CHAPTER –I

1. INTRODUCTION

Nitrogen is the most important nutrient input required for crop production. It constitutes nearly 80% of the atmosphere, yet it cannot be utilized by plants. The plant utilizable form of nitrogen is its most oxidized form, the nitrates, or the most reduced state, the ammonia (Gupta and Sekhon, 1994). Availability of inert nitrogen gas in organic molecules is possible through Biological Nitrogen Fixation (BNF) in which atmospheric nitrogen is converted by the enzyme nitrogenase.

BNF by Legume *Rhizobium* Symbiosis is major source of nitrogen in agriculture for increasing the productivity of legumes and improving soil fertility (Gurung and Serchan, 1997). Nitrogen supplied to crops by BNF reduces our dependence on nitrogenous fertilizers and also builds up soil fertility for succeeding crop. The major nitrogen fixing systems are the symbiotic systems, which can play a significant role in improving the fertility and productivity of low nitrogen soils. The Legume *Rhizobium* Symbiosis is considered the most promising plant-bacteria association for immediate increase in protein yield through BNF (Yami and Khanal, 1988). Nitrogen fixation in root nodules is a symbiotic process between legumes and *Rhizobium* bacteria. Therefore, BNF can make a significant contribution in increasing agricultural productivity and has become important for sustainable agriculture.

Phosphorous is the second major plant nutrient required by the plant in enough quantities for better productivity. In rhizosphere soil, inorganic phosphorous is normally in insoluble form and thus not readily available to plants. Plants acquire this mineral from the environment either directly by their roots or indirectly from mycorrhizal fungi (Singh et al., 2002). For this reason, the mycorrhizal symbiosis is important for plant phosphorous supply since the fungal hyphae extend into the soil and allow roots to explore a larger soil volume (Smith and Read, 1997). The increased phosphorus uptake conferred by the AM symbiosis is beneficial for the functioning of the nitrogenase enzyme of the bacterial symbionts, leading to increased nitrogen fixation and consequently promotion of root and mycorrhizal development (Amora-Lazcano et al., 1998; Johanson et al., 2004; Rabie et al., 2005).

Legumes form tripartite symbioses with arbuscular mycorrhizal fungi (AMF) and rhizobia which influence plant productivity. In Rhizobia-AM-legume tripartite symbiosis relationships, nodulation of Rhizobia and establishment of AM often occur simultaneously and synergically. Besides, Rhizobia provide fixed nitrogen not only to the plant, but also to the fungus. Moreover Rhizobia can also assist in mobilizing nutrients from the soil and improving the growth of infected plants. Reports stated that the presence of AM fungi is known to enhance nodulation and N-fixation by legumes (Amora-Lazecano et al., 1998; Johansson et al., 2004).

In recent years, to meet the demand of nitrogenous fertilizers, the number of chemical industries has been increased. Although, the use of chemical fertilizers is good to increase the crop productivity, however, the progressive rises in their cost and their low efficiency have comprised expensive charges for agricultural product in the developing countries like Nepal. Therefore, it is essential for Nepal to develop alternatives like vermicomposting which can be easily produced in farmer's house by the use of their organic waste.

Vermicomposting is a mixture of worm manure, microbially decomposed matter and some partially decomposed organic matter. Since vermicompost contains a water soluble colloidal wormcast, the NPK content of the vermicompost is higher than of other composts, the higher amount of NPK will help to improve the crop yield by increasing soil fertility and water retention ability (Acharya, 1997). It contains numerous component required for microbial growth and may increase the activity of soil microorganism including rhizobial growth.

Hence, this dissertation work is aimed to study the effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *Piriformospora indica* and vermicompost on different parameters of the growth of bean plant. Rhizobia and mycorrhizae provide nitrogen and phosphorous sources respectively to plants and they also act as dual symbionts in leguminous crops reducing the demand for inorganic fertilizers. Enhancement of legume nitrogen fixation by co-inoculation of rhizobia with AM fungi with the application of fertilizers such as vermicompost is a way to improve nitrogen availability in sustainable agriculture production system.

CHAPTER-II

2. OBJECTIVES

2.1 General Objective

To study the effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *Piriformospora indica* Verma et al. in the growth of bean plant (*Phaseolus vulgaris* L.) grown in a soil treated with vermicompost.

2.2 Specific Objectives

1. To isolate, identify and authenticate *Rhizobium leguminosarum* biovar *phaseoli* from the nodules of bean plant.
2. To study the effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *Piriformospora indica* and vermicompost on different parameters of the growth of bean plant.
3. To study the effect of single inoculation of *Rhizobium leguminosarum* biovar *phaseoli* on different parameters of the growth of bean plant.
4. To study the effect of single inoculation of *Piriformospora indica* on different parameters of the growth of bean plant.
5. To determine the NPK content of bean plant.
6. To determine the percent root colonization by *Piriformospora indica*.

CHAPTER-III

3. LITERATURE REVIEW

3.1 Nitrogen

Nitrogen is the key building block of the protein molecule upon which all life is based; it is thus an indispensable component of the protoplasm of plants, animals, and microorganisms (Martin, 1983). Therefore, without a constant supply of this unavoidable element, life cannot go on. All living things require nitrogen to make proteins needed for life. Although nitrogen makes up about 80% of the air we breathe, most living things cannot use atmospheric nitrogen and require that it be combined or "fixed" with other elements like oxygen and hydrogen before it can be assimilated.

The soil nutrient that plants require in greatest quantity is nitrogen. Despite its abundance in the atmosphere, plants are not able to assimilate the molecular form. The plant utilizable form of nitrogen is its most oxidized form, the nitrates, or the most reduced state, the ammonia (Gupta and Sekhon, 1994). Availability of inert nitrogen gas in organic molecules is possible through BNF.

3.2 Nitrogen fixation

The utilization of nitrogen gas as a source of nitrogen is called nitrogen fixation and is a property of only certain prokaryotes. Nitrogen fixation, the reduction of nitrogen to ammonia, involves a complex enzyme system called nitrogenase, which consists of dinitrogenase and dinitrogenase reductase, metal-containing enzymes found only in certain prokaryotic organisms (Madigan et al., 2000).

Nitrogen fixation occurs both biologically and non-biologically. Non-biological nitrogen fixation or the chemical fixation occurs through the effects of lightening and industry primarily by the Haber-Bosch process while biological nitrogen fixation is carried out by certain prokaryotic microorganisms, known as diazotrophs or nitrogen fixers (Dubey, 1998). Biological Nitrogen Fixation, the microbial conversion of atmospheric nitrogen to plant usable form, helps to

replenish the soil nitrogen pool. It represents the major source of N input in agriculture soil. The biological process of nitrogen fixation is an enormous potential source of nitrogen into natural ecosystem (Gurung and Serchan, 1997).

The total biological nitrogen fixation is estimated to be twice as much as the total nitrogen fixation by non-biological processes. Currently biological nitrogen fixation accounts for perhaps two-thirds of crop nitrogen need worldwide. About 85% of nitrogen fixation on earth is of biological origin and about 60% of biological nitrogen fixation occurs on land and the other 40% in the oceans (Madigan et al., 2000).

3.3 Nitrogen fixation on global scale

The contribution of nitrogen fixation to the global nitrogen cycle probably has not changed for centuries, having been in approximate balance with the de-nitrification process that converts combined nitrogen back to atmospheric nitrogen. Fixation did not occur to excess because BNF is inhibited by the presence of mineral nitrogen. During the past 40 years, the global nitrogen cycle has been affected by the increase in industrial fixation of nitrogen, but the environmental impacts are yet to be measured and assessed. It would be prudent to minimize the further perturbation of the global need as fertilizer for crop production to equal the effects of one ton of nitrogen biologically fixed in a legume crop. Thus, fixed nitrogen from BNF will affect the global nitrogen cycle substantially less than will industrially fixed nitrogen.

It is estimated that out of total annual nitrogen fixation, BNF accounts for 65%, 25% is fulfilled by industrial nitrogen fixation and remaining 10% is compensated by physical means as UV-radiations, volcanic eruption, thunder lightning, electric sparkling (Newton, 2000). The global nitrogen fixation through biological means is around 17.2×10^7 tons annually and 80% of it comes from *Rhizobium*-Legume symbiosis alone (Dadarwal, 1997).

3.4 Biological nitrogen fixation (BNF)

The phenomenon of fixation of atmospheric nitrogen by biological means is known as Biological Nitrogen Fixation or Diazotrophy. BNF is carried out by prokaryotes known as nitrogen fixers or Diazotrophs. Such nitrogen fixers are either free living in soil or exist symbiotically with plants or

associated with rhizosphere of the plant root system, thus contributing directly or indirectly towards the nutrition of plants. Biological nitrogen fixation can be symbiotic or non-symbiotic.

- Non-symbiotic nitrogen fixation- Those microorganisms which utilize gaseous nitrogen directly and independently in the soils are known as non-symbiotic nitrogen fixing organisms and the process by which they fix nitrogen is known as non- symbiotic nitrogen fixation.

- Symbiotic nitrogen fixation- Those microorganisms which utilizes nitrogen indirectly through the mediation of other living organisms in the soil are known as symbiotic nitrogen fixing organisms and the process by which they fix nitrogen is known as symbiotic nitrogen fixation.

3.4.1 Symbiotic nitrogen fixation

Symbiosis is an obligatory relationship between two populations that benefits both populations. A symbiotic relationship is highly specific as one member of the association ordinarily cannot be replaced by another related species. Symbiosis is also known as mutualism. Symbiosis is defined as any stable condition in which two different organisms live together in close physical association for their mutual advantage (Azam, 2001).

Among various nitrogen-fixing organisms, *Rhizobium* is considered most potential bacterium because of its ability to fix atmospheric nitrogen directly inside the root nodules of leguminous plants (Yami and Khanal, 1999). Rhizobium utilizes the free atmospheric nitrogen and synthesizes it into new nitrogenous compounds, which are utilized by plants for their growth; and bacteria get their food from these plants; such a mutual beneficial association of bacteria and plants is referred to as symbiosis.

3.4.2 Rhizobium-legume symbiosis

The most important contribution to BNF comes from the symbiotic association of certain micro-organisms with the roots of higher plants. A classic example is that between leguminous plant and nitrogen fixing bacteria of the genera *Rhizobium, Bradyrhizobium* and *Azorhizobium*. The nitrogen-fixing associations of rhizobia with leguminous plants are of great importance both in

global nitrogen cycling and in agriculture (Evans et al., 1991; Postate, 1992; Somasegaran and Hoben, 1994). BNF by *Rhizobium*-Legume symbiosis is major source of nitrogen in agriculture for increasing the productivity of legumes and improving soil fertility (Gurung and Serchan, 1997). The *Rhizobium*-Legume symbiosis is considered the most promising plant-bacteria association for immediate increase in protein yield through BNF (Yami and Khanal, 1988).

There are about 750 genera and 18,000-19,000 species of leguminous plants of which about 1,300 or more leguminous plant species including cultivated and wild herbs, shrubs and trees are interestingly found to have *Rhizobium* as the micro-symbiont (Subba Rao, 1999). It involves the invasion of the roots of leguminous plants with the appropriate species of *Rhizobium* or *Bradyrhizobium*, resulting in formation of a tumor like growth called **nodule**. Within the nodule, rhizobia are able to convert atmospheric nitrogen to ammonia, which supplies the nitrogen required bacterial and plant growth (Campbell, 1985; Dalton and Mortenson, 1972; Dazzo, 1982; Dillworth and Glenn, 1991; Evans et. al., 1991; Lynch, 1982; Nutman et. al., 1978; Postgate, 1992; Smith, 1982; Schmidt, 1978). Rhizobia also benefit as the plants provide carbohydrates which are required as a source of energy. Also, the carbohydrates produced by the legume plant are transported to the nodules where the rhizobia can use as a source of hydrogen in the conversion of nitrogen to ammonia fixation.

Nitrogen fixation in legumes depends on the formation of nodules by *Rhizobium*. The effectiveness of *Rhizobium*-Legume symbiosis depends upon the specificity of *Rhizobium* strain and responses of host plant. Effective symbiosis requires a more efficient strain of *Rhizobium* for a particular legume. Soil may lack the number of the specific rhizobia that is required for nitrogen fixation. Therefore, artificially multiplied culture of selected microorganisms play a vital role in recycling various important plant nutrients in soil. Thus, *Rhizobium*-Legume symbiosis is of special significance to legume husbandry as seed inoculation with effective strains of *Rhizobium* can meet the nitrogen requirement of the legume to achieve increased yields (Somasegaran and Hoben, 1994).

3.4.3 Physiology of nodule formation

The establishment of *Rhizobium*-Legume symbiosis is a complex process involving interplay of physiological and biochemical processes of both bacterium and host plant. The symbiosis occurs in a host specific way involving several steps of signal exchange between plant and bacteria which lead to the formation of nodules (Nainawatee et al., 1997). The process of nodule formation is the result of a complex sequence of interactions between rhizobia and plant roots (Brewin, 1991;Solheim, 1984).

In the rhizosphere of legumes, rhizobia move towards legume roots due to the presence of chemo-attractants in root exudates. The nature of the substances present in root exudates showing positive chemotaxis depends upon the legume host. Various rhizobia are attracted by amino acids, dicarboxylic acids, flavonoids and betaines. After the chemotaxis, rhizobia attach to the plant root surface. The most favored sites of attachment are the young growing root hairs but attachment can take place on root hairs at other sites also. Initially, rhizobial attachment takes place loosely as single cell and later several cells firmly attach to form a cap structure. The attachment may involve several macromolecules including rhizobial extracellular polysaccharides, lectins of legumes roots and calcium dependent adhesion called **rhicadhesin**. Lectins, plant proteins with high affinity to carbohydrate moieties on the surface of appropriate rhizobial cells, have been identified as specific mediators of the attachment of rhizobia to susceptible root hairs. After the attachment, the root hair curls as a result of the action of the substrates excreted by the bacterium called **Nod factors**. Tryptophan secreted by plant root is metabolized to indole acetic acid (IAA) by rhizobia, and the IAA, together with unknown cofactors probably arising from the host plant roots, initiates hair curling or branching. The root hairs grow around the bacterial cells. Polygalacturonase, secreted by the rhizobia or possibly by the plant roots, depolymerizes the cell wall and allows bacteria to invade the softened plant root tissues.

After the penetration of primary root hair wall, the infection proceeds by the development of an infection tube or thread that is surrounded by the cell membrane and a cellulose wall. Roots cells adjacent to the root hairs subsequently become infected by rhizobia, and Nod factors stimulate plant cell division, eventually leading to the formation of nodules. The first cells of the

developing nodule contain twice the normal number of chromosomes. These tetraploid cells give rise to central nodule cells in which the rhizobia develop to produce nitrogen-fixing tissue.

Within the infected tissue, rhizobia multiply forming unusually shaped and sometimes grossly enlarged cells called **bacteroides**. Bacteroides become surrounded singly or in small groups by portions of the plant cell membrane, called the peribacterial membrane. The space between the bacteroides and the cell membrane gets filled with leghaemoglobin. In morphology, the bacteroides found within the nodule are swollen and irregular, frequently appearing in star, clubbed or branched shape or X or Y shaped. Size and shape of bacteroid vary in different species of *Rhizobium.* During transformation of normal rhizobial cells into bacteroides, the bacterial chromosomes degenerate, eliminating the bacteroides capacity for independent multiplication. The bacteroid cells produce and contain active nitrogenase which carries out fixation of atmospheric nitrogen (Atlas and Bartha, 2001).

3.4.4 Mechanism of nitrogen fixation

BNF in legumes is wholly dependent on the activity on the activity of the enzyme nitrogenase, which is located within the bacteroides of *Rhizobium*. Nitrogenase is a complex multi-component enzyme which reduces nitrogen to ammonia and depends on the energy source from ATP. The nitrogenase complex consists of two dissociating proteins - one termed nitrogenase reductase or Fe protein, which contains four iron and four acid labile sulphur atoms, while the other termed as the nitrogenase or MoFe protein, which contains two molybdenum, 28-32 iron, and approximately 28 acid labile sulphur atoms. Together these two proteins catalyze the reduction of N_2 to NH_3. The source of reductant for these electron transfers is believed to be reduced ferredoxin or flavodoxin. Ferredoxin is Fe-S electron carrier proteins capable of undergoing reversible oxidation and reduction. Flavodoxin is a protein molecule which is found to replace ferredoxin in some reactions. The reactions occur while N_2 is bound to the nitrogenase enzyme complex. The Fe protein is first reduced by electrons donated by ferredoxin. Then the reduced Fe protein binds ATP and reduces the molybdenum-iron protein, which donates electrons to N_2, producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to H_2N-NH_2, and this in turn is reduced to $2NH_3$. The sequence of transfer of electrons to dinitrogen leads to its reduction to diimide, hydrazine and finally to ammonia. Thus formed ammonia is

released from the bacteroides to the host cell where organic forms of nitrogen such as glutamic acid, glutamine, aspartic acid, and alanine are synthesized.

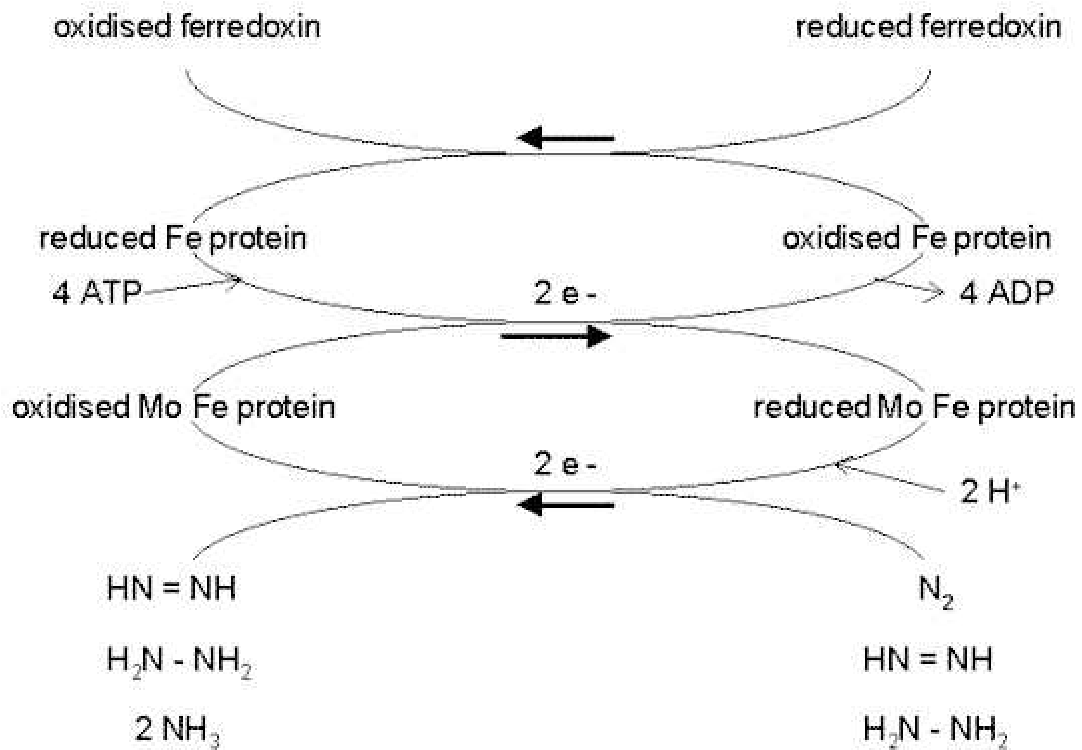

Figure 1: Mechanism of nitrogen fixation

Nitrogen fixation is essentially an anaerobic process and hence the nodule must have a mechanism to exclude oxygen from the bacteroid. This is accomplished by the presence of leghaemoglobin around bacteroides inside nodule tissue. This pigment limits oxygen supply and helps in providing low oxygen tension near the bacteroides with the result that not only the oxygen sensitive nitrogenase is prevented from the damage but also that enough oxygen is available at the site for ATP generation. The presence of this pigment inside nodule makes the nodules pink in color. The amount of leghaemoglobin and the extent of bacteroid tissue in nodules have a direct bearing on the amount of nitrogen fixed by the legumes (Madigan et al., 2000).

3.4.5 Genetics of nitrogen fixation

Rhizobium genetics have thrown light on several gene control steps in the entire symbiotic process. Various bacterial gene involved are *nod*, *nif*, *fix* and nodulin genes. The host specificity of the *Rhizobium* is controlled by the nodulation (*nod* genes); there may be several *nod* genes, one or more of them conferring host specificity and others being common genes, also some *nod* genes are required for root hair curling and for cell division. The gene required for nitrogenase structure and function are called *nif* genes. The symbiotic association of *nif* genes in the *Rhizobium* is dependent on low oxygen concentration which in turn is regulated by another set of gene called *fix* genes. *Fix* gene is essential for nitrogen fixation. Various plant genes which take part in nodule structure and function are collectively known as nodulin genes (Madigan et al., 2000).

3.5 *Rhizobium* spp.

Rhizobia are soil bacteria characterized by their unique ability to infect root hairs of legumes and induce effective nitrogen fixing nodules on roots. Beijerinck in Holland was the first to isolate and cultivate micro-organisms from root nodules of legumes in 1888 which he named as *Bacillus radicicola* which is now classified under the genus *Rhizobium.*

3.5.1 Characteristics

Rhizobia are small to medium sized, non spore forming, gram negative rods (0.5-0.9 by 1.2-3.0 um). They are commonly pleomorphic under adverse growth conditions. Generally motile when young and have bi-polar, sub-polar or peritrichous flagella. Cells often contain granules of polymerized ß-hydroxybutyrate which are refractile under phase contrast, stainable with Sudan black B and soluble in chloroform. Most strains produce gum (extracellular polysaccharide slime) of varying composition.

These bacteria are aerobic, chemoorganotrophs, grows best at 25^0 - 30^0C on complex media, notably with yeast extract; poor growth on peptone agar; slow growth on litmus milk, sometimes with a cleared upper serum zone; indicative of slight proteolytic activity; no coagulation; many able to use nitrate, ammonia, or amino acid as sole source of nitrogen; and utilize dinitrogen primarily in symbiosis with a legume host. Growth on carbohydrate media is usually accompanied by copious extracellular polysaccharide slime. pH range for the growth is 5.0-8.5 but optimum growth is at

neutral to slightly acidic pH (6.8).The chief interest and importance of *Rhizobium* lies in its characteristic ability to invade root hairs of leguminous plants and incite production of root nodules, where in the bacteria occur in pleomorphic forms, bacteroides enclosed singly or in small groups within host membranous sacs. Bacteroides are the ones which fix molecular nitrogen into combined forms utilizable by the host plant. *Phaseolus* bacteroides exhibit few branches in the nodules. Plant infectivity tests are essential for identification. The G+C content of the DNA ranges from 59.1 - 65.5 moles %. (Subba Rao, 2001).

3.5.2 Rhizobium classification

The genus *Rhizobium* has been placed in the family Rhizobacteriaceae in the Bergey's Manual of Determinative Bacteriology. Speciation of *Rhizobium* based on the Linnaean concept has proved difficult and therefore, the cross inoculating grouping based on the classical studies of Fred, Balwin and McCoy is being generally followed. The principle of cross inoculation grouping is based on ability an isolate of a *Rhizobium* to form nodules in a limited number of species of legumes related to one another. All rhizobia that could form nodules on roots of certain legume types have been collectively taken as species. This system has provided a workable basis for the agricultural practice of legume inoculation (Subba Rao, 2001).

