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Proportional analysis of leghaemoglobin concentration in various nodulating plants and intuitive *Rhizobium* species

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ABSTRACT

Hemoglobin is functioning as carriers and storage sources of oxygen in a wide variety of organisms including plants. Plant hemoglobins are found greatest abundance in the nitrogen fixing nodules of legumes. Mostly nodules are formed by bacteria like, Rhizobium or Bradyrhizobium by root colonization. After colonization, the bacteria begin to fix the nitrogen required by the plant. Commonly, the plant nodules are found certain tribes of tropical legumes such as Vigna and on some temperate legumes such as lotus. In this study four tropical legumes such as, Arachis hypogaea, Vigna radiata, Vigna mungo and Mymosa putika plant root nodules were selected and their protein content were analyzed, also 19 Rhizobium species isolated from selected plants root nodules. Among these nodules M. putika root nodules were showed maximum amount of protein (10.65 µg/ml) and Rhizobium GN3 had showed high total protein content (34.95 µg/ml). Leghaemoglobin for all root nodules and rhizobial isolates of respective root nodules were tested by Cyanmethemoglobin method. Among these nodules Leghaemoglobin concentration is high in the root nodules of A. hypogaea GN7, M. putika TN3, V. radiata GG3 and V. mungo BG2 showed that the absorbance value of 0.088, 0.044, 0.024 and 0.020. In conclude that those selected leguminous plants nodules Leghaemoglobin levels highly varied in quantitatively.

Keywords: Leghaemoglobin, Legumes, Nodulation, Root nodule, *Rhizobium*.

INTRODUCTION

Rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root. This zone is about 1 mm wide, but has no distinct edge. Rather, it is an area of intense biological and chemical activity influenced by compounds exuded by the root, and by microorganisms feeding on the compounds. All this activity makes the rhizosphere the most dynamic environment in the soil. Because roots are underground, rhizosphere activity has been largely overlooked, and it is only now that we are starting to unravel the complex interactions that occur. For this reason, the rhizosphere has been called the last frontier in agricultural science (Veeger *et al*., 1981). Legumes are unique among crop plants in their ability to fix nitrogen in symbiotic association with bacteria, mostly belonging to the genera *Rhizobium* and *Bradyrhizobium*. Nitrogen fixation by bacteroids provides ammonia, which is incorporated by the plants into important biomolecules, such as protein, nucleic acids, porphyrins and alkaloids. The symbiosis takes place at the level of the root nodules, tumor - like structures formed as a result of the infection of roots by the bacteria (Andersson *et al.*, 1984).

Leghaemoglobin is a protein which is required for nitrogen fixation by legume nodules in plants. As early as in 1939, the Japanese scientist Kubo discovered that nodules from soybean contained a red colored pigment and demonstrated that it was a haemoprotein. This haemoprotein in N_2- fixing nodules which facilitates O2 diffusion to respiring bacteroid in infected cells. The ATP produced from bacteroid respiration via oxidative phosphorylation is

used to reduce N_2 to form NH3. This capacity of Leghaemoglobin molecules to supply O2 to support bacteroid respiration and ATP production for nitrogenase activity functionally relates the hemoprotein to N_2 fixation (Virtanen *et al.,* 1947). Leghaemoglobin from legume species have been only detected in the infected tissues of root and stem nodules of nitrogen fixing plants (Appleby, 1992; Arredondo Peter *et al*., 1998).

Nitrogen is a limiting nutrient in most environments, with the main reserve of nitrogen in the biosphere being molecular nitrogen from the atmosphere. Molecular nitrogen cannot be directly assimilated by plants, but it becomes available through the biological nitrogen fixation process that only prokaryotic cells have developed. Proliferation of bacteria in soil adhering to the root surface was discovered toward the end of the nineteenth century, at the same time as the discovery of nitrogen fixation. Biological nitrogen fixation is the major source of nitrogen input in agricultural systems. Rhizobia are bacteria that biological elicit on the roots of specific legume hosts the formation of new organs i.e. nodule, within which the bacteria proliferates, differentiate into bacteroids and subsequently the atmospheric nitrogen into ammonia. It can act as a sustainable source of N and can complement or replace fertilizer inputs (Garg and Geetanjali, 2007). The two main agricultural practices to benefit from biological N fixation, crop rotation and intercropping legumes (Fabaceae), and non-fixing plants, were practiced in ancient times, even if the basis for the benefit derived was not understood (Burris, 1993).

