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Ecological Characterization of Indigenous Rhizobium Populations in Rajshahi Area

Nushair, Ali Mohammad

University of Rajshahi

Ecological Characterization of Indigenous *Rhizobium* Populations in Rajshahi Area

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By
Ali Mohammad Nushair

Department of Zoology
University of Rajshahi
Rajshahi 6205
Bangladesh

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Dedicated

To My

Beloved Parents

BRIEF CONTENTS

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The author

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ivRkvnx wekwe `¨vjq
ivRkvnx-6205, evsjv‡`k

Department of Zoology
University of Rajshahi
Rajshahi-6205, Bangladesh
Tel: +880 721 711119
Fax: +880 721 750064

CERTIFICATE

Certified that the work incorporated in the thesis "**Ecological Characterization of Indigenous *Rhizobium* Populations in Rajshahi Area**" submitted by Mr. Ali Mohammad Nushair was carried out by the candidate which was supervised by us. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Prof. Dr. Ananda Kumar Saha)
Principal Supervisor
Department of Zoology
University of Rajshahi
Rajshahi 6205, Bangladesh
email: anandroma@yahoo.com

(Prof. Dr. Md. AnisurRahman)
Co- Supervisor
Department of Zoology
University of Rajshahi
Rajshahi 6205, Bangladesh
email: maanisur@yahoo.com

DECLARATION

I, Ali Mohammad Nushair, hereby declare that the research work incorporated in the thesis entitled **"Ecological Characterization of Indigenous *Rhizobium* Populations in Rajshahi Area"** is an original work was done by me and supervised by Prof. Dr. Ananda Kumar Saha and Prof. Dr. Md. Anisur Rahman, Professors, Department of Zoology and is submitted to the University of Rajshahi for the degree of Doctor of Philosophy in Zoology. Such material has not been submitted to any other University/Institute for any degree. The literature and material that has been obtained from other sources is duly acknowledged in thesis.

(Ali Mohammad Nushair)

ABSTRACT

Rhizobium spp. were isolated from root nodules of Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean on Yeast Extract Mannitol Agar (YEMA) and their morphological, cultural, physiological and biochemical characteristics were studied. It was observed that the colonies were circular, light pink, convex, entire and opaque. The bacterium was gram negative, rod shaped, aerobic, non-spore forming and motile. In this study, all isolates were showed hazy appearance in the motility media and also were positive for Catalase, Citrate Utilization Test, Urea Hydrolysis, Congo red test, Nitrification test, Oxidase Test, Triple Sugar Iron Test , Mac Conkey Agar Test and, motility Tests. The samples were found negative for Methyl Red (MR), Voges-Proskauer (VP), Indole, Starch hydrolysis test, Hydrogen Sulphide production and Hofer's alkaline test. Utilization of different carbon sources is an effective tool to characterize the isolates. In the present study sucrose, fructose, galactose, maltose and mannitol (25 mg Hi-media, India) and 20% solution of glucose, lactose, arabinose and xylose were utilized for this test. All the isolates tested here can ferment sucrose, glucose, fructose, mannitol and produce acid. These carbon sources are generally utilized by bacteria of the genus *Rhizohium*. Fast growing Rhizobial strains utilized a wider variety of carbohydrates than the slow growing strains. Their ability to metabolize a broad range of carbon substrates may be advantageous for survival in soil. Isolates which were named as Lentil, Cow Pea and Bean can be utilized in all the nine sugar and the produced acid. Green Gram, Black Gram, Pigeon Pea, Sweet Pea, Chick Pea cannot ferment maltose sugar. Besides this isolates from Green gram, Black Gram, Pigeon Pea Sweet Pea and Chick Pea fermented all sugar but Green gram and Black Gram cannot fermented galactose, lactose, arabinose and xylose.

Resistance patterns of the isolates to thirteen antibiotics were studied. Screening for antibiotic resistance in our study revealed that most of the strains were resistant to Ampicilin, Erythromycin, Gentamicin, Amoxycillin, Penicillin, Streptomycin and Nalidixic acid. The generalized sensitivity to Mecillinam, Ciprofloxacin, Cotrimoxazole, Pefloxacin, Ceftazidime and Tetracycline were found in our study.

An optimum growth was observed at room temperature (28ºC to 30ºC). The organisms were found to be temperature sensitive as at higher and lower temperatures, a low growth was observed that might be due to a hindrance in the metabolic activity. pH is an important parameter for the growth of the organism. Best growth of *Rhizobium* was reported at pH *i.e.* 7 and very less growth was observed in the medium with pH 5.0 and 9.0. Salt stress may decrease the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function. The experiments also showed that the cells were able to grow in 1% NaCl containing medium but unable to grow on higher concentration, showing that the isolate was sensitive to the salt concentration.

In general, the phenotypic study isolates showed large physiological and biochemical biodiversity. Indeed, the studied strains showed a variable resistance against stress factors, temperature, pH, salinity, resistance to antibiotics and heavy metals, which allowed the selection of good candidates for genetic studies. Several investigators have studied the genetic diversity of *Rhizobium* isolated from several countries around the world. However, it needs I6S rDNA sequencing experiment for further identification of the phenotypic species. These experiments will help to know the relationship between the phenotypic and genotypic identification as well as the phylogenetic position of the identified isolates. 16S rRNA sequencing has helped the description of *Rhizobium* spp. In the studies reported here, the genetic biodiversity of eight represented strains were examined. The genetic study carried out is a quick method that gives a better idea of the diversity of these strains. The 16S rDNA was sequenced to determine the taxonomic position of these strains, and the results revealed that there was a great genetic diversity among the eight rhizobial strains studied. Indeed, sequence analysis of 16S rDNA and subsequent BLASTn analyses indicated that six strains had 95 to 98% and other two bellow similarity with *Rhizobium* spp. The phylogenetic distances indicate that the relationships among this group and the *Agrobacterium* species are as distant as those among the genera *Mesorhizobium* and *Sinorhizobium.* This clearly showed that subgroup belonged phylogenetically to the genus *Rhizobium.* Although the phylogenetic position of

subgroup was not found to be distinct from that of other *Rhizobium* sp. by analysis of the partial 16s rRNA sequences. It was distinguished clearly from the *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY12, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY7, *Rhizobium* sp. SOY12 position by the full sequence of its 16S rRNA genes. So it seems that the partial sequencing of 16S rDNA has limited value in determining phylogenetic relationships among rhizobial species. The BLASTn search results for the partial sequences of the 16S rRNA gene of the isolates revealed that the purity and concentration of extracted DNA were performed at 260/280 nm obtained as 1.77 for Green Gram, 1.74 for Black Gram, 1.74 for Lentil 1.77 for Pigeon Pea,1.85 for Sweet Pea,1.77 for chickPea,1.82 for Cow Pea and 1.84 for Bean and concentration as 42.0, 83.9, 28.9, 49.0, 88.5, 68.8, 86.8 and 69.7 ng/µl for Green Gram , Black Gram, lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean, respectively. Finally the selected eight bacterial isolates were identified up to species as *Rhizobium* sp. through 16S rDNA gene sequencing. There were eight experimental plants from which eight colonies of *Rhizobium* were isolated of which five strains showed similarity among them (*Rhizobium* sp. CCNWYC119) another two strains were also similar (*Rhizobium* sp. SOY12) and only one(*Rhizobium* sp. SOY7) was different based on the nucleotide sequence. The 16S rDNA sequence reveals that the isolated strains Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean are homologous to bacterial strain *Rhizobium* sp.CCNWYC119, *Rhizobium* sp.CCNWYC119, *Rhizobium* sp.SOY12, *Rhizobium* sp.CCNWYC119, *Rhizobium* sp.CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY7, *Rhizobium* sp. SOY12 respectively.

Also the results obtained in this study show some interesting aspects on the growth effects of *Rhizobium* inoculation in Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean which was grown in pots under controlled environment. The results indicate the successful nature of usage of commercial inoculants of *Rhizobia* to improve the growth effects of the pulses studied. Successful results by using commercial inocula of *Rhizobia* were obtained in field grown eight isolates (Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean) showed significant difference between inoculated and control. The no of nodule, fresh

weight of nodule, dry weight of nodule, plant height, pod weight and seed weight were taken. Rhizobial inoculation induced significant changes in plant growth characteristics. This study also showed that enhancement of soil fertility along with the yield of legume plants occurred due to nodulation. Soil treated with *Rhizobium* spp. showed higher values than control regarding total nitrogen and organic matter (%).

ABBREVIATION

%	- Percentages
&	- Punctuation
μg	- Micro gram
μl	- Micro liter
approx.	- approximately
ASM	- American Society for Microbiology
ATP	- adenosine triphosphate
BLAST	- Basic local alignment search tool
bp	- base pair
CFU	- Colony forming unit
d/w	- Distilled water
DNA	- deoxyribonucleic acid
dNTPs	- deoxynucleotide triphosphates
e.g.	- For example
ed.	- Editor, edition, edited, edited by
EDTA	- Ethylene diamine tetra acetic acid
ELISA	- enzyme linked immunosorbent assay
et al.	- And others
etc.	- *Exempli* gratia, for example
F	- Forward
FADH	- flavin adenine dinucleotide
FAO	- food and Agriculture organization of the united Nations
Fig.	- Figure
gm	- Gram
HPLC	- High Performance Liquid Chromatography
hr.	- Hour
i.e.	- *id. est*, that is
ICDDR,B	- International Center for Diarrhoeal Disease Research,Bangladesh

Kb	- Kilo base
Kg	- Kilogram
LAF	- Laminar air flow
LAMP	- loop-mediated isothermal amplillcation
LB	- Luria Bertani
LPS	- lipopolysaceharides
MCA	- MacConkcy agar
MEGA	- Molecular Evolutionary Genetics Analysis
Mg	- milligram
mg	- Milligram
MIC	- minimum inhibitory concentration
Min.	- Minute
Ml	- milliliter
ml	- Milliliter
mM	- millimole
mm	- Millimeter
MRVP	- methyl red Voges-Proskauer
MS	- Mineral salt
NA	- nutrient agar
NADH	- nicotinamide adenine dinucleotide
No.	- Number
OD	- optical density
O-F	- oxidation-fermentation (oxidative-fermentative)
OMP	- outer membrane protein
PCR	- Polymerase chain reaction
PCR	- Polymerase Chain Reaction
pH	- Power potency of hydrogen
ppm	- Parts per million
R	- Reverse
RNA	- Ribonucleic acid
rpm	- Rotation per minute

rRNA	- Ribosnucleic acid
SC	- Simmons citrate
SDS	- Sodium dodecyl sulphate
SIM	- Sulferindole motility
sp.	- Species
spp.	- Species (more than one)
TBE	- Tris base boric acid EDTA
TSI	- Triple sugar iron
U	- Unit
v/v	- Volume/ Volume
viz	- Videlicet(that is to say, namely, to wit)
Viz	- Vizard (namely)/ that is to say
vs	- Versus
W/v	- Weight/ volume
YEMA	- Yeast extract mannitol agar media

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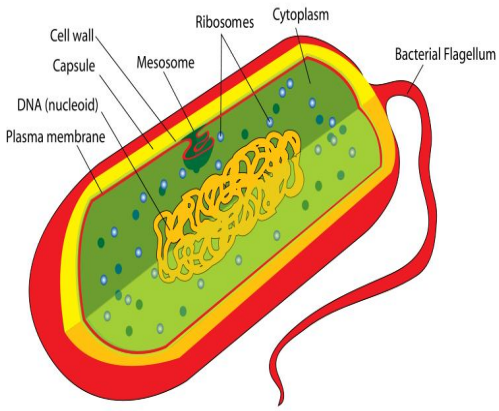
Chapter 1

General Introduction



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Chapter 1

General Introduction

1.1GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1.1 Rhizobacteria

Amongst the soil bacteria there is a unique group called rhizobia that have a beneficial effect on the growth of legumes. Rhizobia are remarkable bacteria because they can live either in the soil or with in the root nodules of host legumes. When legume seeds germinate in the soil, the root hairs come in contact with rhizobia. If therhizobia and the legume are compatible, acomplex process begins during which the rhizobia enter the plant's root hairs. Close to the point of entry, the plant develops a root nodule.Once the relationship between plant and rhizobiais established, the plant supplies the rhizobia with energy from photo synthesis and the rhizobia fix atmospheric nitrogen in the nodule, converting it into a form that the plant can use. Both the plant and the rhizobia benefit from such a relationship called a symbiosis. The rhizobia living in the plant's root nodules are called symbionts.

The complex process by which the rhizobia produce nitrogen for the legume is called biological nitrogen fixation, or BNF. Only rhizobia that are specifically compatible with a particular species of legume can stimulate the formation of root nodules, a process called nodulation. This process has great economic benefit for legume production. As a result, rhizobial inoculants are produced commercially in many countries. Inoculants contain rhizobia isolated from plant nodules and grown (cultured) artificially in the laboratory. Biological nitrogen fixation, (BNF: atmospheric nitrogen fixation through different member of prokaryotes, specifically by diazotrophs) contributes approximately 16% of total nitrogen input in crop land (Ollivier *et al.*, 2011) . Rhizobia are a major contributor to BNF, and the legume- *Rhizobium* symbiosis can fix up to 450 kg N/ha/year (Unkovich Pate, 2000).

Nitrogen is an essential nutrient for plant growth and development. Plants usually depend upon combined, or fixed, forms of nitrogen, such as ammonia and nitratebecause it is unavailable in its most prevalent form as atmospheric nitrogen. Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has led to worldwide ecological problems as well as affects the human health (Vitousek, 1997). Biological nitrogen fixation (BNF) is the cheapest and environment friendly procedure in which nitrogen

fixing micro-organisms, interacting with leguminous plants, fix aerobic nitrogen into soil (Franche *et al*., 2009). *Rhizobium* is the most well known species of a group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria can infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of thebacterium. *Rhizibium* bacteria stimulate the growth of leguminous plants and they are able to fix atmospheric nitrogen into soil by interacting symbiotically withleguminous plants, using the nitrogenase enzyme complex (Kiers *et al*., 2003). The legume-rhizobium interaction is the result of specific recognition of the hostlegume by *Rhizobium*. Various signal molecules that are produced by both *Rhizobia* and the legume confer the specificity (Phillips, 1991). Exopolysaccharide (EPS) produced by *Rhizobium*is one such signal for host specificity during the early stage of root hair infection (Olivares *et al*., 1984). It also protects the cell from desiccation and predation and helps in nitrogen fixation by preventing high oxygen tension (Jarman *et al.,* 1978). Those bacteria which are closely associated with plant roots are called rhizobacteria (Paul and Clark, 1996). According to SubbaRao (1999), the term rhizobacteria implies the ability of certain bacteria to colonize the rhizosphere very aggressively. The plant growth-promoting rhizobacteria (PGPR) and microorganisms in general isolated from the rhizosphere, which have been shown to improve plant health or increase yield. They may exert their effects directly through the production of metaboliles that can act as plant hormones and or indirectly by the suppression of plant pathogens.Plants produce phytohormones, including auxins (primarily indole-3-acetic acid), gibberellins, cytokinis, ethylene, and abscisic acid. These plant growth regulators (PGRs) variously affect the plant's phenology and welfare. Microorganisms produce PGRs that are chemically similar or identical to those produced endogenously by the plant. The bacteria with potential in colonizing seed coats and plant roots are referred to as plant-growth promoting rhizobacteria (PGPR). Such bacteria are currently being used as seed, root, or soil inoculants to achieve biocontrol (Paul and Clark, 1996).

PGPR benefit plants through different mechanisms of action, including e.g., 1) the production of secondary metabolites such as antibiotics, cyanide, and hormone-like substances, ii) the production of siderophores, iii) antagonism to soilborne root

pathogens, iv) phosphate solubilization, and v) dinitrogen fixation. The establishment in the rhizosphere of organism possessing one or more of these characteristics is interesting since it may influence plant growth (Bakker and Schippers, 1987; Weller, 1988).

The ecosystem rhizosphere looks promising as a site for introducing microbes either for plant growth promotion or for biorcmediation of contaminated soils. Unlike bulk soil, which may be considered an oligotrophic environment, the rhizosphere is rich in nutrients, including carbon, and as such provides an ecological niche for those microbes adapted to its unique environment.The plant rhizosphere is an important soil ecological environment for plant-microbe interactions. Itinvolves colonization by a variety of microorganisms in and around the roots, which may result in symbiotic, associative, neutralistic or parasitic relations within the plant, depending on the type of microorganism, soil nutrient status, plant defense system and soil environment (Parma and Dadarwal, 1999). Bacteria, especially species of *Pscudomonas* and *Bacillus* (Guaiquil and Luigi, 1992) found in the rhizosphere of various leguminous crops, may assist in plant colonization by rhizobia and in suppression of plant pathogen (Hassanjadeh, 1991). Interactions between these plant growth promoting rhizobacteria (PGPR) with *Rhizobium* may be antagonistic or synergistic, and the beneficial effects of such interactions could be exploited for economic gains (Glick, 1995; Dubey, 1996) On co-inoculation with symbiotic bacteria, rhizosphere bacteria may increase nodulation through a variety of mechanisms, for example, PGPR could induce phytoalexin production by the plant, creating antibiosis in the rhizosphere for pathogenic organisms, produce siderophores which chelate insoluble cations or colonize root surfaces, thereby out-competing pathogens (Bochow and Fritzsehe 1990; Halverson and Handelsman, 1991).

Leguminous plants (Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean) enrich the soil through symbiotic N_2-fixation from the atmosphere. In some cases, the green crop plants are ploughed into the soil as a green manure. It helps to maintain the soil microflora and plays an important role in biological conservation. During developmental phases of the leguminous plant, environmental factors may become a major constraint to its better yield in Bangladesh. High yield of any crop may be achieved only when a proper combination of variety, environment and agronomic practices is obtained. Plant breeders are trying to breed

high yielding varieties, which may be better adapted to the local environments. It would be of immense help to breeder in quest of improved varieties, and to identify the various morphological and physiological factors governing growth and development. Such factors are important for causing the better yield. But unfortunately, detailed information regarding relationship of developmental stages and seed yield is currently lacking in case of leguminous in context of Bangladesh. Therefore, the strategy for improving agricultural production in developing countries like Bangladesh should be taken into inexpensive, realistic and pragmatic programmes.

Man made fertilizers increase the output of agricultural products and meet the increasing demands of the human population, but that have been accentuated by the limited availability of fertile farmlands. At the same time, the farmlands are being degraded in respect of their fertility. Nitrogen based fertilizer may continue to serve for increasing grain production but efforts should be oriented towards augmenting biological nitrogen fixation mediated by microorganisms. An average area of grain legumes like soybeans, beans, or peas provide sufficient protein for 1000-2000 days for one person, whereas, an average were of plant materials converted to animal protein like beef and poultry provides only for 75-250 days (Burns and Hardy, 1975). As the per capita income increases the demand on animal protein also increases accompanied by a several fold decrease in the intake of vegetable protein. Therefore, in the affluent nations, where the per capita income is high, the demand for fixed nitrogen for conversion into animal protein is higher than that of less affluent nations where the per capita income is low and consequently, the intake of vegetable protein is more pronounced (Subba Rao, 1977) Annual turnover of nitrogen in the biosphere vary from 100 to 200 million tons (Burns and Hardy, 1975; Burris, 1977; Subba Rao, 1977). The ratio between chemically fixed nitrogen and biologically fixed nitrogen ranges approximately from 1:4 to 1:2.5 and within biological fixation, the legume fixation is equivalent to or at least half that of industrial fixation.Estimation of the amounts of N_2 fixed by grain legumes have been made by the International Atomic Energy Agency, Vienna, using labelled nitrogenous fertilizers. Some of the findings obtained by the collaborating scientists indicate that (he values appear to be rather realistic but nevertheless do not minimize the value of legumes in the nitrogen economy of cultivated plants. However, there are both novel and conventional approaches to this problem. In the context of Bangladesh, conventionally, attempts should be made: (1) to

provide efficient strains of rhizobia for inoculating legumes; (2) to evolve varieties of plants responsive to biologically and industrially fixed nitrogen; (3) to overcome the inhibition of fertilizer nitrogen on biological fixed nitrogen; (4) to define agronomic practices leading to belter fixation and conservation of nitrogen in the farm; and (5) to evolve simple practices to conserve water in the farm because optimum moisture is needed for successful nodulation and hence the biological nitrogen fixation in legumes. Legumes have been shown to benefit by the dual action of obligate endophytic enzymes in plant roots and *Rhizobium* in root nodules. The endomycorrhizal fungi appear to influence the uptake of phosphorus which helps in better fixation of nitrogen in root nodules. The biochemical specificity in the legume *Rhizobium* symbiosis happens due to interaction on the root surface involving the two symbionts mediated by carbohydrate binding proteins called 'lectins'.The role of legumes in enriching the fertility of soil is known since the last century. However, scientific demonstrations of the value of legumes in contributing to the nitrogen nutrition of plants were done in the later half of the 19^{lh} century (Fred *et al.,* 1932). The experiments carried out by many scientists proved that nodules on legume roots are responsible for fixing atmospheric nitrogen. Now it is evident that *Rhizobium* plays important role for nodulation in leguminas plants.

1.1.2 Microorganisms in the rhizosphere

Hoffman (1914) reported abundance of bacteria generally adjacent to plant roots than in soil. Many subsequent studies concerned with populations clearly established that microbial cells were much more abundant in the rhizosphere than in the soil (Starkey, 1929, 1931). According to the plate method, the increase in numbers of aerobic bacteria was much higher than that of Actinomycclcs and filamentous fungi. Certain bacterial lyses were more affected by rhizosphere conditions than others. For example, *Agrobacterium radiobactcr* was affected proportionally more than the general bacterialpopulation. However, cells of this bacterium represented but a small portion of the total bacterial population.

The fact that the microbial population is dense on plant roots and this occurs at all stages of plant growth and not only after the plants have died has been verified repeatedly. The importance of the phenomenon is twofold: the plant roots affect microbial development and the plant in turn is affected by the increased activity of the

microorganisms in the rhizosphere. The information is suggestive but, saves for exceptions, is inadequate to indicate the extent to which plant development is affected by the rhizosphere microorganisms, and whether plant growth is enhanced or impaired. Evidence that there is more abundant population of microorganisms in the rhizosphere than in soil rests not only on plate counts but also on visual evidence. By means of the Cholodny buried slide technique it has been shown that not only large roots but small roots and root hairs have large numbers of bacteria on the surface and inside the cells as well (Starkey, 1938). Staining method was used by Rovira (1956) who determined the degree of bacterial development on roots of seedlings grown in a mineral solution on quartz sand.

1.1.3 Unique sources of bacteria

Perhaps, bacteria are found in everywhere, starting from ancient ice cores to the ever active internal organs of higher animals. However, functions of the bacteria vary greatly depending upon their source. Very complex relation between microbial diversity and soil function has been described by scientists. Broadly it is related to conserving biodiversity as well as maintaining a functional biosphere (Nannipieri *et al.* 2003). Soil is enriched by the presence of a number of species of *Rhizobium, Agrobacterium, Azotobacter, Azospirillum, Bacillus, Klebsiella* etc. (Shoebitz *et al.* 2009; Cocking, 2003). A few of these are found in nodules of leguminous plants as nitrifying bacteria aiding in the growth of those plants. Besides of such decent source, there are waste products where bacteria are found in plenty. Amid them many useful organisms are found along with some harmful ones. However, bacterial degradation starts from waste products. Now-a-days, interests have been raised in industrial wastes, which is a good source of many beneficial organisms. Reports are available confirming the presence of *Achromobacter, Alcaligener, Bacillus, Flavobacterium, Micrococcus, Pseudomonas, Thiobacillus, Acinetobacter* in the activated sludge unit of the water treatment industry (Yazdi *et al.* 2001). Few nitrifying organisms like *Nitrosomonaser* and *Nitrobacter* are also available in some industrial effluent (Lightharst & Oglesby, 1989). More importantly, some discrete sources, like ancient and modern ice core archives can be mentioned. Isolates have been found from glacial ice core of Tibet, China that range from 5 to 20000 years in age. Most of these bacteria were spore forming *Bacillus* and *Actinomycetes*. Moreover it is important to consider the numbers and types of native rhizobia in the soil because they can affect there salts of inoculating

legume seeds with introduced rhizobia. Remember that native rhizobial populations are diverse, containing effective and ineffective strains. If there are already many native rhizobia in the soil that can nodulate a legume crop and induce BNF, Rhizobia are more prolific in respect of their microbial diversity. These bacteria help symbiotic relation with legume plants as well as they often draw an impact over their growth performance. Since, bacteria found in Root nodule legume plants, especially *Rhizobium* bacteria have become a tremendous tool in modern day microbial genetics.

1.1.4 Leguminous plants

A legume is a plant in the family fabaceae (or leguminosae), or a fruit of these specific plants. A legume fruit is a simple dry fruit that develops from a simple carpel and usually dehisces (opens along a seam) on two sides. Legumes have been used in agriculture since ancient time and legume seeds or pulses were among the first source of human food and their domestication. Legume plant posses a unique ability to establish symbiosis with nitrogen fixing bacteria of the family Rhizobiaceae. The bacteria belonging to the genera *Rhizobium, Bradyrhizobium, Allorhizobium, Sinorhizobium* and *Mesorhizobium* (Willems, 2006) which are collectively referred to as rhizobia, are able to form nodules on their host plants inside of which they fix-nitrogen. This symbiotic relationship reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops and also enrich soil with nitrogen. Leguminous plants are also of crucial importance as animal feed. Alfaalfa and clovers are grown over extensive areas as forage crops for grazing or as dry hay, and they furnish not only high quality protein but also a variety of biologically active molecules such as vitamins, minerals and other nutrients (Burris and Roberts, 1993). Leguminous plants, like (Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean), Although symbiotic nitrogen fixation by legumes is generally the dominant source of nitrogen input in soil for imparting fertility but also avoid soil stresses, such as temperature, acidity and salinity which pose a severe yield constraint in obtaining plant growth and development (Lawson *et al.,* 1995). In this work, we characterized the diversity of *Rhizobium* isolates by using morphological, biochemical and physiological tests, and to select isolates adapted to the climatic conditions.

Rhizobia are well known for their capacity to establish a symbiosis with legumes, Legumes are unique plants which have the ability to work with certain bacteria *i.e.*

Rhizoibia to gather available nitrogen from the soil atmosphere and convert itto usable ammonia nitrogen and make it available to the plant.They inhabit root nodules, where they reduce atmospheric nitrogen andmakeitavailableto theplant.Biological nitrogen fixation isa componentof sustainableagriculture and Rhizobial inoculants have been applied frequently as bio-fertilizers. Each major legume group is nodulated by different species of *Rhizobium.*Soybeans are nodulated by *Rhizobium japonicum* (Krichner and Buchanan1926). Fred and his associates (1932) recognized eight crossinoculants group inlegumes.The genus *Rhizobium* was erected by Frank (1890) based on its characters to form nodules on roots of legume plants.This property is the only valid test in the identification of the organism. Apart from it some diagnostic features of *Rhizobium* could be conveniently not only to determine and identify the organism but also delinate different species (Graham and Parker1964; Vincent 1970) *Rhizobium japonicum* syn. *Bradyrhizobium japonicum* isassociated with the root nodules of Soybean and fixes 100 kgnitrogen/ha/year (Purohit&Kumar1998). Therefore the attempt has been made to study the morphological and biochemical characters of the bacterium.

Leguminous plants are classified into three major botanical subfamilies of the family Leguminoseae-the Ceasalpinioideae, the Mimosoideae and the Papilionoideae. There are nearly 750 genera and 18,000-19,000 species of leguminous plants of which 500 genera and approximately 10,000 species belong to the subfamily Papilionoideae. Not all legumes bear nodules on their root system and it is known that certain tree forms do not possess them at all. Hardly 16% of Leguminoseae have so far been examined for nodulation of which 95% of Mimosoidese, 26% of Ceasalpinioideae and 90% of Papilionoideae possess root nodules. The legumes form one of the largest families of flowering plants. Leguminosae family constitute 690 genera and 12,800 species (Singh, 1977). When world faces problem of malnutrition, protein-rich crops assume special significance. Pulse crops arc legumes and these are rich in nutrients. India stands first in production and area under legume in the world. The pulses are legumes, which constitute an important source of proteins in the Indian diet. In general pulses contain 20-30% protein, about 60% carbohydrates, 1.0-2.5% fat and are good sources of thiamin, nicotinic acid, calcium and iron (Gupta, 1988).

The leguminous plants through the agency of nodule bacteria fixes atmospheric nitrogen and increases nitrogen content of soil. By considering this role of legumes, in building soil fertility, practice of green manuring started, in earlier years in India. Some of the most common legumes used as green manures in Bangladeshi agriculture are- Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean.Variety of methods have been investigated for studying susceptibility of *Rhizobium* to infect specific legumes. In order to increase Bangladeshi food supplies, to avoid shortage of nitrogenous fertilizers, it would be necessary to accelerate our studies on *Rhizobium* -legume interaction.

When world faces problem of malnutrition, protein-rich crops assume special significance. Pulse crops are legumes and these are rich in nutrients. In general pulses contain 20-30% protein, about 60% carbohydrates, 1.0-2.5% fat and are good sources of thiamin, nicotinic acid, Calcium and iron (Gupta, 1988).Well known legumes include alfalfa, clover, peas, beans, lentils, lupins, mesquite, carob, soybeans and peanuts. legumes are notable in that most of them have symbiotic nitrogen fixing bacteria in structures called root nodules. These crops are short durable and its cultivation is highly profitable. As a result these crops draw special attention to the farmers. By fixing nitrogen, *Rhizobium* increases the soil fertility and also reduces the dependence on synthetic nitrogenous fertilizer which is expensive & injurious. Human beings need many essential food materials to survive on earth and these are protein, carbohydrate, Hit, minerals, vitamins etc. They can obtain those food materials directly from the plants and from these individuals which are also dependent on plants. This way, plants are playing vital role in human life constantly along with few other important functions.

1.1.5 Legume *Rhizobium* association

Rhizobium when come in contact with the roots of leguminous plants penetrates into the root tissues and cause successful infection. This process can occur in three steps.

(i) Recognition of host.

(ii) Attachment and curling of root hair.

(iii) Formation of infection thread and liberation of Rhizobia into root cortex.

Infection and nodule formation is host specific phenomenon. Recently it has been reported that host-bacterium symbiosis may be determined by the binding of host legume lectins to characteristic carbohydrate receptors on the *Rhizobium* cell surface. The lectin is present on root hair which serves as target cells for infection (Dazzo *et al.,* 1979). Root hair curling is caused by secretions of the bacteria. Several workers have suggested the production of indole acetic acid in the rhizosphere of legume (SubbaRao, 1999). Infection thread is essentially of host origin and is composed externally of cellulose and internally of hemicellulosic substances (Rewari and Tilak, 1988).The infection thread penetrates through the cortex and contacts with preformed tetraploid cell, which forms a unit with surrounding diploid cells. The unit undergoes mcristematic division to form the young nodule.

Leghaemoglobin pigment is present in the legume root nodules. Its amount varies with *Rhizobium* strain: Current evidences show that Leghaemoglobin function, as: (i) Site of nitrogen adsorption and reduction, (ii) Specific electron carrier in nitrogen fixation, (iii) Regulator of oxygen supplies, (iv) Carrier of oxygen (Rewari and Tilak, 1988).*Rhizobium* can fix atmospheric nitrogen within root nodules and hence considered as obligatory symbiotic nitrogen fixer. Once *Rhizobium* enters into the roots it derives the nutrients from the host plant. It absorbs carbohydrates present in the root tissue, obtains nitrogen from air and synthesizes the cellmaterial. It passes a part of the nitrogenous compounds synthesized by it (SubbaRao, 1987).Continuous cropping without replenishment of nutrients lead to loss of soil fertility. But due to cultivation of legumes severity of this problem is minimised.Because we know that different species of *Rhizobium* form symbiotic association that promotes fixation of atmospheric nitrogen in the soil. Thus legume-*Rhizobium* association acts as renewable resource of energy.

1.1.6 Taxonomic status of *Rhizobium*

Taxonomic status of *Rhizobium* as well as its classification is still controversialtask. Many authorities tried to solve the problem by reviewing all existing opinions (Elkan, 1992). In fact, Beijerinck (1888) isolated this microorganisms from nodules and proposed the name, *Bacillus radicicola.* But Frank (1889) quickly renamed this as *Rhizobium.* Therefore, a number of uthors provided different types of classification (Dadarwal *et al,* 1977; Elkan, 1992; SubbaRao. 1999; Tate, 2000).There are 750 genera

of legumes, containing 16000-19000 species but of these are economically exploited and grown over large areas, and only a few rhizobia have been studied. Only about 15% of the legume species have been examined for their ability to form nodules with rhizobia (Allen and Allen 1981).

Rhizobium offer taxonomic challenge since many years their characterization was based on a selective interaction with a plant host Beijerinck in Holland was the first to isolate and cultivate a microorganism the nodules of legumes in 1888. He named it *Bacillus radicicola,* which is placed in Bergey's Manual of Determinative Bacteriology under the genus *Rhizobium* (Table 2.1). Early researchers considered rhizobia to be a single species capable of nodulating all legumes. Although others had observed host-*Rhizobium* specificity. It was extensive cross testing on a relatively few legume hosts that led Baldwin and Fred (1927) to propose a taxonomic characterization of rhizobia based on bacteria-plant cross inoculation groups (Eckhard *et al.*,1931; Fred *et al.*,1932). Fred *et al.* (1932) defined these as groups of plants within which the root nodule organisms are mutually interchangeable. The principle of cross-inoculation grouping is based on the ability of an isolate of *Rhizobium* to form nodules in a limited number of species of legumes related to one another. All rhizobia that form nodules on roots of certain legume types have been collectively taken as a species. This system of classification has provided a workable basis for the agricultural practice of legume inoculation. Under this scheme, seven species are recognized which are presented in Table 2.1 (SubbaRao, 1999).

Table1.1: The taxonomic classification of rhizobia according toBergey's Manual of Systematic Bacteriology (Jordan, 1984).

Recognized genera	**Recognized species**
Bradyrhizobium	*B. Japonicum*
Rhizobium	*R, leguminosarum*
	R. leguminosarum bv. *trifolii*
	R. leguminosarum bv. *phaseoli*
	R. leguminosarum bv. *viceae*
	R. mieliloti
	R. loti

Division of rhizobia into groups according to growth rate on laboratory medium. The term "fast growers" commonly refers to rhizobia associated with alfalfa, clover, bean, and pea, since these rhizobia exhibit less than one half the doubling time of slow growers (or <6h). The slow growers are exemplified by soybean and cowpea rhizobia. Graham (1964), using numerical taxonomy, contended that the differences between slow- and fast-growing *Rhizobium* were too great to be based solely on evolutional differentiation of root-nodule bacteria from an organism similar to the present day slow-growing type. Hennecke *et al.* (1985) catalogued the 16S rRNA of the fast and slow-growing rhizobia and concluded that these groupings indeed represented different genetic phyla since the similarity coefficient of the RNA is only 0.53. Thus these groups of rhizobia are less related to each other than to nonsymbiotic relatives. *Rhizobium* is closely related to *Agrobacterium,* whereas the slow-growing rhizobia do not appear related to *Agrobacterium*but rather to *Psudomonas palustris* (Young and Jhonston 1989). Dadarwal *et al.,* (1977) studied seven *Rhizobium* spp. (Cowpea group) isolated from annual wild herbs and desert tree legumes along with *Rhizobium* spp. (Cowpea) isolated from five annual cultivated legumes such as green gram *(Vigna radiata),* black gram *(V. mungo),* red gram *(Cajanus cajan),* ground nut *(Aruchis hypoguc)* and bengal gram *(Ciccr arienlinum)* for serological cross activity among them as well as with *Rhizobium* species of different cross-inoculation groups. Rhizobia of 12 host species formed three somatic serogroups on the basis of cross precipitability for somatic antigens in immuno diffusion plates. The three groups are as follows:

Group 1: Rhizobia of *V. radiata, A. hypogaea, V. mungo, C. cajan Aeschynomene indica.*

Group 2: Rhizobia of Acacia senegal, A. torulis, Prosopis cineraria, and Dolichos lablab.

Group 3: Rhizobia of *Dalbergia sisso, Cicer arietinum,* and *Alysicarpusvaginalis.*

The first group with the exception of *Aeschynomene indica*includes annual, cultivated legumes and their rhizobia are antigenically related to *R. japonicum.* The second group includes all perennial legumes and their rhizobia are more related to *R.meliloti.* The third group consists of three hosts, two of which are perennial wild legumes and the third is the bengal gram. Rhizobia of this group show a great degree of symbiotic as well as antigenic specificity. From the view of a microbiologist, rhizobia constitute a somewhat nondescriptive group of bacteria. Classification schemes based on data derived from use of traditional physiological tests are inadequate to separate the genus

into meaningful groupings. An alternative trait common to rhizobia that provides a reproducible method for their classification that was testable by methods available to early microbiologists is host specificity. The resultant association of strains into groups based on the host specificity is called cross-inoculation groups. Cross-inoculation groups are by definition a collection of leguminous species that develop nodules when exposed to bacteria obtained from the nodules of any member of the group. More than 20 cross- inoculation groups have been established. Of the seven most studied groups, six are sufficiently described to designate the responsible bacteria as species (Tate, 2000). The most- studied cross inoculation groups are:

The alfalfa group: Alfalfa *(Mcdicago* spp.) and sweet clover *(Melilotus* spp.) nodulated by *R. meliloti.*

The clover groups: Clovers *(Trifolium* spp.) nodulated by *R. trifoli*

The pea group: Pea *(Pisum* spp.) nodulated by *R. phaseoli.*

The soybean group: Soybean *(Glycine* spp.) nodulated by *Bradyrhizobium japonicum*and *Bradyrhizobium* spp. (symbionts of the cow pea group) were previously classified as slow growing rhizobia, *Rhizobium japonicum.*

The cowpea group: This group has not achieved a species designation, but these organisms nodulate a variety of legumes including cowpeas *(Vigna* sp.*),* Kudzu *(Pueraria* sp.*),* peanuts *(Arachis* sp.*),* and lima beans *(Phaseolus* sp.*)* However, the following revision has been proposed by the International committee on systematic bacteriology and included in the ninth edition of *Bcrgey's Manual of Determinative Bacteriology* (SubbaRao, 1999).

1.2 BIOCHEMICAL CHARACTERISTICS

Distinguishing *Rhizobium* from its common associate *Agrobacterium*

Agrobacteria happen to be the common contaminant during the isolation of rhizobia. It becomes necessary therefore to ensure their absence while confirming the purity of the rhizobial isolates.

Production of 3 ketolactose: Bernaerts and De Ley (1963) described a test to differentiate Rhizobia from Agrobacterium. According to them *Agrobacterium* produce 3 Ketolactose on lactose containing medium. When the plates are flooded with

Benedict's reagent a yellow ring of precipitate of cuprous oxide is observed around the growth. Neither *Rhizobium* nor *Bradyrhizobium* are able to produce 3 Ketolactose (Gaur and Sen 1981).

Growth in Holer's alkaline medium: Hofer (1935) described the difference in the growth of Agrobacteria and Rhizobia in alkaline medium. He observed that at pH 11, only Agrobacteria could grow. Hence this characterstics was considered suitable in negative selection of rhizobia from agrobacteria (Allen and Allen 1950). However, many contradictory reports have been published which assign only a limited value to the test (Graham and Parker, 1964).

Growth on congo red medium

Initially it was thought that only the Agrobacteria have the ability to absorb Congo red dye to impart orange colour to the colonies (Vincent, 1970). However, it has been observed that many rhizobia especially the fast growers also absorb the dye (Kneen *et al*, 1983). The dye absorbed on the surface of the bacterial cell takes various shades according to the predominance of the ions present in the medium. If the medium was highly alkaline due to predominance of magnesium ions the colonies took fuchsin colour, and blue coloured colonies were observed at pH 3.0 (Hahn, 1966).

Growth on glucose peptone agar

The rhizobia do not use glucose and peptone readily. Hence this test is used to check for non rhizobial contaminants. All rhizobial strains except *Rhizobium meliloti* show little or no growth in the medium without change in pH within 48 hours, whereas agrobacteria grow well, with a pH change in the medium (Kleezkowske *et al*, 1968). Gaur and Sen (1981) in their studies on Cicer rhizobia have shown that most of the strains did not grow and those which showed little growth did not change the pH of the medium in 48hrs. Similar observations were made Subba Rao (1999) mentioned that *Rhizobium* grows poorly in this medium and causes littl e change of pH.

Acid and alkaline reaction

Rhizobial growth on a medium containing a carbon source is accompanied by change in the reaction (acidity or alkalinity) of the medium which depends upon the kind of bacterium and the carbohydrate used. This was first demonstrated by Bialsuknia and

Klott (1923) and subsequently, it was confirmed by many others namely Fred *et al.*,(1932), Jones and Burrows(1969). Acid and alkaline production in yeast extract mannitol agar with bromothymol blue, as culture medium has been employed in taxonomic characterization of rhizobia (Norris, 1965). With mannitol, barring a few exceptions, rhizobia of soybean, lupin and cowpea groups produce alkali; whereas those of medic, clover, pea and bean groups produce acid.

Carbohydrate utilization

In many studies the utilization of different carbon sources of rhizobia have been examined .It appears that all the rhizobia, irrespective of their host group, utilize almost all the carbon sources. If there is any difference it may be quantitative depending upon the strain but not upon the group of rhizobia.

Glucose consumption:

Attempts have been made to work out same method for determination of efficiency of *Rhizobium* spp. from their easily assessable characteristics. Since sugar is used as energy material for fixation of nitrogen by nitrogen fixing organisms, it was suspected that high sugar requirements might be related to their capacity for nitrogen fixation (Gupta and Sen, 1965).The relationship between glucose consumption by *Rhizobium japonicum* and its nitrogen fixation efficiency has been studied by Sen (1965) who observed a significant positive relationship between the two parameters. Gupta *et ai,* (1971) reported wide variation in glucose consumption by strains of *Rhizobium* belonging to the same species.

1.3 PHYSIOLOGICAL ACTIVITY OF THE RHIZOSPHERE BACTERIA

Studies based on cultures isolated from the soil and rhizosphere, it was noted that the bacteria of the rhizosphere are physiologically more active than the soil bacteria. The mere fact that bacterial cells arc more abundant in the rhizosphere than in the soil is evidence that they were more active physiologically in their natural environment, irrespective of their inherent physiological characteristics or what they did in the soil.

Rovira (1956) observed that the rhizosphere isolates generally grow more profusely than the soil isolates in various media. Katzneison and Rauatt (1957) observed that the

rhizosphere bacteria are more active in reduction of methylene blue and resazurin in various media, in production of acid and gas from glucose, in liberation of ammonia from peptone, and in denitrification. They also observed higher oxygen uptake by rhizosphere bacteria while utilizing substrates like glucose, alanine and acetate.

1.3.1 The effects of rhizosphere microorganisms on plants

The opinion has been expressed that each kind of plant has a typical rhizosphere population and inoculation with rhizosphere microorganisms will hasten plant growth and secure vigorous plant development. The activities of some rhizosphere microorganisms, such as phytopathogens, are unfavourable to plant development. The majority of the rhizosphere microorganisms are saprophytes and transform both organic and inorganic material in the rhizosphere. The products of these transformations may be beneficial or injurious. Certain rhizosphere organisms have more direct effect on plants through symbiosis or parasitism. Root colonization and factors affecting microorganisms in soil.

1.3.2 Factors influencing root colonization

Root colonization is influenced by characterstics (rhizosphere competence traits) of the introduced pseudomonad, the rhizosphere microflora, the plant, and the abiotic environment. Bacterial phenazinc antibiotic production was shown to contribute to the long-term survival of fluorescent pseudomonads in soil. The production of specific siderophores and the utilization of broad spectrum of siderophores contribute to the root-colonizing and plant growth-promoting ability of a *Pseudomonas putida* strain (Bakker *et al*, 1986). Chemotactic attraction of pesudomonads towards root-or seed-exudates may be a guide to infection sites where exudation is enhanced. Tolerance to dry soil and low osmotic potential may help some introduced bacteria to survive. Studies with flagella-negative, non-motile mutants revealed that motility either enhanced or had no effect on root colonisation. Cell surface properties, like agglutinability, lipopolysaccarides, and pill may help to support the establishment or the specificity of bacteria-plant association. Composition of root exudates may vary with species, cultivar, and even growth stage of the plant and thus affect root colonisation. Furthermore, interactions with the indigenous rhizosphere microflora and physical and chemical characterstics of the soil may drastically influence the establishment and survival of an introduced PGPR agent.

1.3.3 Environmental factors affecting competition

Environmental factors such as soil type (Ham *et al.*, 1971), temperature (Kluson, *et al.*, 1986), soil moisture (Boonkerd and Weever, 1982) and soil pH (Dughri and Bottomley, 1983) affect the growth of both plants and bacteria. As plants must develop a rhizosphere to support rhizobial growth as well as build a morphologically developed nodule to house the invading rhizobia. Any factor that adversely affects plant growth will also profoundly affect competition.

1.3.4 Soil type

Soil is a reservoir of *Rhizobium*strains and the intrinsic make up of the soil can affect the outcome of competition. Bowen and Rovira (1976) showed that the growth rate of *Rhizobium* in soil without plants is slow as compared to rhizosphere of legumes and observed increase in size of *Bradyrhizobium japonicum* inocula in fallow soil, while a 100-fold increase was noted in the rhizosphere of soybean. Soil acidity affects all aspects of nodulation and nitrogen fixation from survival and multiplication of the rhizobia in soil, through infection and nodulation, to nitrogen fixation. Aluminium and manganese toxicities and deficiencies of calcium, molybdenum, and phosphorous can all contribute to this problem, with their relative importance different in each soil.

Legumes are commonly inoculated with selected strains of rhizobia in the expectation that inoculation will increase nitrogen fixation and crop yield. While dramatic increase in yield became possible, some factors might limit the full expression of the inoculation response. These are failure to establish the inoculums at normal inoculation rates due to competition from the native soil strains or reduced inoculums viability due to stresses such as temperature and desiccation. The plant yield potential may be limited by environment, so that N demand is met by available soil N or through N_2 fixation by less effective soil rhizobia. Sufficient population of soil rhizobia may exist to meet host demand for symbiotic N_2 under non-limiting growth conditions for the host.

However, considering the view points mentioned above much more effort has been made by many scientists in the world to identity the strains of *Rhizobium,* superior to N_2 fixation and tolerant to a variety of soil stresses. But in Bangladesh, there is very little understanding of how native populations of rhizobia determine legume N_2 fixation potential. At the same time report regarding response of inoculation with selected

rhizobia in terms of number and effectiveness of the population for a particular host is also scanty in our country. Thus, the literature regarding responses of legume in relation to effectiveness of *Rhizobium* species along with few other relevant subject matters are reviewed here.

Peat soils are generally dried under sunlight after collection and made them free from any dust materials. Then the soils are ground to fine powder, capable of passing through 200 mesh sieve. Afterwards the soils are heat treated followed by neutralization $CaCO_3$ to raise pH 6.5-7.0. Then pouring in polythene bag the soil materials are sterilized with the help of autoclave. Now a days sterilization by gamma radiation (Cobalt ray) is considered to be most acceptable than autoclave sterilized method. Radiation rate for 1.5-5.0 megarad has been proved to be enough for peat sterilization.

Peat as carrier for rhizobia is more desirable than soil due to its high organic matter content, aeration and higher water holding capacity.Due to higher organic mailer content, aeration and water holding capacity peat is more suitable carrier for rhizobia than soil. Iswaran *el al.*, (1970) found that in peat at 28°C, there was a slight fall in counts in the first week and then, the counts increased while at 25°C there was multiplication up to second week and declined thereafter. In case of the soil at 28°C and 35°C, the counts were appreciable only up to second week and thereafter there was sudden fall. At 40°C there was a rapid death of rhizobia added to soil as compared to peat with no worthwhile recovery after four weeks of storage.

Survival of rhizobia in soil under varying conditions is an important object of study from the point of cultivation of legume and success of inoculation practices. They observed that the counts decreased gradually with advance of time. They found maximum survivability of groundnut *Rhizobium* for 16 weeks in red soil of Coimbatore. However, no correlation was noticed between maximum periods of survival of the strain with any of the soil constituents. They seemed that survivability of *Rhizobium* species in a particular soil to be used as a carrier during preparation of legume inoculants to make it effective and useful.

1.3.5 pH

Legumes are major sources of protein and energy for both humans and domestic animals, and the *legume-Rhizobium* symbiosis is now the most widely managed agricultural system for biological nitrogen fixation. For successful nodulation, soil pH is one of the important factors. Study of the survival of *Rhizobium* in acid soils. They stated that a *Rhizobium* strain nodulating cowpcas did not decline in abundance after it was added to sterile soils at p^H 6.9 and 4.4, and the numbers fell slowly in non-sterile soils pH 5.5 and 4.1. A strain of *R. phasedi* grew when added to sterile soils of pH 4.3 and 4.4. Two *R. meliloti* strains which differed in acid tolerance for growth in culture did not differ in numbers or decline when added to sterile soils at pH 4.8, 5,2 and 6.3. However, the less sensitive strains were found to survive in better condition in limed soil.The basis pH tolerance in neutrophilic species is through the regulation of cytoplasmic pH, with cells maintaining an internal pH of 7.5 to 8.0 against a pH differential with the outside environment of as much as three pH units (Booth, 1985). This is usually achieved through the expulsion of proton, with uptake of potassium ions needed to balance cell charge. Some species also possess a cytoplasmic buffering capacity [20-1000 nmol hydrogen ions per 1 unit per milligram cell protein (Krulwich *et al.,* 1985)] or the ability to regulate cell metabolism and offset the accumulation of acidic end products (Goffwald and Gottschalk, 1985)

Soil pH has an effect on survival and competition of *Rhizobium* in soil. The survival of Rhizobia in acid soils is inversely related to the amount of acid produced, that is, those rhizobia that produce the least acid should survive best in acid soils (Norris, 1965). *Rhizobium* strains vary in their acid tolerance (Karanja and wood, 1988). The failure of legumes to nodulate under acid-soil conditions is common (Brockwell, 1962; Rice *et al.,* 1977), especially in soils of pH less than 5.0. The inability of some rhizobia to persist under such conditions is one cause of nodulation failure (Lowendorf and Alexander, 1983).

Fast- growing rhizobia have generally been considered less tolerant to acid pH than bradyrhizobia, but strains of *Rhizobium loti* and *Rhizobium tropici* have proved highly acid tolerant, with growth in some cases to pH 4.0 (Cooper, 1982). Strains of *Rhizobium meliloti* are particularly affected by acidity, soil pH being the major factor limiting their numbers in soil (Brokwell *et al.,* 1991). Brokwell *et al.,* (1991) found an

average of 89000 organisms per gram in soils where the pH 7.0 or more, but only 37 per gram in soils of pH less than 6.0.

Bushby (1990) found that only *Bradyrhizobium*strains isolated from acid soils had a net positive charge, and postulated a relationship between their surface-charge characterstics and the soil from which they came. Despite this, strains isolated from acid soils are not necessarily pH tolerant (Richardson and Simpson, 1988; but rather are concentrated in more favourable microsites in the soil. Thus, for soil pH 4.2, only 96 of 481 isolates were capable of growth even at pH 4.70 (Richardson and Simpson, 1988).Low soil pH does not allow the rhizobial cells to survive in adequate numbers in free living state. Consequently it becomes inevitable to inoculate the crop in adequate rhizobium (Deka and Azad, 2006).

1.3.6 Temperature

Soil temperature influences the ability of *Rhizobium* to persist in soil. High soil temperature could contribute to high frequency of noninfective isolates in soil. Segovia *et al.* (1991) noted that such noninfective isolates actually outnumbered those that,were infective in the rhizosphere of bean. On inoculation of promiscuously nodulatedculliver of *Glycine mux* with *Vigna*as well as *B. japonicum,* the Vigna-rhizobia were more competitive at high temperatures (24-30°C) (Roughley *et al.,* 1980). Strain differences in nodulating ability at low temperature have also been shown between arctic-zone rhizobia (Ek-Jander and Fahraeus, 1971) while dual occupancy was found reduced from 63% at 8°C to 2% at 25°C, with the proportion of nodules occupied by strain NRG 185 alone increased from 9 to 75% over the same temperature range (Rice and Olsen, 1988).For most rhizobia the temperature for growth in culture is from 28 to 3l°C, with many unable to grow below 10 or at 37°C. However, arctic rhizobia are known to grow well at 10°C (Caudry- Reznick *et al.,* 1986), while the optimum for *R. meliloti* is 35°C (Allen and Allen, 1950).

Temperature affects root-hair infection, bacteroid differentiation and nodule structure, and the functioning of the legume root nodule (Roughly and Dart, 1969). High soil temperatures will also delay nodulation or restrict it to the subsurface region, where temperatures are not as extreme. Munns *et al.,* (1977) found that alfalfa plants grown in desert environments in California maintained few nodules in the top five cm of soil but

were extensively nodulated below this depthand also observed fewer nodules close to the surface in spread plantings than in plantings with dense canopies.Rhizobia are gram negative rods and its speciation is based on the cross-inoculation grouping. Rhizobia that can form nodules on roots of legumes have been collectively taken as a species, particularly in case of the legume *Vigna* and *Arachis* it is known as *Rhizobium* sp. It is also known to survive in soil and in the rhizospheres of legumes as well as non-legumes. *Rhizobium* secretes extracellular polysaccharides which may help in binding soil particles together. They can survive at low temperature and tolerates temperature upto 50°C for more than of few hours. It is sensitive to plant protectants, antibiotics and other agricultural chemicals (Nutman, 1965). *Rhizobium* is more tolerant toward salts than its host legume and therefore, survives in saline soils (Subba Rao *et al.*, 1972, 1974).

Information on the survival of rhizobia seeded in peat and soil or on seed at high temperature exposure is of special significance in tropics as that would determine the success of establishment of effective strains in the soil through seed inoculation. Results of a study in this aspect were reported by Iswaran *et al.* (1970). They found that in peat at 28°C, there was a slight fall in counts in the first week and then, the counts increased while at 35°C there was multiplication upto second week and declined thereafter. In case of the soil at 28°C and 35°C, the counts were appreciable only upto second week and thereafter there was sudden fall. At 40°C there was a rapid death of rhizobia added to soil as compared to peat with no worthwhile recovery after four weeks of storage.Temperature affects growth as well as survival of *Rhizobiwn.* However, it can survive at low temperature and can tolerate temperature up to 50°C for more than a few hours (Subba Rao, 1985). Temperature optimum for nodulation and nitrogen fixation vary between species (and genera) but are within a small range near 30°C (Islam and Dart, 1975).

1.3.7 Salinity

Rhizobia are sensitive to higher salt concentration (Wilson, 1980). They show the capability to grow at 200mM salt concentration, but the growth is more abundant at lower salt concentration. Only few strain of *R. melilotic*an grow at 2% NaCl concentration, but none can grow at 3% NaCl concentration (Graham and Parker, 1964).*Rhizobium* is more tolerant toward salts than its host legume and therefore,

survives in saline soils (Subba Rao *et al.*, 1972, 1974). *Rhizobium* strains from lucerne, moth bean, black gram, green gram and pea were tested by Yadav and Vyas (1971) for their sensitivity to saline-alkaline conditions and pH. Lucerne and pea rhizobia were both salt sensitive (0.2%) and salt resistant (6%). Black gram and moth bean strains were proportionately sensitive to CT and salts of Na^+ and K^+ but those of green gram were stable. Magnesium salts were stimulatory at concentration lower than 1%. For all rhizobia 0.4-0.6%$NaHCO_3$concentration was critical. All the strains survived at pH 10.1, but were inhibited at pH 3.5.The effect of salt stress on growth nodulation and nitrogen accumulation in cowpea *(Vigna sinensis)* and mung bean *(Vigna aureus)* was studied by Balasubramaniam, 1976 in sand culture. Salinity (NaCl) retarded the growth of leaves, stem and roots of both the crops. The relative growth rates of stressed plant parts declined initially but were subsequently higher than those of control for a period, suggesting that the plants tended to adapt to unfavourable environment even while being stressed. The total nodule number, weight and nitrogen content of plant decreased due to salt treatment which interfered with the initiation of nodules but not with their further development. There was a considerable fall in the nitrogen fixation efficiency of mung bean under saline environment but it was not so in cowpea.

Salinity tolerant mutant strains of *Rhizobium*, able to grow and fix nitrogen in symbiosis with lentil in saline soil, were derived from an effective *Rhizobium* strain RL5 by treatment with nitrosoguanidine. Among the five mutants selected, two mutants viz. LM4 and LM1 were resistant to 200 ug ml/ of streptomycin and 1.5% of NaCl. These two mutants also significantly increased number and dry wt of nodules per plant, dry matter yield of the crop and N_2 fixation. Between the two mutants, LM4 was superior to LM1 in symbiotic behaviour (Rai and Prasad, 1983).

Fast growing acid producing *Rhizobium* strain 995 of *Vigna radiata* was screened for growth behaviour in acid, saline and alkaline media. It grew well in yeast extract mannitol broth of wide pH range as well as varying concentrations of NaCI, Na_2S0_4 and $MnCl_2$. Variations in nodulation and nitrogen fixation efficiency occurred on different varieties of green gram, while strain characteristics, were not affected by soil stress (Rai and Prasad, 1984).

1.4 MOLECULAR CHARACTERISTICS

In recent years, molecular tools have been developed to identify and quantify specific microbial cells, or specific microbial activities, in mixed populations. These tools are specifically valuable for analysis of specific bacteria in complex environment samples. Increasingly research has concentrated on the development of marker genes for lagging a particular bacterial species of interest. So that the cells can be specifically identified and monitored.

1.4.1 Molecular Technique

last fifteen years of the twentieth century allowed for an exponential increase in the knowledge of techniques in molecular biology, following the cellular and protein era of the 1970s and 1980s. This explosion of technologies from the primary discipline of molecular biology has had major consequences and has allowed for significant developments in many areas ol the lile sciences, including bacteriology. Although, most modern clinical microbiology diagnostic laboratories rely on a combination of colonial morphology, physiology and biochemical/serological markers, for their successful identification either to the genus level or more frequently to the species level. Molecular methods are increasingly being used.

The aim of this study was to study diversity of *Rhizobium* spp. in agricultural lands of Rajshahi, Bangladesh. Physicochemical properties of soil was studied using standard methods while molecular methods used to study diversity within *Rhizobium* species. Further, population analysis of *Rhizobium* species in relation to genetic diversity was carried out using 16S rDNA- PCR. *Rhizobium* were identified and genetically by determining the %Guanine plus Cytosine content of the whole genome, followed by Restriction enzyme treatment of Polymerase Chain Reaction (PCR) amplified product of 16S rDNA segment was performed. The sequences recognized by the restriction enzymes are distributed at variable intervals in the genome of an organism and also vary in number. The separation carried out by electrophoresis (agarose gel) resulted in specific banding pattern differing within as well as among different species. The technique used was helpful in characterizing *Rhizobium* isolates to be used as inoculants for improving agricultural land quality of Rajshahi, Bangladesh.

Members of the genus *Rhizobium* are symbiotic nitrogen fixing bacteria which are able to invade and form nodules on the roots of leguminous plants. The most dramatic progress in the construction of microbial phylogeny is based on sequencing analysis of the ribosomal genes. The 16S or small subunit ribosomal RNA gene is useful for estimating evolutionary relationshipsamong bacteria because it is slowly evolving and the gene product is both universally essential and functionally conserved (van Berkum and Eardly, 1998). Direct sequencing of genes coding for 16S rRNA (16S rDNA) has been used to establish genetic relationships and to characterize strains at the species or higher level (Laguerre *et al.,* 1996). The full-length sequence analysis of 16S rDNA is one of the most important methods to estimate the phylogeny of rhizobia (Young and Haukka, 1996), while the 900 bp partial 16S rDNA sequencing correlated well with full-length 16S rDNA sequencing (Terefe- work *et al.,* 1998) and has been used for rapid screening of the phylogenetic relationships among a large number of rhizobia.Sequences of 16S rDNA are known to be highly conserved among eubacteria (Woose, 1987) and analysis of genetic variations in this region is not appropriate to differentiate strains within species (Laguerre *et al*., 1996). However, it is very useful for identification of species. Pairs of universal primers, forward and reverse primers, were design for amplification of 16S rDNA regions in most eubacteria. Pairs of universal primers were used to amplify 16S rDNA (Lane, 1991; van Berkum and Fuhrmann, 2000) to ascertain the non-symbiotic isolates belonging to the genus Bradyrhizobium (Pongsilp *et al.,* 2002). Novel nitrogen-fixing symbionts in genera Methylobacterium, Blastobacter, Burkholderia, Ralstonia, Ochrobactrum, Devosia, Phyllobacterium and Herbaspirillum have been discovered by 16S rDNA sequence analysis (Chen *et al.*, 2001; Rivas *et al.,* 2002; Ngom *et al.*, 2004; Valverde *et al.,* 2005; Barrett and Parker, 2006). These Findings suggest that the gene responsible for symbiosis with legumes is transmissible horizontally and functions in a relatively wide range of bacterial taxa (Rivas *et al.,* 2002). Phylogenetic analysis of the 16S rDNA has been constructed in many previous studies. According to Ngom *et al.* (2004), the clusters in the phylogenetic tree, which was constructed based on nearly the full length of 16S rDNA, correlated well with the taxonomy of strains: i) a first cluster contains *Bradyrhizobium* and Blastobacter in Bradyrhizobiaceae;ii) a second cluster contains Ochrobactrum in Brucellaceae; iii)a third cluster consists of two genera Phyllobacterium and Mesorhizobium in Phyllobacteriaceae; iv) a fourth cluster consists of genera *Sinorhizobium, Allorhizobium* and *Rhizobium* In Rhizobiaceae. Besides 16S

rDNA, sequence analysis of 23S or large subunit ribosomal RNA gene has been also studied. However, the 23S rRNA gene has not been extensively used to estimate the genetic relationships among the Rhizobiaceae, but there are several dramatic differences which may be helpful for classification and identification purposes (van Berkum and Eardly, 1998). Terefework *et al.* (1998) reported that the 23S Dendrogram showed deeper branching than the 16S dendrogram and more genotypes were resolved, although in some cases the sequence divergence is not particularly high.The bacterial species from soils of Madhya Pradesh at ten different.

Locations were examined following known biochemical tests and DNA purified from eight strains of Rhizobium was studied. Furthermore, the pattern of similarities as represented by genetic hierarchy was studied using molecular biotyping methods.

1.5 INOCULATION OF RHIZOBIUM ON LEGUMINOUS PLANTS

Pulses like Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean etc are being grown in Bangladesh as winter (Rabi) crops very enormously which meet somewhat the protein requirements of our huge population. Optimum protein content in our daily food is not that easy as most of our poor people need, because they live in sub-human condition. As the per capita availability of animal protein is decreasing in our country, so, it would be wise to obtain more plant protein from pulses for our people by improving the pulses for high yield at low cost.Pulses are second important crop, next to cereals as our food source in Bangladesh, thus it certainly bears impact in socio-economic development of our country. Pulses have high seed protein, such as in lentil - 25.0%, in black gram - 23.4%, in mungbean -23.6%, in cowpea - 28.2%, in pigeonpea - 22.5% and in chickpea - 23.0% (Rahman and Parth, 1988). These are nearly two to three times to that of cereal grains. Secondly, the amino acid composition of pulse protein is such that a mixed diet of cereals and pulses has a biological value more than that of either component alone.

Human beings need many essential food materials to survive on earth and these are protein, carbohydrate, minerals, vitamins etc. They can obtain those food materials directly from the plants and from these individuals which are also dependent on plants. This way, plants are playing vital role in human life constantly along with few other important functions.A major part of the peoples of Bangladesh suffer from malnutrition

due to deficiency of protein in their daily diets. In such cases, pulses may play important role because they contain 20-25% protein, double of that of wheat and three times of that of rice. Pulses contain more protein than eggs, fish or than any fresh food and are generally high in protein content and digestibility of that protein is also high .

Among the important crops of Bangladesh, pulses may be considered as the second most important crop and for a long time pulses particularly lentil, chickpea, black gram, mung bean, pigeon pea etc. are being cultivated as winter crop.During developmental phases of the leguminous plants, environmental factors may become a major constraint to its better yield in Bangladesh. High yield of any crop may be achieved only when a proper combination of variety, environment and agronomic practices is obtained. Plant breeders are trying to breed high yielding varieties, which may be better adapted to the local environments. It would be of immense help to breeder in quest of improved varieties, and to identify the various morphological and physiological factors governing growth and development. Such factors are important for causing the better yield. But unfortunately, detailed information regarding relationship of developmental stages and seed yield is currently lacking in case of black gram in context of Bangladesh. Therefore, the strategy for improving agricultural production in developing countries like Bangladesh should be taken into inexpensive, realistic and pragmatic programmes.

Estimation of the amounts of N_2 fixed by grain legumes have been made by the International Atomic Energy Agency, Vienna, using labelled nitrogenous fertilizers. Some of the findings obtained by the collaborating scientists indicate that (he values appear to be rather realistic but nevertheless do not minimize the value of legumes in the nitrogen economy of cultivated plants. However, there are both novel and conventional approaches to this problem. In the context of Bangladesh, conventionally, attempts should be made: (1) to provide efficient strains of rhizobia for inoculating legumes; (2) to evolve varieties of plants responsive to biologically and industrially fixed nitrogen; (3) to overcome the inhibition of fertilizer nitrogen on biological fixed nitrogen; (4) to define agronomic practices leading to belter fixation and conservation of nitrogen in the farm; and (5) to evolve simple practices to conserve water in the farm because optimum moisture is needed for successful nodulation and hence the biological nitrogen fixation in legumes.

Legumes have been shown to benefit by the dual action of obligate endophytic enzymes in plant roots and *Rhizobium* in root nodules. The endomycorrhizal fungi appear to influence the uptake of phosphorus which helps in better fixation of nitrogen in root nodules. The biochemical specificity in the legume *Rhizobium* symbiosis happens due to interaction on the root surface involving the two symbionts mediated by carbohydrate binding proteins called 'lectins'. The role of legumes in enriching the fertility of soil is known since the last century. However, scientific demonstrations of the value of legumes in contributing to the nitrogen nutrition of plants were done in the later.half of the 19^{lh} century (Fred *et al.*, 1932). The experiments carried out by many scientists proved that nodules on legume roots are responsible for fixing atmospheric nitrogen. Now it is evident that *Rhizobium* plays important role for nodulation in leguminas plants.

Rhizobium improve the yield significantly in many legume crops .Leguminous crop meet up their nitrogen requirement through biological nitrogen fixation, which is dependent on proper growth, development and leghaemoglobin content of the root nodules. Nitrogen is the most deficient nutrient in soil of Bangladesh. Urea, which is used most commonly as nitrogenous fertilizer has now become a costly input for the farmers. *Rhizobium* can minimise the use of urea, which causes injury to soil properties. *Rhizobium* may be also a cheaper substitute for urea in the production of food legume crops (Karim *et al.,* 2001).Response of inoculation depends on soil type, cultivar and effectiveness of *Rhizobium* strains and also its competitive ability with native *Rhizobium* (Dube, 1976). Even then the use of legume based biological nitrogen fixation technology in the form *Rhizobium* inoculants may be an important source of nitrogen for the leguminous crops (Hoque, 1993).

Very little work has been done in Bangladesh regarding root colonization of legumes by rhizobia in relation to improved management practices, required to achieve higher yield of black gram. The present study was designed to determine if the introduced rhizobial strains can colonize, survive and promote the growth and yield of black gram. In addition, a little work has been done on released black gram varieties grown in Bangladesh considering their growth and development patterns and yield potential under different treatments of *Rhizobium*. Therefore, two black gram varieties widely grown in North-Western Bangladesh were selected in the present investigation and imposed different regimes of *Rhizobium* strains on them to understand their growth

pattern during different stages of growth. The study also compared the varieties for final yield and yield components under different *Rhizobium* treatments.

