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IN VITRO SCREENING OF INDIGENOUS RHIZOBIUM ISOLATES OF ARACHIS HYPOGAEA FOR SPECIFIC PLANT GROWTH PROMOTING PHYSIOLOGICAL TRAITS

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ABSTRACT - Plant growth promotion activity by PGPR is a classic example of plant microbe interaction. *In vitro* screening of *Rhizobium* isolates for specific physiological traits were analyzed as the plant growth substances production, PHB assay and antagonistic activity of the rhizobial isolates serves as a selection criterion of the effective strains. These characteristics are generally a prerequisite of good plant growth promoting bioinoculants. In the present investigation of 45 isolates for plant growth promotion, nine isolates (VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2) exhibited increased production of the growth factors tested and antagonistic activity. Seventy-four percent (74%) of the *Rhizobium* species tested produced IAA. When tested for EPS production, 36 µg/mL was observed in *Rhizobium* TRh11 followed by 32 µg/mL in KRh11 & VRh9 respectively. Twenty-seven (27) isolates of 45 strains tested produced an orange halo zone in Chromazurol S agar indicating Siderophore production. When tested for HCN production, 60% of the isolates produced HCN and ammonia production was detected in 35 isolates (75%). Qualitative screening for PHB production indicated that all the 45 isolates demonstrated positive results, and the isolates VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2 displayed maximum absorption. All isolates proficiently fixed nitrogen with substantial variation among the isolate *Rhizobium* TRh11 which fixed higher nitrogen content. The selected nine *Rhizobium* strains were scrutinized for their antagonistic activity against two isolates of pathogenic fungal strains (*Aspergillus niger* and *Fusarium oxysporum*). *Rhizobium* species TRh11 and VRh2 exhibited commendative prohibitive activity which notably

truncated the growth of the fungal isolates tested.

Keywords: PGPR, Siderophore, IAA, Nitrogen fixation, HCN, PHB, *Rhizobium*, Groundnut

I. INTRODUCTION

The use of PGPR constituting a small portion of the rhizobacterial community, is an environmentally sound way of increasing crop yields which profoundly activates plant growth through mechanisms of phosphate solubilization, biological nitrogen fixation, improvement of nutrient uptake, and phytohormone production. Through production of antibiotic, hydrolytic enzymes, Hydrogen Cyanide and Siderophores, biological control of plant pathogens and deleterious microbes could be achieved which improve plant health and growth^{1 & 2}.

Indole Acetic Acid (IAA), a common plant growth promoting hormone helps in fixation of nitrogen at a percentage of 96% and PGPR community could directly enhance plant growth by IAA, cytokinin production, increase nutrient uptake and promote early seedling root³. PGPR produced Hydrogen Cyanide to control growth of different types of pathogens. Available literature revealed that rhizobia also produced Hydrogen Cyanide (HCN). *Rhizobium* species produce variety of exo-polysaccharide (EPS) possessing high moisture holding capacity with iron chelation property serves to maintain moisture in their immediate environment to protect the organism from desiccation and supplies essential energy under nutrient deficit state⁴. The commercialization of microbial EPS for delivery of plant growth regulators and pesticides through encapsulation technology has possible future outcomes. Siderophores production for increasing the uptake of iron by the roots is a profound character of rhizospheric community⁵. Stimulation of



legume *Rhizobium* interaction depends on phytohormones which stimulates root growth to provide sites for rhizobial infection for nodulation. Legume nodulation promotion through flavonoid molecules or flavonoid signal molecules has been extensively studied⁶.

Rhizobium sequester the carbon components provided by the legumes which is stored as Poly-3-Hydroxybutyrate (PHB) granules which benefit the host plants during respiration mechanisms and protects nitrogenase enzyme from oxygen inactivation during the development of seed⁷. PHB can enhance the stress tolerance of rhizobia and help in their own fitness⁸. Many reports indicate the biocontrol ability of rhizobial strain through production of antibiotics and cell wall degrading enzymes⁹. Usage of such strains against soil borne fungi which cause diseases of groundnut can lead to potential control.

One of the major issues in efficient bio fertilizer production using *Rhizobium* species is the quest for strains possessing the unique properties of plant growth induction and defense pathways. The present study discusses the methods used to search an effective strain of *Rhizobium* species capable of producing IAA, Siderophore, Ammonia, Hydrogen Cyanide and the ability to fix increased levels of nitrogen. The strains were also screened for their potential to act as a biocontrol agent and production of PHB granules. The strains identified as *Rhizobium* species through morphological and biochemical studies were analyzed for growth factor promotion factors.

II. MATERIALS AND METHODS

Root nodules of *Arachis hypogaea* plants collected from three different geographical locations of Tamil Nadu were screened for the distribution of *Rhizobium* species. A total of 45 TRh1-TRh15 (Total: 15 strains), KRh1-KRh16 (Total: 16 strains) and VRh1-VRh14 (Total: 14 strains) isolates of *Rhizobium* were then characterized based on their PGPR properties.

2.1. Indole Acetic Acid Production

The IAA production by *Rhizobium* cultures were detected by colorimetric quantification¹⁰. Cultures were grown in 25 mL Luria-Bertani broth amended with 50 µg/mL tryptophan, followed by incubation at $28 \pm 2^\circ\text{C}$ for 48 hours. The cultures were then centrifuged at 10,000 rpm for 15 minutes. Three mL of supernatant was taken for each culture and 2-3 drops of O-phosphoric acid was added. Then 4 mL of Salkowski reagent (1 mL of 0.5 M FeCl_3 in 50 mL of 35% HClO_4) was added to each aliquot. The

samples were then incubated for 25 minutes at room temperature and the absorbance was read at 530 nm. The obtained auxin quantification values were recorded by preparing calibration curve made using IAA as standard (10-100 µg/mL) and the results were recorded as + or – respectively for production or absence of IAA production and the IAA concentration deduced using the IAA calibration curve with standard IAA solutions. IAA standard stock solution was prepared in 50% ethanol at 100µg/mL of IAA. Different standard IAA concentrations were prepared as aqueous solution of IAA ranging from 10µg to 100µg. To each 1 mL of the standard, 2 mL of Salkowski reagent was added. Readings were taken after 25 minutes' incubation at 530 nm by UV-spectrophotometer. Standard graph was prepared by plotting concentration of IAA in micrograms /mL Vs optical density at 530 nm.