Table 1: Classification of *Rhizobium* on the basis of cross-inoculating group (Martin, 1983)

Rhizobium spp.	**Cross-inoculation grouping**	**Legume types**
R. leguminosarum	Pea group	*Pisum, Vicia, Lathyrus, Lens*
R. phaseoli	Bean group	*Phaseolus*
R. trifolii	Clover group	*Trifolium*
R. meliloti	Alfalfa group	*Medicago, Melilotus, Trigonella*
R. lupini	Lupini group	*Lupinus , Ornithopus*
R. japonicum	Soybean group	*Glycine max*
Rhizobium spp.	Cowpea group	*Vigna, Arachis*

However, the ninth edition of Bergey's Manual of Determinative Bacteriology (1983) classifies *Rhizobium* species into fast growers and slow growers.

Table 2: Classification of *Rhizobium* on the basis of growth rate

Fast growers	**Genera of host plants**
GENUS I: *Rhizobium*	
R. leguminosarum biovar - *trifolii* biovar - *phaseoli* biovar- *viceae*	 *Trifolium spp.* *Phaseolus vulgaris* *Pisum*, *Vicia, Lathyrus, Lens*
R. meliloti	*Medicago, Melilotus, Trigonella*
R. loti	*Lupinus, Lotus*
Slow growers	
GENUS II: *Bradyrhizobium*	
Bradyrhizobium japonicum	*Glycine max*
Bradyrhizobium spp. (Vigna)	*Vigna spp.*
Bradyrhizobium spp. (Lupinus)	*Lupinus spp.*

"Fast growers" are likely to have a mean generation time of the order of 2-4 hours, give little detectable growth on agar media in 24 hours, but generally form relatively large (2-4mm diameter), gummy, colorless or white colonies, in 3-5 days. "Slow growers" are likely to have a mean generation time of 6-8 hours, yielding small colonies (1 mm) after 7-10 days; generally colorless, white or cream colored, rarely pink; gumless abundant than in the fast growers, dense and sticky. Based on the ability of the rhizobia to produce acid or alkali on YEMA medium the fast growing *R. phaseoli, R. trifolli, R. leguminosarum* and *R. meliloti* could be grouped as acid producers while the slow growing *R. japonicum, R. lupini* and *Rhizobium spp.* (cowpea) could be grouped as non- acid producers.

3.6 Legumes

The legume, with its symbiotic root nodule bacteria, is the most commonly used BNF system in agriculture. There are more than 13,000 described species of legumes. Of the approximately 3,000 species examined, more than 90 percent form root nodules (in which nitrogen fixation presumably occurs in symbiosis with rhizobia). The role of legumes in enriching the fertility of soil was known through the centuries. Nitrogen fixed by legumes is an essential input for high protein rich food produced in both crops and pasture systems.

Besides nitrogen fixation, some of the legumes like field bean, pea, and lucerne also leave high levels of nitrogen in the soil after cropping which is important for succeeding crops like wheat, maize, and sorghum. This may be the reason why leguminous crops are being traditionally used in Nepal in inter cropping, mixed cropping and in crop rotation with non leguminous crops to enrich soil fertility.

As the cost of chemical fertilizers is increasing and the protein from the animal sources becoming more expensive, the cultivation of legume crop like bush bean would be an answer to the problems in the third world countries of maintaining the soil fertility and producing plant protein rich foods.

3.6.1 *Phaseolus vulgaris*

The common bean (*Phaseolus vulgaris* L.) is the most important legume for human nutrition in developing countries. It is believed to have originated in Southern Mexico and Central America. Common bean (*Phaseolus vulgaris* L.) is known by various names e.g., kidney bean, navy bean, dwarf bean, haricot bean, blue bean, red bean, snap bean, wax bean, French bean, pole bean, bush bean, dry bean, garden bean, rajmah bean and others depending on the type and its uses in particular locality (Neupane et al., 2003).

It is an important cash crop in the hills of mid western region and other areas with similar agro-climatic condition in Nepal. It is grown during rainy season throughout the hilly region as a rain fed crop, whereas it is gaining popularity as winter crop in Terai region. It is an important nutritive vegetable which supplies protein 1.7 g, calcium 50 mg, phosphorous 28 mg, iron 1.7 mg, carotene 132 mg, thiamine 0.08 mg, riboflavin 0.06 mg, vitamin C 24 mg per 100 g of edible pods. The crop has multiple uses as green vegetables, dry beans, sprouted beans, and pulse and as medicinal crop for common ailments. Beans are said to be anti-diabetic and good for bladder burns, cardiac, carminative, depurative, diarrhea, dropsy, dysentery, diabetics, eczema, emollient, hiccups, rheumatism and kidney problems (Bhurer et al., 2003). Besides, it is used as a fodder and green manure crop and bean flour is used for making 'roti 'and 'dhindo'.

3.7 Mycorrhiza

The term mycorrhizae come from the Greek word myco- or fungus and -rhiza or root and literally means fungus root. The mycorrhizal symbiosis is an intimate association which exists between plant root system and certain group of soil fungi (Sahay et al., 1998). The term “mycorrhiza” was coined by Frank in 1885. This relationship benefits the plant growth by enabling a greater proportion of the available nutrients in the soil to be absorbed in the plants. Possible benefits include increased crop yield, protection against certain root pathogens, and increased tolerance to environmental stress and perhaps, most importantly, a reduction in the input of chemical fertilizers (Linderman, 1994).

3.7.1 Types of mycorrhizal fungi

Depending on the plant and fungal species involved as well as distinct morphological patterns, at least seven different types of mycorrhizal associations have been recognized (Singh et al., 2002; Sharma, 2001).

1. Ectomycorrhizae

The fungal members belong to 25 families of Basidiomycotina and 7 families of Ascomycotina. The plant symbiont ranges from Bryophytes to Angiosperms. The Ectomycorrhizae (ECM) are found almost exclusively on woody perennials. They form sheath of fungal mycelium that covers the absorbing root. The presence of “Hartig's” net is a key diagnostic feature of this association (Agerer, 1999). This net of hyphae extends into the cortex but is confined to the intercellular spaces and never penetrates the wall into individual cortical cells although root cap cells may be invaded (Kotte and Oberwinkler, 1986). The fungus never penetrates beyond epidermis.

2. Vesicular-arbuscular mycorrhizae (VAM or AM)

Fungi are confined to the order Glomales of Zygomycotina. The plant symbiont ranges from Bryophytes to Angiosperms. The term Arbuscular mycorrhizae refers to the presence of intracellular structures- vesicles and arbusculars that form in the root during various phases of development. These mycorrhizae are most commonly reported group since they occur in vast taxonomic range of plants, both herbaceous and woody. The AM fungi are in great contrast to the ECM in both anatomical features and morphology. There is no formation of sheath of mycelium and more importantly, intracellular penetration of cortical cells takes place. Internally arbusculars, vesicles, and coils can form within individual cortical cells. Generally, AM causes few changes in the root morphology, but the physiology of the host plant changes significantly. For example tissue concentrations of growth regulating compounds and other chemical constituents change, photosynthetic rates increase. Since AM fungi are major components of the rhizosphere, it is logical that they could affect the incidence and severity of root disease. Much of the literature suggests that the AM fungi reduce soil borne disease or the effect of fungal disease caused by the pathogens. In addition, mycorrhizal plants have greater tolerance to toxic plants, to root

pathogens, drought, high soil temperature, saline soil, adverse soil pH and to transplant shock than non-mycorrhizal plants (Bagyaraj and Verma, 1995).

3. Ericoid mycorrhizae

The fungi belong to Basidiomycotina and one, *Hymenoschyphus*, belongs to Ascomycotina. The ericoid mycorrhizae occur in roots of plants belonging to order Ericales. Rootlets are covered by a loosely woven mesh of dark brown septate hyphae from which branches penetrate the cortical cells, forming intracellular coils invaginating and enclosed by the host's plasmalemma. Hartig's net formed in the intercellular spaces.

4. Arbutoid mycorrhizae

The fungal partner is Basidiomycotina and colonizes members of order Ericales. Fungal sheath is present. Septate hyphae penetrate only the epidermal cells and coil inside it. Hartig's net is present in the intercellular spaces.

5. Monotropoid mycorrhizae

The fungi belong to Basidiomycotina colonizing achlorophyllous member of Angiosperms belonging to family Monotropaceae. These mycorrhizae are very similar to ECM and form a distinct sheath and "Hartig's net". However, they exhibit a distinctive type of intracellular penetration in cortical cells that is unlike other endomycorrhizal types (Duddridge and Read, 1982).

6. Ecto-endomycorrhizae

The fungi belong to Basidiomycotina, which covers both Gymnosperms and Angiosperms plants. The ecto-endomycorrhiozae form a “Hartig's net” in the cortex of the root but develop little or no sheath.

7. Orchidoid mycorrhizae

The fungi belong to Basidiomycotina and mycelia sterlia. These colonize only members of family Orchidaceae (Angiosperm). Septate hyphae enter the cells and develop into coils often called "peltrons".

3.7.2 Functions of mycorrhizal fungi

Mycorrhizal fungus having unique carbon strategies can efficiently couple soil mineralization and nutrient uptake by the plant roots. In many cases the mycorrhizal system actually "bridges" functionally across the rhizosphere, and provides an organic link between the root and the bulk soil. Mycorrhizal plants have an enhanced capability over non-mycorrhizal plants in acquiring nutrients from soil, particularly where the nutrient is poorly soluble and less in concentration. This efficiency in nutrient uptake, primarily through increased absorbing surface area, is likely to be one of the most important features of mycorrhizae (Singh, 2004).

The agricultural significance of arbuscular mycorrhizae lies principally in its ability to assist the plant to absorb inorganic phosphorous from the surrounding soil. Phosphate ions are not very mobile in soil and a phosphate depletion zone often develops around roots when phosphate is scarce. Mycorrhizal hyphae are able to extend from the mycelium within the root to beyond the depletion zone and translocate phosphate directly to the host. Plants with restricted root systems having short stubby roots with few root hairs are generally not very efficient in extracting soil phosphate and tend to benefit considerably from establishment of arbuscular mycorrhizae. The mycorrhizal association often causes a dramatic improvement in phosphate deficient soil absorption of trace element such as zinc and copper can also be enhanced. There is evidence that mycorrhizal plant can contain higher concentration of growth hormones than their non-mycorrhizal equivalent (Singh et al., 2002).

For many land plants mycorrhizal association is one way of generating adequate phosphorous absorption from reserves in soil. The central role of the fungal symbiont in the provision of greater efficiency of phosphate absorption has been shown in vitro and in vivo (Buecking and Heyser, 2001; Harrison et al., 1995; Hodge et al., 2001; Raush et al., 2001). Modification of phosphate uptake properties of roots systems arising from mycorrhizal infection, consequently, depend largely upon development of extrametrical hyphae in soil and then absorption of phosphorous, translocation of phosphorous through hyphae and mycelial development within root tissues and transfer of phosphorous from fungus to root cells (Joner and Johansen, 2000).

3.7.3 *Piriformospora indica*

An axenically cultivable mycorrhiza-like fungus was discovered by Varma and his co-workers (Verma et al., 1998). It was originally found along with arbuscular mycorrhizal spores from the rhizosphere soil samples of spineless cacti and *Cenchuss* spp. (desert grass) growing in North West Rajasthan. The fungus was named *Piriformospora indica*, based on its characteristic pear-shaped spores. Based on the 18S and 28S rRNA analysis and the ultrasuctural details of the septal pores, the phylogenetic relationship of this fungus was established within the Hymenomycetes (Basidiomycotina) and closet to Rhizoctonia group and *Sebacina vermifera* (Varma et al., 1998; Singh, 2004).

P. indica acts as biofertilizer, bioregulator and bioprotector, can be easily mass multiplied on defined synthetic media. *P. indica* is the first symbiotic fungus, known in the literature which can be grown on root of a living plant and under axenic culture (Singh, 2004).

The properties of the fungus *Piriformospora indica* have been patented (Varma A and Franken P, 1997; European patent office, Muenchen, Germany. Patent No. 97121440.8-2105, Nov. 1998). The culture has been deposited at Braunsweich, Germany (DMSNo. 11827).

3.7.3.1 Morphology

P. indica forms inter- and intracellular hyphae in the root cortex, often differentiating into dense hyphal coils (arbusculars-like structures), spore and vesicle-like structures. Like AM fungi, hyphae multiply within the host cortical tissues and never traverse through the endodermis. Likewise, they also do not invade the aerial portion of the plant (stem and leaves). Interestingly, the host spectrum of *P. indica* is very much like AMF. *P. indica* colonizes the roots of wide host plants, both monocots and dicots, including legumes.

The hyphae are highly interwoven, often adhered together and give the appearance of simple intertwined cord. Young mycelia are white and almost hyaline, but inconspicuous zonations are recorded in other cultures. The mycelium is mostly flat and submerged into the substratum. Hyphae are thin- walled and of different diameters ranging from 0.7 to 3.5 micro meter. The septate hyphae often show anastomosis and are irregularly septate. They often intertwine and

overlap each other. In older cultures and on the root surface, hyphae are often irregularly inflated, showing a nodose to coralloid shape and granulated dense bodies is observed. For this reason many cells contain more than one nucleus. Chlamydospores are formed from thin-walled vesicles at the tips of the hyphae. The chlamydospores appear singly or in clusters and are distinctive due to their pear shaped appearance. The chlamydospores are 16-26 µm in length and 10-17 µm in width. The cytoplasm of the chlamydospores is densely packed with granular material and usually contains 8-25 nuclei. Very young spores have thin, hyaline walls. At maturity, these spores have walls up to 1.5 µm thick, which appear two-layered, smooth and pale yellow. Neither clamp connections nor sexual structures are observed (Varma et al., 2001, 2002; Singh, 2004).

3.7.3.2 Cultural characteristics

P. indica can be successfully cultivated on wide range of synthetic solidified and broth media, e.g., MMN1/10, modified *Aspergillus*, M4N, MMNC, MS, WPM, MMN, Malt-Yeast Extract, PDA and *Aspergillus*. Among the tested media, most optimum is *Aspergillus* medium (Kaefer, 1977). The temperature range of *P. indica* is 25-35^{0}C; the optimum temperature and pH being 30^{0}C and 5.8 (4.8-6.8) respectively. On Modified-Melin-Norkran medium sparsely running hyaline hyphae on the agar surface is seen, while on potato dextrose agar deep furrows with a strong adhesion to the agar surface is apparent. This sharp mode of growth is not observed when fortified with malt extract and normal *Aspergillus* medium. In contrast to *Aspergillus* medium, shaking conditions on MMN broth medium invariably inhibit the growth (Varma et al., 2001; Singh 2004).

3.7.3.3 Molecular taxonomy

P. indica produces chlamydospores at the apex of undifferentiated hyphae. Different kinds of substrates are tested to induce sexual development, like young and mature leaves of *Cynodon dactylon* and pollen grains, oat meal, potato, carrot or tomato dextrose agar. Since all these efforts do not lead to the desired results, there are only a few features to characterize the fungus morphologically to group it accordingly to the classical species concept. In order to obtain more information about the systematic position of the new fungus, the ultrastructure of the septal pore and the cell wall are examined.

The cell walls are very thin and showed multilayered structures. The septal spores consist of dolipores within the continuous parenthosomes, which forms the basis for the systematic position within the Hymenomycetes. The dolipores are very prominent with multilayered crosswall and a median swelling mainly consisting of electron transparent material. The parenthosomes are always straight and had the same diameter as the corresponding dolipore. Any kind of pores can not be detected. This means that they are flat discs without any perforation. The parenthosomes consist of an electron dense outer layer and a less dense inner layer, which show an inconspicuous dark line in the median region .The parenthosomes are in contact with ER membranes, which are mostly found near the dolipore (Varma et al, 1998; Singh, 2004).

According to the ultrastructure of the septal pore and the molecular data (analysis of 18S rDNA and 28S rDNA), the fungus is placed within the Hymenomycetes (Basidiomycetes). There is no existing genus that covers all the characters of the new fungus, therefore a new genus was proposed and the fungus was called *Piriformospora indica* (Varma et al, 1998; Singh, 2004).

3.7.3.4 Host spectrum

The host spectrum of *P. indica* is very much like AM fungi. *P. indica* tremendously improves the growth and overall biomass production of diverse hosts including mono-dicots, legumes, and terrestrial orchids, medicinal and economically important plants (Singh, 2004).

P. indica can colonize the roots of host plants as diverse as *Arternisia aannua* (Chinese wormhood), *Bbacopa monnier* (Brahmi), *Cicer arietinum* (chick pea), *Glycine max* (soybean), *Nicotiana tobaccum* (tobacco), *Oryza sativa* (rice), *Petroselinum crispum* (curly parsley), *Pisum sativum* (pea), *Populus tremula* (aspen), *Setaria italica* (thumb millet), *Sorghum vulgare* (millet), *Solanum melongena* (egg plant) and *Zea mays* (maize). Even plants like A*dhatoda vasica* (malabar nut), *Chlorophytum tudersum* (Mexican orange), *Delbergia sisso* (rosewood), *Prosopis julifera* (honey mesquite), *Spilanthus calva* (clove), *Terminalia arjuna* (Arjun tree), *and Withania somnnifera* (winter cherry) promoted in their growth following interaction with *P. indica* (Rai et al., 2001; Singh et al., 2000). Incidentally, the host range of *P. indica* also includes terrestrial orchids like *Dactylorhiza purpurella* (lady orchid), *D. incarnata* (early marsh orchid), *D. majalis* (broad leaved marsh orchid), and *D. fushsii* (spotted orchid). It however fails to establish a

symbiotic relationship with taxa belonging to Brassicaceae, Chenopodiaceae, Cyperaceae, Junaceae, and Proteaceae.

3.7.3.5 Phosphate mobilization and transport

P. indica mediates uptake of phosphorous from the medium and translocates to the host in an energy-dependent process. *P indica* produces significant amounts of acid phosphates for the mobilization of broad range of insoluble forms of phosphate, enabling the host plant the accessibility of adequate phosphorous from immobilized reserves in the soil (Varma et al., 2000). An active involvement of the phosphates in the phosphate metabolism of *P. indica* has been observed. The fungus showed prominent acid phosphates activity in both intra- and extracellular fractions. This was a direct evidence for the involvement of this enzyme in the phosphate metabolism (Sharma, 2000). In recent observation in *Asphodelus fistulosus*, a common weed in South Austria, colonization was more sensitive to P supply (reduced from 60% to just over 30% over the range of P used (Cavagnaro et al., 2003).

3.7.3.6 Biological hardening agent

P. indica promises to be an excellent candidate for biological hardening of micropropagated plantlets as the fungus rendered more than 90% survival rate of transferred plantlets of *Nicotiana tobaccum* (tobacco), *Bacopa manniera* (brahmi) (Sahay and Varma 1999, 2000), *Spithanthus calva* (clove), *Withania somnifera* (Rai et al., 2000) and *Azadirachta indica* (neem) (Singh et al., 2003b) by excessive root proliferation and induction of secondary rootlets, protecting them from transplantation shock and potent root pathogens (Singh et al., 2002a, 2003c; Varma et al., 2000). Therefore, *P. indica* promises as a boon for plant industry.

3.7.3.7 Similarities between *P. indica* and AM fungi

P. indica promises to serve as the substitute of AM fungi to overcome the long- standing enigma of science (Singh, 2004). The functional similarities with AM fungi are the following:

- Broad and diverse host spectrum
- Hyphae extrametrical, inter and intracellular
- Hyphae never invade the endodermis

- Chlamydospores in soil and within cortical tissues
- Sexual stages not seen positive phyto-promotional effects in tested hosts
- Phosphorous mobilizer
- Phosphorous transporter
- Tool for biological hardening of micropropagated plantlets
- Potent biological control agent against root pathogens

3.7.4 Legume-*Rhizobium*-AM symbiosis

Legumes form tripartite symbioses with arbuscular mycorrhizal fungi (AMF) and rhizobia which influence plant productivity. In *Rhizobium*-AM-legume tripartite symbiosis relationships, nodulation of rhizobia and establishment of AM often occur simultaneously and synergically. Besides, rhizobia provide fixed nitrogen not only to the plant, but also to the fungus. Moreover rhizobia can also assist in mobilizing nutrients from the soil and improving the growth of infected plants. Similarly, AMF enhance plant growth by absorbing P from soil and transporting it to the roots (Jakobsen et al., 1992). AMF and rhizobia often act synergistically on infection rate, mineral nutrition, and plant growth.

Reports stated that the presence of AM fungi is known to enhance nodulation and N fixation by legumes (Amora-Lazecano et al., 1998; Johanson et al., 2004). Redente and Reeves (1981) co-inoculated sweetvetch with *G. fasciculatus* and *Rhizobium sp.* and found that co-inoculated plants and those inoculated with *G. fasciculatus* alone produced more aboveground dry matter than those left uninoculated or inoculated with rhizobia alone. Manjunath et al., (1984) inoculated *Leucaena leucocephala* with *G. fasciculatus* and *Rhizobium sp.* and found that plants co-inoculated with AMF and rhizobia had increased root nodulation, mycorrhizal colonization, dry weight, and N and P content compared to plants inoculated with AMF or rhizobia alone. Pacovsky and co-workers (1986) inoculated soybean with *G. fasciculatum* and *Bradyrhizobium japonicum*, and found that co-inoculated plants produced significantly higher shoot biomass than those left uninoculated or inoculated with rhizobia alone. Kucey and Bonetti (1998) reported that field beans inoculated with mixture of native AMF from several Southern Alberta soils enriched

on a strawberry host had up to 54% higher dry matter production than plants not receiving AMF. They also reported that plants co-inoculated with AMF and *R. phaseoli* had a higher N content compared to plants inoculated *R. phaseoli* with alone.

Similarly, Bagyaraj (1984) demonstrated that the AM fungi with other microorganisms are able to enhance the N nutrition of the plant by stimulating the number and activity of free living nitrogen fixers like *Beijernickia* or *Azotobacter* or by increasing nodulation and nitrogen fixing ability of the *Rhizobium*. Manoharacharya (1992) studied the response of AM fungi as biofertilizer in oilseed *Sesamum indicum* L. where he found that AM fungal infection enhanced the plant growth, more yield, and uptake of macro- and micro- nutrients in semi-arid tropical soils. Raut and Ghonsikar (1992) studied the effect of dual inoculation of *Rhizobium* and AM fungi on pigeon pea and found that dual inoculation was more beneficial than the single inoculation of *Rhizobium* or AM fungi to stimulate growth parameters such as nodulation, root shoot growth, fresh and dry weight, yield, and nitrogen uptake. El-Ghaundour et al., (1998) reported increased nodulation and N-content of ground nut due to the dual inoculation of *Bradyrhizobium* strains and AM fungi. They also found that these parameters are affected by the status of P-application and there is a possibility of saving than 50% of recommended N-fertilizer. Shalaby and Hanna (1998) reported that the dual inoculation of *Bradyrhizobium japonicum* either with *Pseudomonas fluorescens* or endomycorrhizal fungi *Glomus mosseae* in soybean significantly increased nitrogen uptake in plants and weight of soybean grain by 20%-30% and 14%-17% as compared to the single inoculation. Hoque et al., (1999) found that the inoculation of soybean plants with *Bradyrhizobium japonicum i*ncreased nodule number, nodule dry weight, chlorophyll content, shoot length, plant dry weight, and grain yield significantly as compared to control. The effect of the inoculation was found to be more significant in presence of sulfur and molybdenum fertilizer. Mestrollet et al., (1999) studied the effect of specific lectin addition on *Rhizobium leguminosarum-Phaseolus vulgaris* symbiosis and found increase in nodule number dry weight of nodule, dry matter of plant and total nitrogen content significantly.

