CHAPTER-I

1. INTRODUCTION

Plants are, by definition, the only higher organisms that can convert the solar energy into stored, usable chemical energy (carbohydrates). Abiotic factors, viz. climate, availability of nutrients or mineral elements, and water are normally considered to have the greatest influence on the plant productivity (Singh *et al*., 2000). Nutrients are fundamental for plant growth in all ecosystems (Johnston and Steen, 2006). Of the twenty mineral elements necessary for plant growth, the biological availability of nitrogen, phosphorous, and potassium is of considerable economic importance because they are the major plant nutrients derived from the soil (Alexender, 1961).

The nitrogen gas that makes up about 79% of the volume of earth's atmosphere is useless to most plants and animals because it is chemically inert and is therefore not a suitable source of the element for most living forms (Deacon, 2006). All plants and animals, as well as most microorganisms depend on a source of combined or fixed nitrogen (ammonia, nitrates or nitrites) in their nutrition. The fixation process is largely a biological one, and bacteria are the organisms capable of causing it, the process known as Biological Nitrogen Fixation (BNF) in which atmospheric nitrogen is converted to ammonia thence into proteins by the enzyme nitrogenase (Deacon, 2006).

Biological nitrogen fixation (BNF) is mediated in part by free-living bacteria (non-symbiotic nitrogen fixation), but the symbiotic fixers are quantitatively more important. The symbiotic fixers of genus *Rhizobium* have received more attention because they form associations with agronomically important leguminous crops. Legumes are equipped with the facility to acquire a major portion of nitrogen directly from atmospheric nitrogen through bacterial fixation (reduction). The bacteria (*Rhizobium* spp.) reside inside the special structures on plant roots i.e. nodules and reduce

atmospheric nitrogen at the expense of carbon supplied by the plant. Thus, by gaining nitrogen from the air and later releasing it to the soil, the legumes are valuable for enriching the soil in nitrogen and hence are used in rotation with other crops quite frequently. Therefore, the legume-*Rhizobium*- plant complex is of great ecological and agronomic significance. The *Rhizobium*-legume symbiosis can generally meet 80% of nitrogen requirement of the legume crop.

As many soils do not contain the appropriate strains of rhizobia, for initial plantings and follow up plantings, it becomes necessary to use the proper inoculant to provide a sufficient population of rhizobia in root nodules for achieving a maximum conversion of free nitrogen (Considine and Considine, 1997).

Phosphorous is one of the most important plant nutrients, next to nitrogen. Phosphorous plays a vital role in several energy transformation and biochemical reactions including BNF (Pant and Singh, 1995). The nitrogenase activity is dependent on ATP for reduction of atmospheric dinitrogen to ammonia (Hayman, 1980). Because of many biotic and aboitic factors which can restrict its mobility in soils, phosphorous is generally considered to be the most important plant-growth limiting factor (Sharma, 2001). Plants acquire this mineral from the environment either directly by their roots or indirectly from mycorrhizal fungi (Singh, 2004). The agricultural significance of the most common and prevalent, arbuscular mycorrhizal (AM) fungi lies principally in its ability to assist the plant to absorb insoluble inorganic phosphorous (Pi) from the surrounding soil. The elaborate network of hyphae beneath the soil surface greatly increases the potential of the root system to absorb nutrients and water. AM fungi, due to the obligate symbiotic mode of growth, non-availability of pure cultures, the commercial production is the greatest bottleneck in the use and application of mycorrhizal biotechnology (Singh, 2004).

A root endophyte was found (gold strike by chance) in a sand desert interior of Rajasthan, which was called for the characteristic spore morphology, *Piriformospora*

indica, which mimics the capabilities of a typical AM fungus (Singh *et al*., 2001; Varma *et al*., 2003). This new wonder fungus improves the growth and overall biomass production of different grasses, trees, legumes, herbaceous species medicinal and economically important plants and can easily be cultivated on a number of complex and synthetic media (Singh *et al*., 2001). Like arbuscular mycorrhizal (AM) fungi, *P. indica* functions as bioregulator, biofertilizer and bioprotector, overcome the water stress, delays the wilting of the leaves and prolongs aging of callus tissues, serves as a strong agent for protection of the tissue culture raised plantlets by overcoming the 'transplantation shock', rendering 100% survival on the host tested (Singh, 2004).

Together with nitrogen and phosphorous, potassium is one of the essential macro minerals for plant survival. Its presence is of great importance for soil health, plant growth and animal nutrition. As such, the element is required in relatively large proportions by the growing plant (Considine and Considine, 1997).

Millions of tons of chemically fixed nitrogen are applied annually. There are several significant environmental reasons to seek alternatives to chemically fixed nitrogen fertilizer: it affects the balance of the global nitrogen cycle, pollutes groundwater, increases the risk of chemical spills, and increases atmospheric nitrous oxide (N_2O), a potent "greenhouse" gas. The increased use of legumes offers the potential for a significant decrease in the need for fertilizer nitrogen, and therefore is a key component of sustainable agricultural systems. The use of biofertilizers is currently gaining interest as a cheap, safe alternate to conventional chemical fertilizers. Symbionts such as rhizobia and mycorrhiza increase the efficiency of nutrient acquisition by plants and hence soil fertility (Sharma, 2001). The management of the legume, symbiotic nitrogen fixing bacteria *B. japonicum* and AM-like fungi *P. indica*, is crucial for attaining higher yields in a sustainable manner. These are extremely important for the SAARC countries like Bangladesh, Bhutan, India, Maldives, Nepal, Pakistan and Sri Lanka where large chunks of land is degraded and has become unfertile for cultivation (Singh, 2004).

CHAPTER-II

2. OBJECTIVES

2.1 GENERAL OBJECTIVES

To study the effect of *Piriformospora indica* as coinoculant with *Bradyrhizobium japonicum* on soybean [*Glycine max* (L.) Merr.] grown in sterilized (autoclaved) and unsterilized (natural) potted soils.

2.2 SPECIFIC OBJECTIVES

i. To study specifically on nodulation, plant growth, aerial and underground parameters.
ii. To measure nitrogen, phosphorous and potassium content in plant tissues and soil samples.
iii. To find out percentage root colonization of *P. indica*.

CHAPTER-III

3. LITERATURE REVIEW

3.1 PLANT NUTRITION

Biotic factors, along with the more obvious abiotic factors such as solar energy, moisture and mineral nutrients, determine and greatly influence the productivity and health of the plants (Singh *et al*., 2000). Plants use inorganic minerals for nutrition and optimum plant growth (Morgan, 2006). The term essential element or mineral nutrient was proposed by Arnon and Stout in 1939. There are actually twenty mineral elements necessary or beneficial for plant growth. The elements, which are required by plant in large amount, are termed macronutrients and three of them are nitrogen, phosphorous and potassium collectively termed NPK (Morgan, 2006).

3.2 NITROGEN

The growth of all organisms depends on the availability of mineral nutrients, and none is more important than nitrogen (N). It is a constituent of all living cells and is a necessary part of all proteins, enzymes and metabolic processes involved in the synthesis and transfer of energy. Besides these, it is also major component of many other biomolecules such as hormones, chlorophyll, vitamins, enzymes, cytochromes and alkaloids. Its metabolism is a major factor in stem and leaf growth (vegetative growth) in plants The functions of N include stimulating plants into rapid, vigorous growth, increasing seed and fruit yield and improving the quality of leaf and forage crops (Morgan, 2006).

There is an abundant supply of nitrogen in the earth's atmosphere-nearly 79% in the form of nitrogen gas. However, nitrogen is unavailable for use by most organisms

because there is a triple bond between the two nitrogen atoms, making the molecule almost inert. In order for nitrogen to be used for growth, it must be "fixed" (combined) in the form of ammonium (NH_4) or nitrate (NO_3) ions. Combined nitrogen is relatively scare in soil and water, often constituting the limiting factor for the development of living organisms and crop yields (Deacon, 2006).

3.3 NITROGEN FIXATION

The turnover of nitrogen through its cycle is estimated to be between 10^8 and 10^9 tons per year. The vast supply of nitrogen gas in the atmosphere and the relatively scarcity of combined nitrogen on the earth's surface suggest that the process of nitrogen fixation is the rate limiting step. The only other natural source of usable nitrogen for plants is from lightening but a relatively small amount of ammonia is produced by this process. Worldwide, lightning may fix 10 million metric tons of N per year, a value that probably has not changed over time. Some ammonia is also produced industrially by the Haber-Bosch process, using an iron-based catalyst, which requires very high pressures and fairly high temperature. Industrial fixation for fertilizer nitrogen has increased from 3.5 million tons in 1950 to 80 million tons in 1989 in response to the needs of high-yielding crops (Hardy, 1993).

3.3.1 Biological nitrogen fixation

Nevertheless, the major conversion of nitrogen into ammonia, and thence into proteins, is achieved by microorganisms in the process called BNF. The total BNF is estimated to be twice as much as the total nitrogen fixation by non-biological processes. Microorganisms have a central role in almost all aspects of nitrogen availability and thus for life support on earth. Microorganisms that fix nitrogen are called diazotrophs. Two kinds of nitrogen-fixing microorganisms are recognized:

Free-living (non-symbiotic) bacteria: Those microorganisms, which utilize gaseous nitrogen directly and independently in the soils such as cyanobacteria, *Anabaena Nostoc, Azotobacter, Beijerinckia, Clostridium* etc.

Mutualistic (symbiotic) bacteria: Those microorganisms, which utilize nitrogen indirectly through the mediation of other living organisms in the soil such as *Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium* and *Sinorhizobium* associated with leguminous plants, and *Spirillum lipoferum,* associated with cereal grasses.

Table 1. Some estimates of the amount of nitrogen fixed on a global scale.

Type of fixation	**N_2 fixed (10^{12} g per year, or 10^6 metric tons per year)**
Non-biological	
Industrial	about 50
Combustion	about 20
Lightning	about 10
Total	**about 80**
Biological	
Agricultural land	about 90
Forest and non-agricultural land	about 50
Sea	about 35
Total	**about 175**

Source: Bezdicek and Kennedy, 1998.

Asymbiotic and symbiotic biological systems fix an estimated 100-175 million metric tons of nitrogen annually into fertilizer nitrogen for which the energy bill is paid by

nature (Burns and Hardy, 1975). In terrestrial habitats, the symbiotic fixation of nitrogen by rhizobia accounts for the largest contribution of combined nitrogen and their rates of fixation are often two to three orders of magnitude (about 40%) higher than rates exhibited by free-living bacteria in soil (Espiritu, 1998). The global nitrogen fixation is through biological means is around $17.2x10^7$ tons annually and 80% of it comes from *Rhizobium*-Legume symbiosis alone. Rhizobia associated with an alfalfa field may fix up to 300 kg N hectare(ha^{-1}) per year, compared to a rate of 0.5-2.5 kg Nha^{-1} per year for free living *Azotobacter* spp. (Dalton, 1980; Burns and Hardy, 1975).

3.4 THE RHIZOBIA

3.4.1. Characteristics

Bergey's Manual of Systematic Bacteriology (Jordan, 1982) describes the rhizobia of the family Rhizobiaceae as cells without endospores, Gram-negative normally rod-shaped (0.5-0.9 by 1.2-3.0 μm), motile, one polar or sub polar flagellum or 2–6 peritrichous flagella, aerobic, with many carbohydrates utilized. Considerable extra cellular polysaccharide slime is usually produced during growth on carbohydrate-containing media. Cells often contain granules of polymerized β-hydroxybutyrate which are refractile under phase contrast, stainable with Sudan black B and soluble in chloroform.

These bacteria are chemo-organotrophs, grows best at 25°C-30°C on complex media, notably with yeast extract; many able to use nitrate, ammonia, or amino acid as sole source of nitrogen; dinitrogen utilized primarily in symbiosis with a legume host, pH range for the growth is 5.0-8.5 but optimum growth is at neutral to slightly acidic.

3.4.2 Classification

Early researchers considered all rhizobia to be a single species capable of nodulating all legumes. Extensive cross testing on various legume hosts led to a taxonomic

characterization of rhizobia based on bacteria-plant cross-inoculation groups (Chen *et al*., 1988).

Traditionally, rhizobia have been divided into two groups according to growth rate, as first suggested by Lohnis and Hansen in 1921. The term “fast growers” commonly refers to rhizobia associated with alfalfa, clover, bean, and pea because, in culture, these grow much faster (less than one-half the doubling time of slow growers or less than 6 hours) than the “slow growers” exemplified by soybean and cowpea rhizobia (generation time greater than 6 hours). Numerous workers have shown that the slow-growing rhizobia are more specific in their carbohydrate requirements than are those of the fast-growing group. Based on the ability of the rhizobia to produce acid or alkali on YMA 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. Dalton (1980) found that fast growers possess G +C content in between 59% and 63% and slow growers possess 61.6% and 65.5%.

Based on the differences between the fast- and slow-growing rhizobia, the International Subcommittee on *Agrobacterium* and *Rhizobium* proposed that the slow-growing rhizobia be separated into a new genus, *Bradyrhizobium* (Jordan, 1982). The fast-growing rhizobia have been placed in the genus *Rhizobium* comprised of five species-*Rhizobium leguminosarum, R. meliloti, R. loti, R. galegae*, and *R. fredii*. Three former species—*R. phaseoli*, *R. trifolii*, and *R. leguminosarum*—have been combined into the species *R. leguminosarum*. *R. fredii* is a new species and has recently been assigned to a new genus, *Sinorhizobium* with the type species being *Sinorhizobium fredii*. This new taxon has yet to be generally accepted but has been approved (Chen *et al*., 1988; Scholla and Elkan, 1984).

Now, according to the classification scheme as presented in *Bergey's Manual of Systematic Bacteriology*, the family *Rhizobiaceae* consists of four genera, two of which include the symbiotic nitrogen-fixing bacteria *Rhizobium* and *Bradyrhizobium*. The genus *Azorhizobium* has been accepted and a proposed new genus *Sinorhizobium* has been approved (Chen *et al*., 1988; Dreyfus *et al*., 1988). A unique photosynthetic *Rhizobium* was isolated and tentatively named *Photorhizobium* but has not yet been validly described.

Table 2: *Rhizobium* and the host plant

Fast growers	**Genera of host plants**
GENUS I: *Rhizobium*	
R. leguminosarium biovar – *trifolii* biovar – *phaseoli*	 *Trifolium sps.* *Phaseolus vulgaris*
R. meliloti	*Medicago, Melilotus,*
R. loti	*Lupinus, Lotus*
Slow growers	
GENUS II: *Bradyrhizobium*	
Bradyrhizobium japonicum	*Glycine max*
Bradyrhizobium spp. *(Vigna)*	*Vigna* spp.
Bradyrhizobium spp.*(Lupinus)*	*Lupinus* spp.

3.5 LEGUMINOSEAE

The Leguminoseae are one of the largest plant families, with worldwide distribution, about 750 genera, and an estimated 16,000 to 19,000 species. The Leguminoseae have traditionally been divided into three distinct sub-families based on floral differences- Mimosoideae, Caesalpinoideae and Papilionoideae. Virtually all species within the Mimosoideae and Papilionoideae are nodulated, but about 70% of the species in the

subfamily Caesalpinoideae are non-nodulated. Although only about 15% (3000) of the total species have been examined for nodulation, more than 90 percent form root nodules in which nitrogen fixation presumably occurs in symbiosis with rhizobia. These species are representative of all three subfamilies of legumes (Allen and Allen, 1981).

In ancient times, the beneficial effects of leguminous species on soil fertility were well recognized. Theophrastus (370–285 B.C.) in his "Enquiry into Plants" wrote as follows: "Of the other leguminous plants the bean best reinvigorates the ground"; and in another section, "Beans … are not a burdensome crop to the ground; they even seem to manure it" (Chen *et al*., 1988).

The presence of nodules on roots of leguminous plants was observed by Malpighi in 1679 but he considered them as insect galls (Rao, 2001). Liebig (1865) thought that legumes with their broad leaves were capable of absorbing ammonia from the atmosphere and thus met much of their needs for nitrogen and also improved nitrogen economy of the soil. Nevertheless, it was not until the middle of the last century that this extraordinary capability of legumes to fix nitrogen by symbiotic bacteria, present in the root nodules was discovered (Azam, 2001). It was only in 1888 that Hermann Hellriegal and Hermann Wilfarth established positively that root nodules contain bacteria capable of fixing nitrogen from the atmosphere. This was quickly followed by the experiments of Beijerinck (1888), who used pure culture techniques to isolate the root nodule bacteria and proved that they were the causative agents of nitrogen assimilation. He proposed the name *Bacillus radicicola* for these organisms. The root nodule bacteria were later renamed *Rhizobium* by Frank (1889). The development of root nodules from root hairs was studied by Ward in 1889 for the first time (Rao, 2001).

3.5.1 Importance of legumes

In many countries, human nutrition is highly dependent on grain legumes for protein. It is estimated that about 20% of food protein worldwide is derived from legumes. The

highest consumption occurs in the former Soviet Union, South America, Central America, Mexico, India, Turkey, and Greece. The dietary use of legumes is quantitatively in the following order: dry bean (*Phaseolus vulgaris*), dry pea (*Pisum sativum*), chickpea (*Cicer arietinum*), broad bean (*Vicia faba*), pigeon pea (*Cajanus cajan*), cowpea (*Vigna unguiculata*) and lentil (*Lens culinaris*). Peanut (*Arachis hypogaea*) and soybean (*Glycine max*) are dominant sources of cooking oil and protein. The importance of legumes in animal feed should not be overlooked. Alfalfa (*Medicago sativa*), clovers (*Trifolium* spp.), stylosanthes (*Stylosanthes* spp.), desmodium (*Desmodiu*m spp.) and other forages are grown extensively and are either grazed or fed as hay or silage. Alfalfa silage furnishes not only roughage and high-quality protein, but also a variety of vitamins, minerals, and other nutrients (Dakora, 2003).

