CHAPTER ONE

INTRODUCTION

1.1 Background

A distinctive characteristic of the majority of legumes is their ability to enter into a nitrogen fixing symbiosis with a distinct group of soil bacteria collectively called root nodules bacteria or the *Rhizobia* (Fred *et al*. 1932; Graham 2008). The *Rhizobia* reduce the atmospheric nitrogen into ammonium which is termed as the biological nitrogen fixation and is more advantageous in the perspective of soil quality. The productivity and sustainability of the world agriculture is significantly enhanced through nitrogen fixation from effectively nodulated legumes (Robson1990). However only certain combination of legumes and Rhizobia result in the formation of effective nitrogen fixing nodules even though many moderately effective and ineffective combination may and do arise. Thus the *Bradirhizobium japonicum* is host specific and nodulate only the species of soybean. Apart from direct benefit from effective nitrogen fixation (Unkovich *et al*.1995) legumes and *Rhizobium* provides added value in weed, pathogen and insect control when rotated with crop in farming system (Reeves and Smith 1975;Latta and Carter 1998) together with improving soil structure and increasing soil organic matter content (Robson1990; O'Hara *et al*. 2002).

The importance of legume crops to world production and compelling needs to exploit the nitrogen fixing potential of those crops have focused attention on technologies for the production of more effective legume inoculants. Most legume inoculants have been prepared by adsorbing broth culture of selected *Rhizobia* on a suitable carrier such as peat, clays, charcoal, lignite, cellulose powder, various powdered crop residues or soil compost mixtures. In 1979, Dommergues *et al.* proposed to entrap rather than adsorb *Rhizobium* cells by incorporating the bacteria in a polymeric gel. The encapsulation of the inoculants with polyacrylamide maintained the suitable moisture content. These formulations of immobilized cells protect the microorganism against the environmental stresses and release them to the soil gradually when the polymers are degraded (Bashan; 1986). Increasing the efficiency of the use of

available soil nitrogen can meet the additional Nitrogen demand by making cereal plants capable of fixing its own nitrogen through close association with diazotropic bacteria will pay off in term of increasing cereal production and helping resource poor farmers as well as saving the environment (Cassman *et al*; 1997, Ladha *et al*. 1997).

Symbiotic Nitrogen fixation is an important source of nitrogen and the various legumes crops and pasture species often fix as much as 200-300 kg Nitrogen per hectare (Peoples et al; 1995). Globally, symbiotic nitrogen fixation has been estimated to amount to at least 70 million metric tons of nitrogen per year (Brockwell *et al*; 1995). In 1990, world consumption of fertilizer Nitrogen is 88 million tones and apart from the consumption of nonrenewable energy sources, environmental pollution from fertilizer Nitrogen escaping the root zones is high because in many cases Nitrogen fertilizers are not used efficiently by crops (Peoples *et al*; 1994). Therefore biological nitrogen fixation is an important and integral component of sustainable agricultural system. Furthermore, biological nitrogen fixation from legumes offers more flexible management than fertilizer nitrogen because the pool of the organic Nitrogen becomes slowly available to non-legumes species (Peoples *et al*; 1995). Concomitant with Nitrogen fixation, the legumes in rotation offers the control of crop disease and pests (Robson, 1990; Graham and Vance; 2000). The Bellagio conference on N_2 fixation (Kennedy and Cocking, 1997) acknowledged that with the decline in the price of manufactured fertilizer in 1990s, biological nitrogen fixation with legumes and Rhizobia, was most likely to remain in extensive rather than intensive agricultural systems. Thus the present study is emphasized for the mass production and immobilization of Rhizobial inoculants in the most effective and cost effective ways of encapsulation.

Biological Nitrogen fixation (BNF)

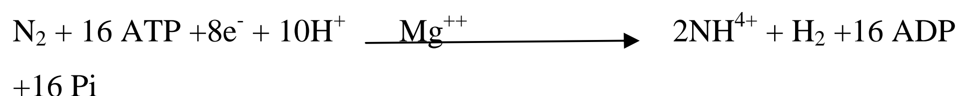
The atmosphere consists of about 78% N_2, however, this large amount of N is not readily plant available due to the strong triple bond and only few processes can transform N_2 into bioavailable form (NO_3^-, NH_4^+,…) namely the Haber-Bosch process, lightning and BNF (Galloway *et al*. 2004). BNF is harnessed by legumes by hosting symbiotic Rhizobium bacteria (as well as other strains)

in specially constructed root nodules (Ledgard and Steele 1992). There the enzyme nitrogenase splits N_2 under strictly anaerobic conditions with high energy input, supplied by labile carbon from the plant (Hardy and Burns 1968). Biological nitrogen fixation (BNF) is an enzymatic process carried out by prokaryotic organisms that transforms atmospheric di-nitrogen (N_2) to forms available for plant growth (Vitousek *et al*. 2002). Legumes form symbiotic relationships with these prokaryotes in root nodules and thus harness the ability to fix nitrogen (N_2) for their own use. For this reason, legumes have been used in agriculture for hundreds of years to overcome N_2 limitations to crop growth, either directly (grain legumes, forage legumes) and/or by providing N_2 for subsequent non-fixing crops (green manure) (Taylor *et al*. 2002).

The reduction of nitrogen gas to ammonia is energy intensive. It requires 16 molecules of ATP and a complex set of enzymes to break the nitrogen bonds so that it can combine with hydrogen. Its reduction can be written as:

$$N_2 + 3H_2 \xrightarrow{\text{Energy}} 2NH_3$$

Fixed nitrogen is made available to plants by the death and lysis of free living nitrogen fixing bacteria or from the symbiotic association of some nitrogen-fixing bacteria with plants. Energetic and ionic characteristics of Biological Nitrogen Fixation can be described as in equation below (Vance, 2008)



$$N_2 + 16\ ATP + 8e^- + 10H^+ \xrightarrow{Mg^{++}} 2NH^{4+} + H_2 + 16\ ADP + 16\ Pi$$

The reaction essentially requires the molybdenum iron (MoFe) and Fe protein of the nitrogenase enzyme, an electron source and a supply of MgATP (Howard and Rees, 1996). The process has to overcome the presence of O_2 released when ATP is generated in the bacteroids (schauser *et al*. 2008), as O_2 inactivates nitrogenase synthesis (Hill et al.1981). This problem is overcome by plant derived leg-hemoglobin which binds with O_2 and facilitates O_2 diffusion through the bacteria at a low and buffered concentration (Burris and Hoas, 1994). Thus LHb acts as carrier of O_2 and helps in accomplishment of bacterial respiration and consequent provision of ATP for Nitrogen fixation. It also creates an O_2 free environment around the active site of nitrogenase which fails to work in presence of O_2. The following diagram shows the mechanism of biological nitrogen fixation by symbiotic bacteria.

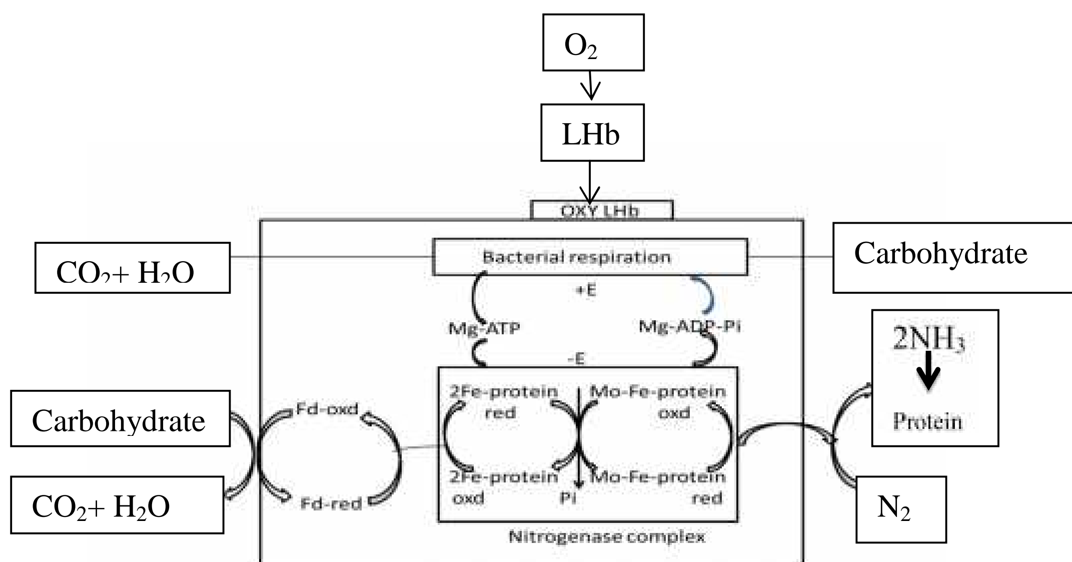


Fig: Mechanism of BNF in the root nodules of leguminous plants (Dubey, R. C. 1993).

Both the metalloproteins, nitrogenase (Mo-Fe protein) and nitrogenase reductase (Fe protein) are essential for nitrogense activity. Fe-protein interacts with ATP and Mg^{++}, and MoFe protein catalyses the reduction of N_2 to NH_3, H^+ to H_2 and acetylene to ethylene. The reduced ferredoxin or flavodoxin serves as the source of reductant for electron transfer during N_2 fixation. From reduced form of ferredoxin (Fd res) electrons flow to Fe-protein which reduced to Mo-Fe-protein with subsequent release of inorganic phosphate, Pi. The nitrogenase complex gets energy from Mg-ATP which in turn is produced from bacterial respiration. Finally, Mo-Fe-protein passes on the electron to reducible substrate i.e. N_2 which in turn gives neutral ammonia or cationic ammonium. This product is transferred through bacterial membrane to host cells, where it is enzymatically converted into many products.