Natalia et al., (2001) studied the management of plant microbe interaction and demonstrated the inoculation of indigenous AM fungi with rhizobial nitrogen fixing bacteria not only enhanced the establishment of key plant species but also increased soil fertility. The dual symbiosis increased

the soil nitrogen content, organic matter and enhanced N transfer from nitrogen fixing to non-nitrogen fixing species associated within the natural succession. Neupane (2003) studied the effect of dual inoculation of AM fungi and *Bradyrhizobium japonicum* in soybean and found that the dual inoculation enhanced the growth, nodulation, nitrogen and phosphorus content and specific P-uptake in soybean plant. Aryal and Fujita (2003) reported that rhizobia and AM fungal inoculation improve growth, nutrient uptake of bean under organic fertilization. They found that dual inoculation significantly increased pod yield, shoot and root dry weight, and shoot N and P compared to control or singly inoculated plants. Jia et al., (2003) studied the influence of *Rhizobium* and arbuscular mycorrhizal fungi on nitrogen and phosphorus accumulation by *Vicia faba* and reported that the synergistic or additive interactions among the components of the tripartite symbiotic association (*Rhizobium*-AMF-broad bean) increased plant productivity.

3.8 Organic fertilizer

Before the introduction of chemical fertilizers in Nepal organic manure was only the sources of fertilizers for enriching soil fertility. The use of appropriate biofertilizer such Biological Nitrogen Fixation, green manuring, farmyard manure, compost and vermicompost and biogas slurry by-products could be achieved the necessity and the importance of integrated nutrient supply system for better soil fertility management that could increase food production on a sustainable basis. While chemical fertilizers introduce extra concentrated supplies of readily available plant nutrients in soil, but the beneficial effect of organic fertilizers predominantly lies in furnishing humus forming material to bring about improvement in soil structure, water holding capacity, microbial population and resistance to soil erosion. Most of the plant nutrients removed by the crops is restored to the soil through the application of organic manures. In addition, the use of organic manure improves the physical, chemical, and biological condition of soils and increases the organic matter content and essential plant nutrient, in particular nitrogen and phosphorus. It contains numerous components required for microbial growth and may increase the activity of soil microorganisms, including rhizobial growth.

3.8.1 Vermicomposting

Vermicompost is a mixture of worm manure, microbially decomposed organic matter and some partially decomposed organic matter. Since vermicompost contains a water soluble colloidal worm cast, the NPK content of the vermicompost is higher than that of other composts. The higher amount of NPK will help to improve the crop yield by increasing soil fertility and water retention ability (Acharya, 1997). Except this, vermicompost also results in the lowering of the C: N ratio, water soluble carbon, and carbohydrates and increases ash percentage and cation exchange capacity.

Finished compost contains a large amount of enzymes, growth hormones, and inorganic compounds in a highly soluble form. Except this, vermicompost also contains large number of microorganisms that are responsible for the decomposition. When such compost is applied to the plants, due to water soluble nature of vermicompost, the nutrients they contain become easily available to plant roots. Along with this, the microbes present in the compost produce different enzymes that are essential for plant growth. They produce antibiotics and toxic substances that show antagonistic activity against certain plant pathogens.

Vermicompost has been ideal organic manure enhancing biomass production of number of crops (Vasudevan and Sharma, 1997; Hidalgo, 1999; Pashanasi et al., 1996). The importance of vermicompost in agriculture, horticulture, waste management, and soil conservation has been reviewed by many workers (Edwards, 1995; Riggle and Holmes, 1994; Kaviraj and Sharma, 2003).

Chan and Griffiths (1998) studied the vermicomposting of pre-treated pig manure and found that worm castings produced had a stimulating effect on the growth of soybean, with an increase in root length, lateral root number, shoot length, and inter-nodal length of seeding plants.

Edwards and Burrows (1988) found that worm-worked composts had better texture and soil enhancing properties, hold typically higher percentage of nitrogen, potassium and might offer plant disease-fighting properties.

Kale et al., (1992) conducted an experiment on the summer crop of paddy variety to test whether it is possible to reduce the use of chemical fertilizer by using vermicompost as organic fertilizer or not. The control plot received the recommended dosage of farmyard manure and the chemical fertilizer. The experimental plot received half the recommended dosage of chemical fertilizer and the vermicompost. At the time of seed setting and two months after the harvest of the crop, the soil samples were analyzed for total microbes, nitrogen fixers, actinomycetes and spore formers. The present mycorrhizal colonization in the plant system was also assessed. Significant increase in the colonization of these microbes in the experimental plot over the control plot was observed. It could be deduced that the vermicompost application had enhanced the activity of these microbes in the soil system. There was high level of total nitrogen in the experimental plot, which comparatively received less quantity of fertilizers.

Kale (1995) studied the nutrient status of vermicompost produced with different organic waste and found organic carbon to be 9.15-17.98%, total nitrogen 0.5-1.5%, available phosphorous 0.1-0.3%, available potassium 0.15% calcium and magnesium 22.70-70mg/100g, copper 2-9.3 ppm, Zinc 5.7-11.5 ppm and available sulphur 128-548 ppm.

Gunathilagaraj and Ravignanam (1996) studied vermicomposting of sericulture waste in the laboratory using *Perionyx excavatus*. Vermicomposting increased the nitrogen content of mulberry (*Morus indica*) leaf litter and silkworm larval litter, phosphorous content was increased in cow dung and sericulture waste by vermicomposting. It also enhanced the potassium, manganese, zinc, and iron content of the mulberry leaf litter.

Rani and Shrivastava (1997) tested vermicompost in pot experiments for its ability to replace a proportion of the urea fertilizer applied to rice, compared to nitrogen fertilizer alone, supplying one third or one quarter of nitrogen as vermicompost increased plant height, grain yield and yield components of rice.

Subler et al., (1998) studied the effects of vermicompost on the germination and growth of flowering plants as well as bedding plants. Consistently, the addition of relatively small amount of worm casting to standard horticultural container mixes, and even to commercially prepared premium quality container media, has resulted in dramatic improvements in plant growth.

Murarkar et al., (1998) conducted an experiment on mulberry crop (*Morus* spp) to investigate the effect of vermicompost in comparison with farmyard manure and fertilizers on the mulberry leaf yield during October 1993 and February 1994 at Akola, India. The treatment of full dose of NPK fertilizers (300:120:120 kg NPK/ha) and vermicompost of 6000 kg/ha and half dose of farmyard manure of 10 cart loads/ha was significantly better than untreated control for increasing the maximum number of branches, height of plants, number of leaves per plant and leaf yield per plant.

Sainz et al., (1998) conducted the application of high amount of vermicompost from composted urban waste to soils might cause a significant reduction of activity of AM fungi, which must be taken into account when using these organic amendments in agricultural systems. The result showed that amendment of soil with 10-15% vermicompost significantly increase dry matter yields of red clover and cucumber plants, compared to treatments where soil was the only substrate. Addition of vermicompost alone increased P and other mineral elements in soil and shoot P, Ca, Mg, Cu, Mn, and Zn concentration but caused a significant reduction on root length colonized by AM fungi in red clover plants.

Ceccanti and Masciandro (1999) conducted a field scale test to evaluate the potential of vermicomposting as an economical/environmental alternative in sludge management. They found vermicompost as high quality humus produced to be used in the field as a soil organic amendment.

Atiyeh et al., (2002) reported that the plant growth regulators and other plants growth influencing materials i.e., auxins, cytokinins, humic substances etc. are produced by microorganisms from vermicompost.

Addision (2004) have found that worm castings improve the condition of the soil and the growth of plants. The castings contain high amounts of nitrogen, potassium, phosphorous, calcium, and magnesium. Worm casts also contain five times more nitrogen, seven times more phosphorous and eleven times more potassium and one and half time more calcium than ordinary soil. The casts are also rich in humic acid, which condition the soil, have a perfect pH balance, and contain plant growth factors.

CHAPTER- IV

4. MATERIALS AND METHOD

4.1 Materials

All the materials that are used to complete this study are listed in Appendix-I.

4.2 Method

The methods followed in this study are given below:

4.2.1 Isolation of *Rhizobium leguminosarum* biovar *phaseoli* from the nodule samples

4.2.1.1 Collection of nodules

Nodules sample were collected from bean plants from Koteshwor at the stage of flowering, so that roots have well developed pink nodules. With the help of spade, a circle with a radius of approximately 15 cm around the plant was marked and this section was cut out to a depth of at least 20 cm or deeper. The plant was slowly lifted out, still using the spade. The soil was carefully removed from the roots with hands. Detaching secondary roots from plants was avoided as nodules may be found on the lateral roots as well as tap root. The whole plant was carefully placed into plastic bag and was brought to the laboratory (Somasegaran and Hoben, 1994). The root system was carefully washed in running tap water to remove the soil particles. Healthy pink undamaged and firm nodules were carefully cut out with a portion of the root attached to the nodule (Subba Rao, 2001).

4.2.1.2 Isolation and purification of *Rhizobium leguminosarum* biovar *phaseoli* from the nodule samples

To isolate *Rhizobium leguminosarum* biovar *phaseoli* from root nodules, collected nodules were washed in the sterilized test tube with sterile distilled water for several times. The nodules were immersed in 95% ethanol for 5-10 seconds and transferred to 3% hydrogen peroxide for 4-6 minutes. The sterilized nodules were again rinsed 5 to 6 times with sterile distilled water in order

to get rid of hydrogen peroxide. The sterilized nodule was crushed in 1ml sterilized distilled water with sterile glass rod in a sterile test tube (Somasegaran and Hoben, 1994).

Gram staining was done from the freshly crushed nodules and microscopic examination with oil immersion was done. The cell morphology was noted.

Isolation and purification of *Rhizobium leguminosarum* biovar *phaseoli* from nodule samples was done by two methods.

A. Streak plate method

One loopful of nodule suspension was streaked on yeast extract mannitol agar (YEMA) plates containing Congo red. YEMA media plates containing Congo red were prepared as mentioned in Appendix-II. The plates were incubated at 26^0C- 28^0C for 3-5 days. Large gummy colonies of rhizobia appeared on the Petri-plates. The rhizobial colonies were then streaked repeatedly on the YEMA plate containing Congo red to obtain the pure culture.

B. Needle method

The surfaced sterilized nodules were first placed on a small piece of filter paper in a sterile Petri-plate. A new piece of filter paper was used for each nodule. The nodule was held with sterile forceps, resting the nodule on sterile filter paper. Then, with the help of sterile scalpel, the nodule was sliced off and with the help of sterile inoculating wire, the inoculum was spotted on the YEMA-CR medium (Somasegaran and Hoben, 1994).The plates were incubated at 26^0C- 28^0C for 3-5 days.

4.2.1.3 Maintenance and preservation of the culture

Once the pure culture was obtained, single bacterial colonies were transferred to YEMA slants. The slants were incubated at 26^0C- 28^0C for 3-5days and finally actively growing slant cultures were stored at 4^0C in the refrigerator.

4.2.1.4 Identification of *Rhizobium leguminosarum* biovar *phaseoli*

Identification of *Rhizobium leguminosarum* biovar *phaseoli* was done by studying the colony characteristics, cell morphology, Gram staining and performing presumptive test (Somasegaran and Hoben, 1994).

A. Motility determination (Aneja, 1996)

Hanging drop method was used to observe bacterial motility. A small amount of Vaseline was placed near each corner of the cover glass with a tooth pick, and then one loopful of organism was transferred in the centre of the cover slip. Depression slide was placed on the cover slip with the colony facing down so that the depression covers the suspension. The completed preparation was examined under the microscopy using oil immersion.

B. Study of colony characteristics

The colony characteristics of the isolate on YEMA media plates were studied and noted.

C. Presumptive test

1. Gram's staining

Gram's staining of the isolates was performed for the presumptive identification of the organism. A thin smear of 24 hours culture was made on clean grease free slide and air dried. The smear was then heat fixed and was flooded with crystal violet for 30 seconds. It was then washed with distilled water and treated with iodine for 1 minute. Then, the smear was decolorized with 95% ethyl alcohol for 10 to 20 seconds and counter stained with safranine for 1 minute. The slide was finally washed with sterile distilled water and air dried. The stained slide was then observed under microscope under 40X magnification and 100X magnification in oil immersion.

2. YEMA containing Congo red

A loopful of rhizobial culture was streaked on YEMA containing Congo red and incubated at 26^{0}C- 28^{0}C for 3-5 days. Rhizobia generally do not absorb Congo red when plates are incubated in the dark. Colonies remain white, opaque, or occasionally pink. Contaminating organism usually absorb red dye.

3. YEMA containing BTB

A loopful of rhizobial culture was streaked on YEMA containing Congo red and incubated at 26^0C- 28^0C for 3-5 days. Slow growing Rhizobia show an alkaline reaction in this medium, turning the dye blue. Fast growing rhizobia show an acid reaction, turning the media yellow (Somasegaran and Hoben, 1994).

4. Glucose peptone agar

A loopful of rhizobial culture was streaked on glucose peptone agar plates and incubated at 26^0C- 28^0C for 3-5 days. No growth or poor growth should be observed in glucose peptone agar (Somasegaran and Hoben, 1994).

4.2.1.5 Authentication of *Rhizobium leguminosarum* biovar *phaseoli*

The bacterial isolates obtained from bean root nodules were authenticated as *Rhizobium* by plant infectivity test. Plant infectivity test was performed in sterile soil using modified Jensen's nitrogen free medium (Subba Rao, 2001). The bean seeds were surface sterilized and sown in a sterilized soil in sterilized small earthen pots. On germination, seedlings were inoculated with 1ml of the broth culture of the isolate. About 20ml of Jensen's nitrogen free nutrient medium was poured in each earthen pot. Uninoculated set was also maintained. The inoculated and uninoculated plants were up rooted after 30 days of seed sowing and the presence and absence of nodules were noted.

4.2.2 Pot culture experiment

4.2.2.1 Collection and preparation of experimental soil

The soil for pot culture experiment was collected from Khumaltar from the depth of 8 inches by using spade. The experimental soil was clay loam which was finely ground and mixed with sand for aeration at the ratio of soil: sand = 3:1. The experimental soil was filled in plastic bags and sterilization was done by autoclaving at 15 lbs pressure at 121^0C for two hours for three consecutive days. The NPK content, pH of the experimental soil was determined.

4.2.2.2 Preparation of earthen pots

Earthen pots were cleaned and swabbed with absolute ethanol and kept for 10 minutes. The pots were filled with 12 kg of prepared experimental soil. The soil in each earthen pot was no more than ½ inch from the top edge of the pot and 50 gm of vermicompost was mixed with the soil in three treatments. The earthen pots were labeled according to the following treatment .There were seven treatments with four replications each.

T1: Control

T2: *P. indica* alone

T3: *Rhizobium* alone

T4: *P. indica* and *Rhizobium*

T5: *P. indica* and vermicompost

T6: *Rhizobium* and vermicompost

T7: *P. indica* and *Rhizobium* and vermicompost

4.2.2.3 Collection of seed

The bean variety (*Phaseolus vulgaris* L.) locally known as ghue simi was used in this study, brought from Annapurna seed bhandar of Kathmandu valley. The seeds were surface sterilized by immersing in 95% ethanol for 5-10 seconds and transferred to 3% hydrogen peroxide for 4-6 minutes. The sterilized seeds were again rinsed 5 to 6 times with sterile distilled water in order to get rid of hydrogen peroxide.

4.2.2.4 Preparation of the inoculum

1. Preparation of *Rhizobium* broth

The yeast extract mannitol broth was prepared as mentioned in Appendix-II. *Rhizobium leguminosarum* biovar *phaseoli* grown on YEMA slants was inoculated in 150 ml sterile YEMA broth in the conical flasks. The flasks containing broth cultures were shaken on a rotary shaker for 5-7 days at 26^0C- 28^0C at 20 rpm. The purity of the fully grown broth culture was tested by gram staining and pH measurement (Somasegaran and Hoben, 1994).

2. Determination of the number of viable cells in YM broth

Eight sterile test tubes containing 9ml of sterile distilled water were taken. Then, 1ml of the broth culture is diluted in step; ten fold each time with the help of sterile pipette. Separate sterile pipette was used for each dilution and 1ml diluted broth culture from 10^4, 10^6, 10^8 dilutions were aseptically transferred to a sterile petri dish. Then 15 to 20ml of YEMA maintained at 45^0C was poured aseptically to each cell suspensions in the petri dish. To dispense the cells evenly, the petri dish was moved gently clockwise and anticlockwise. The plates allowed to solidify and were inverted and incubated at 26^0C- 28^0C for 3 to 5 days.

4.2.2.5 Preparation of *Piriformospora indica* inoculum

PDA plates were prepared as mentioned in Appendix II. *P. indica* cultures were cut into squares with the help of sterilized loops. *P. indica* squares were then placed on the PDA surface and incubated at 25^0C- 27^0C for about 1 week.

4.2.2.6 Sowing of seed

Ten healthy seeds were sown in each pot at a depth of 2 cm of soil. Watering of the plants was done frequently letting the seeds to germinate and grow. Continuous care and proper management were done till plants become ready for harvesting.

4.2.2.7 Application of inoculum

P. indica agar culture disc of size 4 mm in diameter were cut with the help of sterile cork and borer (Singh, 2004). After 7 days of seed germination, the *P. indica* inoculum was carefully placed near the root surface, care was taken so that root was not damaged. After 5 days of the application of *P. indica*, 1ml rhizobial broth was applied to each plant per pot. After 25 days of seed sowing the seedlings were thinned manually leaving eight healthy seedlings each pot.

4.2.3 Growth parameters

4.2.3.1 Nodulation

In the flowering stage (on the 30th day after seed sowing), three plants from each replication were carefully uprooted from the pot and the roots were shaken and rinsed in running tap water. The number of nodules per plant per pot was counted. The remaining five plants were harvested after 50 days of seed sowing and the number of nodules per plant per pot was counted.

4.2.3.2 Shoot length

In the flowering stage (on the 30th day after seed sowing), three plants from each replication were carefully uprooted from the pot and the roots were shaken and rinsed in running tap water. The length of bean plant shoot from the point of cotyledon attachment up to the shoot tip was measured. The remaining five plants were harvested after 50 days of seed sowing and the length of plant were measured.

4.2.3.3 Shoot weight

Three bean plants from each replication in the flowering stage and five plants in harvesting stage were taken and fresh weights were taken. The shoots were wrapped with dry paper respectively and were dried in hot air oven at 80^{0}C for 24 hours. Then, the dry weights of shoots were taken.

4.2.3.4 Root length

In the flowering stage (on the 30th day after seed sowing), three plants from each replication were carefully uprooted from their pot and shaken the roots and rinsed in running tap water. The lengths of bean plant roots from the point of cotyledon attachment up to the main root tip were measured. The remaining five plants were harvested after 50 days of seed sowing and the length of root were measured.

4.2.3.5 Root weight

Three bean plants from each replication in the flowering stage and five plants in the harvesting stage were taken and fresh weights were taken. The roots were wrapped with dry paper respectively and were dried in hot air oven at 80^0C for 24 hours. Then, the dry weights of roots were taken.

4.2.3.6 Number of pods

In the harvesting stage, five plants per pot from each replication were taken and number of pods per plant was counted.

4.2.3.7 Pod weight

In the harvesting stage, five plants per pot from each replication were taken and fresh weight of the bean pods was taken. The bean pods of different treatment were wrapped with dry paper and dried in hot air oven at 80^0C for 24 hours. Then, the dry weights of pods were taken.

4.2.4 NPK analysis of plant

4.2.4.1 Determination of nitrogen content of shoot and root

Nitrogen content of the samples was determined by Kjeldhal method (Pradhan, 1996). About 0.20 gm of dried and ground sample of root and shoot was weighed. About 2 gm of digestion mixture and 10 ml of concentrated sulphuric acid was added to 0.5 gm of the sample in 100ml digestion flask the sample was digested in low heat until frothing stopped. The flasks were swirled at intervals and the digestion was continued till the carbonaceous particle was present and the color was changed to greenish blue. The flasks were allowed to cool and about 40 ml of distilled water was added before the solution starts crystallizing. The solution was then transferred to 100 ml volumetric flask washing the digestion flask with 3-4 lots of small amount of distilled water and the volume was made up to the mark in the volumetric flask. Then, 20 ml aliquot was taken in the distilling flask and 20 ml of 40% NaOH was added and distilled it, collecting the liberated NH_3 in 10 ml 4% boric acid solution containing 2 drops of mixed indicator in 125 ml conical flask. It was

then titrated with 0.05 N HCl. Here, the colour of the mixture indicator gets changed from blue to reddish at the end point. The blank was also run with all the chemicals and process except the sample. Percent Nitrogen content was calculated using following formula:

$$\% \text{ Nitrogen (N)} = \frac{(S-B) \times N \times 14 \times 100 \times 100}{W \times 1000 \times 20}$$

$$= \frac{(S-B) \times N \times 7}{W}$$

Where,

S = Volume of standard acid (ml) used up by sample

B = Volume of standard acid (ml) used up by blank

N = Normality of standard acid

W = Oven dry weight of sample (mg)

14 = Equivalent weight of nitrogen

20 = Aliquot

4.2.4.2 Determination of phosphorous content of shoot and root

Phosphorous content of the samples was determined by Vanadomolybdophosphoric yellow method (Pradhan, 1996). About 1.0 gm of the sample was weighed in 30 ml porcelain crucible. It was then placed in a cool muffle furnace and raised the temperature to 500^0C-525^0C. The sample was ignited at this temperature for 5 hours and then the crucible was removed from the furnace. The residue was dissolved in 3 ml of conc. HCl. It was then evaporated to dryness and 5 ml of 2N HNO_3 was added to dissolve the residue of salts. The solution was then filtered through Whatman No. 31 filter paper into 50 ml volumetric flask and made the volume up to 50 ml. About 5 ml of this diluted solution was taken in the 50 ml volumetric flask and diluted to 35 ml. Then 10 ml of vanadomolybdate reagent was added and diluted to 50 ml with distilled water. The yellow colour was measured after 20 minutes at 420 nm and compared with that of the phosphorous standards.

