CHARACTERIZATION AND EVALUATION OF NATIVE *RHIZOBIUM* OF *LATHYRUS*, GREEN GRAM, CHICKPEA AND BLACK GRAM

M.Sc. (Ag.) Thesis

by

TOPI KAMGO

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY
COLLEGE OF AGRICULTURE
FACULTY OF AGRICULTURE
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CHARACTERIZATION AND EVALUATION OF NATIVE *RHIZOBIUM* OF *LATHYRUS*, GREEN GRAM, CHICKPEA AND BLACK GRAM

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CERTIFICATE - I

This is to certify that the thesis entitled **"Characterization and evaluation of native *Rhizobium* of *Lathyrus*, Green gram, Chickpea, and Black gram"** submitted in partial fulfillment of the requirements for the degree of **"Master of Science in Agriculture" (Agricultural Microbiology)** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur, is a record of the bonafide research work carried out by **Topi Kamgo** under my/our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma or certificate course. All the assistance and help received during the course of the investigations have been duly acknowledged.

13.7.19

Chairman

Date: 13.7.19

THESIS APPROVED BY THE STUDENT'S ADVISORY COMMITTEE

Chairman (Dr. S.B. Gupta) 13.7.19

Member (Dr.Ravindra Soni)

Member (Dr.Anurag)

Member (Dr. Gayatri Chandrakar)

Member (Dr. Tapas Chowdhury)

CERTIFICATE – II

This is to certify that the thesis entitled **"Characterization and evaluation of native *Rhizobium* of *Lathyrus*, Green gram, Chickpea and Black gram"** submitted by **Topi Kamgo** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur, in partial fulfilment of the requirements for the degree of **M.Sc. (Ag.)** in the **Department of Agricultural Microbiology** has been approved by the external examiner and Student's Advisory Committee after oral examination.

Signature External Examiner
(Name Prof. B.R. Maurya, SSAC
BHU, Varanasi.

Date: 27.7.19

Major Advisor

Head of the Department

Faculty Dean

Approved/Not approved

Director of Instructions

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Department of Agricultural Microbiology
College of Agriculture
IGA., Raipur

Dated: ***Topi Kamgo***

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LIST OF ABBREVIATIONS

%	Percent
kg	kilogram
ha	hectare
lb	pound
N	Nitrogen
PSB	Phosphate Solubilizing Bacteria
P	Phosphorus
NH_3	Ammonia
mg	milligram
m	metre
BTB	Bromo Thymol Blue
YEM	Yeast Extract Mannitol Agar
Ndfa	Nitrogen derived from atmosphere
NaCl	Sodium Chloride
ERIC	Entero-bacterial Repetitive Intergeneric Concensus
PCR	Polymerase Chain Reaction
Pb	Lead
Zn	Zinc
LPS	Lipo-polysaacharide
rRNA	Ribosomal RNA
mm	millimetre
Mg	Magnesium
Mn	Manganese
et. al	and co-workers/and others

µg	microgram
Fig.	Figure
NCBI	National Centre for Biotechnological Information
BLAST	Basic Local Alignment Search Tool
MRVP	Methyl Red, Voges-Proskauer
µM	micrometre
DNA	Deoxyribonucleic acid
°C	Degree Celsius
RZT	Root Zone Temperature
PGPR	Plant Growth Promoting Rhizobacteria
RAPD	Random Amplified Polymorphic Rhizobacteria
mM	milliMolar
Cu	Copper
DAS	Days After Sowing
RFLP	Restricted Fragment Length Polymorphism
CFU	Colony Forming Unit
µl	microlitre
WS	Wash solution
rpm	revolutions per minute
TAE	Tris base,acetic acid & EDTA
BNF	Biological Nitrogen Fixation
MSL	Mean Sea Level
ICARDA	International Center for Agriculture Research in the Dry Areas

THESIS ABSTRACT

a) Title of the Thesis	:	Characterization and evaluation of native *Rhizobium* of *Lathyrus*, Green gram, Chickpea and Black gram
b) Full Name of the student	:	Topi Kamgo
c) Major subject	:	Agricultural Microbiology
d) Name and address of the Major Advisor	:	Dr. S.B. Gupta Professor & Head- Agril. Microbiology Department of Agricultural Microbiology, IGKV, Raipur (C.G) 492012
e) Degree to be Awarded	:	Master of Science in Agriculture (Agricultural Microbiology)

Signature of the Major Advisor

13.7.19

Topi Kamgo

Signature of student

Date: 13.7.19

13.7.19

Signature of Head of the Department

ABSTRACT

A study was conducted in 2018-2019 to characterize and evaluate *Rhizobium* isolates of *Lathyrus*, green gram, chickpea and black gram in order to enhance the legume *Rhizobium* symbiosis as well to increase the crop productivity. In this connection, all the isolates were characterized biochemically and tested with N-free sand culture grown in above mentioned different legumes in the Department of Agril. Microbiology, Indira Gandhi Krishi Vishwavidyalaya,Raipur, to select effective

native *Rhizobium* isolates through systematic screening in the present investigation on "Characterization and evaluation of native *Rhizobium* of *Lathyrus*, Green gram, Chickpea and Green gram."

Initially 10 isolates of each crop (*Lathyrus*, green gram, chickpea and black gram) were collected from the repository of Department of Agril. Microbiology, IGKV, Raipur. Further they were qualitatively tested for presence of root nodule forming *Rhizobium* and out of 10 isolates,4 isolates were selected on the basis of their growth performance and purity of the culture, then after selected 4 isolates were characterized for by Gram staining, Indole-production test, MR-VP test, Citrate utilization test, Catalase test, Urease test, Triple sugar Iron agar test, Gelatin liquefaction test, Starch hydrolysis and finally tested for nodulation behavior and biological nitrogen fixation under controlled conditions

Upon exposure to different biochemical tests, all the isolates of *Rhizobium* showed gram negative reaction and similarly, negative results for indole production, catalase and voges-proskauer test.Positive results were observed for citrate utilization. Most of isolates could not liquefy gelatin except isolate no. 3711 of *Lathyrus* and isolate no. 1185 of green gram. Negative results were obtained for starch hydrolysis except isolate no. 1185 of green gram, isolate no. 1068 of chickpea and isolate no.3533 of black gram. Most of the isolates of *Rhizobium* were urease positive except isolate no. 494 of *Lathyrus* and isolate no. 191 of chickpea. Upon triple sugar iron agar test there was no carbohydrate fermentation in all the rhizobial isolates except isolate no. 3711 of *Lathyrus* which only indicated glucose fermentation. The antibiotics streptomycin (10µg/disc) and tetracycline (30µg/disc) had inhibitory effect in all the isolates of *Rhizobium*.

Keeping in view of the findings related to BNF parameters like accumulation of biomass and nitrogen, performance of native *Rhizobium* isolate no. 3693 of *Lathyrus*, isolate no. 257 of green gram, isolate no. 98 of chickpea and isolate no. 3533 of black gram were found to superior among all rhizobial isolates taken for study. These isolates accumulated 3.457, 5.495, 4.941 and 2.845 mg/plant extra amount of atmospheric nitrogen over uninoculated control plant, respectively.

Results of the *Rhizobium* population dynamics studies also supported the effectiveness of the native rhizobial isolate no. 3693 of *Lathyrus*, isolate no. 257 of green gram, isolate no. 98 of chickpea and isolate no. 3533 of black gram. Hence, it was concluded that these native isolates may be the most effective nitrogen fixer for *Lathyrus*, green gram, chickpea and black gram cultivation under agro-climatic conditions of Chhattisgarh.

शोध सारांश

अ.	शोध शीर्षक का नाम	: तिवड़ा, मुँग, चना एवं उड़द के स्थानीय राइजोबियम आइसोलेट्स के विशेषताओं का परीक्षण एवं मुल्यांकन
ब.	विद्यार्थी का नाम	: तोपी कामगो
ग.	प्रमुख विषय	: कृषि सुक्ष्मजीव विज्ञान
घ.	मुख्य सलाहकार का नाम एवं पता	: डॉ. एस.बी.गुप्ता, विभागाध्यक्ष, कुषि सुक्ष्मजीव विज्ञान विभाग, कृषि महाविद्यालय, रायपुर
इ.	उपाधि से सम्मानित किया जाना है	: एम.एस.सी (कृषि)

Topi Kamgo
विद्यार्थी के हस्ताक्षर

13.7.19
प्रमुख सलाहकार के हस्ताक्षर

दिनॉक : 13.7.19

13.7.19
विभागाध्यक्ष के हस्ताक्षर

सारांश

सन् 2018–19 में इंदिरा गॉधी कृषि विश्वविद्यालय के कृषि सुक्ष्मजीव विज्ञान विभाग में तिवड़ा, मुंग, चना तथा उड़द के स्थानीय राइजोबियम की विशेषताओं का परिक्षण एवं उनका उपरोक्त फलीदार पौधों के सहजीवन से वायुमण्डलीय नत्रजन की उपलब्धता एवं इन फलीदार फसलो की उत्पादकता बढाने के लिए अनुसंधान किया गया।

इस अनुसंधान हेतु उपरोक्त फलीदार पौधों के 10–10 स्थानीय राइजोबियम आइसोलेट्स कृषि सुक्ष्मजीव विज्ञान विभाग की रिपोजेटरी से प्राप्त किया गया और उनके ग्रोथ परफॉरमेंस एवं कल्चर की शुद्धता के आधार पर 4 राइजोबियम आइसोलेट्स हरेक फलीदार फसल के लिए चयनित किया गया।

इन चुने हुए राइजोबियम आइसोलेट्स ग्राम स्टेनिंग एवं जैव रसायनिक पैरामीटर जैसे– इंडोल प्रोडक्सन टेस्ट, एम.आर. टेस्ट, साइट्रेट युटीलाईजेसन टेस्ट, कैटेलेस टेस्ट, युरिस टेस्ट, ट्रिपल सुगर आयरन टेस्ट, जिलेटिन लिक्वीफिकेस्न टेस्ट और स्टार्च हाइड्रोलेसि्स का परीक्षण किया गया। इसके उपरांत इन चुने हुए आइसोलेट्स को नियत्रित अवस्था में नाइट्रोजन रहित सेंड कल्चर प्रयोग के तहत उपरोक्त फलीदार फसलों के साथ नोड्युलेशन बिहेवीयर एवं वायुमण्डलीय नत्रजन स्थिरीकरण आदि का गहन अध्ययन किया गया।

विभिन्न जैव रसायनिक परिक्षण में राइजोबियम के सभी आइसोलेट्स ग्राम ऋणात्मकता प्रदर्शित की एवं इण्डोल उत्पादन, केटालेस एवं वोग–प्रोस्कर परीक्षण में ऋणात्मकता पाये गये, अधिकत्तर आइसोलेट्स जिलेटिन लिक्विफिकेशन नही कर पाया, केवल तिवडा के आइसोलेट्स कं 3711 एवं मूंग के आइसोलेट्स कं. 1185 जिलेटिन लिक्विफिकेशन प्रदर्शित किया, उसी तरह मूंग के आइसोलेट्स कं. 1185, चना के आइसोलेट्स कं. 1068 एवं उड़द के आइसोलेट्स कं. 3533 को छोड़कर शेष राइजोबियम आइसोलेट्स स्टार्च हाइड्रोलाइसिस के प्रति ऋणात्मकता प्रदर्शित किया, तिवडा के राइजोबियम आइसोलेट्स कं. 498 एवं चना के 191 को छोड़कर शेष आइसोलेट्स युरियेज परीक्षण में धनात्मक पाये गये। ट्रिपर शुगर आयरन परिक्षण में तिवड़ा के आइसोलेट्स 3711 को छोड़कर सभी राइजोबियम आइसोलेट्स कोर्बोहाड्रेट किण्वनीकरण नही की अतः आइसोलेट्स 3711 केवल ग्लुकोस का किण्वणीकरण किया। प्रतिजैविक स्ट्रेप्टोमाइसिस 10 माइक्रोग्राम एवं टेट्रसाइक्लिन 30 माइक्रोग्राम प्रति डिस्क की मात्रा के सभी राइजोबियम आइसोलेट्स ने निरोधात्मकता प्रदर्शित किया।

वायुमण्डलीय नत्रजन स्थिरीकरण मापदंडो जैसे बायोमास और नत्रजन के संचय से संबंधित निष्कर्षो को ध्यान में रखते हुए स्थानीय राइजोबियम आइसोलेट्स नं. 3693 तिवड़ा, आइसोलेट नं. 257 मूॅग, आइसोलेट नं. 98 चना और उड़द आइसोलेट नं. 3533, अध्ययन में लिए गए अन्य सभी राइजोबियम आइसोलेट्स से श्रेष्ठ पाया गया। राइजोबियम की संख्या गति के अध्ययन के परिणामों ने भी इन स्थानीय राइजोबियम की प्रभावशीलता का समर्थन करते पाया गया। इसलिए यह निषकर्ष निकलता है कि तिवडा के राइजोबियम के स्थानीय आइसोलेट नं. 3693, मूॅग आइसोलेट नं. 257, चना आइसोलेट नं. 98 और उड़द आइसोलेट नं. 3533, छत्तीसगढ़ क्षेत्र की कृषि जलवायु परिस्थितियों में तिवडा, मूॅग, चना और उड़द की खेती के लिए सबसे प्रभावी नत्रजन स्थिरिकारक है।

CHAPTER - I

INTRODUCTION

Pulses are group of plants which belong to the family *fabaceae* (*leguminosae*). In agriculture, legumes are mainly grown for the purpose of human consumption, livestock forage and silage and as soil enriching green manure. Legumes are the third largest land plant family with 751 genera and 19,000 known species after *Orchidaceae* and *Asteraceae*. They supply protein, nutritional minerals, carbohydrates and dietary fibers. They are additionally a splendid source of resistant starch which is broken down by bacteria to produce short chain fatty acids used by the intestinal cells for energy. The regular consumption of pulses can lower the risk of developing metabolic syndrome, blood pressure and cholesterol level.

According to FAO, India is the largest pulse producer in the world and it contributes about 25% of the global pulse production. India grows almost 29.4 million ha of pulses with the annual production of 23.13 million tones and a total productivity of 786 kg/ha. In Chhattisgarh, the total area under pulse production is about 8.83 million hectares with production of 6.69 million tonnes and a total productivity of 858 kg/ha(Anonymous,2016.)

Legumes have symbiotic bacteria called rhizobia within their root nodules. Under ideal conditions, pulse crops have the ability to fix as much as 50-80 per cent of their total nitrogen requirement, with the remaining nitrogen coming from soil or fertilizer. After the harvest of a crop, a significant amount of nitrogen remains in the soil and the rhizobia present in the soil colonizes the legume roots. Thus, converting the atmospheric nitrogen into ammonia. Specific strains of *Rhizobium* are required for the formation of root nodules in legumes to carry the process of nitrogen fixation. Inoculation of legumes with rhizobial strains is beneficial as it increases the crop yield. It is very essential to provide effective screening and selection of the strains

is very important for the development of an inoculum. Presence of many native isolates of *Rhizobium* which can successfully nodulate the legumes have been reported worldwide. The use of host-specific native *Rhizobium* isolates is highly recommended as they can adapt well in the local environment and the soil conditions. The native rhizobial isolates are persistant, have better survival rate and also they can increase the chance of successful nodule formation and can fix nitrogen in the host plant. Determination of rhizobial symbiotic efficiency is required to screen out effective native rhizobial isolates. This is usually carried out in a greenhouse under controlled conditions. Screening of native rhizobial isolates for their nitrogen fixation efficiencies is important in order to achieve maximum legume productivity

The rhizobial inoculations in the seeds are done by coating the seeds with liquid or carrier based inoculant or by treating the soil with the inoculant. The *Rhizobium* bacteria are not mobile in the soil, so the seeds to be inoculated should come in contact with the bacteria for infection within the root hairs. Specific species of *Rhizobium* is required in pulses for nodulation, if wrong species are used than the inoculation will have no beneficial effect in the crop. For example, *Rhizobium* species successful for nodulation in lentil, pea do not nodulate in chickpea.

Formation of nodules and nitrogen fixation are very sensitive to external nitrogen sources such as fertilizers and available soil nitrogen. When the supply of nitrogen available from the soil and fertilizer will increase, the amount of nitrogen fixed decreases. Low levels of available nitrogen may have less influence on nodulation and fixation; but when the degrees of available soil and fertilizer nitrogen are combined they reach approximately 25-30 kg N/ha, any additional nitrogen will minimize nodulation and fixation. Combined levels of soil and fertilizer nitrogen greater than 55 kg N/ha (50 lb.N/acre) can dramatically delay nodulation and decrease the fixation.

In the last few decades, the use of molecular techniques has contributed appreciably to enhance the knowledge of rhizobial diversity. Molecular techniques

have helped to develop easy and quick methods to microbial characterization including works distinguish genera, species and even strains.

In view of the above facts with following objectives, an attempt has been made in the present investigation to characterize and evaluate native rhizobial isolates of *Lathyrus*, Green gram, Chickpea and Black gram that is already present in the repository of Department of Agril. Microbiology, College of Agriculture, Raipur.

The objectives of the investigation are:

i. Biochemical characterization of native *Rhizobium* isolates of *Lathyrus*, Green gram, Chickpea and Black gram.
ii. Biological nitrogen fixing ability of homologous native *Rhizobium* isolates of different legumes under N-free sand culture.

CHAPTER - II

REVIEW OF LITERATURE

The investigation was conducted under controlled conditions to characterize and evaluate the native *Rhizobium* isolates of *Lathyrus*, green gram, chickpea and black gram in the Department of Agril. Microbiology, College of Agriculture, IGKV, Raipur.

The work pertaining to the different aspects of characterization and evaluation of native *Rhizobium* and the other work relevant to present investigation has been covered in the review as follows:

2.1 Rhizobia

Denison *et al.* (2004) reported that the rhizobial strains within species can have three dissimilar genetically determined actions. The rhizobia which are in a mutual relationship with the host plants provides nitrogen while the rhizobia which are parasitic in nature infect the legumes and do not fix nitrogen. The rhizobial strains which are non-symbiotic do not infect the legume plants. A symbiotic (mutualistic or parasitic) *Rhizobium* that thrives in formation of a nodule might produce numerous number of descendants. The non-symbiotic rhizobia can have better reproductive success while the chances of success are very low. Legumes which have the ability to fix very less nitrogen or no nitrogen favors mutualistic strains while the parasitic strains which use plant resources only for their own reproduction do well in association with mutualistic strains.

Poonia (2011) reported that in dry arid soils, legume plants play an important role in their sustainability. Legume have the ability to provide required amount of nitrogen for the physiological growth. In agricultural fields, intensive farming is done with the use of chemical fertilizers which are expensive as well as pose a threat to the environment. Most of the legumes are nodulated by different species of *Rhizobium*. It is a gram negative, fast growing bacteria and has the ability to provide nitrogen.

Increasing the use of bio-fertilizers such as *Rhizobium* is highly recommended so as to decrease the use of chemical fertilizers as well as to save environmental degradation.

Berrada (2014) mentioned that there exists a great diversity among the nitrogen fixing bacteria which has been isolated from different legume plants. Recently, more than 98 species belonging to 14 genera of α- and β- proteobacteria has been reported as rhizobia. The genera of bacteria which are known as legume symbionts include *Mesorhizobium, Rhizobium, Bradyrhizobium, Ensifer* (formerly *Sinorhizobium*),*Microvirga,Azorhizobium,Phyllobacterium,Ochrobactrum,Methylo bacterium, Devosia, Shinella* (class of α-proteobacteria), *Burkholderia, Cupriavidus*(formerly *Ralstonia*) (class of β- proteobacteria) and some γ-proteobacteria. Till now, only 23% of known legumes have been recognized specifically for their symbiotic activity and the majority (88%) have been reported for formation of nodules. To study the rhizobial taxonomy, findings in the new symbionts associated with legume plants is needed for future research.

Laranjo *et al*. (2014) reported that the species belonging to the genera *Mesorhizobium* have high geographical dispersion and as well as have the ability to nodulate legumes such as chickpea and biserrula. The evolution of the rhizobial genomes and the evolutionary relationships among the species can be best studied with the establishment of legume-Mesorhizobia inoculant. The phylogeny of symbiosis genes such as are not compatible with the phylogenies based on basic genes, reflecting rhizobial host range, rather than species bonding. The *Mesorhizobium* species can nodulate new host plants as they have ability to exchange symbiosis genes through lateral transfer of chromosomal symbiosis islands. Phylogenetic study of the *Mesorhizobium* genus on the basis of core and accessory genes reveals complicated evolutionary relationships and a high genomic plasticity, makes the *Mesorhizobium* genus as a suitable model to explore rhizobial genomic evolution and adaptation to different host plants. Further research on genome and transcriptome can be done so as to study the *Rhizobium*- legume association and to develop improved strains of inoculant.

Singh *et al.* (2018) reported that the development in sustainable agriculture can be brought by the use of bio-fertilizers such as *Rhizobium* can decrease the use of inorganic fertilizers and also reduce the severe environmental stresses. The study also revealed the positive influence of rhizobial (*Mesorhizobium*) inoculant on growth characteristics, symbiotic parameters, yield and yield components, nutrient uptake and quality in chickpea. Further, stress in environmental factors such as pH, nutrient availability, temperature, herbicides and moisture have a significant influence on legume -*Rhizobium* symbiosis, nitrogen fixing ability of bacteria and plants.

2.2 Nodulation capacity of *Rhizobium*

Tahir *et al.* (2009) studied the influence of *Rhizobium* inoculation and P fertilization on nodulation, growth and yield characteristics of soybean grown in the presence of starter N fertilizer. Further the results revealed that the benefits of using *Rhizobium* inoculant and P fertilization with reduced level of starter N to increase plant growth, nodulation and seed yield of soybean.

