CHAPTER ONE
INTRODUCTION

Plant protein production is basically reliant upon an adequate supply of water and essential elements, in particular the building blocks of protein, plant available nitrogen. Nitrogen is one of the most common limiting factors for primary production due to its significant presence in most of bio-molecules. It's importance for life can easily be explained as a major component of the master molecules of life (protein), molecules centre for the synthesis of fuel for life (chlorophylls) and a major molecule involved in the production of energy currency (especially ATP) to drive metabolic mill as well as in many growth hormones and vitamins. It's metabolic uses in plant is so dedicately regulated that nitrogen deficiency symptoms is observed first in older parts due to its rapid translocation to the younger parts of the plant . Thus, nitrogen is considered as the structural component of amino acids, which are the building blocks of protein. The plants are generally found to have 10-12% protein of dry weight, but in legumes, protein content exceeds up to 30 percent and of which nitrogen contributes about 16 percent (Kochhar, 1998).

Availability of reduced nitrogen is an important determinant in the growth and development of plants. Unfortunately this huge amount of dinitrogen is chemically inert ($N \equiv N$, $\Delta E^0 = 945.4$ K $Jmol^{-1}$) and cannot be directly utilized by plants. In soil, Nitrogen is available to the roots of the higher plants mostly as nitrate ion (NO_3^-) and ammonium ion (NH_4^+), out of which nitrate is the principle source of nitrogen to the higher plants growing under usual field conditions (Jain, 2001). Even though, nitrate is the principle natural source of nitrogen to plants, many plants are able to utilize ammonical nitrogen readily particularly, when environmental conditions favour high rate of photosynthesis and vigorous plant growth (Noggle and Fritz, 2002). The nitrogen fixed by nodule bacteroids is also an ammonium ion which is incorporated into aspartate by glutamine (1^{st} nitrogen sink) via transamination reaction to give Asparagin. Asparagin is the major nitrogeneous compound detected in the phloem of several legumes. Since nitrogen comprises 79 percent of the earth's atmosphere by weight approx 3.5 to 4×10^5 tons, the plant world may literally be said to be bathing in unavailable nitrogen.

Mainly the conversion of atmospheric nitrogen into the combined forms (-NH_3 and -NO_3) useful in plant nutrition takes place by two natural processes *viz*; Non-biological and biological processes. Non-biological process includes lightening, combustion and volcanism which accounts for about 10 percent of the annual fixation. However, the reaction condition is difficult; the industrial nitrogen fixation is carried out by Haber-Bosch process at high temperature (400-600^{0}c) and pressure (100-200 atm). Industrial nitrogen fixation *i.e.*, ammonia production accounts for about 25 percent of the annual fixation (Newton, 2000). Recently, Newton (2000) has reported that biological nitrogen fixation accounts for about 65 percent of the total nitrogen fixation, thus is therefore provider of fixed nitrogen and so is the major sustainers of the life on this earth.

1.1 Biological Nitrogen Fixation (BNF)

The ability of a plant to supply all or part of its N requirements from BNF in its roots can be a great competitive advantage over non-N_2-fixing neighbours. BNF is the conversion of atmospheric N_2 to ammonium, a form of N that can be utilized by plants. However, BNF is the sole domain of certain bacteria (diazotrophs), which contain nitrogenase, the enzyme complex that catalyzes the conversion of N from the gaseous to the combined form. Nitrogen fixing bacteria are single celled organisms that are essentially miniature urea factories, turning N_2 gas from the atmosphere into plant available amines and ammonium via a specific and unique enzyme they possess (*i.e.*, nitrogenase). Occurrence of N_2 fixing bacteria with higher plants is not uncommon, but in most case these are only 'associations', in which relatively free-living bacteria grow in the rhizosphere, on the rhizoplane, or more rarely, in non-specialized intercellular spaces in plants (Vessey, 2003). Nitrogen fixing bacteria are generally endemic to most soil types (both symbiotic and free living species), however in the natural state they generally only comprise a very small percentage of the total microbial population are often strains with low performance regarding the quantity of nitrogen they can fix. The transfer of fixed N from the bacterium to the plant in these associations is relatively low, and the relationship between the two organisms could be viewed as opportunistic rather than mutualistic. However, in a much smaller proportion of cases across the plant world, the association between plant and bacterium is much more intimate, with the N_2 - fixing bacterium being

housed with specialized plant organs. In these truly mutualistic symbioses, the genetics and physiology of the plant and bacteria are integrated to the extent that the two organisms can appear to function nearly as one.

Nitrogen fixation by *Rhizobium*-legume symbiosis contributes about 50 percent of the available nitrogen in the biosphere and is the largest single input into the nitrogen cycle (Tate, 1995; Vitousek *et. al*, 1997; Batut *et. al*., 2004).

1.1.1 Symbiosis and Symbiotic Nitrogen Fixation

It is only via the action of diazotrophs (residing in soil) that the Nitrogenous compounds enter into the plants metabolic pathways. They reduce N_2 to ammonia and exchange this for reduced carbon compounds from the plant (Michael *et.al.*, 1997). Nitrogen enzyme complex of diazotrophs extracts N_2 from atmosphere and reduces it to NH_3 in a reaction that requires substantial reducing power (electrons) and energy (ATP). The NH_3 is immediately assimilated into amino acids and proteins by subsequent cellular reactions. The reduced carbon is used as fuel for bacterial metabolism including nitrogen fixation and ammonia enables legumes and many non-legumes to grow in soil that have little or no available nitrogen. Probably, the most important form of agricultural nitrogen fixation is carried out by the *Rhizobium*-legume symbiosis in the root nodules (Peter J.lea, 2000). On several occasions, symbioses have evolved between terrestrial plants and N_2 fixing bacteria, leading to the existence of specialized organs on the host plants that provide excellent environments for the prokaryotes to infect, live and fix N_2.

As concerns mount to the growing input of reactive nitrogen into our environment, as part of a "nitrogen cascade", an increased need to understanding biological nitrogen fixation has become of paramount importance.

Although the genetic backgrounds and physiological functioning of these symbioses can be seen as very diverse, there are several developmental and physiological 'imperatives' that must be met for successful symbioses between host plants and their N_2-fixing microsymboints. These imperatives include:

- The ability of the microsymbiont to infect and colonize host plant organs.

- The ability of the host plant to supply energy and nutrients to the microsymbiont.
- The ability of the host plant and microsymbiont to regulate O_2 flux.
- The ability to transfer the fixed N from the microsymbiont to the host.

The classical example of biological nitrogen fixation can be achieved only by establishing symbiotic relationship with root nodule bacterium - *Rhizobium*, process called rhizobial symbiosis. Nodules are only the sites for fixing nitrogen by the diazotrophs. They are inducted by compatible *Rhizobium* species available in the soil. The prerequisite for establishment of legume *Rhizobium* symbiosis is the recognition of both partners. The symbiotic association is host plant specific, *i.e.*, one rhizobial strain infect only a limited number of different hosts. For example, *Rhizobium leguminosarum* bv. vicea can induce formation of dinitrogen fixing nodules on *Pisum, Vicia, Lathyrus* and *Lens*; whereas *R. leguminosarum* bv. trifoli nodulate *Trifolium* spp. A key factor in determining the specificity of nodulation is the structure of nod factors or lipochitinous oligosaccharides (LCO) released by bacterial cells. These organisms, like the legume *Rhizobium* bacteria, need to undergo a rigorous selection process to choose the highest performing strains. The legume is macrosymbiont and the *Rhizobium* is microsymbiont. Plant bacteria starts with exchange of molecular signals, which regulate the expression of genes essential for infection and subsequent step of nodule ontogenesis (Baur *et al.*, 1985, Megias *et al.*, 1993).

The legume root secretes growth stimulating exudates like biotins, thiamine, amino-acids etc. Consequently compatible strains of micro-organisms are stimulated to grow over the other microbes in rhizosphere and form the mucigel. Mucigel denotes the microbial cells and their products together with associated microbial cells and mucilages, organic and inorganic matter in the rhizosphere of root region. The recognization is achieved by plant lectins with unique sugar binding properties. Lectins bind with specific polysaccharides on the *Rhizobium* surface in a manner similar to the formation of enzyme-substrate complex (Law *et al*., 1988). The rhizobial Nod-factors influence the host root growth by causing an increase in amount of root hairs as well as their deformation, *i.e.* curling of root hair (also called shephard's cook) takes place. Subsequent to root hair curling, bacteria penetrate the rhizodermis and development of an infection thread occurs by invagination of root

hair cell wall in the region of curling (Nutmann, 1969). The rhizobia within the infection thread continue to grow and divide down the infection thread and until they reach the base of the root hair where cells are released and engulfed by endocytosis to form the symbiosome (Napoli and Hubbell, 1975; Ridge and Rolfe, 1985; Gage *et al*., 1996).

The infected cortex cells increase in size and divide to form a sphere surrounded by uninfected cell an outer fibrous layer. Within the infected cells the rhizobia divide and enlarge to form bacteroids, which are separated from the plant cytoplasm by the peribacteroid membrance (Michael *et al*; 1997). The organ like structure comprised of peribacteroid membrance and bacteriods is termed as 'symbiosome' and it is the basic nitrogen fixing unit for the nodule.

After the formation of bacteroids, the biosynthesis of nitrogenase and leghaemoglobin starts. Nitrogenase is an oxygen-labile enzyme and so must diazotrophs fix nitrogen only under anaerobic or microaerobic conditions, rhizobia are obligate microaerophiles. Leghaemoglobin (LHb) is the red pigment (due to presence of iron) like haemoglobin present outside the bacteroid membrance (peribacteroid space) but in their close contact. LHb is found only in the healthy nodules. The peribacteroid membrane may separate the bacteroids from the oxygen buffering system. LHb regulates O_2 concentration as bacteroids are aerobic and consume O_2. The conditions where host plants suffer from O_2 deficiency, LHb serves to facilitate the movement of O_2 to O_2 poor tissues in a proper way. At ambient concentration (0.2 atm), O_2 becomes a limiting factor for N_2 fixation and the reduced O_2 diffusion results in prompt inhibition of nitrogenase activity in nodules (Sprent, 1972). Thus LHb acts as a carrier of O_2 and helps in accomplishment of bacterial respiration and consequent provision of ATP for N-fixation. It also creates an O_2 free environment around the active site of nitrogenase which fails to function in the presence of O_2 (Wilbert *et al.*, 1997). Nitrogenase is an enzyme that the diazotrophs possess which helps in the conversion of N_2 to NH_3. This enzyme consists of two brown metalloproteins whose joint action is essential for the reduction of N_2 to NH_3. Nitrogenase is an equilibrium mixture of Mo-fe-protein (Component I) and fe- protein (Component-II) in the ratio 1:2.

Mo-fe-Protein + 2 (fe-protein) $\Leftrightarrow$ Nitrogenase.

Fe-protein interacts with ATP and Mg^{2+}, and Mo-fe-protein catalyses the reduction of N_2 to NH_3, H^+ to H_2 and acetylene to ethylene. The reduced ferredoxin or flavodoxin serves as a source of reductant for electron transfer during N_2 fixation.

The overall stoichiometry of BNF can be represented as follows:

$$N_2 + 16\,ATP + 8H^+ + 8e^- \xrightarrow{Mg^{2+}} 2NH_3 + H_2 + 16ADP + 16P$$

The reduced nitrogen *i.e.,* ammonia cannot be assimilated further by the bacteroids themselves and higher concentration of ammonia within the nodule inhibit the further reduction. So for the continuation of nitrogen reduction so formed ammonia is quickly picked up by the host and assimilated in amino acids. The first step in this assimilation (assimilated in the cytoplasm of the infected cells) in all legumes is catalyzed by glutamine synthetase (GS) which converts ammonia to glutamine which is further converted to glutamate by glautamine oxoglutarate aminotransferase (GOGAT). These enzymes are very active in nodules and maintain ammonia at very low concentrations in the plant cytoplasm and act as a very strong "Sink" driving ammonia efflux from the bacteroid. The assimilated nitrogen is Translocated out of the nodules to other plants parts.

Nodule formation and its structure

In the presence of specific rhizobial species, legume hosts form unique structure (the root nodule) in which nitrogen fixation occurs. Plant and bacterial activities create a nodular environment conductive to both nitrogen fixation and micro aerobic synthesis of ATP by bacteria. Plant metabolism in the nodule generates organic acids that both feed the bacteria and provide carbon skeletons for the N-transport compounds used to transfer fixed (reduced) nitrogen and the rest of the plant. In exchange for the carbon substrates, the bacterial symbionts fix nitrogen and release the resulting ammonia to the plant.

The core of a mature nodule constitutes the 'bacteroid zone' which is surrounded by several layers of cortical cells. The relative volume of bacteroid tissue (16 to 50% of the dry weight of the nodules) is much greater in effective nodules than in ineffective ones. The volume of bacteroid tissue in effective nodules has a direct positive relationship with the amount of nitrogen fixed. Ineffective nodules produced by

ineffective strains are generally small and contain poorly developed bacteroid tissue associated with several abnormalities.

In all ineffective associations, it has been shown that starch accumulates in the uninfected cell and dextran in the infected cell with glycogen in the bacteroid. Effective nodules are generally large and pink (due to leghaemoglobin) with well-developed and organized bacteroid tissue. A fully developed bacteroid has no flagella and is surrounded by three unit membranes. There exists an intracytoplasmic membrane system in the bacteroids of nodules in subterranean clover. The nuclear region of bacteroids appears fragmented and is associated with granular cytoplasm.

The three different hypotheses regarding their formation are:

a) They are formed *de novo* after the release of the bacteria from the infection thread.
b) They are extensions of the endoplasmic reticulum of the host cells, and
c) They are derived from plasmalemma by a process of phagocytosis.

The membrane envelope surrounding the bacteroids is also known as the peribacteroid membrane. The numbers of bacteroids enclosed in membrane envelopes appear to vary from one to many depending on the species of the legume. The multiplication of bacteroids and the formation of the peribacteroid membrane may not be synchronized cell events which result in the variability in the number of bacteroids enclosed by the membrane. The microsymbiont strain determines the particle density and the protein and fatty acid composition of the membrane. The peribacteroid membrane contains nodulins which may play special roles in two-way transport of metabolites between the symbionts.

Determinate and the indeterminate nodules

Rhizobia are capable of forming two distinct types of nodules, determinate and indeterminate, depending on the host plant. Legumes that form determinate nodules are typically *Glycine max, Phaseolus vulgaris* and *L. japonicus* whereas those that form indeterminate nodules are *M. Sativa, N. truncatula, Vicia faba* and *P. sativum*. The main distinguishing characteristic is that determinate nodules lack a persistent

apical meristem and have no obvious development gradient. The infected thread does not continue to extend and instead infection occurs via division of pre-infected cells to give evenly distributed bacteria. Nodule growth occurs by cell enlargement to give nodule a characteristic round shape. In comparison, indeterminate nodules have a persistent apical meristem caused by elongation and branching of the infection thread that continues to divide giving rise to a meristematic zone where new cells are subsequently infected to form new nodule tissue. This causes indeterminate nodules to become elongated and have clear zones at different stages of development.

Factors affecting the nodule development

Factors affecting the formation and longevity of nodules in roots of leguminous plants are:

a) Amount of nitrogen (on addition of nitrogen both the numbers and weight of nodules are reduced).
b) Light and shading.
c) Concentration of inorganic nutrients.
d) Soil temperature (optimum temperature 25-30^{0}c) and
e) Rhizosphere micro-organisms.