The standard curve was prepared by taking 0, 2, 4, 6, 8, 10, 12, and 15 ml of 20 ppm P-standard in 50 ml volumetric flask and dilute to 50 ml mark. Then, the yellow colour was measured in a spectrophotometer after 20 minutes at the same wavelength as the sample. Phosphorous content was calculated using following formula:

$$\text{ppm P in plant tissue} = \frac{\text{ppm P in solution X 10}}{\text{W}}$$

Where, W = Oven dry weight of plant sample and 5 ml aliquot is taken from 50ml dry ash extract

$$\text{\% P in plant tissue} = \frac{\text{ppm P in plant tissue X 100}}{10^6}$$

4.2.4.3 Determination of potassium content of shoot and root

Potassium content of the samples was determined by Flame Photometer Method (Pradhan, 1996). About 1.0 gm of the sample was weighed in 30 ml porcelain crucible. It was then placed in a cool muffle furnace and raised the temperature to 500^0C $\pm$ 25^0C. The sample was ignited at this temperature for 5 hours and then the crucible was removed from the furnace. The residue was dissolved in 3 ml of conc. HCl. It was then evaporated to dryness and 5 ml of 2N HNO_3 was added to dissolve the residue of salts. The solution was then filtered through Whatman No. 31 filter paper into 50 ml volumetric flask and made the volume up to 50 ml. A standard curve of K was prepared by aspirating 0, 5, 10, 15, 20 and 25 ppm K after adjusting full scale deflection of photometer with 25 ppm K, the reading was taken and the graph was drawn. The sample solution aspirated and its reading was noted. K was determined in the sample solution from the graph. Potassium content was calculated using following formula:

$$\text{K\%} = \frac{\text{R X 100 X DF}}{\text{W X } 10^6}$$

Where, R = ppm K in the solution

W = Oven dry weight of sample

DF = Dilution Factor

4.2.5 Determination of percent root colonization

4.2.5.1 Root staining

The roots were stained following the method suggested by Philip and Hayman (1970). Fine feeder roots of bean plant were washed thoroughly in running tap water and cut into 1 cm pieces. They were subsequently treated with 10% KOH solution overnight. Thereafter, the root pieces were washed 3-5 times with distilled water and treated with 1% HCl for 3-4 minutes. The root pieces were then stained with 0.05% trypan blue. The root pieces were finally destained with lactophenol for 1-2 hours. The infected root pieces were examined under microscope at 40 X-100 X magnifications.

4.2.5.2 Assessment of root colonization

For assessment of root colonization by *P.indica*, the slide method proposed by Giovannetti and Mosse (1998) was followed. The root pieces (1 cm long) were selected at random from the stained samples and mounted on microscopic slide in groups of 10. Presence of infection was recorded in each of the 10 pieces and percentage infection was calculated as described by Singh (2004).

$$\% \text{ Colonization} = \frac{\text{No. of root segments colonized} \times 100}{\text{Total no. of root segments observed}}$$

In all 20 roots were examined.

4.2.6 Statistical analysis

In order to test, whether the differences between the treatments were statistically significant or not, one way ANOVA was done using computer program SPSS. Statistical analyses of each treatment are presented in the Appendix-III.

CHAPTER-V

5. RESULTS

5.1 Identification of *Rhizobium leguminosarum* biovar *phaseoli*

Identification of *Rhizobium leguminosarum* biovar *phaseoli* was done by studying the colony characteristics, cell morphology, grams staining and performing presumptive test.

5.1.1 Cultural characteristics of *Rhizobium leguminosarum* biovar *phaseoli*

The nodules aliquot streaked on YEMA containing Congo-red media plates and incubated at 26 ± 2^0C for 3-5 days were examined for the presence of *R. leguminosarum* biovar *phaseoli*. The observed colony was circular, convex with smooth margin and surface, milky- to watery-translucent (colorless), elevation was found to be raised and mucilaginous, usually 2-3 mm in diameter within 3-5 days on YEMA media. The colony of the *R. leguminosarum* biovar *phaseoli* tends to produce excessive amounts of slime, which are extracellular carbohydrates.

5.1.2 Motility determination

Motility determination was carried out by Hanging drop method. The rhizobial isolates were found to be motile.

5.1.3 Presumptive test

5.1.3.1 Gram's staining

On Gram's staining, the isolates were found to be Gram negative. The rod shaped bacterial cells were pinkish in color when observed under oil immersion.

5.1.3.2 YEMA containing Congo red

R. l. biovar *phaseoli* lack the ability to absorb Congo red from a YEMA medium containing Congo-red. They showed rapid growth on YEMA media with acid production. All colonies were translucent and produced abundant amounts of gum.

5.1.3.3 YEMA containing BTB

They significantly acidified the YEMA with bromothymol blue, turning the medium from green to yellow. Thus, *R. leguminosarum biovar phaseoli* isolates were found to be acid producer.

5.1.3.4 Glucose peptone agar

No growth was observed in glucose peptone agar after incubation at 26 ± 2^0C for 3-5 days.

5.2 Authentication of *Rhizobium leguminosarum* biovar *phaseoli*

Plant infectivity test was performed in sterile soil using modified Jensen's nitrogen free medium for authentication of *R. leguminosarum* biovar *phaseoli* isolates. The *R. leguminosarum* biovar *phaseoli* isolates obtained from bean plant nodules were authenticated to be *R. leguminosarum* biovar *phaseoli* by examining their capacity to nodulate the bean plants under bacteriologically controlled condition. The bean plants inoculated with *R. leguminosarum biovar phaseoli* isolates developed nodules on their roots whereas the bean plants without inoculation did not have any nodules on their roots after 30 days.

5.3 Pot culture experiment

The experiment was carried out using sterile soil. The NPK content of the soil was determined before sowing of seed. The NPK content of soil was found to be 0.155 %, 0.063 %, and 0. 015 % respectively and pH of the soil was found to be 6.8.

5.3.1 Effect of dual inoculation along with vermicompost on plant growth in flowering stage

5.3.1.1 Shoot length

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on shoot length are presented in Table 3. It is evident from the table that shoot length was highest in combination of *P. indica* and *Rhizobium* followed by *P. indica* and *Rhizobium* and vermicompost, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* alone, *Rhizobium* alone treatments respectively over the control. The shoot length was

significantly more in combination of *P. indica* and *Rhizobium* as compared to *Rhizobium* and vermicompost, *P. indica* and vermicompost, , *P. indica* alone, *Rhizobium* alone treatments respectively and also with that of control pots. Although no clear cut differences were observed between *P. indica* and *Rhizobium* and vermicompost, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* alone, *Rhizobium* alone treatments, the differences was great from the controls. The percentage increase in shoot length inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 14.25%, 2.88%, 26.58%, 8.32%, 8.26% and 13.58% respectively over the control plants. The shoot length was significantly more in all the treatments as compared to control plants. The results were statistically significant.

5.3.1.2 Shoot weight

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on shoot weight are presented in Table 3. The fresh weight of shoot was significantly more in case of *P. indica* and *Rhizobium* as compared to rest of the treatment. The fresh weight of shoot in treatment with *P. indica* and *Rhizobium* and vermicompost, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* alone, and *Rhizobium* alone was superior as compared to control. The percentage increase in fresh weight of shoot inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 46.27%, 6.46%, 62.58%, 18.98%, 0.35% and 48.66% respectively over the control plants. The results were statistically significant.

The dry weight of shoot was highest in combination of *P. indica* and *Rhizobium* followed by *P. indica* and *Rhizobium* and vermicompost, *P. indica* alone, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *Rhizobium* alone and control treatments respectively. The percentage increase in dry weight of shoot inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 15.64%, 0.68%, 44.21%, 12.92%, 6.80% and 40.13% respectively over the control plants. Although all the treatments showed greater shoot dry weight than the control treatment, the results were not statistically significant.

5.3.1.3 Root length

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on root length are presented in Table 3. It is evident from the table that shoot length was highest in combination of *P. indica* and *Rhizobium* followed by *P. indica* alone, *P. indica* and *Rhizobium* and vermicompost, *P. indica* and vermicompost, *Rhizobium* alone, *Rhizobium* and vermicompost and control treatments respectively. The root length was significantly more in case of *P. indica* and *Rhizobium* as compared to rest of the treatment. The root length in treatment with *P. indica* and *Rhizobium* and vermicompost, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* alone, and *Rhizobium* alone was superior as compared to control. The percentage increase in root length inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium, P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 33.41%, 12.85%,46.26%, 22.07%, 7.47% and 35.86% respectively over the control plants The root system was much branched elaborate in mycorrhizal than non mycorrhizal ones. The results were statistically significant.

5.3.1.4 Root weight

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on root weight are presented in Table 3. The fresh weight of root was significantly more in case of *P. indica* and *Rhizobium* as compared to rest of the treatment. The fresh weight of root in treatment with *P. indica* and *Rhizobium* and vermicompost, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* alone, and *Rhizobium* alone was superior as compared to control. The percentage increase in fresh weight of root inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium, P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 84.72%, 20.72%, 112.50%, 43.05%, 11.11% and 69.44% respectively over the control plants. The results were statistically significant. The results were statistically significant.

The dry weight of root was highest in combination of *P. indica* and *Rhizobium* followed by *P. indica* and *Rhizobium* and vermicompost, *P. indica* alone, *P. indica* and vermicompost,

Rhizobium and vermicompost, *Rhizobium* alone and control treatments respectively. The percentage increase in fresh weight of root inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost 53.34%, 46.67%, 86.67%, 33.34%, 46.67%, and 53.34% respectively over the control plants. Although all the treatments showed higher root dry weight than the control treatment, the results were not statistically significant.

5.3.1.5 Nodulation

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on root nodulation are presented in Table 3. Dual inoculation with *P. indica* and *Rhizobium* and vermicompost showed the best nodulation followed by *P. indica* and *Rhizobium, Rhizobium* and vermicompost, *Rhizobium* alone, *P. indica* and vermicompost, and *P. indica* alone treatments respectively over the control treatments. The nodule number in case of dual inoculated plants was significantly more than the singly inoculated plants and control. Nodules in these plants were predominantly on tap root, large in size and on visible observation look healthy. The results were statistically significant.

Table 3: Effect of dual inoculation along with vermicompost on plant growth in flowering stage

Treatments	Shoot length* (cm)	Root length* (cm)	Shoot weight* (gm)		Root weight* (gm)		Nodule number*
			Fresh	Dry	Fresh	Dry	
T1	31.22±1.13	8.56±1.0	7.11±0.49	1.47±0.23	0.72±0.09	0.15±1.0	1.25±1.5
T2	35.67±2.45	11.42±0.90	10.40±0.87	1.70±0.24	1.33±0.40	0.23±0.90	19.17±2.59
T3	32.12±1.16	9.66±1.17	70.57±1.04	1.48±0.31	0.87±0.32	0.22±1.17	41.92±2.55
T4	39.52±3.0	12.52±2.31	11.56±1.23	2.12±0.59	1.53±0.18	0.28±2.31	52.72±2.85
T5	33.82±5.12	10.45±1.33	8.46±1.31	1.66±0.15	1.03±0.21	0.20±1.33	21.57±1.02
T6	33.80±2.98	9.20±1.75	7.13±0.66	1.57±0.19	0.80±0.19	0.22±1.75	43.90±2.75
T7	35.46±1.64	11.36±1.01	10.57±1.03	2.06±0.85	1.22±0.29	0.23±1.01	53.07±1.20

* Data represents mean of four replications

Table 4: Percentage increase of inoculated over control plants in flowering stage

Treatments	Shoot length (%)	Root length (%)	Shoot weight (%)		Root weight (%)	
			Fresh	Dry	Fresh	Dry
T1	-	-	-	-	-	-
T2	14.25	33.41	46.27	15.64	84.72	53.34
T3	2.88	12.85	6.46	0.68	20.84	46.67
T4	26.58	46.26	62.58	44.21	112.50	86.67
T5	8.32	22.07	18.98	12.92	43.05	33.34
T6	8.26	7.47	0.35	6.80	11.11	46.67
T7	13.58	35.86	48.66	40.13	69.44	53.34

T1- control, T2- *P. indica* alone, T3- *Rhizobium* alone, T4- *P. indica* and *Rhizobium*, T5- *P. indica* and vermicompost, T6- *Rhizobium* and vermicompost, T7- *P. indica* and *Rhizobium* and vermicompost

5.3.2 Effect of dual inoculation along with vermicompost on plant growth in the harvesting stage

5.3.2.1 Shoot length

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on shoot length in harvesting stage are presented in Table 5. The shoot length was highest in combination of *P. indica* and *Rhizobium* and vermicompost followed by *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* alone, *Rhizobium* alone treatments respectively over the control. The shoot length was significantly higher in case of *P. indica* and *Rhizobium* and vermicompost as compared to rest of the treatment. The shoot length in treatment with *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *P. indica* alone, *Rhizobium* and vermicompost, and *Rhizobium* alone was superior as compared to control. The percentage increase in shoot length inoculated with *P. indica* alone,

Rhizobium alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 6.25%, 0.43%, 8.03%, 7.86%, 6.77% and 21.97% respectively over the control plants. The results were statistically significant.

5.3.2.2 Shoot weight

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on shoot weight in harvesting stage are presented in Table 5. The shoot fresh weight was highest in combination of *P. indica* and *Rhizobium* and vermicompost followed by *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *P. indica* alone, *Rhizobium* and vermicompost, *Rhizobium* alone and control treatments respectively. Dual inoculation with or without vermicompost showed significantly highest shoot fresh weight as compared to rest of the treatment. The percentage increase in fresh weight of shoot inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* vermicompost, *P. indica* and *Rhizobium* and vermicompost was 31.49%, 18.83%, 47.66%, 34.25%, 25.68% and 54.32% respectively over the control plants. The results were statistically significant. The results were statistically significant.

The shoot dry weight was highest in combination of *P. indica* and *Rhizobium* and vermicompost followed by *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *P. indica* alone, *Rhizobium* and vermicompost, *Rhizobium* alone and control treatments respectively. Dual inoculation with or without vermicompost showed significantly higher shoot dry weight as compared to rest of the treatment. The percentage increase in dry weight of shoot inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 23.20%, 16.02%, 50%, 41.0%, 16.62% and 83.92% respectively over the control plants. The results were statistically significant.

5.3.2.3 Root length

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on root length in harvesting stage are presented in Table 5. The root length was highest in combination of *P. indica* and *Rhizobium* followed by *P. indica* and *Rhizobium* and vermicompost, *P. indica* and vermicompost, *P. indica* alone *Rhizobium* and vermicompost, *Rhizobium* alone treatments respectively over the control. The shoot length was significantly higher in case of *P. indica* and *Rhizobium as* compared to rest of the treatment. The percentage increase in root length inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 45.73%, 36.43%, 65.64%, 47.43%, 44.95% and 61.74% respectively over the control plants The results were statistically significant.

5.3.2.4 Root weight

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on root length in harvesting stage are presented in Table 5. The fresh weight of root was significantly more in case of *P. indica* and *Rhizobium* and vermicompost as compared to rest of the treatment. The fresh weight of root in treatment with *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* alone, and *Rhizobium* alone was superior as compared to control. . The percentage increase in fresh weight of root inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 84.70%, 8.23%, 129.41%, 88.20%, 15.29% and 152.94% respectively over the control plants. The results were statistically significant.

The dry weight of root was highest in combination of *P. indica* and *Rhizobium* and vermicompost followed by *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* alone, *Rhizobium* alone and control treatments respectively. Dual inoculation with or without vermicompost showed significantly higher root dry weight as compared to rest of the treatment. The percentage increase in fresh weight of root inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 57.14%, 33.33%,

109.52%, 76.19%, 57.14% and 147.61% respectively over the control plants. The results were statistically significant.

5.3.2.5 Pod number

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on pod number in harvesting stage are presented in Table 5. The pod number was highest in combination of *P. indica* and *Rhizobium* and vermicompost followed by *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *P. indica* alone, *Rhizobium* and vermicompost, *Rhizobium* alone and control treatments respectively. The pod number was significantly highest in dual inoculated plants with or with out vermicompost compared to rest of the treatment. The percentage increase in pod number inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 76.66%, 58.66%, 104%, 93.33%, 73.33% and 107.33% respectively over the control plants. The results were statistically significant.

5.3.2.6 Pod weight

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on pod weight in s harvesting stage are presented in Table 5. The fresh weight of pod was significantly higher in case of *P. indica* and *Rhizobium* and vermicompost as compared to rest of the treatment. All the treatment showed significant increase in pod fresh weight as compared to control. The percentage increase in fresh weight of pod inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 44.44%, 20.72%, 68.76%, 64.56%, 54.05% and 84.08% respectively over the control plants The results were statistically significant.

The pod dry weight was highest in combination of *P. indica* and *Rhizobium* and vermicompost followed by *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* alone, *Rhizobium* alone and control treatments respectively. Dual inoculation with or without vermicompost showed significantly higher pod dry weight as compared to rest of the treatment. The percentage increase in fresh weight of pod inoculated with

P. indica alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 37.77%, 28.88%, 102.22%, 104.44%, 77.78% and 160% respectively over the control plants The results were statistically significant.

Table 5: **Effect of dual inoculation along with vermicompost on plant growth in harvesting stage**

Treatments	**Shoot* length (cm)**	**Root length* (cm)**	**Shoot weight* (gm)**		**Root weight* (gm)**	
			Fresh	**Dry**	**Fresh**	**Dry**
T1	34.22±2.71	15.37±0.91	10.51±1.20	1.68±0.34	0.85±0.12	0.21±0.04
T2	36.36±1.56	22.40±2.75	13.82±1.05	2.07±0.32	1.57±0.45	0.33±0.04
T3	34.37±1.67	20.97±3.12	12.49±1.71	1.95±0.31	0.92±0.06	0.28±0.05
T4	36.97±1.09	25.46±1.27	15.52±0.88	2.52±0.53	1.95±0.20	0.44±0.05
T5	36.91±2.52	22.66±1.48	14.11±1.06	2.37±0.52	1.6± 0.56	0.37±0.04
T6	36.54±1.48	22.28±2.83	13.2±1.54	1.96±0.34	0.98±0.17	0.33±0.05
T7	41.74±2.30	24.86±1.66	16.22+0.88	3.09±0.86	2.15±0.36	0.52±0.03

Contd…

Table 5: **Effect of dual inoculation along with vermicompost on plant growth in harvesting stage (contd…)**

Pod number*	Pod weight* (gm)	
	Fresh	Dry
3±0.40	3.33±0.79	0.45±0.04
5.3±0.85	4.81±0.55	0.62±0.04
4.76±1.03	4.02±0.87	0.58±0.04
6.12±0.51	5.62±0.51	0.91±0.08
5.8±1.06	5.48±0.44	0.92±0.06
5.2±0.42	5.13±0.58	0.80±0.03
6.22±0.63	6.13±0.43	0.17±0.28

* Data represents mean of four replications

Table 6: Percentage increase of inoculated over control plants in harvesting stage

Treatments	Shoot length (%)	Root length (%)	Shoot weight (%)		Root weight (%)		Pod number (%)	Pod weight (%)	
			Fresh	Dry	Fresh	Dry		Fresh	Dry
T1	-		-	-	-	-	-	-	-
T2	6.25	45.73	31.49	23.2	84.70	57.14	76.66	44.44	37.77
T3	0.43	36.43	18.83	16.02	8.23	33.33	58.66	20.72	28.88
T4	8.03	65.64	47.66	50	129.41	109.52	104	68.76	102.22
T5	7.86	47.43	34.25	41.0	88.20	76.19	93.33	64.56	104.44
T6	6.77	44.95	25.68	16.62	15.29	57.14	73.33	54.05	77.78
T7	21.97	61.74	54.32	83.92	152.94	147.61	107.33	84.08	160

T1- control, T2- *P. indica* alone, T3- *Rhizobium* alone, T4- *P. indica* and *Rhizobium*,
T5- *P. indica* and vermicompost, T6- *Rhizobium* and vermicompost, T7-. *P. indica* and *Rhizobium* and vermicompost

5.3.3 Effect of dual inoculation along with vermicompost on nitrogen, phosphorous and potassium uptake by plants

5.3.3.1 Nitrogen

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on nitrogen uptake by shoot and root in harvesting stage are presented in Table 7. The Nitrogen content of shoot was significantly higher wherever dual inoculation was implemented as compared to rest of the treatment. Plants treated with *Rhizobium* alone or *Rhizobium* and vermicompost gave more nitrogen content as compared to plant treated with *P. indica* alone or *P. indica* and vermicompost. All the treatment showed significant increase in shoot nitrogen uptake as compared to control. The percentage increase in nitrogen content of shoot inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and

vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 18.285%, 49.14%, 71.42%, 48%, 58.28%, and 78.85% respectively over the control plants. The results were statistically significant. Similar results were observed with nitrogen content of root. The percentage increase in nitrogen content of root inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 22.52%, 53.15%, 121.16%, 34.23%, 68.46%, and 156.75% respectively over the control plants.

5.3.3.2 Phosphorous

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on phosphorous uptake by shoot and root in harvesting stage are presented in Table 7. The phosphorous content of shoot and root was highest in combination of *P. indica* and *Rhizobium* and vermicompost followed by *P. indica* and vermicompost, *P. indica* and *Rhizobium*, *P. indica* alone, *Rhizobium* and vermicompost, *Rhizobium* alone and control treatments respectively. Plants inoculated with *P. indica* singly or dually gave more phosphorous content than rest of the treatment. However, all the treatment showed significant increase in shoot and root phosphorous uptake as compared to control. The percentage increase in phosphorous content of shoot inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 39.39%, 24.24%, 60.60%, 27.27% and 72.72% respectively over the control plants. Similarly, the percentage increase in phosphorous content of root inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 42.10%, 15.78%, 57.89%, 68.42%, 31.57% and 105.26% respectively over the control plants. The results were statistically significant.

5.3.3.3 Potassium

The data on the effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on shoot and root potassium uptake in harvesting stage are presented in Table 7. It is evident from the table that shoot length was highest in combination of *P. indica* and *Rhizobium* followed by *P. indica* and *Rhizobium* and vermicompost, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* alone, *Rhizobium* alone treatments respectively over the control. Dual inoculated plants with or without vermicompost showed significantly higher shoot and root potassium uptake as compared to rest of the treatment. The percentage increase in potassium content of shoot inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 21.23%, 13.01%, 43.15%, 36.64%, 25.34% and 51.71% respectively over the control plants. Similarly, the percentage increase in potassium content of root inoculated with *P. indica* alone, *Rhizobium* alone, *P. indica* and *Rhizobium*, *P. indica* and vermicompost, *Rhizobium* and vermicompost, *P. indica* and *Rhizobium* and vermicompost was 24.10%, 12.82%, 49.74%, 42.56%, 33.38%, and 66.15% respectively over the control plants. The results were statistically significant.

Table 7: Effect of dual inoculation along with vermicompost on nitrogen, phosphorous and potassium uptake by plants in harvesting stage

Treatments	Nitrogen (%)*		Phosphorous (%)*		Potassium (%)*	
	Shoot	Root	Shoot	Root	Shoot	Root
T1	1.75	1.11	0.33	0.19	2.92	1.95
T2	2.07	1.36	0.46	0.27	3.54	2.42
T3	2.61	1.70	0.41	0.22	3.30	2.20
T4	3.0	2.46	0.53	0.30	4.18	2.92
T5	2.59	1.49	0.55	0.32	3.99	2.78
T6	2.77	1.87	0.42	0.25	3.66	2.64
T7	3.13	2.85	0.57	0.39	4.43	3.25

Table 8: Percentage increase of inoculated over control plants

Treatments	Nitrogen (%)*		Phosphorous (%)*		Potassium (%)*	
	Shoot	Root	Shoot	Root	Shoot	Root
T1	-		-	-	-	-
T2	18.28	22.52	39.39	42.10	21.23	24.10
T3	49.14	53.15	24.24	15.78	13.01	12.80
T4	71.42	121.16	60.60	57.89	43.15	49.74
T5	48	34.23	66.67	68.42	36.64	42.56
T6	58.28	68.46	27.27	31.57	25.34	33.38
T7	78.85	157.75	72.72	105.26	51.71	66.15

* Data represents mean of four replications

T1- control, T2- *P. indica* alone, T3- *Rhizobium* alone, T4- *P. indica* and *Rhizobium*, T5- *P. indica* and vermicompost, T6- *Rhizobium* and vermicompost, T7-. *P. indica* and *Rhizobium* and vermicompost

5.3.4 Percent root colonization

The data effect of dual inoculation of *R. leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on Percent root colonization by is given in table 9. It is evident from the table that Percent root colonization by *P. indica* was highest in combination of *P. indica* and *Rhizobium* and vermicompost followed by *P. indica* and *Rhizobium*, *P. indica* and vermicompost, and *P. indica* alone treatment respectively. Plant receiving the dual inoculation of *P. indica* and *Rhizobium* with or without vermicompost showed higher Percent root colonization by *P. indica* than the plant receiving single inoculation of *P. indica* and control both in flowering (30 days) and harvesting

stage (45 days). Percent root colonization by *P. indica* was found to be increased in fruiting stage than in flowering stage.

