ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR IDENTIFICATION OF *BRADYRHIZOBIUM JAPONICUM* STRAIN IN THE NODULES OF SOYBEAN FROM KATHMANDU VALLEY

A Dissertation submitted to

Central Department of Botany, Tribhuvan University

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Submitted by:

Sishir Panthi

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CENTRAL DEPARTMENT OF BOTANY

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NEPAL

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CERTIFICATE

This is to certify that the M.Sc. dissertation work entitled "**Enzyme-Linked Immunosorbent Assay (ELISA) for Identification of Bradyrhizobium japonicum strain in root nodule pf soybean grown in Kathmandu Valley**" submitted by **Mr. Sishir Panthi** has been carried out under our supervision. The entire work is based on the laboratory research, and results of his research work have not been published or submitted for any other degree. We recommend this dissertation work to be accepted for the partial fulfillment of Master's Degree in Botany (Plant Biotechnology and Biochemistry).

……..

Prof. Dr. **Vimal Narayan Prasad Gupta**

Supervisor

Central Department of Botany

Tribhuvan University

Kirtipur, Kathmandu

……..

Prof. Dr. Vishwanath Prasad Agrawal

(Co-supervisor)

Executive Director

Research Laboratory for Agricultural Biochemistry and Biotechnology (RLABB)

Phone: 4331322, 4332636, Fax: +977-1-4333515, Post Box: 26429 E-mail: info@cdbtu.edu.np, Webpage: www.cdbtu.edu.np

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LETTER OF APPROVAL

This dissertation paper entitled **"Enzyme-linked Immunosorbent Assay (ELISA) for Identification of *Bradyrhizobium japonicum* Strain in the Nodules of Soybean from Kathmandu Valley"** submitted at the Central Department of Botany, Tribhuvan University by **Mr. Sishir Panthi"** has been accepted for the partial fulfillment of requirements for Master of Science in Botany.

EXPERT COMMITTEE

……..	……..
(Supervisor)	(Co-supervisor)
Prof. Dr. Vimal Narayan Prasad Gupta	**Prof. Dr. Vishwanath Prasad Agrawal**
Central Department of Botany	Executive Director
Tribhuvan University	RLABB
……	……
(External Examiner)	(Internal Examiner)
Dr. Rajani Malla	**Prof. Dr. Sanu Devi Joshi**
Associate Professor	Central Department of Botany
Central Department of Biotechnology	Tribhuvan University
Tribhuvan University	

……..

Prof. Dr. Krishna Kumar Shrestha

(Head of Department)

Central Department of Botany

Tribhuvan University

Date of Examination: 19th November, 2009

Phone: 4331322, 4332636, Fax: +977-1-4333515, Post Box: 26429 E-mail: info@cdbtu.edu.np, Webpage: www.cdbtu.edu.np

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Sishir Panthi
panthi.sishir@gmail.com

ABSTRACT

ELISA technique was applied for the identification of *Bradyrhizobium japonicum* and to study their somatic antigen diversity. *B. japonicum* was isolated from the nodules of soybean grown on Tangla soil and identified. Somatic antigen of *B. japonicum* was prepared and immunized to Newzealand white rabbit to raise polyclonal antibody against it. Indirect ELISA was optimized and immuno-crossreacrtivity of the strains from the different localities was observed. The optimal dilution of the antisera was found to be 1000 folds and secondary antibody to be 2000 folds. The cut off value for the colour observation at OD_{450} was 0.76nm. Mulpani strains have similar somatic antigen showing 97% crossreactivity while the Dhobighat strains shows the least crossreactivity with 19%. Crossreactivity of the strains from same locality was also not consistent. Champasingh strain show high variation up to 40% (22-62%) while Panga strains show 9% (26-34%). Different localities of the same agro climatic region possess different strain of *B. japonicum* as well as different nodule of single plant also gets infected by different strains.

CONTENTS

ACRONYMS

ATP	Adenosine Triphosphate
B_{101}	*Bradyrhizobium japonicum* strain from Tangla
BNF	Biological Nitrogen Fixation
BSA	Bovine serum albumin
CDB	Central Department of Botany
CFA	Complete Freund's adjuvant
CR	Congo red
ELISA	Enzyme linked immunosorbent assay
HRP	Horse raddish peroxidase
IFA	Incomplete Freund's adjuvant
iP	Inorganic phosphate
kDa	Kilodalton
N/N_2	Nitrogen
NARC	National Agricultural Research Council
nm	Nanometer
OD	Optical density
PBS	Phosphate buffer saline
pH	Negative logarithm of hydrogen ion concentration
RLABB	Research Laboratory for Biochemistry and Biotechnology
rpm	Round per minute
TMB	Tetramethyl benzidine
YMA	Yeast extract mannitol agar
YMB	Yeast mannitol broth

CHAPTER I

INTRODUCTION

1.1 Background

Nitrogen is commonly the most limiting element in agricultural production and or the most the expensive to purchase as fertilizer. Though the atmosphere contains 78% of nitrogen, it is stable gas normally unavailable to plant. It is chemically inert (N≡ N, ΔE=954.4 $KJmol^{-1}$) and cannot be directly utilized by plant. Mainly the conversion of atmospheric nitrogen into the combined form like NH_3 and NO_3 takes places by two process viz., Non-biological and Biological. Non biological process includes lightning combustion and volcanism which account for about 10% of annual fixation and industrial fixation by Harber-Bosh process which account for about 25%. Biological nitrogen is fixed by a range of prokaryotic organism which account for 65% (Newton, 2000). Global estimate of biologically fixed nitrogen is about 175 million tons and 40% of this amount is fixed by legume-*Rhizobium* symbiosis (Espiritu, 1999). About 60% of total nitrogen fixation by *Rhizobium* is contributed by agro ecosystem and remaining from forest.

BNF is unique to bacteria, plant that fix N_2 must be associate with bacteria. Legumes are able to utilize this atmospheric nitrogen through associate with bacteria. Rhizobia reduce the atmospheric nitrogen into NH_4^+ in the soil that is used in the synthesis of glutamate and aspartate. The overall BNF can be summarized in the following equation (Stryer, 1995).

$$N_2 + 8e^- + 16ATP + 16H_2O \longrightarrow 2NH_3 + H_2 + 16ADP + 16iP + 8H^+$$

The major nitrogen fixing system is the symbiotic systems, which can play a significant role in improving the fertility and productivity of low-N soils. The legume-*Rhizobium* symbioses have received most attention and have been examined extensively. Deficiency in mineral nitrogen often limit plant growth, and so symbiotic relationship 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 ecosystem. Legume-*Rhizobium* symbioses are the primary source of fixed nitrogen in land based ecosystem and can proved 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.

In the presence of specific rhizobial species, legume host form unique structure, the root nodule, in which nitrogen fixation occurs. The process begins with curling of the root hair. Subsequent to the curling, infection thread is formed by a process of invagination of the hair cell wall in the region of curling (Nutman, 1969). The infection thread grows toward the cortex and ramifies throughout the central portion of cortex. Simultaneously the cortical cell division commences leading to the formation of nodule. The nodule developed in legume-*Rhizobium* symbioses are of determinate type. Rhizobia loose cell wall and become substantially enlarged and pleomorphic. Each enlarged pleomorphic bacterium is referred as bacteriod. These bacteriod are separated from the plant cytoplasm by peribacteriod membrane (Michael *et al*., 1997). These rhizobia feed its host legume with ammonia in the expense of shelter and reduced substrates. Thus both the symbionts faithfully negotiate the biological nitrogen fixation in legume-*Rhizobium* symbioses (Alexander, 1997)

Soybean is one of the oldest known food crop to man. It has been grown for centuries in Nepal. It was mainly grown in the mid-hills at an altitude of 3000-5000 ft. but has later started to be grown in other parts of Nepal. It is intercropped with maize, millet, rice, pigeon peas etc. It contains 40-42% good quality protein and 18-22% oil comprising up of 85% unsaturated fatty acid and is free from cholesterol. Soybean not only contains high quality protein, but their protein content is much higher than that of other food and it also contains 12% carbohydrate (Achakzai *et al*., 2003). It is recognized all over the world as an efficient producer of plant protein, hence the necessity to promote its popularity in developing countries to meet protein deficiency. Soybean occupies 21,003 ha. of cultivated land and average yield is only 903.47 kg ha^{-1} (NG, 2065).

The country's average yield is very low due to several factors and most important among them is the absence or low population of efficient *Bradyrhizobium japonicum* strain in the soil. So to increase the yield, effective *B. japonicum* have to be inoculated. A 40-50% increase in yield due to inoculation has been very common.

Occasionally, yield responses in the range of 100-200% have been noted (Aslam *et al*., 2000). Inoculation of soybean is a significant way of manipulating rhizobia for improving the crop productivity and soil fertility (Keyser & Li, 1992). This inoculant is produced by the National Agricultural Research Council (NARC) but only in a small scale. Some private firm has also started the production of inoculum but the awareness programmed is not conducted. Farmers have not been educated to benefits of the inoculation and have little concept of the practice.

For inoculum preparation, characterization and selection of effective *Bradyhizobium japonicum strain* is must. The characterization of the strain from different niches provides useful information on the ecological patterns of distribution, and opportunities for the selection of best strain. Effective strains can neither be selected by the general morphology nor by cross inoculation grouping. Serological marker like agglutination, immunofluorescence, ELISA, immunoblot and other methods like SDS-PAGE, Nucleic acid hybridization (DNA-DNA and DNA-RNA hybridization) etc are used for the strain differentiation. The choice of the marker system and an awareness of its limitation are as important to the success of an experiment as the isolation and application of the markers (Schwinghamer & Dudmann, 1980). Marker system which are used in field ecological experiment for the study of legume-*Rhizobium* symbiosis need to be specific, sensitive, reliable, reproducible, rapid easy to use and of relative low cost. The ELISA technique offers sensitivity, specificity, speed and ease of use (Renwick *et al*., 1985). It has been used successfully in clinical and immunological studies (Voller *et al*., 1978) and also for the identification of bacterial and plant pathogen (Sunders and French, 1983).