Translocation of the fixed Nitrogen

The products of N_2-fixation in legume nodules are exported to the leaves and the shoot via the xylem of the plant (Pate and Atkins, 1983). These products are generally the amides asparagine and glutamine or the ureides allantoin and allantoic acid (Atkins, 1991).

Although in most vascular plant species, the major transport form of reduced/organic nitrogen is as amino acids (including amides), tropical, sub-tropical and temperate legumes like Cowpea (*Vigna unguliculata*), Soybean (*Glycine max*), French bean (*Phaseolus vulgaris)* and Broad bean (*Vicia faba*) transport large amounts of these nitrogenous compounds called ureides. The dominant forms of ureides in these species are allantoin and allantoic acid (Pate *et al.*; 1980). Ureides can comprise upto 90% of the total nitrogen transported in the xylem of nitrogen fixing tropical legumes (Herridge *et al*., 1978; Pate *et al.*, 1980) and can be stored in high amounts in the different plant organs (Matsumoto *et al*., 1977 a; Streeter, 1979; Layzell and LaRue,

1982). Due to high concentrations in the vascular system and in certain plant tissues, ureides are interpreted to have an important function in nitrogen transport and nitrogen storage in tropical legumes. In addition to nitrogen from root nodules, N assimilated from soil combined nitrogen and from N cycling within the plant will all be present in the transpiration stream.

The N contained in these ureides originates predominantly from N_2 fixation. The ureide concentrations in plant tissue have been suggested to indicate the rate of N_2 fixation. Estimates of N_2-fixation made by the analysis of ureides have the advantages of being simple, inexpensive, rapid and non-destructive to test plants.

In vascular plants, nitrogen cycles continuously as amino compounds in the phloem and xylem saps. Phloem transport to the root delivers amino-acids that may be used for the growth of roots, stored or recycled back to the shoot in the xylem stream (Layzell *et al.*, 1981). The amount or composition of amine compounds in the phloem sap flowing into nodules may signal plant status and mediate changes in nodule growth and activity (Parsons *et al.*, 1993). This feedback inhibition of nitrogen fixation may operate regardless of the origin of N (soil, stored or N_2-fixation) although the study of this process is complicated by these possible origins of N, translocated in plants.

The synthesis of ureides occurs mainly in root nodules via the coordination of the plant bacteria association (Atkins and Smith, 2000). After nitrogen fixation in bacteriods of infected root cells, ammonia (NH_3), ammonium (NH_4^+), or amino acids are released or transported from the symbiosome into the cytosol, where they are utilized for Gln synthesis (Smith and Emerich, 1993, Day *et al.*, 2000; Ladwig *et al.*, 2003). After formation of Gln, the nitrogen goes via the *de novo* purine synthesis pathway in the plastids (Shelp *et al.*, 1983) or mitochondria (Atkins *et al.*, 1997), followed by purine degradation via Xanthine in the plastids (Schubert, 1986) or cytsol of infected or uninfected root cells (Matsumoto *et al.*, 1977 c; Atkins *et al.*, 1980; Shelp *et al.*, 1983). The ureides allantoin is finally synthesized in the peroxisomes of non infected root cells from the purine degradation product uric acid (Hanks *et al.*, 1981). Allantoic acid is probably produced in the smooth endoplasmic reticulum of the non-infected cells after import of allantoin into this compartment (Hanks *et al.*, 1981). From the place of synthesis, allantoin and allantoic acid are transferred into the

cytosol and are then transported to the root xylem and phloem for long distance transport (McClure and Israel, 1979; Streeter, 1979; Atkins *et al.*, 1982).

1.2 *Phaseolus vulgaris* L.cv. Trishuli (Field bean)

Highly polymorphic species; annual herb; erect and bushy, 20-50 cm tall, or twining with stems 1.5 - 3m long; with a taproot and nitrogenous nodules; leaves alternate, green or purple, trifoliate, stipulate, petiolate, a marked pulvinus at base; leaflets ovate, entire; acuminate 5-15 cm long, 3-11 cm wide; flowers in lax, axillary few-flowered (12) racemes, zygomorphic, variegated, white, pink, or purple, usually glabrous, sometimes puberulent, beak prominent; seeds white, red, tan, purple, grey or black, often variegated, reniform, oblong or globose, up to 1.5 cm long, endosperm absent; germination phanerocotylar.

1.3 *Pisum sativum* L.

Annual, often climbing, stem glabrous, glaucous. Leaf paripinnately compound, rachis ending in a branched tendril, leaflets 2-8, entire or dentate; stipules 1.5-8 cm long, obliquely ovate, toothed at least below, semi-amplexicaul at the base, peduncle ½ to twice as long as the stipule, 1-3 flowered. Calyx 8-15 mm long, teeth longer than the tube, subequal. Corolla white or vexillum lilac and reddish purple Vexillum 16-30 mm long. Fruit 40-70 mm long, 12-17 mm broad.

1.4 *Vicia faba* L. (Broad bean)

A rigid, erect plant of 0.5-1.7 in tall, with stout stem with a square cross-section. The leaves are 10-25 cm long, pinnate with 2-7 leaflets and of distinct glaucous greygreen colour; flowers are 1-2.5 cm long, with five petals, the standard petal white, the using petals white with a black spot and the keel petals white. The fruit is a broad leathery pod, green maturing blackish-brown, with a densely downy surface; in the wild species, the pods are 5-10 cm long and 1 cm diameter. Each pod contains 3-8 seeds; round to oval and 5-10 mm diameter in the wild plant.

1.5 Source of Nitrogen

Ammonium nitrate and urea are widely used for the source of nitrogen in the soil. Urea (NH_2CoNH_3) contains 46% N. Urea is highly soluble in water and readily

absorbed via the leaves. It is also called carbamide. In soil, urea is readily couverted by an enzyme urease to ammonium carbonate, which is unstable and release free ammonia. The amount of urea required is 60-120 $kgha^{-1}yr^{-1}$ (50 $mgkg^{-1}$ of soil). The nitrogen application does increase the soil acidity and the lime requirement. Low rate of application involves low quantities of nitrogen required by the plants with large pool of soil nitrogen available.

1.6 Rationale

In the light of national perspectives and the guidelines provided in National conservation strategy for Nepal, it is imperative to work in this field of Biological Nitrogen Fixation keeping in mind the cost of chemical fertilizer and the status of soil fertility of the hills in particular, we are required to find out an alternative method to enrich the soil in order to achieve enhanced crop productivity on a sustainable basis.

In recent days, gradually more increasing of the chemical fertilizers have deteoriated the quality of the soil and left severe impacts on the living organisms. So, in order to check the deteoriating quality of soil, use of the biofertilizers over the chemical fertilizers is a must. Thus, the role of present day scientists is to improve and increase the natural products to fulfill ever-increasing need of the world without any harmful impact on environment, economy and other aspect of the society. Several practices have been applied to increase the productivity in the agronomic field.

So, for the betterment of the soil and the microbial fixation of nitrogen, legumes are widely used. Legumes play a key role in nitrogen fixation by allowing the nitrogen fixing bacteria to reside in its roots forming enlarged structures called nodules. Most of the impact with the use of micro organisms as bio-fertilizers has been directed towards understanding of the biological nitrogen fixation that occurs in the symbiotic system of legume and bacteria of Rhizobiaceae family. Legumes have probably received more attention to date due to their higher nutritional value and major role in improvement of soil fertility.

In the context of Nepal, nothing new has been done in the determination of ureides, allantoin and allantoic acid. So, as such, it is a completely new work. With this research, it will help to open the door of opportunity on this field for the advance

research. With the estimation of the ureides, allantoin and the allantoic acid, the amount of the nitrogen fixed and translocated to the aerial parts from the nodules can be known. The more is the concentration of these compounds in the aerial parts, the more is the amount of Nitrogen fixed up by the nodules. The size and number of nodules indicates the intensity of Rhizobia in the soil. Since legumes are the good source of protein, it can overcome the large amount of nutrients and for this Rhizobia play a key role. After the senescence of nodules, the entrapped Rhizobia are ultimately released to the environment and they in turn get incorporated on the next compatible leguminous plant.

Greater is the amount of ureides translocated to the aerial parts, greater are the nitrogenous molecules incorporated to form different compounds and in turn the greater amino-acids. Amino-acids are the building blocks of proteins. The present investigation is also concerned with the symbiotic effectiveness of two rhizobial isolates taken from two different climatic regions; Manang and Kathmandu valley. These rhizobial isolates are also used to check the cross-inoculation grouping of legumes.

1.7 Hypothesis

- The amount of the ureides translocated to the aerial parts of the plant is equivalent to the amount of the N fixed by the nodules.

1.8 Objectives

a) Overall objectives: Present investigation aims to fulfill the following objectives:

i) Isolation of rhizobial strains from the legume root nodules of Manang and Kathmandu valley.

ii) Authentication of the rhizobial isolates.

iii) To estimate the gram characteristics by gram staining.

iv) To determine the cross-inoculation effect.

b) Specific Objectives: Specific objectives of this research include:

i) To estimate the total ureides transported via the xylem stream.

ii) To estimate the total Nitrogen content.

iii) To compare the symbiotic effectiveness of both the rhizobial isolates.

1.9 Limitations

- Time constraints.
- Unavailability of the necessary literatures.

CHAPTER TWO
LITERATURE REVIEW

Plants are capable of absorbing the nutrients of different forms by the conversion of gaseous nitrogen into the absorbable form by different process. A very small amount is converted into nitric and by electric discharge (Vistanen and Miethinen, 1963).

Industrially, Haber process reduces N≡N into ammonia at the expense of the energy. However, a major quantity of N_2 is reduced by a natural process called Biological Nitrogen fixation. This accounts 90% of the global nitrogen fixation (Allen and Allen, 1958). The symbiotic nitrogen fixation is one of the efficient and environment friendly means of biological nitrogen fixation and is accomplished by two biological identities, *i.e.*, micro-symboiont (rhizobia) and macro-symbiont (legume).

Since long, legumes have been used as a green manure to increase the soil fertility. Subba Rao (1999) showed that legumes can obtain nitrogen from air when grown in soil which was not heated (sterilized).

The innate ability of legumes to fix atmospheric nitrogen was revealed by Herman Hellerigel and Herman Wilfarth in 1886 and 1888 for the first time. Beijerink isolated and cultivated the micro-organism from the legume root nodules in 1888 and named them as *Bacillus radiciola*, which is now placed under the genus *Rhizobium*. The interesting and exciting scientific endeavours revealed the most of the physiological, agronomic as well as molecular aspects of the legume *Rhizobium* into amino-acids, which is turn, are synthesized into proteins.

Formations of spores or resting bodies are set to be established for the bacterial species. The cell can be motile by one or more flagella and produce copious amounts of polysaccharide slime in most of the cases. In culture, the optimum temperature ranges from 25-30^{0}c and the lethal temperature is between 30^{0}c to 40^{0}c depending upon species and strain (Nutman and Skinner, 1968).

2.1 *Rhizobium*

Rhizobium are fast-growing root nodule bacteria of medium sized, having sub-polar flagella, rod-shaped cells, 0.5-0.9 μm in width and 1.2-3.0 μm in length (Somasegaran and Hoben, 1994). It has a cell-wall membrane overlying an intermediate (rigid) layer of variable thickness. The protoplasm enclosed in a unit membrane, contain a prokaryotic nucleus, ribosome and sometimes contain polyhydroxy butyric acid, storage granules (Burton, 1980).

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2.2 *Rhizobium* Classification

Bacteria belonging to the genus *Rhizobium* live freely in soil and in the root region of both lenguminous and the non-leguminous plants. However, they can enter into the symbiosis only with leguminous plants, by infecting their roots and forming nodules on them, the only exception being root nodulation in Trema (*Parasponia*) by a *Rhizobium* sp.

When a nodule becomes senescent after a period of nitrogen fixation, decay of tissue sets in liberating motile forms of *Rhizobium* into soil which normally serve as a source of inoculums for the succeeding crop of a given species of legume. The genus *Rhizobium* has been placed in Bergey's Mannual of Determinative Bacteriology in such diverse families as Azobacteriaceae, Myxobacteriaceae and Pseudomonadaceae.

The principle of cross-inoculation grouping is based on the ability of an isolate of *Rhizobium* to form nodule in a limited number of species of legumes related to one another. All rhizobia that could form nodules on roots of certain legume types have been collectively taken as a species (Subba Rao, 1999). This system of classification has provided a workable basis for the agricultural practice of legume inoculation.

The system of cross-inoculation of rhizobia is not perfect since bacteria have been found to cross-infect or interchange between groups. However, until a better system

of classification has been perfected, it appears as if we have to be content with the cross inoculation grouping as a convenient and workable method of classifying root nodule bacteria into species.

Table: Cross-Inoculation Grouping of Legumes and Rhizobia

Rhizobia	Cross Inoculation Group	Legumes in Cross Inoculation Group
Genus I : *Rhizobium*		
Rhizobium leguminosarum		
bv. viceae	Pea	*Vicia, Pisum, lens, Lathyrus*
bv. trifoli	Clover	*Trifolium* sp (with exception)
bv. phaseoli	Bean	*Phaseolus* spp. (temperate spp. *Vulgaris, angustifolus, multiflorus)*
R. meliloti	Alfalfa	*Melilotus/Medicago/Trigonella*
R. loti	Lotus	*Lupinus/Antyllis/Leucaea* and many other tropical tree legumes.
R. galegae		*Galega orientalis*
R. fredii	Soybean	*Glycine* spp., *leucaena* spp., *Prosopis* spp., *Robinia* spp.
Rhizobium sp	Chickpea	*Cicer arientinum*
Genus II : *Bradyrhizobium*		
B. japonicum	Soybean	*Glycine* spp. (*G. max* and *G. soja*)
Bradyrhizobium spp.	Cowpea	*Cajanus cajan, Arachis hypogaea, Phaseolus lunatus, Vigna umbellate* and many tropical legumes

Source : Somasegaran and Hoben, 1994.

Out of many criteria's *i.e.*, rDNA mapping, growth kinetics, acid production activity etc, cross inoculation grouping is one of the most convincing systems of classification for *Rhizobium*.

2.3 Symbiotic Properties

Schiffmann, 1958: Vincent (1980) studied the values of nitrogen contents of the roots (Lucerne) and they found that the higher percent of nitrogen content associated with the effectively nodulated plants (2.7 - 3.7%) compared with the severely limited uninoculated controls (1.3-2.5%).

Bergersen (1974) studied about the formation of bacteroids and their function in legume root nodules and revealed that about 25-30% of interior tissue of soybean nodule forms the bacteroidal zone occupied by bacteroids, which are essential for N_2 fixation.

Cho *et al.*, (1985) isolated mutants capable of more rapid nodulation of soybean seedlings. Competitiveness of these mutants under field condition, however, remained to be determined.

Gupta (1985) comparatively studied the different strains of rhizobia on nodulation, nitrogen fixation and nitrogen metabolism of *Vigna mungo* and found that isolate '2' was highly productive followed by '6' on the basis of plant biomass, nodule weight, nitrogenase, ATPase and reducing carbon content.

Triplett (1986) developed two methods for the detection of altered ureides metabolism in legume nodules. Both of the developed techniques were based on the positive correlation between the presence of high Xanthine dehydrogenase specific activity in nodules and the ability of those nodules to produce the ureides, allantoin and allantoic acid.

Poudyal, S.P. (1992) studied on isolation, characterization and inoculation response studies on French bean (*Phaseolus vulgaris* L.) root nodule *Rhizobium* from different locations of Kathmandu valley and found that local isolate of Indrayani to be the most effective among the other.