Table 9: Effect of dual inoculation along with vermicompost on percent root colonization

Treatments	**Percent root colonization (%)***	
	30 days	**50 days**
T1	0	0
T2	65	85
T3	0	0
T4	72	90
T5	69	85
T6	0	0
T7	75	95

*Data represents average of 20 root segments

T1- control, T2- *P. indica* alone, T3- *Rhizobium* alone, T4- *P. indica* and *Rhizobium*, T5- *P. indica* and vermicompost, T6- *Rhizobium* and vermicompost, T7-. *P. indica* and *Rhizobium* and vermicompost

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

This study was conducted during February 2005 to July 2005 in the biotechnology laboratory, RONAST for the purpose of studying the effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* on the growth of *Phaseolus vulgaris* L. grown in soil treated with vermicompost.

6.1.1 Identification of *Rhizobium leguminosarum* biovar *phaseoli*

The identification of *R. leguminosarum* biovar *phaseoli* was based on the colony characteristics, cell morphology, grams staining, and presumptive test. The colony was circular, convex, with smooth margin and surface, milky- to watery- translucent (colorless), elevation was found to be raised and mucilaginous, usually 2-3 mm in diameter within 3-5 days on of incubation YEMA media. The most dominate type of colony was large mass of white slime that covered more than 50% of the plate, with large patches of single colonies of the same morphology. In addition, there were two other colony types that probably originated from the nodule and are also capable of growth in this medium under the same incubation techniques. Hence, we cannot exclude the possibility of contamination from the organisms in the soil. Schwinghamer and Dudman (1980) suggested that the colony type in culture provides the much convenient means of strain recognition. Somasegaran and Hoben (1994) stated that the strains of *Rhizobium* can be described according to their growth in solid and liquid media. The size, shape, color, and texture of colonies and the ability to alter the pH of the medium are generally stable characteristics in defining strain or isolates. The rhizobial isolates was found to be motile. The motility was determined by Hanging drop method.

On gram staining, the rhizobial isolates were found to be gram negative, rod shaped bacteria. The rhizobial isolates didn't absorb Congo red when YEMA plates containing Congo red were incubated in dark. Colonies remained white, opaque, or occasionally pink. In YEMA-BTB media,

the rhizobial isolates showed an acid reaction, turning the medium yellow which is only produced by fast growing rhizobia. On glucose peptone agar, the rhizobial isolates showed no growth. Thus, based on the colony characteristics, cell morphology, grams staining, and presumptive test, the rhizobial isolates isolated from bean nodules can be identified as of *R. leguminosarum* biovar *phaseoli.*

The rhizobial isolates isolated from bean nodules were authenticated as *R. leguminosarum* biovar *phaseoli* by performing plant infectivity test in sterile soil using modified Jensen's nitrogen free medium. Somasegaran and Hoben (1994) stated the importance of determining that the isolate is a pure culture which can form nodules on legume roots cannot be over stressed. It proves the authenticity of a pure culture of *Rhizobium*. The bean plants inoculated with *R. l.* biovar *phaseoli* isolates developed nodules on their roots whereas the bean plants without inoculation did not have any nodules on their roots after 30 days. The inoculated bean plants were healthy and the root nodules were pink in color.

6.1.2 Pot culture experiments

The experiment was carried out using sterile soil so that all the indigenous microorganisms would kill out and exact effect of dual inoculation on the plant growth could be seen. The experimental soil and sand were mixed in the ratio of 2:1. This was done to make the soil less compact, which improves the soil aeration as well as facilitates the handling of experimental sets during uprooting the plants and washing of the roots The growth parameters selected to study the effect of *R. leguminosarum* biovar *phaseoli and P. indica* and vermicompost either individually or in combination were number of nodules, shoot length, shoot weight, root length, root weight, number of pods , pod weight, shoot and root nitrogen uptake, shoot and root phosphorous uptake, and shoot and root potassium uptake respectively.

6.1.2.1 Effect of dual inoculation along with vermicompost on plant growth

Legumes form tripartite symbioses with arbuscular mycorrhizal fungi (AMF) and rhizobia which influence plant productivity. In Rhizobia-AM-legume tripartite symbiosis relationships, nodulation of rhizobia and establishment of AM often occur simultaneously and synergically. Besides, rhizobia provide fixed nitrogen not only to the plant, but also to the fungus. Moreover

Rhizobia can also assist in mobilizing nutrients from the soil and improving the growth of infected plants. Reports stated that the presence of AM fungi is known to enhance nodulation and N fixation by legumes (Amora-Lazecano et al., 1998; Johansson et al., 2004). AM fungi and rhizobia often act synergistically on infection rate, mineral nutrition, and plant growth. In our experiment, we observed the effect of dual inoculation of *R l* biovar *phaseoli* and *P. indica* on the growth of *Phaseolus vulgaris* L. grown in soil treated with vermicompost.

The numbers of nodules, shoot length, shoot weight, root length and root weight was recorded during the flowering period and in the harvesting period, shoot length, shoot weight, root length, root weight, number of pods , pod weight, shoot and root nitrogen uptake, shoot and root phosphorous uptake, and shoot and root potassium uptake was recorded respectively.

It has been found that the nodulation and nitrogen fixation attains its peak point around the flowering period and beyond which nodule senescence takes place. Dual inoculation with *P. indica* and *Rhizobium* and vermicompost showed the best nodulation .This might be due to the reason that vermicompost contain numerous component required for microbial growth and may increase the activity of soil microorganism including rhizobial growth. Rhizobial inoculation positively influenced nodulation of plants. The nodule number in case of plants inoculated with *Rhizobium* individually or in combination with vermicompost was significantly more than the plants inoculated with *P. indica* individually or in combination with vermicompost and control plants. This might be due to the reason that *Rhizobium* interacted more synergistically with the legume as compared to *P. indica*. In case of dual inoculation, both IAA (due to rhizobia) and photosynthate (due to *R. leguminosarum* biovar *phaseoli* and *P. indica*) might have increased due to availability of more nitrogen and phosphorous for normal physiological events and ultimately increased nodule number. Bhuiyan et al., (1998) reported the significant increase in nodule number, nodule weight in soybean by *Bradyrhizobium* inoculation. Nodules in these plants were predominantly on tap root, large in size, pink and on visible observation look healthy indicating active nitrogen fixation. The number of nodules in control should be zero but the presence of small number of inactive nodules might be due to the contamination through water or there might be left few spores of bacteria during autoclaving. *Bacillus spp* are among the bacteria most often isolated from plant tissues and they have been found to exist in soybean seed and tissue and could

survive common surface sterilization procedures (Tenne and Sinclair, 1977). Spore forming *Bacillus* strains were isolated from surface disinfected soybean root nodule and showed growth promoting effects on soybean plants (Tenne and Sinclair, 1977).

In the flowering stage, shoot length was highest in combination of *P. indica* and *Rhizobium*. Although no clear cut differences were observed between other treatments, the differences were significantly higher from the controls. In the harvesting stage, the shoot length was highest in plants receiving dual inoculation along with vermicompost. The control plants became shorter during this time whereas *Rhizobium* treated or *P. indica* treated or dual inoculated plants with or without vermicompost grew significantly better. Better positive effect of inoculation was observed during harvesting stage. This can be attributed to the fact that symbiosis between *P. indica* and *Rhizobium* was well established in harvesting stage than in flowering stage. Analysis of shoot fresh weight in flowering stage indicates that the plant receiving dual inoculation with or without vermicompost grew much better than plant receiving single inoculation and plant receiving single inoculation grew much better than control plants. The results were statistically significant at 5% level of significance. Although all the treatments showed greater shoot dry weight than the control treatment, the results were not statistically significant. In the harvesting stage, dual inoculation with or without vermicompost showed significantly highest shoot fresh weight and dry weight as compared to rest of the treatment. Singh *et al*. (2001) reported significant increment in shoot length when *Spilanthes calva* and *Withania somnifera* were inoculated with *P. indica*. Rai and Varma (2005) reported a significant root and shoot biomass increment by 331.42% and 140.9% respectively over controls when *Adhatoda vasica* Nees was inoculated with *P. indica*

The root length was significantly more in case of *P. indica* and *Rhizobium* as compared to rest of the treatment in the flowering as well as in harvesting stage. The root system was much branched and elaborate in mycorrhizal than non mycorrhizal ones. In the flowering stage, the fresh weight of root was significantly more in case of *P. indica* and *Rhizobium* as compared to rest of the treatment. Although all the treatments showed higher root dry weight than the control treatment, the results were not statistically significant. A significantly higher value for root fresh weight and dry weight was recorded for those receiving dual inoculation with or without vermicompost in the

harvesting stage. This might be due to the high number of lateral roots and root hairs, which eventually increased the root fresh weight and dry weight. Singh (2004) reported that *P. indica* inoculated maize plants produced more root dry matter than control plants. Singh *et al*. (2001) found that the over all root biomass of the *P. indica* inoculated *Spilanthes calva* and *Withania somnifera* plants was higher than the corresponding controls. In addition, the fresh and dry weight of the roots of *P .indica* treated *Azadirachta indica* plants showed 31.03 and 66.66% increase over corresponding control respectively. The results were statistically significant at 5% level of significance.

All treatments produced significantly more pods as compared to control. Plants inoculated with *P. indica* and *Rhizobium* was significantly superior with respect to pod production. Maximum yield was noted wherever; dual inoculation along with vermicompost was implemented. Dual inoculation with or without vermicompost showed significantly higher pod fresh weight and dry weight as compared to rest of the treatment. The results were statistically significant.

Hence, it can be concluded that in our experiment, dual inoculation along with vermicompost was found to be more effective than rest of the treatment. In general, better positive effects of inoculation were observed in vermicompost treated plants than in vermicompost non treated plants suggesting higher dependency of vermicompost treated plants on these symbionts for better growth and development. The high percentage of available nutrients in vermicomposts and acceleration of mycorrhizal development through earthworms may be the factors responsible for enhanced plants growth with vermicomposts. A similar result was observed by Aryal and Fujita (2003); they reported that rhizobia and AM fungal inoculation improve growth, nutrient uptake of bean under organic fertilization. They reported that the dual inoculation significantly increased nodulation, shoot and root dry weight, shoot N and P, pod yield of *Phaseolus vulgaris* L. compared to control or singly inoculated plants. Under fertilized conditions, nodulation, pod yield, AMF colonization, and spore population were generally more pronounced in dually inoculated organic plants than in chemical plants and control plants. Neupane (2003) studied the effect of dual inoculation of AM fungi and *Bradyrhizobium japonicum* in soybean and found that the dual inoculation enhanced the growth, nodulation, nitrogen and phosphorus content and specific P uptake in soybean plant. Raut and Ghonsikar (1992) studied the effect of dual

inoculation of *Rhizobium* and AM fungi on pigeon pea and found that dual inoculation was more beneficial than the single inoculation of *Rhizobium* or AM fungi to stimulate growth parameters such as nodulation, root shoot growth, fresh and dry weight, yield, and nitrogen uptake.

6.1.2.2 Effect of dual inoculation along with vermicompost on nitrogen, phosphorous and potassium uptake by plants

Nitrogen is the most important nutrient input required for crop production. In the soils, nitrogen is available for plants in the inorganic form (as NH_4^+ and NO_3^-) but most N is in the organic form, often occurring in complex molecules (Carling et al., 1978; Oliver et al., 1983). Adequate level of nitrogen is essential for proper plant growth. BNF by Legume-*Rhizobium* Symbiosis is major source of nitrogen in agriculture for increasing the productivity of legumes and improving soil fertility (Gurung and Serchan, 1997). Reports stated that the presence of AM fungi is known to enhance nodulation and N-fixation by legumes (Amora-Lazecano et al., 1998; Johansson et al., 2004), thus enhancing the N-uptake plants. In our experiment, the nitrogen content of shoot and root was significantly higher wherever dual inoculation was implemented as compared to rest of the treatment. Plants treated with *Rhizobium* alone or *Rhizobium* and vermicompost gave more nitrogen content as compared to plant treated with *P. indica* alone or *P. indica* and vermicompost. This might be due to the reason that plants inoculated with *Rhizobium* developed more nodules and nodule is the site of nitrogen fixation which attributed to high uptake of nitrogen by plants. Rhizobial inoculation positively influenced nitrogen uptake of plants. Similar results were obtained by Biswas et al., (2000), Shalaby and Hanna (1998). Biswas et al., (2000) reported that rhizobial inoculation significantly increased uptake of N, P, K, and Fe by rice plants compared with uninoculated controls. Shalaby and Hanna (1998) reported that the dual inoculation of *Bradyrhizobium japonicum* either with *Pseudomonas fluorescens* or endomycorrhizal fungi *Glomus mosseae* in soybean significantly increased nitrogen uptake in plants and weight of soybean grain by 20%- 30% and 14%-17% as compared to the single inoculation.

Phosphorous is an important element which influences Rhizobia-AM-legume tripartite symbiosis. The importance of phosphorous in Rhizobia-AM-legume tripartite symbiosis cannot be overstated because 16 ATP molecules are required for the reduction of one molecule of nitrogen to ammonia (Bieleski and Ferguson, 1975; Theodorou and Plaxton, 1993). Phosphate is present in the soil in

different forms, with an organic and a mineral pool (Holford, 1997). The form of P most readily accessed by plants is Pi (inorganic Phosphate), because of different factors like adsorption, precipitation, or conversion into organic forms, 80-99% of the phosphorus is barely available for plant uptake. For this reason, the mycorrhizal symbiosis is important for plant P-supply since the fungal hyphae extend into the soil and allow roots to explore a larger soil volume (Smith and Read, 1997). In our experiment, the phosphorous content of shoot and root was highest in combination of *P. indica* and *Rhizobium* and vermicompost. Mycorrhizal inoculation positively influenced phosphorous uptake of plants. Plants inoculated with *P. indica* singly or dually gave more phosphorous content than rest of the treatment. This can be attributed to the fact that *P. indica* mediates uptake of phosphorous from the medium and translocates to the host in an energy-dependent process. *P. indica* produces significant amounts of acid phosphates for the mobilization of broad range of insoluble forms of phosphate, enabling the host plant the accessibility of adequate phosphorous from immobilized reserves in the soil (Varma et al., 2000). The increased phosphorus uptake conferred by the AM symbiosis is beneficial for the functioning of the nitrogenase enzyme of the bacterial symbionts, leading to increased nitrogen fixation and consequently promotion of root and mycorrhizal development (Amora-Lazcano et al., 1998; Johanson et al., 2004; Rabie et al., 2005)

Potassium promotes the root growth in plants and assists the absorption of minerals and other elements. It also helps plants to develop resistance to disease and pests and to cope with adverse weather condition. In our experiment, dual inoculated plants with or without vermicompost showed significantly higher shoot and root potassium uptake as compared to rest of the treatment.

Hence, in our experiment dual inoculation with *P. indica* and *Rhizobium* along vermicompost was found to increase NPK uptake by plants as compared to single inoculation and control. It is already known that these symbioses, rather than compete with each other for plant resources, complement each other by enhancing the plant's nutrient acquisition strategies. Acharya (1997) reported that vermicompost contains higher amount of NPK and it will help to improve the crop yield by increasing soil fertility and water retention ability and increase NPK uptake by plants.

Hence it can be concluded that the result of our experiment is consistent with the fact that mycorrhizal and nodule symbioses often act synergistically on infection rate, mineral nutrition, and plant growth (Belimov et al., 1999; Patreze and Cordeiro, 2004).

6.1.2.3 Percent root colonization

Measurements of the extent to which roots are mycorrhizal have in past been used to indicate the abundance of mycorrhizal fungi in soil (Sparling and Tinker, 1978; Hayman and Stovold, 1979). Singh (2004) stated that root colonization denotes an active symbiotic phase. In our experiment, percent root colonization by *P. indica* was significantly higher in plants inoculated with *Rhizobium* and *P. indica* with or without vermicompost. Control and the plants without *P. indica* inoculation were devoid of any root colonization as the soil was sterile. Percent root colonization by *P. indica* was found to be increased in fruiting stage than in flowering stage. Spore number in agricultural soils can be affected by management practices. First, both nitrogen and phosphorous fertilizers can depress spore numbers (Hayman, 1980) but the fertilizers can increase spore numbers where plant growth is increased. Rai and Verma (2005) reported that rapid proliferation of roots was recorded in *A. vasica* with an important root colonization estimated to 95% after 6 months. Singh (2004) reported highest root colonization in maize plants inoculated with *P. indica* than other mycorrhiza.

6.2 Conclusion

Hence, the effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* on the growth of *Phaseolus vulgaris* L. grown in soil treated with vermicompost was studied conducting pot culture experiment under sterile condition. In the flowering stage, dual inoculation with *Rhizobium* and *P. indica* significantly increased shoot length, root length, shoot fresh weight, and root fresh weight as compared to rest of the treatment. However, the results were not statistically significant for shoot and root dry weight, although all the treatments showed greater shoot and root dry weight than the control treatment. Similarly, in the harvesting stage, dual inoculation along with vermicompost significantly increased shoot length, root length, shoot weight, root weight, pod number and pod weight as compared to rest

of the treatment. Dual inoculated plants with or without vermicompost showed significantly higher shoot and root NPK uptake as compared to rest of the treatment. Percent root colonization by *P. indica* was significantly higher in dual inoculated plants with or without vermicompost. Percent root colonization by *P. indica* was found to be increased in fruiting stage than in flowering stage. Further, study on the effect of dual inoculation should be carried out on growth of different legume crop under varying agro climatic conditions in the fields.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

1. A pot culture experiment was conducted to study the effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* on the growth of *Phaseolus vulgaris* L. grown in soil treated with vermicompost. The effect of dual inoculation was studied with respect to number of nodules, shoot length, shoot weight, root length, root weight, number of pods, pod weight, shoot and root nitrogen uptake, shoot and root phosphorous uptake, and shoot and root potassium uptake and mycorrhizal colonization respectively.

2. In the flowering stage, dual inoculated plants with or without vermicompost showed the best nodulation compared to other treatments. Rhizobial inoculation positively influenced nodulation of plants. Dual inoculation with *Rhizobium* and *P. indica* significantly increased shoot length, root length, shoot fresh weight, and root fresh weight as compared to rest of the treatment. However, the results were not statistically significant for shoot and root dry weight, although all the treatments showed greater shoot and root dry weight than the control treatment.

3. In the harvesting stage, dual inoculation along with vermicompost significantly increased shoot length, root length, shoot weight, and root weight as compared to rest of the treatment. The root system was much branched and elaborate in mycorrhizal than non mycorrhizal ones. Maximum yield was noted wherever; dual inoculation along with vermicompost was implemented. A significantly higher value for pod fresh weight and dry weight was recorded for those receiving dual inoculation along with vermicompost. In general, better positive effects of dual inoculation were observed in plants grown in vermicompost treated soil than vermicompost non treated soil.

4. Dual inoculated plants with or without vermicompost showed significantly higher shoot and root NPK uptake as compared to rest of the treatment. Rhizobial inoculation positively

influenced nitrogen uptake of plants whereas mycorrhizal inoculation positively influenced phosphorous uptake of plants.

5. Percent root colonization by *P. indica* was significantly higher in dual inoculated plants with or without vermicompost. Control and the plants without *P.indica* inoculation were devoid of any root colonization. Percent root colonization by *P. indica* was found to be increased in fruiting stage than in flowering stage.

7.2 Recommendations

1. Study on the effect of dual inoculation should be carried out on growth of different legume crop under varying agro climatic conditions in the fields.

2. *P. indica* can be used for the field experiments like in plants of economic importance under agricultural climatic conditions. Further transformation studies can be made to make *P. indica* resistant against temperature, fungicides, insecticides, pesticides, heavy metals, antibiotics, salinity, etc.

3. The screening of most effective *R. leguminosarum* biovar *phaseoli* strains could be the major break through for increasing bean production in Nepal. Thus, still extensive research works should be carried out to screen most effective strain. Further, improved strains and new species of nitrogen fixing microorganisms should be investigated in order to reduce the use of chemical fertilizer in fields.

4. The microbes present in the vermicompost produce antibiotics and toxic substances that show antagonistic activity against certain plant pathogens. Thus study of antagonistic activity of vermicompost against plant pathogens should be carried out.

5. Comparative study on the use of chemical fertilizer and vermicompost should be carried out.

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APPENDIX-I

LIST OF MATERIALS

1.1 Equipments

1. Autoclave (Life steriware, India)
2. Electric Balance (Denver instrument Co. Pvt. Ltd., USA)
3. Flame photometer
4. Grinder (Relsh GmbH, West Germany)
5. Hot air oven (Universal, India)
6. Incubators (Universal, India)
7. Kjeldhal apparatus
8. Microscope (Olympus, Japan)
9. Laminar flow cabinet (Bassaire, 96 Module, England)
10. pH meter (TOA pH meter HM-10P)
11. Refrigerator (Minsk 15)
12. Spectrophotometer
13. Vortex shaker (AX 2000)
14. Water bath shaker (Narang, India)

1.2 Microbiological media

1. Glucose peptone Agar
2. Jensen's free nitrogen medium
3. Potato dextrose agar (Hi -media)
4. Yeast mannitol agar- Bromothymol blue (YEMA-BTB)
5. Yeast mannitol agar- Congo red (YEMA-CR)
6. Yeast mannitol broth

1.3 Glasswares

1. Beakers
2. Conical flasks
3. Concave slides
4. Burette
5. Digestion apparatus
6. Distillation apparatus
7. Funnel
8. Kjeldhal digestion flasks
9. Volumetric flasks
10. Measuring cylinder
11. Petri plates
12. Pipettes
13. Reagent bottles
14. Screw capped test tubes
15. Microscopic slides

1.4 Miscellaneous

1. Aluminum foil
2. Inoculating loop
3. Soap
4. Blotting paper
5. Inoculating wire
6. Tissue paper for microscopy
7. Cotton roll
8. Measuring scale
9. Transport tray
10. Cork and borer
11. Parafilm roll
12. Vaseline
13. Dropper
14. Sticker

1.5 Chemicals/Reagents

1. Bromothymol blue
2. Boric acid 4%
3. Concentrated sulphuric acid
4. Congo red
5. Crystal violet
6. Digestion mixture :grind and mix 10 grams of copper sulphate with 200 grams of sodium sulphate
7. Ethanol
8. Hydrochloric acid
9. Iodine
10. Lactophenol
11. Mixed indicator: Dissolve 0.5 gram bromocresol green and 0.1 gram methyl red in 100 ml of 95% ethanol
12. Nitric acid
13. Potassium hydroxide
14. Safranin
15. Sodium hydroxide 40%
16. Standard K solution
17. Standard P solution
18. Trypan blue
19. Vandomolybdate

APPENDIX-II

I. COMPOSITION AND PREPARATION OF MEDIA

1. Glucose peptone agar

Ingredients	Gram/liter
Peptone	10.0
Bromocresol purple (1% alcoholic solution)	10.0
Agar	15.0
Distilled water	1 litre

The medium was boiled to dissolve the medium completely. It was then sterilized by autoclaving at 15 lbs. pressure (121^0C) for 15 minutes.

