CHAPTER ONE
INTRODUCTION

Unique behaviour of *Rhizobium* in responsible for the conversion of atmospheric nitrogen into the combined form of organic compound. 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 to 12% protein of their dry weight, but in legumes, protein content exceeds upto 30% and of which nitrogen contributes about 16% (Kochhar, 1998).

Though the atmosphere is the major reservoir of nitrogen (78% by volume), only limited class of prokaryotic microorganism have potentiality for fixation of atmospheric nitrogen. Biological nitrogen fixation is the part of biogeochemical nitrogen cycle which drives conversion of atmosphere N_2 to ammonia by outstanding potentiality of biological organisms. Nitrogen is fixed by non biological natural process including lightening, combustion and volcanism which accounts for about 10% of the annual fixation. Industrial nitrogen fixation i.e., ammonia production accounts for about 25% of the annual fixation (Newton, 2000). Recently, Newton (2000) has reported that biological nitrogen fixation accounts for about 65% of the total nitrogen fixation, thus is therefore provider of fixed nitrogen and so is the major sustainer of the life on this earth.

The major nitrogen fixing systems are the symbiotic systems, which can play a significant role in improving the fertility and productivity of low-N soils. The legume-*Rhizobium* symbiosis have received most attention and have been examined extensively. Deficiency in mineral nitrogen often limits plant growth, and so symbiotic relationships have evolved between plants and a variety of nitrogen fixing organisms (Freiberg *et al.*, 1997). Successful legume-*Rhizobium* symbioses definitely increase the incorporation of BNF into soil ecosystems. legume- *Rhizobium* symbioses are the primary source of fixed nitrogen in land based systems and can provide well over half of the biological source of fixed nitrogen (Tate, 1995). Atmospheric N_2 fixed symbiotically by the association between *Rhizobium* species and legumes represent a renewable source of N for agriculture.

The prerequisite for establishment of legume- *Rhizobium* symbiosis is the recognition of both partners. 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 (Bauer *et al.,* 1985, Megias *et al.,* 1993). The legume root secretes growth stimulating exudates like biotins, thiamine, amino acids etc. Consequently compatible strains of microorganisms are stimulated to grow over the other microbes in rhizosphere and from the mucigel. The recognition 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 takes place. Subsequent to root hair curling, bacteria perpetrate the rhizodermis and development of an infection thread occurs by invagination of root hair cell wall in the region of curling (Nutman, 1969). The infection thread grows towards the cortex and ramify throughout the central part of the root cortex. Simultaneously the cortical cell division commences leading to the formation of nodule. The nodule developed in legume- *Rhizobium* symbiosis are of determinate type. Rhizobia loose their cell wall and become substantially enlarged and pleomorphic. Each enlarged pleomrphic bacterium is referred as bacteriod. After the formation of bacteriods, the biosynthesis of nitrogenase and leghaemoglobin starts. The *Rhizobium* feeds its host legume with ammonia in the expense of shelter and reduced substrates. Thus, both the symbionts faithfully negotiates the biological nitrogen fixation in legume- *Rhizobium* symbiosis (Alexander, 1997).

Rhizobial symbiosis with leguminous plants is the classical example and most studied field of biological nitrogen fixation. There are nearly 750 genera and 18,000-19,000 species of leguminous plants of which, about 13,000 or more leguminous plant species including cultivated and wild herbs, shrubs and trees are interestingly found to have *Rhizobium* as the microsymbiont (Subba Rao, 1999).

The ability of certain rhizobia to infect and nodulate particular groups of legume species is important in the classification of rhizobia. Rhizobia are generally classified according to a host-based system. In this host based system legumes have been assembled into cross inoculation groups, which are useful in organizing the diverse legumes and their rhizobial partners (Somasegaran and Hoben, 1994).

Rhizobia	**Cross Inoculation Groups**	**Legumes in Cross-inoculation Groups**
Genus I : *Rhizobium*		
* *Rhizobium leguminosarum* bv. viceae	Pea	Peas, vetches, lentils
* *R. leguminosarum* bv. trifolii	Clover	Clovers
* *R. leguminosarum* bv. phaseoli	Bean	Common beans, Scarlet runner beans
* *R. meliloti*	Alfalfa	Alfalfa/medics, fenugreek
* *R. loti*	Lotus	Lupines, kidney vetch, trefoils,
* *R. galeage*		Goat's rue
* *R. fredii*	Soybean	Soybean
* *Rhizobium* spp.		Leucanea, sesbania
* *Rhizobium* sp.	Chickpea	Chickpea
Genus II : *Bradyrhizobium*		
* *Bradyrhizobium japonicum*	Soybean	Soybean
* *Bradyrhizobium* spp.	Cowpea	Pigeon pea, peanut, cowpea, hyacinth bean

The legumes involved during the present investigation includes *Argyrolobium roseum*, *Trigonella corniculata*, *Phaseolus vulgaris*, *Trifolium repens*, *Indigofera* spp., *Desmodium* spp., *Dolichos lablab* and *Glycine max* (Appendix I).

Bacteria responsible for the formation of morphologically defined nodules on the roots of members of the family leguminosae constitute the genera *Rhizobium* and *Bradyrhizobium*. *Rhizobium* and *Bradyrhizobium* are the nitrogen fixing microsymbiont and belongs to the family Rhizobiaceae. (Somasegaran and Hoben, 1994). These symboitic bacteria are motile, rod shaped gram negative and ranges 0.5×0.9×1.2–3.0cm in size. They produce characteristic translucent

to opaque, white raised and smooth bacterial colonies on artificial medium like Yeast extract Mannitol Agar (YMA) and bacterial colonies does not absorb the colour of Congo red when cultured in dark. The genus *Rhizobium* includes fast growing bacteria with polar flagella while *Bradyrhizobium* is a slow growing bacteria with sub-polar flagella. Optimum temperature requirement for growth ranges from 25–30°C and the lethal temperature lies between 30–40°C depending upon the strain (Nutman, 1972). The phylogenetic relatedness of the agriculturally important species in the order *Rhizobiales* was estimated by comparative 16S rRNA and *dnaK* sequence analyses. Among two groups, one group consisted of *Mesorhizobium loti* and *Mesorhizobium ciceri,* and the other groups consisted of *Agrobacterium rhizogenes, Rhizobium tropici, Rhizobium etli,* and *Rhizobium leguminosarum.* (Sulander et al., 2005)

Rhizobia are the facultative microsymbionts that live as normal components of the soil microbial population when not living symbiotically in the root nodules of the host legume. Rhizobial strains of a given locality are likely to be diverse, whether this is judged on their surface antigens or patterns of symbiotic effectiveness with different hosts (Vincent, 1974). There can be some broad geographical relationships in connection with surface antigens (Allen and Allen, 1950) and where symbiotic effectiveness reflects host influence (Vincent 1974).

Direct quantitative recovery of rhizobia from the soil is not sufficiently dependable for regular use, although a complex selective medium may serve a particular purpose (Pattison and Skinner, 1974). The susceptibility of nodulation of an appropriate host plant can be used in conjugation with appropriately diluted samples and probability tables to arrive at a reasonable estimate (Vincent, 1970). An indirect counting procedure based on plant infection and application of a most probable number (MPN) estimate determines the population of rhizobia in the soil. The MPN count is often used to determine the number of rhizobia present in the soil.

Nepal is an agro major country and about 94% of its labour force is engaged in agricultural practices. Although the economy of the country largely depends upon the agricultural sector for the income and employment generation and agriculture contribute about 43% of the total national GDP, the absolute performance of agriculture is quite disappointing (FAO, 1997). Legumes have probably received more attention to date due to their nutritional value and major role in improvement of soil fertility.

In context to Nepal, estimation of *Rhizobium* in particular altitude is a completely new work. This research aims to provide a new information on rhizobial ecology in the high Himalayan habitat like Manang. The estimation of rhizobial population in cropland of the Trans-Himalayan region is much of importance for the cultivation and production of legumes. The MPN method gives the actual number of rhizobia present in each locality. Since legumes are the good source of protein, it can overcome the large amount of nutrient. For the better growth of the legumes, the rhizobia play a key role. Larger the number of rhizobla in soil, larger is the production of legumes. The present investigation is also, concentrated on the effectiveness of the rhizobial strains of Manang as well as the rhizobial population based on different cross inoculation group of legumes, in each altitude i.e., with about 100m altitudinal difference ranging from Khangsar Goth (4190m) to Chame (2705m).

1.1 Objectives

Present investigation has been carried out to fulfill the following objectives.

1. Isolation of rhizobial strains from the nodules of wild and cultivated legumes of Manang.
2. Authentication of the isolates.
3. Determination of Most Probable Number (MPN) of rhizobia in the soils of Manang (2705m to 4190m) at an altitudinal differences of 100m.

1.2 Limitations

The remoteness, difficult terrain, harsh climate of study area and adverse political situation of the country were the main problems during the work.

CHAPTER TWO

LITERATURE REVIEW

Till the date large number of researches have been carried in the field of legume - *Rhizobium* symbiosis and now it has become the most interesting field for most of scientists throughout the world.

Beijrinck (1888) in Holland was the first to isolate and culture the microorganism from the nodules of legumes, he named it *Bacillus radicicola* which is placed in Bergey's manual of systematic bacteriology under the genus *Rhizobium* (Subba Rao, 1999).

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

Dart (1974) studied biochemical effects of *Rhizobium* in nodule formation and reported that exudates cytokinin, polymixin B etc. of *Rhizobium* resulted in root curling during nodule formation.

Quispel (1974) found that the development of insufficient amount of bacteroid leads to ineffective or partially effective nodules which affect the N_2 fixation and development of nodules and bacteroids is governed by carbohydrate supply to the nodule bacteroid and transfer of the fixation products. The carbohydrate supply was compensated by host photosynthesis. Out of total photosynthate, 23% transported to the nodules, 42% to supporting roots and in nodules, 5% consumed in growth, 12% in respiration and 25% used for the nitrogen assimilation.

Vincent (1974) provided the representative evidence of N_2 fixation by various legumes and found that N_2 fixed by soybean was 20–200 kg/ha. The characteristic that quantifies a bacterium for to be the *Rhizobium* is its capacity to form morphologically defined effective nodule on the root of leguminous host.

Dalton (1980) found that the microsymbiont in legume root nodule was normally rhizobia and depending upon growth patterns in yeast extract medium classified rhizobia into two classes. The so called 'fast growers' give abundant growth after 3-5 days at 30°C. They possess G+C content in between 59 and 63%. The 'slow growers' which produce moderate growth after 10 days possess G+C content in between 61.6 and 65.5%.

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

Evans *et al.* (1982) studied hydrogen recycling in legumes and reported that about 22% of strain of *R. japonicum* posses a capacity to synthesize the H_2 recycling system and as a consequence nodule formed by such strains internally recycle the H_2 produced by the bacteroid nitrogenase reaction.

Kondorosi *et al.* (1982) successfully transferred the megaplasmid of *R. meliloti* into other *Rhizobium* species and *Agrobacterium tumefaciens* with the result that the transconjugants became able to form nodules or nodule like structures on alfalfa.

Sadowsky *et al.* (1983) compared 11 fast growing soybean *Rhizobium* with 7 isolates of slow growing *R. japonicum* and found both groups to be positive for catalase, oxidase, urease, penicillinase and nitrate reductase.

Herridge *et al.* (1984) studied that effect of *Rhizobium* and soil nitrate concentration on the establishment and functioning of the soybean symbiosis. It was found that increasing the rates of inoculant applied to the seeds causes larger population and more extensive distribution of *Rhizobium* in the rhizosphere, which improved nodulation and nitrogen fixation as well as the residual populations in the soil after harvest. They also reported that the root system exposed to low concentrations of nitrate would nodulate first.

Keyser *et al.* (1984) reported the existence of fast growing *Rhizobium* isolates from soils and soybean nodules collected from China, which were different

from slow growing *Bradyrhizobium* in terms of microbilogical, genetic and symbiotic properties. Since central China is considered as the gene centre of *Glycine max* and *G. soja*, they concluded that it would also be the gene centre of two types of soybean rhizobia.

Prasad (1984) studied the effect of cytozyme on seedling growth of *Phaseolus mungo* and found its stimulatory effect of cytozyme on seedling growth of *Phaseolus*.

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.

Buttery and Dirks (1986) analyze the effectiveness of *Rhizobium* strain and soybean cultivar on plant and nodule weight and acetylene reduction rates between 33 to 41 days. Cultivar Blackhawk with all strains of *Rhizobium* and strain 110 and CB 1809 with all cultivar of soybean seemed to be most effective for nitrogen fixation.

Ta *et al.* (1986) studied the biochemistry of N_2 fixation and reported that the first stable product of N_2 fixation in legume nodule is ammonia.

Woomer *et al.* (1988) studied on six legume - *Rhizobium* systems to test the reliability of the most probable number (MPN) technique for enumerating rhizobia introduced into 14 sites representing four soil orders. The range of transition values were compared for each species and for each soil. They found that the acceptability of the MPN data obtained for a host species was related to rooting habit and time to nodulation. Comparison of data for each soil indicated that despite large differences in characteristics, the soil was not a major source of variability in the MPN counts.

Bottomley and Dughri (1989) studied the population size and distribution of *R. leguminosarum* bv. trifolii in relation to soil bacteria and soil depth (0-20cm). The result revealed that the increase in density of the small cell population correspond to a significant increase in soil bulk density (1.07 to

1.21g cm). The percent contribution of the <0.4-mum direct count to individual serogroup totals increased with soil depth by approximately 2 fold for serogroups 17 and 21, and 12 fold for serogroups 6 and 36.

Bottomley and Maggard (1990) studied about the percentage of viable cells within soil rhizobia populations measured by the immunofluorescence direct count method. The direct viable count method was combined with immunofluorescence to compare the percent viability and kinetics of appearance of elongated cells with serotypes of a soil population of *R. leguminosarum* bv. trifolii. They found that the majority of these organism were viable as observed by immunofluorescence. They obtained the evident that subpopulation within the soil rhizobia community were in different states of competence to respond to substrate.

Razakafoniaina and Schmidt (1992) studied the effect of symbiotic nitrogen fixation by *Rhizobium* on yield and protein content in grains of groundnut (*Vigna subterranea*). Among 18 rhizobial strains studied, two were proved adequate for the improvement of crop yield and crude protein content in the grain of two groundnut varieties.

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 was the most effective among the others.

Smith and Wollum (1993) studied the attachment process of *Bradyrhizobium* to roots of soybean and reported that lectins originating from rhizobia may also be involved in the attachment process.

Odee *et al.* (1995) studied the rhizobial population and nodulation status of both indigenous (mainly *Acacia* species) and some introduced woody legume species were assessed under glasshouse conditions in soils collected from 12 sites located in different ecological zones of Kenya. The population among the sites, as estimated by the MPN technique varied from 3.6 to $> 2.3 \times 10^5$ cells g^{-1} of soil. Nodulation of most species showed interplant and intraspecific variability with a single soil source.