The ELISA technique was first applied to the identification of *Rhizobium* by Kishinvesky & Bar-Joseph, 1978 and various scientist have modified the technique in attempting to make it more sensitive (Berger et al., 1979; Morley and Jones, 1980; Furhmann and Wollum II, 1985). ELISA is an immunoassay utilizing enzyme conjugated antibodies, with antigen or antibody bound to a solid support. The assay measures change in enzyme activities proportional to the antigen or antibody concentration involved in the underlying immune reaction. In indirect ELISA, the antigen is immobilized in the wells of polystyrene microtiter plates and is reacted with specific (primary) antibodies. Primary antibodies bound to the antigens in the wells

are reacted with enzyme conjugated secondary antibody that binds specifically to the primary antibodies. Reaction with an enzyme substrate causes a colour change, the intensity of which corresponds to the amount of primary antibody present (Somasegaran and Hoben, 1994).

Hence ELISA can be effectively used to study cross reactivity of a given type of antigen. Identical antigens possess 100% crossreactivity with the given antisera and non identical ones don't show any degree of crossreactivity. The diversity of the given antigen and organisms i.e. *Bradyrhizobium japonicum* in a given population can be studied by their crossreactivity among themselves. Thus ELISA can be used to screen the most effective strain from the rest of population and this strain can be used for the preparation of inoculant for the better yield of soybean.

1.2 Objectives

General objective

This research work aims to study the somatic antigens diversity among the *Bradyrhizobium japonicum* strains of soybean grown in different localities of Kathmandu Valley.

Specific objectives

- To isolate and characterized *B. japonicum*
- To prepare the somatic antigen of the strain.
- To immunize *B. japonicum* in NewZealand white rabbit to produce polyclonal antisera.
- To study the immuno-crossreactivity of somatic strain from different strains of *B. japonicum* using polyclonal antisera.

CHAPTER II

LITERATURE REVIEW

2.1 Description of *Bradyrhizobium japonicum*

Bradyrhizobium japonicum is nitrogen fixing symbiont of (*Glycine max)* and belongs to family Rhizobiaceae. It is gram negative, non spore forming, aerobic and slow growing bacteria in artificial medium. It is motile rod shaped cell ranges from 0.5-0.9x1.2-3.0 µm in size. It produce characteristic translucent to opaque, white, raised colony on artificial medium like Yeast extract Mannitol Agar (YMA). It doesn't absorb the colour of Congo-Red (CR) when cultured in dark. Optimum temperature requirement for growth ranges from 27 ± 2^0C and lethal temperature lies between 30-40^0C depending up on the strains (Nutman, 1969).

2.1.1 Metabolism

Bradyrhizobium japonicum synthesizes a varities of self surface carbohydrate these include lipopolysaccharide, capsular polysaccharides, exopolysaccharides, nodulepolysaccharides, lipochitine oligosaccharides and cyclic glucans, some of which may provide important function to symbiosis. It uses these carbohydrate structures to obtain carbon sources from the soybean plant as well as gain entry. *Bradyrhizobium japonicum* uptakes trehalose the most rapidly and convert it to CO_2 (Steppo, 1986)

2.1.2 Genome structure

Bradyrhizobium japonicum genome has been completely sequenced. It is made up of a single circular chromosome of 9105828 bp and has no plasmid. The average GC content is 64.1%. It has 8317 potential protein codding genes, 1 set of rRNA and 50 sets of tRNA genes (Kaneko *et al.*, 2002).

2.1.3 Historical Development

Nitrogen fixing bacteria, present in root nodules of Legumes, were first time isolated by Duch Scientist M. W. Beijerink in 1888 and named it as *Basillus radicicolla*. It was then placed in genus *Rhizobium*. Later on, Jordan (1982) divided the genus into two groups, fast growing and slow growing. Fast growing rhizobia were placed under

previous genus whereas slow growing rhizobia were placed under new genus *Bradyrhizobium*.

2.1.4 Classsification

The ability of certain rhizobia to infect and nodulated particular group of legumes is important in the classification of rhizibia. Rhizobia are generally classified according to the host based system. The cross inoculation system has provided a useful classification system for rhizobial strain. The principle of cross inoculation grouping is based on the ability of an isolate of rhizobium to form nodule in a limited number of species of legumes related to one another. All rhizobia that could form nodules on root of certain legumes have been collectively taken as species (Rao, 1999). This system of classification has provided a workable basis for the agricultural practice of legume inoculation.

Table 1: Cross inoculation grouping of legume and rhizobia

Rhizobia	**Cross inoculation group**	**Legume in cross inoculation group**
Genus I *Rhizobium*		
Rhizobium leguminosarum bv. *viceae*	Pea	*Vicia faba, Pisum sativum, Lens esculentum, Lathyrus odoratus*
R. leguminosarum bv. *trifoli*	Clover	*Trifolium* spp.
R. leguminosarum bv. *phaseoli*	Bean	*Phaseolus vulgaris, P. coccinus,*
R. meliloti	Alfalfa	*Medicago* spp., *Melilotus* spp.,*Trigonella foenumgraecum*
R. loti	Lotus	*Lotus corniculatus, L. tenuis, Lupinus densiflorus,*
R. galega	Soybean	*Galega orientalis*
R. fredii	Soybean	*Glycine max*
Rhizobium sp.	Soybean	*Glycine max, Lecuna* spp., *Sesbania grandiflora*
Rhizobium sp.	Chickpea	*Cicer arietinum*
Genus II *Bradyrhizobium*		
Bradyrhizobium japonicum	Soybean	*Glycine max*
Bradyrhizobium sp.	Cowpea	*Cajanus cajan, Arachis hypogaea, Vinga* spp., *Phaseolus lunatus* and many tropical legumes

Source: Somasegaran and Hoben, 1994.

2.2 Immunology

2.2.1 Antigen

Any substance that provokes an immune response when introduced into the tissue of an animal or human is referred to as antigen. In rhizobial work whole cell is used antigen. Antigen of rhizobia can be categorized into somatic, flagellar and capsular depending upon their derivation. Somatic antigens (O antigen) are closely related to the rhizobial cell wall. Some somatic antigen may be tightly bound to the cell wall. They are not removed by washing the cell. Therefore, these antigens are only detected when whole cell of rhizobia react with antibody. Flagellar (H antigen) of the rhizobia are also antigenic. The capsular (K antigen) are surface antigen and found outside the cell. Cultured cell as well as nodule can be used as antigen for strain identification (Sommasegaran and Hoben, 1994).

2.2.2 Antibody production

Antibodies are the protein complex produced by the immune system. There are five different classes of antibodies or immunoglobulins IgA, IgD, IgE, IgG, IgM. There are two major structural fragments of IgG called Fc and Fab region (fig. 1). The antigen binding fragment Fabs of IgG immunoglobuns are the two regions at the top of molecule, are the site of antibody antigen interaction. The Fc region is the tail of antibody which is recognized by the host (Dowd and Maier, 2000). For rhizobial research, the most important of these are IgG and IgM antibodies. The ratio of IgG and IgM can vary with time, immunization route and form of antigen (Somaseggaran and Hoben, 1994).

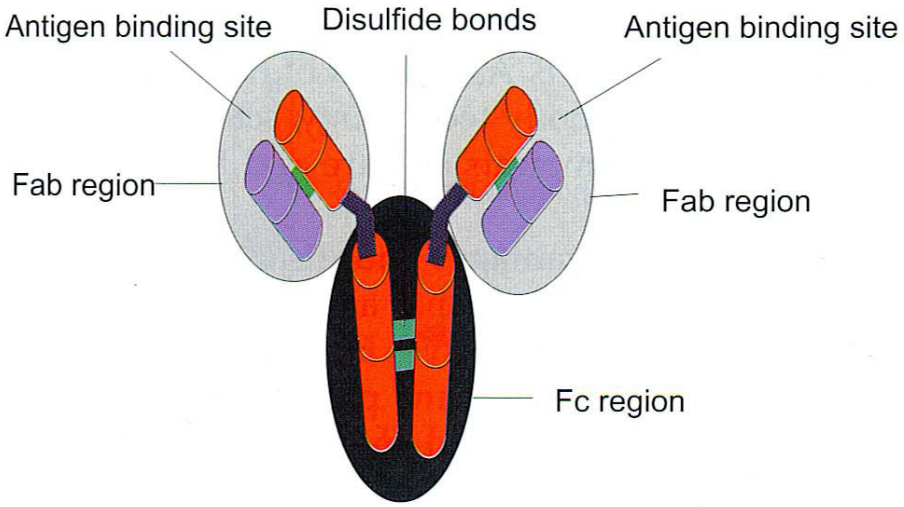


Fig. 1: Schematic representation of an IgG antibody, showing the various regions associated with the antigen/antibody interaction.

Antibody formation consists of two phases an induction and production phase. Specific information for the anamnestic response is stored in the lymphoid cell. The magnitude of immune response depends upon the various factors namely:

- Choise of animal
- Form and dose of antigen
- Adjuvant
- Route of immunization
- Immunization schedule

2.2.3 Choice of animal

Rabbits, goat, sheep, horse, mouse are generally employed for production of antibodies (Talwar and Gupta, 1997) but for rhizobial research rabbits are commonly used for immunization (Somaseggaran and Hoben, 1994).

2.2.4 Form and dose of antigen

Antigens may be of several types, soluble and particulate proteins, viruses, subcellular particulates and entire complex such as tumor cells is used as antigen. In rhizobial serology, both cultured cell and legumes root nodule are used as antigen for strain identification (Means *et al*., 1964). Somatic antigen of rhizobia is more specific than flagellar and internal antigen (Vincent, 1982). Cultured cells suspended in PBS at 10^9 cells ml^{-1} are heated at 100°C in water bath to inactivate the falgellar antigen (Kishinvesky *et al*., 1993)

2.3 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA, enzyme linked immunosorbent assay, is a highly versatile and sensitive analytical procedure for qualitative or quantitative determination of antibodies and almost any kinds of antigens. Three principal types of antibodies are generally recognized: direct ELISA, indirect ELISA and sandwitch ELISA.