1.2 Systamatic classification

Kingdom: Bacteria

Phylum: Protobacteria

Class: Alphaprotobacteria

Order: Rhizobiales

Family: Bradyrhizobiaceae

Genus: *Bradyrhizobium*

Species: *B. japonicum* (Jordan 1984)

1.3 Rhizobia

Rhizobia are the gram negative, rod shaped, aerobic and heterotrophic soil bacteria, which includes genera *Rhizobium*, *Bradyrhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium,* and *Azorhizobium*, which are able to form symbiosis with leguminous plants. They are facultative symbionts that have adapted to persists for long period in soil in a free living state if the suitable legume host is absent (Graham 2008). They could form the specialized organs, called nodules, on roots or stems of their hosts. Rhizobia inside nodule could reduce atmospheric nitrogen and make it available to the plant. Symbiotic rhizobia are common colonizers of the rhizosphere of both legume and non-legume plants and in addition to legumes they are also endophytes of several non-legumes like rice and maize (Sessitsch *et al*. 2002). However, non-symbiotic rhizobia can also be present in soil (Sullivan *et al*. 1996).

In the old system of classification the *Rhizobium* fall into two groups based on their growth characteristics i.e. fast growing and the slow growing *Rhizobium*. Fast growing *Rhizobium* are acid producers which develop pronounced turbidity in liquid media within 2-3 days and have the mean doubling time of 2-4 hours. The cells are rod shaped to pleomorphic, 0.5-0.9 microns in

diameter and 1.2 to 3.0 micron long, and are motile by 2-6 peritrichus flagella. Whereas slow growing *Rhizobium* are alkali producing Rhizobia and require 3-5 days to produce moderate turbidity in liquid media and have the mean doubling time of 6-7 hours. The cells are predominantly rod shaped and motile by a single polar or sub-polar flagellum (somasegaren and Hoben; 1994).

1.4 History of Rhizobial inoculant

Since 1886, Hellriegel and Wilfarth discover that bacteria caused the formation of the nitrogen-fixing nodules. Then, the isolation of rhizobia from the nodules as pure cultures opened the way for artificial inoculation to replace the 'soil transfer' method, which dry soil, from a location where the legume had been grown previously, was coated onto the seed just before sowing (Date, 2001). This dust method was modified to the "soil-paste or muddy water process", in which the soil was mixed with water before pouring over the seed (Burlison *et al.* 1930). The first commercial pure (agar) culture inoculants have been patented by Nobbe and Hiltner in 1896. Their patented culture was placed on the market under the name Nitragin, which consist of a pure culture of desired strain of rhizobia grown in flat glass bottle containing only a small amount of solid gelatin medium. This material was either to be applied to seed or mixed with soil and spread over the field (Smith, 1992). Then, solid carrier such as soil or peat was first suggested in 1914 (Date, 2001). Present day inoculants production techniques have been changed from those of the early 1900s. Even many types of inoculant have been investigated; peat is the best carrier and is widely accepted in the inoculant industry. However, the challenge today is to develop further improved inoculant formulations and methods of application.

History of Rhizobial inoculants in Nepal

In Nepal Rhizobial inoculants has been used from few years. Rhizobial inoculants has produced in soil science department of Nepal Agricultural Research Council. This was studied and conducted by Sanu kesheri Bajracharya. Powder inoculums were made in soil and goal mixture in 3:1 ratio. But for the research proposes liquid inoculums is being used. The work was performed under the supervision of Soil Science Department and Farmer

Centered Agricultural Resource Management (FARM), Asian Bio-Technology and Bio- Diversity Sub–Program Nepal (Annual reports of Soil Science Department of Nepal Agricultural Research Council). Some researchs has been done on the effect of the peat based inoculums of the *Bradyrhizobium japonicum* on the *Glycine max* in the university researches.

Bacterial fertilizer packets production and distribution in NARC in 2069/070

S. N	Types of crops	No. of packets	
		Production	No. production
1	Lentil	800	554
2	Chickpea	473	365
3	Soybean	439	390
4	Pigeon pea	370	273
5	Pea	106	23
6	Black gram	74	50
7	Mung bean	50	40
8	Peanut	50	10
9	Cowpea	50	12
10	Bean	43	30
11	Stylo	460	345
12	White clover	311	39
13	Lucerne	284	30
14	Berseem	180	40
15	Vetch	155	40
16	Siratro	51	30
17	Azotobacter	204	106
	Total	**4100**	**2377**

In the year 2069/070, total of 4100 packets of bio-fertilizer including different microorganism each of 40 gm were produced by the Soil Science Division of NARC and only 2377 packets were distributed which is only 57.98 % of the total production.

1.5 The need for inoculation

Although rhizobia seem to be widely distributed in the soil, however soil indifferent places contains different strain of rhizobia and these rhizobia may not effective for nitrogen fixation, and may not appropriate for legume need to plant. Some soil may have effective rhizobial strain, but the number of rhizobia is low or containing higher number of ineffective strain (Herridge *et al*., 2002). Inoculation of legume seed is a simple and practical means of ensuring effective nitrogen fixation. However, to answer the question “Is inoculation of seed necessary?” is critical, even the using of rhizobial inoculant is not necessary in that area (over inoculating). Therefore, Allen (1958) has listed four indicators that, if positive, the inoculation would be beneficial:

- The absence of the same or symbiotically-related legume in the Immediate past history of the land
- Poor nodulation when the same crop was grown on the land previously
- When the legume followed a non-legume in the rotation
- When the land was undergoing reclamation

1.6 Types of inoculants

1.6.1 Solid-based inoculants

Solid-based inoculants form the vast majority of cultures in the market. A history of the development of the legume inoculants based on solid carriers, mainly soil and peat, but also other materials have been studied, such as soils, clays, coal, charcoal, perlite, rock phosphate, bagasse, filter mud, vermiculite, and ground plant residues (Brockwell, 1982; Stephens and Rask, 2000). However, the best researched and the most frequently used material for inoculant production is peat. A large number of studies have shown that peat provides better protection for rhizobia in the package and on inoculated seed than other materials due to peat being rich in organic matter and mineral (Somasegaran and Hoben, 1994). For commercial purposes, a safe storage period of 6 months can be expected from peat-based inoculants (Roughley and Vincent, 1967). The type of peat affects the number of rhizobial cells which

develop, and their subsequent survival during storage. Notably, the chemical analysis of the peat may not always confirm its quality as a suitable material. The acceptable peat can be made only on actual tests of its suitability for growth and survival of particular strains of rhizobia. Moreover, its suitability can also vary depending on source and batch in each production (Roughley and Vincent, 1967). The production of peat inoculants is complicated and need many steps, started by selection and screening to remove debris, such as stones, roots, and then drying with forced air. Drying temperature should not exceed 100°C as higher temperatures can degrade peat and release toxic substances, which can also restrict subsequent growth and survival of rhizobia. The peat is then ground in a high speed hammer mill and passes through a shifting machine. Peat with a particle size of 10-40 µm is collected for seed coating, and peat with particle size of 500-1500 µm is used for production of soil implant (granular form) inoculant. Most peats are too acidic to use as carrier without prior neutralization. Therefore, peat must be neutralized with precipitated CaCO3 (pH 6.5-7.0). This neutralizing agent is usually added to the peat before milling (Somasegaran and Hoben, 1994). Both sterilized and unsterilized peats are used in commercial production systems. Peat can be sterilized by autoclaving at 15 lb pressure, 121°C for at least 60 minutes. However, heat sterilization of some peat has been found to produce undesirable changes and to release toxins. Therefore, Gamma-irradiation sterilization is preferred. This sterilization cause sterile peat production more costly than non-sterile peat.

1.6.2 Liquid inoculants

According to complicated processing of solid-based inoculant, liquid inoculants are ideal because preparation and handling are simple and purity easily confirmed. In the past, commercial liquid inoculants have been marketed only sporadically, basically because of the difficulties which arise in maintaining biological control after the cultures leave the manufacturer (Brockwell, 1982). Manufacturers seem to have overcome the problem of deterioration by concentrating the broth inoculant with centrifugation, placing it in plastic containers, freezing it, and transporting it to the user in a frozen state packed in dry ice. Then, the concentrated rhizobia are diluted with water before use. Nevertheless, the shelf life and the need of cool temperature

storage are limited the use of liquid inoculant (Stephens and Rask, 2000). However, the liquid inoculants currently available display various broth formulations, each the result of individual producer's research and development activities. Brockwell and Bottomley (1995) has been reported the liquid inoculants which packaged in dispenser bottles, and can be used as a seed inoculant or for delivery directly into seed bed. This form of liquid inoculant has good storage characteristics in the bottle or on the seed, adheres tenaciously to the seed coat without the need for adhesive, and gives rise to nodulation and N_2fixation as good as can be obtained with peat inoculant. Moreover, several compounds have been studied for their protective function and added to liquid inoculant for promoting the survival of rhizobia after inoculation. For example, polyvinylpyrrolidone (PVP) is the polymer that may bind toxic compounds release from seed coat, and also has high water binding capacity, which appears to slow drying of the inoculant after application to seed. FeEDTA and glycerol are added to supplement iron and carbon source to the rhizobia, glycerol can also protect cells from the effects of desiccation (Singleton et al., 2002). Therefore, liquid inoculation formulation with other additives can be moved toward for their commercialization instead of peat-based inoculant, which true peat are rare and can be exhausted in the future.

1.6.3 Polymer-gel entrapped inoculants

Possibly, the most innovative technology for rhizobial inoculants in recent times involves the encapsulation of the bacteria in polymer microcapsules, beads or pellets. Polyacrylamide and alginate are the 2 most commonly tested gels. The inoculant is formulated by mixing a culture of the bacteria with a hardening or gelling compound (adjuvant) in a polymer solution. Bulking or nutritive additives such as bran or clay can be added and the mixture added drop-wise into a solution containing a calcium salt to solidify and form the gel pellets. Particle size and shape can be controlled by varying the drop-forming system. The particles are then dried for ease of packaging and handling. Hardness, moisture uptake or release and rate of degradation of the beads can be adjusted by varying the amount and grade of adjuvant and polymer used to prepare the pellets (Bashan, 1986). Particles of polyacrylamide-entrapped *Rhizobium* (PER) (Dommergues *et al.* 1979) resulted in an inoculant that compared very favorably with peat-based inoculant when kept at suitable

moisture content. Rhizobia entrapped in a similar way with alginate (AER, alginate-entrapped *Rhizobium*) or a mixture of xanthan and carob gum (XER, xantan-entrapped *Rhizobium*) made satisfactory inoculants and were not different to PER in the wet form after 50 days of storage at 28°C. Dried PER, AER and XER formulations were of poorer quality (number of cells per unit) and were not consistently better than peat either as soil or seed inoculant (Jung et al., 1982). Although polymer-entrapped rhizobia formulations can provide an alternative to powdered organic carrier-based inoculants, they have the disadvantage that the best results are obtained when they are maintained in their 'wet' condition (Tittabutr, 2005). Drying caused significant loss of viability of the same order as in dried peat and on a volume basis had fewer viable cells (Date, 2001). Drying PER appeared to be harmful since nodulation and total N of aerial parts were markedly decreased when dried (Dommergues *et al*., 1979).