Yadav (1995) isolated the *R. leguminosarum* bv. viceae from the lentil and inoculated to lentil. It was found that inoculated cases resulted in higher biomass and seed yield compared to area application (60kg/ha)

Hartmann *et al.* (1998) studied the genotypic diversity of *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) strains isolated directly from a soil and from nodules of alfalfa (*Medicago sativa*) grown in the same soil using an elective medium and colony hybridization was assessed by three molecular methods. They found that the dominant plasmid type in nodule isolates (30% of the population), carrying a large plasmid of 230kb, is poorly represented among soil isolates (8%). The result suggested that plasmid might be involved in the compititiveness of *S. meliloti* strains.

Mendes and Bottomley (1998) studied the distribution of a population of *R. leguminosarum* bv. trifolii among different size classes of soil aggregates by using most probable number (MPN) technique and found that the *Rhizobium* soil population was heterogenously distributed across the different size classes of soil aggregates, and the distribution was influenced by cover crop treatment and sampling time.

Carranca and Rolston (1999) studied the biological nitrogen fixation in some legumes. They found that legumes derive more than 60% of their nitrogen from the atmosphere under favourable soil conditions. The annual rate of N_2 fixation was found 76 - 125kg/ha and 31–107 kg/ha by uninoculated faba bean and peas respectively.

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

Sato *et al.* (1999) studied the regulation of nodule development and nitrogen fixation. They observed that insufficient supply of photosynthate to the growing nodule retard the nodule growth but auto-regulatory control plays an important role in optimizing the nodule number where nodule growth and N_2 fixation activity can be maximized.

Hungria and Milton (2000) studied the effects of environmental factors in grain legumes for nitrogen fixation in the tropics and found that high temperature and moisture deficiency are major factor of nodulation failure, affecting all stages of symbiosis and limiting rhizobial growth and survival in tropical soil.

Palmer and Young (2000) studied the higher diversity of *Rhizobium leguminosarum* bv. viciae populations in arable soils than in grass soils and concluded that rhizobial diversity can be affected by differences between chromosomal types, *rep C* profiles and combined genotypes.

Hungria *et al.* (2001) isolated the effective fast growing *Rhizobium* strains with doubling time 85–225 minutes and acidic reaction in YEM medium from soybean nodules in Brazil.

Jiang *et al.* (2001) studied the myo-inositol dehydrogenase activity for efficient nitrogen fixation and nodulation of soybean. They found high activity of myoinositol dehydrogenase for the better nitrogen fixation and nodulation.

Maskey *et al.* (2001) performed the onfarm measurements of nitrogen fixation by winter and summer legumes in Hill and Terai regions of Nepal and estimated that approximately 3,000 tons nitrogen was fixed annually in Nepal by legumes valued at US$15 million.

Andrade *et al.* (2002) studied the diversity of *Phaseolus* nodulating rhizobial population in Brazil and concluded that the rhizobial population is altered by liming of acid soils planted with *Phaseolus vulgais* L.

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.

Pant and Prasad (2004) studied the effectiveness of different *Bradyrhizobium* isolates for nitrogen fixation in soybean and found that the effectiveness of *Bradyrhizobium* isolate KH_1, isolated from the root nodules of *G. max* (L)

Merr. cv. Sathiya collected from Khumaltar, Kathmandu was more than that of *Bradyrhizobium* isolates TU_1 and TU_2 isolated from root nodules collected from Kirtipur, Kathmandu. The results showed the variable effectiveness of *B. japonicum* strains for N_2-fixation in soybean.

Zribi *et al.* (2004) studied the distribution and genetic diversity of rhizobia nodulating natural population of *Medicago truncatula* in tunisian soils and showed a clear distinction between the two *Sinorhizobium* species i.e. *S. meliloti* and *S. medicae* showing the higher diversity for *S. meliloti*.

Duodu *et al.* (2006) studied the genetic diversity of a natural population of *R. leguminosarum* bv. trifolii from field nodules and by a plant infection technique and suggested that the sample of clover *Rhizobium* strains from soil dilutions may not be representative of the field nodulating population.

Lapinskas (2007) studied the effect of acidity on the distribution and symbiotic efficiency of rhizobia in Lithuanian soils and presented the relationship between the soil pH and the nitrogen fixing capacity of rhizobia. He demonstrated a positive effect of liming of acid soils in combination with inoculation of legumes on the efficiency of symbiotic nitrogen fixation.

Romdhane *et al.* (2007) studied on the competition for nodule formation between introduced strains of *Mesorhizobium ciceri* and the native population of rhizobia nodulating chickpea (*Cicer arietinum*) in Tunisia and resulted that the local strain gave a significant increase in nodule number and shoot dry yield in all experimental fields for the three cultivars used.

CHAPTER THREE

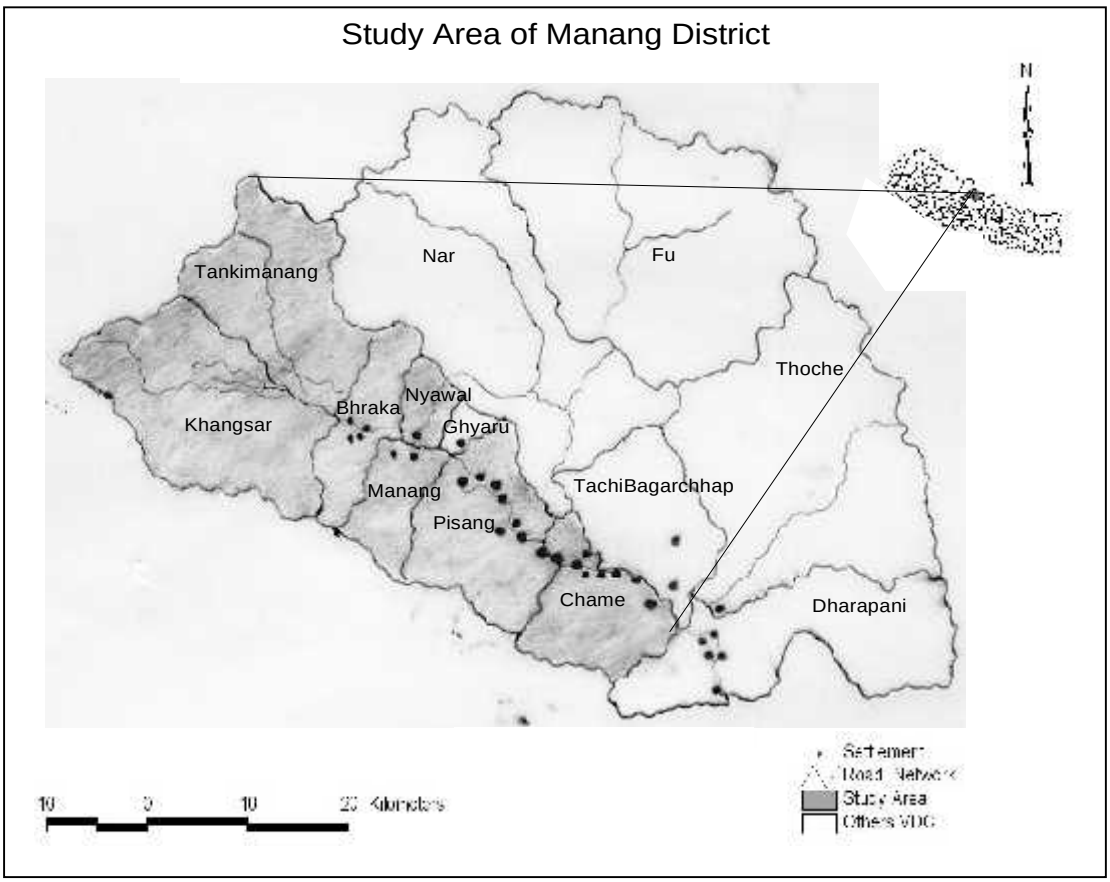
STUDY AREA

3.1 Location and Physiography

Manang i.e., the hidden paradise behind the Himalaya, is located in the north central part of Nepal at 28°27' – 28°54' N latitude and 83°49'–84°34' E longitude. The altitude varies from 1,830m asl (6,000 feet) to 8,029m asl (26,500 feet). It is situated entirely within the Annapurna Conservation Area. It's unique geographical terrain is bounded by the Tibet to the north and high mountain chains to the west, south and east. High mountains occupy most of the surface area of this region. These mountains are interrupted by the main flow of the Marsyangdi river, and these have two tributaries, i.e. the Nar and Dudh-khola, which drain the entire area towards the south. Due to the different geological and geomorphological conditions, various valley forms have had decisive influence in the settlement pattern and economic activities within the area. These form the basis for the territorial sub-division of Manang into three distinct regions, viz. Gyasumdo (lower region), Nyeshang valley (Western region) and Nar and Phoo valley (Northern region).

3.2 Study Location

The present study has been carried out in Gyasumdo and Nyeshang valley which comprises the lower and upper western part of the Manang respectively. It extends from chame, the district headquarter (2705m asl) to Khangsar Goth (4190m asl). Specific study area includes Chame, Taleku, Bhratang, Pisang, Upper Ngawal, Humde, Bhraka, Manang, Tanki Manang, Khangsar and Khangsar Goth. The physiography of these location are given in Appendix II (Table 1).



Source: Department of Survey.

3.3 Climate

The extreme difference in altitude and aspect with different landscape results in a great variation in climate of Manang. The climate ranges from temperate to cold alpine type. Climate of study site is cold sub-alpine in nature. Since the area lies in north of massive Anapurna range, thus receives little of monsoon rain that comes from southeast. Mean annual precipitation is 400mm (Anonymous, 1995). There is decreasing moisture from east to west and south facing slopes are significantly drier than those facing north (Bhattarai *et al.*, 2004). Snow is common during winter. The meterological data of the year 2006 shows that the highest maximum temperature reached utp 21.6°C during August and the lowest minimum temperature reached upto –1.4°C during December. Similarly, highest precipitation was recorded during July (366.2mm) followed by August (287mm) and May (101mm). Monthly average maximum and minimum temperature as

well as the precipitation of Chame in the year 2006 is given in Appendix II (Table 2).

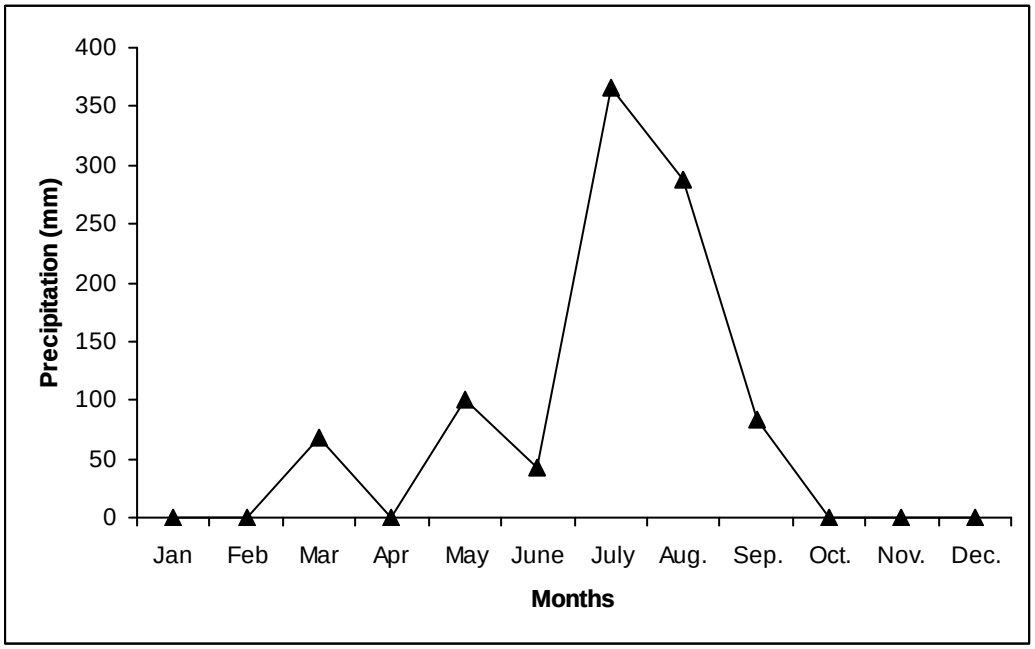


Fig. 1 : Monthly Average Precipitation of Chame (2006)

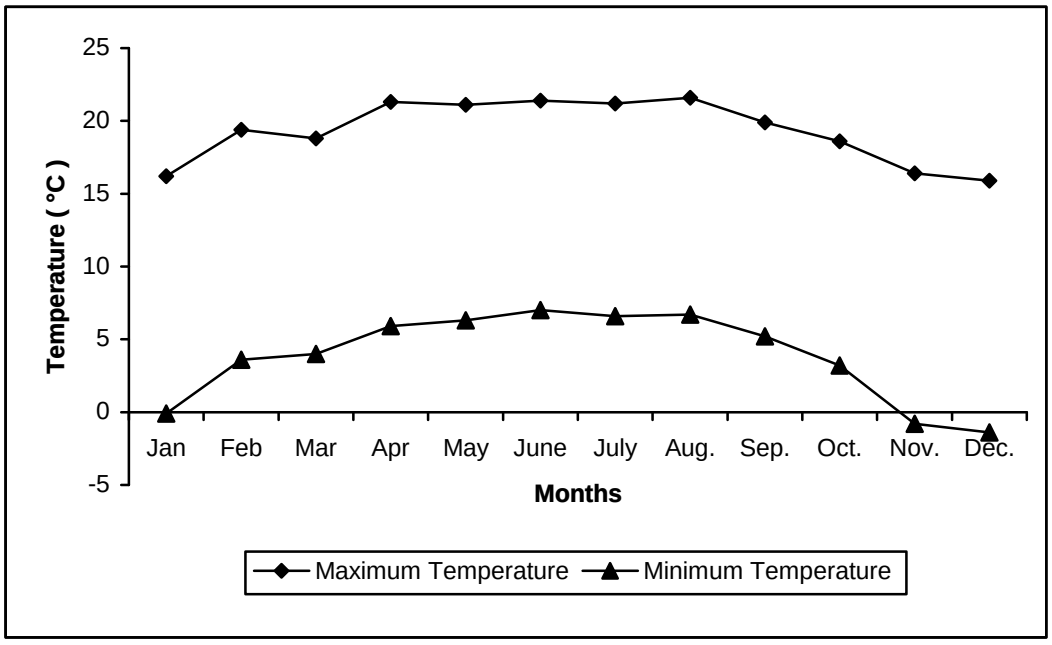


Fig. 2 : Monthly Average Maximum and Minimum Temp. of Chame (2006)

3.4 Cropping System

Wheat, buckwheat and potato are the main crop plants grown in upper Manang. Rice is not grown in this area. Few legumes such as *Trigonella* spp.,

Foenumgreaccum spp., *Phaseolus vulgaris*, *Cicer arietinum*, *Pisum* spp. etc are also grown. The farmers practice crop rotation and they rotate wheat with buckwheat and potato. During the year of buckwheat cultivation, they apply compost manure made up of leaf litter and cattle dung. The fertilizer overleft is used by buckwheat or potato in successive years. Such practice is considered better even in modern agriculture which helps in proper utilization of nutrients added in crop field. The crops here are sown during March/April and harvested during the months of September/ October. The cropping system is influenced by the decision of lama and local headman. Also the cropfield of upper Manang enjoy the presence of luguminous weeds. Some weeds of wheat field in upper Manang are *Medicago sativa, M. lupulina, Trigonella emodi, Argyrolobium roseum* and *lens culinaris* (Sharma, 2007).