2. Jensen's free nitrogen medium

Ingredients	Gram/liter
$CaHPO_4$	1.0
K_2HPO_4	0.2
$MgSO_4.7H_2O$	0.2
NaCl	0.2
$FeCl_3$	0.1
*Trace element stock solution	0.1
Distilled water	1 litre

*A stock solution of trace elements consists of the following:

Ingredients	Gram/liter
H_3BO_3	2.86
$MgSO_4.4H_20$	2.03
$ZnSO_4.7H_2O$	0.22
$CuSO_4.5H_20$	0.08
$Na_2MoO_4.2H_2O$	0.14

Stock solution of trace elements was prepared and stored at 4^0C. All the constituents of modified Jensen's N-free medium were mixed one by one avoiding any precipitation and volume was adjusted to 1 liter. It was then sterilized by autoclaving at 15 lbs. pressure (121^0C) for 15 minutes.

3. Potato Dextrose Agar (PDA)

Ingredients	Gram/liter
Potato peel	200.0
Dextrose	20.0
Agar	20.0
Distilled water	1000 ml

The medium was boiled to dissolve the medium completely. It was then sterilized by autoclaving at 15 lbs. pressure (121^0C) for 15 minutes.

4. Yeast mannitol agar- Congo Red (YEMA-CR)

Ingredients	Gram/liter
Mannitol	10.0
Dipotassium hydrogen phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Calcium carbonate	4.0
Yeast extract	1.0
Congo red (1% solution)	2.5 ml
Agar	20.0
Distilled water	1000 ml

The medium was boiled to dissolve the medium completely. It was then sterilized by autoclaving at 15 lbs. pressure (121^oC) for 15 minutes.

5. Yeast mannitol agar- Bromothymol Blue (YEMA-BTB)

Ingredients	Gram/liter
Mannitol	10.0
Dipotassium hydrogen phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Calcium carbonate	4.0
Yeast extract	1.0
Bromothymol blue (1%solution)	2.5 ml
Agar	20.0
Distilled water	1000 ml

The medium was boiled to dissolve the medium completely. It was then sterilized by autoclaving at 15 lbs. pressure (121^0C) for 15 minutes.

6. Yeast mannitol broth

Ingredients	Gram/liter
Mannitol	10.0
Dipotassium hydrogen phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Calcium carbonate	4.0
Yeast extract	1.0
Distilled water	1000 ml

The medium was boiled to dissolve the medium completely. It was then sterilized by autoclaving at 15 lbs. pressure (121^{0}C) for 15 minutes.

II. COMPOSITION AND PREPERATION OF REAGENTS

1. Gram's staining reagent

(a) Crystal Violet solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml
Distilled Water (D/W)	1 liter

Preparation: In a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Finally the volume was made 1 litre by adding D/W.

(b) Lugol's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

Preparation: To 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1 liter by adding D/W.

(c) Acetone-Alcohol Decoloriser

Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled Water	25 ml

Preparation: To 25 ml D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

Preparation: In a clean piece of paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then 1 litre D/W was added to the bottle and mixed well until safranin dissolved completely.

2. Root staining reagent

a. Lactophenol

Phenol	150g
Water	150ml
Lactic acid	125ml
Glycerol	125ml

b. Trypan blue

Trypan blue	0.1g
Lactophenol	100ml

APPENDIX-III

STATISTICAL ANALYSES

A. Flowering stage

1. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on shoot length (cm)

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	31.95	31.30	29.60	32.05	31.22
T2	38.40	36.80	32.75	34.80	35.68
T3	31.15	33.35	32.90	31.10	32.12
T4	35.15	41.70	41.25	40.0	39.52
T5	29.45	29.65	39.80	36.40	33.82
T6	37.45	30.90	34.35	32.25	33.81
T7	35.19	36.30	33.30	37.10	35.47

Test Statistics is one way ANOVA which was done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	179.587	29.931	3.769	0.03
Within groups	21	166.792	7.942		
Total	27	346.379			

Since p-value is less than 0.05. i.e., ℑ|at 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on shoot length at ℑ|= 5% level of significance.

2. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on root length (cm)**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	8.70	8.50	9.75	7.30	8.56
T2	12.45	11.50	10.25	11.50	11.42
T3	9.25	10.95	10.20	8.25	9.66
T4	12.20	11.90	10.25	15.75	12.52
T5	10.70	8.50	11.30	11.30	10.45
T6	9.30	9.20	7.0	11.30	9.20
T7	9.85	11.70	11.85	12.05	11.36

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments**.**

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	47.336	7.889	3.823	0.02
Within groups	21	43.336	2.064		
Total	27	9.0672			

Since p-value is less than 0.05. i.e., ℑ|= 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on root length at ℑ|= 5% level of significance.

3. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on fresh weight of shoot (gm).

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	7.75	6.90	7.20	6.60	7.11
T2	10.95	11.20	9.25	10.20	10.40
T3	7.95	6.35	8.80	7.20	7.57
T4	7.95	10.90	9.45	10.0	11.56
T5	8.75	6.60	8.80	9.70	8.46
T6	7.75	6.20	7.40	7.20	7.13
T7	10.25	9.25	11.40	11.40	10.57

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments

<table>
<tr><th>Source of variation</th><th>Degrees of freedom</th><th>Sum of squares</th><th>Mean sum of squares</th><th>F value</th><th>Significance</th></tr>
<tr><td>Between groups</td><td>6</td><td>53.817</td><td>8.970</td><td rowspan="3">9.122</td><td rowspan="3">0.0</td></tr>
<tr><td>Within groups</td><td>21</td><td>20.648</td><td rowspan="2">0.983</td></tr>
<tr><td>Total</td><td>27</td><td>74.465</td></tr>
</table>

Since p-value is less than 0.05. i.e., ℑ|= 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on fresh weight of shoot at ℑ|= 5% level of significance.

4. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on dry weight of shoot (gm).**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	1.40	1.25	1.45	1.80	1.47
T2	1.45	2.0	1.55	1.80	1.70
T3	1.90	1.40	1.15	1.50	1.47
T4	1.25	2.30	2.60	2.35	2.12
T5	1.72	1.50	1.85	1.60	1.66
T6	1.65	1.75	1.30	1.60	1.57
T7	1.85	3.20	2.05	1.15	2.06

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	1.677	0.279	1.444	0.245
Within groups	21	4.065	0.194		

Total	27	5.742			

Since p-value is greater than 0.05. i.e., ℑ|= 5% level of significance. We accept H_0 and reject H_1. Therefore, there is no significant difference between treatments. Therefore, different methods of treatment have no significant effect on dry weight of shoot at ℑ|= 5% level of significance.

5. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on fresh weight of root (gm).**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	0.65	0.75	0.65	0.85	0.72
T2	1.50	1.30	0.80	1.75	1.33
T3	0.8	0.45	1.15	1.10	0.87
T4	1.50	1.30	1.75	1.60	1.53
T5	1.20	0.75	1.0	1.20	1.03
T6	1.0	0.55	0.75	0.90	0.80
T7	1.45	0.80	1.30	1.35	1.22

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	2.179	0.363		

Within groups	21	1.438	0.068	5.304	0.002
Total	27	3.617			

Since p-value is lesser than 0.05. i.e., ℑ|= 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on fresh weight of root at ℑ|= 5% level of significance.

6. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on dry weight of root (gm).**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	0.10	0.15	0.15	0.20	0.15
T2	0.25	0.20	0.30	0.20	0.23
T3	0.25	0.20	0.20	0.25	0.22
T4	0.25	0.30	0.40	0.20	0.28
T5	0.15	0.30	0.15	0.20	0.20
T6	0.30	0.25	0.15	0.20	0.22
T7	0.20	0.30	0.30	0.15	0.23

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments

Source of	Degrees of	Sum of	Mean sum of	F value	Significance

variation	freedom	squares	squares		
Between groups	6	0.04179	0.069	1.814	0.145
Within groups	21	0.08063	0.038		
Total	27	0.12242			

Since p-value is greater than 0.05. i.e., ℑ|= 5% level of significance. We accept H_0 and reject H_1. Therefore, there is no significant difference between treatments. Therefore, different methods of treatment have no significant effect on dry weight of root at ℑ|= 5% level of significance.

7. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on number of nodules.**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	0	2	3	0	1.25
T2	20.40	21.20	19.70	15.40	19.17
T3	40.20	41.70	45.60	40.20	41.92
T4	50.80	54.70	49.80	44.60	52.72
T5	21.20	22.80	21.90	20.40	21.57
T6	42.80	47.40	44.50	40.90	43.90
T7	54.50	51.80	53.60	52.40	53.07

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	9277.145	1546.191	321.016	0.0
Within groups	21	101.148	4.817		
Total	27	9378.293			

Since p-value is lesser than 0.05. i.e., ℑ|at 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on number of nodules at ℑ|= 5% level of significance.

B. Fruiting stage

8. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on shoot length(cm)

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	33.40	32.50	32.75	38.25	34.22
T2	37.72	35.65	34.50	37.60	36.36
T3	33.55	32.50	36.25	35.21	34.37
T4	36.25	37.50	35.90	38.25	36.97
T5	39.25	33.46	36.75	38.20	36.91
T6	36.40	38.45	36.50	34.82	36.54
T7	38.60	42.44	41.83	44.10	41.74

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	148.780	24.797	6.274	0.001
Within groups	21	82.997	3.952		
Total	27	231.778			

Since p-value is less than 0.05. i.e., ℑ|at 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on shoot length at ℑ|= 5% level of significance.

9. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on root length(cm)

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	14.25	15.60	16.45	15.2	15.37
T2	20.65	24.25	19.50	25.20	22.40
T3	19.25	17.65	22.40	24.60	20.97
T4	27.20	25.65	24.40	24.62	25.46
T5	20.65	22.45	23.62	23.92	22.66
T6	19.45	20.26	24.42	25.0	22.28
T7	25.25	26.94	24.25	23.0	24.86

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments**.**

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	262.933	43.822	9.363	0.0
Within groups	21	98.297	4.680		
Total	27	361.220			

Since p-value is less than 0.05. i.e., ℑ|at 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on root length at ℑ|= 5% level of significance.

10. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on fresh weight of shoot (gm).

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	12.24	9.92	10.40	9.50	10.51
T2	14.65	13.15	14.80	12.70	13.82
T3	11.91	10.47	14.50	13.10	12.49
T4	15.43	13.20	16.15	14.30	15.52
T5	14.30	15.50	13.67	13.0	14.11
T6	12.25	13.20	15.40	12.0	13.21
T7	16.65	15.70	17.25	15.30	16.22

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	86.847	14.475	9.544	0.0
Within groups	21	31.848	1.517		
Total	27	118.696			

Since p-value is less than 0.05. i.e., ℑ|= 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on fresh weight of shoot at ℑ=5% level of significance.

11. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica along* with vermicompost on dry weight of shoot (gm).**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	2.0	1.20	1.72	1.80	1.68
T2	2.27	2.43	1.75	1.86	2.07
T3	1.78	2.40	1.68	1.97	1.95
T4	3.30	2.22	2.11	2.46	2.52
T5	1.90	2.61	2.0	3.0	2.37
T6	1.55	1.90	2.0	2.40	1.96

T7	3.60	4.10	2.50	2.17	3.09

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	5.289	0.882	3.370	0.017
Within groups	21	5.493	0.262		
Total	27	10.782			

Since p-value is less than 0.05. i.e., ℑ|= 5% level of significance. We reject H_0 and accept $H_{1.}$ Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on dry weight of shoot at ℑ|=5% level of significance.

12. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on fresh weight of root (gm).**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	1.0	0.80	0.90	0.72	0.85
T2	1.55	1.96	0.95	1.85	1.57
T3	1.0	0.95	0.90	0.86	0.92
T4	2.02	1.76	2.22	1.82	1.95
T5	1.46	2.29	1.72	0.93	1.60

T6	0.87	1.25	0.92	0.90	0.98
T7	2.25	2.62	1.90	1.84	2.15

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	6.517	1.086	10.162	0.0
Within groups	21	2.245	0.107		
Total	27	8.761			

Since p-value is lesser than 0.05. i.e., ℑ=5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant on fresh weight of root at ℑ=5% level of significance.

13. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on dry weight of root (gm).**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	0.26	0.23	0.16	0.22	0.21
T2	0.40	0.31	0.29	0.34	0.33
T3	0.29	0.34	0.30	0.20	0.28
T4	0.45	0.52	0.38	0.42	0.44
T5	0.42	0.36	0.31	0.40	0.37
T6	0.34	0.29	0.41	0.31	0.33
T7	0.49	0.55	0.56	0.51	0.52

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	0.250	0.0416	16.897	0.0
Within groups	21	0.052	0.00247		
Total	27	0.302			

Since p-value is lesser than 0.05. i.e., ℑ=5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant on dry weight of root at ℑ=5% level of significance.

14. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on number of pods.**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	3.0	3.50	2.50	3.0	3.0
T2	5.0	6.50	4.50	5.20	5.30
T3	5.0	5.75	3.50	4.80	4.76
T4	7.50	5.0	6.0	6.0	6.12
T5	7.0	4.50	6.20	5.50	5.80
T6	5.70	5.0	4.80	4.50	5.20
T7	6.40	7.0	6.0	5.50	6.22

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

<table>
<tr><th>Source of variation</th><th>Degrees of freedom</th><th>Sum of squares</th><th>Mean sum of squares</th><th>F value</th><th>Significance</th></tr>
<tr><td>Between groups</td><td>6</td><td>29.238</td><td>4.873</td><td rowspan="3">7.518</td><td rowspan="3">0.0</td></tr>
<tr><td>Within groups</td><td>21</td><td>13.612</td><td rowspan="2">0.648</td></tr>
<tr><td>Total</td><td>27</td><td>42.850</td></tr>
</table>

Since p-value is lesser than 0.05. i.e., $\Im$=5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant on number of pods at $\Im$ = 5% level of significance.

15. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on fresh weight of pod.**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	3.35	4.13	2.25	3.60	3.33
T2	5.0	4.50	4.25	5.50	4.81
T3	4.45	3.0	3.65	5.0	4.02
T4	5.70	5.55	6.25	5.0	5.625
T5	6.0	5.25	5.70	5.0	5.48
T6	4.80	5.75	5.50	4.50	5.13
T7	6.40	6.20	5.50	6.45	6.13

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

<table>
<tr><th>Source of variation</th><th>Degrees of freedom</th><th>Sum of squares</th><th>Mean sum of squares</th><th>F value</th><th>Significance</th></tr>
<tr><td>Between groups</td><td>6</td><td>22.718</td><td>3.786</td><td rowspan="3">9.790</td><td rowspan="3">0.0</td></tr>
<tr><td>Within groups</td><td>21</td><td>8.122</td><td rowspan="2">0.387</td></tr>
<tr><td>Total</td><td>27</td><td>30.840</td></tr>
</table>

Since p-value is lesser than 0.05. i.e., ℑ=5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant on fresh weight of pod at ℑ=5% level of significance.

16. **Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on dry weight of pod.**

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	0.45	0.43	0.41	0.52	0.45
T2	0.64	0.60	0.56	0.55	0.58
T3	0.67	0.64	0.61	0.57	0.62
T4	0.90	0.80	1.0	0.95	0.91
T5	0.95	0.84	1.0	0.90	0.92
T6	0.80	0.82	0.84	0.75	0.80
T7	1.0	1.6	1.1	1.0	1.17

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	1.454	0.242	16.665	0.0
Within groups	21	0.305	0.014		
Total	27	1.759			

Since p-value is lesser than 0.05. i.e., ℑ=5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant on dry weight of pod at ℑ=5% level of significance.

17. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on nitrogen content of shoot (%).

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	1.87	1.45	1.92	1.77	1.75
T2	1.94	2.21	1.82	2.32	2.07
T3	2.52	2.14	3.06	2.72	2.61
T4	3.24	2.94	2.72	3.12	3.0
T5	2.54	2.81	2.40	2.62	2.59
T6	2.62	2.71	2.94	2.82	2.77
T7	3.45	3.22	2.96	2.90	3.13

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

<table>
<tr><th>Source of variation</th><th>Degrees of freedom</th><th>Sum of squares</th><th>Mean sum of squares</th><th>F value</th><th>Significance</th></tr>
<tr><td>Between groups</td><td>6</td><td>5.587</td><td>0.976</td><td rowspan="3">16.662</td><td rowspan="3">0.0</td></tr>
<tr><td>Within groups</td><td>21</td><td>1.230</td><td rowspan="2">0.058</td></tr>
<tr><td>Total</td><td>27</td><td>7.087</td></tr>
</table>

Since p-value is less than 0.05. i.e., ℑ= 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on nitrogen content of shoot at ℑ=5% level of significance.

18. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on nitrogen content of root (%).

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	1.12	1.0	1.14	1.18	1.11
T2	1.24	1.50	1.31	1.42	1.36
T3	1.65	1.53	1.93	1.69	1.70
T4	2.62	2.34	2.09	2.81	2.46
T5	1.53	1.65	1.39	1.42	1.49
T6	1.93	1.73	2.02	1.82	1.87
T7	2.74	2.69	3.20	2.80	2.85

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

<table>
<tr><th>Source of variation</th><th>Degrees of freedom</th><th>Sum of squares</th><th>Mean sum of squares</th><th>F value</th><th>Significance</th></tr>
<tr><td>Between groups</td><td>6</td><td>9.281</td><td>1.547</td><td rowspan="3">46.804</td><td rowspan="3">0.0</td></tr>
<tr><td>Within groups</td><td>21</td><td>0.694</td><td rowspan="2">0.033</td></tr>
<tr><td>Total</td><td>27</td><td>9.975</td></tr>
</table>

Since p-value is less than 0.05. i.e., ℑ = 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on nitrogen content of root at ℑ|= 5% level of significance.

19. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on phosphorous content of shoot (%).

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	0.33	0.28	0.39	0.35	0.33
T2	0.48	0.46	0.50	0.42	0.46
T3	0.44	0.40	0.43	0.39	0.41
T4	0.51	0.63	0.49	0.50	0.53
T5	0.62	0.59	0.52	0.49	0.55
T6	0.47	0.38	0.39	0.45	0.42
T7	0.58	0.67	0.55	0.51	0.57

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

<table>
<tr><th>Source of variation</th><th>Degrees of freedom</th><th>Sum of squares</th><th>Mean sum of squares</th><th>F value</th><th>Significance</th></tr>
<tr><td>Between groups</td><td>6</td><td>.182</td><td>0.030</td><td rowspan="3">11.586</td><td rowspan="3">0.0</td></tr>
<tr><td>Within groups</td><td>21</td><td>0.055</td><td rowspan="2">0.0026</td></tr>
<tr><td>Total</td><td>27</td><td>0.237</td></tr>
</table>

Since p-value is less than 0.05. i.e., ℑ=5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on phosphorous content of root at ℑ=5% level of significance.

20. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on phosphorous content of root (%).

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	0.19	0.21	0.18	0.20	0.195
T2	0.26	0.29	0.23	0.30	0.27
T3	0.20	0.24	0.19	0.25	0.22
T4	0.25	0.32	0.39	0.27	0.30
T5	0.32	0.37	0.27	0.34	0.32
T6	0.31	0.27	0.22	0.21	0.25
T7	0.41	0.39	0.37	0.40	0.39

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	0.108	0.018	12.478	0.0
Within groups	21	0.031	0.0014		
Total	27	0.139			

Since p-value is less than 0.05. i.e., $\Im$= 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on phosphorous content of root at $\Im$=5% level of significance.

21. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on potassium content of shoot (%).

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	2.80	3.02	3.19	2.70	2.92
T2	3.41	3.62	3.59	3.56	3.54
T3	3.31	3.25	3.34	3.30	3.30
T4	4.09	3.97	4.14	4.54	4.18
T5	3.81	4.03	4.14	3.98	3.99
T6	3.92	3.02	4.09	3.64	3.66
T7	4.41	4.14	4.56	4.62	4.43

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Significance
Between groups	6	6.538	1.090	18.828	0.0
Within groups	21	1.215	0.057		
Total	27	7.754			

Since p-value is less than 0.05. i.e., ℑ= 5% level of significance. We reject H_0 and accept H_1. Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on potassium content of root at ℑ=5% level of significance.

22. Effect of dual inoculation of *Rhizobium leguminosarum* biovar *phaseoli* and *P. indica* along with vermicompost on potassium content of root (%).

Treatment	Pot 1	Pot 2	Pot 3	Pot 4	Mean
T1	1.94	2.10	1.80	1.96	1.95
T2	2.40	2.43	2.24	2.63	2.42
T3	2.23	1.96	2.50	2.12	2.20
T4	2.74	2.69	3.47	2.80	2.92
T5	3.01	2.64	2.54	2.94	2.78
T6	2.46	2.54	2.92	2.64	2.64
T7	3.24	3.31	2.98	3.50	3.25

Test Statistics is one way ANOVA which is done by using computer program SPSS.

Here,

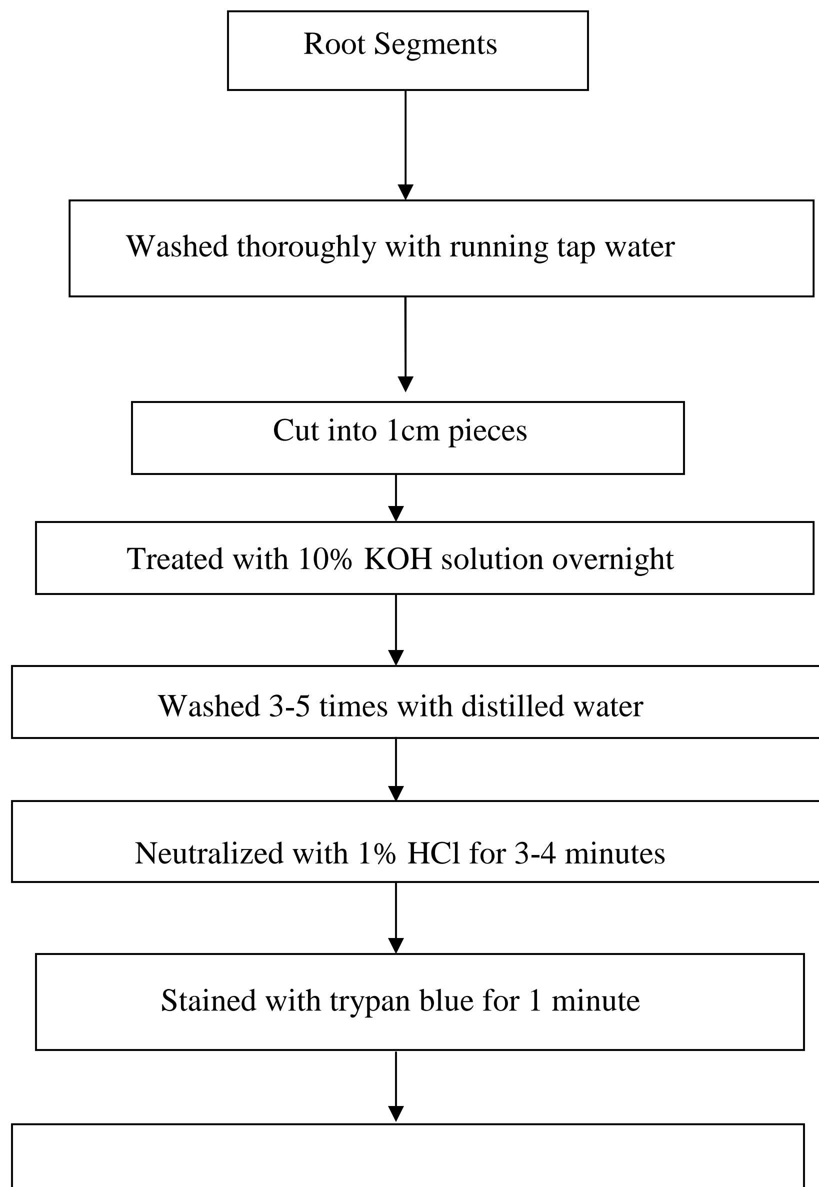
Null hypothesis, H_0: There is no significant difference between treatments. i.e., There is no treatment effect.