Most of the fixed N in legumes is harvested and fed to animals, but evidence from a number of experiments using different methodologies indicates that legumes can deposit significant amounts of N in the soil during growth. Fixed N can also be transferred to intercropped non-legumes in the case of mixed cropping systems, or to following crops in the case of crop rotation (Mahieu, 2007). Nitrogen fixation is energy-demanding process and a constant supply of oxygen to the bacterial oxidases must be maintained. Because the high affinity of leghaemoglobin for O2 and of its high concentration in nodules, it was postulated that leghaemoglobin functions by facilitating the diffusion of O2 to the bacteroids, thus leghaemoglobin is essential for nitrogen fixation (Bergersen *et al.,* 1980). The prime objective of this study is to estimate the leghaemoglobin concentration in selected legumes plant root nodules as well as *Rhizobium* from selected legumes plant root nodules by cyanomethaemoglobin method.

MATERIALS AND METHODS

Sampling

Different types of root nodules such as *Arachis hypogea,Vigna mungo, Vigna radiata* and *Mymosa putika* were collected from Mannargudi in Thiruvarur District of Tamil Nadu, India. Plants are carefully uprooted from the field and are brought to the laboratory in aseptic condition and processed for rhizobacteria isolation.

Isolation of *Rhizobium*

Nodules were collected from the roots and washed with sterile water followed by surface treatment with 95% alcohol and again with sterile water. The washed nodules were surface sterilized with 0.1% mercuric chloride for 2-3 min and again washed for at least 10 times with sterile water as to remove the traces of mercuric chloride. The nodules were transferred in culture tube half filled with sterile water and crushed with a sterile glass rod to obtain a milky bacterial suspension. After serial dilution was made and the suspension was streaked on Yeast extract mannitol (YEMA) agar plates and incubated for 2-3 days at 28°C. A single colony was taken from agar plates and it was streaked on freshly prepared YEMA plates to obtain the pure culture (Gachande and Khansole, 2011).

Gram's Staining

A loopful of culture was taken in a clean glass slide and then smeared. The cells are fixed by passing the slide gently over a flame. The smear is then stained with crystal violet solution for about 2-3 min. The slide is washed with tap water. A few drops of Gram's iodine solution is added to the slide and kept up to 2min. The slide is again washed in tap water. The slide is kept in a slanting position and washed with alcohol which is added dropwise. This is continued until all free blue color has been removed (upto 3sec). The slide is again washed in tap water. The smear is then flooded with a safranin for one minute. It is then washed in tap water and air dried. The cells are observed under high power of the microscope and then through oil immersion objective (Bergey *et al.,* 1994).

Biochemical and Physiological Characters

The biochemical tests were carried out in growth medium at 28ºC for 48h incubation. All the tests were carried out with duplicates.

Catalase Activity

48 hours old rhizobacterial isolates were flooded with hydrogen peroxide (H2O2) and observed for liberation of effervescence of oxygen around the bacterial colonies according to Graham and Parker (1964).

Oxidase Activity
Few drops of p-aminodimethylaniline oxalate were added on the surface of isolates on YEM agar and observed for the production of color according to Kovacs (1956).

Growth on Glucose Peptone Agar
Glucose peptone agar (GPA) plates were streaked with isolated strains and incubated. Presence of growth was observed after 48h according to Vincent (1970).

Citrate Utilization
Citrate utilization ability was determined, by replacing mannitol from YEM agar with equal amount of sodium citrate and bromothymol blue (25mg/l). The plates with modified media were inoculated and then incubated for 48h (Koser, 1923).

Protein Estimation
The protein estimation was determined by Lowry's method (Lowry's, 1951). Different dilutions of BSA solution are prepared by mixing BSA and water in a tube. The final volume of the each test tubes in 5ml. The BSA range is 0.05 to 1mg/ml. From these different solutions pipette out 0.2ml protein solution to different test tube and add 2ml of alkaline copper sulphate reagent. Mix the solution well. This solution is incubated at room temperature for 10min. Then add 0.2ml of Folins-ciocalteous solution to each tube and incubated for 30 min. Zero the calorimeter with blank and take the optical density at 660nm.

Leghaemoglobin Assay
Leghaemoglobin concentration was determined by the cyanmethemoglobin method by Wilson and Reisenauer (1962). 50 to 100mg nodules were collected and it was crushed in 9 volumes of Drabkin's solution in a microfuge tube with a glass rod, and then the tube was centrifuged at 12,000 for 15min. Supernatant was filtered through a 0.2µm syringe filter. The filtrate was taken in a micro cuvette and its absorbance is noted in a spectrophotometer at wavelength 540 nm.

RESULTS

Isolation of *Rhizobium*
Totally 19 bacterial colonies were isolated from the root nodules of *A. hypogea*, *M. putika*, *V. mungo* and *V. radiate*, root nodules based on their white colour mucoid colony appearance on the congored containing YEMA medium. These 19 isolates were analyzed for their morphological and biochemical characterization based on the results they were identified as *Rhizobium* (Table 1 & 2).