2.2. Screening the isolates for production of EPS¹¹

Rhizobium sp. isolates were screened for EPS production by growing it on CRYEMA at 28°C for 48 hours and observed for the formation of gummy/mucoid colonies of *Rhizobium*. EPS production in liquid media was carried out by separately growing the 6×10^6 cells mL^{-1} of *Rhizobium* in YEM Broth (M1824, HiMedia, India) at 28°C for 8-10 days with constant shaking at 120 rpm. Following the incubation, inoculated flasks were observed for change in the rheology. Increase in the viscosity of broth was taken as an indicator of EPS production. After incubation, culture broth was centrifuged at 3500 rpm for 30 minutes. One mL of supernatant was mixed with two volumes of chilled acetone. The crude polysaccharide developed was collected by centrifugation at 3500 rpm for 30 minutes. The EPS was washed with distilled water and acetone alternately and transferred into a filter paper and weighed after drying overnight at room temperature. Results were tabulated according to the weight measured after overnight incubation and expressed as µg/mL.

2.3. Siderophore Production¹²

Siderophore (iron chelator) production of the isolates were determined using Chromazurol S (CAS) method developed by Schwyn and Neilands, 1987. The media with dye was poured into Petri plates and the *Rhizobium* isolates were streaked onto the plates and incubated for six days at $28 \pm 2^\circ\text{C}$. Formation of an orange halo zone indicated the Siderophore (iron-chelator) production and they were grouped based on diameter of the halo, i.e., 10mm as strong reaction (++), moderate growth (+) and no zone formation (-).



2.4. Hydrogen cyanide production¹³

Nutrient agar (M001, HiMedia, India) was modified by supplementing with 4.4 g/l of glycine and the isolates were streaked on modified agar plates. A Whatman No.1 filter paper soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. After sealing the plates with Para film, incubation at 36±2 °C for four days followed by development of orange to red color confirms Hydrogen Cyanide (HCN) production (Lorck, 1948).

2.5. Nitrogen fixing efficacy: Estimation of total nitrogen fixed by Micro-Kjeldahl method

Micro-Kjeldahl's method was used for estimating the nitrogen fixing efficacy of rhizobial isolates. The rhizobial cultures grown in 10 mL of YEM Broth were incubated for 48 hours in a rotary shaker at room temperature. Cell concentrations were determined at 10⁵ cfu/mL of each isolate by plate counts on YEM Agar¹⁴. The uninoculated media serves as control. The concentration of nitrogen fixed was then evaluated and estimated by Kjeldahl method.

2.5.1. Sample preparation, Distillation, Digestion process and Titration

After incubation period, the media containing isolates were digested by adding the salt mixture, transferred to boiling tubes and a 0.5g selenium mixture catalyst (50:10:1 ratio of Potassium Sulphate, Copper Sulphate and metallic selenium) added followed by 3.5 mL of concentrated sulphuric acid to initiate the digestion process. The set-up was left in digestion chamber for two hours. The digested samples after retrieving from the chamber in a time period of two hours, were allowed to cool for 30 minutes, transferred to distillation flask and 40 mL of 10N NaOH were added to proceed with the distillation process. NH₃ released was collected into 20 mL of 1% Boric acid and titration done against 0.01 M HCl. The quantities of nitrogen reported represent the average of duplicate cultures after deducting the average of duplicate cultures¹⁵. The total nitrogen percentage was calculated by the following formula:

Percentage of nitrogen in the sample =

$$\frac{(\text{sample titre} - \text{blank titre}) \times 1.4007 \times \text{Normality of HCl} \times 100}{\text{Weight of the sample} \times 1000}$$

2.6. Ammonia Production

The rhizobial isolates were tested for the production of ammonia in peptone water following the method of Cappuccino & Sherman¹⁶, 1996. Nessler's reagent was added to fresh cultures of the isolates

grown in peptone water for 48 hours at room temperature. Development of brown to yellow color indicates positivity for ammonia production.

2.7. Qualitative and Quantitative Screening for the production of PHB using Sudan Black Staining Technique

The rhizobial strains obtained from root nodules of groundnut plant were screened for the PHB production by viable colony staining technique. The cultures were grown on Minimal Salt Medium (M1253, HiMedia, India) supplemented with glucose (2%) as a sole carbon source and incubated at 30°C for 48 hours. The plates were flooded with Sudan black stain after incubation to detect the secretion of intracellular lipid granules. The qualitative screening method of PHB production of the isolates based on Sudan black absorption pattern by the rhizobial strains on the agar plates were characterized as Maximum, Moderate and Minimum¹⁷.

2.7.1. Extraction of Poly-β-hydroxybutyrate granules

The *Rhizobium* strains which showed maximum production of PHB based on qualitative screening methods of PHB production were subjected to quantification of PHB by centrifuging the bacterial cells containing the polymer at 10,000 rpm for 10 minutes and the pellet was resuspended into alkaline sodium hypochlorite solution (5.25% - 5.5%) which is maintained at a pH of 10.0-10.5. The suspension was incubated at room temperature for one hour and centrifugation was carried out again at 10,000 rpm for 10 minutes followed by discarding the supernatant. Using water, alcohol and acetone, the cell pellet containing PHB was washed. The extracted polymer was dried for two hours at 105°C and weighed. Dry weight of extracted polymer was expressed as g/L. The results indicate that a relationship might exist between dry cell weight and dry weight of PHB. Residual biomass was estimated as the difference between dry cell weight and dry weight of PHB^{17 & 18}.

2.7.2. Estimation of Dry Cell Weight (DCW)

The MSM medium with rhizobial growth was collected and centrifuged at 10,000 rpm for 15 minutes after incubation at 37°C for 48 hours. Supernatant was discarded and the cell pellet was washed twice in deionized water and recovered^{19 & 20}. The cell pellet was dried for 24 hours at 100°C and the total bacterial cell dry weight was determined as g/L.