2.3.1. Indirect ELISA to determine Antigen.

Solid phase immobilization of antigens or antibodies is achieved by linking them, through adsorption to a suitable matrix. Such immobilization makes use of the capacity of various plastic (e.g. polyvinylchloride or polystyrene) to adsorb protein as monomolecular surface layers without significantly altering their immunological properties. The reactants are usually adsorbed onto the wells of 96 well microtiter plates of polystyrene, an adsorption characterized by strong hydrophobic binding and slow dissociation rates. After coating with antigen or antibody, the residual protein binding capacity of the solid matrix is blocked by exposing it to an excess of unrelated protein such as gelatin, bovine serum albumin (BSA), egg albumin. Coating of the microtitration plates is followed by addition of the specific antibody against the solid-phase test antigen. Then follows addition of antiglobulin-enzyme conjugate and a substrate; generating a coloured product when hydrolyzed. The resulting change in colour may be recorded visually or spectro-photomertically to determine the conjugate fixed, which itself is proportional to the antibody level in the test sample. ELISA technique can be used to detect either antigen or antibody qualitatively or quantitatively.

Before testing, the ELISA condition must always be optimized which depends on different factors such as ELISA plate (solid phase), antigen dilution, antibody dilution, enzyme system (enzyme conjugated secondary antibody), substrate used etc. A small amount of enzyme can react with much larger amount of substrate to produce detectable levels of break-down product in a given time. Enzymes which bring about chromogenic or fluorescent change in their substrates are selected for enzyme immunoassay. Antibodies are conjugated or labeled with an enzyme by addition of glutaraldehyde in such way that resulting conjugate retain both immunological and enzyme activity (Voller *et al*., 1978). A variety of enzyme have been used, including glucose oxidase, β-d-galactosidase, alkaline phosphatase, horse-radish peroxidase

(Engvall and Perlmann, 1971; Kato *et al*., 1976). Horse radish peroxidase (HRP), one of the most widely used enzyme system, is a 40 kDa protein that catalyzes the oxidation of substrates by hydrogen peroxidase, resulting in a coloured fluorescent product or the release of light as a byproduct. HRP function optimally at a near neutral pH and is inhibited by cyanides, sulphides and azides.

Substrates are usually chosen to give a coloured product following enzymatic degradation. For visually read ELISA test, an insoluble or partly insoluble substrates like 5-amino-salicylic acid (5AS) or diaminobenzidine (DAB) may be used. However for all quantitative ELISA test the substrate product must be soluble. Ortho-phenyl-diamine is suitable for use with peroxidase conjugate although it is light sensitive and is reputed to have mutagenic properties. For alkaline phosphates conjugates para-nitrophenyl phosphate is an excellent substrate. It is safe readily available in tablet form and give a stable yellow product (Voller *et al*., 1978). TMB (3, 3', 5, 5'-tetramethyl benzidine) is the most common chromogenic substrates for use in ELISA with peroxidase conjugate. TMB is non carcinogenic analog of benzidine which is used as a substrate for immunoassays using horse-radish peroxdase conjugates. When the reaction stopped, the colour turns from blue to yellow and is read at 450nm (Schneider *et al*., 2004)

Schmidt *et al*. (1968) explored the application of fluorescent antibody (FA) technique to study the rhizobia as frees living soil bacteria. Antiserum to particular strains of *Rhizobium japonicum* proved specific to both agglutination and FA tests. The FA technique also detected antibody reacting bacteria in the field whose rhozobial content was unknown.

Berger *et al*. (1979) described the colorimetric method to identify strains of *Rhizobium* spp. in pure culture and lentil nodule. The test was based on an indirect enzyme linked immunosorbent assay using strain specific rabbit antisera and sheep antirabbit globulin conjugated to alkaline phosphatase.

Furhmann and Wollium II, (1985) developed a simple, reliable and flexible modification of the indirect enzyme linked immunosorbent assay for the identification of *Rhizobium japonicum* antigen from cultures and nodules. Their procedure

emphasizes on efficient use of time, reagent, adaptability to variously equipped laboratories and maintenance of sensitivity levels that are adequate for ecological studies.

Ayanaba *et al*. (1986) evaluated three antisera and four enzyme conjugates for the detection of Bradyrhizobium *japonicum* by indirect enzyme linked immunosorbent assay in microtitter plates. Nitrocellulose membrane sheets were then evaluated as an alternative support media by using same combination. Antisera to strain USDA 110 raised in rabbit , goat or sheep was reacted in microtitter plates with alkaline phosphatase conjugate to protein A, goat anti rabbit (GAR), sheep anti rabbit (SAR) or rabbit anti goat (RAG) IgG. Culture or nodule containing homologous rhizobia were detected with equal sensitivity when protein A, GAR or SAR was reacted with 5µg of protein IgG per ml or a 1:800 titer of antisera from rabbit but not goat or sheep. RAG reacted with IgG or antisrea from goat or sheep.

Schimidt *et al*. (1986) examined the diversity within a group of 79 isolates of *Bradyrhizobium japonicum* reactive to fluorescent antibodies (FAs) prepared against *B. japonicum* USDA123 and found that serogroup 127 and 129 shared antigenic determinants with serogroup 123 but not with each other.

Wright *et al*., (1986) produced the monoclonal antibodies for identification of strains of *Rhizobium trifolii.* This antibody was used in an indirect ELISA to differentiate strain 162 x 95 from naturalized strain in the Appalachain region and found that there are four or five Californian strains not the ten as reported earlier.

Martenson *et al*., (1989) investigate the influence of different N level in soil in strain competition to select the suitable strains for successful inoculation of soybean at high N levels. Strain E_{110} dominates the symbiotic relationship even if other strains are also present in the nodule. However, at high N fertilizer level strains E_{110} decreases the plant yield in contrast to E_{104} which they recommended as inoculants at increased level of soluble soil nitrogen.

Graham *et al*., (1991) reviewed the status of *Rhizobium* taxonomy and proposed minimal standard for the description of future genera and species belong to stem and

root nodulated bacteria. The change in the taxonomy of this group should be on the basis of both phylogenetic and phenotypic (symbiotic, cultural, morphological, physiological) traits and following studies with a relatively large number of strains.

Trottier *et al*., (1992) reviewed each parameter involved in the indirect ELISA protocol to achieve maximal assay performance and modified it for serodiagnosis of *Actinobacillus pleuropneumoniae*. They found Nunc 475-904 and 269-620 immunoplates were best in term of high positive to negative ratio and low variability. Presence of magnesium ion ($Mg^{+2,}$ 0.02M) resulted in the two fold increase in nonspecific background. An optimal screening dilution of sera was established at 1:2000. One hour incubation period for test serum was found optimum.

Kishinvesky *et al*., (1993) determined the serological properties of indigenous peanut rhizobial population in different peanut growing region of Thailand. They used ELISA and antibody adsorption test to determine the minimal somatic antigen constituent of 243 strains of *Bradyrhizobium* spp. Using 12 antisera. They identified 29 serogroup among which 80% of the isolates tested had at least one heat stable antigen common with strain 280A, forming the so called 280A serocluster. The serological properties of indigenous bradyrhizobia were not related to the cropping history of the cultivated field from which they were isolated.

Hoben *et al*. (1994) evaluated the production of antisera by immunizing a single rabbit with an antigen prepared by mixing antigenically distinct species of rhizobial and found that antisera contained antibodies for all there rhizobial species. The cross absorbed and the mixed rhizobial species antisera were found to be similar in quality for strain identification by agglutination, fluorescent antibody, immunoblot and ELISA. Cost for the production of antisera by immunization with mixed rhizobial species antigens was estimated to be reduced by 66%.

Leung *et al*. (1994) identified the major nodule occupying serotypes of *Rhizobium leguminosarum* bv *trifolii* and compared the population densities and dynamics of the both major and minor nodule occupying serotypes in the host and non host rhizospheres. They found the existence of 13 antigenically distinct serotypes of *R. leguminosarum* bv *trifolii* among which one serotype, AS6, occupies ≥50% of root nodule formed on field grown subclover.

Madrzak *et al*. (1995) investigated diversity among field population of *Bradyrhizobium japonicum* isolates in Poland. Of the ten field sites examined, only four contained population of indigenous *B*. *japonicum*. Serological analysis indicated that 87.5% of polish *B*. *japonicum* tested were in serogroup 123 and 129 while seven, 12.5%, isolates tested belongs to their own serogroups.

Yokoyama *et al*. (1999) reported the existence of phenotypic difference between the Thai soybean bradyrhizobia and soybean bradyrhizobia isolated from the temperate region using serological and intrinsic antibiotic resistance analysis. 94 Thai strains were compared with those of USDA and Japanese strains. Indirect ELISA test for each Thai strain were performed against polyclonal antisera prepared against 15 USDA standard serotypes strains of *Bradyrhizobium japonicum* and *B*. *elkanii*. Among 96 Thai strains tested, 36 which were previously identified as *B*. *elkanii*, with the exception of one strain were strongly responsive to an antiserum prepared against USDA 31. The remaining 58 strains, with the exception of two strain, show multiple cross reaction which were peculiar to Thai strains.

Pant (2003) evaluated the effectiveness of Bradyrhizobium isolates on seedling growth of soyabean as revealed by shoot and root length, shoot and root dry weight and nitrogen content in shoot in inoculated and control plants. All isolates were found effective over the control. The effectiveness of KH was found more than TU1, TU2.

Botha et *al*. (2005) examined the size, effectiveness and diversity of lupine nodulating rhizobia at five different localities in Western Cape of South Africa. Purified antibodies were used to detect the strain VK_{10} in nodules by an indirect ELISA method. Positive ELISA reactions were obtained when antiserum preparation against VK7 and VK10 respectively was tested against the heterologus strains. Both antisera also reacted positively with strain WU 425, WSM 425 and Lup 025.

Thapa (2006) optimized the ELISA for crystal protein detection and studied its immunocrossreactivity using polyclonal antisera. The optimal dilution of the polyclonal antibody was 1000 folds. Of the total 86 endotoxins producing isolates 31 were 25-30% crossreactive, 6 each were 75-80% and 85-90% crossreactive and 4 were 80-85% crossreactive. Only 3 were more than 90% crossreactive.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

All the chemicals and the equipments used are listed in the Annex I.