1.7 Justification of the study

Soybean is the most important grain legume in the world occupying 78.9 million hectare during the year 2001-2002(Agbioforum review 2005). (Boonkerd 2002) investigated the soybean productivity and economic gain due to different cultural practices and observed more than 50% increase in soybean yield due to the *Bradyrhizobium japonicum* application as shown in the table below:

Table 1.1: soybean productivity and economic gain due to rhizobial inoculants application

Treatments	Yields (kg/ha)	Net benefit over control(US$/ha)
Control (without Rhizobial and chemical fertilizer)	803	0
Rhizobial inoculants only	1228	126.7
Rhizobial inoculants +P(56.3kg/ha)+K(37.5kg/ha)	1352	109.7
N:P:K(75:56:37 kg/ha)	1224	26.5

Modified from Boonkerd (2002)

It was found in the experiment that 1250 gm of inoculants could replace 179.2 kg urea fertilizer or 200 gm of *Bradyrhizobium* inoculants for 28.6 kg of urea. These result demonstrate that *Bradyrhizobium* inoculation is necessary for enhancing soybean yields and is more beneficial than the urea in the perspective of its cost.

Rhizobial inoculants can be immobilized in different materials. The material for peat based carrier is obtained from a naturally occurring organic material. The supply of peat based organic material is limited. Even other solid materials such as lignite, charcoal, coir dust and compost of various agricultural wastes have been used instead of peat but their performance characteristics are not equivalent to peat based inoculants product (Singleton *et al.* 2002). Therefore it is important to immobilize the *Rhizobium* in any other suitable form as sodium alginate encapsulation. In solid and liquid inoculants three basic contaminant types were observed, such as bacteria, actinomycetes, and fungi. These include the possibilities of pathogenicity to human, animal, plant or rhizobia, which reduce the effectiveness of inoculant (Olsen *et al*. 1996). Thus it is necessary to immobilize the bacterial cells in the form of encapsulated beads made in aseptic condition which prevents the contaminants and well as preserved the cells for several months without losing their viability. Also the encapsulated beads are easy to handle, to use and to do packaging and distribute to the farmers.

Generally, soybean is used in the food industry for flour, oil, cookies, candy, milk, vegetable cheese, lecithin and many other products. The micro-symbiont of soybean; *Bradyrhizobium japonicum* is the highly efficient N_2 fixer forming symbiotic association with soybean. According to Unkovich and Pate (2000), the amounts of N_2-fixed (kg /ha) by soybean have been up to 450 Kg N ha-1. Thus soybean production depends on its symbionts for a large part of its N_2 requirements for effective growth and dry matter production. Studies carried out by Wasule *et al*. (2007) and Son *et al*. (2007) clearly revealed that co-inoculation of *Bradyrhizobium* and phosphate solubilizing microorganism significantly improved soybean growth and its yield components as compared with the sole application of *Bradyrhizobium* or phosphate solubilizing

microorganism. In *Bradyrhizobium* there are strains tolerant to high temperatures, desiccation, acidity (reviewed in Graham, 1992), and strains that fix nitrogen as free-living bacteria under low levels of oxygen (Keister, 1975).

Unbalanced use of chemical fertilizers had led to a reduction in soil fertility and to environmental degradation (Gyaneshwar *et al.* 1998) and the cost of chemical fertilizers has increased so that it is unaffordable for farmer of developing country such as Nepal. As a consequent, there has recently been a growing level of interest in environmentally friendly sustainable agricultural practices including organic farming systems (Rigby and Caceres, 2001; Lee and Song, 2007). For example, *Rhizobium* and phosphate solubilizing microorganisms would reduce the need for N_2 and P chemical fertilizers and decrease adverse environmental effects. Therefore, in the development and implementation of sustainable agriculture techniques, bio-fertilization is of great importance in alleviating environmental pollution and the deterioration of nature (Elkoca *et al.* 2008). A tightening of the agricultural N_2 cycling to reduce N losses and an increase of N_2 inputs through BNF to replace artificial fertilizer N_2 use can help achieve this goal while at the same time maintaining agricultural production and reducing greenhouse gas emissions and energy consumption for the production of artificial N fertilizers (Bohlool *et al.* 1992, Galloway *et al.* 2008, Peoples *et al.* 2009).

1.8 Objectives of the study

Broad objective

- Immobilization of *Bradyrhizobium japonicum* by encapsulating them with sodium alginate and sucrose as additives and test their viability and effect on *Vigna unguiculata*.

Specific objectives

- To isolate and identify the *Bradyrhizobium japonicum* in the laboratory
- To immobilize mass culture of *Bradyrhizobium japonicum* by encapsulating them with sodium alginate and mixing sucrose as additive.

- To test the effect of immobilized *Bradyrhizobium japonicum* on *Vigna unguiculata* and the viability of beads on room temperature.

1.9 Limitation of the study

- The growth rate of the bacterial strain during mass culture could not be reported because of the lack of the measuring instrument i.e. neither colony counter nor the hemi cytometer.
- Due to lack of proper green house, the *Vigna unguiculata* could not grow well in the off season i.e. winter season and the leaves turn yellow and fall down. Thus the study could not proceed towards the grain yield, dry weight of the plant and the number of nodules.
- So many biochemical tests which are more effective for identification could not be done because of the lack of the chemicals in the laboratory.

1.10 Hypothesis

Null hypothesis(H0):

There is no significant difference between the shoot length of the *Vigna unguiculata* in the encapsulated *Bradyrhizobium japonicum* inoculated and the un-inoculated plant.

Alternative hypothesis(H1):

The shoot length of the *vigna unguiculata* in the encapsulated *Bradyrhizobium japonicum* inoculated plant is greater than that of the un-inoculated plant.

CHAPTER TWO

LITERATURE REVIEW

Beijerinck (1888) stated that *Rhizobium* was isolated from root nodule of the leguminous plants and established that they were responsible for the process of nitrogen fixation.

Sears OH and Carroll WR(1927); Van Rensburg *et al*.(1976) Leonard LT (1923) confirmed that Rhizobium japonicum forms a N_2-fixing symbiosis with *Glycine max* L. Merr.(soybean), but these bacteria also nodulate *Vigna unguiculata* (cowpea) and *Macroptilium atropurpurem* (siratro) .

Heller,(1941); Annear,(1956,1962); Vincent,(1958) reported in their early studies on the freeze-drying of bacteria, the nature of the suspending media was identified as an important aid to survival.

Heller,(1941); Appleman and Sears, (1944); Annear,(1956, 1962); Redway and Lapage, (1974); Dye,(1982) noticed that extensive research has been carried out on the use of bacterial nutrients as suspending agents for freeze-drying and storage of cells.

Heller (1941) investigated the protective effects of crystalline compounds and colloids during desiccation of *Streptococcus pyogenes* C203 and *Escherichia coli.* He concluded that survival was related to the assimilability and solubility of the compound.

Vincent (1958) reported 24–44% of cells suspended in a 10% sucrose solution survived primarily drying whereas only 0.1% survived when suspended in water. Sucrose was a superior suspending agent to sorbitol, mannitol, lysine, amino acid mixtures, milk and yeast mannitol broth. The poor survival of rhizobia on seeds and beads was improved by the addition of sucrose and also found that the incorporation of 10% sucrose in YEM broth improved the survival on glass beads compared with un-amended broth.

McLeod and Roughley (1961) studied on the freeze dried cultures as the commercial legume inoculant and found that the incorporation of 10% sucrose into yeast mannitol broth improved the survival on glass beads compared with un-amended broth.

Martinez-de Drets and Arias, (1972) studied on the enzymatic basis for differentiation of *Rhizobium* on fast and slow growing group and found that slow growing *Bradyrhizobia* cannot use sugars produced from starch.

Subba,Rao and Tilak (1977) had mentioned on their study on biofertilizer that there is more yield of subsequent crops in *Rhizobium* inoculated fields than in un-inoculated control.

Hale and Mathers (1977) demonstrated the water insoluble form of PVP defined as polyvinyl polypyrrolidone, PVPP, and water soluble form of PVP adsorbed toxic seed exudates from clover seeds. PVP can form insoluble complexes with phenols and the amounts bound were from 31 to 44% of the dry weight of polymer. PVP is a strong proton acceptor and therefore has a high capacity for binding phenolics. Moreover, phenolic binding was also observed in proteins such as casein and synthetic polyamides.

Hale and Mathers (1977) demonstrated phenolic adsorption by several materials. In addition, antibiotic activity of toxic seed diffusates was also suppressed by activated charcoal and skim milk powder. Evidently, the various additives applied during seed inoculation have provided some protection to rhizobia. However, polymers need to be selected carefully so that individual properties can be isolated and specific affects attributed to them. Desirable properties may then be optimized contributing to an overall positive effect on survival.

Lloyd (1979) studied on the seed coating technique and patented water-soluble polyvinylpyrrolidone (PVP) as a seed-coating agent which is a synthetic vinyl polymer produced by free radical vinyl polymerization of the monomer vinylpyrrolidone.

Gulati,(1979) found that moreover, agricultural wastes, such as malt extract, whey, and molasses have been reported to use for alternative ingredients for development of the inoculant industry.