CHAPTER FOUR

MATERIALS AND METHODS

Present research work was conducted in Manang and in Central Department of Botany, Tribhuvan University, Kirtipur. Chemicals and glasswares were provided by TU-NUFU project and CDB, T.U.

I. Materials

4.1 Source of seeds

Seeds of *Phaseolus vulgaris*, *Dolichos lablab*, *Desmodium* spp. *Trifolium repens*, *Glycine max* and *Trigonella corniculata* were borrowed from the National Agricultural Research Centre (NARC), Agronomy Division, Khumaltar, Kathmandu.

4.2 Laboratory facilities

Research work was carried out in the laboratory of CDB, T.U., Biotechnology unit. TU-NUFU project and Biotechnology unit, CDB, T.U. provided the chemicals and glasswares for the research.

II. Methods

4.1 Isolation of pure culture

4.1.1 Collection of nodules

Root nodules of *Argyrolobium roseum*, *Phaseolus vulgaris*, *Trifolium repens* and *Indigofera* spp. were collected from four different sites of Chame, Manang. The altitudinal variation and physiography of the site for nodule collection is given in Appendix II (Table 3). Effective and healthy root nodules were collected from young, healthy and green plants. Excised nodules were washed in tap water and collected in silica gel crystals (desiccant) containing nodule collection vials. The vials were labelled and brought to the laboratory of Biotechnology unit, CDB, TU for further investigation and were stored at 4±1°C for short term storage.

4.1.2 Preparation of stock solution

4.1.2.1 Congo red (CR) stock solution

Stock solution of Congo red was prepared by dissolving 0.25g Congo red dye in distilled water to make final volume 100ml. Stock solution was stored at 4°C in a well labelled brown bottle in a refrigerator.

4.1.2.2 Bromothymol blue (BTB) stock solution

100ml of BTB stock solution was prepared by dissolving 0.25g BTB in 100ml distilled water. Stock solution was stored at 4°C in a well labelled brown bottle in a refrigerator.

4.1.3 Preparation of Yeast Extract Mannitol Agar-Congo Red (YEMA-CR) medium

Different strains of rhizobial isolates were obtained by crushing the root nodules. The root nodule extract were cultured on Yeast extract Mannitol Agar culture medium supplemented with red dye, Congo red (YEMA-CR). The composition of YMA-CR medium according to Vincent, 1970 is as follows:

Constituents	**Amount (Per litre)**
Mannitol	10.0g
Dipotassium Hydrogen Phosphate (K_2HPO_4)	0.5g
Magnesium Sulphate ($MgSO_4.7H_2O$)	0.2g
Sodium Chloride (NaCl)	0.1g
Yeast Extract	0.5g
Agar Powder	15g
Congo red (stock)	10ml

All the required amount of constituents were first dissolved in 500ml distilled water. First of all, salts were dissolved, then mannitol and yeast extract were added. Continuous stirring was done to dissolve all the constituents. 10ml of stock solution of Congo red was added to the solution and the final volume was made 1000ml. The pH of the solution was adjusted to 6.8±0.1 with the help of 0.1N NaOH and 0.1N HCl. Finally 15 gram of agar powder was added

to the broth and YEMA-CR medium was autoclaved at 121°C (15 lbs) for 15 minutes. This sterilized YEMA-CR medium was used for the isolation of rhizobial isolates from different root nodules.

4.1.4 Culture of root nodule extract on YEMA-CR media

Root nodule extract was cultured in YEMA-CR culture medium by loop plating method under aseptic conditions. The *Phaseolus vulgaris, Trifolium repens, Argyrolobium roseum* and *Indigofera* spp. root nodules were soaked separately in sterilized distilled water at room temperature. Water soaked nodules were immersed in 70% ethanol for 30 seconds followed by 3% hydrogen peroxide for 4 minutes. Nodules were then washed with sterile water for 5 times. Nodules were continuously agitated in all the steps for the complete sake of better sterilization. Subsequently, sterilized nodules were kept in sterilized petriplates separately for different plant nodule samples.

After the surface sterilization, about 10 healthy nodules were crushed in the petriplate with the help of sterilized glass rod dropping 1ml sterilized distilled water. One loopful of the nodule suspension was streaked on a yeast mannitol agar (YEMA) plate containing Congo red (CR). All four culture settings were symbolized on the basis of number and strain as R101, R103, R109 and B111 to the nodule samples of *Argyrolobium roseum, Phaseolus vulgaris, Trifolium repens* and *Indigofera* spp. respectively. After the completion of the streaking, all the plates were incubated at 28±2°C in an inverted position and left for the bacterial growth in an incubator. The *Rhizobium* and *Bradyrhizobium* culture were left for 7 and 10 days respectively.

4.1.5 Isolation of rhizobial colonies and maintenance of pure culture

Rhizobial colonies grown in YEMA-CR master plate were isolated in sterile condition under laminar air flow cabinet. Single creamy white, translucent and raised colony of *Rhizobium* and *Bradyrhizobium* was picked up and streaked in a new YEMA-CR plates. Each new YEMA-CR plates were streaked with single rhizobial colony with the help of flame sterilized inoculation loop and plates were incubated upside down for 7 days (for *Rhizobium*) and 10 days (for *Bradyrhizobium*) at 28±2°C in an incubator for proper bacterial growth.

To obtain pure rhizobial colonies, the colonies from uncontaminated previous plates were restreaked in a new YEMA-CR plates. As the pure culture of rhizobial isolates were obtained, single bacterial colonies were transferred to the YEMA-CR slants. The slants were incubated for at 28±2°C and finally pure rhizobial culture containing YEMA-CR slants were stored at 4°C for short term storage.

4.2 Characterization of rhizobial isolates

The bacterial isolates obtained from *Argyrolobium roseum*, *Phaseolus vulgaris* and *Trifolium repens* root nodules were characterized as *Rhizobium* and *Indigofera* spp. as *Bradyrhizobium* by studying their presumptive characteristics.

4.2.1 Colony characteristics and growth response on YEMA-CR medium

Sterilized root nodule extract was cultured on YEMA-CR medium and duration of bacterial colony growth, morphology of colony, nature of bacterial colony with Congo red were observed for all the isolates.

4.2.2 Acid/Alkali production characteristics

The isolates were studied for its behaviour of acid and alkali production. Both solid and liquid media were used for the purpose.

4.2.2.1 Acid/Alkali production on solid (YEMA-BTB) medium

The bacterial isolates were cultured on YEMA-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 on the plates containing YEMA-BTB. YMA-BTB plates were labelled and incubated at 28±2°C for their growth period.

4.2.2.2 Acid/Alkali production on liquid medium

YEM broth with the usual pH 6.8 was adjusted using 0.1N NaOH and 0.1N HCl depending upon the cases. They were then inoculated with one loopful of 72 hour old culture and placed in horizontal shaker (28±2°C and 120 strokes min^{-1}) for 0, 24, 48, 72 and 96 hours. Resulted cultures in each time period

were then subjected to centrifugation (2000 rpm, 15 min) and the supernatant was used to estimate p^H by a p^H meter. A periodic estimate with fresh culture was accomplished.

4.2.3 Gram staining

The presumptive test of rhizobial isolates towards Gram stain and their morphological characteristics were studied by Gram staining. Different Gram stain reagents (solutions) were freshly prepared. The composition of Gram stain solutions according to Vincent, 1970 is as follows:

I. Crystal violet solution

Crystal violet	10g
Ammonium oxalate	4g
Ethanol	100ml
Distilled water	400ml

II. Iodine solution

Iodine	1g
Potassium iodide	2g
Ethanol	25ml
Distilled water	100ml

III. Alcohol (iodinated)

Iodine solution (II)	5ml
Ethanol	95ml

IV. Counter stain

2.5% safranin in ethanol	10ml
Distilled water	100ml

All the Gram stain solutions were prepared appropriately and kept accordingly. The heat fixed bacterial smears were treated with Gram stain solutions appropriately. The smears were prepared by heat fixing a loop of rhizobial cells. Bacterial smears on the slide were stained with crystal violet solution (I) for one minute and gently washed with distilled water and drained

off excess water. The smears were flooded with Iodine solution (II) for one minute followed by washing with decoloriser i.e., iodinated alcohol (III) for five minutes. Finally, the smears were flooded with counter stain (IV) for five minutes. The slides were then washed with tap water and freshly prepared slides were examined directly under oil immersion.

4.2.4 Multiplication of Bacterial Cells

The population of bacterial cells was determined in each 24 hours throughout the duration of the experiment. One loopfull of the 72 hours old rhizobial isolate was inoculated in the sterilized 125ml YMB in aseptic condition and placed in horizontal shaker (27±1°C and 120 strokers per min.). In each 24 hours 1ml broth was pipetted out and ten fold dilution series was performed. The extent of the dilution for the bacterial count (to be plated) was 10^{-11}. 0.06ml inoculum was pipetted from the 10^{-11} dilulion series and pour plate count method was used to perform viable counts for each culture on the difference of 24 hours. This method was applied separately for R101, R103, R109 and B111. The experiment was terminated after 96 hours for R101, R103 and R109, and after 120 hours for B111.

4.3 Authentication of rhizobial isolates by infection test

The bacterial isolates obtained from different plant nodules were set for the authentication. Incase of *Phaseolus vulgaris* and *Trifolium repens*, their own seeds were used whereas incase of *Argyrolobium roseum* and *Indigofera* spp. the seeds of *Trigonella corniculata* and *Desmodium* spp. were used respectively on the basis of cross inoculation group of legumes.

4.3.1 Seeds, filter papers and growth pouches sterilization

Healthy seeds were used for the purpose of experiment. Each seeds were surface sterilized by placing the seeds in 95% ethanol for 10 seconds to remove waxy materials and then transferred to 3% hydrogen peroxide solution for 4 minutes. They were washed with several changes in sterile water and dipped in fresh amount of sterile water and placed at 4°C in refrigerator to imbibe for 24 hours.

Filter paper and growth pouches (size 16×18cm) were sterilized by autoclaving them at 121°C (15 lbs) for 15 minutes. Filter papers were wrapped with aluminum foils while plastic pouches were kept in the water containing plastic bag.

4.3.2 Seed germination on Agar water medium

Well imbibed seeds were grown in autoclaved petridishes containing 0.75% (w/v) water agar (Somasegaran and Hoben, 1994). Carefully each seed was manually half pushed into the agar water medium with equal seed to seed spacing. Petridishes were incubated at 30°C for 4 days in an upside down position in order to get straight morphology of radide.

4.3.3 Preparation of liquid rhizobial inocula

Rhizobial isolates were maintained in liquid medium i.e., Yeast Extract Mannitol Broth (YEMB) for inoculation on their specific host. YEMB was prepared according to Vincent (1970). 125ml of YMB was dispensed on each conical flask and sealed with aluminium foil and autoclaved for 15 minutes at 121°C (15 lbs). The separate autoclaved YMB flasks each for single bacterial species were then inoculated with 3 different rhizobial and a single bradyrhizobial isolate. The separate specific rhizobial colonies from the YEMA-CR slants were transferred to the YEMB flasks. 10ml of sterilized YEMB was dispensed into the YEMA-CR slant and the bacterial colonies of the slants were scrapped with the help of sterilized inoculation loop. The YEMB along with the bacterial colonies was then transferred into the conical flask and again sealed with sterilized muslin wrapped cotton plug. All the activities were performed in laminar air flow cabinet. The inoculated YEMB flasks were then incubated at 28±2°C for 7 days (for *Rhizobium*) and for 10 days (for *Bradyrhizobium*) in horizontal shaker with 120 rpm of rotation frequency.

4.3.4 Preparation of modified Jensen's N-free medium

The seedlings grown in sterile growth pouches were supplied with nitrogen free nutrient solution. The composition of modified Jensen's N-free medium is given below (Roughley, 1984, Somasegaran and Hoben, 1994).

Constituents	**Per litre**
Calcium Hydrogen Phosphate ($CaHPO_4$)	1.0g
Dipotassium Hydrogen Phosphate (K_2HPO_4)	0.2g
Magnesium Sulphate ($M_gSO_4 . 7H_2O$)	0.2g
Sodium Chloride (NaCl)	0.2g
Ferric Chloride ($FeCl_3$)	0.1g
Trace Elements Stock Solution	1.0ml

Composition of trace elements stock solution

Constituents	**Gram/litre**
Boric Acid (H_3BO_3)	2.86
Manganese Sulphate (MnSO4 . 4H2O)	2.03
Zinc Sulphate ($ZnSO_4 . 7H_2O$)	0.22
Copper Sulphate ($CuSO_4 . 5H_2O$)	0.08
Sodium Molybdate ($Na_2MoO_4 . 2H_2O$)	0.14

Stock solution of trace elements was prepared and stored at 4°C. All the constituents of modified Jensen's N-free medium were mixed one by one avoiding any precipitation and volume was adjusted to 1 litre. The pH of the medium was adjusted to 6.8±0.1 with the help of 0.1N NaOH and 0.1N HCl. The medium was autoclaved at 121°C (15 lbs) for 15 minutes and sterilized medium was then applied in seedling growing growth pouch.

4.3.5 Inoculating rhizobial count

This process was done as similar to 4.2.4. Instead of 0.06ml, 1ml was pipetted out and pour plate count method was applied for the bacterial count after 5 and 7 days of culture of the respective bacterial strains.

4.3.6 Infection test on growth pouch

The germinated seeds were placed into the folds of filter paper in the sterilized growth pouches containing 30-40ml nitrogen free nutrient solution (Evans *et al.,* 1972). Only one seed was used per bag with its radicle downward piercing

into the hole made by fine sterilized forceps. These pouches were arranged into wooden rack specially prepared for the purpose.

Each pouch containing the germinated seeds of *Trigonella corniculata, Phaseolous vulgaris* and *Trifolium repens* were applied separately with one ml of 5 days old rhizobial broth culture obtained from R101, R103 and R109 respectively. Similarly germinated seed of *Desmodium* spp. was applied with one ml of 7 days old bradyrhizobial broth culture (obtained from B111) per pouch. This application of the inoculum was performed at the junction of radicle and plumule. The assembly was placed in well aerated and light conditioned green house maintaining the light of 12000 lux luminous intensity. The nodulation was checked after three weeks.

4.4 Estimation of Most Probable Number (MPN)

The MPN count is used to determine the number of rhizobia present in soil. For this purpose, four legumes i.e., *Trigonella corniculata*, *Phaseolus vulgaris*, *Glycine max* and *Dolichos lablab* were planted for the determination of the population of *R. meliloti*, *R. leguminosarum* bv. phaseoli, *B. japonicum* and *Bradyrhizobium* spp. on the basis of nodule formation by the soil bacteria. The MPN of rhizobia from the soil was determined by the method as described by Somasegaran and Hoben (1994) and calculated from Table $VIII_2$ of Fisher and Yates (1963).

$$X = \frac{m \times d}{v}$$

where,

X = MPN per gram of inoculant

m = Most likely number

d = The lowest dilution used in the series

v = Aliquot used for inoculation

Since the soil was diluted 1:10 before the actual dilution series was started, the final result was multiplied by 10.