Younesi *et al.* (2013) studied on rhizobacteria co-inoculated with *Sinorhizobiummeliloti* which were tested for their capacity to decrease the negative effects of low root zone temperature (RZT) on alfalfa (*Medicago sativa* L.) nodulation and nitrogen fixation. The results indicated that the nodulation was repressed below suboptimal root zone temperatures. At low temperature, PGPR strains resulted in an increase in the number of nodules formed and the amount of nitrogen fixed when co-inoculated with *S. meliloti*. The negative effects of low temperatures on nodulation and nitrogen fixation in alfalfa plants are minimized by co-inoculation with *Rhizobium* and *Pseudomonas.*

Hussain *et al.* (2014) studied on effect of P fertilizers and *Rhizobium* inoculation on the nodulation, growth and yield of Mung bean (*Vigna radiata)* under pot condition at University of Agriculture, Faisalabad. Seeds of mung bean (NM-92) were inoculated with *Rhizobium phaseoli* and phosphorus was applied along with it. It was observed that the combined use of P and rhizobial inoculant increased the number of nodules per plant and height of the plants significantly. Therefore,

100% of recommended phosphorus along with *Rhizobium* inoculation was found to be best treatment for the above experiment.

Khan and Prakash (2014) conducted an experiment to study the influence of *Rhizobium* inoculation, zinc, molybdenum on nodulation, yield, nutrient uptake and nutrient restoration in summer urd bean (*Vigna mungo* L.) atUniversity of Agriculture and Technology, Narendra Nagar (Kumarganj), Faizabad. The results showed increase in number of nodules and dry weight of the rood nodules per plant, seed and stover yield, seed protein content, nitrogen, zinc and molybdenum uptake of urd bean with *Rhizobium* inoculation.

Singh *et al.* (2014) carried out an investigation to determine the influence of microbial inoculants (un-inoculated control, *Rhizobium* CAT4059, *Rhizobium* CAT-5078, PSB) and nitrogen levels on growth and nodulation of chickpea (*Cicer arietinum*). Application of N improved the plant height and nodulation such as number of nodules, fresh weight of nodules and dry weight of nodules. Results also revealed that seed inoculation with rhizobialsohadsignificant influence on the plant height, number of nodules, fresh weight of nodules and dry weight of nodules.

Alam *et al.* (2015) conducted an experiment on the influence of *Rhizobium* sp. BARIRGm901 inoculation on nodulation, nitrogen fixation and yield of soybean (*Glycine max*) genotypes. Soybean plant genotypes inoculated with *Rhizobium* sp. BARIRGm901 formed greater nodule numbers, nodule weight, shoot and root biomass, and plant height than non-inoculated plants. Likewise, the inoculated plants also revealed significantly enhanced activity of nitrogenase contributing to higher nitrogen fixation and assimilation.

Mweetwa *et al.* (2016) carried out an investigation on nodulation, nutrient uptake and yield of common bean dual inoculated with *Rhizobium tropici* and *Trichoderma harzanium*. After 51 days of planting, the nodule effectiveness and nodulation were determined. The study revealed that soils ameliorated with inorganic N slows down the process of nodulation even in presence of abundant inorganic P. Inoculation of common bean with *Rhizobia* and *Trichoderma* either alone or in combination increases the number of nodules and effectiveness per plant but is unable to increase N and P accumulation, biomass or grain yield.

Kumari *et al.* (2017) studied on effect of salinity and seed inoculation with *Rhizobium* on nodulation and leghemoglobin content in fenugreek. The results revealed that the nodule growth decreased with increase in salinity. Seeds inoculated with *Rhizobium* ameliorated the harmful effect of salinity at all levels besides improving the fertility of soil and nodule growth by nitrogen fixation.

Abitew and Kibret (2017) conducted study on the influence of *Bradyrhizobium japonicum*, N and P Fertilizers on Growth, Nodulation, Yield and Yield Components of Soybean. The results indicated that inoculation of *B. japonicum* and P application alone or in combination with *B. japonicum*, or P with small dose of N fertilizer can increase the growth, yield and rate of nitrogen fixation.

Kumar *et al.* (2017) conducted a study on influence of inoculation of *Rhizobium* and PSB on growth and nodulation of *Acacia nilotica*. The results revealed that the dual inoculation of *A. nilotica* with *Rhizobium* and PSB in addition with 25% of N gave increased shoot length, collar diameter, shoot dry mass accumulation and also significantly higher nodulation (more number of nodules, increase in nodule dry weight) was recorded.

Samudin and Kuswantoro (2018) investigated on the effect of *Rhizobium* inoculation on nodulation and growth of soybean (*Glycine max* (L.) Merrill) germplasm. The results revealed no interaction between the *Rhizobium* inoculation and the genotype. The result of inoculation was shown by the number of nodules, nodule dry weight, root length, and root dry weight and without *Rhizobium* inoculation the root length was higher but the root dry weight was lower.

Khaitov (2018) carried out investigation on effect of *Bradyrhizobium* inoculation and magnesium application on growth, nodulation, symbiotic nitrogen (N) fixation as well as N nutrition status of soybean in hydroponics under greenhouse conditions. The availability of Mg was essential for the uptake of N by the plants as well as in improving nodulation process in roots by *Bradyrhizobium.*Inoculation with rhizobial inoculants was effective in formation of nodules, increased soybean shoot, root biomass and yield, as well as plant N nutrient status.

2.3 Nitrogen fixation

Cheng (2008) reported that the plants especially legumes fix nitrogen through symbiotic anaerobic microorganisms mainly rhizobia. The process of biological nitrogen fixation involves enzyme dinitrogen which catalyzes the inert atmospheric nitrogen into ammonia i.e., NH_3. The nitrogenases are usually oxygen liable. The development of nitrogen fixing plants is a long term effort due to the requirement of assemblage of complex enzyme and anaerobic conditions. With the advancing protein catalyst, the anaerobic enzymes have been replaced in many reactions with efficient and irreversible aerobic version. Evolutionary relationships exist in nature such as an enzyme catalyzing a highly specific oxygen dependent reaction has an oxygen independent part which carries out reactions in absence of oxygen.

Mohammadi *et al.* (2012) described that the growth, metabolic activity and survival of nitrogen fixing bacteria is greatly affected by the change in temperature, pH, water status, and nutrient availability. The permeability of nodules is decreased by nitrogenase inhibition which results in accumulation of oxygen in the infected zones. In dry season, the legume nodulation is poor in arid soils due to the decreasing population of the rhizobia in the soil. With the subsequent increase in soil N, the nitrogen fixing ability decreases with the decrease in age of the legumes. The attachment of the rhizobia to the roots is greatly affected by the calcium deficiency. The high temperature in roots has great impact on nitrogen fixing ability, legume growth and infection and also the nodulation in bacteria is influenced by the mycorrhizal formation. New techniques for development of genomics and proteomics of micro and macro symbionts needs to be engineered as a response to different environmental stresses.

Roychowdhury *et al.* (2013) reported that the legume- rhizobia symbiosis plays a major role in the increasing cultivation of legumes as the atmospheric nitrogen fixed by the microorganisms is more than that of the nitrogen fertilizers which is bought and applied by the farmers in the agricultural fields. Thus, the symbiosis between the rhizobia and legume plants are quite economical and can improve the soil fertility and increase the crop production.

Shamseldin (2013) reported that the process of symbiotic nitrogen fixation requires the *nif* genes and the *fix* genes present in the rhizobia. The process of nitrogen fixation is catalyzed with the help of enzyme nitrogenase which is a complex encoded with *nif*DK and *nif*H genes. The enzyme nitrogenase is composed of sub unit I, subunit II an iron containing protein and molybdenum-iron protein(MoFe). *Nif*D and *nif*K genes encodes the Mo-Fe protein subunit and to activate the Mo-Fe protein it requires Fe-Mo cofactor.

Suleiman *et al.* (2014) reported that symbiotic nitrogen fixation is one of the key processes involved in expansion of sustainable agriculture in which nitrogen present in atmosphere is converted into ammonia with the help of enzyme nitrogenase. It is attained by the bacteria present in the root nodules of the leguminous plants. This results in a mutual relationship between the host plant and the rhizobia, the host plant supplies carbon and energy sources for growth and other functions to rhizobia while the rhizobia fix the atmospheric nitrogen. Thus, the process of symbiotic nitrogen fixation is economical and ecologically reliable means of reducing use of external inputs in agriculture.

Zahran (2015) reported that the primary source of nitrogen for most agricultural soils including arid regions is biological nitrogen fixation. The symbiotic system is one of the nitrogen fixing system which plays a major role in increasing the fertility and productivity condition of low nitrogen soils. The symbiotic activity of most of the rhizobia is affected by severe environmental stresses such as drought stress, salt stress, alkalinity, acidity, nutrient deficiency, fertilizers, heavy metals, and pesticides while some of the rhizobial strains are tolerant to stress effects due to their effective symbiosis between the host legumes. The addition of organic fertilizers such as manure, sewage sludge and inorganic fertilizers for reclamation and improving the fertility of soil in arid lands is quite expensive and is one of the sources of pollution. The Legume-*Rhizobium* symbiosis is the best solution for improving the fertility of soils in arid regions.

De Bruijn (2015) described nitrogen fixation as one of the key processes involved in the nitrogen cycle where the nitrogen present in the atmosphere is converted into organic nitrogen with the aid of some particular bacteria and

cyanobacteria. The bacteria which can fix atmospheric nitrogen lives in symbiotic relationship with the legume plants such as beans, clover, peas and some tropical trees in the nodules of the roots. Due to the activity of these microbes there is improvement in soil fertility and there is adequate supply of nitrogen required for the plant growth and function.

Geddes *et al.* (2016) reported that every year around 380 terra grams of nitrogen are fixed by the process of biological, atmospheric and industrial nitrogen fixation. In the current world agricultural scenario, the use of reduced nitrogen produced by the Haber- bosch process is extensive but the reduced nitrogen produced from fossil fuel is not sustainable. The process of biological nitrogen fixation is carried out by the diazotrophic microorganisms which have the ability to fix atmospheric nitrogen with the help of enzyme nitrogenase mostly it is due to the symbiotic association between the diazotrophs and the host plants.

Masson-Boivin *et al*. (2018) described that the evolutionary and ecological success of rhizobia which has been achieved due to its evolving intracellular survival and symbiotic nitrogen fixation ability in legumes. The rhizobial symbiosis has spread horizontally to hundreds of bacterial species and geographically throughout the globe inspite of its dual capacity of intracellular survival. Therefore, new techniques and tools can be engineered for crop improvement assisted by microbes related to plant associated microbiota and development of nitrogen fixing ability in cereals to revolutionize world agriculture scenario.

2.4 Variation in symbiotic nitrogen fixation by legumes

Bowman *et al.* (1996) investigated on the symbiotic N -fixation in alpine tundra based on their ecosystem input and variation in fixation rates among communities. To determine the rate of nitrogen fixation, abundance of N isotopes (δ N) in nature were measured in field collected *Trifolium* and reference plants and in *Trifolium* plants grown in N-free medium in a growth chamber. However, the results showed that the *Trifolium* species to meet their nitrogen requirement relied largely on atmospherically fixed nitrogen varying from 70% to 100%. Differences in the contribution of the *Trifolium* species to community cover resulted in a wide range

of annual N inputs from fixation, from 127 mg m^{-2} $year^{-1}$ in wet meadows to 810 mg m^{-2} $year^{-1}$ in fellfields.

Icgen *et al.* (2002) evaluated the symbiotic effectiveness of the *Rhizobiumcicer* strains found in Chickpea.The parameters taken for plant productivity were shoot dry weight, nodule number, nodule dry weight, protein and total nitrogen contents. In single infection, the maximum increase in total nitrogen content was only 3.5-fold whereas an increase of 35-fold was recorded for multiple infections. The double infection with Y-29 and 385 as well as the triple infection with Y-29, 620 and 3379 gave rise to the maximum values.

Fening *et al.* (2002) studied the variation in symbiotic effectiveness of cowpea bradyrhizobia in soils of Ghana. Total of 100 isolates were examined varied with expected values for effectiveness, relative to an un-inoculated control, ranging from 23.5 to 118%. The isolates ranged from ineffective to highly effective, but most of them (68%) were being ranked as moderately effective and the highly effective ones constituted only 26% of the 100 isolates studied. Diversity of the isolates in symbiotic effectiveness was observed when compared to the fertilized N control or with a standard strain of *Bradyrhizobium* species. Some of the isolates revealed high N_2 fixing capabilities that were comparable to the N fertilized plants equivalent to 70kg/ha of inorganic N and some of them showing superiority in symbiotic effectiveness relative to the standard strain.

Rodino *et al.* (2005) studied on the variability in symbiotic nitrogen fixation among white landraces of common bean from the Iberian Peninsula. The nitrogen dependent growth of various bean accessions, belonging to the great northern, caparron, white kidney and cannellini market classes, was compared in glasshouse, under optimized symbiotic conditions with a *Rhizobium tropici* inoculant.The germplasm study resulted in genotypic variation for (i) nodulation with PHA0184, PHA-0267, PHA-0290, PHA-0034 and PHA-0276 having the highest nodule biomass, (ii) Nitrogen-dependent plant-growth with PHA-0013, PHA-0014, PHA-0290 and PHA-0838 having the highest shoot biomass and (iii) the ratio of the shoot growth as a function of nodule growth with PHA-0034, PHA-0053, PHA-0184, PHA-0267 and PHA-0276 having the highest value.

Schipanski *et al.* (2010) studied the variability in soybean nitrogen fixation across ecosystems. The soybean dependence on nitrogen fixation varied from 36% to 82% and total nitrogen fixed in aboveground biomass ranged from 40 to 224 kg N ha^{-1}. The results concluded that the soil N uptake by nitrogen fixing soybeans comparative to the non-nodulating ones increased as soil N decreased, proposing that nitrogen fixation resulted in an increase of soil N scavenging in low fertility fields. In addition, the internal regulation of nitrogen fixation was found to be weak due to the inhibitory effects of soil N availability were secondary to the environmental and site characteristics, such as soil texture and corresponding soil characteristics that varied with texture, which affected soybean biomass, total N fixation, and net N balance.

Buchi *et al.* (2015) investigated on the biological nitrogen fixation by legumes cultivated as cover crops. Two field experiments were done to check the biomass production and nitrogen content of 19 legumes and two non-legumes. The proportion of nitrogen derived from atmospheric N_2 (% Ndfa) was assessed using the 15N natural abundance method A pot experiment was also set up to determine the species-specific B values necessary to apply this method. Results concluded that some species produced a significant amount of biomass in 3 months, up to 6.86 t/ha for *Vicia faba*. 5 species, *Lathyrus sativus*, *Pisum sativum*, *Vicia sativa*, *Vicia villosa*, and *V. faba,* acquired more than 100 kg/ha of N through biological fixation. Some amount of nitrogen was also made available through soil. % Ndfa values exhibited high variability between and within species, varying from 0 % to almost 100 %.

Razzaque *et al.* (2016) carried out an investigation on the nodulation, biological nitrogen fixation and yield potential of genotypes of mung bean under varying levels of N application under pot culture experiment.10 mung bean genotypes viz. IPSA 12, GK 27, IPSA 3, IPSA 5, ACC12890055, GK 63, ACC12890053, BU mug 4, BARI Mung 6 and Binamoog 5, were treated with six levels of N (0, 20, 40, 60, 80 and 100 kg N ha^{-1}). The results concluded that the genotype IPSA 12 at 40 kg N ha^{-1} produced the maximum number of nodules (14.54 $plant^{-1}$),the highest nitrogen fixation (2.684 μmol C_2H_4) and highest seed yield

(14.22 g plant-1). The genotype ACC12890053 showed lowest nodulation (6 plant^{-1}), nitrogen fixation (1.134) and seed yield (7.33 g plant^{-1}).

2.5 Biochemical characterization of *Rhizobium*

Kucuk and Kivanc (2008) studied characterization of 28 *Rhizobium* isolates of chickpea based on their morphological, cultural and biochemical characteristics. The strains were found to be producing abundant extracellular polysaccharides, showed tolerance to 0.5M NaCl and temperature of 40°C. The strains were found to be resistant to antibiotics such as streptomycin, kanamycin, erythromycin, chloramphenicol and penicillin.

Sharma *et al.* (2010) studied twenty-two isolates of *Rhizobium* collected from different soybean growing areas and eight reference strains for their biochemical and metabolic characterization. Out of 22 isolates, 18 isolates grew fast and the rest were slow growers on the basis of bromo-thymol blue test. Some of the rhizobial strains grew well in glucose peptone agar medium. Some of the isolates showed positive result for ketolactose test. Exceptional metabolic diversity was observed among the rhizobial strains based on the C- sources utilization (15 carbohydrates). The clustering and matching analysis showed many of the isolates were same as that of slow growing reference strains while few with fast growing reference strains. Further the practice of polyphasic method along with both molecular and biochemical techniques should be employed to characterize the native soybean rhizobia.

Deora and Singhal (2010) described the characterization of isolates of *Rhizobium* isolates from the root nodules of *Vigna radiata*. The rhizobial strains produced mucous, were gram-negative and rod shaped in nature. No growth was observed in the isolates when treated with 0.1% methylene blue and lactose. It utilized glucose and starch as the main carbon source. The *Rhizobium* strains showed negative result for starch hydrolysis test, catalase test, urease test and were sensitive to antibiotics such as tetracycline, kanamycin and streptomycin. The strains showed antibacterial activity against *Streptococcus, E. coli* and *Pseudomonas.* Immobilization of *Rhizobium* can be done in the fields by using charcoal as the carrier which can be further applied as a biofertilizer.

Gachande and Khansole (2011) isolated *Rhizobium japonicum* and *Bradyrhizobium japonicum* from the root nodules of soybean (*Glycine max* L.) was cultured in YEMA medium to study the morphological, cultural and biochemical characteristics. The colonies were observed to be circular, light pink, convex and opaque. It was aerobic, non -spore forming, rod shaped and motile bacterium. The isolates indicated negative result for Methyl Red, Voges- Proskaur, gelatin hydrolysis, indole production, carbohydrate utilization and hydrogen sulphide production. It indicated positive test for citrate utilization, catalase and ammonia production from peptone and urea.

Singh *et al.* (2011) studied the characterization of rhizobial strain isolated from the root nodules of fenugreek. The nature of the isolates was rod shaped, gram negative, acid and mucous producing. They grew well in optimum temperature of 29.4°C and pH of 7. The bacteria showed sensitivity to antibiotics such as chloramphenicol, kanamycin and streptomycin. It utilized glucose, sucrose and starch as the main carbon source. The species of *Rhizobium* isolated from the nodules of fenugreek have the ability to produce enzymes such as amylase and cellulose. The activity of the organism was not affected when immobilized in agar and agarose instead showed increase in biomass yield and production of enzymes. The *Rhizobium* can be inoculated in the soil as bio-fertilizer by using charcoal as the carrier.

Shahzad *et al.* (2012) studied the isolation of biological nitrogen fixing *Rhizobium* from root nodules of Alfalfa (*Medicago sativa*) plant. 50 nodule samples were collected from different regions of District Quetta Baluchistan, Pakistan and were cultured on differential media Bromo thymol Blue (BTB) with Yeast Extract Mannitol (YEM). 25 isolates were reported to be *Sinorhizobium meliloti* based on biochemical and sugar fermentation tests. The study confirmed the presence of species of *Rhizobium* in the locality.

Bhatt *et. al* (2013) isolated nine different rhizobial isolates from nodules of mung bean which were collected from different mung bean cultivating areas of Gujarat. The collected isolated when tested were gram negative in nature and indicated negative test upon starch hydrolysis. All the isolates had the ability to

reduce nitrate to nitrite and not any of the test isolates produced hydrogen sulphide gas. When grown on 2% NaCl the isolates showed varied growth while it indicated poor growth upon glucose consumption and utilization of nitrogenous compound.

Bhattacharya *et al.* (2013) studied the isolation of *Rhizobium* species collected from the root nodules of *Pisum sativum* (pea) and characterized them, based on physiological and biochemical characteristics. The isolated *Rhizobium* produced mucous, were gram negative and rod shaped in nature. They showed sensitivity to temperature and pH. The isolates utilized glucose and starch as the carbon source. They showed negative result for starch hydrolysis and were sensitive to antibiotics like tetracycline, kanamycin.

Alshaharani and shetta (2014) isolated 30 root nodulating bacteria from the roots of *Acacia ampliceps* (Maslin), *A. ehrenbergiana* (Hayne.), *A. saligna* (Labill.), *A. seyal* (Del.), *A. tortilis* (Forssk.), *A. tortilis subsp. raddiana* (Savi.), *Leucaena leucocephala* (Lam.) and *Vicia faba* (L.) trees growing in the Riyadh region. The isolates were characterized on the basis of their phenotypic and biochemical properties by taking into account the colony appearance, growth rate, resistance to antibiotics and heavy metals, and tolerance to salinity, extreme temperature and pH. Same colony morphology was observed and the isolates grew well in Yeast Extract mannitol and Tryptone yeast agar but not in MGS media. Diversification among the isolates and different pattern of resistance against abiotic stresses were observed. The isolates could tolerate temperature upto 37°C, grew well in pH 5.5-8.5 and at high NaCl concentration (2% w/v) and also exhibited sensitivity to wide range of antibiotics.

Malisorn and Prasarn (2014) studied on the isolation and characterization of *Rhizobium* isolates from the root nodules of the legume plants. The strains were characterized based on the morphological and biochemical properties. Some of the isolates were fast growers, had white and pink colonies. All the isolated strains were rod shaped, gram negative and produced poly hydroxyl butyrate. The isolates utilized glucose as the core carbon source.

Deshwal and Chaubey (2014) carried out an investigation on isolation and characterization of *Rhizobium leguminosarum* from Root nodule of *Pisum sativum*

L. the isolates were characterized based on specific biochemical tests. The strains were fast growing, gram negative and did not absorb the red color when cultured in YEMA medium. They showed positive results for urea hydrolysis (9.87%), gelatinase activity (12.34%) and precipitation in calcium glycerol phosphate (14.81%). Further, it was concluded that the isolated strains were of *Rhizobium leguminoserum*.

Rai *et al.* (2015) studied biochemical characterization in 18 *Rhizobium* strains which were isolated from the nodules of French bean collected from different areas of North Bengal and Sikkim. The isolates were allowed to grow in Yeast Extract Mannitol medium. The isolates indicated negative result to ketolactose, gelatinase, cellulose and catalase test while they were found to be glucose fermenters once grown in triple sugar iron agar media. 100% of the isolates had the ability to grow in medium containing dextrose. Under the carbohydrate utilization test, the strains formed clusters based on their geographical location. The study also revealed chemical and behavioral diversity among the *Rhizobium* strains.