Micke (1993) while working on topic 'Mutation breeding of grain legumes to enhance biological nitrogen fixation' he observed many mutants varieties of common bean have been possessed many different improved characters. Thus he concluded that mutation breeding appears to be an appropriate approach not only for genetic improvement of grain legumes in general but also for improving their symbiotic nitrogen fixation.

Somasegaran and Hoben (1994) reviewed plastic pouch plant infection test methods. It is one of the best methods applied for infection test.

Hoque and Scattar (1998) and Hogue *et al.,* (1999) found that soybean plants inoculated with *B. japonicum* increased nodule number, nodule dry weight, chlorophyll content, shoot length, plant dry weight and grain yield significantly as compared to uninoculated plants. The inoculation effect in presence of sulphur and molybdenum fertilizer was found more significant.

Gupta *et al.,* (1999) studied the production of cellulolytic enzyme by *Bradyrhizobium* sp. mungbean. They found that the enzyme cellulose produced by rhizobia facilitate their entry into the plant root cells to infect the host.

Lodwig *et al.,* (2003) studied the amino acid cycling between rhizobia and host in nodules that controls the symbiotic N_2 fixation within the nodules and suggested that a complex amino-acid cycle is essential for symbiotic N_2-fixation by *Rhizobium* in root nodules. It was revealed that the plant provides amino acids to bacteroids enabling them to shut down their ammonium assimilation, in return bacteroids act like a plant organelle to cycle amino acids back to the plant for asparagine synthesis.

Vessey *et al.,* (2004) revealed the mutualistic symbiosis between legumes and rhizobia, actinorhizal plants and *Frankia, Parasponia* sp. and rhizobia, and cycads and cyanobacteria and they found a great diversity in many aspects of these root-based N_2 - fixing symbioses, though similarities exists in the genetics, development and functioning of the symbioses. Each symbiosis was admired for the elegant means by which the host plant and microsymbiont integrate to form the mutualistic relationship so important to the functioning of the biosphere.

Werner *et al.,* (2005) isolated seven *Rhizobium etli* strains from Egyptian soils, out of which strain EBRI21 and EBRI26 are highly tolerant to salt concentration up to 4% NaCl. A positive correlation was found between the salt tolerance and adaptation to alkaline p^H (9). They also added, the stress of alkalinity had a less detrimental effect on nodulation and N_2 fixation than stress of salinity.

2.4 Enzymatic Effect

Triplett *et al.* (1980) suggested that Xanthine-oxidizing enzyme in the nodule is an NAD^+-dependent xanthine dehydrogenase which is present only in the cytosol fraction. They found that the NADH production by this enzyme played a critical and energy conserving role in the ureides synthetic pathway. Also, cytosol xanthine dehydrogenase activity was sufficient for the metabolism of fixed N because the calculation on N_2 fixation and xanthine oxidations were similar during the experiment.

Rosenbery *et al.* (1981) studied the genetics of *Rhizobium* species and reported that *Rhizobium* consisted of a megaplasmid in cell which possess numerous genes coding for nodulation (nod genes) and nitrogen fixation (nif genes) and both are closely located.

Shelp *et al.* (1983) studied the role of Inosine Monophosphate oxidoreductase in the formation of ureides in Nitrogen fixing nodules of Cowpea and finally revealed that *in vivo* inosine monophosphate oxidation rather than dephosphorylation is the predominant metabolic route leading to ureides synthesis and that inosine monophosphate provides the link between *de novo* purine nucleotide synthesis in the plastid and ureides production in the plant cell cytosol.

Triplett (1986) developed two methods for the detection of altered ureides metabolism in legume nodules. Both of the developed techniques were based on the positive correlation between the presence of high Xanthine dehydrogenase specific activity in nodules and the ability of those nodules to produce the ureides, allantoin and allantoic acid.

Gupta *et al.* (1999) studied the production of cellulolytic enzyme by *Bradyrhizobium* sp. mungbean. They found that the enzyme cellulose produced by rhizobia facilitate their entry into the plant root cells to infect the host.

2.5 Ureides (allantoin and allantoic acid)

Fujihara *et al.*, (1977) studied the role of allantoin and the influence of nodulation on its production in soybean plants. They found that in the stem allantoin - N markedly increased after the flowering period and then decreased during seed formation.

Allantoin was accumulated in the pod during pod formation, while in the seed the main N-compounds were amino-acids, the allantoin concentration was very low.

Herridge *et al.,* (1978) while working on Cowpea (*Vigna unguiculata* (L.) Walp.) found that 60-80% of xylem-borne N consisted of ureides; the remainder was glutamine, asparagine and amino acids. They also observed that allantoin predominated in the soluble N fraction of nodules and fruits, while allantoin and allontoic acid were present in approximately equal proportions in xylem exudates, stems and petioles.

Woo *et al.,* (1980) studied the biosynthesis of ureides from Purines in a cell-free system from nodule extracts of Cowpea and found that the ureides synthesis to be confined to soluble extracts from the bacteroid-containing tissue, was stimulated by puridine nucleotides and intermediates of the pathways of aerobic oxidation of ureides, but was completely inhibited by allopurinol, a potent inhibitor of xanthine dehydrogenase.

Thomas *et al.*, (1980) examined the capacity of the shoot tissues to assimilate allantoin via allantoinase during leaf and fruit development in nodulated soybeans. They found the specific activity of allantoinase in leaves peaked during pod formation and early seed filling while in developing fruits allantoinase activity in the seeds was 2 to 4 times that in the pots. They recorded the highest amounts of ureides in the pith and xylem of stem tissues and in developing pod walls.

McClure *et al.,* (1980) evaluated the relative ureides content of xylem sap as an indicator of N fixation in soybeans and found the significant decreased in the total N concentration of xylem sap from plants which are totally dependent upon N_2 fixation as their source of N, but no significant decrease was observed in the total N concentration of sap from the plants that are dependent upon both N_2 fixation and uptake of exogenous nitrate. They found the relative ureides content of xylem sap from plants were totally dependent upon N_2 fixation and was shown to be insensitive to the changes in the exudation rate and total N concentration of xylem sap brought about by diurnal changes in environmental factors.

Reynolds *et al.,* (1982) performed an experiment comparing between ureides and amide transporting plants. They revealed that enzymes of purine oxidation were found to be present in significant quantities only in ureides-transporting pintobean and

soybean nodules. The levels of these enzymes were low in lupin, but this amide-exporter had significantly higher levels of asparagine synthetase.

Berkum *et al.,* (1983) performed ureides analysis of soybean with a modified and simplified automated analysis to determine allantoin concentration in rat urine. They revealed that keto acids did not significantly interfere with the determination of ureides except for the glyoxylic acid in extracts of fresh soybean tissue and the interference caused by glyoxylic acid was avoided by adding phenylhydrazine HCl to solution of NaOH used for alkaline hydrolysis of allantoin.

Polayes *et al.,* (1984) observed the patterns of activity for uricase and allantoinase, enzymes involved in ureides synthesis, and revealed that they were positively correlated with the accumulation of ureides in the roots and cotyledons. They also found allopurinol and azaserine inhibiting the ureides production in 3-days old cotyledons while no inhibition was observed in the roots. They also revealed that inhibition of ureides accumulation by allopurinol but not azaserine in 8-days-old cotyledons suggested that ureides in these older cotyledons arose via nucleotide breakdown.

Shelp *et al.,* (1984) studied the ureides metabolism in leaves of Nitrogen-fixing soybean plants and found out that urea dependent NH_3 and CO_2 production in the dark showed an approximately 2:1 stoichiometry and was decreased to less than 11% of the control in the presence of 50 millmolar acetohydroxamate. They finally came to a conclusion that almost complete sensitivity of NH_3 and CO_2 production from allantoin and urea metabolism to acetohydroxamate, together with the observed stoichiometry, indicated a path of ureides assimilation via allantoate, ureidoglycolate and glyoxylate with the production of two urea molecules.

Schubert and Coker (1984) studied carbon metabolism in soybean roots and nodules and found that primary products of N_2 fixation exported from nodule are ureides, allantoin and allantoic acid. Ammonium itself does not transported directly and eventually incorporated into amino acid. The presence of nitrate or ammonium in the rooting medium decreases N_2 fixation and ureides transport increasing amino acid synthesis and transport.

Schuller *et al.,* (1985) studied the effect of nitrate on N_2 fixation by three different techniques, acetylene reduction, N_2 fixation and relative abundance of ureides in xylem exudates which gave the similar results. They finally concluded that when

nitrate is supplied to an established symbiosis, inhibition of nodulated root N_2 fixation precedes the loss of the potential of bacteroids to fix N_2.

Imsande (1986) studied the degradation and utilization of exogenous allantoin by intact soybean root. During the experiment, it was revealed that the decomposed nodules release allantoin in the surrounding soil and the root exogenous allantoin was rapidly degraded. The hydrolysis of urea to ammonia took very slowly and the urea seemed to be taken up by the intact soybean root.

Winkler *et al.,* (1986) studied the metabolism of allantoin in intact leaf tissue to elucidate the pathway of allantoin catabolism and its regulation and finally found the consistency with the pathway of catabolism including allantoate, ureidoglycolate and glyoxylate.

Kessel *et al.,* (1988) studied the ureides production by N_2 - fixing and non-N_2-fixing leguminous trees. They found that certain non-N_2-fixing species (*i.e., Tamarindus indica* and *Adenanthera pavoninia*) producied significant amount of ureides. They also revealed that several N_2 fixing species (*i.e., Mimosa scabrella, Sesbania grandiflora, Acacia mearnsii* and *Gliricida sepium*) grown on mineral-N had higher absolute amount of ureides in both extracts and exudates that did most nodulated species.

Herridge *et al.,* (1990) performed the ureides assay for measuring Nitrogen fixation by nodulated soybean calibrated methods and found positive effect of nitrate supply on concentration of nitrate in saps and extracts and a negative effect on ureides and on nitrate in saps and extracts and a negative effect on ureides and on the proportion of plant N derived from Nitrogen fixation. The relative abundance of ureides - N in root-bleeding sap, vacuum-extracted sap and stem extracts and the proportion of plant N, derived from nitrogen fixation between successive samplings were highly correlated.

Thumfort *et al.,* (1999) predicted a distinctively compartmentalized distribution of [O_2] between uninfected and infected cells; with high O_2 concentrations for an uninfected cell being consistent with and necessary for, efficient operation of uricase and ureides synthesis and low O_2 concentrations across most of the infected cell providing a suitable environment for N_2-fixation.

Pelissier *et al.,* (2004) studied PvUPS1, an allantoin transporter in nodulated roots of French bean and found that PvUPS1 transported allantoin but also binded its

precursors xanthine and uric acid. They revealed that PvUPS1 expressed via out the plant body in beans with strongest expression in nodulated roots, source leaves, pods and seed coats while in roots, PvUPS1 expression was dependent on the status of nodulation, with highest expression in nodules and roots of nodulated plants compared with non-nodulated roots supplied with ammonium nitrate or allantoin.

Tajima *et al.,* (2004) found the ureides reaching to 60-75% of soluble nitrogen in the xylem sap of Soybean in reproductive phase. They came to the conclusion that the nodules infected cells (plastid and mitochondria) and uninfected cells (peroxisome) shares *de novo* purine biosynthesis and urate oxidation to produce ureides respectively.

Atkins and Smith (2007) studied the translocation (Assimilates, nutrients and signaling molecules) in legumes. They revealed that the signal was systemic. They identified a number of mutants, super-or hypernodulators (HAR 1 in *Lotus japonicus*, GmNARK in Soybean, SYM29 in *Pisum sativum* and SUNN in *Medicago truncatula*. They found the gene responsible for super nodulation phenotype in a number of legumes as a leu-rich repeat receptor-like kinase similar to CLV1 regulating shoot and loral proliferation in *Arabidopsis*.

Hayat *et al.,* (2008) estimated the N_2-fixation of Mung bean and Mash bean via xylem ureides technique and found the relative abundance of ureides-N in the xylem sap of beans to be in the range of 30-60%. Also the correlation between different legume parameters showed that N_2-fixation to be positively correlated with all legume parameters.

CHAPTER THREE
MATERIALS AND METHODS

3. Materials

3.1 Sources of *Vicia faba* L.

Seeds of *Vicia faba* were purchased from the local farmers of Kathmandu valley and the adjoining areas to it. Morphologically healthy and uniform seeds were selected so as to have uniformity in the initial plant growth.

3.2 Laboratory facilities

The present research work was carried out in Central Dept. of Botany, Tribhuvan University, Kirtipur and the laboratory of this institution provided all the necessary requirements.

3.3 *Rhizobial* isolation and their analysis

3.3.1 General

The *Rhizobium* inocula for inoculation on *Vicia faba* was taken from the rhizobial isolates {(*i.e., Phaseolus vulgaris* from Manang (a trans-Himalayan region) and *Pisum sativum* from Kathmandu valley)}. This helped a lot in maintaining the diversity of the rhizobia.

3.3.2 Collection of Nodules

The healthy plants with vigorous growth were uprooted with soil after 30 days of seed sown. Each plant from the respective bed was washed by immersing in water along with the soil and the healthy red nodules were detached from the root along with the small portion of the root as well. These nodules were then transferred in well labeled vials with silica gel crystals and were stored at 4 ± 1^0c for short term storage.

3.4 Preparation of Stock Solution

3.4.1 Congo Red (CR) Stock Solution

Stock solution of Congo red was prepared by dissolving 0.25 g Congo red dye in distilled water to make final volume 100 ml. This stock solution was then stored at 4^0c in a well-labeled brown bottle and kept in a refrigerator.

3.4.2 Bromothymol blue (BTB) stock Solution

Stock solution of Bromothymol blue was prepared by dissolving 0.25g BTB in 100 ml distilled water. This stock solution was stored at 4^0c in a well labeled brown bottle and kept in a refrigerator.

3.4.3 Preparation of the Culture Medium

For the culture of the *Rhizobium in vitro*, Vincent (1970) proposed a best rhizobial growth supporting medium. This medium is known as Yeast extract mannitol Agar-Congo Red (YMA-CR) medium. The chemical constituents of YMA-CR have been tabulated in Annex 2 (2.1).

All the required amount of constituents was weighed for one liter and dissolve one by one with continuous stirring. The formation of the precipitate was completely avoided. If the chemicals don't dissolve, certain heat may also be applied just to dissolve it. After the chemicals were added, the pH of the solution was observed and was maintained to 6.8 with 0.1 N NaOH and 0.1 N HCl. Finally 10 ml of stock solution of Congo Red was added to the solution and the final volume was made 1000 ml. The solution was then heated till boiling and then agar (15 g) was added and stirred well to homogenize. This medium was autoclaved at 121^0c (15 lbs psi) for 15 minutes. This sterilized YMA-CR medium was used for the isolation of rhizobial isolates from different root nodules.

3.4.4 Isolation of *Rhizobium* (Pure Culture)

Nodules from the healthy and matured plants were taken along with the small section of the root as well. Big and brown red nodules were selected and these were then washed with surface sterilizing chemicals. The process was carried out in laminar air flow cabinet under the complete aseptic condition. For surface sterilization the nodules were first immersed in 90 percent ethanol for 10 sec and were then transferred to 3% (v/v) solution of sodium hypochlorite for 4 minutes. These were

then finally washed with sterile distilled water for about six folds to get rid of the steriliant.

In the mean time, about 20 ml of melted YMA-CR medium was poured on each petriplates and was left for solidification. At the same time, the sterilized nodules were cut transversely and with the help of inoculation loop the inner residing bacteria were taken out and streaked in the plates. The streaking process was of continuous type. After each streaking, the inoculation loop was flame sterilized. All streaked plates were incubated in inverted position at 28 $\pm$ 1^0c for five days inside an incubator on complete darkness.

