STUDIES ON THE PGPR ACTIVITY AND DIVERSITY OF RHIZOBIAL ISOLATES FROM SOIL

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Thesis submitted in part fulfillment of the requirements for the degree of

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CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON SURVIVAL OF INOCULATED RHIZOBIUM IN GROUNDNUT(*Arachis hypogaea* L.)"** submitted in part fulfillment of the requirements for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **AGRICULTURALMICROBIOLOGY** to the Tamil Nadu Agricultural University, Coimbatore is a record of bonafide research work carried out by **Miss.RAJASUNDARI K** . under my supervision and guidance and no part of the thesis has been submitted for the award of any degree, diploma, fellowship or other similar titles and that the work has not been published in part or full in any scientific or popular journal or magazine.

Place : Coimbatore

Date :

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DATE:

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*I wish to place my deep sense of profound gratitude, praise, glory and honour to **Lord Almighty** for bestowing His blessings and grace upon me to understand and unravel the mysteries of His wonderful creation and also enabling to give life to this piece of task.*

With full respect and from my deepest heart…

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(K.RAJA SUNDARI)

ABSTRACT

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The present study was undertaken to study the rhizobial diversity and isolate efficient *Rhizobium* having growth promoting activities from the TNAU farms. Nine soil rhizobia isolated from different field locations, two soil rhizobia isolated from ARS Aliyarnagar and three reference rhizobial cultures obtained from Department of microbiology, Tamil Nadu Agricultural University, Coimbatore were used. These isolates were authenticated as *Rhizobium* by microscopic examination, conducting various biochemical studies and plant infection test. Growth rate ranged from 0.154 to 0.180, and generation time ranged from 5.5 to 6.2 h. Rhizobial isolate ALN 7 produced higher amount of water soluble (3.61 mg ml^{-1}) and alkali stable (1.20 mg ml^{-1}) polysaccharides followed by isolate ALN 2. Isolate ALN 7 produced 3.5 µg ml^{-1} IAA and 3.3 µg ml^{-1}cytokinin. Intrinsic antibiotic resistance levels of the fourteen rhizobial cultures were determined for the antibiotics like erythromycin, kanamycin, gentamycin rifampicin and streptomycin. Rhizobial isolate ALN 7 was found to have resistance for most of the antibiotics. The effect of introduced rhizobial isolate ALN 7 was evaluated on groundnut crop and found that significantly superior in increasing

the soil nitrogen, available phosphorus, plant growth, nodulation, and yield over uninoculated control. Soil urease, phosphatase, dehydrogenase activity showed significant positive correlation with soil microbes *viz.*, total bacteria, fungi, actinomycetes and phosphobacteria. It also induced more leghaemoglobin production than control. Analysis of the SDS-PAGE protein profile of different rhizobial cultures revealed characteristic banding pattern for the rhizobial cultures. The size of the subunits separated were of the range of 14 KDa to 97 KDa. DNA amplification on finger printing study of the rhizobial isolates showed finger printing pattern characteristic of each culture and revealed clear polymorphism and genetic diversity among the rhizobial cultures. Dendrogram analysis revealed that high diversity occurred among the rhizobial isolates.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ml	mililitre
h	hour
g	gram
l	litre
mg	miligram
cm	centimetre
min	minute
BTB	Bromothymol blue
w/v	Weight/Volume
SEd	Standard deviation
CD	Critical difference
OD	Optical dencity
N	Nitrogen
P	Phosphurs
K	Potassium
µ	micron
µg	microgram
IAA	Indole acetic acid
ppm	Parts per million
rpm	Revolution per min
DAS	Days after sowing

CHAPTER I

INTRODUCTION

Legumes are the main protein source in many developing countries; the area cultivated with these plants world over is only about 25 per cent of the area that is used for cereal crop production. Groundnut is a major leguminous oil seed crop is grown in 10.8 lakh hectares in Tamil Nadu. Given escalating growth, land degradation and increasing demands for foods, achieving sustainable agriculture and viable agricultural systems is critical to the issue of food security and poverty alleviation in most, if not all, developing countries. It is fundamental to the sustained productivity and viability of agricultural systems worldwide.

The natural process of biological nitrogen fixation (BNF) constitutes an important source of nitrogen for crop growth and protein production in many soils and ecosystems. It therefore provides a major alternative to the use of commercial nitrogen fertilizer in agriculture. It has recently been estimated that global terrestrial BNF ranges between 100 and 290 million tons of nitrogen per year of which 40-48 million tons of N per year is estimated to be biologically fixed in agricultural crops and fields. In comparison, 83 million tons per year are currently fixed industrially for the production of fertilizer. Rhizosphere, the narrow zone of soil surrounding the root is under the immediate influence of the root system and is rich in nutrients when compared with the bulk soil, due to the accumulation of a variety of organic compounds released from the roots by exudation, secretion and deposition. This region is of most importance since it determines the survival of the introduced microbial inoculants for successful effect on crop growth.

Several opportunities to enhance BNF inputs are available across different agro-ecosystems and socio-economic conditions, *inter alia:* through altering the number of effective symbiotic or associated organisms in the system (inoculation); screening and selection of appropriate legume crop (selecting high BNF species well adapted to environmental conditions); and management practices that enhance N_2 fixation and recycling of net N_2 inputs into the cropping system.

Inoculation of different legume crops with inoculants has led to increase in grain yield from 18 to 62 per cent. However responses are often inconsistent in farmers' field due to the poor survival and establishment of inoculant strain in the rhizosphere of inoculated seedling. Inoculation rates higher than 10^5 cells $seed^{-1}$ are usually required for high nodulation, nitrogen fixation and grain yield (Rice *et al*., 2001). Several microorganisms native to the rhizosphere also influence the activity of inoculants strain and significantly regulate the establishment of the introduced strains. Usually the population of indigenous rhizobia ranges between 10^4-10^7cells g^{-1} in fallow and legumes grown soils.

Below- ground biodiversity determines resource use efficiency, as well as the sustainability and resilience of low-input agro-ecological systems, which ensure the food security of much of the world's population, the identification and promotion of the transfer of technologies for the detection of symbiotic soil microorganisms and their uses in plant nutrition. The estimation of potential and actual economic gains associated with reduced use of nitrogen and phosphorus chemical fertilization of crops with the enhanced use and conservation of symbiotic soil microorganisms; identification and promotion of best cultures for more sustainable agriculture and of conservation measures to conserve symbiotic soil microorganisms or to promote their reestablishment.

Specific objectives for this thesis are:

1. To establish the identity and diversity of the rhizobial species associated with TNAU farm soils.
2. To determine the efficacy of the symbiotic associations of rhizobial species with groundnut crop, by investigating their PGPR activity and nodulation capacity.
3. To investigate possible genetic diversity between rhizobial isolated species.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Groundnut (***Arachis hypogaea*** L) has traditionally been an important oil seed crop in India. It is a legume nodulated by rhizobia of cowpea cross inoculation group (Graham and Donawa, 1982). The genus *Arachis* belongs to the tribe **Aeschynomenaae** (**leguminosae**, subtribe, **papilonoideae**) with 22 described and possibly 40 nondescribed species (Gregory *et al.*, 1973).

Nodules were noted on groundnut roots by Poiteau as early as 1853. Burrill and Hansen during 1917 classified the groundnut root nodule bacteria into the "Cowpea cross-inoculation group'' (Allen and Allen, 1981). Unlike many legumes that are nodulated only by a specific group of rhizobia, groundnut is nodulated by rhizobia that also nodulate several species of tropical leguminous plants (Allen and Allen, 1981). Dadarwall *et al.* (1974) and Wange (1989) observed that rhizobia from many wild species of **Arachis** nodulated ***Arachis hypogaea*** L. However, there is some degree of specificity in nodulation, since *Rhizobium* isolated from many other legumes do not nodulate on groundnut (Nambiar, 1988).

Rhizobia are gram negative, rod shaped, motile bacteria belonging to the family Rhizobiaceae. They are aerobic chemoorganotrophs grow well in the presence of oxygen and utilize relatively simple carbohydrates and amino acid (Somasegaran and Hoben, 1994). They are able to establish effective nitrogen fixing symbiosis system with legume plants, in the specialized structure called root nodules. In the symbiotic process they reduce atmospheric nitrogen to ammonia using the enzyme nitrogenase and supply this essential nutrient to the host plant.

2.1. Characterization

The genus *Rhizobium* is well characterized by its novel association with legumes for fixing atmospheric nitrogen. Beijerinck (1888) was the first to isolate and cultivate the microorganisms from the nodules of legumes and named it as *Bacillus radicicola* which is now member of the genus *Rhizobium* (Jordan and Allen, 1994).

The present classification of root nodules bacteria as described in Bergy's Manual of Systematic Bacteriology separates the fast and slow growing microorganisms into two genera *Rhizobium* and *Bradyrhizobium*. The fast growing rhizobia is divided into three species, *Rhizobium leguminosarum*, *Rhizobium meliloti*, and *Rhizobium loti* with *Rhizobium leguminosarum* comprising three biovars *Rhizobium leguminosarum* biovar trifolii, *Rhizobium leguminosarum* biovar phaseoli and *Rhizobium leguminosarum* biovar viciae. In addition, fast growing rhizobia that nodulate soybeans have recently been assigned to a new species, *Rhizobium fredii* and slow growers are placed under the genus *Bradyrhizobbium* (Jordan, 1982). It is an expanding field because more and more rhizobia are isolated and characterized. The classification based on Zakhia and Lajudie (2001) is presented in Table 1. Sy *et al.*, (2001) reported that some rhizobia isolated from *Crotalaria* sp. in Senegal were physiologically different from all recognized rhizobia. They from a new species in the *Methylobacterium* genus. For which the name *M.nodulans* has been proposed.

2.2. Acid / alkali production

The acid production in culture media is an indication of acid sensitivity (Norris, 1965; Brockwell *et al.,* 1966; Bromfield and Jones, 1980). Bromfield and Kumar Rao (1983) studied the slow and fast growing rhizobial isolates of *Cajanus cajan* and *Cicer arietinum* for their ability to produce acid/alkali and reported that slow growing rhizobial isolates of *Cajanus cajan* produced alkali and the fast growers produced acid, whereas the fast and slow growing rhizobial isolates of *Cicer arietinum* produced only acidic reaction.

Acid or alkali production is a marked by variable strain characteristic and many studies have shown that it is influenced by changes in composition of the growth medium (Lange, 1961; Jarvis *et al.,* 1977; Parker *et al.,* 1977 and Date and Haliday, 1979). Cooper (1982) found that acid or alkali can be generated in a single medium by one strain at different phases of the growth cycle.

2.3. Polysaccharide production

The polysaccharides are composed of oligosaccharide repeat units that are assembled on a ployisoprenyl-pyrophosphate lipid carrier at the cytoplasmic face of the inner

membrane by a sequential transfer of specific glycosyltransferases. The oligosaccharides are subsequently translocated, polymerized, and secreted outside the cell (Whitfield *et al.,* 1997).

Polysaccharides of *Rhizobium* have been studied from the time of Ljunggren and Fahareus (1959). They considered that these substances may be the agents of symbiotic specificity. *Rhizobium* species produce variety of cell surface polysaccharides including exopolysaccharide, capsular polysaccharides, and liposaccharides (Becker and Puhler, 1998; Kannenberg *et al.,* 1998).

Sanders *et al.* (1978) reported that mutant strains of *Rhizobium leguminosarum* which produced less amount of extra cellular polysaccharide failed to nodulate host pea plant. The inability to infect the roots for nodulation and diminished nodule production were correlated to the quantity of polysaccharide produced (Napoli and Alberstein, 1980).

Pant and Gangwar (1984) reported that *Rhizobium trifolii* isolates, in general were found to produce maximum polysaccharide in sucrose and minimum in glucose medium. However, the amounts of polysaccharide were not correlated with the efficiency of the isolates to form nodules in the host plant.

Rhizobium sp. NGR234 has a broad host range and it nodulates more than 112 genera of the Leguminosae, as well as the nonlegume *Parasponia andersonii* (Pueppke and Broughton, 1999). The promiscuity of this strain can be partly explained by the specific symbiotic factors it produces. When interacting with plants, NGR234 synthesizes a large family of Nod-factors (Broughton *et al*., 2000; Perret *et al.,* 2000) as well as rhamnose rich LPS (Marie *et al.,* 2003) that contain a modified core oligosaccharide to which is linked an O-antigen called rhamnan (Fraysse *et al*., 2002). Deletion of genes encoding enzymes involved in rhamnan synthesis leads to the formation of nodules with reduced nitrogen fixation ability on the roots of *Vigna unguigulata* and other legumes (Broughton *et al.,* 2000).

Bacterial exo - polysaccharide (EPS) has been shown to be a critical factor in symbiotic interactions of rhizobia with leguminous plants that form indermediate type of nodules, for example, *Leucaena, Medicago, Pisum, Trifolium, and Vicia* spp.

(Becker and Puhler, 1998). *Rhizobium leguminosarum bv. trifolii* produces an acidic EPS that is important for the induction of nitrogen-fixing nodules on clover (Mazur *et al.,* 2002). Purified low molecular weight (LMW)) succinoglycan or galactoglucan (EPSII) can partially suppress the nodule invasion defect of EPS-deficient mutants (Gonzales *et al.,* 1996; Wang *et al.,* 1999). A study conducted by Kucuk *et al*. (2006) reported that 50 isolates from bean plants were characterized as producing copious amounts of exopolysaccharide slime containing granules of poly-β- hydroxybutyrate.

2.4. Growth rate of Rhizobial cultures

The genus *Rhizobium* which fixes nitrogen in root nodules of leguminous plants is divided into two main groups based on their growth rate in an appropriate complete medium. The fast growing strains have a mean generation time of approximately 3 h in a rich medium at 30°C under aerobic conditions and the other slow growing strains has a generation time of about 6-7 h under the same condition (Gara and Shanmugam, 1978). The growth rate of rhizobial strains isolated from *Acacia* ranged from very fast (54 min) to very slow (16 h and 15 min) studied by Zerhari *et al*. (2000). Pant and Gangwar (1984) studied the correlation between the growth rate and the efficiency of nitrogen fixation. He found a significant positive correlation between cell populations at 92 h and the efficiency. The generation time of *Bradyrhizobium japonicum* (344-461 minute) was reported by Pelczar *et al*. (2005).

2.5. Production of plant growth promoting substances by *Rhizobium* sp

Many rhizosphere associated microorganisms are involved in the production of phytohormones. Rhizobia are important members of plant growth promoting rhizobacteria (PGPR) showing several plant growth promoting activities (Glick, 1995).

2.5.1. Indole acetic acid production

Production of plant growth promoting substances like indole acetic acid (IAA) from tryptophan by rhizobia was demonstrated by Georgi and Beguin (1939). According to Tanner and Anderson (1963) rhizobia have the ability to reduce nitrate to nitrite and convert tryptophan to indole actic acid.

The effectiveness of the isolates of *Rhizobium* sp. in nodulating groundnut was correlated positively with IAA producing ability of the same. Reports have been to indicate the correlation between IAA production *in vitro* and the symbiotic efficiency of the rhizobia (Vidyasekaran *et al.*, 1973).

Rhizobium may be able to produce plant growth regulators or stimulated production of those compounds by plant roots of non legumes. (Triplett, 1986).

Thiman (1936) demonstrated that nodules contain high levels of auxin. Later Chen (1938) found that *Rhizobium* cultures produce auxin when grown on the proper substrates. Vasanthakumar and McManus (2004) reported that IAA production by members of the *Agrobacterium* / *Rhizobium* ranged from 0.02 μg ml^{-1} to 3.7 μg ml^{-1}.

Sinorhizobium meliloti PP3 isolates found to release good amount of IAA. 9.99 per cent increase in shoot length was recorded with seeds inoculated with *Sinorhizobium meliloti* PP3 isolate and 61.4 per cent increase in shoot weight was recorded with *Sinorhizobium meliloti* PP3 in *Cajanus cajan*. Still, IAA production increased about 50 per cent when it is mixed with *Burkholderia* sp. (Pandey and Maheswari, 2007) as mixed inoculants interact synergistically which yield better and quick results (Bashan, 1998).

2.5.2. Cytokinin

Adenosine is the precursor for cytokinin and promotes cell division, cell enlargement and tissue expansion (Salisbury, 1994). The effects of exogenously applied cytokinin on plants are numerous, the most notable of which is enhanced cell division, root development and root formation.

Plant associated microorganisms have been found to produce over 30 growth promoting compounds belonging to cytokinin group. As many as 90 per cent of microorganisms found in rhizosphere release cytokinin (Barea *et al*., 1976). Some of PGPR producing cytokinin are *Azospirillum* (Cacciari *et al*., 1989), *Rhizobium* (Upadhaya *et al*., 1991) and *Rhizobium legumonisarum* (Noel *et al*., 1996). Zahir *et al*. (2001) proposed the hypothesis that bacterially supplied cytokinin to the soil can

improve the growth and yield of treated plants as increase in yield and N, P, K content of grain after exogenous application of cytokinin in rice fields.