Upadhayay *et al.* (2015) isolated 15 rhizobial strains from root nodules of *Pisum sativum* L. and *Lens culinaris* L. collected from fields a tdifferent locations of Norman E. Borloug Crop Research Center, G. B. P. U. A. & T., Pantnagar, Uttarakhand and were characterized by some standard tests. When tested the strains were gram negative in nature and when grown in YEMA media containing congo-red, it did not absorb the red color of the media. The yellowish ring of Cu2o was not formed upon ketolactose test. On glucose peptone medium, the isolates indicated poor or no growth which is indicative character of rhizobia. Out of 15 strains, thirteen were fast growing and the rest eight were slow grower which was confirmed by bromo-thymol blue test. The results of the biochemical tests confirmed that the isolated strains were of *Rhizobium* species.

Kapembwa *et al.* (2016) studied the isolation and characterization of *Rhizobium* isolates of soybean collected from uncultivated and cultivated soils of 3 agro-ecological areas of Zambia. 61 isolates were cultured on Yeast Extract Mannitol (YEM) agar medium. Transparent colonies were observed in region I and region III while pink colonies were only visible in region III. The isolates were

mucous producing, gram- negative and rod- shaped in nature. The isolates utilized glucose as the source of carbon. 59 isolates were fast growers while 2 isolates from the cultivated soils of the region II were slow growing based on the Bromo thymol (BTB) test. The results indicated the 59 fast growers could be *Ensifer fredii* or/and *Rhizobium tropici* rather than *Bradyrhizobium.*

Paudyal and Gupta (2017) isolated ten *Rhizobium* strains from the root nodules of legume *Mucuna pruriens* (L.) found in the foothills of Himalaya. All the isolated strains were from different regions were morphologically, biochemically and physiologically characterized based on the Bergey's Manual of systematic Bacteriology.Test for antibiotic sensitivity were done in which the strains indicated high sensitivity to amoxicillin and least to erythromycin. The isolated bacteria were confirmed to be species of *Rhizobium meliloti* due to its generation time, ability to utilize carbon source, DNA base composition and its antibiotic resistance.

Wadhwa *et al.* (2017) studied the isolation and identification of *Rhizobium* from the root nodules of *Cicer arietinum* by using CRYEMA medium. The bacterium did not absorb red color when cultured on YEMA medium and the milky white colony having spherical surface were isolated. The isolated strain showed positive results for catalase, oxidase and bromo-thymol blue test while it showed negative results for lipase test, starch hydrolysis, caseinase and lysine decarboxylase which confirmed that the isolated strain was of species of *Rhizobium*. The growth of strains was best in pH 6-7 temperature 28℃-30℃ and 1% salt concentration. The strains were resistant to antibiotic, metal salts and salinity. The study also indicated that the crop chickpea would perform better if inoculated with strains of *Rhizobium* and would lessen the environmental threat caused by the use of synthetic nitrogen fertilizers.

Hamza *et al.* (2017) studied the isolation and characterization of *Rhizobium* isolates from the rhizosphere and nodules collected from the study area. Isolation of the *Rhizobium* was done by culturing in Yeast Extract Mannitol Agar medium. Based on the MR-VP and starch hydrolysis test, the isolates LLsm1, CPsm1, CPnm1 and Esm1 showed negative results. All the isolates showed positive results for catalase test and negative results for citrate utilization test. The strains produced indole, were gram negative, rod shaped and fast growing in nature. The isolates did

not absorb the red color of the congo red and no growth was observed in YEMA with 2% NaCl. The isolates were confirmed to be species of *Rhizobium* and plant growth promoting bacterial strains.

2.6 Molecular characterization of *Rhizobium*

Hinge *et al.* (2009) conducted study on molecular characterization on rhizobial strains collected from different regions of Gujarat. Molecular characterization based on repetitive DNA sequence especially, ERIC sequence (Enterobacterial Repetitive Intergeneric Consensus) were done together with two known *Rhizobium* strains, one commercial culture (GSFC, Vadodara), five standard strains *of Rhizobium* and one standard strain of *Agrobacterium tumefaciens.* The results revealed that ERIC-PCR could be used for representing the genetic diversity and to determine phylogenetic relationships among strains.

Ogutcu *et al.* (2009) determined the phenotypic and genotypic differences in *Rhizobium leguminosarum subsp. ciceri* strains isolated from perennial wild chickpeas (*Cicer anatolicum*) from high altitudes in mountains of Erzurum, Eastern Anatolia, Turkey. For the purpose of genotypic characterization and phylogenetic analysis of the *Rhizobium* isolates rep-PCR (ERIC-, REP- and BOX-PCR) fingerprinting methods were used. The study revealed a high intra-species diversity among the strains in terms of rep-PCR (ERIC-, REP- and BOX-PCR) profiles.

Al-Judy and Majeed (2013) studied on the characterization of 10 rhizobial isolates collected from the root nodules of locally cultivated Chickpea or non- local isolate from ICARDA. The identification and characterization of the isolates was done based on the colony morphology and biochemical tests such as gram staining, catalase and oxidase test.Genetic diversification among the isolates was determined by RAPD (Random Amplified Polymorphic DNA)-PCR (Polymerase Chain Reaction) finger printing by using five primers.The results from the RAPD indicated high capacity to detect genetic polymorphism in rhizobia and ability to generate unique bands (marker) in Shiekhan 3(10) bands and Mosle (8) bands isolates of chickpea.

Vinay and Kiran (2013) studied the rhizobial diversity in the agricultural fields of Madhya Pradesh. Standard methods were used to study the physiochemical property of the soil and molecular methods were used to study the genetic diversification within the species. The 16S rDNA-RFLP PCR was used for population analysis relating to genetic diversity of the *Rhizobium* species. The study on genetic variability with molecular techniques along with biochemical analysis helped in identifying and defining the phylogeny amongst *Rhizobium* isolates. Therefore, suggested that study on gene sequencing can be done in the strains of *Rhizobium* to be used as biofertilizer.

El-Zanaty *et al.* (2014) isolated 11 isolates of *Rhizobium leguminosarum symbiovar. Viciae* from root nodules of *Vicia faba L.* which were collected from 11 fields belonging to different regions in Egypt to study the genetic diversification among the species using 16S rRNA gene partial sequence. Two groups of isolates were formed based on phylogenetic analysis and the genetic distance between the isolates were variable. The highest genetic distance was observed between the isolates RL6 of North Sinai and RL8 of Dakhalia, and the shortest distance was between isolates RL9 of Giza and RL10 of Sharkia. The isolates were evaluated based on tolerance to heavy metal (Cu, Pb, Zn) concentrations of 0.5, 1 and 2mM. The ability of resistance to heavy metal decreases with increasing concentration. No growth was observed in the media with addition of Zn and Mn at highest concentration 2mM while only 27% isolates survived in the growth media containing Pb at same concentration.

Sunega *et al.* (2016) conducted study on molecular characterization of rhizobial isolates and he further concluded that sequencing partially amplified 16S rDNA of three rhizobial isolate from cv. ICC4993 (R) and one from cv. HC5 showed more than 98% similarity with *Mesorhizobium muleiense* and *Mesorhizobium mediterraneum*. The phylogenetic analysis of 16S rRNA partial sequence showed 11 monophyletic clades.

Akter *et al.* (2016) studied the molecular characterization of *Rhizobium* isolates in *Sesbania bispinosa*. The characterization and identification of isolates were done through biochemical test. Correct size for amplification for *nifH* gene and

nodC gene were obtained which were indicative of nodulation and nitrogen fixing capacity. The results revealed existence of genetic diversity among the isolates with respect to LPS expression and had the phosphate solubilizing capacity as well as presence of plasmids in the isolates. Therefore, *Rhizobium* is a suitable choice as biofertilizer in *Sesbania bispinosa.*

Cardoso *et al.* (2016) studied on the characterization of *Rhizobium* isolates collected from nodules of wild genotypes of common beans to estimate the tolerance to salinity and temperature, the genetic diversity and symbiotic nitrogen fixing ability. Genotypic characterization was done based on BOX-PCR, REP-PCR markers and 16S rRNA sequencing. 65% of the isolates indicated an approximately 66% similarity with *R. tropici* CIAT899 and *R. tropici* H12 on the basis of genetic characterization. Cluster analysis based on tolerance to salinity and temperature revealed *R. tropici* CIAT899 and *R. tropici* H12 with a similarity level of 76%. And also, 20% of the isolates showed better or similar symbiotic nitrogen fixing ability with the *Rhizobium* reference strain (R. tropici CIAT899).

Nahar *et al.* (2017) studied the biochemical and molecular characteristics of *Rhizobium* isolates of *Sesbania bispinosa.* The isolates showed resistance to antibiotics such as cloxacillin and penicillin G. Some of the strains indicated positive results for *nodC* and *nifH gene* amplification which are present in *Rhizobium* species. Genetic diversity among the strains were evaluated by comparing the sequences of 16S rRNA. the study identified the ideal strain of *Rhizobium* which can be inoculated in the soil as biofertilizer and improve the sustainable agriculture.

Moghaddam *et al*. (2018) isolated 63 *Sinorhizobium* isolates from the root nodules of alfalfa plants in Iran to study the characterization of phenotypic and molecular characteristics. The isolates were cultured in Yeast Extract Mannitol agar and confirmed by plant infection test. The isolates were mucoid producing, gram-negative and convex at edges. All the isolates showed positive result for catalase and oxidase test. Some of the isolates showed tolerance to salinity, acidity, temperature and heavy metal stresses. amplified using two primers. The bacterial isolates were confirmed using PCR and PCR-RFLP techniques for *Sinorhizobium meliloti.*

Sankhala *et al.* (2018) examined the *Crotalaria burhia* Buch. - Ham.ex Benth. in search of potential rhizobial species. 72 isolates were isolated from the root nodule of *C. burhia* out of which 51 isolates were evaluated for genetic diversity on the basis of ARDRA and RAPD patterns. BLASTn sequence results based on 16S rRNA gene of specific 13 isolates representing four ARDRA types revealed that they were related to genera *Ensifer, Rhizobium and Bradyrhizobium.* The study suggested that *C. burhia* is nodulated by strains of *Ensifer* and *Bradyrhizobium* in alkaline soil of Thar desert and the strains can be efficiently cross- nodulated with *Vigna radiata.*

CHAPTER - III
MATERIALS AND METHODS

The present study entitled **"Characterization and evaluation of native *Rhizobium* of *Lathyrus*, Green gram, Chickpea and Black gram."** was carried out during 2018-19 at the Department of Agricultural Microbiology, Indira Gandhi Krishi Vishwavidyalya, Raipur. A brief description of the materials used and the techniques adopted during the course of study are presented in this chapter.

3.1 LOCATION AND CLIMATE

3.1.1. Location of Experimental site

The experiments were conducted in Department of Agricultural Microbiology, IGKV Raipur in order to characterize and evaluate *Rhizobium* isolates of *Lathyrus,* green gram, chickpea and black gram.

3.1.2. Physiography

The experimental site is located in plains of Chhattisgarh at latitude 21°16'N and' 81°36' E longitude with an altitude of 298.58 meter above mean sea level (MSL).

3.1.3. Climate

Raipur comes under dry sub humid region, receiving an average rainfall of 1200 - 1400 mm out of which about 85 per cent is received during the rainy season (June to September) and the rest 12 per cent during winter season (October – February). The place experiences a short mild winter, January being the coolest. May being the hottest month.

Soil surface temperature of this region crosses 60°C, air temperature touches the48°C and humidity drops up to 3 to 4 per cent during summer season and mercury level drops to as low as 6°C during December and January.

3.2 EXPERIMENTAL DETAILS

The experiment was conducted in the laboratory and glass house under controlled conditions.

3.2.1. Collection of isolates

Native rhizobial isolates of *Lathyrus,* green gram, chickpea and black gram respectively were collected from the repository of Department Agricultural Microbiology, College of Agriculture Raipur, Chhattisgarh.

3.2.2 Retrieving of cultures

The *Rhizobium* slants of *Lathyrus,* green gram, chickpea and black gram respectively were collected from the repository of Department of Agricultural Microbiology, College of Agriculture, Raipur. Yeast Extract Mannitol-Agar medium (YEMA) was used for retrieving of *Rhizobium* cultures. With the help of an inoculating loop, streaking was done in the YEMA plates and incubated at 28°C temperature.

3.2.3 Purity test of the cultures

3.2.3.1 Congo-red test

Congo red was added aseptically to each litre of Yeast Extract Mannitol-Agar medium just before using it. Media was sterilized by autoclaving at 15 lbs pressure for 15 mins. In general, the *Rhizobium* takes up the dye weekly while many of the soil bacteria take it up easily.

3.2.3.2 Growth in Alkaline medium

Selective species of *Agrobacterium* were detected by drawing streaks on Hoffer's Alkaline Medium where *Rhizobium* doesn't grow.

Hoffer's Alkaline media was prepared by adding YEMA with 1ml/litre of Thymol Blue (1.6% solution) and adjusted to pH 11.

On the slants, the growth of *Rhizobium* and the change in color of indicator was observed upto 15 days.

3.2.3.3 Ketolactose test

Most of the strains of *Rhizobium* species have been found to produce 3-ketolactose in lactose containing medium . The composition of media used for this test was same as that of Yeast Extract Mannitol Agar except mannitol is replaced by lactose.

The media was poured in plates and on solidification, the inoculum was streaked over it. After incubation when sufficient growth was observed, the plates were flooded with Benedict's reagent.

Development of yellow ring of cuprous oxide around the growth of organism was indicative of *Agrobacterium* contamination.

3.2.4 Colony Morphological characteristics

One ml of appropriate dilution of *Rhizobium* isolates was transferred into the petri plates containing YEMA with congo red medium. The colony characters viz margin, elevation, form and color were observed on agar medium and recorded.

3.2.5. Biochemical characterization of collected Isolates

The *Rhizobium* isolates were characterized by Gram staining, Indole-production test, MR-VP test, Citrate utilization test, Catalase test, Urease test, Gelatin liquefaction test , Triple sugar Iron agar test , starch hydrolysis.

3.2.5.1. Gram staining

The gram staining was performed and the bacteria were classified into two major groups: the gram- positive and the gram- negative. The gram staining was carried out as per the following procedure.

Thin smears of bacteria were made on the glass slides and they were allowed to air dry followed by heat fixing the smears. Then the smears were covered with crystal violet for 30 seconds followed by washing the slides with distilled water for not more than 2 seconds to remove excess stain. Then the smear was covered with Gram's iodine solution for 60 seconds. The iodine solution was washed off with 95 percent ethyl alcohol. Ethyl alcohol was added drop by drop until no more color was flowing out of the smear. Again the slides were washed with distilled water and allowed to drain. Safranin was applied to the smears for 30 seconds. Again washed with distilled water and blotted dry with absorbent paper and were allowed to air dry. The air dried slides were observed under microscope. The bacteria that appeared purple were referred to as Gram positive while the bacteria that appeared pink were described as Gram- negative (Aneja, 2003).

3.2.5.2. Indole- production test

The Indole production test was carried out by inoculating the bacterial culture under investigation into the tryptone broth and incubated at 35℃ for 48 hours. After the period of incubation, kovac's reagent was added to each tube to detect the Indole production. The positive test for Indole production was development of cherry red color at the top layer of the tube.

3.2.5.3. MR-VP test

3.2.5.3.1 Methyl Red test

Tubes containing MRVP broth were inoculated with the bacterial culture under investigation and incubated at 35℃ for 48 hours. After incubation, 5 drops of methyl red were added. Positive reaction for the test was indicated by development of red color in the tubes.

3.2.5.3.2 Voges-Proskauer test

Tubes containing MRVP broth were inoculated with pure culture of the test organism and incubated at 35℃ for 48 hours. After incubation, 12 drops of napthol solution and 2-3 drops of 40 percent KOH was added to the tubes. Development of crimson to ruby pink color on the surface was indicative of the positive test.

3.2.5.4 Citrate utilization test

Tubes with Simmon's citrate agar slants were inoculated with the bacterial culture and incubated at 37℃ for 48 hours. The change of color of the incubated slants from green to blue was indicative of citrate positive test.

3.2.5.5 Catalase test

Trypticase soy agar slants were inoculated with the pure culture and incubated at 35℃ for 24-48 hrs. After incubation, 3-4 drops of hydrogen peroxide were allowed to flow over the growth of the slant culture. Production of bubbles of oxygen within one minute after addition of hydrogen peroxide was indicative of catalase positive test.

3.2.5.6 Urease test

Urea agar slants were inoculated with the bacterial culture and incubated for 24-48 hours at 37℃. Urease positive test was indicated by development of deep pink coloration of the medium.

3.2.5.7 Gelatin liquefaction

A heavy inoculum of the test bacteria was inoculated into the nutrient gelatin medium and incubated at 30℃ for 48 hours. After the period of incubation, the gelatin tubes were placed in refrigerator (4℃) for 15-30 minutes to check the gelatin liquefaction. Partial or total liquefaction of the inoculated tube after being exposed to cold temperature was indicative of the positive test.

3.2.5.8 Triple sugar iron agar test

Bacterial cultures were inoculated into the triple sugar iron agar slants by stabbing through the center of the medium to the bottom and then streaked over the surface followed by incubating at 37°C for 2-5 days. The appearance of an alkaline slant (red) and acid slant (yellow) butt after incubation indicates that the organism is able to ferment glucose but it is unable to ferment lactose and glucose or either of both. If hydrogen sulfide is formed, then the butt shows black color.

3.2.5.9 Starch hydrolysis

Hydrolysis of starch is done to detect the ability of the enzyme to produce amylase. The test organism was inoculated in starch agar plate by using inoculating loop making small central line or streak in the plate. The bacterial inoculated plates were incubated for 48 hours at 37°C. After the period of incubation, the surface of the plates was flooded with iodine solution for about 30 seconds and the excess of iodine was discarded. The plates were examined for the color change. A clear zone surrounding the microbial colonies is indicative of positive result for starch hydrolysis.

3.2.6 Study on Antibiotic Susceptibility

The isolates of *Rhizobium* were studied for sensitivity to exposure of different antibiotics. The antibiotic resistance of the strains was assayed with the help of agar diffusion method using filter paper discs impregnated with the test antibiotics in different concentrations (Antoun *et al.*, 1982; Date and Hurse,1991). The actively growing cultures of the test *Rhizobium* isolates were prepared in YEM broth. Sterilized petri plates containing solidified YEMA medium were surface inoculated with 0.5 ml of actively growing culture of the test strain and spread uniformly by means of a sterilized spreader. Then the inoculated plates were incubated for 1 to 2 hours to allow the inoculum to be absorbed in the solidified medium.

Antibiotic disc of Streptomycin 10 µg, Penicillin 10µg, Tetracycline 30µg and Ampicillin 10µg were tested separately by placing the disc on the surface of

inoculated agar plates using sensi- disc dispenser. Plates were incubated for 4-5 days at 28 ± 2°C. After the incubation period, the inhibition zone was observed.

3.2.7. Glass house experiment

The study was aimed at identifying effective rhizobial isolates for *Lathyrus*, green gram, chickpea and black gram cultivation. The experiment was conducted in glass house condition under sand culture devoid of nitrogen source. The experiment was specially conducted for testing the biological nitrogen fixing ability of isolates of *Rhizobium* for different legumes.

In this experiment four selected isolates of each from *Lathyrus*, green gram, chickpea and black gram were compared with one uninoculated control under completely randomized design. Each treatment was replicated four times.

3.2.7.1. Sand culture

For the purpose of selection of effective *Rhizobium* isolates, finely graded sterilized river sand (20 lb. pressure per inch2 for 2 hours) was filled in upper portion of Leonard's self-irrigating assembly of glass and the lower portion was filled with N-free nutrient solution which acted as a source of nutrients for the crops.

3.2.7.2. Surface sterilization of seeds

Healthy seeds were taken to conduct the experiment. Uniform sized seeds were rinsed with 95% ethanol and then immersed in 0.1% mercuric chloride for about 4 minutes. The seeds were then washed thoroughly with double distilled water for atleast five times.

3.2.7.3. Treatments details

The following treatments were set up in the glass house under controlled conditions.

Table: 3.1 Treatment details carried out in *Lathyrus*

Treatments	***Lathyrus* rhizobial isolates**
T1	Control (uninoculated)
T2	*Lathyrus* -3
T3	*Lathyrus*-494
T4	*Lathyrus*-3693
T5	*Lathyrus*-3711

Table: 3.2Treatment details carried out in green gram

Treatments	**Green gram rhizobial isolates**
T1	Control (uninoculated)
T2	Mung-125
T3	Mung-257
T4	Mung-1185
T5	Mung-3492

Table: 3.3 Treatment details carried out in chickpea

Treatments	**Chickpea rhizobial isolates**
T1	Control (uninoculated)
T2	Gram-98
T3	Gram-191
T4	Gram-1052
T5	Gram-1068

Table: 3.4 Treatment details carried out in black gram

Treatments	Black gram rhizobial isolates
T1	Control (uninoculated)
T2	Urad-577
T3	Urad-1168
T4	Urad-3533
T5	Urad-3536

3.2.7.4. Sowing, handling and irrigation

The surface sterilized seeds of different legumes were inoculated before sowing by measure quantity of YEM-rhizobial suspension. The seed of the control bottles received the same amount of YEM broth but without rhizobial population. Amount of mature YEM-rhizobial suspensiom was fixed to ensure at least 10^4 viable cells were received by each seed (Nambiar,1985). Four seeds were sown in each bottles (date of sowing 18-10-2018). After some days only 2 plants were maintained by thinning out the extra seedlings. Then the glass assemblies were properly tagged and labeled. Timely and uniform irrigation were provided to all the bottles by N-free Mcknight seedlings nutrient solution as and when required (Vincent,1970)

Composition of Mcknight seedling N- free nutrient solution

Solution A	**mg 100 ml^{-1} Distilled water**
Boric acid (H_3BO_3)	2.86
Manganese Sulphate ($MnSO_4.4H_2O$)	1.54
Zinc Sulphate ($ZnSO_4.7H_2O$)	0.22
Copper Sulphate ($CuSO_4.5H_2O$)	0.08
Molybdic acid (H_2MoO_4)	0.09

Solution B **Distilled water**	**mg 100 ml^{-1}**
Ferric Chloride ($FeCl_3$)	16.8
EDTA	2.0

Solution C **Distilled water**	**mg 100 ml^{-1}**
Calcium Sulphate ($CaSO_4$)	24.0
Magnesium Sulphate ($MgSO_4$)	4.0
Potassium Dihydrogen Phosphate (KH_2PO_4)	4.0
Potassium chloride (KCl)	6.0

To the 960 ml of solution C, 20 ml each of solution A and B were added and whole solution was diluted 20 times. The pH of the nutrient solution was adjusted at 7.0. For sterilization, the nutrient solution was autoclaved at 15 lb/inch^2 pressure for 30 minutes (Katre *et al.*, 1997).