3.4.5 Maintenance of Pure Culture

On an interval of 5 days, rhizobial colonies grown in the YMA-CR master plates were then isolated in completely sterile condition under the laminar air flow cabinet. The translucent, milky raised, round and flat colonies containing plates were selected for sub culture and also to preserve as the master plate. The isolated, translucent and raised colony was picked up and was streaked on the new YMA-CR plate with the help of the sterilized inoculation loop. This was then incubated at 28$\pm1^0$c for 5 days.

As the pure cultures of rhizobial isolates were obtained, single bacterial colonies were transferred to the YMA-BTB slants. The slants were incubated for 5-7 days at 28$\pm1^0$c and finally pure *Rhizobium* culture containing YMA-BTB slants were stored at 4^0c for inocula production.

3.4.6 Characterization of rhizobial isolates

3.4.6.1 Gram Staining

The test of the rhizobial isolates towards the Gram stain and their morphological characteristics were studied by Gram staining. Staining is a bio-chemical technique of adding a class-specific (DNA, Proteins, lipids, carbohydrates) dye to a substrate to qualify or quantify the presence of a specific compound. It is similar to fluorescent tagging. Gram staining uses crystal violet to stain cell-walls, iodine as a mordant, fuchsin or safranin counter stain to mark all bacteria.

3.4.6.2 Component of the Bacterial Cell Wall

To differentiate the Gram positive and the negative bacteria, this gram staining procedure was employed. This gram staining generally differentiates the bacteria having a thick layer of peptidoglycan (Gram positive) and lacks the secondary membrane and a lip polysaccharide layer found in gram negative bacteria. This rigid structure provides the rigid framework around the cytoplasmic membrane and provides the full protection against the adverse environment. Peptidoglycan is a huge polymer of interlocking chains of identical monomers connected by interpeptide bridges.

Peptidoglycan makes up as much as 90% of the thick compact cell in Gram positive cell whilst the cell walls of Gram negative bacteria are more chemically complex, thinner and less compact.

3.4.6.3 Staining Mechanism

For staining, four general basic steps are followed, which include applying a primary stain (Crystal violet) to a heat-fixed smear of a bacterial culture followed by the addition of a mordant (Gram's iodine), rapid decolorization with alcohol or acetone, and counterstaining with safranin or fuchsin.

Generally the crystal violet (CV) gets dissociation in aqueous solution to give rise to CV^+ and chloride (Cl^-) ions which penetrate via the cell-wall and cell-membrane of both Gram positive and Gram negative cells. The positive ion (CV^+) interacts with negatively charged components of bacterial cells and stains the cell purple. The addition of iodine (I^- or I_3^-) interacts with CV^+ and forms the large complexes of crystal violet and iodine (CV-I) within the inner and the outer layers of the cell. On the addition of decolorizer like alcohol or acetone, it interacts with the lipids of the cell membrane. A Gram-negative cell will loose its outer membrane and the peptidoglycan layer is left exposed. The CV-I complexes are washed from the Gram-negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. The large CV-I complexes become trapped within the Gram-positive cell due to the multilayered nature of its peptidoglycan. The decolorization step is very critical and it must be timed correctly; the crystal violet stain will be removed from both Gram-positive and negative cells if the decolorizing agent is left for some time (within a second as well).

After decolorization, the Gram-positive cell remains purple and the Gram-negative cell loses it's purple color-counter stain, which is usually positively-charged safranin or basic fuchsin, is applied last to give decolorized Gram negative bacteria a pink or red color.

3.4.6.4 Steps followed on Gram-Staining

The composition of Gram staining solutions according to Vincent, 1970 is tabulated in the Annex 3.

For the process of gram staining, firstly a colony of the pure culture was taken by the help of the sterile loop and was diluted in sterile water. The diluted culture was evenly smeared on a clean glass slide with the help of another clean slide and was allowed to air dry. Then the crystal violet solution (A) was applied on dried smear on glass slide for one minute followed by the gentle washing in the running tap water. Then the solution (B) *i.e.* Gram's iodine was applied for one minute followed by washing with ethyl-alcohol for 10-20 seconds with slight agitation. Finally, the counter stain (Safranin) was applied for one minute followed by gentle washing in running tap water. The slide was then allowed to air dry and finally examined under a light microscope under oil immersion at high power. Both of the rhizobial isolates were similarly examined.

3.4.7 Authentication of rhizobial isolates by infection test

For the conformation of the presence of the particular bacteria, the plant infection test must be carried out. Usually the plants with the same cross-inoculation groups are employed for this. All the methodologies generally performed are presented below:

Sterilization of Seeds, filter papers and growth pouches

Surface sterilizing legume seed is dependant on the purpose and nature of the experiment. Seeds of the *Vicia faba* were taken and 3% hydrogen peroxide was employed as a sterilant. These sterilized seeds were then washed with six folds of sterilize distilled water (st.dH_2O) ensuring no traces of sterilants to be present. Healthy seeds were then taken and were left for germination.

Similarly, the filter papers were sterilized, on the hot air oven and the growth pouches by the moist steam under pressure inside the autoclave at 121^0c.

Germination of Seeds

Surface sterilized seeds were allowed to germinate by imbibing them at 5^0c in refrigerator. These were then placed on sterile Petri dishes containing water agar (0.75% w/v) medium at equidistant from each other and were left on incubator at 28 ± 1^0c for five days.

Preparation of liquid rhizobial Inocula

Rhizobial isolates were maintained in liquid medium *i.e.* Yeast Extract Mannitol Broth (YMB) for inoculation in their specific host. The chemical constituents required were followed as provided by Vincent (1970). For each isolate, 250 ml inocula were made in two conical flasks by shaking inoculated broth on horizontal shaker (120 ppm) as mentioned to find the generation time. All the activities were performed in laminar air flow cabinet. The inoculated YMB flasks were then incubated at 28 ± 1^0c for 5 days in horizontal shaker.

Preparation of Nitrogen-free Nutrient Solution

Presence of nitrogen generally inhibits the formation of nodules. So the N-free medium should only be employed for the nodulation to take place. Many of the workers have proposed different nutritional ingredients for the preparation of the medium, viz. Fahraeus N-free medium (1957), Modified Jensen's N-free medium (Roughley, 1984), N-free Nutrient solution (Broughton and Dilworth, 1970), etc. Among these, modified Jensen's N-free medium (Roughley, 1984) was employed. The chemical constituents required are tabulated in the Annex 2 (2.2).

These chemical constituents are dissolved in sterile distilled water one by one avoiding precipitation. If the chemicals do not dissolve by manual shaking then little amount of heat may also be applied just making sure to dissolve chemicals.

Infection test on sterile sand

Infection is preceded by a deformation of root hairs and the forming of an infection thread that can be observed directly under the microscope.

For testing the infection on sterile sand, firstly the sand was fully autoclaved at 121^0c for 30 min and was left to cool. It was then filled in the polythene bags and was supplied up with the Nitrogen free Nutrient solution. The seeds that were surface

sterilized were then allowed to germinate and the infection was observed. Infection of the roots can be obtained within an interval of three weeks.

Infection test on Growth Pouch

The nitrogen free nutrient media was autoclaved at 121^0c (15 lbs) for 30 minutes and left for cooling. Approximately, 40 ml of the Jensen's medium was poured on twenty four polythene bags containing the rolled filter paper. This filter paper placed was to support the seeds and the radical of the seeds were then introduced making a pore and the respective inoculums of the bacteria was applied (1 ml broth to each PVC bags). Six of the PVC bags were not inoculated with any bacteria (control). At every 5 days, 10 ml of sterile distilled water was poured to each bag to compensate the excessive dessication. This was all performed inside the temperature controlled green house.

3.4.8 Acid/Alkali production characteristics

The isolates were studied for its behavior of acid and alkali production. Both solid and liquid media were employed for this purpose.

Acid/Alkali production on Solid (YMA-BTB) medium

The bacterial isolates were cultured on YMA-BTB medium to observe the acid and alkali producing nature of rhizobial isolates. Single bacterial colony from the pure culture of all the isolates were streaked (continuous streaking) on the plates containing YMA-BTB. YMA-BTB plates were labeled and incubated in inverted position at 28 ± 1^0c for their growth period.

Acid/Alkali production on liquid medium

YMB broth with the usual p^H 6.8 was adjusted using 0.1N NaOH and 0.1N HCl depending upon the cases. This broth was poured (125 ml) in each of the two 250 ml conical flasks. They were then inoculated with one loopful of 72 hours old culture and placed in the horizontal shaker (28 ± 1^0c and 120 strokes min^{-1}) for 0, 28, 48, 72 and 96 hours. Resulted cultures in each time period were then subjected to centrifugation (2000 rpm for 15 minutes) and the supernatant was used to estimate p^H by a p^H meter. A periodic estimate with fresh culture was accomplished.

3.4.9 Mean generation time

The time required for a doubling of a given cell population or one cell to become two is referred to as the generation time or doubling time (Somasegaran and Hoben, 1994). The data for mean generation time calculation was taken out by both the methods; viz; pour plate method and drop plate method (Miles and Misra, 1938; Vincent, 1970).

Pour-Plate method

For the estimation of the population of the bacterial cells by pour plate method, one loopful of the 72 hours old rhizobial isolates was inoculated in the sterilized 125 ml YMB in aseptic condition placed in horizontal shaker (28 ± 1^0c and 120 strokes min^{-1}). This was then examined regularly at an interval of every 24 hours. For this, one ml of the broth was pippeted out and ten fold dilution series was performed. The extent of the dilution for the bacterial count (to be plated) was 10^{-11}. Inoculum of 0.06 ml was pipetted out from the 10^{-11} dilution series and pour plate method was used to perform viable counts for each culture on the difference of 24 hours. This method was applied separately for both the isolates and was terminated after the 96 hours of the experiment commencement.

Drop-plate counting and inoculation of YMB medium

The liquid medium (*i.e.*, YMB) was prepared (constituents as per Somasegaran and Hoben, 1994) and its p^H adjusted approximately to 6.8 by the help of 0.1 N HCl and 0.1N NaOH. 125 ml of the liquid medium was poured in each of four 250 ml conical flasks and was autoclaved. After cooling this autoclaved media was then inoculated by a much diluted (0.5 ml) pure culture of the isolates separately. Counting of the *Rhizobium* was done from the zero time, so the flasks for the zero time was done separately whilst the others were shaked on horizontal shaker at temperature of 28 ± 1^0c and 120 strokes per minute.

Pasture-pipette Calibration

For dropping of the rhizobial isolates, pasture pipettes were employed. Firstly, ten Pasteur pipettes were calibrated for volume per drop. For this, 100 drops of tap water was collected from the pipette in 10 ml measuring cylinder and the final volume occupied was divided by 100 to obtain volume of each drop. For *e.g.*, 14 gauge pasture pipette tip contains 0.03 ml per drop. Therefore, 1 ml contains 33 drops.

Preparation of dilution series

In order to prepare the dilution series, one ml inoculated YMB colonies from one conical flask were diluted by 9 ml sterile water to get concentration of 10^{-1}. Similarly, dilutions up to 10^{-10} were made.

Calculation of Mean generation time

For calculating the mean generation time, following formula (Power and Dagniwala, 1982) was employed.

$G = t/N$

$$= \frac{t \log 2}{\log B_n - \log B_0}$$

Where,

t = Time of incubation

G = Generation time

B_o = Number of organisms at zero time

B_n = Number of organisms after n generation

N = Number of generation

3.4.10 Growth Rate Constant («)

During the exponential (or logarithmic) growth phase, a bacterial culture mimics a first-order chemical reaction, *i.e.*, the rate of increase of cells is proportional to the number of bacteria present at that time. The constant of proportionality, μ is an index of the growth rate and is called the growth rate constant.

Rate of increase of cells = μ × no. of cells

The value of μ can be determined from the following equation:

$\text{In } N_t - \text{In } N_0 = \mu (t - t_0)$

Where,

$\text{In } N_t$ = natural log of the no. of cells at time't'.

$\text{In } N_0$ = natural log of the no. of cells at time zero (t)

$(t - t_0)$ = time interval

For easier, it can be converted to:

$\text{Log}_{10}N - \log_{10}N_0 = (\mu I 2.303) (t - t_0)$

Alternatively,

$$\mu = ((\log_{10}N - \log_{10}N_0)2.303) / (t - t_0)$$

Relation of '«' and 'g'

$\mu = In2/g = 0.693/g$

3.4.11 Experimentation

3.4.11.1 Laboratory Experimentation

Collection and preparation of soil for potting

Soil from a depth of 10-15 cm was taken out with the help of the steel spade and the sampling of the soil was taken randomly within a soil type. The large pieces of soil were broken with the help of the wooden mallet. The soil was then spread and was allowed to air dry. The soil was thoroughly mixed and was shifted using a 5 mm mesh screen. The p^H of the soil was recorded using its small sample. The p^H was adjusted to 6.0 - 6.5 by adding lime (if the soil is acidic). The soil and lime were thoroughly mixed and were allowed to equilibrate for at least 7 days covering it with a polythene sheet.

Experiment using earthen pots

Strong earthen pots of 15-16 cm diameter and 18 cm height with a capacity of just over 3 liters and with at least one hole at the bottom were obtained. Plastic bags of suitable size and similar thickness were used as inner liners for the pots punching the holes for proper drainage at the bottom. Pots of this size hold 4.4 - 4.7 kg of soil high in organic matter. Approximately 4.5 kg of soil was weighed and was placed in the pot.

Before placing the soil in the pots, a little amount of coarse granules (stone chips) were placed at the bottom of the pots to facilitate the drainage. After the soil was placed in the pots, the soil was again covered with the chips in order to check the unwanted growth of the weeds.

3.4.11.2 Experimental layout

Table 1: (Treatment combinations 4 × Rhizobial Isolates 2) ×4 replications

S.N.	Treatment Combinations	Rhizobial Isolates							
		Replications							
		R_1				R_2			
		I	II	III	IV	I	II	III	IV
1	I + N								
2	I - N								
3	O + N								
4	O - O								

Morphologically similar and healthy seeds of the same variety of *Vicia faba* were taken for this experiment. Seeds were surface sterilized by using sodium hypochlorite and were allowed to germinate, as mentioned in the infection test. Well germinated six seeds were planted in each pot and covered with the sterilized pebbles. Only three similar plants were kept after germination in a triangle shaped at their ends and the remaining were uprooted. In each pot, different kinds of treatments were performed, *i.e.*, first treatment inoculation with Nitrogen, second inoculation without nitrogen, third nitrogen without inoculation and the fourth was without nitrogen and inoculation (*i.e.*, control). Four replications of each treatment were performed for both the rhizobial isolates. For inoculation, 1 ml of diluted liquid inoculums was used on five days of germination and as a nitrogen source, urea (Co $(NH_2)_2$), 50 mg kg^{-1} of soil *i.e.* 250 mg for 5 kg of soil) was employed. This is depicted in the experimental layout table.

Plant Care

Intensive plant care was done in a closed green house chamber and the plants were well nourished with modified Jensen's N-free medium at an interval of 10 days and were watered (tap water) in every alternate day. Weeds and other things except the *Vicia faba* were uprooted and removed regularly.

3.4.11.3 Study parameters

The parameter as suggested by Somasegaran and Hoben, 1994 were employed which are reliable to verify biological nitrogen fixation.