2.7.3. Estimation of PHB from the selected isolates

Intracellular PHB accumulated is estimated using the following formula and expressed as



percentage:

$$PHB \text{ accumulation } (\%) = \frac{\text{Dry Weight of extracted PHB (g/L)}}{DCW (g/L)} \times 100$$

2.8. Antagonistic activity of *Rhizobium* against fungal pathogens

The antifungal effect of nine (VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2) rhizobial isolates selected based on the preliminary screening methods of production of cell wall degrading enzymes and identification properties of potential PGPR traits against pathogenic fungi (*Aspergillus niger* and *Fusarium oxysporum*) was estimated by well diffusion method developed by Arfaoui method²¹. *In vitro* antagonistic activity was performed on Potato Dextrose Agar (PDA- M096, HiMedia, India) in Petri plates by using dual culture technique. Isolates of *Rhizobium* sp. were streaked across the two edges of the plates and a disc of fungal growth of *Fusarium oxysporum* and *Aspergillus niger* cut from the edge of a seven day old cultures were placed in the center of the plates. Ampicillin antibiotic was used as a positive control and saline as negative control. The plates were incubated for seven days at 28°C for antifungal activity and the percent growth inhibition was recorded using the formula,

$$\% \text{ inhibition} = \frac{(R-r)}{R} \times 100$$

where, *R* – Radius of the fungal colony opposite to the antagonist

r – Radius of the fungal colony towards the antagonist

III. RESULTS

Plant growth promotion activity by PGPR is a classic example of plant microbe interaction. In the forthcoming section, the plant growth substances production, PHB assay and antagonistic activity of the rhizobial isolates were discussed which serves as selection criterion of the effective strains. These characteristics are generally a prerequisite of good plant growth promoting bio-inoculant.

3.1. IAA production

In the present study, it was observed that the maximum production of IAA was observed in nine isolates (VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2). The isolated strains and MTCC strain synthesized IAA in the presence of tryptophan.

In IAA production, the amount of IAA produced by the promising isolates were: TRh2: 96 (µg/mL), TRh7: 97.5 (µg/mL), TRh11: 95.5 (µg/mL),

KRh3: 94.5 (µg/mL), KRh7: 92.5 (µg/mL), KRh11: 95.5 (µg/mL), VRh2: 92.5 (µg/mL), VRh4: 96 (µg/mL), VRh9: 95 (µg/mL) and MTCC: 72.5 (µg/mL) respectively (Table 1). Among the 45 isolates, nine strains produced higher concentrations of IAA above the levels elaborated by the reference strain. The distribution of IAA production in comparison with the reference strain (control strain MTCC), which elaborated an IAA amount of 72.5 µg/mL, the rhizobial isolates of Tiruvallur district, showed high production of IAA. Similar results were observed in few rhizobial isolates of Kanchipuram and Vellore which produced increased amounts of IAA. It is evident from the results, the dependency of IAA production on the availability of tryptophan which proves the fact of adaptability of tryptophan dependent pathway by microbes. The isolates in the current study varied in their intrinsic ability to produce IAA as the production varied under the same condition.

3.2. Exo-Polysaccharide (EPS) production

Formation of mucoid gummy colonies on YEMA and increase in the viscosity of YEMB indicated the ability of *Rhizobium* sp. to produce EPS. Totally nine strains along with MTCC produced maximum EPS after 72 hours of incubation. VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2 isolates produced EPS at higher levels when compared to other strains.

A maximum EPS production of 36 µg/mL was observed in TRh11 followed by 32 µg/mL in KRh11 & VRh9 respectively, whereas, MTCC (45 µg/mL) has produced the high amount of EPS. Figures 1.1, 1.2 & 1.3 indicates the EPS production results of the rhizobial isolates in comparison with the reference strain indicated by the diagonal cutoff. Among the Tiruvallur isolates, TRh2, TRh7 and TRh11 secreted 28 µg/mL, 31 µg/mL and 36 µg/mL respectively. Kanchipuram isolates KRh3, KRh7 and KRh11 elaborated 29 µg/mL, 28 µg/mL and 32 µg/mL distinctively. Vellore isolates VRh2, VRh4 and VRh9 at disparate levels, produced 26 µg/mL, 30 µg/mL and 32 µg/mL of EPS respectively. Even though the figures indicate that EPS production was high in *Rhizobium leguminosarum* MTCC 99, isolated native strains also produced equivalent amount of EPS. High amount of EPS production was mostly by the fast growing strains and lowest amount produced by the slow growing strains. Intermediate values were obtained with few fast and slow growing strains in the present study.

3.3. Siderophore production

Another interesting trait of *Rhizobium* is the ability to secrete siderophores (ferric-specific ligands)



which chelates ions and other metals contributing to disease suppression and acquisition of ferrous to plants for increasing the crop growth. Amongst the overall isolates (45), only nine (VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2) isolates along with MTCC were able to produce maximum siderophores (good growth: ++)(which were indicated by moderate yellow-orange halo around their colonies due to removing the color from CAS dye-Fe III-complex) and low Siderophore (expressed as a weak zone) (Table 2). Few isolates depicted negative results for Siderophore production (TRh1, TRh4, TRh6, TRh10, TRh12, TRh15, KRh2, KRh5, KRh6, KRh8, KRh9, KRh13, KRh14, VRh5, VRh6, VRh7, VRh13 and VRh14). The district wise percentage production of the isolates was 64% > 60% > 54% of Vellore, Tiruvallur and Kanchipuram respectively.

Table 1: IAA production of the isolated strains of *Rhizobium* from *Arachis hypogaea*

Isolates	IAA production (µg/mL)	Isolates	IAA production (µg/mL)	Isolates	IAA production (µg/mL)
TRh1	21.5	KRh1	81	VRh1	70
TRh2	96	KRh2	80.5	VRh2	92.5
TRh3	22	KRh3	94.5	VRh3	30
TRh4	17	KRh4	87.5	VRh4	96
TRh5	80.5	KRh5	74	VRh5	25
TRh6	66.5	KRh6	68	VRh6	45.5
TRh7	97.5	KRh7	92.5	VRh7	80
TRh8	66.5	KRh8	46.5	VRh8	24
TRh9	95.5	KRh9	25	VRh9	95
TRh10	16.5	KRh10	38	VRh10	18
TRh11	95.5	KRh11	95.5	VRh11	67
TRh12	18	KRh12	55	VRh12	52
TRh13	87.5	KRh13	68	VRh13	48
TRh14	20.0	KRh14	70	VRh14	40
TRh15	15.5	KRh15	75		
		KRh16	40		
<i>Rhizobium leguminosarum</i> MTCC 99	72.5				