4.4.1 Collection of soil

Soil from cultivated land of Manang were collected from an altitudinal difference of about 100m from Khangsar Goth (4190m) to Chame (2705m) Appendix II (Table 1). Quadrat size of $10\times10m^2$ was led to collect the soil samples. 0.5kg soil from each corner and from the centre of the quadrat was collected. They were mixed together. Fine soil particles were separated through the metallic seive from each altitude. 0.5kg soil was kept in air tight plastic bag and stored at low temperature i.e., 4°C for further investigation.

4.4.2 Sterilization of seed and seed germination

The method was similar as described in 4.3.1 (for seed sterilization) and 4.3.2 (for seed germination).

4.4.3 Sterilization of sand, pebbles and growth pouches

Sand and pebbles were sterilized by autoclaving at 121°C (15 lbs) for 15 minutes whereas the growth pouches were sterilized as described in 4.3.1. The sterilized sand and pebbles were kept in a air tight plastic bags and stored in a sterile room.

4.4.4 Soil dilution

100g of soil was diluted in 900ml of sterile water. A fourfold dilution series with four replications was prepared ranging from 4^{-1} to 4^{-8} (Somasegaran and Hoben, 1994).

4.4.5 Plantation

The growth pouches containing sterilized sand were used for the transfer of germinated seed. The straight radicle with its tip was penetrated inside the sand. An aliquot of 2ml from each dilution series was poured in the junction between the radicle and the plumule of the particular plant by the help of pipette.

The pouches containing the seedlings were covered by the pebbles inorder to avoid volunteer infection. These were then placed in green house. 30%

moisture was maintained by regular watering. The experiment was terminated after 3 weeks.

4.4.6 Nodulation

The presence, absence and the number of nodules were observed after 3 weeks. The plants were carefully uprooted and removed from the pouches. Adhering sand particles were removed carefully and the roots were then thoroughly washed in tap water. All the distinct visible nodules were noted one by one for all the experimental sets.

4.5 Evaluation of N_2-fixing efficiency of rhizobial strains from the soils of Manang and Kathmandu

The nitrogen fixing efficiency of different rhizobial strains from two different localities i.e., Chame (Manang) and Kirtipur (Kathmandu) was examined on the basis of total nitrogen content in root nodule. For this purpose, seeds of *Trigonella corniculata, Phaseolus vulgaris, Dolichos lablab* and *Glycine max* were used.

4.5.1 Legume plantation on Manang and Kathmandu soil

The sterilized seeds (as method described in 4.3.1) of the above described legumes (in 4.5) were planted on separate growth pouches containing 25% soil sample of Manang and 75% sterilized sand. The same method was applied for the soil of Kathmandu.

4.5.2 Nitrogen content in root nodules

The total nitrogen content in dry samples of root nodules of different legumes planted on the 30^{th} day was estimated by modified Kjeldahl method (Bergerson, 1980, PCARR, 1980).

$$\text{Nitrogen (\%)} = \frac{(V_S - V_B) \times N \times 14}{\text{wt. of sample (mg)}} \times 100$$

where, V_S = Volume of standard HCl required for sample

V_B = Volume of standard HCl required for blank

N = Normality of standard HCl

4.6 Soil analysis

Soil samples collected from different spots (Appendix II, Table 1) were analyzed in the laboratory of Agricultural Trading Centre, Lalitpur. Soil was analyzed for p^H, moisture, total nitrogen, total phosphorous, total potassium and organic matter. In case of soil p^H and moisture, these were measured directly in the field.

4.6.1 Soil p^H

Soil acidity is a major factor determining the occurrence of particular rhizobia according to their relative sensitivity. The particular p^H measured in a soil is caused by a particular set of chemical conditions. Therefore, the determination of soil p^H is one of the most important test that can be made to diagnose plant growth problems. Rhizobial strains may differ somewhat with respect to optimum p^H conditions, most strains grow well at 6.0 - 7.0 (Jensen, 1942). Soil p^H was measured by using the p^H meter called soil p^H and moisture tester (Model DM 15), Takamura Electric Works Ltd. Japan at the field.

4.6.2 Soil moisture

The direct relation of soil water on rhizobia is a major factor determining the rise and fall of the soil population. Water shortage is more serious than periods of excess, particularly when combined with high soil temperature and lack of soil colloids. Soil moisture was measured by the same instrument that measured the p^H.

4.6.3 Nitrogen (N)

Nitrogen is an integral component of many compounds essential for plant growth process including chlorophyll and many enzymes. Total nitrogen content of the soil was determined by modified kjeldahl method as method described by Gupta (2000).

$$\text{Nitrogen (\%)} = \frac{(V_S - V_B) \times N \times 14}{\text{wt. of sample (mg)}} \times 100$$

where,

V_S = Volume of standard HCl required for sample

V_B = Volume of standard HCl required for blank

N = Normality of standard HCl.

4.6.4 Phosphorus (P)

Phosphorus has been called "the key to life" because it is directly involved in most life processes. Phosphorus occurs in the soil in both organic and inorganic forms. Available phosphorus was determined by using Olsen's modified carbonate method (PCARR, 1980).

Phosphors (kg/ha) = R × F

Where,

F = Coefficient factor, calculated from blank solution

K = Reading in spectrophotometer (coefficient factor = 3615. 15)

4.6.5 Potassium (K)

Potassium is the third most likely nutrient element to limit plant growth and is therefore a very common constituent of fertilizers. Plant absorb large amounts of potassium, all of it in the form of k^+ ion. It was measured by flame photometer method.

$$\text{Potassium (kg/ha)} = \frac{R20}{7} \times 1.2 \times 2$$

Where,

R = Reading in photometer

4.6.6 Organic matter

Soil organic matter consist of plant, animal and microbial residues in various stages of decay. Organic matter decomposition releases nutrients in soil. The amount of organic matter content affects the water retention of the soil. A peaty soil, high in organic matter, holds considerably more water than does a mostly inorganic soil (Chapman and Reiss, 1995). Organic matter contains about five percent total nitrogen, so it serves as a store home for reserve

nitrogen. Organic matter supplies energy and all building constituents for most micro-organisms so it is a critical factor in soil fertility.

Soil organic carbon is one of the constituents of soil organic matter. Soil organic matter roughly contains 58% organic carbon.

Soil organic carbon and organic matter was determined by Walkely and Black (1934) rapid titration method as described by Gupta (200).

$$\text{Soil organic carbon}(\%) = \frac{0.003 \times 5\ (\text{Blank reading} - \text{Titration reading})}{\text{Blank reading} \times \text{wt. of soil (g)}} \times 100$$

The organic carbon obtained by above method was multiplied by a factor 1.3 based on assumptions that there was 77 percent recovery.

Organic carbon = Organic carbon estimated × 1.3

To determine organic matter content of the soil, this organic carbon was multiplied by Van Bemmelen factor of 1.724 because organic mater contains 58% organic carbon.

4.7 Statistical Analysis

Statistical analysis was carried out by using analysis of variance (ANOVA). The procedure of ANOVA was performed by using the standard formula (Bailey, 1994). F-distribution analysis was carried out to find out the level of significance.

The hypothesis assumed was :

For altitude :

$^{A}H_o$ = No significant relationship between altitude and no. of bacteria.

$^{A}H_1$= Significant relationship between altitude and no. of bacteria.

For types of Bacteria :

$^{B}H_o$ = No significant relationship between types and no. of bacteria.

$^{B}H_1$ = Significant relationship between types and no. of bacteria.

At, 5% significance level

when $F_{cal} > F_{tab\ 5\%}$; null hypothesis rejected,

when $F_{tab\ 5\%} > F_{cal}$; null hypothesis accepted.

CHAPTER FIVE

RESULT

In the conformity of various methods described, the results obtained to address the objectives of the study are presented as follows:

5.1 Isolation of rhizobial isolates

The distinct, creamy white to translucent, circular and convex, raised rhizobial colonies were obtained after 7 days (for R101, R103, and R109) and after 10 days (for B111) in YEMA-CR plates inoculated with previously described root nodule extract (Photoplate I, Photo 1 - 4). Pure culture of rhizobial isolates were obtained by subsequent restreaking and pure rhizobial isolates were maintained in YEMA-CR slants. The liquid rhizobial inocula i.e., Yeast Extract Mannitol Broth (YEMB) was obtained after 7 and 10 days for *Rhizobium* and *Bradyrhizobium* respectively which was highly turbid and milky white in colour. These pure cultures were subjected to characterization, authentication and Gram staining.

5.2 Characterization of rhizobial isolates

Different rhizobial isolates were characterized by studying their presumptive morphological and physiological characteristics. The inferences obtained are summarized in the following sub titles.

5.2.1 Colony characteristic and growth response on YEMA-CR medium

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

5.2.2 Acid/Alkali production characteristics

The pure rhizobial isolates showed different characteristics when grown in solid and liquid media.

5.2.2.1 Acid/Alkali production on solid (YEMA-BTB) medium

The blue colour of Bromothymol blue (BTB) was changed to yellow after the sufficient growth of rhizobial colonies on YEMA-BTB plates. Thus all the rhizobial isolates from R101, R103 and R109 were found to be acid producing in the culture after 7 days when incubated in 28±2°C. The bradyrhizobial isolate i.e. B111 cultured after 10 days was found to be alkali producing in YEMA-BTB plates turning the color of the media to light blue (Photo 6).

5.2.2.2 Acid/Alkali production in liquid medium

In order to estimate the amount of acid and alkali produced by different isolates. The broth culture technique was used. The change in pH of the broth was observed during the experiment which is shown in Fig. 3; Appendix II, Table 4. *R. leguminosarum* bv. phaseoli was found to be the most acid producing (pH 4.1) and *Bradyrhizobium* spp. was the alkali producing (pH 7.8) strain.

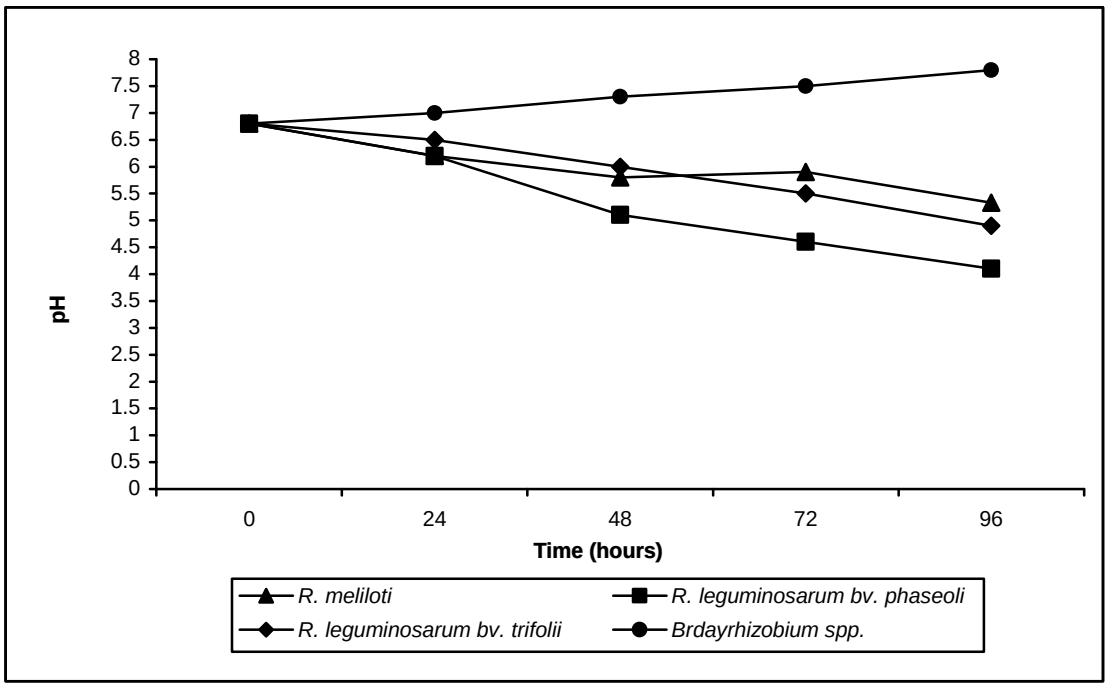


Fig. 3 : Change in pH of broth of rhizobia in different time intervals.

5.2.3 Gram staining

All four rhizobial isolates (R101, R103, R109 and B111) were found to be Gram negative. The rod shaped bacterial cells were pinkish in colour when observed

under oil immersion. Gram negative character of the isolates were taken as the presumptive test for *Rhizobium and Bradyrhizobium* (Photoplate I, Photo 5).

5.2.4 Multiplication of bacterial cells

There was a rapid multiplication of cells of R103 and reached upto 37.51 × 10^{13} cells in 96 hours. There was decrease in multiplication of cells of R101 and R109 after 96 hours. Similarly the multiplication rate of B111 was almost constant after 96 hours. This decrease pattern of multiplication showed a hyperbolic curve (Fig. 4; Appendix II, Table 5). The bacterial population during study was very high so logarithm transformation was used to reduce the data into small values. As a result it is easy to analyze and data changes to linear form.

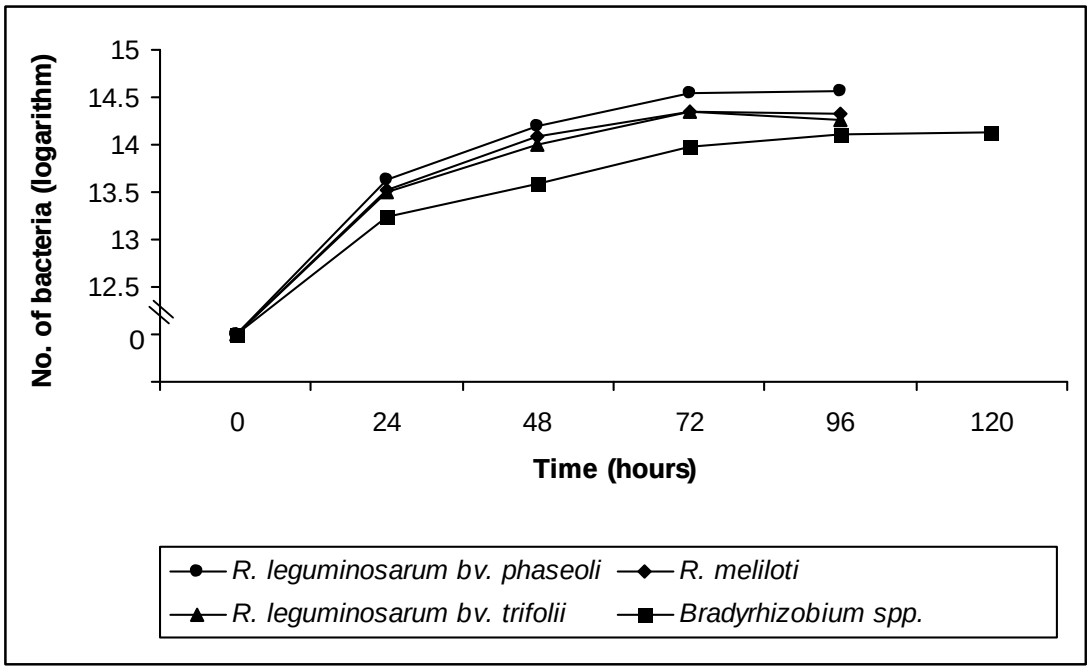


Fig. 4 : Multiplication of bacterial cells in different time intervals.