The view that symbiotic legumes benefit companion and subsequent plant species in intercrop and rotation systems as well as on pest and pathogen control. is now well accepted. Similarly, leguminous plant fix large amounts of N in tropical environments, and are also valued mainly for their contribution of N to non-leguminous crop species (Dakora, 2003). Non-legumes deplete the nitrogen in the soil whereas leguminous crops can restore nitrogen, primarily as organic forms that are not readily leachable which can substantially increase N supply in the soil-plant system Growing legumes improves soil quality through their beneficial effects on soil biological, chemical and physical conditions (Shakya, 2004). At present, the value of leguminous species in crop rotation stands established beyond any doubt (Azam, 2001).

In Nigeria, after a continuous cropping for 4 years on an area that had been under bush fallow for over 12 years, the inclusion of a legume in the crop rotation showed an important residual effect in terms of N supply to the monocropped maize while no residual effects were obtained from chemical fertilizer application (Shakya, 2004).

According to Vance (2001), properly managed alfalfa (*Medigo sativa*)- corn (*Zea mays*) rotations in the U.S upper Midwest (Corn Belt) could reduce fertilizer inputs by up to

25% without loss of production and give a realized net return of $70 to $90 million. Depending upon the management and cropping system, legume green manures have the potential to replace more than 100 kg Nha^{-1} for a subsequent grain crop. This equates to savings of between $60 to $90 ha^{-1} in N fertilizer.

According to Shakya (2004), introducing more leguminous crops in the pure stands as well as in association would help in maintaining soil fertility especially in improving the N status of the soils in the hills of Nepal.

3.6 THE SOYBEAN

Literature suggests that soybean originated in the northern provinces of China and was first described in about 2000 B.C as one of the most important cultivated legumes and one of the five scared grains essential to Chinese civilization. It began to be cultivated there for its seed about 1100B.C. From this primal centre, the soybean spread to Korea, Japan, Southern China, and other countries in Southeast Asia. Soybeans were first introduced to Europe and North America as a forage crop (Caldwell, 1973).

The soybean is classified as a member of the family Leguminoseae, the sub-family Papilionoideae, the genus *Glycine*, the subgenus *soja* (the other subgenera are *bracteata* and *Glycine*), and the species *max*. *G. ussuriensis* Regel and Maack is considered to be the progenitor of cultivated *G. max* (L.) Merrill (Johanson and Berhard, 1963).

The soybean is a dicotyledonous plant that exhibits epigeal emergence with alternate trifoliate leaves except at the first two nodes. Leaves, stems and pods are covered with a gray or brown pubescence, which is very noticeable at maturity. Flowers are either purple or white in colour, with purple being dominant with 6-7 mm in length and are self-pollinated. Pods may be black, brown or tan at maturity. Seeds are spherical with seed coats yellow in common, but green, black, brown and bi-colored can also be found (Naeve, 2005).

Soybean is grown in many areas of the globe in both tropical and temperate climates. It can yield, on average, between 3 to 5 tons per hectare, given good soil and water conditions (Gresshoff, 1990). The soybean was used as a basic food and a source of medicinals from the Middle ages until the early 1700s when greater interest was shown in the legume. Owing to the high nutritional as well as commercial value, the world's soybean cultivation and production has been increased about 10.5 folds within last 50 years i.e. from 17 million tons in 1950 to 179 million tons in 2002 (FAO, 2002).

Soybean is an ancient and the fifth important legume crop of Nepal, grown in tropical to temperate region of an elevation from 500-3000 m, mainly in the mid-hills as inter and or mixed crop with maize during summer and on the bunds of paddy fields under upland conditions. Plant can grow between 25°C to 30°C at pH 6-7 and rainfall 500 to 750 mm. The cultivation of soybean in terai and inner terai has been growing in recent years due to its commercial value in oil and cattle feed industries. The soybean cultivars Sathiya, Hardee (1978), Hill (1978), Ransom(1987), Seti (1990), Cobb (1990), Lumle-1 (1996) and Tarkari Bhatmas (2004) have been reported in Nepal (MoA and C, 2005).

Table 3. Production of soybean in Nepal

Year	**Area (ha)**	**Production**	**Yield (kgha^{-1})**
2003/2004	22073	19,363 (metric tones)	877
2002/2003	21450	18,681 (metric tones)	871

Source: Ministry of Agriculture and Cooperatives 2005, Nepal

3.6.1 Importance of soybean

Soybean is mainly produced for protein and oil contents. It contains 40-42% good quality proteins having high content of essential amino acids and 18-22% oil comprising up of 85% unsaturated fatty acid that is free from cholesterol. Therefore, it

is highly desirable in human diet and animal nutrition. Soybeans are capable of producing the greatest amount of protein per unit of land of any major plant or animal source as food by people today **(**Considine and Considine, 1997). Soybean builds up the soil fertility by fixing large amount of atmospheric nitrogen through the root nodules and also through the leaves that fall on the ground at maturity (Poudyal, 2005).

Vincent (1974) provided the representative estimate of N fixation by various legumes and reported that N fixed by soybean was 20-200 $kgha^{-1}$. Sauer (1993) reported that rotating maize with soybeans reduced the need for N fertilizers. Peel (1998) while working in North Dakota showed that soybeans had a residual N yield of 100 kg Nha^{-1} for subsequent crops, Lindemann and Glover (1999) estimated that the N fixation by soybeans ranged from 200 to 617 kg Nha^{-1} (Vance, 2001). Praharaj and Dhingra (2001) concluded that *Bradyrhizobium* inoculation significantly enhanced the efficiency of BNF in soybean and fixed an additional 66.1-74.7 kg Nha^{-1} over control and this resulted an increased yield of wheat by 60 $kgha^{-1}$ on the following year.

3.7 SYMBIOSIS

The term symbiosis was first used by Frank in 1877 to describe the regular coexistence of different organisms such as fungi and algae in lichens. Symbiosis is defined as any stable condition in which two different organisms live together in close physical association for their mutual advantage (Azam, 2001) In recent years, symbiosis is defined as the living together of differently named organisms (Sharma, 2001).

3.7.1 Legume-*Rhizobium* symbiosis

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. In this symbiosis, the plant provides the bacterium with a

"safe" environment and a steady supply of carbon for energy and growth. This carbon source is referred to as photosynthate and in most *Rhizobium*-legume symbiosis; photosynthate refers to the dicarboxylic acids, succinate, fumarate and malate. In return, the bacteria provide the plant with fixed nitrogen, which is readily utilized, by the plant. The result of this symbiosis is a dramatic increase in plant production without the need for adding external fertilizer (Deacon, 2006).

3.7.2 Initiation of nitrogen fixing symbiosis

Since the Rhizobia are motile, they actually swim to the rhizosphere of their host plant chemotactically. Phenolic compounds secreted by the roots, e.g. luteolin, serve as signals. Once they reach the host root system, nitrogen-fixing symbiosis of legume plants and *Rhizobium* is initiated by recognition by the host plants of lipochitin oligosaccharides signal molecules (Nod factors) produced by *Rhizobium*. The host specificity is determined by at least two steps of the mutual signal exchange between the plants and microsymbionts. First, bacterial nodulation (Nod D) genes are activated in response to plant-secreted signal molecules such as flavinoids, betaines and aldonic acids in the seed and root exudates resulting in biosynthesis and secretion of lipo-chitooligosaccharides (LCOs) by rhizobia. In the second step, LCOs elicit nodule formation on the host plant roots and trigger the infection process (Umehara and Kouchi, 1999). In plants such as alfalfa and wild soybean (*Glycine soja*), genuine nodule structures are shown to be induced by the application of Nod factors alone without bacteria (Souleimanov *et al*., 2002).

The earliest responses of legume plants to Nod factors appear on root hair cells, i.e., membrane depolarization, alkalization of extra cellular fluid, and transient Ca^{++} influx into root hair cells, followed by oscillation of Ca^{++} influx/efflux (Ca-spiking). Concomitantly, root hairs show typical morphological changes, such as tip swelling, multiple tip growth (branching), and abundant deformation. These responses prepare the rhizobial infection process.

Rhizobia enter the roots of legumes either through root hairs or directly at the point of emergence of lateral roots. The root cap cells and probably also the epidermal cells secrete large amounts of polysaccharide, lectins of legumes roots and calcium dependent adhesion called rhicadhesin. Lectins have high affinity to carbohydrates moieties on the surface of appropriate rhizobial cells and have been identified as specific mediators of the attachment of rhizobia to susceptible root hairs. 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. Curling or controlled growth and branching of root hairs is the first visible plant response to *Rhizobium* (Nutman *et al.*, 1959).

In the first stage of infection, bacteria grow very profusely at the tip of the root hair and form a long filament in the root hair called infection thread. This thread reaches the endodermis and pericycle area through cortex tissue. Cells of this area go on dividing and form a tumor like young nodule. The precise exchange of molecular signals between *Bradyrhizobium japonicum* and the host plant is essential for the development of effective root nodules. Depending upon the host plant and partly on the *Rhizobium* strain, many infections cease before reaching the base of the root hair. Others cease at the base of the hair and in the root cortex (Sharma, 2001).

3.7.3 Structure and function of nodule

The outermost layer of the nodule constitutes the bacteroid zone, which is enclosed by several layers of cortical cells. Anatomically, a nodule is made of thin walled parenchymatous cells, which are filled up with the nitrogen-fixing organisms. Inside the nodule, the Rhizobia change their form to cells called bacteroids which are swolled and irregular, frequently appearing in star, clubbed or branched shape or X or Y shaped. The

bacteriods carry out fixation of atmospheric nitrogen which is subsequently made available to the host plant. When the host plants die, their nitrogen is returned to the soil for use by other organisms (Atlas and Bartha, 2005). The rate of nitrogen fixation of nodule is directly proportional to the volume of the effective nodule (Sharma, 2001).

3.7.4 Mechanism of BNF

Biological nitrogen fixation can be represented by the following equation, in which two moles of ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of (ATP) and a supply of electrons and protons (hydrogen ions):

$$\mathbf{N_2 + 8H+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi}$$

This reaction is performed exclusively by prokaryotes (the bacteria and related organisms), using an enzyme complex termed nitrogenase**.** This enzyme consists of two proteins - nitrogenase reductase or an iron (Fe) protein that contains four iron and four acid labile sulphur atoms and a molybdenum-iron protein that contains two molybdenum, 28-32 iron, and approximately 28 acid labile sulphur atoms. 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) N=NH is reduced to $H_2N\text{-}NH_2$, and this in turn is reduced to $2NH_3$.

The nitrogenase enzyme complex is highly sensitive to oxygen, which is inactivated when exposed to oxygen, because this reacts with the iron component of the proteins. In *Rhizobium*, the root nodules contain oxygen-scavenging molecules such as leghaemoglobin, which shows as a pink colour when the active nitrogen-fixing nodules are cut open. Leghaemoglobin is a macromolecule synthesized by both symbiotic partners, the rhizobia and the host plant. *Rhizobium* synthesizes the heme portion, and

the plant the globin. The function of leghaemoglobin in nodules is to reduce the amount of free oxygen by binding to oxygen and generating thus oxygen- free areas within the roots of plants where the bacterial nitrogen-fixing enzyme nitrogenase can become active and thereby protecting it, which is irreversibly inactivated by oxygen (Deacon, 2006). Yet the bacteria always remain separated from the host cytoplasm by being enclosed in a membrane - a necessary feature in symbiosis (Sharma, 2001).

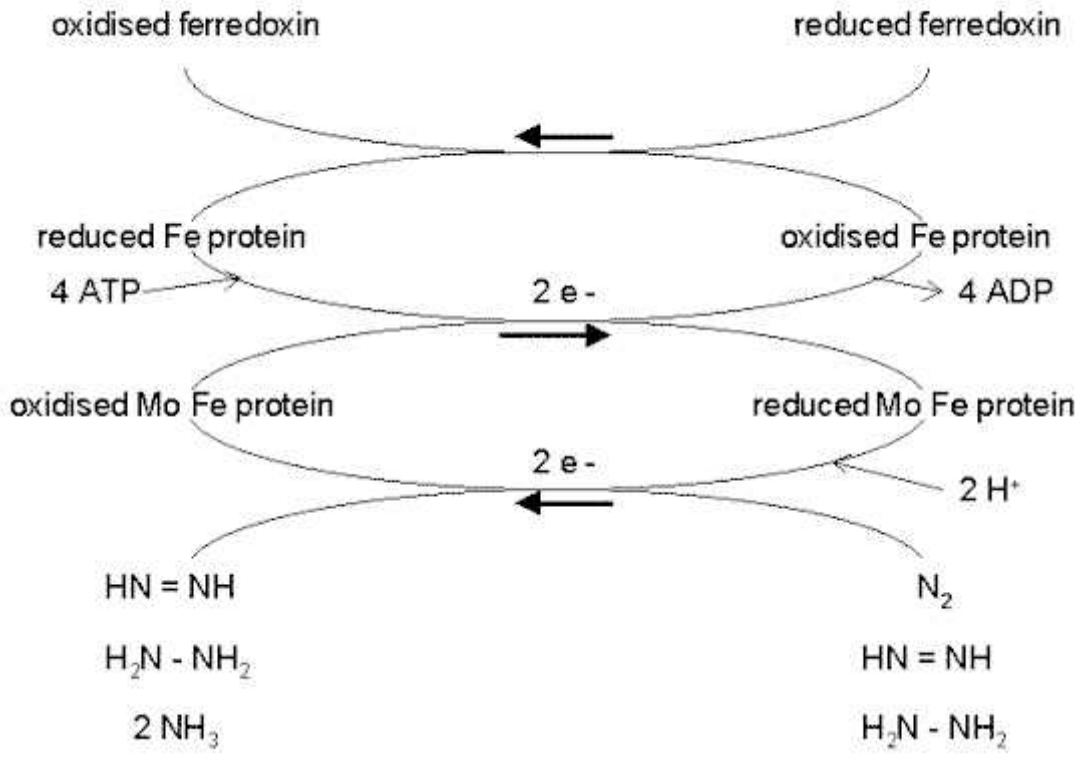


Figure 1. Mechanism of nitrogen fixation (Deacon, 2006)

Various bacterial genes involved are nod, nif, fix and nodulin genes. The host specificity of the *Rhizobium* is controlled by the nodulation (nod genes). The gene required for nitrogenase structure and function are called nif genes. The nif genes in the *Rhizobium* are regulated by another set of gene called fix genes. Fix gene is essential for nitrogen fixation. Various plant genes that take part in nodule structure and function are collectively known as nodulin genes (Madigan *et al*., 2000).

3.7.5 Factors Limiting BNF

Interactions between the microsymbiont and the plant are complicated by edaphic, climatic, and biotic factors. These factors affect either the microsymbiont, the host-plant, or both (Mulongoy, 1992).

The six main edaphic factors limiting BNF are excessive soil moisture, drought, soil acidity, phosphorus deficiency, excess mineral N, and deficiency of Calcium, Molybdenum, Cobalt and Boron. The two important climatic determinants are temperature and light. Biotic factors are the absence of the required rhizobia species and excessive defoliation of host plant, crop competition, and insects and nematodes.

3.8 PHOSPHORUS

Phosphorous is ubiquitous element, found throughout the plant and animal worlds and occurring in all tissues and organs. In young plants, phosphorus is the most abundant in tissue at the growing point. It is readily translocated from older tissue to younger tissue, and as plants mature, most of the element moves into the seeds and/or fruits. Phosphorus is responsible for utilization of starch and sugar, cell nucleus formation, cell division and multiplication, fat and albumin formation and transfer of heredity. It is essential for flower and fruit formation. Deficiency symptoms are purple stems and leaves; maturity and growth are retarded. Premature drop of fruits and flowers may often occur (Johansson *et al*., 2004).

3.9 MYCORRHIZA

Mycorrhiza comes from the combination of two words, one Greek: *mikes* (fungus) and the other Latin: *rhiza* (roots). Mycorrhiza, literally translated ‘root fungus’ was coined by Frank in 1885, refers to the symbiotic association that exists between plant root system and fungi. Probably the roots of the majority of terrestrial plants are mycorrhizal

(Sahay *et al.*, 1998). It has now established that more than 6000 fungal species are capable of establishing mycorrhiza approximately with 2,40,000 plant species belonging to the Zygomycotina, Ascomycotina and Basidomycotina (Singh, 2004).

3.9.1 Types of associations

Depending on the plant and fungal species involved as well as distinct morphological patterns, seven different types of mycorrhizal associations have been recognized. They are vesicular-arbuscular mycorrhizae (VAM or AM or AMF), ectomycorrhizae, ectendo, arbutiod and monotropoid, orchid, ericoid and the Australian lily Thysanotus). All the groups of mycorrhizal fungi build a living bridge between plant root and bulk soil in most ecosystems (Singh, 2004; Singh *et al*., 2002; Sharma, 2001).

3.9.2 Functions of mycorrhizal fungi

As all mutualistic beneficial co-operations, both partners (fungi and plant) have advantages of the symbiosis. The plant-acquired carbon from photosynthesis is traded for various mycorrhizal benefits to the host plant. The fungal mycelium that extends from the root surfaces into the soil matrix captures nutrients from soil solution. The minuscule diameter of the fungal hyphae increases the surface area that the plants are able to utilize for their nutrient acquisition (Rai, 2006). This is likely to be one of the most important features of mycorrhizae (Singh, 2004).

3.9.3 Vesicular-arbuscular mycorrhizae

These are associations where Zygomycete fungi in the order Glomales produce intracellular structures-arbuscles and vesicles within roots during various phases of development. The plant symbiont ranges from Bryophytes to Angiosperms. These mycorrhizae are most commonly reported group since they occur on a vast taxonomic range of plants, both herbaceous and woody (Singh, 2004; Malla *et al*., 2002).

Members of more than of 80% of extant vascular plant families are capable of forming the AM with as few as 150 fungal species. AM colonization of plants has been observed over a wide range of soil pH, soil phosphate levels and salinity. It is generally assumed that the arbuscule is a key structure in AM symbiosis and that the interface between arbuscules and the invaginated plant cell membrane, the so-called periarbuscular membrane, is the site of phosphate, and possibly carbon, exchange between the symbionts.