Table 2: Siderophore production of the rhizobial isolates

Isolates	Siderophore	Isolates	Siderophore	Isolates	Siderophore
TRh1	-	KRh1	+	VRh1	+
TRh2	++	KRh2	-	VRh2	++
TRh3	+	KRh3	++	VRh3	+
TRh4	-	KRh4	+	VRh4	++
TRh5	+	KRh5	-	VRh5	-
TRh6	-	KRh6	-	VRh6	-
TRh7	++	KRh7	++	VRh7	-
TRh8	+	KRh8	-	VRh8	+
TRh9	+	KRh9	-	VRh9	++
TRh10	-	KRh10	+	VRh10	+
TRh11	++	KRh11	++	VRh11	+
TRh12	-	KRh12	+	VRh12	+
TRh13	+	KRh13	-	VRh13	-
TRh14	+	KRh14	-	VRh14	-
TRh15	-	KRh15	+		
		KRh16	+		
<i>Rhizobium leguminosarum</i> MTCC 99	++				
Total Positives	9(60%)		9(56%)		9(64%)

TRh- Rhizobial strain isolated from Tiruvallur;
KRh-Rhizobial strain isolated from Kanchipuram;
VRh-Rhizobial strain isolated from Vellore

Figure 1.1: EPS production (µg/mL) by *Rhizobium* isolates from *Arachis hypogaea* of Tiruvallur district (TRh1-TRh15)

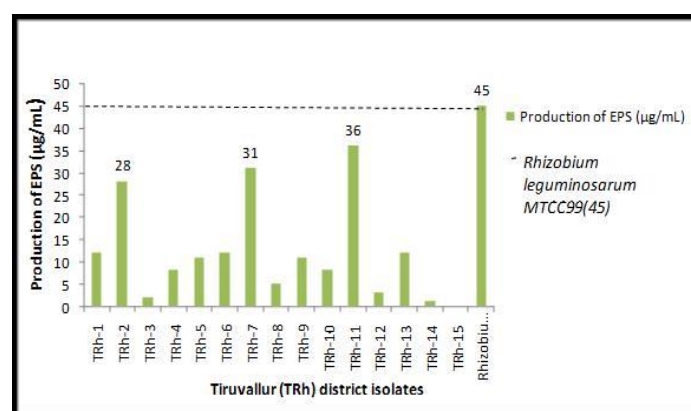


Figure 1.2: EPS production (µg/mL) by *Rhizobium* isolates from *Arachis hypogaea* of Kanchipuram district (KRh1-KRh16)

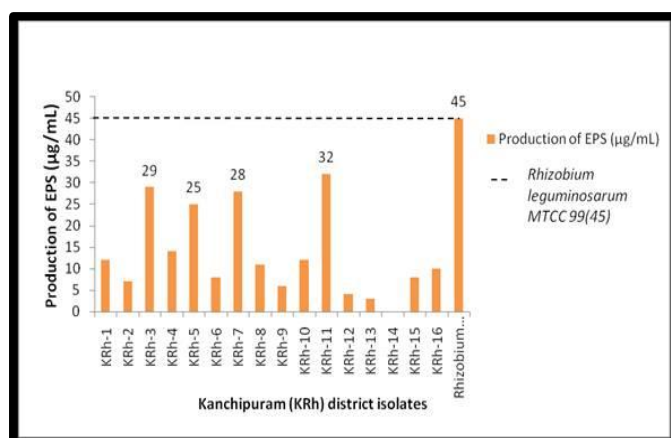
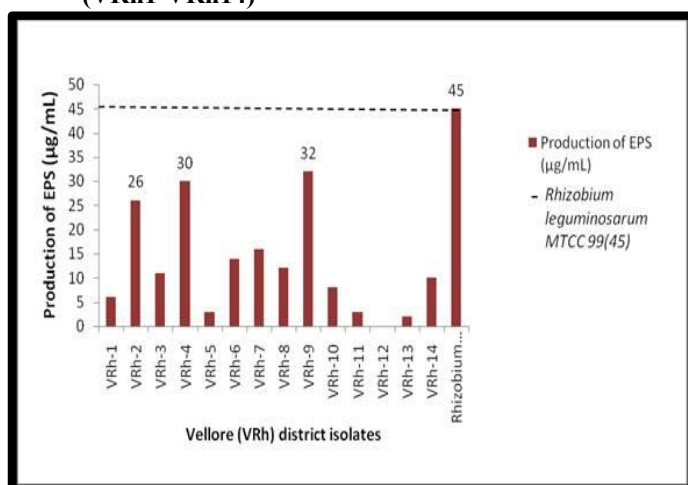


Figure 1.3: EPS production (µg/mL) by *Rhizobium* isolates from *Arachis hypogaea* of Vellore district (VRh1-VRh14)



3.4. Hydrogen Cyanide production

Hydrogen Cyanide production was detected in twenty-seven rhizobial isolates and the data indicates maximum production by Tiruvallur isolates (80%) followed by Kanchipuram (56%) and Vellore districts (43%). Out of 45 *Rhizobium* isolates, maximum Hydrogen Cyanide production was observed in nine strains (VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2) indicated as ++ along with MTCC (Table 3). Seventeen strains of *Rhizobium* isolates (TRh1, TRh3, TRh5, TRh6, TRh8, TRh9, TRh10, TRh12, KRh4, KRh8, KRh10, KRh12, KRh14, KRh15, VRh5, VRh10 and VRh11) elaborated low cyanide production revealed as a weak orange red pigmentation (+), Whereas no Hydrogen Cyanide production (-) was observed in strains TRh13, TRh14, TRh15, KRh1, KRh2, KRh5, KRh6, KRh9, KRh13, KRh16, VRh1, VRh3, VRh6, VRh7, VRh8,

VRh12, VRh13 and VRh14.