3.1.1 Source of seeds

Seeds of Soybean (*Glycine max* (L.) Merr.) was purchased from farmer of Tangla, Kirtipur municipality.

3.1.2 Laboratory facilities.

Research work was carried out in the laboratory of CDB, TU, Biotechnology unit and RLABB.

3.2 Methods

3.2.1 Collection of soil

Soil from the cultivated land of kathmandu valley was collected. It was collected from Champasingh, Mulpani, Dhapakhel, Tangla, Panga, Dhobighat area. Quadrate size of 10x10 m^2 was led to collect the soil samples. Soil from the each corner and from the centre of the quadrate was collected and mixed together 10 Kg soil was kept in tight plastic bag and brought to laboratory (Zobel *et. al.*, 1987)

3.2.2 Plantation

Earthen pots of medium sized were taken and was filled with soil collected from the different localities. Three pots were filled from each locality and labelled. Morphologically similar and healthy seeds of Ransom variety of soybean (*Glycine max* (L.) Merr.) were planted four in each pot. Thinning was done and two plants were left in each pot. Intensive plant care was done in a closed green house chamber and plants were watered in every alternate day. Weeds were uprooted regularly.

3.2.3 Collection of nodule

Root nodules were collected from the *Glycine max* (Soybean) grown on the Tangle soil. Root nodules from other localities were collected at the time of ELISA test.

Effective and healthy root nodules were collected from young healthy green plants. Excised nodule were washed in tap water, shade dried for one hour and collected in vials with silica gel crystal. The vials were stored at 4±1^{o}C for short term storage.

3.2.4 Preparation of stock solution

Congo red (CR) stock solution

Stock solution of congo red was prepared by dissolving 0.25 g of CR in distilled water to make final volume 100 ml. Stock solution was stored at 4^{o}C in well labeled brown bottle in refrigerator (Annex II).

Bromothymol blue (BTB) stock solution

BTB stock solution was prepared by dissolving 0.25 g BTB in distilled water and made its final volume to 100 ml. This stock was also stored at 4^{o}C in a well labeled brown bottle and kept in a refrigerator (Annex II).

3.2.5 Preparation of culture medium

For the culture of the *Bradyrhizobium* in vitro, Yeast extract Mannitol Agar Congo Red (YMA-CR) was prepared. The chemical constituents of YMA-CR have been tabulated in Annex II

All the required amount of constituent was weighted for one liter and dissolve one by one with continuous stirring. The formation of precipitate was completely avoided. 10 ml. of CR was added to the solution and the final volume was made 1000ml. the pH of the solution was adjusted 6.8 ± 0.1 with the help of 0.1N HCl or 0.1N NaOH. Finally 15 g of agar powder was added to the broth and YMA-CR was autoclaved at 121^{o}C for 15 min. this sterilized YMA-CR medium was used for isolation of rhizobial isolates from different root nodules (Vincent, 1970).

3.2.6 Isolation of *Bradyrhizobium* pure culture

Nodules from the healthy and matured plants were taken along with the small section of root as well. Big and brown, red nodules were selected and these were then washed with surface sterilized chemicals. The process was carried out in laminar air flow cabinet under the complete aseptic condition. For surface sterilization the nodules were first immersed in 90% ethanol for 10 sec and were then transferred to 30 % (v/v) solution of sodium hypochlorite for four minutes. These were then finally washed with sterile distilled water for bout six folds to get rid of the steriliant.

In the mean time about 20 ml of melted YMA-CR medium was poured on each Petridish and was left for solidification. At the same time, the sterilized nodules were cut transversely and with the help of inoculation loop the inner residing bacteria were taken out and streaked in the plates. The streaking process was of continuous types. After each streaking, the inoculation loop was flamed sterilized. All streaked plates were incubated in inverted position at 28±1^{o}C for seven days inside the incubator on complete darkness.

3.2.7 Maintenance of pure culture

On the interval of seven days, bradyrhizobial colonies grown on master plates were then isolated in completely sterile condition. The translucent, milky, raised, round colony of *Bradyrhizobium* was picked up and streaked in a new YMA-CR plates. Each new YMA-CR plates were streaked with single bradyrhizobial colony with the help of flamed sterilized inoculation loop and plates were incubated upside down for seven days at 28±1^{o}C in incubator for proper bacterial growth.

As the pure culture of brayrhizobial isolates were obtained, single bacterial colony was transferred to YMA-BTB slants. The slants were incubated for seven days at 28±1^{o} C and finally pure *Bradyrhizobium* culture containing YMA-BTB slants were stored at 4^{o} C for short term storage.

3.2.8 Characterization of bradyrhizobial isolates.

The bacterial isolates obtained from the nodules of soybean (*Glycine max*) were characterized.

3.2.8.1 Colony characteristics and growth response on YMA-CR medium

Sterilized root nodule extract was cultured on YMA-CR medium and duration of bacterial colony growth, morphology of colony and nature of bacterial colony with Congo red were observed. Colony characteristics were studied according to Bergey's manual of systematic bacteriology.

3.2.8.2 Acid/Alkali producing characteristics

The bacterial isolate was cultured on YMB-BTB medium to observe the acid or alkali producing nature of isolates. Single bacterial colony from the pure culture of isolates was streaked on the plates containing YMB-BTB. These inoculated plates were incubated at 28±1^{o}C for their growth period.

3.2.8.3 Gram staining

The presumptive test of bradyrhizobial toward the Gram stain and their morphological characteristics were studied by Gram staining. Different Gram staining reagents were freshly prepared. The chemical constituents of Gram staining have been tabulated in Annex III.

3.2.9 Authentication of pure culture

For the conformation of the presence of the particular bacteria, the plant infection test must be carried out. Usually the plants with same inoculation group are employed for this purpose. All the methodology generally performed is presented below.

3.2.9.1 Sterilization of seeds, filter paper and growth pouches

Surface sterilization of legume seed depends on the purpose and nature of experiment. Seeds of soybean (*Glycine max*) were taken and 3% hydrogen peroxide was employed as steriliant. The sterilized seeds were then washed with six folds of sterilized distilled water ensuring no traces of sterilianat to be present. Similarly filter paper was sterilized on hot air oven and growth pouches were autoclaved at 121^{o}C for 15 minutes

3.2.9.2 Germination of seeds on Agar water medium.

Surface sterilized seeds are allowed to imbibe by dipping them on water at 5^{o}C. Well imbibed seeds were grown on petridish containing 0.75% (w/v) water agar. (Somasegaran and Hoben, 1994). Petridish were incubated at 28^{o}C for four days in an upside down position in order to get straight morphology of radicle.

3.2.9.3 Preparation of inoculum.

Bradyrhizobial isolates were maintained in the liquid medium i.e. Yeast Extract Mannitol Broth (YMB) for inoculation on their specific host. YMB was prepared according to Vincent, 1970 as mentioned elsewhere.

3.2.9.4 Preparation of modified Jensen's N-free medium.

Modified Jensen's N-free medium was prepared according to Roughley, 1984. The chemical constituents are tabulated in Annex II.

3.2.9.5 Infection test on growth pouches

The germinating seeds were placed into the folds of filter paper in the sterilized growth pouches containing 30-40 ml N-free nutrient solution.Small hole was made in the center of the paper. Only one seed was used per pouch with its radicle downward piercing into the hole. These pouches were arranged into wooden racks specially prepared for the purpose (Evans *et al*., 1972).

Each pouch contains the germinating seeds of soybean. Eight pouches were used. Among eight growth pouches, four pouches were inoculated with 1 ml of broth while the remaining four pouches were not used as control. In the interval of every five days 10ml of sterile distilled water was added to each pouch to compensate the excess desiccation. The work was conducted in green house.

3.2.10 Immunology

3.2.10.1 Preparation of Antigen

Whole cell of *Bradyrhizobium japonicum* was used as antigen. For antigen preparation, *Bradyrhizobium* was cultured in liquid medium i.e. YMB. Cells in Post logarithmic phase were used and broth culture was transferred to sterile centrifuge tube. It was centrifuged at 7000 rpm for 5 min. The supernatant was discarded and the pellet was suspended in saline (0.85%). This sequence of centrifugation and resuspension was repeated for three times. Finally the cell concentration was adjusted to approximately $1x10^9$ cells ml^{-1} by using Mc Farland standards (Annex V). Only somatic antigens were used so flagellar antigen were inactivated by heating the antigen in water bath at 100^oC for one hour (Somasegaran and Hoben, 1994).