1.6 ECOLOGICAL ASPECTS OF RHIZOBIA

Rhizobia are ecologically important soil bacteria because they fix atmospheric nitrogen in symbiosis with legumes. Bio-inoculant of efficient, effective and competent *Rhizobium* strains are often used as bio-fertilizer for monitoring soil fertility (Subba Rao, 1977).In global terms the nodulated legume is the most important for biological N_2 fixation. Legumes able to secure their nitrogen in this way are to be found from subarctic to temperate and tropical zones (Allen *et al*., 1963). But work on tropical legumes is accelerating and has added significance because solutions to problems associated with these regions are so important to many developing countries (Vincent, 1977). The N_2 fixing legume has distinct ecological advantage as colonizer of N soils; it provides first protein for man and his animals, without dependence on expensive N-fertilizer and is likely to build up, or at least maintain soil fertility.

Encouragement of *Rhizobium* legume symbioses development does increase soil fixed nitrogen resources. The balance between nitrogen inputs and outputs to land systems and the identity of the sources of this fixed nitrogen (Burns and Hardy, 1975) support the conclusion that *Rhizobium* legume symbioses are a major source of fixed nitrogen in land-based systems (Tate, 2000). The amount of N_2 fixed by an effectively nodulated legume is a function of the amount of plants growth and the extent to which other factors of soil, climate, and photosynthesis permit the system potential to be achieved.

*Rhizobium*significantly improve the yield in many legume crops. Response to inoculation depends on soil type, cultivar and effectiveness of *Rhizobium* strains and its competitive ability with native *Rhizobium* (Dube, 1976). *Rhizobium* can minimize the use of synthetic nitrogen fertilizer which is rather expensive and causes injury to soil properties. Leguminous crop meet up their nitrogen requirement through biological nitrogen fixation, which is dependent on the proper growth, development and also the leghaemoglobin content of the root nodules.

In developing countries, the construction of new nitrogen fertilizer plants is not only expensive but time consuming. Farmers in many parts of Africa do not use inorganic nitrogenous fertilizers because they are imported and expensive. Therefore, the strategy

for improving agricultural production in developing countries should take into account inexpensive, realistic and pragmatic programmes to augment biological nitrogen fixation Bangladesh soils are quite poor in nutrient elements and the yield levels (national average) of major crops are very low compared to other Asian countries of the world. The supply of the nutrients in available form in adequate quantity is essential for higher crop production in intensive agriculture. The synthetic fertilizers are costly and in scarcity due to non availability and high prices of raw materials and growing energy crisis. Besides this, the poor economy of most of the farmers does not allow them to use recommended doses of various fertilizers while the imbalanced use of synthetic fertilizers create environmental pollution.

The role of microorganisms in building and conserving soil fertility is widely accepted in modem agriculture. The beneficial effect of rhizobial inoculates in increasing yield at leguminous crops, results from the activity of its root nodule bacteria, which fix atmospheric nitrogen making it available for the plants. The biofertilizer technology is most popular in modern agriculture, the benefits at this technology in not only utilized in developed countries like USA, Australia, France, Germany, etc. But also in developing and neighbouring countries like India, Thailand, Myanmer, the Philippines, etc. Both Government and Non organizations are producing inoculates in these countries in augmenting crop productivity and to improve soil physical, chemical and biological properties. The technology is gaining momentum at the face of energy crisis prevailing in the modern world. The harmful effect at chemical fertilizers on soils, sky rocketing prices and the increasing gap between supply and demand of the fertilizers, the green house effect, etc. have increased the need of developing indigenous biological renewable resources that could reduce the environmental hazards, lower down the cost of cultivation, improve the soil health and increase the crop yield substantially. Hence, an attempt has been taken to isolate the *Rhizobium* sp. to fix atmosphering nitrogen and thus not only increase the production of inoculated Crops, but also leave a fair amount of nitrogen in the soil, which benefit the subsequent Crops.

Rhizobia are aerobic rod shaped, motile, gram negative, and non spore forming bacteria. Genus *Rhizobium* consists of two groups, (Fast growers and slow growers) based upon their growth rates. Fast growing species can use wide range of carbohydrates and organic acids for their growth while slow growers require restricted

range of substrates for their growth rate. Fast growers possess peritrichous flagella while slow-growers have sub polar flagella (Sprent and Sprent, 1990). *Rhizobium* grows on yeast extract mannitol agar (YEMA) and produces small to medium sized colonies. On medium containing congo red, they produce colourless to faint pink colonics, whereas colonies of contaminants are often deep red. Fast growers produce acid on yeast extract mannitol agar, which includes *Rhizobium loti*, *Rhizobium leguminosarum* and *Rhizobium meliloti.* While slow growing strains produces alkali on yeast extract mannitol agar, which includes *Bradyrhizobium japonicum* and *Bradyrhizobium spp.* (SubbaRao, 1987).

Rhizobium is known to survive in soil in rhizosphere of legumes and Otherplants. *Rhizobium* secretes extracellular polysaccharides, which may help in binding soil particles together. *Rhizobium* is able to enter into symbiotic relationship with legumes. They fix atmospheric nitrogen and thus not only increase the production of inoculated crops, but also leave a fair amount of nitrogen in the soil, which benefit the subsequent crops.*Rhizobium* can survive at low temperature and tolerate temperature upto50°C for more than a few hours (SubbaRao, 1985). It is sensitive to antibiotics other agricultural chemicals (Nutman, 1965). Several soil microorganisms and bacteriophages are known to inhibit the growth of rhizobia (Chonkar and Subba Rao, 1966).

1.7 IMPORTANCE IN AGRICULTURE

In view of legumes cultivation and success of inoculation practices survival of rhizobia in soil is an important subject of study. Rhizobia may survive in soil for a considerable number of years even in absence of host plants unless they are destroyed by unfavourable conditions. Thus, ability of the rhizobia strain for nodulation under varying conditions of soil from different agro-ecological zones of Bangladesh may be an important aspect of this study.

A major part of the peoples of Bangladesh suffer from malnutrition due to deficiency of protein in their daily diets. In such cases, pulses may play important role because they contain 20-25% protein, double of that of wheat and three times of that of rice. Pulses contain more protein than eggs, fish or than any fresh food and are generally high in protein content and digestibility of that protein is also high.Although much of the nitrogen is removed when protein-rich grain or is harvested significant amounts can

remain in the soil for future crops. This is especially important when nitrogen fertilizer is not used, as in organic rotation schemes or some less-industrialized countries. Nitrogen is the most commonly deficient nutrient in many soils around the world and it is the most commonly supplied plant nutrient of nitrogen through fertilizers has severe environmental concerns. Among the important crops of Bangladesh, pulses may be considered as the second most important crop and for a long time pulses particularly Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean are being cultivated as winter crop. In Bangladesh, pulses are grown as kharif crop in some areas mainly in the north/north-west part of the country. It is successfully cultivated in the districts of Chapai Nawabganj, Rajshahi, Pabna, Jessore, Jhenaidah and Faridpur.In Bangladesh, this crop is normally sown in the first week of August to second week of September and harvested in the beginning of November to early December.

The soils of Bangladesh, especially Rajshahi regions are deficient in nitrogen fixing bacteria *(Rhizobium* sp.) which causes poor yield of legumes (Bhuiyan *et al.,* 1998; Khanam *et al.,* 1994). The Bangladesh Agricultural Research Institute (BAR1) has developed a good number of varieties of chickpea. There is a good possibility to increase its production by exploiting better colonization of the roots and rhizospheres through application of effective nitrogen fixing bacteria to the seed or to the soil. This can minimize the uses of nitrogenous fertilizer, which is very costly in this country. Using high yielding varieties of chickpea along with use of effective rhizobial strains can enhance the yield.

But information regarding the survival of *Rhizobium* due to varying conditions of the soil is lacking. Information regarding the soil constituents like organic matter, pH, soluble salts, available phosphorous, potassium, sulphur, zinc, total nitrogen *etc.* which may have different sort of effects on the nodulation, is not also known. The present study may enrich our idea and also may be helpful for conducting further research at various levels.

At the end of the last century symbiotic nitrogen fixing bacteria of *Rhizobium* sp. gained importance in Bangladesh. Encouraging results are being recorded on the yield of cereals and legumes as different strains of *Rhizobium* sp. are being used at institutional level for soil and seed treatment. However, while reviewing the work on

Rhizobium sp. it may be said that actual experimental analysis in Bangladesh is very difficult because of the scarcity of those works in published form and as well as their inadequate statistical evaluation.

1.8 THE OBJECTIVES OF THE RESEARCH WORK

I. Isolation and characterization of *Rhizobium* from Leguminous plants at Rajshahi.

II. To identify *Rhizobium* spp. in Rajshahi area.

III. Physiological characterization on the basis of pH, temperature and salt tolerance.

IV. Biochemical characterization of *Rhizobium.*

V. Extraction of genomic DNA and PCR amplification of 16S rDNA.

VI. Estimation of genomic DNA.

VII. Effects of *Rhizobium* inoculants on Leguminous plants.

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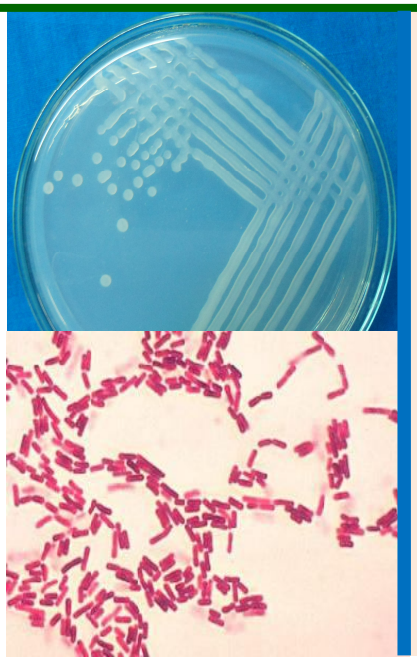
Chapter 2

Isolation and Biochemical Characterization of *Rhizobium* Spp.



CONTENTS

Chapter 2

Isolation and Biochemical Characterization of *Rhizobium* spp.

2.1 INTRODUCTION AND LITERATURE REVIEW

Soil contains many types of microorganisms such as bacteria, actinomycetes, fungi, and algae, which are important because they affect the physical, chemical and biological properties of soil. Amongst the soilbacteria a unique group called Rhizobia has a beneficialeffect on the growth of plants. It can live either in the soilor within the root nodules of host legumes. The bacteria colonize within root nodules, where it converts atmospheric nitrogen to ammonia and provides organicnitrogenous compounds to the plants. In legumes and fewother plants, the bacteria live in small outgrowths on theroots called nodules. Within these nodules, the bacteriado nitrogen fixation, and the plant absorbs the ammonia (Oblisami, 1995). The soluble form of nitrite and nitrate can be assimilated by plant roots and utilized insynthesizing proteins and nucleic acids. This form ofnitrogen can be converted to ammonia by plants, animals and microorganisms. Animals return nitrogenous wastes to the environment as uric acids . (Atlas, 1998)

Nitrogen is an essential nutrient for plant growth and development. Plants usually depend upon combined, or fixed, forms of nitrogen, such as ammonia and nitratebecause it is unavailable in its most prevalent form as atmospheric nitrogen. Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has led to worldwide ecological problems as well as affects the human health (Vitousek, 1997). Biological nitrogen fixation (BNF) is the cheapest and environment friendly procedure in which nitrogen fixing micro-organisms, interacting with leguminous plants, fix aerobic nitrogen into soil (Franche *et al.,* 2009). *Rhizobium* is the most well known species of a group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria can infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of thebacterium. *Rhizibium* bacteria stimulate the growth of leguminous plants and they are able to fix atmospheric nitrogen into soil by interacting symbiotically with leguminous plants, using the nitrogenase enzyme complex (Kiers *et al.,* 2003). The legume-rhizobium interaction is the result of specific recognition of the hostlegume by *Rhizobium*. Various signal molecules that are produced by both

Rhizobia and the legume confer the specificity (Phillips, 1991). Exopolysaccharide (EPS) produced by *Rhizobium* is one such signal for host specificity during the early stage of root hair infection (Olivares *et al.*, 1984). It also protects the cell from desiccation and predation and helps in nitrogen fixation by preventing high oxygen tension (Jarman *et al.*, 1978)

Rhizobium inhabits the soil and possess the power of fixing atmospheric nitrogen in symbiotic association with legumes. They enter the root hairs, and multiply in the roots where they form nodules and ultimately leguminous plants enrich soil with nitrogen. The first exact experiments which proved that legumes benefit the soil by enrichment were in 1838 at Alsace (Gallon and Chaplin, 1987).The work of Hellrigel and Wilfarth (1988) conclusively showed that legumes could utilize atmospheric nitrogen which was dependent upon bacteria present in the nodules: Subsequently, Beijerinck (1888) isolated the nodule bacterium and called it *Rhizobium radicicola.* The name was subsequently changed to *Rhizobium leguminosarum* (Gallon and Chaplin, 1987).

Rhizobia are aerobic rod shaped, motile, gram negative, and non spore forming bacteria. Genus *Rhizobium* consists of two groups, (Fast growers and slow growers) based upon their growth rates. Fast growing species can use wide range of carbohydrates and organic acids for their growth while slow growers require restricted range of substrates for their growth rate. Fast growers possess peritrichous flagella while slow-growers have sub polar flagella (Sprent and Sprent, 1990). *Rhizobium* grows on yeast extract mannitol agar (YEMA) and produces small to medium sized colonies. On medium containing congo red, they produce colourless to faint pink colonics, whereas colonies of contaminants are often deep red. Fast growers produce acid on yeast extract mannitol agar, which includes *Rhizobium loti, Rhizobium leguminosarum* and *Rhizobium meliloti.* While slow growing strains produces alkali on yeast extract mannitol agar, which includes *Bradyrhizobium japonicum* and *Bradyrhizobium spp.* (SubbaRao, 1987).

2.1.1 Rhizobia Classification and Characterization

Rhizobia generally refer to alpha-proteobacteria of the genus *Rhizobium* which has thegeneral characteristic of being able to enter into nitrogen-fixing symbiotic relationships with leguminous plants (Valerie & Sharon, 1999). The first recorded

isolation and description of this genus of bacteria was reported in 1888 by Beijerinck who described that isolations ofbacteria from root nodules of plants had led to the establishment that they were the causativeagent of nitrogen fixation in legumes. Beijerinck named these bacteria *Bacillus radiciola* (Kaisa *et al.,* 1996). The genus name was changed a year later to *Rhizobium* with only onespecies identified, the *Rhizobium leguminosarum* (Tindall, 2008). According to Tindall (2008), as interest into the nitrogen fixation phenomenon increased in the 20th century, more nitrogen fixing symbiotic bacteria were found and later classified into the family *Rhizobiacaea* which to date contains the genera *Rhizobium*, *Mesorhizobium, Ensifer* and *Bradyrhizobium*. *Ensifer* has recently gained prominence over *Sinorhizobium* asthe latter was found to be simply a recombination of the former (Tindall, 2008). The genera*Rhizobium* and *Bradyrhizobium* are particularly known to be the nitrogen fixing bacteria(Sofie *et al.*, 2011). The genus *Bradyrhizobium* was created by dividing the genus *Rhizobium*into two genera of nitrogen fixing bacteria based on their growth characteristics on yeastmannitol. Fast growing (2 to 3 incubation days) and acid producing bacteria are classified as*Rhizobium* (Valerie & Sharon, 1999) while the slow growing strains (8 days) typicallyalkalize their environment and are classified into the *Bradyrhizobium* genus (Samrudhi *et al.*, 2013). At the time the *Bradyrhizobium* genera were classified, there was only one species, the *Bradyrhizobium japonicum* which was primarily in the soybean cross inoculation group. To date 16 species of *Bradyrhizobium* have been accepted onthe official List of Prokaryotic names with Standing Nomenclature (LPSN) namely *betae,canariense, cytisi, daqingense, denitrificans, diazoefficiens, elkanii, huanghuaihaiense, iriomotense, japonicum, jicamae, lablabi, liaoningense, oliotrophicum, pachyrhizi,* and *yuanmingense* (LPSN, 2013). Pinton (2008) discusses that the rhizobia-legume symbiosis is affected by the same environmental factors that affect all microorganisms in the soil such as water stress, pH stressand competition. Pinton (2008) further states a hypothesis that symbiotic interaction does notstart with a carbon/nitrogen exchange, which takes place only in already developed nodules, 8 but with the supply of essential trace elements (like Molybdenum and Iron) by the host plants to the rhizobia in a competition-limiting environment. Aluminum, manganese toxicity and phosphorus deficiency also affect symbiotic effectiveness. Like other species of bacteria, Rhizobia are characterized according to phenotypic expressions in culture of particular importance in agriculture is its ability to tolerate low pH and high

salinity environments.Competition dynamics in the soil determine the efficacy of rhizobia to survive in its environment (Bohlool *et al.,* 1992). Altering of nutrient levels (*i.e.* P) ultimately changesthese competition dynamics and equilibrium states. Salinity is an important factor that affectsthe survival of *Rhizobium* in the soil and one that is most likely to be of utmost importance inthe semi-humid and semi-arid regions of Africa at large and Malawi in particular. In salinity tests by Keneni *et al.,* (2010) on indigenous and exotic strains, it was shown that *Rhizobium*from both sources was not able to survive NaCl concentrations higher that 5%. In addition tosalinity, Samrudhi *et al.* (2013) state that the other factors affecting symbiosis are the soil pH,nutrient deficiency as well as mineral and heavy metal toxicity, temperature extremes whilesingling out salinity as a particularly important condition limiting rhizobium growth due to its effects on plant growth hence on the symbiosis although it is noted that *Rhizobium* have shown a degree of tolerance to salinity. Keneni *et al.,* (2010) discuss the importance of usingsalinity tolerant strains as their enhancement on the nodulation is better than other strains.The authors illustrate the importance of pH in the rhizobium-legume symbiosis where exoticrhizobia were not able to survive pH levels below 5.5 while the indigenous ones were able toform nodules in a pH range of 4 - 7 which suggested more robustness on the part of the indigenous strains. Mensah *et al.,* (2006) report the optimum pH level of cowpea rhizobia which is in the same cross-inoculation group as soybean to be in the range of 6 - 7. Salinity and pH tolerance are of particular interest for this research and will be used to groupindigenous rhizobium strains into presumptive species and estimate a Shannon diversity index.

One *Rhizobium* strain is distinguished from another in culture by its morphological characteristics and reactions. Somasegaran & Hoben (1994) explain the aspects of distinction of rhizobia as they form in culture, the size, colour, gram reaction as well as its characteristic growth in a range of media *e.g.* acidic or alkaline media. Rhizobia morphology is often described by the type of shape their colonies form. Vertical colony shapes can range fromflat, domed and conical. When growing below the surface of the agar, colonies are typically shaped like a lens (Somasegaran & Hoben, 1994; Woomer *et al.*, 2011).In terms of colourand texture, colonies are typically opaque white or they may be milky to watery-translucent.The variation in growth rate is such that the fast-growing rhizobia typically take 3 - 5 days toproduce visible colonies on agar with colony size of about 1mm while the slow-growing ones usually take 5 - 7 days with colonies of 4 - 5

mm (Somasegaran & Hoben, 1994; Woomer *et al.*, 2011). The Gram stain test also helps to presumptively filter rhizobia from other types of bacteria. Woomer *et al.* (2011) explains that Gram-positive organisms retain the crystal violetstain after treating with iodine and washing with alcohol, and appear dark violet after stainingwhile gram-negative organisms lose the violet stain retain a red coloration of Safranin whichis used as a counter-stain. Rhizobia are typically gram negative. Another way ofcharacterizing bacteria is using serological methods (Swift & Bignell, 2001) where the rhizobia acting as an antigen is responded to by an antibody cultured/produced in rabbits (Somasegaran & Hoben, 1994; Woomer *et al.*, 2011). Sessitsch *et al.,* (2002) report that *Rhizobium leguminosarum* strains have been shown to produce bacteriocins antibiotics that are active against closely related strains or species. The author notes that this is amechanism deployed for reducing competition for root colonization. To survive, competingrhizobia have to develop differential resistance to antibiotics therefore intrinsic antibiotic resistance is an important genetic marker that has been used to recognize different groups orrhizobia, some of which are considered members of separate species (Dupuy *et al.,* 1994). Keneni *et al.,* (2010) used growth media amended for different concentrations of *Streptomycin, Chloramphenicol, Rimfampenicillin, Oxytetracycline, Penicillin* and *Tetracycline* to establish antibiotic resistance patterns of indigenous and exotic rhizobiaspecies as part of characterization which would enable the evaluation of exotic rhizobia'scompetitiveness to the indigenous rhizobia. This study found that indigenous rhizobia weremore resistant to the said antibiotics than the exotic strains. According to Sessitsch (2002),different rhizobia species also utilize different carbon sources in the rhizosphere as part ofmanipulation of competition dynamics. Scientists have also used these properties tocharacterize rhizobia. PCR techniques are also used to produce the genetic fingerprint ofbacteria for definitive characterization and species identification (Swift & Bignell, 2001).

2.1.2Taxonomic status of *Rhizobium*

Taxonomic status of *Rhizobium* as well as its classification is still controversial task. Many authorities tried to solve the problem by reviewing all existing opinions (Elkan, 1992). In fact, Beijcrinck (1888) isolated this microorganisms from nodules and proposed the name, *Bacillus radicicola.* But Frank (1889) quickly renamed this as *Rhizobium.* Therefore, a number of authors provided different types of classification

(Dadarwal *et al.*, 1977; Elkan, 1992; Subba Rao. 1999; Tate, 2000).There are 750 genera of legumes, containing 16000-19000 species but of these are economically exploited and grown over large areas, and only a few rhizobia have been studied. Only about 15% of the legume species have been examined for their ability to form nodules with rhizobia (Allen and Allen 1981). *Rhizobium* offer taxonomic challenge since many years their characterization was based on a selective interaction with a plant host. Bijerinck in Holland was the first to isolate and cultivate a microorganism the nodules of legumes in 1888. He named it *Bacillus radicicola,* Which is placed in Bergey's Manual of Determinative Bacteriology under the genus *Rhizobium* (Table 2.1). Early researchers considered rhizobia to be a single species capable of nodulating all legumes. Although others had observed host- *Rhizobium* specificity . It was extensive cross testing on a relatively few legume hosts that led Baldwin and Fred (1929) to propose a taxonomic characterization of rhizobia based on bacteria-plant cross inoculation groups (Eckhard *et al.*,1931; Fred *et al.*,1932). Fred *et al.*,(1932) defined these as groups of plants within which the root nodule organisms are mutually interchangeable. The principle of cross-inoculation grouping is based on the ability of an isolate of *Rhizobium* to form nodules in a limited number of species of legumes related to one another. All rhizobia that form nodules on roots of certain legume types have been collectively taken as a species. This system of classification has provided a workable basis for the agricultural practice of legume inoculation.

Table 2.1: Cross inoculation groups of *Rhizobium.*

***Rhizobium* spp.**	**Cross-inoculation group**	**Legume types**
R. leguminosarum	Pea	*Pisum, Vicia, Lens*
R. phaseoli	Bean	*Phaseolus*
R. trifolii	Clover	*Trifolium*
R. meliloti	Alfalfa	*Melilotus,Medicago, Trigonella*
R. lupini	Lupini	*Lupinus, Orinthopus*
R. japonicum	Soybean	*Glycine*
R. sp.	Cowpea	*Vigna, Arachis*

Division of rhizobia into groups according to growth rate on laboratory medium. The term "fast growers" commonly refers to rhizobia associated with alfalfa, clover, bean, and pea, since these rhizobia exhibit less than one half the doubling time of slow growers (or <6h). The slow growers are exemplified by soybean and cowpea rhizobia. Graham (1964), using numerical taxonomy, contended that the differences between slow- and fast-growing *Rhizobium* were too great to be based solely on evolutional differentiation of root-nodule bacteria from an organism similar to the present day slow-growing type. Hennecke *et al.*, (1985) catalogued the 16S rRNA of the fast and slow-growing rhizobia and concluded that these groupings indeed represented different genetic phyla since the similarity coefficient of the RNA is only 0.53. Thus these groups of rhizobia are less related to each other than to nonsymbiotic relatives. *Rhizobium* is closely related to *Agrobacterium,* whereas the slow-growing rhizobia do not appear related to *Agrobacterium* but rather to *Psudomonas palustris* (Young and Jhonston 1989). However, Elkan (1992) presented summary of the differences between the slow-and fast growing rhizobia (Table 2.3).

Table 2.2: Summary of differences between fast- and slow-growing rhizobia.

Charaetersties	**Fast- growing**	**Slow- growing**
Generation time	< 6 h	>6h
Carbohydrate nutrition	Uses pentose, hexoses, and mono-, di-, and trisaccharides.	Usually solely pentoses and hexoses
Metabolic pathway	EMP low aetivity, Strain specific, ED main pathway, TCA fully active, PP pathway	EMP low aetivity ED main pathway TCA fully aetiveHexose cycle
Flagellation	Peritriehous	Subpolar
Symbiotic gene location	Plasmid and Chromosome	Chromosome
Nitrogen fixation gene location	nifH. nifD, nifK on same operon	nifD, nit'K, nifH on specific operon
Intrinsic antibiotic resistance	Low	High

ED, Entner-Doudoroft pathway ; EMP, Embedon- Meyerhof-Parnas Pathway ; pp, Pentose phosphate pathway; TCA, Tricarboxylic acid cycle.

The genus *Rhizobium* consist of three reorganized species: *R. leguminosarum,* incorporating three biovars viz. biovar *trifolii,* biovar *phaseoli,* and biovar *viceae; R. meliloti;* and *R. loti.* The reorganization combines into one the former species of *R. legumiribsarum, R. trifolii,* and *R. phaseoli.* The fast-growing members of the cowpea rhizobia and the former species *R. lupines* have been included in the species *R. loti.* The new genus, *Bradyrhizobium,* is made up of one species, *B. japonicum,* which consists of the former species *R. japonicum,* plus the slow-growing members of the cowpea rhizobia. Two more genera have been added to the family Rhizobiaceae. They are *Sinorhizobium* and *Azorhizobium,* nodulating soybean and *Sesbania,* respectively (Table 2.3).

Table 2.3: Classification of rhizobia (Elkan, 1992).

Rccognized genera	**Recognized species**
Bradyrhiozobium(Jordan 1982)	*B. japonicum*(Jordan 1982)
Rhizobium (Jordan 1982)	*R. leguminosarum*(Jordan 1982)
	R. mcliloti(Jordan 1982)
	R. loti (Jordan 1982)
	R. galegae(Lindstrom 1989)
	R. tropici(Martinez *et al*., 1991)
	R. huakuii(Chen *et al*., 1991)
Azorhizobium(Dreyfus *et al.* 1988)	*A. caulinodaris*(Dreyfus *et al.* 1988;
Sinorhizobium(Chen *et al.* 1988)	*S. fredii* (Chen *et al.,* 1988)
	S. xinijiangensis(Chen *et al.,* 1988)

Nitrogen is an essential nutrient for plant growth and development. Plants usually depend upon combined orfixed forms of nitrogen, such as ammonia and nitrate because it is unavailable in its most prevalent form asatmospheric nitrogen. Much of this nitrogen is provided tocropping systems in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has led toworldwide ecological problems as well as affects thehuman health (Vitousek, 1997). Biological nitrogenfixation (BNF) is the cheapest and environment friendly procedure in which nitrogen fixing micro-organisms interacting with leguminous plants, fix aerobic nitrogeninto soil (Franche *et al.,*2009).Among plant-microbe interactions, legume–*Rhizobium* interactions are uniquebecause they supply 80-90% of total nitrogen requirement of legumes. It

involves a complex interactionamong host, microbial symbiont and environment. Amongnitrogen fixing systems, legume-rhizobium symbiosis isone of the most promising and the bacterial species of *Rhizobium* complex are very important (Sprent,2001) rather *Rhizobium* is the most well known species of a group of bacteria that acts as the primary symbioticfixer of nitrogen. These bacteria can infect the roots ofleguminous plants, leading to the formation of lumps ornodules where the nitrogen fixation takes place. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. This symbiosis reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops (rhizobium as biofertilizer). *Rhizibium* bacteriastimulate the growth of leguminous plants and they areable to fix atmospheric nitrogen into soil by interacting symbiotically with leguminous plants, using thenitrogenase enzyme complex (Kiers *et al*., 2003). The legume-*Rhizobium* interaction is the result of specificrecognition of the host legume by *Rhizobium*. Various signal molecules that are produced by both *Rhizobia* andthe legume confer the specificity (Phillips, 1991). Exopolysaccharide (EPS) produced by *Rhizobium* is one such signal for host specificity during the early stage ofroot hair infection (Olivares *et al.*, 1984). It also protectsthe cell from desiccation and predation and helps in nitrogen fixation by preventing high oxygen tension (Jarman *et al.*, 1978). In addition, *Rhizobium* strains secrete growth hormones like indole acetic acid (IAA), which shows positive influence on plant growth and alsoplays an important role in the formation and development of root nodules (Nutman, 1977).The present study was carried out to isolate and characterize *Rhizobium* sp. morphologically andbiochemically and to prepare cost effective biofertilizerfrom the established cultures of rhizobia and to create awareness among farmers to cultivate leguminous (croprotation) plants and use biofertilizers for better agricultural and economic growth.

2.1.3 Biochemical and physiological characterstics

Distinguishing *Rhizobium* from its common associate *Agrobacterium*

Agrobacteria happen to be the common contaminant during the isolation of rhizobia. It becomes necessary therefore to ensure their absence while confirming the purity of the rhizobial isolates.

Production of 3 ketolactose: Bernaerts and De Ley (1963) described a test to differentiate Rhizobia from Agrobacterium. According to them *Agrobacterium* produce 3 Ketolactose on lactose containing medium. When the plates are flooded with Benedict's reagent a yellow ring of precipitate of cuprous oxide is observed around the growth. Neither *Rhizobium* nor *Bradyrhizobium* are able to produce 3 Ketolactose (Gaur *et al.*, 1973 and Gaur and Sen 1981).

Growth in Holer's alkaline medium: Hofer (1935) described the difference in the growth of Agrobacteria and Rhizobia in alkaline medium. He observed that at pH 11, only Agrobacteria could grow. Hence this characterstics was considered suitable in negative selection of rhizobia from agrobacteria (Allen, 1950). However, many contradictory reports have been published which assign only a limited value to the test (Graham and Parker, 1964; Basak and Goyal, 1980).

Growth on congo red medium

Initially it was thought that only the Agrobacteria have the ability to absorb Congo red dye to impart orange colour to the colonies (Dye 1979, Vincent, 1970). However, it has been observed that many rhizobia especially the fast growers also absorb the dye (Kneen *et al*, 1983). The dye absorbed on the surface of the bacterial cell takes various shades according to the predominance of the ions present in the medium. If the medium was highly alkaline due to predominance of magnesium ions the colonies took fuchsin colour, and blue coloured colonies were observed at pH 3.0 (Hahn, 1966).

Growth on glucose peptone agar

The rhizobia do not use glucose and peptone readily. Hence this test is used to check for non rhizobial contaminants. All rhizobial strains except *Rhizobium meliloti* show little or no growth in the medium without change in pH within 48 hours, whereas agrobacteria grow well, with a pH change in the medium (Kleezkowske *et al.*,1968). Gaur and Sen (1981) in their studies on Cicer rhizobia have shown that most of the strains did not grow and those which showed little growth did not change the pH of the medium in 48hrs. Similar observations were made by Subba Rao (1999) mentioned that *Rhizobium* grows poorly in this medium and causes little change of pH.

Acid and alkaline reaction

Rhizobial growth on a medium containing a carbon source is accompanied by change in the reaction (acidity or alkalinity) of the medium which depends upon the kind of bacterium and the carbohydrate used. This was first demonstrated by Bialsuknia and Klott (1923) and subsequently, it was confirmed by many others namely Fred *et al,* (1932). Raju (1938), Johnson and Allen (1952).Jones and Burrows (1969). Acid and alkaline production in yeast extract mannitol agar with bromothymol blue, as culture medium has been employed in taxonomic characterization of rhizobia (Norris, 1965). With mannitol, barring a few exceptions, rhizobia of soybean,lupin and cowpea groups produce alkali; whereas those of medic, clover, pea and bean groups produce acid.

Carbohydrate utilization

In many studies the utilization of different carbon sources of rhizobia have been examined (Table 2.5). It appears that all the rhizobia, irrespective of their host group, utilize almost all the carbon sources. If there is any difference h may be quantitative depending upon the strain but not upon the group of rhizobia.

Glucose consumption

Attempts have been made to work out same method for determination of efficiency of *Rhizobium* spp. from their easily assessable characteristics. Since sugar is used as energy material for fixation of nitrogen by nitrogen fixing organisms, it was suspected that high sugar requirements might be related to their capacity for nitrogen fixation (Gupta and Sen, 1965).

The relationship between glucose consumption by *Rhizobium japonicum* and its nitrogen fixation efficiency has been studied by Sen (1965) who observed a significant positive relationship between the two parameters. Gupta *et al.,* (1971) reported wide variation in glucose consumption by strains of *Rhizobium* belonging to the same speciesalso found more glucose consumption in temperature-tolerant than in temperature-sensitive *Rhizobium* strains of Black gram, Cow pea and Pigeon pea.

2.1.4 Antibiotic sensitivity

Most rhizobia are sensitive to a wide spectrum of antibiotics, and many workers (Davis, 1962; Graham, 1963; Cole and Elkan, H979; Nimbalkar, 1986) have tested a large number of fast and slow-growing ihizobia against them. The strain to strain variation within a rhizobial group is large enough to make any generalization. Graham (1963) observed that slow growing rhizobia were less susceptible to antibiotics than the fast-growing ones. Sinclair and Eaglesham (1984) studied the intrinsic antibiotic resistance of 128 strains and observed multiple resistance against rifampicin, gentamycin and penicillin by most of the strains having wet colonies (translucent, copious watery slime, confluent with raised elevation) whereas the strains having dry colonies (opaque, paste like, granular, punctiform with raised elevation) showed resistanc e against only one or two antibiotics. Intrinsic antibiotic resistance to a range of antibiotics may contribute to the saprophytic competence of a strain introduced into a soil as an inoculant. The intrinsic antibiotic resistance pattern is generally used for strain identification in ecological studies (Gupta *et al.,* 1983).

2.1.5 Plant infectivity and cross inoculation studies

Plant infection test: The biochemical tests which distinguish *Rhizobium* from *Agrobacterium* and other nonrhizobial contaminants, include Ketolactose test, growth in Hofer's alkaline medium, growth on glucose peptone agar and Congo red medium. These are of limited value and offer only presumtive evidence that the root nodule bacteria under study are *Rhizobium* and not *Agrobacterium* (Gaur *et al.,* 1973 and Graham, 1976). According to Allen and Allen (1981) for building up of a rhizobial collection, infectivity test becomes a vital requirement, where in the ability of the isolates to form nodules on its homologous host is confirmed. Dilworth and Parker (1969) have started that *Rhizobium* was a free living nitrogen fixer and it formed symbiotic association with the legume in the course of evolution.

Cross inoculation Studies: Fred *et al.,* (1932) have developed the rhizobial classification which is based on the principle that each species of *Rhizobium* would only nodulate plants within a cross inoculation group. Thus within each cross inoculation group rhizobia from one plant will nodulate all other plants and vice versa. Various workers have carried out cross inoculation studies and placed the legume

species into different groups (Carrol, 1934; Conklin, 1936; Allen and Allen,1936; Shinde, 1981).The system of cross inoculation grouping of rhizobia is however not perfect since bacteria have often been found to cross infect or interchange between the groups as has been shown by Allen and Allen (1939); Wilson (1944); Lange (1961); Trinick (1965); Grahm (1976).

Vincent (1970) has been shown that there is greatest difficulty in classifying cowpea rhizobia as some of them show wide degree of cross invasiveness and others show close host specificity. Various workers have tried alternative methods to overcome the difficulties encountered in the system of cross inoculation grouping e.g. Computer analysis (Subba Rao, 1977), Adansonian analysis (Graham, 1964), DNA base ratio studies and hybridization (De Ley, 1968). Each of them has some drawbacks. Vincent (1970) however has started that the present cross inoculation grouping is a suitable starting point ;o establish relationship between the host and its microsymbiont. Somasegaran and Hober (1985) have also said that the host dependent cross inoculation group system is the best practical system currently available inspite of the criticism levied against it.

2.1.6 Role of Bacteria

Amongst the soil bacteria there is a unique group called rhizobiathat have a beneficial effect on the growth of legumes. Rhizobia are remarkable bacteria because they can live either in the soil or within the root nodules of hostlegumes.When legume seeds germinate in the soil, the root hairs come in contact with rhizobia. If the rhizobia and the legume are compatible, a complex process begins during which the rhizobia enter the plant's root hairs. Close to the point of entry, the plant develops a root nodule.Once the relationship between plant and rhizobia is established, the plant supplies the rhizobia with energy from photosynthesis and the rhizobia fixatmospheric nitrogen in the nodule, converting it into a form that the plant can use. Both the plant and the rhizobia benefit from such a relationship called a symbiosis. The rhizobia living in the plant's root nodules are called symbionts.The complex process by which the rhizobia produce nitrogen for the legume is called biological nitrogenfixation, or BNF. Only rhizobia that are specifically compatible with a particular species of legume can stimulate the formation of root nodules, a process called nodulation. This process has

great economic benefit for legume production. As a result, rhizobial inoculantsare produced commercially in many countries. Inoculants contain rhizobia isolated from plant nodules and grown (cultured) artificially in the laboratory.

2.1.7 Research Activities

In this research, we set up some goals to be achieved through the progress of work. The activities planned for the current investigation were as follows.

I. Isolation of bacteria from the Legumenous plantsof Rajshahi. The eight legume plants selected for the present research were Black Gram, Green Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean.

II. Isolation of root nodule bacteria.

III. Identification of the isolated bacteria.

IV. To ensure culture and preservation of the isolated bacteria.

2.2 MATERIALS AND METHODS

To conduct the present research following materials and equipments were used.

2.2.1 Study Area

This study was conducted in the districtRajshahi, Bangladesh . Rajshahi is a city found in Rajshahi, Bangladesh. It is located 24.37 latitude and 88.60 longitude, Altitude (feet) 101and it is situated at elevation 23 meters above sea level. Rajshahi has a population of 700,133 making it the biggest district of Rajshahi Division.

Rajshahi Division is one of the seven administrative divisions of Bangladesh. It has an area of 18,174.4 km² and a population at the 2011 Census of 18,329,000 (preliminary figures). Rajshahi Division consists of 8 districts, 70 Upazilas (the next lower administrative tier) and 1,092 Unions (the lowest administrative tier). This division is characterized by its cheap labour force. It has an excellent rail and road communication infrastructure. The divisional capital of Rajshahi is only six-seven hours road journey away from Dhaka, the capital city. (Wikipedia)

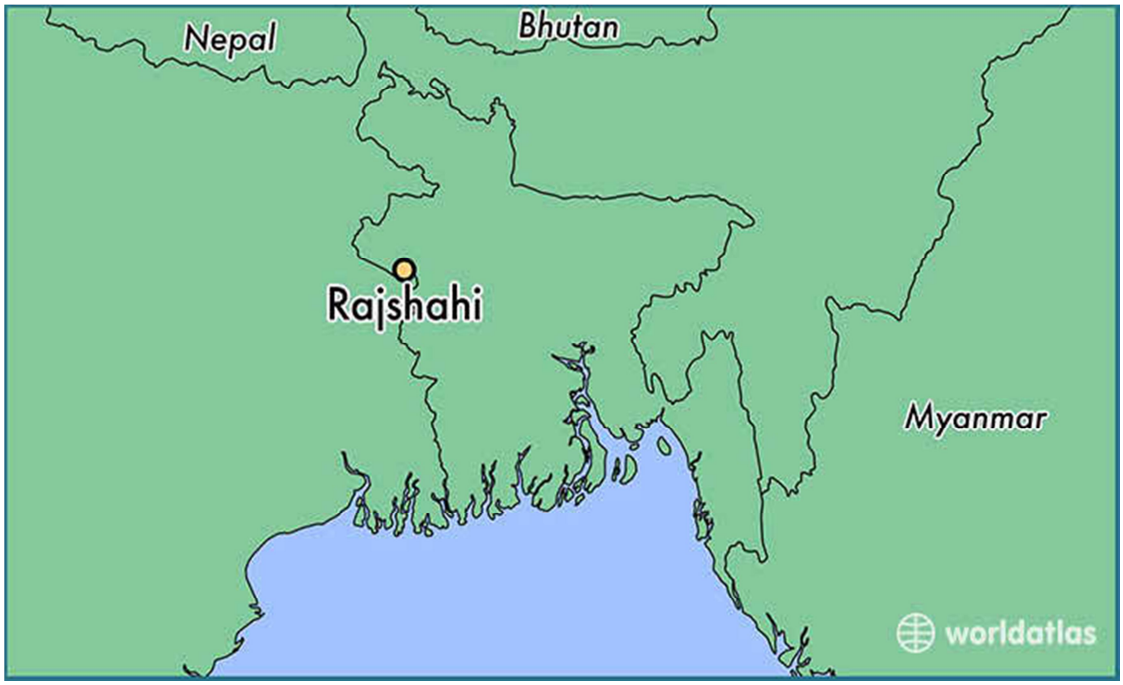


Figure 2.1: Study Area, Rajshahi , Bangladesh.

Selected area

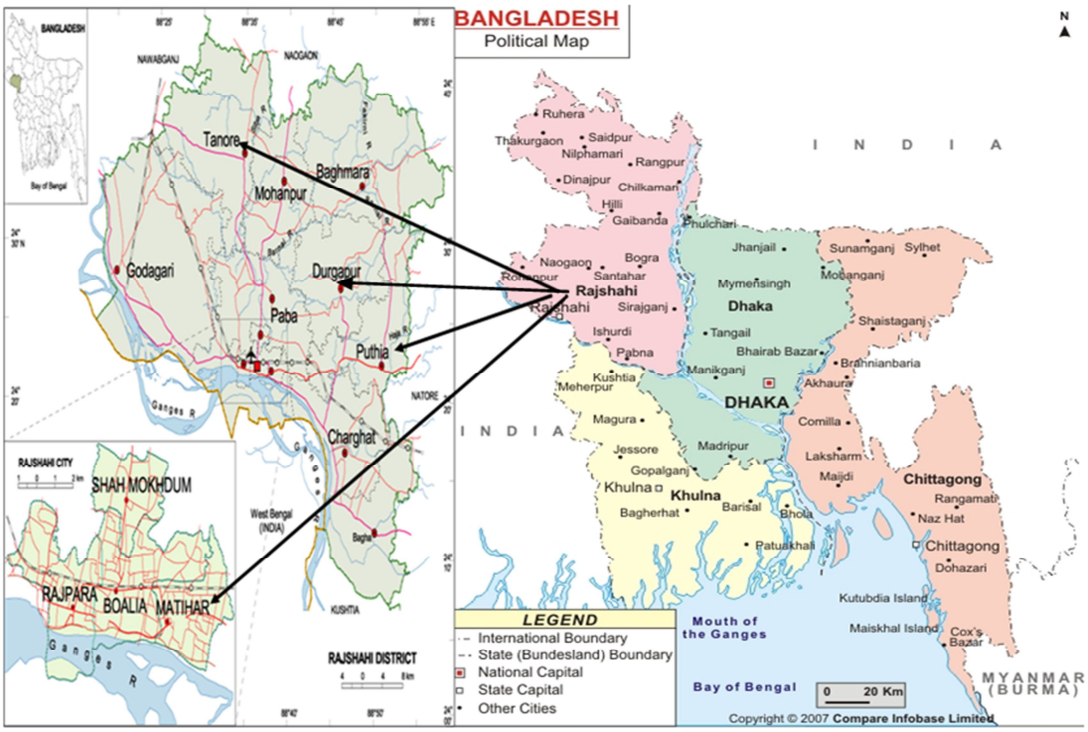


Figure 2.2: Selected area of Rajshahi.

2.2.2 Bacterial Source

Legumes plant was the source of bacteria for the present research. Bacteria were isolated from eight different Legumes plant. These Legumes plant were collected from the Rajshahi area. We targeted eight legumes plant such as Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean

Bacterial sources

Green gram

Black Gram

Lentil

Pigeon pea

Sweet pea

Cow pea

Chick pea

Bean

Plate 2.1: Nodules collected from different types of legumes.

2.2.3 Other materials

Chemicals

Peptone, yeast extract, sodium chloride, bacteriological agar, absolute, 95% and 70% alcohol, methylated spirit, crystal violet, grams iodine, safranin, glycerine, Macconkey agar, 30% hydrogen peroxide, potassium biphosphate, potassium dihydrogen phosphate, dextrose (glucose), methyl red, α-naphthol, potassium hydroxide, ready mix of SIM medium and Simmon's citrate agar, urea, starch and phenol red.

Glass Wares

Test tubes, petri dishes of various capacities, glass spreader, conical flasks (100 ml, 250 ml, 500 ml), beakers (50 ml, 100 ml, 200 ml, 500 ml), measuring cylinder (25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1000 ml), glass pipettes (0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, 5.0 ml, 10.0 ml), microscopic slides, funnel.

Electronic Instruments

Magnetic stirrer, centrifuge, analytical loading single pan balance, distilled water plantation, digital pH meter, autoclave, microwave oven, laminar air flow cabinet, incubator, incubator rotator, freezer and refrigerator.

Nichrome loop, scissors, spirit lamp, scalpel handles with blades, various sizes of forceps, micro pipettes, tips, eppendorf tubes, pipette pumps, parafilm, aluminium foil, cotton, water proof marker pen, paper towel, tissue paper, filter paper, spatula, brown paper, test tube racks, trolley and waste basket.

2.3 METHODS

Methods that are followed in the current research are described below with suitable headings.

2.3.1 Culture Media Preparation

Two types of culture media were used for the bacterial growth in the present research.

LB Broth Medium

LB broth medium was used for the liquid culture of the bacteria.The composition of LB broth medium is given below.At first a 250 ml beaker was taken and little amount of distilled water (approximately 50 ml) was poured into it with the help of a measuring cylinder. Now all the necessary ingredients were measured using an electric balance and were dissolved in distilled water in the beaker one after one by shaking gently gentle shake. Then pH of the medium was adjusted to 6.8 to 7.0 using a pH meter with the help of 0.1 M sodium hydroxide and 0.1 M hydrochloric acid. Dissolving and pH adjustment were assessed by a magnetic stirrer. The medium was then transferred to a measuring cylinder and distilled water was added carefully to make the total volume 100 ml. The liquid medium was now transferred to a 250 ml conical flask, sealed with cotton plug and parafilm and autoclaved at 121°C for 30 minutes.

Chemical composition of nurient agar media

Peptone..... 1gm.
Yeast extract.....0.5 gm.
NaCl.....0.5 gm.
Distilled water.....100 ml.
pH.....7.2.
Ager.....5 gm.

LB Agar Medium

Composition and protocol required for LB agar medium were the same as the broth medium; but right after the pH and final volume adjustment, the medium was kept into a 250 ml beaker and 03g/100 ml bacteriological agar was added to it. Agar was dissolved by gradual heating through a microwave oven for 5 minutes. Then the beaker was sealed with cotton plug and autoclaved at 121°C and 1.5 atmosphere pressure for 30 minutes. The LB agar medium was taken under laminar airflow and poured into autoclaved petridishes under sterilized condition. 100 ml medium was sufficient to make 5 plates having 0.5 cm medium thickness.

Chemical compostion of yeast extract mannitol agar media (YEMA)

K_2HPO_4 .....0.5g

$MgSO_4.7H_2O$.....0.2 gm

NaCl.....0.2 gm

$CaCO_3$..... 0.2gm

$FeCl_3$. $6H_2O$.....0.01 gm

Mannitol.....10 gm

Yeast extract.....0.4 gm

Agar (Difeo, USA).....15 gm

Deionized water to 1 liter was used. The pH of the medium was adjusted to 7.0 with HCl solution.

2.3.2 Isolation and identification of root nodule bacteria

A healthy, unbroken nodule from the roots of tree legume, Black Gram,Green Gram,Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean from Rajshahi, Bangladesh was selected for isolation of root nodule bacteria. It was cut from the root leaving a small piece of the root attached to it to avoid possible exposure of the root nodule bacteria to the disinfectant. It was then washed thoroughly in tap water to remove gross surface contamination and then surface sterilized by immersing it in 0.1% w/v mercuric chloride for 2 minutes and then 95% alcohol for 2-3 minutes. It was finally washed thoroughly in six changes of sterile distilled water to remove traces of the disinfectant. Each nodule was then crushed in a small aliquot of sterile physiological saline in a sterile test tube (100x10 mm) with the help of a sterile glass rod and then milky fluid was streaked on sterile Yeast Extract Mannitol Agar (YEMA) containing Congo red (1% congo red, 2.5 ml in 100 ml YEMA). The plates were incubated at 28°C for five days. The typical well isolated white, raised colonies were picked up and suspended in sterile physiological saline in a test tube and streaked on YEMA plates for purification. Well isolated typical single colonies wererestreaked on freshly prepared YEMA plates .A well isolated typical colony, from each plate was picked up and subcultured on YEMA slants for maitainance in order to obtain pure cultures and futher studies

2.3.3 Methods of Isolation and Maintenance of Pure Culture

The process of screening a pure culture by separating one type of microbes from a mixture is called isolation. A culture containing only one species of microbe is called pure culture. In a mixed culture, a particular species is present in small numbers in comparison to the numbers of others. The method which has been followed is given below.

Isolation by Streaking:

The streaking technique consist of the following steps:

i) Hold the broth culture containing tube in left hand and shake it.

ii) Sterilize the wire loop of the inoculation needle on burner flame.

iii) Remove the cotton plug of the broth culture tube by little finger of right hand.

iv) Flame the mouth of the test tube immediately.

v) Inside the test tube insert the wire loop to form a thin film and replace the cotton plug.

vi) The thin film in the loop is streaked in either a zigzag manner by removing the loop backwards and forwards firmly. Care should be taken that loop should not be firmly pressed against the agar surface.

vii) Incubate the petridish in incubator at a required temperature.

viii) Growth of the bacteria will be visible (after an overnight incubation) on the streaked marks.

2.3.4 Isolation of Pure Bacterial Colonies through Streaking

The single colonies were observed on these plates after overnight incubation. Single colonies were subjected to streaking to replicate the bacterium on other agar plates and to find out the single and pure culture. This was done through streak plate technique by a flame sterilized nichrome loop. Tip of the loop was gently touched on the single colony and then first series of streaking was done on another agar plate. Then the loop was flamed on the spirit lamp and second set of streaks was made from the first series. The procedure was repeated for a few times. All the works were done under laminar airflow and by keeping the plate very close to the flame of spirit lamp to avoid

contamination. The plates were sealed with parafilm and incubated at 37°C for 24 hours. Thewhole isolation procedure was repeated twice to ensure the experimental Legumes plant as the appropriate source of the single isolate.

Maintenance of the Isolated Pure Bacterial Culture

After isolating the single colony, maintenance was required to preserve pure bacterial culture. Following two techniques were applied to maintain the pure culture.

Maintaining through Subculture by Streaking

After identifying the pure bacterial colonies, plates were stored carefully in 4°C into a refrigerator and were subjected to subculture by streaking in every 10 days to maintain the pure bacterial culture for the ongoing research experiments. Procedure of streaking was same as described earlier.

Glycerine Stock Preservation

To prepare a glycerin stock of pure bacterial culture, we made LB broth medium according toand autoclaved eppendorf tubes, micropipette blue tips and glycerin. The liquid culture was inoculated by taking an isolated single colony from the agar plate culture with a flame burnt loop and putting it into the culture. The culture was incubated in an incubator rotator overnight at 37°C and 160 rpm. Now 1.0 ml of liquid culture was taken to an eppendorf tube with the help of a micropipette under laminar airflow and then subjected to centrifugation at 2000 rpm for 2 minutes. The pellet was taken and supernatant was drained out on a paper towel. The procedure was repeated until adequate amount of pellet was found. Finally, 1.0 ml of glycerin was added to it gradually by micropipette. The tube was sealed with parafilm and stored at –4°C in a freezer.

2.3.5 Characterization of the isolates

Cultural characterization : The isolates were inoculated both on solid and liquid YEMA medium. Colony characterstics of the isolates on YEMA medium (with Congo red) were noted after 3-5 days incubation at 28°C. The growth pattern in the liquid medium was observed.

Morphological characterization: The isolates from both liquid and solid media were gram stained (Vincent, 1970).After 48 hrs of incubation, colony morphology of isolates was recorded on YEMA plates. The observed colonies were characterized as per method described by Aneja , 2003), it include colour, size, shape, margin, elevation, opacity and consistency and observed under microscope to record morphology and gram characters of isolates. The motility of the isolates from both media was observed by hanging drop method under phase contrast microscope (Nikon, Japan).

Colony and cell morphology: Bacterial strains from overnight grown cultures in YEMA (Vincent, 1970) broth were spread on YEMA agar plates and incubated at 30 for 24 h. After 24 h the colour and shape of colonies was noted. Cell motility and shape of single colony was observed under light microscope (Nikon, Japan).

Colony Morphology

The isolates were inoculated both on solid and liquid YEMA medium. Colony characteristics of the isolates on YEMA medium were noted after 24 hrs incubation at 28^{o}C. The growth pattern in the liquid medium was observed.

Cell Morphology

The shape and size of the cells were observed by the Microscope. Motility test can be used to check for the ability of bacteria to migrate away from a line of inoculation. To perform this test, the bacterial sample is inoculated into SIM or motility media (mannitol) using a needle. Simply stab the media in as straight a line as possible and withdraw the needle very carefully to avoid destroying the straight line. After incubating the sample for 24-48 hours observations can be made. Observe if the bacteria have migrated away from the original line of inoculation.

- If migration away from the line of inoculation is evident then the test organism is motile (positive test result)
- Lack of migration away from the line of inoculation indicates a lack of motility (negative test result).

2.3.6 Gram Staining Technique

The gram staining technique was used for the observation of the morphological characteristics and identification of bacterium. Gram staining experiment of the isolated bacteria was done in three steps.Gram staining was done by the method of Vincent 1970. Arora, 2003.

Preparation of the Staining Solutions

Following stains were prepared for the gram staining experiment of the isolated bacteria.

i) 0.5% crystal violet solution

ii) Lugol's iodine solution (1% iodine solution)

iii) 1% safranin solution

Procedure

i) A drop of sterilized distilled water was taken on the middle of the clean slide. Then a loopful bacterial suspension was transferred to the sterilized drop of water and very thin film was prepared on the slide by spreading uniformly.

i. The slide with fixed bacterial film was flooded with crystal violet solution for 30 second and washed thoroughly with gentle stream of tap water.
ii. The slide was flooded with iodine solution for 1 minute and washed thoroughly with gentle stream of tap water.
iii. The slide was then washed with alcohol (95%) for 10-15 second.
iv. Alcohol was washed thoroughly with gentle stream of tap water.
v. The slide was covered with safranin for 1 minute.
vi. Washed with tap water and bloated dry.
vii. Examined under microscope.

Potasium hydroxide string test (alternative test for Gram reaction)

A loop full growth of bacterial colony was emulsified on the surface of a glass slide in suspension of 3% KOH.The suspension was stirred continuously for 60 seconds after which the loop was gently pulled from the suspension .The test was considered positive if string occurred with in first 30 seconds after mixing the bacteria in KOH solution. Add a heavy innoculum of pure culture of bacteria grown on a solid medium to a drop of 3 % KOH solution on a clean glass slide Stir for about one minute , Occationally lifting the loop to look for thickening and stringing of the slurry.

Interpretation of findings:

Gram positive bacteria did not appear to change the viscosity of the KOH solution.

Gram negative bacteria caused the KOH solution to become stringy or mucoid in appearance and consistency.

2.3.7 Motility Test (Hanging drop method)

Reagent and Equipment

i) Liquid bacterial culture.

ii) Microscope slide with a central depression.

iii) Cover slip.

Procedure

i. Fresh broth cultures of bacteria were taken in a test tube.
ii. The cultures were centrifuged and supernatant was removed carefully and the cell pellet was suspended by saline solution.
iii. A small drop of suspended bacterial solution was placed into the central depression of a slide.
iv. The slide was covered with cover slip.
v. Examined microscopically.

A darting, zigzag, tumbling or other organized movement indicated positive results and negative results indicated no movement or Brownian motion only.

2.3.8 Biochemical Studies

Biochemical test was performed to identify the species of the bacterium. For biochemical identification liquid culture and plate culture are required. One liquid culture and one nutrient agar culture are sufficient for the entire series of tests for one single species. That's why at first we made both the liquid medium and plate medium respectively. These cultures were used for all the biochemical tests.

The different biochemicalcharacterizations were done namely,Catalase test, Indol test, Methyl red test,Vogus Proskar test, Citrate utilization test,Nitrate reductase test,Sugar fermentation test, H2S productiontest as described by Lowe (1962).

All the collected samples were selectedbased on their morphological and biochemical characteristics. The culture was tested for thefollowing biochemical tests; Catalase, Citrate, Methyl Red test, Vogues Proskuer, Indole asdescribed by (Lowe,1962), Urease, Nitrate reduction, Starch hydrolysis, Gelatin hydrolysis andMotility by (Arora, 2003), Oxidase, Hydrogen sulphide production as shown by (Sadowsky,1983) and Nitrogenase test by (Wilcockson,1978). The culture was also subjected to sugarfermentation tests with glucose, mannose, xylose, galactose, raffinose, mannitol andtrehaloseby (Oblisami, 1995).

Chemical compostion of yeast extract mannitol agar media

K_2HPO_4 .....0.5g

$MgSO_4.7H_2O$.....0.2 gm

NaCl.....0.2 gm

$CaCO_3$..... 0.2gm

$FeCl_3$. $6H_2O$.....0.01 gm

Mannitol.....10 gm

Yeast extract.....0.4 gm

Agar (Difeo, USA).....15 gm

Deionized water to 1 liter was used. The pH of the medium was adjusted to 7.0 with HCl solution.

Chemical composition of nurient agar media

Peptone..... 1gm.

Yeast extract.....0.5 gm.

NaCl.....0.5 gm.

Distilled water.....100 ml.

pH.....7.2

Ager.....5 gm.

Triple Sugar Iron (TSI)

Test Checks simultaneously for the production of hydrogen sulfide (H_2S), acid fermentation of lactose, sucrose, glucose and gas production from glucose.

Procedure

Suspend 64.52 grams in 1000ml (= 9.678 into 150ml) dH_2O. Heat to boiling to dissolve the medium completely. Mix well and distribute into test tube. Sterilize by autoclaving at 15 Ibs pressure (121^oc) for 15 minutes.

Results

- H_2S production: Positive (+) if a black precipitate of iron sulfide forms.
- Lactose/sucrose fermentation: Positive (+) for yellow colour of slant and negative (-) for red slant.
- Glucose fermentation: Positive (+) if medium changes to yellow, negative (-) if no change occur.
- Gas production: Gas bubble captured in the bottom part of the medium are observed. Sometimes gas production is so high medium pushed up to the cap.
- Peptone utilization:

Citrate Utilization Test: Some bacteria may be able to use organic compounds other than sugars as their sole source of carbon. Simons Citrate agar is a medium containing citrate as the sole carbon sources and ammonium salts as the sole nitrogen sources.

Organisms that metabolize citrate utilize the ammonium salts releasing ammonia and increasing the pH of the medium. Bromothymal blue is present in the medium as the indicator dye.

For the Citrate utilization test, slope culture with a 1 inch butt of Simon's citrate agar was inoculated by streaking over surface with a wire needle and incubated at 28^0C for 1-2 days. The color of the medium changed from green to bright blue due to the utilization of citrate and when citrate is not utilized, the color of the medium remains unchanged.Testing the ability of bacteria to utilize sodium or ammonium citrate as sole carbon source.

Procedure

A well isolated colony was picked and dispersed on medium surface (Slant of test tube), incubate 24 hours at 37 ^{o}c.

Results

- Reaction is positive (+) if medium colour turn from green to blue.
- Reaction is negative (-) if medium remain green.

Catalase test

This test was performed to study the presence of catalase enzyme in bacterial colonies. Rhizobial colonies (24 h old) were taken on glass slides and one drop of H_2O_2 (30 %) was added. Appearance of gas bubble indicated the presence of catalase enzyme. (MacFaddin, 1980).Catalase is an enzyme that splits hydrogen peroxide into water and oxygen. Hydrogen peroxide is produced as a byproduct of respiration and is lethal if it accumulates in the cell. All respiring organisms therefore must have some mechanism for detoxification. Catalase is one of the common methods. When one drop of 30% hydrogen peroxide is added over a colony of catalase- producing bacteria, it is broken down and the oxygen that is produced can be seen as bubbles. Bubble production indicated positive result.

Hydrogen peroxide and oxygen radicals used in bacterial electron transport chains of aerobic and facultative anaerobic respiration are toxic compounds. Catalase is an

enzyme present in most of the organisms and is involved in decomposition of the hydrogen peroxide in H_2O and O_2. If catalase is present, bubbles will form from the oxygen that is made in the reaction: 2 H_2O_2 + catalase = > $2H_2O$ + O_2 + catalase. Some bacteria can't produce catalase so, in bacteriology this test is used together with other tests for bacterial identification.

Procedure

A 3% hydrogen peroxide solution was prepared and one drop was put on a slide.Then a well isolated colony was harvested and immersed the loop in the solution on the slide.

Results

Observe the quick effervescence for a positive reaction. The absence of bubbling is considered negative.

Oxidase Test

Oxidase test was performed to determine the presence of oxidase enzyme in bacterial isolates (Steel, 1961). Kovac's reagent (1% N, N, N.N-tetramethyle-pphenylene diamine) was dissolved in warm water and stored in dark bottle. A strip of filter paper was dipped in this reagent and air-dried. With the help of sterile wire loop, one-day-old rhizobial colonies from agar plates were transferred on this filter paper strip. The oxidase positive colonies turned lavender colored which became dark purple to black in color within 5 min.

Oxidase test was performed to detect the presence of cytrocrome oxidase in the organism. This test was carried out by wet paper method. A strip of Whatman filter paper no.1 was soaked with 2-3 drops of dyes. A single colony was picked up with a sterile toothpick and rubbed on to the paper. A positive result was recognized by a dark purple color, which developed within 5-10 sec.

Some bacteria can produce cytochrome C oxidase located in their membrane which can catalyse the transport of electrons from donor compounds to electron acceptors (oxygen). This respiratory system is present in aerobic bacteria which are capable to use oxygen as the final hydrogen receptor.

Procedure

There are several ways to perform oxidase test: using filter paper, using a swab or adding the reactive directly on plate.

Filter paper method: Soak a piece of filter paper in Kovacs reagent (N, N, N´, N´-tetra-methyl-p- phenylenediamine dihydrochloride). A colony was picked and dispersed on filter paper.

Results

The dye is reduced to deep purple colour if the test is positive.

Sulfide indole motility test (SIM)

To differentiate between bacteria based on three tests: sulfur reduction (cysteine desulfurase), indole production (tryptophanase), and motility.

Procedure:

1. Inoculate a SIM tube with the organism to be tested.
2. Incubate for 48 hours.
3. Add kovacs reagent to the top of the tube, pink/red color formation indicates indole formation.
4. Black percipitate in the medium indicates hydrogen sulfide formation.
5. Growth away from the stab line indicates motility.

Results

A positive H_2S test is denoted by a blackening of the medium along the line of inoculation. A negative H_2S test is denoted by the absence of blackening.

A positive motility test is indicated by a diffuse zone of growth flaring from the line of inoculation.A negative motility test is indicated by growth confined to the stab line.A positive test for indole is denoted when a pink to red color band is formed at the top of the medium after addition of Kovacs Reagent. A yellow color denotes a negative indole test after addition of Kovacs Reagent.

Production of hydrogen sulphide

The test was performed in SIM medium which was prepared by a ready mix of SIM medium, Bio Lab, Hungary. 2.4 g of medium was heat dissolved in 100 ml distilled water and distributed in test tubes. Tubes were sealed with cotton plugs and autoclaved at 121°C for 15 minutes. Then inoculation was done with different samples and incubation was done for 48 hours with loosen caps. Observation was done accordingly.

Indole tests

For the Indole, one loopful fresh bacterial culture was inoculated in SIM (Simmon's citrate media ,Bio Lab, Hungary) broth medium and incubated at 28^0C for 1-2 days, after incubation, Kovac's solution was added and shaken vigorously for one minute. A red color in the reagent layer indicated positive reaction.

Methyl Red Test

Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose. Types and proportion of fermentation products produced by anaerobic fermentation of glucose.

Methyl Red (MR) test detects acid production to a sufficient degree (below pH 4.5) from glucose. One ml of fresh bacterial culture grown in glucose phosphate medium was taken in a test tube. Five drops of methyl red reagent was added and read immediately. Positive tests are light red and negative are yellow.

Procedure

Inoculate MR-VP broth tube with one loopful of fresh inoculums. Incubate at 37°C, 24-28 hours or until turbid growth is observed. Add about 5 drops of the methyl red indicator solution to the tube.

Result : A positive reaction is indicated, if the color of the medium changes yellow to red within a few minutes. A negative reaction is indicated, if the color of the medium remains unchanged.

Voges-Proskauer Test

For this test, V.P. medium in each was inoculated with 48-72 hours old culture and incubated at 37°C for 24-48 hours. After incubation 3 ml of 5% alcoholic α-naphthol solution was added to each tube followed by 1 ml of potassium hydroxide creatine solution. The tubes were then shaken vigorously for 1-2 minutes. Appearance of crimson ruby color in the tube indicated positive result that indicated the production of acetyl methyl carbinol (acetoin).Test checks for the presence of acetoin (acetyl methyl carbinol), butylenes glycol and finally siacetyl resulted from bacterial decomposition of carbon compounds (glucose). Diacetyl in the presence of strong KOH solution reacts with proteins to give a pink coloration.

Procedure

Inoculate VP broth tube with one loopful of fresh inoculums. Incubate at 37^0C, 24-28 hours or until turbid growth is observed. Add Barritt's reagent A (alpha naphthol) and reagent B (40% KOH).

Result

Reaction is positive if a red color is seen within 30 minutes. Reaction requires oxygen presence and it only appears on the surface of the medium unless the tube is vigorously shaken.

Urea hydrolysis

Testing the ability to produce urease, an enzyme that hydrolyse urea providing a source of nitrogen for bacteria.

Sterilize the glucose and urea solutions by filtration prepare the basal medium without glucose or urea. Adjust the pH and sterilize by autoclaving in a flask at 121^0C for 15 min. Cool to about 50^0C add the glucose and urea and tube the medium as slant. The medium used to detect urease production by the bacteria. Red colour after incubation indicate urease production.

Procedure

Pick a well isolated colony and inoculate medium. Incubate 24 hours at 37^0C.

Results

Reaction is positive if the medium become red (urea is decomposed resulting ammonia which changes the pH).

Oxidation Fermentation Test (O/F Test)

This test is performed to test whether glucose utilization in bacteria takes place in presence of oxygen (i.e. oxidatively or aerobically) or in absence of it (i.e. fermentatively anaerobically) (Fig 9).A bacteria able to ferment glucose must be able to oxidize it. But reverse may not be true. If glucose is utilized in either way, acid is produced changing the colour from purple (due to bromocresol purple used in media) to yellow by lowering pH (Holt et al., 1994).

Procedure:

a) Hugh-Leifson Glucose Broth (HLGB) with glucose, bromocresol purple, as main component and pH 7.4 is prepared. Less agar is used to get semisolid media to facilitate stab. Then distributed into test tubes and sterilized.

b) Inoculation is done by stabbing of loop.

c) Over one set of tubes paraffin is poured to give anaerobic condition and then incubated for 24 hours and the other set incubated.

Results

- Colour changed from purple to yellow in both tubes: fermentative.
- Colour changed only in tubes without paraffin: oxidative.
- No colour change in any tube: microorganism is inert to the media.