Table 3: Hydrogen Cyanide production by *Rhizobium* isolates from *Arachis hypogaea*

Isolates	HCN	Isolates	HCN	Isolates	HCN
TRh1	+	KRh1	-	VRh1	-
TRh2	++	KRh2	-	VRh2	++
TRh3	+	KRh3	++	VRh3	-
TRh4	+	KRh4	+	VRh4	++
TRh5	+	KRh5	-	VRh5	+
TRh6	+	KRh6	-	VRh6	-
TRh7	++	KRh7	++	VRh7	-
TRh8	+	KRh8	+	VRh8	-
TRh9	+	KRh9	-	VRh9	++
TRh10	+	KRh10	+	VRh10	+
TRh11	++	KRh11	++	VRh11	+
TRh12	+	KRh12	+	VRh12	-
TRh13	-	KRh13	-	VRh13	-
TRh14	-	KRh14	+	VRh14	-
TRh15	-	KRh15	+		
		KRh16	-		
Rhizobium leguminosarum MTCC 99	++				
Total Positives	12(80%)		9(56%)		6(43%)

3.5. Nitrogen Fixation Assay

The varied nitrogen fixation capacity of the rhizobial isolates obtained from root nodules has showed proven ability of nitrogen fixing capacity of the isolates. Nitrogen fixing ability, being the prominent characteristic of host rhizobial cells was well established with the results obtained as all the strains fixed nitrogen. It was observed that the maximum total nitrogen fixed by only nine isolates (VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2) and remaining isolates fixed minimum nitrogen level in comparison with the reference strain (Figure 2.1, 2.2 and 2.3). Out of the 45 isolates, TRh11 and VRh2 fixed nitrogen at a rate of 0.52% and 0.53% on par with *Rhizobium leguminosarum* MTCC 99 strain (0.54%).

Figure 2.1: Amount of nitrogen fixed (%) by the *Rhizobium* isolates from *Arachis hypogaea* of Tiruvallur district (TRh1-TRh15)

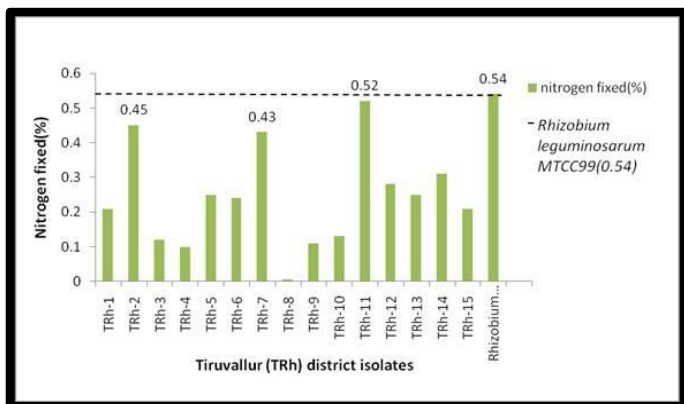


Figure 2.2: Amount of nitrogen fixed (%) by the *Rhizobium* isolates from *Arachis hypogaea* of Kanchipuram district (KRh1-KRh16)

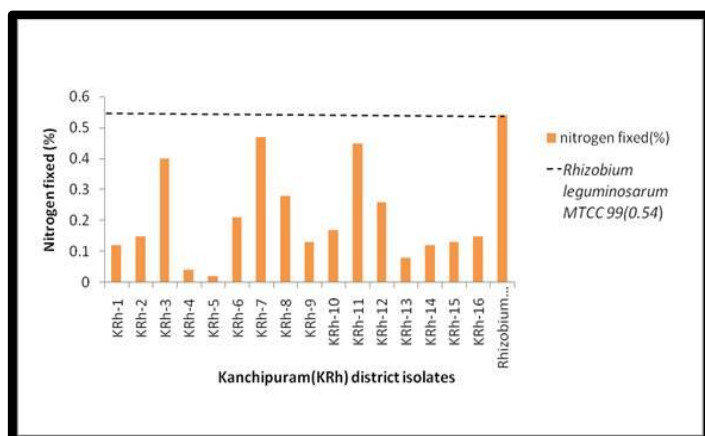
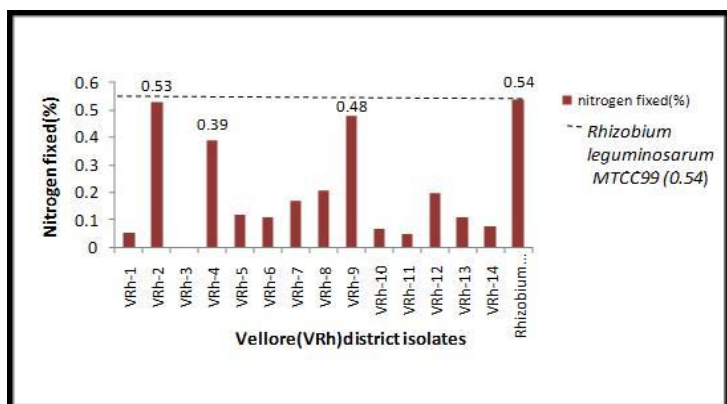


Figure 2.3: Amount of nitrogen fixed (%) by the *Rhizobium* isolates from *Arachis hypogaea* of Vellore district (VRh1-VRh14)

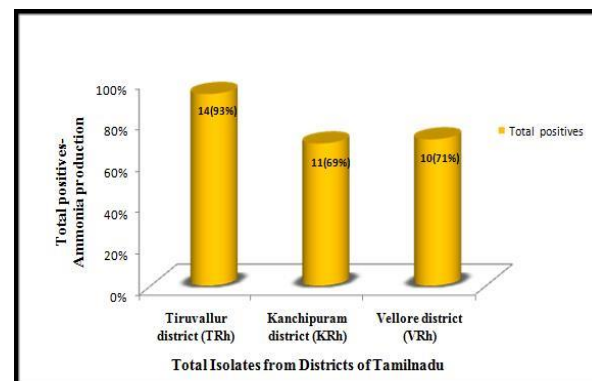


3.6. Ammonia Production

The rhizobial isolates were screened for the production of ammonia, an important trait of PGPR which indirectly influences the plant growth and the

data unveiled authenticates the ammonia production ability of majority of the isolates. Out of the 45 strains tested, 35 (75%) were positive for ammonia production. The district wise data of rhizobial isolates positive for ammonia production is presented in figure 3 reveals that Tiruvallur strain showed 93% followed by Vellore (71%) and Kanchipuram (69%).

Figure 3: Percentage of *Rhizobium* isolates from *Arachis hypogaea* positive for ammonia production



3.7. PHB production

3.7.1. Qualitative screening for PHB producing rhizobial isolates

To reveal the production of PHB through Sudan black absorption, growth pattern of isolates by viable colony staining technique was performed and the results were tabulated. Out of 45 *Rhizobium* isolates, nine isolates showed strong Sudan black absorption (+++) PHB. Further, six isolates scored moderate absorption (++) and 27 isolates displayed less absorption of Sudan black (+). Rhizobial isolate KRh13 failed to absorb Sudan black which is indicative of absence of PHB production (Table 4). Based on the qualitative results, the efficient PHB producing strains were tested for quantitative methods of production of PHB granules.