The role of cytokinin in lateral root formation and symbiosis in *Lotus japonicus* was studied by Lohar *et al.* (2004). Features of lateral root and nodule development in legumes have been compared (Hirch, 1992 and Koltai *et al.,* 2001) and plant hormone cytokinin has been implicated in the formation of both organs (Hirsen *et al.,* 1995 and Schmulling, 2002). The higher quantities of cytokinin (11.2 mg ml^{-1}) produced in *Bradyrhizobium* was reported by Uma (2003).

2.5.3. Phosphate solubilization

Phosphorus (P) is one of the major plant growth-limiting nutrients although it is abundant in soils in both inorganic and organic forms. Phosphate solubilizing micro-organisms (PSMs) are ubiquitous in soils and could play an important role in supplying P to plants in a more environmentally friendly and sustainable manner. Although solubilization of P compounds by microbes is very common under laboratory conditions, results in the field have been highly variable. This variability has hampered the large-scale use of PSMs in agriculture (Gyaneshwar *et al*., 2002). Soil micro organisms are involved in a range of processes that affect P transformation and thus influence the subsequent availability of phosphate to plant roots (Richardson, 2001).

In the rhizosphere region 20-40 per cent of total microbial population accounts for phosphate solubilizers (Swaby and Sperber, 1959). Chandrasekaran (1969) reported high degree of inorganic phosphate solubilization by rhizosphere bacteria isolates compared to non rhizosphere bacterial isolates. Mohammad (1984) reported that *Rhizobium leguminosarum* solubilizes phosphate from organic compounds through the action of phosphatase enzyme. Rhizobial strains when it is applied along with Arbuscular Mycorrhizal fungi, increased phosphorus uptake efficiency as it related to the compatible paring of these two microorganisms (Zarei *et al*., 2006). Strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are among powerful P solubilizers (Rodriguez and Fraga, 1999). The highest P solubilization activity was obtained with two strains P31 and R1 of *Rhizobium leguminosarum bv. Phaseoli.* The two selected rhizobia were superior root colonizes of maize and lettuce as compared

to other P-solubilizing bacteria studied (Chabot *et al*., 1996 a). Peat or bran inoculants of *Rhizobium leguminosarum* RCR 1044/ *Aspergillus niger* and *Bradyrhizobium* USDA 3447/ *Aspergillus niger* significantly increased drymatter yield, nitrogen, and phosphrous content of faba bean and soybean, respectively when grown in alkaline soil amended with rock phosphate (Abd- Alla *et al.*, 2001).

2.6. Intrinsic antibiotic resistance

The use of antibiotic resistant strains of *Rhizobium* as a method of identification has received much attention. Pinto *et al.* (1974) differentiated *Rhizobium meliloti* strains in the nodules from *Medicago sativa* grown under laboratory condition by using levels of natural resistance to kanamycin and streptomycin.

Kremer and Peterson (1982) determined the pattern of resistance and susceptibility to different levels of antibiotics for both fast and slow-growing rhizobia. However, Graham (1963) and Pankhurst (1977) reported that slow-growing rhizobia of cowpea and soybean groups were more tolerant to antibiotics than fast-growers.

Date and Hurse (1992) recorded spontaneous resistance to rifampicin (30 μg ml^{-1}) and streptomycin (500 μg ml^{-1}) in strains of *Bradyrhizobium* those had similar growth and rhizosphere colonizing abilities, but they were not equally effective in nitrogen fixation or competitive in nodule formation in *Desmodium intortum*.

Rhizobial isolates obtained from 10 wild legumes of sal forest ecosystem in subtropical north western Himalayas were evaluated for their intrinsic antibiotic resistance with different concentrations of six antibiotics *viz.*, streptomycin, gentamycin, rifampicin, chloramphenicol, neomycin and tetracycline, the results suggested that intrinsic antibiotic patterns were both strain specific and antibiotic specific (Subramanian and Babu, 1993).

Resistance to streptomycin has been studied by Vincent (1970) for ecological studies involving survival of *Rhizobium* strains or competition between strains in rhizosphere of legume plants. Schwinghamer and Dudman (1973) compared between spectinomycin and streptomycin resistance in ecological or genetic studies with

rhizobia. Moreover, in a study by Rodriguez *et al.* (1987) on tolerance to streptomycin and novobiocin, it was evident that existence of clear differences in the tolerance or resistance between variants from the same strain.

Keyser and Munns (1979) and Ayanaba and Wong (1992) reported that, strains of cowpea *Rhizobium* from strongly acidic soils were more resistant to higher concentrations of antibiotics than strains from less acidic soils .

Germell and Roughley (1993) developed a method for studying the population dynamics of rifampicin resistant rhizobia even when they were less in numbers compared with naturalized strains in soil. The inhibitory effect of amoxicillin, ampicillin, colaxicillin, norbactin, oxytetracycline and tetracycline at a concentration of 50, 100, 150, 250, 500 and 1000 ppm respectively on *Rhizobium leguminosarum* was studied and inferred that rhizobial strains shows sensitivity at high dose for few antibiotics while at lower dose, *Rhizobium* sp. was found to be more tolerant to different antibiotics (Mishra and Bhattacharya, 1994). *Rhizobium* TNAU 14 was found to have resistance for ciproflaxin at 20 μg ml^{-1} and nalidixic acid at 30 μg ml^{-1} (Thavachelvam, 2001).

Kucuk *et al*. (2006) isolated 30 isolates from bean (*Phaseolus vulgaris* L.) which showed an intrinsic resistance to the antibiotics chloramphenicol (50 μg ml^{-1}), streptomycin (100 μgml^{-1}), kanamycin (10 μg ml^{-1}), and erythromycin (30 μg ml^{-1}). Zilli *et al.* (2004) reported that isolate *Bradyrhizobium japonicum,* showed high intrinsic resistance to antibiotics *viz.*, gentamycin sulphate(40 μgml^{-1}), streptomycin sulphate (100 μg ml^{-1}) and kanamycin (30 μg ml^{-1}).

2.7. Protein profile study

Noel and Brill (1980) indicated that the subjective comparison of complex patterns of proteins made definite identification of closely related strains by the sodium dodecyl sulphate polyaccrylamide gel electrophoresis (SDA-PAGE) technique. SDS-PAGE analysis has the advantage that it supplies specific genetic information which is readily detectable in different test samples (Harrison *et al*., 1989). This technique was used to analyse the *Rhizobium japonicum* populations of two soybean fields in Wisconsin. In 1986, Kamiker and Brill analysed the whole cell

protein profile of *Bradyrhizobium* sp. by the SDS –PAGE technique and constructed proteinogram based on the profile, which showed identical protein pattern between isolates.

Host plant preference for nodulating strains and strain competitiveness within two natural soil populations of *Rhizobium leguminosarum biovar trifolii* were investigated using the techniques of polyacrylamide gel electrophoresis. Membrane protein and lipopolysaccharide profile of *Rhizobium leguminosarum biovars viciae, trifolii and phseoli, Rhizobium meliloti and Agrobacterium tumefaciens* strains were analyzed and compared by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Differencies in the profiles were used to distinguish all the strains tested in the study (De Maagd *et al*., 1988).

Moreira *et al*. (1993) characterized rhizobia, isolated from different divergence groups of tropical legumes by comparative poly acrylamide gel electrophoresis of their total proteins, and isolated several rhizobial strains from root nodules of *Phaseolus coccinens* and characterized these strains based on their SDS-PAGE protein banding patterns. Salinity induced changes in the protein profile of *Rhizobium* sp. was studied by SDS-PAGE method. The difference in the protein profiles was more marked in salt grown cells than the control (Saxena *et al*., 1996). Yang and Lin (1998) analysed cellular protein profile of *Rhizobium fredii* by SDS-PAGE to distinguish several strains of Taiwanese soils.

2.8. Ecology of rhizobia

Rhizobia are facultative microsymbionts that live as normal components of the soil microbial population when not living symbiotically in the root nodules of the host legume. Besides the root nodule they are mostly found on the rhizoplane, and close to the root surface and to lesser extent in the non-rhizosphere soil. Rhizobia pass through various pleomorphic stages in its life cycle *viz.,* short rods and long rods with flagellation (swarming cells) exist in soil, infection thread present in root hair and bacteriods seen only in nodule structures. In the absence of the host, free living rhizobia are in their saprophytic phase and compete with other soil microflora for the nutrient resources.

Populations of naturalized rhizobia as well as introduced rhizobia have been shown to differ in their tolerance to the major environmental factors, which affect the persistence and survival of the individual species in the soil (Vidor and Millar, 1980). The most problematic environments for rhizobia are marginal lands with low rainfall, extremes of temperature and acid soils with low nutrient status and poor water holding capacity (Bottomley, 1992 and Brockwell *et al.,* 1995). They thrive at the temperature of 25-30˚C and the pH of 6.0 – 7.0.

The indigenous population is adapted to the soil environmental factors like soil structure, nutritional availability, moisture, pH, salinity and temperature where as it is alien to the introduced species. But which may be alien to the introduced species changes in soil pH have been found to alter the relative proportion of nodules formed by competing strains of *Rhizobium*. However, the manipulation of pH may not always favor nodule formation by effective rhizobia (Russell and Jones, 1975).

The success of rhizobial innoculant is generally reduced in the presence of a large indigenous soil microflora. Danso *et al.,* (1975) reported that the rhizobial population was decreased due to the presence of predatory protozoan. The use of pesticides such as thiram that suppress predatory protozoa resulted in enhancing the nodulation of Phaseolus *by Rhizobium phaseolus* (Ramirez and Alexander, 1980).

2.9. Native rhizobia

The abundance of indigenous populations of rhizobia in soil has been reported to vary between <10 and 10^7 g^{-1} of soil for a particular species. The density of rhizobia in nodules is about 10^9 cells g^{-1}, several orders of magnitude greater than that in soils.

Johnson *et al*. (1965) reported that when different strains of *Bradyrhizobium japonicum* were applied to soil having naturalized rhizobia, the nodules formed at normal rate of inoculum were hardly five per cent with the inoculated strain. A few strains showed increased nodule occupancy with increasing dose of inoculum whereas others did not show increase in nodule occupancy suggesting specific strain competence as a factor for nodulation competition irrespective of their population.

Weaver and Frederick (1974) showed that the percentage of nodules formed by an inoculant strain applied to soil containing native *Bradyrhizobium japonicum* population varied proportionally with the logarithm of number of bacteria in the inoculum. The inoculation of rhizobia at the rate of 10^4 cells seed^{-1} in soils having more than 10^3 rhizobia g^{-1} soil failed to compete with native rhizobia. In order to obtain 50 per cent nodule occupancy by an introduced strain, an inoculum dose, 1000 times that of rhizobial population is required (Weaver and Frederick, 1974).

Roughley *et al*. (1976) introduced *Rhizobium trifoli* strain at the rate of $2x10^6$ cells seed $^{-1}$ in a soil containing naturalized rhizobial population ranging from 0 to $9x10^6$ rhizobia g^{-1} soil. The introduced rhizobia formed 100 per cent noduation at sites where native rhizobia were absent. At sites having greater number of naturalized rhizobia, the introduced rhizobia formed sufficient nodules in the first year but in subsequent years, the introduced rhizobia disappeared, suggesting the necessity of continuous use of superior strains for nodulation. The host plants have been reported to promote free-living rhizobial population, probably due to release of bacteria from senescent nodules or due to release of plant exudates, which are utilized preferentially by rhizobia (Kucey *et al*., 1989).

The quantification of nodulation competitiveness has been considered important in predicting the level of inoculation required to establish inoculant strains successfully in soils containing indigenous rhizobia. Dowdle and Bohlool (1987) studied competition between *Bradyrhizobium japonicum* and *Rhizobium fredii* for nodulation of soybean by using three different soils in pots. Two soils were from soybean field with no history of *Rhizobium* inoculation and one soil from rice field with no history of soybean cultivation. Inoculation did not result in significant increase in nodule number on plants in any soil. These studies showed that *Rhizobium* strains indigenous to particular soils are more competitive than alien strains in nodulation of their soybean host. Thies *et al*. (1991) showed that the competitive success of inoculant rhizobia and crop response to inoculation was inversely related to indigenous population size in Hawaiin soils.

The studies by Moawad *et al*. (1983), Robert and Schmidt (1983), Bushby (1982) and Leung *et al*. (1994) suggested that the assumption of a positive correlation

between the abundance of rhizobial strains in nodules and in the surrounding soil may not be valid always.

2.10. Symbiotic efficiency of *Rhizobium* on biomass production and nitrogen fixation

Inoculation success of *Rhizobium* in legumes at field levels has been well documented from time to time. Remarkable increase in groundnut pod yields has been achieved through inoculation with *Rhizobium*; (Staphorst *et al.*, 1975; Stoddard, 1978 and Nambiar, 1985). Geneally groundnut has been found to nodulate freely and effectively with a wide range of cowpea type *Rhizobium* strains (Buchanan and Gibbon, 1974).

Graham and Donawa (1982) screened 19 effective nitrogen fixing rhizobia in groundnut. They found that inoculation with effective rhizobial strains increased yields by 64 to 82 percent. Inoculation has increased shoot dry weight, kernel weight and pod weight than the uninoculated treatment. Raverkare *et al*., (1988) observed that inoculation of the rhizobial strain NC92 in *Arachis villosa* had resulted about two fold increases in shoot nitrogen content than the uninoculated control.

Somasegaran *et al*. (1990) evaluated 23 strains of rhizobia from 14 leguminous species on a Thai cultivar of bambarra groundnut in a Leonard jar trails and found that the symbiosis ranged from completely ineffective through moderate effectiveness to full effective and *Bradyrhizobium* TAL 169 isolated from *Vigna unguiculata* ranked most effective.

Rroco *et al*. (2003) reported that plants cultivated on the sterile soil were significantly retarded in root and shoot growth due to insufficient Fe supply. Rubin and Paolillo (1984) also found that sterilized soils that inhibited germination of spores of the fern, *Onoclea sensibilis*.

Bremer *et al.* (1990) studied the effect of *Rhizobium leguminosarum* lentil in relation to number of nodules, shoot weight and nitrogenase activity and reported that inoculation had increased yield up to 135 per cent and total N fixed ranged from 10 to 764 kg ha^{-1}. There are significantly more nodules on the inoculated pigeon pea plants

(21-298 nod pl^{-1}) than on non inoculated plants (3-54 nod pl^{-1}). This indicated an increased longevity of the nodules of inoculated plants with clear benefits for increased nitrogen fixing potential and production (Grange *et al*., 1999).

Kumar Rao *et al*., (1987) reported that nodulation continued to increase from 20 DAS until 60 – 80 DAS, followed by a decline, and indicated the beneficial effect of seed inocuation with *Rhizobium* and observed that an enhanced soybean yield by 11 per cent alone over the recommended fertilizer. Inoculation of *Rhizobium* increased the N content, dry matter in seed and vegetative parts, N harvest index and seed yield of soybean (Tashin Sogul, 2006). Chabot et al. (1996) found an increase in dry matter in lettuce crop on inoculation with *Rhizobium leguminosarum bv phaseoli* and also higher phosphate concentration in treated plants than uninoculated plants. Paul and Shende (2000) suggested that as much as 94 per cent of fixed nitrogen is exported to host plants and the amount of nitrogen fixed by groundnut and grain legumes from 25-200 kg ha^{-1} in 60-120 days. Dakora (1985) reported increased nodulation, kernel weight and nitrogen fixation in groundnut by 61.2, 60 and 65 percent over control when complete fertilizes were added.

Groundnut derived 18.2-24.7 per cent of total nitrogen from symbiotic nitrogen fixation of *Bradyhizobium*. Toan and Hein (2002) reported that *Rhizobium* could replace 15 kg ha^{-1} of nitrogen requirement in groundnut.

2.11. Effect of nitrogen fertilizer on the *Rhizobium*-legume symbiosis:

Nitrogenase and hydrogenase catalysed reactions are subject to external stimuli (Maier *et al.,* 1978). Various inhibitors like azide, carbon, monoxide etc. (Burris, 1979) have been shown to be potent inhibitors of nitrogenase activity in a variety of nitrogen fixing microorganisms.

The effect of combined nitrogen on the physiology of the *Rhizobium* legume symbiosis has been the subject of many studies (reviewed by Gibson 1976; Munns 1977; Rigaud, 1981). Addition of mineral nitrogen in soil or synthetic media affects both nodulation and nitrogen fixation depending on the concentration and the form of nitrogen compounds, the time of application the cultivation conditions, and finally the type of plant and bacteria used. The number of root nodule per plant grown at 1.5 mM

NH_3 was about 50 per cent that of control plants. Root nodules were not observed in plants grown at 3.0 mM NH_4NO_3 or more (Moudiongui and Rinaudo, 1987). Combined treatment of *Rhizobium*, simazine and nitrogen increased the pod yield to the extent of 70 per cent over control in *Cicer* arietinum(Rigaud, 1981)

Rhizobium inoculation significantly increased the total nodule number per plant, 100 seed weight, yield and protein content of seed of *Cicer arietinum*. Inoculation with *Rhizobium* strain TAL 1148 resulted in a significant increment in most of the parameters studied (El Hadi and Elsheikh, 1999), and also reported that *Rhizobium* strain TAL 1148 increased yield by 72 and 70 per cent, where as, 50 kg N ha^{-1} increased it by 70 and 69 per cent in the first and second seasons respectively.