5.3 Authentication of rhizobial isolates

Plant infection test was carried out in growth pouches of 5 days old rhizobial isolate of R101, R103 and R109; and 7 days old Bradyrhizobial isolate (B111). The physical appearance of the nodules appeared on the root system of *Trigonella corniculata* by the isolate R101, *Phaseolus vulgaris* by the isolate R103, *Trifolium repens* by the isolate R109 confirmed the isolates to be *R.*

meliloti, *R. leguminosarum* bv. phaseoli and *R. leguminosarum* bv. trifolii respectively; and similarly for *Desmodium* spp. by the isolate B111 confirmed the isolate to be *Bradyrhizobium* spp. (Photoplate II, Photo 7, 8, 9, 10).

The viable counts of the rhizobia inoculated for plant infection were:

Rhizobial isolates	**Viable count**
R101	55×10^{11} cells ml^{-1}
R103	58×10^{11} cells ml^{-1}
R109	43×10^{11} cells ml^{-1}
B111	12×10^{11} cells ml^{-1}

5.4 Estimation of Most Probable Number (MPN)

After three weeks of plantation and inoculation with soil dilution, the nodules appeared on the lateral roots showing the legume-*Rhizobium* symbiosis. Each plant was carefully studied for the presence or absence of the nodule. The plants which were inoculated with 2ml aliquot of 4^{-8} dilution series did not nodulated whereas above this series nodulated depending upon the dilution of the soil. From the total number of nodulated units (Appendix IV) the most likely number (m) of *Rhizobium* and *Bradyrhizobium* were estimated by plant infection. Finally MPN was determined. The highest population of *R. leguminosarum* bv. phaseoli was found in spot 15 i.e., Chame (70×10^3 cells g^{-1} soil), *R. meliloti* in spot 14 and 15 i.e., Taleku and Chame (48×10^3 cells g^{-1} soil). *Bradyrhizobium japonicum* in spot 15 i.e., Chame (40×10^2 cells g^{-1} soil) and *Bradyrhizobium* spp. in spot 15 i.e., Chame (112×10^2 cells g^{-1} soil). The population of bacteria was lower at higher altitude i.e., Khangsar Goth. The population was found greater with the decrease in altitude. The detailed information of the bacterial population with respect to altitude is given in Fig. 5; Appendix II, Table 6.

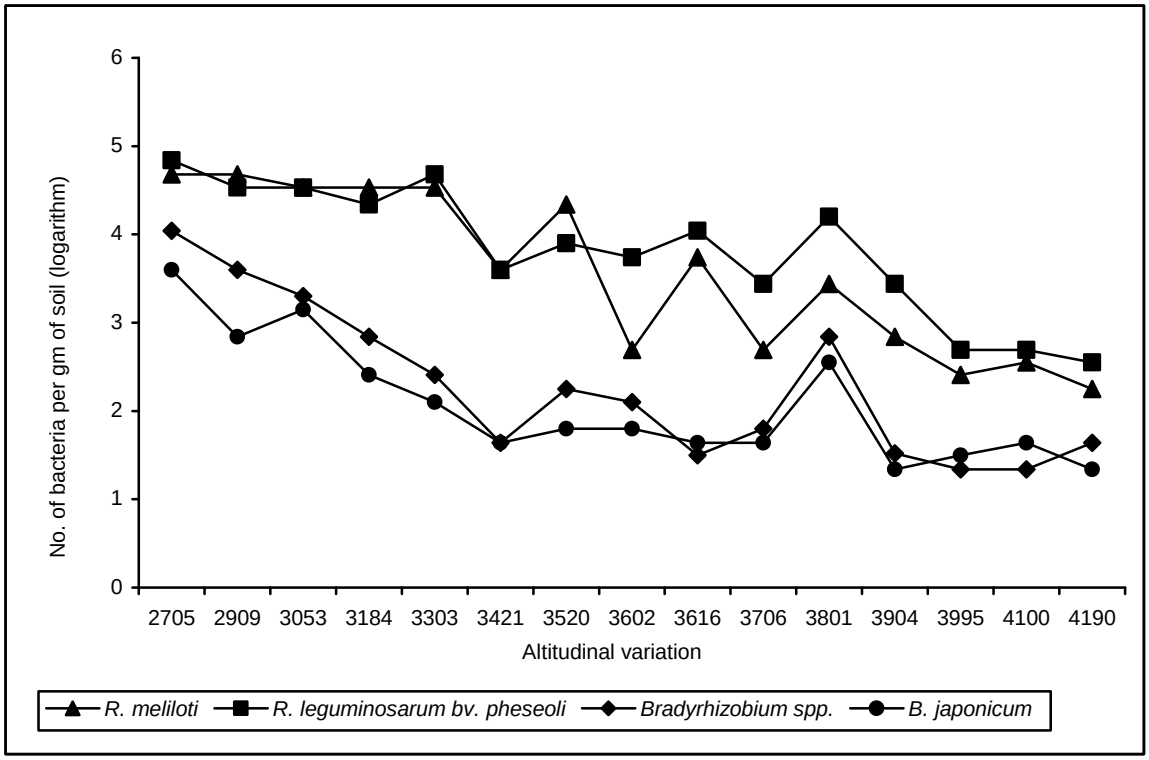


Fig. 5 : Most Probable Number (MPN) of different types of bacteria based on altitudinal variation

5.5 Evaluation of effectiveness of rhizobial strains from soil of Manang and Kathmandu

The total nitrogen content of the nodules from the legumes (described in 4.5) grown in soil of Manang and Kathmandu were compared among the same genus of legume. Among the grown plants the highest nitrogen content i.e. 5.24% was found in nodules of *Phaseolus vulgaris* grown in the soil of Manang while that of same plant, planted in soil of Kathmandu was 4.76%. The lowest percentage of nitrogen content in root nodule was found in *Glycine max*, i.e., 3.98% grown in soil sample of Manang while that from Kathmandu was 5.01%. *Trigonella corniculata* and *Dolichos lablab* remained in the intermediate position between *Phaseolus vulgaris* and *Glycine max*. The total root nodule nitrogen content of each species from two localities is shown in Fig. 6; Appendix II, Table 7.

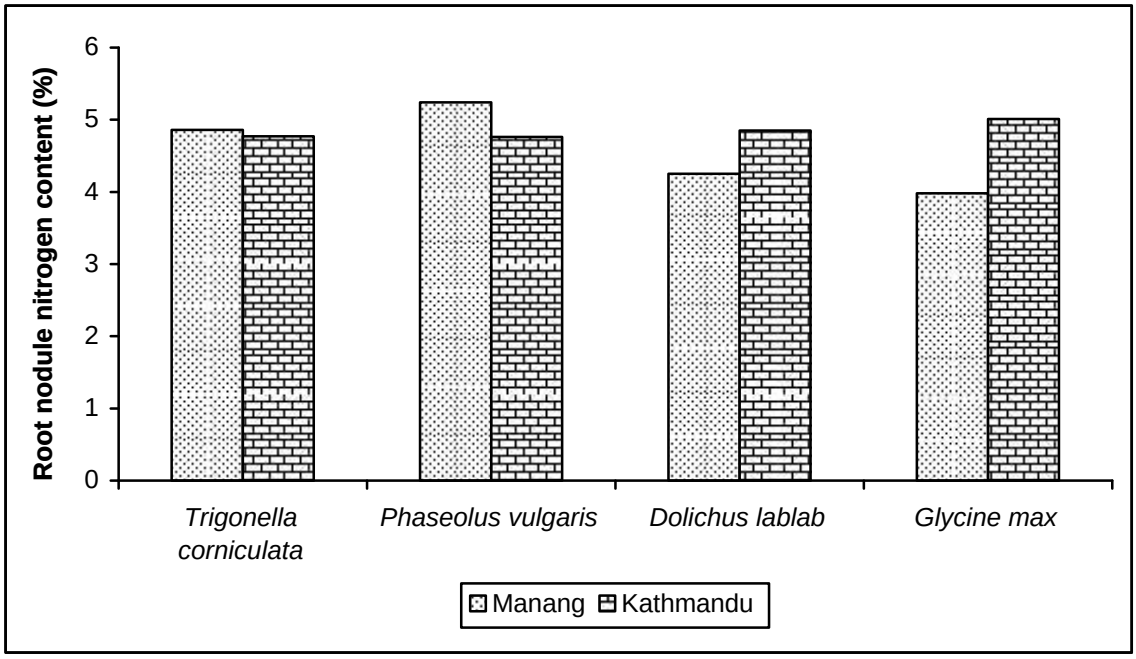


Fig. 6: Root nodule nitrogen content (%) of different legumes from two different localities (i.e., Manang and Kathmandu)

5.6 Soil Analysis

5.6.1 Soil pH

The pH of the soil sample in the study area was slightly acidic. The highest value of pH was found in spot no. 5 i.e., 3801m (pH 6.82) where as the least pH was found in spot no. 15 i.e., 2705m (pH 6.16). Spot wise soil pH is shown in appendix II, Table 8.

5.6.2 Soil moisture (%)

The soil moisture was found highest in spot no. 10 i.e., 3303m (3.46%) whereas the lowest value was found in spot no. 5 i.e., 3801m (1.6%). Spot wise soil moisture is shown in Appendix II, Table 8.

5.6.3 Nitrogen (%)

The maximum value of total nitrogen content was in spot no. 5 i.e., 3801m (0.45%). Similarly the minimum value was in spot no. 12 i.e., 3184m (0.04%). The spot wise nitrogen content is given in Appendix II, Table 8.

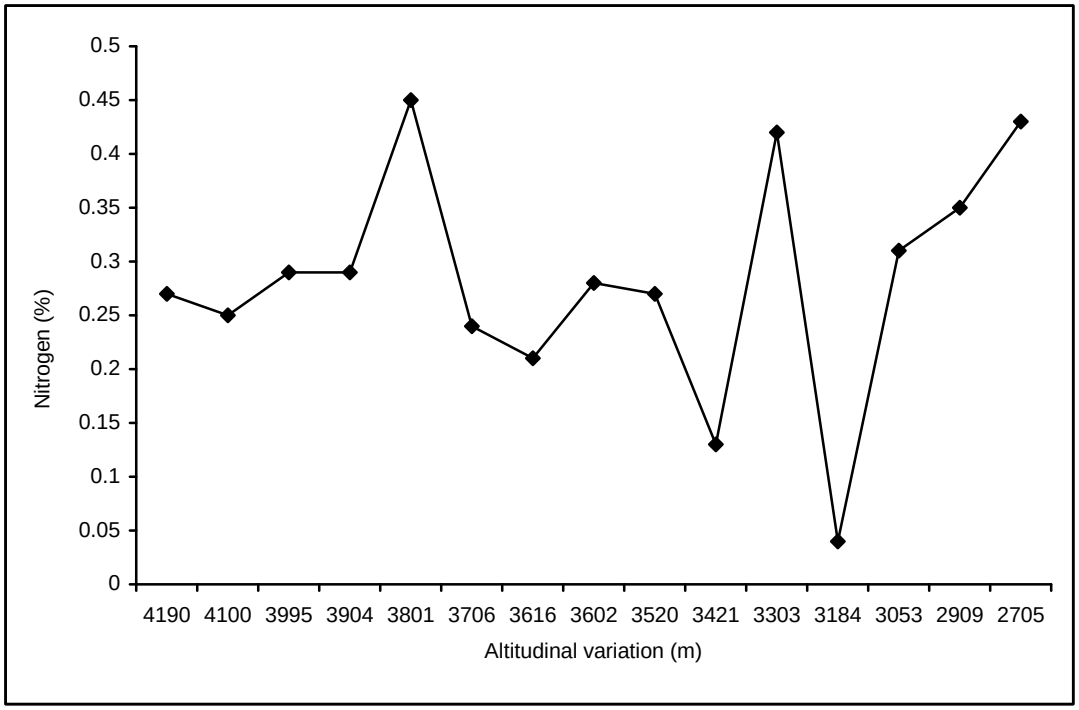


Fig. 7: Nitrogen content (%) of soils from different altitudes of Manang.

5.6.4 Phosphorus (kg/ha)

The highest value of total available phosphorus was found in spot no. 5 i.e., 3801m (110.85 kg/ha) and the lowest value was found in spot no. 9 i.e., 3421m (10.15 kg/ha). The total available phosphorus (kg/ha) of different spots is shown in Appendix II, Table 8.

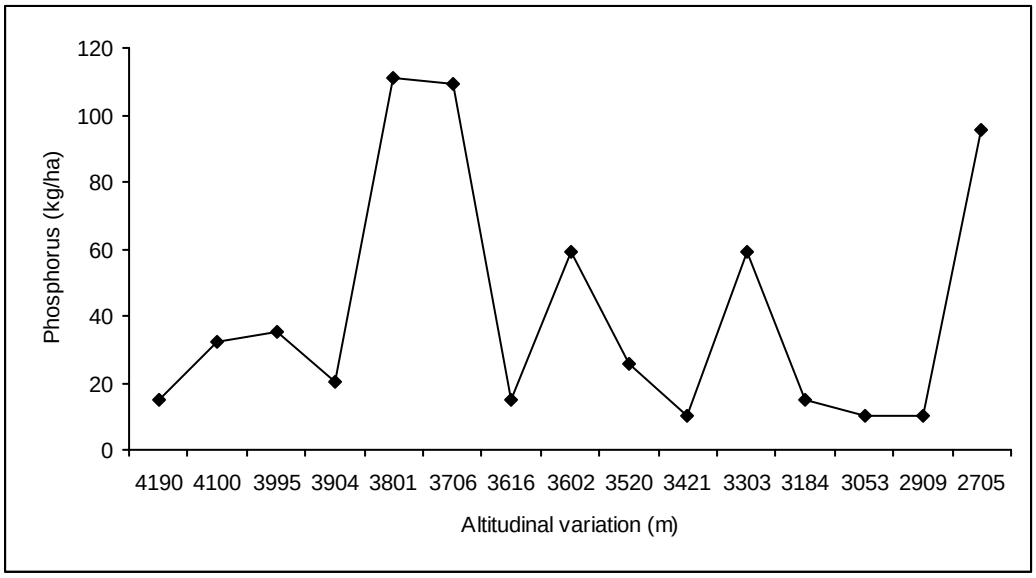


Fig. 8: Total available phosphorus (kg/ha) of soils from different altitudes of Manang.

5.6.5 Potassium (kg/ha)

The highest available potassium was found in spot no. 6 i.e., 3706m (705.6 kg/ha) whereas the lowest value was found in spot no. 13 i.e., 3053m (124.8kg/ha). Potassium (kg/ha) in different spots is shown in Appendix II, Table 8.