Table 1. *Rhizobium* isolates from collected samples

Sl. No	Sample	Name	No. of isolates
1.	*Arachis hypogea*	Ground nut	8
2.	*Mymosa putika*	Touch me not	4
3.	*Vigna mungo*	Black Gram	4
4.	*Vigna radiata*	Green Gram	3

Morphological Characterization
The rhizobial colonies from the root nodules were plated on the YEMA medium and the colony morphology of the isolates were observed. Based on the observation those isolates were showed the same colony characteristic, after 48h of incubation. The colonies were milky white, mucoid, translucent, circular in shape shiny, raised and 2-4mm in diameter. The isolated colonies were named on the basis of their isolated plant name likewise *A. hypogea* isolates were named as GN1 to GN8, *M. putika* isolates were named as TN1, TN2, TN3 and TN4, *V. mungo* isolates were named as BG1, BG2, BG3 and BG4, *V. radiata* isolates were named as GG1, GG2 and GG3.

Protein Estimation from Root Nodules
Root nodules were subjected to protein estimation according to Lowry's method. Root nodules of *A. hypogea*, *V. mungo*, *V. radiata* and *M. putika* showed 5.6, 7.1, 3.75 and 10.65 µg/ml of protein content respectively. Among this, root nodules of *M. putika* showed high protein content. Hence, the result of protein estimation denoted as the protein content is high in the *V. mungo* root nodules (Fig.1).

Protein Estimation of *Rhizobium* Isolates
Totally 19 bacterial isolates were subjected to protein estimation by Lowry's method. Protein content of *A. hypogea* root nodules bacterial isolates namely GN1 to GN8 were identified as 25.8, 26.1, 34.95, 24.6, 15.95, 25.85, 25.9 and 24.65µg/ml respectively. High amount of protein content was present in GN3 nodule isolates (Fig.2).

Figure 1. Protein estimation of root nodules

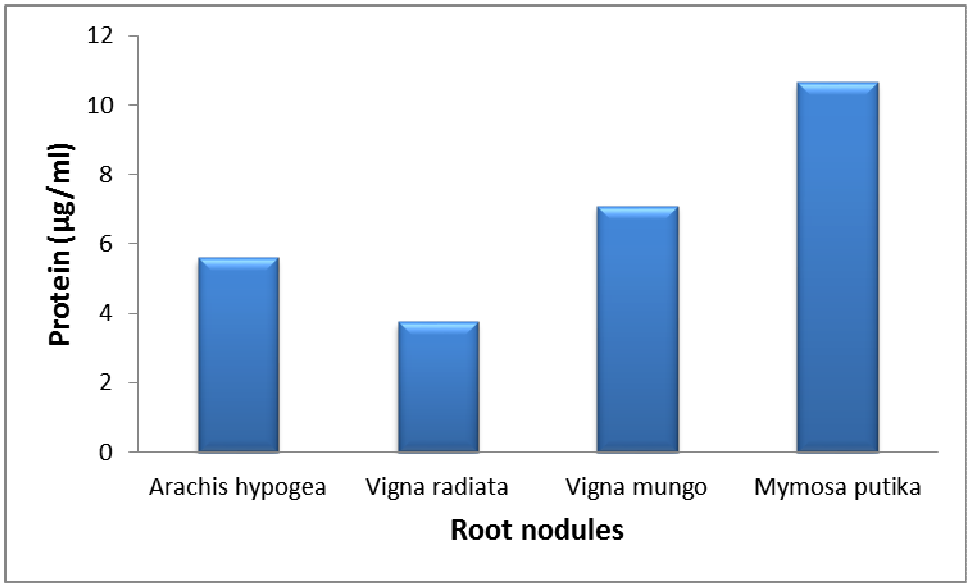
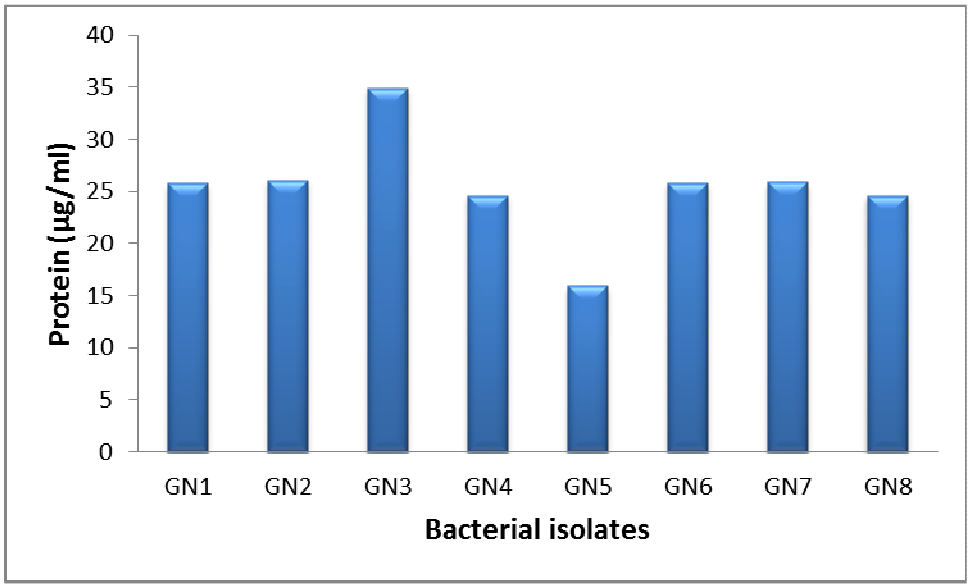