2.12. Soil enzyme activity

Biological activities in soil takes place with help of enzymatic processes. The level of enzyme activity is used as indicator of soil fertility (Skujins, 1978). Quantitative measurement of soil enzyme activities can contribute to our understanding of these biological transformations by allowing us to evaluate the activity present in soil (Tiwari *et al*., 1989). Soil enzyme is also found to have important role in the rhizosphere activity (Dakora *et al*., 2002).

2.12.1. Urease

Urease plays an important role in germination and in seedling's nitrogen metabolism. It may function coordinately with arginase in the utilization of seed protein reserves during germination (Polacco and Holland, 1996). Urea was the first organic molecule to be synthesized and jack bean urease was the first enzyme ever to be crystallized (Sirka and Brodzik, 2000). Due to urease activity, bacteria are able to use as a sole nitrogen source (Mulrooney *et al*., 1989).

The primary role of urease is to allow the organism to use external or internally generated urea as a nitrogen source (Mobley *et al*., 1995). In plants, urease is the only enzyme that is able to recapture nitrogen from urea (Polacco and Holland, 1996).

Urea added to soil as fertilizer is hydrolyzed enzymatically by soil urease ($NH_2CO\ NH_2 + H_2O\ 2NH_3 + CO_2$), resulting in the formation of ammonia and carbon

dioxide. Urease activity in the soil can be used as an indicator for total biological activity and for soil fertility. Urease activity is increased by the addition of readily decomposable organic material under aerobic condition and it is largely dependent upon the organic carbon content of the soil. (Beri *et al.*, 1989).

Soil urease activity increased after the addition of organic substances promoting microbial growth but subsequently decreased and eventually stabilized (Zantua and Bremner, 1976). Type of vegetation and type of organic matter added to the soil also influence the urease activity. There is a positive correlation of urease activity with organic matter content accounted for most of the variation in the soil urease activity. Increased activity of glutamine enzyme in the roots of a legume plant, *Lotus japonicus*, leads to decreased plant biomass production, possibly due to limited nitrate uptake (Limami *et al*., 1999).

2.12.2. Phosphatase

Soil always contains considerable amount of total phosphates which is protected by different soil components. Barber and Lynch (1976) observed that phosphatase activity in rhizosphere soil increase due to release by growing microbial biomass utilizing root exudates and by the direct release of extra cellular phosphates from the roots of growing plants (Bieleskli, 1978). Phosphatase enzymes are present in a larger proportion in soil as ester phosphate. Phosphatases are classified into alkaline and acid phosphatase. In complex organic compounds, alkaline and acid phosphatase gets immobilized in soil clays and humates there by constituting a persistent extacellular catalytic activity (Burns, 1986). Phosphatase activity is often not correlated with the degree of phosphate in natural system.

The increased phosphatase activity in summer was attributed to high temperatures and bacterial population numbers (Chhonkar and Tarafdar, 1984). In rainy season the low concentration of phosphatase was found to be due to low microbial populations resulted in low concentrations of phosphorus. Tarafdar *et al.* (1989) reported that phosphatase activity showed an insignificant positive correlation with soil available phosphorus. Phosphatase is found to mediate the release of inorganic phosphorus from organically bound phosphorus returned to soil as litter and other organic debris (Jha *et al.*, 1992). Li *et al*. (2004) reported that soil acid phosphatase activity in the

rhizosphere of chickpea was also significantly higher than maize regardless of phosphorus sources. Organic phosphorus sources can be utilized by the plant after they are hydrolyzed by phosphatase (Gilbert and Knight, 1999). Agro forestry species with high acid phosphatase activities can mobilize and utilize organic phosphorus in the soil (George *et al*., 2002a).

2.12.3. Dehydrogenase

Soil dehydrogenase activity is a measure of microbial biomass. Dehydrogenase is universal enzyme that belongs to the group of oxidoreductase enzymes, produced by all organisms and linked with respiratory processes. It is found that there is no preferential stimulations of any particular group of soil microorganisms (Stevenson, 1959) because it does not add any additional substrate to the soil.

Ross (1970) stated that dehydrogenase activity appeared to be more dependants on the metabolic state of the soil or on the biological activity of the microbial population than on any free enzyme present. Dehydrogenase enzymes appear to be linked with microbial activity associated with initial break down of organic matter (Ross, 1971). Soil dehydrogenase is considered to be an index of endogenous soil microbial activity (Moore and Rusell, 1972). The level of enzyme using the end product as a measure can be used as an index of total microbial activity (Skujins, 1976). It is a measure of the intensity of microbial metabolism in soil and thus the microbial activity of soil (Tabatabai, 1982). Measurement of the activity of intracellular dehydrogenase provides information on the biological activities on soil microorganisms (Dkhar and Mishra, 1983). Dehydrogenases are believed to be intracellular enzymes mainly linked with microbial respiratory processes (Bolton *et al*., 1985).

Soil dehydrogenase activity is a useful indicator of microbial metabolism levels and hence a parameter for the activity of soil microflora and for microbial biomass (Benckiser *et al*., 1984). Viswanath *et al*. (1997) suggested that Triphenyl Tetrazolium Chloride (TTC) dehydrogenase technique for measuring the biological activity could be applicable to coarse textured and more open soils. They also indicated that fungi contributed to the soil dehydrogenase activity as well. Sajjad *et al*.

(2002) observed plant residues had a variable effect on dehydrogenase activity suggesting that even a similarity in chemical composition might not induce similar changes in microbial functions.

2.13. Leghaemoglobin

The role of leghaemoglobin is to maintain and regulate the supply of oxygen to respiring *Rhizobium* bacteria at a level that does not damage the oxygen-sensitive nitrogenase enzyme (Appleby, 1982; Davies *et al.,* 1999 and Appleby, 1974). It is well documented that the leghaemoglobin content of most legume root nodules is positively correlated with their nitrogen fixing ability (Appleby, 1984). Although some mutant strains of rhizobia have the ability to form nodules containing leghaemoglobin or its apoprotein but unable to fix nitrogen (Schwinghamer *et al*., 1970, Maier and Brill, 1976 and Bisseling *et al.,* 1980). The complementary situation, of nitrogen fixing nodules containing no leghaemoglobin, has not been recorded (Appleby, 1984). The purification of leghaemoglobin is generally achieved by extraction of nodules with aqueous buffer, ammonium sulphate precipitation.

2.14. Occurrence and significance of ureides in legumes

N2- fixing legumes move fixed N from the nodules to the aerial portions of the plants primarily in the form of ureides, allantoin and allantoate. Ureide biochemistry includes two additional enzymatic steps in the conversion of uric acid to allantoin in the nodule and the mechanism of allantoin and allantoate breakdown in leaf tissue (Christopher *et al*., 2006). *De novo* synthesis of purines is the main route for ureide formation in nodules (Schubert, 1986; Atkins and Smith, 2000). Ureides do not accumulate to high levels in seeds (Streeter, 2005).

Urate is derived from xanthine dehydrogenase action in infected cells of the nodule. Urate is then moved to the uninfected cells of the nodule where it is oxidized in microbodies, eventually forming allantoin (Smith and Atkins 2002). Khadri *et al*. (2001) reported that the inhibition of enzymatic activities related to ammonium assimilation of the GS/GOGAT pathway promote other enzyme activities (GDH and AAT). The inhibition of enzymes related to purine catabolism (xanthine dehydrogenase and urease) decreased the ureide content and alternatively the amino

acid content increase in the nodules of common bean. *Phaseolus vulgaris* is considered as a legume that could transport and store nitrogen as ureides (Hansen *et al*., 1993; Hongria *et al*., 1987).

Nitrogen fixation in soybean is especially sensitive to soil drying. The basis of sensitivity appears to be related to the fact that ureides are transported from the nodules, and to the ureide concentrations increase with water deficits in leaves resulting in an apparent feedback to nodules involving ureides that inhibit activity (Serraj and Sinclair 2003).

The principal compounds transported from nodules to the shoot are the ureides, allantoin and allantoic acid (Sprent, 1980; Thomas and Schrader, 1981; Atkins, 1982). Allantoin and allantoic acid have been observed in different parts of the plant belonging to various genera of leguminaseae. These compounds were synthesized in nodules and roots translocating these isolutes to different parts of the host plant which could be correlated with effective nitrogen fixation (Pate, 1973). Matsumoto *et al.* (1977a) studied the distribution and change in the contents of alantoin and allantoic acids in developing nodulating and non nodulating soybean plants. The allantoin levels in stems, roots, and nodules of nodulating variety increased with the growth and attained a maximum at the green pod stage and then decreased. On the other hand the organs of non-nodulating variety accumulated little allantoin over the different growth periods. The allantoin level was highest in the stems of nodulating variety soybean (14.8 mg g^{-1} of tissue). Matsumoto *et al*. (1977).

Xylem sap composition of nodulated and non-nodulated cowpea was examined by Pate *et al.* (1980). The quantity of nitrate and ureide stored in leaflets, stem, petioles and roots of cowpea varied in a complex manner with level of nitrate added and the presence or absence of nitrogen fixation.

Triplett (1986) indicated that *Glycine max* and *Glycine soja* plants were found to produce ureides when plants were inoculated with fast growing *Rhizobium fredii* and he also observed the decline in ureide level when nitrate and ammonia were added to the nutrient solution. Experiments in which ureide was fed to soybean plants showed that N_2 fixation activity was readily inhibited as a result of increased ureide concentration in the plant (Vadez and Sinclair 2000).

The possibility of differing catabolic enzymes for allantoic acid leading to differences in N_2 fixation sensitivity to water deficits opens the possibility of genotypic segregation based on ureide degradation characteristics. Ureide accumulation (Purcell *et al*., 2000) and degradation (Vadez and Sinclair, 2000) in the tolerant cultivar Jackson is insensitive to Mn concentration in the leaves, indicating the presence of allatoate amidinohydrolase. Further, Vadez and Sinclair (2001) compared ureide accumulation in leaves of nine soybean cultivars with varying sensitivity of N_2 fixation to water deficit.

2.15. Groundnut *Rhizobium* diversity

Soil microorganisms play an important role in soil process that determines plant productivity. For successful functioning of introduced microbial bioinoculants and their influence on soil health, exhaustive efforts have been made to explore soil microbial diversity of indigenous community, their distribution and behaviour in soil habitat (Hill,2000).

Sahgal and Johri (2003) outlined the current status of rhizobial taxonomy and enlisted 36 species distributed among seven genera (*Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Methylobacterium, Rhizobium, and Sinorhizobium*) derived, based on the polyphasic taxonomic approach. Ogasawara *et al.* (2003) reported new species, *Sinorhizobium abri* from *Abrus precatorius* and *Sinorhizobium indiaense* from *Sesbania rostrata.* Genetic diversity amongst rhizobia of five medicinal plants of the sub Himalayan region was reported by Pandey *et al.* (2004)

Genetic diversity of five Sinorhizobia from medicinal legume, *Mucuna pruriens* was investicated using ARDRA Analysis. Restriction patterns produced by amplified DNA coding for 16S rDNA(ARDRA) and were placed into two genotypes. All five isolates belonged to a single cluster. A distinct similarity was observed between the RAPD and 16S rDNA RFLP Analysis.

Randomly amplified polymorphic DNA (RAPD) profiles have provided new tools for investigating genetic polymorphism. This method was used by Van Rossum *et al. (*1995) for genetic analysis of *Bradyrhizobium* strains nodulating *Arachis hypogea* and nodule isolates of *Arachis* sp. (Khbaya *et al.* (1998). Van Rossum *et al.*

(1995) observed greater genetic diversity by RAPD finger printing than that compared to rDNA sequence comparision while studing genetic diversity in *Bradyrhizobium* strains nodulating peanut. Random primers have been used to determine variation among legume symbionts by RAPD (Selenska-Pobell *et al*., 1995).

Fall *et al.* (2003) identified cowpea varieties with high nitrogen fixing potential, to verify the pattern of variation within cowpea breeding lines in Senegal, and to evaluate the applicability of ARA and RAPD techniques in the screening of cowpea varieties. Wazael *et al.* (2004) observed diversity with in 100 selected accessions of bambara groundnut from Tanzania was assessed with 49 polymorphic AFLP bands.

Zilli *et al*. (2004) reported that, in areas where neither soybean, nor cowpea had ever been planted, the highest diversity values were found. On the other hand the presence of a leguminous crop tended to decrease the diversity of the *Rhizobium* population.

CHAPTER III

MATERIALS AND METHODS

3.1. General

3.1.1. Location

The experiments were conducted at the Department of Agricultural Microbiology, TamilNadu Agricultural University, Coimbatore.

3.1.2. Glassware

All the glassware used for the experiments were cleaned first with soap water and then soaked in 6.5 percent chromic acid and finally washed with water. The glassware thus cleaned were rinsed once with distilled water and sterilized before use.

3.1.2. Chemicals used in the study

The chemicals used were of analytical grade obtained from Hi-Media, Fisher and E.Merck.

3.1.4. Sterilization techniques

Glasswares were sterilized in a hot air oven at 180° C for three hours. All growth media, broth and water blanks were sterilized in an autoclave at 121°C for 15 min. Vitamin solution growth factors and antibiotics were filter sterilized using Sartorius 0.22 μm nitrocellulose membrane filters. Isolation, purification, inoculation and other microbiological works were carried out in laminar air flow chamber.

3.1.5. Rhizobial cultures

The standard rhizobial cultures used in this study were (TNAU14, COG 15, SOB 1) obtained from the culture collection center of the department of Agricultural Microbiology, TamilNadu Agricultural University, Coimbatore.

3.2. Maintenance of rhizobial strains

The rhizobial strains were maintained on yeast extract mannitol agar (YEMA) slants at 4°C (Vincent, 1970). Yeast extract mannitol broth was inoculated with the rhizobial strains and incubated at room temperature for 3 days in an orbital shaker and this culture was used for further study.

3.2.1. Isolation of rhizobia from soil

Soil samples were collected from different fields of the Tamilnadu Agricultural University. Different cropping areas were chosen in each location *viz.,* continuously cropped with groundnut, legumes, non-legumes and uncultivated one. These soil samples (1g) were serially diluted then transferred to YEMA medium and plates were incubated at room temperature for 3-4 days. Predominant colonies were selected for further study. The isolates were purified and stored at 4°C in YEMA slants.

3.3. Characterization of different strains of rhizobia

Characterization of different strains of rhizobia were carried out by following the methods of Somasegaran and Hoben (1994).

3.3.1. Gram staining

Gram staining was carried according to the procedure described by Vincent (1970).

3.3.2. Growth on Congored yeast extract mannitol agar (Vincent, 1970)

One ml of appropriate dilution on *Rhizobium* was transferred in to the Petri plates containing YEMA with Congo red medium. The plates were incubated at room temperature for 3-4 days. The phenotypic and growth patterns were observed.

3.3.3. Growth on glucose peptone agar (Vincent, 1970)

Rhizobia were streaked on glucose peptone agar medium and incubated at room temperature for three days.

3.3.4. Growth on Hofers Alkaline broth (Hofer, 1935)

A loopful of the rhizobial inoculum was inoculated in to 50 ml of the hoofers alkaline broth (Annexure I) and incubated in the shaker at room temperature. Absence of growth in the Hofer's alkaline broth indicates *Rhizobium*.

3.3.5. Growth on lactose agar (Bernaetz and De ley, 1963)

The rhizobial isolates were streaked on petriplates containing lactose agar medium (Annexure I) after the growth, Benedict's reagent was poured over the agar medium. Absence of yellow colour was considered as positive for *Rhizobium*.

3.3.6. Growth on YEMA + Bromothymol blue medium (Norris, 1965)

The rhizbial isolates were streaked on yeast extract mannitol agar plates containing bromothymol blue (Annexure I). The acid production will be indicated by yellow colour and the alkali production will be indicated by blue colour.

3.3.7. Polysaccharide production

To the 100 ml of YEM broth, 2 ml of the standard inoculum (10^9cells ml^{-1}) was added and incubated at 30° C for 72 h grown culture was used for estimating water soluble and alkali stable polysaccharide as described by (Sutherland and Wilkinson,1971).

3.3.7.1. Water soluble polysaccharide

The broth culture was centrifuged at 5000x g for 15 minutes. The cell pellet was set aside for the analysis of alkali stable fraction of polysaccharide.

To the 20 ml of the supernatant an equal volume of 90 percent ethanol was added mixed well and placed at 4°C over night to precipitate the water soluble portion of polysaccharide. Then it was centrifuged at 7000 rpm for 15 minutes. The pellet was dissolved in one percent acetic acid and the polysaccharide content was estimated by anthrone reagent method.

3.3.7.2. Alkali stable polysaccharide

The harvested cell pellet was suspended in 5 ml of distilled water and added with 5 ml of 0.1 N KOH then it was boiled 30 minutes and cooled to the room temperature. The suspension was centrifuged at 5000 rpm for 10 minutes; the supernatant was retained and neutralized with 0.1N HCl for the estimation of alkali stable polysaccharide.