Jung *et al*., (1982); Paul *et al*.,(1993)reported that several studies have used alginate as the encapsulating material as it forms beads and also noticed that

soil inoculants must be used in a dry form, but the drying of alginate beads reduces viable cells numbers to about 1% of the original cell count .

Singleton *et al*. (1992) had studied on effect of salinity on rhizobial growth survival and reported that increasing salt concentration may have detrimental effects on rhizobial population.

Thornton and Davey,(1983); Richardson and Simpson,(1989) studied on the acid tolerance by *Rhizobium trifolii* and found that slight change in pH alone can significantly affect the growth of root nodule bacteria.

Stowers, (1985) studied on carbon metabolism in *Rhizobium* species and concluded that generally, fast-growing rhizobia can utilize a variety of sugars such as glucose, sucrose, maltose, whereas *Bradyrhizobia* appear to be nutritionally fastidious. He also showed that *Bradyrhizobium* can use arabinose, gluconate and some sugar alcohols, such as mannitol and glycerol as a preferred carbon source. Mannitol is commonly used for cultivation both fast- and slow-growing rhizobia. He also reported that fast-growing rhizobia are unable to use disaccharides, trisaccharides, and organic acids for growth. Mannitol is the traditional carbon source for all rhizobia.

Stanier *et al*. (1985) formulate a formula to calculate the specific growth rate of the bacteria as mentioned below:

$$\text{Specific growth} = \frac{\text{Log OD1} - \text{Log OD0}}{\text{T1} - \text{T0}} \times 2.303$$

Where:

Log OD1 = Log value of O. D. of culture at time T hour

Log OD0 = Log value of O. D. of culture at time T0 hour

Smith,(1987) studied on the production and quality control and mentioned that Sucrose is normally use for fast-grower rhizobia for commercial mass culture production and also reported that inoculants are perishable product which should not be submitted to conditions that enhance drying, such as increase temperatures and direct sunlight.

Cleyet-Marel,(1988) studied on the seed inoculation and inoculants technology and reported that legume inoculation with rhizobial inoculant is one way to ensure that specific rhizobial strain for that host plant is present in

the soil at the proper time and in sufficient number to assure a quick and effective nodulation and efficient subsequent nitrogen fixation.

El-Mokadem,(1991) studied on the salt response of clover and alfa alfa inoculated with salt tolerant strain of rhizobia and resulted that salt tolerant rhizobia may have the potential to improve yield of legumes under salinity stress.

Lie *et al*. (1992) on their study ‘cultivation of *Bradyrhizobium* on sucrose pre-fermented with yeast’ mentioned that the alkaline-reacting salt, $CaCO_3$ was added to maintain alkaline condition.

Smith (1992) has noted that the raw materials chosen for use in conventional inoculants are determined by availability, consistency of quality and cost. The carrier must display two fundamental properties; it must support growth of the target organism and maintain desired populations of inoculant strains over an acceptable time period. To achieve these goals a carrier must also display high water holding capacity and retention characteristics, display chemical and physical uniformity and be non-toxic to inoculant strains and environmentally safe.

Paul *et al*. (1993) studied on the survival of alginate entrapped cells of *Azospirillium lipoferum* during dehydration and storage in relation to water properties and found that the behavior of bacterial cells to encapsulation is strongly species dependent.

Catroux et al. (1996) studied on the survival of manufactured inoculants of *Bradyrhizobium japonicum* and evaluated pure industrial products, stored up to 8 years, at 20°C. Peat and liquid *B. japonicum* inoculants can provide 109 viable *B. japonicum* per gram or per ml (determined by plate counts) after 4 – 6 years storage. These data demonstrated that high standards of inoculant quality could be reached.

Saiprasad, (2001) reported that Sodium alginate was the most accepted hydro-gel and frequently used as a matrix for the synthetic seeds because of its low toxicity, low cost, quick gellation and biocompatibility characteristics.

Zahran, (2001) had studied on diversity, taxonomy, ecology, nitrogen fixation and biotechnology of *rhizobia* from wild legumes and found that the use of effective rhizobia from wild non-crop legumes as a bio-inoculant to crop plants was proven to increase nodulation and nitrogen fixation.

Matiru & Dakora (2004), found that *Rhizobium* form intimate symbiotic relationship with legumes by responding chemotactically to flavonoid molecules released as signal by legume host. The plant compounds induce the expression of nodulation gene in *Rhizobia* which in turn produce lipochitooligosachharide (LCO) signals that trigger mitotic cell division in roots leading to nodule formation and demonstrated the ability of *Rhizobium* to colonize roots of non-legumes and act as phytohormone producer, phosphate solubilizer and to some extent as nitrogen fixer.

Deaker *et al*. (2004).Proved the sucrose to be a superior suspending agent to glucose, xylose, tryptophan, salicin, saline and water for both species.

Pokherel R.R and Prasad B.N (2005) have studied on the 'Interactive effect of *Bradyrhizium japonicum* and *Azospirillum brasilense* on germination , growth, N-fixation and the yield of soybean' and reported that the single inoculated plant with the *Bradyrhizobium japonicum* showed the increasing tendency in the yield parameters with 1.127 times more over the control.

Aryal R.R and Prasad B.N (2005) had studied on the effect of *Bradyrhizobium japonicum* on germination and the seedling growth of the soybean and found the increase in the embryo axis dry weight and germination and seedling growth but the decrease in the weight of the cotyledon in the inoculated over the control.

Adewusi *et al*. (2008).studied and found that Rhizobial inoculation increases nodule biomass thus encourages sustainable environmental friendly agriculture by responding perfectly in biological nitrogen fixation

Graham (2008) studied on the ecology of root nodule bacteria of legumes and found that *Rhizobia* are facultative symbionts that have adapted to persist for long period in soil in a free living state, if a suitable legume host is absent.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

The materials used for the present study were root nodules of *Glycine max* (white seeded species) grown at the earthen pot at Putalisadak. The seed of *Glycine max* and the *Vigna unguiculata* were taken from the market for the test. The necessary equipment and the chemicals required for the completion of the present study were provided from the Biotechnology and Biochemistry unit of Central Department of Botany.

3.2 Composition of YEMA Media

Ingredients	Gms / Litre
Yeast extract	1.000
Mannitol	10.000
Dipotassium hydrogen phosphate	0.500
Magnesium sulphate	0.200
Sodium chloride	0.100
Congo red	0.025
Calcium carbonate	4.000
Agar	20.000
Distilled water	1 ltr

Final pH (at 25°C) 6.8±0.2 (Dubey and Maheshwari, 2002).

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3.3 Preparation of YEMA media

- All the ingredients required for the preparation of the YEMA media except agar and Congo red are dissolved in the 950ml of the sterilized water.
- Congo red is dissolved separately in the next conical flask in 50 ml of water and sterilized them separately.
- Then pH is maintained to 6.8-7.0

- Agar is added in the mixture of the ingredients and sterilized in Autoclave at 121 degree celcius and 15 lb pressure for fifteen minutes.
- From the Autoclave, media is directly taken to the Laminar Air Flow Chamber and Congo red is mixed with the ingredients mixture and poured in the sterilized petri plates and allow it to cool down.
- Finally the media is ready for the inoculation of the *Rhizobia*.

3.4 Isolation of *Bradyrhizobium japonicum*

3.4.1 Collection of root nodules:

The roots of the soybean (white seeded species) were collected from Putalisadak, Kathmandu which were cultivated in the pots at the rooftops. The soil from the root was removed by washing with tap water. Then only the fresh, turbid, matured and pinkish colored nodules were selected and collected on the beaker. Only 0.2 gm of nodules were taken for the present study.

3.4.2 Surface sterilization of the root nodules:

- Root nodules were rinsed with tap water to remove the soil particle followed by rinsing with detergent and few drops of tween twenty for 1 hour in the running tap water.
- Roots nodules were dipped in 95% ethanol for 5-10 seconds under laminar air flow chamber and transferred to 2.5% sodium hypoclorite for 2-4 minutes. Then rinsed with sterile water for five times.

3.4.3 Preparation of the inoculants

- The root nodules were crushed in 1ml of sterile water in the test tube with the sterile glass rod.
- Then the solution was made 10 ml by adding sterile water.
- With the help of the pipette, 1 ml of the solution was taken in the next test tube and the final volume was 10 ml by adding 9 ml of the sterile water to make 10^{-1} dilution of the solution.
- Similarly the solution was mesh up to the serial dilution of 10^{-6} by transferring 1ml solution from the former test tube to the next one.

- From each of the serial dilution, 0.5 ml solution was taken and inoculated in the YEMA media by spreading with the help of L-shaped glass rod.

Finally the plates were incubated at 30 ^{0}C in dark in inverted position for 4 days. To isolate the pure culture of *Rhizobia*, only red colony from 4th day culture were taken with the inoculating loop and streaked in the YEMA media with Congo red and incubated at same condition as before.

3.5 Identification of *Rhizobium*

The species of *Rhizobium* were identified on the basis of its host as well as some biochemical tests as mentioned below:

3.5.1 Catalase production test: (lowe, 1992)

The dark red portion of an 18 to 24 hours pure colony was picked with the help of an inoculating loop and placed in the clean glass watch. Then few drops of the 3% H_2O_2 were added over the organism on the watch glass with the help of the Pasteur pipette. The immediate emergence of bubbles shows the production of catalase.

3.5.2 pH tolerance test:

YEM broth was prepared without adding the agar in the YEMA media and adjusted to different pH as 4.5, 7, 9 and 9.5 by add HCl and NaOH. Then the media was sterilized and *Rhizobium* strain was inoculated and incubated for 14 days at 30 degree Celsius and observed the growth of the *rhizobia.*

3.5.3 NaCl tolerance test:

YEMA plates with different concentration of NaCl (1%, 2%, and 4%) was prepared, sterilized and inoculated with *Rhizobium* and incubated for 14 days at 30 degree Celsius and observed the specific growth of the *Rhizobium.*

3.5.4 Penicillin resistance test:(Kirby-Bauer Method) (Shah *et al*. 2009)

- YEMA plates were prepared and placed right side up in an incubator at 37 ^{0}C for 10 to 20 minutes with the cover adjusted so that the slides are slightly opened.