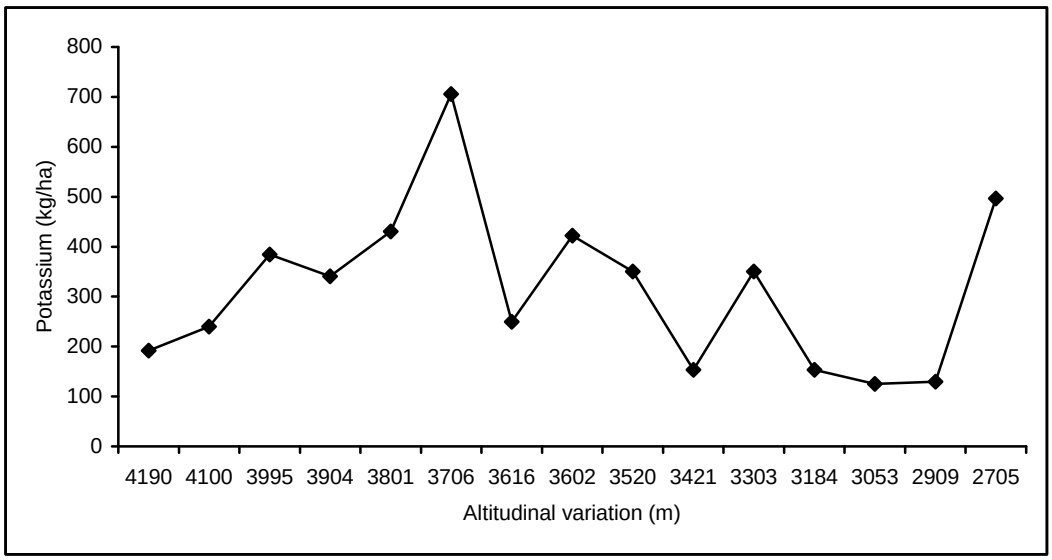


Fig. 9 : Total available potassium (kg/ha) of soils from different altitudes of Manang.

5.6.6 Organic matter (%)

The highest organic matter (%) was contained in soil of spot no. 5 i.e., 3801m (8.93%) whereas it was lowest in spot no. 12 i.e., 3184m (0.86%). Organic matter (%) in different spots in shown in Appendix II, Table 8.

Summary of soil parameters in study sites

Soil character	Range
Soil pH	6.16 to 6.82
Soil moisture (%)	1.6 to 3.46
Nitrogen (%)	0.04 to 0.45
Phosphorus (kg/ha)	10.15 to 110.85
Potassium (kg/ha)	124.8 to 705.6
Organic matter (%)	0.86 to 8.93

5.7 Statistical analysis

The F Ratio (calculated) obtained from the calculation was compared with the F Ratio (Tabular 5%).

For altitude;

$F_{(14, 42)cal} = 3.53$ and $F_{(14, 42)tab\ 5\%} = 1.92$

Here, $F_{cal} > F_{tab\ 5\%}$

Hence, $^{A}H_o$ is rejected i.e., the research revealed that there is significant relation between the altitude and the number of bacteria.

Furthermore, for types of bacteria;

$F_{(3, 42)cal} = 1.01$ and $F_{(2, 42)tab\ 5\%} = 2.84$

Here, $F_{cal} < F_{tab\ 5\%}$

Hence, $^{B}H_o$ is accepted i.e., the research showed that there is no significant relation between the types and no. of bacteria. The no. of bacteria varies significantly ($P < 0.05$) in between the 3303m and 3421m altitudinal range. However in rest of altitudinal ranges (2705m to 3303m) and (3421m to 4190m) the no. varies insignificantly ($P > 0.05$). Finally, the altitudinal response to the no. of bacteria here found is in the order 2705m > 2909m > 3053m > 3184m < 3303m >> 3421m < 3520m > 3602m < 3616m > 3706m < 3801m > 3904m > 3995m < 4100m > 4190m ($P < 0.05$; LDS = 15251) Appendix III.

CHAPTER SIX

DISCUSSION

In the present study, four rhizobial strains i.e., R101, R103, R109 and B111 were isolated from the *Argyrolobium roseum, Phaseolus vulgavis, Trifolium repens* and *Indigofera* spp. root nodules from the plants of Chame (Manang) to observe their characteristics. Furthermore, soil from different altitude of Manang were collected to estimate the population of rhizobia and the effectiveness of bacterial strain from the soil of Chame. Effective root nodules of the four legumes were collected from the site and rhizobia were cultured and isolated using YEMA-CR media (Vincent, 1970, Dalton, 1980). Rhizobial isolates were purified by continuous restreaking of single rhizobial colony and such restreaking was performed several times for each isolate (Ogg and Sharma, 1977, Kaushik *et al.,* 2003). The shorter duration of colony development of R101, R103 and R109 showed the fast growing nature of *Rhizobium* where as the longer duration of colony development of B111 showed the slow growing nature of *Bradryrhizobium*.

Schwinghamer and Dudman (1980) suggested that the colony type in culture provides the much convenient direct means of strain recognition. The creamy white appearance of rhizobial colonies in YEMA medium with Congo-red is due to the fact that YEMA plates had been incubated in dark and this is in accordance with Somasegaran and Hoben (1994) that rhizobia do not absorb Congo-red when incubated in dark but the contaminant organisms usually absorb red dye. Rhizobia can absorb red dye if plates are exposed to light during incubation or exposed to light for an hour or more after growth has occurred.

The selected rhizobial isolates were characterized in the conformity of acid and alkali production, Gram staining as well as the multiplication of bacterial cells in different time intervals. The blue color of bromothymol blue (BTB) after 7 days of rhizobial culture on YEMA-BTB changed into yellow. This indicated the acid producing nature of *Rhizobium*. Similarly the blue color of BTB after 10 days of bradyrhizobial culture on YEMA-BTB plates changed into light blue. This indicated the alkali producing nature of *Bradyrhizobium*.

According to Somasegaran and Hoben (1994), a blue color, indicating alkali reaction on BTB, obtained only in slow growing *Bradyrhizobium* spp. and yellow color is usually produced by fast growing *Rhizobium* spp. Similarly, acid/alkali producing behaviour of rhizobia was also analyzed through broth culture. R101, R103 and R109 showed the decrease in p^H proving that they were acid producing. Among these three, R103 (*R. leguminosarum* bv. phaseoli) was considered the most acid producing bacteria. Beginning from p^H 6.8 at 'O' hour the p^H was changed to 4.1 at 96 hours. The p^H of broth of R101 and R109 were changed to 5.2 and 5.4 respectively in 96 hours. In contrast the p^H of the broth of B111 (*Bradyrhizobium* spp.) was increased and changed to 7.8 in 96 hours. These reasons also gives the conformity for the acid and alkali producing behaviour of rhizobia. The Gram staining test further supported that the rhizobial isolates under examination were the rhizobia as all the isolates showed gram negative response that is also the characteristic feature of rhizobia (Pelczar *et al.*, 1993). From the study of multiplication of bacterial cells in different time intervals, there was a rapid multiplication of cells from 24 hours to 72 hours for different strains (R101, R103 and R109) of bacteria. But after 72 hours there was no more multiplication of cells of R103, whereas in case of R101 and R109 the multiplication rate was lower down in 96 hours. In case of *Bradyrhizobium* spp., there was no much multiplication of cells as those of rhizobia. The multiplication rate was almost constant after 96 hours. (Appendix II, Table 5) This rapid multiplication rate was due to the availability of nutrient for their growth and fall in multiplication rate of bacterial cells was due to the deficiency of nutrient in the medium.

The rhizobial isolates i.e., R101, R103, R109 and B111 obtained were authenticated to be the endosymbiont of *Trigonella corniculata*, *Phaseolus vulgaris*, *Trifolium repens* and *Desmodium* spp. root nodules respectively and confirmed as *R. meliloti*, *R. leguminosarum* bv. phaseoli, *R. leguminosarum* bv. trifolii and *Bradyrhizobium* spp. This was performed by infection test in sterile plastic pouch and filter paper using sterilized modified Jensen's N-free medium (Somasegaran and Hoben, 1994). According to Brockwell (1980), an isolate cannot properly be regarded as species of *Rhizobium* until its identity has been confirmed and bacteriologically controlled plant infection tests are

usually used for authentication of strains. Generally, three symbiotic criteria i.e., nodulation, nitrogen fixation and level of effectiveness can be employed for strain identification. The infection test revealed that the seedlings growing on inoculated pouches were having distinct nodules on their roots. All the root were healthy with internally pinkish color when sliced, which was due to the leghaemoglobin produced within the nodule. This denotes that the nodules so formed were effective one (Bergerson, 1974). In the infection test, the nitrogen free nutrient medium was supplied for the growing legume seedlings to create the nitrogen deficient condition, which stimulate the bacterial nod gene expression to initiate nodulation leading to nitrogen. Quispel (1974) suggested that the presence of sufficient combined nitrogen interfere the nod gene expression and ultimately nodulation and nitrogen fixation. Schubert and Coker (1984) also found that the presence of nitrate or ammonia in the rooting medium decreased the nitrogen fixation.

There was significant variation in bacterial population in different spots ranging from 2705m asl to 4190m asl. Among the four bacteria i.e., *R. meliloti*, *R. leguminosarum* bv. phaseoli, *Bradyrhizobium japonicum* and *Bradyrhizobium* spp., the highest number of bacterial population was of *R. leguminosarum* bv. phaseoli, *R. meliloti* stood in the second position. *Bradyrhizobium* spp. stood in the third position and *B. japonicum* remained the last. Khangsar Goth (4190 m) showed very little population of bacterial cells and remained almost constant upto 3995m. Spot no. 5 i.e., Khangsar (3801m) showed the satisfactory presence of bacterial cells. Similarly spot no. 11 (Ngawal, 3616m), spot no. 8 (Manang, 3520), spot no. 10 (Humde, 3303m) showed the large presence of nodulating bacteria. From spot no. 12 (Pisang, 3184m) onwards there was increase in number of bacterial cells upto spot no. 15 (Chame, 2705m). The cultivated land of Chame contained the highest population of *R. leguminosarum* bv phaseoli i.e., 70×10^3 cells g^{-1} of soil, *R. meliloti* i.e., 48×10^3 cells g^{-1} soil, *Bradyrhizobium* spp. i.e., 112×10^2 cells g^{-1} soil and *B. japonicum* i.e., 40×10^2 cells g^{-1} soil. To test the compliance probability of a dilution series, the range of transition was compared with a tabular value applicable to the dilution series or replicate combination (Somasegaran and Hoben, 1994). From the distribution of bacterial cell

population it was seen that, as the altitude of the cultivated land increases, the bacterial cell population decreases and vice versa (Appendix II, Table 6). It was also seen that the highest number of bacteria was found in those cultivated land where legumes are mostly grown.

The nitrogen content in root nodule of different legumes grown in soil of Manang and Kathmandu was recorded for the evaluation of symbiotic effectiveness of rhizobia from Manang and Kathmandu. *Phaseolus vulgaris* root nodule contained the highest proportion of nitrogen content (5.24%) from the soil of Manang. Similarly *Glycine max* root nodule contained 5.01% nitrogen from the soil of Kathmandu. The nitrogen content in nodule of *Trigonella corniculata* was found almost similar from the soil of Manang and Kathmandu separately. In case of *Dolichos lablab* nitrogen content was found higher in those nodule obtained from the soil of Kathmandu. The effectiveness of bacterial cell was reverse in case of plants *Phaseolus vulgaris* and *Glycine max*. *R. leguminosarum* bv. phaseoli was found to be most effective strain and *B. japonicum* was found to be least symbiotically effective in Manang and vice versa in Kathmandu. Mahamdi *et al.* (1999) also revealed the genotype diversity and variable symbiotic efficiency of rhizboial isolates. They found that, out of 160 isolates only few were having symbiotic effectiveness and most were ineffective. Bhattarai and Maskey (1992) also revealed the differential symbiotic efficiency among different *B. japonicum* strains collected from different sites of Nepal and found that *B. japonicum* strain collected from Nuwakot was the most effective of all.

The nitrogen content of the soil is higher (0.04 to 0.45%), phosphorus content of soil ranged from 10.15 to 110.85kg/ha, while potassium content ranged from 124.8 to 705.6kg/ha. Soil organic matter content is between 0.86 to 8.93% (Appendix II, Table 8). NARC (2002) published standards of soil nitrogen for Terai and Midhills, into four categories namely very low, low, medium, high and very high (However, our area does not lie in midhills, for the case of comparison data of midhill is taken here). Soil was found to be slightly acidic (6.16 to 6.82). Similarly the soil moisture ranged 1.6 to 3.46%. The soil nutrient of Khangsar (spot no. 5) is found to contain relatively higher

amount of soil nutrient particularly the nitrogen, phosphorus and organic matter. The reason behind such higher content may be due to farmer's continuous supply of compost manure containing pine needles and the decomposition and mineralization. According to Brady (1984) the conducive condition for rapid decomposition and mineralization include a near neutral p^H, sufficient soil moisture and a good temperature (25 – 30°C). The soil acidity is also variable according to the altitude but such acidity is suitable for the growth of the bacterial cell. In overall, those cultivated land where legumes are continuously grown contain the moderate nutrient content but in some cultivated lands some portion of these are influenced by the chemical fertilizer, especially in the settlement areas.

Statistical analysis (ANOVA) was performed for the bacterial population with respect to its altitude. From the analysis it showed that, there is a significant relation between the altitude and the bacterial population. As from the fig. 4 also, it is revealed that as the height increases the population of bacteria decreases. This relation does not depend upon the types of bacteria but only on the population of bacteria. The decrease in bacterial population on increase in altitude may be due to the dry condition of soil, deficiency of nutrient condition and adverse climatic condition for the growth of bacteria.

CHAPTER SEVEN

CONCLUSION AND RECOMMENDATION

7.1 Conclusion

From the present investigation, the rhizobia isolated from the legumes of Manang were characterized. Colony characteristics, acid or alkali producing behaviour, Gram staining and multiplication rate of bacteria were observed. For the conformity of the specific rhizobia infecting their host legume, their authentication was performed. *R. leguminosarum* bv. phaseoli was found to be the most abundant bacteria in comparison to *R. meliloti, Bradyrhizobium* spp. and *B. japonicum*. *R. meliloti* stood in the second position, *Bradyrhizobium* spp. remained in the third position and *B. japonicum* remained the last. Further more, it is obvious that as the altitude increases the population of bacteria decreases and vice versa. But within the same altitude the population of bacteria may remain diverse depending upon the types of bacteria. From the comparative study of root nodule nitrogen content of different legumes grown in soils of Manang and Kathmandu, the *Phaseolus vulgaris* grown in soil of Manang contained the highest percentage of nitrogen in root nodule indicating the highest effectiveness of *R. leguminosarum* bv. phaseoli. Similarly *B. japonicum* was found to most effective microsymbiont in the soil of Kathmandu.

Therefore the present investigation opens an array of avenews of work on legumes and their microsymbiont as well as their population on different geographical locations mainly at the high altitudes.

7.2 Recommendations

Depending upon the findings of the study, following recommendations has been made.