3.3.7.3. Estimation of total reducing sugar

The polysaccharide portions of both water soluble and alkali stable were estimated in terms of total reducing sugars by anthrone reagent method (Dubois *et al.,* 1951). One ml of sample was taken in the test tube and to this 4 ml of anthrone reagent was added. Then the mixture was heated for 10 min and cooled rapidly. The intensity of the color developed (green to dark green) was read at 520 nm in spectrophotometer. The quantity of polysaccharides present in the sample was determined by referring to the standard graph prepared with glucose as standard.

3.3.8. *Invitro* growth study

The overnight grown rhizobial strains served as inocula for conducting the growth experiments. The OD of the inocula was adjusted to 1.0 at 540 nm Bausch and Lamb Spectronic 20 Spectrophotometer. Two ml of inocula were inoculated in to 100 ml of yeast rextract mannitol broth and incubated over a gyrotary shaker at 28°C, at different time interval of 0, 12, 24, 36, 48, 72, 84 and 96 h, the growth was determined by measuring the absorbance at 540 nm, against appropriate blank in a Bausch and Lamb Spectronic 20 spectrophotometer (Glenn *et al.,* 1980).

$$\text{Growth rate (K)} = \frac{\log_{10} X_t - \log_{10} X_0}{0.301Xt}$$

Generation time = 1/ Growth rate

3.3.9. Plant infection test

3.3.9.1. Seed surface sterilization

The seeds were placed in the sterile 250 ml conical flask and rinsed with 95 percent alcohol for 10 seconds to remove waxy material, alcohol was drained off. Then 30 percent hydrogen peroxide solution was added and the contents were mixed contact. After 2 minutes hydrogen peroxide solution was drained off and the seeds were rinsed with six changes of sterile water. Aseptic procedure was maintained throughout the rinsing. Sufficient water was poured to submerge the seeds and placed in the refrigerator for four hours to imbibe. After the imbibition, the seeds were placed on moist germination paper in Petri plates and incubated under dark conditions for pre-germination.

3.3.9.2. Growth pouches

The poly propylene (16x18 cm) bags were used as growth pouches. In side the pouches, germination paper towel was inserted with fold at the top in such a way that it will form bed pouch for the seed placement. Over this, three holes were made at an appropriate distance. Fourty ml of the nutrient solution was poured into the pouches and sterilized in the autoclave. The germinated seeds were placed by keeping the radicle inside the hole made in the germination paper. After two days of the establishment, one ml of inoculum was poured on to the root surface. (Somasegaran and Hoben 1994).

3.4. Plant growth promoting activity of the isolates in groundnut

The isolates were screened for their plant growth promotion activities by conducting the following experiments.

Production of IAA

Production of cytokinin

3.4.1. Estimation of Indole acetic acid

Spectrophotometric estimation of auxin production by bacteria (Bar and Okon, 1993).

3.4.1.1. Procedure

Luria Bertani medium (Annexure I) amended with 100 µg ml^{-1} of tryptophan was prepared and then sterilized. One ml culture was inoculated and incubated for one week at room temperature in shaker. The flasks were covered with black paper. At desired interval, 25 ml sample was with drawn and cells spun at 5000 rpm for 15 min.

3.4.1.2. Extraction process

The cell free filtrate was adjusted to pH 2.8 with 1N HCl. Equal volume of peroxide free cold diethyl ether was added and the contents shaken in separating funnel and allowed to stand for 4 h at 4°C with intermittent shaking. The aqueous phase was separated from the solvent phase. At 4 h interval, two or more ether extraction was done. Solvent phases were pooled and evaporated to dryness in vacuum. The residue was dissolved in 2 ml methanol for analysis.

3.4.1.3. Analysis

A quantity of 0.5 ml sample was taken in a test tube. 1.5 ml distilled water and 4 ml of salper's reagent (Annexure I) were added and incubated for 1 h at 28 C. the intensity of pink colour developed was read using spectrophotometer at 535 nm. By referring to a standard graph, the quantity of indole acetic acid in the sample was determined.

3.4.2. Bioassay for cytokinin

3.4.2.1. Radish cotyledon assay (Letham, 1971)

Radish seeds were surface sterilized with 0.5% sodium- hyphochlorite solution and after rinsing with sterile water allowed to germinante on blotter paper in darkness at 24-25°C for 3 days. Cotyledons of uniform weight were selected and placed on filter paper in 9 cm petriplates. Ten ml of 2 mM potassium phosphate buffer

of pH 5.9 followed by 1 ml of cell free culture extract prepared earlier were added to petriplates. Similar set of experiment was conducted with various concentrations of benzyl amino purine *viz.*, 2,4,6,8 and 10 µg ml^{-1} instead of culture extract. Cotyledons were incubated under fluorescent light for 3 days at 24°C. The weights of the cotyledons were recorded after drying with blotting paper and a dosage response curve was drawn.

3.5. Intrinsic antibiotic resistance (IAR)

Different rhizobial strains were grown in tryptone yeast extract broth (Annexure I) for 48h at 30°C with shaking and 1.5 ml culture was taken in microfuge tube and centrifuged at 10,000 rpm for five minutes. The pellet was washed thrice with saline solution and suspended in one ml of sterile saline solution. To 50 ml of sterilized and cooled media appropriate concentration of antibiotics were added and poured into sterile petri plate. After solidification, plates were divided into 20 squares by forming labeled grid on the back of petri plate and by the using sterile tooth picks each square was inoculated with different rhizobial strains under sterile condition. Control plates were maintained separately excluding the antibiotics in the medium. After 2-3 days of incubation, the growth was recorded.

Antibiotics used and their concentration in µg ml^{-1}

Gentamycin: 25, 50, 100, 150, 200, 250, and 300

Kanamycin: 25, 50, 100, 150, 200, 250, and 300

Rifampicin: 25, 50, 100, 150, 200, 250, and 300

Streptomycin: 25, 50, 75,100,150,200,250, and 300

Erythromycin: 25, 50, 100, 150, 200, 250, and 300

3.6. Protein profile study

The total cellular protein of the rhizobial strains was analyzed for their polypeptide patterns in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

3.6.1. Protein extraction (Bhaduri and Demchick, 1983; Kamiker and Brill, 1986; Yang, 1998)

To obtain cellular protein, rhizobia were cultured in tryptone yeast extract broth (annexure I) at 30°C with shaking for 3 days. The cells were harvested by centrifugation and 1000 rpm for 15minutes. The pellets were washed in 10mM Tris HCl (PH 7.6) and incubated with ice cold acetone for 10 minutes. After washing with Tris buffer, the pellet was suspended in 1:1 mixture of 10Mm Tris HCl (pH 7.6) and SDS sample buffer (Annexure IV). The cells were lysed in a steam cabinet for 5 minutes and allowed to cool immediately. The broken cells were mixed vigorously to break clumps of DNA and centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was stored at -20°C for further use.

3.6.2. Estimation of protein content

The protein content was determined as per Lowry's method (Lowry *et al.*, 1951).

Materials required

Reagent A, B, C, D (Annexure IV)

3.6.2.1. Working standard

Ten ml of stock solution (50mg Bovine serum albumin in 50ml) was diluted to 50 ml with distilled water. One ml of this solution contains 200 mg protein.

3.6.2.2. Estimation

Five hundred µl of sample was taken in test tubes and the volume was made up to 1 ml. To each tube, five ml of reagent C was added mixed well and allowed to stand for 10 minutes. Then 0.5 ml of reagent D was added, mixed well and incubated at room temperature in the dark for 30 minutes. The intensity of blue color developed was read in spectrophotometer at 660 nm against appropriate blank. The protein content was calculated by referring to a standard curve prepared with bovine serum albumin.

3.6.3. SDS-PAGE

The protein profile of different strains of rhizobia was carried out by following the method of Laemmeli (1970) and Kamiker and Brill (1986).

The electrophoresis was carried out in a vertical slab gel unit in a continuous system. The gel plates were cleaned thoroughly with soap water and alcohol, and then assembled in the casting tray and casted with separating gel mixture (Annexure IV) over this stacking gel mixture was (Annexure IV) poured and the comb was placed on the top of the sandwich to form slots or wells. The wells were rinsed with electrode buffer before loading 40 µl sample per well.

3.7. Symbiotic potential of introduced *Rhizobium*

A pot culture experiment was conducted at the Department of Agricultural microbiology, Tamilnadu agricultural university, Coimbatore, to study and monitor the effect of the *Rhizobium* ALN 7 on groundnut. The treatments were as follows.

T_1 - uninoculated control

T_2 - starter N+ sterilized soil

T_3 - *Rhizobium* isolate ALN 7 + sterilized soil

T_4 - *Rhizobium* isolate ALN 7 + starter N + sterilized soil

T_5 - Starter N + unsterilized soil

T_6 - *Rhizobium* isolate ALN 7 + unsterilized soil

T_7 - *Rhizobium* isolate ALN 7 + starter N + unsterilized soil

Design- Completely Randomized Design (CRD)

Crop - Groundnut (Variety: CO 4)

Starter Nitrogen - 20 kg ha^{-1}

Each treatment was replicated 5 times. Before sowing, the seeds were surface sterilised and treated with inoculum of respective strains. Five plants were maintained per pot during the study period. Biometric parameters like shoot length, root length, plant dry weight, nodule number, nodule dry weight, nitrogen content of shoot and root were recorded. The soil rhizobial population was enumerated at 30 days intervals.

3.7.1. Shoot length

The shoot length of the groundnut plant was measured from the collar region to the top of the plant and expressed in cm.

3.7.1.2. Root length

Root length was measured from the collar region to the tip of the root and expressed in cm.

3.7.1.3. Plant dry weight

The plants were dried in the hot air oven at 70°C to a constant weight and the dry weight was expressed as g pl^{-1}.

3.7.1.4. Number of nodules

Uprooted plants were washed free of adhering soil particles under a steam of tap water and the number of nodules were counted and expressed as number pl^{-1}.

3.7.1.5. Nodule dry weight

The separated nodules were oven dried at 70°C to a constant weight and the dry weight was recorded and expressed as mg pl^{-1}.

3.7.1.7. Enumeration of *Rhizobium* in pot soil

The population of introduced *Rhizobium* isolates in the pot culture soil was monitored by enumerating the viable rhizobial cells at fifteen days intervals. The total bacterial population was also enumerated by standard serial plate method.

3.7.1.8. Enumeration *Rhizobium* by plant infection method (Brockwell, 1980; Somasegaran and Hoben, 1994)

The plant infection count, by most probable number (MPN) method was followed to determine the number of viable and infective rhizobia in the soil. For this purpose growth pouches were used. The soil inoculum was serially diluted and 1 ml of respective dilution was inoculated in the pouches containing pre germinated seeds. Then the growth pouches were arranged in the wooden rack and maintained in the green house. Thirty days after inoculation the pouches were examined for the presence of nodules. The nodulated units (pouch with respective dilution along with replications) were recorded and the population was calculated using the formula.

$$\text{Population} = \frac{m \times d}{V} \times rhizobia\ g - 1$$

Where

m= most likely number (referred from MPN table – Annexure– II)

d = Reciprocal of the middle dilution used.

v = volume of inoculum used.

3.8. Characterization of experimental soil

3.8.1. Soil nitrogen

Nitrogen content of the samples was determined by the alkaline permanganate method (Subbiah and Asija, 1956). A 5 g of soil was taken in distillation flask and 25 ml of 0.32 percent $KMnO_4$ and 25 ml of 2.5 percent NaOH was added to the soil. 20 ml of 2 % boric acid and a drop double indicator was taken in a beaker and kept near the delivery end. The distillation was carried out for 3 min. and the liberated ammonia was collected and titrated against 0.02 N sulphuric acid. From the titer value the available nitrogen was calculated.

3.8.2. Soil phosphorus

Available phosphorus of the samples was determined by colorimetric method (Olsen's method) Olsen's *et al.,* (1954). A 5 g of soil was taken in a shaking bottle. 50 ml of 0.5M sodium bicarbonate and a pinch of activated carbon were added to the soil. And shaked for 30 min. the extract was filtered using Whatman no. 40 filter paper. 5 ml of filtrate was pipette out in to a 25 ml volumetric flask and 4 ml of reagent B was added and made up to 25 ml and left for 30 min for colour development. The absorbance value of the colour developed in the sample was read at 660 nm in colorimeter and the available phosphorus was calculated from the standard curve.

3.9. Effect of enzymatic activity in experimental soil

3.9.1. Urease

Ten grams of dry and sieved soil was taken in 100 ml volumetric flask. To this 1.5 ml toluene was added. Mixed well and incubated for 15 minutes. Then 10 ml of 10 % urea solution and 20 ml of citrate buffer was added. Mixed thoroughly, stoppered and incubated for 3 h at 37 ^{0} C in an incubator. Then volume was made up to 100 ml with distilled water and mixed by shaking immediately. The contents were filtered. One ml of filtrate was pippetted out in 50ml volumetric flak. To this 9 ml of distilled water, 4 ml of phenate and 3 ml of NaOCl were added. Mixed well and allowed to stand for 20 minutes. The volume was made up to 50 ml and mixed well. The bluish green colour developed was read at 630 nm. Simultaneously a blank also prepared (without urea solution). The concentration of urease in the sample was obtained from the standard graph using diammonium sulphate (Halstead, 1964). The urease activity was expressed as microgram of NH_4 –N released per gram of soil per hour ($\mu g\ NH_4$- N g^{-1} Soil h^{-1}).

3.9.2. Phosphatase

Five grams of soil sample was taken in boiling tube. To this 10 ml of distilled water, 0.25 ml of toluene and 1ml of 10 mM p- nitrophenyl phosphate (PNPP) were added. The mixture was incubated at room temperature for 1 h and then added with 5

ml of 0.5 M $CaCl_2$ and 20 ml of 0.5 M NaOH. The content was filtered using Whatman No.42 and volume made up to 50 ml with distilled water. The colour intensity read at 420 nm. The concentration of phosphatase was obtained from standard graph. (Tabatabai and Bremner, 1969). The phosphatase activity was expressed as microgram of p – nitrophenol released per gram of soil per hour (µg P-nitrophenol g^{-1} soil h^{-1}) with reference to a standard curve of p- nitrophenol.

3.9.3. Dehydrogenase

Five grams of soil sample was taken in boiling tube. To this 1 ml of 3 % 2, 3, 5-Tri phenyl tetrazolium chloride was added. Then 1 ml of 1 percent glucose and 2.5 ml of distilled water was added. Incubated for 24 h. After that 10 ml methanol was added. Incubated for another 5 h. The content was filtered through Whatman No 1 filter paper. The samples were washed thoroughly with methanol. The red colour developed was read at 485 nm. (Casida *et al*., 1964). The dehydrogenase activity was expressed as microgram of triphenyl farmazon formed per gram of soil per hour. (µg TPF g^{-1}soil h^{-1}). The concentration of dehydrogenase in the sample was obtained from the standard graph using triphenyl farmazon.

3.10. Estimation of Allantoin in different parts of the groundnut plant

Distilled water was added to 0.25 ml of sap sample to a volume of exactly 0.75 ml. Inorder to hydrolyse all allantoin to allantoic acid, 0.25 ml of 0.5 mol l^{-1} sodium hydroxide solution was added and heated to 100° C for 8 minutes. The mixture was cooled in an ice bath and 0.25 ml of 0.65 mol l^{-1} hydrochloric acid was added and heated at 100° C for 4 minutes to hydrolyze the allantoic acid to glyoxylate and urea.

The solution was again cooled in an ice bath and then 0.25 ml of ice cold phosphate buffer pH 7.0 (0.4 mol^{-1} NaH_2PO4 – Na_2HPO4) and 0.25 ml of a solution of Phenylhydrazinehydrochloride(66 mg in 20 ml of water, freshly prepared) were added. After standing at room temperature for 5 minutes to allow formation of glyoxylic acid phenyl hydrazone the solution was finally cooled again and 1.25 ml of cold concentrated HCl and 0.25 ml of a freshly prepared aqueous solution of potassium ferric cyanide (1.7 percent w/v) was added. After standing at room

temperature for another 10 minutes to form the 1, 5 diphenyl formazon. The absorbance of the solution was read at 535 nm (Vogels and Van Der Drift, 1970).

3.11. Leghaemoglobin assay (Dilworth, 1980)

Purified preparation of leghaemoglobin was conveniently assayed by the pyridine haemochromogen method (Dilworth, 1980). Equal volumes of 4.2 M pyridine in 0.2M NaOH and leghaemoglobin solution were mixed and the resulting haemochrome reduced with a few crystals of sodium dithionite. Absorbance at 556 nm was measured against a reagent blank and converted to leghaemoglobin concentration using $\Sigma 556nm_{mM} = 34.6$ mg cm^{-3}.

In less purified preparations, one cuvette of the leghaemoglobin (lb) alkaline pyridine reagent was oxidized with minimal amount of $Fe(CN)_6$ and another reduced as above with dithionite. The value of A_{556} nm minus A_{539} nm was determined and used to calculate leghaemoglobin concentration from $\Delta \Sigma_{mM} = 23.4$ (Bergersen *et al.*, 1973).