Mc Conkey Agar

For the selective isolation, cultivation and differentiation of coliformed enteric pathogens based on the ability to ferment lactose. Lactose- fermenting organisms appear as red to pink colonies. Lactose non-fermenting organisms appear as colorless or transparent colonies. For Bacterial growth indicated positive the result**.**MCA test was performed on MCA plate prepared from ready mix of MCA, Bio Lab, Hungary. 5 g of

MCA powder was taken in 100 ml of distilled water in a beaker and heat dissolved in a microwave oven. The medium was then autoclaved at 121°C for 20 minutes and distributed in petri plates. After condensation, the plates were subcultured by streaking with fresh bacteria culture grown on nutrient agar. Incubation was done at 37°C and observation was done after 24 hours.

Carbohydrate utilization test

The isolates were also examined for fermentation of the various sugarsincluding Glucose, Mannitol, Galactose, Raffinose,Trehalose, Mannose, Xylose, Cellebiose. One percent01% aqueous stock solution of the test sugars wasprepared in small tubes while for sialicin 4% sugar solution was prepared and sterilized as mentioned by(Hugh, 1953,Oblisami , 1995) In order to find out the ability of the isolates to utilize different carbohydrates, the cultures were inoculated in MS medium containing different carbohydrates *viz.* glucose, galactose, mannose, arabinose, maltose, sucrose, lactose, manitol and cellulose. The final concentration of the carbohydrates is 1%. The tubes were incubated at 28°C for 3-5 days and observed for any growth.In order to find out the ability of the isolates to utilize different carbohydrates, the cultures were inoculated in the YEMA base, containing different carbohydrates, viz. arabinose, dextrose (glucose), galactose, mannitol, mannose, maltose, glycerol, raffinose, sucrose, inositol and xylose in the final concentration of 1%. The tubes were incubated at 28°C for 3-5 days and observed for any growth (Stowers and Eaglesham, 1983).Phenol Red Broth Base is used as a basal medium to which carbohydrates are added for determination of fermentation reactions of pure cultures of microorganisms.

Procedure:

Suspend the ingredient (peptone, NaCl, Beaf extract and phenol red) into distilled water, mix well. Heat if necessary to ensure complete solution. Distribute 5mL of media in fermentation tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 30 minutes. Aseptically add filter sterilized carbohydrate solution or carbohydrate disc to sterile basal medium. Incubate the tube at 37^0C for 24-36 hours.

a) **Using carbohydrate stock solution:**Dilute 2 gm of carbohydrate in 10 mL distilled water. Then filter the solution and add 300 µL of stock solution to each tube (5 mL) to reach the concentration at 5-10%.

b) **Using carbohydrate disc**:Dispense the carbohydrate disc (25 mg/disc) in 5mL phenol red broth solution.

Results

If the solution is turned yellow it means positive result and if the solution color is unchanged it means fermentation not occurred.

Growth on congo red YEMA

Colony colour of the isolates on YEMA with congo red (0.25 ml of 1% Congo red in 100 ml of the medium) was noted after 5 days of incubation at 28°C (Appendix 2).

Growth on Hofer's alkaline medium

The ability of the isolates to grow in Hofer's alkaline medium (Hofer, 1935) at pH 11.0 was tested (Appendix 2).

Hofer's Alkaline Medium (Hofer 1935)

The reaction of Yeast extract mannital medium is raised to pH 11.0 with IN NaOH, 1 ml of 1.6% thymol blue per litre 10 added.

Glucose Peptone Agar medium

Rhizobia cannot utilize peptone whereas *Agrobacterium*can utilize and grow very fast on this medium. Thecomposition of the medium used during theexperimentation contained Glucose-10g, Peptone-20g, NaCl-5 g, Agar–Agar- 20 g, distilled water-1000 ml andpH is maintained at pH-7.2. In this medium 1 ml of 1.6 %Bromocresol purple per liter was added and autoclaved forthree consecutive days. Rhizobia showed poor or no growthafter 24 hrs with neutral or alkaline reaction Presence of*Agrobacterium* was acknowledged on the basis ofmaximum growth and acid reaction. Colour change toyellow due to production of acid by *Agrobacteria* and othercontaminants of the medium (Kleczkowska and Nutman1968).

Ketolactose test

Ketolactose medium was prepared by replacing mannitolwith lactose in YEMA medium. Sterilization was achievedby steaming for a period of 30 min for two successive days.After sterilization the medium was allowed to cool at roomtemperature (30 ± 2^0C) and stored at the same temperaturefor 3 to 4 days to check the contamination and confirm itssterility. A loopful of the inoculum from a fully grownculture slant (7 days old culture) was transferred to a petriplate containing the ketolactose agar medium. Afterincubation for 5 to 7 days at 27 – 30^0C the plates wereflooded with a shallow layer of Benedict‟s solution. Thecomposition of Benedict‟s solution consisted of twosolutions: Solution A (Sodium citrate-173 g; Anhydroussodium carbonate-100 g; Distilled water-600 ml) andSolution B (Copper sulphate-17.3 g; Distilled water-100ml). The solutions A and B were prepared separately andlater solution B was mixed with A and then filtered. Theresultant solution was clear and having transparent bluecolour. After pouring the Benedict's solution into abovementioned plates the excess of solution was drained off andthe plates were incubated for one hour at 30 ± 1^0C withoutany disturbance. The yellow colouration around bacterialcolonies confirms the presence of *Agrobacterium*(Bernaerts and Delay 1963).

Nitrification test

Composition

$(NH_4)_2SO_4$-1.0 g

K_2HPO_4-1.0 g

NaCl-2.0 g

$MgSO_47H_2O$-0.50 g

$FeSO_4$-Trace

Distilled water-1000 ml

Preparation

Dissolve the ingredients in water. Adjust the pH. Distribute in the tubes. Steam sterilize the medium at 121^0C for 20 min.

Use: To test ammonia to nitrite(Nitrification) conversion ability of bacteria. Presence of nitrite can be tested by using an appropriate colorimetric reagent

Diphenylamine reagent (Nitrate test)

Diphenylamine-0.7 g

Sulfuric acid (conc.)-60 ml

Distilled water -28.8 ml

Preparation

Dissolve Diphenylamine in a mixture of sulfuric acid and water . Cool and add slowly 11.3 ml of concentrated hydrochloric acid .After the solution has stood for 12 hours some of the base separates , showing that reagent is saturated .

Use

Used in detection of nitrate in the medium. This reagent produces a blue black clour in the presence of either nitrites or nitrates.

Starch hydrolysis test

For starch hydrolysis test, a starch broth medium was prepared by dissolving the ingredients shown in the 100 ml distilled water. The medium was distributed in test tubes in 5 ml amount and autoclaved at 121°C for 20 minutes. Now the tubes were inoculated and incubated at 37°C for 3 days. After incubation, 1 ml of gram's iodine was added to them and results were recorded at once.

Starch(Soluble)- 20 g

Peptone – 5.0 g

Beef extract – 3.0 g

Agar – 20.0 g

Distilled water- 1000 ml

pH – 7.0

Preparation

Dissolve the all ingredients in distilled water. Adjust the pH. Add agar .Steam sterilize the medium at 100^0C for 1 hour.

Use

To study the starch hydrolysis by microorganisms. After incubation , flood the plate with Gram's iodine. Amylolytic colony will be sourrounded by clear zone against purpelle coloured background.

2.3.9 Antibiotic sensitivity test

The patterns of sensitivity and resistance of rhizobial cultures to different antibiotics were tested by the disc method using antibiotic disc on YEMA medium.

The isolated bacterial strains were grown overnight in nutrient broths that were placed in the shaker at 28°C temperature and 120 rpm. for the antibiotic sensitivity test. A serial dilution technique was made for the test respective. YEMA medium plates were dried at 28°C. Antibiotics disks were placed centrally on the respective plates and incubated overnight at 28°C (Table 2.1) after overnight incubation than the zone was observed of the plate and measured the zone with the helping of mm scale

The patterns of sensitivity and resistance of rhizobial cultures to 13 different antibiotics was tested by the disc method using antibiotic disc of Span Diagnstic and Himedia on YEMA medium. After incubation three days at 28°C, the diameter of the inhibition zone, if any was measured.

Table 2.4: Shows antibiotic disks containing different concentration of antibiotic that were used in the antibiotic sensitivity test.

Antibiotics	ConcentrationDose(µg)
Mecillinam	25µg
Ampicilin	10µg
Ciprofloxacin	5µg
Erythromycin	15µg
Gentamicin	10µg
Cotrimoxazole	25µg
Amoxycillin	10µg
Penicillin	10µg
Pefloxacin	5µg
Ceftazidime	30µg
Streptomycin	10µg
Nalidixic acid	30µg
Tetracycline	30µg

2.4 RESULTS

2.4.1 Isolation of Bacteria

Rhizobium was isolated from the nodules of legume, in order to analyze its characterization *i.e.* Study of cultural, morphological, physiological, biochemical and antibiotic sensitive characters.According to Dye (1979) and Vincent (1970) *Rhizobium* grows on yeast extract mannitol agar (YEMA) and produce small to medium sized colonies, usually smaller than 2 mm. On YEMA the colonies of *Rhizobium* are circular, cream colored, sticky, raised and translucent structure. On YEMA containing congo red, produce colorless to faint colonies, whereas colonies of contaminants are often deep red or orange. Colony characteristics of isolate (table- 2.5) were similar to those of *Rhizobium* as mentioned above. These similarities indicate that the isolate is *Rhizobium* sp.

Total eight isolates were isolated from the nodules of tree legume, Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean.These isolates were subjected to characterization. They were alike as regards to their cultural, morphological, biochemical and antibiotic sensitive characters (Table 2.5, 2.6, 2.8).

Table 2.5: Colony morphology of isolated (*Rhizobium* spp.)

Characters	Eight Isolated (*Rhizobium* spp.)
Size	>2mm
Shape	Circular
Colour	Creamy white
Consistency	Sticky water
Margin	Entire
Elevation	Raised ,Convex
Opacity	Semi Translucent
Mucoid	Mucoid
Texture	Gummy
Surface	Smooth

Observations made after 72h growth on YEMA at 28°C

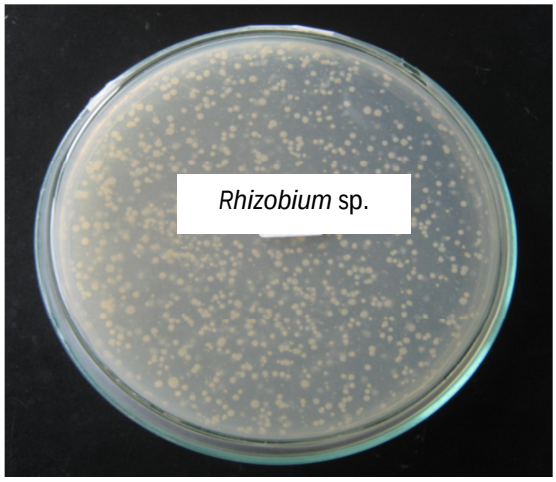


Plate 2.2: Mother culture of *Rhizobium* spp.

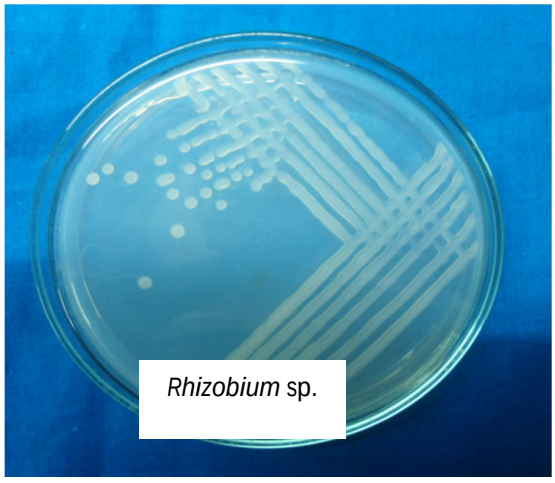


Plate 2.3: Isolated colony of *Rhizobium* sp.

2.4.2 Gram Staining Results

After preparing the slides, we visualized it under a light microscope. Gram positive bacteria show a violet color under the light microscope; while a gram negative result is confirmed by a pink color during microscopy.

All sample showed a pink color during microscopy, which proved them as gramnegative bacteria.

Table 2.6:Cell morphology: Cell morphology of the strain was studied and observation are described of isolated strains.

Strain Name	Gram character	Shape	Motility
Green Gram	-ve	rods	Motile
Black Gram	-ve	rods	Motile
Lentil	-ve	rods	Motile
Pigeon Pea	-ve	rods	Motile
Sweet Pea	-ve	rods	Motile
Chick Pea	-ve	rods	Motile
Cow Pea	-ve	rods	Motile
Bean	-ve	rods	Motile

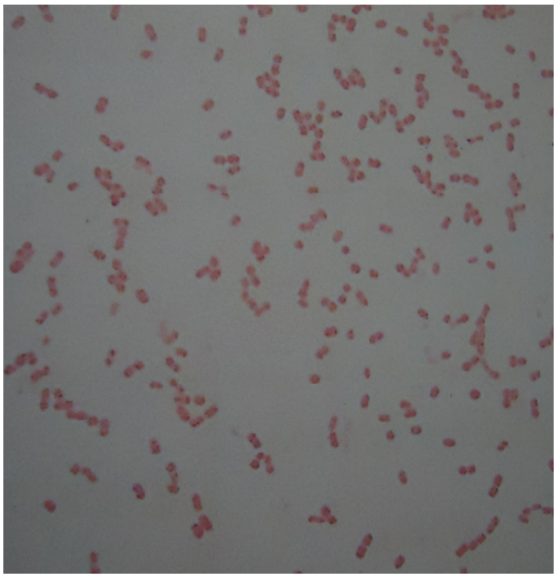


Plate 2.4: Gram characters and shapes of the *Rhizobium* sp.

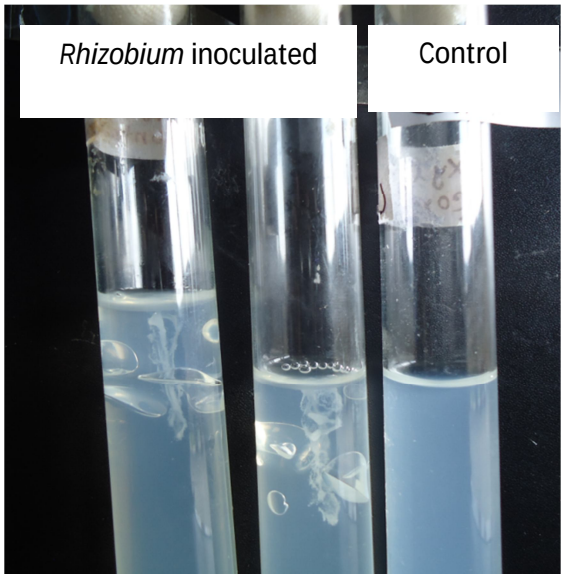


Plate 2.5: Motility test of *Rhizobium* sp.

2.4.3 Results of Biochemical Tests

Results of biochemical tests have been outlined below and given in below.

Ketolactose test

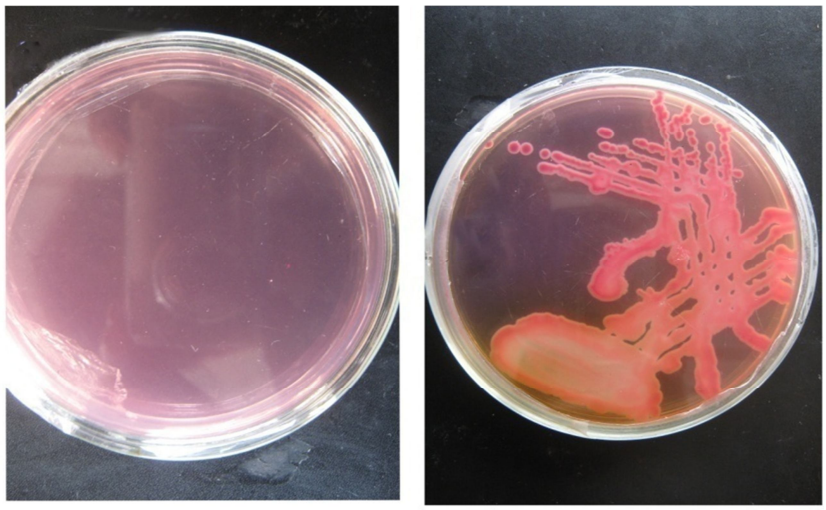
The yellow colouration around bacterial colonies confirms the presence of *Agrobacterium* (Bernaerts and Delay 1963). Similarly all the eight isolates did not produce yellow coloration around their colonies indicating that *Agrobacterium* was absent and all of them were *Rhizobium.*

Glucose Peptone Agar medium

Rhizobia cannot utilize peptone whereas *Agrobacterium* can utilize and grow very fast on this medium Rhizobia showed poor or no growth after 24 hrs with neutral or alkaline reaction. Presence of *Agrobacterium* was acknowledged on the basis of maximum growth and acid reaction. Colour change to yellow due to production of acid by *Agrobacteria* and other contaminants of the medium (Kleczkowska and Nutman 1968). The results indicate that all the isolates showed a negative growth on glucose-peptone agar.

Macconkey Agar Test

Gram negative bacteria grow on MCA Plates, while gram positive ones do not grow on MCA. All sample grew on MCA,and that's why it was gram negative. MCA test results are showed in Plate no 2.6



Control ***Rhizobium* sp.**

Plate 2.6: Macconkey Agar Test of *Rhizobium* sp.

Catalase Test

A positive result of catalase test is obtained by instant bubble formation, while negative result is confirmed by the lack of bubbles.All Sample formed bubble and thus was identified as catalase positive. Catalase test results of the all samples are presented in Plate 2.7

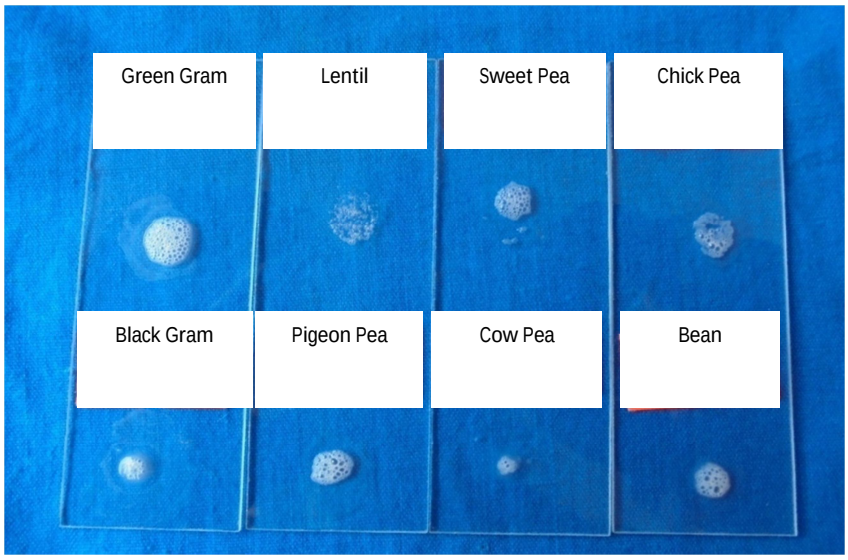


Plate 2.7: Catalase activities of isolated *Rhizobium* spp.

Methyl Red (MR) Test

After addition of methyl red to 7 days old MRVP liquid culture, positive result is showed by a red coloration, while negative by yellow color. A negative result has to be incubated for additional 48 hours.All Sample showed no color change and thus was identified as MR negative.MR test results of the five samples are shown in Plate 2.8.

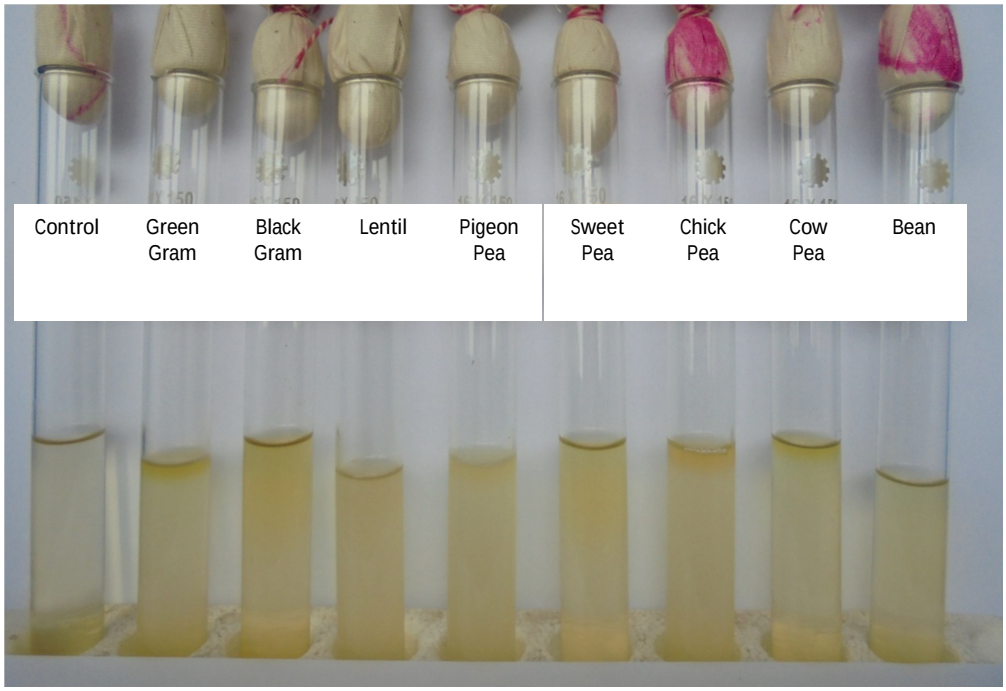


Plate 2.8: Methyl Red (MR) Test.

VP Test

A positive result is exhibited by a red color, while negative by a yellow to brown color after adding Barrit's reagent A and B respectively to a two days old culture in MRVP broth.All sample showed brownish color and that's why it was VP negative. VP test results of the All samples have been exhibited in Plate 2.9

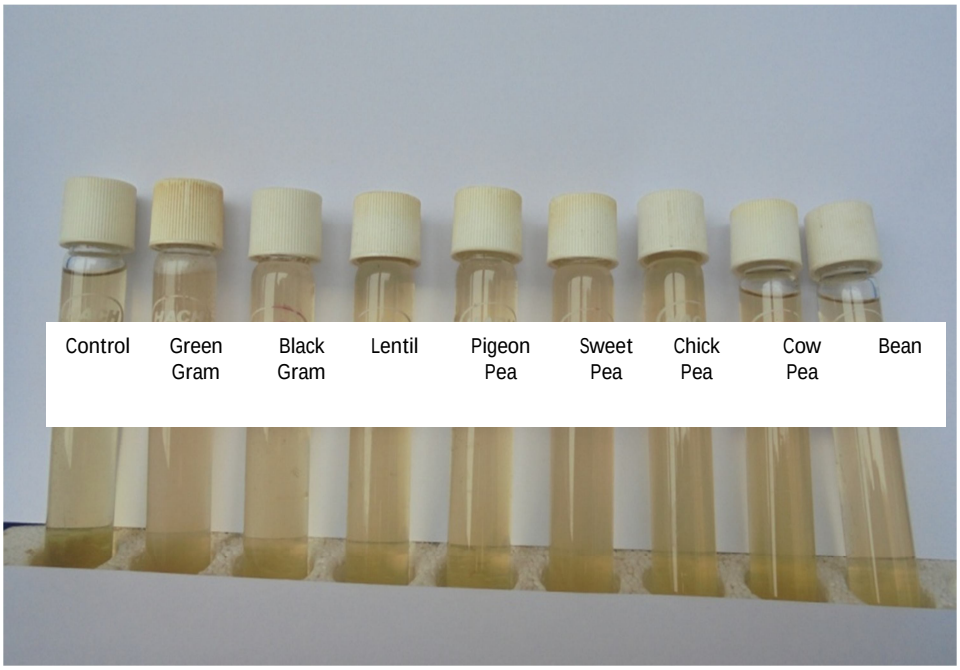


Plate 2.9: Voges-Proskauer reaction of the isolated *Rhizobium* sp.

Hydrogen Sulfide Production Test

A positive result of sulfide production is interpreted by the formation of black precipitation. None of the samples produced any blackening of the medium and thus all were hydrogen sulfide negative. Hydrogen sulfideproduction test results have been shown in **Plate 17.**

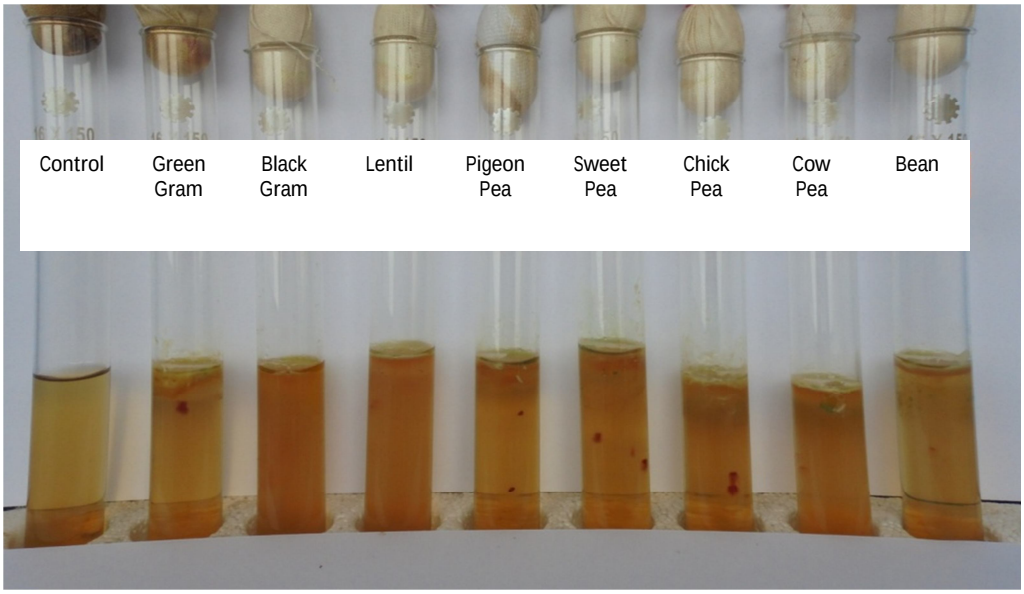


Plate 2.10: Hydrogen Sulfide Production Test of the isolated *Rhizobium* sp.

Indole Test

A positive result is derived by a pink colored ring formation at the top of the medium after adding Kovac's reagent. All sample showed no color change and thus were identified as indole negative. Photographs of indole test result of the five samples are given in **Plate 2.11**

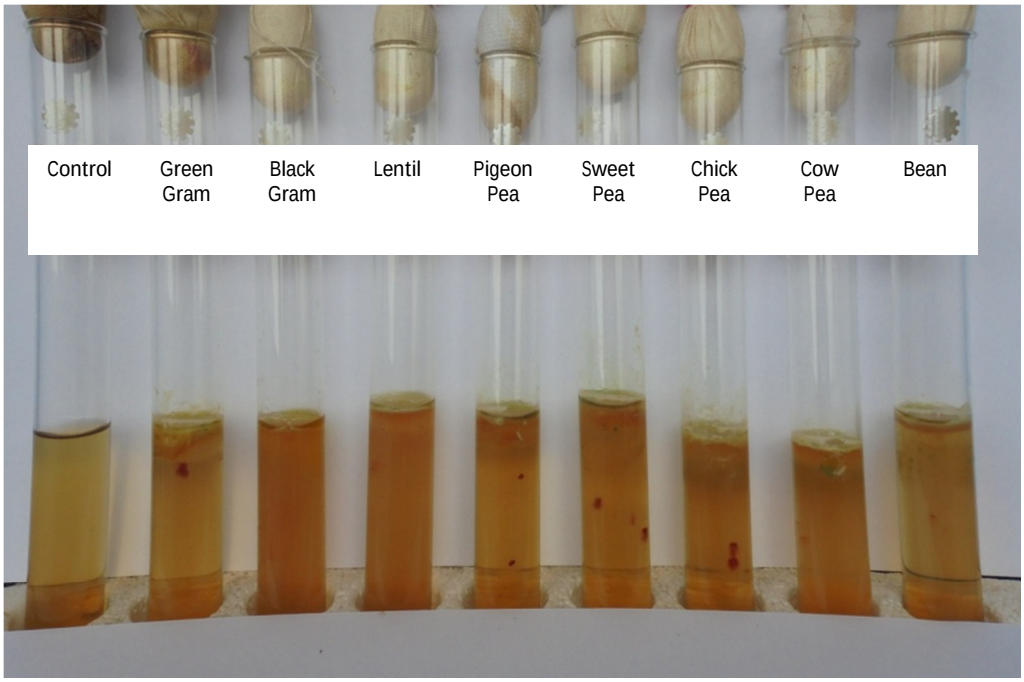


Plate 2.11: Indole Test of the isolated *Rhizobium* sp.

Starch Hydrolysis Test

A positive result of starch hydrolysis is notified by a brown color, while starch non-utilizing bacteria exhibit a dark violet color after adding Gram's Iodine to 2-3 days old culture. All sample showed brown color and that's why they were positive. Starch Hydrolysis test results of the All samples are shown in Plate 2.12.

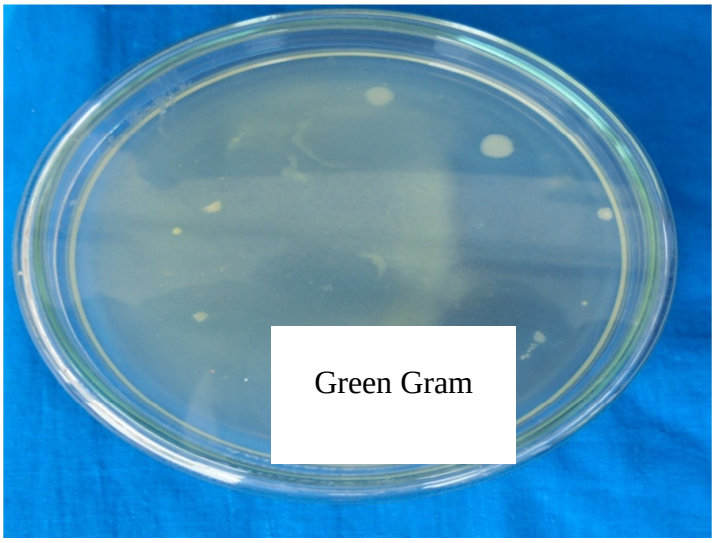


Plate 2.12: Starch Hydrolysis Test of the isolated *Rhizobium* sp.

Urease Test

Positive urease result is interpreted by a pink color, while negative by no color change of the medium. All samples were found to be positive .Results have been shown in **Plate2.13.**

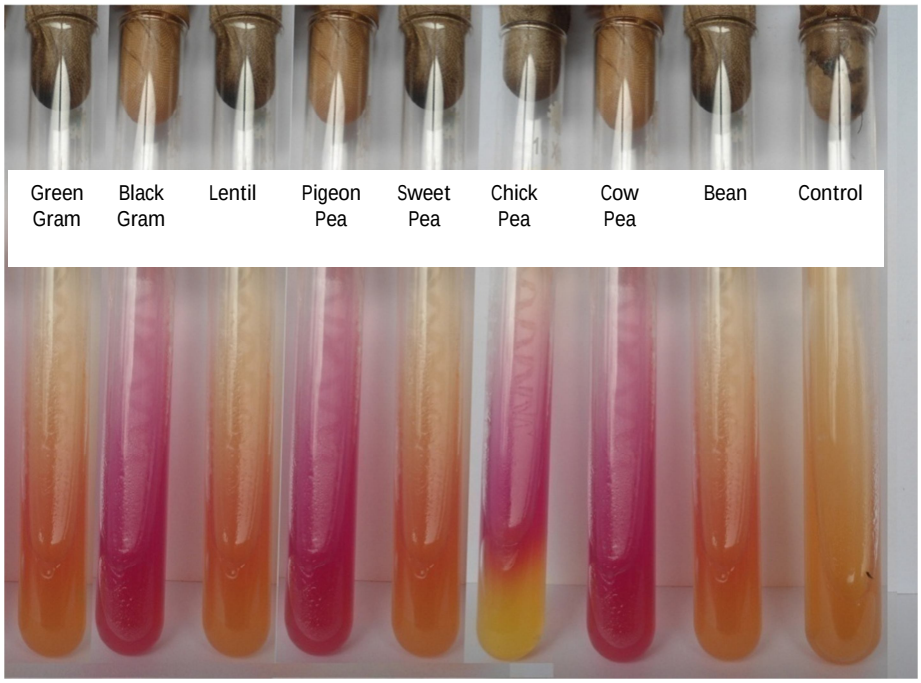


Plate 2.13: Urease Test of the isolated *Rhizobium* sp.

Citrate Utilization Test

Citrate utilizing bacteria transforms Simmon's citrate medium dark blue from forest green. However in this study none of the all samples was found to be citrate positive. Results are exhibited in Plate 2.14.

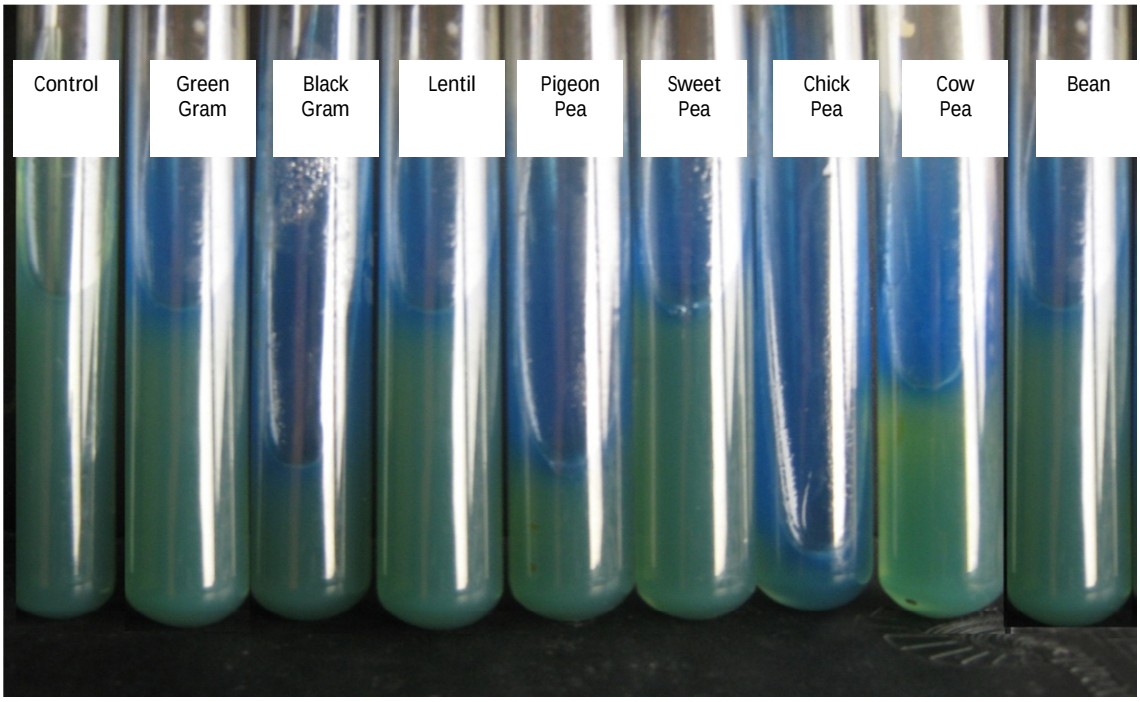


Plate 2.14: Citrate Utilization Test of the isolated *Rhizobium* sp.

Triple Sugar Iron Test

TSI test was performed on Test tube slant prepared from ready mix of TSI , Bio Lab, Hungary. Suspend 64.52 grams in 1000ml of distilled water in a beaker and heat dissolved in a microwave oven. The medium was then autoclaved at 121°C for 20 minutes and distributed in Test tube. After condensation, the Testtube were subcultured by streaking with fresh bacteria culture grown on YEMA agar. Incubation was done at 28°C and observation was done after 24 hours.

Table 2.7: Triple Sugar Iron Test Growth Characteristics of the isolates(N=8) on Triple Sugar Iron (TSI) agar

Isolates	H_2S production	Lactose / sucrose fermentation	Glucose fermentation	Gas production	Peptone utilization
Green Gram	-	-	-	-	+
Black Gram	-	-	-	-	+
Lentil	-	+	+	+	+
Pigeon Pea	-	-	-	-	+
Sweet Pea	-	-	-	-	+
Chick Pea	+	+	+	+	+
Cow Pea	-	-	-	-	+
Bean	-	+	+	+	+

(+) Growth, (-) No Growth

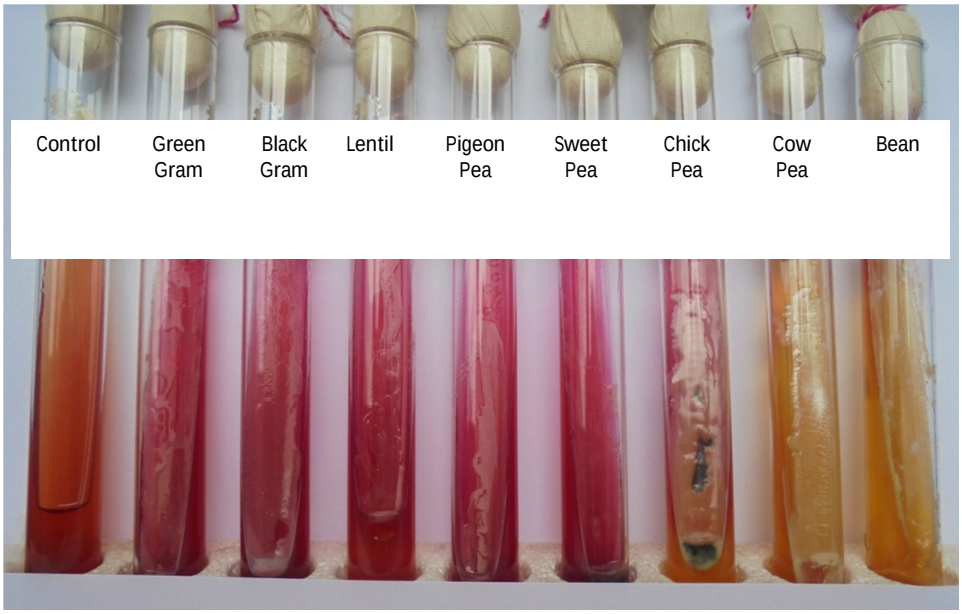


Plate 2.15: Triple Sugar Iron Test of the isolated *Rhizobium* sp.

Oxidase Test

Some bacteria can produce cytochrome C oxidase located in their membrane which can catalyse the transport of electrons from donor compounds to electron acceptors (oxygen). This respiratory system is present in aerobic bacteria which are capable to use oxygen as the final hydrogen receptor.

The pathway used to metabolise a sugar under aerobic conditions differ from those used under anaerobic conditions. When oxygen is present, the process is called oxydation, and when oxygen is absent it is called fermentation.This test provides insight in to the ability of the bacterium to use glucose by both methods.

In the presence of oxygen the cytochrome C enzyme oxidizes the phenylenediamine reagent, resulting indophenols, a purple-blue compound.

Positive Oxidase result is interpreted by a Blue color, while negative by no color change of the medium. All sample were found be positive .Results have been shown in Plate 2.16.

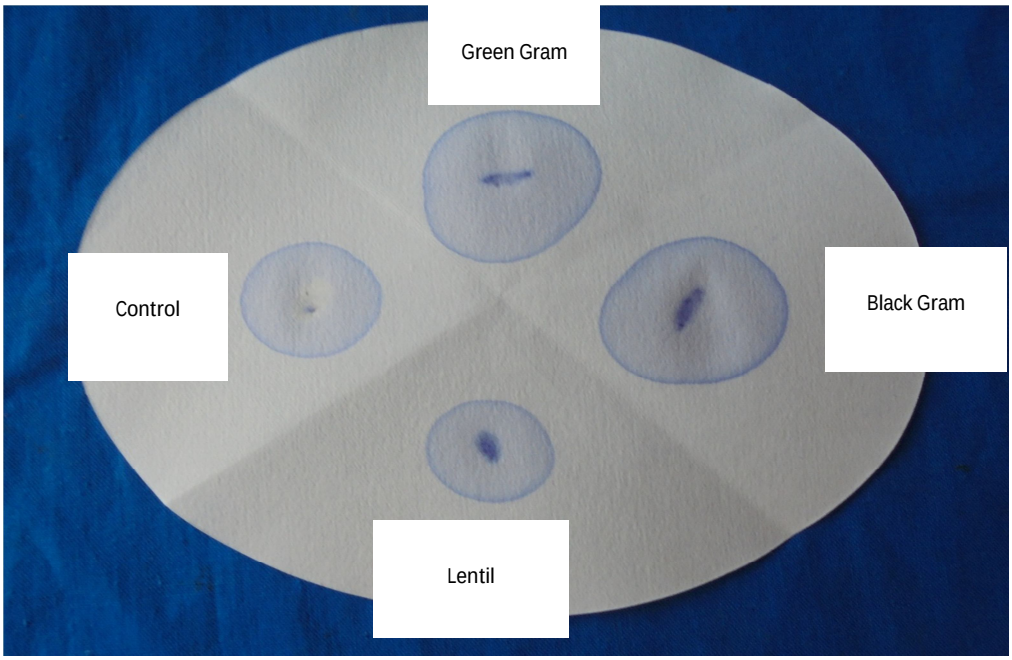


Plate 2.16: Oxidase Test of the isolated *Rhizobium* sp.

Nitrification test

Used in detection of nitrate in the medium. This reagent produces a blue black clour in the presence of either nitrites or nitrates.A positive result of Nitrification test is notified by a blue black color, while nitrate non-utilizing bacteria exhibit no color after adding Diphenylamine in a mixture of sulfuric acid and water. After the solution has stood for 12 hours andall sample are shownthe base separates , showing that reagent is saturated that's why they were positive for nitrification test.

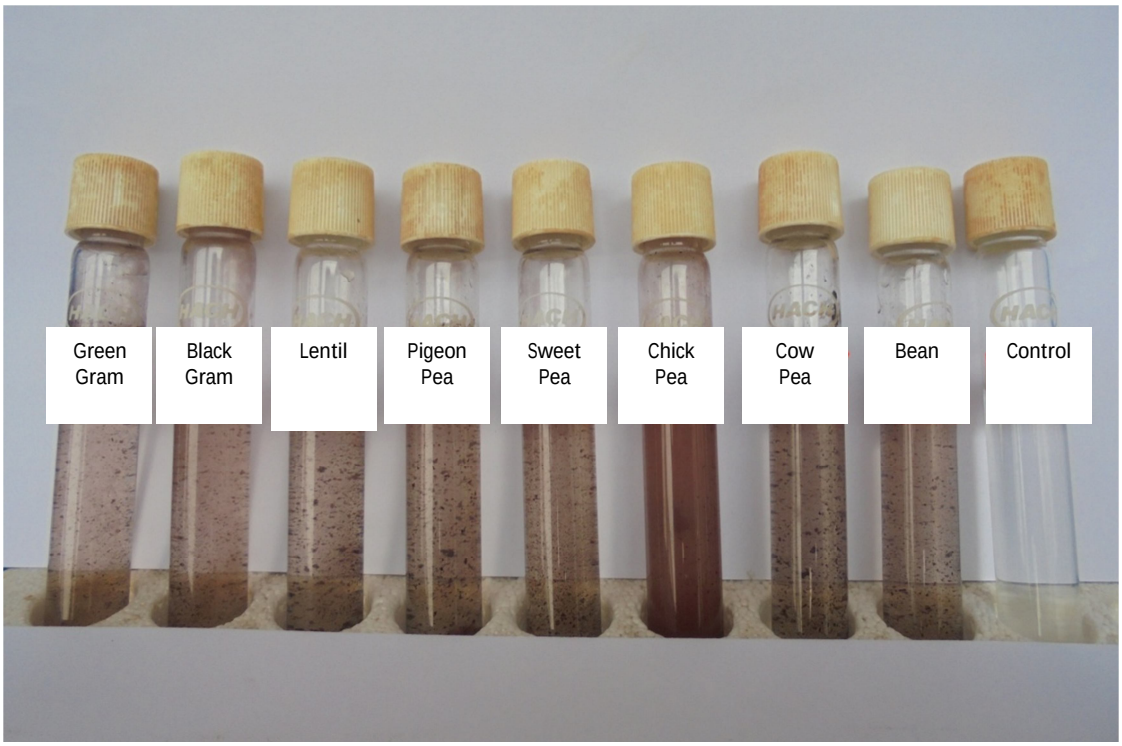


Plate 2.17: Nitrification test of *Rhizobium* sp.

Hofer's alkaline test

Growth in Holer's alkaline medium: Hofer (1935) described the difference in the growth of Agrobacteria and Rhizobia in alkaline medium. He observed that at pH 11, only Agrobacteria could grow. Hence this characterstics was considered suitable in negative selection of rhizobia from agrobacteria

It could be seen that none of the isolates grew at pH 11.0 in the Hofer's alkaline medium indicating that they do not belong to the genus *Agrobacterium* (Gaur and Sen, 1981).

Plate 2.18: Hofer's alkaline test of *Rhizobium* sp.

The ability of the isolates to grow in Hofer's alkaline medium (Hofer, 1935) at pH 11.0 was tested and all isolated sample showed negative growth in Hofer's Alkaline Medium (Hofer 1935). The results indicate that all the isolates showed a negative growth on Hofer‟s Alkaline medium.

Growth on Congo red medium

The isolate grew luxuriantly on YEMA with congo red. In addition the colonies were white, translucent, glistening. All these features indicate and they were not stained, showing that they did not belong to the genus *Agrobacterium* (Dye, 1979; Vincent, 1970).Congored test positive for all the isolates.positive result is showed no colour chang, while *Agrobacterium* showed pink color.

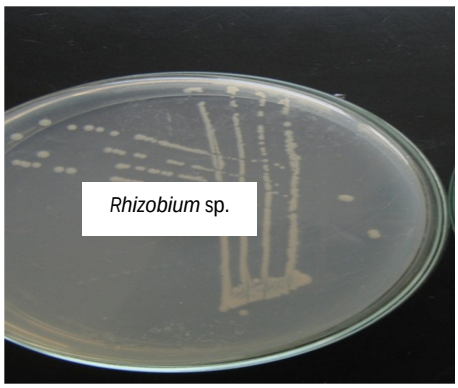


Plate 2.19: Congo red test of *Rhizobium* sp.

Table 2.8: Biochemical tests result.

Test performed	Isolates							
	Green Gram	Black gram	Lentil	Pigeon Pea	Sweet Pea	Chick Pea	Cow Pea	Bean
MacConkey Agar Test	+	+	+	+	+	+	+	+
Catalase Test	+	+	+	+	+	+	+	+
Methyl Red Test	-	-	-	-	-	-	-	-
Voges-Proskauer Reaction	-	-	-	-	-	-	-	-
Indole Test	-	-	-	-	-	-	-	-
Starch hydrolysis test	-	-	-	-	-	-	-	-
Urea Hydrolysis	+	+	+	+	+	+	+	+
Citrate Utilization Test	+	+	+	+	+	+	+	+
Triple Sugar Iron Test	+	+	+	+	+	+	+	+
Oxidase Test	+	+	+	+	+	+	+	+
Nitrification test	+	+	+	+	+	+	+	+
Hofer's alkaline test	-	-	-	-	-	-	-	-
Congo red test	+	+	+	+	+	+	+	+

(-) No Growth, (+) Growth

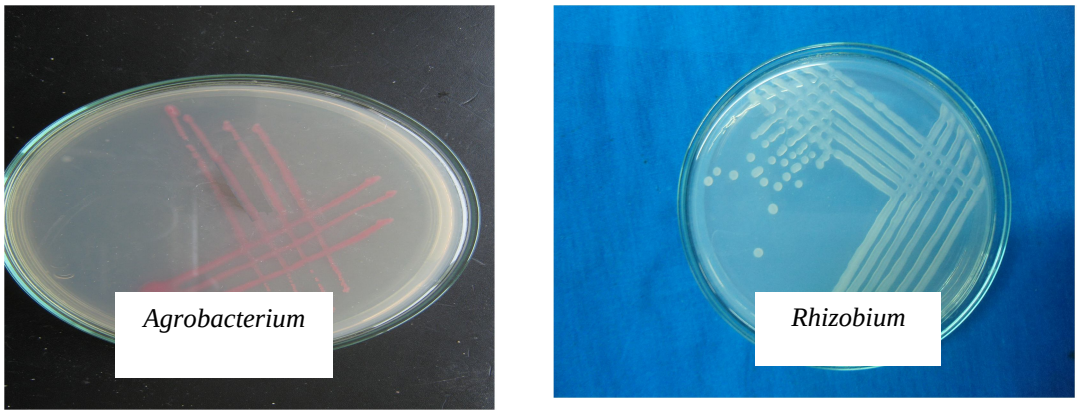
In order to distinguish the isolates from the common contaminant *Agrobacterium* following tests were carried out.

The isolates were subjected to growth in Hofer's alkaline medium, growth on congo red medium, growth on glucose peptone agar, to see whether they belong to the genus *Rhizobium or Agrobacterium.*

Table 2.9: Differentiation tests for *Agrobacterium* and *Rhizobium* sp.

Organisms	*Rhizobium* sp.	*Agrobacterium*
Colonies on Congo red	Colourless	Colour
In Glucose peptone	++	-
In Salt 2% growth	+	++
Hofer's alkaline test (pH 11) growth	-	+

(-) No growth, (+) Growth & (++) Good growth



Pate 2.20: Distinguish between *Rhizobium* and *Agrobacterium* bacteria on Congo red medium.

Carbohydrate utilization

The results in this present investigation support the findings of Allen and Allen (1950) and Graham and Parker (1964) who showed carbohydrate utilization differences between fast and slow growing rhizobia are not fastidious like slow-growers with report to their carbohydrate requirements. The ability of fast growing rhizobia to utilize a number of carbohydrates has been reported earlier (Trinick, 1980; Broomfield and Kumar Rao, 1983; Stowers and Eagleshan, 1984).

Dissacharides lactose, sucrose, and trehalose were not utilized by slow growers. Elkan and Kwick (1968) while examining the carbohydrate response of 36 *R. japonicum* strains, found that these strains grew poorly or not at al on carbohydrates such as sucrose or lactose. Finally they concluded that the inability to utilize disaccharide was one of the pecularities of slow growing nodule bacteria. Martinez-de-Drets and Arias (1972) have proposed an enzymetic base to explain the difference exhibited by fast and slow growing rhizobia in the utilization of different carbohydrates and that is the presence of NADP-6-phosphogluconate dehydrogenase only in the first growing rhizobia. Sadowsky *et al.,* (1983) found that disaccharides were utilized only by fast growing strains. According to Gleen and Dilworth (1981) slow-growing rhizobia tend to lack both upotake-systems and catabolic enzymes for disaccharides.

The nodulation test for all these isolates indicated that they are able to nodulate cowpea legume. This confirms their identification as *Rhizobium* of cowpea group. These isolates caused acid reaction on growth on YEMA containing bromothymol blue. Growth rate study of these isolates was performed. The average generation time was 1 h. (Fig. l.l).They are resistant to large number of antibiotics and are able to utilize wide range of carbohydrates. All these characteristics support inclusion of the cowpea Rhizobia under fast growing *Rhizobium* sp. *i.e. R. loti*

Acid production from sugars:(Carbohydrate /Sugar utilization / Fermentation test)

Sucrose,fructose,galactose, maltose and mannitol (25 mg Hi-media, India) and 20% solution of glucose, lactose, arabinose and xylose were utilized for this test. All the isolates tested here can ferment sucrose, glucose, Fructose, mannitol and produce acid. Isolates which were named as lentil, Cow pea and Bean can utilized all the nine sugar and the produced acid. Green Gram, Black Gram, Pigeon Pea, Sweet Pea Chick Pea can not ferment maltose sugar. Besides this isolates from Green Gram, Black Gram, Pigeon Pea, Sweet Pea and Chick Pea fermented all sugar but Green gram and black gram cannot fermented galactose lactose arabinose and xylose.

Table 2.10: Acid production from sugars:(Carbohydrate /Sugar utilization / Fermentation test)

Carbohydrate Uttilization test	Green Gram	Black gram	Lentil	Pigeon Pea	Sweet Pea	Chick Pea	Cow Pea	Bean
Glucose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Galactose	-	-	+	+	-	+	+	+
Maltose	-	-	+	-	-	-	+	+
Sucrose	+	+	+	+	+	+	+	+
Lactose	-	-	+	+	+	+	+	+
Arabinose	-	-	+	+	+	+	+	+
Xylose	-	-	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+

(-) No growth, (+) Growth

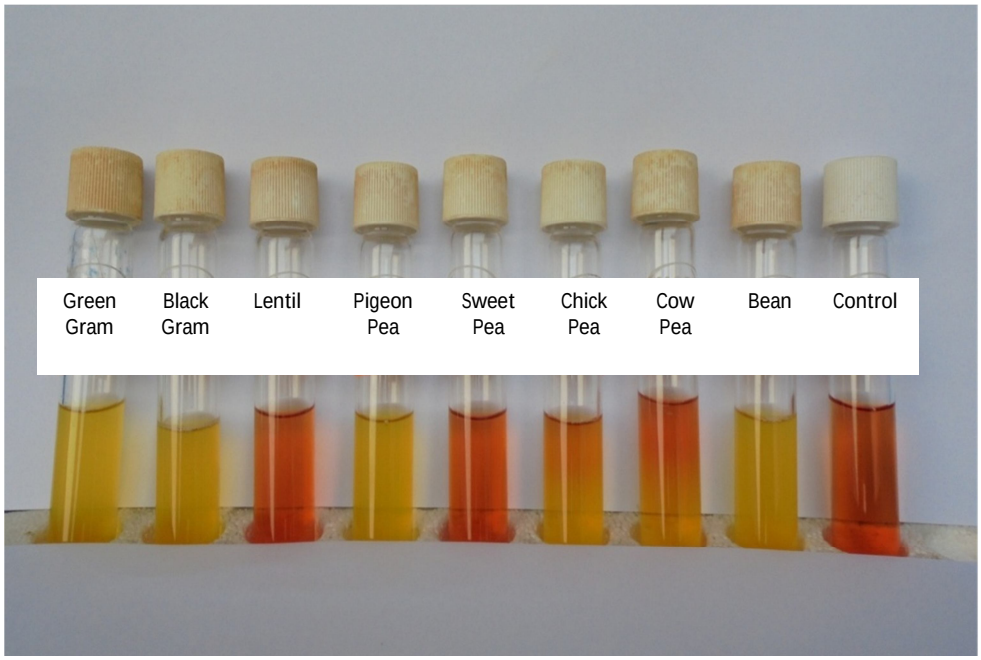


Plate 2.21: Sugar utilization test of the isolated *Rhizobium* sp.

Plate 2.22: Fermentation test of the isolated *Rhizobium* sp.

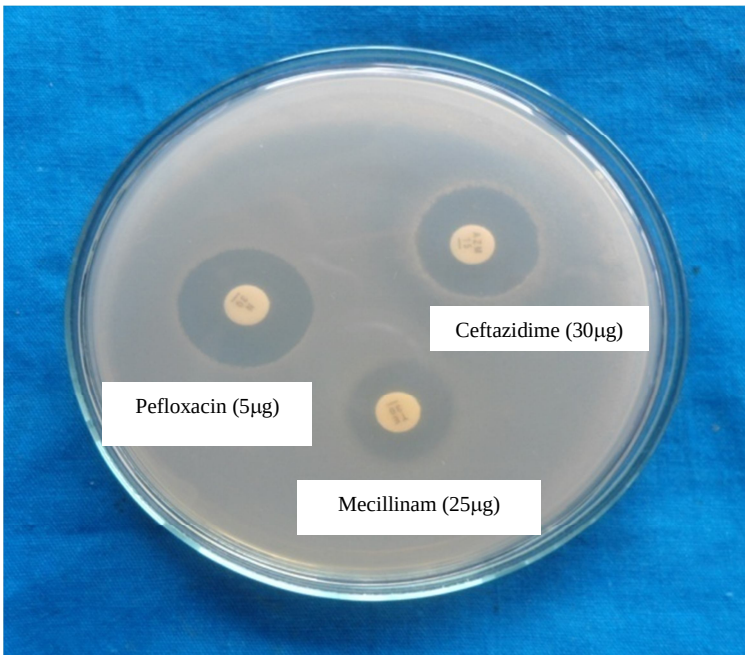
2.4.4 Antibiotic Sensitivity

The intrinsic antibiotic resistance pattern is generally used for strain identification in ecological studies. The patterns of sensitivity and resistance of isolating bacterial cultures to 13 different antibiotics were tested by the disc method using on nutrient broth medium. After incubation overnight at 28°C. The diameter of the inhibition zone, if any was measured. Antibiotic sensitivity is presented in the (Table 3.5). From the table it is evident that the isolated bacteria is resistant to 07 antibiotics out of 13 antibiotics

Table 2.11: Antibiotic sensitivity tests of *Rhizobium* sp.

Antibiotics	Green Gram	Black Gram	Lentil	Pigeon Pea	Sweet Pea	Chick Pea	Cow Pea	Bean
Mecillinam (25μg)	S 20mm	S 20mm	S 17mm	S 20mm	S 20mm	S 20mm	S 22mm	S 17mm
Ampicilin (10μg)	R	R	R	R	R	R	R	R
Ciprofloxacin (5μg)	S 28mm	S 28mm	S 25mm	S 28mm	S 28mm	S 28mm	S 30mm	S 25mm
Erythromycin (15μg)	R	R	R	R	R	R	R	R
Gentamicin (10μg)	R	R	R	R	R	R	R	R
Cotrimoxazole (25μg)	S 24mm	S 24mm	S 20mm	S 24mm	S 24mm	S 24mm	S 26mm	S 20mm
Amoxycillin (10μg)	R	R	R	R	R	R	R	R
Penicillin (10μg)	R	R	R	R	R	R	R	R
Pefloxacin (5μg)	S 18mm	S 18mm	S 15mm	S 18mm	S 18mm	S 18mm	S 21mm	S 15mm
Ceftazidime (30μg)	S 20mm	S 20mm	S 18mm	S 20mm	S 20mm	S 20mm	S 23mm	S 18mm
Streptomycin (10μg)	R	R	R	R	R	R	R	R
Nalidixic acid (30μg)	R	R	R	R	R	R	R	R
Tetracycline (30μg)	S 20mm	S 17mm	S 18mm	S 20mm	S 16mm	S 20mm	S 20mm	S 18mm

S= Sensitive, R= Resistant, mm=Diameter of the clear Zone



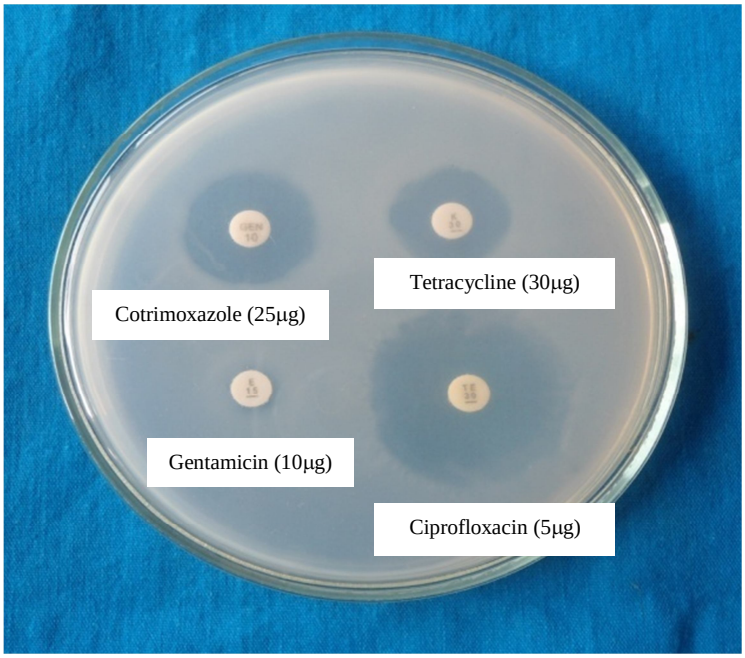


Plate 2.23: Antibiotic sensitivity tests of *Rhizobium* sp.

2.5 DISCUSSION

The nitrogen fixing bacteria can be isolated directly from the root nodules of the host plant or from the soil (Geniaux *et al.,* 1993), using yeast extract mannitol selective culture media (YEM) (Handley *et al.,* 1998; Castro et al., 2003; Kukuc *et al.,* 2006) Al-Mujahidy *et al.,* 2013.We isolated nitrogen fixing bacteria from adjoining soil samples of legumes. Bromthimol blue (BTB) was used as indicator in order to detect the multiplication of the nitrogen fixing bacteria. The yellow halos around the colonies on blue were observed after three days. Yellow color was produced due to the production of acid from nitrogen fixing bacteria. Their morphological shapes were observed under light microscope. Gram staining of these microbes was performed and was observed microscopically. Total eight isolates were selected as nitrogen fixing bacteria from which isolates from Black Gram, Green Gram, Lentil, PigeonPea , Sweet Pea, Chick Pea, Cow Pea and Beanidentical to *Rhizobium* in colony morphology**,**The colonies of *Rhizobium* sp. Were observed on YEMA plates after completion of two days incubation at 30^0C. Eight isolate were obtained designated as Black Gram, Green Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean. These eight isolates were rod shaped and gram negative in nature. The conformations of this *Rhizobium* sp. were done by specific biochemical tests viz. Catalase, Indol test, Methyl red test, Vogus Proskar test, Citrate test, Gelatinase test, Sugar fermentation testwere chosen for further characterization. Regarding Various biochemical tests were performed to screen the particular speciesof *Rhizobium*. Interestingly, the five *Rhizobium* general showed nearly same features and the cellular morphological analysis, five isolates were proved as rod shape and gram negative. These 5 strains were considered as *Rhizobium* genera (Garrity, 1982).

In the present study According to Dye (1979) and Vincent (1970) *Rhizobium* grows on yeast extract manitol agar (YEMA) and produce small to medium sized colonies, usually smaller than 2 mm. *Rhizobium* can not grow at pH 11.0 in the Hofer's alkaline medium (Gaur and Sen, 1981). The rhizobial growth on glucose peptone agar medium is usually poor and causes little change in pH (Subba Rao, 1999). *Rhizobium* isolated from nodules of , Green Gram , Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean is considered as *Rhizobium* sp. The fast growing members of the cowpea group rhizobia and the species of *Rhizobium lupini* have been included in

the species *Rhizobium loti* (Subba Rao, 1999).*Agrobacterium* happens to be a common contaminant during the isolation of rhizobia. To ensure their absence, growth in Hofer's alkaline medium, growth in congo red medium, production of 3-ketolactose, growth on glucose peptone agar (GPA), salt tolerance, carbohydrate utilization tests were done.

Colonies of *Rhizohium* were obtained on YEMagar mediumafter incubation at 28^0 C for 24 hrs which is isolated from Black Gram, Green Gram, Lentil, Pigeon Pea ,Sweet Pea, Chick Pea, Cow Pea and Bean. The colonies were large (2-4 mm indiameter) mucilaginous, circular, raised and smooth edges, glistening translucent orwhite with musky odor, these findings are in line with Aneja (2003), Vincent, (1970), Holt *et al*., (1994), Hussain *et al.,* (2002).Shahzad, *et al., (*2012); Oblisami (2005) who also isolated the *Sinorhizobium meliloti* from alfalfa with same Characteristics. Microscopic examination revealed that the isolates were rod shaped and gram negative in nature which resembles the findings of Keyser (1982), Anand and Dogra (1991), Singh *et al.*, (2008).

All the strains showed growth in three days and turned the yeast extract mannitol agar mediacontaining bromothymol blue to yellow color showing that all were fastgrowers and acid producers (Alemayehu, 2009 and Singh *et al.*, 2011). The colonies were large (2-4 mm in diameter) mucilaginous, circular, convex with smooth edges, glistening translucent or white and precipitated calcium glycerophosphate present in YEM agar (Vincent, 1970; Holt *et al*., 1994). Microscopic examination revealed that the isolates were rod shaped and gram negative in nature (Keyser, 1982; Anand and Dogra, 1991; Singh *et al*., 2008). In this study, all isolates were oxidase, catalase and urease positive and unable to utilize citrate, which complements the finding of Lupwayi and Hague, (1994). Eight isolates are identified and subjected to biochemical analysis. Biochemical characteristics among the Eight selected isolates indicated that they were closely related to *Rhizobium* species.

In this study, all isolates were. showed hazy appearance in the motility media and also were positive for Catalase Citrate Utilization Test ,Urea Hydrolysis , Congored test, Nitrification test ,Oxidase TestTriple Sugar Iron Test ,MacConkey Agar Test and, motility Tests. oxidase, catalase and urease positive, which complements the finding of Lupwayi and Haque, (1994).The isolates were found negative for Methyl Red (MR)

and Voges-Proskauer (VP) reaction. These findings are in close agreement with Elsheikh and wood (1986).

The samples were found negative for Methyl Red (MR), Voges-Proskauer (VP), Indole, Starch hydrolysis test, Hydrogen Sulphide production, and Hofer's alkaline test tests (Table-2.8). Our these findings are in close agreement with Elsheikh and wood (1989) who also characterized the rhizobium from soil and sunflower root nodules with the same positive biochemical tests. Similarly Oblisami (1995) also studied the nodulation pattern in forage legume bacteria by screening through the same tests results and Singh *et al.,*(2008) characterized rhizobium strain from the roots of *Trigonella foenumgraecum.*

In the present investigation it was found that, samples gives positive results for Catalase and Motility tests while, negative for Methyl red test, Vogus Proskar test, Indol test, Citrate utilization test, Hydrogen sulphide production (Table-2.8). Our result shows close conformity with findings of Patil1, *et al.,* (2014);Shahzad*et al.,* (2012). These results conformed the isolated bacterial strains are *Rhizobium* sp. Previously, Erum (2008), Singh (2008) and Shahzad *et al.,* (2012) mentioned that sugar tests are positive during isolation and characterization of *Rhizobium meliloti*.

The bacterium showed well-marked growth on YEMA medium at pH 7.0. However poor growth was observed on Hofer's medium. Mahana *et al.,(2000)* reported that the *Rhizobium* isolated from *Vigna mungo* L. showed marked variations in growth with respect to time period on YEMA while they do not show any growth on Hofer's alkaline medium at pH 10.0with slight growth and evolution of gas and acid production. According to Ditmer (1930) those Rhizobia, which produce acid are considered to be advanced type and those, which produce alkali are ancient type The bacterium showed positive test for Citrate, production of ammonia and catalase activity. Nitrate is reduced to nitrite producing ammonia. Neal and Walker (1935) suggested rapid nitrate utilization by slow growing root nodule bacteria. Mahana *et al.* 2000 reported catalase activity in some isolates from *Vigna mungo* .The bacterium is negative for MR-VP and indole reaction. Similarly, Grahm and Parker (1964) did not observe MR reduction in all the isolates of seven rhizobia groups. While Basak and Goyal (1980) also reported that none of the rhizobial isolates of of seven groups produces indole.

Utilization of different carbon sources is an effective tool to characterize the isolates (Mirza *et al.,* 2007: Erum and Bano, 2008). In the present study sucrose, fructose, galactose, maltose and mannitol (25 mg Hi-media, India) and 20% solution of glucose, lactose, arabinose and xylose were utilized for this test. All the isolates tested here can ferment sucrose glucose, fructose, mannitol and produce acid. These carbon sources are generally utilized by bacteria of the genus *Rhizohium* (Stowers 1983). Sadowsky *et al.* (1983) and Anand and Dogra (1991) also observed that fast growing Rhizobial strains utilized a wider variety of carbohydrates than the slow growing strains. Their ability to metabolize a broad range of carbon substrates may be advantageous for survival in soil. Isolates which were named as lentil ,cow pea and bean can utilized all the nine sugar and the produced acid. Green Gram , Black Gram, Pigeon Pea, Sweet Pea, Chick Pea can not ferment maltose sugar. Besides this isolates from Green Gram , Glack Gram,Pigeon Pea, Sweet Pea and Chick Pea fermented all sugar but Green Gram and Black Gram cannot fermented galactose lactose arabinose and xyols. These findings corroborate with the results of Oblisami (1995); Michael (2006); Singh (2008) and Erum (2008)who also reported these sugar tests positive during isolation and characterization of *rhizobium* sp.