- To achieve the goal of improvement in agricultural production and forest security through sustainable agricultural system without harming the environment, nitrogen needs in the cereal field can be achieved by the application of legumes.
- Bare land should be applied by the legume cultivation to increase the rhizobial population. The farmers should be encouraged to apply legumes in the crop field to increase the agricultural productivity.
- Commercialization of rhizobial strains should be promoted to increase the rhizobial population (biofertilizer) and reduce the chemical fertilizer.

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APPENDIX I

***Argyrolobium roseum* (Cambess) Jaub. and Spach**

Small much branched prostrate herbaceous plant. Steams 10cm to 30cm. Leaflet obovate, 4 -10 × 2-6mm, base cuneate, margin entire, apex round. Flower red, borne in axillary stalked, 2-few flowered clusters. Pedicels much longer than leaves. Calyx 2-lipped; tube c4mm; teeth c4mm. Petals c1cm. Pods 2-3×0.2-0.4cm.

***Trigonella corniculata* (L.) L. cv. common**

Annual, stems up to 55cm; leaflets oblong to obovate, 10–30×8–10mm, obtuse or retuse, base cuneate, margin denticulate in upper part; petioles 1–5cm; stipules lanceolate, 5–8mm, coarsely toothed; racemes 8–15-flowered, peduncles 3 –6cm; clayx c3mm, glabrous or with a few hairs between teeth; standard obovate, 6–8mm, emarginate, wings oblong c4mm, keel almost as long as standard; pods linear 10–15×3mm, curved, compressed, 3–5-seeded.

***Phaseolus vulgaris* L. cv. Trishuli**

Stem pubescent, leaflets ovate, 6–10×–5–9cm acuminate peduncles 3–8(–15)cm, bracts conspicuous, ovate 7–8×4–5mm, subtending base of calyx; petals white or purplish, standard suborbicular 10–15×10–15mm, wings obovate 15–20×5–11mm, keel c10mm; pods 9–18×1–1.5cm, smooth or finely striated; seeds oblong or reniform, 8–17×5–7mm white, purplish brown to black or variously spotted and mottled.

***Trifolium repens* L.**

Creeping perennial, stems frequently rooting at nodes. Leaves digitately 3-foliate, leaflets obovate or obcordate 1–3×0.7–2cm, apex rounded or emarginate, base cuneate, margin finely denticulate, glabrous, usually with whitish V-shaped marking on upper surface; petioles 4–16cm; stipules membranous, sheathing, upper parts lanceolater, 1.5-2cm, subulate at apex. Flower heads 2–2.5cm diameter, peduncles 6–30cm, pedicels 2–4mm. Calyx tube 2–3mm, teeth lanceloater 3–4mm, upper pair longer than lower ones. Petals white, 7–10mm. Pods oblong, 4–5mm, seeds 3–4.

***Indigofera* L.**

Annual herbs or perennial shrubs, sometimes trees, usually bearing appressed medifixed, branched hairs. Leaves odd-pinnate, sometimes digitately 3-foliate or

simple; stipels sometimes present; stiuples small, linear-lanceolate or subulate. Flowers in lax or dense axillary racemes or clusters, bracts mostly small, deciduous. Calyx tube campanulate, teeth 5, usually subequal. Standard broadly elliptic, ovate or suborbicular, wings oblong or obovate, slightly adherent to keel, keel straight or curved, lanceolate or oblanceolate, obtuse at apex with a short spur or puch on each side near base. Stamens diadelphous. Pods linear or oblong, straight or curved, terete, rarely subglobose, transversely septate between seeds.

***Glycine max* (L.) Merrill cv. Seti**

Stems 30–200cm, erect, spreading brownish hirsute. Leaflets ovate, 5–9(–14)×3–6(–10)cm, acute, base cuneate or rounded, appressed pubescent, stipels narrowly lanceolate 1–3.5mm,; petioles 7–20cm; stipules ovate 3–7mm. Racemes usually 5–8-flowered; bracteoles narrowly lanceolate 2–3mm. Calyx 5–7mm, brown hirsute. Petals purplish or white. 6–8mm, wings narrowly oblong, longer than keel. Pods oblong 4–5 × 0.75–1.2cm, hirsute; seeds 2–3, 6–10×5–7mm, creamy-white or blackish.

***Dolichos lablab* (L.) Sweet**

Annual, leaflets elliptic or obovate, 1–5(–8)×0.7–3(–7.5)cm, subacute or obtuse, base rounded, pubescent,; stipels filiform 2mm; stipules lanceolate 4–8mm, veiny. Flowers subsessile, in clusters of (1–) 2–3(–5), acuminate. Petals yellow or greenish, standard obovate-oblong, 6–12×4 –7mm, usually with a central purple spot, lamelliform appendages c5mm, wings 4–10×1mm, keel slightly curved 5–10×1.5mm. Pods 3–5.5×0.5 –0.8cm, pubescent; seeds blackish 3–6.5×3–5mm.

***Desmodium* D.**

Shrub upto 2m. leaves 3 foliate, leaflets elliptic or bobovate, 2–5×1–2cm, obtuse, base rounded, appresed greyish pubescent beneath. Petioles 1–2cm, stipules lanceolate, 7–10×2–3mm, Rescemes elongate, axillary or terminal. Calyx 2.5 – 3mm. Petal purplish 4.5 – 8mm, standard elliptic to suborbicular 4 – 6 broad, wings and keel oblong or obovate, blades 3.5 – 5 × 1.5 – 2.5mm. Pods undulate along lower suture, sparsely pubescent with hooked hairs.

APPENDIX II

Table 1 : Physiography of the study area

S.N.	Spot No.	Location	Altitude (m)	Latitude	Longitude
1	1	Khangsar Goth	4190	28°41'766"	83°58'334"
2	2	Above Khangsar	4100	28°41'573"	83°58'436"
3	3	Above Khangsar	3995	28°41'508"	83°58'419"
4	4	Above Khangsar	3904	28°41'283"	83°58'440"
5	5	Khangsar	3801	28°41'180"	83°58'380"
6	6	Above Tanki Manang	3706	28°41'284"	84°00'791"
7	7	Tanki Manang	3602	28°41'196"	84°00'846"
8	8	Manang	3520	28°41'175"	84°00'987"
9	9	Bhraka	3421	28°39'249"	84°02'538"
10	10	Humde	3303	28°38'287"	84°05'432"
11	11	Upper Ngawal	3616	28°38'982"	84°06'052"
12	12	Pisang	3184	28°36'920"	84°08'836"
13	13	Bhratang	3053	28°33'782"	84°12'682"
14	14	Taleku	2909	28°33'338"	84°13'826"
15	15	Chame	2705	28°33'209"	84°14'346"

Table 2: Monthly average precipitation, maximum and minimum temperature of Chame (2006)

S.N.	Months	Precipitation (mm)	Maximum Temp. (°C)	Minimum Temp. (°C)
1	January	0.0	16.2	-0.1
2	February	0.0	19.4	3.6
3	March	67.7	18.8	4.0
4	April	0.0	21.3	5.9
5	May	101.0	21.1	6.3
6	June	42.0	21.4	7.0
7	July	366.2	21.2	6.6
8	August	287.0	21.6	6.7
9	September	84.0	19.9	5.2
10	October	0.0	18.6	3.2
11	November	0.0	16.4	-0.8
12	December	0.0	15.9	-1.4

Source: Department of Meterology.

Table 3: Physiography of the site of nodule collection

S.N.	Tag. no.	Legumes	Plant status	Altitude (m)	Latitude	Longitude
1	101	*Argyrolobium roseum*	Wild	2720	28°33'174"	84°14'27"
2	103	*Phaseolus vulgaris*	Cultivated	2705	28°33'209"	84°14'346"
3	109	*Trifolium repens*	Wild	2720	28°33'197"	84°30'95"
4	111	*Indigofera* spp.	Wild	2720	28°33'159"	84°30'962"

Table 4:Change in p^H of the broth of rhizobia in different time intervals

Bacterial strain	Change in p^H (hours)				
	0	24	48	72	96
R101 (*R. meliloti*)	6.8	6.2	5.8	5.9	5.2
R103 (*R. leguminosarum* bv. phaseoli)	6.8	6.2	5.1	4.6	4.1
R109 (*R. leguminosarum* bv. trifolii)	6.8	6.3	6.0	5.8	5.4
R111 (*Bradyrhizobium* spp.*)*	6.8	7.0	7.3	7.5	7.8

Table 5: Multiplication of bacterial cells in different time intervals

S.N.	Bacterial strains	No. of bacteria					
		0 hr.	24 hr	48 hr	72 hr	96 hr	120 hr
1	R101	0	3.48×10^{13}	12.11×10^{13}	22.57×10^{13}	21.24×10^{13}	-
2	R103	0	4.48×10^{13}	15.60×10^{13}	34.69×10^{13}	37.51×10^{13}	-
3	R109	0	3.15×10^{13}	9.96×10^{13}	22.07×10^{13}	18.26×10^{13}	-
4	B111	0	1.82×10^{13}	3.98×10^{13}	9.62×10^{13}	12.61×10^{13}	13.94×10^{13}

Table 6: Most Probable Number (MPN) of different types of bacteria based on altitudinal variation

Spot No.	Location	Altitude (m)	MPN of bacteria based on different legumes			
			R. meliloti (hp: *Trigonella*) *corniculata*	*R. leguminosarum* bv. phaseoli (hp: *Phaseolus vulgaris*)	*Bradyrhizobium spp.* (hp : *Dolichors lablab*)	*Bradyrhizobium japonicum* (hp : *Glycine max*)
1	Khangsar Goth	4190	178	36×10^{1}	44	22
2	Above Khangsar	4100	36×10^{1}	50×10^{1}	22	44
3	Above Khangsar	3995	26×10^{1}	50×10^{1}	22	32
4	Above Khangsar	3904	70×10^{1}	28×10^{2}	32	22
5	Khangsar	3801	28×10^{2}	160×10^{2}	70×10^{1}	36×10^{1}
6	Above Tanki Manang	3706	50×10^{1}	28×10^{2}	64	44
7	Tanki Manang	3602	50×10^{1}	56×10^{2}	126	64
8	Manang	3520	22×10^{3}	80×10^{2}	178	64
9	Bhraka	3421	40×10^{2}	40×10^{2}	44	44
10	Humde	3303	34×10^{3}	48×10^{3}	26×10^{1}	126
11	Upper Ngawal	3616	56×10^{2}	112×10^{2}	32	44
12	Pisang	3184	34×10^{3}	22×10^{3}	70×10^{1}	26×10^{1}
13	Bhratang	3053	34×10^{3}	34×10^{3}	20×10^{2}	142×10^{1}
14	Taleku	2909	48×10^{3}	34×10^{3}	40×10^{2}	70×10^{1}
15	Chame	2705	48×10^{3}	70×10^{3}	112×10^{2}	40×10^{2}

Note: hp - host plant.

Table 7: Root nodule nitrogen content (%) of different legumes from two different localities i.e., Manang and Kathmandu

S.N.	Legumes	Root nodule nitrogen content (%)	
		Manang	Kathmandu
1	*Trigonella corniculata*	4.86%	4.77%
2	*Phaseolus vulgaris*	5.24%	4.76%
3	*Dolichos lablab*	4.25%	4.85%
4	*Glycine max*	3.98%	5.01%

Table 8: Sport wise soil p^H, moisture, nitrogen, phosphorus, potassium and organic matter content of Manang

Spot No.	Altitude (m)	p^H	Moisture (%)	Nitrogen (%)	Phosphorus (kg/ha)	Potassium (kg/ha)	Organic matter (%)
1	4190	6.54	2.32	0.27	15.15	192	5.40
2	4100	6.35	5.25	0.25	32.2	240	5.25
3	3995	6.36	2.65	0.29	35.12	384	5.76
4	3904	6.44	2.56	0.29	20.33	340.8	5.76
5	3801	6.82	1.60	0.45	110.85	430.4	8.93
6	3706	6.72	1.74	0.24	109.12	705.6	4.75
7	3602	6.26	3.21	0.28	59.1	422.4	5.61
8	3520	6.6	2.20	0.27	25.5	350.4	5.47
9	3421	6.74	1.52	0.13	10.15	153.6	2.66
10	3303	6.17	3.46	0.42	59.1	350.4	8.57
11	3616	6.56	2.00	0.21	15.16	249.6	4.10
12	3184	6.54	2.28	0.04	15.15	153.6	0.86
13	3053	6.58	2.22	0.31	10.2	124.8	6.10
14	2909	6.64	1.94	0.35	10.2	129.6	7.13
15	2705	6.16	2.24	0.43	95.25	496.8	8.71

APPENDIX III

Table 1 : Data for ANOVA containing different types of rhizobia at different altitudes.

Altitude (m)	MPN of bacteria				**Total bacteria (T)**	Mean (X)
	R. meliloti	*R. leguminosarum* bv. phaseoli	*Bradyrhizobium* spp.	*B. japonicum*		
4190	178	36×10^1	44	22	**604**	151
4100	36×10^1	50×10^1	22	44	**926**	231.5
3995	26×10^1	50×10^1	22	32	**814**	203.5
3904	70×10^1	28×10^2	32	22	**3554**	888.5
3801	28×10^2	160×10^2	70×10^1	36×10^1	**19860**	4965
3706	50×10^1	28×10^2	64	44	**3408**	852
3616	56×10^2	112	32	44	**16876**	4219
3602	50×10^1	56×10^2	126	64	**6290**	1572.5
3520	22×10^3	80×10^2	178	64	**30242**	7560.5
3421	40×10^2	40×10^2	44	44	**8088**	2022
3303	34×10^3	48×10^3	26×10^1	126	**82386**	20596.5
3184	34×10^3	22×10^3	70×10^1	26×10^1	**56960**	14240
3053	34×10^3	34×10^3	20×10^2	142×10^1	**71420**	17855
2909	48×10^3	34×10^3	40×10^2	70×10^1	**86700**	21675
2705	48×10^3	70×10^3	112×10^2	40×10^2	**133200**	33300
Total	**234898**	**259760**	**19424**	**7246**	**521328**	**130332**

No. of Altitude = k = 15

Types of Bacteria = b = 4

No. of replicates studied per altitude = n = 1

Grand total of all altitude bacteria (GT) = 521328

Grand mean of all altitude bacteria (X) = 130332

Correction factor (CF) = $\frac{(GT)^2}{bkn}$ = 4529714726

Total sum of squares (SS) = $\Sigma X^2 - CF$ = $1.476109731\times 10^{10}$

Altitude sum of squares (SST) = $\frac{\Sigma T^2}{bn} - CF$ = 5999472436

Bacterial sum of squares (SSB) = $\frac{\Sigma B^2}{kn} - CF$ = 3675760227

Error sum of squares (SSE) = SS – (SST + SSB) = 5085864647

Table 2 : ANOVA chart for different types of bacteria at different altitudes.

Sources of variation	Sum of squares	df	Mean squares	F Ratio (calculated)	F Ratio (Tabular 5%)
Altitude	5999472436	14	428533745.4	3.53891001*	1.92
Bacteria types	3675760227	3	122525409	1.01183722#	2.84
Error	5085864647	42	121092015.4		
Total	1.476109731×10^{10}	59			

* = Significant # = non significant

We analyze only for the significant case.