The plants were first harvested at 20 days after plantation and repeated at every 10 days ending on 40 days. At first, four plants of each treatment were uprooted *i.e.* one plant from one earthen pot with one each from four replications. The plants within PVC were kept in water inside water bucket. The plants with undamaged roots and nodules were again cleaned in clean water. The plants of each treatment were kept in labeled paper and kept for the data collection.

Nodule Induction

The total nodules present on the crown root zone (regarded as 5 cm, below the first lateral root) Corbin *et al.*, (1977) were counted and recorded for each plant from the

first harvest. The nodules that were visible were detached with forceps and were counted and were kept for preservation covered with the aluminum foil.

Shoot length

After each plant harvest, length of the plant part above the cotyledon up to the apex of the plant was recorded as the shoot length. The experiment was carried out till the last harvest of the plant.

Biomass

Nodules Biomass

All the preserved nodules were well labeled and was then oven dried at 70^0c for 48 hours. The oven dried nodules were weighed and the data were recorded for the total biomass contents of nodules for each sample of each treatment (Bergersen, 1980).

Shoot biomass

The harvested plants were derooted and the remaining shoots were wrapped in a well labeled aluminium foil and were allowed to dry at 70^0c for 48 hours to get shoot biomass.

3.4.11.3 Ureides Analysis

For estimating the ureides assimilated and translocated, the experimental layout was just as similar to that of the previous experiments. The difference is that the numbers of plants in the pots were six and were analyzed at an interval of every five days starting from 20 days after sowing to 45 days performing six run of experiments.

The principles for the ureides analysis is based on the colorimetric method as described by Young and Conway, (1942) in which allantoin is first hydrolysed under a weak alkaline solution at 100^0c to allantoic acid which is hydrolyzed to urea and glyoxylic acid in a weak acid solution. Glyoxylic acid thus formed reacts with phenylhydrazine hydrochloride to produce a phenylhydrazone derivative of the acid. The product forms an unstable chromophore with potassium ferricyanide and the color is read at 522 nm.

The procedure followed by Young and Conway requires critical timing of the reactions and the reading of standard and samples absorbance must be done within a shortest possible time-span, since absorbance decreases with time. Therefore, few samples in duplicates should be processed in each run. A set of standards and a blank (using dH_2O) in duplicate are processed.

Control of Standards

Standard Preparation

For accuracy of weighing on balance, a large quantity of each working standard solution was made. For this, 50 mg of allantoin was weighed and was transferred to a 500 ml volumetric flask. This was dissolved in about 100 ml 0.01 M NaOH, and topped out to volume with dH_2O. The addition of NaOH is only to help to dissolve allantoin.

For preparing 50 ml of the working standards 10, 20, 30, 40, 50 and 60 mgl^{-1} respectively, stock solution of weight 5, 10, 15, 20, 25 and 30 g was accurately weight into 50 ml volumetric flask and was topped up to volume with dH_2O. Each working standards were stored as small aliquots in the freezer and only the necessary quantities were thawed and the remaining over were discarded.

Procedure followed for Ureides Analysis

Firstly, the plants grown under the aseptic conditions and inoculated with the different rhizobial strains were uprooted at different time intervals starting from the twenty days after sowing. A portion of the stem (approximately 3.0 cm) above the ground was taken and grinded finely by the mortar and the pestle.

Then, one ml each of the plant sample (the grinded mixture), QC sample (*i.e.*, the test sample for measuring out the consistency), standard or distilled water (blank) were pipette out and put into the 15 ml tubes. These individual tubes were then treated with 5 ml of distilled water and 1 ml of 0.5 M NaOH followed by vigorous shaking or using a vortex mixture. NaOH was used for the color development at room temperature.

These test tubes were then placed in the boiling water bath for approximately seven minutes followed by cooling the tubes in cold water. To each tube 1 ml of HCl (0.5

M) was added adjusting the pH within 2-3. The tubes were then treated with 1 ml of the phenylhydrazine solution and were again transferred to the boiling water bath for exactly 7 minutes. The tubes were then removed from the hot water bath and were placed immediately in the icy alcohol bath for 10 minutes. The fresh solutions of phenylhydrazine HCl and potassium ferricyanide should only be applied to get the better results.

Keto-acids did not significantly interfere with the determinations of ureides except for glyoxylic acid in extracts of fresh broad bean tissue. The interference caused by glyoxylic acid was avoided by adding phenylydrazine HCl to the solution of NaOH used for alkaline hydrolysis of allantoin (Peter *et al.*, 1983).

The cooled tubes were treated with 3 ml of precooled concentrated HCl and 1 ml of Potassium ferricyanide. This was operated in a fume cupboard within the shortest possible time span. This was then mixed thoroughly and was transferred to 4.5 ml cuvettes at room temperature. The absorbance with blank (or distilled water) was adjusted at 522 nm and the absorbance was taken for each tubes exactly after 20 min of addition of potassium ferricyanide reagent. The absorbances for the different tubes were done quickly without pausing and within the shortest time span since the color fades out gradually. Finally, the absorbance readings for the different tubes were recorded.

These data's were then used to plot out the standard curve. The allantoin standard curve was then plotted with allantoin concentration (mgl^{-1}) on x-axis to optical density (at 522 nm) on Y-axis.

Calculation of the Concentrations

For calculating the concentration of the QC sample and the samples from the measured absorbance, Young and Conway (1942) formulated a formula,

i.e., $C = (Y - a) \div b \times f$

Where,

C = concentration of the unknown

Y = absorbance

a = Intercept of the standard curve

b = slope of the standard curve

f = dilution factor

Average of the duplicates were calculated and the percentage difference in duplication were calculated by using the formula

Percent difference (%) = [Replicate 1-Replicate 2] ÷mean of two replicates×100

The percent difference between two replicates should not exceed more than 10%.

This experiment was repeated at an interval of every five days starting from 20 days after sown. These data's were then plotted on the allantoin calculation database and also shifted to the quality control database.

3.4.11.4 Total Nitrogen Content (%)

Nodules Nitrogen

The total nitrogen (%) of nodules was determined by Micro-Kjeldahl method (PCARR, 1980). The chemicals needed for this experiment is tabulated in the Annex 4. This method includes following three steps:

a) Digestion

Two hundred and fifty milligram (mg) of grinded nodule sample was passed via 0.425 mm sieve and was transferred to a dry Kjeldahl digestion flask of 300 ml. Then it was digested in 6 ml conc. H_2SO_4 with one gram of mixed catalyst (prepared by mixing $CuSO_4$, K_2SO_4 and Selenium in 100:10:1 ratio). Then Kjeldahl digestion flask was heated first by adjusting heating mantle knob on 2 and after 10 minutes the knob was adjusted to 7 for an hour until apple green clear solution appeared in the Kjeldahl flask. Then the flask was removed immediately from the mantle and allowed to cool down for 30 minutes. To the digest, 60 ml of dH_2O was added and the mixture was shaken well. As a result, total nitrogen gets converted into ammonium sulphate. A blank without plant sample was also run for each batch of plant samples digested via this process.

b) Distillation

The diluted digest of Kjeldahl digestion flask was now transferred to Kjeldahl distillation flask. A beaker of 100 ml capacity with 10 ml of boric acid indicator was placed below the nozzle of the condenser in such a way that the end of the nozzle dipped into the indicator. 30 ml of 40% NaOH solution was poured into the

distillation flask and heated on heating mantle. The ammonia evolved as a result of reaction was trapped in 10 ml boric acid (4%) indicator due to which the pink color of indicator changed into green. The distillate was collected in indicator until the volume became more than 40 ml in boric acid indicator containing beaker.

c) Titration

The NH_3 trapped in distillate was titrated with 0.1N HCl solution and the end point was noted by observing changing of the color of the indicator from green to pink. The volume of HCl consumed in titrating distillate was recorded. The volume of acid consumed by both blank and nodules samples were noted and on the basis of which the total nitrogen content (%) of the plant sample was calculated by using following formula:

$$\text{N (\%)} = \frac{14 \times N \times (S - B) \times 100}{M}$$

Where, N = Normality of HCl

S = Volume of HCl consumed with plant sample (ml)

B = Volume of HCl consumed with blank (ml)

M =Mass of plant material (mg)

Shoot N (%)

For the analysis of the shoot Nitrogen, the sample process was employed same as that for nodules nitrogen.

Leaf N (%)

Nitrogen content analysis of the leaf blade along with the petiole was carried out and the process was same as that of nodules nitrogen.

3.5 Statistical Analysis

3.5.1 Analysis of Variance (ANOVA)

Statistical analysis for analysis of variance was done according to the methodology as suggested by Somasegaran and Hoben (1994).

Table 2: General Table for Analysis of Variance

Source of Variation	Sum of Squares	df	Mean Square	F Ratio
Treatment	SST	k - 1	$\frac{SST}{(k-1)}$	$\frac{SST}{SSE} \times \frac{(bkn-k-b+1)}{(k-1)}$
Blocks	SSB	b - 1	$\frac{SSB}{(b-1)}$	$\frac{SSB}{SSE} \times \frac{(bkn-k-b+1)}{(b-1)}$
Error	SSE	bkn - k-b+1	$\frac{SSE}{(bkn-k-b+1)}$	
Total	SS	bkn-1		

Where, no. of treatment combinations = k

Types of bacteria blocks = b

No. of replicates per treatment per block = n

Grand total (summation of all treatments) = GT

Correction factor (CF) = $\frac{(GT)^2}{bkn}$

Total sum of squares (SS) = Σx^2 - CF

Isolates sum of squares (SST) = $\frac{\sum T^2}{bn} - CF$

Bacterial sum of square (SSB) = $\frac{\sum B^2}{kn} - CF$

Error sum of squares (SSE) = SS - (SST + SSB)

The calculated value of f-ratio was compared with tabulated value at 5% level of significance. The variable with ($p < 0.05$) was considered significant and $LSD_{0.05}$ value was calculated to locate the significant effects.

$$LSD_{0.05} = t_{0.05} \sqrt{\frac{2S^2}{No.\ of\ \ replications}}$$

Where,

$t_{0.05}$ = the tabulated value of t for degrees of freedom for error at the 5% probability level.

S^2 = Mean square for error.

n = number of replications.

The LSD values were compared with the difference in marginal mean of variables. According to which, inference was made for variance in variables.

3.5.2 Correlation of Variances

The correlations express the relationship or inter dependence of two sets of variables upon each other in such a way that the changes in the magnitude of one variable are accompanied by the changes in the magnitude of another variable. Correlation is the numerical measurement showing the intensity of relation between two variables. One variable may be called independent and other dependent variable.

This is calculated by using Karl Pearson's coefficient of correlation (Harold and Kiess, 1996)

$$r = \frac{Co\,\text{var}\,iance\ of\ \ xy}{\sqrt{\text{var}\,iance\ of\ \ x}\ \sqrt{\text{var}\,iance\ of\ \ y}} \text{ ---------- (i)}$$

$$\text{or, } r = \frac{n\sum xy - \sum x \sum y}{\sqrt{n\sum x^2 - \left(\sum x\right)^2}\ \sqrt{n\sum y^2 - \left(\sum y\right)^2}} \text{ ---------- (ii)}$$

Where,

n = number of variables

x & y = different variables

Σx = sum of variables of x

Σy = sum of variables of y

Σx^2 = sum of the square of x

Σy^2 = sum of the square of y

Σxy = sum of the multiple of x and y

CHAPTER FOUR
RESULTS

4.1 Isolation of Rhizobial Isolates

The two rhizobial isolates of two climatic regions *i.e.*, Manang (trans-himalayan region) and Kathmandu Valley were isolated and their pure culture was extracted by pour plate (YMA-CR), plating exhibited same physical characteristics of colonies *i.e.*, the individual mature colony had entire margin, milky translucent, glummy with convex outer surface. Within five days at a temperature of 28 ± 1^0c a mature colony with its diameter of 0.1 - 1.1 cm was grown. The liquid rhizobial inocula *i.e.* Yeast Extract Mannitol Broth (YMB) obtained after five days were highly turbid and milky white in color.

4.2 Characteristics of Rhizobial Isolates

The different rhizobial isolates were characterized by studying their presumptive morphological and the physiological characteristics.

4.2.1 Colony Characteristics and Growth Response on YMA-CR Medium

All the bacterial colonies grown in YMA-CR plates were creamy white to translucent, watery, smooth, circular and raised. The bacterial colonies did not absorb the red color of Congo red when cultured in dark which is the characteristics feature of rhizobial colonies.

4.2.2 Growth-response on YMA-BTB Medium

The blue color of bromothymol blue (BTB) was changed to yellow after the significant growth of the rhizobial colonies on YMA-BTB plates. Thus, both of the rhizobial strains (*i.e.*, R_1 and R_2) were found to be acid producing in the culture within an interval of 5 days when incubated in dark at 28 ± 1^0c.

4.2.3 Acid/Alkali Production in Liquid Medium

For estimating whether the rhizobial isolates to be acid or alkali producing, the broth culture technique was generally employed. The change in p^H of the broth was observed throughout the experiment which is illustrated in fig. 1, Annex 8. Table 3 shows that *R. leguminosarum* bv. phaseoli to be more acid producing than the other rhizobial isolate *i.e., R. leguminosarum* bv. viceae.

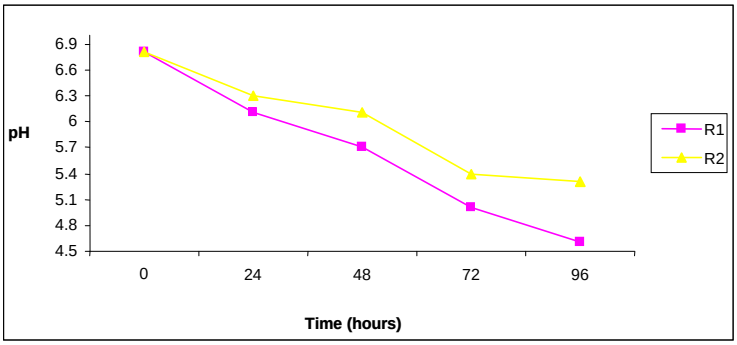

Figure 1: Change in pH of broth of rhizobia in different time intervals.

4.2.4 Gram Staining

Testing of the rhizobial isolates towards the Gram Stain and their morphological characters by Gram Staining reveal that both of the isolates R_1 and R_2 were Gram negative. Both of them did not take violet color of crystal violet but took pink color of safranin; thus appeared pink rod shaped when observed under oil immersion. (Photo plate 2: photo 6)

4.2.5 Mean Generation Time

Counting of the rhizobial population of different time intervals were carried out simultaneously by drop plate and pour-plating method. However, the drop plating method showed the best result. These datas were used to calculate the mean generation time. The mean generation time was calculated for the exponential phase only (Somasegaran and Hoben, 1994) and the mean generation time was plotted between (log (n) = no. of cells) and time which is illustrated below:

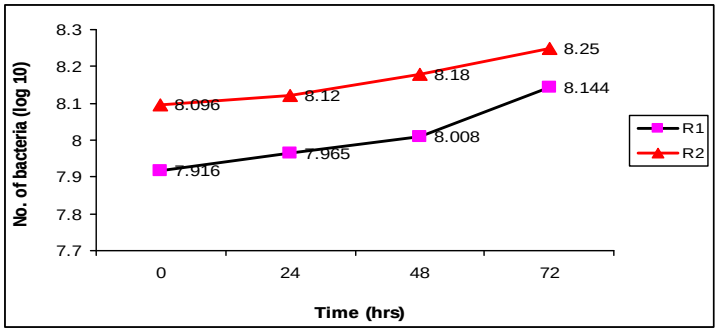

Figure 2: Multiplication of bacterial cells in different time intervals.