- Each plates were labeled with the name of test organism to be inoculated.
- A sterile cotton swab was dipped into a test culture and removes excess inoculums by pressing the saturated swab against the inner wall of the beaker containing the test organism.
- Using the swab, the entire agar surface was streaked horizontally and vertically to ensure a heavy growth over the entire surface.
- The culture plates were allowed to dry for about 5 minutes.
- Using the aseptic technique the penicillin disc was applied on the agar surface bu using sterile forceps. Each disc were kept at least 15 mm from the edge of plate
- Each disc were gently pressed down with the sterile forceps to endure that the disc adhere to the surface of the media.
- The plate cultures were then incubated in an inverted position for 24 to 48 hours at 30^0C.
- Finally all the plates were examined for the presence or absence of a zone of inhibition surrounding each disc.

3.5.5 Nodulation test:(Dubey R.C1993)

- The seeds of soya bean were taken and surface sterilized in running tap water followed by dipping in 95% ethanol for 1 minutes.
- Seeds were then washed with 6 consecutive washing with sterilized water.
- Then the earthen pots along with 1:1 ratio of sand and soil were sterilized in Hot Air Oven at 160^0C for three hours.
- The sticking solution was made by adding 10% sucrose in distilled water which was first heated and then cooled to make sticky.
- The Rhizobial inoculants of 4 days culture were added in the sticker solution to make slurry.
- The seeds of soybean were mixed in that slurry and stirred completely to make the inoculants attached on the seeds.
- Seed were then taken out and rolled on the $CaCo_3$to maintain the alkalinity, the process is called pelleting.

- The seeds were then dried in the air and sown in the sterilized earthen pots at the depth of one inch.
- Similarly the seeds without inoculating the Rhizobia are also sown in the next pot.
- Finally the pots were watered and covered with transparent polyethylene sheet and tied around the pots. The polyethylene were made to have alots of holes for watering as well as for providing ventilation and kept in the green house.
- They were watered regularly and observed for the nodulation when the plant becomes 10-15cm high.
- The presence of nodules in the inoculated plants and absence in un-inoculated plants shows the positive result of the respective Rhizobial strain.

3.5.6 Color change of BTB:

YEMA plates containing BTB were prepared similarly as the YEMA plates with Bromothymol Blue and inoculated with test organism and incubated at 30^0C and observed the color change after 4-5 days. The appearance of blue color shows that the rhizobial strain is slow growing and the appearance of the yellow color shows that the rhizobial strain is of fast growing type.

3.6 Mass production of *Rhizobium*

3.6.1 Starter culture of Rhizobium

YEM broth medium (100ml) was prepared and autoclaved by transferring in a flask. Thereafter, pure *rhizobium* colony was transferred into sterilized YEM broth. Inoculated YEM broth was incubated at the water bath at 30^0C for four days. This was the starter culture of the Rhizobium.

3.6.2 Mass culture of Rhizobium

For the mass culture of *Rhizobium*, YEM broth was prepared in the large quantity in the conical flask and sterilized as mentioned before. The PH was maintained 6.5 to 7.0. Then the following steps were done:

- The sterilized YEM broth was inoculated with the broth of starter culture prepared in advance,
- Then it was incubated for 3-4 days on the water bath at 30^0C.
- The culture was tested for the purity by inoculating in the YEMA plates staining with Congo red,
- The broth culture was then transferred to the large flask and incubated for 4-9 days for the bacterial growth.

3.7 Encapsulation of *Rhizobium* (Jung G.*et al*. 1982)

The *Rhizobium* were immobilized by encapsulating with sodium alginate along with different concentration of sucrose as their nutrient. The methods used for the encapsulation of the *Rhizobium* are as follows:

- Beads were prepared aseptically in laminar air flow chamber by using dropper and the micropipette.
- From the mass culture of Rhizobium of 4-9 days, 25ml of broth was taken in four different beaker.
- Then the 2% sodium alginate was weighted and mixed in the broth in each beaker.
- The sucrose concentration of 1%, 2%, 5% and 10%was added in different beaker and leveled them.
- The sterilized magnet was kept in the beaker and covered with the aluminum foil.
- Then the beaker was kept on magnetic stirrer at 250 rpm for 8 minutes in order to dissolve the sodium alginate and the sucrose.
- On the other hand the solution of the 0.2M $CaCl_2$ was prepared in the 1 ltr beaker.
- The solution of the inoculums, sucrose and the sodium alginate was allowed to settle down for few minutes so that the air bubbles get disappeared.
- Then the mixture was dropped from about 30cm height by using the blunt ended pipette and collected in beaker containing 0.2M $CaCl_2$ solution.

- The rounded beads being formed in the beaker were stirred regularly to prevent them from being attached with each other.
- After 30 minutes beads were formed which were taken out from the $CaCl_2$ solution by filtering with muslin cloth and kept in the filter paper to be air dried and left overnight.
- Finally the air dried bead was kept in the lead closed culture tubes for further use to test their viability.

3.8 Cross inoculation of encapsulated beads to *Vigna unguiculata*.

To observe the effect of the encapsulated beads of the *Bradyrhizobium japonicum* obtained from the soybean to the *Vigna unguiculata*, the beads were cross inoculated in the seed of the *Vigna unguiculata* and observe for the differences in the shoot length of the germinated plant. The methods used for this study are as follows:

- The seeds of the cow pea were taken and surface sterilized with detergent and the few drops of the Tween 20 in the running tap water for one hour.
- Again they were treated with 95% alcohol for 1 minute followed by the 1% sodium hypo chloride for 3-4 minutes.
- Then the seeds were washed with the sterilized water for five times.
- On the other hand, the earthen pots and the mixture of soil and sand in 1:1 ratio, were sterilized in the Hot Air Oven at 160^0C for three hours.
- The soil mixtures in the pots were moistened with the sterilized water and allow to cool down.
- Then the seeds of the cowpea were sown in the pots (with 6 replicates in each pots) in which the encapsulated beads were also inoculated prior to sowing of seeds.
- Similarly the seeds were sown in the next pots without inoculating the encapsulated beads.
- The earthen pots were then covered with the polythene bags making a lot of holes for watering them and transferred to the green house. It was done to maintain the humidity for the seeds.

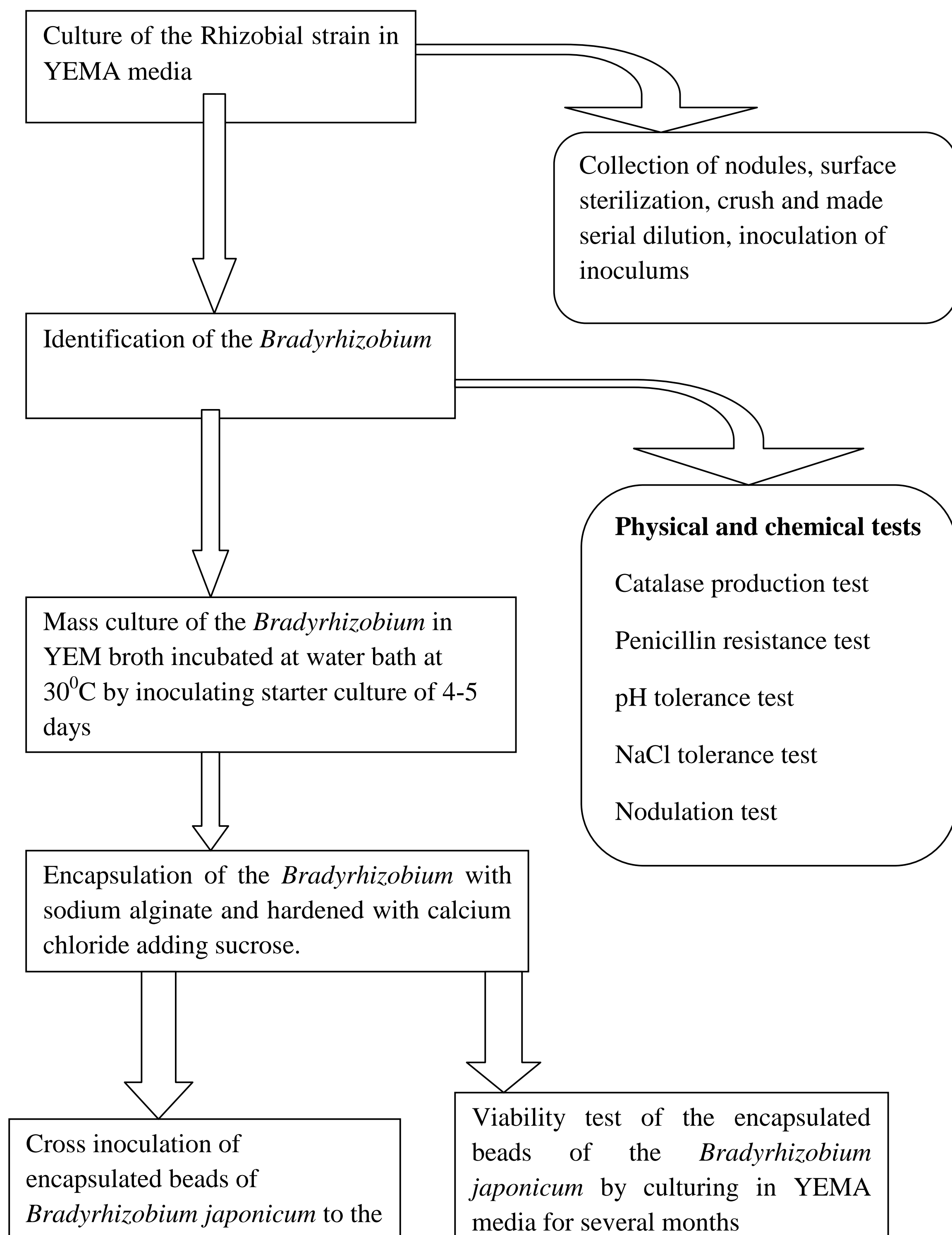
- The pots were labeled to distinguish that whether they are inoculated or un-inoculated.
- After the germination of the seeds, each plants were labeled with separate name and measure their height periodically and data was taken and analyzed.