3.7.2. Extraction and Quantification of PHB

Table 5 elaborates the yield of PHB in efficient *Rhizobium* strains (VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2) selected based on the qualitative screening for PHB production. Extracted PHB concentration and percentage yield of PHB was observed on a higher scale for TRh11 and VRh2 isolates as it yielded 30.01% and 29.88% respectively when compared with MTCC strain (Figure 4 & 5). The results indicate that a significant relationship might exist between the dry cell weight and PHB production.

Table 4: Qualitative screening for PHB producing *Rhizobium* isolates from *Arachis hypogaea*

Isolates	PHB production	Isolates	PHB production	Isolates	PHB Production
TRh-1	+	KRh-1	+	VRh-1	+
TRh-2	+++	KRh-2	++	VRh-2	+++
TRh-3	+	KRh-3	+++	VRh-3	+
TRh-4	++	KRh-4	+	VRh-4	+++
TRh-5	+	KRh-5	+	VRh-5	+
TRh-6	+	KRh-6	+	VRh-6	++
TRh-7	+++	KRh-7	+++	VRh-7	+
TRh-8	++	KRh-8	+	VRh-8	+
TRh-9	+	KRh-9	+	VRh-9	+++
TRh-10	+	KRh-10	+	VRh-10	+
TRh-11	+++	KRh-11	+++	VRh-11	+
TRh-12	+	KRh-12	+	VRh-12	+
TRh-13	++	KRh-13	-	VRh-13	+
TRh-14	+	KRh-14	+	VRh-14	+
TRh-15	+	KRh-15	+		
		KRh-16	++		
<i>Rhizobium leguminosarum</i> MTCC 99	+++				

+++ indicates maximum Sudan black absorption; ++ indicates moderate Sudan black absorption; + indicates less absorption of Sudan black on minimal agar plates

Figure 4: PHB concentration of the selected *Rhizobium* isolates (g/L) from *Arachis hypogaea*

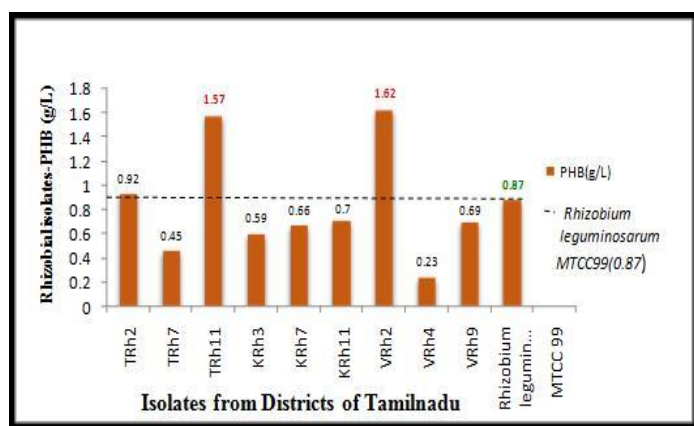


Figure 5: Yield % of PHB produced by the selected *Rhizobium* isolates of *Arachis hypogaea*

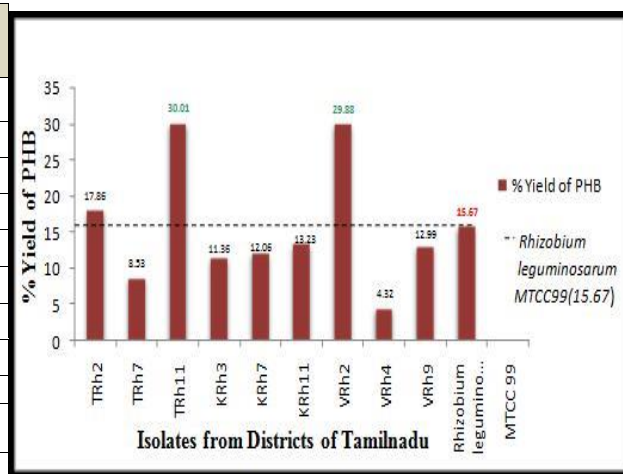


Table 5: Quantification of PHB by the selected *Rhizobium* isolates of *Arachis hypogaea*

Rhizobial isolates	DCW (g/L)	PHB (g/L)	% Yield of PHB
TRh2	5.15±0.17	0.92±0.09	17.86
TRh7	5.27±0.13	0.45±0.12	8.53
TRh11	5.23±0.15	1.57±0.15	30.01
KRh3	5.19±0.11	0.59±0.17	11.36
KRh7	5.47±0.19	0.66±0.13	12.06
KRh11	5.29±0.12	0.70±0.16	13.23
VRh2	5.42±0.11	1.62±0.12	29.88
VRh4	5.32±0.16	0.23±0.11	4.32
VRh9	5.31±0.13	0.69±0.14	12.99
<i>Rhizobium leguminosarum</i> MTCC 99	5.55±0.18	0.87±0.16	15.67

(Average of three values presented as Mean±SD)

3.8. Antagonistic activity of *Rhizobium* against fungal pathogens

In vitro antagonistic potential of the selected nine *Rhizobium* isolates (VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2) along with MTCC reference strain were tested against *Aspergillus niger* and *Fusarium oxysporum* in dual culture under *in vitro* conditions and the percentage of inhibition was recorded. After five days of incubation, inhibition zone was clearly visible. All isolates were found to be inhibitory against fungal strains, yet, maximum inhibition potential was exhibited by two rhizobial isolates TRh11 and VRh2. *Rhizobium* TRh11 isolate recorded a growth inhibition percentage of 58% and



59% against *Fusarium oxysporum* and *Aspergillus niger* respectively. *Rhizobium* VRh2 isolate exhibited 58% and 63% growth inhibition against *Fusarium oxysporum* and *Aspergillus niger* respectively. The other isolates showed minimal growth inhibition when compared to TRh11 and VRh2. The percentage of growth inhibition of the selected fungal strains depicted in figures 6.1 & 6.2 infers that native *Rhizobium* isolates especially TRh11 and VRh2 could be considered for bio control activity.