The beneficial effects of arbuscular mycorrhizae on plant growth have often related to the increase in the uptake of no mobile nutrients from the soil such as phosphorus by the direct activity of the extramatrical mycelium that allows the exploration of the soil volume (Illmer *et al*., 1995). The AMF acquire not only phosphorus, but also N. It also provides increased protection against environmental stresses, including drought, cold, salinity and pollution. In addition, symbiosis tends to reduce the incidence of root diseases and minimizes the harmful effect of certain pathogenic agents. By and large, the growth and health of colonized plants is improved (Marschner and Dell, 1994).

A major obstacle that in the utilization of these soil microorganisms is their obligate symbionts status. These fungi can grow only in the presence of a living plant, which means that their propagation requires the use of pot cultures. One most positive point is that the AMF are not specific in terms of the partner plant they choose, which means that the same fungus can be grown on a large number of plant species (Dadarwal, 1997).

3.9.4 *Piriformospora indica*

To fill the gap of obligate photosymbiont, the AM like fungi- a new root endophyte, *Piriformospora indica*, which can be easily cultivated on a number of complex and synthetic media- has been described by Varma and his collaborators (Singh, 2004).

It was originally found (gold strike by chance) along with arbuscular mycorrhizal spores from the rhizospheric soil samples of spineless cacti and *Cenchrus* spp. (desert grass) growing in the sandy deserts of interior of North-West Rajasthan, India. Based on the morphological features and its characteristics pear-shaped chlamydospores, the fungus was named *Piriformospora indica*. The culture of *Piriformospora indica* was patented and deposited at Germany (Singh *et al*., 2001; Varma *et al*., 2001).

3.9.4.1 Molecular Taxonomy

In order to obtain more information about the systematic position of the new fungus, the ultrastructure of the septal pore and the cell wall were examined. The cell walls were very thin and showed multilayered structures. The septal pores consisted of dolipores within the continous parenthosomes, which forms the basis for the systematic position within the Hymenomycetes. The dolipores were very prominent, with multilayered crosswall and a median swelling mainly consisting of electron transparent material.

In median sections of the septal pores, the parenthosomes were always straight and had the same diameter as the corresponding dolipore. Parenthosomes were flat discs without any detectable perforation and consisted of an electron dense outer layer and a less dense inner layer, which showed an inconspicuous dark line in the median region .They were in contact with ER membranes, which were 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 and 28S rDNA), the fungus was 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*. Due to primitive similarities to Zygomycetes, this fungus was termed as AM-like-fungi (Varma *et al*., 1998; Singh, 2004).

3.9.4.2 Fungal 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-AMF, 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).

The hyphae were highly interwoven, often adhered together and gave the appearance of simple intertwined cord. Young mycelia were white and almost hyaline, but inconspicuous zonations were recorded in other cultures. The mycelium was mostly flat and submerged into the substratum. Hyphae were thin- walled and of different diameters ranging from 0.7 to 3.5 µm. The septate hyphae often showed anastomosis and were irregularly septate. They often intertwined and overlapped each other. In older cultures and on the root surface, hyphae were often irregularly inflated, showing a nodose to coralloid shape and granulated dense bodies was observed. For this reason, many cells contained more than one nucleus (Singh *et al*., 2001).

Chlamydospores were formed from thin-walled vesicles at the tips of the hypha and appeared singly or in clusters and were distinctive due to their pear shaped appearance. They were 16-26 µm in length and 10-17 µm in width. The cytoplasm was densely packed with granular material and usually contained 8-25 nuclei. Very young spores had thin, hyaline walls. At maturity, these spores had walls up to 1.5 µm thick, which appeared two -layered, smooth and pale yellow. Neither clamp connections nor sexual structures could be observed (Varma *et al*., 2001; Singh, 2004).

3.9.4.3 Cultural characteristics

P. indica can be successfully cultivated on wide range of synthetic solidified and broth media, e.g., modified aspergillus, M4N, Murashige and Skoog (MS), woody plant

medium (WPM), modified Merlin-Norkrans (MMN), MMN1/10, MMNC, Malt-Yeast Extract and potato dextrose agar (PDA). Among the tested media, most optimum was aspergillus (Kaefer, 1977). The temperature range of *P. indica* is 25-35°C; the optimum temperature and pH being 30°C and 5.8 (4.8-6.8), respectively. On MMN medium sparsely running hyaline hyphae on the agar surface was seen, while on PDA deep furrows with a strong adhesion to the agar surface was apparent. This sharp mode of growth was not observed when fortified with malt extract and normal aspergillus medium. In contrast to aspergillus medium, shaking conditions on MMN broth medium invariably inhibited the growth. On Moser b medium, the colonies appear compact, wrinkled with furrows and constricted (Varma *et al*., 2001; Singh 2004).

3.9.4.4 Host spectrum

Interestingly, the host spectrum of *P. indica* is very much alike AMF. It can colonize the roots of host plants as diverse as economically important crops, medicinal forests and ornamental plants such as *Artemisia annua, Bacopa monnieri, Cicer arietinum, Glycine max, Nicotiana tabacum, Oryza sativa, Petroselinum crispum, Pisum sativum, Populus tremula, Setaria italica, Sorghum vulgare, Solanum melongena*, and *Zea mays.* Even plants like *Adhatoda vasica Chlorophytum tuberosum, Dalbergia sisso, Prosopis julifera, Tagetes erecta, Terminalia arjuna, Withania somnifera* and *Spilanthus calva* stand promoted in their growth following interaction with *P. indica* (Singh *et al*., 2000; Rai *et al*., 2001; Singh, 2004; Rai and Varma, 2005).

One striking difference is that unlike AMF, the host range of *P. indica* also includes terrestrial orchids like *Dactylorhiza purpurella, D. incarnate, D. majalis,* and *D. fuchsia* (Singh *et al*., 2001). It, however, fails to establish a symbiotic relationship with taxa belonging to Brassicaceae, Chenopodiaceae, Cyperaceae, Junaceae, and Proteaceae or with lupines and Cruciferae. But exceptions are those belonging to the members of Cruciferae and some members of Chenopodiaceae and Amaranthaceae. In vitro studies recorded that *P. indica* profusely interact with the root system of the crucifer plants viz.,

mustard (*Brassica junaceae*), spinach (*Spinaceae oleraceae*), cabbage (*Brassica oleraceae* var *capitata*) (Kumari *et al*., 2003). A recent report indicated the ability of *P. indica* to colonize the rhizoids of a liverwort (bryophyte). The fungus interacts also with the non-mycorrhizal host *Arabidopsis thaliana* (Singh *et al*., 2001; Singh, 2004).

3.9.4.5 Functions

P. indica promises to be an excellent candidate for biological hardening of micro propagated plantlets. The survival rate of *P. indica* inoculated plantlets of *Nicotiana tabacum* L. (tobacco) was 95%, but only 57% of untreated plantlets survived the transfer to pots (Varma *et al*., 1998). Fungus also rendered more than 90% survival rate of the transferred plantlets of *Bacopa monniera* L. Wett., *Spithanthus calva* DC, *Withania somnifera* L. and *Azadirachta indica* A JUSS by protecting them from 'transplantation shock'. Recent experiments on the tissue culture-raised coffee (*Coffea arabica* L.) plants have shown very promising results (Singh *et al*., 2000; Rai and Varma, 2005).

P. indica produces significant amounts of acid phosphatases for the mobilization of a broad range of insoluble, condensed or complex forms of phosphate, enabling the host plant the accessibility of adequate phosphorus from immobilized reserves in the soil in an energy dependent process. The fungus showed prominent acid phosphates activity in both intra- and extra cellular fractions. The expression of a phosphate repressible isoform of phosphatase, whose expression was dependent on the external phosphate supply, gave a direct evidence for the involvement of this enzyme in the phosphate metabolism (Singh *et al*., 2000).

P. indica completely blocked growth of root and seed pathogen *Gaeumannomyces graminis* which indicated that *P. indica* acted as a potential agent for biological control of root diseases, however, chemical nature of the inhibitory factor is still unknown (Singh, 2004).

3.9.4.6 Functional similarities of *P. indica* with AMF

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

- broad and diverse host spectrum
- hyphae extramatrical, inter- and intracellular
- hyphae never invade the endodermis
- chlamydospores in soil and within cortical tissues
- sexual stages not seen
- positive phytopromotional effects on tested hosts
- phosphorus mobilizer.
- phosphorus transporter
- tool for biological hardening of micro propagated plantlets
- potent biological control agent against root pathogens

3.10 POTASSIUM

Together with nitrogen and phosphorous, potassium is one of the essential macro minerals for plant survival. Potassium is required in adequate amounts in several enzymatic reactions, particularly those involving the adenosine phosphates, for formation of sugars, starches, carbohydrates, protein synthesis and cell division in roots and other parts of the plant. The meristematic tissues in general are particularly rich in potassium (Considine and Considine, 1997). It helps to adjust water balance, improves stem rigidity and cold hardiness, enhances flavor, color and oil content of fruit and vegetable crops. Its deficiency may produce both gross and microscopic changes in the structure of plant which include leaf damage, high or low water content and mottled, spotted or curled leaves, decreased photosynthesis, disturbed carbohydrate metabolism, low yields, low protein content and other abnormalities. Potassium that is dissolved in

soil water plus that held on exchange sites on clay particles (exchangeable K) is considered readily available for plant growth (Rehm and Schmitt, 1997).

Bacteria that live in the nodules on the roots of leguminous plants need an ample supply of potassium to function efficiently. Many legumes are much more responsive to applied potassium than other plants because they have less extensive root systems and are less able to scavenge nutrients from the soil (Johnston and Steen, 2006).

3.11 INOCULATION PRACTICES

Inoculation is the introduction of specific microorganism into the soil. Salfield in 1860's demonstrated that legume growth is benefited by the transfer of soil from a field that had previously grown the same plant. This was the first form of crude inoculation. Pure bacterial preparations were not added to seed prior to planting until 1890, when Nobbe and Hiltner demonstrated the procedure (Considine and Considine, 1997) but were soon replaced by sterilized soil impreginated with rhizobia, then by peat coated with agar, and finally in the early 1920's by peat alone impregnated with rhizobia. Since then the use of rhizobial cultures in the establishment of legumes has been widely recognized (Oad *et al*., 2002).

Artificial seed inoculation is often needed to restore population of effective strains of *Rhizobium* near the root zone to ensure high yields. Rhizobia have been used commercially as seed inoculants in the form of seed coatings for over one hundred years. Currently, about 80% of alfalfa grown in the United States is inoculated with rhizobia prior to planting (Vance, 2001).

Co-inoculation or dual inoculation is the practice of application of two or more types of microorganisms in the crop fields and this practice is found to be more efficient than that of single inoculation. Generally single inoculation practice helps to supply only one

type of nutrient to the plant through only one way but co-inoculation helps in supply of many nutrients to the plant through different ways (El-Ghandour *et al.*, 1998).

The tripartite symbiosis between leguminous plants, *Rhizobium* spp. and VAM fungi has been the subject of intensive research in recent years (Thiagarajan and Amed, 1993) and is the most efficient combination for growth promotion and for gaining higher yield of crops, vegetables, and trees. 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).

Raut and Ghonsikar (1992) reported that dual inoculation of *Rhizobium* and AM fungi on pigeon pea was more beneficial than the single inoculation of *Rhizobium* or AMF to stimulate growth parameters such as nodulation, root shoot growth, fresh and dry mass, yield, and nitrogen uptake. Singh and Singh (1993) reported that inoculation with *Bradyrhizobium*, VAM and phosphate solubilizing microorganisms in combinations of any two or three in soybean resulted in significant increases in nodulation, plant growth, mycorrhization, N and P uptake, available soil P and grain and straw yields. Pant and Singh (1995) conducted the two-year field experimentation on the co-inoculation of phosphate solubilizers with *B. japonicum* and reported a positive and significant impact on nodulation and seed yield of soybean. Abdelgadir (1998) investigated the symbiosis of twelve different VAM fungi on two different soybean varieties and reported that depending on plant variety, measurements of plant fresh weight revealed synergistic, antagonistic and no interactions between *Bradyrhizobium* strains and *Glomus* species. El-Ghaundour *et al*. (1998) reported increased nodulation and N content of ground nut (*Arachis hypogea*) due to the dual inoculation of *Bradyrhizobium* strains and AM fungi and concluded that there was a possibility of saving than 50% of recommended N-fertilizer. Shalaby and Hanna (1998) reported the dual inoculation of *Bradyrhizobium japonicum* either with *Pseudomonas fluorescens* or endomycorrhizal fungi *Glomus mosseae* in soybean significantly increased nitrogen uptake in plants and also the productivity of soybean grain by 20%- 30% and 14%-17% as compared to the single

inoculation. Hoque *et al*. (1999) observed 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. Hazarika *et al*. (1999) reported that the inoculation of black gram with efficient AM fungi in phosphorous deficient soil of Asam greatly improved plant growth, nutrient uptake and yield.

Similarly, Giri *et al*. (2000) studied the inoculation of *Sesbania aegyptidea* seedlings with *Glomus macrocarpum* and showed that there was significant increase on biomass, nutrient uptake and primary establishment of seedlings. Nalalia *et al*. (2001) concluded that 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. Ruiz-Lozano *et al*. (2001) concluded that AM symbiosis can protect legume plants against the detrimental effect of premature nodule senescence induced by drought stress.

According to Singh *et al*. (2001), fresh and dry weights of both underground and aboveground parts, basal and leaf area, number of inflorescence and flower and seed production of *P. indica* inoculated *Spilanthes calva* and *Withania somnifera* plants were higher than the corresponding controls and so did their net primary productivity and also *Azadirachta indica* inoculated with *P. indica* drastically improved plant height and total biomass in contrast to uninoculated controls when conducted both under natural (unautoclaved) and sterile (autoclaved) potted soils. Oad *et al*. (2002) concluded that the inoculation of *R. japonicum* exhibited the positive change in terms of enhanced growth and seed yield of soybean crop. Varma *et al*. (2002) identified a model plant, *Setaria italica* (monocot) the smallest genome next to rice was highly promoted by interacting with *P. indica*. Aryal and Fujita (2003) reported that rhizobia and AM fungal inoculation significantly increased pod yield, shoot and root dry weight, and shoot N

and P compared to control or singly inoculated bean plants. Jia *et al*. (2004) 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. Johansson *et al*. (2004) and Rabie and Almadini (2005) showed that dual inoculation with AM fungi and N-fixing bacteria can support both needs for nitrogen and phosphorus and increase the growth of host plant.

Singh (2004) reported that *P. indica* inoculated maize plants produced more root and shoot biomass than control plants. Rai and Varma (2005), while working with *P. indica* inoculated *Adhatoda vasica* Nees, reported a significant root and shoot biomass increment over controls. Shende *et al*. (2006) observed *P. indica* inoculated *Withania somnifera* plants produced more root and shoot dry weight than control plants on every 15 days after plantation until 60 days.

In Nepal, research work on bacterial fertililizers was initiated around late sixties by Department of Agriculture (DOA) at Division of Soil Science and Agricultural Chemistry (DSSAC), Khumaltar. In order to strengthen the research work in this field, Nepal Academy of Science and Technology (NAST) carried out a Biofertilizer Project in collaboration with DOA at DSSAC from 1985 to 1988 with the financial assistance of International Foundation for Science (IFS), Sweden. Results from this project showed the increase in broad bean yield up to 67.27% and 63.95% respectively due to inoculation of *Rhizobium* (Rh) strains like Rh-2002 and Rh-2010 (Shakya, 2004).

Maskey and Bhattarai (1986) reported 17% increment in soybean yield and 60% increment in lentil yield when inoculated with *Rhizobium* strain. Sthapit (1987) reported increased bean yield by 5-60% when inoculated with *Rhizobium japonicum* under varying conditions at Lumle. Similarly, inoculation of lentil seeds with *Rhizobium leguminosarium* significantly increased yield by 17.84% over uninoculated plot in rice-wheat systems at shera. Bhattarai (2000) studied the effect of co-inoculation of

Azotobacter chrococcum amd *Bradyrhizobium japonicum* on the soybean plants and reported significant increase on plant growth, dry matter of plant and nitrogen content over the control. Maskey *et al*. (2001) performed the on-farm measurements of nitrogen fixation by winter and summer legumes in Hill and Terai regions of Nepal and estimated that approximately 3,000 tons of nitrogen was fixed annually in Nepal by legumes valued at US$ 15 million. Neupane (2003) studied the effect of dual inoculation of AM fungi and *Bradyrhizobium japonicum* in soybean and reported that the dual inoculation enhanced growth, nodulation, nitrogen and phosphorus content and specific P uptake in soybean plant.

3.12 SIGNIFICANCE OF BIOFERTILIZER IN NEPAL

The overwhelming majority of Nepal's people engage in agriculture (80%), which contributes about 40% of the GDP (Khadka, 2006). Farmers use different chemical fertilizers without being aware of its consequences. Though uses of these chemical fertilizers give satisfactory result in the beginning, it causes degradation and infertility of soil at the end. Thus, causes less cultivation. These can be overcome by the use of biofertilizer, which are natural, beneficial, and ecologically and user-friendly that help in optimum cultivation of different agricultural products without the risk of soil degradation, infertility and soil erosion maintaining the mineral economy of nature. For this reason, in context of Nepal, we should promote to rescue farmers and nation from the consequences of chemical fertilizers and protect cultivated land by using bio-fertilizers enhancing indigenous knowledge and practice of the people. Overall, it will bring more benefit to farmers, producers and consumers and contribute substantially to our economy which is the only alternative for sustainable agriculture.

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 MATERIALS

Materials used in this study are given in Appendix-III.

4.2 METHODS

The present study was carried out from March to September, 2005. The pot experiments were established at the garden of Central Department of Botany (CDB), Tribhuvan university (TU), Kirtipur.

The experiment was divided into two parts;

1) Using sterilized (autoclaved) soil.
2) Using unsterilized (natural) soil.