3.12. Genetic diversity of Groundnut rhizobia

3.12.1. Genomic DNA extraction of rhizobial isolates

Procedure

The total genomic DNA of rhizobial isolates was isolated by following the Ivanova *et al.,* (2000) with slight modifications. The rhizobial cultures were grown in TY medium (Annexure I) at incubator shaker at 150 rpm and 30°C for 48 h. About 25 ml of actively grown rhizobial cultures were centrifuged at 6000 rpm; 5 min at 4°C to harvest the cell and suspended the cell pellets in 1 ml of TE and 0.5 ml of 1-butanol and mixed well. Centrifuged the contents at 6000 rpm; 5 min at 4°C and the supernatant discarded. The cell pellets were resuspended in 2 ml of TE buffer and centrifuge at 6000 rpm; 5 min at 4°C to remove trace of butanol. The cell pellets were added with 1 ml of TE buffer and 100 µl lysozyme (10 mg ml^{-1} freshly prepared) and incubated at room temp. for 5 min. 100 µl of 10 per cent SDS and 25 µL of 100µg ml^{-1} proteinase K were added, mixed well and incubated at 37°C for 1 h. After incubation, the cell lysates were added with 200 µl of 5 M NaCl; 150 µl of CTAB (10

percent stock) and incubated at 65°C for 10 min. Deproteinized cell lysate with 1 ml of Phenol:choloroform mixture and centrifuged at 6000 rpm; 10 min at 4°C. The aqueous layer was transferred carefully to new 1.5 or 2.0 ml microfuge tube, the volume was noted; added 0.6 volume of ice-cold isopropanol and incubated at -20°C for overnight. The precipitated DNA was pelletized by centrifuge at 12000 rpm; 15 min at 4°C. After discarding the supernatant, the pellet was vacuum dried for 30 min.and resuspended in 100 µl of TE buffer. One µl of DNAse free RNAse (10 mg ml ml^{-1} stock) was mixed by swirling, incubated at 37°C and store at -20°C for further use.

3.12.1.1. Genetic diversity of rhizobial isolates by Randomly Amplified Polymorphic DNA

Two RAPD primers (OPQ1 and OPM10) were used in this study. RAPD-PCR reactions were carried out as described elsewhere (Guthrie *et al.*, 1992). RAPD PCR reaction mixture (25 μl) contained 25 ng of genomic DNA, 100 μM each of dNTPs, 1 μM primer, 2.5 μM $MgCl_2$ and 1U of Taq polymerase. PCR reactions were performed in PCR thermal cycler (Eppendoff Mastercycler, German) with the programme as: initial denaturation of 95°C for 3 min followed by 35 cycles of denaturation at 94°C for one min; annealing at 36°C for 45 s; extension at 75°C for one min and final extension at 72°C for 7 min. PCR products were resolved in 1.5% agarose gel, stained with ethidium bromide and documented in Alpha imager TM1200 documentation and analysis system. Strong and clear RAPD bands were scored as primer wise and similarity and clustering analysis were done using the software, NTSYS-PC 2 package (Numerical taxonomy analysis program package, Exeter software, USA). Similarity among the strains was calculated by Jaccard's coefficient (Jaccard 1912) and dendrogram was constructed using UPGMA method (Nei and Li 1979).

3.13. Statistical analysis

The results of the experiments were subjected to statistical scrutiny as per the methods detailed by the Panse and Sukhatme (1985).

CHAPTER - IV

EXPERIMENTAL RESULTS

4.1. Isolation of *Rhizobium* from soil

Rhizobia cultures from the different soil samples were enumerated as per the procedure of Vincent (1970). Predominant colony from each sample source only was taken for further studies. Nine isolates were obtained from different field soils of Tamil Nadu Agricultural University, Coimbatore, tenth and eleventh isolate was obtained from field soils of Aliyar Nagar (Table 2). The isolates were purified and transferred to yeast extract mannitol agar slants and maintained until further use. Three more cultures are obtained from the culture collections of the Department of Agricultural Microbiology, Tamil Nadu Agricultural University. The details of the isolates are furnished in Table 3.

4.2 Authentication and characterization of rhizobial isolates used in the study

The rhizobial isolates used in the study were subjected to various cultural and biochemical characterization and the results are given in the Table 4.

The colonies formed on the surface of YEMA medium was translucent to white opaque, discrete, round, varying from flat to convex from with smooth margin. All the isolates were found to be gram negative. These isolates produced yellow colouration when inoculated in the YEMA medium containing bromothymol blue indicating that they are acid producers. In the lactose agar medium these isolates failed to form yellow colouration, when Benedict's reagent was added. In the glucose peptone agar, poor growth was observed and in Hofer's alkaline broth no growth was seen. Plant infection test carried out with 11 soil isolates along *Rhizobium* sp. TNAU 14, *Rhizobium* sp COG 15 and *Rhizobium* sp SOB1 on both green gram and groundnut indicated that all the strains were capable of infecting green gram and groundnut hosts (Plate 2)

4.3. Polysaccharide production

The rhizobial isolates were tested for the production of both water - soluble and alkali stable polysaccharides in the yeast extract mannitol broth. The results are presented in the Table 5.

4.3.1. Water soluble polysaccharide

The amount of water soluble polysaccharide production varied from 1.65 mg ml^{-1} to 3.61 mg ml^{-1}. The maximum amount was recorded by the soil isolate of *Rhizobium* ALN 7 (3.61 mg ml^{-1}) and the minimum amount was produced by the soil isolate EB 34.

4.3.2. Alkali stable polysaccharide

The alkali stable polysaccharide production by these isolates varied from 0.065 mg ml^{-1} to 1.200 mg ml^{-1}. The maximum amount was produced by soil isolate of *Rhizobium* ALN 7 and the minimum amount was recorded in WLO 2.

4.4. *Invitro* growth of rhizobial isolates

Invitro growth rate of the rhizobial isolates and generation time are given in the Table 6 and 7. All the rhizobial strains grew well, though to varying levels in the YEM liquid medium. While most of the isolates reached stationary phase of growth at 60 h after inoculation, EB34, TNAU 14, *Rhizobium* ALN 2 and *Rhizobium* ALN 7 isolates reached stationary phase of growth at 72 h. The growth rate of *Rhizobium* isolates found to be varied from 0.154 to 0.180 and it was also expressed in generation h^{-1} ranged from 5.5 to 6.2 h (Table 7, Fig 1).

4.5. Production of plant growth promoting substances by rhizobial isolates

Productions of plant growth promoting substances by various rhizobial isolates were tested by bio assays. Cytokinin production was tested by radish cotyledon assay.

4.5.1. Estimation of Indole acetic acid

Quantity of IAA produced was tested for rhizobial isolates. The results of the experiments are presented in Table 8, Fig 2. Quantity of IAA produced varied between 1.4 μg ml $^{-1}$ to 3.5 μg ml $^{-1}$. Among the isolates, *Rhizobium* ALN 7, produced higher quantity of IAA (3.5 μg ml $^{-1}$) followed by *Rhizobium* ALN 2 (3.4 μg ml $^{-1}$) and COG 15 and SOB 1(3.2 μg ml^{-1}). Compared to other isolates CBS12 produced the least amount (1.4 μg ml^{-1}) of IAA.

4.5.2. Cytokinin production

Cytokinin production was tested by radish cotyledon assay; the results are presented in Table 9, Fig 3, Plate 3. Cytokinin production was tested by measuring the weight increase of radish cotyledons due to culture extracts of rhizobia and compared with corresponding effect due to the application of standard benzyl amino purine (BAP). The quantity of BAP production varied between 0.71 μg ml^{-1} to 3.3 μg ml $^{-1}$ (Table 8). Among the isolates *Rhizobium* ALN 7 produced substantial quantity of BAP (3.3 μg ml-^{1}).

4.6. Intrinsic antibiotic resistance (IAR)

The intrinsic antibiotic resistance level of the different rhizobial isolates was determined for the antibiotics like streptomycin, erythromycin, rifampicin, Kanamycin, gentamycin, and ciproflaxin (Table 10). The concentration of antibiotics that significantly inhibited the growth varied with antibiotics as well as with isolates. *Rhizobium* sp TNAU 14 isolate tolerated streptomycin, at 300 μg ml^{-1} and ciproflaxin at 30 μg ml^{-1}. Most of the soil isolates tolerated streptomycin at 300 μg ml^{-1} and ciproflaxin at 30 μg ml^{-1}. *Rhizobium* isolated from ARS Aliyar nagar was tolerant to most antibiotics.

4.7. Protein profile analysis

Total cellular protein of the 14 rhizobial isolates were analyzed in 12 per cent SDS - PAGE and the molecular size of these proteins ranged from 14 to 97 K Da. The results indicated that the banding pattern in the protein profile were unique in nature among these rhizobial isolates and majority of the proteins have the molecular

size ranging between 43 and 97 K Da. The strains share common banding pattern in the range of 43 - 97 K Da. However, among the isolates, variations were observed in the proteins at, higher and lower molecular sizes in the profile. (Plate 4).

4.8. Pot culture experiment

Among the different isolates obtained. Isolate *Rhizobium* ALN 7 should maximum PGPR Activity hence it was used as inoculant for groundnut under pot culture condition for further studies. (Plate 5).

4.8.1. Enumeration of rhizosphere microbial population of groundnut under potculture condition

The rhizosphere population of total bacteria**,** fungi, actinomycetes and phosphobacteria was enumerated on 30^{th}, 60^{th} and 90^{th} day. The results are presented in Table 11.

4.8.1.1. Total Bacteria

Among treatments, T_2 (starter N + sterilized soil) recorded the minimum bacterial population (5.4 cfu x 10^6 g^{-1} soil) on 30^{th} day, and T_7 (*Rhizobium* ALN 7 + starter N+ unsterilized soil) recorded the maximum population (50.3 cfu x 10^6 g^{-1} soil) on 60^{th} day.

4.8.1.2. Total Fungi

The fungal population ranged from 3.13 cfu x 10^4 g^{-1} soil (T_2) to 10 cfu x 10^4 g^{-1} soil (T_7) both were recorded on 30^{th} day.

4.8.1.3. Total Actinomycetes

Highest population of actinomycetes (37.7 cfu x 10^3 g^{-1} soil) was recorded in T_7 (*R*hizobium ALN 7 +starter N+ unsterilized soil) on 60th day, and the least population (5.71 cfu x 10^3 g^{-1} soil) was recorded in T_2 (starter N + sterilized soil) on 30^{th} day.

4.8.1.4. Total Phosphobacteria

T_7 (*R*hizobium ALN 7 + starter N+ unsterilized soil) had the maximum phosphobacterial population (40.3 cfu x 10^3 g^{-1} soil) recorded on 60^{th} day and the T_2 (starter N + sterilized soil) had the least total phosphobacteria (6.7 cfu x 10^3 g^{-1} soil) recorded on 90^{th} day.

4.8.1.5. Enumeration of rhizobial population of groundnut under pot culture condition

Population dynamics of both introduced *Rhizobium* and nitrogen and the soil rhizobial population was enumerated at 30 days interval upto 90 days by plant infection (MPN) method and the results are given in the fig 4.The MPN count was ranged from 0.018 x 10^2 to 5.5 x 10^3. The population of introduced isolates gradually increased over the uninoculated control.

4.9. Effect of *Rhizobium* ALN 7 inoculation of groundnut - pot culture experiment

The effect of *Rhizobium* ALN 7 on soil characteristics was examined through a pot culture experiment conducted for 90 days.

4.9.1. Soil available nitrogen

The effect of inoculated *Rhizobium* ALN 7 and applied nitrogen on mineral N content in soil is presented in Table 12, Fig 5. The mineral N content varied from 54 (T_2) to 401 kg ha^{-1}(T_7) in soil.

Initially this soil had low amount (54 kg ha^{-1}) of mineral N upon *Rhizobium* and starter *N*itrogen application, the mineral N content was significantly increased in soil. In all treatments, the mineral N content remarkably increased up to 60 days of growth and thereafter was found decreased in soil. The soil that received (T_7) highest amount of mineral N content (401 kg ha^{-1}) at 60^{th} day of growth period.

4.9.2. Soil available phosphorus

The changes in amount of soil phosphorus content during 90 days of growth are presented in Table 13, Fig 6.

In the treatments, T7 (*Rhizobium* ALN 7 + starter N+ unsterilized soil) recorded highest available phosphorus content (65 kg ha^{-1}) on 60^{th} day and T_2 (starter N + sterilized soil) recorded the lowest available phosphorus (26 kg ha^{-1}) on 0^{th} day. Among different days of interval, on 60^{th} day each treatment had recorded the high phosphorus content than other recorded days.

4.9.3. Effect of *Rhizobium* on enzyme activities in soil

4.9.3.1 Urease activity

The Urease activity in soil as influenced by the application of *Rhizobium* during 90 days growth is given in Table 14, Fig 7.

The application of *Rhizobium* had significantly improved the urease activity in soil. Initially, the urease activity varies from 0.93 (T_2) to 3.39 μg NH_4-N g^{-1} soil h^{-1} (T_7). During growth period, the urease activity was significantly enhanced upto 60^{th} day (1.04 to 6.27 μg NH_4-N g^{-1} soil h^{-1}) and thereafter showed a decreasing trend.

4.9.3.2 Phosphatase activity

Similar to Urease activities, the Phosphatase activity was also found to be markedly influenced by the *Rhizobium* application (Table 15, Fig 8).

At all time, T_2 (starter N + sterilized soil) recorded lesser activities (1.28 to 1.40 μg P-nitrophenol g^{-1} soil h^{-1}) while soil with *Rhizobium* ALN 7 + starter N + unsterilized soil (T_7) recorded greater activities (ranging form 3.75 to 6.62 μg P-nitrophenol g^{-1} soil h^{-1}). Irrespective of treatment the phosphatase activities were notably enhanced during growth up to 60^{th} day (1.40 to 6.62 μg P-nitrophenol g^{-1} soil h^{-1}) and after wards no further improvement was observed.

4.9.3.3. Dehydrogenase activity

The effect of *Rhizobium* application on dehydrogenase activity in soil is presented in Table 16, Fig 9. Remarkable improvement in the dehydrogenase activities was observed due to the *Rhizobium* and *started N*itrogen application. Initially at 0 day, the activity of dehydrogenase enzyme varied between 1.14 to 2.67 μg TPF g^{-1} soil h^{-1}), the minimum (1.93 to 2.03 μg TPF g^{-1} soil h^{-1}) being observed in T_2 (*Rhizobium* ALN 7 + starter N+ sterilized soil) and the maximum in soil with the application of *Rhizobium* and *started N*itrogen with unsterilized soil (T_7). During the growth period, the dehydrogenase activity was found to be marginally increased upto 60^{th} day (1.42 to 3.03 μg TPF g^{-1} soil h^{-1}) and there after mostly remained unchanged.

4.9.3.3.1 Correlation coefficient of microbial population with enzyme activity of soil

Soil urease, phosphatase, dehydrogenase activity showed significant positive correlation with soil microbes *viz.*, total fungi, total bacteria, total actinomycetes and total phosphobacteria. Total soil bacteria were significantly correlated with urease and dehydrogenase at 0.01 per cent probability. Total soil fungi were correlated with phophatase and dehydrogenase at 0.05 per cent probability. Total soil actinomycetes were significantly correlated with phophatase and dehydrogenase at 0.01 per cent probability level. Similarly, Total soil phosphobacteria were correlated with urease and phosphatase at 0.01 and 0.05 per cent significant level respectively (Table 17).

4.10.1 Growth characters

Bacterization of groundnut seeds with the rhizobial isolates and nitrogen promoted the shoot length, root length, and dry matter weight of the plant at all stages of sampling when compared to uninoculated control treatment. At 60^{th} day, groundnut plant received (T_7 unsterilized soil + *Rhizobium* ANL 7 + starter N) recorded maximum shoot length (25.13 cm pl^{-1}), maximum root length (22.16 cm $p1^{-1}$) and more drymatter weight (17.31 g Pl^{-1}). The symbiotic effectiveness was well established by the T_7 (107.85%) and was followed by T_6 (104.17%) and T_5 (100.99%) (Table 18, Plate 6).

4.10.2 Nodulation and yield characters

All the treatments of inoculated *Rhizobium* ALN 7 formed more number of root nodules 14.88, 36.10 and 64.35 pl^{-1} at 30^{th}, 60th and 60^{th} days after sowing respectively.

At 60^{th} day, groundnut plant received T_7 (Unsterillized soil + *Rhizobium* ALN 7 + starter N) recorded more nodule dry weight (1.965 g pl^{-1}) when compared to the uninoculated control (T_1) (1.12 g pl^{-1}) (Table 20).

4.10.3. Leghaemoglobin

The leghaemoglobin content of the nodule due to the inoculation of *Rhizobium* ALN 7 and starter nitrogen was T_7 (5.34 mg g^{-1} of tissue) T_6 (5.09 mg g^{-1} of tissue) and T_5 (5.05 mg g^{-1}) of tissues were on par and were superior to other treatments. (Table 19).