Figure 2. Protein content of *A. hypogea* root nodule isolates



Vigna radiata

The protein content of *V. radiata* root nodules bacterial isolates namely GG1, GG2 and GG3 were identified as 2.55, 5.85 and 5.7 µg/ml respectively. Among the three isolates GG2 isolates shows high (5.85 µg/ml) of protein content (Fig.3).

Vigna mungo

The protein estimated of *Vigna mungo* root nodules bacterial isolates namely BG1, BG2, BG3 and BG4 were identified as 2.45, 2.6, 3.4 and 3.6 µg/ml respectively. High amount of protein content was present in BG4 nodule isolates (Fig.4).

Mymosa putika

The protein content of *Mymosa putika* root nodules bacterial isolates namely TN1, TN2, TN3 and TN4 were identified as 7.95, 7.6, 8.1 and 7.5µg/ml respectively. High amount of protein content was present in TN3 nodule isolates (Fig.5).

Leghaemoglobin Assay

Leghaemoglobin constitute a buffering mechanism in legume root nodules serving to minimize the O_2 gradient through the

infected tissue and to provide sufficient O_2 for bacteroids respiration, albeit at an extremely low O_2 concentration (Fig. 6).

Figure 3. Protein content of *Vigna radiata* root nodule

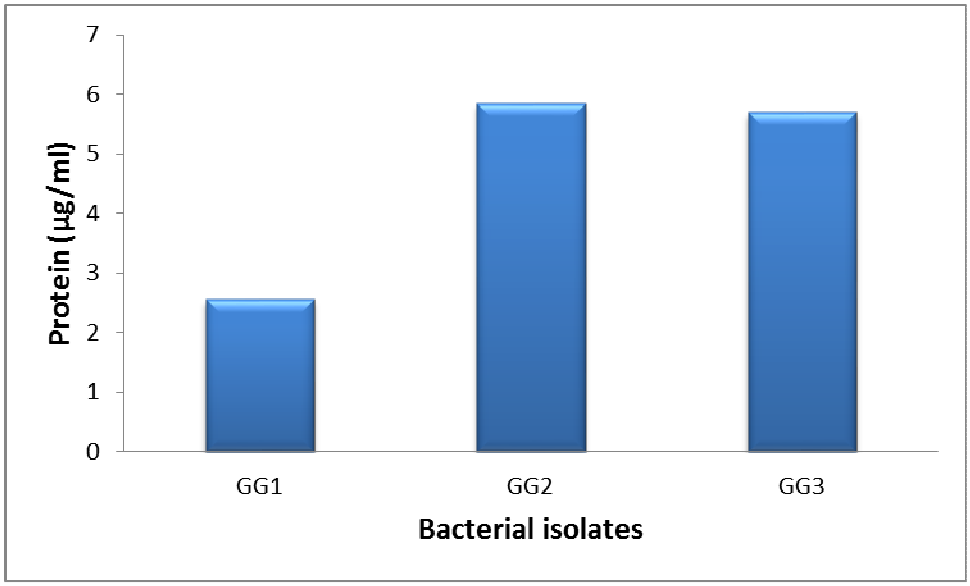
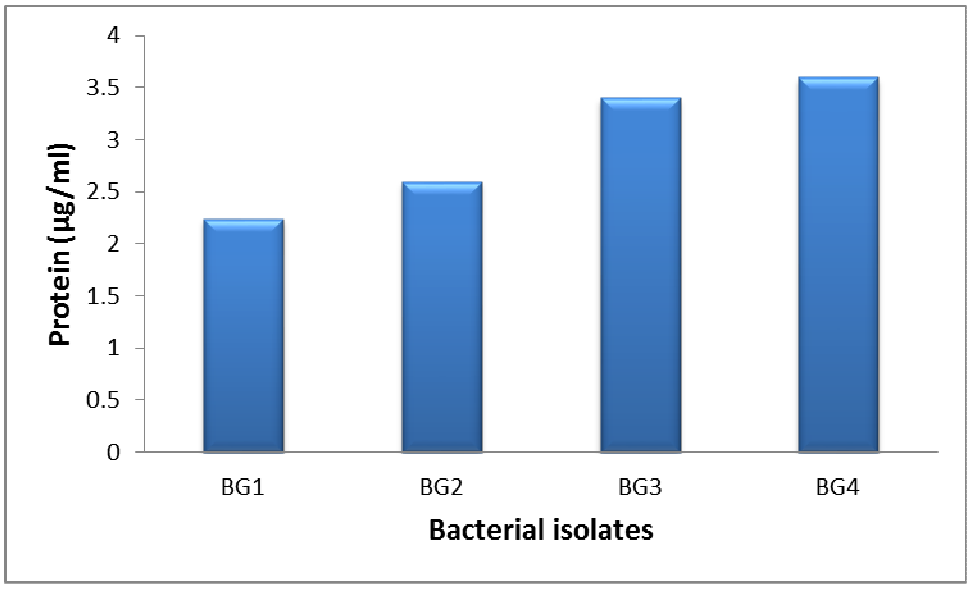