4.2.1 Collection of soil

The experimental soil was collected from the surface of garden of CDB, TU, Kirtipur. The experimental soil was mixed with river bed sand for aeration at the ratio of 1:2 (sand: soil).

4.2.2 Host plant

The study was conducted on a pot trial with *Glycine max* (L.) Merr. local cultivar 'Sathiya' as the host plant.

4.2.3 Source of soybean [*Glycine max* (L.) Merr. cv 'Sathiya']seeds

Seeds of soybean were kindly provided by Agronomy division, National Agriculture Research Council (NARC), Khumaltar, Lalitpur.

4.2.4 Source of *B. japonicum*

Pure culture of *B. japonicum* maintained on slants with Yeast extract Mannitol agar with congo red (YMA-CR) was kindly provided by CDB, TU.

4.2.4.1 Preparation of *B. japonicum* inoculum

Yeast extract Mannitol broth (YMB) (Vincent, 1970) was prepared and 75 ml was dispensed into three 250 ml conical flasks and autoclaved. After cooling down, 10 ml of sterilized YMB was dispensed into YMA-CR slant and the bacterial colonies of the slants were scrapped with the help of sterilized inoculation loop. The YMB along with bacterial colonies was then transferred into the conical flask and sealed with aluminum foil. The inoculated flasks were incubated on water bath shaker at 200 revolutions per minute at 28°C for 7-10 days. The number of bacteria in each flask was determined by serial dilution agar plating method using YEMA media.

4.2.5 Source of *P. indica*

Pure culture of *P. indica* was kindly provided by CDM, TU.

4.2.5.1 Cultivation of *P. indica*

One circular disc (1 cm in diameter) of *P. indica* was aseptically transferred with the help of sterilized cork borer onto centre of the petriplate containing solidified Potato Dextrose Agar medium and incubated at 30±2°C in dark for 7 days (Singh, 2004).

4.2.6 Pot preparation for the experiment using sterilized (autoclaved) soil

The earthenware pots having 23 cm diameter and 16 cm length were washed neatly with tap water. Soil mixture from 4.2.1 was sterilized by autoclaving at 121°C for two hours for three consecutive days. Pots were sterilized with 70 % (v/v) ethanol. Five kgs of the autoclaved soil was filled in the pots, leaving one inch from the top edge of the pot. The soil samples were previously analyzed for NPK.

4.2.7 Pot preparation for the experiment using unsterilized (natural) soil

The earthenware pots having 23 cm diameter and 16 cm length were washed neatly with tap water. Five kilograms (kgs) of field soil from 4.2.1 was filled in the pots, leaving one inch from the top edge of the pot. The soil samples were analyzed for NPK before.

4.2.8 Treatments

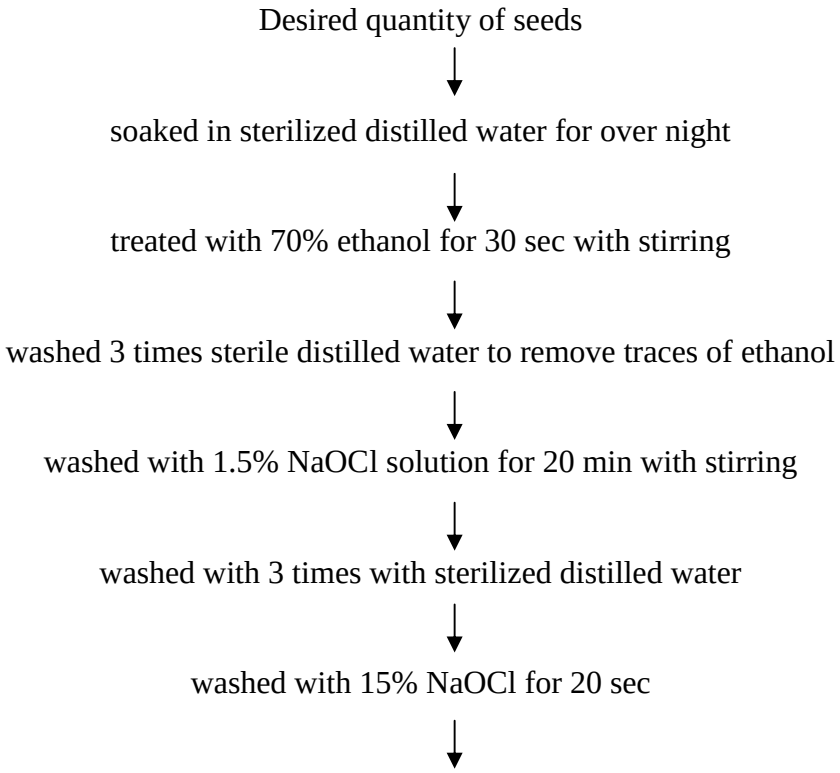
The following treatments were imposed in a completely randomized design with five replications for each set of sterilized and unsterilized soil.

1. Control plants without *Bradyrhizobium japonicum* and *Piriformospora indica*
2. Plants inoculated with *Bradyrhizobium japonicum*
3. Plants inoculated with *Piriformospora indica*
4. Plants inoculated with *Bradyrhizobium japonicum* and *Piriformospora indica*

4.2.9 Surface sterilization of seeds

Healthy seeds were soaked in sterile distilled water overnight in a flask and next day water was decanted off. The seeds were treated with 70% ethanol for one minute with constant stirring and ethanol was discarded and then seeds were washed thoroughly with distilled water. The seeds were transferred to another sterile flask and treated with

1.5% sodium hypochlorite (NaOCl) for 20 seconds and then washed six times with sterile distilled water to remove traces of NaOCl. The entire process was performed under sterile laminar flow bench to prevent contamination.



Flow chart No. 1. Protocol for surface sterilization of seeds

4.2.10 Pot culture trial

The seeds were sown at the rate of 8 seeds per pot in both cases. In the set containing sterilized soil, seeds were surface sterilized while in the set containing unsterilized soil seeds were not sterilized. The seeds were sown at a depth of 2.5 cm-4 cm. Watering was done frequently letting the seeds to germinate and grow. After five days of seed sowing when seeds began to germinate, seeds with poor germination were uprooted and thinning was done leaving only the healthy plants.

In case of the set using sterilized soil, after 10 days of seeds sowing plants were supplied with 1:10 strength of Hoagland Solution (Hogland and Arnon,1938, Appendix-II) on every alternate week consisting phosphate and devoid of phosphate nutrients for 10 weeks at the rate of 5 ml per plant.

4.2.11 Microbial inoculations

After one week of seed sowing, one circular disc (1 cm in diameter) with actively growing hyphae of *P. indica* was inoculated near the root of each plant at the depth of 2 cm from the soil surface (Singh, 2004).

After five days of inoculation of *P. indica*, 2 ml of broth culture (10^7 cells/ml) of *B. japonicum* was inoculated per plant near the root surface.

4.2.12 Observation

Growth parameters, namely nodule number and nodule fresh and dry matter, root length, root fresh and dry matter, shoot length, shoot fresh and dry matter were recorded at every 15 days interval i.e., on 21^{st}, 35^{th} , 50^{th} and 65^{th} from the date of plantation. For this, the soybean plants were carefully uprooted and removed from the pots, adhering soil particles were removed. Roots were thoroughly washed in tap water and air-dried for fresh weight. Experiments were recorded in five replicates from each group.

4.2.12.1 Nodulation

All the distinct visible and healthy pink nodules were detached from the root. The number of root nodules per plant was counted one by one for all experimental sets. All the nodules were oven dried at 70°C for 24 hours and nodule dry matter was measured accordingly.

4.2.12.2 Root growth parameter

The length of soybean root from the point of cotyledon attachment up to the main root tip was measured. Roots were oven dried at 70°C for 24 hours and root dry matter was measured for all experimental sets.

4.2.12.3 Shoot growth parameter

The length of soybean shoot from the point of cotyledon attachment up to the shoot tip was measured. The fresh weight was taken and the shoots were oven dried at 70°C for 24 hours and shoot dry matter was measured for all experimental sets.

4.2.13 Staining of root samples

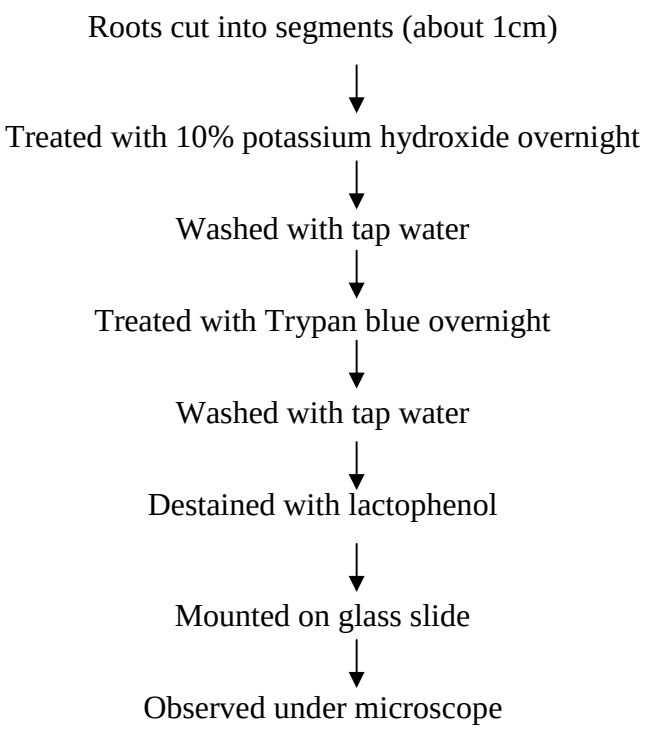
The percentage of root colonization was determined on 21^{st}, 35^{th}, 50^{th} and 65^{th} day after plantation and also after harvesting after maturity only in case of the experiment using sterilized soil. For this, at each observation, some plants were harvested randomly per treatment and separated from residual soil by washing thoroughly under running tap water. These were cut in small fragments (about 1 cm) and subsequently treated with 10% potassium hydroxide overnight. the root-pieces were washed with tap water and stained with trypan blue overnight. Finally, they were washed with tap water and destained with lactophenol. The destained root segments were mounted on a glass slide and observed under a light microscope.

4.2.13.1 Assessment of root colonization

The root pieces (1 cm long) were selected at random from the stained samples and mounted on a microscopic slide in groups of 10. Presence of infection was recorded in each of the 10-pieces. In all 50 root segments were examined and percentage colonization was calculated according to Singh (2004).

Calculation:

$$\text{Percent colonization } X\frac{\text{Number of colonized root segments}}{\text{Total number of segments examined}}|100$$



Flow chart No. 2. Protocol for root staining (Singh, 2004)

4.2.14 Soil analysis

Analysis of soil was done before soybean sowing and on 50th day after plantation and after harvesting to observe the effects of microbial inoculations on soil NPK content.

4.2.14.1 Determination of total nitrogen in soil

A. Digestion: One gram (g) of soil sample was weighed in 50 ml Kjeldahl digestion flask. 2 gram of catalyst digestion mixture was added followed by 10 ml of concentrated sulphuric acid and few pieces of broken porcelain. The soil was mixed

with sulphuric acid by swirling the flask and heated gently until frothing stopped. The heat was increased gradually until the acid boiled. Digestion was continued for 1-1.5 hours until the colour of the mixture changed to green blue. The flask was cooled and 20 ml of distilled water was added before the solution started to crystallize. The solution was transferred in a 100 ml volumetric flask, leaving the sand in the digestion flask and volume was made.

B. Distillation: Twenty ml of 40% NaOH was added in 20 ml of aliquot in a distillation flask and distillation was performed collecting the liberated ammonia in 10 ml 4% boric acid solution containing 2 drops of mixed indicator in a conical flask. It was then titrated with 0.01N hydrochloric acid.

Calculation:

$$\%N \text{ X} \frac{7 \mid n \mid (T - B)}{S}$$

where, n=Normality of acid

T = Volume of acid used in titration

B = Volume of acid used in blank

S = Weight of sample taken

4.2.14.2 Determination of available phosphorous

This was determined according to modified Olsen's bicarbonate method. Two and half g of air dried soil sample <2 mm sieve was weighed in a 100 ml polythene bottle. One teaspoon of activated charcoal and 50 ml of 0.5N $NaHCO_3$ extracting solution was added and shaken for 30 minutes in a shaker and filtered through Whatman No. 42 filter paper. Ten ml of aliquot of the filtrate was pipetted in a 50 ml volumetric flask and acidified with 5N H_2SO_4 to pH 5.0 using p-nitrophenol indicator till the yellow colour just disappeared. It was shaken gently after each addition of acid. Acid was further added, drop wise until the colour changes from yellow to colorless. Distilled water was

added washing down the sides of volumetric flask to 40 ml followed by 8 ml of reagent 17 (appendix-II). Volume was made up to the mark and shaken well. Maximum intensity of blue colour was obtained in 10 minutes. A blank was included by shaking the extracting solution without soil including all the reagents added in each step. The colour intensity was measured after 10 minutes using red filter (660 nm).

A standard curve was prepared by taking 0,1,2,4,6,8,10,12 and 15 ml of 2 ppm phosphate (P) standard solution in 50 ml volumetric flask adding $NaHCO_3$ extracting solution and preceded exactly like in test solution.

Calculation:

$$\text{ppm P in soil} = \text{ppm P in solution} \times \frac{50}{10} \times \frac{50}{2.5}$$

$$= \text{ppm P in solution} \times 100$$

$$P_2O_5 \text{ Kgha}^{-1} = \text{ppm P in soil} \times 2.24 \times 2.3$$

where, 2.24 = conversion factor for ppm in soil to Kgha^{-1}

2.3 = conversion factor for P to P_2O_5

4.2.14.3 Determination of available potassium

Two gm of air dried soil was weighed in a conical flask, 20 ml of neutral ammonium acetate was added and shaken for 5 minutes and filtered through Whatman No. 42 filter paper. A standard curve of K using different working standard solutions 0,5,10,15,20 and 25 ppm K were prepared and aspirated after adjusting full scale deflection of flame photometer with 25 ppm K. The reading was noted and a standard curve was drawn. The soil solution was aspirated, its reading was noted and K in the soil solution was determined from the standard curve.

Calculation:

$$K_2O \text{ Kg/ha XR (ppm)} \mid \frac{20}{2} \mid 1.2 \mid 2 \mid 1.2 \text{ XR} \mid 26.88$$

where, R = potassium of soil extract in ppm from the standard curve

1.2 = conversion factor for K to K_20

2 X 1.12 = conversion factor from ppm to $Kgha^{-1}$

20/2 = dilution factor

4.2.15 Plant tissue analysis

Plant root and shoot were analyzed for NPK contents to observe the effects microbial inoculations on 50^{th} day after plantation.

4.2.15.1 Preparation of plant samples

Plant samples collected were first cleaned of soil particles with minimum amount of distilled water. The sample was dried immediately in a forced draft oven at 70°C by thinly spreading the samples. The dried samples were ground in grinding mill such that the ground plant sample was able to pass through 0.5 mm sieve.

4.2.15.2 Determination of total nitrogen

The total nitrogen content of plant tissues was estimated by Kjeldahl method as that of soil samples except the weight of plant tissues i.e. 0.20 g of plant sample was taken.

4.2.15.3 Preparation plant tissue extract

One g of plant sample was weighed in 30 ml porcelain crucible. It was placed in cooled muffle furnace and the temperature was raised to 500^oC±25°C. The samples were

ignited at that temperature for 5 hours. Then 3 ml of 5N HNO_3 was added and evaporated to dryness. It was again placed in cooled muffle furnace at 400°C for about 15 minutes. The sample was again taken out from furnace, cooled, moisten with distilled water and 3 ml of concentrated hydrochloric acid was added. It was allowed to evaporate to dryness on low heat plate and allowed to bake for one hour to dehydrate the silica. It was removed from the hot plate; 5 ml of 2N hydrochloric acid was added, heated to warm and stirred with policeman to dissolve the residue of salts. The solution was filtered through Whatman No 31 filter paper into 50 ml volumetric flask, washing the crucible and funnel with hot distilled water. It was cooled and the volume was made and mixed. The filtrate was labeled as plant tissue extract (A).

4.2.15.4. Determination of total phosphorus

This was determined according to Vandomolybdophosphoric yellow method**.** The standard curve was prepared by taking 0,2,6,8,10,12 and 15 ml of 20 ppm P standard solution in 50 ml volumetric flask and diluted to 35 ml and mixed, 10 ml of vandomolybdate reagent was added and diluted to the mark. It was mixed and the yellow colour was measured after 20 minutes at 420 nm. Five ml of plant tissue extract (A) was taken in 50 ml volumetric flask and diluted to 35 ml. 10 ml of vandomolybdate reagent was added and diluted to 50 ml with distilled water and mixed. The yellow colour was measured after 20 minutes at 420 nm and P content was determined from the standard curve.

Calculation:

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

where, W = oven dry weight of plant sample

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

4.2.15.5 Flame photometric determination of potassium

The series of standards 0,1,2,4,6,8 and 10 ppm were prepared and readings read, with the highest standard to correspond to 100 reading, using the K filter and the standard curve was drawn. The plant tissue extract (A) was diluted to contain 2 to 8 ppm K per ml and the flame photometer reading was determined The K content was determined from the standard curve.

Calculation:

$$K\% \; X \frac{R \mid 100}{W \mid 10^6} \mid DF$$

where, R = ppm K in the solution

W= oven dry weight of sample

DF= dilution factor

4.2.16. Statistical Analysis

Data for plant growth parameters were subjected to one-way analysis of variance (ANOVA) using the program SPSS-11.5 (Statistical Package for Social Scientists) at 5% level of significance i.e., $P<0.05$.

CHAPTER-V

5. RESULT

5.1 Analysis of experimental soil

The experimental soil was analyzed before soybean sowing. i.e. the soil was analyzed before and after autoclaving.