4.10.4. Allantoin

The results on quantification of ureide nitrogen in different parts of the groundnut plant as influenced by the inoculation of *Rhizobium* ALN 7 with nitrogen are given in Table 20, Fig 10. All the groundnut plants inoculated with *Rhizobium* ALN 7 showed more allantion content in all the plant parts than those of the uninoculated control treatment.

The accumulation of allantoin was maximum in the stem followed by leaves and roots in all the treatments where as in the uninoculated control plants, root recorded higher allantion production followed by leaves and the least in the stem, the accumulation of allantoin rhizobial strains by T_7 and T_6 was on par and superior to other treatment (15.88 mg g^{-1} to tissue and 15.50 mg g^{-1} of tissue respectively) this was followed by T_5 (15.25 mg g^{-1} of tissue). In the leaves, the treatment T_6 (5.86 mg g^{-1} of tissue), T_5 (5.75 mg g^{-1} tissue) and T_7 (5.69 mg g^{-1} of tissue), accumulated more allantoin than other treatments. (Table 20).

4.11. Genetic diversity of ground nut *Rhizobium* isolates

The DNA extracted from different *Rhizobium* isolates was resolved in 0.8 per cent agarose gel. Totally 10 groundnut *Rhizobium* isolates were studied for their polymorphism at molecular level. The electrophoretic profile of groundnut *Rhizobium* isolates generated by RAPD-PCR by using the primer OPQ 1and OPM 10. From electrophoretic banding pattern, cluster analysis was carried out using Jaccard similarity co-efficient. From the result, it was found that *Rhizobium* isolates EB 34, SOB 1, EB 36 and MBS 19 were clustered together to form cluster 1 followed by *Rhizobium* isolates MBS 9, TNAU 14, COG 15 were belong to cluster 2 and CBS 9 and ALN 2 formed the third cluster. Isolate CBS 12 was found to have more divergence (>90 per cent) to make the separate cluster. COG 15 with TNAU 14 and SOB 1 with *Rhizobium* ALN 7 were clustered together with more than 50 per cent similarity. The results are presented in Fig 11, plate 7.

CHAPTER-V

DISCUSSION

In the present study nine soil rhizobia were isolated from different field location of Tamil Nadu Agricultural University. Tenth and eleventh from field of ARS Aliyar Nagar by plant infection method. Along with these soil isolates three reference rhizobial cultures available in the department of agricultural microbiology were used. The isolates and were subjected to microscopic observation and found to be rod shaped and gram negative in reaction. The colony characters on congo red agar were white translucent appearance with varying quantity of slime production. The shape was discrete, round with smooth margin. There was no growth in Hofer's alkaline broth and exhibited poor growth in glucose peptone agar medium. All the cultures were positive for plant infection study indicating that the cultures used were *Rhizobium* (Somasegaran, 1994)

Acid or alkali production is a marked by variable strain characteristic and many studies have shown that it is influenced by changing the composition of the growth medium (Lange 1961; Jarvis *et al*., 1977; Parker *et al*.,1977 and Date and Halliday, 1979). The acid production in culture media is an indication of acid sensitivity (Norris, 1965). The acid or alkali could be generated in a single medium by one strain at different phases of the growth cycle (cooper, 1982). All the selected rhizobial isolates produced acid after 72 h of growth and variation in the final pH ranged from 5.1 to 6.6 in the yeast extract mannitol broth. From the result all the slow growing isolates produce only acidic reaction. The result is in consonance with the studies of Bromfield and Kumar Rao (1983).

The growth rate is an important criteria for characterization. The generation time has been reported for fast growing bacteria are approximately 3 h and for slow growing bacteria is 6-7 h (Gara and Shanmugam 1978). All isolates used in this study has a generation time ranged from 5.6 to 6.2 h gen^{-1} indicating the soil isolates are of slow growing nature. The growth rate is directly correlated with nitrogen fixing ability (Pant and Gangwar 1984).

Polysaccharide production is a typical characteristic feature of *Rhizobium.* Many authors have correlated, the exopolysaccharide production to that of nodulating ability and nitrogen fixing ability (Diwnie and Johnson, 1986; Noel *et al.,* 1986 and Abdrezel mazur *et al.,* 2002). In the present study, all the fourteen isolates tested positive for polysaccharide production and produced both type of polysaccharides at varying quantities, the ability of rhizobia to produced polysaccharides has been documented by severel authors (Zevenhuizen, 1973, Duman, 1981, Kucuk *et al*., 2006). The *Rhizobium* isolate ALN 7 produced maximum of 3.61 mg ml^{-1} water soluble polysaccharide and 1.200 mg ml^{-1} of alkali stable polysaccharide. Earlier work indicated good correlation between polysaccharide production and nodule formation in *Rhizobium* sp (Dazzo and Habbel, 1975) and the present study did show such relationship. Thavaselvam (2001) found correlation between polysaccharide production and nodule formation in *Rhizobium* sp TNAU14.

Indole acetic acid production by rhizobial isolates demonstrated by several workers (Dullart, 1967). The effectiveness of the isolates of *Rhizobium* sp. in nodulating groundnut was correlated positively with IAA producing ability of the same. Vidyasekaran *et al.* (1973) reported positive correlation between IAA production *in vitro* and the symbiotic efficiency of the rhizobia. All the 14 *Rhizobium* isolates tested produced IAA. Antoun *et al*. (1998) reported that 58 percentage of 266 *Rhizobium* isolate produce IAA. *Rhizobium* isolate ALN 7 was found to have high indole acetic acid production consequently resulted to high nodulation, and root and shoot growth in grondnut. Pandey *et al*., (2007) correlated the IAA producing ability of *Rhizobium meliloti* PP3 to the shoot length in *Cajanus cajan*. IAA production by *Rhizobium* isolates in the range of 1.4 µg ml^{-1} to 3.5 µg ml^{-1}. Similar result (3.7 µg ml^{-1}) on IAA production by *Rhizobium* sp. is reported by vasanthakumar and Mcmanus (2004). *Sinorhizobium meliloti* PP3 reported to produce 80 µg ml^{-1} IAA after 168 h of incubation (Pandey and Mahaswari 2007).

As many as 90 per cent of microorganisms found in the rhizosphere release cytokinin (Barea *et al.,* 1976). Phillips and Torry (1970) reported *Rhizobium japonicum* is capable of producing cytokinin. Production of cytokinin by *Rhizobium* sp. and *Rhizobium leguminosarum* were earlier reported by Noel *et al*. (1996) and Biswas *et al*. (2000). Among all the isolates, *Rhizobium* ALN 7 recorded high

cytokinin production, there by producing maximum shoot and root length in Groundnut and the results are in agreement with the findings of Kalaivani (1998).

The antibiotic resistance pattern of the *Rhizobium* is unique in nature. The rhizobial isolates from soil were resistant to 300 μg ml^{-1} of streptomycin and gentamycin. Similar result was obtained by Thavachelvam (2003). Kucuk *et al.* (2006) reported that *Rhizobium trifolii* was resistant to kanamycin (150 μg ml^{-1}) erythromycin (150 μg ml^{-1}) and rifampicin (150 μg ml^{-1}). Exopolysaccharides and lipopolysaccharides influence the transport of antibiotics into bacteria (Segovia *et al*., 1993). The basis for the correlation found here between antibiotic resistance and rhizobial colony morphology remains to be determined, but it is probable that the *Rhizobium* that showed a high level of resistance did not take up the antibiotics. Streptomycin, erythromycin, chloramphenicol, and kanamycin were generally more effective against the wet *Rhizobium* isolates than they were against the dry ones: however, most of the wet and dry isolates were resistant to relatively high levels of streptomycin and chloramphenicol (Kucuk *et al.*, 2006). *Rhizobium* isolate ALN 7 was found to be resistance to most of the antibiotics than other isolates since it had been classified as slow growing bacteria. Graham (1963) and Pankhurst (1977) reported that slow growing rhizobia were more tolerant to antibiotics than fast growing rhizobia. Although several strains showed resistance towards the different antibiotics tested, there was no identical pattern of resistance among any of these strains, consequently this facilitated for differentiating the *Rhizobium* isolate.

SDS-PAGE is used for subjective comparison of complex patterns of protein made definite identification of closely related strains (Noel and Brill, 1980). Zahran *et al*., (2003) used SDS-PAGE profile of whole cell protein along with FAME for classification of *Rhizobium* from wild legumes. Current study of rhizobia isolates revealed distinctive protein profile and no two isolates has same pattern. Sodium dodecyl sulphate (SDS-PAGE) gel electrophoresis pattern was used frequently to characterize strains of *Rhizobium* spp. (Noel and Brill, 1980 and Dughri and Bottomly, 1985). Sylla *et al*., (2002) employed SDS-PAGE in addition to other tools in studying 50 isolates of native sudanean and savalion region of Senegal for characterization. He further grouped slow growing rhizobial isolate is one major

cluster containing *Bradyrhizobium* sp. as reference strain. From the analysis it is clear that most of the genetic diversity was found with the population of soil isolates.

An increasing interest has emerged with respect to the importance of microbial diversity in soil habitats. The extent of the diversity of microorganisms in soil seens to be critical for the maintenance of soil habitat and quality, as a wide range of microorganisms are involved in important soil function. For successful functioning of introduced microbial bioinoculants and their influence on soil health, exhaustive efforts have been made to explore soil microbial diversity of indigenous community, their distribution and behaviour in soil habitat (Hill, 2003). Diversity is important for genetic characterization with different nitrogen fixing capacities. Using diversity analysis, novelties of new *Rhizobium* sp. with high nitrogen fixing potential could be revealed which have not identified ever before. Genomic DNA finger printing using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating between closely related bacteria. Ten selected rhizobial isolates, representing both the different morphological groups and the areas where soil samples have been collected, were analyzed by RAPD. Rhizobial isolates of cluster 1(ALN 7 , SOB 1, EB 36, MBS 19) is away from the cluster IV (CBS 9 and *Rhizobium* ALN 2) that revealed *Rhizobium* isolates belonged to cluster 1 and cluster IV would be highly diverse.

The results indicate that RAPD is discriminative and efficient method for differentiating and studying genomic diversity of *Rhizobium* strains. RAPD finger printing was used for identification and the assessment of genetic diversity with in the field population of *Bradyrhizobium japonicum* by Sikora *et al*. (1997). From the analysis it is clear that most of the genetic diversity was found with in the population of soil isolates.

Soil enzyme activity measured in these assays represents enzymes associated with living organism, especially dehydrogenase activity (Burns, 1982). Urease activity in the soil can be used as an indicator for total biological activity and for soil fertility. Among all treatments, T_7 has maximum urease activity. Similarly nitrogen content was maximum in plant treated with *Rhizobium* ALN 7 (T_7). As it was reported that urease activity is correlated with nitrogen source utilization (Mulrooney *et al*., 1989). Higher Urease activity may be due to the presence of total microbes associated

with decaying organic matter (Limami *et al*., 1999). There was a significant positive correlation between Urease activity and total microbial population at 0.05 percent probability level.

Soil phosphatase activity estimated ranged from (1.28 to 6.34 µg P nitrophenol g^{-1} soil h^{-1}). Phosphatase activity was found to be more in T_7. There was a significant positive correlation between phosphatase activity and total microbial population at 0.01 percent probability level (Chhonkar and Tarafdar 1984). Haldar and Chakrabarthy (1993) reported large number of *Rhizobium* and *Bradyrhizobium* are able to solubilize inorganic phosphates. Abd – Alla (1994) showed that strain TAL 1236 of *Rhizobium leguminosarum bv. Viciae* contributed significantly to the release of phosphorus from organic compounds through the action of acid and alkaline phosphatase. As phosphatase is directly correlated with available phosphorus (Ponmurugan and Gopi 2006), the phosphorus content of the T_7 was found to be high (57 to 64 kg ha^{-1}) since it had high phosphatase activity. The variation in phosphatase activity of the soil may be due to variation in plant species composition as reported by Beck (1974).

Soil dehydrogenase is an index of endogenous soil microbial activity (Moore and Rusell, 1972). Dehydrogenases are believed to be intracellular enzymes mainly linked with microbial respiratory processes (Bolton *et al*., 1985). Soil dehydrogenase activity estimated ranged from 1.04 to 3.03 µg TPF g-1 soil h^{-1} , dehydrogenase activity showed significant positive correlation with total microbial population (Jha *et al*., 1992). High dehydrogenase activity (2.67 to 3.03 µg TPF g-1 soil h^{-1}) has been related to high bacterial and fungal population. Sometimes high dehydrogenase activity has been related to high nitrogen and organic carbon content.

The endogenous enzyme activity was found to be higher in the T_7 than others. Over all, these enzyme assays are indicative of greater microbial activity in the soil. Nevertheless increased level of enzyme activity observed in T_7 soils agree with total microbial population suggesting the potential for enhanced microbial activity. Similar results were obtained by Benoit *et al*., (1999).

Inoculation of seeds with *Rhizobium* is known to increase nodulation, nitrogen uptake, growth and yield of groundnut. *Rhizobium* inoculation tended to increase the

growth and dry pod yield of the groundnut cultivars (11.19 g pl^{-1}). Except in treatments containing sterilized soils where stunted growth was observed similar results were obtained y Rubin and Paolillo (1984), and Rroco et al. (2003). This supremacy of symbiotic nitrogen is explained as nitrogen fixed by symbiosis is already in the organic reduced form and, hence, more readily available for plant metabolism (Sogut, 2006). In the absence of symbiotic nitrogen, plants must spend a lot of energy to takeup nitrates the most abundant form of nitrogen in the soil during the growth period of groundnut and reduce them to the level of NH_3. Thus although soil nitrogen availability was adequate; inoculation resulted in greater dry matter and nitrogen accumulation in vegetative tissue of groundnut. The soybean *Bradyrhizobium* symbiosis can be c. 300 kg N ha^{-1} under good conditions (Keyser and Li, 1992). Thus nitrogen fertilization is normally not recommended for cultivation of soybean, since under favorable conditions it is able to grow well as soil nitrogen plus nitrogen derived from symbiotic fixation. There are however, instances in which nitrogen fertilization (starter dose) has been added to ensure maximum pod yield .Similar result were observed by Hardarson and Zapata (1984) in soybean.

There are significantly more nodules on the inoculated plants than on non-inoculated plants, nodule numbers are also found increasing with increasing DAS. This indicated an increase in longevity of the nodules of the inoculated plants with clear benefits for increased nitrogen fixing potential and production (Grange *et al*., 1999). Inoculation of *Rhizobium* ALN 7 increased, all the yield parameters than the uninoculated control. Concurrent result was reported by E1 Hadi (1999) and Liadam *et al*. (1977).

The maximum leghaemoglobin content was recorded in T_7 this leads to higher pod yield as it is well documented that the leghaemoglobin content of most legume root nodules is positively correlated with their nitrogen fixing ability (Appleby, 1984). The principal compounds transported from nodules to the shoot are the ureides, allantoin and allantoic acid (Sprent, 1980). Similar results are obtained in the present study also. It has been reported that allantoin levels in stem, root and leaves increased with the growth and attained a maximum at green pod stage of soybean plants (Matsumoto *et al*. (1977a).

Antoun *et al*. (1998) reported 54 per cent of 266 *Rhizobium* isolates show phosphorus solubilizing activity. Inoculation of plants with phosphate solubilizing bacteria frequently stimulates plant growth by increasing phosphorus uptake (Chabot *et al*., 1993 and Kucoz *et al*., 1989). Reduced phosphorus supply decreased total nitrogen fixation (Tanz *et al*., 2001). Milkanova (2002) reported that *Rhizobium* sp. are efficient phosphate solubilizers. Chabot *et al*. (1996a) reported significant increases in dry matter yield by using phosphate solubilizing *Rhizobium leguminosarum bv. Phaseoli.*

There are significantly more nodules on the inoculated plants than on non-inoculated plants, nodule numbers are also found increasing with increasing days after sowing . *Sinorhizobium meliloti* PP3 found to solubilize 10.95 mg ml^{-1} phosphorus in the 168 h time (Pandey and Maheswari, 2007). Which confirm the result of present study. Seed inoculation with P solubilizing *Rhizobium* and *Bradyrhizobium* is known to improve solubilization of fixed soil phosphorus and applied phosphates resulting in higher pod yield. This is in consorance with the findings of Abd-Alla (1994).

From the study undertaken the rhizobial isolate ALN7 crop was superior in increasing the plant growth, nodulation and yield of groundnut crop (CO4) over uninoculated control.

CHAPTER - VI

SUMMARY

The salient findings of the experiments conducted are presented below:

- Eleven soil rhizobial isolates and three standard rhizobial cultures were authenticated as *Rhizobium* species and used in the study.

- Growth rate and generation time for the fourteen rhizobial cultures was assessed and found that growth rate of all ranged from 0.154 to 0.180. And generation time ranged from 5.5 to 6.2 h .

- Production of water soluble and alkali stable polysaccharides by the fourteen rhizobial cultures were assessed and found that rhizobial isolate ALN 7 produced higher amount of water soluble (3.61 mg ml^{-1}) and alkali stable (1.20 mg g^{-1}) polysaccharides followed by isolate *Rhizobium* ALN 7.