3.2.10.2 Laboratory animal

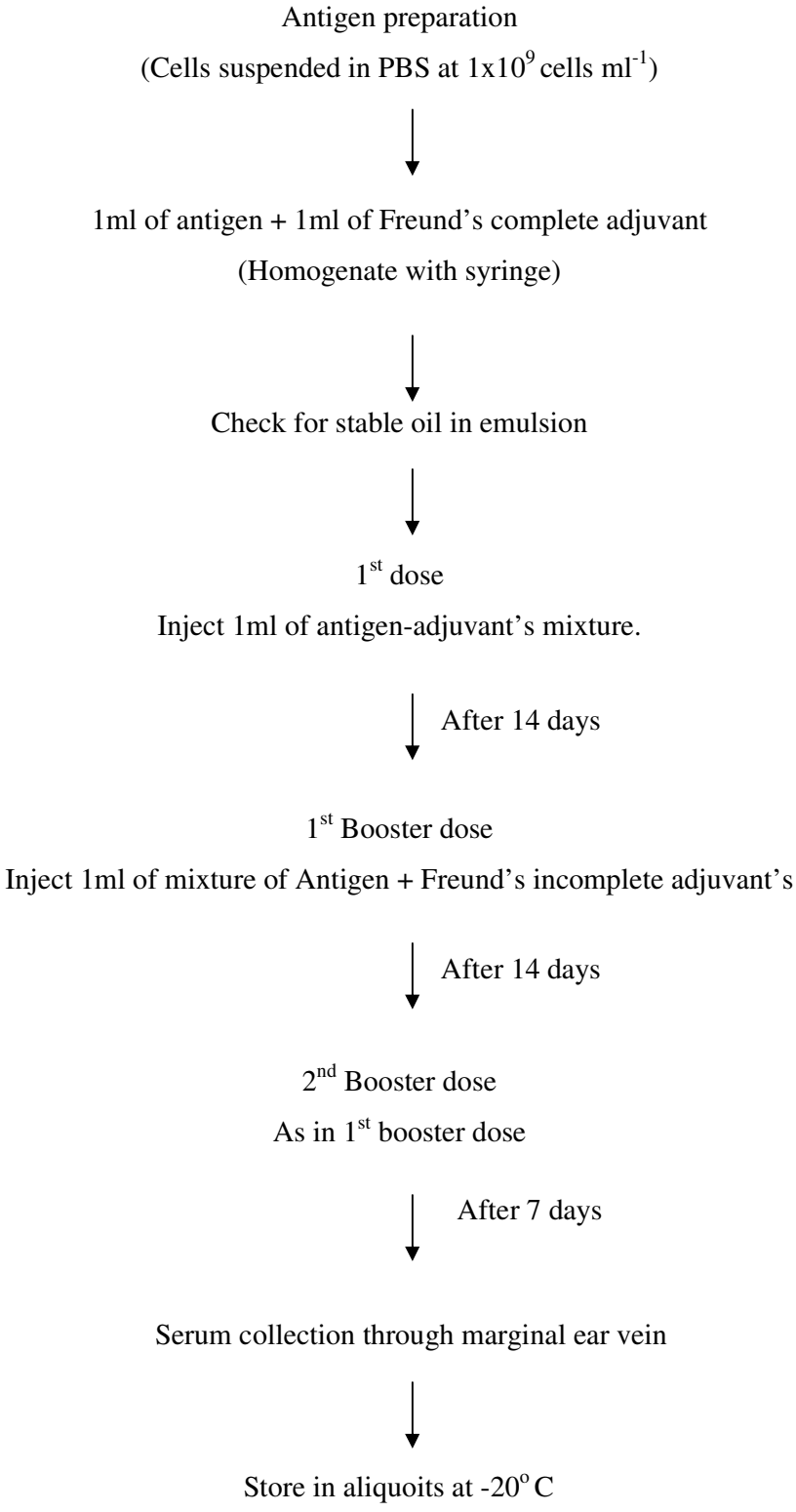
A pair of New Zealand white rabbits weighing 2 kg of 2-3 months was purchased from NARC. The rabbit were tested for parasites and found free from them.

3.2.10.3 Immunizing the rabbit

Various injection schedules have been used to produce antisera of sufficiently high titers. Before immunization 5ml of blood was taken out for control. Immunization schedule was followed according to Somasegaran and Hoben, 1994.

From the above prepared antigen two ml was pipetted out in 50 ml serum bottle. Two ml of Freund's complete adjuvants was also pipetted out and it was mixed with the antigen. The mixture was emulsified with the syringe by taking in and ejecting out several times. The antigen and adjuvants was completely mixed. 1ml of antigen adjuvant emulsion was injected into the thigh muscles of hind leg rabbit. The rabbit was marked with tag in the ear. Then it was kept with proper care. Two weeks after the first injection, i.e. in 14^{th} day, second dose of injection was given. Second dose consists of 1 ml of antigen with Freund's incomplete adjuvant. Third injection was given after four weeks of the first injection, i.e. in 28^{th} day. Third dose also contain the same constituent abs second dose. Seven days after the last injection, i.e. in 35^{th} day, animal was bleed through the marginal ear vein. About 20 ml of blood was taken. The blood was incubated at 56^{o}C for 30 minutes for clotting and refrigerated at 4^{o}C for overnight. Then the serum was decanted and centrifuged at 5000 rpm for 10 minutes. Centrifugation was done to make serum free from Red Blood Corpuscles (RBC). Aliquots of antisera (0.5–1.0 ml) were stored at -20^{o}C.

Fig. 2: Flow chart for immunization schedule (Somasegaran and Hoben, 1994).



3.2.11 Buffer preparation

All the buffers used in indirect ELISA were prepared according to Voller *et al.* (1980). Buffer and their chemicals constituent are tabulated in Annex IV

3.2.12 Optimization of ELISA

3.2.12.1 Choice of solid phase

The solid phase used was cobalt irradiated polystyrene microplates (Nunc Immunoplates I, Nunc, Denmark).

3.2.12.2 Optimal antigen coating dose.

The cells of *Bradyrhizobium japonicum* was suspended in coating buffer with the cell concentration of 1×10^9 cells ml^{-1} using Mc Farland Nephalometer Standard. This was used as antigen. For the control PBS only was coated. Positive control consists of PBS plus immunized sera plus secondary antibody. While negative control consists of antigen plus pre-immunized sera plus secondary antibody. The optimization of primary and secondary antibody was done by checkerboard titration (Voller *et. al.*, 1976).

3.2.12.3 Antigen coating

Antigen, cell concentration of 1×10^9 cells ml^{-1}, suspended in coating buffer was coated in microplate titer. It was covered wit parafilm and incubated at 4^oC overnight. Parafilm was removed and washed with washing buffer, PBS-T (Annex IV) for three times. Then the plates were dried by clean towel.

3.2.12.4 Checkerboard titration.

Titration was done according to Voller *et al.*, 1976. Primary antibody (immunized sera) and secondary antibody (antirabbit-goat-IgG) conjugated with HRP were optimized during checker board dilution. Different dilution of primary antibody ranges from 1:100, 1:1000, and 1:10000. The anti-rabbit goat antibody conjugated with HRP, secondary antibody, was used at the dilution of 1:1000, 1:2000, and 1:10000.

3.2.12.5 Blocking

To block nonspecific binding sites, 200µl of blocking buffer (Annex IV) was added to each wells and incubated for one hour at 30^oC. Then washing step was repeated.

3.2.12.6 Reacting the antigen with primary antibody

The antigens immobilized on the surface of wells were incubated with the different dilution of primary antibody. Primary antibody was diluted in PBS at 1:100, 1:1000, and 1:10000. About 100 µl of primary antibody of different concentration was added. Then it was covered with parafilm and was incubated at room temperature for two hour. After incubation parafim was removed and washing step was repeated.

3.2.12.7 Addition of secondary antibody

The secondary antibody used was goat ant-rabbit (GAR) conjugated with HRP. GAR-HRP was diluted at 1:1000, 1:2000 and 1:10000 in PBS. About 100 µl of different dilution of GAR-HRP was added. Then it was covered with parafilm and was incubated at 30^{o}C for two hour. After incubation washing step was repeated.

3.2.12.8 Substrate addition

Substrate solution was made by mixing solution A and solution B. Solution A consists of tetramethyl benzidine and solution B consists hydrogen peroxidase. Both the solution was mixed in equal volume and 100 µl of the solution was added on each well. Then it was incubated at room temperature for half an hour. The substrate solution was prepared and used on the same day.