Alternative hypothesis, H_1: There is significant difference between treatments.

<table>
<tr><th>Source of variation</th><th>Degrees of freedom</th><th>Sum of squares</th><th>Mean sum of squares</th><th>F value</th><th>Significance</th></tr>
<tr><td>Between groups</td><td>6</td><td>4.736</td><td>0.789</td><td rowspan="2">15.145</td><td rowspan="2">0.0</td></tr>
<tr><td>Within groups</td><td>21</td><td>1.094</td><td rowspan="2">0.052</td></tr>
<tr><td>Total</td><td>27</td><td>5.830</td><td></td><td></td></tr>
</table>

Since p-value is less than 0.05. i.e., ℑ= 5% level of significance. We reject H_0 and accept H_1.

Therefore, there is significant difference between treatments. Therefore, different methods of treatment have significant effect on potassium content of root at ℑ=5% level of significance.


Root Segments
Washed thoroughly with running tap water
Cut into 1cm pieces
Treated with 10% KOH solution overnight
Washed 3-5 times with distilled water
Neutralized with 1% HCl for 3-4 minutes
Stained with trypan blue for 1 minute

Destained with lactophenol for 1-2 minutes

Mounted in lactophenol on a glass slide

Figure 2: Flow diagram for root staining procedure

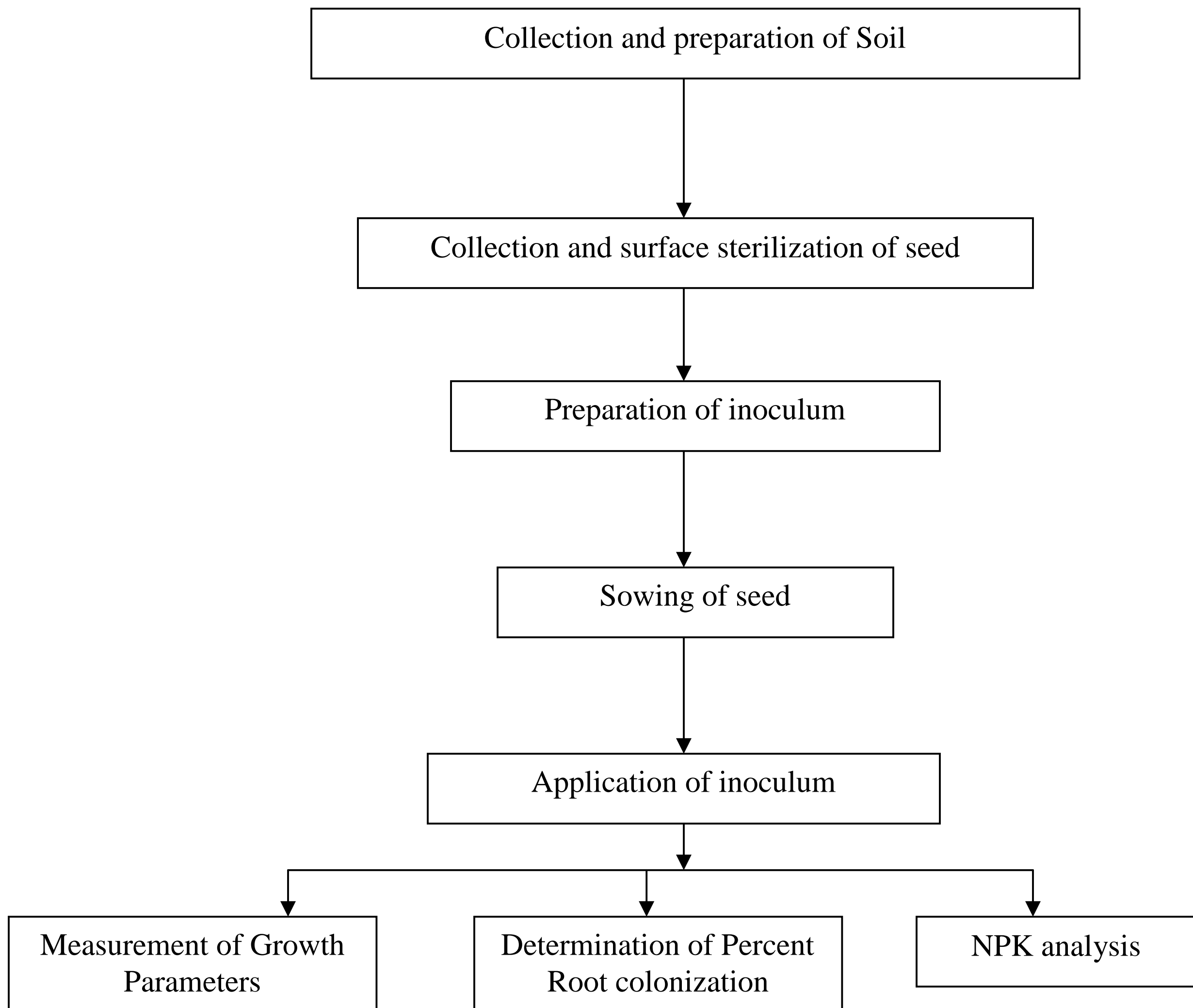


Figure 3: Flow diagram for pot culture experiment

Figure 5: Effect of dual inoculation along with vermicompost on shoot length at flowering stage

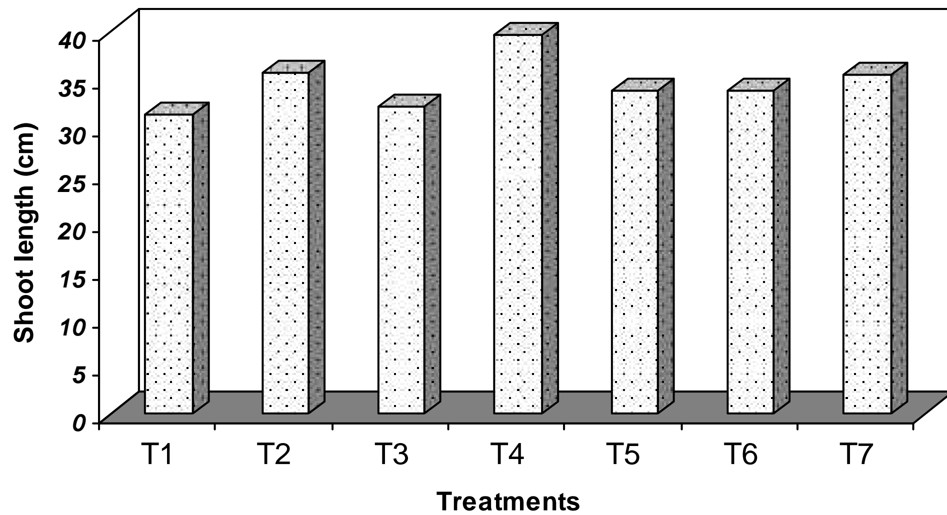
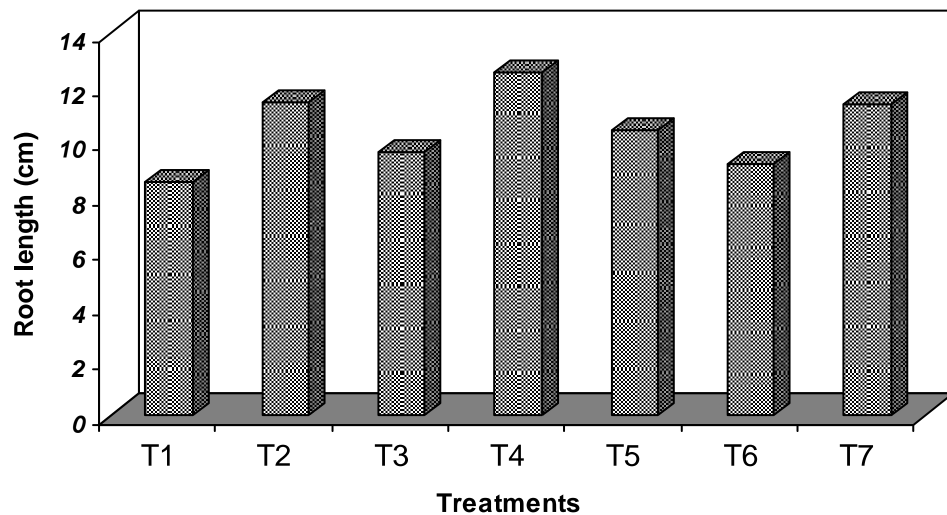


Figure 6 : Effect of dual inoculation along with vermicompost on root length at flowering stage



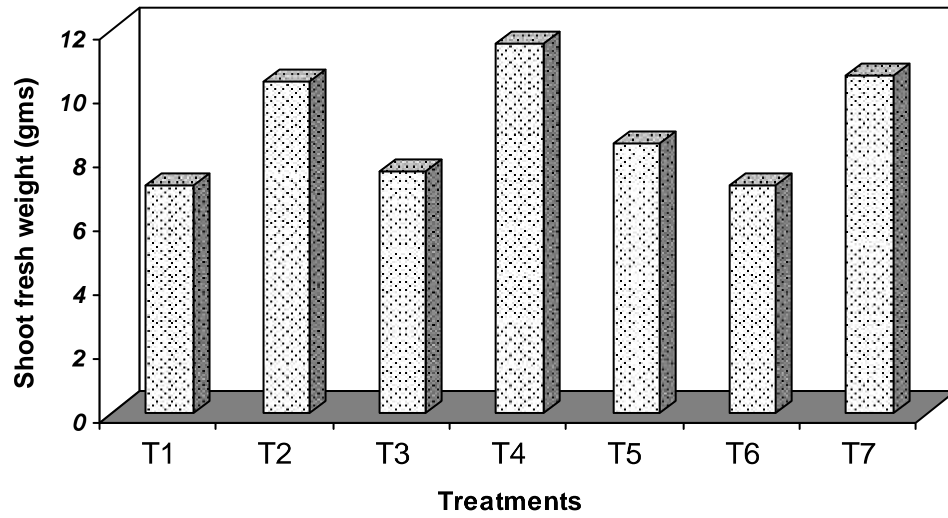


Figure 7: Effect of dual inoculation along with vermicompost on shoot fresh weight at flowering stage
Shoot fresh weight (gms)
12
10
8
6
4
2
0
T1
T2
T3
T4
T5
T6
T7
Treatments

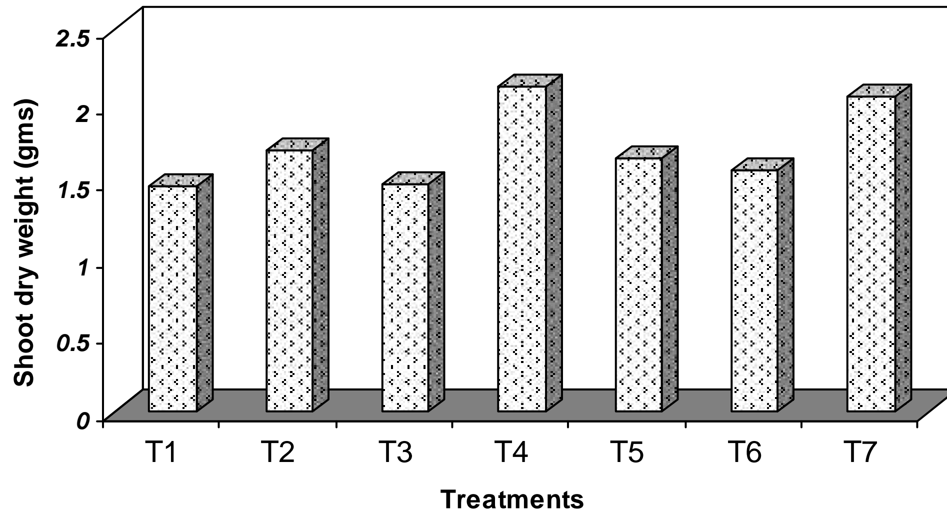


Figure 8: Effect of dual inoculation along with vermicompost on shoot dry weight at flowering stage
Shoot dry weight (gms)
2.5
2
1.5
1
0.5
0
T1
T2
T3
T4
T5
T6
T7
Treatments

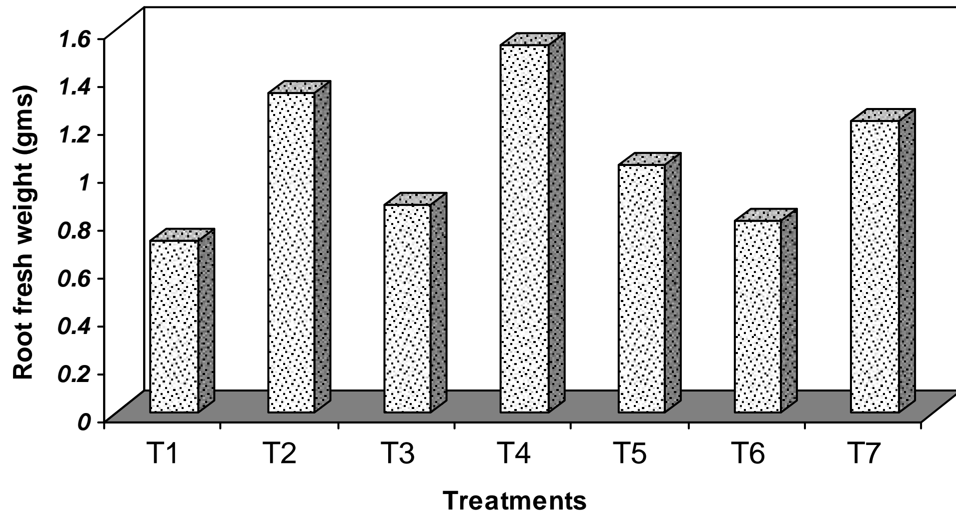


Figure 9: Effect of dual inoculation along with vermicompost on root fresh weight at flowering stage
Root fresh weight (gms)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
T1
T2
T3
T4
T5
T6
T7
Treatments

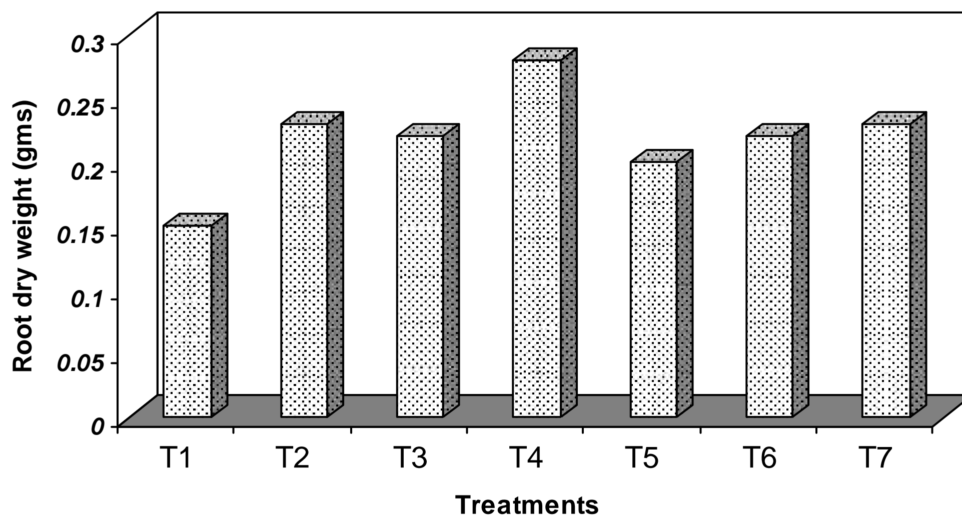


Figure 10: Effect of dual inoculation along with vermicompost on root dry weight at flowering stage
Root dry weight (gms)
0.3
0.25
0.2
0.15
0.1
0.05
0
T1
T2
T3
T4
T5
T6
T7
Treatments

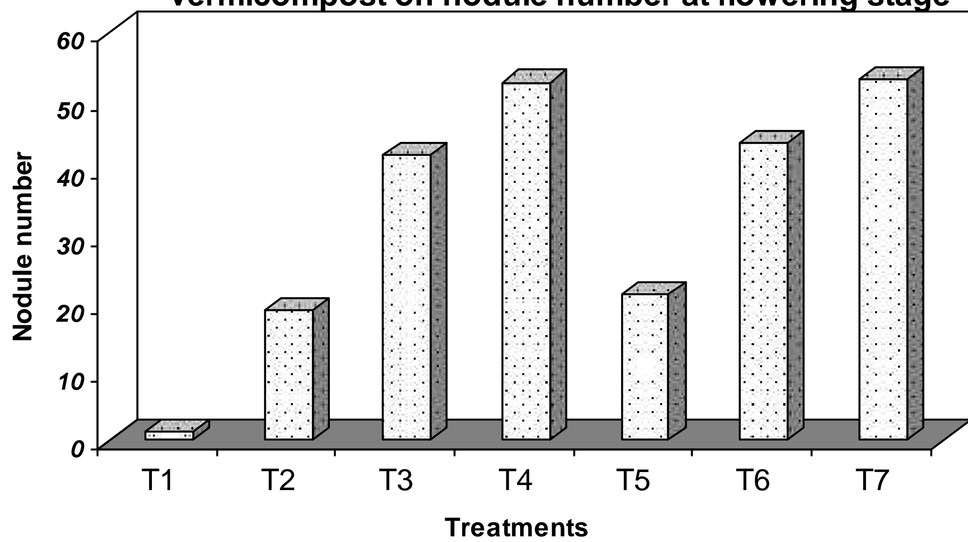


Figure 11 : Effect of dual inoculation along with vermicompost on nodule number at flowering stage
Nodule number
0
10
20
30
40
50
60
T1
T2
T3
T4
T5
T6
T7
Treatments

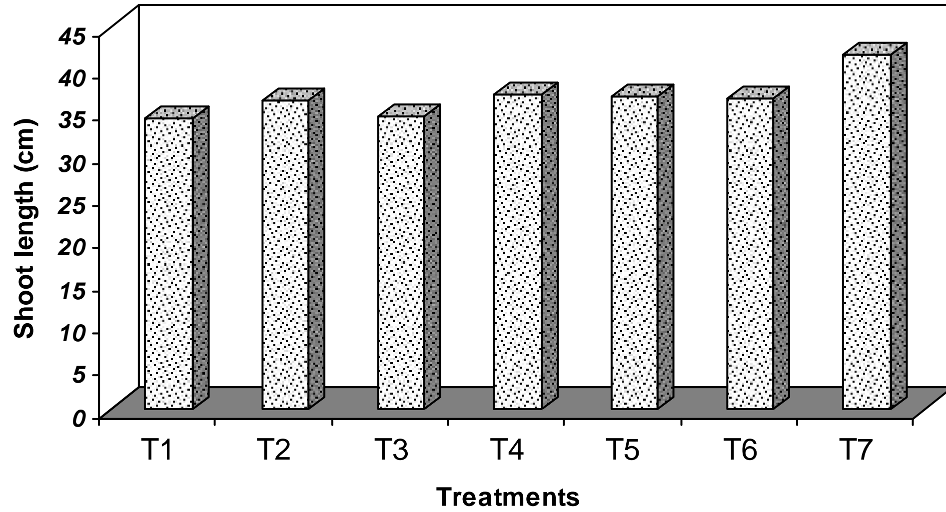


Figure 12 : Effect of dual inoculation along with vermicompost on shoot length in harvesting stage
Shoot length (cm)
0
5
10
15
20
25
30
35
40
45
T1
T2
T3
T4
T5
T6
T7
Treatments

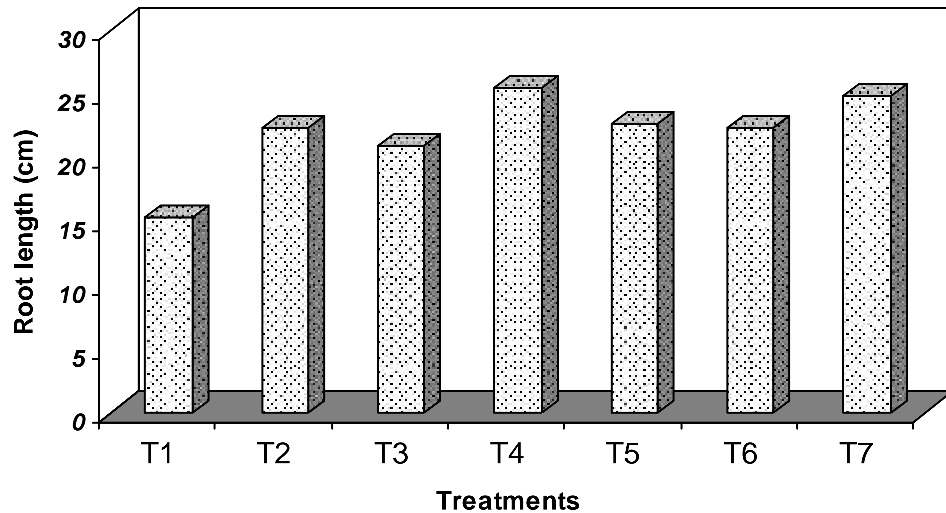


Figure 13 : Effect of dual inoculation along with vermicompost on root length in harvesting stage
Root length (cm)
0
5
10
15
20
25
30
T1
T2
T3
T4
T5
T6
T7
Treatments

Figure 14 : Effect of dual inoculation along with vermicompost on shoot fresh weight in harvesting stage