4.3 Authentication of the Rhizobial Isolates

For the confirmatory test of the isolates to be *Rhizobium*, the experiment was performed in growth pouches of 5 days old rhizobial isolate of R_1 and R_2 at different dilution range *i.e.*, dilution of 4, 6, 8 and 10. The physical appearance of nodules on the root system of *Vicia faba* L. confirmed the isolates to be *Rhizobium.* Photo plate 1 (Plate 9), Photoplate 2 (photo 8)

For the authentication of native rhizobial isolates, plant infection test was also carried in sterile sand. The rhizobial isolates obtained from *Phaseolus vulgaris* L. and *Pisum sativum* L. nodules were authenticated to be the *R. leguminosarum* strains by examining their capability to nodulate the *Vica faba* L. (Broadbean) seedings under bacteriologically controlled condition (Photo plate 1; photo 1, 2, 4). The broad bean seedlings without inoculation did not have any traces of nodules on their roots after 21 days with some exceptions. (Photo plate 1, photo 5)

4.4 Study Parameters

4.4.1 General

Two of the rhizobial isolates were taken from two different locations and were tested on an entirely different plant. Rhizobial isolates were collected from *Phaseolus vulgaris* L. and *Pisum sativum* L. and were authenticated on *Vicia faba* L.

4.4.2 Nodules Induction

Counting of the nodules was done after 20 DAS. At 20 DAS, isolate R_1 had the greater effect on nodulation than that of R_2 isolate.

On first isolate (*i.e.*, R_1), treatment combinations of R_1 (I-N), R_1 (I-N) and R_1 (I + N) had the maximum values for the nodules induction at 20, 30 and 40 days respectively.

Similarly, on the second isolate (*i.e.*, R_2), treatment combinations of R_2 (I-N), R_2 (I-N) and R_2 (I + N) had the maximum values for nodule induction at 20, 30 and 40 days respectively.

At 40 DAS, the nodules collected were globose and streaked dull blood red colored. The two isolate showed significance variance at 5% level of significance. The analysis of variance showed the significant difference in different treatment combinations and blocks (treatment = 13.350; Blocks = 3.163; $P<0.05$; LSD = 6.362).

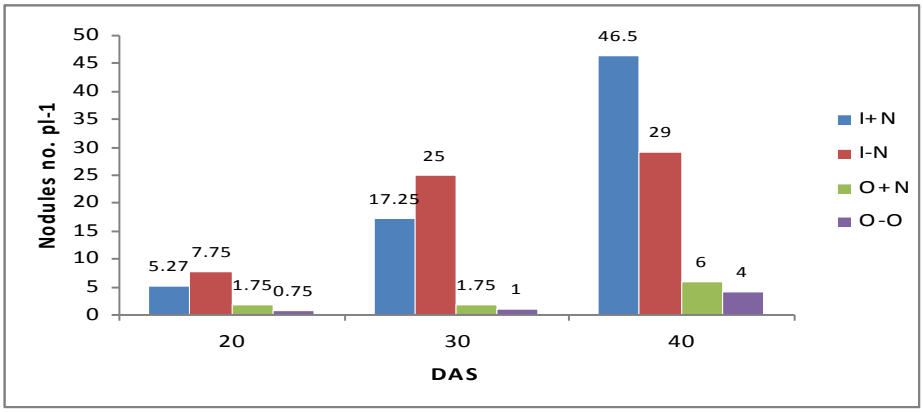

Figure 3: Effect of rhizobial isolate (R_1) on nodules number.

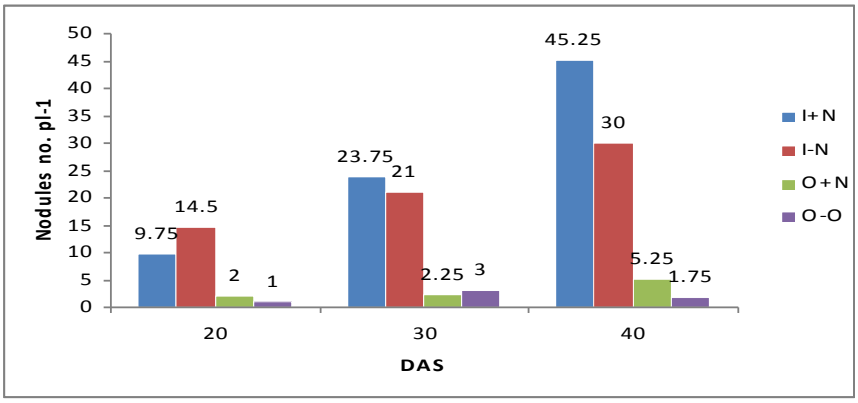

Figure 4: Effect of R_2 on nodules number

4.4.3 Shoot Length

As far as the length of shoot is considered, it was revealed that the inoculation of the first rhizobial isolate (R_1) had maximum values for the treatment combinations of R_1 (0-0), R_1 (I+N) and R_1 (0+N) and the least values of R_1 (I + N), R_1 (I-N) and R_1 (0-0) at 20, 30 and 40 days respectively.

Similarly, inoculation of the second rhizobial isolate (R_2) had the maximum values (length) for the treatment combinations of R_2 (0+N) and least for R_2 (0-0) on every run of experiment.

Analysis of variance for the shoot length of both the rhizobial isolates were found to have the significant differences in different treatments and the blocks (Treatments = 4.462; blocks: 12.573; $P < 0.05$; LSD = 4.566).

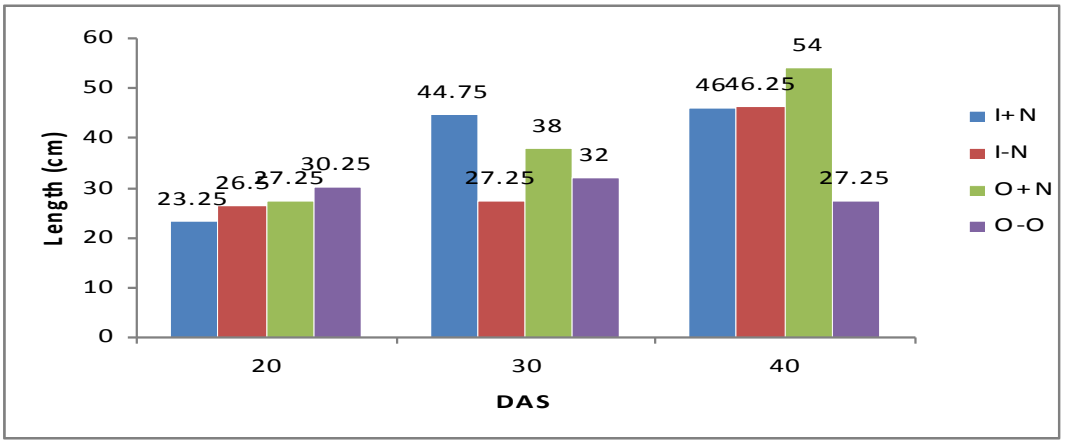

Figure 5: Effect of R_1 on shoot Length

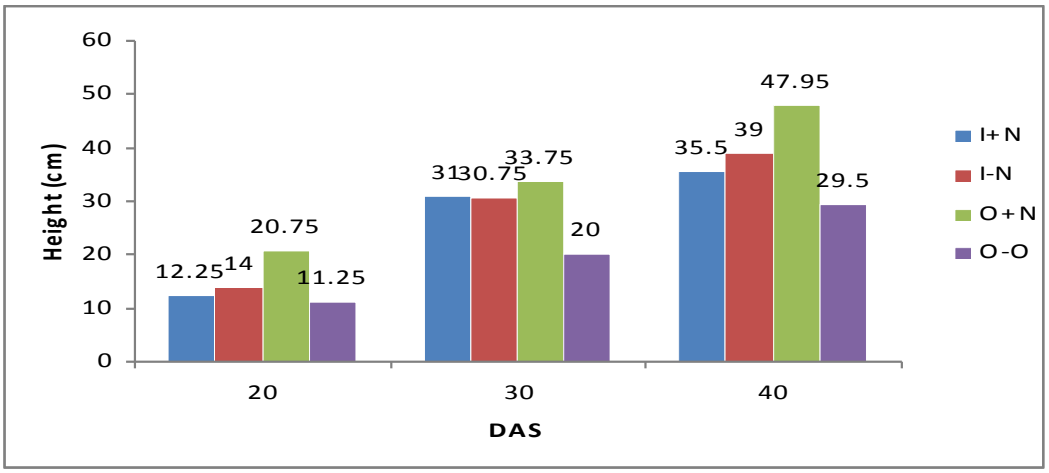

Figure 6: Effect of R_2 on shoot length

5.4.4 Roots Length

Increment in length of the roots was observed on the test plant by the inoculation of the rhizobial inoculums. By the inoculation of the first rhizobial inoculum (R_1) on test plant, treatment combination of R_1 (I-N) showed the maximum value at 20, 30 and 40 days after sown.

Similarly while observing the length of the roots by the second rhizobial inoculums (R_2) on the test plant showed that the treatment combinations of R_2 (0+N) to be maximum at 3 run of experiments *i.e.*, at 20, 30 and 40 DAS.

The analysis of variance revealed the insignificant difference within the treatment combinations (1.667) while significant difference on blocks (26.102) with LSD value of 8.00 cm.

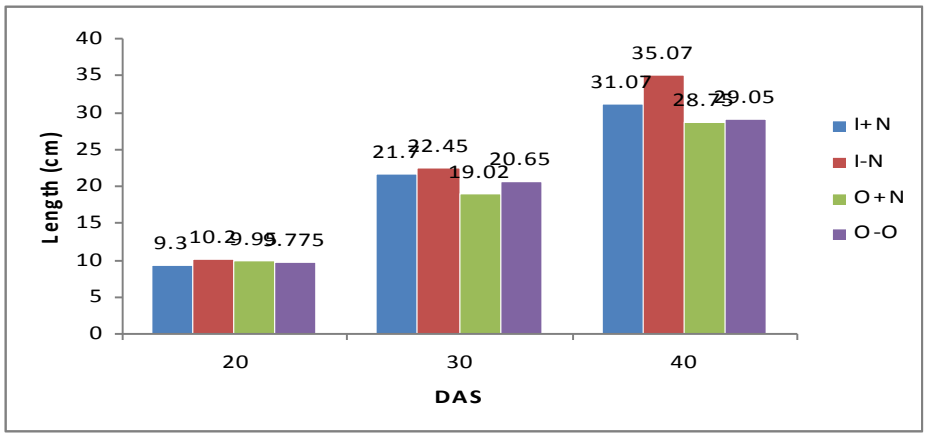

Figure 7: Effect of R_1 on roots length at different time interval

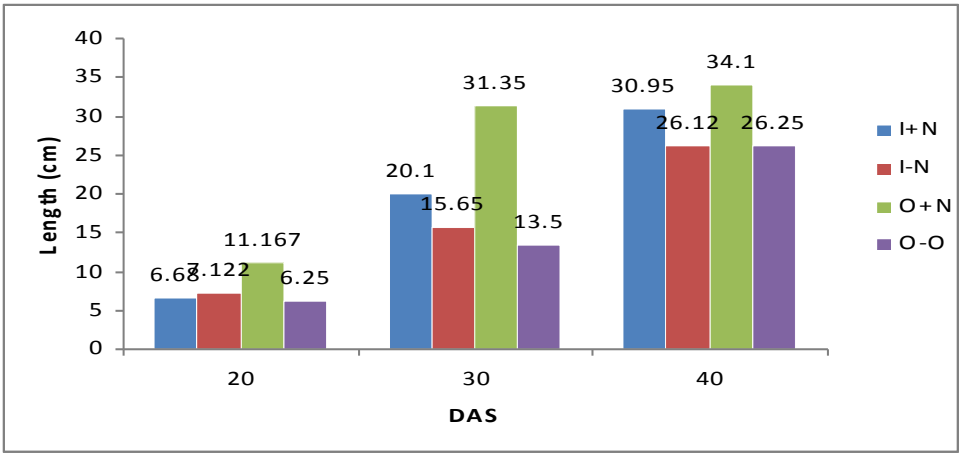

Figure 8 : Effect of R_2 on roots length at different time interval

4.4.5 Biomass

Biomass refers to the dry weight of plant materials dried in hot air oven at 78^0c for 48 hours.

4.4.5.1 Nodules

The increment of nodules biomass was recorded in each experiment starting from 20 DAS and ending at 40 DAS. At 40 DAS, for both rhizobial incoculum, treatment combinations (I+N) had maximum effect on nodule biomass while the control had the minimum effect.

The sequences of inoculums effects of R_1 and R_2 at 40 DAS was found in the order (I + N > I - N > 0 + N > 0 - 0) and for the analysis of variance showed the insignificant difference in treatment combinations and the blocks (treatments = 1.98; blocks = 0.934; P > 0.05; LSD = 0.479g)

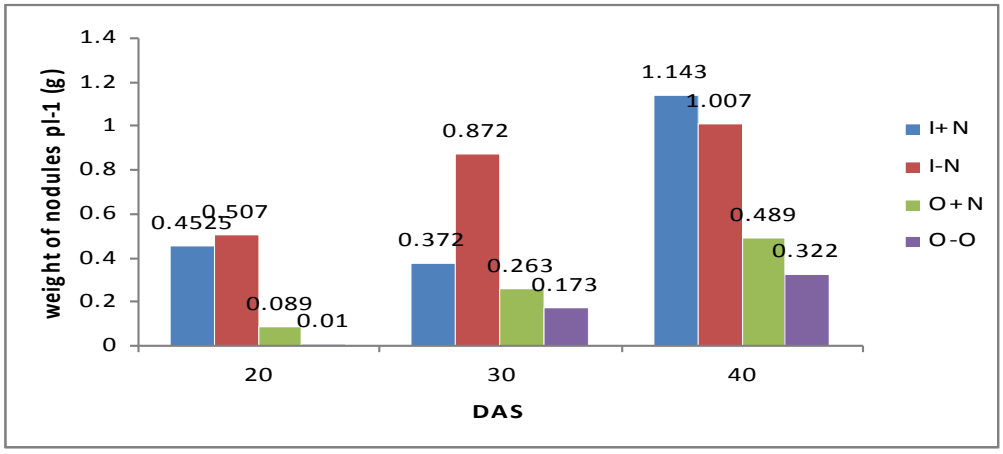

Figure 9: Effect of R_1 on nodules biomass

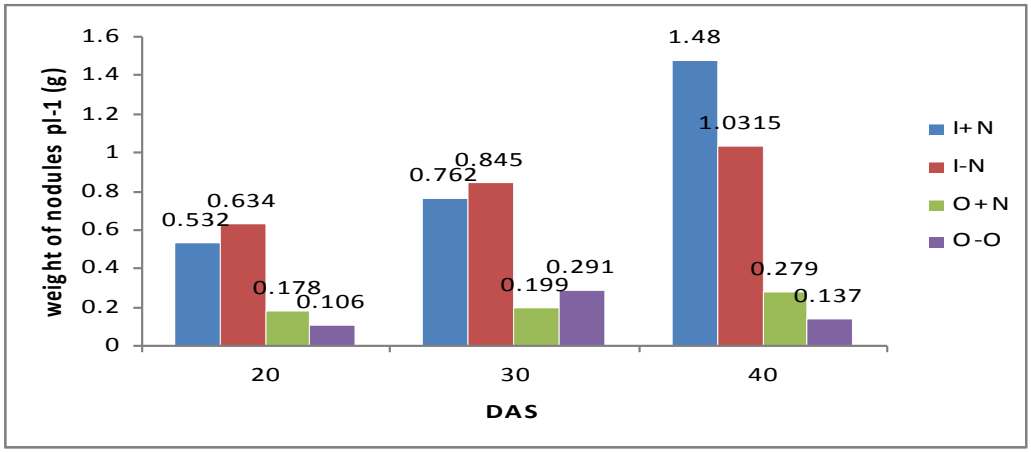

Figure 10: Effect of R_2 on nodules biomass

5.4.5.2 Shoot Biomass

Shoot biomass was calculated at every 10 days starting from 20 DAS. For R_1 the highest value recorded was for the treatment combinations of (I+N), (0+N) and (O+N) at 20, 30 and 40 DAS respectively. Similarly, for R_2 the highest value recorded was for the treatment combinations of (I+N), (0-0) and (0-0) at 20, 30 and 40 DAS respectively.