- All rhizobial isolated tested produced indole acetic acid (IAA) in varied quantity. Isolate *Rhizobium* ALN 7 produced higher quantity of IAA (3.5 μg ml^{-1}).

- Rhizobial isolate ALN 7 produced greater amount of cytokinin (3.31 μg ml^{-1}) followed by *Rhizobium* ALN 2 (3.1 μg ml^{-1}).

- Intrinsic antibiotic resistance level of the fourteen rhizobial cultures was determined for the antibiotics like erythromycin, kanamycin, gentamycin, rifampicin and streptomycin at low concentration. Rhizobial isolate ALN 7 was found to have resistance as scored by using IAR for most of the antibiotics.

- The effect of introduced rhizobial isolate ALN 7 was evaluated on groundnut crop and found that significantly superior in increasing the soil nitrogen, plant growth, nodulation, and yield over uninoculated control.

- Soil urease, phosphatase, dehydrogenase activity showed significant positive correlation with soil microbes *viz.*, total bacteria, fungi, actinomycetes and phosphobacteria.

- Bacterization of groundnut seeds with rhizobial isolate ALN 7 induced more leghaemoglobin production than control. The rhizobial strains inoculated plant had more allantion in stem, leaves and root than the uninoculated plants.

- The analysis of the SDS-PAGE protein of different rhizobial cultures revealed characteristic banding pattern for the rhizobial cultures. The size of the subunits separated were of the range of 14 KDa to 90 KDa character banding pattern for rhizobial isolates were observed.

- DNA amplification on finger printing study of the rhizobial cultures showed finger printing pattern characteristic of each culture and revealed clear polymorphism and genetic diversity among the rhizobial cultures. The oligonucleotide primers differed in their usefulness in detecting polymorphisms among the cultures and between the cultures. The primers OPM 10 and OPQ1 generated markers for rhizobial cultures. These markers could be further used to identify and establish genetic purity of the different strains in the rhizobial inoculum. Dendrogram analysis revealed that high diversity occurred among the rhizobial cultures.

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Table 1. Classification of nitrogen fixing bacteria forming symbiosis with legume plants

Species	Hostplant	References
Rhizobium		Frank(1889)
Rhizobium leguminosarum	*Pisum sativum, Vicia, Lathyrus,*	Frank(1889) and
biovar viciae	*Lens*	Jordan (1984)
biovar trifolii	*Trifolium pretense*	
biovar phaseoli	*Phaseolus vulgaris*	
Rhizobium tropici		
Type II A	*Phaseolus vulgaris,* *leucaena leucocephala*	Martinez-romero *et al.* (1991)
Type II B	*Phaseolus vulgaris,* *leucaena leucocephala*	
Rhizobium etli		Segovia *et al*.(1993),
biovar phaseoli	*Phaseolus vulgaris*	Hernandez-Lucas *et al.* (1995)
biovar mimosae	*Mimosa affinis, ,* *leucaena leucocephala,* *Phaseolus vulgaris* L.	Segovia *et al*.(1993) and Wang *et al*.(1999)
Rhizobium hainanese	*Desmodium sinuatum and* other plants of arid regions.	(Chen *et al*., 1997)
Rhizobium gallicum		Amerger *et al*.(1997)
biovar gallicum	*Phaseolus vulgaris*	
biovar phaseoli	*Phaseolus vulgaris*	
Rhizobium mongolense	*Medicago ruthencia*	(Van Berkum et al., 1998)
Rhizobium galegae		Lindstrom (1989)
biovar orientalis	*Galega orientalis*	Nick *et al*. (1998)
biovar officinalis	*Galega officinalis*	Nick *et al*.(1998)
Rhizobium giardinii		Amarger *et al*.(1997)
biovar giardinii	*Phaseolus vulgaris*	Amarger *et al*.(1997)
biovar phaseoli	*Phaseolus vulgaris*	Amarger *et al*.(1997)
Rhizobium huautlense	*Sesbania herbacea*	Wang *et al*.(1998)
Mesorhizobium		
M. loti	*Lotus corniculatus*	Jarvis *et al*.(1982)
M. huakuii	*Astragalus sinicus, Acacia*	Chen *et al*. (1991)
M. ciceri	*Cicer arietinum*	Nour *et al*.(1994)
M.tianshanense	*Glycyrrhiza pallidiflora* and other tropical plants	Chen *et al*.(1995)
M. mediterraneum	*Cicer arietinum*	Nour *et al*.(1995)
M. Plurifarium	*Acacia, prosopis*	De Lajudie *et al*.(1998)
M. amorphae	*Amorpha fruticosa*	Wang *et al*.(1999)

Species	Hostplant	References
Sinorhizobium		Chen *et al*.(1988) and De Lajudie *et al*.(1994)
S.meliloti	*Medigaco, Melilotus, Trigonella*	Dangeard 1926; De Lajudie *et al*.(1994) and Jordan (1984)
S.fredii		De Lajudie *et al*.(1994) and
chemovar fredii	*Glycine max*	Jordan (1984)
chemovar siensis	*Glycine max*	Jordan (1984)
S.sahelense	*Sesbania spp.*	De Lajudie *et al*.(1994)
biovar acaciae	*Acacia spp.*	Biovin *et al*.(1999)
biovar sesbaniae	*Sesbania spp.*	Biovin *et al*.(1999)
S. terangae		De Lajudie *et al*.(1994)
biovar acaciae	*Acacia spp.*	Lortet *et al*.(1996)
biovar sesbaniae	*Sesbania spp*	Lortet *et al*.(1996)
S. medicae	*Medicago*	Rome *et al*.(1996)
S. kostiense	*Acacia, prosopis*	Nick *et al*. (1999)
S. arboris	*Acacia, prosopis*	Nick *et al*. (1999)
Azorhizobium		Dryfus *et al*. (1988)
Azorhizobium caulinodans	*Sesbania rosrtata*	Dryfus *et al*. (1988)
Azorhizobium sp.	*Sesbania rosrtata*	Rinaudo *et al*.(1991)
Bradyrhizobium		Jordan (1982)
B.japonicum	*Glycine max, Glycine soja*	Jordan (1982), Kirchner(1896)
B. elkanii	*Glycine max*	Kuykendall *et al*.(1992)
B. liaoningense	*Glycine max, Glycine soja*	Xu *et al*.(1995)
Bradyrhizobium sp.	*Vigna,Lupinus,Mimosa*	Jordan (1984)
	Acacia,	Dupuy *et al*. (1994)
	Asechynomene	Alazard (1985) and Young *et al*. (1991)
Allorhizobium		De Lajudie *et al*.(1998)
Allorhizobium undicola	*Neptunia natans*	De Lajudie *et al*.(1998)

Table 4. Biochemical characterization of rhizobial isolates used in the study

S.No	*Rhizobium* isolates	Gram's reaction	Growth on				Acid/Alkali production	Polysaccharide production	Plant infection (Green gram)	Plant infection groundnut
			CYEMA	Hofer's Alkaline	Lactose Agar	Glucose peptone				
1.	EB 36	-	White translucent	NG	NC	PG	Acid	+ +	*	*
2.	EB 34	-	White translucent	NG	NC	PG	Acid	+ +	*	*
3.	CBS 9	-	White translucent	NG	NC	PG	Acid	+ +	*	*
4.	CBS 12	-	White translucent	NG	NC	PG	Acid	+ +	*	*
5.	MBS 9	-	White translucent	NG	NC	PG	Acid	+ +	*	*
6.	MBS19	-	White translucent	NG	NC	PG	Acid	+	*	*
7.	WLO 2	-	White translucent	NG	NC	PG	Acid	+	*	*
8.	FB 37	-	White translucent	NG	NC	PG	Acid	+ +	*	*
9.	MBS 7	-	White translucent	NG	NC	PG	Acid	++	*	*
10.	ALN 2	-	White translucent	NG	NC	PG	Acid	++	*	*
11	RHIZOBIUM ALN 7	-	White translucent	NG	NC	PG	Acid	++	*	*
12.	TNAU 14	-	White translucent	NG	NC	PG	Acid	+ + +	*	*
13.	COG 15	-	White translucent	NG	NC	PG	Acid	+ + +	*	*
14.	SOB 1	-	White translucent	NG	NC	PG	Acid	+ +	*	*

- : Negative +: low *: Positive NG: No growth

PG: Poor growth + +: Medium + + +: high NC: No colour

Table 6. *Invitro* growth of rhizobia strains in YEM liquid medium. O.D. value at 540 nm

Sl.No.	*Rhizobium* isolates	Incubation period in hour							
		0	12	24	36	48	60	72	84
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	EB 36	0.008	0.105	0.315	0.490	0.620	0.750	0.755	0.755
2	EB 34	0.006	0.081	0.345	0.538	0.658	0.602	0.610	0.610
3	CBS 9	0.006	0.121	0.357	0.552	0.620	0.720	0.728	0.728
4	CBS 12	0.004	0.105	0.267	0.602	0.715	0.730	0.732	0.732
5	MBS 9	0.009	0.032	0.276	0.390	0.510	0.520	0.550	0.550
6	MBS 19	0.007	0.085	0.315	0.510	0.630	0.750	0.750	0.750
7	WLO 2	0.008	0.090	0.290	0.530	0.580	0.630	0.635	0.635
8	FB 37	0.009	0.061	0.234	0.692	0.755	0.768	0.770	0.770
9	MBS 7	0.007	0.095	0.315	0.460	0.580	0.630	0.630	0.630
10	ALN 2	0.006	0.085	0.315	0.530	0.580	0.580	0.610	0.610
11	ALN 7	0.006	0.085	0.315	0.530	0.560	0.580	0.610	0.610
12	TNAU 14	0.006	0.095	0.215	0.390	0.560	0.580	0.610	0.610
13	COG 15	0.007	0.085	0.315	0.510	0.630	0.750	0.750	0.750
14	SOB 1	0.006	0.090	0.180	0.460	0.580	0.620	0.622	0.622

Table 10. Intrinisic antibiotic resistance levels of different rhizobial isolates

S.No	Rhizobial isolates	Antibiotic concentration in μg ml^{-1}											
		Streptomycin		Erythromycin		Rifamycin		Kanamycin		Gentamycin		ciproflaxin	
		250	300	150	200	100	150	150	200	250	300	25	30
1.	EB 36	+	-	+	-	+	+	+	+	+	+	-	-
2.	EB 34	+	-	+	+	+	-	+	+	+	+	-	-
3.	CBS 9	+	-	+	-	+	-	+	+	+	+	-	-
4.	CBS 12	+	-	+	-	+	-	+	+	+	-	-	-
5.	MBS 9	+	+	+	+	+	+	+	-	+	+	-	-
6.	MBS19	+	-	+	-	+	-	+	-	+	-	-	-
7.	WLO 2	+	-	+	-	+	-	+	-	+	-	-	-
8.	FB 37	+	-	+	+	+	+	+	+	+	-	-	-
9.	MBS 7	+	-	+	+	+	-	+	+	+	+	+	+
10.	ALN 2	+	-	+	+	+	-	+	+	+	+	+	-
11.	ALN 7	+	-	+	+	+	+	+	+	+	+	+	-
12.	TNAU 14	+	-	+	+	+	+	+	+	+	+	+	-
13.	COG 15	+	-	+	-	+	+	+	+	+	+	-	-
14.	SOB 1	+	+	+	-	+	+	+	+	+	+	+	+

+ Positive for growth
- Negative for growth

Table 18. Effect of introduced *Rhizobium* ALN 7 inoculation on the growth of groundnut

Treatment	Shoot length (cm pl $^{-1}$)			Root length (cm pl $^{-1}$)			Plant dry weight (g pl $^{-1}$)			Symbiotic effectiveness in per cent
	30*	60*	90*	30*	60*	90*	30*	60*	90*	
T_1- uninoculated control	13.60	22.16	24.26	10.10	17.66	19.26	10.72	14.22	16.05	-
T_2-Starter N+ sterilized soil	6.30	13.60	14.25	9.12	13.32	14.35	4.08	8.11	11.05	68.85
T_3-*Rhizobium* ALN 7+ sterilized soil	7.41	13.71	16.64	9.38	13.66	14.85	4.12	8.17	12.09	75.32
T_4- *Rhizobium* ALN 7 + starter N+ sterilized soil	8.41	14.82	17.14	9.43	14.36	15.26	4.16	10.22	13.10	81.62
T_5-Starter N+ unsterilized soil	14.85	22.23	24.72	10.15	18.92	20.33	10.23	14.26	16.21	100.99
T_6- *Rhizobium* ALN 7 + unsterilized soil	15.05	23.58	24.79	10.20	19.58	21.35	10.24	14.32	16.72	104.17
T_7- *Rhizobium* ALN 7 +starter N+ unsterilized soil	16.20	23.96	25.13	10.31	19.63	22.16	11.24	16.10	17.31	107.85
SE(d)	0.137	2.416	0.432	0.487	0.191	0.209	0.053	0.115	0.089	0.431
CD	0.300	5.172	0.942	1.044	0.417	0.455	0.116	0.252	0.195	0.960

* Days after sowing

Table 19. Effect of inoculation of *Rhizobium* ALN 7 on the nodulation and yield characters of groundnut

Treatment	Nodule number pl $^{-1}$			Nodule dry weight(mg pl $^{-1}$)			Yield of groundnut		Leghaemoglobin (mg/g nodule tissue
	30*	60*	90*	30*	60*	90*	No of pod pl^{-1}	Dry pod yield g pl^{-1}	
T_1- uninoculated control	7.49	22.07	24.51	0.049	0.760	1.12	5.0	4.30	3.23
T_2- Starter N+ sterilized soil	0.22	14.07	16.22	0.018	0.390	0.760	3.0	1.97	0.47
T_3-*Rhizobium* ALN 7+ sterilized soil	0.47	14.51	16.27	0.018	0.540	0.823	4.0	3.17	1.49
T_4- *Rhizobium* ALN 7 + starter N+ sterilized soil	0.92	16.26	16.27	0.029	0.570	0.925	4.0	3.25	1.88
T_5- Starter N+ unsterilized soil	10.52	30.11	52.31	0.059	0.607	1.623	8.0	7.39	5.05
T_6- *Rhizobium* ALN 7 + unsterilized soil	12.82	33.98	55.21	0.064	0.889	1.735	10.0	8.79	5.09
T_7- *Rhizobium* ALN 7 +starter N+ unsterilized soil	14.88	36.10	64.35	0.066	0.997	1.965	12.0	11.19	5.34
SE(d)	0.181	1.384	2.622	0.010	1.126	1.933	0.065	0.169	0.222
CD	0.395	3.017	5.713	0.023	2.454	4.212	0.138	0.298	0.485

* Days after sowing

Table 11.Enumeration of total bacteria, fungi and actinomycetes present in the rhizosphere of groundnut under pot culture condition.