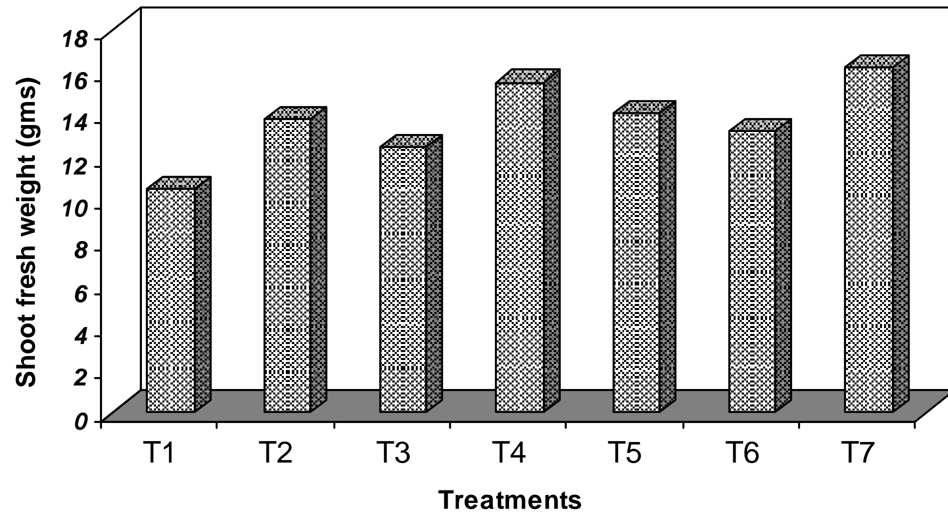
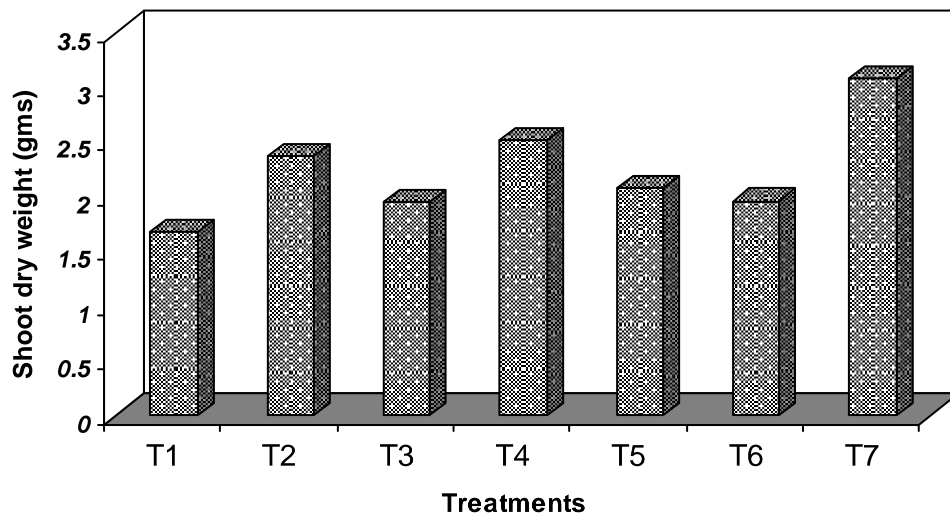


Figure15 : Effect of dual inoculation along with vermicompost on shoot dry weight in harvesting stage



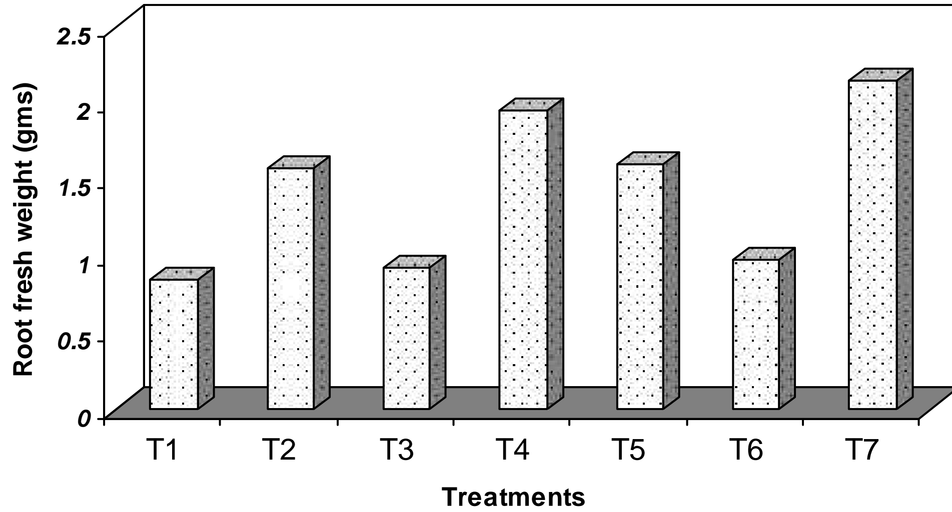


Figure 16 :Effect of dual inoculation along with vermicompost on root fresh weight in harvesting stage
Root fresh weight (gms)
2.5
2
1.5
1
0.5
0
T1
T2
T3
T4
T5
T6
T7
Treatments

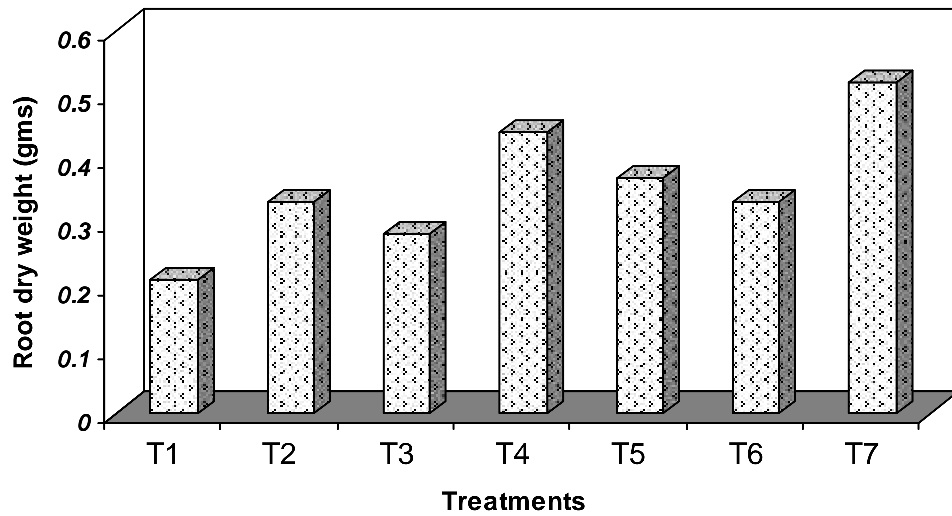


Figure 17 :Effect of dual inoculation along with vermicompost on root dry weight in harvesting stage
Root dry weight (gms)
0.6
0.5
0.4
0.3
0.2
0.1
0
T1
T2
T3
T4
T5
T6
T7
Treatments

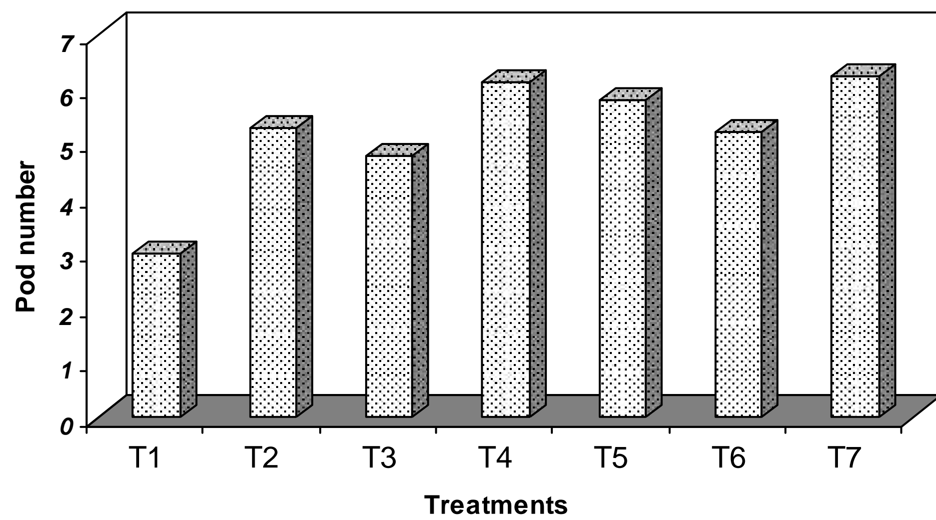


Figure 18 : Effect of dual inoculationalong with vermicompost on pod number in harvesting stage
Pod number
0
1
2
3
4
5
6
7
T1
T2
T3
T4
T5
T6
T7
Treatments

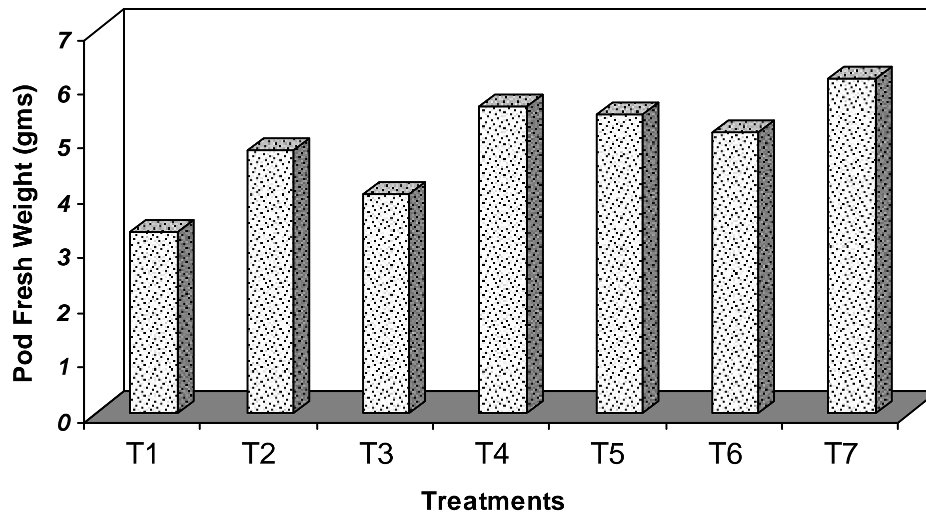


Figure 19 :Effect of dual inoculation along with vermicompost on pod fresh weight in harvesting stage
Pod Fresh Weight (gms)
0
1
2
3
4
5
6
7
T1
T2
T3
T4
T5
T6
T7
Treatments

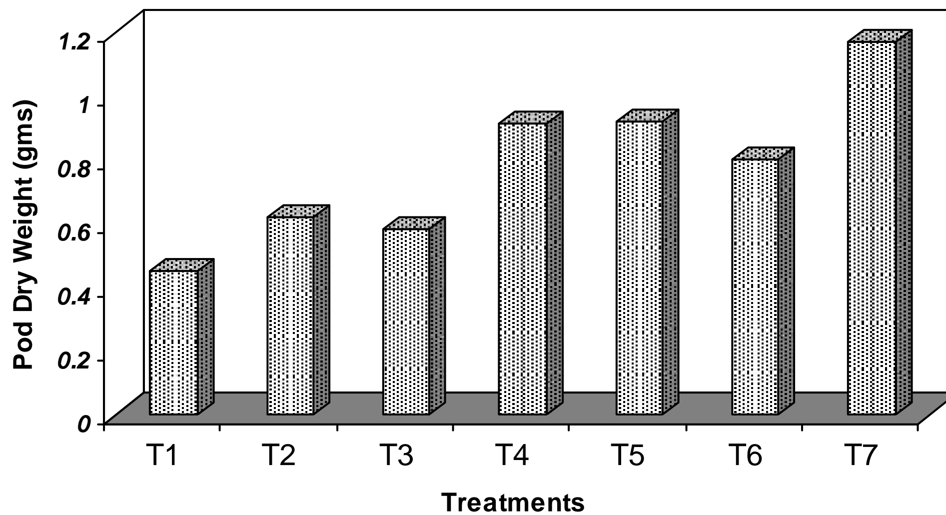


Figure 20 : Effect of dual inoculation along with vermicompost on pod dry weight in harvesting stage
Pod Dry Weight (gms)
0
0.2
0.4
0.6
0.8
1
1.2
T1
T2
T3
T4
T5
T6
T7
Treatments

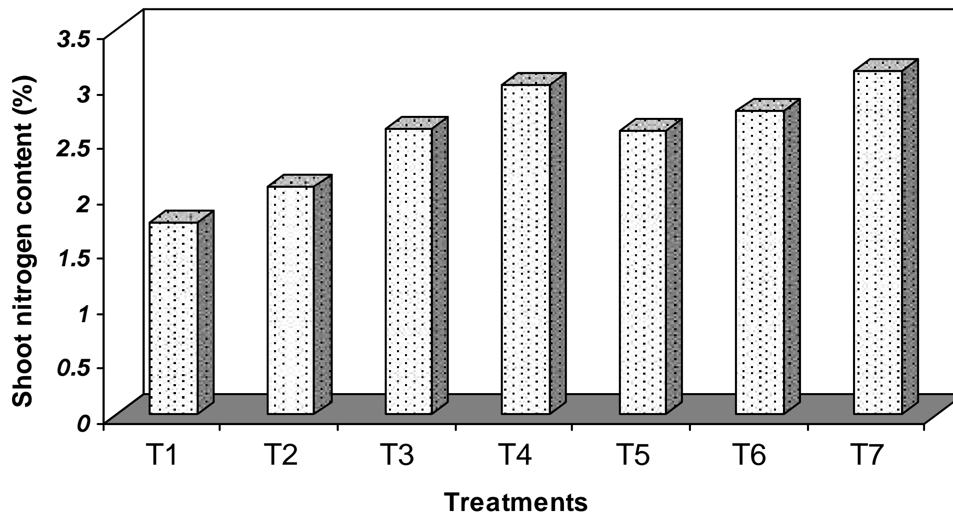


Figure 21 :Effect of dual inoculation along with vermicompost on nitrogen content of shoot
Shoot nitrogen content (%)
3.5
3
2.5
2
1.5
1
0.5
0
T1
T2
T3
T4
T5
T6
T7
Treatments

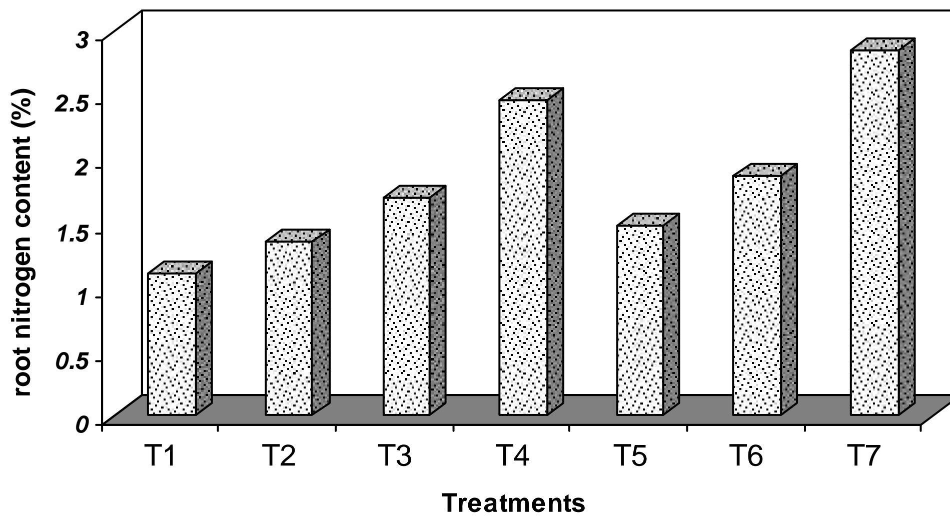


Figure 22 : Effect of dual inoculation along with vermicompost on nitrogen content of root
root nitrogen content (%)
3
2.5
2
1.5
1
0.5
0
T1
T2
T3
T4
T5
T6
T7
Treatments

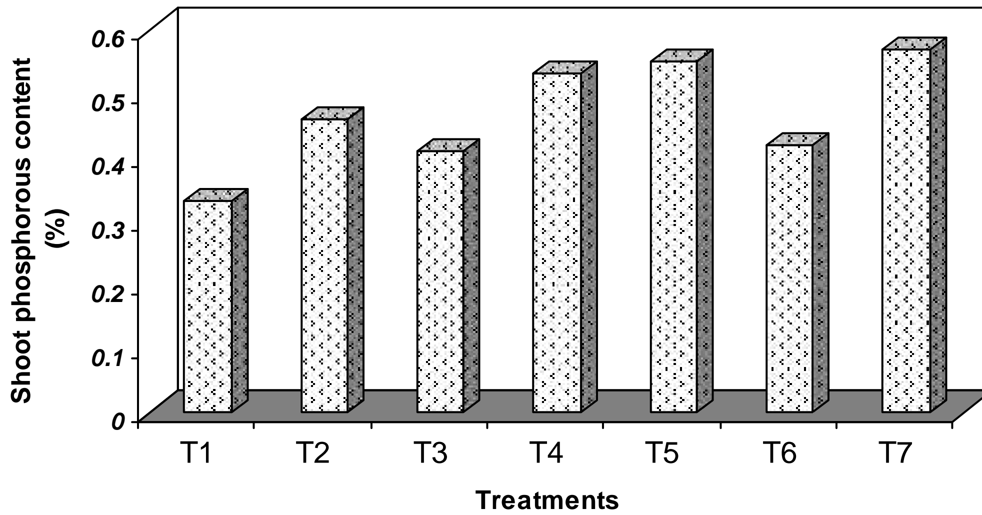


Figure 23 :Effect of dual inoculation along with vermicompost on phosphorous content of shoot
Shoot phosphorous content (%)
0.6
0.5
0.4
0.3
0.2
0.1
0
T1
T2
T3
T4
T5
T6
T7
Treatments

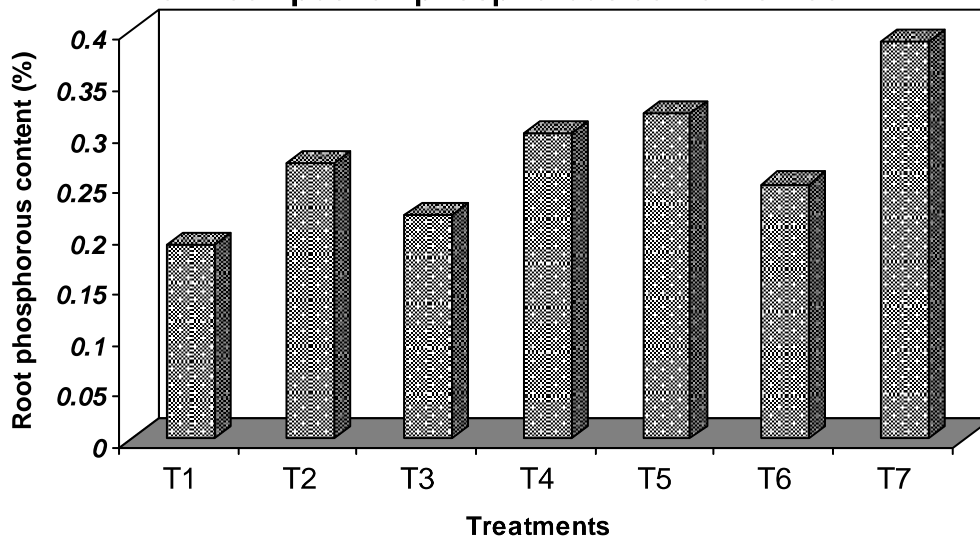


Figure 24: Effect of dual inoculation along with vermicompost on phosphorous content of root
Root phosphorous content (%)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
T1
T2
T3
T4
T5
T6
T7
Treatments

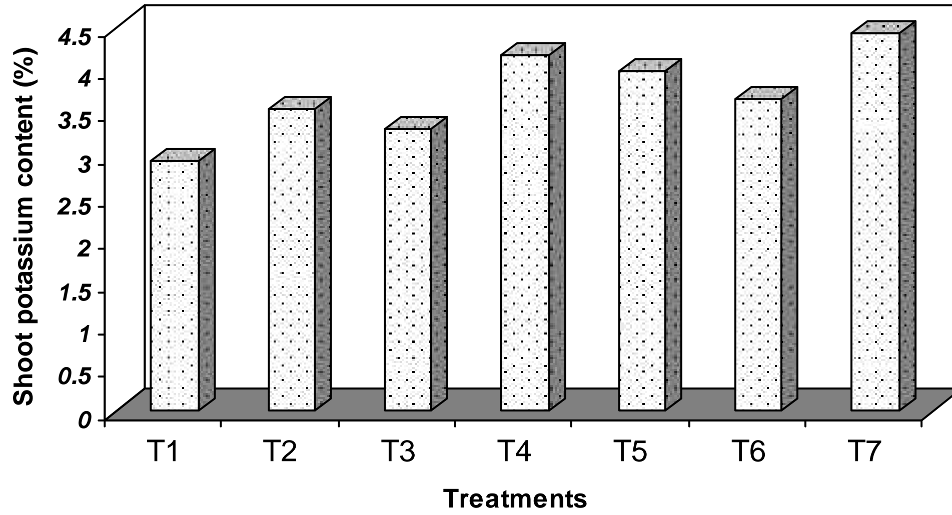


Figure 25 :Effect of dual inoculation along with vermicompost on potassium content of shoot
Shoot potassium content (%)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
T1
T2
T3
T4
T5
T6
T7
Treatments

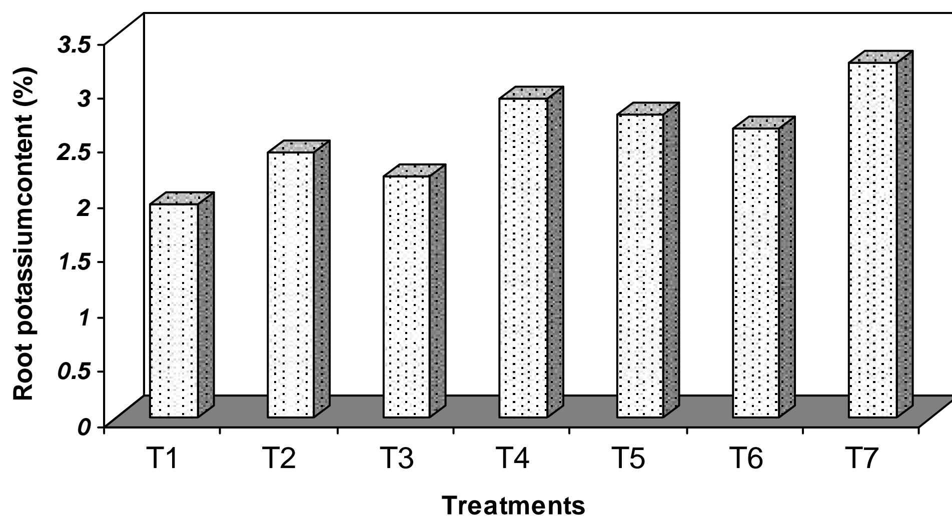


Figure 26 : Effect of dual inoculation along with vermicompost on potassium content of root
Root potassiumcontent (%)
3.5
3
2.5
2
1.5
1
0.5
0
T1
T2
T3
T4
T5
T6
T7
Treatments

Photograph 1 Growth of *Rhizobium leguminosarum biovar phaseoli* on Yeast Mannitol Congo Red Agar

Photograph 2 Growth of *Rhizobium leguminosarum biovar phaseoli* on Yeast Mannitol Bromothymal Blue Agar

Photograph 5 Growth of *Piriformospora indica* on Potato Dextrose Agar

Photograph 6 Spore of *Piriformospora indica* under 40X

Photograph 7 Effect of dual inoculation on plant growth in flowering stage

Photograph 8 Effect of dual inoculation on root growth and nodulation in flowering stage

Photograph 9 Effect of dual inoculation along with vermicompost on plant growth in flowering stage

Photograph 10 Effect of dual inoculation along with vermicompost on root growth and nodulation in flowering stage

Photograph 11 Effect of dual inoculation on plant growth in harvesting stage

Photograph 12 Effect of dual inoculation on root growth and nodulation in harvesting stage

Photograph 13 Effect of dual inoculation along with vermicompost on plant growth in harvesting stage

Photograph 14 Effect of dual inoculation along with on root growth in harvesting stage

Photograph 3 Yeast Mannitol Broth inoculated with *Rhizobium leguminosarum biovar phaseoli*

Photograph 4 *Rhizobium leguminosarum biovar* phaseoli cells after gram staining under 100X

Photograph 15 Experimental bean plants under treatment in flowering stage

Photograph 16 Experimental bean plants under treatment in flowering stage