Rhizobial cells were able to grow on the GPA media showing the utilization of glucose as the carbon source by the *Rhizobium*. It is a confirmatory test for *Rhizobium* and these are able to utilize glucose as carbon source (Deora *et al.,* 2010).Result of carbohydrate utilization test support the findings of Allen and Allen (1950) and Graham and Parker (1964) who showed differences of carbohydrate utilization between fast and slow growing rhizobia. Dissacharides lactose, sucrose, and trehalose were not utilized by slow growers. Sadowsky *et al.,* (1983) found that disaccharides were utilized only by fastgrowing strains. According to Gleen and Dilworth (1981) slow-growing rhizobia tend to lack both uptake-systems and catabolic enzymes for disaccharides. All these evident reveals that the isolate was a member of the fast growing rhizobia. Subba Rao (1999) reported that the number of viable rhizobia tend to diminish in pre-inoculated seeds stored indefinitely. Many leguminous seeds contain water-soluble toxic compound which adversely affect the viability of rhizobia (Millington, 1995). It may be a cause of lower viability of *Rhizobium* on preinoculated legume seeds than in preinoculated soil. Saha and Kapadnis (2001a) reported that there existed a significant difference in bacterial population on seeds by using different adhesives. The count of

Rhizobium transformant decreased almost equally in both sterile and natural black and poita soil but the rate of decline was relatively more in natural soils than in sterile soils (Saha and Kapadnis, 2001b). It was reported that the viability of *R.leguminosarum* in natural soil was greatly affected by certain protozoa, fungi and bacteriophages (Subba Rao, 1999). Increasing evidence supports the view that bacteria inoculated on seeds colonize roots emerging from the germinating seed and then moves along the roots passively (Dijikstra *et al.,* 1987). Therefore, the density of bacteria applied to seeds may determine bacterial root colonization efficacy.

Utilization of different carbon sources is an effective tool to characterize the isolates (Mirza *et al*., 2007; Erum and Bono, 2008). All isolates obtained from barseem clover nodules were able to utilize glucose, galactose, rhamnose, sorbitol, arabinose, xylose, lactose, sucrose and glycerol as carbon sources. These carbon sources are generally utilized by bacteria of the genus *Rhizobium* Sadowsky *et al*., (1983) and Anand and Dogra (1991) also observed that fast growing Rhizobial strains utilized a wider variety of carbohydrates than the slow growing strains. Their ability to metabolize a broad range of carbon substrates may be advantageous for survival in soil. Only Black Gram,Green Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean. isolates obtained in this study were able to use dextrin as a carbon source, which is in accordance with other works indicating that dextrin is rarely utilized by *Rhizobium* (Jordan, 1984). These results further confirm the taxonomic position of *Rhizobium* isolates. Moreover morphological, biochemical, physiological and *in vivo* infectivity of the strains were found similar to that of *R. trifolii*.

Resistance patterns of the isolates to thirteen antibiotics were studied. Screening for antibiotic resistance in our study revealed that most of the strains were resistance to Ampicilin, Erythromycin, Gentamicin, Amoxycillin, Penicillin, Streptomycin and Nalidixic acid. It is in accordance to Hungaria *et al.*,(2000) . The generalized sensitivity to Mecillinam Ciprofloxacin Cotrimoxazole Pefloxacin Ceftazidime and Tetracycline in our study agrees with the results of Jordan(1984) for the genus *Rhizobium* and Hagedorn(1979) for *R.leguminosarum* by *trifolii*. Screening for antibiotic resistance in our study revealed that most of the strains were resistance to streptomycin, chloramphenicol, ampicillin, erthyromycin, kanamycin, polymyxin, nalidixic acid, gentamycin and neomycin and least to vancomycin, rifampicin, cephalothin and

tetracycline. It is in accordance to Hungaria *et al.*, (2000) that this character is due to three known determinants of bacterial permeability to antibiotics: hydrophobicity, electrical charge and amount of the antibiotic. Streptomycin resistance, when caused by a chromosomal mutation, has been shown to be due to an alteration of specific protein on the 30S ribosomal subunit to which streptomycin binds in the sensitive cell. Another class ofstreptomycin resistant bacteria owes their resistance to the presence of plasmid which mediates either the acetylation, adenylation or phosphorylation of the drug molecule, itself. According to Holt *et al.,* (1994) spontaneous mutants for resistance to most antibiotics are a component of all "wild type" strain. Resistance to streptomycin may be greater than 10 times that of "wild type",where as resistance to other antibiotics were generally of a lower order (2 or 3 times). Therefore, streptomycin resistant mutants, those are usually, effective are important in ecological field studies on strain competition. Occurrence of higher resistance to antibiotics like penicillin, streptomycin and erythromycin was reported by Kahlon (1980). Similarly Kucuk and Kivnac, (2008) observed higher resistance of rhizobia against streptomycin, chloramphenicol, erthyromycin, kanamycin and penicillin.The generalized sensitivity to tetracycline, Mecillinam, Ciprofloxacin, Cotrimoxazole, Pefloxacin and Ceftazidime in our study agrees with the results of Jordan (1984) for the genus *Rhizobium* and Hagedorn (1979) for *R. leguminosarum*bv *trifolii*. Sensitivity of isolates to antibiotics may be due to the fact that these bacteria have not been exposed to these antibiotics in natural environments. Depending on the difference in antibiotic resistance pattern, this technique could be successfully employed for the field of ecological studies particularly in the recovery and enumeration of rhizobia introduced into soil with regard to carbon utilization, it has been established that *Rhizobium* is able to utilize a wide variety of carbon sources for growth and have several pathways available for carbon catabolism (Stowers, 1983).

It can easily be concluded from this study thatsoil of different location of Rajshahi City is equally suitable for the rotational cultivation of leguminous fodder. There is dire need to createawareness among agriculture farmers to cultivate certainleguminous fodders in the area that will not only improvetheir socio-economical status but will also be helpful to put on pedestal the national economy.

2.6 CONCLUSION

Rhizobia are aerobic rod shaped, motile, gram negative, hetrotrophic, and non-spore forming bacteria. They are motile when young and have bipolar, subpolar or peritrichous flagella. Rhizobia fix atmospheric nitrogen and thus not only increase the production of inoculated crops, but also leave a fair amount of nitrogen in the soil, which benefit the subsequent crops. This successful symbiotic association requires the survival of rhizobia in sufficient number as free living bacteria in the soil ecosystem . Nitrogen is the most deficient nutrient in Bangladesh soils. Urea, which is the most commonly used nitrogenous fertilizer, has now become a costly input for most of the farmers. As such, *Rhizobium* inoculants may be used as a cheaper substitute for urea in the production of food legume crops. The beneficial effect of rhizobial inoculates in increasing yield of leguminous crops results from the activity of its root nodule bacteria, which fix atmospheric nitrogen making it available for the plants. Inoculation types and many other factors usually affect the nodulation, nitrogen fixation and plant growth. For crop growth minimum inoculum level is necessary to obtain beneficial effects showed that at increasing inoculum densities increasing numbers of *Rhizobium leguminosarum* biovar trifolii survive under stress conditions in liquid medium. Also mentioned that dense populations are killed to lesser extent than sparse populations. The inhibitory or stimulatory effects of soil microorganisms such as bacteria, fungi and actinomycetes on *Rhizobium* are known. Culture filtrates of fungi isolated from soil and those isolated from washed nodules often inhibit the growth of Rhizobia. The failure of nodulation in certain parts of Western Australian soil has been attributed to the presence of microorganisms antagonistic to Rhizobia. It is sensitive to antibiotics and other agricultural chemicals. In the present study an attempt has been made to isolate *Rhizobium* from Green Gram , Black Gram, Lentil, Pigeon Pea , Sweet Pea, Chick Pea, Cow Pea and Bean to study the effects of its inoculation on nodulation and growth.

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Chapter 3

Physiological Characterization of Isolated *Rhizobium* Spp.

CONTENTS

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Physiological Characterization of Isolated *Rhizobium* spp.

3.1 INTRODUCTION AND LITERATURE REVIEW

3.1.1 Physiological Characterization

An ordered increase in the size of an individual is considered as growth for multicellular organisms like plant, fish or human being. On the other hand, growth in unicellular microorganisms such as bacteria is interpreted in terms of an increase in the size of a given population. This is expressed as an increase in either the number of individuals or the total amount of biomass. Biomass is the total amount of cellular material in a system.

However, the growth by any mean is controlled by some factors, e.g. pH, temperature, salt concentration etc. Nevertheless, each and every microbe has its' own defined favorable combination for the smoothest growth. Physiological characterization deals with the estimation of the suitable most condition for the growth of microorganism (Hogg, 2005).

3.2 The factors affecting rhizobia population, legume and nitrogen fixation

Nitrogen fixing rhizobia cannot express their full potential in fixing nitrogen if the environment and the plant is in poor state (Balasubramanian, *et al.,* 1976 and Elsheikh, *et al.,* 1990). The process of nitrogen fixation depends much on the functional state of the legume plant (LaRue, *et. al.*1981) and the optimum environmental conditions supporting the macro and microsymbionts. The nitrogen fixing rhizobia vary in their tolerance to major environmental factors (Graham, 1992). Environmental stresses can affect the host plant and symbiotic rhizobia. The most threatening environments for rhizobia functions are marginal lands with low rainfall, acidic soils with poor water holding capacity, nutrient stress and temperature extremes. Zahran (1999).

3.2.1 Temperature Extremes

High temperatures have effects on the root nodule structure, function and root hair infection Graham, 1992. The best temperature for nodule functioning in common beans (*Phaseolus vulgaris*) is between 25°C-30°C (Figueiredo, *et al.,*2008 and Alexandre, *et al.,* 2013).The optimum temperature range for rhizobia has been reported to be 25°C to 31°C in culture media but rhizobia isolated from hot and dry Sahel Savannah

environment were reported to grow well at 40°C Zahran, (2001). The favorable recommended temperature for root hair development and large number of infection is between 15 and 20°C. The limits of low temperature for crops native to temperate region is 2oC while for tropical species is 10°C (Naeem, *et al.,* 2008). Legume species can fix nitrogen at different critical levels of temperature such as 35°C - 40°C for soybeans and cowpeas, and 30°C (Michiels, *et al*., 1994) for peas .The 68 rhizobia species isolated in Egypt grew at temperature ranging from 20°C - 35°C though some species grew at 35°C - 40°C and at maximum of 50°C (Zahran, *et al.,* 2012) Successful isolation of high temperature tolerant rhizobia for common bean has been reported in various regions (107). The rhizobia population in relation to temperature for across different zones in Africa still needs more research. The low temperature experienced at the highlands and high temperature with low moisture content in lowlands has resulted into varied crop yield from one season to another. Therefore, isolation of native rhizobia species from extreme temperature range as those found in Africa is essential for obtaining temperature tolerant rhizobia species for improvements of legume yield.

3.2.2 Drought (Soil Moisture Deficiency)

Both rhizobia and legume can exist in soils with low moisture levels with the lowest population densities reported in most desiccated environments Zahran, (1999). Drought reduces the rhizobia population in soils, inhibits nodulation and N fixation. Nitrogen fixation process is highly sensitive to the deficiency of soil moisture (Ledgard, *et al.,* 1992). Rhizobia population in relation to drought has received little attention in Tanzania and Africa at large. Most dry lowlands in Africa are characterized with low moisture content and high annual temperature range. Therefore, successful isolation of rhizobia from such environment will definitely result in obtaining good rhizobia candidates for establishing successful symbioses in drought environments useful for production of common bean and other legumes.

3.2.3 Soil Acidity and Related Stress

Soil acidity and related problems of manganese and aluminum toxicity as well as calcium deficiency seriously affect nodulation, N fixation and plant growth (Eaglesham, *et al.,* 1984). Nitrogen fixation by rhizobia to most leguminous plants is effective at neutral or slightly acidic soils (Francis, 1982). Bordeleau, *et al.,*1994.

Researchers have reported that most legume species fail to nodulate at pH less than 5.0 because cannot withstand acidic condition. A study conducted in Kenya revealed that common beans (*Phaseolus vulgaris*) can be nodulated by both rhizobia from low pH (acidic soils) and high pH soils (Graham, 1992 and Bordeleau, *et al.*,1994). Rhizobia isolated in Egypt grew at pH ranging from 6 - 8 with some being able to 2 tolerate acidic pH ranging from 3.5.to 4.0 and alkalinity at pH ranging from 9 – 10 (Zahran, *et al.*, 2012). *Rhizobium tropici*is a species adapted to acidic soils than any other species of *Rhizobium* reported to nodulate *Phaseolus vulgaris* (Kellman, 2008).Therefore isolation of rhizobia from varied locations with wide range of soil pH is a pavement to acquire effective native rhizobia tolerant to low and high soil pH.

3.2.4 Salt and Osmotic Stresses

Researchers have reported the detrimental effect of salt on growth and survival of rhizobia (Bouhmouch, *et al.*, 2005 and Singleton, *et al.*, 1982). The response of legume to salinity varies much depending on soil properties, climatic conditions and plant growth stage (Bernstein, *et al.*, 1974 and Maas, *et al.*, 1977). Salt and osmotic stress can affect the initial stage of legume-rhizobial interaction and nodule formation than it does to rhizobia (Sulieman, *et al.*, 2013).The root hair formation on plants is more sensitive to salt than rhizobia cells hence rhizobia can tolerate salinity from 4.5 to 5.2 dsm -1 Saxena. 1992. Therefore, isolation and estimation of the population density in relation to salt and osmotic stress for native rhizobia nodulating common beans, cowpeas, soybeans and peas across favorable to unfavorable rhizobia growth conditions across Africa is a means for improvement of legume production.

3.3 Impact of temperatures on bacterial growth

Bacteria are able to grow over a wide range of temperatures, from around freezing to above boiling point. For any organism, the minimum and maximum growth temperatures define the range over which growth is possible which is typically about 25–30°C. Growth is slower at low temperatures because enzymes work less efficiently and also because lipids tend to harden and there is a loss of membrane fluidity. Growth rates increase with temperature until the optimum temperature is reached and then the rate falls again. The optimum and limiting temperatures for an organism are a reflection of the temperature range of its enzyme systems, which in turn are determined by their

three-dimensional protein structures. The optimum temperature is generally closer to the maximum growth temperature than the minimum. Once the optimum value is passed, the loss of activity caused by denaturation of enzymes causes the rate of growth to fall away sharply. The majority of microorganisms achieve optimal growth at middling temperatures of around 25–40°C and are called mesophiles. In contrast, the thermophiles can thrive at much higher temperatures. Typically, these would be capable of growing within a range of about 40–80°C, with an optimum around 50–65°C. Extreme thermophiles have optimum values in excess of this and can tolerate temperatures in excess of 100°C. A member of the primitive bacterial group called the *Archaea* was reported to grow at a temperature of 121°C. Psychrophiles occupy the other pole of the temperature range; they can grow at 0°C, with optimal growth occurring at 15°C or below. Such organisms are not able to grow at temperatures above 25°C or so. Psychrotrophs, on the other hand, although they can also grow at 0°C, have much higher temperature optima (20–30°C). Members of this group are often economically significant due to their ability to grow on refrigerated food stuffs. In the laboratory, appropriate temperatures for growth are provided by culturing in an appropriate incubator. These come in a variety of shapes and sizes, but all are thermostatically controlled and generally hold the temperature within a degree or two of the desired value (Madigan and Marrs, 1997).

Soil temperature influences the ability of *Rhizobium* to persist in soil. High soil temperature could contribute to high frequency of noninfective isolates in soil. (Roughley *et al.,* 1980) noted that such noninfective isolates actually outnumbered those that, were infective in the rhizosphere of bean. On inoculation of promiscuously nodulated culliver of *Glycine mux* with *Vigna* as well as *B. japonicum,* the Vigna-rhizobia were more competitive at high temperatures (24-30°C). Strain differences in nodulating ability at low temperature have also been shown between arctic-zone rhizobia (Ek-Jander and Fahraeus, 1971) while dual occupancy was found reduced from 63% at 8°C to 2% at 25°C, with the proportion of nodules occupied by strain NRG 185 alone increased from 9 to 75% over the same temperature range (Rice and Olsen, 1988).

For most rhizobia the temperature for growth in culture is from 28 to 3l°C, with many unable to grow below 10 or at 37°C. However, arctic rhizobia are known to grow well

at 10°C (Caudry- Reznick *et al.,* 1986), while the optimum for *R. melilotii*s 35°C (Allen and Allen, 1950).

Rhizobial survival in soil exposed to high temperatures has been shown to vary with the montmorillonitc content of the soil (Marshall, 1964), to be greater in soil aggregates than in non aggregate soil (Ozawa *et al.,* 1988), and to favoured by dry rather than moist conditions. Ten inoculant strains examined by (Somasegaran *et al.,* 1984) showed a gradual decline in population during eight weeks incubation at 37°C, while exposure to 46°C was lethal to all strains in less than two weeks (Wilson and Trang, 1980).

Temperature affects root-hair infection, bacteroid differentiation and nodule structure, and the functioning of the legume root nodule (Roughly and Dart, 1969). High soil temperatures will also delay nodulation or restrict it to the subsurface region, where temperatures are not as extreme. Munns *et al.* (1977) found that alfalfa plants grown in desert environments in California maintained few nodules in the top five cm of soil but were extensively nodulated below this depth. Graham and Roses (1978) observed fewer nodules close to the surface in spread plantings than in plantings with dense canopies. Rhizobia are gram negative rods and its speciation is based on the cross-inoculation grouping. Rhizobia that can form nodules on roots of legumes have been collectively taken as a species, particularly in case of the legume *Vigna* and *Arachis*it is known as *Rhizobium* sp. It is also known to survive in soil and in the rhizospheres of legumes as well as non-legumes. *Rhizobium* secretes extracellular polysaccharides which may help in binding soil particles together. They can survive at low temperature and tolerates temperature upto 50°C for more than of few hours. It is sensitive to plant protectants, antibiotics and other agricultural chemicals (Nutman, 1965). *Rhizobium* is more tolerant toward salts than its host legume and therefore, survives in saline soils (SubbaRao *et al.,* 1972, 1974). Information on the survival of rhizobia seeded in peat and soil or on seed at high temperature exposure is of special significance in tropics as that would determine the success of establishment of effective strains in the soil through seed inoculation. Results of a study in this aspect were reported by Iswaran *et al.,* (1970). They found that in peat at 28°C, there was a slight fall in counts in the first week and then, the counts increased while at 35°C there was multiplication upto second week and declined thereafter. In case of the soil at 28°C and 35°C, the counts were appreciable only upto second week and thereafter there was sudden fall. At 40°C there was a rapid

death of rhizobia added to soil as compared to peat with no worthwhile recovery after four weeks of storage.

Temperature affects growth as well as survival of *Rhizobiwn.* However, it can survive at low temperature and can tolerate temperature up to 50°C for more than a few hours (SubbaRao, 1985). Temperature optimum for nodulation and nitrogen fixation vary between species and genera but are within a small range near 30°C (Islam and Dart, 1975: Date, 1989; Date and Ratcliff, 1989).

3.4 Impact of pH on bacterial growth

Bacteria are strongly influenced by the prevailing pH of their media or surroundings like water or soil. Minimum, optimum and maximum values of pH for growth of a particular type of bacteria can be conferred. Most bacteria grow best around the neutral pH which is actually 7.0. Slightly alkaline conditions are preferred by many bacteria known as alkalophilic while relatively few are tolerant to acid conditions which are called to be acidophilic. The reason for the growth rate falling away either side of the optimum value is due to alterations in three-dimensional protein structure. The pH value of growth media is adjusted to the desired value by the addition of acid or alkali during its preparation. The metabolic activities of microorganisms often means that they change the pH of their environment as growth proceeds, so it is important in a laboratory growth medium that a desirable pH is not only set but maintained. This is achieved by the use of an appropriate buffer system. Phosphate buffers are widely used in the microbiology laboratory; they enable media to minimize changes in their pH when acid or alkali is produced (Moat *et al*., 2002) ported due to this novel ability (Margesin and Schinner, 2001).

Soil pH has an effect on survival and competition of *Rhizobium* in soil. The survival of Rhizobia in acid soils is inversely related to the amount of acid produced, that is, those rhizobia that produce the least acid should survive best in acid soils (Norris, 1965). *Rhizobium* strains vary in their acid tolerance (Graham *et at.*,1982). The failure of legumes to nodulate under acid-soil conditions is common (Brockwell, 1962; *Rice et al.*, 1977), especially in soils of pH less than 5.0. The inability of some rhizobia to persist under such conditions is one cause of nodulation failure (Lowendorf and Alexander, 1983).Fast- growing rhizobia have generally been considered less tolerant

to acid pH than bradyrhizobia, but strains of *Rhizobium loti* and *Rhizobium tropici* have proved highly acid tolerant, with growth in some cases to pH 4.0 (Cooper, 1982). Strains of *Rhizobium meliloti* are particularly affected by acidity, soil pH being the major factor limiting their numbers in soil (Brokwell *et al.,* 1991). Brokwell *et al.,* (1991) found an average of 89000 organisms per gram in soils where the pH 7.0 or more, but only 37 per gram in soils of pH less than 6.0. Bushby (1990) found that only *Bradyrhizobium* strains isolated from acid soils had a net positive charge, and postulated a relationship between their surface-charge characterstics and the soil from which they came. Despite this, strains isolated from acid soils are not necessarily pH tolerant (Richardson and Simpson, 1988), but rather are concentrated in more favourable microsites in the soil. Thus, for soil pH 4.2, only 96 of 481 isolates were capable of growth even at pH 4.70 (Richardson and Simpson, 1988).

The basis pH tolerance in neutrophilic species is through the regulation of cytoplasmic pH, with cells maintaining an internal pH of 7.5 to 8.0 against a pH differential with the outside environment of as much as three pH units (Booth, 1985). This is usually achieved through the expulsion of proton, with uptake of potassium ions needed to balance cell charge. Some species also possess a cytoplasmic buffering capacity [20-1000 nmol hydrogen ions per unit per milligram cell protein (Krulwich *et al.*, 1985)] or the ability to regulate cell metabolism and offset the accumulation of acidic end products (Goffwald and Gottschalk, 1985). Several studies have reported "acid-shock" proteins, which are synthesized in greater amount or detected under acid growth conditions (Taglicht *et al.,* 1987; Hickey and Hirschfield, 1990). Genetic studies of tolerance to low pH suggested chromosomal location of genes encoding acid shock proteins (Chen *et al.,* 1991).The failure of rhizobia to nodulate under acid- soil conditions is common (Rice *et al.,* 1977), especially in soils of pH less than 5.0. The inability of these rhizobia to persist under such conditions is one cause of nodulation failure (Lowendorf and Alexander, 1983), but poor nodulation can be demonstrated. The pH-sensitive stage early iii the infection process and nodulation is inhibited when plants are expofedtp acidity during the period of root- hair extension and curling (Munns, 1968; Lie, 1969)

Different soil properties such as texture, permeability, water retention capacity, pH and organic matter context are known to influence the survival of rhizobia in soil during

storage. Juwarkar *et al.,* (1986) made a study on survivability of *Rhizobium* in stored soils of India. From their findings on nodulation, it appeared that black gram rhizobia were adversely influenced by high pH, low organic carbon and high EC. However, these factors had no influence on the cowpea rhizobia. Red gram rhizobia were exclusively confined to black soils and preferred slightly higher pH. The EC and organic carbon content of the soils did not have much influence on them.

Legumes are major sources of protein and energy for both humans and domestic animals, and the *legume-Rhizobium* symbiosis is now the most widely managed agricultural system for biological nitrogen fixation. For successful nodulation, soil pH is one of the important factors. Lowendorf *et al.,* (1981) studied survival of *Rhizobium* in acid soils. They stated that a *Rhizobium* strain nodulating cowpcas did not decline in abundance after it was added to sterile soils at pH 6.9 and 4.4, and the numbers fell slowly in non-sterile soils pH 5.5 and 4.1. A strain of *R. phased* grew when added to sterile soils of pH 4.3 and 4.4. Two *R. meliloti* strains which differed in acid tolerance for growth in culture did not differ in numbers or decline when added to sterile soils at pH 4.8, 5,2 and 6.3. However, the less sensitive strains were found to survive in better condition in limed soil.

3.5 Impact of salinity on bacterial growth

Salinity is one of the major environmental factors deleterious to plant growth and yield (Allakhverdiev *et al*., 2000). Khewra Salt Range is situated in the foothills of theSalt Range. It is located between longitudes 073 00, 26.9 E and latitudes 32 39, 03.4 N. The vegetation of region is exposed to severe habitat losses due to high EC and pH. Increasing salt concentration may have detrimental effects on rhizobial population (Singleton *et al.,* 1982). Salt tolerant rhizobia may have the potential to improve yield of legumes under salinity stress (El-Mokadem, 1991). Rhizobial inoculation increases nodule biomass thus encourages sustainable environmental friendly agriculture by responding perfectly in biological nitrogen fixation (Adewusi *et al*., 2008). Various researches demonstrated the ability of *Rhizobium* to colonize roots of non-legumes(Matiru & Dakora, 2004) and act as phytohormone producer, phosphate solubilizer and tosome extent as nitrogen fixer (Afzal & Bano, 2008). Various phenotypic and genotypic methodologies are being used to identify and characterize

bacteria. Although phenotypic methods play a significant role in identification but the molecular methods are more reliable and authenticated for identification and to study genetic diversity of bacterial isolates. Major molecular techniques include PCR (Polymerase chain reaction), RAPD (randomly amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), SSR (single sequence repeats) and 16S-rRNA gene sequencing. RAPD is the most reliable, rapid and practical method (Mehmood *et al*., 2008) used for phylogenetic relationships among and within closely related species. This paper reports the isolation of *Rhizobium* isolates from Khewra salt range and Attock and their characterization on the basis of morphological, biochemical and molecular characters.

Nitrogen is one of the most essential nutrients required for growth and development of plants. The nitrogen can be provided for plants in the form of chemical fertilizers and biological fixation. Among these two, biological nitrogen fixation is the less costly and ecofriendly. According to Franche *et al.,* (2009) leguminous plants fix atmospheric nitrogen into soil with the help of nitrogen fixing micro-organisms. *Rhizobium* acts as primary symbiotic fixer of nitrogen by infecting root of leguminous plants. Kiers *et al.,* (2003) state that, *Rhizobium* bacteria stimulates the growth of leguminous plants by fixing atmospheric nitrogen into soil by symbiotically interaction. Several environmental stresses may affectthe nitrogen fixation in plants. It includes salinity, water stress, soil pH, temperature and heavy metals (Kucuk and Kivanc 2008). Waraporn Payakapong (2006) reported that, nearly 40% of total world land is affect due to salinity. Most of the leguminous plants are more sensitive to salinity and they require slightly acidic soil for N2 fixation. According to Zahran, (1999) salt stress directly affects symbiosis than free living *Rhizobia*. Thus, the isolated salt tolerance *Rhizobia* would be the highly important inoculums to improve the growth and development of the leguminous plants under saline environment.

Salt tolerance indicates at the ability of a bacterium to survive under high salt concentration. Such organism, therefore, considered as halotolerant. However, there is another kind of organism which requires salt for metabolism. This salt loving bacterium is known as halophilic. The presence of salt in the culture medium affects the growth of bacteria significantly. A considerable amount of variation is noticed with the rise in the salt level. Presence of salt often inhibits the growth of many bacterial organisms and

therefore, salt has been used as a preservative. Truly the salt tolerant character of bacteria is a potential finding in today's modern biotechnology. An important diversity of their utilization is often reported due to this noble ability. (Margesin and Schinner, 2001). *Rhizobium* is more tolerant toward salts than its host legume and therefore, survives in saline soils (SubbaRao *et al.*, 1972, 1974).

Rhizobium strains from lucerne, moth bean, black gram, green gram and pea were tested by Yadav and Vyas (1971) for their sensitivity to saline-alkaline conditions and pH. Lucerne and pea rhizobia were both salt sensitive (0.2%) and salt resistant (6%). Black gram and moth bean strains were proportionately sensitive to CT and salts of Na^+ and K^+ but those of green gram were stable. Magnesium salts were stimulatory at concentration lower than 1%. For all rhizobia 0.4-0.6% $NaHCO_3$concentration was critical. All the strains survived at pH 10.1, but were inhibited at pH 3.5.

The effect of salt stress on growth nodulation and nitrogen accumulation in cowpea *(Vigna sinensis)* and mung bean *(Vigna aureus)* was studied by Balasubramaniam and Sinha (1976) in sand culture. Salinity (NaCl) retarded the growth of leaves, stem and roots of both the crops. The relative growth rates of stressed plant parts declined initially but were subsequently higher than those of control for a period, suggesting that the plants tended to adapt to unfavourable environment even while being stressed. The total nodule number, weight and nitrogen content of plant decreased due to salt treatment which interfered with the initiation of nodules but not with their further development. There was a considerable fall in the nitrogen fixation efficiency of mung bean under saline environment but it was not so in cow pea.

Salinity tolerant mutant strains of *Rhizobium,* able to grow and fix nitrogen in symbiosis with lentil in saline soil, were derived from an effective *Rhizobium* strain RL5 by treatment with nitrosoguanidine. Among the five mutants selected, two mutants viz. LM4 and LM1 were resistant to 200 µg /ml of streptomycin and 1.5% of NaCl. These two mutants also significantly increased number and dry wt. of nodules per plant, dry matter yield of the crop and N_2 fixation. Between the two mutants, LM4 was superior to LM1 in symbiotic behaviour (Rai and Prasad, 1983). Fast growing acid producing *Rhizobium* strain 995 of *Vigna radiate* was screened for growth behaviour in acid, saline and alkaline media. It grew well in yeast extract mannitol broth of wide pH range as well as

varying concentrations of NaCI, Na_2S0_4 and $MnCl_2$. Variations in nodulation and nitrogen fixation efficiency occurred on different varieties of green gram, while strain characteristics, were not affected by soil stress (Rai and Prasad, 1984).

Three strains of *Rhizobium* able to fix N_2 in symbiosis with lentils in saline soil were screened. Nodulation pattern, N_2 fixation and grain yield were influenced by *Rhizobium* strain and lentil genotype. Genotypes DL-443 and Pant L-406 were found to be more salt tolerant than others and gave the highest grain yield (Rai *et al.,* 1985). Rhizobia are sensitive to higher salt concentration (Wilson, 1994). They show the capability to grow at 200mM salt concentration, but the growth is more abundant at lower salt concentration (Rafiq, 1997). Only few strain of *R. meliloti* can grow at 2% NaCl concentration, but none can grow at 3% NaCl concentration (Yadav and Vyas, 1973; Graham and Parker, 1964)

3.6 Research activities

In the present research, we tried to fulfill the following goals.

i. Observing growth performance under different pH and optimization of pH for the maximum growth of the five isolated samples.
ii. Observing growth performance under different incubation temperatures and optimization of suitable temperature for the maximum growth of the five isolated samples.
iii. Conferring the salt tolerant ability and observing growth performance under different salt concentrations.

3.7 Materials and Methods

3.7.1 Materials

The culture was tested for the environmental parameter. Salt tolerance test to check the growth of Rhizobium on 1%, 2%, 3% and 4% salt containing nutrient broth. pH tolerance test was carried out in nutrient broth by adjusting the pH range from 5 to 9. Growth at range of temperatures that were also investigated was at 20ºC, 28ºC and 37ºC .To conduct this part of research following materials and equipments were used.

Bacteria

Single bacterial culture isolated from Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean.

Chemicals

Mannitol, yeast extract, sodium chloride, K2HPO4 , 70% alcohol, methylated spirit.All the chemicals, used in the research work, were purchased from Fluka AG (Buchs, Switzerland); BDH Ltd. (poole, England) and from Merck (Darmstadt, Germany) and were of analytical grade. Components of bacteriological growth media were obtained from Biolab (Hungary), UNIPATH Ltd. (England), DIFCO Laboratories, USA and BDH chemical Ltd. England**.**

Glass Wares

Test tubes, conical flasks (250 ml), beakers (50 ml, 100 ml, 200 ml, 500 ml), measuring cylinder (25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1000 ml).

Electronic Instruments

Spectrophotometer, magnetic stirrer, analytical loading single pan balance with precision of 0.001 g, distilled water plantation, digital pH meter, autoclave, laminar air flow cabinet, incubator rotator.

Other Materials

Nichrome loop, spirit lamp, parafilm, aluminium foil, cotton, water proof marker pen, paper towel, tissue paper, filter paper, brown paper, test tube racks, spatula, trolley and waste basket.

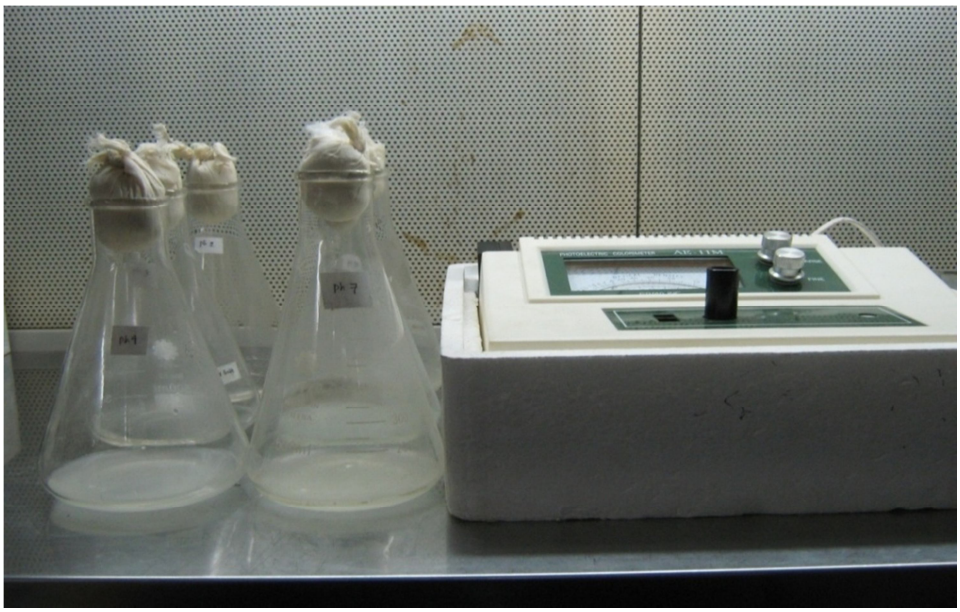


Plate 3.1: Spectrophotometer.

3.8 METHODS

The part deals with all the methods that were applied during the characterization of the isolated bacteria.

Determination of Bacterial Growth Curve

Bacterial growth curve is a part of the characterization which is determined by the means of optical density (OD). Optical density is directly proportional to the number of bacterial cells in the liquid culture. OD was measured by a spectrophotometer. However, bacterial growth can be affected by the pH of the medium and by the temperature of the incubator. That's why we observed the diversity of bacterial growth by applying different pH and temperatures. We also made an assessment on the salt tolerance ability of the isolated samples.

Physico-Chemical Parameters

Physico-chemical parameters like temperature, pH and salt tolerance were analyzed.

Bacterial Growth in Different Temperatures

Again liquid medium was prepared according to Yeast Extract Mannitol Broth medium but this time one culture from each bacterial sample was prepared. This culture was

incubated at a fixed temperature of 28°C and data were collected exactly in the same manner as YEM Broth medium. Now, the experiment was repeated at the temperature of 20°C, 28°C, and 37°C. Thus the performance of the bacterium was observed at different temperatures. Growth curve was derived according to

Effects of Temperature on bacterial growth

The temperature is an important factor to which bacteria show a wide pattern on growth behavior. The procedure for study the effect of temperature on bacterial growth is as follows:

1. 3ml of the medium was taken in different test tubes
2. 100µl of the overnight culture (Luria Bertani, LB) was dispensed into the conical flask.
3. The test tubes were incubated at 3 different temperatures i.e. 20°C, 28°C, 37°C for 24 hours respectively.
4. OD was taken at 660nm using Spectrophotometer (ELICO BL 200 B10 Spectrophotometer).

The procedure for study the effects of pH on bacterial growth is as follows

Liquid medium was prepared according to Yeast Extract Mannitol Broth medium However, 3 different media were prepared in 3 different conical flasks having pH of 5, 7 and 9. 0.1 M sodium hydroxide and 0.1 M hydrochloric acid were used to adjust the pH. Inoculation was done in every medium from the same culture according to Yeast Extract Mannitol Broth medium. Thus we got 6 different cultures of the same bacterium having 3 different pH. Now, instead of incubating them overnight at 28°C, we took the reading of OD at 660 nm wavelength with a spectrophotometer. Thus, in first 12 hours, we got 3 readings. Next data were taken at 24 hours, 36 hours, 48 hours and 72 hours. A growth curve can be drawn by putting the time of observation in X- axis and OD in Y- axis.

Estimation of pH tolerance of bacterial isolates

The pH tolerance test was conducted to study the cardinal pH of the arsenic resistant bacteria. To analyzed the effect of pH variation on the growth of microorganism, media

were prepared with pH 4.0 and 9.0. After incubation of 48 hours growth was determined by measuring the optical density at 660 nm. The procedure for estimation of pH tolerance is as follows:

1. 100 ml of the nutrient medium was taken in different 250 ml conical flask and the pH was adjusted from 5-9 respectively with the help of 1N HCl, 1N NaOH.
2. 100µl of the overnight culture (nutrient broth) was dispensed into the conical flask and incubated at 28°C for 5 days.
3. OD was taken at 660nm using Spectrophotometer (ELICO BL 200 B 10).

The procedure for study the effects of Salinity on bacterial growth is as follows:

Variation in growth of bacteria can be possessed by the concentration of salt of the medium. Sodium chloride has an impact on growth of many bacterial species. There are many bacteria which cannot tolerate higher concentration of sodium chloride; however a significant number of aquatic bacteria are tolerant to higher concentration of the salt. To determine salt tolerance and growth under variable salt concentration, A bacterial suspension was prepared from the pure isolates of *Rhizobium* and further inoculated into freshly prepared YEM broth supplemented with different concentration of NaCl (1-4%) for salt tolerance study. we prepared five 100 ml liquid media according to Yeast Extract Mannitol Broth medium without adding sodium chloride. Now we added 1g, 2g, 3g and 4g sodium chloride to those media to get4 liquid media with 1%, 2%, 3%, and 4% and sodium chloride concentration respectively. We inoculated those four media with the same bacteria sample. They were incubated at 28°C. OD was recorded and growth curve was formulated according to Yeast Extract Mannitol Broth medium (Mansah *et al.,* 2006).The process was repeated for the other 07 samples.

1. 5ml of the fresh yeast extract mannitol broth medium was taken in different spectrophotometric test tubes and the salt concentration was adjusted by adding 1%, 2% , 3% and 4%.(wt/v) Nacl.
2. 100 µl of the 24 hours culture (YEM Broth) was dispensed in to the spectrophotometric test tubes and incubated at 28^0 C for four days.
3. OD was taken at 660nm using Spectrophotometer (ELICO BL 200 B10 Spectrophotometer).

3.9 RESULTS

The prokaryotes exist in nature under an enormous range of physical conditions such as $o_2$concentration, Hydrogen ion concentration (pH) an temperature. The exclusion limits of life on the planet, with regard to environmental parameters, are always set by some microorganisms, most often a prokaryotes, and frequently and archaeon. Applied to all microorganisms is a vocabulary terms used to describe their growth within a range of physical conditions. A thermophile grows at high temperatures, anacidophile grows at low pH, an osmophile grows at solute concentration, and so on. Optimization of bacterial growth is one of the major constraints in microbiological laboratories. It takes a series of work and difficulties hampered in many ways when there is possibility and chance of contamination. On the basis of physiological characteristics of the isolates eight were chosen (Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean) for optimization at different temperature, pH and salinity. As the isolates were collected from the temperate region they showed their optimum growth in the range of temperature 28-30^0 C, pH 6.8- 7.5 and salinity 1% (figure.) All the eight isolates comfortable to grow at pH 7, 1% salinity and temperature at 30^0 C. The results obtained through characterization have been elaborated under the following heads.

3.9.1 Effect of Temperature, pH and Salinity on Bacterial Growth

The bacterial growth depends on temperature and pH. The optimum pH for the growth of the isolates was 7.0 and bacteria also grow in other pH value range to 5.0 – 9.0 (Table 3.2 and Figure 3.1).The optimum temperature 28°C for the growth of bacteria and growth rate was moderately low in other temperature value like 20°C and 37°C (Table 3.3 and Figure 3.2

The isolates were able to grow well at 1 % NaCl concentration (table no graph noIsolated bacteria were grown in different media having different temperature, different pH, and different salt lebel to asses it's effect on growth. This work also help to optimize the suitable temperature, pH and salt for each of the bacterium. The results have been described below.

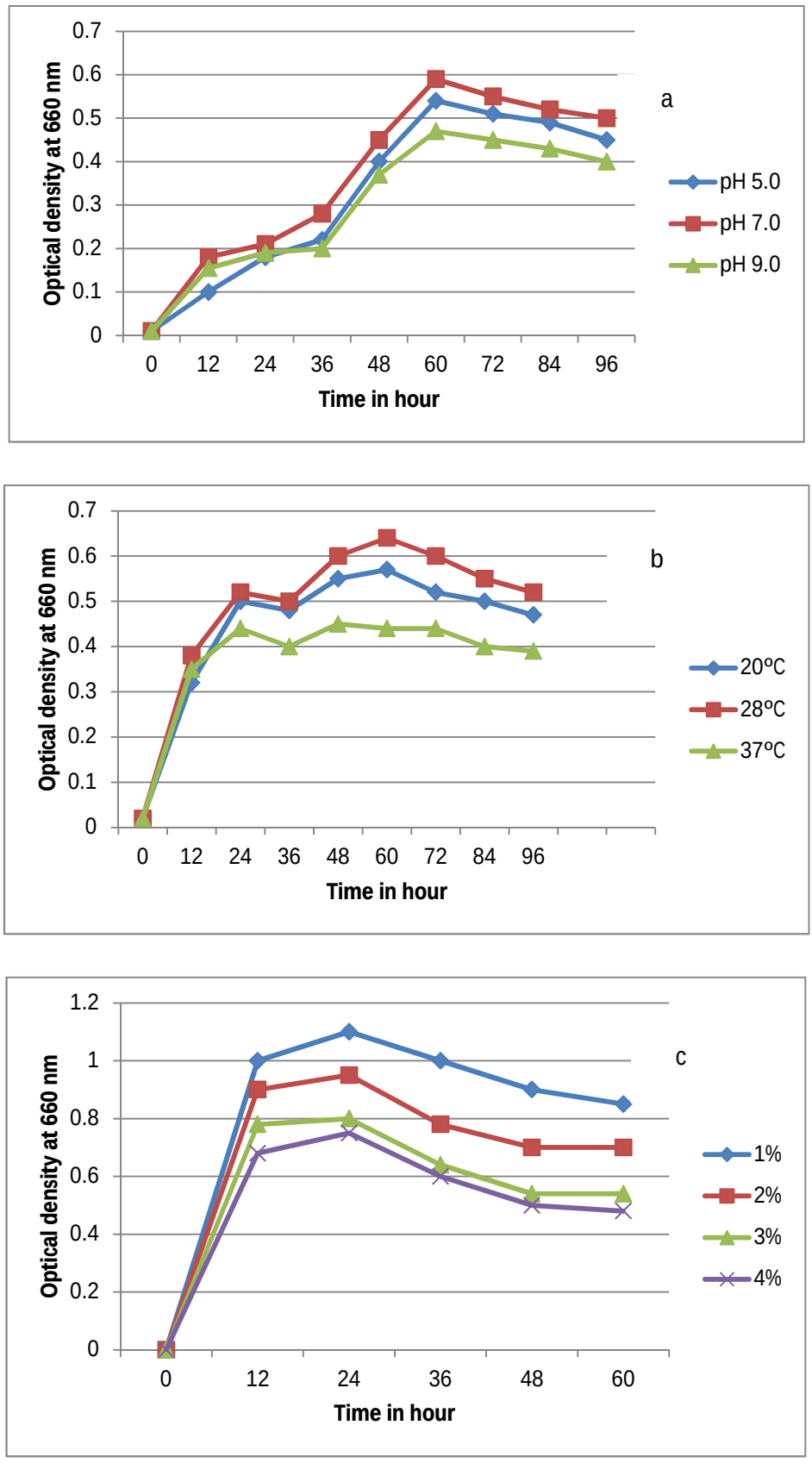
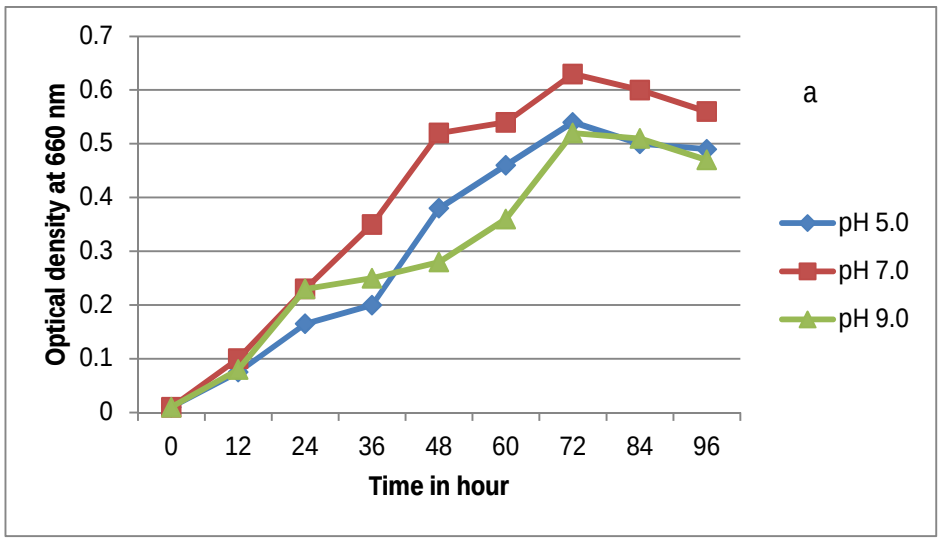
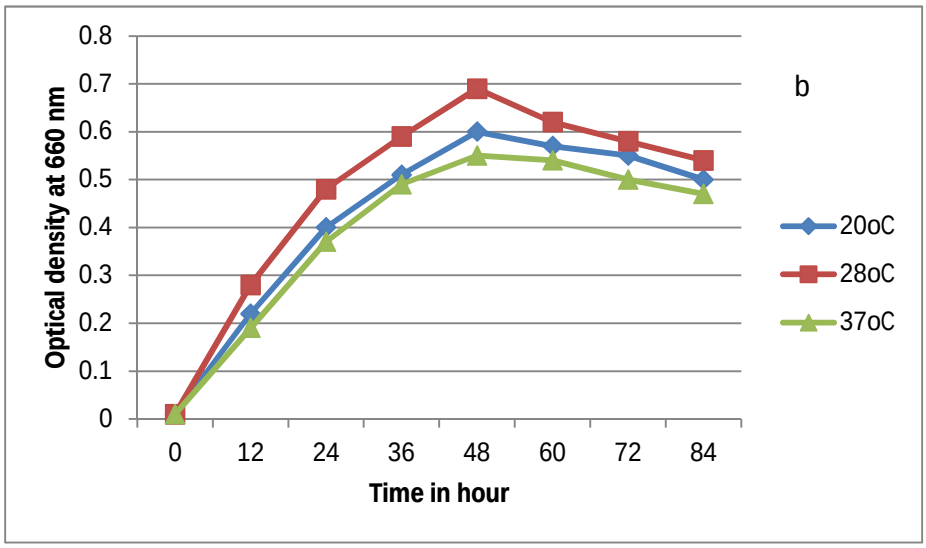


Figure 3.1: Growth characteristics of the isolates Green Gram at different a) temperature, b) pH, and c) salinity**.**





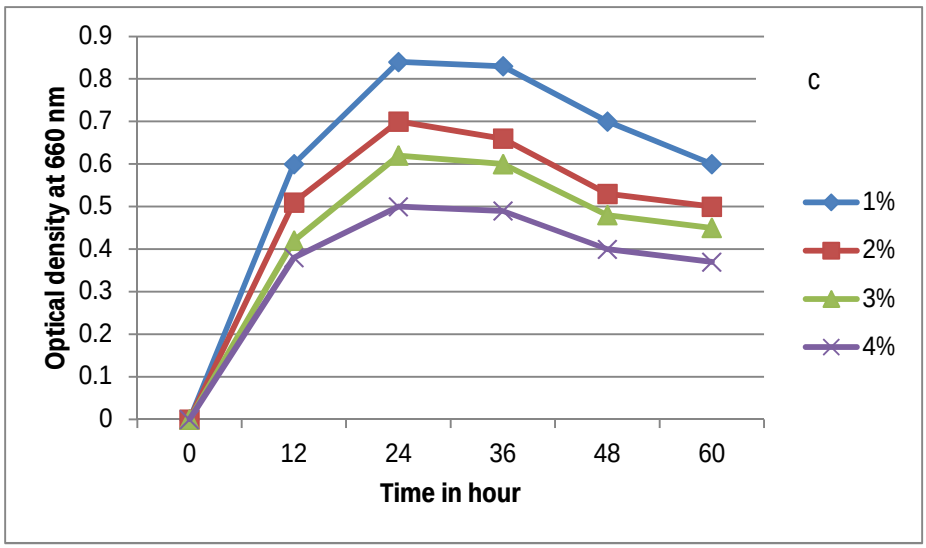


Figure 3.2: Growth characteristics of the isolates Black Gram at different a) temperature, b) pH, and c) salinity.

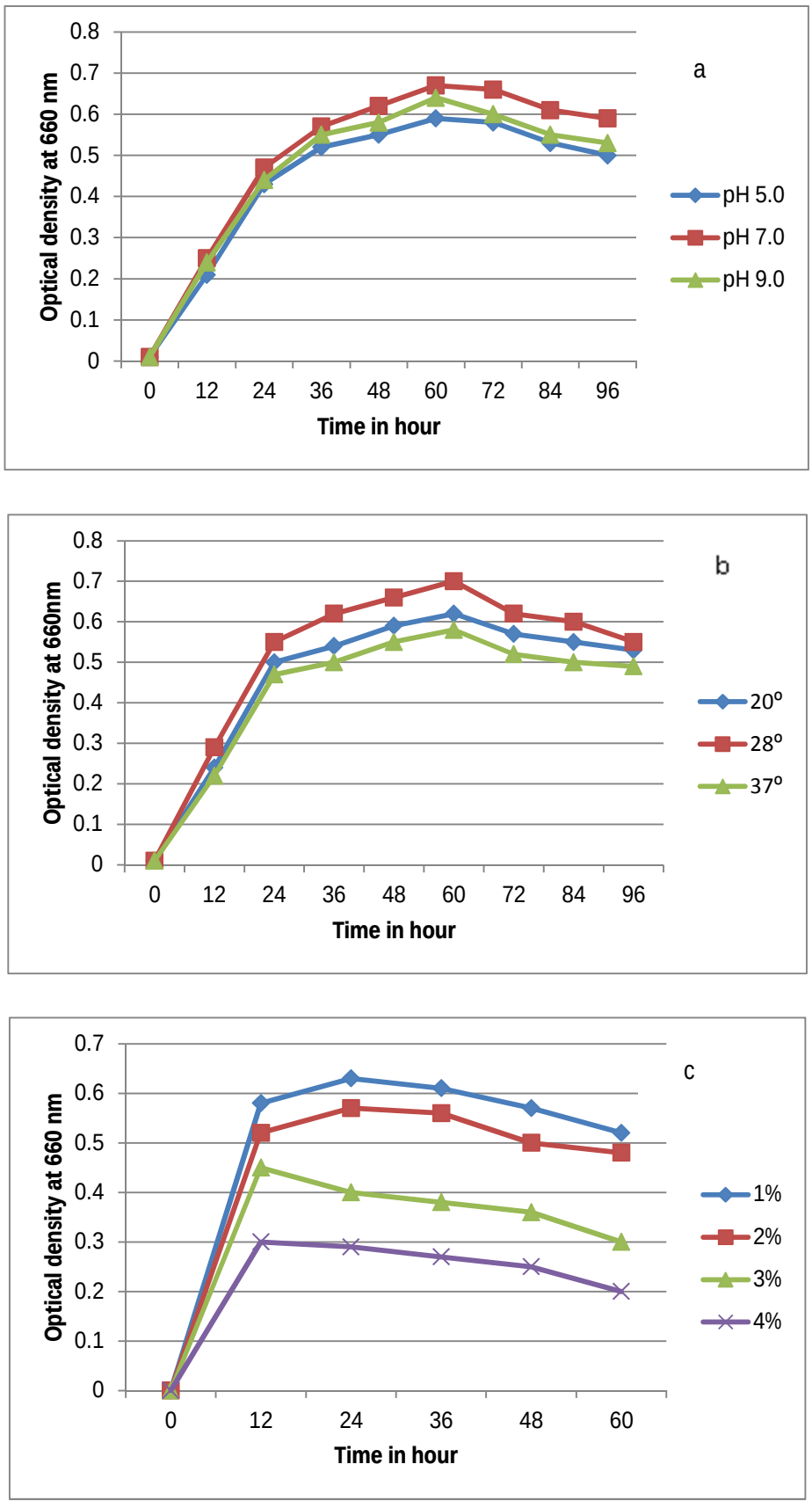


Figure 3.3: Growth characteristics of the isolates Lentil at different a) temperature, b) pH, and c) salinity.

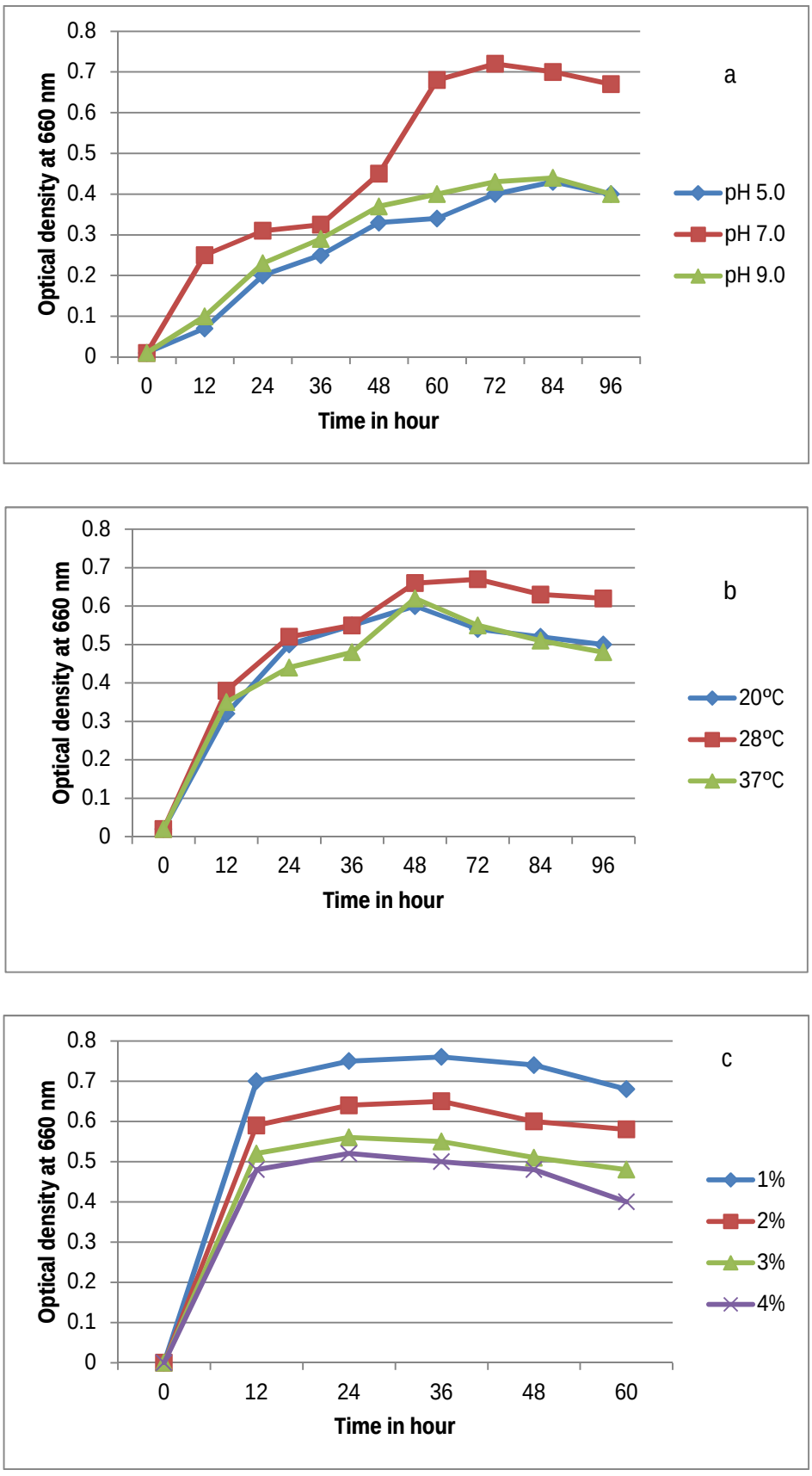


Figure.3.4: Growth characteristics of the isolates Pigeon Pea at different a) temperature, b) pH, and c) salinity.

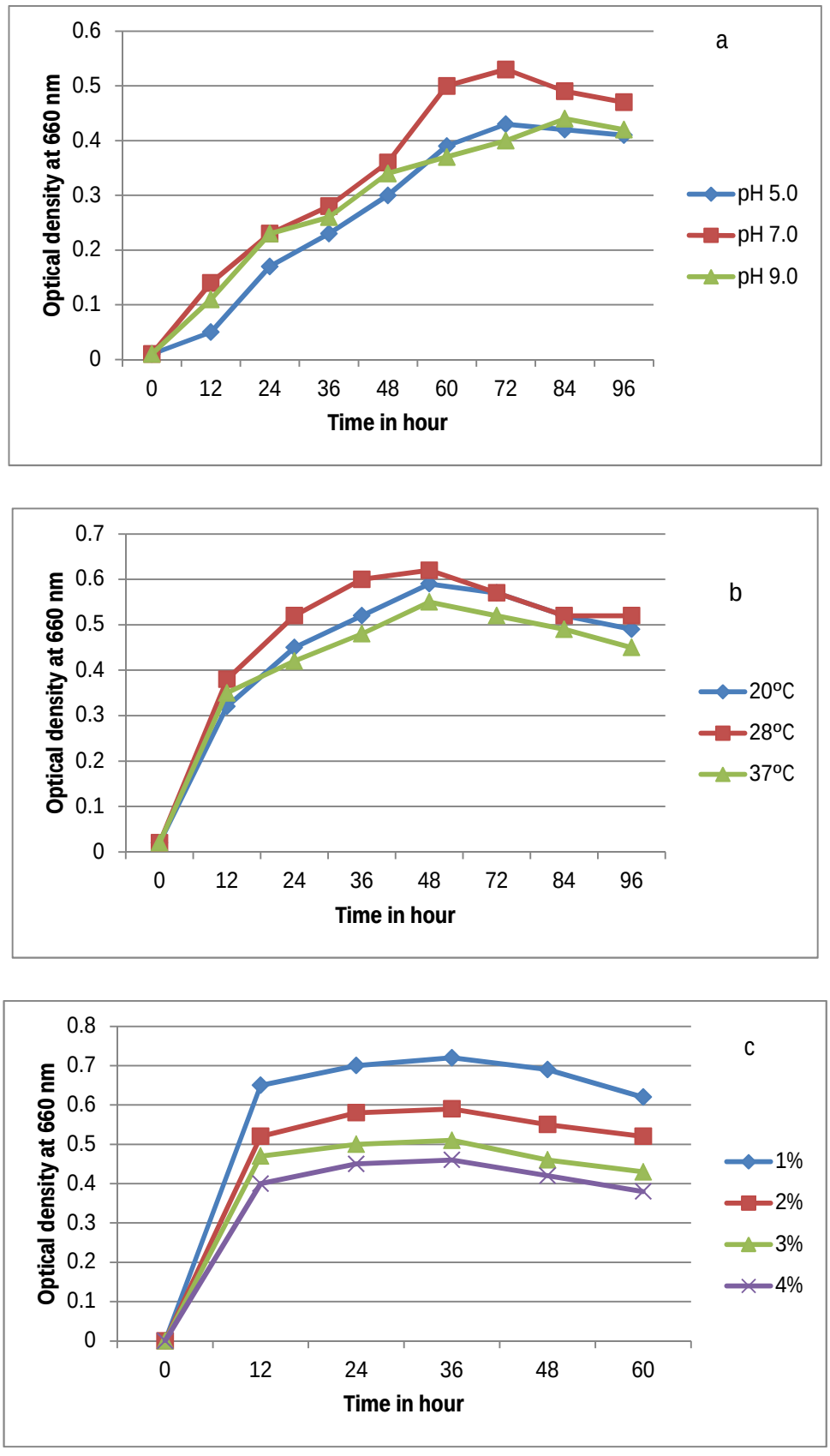


Figure.3.5: Growth characteristics of the isolates Sweet Pea at different a) temperature, b) pH, and c) salinity.

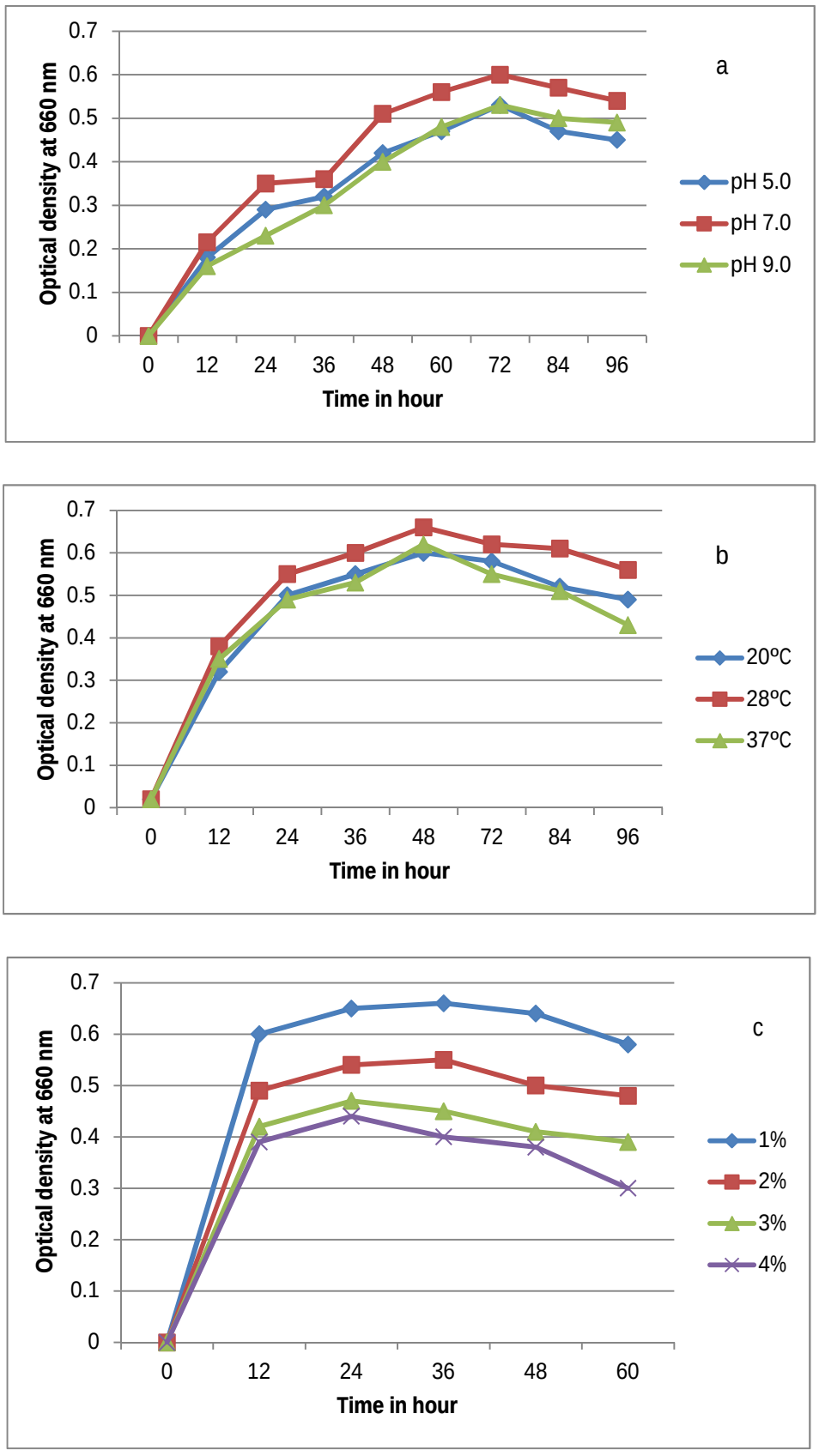


Figure.3.6: Growth characteristics of the isolates Chick Pea at different a) temperature, b) pH, and c) salinity.

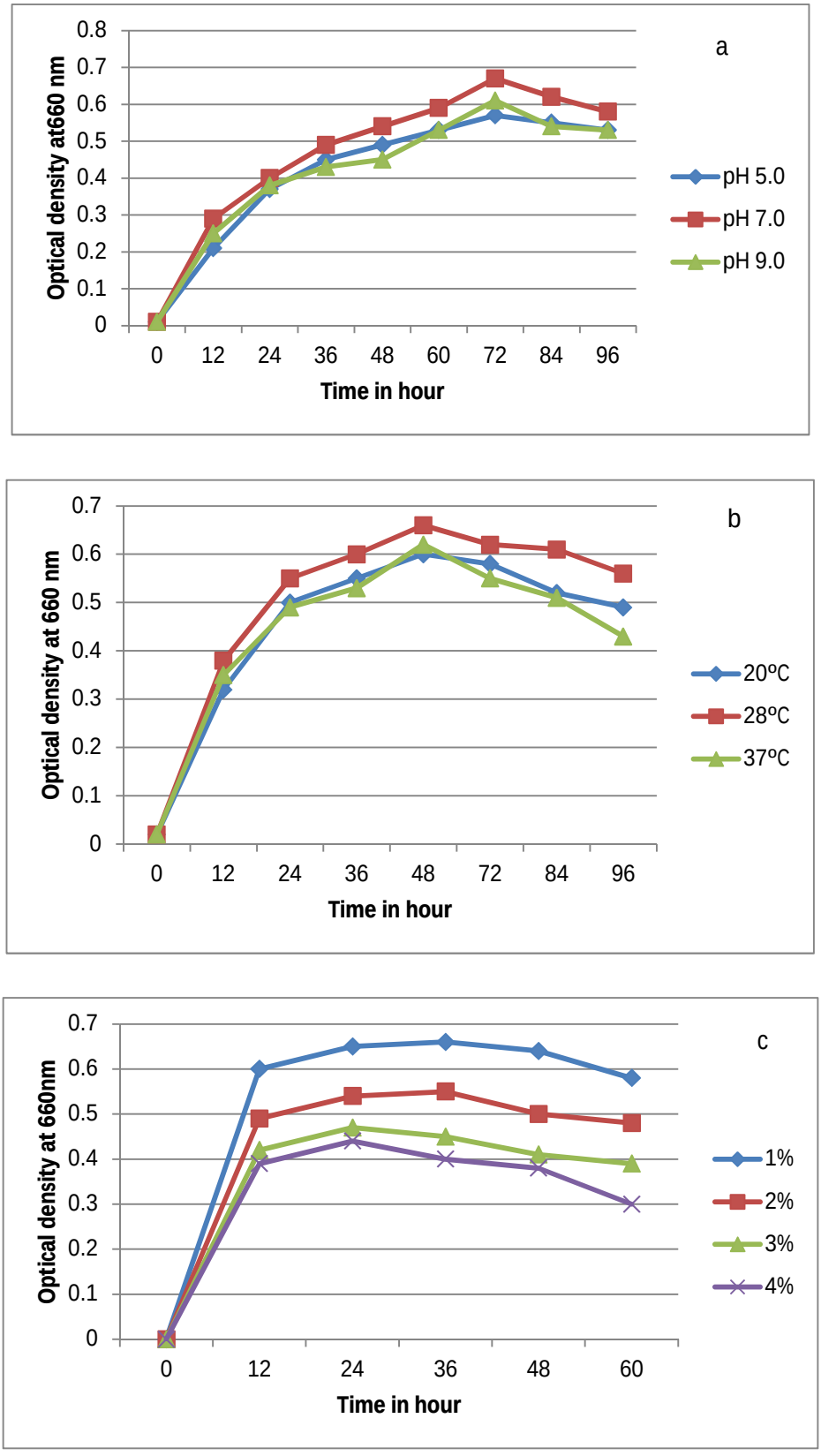


Figure 3.7: Growth characteristics of the isolate Cow Pea at different a) temperature, b) pH, and c) salinity.