Figure 6.1: Growth of inhibition (%) of effective *Rhizobium* isolates against *Fusarium oxysporum* (Values are replicates of three values (Mean $\pm$ SD))

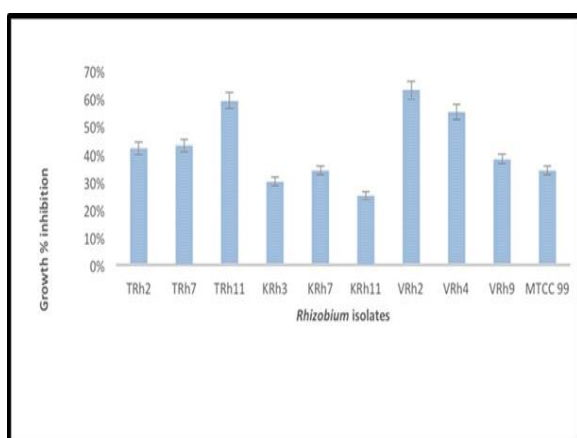
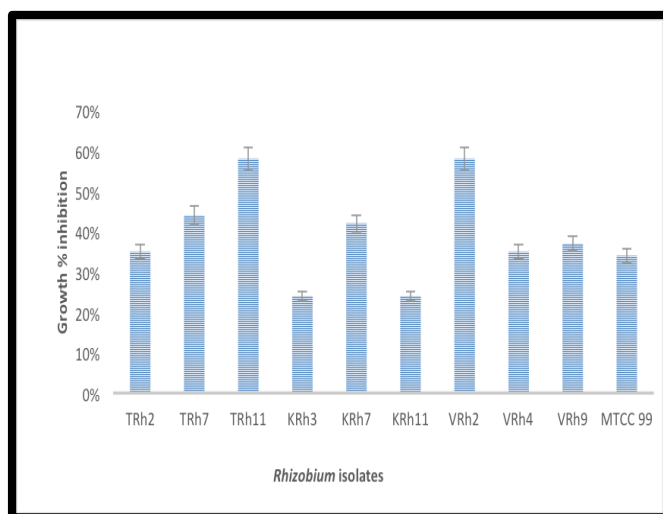


Figure 6.2: Growth of inhibition (%) of effective *Rhizobium* isolates against *Aspergillus niger* (Values are replicates of three values (Mean $\pm$ SD))



IV. DISCUSSION

Mobilization of nutrients, production of IAA, EPS, HCN, Siderophore, ammonia with enhanced stress resistance and solubilization of phosphates are the primary mechanisms of plant growth promotion habituated by rhizobia.

Chemical substances occurring naturally, possessing indole nucleus are termed as Indole Acetic Acid (IAA). This phytohormone acting as a regulator of the physiological activities and growth in plants is widely produced by symbiotic bacteria²². In plants, lateral root initiation, cell enlargement and division are controlled by IAA²³ and 74% of the rhizobacteria tested produced IAA²⁴. In the present investigation of 45 isolates, nine isolates (VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2) showed increased production of IAA when compared to the standard strain (Table 1). In the current study, varied IAA production levels by strains of *Rhizobium* is dependent on cultural conditions as reported by many workers. This enhanced IAA production capability not only helps in the establishment of effective symbiosis but also promotes the plant growth²⁵. Screening for the production of IAA is an important strategy while developing bio-inoculants for promoting growth of plants²⁶.

Polysaccharide production is a marked characteristic of rhizobia and involved in infection process and nodule formation. Polysaccharides also protect rhizobia in the soil against deleterious biotic and abiotic stress factors and also restrict the oxygen diffusion through the nodular cells to protect the oxygen sensitive nitrogenase in the nodules²⁷. Most of the rhizobial isolates secreted EPS in the current study. Among the 45 strains tested, the maximum EPS production was observed in nine isolates (VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2). This finding implies that such strains when used as bio-inoculants could improve the symbiotic association and colonize the rhizosphere of groundnut effectively. Many experiments have shown that rhizobia secreting EPS are good colonizers²⁸ of legumes and non-leguminous plants²⁹. Desiccation survival of rhizobia required an increased EPS production³⁰. Rhizobial adaptation³¹ to changing environmental conditions³² and for interaction with legumes to initiate³³ and form infectious threads is dependent on EPS.

The present findings showed that 27 isolates of 45 strains tested were positive for Siderophore production which helps in iron acquisition and possess biocontrol ability. The maximum good growth recorded and expressed as “++”, was observed in nine *Rhizobium* isolates compared to MTCC (Table 2). Few



isolates tested negative for Siderophore production might use alternate chelating agents to facilitate iron transfer. Production of Hydrogen Cyanide and Siderophores are important attributes of PGPR that influence plant growth indirectly and strengthens the host disease mechanism. Bacterial siderophores have been demonstrated to have direct benefits to plant growth promotion by acting as a direct source of iron³⁴ and making it available to plants³⁵. Thus, cyanide and siderophores have an important role in the biocontrol activity against soil borne phytopathogens, beside the essential function of siderophores in the improvement of iron nutrition. 32 µg/mL of hydroxymate siderophores³⁶ production by *M. loti* after 48 hours of incubation was observed which could be attributed to the growth inhibition of *Sclerotinia sclerotiorum* in dual culture technique. Production of Siderophore results in competition based exclusion of siderophore non-producer pathogens from the rhizosphere region due to lack of iron depletion required for hyphal growth and germination. A rhizosphere isolate *Bacillus subtilis* BN1³⁷ inhibited the growth of *M. phaseolina* up to 60 %.

Of all the tested *Rhizobium* isolates (45) for Hydrogen Cyanide, nine isolates showed excellent production and few isolates resulted in moderate production levels (Table 3). Presence or absence and intensity of Hydrogen Cyanide production can play a significant role in antagonistic potential of bacteria against phytopathogens. Production of Hydrogen Cyanide was an important trait in a PGPR in controlling fungal diseases in wheat seedlings under *in vitro* conditions³⁸. The ability of *Rhizobium* and *Bradyrhizobium* strains to secrete hydrogen cyanide is a notable feature³⁹.

