03	0.971+0.003
VBRS-5	T0	14.3+0.016	6+0.012	1.8+0.013	0.009+0.010	0.019+0.03	0.065+0.012
	T1	21.3+0.012	10.5+0.013	5+0.013	0.016+0.010	0.027+0.03	0.078+0.018
	T2	26.5+0.018	12.2+0.013	10+0.01	0.029+0.010	0.033+0.03	0.932+0.018
VBRS-6	T0	13.6+0.003	5.3+0.013	1.0+0.012	0.007+0.010	0.015+0.03	0.062+0.021
	T1	17.5+0.038	8.5+0.011	5+0.011	0.012+0.010	0.026+0.03	0.069+0.009
	T2	25.2+0.031	16.8+0.012	10+0.013	0.022+0.010	0.029+0.03	0.943+0.016
VBRS-7	T0	12.7+0.021	6.5+0.011	1.5+0.013	0.006+0.010	0.012+0.03	0.053+0.032
	T1	19.5+0.016	9.2+0.013	4.5+0.011	0.013+0.010	0.020+0.03	0.067+0.012
	T2	24.2+0.023	13.9+0.013	10+0.013	0.027+0.010	0.024+0.03	0.834+0.023
VBRS-8	T0	15.3+0.015	7.5+0.013	5.5+0.012	0.021+0.010	0.027+0.03	0.081+0.012
	T1	22.1+0.023	12.7+0.013	8.7+0.012	0.039+0.010	0.038+0.03	0.108+0.015
	T2	39.8+0.020	20.5+0.013	23.2+0.013	0.047+0.010	0.045+0.03	0.197+0.002
VBRS-9	T0	12.5+0.013	3.9+0.010	1.8+0.012	0.007+0.010	0.0170.03	0.056+0.032
	T1	19.7+0.022	5.5+0.021	3.5+0.011	0.009+0.010	0.023+0.03	0.059+0.012
	T2	26.8+0.014	11+0.011	9.0+0.011	0.013+0.010	0.029+0.03	0.886+0.023
VBRS-10	T0	13.8+0.031	4.1+0.010	1.3+0.012	0.005+0.010	0.022+0.03	0.059+0.031

	T1	19.5+0.018	8.5+0.021	5.8+0.012	0.012+0.010	0.029+0.03	0.068+0.011
	T2	24.7+0.015	13.9+0.011	10.5+0.011	0.019+0.010	0.034+0.03	0.946+0.013

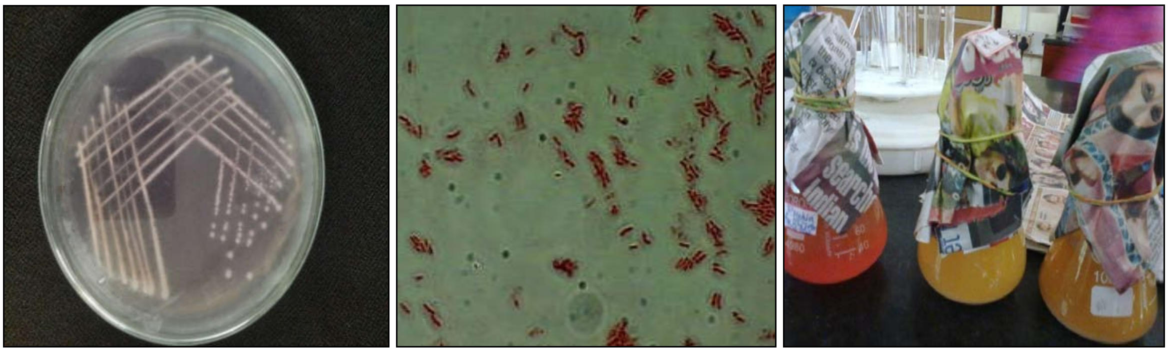

Fig 1: Images on VBRS strains and Mass culture.

Plant growth promoting Rhizobacteria (PGPR) are soil resident that are capable to colonize plant roots, incite plant growth, and augment crop yields (Kasa *et al*., 2020). *Rhizobium* is an significant microorganism for the environment reason of its nitrogen-fixing capability when in symbiotic relevance with plants (mainly legumes). This lesson confirmed that the root nodules of chickpea plants asylum the nitrogen-fixing bacterium- *Rhizobium* (Zeenat *et al*., 2017) [17]. The degree of specificity between leguminous plants and rhizobia is highly variable (Debojyoti *et al*., 2015) [3]. It also proved that these plants (*Cicer arietinum*) when inoculated with *Rhizobium* isolates create nodule after 26 days that are authentically identified that isolates are *rhizobia* species. After 50 days when nodule section that are showed red color that are conformed active rhizobia species. The potential rhizobia isolates are treated at plant growth enhancement experiment. This isolates will comprehensively extend agricultural production, if they are often used to inoculate legume plants basically *Cicer aritinum*, thereby abatement the environmental intimidation of artificial nitrogen fertilizers.

Conclusion

Symbiotic process for biological nitrogen fixation (BNF) in agriculture are most hopeful. Accordingly, the current study was directed for the isolation, identification, and Characterization of nitrogen fixing bacterial isolates of *Rhizobium* characterization of nitrogen-fixing bacterial isolates of *Rhizobium* from the legume plant of chickpea which grows extensively in almost every area of India. In this experiment screened potential (*VBRS*-8) *Rhizobium* isolates were identification by morphological characteristics, biochemical test and plant nodulation check test and Plant growth enhancement by *Rhizobium* bacteria in Leonard jar experiment (Rajeswari *et al*., 2017) [11]. In future this isolates are experiment carried out in field experiment and prepare *Rhizobium* inoculum are use as biofertilizer which can help agricultural sector in India.

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