The analysis of variance of shoot biomass of two rhizobial isolates on treatments and blocks were found to be insignificant. The sequences of result for the significance analysis for R_1 was in the order [R_1 (O+N) > R_1 (I+N) > R_1 (I-N) > R_1 (O-O); P > 0.05] and for R_2 was [R_2 (O+N) > R_2 (O-O) > R_2 (I+N) > R_2 (I-N); P > 0.05] (treatments : 0.493; Blocks : 2.165; P > 0.05; LSD = 0.199g).

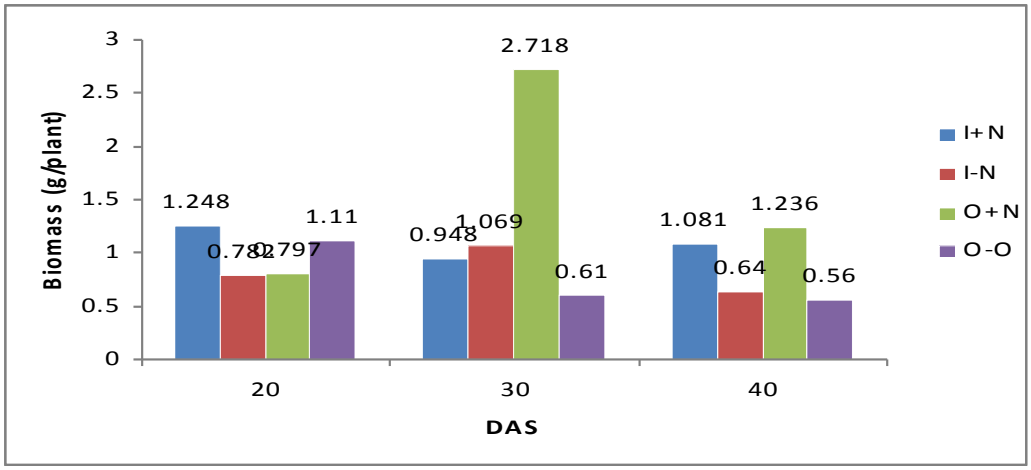

Figure 11: Effect of R_1 on shoot biomass

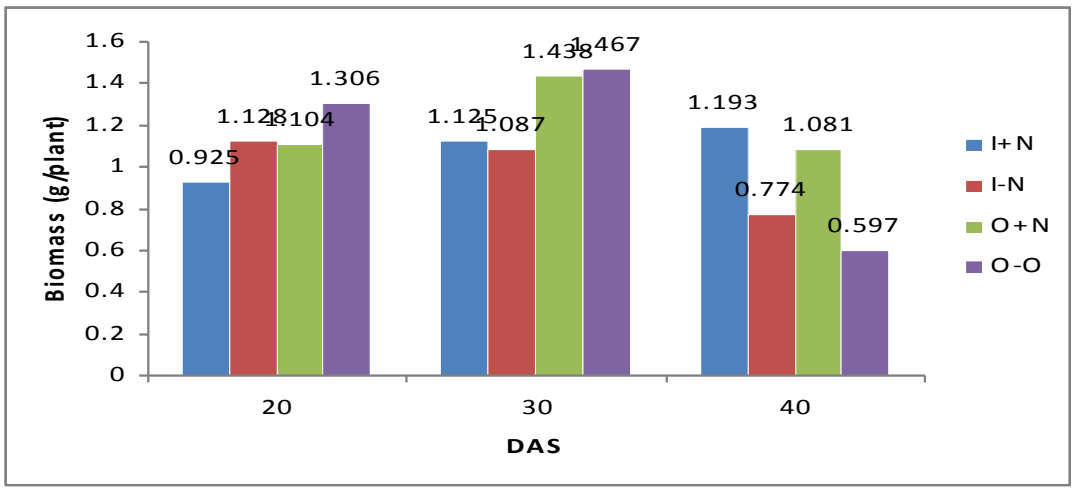

Figure 12: Effect of R_2 on shoot biomass

4.4.6 Total Nitrogen

The estimation of Nitrogen was done separately for the different parts of the plant and the used parts of the plants were nodules, stems and leaves (leaf blade with petiole).

4.4.6.1 Nodules N (%)

Estimation of the nodules N was performed by Micro-Kjeldahl method (PCARR, 1980). The experiment was carried out on the nodules collected in an interval of 10 days starting from the 20 DAS.

The percentage of nodules N for R_1 was in the order (I+N) > (O - O) > (O + N) > (I - N) and for R_2 (O + N) > (I + N) > (I - N) > (O - O). The analysis of variance showed the insignificant differences in both the treatment combinations and the blocks (Treatments = 1.258; blocks 1.41; P > 0.05; LSD = 0.251%).

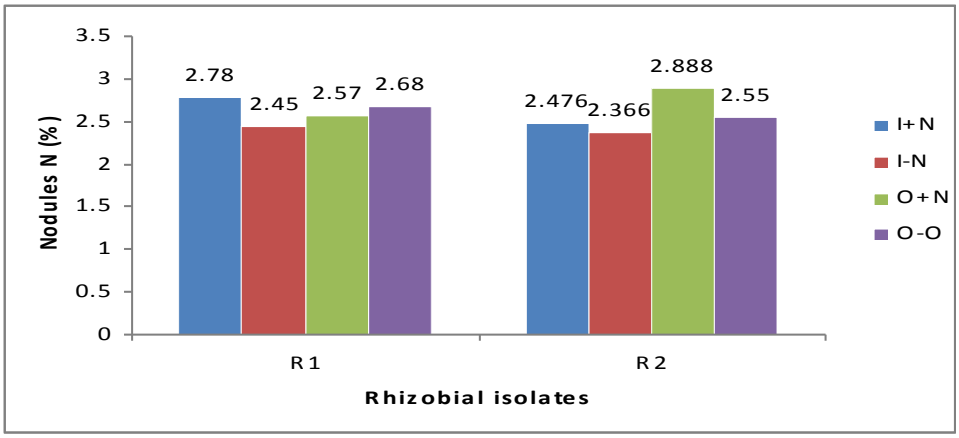

Figure 13: Estimate of nodules N (%)

4.4.6.2 Stem N (%)

Estimate of stem N was done as similar to that of the nodules. The percentage of the stem N for R_1 was found out in the order of (I - N) > (O + N) > (I + N) > (O - O) and that of R_2 in the order of (O + N) > (I + N) > (O - O) > (I - N). The analysis of variance of both the rhizobial isolates on treatments and blocks were found out to be non-significant (Treatment = 1.328; Blocks = 0.859; P > 0.05; LSD = 0.202%).

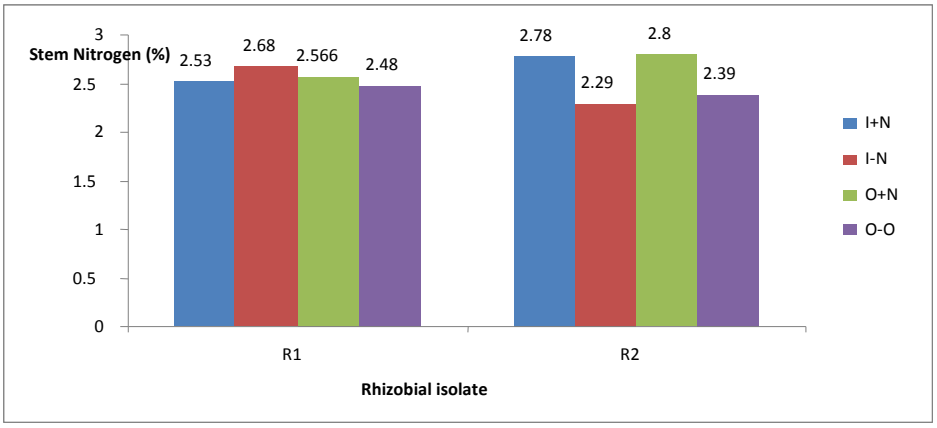

Figure 14: Estimate of stem N (%)

4.4.6.3 Leaf N (%)

Nitrogen estimation of leaf for both the isolates was found to be in the order to (I + N) > (I - N) > (0 + N) > (0 - 0). The analysis of variance of rhizobial isolates on treatments was found to be significant and within the blocks to be insignificant (Treatments = 4.028; Blocks = 0.864; LSD = 0.311).

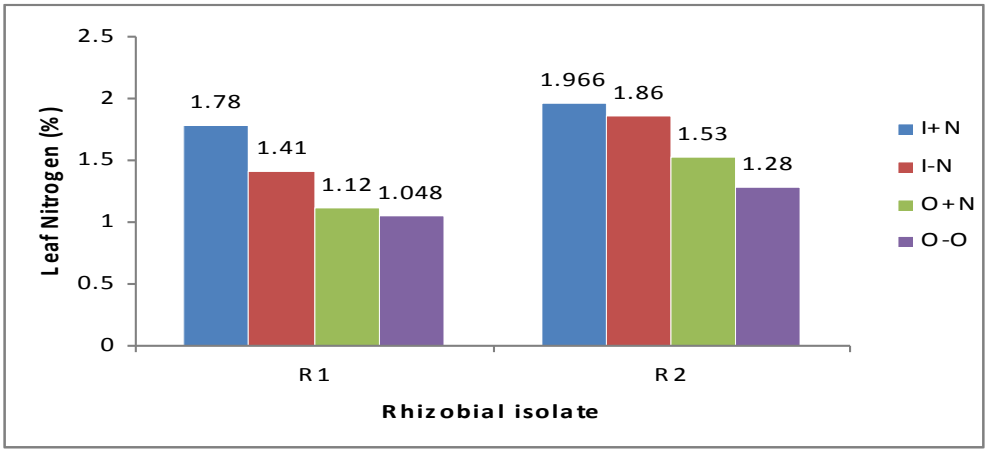

Figure 15: Estimate of leaf N (%)

4.4.7 Total Ureides (Allantoin and Allantoic Acid) Content

The estimation of ureides was performed according to the principle as describe by Young and Conway. The plants with different treatment combinations on different time intervals of time were taken out for the experiment. Plants with control (without treatments) and the QC sample were also simultaneously analyzed. Six run of the experiment were carried out at the interval of 5 days each starting from the 20 days after sowing and ending at 45 DAS.

Firstly, the standard curve of allantoin was obtained at each run of the experiment followed by the significant change in the concentration of the allantoin on the different treatment combinations. The standard curve of the allantoin observed at each run was linear and with the increase of the concentration of the allantoin, the absorbance was also increased (Annex 8).

According to the data's obtained, the concentration of ureides kept on increasing severely at the first and a gradual increase to last of the experiment. The translocation of the ureides (allantoin and allantoic acid) was higher in the inoculums provided of *Pisum sativum* L. than in *Phaseolus vulgaris* L.

And also, according to the calculation of the variance of ureides (allantoin and allantoic acid) of both the rhizobial isolates, it was revealed that there was significant difference between two population variances (Treatments = 6.633; Blocks = 3.502; $P < 0.01$; LSD = 52.146).

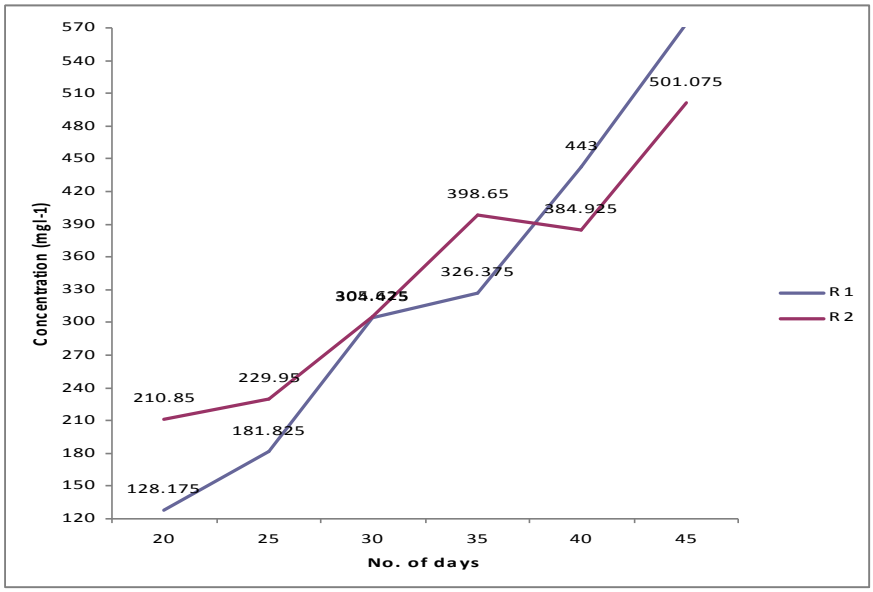

Figure 16: Pattern of change in total ureides content with plant age and inoculant rhizobia.

4.4.8 Symbiotic Effectiveness

Data's of plant biomass produced under inoculated and uninoculated as well as nitrogen fertilizer (urea) used and not used (control) treatment conditions were used to calculate symbiotic effectiveness of both the rhizobial isolates (R_1 and R_2). The rhizobial inoculation and the fertilizer nitrogen application showed no significant difference ($P>0.005$). However, plant age significantly affected the symbiotic effectiveness reaching the maximum at 4^{th} week of plant growth. However, the result showed the similarity between the inoculums of the rhizobial isolates and the urea used (50 mg kg^{-1} soil) when tested in the soil condition of Kathmandu.

CHAPTER FIVE
DISCUSSION AND CONCLUSION

5.1 Discussion

Nitrogen fixing bacteria are single celled organisms that are essentially miniature urea factories, turning N_2 gas from the atmosphere into plant available amines and ammonium via a specific and unique enzyme they possess called nitrogenase. These organisms need to undergo a rigorous selection process to choose the highest performing strains. Also, they need to be inoculated into the crop to achieve threshold levels where their numbers are great enough to effect significant nitrogen production. Legumes are very important in rejuvenating the soil.

The endosymbiont of Pea and Bean root nodules has been found to have major contribution in nitrogen supply to the growing legumes. Soil inoculated with the effective bacterial population is found to have more N-fixation than the other. Hence, inoculation of legume especially for better crop of nitrogen from compatible and efficient rhizobial strains has got popularity all over the world. However, the screenings of more efficient strains of rhizobia are done on the basis of many characteristics *e.g.*, resistant against salt, toxic metals, temperatures etc, but for every characteristic nitrogen crop is the essential determinants to satisfy their authenticity.

N_2 fixing bacteria use plant carbon as a high calorie energy source to fuel the biological reaction that converts N_2 gas into plant available N compounds. Whether be it the soil dwelling species or the endophyte N fixing species, the plant is controlling the amount of energy (plant carbon) the N fixing bacteria receive to perform their N fixation function. As such, the quantity of nitrogen being fixed for plant use is controlled by the plant itself.