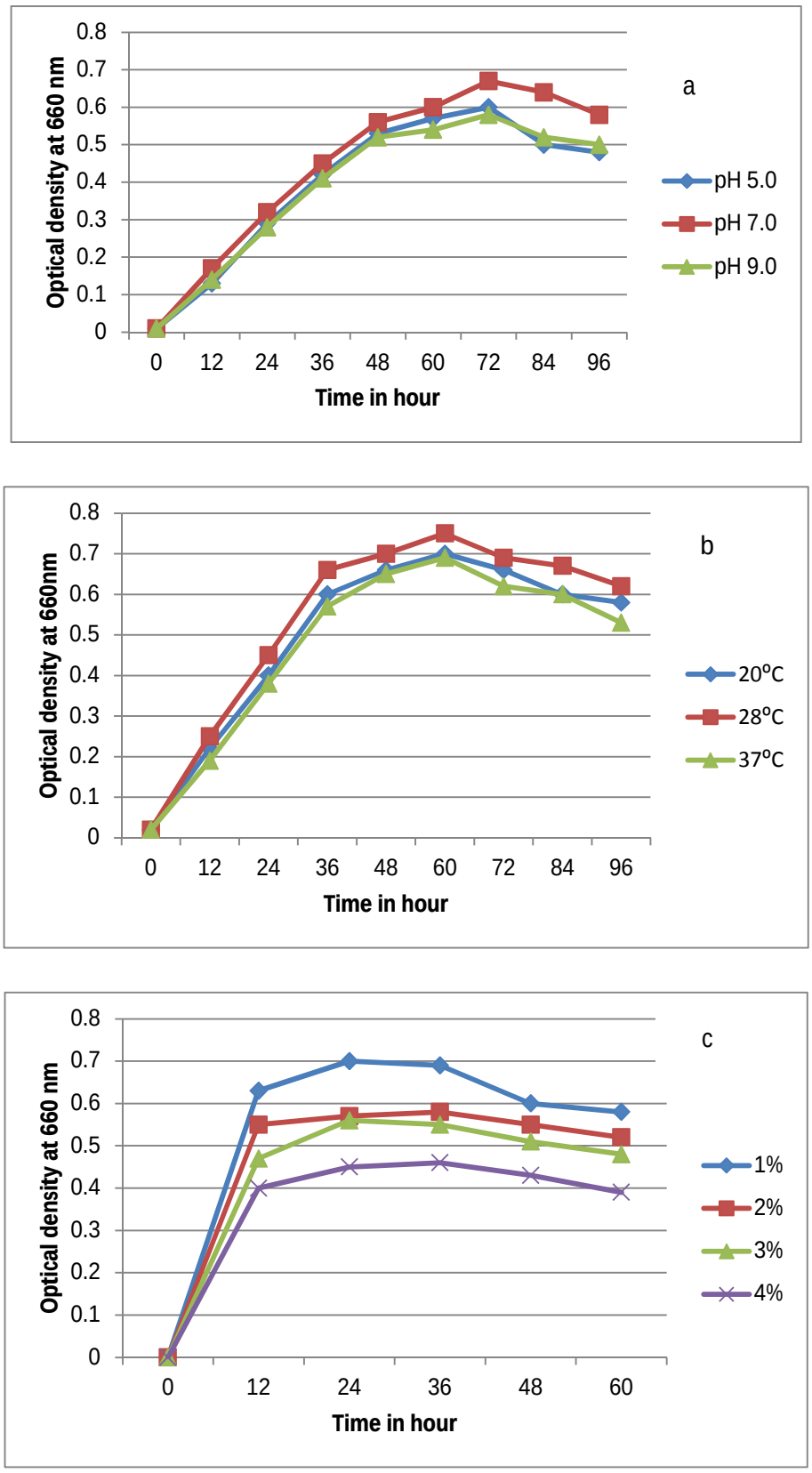


Figure 3.8: Growth characteristics of the isolates Bean at different a) temperature, b) pH, and c) salinity.

Table 3.1: Optimum Condition Based on pH, Temperature and salt of the isolated *Rhizobium* sp.

Samples	Optimum Condition		
	pH	**Temperature(c)**	**Salt Level**
Rhizobium sp. CCNWYC119 Green Gram	7	28^0	1%
Rhizobium sp. CCNWYC119 Black Gram	7	28^0	1%
Rhizobium sp. SOY12 16S Lentil	7	28^0	1%
Rhizobium sp. CCNWYC119 Pigeon pea	7	28^0	1%
Rhizobium sp. CCNWYC119 Sweet Pea	7	28^0	1%
Rhizobium sp. CCNWYC119 Chick Pea	7	28^0	1%
Rhizobium sp. SOY7 Cow Pea	7	28^0	1%
Rhizobium sp. SOY12 Bean	7	28^0	1%

3.10 DISCUSSION

Physiological characterization was a very important part of the present research. The characterization of the isolated samples was done by physiological means. To complete the purpose we planned an experimental design to standardize the suitable most pH of the medium and temperature for the proper growth of the isolated bacteria. Experimental pH of the media was adjusted to 5.0 to 9.0 and temperatures were 20°C to 37°C. We also made a profile of their behavior in different sodium chloride concentrations, by adding 1% to 4% sodium chloride. The growth of the bacteria was estimated by the value of optical density (OD) at 660 nm. Data were recorded until it reaches the maximum and gets fixed.

An optimum growth was observed at room temperature (28ºC to 30ºC). The organisms were found to be temperature sensitive as at higher and lower temperatures, a low growth was observed that might be due to a hindrance in the metabolic activity. pH is an important parameter for the growth of the organism. Best growth of *Rhizobium* was reported at neutral pH i.e. 7 and very less growth was observed in the medium with pH 5.0 and 9.0.This may be explained with the help of the study carried out by Kucuk *et al*., in 2006 reporting that salt stress significantly reduces nitrogen fixation and nodulation in legumes. In addition, Hashem *et al.,* in 1998 had proposed that salt stress may decrease the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events, such as chemo taxis and root hair colonization, thus directly interfering with root nodule function. The experiments also showed that the cells were able to grow in 1% NaCl containing medium but unable to grow on higher concentration, showing that the isolate was sensitive to the salt concentration.

In this investigation we recorded the most suitable Temperature for the growth of all the isolated bacterial samples which was Optimization of incubation temperature, therefore, was a characteristic feature decided by us in this study. Hence, all the samples of ours exhibited their best performance at 20°C and 28°C. We also found exceptions and similarities in this case while retrieving literatures. For example, Kucuk *et al*., (2006); Baoling *et al.,* (2007) found 28°C as the best incubation

temperature while growing their eight isolated *Rhizobium* samples. However there were enough literatures which exhibited similarities with our reports. Furthermore, we observed a lack in growth of every sample when theincubation temperature was decreased. Without any exception, every single sample exhibited weakest growth at 37°C. Its' worst performance curve at 37°C very close to the best one at 28°C. Again some clear breaks were observed in the growth curve in case of all eight isolated *Rhizobium* spp. showed as the curves of 20°C, 28°C and 37°C very close to each other. However, the adequate temperature was recorded 28°C which far away from them. After obtaining the effect of pH and temperature, we observed that *Rhizobium* starts growth at a delayed phase. Temperature conditions have a great effect on rhizobial growth and symbiotic performance (Zahran, 1999). As shown in Graph 3.1-3.8, maximum growth of all tested strains were obtained at 28 °C. Below and above those values, the isolates that grew decreased. None of the isolates showed growth above 38°C. According to Jordan, (1984), the maximum temperature reported for *R. leguminosarum* bv *trifolii* is 30°C, However, temperature range is highly strain dependent for genus *Rhizobium* (Jordan, 1984). Nevertheless, survival under higher temperatures does not mean efficiency in nitrogen fixation. Rhizobial strains obtained from hot and dry environments that grew up to 45°C lost their ineffectiveness. Screening of *R. leguminosarum* bv *phaseoli* showed that some strains were able to nodulated *Phaseolusvulgaris* at high temperature (35°C and 38°C) but nodules formed at higher temperatures were ineffective and plants did not accumulate nitrogen in shoots (Zahran, 1999). Although critical temperatures for efficiency in nitrogen fixation for clovers have been reported to be around 30°C, this winter annual clover is exposed to temperatures in the range of 10°C up to 36°C. Despite the fact that high soil temperatures usually results in the formation of ineffective nodules, several strains of rhizobia have been reported to be heat tolerant and to form effective symbiosis with their host legumes. The selection of heat resistant isolates may be relevant for cultivation of ball clover, which is sown during late fall when temperatures can reach 36°C. Likewise, Harun *et al.,* (2009) observed that all lentil nodulating rhizobia strains from Bangladesh grew well at 33°C and one strain could grow at 38°C. Increased temperature optima of these isolates may be beneficial for its application in temperature stressed conditions as symbiotic performance of different rhizobial strains under temperature stress has been correlated with their ability to

grow in pure culture at elevated temperature (Hungaria, 2000). Though correlation between climatic region of origin area of each isolates and tolerance to low or high temperature was not carried out their temperature tolerance might be related to their origin soil temperature as tropical soil could have wide range of temperature. So we could conclude that the isolated strain of *Rhizobium* were tolerant to high temperature pH is another important physiological parameter for bacterial growth. The most suitable pH for the growth of all the isolated bacterial samples which was 7.0 Graham, (1982); Gao *et al.,* (1994).They found that pH 7.0 was most suitable for the bacterium. Perhaps, besides formulating the best pH for the culture media, our research led us towards some noteworthy issues. We observed that every sample showed their worst performance at pH 9.0; It showed the worst performance at pH 9.0; however the curve was not too far from the best one. Eventually, all the samples get to the stationary phase which takes 36 hours to reach the stationary phase. Considering the pattern of the growth curve of the maximum performance, we can easily see *Rhizobium* showed the usual nature, where growth is visualized in a smooth way after the lag phase.

pH is an important parameter for the growth of the organism. Slight variations in pH of medium might have enormous effect on the on the growth of organism. Superior growth of *Rhizobium* has been reported at neutral pH. Results showed that cells were able to grow only at pH 7.0 at 30ºC temperature. No growth was observed in medium with pH 4.0 and 9.0. Similar observation were made by Deora *et al.*, 2010, Baoling *et al.*, 2007.

As shown in Figure (3.1-3.8), The Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean nodulating rhizobia tested showed a wide diversity in their different pH tolerance. All tested isolates grew in mildly acid pH (5.0) to neutral pH and slightly alkaline pH (9.0) but the most suitable pH for the growth of all the isolated bacterial samples which was 7.0.This might be related to less saline nature of the soil from which most isolates were recovered. Fast-growing rhizobia strains which could tolerate 2% NaCl were capable of growing at pH 9.5 (Sadowsky et al., 1983). Similarly, an apparent positive correlation was observed between salt tolerance of the isolates and their alkaline pH tolerance(R=0.78). Harun *et al.,* (2009) also found that there is great variation among lentil nodulating rhizobia with respect to growth and

survival in acidic and alkaline conditions. They showed that they can grow well at acidic pH as low as pH 4 and alkaline pH as high as pH 10. Generally, large variation was observed among fast-growing rhizobial isolates with regard to growth in relation to pH of the medium (Graham and Parker, 1964). There might be a relation between pH of origin of isolates and their acid and alkaline pH tolerance. However, in this study such kind correlation was not statistically significant. Moreover, metal (Al and Mn) toxicity tolerance of isolates was tested at pH5.0 and all tested isolates were found to be sensitive to very low concentration. About 23% of them tolerated both high and low concentrations of Mn at pH 5.0. Since most Ethiopian soils are acidic like any other tropical soil where associated metal toxicity expected to prevail such isolates are very important to use as inoculants.

Results also showed that most of the isolates are acid adapted, capable of surviving at pH values lower than the pH range between 4.5 and 9.5, as reported for the genus *Rhizobium* by Jordan (1984). The fact that different strains of the same species may vary widely in their pH tolerance has been demonstrated previously (Glenn and Dilworth, 1994; Correa and Barneix, 1997). Some Rhizobial isolates can be shown more sensitive to low pH than their host and this affects the establishment of the symbiosis, limiting the survival and persistence of the rhizobia (Zahran, 1999). The performance of some clover-*Rhizobium* symbiosis under acidic conditions is best when the Rhizobial strains were isolated from acidic soils (Zahran, 1999). Therefore, selection of acid-tolerant rhizobia to inoculate legume hosts under acidic conditions may help the establishment of the symbiosis and also may improve the acid tolerance of legume. In our study50% of the isolates grow at pH 4.0 which is lower than the pH tolerated by clover rhizobia. Such low tolerance has been previously reported for some strains of rhizobia that nodulates arrow leaf clover, which can survive and even increase in numbers at pH 4.2 (Weaver *et al*., 1985). The ability of isolates to utilize a broad range of carbon substrates is also related to the survival of these isolates under acidic environments. Rhizobia are capable of metabolizing different carbon sources so that the products ameliorate the environmental pH (Glenn and Dilwarth, 1994). Under acidic conditions the catabolism of organic acids and amino acids leads to alkalinisation (Ibekew *et al*., 1997) and this buffering action may help in the establishment of the legume in acidic soils. This feature can make these strains more competent in acidic soils, thus

it is important to couple the results of our investigation with the selection of breeds or varieties of acid tolerant barseem clover, in order to establish and maintain symbiosis in soil with low pH.

We also produced results of the growth of the bacteria at different salt (sodium chloride) concentrations. We observed that every single isolate showed their best performance at 1 % salt level. Their performance was negatively proportional to the salt concentration and thus all the bacteria showed lowest performance at 4% salt level. Such salt tolerance test has been done by Soil temperature, physical and chemical composition, moisture content in soil varies within small areas and these variations alfect the populations of the soil inhabitants. Therefore, differences in response to wards salinity, pH and temperature are expected. It is known that salt stress significantly reduces nitrogen fixation and nodulation legumes. Hashem *et al.* (1998) proposed that salt stress may decrease the efficiency of the *Rhizobium* legume symbiosis by reducing plant growth and photosynthesis, and hencc nitrogen demand by decreasing survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as colonization, thus directly interfering with root nodule formation. Present study indicated that all isolates were able to grow in salt concentrations up to 4% NaCl. The salt inhibitory concentrations varied among strains. All strains in this study were able to tolerate1-4% NaCl, which is in accordance with the characteristics of fast growing *Rhizobium* reported by Holt *et al*., (1994).He and his team isolated eight bacterial strains from activated sludge and when subjected to different salt levels, they produced maximum growth at 1% salt level. However, in their case, the growth gradually decreased with a rise in the salt concentration. A very noteworthy outcome of our research is that almost every sample grows well even at 1% salt level. Several studies also reported that rhizobial isolates exhibit a large range of sensitiveness to salinity (Singleton *et al.*, 1982). It is often believed that saline soils naturally select strains more tolerant to salinity.

The data in Table 3.1 show that Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean rhizobia exhibited less diversity in their salt tolerance. The salt inhibitory concentrations varied among strains. Generally, tolerance to sodium chloride (NaCl) was observed since all isolated tested rhizobia could grow

well with 1% NaCl. However, at higher concentrations (4%) all the bacteria showed lowest performance. Colonies of *Rhizobium* sp. were obtained on YEMA medium after incubation at 28±2ºC for two days. General microscopic view of the isolates showed them to be rod cells and germ negative in nature. It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumens. Hashem *et al.*,(1998) have proposed that salt stress may decrease the efficiency of the Rhizobium legume symbiosis by reducing plant growth and photosynthesis survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function.

Some rhizobial isolates have been shown to grow under high salt conditions 4-5% (Kucuk *et al.,* 2006). Results indicated that cells were able to grow on 1% NaCl containing medium but unabled to grow on higher concentrations, showing that the isolate was sensitive to salt.

Present study indicated that all isolates were able to grow in salt concentrations up to 1% (w/v) NaCl. The salt inhibitory concentrations varied among strains. Indeed, tolerance to sodium chloride was found since all isolated of the tested rhizobia continued to grow with 1-4 % NaCl (w/v). However, at higher concentrations, the percentage of tolerant strains decreased rapidly and some *Rhizobium* sp. showed moderate growth in 5% NaCl. The ability of some strains of *R. leguminosarum bv trifolii* to grow under NaCl concentrations up to 350 mM in broth culture has been reported previously (Zahran, 1999). Kassem *et al.,* (1985) observed that strains of *R. meliloti* are able to grow in the presence of 4.5% NaCl, similarly Kucuk *et al.,* (2006) reported that some *Rhizobial* isolates grown under 4.5% NaCl. Our study, therefore reported the isolation of strains highly tolerant to high salt concentrations. Salt tolerant rhizobia may have the potential to improve yield of legumes under salinity stress (El-Mokadem *et al.,* 1991).

The presence of the strains growing under stressed laboratory conditions in our study indicates their significance in contributing biologically fixed nitrogen to stressful ecosystems. This shows the possibility of screening tolerant strains from the soil where they are naturally selected (O'Hara *et al.,* 2002). The presence of tolerant strains

becomes more interesting since the selected strains are good for nodulation and plant growth. This shows the possibility of getting effective stress tolerant strains through rigorous screening and characterization to exploit biological nitrogen fixation in low-input agricultural systems

When the isolated strain of *Rhizobium* was inoculated on YEMA plates having 1%, 2% and 3% NaCl and then incubated at 28°C for 24 hr., growth was observed on all the three concentrations on NaCl. Thus we could conclude that the isolated strains of bacteria are tolerant to high salt (NaCl) concentrations. But according to Hashem *et al.*, (1998) salt stress may decrease the efficiency of the Rhizobium-legume symbiosis by reducing plant growth and photosynthesis survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function. The isolated, pure culture of *Rhizobium* was used to prepare the biofertilizer and the most important of all, following such practices can improve their socioeconomical status which is a need of hour in remote area like Rajshahi, isolate may be beneficial to improve growth and development of leguminous plants under salinity condition.

Moreover morphological, biochemical, physiological and *in vivo* infectivity of the strains were found similar to that of *R. trifolii*. Soil temperature, physical and chemical composition, moisture content in soil varies within small areas and these variations affect the populations of the soil inhabitants. Therefore, differences in response towards salinity, pH and temperature are expected. It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumes. Hashem*et al.*, (1998) proposed that salt stress may decrease the efficiency of the *Rhizobium* legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand by decreasing survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as colonization, thus directly interfering with root nodule formation.

3.11 CONCLUSION

Different samples showed the difference in their nature as the suitable most pH for their growth varied in an outstanding way. It was found that *Rhizobium* performed most at pH 5, 7 and 9. Among them pH 7 was good for the growth of all the samples analyzed. However, all the samples showed a decrease in the growth with a rise in the pH.

Adequate temperature for the growth of bacteria also varied greatly and provided some significant results. Nevertheless, all the bacteria showed their best performance at 28°C. Moreover, all the bacteria samples showed their growth with the decrease in the culture temperature. Least growth of every bacterium sample was observed at 37°C.

Salt (sodium chloride) tolerance test exhibited a diversified result for the different samples. All the samples grew well at the medium having 1% salt concentration. 4% salt level to be the worst for all the sample. Therefore, all eight isolates may be beneficial to improve growth and development of leguminous plants under salinity condition.

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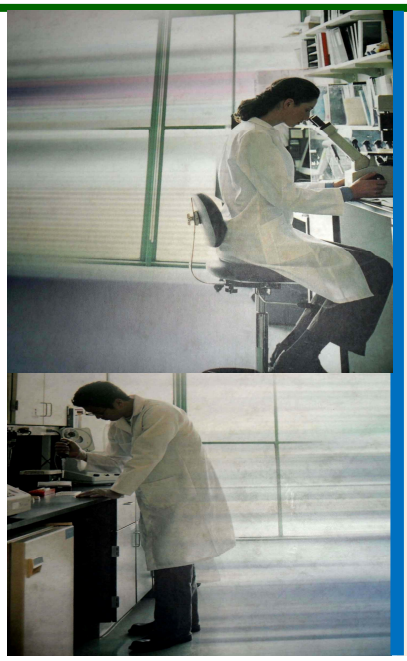
Chapter 4

16S rDNA Partial Genomic Sequence Analysis of Phylogenetic Tree of Isolated *Rhizobium* Bacteria



CONTENTS

Chapter 4

16S rDNA Partial Genomic Sequence Analysis of Phylogenetic Tree of Isolated *Rhizobium* Bacteria

4.1 INTRODUCTION AND LITERATURE REVIEW

Microbial diversity plays a vital role for maintaining the ecosystem functions which support life on earth. There are over 1.7 million strains registered and stored in World Data Centre for Microorganisms. Microbial interactions with plants together with cell signaling are known as plant microbial interaction (Hooper and Gordon, 2001). This interaction results in revealing important information and application in the field of biofertilizer, biofilming, bioinoculant and bioprocessing. In recent period, their importance in different capacities has been highlighted such as phosphate solubilization, nitrogen fixation, induced systematic resistance and plant growth improvements (Hayat *et al*., 2010; Berg, 2009; Choi *et al*., 2008; Rodriguez *et al*., 2004). Still a lot more to be discovered which may be linked to unearthing novel discoveries, identification, studying their potential role in biodegradation, reclamation of polluted soils and industrial waste managements.

In soil, the major microbial activity is restrained to organic matter decomposition in the rhizosphere (Lynch, 1990). Plant and soil type, both have influence on the microbial diversity and community structure in the rhizosphere (Liu and Sinclair, 1993). Rhizobacteria colonize and proliferate on all ecological niches of plant roots at all stages of plant growth, in the presence of a competing microflora (Antoun and Kloepper, 2001). Soil contains nitrogen-fixing bacteria to fix atmospheric nitrogen to supply the partial needs of growing plants. In the association of rhizobia and its host plant, the bacteria enter into the cortex and induced nodule formation, reproduce and eventually differentiate into bacteriods, which further produce nitrogenase enzyme complex and convert atmospheric nitrogen into ammonia in presence of low oxygen concentration created by nodule. Plants provide carbon source to bacteria in return of nitrogen fixation (Berg, 2009).

Limited studies are available on the identified bacterial natural resources of Rajshahi, Bangladesh. The present study was undertaken to explore the potential of rhizobacteria and nodulating bacteria collected and isolated from field grown crops, leguminous plants. This study is also an attempt to identify and characterize the bacterial strains by morphological, biochemical, physiological and molecular methods that play an important role in plant growth promotion.

Root associated bacteria were isolated from the Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean from the Rajshahi by using YEMA medium. Various tests were preformed for the identification of isolates, but for identification upto genus level the 16S rDNA analysis was performed. Then these organisms were evaluated for their plant growth promoting activities by *in-vitro* inoculation to legume plants. Results obtained during characterization and greenhouse studies indicated its good candidates as PGPR and also showed good response in legumes for growth promotion.Molecular phylogeny extends our knowledge regarding organism relationships and provides the foundation for the conventional identification techniques (Singh *et al.*, 2007). Soil microorganisms play important role in soil formation, fertility and so also productivity. Nitrogen is an important element to support plant growth. Plants depend on soil microorganisms for fixed nitrogen.

The aim of this study was to study diversity of *Rhizobium* spp. in agricultural lands of Rajshahi, Bangladesh. Physicochemical properties of soil was studied using standard methods while molecular methods used to study diversity within *Rhizobium* species. Further, population analysis of *Rhizobium* species in relation to genetic diversity was carried out using 16S rDNA- PCR. *Rhizobium* were identified and genetically by determining the %Guanine plus Cytosine content of the whole genome, followed by restriction enzyme treatment of Polymerase Chain Reaction (PCR) amplified product of 16S rDNA segment was performed. The sequences recognized by the restriction enzymes are distributed at variable intervals in the genome of an organism and also vary in number. The separation carried out by electrophoresis (agarose gel) resulted in specific banding pattern differing within as well as among different species. The technique used was helpful in characterizing *Rhizobium* isolates to be used as inoculants for improving agricultural land quality of Rajshahi, Bangladesh.

Members of the genus *Rhizobium* are symbiotic nitrogen fixing bacteria which are able to invade and form nodules on the roots of leguminous plants. The most dramatic progress in the construction of microbial phylogeny is based on sequencing analysis of the ribosomal genes. The 16S or small subunit ribosomal RNA gene is useful for estimating evolutionary relationships among bacteria because it is slowly evolving and the gene product is both universally essential and functionally conserved (van Berkum and Eardly, 1998). Direct sequencing of genes coding for 16S rRNA (16S rDNA) has

been used to establish genetic relationships and to characterize strains at the species or higher level (Laguerre *et al.,* 1996). The full-length sequence analysis of 16S rDNA is one of the most important methods to estimate the phylogeny of rhizobia (Young and Haukka, 1996), while the 900 bp partial 16S rDNA sequencing correlated well with full-length 16S rDNA sequencing (Terefework *et al.,* 1998) and has been used for rapid screening of the phylogenetic relationships among a large number of rhizobia.Sequences of 16S rDNA are known to be highly conserved among eubacteria (Woose, 1987) and analysis of genetic variations in this region is not appropriate to differentiate strains within species (Laguerre *et al.,* 1996). However, it is very useful for identification of species. Pairs of universal primers, forward and reverse primers, were design for amplification of 16S rDNA regions in most eubacteria. Pairs of universal primers were used to amplify 16S rDNA (Lane, 1991; van Berkum and Fuhrmann, 2000) to ascertain the non-symbiotic isolates belonging to the genus Bradyrhizobium (Pongsilp *et al.,* 2002). Novel nitrogen-fixing symbionts in genera Methylobacterium, Blastobacter, Burkholderia, Ralstonia, Ochrobactrum, Devosia, Phyllobacterium and Herbaspirillum have been discovered by 16S rDNA sequence analysis (Rivas *et al.,* 2002; Ngom *et al.,* 2004; Valverde *et al.,* 2005; Chen *et al.,* 2006). These Findings suggest that the gene responsible for symbiosis with legumes is transmissible horizontally and functions in a relatively wide range of bacterial taxa (Rivas *et al.,* 2002). Phylogenetic analysis of the 16S rDNA has been constructed in many previous studies. According to Ngom *et al.,* (2004), the clusters in the phylogenetic tree, which was constructed based on nearly the full length of 16S rDNA, correlated well with the taxonomy of strains: i) a first cluster contains *Bradyrhizobium* and Blastobacter in Bradyrhizobiaceae;ii)a second cluster contains Ochrobactrum in Brucellaceae; iii)a third cluster consists of two genera Phyllobacterium and Mesorhizobium in Phyllobacteriaceae; iv) a fourth cluster consists of genera Sinorhizobium, Allorhizobium And *Rhizobium* In Rhizobiaceae. Besides 16S rDNA, sequence analysis of 23S or large subunit ribosomal RNA gene has been also studied. However, the 23S rRNA gene has not been extensively used to estimate the genetic relationships among the Rhizobiaceae, but there are several dramatic differences which may be helpful for classification and identification purposes (van Berkum and Eardly, 1998). Terefework *et al.,* (1998) reported that the 23S Dendrogram showed deeper branching than the 16S dendrogram and more genotypes were resolved, although in some cases the sequence divergence is not particularly high.The bacterial species from

soils of Madhya Pradesh at ten different locations were examined following known biochemical tests and DNA purified from eight strains of *Rhizobium* was studied. Furthermore, the pattern of similarities as represented by genetic hierarchy was studied using molecular biotyping methods.

The Leguminosae is one of the largest families of plants, with over 18000 species classified into around 650 genera, representing approximately one-twelfth of all known flowering plants and occupying nearly all terrestrial biomes (Polhill& Raven, 1981). Many species within this family are capable of establishing symbioses with a group of bacteria collectively called rhizobia, of which the most important feature is the capacity for fixing atmospheric nitrogen (N) (Allen &Allen, 1981).Until 1982, all bacteria isolated from root nodules were classified in the genus *Rhizobium*, and speciation was based on the formation of nodules with certain host plants, establishing the 'cross-inoculation group concept (Fred *et al.*, 1932; Jordan, 1982). Based on morphological and physiological patterns, the bacteria were then split into the genera Bradyrhizobium, which included relatively slow growers that produced an alkaline reaction in culture medium with mannitol as carbon source, and Rhizobium, which contained fast-growing acid producers (Jordan, 1982, 1984). Initially, *Bradyrhizobium japonicum* was the only described species within the genus (Jordan, 1982, 1984), but reports of a large genetic and physiological variability among strains that nodulate soybean (*Glycine max*) led to the description of *Bradyrhizobium elkanii* a few years later (Kuykendall *et al.*, 1992)Ribosomal sequences, with the emphasis on the region that encodes the 16S rRNA, have become the tool of choice in molecular taxonomy for tracing bacterial phylogenies (Woese, 1987; Garrity& Holt, 2001). Partial or complete 16S rRNA gene sequences, which have also been used extensively for studying the phylogeny of rhizobia (e.g. Young *et al.*, 1991; Oyaizu *et al.*, 1992; Urtz&Elkan, 1996; Moreira *et al.*, 1998; Vinuesa *et al.*, 1998; Wang *et al.*, 1999; Chen *et al.*, 2000; JaraboLorenzo *et al.*, 2000), have contributed to the recent descriptions of four new genera and several rhizobial species. However, there are reports showing that, despite a high level of diversity in morphological, physiological and genetic properties, diversity is low in the 16S rRNA gene sequences of strains of Bradyrhizobium investigated so far. (Urtz&Elkan, 1996; Vinuesa *et al.*, 1998; Chen *et al.*, 2000; van Berkum&Fuhrmann, 2000; Willems *et al.*, 2001; Qian *et al.*, 2003).The 23S rRNA is a long fragment of about 2.3 kb; it therefore contains more information than the 16S rRNA and has proven

to be useful in the speciation of several genera of bacteria , including rhizobia (Tesfaye *et al.,* 1997; Terefework *et al.,* 1998; Tesfaye & Holl, 1998; Qian *et al.,* 2003). Furthermore, as the rate of sequence change seems to be faster in the 23S rRNA than in the 16S rRNA gene the former may be more valuable for delineating close relationships (Wang & Martinez-Romero, 2000).When bacterial speciation is not clarified by 16S rRNA gene sequencing, analysis of sequences of the 16S–23S rRNAintergenic spacer (IGS) has also proven to be useful, as the usually long sequence and the greater variability make the region particularly interesting for phylogenetic studies (Laguerre *et al.,* 1996; Vinuesa *et al.,* 1998; van Berkum&Fuhrmann, 2000; Willems *et al.,* 2001).

Sequencing analysis of ribosomal genes of several strains can be very expensive; however, PCR based associated with ribosomal genes may be convenient for phylogenetic studies, generally showing high reproducibility and good agreement with partial or complete gene sequencing (Laguerre *et al.,* 1996; Vinuesa *et al.,* 1998; Wang *et al.,* 1999; Abaidoo *et al.,* 2000; Jarabo-Lorenzo *et al.,* 2000). Although it has been suggested that Bradyrhizobium is the ancestor of all rhizobia (Norris, 1965) and strains have been isolated from a variety of legumes distributed worldwide, most studies on diversity and genetics have been performed with fast-growing rhizobia. Furthermore, as has been pointed out since the pioneering studies of ribosomal genes, it seems that there are many more varieties of rhizobiain the tropics and subtropics than in temperate regions (Oyaizu *et al.,* 1992; Vinuesa *et al.,* 1998). Indeed, bradyrhizobia seem to represent the majority of isolates from leguminous trees in Brazilian tropical forests (Moreira, 1991, 2000). In addition, a high level of diversity among strains has been reported in a few studies performed in South America (Urtz & Elkan, 1996; Chen *et al.,* 2000; Menna, 2005).

However, the diversity of these bacteria is still far from clear compared with the great number and vast distribution of their leguminous hosts. Also, in these studies, rhizobia were mainly isolated from a small proportion of legumes, mainly crops, such as soybean, common bean and alfalfa. Recently, some rhizobia have been characterized from wild and tree legumes and severalnovel taxa were proposed on the basis of these studies (Wei *et al.,* 2002). Therefore, the characterization of more isolates from different leguminous species is necessary in order to understand the diversity and evolution of rhizobia.

In this research, nodule isolates from Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean were characterized. The aims of the research were to examine the diversity and to clarify both the phenotypic and genetic analyses.

4.1.1 Molecular Identification

Last fifteen years of the twentieth century allowed for an exponential increase in the knowledge of techniques in molecular biology, following the cellular and protein era of the 1970s and 1980s. This explosion of technologies from the primary discipline of molecular biology has had major consequences and has allowed for significant developments in many areas of the life sciences, including bacteriology.

Although, most modern clinical microbiology diagnostic laboratories rely on a combination of colonial morphology, physiology andbiochemical/serological markers, for their successful identification either to the genus level or more frequently to the species level. Molecular methods are increasingly being used. For example, gave a description and mentioned that with the use of 16S rDNA sequencing. 215 novel bacterial species, 29 of which belong to novel genera, have been discovered from human specimens in the past 7 years of the 21st century (2001-2007) through numerical taxonomy studies involving large numbers of isolates (e.g., 197 cultures investigated in one study (Grimes *et al.*, 1993); 473 isolates studied in another to culturc- independent molecular approaches (e.g., partial sequencing of the 16S rRNA gene. The benefit of the latter is the recognition of organisms that may or may not be calculated by conventional techniques Sometimes, the phonetic approach has centered on the use of rapid systems, such as BIOLOG or MIDI (Nedoluha and Westhoff 1997a, b). It is encouraging that some comparative studies have pointed to congruence between phenolypic and molecular analyses. Bacterial taxonomy has progressed from reliance on highly artificial culture-dependent techniques involving the study of phenotype (including morphological, biochemical and physiological data) to the modern applications of molecular biology, most recently 16S rRNA gene, sequencing, which gives an insight into evolutionary pathways phylogenctics. There is evidence that molecular techniques have been used with increasing regularity for bacterial pathogens. Molecular methods, namely sequencing of the 16S rRNA gene, permit the study of evolutionary relationships, i.e. phylogenctics. Which may be viewed as phylogenetic trees which are interpreted by cladistics and used in defining taxa.

4.1.2 Based diagnostics

Developments in bacterial ecology and industrial biotechnology are severely hampered by the lack of reliable identification system (Bull *et al.*, 1993. Goodfellow and O'Donnell, 1993). To obtain a phenolypic description requires long and fastidious work, which does not always warrant satisfactory identifications of bacterial species. Following determination of the structure of DNA by Watson and Crick (1953). Studies in bacteriology have seen a major shift from functional to molecular techniques for identifying bacteria. Phylogenetic relationships among various organisms and their identification now can be derived from degree of DNA relatedness of their genomes: two closely related organisms share significant homologies, while distant organisms display low homologies. DNA-DNA relatedness is best suited for identification of closely related species or strains within a single species no doubt.

Presently, a direct comparison of rRNA genes sequence is probably the most powerful tool for the identification of many bacteria (Stackebrandt and Goodfellow, 1991). These genes have acquired paramount relevance for the study of bacterial evolution and phylogeny. Indeed, rRNA genes (rDNA) are present and expressed in all bacterial species, are truly homologous in all organisms, are easily sequenced and now offer a large and ever increasing database of sequences and allow the identification of bacteria (Amann *et al.*, 1994).

The sequences of gene coding for 16S rDNA is also a powerful tool for deducing phylogenetic and evolutionary relationships among eubacteria, archaebacltria and eucaryotic organisms because of their high information content, conservative nature and universal distribution (Lane *et al.*, 1985. Woese, 1987). The 16S rDNA sequence analysis is a standard method for the investigation of their phylogenetic relationships. even though some closely related species may have only a few differences in their 16S rDNA sequences, a phylogenetic tree can be established to give them an exact taxonomic position (Collins *et al.*, 1991).

This studies was therefore undertaken to sequenced 16S rRNA gene of isolated bacteria identified in previous chapter in an attempt to i) clarify the intragcneric relationships of identified isolates and compare the sequence similarities with Genebank databases; ii) to know the taxonomic position of the identified isolates and iii) identify regions in 16S rRNA genes whieh will have a value to develop probe in future.

4.1.3 Research Activities

In this research, we set up some goals to be achieved through the progress of work. The activities planned for the current investigation were as follows.

1. Extraction of genomic DNA and PCR amplification of 16S rDNA.
2. Purity and concentration of the isolated *Rhizobium* bacteria.
3. 16S rDNA partial genomic sequence analysis of phylogenetic of *Rhizobium* bacteria.
4. Identification of the isolated *Rhizobium* strain.

4.2 MTERIALS AND METHODS

4.2.1 Materials

To conduct the present research following materials and equipments were used.

Table 4.1: Apparatus details used for molecular identification of the isolates (N=8)

Name ofthe Apparatus	Model	Company	Country of origin
Centrifiuge	WiseSpin CF-10	Wisd Laboratory Instruments	Germany
Gel electrophoresis	Mini-Sub Cell GT Cell	Bio-Rad Laboratories, Inc.	USA
Thermal Cycler	Gene Atlas 482/485	Astec	Japan
Gel documentation	Alfalmager MINI	ProteinSimple	California, USA
Spectrophotometer	NanoDrop 2000	Thermo Scientific	USA
Sequencer	Genetic Analyzers 3130	Applied Biosystems	USA
Orbital Shaker	VS 201D	Vision Scientific	Korea

Chemicals:

Genomic DNA was extracted from the bacterial cells using TIANamp Bacteria DNA kit (Tiangen, China).

Table 4.2: Primers and theirsequence used in this study.

Primers*	Sequences 5′ to 3′	Target Group	Reference
16S-Forward (8f)	AGAGTTTGATCCTGGCTCAG	Universal	Turner *et al*., 1999
16S-Reverse (806r)	GGACTACCAGGGTATCTAAT	Universal	McBain *et al.,* 2003

Note:* Numbered primers are named for the approximate position on the *E. coli* 16S rRNA molecule.

The PCR products were purified using TIANquick Midi purification kit (Tiangen, China)

4.3 METHODS

4.3.1 Principle of PCR

Polymerase Chain Reaction(PCR) is an enzyme-driven, primer-mediated. Temperature-dependent process for replicating a specific DNA sequence *in vitro.* The principle of PCR is based on the repetitive cycling of three simple reactions, the conditions of which vary only in the temperature of incubation. The three simple reactions include

1. Denaturing: When the temperature is raised to around 95°C, template DNA double strand is separated to two single strands.

2. Annealing: When the temperature reduces to approx. 55°C, two specific oligonucleolide primers bind to the DNA template complemcntarily.

3.Extension: When the temperature rises to 72°C, DNA polymerase extends the primers at the 3' terminus of each primer and synthesizes the complementary strands along 5'to3' terminus of each template DNA using deoxynucleotides containing in

media. After extension, two single template DNA strands and two synthesized complementary DNA strands combine together forming two new double strand DNA copies. After extension the reaction will repeat above steps. Each copy of DNA may then serve as another template for further amplification. PCR products will be doubled in each cycle. After n cycles (approx. 30) the final PCR products will have 2n copies of template DNA in theory and it just needs few hours. The 16S rRNA gene of the isolated bacteria were amplified by a polymerase chain reaction (PCR) With the forward and reverse primers which correspond to nucleotidcs 8F-1492R, 8F-806R.16S rRNA gene sequence, respectively. The amplified PCR product was cloned and single forward strand was add lysozyme to 20 mg/ml) methods that are followed in the current research are described below.

4.3.2 gDNA isolation protocol

Genomic DNA isolation protocol

Genomic DNA was extracted from the bacterial cells using TIANamp Bacteria DNA kit(Tiangen, China) and purified according to the manufacture′s instruction.

Free-living soil *bacteria* beneficial to plant growth, Genomic *DNA* extraction The isolate chromosomal *DNA* was extracted using a TIANamp*Bacteria DNA kit*(*Tiangen, China*) according to the Bacillus, *Rhizobium*, and Streptomyces (Bashan 1998).

4.3.3 Agarose gel electrophoresis

The amplification products were separated by electrophoresis of 10 µl. (7 µl PCR product 3 µl loading dye, Bromothymol blue) of the reaction product in 1.0% agarose gel (wv^{-1}) in Tris-Borate buffer (0.089M Tris, O.O89M boric acid, and 0.002M EDTA, pH 8), stained with ethidium bromide (1.6 mg/ml). The gel electrophoresis was carried out at 70 V at room temperature for ~ 1.0 hour in electrophoresis unit (Bio-Rad, USA) and DNA bands were visualized using UV transilluminator in gel documentation system. A 1 kb DNA ladder was used as molecular weight markers.

4.3.4 PCR amplification and purification of the product

Polymerase chain reaction (PCR): In the Polymerase Chain Reaction the DNA to be amplified is denatured by heating the sample in the presence of DNA polymerase and excess dNTPs, the oligonucleotides that hybridize specifically to the target sequence can prime new DNA synthesis. The first cycle is characterized by a product of indeterminate length; however, the second cycle produces the discrete "short product" which accumulates exponentially with each successive round of amplification. This can lead to the many million fold amplification of the discrete fragment over the course of 20 to 30 cycles.

Primers used for amplification

Both sets of primers were designed from 8f and 806r, which are capable of amplifying 16S from a wide variety of bacterial taxa. Primers used for the amplification of 16S rDNA from all the strains.

Table 4.3: List of primers used for PCR amplification and sequencing of 16S rDNA from isolates.

Primers*	Sequences 5′ to 3′	Target Group	Reference
16S-Forward (8f)	AGAGTTTGATCCTGGCTCAG	Universal	Turner *et. al.,* 1999
16S-Reverse (806r)	GGACTACCAGGGTATCTAAT	Universal	McBain *et al.,* 2003

The primers are frequently used as universal for genomic bacterial DNA. The PCRs used here were performed in final volume of 50µl containing10µl of DNA,5µl of 10×PCR buffer (50 mM ,KCL 2 mM $MgCL_2$,10 mMTris HCL) 2.0 µl of dNTPs (5mM),2.0 µl of a 5mM solution of each primer , 0.5 µl of *Taq*DNA polymerase at 5 U/µl, and 28.5 µl of double-distilled sterile water. We used 1 cycle of denaturation at 95° C for 40s, anneling at 49° C for 45s, and extension at 72° C 1:30 min, and a final extension round at 72° C for 10 min. The PCR amplicons are separated electrophoretically in a 1% agarose gel and visualized after ethidium bromide staining.

Agarose gel electrophoresis

The amplified products were separated were by electrophoresis of 10 µl (7 µl PCR product 3 µl loading dye, Bromophenol blue) of the reaction product in 1.0 % agarose gel (WV^{-1}) in Tris- Boret buffer (0.089M Tris, 0.089 M boric acid , and 0.002M EDTA pH 8) stained with ethidium bromide (1.6 mg/ml). The gel electrophoresis was carried out at 70 V a room temperature for~ 1.0 hour in electrophoresis unit (Bio- Rad, USA) and DNA bands were visualized using UV transilluminator in gel documentation system. A 1 kb DNA ladder was used molecular weight markers.

4.3.5 PCR product purification protocol

PCR product purification

The PCR products were purified using TIANquick Midi purification kit(Tiangen, China)according to the manufacture′s protocol.

4.3.6 Estimation of DNA

The total DNA yield and quality were determined spectrophotometrically by NanoDrop 2000(Thermo Scientific, USA)

4.3.7 DNA sequencing

In 1977 two different methods for sequencing DNA were developed, namely, the chain termination method and the chemical degradation method. Both methods were equally popular to begin with, but, the chain termination method soon become more popular and this method is more commonly used today. This method is based on the principle that single-stranded DNA molecules that differ in length by just a single nucleotide can be separated from one another using polyacrylamide gel elcctrophorcsis. The fixed laser beam excites the fluorescently labeled DNA bands and the light emitted is detected by sensitive photodetectors. DNA sequence data is the most accurate and definitive way to identify microbes because the microbes may be identified by base pair to base pair of fhe nucleic acid. The DNA sequences of the variable regions form the basis of phylogenetic, classification of microbes. By sequencing broad range PCR products, it is possible to detect DNA from almost any bacterial species. After comparing the resulting sequences with known sequences in GenRank or other databases, the identity of the unknown bacteria can be revealed. Since the 1990s 16S

rDNA sequencing has become a powerful tool, which is used more and more in microbial detection and identification algorithms, especially for unusual, non-culturable, fastidious and slow growing pathogens, or after antibiotics that have been administered to the patient. Such a technique as this is becoming a routine method of detection and Sanger sequencing work flow using dye terminator technology was followed for the present study sequencing analysis was performed on a~800 bp PCR product . The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit . The 16S rRNA genes in the Gene Bank by using the NCBI Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nih.gov/BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model . The phylogenetic trees were formed using Weighbor (Weighted Neighbor Joining: A likelihood-Based Approach to Distance - Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000.The 16S rRNA gene sequences were deposited to Genbank using BankIt submission tool.

4.3.8 16SrRNA Sequencing and Data Analysis

Sequencing analysis was performed on~800 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and BigDye Terminator version 3.1 cycle sequencing kit. The 16S rRNA sequences were aligned and compared with other 16Sr RNA genes in the GenBank by using the NCBI Basie Local Alignment Search Tools BIASTn program (Http://www.ncbi.nlm.nih.gov/BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees created using Weighbor (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000. The 16S rRNA gene sequences have been deposited to (Genbank using Banklt submission tool and has been assigned with NCBI accession numbers. The PCR product was sequenced in single directions and the sequences obtained were checked using Chromas (version 2.4). The 16S rDNA sequences were compared with sequences deposited in the Genbank and EMBL. Searching for homologous sequences in data bank was carried out using blastn analysis (Altschul *et. al*., 1990; http://www.ncbi.nlm.nih.gov/blastn). The sequences used for the multiple alignments were retrived from Genbank and EMBL.

4.3.9 Phylogenetic Analysis

The phylogenetic trees were constructed by the multiple alignments of all the strains and the related species using Neighbor-Joining algorithms using the Jukes-cantor model in Mega VI software (version 6.0). The level of support for the phylogenies derived from neighbor-joining analysis was gauged by 500 bootstrap replicates.

Phylogenetic analysis is the process used to determine the genetic evolutionary connections between species. The results of an analysis can be drawn in a hierarchical diagram called a cladogram or phylogram (phylogenetic tree). The branches in a tree are based on the hypothesized evolutionary relationships (phylogeny) between organisms. Each member in a branch, also known as amonophyletic group, is assumed to be adescendent from a common ancestor. Although originally, phylogenetic trees were created using morphological characteristics, the development of molecular biology has provide a molecular mean for determination the evolutionary relationships through matching patterns in nucleic acid and protein sequences during evolution, it is very common for agene to be duplicated. The copies continue to evolve separately, resulting in at least two similar instances of the same gene along the genome of a species (Saitou and Nei 1987).

Procedure of nueleotide sequence analysis

- Nueleotide sequence from genetic analyzer
- BLASTn search performed through NCBl BLAST search engine
- Select 25 blast match from the first match except uncultured bacterium clone and complete sequence of any matched bacterial strain
- Distance to tree result (this tree was produced using BLAST pairwise alignments)

BLAST computes a pair wise alignment between a query and the database sequences searched. It does not explicitly compute an alignment between the different database sequences (i.e., does not perform a multiple alignment). For purposes of this sequence tree presentation an implicit alignment between the database sequences is constructed, based upon the alignment of those (database) sequences to the query. It may often occur that two database sequences align to different parts of the query, so that they barely overlap each other or do not overlap at all. In that case it is not possible to

calculate a distance between these two sequences and only the higher scoring sequence is included in the tree.

Tree method

- Neighbor joining
- Maximum sequence difference: 0.75
- Sequence level: Taxonomic name (sequence ID)
- Collapse mode: Custom, distance showed
- Download unrooted tree as Newick formal

Download

Downloads the guide tree into a text file in Newick or Nexus format (recognized by popular phylogenetic analysis software). Node labels can be changed with the Sequence Label option (below, to the right).

Note: Newick and Nexus formats do not support collapsed subtrees, therefore fully expanded tree is always downloaded.

- Open the Newick format tree and edit with MEGA version 6.0 software.

4.4 RESULTS

4.4.1 Molecular identification

Genomic DNA extraction and gel electrophoresis: Genomic DNA from the 18 hours old broth culture of the 8 isolates was extracted and gel electrophoresis was carried out to confirm its quality (figure). We found a bright single band with high molecular weight and less smearing which was seen on top of the 10 kb band of 1 kb plus DNA ladder indicative of good quality DNA. We used this genomic DNA for our further molecular biology experiments.

It was initially diluted the genomic DNA and used 1 µl of this diluted genomic DNA for PCR. We used two different primers in following combination to find out the best pair of primers to amplify the 16S rRNA gene 8F-1492R and 8F-806R. Each and every PCR products were run through 1.0% agarose gel electrophoresis and visualized under

UV transilluminator. The combination of 8F-806R and 8F-1492R gave the more contrast single band in between 700-800 bp in comparison to 1 kb plus ladder Subsequently, PCR was carried with an increased volume (50 µl) using 8F-806R.PCR product was purified using TIANquick midi purification kit and preapared for sequencing PCR.As it was the objective to sequence the clone only from one end of the gene we further carried outPCRuusing the purified PCR product as template and one primer each time. Among forward 8F and reverse 806R primers, only 8F generated brilliant single band and thus help us to decide to sequence our PCR product using 8F primer (fig 4.1-4.3). Before big dye cycle sequencing use big dye terminator Version 3(BD- V3). The purity and concentration of the purified PCR products were measure as it is needed fof dilution. The results are presented in table 4.4 for each every isolates.The sequencing was carried out in an ABI prism 3130 genetic analyzer and the obtained sequences were shown in Appendix 3-10 in FASTA format.

For a more precise classification, a sequence analysis of the 16S rRNA was carried out. The blast search results for the partial sequences of the 16S rRNA gene of the isolates revealed that five isolates Green Gram , Black Gram, Pigeon Pea Sweet Pea and Chick Pea are from the same species *Rhizobium* sp. CCNWYC119 with same strains. They had 95, 98, 98, 98 and 98% similarity with that of *Rhizobium* sp. CCNWYC119, with sequence IDDQ674859.1, DQ674859.1, DQ674859.1, DQ674859.1 and DQ674859.1, respectively another two isolates Lentil and Bean were similar with *Rhizobium* sp. SOY12.They had 79 and 85% similarity with that of *Rhizobium* sp. SOY12, with sequence ID KF008236.1 and KF008236.1 respectively and The isolates cow Pea was the only member *Rhizobium* sp. SOY7 which got 96% similar *Rhizobium* sp. SOY7, with sequence ID KF008235.1 . The strain is considered as 1st reported strain and named as *Rhizobium* sp.BDRAJGG101, *Rhizobium* sp. BDRAJBG102, *Rhizobium* sp. BDRAJL103, *Rhizobium* sp. BDRAJPP104, *Rhizobium* sp. BDRAJSP105, *Rhizobium* sp. BDRAJCP106, *Rhizobium* sp. BDRAJCP106, *Rhizobium* sp. BDRAJCP107 and *Rhizobium* sp. BDRAJB108.

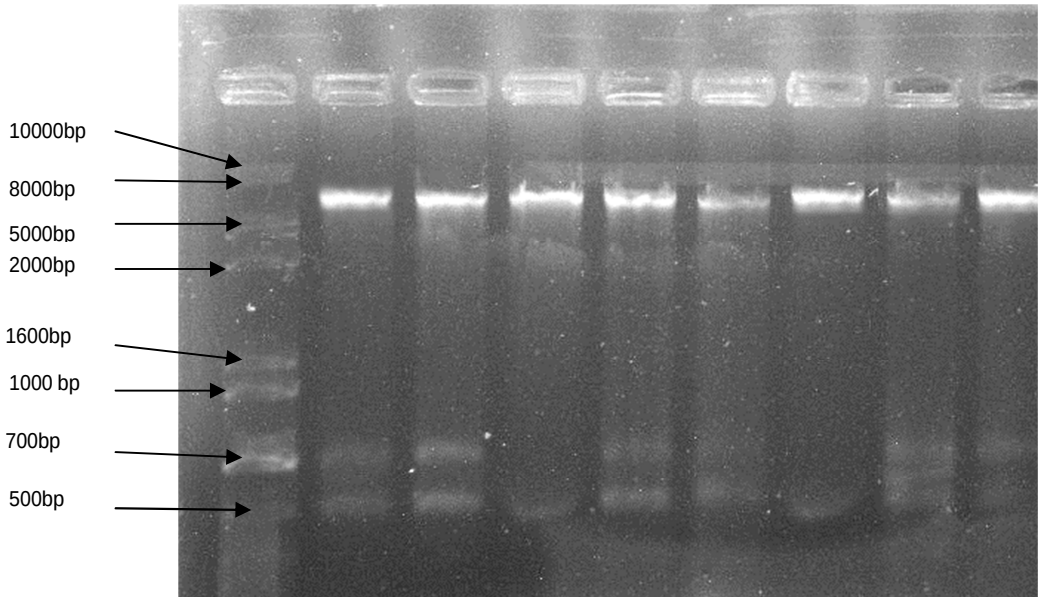


Figure 4.1: Genomic DNA of (8) isolates of the bacteria obtained from legume plant using TIANamp Bacteria DNA kit. DNA was run on 1% agarose gel containing Ethidiumbromide.Lane 1: Tiangen 1 kb plus DNA ladder, Lane 2-9: Isolates 1-8.

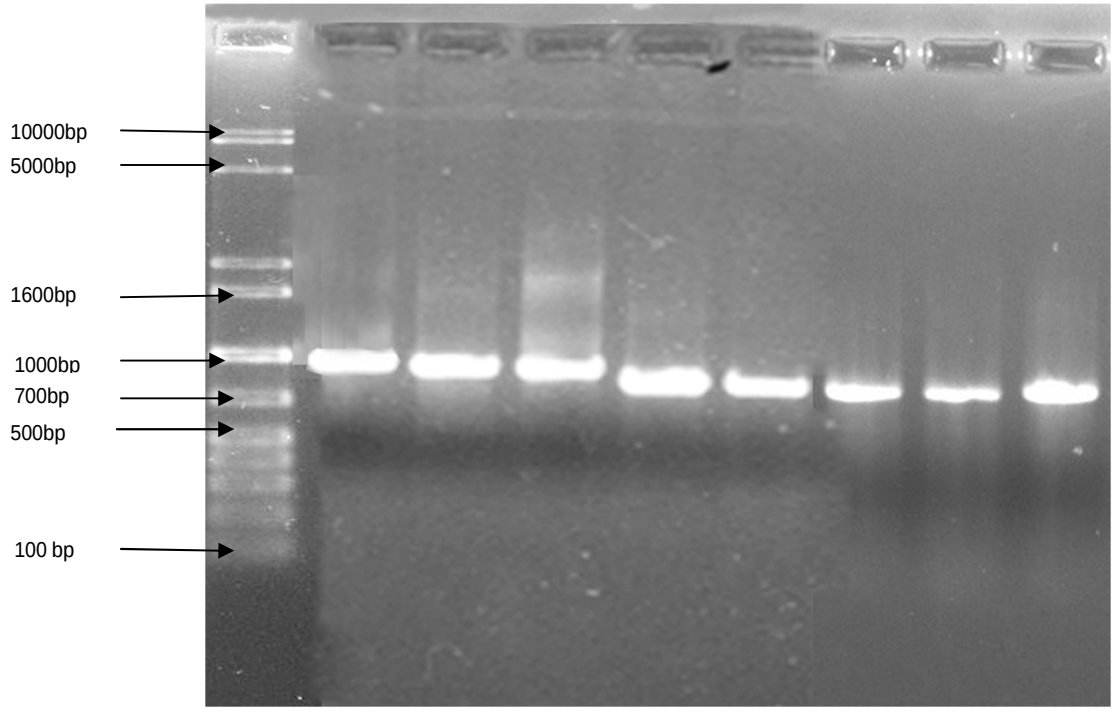


Figure 4.2: PCR products of (8) isolates of the bacteria obtained from legume plant using 8F and 806R primers. DNA was run on 1% agarose gel containing Ethidiumbromide. Lane 1: Tiangen 1 kb plus DNA ladder, Lane 2-9: Isolates 1-8.

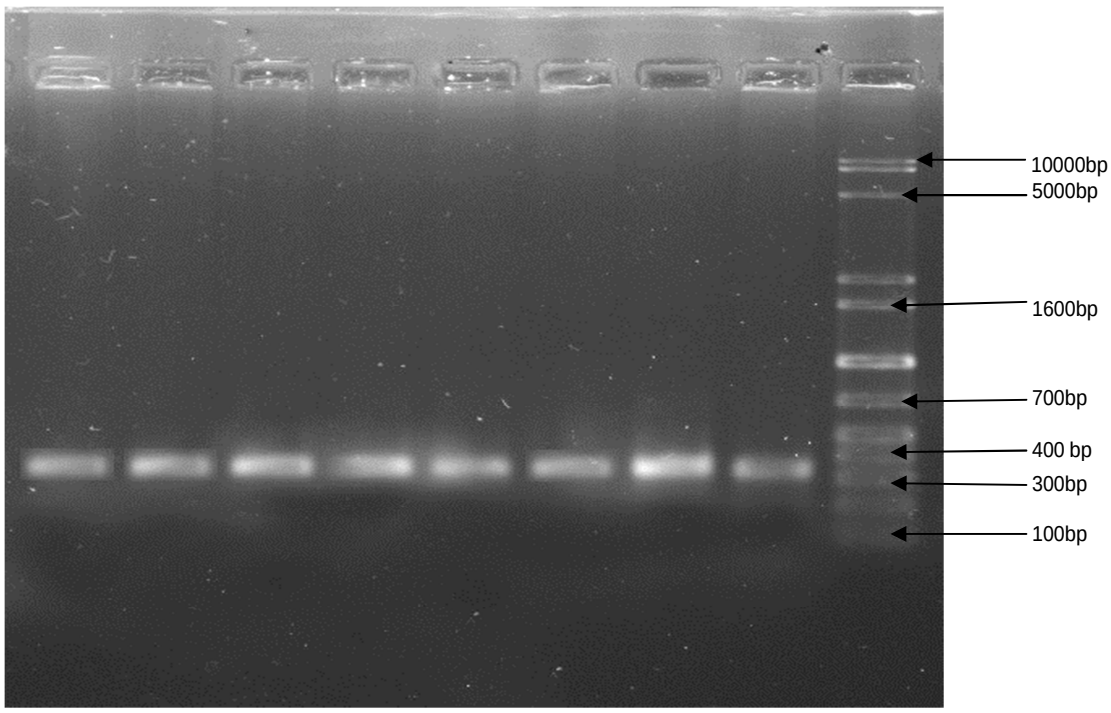


Figure 4.3: Purified PCR products using 8F primer generated brilliant single band of 8 isolates of the bacteria obtained from legume plants and DNA was run on 1% agarose gel containing Ethidium bromide. Lane 1: Tiangen 1 kb plus DNA ladder, Lane 2-9: Isolates 1-8.

Table 4.4: Purity and concentration of the isolates

Isolates	Concentration (ng/µl)	Purity (260/280nm)
Green Gram	42.0	1.77
Black Gram	83.9	1.74
Lentil	28.9	1.74
Pigeon Pea	49.0	1.77
Sweet Pea	88.5	1.85
Chick Pea	68.8	1.77
Cow Pea	86.8	1.82
Bean	69.7	1.84

4.4.2 Sequences of 16S rDNA

The amplified 16S rDNA fragments of the three isolates were sequenced only in one direction by using forward (fD1) primer. The sequencing data obtained as chromas file and the consensus sequebce was used to perform blastn. The sequences obtained were 948 bp (Green Gram), 977 bp (Black Gram),783 bp (Lentil), 763 bp (Pigeon Pea) , 770 bp (Sweet Pea), 921 bp(Chick Pea), 769bp (Cow Pea) and 797 bp (Bean) respectively.

Representative chromatogram of samples:

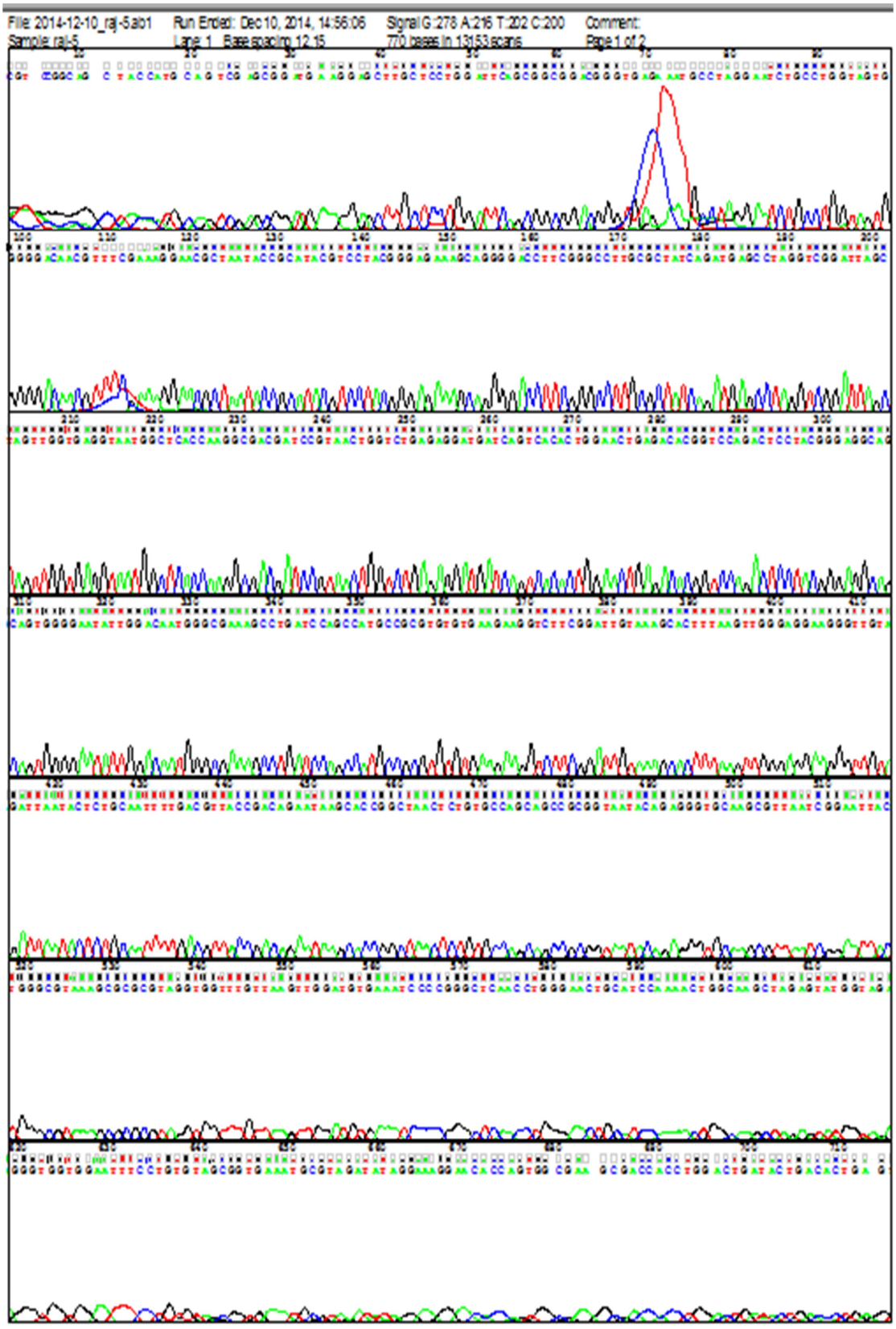


Figure 4.4: Chromatogram of the bacterial isolates Sweet Pea.

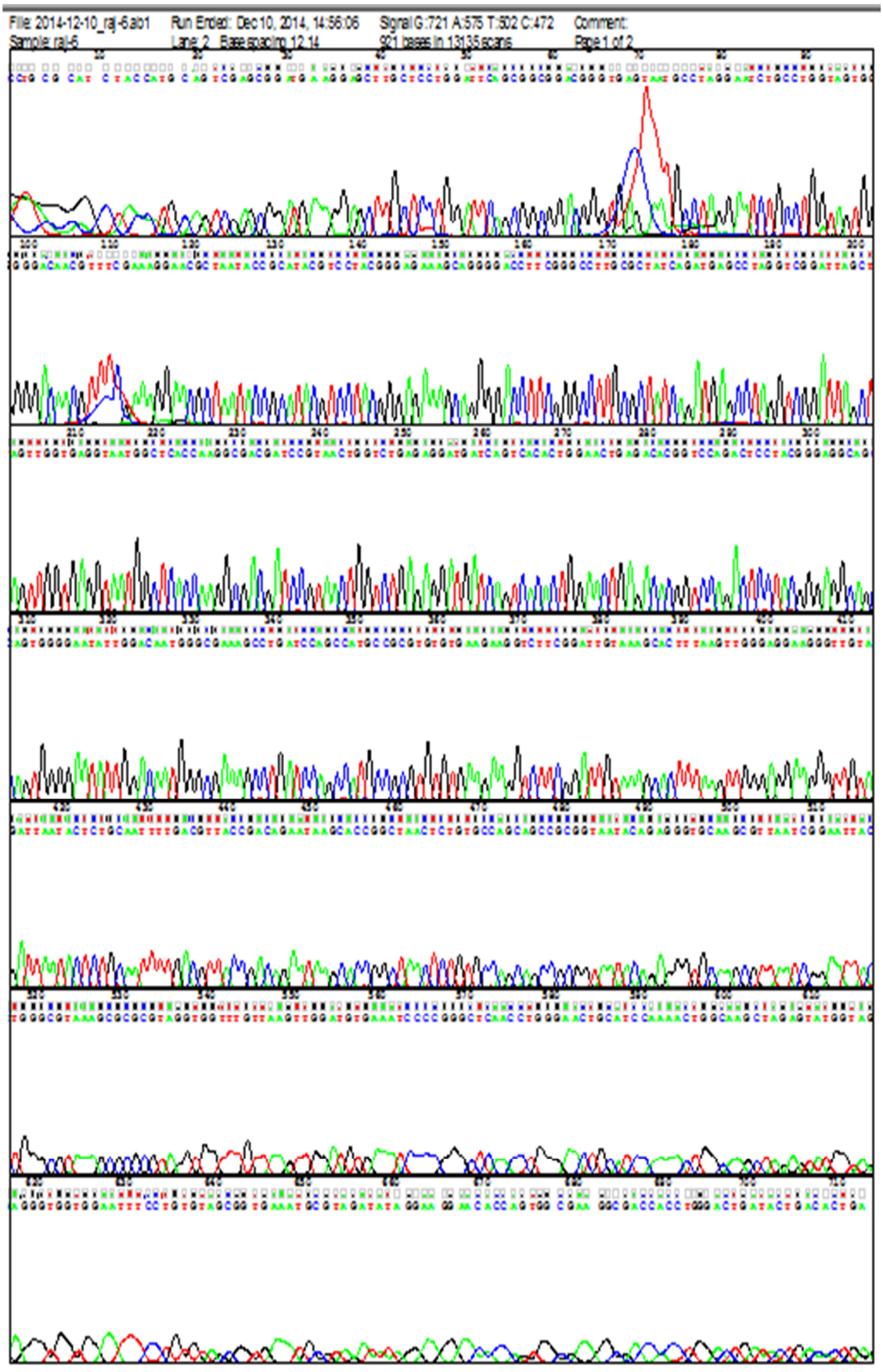


Figure 4.5: Chromatogram of the bacterial isolates Chick Pea.