Figure 4. Protein content of *V. mungo* root nodule isolates



It was determined by the cyanmethemoglobin method and absorbance value of ghaemoglobin content of *A. hypogea*, *V. radiata*, *V. mungo* and *M. putika* nodules were identified as 0.037, 0.127, 0.612 and 0.153. Among this *V. mungo* had showed higher leghaemoglobin level in comparison with others.

Arachis hypogea

Absorbance value of leghaemoglobin content of *A. hypogea* root nodules bacterial isolates namely GN1to GN8 were identified as 0.083, 0.073, 0.079, 0.076, 0.038, 0.072, 0.088 and 0.061 respectively. Leghaemoglobin concentration is high in GN7 bacterial isolates (Fig.7).

Vigna radiata

Absorbance value of leghaemoglobin content of *V. radiata* root nodules bacterial isolates namely GG1 to GG3 were identified as 0.020, 0.048 and 0.056 respectively. Leghaemoglobin concentration is high in GG3 bacterial isolates (Fig.8).

Vigna mungo

Absorbance value of leghaemoglobin content of *V. mungo* root nodules bacterial isolates namely BG1 to BG3 were

identified as 0.016, 0.020, 0.018 and 0.014 respectively. Leghaemoglobin concentration is high in BG2 bacterial isolates (Fig.9).

Figure 5. Protein content of *M. putika* nodule isolates

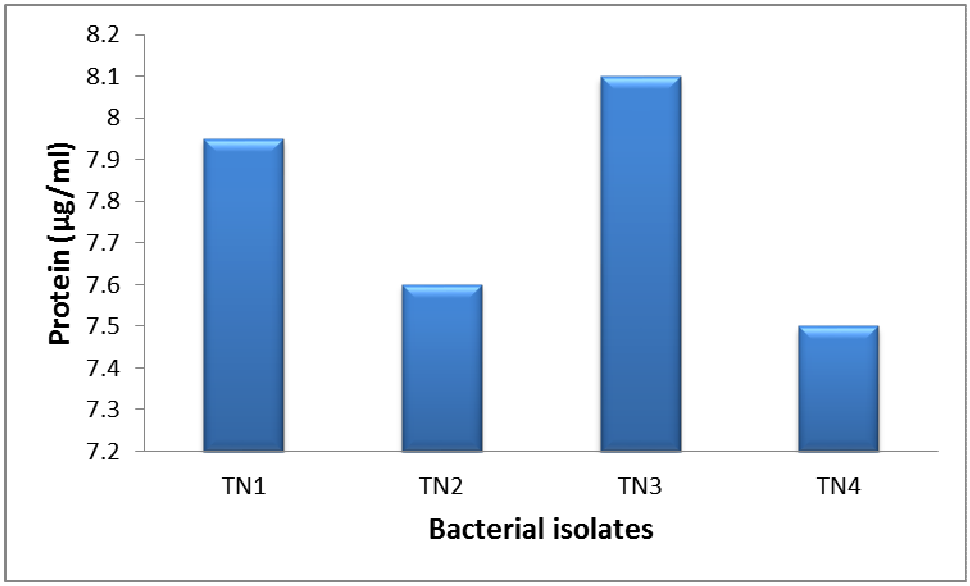
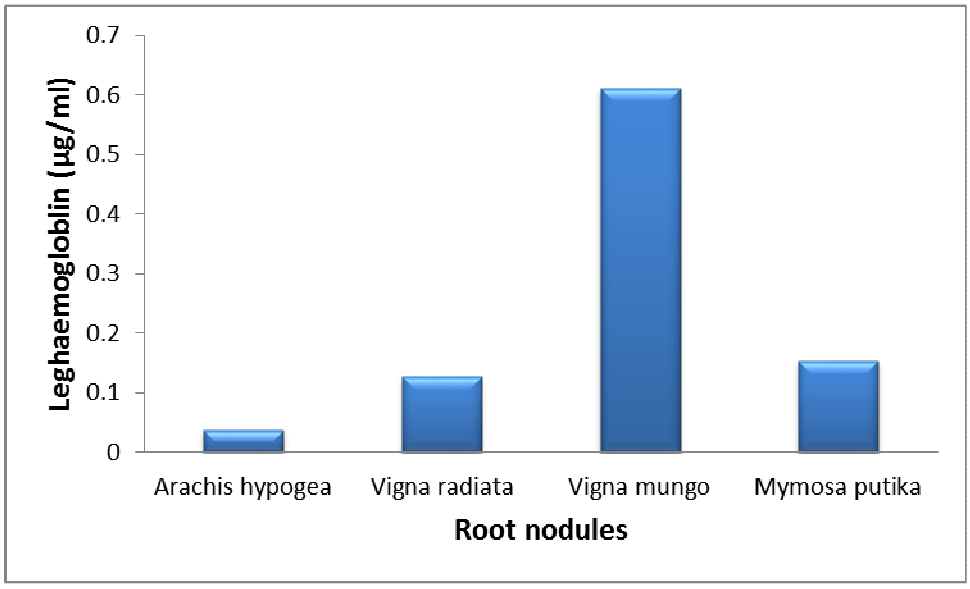