3.2.7.5. Observations recorded during the Experiment

3.2.7.5.1 Plant height at 30 and 45 DAS

3.2.7.5.2 Fresh weight at 45 DAS

3.2.7.5.3 Dry weight at 45 DAS

3.2.7.5.4 Study of Nodulation and *Rhizobium* population Dynamics

3.2.7.5.5 Bio-chemical characterization of *Rhizobium* isolates

3.2.7.6 Harvesting

The glass house legume plants were uprooted at 45 DAS on reaching maturity (date of harvesting 12-01-2019). The plants sample were over dried at 60 to 65°C up to the attainment of certain weight. The weight of the dry samples was recorded.

3.2.8. Chemical analysis of plant

Nitrogen: The nitrogen content in plant samples were estimated by micro-kjeldahl method as described by Jackson (1973) using Gerhardt auto digestion and distillation system (Vapodest -30).

3.2.9. Microbial analysis of sand

Microbial analysis of sand was done by serial dilution plating method (Subba Rao,1988). Sand samples upto 5-10 cm depth were drawn out with the help of sterilized spoon from each pot at different stages of the crop growth. The sampling of sand was done at 30 and 45 DAS.

Soon after sampling, the sand samples were kept in polythene bags to prevent the moisture loss and were properly tagged, sealed and stored in refrigerator for quantitative estimation of *Rhizobium.*

Microbiological estimation with respect to rhizobial count in the sand samples were done by dilution plate method (Subba Rao, 1988). For rhizobial counting, the serial dilutions of the samples were done by taking 1gm of sand sample in 9ml sterilized water in a dilution tube (Tuladhar,1983) and it was kept in shaker for about 30 minutes for shaking. After shaking, the dilution tube (No. 1) was kept for 30 minutes allowing the sand particles to settle down. In this way,10^1 dilution of sand sample was obtained. Now 1.0 ml of the rhizobial suspension from the dilution tube No. 1 was drawn with the help of autopipette and transferred to another dilution No. 2 containing 9 ml sterilized water resulting in 10^2 dilutions. It was again kept in the shaker for about 5 minutes. Again 1.0 ml was drawn from dilution No. 2 for 10^3 dilutions and in this way serial dilution of sand sample was carried up to desirable dilution and finally a complete set of desirable dilutions of sand sample was obtained. Similarly, population density of *Rhizobium* in mature YEM broth was also determined.

Yeast Extract Mannitol Agar Media (YEMA) for *Rhizobium* (Subba Rao,1988)

Composition of the medium

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.\ 7H_2O$	0.2 g

NaCl	0.1 g
Yeast Extract	1.0 g
Agar	15.0 g
Distilled water	1000 ml
Congo red solution (1%)	2.5 ml
pH	7.0

About 20 ml of the sterilized and partially cooled media was poured into the sterilized plates containing 1 ml aliquot of appropriate dilution at the bottom which was drawn out of the dilution tube with the help of sterilized tips of autopipette and the petri-plates were incubated at 28℃ in the incubator. Counting of rhizobial colonies was done after 24 hours of period of incubation. Counted colonies were marked using an instant marker to avoid repeated counting of the colonies and the process of counting was continued up to 7 days of incubation. Colony counting was done in the colony counter.

Plating of each samples was done in duplicate and mean values were worked out of each samples. One control was also incorporated with each set of plating. After counting of colonies, rhizobial population was calculated on the basis of per gm of dry sand using following formula (Schmidt and Caldwell ,1967). rhizobial population density in the YEM broth was estimated by using the same formula.

Number of rhizobia per gm of oven dry sand

$$= \frac{\text{No. of colony forming unit (CFU) x dilution}}{\text{Dry weight of 1 gm moist sand sample x aliquot taken}}$$

Number of rhizobia/ml of matured YEM broth

$$= \frac{\text{No. of colony forming unit (CFU) x dilution}}{\text{Aliquot taken}}$$

The operation of making serial dilution, setting up of agar plates, inoculate appropriate amount of media, was done in sterilized atmosphere of laminar flow. Characterization study for confirmation of *Rhizobium* isolates was completed by using Gram's staining technique, microscopy through phase contrast microscope Leica DMBRE etc. before using them in the experiments as *Rhizobium* isolates of *Lathyrus*, green gram, chickpea and black gram.

3.2.10. Microbial analysis of seed

The population on inoculated seeds was also determined similar to that of population density of sand.

Number of rhizobia per gm of oven dried seed

$$= \frac{\text{No. of colony forming unit (CFU) x dilution}}{\text{Dry weight of 1 gm moist seed sample x aliquot taken}}$$

3.2.11. Statistical analysis:

All the pre and post- harvest observations were recorded and tabulated in a systemic manner. The final observations were statistically analyzed by completely randomized design (Panse and Shukhatme,1978)

CHAPTER - IV

RESULTS AND DISCUSSION

The present investigation was conducted at the Department of Agril. Microbiology, IGKV, Raipur during 2018-2019 in order to select effective native *Rhizobium* isolates of *Lathyrus,* green gram, chickpea and black gram. In this connection morphologically and biochemically characterized *Rhizobium* isolates ware further properly screened through N-free sand culture experiment. The plants were raised in the N-free sand culture from seeds inoculated with different *Rhizobium* isolates to observe variations in biologically fixed amount of nitrogen during legume - *Rhizobium* symbiosis. The results of the present investigation are as follows.

Lathyrus, green gram, chickpea and black gram are the popular legumes which is cultivated in most parts of the country but availability of location specific effective *Rhizobium* isolates is very limited. Therefore, in order to select effective *Rhizobium* isolates,40 local *Rhizobium* isolates, 10 of each of *Lathyrus*, green gram, chickpea and black gram were collected from the repository of Department of Agril. Microbiology, IGKV, Raipur. All the isolates were confirmed and screened by growth performance, colony morphology, Gram's staining and other biochemical tests before using them in the N-free sand culture experiments as *Rhizobium* isolates of *Lathyrus*, green gram, chickpea and black gram. Out of 10 *Rhizobium* isolates of each legume only 4 isolates were selected for biochemical characterization and the sand culture experiment.

4.1 Common morphological characteristics of the *Rhizobium* isolates of *Lathyrus,* green gram, chickpea and black gram

The observations based on the morphological characteristics are presented in the Table 4.1(Plate 1). The *Rhizobium* isolates when cultured on Yeast Extract Mannitol Agar (YEMA) with congo red, produced semi-translucent, nearly circular, convex, raised and mucilaginous colonies in addition to some white colonies

pink colonies were also observed. Upon Gram staining the isolates retained red color which indicated that they were Gram negative. The confirmation of the isolate was performed using sub culturing method. The microscopic examination of all the isolates was found to be Gram negative, rod shaped and motile. Datta *et al.*, (2015) also reported that *Rhizobium* was Gram negative, motile, rod shaped and showed convex elevation in Yeast Extract Mannitol medium.

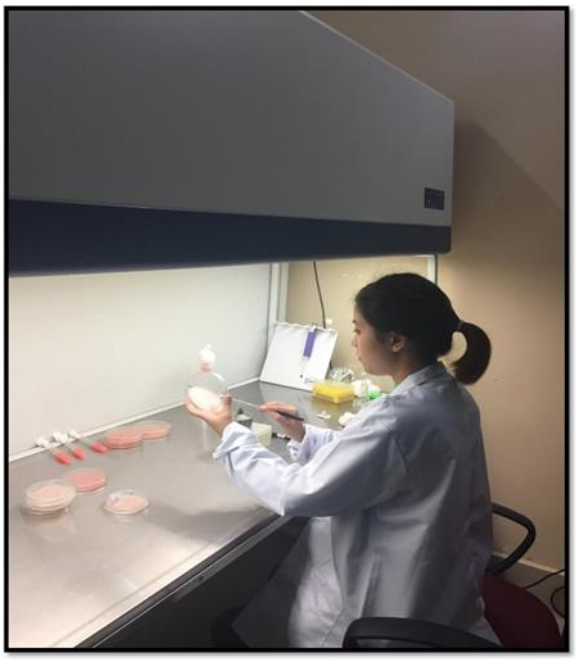
Table 4.1: Common morphological characteristics of the native *Rhizobium* isolates

Sl. No.	Characters	Observations
1	Colony Shape	Circular
2	Colony color on YEMA media	White/light pink(mucilaginous & semi-translucent)
3	Elevation	convex
4	Motility	Motile
5	Bacterium shape	Rod shaped
6	Gram's nature	Gram negative

4.2 Biochemical characterization

The selected isolates of *Rhizobium* were subjected to different test for biochemical characterization. One of the main criteria used to characterize the different isolates of *Rhizobium* is through biochemical test as described by Rai *et al.*, 2014.

The isolates when allowed to grow on YEMA medium did not absorb the red colour of the congo red (Patil & Kamble.,2016). The strains of *Rhizobium* did not show any growth in Hoffer's alkaline medium and lactose containing medium (Agrawal *et al.*, 2012). Mahana *et al.* 2000 also reported similar results from



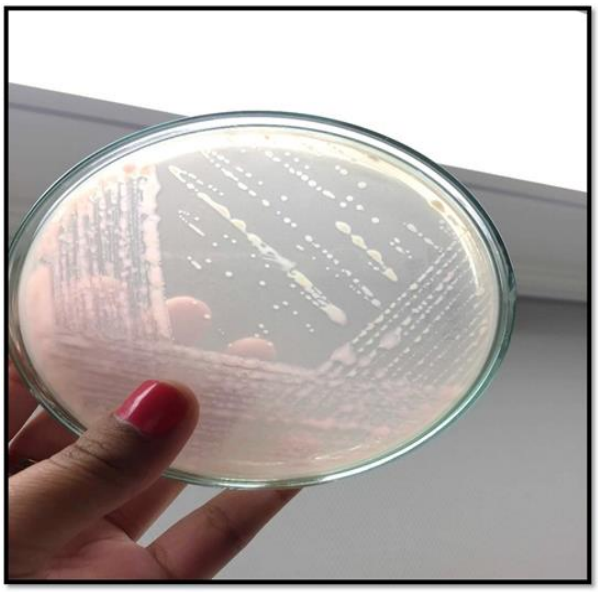


Plate 1. General view of purification work and native *Rhizobium* isolates

Rhizobium isolates, isolated from *Vigna mungo*. The test samples of *Rhizobium* indicated negative results for indole production, voges-proskauer test (Table 4.2,4.3,4.4,4.5 and Plate 2) as described by Shahzad *et al.*,2012. While, these isolates showed positive results for citrate utilization (Table 4.2, 4.3, 4.4, 4.5 and Plate 3). Gachande and Khansole (2011) also studied the characterization of *Rhizobium* in the root nodules of soybean through same biochemical test and reported similar type of results. Most of the isolates of *Rhizobium* were urease positive (Table 4.2,4.3,4.4,4.5 and Plate 4) similar to that of findings of Aneja,1996; Singh *et al.*, 2011; Gauri *et al.*,2011; Rai *et al.*, 2014. But few of the strains gave negative results i.e, isolate no. 494 of *Lathyrus* and isolate no. 191 of chickpea. All the isolates of *Rhizobium* gave negative results for catalase test (Table 4.2, 4.3,4.4,4.5 and Plate 3) which was in close agreement with Deora and Singhal 2010; Rai *et al.*,2014. Most of the isolates indicated negative results for the gelatin liquefaction as described by Hunter *et al.*,2007; Singh *et al.*,2008. Deshwal & Chaubey,2014. Haseem *et al.* 1998 also reported same type of results by studying the *Rhizobium* from soil and roots through same biochemical tests. But some rhizobial isolates showed positive results for gelatin liquefaction namely, isolate no. 3711 of *Lathyrus* and isolate no. 1185 of Green gram. Most of the test isolates of *Rhizobium* gave negative results for starch hydrolysis test (Table 4.2, 4.3, 4.4,4.5 and Plate 4) similar to that of findings of Aneja,1996; Deka & Azad, 2006; Gachande & Khansole,2011.Wadhwa *et al.* 2017 also characterized the *Rhizobium* from chickpea and reported similar findings by the same biochemical tests. Few of the isolates of *Rhizobium* indicated positive results for starch hydrolysis i.e, isolate no. 1185 of green gram, isolate no. 1068 of chickpea and isolate no.3533 of black gram.

4.2 Common biochemical characterization of *Lathyrus* native *Rhizobium* isolates

Sl. No.	Isolates	Biochemical characterization							
		Indole production	**Methyl red test**	**Voges-proskauer test**	**Citrate utilization**	**Catalase**	**Urease**	**Gelatin liquefaction**	**Starch hydrolysis**
1	*Lathyrus* -3	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
2	*Lathyrus*-494	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve
3	*Lathyrus*-3693	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
4	*Lathyrus*-3711	-ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve

Table 4.3 Common biochemical characterization of the green gram native *Rhizobium* isolates.

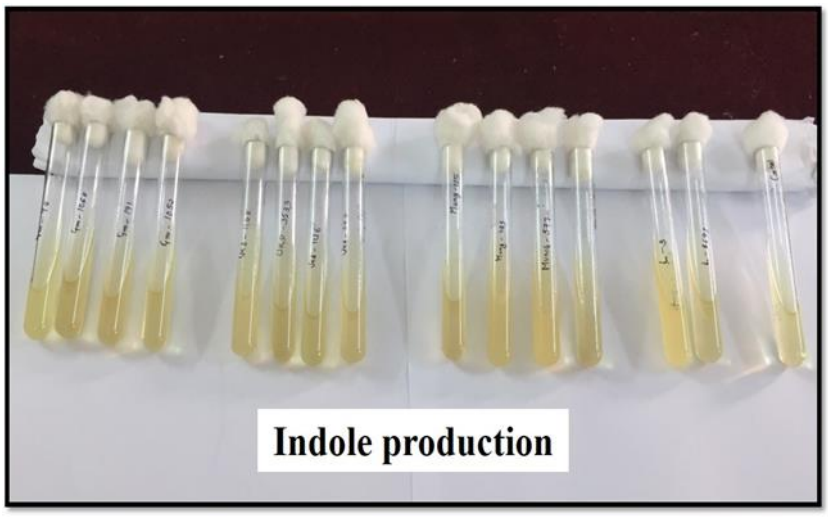
Sl. No.	Isolates	Biochemical characterization							
		Indole production	**Methyl red test**	**Voges-proskauer test**	**Citrate utilization**	**Catalase**	**Urease**	**Gelatin liquefaction**	**Starch hydrolysis**
1	Mung-125	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
2	Mung-257	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
3	Mung-1185	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve
4	Mung-3492	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve

Table 4.4 Common biochemical characterization of the chickpea native *Rhizobium* isolates.

Sl. No.	Isolates	Biochemical characterization							
		Indole production	**Methyl red test**	**Voges-proskauer test**	**Citrate utilization**	**Catalase**	**Urease**	**Gelatin liquefaction**	**Starch hydrolysis**
1	Gram - 98	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
2	Gram-191	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve
3	Gram-1052	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
4	Gram-1068	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve

Table 4.5 Common biochemical characterization of the black gram native *Rhizobium* isolates

Sl. No.	Isolates	Biochemical characterization							
		Indole production	**Methyl red test**	**Voges-proskauer test**	**Citrate utilization**	**Catalase**	**Urease**	**Gelatin liquefaction**	**Starch hydrolysis**
1	Urad - 577	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
2	Urad-1168	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
3	Urad-3533	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
4	Urad-3536	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve



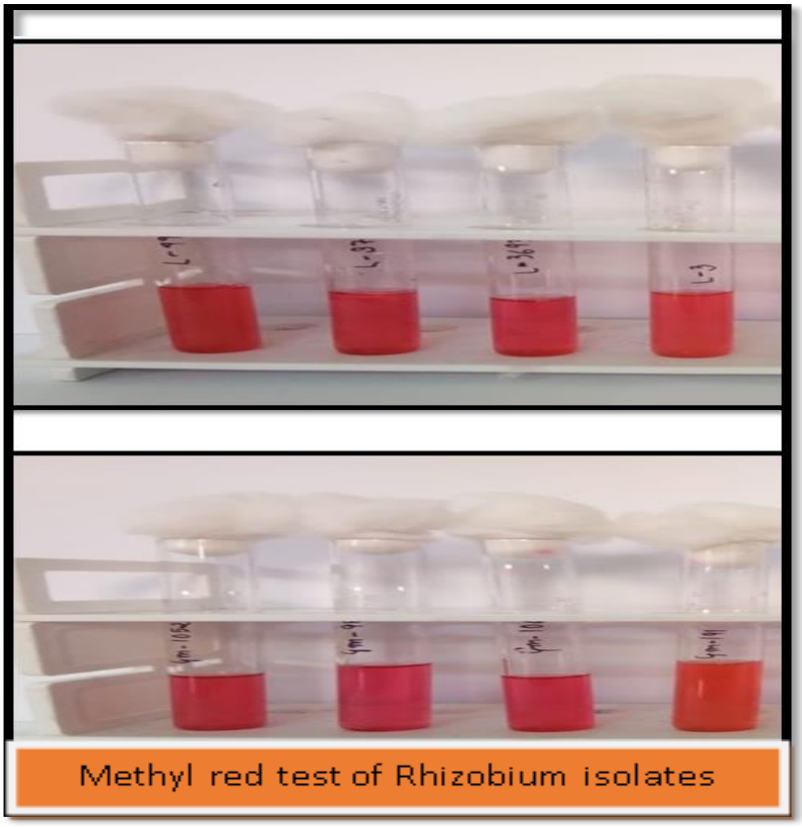


Plate 2. General view of biochemical analysis (indole production and methyl red test) of purified *Rhizobium* isolates

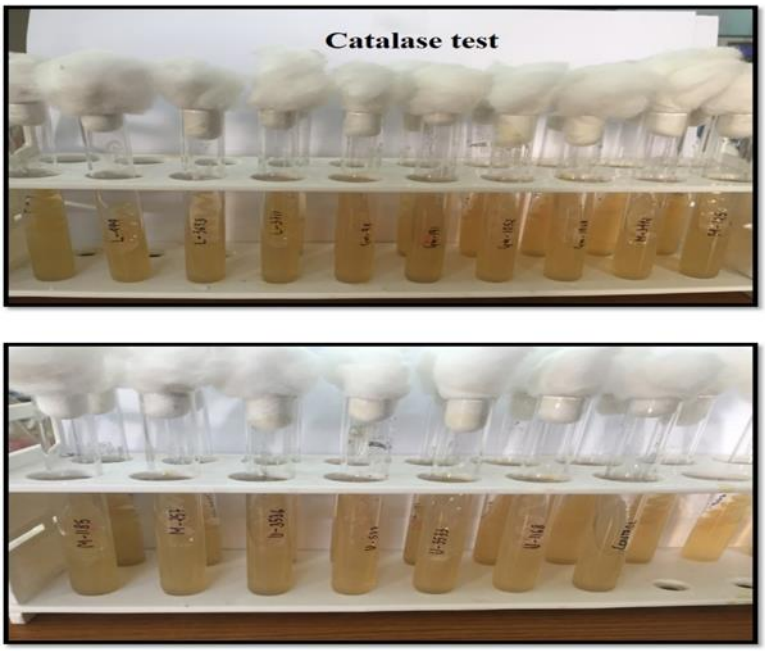
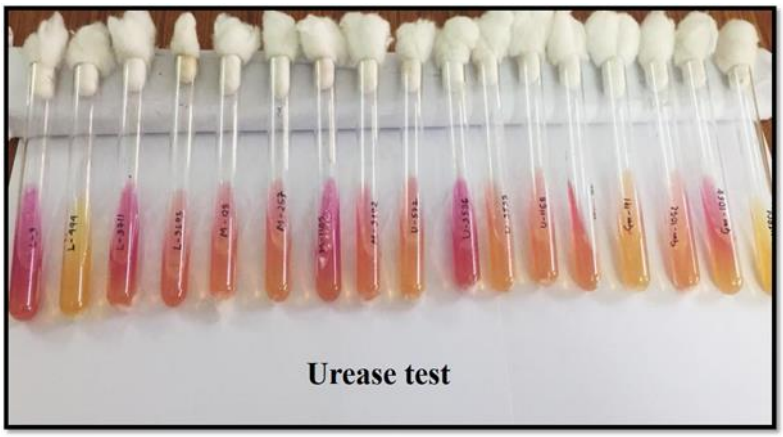


Plate 3. General view of biochemical analysis (citrate and catalase test) of purified *Rhizobium* isolates



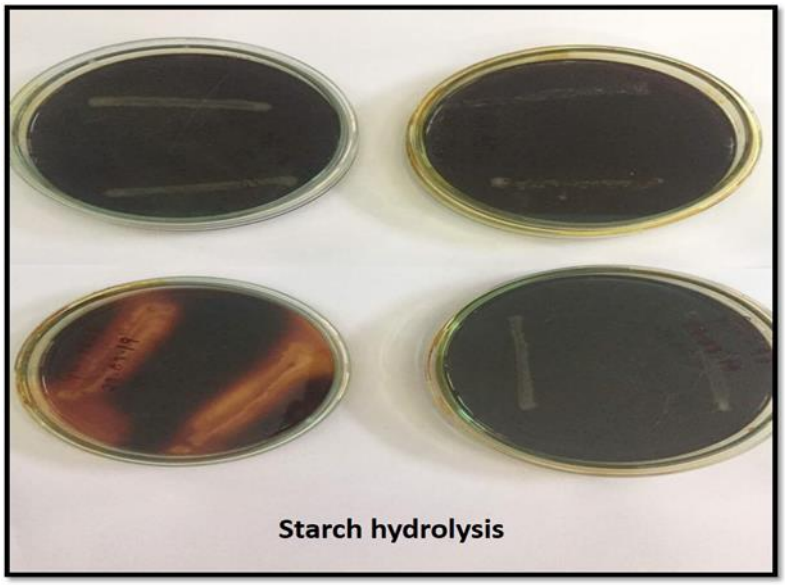


Plate 4. General view of biochemical analysis (Urease and hydrolysis of Starch) of purified *Rhizobium* isolates.