Table 4. Physico-chemical properties of the experimental soil

Soil	%Nitrogen	Available phosphorus P_2O_5 ($Kgha^{-1}$)	Available potassium K_2O ($Kgha^{-1}$)
sterilized	0.07	39.9	61.9
unsterilized	0.06	35.0	83.9

5.2 Effect of co-inoculation of *B. japonicum* and *P. indica* on growth parameters of soybean when the treatments were conducted under sterilized (autoclaved) and unsterilized (natural) potted soils at different days after plantation

5.2.1 Effect of microbial inoculants on nodulation

5.2.1.1 Nodule number

In the pot experiment using sterilized soil, on all the days observed, the maximum nodule number was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *B. japonicum*, single inoculation of *P. indica* and uninoculated control (Table 5). Significant difference among the treatments

occurred at P<0.05 (Appendix IV). The increment in nodule number of plants inoculated with *B. japonicum* and *P. indica*, *B. japonicum* alone and *P. indica* alone over uninoculated control on 21st day was 1350.0%, 550.0% and 350.0% respectively; on 35th day it was 236.36%, 196.96% and 121.21% respectively; on 50th day, it was 329.16%, 216.66% and 145.83% respectively and on 65th day, it was 309.61%, 230.76% and 176.92% respectively (Appendix V).

In the pot experiment using unsterilized soil, on all the days observed, the maximum nodule number was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 5) and significant difference among the treatments occurred at P<0.05 (Appendix IV). The increment in nodule number of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 475%, 262.5% and 87.5% respectively; on 35th day it was 224.0%, 178.0% and 118% respectively; on 50th day it was 109.0%, 83.0% and 64% respectively and finally on 65th day it was 81.10%, 59.05% and 49.60% respectively (Appendix V).

5.2.1.2 Nodule fresh weight

In the pot experiment using sterilized soil, on all the days observed, the maximum nodule fresh weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *B. japonicum*, single inoculation of *P. indica* and uninoculated control (Table 6) and significant difference among the treatments occurred at P<0.05 (Appendix IV). The increment in nodule fresh weight of plants inoculated with *B. japonicum* and *P. indica*, *B. japonicum* alone and *P. indica* alone over uninoculated control on 21st day was 685.71%, 533.92%, 471.42% respectively; on 35th day it was 631.33%, 455.76% and 304.97% respectively; on 50th day, it was 481.0%, 436.31% and 413.96% respectively and on 65th day it was 530.33%, 451.65% and 312.32% respectively (Appendix V).

In the pot experiment using unsterilized soil, on all the days observed, the maximum nodule fresh weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica* , single inoculation of *B. japonicum* and uninoculated control (Table 6) and significant difference among the treatments occurred at P<0.05 (Appendix IV). The increment in nodule fresh weight of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 154.34%, 134.78% and 126.08% respectively; on 35th day it was 266.66%, 145.0% and 117.22% respectively; on 50th day it was 72.82%, 72.0% and 47.12% respectively and on 65th day it was 80.90%, 78.88% and 76.31% respectively (Appendix V).

Table 5. Effect of microbial inoculants on nodule number of soybean grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatments	Nodule number/plant			
		21 days	35 days	50 days	65 days
Sterilized	Uninoculated control	0.40±0.55	6.60±1.67	9.60±1.52	10.40±0.55
	B. japonicum	2.60±0.89	19.60±3.71	30.40±2.88	34.40±3.78
	P. indica	1.80±0.84	14.60±2.79	23.60±3.65	28.80±3.77
	B. japonicum + *P. indica*	5.80±1.10	22.20±4.97	41.20±5.93	42.60±4.28
Unsterilized	Uninoculated control	1.60±0.55	10.00±1.22	20.00±6.04	25.40±5.41
	B. japonicum	3.00±0.71	21.80±7.66	32.80±5.31	38.00±8.77
	P. indica	5.80±1.30	27.80±5.17	36.60±2.88	40.40±3.91
	B. japonicum + *P. indica*	9.20±0.84	32.40±4.98	41.80±4.21	46.00±8.54

Data showed the average of independent five replicate readings

5.2.1.3 Nodule dry weight

In the pot experiment using sterilized soil, on all the days observed, the maximum nodule dry weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *B. japonicum*, single inoculation of *P. indica* and uninoculated control (Table 7) and significant difference among the treatments occurred at $P<0.05$ (Appendix IV). The increment in nodule dry weight of plants inoculated with *B. japonicum* and *P. indica*, *B. japonicum* alone and *P. indica* alone over uninoculated control on 21st day was 492.59%, 459.25% and 348.14% respectively; on 35th day it was 468.75%, 318.75% and 243.75% respectively; on 50th day, it was 473.42%, 356.87% and 317.24% respectively and on 65th day, it was 576.13%, 343.18% and 271.59% respectively (Appendix V).

In the pot experiment using unsterilized soil, on all the days observed, the maximum nodule dry weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica* , single inoculation of *B. japonicum* and uninoculated control (Table 7) and significant difference among the treatments occurred at $P<0.05$ (Appendix IV). The increment in nodule dry weight of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 184.23%, 154.35% and 107.88% respectively; on 35th day, it was 215.39%, 161.90% and 139.04% respectively; on 50th day, it was 64.91%, 53.97% and 31.14% respectively and on 65th day, it was 124.58%, 65.48% and 27.65% respectively (Appendix V).

5.2.2 Effect of microbial inoculants on root growth parameters

5.2.2.1 Root length

In the pot experiment using sterilized soil, on all the days observed, the highest root length was produced by the control plants followed by dual inoculation of *B. japonicum*

and *P. indica*, single inoculation of *P. indica* and single inoculation of *B. japonicum* (Table 8). Significant difference among the treatments occurred at P<0.05 (Appendix IV). The decrement in root length of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 9.44%, 22.76% and 24.45% respectively; on 35th day, it was 3.48%, 4.17% and 9.52% respectively; on 50th day, it was 7.0%, 7.04% and 14.26% respectively and finally on 65th day it was 2.85%, 4.27% and 12.21% respectively (Appendix V).

In the pot experiment using unsterilized soil, on all the days observed, the longest root length was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 8) and significant difference among the treatments occurred at P<0.05 (Appendix IV). The increment in root length of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 42.85%, 40.92% and 27.49% respectively; on 35th day, it was 49.43%, 26.21% and 14.60% respectively; on 50th day, it was 57.19%, 41.32% and 25.83% respectively and finally on 65th day it was 41.31%, 26.75% and 21.70% respectively (Appendix V).

5.2.2.2 Root fresh weight

In the pot experiment using sterilized soil, on all the days observed, the maximum root fresh weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 9) and were statistically significant at P<0.05 (Appendix IV). The increment in root fresh weight of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 61.11%, 22.22% and 10.18% respectively; on 35th day, it was 83.08%, 25.37% and 15.42% respectively; on 50th day, it was 71.17%, 34.23% and

15.31% respectively and on 65th day, it was 45.17%, 39.66% and 21.03% respectively (Appendix V).

In the pot experiment using unsterilized soil, on all the days observed, maximum root fresh weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 9) and were statistically significant at $P<0.05$ (Appendix IV). The increment in root fresh weight of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 81.81%, 69.69% and 12.12% respectively; on 35th day it was 75.67%, 67.56% and 24.32% respectively; on 50th day it was 65.51%, 63.21% and 54.02% respectively and on 65th day it was 91.14%, 55.06% and 18.99% respectively (Appendix V).

Table 6. Effect of microbial inoculants on nodule fresh weight of soybean grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatments	Nodule fresh weight (mg/plant)			
		21 days	35 days	50 days	65 days
Sterilized	Uninoculated Control	1.12±1.54	17.68±2.34	35.8±6.87	42.20±5.12
	B. japonicum	7.10±2.59	98.26±12.51	192.0±35.64	232.80±26.49
	P. indica	6.40±1.88	71.60±11.37	184.0±22.75	174.00±55.95
	B. japonicum + *P. indica*	8.80±0.65	129.30±8.61	208.0±23.87	266.00±53.67
Un-sterilized	Uninoculated Control	9.20±3.03	36.0±4.05	111.36±31.17	358.22±84.35
	B. japonicum	20.80±1.92	78.20±18.15	163.84±32.51	631.60±27.61
	P. indica	21.60±3.29	88.20±7.33	191.54±41.58	640.80±73.55
	B. japonicum + *P. indica*	23.40±3.36	132.0±21.68	192.46±42.53	648.04±86.00

Data showed the average of independent five replicate readings.

Table 7: Effect of microbial inoculants on nodule dry weight number of soybean grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatments	Nodule dry weight (mg/plant)			
		21 days	35 days	50 days	65 days
Sterilized	Uninoculated control	0.54±0.75	6.40±2.30	8.58±1.13	17.60±2.41
	B. japonicum	3.02±1.34	26.80±8.96	39.20±13.16	78.00±16.11
	P. indica	2.42±0.61	22.0±9.35	35.80±4.49	65.40±15.34
	B. japonicum + *P. indica*	3.20±0.94	36.4±3.51	49.20±11.26	119.0±27.02
Un-sterilized	Uninoculated control	4.82±1.99	12.60±3.91	31.98±7.32	84.60±17.84
	B. japonicum	10.02±2.21	30.12±2.80	42.90±6.29	108.0±16.43
	P. indica	12.26±1.58	33.0±0.15	49.24±10.17	140.0±14.14
	B. japonicum + *P. indica*	13.70±1.67	39.74±9.41	52.74±13.38	190.0±23.45

Data showed the average of independent five replicate readings.

Table 8: Effect of microbial inoculants on root length of soybean grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatment	Root length (cm/plant)			
		21 days	35 days	50 days	65 days
Sterilized	Uninoculated control	41.30±4.32	46.42±1.57	48.52±2.17	49.10±0.91
	B. japonicum	31.20±1.79	42.00±1.58	41.60±2.70	43.10±2.56
	P. indica	31.90±1.21	44.48±1.24	45.10±2.09	47.0±2.00
	B. japonicum + *P. indica*	37.40±1.95	44.80±1.26	45.12±1.77	47.70±2.11
Un-sterilized	Uninoculated Control	18.62±1.04	26.70±1.20	27.10±1.56	30.50±1.62
	B. japonicum	23.74±2.05	30.60±2.61	34.10±4.53	37.12±4.66
	P. indica	26.24±2.61	33.70±5.63	38.30±1.71	38.66±1.94
	B. japonicum + *P. indica*	26.60±3.13	39.90±4.83	42.60±3.58	43.10±2.28

Data showed the average of independent five replicate readings.

Table 9: Effect of microbial inoculants on root fresh weight of soybean grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatments	Root fresh weight (g/plant)			
		21 days	35 days	50 days	65 days
Sterilized	Uninoculated Control	1.08±0.22	2.01±0.60	2.22±0.35	2.90±0.50
	B. japonicum	1.19±0.08	2.32±0.89	2.56±0.69	3.51±0.40
	P. indica	1.32±0.28	2.52±0.71	2.98±0.50	4.05±0.32
	B. japonicum + P. indica	1.74±0.59	3.68±0.48	3.80±0.72	4.21±0.63
Un-sterilized	Uninoculated Control	0.33±0.05	0.37±0.07	0.87±0.10	1.58±0.41
	B. japonicum	0.37±0.05	0.46±0.05	1.34±0.36	1.88±0.22
	P. indica	0.56±0.10	0.62±0.07	1.42±0.45	2.45±0.27
	B. japonicum + P. indica	0.60±0.08	0.65±0.04	1.44±0.26	3.02±0.23

Data showed the average of independent five replicate readings.

5.2.2.3 Root dry weight

In the pot experiment using sterilized soil, on all the days observed, the highest root dry weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table10). There was significant difference among the treatments at $P<0.05$ on 50^{th} and 65^{th} day (Appendix IV). The increment in root dry weight of plants inoculated with of *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated control on 21^{st} day was 31.25%, 25.0% and 18.75% respectively; on 35^{th} day it was 39.22%, 33.33% and 5.88% respectively; on 50^{th} day, it was 48.15%, 42.59% and 18.52% respectively and finally on 65^{th} day it was 67.79%, 59.32% and 15.25% respectively (Appendix V).

In the pot experiment using unsterilized, on all the days observed, the highest root dry weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation *B. japonicum* and uninoculated control (Table10) and the results were statistically significant at $P<0.05$ (Appendix IV). The increment in root dry weight of plants inoculated with *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 57.14%, 42.86% and 28.57% respectively; on 35th day it was 123.08%, 92.31% and 38.46% respectively; on 50th day it was 66.67%, 63.33% and 46.67% respectively and finally on 65th day it was 72.22%, 64.81% and 18.52% respectively (Appendix V).

Table 10: Effect of microbial inoculants on root dry weight of soybean grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatments	Root dry weight (g/plant)			
		21 days	35 days	50 days	65 days
Sterilized	Uninoculated Control	0.16±0.05	0.51±0.04	0.54±0.09	0.59±0.08
	B. japonicum	0.19±0.06	0.54±0.15	0.64±0.08	0.68±0.12
	P. indica	0.20±0.06	0.68±0.03	0.77±0.11	0.94±0.07
	B. japonicum + *P. indica*	0.21±0.08	0.71±0.23	0.80±0.09	0.99±0.27
Un-sterilized	Uninoculated Control	0.07±0.01	0.13±0.03	0.30±0.11	0.54±0.10
	B. japonicum	0.09±0.02	0.18±0.03	0.44±0.07	0.64±0.09
	P. indica	0.10±0.02	0.25±0.05	0.49±0.08	0.89±0.13
	B. japonicum + *P. indica*	0.11±0.03	0.29±0.07	0.50±0.11	0.93±0.05

Data showed the average of independent five replicate readings.

5.2.3 Effect of microbial inoculants on shoot growth parameters

5.2.3.1 Shoot length

In the pot experiment using sterilized soil, on all the days observed, the highest shoot length was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation *B. japonicum* and uninoculated control (Table 11). The results were statistically significant at $P<0.05$. (Appendix IV).The increment in shoot length of plants inoculated with *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated on 21st day was 39.46%, 24.49% and 8.57% respectively; on 35th day it was 41.94%, 31.55% and 12.62% respectively; on 50th day it was 43.49%, 36.0% and 21.90% respectively and finally on 65th day it was 45.36%, 36.45% and 26.48% respectively (Appendix V).

In the pot experiment using unsterilized, on all the days observed, the highest shoot length was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation *B. japonicum* and uninoculated control (Table 11) There was significant difference among the treatments at $P<0.05$ on 35th, 50th and 65th day (Appendix IV). The increment in shoot length of plants inoculated with *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 29.64%, 29.34% and 25.75% respectively; on 35th day it was 60.23%, 58.91% and 10.89% respectively; on 50th day, it was 61.09%, 48.25% and 8.95% respectively and finally on 65th day it was 68.58%, 65.48% and 37.47% respectively (Appendix V).

5.2.3.2 Shoot fresh weight

In the pot experiment using sterilized soil, on all the days observed, the maximum shoot fresh weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum*

and uninoculated control (Table 12) and the results were statistically significant at P<0.05 (Appendix IV). The percent increment in shoot fresh weight of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 54.04%, 34.78% and 29.19% respectively; on 35th day it was 46.65%, 41.75% and 25.52% respectively; on 50th day it was 66.19%, 64.79% and 55.63% respectively and finally on 65th day it was 75.62%, 64.35% and 36.04% respectively (Appendix V).

In the pot experiment using unsterilized soil, on all the days observed, the maximum shoot fresh weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 12) and were statistically significant at P<0.05 (Appendix IV). The increment in shoot fresh weight of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 83.92%, 78.57% and 55.36% respectively; on 35th day, it was 120.0%, 80.95% and 17.14% respectively; on 50th day it was 94.01%, 93.63% and 55.43% respectively and finally on 65th day, it was 70.10%, 61.87% and 28.67% respectively (Appendix V).

5.2.3.3 Shoot dry weight

In the pot experiment using sterilized soil, on all the days observed, the highest shoot dry weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 13) and were statistically significant at P<0.05 (Appendix IV). The increment in shoot dry weight of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 44.12%, 38.24% and 11.76% respectively; on 35th day, the it was 24.37%, 21.85% and 9.24% respectively; on 50th day it was 34.71%, 22.94% and

10.59% respectively and finally on 65th day it was 98.59%, 93.90% and 65.26% respectively (Appendix V).

In the pot experiment using unsterilized soil, on all the days observed, the highest shoot dry weight was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 13) and significant difference among the treatments occurred at P<0.05 (Appendix IV). The increment in shoot dry weight of plants inoculated with of *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated control on 21st day was 100%, 81.82% and 45.45% respectively; on 35th day it was 110.81%, 75.68% and 16.22% respectively; on 50th day it was 131.94%, 74.99% and 52.78% respectively and finally on 65th day it was 123.30%, 105.11% and 64.77% respectively (Appendix V).

Table 11: Effect of microbial inoculants on shoot length of soybean grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatments	Shoot length (cm/plant)			
		21 days	35 days	50 days	65 days
Sterilized	Uninoculated Control	14.70±1.30	20.60±1.22	31.50±1.22	32.10±3.78
	B. japonicum	15.96±2.05	23.20±1.14	38.40±2.41	40.60±0.81
	P. indica	18.30±1.51	27.10±0.89	42.84±3.42	43.80±3.35
	B. japonicum + *P. indica*	20.50±2.15	29.24±1.96	45.20±4.66	46.66±2.14
Un-sterilized	Uninoculated Control	6.68±1.27	12.12±1.74	25.70±2.77	25.78±0.89
	B. japonicum	8.40±0.65	13.44±0.60	28.0±1.51	35.44±1.81
	P. indica	8.64±1.71	19.26±1.32	38.10±1.68	42.66±1.98
	B. japonicum + *P. indica*	8.66±1.40	19.42±2.79	41.40±1.14	43.46±1.44

Data showed the average of independent five replicate readings

Table 12: Effect of microbial inoculants on shoot fresh weight of soybean grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatments	shoot fresh weight (g/plant)			
		21 days	35 days	50 days	65 days
Sterilized	Uninoculated Control	1.61±0.31	3.88±0.93	4.26±0.82	7.63±0.32
	B. japonicum	2.08±0.51	4.87±0.66	6.63±1.40	10.38±0.85
	P. indica	2.17±0.40	5.50±1.42	7.02±0.82	12.54±1.40
	B. japonicum + *P. indica*	2.48±0.34	5.69±0.84	7.08±0.57	13.40±1.99
Un sterilized	Uninoculated Control	0.56±0.08	1.05±0.26	2.67±0.70	7.29±1.86
	B. japonicum	0.87±0.12	1.23±0.24	4.15±0.66	9.38±1.20
	P. indica	1.0±0.22	1.90±0.36	5.17±0.15	11.80±1.13
	B. japonicum + *P. indica*	1.03±0.06	2.31±0.64	5.18±0.80	12.40±1.30

Data showed the average of independent five replicate readings.