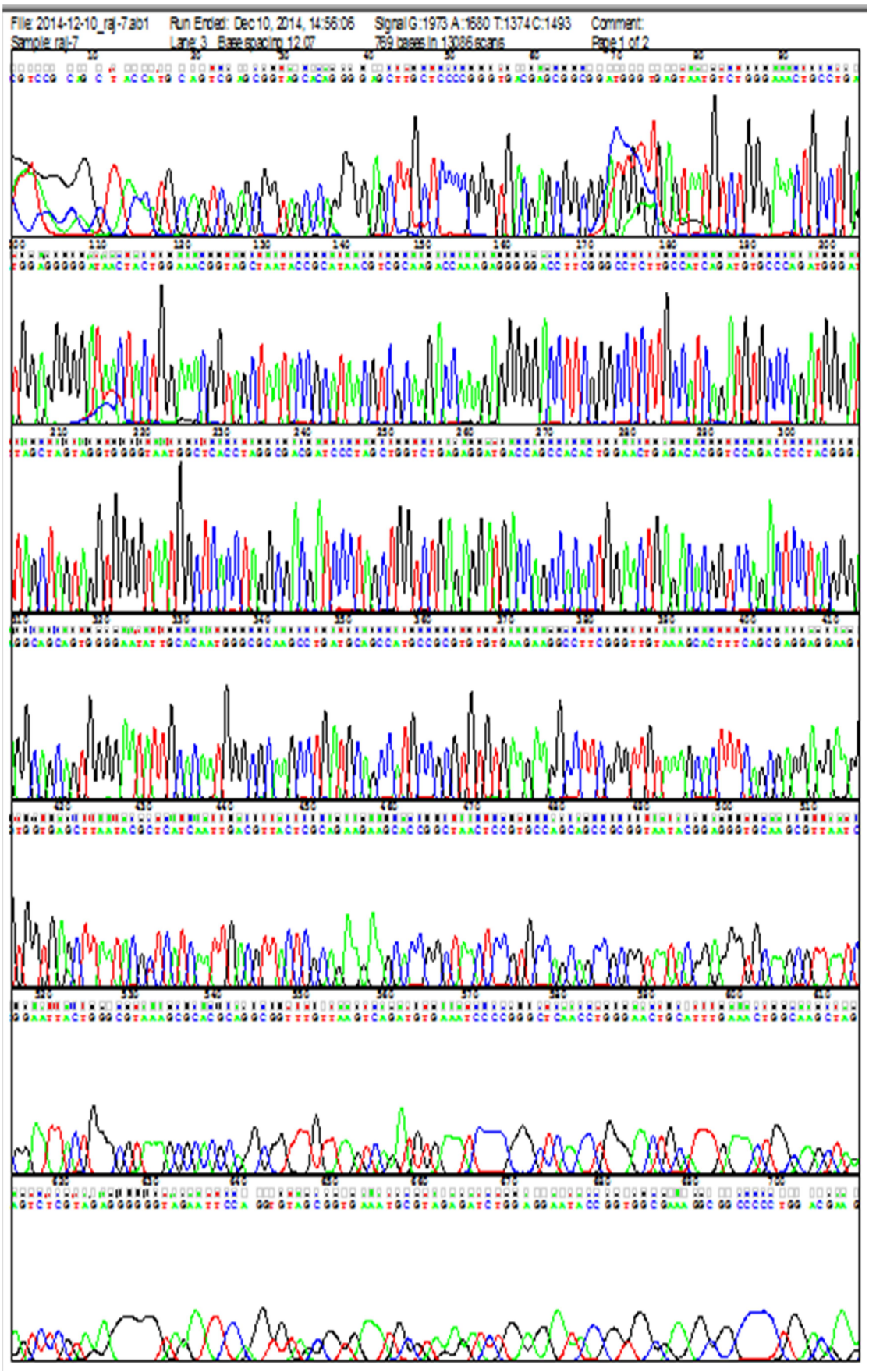


Figure 4.6: Chromatogram of the bacterial isolates Cow Pea.

Table 4.5: Isolates with 16S rDNA sequence match results found in NCBI database.

Isolates	Resembles with NCBI BLASTn suite			
	1st matching	**Identities**	**Sequence ID**	**Strain Identifier**
Green Gram	*Rhizobium* sp. CCNWYC119	95%	DQ674859.1	*Rhizobium* sp.BDRAJGG101
Black Gram	*Rhizobium* sp. CCNWYC119	98%	DQ674859.1	*Rhizobium* sp. BDRAJBG102
Lentil	*Rhizobium* sp. SOY12	79%	KF008236.1	*Rhizobium* sp. BDRAJL103
Pigeon Pea	*Rhizobium* sp. CCNWYC119	98%	DQ674859.1	*Rhizobium* sp. BDRAJPP104
Sweet Pea	*Rhizobium* sp. CCNWYC119	98%	DQ674859.1	*Rhizobium* sp. BDRAJSP105
Chick Pea	*Rhizobium* sp. CCNWYC119	98%	DQ674859.1	*Rhizobium* sp. BDRAJCP106
Cow Pea	*Rhizobium* sp. SOY7 ,	96%	KF008235.1	*Rhizobium* sp. BDRAJCP107
Bean	*Rhizobium* sp. SOY12	85%	KF008236.1	*Rhizobium* sp. BDRAJB108

BLAST computes a pair wise alignment between a query and the database sequences searched. It does not explicitly compute an alignment between the different database sequences (i.e. does not perform a multiple alignment). For purposes of this sequence tree presentation an implicit alignment between the database sequences is constructed, based upon the alignment of those (database) sequences to the query. It may often occur that two database sequences align to different parts of the query, so that they barely overlap each other or do not overlap at all. In that case it is not possible to calculate a distance between these two sequences and only the higher scoring sequence is included in the tree.

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1166 bits(1292)	0.0	714/751(95%)	6/751(0%)	Plus/Plus

```
Query  16   CATGCA-GTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGAA  74
            ||||||  |||||||||  |||||  ||||||||  ||      |||||||||||||||  |
Sbjct  4    CATGCAAGTCGAGCGGATGAGAGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTA  63

Query  75   AAGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCA  134
            | ||||||||||||||||||||||||||| ||||||  ||||  | ||||||||||||||
Sbjct  64   ATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCA  123

Query  135  TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT  194
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124  TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT  183

Query  195  CGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG  254
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  CGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG  243

Query  255  AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG  314
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG  303

Query  315  GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC  374
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC  363

Query  375  TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTT  434
            |||||||||||||||||||||||||||||||||||  ||| |||||||||  |||  |||
Sbjct  364  TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTT  423

Query  435  TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA  494
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA  483

Query  495  GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT  554
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484  GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT  543

Query  555  GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTAT  614
            |||||||||||||||||||||||||||||||||||| ||||||||  |   |||||||||
Sbjct  544  GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTAT  603

Query  615  GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGGAACA  674
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  604  GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAA-GGAACA  662

Query  675  CCAGTGGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGG  734
            |||||  |||||||||||||||||||||  |||||||||||||||||||||||||||  ||||
Sbjct  663  CCAGT-GGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGT-GGGG  720

Query  735  AGC-AACA-GAATAGATACCCTAGTAGTCCA  763
            ||| |||| || |||||||||| ||||||||
Sbjct  721  AGCAAACAGGATTAGATACCCTGGTAGTCCA  751
```

Figure 4.7: Pair wise alignment between *Rhizobium* sp. CCNWYC119 (1st match) and Green Gram.

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1238 bits(1372)	0.0	716/729(98%)	5/729(0%)	Plus/Plus

```
Query  15   CATGCA-GTCGAGCGGATGA-AGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGCGTA  72
            ||||||  ||||||||||||| |||||||||||||||||||||||||||||||||| |||
Sbjct  4    CATGCAAGTCGAGCGGATGAGAGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTA  63

Query  73   TTGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCA  132
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64   ATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCA  123

Query  133  TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT  192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124  TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT  183

Query  193  CGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG  252
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  CGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG  243

Query  253  AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG  312
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG  303

Query  313  GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC  372
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC  363

Query  373  TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTT  432
            ||||||||||||||||||||||||||||||||||  ||| ||||||||| |||  |||
Sbjct  364  TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTT  423

Query  433  TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA  492
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA  483

Query  493  GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT  552
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484  GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT  543

Query  553  GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTAT  612
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  544  GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTAT  603

Query  613  GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAAGGAACA  672
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  604  GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGG-AAGGAACA  662

Query  673  CCAGTGGCGAAGGCGACCACCTGGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGG  732
            |||||||||||||||||||||| |||||||||||||||||||||||||||||| ||||
Sbjct  663  CCAGTGGCGAAGGCGACCACCT-GGACTGATACTGACACTGAGGTGCGAAAGCGT-GGGG  720

Query  733  AGCAAACAG  741
            |||||||||
Sbjct  721  AGCAAACAG  729
```

Figure 4.8: Pair wise alignment between *Rhizobium* sp. CCNWYC119 (1st match) and Black Gram.

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
596 bits(660)	2e-169	578/730(79%)	16/730(2%)	Plus/Plus

```
Query  16   CATGCA-GTCGAGCGGCAGCGGAGGTAGCTTGTTACTTTGCCGGAGAGCGGCGGTTTGAA  74
            |||||| ||||||||| | |  ||| |||||| | ||  |  |  |||||||||   |
Sbjct  21   CATGCAAGTCGAGCGGTAACACAGGGAGCTTGCTCCTGGGT-GACGAGCGGCGGACGGGT  79

Query  75   GAGAAGTGTCTGGGAAAGTGCCTGATGGAGGGGTATCCCTTGTGGAAACGGTATCTAATA  134
            ||| | |||||||||||| ||||||||||||||| ||  ||  |||||||||||| ||||||
Sbjct  80   GAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATA  139

Query  135  CCGCATAGTGTCACAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCCTCATATGTGCC  194
            |||||||  ||| ||||||||||||||||||||||||||||||||||| ||| |||||||
Sbjct  140  CCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCC  199

Query  195  CATATGGTATTATCTAGTATGTGGGGTAACGTCTCCTCTACGCCACTATCCCTATCTGGT  254
            || |||| |||| |||||| |||||||||| |||  ||| || || ||||||| |||||
Sbjct  200  CAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGT  259

Query  255  CTGAGAAGATCACCACCCACTCTGAAACTGACACACGCCCCACACTCCTACGGGGGGCAG  314
            |||||| ||| |||| |||| ||| |||||| |||||  ||| |||||||||| |||||
Sbjct  260  CTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG  319

Query  315  CTGTGGGGAATTTTGCACAATGGGCGCGACTCTTATGC-CCCCTGCCGTGTGTGTGAAAA  373
            | ||||||||| |||||||||||||| |  || ||||  || ||||| |||| |||| |
Sbjct  320  CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGA  379

Query  374  AGGCCTTCGGTTTGTAAAGTTCTCTCCGCGGAGGAGAAGGCGTTGAGGTTTATACTCTCC  433
            |||||||||| ||||||||| || || |||| |  |||||||||||||| |||  |||
Sbjct  380  AGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGTTGAGGTTAATAACCTCA  439

Query  434GCTAGTGACTTCACCCGCaaaaaaaGCACCGTCTATCTCTGTGCCAACACGCGCGATAAT  493
            || | |||| | |||||||| || |||||||| ||| ||| |||||| ||  |||| ||||
Sbjct  440  GCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT  499

Query  494  ACAGAGGGTGCACGCGTAACTCGATATTACTGGGCGTAACGCACACGCGCGCGCTCTCTC  553
            || ||||||||| |||| | |||  |||||||||||||| || |||||  ||| ||| ||
Sbjct  500  ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTC  559

Query  554  AAGTGAGATGTGAACTCCGCGGTCTCACCGTGGGATCTGCTTTCAAATCTGGCGGTCTAG  613
            ||||  |||||||| ||| ||| |||| | ||||| |||| ||| || ||||| | ||||
Sbjct  560  AAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAG  619

Query  614  ACTCGTGTAGAGGGGGGTAGATCTACGTGTGTAGCTGAGGATATGCGTAGATACTCGTAG  673
            | || |||||||||||||||  | |  ||||||| |  || ||||||||| | || | |
Sbjct  620  AGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGC-GGTGAAATGCGTAGAGA-TC-TGG  676

Query  674  AGGTTATCACCTGTCGACGAGCGCGG--CCCTGTGACACAGAGTAGCTGACTGCTCAGTG  731
            |||  |  ||| || | |||  ||||  ||||| |||| |||    ||||| ||||||
Sbjct  677  AGG--AATACCGGT-GGCGAAGGCGGCCCCCTGGGACAAAGA----CTGAC-GCTCAGGT  728

Query  732  TCGCAAGCGT  741
              || ||||||
Sbjct  729  GCGAAAGCGT  738
```

Figure 4.9: Pair wise alignment between *Rhizobium sp*. SOY12 (1st match) and Lentil.

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1240 bits(1374)	0.0	722/737(98%)	6/737(0%)	Plus/Plus

```
Query  14   CATGCA-GTCGAGCGGATGAGAG-AGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTA  71
            |||||| |||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  4    CATGCAAGTCGAGCGGATGAGAGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTA  63

Query  72   ATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCA  131
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64   ATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCA  123

Query  132  TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT  191
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124  TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT  183

Query  192  CGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG  251
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  CGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG  243

Query  252  AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG  311
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG  303

Query  312  GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC  371
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC  363

Query  372  TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTT  431
            |||||||||||||||||||||||||||||||||  ||| ||||||||| |||  |||
Sbjct  364  TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTT  423

Query  432  TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA  491
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA  483

Query  492  GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTT  551
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  484  GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT  543

Query  552  GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTAT  611
            |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  544  GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTAT  603

Query  612  GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGG-ACAC  670
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  604  GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACAC  663

Query  671  CAGTGGCGAACGCGACCACCTGGACTGATACTGACACTGA-GTGCGAAAGCGGTGGGGAG  729
            |||||||||| |||||||||||||||||||||||||||| |||||||||| ||||||||
Sbjct  664  CAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGC-GTGGGGAG  722

Query  730  CAAACA-GATTAGATAC  745
            |||||| ||||||||||
Sbjct  723  CAAACAGGATTAGATAC  739
```

Figure 4.10: Pair wise alignment between *Rhizobium* sp. CCNWYC119 (1st match) and Pigeon Pea.

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1258 bits(1394)	0.0	725/737(98%)	5/737(0%)	Plus/Plus

```
Query  15   CATGCA-GTCGAGCGGATGA-AGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGAA  72
            |||||| ||||||||||||| |||||||||||||||||||||||||||||||||||| |
Sbjct  4    CATGCAAGTCGAGCGGATGAGAGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTA  63

Query  73   ATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCA  132
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64   ATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCA  123

Query  133  TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT  192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124  TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT  183

Query  193  CGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG  252
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  CGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG  243

Query  253  AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG  312
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG  303

Query  313  GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC  372
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC  363

Query  373  TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTT  432
            |||||||||||||||||||||||||||||||||||  ||| ||||||||| |||  |||
Sbjct  364  TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTT  423

Query  433  TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA  492
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA  483

Query  493  GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT  552
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484  GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT  543

Query  553  GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTAT  612
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  544  GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTAT  603

Query  613  GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAAGGAACA  672
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  604  GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGG-AAGGAACA  662

Query  673  CCAGTGGCGAA-GCGACCACCTGGACTGATACTGACACTGA-GTGCGAAAGCGTGGGGAG  730
            |||||||||||| |||||||||||||||||||||||||||| |||||||||||||||||
Sbjct  663  CCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAG  722

Query  731  CAAACAGGATTAGATAC  747
            |||||||||||||||||
Sbjct  723  CAAACAGGATTAGATAC  739
```

Figure 4.11: Pair wise alignment between *Rhizobium* sp. CCNWYC119 (1st match) and Sweet pea.

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1249 bits(1384)	0.0	719/730(98%)	5/730(0%)	Plus/Plus

```
Query  14   CATGCA-GTCGAGCGGATGA-AGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTA  71
            ||||||  ||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct  4    CATGCAAGTCGAGCGGATGAGAGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTA  63

Query  72   ATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCA  131
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64   ATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCA  123

Query  132  TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT  191
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124  TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT  183

Query  192  CGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG  251
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  CGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG  243

Query  252  AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG  311
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG  303

Query  312  GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC  371
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC  363

Query  372  TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTT  431
            |||||||||||||||||||||||||||||||||||  ||| ||||||||| |||  |||
Sbjct  364  TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTT  423

Query  432  TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA  491
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA  483

Query  492  GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT  551
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484  GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT  543

Query  552  GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTAT  611
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  544  GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTAT  603

Query  612  GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACAC  671
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  604  GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACAC  663

Query  672  CAGTGGCGAAGGCGACCACCTGGGACTGATACTGACACTGA-GTGCGAAAGCGT-GGGAG  729
            |||||||||||||||||||| |||||||||||||||||| |||||||||||| |||||
Sbjct  664  CAGTGGCGAAGGCGACCACCT-GGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAG  722

Query  730  CAAACAGGAT  739
            ||||||||||
Sbjct  723  CAAACAGGAT  732
```

Figure 4.12: Pair wise alignment between *Rhizobium* sp. CCNWYC119 (1st match) and Chick Pea.

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1175 bits(1302)	0.0	705/733(96%)	6/733(0%)	Plus/Plus

```
Query  14   CATGCA-GTCGAGCGGTAGCACAGGGGAGCTTGCTCCCCGGGTGACGAGCGGCGGATGGG  72
            |||||| |||||||||||| ||||||| ||||||||||  |||||||||||||||||| |||
Sbjct  32   CATGCAAGTCGAGCGGTAACACAGGG-AGCTTGCTCCT-GGGTGACGAGCGGCGGACGGG  89

Query  73   TGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAAT  132
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90   TGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAAT  149

Query  133  ACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGC  192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  150  ACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGC  209

Query  193  CCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGG  252
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  210  CCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGG  269

Query  253  TCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA  312
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  270  TCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA  329

Query  313  GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAG  372
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  330  GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAG  389

Query  373  AAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAGCTTAATACGCTC  432
            |||||||||||||||||||| |||||||||| ||||||||| | |||| ||||||  |||
Sbjct  390  AAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGTTGAGGTTAATAACCTC  449

Query  433  ATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA  492
            | | |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  450  AGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA  509

Query  493  TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGT  552
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  510  TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGT  569

Query  553  TAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTA  612
             ||||| |||||||||||||||||||||||||||||||| |||||||||| ||||
Sbjct  570  CAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTA  629

Query  613  GAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAG  672
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  630  GAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAG  689

Query  673  GAATACCGGTGGCGAAAGGCGGCCCCCTGGACGAAGACTGACGCTCA-GTGCG-AAGCGT  730
            ||||||||||||| |||||||||||||| |||||||||||||| ||||| ||||||
Sbjct  690  GAATACCGGTGGCG-AAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGT  748

Query  731  GGGGAGCAAACAG  743
            |||||||||||||
Sbjct  749  GGGGAGCAAACAG  761
```

Figure 4.13: Pair wise alignment between *Rhizobium* sp. SOY7 (1st match) and Cow Pea.

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
737 bits(816)	0.0	571/669(85%)	14/669(2%)	Plus/Plus

```
Query  15   CATGCA-GTCGA-CGGTAGCACAGAGAGCTTGCTTTCGTGTGACGAGTGGCGGACGGGGG  72
            |||||| ||||| ||||| ||||| |||||||||   | ||||||| |||||||||| |
Sbjct  21   CATGCAAGTCGAGCGGTAACACAGGGAGCTTGCTCCTGGGTGACGAGCGGCGGACGGGTG  80

Query  73   AGGAATGTCTGGCAAACTGGCTGATGGAGGGGGATAACCCCTGGGAACGGTAACTCATAC  132
            || ||||||||| |||||| |||||||||||||||||  |||| ||||||| || ||||
Sbjct  81   AGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATAC  140

Query  133  CGCGTAACGTCGCAAGACCAAAGAGGGGGACCTTCCGGGCTCTTGCCATTTAATGTGCCC  192
            ||| ||||||||||||||||||||||||||||| || ||||||||||   ||||||||
Sbjct  141  CGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCC  200

Query  193  CGATGGGATTAGCTAGTGATGGGGGGAACGGCTCACCTAAGCGACCATCCCTGGCTGGTC  252
             ||||||||||||||||    |||| |||||||||||| ||||| |||||| |||||||
Sbjct  201  AGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTC  260

Query  253  TGAAAGGATGACCACCCTCACTGTGACTGAGACACGGTCCTCACTCCTACGGGAGGCATG  312
            ||| |||||||||| || |||||  ||||||||||||||  ||||||||||||||||
Sbjct  261  TGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC  320

Query  313  GGTGGGGAATATTGCACAATGGGCGCCAGCCTGATGCATCCATGCCGCGTGTATGAAGAA  372
             |||||||||||||||||||||||| |||||||||| ||||||||||||||||||||||
Sbjct  321  AGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAA  380

Query  373  TGCCTTTTGGTTGTAAAGTACTTTTGGCGGGAAGGAAGGCGGTTAGGTTAATAACCTCTT  432
             |||||  |||||||||||||||  ||||| ||||||||| | ||||||||||||||
Sbjct  381  GGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGTTGAGGTTAATAACCTCAG  440

Query  433  TGATTGACCTTACCCACAAAACAAGCACCGGCTAACTCCGTGCCACCAGCCGCGTTCATA  492
             ||||||| |||||| || || |||||||||||||||||||||| |||||||| | |||
Sbjct  441  CGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA  500

Query  493  CGGAGGGTGCGTGCGTTAATCTTACTTACTGTGCGTAAAGCGCACGCAGGCGGTCTGTCA  552
            ||||||||||  |||||||||  | |||||| ||||||||||||||||||||||||||||
Sbjct  501  CGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCA  560

Query  553  AGTATGTATGTGAAATCCCC---CTCGATCTCCTGGGAACTGCATTCCTGACTGGC-TGC  608
            |||  | |||||||||||||   ||| |   |||||||||||||||   ||||||  ||
Sbjct  561  AGT-CGGATGTGAAATCCCCGGGCTCAA---CCTGGGAACTGCATTCGAAACTGGCAGGC  616

Query  609TATATTCTTGTAGAgggggggTATAATTCTTGGGTGTAGCGGTATAAATTGCGTATAGAG  668
            || | ||||||||| |||||||| |||||   ||||||||||  | || |||||| |||
Sbjct  617  TAGAGTCTTGTAGA-GGGGGGTAGAATTC-CAGGTGTAGCGG--TGAAATGCGTAGAGAT  672

Query  669  CTGGAAGAA  677
            ||||| |||
Sbjct  673  CTGGAGGAA  681
```

Figure 4.14: Pair wise alignment between *Rhizobium* sp. SOY12 (1st match) and Bean.

The pairwise alignment of the first match of each isolates Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean are shown in fig 4.7-4.14.

4.4.3 Phylogenetic analysis and identification of the strains

Phylogenetic tree were constructed from pair wise alignment between the BLAST related sequences for each *Rhizobium* strain strains. A total of 25 related blast sequences randomly selected for constructing phylogenetic tree. Neighbour joining algorithm used to produce a tree from given distances (or dissimilarities) between sequences (Saitou and Nei, 1987). Distances between sequences were analyzed from the NCBI website (http://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi?) and the unrooted tree date downloaded as Newick format.The unrooted tree opened in MEGA VI phylogenetic tree software then edited and resizing (Tamura *et al.,* 2013). The phylogenetic positions of all isolates within different subgroups were investigated by comparing their 16S rDNA sequences to those representatives of various genera. Three different groups can be seen from the tree: It is evident from the phylogenetic tree that the isolated strains Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean are homologous to bacterial strain *Rhizobium* sp.CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY12, *Rhizobium* sp.CCNWYC119, *Rhizobium*sp.CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY7, *Rhizobium* sp. SOY12 respectively. The distance was indicated at the branches and its nodes.

Sample 1 - Green Gram

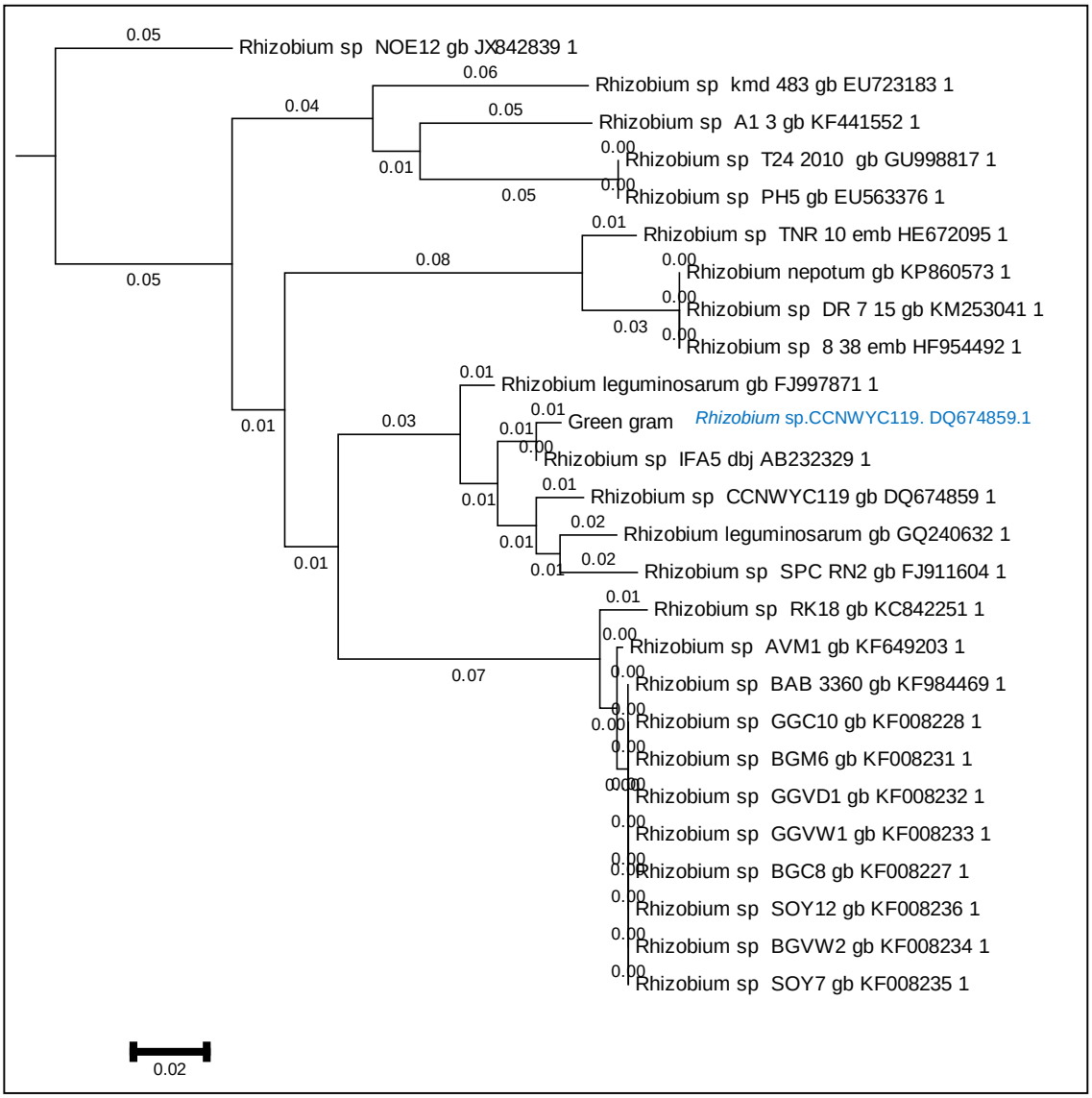


Figure 4.15: Unrooted Phylogenetic tree showing the genetic relationship among the cultivated bacteria Green Gram and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.02 = 2% difference among nucleotide sequences.

Sample2- Black Gram

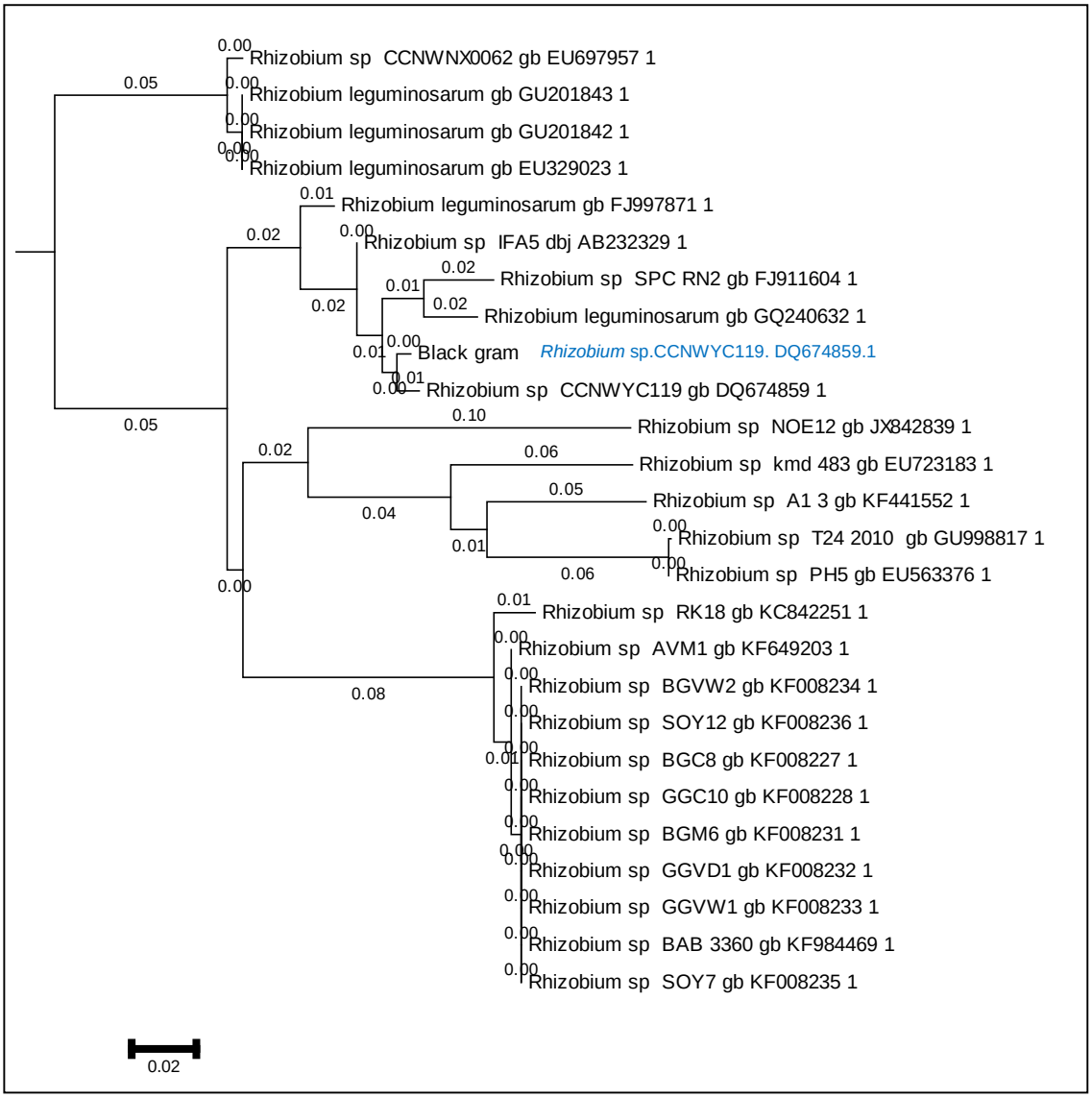


Figure 4.16: UnrootedPhylogenetic tree showing the genetic relationship among the cultivated bacteria Black Gram and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.02 = 2% difference among nucleotide sequences.

Sample 3- Lentil

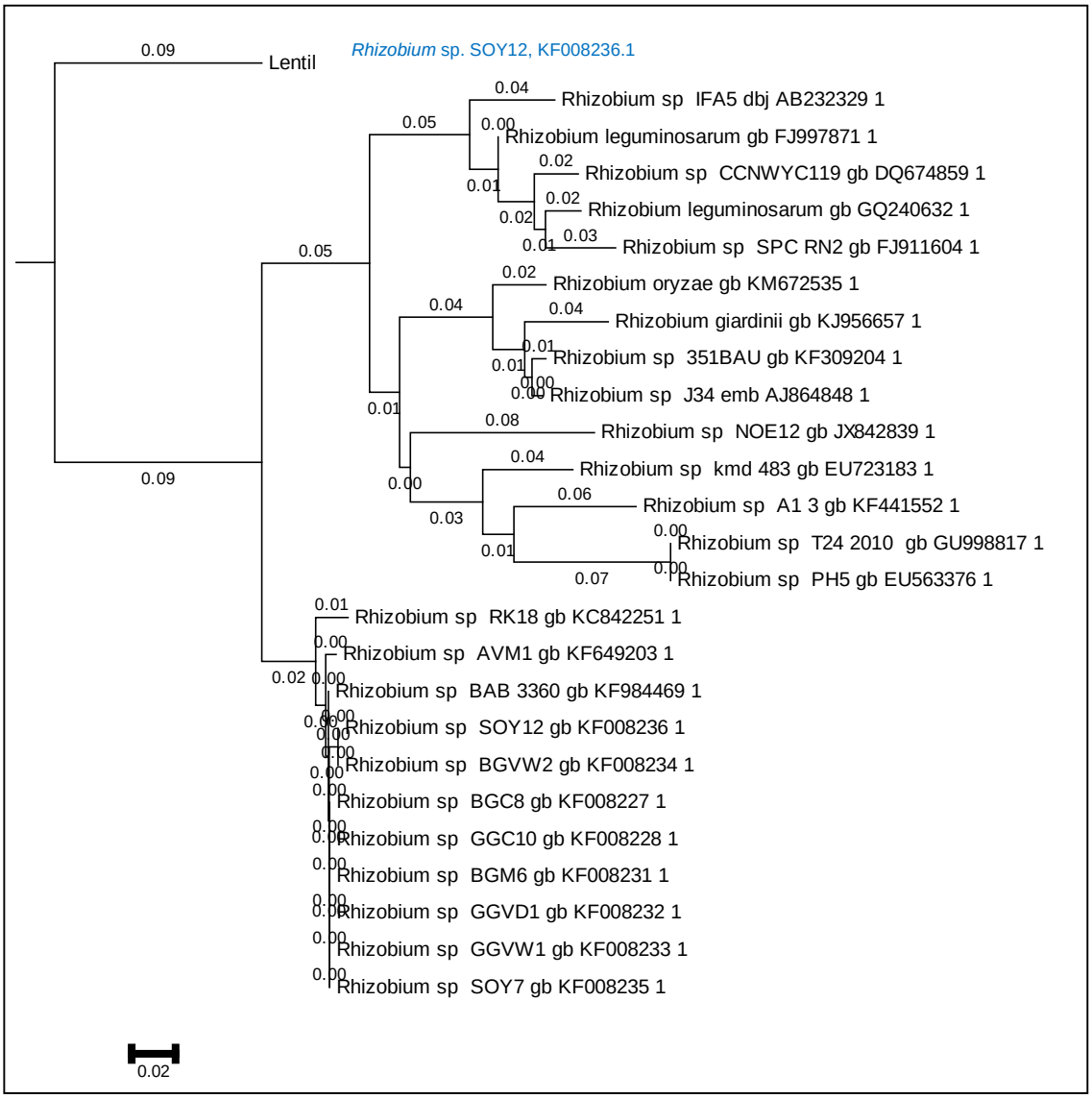


Figure 4.17: Unrooted Phylogenetic tree showing the genetic relationship among the cultivated bacteria Lentil and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.02 = 2% difference among nucleotide sequences.

Sample 4 -Pigeon Pea

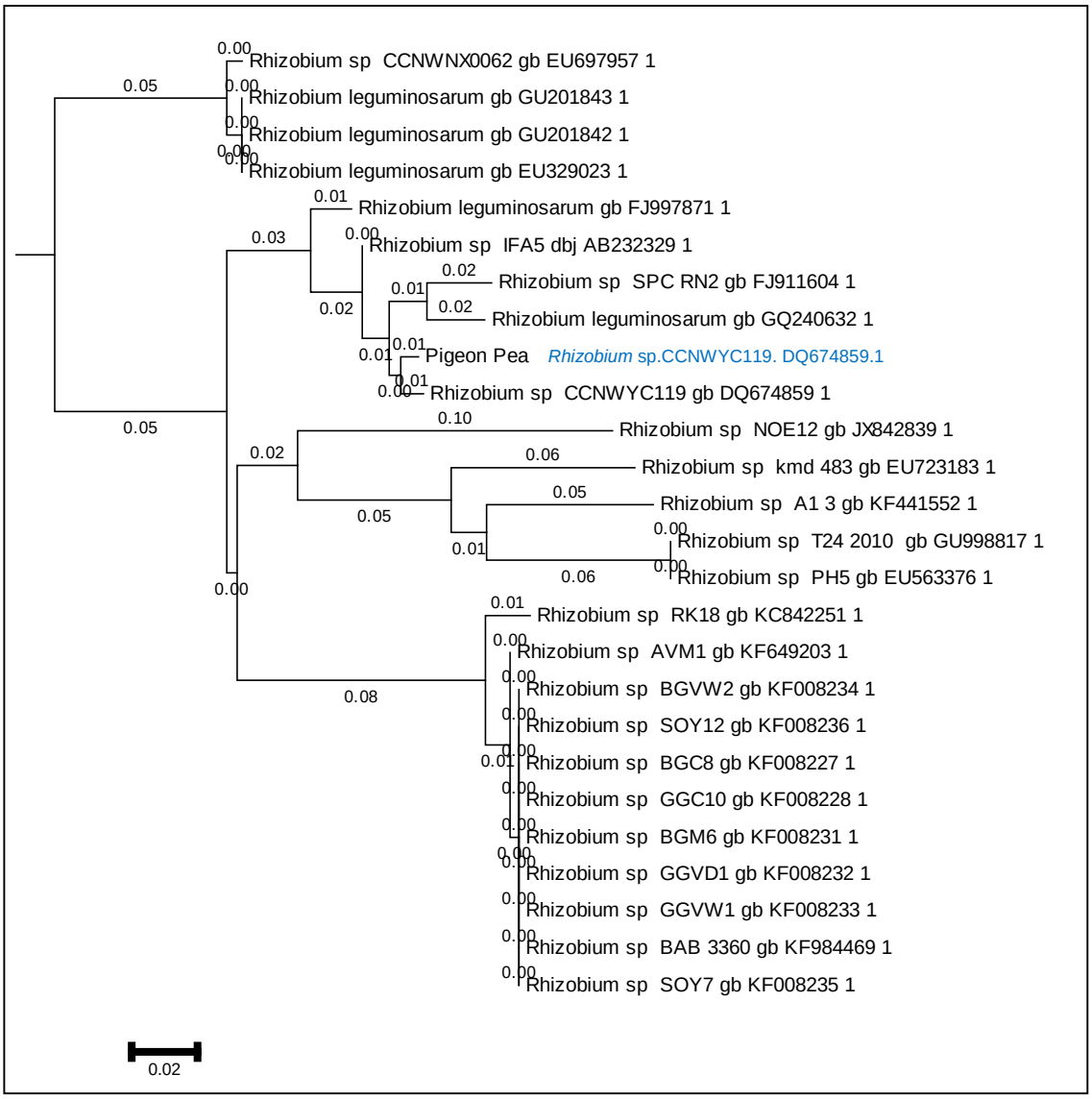


Figure 4.18: Unrooted Phylogenetic tree showing the genetic relationship among the cultivated bacteria Pigeon Pea and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.02 = 2% difference among nucleotide sequences.

Sample 5 - Sweet Pea

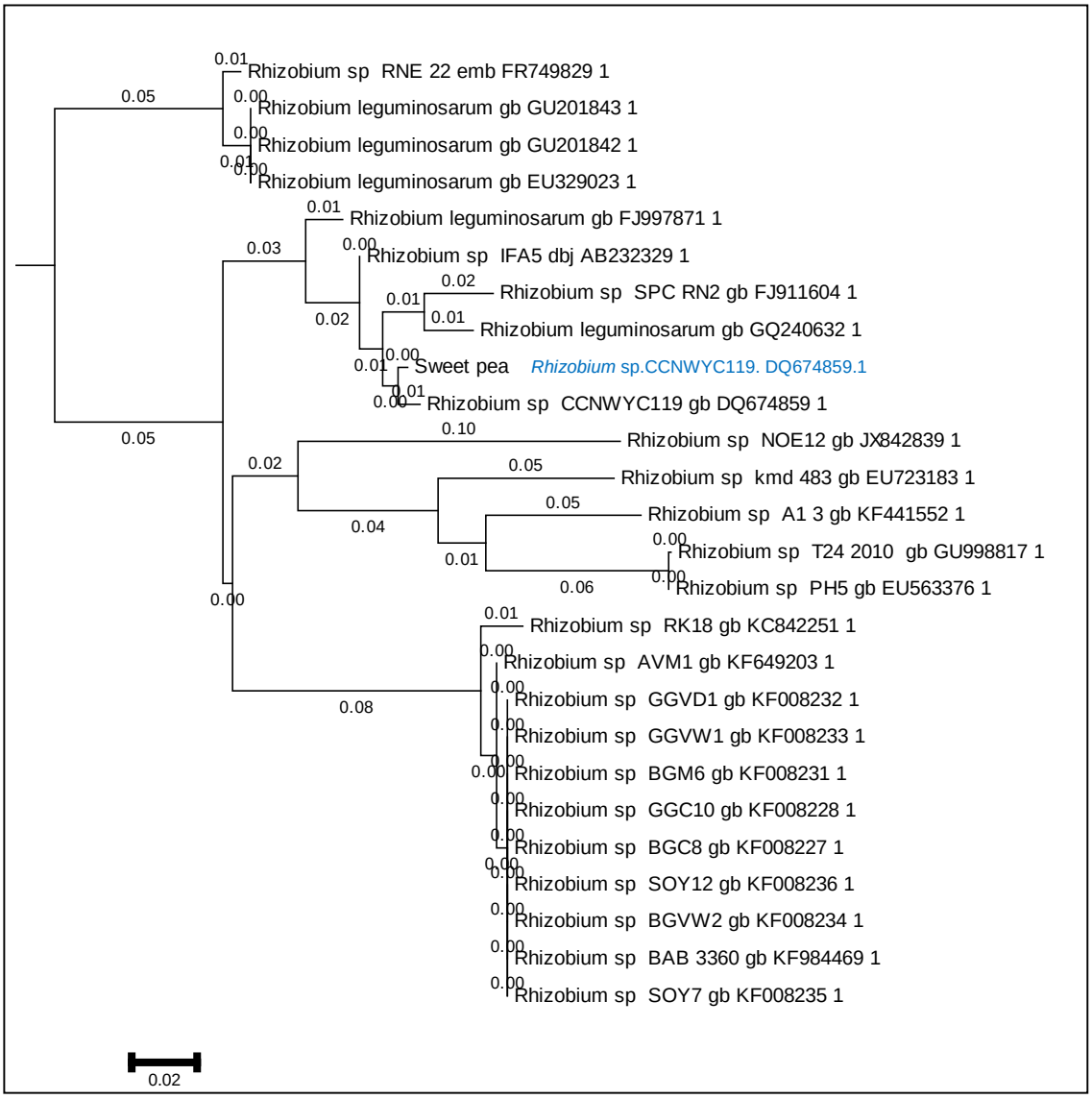


Figure 4.19: Unrooted Phylogenetic tree showing the genetic relationship among the cultivated bacteria Sweet Pea and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.02 = 2% difference among nucleotide sequences.

Sample 6- Chick Pea

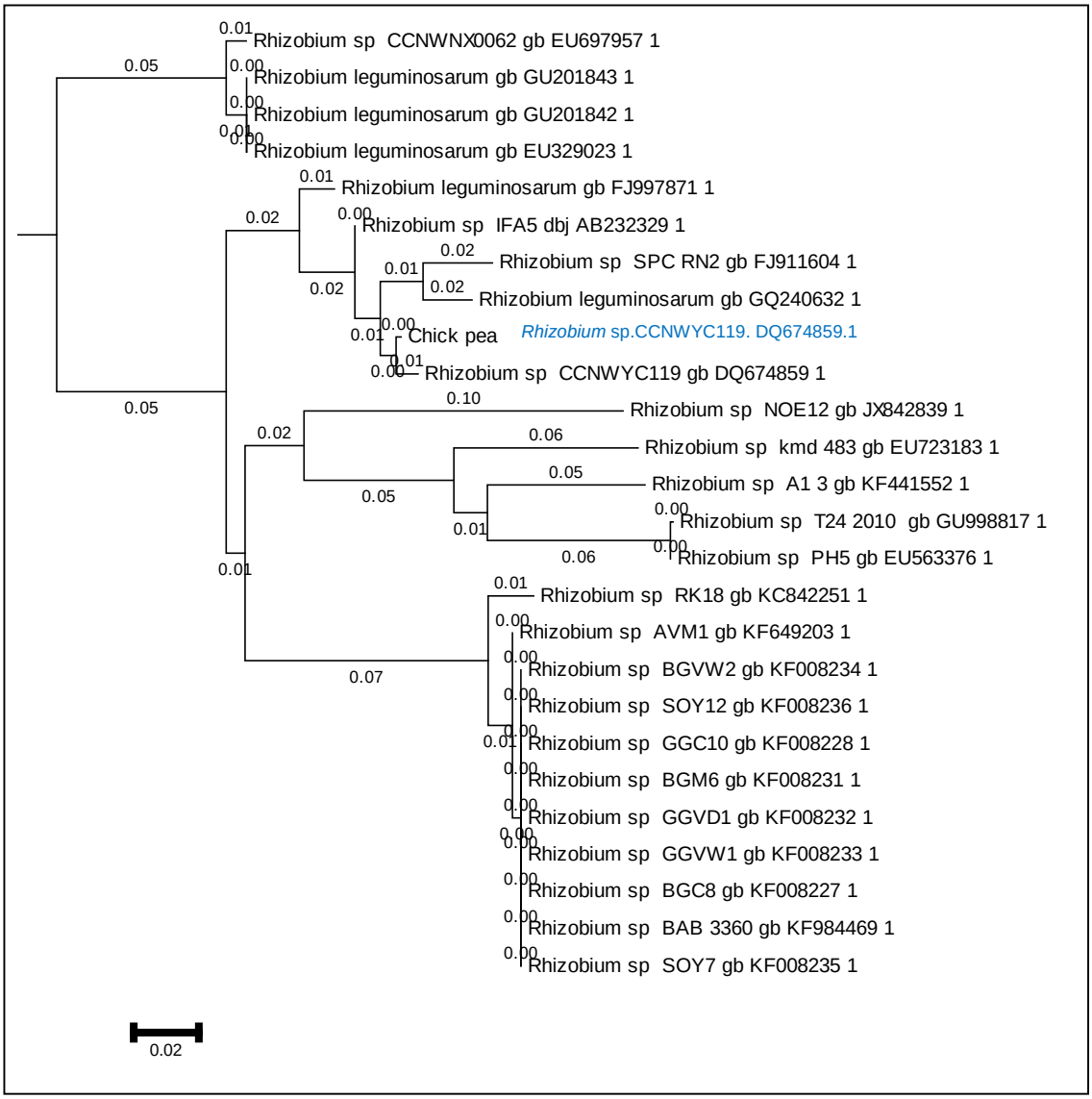


Figure 4.20: Unrooted Phylogenetic tree showing the genetic relationship among the cultivated bacteria Chick Pea and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.02 = 2% difference among nucleotide sequences.

Sample 7 - Cow Pea

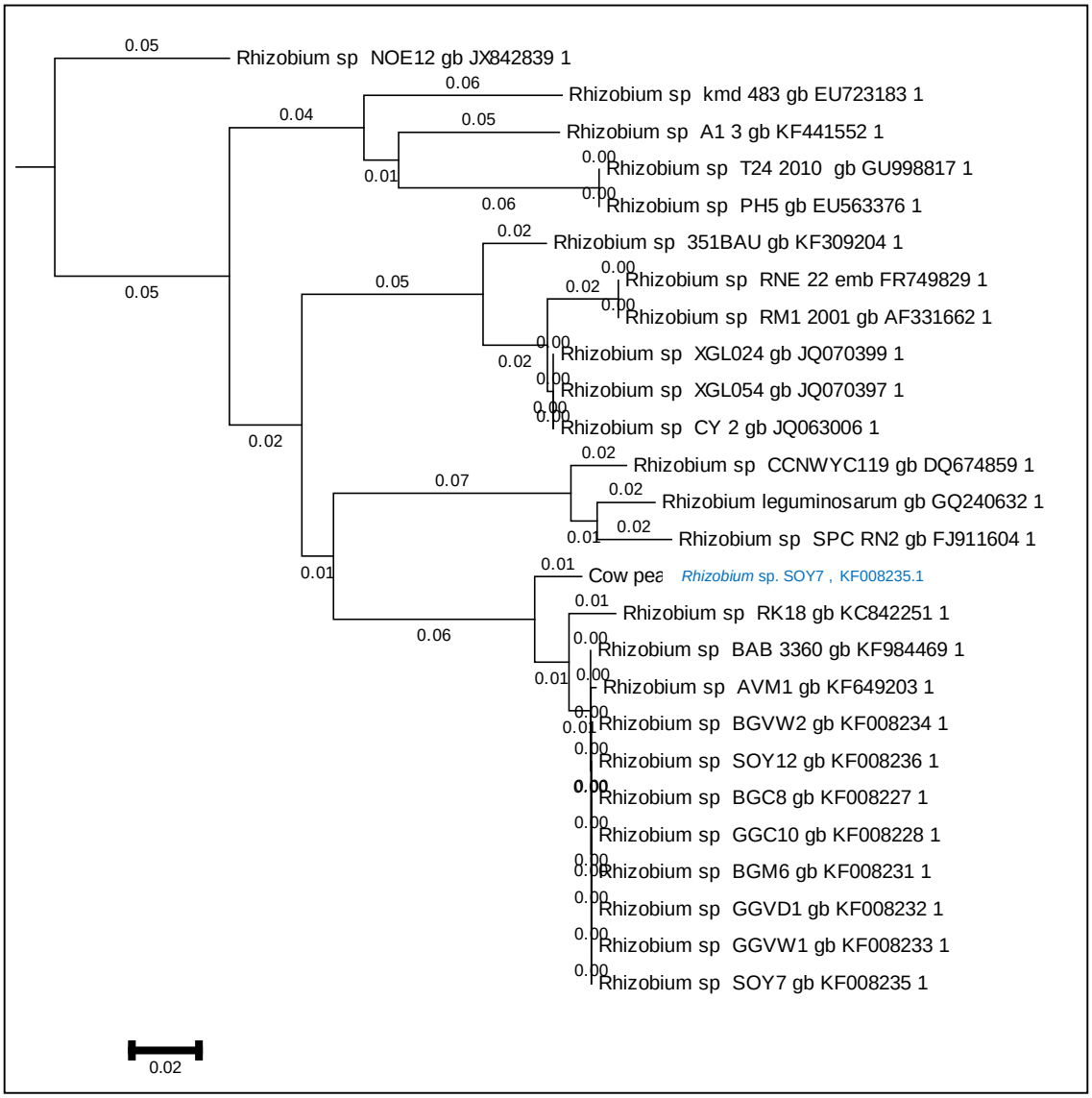


Figure 4.21: Unrooted Phylogenetic tree showing the genetic relationship among the cultivated bacteria Cow pea and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.02 = 2% difference among nucleotide sequences.

Sample 8- Bean

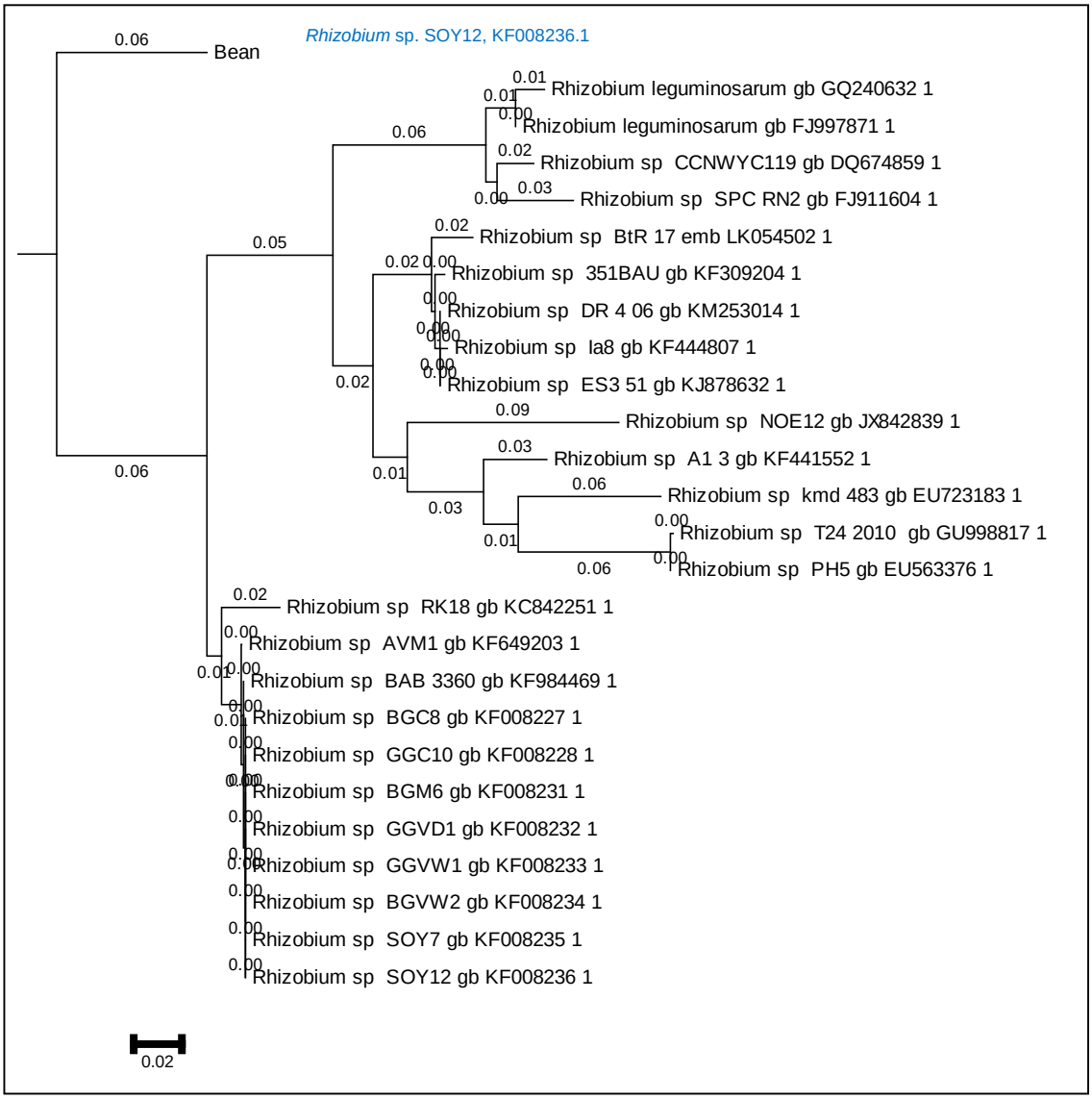


Figure 4.22: Unrooted Phylogenetic tree showing the genetic relationship among the cultivated bacteria Bean and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.02 = 2% difference among nucleotide sequences.

4.5 DISCUSSION

In general, the phenotypic study showed large physiological and biochemical biodiversity. Indeed, the studied strains showed a variable resistance against stress factors, temperature, pH, salinity resistance to antibiotics and heavy metals, which allowed the selection of good candidates for genetic studies. Several investigators have studied the genetic diversity of *Rhizobium* isolated from several countries around the world (Amanuel *et al.,* 2000; Mutch *et al.,* 2003; Mutch and Young, 2004; Tian *et al.,* 2007; Shamseldin *et al.,* 2009).

Identification of bacteria in most microbiological laboratories of the third world depends on their phenotypic properties because of the unavailability of molecular techniques due to financial and technical support. Genetic diversity in tropical rhizobial species is still poorly known. A false or weak positive and negative reaction, which often occurs in routine laboratories, leads a misidentification of bacterial species. The phenotypic properties presented herein, might he a useful phenotypic scheme to identify the bacteria from legumes plants in the microbiological laboratories. However, it needs I6S rDNA sequencing experiment for further identification of the phenotypic species. These experiments will help to know the relationship between the phenotypic and genotypic identilication as well as the phylogenctic position of the identified isolates. 16S rRNA sequencing has helped the description of *Rhizobium* sp.

The identification of bacterial species in most bacteriological laboratories is based on phenotypic properties. Although this method has been quite successful, it has not been precise enough to distinguish closely related species. Bacterial genus, with wide phenotypic variations, the problem becomes aggravated resulting in confusion of the identification, which hampered its clinical significance. It would seem that phenotypie identification based on routine procedures in most laboratories may not be sufficient to identify bacteria to the species level. Among the molecular techniques PCR is undoubtedly the most efficient tool the diagnosis of bacteria. Several protocols were applied, but due to the lack of' data on the genome sequence of *Rhizobium* sp. from legume plants.Amplification of the 16S rDNA gene region is a simple technique to he performed for the characterization of large numbers of isolates. Data from this study show the existing genetic variation within a Supposedly conserved gene, 16S rDNA, and they can explain the diflicultyof establishing diagnostic protocols for molecular

characterization of the studied strains. In this study, bacteria were first identified comparing the biochemical properties then the phenotypic identificaion was confirmed by genotypic identification. It appears from our study presented herein. There are not sufficient phenotypic properties, which can identify bacterial isolates with confidence Present study, however, concentrated on the nitrogen fixing strains. Hofer's alkaline broth test conducted is because *Agrobacterium* grows at higher pH levels and not rhizobia. The isolates strains failed to utilize peptone when were Grown on glucose peptone agar medium. *Rhizobium* respond negatively ketolactose test. Microscopic observations on pure culture cells confirmed the gram-negative nature. Bacterial cells once inoculated on pre-sterilized yeast extract Mannitol Agar (YEMA) produced white, translucent glistering Colonies with entire margin soil samples from Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Beanwere subjected to the above mentioned biochemical parameters. The composition of DNA in bacterial genome is similar as it shows presence of all the four defined bases. The helix of DNA with double stranded structure shows pairing between A+T and G+C, thus (A+T)/ (G+C) ratio or (G+C) content reflects compatibility of microbial strain in relation to evolutionary stress. The G+C content is examined in the form of temperature of melting (Tm). The bases are joined by hydrogen bonds and show regular pairing. It is obvious that the DNA with higher G+C content will stand higher melting temperature as more energy is needed to separate the doublestranded DNA. The melting temperature thus is calculated by observing midpoint of the rising curve. The optical density of DNA shows further use in the presence of greater amount of G+C content. In all the organisms ranging between eukaryotes to prokaryotes, the highest degree of variation is observed in case of microbes (between 25-85%).

The composition of G+C content with slight variation shows similar base sequences thus giving emphasis on relatedness among species in contrast to dissimilarity as observed in PCR-based observations. Samples analyzed presently, amongst eight strains *i.e.*, Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean. Thus present observation as listed in provides most similarity amongst microbial isolates in relation to DNA as a parameter. Furthermore, the pattern of genetic diversity was studied using known and Genotyping of the isolates was done by using molecular methods. Taq I restriction enzyme when used to have 16S rRNA digestion the group of strains showed variation in pattern on DNA profiling studies

with 1.0 % agarose (Fig. 4.1,4.2 &4.3). In the presence of Taq I the digestion of 16 S rDNA showed bands between 700 bp and 1000bp. DNA profile once put on computation data on BLASTn search performed through NCBl BLAST search engine and Nueleotide sequence from genetic analyzer.

In the studies reported here, the genetic biodiversity of eight represented strains were examined .The genetic study carried out is a quick method that gives a better idea of the diversity of these strains. The 16S rDNA was sequenced to determine the taxonomic position of these strains, and the results revealed that there was a great genetic diversity among the eightrhizobial strains studied. Indeed, sequence analysis of 16S rDNA and subsequent BLASTn analyses indicated that six strains had 95 to 98% and other two bellow similarity with *Rhizobium* sp.

The variable fragment of 16s rDNA amplified by using primers 8F and 806R primers (08) on DNAfrom strain eight was sequenced in our research .The sequence of that fragment in the full sequence obtained in this research is identical with the partial sequencing result. An unrooted phylogenetic tree was generated by the neighbor-joining method based on a comparison of the 16s rRNA gene sequences of all available rhizobial species and related bacteria. Five different primers were used and combination of 8F-806Rand 8F- I492R gave more contrast single band in between 700 - 800bp in comparison to 1 kb plus DNA ladder. PCR was carried out with an increased volume (50 µl) using 8F-806R. PCR products were purified and prepared for sequencing PCR. The BLASTn search results for the partial sequences of the 16S rRNA gene of the isolates revealed Purity and concentration of extracted DNA were performed at 260/280 nm obtained as 1.77 for Green Gram, 1.74 for Black Gram, 1.74 for Lentil 1.77 for Pigeon Pea, 1.85 for Sweet Pea, 1.77 for chick Pea, 1.82 for Cow Pea,and 1.84 for Bean and concentration as 42.0, 83.9, 28.9, 49.0, 88.5, 68.8, 86.8 and 69.7 ng/µl for Green Gram , Black Gram, lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean, respectively. Finally the selected eight bacterial isolates were identified up to species as *Rhizobium* sp. through 16S rDNA gene sequencing. There were eight experimental plants from which eight colonies of Rhizobium were isolated of which five strains showed similarity among them (*Rhizobium* sp. CCNWYC119) another two strains were also similar (*Rhizobium* sp. SOY12) and only one (*Rhizobium* sp. SOY7) was different from based on the nucleotide sequence.The 16S

rDNAsequence reveals that the isolated strains Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Beanare homologous to bacterial strain *Rhizobium* sp.CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY12, *Rhizobium* sp.CCNWYC119, *Rhizobium*sp.CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY7, *Rhizobium* sp. SOY12 respectively.

PCR amplicons of 16S rDNA was sequenced and analyzed using bioinformatics softwares. Distance Matrix based on Nucleotide Sequence Homology and alignment using combination of NCBI GenBank and The sequences were submitted to NCBI data Bank having accession no s. Sequence ID DQ674859.1, DQ674859.1, KF008236.1, DQ674859.1, DQ674859.1, DQ674859.1, KF008235.1 and KF008236.The cultures were identified as *Rhizobium* sp. CCNWYC119, *Rhizobium* sp.CCNWYC119, *Rhizobium* sp. SOY12, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp.CCNWYC119, *Rhizobium* sp.CCNWYC119, *Rhizobium* sp. SOY7, *Rhizobium* sp.SOY12 respectively.

The phylogenetic distances shown in Fig (4.15-4.22) indicate that the relationships among this group and the *Agrobacterium* species are as distant as those among the genera Mesorhizobium and Sinorhizobium this clearly showed that subgroup belonged phylogenetically to the genus *Rhizobium.* Although the phylogenetic position of subgroup was not found to be distinct from that of other *Rhizobium* sp. by analysis of the partial 16s rRNA sequences. Wang, *et al.*,1994, it was distinguished clearly from the *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY12 ,*Rhizobium* sp. CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY7, *Rhizobium* sp. SOY12 position by the full sequence of its 16s rRNA genes. So it seems that the partial sequencing of 16s rDNA has limited value in determining phylogenetic relationships among rhizobial species.

Based upon 16S rRNA gene sequences analysis, strains Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean were identified as *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY12 , *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY7 and *Rhizobium* sp. SOY12. Comparative sequence analysis of 16S rRNA is currently the most widely used approach for the reconstruction of microbial phylogeny (Rasche *et al.*, 2006).

In our study, we found that all the strains showed sequence similarity of 95 -98 % or less (Table 4.5). This low sequence similarity of the strain Lentil (79%) and Bean (85%) with the closely related members of *Bacillus* gives a further opportunity to investigate these strains taxonomically for delineation of possible novel species; however, the taxonomic studies are beyond the scope of this manuscript. 16S rRNA gene sequence of bacterial strains with similarity less than 97% can be declared as novel after complete taxonomic characterization as reported by Lim *et al.,* (2006).Our strains revealed diverse morphological, physiological and biochemical behavior. The idea here was not only to identify but also to find some promising strain with unique traits such as potential candidates to solubilize phosphate, induced systematic resistance and plant growth improvements.

In this study, the symbiotic association of *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. CCNWYC119, *Rhizobium* sp. SOY12, *Rhizobium* sp. CCNWYC119 , *Rhizobium* sp. CCNWYC119 , *Rhizobium* sp. CCNWYC119 , *Rhizobium* sp. SOY7 and *Rhizobium* sp. SOY12. With legumes of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean and symbiotic association of Rhizobia and leguminous plant has been reported for the first time the Identifier Strain are *Rhizobium* sp. BDRAJGG101, *Rhizobium* sp. BDRAJBG102, *Rhizobium* sp. BDRAJL103, *Rhizobium* sp. BDRAJPP104, *Rhizobium* sp. BDRAJSP105, *Rhizobium* sp. BDRAJCP106, *Rhizobium* sp. BDRAJCP107 and *Rhizobium* sp. BDRAJB108 in Rajshahi region of Bangladesh.

Bangladesh is a developing country facing malnutrition problems.This is why So protein-rich crops might play a significant role to meet up this problem. Pulse crops are legumes containing enriched nutrients might be an important source of plant protein.There were eight experimental plants from which eight colonies of *Rhizobium* were isolated of which five strains showed similarity among them (*Rhizobium* sp. CCNWYC119) another two strains were also similar (*Rhizobium* sp. SOY12) and only one(*Rhizobium* sp. SOY7) was different from based on the nucleotide sequence. Around 5.2% of cultivable lands are subjecte to legume cultivation. As a bio-fertilizer, *Rhizobium* significantly improves the yield in many legume crops by fixing atmospheric nitrogen in symbiosis with legumes. So, in order to accelerate the increase of production of legume cropsfor the uplift of protein status nationally as wellas internationally it is important to study the ecological characterization and identification the *Rhizobium* spp.

4.6 CONCLUSION

On the basis of different physiological and biochemical parameter studied, it seems all the strain of *Rhizobium* sp. are same. Findings on the isolated Rhizobium strains from agricultural soils of Rajshahi, Bangladesh. with regard to the level of gene sequences will help establish the improved strains as biofertilizers. The utility of present observation falls with relative similarity between patternsof genome i.e., alignment of bases of DNA, although preference was given to the pattern of blastn search performed through NCBl BLAST search engine and Nueleotide sequence from genetic analyzer. Thus, the present observations give an insight on molecular orientation of Rhizobium species occurring naturally in the agricultural soils of Rajshahi, Bangladesh.

The present study deals with investigating the bacterial diversity in root nodules and rizosphere in highly diversified agricultural areas of Bangladesh. We attempted to culture indigenous microbes collected from these areas. Their identification based on molecular analysis gives us an edge to have more cultured microorganisms with their taxonomy from indigenous environments. The microbial diversity can prove to be a valuable future resource in various industrial and biotechnological processes. Such microbes can also be used as a source of gene(s) that can increase phosphorus and nitrogen uptake in different crop species through genetic transformation.Bangladesh is a developing country facing malnutrition problems. This is why so protein-rich crops might play a significant role to meet up this problem. Pulse crops are legumes containing enriched nutrients might be an important source of plant protein.