4.2.1 Triple sugar iron agar test

The triple sugar iron test was done to detect the ability of the organism to ferment sugars and to produce hydrogen sulfide. If only glucose is fermented, acid production in bottom of tube shows yellow color, but if either Sucrose or Lactose is fermented, acid is produced showing both bottom and slant yellow. While no fermentation occurs when slant and bottom is red.

Table:4.6 Carbohydrate fermentation of the rhizobial isolates of *Lathyrus* upon Triple Sugar Iron Agar test

Sl.No.	Name of the isolates	Results (slant/bottom)	Interpretation
1.	*Lathyrus*-3	Red/Red	No fermentation
2.	*Lathyrus*-494	Red/Red	No fermentation
3.	*Lathyrus*-3693	Red/Red	No fermentation
4.	*Lathyrus-3711*	Yellow/Yellow	Sucrose or Lactose fermentation

Table:4.7 Carbohydrate fermentation of the rhizobial isolates of green gram upon Triple Sugar Iron Agar test

Sl.No.	Name of the isolates	Results (slant/bottom)	Interpretation
1.	Mung -125	Red/Red	No fermentation
2.	Mung -257	Red/Red	No fermentation
3.	Mung -1185	Red/Red	No fermentation
4.	Mung -3492	Red/Red	No fermentation

Table:4.8 Carbohydrate fermentation of the rhizobial isolates of chickpea upon Triple Sugar Iron Agar Test

Sl.No.	Name of the isolates	Results (slant/bottom)	Interpretation
1.	Gram-98	Red/Red	No fermentation
2.	Gram-191	Red/Red	No fermentation
3.	Gram-1052	Red/Red	No fermentation
4.	Gram-1068	Red/Red	No fermentation

Table:4.9 Carbohydrate fermentation of the rhizobial isolates of black gram upon Triple Sugar Iron Agar Test.

Sl.No.	Name of the isolates	Results (slant/bottom)	Interpretation
1.	Urad - 577	Red/Red	No fermentation
2.	Urad - 1168	Red/Red	No fermentation
3.	Urad - 3533	Red/Red	No fermentation
4.	Urad - 3536	Red/Red	No fermentation

Most of the selected isolates of *Rhizobium* produced red slant and red bottom when exposed to TSI agar test which was indicative of no carbohydrate fermentation (Table 4.6, 4.7, 4.8, 4.9 and Plate 5) except the isolate no. 3711 of *Lathyrus* produced yellow slant and yellow bottom (Table 4.6 and Plate 5) which indicates the fermentation of lactose/sucrose similar to that of finding of Singh *et al.* 2008.

4.3 Study on Antibiotic Susceptibility

The susceptibility of selected rhizobial isolates of *Lathyrus*, green gram , chickpea and black gram against streptomycin, tetracycline, penicillin and ampicillin was

determined with the help of antibiotic disc test and the susceptibility of the isolates were observed as indicated in Table 4.10, Table 4.11, Table 4.12 and Table 4.13

Table 4.10: Antibiotic Susceptibility of *Rhizobium* isolates of *Lathyrus*

Sl. No.	Name of the isolates	Antibiotics			
		Streptomycin (10µg/disc)	**Penicillin (10µg/disc)**	**Ampicillin (10µg/disc)**	**Tetracycline (30µg/disc)**
1	*Lathyrus*-3	**+ve**	**-ve**	**+ve**	**+ve**
2	*Lathyrus*-494	**+ve**	**+ve**	**-ve**	**+ve**
3	*Lathyrus*-3693	**+ve**	**+ve**	**-ve**	**+ve**
4	*Lathyrus*-3711	**-ve**	**-ve**	**-ve**	**+ve**

+ve: Susceptible, -ve : Resistance

Table 4.1 : Antibiotic Susceptibility of *Rhizobium* isolates of green gram.

Sl. No.	Name of the isolates	Antibiotics			
		Streptomycin (10µg/disc)	**Penicillin (10µg/disc)**	**Ampicillin (10µg/disc)**	**Tetracycline (30µg/disc)**
1	Mung -125	**+ve**	**+ve**	**-ve**	**+ve**
2	Mung -257	**+ve**	**+ve**	**-ve**	**+ve**
3	Mung-1185	**+ve**	**+ve**	**-ve**	**+ve**
4	Mung-3492	**+ve**	**+ve**	**-ve**	**+ve**

+ve: Susceptible, -ve : Resistance

Table 4.12: Antibiotic Susceptibilty of *Rhizobium* isolates of chickpea.

Sl. No.	Name of the isolates	Antibiotics			
		Streptomycin (10µg/disc)	Penicillin (10µg/disc)	Ampicillin (10µg/disc)	Tetracycline (30µg/disc)
1	Gram-98	+ve	-ve	-ve	+ve
2	Gram –191	+ve	-ve	-ve	-ve
3	Gram-1052	+ve	-ve	-ve	+ve
4	Gram-1068	+ve	+ve	+ve	+ve

+ve: Susceptible, -ve : Resistance

Table 4.13: Antibiotic Susceptibility of *Rhizobium* isolates of black gram

Sl. No.	Name of the isolates	Antibiotics			
		Streptomycin (10µg/disc)	Penicillin (10µg/disc)	Ampicillin (10µg/disc)	Tetracycline (30µg/disc)
1	Urad-577	+ve	-ve	-ve	+ve
2	Urad-1168	+ve	-ve	-ve	+ve
3	Urad-3533	+ve	-ve	-ve	+ve
4	Urad- 3536	-ve	-ve	-ve	+ve

+ve: Susceptible, -ve : Resistance

The results presented in the Table 4.10 indicate that most of the isolates of *Lathyrus* were susceptible to tetracycline (30µg/disc) and streptomycin (10µg/disc) and few of the strains were sensitive to ampicillin (10µg/disc) i.e. isolate no.3 and two isolates were sensitive to penicillin (10µg/disc) namely isolate no. 3693 and isolate no. 494.

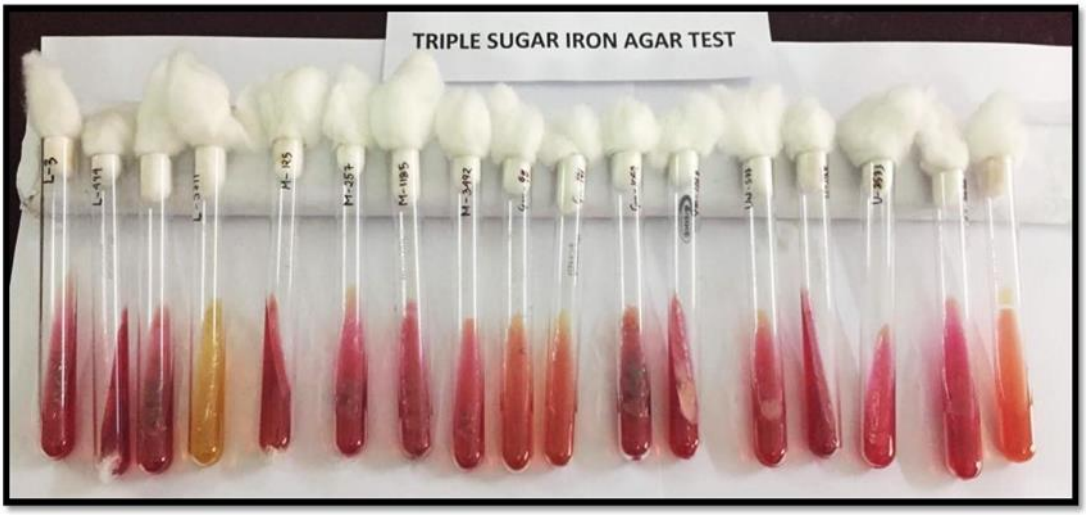
Similarly the data presented in Table 4.11 reveal that all the selected isolates of green gram were susceptible to streptomycin (10µg/disc), tetracycline (30µg/disc) and penicillin (10µg/disc) while all were insenstitive to ampicillin (10µg/disc) .

Data of Table 4.12 further revealed that all the selected isolates of chickpea were susceptible to streptomycin (10µg/disc) and tetracycline (30µg/disc), only one of the

strain was sensitive for ampicillin (10µg/disc) and penicillin (10µg/disc) i.e, isolate no. 1068. All other isolates indicated negative results for ampicillin (10µg/disc) and penicillin (10µg/disc).

Likewise, data in Table 4.13 indicates that the selected isolates of black gram were susceptible to streptomycin (10µg/disc) and tetracycline (30µg/disc) but gave negative results for ampicillin (10µg/disc) and penicillin (10µg/disc) sensitivity.

There was a formation of clear inhibition zone in the plates exposed with these antibiotic discs (plate 5) as described by Bhattacharya *et al.*,2013. Paudyal and Gupta, 2017 also reported similar type of results while studying the characterization of rhizobia isolated from root nodules of velvet bean.



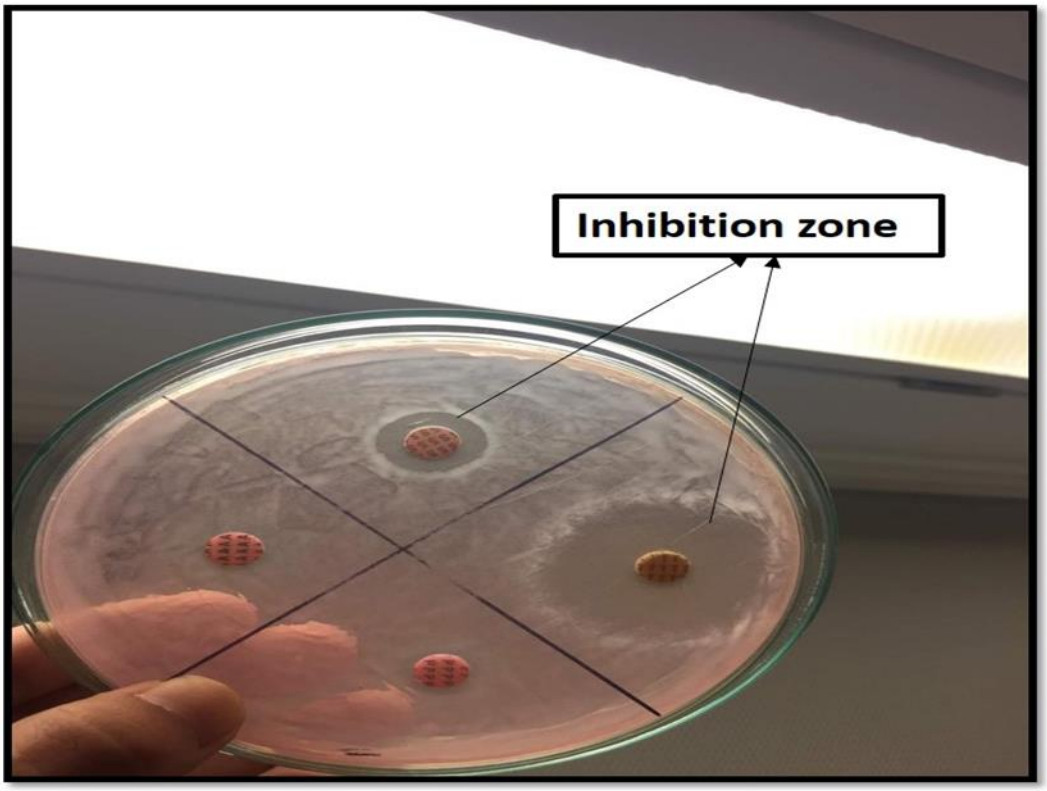


Plate 5. TSI test and antibiotic susceptibility test for purified *Rhizobium* isolates

4.4 Glass House Sand Culture Experiment

Selected 4 native *Rhizobium* isolates of each of *Lathyrus,* green gram, chickpea and black gram were used for variations in Legume- *Rhizobium* symbiosis over plants raised from uninoculated seeds under N-free sand culture conditions in glass house (Plate 6).

4.4.1 Plant height study

Data presented in the Table 4.14,4.15,4.16,4.17 and Fig. 4.1,4.2,4.3,4.4(Plate 7) revealed that the plant height of inoculated *Lathyrus,* green gram, chickpea and black gram at 15, 30 and 45 DAS increased significantly over its uninoculated control.

At 15 DAS, the highest plant height observed was 18.50 cm in *Lathyrus* due to inoculation of rhizobial isolate no. 3711 and lowest plant height observed was 15.25 cm due to inoculation of rhizobial isolate no. 494 (Table 4.14 and Fig.4.1). In green gram, the highest plant height observed at 15 DAS was 24.37 cm due to inoculation of rhizobial isolate no. 125 and lowest plant height was 21.25 cm due to inoculation of rhizobial isolate no.125(Table 4.15 and Fig. 4.2). The highest plant height observed in chickpea at 15 DAS was 32.37 cm due to inoculation of rhizobial isolate no.98 and lowest plant height was 30 cm due to inoculation of isolate no. 1068 (Table 4.16 and Fig. 4.3). In black gram, the highest plant height observed at 15 DAS was 18 cm due to inoculation of isolate no. 3533 while lowest plant height observed 16.12 cm due to inoculation of isolate no. 3536(Table 4.17 and Fig 4.4).

Lathyrus plants inoculated with rhizobial isolate no. 3693 produced taller plant of 23.37 cm than other isolates at 30 DAS. Among the isolates of *Lathyrus* the lowest plant height observed at 30 DAS was 17.25 cm due to the inoculation of rhizobial isolate no. 494 (Table 4.14 and Fig. 4.1). In green gram, the maximum height attained at 30 DAS was found to be 29.50 cm due to the inoculation of rhizobial isolate no. 257 and the lowest plant height attained recorded was 25.37 cm due to inoculation of rhizobial isolate no. 3492 (Table 4.15 and Fig. 4.2). In chickpea, the highest plant height recorded at 30 DAS was 38.25 cm due to the inoculation of rhizobial isolate no. 98 and the lowest plant height attained was 36 cm due to inoculation of rhizobial isolate no. 191 (Table 4.16 and Fig. 4.4). In black gram, the

maximum plant height recorded was 22.12 cm due to the inoculation of rhizobial isolate no. 3533 and the lowest plant height recorded was 19.12 cm due to the inoculation of rhizobial isolate no. 577(Table 4.17 and Fig 4.4).

At 45 DAS, the plant height revealed significant effect of seed inoculation with root nodule bacteria. The data indicated that the tested rhizobial isolates gave significantly higher plant height values over control. In *Lathyrus*, the maximum height attained was 36.25 cm due to the inoculation of isolate no. 3711 and the lowest value of plant height recorded was 24.37 cm due to inoculation of isolate no. 3(Table 4.14 and Fig. 4.1). In green gram, the maximum height recorded was 32.50 cm due to inoculation of isolate no. 125 and the lowest plant height observed was 27.12 cm due to the inoculation of isolate no. 3492 (Table 4.15 and Fig. 4.2). In chickpea, the maximum height attained was 59.25 cm due to inoculation of isolate no. 1052 and the lowest value of plant height was 51.50 cm due to inoculation of isolate no. 1068 (Table 4.16 and Fig. 4.3). In black gram, the highest value of plant height recorded was 31.25 cm due to inoculation of isolate no. 3533 and the lowest value of the plant height observed was 27.12 cm due to inoculation of isolate no. 1168 (Table 4.17 and Fig. 4.4).

These findings of present investigation were supported by Alam *et al.* (2015), Khaitov *et al.*(2015), Dongare *et al.*(2016), Choudhary *et al.*(2017) ,Rehan *et al.*(2018),Singh *et al.* (2018),Eshetu *et al.*(2018). Meena (2016) mentioned that *Rhizobium* inoculation in *V. radiata* resulted in significant increase in height against uninoculated control.

Table 4.14: Effect of native *Rhizobium* isolates on plant height of *Lathyrus* at 15 DAS ,30 DAS and 45 DAS under sand culture glass house conditions.

Treatments	Name of the isolates	Plant height (cm) at		
		15 DAS	30 DAS	45 DAS
T1	Control (uninoculated)	13.00	16.25	21.25
T2	*Lathyrus*- 3	16.12	19.00	24.37
T3	*Lathyrus*-494	15.25	17.25	25.50
T4	*Lathyrus* - 3693	17.37	23.37	30.12
T5	*Lathyrus* -3711	18.50	22.12	36.25
	C.D (5%)	3.00	3.19	3.74

Table 4.15: Effect of native *Rhizobium* isolates on plant height of green gram at 15 DAS,30 DAS and 45 DAS under sand culture glass house conditions.

Treatments	Name of the isolates	Plant height (cm) at		
		15 DAS	30 DAS	45 DAS
T1	Control (uninoculated)	20.25	24.12	26.50
T2	Mung -125	24.37	28.37	32.50
T3	Mung-257	23.25	29.50	30.25
T4	Mung -1185	21.25	28.12	30.12
T5	Mung -3492	21.37	25.37	27.12
	C.D (5%)	2.63	2.81	2.40

Fig 4.1. Effect of different *Rhizobium* isolates of *Lathyrus* on plant height under glass house condition at different DAS.

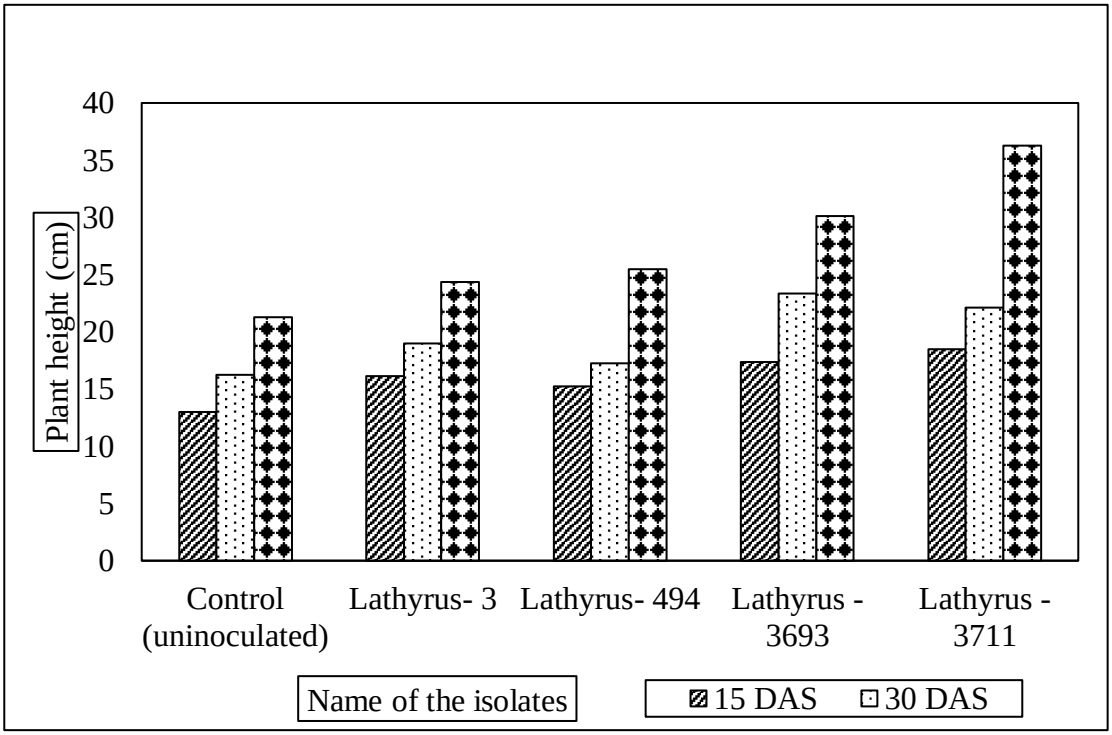


Fig.4.2 Effect of different *Rhizobium* isolates of green gram on plant height under glass house condition at different DAS.

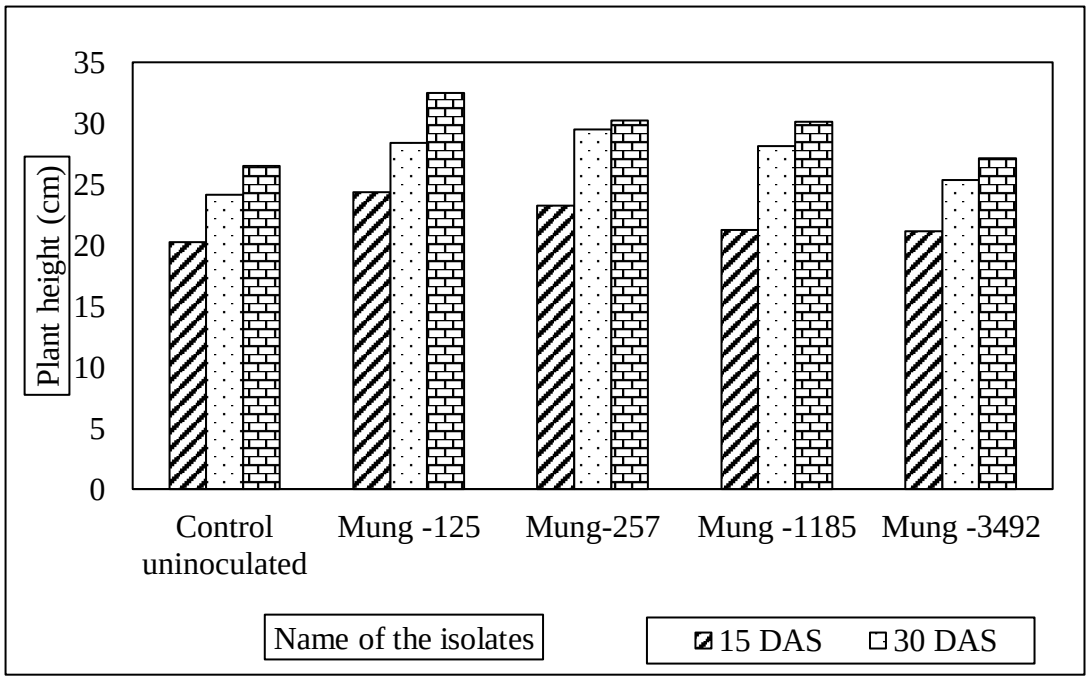


Table 4.16: Effect of native *Rhizobium* isolates on plant height of chickpea at 15 DAS, 30 DAS and 45 DAS under sand culture glass house conditions.