LSD for altitude $(LSD)_{0.05} = T_{0.05}\sqrt{\frac{2\times \text{mean square for error}}{\text{No. of replications}}}$

$$=1.960\sqrt{\frac{2\times121092015.4}{4}}$$

= 15251.01 bacteria

APPENDIX IV

Data obtained for the MPN enumeration of different rhizobia in field soils using different legumes as the trap host.

Test legume: *Trigonella corniculata.*

Spot No. 1 Altitude: 4190m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	3	+
4^{-3}	-	+	-	-	1	+
4^{-4}	+	-	-	-	1	+
4^{-5}	-	+	-	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	10	

Spot No. 3 Altitude: 3995m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	-	+	+	3	+
4^{-3}	-	+	-	-	1	+
4^{-4}	-	+	-	+	2	+
4^{-5}	-	-	+	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	11	

Spot No. 2 Altitude: 4110m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	-	+	-	+	2	+
4^{-4}	+	-	-	-	1	+
4^{-5}	-	+	-	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	12	

Spot No. 4 Altitude: 3904m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	-	+	+	3	+
4^{-3}	+	-	-	-	1	+
4^{-4}	-	+	-	+	2	+
4^{-5}	-	+	-	+	2	+
4^{-6}	+	+	-	-	2	+
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	14	

Spot No. 5 Altitude: 3801m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	-	-	2	+
4^{-4}	+	-	+	+	3	+
4^{-5}	+	-	-	+	2	+
4^{-6}	+	+	+	-	3	+
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	18	

Spot No. 6 Altitude: 3706m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	-	-	+	2	+
4^{-3}	-	+	+	+	3	+
4^{-4}	+	-	-	+	2	+
4^{-5}	+	-	-	-	1	+
4^{-6}	-	+	-	-	1	+
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	13	

Spot No. 7 Altitude: 3602m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	-	3	+
4^{-3}	+	-	-	+	2	+
4^{-4}	-	-	+	+	2	+
4^{-5}	+	-	-	+	2	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	13	

Spot No. 8 Altitude: 3520m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	-	+	+	+	3	+
4^{-6}	+	-	+	+	3	+
4^{-7}	-	+	-	+	2	+
4^{-8}	-	-	-	-		
				Total	24	

Spot No. 9 Altitude: 3421m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	-	+	+	3	+
4^{-3}	+	+	+	+	4	
4^{-4}	-	+	-	-	1	+
4^{-5}	+	-	+	-	2	+
4^{-6}	+	+	-	+	3	+
4^{-7}	-	+	-	+	2	+
4^{-8}	-	-	-	-		
				Total	19	

Spot No. 11 Altitude: 3616m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	-	+	+	3	+
4^{-5}	+	-	+	-	2	+
4^{-6}	-	+	-	+	2	+
4^{-7}	+	-	-	-	1	+
4^{-8}	-	-	-	-		
				Total	20	

Spot No. 10 Altitude: 3303m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	+	+	+	4	
4^{-6}	-	+	+	+	3	+
4^{-7}	+	-	-	+	2	+
4^{-8}	-	-	-	-		
				Total	25	

Spot No. 12 Altitude: 3184m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	-	+	+	3	+
4^{-4}	+	+	+	+	4	
4^{-5}	+	+	+	+	4	
4^{-6}	+	+	+	+	4	
4^{-7}	-	-	-	+	2	+
4^{-8}	-	-	-	-		
				Total	25	

Spot No. 13 Altitude: 3053m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	+	+	+	4	
4^{-6}	+	+	+	-	3	+
4^{-7}	+	-	+	-	2	+
4^{-8}	-	-	-	-		
				Total	25	

Spot No. 15 Altitude: 2705m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	+	+	+	4	
4^{-6}	+	+	+	-	3	+
4^{-7}	-	+	+	+	3	+
4^{-8}	-	-	-	-		
				Total	26	

Spot No. 14 Altitude: 2909m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	+	+	+	4	
4^{-6}	+	+	+	-	3	+
4^{-7}	+	-	+	+	3	+
4^{-8}	-	-	-	-		
				Total	26	

Test legume: ***Phaseolus vulgaris***

Spot No. 1 Altitude: 4190m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	-	3	+
4^{-3}	-	+	-	+	2	+
4^{-4}	-	+	+	-	2	+
4^{-5}	+	-	-	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	12	

Spot No. 3 Altitude: 3995m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	-	+	+	+	3	+
4^{-3}	-	+	-	+	2	+
4^{-4}	-	+	-	+	2	+
4^{-5}	-	+	-	+	2	+
4^{-6}	-	-	-	-		+
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	13	

Spot No. 2 Altitude: 4110m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	-	+	+	+	3	+
4^{-3}	+	-	+	-	2	+
4^{-4}	+	-	+	-	2	+
4^{-5}	-	+	-	-	1	+
4^{-6}	-	-	+	-	1	+
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	13	

Spot No. 4 Altitude: 3904m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	-	+	+	+	3	+
4^{-4}	+	+	+	-	3	+
4^{-5}	-	+	+	-	2	+
4^{-6}	+	-	+	-	2	+
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	18	

Spot No. 5 Altitude: 3801m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	-	+	+	3	+
4^{-6}	+	+	-	+	3	+
4^{-7}	+	-	-	-	1	+
4^{-8}	-	-	-	-		
				Total	23	

Spot No. 6 Altitude: 3706m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	-	+	3	+
4^{-4}	+	+	-	+	3	+
4^{-5}	+	-	+	-	2	+
4^{-6}	-	-	+	-	1	+
4^{-7}	-	+	-	-	1	+
4^{-8}	-	-	-	-		
				Total	18	

Spot No. 7 Altitude: 3602m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	-	+	3	+
4^{-4}	-	+	+	+	3	+
4^{-5}	-	+	+	-	2	+
4^{-6}	+	-	+	+	3	+
4^{-7}	-	+	-	-	1	
4^{-8}	-	-	-	-		
				Total	20	

Spot No. 8 Altitude: 3520m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	-	3	+
4^{-5}	-	+	+	+	3	+
4^{-6}	-	+	-	+	2	+
4^{-7}	-	+	-	-	1	+
4^{-8}	-	-	-	-		
				Total	21	

Spot No. 9 Altitude: 3421m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	-	+	3	+
4^{-4}	-	+	-	+	2	+
4^{-5}	+	+	-	+	3	+
4^{-6}	+	-	+	-	2	+
4^{-7}	+	-	-	-	1	+
4^{-8}	-	-	-	-		
				Total	19	

Spot No. 10 Altitude: 3303m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	+	+	+	4	
4^{-6}	+	+	-	+	3	+
4^{-7}	+	+	+	-	3	+
4^{-8}	-	-	-	-		
				Total	26	

Spot No. 11 Altitude: 3616m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	+	-	+	3	+
4^{-6}	-	+	-	+	2	+
4^{-7}	+	-	-	-	1	+
4^{-8}	-	-	-	-		
				Total	22	

Spot No. 12 Altitude: 3184m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	+	+	+	4	
4^{-6}	+	+	+	-	3	+
4^{-7}	+	-	-	-	1	+
4^{-8}	-	-	-	-		
				Total	24	

Spot No. 13 Altitude: 3053m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	+	+	+	4	
4^{-6}	+	-	+	+	3	+
4^{-7}	-	+	-	+	2	+
4^{-8}	-	-	-	-		
				Total	25	

Spot No. 15 Altitude: 2705m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	+	+	+	4	
4^{-6}	+	+	+	+	4	
4^{-7}	+	+	+	-	3	+
4^{-8}	-	-	-	-		
				Total	27	

Spot No. 14 Altitude: 2909m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	3	+
4^{-5}	+	+	+	+	4	
4^{-6}	+	+	+	+	4	
4^{-7}	+	-	+	-	2	+
4^{-8}	-	-	-	-		
				Total	25	

Test legume: ***Dolichos lablab***

Spot No. 1 Altitude: 4190m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	-	3	+
4^{-2}	+	-	+	-	2	+
4^{-3}	+	-	-	-	1	+
4^{-4}	-	-	-	-		
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	6	

Spot No. 3 Altitude: 3995m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	-	-	1	+
4^{-2}	-	+	-	-	1	+
4^{-3}	+	-	-	-	1	+
4^{-4}	-	+	-	-	1	+
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	4	

Spot No. 2 Altitude: 4110m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	+	-	2	+
4^{-2}	-	+	-	-	1	+
4^{-3}	-	-	-	+	1	+
4^{-4}	-	-	-	-		
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	4	

Spot No. 4 Altitude: 3904m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	+	-	2	+
4^{-2}	+	-	-	-	1	+
4^{-3}	-	+	-	-	1	+
4^{-4}	-	-	+	-	1	+
4^{-5}	-	-	-	-		+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	5	

Spot No. 5 Altitude: 3801m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	-	+	+	3	+
4^{-4}	+	-	+	-	2	+
4^{-5}	-	+	-	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	14	

Spot No. 7 Altitude: 3602m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	+	+	3	+
4^{-2}	-	+	-	+	2	+
4^{-3}	-	+	+	-	2	+
4^{-4}	+	-	-	-	1	+
4^{-5}	-	-	+	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	9	

Spot No. 6 Altitude: 3706m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	-	3	+
4^{-2}	-	+	-	+	2	+
4^{-3}	-	-	+	-	1	+
4^{-4}	-	+	-	-	1	+
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	7	

Spot No. 8 Altitude: 3520m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	-	+	+	3	+
4^{-3}	+	-	-	-	1	+
4^{-4}	-	+	-	-	1	+
4^{-5}	+	-	-	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	21	

Spot No. 9 Altitude: 3421m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	-	3	+
4^{-2}	+	-	+	-	2	+
4^{-3}	-	+	-	-	1	+
4^{-4}	-	-	-	-		
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	6	

Spot No. 11 Altitude: 3616m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	-	+	3	+
4^{-2}	+	-	-	-	1	+
4^{-3}	-	-	-	+	1	+
4^{-4}	-	-	-	-		
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	5	

Spot No. 10 Altitude: 3303m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	-	+	+	3	+
4^{-3}	+	-	+	-	2	+
4^{-4}	-	-	-	+	1	+
4^{-5}	-	+	-	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	11	

Spot No. 12 Altitude: 3184m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	-	+	3	+
4^{-3}	+	-	+	-	3	+
4^{-4}	-	+	-	+	2	+
4^{-5}	+	-	+	-	2	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	14	

Spot No. 13 Altitude: 3053m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	-	+	+	3	+
4^{-4}	+	-	+	+	3	+
4^{-5}	-	+	-	+	2	+
4^{-6}	+	-	-	-	1	
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	17	

Spot No. 15 Altitude: 2705m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	+	4	
4^{-5}	+	-	+	+	3	+
4^{-6}	-	+	-	+	2	+
4^{-7}	+	-	-	-	1	+
4^{-8}	-	-	-	-		
				Total	22	

Spot No. 14 Altitude: 2909m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	-	+	+	3	+
4^{-5}	+	-	+	+	3	+
4^{-6}	-	+	-	-	1	+
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	19	

Test legume: ***Glycine max***

Spot No. 1 Altitude: 4190m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	-	+	+	-	2	+
4^{-2}	+	-	-	-	1	+
4^{-3}	-	-	-	+	1	+
4^{-4}	-	-	-	-		
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}						
				Total	4	

Spot No. 3 Altitude: 3995m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	+	-	2	+
4^{-2}	-	+	-	+	2	+
4^{-3}	-	-	+	-	1	+
4^{-4}	-	-	-	-		
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	5	

Spot No. 2 Altitude: 4100m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	+	+	3	+
4^{-2}	-	+	-	-	1	+
4^{-3}	+	-	-	-	1	+
4^{-4}	-	-	+	-	1	+
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	6	

Spot No. 4 Altitude: 3904m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	-	-	1	+
4^{-2}	-	+	-	-	1	+
4^{-3}	+	-	+	-	2	+
4^{-4}	-	-	-	-		
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	4	

Spot No. 5 Altitude: 3801m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	-	+	-	2	+
4^{-3}	-	+	+	+	3	+
4^{-4}	+	-	-	-	1	+
4^{-5}	+	-	-	-	1	+
4^{-6}	-	+	-	-	1	+
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	12	

Spot No. 6 Altitude: 3706m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	+	-	2	+
4^{-2}	-	+	-	-	1	+
4^{-3}	+	-	-	-	1	+
4^{-4}	-	+	-	-	1	+
4^{-5}	-	-	+	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	6	

Spot No. 7 Altitude: 3602m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	+	+	3	+
4^{-2}	+	-	+	-	2	+
4^{-3}	-	+	-	-	1	+
4^{-4}	-	-	+	-	1	+
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	7	

Spot No. 8 Altitude: 3520m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	-	-	2	+
4^{-2}	-	+	-	-	1	+
4^{-3}	-	-	+	-	1	+
4^{-4}	+	+	-	-	2	+
4^{-5}	-	-	+	-	1	
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	7	

Spot No. 9 Altitude: 3421m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	+	-	2	+
4^{-2}	-	+	+	-	2	+
4^{-3}	+	-	-	-	1	+
4^{-4}	-	-	+	-	1	+
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	6	

Spot No. 11 Altitude: 3616m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	-	+	-	2	+
4^{-2}	-	+	-	-	1	+
4^{-3}	+	-	+	-	2	+
4^{-4}	-	+	-	-	1	+
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	--	-	-		
4^{-8}	-	-	-	-		
				Total	6	

Spot No. 10 Altitude: 3303m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	-	3	+
4^{-2}	+	+	+	-	3	+
4^{-3}	+	-	+	-	2	+
4^{-4}	-	+	-	-	1	+
4^{-5}	-	-	-	-		
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	9	

Spot No. 12 Altitude: 3184m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	-	+	3	+
4^{-2}	-	+	+	+	3	+
4^{-3}	+	-	-	+	2	+
4^{-4}	-	+	+	-	2	+
4^{-5}	-	+	-	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	11	

Spot No. 13 Altitude: 3053m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	-	+	+	+	3	+
4^{-3}	+	-	+	+	3	+
4^{-4}	+	-	+	-	2	+
4^{-5}	+	-	+	+	3	+
4^{-6}	-	+	-	-	1	+
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	16	

Spot No. 15 Altitude: 2705m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	+	+	+	+	4	
4^{-4}	+	+	+	-	3	+
4^{-5}	+	-	-	+	2	+
4^{-6}	-	+	-	-	1	+
4^{-7}	-	-	-	+	1	+
4^{-8}	-	-	-	-		
				Total	19	

Spot No. 14 Altitude: 2909m

Dilution	Nodulation Replications				No. of nodulated units	ROT
	I	II	III	IV		
4^{-1}	+	+	+	+	4	
4^{-2}	+	+	+	+	4	
4^{-3}	-	+	+	+	3	+
4^{-4}	+	-	+	-	2	+
4^{-5}	-	+	-	-	1	+
4^{-6}	-	-	-	-		
4^{-7}	-	-	-	-		
4^{-8}	-	-	-	-		
				Total	14	

Note: + = Presence of nodule; – = absence of nodule