Table 13: Effect of microbial inoculants on shoot dry weight of soybean plant grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatments	Shoot dry weight (g/plant)			
		21 days	35 days	50 days	65 days
Sterilized	Uninoculated control	0.34±0.09	1.19±0.10	1.70±0.25	2.13±0.43
	B. japonicum	0.38±0.05	1.30±0.11	1.88±0.22	3.52±0.47
	P. indica	0.47±0.08	1.45±0.11	2.09±0.39	4.13±0.74
	B. japonicum + *P. indica*	0.49±0.09	1.48±0.13	2.29±0.35	4.23±0.66
Un-sterilized	Uninoculated Control	0.11±0.04	0.37±0.13	0.72±0.21	1.76±0.17
	B. japonicum	0.16±0.02	0.43±0.08	1.10±0.21	2.90±0.17
	P. indica	0.20±0.05	0.65±0.12	1.26±0.31	3.61±0.37
	B. japonicum + *P. indica*	0.22±0.07	0.78±0.13	1.67±0.25	3.93±0.62

Data showed the average of independent five replicate readings.

5.2.4 Effect of microbial inoculants on root and shoot NPK of soybean grown in sterilized soil on 50th day after plantation

5.2.4.1 Root and shoot nitrogen

The maximum root and shoot nitrogen content were produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *B. japonicum*, single inoculation of *P. indica* and uninoculated control (Table 14).

The increment in root nitrogen content of plants inoculated with *B. japonicum* and *P. indica*, *B. japonicum* alone and *P. indica* alone over uninoculated control was 44.64%, 32.14% and 28.57% respectively (Appendix V). Similarly, the increment in shoot nitrogen content of *B. japonicum* and *P. indica*, *B. japonicum* alone and *P. indica* alone over uninoculated control was 63.33%, 26.67% and 20.0% respectively (Appendix V).

5.2.4.2 Root and shoot phosphorus

The maximum root and shoot phosphorus content was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 14).

The increment in root phosphorus content of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control was 235.56%, 163.70% and 94.07% respectively (Appendix V). Similarly, the percent increment in shoot phosphorus content of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control was 124.09%, 109.09% and 80.91% respectively (Appendix V).

5.2.4.3 Root and shoot potassium

The maximum root and shoot potassium content was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 14).

The increment in root potassium content of content of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control was 106.09%, 49.82% and 13.80% respectively (Appendix V). Similarly, the increment in shoot potassium content of plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control was 128.79%, 63.03% and 24.24% respectively (Appendix V).

Table 14: Effect of microbial inoculants on root and shoot NPK content of soybean plant grown in sterilized soil on 50th day after plantation

		ROOT			SHOOT		
Soil	**Treatment**	**%N**	**%P**	**%K**	%N	%P	%K
Sterilized	Uninoculated control	0.56	0.135	0.558	1.50	0.220	0.660
	B. japonicum	0.74	0.262	0.635	1.90	0.398	0.820
	P. indica	0.72	0.356	0.836	1.80	0.460	1.076
	B. japonicum + *P. indica*	0.81	0.453	1.150	2.45	0.493	1.510

5.2.5 Effect of microbial inoculants on root and shoot NPK content of soybean plant grown in unsterilized soil on 50th day after plantation

The maximum root and shoot NPK contents were produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 15).

5.2.5.1 Root NPK

The increment in root nitrogen content of plants inoculated with *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated control was 36.43%, 35.71% and 12.86% respectively. The increment in root phosphorus content of plants inoculated with *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated control was 76.12%, 43.28% and 10.45% respectively. The increment in root potassium content of plants inoculated with *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated control was 94.10%, 57.86% and 29.48% respectively (Appendix V).

5.2.5.2 Shoot NPK

The increment in shoot nitrogen content of plants inoculated with *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated control was 32.56%, 16.74% and 14.42% respectively. The increment in shoot phosphorus content of plants inoculated with *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated control was 18.45%, 13.59% and 2.91% respectively. The increment in shoot potassium content of plants inoculated with *B. japonicum* and *P. indica, P. indica* alone and *B. japonicum* alone over uninoculated control was 67.52%, 35.05% and 29.58% respectively (Appendix V).

Table 15. Effect of microbial inoculants on root and shoot NPK content of soybean grown in unsterilized soil on 50th day after plantation

		ROOT			SHOOT		
Soil	**Treatment**	**%N**	**%P**	**%K**	**%N**	**%P**	**%K**
Un-sterilized	Uninoculated Control	1.40	0.067	0.458	2.15	0.103	0.622
	B. japonicum	1.58	0.074	0.593	2.46	0.106	0.806
	P. indica	1.90	0.096	0.723	2.51	0.117	0.840
	B. japonicum + P. indica	1.91	0.118	0.889	2.85	0.122	1.042

5.2.6 Effect on soil nitrogen (N), available phosphorous (P_2O_5) and available potassium (K_2O) content of soybean in sterilized soil on 50th day after plantation and after harvesting after maturity

5.2.6.1 Soil N

On the days observed, the maximum soil nitrogen content was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *B. japonicum*, single inoculation of *P. indica* and uninoculated control (Table 16). The increment in soil nitrogen content of plants inoculated with *B. japonicum* and *P. indica*, *B. japonicum* alone and *P. indica* alone over uninoculated control on 50th day was 100.0%, 75.0% and 50.0% respectively and after harvesting, was 83.33%, 50% and 33.33% respectively (Appendix V).

5.2.6.2 Soil available phosphorous (P_2O_5)

On the days observed, the maximum soil available phosphorus was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control. (Table 16). The increment in soil available phosphorus of dual inoculation *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 50th day was 115.74%, 55.55% and 37.04% respectively and after harvesting, was 151.0%, 119.50% and 49.0% respectively (Appendix V).

5.2.6.3 Soil available potassium (K_2O)

On the days observed, the maximum soil available potassium was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 16). The increment in soil available potassium of dual inoculation of *B.*

japonicum and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 50th day was 47.97%, 38.43% and 20.78% respectively and after harvesting, was 39.81%, 39.08% and 19.66% respectively (Appendix V).

Table 16: Effect on soil (N), available phosphorous (P_2O_5) and available potassium (K_2O) content of soybean in sterilized soil on 50th day after plantation and after harvesting after maturity

Soil	Treatment	50 days			After harvest		
		%N	P_2O_5 ($Kgha^{-1}$)	K_2O ($Kgha^{-1}$)	%N	P_2O_5 ($Kgha^{-1}$)	K_2O ($Kgha^{-1}$)
Sterilized	Uninoculated Control	0.04	10.80	76.50	0.06	20.00	82.40
	B. japonicum	0.07	14.80	92.40	0.09	29.80	98.60
	P. indica	0.06	16.80	105.90	0.08	43.90	114.60
	B. japonicum + *P. indica*	0.08	23.30	113.20	0.11	50.20	115.20

5.2.7 Effect on soil (N), available phosphorous (P_2O_5) and available potassium (K_2O) content of soybean grown in unsterilized soil on 50th day after plantation and after harvesting after maturity

On the days observed, the highest soil nitrogen, available phosphorous (P_2O_5) and available potassium (K_2O) content was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control (Table 17).

5.2.7.1 Soil N

The increment in soil nitrogen content of dual inoculation of *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 50th day

was 140.0%, 120.0% and 80.0% respectively and after harvesting, was 87.5%, 62.5 % and 25.0% respectively(Appendix V).

5.2.7.2 Soil available phosphorous (P_2O_5)

On the days observed, the highest soil available phosphorus was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, by single inoculation of *B. japonicum* and uninoculated control. The increment in soil available phosphorus of dual inoculation of *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 50th day was 219.34%, 137.02% and 82.32% respectively and after harvesting, was 171.86%, 111.69% and 54.98% respectively (Appendix V).

5.2.7.3 Soil available potassium (K_2O)

On the days observed, the highest soil available potassium was produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control. The increment in soil available potassium of dual inoculation of *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone over uninoculated control on 50th day was 32.24%, 24.12% and 8.11% respectively and after harvesting, was 28.40%, 23.73% and 7.40% respectively (Appendix V).

5.2.8 Percent colonization of roots of soybean grown in sterilized potted soil on different days after plantation

Root colonization on soybean by single inoculation of *P. indica* was 30% on 21st day, 43.33% on 35th day, 53.33% on 50th day, 73.33% on 65th day and 76.66% after harvesting after maturity while that of dual inoculation of *B. japonicum* and *P. indica* was 36.66% on 21st day, 50.0% on 35th day, 66.66% on 50th day, 80% on 65th day and finally 86.66% after harvesting after maturity (Table 18).

Table 17: Effect on soil (N), available phosphorous (P_2O_5) and available potassium (K_2O) content of soybean grown in unsterilized potted soil on 50th day after plantation and after harvesting after maturity

Soil	Treatment	50 days			After harvest		
		%N	P_2O_5 ($Kgha^{-1}$)	K_2O ($Kgha^{-1}$)	%N	P_2O_5 ($Kgha^{-1}$)	K_2O ($Kgha^{-1}$)
Un-sterilized	Uninoculated control	0.05	18.10	91.20	0.08	23.10	98.60
	B. japonicum	0.09	33.00	98.60	0.10	35.80	105.90
	P. indica	0.11	42.90	113.20	0.13	48.90	122.00
	B. japonicum + *P. indica*	0.12	57.80	120.60	0.15	62.80	126.60

Table 18. Percent root colonization of soybean in sterilized (autoclaved) soil after inoculation with *P. indica* at different days after plantation

	Days				After Harvesting
Treatment	**21**	**35**	**50**	**65**	
Uninoculated Control	–	–	–	–	–
B. japonicum	–	–	–	–	–
P. indica	30.0%	43.33%	53.33%	73.33%	76.66%
B. japonicum + *P. indica*	36.66%	50.0%	66.66%	80.0%	86.66%

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

It is generally recognized that nitrogen fixation by leguminous plants is one of the most important routes by which the molecular nitrogen of the atmosphere enters the food chains of the biosphere. Associative and symbiotic nitrogen fixing bacteria and AM fungi are common beneficial microbes of leguminous plants (Groppa *et al.,* 1999).

The pot experiment was performed to evaluate the possible beneficial effects of single inoculation of *B. japonicum* and *P. indica* and also the effect when both are inoculated (co-inoculated or dual inoculated) using soybean [*Glycine max* (L.) Merr. local cultivar "Sathiya"] as a host plant in terms of growth parameters viz., nodule number, nodule fresh and dry weight, root length, root fresh and dry weight, shoot length, shoot fresh and dry weight, plant NPK content and soil fertility in terms of NPK content.

Glycine max (L.) Merr. cultivar "Sathiya" is a local variety and can be grown in mid hills. Due to its seed quality (taste), seed size and maturity period it is more popular among farmers than other new varieties (Neupane, 2003). It is most unfortunate that an agricultural country like Nepal has not given any thought for the processing of soybean for the production of protein rich food despite the known fact that the majority of the Nepalese population is consuming protein deficient food. Moreover, animal protein is an expensive commodity that average Nepali citizen cannot afford to buy as part of their daily diet. It is high time for our industrialists and government to seriously consider the need for establishing soybean-based food industries. The only thing is to consolidate our efforts to the realization that we have a potential to grow more soybeans, and this environmental-friendly crop is needed for better national and global health (Lekhak, 2001).

The experiment was divided into two sets, one set using sterilized (autoclaved) soil and other set using unsterilized (field) soil. This was done because using unsterilized soil, the response of inoculated microorganisms whether positive or negative on the host plant could be studied under natural conditions and by this, results could be expected as that of field. Another set was carried out using sterilized soil because inoculation of pure microbial cultures in sterilized soil allowed us to directly examine the effects of these microorganisms since there would be absence of other biological lives. Soil sterilization was done to further separate biotic verses abiotic soil factors.

The field soil and riverbed 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 (Dhami, 2004). Since all the pots were having same ratio of sand and soil, it might not have variable effect on experimental sets.

In the experiment using sterilized soil, the sterilization of pots, soil and seeds were done to avoid the contamination of experimental sets by other microorganisms that might be adhering in earthen pots or in the seed surface. The inoculated and uninoculated pots were kept at sufficient distance to minimize the chances of cross contamination.

The effect of microbial inoculants in terms of nodule number, nodule fresh and dry weight, root length, root fresh and dry weight, shoot length, shoot fresh and dry weight, in both sets were recorded on 21^{st}, 35^{th}, 50^{th} and 65^{th} day after plantation. Plant NPK content in both sets was recorded on 50^{th} day. NPK contents in soil were analyzed before soybean sowing, on 50^{th} day and after harvesting the plant after maturity. The above time period was selected depending on the various stages of plant growth. Soybean development is characterized by two distinct growth phases. The first is the vegetative phase (V) that covers development from emergence through flowering. The second is the reproductive (R) stages from flowering through maturation (Naeve, 2005). Therefore, the first two, i.e. 21 and 35 days represent the vegetative growth phase and

the later two i.e. 50 and 65 days represent the reproductive growth phase. The nitrogen fixation and nodulation attains its peak around the flowering period and beyond flowering period and pod formation, nodule senescence and loss of chlorophyll takes place.

Soil is one of the most dynamic sites of biological interactions in nature. It is the region in which occurs many of the biochemical reactions concerned in the destruction of organic matter (plants, animals and microbes), in the weathering of rock minerals and in the nutrition, growth and productivity of agricultural crops (Alexender, 1961). Hence the characteristics of soil i.e. nitrogen, phosphorous and potassium, collectively termed NPK were analyzed.

The soybean seeds were sown at the depth of 2.5-4 cm because deeper sowing exposes the seedling to the greater risk of damage from soil borne pathogens and poor emergence of those cultivars with short hypocotyls (Upfold and Olechowski, 1994).

The broth culture of *B. japonicum* was incubated at 28°C for 7-10 days, which is the optimum incubation for slow growing rhizobia (Somasegaran and Hoben, 1994). The broth culture used to inoculate soybean contained 10^7 cfu/ml bradyrhizobial cells as found by serial dilution agar plating method. Because literatures suggest that for plant inoculation, the cultures should reach mid-log phase, which is equivalent to 10^7-10^8 cells per ml (Zhang *et al*., 2002).

On all the days observed, compared with control alone, co-inoculation with *B. japonicum* and *P. indica* increased nodule number, nodule fresh and dry weight, shoot length, root and shoot fresh and dry weight, soil NPK and root and shoot NPK in both sets using sterilized and unsterilized soil.

In the experiment using sterilized soil, nodule number was highest in dual inoculated plants followed by *B. japonicum* alone, *P. indica* alone and control. In plants with single

inoculation of *B. japonicum*, increased in number of nodules might be due to the presence of higher number of *B. japonicum* cells in rhizosphere due to inoculation and eventually more bradyrhizobia can infect the host root resulting in higher number of nodules. *P. indica* inoculation also increased nodulation in compared to control. Crush (1974) obtained direct evidences of mycorrhizae increasing nodulation in legume.

In the experiment using unsterilized soil, nodule number was highest in dual inoculated plants followed by *P. indica* alone and *B. japonicum* alone and uninoculated control. Nodulation in uninoculated plant might be due to the presence of existing indigenous rhizobia as many soils already harbor native rhizobial strains of different competitiveness and effectiveness and also many rhizospheric microorganisms since the soil was not sterilized. Similar results were obtained by Darmola *et al*. (1993). Bai *et al*. (2003) found that some rhizospheric microorganisms such as *Bacillus* spp. (*B. azotoformans, B. brevis, B. circulans, B. insolitus, B. megaterium* and *B. subtilis*) promoted clover nodulation when co-inoculated with *Rhizobium leguminosarium.* In plants with single inoculation of *B. japonicum*, inoculated bradyrhizobia increased nodule number than control plants but less than *P. indica* inoculated plants because there might be presence of many strains of native bradyrhizobia and antagonists in soil which competed with inoculated bradyrhizobia but in plants inoculated with *P. indica* alone, inoculated organism might have acted synergistically with native bradyrhizobia so there was increased in nodule number. In dual inoculated plants, inoculated microorganisms might have positive influence upon rhizospheric microorganisms and enhance nodule formation.

The fresh and dry weight of nodules per plant have been found higher in dual inoculated plants, which are obviously due to the bigger and higher number of nodules. Thus, the size, and number of nodules was found to be correlated with each other. *B. japonicum* and *P. indica* might have produced plant growth regulators or other activators by interacting with rhizospheric microorganisms that caused enhancement in nodulation in unsterilized soil than in sterilized soil. On the other hand, other 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). Exudates from a host root especially, isoflavinoids enhance spore germination, elongation, and branching of hyphae in AM fungi and it has been shown that isoflavinoids are one of the factors responsible for initiation of nod factor (Albrecht *et al*., 1998). In case of dual inoculation, both IAA (due to rhizobia) and photosynthate (due to *B. japonicum* and *P. indica*) might have increased due to availability of more nitrogen and phosphorous for normal physiological events and ultimately increased nodule number. Whether *P. indica* promotes nodulation through similar or different mechanisms remains to be investigated.

In both the sets using sterilized and unsterilized soil, co-inoculated plants had more nodules on the main root, while less on lateral roots. Nodules were also large, pink and looked healthy indicating active nitrogen fixation. The increase in nodulation may be attributed to relatively high amounts of available phosphorous in soil of dual inoculated plant, as suggested by Mosse *et al*. (1976) who reported increased nodulation with increasing phosphorus content. Groppa *et al*. (1999) observed similar results when soybean plants were co-inoculated with *B. japonicum* and *Azospirillum brasilense* in sterilized soil.