3.9 Viability tests of the encapsulated beads of *Bradyrhizobium japonicum* (modified from Jung G.*et al*. 1982)

The sodium alginate encapsulated beads hence prepared were stored in the air tight culture tube at room temperature for the further viability test. The methods applied in the viability test of the beads are mentioned below:

- YEMA media was prepared and sterilized as mentioned before.
- With the sterilized forceps the beads were inoculated in the surface of the media.
- The beads with different sucrose concentration were inoculated in different plates for testing the viability.
- Then they were incubated at 30^0C for 48 to 72 hours in the incubator.
- The plates were observed for the formation of the rhizobial colony in the surface of the media.
- The same process was repeated at the interval of 2 weeks, 4 weeks, 6 weeks, 8 weeks, 10 weeks, and so on up to 7 months.

Flow chart of the methodology



CHAPTER FOUR

RESULTS

The *Bradyrhizobium* were isolated in the YEMA media and the number of colonies formed in the plates were enumerated and the average number of the cell forming unit were calculated .Similarly, the isolated cells were tested with different biochemical tests for their identification. Finally they were undertaken for mass production and immobilized in encapsulated form with sodium alginate mixing sucrose as the nutrient. The beads were cross inoculated and also their viability was tested. In this whole procedure so many results have been observed which are mentioned in this portion.

1. Isolation and enumeration of the colonies

The serially diluted solution of the inoculants was inoculated in the YEMA plates and the number of colonies formed in each plates were counted and the number of colony forming units present in each ml of inoculants were calculated by using the following formula mentioned below:

$$\text{Dilution factor} = \frac{\text{volume of the sample used}}{\text{Total volume of sample and the diluents}}$$

$$\text{Number of organism} = \text{dilution} \times \text{number of colonies}$$

$$\text{Number of CFU per ml} = \frac{\text{Number of organisms formed in average}}{\text{Inoculums size} \times \text{dilution}}$$

Here one colony is considered as one colony forming unit.

Table 1: Enumeration of organism by spread plate technique.

S.N	Dilution factor	Inoculums size(ml)	No. of colonies per plate	No. of organisms (dilution ×number of colonies)	Average no. of organisms	Number of c.f.u. per ml
1.	10^{-1}	0.5	339	33.9		
2.	10^{-2}	0.5	76	0.76		
3.	10^{-3}	0.5	17	0.017	578×10^{-2}	
4.	10^{-4}	0.5	2	0.0002		1156×10^{-2}
5.	10^{-5}	0.5	5	0.00005		
6.	10^{-6}	0.5	1	0.000001		

The enumeration of the number of colonies formed in different plates with differently diluted inoculums, results that the plate with first serially diluted inoculums contains the highest number of colonies i.e 339 and the 6th dilution inoculums contains the lowest number of colony i.e 1. Similarly the other serial dilution contains descending number of the colonies except the 4th which contains less colonies than 5th dilution inoculums. From the calculation it was resulted that an average of the 578×10^{-2}rhizobial cells is formed from the inoculums size of 0.5 ml. From this it was calculated that altogether 1156×10^{-2} colony forming units were obtained from 1 ml of original inoculums.

Shape of organism on the plate:

In the first plate many colonies were formed by the inoculation of the *Bradyrhizobium* inoculums which were irregular shaped and some were concentric and spreading. Some colonies were large enough and some were too small. The colony formed after re-streaked had shown smooth, the raised and convex shape at the place of the streak (Fig; 3 &5)

Color and texture:

The colonies were watery translucent with dark red rib like marking in the centre of the streak. Their color was noticed pinkish red on the plate of re–streak (fig 06).

Size of colony:

The size of the colonies in average was 4-5 mm and the maximum colony was achieved in 6-8 days of culturing as it was noticed under visual estimation.

Biochemical tests:

Many biochemical tests have performed which conformed the isolated bacterial strains as the *Bradyrhizobium japonicum*. The results of these tests can also be kept as the properties of the *rhizobium* of present study. The results of the biochemical tests are shown in the table below:

Table 2: Biochemical tests on *Rhizobium*

S.N	Biochemical tests	Result	Remarks
1.	Catalase production test	+ Ve	
2.	Penicillin resistance test	-Ve	
3.	**pH tolerance test**		
	pH 4.5	+Ve	
	pH 7	+Ve	Slow growing rhizobia
	pH 9	-Ve	
	pH 9.5	-Ve	
4.	**NaCl tolerance test**		
	1% NaCl	Extreme	
	2% NaCl	More	
	4% NaCl	less	
5.	Color change of BTB	Yellow	
6.	Nodulation test	+Ve	

+Ve = positive, -Ve= negative

The different biochemical test shows the varied results. The catalase production test shows the immediate appearance of the bubbles in 3% hydrogen peroxide which conforms that the Rhizobial strain under study can produce the catalase. The penicillin resistance test results into the zone of clearance around the penicillin disc (or formation of the ring). This proves that the *Bradyrhizobium japonicum* have the less susceptibility with the penicillin. The different pH shows the different result for their tolerance. The *Bradyrhizobium japonicum* shows the good growth at pH 4.5 and pH 7 but the growth was not obtained at pH 9 and pH 9.5. Thus it can be said that the *Bradyrhizobium japonicum* has tolerance to the acidic pH but they have not tolerance to the basic pH. Similarly the NaCl tolerance is also different at different concentration. The present studied bacteria have showed the extreme tolerance to 1% NaCl and more tolerance to the 2%NaCl than the 4% NaCl which shows very less or almost negligible tolerance. The green color of the YEMA with Bromothymol blue was changed into the yellow color. Finally the nodulation test shows the positive test with the formation of the root nodules while germinating in the own host. Those results point the bacterial strain as the slow growing species of the *Rhizobium* except the result of the color change of BTB.

Mass culture

The rhizobial strain cultured in the large conical flask by transferring the starter culture shows the formation of the thick mass at the surface of the broth as a single colony but below the surface no colonies were observed. The colonies were reddish in color but the broth below was seen watery. This shows that Congo red is being absorbed by the *Rhizobium*.

Immobilization of Rhizobial strain

As the Rhizobial strain was immobilized by encapsulating in the beaded form with sodium alginate hardened by $CaCl_2$and mixing the sucrose as the additives, the number of beads formed from every 25 ml of broth were enumerated and the beads formed per liter of the broth was calculated which is mentioned below in the table:

Table 3: Number of beads formed from the 25ml of cultured solution in different concentration of sucrose.

S.N	% of sodium alginate ($C_{14}H_{22}O_{13}$)	% of sucrose	Calcium carbonate $CaCl_2$(M)	Beads per 25ml	Average beads
1.	2%	10%	0.2	169	
2.	2%	5%	0.2	127	
3.	2%	3%	0.2	126	137
4.	2%	2%	0.2	146	
5.	2%	1%	0.2	117	

The number of beads formed from every 25 ml of the cultured broth was different .An average of 137 beads were formed from 25 ml of the solution. Thus 548 beads can be made from 100 ml of broth culture and 5480 beads can be made from one liter of the broth.

Viability tests of the encapsulated beads

The encapsulated beads of the *Bradyrhizobium japonicum* were kept in the sealed bottle and they were tested periodically for the viability of the bacterial cells. The result of the viability test done up to 190 days is shown in the table below:

Table 4: viability test of the encapsulated beads of *Rhizobium japonicum*

S.N	Periods of viability test (In Days)	Concentration of the sucrose				
		1%	2%	3%	5%	10%
1	7	+	+	+	+	+
2	20	+	+	+	+	+
3	50	+	+	+	+	+
4	75	+	+	+	+	+
5	100	+	+	+	+	+
6	120	+	+	+	+	+
7	145	+	+	+	+	+
8	170	+	+	+	_	_
9	190	+	+	+	_	_

*(+)means viable and (–) means not viable (beads were tested in each test)

The result of the viability tests shows the diversified results. The beads prepared at different sucrose concentration had shown the viability up to several months. The beads with the lower sucrose concentration i.e. 1%, 2% and 3% had shown the good viability to for 190 days of the experiment whereas the beads with 5% and 10% sucrose concentration had shown the viability up to only 145 days of preparation. On the experiment done on 170th day and 190th day, the Rhizobial strain was absent and the zone clearance rings were observed around the beads on the YEMA plates. From this it can be concluded that the *Bradyrhizobium japonicum* can survive for the long time in encapsulated form at low sucrose concentration than the high concentration of the sucrose at normal room temperature. Furthermore the sucrose had maintained the moisture level of the sodium alginate encapsulated beads.

Effects of encapsulated beads of the *Bradyrhizobium japonicum* on *Vigna unguiculata* (cow pea)

The beads of the *Bradyrhizobium japonicum* were cross inoculated with the seeds of the *Vigna unguiculata* and the shoot length of the germinated plants was observed and compared with the control (un-inoculated). The data so obtained were arranged and analyzed as mentioned below in the table:

Table 5: Growth of *Vigna unguiculata* co-inoculated with the beads of *Bradyrhizobium japonicum*

S.N	conditions of the data taken	plant samples	length of the plants in the three different sampling units		
			Inoculated I	Inoculated II	control
1		a1	14.4	14.3	11.9
2		b1	13.9	14.6	11.6
3	Inside the green house	c1	13.8	12.5	11.4
4		d1	13.3	13.7	8.9
5		e1	13.2	12.3	10.7
6		f1	12.2	12.5	10.6
		mean	13.46	13.31	10.85
1		a1	17	19	14.3
2		b1	17.5	22	15.2
3	In the shaded areas	c1	19.5	20.5	14.8
4		d1	22	17.5	14.3
5		e1	18	17	13.5
6		f1	20	21.5	14.1
		mean	19	19.58	14.36
1		a1	28.5	27.2	21.3
2		b1	25.5	24.3	22.1
3	In the external environment	c1	26.7	30.1	22.2
4		d1	27	24.9	21
5		e1	24	25.6	20.3
6		f1	23.9	25.9	22.1
		mean	25.93	26.33	21.5

Inoculated I: treated with beads containing 1% sucrose.