The second most important biological process in the earth is nitrogen fixation which has assumed greater significance as it is the major source of fixed nitrogen. Biological nitrogen fixation (BNF) contribute to groundnut productivity both directly, or indirectly by improving sustainability or inducing soil fertility in the agricultural system by adding nitrogen to the soil and to the crops. Nitrogen fixation by rhizobia and its supply to the legumes through nodule formation is of great importance as it is concerned as one of the PGPR traits. In the present study, total nitrogen assay by Micro Kjeldahl method evaluated the levels of nitrogen fixed easily. It was found that VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2 isolates along with MTCC showed a marked increase in nitrogen fixation. The research results show considerable variation in the percentage of nitrogen fixed by the isolated strains. Among the nine isolates which efficiently fixed more nitrogen, VRh2 and TRh11 fixed nitrogen at a percentage range

of 0.52-0.53% equivocal to the MTCC standard (figures 2.1, 2.2 & 2.3). Literature supports that the symbiotic interactions between a legume and rhizobia resulting in a unique, nitrogen fixating plant organ, the nodule symbiotic nitrogen fixation in legumes is well known, which help to reduce the application of inorganic nitrogen and can also play a major role as green manure in improving the soil fertility⁴⁰ & ⁴¹. In peanuts, the potential of enhancing nitrogen fixation at higher levels is associated with these symbiotic associations. Seventy million metric tons of fixed nitrogen per year accounting to 40 % of the total N fixed on earth is contributed by *Rhizobium* - legume association⁴². The potential of native rhizobia isolates in enhancing nitrogen fixation and bean production thereby adding value to agroecosystem has been proved⁴³.

Ammonia produced by the rhizobia can be taken up by the plants as a source of nitrogen for their growth. The ammonia production results of the test isolates showed varied outcome (Figure 3). 78% of the isolates were able to produce ammonia even though all the isolates fixed nitrogen at a specific concentration. This suggests that it is vital to select a nitrogen fixer with ammonia production in a biofertilizer consortia for agriculture practices.

Current scenario in the agricultural sector involving rhizobial biofertilizers over commercial chemical fertilizers reveals that rhizobial inoculants have notable qualities with addition to nitrogen fixing capacity with enhanced nodulation, such as production of plant growth promoting hormones like Indole acetic acid (IAA), secretion of siderophores and solubilization of phosphates etc. Exopolysaccharide (EPS) produced by *Rhizobium* is one such signal for host specificity during the early stage of root hair infection⁴⁴. It also protects the cell from desiccation and predation and helps in nitrogen fixation by preventing high oxygen tension⁴⁵. Nutman documented the positive influence of plant growth by indole acetic acid⁴⁶. The production of EPS and IAA are considered as important traits of plant growth promoting rhizobacteria which indicates the achievement of active biological nitrogen fixation in the presence of rhizobial isolates which correlates with the findings in this research. Similar observations have also been reported in several plant species previously⁴⁷ & ⁴⁸. These observations of the current work revealed the potentiality of isolated strains to increase nitrogen content through production of phytohormones may contribute to peanut growth promotion by suitable strains.

PHB synthesized by many species of rhizobacteria has shown to improve survivability during starvation. Furthermore, it helps the bacteria to



tolerate extreme high temperatures⁴⁹, desiccation⁵⁰, osmotic stress and hydrogen peroxide exposure⁵¹. Most efficient PHB producing bacteria belong to the genus *Rhizobium*⁵². The polymer accumulation inside the cells is an attribute of nutrient imbalance and its synthesis is related to energy requirements of the cell. Qualitative screening for PHB production indicated that all the 45 isolates showed positive results, and the isolates VRh9, KRh7, TRh11, VRh2, KRh11, TRh7, KRh3, VRh4 & TRh2 showed maximum absorption (Table 4). Based on the qualitative screening, nine isolates were subjected to quantitative method to estimate the yield of PHB. The findings suggest that TRh11 and VRh2 produced 30.11% and 29.88% respectively than the reference strain (Table 5). The competing effect of nodulation is affected due to disrupted PHB gene synthesis in rhizobia⁵³ & ⁵⁴. As rhizobial fitness is crucial during starvation, the capability to synthesize and degrade PHB by *Rhizobium* may improve by stabilizing cellular redox conditions.

To check the efficacy of antagonism of selected rhizobial isolates against soil borne fungal isolates (*Aspergillus niger* and *Fusarium oxysporum*) infecting groundnut plant, dual culture method was adapted and the percentage of inhibition of growth was recorded (Figures 6.1 & 6.2). As a result of the plate assay, some rhizobial isolates curtailed the growth of pathogenic fungi tested and were found to be highly inhibitory to *Aspergillus niger* and *Fusarium oxysporum*, whereas other strains showed only nominal antifungal activity. Rhizobial Strains VRh11 and TRh 2 suppressed the growth of tested fungi at higher percentage when compared to other strains tested. Similar results were observed by Antoun *et al.*, 1998, who found that 49 of his rhizobial strains inhibited the growth of *Fusarium oxysporum*⁵⁵. By inoculating the strains of antifungal ability, a significant reduction in Damping-off or Wilt disease of groundnut plant could be achieved thereby reducing the percentage of crop loss. Such biocontrol agents have also been reported to produce toxic metabolites, enzymes or volatile compounds which have inhibitory effects on soil-borne pathogens⁵⁶. One of the mechanisms adapted by rhizobia to control fungal pathogens is production of siderophores, cell wall degrading enzymes and cyanogenic compounds. The strains which showed positive results for phosphate solubilization, cyanide production, siderophore production and produced cell wall degrading enzymes, gave the highest percentage of inhibition when tested for antagonistic activity and classified as the most effective isolates among the others in dual culture tests. The biocontrol ability of sixteen native isolates

of *Mesorhizobium* strains *in vitro* against *Fusarium* species has been identified in previous studies⁵⁷.

The ability of tested *Rhizobium* isolates to exhibit some PGP properties was evaluated under *in vitro* conditions in the present investigation. On the basis of the data obtained, it could be concluded that two of the *Rhizobium* isolates (VRh2 and TRh11) excelled in nitrogen-fixation performance, growth factor production studies, antagonistic activity and PHB production.

V. CONCLUSION

The current study paves the way for the ideal selection of *Rhizobium* possessing PGPR properties and proven antagonistic activity for their consistent performance on the growth and yield of groundnut under field conditions. Selection of the appropriate rhizobial inoculant improves nitrogen fixation and food production. Considering every perspective, the present study was mainly taken up to understand the impact of rhizobial bioinoculants isolated from the native lands of Tamil Nadu and to use it as a biofertilizer for stimulating the growth of groundnut crop. The ability of tested *Rhizobium* isolates exhibiting some PGP-properties, namely, IAA production, EPS, siderophores, ammonia, HCN, nitrogen fixation and antagonistic activity evaluated under *in vitro* conditions proved the efficacy of strains which indicates that inoculation of native *Rhizobium leguminosarum* strain TRh11h exemplifies beneficiary response on groundnut.

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