Figure 6. Leghaemoglobin concentration in various nodulating plants (Ab)



Mymosa putika

Absorbance value of leghaemoglobin content of *M. putika* root nodules bacterial isolates namely TN1 to TN4 were identified as 0.016, 0.032, 0.044 and 0.028 respectively. Leghaemoglobin concentration is high in TN3 bacterial nodule isolates (Fig.10).

From the result of leghaemoglobin assay result is in the following order *A. hypogea*, *M. putika*, *V. radiata* and *V. mungo* respectively. Thus the result suggested that, the protein as well as hemoglobin content is high in the *A. hypogea* root nodules and *Rhizobium* isolated from the root nodules of *A. hypogea*.

Figure 7 . Leghaemoglobin concentration of *Arachis hypogea* nodule isolates

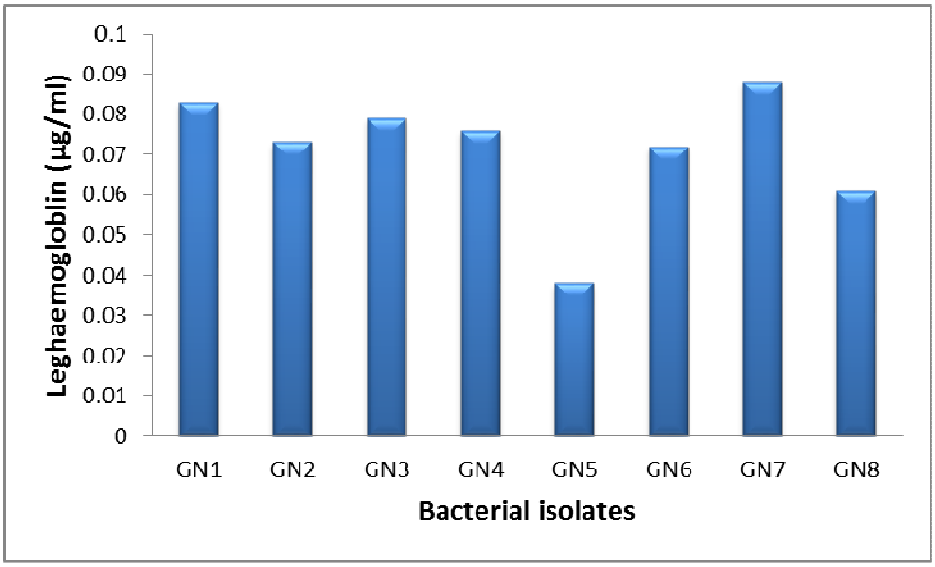


Figure 8. Leghaemoglobin concentration of *V. radiata* nodule isolates

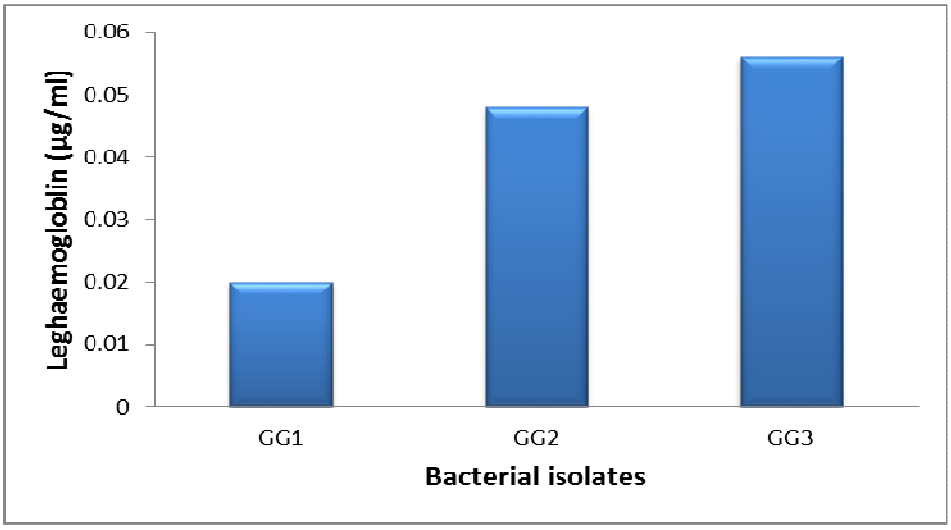


Figure 9. Leghaemoglobin concentration of *V. mungo* nodule isolates

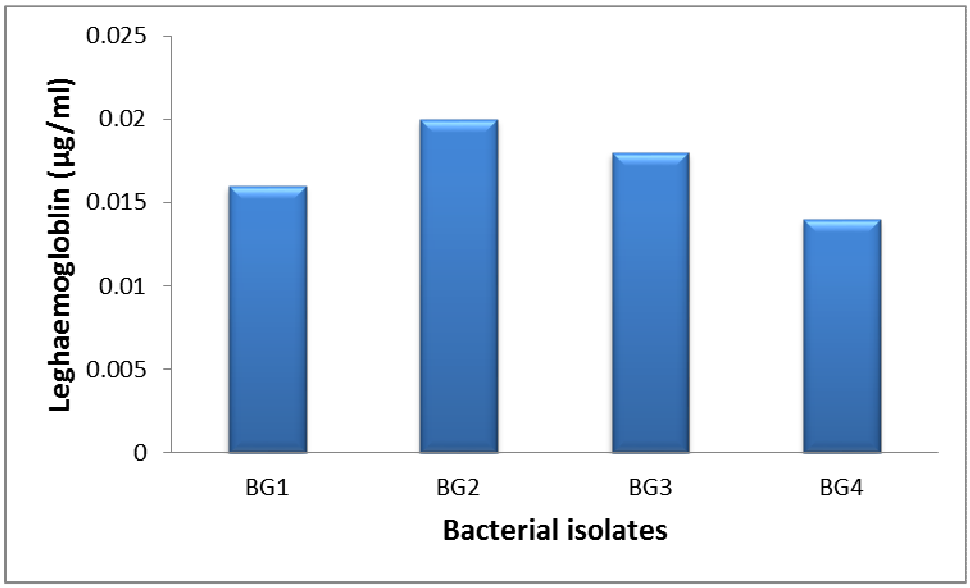


Figure 10. Leghaemoglobin concentration of *M. putika* nodule isolates

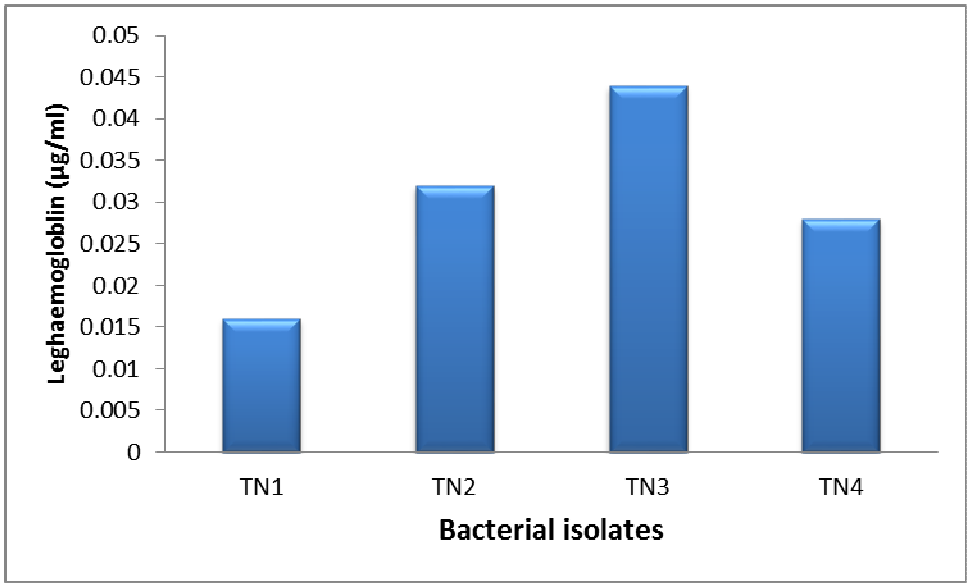


Table 2. Biochemical characterization of *Rhizobium* sps

S.No.	Isolates	Indole	MR	VP	Citrate	TSI	Catalase	Oxidase	Gram's staining
1.	GN1	-ve	- ve	- ve	- ve	ALS,AB	+ ve	+ ve	_ ve
2.	GN2	-ve	-ve	- ve	- ve	ALS,AB	+ve	+ve	-ve
3.	GN3	-ve	-ve	- ve	- ve	ALS,AB	+ve	+ ve	-ve
4.	GN4	-ve	-ve	- ve	-ve	ALS,AB	+ve	+ve	-ve
5.	GN5	- ve	-ve	-ve	-ve	ALS,AB	+ ve	+ve	-ve
6.	GN6	-ve	-ve	-ve	-ve	ALS,AB	+ ve	+ ve	-ve
7.	GN7	-ve	-ve	-ve	-ve	ALS,AB	+ve	+ve	-ve
8.	GN8	-ve	-ve	-ve	-ve	AB,ALS	+ve	+ve	-ve
9.	TN1	-ve	-ve	-ve	-ve	AB,ALS	+ve	+ve	-ve
10.	TN2	-ve	-ve	-ve	-ve	AB,ALS	+ve	+ve	-ve
11.	TN3	-ve	-ve	-ve	-ve	AB,ALS	+ve	+ve	-ve
12.	TN4	-ve	-ve	-ve	-ve	AB,ALS	+ve	+ve	-ve