Inoculated II: treated with beads containing 10% sucrose.

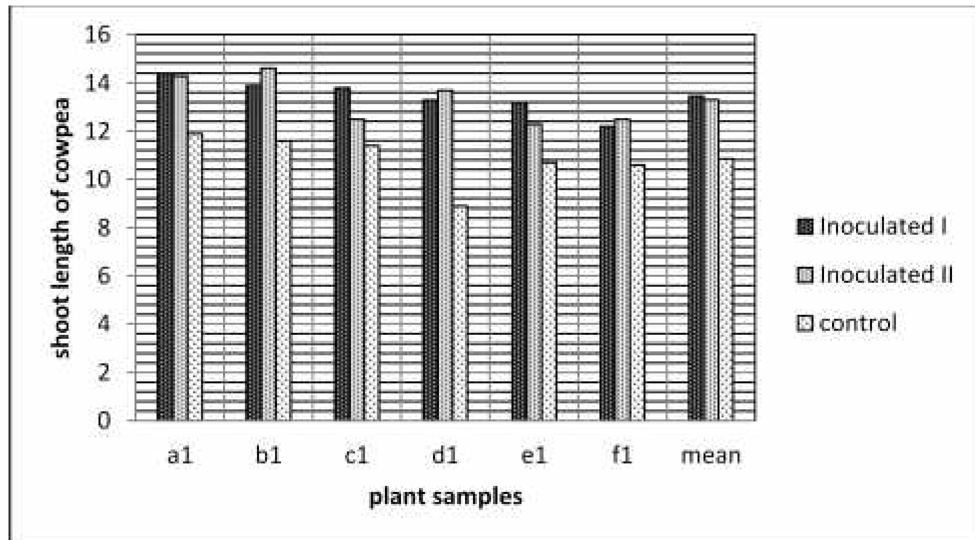


Figure 1 shoot length of cowpea in green house condition

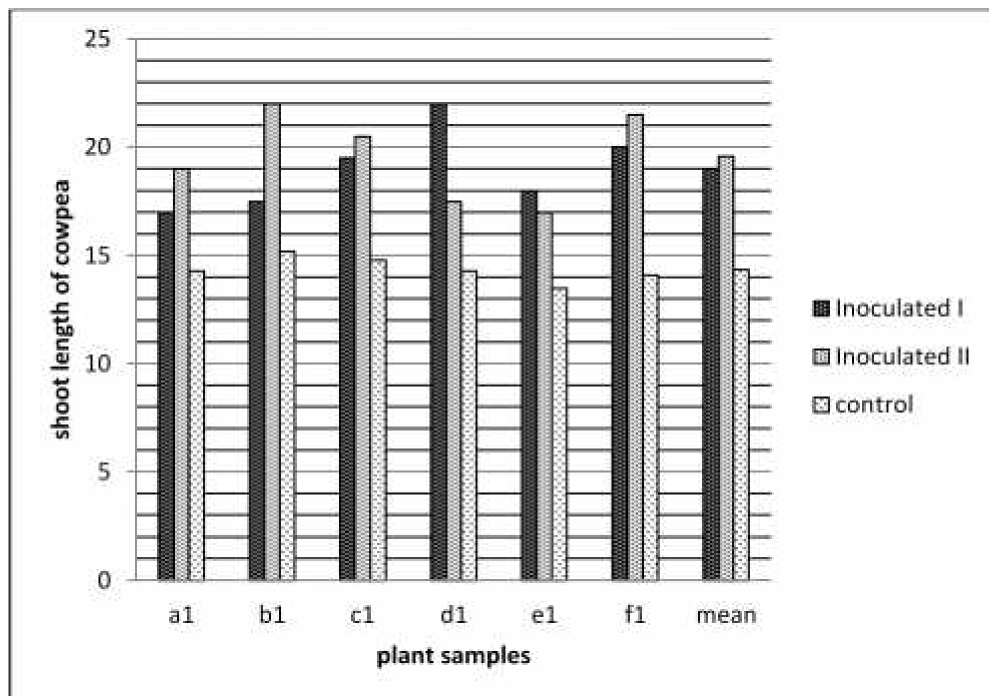


Figure 2 shoot length of cowpea in shaded area

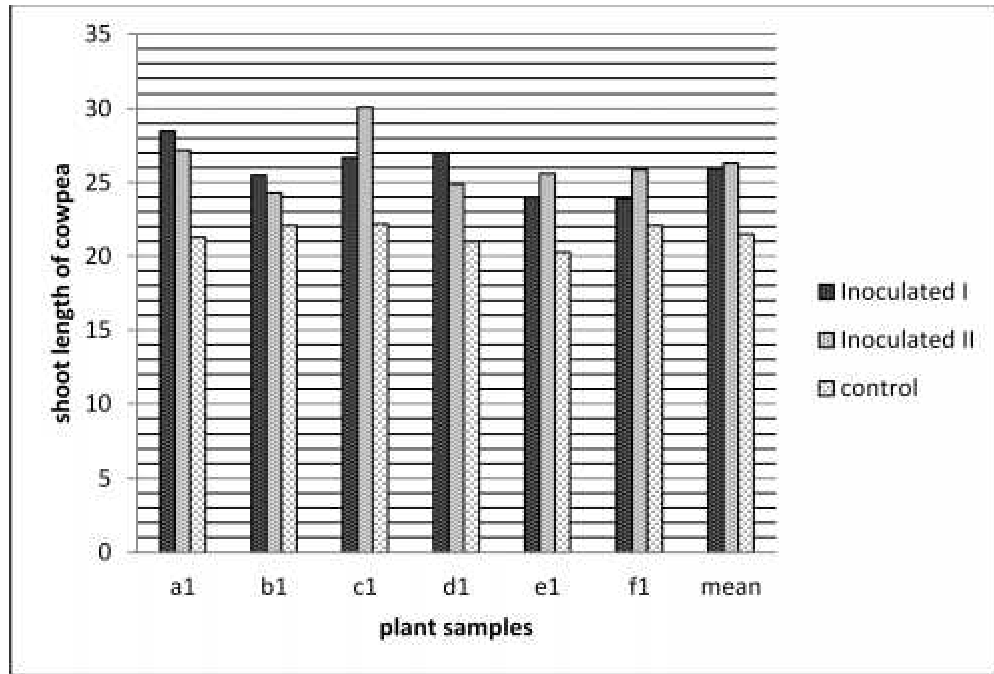


Figure 3 shoot length of cowpea in external environment

The data obtained on the shoot length of the cowpea are not normal and do not support the requirements of the parametric test and thus the non-parametric test was performed for the analysis of the data. In each of the three conditions the average of the data shows that the shoot length of the inoculated first and inoculated second is greater than the un-inoculated one. Similarly the Mann-Whitney U test (analyzed by spss-16 at 95 % level of significance) gives the different p value when inoculated first, inoculated second and the un-inoculated (control) samples were compared which is given below:

Table 6: Result of Mann-Whitney U test performed on shoot length.

Compared samples	Inside the green house (P-value)	In the shaded area (P-value)	In the outer environment(P-value)
Inoculated 1st and inoculated 2nd	0.59	0.69	0.69
Inoculated 2nd and control	0.002	0.002	0.002
Inoculated 1st and control	0.002	0.002	0.002

The p- value obtained from comparison of inoculated 1st and inoculated 2nd inside the green house condition is 0.59, in shaded area is 0.69 and in external environment is also 0.69 whereas p-value of inoculated 2nd and un-inoculated, and inoculated 1st and un-inoculated in all three condition is 0.002. It means that 0.59 and 0.69 are more than 0.005. Hence there is no significant difference between the shoot lengths of the samples inoculated I and the inoculated II in every environmental conditions but there is the significant difference in the shoot length of inoculated I and un-inoculated and the inoculated II and the un-inoculated samples in each conditions.

Hypothesis testing

From the above results it can be concluded that the Null hypothesis is regected. It means that there is the significant difference between the shoot length of the cowpea in the inoculated and the un-inoculated samples. It concluded that the strain of the *Bradyrhizobium japonicum* has positive effect on shoot length when cross inoculated with the *Vigna unguiculata.*

CHAPTER FIVE

DISCUSSION

Present study was carried on *Bradyrhizobium japonicum* and it was based on the *Rhizobium* present on the root nodules of the soybean species found in Nepal. Different methods and the materials were used for the isolation, identification, mass culture, immobilization and viability tests and their effect on the growth of cowpea in terms of the shoot length of the plant. The data obtained have been discussed with the relevant information and the similar works carried out by the different investigators. Very few works have been done in Nepal but several works have been done by the foreign researcher. From the present study performed on the *Bradyhzobium japonicum*,so varied responses were obtained regarding isolation, enumeration, identification, the beads preparation, test of their viability and the effect of the *Bradyrhizobium* beads to the cowpea.

The enumeration by spread plate method result into random result where 1st dilution have more number of colonies and the 6th dilution have the lowest number of colonies but the other have the ascending number of the colonies except the 4th serial dilution which have the less number of the colonies than the 5th dilution which alter from the principle of the serial dilution. Such result of formation of the colonies from the serially diluted samples may have obtained because of the low spreading of the bacterial cells. The calculation shows that altogether 1156×10^{-2}colony forming units are present in the 1 ml of the original sample obtained from the root nodules.