Treatments	Name of the isolates	Plant height (cm) at		
		15 DAS	30 DAS	45 DAS
T1	Control (uninoculated)	28.25	33.37	45.00
T2	Gram- 98	32.37	38.25	57.35
T3	Gram-191	30.25	36.00	52.50
T4	Gram-1052	31.50	37.37	59.25
T5	Gram -1068	30.00	36.50	51.50
	C.D (5%)	N/S	N/S	5.41

Table 4.17: Effect of native *Rhizobium* isolateson plant height of black gram at 15 DAS,30 DAS and 45 DAS under sand culture under glass house conditions.

Treatments	Name of the isolates	Plant height (cm) at		
		15 DAS	30 DAS	45 DAS
T1	Control (uninoculated)	13.12	17.50	25.12
T2	Urad - 577	16.37	19.12	28.25
T3	Urad - 1168	17.50	20.25	27.12
T4	Urad - 3533	18.00	22.12	31.25
T5	Urad - 3536	16.12	19.50	30.50
	C.D (5%)	3.15	N/S	3.25

Fig 4.3 Effect of different *Rhizobium* isolates of chickpea on plant height under glass house condition at different DAS.

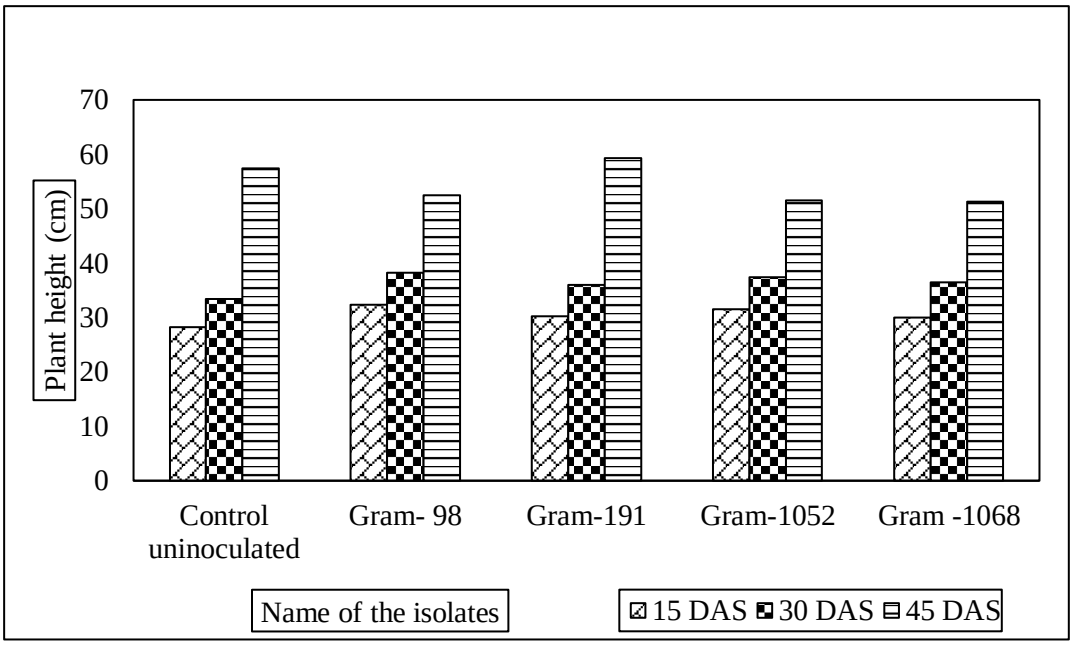


Fig 4.4 Effect of different *Rhizobium* isolates of black gram on plant height under glass house condition at different DAS.

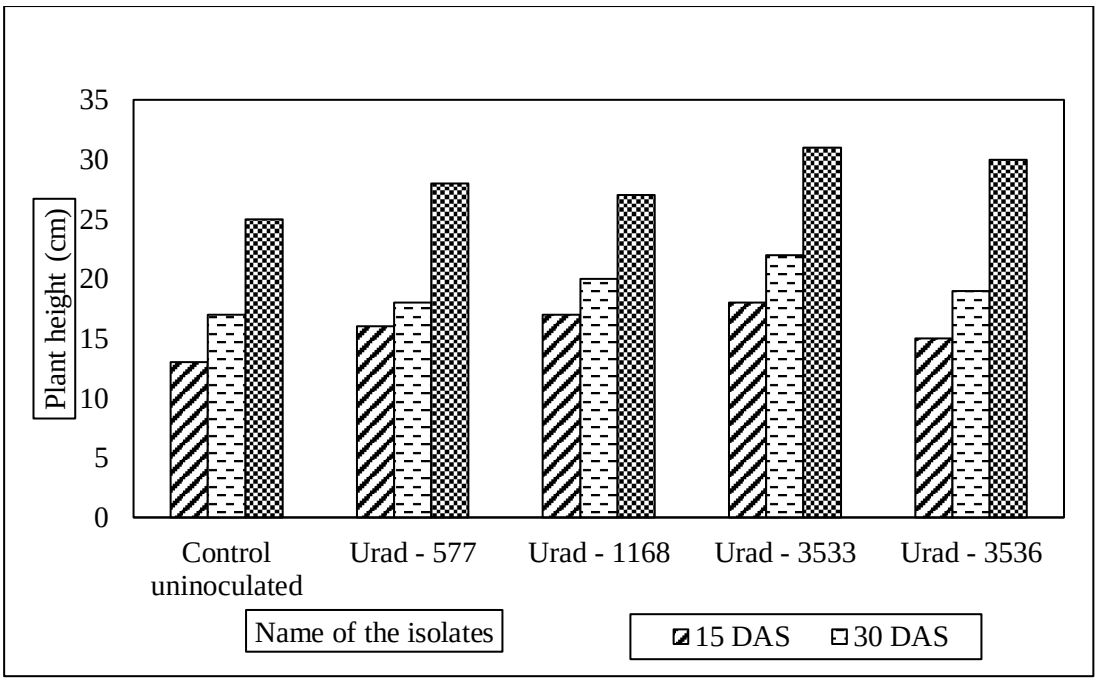


Plate 6. General view of sand culture glass house experiment of legume-*Rhizobium* symbiosis

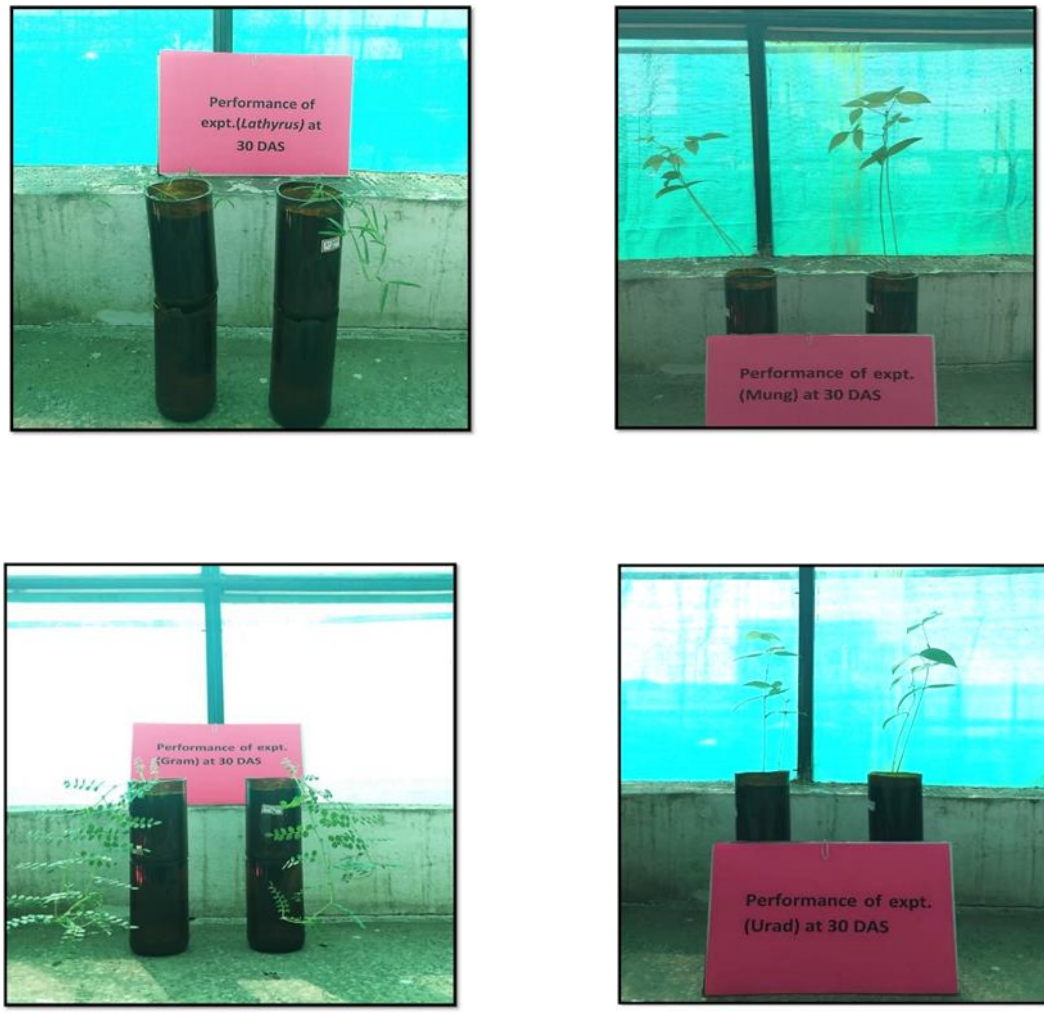


Plate 7. Growth performance of plants, inoculated with different *Rhizobium* isolates

4.4.2. Nodulation study

Results of Table 4.18 and Fig. 4.5 indicate that highest nodulation in *Lathyrus* was observed in plants raised from seeds inoculated with the isolate no. 3693 followed by isolate no. 3711. There was no nodule formation in the control plants. Number of nodules/plant at 45 DAS was observed in between zero to 7.37. Among all tested isolates, the lowest number of nodules/plant was observed 4.00 due to inoculation of isolate no. 3 and isolate no. 494 while the highest number of nodules was associated with plants raised from seeds inoculated with isolate no. 3693.

Similarly, results of Table 4.19 and Fig. 4.6 indicate that highest nodulation in green gram was observed in plants raised from seeds inoculated with the isolate no. 257 followed by isolate no. 125. There was no nodule formation in the control plants. Number of nodules/plant at 45 DAS was observed in between zero to 12. Among all tested isolates, the lowest number of nodules/plant was observed 6.5 due to inoculation of isolate no. 3492 while the highest number of nodules was associated with plants raised from seeds inoculated with isolate no.257.

Results of Table 4.20 and Fig. 4.7 indicate that highest nodulation in chickpea was observed in plants raised from seeds inoculated with the isolate no. 98 followed by isolate no. 1052. There was no nodule formation in the control plants. Number of nodules/plant at 45 DAS was observed in between zero to 13.12. Among all tested isolates, the lowest number of nodules/plant was observed 9.25 due to inoculation of isolate no. 191 while the highest number of nodules was associated with plants raised from seeds inoculated with isolate no.98.

Likewise, in black gram results of Table 4.21 and Fig. 4.8 indicate that highest nodulation in chickpea was observed in plants raised from seeds inoculated with the isolate no. 3533 followed by isolate no. 1168. There was no nodule formation in the control plants. Number of nodules/plant at 45 DAS was observed in between zero to 11.50. Among all tested isolates, the lowest number of nodules/plant was observed 7.00 due to inoculation of isolate no. 3536 while the highest number of nodules was associated with plants raised from seeds inoculated with isolate no.3533.

The results observed are in line with that of Ravikumar, (2012) who mentioned that the nodulation in *Vigna mungo* and *Vigna radiata* increased significantly due to *Rhizobium* inoculation. Gupta et. al. (2005), Javaid *et al.* (2009), Mweetwa *et al.*(2014),Mohammad and Hassan (2015) also revealed that seed inoculation with *Rhizobium* increase the number of nodules per plant compared to uninoculated.

4.4.3 Plant biomass and N-uptake study:

Data of Table 4.18 and Fig. 4.5 clearly indicate that highest fresh weight of shoot in *Lathyrus* was observed in plants raised from seeds inoculated with isolate no. 3693 followed by isolate no. 3711. Fresh weight of shoot recorded at 45 DAS were 1.80, 1.74, 2.00, 1.87 g/plant due to inoculation of seeds with isolate Nos. 3, 494, 3693 and 3711 respectively. Among the inoculated plants. the lowest value of fresh weight of shoot recorded was 1.74 g/plant while the highest value recorded was 2.00 g per plant. Uninoculated control plant gave fresh weight of shoot 1.27 g/plant.

Data presented in Table 4.18 and Fig.4.5 reveal that the highest dry biomass of shoot recorded in *Lathyrus* was 0.27 g/plant due to seeds inoculated with *Rhizobium* isolate no. 3693. Dry weight of shoots recorded at 45 DAS was 0.22, 0.19, 0.27, 0.26 associated with seeds inoculated with isolate Nos. 3, 494, 3693 and 3711 respectively. Among the inoculated plants, the lowest value of dry weight of shoot recorded was 0.19 g/plant while the highest value recorded was 0.27 g per plant. Uninoculated control plants gave dry weight of shoot 0.14 g/plant

Data in Table 4.18 and Fig. 4.5 clearly indicate that plant N- content in *Lathyrus* at 45 DAS significantly increased from 0.79 percent to 1.69 percent. The plant N-content recorded was 1.64, 1.64, 1.69 and 1.66 percent due to seed inoculation with *Rhizobium* Isolate Nos. 3, 494, 3693 and 3711 respectively. The highest value of N-content recorded was 1.69 percent due to seeds inoculated with isolate no. 3693 while lowest value recorded was 0.79 percent in uninoculated control under glass house conditions.

Data presented in Table 4.18 and Fig. 4.5 further revealed that the highest N-uptake observed in *Lathyrus* was 4.563 mg per plant due to seeds inoculated with isolate no. 3693. Values of N-uptake at 45 DAS recorded were 3.608, 3.116, 4.563 and 4.316 mg per plant due to seed inoculation with isolate Nos. 3, 494, 3693 and 3711 respectively. The lowest value of N- uptake recorded was 1.106 mg per plant in uninoculated control under glass house-sand culture condition.

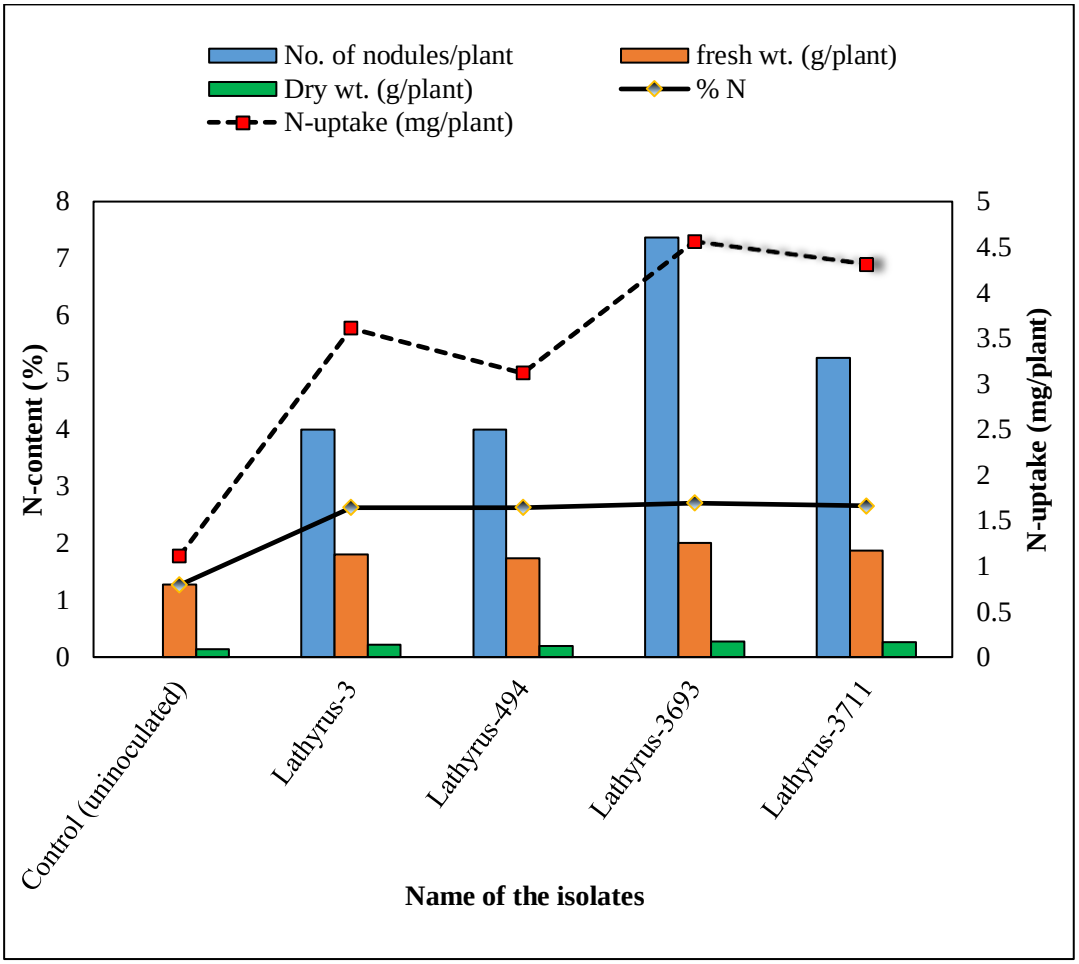
Further, the data presented in Table 4.18 and Fig. 4.5 indicated that the highest extra biologically fixed amount of nitrogen observed among the isolates of *Lathyrus* was 3.457 mg/plant due to inoculation of isolate no. 3693 and lowest was 2.010 mg/plant due to inoculation of isolate no. 494.

Table 4.18: Effect of native *Rhizobium* isolates on nodulation, biomass and nitrogen accumulation by *Lathyrus* at 45 DAS

Treat-ments	Name of the isolates	No. of Nodule /plant	Shoot biomass accumulation		N accumulation		
			Fresh wt (g/plant)	Dry wt (g/plant)	% N	N-uptake (mg/plant)	BFAN* by inoculated plant (mg/plant)
T1	Control (uninoculated)	0.00	1.27	0.14	0.79	1.106	----
T2	*Lathyrus*-3	4.00	1.80	0.22	1.64	3.608	2.502
T3	*Lathyrus*- 494	4.00	1.74	0.19	1.64	3.116	2.010
T4	*Lathyrus*-3693	7.37	2.00	0.27	1.69	4.563	3.457
T5	*Lathyrus*-3711	5.25	1.87	0.26	1.66	4.316	3.210
	C.D (5%)	1.90	0.15	0.06	0.06	0.381	

*** BFAN indicates biologically fixed amount of nitrogen in plant shoot**

Fig.4.5: Effect of native *Rhizobium* isolates on nodulation, biomass and nitrogen accumulation by *Lathyrus* at 45 DAS



Similarly, data of Table 4.19 and Fig. 4.6 clearly indicate that highest fresh weight of shoot in green gram was observed in plants raised from seeds inoculated with isolate no. 257 followed by isolate no. 125. Fresh weight of shoot recorded at 45 DAS were 1.92,1.97,1.88,1.63 g/plant due to inoculation of seeds with isolate Nos. 125, 257, 1185 and 3492 respectively. The lowest value of fresh weight of shoot recorded was 1.39 g per plant in uninoculated control while the highest value recorded was 1.97 g per plant due to seed inoculation with isolate no. 257.

Data presented in Table 4.19 and Fig.4.6 reveal that the highest dry biomass of shoot recorded in green gram was 0.40 g/plant due to seeds inoculated with *Rhizobium* isolate no. 257. Dry weight of shoots recorded at 45 DAS was 0.39, 0.40, 0.37, 0.33 associated with seeds inoculated with isolate Nos.125, 257, 1185 and 3492 respectively. Among the isolates, the lowest value of dry weight of shoot recorded was 0.33 g per plant while the highest value recorded was 0.40 g per plant. Uninoculated control gave dry weight of shoot 0.21 g/plant.

Data in Table 4.19 and Fig. 4.6 clearly indicate that plant N- content in green gram at 45 DAS significantly increased from 0.85 percent to 1.82 percent. The plant N- content recorded was 1.77, 1.82, 1.71 and 1.69 percent due to seed inoculation with *Rhizobium* Isolate Nos.125, 257, 1185 and 3492 respectively. The highest value of N- content recorded was 1.82 percent due to seeds inoculated with isolate no. 257 while lowest value recorded was 0.85 percent in uninoculated control under glass house conditions.

Data presented in Table 4.19 and Fig. 4.6 further revealed that the highest N-uptake observed in green gram was 7.28 mg per plant due to seeds inoculated with isolate no. 257. Values of N-uptake at 45 DAS recorded were 6.903, 7.28, 6.327 and 5.577 mg per plant due to seed inoculation with isolate Nos.125, 257, 1185 and 3492 respectively. The lowest value of N- uptake recorded was 1.785 mg per plant in uninoculated control under glass house-sand culture condition.