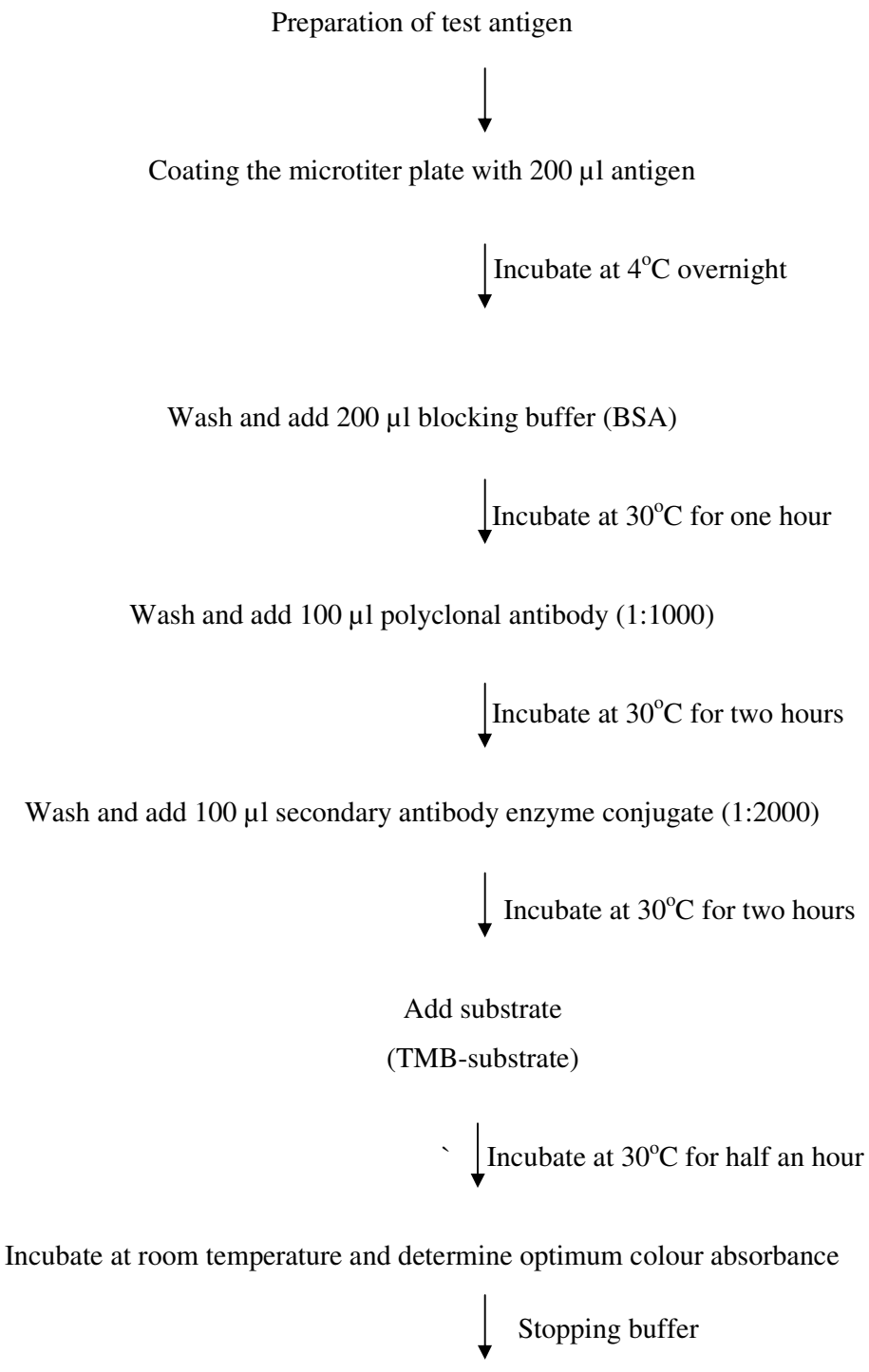
3.2.13 Preparation of Test Antigen

Fresh nodules from soybean (*Glycine max*) were used as test antigen. Nodules were collected from the soybean grown on the different soil samples of Kathmandu valley. From each samples nine nodules were collected. These nodules were washed and surface sterilized. They were placed in microtiter plates serially and their position was noted. Then 200µl of sterile saline was added to each nodule in the well. Microtiter plate was kept in water bath at 100^{o}C for 20 minutes to inactivate the flagellar antigen. Then saline from the well was removed by multiple pipettor and 200 µl of coating buffer was added to all nodules. Nodules were gently pressed with sterile toothpick to squeeze the bacteriod. Nodule residue was removed with the same toothpick. Different toothpick was picked for different nodule.

3.2.14 Immunocrossreactivity

100 µl of each nodule antigen was transferred into the wells of new microtiter plates. Positive control consisting of PBS only and Negative control consisting prebreeding serum was also added. Wells were sealed with transparent tape and were incubated at $4^{o}C$ overnight. Washing step and blocking of non specific binding sites was same as that in optimization of ELISA. After blocking the antigen, the plate was washed. Then polyclonal antibody of 1000 folds dilution was added in each well and incubated at $30^{o}C$ for two hours. After it plate was washed and secondary antibody of 2000 folds dilution was added and incubated at $30^{o}C$ for two hours and washing step was repeated. Horse radish peroxidase (HRP) was used as substrate. About 100 µl of HRP was added to each well. It was left for about 30 minutes to develop green colour. When the colour develops, 50 µl of 3N H_2SO_4 was added to each well to stop the reaction. Finally the colour intensity was measured by reading the absorbance at 450 nm at ELISA reader.

Fig. 3: Flow chart for Immunocrossreactivity of somatic antigen of *Bradyrhizobium japonicum*



CHAPTER IV

RESULTS

4.1 Isolation of native *Bradyrhizobium japonicum* isolates

The distinct, creamy white to translucent circular and convex, raised bradyrhizobial colonies of about 1.5 to 3 mm diameter were obtained after 7 days in YMA-CR plates inoculated with soybean root nodule extract. Pure culture of *Bradyrhizobium japonicum* isolates was obtained by subsequent restreaking and pure *B. japonicum* isolates were maintained in YMA-CR slants. The liquid bradyrhizobial inocula i.e. YMB obtained after eight days was highly turbid and milky white in colour. The pure culture, here after referred as native isolates were subjected to characterization, authentication and antigen preparation.

4.2 Characterization of *Bradyrhizobium japonicum* isolates

Bradyrhizobial isolates were characterized by studying its presumptive morphological and physiological characteristics.

4.2.1 Colony characteristics and growth response on YMA-CR

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

4.2.2 Growth response on YMA-BTB medium

The blue colour of Bromothymol blue (BTB) was retained after the significant growth of the bradyrhizobial colonies on YMA-BTB plates. Thus the bradyrhizobial isolates was found to be alkali producing in the culture.

4.2.3 Acid / Alkali producing in liquid medium

For estimating whether the bradyrhizobial isolates to be acid or alkali producing, the broth culture technique was employed. The change in the pH of the broth was observed throughout the experiment which is illustrated below.

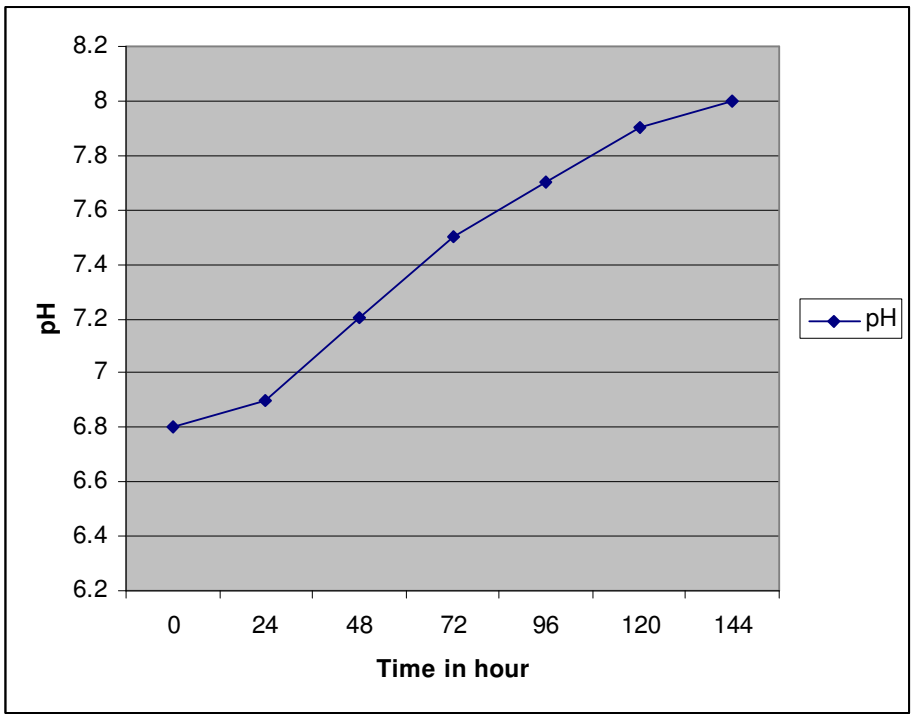


Fig. 4: Change in pH of broth of bradyrhizobia in different time interval

4.2.4 Gram staining

Bradyrhizobial isolate was found to be gram negative rod.

4.3 Authentication of Bradyrhizobial isolates

Plant infection test was carried out in growth pouch for authentication of isolates. The isolates obtained from soybean nodules were authenticated to be the *Bradyrhizobium japonicum* strain by examining their capability to nodulate the soybean seedling under bacteriological controlled condition. The isolates nodulated the soybean roots grown on growth pouches where as soybean seedling without inoculation did not have any nodule on their roots after 21 days.

4.4 Optimization of ELISA

Optimization of ELISA was carried out at different concentration of primary antibody (antisera) and secondary antibody (conjugates). Using 200µl of antigen, having cell concentration of $1x10^9$cell ml^{-1}, the optimal dilution of the primary and secondary antibodies was found to be 1000 and 2000 folds respectively. The cut off value for colour observation was determined at OD_{450} and found to be 0.76.

Table 2: Optimization of polyclonal antisera and secondary antibody enzyme conjugate against somatic antigen.

S.N.	Coating samples	Polyclonal antisera	Secondary antibody	O.D [b] 450 nm
1.	Positive control Blank (PBS) only	1:100	1:1000	0.00
2.	Negative control Preimmunized serum	1:100	1:1000	0.05
3.	Antigen [a] Somatic antigen of *Bradyrhizobium japonicum* (TAN 01)	1:100	1:1000	1.15
			1:2000	0.93
			1:10000	0.07
		1:1000	1:1000	0.82
			1:2000	**0.76**
			1:10000	0.05
		1:10000	1:1000	0.09
			1:2000	0.10
			1:10000	0.01

a = the microtiter plates were coated with 200 µl of antigen, having cell concentration of $1x10^9$ cell ml^{-1}

b = average value of three set of corresponding assay

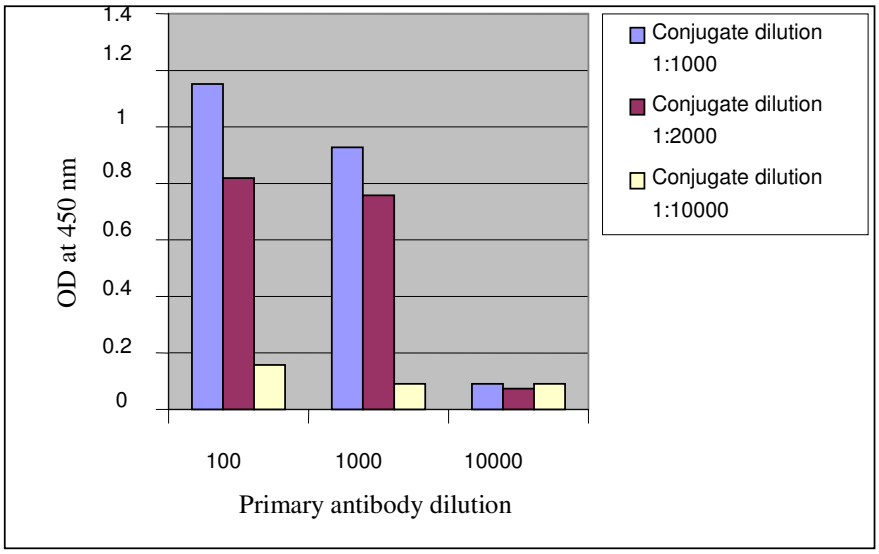


Fig. 5: Optimization of primary antibody and conjugate against somatic antigen of Bradyrhizobium japonicum

4.5 Immunocross-reactivity

For the study of cross-reaction, primary antibody produced against B_{101} was put against nodule antigen of soybean coated in microtiter plate. During subsequent immunoassay cross-reactivity was confirmed by measuring optical density against them. The highest crossreactivity was shown by nodules grown on the soil of Mulapani where as lowest crossreactivity was shown by Dhobighat. The result is shown in table.