For the identification of the bacterial species present in the root nodules of the soybean, different tests have been performed. Different biochemical tests performed for present study reveals that the strain of the *Rhizobium* under study was the slow growing species. The catalase production test of the *Bradyrhizibium japonicum* shows the positive result which is adjacent to the Rhizobial isolates of the alfalfa as in the biochemical characterization performed by Shahzed, F. *et al.*(2012). It means that the Rhizobial isolates of the present study contain the catalase enzyme which decomposes the hydrogen peroxide to release oxygen. This conforms that the *Bradyrhizobium japonicum*

is the cytochrome containing aerobic bacteria as described by Buchanan and Gibbons (1974) that *Rhizobia* are aerobic bacteria utilizing oxygen as the terminal electron acceptor. Similarly the Rhizobial isolates of the present study shows the less resistance to the penicillin disc 10µg which indicate that penicillin is effective to the *Bradyrhizobium japonicum* which reduced the growth of the *rhizobium* showing the antimicrobial activity to rhizobia. The pH tolerance test performed for the present study shows that the rhizobial isolates can tolerate the low pH but cannot tolerate the high pH. It means that *Bradyrhizobium japonicum* is the acid tolerant species of the rhizobium. As Thornton and Davey,(1983); Richardson and Simpson,(1989) mentioned that slight change in pH alone can significantly affect the growth of root nodule bacteria ,the *Bradyrhizobium* shows the high growth in pH 4.5 and 7 whereas it can’t grow in pH 9 and and 9.5. The concentration of the sodium chloride also effects the growth and the survival of the *Rhzobium* species. As mentioned by Singleton *et al*.(1982) that increasing salt concentration may have detrimental effects on rhizobial population, the *Bradyrhizobium japonicum* grow well in the 1% and 2% NaCl but do not grow well in 4% NaCl concentration and it has also vital role in the cell viability for 7 weeks in the YEMA plates. Also the nodulation test shows the positive result of the present study confirmed the isolates as the *Bradyrhizobium japonicum* since it is host specific. The color change of BTB to yellow showed that it is the fast growing species but the all other results biochemical tests points it as the slow growing bacteria.
The mass culture of the Rhizobial isolates of the present study shows the dense growth of the bacteria at 7-9 days of the inoculation forming the dense mass at the surface of the YEMA broth. It also indicate that it is the slow growing species of *Rhizobium* since the fast growing species grow densely at 4-6 days of inoculation at the temperature of 30^0C.

The cultured mass of the *Rhizobium* was immobilized in the form of the encapsulated beads by using the sodium alginate extracted from algae as studied by Mc Neely W.H and Pettitt D.J (1973). The preparation of encapsulated beads of *Rhizobium* was not easier and it has many limitations in its procedure. The missing of one step hampers severely the formation of

beads. The height of dropping, time and rotation of magnetic stirrer are the most important factors. An average of 137 beads was prepared from 25ml of broth. Thus 548 beads per 100 ml can be prepared within the limitation of time and rotation of magnetic stirrer. The less rotation and the over rotation results in the deformation of beads. The large volume of the inoculant in the small beaker with small magnet could not dissolve the sodium alginate and hence the beads formation is effected which could not remain in the beaded form for the longer period of time at room temperature and dissolves itself. As Saiprasad, (2001) reported that Sodium alginate was the most accepted hydro-gel and frequently used as a matrix for the synthetic seeds because of its low toxicity, low cost, quick gelation and biocompatibility characteristics, it was used as the gelling agent along with the sucrose as the additives for their survival on the basis of study performed by Vincent (1958) and found that 24-44% of cells suspended in a 10% sucrose solution survived primarily drying whereas only 0.1 % survived when suspended in water. 2% of sodium alginate was found to be the best for the encapsulation which are hardened by 0.1M $CaCl_2$ as noticed by Kierstan, M. and Bucke, C. (1977).

When the beads encapsulated with sodium alginate were stored at the room temperature and tested for their viability, they showed the viable cells for six months. The air dried beads kept sealed in the culture tube have maintain their beaded structure for several months. The different sucrose concentration mixed as the additives for their survival have played the important role. In 1%, 2% and 3% sucrose concentration the cells were viable for 190 days of inoculation whereas in 5% and 10% sucrose concentration the cells were survived only for 145 days. Mcleod had found that the incorporation of 10% sucrose in yeast Mannitol broth improved the survival on glass beads compared with un-amended broth cited in Vincent (1958) but the survival of the *Bradyrhizobium japonicum* is less at higher sucrose concentration and vice versa. It shows that the sucrose at low concentration maintains the moisture content and support for the viability of the *rhizobium* whereas the higher concentration of the sucrose effects their survival after few months. Thus it can be said that beads of *Bradyrhizobium japonicum* prefers the lower concentration of the sucrose.

Sears, O. H. and Carroll W. R. (1927); Vanrensburg *et al*. (1976) Leonard L. T. (1923) confirmed that *Rhizobium japonicum* forms a N2-fixing symbiosis with Glycinemax L. Merr.(soybean), but these bacteria also nodulate *Vigna unguiculatum* (cowpea) and *Macroptilium atropurpurem* (siratro). Based on these findings the effect of the *Bradyrhizobium japonicum* on cowpea was studied in the present work to compare the increase in the shoot length of the cowpea when inoculated with the rhizobial beads with that of un-inoculated ones. As the data were taken from the green house condition, shaded areas and the external environment and compared the inoculated and the un-inoculated samples and analyze them by using Mann-whitney U test. From analysis, it was noticed that the inoculated samples and the un-inoculated samples has significant difference in their shoot length. Similar work had been done on the basis of plant fresh weight and nodule formation by Harold H. Keyser, Peter Van Berkum, and Deane F. Weber (1982)and had shown that ten strains of *R. japonicum* were effective on cowpea cv Pinkeye Purple Hull and all 20 strains tested produced nodules. Nodule mass ranged from 0.12 g fresh weight/plant for USDA117 to 0.68 g fresh weight/plant for USDA136. Those plants nodulated with the ineffective strains produced light green foliage in contrast to the yellow leaf color of the un-inoculated controls suggesting low rates of N2 fixation, although plant dry weights were similar. Present study focused on growth of *Vigna unguiculata* in terms of shoot length also shows the effect of encapsulated *Bradyrhizobium japonicum* on them.

CHAPTER SIX

CONCLUSION

The following conclusions can be drawn from the findings of the present investigation:

- The *Bradyrhizobium japonicum* can be isolated from the matured and pinkish root nodules of the soybean and enumerated by using the spread plate methods. One milliliter of the original samples contains the 1156×10^{-2} colony forming units. Thus with the continuous subculture of the isolated bacterial colony, the pure culture of the *Rhizobium* was isolated using YEMA media.
- For the identification, many physical and biochemical tests were done and the bacterial isolate were characterized. *Bradyrhizobium japonicum* is the slow growing, aerobic species of the *rhizobium* which can tolerate only the acidic pH and low concentration of the NaCl. It can produce catalase and nodulated its own host. It is less resistance to the penicillin disc. Thus penicillin can be used as the anti-microbe to the *Bradyrhizobium japonicum.*
- In the absence of the fermenter, they can be cultured in the water bath at 30^0C in the closed conical flask for the mass cultivation. Though the proper aeration can't be made, the rhizobial strain can survive inside the closed conical flask in spite of the Erin-Meyer flask.
- Beside soil, peat, charcoal as the solid inoculants and the broth as liquid inoculant, the rhizobial inoculants can be immobilized in the form of encapsulated beads by using 2% sodium alginate, 1-3% sucrose as additives and 0.1M $CaCl_2$ as the hardening substances. This maintains the moisture content of the beads as well as prevents the contaminants and preserved the cells for several months. This study concludes that the encapsulated beads with sucrose (1-3%) as the additives can be viable for more than 190 days where as with the 5% and 10% sucrose cells survive only for five months. Thus it can be concluded that low sucrose concentration is good for encapsulation.

- Altogether 580 beads can be prepared by the 100 ml of the Rhizobial inoculants prepared in YEM broth. It concluded that 100 ml of broth is enough for more than 580 plants to be inoculated.
- The beads of the *Bradyrhizobium japonicum* when inoculated into *Vigna unguiculata* shows positive effect in the shoot length of the plants. Thus it can be conclude that the *Bradyrhizobium japonicum* could be used as the bio-fertilizer for the *Vigna unguiculata*.

CHAPTER SEVEN

RECOMMENDATION

The following recommendations can be drawn from the present study:

To the government level

- The industrial production of bio-fertilizer could be done by immobilizing the *Bradyrhizobium japonicum* in the form of encapsulated beads using sodium alginate for gelling and sucrose as the additives and $CaCl_2$ as the hardening substances. Such product can be stored for more than six months at room temperature before distribution to the farmers.

To the farmers

- The farmer could use the encapsulated beads of the *Bradyrhizobium japonicum* to the Glycine max L. Merr. As well as *Vigna unguiculata*. They should keep such encapsulated bio-fertilizer in the dry places.

To the student researcher

- Study can be done to differentiate the biochemical characteristics of the *Bradyrhizobium* isolated from the same host taken from different climatic zones.
- The encapsulated beads can be viable for more than six months. Further study can be carried out on the requirement of the beads for the effective production for particular space of the land(kg/hectare).
- Since the *Bradyrhizobium* can fix the atmospheric nitrogen into the ammonia and nitrate, the further study can be carried on to find out the amount of nitrogen reduced by the *Bradyrhizobium japonicum.*
- The upcoming researcher could extend this work to measure the growth parameters such as dry weight, number of nodules per plants, grain yields of *Vigna unguiculata.* But this study should be done during the spring to monsoon season.

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ANNEX

(photo plates)

Photo plate I

Fig 01: Root nodules of soybean

Fig 02: Mass culture of *Bradyrhzobium japonicum.*

Fig 03: Isolation of Rhizobial strain

Fig 04: Catalase test of Rhizobial strain

Fig 05: pH tolerance test

Photo plate II

Fig 06: NaCl tolerance test

Fig 07: Penicillin resistance test

Fig 08: BTB change test

Fig 09: Encapsulated beads of *Bradyrhizobium japonicum*

Fig 10: Beads with different sucrose concentration kept for viability test.

Photo plate III

Fig 11: Viability test of beads

Fig 12: Colony of rhizobia formed by encapsulated beads.

Fig 13: Rashes shown by 5% sucrose contained beads at 170 day

Fig 14: Nodulation test on *Glycine max*.

Fig 15: Growth of *Bradyrhizobium japonicum* from beads.

Photo plate IV

Fig 16: *Vigna unguiculata* germinated in earthen pots in the external environment.

Fig 17: Growth of *Vigna unguiculata* in shaded area.

Fig 18: Germination of Vigna unguiculata by maintaining green house condition.