Further, the data presented in Table 4.19 and Fig. 4.6 indicated that the highest extra biologically fixed amount of nitrogen observed among the isolates of

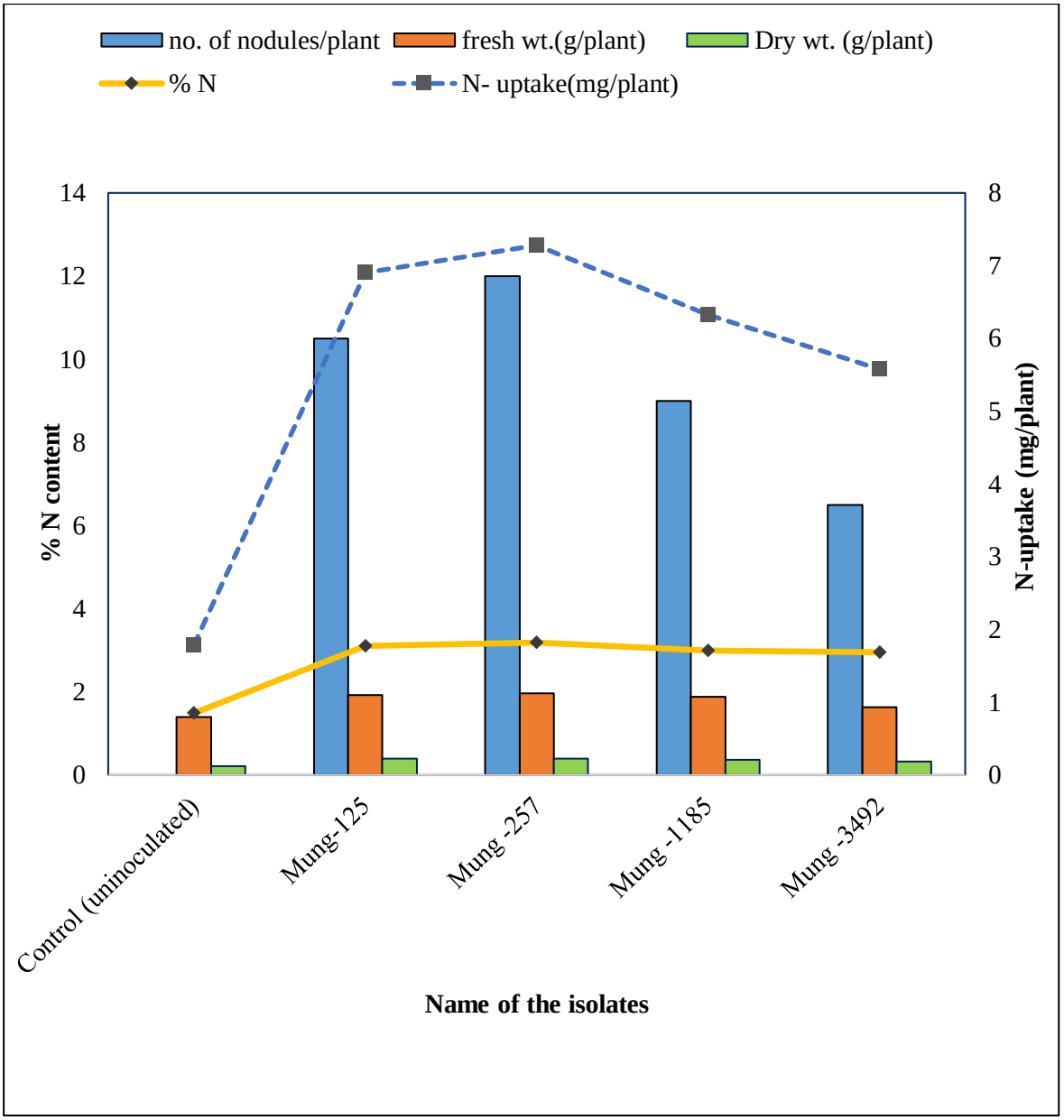
green gram was 5.495 mg/plant due to inoculation of isolate no. 257 and lowest was 3.792 mg/plant due to inoculation of isolate no. 3492.

Table 4.19: Effect of native *Rhizobium* isolates on nodulation, biomass and nitrogen accumulation by green gram at 45 DAS

Treat-ments	Name of the isolates	No. of Nodule/ plant	Shoot biomass accumulation		N accumulation		
			Fresh wt (g/plant)	Dry wt (g/plant)	% N	N- up take (mg/plant)	BFAN* by inoculated plant (mg/plant)
T1	Control (uninoculated)	0.00	1.39	0.21	0.85	1.785	-----
T2	Mung-125	10.5	1.92	0.39	1.77	6.903	5.118
T3	Mung -257	12.0	1.97	0.40	1.82	7.28	5.495
T4	Mung -1185	9.0	1.88	0.37	1.71	6.327	4.542
T5	Mung -3492	6.5	1.63	0.33	1.69	5.577	3.792
	C.D (5%)	2.83	0.12	N/S	0.08	0.387	

*** BFAN indicates biologically fixed amount of nitrogen in plant shoot**

Fig.4.6: Effect of native *Rhizobium* isolates on nodulation, biomass and nitrogen accumulation by green gram at 45 DAS



Similarly, data of Table 4.20 and Fig. 4.7 clearly indicate that highest fresh weight of shoot in chickpea was observed in plants raised from seeds inoculated with isolate no. 98 followed by isolate no. 1052. Fresh weight of shoot recorded at 45 DAS were 1.92,1.62,1.84,1.73 g/plant due to inoculation of seeds with isolate Nos. 98, 191, 1052 and 1068 respectively. The lowest value of fresh weight of shoot recorded was 0.83 g per plant in uninoculated control while the highest value recorded was 1.92 g per plant due to seed inoculation with isolate no. 98.

Data presented in Table 4.20 and Fig.4.7 reveal that the highest dry biomass of shoot recorded in chickpea was 0.46 g/plant due to seeds inoculated with *Rhizobium* isolate no. 98. Dry weight of shoots recorded at 45 DAS was 0.46, 0.39, 0.45, 0.43 associated with seeds inoculated with isolate Nos.98, 191, 1052 and 1068 respectively. The lowest value of dry weight of shoot recorded was 0.27 g per plant in uninoculated control while the highest value recorded was 0.46 g per plant.

Data in Table 4.20 and Fig. 4.7 clearly indicate that plant N- content of chickpeaat 45 DAS significantly increased from 0.93 percent to 1.82 percent. The plant N- content recorded was 1.62, 1.58, 1.59 and 1.58 percent due to seed inoculation with *Rhizobium* Isolate Nos.98, 191, 1052 and 1068 respectively. The highest value of N- content recorded was 1.62 percent due to seeds inoculated with isolate no. 98 while lowest value recorded was 0.93 in uninoculated control under glass house conditions.

Data presented in Table 4.20 and Fig. 4.7 further revealed that the highest N-uptake observed in chickpea was 7.452 mg per plant due to seeds inoculated with isolate no. 98. Values of N-uptake at 45 DAS recorded were 7.452, 6.162, 7.155 and 6.794 mg per plant due to seed inoculation with isolate Nos.98, 191, 1052 and 1068 respectively. The lowest value of N- uptake recorded was 2.511 mg per plant in uninoculated control under glass house-sand culture condition.

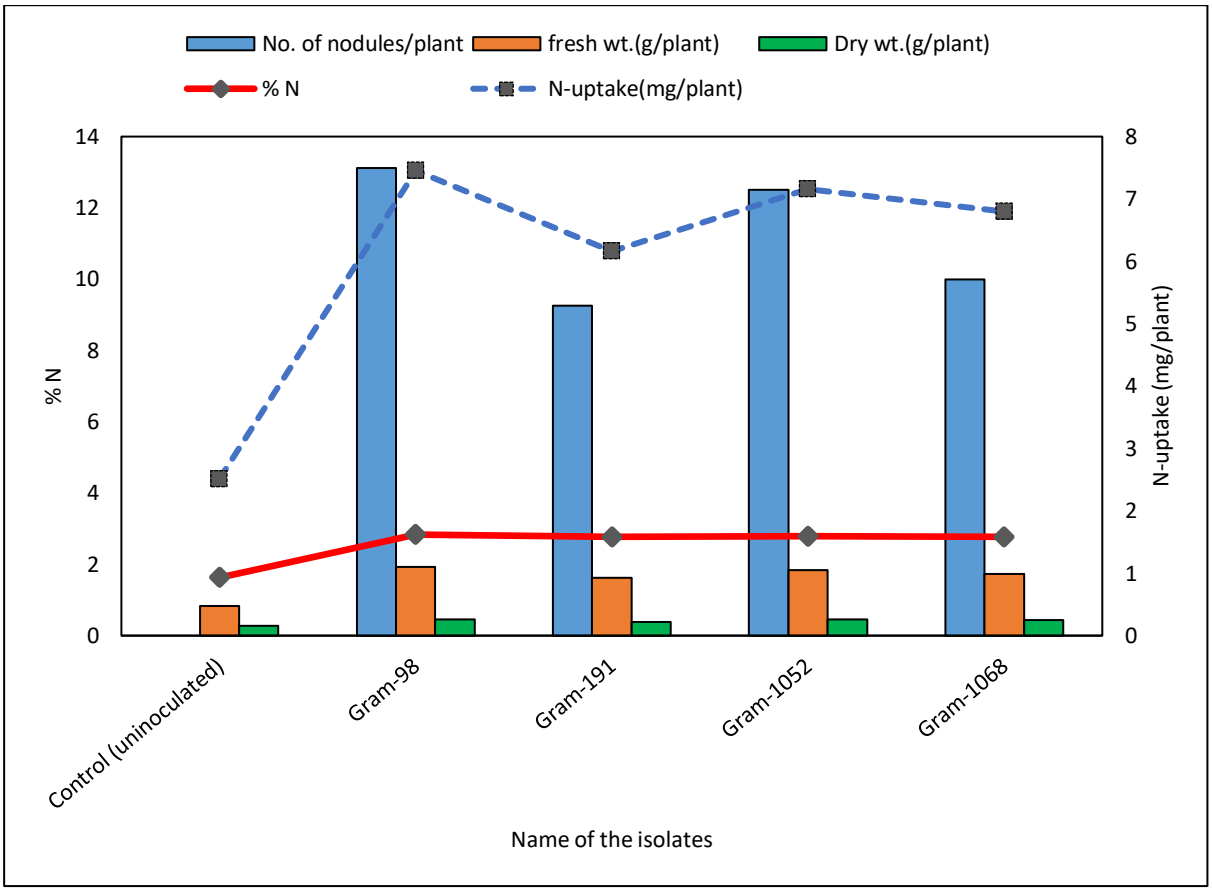
Further, the data presented in Table 4.20 and Fig. 4.7 indicated that the highest extra biologically fixed amount of nitrogen observed among the isolates of chickpea was 4.941 mg/plant due to inoculation of isolate no. 98 and lowest was 3.651 mg/plant due to inoculation of isolate no. 191.

Table 4.20: Effect of native *Rhizobium* isolates on nodulation, biomass and nitrogen accumulation by chickpea at 45 DAS

Treat-ments	Name of the isolates	No. of Nodule /plant	Shoot biomass accumulation		N accumulation		
			Fresh wt (g/plant)	Dry wt (g/plant)	% N	N-uptake (mg/plant)	BFAN* by inoculated plant (mg/plant)
T1	Control (uninoculated)	0.00	0.83	0.27	0.93	2.511	-----
T2	Gram-98	13.12	1.92	0.46	1.62	7.452	4.941
T3	Gram-191	9.25	1.62	0.39	1.58	6.162	3.651
T4	Gram-1052	12.50	1.84	0.45	1.59	7.155	4.644
T5	Gram-1068	10.00	1.73	0.43	1.58	6.794	4.283
	C.D (5%)	1.08	0.06	0.05	0.05	0.111	

*** BFAN indicates biologically fixed amount of nitrogen in plant shoot**

Fig. 4.7: Effect of native *Rhizobium* isolates on nodulation, biomass and nitrogen accumulation by chickpea at 45 DAS



Similarly, data of Table 4.21 and Fig. 4.8 clearly indicate that highest fresh weight of shoot in black gram was observed in plants raised from seeds inoculated with isolate no. 98 followed by isolate no. 3533. Fresh weight of shoot recorded at 45 DAS were 1.44, 1.41, 1.52, 1.34 g/plant due to inoculation of seeds with isolate Nos. 577, 1168, 3533 and 3536 respectively. The lowest value of fresh weight of shoot recorded was 1.11 g per plant in uninoculated control while the highest value recorded was 1.52 g per plant due to seed inoculation with isolate no. 3533.

Data presented in Table 4.21 and Fig.4.8 reveal that the highest dry biomass of shoot recorded in black gram was 0.25 g/plant due to seeds inoculated with *Rhizobium* isolate no. 3533. Dry weight of shoots recorded at 45 DAS was 0.22, 0.24, 0.25, 0.19 associated with seeds inoculated with isolate Nos.577, 1168, 3533 and 3536 respectively. The lowest value of dry weight of shoot recorded was 0.12 g per plant in uninoculated control while the highest value recorded was 0.25 g per plant.

Data in Table 4.21 and Fig. 4.8 clearly indicate that plant N- content in black gram at 45 DAS significantly increased from 0.78 percent to 1.51 percent. The plant N- content recorded was 1.47, 1.50, 1.51 and 1.45 percent due to seed inoculation with *Rhizobium* isolate Nos.577, 1168, 3533 and 3536 respectively. The highest value of N- content recorded was 1.51 percent due to seeds inoculated with isolate no. 3533 while lowest value recorded was 0.78 in uninoculated control under glass house conditions.

Data presented in Table 4.21 and Fig. 4.8 further revealed that the highest N-uptake observed in black gram was 3.775 mg per plant due to seeds inoculated with isolate no. 3533. Values of N-uptake at 45 DAS recorded were 3.234, 3.60, 3.775 and 2.755 mg per plant due to seed inoculation with isolate Nos.577, 1168, 3533 and 3536 respectively. The lowest value of N- uptake recorded was 0.93 mg per plant in uninoculated control under glass house-sand culture condition.

Further, the data presented in Table 4.21 and Fig. 4.8 indicated that the highest extra biologically fixed amount of nitrogen observed among the isolates of black

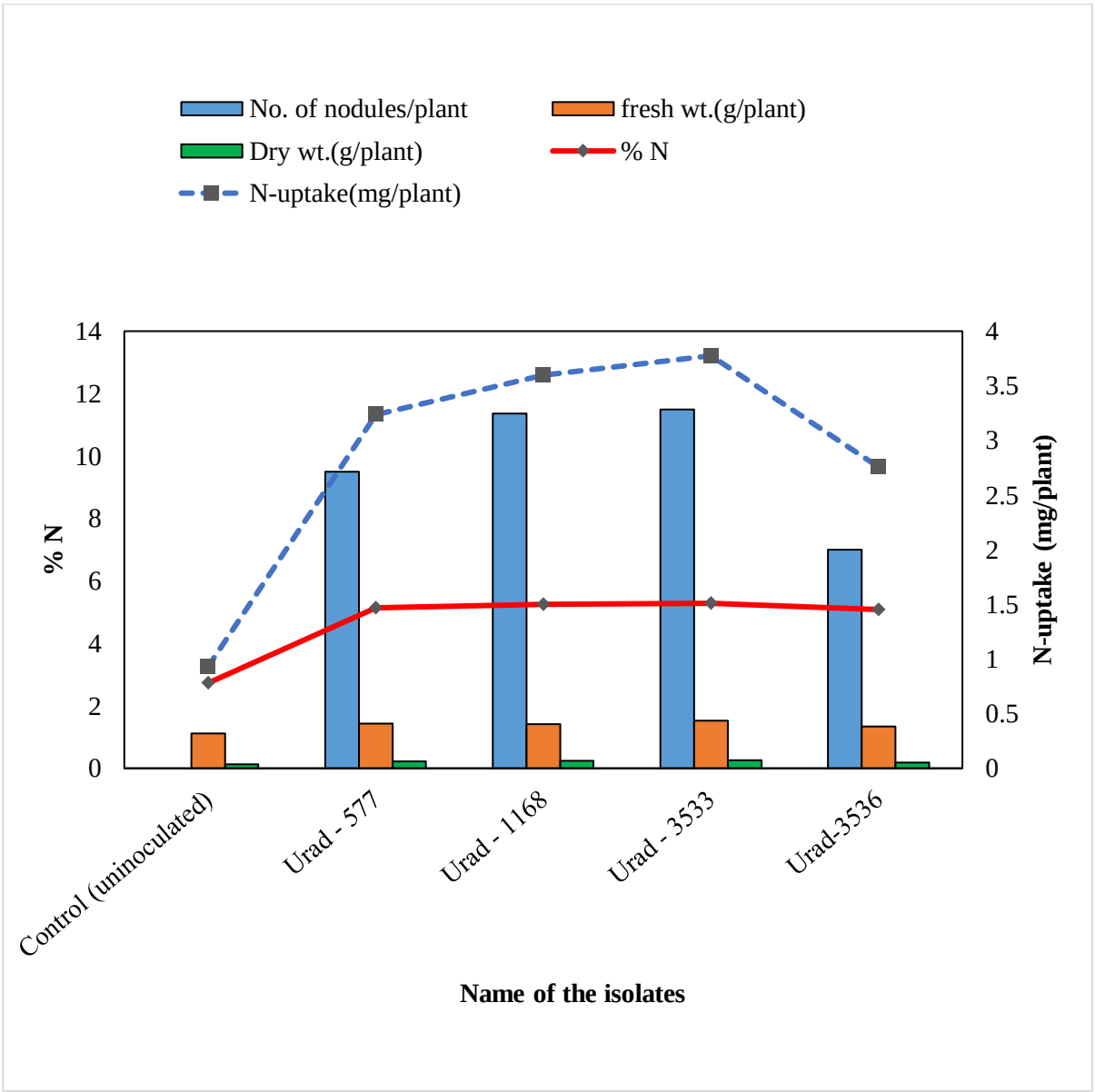
gram was 2.845 mg/plant due to inoculation of isolate no. 3533 and lowest was 1.825 mg/plant due to inoculation of isolate no. 3536.

Table 4.21: Effect of native *Rhizobium* isolates on nodulation, biomass and nitrogen accumulation by black gram at 45 DAS

Treat-ments	Name of the isolates	No. of Nodule /plant	Shoot biomass accumulation		N accumulation		
			Fresh wt.(g/ plant)	Dry wt.(g/ plant)	% N	N- up take (mg/plant)	BFAN* by inoculated plant (mg/plant)
T1	Control (uninoculated)	0.00	1.11	0.12	0.78	0.93	------
T2	Urad - 577	9.50	1.44	0.22	1.47	3.234	2.304
T3	Urad - 1168	11.37	1.41	0.24	1.50	3.600	2.670
T4	Urad - 3533	11.50	1.52	0.25	1.51	3.775	2.845
T5	Urad-3536	7.00	1.34	0.19	1.45	2.755	1.825
	C.D (5%)	1.86	0.06	0.02	0.04	0.058	

*** BFAN indicates biologically fixed amount of nitrogen in plant shoot**

Fig.4.8: Effect of native *Rhizobium* isolates on nodulation, biomass and nitrogen accumulation by black gram at 45 DAS



Results of present investigation are in close agreement with Gupta *et al.* (1995), Javaid (2009), Mweeta *et al.* (2014), Alam *et al.*(2015) , khaitov *et al.*(2015) ,Eshetu *et al.* (2018) .and Singh *et al.*(2018). Further Benidire *et al.*(2017), Massawe *et al.* (2017) also mentioned that there was a significant increase in N- uptake in legume plants due to *Rhizobium* inoculation.

Plate 7. Estimation of biologically fixed amount of N

4.5 Rhizobial population Dynamics

Data presented in the Table 4.22 and Fig. 4.9 indicate that the rhizobial population density increased significantly in *Lathyrus* due to inoculation of isolate no. 3693 followed by isolate no. 3711 at both the stages of crop growth (30 DAS and 45 DAS). At 30 DAS rhizobial population density varied significantly from 1.45 x 10^4 to 1.53 x10^4 viable cells per gram of rhizosphere sand because of seed bacterization with different native isolates. Similarly, at 45 DAS, it varied from 2.26 x 10^4 to 2.68 x 10^4 per gram of rhizosphere sand due to rhizobial inoculation. The highest rhizobial population density in *Lathyrus* was estimated to be 1.53 x10^4 at 30 DAS and 2.68 x 10^4 at 45 DAS. There was no rhizobial population count in uninoculated control. The performance of native isolate no. 3693 of *Lathyrus* was considered to be superior among all tested native rhizobial isolates under glass house study.

Similarly, data presented in Table 4.23 and Fig. 4.10 indicate that the rhizobial population density increased significantly in green gram due to inoculation of isolate no. 257 at both the stages of crop growth (30 DAS and 45 DAS). At 30 DAS rhizobial population density varied significantly from 3.63 x 10^4 to 3.97 x10^4 viable cells per gram of rhizosphere sand because of seed bacterization with different native isolates. Similarly, at 45 DAS, it varied from 5.48 x 10^4 to 5.88 x 10^4 per gram of rhizosphere sand due to rhizobial inoculation. The highest rhizobial population density in Green gram was estimated to be 3.97 x10^4 at 30 DAS and 5.88 x 10^4 at 45 DAS. There was no rhizobial population count in uninoculated control.The performance of native isolate no. 257 of green gram was considered to be superior among all tested native rhizobial isolates under glass house study.

Table 4.22 Effect of *Lathyrus* seed inoculation by different native *Rhizobium* isolates on their population dynamics during 30 DAS and 45 DAS under glass house conditions.

Treatments	Name of the isolates	*Rhizobium* population (X 10^4) per gram of rhizosphere sand	
		30 DAS	45DAS
T1	Control (uninoculated)	0	0
T2	*Lathyrus*-3	1.47	2.48
T3	*Lathyrus*-494	1.45	2.26
T4	*Lathyrus*-3693	1.53	2.68
T5	*Latyhrus* -3711	1.49	2.61
C.D (5%)		0.42	0.57

Table 4.23: Effect of green gram seed inoculation by different native *Rhizobium* isolates on their population dynamics during 30 DAS and 45 DAS under glass house conditions.

Treatments	Name of the isolates	*Rhizobium* population (X 10^4) per gram of rhizosphere sand	
		30 DAS	45DAS
T1	Control (uninoculated)	0	0
T2	Mung-125	3.76	5.64
T3	Mung-257	3.97	5.88
T4	Mung -1185	3.71	5.52
T5	Mung -3492	3.63	5.48
C.D (5%)		0.40	0.50

Fig.4.9: Effect of *Lathyrus* seed inoculation by different native *Rhizobium* isolates on their population dynamics during 30 DAS and 45 DAS under glass house condition.