S.N.	Localities of soil samples	Nodules	Mean O.D. at 450nm	Crossreactivity (%)
1.	Blank (PBS)	-	0.0	0.0
2.	Positive control	B101	0.73	100
			0.72	
			0.72	
3.	Negative control	B101	0.04	5.47
4.	Champasingh	Nodule 1	0.45	62.5
		Nodule 2	0.23	31.94
		Nodule 3	0.16	22.22
5.	Mulpani	Nodule 1	0.61	84.72
		Nodule 2	0.70	**97.22**
		Nodule 3	0.48	63.3
6.	Dhapakhel	Nodule 1	0.43	59.72
		Nodule 2	0.27	37.5
		Nodule 3	0.18	25.0
7.	Panga	Nodule 1	0.18	25.0
		Nodule 2	0.25	34.72
		Nodule 3	0.17	26.71
8.	Dhobighat	Nodule 1	0.21	29.17
		Nodule 2	0.14	**19.44**
		Nodule 3	0.26	36.11

CHAPTER V

DISCUSSION

In the present study *Bradyrhizobium japonicum* B_{101} strains was isolated from soybean (*Glycine max* (L.) Merr.) root nodules from the plants grown on Tangla soil. This isolate was used as antigen for the production of antibody. Soil samples from different localities of Katmandu valley were collected. Soybean was grown on these soils and nodule was collected. Nodules of different soil may contain different *B. japonicun* strains. These strains may share some common antigenic determinant which was studied by cross-reactivity of these strains with the antibody raised against $B_{101.}$

Effective soybean root nodules were collected from Tangla soil and bradyrhizobia was cultured and isolated in YMA-CR media (Vincent, 1970; Dalton, 1980). *B. japonicum* isolates was purified by continuous streaking of single bradyrhizobial colonies and such streaking was performed four times (Ogg and Sharma, 1977; Kaushik *et al*., 2003). Bradyrhizobial colonies of 1.5-2mm diameter were obtained after seven days culture in YMA-CR plates. The longer duration of colony development showed the slow growing nature of bradyrhizobia. Dalton (1980) found that *Rhizobium japonicum*, the soybean root nodule rhizobia is slow growing and produce moderate growth after 10 days. Schwinghamer and Dudman (1980) suggested that the colony type in culture provides the much convenient direct means of recognition. The creamy white appearance of bradyrhizobial colonies in YMA medium with CR is due to the fact that YMA plates had been incubated in dark and this is in accordance with Somasegaran and Hoben (1994) that bradyrhizobia do not absorb CR when incubated in dark but the contaminant organism usually absorb red dye. *B. japonicum* can absorb red dye if plates are exposed to light during incubation.

Characterization of bradyrhizobial isolates was performed. Along with the study of morphological characters, other characters such as acid or alkali producing, Gram staining, multiplication rate and ability to nodulate soybean were also observed. Authentication of the bradyrhizobial isolates toward the Gram stain showed the Gram negative response, a characteristics feature of bradyrhizobia (Pleczar *et al*., 1993).

The blue colour of BTB retain even after eight days of bradyrhizobial culture on YMB-BTB plate indicating the alkali producing nature of *B. japonicum*. According to Somasegaran and Hoben (1994) a blue colour, indicating alkaline reaction on BTB is obtained only in slow growing *Bradyrhizobium* spp. Yellow colour is usually produced by fast growing *Rhizobium* spp. Acid/alkali producing behaviour of bradyrhizobia was also analyzed through broth culture. The pH of broth increased from 6.8 to 7.4 and 7.9 in 72 hrs. and 120 hrs. respectively. This result also suggests the further conformity for alkali producing behavior of rhizobia.

The bradyrhizobial isolates B_{101} was authenticated to be the endosymbiont of soybean root nodule and confirmed as *Bradyrhizobium japonicum*. This was performed by infection test in sterile plastic pouches and filter paper using modified N free medium (Somasegaran and Hoben, 1994). According to Brockwell (1980) an isolate cannot be regarded as a 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 character i.e. nodulation, nitrogen fixation and level of effectiveness can be employed for strain identification. The infection test revealed that the seedling grown on inoculated pouches possess distinct nodule on their roots where as nodule was absent on the roots of seedling grown in control. i.e. uninoculated pouches. All the roots healthy and internally pinkish colour when sliced, which was due leghaemoglobin produced within the nodule. Large, brown nodule with pinkish internally denotes effective nodule which can fix the atmospheric nitrogen (Bergerson, 1974). The plant in growth pouch was supplied with modified Jensen's N-free nutrient medium (Roughley, 1984). The nitrogen deficient condition stimulates the nod gene expression which led into nodulation of root of host plant. Quespel (1974) suggested that the presence of sufficient combined nitrogen interfere the nod gene expression and ultimately nodulation and nitrogen fixation.

The objective of present study was also to optimize the ELISA. The success of ELISA depends upon the various factors. The solid phase, concentration and volume of antigens, optimal dilution of specific antibody (immunized sera) and secondary antibody enzyme conjugate, incubation time, substrate etc. regarding the solid phase disposable polystyrene microtiter plates were used for convenient (Voller *et al.*, 1974). Although various carrier materials can be used, it is essential that each new

type is thoroughly tested to find the amount and reproducibility of uptake of the antigen or the antibody since the variables influences the result of a test (Bidwell *et al*., 1977).

B_{101} strain was used for the antibody production. Culture of post logarithmic phase was harvested and suspended in PBS. The concentration of antigen is important to provoke the antibody formation. Cell concentration of $1x10^9$ cells ml^{-1} was used as antigen for antibody production. Antigen emulsified with adjuvant does enhance the level of antibody. Since somatic antigens are more specific than flagellar antigens, it was steamed to inactivate the flagellar one. Antigen ($1x10^9$ cells ml^{-1}) was mixed with Freund's complete adjuvant to produce the higher quality antiserum having higher titer. Furhmann and WolliumII (1985) revealed that antigen concentration had essentially no effect on the final absorbance value within the visible turbidity. So for optimization single concentration of antigen was used. Incubation time of more then one hour after coating the antigen generally doesn't affect final absorbance. Optimal dilution of specific antibody gives better result.

The appropriate concentration may differ according to the nature of antigen and methodology used. The optimal dilution was determined by using chequerboard titration (Trottier *et al*., 1992; Voller *et al*., 1980). Primary antibody was diluted in PBS. A series of dilution of antibody viz., 1:100, 1:1000, 1:10000 were titrated as first antibody against a series of dilution of conjugate (anti-rabbit IgG conjugated with HRP) viz., 1:1000, 1:2000, 1:10000. The concentrations of antibodies were indirectly assayed by respective degree of colour development after adding TMB substrate in the wells. ELISA reader was used to read absorbance characteristics at 450 nm (Schneider *et al*., 2004). Simultaneously the visible colour change as average cut-off value was observed to be $OD_{450\ 0.76}$. Thus, the primary and secondary antibody concentration was defined 1:1000 and 1:2000 respectively as optimal. Below $OD_{450\ 0.76}$ there was not clearly visible change in colour to naked eye. Trottier *et al*., 1992 choose the optimal dilution which demonstrated a maximum positive test with negative control using minimal concentration of polyclonal antibody and conjugate. The optical density value obtained from the test samples should be remarkably higher than negative control serum (Voller et al., 1976). OD of negative control found to be 0.05 where as for positive test it was between 1.15-0.09. The lowest positive test was also found to be higher than that of negative control.

After optimization of ELISA, it was used for the antigenic determination of nodule on the soil samples of different localities. Antibody was raised against the standard strain, $B_{101.}$ So the nodule of Tangla was taken as positive test. The OD of Tangla nodule i.e.0.72 was taken as 100 percent cross-reactive. The OD of different nodules was compared with that of standard strain and their respective cross-reactive percentage was calculated. All the nodules tested were found to be positive. All the nodules have the OD value higher than that of negative control (preimmunized sera). This implies all the nodules possess at least one common antigen with standard strains. The result shows a great variation in cross-reactivity. Among nodules grown on other five localities, Mulpani shows highest cross-reactivity i.e. 97.22 % and least cross-reactivity, 19.44 % was observed in Dhobighat. There was also a difference in the cross-reactivity of different nodules of the same plant. In Champasingh, the highest and lowest cross-reactivity was found to be 62.50% and 22.22% respectively, having a difference of nearly 40%. Similarly the difference between highest and lowest cross-reactivity was found to be: Mulpani 40 %, Dhapakhel 35%, Dhobighat 17 % and Panga 9%.

The cross-reactivity of different nodule antigen to the antibody was in order of Mulpani, Chamoasingh, Dhapakhel, Dhobighat and Panga. Mulpani nodule shows highest cross-reactivity because it shared maximum antigenic determinants with standard strain. Nodule having a similar antigen cross reacts with antibody and show high cross-reactivity (Noel and Brill, 1980). Next to Mulpani, the highest cross-reactivity was shown by Champasingh with 62.5%. But it also shows the great variation in cross-reactivity of different nodule ranging from 62.5-22.2 %. The greater variation in Champasingh, 40% reveals that same plant was infected by different strains of *Bradyrhizobium japonicum*. These strains possess diverse antigenic determinants. Some nodule of Champasingh share maximum antigen with standard while some other nodules share least antigenic determinants.

The least variation in cross-reactivity was found in the nodule grown on Panga soil. The highest cross-reactivity was 34.72 % and lowest was 25 % with a difference of 9.72 %. Although it differs in the antigenic determinants with standard showing less cross-reactivity but the locality contain similar type of strain. Nodules of Panga were infected by same strain. Similar result was found in Dhobighat. Nodules of Dhobighat

posses the least cross-reactivity i.e.19.44% with OD_{450} of 0.14. This value is slightly higher than negative control (0.06). This implies that some nodules of Dhobighat don't show or show very little cross-reactivity with standard whiles other nodule of same locality show the cross-reactivity upto 36 %. It also denotes that nodules of same plant were infected by different strains.