13.	BG1	-ve	-ve	-ve	-ve	AB,ALS	+ve	+ve	-ve
14.	BG2	-ve	-ve	-ve	-ve	ALS,AB	+ve	+ve	-ve
15.	BG3	-ve	-ve	-ve	-ve	AB,ALS	+ve	+ve	-ve
16.	BG4	-ve	-ve	-ve	-ve	AB,ALS	+ve	+ve	-ve
17.	GG1	-ve	-ve	-ve	-ve	ALS,AB	+ve	+ve	-ve
18.	GG2	-ve	-ve	-ve	-ve	AB,ALS	+ve	+ve	-ve
19.	GG3	-ve	-ve	-ve	-ve	AB,ALS	+ve	+ve	-ve
Note: AB – Acid butt, ALS – Alkaline slant									

DISCUSSION

Earlier studies have shown that the legume root nodule the site of nitrogen fixation, and depend upon plant and bacterial cells. The oxygen binding protein leghaemoglobin found high concentration in nodules; it facilitates the diffusion of oxygen to the rapidly respiring bacteroids and concomitantly buffers the free O_2 concentration at an extremely low tension. Previous studies of legume nodules have indicated that formation of the heme moiety of leghaemoglobin is a function of the bacterial symbiont. Wittenberg *et al.*, 1980 identified that *hemA* mutant of *Bradyrhizobium japonicum* cannot carry out the first step in heme biosynthesis forms fully effective nodules on soybeans.

In our studies totally 19 rhizobacterial colonies were isolated from the root nodules of *A. hypogea*, *M. putika*, *V. mungo* and *V. radiate* and that bacterial were characterized biochemically as *Rhizobium* species. Gram negative rods with milky white, mucoid, translucent, circular in shape shiny, raised and were observed in 2-4 mm diameter. These findings are in line with Hussain *et al*. (2002); Oblisami (2005). The *Rhizobium* strain S24 and M11 of green gram were earlier found to be symbiotic effectively on five cultivars of green gram, with strain M11 producing significantly higher amount then strain S24 (Dadarwal, 1980). Leghaemoglobin content in nodules of all the species inoculated with Strain M11 was higher as compared to strain S24 of green gram.