In the experiment using sterilized soil, a lower number of nodules were found on the root of control treatments not inoculated with selected microorganisms, although the soil was autoclaved and the seeds and pots were surface sterilized. This showed that some plants were contaminated with indigenous rhizobia and probably other microorganisms. The main explanation of this contamination might be that the irrigation water possibly contained nitrogen-fixing bacteria. Comparable results have been reported by Founoune *et al*. (2002). *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. Spore forming *Bacillus*

strains were isolated from surface disinfected soybean root nodule and showed growth promoting effects on soybean plants (Bai *et al*., 2003).

This work was performed only on the nodules attached to the roots and did not take into account the detached nodules.

In the experiment using sterilized soil, longest root length was observed with control plants followed by plants inoculated with *B. japonicum* and *P. indica*, *P. indica* alone and *B. japonicum* alone. Dual inoculated plants produced more root length than either *B. japonicum* alone or *P. indica* alone but less than control but had more branched (lateral) roots than all others. However, in the experiment using unsterilized soil, root length was found highest in dual inoculated plants followed by *P. indica* alone, *B. japonicum* alone and uninoculated control. Dual inoculated plants had more branched root and drier root weight while control plants were found to contain shortest root length and lowest dry weight with least branching and lateral roots. Neupane (2003) reported the similar results when *Glycine max* (L.) Merr. cv Sathiya was inoculated with VAM and *B. japonicum* in sterilized soil.

Higher number of effective nodules in dual inoculated plants obviously contains large number of active bacteriods, which ultimately produce higher amount of IAA. IAA remains the most likely candidate for the endogenous promoter of root elongation. However, over production of IAA inhibit root elongation (Scott, 1984). Therefore, in experiment using sterilized soil, there were not other microorganisms to share the produced IAA. Therefore, single inoculation of *B. japonicum* had shortest root length.

In both sets using sterilized and unsterilized soil, microbial inoculations increased root biomass on all the days observed. Highest fresh and dry weight was found in dual inoculated plants followed by or *P. indica* alone, *B. japonicum* alone and control plants. Root fresh and dry weight of dual inoculated plant was highest which might be due to

the high number of lateral roots and root hairs, which eventually increased the root dry weight.

In the experiment using sterilized soil, dual inoculated plants had highest root dry weight although they had shorter root length than control plants. Similar results have been obtained by Neupane (2003). According to Dart (1974), rhizobia are also responsible for cytokinin production during root hair curling that is responsible for stimulating more lateral root and according to Singh *et* al. (2001), *P. indica* is also known to produce phytohormones. The availability of more NPK in roots of dual inoculated plants might have positive role in root dry matter content regardless of root length (Dart, 1974). Mycorrhiza colonization also protect the roots from the soil pathogens (Kung, 2006) and, therefore could have lead to an increase in not only the root growth and nutrient acquisition of the host roots, but also the number of surviving roots. This in turn could have enhanced higher plant growth rate resulting more roots per plant. Plant roots are, however, extraordinarily complex due to variations in time, for e.g., due to changing environmental conditions of nutrients and micronutrient supply, salinity and water stress etc (Lauchli and Bieleski, 1983).

P. indica are able to enhance the absorption of nutrients from the soil (Singh, 2004) which could have moved to the roots principally by mass flow, in addition to those, which could have diffused through the soil slowly. This could have resulted in a higher root biomass in *P. indica* inoculated plants than in *B. japonicum* inoculated plants and control plants. Shende *et al*. (2006) found that *P. indica* inoculated *Withania somnifera* plants produced more root dry weight than control plants on every 15 days after plantation until 60 days. 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 treatments were conducted under both natural (unautoclaved) and sterile (autoclaved) potted soils.

The effect of microbial inoculants on the height increment was obvious on visual comparison from the 35th day in both experiments using sterilized and unsterilized soil, as (Table 11) shows a significant height increment in dual inoculated. The taller, more branched and healthier plants were observed on dual inoculated but not much different than single inoculation of *P. indica*. The results obtained indicated the influence of soybean on microbial symbiosis. The enhanced height increment in dual inoculated plants could be attributed to the *P. indica* colonization in presence of *Bradyrhizobium japonicum.* Dual inoculation produced more and healthy nodules, which in turn translocated nitrogen from lower parts to the growing tips and enlarged the height of the plants as found by Hanway and Weber (1971).

In both experiments using sterilized and unsterilized soil, shoot length was higher in *P. indica* inoculated plants than *B. japonicum* inoculated plants. Mycorrhizal infection is known to enhance plant growth by increasing nutrients uptake (Marschner and Dell, 1994). *P. indica* is reported to enhance plant growth rate through an increase in nutrient uptake, especially phosphorus that is relatively immobile in soils (Singh *et al*., 2001; Varma *et al*., 2000). *P. indica* inoculation could have enhanced soybean to absorb more nutrients via an increase in the absorbing surface area. Singh *et al*. (2001) and Malla *et al*. (2002) reported significant increment in shoot length when *Spilanthes calva* and *Withania somnifera* were inoculated with *P. indica*. Rai and Varma (2005), while working with *P. indica* inoculated *Adhatoda vasica* Nees, reported a significant root and shoot biomass increment by 331.42% and 140.9% respectively over controls.

Microbial inoculations increased the shoot biomass yield over controls on all the days observed in both experiments using sterilized and unsterilized soil. The increased shoot biomass production by the inoculated plants could be attributed to enhanced inorganic nutrition absorption and greater rates of photosynthesis in inoculated plants (Allen and

Allen 1981; Cooper, 1985). It seems likely that in this study dual inoculation increased nutrient uptake by shortening the distance the nutrients had to diffuse from the soil to the roots giving rise to a higher root and shoot biomass increment with a uniform growth.

Many authors have reported a significant increment in shoot biomass, after inoculating the plants with mycorrhiza and/or *Rhizobium*. Neupane (2003) reported an increment in shoot dry weight when *Glycine max* (L.) Merr. cv Sathiya was inoculated with VAM and *B. japonicum.* Dhami (2004) while working with inoculated *Glycine max* (L.) Merr. cv "Sathiya" with *B. japonicum* reported a significant increment in plant growth parameters. Singh *et al*. (2001) and Malla *et al*. (2002) reported significant increment in shoot fresh and dry weight of *P. indica* inoculated *Azadirachta indica* by 28.14% and 46.25% respectively over the control. A growth study of *P .indica* with *Azotobacter, Azospirillum* spp., *Bradyrhizobium* spp. and *Bacillus coagulans,* on a defined solidified medium have indicated that the fungus interacted positively with these microorganisms (Singh *et al.*, 2001).

Root colonization was assessed on 21st, 35th, 50th and 65th days after plantation in the experiment using sterilized soil, as many other endophytic fungi might be present in natural condition. In a number of the evaluation periods, root colonization by *P. indica* was observed by 21 days after plantation. Inoculation of *P. indica* simultaneously with *B. japonicum* resulted in percent colonized root of soybean higher than those where only *P. indica* was inoculated alone. Therefore, the inocula used were viable and the symbionts were compatible.

As shown in Table 18, inoculating soybean with *P. indica* resulted into a root colonization ranging from 30% on 21st day to 76.66% after harvesting after maturity while that of dual inoculation ranged from 36.66% to 86.66%. There was no mycorrhizal contamination as evident in the non-inoculated plants (control) which showed 0% colonization. Hayman (1980) reported the leguminous plant species that

usually develop nodules in association with *Rhizobium* (*Bradyrhizobium, Azorhizobium*) are also characteristically colonized by VAM. Root-exudates flavinoids that activate rhizobial *nod* genes can also stimulate growth of mycorrhizal fungi prior to infection.

Singh (2004) observed percentage colonization of maize plants by *P. indica* ranged from 73% on 28th day to 90% 42nd day. Shende *et al*. (2006) recorded 76.06% root colonization when *P. indica* alone was inoculated but in combinations with *Trichoderma viridae* and *Pseudomonas fluorescence,* root colonization increased up to 93.33% after 60 days. Rai and Varma (2005) found that root-colonization of *Adhatoda vasica* Nees by *P. indica* increased with time from 53% after 2 months to 95% after 6 months. Similarly, Kumari *et al*. (2003) found root-colonization of *Brassica oleraceae* var *capitata*, *Brassica juncea* and *Spinaceae oleraceae,* which are known as conventional non-host for AM fungi, by *P. indica* after 45 days were 70%, 50% and 35% respectively. Singh *et al*. (2001) found that inoculating *Spilanthes calva* and *Withania somnifera* plants with *P. indica* resulted into 62 and 73% root colonization over controls respectively.

The extent to which typical VAM fungi colonize root systems varies with species of plant (Smith and Bowen 1979; Kung, 2006) and environmental factors, the most important being the age of the plants, the level of phosphate in the soil relative to the requirements of the plant and the capacity of the population of mycorrhiza propagules in the soil to form mycorrhiza. Soybean is a nodulating legume and *Rhizobium* could have posed some threat in competing with *P. indica* for infection sites and for carbohydrates.

The results indicated that the inoculation of *B. japonicum and P. indica* affected the colonization percentage relative to single inoculations throughout the experimental period. The increment in percentage colonization with time period was observed. This could have been, since the root system infected normally increases with time

sigmoidally (Kung, 2006). Seasonal patterns in the formation of mycorrhiza have also been said to vary considerably from year to year (Allen and Allen, 1989). Furthermore, root colonization by mycorrhizal fungi may be inhibited by chemical compounds produced by the host, by mechanical barriers, by competition for carbohydrates etc (Santos *et al*., 2001).

In the experiment using sterilized soil, on 50^{th} day and after harvesting, highest nitrogen content was found in soil of co-inoculated plant followed by *B. japonicum* alone and P .indica alone but in unsterilized soil, it was followed by *P .indica* alone and *B. japonicum* alone. Increase in percent nitrogen due to dual inoculation might be attributed to increase in the number and dry weight of nodules as highest nodule number and nodule dry weight were recorded in dual inoculated plant in both cases and also due to release of nitrogenous exudates from roots, root nodules or degradation of nodules. It is found that as the pod filling initiates simultaneously nodule senescence switches on and ultimately they are degraded which eventually increases soil nitrogen content (Dhami, 2004). Similar results were obtained by Azcon *et al.* (1979) when VAM and *Rhizobium* together increased nodulation and nitrogen fixation in legumes grown under greenhouse and field conditions. Bigger nodule size is associated with active symbiotic relationship and nitrogen fixation between *Rhizobium* and the host plant (Oad *et al*., 2002) because improvement of phosphorous uptake by the host plant resulting from mycorrhizal symbiosis enhances nodulation and nitrogen fixation (Founoune *et al*., 2002). In legumes, mycorrhiza supplies the plant and the rhizobacteria with phosphorus, which is essential for the enzymes involved in the N fixation process and nitrogen fixation further promotes mycorrhizal development (Johansson *et al*., 2004). As reported by Packer and Clay (2003), mycorrhizal snowbrush plants fixed nearly twice as much as nitrogen as non-mycorrhizal plants.

In unsterilized soil, control plants also showed increase in nitrogen content after maturity than before soybean sowing. This shows that soybean planting in unsterilized

soil increases soil nitrogen content even without microbial inoculation but if inoculation is done then there is further increase in nitrogen content. Increase in nitrogen content due to microbial inoculation might be due to the positive interaction of inoculated microorganisms with indigenous microflora of the experimental site. Plants with single inoculation of *P. indica* showed more nitrogen than single inoculation of *B. japonicum* This might be because soil contained indigenous nitrogen fixing organisms (free or symbiotic) which on interacting synergistically with inoculated *P. indica*, increased nitrogen concentration. In case of co-inoculation, inoculated microorganisms and those already present in soil might have interacted synergistically and fixed more nitrogen. Comparable observations have been reported by Founoune *et al*. (2002) for the dual effect of AM and *Rhizobium* with *Acacia* species. Bagyaraj (1984) demonstrated that the AM fungi together with other microorganisms are able to enhance the nitrogen nutrition of the plant by stimulating the number and activity of free-living nitrogen fixers like *Beijerinkia* or *Azotobacter* in soil or by increasing nodulation and nitrogen fixing ability of the *Rhizobium*. Nitrogen content was found less in *B. japonicum* alone than in *P. indica* alone because the number of rhizobia present in the soil, their effectiveness, and their often-superior ability to compete with an inoculant strain determine the success of inoculum. In the literature, both the success and failure of *Rhizobium* inoculation in the unsterilized (field) soil have been reported.

In the experiment using sterilized soil, plants inoculated with *B. japonicum* alone showed more nitrogen than *P. indica* alone inoculated plants because the inoculated *B. japonicum* did not have to compete with any existing native bradyrhizobia since the soil was sterilized. Control plants were found to deplete soil nitrogen content after harvesting because there wasn't sufficient number of microorganisms present to fix atmospheric nitrogen.

In this study, the microsymbionts inoculation increased the concentration of soil phosphorus compared with non-inoculated ones in both sterilized and unsterilized soil, highest being found in dual inoculated plant followed by *P. indica* alone, *B. japonicum*

alone and uninoculated control. In most situations, the primary effect of endomycorrhizae is to improve the phosphate uptake by the host plant, through the transport from the soil by the fungal mycelium. An active involvement of the phosphatases in the phosphate metabolism of *P. indica* has been observed. Acid phosphatases have been observed to be active in *P. indica* mycelium (Malla, 2003). The molecular mechanisms underlying the uptake and translocation of nutrients, especially phosphate, are to be investigated (Singh *et al*., 2000).

Johansson *et al*. (2004) and Rabie and Almadini (2005) showed that dual inoculation with AM fungi and N-fixing bacteria (NFB) can support both needs for nitrogen and phosphorus and increase the growth of host plant. These authors reported that in bacterial-AM-legume tripartite symbiosis relationships nodulation of nitrogen fixing bacteria and establishment of AM often occur simultaneously and synergistically. Many reports have also indicated the P-solubilizing activity of some NBF (Johri *et al*., 1999). Besides, NFB provide fixed N not only to the plant, but also to the fungus. Pant and Prasad (2004) had shown the positive response of *Bradyrhizobium*-soybean symbiosis to phosphorus application.

On 50^{th} day and after harvesting, highest potassium content was found in soil of co-inoculated plant followed by *P. indica* alone *B. japonicum* alone and control in both sterilized and unsterilized soil. Bacteria that live in the nodules on the roots of leguminous plants need an ample supply of potassium to function efficiently and fix atmospheric nitrogen for the host plant (Johnston and Steen, 2006).

The successful diagnosis of the nutrient status of a plant based on plant analysis depends on the closeness of the relationship between nutrient concentrations at a certain stage of development and growth or yield (Lauchli and Bieleski, 1983).The agriculturally important symbiotic microorganisms play a remarkable role in nutrient (NPK and microelements) acquisition for plants. In pursuit of that goal, various workers

have used AM fungi and nitrogen fixing bacteria, a single inoculants and in combination with each other's in various plants. These symbiotic organisms have high ability to increase NPK as well as other nutrients in inoculated plants.

Inoculating soybean with *B. japonicum* and *P. indica* increased plant tissue nutrients concentration as Table 15 showed plant tissue NPK concentration was much higher in the co-inoculated plants than non-inoculated ones and single inoculation in both sterilized and unsterilized soil. VAM infection has been reported to increase the concentration of nutrients in the plant tissues (Smith and Bowen, 1979).

The higher plant tissue nitrogen content in co-inoculated plants could be attributed to hyphae uptake. Marschner and Dell (1994) reported that the existence of extra-radical hyphal bridges between individual plants permits transfer of nutrients such as nitrogen and also the inorganic sources of ammonium. The two have reported that about 24% of the total nitrogen uptake in mycorrhizal plants could be attributed to uptake and delivery by the external hyphae.

The higher phosphorus concentration in the inoculated plants could be attributed to a higher nutrients absorption rate by inoculated plants. The phosphorus content of control plant was much lower than that of other treatments. Several authors have reported that mycorrhizal roots are able to absorb several times more phosphate than non-inoculated roots from soils and from solutions and same might be the case for *P. indica* (Singh, 2004; Malla *et al*., 2002). Increased efficiency of phosphorus uptake by *P. indica* could have led to higher concentrations of phosphorus in the plant tissues and this increased in presence of *B. japonicum.* Smith and Bowen (1979) reported that the inflows of phosphorus to mycorrhiza roots can be greater than inflows to comparable non-mycorrhiza roots by up to 2-5 times. The same could be said of the higher potassium concentration in inoculated plants. In a compartment pots experiment, Li *et al.* (1991) demonstrated that about 10% of the total potassium uptake in mycorrhizal coach grass

was due to hyphal uptake and transport. Field studies have shown that inoculating groundnut cv. Florunner with different rhizobial strains stimulated greater accumulation of calcium, phosphorus, magnesium, potassium, zinc and other nutrient elements in seeds and nodules relative to that in uninoculated controls (Dakora, 2003). Several authors have noted that potassium uptake by soybean is influenced in response to different level of applied nitrogen. The results of this study demonstrate the beneficial influence of combined or single effect of *B. japonicum* and *P. indica* symbiosis on NPK accumulation in soybean.

Phosphorus accumulated in the shoot was higher than in the root in plants grown in both sterilized and unsterilized soil. Since the phosphorus is utilized largely in young, meristematic cells of the growing regions for the formation of nucleoproteins and other phosphorus-containing compounds, it would seem logical for the roots to contain less phosphorus than shoots. Phosphorus from roots might have transported to the fast growing shoots.

An increase in nutrient concentration, especially phosphorus and potassium by plants grown in sterilized soil could have resulted in relief of nutrients stress and an increase in photosynthetic rate, which obviously could have given rise to an increase in plant growth. This possibility needs to be investigated through additional research.