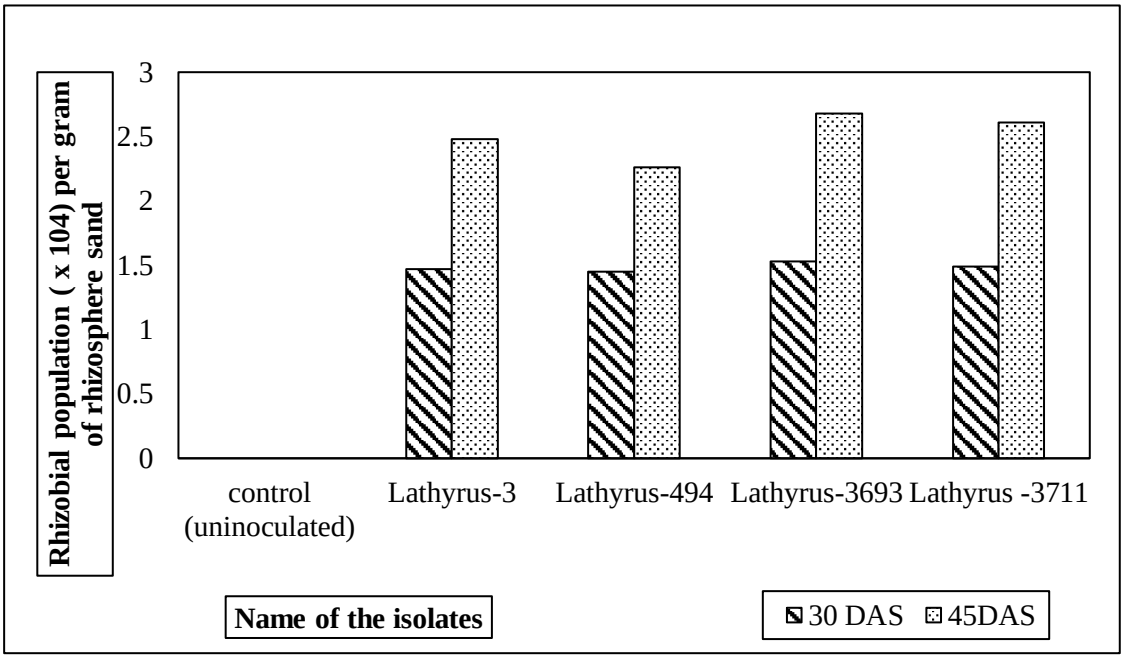
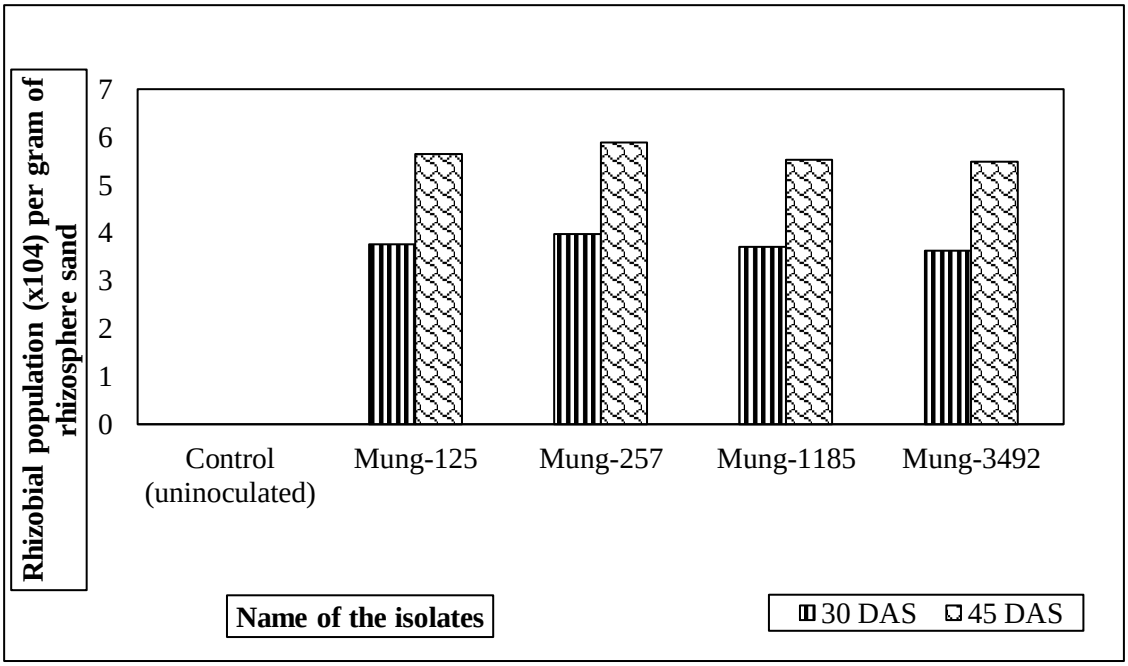


Fig. 4.10: Effect of green gram seed inoculation by different native *Rhizobium* isolates on their population dynamics during 30 DAS and 45 DAS under glass house conditions.



Data presented in Table 4.24 and Fig. 4.11 indicate that the rhizobial population density increased significantly in Chickpea due to inoculation of isolate no. 98 at both the stages of crop growth (30 DAS and 45 DAS). At 30 DAS, rhizobial population density varied significantly from 1.74 x 10^4 to 1.98 x10^4 viable cells per gram of rhizosphere sand because of seed bacterization with different native isolates.

Similarly, at 45 DAS, it varied from 2.39 x 10^4 to 2.98 x 10^4 per gram of rhizosphere sand due to rhizobial inoculation. The highest rhizobial population density in chickpea was estimated to be 1.98 x10^4 at 30 DAS and 2.98 x 10^4 at 45 DAS. There was no rhizobial population count in uninoculated control. The performance of native isolate no. 98 of chickpea was considered to be superior among all tested native rhizobial isolates under glass house study.

Likewise,data presented in Table 4.25 and Fig. 4.12 indicate that the rhizobial population density increased significantly in black gram due to inoculation of isolate no. 3533 at both the stages of crop growth (30 DAS and 45 DAS). At 30 DAS,rhizobial population density varied significantly from 1.53 x 10^4 to 2.22 x10^4 viable cells per gram of rhizosphere sand because of seed bacterization with different native isolates. Similarly, at 45 DAS, it varied from 2.20 x 10^4 to 3.13 x 10^4 per gram of rhizosphere sand due to rhizobial inoculation. The highest rhizobial population density in black gram was estimated to be 2.22 x10^4 at 30 DAS and 3.13x 10^4 at 45 DAS. There was no rhizobial population count in uninoculated control.The performance of native isolate no. 3533 of black gram was considered to be superior among all tested native rhizobial isolates under glass house study.

Table 4.24: Effect of chickpea seed inoculation by different native *Rhizobium* isolates on their population dynamics during 30 DAS and 45 DAS under glass house conditions.

Treatments	Name of the isolates	*Rhizobium* population (X 10^4) per gram of rhizosphere sand	
		30 DAS	45 DAS
T1	Control (uninoculated)	0	0
T2	Gram-98	1.98	2.98
T3	Gram-191	1.74	2.39
T4	Gram- 1052	1.92	2.70
T5	Gram-1068	1.89	2.64
C.D (5%)		0.47	0.34

Table 4.25: Effect of black gram seed inoculation by different native *Rhizobium* isolates on their population dynamics during 30 DAS and 45 DAS under glass house conditions.

Treatments	Name of the isolates	*Rhizobium* population (X 10^4) per gram of rhizosphere sand	
		30 DAS	45DAS
T1	Control (uninoculated)	0	0
T2	Urad-577	1.66	2.34
T3	Urad- 1168	1.74	2.59
T4	Urad-3533	2.22	3.13
T5	Urad-3536	1.53	2.20
C.D (5%)		0.32	0.54

Fig. 4.11: Effect of chickpea seed inoculation by different native *Rhizobium* isolates on their population dynamics during 30 DAS and 45 DAS under glass house conditions.

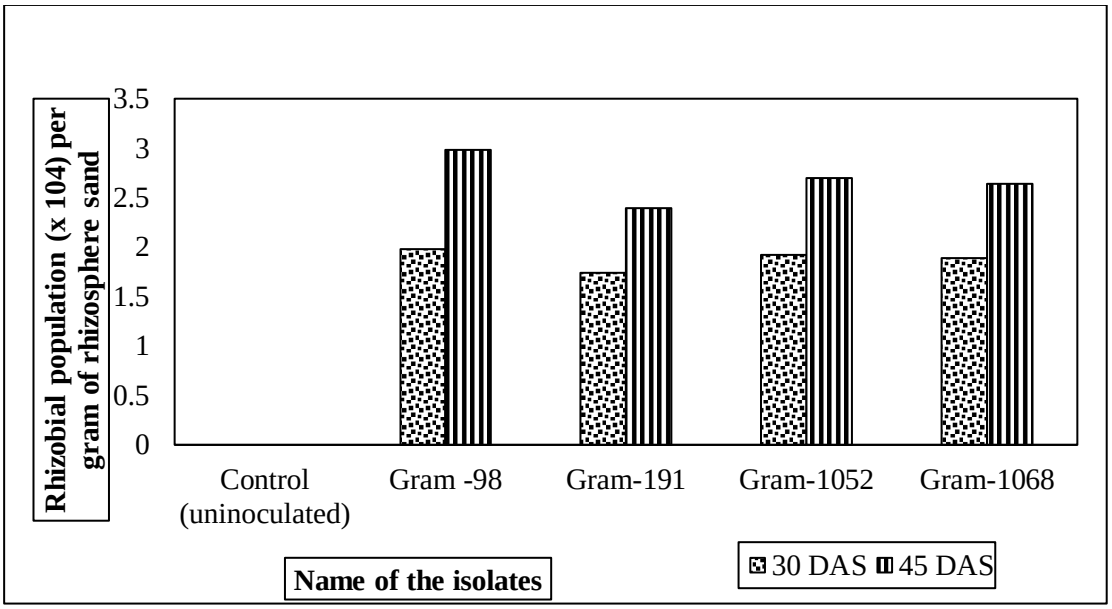
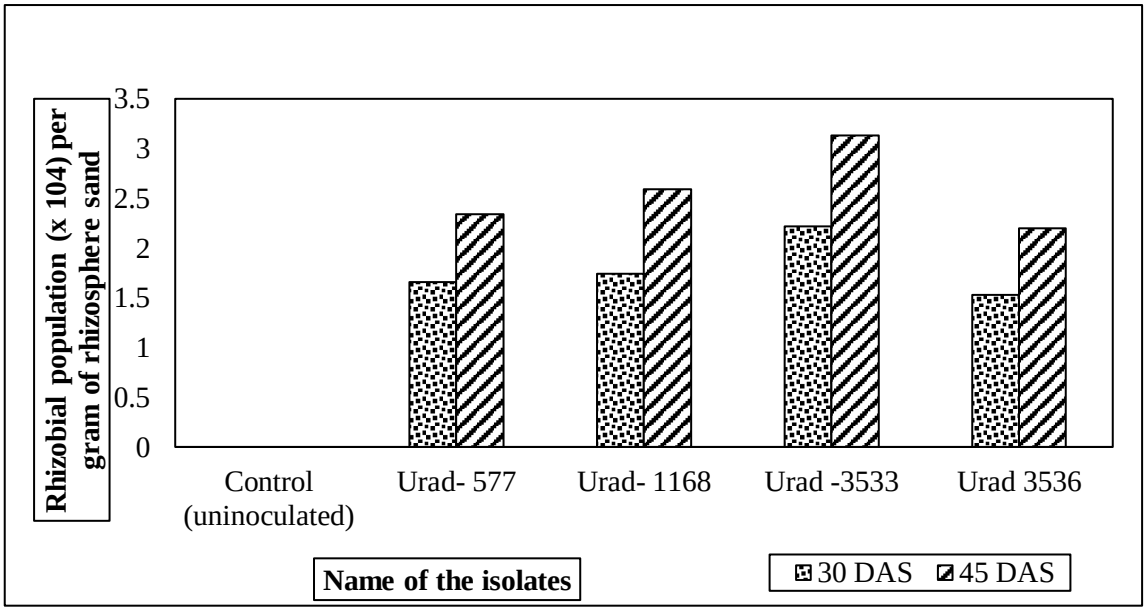


Fig. 4.12:Effect of black gram seed inoculation by different native *Rhizobium* isolates on their population dynamics during 30 DAS and 45 DAS under glass house conditions.



Similar, results were observed by Gupta *et al.* (1992), Prasad (1997) and Bhagat *et al.*(2014). They mentioned that the rhizobial population increased significantly upto flowering stage of crop growth due to higher degree of rhizosphere effect. Hynes *et al.*(2001) reported that rhizobial population increases considerably in crop growing season in inoculated plants while in uninoculated control there is no increase in population .

Keeping in view of the findings related to BNF parameters like accumulation of biomass and nitrogen, performance of native *Rhizobium* isolate no. 3693 of *Lathyrus*, isolate no. 257 of green gram, isolate no. 98 of chickpea and isolate no. 3533 of black gram were found to superior among all rhizobial isolates taken for study. These isolates accumulated 3.457, 5.495, 4.941 and 2.845 mg/plant extra amount of atmospheric nitrogen over uninoculated control plant, respectively. Results of the *Rhizobium* population dynamics studies also supported the effectiveness of the native rhizobial isolate no. 3693 of *Lathyrus*, isolate no. 257 of green gram, isolate no. 98 of chickpea and isolate no. 3533 of black gram. Hence, it was concluded that these native isolates may be the most effective nitrogen fixer for *Lathyrus*, green gram, chickpea and black gram cultivation under agro-climatic conditions of Chhattisgarh.

CHAPTER - V

SUMMARY AND CONCLUSIONS

With the increasing demands from the growing population, there is a need to develop ecologically sustainable agriculture system in order to maintain the agricultural productivity. One of important factors for sustainable agricultural productivity is the efficient management of nitrogen present in the environment. The symbiosis between legumes and root nodule bacteria can fulfill upto 80% of nitrogen requirement of legumes. The direct input of atmospheric nitrogen to the agricultural system is through biological nitrogen fixation. The control of some crop diseases as a consequence of involving legumes in crop rotations can also be accomplished through BNF.

Present study deals with "characterization and evaluation of native *Rhizobium* of *Lathyrus*, Green gram, Chickpea and Black gram" to determine the effective isolates of *Rhizobium*. Biochemical characterization and glass house studies of different legumes were carried out without mineral source of nitrogen under sterilized sand condition in Dept. of Agril. Microbiology, IGKV, Raipur.

The following are the salient findings of the study:

- Morphological characterization of the selected isolates of *Lathyrus*, green gram, chickpea and black gram based on Gram staining revealed that all the isolates of *Rhizobium* were gram negative, rod shaped.
- The biochemical study revealed that all the selected isolates of *Lathyrus*, green gram, chickpea and black gram gave negative results for indole production, Voges-proskauer test, catalase test. Most of the isolates were negative for gelatinase activity namely *Rhizobium* isolate no. 3711 of *Lathyrus* and *Rhizobium* isolate no. 1185 of green gram. Upon citrate utilization test and methyl-red test all the isolate gave positive results. As for urease activity, positive results were observed in all the selected isolate of the crop under study except isolate no. 494 of *Lathyrus* and isolate no. 191 of chickpea. Further most of the selected rhizobial isolates were

negative for starch hydrolysis except rhizobial isolate no. 1185 of green gram, isolate no. 1068 of chickpea and isolate no. 3533 of black gram. Upon triple sugar iron agar test, none of isolates indicated carbohydrate fermentation except rhizobial isolate no. 3711 of *Lathyrus* which revealed gliucose fermentation.

- Antibiotic sensitivity test revealed that all the isolates of the crop under study were sensitive to streptomycin (10µg/disc) and tetracycline (30µg/disc). While only few were sensitive to ampicillin and penicillin.
- Nodulation under the glass house conditions increased significantly in the crop under study raised from seeds inoculated with tested *Rhizobium* isolates. There was no nodule formation in uninoculated plants as they were raised under completlety sterilized conditions of sand culture.
- N-uptake by plants grown in glass house condition increased from 3.608, 3.116, 4.563 and 4.316 mg per plant due to seed inoculation with isolate Nos. 3, 494, 3693 and 3711 respectively in *Lathyrus*. Highest N-uptake was reported in isolate no. 3693 while in unioculated it was 1.106 mg per plant. The values of N-uptake in green gram were 6.903, 7.28, 6.327 and 5.577 mg per plant due to seed inoculation with isolate Nos. 125, 257, 1185 and 3492 respectively. Highest value was observed in isolate no. 257 while in uninoculated it had a lowest value of 1.785 mg per plant. Similarly, in chickpea the N-uptake values recorded were 7.452, 6.162, 7.155 and 6.794 mg per plant due to seed inoculation with isolate Nos. 98, 191, 1052 and 1068 respectively. The highest value recorded was 7.452 mg per plant in plants inoculated with isolate no. 98 while lowest was 2.511 mg per plant in uninoculated. Likewise, in black gram, the N-uptake values recorded were 3.234, 3.60, 3.775 and 2.755 mg per plant due to seed inoculation with isolate Nos. 577, 1168, 3533 and 3536 respectively. The lowest value of N-uptake recorded was 0.93 mg per plant in uninoculated control.
- In glass house conditions, *Rhizobium* population density increased significantly from 30 DAS to 45 DAS. Highest rhizobial population density recorded in *Lathyrus* was due to inoculation of rhizobial isolate no. 3693

and it showed maximum increase in population count from 1.53 $x10^4$ to 2.68 x 10^4 viable *Rhizobium* cells per gram of rhizosphere sand. In green gram, the highest rhizobial population density recorded was due to inoculation of isolate no. 257 and the increase in population count recorded was 3.97 $x10^4$ to 5.88 x 10^4 *Rhizobium* cells per gram of rhizosphere sand. Similarly, the highest rhizobial population density recorded in chickpea was due to inoculation of isolate no. 98 and the increase in population count reported was 1.98 $x10^4$ to 2.98 x 10^4 *Rhizobium* cells per gram of rhizosphere sand. The highest rhizobial population density was recorded in black gram was due to inoculation of isolate no.3533 and the increase in population count recorded was 2.22 $x10^4$ to 3.13 x 10^4 *Rhizobium* cells per gram of rhizosphere sand.

- Keeping in view of the findings related to BNF parameters like accumulation of biomass and nitrogen, performance of native *Rhizobium* isolate no. 3693 of *Lathyrus*, isolate no. 257 of green gram, isolate no. 98 of chickpea and isolate no. 3533 of black gram were found to superior among all rhizobial isolates taken for study. These isolates accumulated 3.457, 5.495, 4.941 and 2.845 mg/plant extra amount of atmospheric nitrogen over uninoculated control plant, respectively. Results of the *Rhizobium* population dynamics studies also supported the effectiveness of the native rhizobial isolate no. 3693 of *Lathyrus*, isolate no. 257 of green gram, isolate no. 98 of chickpea and isolate no. 3533 of black gram. Hence, it was concluded that these native isolates may be the most effective nitrogen fixer for *Lathyrus*, green gram, chickpea and black gram cultivation under agro-climatic conditions of Chhattisgarh.

SUGGESTIONS FOR FUTURE RESEARCH WORK

Rhizobium have been said to contribute up to almost 80% of overall nitrogen demand of leguminous plants but much work has not been done yet to search and identify crop specific effective *Rhizobium* isolates native to particular region. In the light of findings of present study, future line of work is suggested as follows:

- The promising isolates of *Rhizobium* need be atleast examined for three years in farmer's field to ensure long term growth performance of *Lathyrus*, green gram, chickpea and black gram in response to rhizobial inoculation.
- Efforts needs be made to increase availability of location specific native effective *Rhizobium* cultures at right time among farmers.
- Keeping in view of the increase in input cost of nitrogenous chemical fertilizers for optimal crop production, massive efforts must be made to disseminate *Rhizobium* culture among the farmers.
- Apart from atmospheric nitrogen fixing ability of *Rhizobium*, efforts should be made to observe their effect on plant population of different leguminous crops under field conditions.
- Molecular sequencing and identification of efficient *Rhizobium* strain may be needed to increase effect of N fixation and boost the quality and growth of crop.
- There is an urgent need of setting up mass production unit for location specific *Rhizobium* so that effective *Rhizobium* culture can be made available timely to the farmers.

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APPENDIX A

Chemical Composition of Media

1.Composition of Nutrient Broth

Peptone - 5 gm
Beef Extract - 3 gm
Nacl – 5 gm
Distilled water – 1000 ml

2. Composition of MRVP broth

Peptone - 7 gm
Dextrose/Glucose - 5 gm
Potassium phosphate – 5 gm
Distilled water – 1000 ml
For methyl red test, add 0.2% methyl red solution.

3. Composition of Simmon's citrate Agar

Ammonium dihydrogen phosphate - 1 gm
Dipotassium phosphate - 1 gm
Sodium chloride -5 gm
Sodium citrate - 2 gm
Magnesium sulphate - 0.2 gm
Agar – 15 gm
Bromothymol blue - 0.8 gm
Distilled water – 1000 ml

4. Composition of Trypticase soy agar

Trypticase – 50 gm
Phytone – 5 gm
Sodium chloride – 5 gm
Agar – 15 gm
Distilled water – 1000 m

5. Composition of urease test broth

Yeast extract- 0.1gm
Urea – 20 gm
Monopotassium phosphate- 0.091 gm
Disodium phosphate - 0.095gm
Phenol red - 0.01 gm
Agar- 15 gm
Distilled water – 1000 ml

6. Composition of gelatin agar media.

Bactopeptone – 5 gm
Beef extract – 3 gm
Sodium chloride – 5 gm
Gelatin – 4 gm
Agar – 15 gm
Distilled water – 1000 ml

7. Composition of starch agar media

Soluble starch – 10 gm
Beef extract – 3 gm
Peptone – 5 gm
Agar – 15 gm
Distilled water – 1000 ml

8. Composition of TSI agar media

Peptone – 20 gm
Nacl - 5 gm
Lactose – 10 gm
Sucrose – 10 gm
Dextrose – 10 gm
Ferrous ammonium sulphate – 0.2 gm
Sodium thiosulphate – 0.2 gm
Phenol red – 0.0025 gm
Agar – 15 gm
Distilled water – 1000

RESUME

Name	Topi Kamgo
Date of birth	10-09-1994
Present Address	Kadambari girls hostel, krishak nagar, IGKV, Raipur.
Phones	7477057231
E. mail	kamgotopi@gmail.com
Permanent address	Village- kamki,PO/PS Kamba,Dist. West siang, Arunachal Pradesh-791001

Academic Qualification

Degree	Year	University
B.Sc Agriculture	2017	CAU,IMPHAL
M.Sc.(Ag.) in Agricultural Microbiology	2019	IGKV,RAIPUR

Professional Experience (If any) : Rural Agricultural Work Experience

Membership of Professional Societies (If any) :

Awards / Recognitions (If any) :

Publications (If any) :

Signature