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Chapter 5

Effects of *Rhizobium* on Nodulation of Different Types of Leguminous Plants

CONTENTS

Chapter 5

Effects of *Rhizobium*on Nodulation of Different Types of Leguminous Plants

5.1 INTRODUCTION AND LITERATURE REVIEW

Rhizobia are Gram negative bacteria that exist in a symbiotic relationship with several grain legumes as a host plants. In this association, the host plant provides the bacteria symbiont with sugars and a protected environment, while the bacteria fix nitrogen from the air and make it available to the plant in the form of ammonia (FAO, 1985; Kiers *et al.,* 2002). In the Rajshahi, Bangladesh where the population diet is mostly based on cereals, there is a need to improve the production of common grain legumes such as Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, CowPea and Bean inoculation technology, if full benefit from these crops is to be achieved in terms of maximum yield and soil fertility. Legumes play a critical role in natural ecosystems, agriculture, and agroforestry, where their ability to fix N in symbiosis makes them excellent colonizers of low-N environments, and economically friendly crop, pasture, and tree species (Jensen and Hauggaard-Nielsen, 2002). In addition to its role as a source of protein in the diet, N from legume fixation is essentially "free" for use by the host plant and by associated or subsequent crops (Kiers *et al.,* 2002). Legumes also have positive effects on agriculture by adding and recycling biologically fixed N, enhancing nutrient uptake, reducing greenhouse gas emissions by reducing N-fertilizer use, and breaking non-legume crop pest cycles (Zahran, 1999; Lupwayi and Kennedy, 2007). Legume yields unfortunately continue to lag behind those of cereals. A research orientation that better balances the needs of sustainable oriented agriculture with the breakthrough low-cost technologies such as rhizobial inoculation is required. Most grain legumes produced by small holder farmers are not often inoculated with rhizobia or applied with fertilizers (Ogendo and Joshua, 2001).

To date, there has been only limited commercial use of plant growth-promoting bacteria in agriculture and horticulture (Vessey, 2003). However, with recent progress towards understanding the mechanisms that these organisms utilize to facilitate plant growth, the use of plant growth-promoting bacteria is expected to continue to increase worldwide (Arshad And Frankenberger, 1993; Cheng,2003). Generally, grain legumes do not need additional nitrogen if the seed is effectively inoculated at planting. Inoculation with the most efficient N-fixing bacteria for the type of legume is necessary to help maximize nodulation and N-fixing ability (Pal and Shehu, 2001). In fact, rhizobia are randomly distributed in growing soils where they compete with other

indigenous strains which often form less efficient nodules (Simms and Taylor, 2002). However, the mechanisms which determine the relative competitiveness among strains are still poorly understood (Kober*et al.,* 2004). The study of *Glycine max*-rhizobia association in the Guinea-savannah zone of Cameroon has revealed the efficiency of indigenous strains over the exotic ones (Megueni*et al.,* 2006), although these later showed efficiency in other agroecological zones for *Vignaunguiculata* (Ngakou, 2007). Rhizobia are fairly specific as to which legumes they will infect, form nodules on the roots of, and for which they will fix nitrogen (Lupwayi and Kennedy, 2007). In order to ensure the availability of the correct effective strain specific to a legume plant species, an inoculation practice should be performed, particularly when the legume being planted has not been grown in that field in at least the 1079 past three years or with every planting of a high value crop (Zdor and Pueppke, 1990). Researchers have sought for ways of improving nitrogen fixation and enhancing yield of grain legumes by selecting efficient strains for production of inoculants (Hynes *et al.,* 2001). Vance and Graham (1995) emphasized the importance of understanding the characteristics of indigenous rhizobia populations as the prerequisite for inoculants production. These characteristics would be considered in the selection of strains which would be most adapted in a particular region.The objectives of this study were to characterize and produce the native population of rhizobia nodulating eight grain legumes in soil types of Rajshahi. During this study, the most efficient strains would be selected and used to produce rhizobia biofertilizers. Such fertilizers are expected to improve the growth of grain legumes in the region.

Pulses like Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean etc. are being grown in Bangladesh as winter (Rabi) crops very enormously which meet somewhat the protein requirements of our huge population. Optimum protein content in our daily food is not that easy as most of our poor people need, because they live in sub-human condition. As the per capita availability of animal protein is decreasing in our country, so, it would be wise to obtain more plant protein from pulses for our people by improving the pulses for high yield at low cost.

Pulses are second important crop, next to cereals as our food source in Bangladesh, thus it certainly bears impact in socio-economic development of our country. Pulses have high seed protein, such as in lentil - 25.0%, in black gram - 23.4%, in mungbean -

23.6%, in cowpea - 28.2%, in pigeonpea - 22.5% and in chickpea - 23.0% (RahmanandParth, 1988). These are nearly two to three times to that of cereal grains. Secondly, the amino acid composition of pulse protein is such that a mixed diet of cereals and pulses has a biological value more than that of either component alone.

A major part of the peoples of Bangladesh suffer from malnutrition due to deficiency of protein in their daily diets. In such cases, pulses may play important role because they contain 20-25% protein, double of that of wheat and three times of that of rice. Pulses contain more protein than eggs, fish or than any fresh food and are generally high in protein content and digestibility of that protein is also high.Human beings need many essential food materials to survive on earth and these are protein, carbohydrate, fat, minerals, vitamins etc. They can obtain those food materials directly from the plants and from these individuals which are also dependent on plants. This way, plants are playing vital role in human life constantly along with few other important functions. Among the important crops of Bangladesh, pulses may be considered as the second most important crop and for a long time pulses particularly Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean etc are being cultivated as winter crop.

5.1.1 Leguminous plants

Leguminous plants include many important species that are used as food and fodder crop throughout the world. They can provide their own nitrogen requirements through nitrogen fixation in symbiosis with soil bacteria collectively known as rhizobia. These bacteria form root nodules on leguminous plants and convert atmospheric N into a form usable by plants. Application of effective rhizobial strains as biofertilizers to improve legume production is an important approach in sustainable agriculture.Many studies have recently shown that inoculation with some plant growth-promoting bacteria (PGPB), increases growth and yield in great number of plants including legumes. PGPB can contribute to plant growth in different manners: by increasing nitrogen uptake, synthesis of phytohormones (auxin, cytokinin), minerals solubilization and iron chelation. PGPB also have beneficial effects on legume growth, and at least some strains enhance nodulation and nitrogen fixation by affecting interactions between plant and rhizobia. Available reports indicate improved legumes yield, health and nodulation when co-inoculated with PGPB, compared to inoculation with rhizobium alone.

Green Gram

Green Gram (*Vigna radiate* L.) constitutes the important group of grain legumes which form a major source of dietary proteins of high biological value, energy, minerals and vitamins (Taylor *et al.,* 2005). Inoculation of *Rhizobium* sp. causes a greater increase in growth and yield and the number of nodules per root system is significantly higher in plants inoculated with *Rhizobium* sp. compared to plants without *Rhizobium* sp. under field condition (Akhtar*et al.,* 2009). Soil microorganisms produce quite a number of extra cellular enzymes to decompose the complex organic matter before it is absorbed as a source of energy. Seasonal variations in enzymes activities in forest soils are seen to bear correlation with the counts of fungi and bacteria (Kathiresan and Selvam, 2006; Richard *et al.,*2007). The present study is to isolate and characterize*Rhizobium* from leguminous plants and induce the growth of black and green gram by inoculation of *Rhizobium* as bio-fertilizer.

Black Gram

Black Gram (*Vignamungo)* is the important pulse crop in Bangladesh. It is annual pulse crop and native to central Asia. It is also extensively grown in West Indies, Japan and other tropics/subtropical countries. Black Gram seeds are highly nutritious containing higher amount of protein (24-26%) and are reported to be rich in potassium, phosphorus and calcium with good amount of sodium. In Bangladesh, about 10.5% of pulses are obtained from black gram and it is grown as kharif crop in some areas mainly in the north/north-west part of the country. It is successfully cultivated in the districts of Rajshahi,ChapaiNawabganj,Pabna, Jessore, Jhenaidah and Faridpur.

Lentil

Lentil (*Lens culinaris*L.) occupies the top position in terms of popularity and has been placed second in respect of area and production in Bangladesh. Lentil is one of the oldest annual grain legumes consumed and cultivated in the world. Originating from South western Asia as early as 6000 B.C., lentil is rich in proteins and contains high concentrations of essential amino acids like isoleucine and lysine, as well as other nutrients like dietary fiber, folate, vitamin B, and minerals. Lentil is widely consumed in various parts of the world as loaves, soups, pies, curries etc., especially in vegetarian

cultures. It is also an important source of dietary protein in the Mediterranean and South Asian regions. Lentil is a cool season pulse crop and is also relatively tolerant to drought. Lentil is an important pulse crop grown widely through out the world (Ford and Taylor, 2003; Erskine, 1997).Especially in Bangladesh, India, Canada and Turkey.It is cultivated during rabi season under rainfed condition. About 80% of total lentil in the country is grown in Faridpur, Kustia, Jessore, Rajshahi and Pabna. The yield of lentil is very poor. There is a great possibility to increase its production by exploiting better colonization of their root and rhizosphere through *Rhizobium* bacteria, which can reduce nitrogenous fertilizer use and protect environment. But Bangladesh soils have lack of sufficient and effective *Rhizobium* strains in most of the cases. Moreover, degradation of *Rhizobium* occurs regularly. So, collection and screening of new *Rhizobium* strains and their sub-culturing and testing are necessary for quality inoculant production. For this reason, indigenous *Rhizobium* strains were collected from different AEZs of Bangladesh for lentil and were screened. Their efficiency in lentil production needs to be tested. The present study was, therefore under taken to find out the effectiveness of some *Rhizobium* strain/(s) on lentil at Rajshahi, Bangladesh.

Pigeon Pea

Pigeon Pea (*Cajanuscajan* L.) is the only crop member of the Cajaninae tribe (Hancock, 2004). This species is a very rustic crop that can grow vigorously in soils with low fertility, and for this reason, is cultivated mainly in marginal lands in BangladeshIndeed, Pigeon Pea is able to associate with a large diversity of indigenous rhizobia in soil. To exploit the BNF potential of this crop, the selection and evaluation of new rhizobial strains from different areas where pigeonpea is cropped must be carried out. The slow and fast growing pigeonpea rhizobia present great genetic and metabolic diversity and are likely to have new species among the culture collections worldwide (Ramsubhag*et al.,* 2002; Fernandes Jr. *et al.,* 2012). In addition to being efficient in fixing nitrogen in field conditions, pigeonpea rhizobia also present other biotechnological applications, such as biopolymer production and enzymatic activity (Fernandes Jr. *et al.,* 2012).

Sweet pea

Sweet Pea (*Lathyrussativus* L.), locally known as Khesari, is a protein-rich legume grown in harsh conditions of dry to lowlying, water-logged and flooded land. The crop is widely cultivated in Bangladesh, India, Myanmar, Nepal and Pakistan in cold winter months under rainfed condition. It is also grown to a small extent in the middle Eastern countries, Southern Europe, and parts of Africa and South America. Among the pulses, it occupies the highest area (33%) and production (34%). Sweet Pea is the hardiest of the pulse crops because it can tolerate flooding, drought, and moderate soil salinity. This attribute has made it a very popular pulse crop as food and cattle feed among the poor farmers of Bangladesh. It is commonly cultivated as a relay crop in the wet rice fields without any input and care.

Chick Pea

Chick Pea *(Cicerarietinum*L.) is a major pulse crop in Bangladesh. The average yield of chickpea is low which can be attributed to lack of high yielding varieties and suitable rhizobial strains capable of fixing high atmospheric nitrogen. The soils of Bangladesh, especially Rajshahi regions are deficient in nitrogen fixing bacteria *(Rhizohium*spp.) which causes poor yield of chickpea (Bhuiyan*et al.,* 1998; Khanam*etal.,* 1999). The Bangladesh Agricultural Research Institute (BARI) has developed a good number of varieties of chickpea. There is a good possibility to increase its production by exploiting better colonization of the roots and rhizospheres through application of effective nitrogen fixing bacteria to the seedor to the soil. This can minimize uses of nitrogenous fertilizer, which is very costly in this country. Using high yielding varieties of chickpea along with use of effective rhizohial strains can enhance the yield. Nutritionally, chickpea is relatively free from various antinutritional factors, has a high protein digestibility and is richer in phosphorus and calcium than other pulses.Because of its higher fat content and better fiber digestibility, chickpea holds great promise as a protein and calorie source for animal feed for both ruminants and nonruminants. *Rhizobium* inoculants significantly improves yield in many leguminous crops and can minimize the use of synthetic nitrogenous fertilizer, which is rather expensive and deteriorates soil properties. The crops thus improve soil and economize crop production not only for themselves but also for the next cereals or non-legume crop grown in the

rotation and thereby reducing the requirement of added nitrogen fertilizers. Research report showed that *Rhizobium* inoculation of pea plants added 80 kg N/ha over uninoculated control. Biological nitrogen fixation and seed yield of soybean significantly increased due to inoculation with *Rhizobium*. Field trials conducted in Bangladesh showed that grain yield of chickpea increased by 37 to 119% with *Rhizobium* inoculation. The number and mass of nodules of chickpea also increased with rhizobial inoculation a good number of varieties of chickpea have been developed in Bangladesh over the past years. Some of these varieties are being cultivated by the farmers of Bangladesh but their response to *Rhizobium* inoculation in respect to nodulation, dry matterproduction and nitrogen fixation has not been tested. Biological nitrogen fixation technology may be an important tool to increase production of pulses especially chickpea. Keeping these facts in mind the present investigation was, therefore, carried out to characterize some *Rhizobium* strains isolated from the root nodules of chickpea and to assess their effectiveness in respectof nodulation, dry matter production and nitrogen fixation in host legume.

Cow Pea

Cow Pea (*Vignaunguiculata*)is one of the main grain legume crops in Bangladesh. It is a high protein content food crop. As pea is a short durable crop. Its cultivation is highly profitable and preferable to the farmers. Inclusion of peas in crop rotation helps improvement of soil fertility and yield of the succeeding crops. Pea like other legumes is capable of fixing and utilizing atmospheric nitrogen through symbiotic relationship with *Rhizobium* bacteria at the root of the crop. *Rhizobium* inoculants significantly improves yield in many leguminous crops and can minimize the use of synthetic nitrogenous fertilizer, which is rather expensive and causes injury to soil properties. The crop thus improves soil and economizes crop production not only for itself but also for the next cereals or non-legume crops grown in the relation and there by reducing the requirement of added nitrogen fertilizers. Many researchers have reported the beneficial effects of inoculation of grain legumes. Seed inoculation with *Rhizobium* strains is known to influence nodulation and growth of pea.Most soils of Bangladesh contain appreciable number of ineffective *Rhizobium* strain. But the number of *Rhizobium* strains effective on Cow pea is very scarce in soils of Bangladesh.Keeping these facts in mind the present investigation was, therefore, carried out to characterize some

Rhizobium strain isolated from root nodules and to assess their effectiveness in respect of nitrogen fixation in Cow pea.

Beans

Beans(*Phaseolus vulgaris*)are the most important grain legumes for direct human consumption in the world. In nutritional terms, beans are great protein source and they are rich in minerals (especially iron and zinc) and vitamins. Symbiotic nitrogen fixing potential in common bean is considered to be low in comparison with other legumes. However, owing to genotypic variability for traits associated with N2 fixation potential selection has produced breeding lines able to fix high levels of N. The selection of particular rhizobial strains with high nitrogen fixing potential is also important. Phosphorus has a considerable influence on the legume-rhizobia symbiosis. Positive effects of P on nodulation and nitrogenase activity in common bean were observed. Nitrogen fixation in common bean is more affected by P deficiency than in other legume crops. Taking into account that some PGPB possess ability of phosphate solubilization, they could be useful in bean production improvement by increasing P content in the soil and enhancing nodulation and N fixation.

The soils of Bangladesh, especially Rajshahi regions are deficient in nitrogen fixing bacteria *(Rhizohium*spp.) which causes poor yield of pulse crop especially chickpea (Bhuiyan*et al.,* 1998; Khanam*etal.,* 1999). The Bangladesh Agricultural Research Institute (BARI) has developed a good number of varieties of pulse crops. There is a good possibility to increase its production by exploiting better colonization of the roots and rhizospheres through application of effective nitrogen fixing bacteria to the seed or to the soil. This can minimize uses of nitrogenous fertilizer, which is very costly in this country. Using high yielding varieties of chickpea along with use of effective rhizohial strains can enhance the yield. The present investigation was undertaken to study the response of different chickpea genotypes to *Rhizobium* inoculation in High Ganges River Floodplain soil of Bangladesh.

Food is the primary necessity of life. It serves the physiological functions related to supply of energy, building and maintaining the cells and tissues and regularity of body processes. These needs are satisfied by the nutrients present in the food. Mainly, cereals and pulses are the basic vegetarian food source for Bangladeshi society. Among this,

the pulses are designated as poor man□s meat as they contain considerable amount of proteins. Legumes are the sources of pulses. The evidence for the evolution of the legumes (Fabaceae), the third largest family of flowering plants, is fragmentary, at least based on fossil evidence. There are no obviously identifiable nodules associated with fossils that can be accurately described.An increase in the human population has been witnessed during the past four decades concurrently increasing the demand of food. This increase in the demand for food has led tothe need for improvements better plant nutrition to get higher yields. One solution lies in theuse of commercial man-made fertilizers. The use of these fertilizers has facilitated plantproduction at relatively low costs by using highly productive and intensive agriculturalsystems. The intensive use of organic or chemical fertilizers not only increases agriculturalyield but also causes degradation of soil health and environment quality. The use of theseinorganic fertilizers has increased exponentially since a few decades in a bid to counter theever increasing consumer demand. The use of nitrogen (N) fertilizer has increased by almostnine folds and phosphorus (P) fertilizer by more than four folds till recently. Mekki,*et al*.,2005. However, theabundant use of fertilizers and highly productive systems has led to environmental problemslike deterioration of soil quality, surface water, groundwater, air pollution, reducedbiodiversity and suppressed ecosystem function Brockwell, *et al.,*1995.Biofertilizers, also known as microbial inoculants, are the fertilizers that help plants meettheir nutritional requirements through biological fixation of essential elements as well as byenhancing rate of decomposition. This is done by supplementation with nitrogen,phosphorus and plant growth promoting rhizobacteria (PGPR) Singh, *et al.,*1991. These are carrier basedmicrobial preparations that contain beneficial microorganisms in a viable state intended forseed or soil application, which enhance plant growth through nutrient uptake and/or growthhormoneproduction.Rhizobia are a genetically diverse and physiologically heterogeneous group of bacteria thatelicit nodule formation on legumes. The most commonly studied genera are *Rhizobium*,*Bradyrhizobium*, *Azorhizobium*and *Sinorhizobium*. They are ubiquitous part of the soilmicro-flora and in a free-living state in the rhizosphere of legumes until the point ofnodulation. The nature and properties of soils allows billions of organisms to co-exist. Theability to form symbiotic relationships with members of the plant family *Fabaceae*is a uniquefeature associated with bacteria belonging to the family *Rhizobiacea* .Denarie,*et al.,*1992*e*. Rhizobia elicit ontheir host the formation of nodules in which they

fix nitrogen and thus, providing nutrition tothe plantDenarie, *et al.,*1992.However, under some conditions, nodulation may not occur due to the lack of suitablerhizobial strains as some soils remain void of it. For instance, acidic soils generally containno or low population densities of the alfalfa rhizobialsymbiont*Sinorhizobiummeliloti*,whereas basic soils contain a low inoculum potential of *Bradyrhizobium*sp., a rhizobialsymbiont of *Lupinus*spp.Kaur, *et al.,*2012. Under such conditions, inoculation with compatible rhizobia islikely to prove highly advantageous.Extensive work is being carried out to identify new species of endophytic yeasts as biocontrolagent or as a biofertilizer.

The rhizobia, are soil microorganisms that can interact with leguminous plants to form root nodules within which conditions are favourable for bacterial nitrogen fixation. Legumes allow the development or very large rhizobial populations in the vicinity of their roots. Infection and nodule formation require the specific recognition of host and *Rliizobium,* probably mediated by plant lectins. Penetration of the host by a compatible *Rhizobium* species usually provokes host root cell division to form the nodule, and a process of differentiation by both partners then ensues. In most cases the rhizobia alter morphologically to from bacteroids, which are usually larger than the free-living bacteria and have altered cell walls. At all stages during infection, the bacteria are bounded by host cell plasmalemma. The enzyme nitrogenase is synthesized by the bacteria and if leghaemoglobin is present, nitrogen fixation will occur. Leghaemoglobin is a product of the symbiotic interaction, since the globin is produced by the plant while the haem is synthesized by the bacteria. In the intracellular habitat the bacteria are dependent upon the plant for supplies of energy and the bacteriods. in particular, appear to differentiate so that they are no longer able to utilize the nitrogen that they fix. Regulation of the supply of carbohydrate and the use of the fixed nitrogen thus appear to be largely governed by the host (Beringer*et al.,* 1979).Survival of rhizobia in soil under varying conditions is an important object of study from the point of cultivation of legume and success of inoculation practices (Pant and Iswaran, 1970). They observed that the counts decreased gradually with advance of time. They found maximum survivability of groundnut *Rhizobium* for 16 weeks in red soil of Coimbatore. However, no correlation was noticed between maximum period of survival of the strain with any of the soil constituents. They seemed that survivability of*Rhizobium* species in a particular soil to be used as a carrier during preparation of legume inoculants to make it effective and useful.

Peat as carrier for rhizobia is more desirable than soil due to its high organic matter content, aeration and higher water holding capacity. Information on the survival of rhizobia seeded in peat and soil or on seed at high temperature exposure is of special significance in tropics as that would determine the success of establishment of effective strains in the soil through seed inoculation. Results of a study in this aspect were reported by Iswaran*et al.* (1970). They found that in peat at 28°C, there was a slight fall in counts in the first week and then, the counts increased while at 35°C there was multiplication upto second week and declined thereafter. In case of the soil at 28°C and 35°C, the counts were appreciable only upto second week and thereafter there was sudden fall. At 40°C there was a rapid death of rhizobia added to soil as compared to peat with no worthwhile recovery after four weeks of storage.The possibility that factors in addition to soil reaction might explain the ecology of rhizobia in acid soils was discussed using as examples a) the influence of exchangeable bases, water content and cultivation on the distribution of rhizobia in soil; and b) pure culture studies of acid tolerance, production of acidic by-products and the reduction of effectiveness promoted by high concentrations of Manganese (Holding and Lowe, 1971).

Moisture is an important factor affecting the survival of rhizobia in soil and its survivability on the inoculated plant is very important for nodule formation and competitive ability of the inoculated strain. Kumar *et al.*(1986) studied survival of two chickpea *Rhizobium* strains on the seed surface under five moisture levels (10, 20, 30, 40 and 50%) of the potted soil in a greenhouse. Viable cell number of *Rhizobium* on inoculated seeds at the time of sowing was 10 cells/seeds. However, after 2 days of sowing viable population on the inoculated seeds decreased tremendously at all the moisture levels. To examine the effect of different moisture regimes on nodulation and effectiveness of symbiosis, chickpea was inoculated with *Rhizobium* strains TAL520 and G4 and sown in potted soil. Soil having 10 to 50%, moisture significantly increased the nodulation, shoot dry weight and nitrogen uptake. Performance of G4 was better at lower levels of moisture while at higher moisture TAI-620 performed better. However, nitrogen uptake was slightly more at all the moisture levels inoculated with TAI-620. In general, performance of inoculated strains was appreciably better in sterilized soil than unslerilized (Kumar and Pareek, 1984).

Seed treatment is a common method of adding nitrogen fixing bacteria (i.e., *Rhzsobium*spp.) to the rhizosphere Chao *et al.* (1988) obtained a total of 220 bacterial isolates from pea rhizosphere and non-rhizosphere samples. Of these, 100 isolates were tested for their agglutinin reaction against pea root exudate. Percentage of positive agglutination of bacteria samples isolated from non-rhizosphere sample was significantly lower than that of rhizosphere sample. Bacteria that could be agglutinated by pea root exudates followed the downward growth of the pea root through the soil profile. Greater abilities of such bacteria to colonize the pea rhizosphere were indicated by (heir higher rhizosphere-colonizing ratios.

Legumes have been shown to benefit by the dual action of obligate endophytic enzymes in plant roots and *Rhizobium* in root nodules. The endomycorrhizal fungi appear to influence the uptake of phosphorus which helps in better fixation" of nitrogen in root nodules. The biochemical specificity in the legume *Rhizobium*symbiosis happens due to interaction on the root surface involving the two symbionts mediated by carbohydrate binding proteins called 'lectins'. The role of legumes in enriching the fertility of soil is known since the last century. However, scientific demonstrations of the value of legumes in contributing to the nitrogen nutrition of plants were done in the later.half of the 19^{lh} century (Fred *et al.,* 1932). The experiments carried out by many scientists proved that nodules on legume roots are responsible for fixing atmospheric nitrogen. Now it is evident that *Rhizobium* plays important role for nodulation in leguminas plants.

Rhizobia are gram negative rods and its speciation is based on the cross-inoculation grouping. Rhizobia that can form nodules on roots of legumes have been collectively taken as a species, particularly in case of the legume *Vigna*and *Arachis*it is known as *Rhizobium* sp. It is also known to survive in soil and in the rhizospheres of legumes as well as non-legumes. *Rhizobium* secretes extracellular polysaccharides which may help in binding soil particles together. They can survive at low temperature and tolerates temperature upto 50°C for more than of few hours. It is sensitive to plant protectants, antibiotics and other agricultural chemicals (Nutman, 1965). *Rhizobium* is more tolerant toward salts than its host legume and therefore, survives in saline soils (SubbaRao*et al.,* 1972, 1974).

Rhizobia are usually maintained by sub-culturing at frequent intervals on yeast extract manitol agar (YEMA) medium. Its lypholyzed permanent cultures are maintained for reconstitution into agar based cultures, as and when necessary.However, agar based inoculants have become outdated except for small scale experiments. Broth cultures are being maintained in European countries. Regarding agronomic practices, its success and failure at field levels have been well documented in literature from time to time (Taha*et al.,* 1967a,b; Ham *et al,* 1976; SubbaRao, 1976, 1979; Vojinovic, 1976; SubbaRao and Tilak, 1977).However, responses to *Rhizobium* inoculation have been demonstrated with principal grain legumes such as arhar*(Cajanuscajan),* gram *(Cicerarietinum),* black gram *(Vignamungo),* and broad bean by Taha*et al.,*(1967a, 1976).

Legumes are known to leave behind some residual nitrogen in soil and maximum residual effect was seen in case of the yield of subsequent crop over corresponding uninoculated controls (SubbaRao and Tilak, 1977).Thus, the use of rhizobial cultures in the establishment of legumes has been widely recognized, especially in areas where indigenous nodulation has been found to be inadequate.*Rhizobium*improve the yield significantly in many legume crops (David, 1991). Leguminous crop meet up their nitrogen requirement through biological nitrogen fixation, which is dependent on proper growth, development and leghaemoglobin content of the root nodules ' (Chowdhury,*et al.,*1998).

Nitrogen is the most deficient nutrient in soil of Bangladesh. Urea, which is used most commonly as nitrogenous fertilizer has now become a costly input for the farmers. *Rhizobium* can minimise the use of urea, which causes injury to soil properties. *Rhizobium* may be also a cheaper substitute for urea in the production of food legume crops (Karim*et at.* 2001).Response of inoculation depends on soil type, cultivar and effectiveness of *Rhizobium* strains and also its competitive ability with native *Rhizobium* (Dube, 1976). Even then the use of legume based biological nitrogen fixation technology in the form *Rhizobium* inoculants may be an important source of nitrogen for the leguminous crops (Hoque, 1993). Beneficial effects of inoculation with *Bradyrhizobium*and*Rhizobium* bacteria on legumes crops have been reported (Islam *et al.,* 1987; Hoque*et al.,*1988).

When legume seed is inoculated, it is necessary to maintain a high number of viable rhizobia on the seed with minimum mortality during the time lag between inoculation and sourcing. For this purpose, Iswaran and Chhonkar (1971) used two methods of inoculation (Slurry and Sprinkle). They counted rhizobia immediately after inoiculation and 24 hr after storage at 28°C and expressed as 10^3 cells per seed. They found gum arabic to be superior adhesive for survival of *Rhizobium.* They also stated that it was advantageous to sow the inoculated seeds as soon as possible after inoculation.

Pot culture and field experiments with 11 treatments in gram were conducted by Patil and Medhane (1974) in order to find out an efficient strain of rhizobia for seed inoculation in future. Results of both the experiments were statistically significant. All the isolates of *Rhizobium* although, were superior over control, did not perform equally in the field. Seed inoculation with different strains of *Rhizobium* in gram increased the dry weight as well as grain yield significantly.

Effect of various doses of *Rhizobium* inoculant on nodulation and yield in Bengal gram was studied under field conditions. The results revealed increased nodulations and other yield parameters over control due to increased doses of *Rhizobium* inoculant although, differences were meager (Jagdale,*et al.,* 1980).A field experiment was conducted by Vaishya*et al.,*(1982) for two years to study the effect of *Rhizobium* inoculation with strain MI on nodulation and grain yield of twelve cultivars of mung bean. The statistical analysis of pooled data of the experiment revealed that inoculation had increased nodule number, nodule weight and grain yield significantly. The yield increase due to inoculation ranged from 4.35% with PusaBaisakhi to 168.21% with J-10, with a mean value of 48.28% than the control. No significant interaction was observed with respect to either nodulation or yield.

Rhizobium inoculation significantly increased number of nodules, plant height, green fodder and dry matter yield over uninoculated control. There was appreciable increase due to inoculation in yield of green fodder and dry matter yield in case of cowpea 85 followed by cowpea 74. The different varieties behaved differently to inoculation with the same strain *of Rhizobium.* Out of eight varieties tested, only cowpea 85 formed the best combination with the efficient strain C41 of *Rhizobium* used for seed inoculation (Sahoo*et al.,* 1984).In order to study nodulation pattern in gram, a field experiment

with different *Rhizobium* strains was conducted on the agricultural college farm, Pune (India), Efficient *Rhizobium* cultures of gram were obtained from the different institutions of India. Obtained results were analysed statistically and found to be significant. The result also revealed that all the strains showed superiority over the control. The strains viz., 5, 6 and 7 gave significantly more number of nodules and dry weight.The effect of legume inoculants on the yield and nitrogen uptake by green gram *(Vignaradiata*L. Wilczek) was studied in alkaline soil. *Rhizobium* inoculants from five different Indian institutes were used. Nodule number and nodule mass increased by inoculation with legume inoculant while straw yield and N uptake were not significantly affected by any of the cultural treatments. Only grain yield was found to be enhanced when *Rhizobium* inoculants from Hisar end Pantnagar were applied (Rao and Batra, 1985.

Legumes are commonly inoculated with selected strains of rhizobia in the expectation that inoculation will increase nitrogen fixation and crop yield. While dramatic increase in yield became possible, some factors might limit the full expression of the inoculation response. These are failure to establish the inoculums at normal inoculation rates due to competition from the native soil strains or reduced inoculums viability due to stresses such as temperature and desiccation. Legumes play important role in agriculture because of their symbiotic relationship with rhizobia and this is why, inoculation of legume seeds with rhizobia is common practice in different countries of the world. But results vary from soil to soil because of the deficiency of native rhizobia. Germida (1988) assessed the ability of indigenous *Rhizobium leguminosarum*and *R. meliloti*to use organic nutrients as growth substance in soil. Nutrient amendments, stimulated growth of indigenous rhizobia but the growth response varied for each rhizobial strain depending on the nutrient, nutrient concentration and the soil.

In Bangladesh symbiotic nitrogen fixing bacteria of *Rhizobium* spp. gaining importance gradually from last part of the last century. Different strains of *Rhizobium* sp. are being used at institutional level for soil and seed treatment and spectacular results are being recorded on the yield of cereals and legumes. However, while reviewing the work on *Rhizobium* sp. it may be said that actual analysis of Bangladesh experiment is very difficult because of the scarcity of those works in published form and also of statistical evaluation.

The literature reviewed here indicates that legumes allow the development of very large rhizobial populations in the vicinity of their roots. Infection and nodule formation require the specific recognition of the host and *Rhizobium.* Penetration of the host by a compatible *Rhizobium* species usually provokes host mot cell division to form the nodule and a process of differentiation by both partners then ensues. In most cases, the rhizobia alter morphologically to form bacteroids, which are usually larger than the free-living bacteria and have altered cell walls. At all stages during infection, the bacteria are bounded by host cell plasmalemma. The enzyme nitrogenase is synthesized by the bacteria and, if leghaemoglobin is present, nitrogen fixation will occur. In the intracellular habitat the bacteria are dependent upon the plant for supplies of energy and the bacteroids, in particular, appear to differentiate so that they are no longer able to utilize the nitrogen that they fix. Regulation of the supply of carbohydrate and the use of the fixed nitrogen thus appear to be largely governed by the host.At the end of the last century symbiotic nitrogen fixing bacteria of *Rhizobium* sp. gained importance in Bangladesh. Encouraging results are being recorded on the yield of cereals and legumes as different strains of *Rhizobium* sp. are being used at institutional level for soil and seed treatment. However, while reviewing the work on *Rhizobium* sp. it may be said that actual experimental analysis in Bangladesh is very difficult because of the scarcity of those works in published form and as well as their inadequate statistical evaluation.

5.1.2 Inoculation and Biological Nitrogen Fixation in Legumes

Nitrogen fixation through the Rhizobium-legume symbiosis is an important source of nitrogen in marginalized farmsteads in terms of size, income and distance from markets(Giller, 2001). Such farmsteads which are not able to provide sufficient nitrogen to their soilssometimes find themselves with BNF as the only feasible source of nitrogen. Thesignificance of nitrogen fixation is put into perspective when consideration is taken of thepotential quantities of fixed nitrogen per hectare of different legumes.During the 20th century, scientists found out that there was legume group-*Rhizobium*specificity in nodule formation which led to the development of the cross inoculation groupconcept where one rhizobia species from one plant in a cross-inoculation group is supposedtonodulate all other plants in the group (Willens, 2006). Cross inoculation groups of rhizobia are particularly important in the production ofrhizobia-based inoculants for legumes since they aid in strain selection for the

inoculants.Some rhizobia are able to form effective nodules on a broad range of hosts while some arespecific to certain hosts. The same scenario happens in plants whereby some are able toassociate with many rhizobia strains while some also nodulate with specific strains. Plantcultivars that form nodules with a broad range of rhizobia strains are generally calledpromiscuous cultivars (Elkan& Upchurch, 1997).

In Bangladesh, this crop is grown on well drained, sandy loam alluvial to clay loam soils. It can not be cultivated in poorly drained low lands. Chickpeas are drought tolerant and can be grown on high lands. Their roots can go deep up to 120cm in search of water. Chickpeas show specificity of adaptation to agro-ecological conditions although they are grown in diverse climatic conditions in different parts of the world. Common (Desi) types are generally better adapted to the East Asian conditions than the Kabulis. The Kabuli type is better adapted to the-Middle East and Mediterranean regions for spring planting. These perform better than Desi types with late planting in the Indian sub-continent also.The inhibitory or stimulatory effects of soil microorganisms such as bacteria, fungi and actinomycetes on *Rhizobium*are known. Culture filtrates of fungi isolated from soil and those isolated from washed nodules often inhibit the growth of Rhizobia. The failure of nodulation in certain parts of world has been attributed to the presence in soil of microorganisms antagonistic to Rhizobia. It is sensitive to antibiotics and other agricultural chemicals (Nutman, 1965).Fungicides, herbicides and other plant protectants might have toxic effect to Rhizobia and reduce the inoculums in soil. The susceptibility of *Rhizobium* to these chemicals differs among different species.

More importantly, Legumes increases soil fertility. The higher protein content legume is directly correlated with the presence of nodules on its roots containing nitrogen fixing bacteria *(Rhizoblum*sp.) which live in symbiotic association with the plant. Because of the ability of utilizing atmospheric nitrogen by fixing it in some forms or other, chickpea increases the nitrogen content and availability in the soil and ultimately the fertility of the soil is improved.Unfortunately this important crop is much neglected and little work has been done so far for its improvement in this country. Its yield per acre is low in our country. It is generally cultivated in Bangladesh as a low yielding rain fed rabi crop with poor field management, whereas it is being cultivated extensively in Western Asia, Middle East and also in India and Pakistan.Compared to

other Asian countries soils in Bangladesh are quite poor in nutrient elements and yield level of major crop because of the poor economy of most of the farmers. They can not use recommended doses of various fertilizers. In modern aquiculture role of rhizobial inoculates, in increasing yield of leguminous crop by fixation of atmospheric nitrogen, has become a popular subject. From this point of view, the bacterization technology may fulfil the demand of chemical fertilizers.

In view of legumes cultivation and success of inoculation practices survival of rhizobia in soil is an important subject of study. Rhizobia may survive in soil for a considerable number of years even in absence of host plants unless they are destroyed by unfavourable conditions. Thus, ability of the rhizobia strain for nodulation under varying conditions of soil from different agro-ecological zones of Bangladesh may be an important aspect of this study.

The soils of Bangladesh, especially Rajshahi regions are deficient in nitrogen fixing bacteria *(Rhizobium* sp.) which causes poor yield of pulse crops (Bhuiyan*et al.,*1998; Khanam*at al.,*1994). The Bangladesh Agricultural Research Institute (BARI) has developed a good number of varieties of pulse crops. There is a good possibility to increase its production by exploiting better colonization of the roots and rhizospheres through application of effective nitrogen fixing bacteria to the seed or to the soil. This can minimize the uses of nitrogenous fertilizer, which is very costly in this country. Using high yielding varieties of chickpea along with use of effective rhizobial strains can enhance the yield.

However, considering the view points mentioned above much more effort has been made by many scientists in the world to identity the strains of *Rhizobium,* superior to N_2 fixation and tolerant to a variety of soil stresses. But in Bangladesh, there is very little understanding of how native populations of rhizobia determine legume N_2 fixation potential. At the same time report regarding response of inoculation with selected rhizobia in terms of number and effectiveness of the population for a particular host is also scanty in our country. Thus, the literature regarding responses of legume in relation to effectiveness of *Rhizobium* species alongwith few other relevant subject matters are reviewed here.

Very little work has been done in Bangladesh regarding root colonization of legumes by rhizobia in relation to improved management practices, required to achieve higher yield of black gram. The present study was designed to determine if the introduced rhizobial strains can colonize, survive and promote the growth and yield of black gram. In addition, a little work has been done on released black gram varieties grown in Bangladesh considering their growth and development patterns and yield potential under different treatments of *Rhizobium*. Therefore, two black gram varieties widely grown in North-Western Bangladesh were selected in the present investigation and imposed different regimes of *Rhizobium* strains on them to understand their growth pattern during different stages of growth. The study also compared the varieties for final yield and yield components under different *Rhizobium* treatments.

But information regarding the survival of *Rhizobium* due to varying conditions of the soil is lacking. Information regarding the soil constituents like organic matter, pH, soluble salts, available phosphorous, potassium, sulphur, zinc, total nitrogen *etc.* which may have different sort of effects on the nodulation, is not also known. The present study may enrich our idea and also may be helpful for conducting further research at various levels.

5.1.3 Research Acvities

In the present research, we tried to fulfill the following goals.

I. Effects of *Rhizobium* inoculants onLeguminousplants.
II. Analysis of *Rhizobium* inoculated soil.

5.2 MATERIALS AND METHODS

Materials

The plant materials used in the present investigation were eight varieties of Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean. These were collected form Regional Pulse Research Station of Bangladesh Agricultural Research Institute (BARI), Isurdi, Pabna. Gram negative soil bacteria used in this study was *Rhizobium* sp. capable of effective symbiosis with legumes for nitrogen fixation. Three strains of *Rhizobium* spp., e.g., *Rhizobium* sp.CCNWYC119,*Rhizobium* sp. SOY12 and *Rhizobium* sp. SOY7,were used for nodulation in rhizosphere of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and

Bean.Apart from these, the soils used for plantation in plastic pot were sterilized, dried well and however, this event is described in method of this chapter.In this experiment gram negative soil bacteria *(Rhizobium* sp.) was used for effective symbiosis with legumes for nitrogen fixation. For bacterial strain *(Rhizobium* spp.) nodules of legumes were collected from the Held of RajshahiareaNext, YEMA (Yeast Extract Mannitol Agar) and LB (Liquid Broth) liquid medium were used in the laboratory for isolation and culture of the bacterial strain, respectively .The components along with their proportions of these two culture mediums are given in table-1 and table-2, respectively. Cultured strain was preserved in laboratory under optimum conditions for the experiment.

Table 5.1: Composition of YEMA medium.

Components	Amount (g/L)
K_2HPO_4	0.5 g
$MgSO_4$ 7 H_2O	0.2 g
NaCl	0.1 g
Mannitol	10.0 g
Yeast extract	0.5 g
Agar	20 g
Distilled water	1 litre

Table-5.2: Composition of Liquid Broth (LB) medium.

Components	Amount (g/L)
K_2HPO_4	0.5g
$MgSO_4$ 7 H_2O	0.2 g
NaCl	0.1 g
Mannitol	10.0 g
Yeast extract	0.5 g
Distilled water	01 litre

For the inoculation of seed with *Rhizobium* sp. peat soil was used as carrier and IT. classes as adhesive.

5.2.1 Production of RhizobialBiofertilizer

Biofertilizer is generally comprised of microbes and culture media and microbes are generally produced using agar culture in the laboratory. But the following properties of the microbes arc essential in view of the biofertillizer production.

i. The microbe which is to be used as biofertilizer that must be capable of nodulation in the rhizosphere of the particular crop.

ii. Under the same soil and weather where the crop plants will be grown, the microbes will be able there to fix nitrogen by nodulation.

iii. That microbe will be preferred as biofertilizer which can tolerate different level of temperature, acidity or alkalinity with different varieties of a crop compared to that of other microbes in the same field.

iv. With the help of which microbe biofertilizer will be prepared, that microbe would be move strong than the other native strain living in the field of a particular area.

v. Higher ability of nodulation for nitrogen fixation and tolerance to insecticide of the strains should be notable feature.

5.2.2Culture Media

Generally two types of culture medium are used for preparing biofertilizer and these are (i) liquid medium and (ii) solid medium.

For different advantages peat based biofertillizer is considered to be best. Survivability for long time, easy of application and availability of a strain depend on the culture medium which possess the following properties.

i. That culture medium must have high amount of carbon and enough water holding capacity.

ii. The pH range of the medium should be 6.5 - 7.0 and that will remain unchanged.

iii. The culture medium will be free from any poisonous substances and can be converted into fine particles easily.

iv. Physical and chemical properties of it should be durable.

v. Most available and very light in weight.

5.2.3 Quality of Biofertilizer

Quality of biofertilizer depends upon the number of both expected and unexpected microbes which are able to produce nodule in a particular crop in relation to application of each gmbiofertilizer. Generally during applications there should be 10^6number of active microbes per each gm peat. However, based on effectiveness of microbes it is maintained following the previous records seconds in different countries. For examples, in Canada, Australia and Holland the number of microbes varies from 10^6-10^9 per gm peat. In Uruguay, India and Brazil its minimum number is 10^8 per gmpeat.Sometimes the quality of microbes is measured on the basis of the number of microbes per seed just after inoculation of the seeds with biofertilizer. For example, in case of small seeds (mung, letitil etc.), medium sized seeds (black gram, red gram etc.) and large sized seeds (soybean, chickpea, peas etc.) the number of microbes should be 1000, 10,000 and 100,000, respectively after inoculation.

Burton (1978) has recommended the microbes number 1000 per seed in case of fast germinating seeds and 100,000 per seed for slow germinating seeds. However, after sterilization it should not exceed above 0.001% for expected microbe and in case of unsterilized medium it should be above 1000 per gm.

Production Procedure

It covers preparation of broth culture and that of peat soil.

Broth Culture

This is that well known culture medium where the strain is cultivated and this liquid medium is sterilized under the pressure of 15 lb (121°C temperatures) and then selected strains are grown in the cold liquid medium. After growing of enough number of microbes (>10^6 - 10^9/ml) certain amount of the liquid medium are mixed with the sterilized peat soil. The amount of the liquid medium is determined, as it contains 60% moisture. Under this condition the microbes are allowed for multiplication at least for 5-7 days and thus, the numbers of microbes are increased in member, satisfactory for application.

5.2.4 Selection and Processing of Carrier Material

The carrier material used was fine activated molasses. Then it was spread on a clean, dry, glass tray and the bacterial cultures were added to the sterilized carrier materials and manually mixed in the ratio of 1:1. After proper mixing with activated charcoal, it was left for 5 days by covering the trays at 24ºC. Thereafter, *Rhizobium* inoculants could either be used directly or packed and stored. The packets were stored in a cool place away from the heat or direct sunlight.

5.2.5 Pot Experiment

The efficiency of the biofertilizer was tested by running a pot culture experiment and monitoring the growth of Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean. plants. The experiment was arranged in two treatments (control i.e. without addition of biofertilizer and test i.e. application of *Rhizobium spp*combined with mollases). Seeds of Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean.were mixed in the prepared slurry and allowed to stand still for 30 mins. Test and control seeds were directly sown into their respective pots. Each pot contained about 500 gm of soil. The phenotypic parameters i.e. stem height; root length and the wet weight of the plants were measured after the 15 th day from sowing. Soil used for the whole experiment was taken from a Rajshahi, Bangladesh. The plants were kept in a polyhouse with humidity ranging from 70 – 80%. The temperature of the polyhouse was not controlled and varied with local conditions (max: 32ºC, min: 15ºC).

5.2.6 Peat soil

Peat soils are generally dried under sunlight after collection and made them free from any dust materials. Then the soils are ground to fine powder, Afterwards the soils are heat treated followed by neutralization $CaCO_3$ to raise pH 6.5-7.0. Then pouring in polythene bag the soil materials are sterilized with the help of autoclave. Now a days sterilization by gamma radiation (Cobalt ray) is considered to be most acceptable than autoclave sterilized method.

Diluted broth having microbes *(Rhizobium* cell population) is blended with the peat carrier so as to bring the final moisture content of peat to 35-40% on wet basis. These carrier based microbes are then used for inoculation of particular crop seeds.

Plate 5.1: Peat soil.

Preparation of soil

Collected of soils were sun dried for 7 days. Then it was made free from plant debris and other waste and dust materials. Later it was grounded manually into fine particle. Finally, it was kept in an electric oven at 60℃ for 12 hours to make it more dry and Tree of native microorganisms. Dried soils were then fairly mixed with compost proportionately for use.

Soil properties

Soil samples used in this experiment were analyzed by the Regional Laboratory, Soil Resource Development Institute (SRD1), Shyampur, Rajshahi. The prepared ground sieved soil samples (without compost) were transferred to air tight plastic container and then labeled properly. The supplied samples, subsequently, were used for analysis of pH and nutrient contents of soils by the methods directed by SRDI.

5.2.7 Isolation of *Rhizobium* from nodules

Plants Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean etc. were carefully uprooted and the root system was washed in running water to remove soil particles. Healthy, unbroken, firm and preferably pink nodules were selected and washed in water. They were immersed in 0.1% acidified $HgCl_2$ for 4-5 minutes (or 3-5% H_2O_2). After surface sterilization with $HgCl_2$, nodules were washed repeatedly with distilled water and clipped in 70% ethanol for a while followed by more washing with distilled water.

The nodules were crushed in a mortar and pestle with few drops of H_2O. The fluid from the crushed nodules was spread on the surface of YEMA plates with glass rod. The plates were incubated up to 10 days in an incubator at 26°C. Large and small gummy colonies of bacteria emerged in 4-5 days.

Preservation of rhizobial strain

Then bacteria were transferred and subcultured on YEMA plates for several times until the pure colonies were obtained. From the pure colony *Rhizobium* were again transferred in to conical flasks containing liquid broth medium (pH 7.5) and were placed on a shaker for 24 hours at 155 rpm at 30°C. After shaking bacterial strain containing LB medium was preserved at 4°C in a refrigerator for use.

Plate 5.2: Broth culture of *Rhizobium* sp.

5.2.8 Experimental design

This experiment was laid out with a control and three replications. A total of 32Plastic pots were filled up with the prepared soils, of which four pots were used for each legume type. Liquid cultured media containing *Rhizobium* strain was then sprayed thoroughly in inner part (1-1.5 inches below the surface) of soil in three pots of each soil type and the remaining one was used as control. Finally, collected fresh and dry seeds of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean etc.were sown in all the 32 pots prior to inoculation with rhizobial strain, 08-10 seeds in each pot, for growing. During the experiment the soils in pots were kept moistened very slightly.

5.2.9 Seed inoculation

Seed inoculation was done by slurry method and its different steps are as follows:

i. 100grams healthy and dry seeds of legume were mixed with 5gms of adhesive like molasses in a big test tube under a dry and shady condition.

ii. Then these seeds were separated equally in different petridishcontainers: Subsequently, seeds were inoculated with the strains of *Rhizobium* sp. All the seeds were inoculated and every seed receives equal amount of biofertilizer on its coat.

iii. These seeds were then dried for a while in a dry and shady place

iv. The inoculated seeds were sown in the pots in a shady place.

v. Then the soils in pots were made moistened very slightly.It may be mentioned here that the biofertilizers were used within 90 days of its production. After sowing, each pot was labelled according to soil types and treatment.

Plate 5.3:*Rhizobium* inoculation of leguminous seed.

Crop management

The crop was always kept free from weeds. An insecticide named Nagos 100 EG was sprayed gently on the leaves at the rate of 1 ml/litre water. Spading was done whenever necessary. As legume is grown under rain fed condition, no heavy irrigation was applied during this investigation.

5.2.10 Growth attribute

Over the period of investigation seedling heights of all the experimental plants were measured and recorded at three different time intervals of maturity like 15, 25 and 40 days after sowing (DAS) before the final harvest.

Plate 5.4: Cultivation ofLeguminous Plants.

5.2.11Yield and yield components

At physiological maturity of the plants the harvest was done. Five plants each from a control and three treated replications of each of the four soil types were harvested for determination of the grain and biological yield and their components. The following characters were recorded at the final harvest:

1. Plant heights at maturity (cm)
2. 50 pods weight (gm)
3. 100 seed weight (gm)
4. Nodules/plant
5. Fresh weight of nodules/plant
6. Dry weight of nodules/plant

5.2.12 Soil analysis

Soil materials were collected from Rajshahi area. Surface soil (0-5cm depths) were collected and dried at room temperature (30+ 3°C) ground well and sieved through 20-mesh sieve and supplied to the soil analysis Institute at Rajshahi.

Plate 5.5: *Rhizobium* inoculated soil.

5.2.13 Statistical Analysis

In this study, also an attempt has been made to compare the data using the "t" test.Theobtained"t" value for each of the parameter was compared with the table at probability level of at all degrees of freedom (0.05) to find out the significanceof*Rhizobium* inoculation. The "t" tables of Panse and Sukhatme (1967) were referred for comparision.The experiment data was taken from a total of eight leguminous plant each having 05 treated plants and 05 control plants. All the data obtained from the pot experiment was analyzed using Graph Pad Prism 5. Paired T test was carried out between the control values and treated values of plant height,pod weight, seed weight ,no of nodules,fresh weight of nodule and dry weight of nodule respectively. The graphs were plotted in MS Excel with the error bars representing standard error as calculated from Graph Pad Prism 5.

5.3 RESULTS

The results obtained in the study arepresented in tables5.3-5.18 .The results show that in Green Gram, Black Gram, Lentil, Pigeon Pea , Sweet Pea, Chick Pea, Cow Pea and Bean studied, the inoculated plantspossessed greater height, greater fresh weight, greaternumber of nodules, greater number of pods, greater length of pods, greater seedweight, over their respective controls and also soil analysis. The percentageincrease in all these parameters were satisticallyanalyzed using „t test and was found that in eight thepulses studied the *Rhizobial* inoculation produced astatistically significant increase over controls as theprobability values were lower than the table "t" values(0.05).

5.3.1 Soil analysis

Before setting of the experiment and after harvest of the crop data on soil nutrients were recorded. Soils of different region may response differently in terms of nutrient as well as soil bacteria *Rhizobium.* This is why, different legume soils were Collected from Rajshahi area and used in the present experiment. The recorded data properties of different legume plant soil before and after inoculated with *Rhizobium* strain given below

Table 5.3: Properties of Green Gram soil types before and after treatment with *Rhizobium* strain.

Analysis of Soil of Green Gram

Parameters	Units	Before use	Control	Inoculated
pH		8.2	8.3	8.3
Organic mater	%	1.22	1.28	1.47
Potassium	Cmol/kg	0.18	0.24	0.29
Total Nitrogen	%	0.07	0.07	0.09
Phosphorous	ppm	11.5	15.7	15.7
Sulphar	ppm	15.7	26.4	49
Zinc	ppm	2.47	3.36	2.69

Table5.4: Properties of Black Gram soil types before and after treatment with *Rhizobium* strain.

Analysis of Soil of Black Gram

Parameters	Units	Before use	Control	Inoculated
pH		8.2	8.2	8.3
Organic mater	%	1.22	1.35	1.70
Potassium	Cmol/kg	0.18	0.32	0.28
Total Nitrogen	%	0.07	0.08	0.10
Phosphorous	ppm	11.5	13.5	26.8
Sulpher	ppm	15.7	58.1	64.2
Zinc	ppm	2.47	11.61	5.80

Table 5.5: Properties of Lentil soil types before and after treatment with *Rhizobium* strain.

Analysis of Soil of Lentil

Parameters	Units	Before use	Control	Inoculated
pH		8.2	8.5	8.4
Organic mater	%	1.22	1.23	1.47
Potassium	Cmol/kg	0.18	0.18	0.18
Total Nitrogen	%	0.07	0.07	0.09
Phosphorous	ppm	11	11.1	22.2
Sulpher	ppm	15	11.3	7.8
Zinc	ppm	2.47	5.42	4.36

Table5.6: Properties of Pigeon Pea soil types before and after treatment with *Rhizobium* strain.

Analysis of Soil of Pigeon Pea

Parameters	Units	Before use	Control	Inoculated
pH		8.2	8.3	8.3
Organic mater	%	1.22	1.40	1.63
Potassium	Cmol/kg	0.18	0.28	0.23
Total Nitrogen	%	0.07	0.08	0.10
Phosphorous	ppm	11.5	25.5	25.9
Sulpher	ppm	15.7	28.7	41.1
Zinc	ppm	2.47	4.94	5.20

Table5.7: Properties of Sweet Pea, soil types before and after treatment with *Rhizobium* strain.

Analysis of Soil of Sweet Pea,

Parameters	**Units**	**Before use**	**Control**	**Inoculated**
pH		8.2	8.2	8.4
Organic mater	%	1.22	1.29	1.64
Potassium	Cmol/kg	0.18	0.24	0.28
Total Nitrogen	%	0.07	0.08	0.10
Phosphorous	ppm	11.5	18.7	25.4
Sulpher	ppm	15.7	33.9	27
Zinc	ppm	2.47	3.59	3.9

Table5.8: Properties of Chick Pea, soil types before and after treatment with *Rhizobium* strain.

Analysis of Soil Chick Pea

Parameters	**Units**	**Before use**	**Control**	**Inoculated**
pH		8.2	8.4	8.2
Organic mater	%	1.22	1.15	1.45
Potassium	Cmol/kg	0.18	0.32	0.27
Total Nitrogen	%	0.07	0.08	0.10
Phosphorous	ppm	11.5	13.5	29.3
Sulpher	ppm	15.7	7.8	28.9
Zinc	ppm	2.47	3.89	3.36

Table5.9: Properties of Cow Pea, soil types before and after treatment with *Rhizobium* strain.

Analysis of Soil Cow Pea

Parameters	Units	Before use	Control	Inoculated
pH		8.2	8.3	8.3
Organic mater	%	1.22	1.40	1.63
Potassium	Cmol/kg	0.18	0.28	0.23
Total Nitrogen	%	0.07	0.08	0.10
Phosphorous	ppm	11.5	25.5	25.9
Sulpher	ppm	15.7	28.7	41.1
Zinc	ppm	2.47	4.94	5.20

Table 5.10: Properties of Bean soil types before and after treatment with *Rhizobium* strain.

Analysis of Soil of Bean

Parameters	**Units**	**Before use**	**Control**	**Inoculated**
pH		8.2	8.5	8.5
Organic mater	%	1.22	1.28	1.42
Potassium	Cmol/kg	0.18	0.24	0.25
Total Nitrogen	%	0.07	0.05	0.07
Phosphorous	ppm	11.5	12.3	34.4
Sulpher	ppm	15.7	16.1	6.9
Zinc	ppm	2.47	4.69	6.26

5.3.2Soil status after crop harvest

Soil tests were carried out for different plant (Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean) soils after harvest of the crop in two forms: control and treated.

In case of soil pH treated soils showed higher values than control. Compared to those of soil the values for organic matter in control and treated form of eight type of legume plant soil were not always high, but treated soils showed higher values than control.

On the contrary, in case of total nitrogen such differences were found among the values recorded for control and inoculated. such difference pattern of results like total nitrogen was obtained in case of potassium (K). It was to increase only in case of treated soil.In case of phosphorous (P) the values recorded for treated soil of eight different types of plant soil were high than control. Also the values recorded for sulphur were found to increase in all cases when compared to that all eight types of legumes soil. However, the highest values were found in all the eight plant inoculated soil.

In case of zinc (Zn),Alllegumeplantsinoculated soil showed greater values than control and the values for this nutrient element were found to increase.

5.3.3 Growth Analysis

Growth analysis has been done in the present study as it relates the influence of *Rhizobium* sp. and different inoculation on the growth and yield of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean.

Significant at $P < 0.05$ The results of present study also showed that the seed inoculation caused an increase in the number of root nodules in inoculated plants might have increased the production over controls as its evident from tables and text figs. 1 and 2. All grown and yield parameters increased of both pulses over No. of root nodules

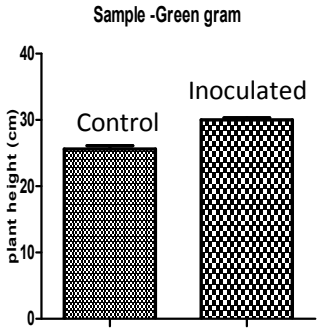
The recorded data, Effect of *Rhizobium* inoculation on various growth parameters of legume plants given below.

Table 5.11: Effect of *Rhizobium* inoculation on various growth parameters in Green Gram.

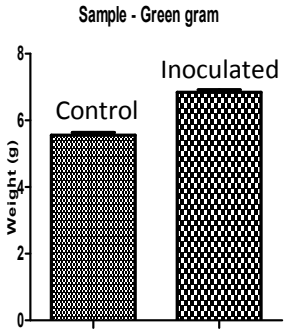
	Length (cms)	**50 pod wt. (gms)**	**100 seed wt. (gms)**	**No. of Nodules/ Plant**	**Fresh wt. of nodules (gms)**	**Dry wt. of nodules (gms)**
Inoculated	30.00 ± 0.3162 N=5	5.400 ± 0.1789 N=5	6.840 ± 0.07483 N=5	19.20 ± 0.5831 N=5	0.2760 ± 0.005099 N=5	0.1960 ± 0.002449 N=5
Control	25.60 ± 0.5099 N=5	4.200 ± 0.08944 N=5	5.560 ± 0.07483 N=5	13.20 ± 0.5831 N=5	0.0800 ± 0.003162 N=5	0.0480 ± 0.003742 N=5
Degrees of freedom	08	08	08	08	08	08
Calculated value. P=0.05	< 0.0001 ***	0.0003 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***

***Rhizobium*Inoculated** **Control**

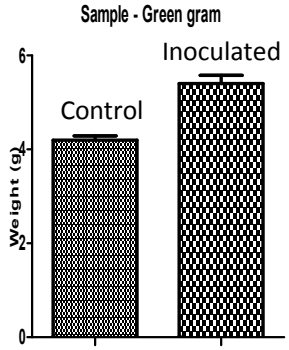
Plate 5.6: Number of nodules of Green Gram.



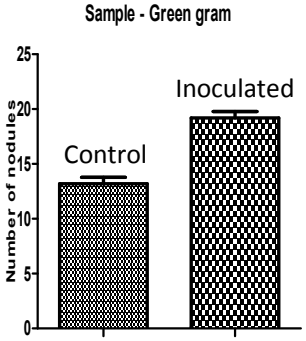
significant difference of Green gram plant height between control and inoculated



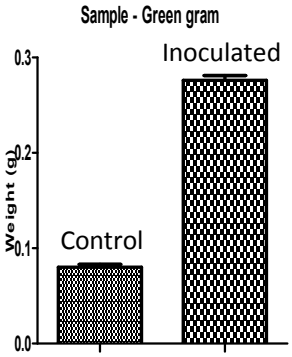
significant difference of Green gram 100 seed weight between control and inoculated



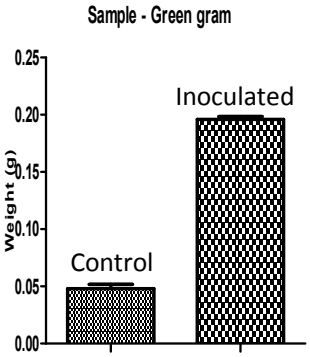
significant difference of Green gram 50 Pod weight between control and inoculated



significant difference of Green gram no of nodules between control and inoculated



significant difference of Green gram fresh wt of nodules between control and inoculated



significant difference of Green gram Dry wt of nodules between control and inoculated

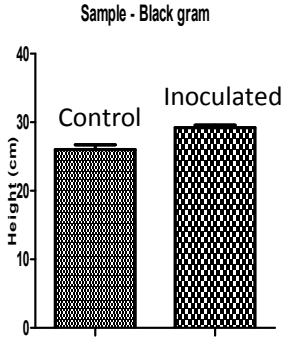
Figure 5.1: Graphical presentation, Effect of *Rhizobium* inoculation on Green Gram.

Table 5.12: Effect of *Rhizobium* inoculation on various growth parameters in Black Gram.

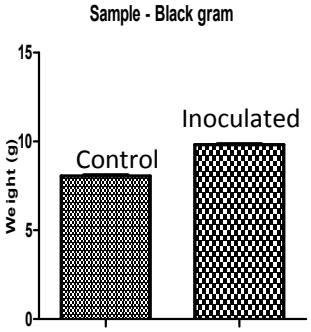
	Length (cms)	**50 pod wt. (gms)**	**100 seed wt. (gms)**	**No. of Nodules/ Plant**	**Fresh wt. of nodules (gms)**	**Dry wt. of nodules (gms)**
Inoculated	29.20 ± 0.3742 N=5	6.280 ± 0.03742 N=5	9.820 ± 0.03742 N=5	15.40 ± 0.8124 N=5	0.2500 ± 0.02074 N=5	0.1720 ± 0.01934 N=5
Control	26.00 ± 0.7071 N=5	5.060 ± 0.04000 N=5	8.060 ± 0.04000 N=5	9.200 ± 0.3742 N=5	0.0780 ± 0.008602 N=5	0.0540 ± 0.005099 N=5
Degrees of freedom	08	08	08	08	08	08
Calculated value. P=0.05	0.0039 **	< 0.0001 ***	< 0.0001 ***	0.0001 ***	< 0.0001 ***	0.0004 ***

***Rhizobium*Inoculated** **Control**

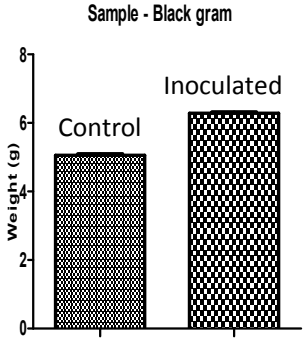
Plate 5.7: Number of nodules of Black Gram.



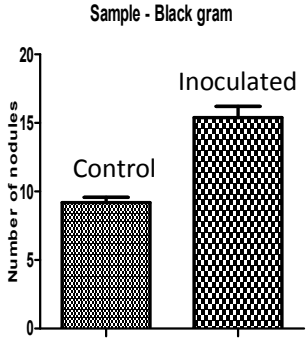
significant difference of Black gram plant height between control and inoculated



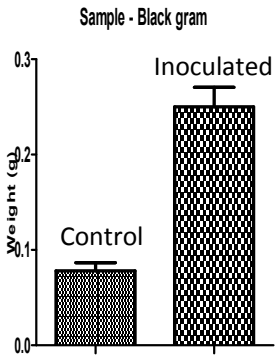
significant difference of Black gram 100 seed weight between control and inoculated



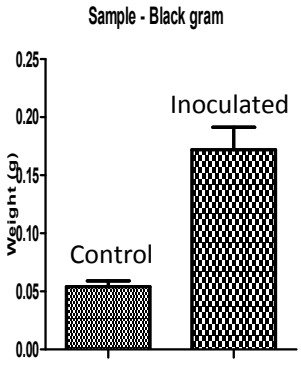
significant difference of Black gram 50 Pod weight between control and inoculated



significant difference of Black gram no of nodules between control and inoculated



significant difference of Black gram fresh wt of nodules between control and inoculated



significant difference of Black gram Dry wt of nodules between control and inoculated

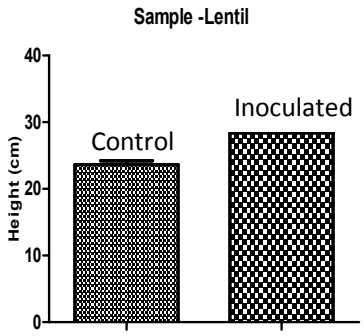
Figure 5.2: Graphical presentation, Effect of *Rhizobium* inoculation on Black gram.

Table 5.13: Effect of *Rhizobium* inoculation on various growth parameters in Lentil.

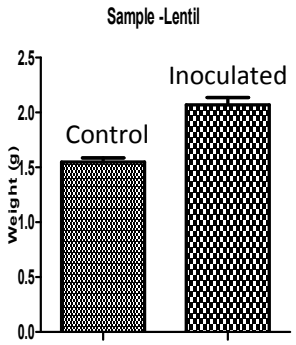
	Length (cms)	**50 pod wt. (gms)**	**100 seed wt. (gms)**	**No. of Nodules/ Plant**	**Fresh wt. of nodules (gms)**	**Dry wt. of nodules (gms)**
Inoculated	28.40 ± 0.2449 N=5	2.210 ± 0.03674 N=5	2.070 ± 0.06633 N=5	22.00 ± 1.378 N=5	0.2540 ± 0.01568 N=5	0.1030 ± 0.009165 N=5
Control	23.60 ± 0.6000 N=5	1.560 ± 0.01871 N=5	1.550 ± 0.03701 N=5	12.40 ± 0.5099 N=5	0.0870 ± 0.008888 N=5	0.0444 ± 0.001965 N=5
Degrees of freedom	08	08	08	08	08	08
Calculated value. P=0.05	< 0.0001 ***	< 0.0001 ***	0.0001 ***	0.0002 ***	< 0.0001 ***	0.0002 ***

***Rhizobium*Inoculated** **Control**

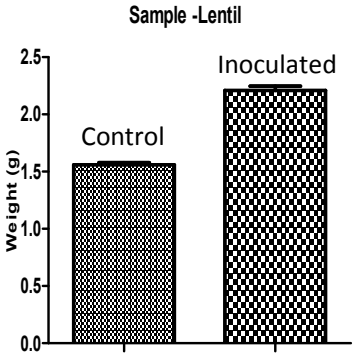
Plate 5.8: Number of nodules of Lentil.