Soybean nodules are generally infected by *Bradyrhizobium japonicum* which are slow growing. But there has been report that it is also infected by fast growing rhizobia. Fast growing rhizobia have been isolated from soybean root nodules collected in China. These new isolates are physiologicaliy distinct from slow growing soybean rhizobia (Keyser *et al*., 1981). So soybean nodule can get infected either by fast growing rhizobia or slow growing Rhizobia. There is a specific interaction between soybean genotypes and *Rhizobium japonicum* serogroups in nodule formation. Plant genotype affects the acceptance of *Rhizobium japonicum* strains of specific serogroups. Closely related genotypes had similar distribution of *Rhizobium japonicum* in the nodules (Cladwell and Vest, 1968).

CHAPTER SIX

CONCLUSION AND RECOMENDATIONS

6.1 Conclusion

Ecological study of *Bradyrhizobium japonicum* requires the identification of specific *B. japonicum* strains in culture as well as nodule. ELISA has found application in Rhizobium research. The procedure is simple, flexible and efficient with respect to time, reagent and equipment yet retains a high level of sensitivity. In present study, bradyrhizobial isolates B_{101} was characterized as slow growing, alkali producing, and gram negative bacteria. For the conformity of the specific bradyrhizobial infecting their host legumes, their authentication was performed.

The primary antibody and conjugate were optimized and optimal dilutions were found to be 1000 and 2000 folds respectively. *Bradyrhizobium japonicum* of the same agro climatic region also varies significantly. There was a great difference in the cross reactivity of strains to that of standard. Nodules grown on soil of Mulpani have similar antigenic determinants showing 97% cross-reactivity while Dhobigahat shows the least cross-reactivity with 19%. Strain diversity was observed not only in the different localities of same agro climatic region but also within the same locality. Champasingh shows wide range cross-reactivity from 62.5-22.2% with a difference of 40%. Least variation was observed in Panga with a difference of 9%.

In a nutshell, soybean grown within the same agro climatic region may contain different strain of *Bradyrhizobium japonicum* and even the nodules of same plant may be infected by different strains.

6.2 **Recommendation**

1. Screening of the most effective strains of *Bradyrhizobium japonicum* should be carried out from the every parts of the country where soybean is grown.

3. Inoculum of the most effective strain of *Bradyrhizobium japonicum* should be prepared and it should be well accessed to the local farmers.

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Annex I

Equipments

Autoclave	Life, Portable steam stericlave-800
Centrifuge	Fisher Scientific Microcentrifuge , 235C
Electric balance	Mettler Instrumental Corp.
ELISA plate	
ELISA reader	
Horizontal shaker	
Hot air oven	Memmert
Incubator	Nakamira Scientific Co. Ltd.
Laminar air flow cabinet	The Baker Company EG-3252
Multiple pipette	
Vertexer	Lab. line Instrumental, Inc.

Chemicals

Agar agar
Alcohol
Ammonium oxalate
Barium sulphate
Boric acid
Bovine serum albumin
Bromothymol blue
Calcium hydrogen phosphate
Congo red
Copper sulphate
Crystal violet
Dipotassium hydrogen phosphate
Ferric chloride
Hydrochloric acid
Hydrogen peroxide
Iodine
Magnesium sulphate
Manganese sulphate
Mannitol
Potassium chloride
Potassium orthophosphate
Potasssium iodide
Safranin
Silica gel
Sodium chloride
Sodium hydroxide
Sodium molybdate
TMB
Tween 20
Yeast extract
Zinc sulphate

Annex II

1 Composition of different culture media

- Yeast extract Mannitol Broth (YMB). (Vincent, 19700

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
Distilled water	1.0 liter

- Yeast extract Mannitol Agar (YMA)

YMB	1.0 liter
Agar	15.0g

- Congo-Red (CR) stock solution

Congo-Red	0.25g
Distilled water	1.0 liter

- Bromothymol Blue (BTB)

Bromothymol blue	0.5 ml
Ethanol	100 ml

2. Modified Jensen's N-free medium

Calcium Hydrogen Phosphate ($CaHPO_4$)	1.0g
Dipotassium Hydrogen Phosphate (K_2HPO_4)	0.2g
Magnesium Sulphate ($MgSO_47H_2O$).	0.2g
Ferric Chloride ($FeCl_3$)	0.1g
Trace element stock solution	1 ml
Distilled water	1 liter

Composition of trace element stock solution

Boric acid (H_3BO_3)	2.86g
Manganese Sulpahte ($MnSO_4.4H_2O$)	2.03g
Zinc Sulphate ($ZnSO_4.7H_2O$)	0.22g
Copper Sulphate ($CuSO_4.5H_2O$)	0.08g
Sodium Molybdate ($Na_2MoO_4.2H_2O$)	0.14g
Distilled water	1.0 liter

Annex III

Composition of Gram staining reagent (Vincent, 1970).

1. Crystal violet solution

Crystal violet	1.0g
Ammnonium Oxalate	0.4g
Ethanol	10 ml
Distilled water	50 ml

2. Iodine solution

Iodine	0.5g
Potassium iodide	1.0g
Ethanol	12.5 ml
Distilled water	50 ml

3. Alcohol

Ethanol	95 ml
Distilled water	5 ml

4 Counter strain

2.5% safranin in ethanol	10 ml
Distilled water	90 ml

Annex IV

Buffers solution of ELISA

1. Saline

Sodium Chloride (NaCl)	8.5g
Distilled water	1 liter

2. Coating Buffer (Phosphate Buffer Saline, PBS) pH 7.2

Sodium Chloride	8.5g
Potassium orthophosphate (K2HPO4)	0.15g
Potassium Chloride (KCl)	0.2g
Distilled water	1 liter

3. Washing Buffer

PBS	1liter
Tween 20	0.5 ml

4. Blocking Buffer

PBS	liter
Bovine Serum Albumin (BSA)	10ml.

5. Stopping buffer

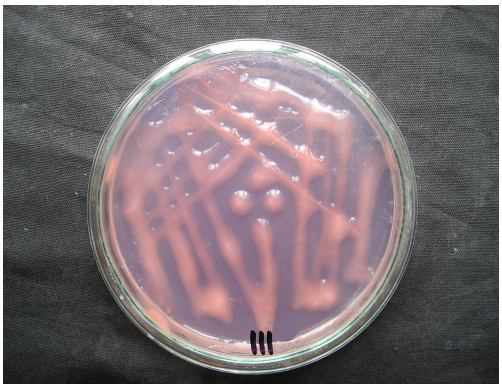
Conc Sulphuric acid (H_2SO_4)	3N

Annex V

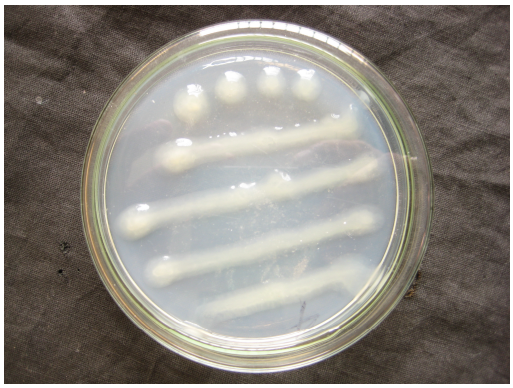
Mc Farland Barium Sulphate Standard.

Tube	Barium chloride 1% solution (ml)	Sulphuric Acid 1% solution (ml)	Approximate density of bacteria ($Cellsml^{-1}$)
1	0.1	9.9	3×10^8
2	0.2	9.8	6×10^8
3	0.3	9.7	9×10^8
4	0.4	9.6	1.2×10^9
5	0.5	9.5	1.5×10^9
6	0.6	9.4	1.8×10^9
7	0.7	9.3	2.1×10^{10}

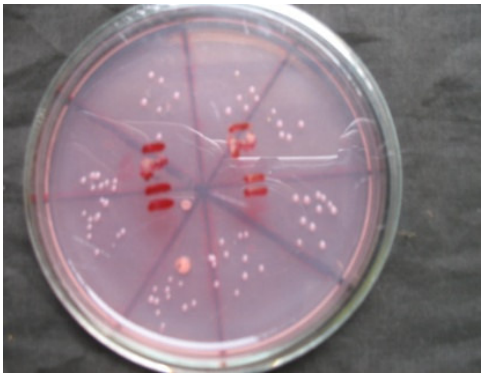
PHOTO PLATE I



Pure culture in YMA-CR.



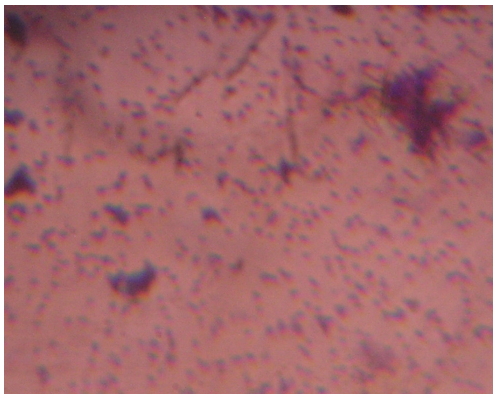
Pure culture in YMA.



Counting of Bradyrhizobial colonies.

Broth culture

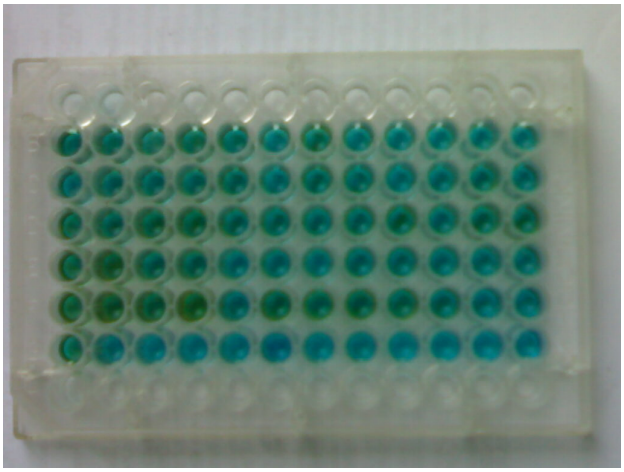
Slants culture



Gram staining

Photo Plate II

Authentication of *Bradyrhizobium japonicum* on growth pouch.



Immunocrossreactivity of different strain with antibody raised against standard B_{101}