Treatment	Total bacteria (CFU x10^6 g^{-1} of soil)			Total Fungi (CFU x10^4 g^{-1} of soil)			Total Actinomycetes (CFU x10^3 g^{-1} of soil)			Total Phosphobacteria (CFU x10^3 g^{-1} of soil)		
	30*	60*	90*	30*	60*	90*	30*	60*	90*	30*	60*	90*
T_1-uninoculated control	32.7	40.2	22.7	7.7	7.0	5.3	16.0	30.3	22.0	19.0	32.0	22.7
T_2-Starter N+ sterilized soil	6.7	12.3	5.4	3.13	3.73	3.50	5.71	14.3	12.7	8.4	9.1	6.7
T_3-*Rhizobium* ALN 7+ sterilized soil	7.9	13.4	6.1	4.51	4.41	4.12	6.30	14.7	13.3	9.1	10.7	7.5
T_4- *Rhizobium* ALN 7 + starter N+ sterilized soil	9.3	14.0	7.4	4.70	5.10	4.37	7.41	16.0	14.1	10.3	11.3	8.4
T_5-Starter N+ unsterilized soil	32.3	46.3	25.7	8.7	7.0	7.2	17.0	32.0	22.3	20.3	34.7	23.0
T_6- *Rhizobium* ALN 7 + unsterilized soil	34.7	48.3	26.4	9.3	7.3	8.4	18.4	34.0	23.7	24.7	38.0	29.7
T_7- *Rhizobium* ALN 7 + starter N+ unsterilized soil	38.0	50.3	28.3	10	7.5	5.7	19.2	37.7	24.7	26.7	40.3	31.3
SE(d)	0.75	2.26	1.60	0.43	0.472	0.46	0.82	2.75	1.60	0.62	2.34	1.12
CD	1.64	4.93	3.47	0.93	1.03	0.99	1.94	5.10	3.52	1.31	5.17	2.33

* Days after sowing

Table 2. Isolation of *Rhizobium* from different soils of groundnut field

S.No.	Source of soil sample	No.of rhizobia (CFU $x10^6$ g^{-1} of soil)
1.	Eastern block field No.36	13.70
2.	Eastern block field No.34	15.72
3.	Cotton Breeding station field No:9	15.00
4.	Cotton Breeding station field No:12	13.71
5.	Millet Breeding station field :8	14.70
6.	Millet Breeding station field :9	8.00
7.	Wet land field No:2	13.20
8.	Eastern block field No:37	12.70
9.	Millet Breeding station field No:7	11.51
10.	Aliyar Nagar	16.40
11.	ARS - Aliyar Nagar	17.30

Table 3. Rhizobial isolates used in this study

S.No.	Source of soil sample	Isolate no selected for further study
1.	Eastern block field No.36	EB 36
2.	Eastern block field No.34	EB 34
3.	Cotton Breeding station field No:9	CBS 9
4.	Cotton Breeding station field No:12	CBS 12
5.	Millet Breeding station field :8	MBS 9
6.	Millet Breeding station field :9	MBS19
7.	Wet land field No:2	WLO 2
8.	Eastern block field No:37	FB 37
9.	Millet Breeding station field No:7	MBS 7
10.	Aliyar Nagar	ALN 2
11	ARS - Aliyar Nagar	ALN 7
12.	Department of Agrl. Microbiology	TNAU 14
13.	Department of Agrl. Microbiology	COG 15
14.	Department of Agrl. Microbiology	SOB 1

Table 5. Polysaccharide production by different rhizobial isolates

S.No.	*Rhizobium* isolates	Water soluble polysaccharide (mg ml^{-1})	Alkali stable polysaccharide (mg ml^{-1})
1.	EB 36	2.30	0.221
2.	EB 34	1.65	0.095
3.	CBS 9	2.07	0.101
4.	CBS 12	2.12	0.135
5.	MBS 9	2.41	0.147
6.	MBS19	2.30	0.127
7.	WLO 2	1.68	0.065
8.	FB 37	2.75	0.130
9.	MBS 7	2.66	0.149
10.	ALN 2	3.53	0.217
11	ALN 7	3.61	1.200
12.	TNAU 14	3.17	0.207
13.	COG 15	2.57	0.140
14.	SOB 1	2.90	0.375

Table 8. Estimation of Indole Acetic Acid by rhizobial isolate

S.No.	*Rhizobium* isolates	Concentration of IAA ($\mu g\ ml^{-1}$)
1.	EB 36	2.0
2.	EB 34	1.58
3.	CBS 9	2.7
4.	CBS 12	1.4
5.	MBS 9	2.1
6.	MBS19	1.7
7.	WLO 2	2.0
8.	FB 37	3.2
9.	MBS 7	2.1
10.	ALN 2	3.4
11.	ALN 7	3.5
12.	TNAU 14	3.0
13.	COG 15	3.2
14.	SOB 1	3.2

Table 9.Cytokinin production by rhizobial isolates

S.No.	*Rhizobium* isolates	Fresh weight mg 5^{-1} cotyledons	Concentration of benzyl amino purine ($\mu g\ ml^{-1}$)
1.	EB 36	41.5	0.74
2.	EB 34	43.3	1.2
3.	CBS 9	44.6	1.8
4.	CBS 12	41.0	0.71
5.	MBS 9	43.9	1.9
6.	MBS19	43.7	1.6
7.	WLO 2	45.1	2.9
8.	FB 37	44.9	2.1
9.	MBS 7	42.7	0.9
10.	ALN 2	45.7	3.1
11.	ALN 7	47.5	3.3
12.	TNAU 14	43.3	1.2
12.	COG 15	44.6	1.8
14.	SOB 1	41.5	0.72

Table 12. Effect of *Rhizobium* ALN 7 inoculation on mineral nitrogen content of soil

Treatments	Nitrogen content (kg ha^{-1})			
	*0 DAS	*30 DAS	*60 DAS	*90 DAS
T1-uninoculated control	156	203	256	126
T2-Starter N+sterilized soil	54	98	131	50
T3-*Rhizobium* ALN 7+sterilized soil	62	117	142	59
T4 - *Rhizobium* ALN 7+starter N+ sterilized soil	81	121	149	80
T5- Starter N+ unsterilized soil	197	253	360	113
T6- *Rhizobium* ALN 7 + unsterilized soil	224	280	378	157
T7- *Rhizobium* ALN 7+starter N+unsterilized soil	236	310	401	194
SE(d)	1.79	2.28	1.25	1.23
CD	3.65	5.13	3.92	3.17

*Days after sowing

Table 13. Available Phosphorus content of soil

Treatments	Available phosphorus (kg ha^{-1})			
	*0 DAS	*30 DAS	*60 DAS	*90 DAS
T1-uninoculated control	41	45	45	43
T2-Starter N+sterilized soil	26	29	32	30
T3-*Rhizobium* ALN 7+sterilized soil	29	33	37	35
T4 - *Rhizobium* ALN 7+basal N+sterilized soil	31	39	41	39
T5- Starter N+ unsterilized soil	51	53	60	59
T6- *Rhizobium* ALN 7 +unsterilized soil	53	59	63	62
T7- *Rhizobium* ALN 7+starter N+unsterilized soil	57	62	65	64
SE(d)	0.784	0.791	0.890	0.875
CD	0.708	1.725	1.940	1.908

*Days after sowing

Table 14. Soil Urease activity

Treatments	Soil urease activity (μg NH_4-N g^{-1} Soil hr^{-1})			
	*0 DAS	*30 DAS	*60 DAS	*90 DAS
T1-uninoculated control	2.16	2.73	3.48	2.95
T2-Starter N+sterilized soil	0.93	1.07	1.04	1.37
T3-*Rhizobium* ALN 7+sterilized soil	0.99	1.17	2.13	1.84
T4 - *Rhizobium* ALN 7+starter N+sterilized soil	1.65	2.61	3.39	2.01
T5- Starter N+ unsterilized soil	2.32	3.48	4.04	3.84
T6- *Rhizobium* ALN 7 +unsterilized soil	3.06	4.04	4.48	4.05
T7- *Rhizobium* ALN 7 + starter N+unsterilized soil	3.39	4.32	6.27	5.03
SE(d)	0.253	0.338	0.436	0.366
CD	0.544	0.726	0.935	0.786

*Days after sowing

Table 15. Soil Phosphatase activity

Treatments	Phosphatase activity (µg P-nitrophenol g^{-1} soil hr^{-1})			
	*0 DAS	*30 DAS	*60 DAS	*90 DAS
T1-uninoculated control	2.76	3.14	5.28	4.45
T2-Starter N+sterilized soil	1.28	1.36	1.40	2.28
T3-*Rhizobium* ALN 7+sterilized soil	1.89	2.06	2.33	2.63
T4 - *Rhizobium* ALN 7+starter N+sterilized soil	2.01	2.11	2.70	1.35
T5- Starter N+ unsterilized soil	3.10	4.35	5.68	4.87
T6- *Rhizobium* ALN 7 +unsterilized soil	3.45	4.83	5.92	5.53
T7- *Rhizobium* ALN 7+starter N+unsterilized soil	3.75	5.76	6.62	6.34
SE(d)	0.308	0.416	0.527	0.481
CD	0.660	0.893	1.131	1.033

* Days after sowing

Table 16. Soil Dehydrogenase activity

Treatments	Soil dehydrogenase activity (μg TPF g^{-1}soil hr^{-1})			
	*0 DAS	*30 DAS	*60 DAS	*90 DAS
T1-uninoculated control	2.36	2.59	2.59	2.54
T2-Starter N+sterilized soil	1.14	1.37	1.42	1.38
T3-*Rhizobium* ALN 7+sterilized soil	1.73	1.89	1.89	1.84
T4 - *Rhizobium* ALN 7+starter N+sterilized soil	1.93	2.03	2.03	1.98
T5- Starter N+ unsterilized soil	2.53	2.67	2.67	2.61
T6- *Rhizobium* ALN 7 +unsterilized soil	2.61	2.93	2.93	2.87
T7- *Rhizobium* ALN 7+starter N+unsterilized soil	2.67	3.03	3.03	2.95
SE(d)	0.247	0.269	0.273	0.266
CD	0.531	0.579	0.586	0.571

*Days after sowing

Table 17.Correlation coefficient values(r)

Pair of Attributes	R
Total bacteria Vs urease	0.642**
fungi Vs urease	0.513*
Actinomycetes Vs urease	0.639*
Phosphobacteria Vs urease	0.824*
Total bacteria Vs phosphatase activity	0.821*
Fungi Vs phosphatase activity	0.543**
Actinomycetes Vs phosphatase activity	0.732*
Phosphobacteria Vsphosphataseactivity	0.821*
Total bacteria Vs dehydrogenase activity	0.754**
Fungi Vs dehydrogenase activity	0.592*
Actinomycetes Vs dehydrogenase activity	0.645**
Phosphobacteria Vs dehydrogenase activity	0.724**

* $P<0.05$, ** $P<0.01$

Table 20. Effect of inoculation of *Rhizobium* ALN 7 on the production of allantoin in the groundnut plant in the pot culture studies.

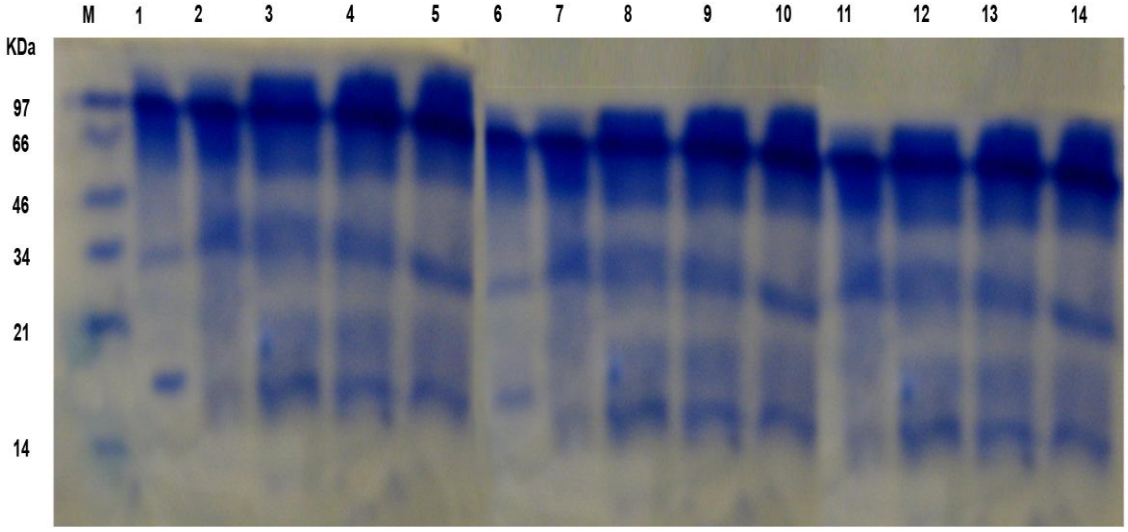
Treatment	Allantoin (mg g^{-1} of tissue)		
	Leaves	stem	Root including nodule
T1-uninoculated control	4.45	12.88	4.44
T2-Starter N+sterilized soil	0.930	0.06	1.10
T3-*Rhizobium* ALN 7+sterilized soil	1.14	2.23	2.70
T4 - *Rhizobium* ALN 7+starter N+sterilized soil	1.72	3.63	2.90
T5- Starter N+ unsterilized soil	5.75	15.25	4.78
T6- *Rhizobium* ALN 7 +unsterilized soil	5.86	15.50	4.69
T7- *Rhizobium* ALN 7+starter N+unsterilized soil	5.69	15.88	4.99
SE(d)	0.194	0.637	0.153
CD	0.422	1.389	0.335

Table 7. Generation time of different rhizobial isolates

S.No.	*Rhizobium* isolates	Growth rate(k)	Generation time (t) h
1.	EB 36	0.168	5.9
2.	EB 34	0.170	5.8
3.	CBS 9	0.180	5.5
4.	CBS 12	0.178	5.6
5.	MBS 9	0.168	5.9
6.	MBS19	0.164	6.0
7.	WLO 2	0.175	5.6
8.	FB 37	0.168	5.9
9.	MBS 7	0.164	6.0
10.	ALN 2	0.154	6.2
11.	ALN 7	0.175	5.6
12.	TNAU 14	0.170	5.8
13.	COG 15	0.170	5.8
14.	SOB 1	0.169	5.9

Plate 5. Pot culture experiment to study the effect of introduced *Rhizobium* ALN 7 on groundnut

Plate 4. Protein profile of different rhizobial isolates on SDS-PAGE



Lane 1 : EB 36
Lane 2 : EB 34
Lane 3 : CBS 9
Lane 4 : CBS 12
Lane 5 : MBS 9
Lane 6 : MBS 19
Lane 7 : WLO 2
Lane 8 : FB 37
Lane 9 : MBS 7
Lane 10 : ALN
Lane 11 : ALN 7
Lane 12 : TNAU 14
Lane 13 : COG 15
Lane 14 : SOB 1

Plate 3. Cytokinin production by *Rhizobium* isolates (Radish cotyledon assay)

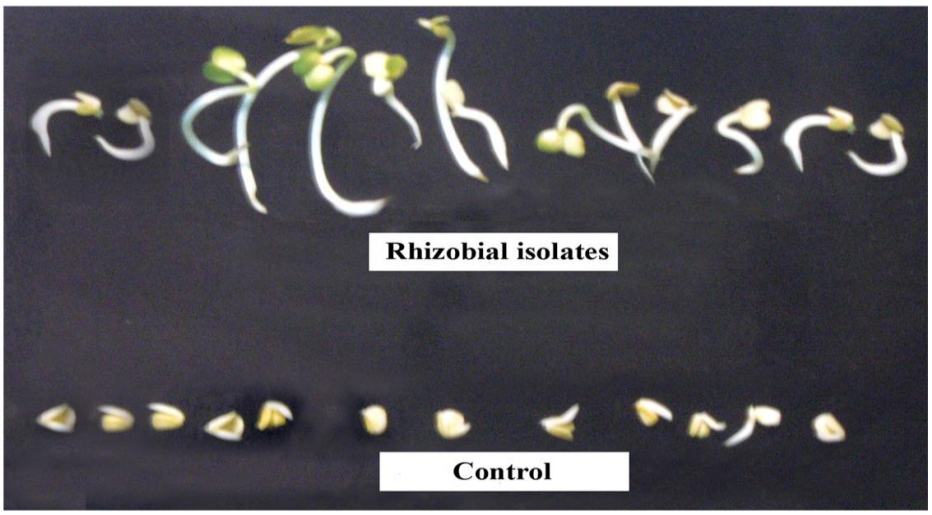


Plate 1. Grams stained *Rhizobium* ALN 7 cells

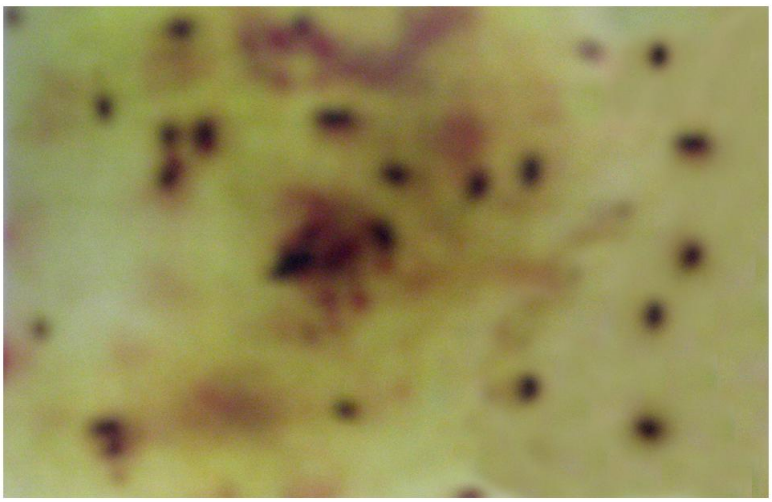
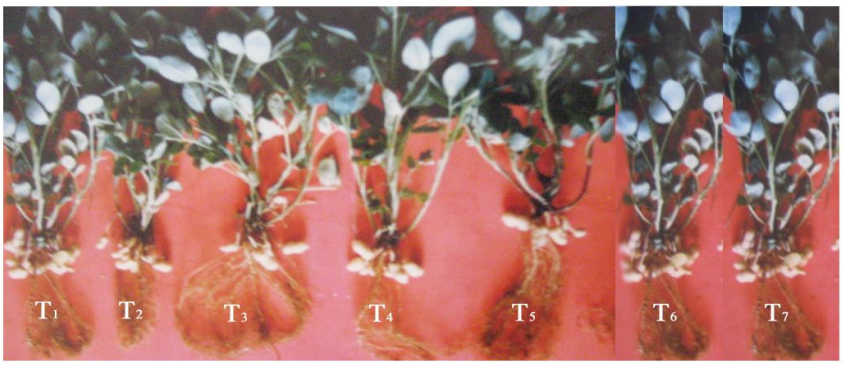


Plate 2. Plant infection test for testing nodule forming ability of Rhizobia

Growth pouch method

Plate 6. Effect of *Rhizobium* ALN 7on growth of groundnut

Plate 7. Effect of *Rhizobium* ALN 7 on nodulation and yield of groundnut



T1 - Control
T2 - Basal N + sterilized soil
T3 - ALN 7 + stertilized soil
T4 - ALN 7 + basal N + sterilized soil
T5 - Basal N + unsterilized soil
T6 - ALN 7 + unsterilized soil
T7 - ALN 7 + basal N + unsterilized soil