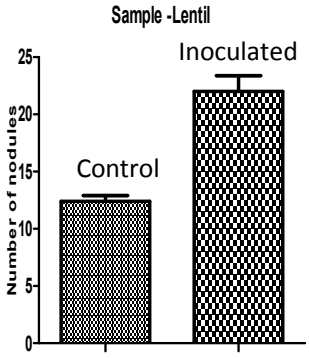
significant difference of Lentil plant height between control and inoculated



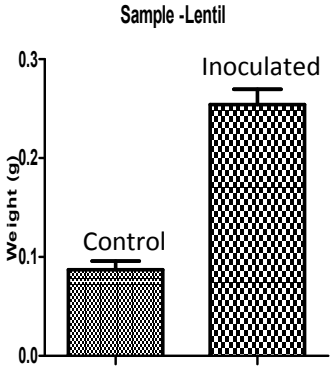
significant difference of Lentil 100 seed weight between control and inoculated



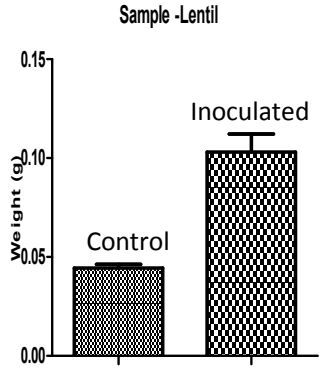
significant difference of Lentil 50 Pod weight between control and inoculated



significant difference of Lentil no of nodules between control and inoculated



significant difference of Lentil fresh wt of nodules between control and inoculated



significant difference of Lentil dry wt of nodules between control and inoculated

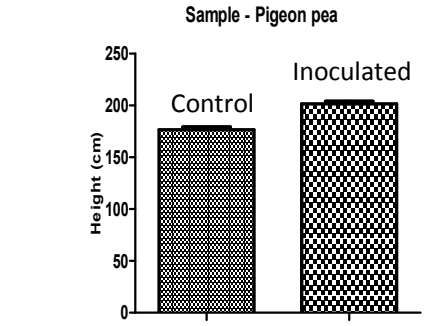
Figure5.3: Graphical presentation, Effect of *Rhizobium* inoculation on Lentil.

Table 5.14: Effect of *Rhizobium* inoculation on various growth parameters in Pigeon Pea.

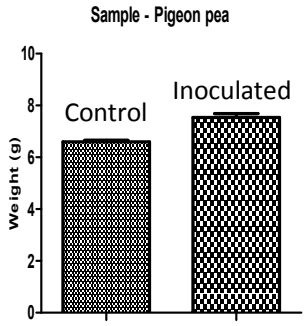
	Length (cms)	**50 pod wt. (gms)**	**100 seed wt. (gms)**	**No. of Nodules/ Plant**	**Fresh wt. of nodules (gms)**	**Dry wt. of nodules (gms)**
Inoculated	201.6 ± 2.462 N=5	12.78 ± 0.2059 N=5	7.540 ± 0.1503 N=5	205.4 ± 1.720 N=5	20.00 ± 0.4472 N=5	13.00 ± 0.3162 N=5
Control	176.6 ± 2.731 N=5	11.32 ± 0.1068 N=5	6.600 ± 0.05477 N=5	171.0 ± 1.612 N=5	14.80 ± 0.3742 N=5	9.800 ± 0.3742 N=5
Degrees of freedom	08	08	08	08	08	08
Calculated value. P=0.05	0.0001 ***	0.0002 ***	0.0004 ***	< 0.0001 ***	< 0.0001 ***	0.0002 ***

***Rhizobium*Inoculated** **Control**

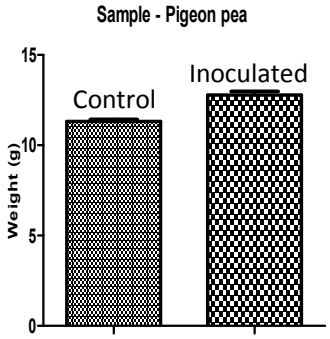
Plate 5.9: Number of nodules of Pigeon Pea.



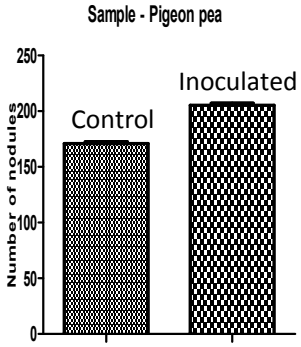
significant difference of Pigeon pea plant height between control and inoculated



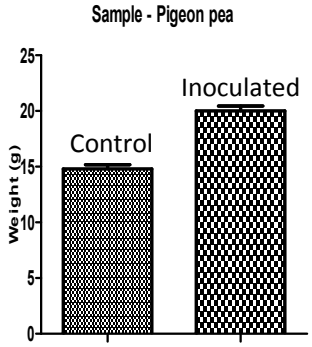
significant difference of Pigeon pea 100 seed weight between control and inoculated



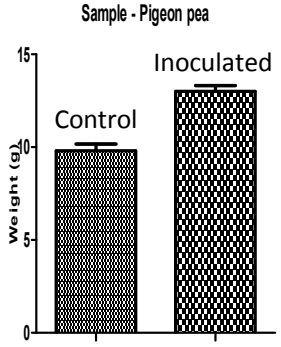
significant difference of Pigeon pea 50 Pod weight between control and inoculated



significant difference of Pigeon pea no of nodules between control and inoculated



significant difference of Pigeon pea fresh wt of nodules between control and inoculated



significant difference of Pigeon pea dry wt of nodules between control and inoculated

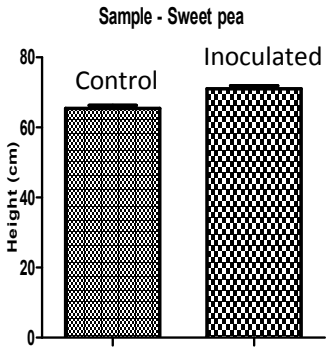
Figure 5.4: Graphical presentation, Effect of *Rhizobium* inoculation on Pigeon pea.

Table 5.15: Effect of *Rhizobium* inoculation on various growth parameters in Sweet Pea.

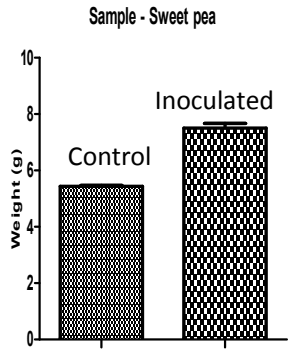
	Length (cms)	**50 pod wt. (gms)**	**100 seed wt. (gms)**	**No. of Nodules/ Plant**	**Fresh wt. of nodules (gms)**	**Dry wt. of nodules (gms)**
Inoculated	71.00 ± 0.8944 N=5	6.790 ± 0.1269 N=5	7.500 ± 0.1673 N=5	17.00 ± 0.7071 N=5	0.2700 ± 0.02470 N=5	0.2120 ± 0.01497 N=5
Control	65.40 ± 0.9274 N=5	5.780 ± 0.02550 N=5	5.440 ± 0.02915 N=5	11.80 ± 1.068 N=5	0.1500 ± 0.02702 N=5	0.1040 ± 0.02015 N=5
Degrees of freedom	08	08	08	08	08	08
Calculated value. P=0.05	0.0025 **	< 0.0001 ***	< 0.0001 ***	0.0036 **	0.0112 *	0.0026 **

***Rhizobium* Inoculated** **Control**

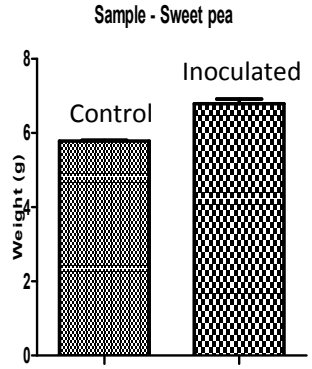
Plate 5.10 - Number of nodules of Sweet Pea.



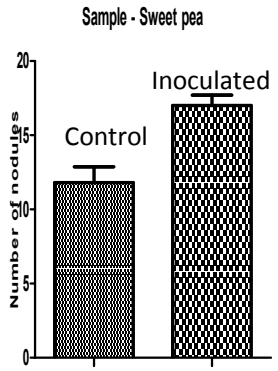
significant difference of Sweet pea plant height between control and inoculated



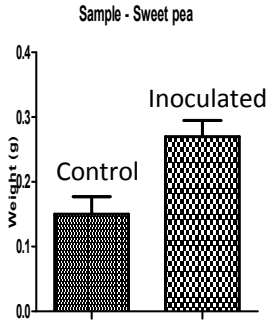
significant difference of Sweet pea 100 seed weight between control and inoculated



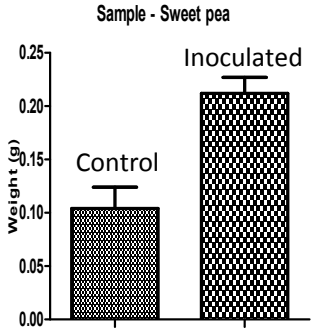
significant difference of Sweet pea 50 Pod weight between control and inoculated



significant difference of Sweet pea no of nodules between control and inoculated



significant difference of Sweet pea fresh wt of nodules between control and inoculated



significant difference of Sweet pea dry wt of nodules between control and inoculated

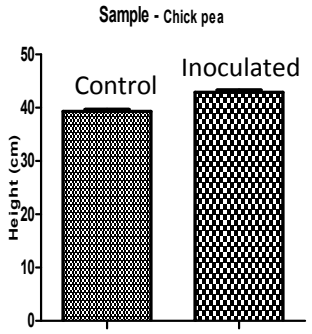
Figure 5.5: Graphical presentation, Effect of *Rhizobium* inoculation on Sweet Pea.

Table 5.16: Effect of *Rhizobium* inoculation on various growth parameters in Chick Pea.

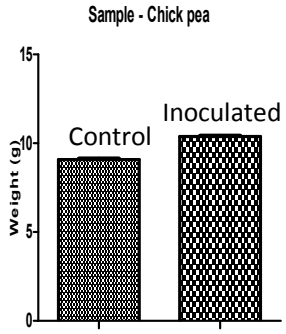
	Length (cms)	**50 pod wt. (gms)**	**100 seed wt. (gms)**	**No. of Nodules/ Plant**	**Fresh wt. of nodules (gms)**	**Dry wt. of nodules (gms)**
Inoculated	42.90 ± 0.4000 N=5	7.720 ± 0.08602 N=5	10.38 ± 0.05831 N=5	14.00 ± 0.5477 N=5	0.1920 ± 0.003742 N=5	0.1440 ± 0.002449 N=5
Control	39.30 ± 0.3742 N=5	6.540 ± 0.05099 N=5	9.080 ± 0.07348 N=5	7.000 ± 0.3162 N=5	0.0540 ± 0.005099 N=5	0.0400 ± 0.003536 N=5
Degrees of freedom	08	08	08	08	08	08
Calculated value. P=0.05	0.0002 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***

***Rhizobium*Inoculated** **Control**

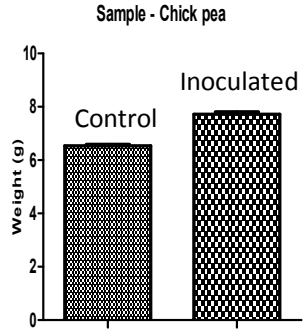
Plate 5.11: Number of nodules of Chick Pea.



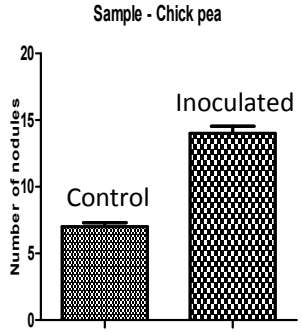
significant difference of Chick pea plant height between control and inoculated



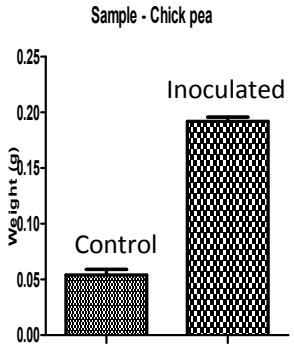
significant difference of Chick pea 100 seed weight between control and inoculated



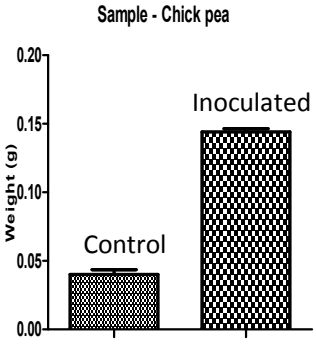
significant difference of Chick pea 50 Pod weight between control and inoculated



significant difference of Chick pea no of nodules between control and inoculated



significant difference of Chick pea fresh wt of nodules between control and inoculated



significant difference of Chick pea dry wt of nodules between control and inoculated

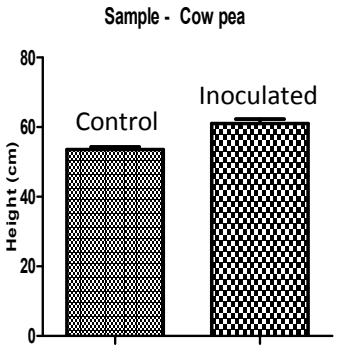
Figure 5.6: Graphical presentation, Effect of *Rhizobium* inoculation on Chick Pea.

Table 5.17: Effect of *Rhizobium* inoculation on various growth parameters in CowPea.

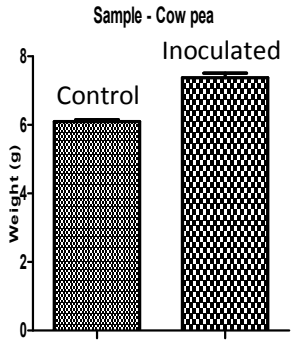
	Length (cms)	**50 pod wt. (gms)**	**100 seed wt. (gms)**	**No. of Nodules/ Plant**	**Fresh wt. of nodules (gms)**	**Dry wt. of nodules (gms)**
Inoculated	61.00 ± 1.304 N=5	5.348 ± 0.05004 N=5	7.376 ± 0.1335 N=5	36.60 ± 0.9274 N=5	0.1820 ± 0.009165 N=5	0.0700 ± 0.004472 N=5
Control	53.60 ± 0.6782 N=5	4.570 ± 0.02000 N=5	6.096 ± 0.05154 N=5	30.80 ± 0.7348 N=5	0.1260 ± 0.006000 N=5	0.0440 ± 0.008124 N=5
Degrees of freedom	08	08	08	08	08	08
Calculated value. P=0.05	0.0010 **	< 0.0001 ***	< 0.0001 ***	0.0012 **	0.0009 ***	0.0231 *

***Rhizobium*Inoculated** **Control**

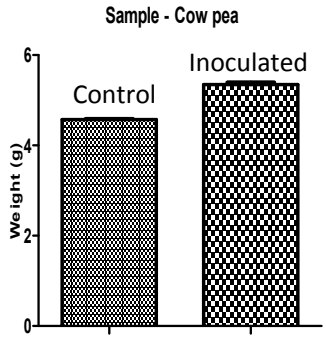
Plate 5.12: Number of nodules of Cow Pea.



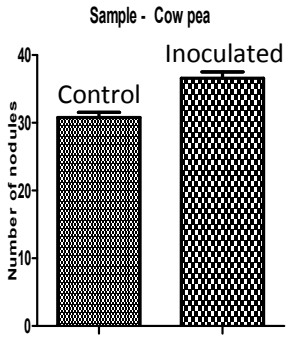
significant difference of Pea plant height between control and inoculated



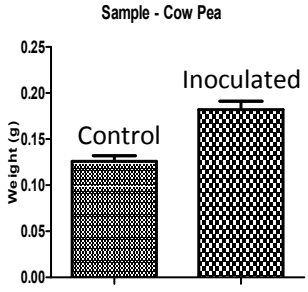
significant difference of Pea 100 seed weight between control and inoculated



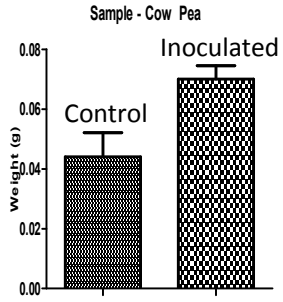
significant difference of Pea 50 Pod weight between control and inoculated



significant difference of Pea no of nodules between control and inoculated



significant difference of Pea fresh wt of nodules between control and inoculated



significant difference of Pea dry wt of nodules between control and inoculated

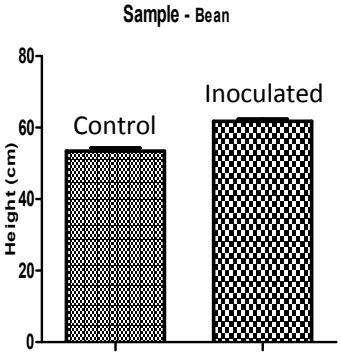
Figure 5.7: Graphical presentation, Effect of *Rhizobium* inoculation on Cow Pea.

Table 5.18: Effect of *Rhizobium* inoculation on various growth parameters in Bean

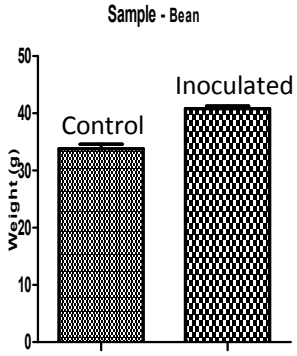
	Length (cms)	**50 pod wt. (gms)**	**100 seed wt. (gms)**	**No. of Nodules/ Plant**	**Fresh wt. of nodules (gms)**	**Dry wt. of nodules (gms)**
Inoculated	61.80 ± 0.5831 N=5	430.0 ± 6.124 N=5	40.80 ± 0.4899 N=5	72.40 ± 2.502 N=5	3.430 ± 0.07517 N=5	2.328 ± 0.08476 N=5
Control	53.40 ± 0.9274 N=5	380.0 ± 3.536 N=5	33.80 ± 0.8000 N=5	49.20 ± 1.685 N=5	2.246 ± 0.04238 N=5	1.594 ± 0.05372 N=5
Degrees of freedom	08	08	08	08	08	08
Calculated value. P=0.05	< 0.0001 ***	0.0001 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***

***Rhizobium*Inoculated** **Control**

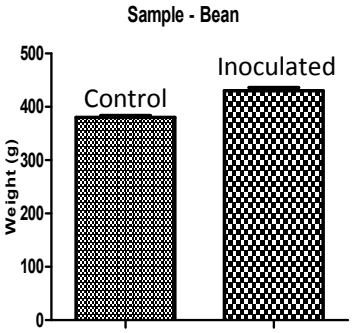
Plate 5.13: Number of nodules of Bean.



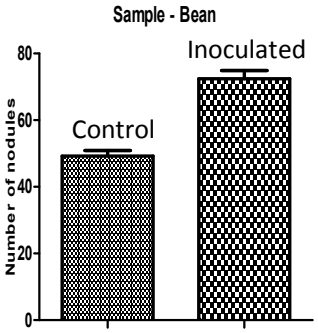
significant difference of Bean plant height between control and inoculated



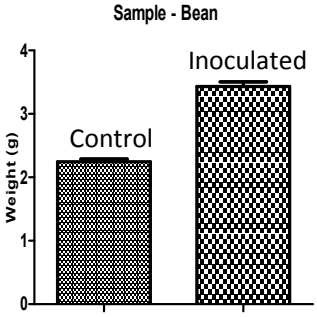
significant difference of Bean 100 seed weight between control and inoculated



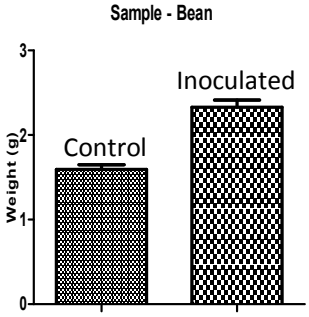
significant difference of Bean 50 Pod weight between control and inoculated



significant difference of Bean no of nodules between control and inoculated



significant difference of Bean fresh wt of nodules between control and inoculated



significant difference of Bean dry wt of nodules between control and inoculated

Figure 5.8: Graphical presentation, Effect of *Rhizobium* inoculation on Bean.

5.3.4 Effects of *Rhizobium* inoculation on leguminous plants

Plant height

In this case, all types of soil both for inoculated and control showed more or less similar results. Plants height of control and treated soil for Green Gram gave average values Green Gram(30 cm and 25.60 cm), , Black Gram, (29.2 cm and 26 cm), Lentil, (28.4 cm and 23.6 cm), Pigeon Pea (201.6 cm and 176 cm), Sweet Pea, (71 cm and 65 cm), Chick Pea, (42.9 cm and 39.3 cm), Cow Pea(61 cm and 53.6 cm), and Bean(61.8 cm and 53.40 cm), . In case of control, treated soils showed nearly similar results.

Plate 5.14: Plant height of lentil.

Dry weight of 50 pods

Here, also inoculated always showed higher result than control. For this character, inoculated soil of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean gave highest average value (5.4, 6.2 , 2.2 , 12.7, 6.7, 7.7, 5.3 and 430 g) and Control soil of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean gave average value were (4.2, 5.0, 1.56, 11.32, 5.7, 6.5, 4.5 and 380 g).

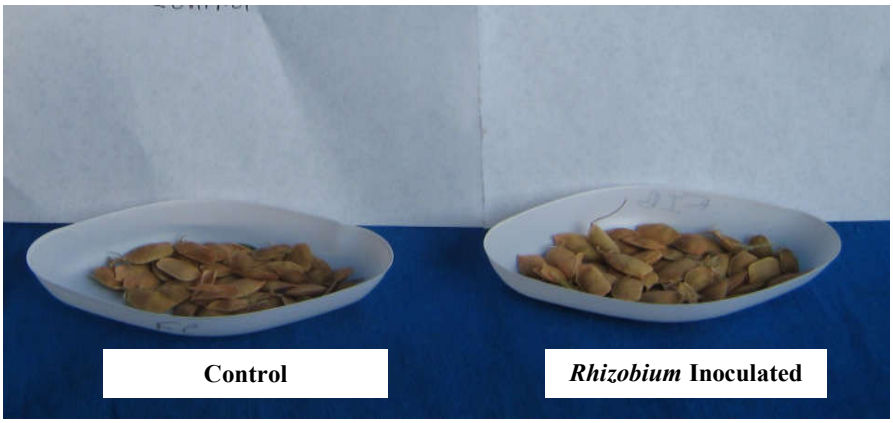


Plate 5.15:50 pod of lentil.

Average weight of 100 seeds

Here, also inoculated always showed higher result than control. For this character, treated soil of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Beangave highest average value (6.8, 9.8, 2.07,7.5,7.5,10.3,7.3, and 40.8 g) and control soil of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean gave average value were (5.5,8.0,1.55,6.6,5.4,9.0,6.0,and33.8 g).

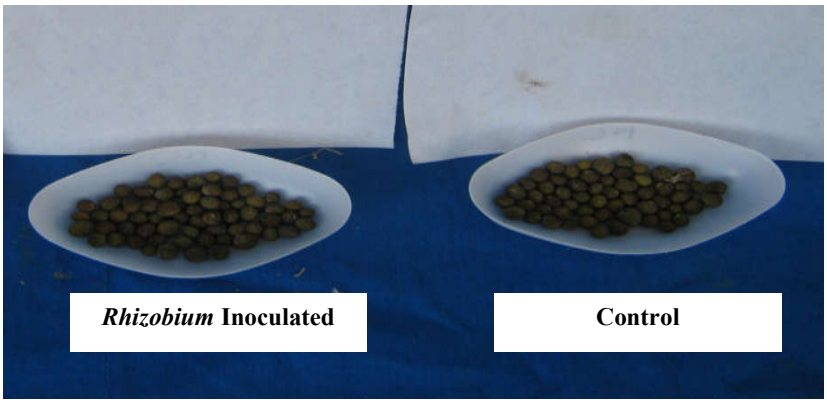


Plate 5.16: 100 seeds of Black Gram.

Number of nodules/plant

Nodulation of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Beanplants in inoculated soil (19.2,15.4,22,205,17,14,36.6,and72.40) showed highest value than control (13.2,9.2,12.4,171,11.8,07,30.8 and 49.2). Plants of the inoculated soil always showed more nodulation than uninoculated control soil.

Fresh weight of nodules

Fresh weight of nodules of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean plants in inoculated soil 0.27,0.25,0.25,20,0.27,0.19,0.18 and3.4g) showed highest value than control (0.08,0.078,0.08,14.80,0.15,0.05,0.12 and 2.24g). Average fresh weight of the plants nodules of the treated soil always showed greater value than uninoculated control soil.

Dry weight of nodules

Dry weight of nodules of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean plants in treated soil (0.19,0.17,0.10,13,0.21,0.14,0.07 and 2.3 g) showed highest value than control (0.048,0.05,0.044,9.8,0.10,0.04,0.04and 1.59 g). Average dry weight of the plants nodules of the treated soil always showed greater value than uninoculated control soil.

5.4 DISCUSSION

The results obtained in this study show some interesting aspects on the growth effects of *Rhizobium* inoculation in Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean which was grown in pots under controlled environment. The results indicate the successful nature of usage of commercial inoculants of *Rhizobium* to improve the growth effects of the pulses studied. Successful results by using commercial inocula of *Rhizobium* were obtained in field grown soybeans (Sharma and Tilak, 1974; Kapur*et al.,* 1975; Dev and Tilak, 1976). Eight isolates, Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean showed significant difference between inoculated and control .each other in No of nodule, fresh weight of nodule, dry weight of nodule, plant height,pod weight and seed weight. Rhizobialbial inoculation induced significant changes in plant growth characteristics. (Sharma and Tilak, 1974; Kapur*et al.,* 1975; Dev and Tilak, 1976). Inoculation of *Rhizobium* sp. causes a greater increase in growth and yield and the number of nodules per root system is significantly higher in plants inoculated with *Rhizobium* sp. compared to plants without *Rhizobium* sp. under field condition (Akhtar*et al.,* 2009) . Soil microorganisms produce quite a number of extra cellular enzymes to decompose the complex organic matter before it is absorbed as a source of energy. Seasonal variations in enzymes activities in forest soils are seen to bear correlation with the counts of fungi and bacteria (Kathiresan and Selvam, 2006;). The present study is to isolate and characterize *Rhizobium* from leguminous plants and induce the growth of black and green gram by inoculation of *Rhizobium* as bio-fertilizer. The effect of *Rhizobium* inoculation on number of nodules per plant of pea was significant (Table 5.11- 5.18). Plant inoculated with *Rhizobium* strains produced significantly higher number of total and effective nodule as compared to that of uninoculatedcontrol.Solaiman and Rabbani (2006) observed that pea inoculated with *Rhizobium* inoculant produced the highest number of nodules at pre-flowering and pod filling stages. The highest number of effective and total nodule was obtained with isolates.The plant height of eight leguminous plants was significantly influenced by rhizobialinoculants .The highest plant height was recorded inoculated rhizobial plants. All the isolates, which was statistically similar. The lowest plant height was obtained in uninoculated control. This result resembles with that of Hossain and Solaiman(2004) who stated that plant height of mungbean increased significantly due to inoculation seeds with *Rhizobium* isolates.

There was a significant variation in dry weight of nodule of eight leguminous plants of pea with different *Rhizobium*isolates.*Rizobium*inoculated with Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean produced the highest dry weight of effective and total nodule.The lowest dry weight of total nodule was obtained with the control plant whose effect was statistically similar result in agreement with Solaiman and Rabbani (2006) and who reported that *Rhizobium* inoculant significantly increased dry weight of nodules per plant in edible-podded leguminous plants.Further the *Rhizobium* inoculums used was containing charcoal as a carrier. The significantincreases obtained due to *Rhizobial* inoculation might have been due to better physico chemical properties of charcoal as explained by SubbaRao and Tilak (1982), who obtained significant increases in grain yield in their test plants where in they used charcoal as Rhizobial carrier. Further results also indicate the success of seed inocula of Rhizobia in the pulses tested. Burton (1979) has stated that the seed inoculum is done to introduce sufficient viable N Ravikumar, 2012.N2 fixing Rhizobia in to the area where the seed will germinate to assure effective nodulation.

Effects of varieties on nodule number, Fresh nodule weight,Dry nodule weight ,plant height, 50 pod weight and 100seed weight, have been presented in (Table 5.11-5.18).All the inoculated legume plant varieties studied, gave significantly higher on nodule number, fresh nodule weight, dry nodule weight, plant height, 50 pod weight and 100 seed weight yield.

Khanam*et al.,*(1994) in Bangladesh and Gupta and Namdeo (1986a) have also reported varied in nodulation and yield of chickpea due to use of different varieties. In the present experiment, BARI Chola-3 produced the highest nodule number and nodule weight, while BARI Chola-4 gave the highest seed yields which were significantly higher over all other varieties in 2002-03, but identical to other varieties in 2003-04. In another study, EusufZai*et al.,*(1999) found significantly more nodules in variety BARI Chola-6.

Effects of rhizobial inoculation on nodule number, nodule weight, 100 seed weight, stover yield and seed yield have been presented in (Table 5.11- 5.18). Inoculated plants gave significantly higher nodule number, nodule weight, stover yield and seed yield compared to uninoculated control. Khanam*et al.,*(1994) reported that inoculation with

Rhizobium strains gave higher nodule number, nodule dry weight, stover yield and seed yield compared to uninoculated plants. Bhuiyan*etal.,*(1998) found that *Rhizobium* inoculation increased nodulation and seed yields upto 35%.Gupta and Namdeo (1996b) found that seed inoculation with *Rhizohizim*increased chickpea seed yields by 9.6-27.9%. Interaction effects of varieties and rhizobial inoculation on nodule number, nodule weight, 1000-seed weight, stover yield and seed yield have beenpresented in Table 4. The highest nodule number, nodule weight and stover yield .were recorded with BARI Chola-3 with inoculation, but the highest seed yield was observed in inoculated BARI Chola-4 (16.0% higher in 2002-03 and 11.6% higher in 2003-04 over uninoculated control). Response to *Rhizohium*inoculation was more pronounced in BARI Chola-5which was 21.6% higher over uninoculated control in 2002-03 and 11.7% higher in 2003-04. Positive and significant correlations of nodule number were observed with nodule weight and 1000-seed weight and stover yield in both years. On the contrary, there was no significant correlation between seed yield and nodule number or weight. The nitrogen content of soil was below critical level in both the years (Table 1). Hence, inoculation with *Rhizobium* strains no. RCa-220 gave higher nodule number and weight in BARI Chola-3, but higher seed yields in BARI Chola-4 variety. Again, the seed yield varied significantly with the varieties in 2002-03 showing the highest result recorded by BARI Chola-4 and the lowest by BARI Chola-3. No significant difference was observed between varieties in 2003-04.

Inoculation in Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean enhanced the plant soil properties than control (Table 5.4-5.11) in line with(Tabatabai, 1994).

Soil reaction is the most important characteristic influencing the physical and chemical properties of soil. Plant growth and microorganism activity depends upon soil reaction and possible condition of the soil i.e.soil acidity, neutrality and alkalinity. On the contrary, excess to mineral nitrogen often limits plant growth. This is why properties of different types plant soil were tested and used accordingly in the present study. Alongside nitrogen fixing bacteria were used which can fix atmospheric nitrogen within the root nodules.

However, the present study reflects primarily the soil properties due to absence and presence of soil bacteria *Rhizobium.* The values recorded for total nitrogen in all the cases of this study indicates also the low level of fertility, though potassium (K) was medium in all the four types of soils before and after use in this experiment. On the other hand, phosphorous (P), sulphur (S) and zinc (Zn) status were very high according to BARC (2005).However, the present study deals mainly with the response of *Rhizobium* nodulation, and growth and crop yield of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean. It may be mentioned that Pahwa and Fatil (1983) found increased plant height of *Dolichos lablab* due to inoculation of pasture legumes with cowpea *Rhizobium.* Sing *et al.* (1984) found notable variation for biological nitrogen fixation at different growth stages of *Pisumsativum.*

In the present study plants grown in control and inoculated soil of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean showed highest values for plant height at maturity. Pawar and Ghulghule (1977) stated that branches/plants, number of pods and thousand seed weight of *Vigna radiate* were not found to be affected due to treatment with *Rhizobium.* For days to flower chickpea plants from all types of treated soils took fewer days compared to those of control. Plants grown in Padma soil particularly in control took fewest days to flower.Gowda*et al.* (1979) reported increased fresh weight of pods per plant in cowpea due to inoculation with lignite based culture of *Rhizobium.*

Rhizobium strains differ in their ability to nodulate and in their capacity t**o** fix nitrogen. Response to *Rhizobium* inoculation has been demonstrated by pot experiments in green houses, but field inoculation has given inconsistent results. This may be due to different population levels of native and added strains of *Rhizobium* and due to the competitive ability of native *Rhizobium* with the added strain (Rupelaand Dart, 1980). Symbiotic nitrogen fixation by *Rhizobium* meets the N requirement of the crop and leaves 40-108 kg/ha in the soil (SubbaRao, 1976). It is a matter of concern that reliable data is not available on the yield contributing potential and N contributing potential of rhizobia, despite the considerable investment and research attention this subject has received. Such information is needed to guide investment on fertilizer inputs.The highest value for fresh weight of nodules was found in the plants when grown in treated soil. The same soil showed the highest value for dry weight of nodules also. Dry weight of

nodules per plant compared to number of nodules was more closely related to seed yield as reported by Khurana*et al.,(*1984).Studies conducted in Bangladesh have been inconclusive and fragmented regarding nodulation and nitrogen fixation. Hossain (1999) reported that nitrogen up to 20ib/acre increase the number and weight of nodules. Similarly, P_2O_5 and K_2O have been reported to improve nodulation. The amount of N fixed by chickpea varied from 12.4 to 22.4 kg N/ha, in comparison to Sudan grass. Studies on survival of rhizobial strains indicated that the strain BAU-16 had longer viability under room temperature, up to 75 days. Both peat and soil are equally good as carriers of *Rhizobium* (Bhuiya*et al.,*2008).The present study deals with soil status of eight different plants and yield potential of Green gram,Black gram and Bean due to use of *Rhizobium* which indicates that all the three types of soil were very poor in terms of fertility and the yield of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean was, considerably, due to nodulation by the *Rhizobium.*Soil productivity is the ability of a soil for producing a specified plant under a specified system of management. It is usually expressed in terms of crop yield and that (soil status and yield in treated soil compared to that of control soil) has been possible to some extent in this study mainly due to use of*Rhizobium.*However, the present study also indicate that chemical fertilizers may be used in a reduced rate by the farmers in future for increasing grain production, but considering the productivity of the soil of Bangladesh efforts should also be oriented towards augmenting biological nitrogen fixation mediated by soil microorganisms.

5.5 CONCLUSION

Isolation and testing effectiveness of nitrogen fixing rhizobia from mega-biodiversity ecosystem of Bangladesh as well as monitoring the factors affecting the rhizobia, legume and symbiosis providing effective rhizobia is essential as it may result into the identification of super inoculants for improving legume growth and yield and later providing economic benefit to legume producers.Among the nitrogen fixing microorganisms, the role of *Rhizobium* is quite significant.In this work, a novel biofertilizer was developed using a species of *Rhizobium*, which was isolated from the root nodules of Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean plant. It was characterized with the help of morphological and

biochemical tests. The isolated strain was proven to be of *Rhizobium* spp by DNA sequencing. Using this strain, a biofertilizer was successfully produced using activated charcoal as the carrier and its effect was studied on Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean in combination with *S.cerevisiae*. An overall increase in the growth of the plant treated with the formulation was observed. Increase in biomass was observed in plant length , pod weight seed weight , number of nodules per plant , fresh weight of nodule and dry weight of nodule of the Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean plants.

From the above results it can be concluded that the bacteria isolated from root nodules of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean plants is a species of *Rhizobium*. The isolate is also a fast growing rhizobial species and able to form remarkable number of healthy nodules on inoculated crops. Result of inoculation test indicates that bacterization of seeds with adhesive is the most suitable among the inoculation methods used in this experiment and inoculation is responsible for good.These results indicate a significant increment in the productivity of plants treated with the newly developed biofertilizer. This novel biofertilizer can be produced at a higher commercial level and its effect checked on various high yielding agricultural plants. It is necessary to continue researching in this field as it has the potential to be highly profitable for farmers as well as provide a way to a more sustainable future.

So inoculation of Green Gram , Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean with *Rhizobium,* enhanced plant growth by providing a balanced nutrient supply due to their beneficial association with root system of the host plant. Therefore, more research and emphasis is required to popularize this cheap and eco-friendly technology for majority of farmers in Bangladesh.

5.6 REFERENCES

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Chapter 6

Summary, Conclusion and Recommendations

SUMMARY

The present study was carried out from March 2012 to February 2015 to isolate and characterize the bacteria which were responsible for nodulation in legume plants. samples were collected from Rajshahi, Bangladesh. Legumes (Green Gram, Black Gram, Lentil, Pigeon Pea, Sweet Pea, Chick Pea, Cow Pea and Bean) were collected and immediately brought to the Genetics and Molecular Biology Laboratory, Department of Zoology, University of Rajshahi. The isolates were primarily differentiated on the basis of their colony size and colour. Then sub-cultured on regular basis on Yeast extract mannitol agar medium and Yeast extract mannitol broth to get pure cultures for further tests.

Morphological, physiological and biochemical characteristics were done through Gram-staining, KOH stringing, lactose fermentation, citrate utilization, catalase, cytochrome-oxidase, oxidative-fermcntative, growth on triple sugar iron agar, sulfur reduction,nitrification test, alkaline test, congo red test, indole production, motility investigation, urea hydrolysis, methyl red and voges-Proskauer reaction, carbohydrate utilization, and antibiotic susceptibility test and growth optimization of the isolates have been done at temperatures from 28°C, at pH from 07 and at salinity 1 to 4%.

Genomic DNA was extracted from each of eight isolates using TIANamp Bacteria DNA kit (Tiangen, China) for molecular identification through I6S rRNA gene sequence. PCR was done with extracted genomic DNA using several sets of forward and reverse primers. PCR products were run through 1.0% agarose gel containing ethidium bromide and visualized under UV-transilluminator. Amplicons were purified using TIANquick Midi purification kit (Tiangen, China) and purity and concentration were measured using spectrophotometer Nanodrop 2000. With the obtained sequence from ABI Prism genetic analyzer NCBI nucleotide BLASTn search tool was used to find the possible matches deposited on databases. Bacterial identity was found according to the first match and FASTA sequences were downloaded and phylogenetic trees were constructed. Trees of the isolates were produced using BLAST pair wise alignments and neighbor joining tree method was used. Unrooted tree was downloaded as Newick in format and trees were edited with MEGA version 6.0 software.

The isolates designated as Legumes (Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean). Colony sizes were large for all the isolates and shapes.The colonies were large (>2mm indiameter) mucilaginous, circular, raised and smooth, edges, glistening translucent or white with musky odor, colony. Microscopic examination revealed that the isolates were rod shaped and gram negative in nature. In this study, all isolates were showed hazy appearance in the motility media and also were positive for Catalase, Citrate Utilization Test, Urea Hydrolysis, Congo red test ,Nitrification test ,Oxidase TestTriple Sugar Iron Test ,Mac Conkey Agar Test and, motility Tests. oxidase, catalase and urease positive and the samples were found negative for Methyl Red (MR), Voges-Proskauer (VP), Indole, Starch hydrolysis test, Hydrogen Sulphide production, and Hofer's alkaline test tests

Utilization of different carbon sources is an effective tool to characterize the isolates. In the present study the isolate Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean obtained from nodules were able to utilize glucose, galactose, arabinose, xylose, lactose, sucrose, maltose, mannitol and fructose as carbon sources. These carbon sources are generally utilized by bacteria of the genus *Rhizohium*. also observed that fast growing Rhizobial strains utilized a wider variety of carbohydrates than the slow growing strains. Their ability to metabolize a broad range of carbon substrates may be advantageous for survival in soil. Among other isolates obtained in this study.

Resistance patterns of the isolates to thirteen antibiotics were studied. Screening for antibiotic resistance in our study revealed that most of the strains were resistance to Ampicilin, Erythromycin, Gentamicin, Amoxycillin, Penicillin, Streptomycin and Nalidixic acid. The generalized sensitivity was found to tetracycline, Mecillinam, Ciprofloxacin, Cotrimoxazole, Pefloxacin and Ceftazidime in our study.

Isolates showed their optimum growth in the range of temperature 28-30°C, pH 6.8 – 7.0 and salinity at 1%.Five different primers were used and the combination of 8F-806Rand 8F- I492R gave more contrast single band in between 700 - 800bp in comparison to 1 kb plus DNA ladder. PCR was carried out with an increased volume (50 µl) using 8F-806R. PCR products were purified and prepared for sequencing PCR. The BLAST search results for the partial sequences of the 16S rRNA gene of the isolates revealed purity and concentration of extracted DNA were performed at 260/280

nm obtained as 1.77 for Green Gram,1.74 for Black Gram, 1.74 for Lentil 1.77 for Pigeon Pea,1.85 for Sweet Pea,1.77 for chickPea,1.82 for CowPea,and 1.84 for Bean and concentration as 42.0, 83.9, 28.9, 49.0, 88.5, 68.8, 86.8 and 69.7 ng/µl for Green Gram , Black Gram, lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean, respectively. Finally the selected eight bacterial isolates were identified up to species as *Rhizobium* sp. through 16S rDNA gene sequencing. There were eight experimental plants from which eight colonies of *Rhizobium* were isolated of which five strains showed similarity among them (*Rhizobium* sp. CCNWYC119) another two strains were also similar (*Rhizobium* sp. SOY12) and only one (*Rhizobium* sp. SOY7) was different from based on the nuleotide sequence.

This study also shows that enhancement of soil fertility along with the yield of legume plants occurred due to nodulation. In this study show some interesting aspects on the growth effects of *Rhizobium* inoculation in Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean which was grown in pots under controlled environment. The results indicate the successful nature of usage of commercial inoculants of *Rhizobia* to improve the growth effects of the pulses studied. Successful results by using commercial inocula of *Rhizobia* were obtained in field grown eight isolates(Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean) showed significant difference between inoculated and control . Each other in No of nodule, fresh weight of nodule, dry weight of nodule, plant height, pod weight and seed weight. Rhizobial inoculation induced significant changes in plant growth characteristics. This study also showed that enhancement of soil fertility along with the yield of legume plants occurred due to nodulation. Soil treated with *Rhizobium* spp. showed higher values than control regarding total nitrogen and organic matter (%).

Bangladesh is a developing country facing malnutrition problems.Protein-rich crops might play a significant role to meet up this problem. Pulse crops are legumes containing enriched nutrients might be an important source of plant protein.

Around 5.2% of cultivable lands are subjecte to legume cultivation. As a bio-fertilizer, *Rhizobium* significantly improves the yield in many legume crops by fixing atmospheric nitrogen in symbiosis with legumes. So in order to accelerate the increase of production of legume crops for the uplift of protein status nationally as well as internationally it is important to study the ecological characterization and identification the *Rhizobium* spp.

CONCLUSION

Bangladeshi soils harbor highly efficient nitrogen-fixing lentil nodulating rhizobia which are diverse in their morphological, physiological and symbiotic characteristics. Our study also shows that Rajshahi has indigenous rhizobia which exhibit a wide diversity in tolerance to acidity, salinity and antibiotics. During this study methods used for characterizing and distinguishing rhizobial strains were morphological, physiological and symbiotic. However, these traditional methods of rhizobial characterization frequently fail to identify strains within a species. Hence, such kinds of study must be substantiated by PCR based molecular methods and 16S rRNA sequence analysis so as to obtain a better understanding of microbial diversity and strain identification.

Rhizobia are aerobic rod shaped, motile, gram negative, hetrotrophic, and non-spore forming bacteria. They are motile when young and have bipolar, subpoler or peritrichous flagella. Rhizobia fix atmospheric nitrogen and thus not only increase the production of inoculated crops, but also leave a fair amount of nitrogen in the soil, which benefit the subsequent crops. This successful symbiotic association requires the survival of rhizobia insufficient number as free living bacteria in the soil ecosystem. Nitrogen is the most deficient nutrient in Bangladesh soils. Urea, which is the most commonly used nitrogenous fertilizer, has now become a costly input for most of the farmers. As such, *Rhizobium* inoculants may be used as a cheaper substitute for urea in the production of food legume crops. The beneficial effect of rhizobial inoculates in increasing yield of leguminous crops results from the activity of its root nodule bacteria, which fix atmospheric nitrogen making it available for the plants. Inoculation types and many other factors usually affect the nodulation, nitrogen fixation and plant growth.

For crop growth minimum inoculum level is necessary to obtain beneficial effects. The inhibitory or stimulatory effects of soil microorganisms such as bacteria, fungi and actinomycetes on *Rhizobium* are known. In the present study an attempt has been made in the present investigation to isolate *Rhizobium* from Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean to study the effects of its inoculation on nodulation and growth.

Among the nitrogen fixing microorganisms, the role of *Rhizobium* is quite significant. In this work, a novel biofertilizer was developed using a species of *Rhizobium*, which was isolated from the root nodules of Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean plant. It was characterized with the help of morphological and biochemical tests. The isolated strain was proven to be of *Rhizobium* spp by DNA sequencing. Using this strain, a biofertilizer was successfully produced using activated charcoal as the carrier and its effect was studied on Green Gram, Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean in combination with *S. cerevisiae*. An overall increase in the growth of the plant treated with the formulation was observed. Increase in biomass was observed in plant length , pod weight seed weight , number of nodules per plant , fresh weight of nodule and dry weight of nodule of the Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean plants.

From the above results it can be concluded that the bacteria isolated from root nodules of Green Gram , Black Gram, Lentil, Pigeon Pea Sweet Pea, Chick Pea, Cow Pea and Bean plants is a species of *Rhizobium*. The isolate is also a fast growing rhizobial species and able to form remarkable number of healthy nodules on inoculated crops. Result of inoculation test indicates that bacterization of seeds with adhesive is the most suitable among the inoculation methods used in this experiment and inoculation is responsible for good.

These results indicate a significant increment in the productivity of plants treated with the newly developed biofertilizer. This novel biofertilizer can be produced at a higher commercial level and its effect checked on various high yielding agricultural plants. It is necessary to continue researching in this field as it has the potential to be highly profitable for farmers as well as provide a way to a more sustainable future.

It is inferred from the present investigation that association of microbes depends on developmental stage of plants and on its root architecture. A remarkable diversity of *Rhizobium* was evident with regard to water stress. There were differences in carbon/nitrogen utilization among the isolates collected from different moisture regimes. Isolates obtained from roots and rhizosphere of plants growing at irrigated field area and from plants growing under well watered conditions showed maximum utilization of carbon/nitrogen, while the isolates obtained from roots and rhizosphere

of plants growing at arid field area and under induced water stress showed least utilization efficiency. Association of microbes depends on developmental stage of plants and on its root architecture. Anthesis stage of plants is more critical with regard to plant microbe interaction as maximum phytohormone production has also been showed by isolates obtained from roots and rhizosphere of plants growing at irrigated field area and from plants growingunder well watered conditions. For the better performance the inoculums should be applied at both stages particularly on anthesis with regard to the degree of associations with plants as well as phytohormone production on the basis of better phytohormone production and the affects of water stress were more pronounced at anthesis stage on survival efficiency and phytohormone production *Azospirillum* can be better adapted to water stress conditions as it has greater magnitude of hormone production. isolated from moisture stressed conditions arid region have potential for increased tolerance to water stress if used as an inoculum to promote plant growth on stressed sites particularly semiarid and arid regions of the world.

PGPRs are the potential tools for sustainable agriculture and trend for the future. For this reason, there is an urgent need for research to clear definition of what bacterial traits are useful and necessary for different environmental conditions and plants, so that optimal bacterial strains can either be selected and or improved. Combinations of beneficial bacterial strains that interact synergistically are currently being devised and numerous recent studies show a promising trend in the field of inoculation technology. These findings allow us a new scope for extensive research in Agricultural Biotechnology.

RECOMMENDATION

The studies reviewed here have provided clear evidence of the considerable effort being made to select legume verities and rhizoidal inoculate that can fix nitrogen and generate competitive crop yields in degraded and marginal stress-affected soils. Furthermore, better understanding the physiological and molecular mechanisms involved in the tolerance to environmental stresses in giving rise to numerous biotechnological approaches aimed at obtaining improved legumes and rhizobia with enhanced tolerance to abiotic stress, paying particular attention to the sensitive nitrogen-fixing activity.

In a context of global and climate change, with a growing population and an increasing demand for food and feed, the importance of legumes in sustainable agriculture, and particularly in the reclamation of marginal lands, seems beyond any doubt. Needs might diverge in different parts of the glove, often in close connection with the regional development, the natural and acquired richness of the countries and the level of wellbeing, environmental awareness or unacceptable poverty of their peoples. For different and often cumulative reasons, sustainable agriculture in an ever increasing and universal need. While developing countries focus on providing sufficient food to their population, which requires both increasing yields and reclaiming yet-unexploited marginal lands, developed states are increasingly aware of the importance of the quality of their food and of the need to restore degraded environments. In either case, improved legumes are equall advantageous. High yields without the added costs of nitrogen fertilization are becoming a must for precarious economies whereas chemical-free, organic foods and feed are in increasing demand in advanced societies.

Given that research in the field of legume and inoculants stress tolerance in increasingly necessary, it appears the field experimentation is still insufficient, and laboratory and greenhouse results must be contrasted in cultivation. Despite some success stories, much still remains to be achieved in this respect, which is necessarily associated with technology transfer and the spread of knowledge to farmers, including the acquisition of agricultural management practices to maximize biological nitrogen fixation, such as intercropping or limited tillage. Selecting locally adapted legumes and rhizoid proves to be essential and as such, small scale approaches are indispensable. Regarding transgenic approaches, the perspectives are extremely promising as our understanding of the mechanism involved in stress tolerance is advancing in great

bounds. Transcriptomic approaches are being complemented by proteomics, metabolic and gens regulation studies, and new genes and traits are being made available to engineer tolerance in the extremely complex legume *Rhizobium* soil climate system. Thus research is under way and unprecedented local and global success in expected.

Others given below

1. There is need for extensive agro ecological zone evaluation for eight rhizobia because rhizobia isolates are site-specific.
2. Cultivar selection should be a major component of future work related to the pulse crops rhizobia symbiosis in Bangladesh.
3. Continued research on the effectiveness of commercially available rhizobia inoculants in Bangladeh is crucial for the farmer to get the best for yield increase.
4. Further research; molecular characterization of eight isolates should be done for appropriate recommendation to farmers.
5. To avoid pot inoculation, there should be specific inoculants for specific legume variety. Therefore, consideration of legume variety when producing inoculants is crucial since differences in response to the commercial standard strain was evident
6. *Rhizobium* biofertilizers are recommended for grain legumes to improve the productivity and to augment soil nitrogen status. A good strain of *Rhizobium* should beused in biofertilizer production which is capable offorming effective nitrogen fixing nodules on legumes. *Rhizobium* cells were immobilized on carriers, which is aninert material used for mixing with broth so that inoculants can easily be handled, packed, stored, transported and used. The broth containing *Rhizobial* cells were mixed with carrier. The moisture content was maintained at 35-40%. During this period, *Rhizobium* cells multiplied, by a process called curing. Thereafter, *Rhizobium* inoculants were used, packed and stored. *Rhizobium* inoculation is a well known agronomic practice to ensure adequate nitrogen fixation of legumes instead of N-fertilizers.

Co inoculation benefits the plant growth. Quantitative analysis revealed that sufficient amount of growth on the basis of viable cell count was recorded in both the forms of *Rhizobium* biofertilizer.

However, Quality of biofertilizer is one of the most important factors resulting in their success or failure and acceptance or rejection by end-user, the farmers. Basically, quality means the number of selected microorganism in the active form per gram or milliliter biofertilizer. Quality standards are available only for *Rhizobium* in different countries. Quality has to be controlled at various stage of production (during mother culture stage, carrier selection, broth culture stage, mixing of broth and culture, packing and storage). When solid biofertilizers were assessed for quality check; both the inoculants exhibited improved properties. Sufficient amount of nitrogen and carbon content was found in both the strains. Neutral pH, sufficient moisture and moderate electrical conductivity revealed that both the inoculants were effective to be used in pot culturetest. Use of the Rhizobial strains as biofertilizer in the field is completely safe based on decades of research and application as biofertilizers and does not harm the environment as they do nitrogen fertilizers are usually applied. The preparation of *Rhizobium* bacteria for use as biofertiliziers and application of planting is simple and very inexpensive. So the replacement of chemical fertilizer nitrogen represents a significant economic benefit for fermers. Additionally the seed produced has better nutritional characteristics for the general consumer.

The present study indicated the presence of indigenous population of *Rhizobium* spp. in different field sites in Rajshahi, Bangladesh. These indigenous strains were genetically divers from reference and type strains used in this study. Symbiotic efficiency as well as other symbiotic properties of this strains should be further investigated. This study provides an ecological frame work that can be used for selection of efficient Rhizobial strains that are adapted to local environmental factors and could be used in production of high quality rhizobial inoculants.

APPENDICES

Appendix 1: Apparatus used in this study (image source internet)

Incubator

Hot water bath

Orbital shaker

Photoelectric colorimeter

Hot plate and magneticstirrer

Mini centrifuse

Table top centrifuse

Gel electrophoresis unit

Thermal cycle

Gel documentation

Nanodrop spectrophotometer

Sequencer

Appendix2:Culture media used in this study

Yeast extract mannitol agar (YEMA)

K_2HPO_2	: 0.5g
$MgSo_{4,}\ 7H_2O$	: 0.2 gm
NaC1	: 0.1 gm
Mannitol	: 10.0 gm
Yeast extract	: 1gm
Distilled water	: 1000ml
Agar	: 20.0gm

congored 1% sol 2.5 ml (only for solid medium during isolation)

Hofer's Alkaline Medium (Hofer 1935)

The reaction of Yeast extract mannital medium is raised to pH 11.0 with IN NaOH, 1 ml of 1.6% thymol blue per litre 10 added.

YEMA with congo-red Medium

Congo-red (2.5 ml/1 of 1% solution) is incorporated into yeast extract mannitol medium.

Peptone-glucose agar

Glucose	: 5.0 gm
Peptone	: 10.0gm
Agar	: 10-15gm
Distilled water	: 1000ml

Lactose medium

Lactose is substituted to mannitol in the yeast extract mannitol medium

LB(Luria Bertini) medium

Tryptone:1g

NaC1	: 0.1 g
Yeast extract	: 1g
Distilled water	: 1000ml
pH	: 7.2

Appendix 3:Obtained nucleotide sequences of the isolates Green Gram (from Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

CGAGCGGCAGTCTACCATGCAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGC
GGACGGGTGAGAAAAGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGA
CGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAG
ATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTA
ACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTG
AAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTC
TGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA
ATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGT
TAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAG
AGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGG
AACACCAGTGGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTG
GGGGAGCAACAGAATAGATACCCTAGTAGTCCAGGAGGTCGCCTTCGCCACTGGGTGTTCC
TTCTATATCTACGCCATTTCACCGCTACACAGTAATCACCCACCCTCTACATACTCTAGTCAG
TCAGTTTTGCAATGCAGTTCCCAGGATGAGCCCGGGGATTCCACTCCACCTAACCACACATC
GCGCGCATAGCCCCGAGTATTTCCGGATTTAG

Appendix 4:Obtained nucleotide sequences of the isolates Black Gram(from Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

CATAGCGCATCTACCATGCAGTCGAGCGGATGAAGGAGCTTGCTCCTGGATTCAGCGGCGG
ACGGGTGCGTATTGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGC
TAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATG
AGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACT
GGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGC
AATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATA
CAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAA
GTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGT
ATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAAGGAAC
ACCAGTGGCGAAGGCGACCACCTGGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGG
GAGCAAACAGATAGAATCTCTGTGTGTAATTAAGAGGTTGTCCCCCTTCGCCCACTGGGTGT
TCCTTCCTATATCTACGACGTCTTTCCCACTGCAAGCAAGTTAATTCCCTCCAACCGTCATCA
CTCGCAAGCTTGAAGTCATTCGCATGTAGATCCCGGGAATTGACTCCGAGTATATATAACAC
TACGCCGTCTTACACATCACTTCGATGACCTTGGCCTTGGATATTACTGCGCGCGACTG

Appendix 5: Obtained nucleotide sequences of the isolates Lentil (from Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

GGTACCGGCGTCTACCATGCAGTCGAGCGGCAGCGGAGGTAGCTTGTTACTTTGCCGGAGA
GCGGCGGTTTGAAGAGAAGTGTCTGGGAAAGTGCCTGATGGAGGGGTATCCCTTGTGGAAA
CGGTATCTAATACCGCATAGTGTCACAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCC
TCATATGTGCCCATATGGTATTATCTAGTATGTGGGGTAACGTCTCCTCTACGCCACTATCCC
TATCTGGTCTGAGAAGATCACCACCCACTCTGAAACTGACACACGCCCCACACTCCTACGG
GGGGCAGCTGTGGGGAATTTTGCACAATGGGCGCGACTCTTATGCCCCCTGCCGTGTGTGTG
AAAAAGGCCTTCGGTTTGTAAAGTTCTCTCCGCGGAGGAGAAGGCGTTGAGGTTTATACTCT
CCGCTAGTGACTTCACCCGCAAAAAAAGCACCGTCTATCTCTGTGCCAACACGCGCGATAA
TACAGAGGGTGCACGCGTAACTCGATATTACTGGGCGTAACGCACACGCGCGCGCTCTCTC
AAGTGAGATGTGAACTCCGCGGTCTCACCGTGGGATCTGCTTTCAAATCTGGCGGTCTAGAC
TCGTGTAGAGGGGGGTAGATCTACGTGTGTAGCTGAGGATATGCGTAGATACTCGTAGAGG
TTATCACCTGTCGACGAGCGCGGCCCTGTGACACAGAGTAGCTGACTGCTCAGTGTCGCAA
GCGTCGTGGGGGCGCCCCCGGTTGGGTCTGGGGTTAGTTGACCAAA

Appendix 6: Obtained nucleotide sequences of the isolates Pigeon Pea(from Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

CGTGGGCAGCTACCATGCAGTCGAGCGGATGAGAGAGCTTGCTCCTGGATTCAGCGGCGGA
CGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCT
AATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGA
GCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTG
GTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGA
AGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCA
ATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC
AGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAG
TTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTA
TGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGACACC
AGTGGCGAACGCGACCACCTGGACTGATACTGACACTGAGTGCGAAAGCGGTGGGGAGCA
AACAGATTAGATACGTGTGGTAATATTCAGGG

Appendix 7: Obtained nucleotide sequences of the isolates Sweet Pea(from Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

CGTCCGGCAGCTACCATGCAGTCGAGCGGATGAAGGAGCTTGCTCCTGGATTCAGCGGCGG
ACGGGTGAGAAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGC
TAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATG
AGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACT
GGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGC
AATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATA
CAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAA
GTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGT
ATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAAGGAAC
ACCAGTGGCGAAGCGACCACCTGGACTGATACTGACACTGAGTGCGAAAGCGTGGGGAGC
AAACAGGATTAGATACGTGTGGGAGTGTTCACGAGGGGG

Appendix 8: Obtained nucleotide sequences of the isolates Chick Pea(from Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

CCTGCGCATCTACCATGCAGTCGAGCGGATGAAGGAGCTTGCTCCTGGATTCAGCGGCGGA
CGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCT
AATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGA
GCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTG
GTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGA
AGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCA
ATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC
AGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAG
TTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTA
TGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACAC
CAGTGGCGAAGGCGACCACCTGGGACTGATACTGACACTGAGTGCGAAGCGTGGGAGCAA
ACAGGATAGAACTCGTGTGAGATGACCACAAGTTGGCCCTTGCGCAATGGGTGTTTCCTTCT
CATATTCTACCGCAGTTCCCCCTGACACGAAGATTCCATCACCTCATACTAACTTACTGCAG
TTTGGAAGTTCTGTCAGGTGACCGCATATTCAACTGACTTAACAACACTTAGCGCTTCTTTA
GCA

Appendix 9: Obtained nucleotide sequences of the isolates Cow Pea (from Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

CGTCCGCAGCTACCATGCAGTCGAGCGGTAGCACAGGGGAGCTTGCTCCCCGGGTGACGAG
CGGCGGATGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAAC
GGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCAT
CAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCC
CTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT
GTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAGCTTAATA
CGCTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCG
GTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTT
TGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGC
TAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGA
GGAATACCGGTGGCGAAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGTGCGAAGCGTG
GGGAGCAAACAGATAGACACGTGGGTGTGTAAAGAAAA

Appendix 10:Obtained nucleotide sequences of the isolates Bean (from Rajshahi) from ABI Prism 3130 genetic analyzer in FASTA format.

AATAATAAAATAGTGGGTTCAGAACAAAAAAACGTCCGCAGTCTACCATGCAGTCGACGGT
AGCACAGAGAGCTTGCTTTCGTGTGACGAGTGGCGGACGGGGGAGGAATGTCTGGCAAACT
GGCTGATGGAGGGGGATAACCCCTGGGAACGGTAACTCATACCGCGTAACGTCGCAAGACC
AAAGAGGGGGACCTTCCGGGCTCTTGCCATTTAATGTGCCCCGATGGGATTAGCTAGTGAT
GGGGGGAACGGCTCACCTAAGCGACCATCCCTGGCTGGTCTGAAAGGATGACCACCCTCAC
TGTGACTGAGACACGGTCCTCACTCCTACGGGAGGCATGGGTGGGGAATATTGCACAATGG
GCGCCAGCCTGATGCATCCATGCCGCGTGTATGAAGAATGCCTTTTGGTTGTAAAGTACTTT
TGGCGGGAAGGAAGGCGGTTAGGTTAATAACCTCTTTGATTGACCTTACCCACAAAACAAG
CACCGGCTAACTCCGTGCCACCAGCCGCGTTCATACGGAGGGTGCGTGCGTTAATCTTACTT
ACTGTGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTATGTATGTGAAATCCCCCTCGATCTC
CTGGGAACTGCATTCCTGACTGGCTGCTATATTCTTGTAGAGGGGGGGTATAATTCTTGGGT
GTAGCGGTATAAATTGCGTATAGAGCTGGAAGAAACAATCGGGTGACGGAAGGCCGGCCCT
CCTCTCGTACTACATAACTGTTCACCTCTTGGTGCAAAAGCGTGGGGGGAGCACGACAATG

Appendix 11: Data, effects of *Rhizobium* inoculation on Green Gram.

Plant height (cm)		**50 pod wt (g)**		**100 seed wt (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
26.	30.	4.4	6.0	5.4	7.0
25.	31.	4.2	5.6	5.6	6.8
24.	30.	4.0	5.2	5.4	6.6
26.	29.	4.4	5.0	5.8	7.0
27.	30.	4.0	5.2	5.6	6.8

Number of Nodules per plant		**Fresh wt of nodules (g)**		**Dry wt of nodules (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
12	18	0.08	0.28	0.05	0.19
14	20	0.08	0.28	0.05	0.20
15	21	0.09	0.29	0.06	0.20
13	18	0.07	0.27	0.04	0.20
12	19	0.08	0.26	0.04	0.19

Appendix 12: Data, effects of *Rhizobium* inoculation on Black Gram.

Plant height (cm)		**50 pod wt (g)**		**100 seed wt (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
25.	29.	5.0	6.30	8.0	9.8
26.	30.	5.1	6.30	8.1	9.9
24.	28.	5.2	6.40	8.2	9.9
27.	29.	5.0	6.20	8.0	9.7
28.	30.	5.0	6.20	8.0	9.8

Number of Nodules per plant		**Fresh wt of nodules (g)**		**Dry wt of nodules (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
8.	13.	0.07	0.19	0.05	0.12
9.	15.	0.05	0.22	0.04	0.13
10.	16.	0.08	0.25	0.05	0.20
9.	18.	0.09	0.29	0.06	0.21
10.	15.	0.10	0.30	0.07	0.20

Appendix 13: Data, effects of *Rhizobium* inoculation on Lentil.

Plant height (cm)		**50 pod wt (g)**		**100 seed wt (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
22.	28.	1.55	2.25	1.65	1.90
23.	28.	1.60	2.10	1.62	2.00
25.	29.	1.50	2.15	1.51	2.20
23.	28.	1.55	2.25	1.45	2.25
25.	29.	1.60	2.30	1.52	2.00

Number of Nodules per plant		**Fresh wt of nodules (g)**		**Drywt of nodules (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
11.	19.	0.090	0.25	0.047	0.130
14.	24.	0.070	0.29	0.050	0.120
13.	22.	0.075	0.22	0.040	0.090
12.	26.	0.080	0.22	0.040	0.090
12.	19.	0.120	0.29	0.045	0.085

Appendix 14: Data, effects of *Rhizobium* inoculation on Pigeon Pea.

Plant height (cm)		50 pod wt (g)		100 seed wt (g)	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
180.	200.	11.6	13.4	6.6	7.10
175.	210.	11.3	13.0	6.5	7.40
169.	203.	11.2	12.8	6.8	8.00
174.	195.	11.0	12.5	6.6	7.50
185.	200.	11.5	12.2	6.5	7.70

Number of Nodules per plant		Fresh wt of nodules (g)		Dry wt of nodules (g)	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
170.	210.	15.	21.	10.	13.
166.	204.	14.	19.	11.	12.
175.	200.	16.	20.	9.	13.
170.	205.	15.	21.	10.	14.
174.	208.	14.	19.	9.	13.

Appendix 15: Data, effects of *Rhizobium* inoculation on Sweet Pea .

Plant height (cm)		**50 pod wt (g)**		**100 seed wt (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
65.	74.	5.80	6.30	5.40	7.2
64.	72.	5.70	6.80	5.35	7.3
67.	70.	5.85	6.90	5.50	7.2
68.	69.	5.80	6.95	5.45	7.8
63.	70.	5.75	7.00	5.50	8.0

Number of Nodules per plant		**Fresh wt of nodules (g)**		**Dry wt of nodules (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
9.	18.	0.08	0.29	0.06	0.210
12.	16.	0.18	0.25	0.13	0.210
13.	19.	0.19	0.35	0.14	0.250
15.	15.	0.21	0.20	0.14	0.230
10.	17.	0.09	0.26	0.05	0.160

Appendix 16: Data, effects of *Rhizobium* inoculation on Chick Pea.

Plant height (cm)		**50 pod wt (g)**		**100 seed wt (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
40.0	43.0	6.4	7.5	9.0	10.5
39.0	42.0	6.5	7.6	9.3	10.3
40.0	43.5	6.6	7.8	9.0	10.2
38.0	42.0	6.7	8.0	9.2	10.5
39.5	44.0	6.5	7.7	8.9	10.4

Number of Nodules per plant		**Fresh wt of nodules (g)**		**Dry wt of nodules (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
6.	12.	0.04	0.18	0.030	0.14
7.	15.	0.05	0.20	0.040	0.15
7.	14.	0.05	0.19	0.035	0.14
8.	14.	0.07	0.19	0.050	0.15
7.	15.	0.06	0.20	0.045	0.14

Appendix 17: Data, effects of *Rhizobium* inoculation on Cow Pea

Plant height (cm)		**50 pod wt (g)**		**100 seed wt (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
55.	60.	4.50	5.34	6.24	7.84
52.	61.	4.60	5.40	6.04	7.24
54.	62.	4.55	5.20	6.00	7.50
55.	65.	4.60	5.30	6.20	7.10
52.	57.	4.60	5.50	6.00	7.20

Number of Nodules per plant		**Fresh wt of nodules (g)**		**Dry wt of nodules (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
30.	38.	0.12	0.18	0.05	0.06
29.	37.	0.12	0.18	0.06	0.08
30.	39.	0.15	0.20	0.03	0.08
32.	35.	0.12	0.15	0.02	0.07
33.	34.	0.12	0.20	0.06	0.06

Appendix 18 Data, effects of *Rhizobium* inoculation on Bean .

Plant height (cm)		**50 pod wt (g)**		**100 seed wt (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
55.	60.	375.	415.	34.	42.
56.	63.	380.	420.	32.	40.
52.	62.	390.	430.	36.	40.
53.	63.	385.	435.	35.	40.
51.	61.	370.	450.	32.	42.

Number of Nodules per plant		**Fresh wt of nodules (g)**		**Dry wt of nodules (g)**	
control	**inoculated**	**control**	**inoculated**	**control**	**inoculated**
49.	65.	2.22	3.20	1.62	2.100
55.	70.	2.40	3.35	1.75	2.200
50.	80.	2.26	3.65	1.65	2.540
47.	72.	2.20	3.45	1.50	2.300
45.	75.	2.15	3.50	1.45	2.500