All the rhizobium isolates were streaked on Bromothymol blue added YEMA selective media for further confirmation. Similarly the rhizobium isolates were showed positive for motility, Catalase and Oxidase test and showed negative for Indole, Methyl Red, Voges-Proskauer, Indole and Citrate utilization test. Our these findings are in close agreement with Elsheikh and wood (1989); Javed and Asghari (2008) who also characterized the rhizobium from soil and sunflower root nodules with the same positive biochemical tests.

To synthesis the role of leghaemoglobin in symbiotic nitrogen fixation it is important to know the state of leghaemoglobin in the functioning legume root nodule. Jones (1982) reported leghaemoglobin from soybean nodule extracted at pH 5.6 with ferric hemochrome. Crepon (2006) identified leghaemoglobin from soybean root nodules as a complex, leghaemoglobin a-X of ferric leghaemoglobin with a low molecular weight ligand X. In this studies, leghaemoglobin content varies from 0.22 to 0.59 mg per nodule in different cultivars (Mathur *et al.,* 1989), this results supports in our study the leghaemoglobin content was vary 0.34 to 0.78 mg and this content is more in nodules on plants grown in light than those grown darkness (Elsheikh and Wood, 1995). The leghaemoglobin also influenced the *Rhizobium* strain that forms the nodule and use of phosphorus fertilizers of *Rhizobium*. Stougaard (2000) reported that the use of phosphorus fertilizers had significantly increased in dry weight and leghaemoglobin content of root nodules. In this study, the result suggested that, the protein as well as hemoglobin content high in the *A. hypogea* root nodules and *Rhizobium* isolated from the root nodules of *A. hypogea*.

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