For instance, when there is limited soil moisture, nitrogen fixation slows down dictated by the plants diminishing nitrogen requirements and subsequent fugal supply

of carbon to the bacteria. When soil conditions are optimum, nitrogen fixation is maximized by the plant's increasing supply of carbon to the bacterial colony, in turn increasing N fixation to meet the increasing nitrogen requirements. It works like a feedback system and assures the plant receives just the right amount of nitrogen it requires based on the growing conditions at the time.

The quantity of N fixed in nature is about 230×10^6 Tm per year (White, 2006), of this, approximately 87% is the result of biological N_2 fixation, either via symbiotic association (80%) or by free living organism (20%) (Gutschick 1980; Vance *et al.*, 1998). The current annual world industrial production of ammonia exceeds 130×10^6 Tm per year (Bakemier *et al.*, 1997), most of which is being produced by the costly Haber-Bosch process.

Present research envisages the ureides concentration and the symbiotic potentiality of two isolates considered on a single plant *Vicia faba* L. Symbiosis, induction of nodules and fixation of nitrogen are reported to be complex and variables (Allen and Allen, 1958). Strains of *Rhizobium* and the genotypes/varieties of a legume exert significant influences that determine the growth response of the host (Bergersen and Nutman, 1957; Caldwel, 1969).

Authentication of the isolates was carried out using presumptive and confirmative tests such as gram staining, microscopic observations, colony characters and plant infection (nodule induction). Legume *Rhizobium* symbiosis is significant for the increment of soil fertility in order to sustain nitrogen nutrient needs of succeeding crops in agronomic system. Adoption of legume *Rhizobium* biotechnology provides the solution to many types of ecosystem managements and conservation sustaining a healthy environmental condition.

Effective plant root nodules of *Pisum sativum* and *Phaseolus vulgaris* were collected from Kathmandu and Manang and the *Rhizobium* were cultured and isolated using the YMA media (Vincent, 1970; Dalton, 1980). *R. leguminosarum* isolates were purified by continuous restreaking of single rhizobial colony and such restreaking was performed till the pure culture was obtained for each isolate (Ogg and Sharma, 1977; Kaushik *et al.*, 2003). Rhizobial colonies of 0.8 - 1 cm diameter were obtained after 5

days of culture in YMA plates. The shorter duration of colony development showed the fast growing nature of *Rhizobium*.

Characterization of the rhizobial isolates were performed in the conformity of the acid and alkali production, Gram staining as well as the multiplication of the bacterial cells in different time intervals. The blue color of bromothymol (BTB) changed into yellow after 5 days of rhizobial culture on YMA-BTB indicating the acid producing nature of *Rhizobium.* Similarly, acid/alkali producing behaviour of rhizobia was also analyzed through broth culture. Both R_1 and R_2 showed the decrease in p^H providing that both the isolates were acid producing. Between these two, R_1 isolate was found to be more acid producing then the R_2 isolate. The p^H of broth of both the isolates was changed to 4.6 and 5.3 from 6.8 at 96 hours for R_1 and R_2 respectively. These reasons also suggest the conformity for the acid producing behaviour of rhizobia.

Authentication of the rhizobial isolates towards the Gram stain and their morphological characteristics were studied by Gram staining which reveal the Gram negative response, a characteristic feature of Rhizobia (Pelezar *et al.*, 1993; Somasegaran and Hoben, 1994). For the multiplication of bacterial cells in different time intervals it was found that the changes in number was much swift and among them R_2 multiplied more rapidly than R_1. This rapid multiplication rate was due to the availability of nutrients for their growth.

The plant in growth pouch was supplied with modified Jensen' N-free nutrient medium (Roughley, 1984). The nitrogen deficient conditions stimulate the bacterial nod gene expression which leads into nodulation of root of host plant. The positive test results for all isolates helped to confirm the suspected rhizobial isolates were isolates of genetically compatible rhizobia (Gibson, 1963; Vincent, 1970; Bergerson, 1980; Somasegaran and Hoben, 1994). Quispel (1974) suggested that the presence of sufficient combined nitrogen interfere the nod gene expression and ultimately nodulation and nitrogen fixation.

The accumulation of ammonium ion (NH_4^+) is poisonous to plant cells and is therefore immediately incorporated into amino acids (Day *et al.*, 2001). The main pathway for NH_4^+ assimilation involves two enzymes; glutamine synthase, which catalyzes the incorporation of NH_4^+ into glutamate, and NADH-glutamate synthase

which transfers the amide group of glutamine to α-ketoglutarate (Miflin and Habash, 2002). Assimilated NH_4^+ is exported from the nodules to the shoot.

Legumes with indeterminate nodules transport N is the form of amides, such as glutamine and asparagines, whereas legumes with determinate nodules transport N as ureides, such as allantoin, allantoic acid and citruline (Smith and Atkins, 2002). Ammonia derived from symbiotic fixation is converted into ureides, allantoin and allantoic acid in the nodule and then transported to the shoot in the chemical form in the transpiration stream; in contrast N taken up from the soil, which is primarily nitrate is transported either directly as nitrate or is assimilated into the amino acids asparagines or glutamine in the root prior to the transport (Herridge and People, 1990).

In glass house experiment, formation of nodules in legume root was found affected by environmental factors *e.g.* physical, p^H and nutritional (Gibson, 1967; Gates, 1974). During the experiment the needs of other requirements for the better symbiosis and nitrogen fixation were taken into consideration. Two isolates, four different treatments and four replications of each were carried out for two sets, firstly for the ureides estimation and secondly for the other different parameters like nodules number, nodules biomass, stem N, leaf N, Nodule N, plant biomass (for observing symbiotic effectiveness). The experimental soil was heat sterilized and some of the plants without inoculums also bear the nodules at different days. In uninoculated cases, *i.e.*, control provided fewer numbers of smaller dull white nodules on the root, possibly due to the infection of the local rhizobial population in the soil.

Plants with inoculums had a good deal of effective nodules on their root systems at 40 DAS. Visible nodules were observed at 20 DAS. The delay in nodulation might be due to low exposure of sunlight and continuous cloud during the initial period of the experiment. The low light exposure leads into low synthesis of photosynthetas (Carbohydrates). The carbohydrates supplied by host plants used by bacteroids as a skeleton for incorporation of NH_3 and derive energy via respiration which is utilized in nitrogen fixation and also run their own metabolism (Aslam *et al.*, 1993). Plants from low seed weight born little nodules at 20 DAS. Since small seeded plants produce low plants biomass and low photosynthetic areas at the beginning days 20

DAS thus low initial nodulation at 20 DAS may be interpreted in terms of low photosynthetas available for bacteriods.

Deswal *et al.*, (2003), revealed that nodulation depends on the production of IAA by invading rhizobia. The variation in nodules number along the compatible host inoculated with different isolates might be due to their variable infectivity and the isolate R_1 with treatment combination of inoculums and nitrogen have the highest infectivity under the prevailing conditions. When nitrate is supplied to an established symbiosis, inhibition of nodulated root N_2 fixation precedes the loss of the potential of bacteroids to fix N_2.

The nitrogen content of the root nodule, stem and leaf blades with petioles were recorded for the evaluation of symbiotic effectiveness of rhizobia of Kathmandu and Manang. For nodules nitrogen, R_2 (I-N) treatment combination showed the highest proportion of nitrogen content (2.88%) than the other isolate R_2 (which have the highest nitrogen for the treatment combination I+N, 2.78%). Similarly, the nitrogen content of the stem revealed the highest value to be of the R_2 on the application of nitrogen without inoculums (2.8% N). The rhizobial isolate R_1 possess the highest value at treatment combination of I-N (2.68% N). Likewise, while examining the N of leaves blades (with petioles), it was revealed that the treatment combination of inoculums with N gave the maximum value for both isolates (1.78 % and 1.96% N for R_1 and R_2 resp.).

Togri *et al.*, (1995) found that the total nitrogen continue to increase until maturity (70 DAS). The variation in plant biomass, shoot N, Nodule biomass and nodules N in inoculated plants might be due to the variable degree of symbiotic potentiality of the *Rhizobium* isolates. Wilbert *et al.*, (1997) pointed out that the composition of exopolysaccharides (signal molecule for legume - *Rhizobium* symbiosis) produced by *Rhizobium* spp. differ from strain to strain. It has also been suggested that all the *R. leguminosarum* strains do not have the similar symbiotic efficiency.

The nodules of plants incorporated a major proportion of their fixed N into ureides, which recently fixed N was exported from nodules to the shoot in the xylem largely as ureides, and that recent photosynthate donated carbon to synthesis of ureides in the nodulated roots (Herride *et al.*, 1978). The relative ureides content of xylem sap from

plants totally dependent upon N_2 fixation was shown to be insensitive to changeurs in the exudation rate and total N concentration of xylem sap brought about by diurnal changes in environmental factors (Mcclure *et al.*, 1980). The dominant form of soluble N in stem and leaf extracts and xylem sap is a-amino-acids.

Ureides production is not essential for completing legume plant life cycle and the accumulation of ureides is inhibitory for nitrogen fixation. Tajima *et al.*, (2004) revealed that in higher plants ureides (allantoin and allantoic acid) are detected in low concentrations in various tissue, but high concentration of ureides were detected only in restricted tissues like senesced leaves. Unusual and significant ureides accumulation in legume plants was found by them in root nodule extract.

The calculations assumed that fixed N was exported to shoots as the compounds indicated by xylem sap analysis and the extent of metabolism of incoming ureides was assessed as the difference between the amount imported and the amount accumulated in the shoot over a specific period of growth. Decomposed nodules may release allantoin into surrounding soil and if the released allantoin were to be taken up by the plant without degradation, it is possible that exogenous allantoin might repress subsequent nodulation (Imsande, 1986). A nodulated legume that remains green during allopurinol treatment also lacks ureides synthesis since the leaves of ureides-producing legumes are very chlorotic following allopurinol treatment (Triplett, 1986).

Ureides were *de novo* synthesized from purines in the cotyledons and as the cotyledons began to senesce, formation of ureides from pre-existing nucleic acids increased and *de novo* purine synthesis declined (Polayes *et al.*, 1984). Over supply of nitrates leads to the production of the shorter chain, incomplete protein compounds that weaken the plant. Higher leaf nitrates result in a watery, softer plant, much more prone to insect and disease attack and actually poisonous to animals, including humans (Nitrate poisoning).

Ureides are made up of four C and four N atoms, having a C: N ratio of 1.0 compared with 2.5 and 2.0 for Gln and Asn respectively. Thus, less C is required for translocation of fixed N as ureides of amide-forming versus ureides forming species indicate that this saving of translocated C could be significant.

Despite the putative economy of C use in translocating N in the form of ureides, they are not as suitable as Asn and Gln as a source of N for amino acid and protein synthesis. Their metabolism in the shoot involves release of all N as NH_3, with the need for reassimilation. Similarly, most of the ureides- C is released as CO_2. As a consequence, little xylem-borne ureidess is transferred as such to phloem in leaves but rather its N is reassimilated to Asn and Gln, and these constitute the principal N solutes of the assimilate stream in ureides forming legumes.

Statistical analysis was performed for nodulation, shoot length, shoot dry matter, N content for all the experimental sets. According to Somasegarm and Hoben (1994), shoot weight is the most reliable parameter and is routinely used as an indicator of relative stain effectiveness. The correlation output revealed the positive correlation between nodule no. and the growth parameters.

At 40 DAS, the nodules N and the shoot N of different isolates were between 2.3% - 2.8% which is near the range of the % N found in effectively nodulated plants of Lucern (2.7 - 3.7) (Schiftman's 1958; Vincent, 1970) and this combination of Lucern - rhizobial symbiotic system is one of the highest nitrogen fixing biological system in the world of legumes after leucaena FAO, 1984).

The calculation of the F-test between the two rhizobial isolates also showed the significant differences on each of the experiments.Some of the slight deviation from the normal path are due to the unequal light distribution, variability of the nutrients available, soil p^H and other inorganic compounds present in the soil. The ureides translocated between the two rhizobial isolates were also highly correlated.

As far as the translocation of the total ureides (Allantoin and Allantoic acid) is concerned the concentration of ureides kept on increasing constantly (more or less) till the 45 DAS. At 45 DAS, the first isolate (R_1) has more accumulation of the ureides (574.05 mgl^{-1}) than the next isolate R_2 (501.075 mgl^{-1}) when detected through xylem bleedings technique. This may be due to the quality of the soil (soil composition), because it was taken from the shade area with more litters, since inoculation with isolate R_2 resulted into production of higher plant biomass, nodule biomass, stem N and leaf N than other isolates considered for this experiment. Thus, it can be

concluded that isolate R_2 was more effective than other for biological nitrogen fixation in present research.

Factors Found Limiting BNF

1. Edaphic Factors
    - Excessive soil moisture, drought, soil acidity, P deficiency, excessive mineral and deficiency of Ca, Mo, Co and B.
2. Climatic Factors
    - Extreme temperature, availability of light.
3. Biotic Factors
    - Excessive defoliation and insects and nematodes.

5.2 Concluding Remarks

The rhizobia-legume symbiosis is the major source of N_2 into agricultural systems and represents an economical and environmental friendly alternative to chemical fertilization. Taking into account that legumes provide 25-35% of the worldwide protein intake (Hernandez, 2002) the improvement of legume N_2 fixation by genetically manipulating the symbiotic partners will be considerable benefit for agriculture.

Legume root nodules are formed as a result of a complex exchange of molecular signals between the rhizobia and the plant. Characterization of the rhizobial isolates isolated from the legumes of Kathmandu valley and Manang was performed and it was revealed that both the isolates were acid producing, intermediate fast growing and gram negative.

For the conformity of the specific rhizobia infecting their host legume, their authentication was performed. Both the rhizobial isolates were able to nodulate the test plant of the same cross inoculation grouping (*Vicia faba* L.). Analysis of the efficiency of biological nitrogen fixation for different isolates, isolated from the plants of two different Agro climatic regions revealed that the second isolate (R_2) was the

most efficient for influxing N to the Broadbean. Thus the test plant accumulated higher biomass with the inoculums of second isolate and is positively correlated with nodule biomass, numbers and nitrogen content at 40 DAS.

Between the two rhizobial isolates, there were insignificant variances in marginal mean as observed in different parameters like plant N plant biomass, nodules N, nodules biomass etc. For the translocation of the total ureides, the first isolate (R_1) had more accumulation and translocation of the ureides nitrogen than R_2. From the comparative study of N content of root, shoot and stem, plants inoculated with the second isolate (R_2) contained the highest percentage of N indicating the highest effectiveness of *R. legumiminosarum* bv. phaseoli while the ureides translocation data shows the maximum value for the first isolate (R_1) at 45 DAS.

In a nutshell, it can be said that the present investigation opens an array of avenues of work on legumes and their microsymbiont as well as their nitrogen fixing capacity on the different types of soils and the nitrogen fixing capacity varies on the different plants by the different isolates.

5.3 Recommendations

Policy recommendation

- Advocacy on the application of legume cultivation on the ground levels to the needy farmers should be done.
- Joint research programme of T.U. with NARC, NAST and other relevant agencies to promote rhizobial technology in Nepal should be performed.

Scientific Recommendation

- Access of general farmers to the rhizobial inoculum should be done while cultivating the bare land.
- Amount of N_2 fixed by diazotrophs should be carried out routinely and the low nitrogen fixing strains should be replaced by high and effective N_2 fixing rhizobial strains.

- Application of the appropriate tools should be employed and the excessive use of the chemical fertilizers should be discouraged.

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