Characteristics of the site into which a seed disperses affect germination, seedling growth, and survival. The effects of site on plant fitness are due in part to the local vegetation. Species-specific alteration of chemical and physical properties of soil has frequently been documented. Soil alteration may result from site-specific differences in patterns of nutrient acquisition and use, modification of moisture and temperature (Packer and Clay, 2003). As a result, the micro site quality may vary for each individual species of both plant and microorganism. Plant species can also differ in the microbial soil communities they host. When soil microorganisms are dependent on host plant

composition, variation in plant species abundance and composition can result in different soil communities. Positive feedback occurs when the presence of a plant or plant population makes condition more favorable for individual of same species, e.g., symbiotic associations with rhizobia or mycorrhizal fungi (Bever, 1994). On the other hand, negative interactions between plants and their associated soil biota may have important implications for species coexistence and succession (Parker and Clay, 2003).

The difference in the growth parameters observed between sterilized and unsterilized soil in our study might be in part due to the nutrient effects although our aim was not to compare the growth parameters between unsterilized and sterilized condition. Our aim was only to find out whether the inoculated microorganisms have any beneficial effects on the host plant when grown on sterilized and unsterilized (field) natural soil. However, results of this study suggest that plant modification of their microbial soil community affected its growth parameters. Varieties of effects were observed after autoclaving the soil. Stem and root masses may have increased in sterilized soil due to the elimination of pathogens and nutrient release from microbial biomass. Alternatively, biomass may have increased in response to nutrients released during soil sterilization. In this experiment, it was found that sterilized soil had increased nitrogen and phosphorus relative to unsterilized soil after autoclaving. Similar results were reported by Packer and Clay (2003). In addition, plants were provided with nutrient solution every week in the set using sterilized soil.

Studies using sterilization treatments have found both increases and decreases in plant biomass. As reported by Packer and Clay (2003) and Putten *et al*. (1993), the biomass of sand dune grass Ammophila arenaria improved when soil previously cultured by A. arenaria was sterilized. The same effect was not observed when beach sand was sterilized. Similarly, blackbutt (Eucalyptus pilularis) seedlings were severely inhibited in blackbutt forest soil, but this inhibition was overcomed by gamma irradiation of the soil. Apple trees, which commonly suffer poor survival and/or growth when grown on

sites previously planted with apple, showed enhanced growth when orchard soils were sterilized. Removal of soil biota has also been found to inhibit plant growth relative to unsterilized field soil, suggesting an important role for soil borne microbial mutualists like mycorrhizal fungi (Packer and Clay, 2003).

In determining plant yields, as defined by the amount of either fresh or dry matter or certain constituents (e.g. sugar, starch, protein) produced per unit area, photosynthesis is the most common pre requisite. There is a close relationship between an increase in photosynthetic active surface area and an increase in fresh and dry weight. Here mineral nutrients determine yield, depending on their function as constituents of organic compounds, enzyme activators, osmoregulators etc (Laiichii and Bieleski, 1983). The increase in growth mainly plant height in sterilized soil may be due to the factor of photosynthesis production which was higher due to above factors. Here, co-inoculation with *B. japonicum* and *P. indica* generally promoted soybean plant growth under either sterilized or unsterilized soil although the aim was not to compare the growth parameters between sterilized and unsterilized condition.

6.2 CONCLUSION

The current study showed that inoculating soybean with *B. japonicum* and *P. indica* enhanced growth performance of soybean plants when grown in sterilized and unsterilized soil during pot culture experiment. The inoculation, whether singly or dual, resulted in an increment in growth parameters over the control plants on all the days observed.

The present study indicated that microsymbionts inoculation increased the concentration of NPK in soybean plant tissue and soil compared with uninoculated one. The beneficial effects of the microsymbionts, observed in this study, arouse an

interest in considering the role of *Rhizobium-P. indica*-plant tripartite symbiosis in

plant growth promotion and increasing soil nutrient status. We can conclude that the soybean-*B. japonicum-P. indica* symbiosis becomes even more important in sustainable agricultural systems. However, the data obtained from this study are insufficient to generalize the conclusion and the applicable uses of biofertilizer still remain to be discussed for different associations in various plant species and families in different types of soil.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 SUMMARY

The effect of dual inoculation of *Bradyrhizobium japonicum* and *Piriformospora indica* on the host plant soybean [*Glycine max* (L.) Merr. cv "Sathiya"] in pot cultures were investigated using sterilized (autoclaved) and unsterilized (natural) soil. The effects were recorded on 21st, 35th , 50th and 65th day from the date of plantation with respect to number, fresh and dry weight of nodules, root length, root fresh and dry weight, shoot length, shoot fresh and dry weight. Root and shoot NPK contents were recorded on 50th day while soil NPK contents were recorded on 50th day and after harvesting after maturity. Percent root colonization was recorded on all the days and after harvesting after maturity.

The findings of the study are summarized as follows:

1. When the treatments were conducted under sterilized and unsterilized potted soils, on all the days observed, dual inoculation increased nodule number, nodule fresh and dry weight, root fresh and dry weight, shoot length, shoot fresh and dry weight. However, the results were not statistically significant for root dry weight in case of sterilized soil on 21st and 35th day and shoot length in case of unsterilized soil on 21st day, although all the treatments showed greater value than the control treatment. On rest of the days, the results were statistically significant.

2. When the treatments were conducted in sterilized potted soil, on the days observed, the maximum root, shoot and soil nitrogen content were produced by the plants receiving dual inoculation of *B. japonicum* and *P. indica* followed by *B. japonicum* alone, *P. indica* alone and uninoculated control. The maximum root, shoot and soil phosphorus and potassium content were found in the dual inoculated plants followed by single inoculation of *P. indica*, single inoculation of *B. japonicum* and uninoculated control.

3. When soybean plants were grown in unsterilized potted soil, on the days observed, the maximum root, shoot and soil NPK contents were produced by the dual inoculated plants followed by single inoculation of *P. indica* alone, *B. japonicum* alone and uninoculated control.

4. The inoculation of *B. japonicum and P. indica* affected the root colonization percentage relative to single inoculation of *P. indica* on all the days observed. This was done for sterilized soil only. The increment in percentage root colonization with time period was observed.

7.2 RECOMMENDATIONS

Depending upon the findings of this study, following recommendations have been made:

1. In addition to the physiological and biochemical parameters tested to evaluate plant reaction to microbial inoculants in this study, other parameters such as maturation, photosynthesis, sugar content , yield etc should also be investigated. Further researches should be carried out to understand the molecular basis of plant-microbes interaction.

2. The plant growth promotion should be tested in field because the field environment is generally much more stressful, and the conditions are more complex there. The results obtained in pot experiment do not necessarily reflect the potential for plant growth promotion in field.
3. Since *B. japonicum* and *P. indica* can be cultured in *in vitro*, methodology for the processing and propagation of strains in *in vitro* culture should be developed by concerned authorities so that they can be commercially produced and can be accessible throughout the country for better production of crops.

4. Emphasize on the use of microbial biofertilizer to improve soil nitrogen and phosphorus contents in order to gradually integrate this technology with conventional agricultural practices.

5. Additional research and methodology should be carried out for practical evaluation of inoculant potential of *B. japonicum* and *P. indica* on various legumes under varying agroclimatic conditions in different fields for agricultural development.

6. Scientific collaborative projects among research-based organizations should be pursued by combining experience from various disciplines including exchange visits for collaborators.

CHAPTER-VIII

8. REFERENCES

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APPENDIX-I

COMPOSITION AND PREPARATION OF CULTURE MEDIA

1. Yeast extract Mannitol Broth (YMB) (Vincent, 1970)

Constituents	**grams/litre**
Mannitol	10.0
Dipotassium hydrogen phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Yeast extract	0.5
Distilled water	1.0

Preparation: The salts were dissolved in 1 litre of distilled water. Mannitol and yeast extract were added and dissolved by continuous stirring. pH was adjusted to 6.8 with 0.1N NaOH and 0.1N HCl. It was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121^{o}C.

2. Yeast extract Mannitol Agar with Congo Red indicator (YMA -CR)

Constituents	**grams/litre**
YMB	1 litre
Agar	15 g
Congo red stock solution	10 ml

Preparation: YMB was prepared. Agar was added and shaken to suspend evenly. Congo red was added. It was autoclaved at 15 lbs pressure for 15 minutes at 121^{o}C.

3. Potato Dextrose Agar (Martin, 1950)

Composition	**grams/litre**
Potato peel	200.0
Dextrose	20.0
Agar	15.0
Distilled water	1.0

Preparation: Skin of potatoes was peeled off, cut into small pieces and boiled (200 g) in 500 ml of water, till they were easily penetrated by a glass rod. It was filtered through cheese cloth and dextrose was added to the filtrate. Agar was dissolved and the required volume (1 litre) was made up by the addition of water. The medium was autoclaved at 15 lbs pressure for 20 minutes.

APPENDIX-II

COMPOSITION AND PREPARATION OF STAINS AND REAGENTS

1. Trypan blue (Phillips and Hayman,1970)

Trypan blue	0.1 g
Lactophenol	100 ml

2. Lactophenol (Phillips and Hayman, 1970)

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

3. Hogland solution (Hogland and Arnon, 1938)

Chemicals	**Concentrations (mg/l)**
Macro-nutrients	
$MgSO_4.7H_2O$	490
$Ca(NO_3)_2.4H_2O$	492
KNO_3	1002
$NH_4H_2PO_4$	230
Micro-nutrients	
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22

H_3BO_3	2.86
$CuSO_4.H_2O$	0.08
$NaMoO_4$	0.09

Iron source

$FeSO_4.7H_2O$	31

Preparation: All ingredients were dissolved separately in double distilled water and then mixed. pH 6.7

4. 70% Ethanol

Absolute alcohol	70 ml
Distilled Water	30 ml

Preparation: Ethanol was dissolved in distilled water.

5. 1.5% Sodium hypochlorite

Sodium hypochlorite	1.5 ml
Distilled water	100 ml

Preparation: Sodium hypochlorite was dissolved in distilled water and volume made to 100 ml.

6. 10% Potassium hydroxide

Potassium hydroxide	10 g
Distilled water	100 ml

Preparation: Potassium hydroxide was dissolved in distilled water and volume made to 100 ml.

7. Congo red stock solution

Congo red	250 mg
Distilled water	100 ml

Preparation: Congo red was dissolved in 100 ml distilled water.

8. Digestion mixture (catalyst) for total nitrogen determination

Copper sulphate ($CuSO_4$)	10 g
Sodium sulphate (Na_2SO_4)	200 g

Preparation: $CuSO_4$ and Na_2SO_4 were mixed and grinded.

9. 40% Sodium hydroxide

Sodium hydroxide (pellets)	400 g
Distilled water	1000 ml

Preparation: Sodium hydroxide was dissolved in distilled water and cooled.

10. Mixed indicator

Bromocresol green	5 g
Methyl red	1 g
95% ethanol	100 ml

Preparation: Bromocresol green and methyl red was dissolved in ethanol.

11. 4% Boric acid

Boric acid crystal	40 g
Distilled water	1000 ml

Preparation: Boric acid crystal was dissolved in distilled water.

12. 0.01N Hydrochloric acid (HCl)

Concentrated hydrochloric acid	
(specific gravity 1.19)	17 ml
Distilled water	2000 ml

Preparation: Conc. HCl was diluted with distilled water to 2 litre-(A). 20 ml of solution was standardized with Na_2CO_3 and normality was calculated. The solution (A) was diluted according to its strength to give 0.01N HCl.

13. 0.05N HCl

Conc. HCl	17 ml
Distilled water	2000 ml

Preparation: 17 ml conc. HCl was diluted with distilled water to 2 litre-(A). 20 ml of solution was standardized with 0.5N Na_2CO_3 and normality was calculated. The solution (A) was diluted according to its strength to give 0.05N HCl.

14. Extracting Solution pH 8.5

$NaHCO_3$ (Sodium bicarbonate)	210 g
Distilled water	5 litre

Preparation: $NaHCO_3$ was dissolved in distilled water. pH was adjusted to 8.5 with 0.5N sodium hydroxide or sulphuric acid.

15. 5N Sulphuric acid (H_2SO_4):

Concentrated A.R. H_2SO_4	35 ml
Distilled water	250 ml

Preparation: Conc. A.R. H_2SO_4 was diluted to 250 ml.

16. Ammonium molybdate:

Ammonium molybdate .	12 g
Antimony potassium tartarate	0.298 g
5N H_2SO_4	1000 ml
Distilled water	250 ml

Preparation: Ammonium molybdate was dissolved in 250 ml distilled water. Antimony potassium tartarate was dissolved in 100 ml distilled water. Both the solutions were added to 1000 ml of 5N H_2SO_4, mixed thoroughly and made to 2 litre. It was stored in pyrex glass bottle and kept in a dark and cool temperature.

17. Ascorbic acid

Ascorbic acid	1.056 g
Reagent 17	200 ml

Preparation: 1.056 g of ascorbic acid was dissolved in [reagent (16)].

18. Standard P (phosphate) Solution

a. Primary Standard 50 ppm P

Potassium dihydrogen phosphate (KH_2PO_4)	0.2195 g
7N H_2SO_4	25 ml

Preparation: Potassium dihydrogen phosphate dried at 40^oC ,was dissolved in about 400 ml distilled water in one litre volumetric flask. About 25 ml of 7N H_2SO_4 was added to it and the volume made to 1 litre.

b. **Secondary standards, 2 and 20 ppm P**

Preparation: 20 ml 50 ppm stock solution was diluted to 500 ml in a volumetric flask for the 2 ppm P. 100 ml of 50 ppm P stock solution was diluted to 250 ml for 20 ppm P.

19. p-Nitrophenol indicator 0.25%

p-nitrophenol	0.25 g
Distilled water	100 ml

Preparation: p-nitrophenol indicator was dissolved in 100 ml of distilled water.

20. 1N Ammonium acetate pH 7.0

Ammonium acetate	77 g
Distilled water	1000 ml

Preparation: Ammonium acetate was dissolved in 1000 ml of distilled water.

21. K (potassium) standard (stock solution)- 100 ppm K

KCl	0.1905 g
Distilled water	1000 ml

Preparation: 0.1905 g KCl, dried at 40^{o}C, was dissolved in 1000 ml volumetric flask and volume made up.

22. Working standard K solution for soil

Preparation: 0,5,10,15,20 and 25 ml of solution (21) was taken in 100 ml volumetric flask and diluted with 1N ammonium acetate pH 7 solution to the mark-0,5,10,15,20 and 25 ppm K.

23. Working standard K solution for plant nutrient analysis

Preparation: 0,1,2,4,6,8 and 10 ml of solution (21) was taken in 100 ml volumetric flask. 10 ml of 1N H_2SO_4 or 1N nitric acid was added depending upon the acid system of sample and diluted to the mark with distilled water to give 0,1,2,4,6,8 and 10 ppm K in the acid system.

24. 5N Nitric acid:

Conc. nitric acid specific gravity 1.42	320 ml

Distilled water	1000 ml

Preparation: Conc. nitric acid was diluted to 1000 ml with distilled water.

25. 4N HCl

Conc.HCl	172 ml
Distilled water	500 ml

Preparation: Conc. hydrochloric acid was diluted to 500 ml with distilled water.

26. Ammonium molybdate-vanadate solution

Ammonium molybdate [$NH_4 6Mo_7O_{24}.4H_2O$]	25 g
Ammonium metavanadate (NH_4Vo_3)	1.25 g
Concentrated nitric acid	250 ml

Preparation: $NH_4 6Mo_7O_{24}.4H_2O$ was dissolved in 400 ml distilled water. NH_4Vo_3 was dissolved in 300 ml of boiling distilled water. It was cooled and concentrated nitric acid was added and cooled. The two solutions mixed and diluted to 1litre with distilled water.

APPENDIX-III

Materials

Autoclave	: Life, Portable steam stericlave-800, India
Beakers	: Borosil
Burettes	: Borosil
Conical flask-125ml	: Borosil
Conical flasks	: Borosil
Distilling apparatus	: Borosil
Electronic balance	: Scaltec
Erlenmeyer flask 100ml	: Borosil
Flame photometer	: Jenway
Funnel	: Borosil
Hot air oven	: Narang Scientific Works Pvt. Ltd, India
Incubator	: Indoexim, India
Kjeldahl flask holder	: Elite, India
Kjeldhal digestion flask-50ml	: Borosil
Measuring cylinder	: Borosil
Microscope	: Olympus Optical Co. Ltd, Japan
Muffle furnace	: Vinay Trading Ltd. India
N-Digestion apparatus	: Elite, India
Petridishes	: Borosil
Photomicrograph	: Olympus Optical Co.Ltd, Japan
Pipettes	: Borosil
Porcelain crucible 30 ml	:

Refrigerator	: Haier
Spectrophotometer	: Jenway
Volumetric flask-100ml	: Borosil
Vortex shaker	: Jindal, India
Water bath shaker	: Type OLS200 Ser No. 8Q0048002, Grant Instruments (Cambridge) Ltd, England
Whatman No. 42 filter paper	: Whatman International Limited

Chemicals

Agar agar	: Hi-media Laboratory
Ammonium acetate	: Qualigens Fine Chemicals, India
Ammonium dihydrogen phosphate	: Merk Ltd, India
Ammonium metavanadate	: Qualigens Fine Chemicals, India
Ammonium molybdate	: Qualigens Fine Chemicals, India
Antimony potassium tartarate	: Qualigens Fine Chemicals, India
Ascorbic acid	: Qualigens Fine Chemicals, India
Boric acid	: Ranbaxy Fine Chemicals Ltd, India
Bromocresol green	: Ranbaxy Fine Chemicals Ltd, India
Calcium nitrate	: Ranbaxy Fine Chemicals Ltd, India
Concentrated hydrochloric acid (AR)	: Qualigens Fine Chemicals, India
Concentrated Nitric acid	: Qualigens Fine Chemicals, India
Concentrated Sulphuric acid (LR)	: Qualigens Fine Chemicals, India
Copper sulphate	: Ranbaxy Fine Chemicals Ltd, India
Dextrose	: Qualigens Fine Chemicals, India
Disodium hydrogen phosphate	: S.D Fine Chemicals Ltd. Boisar
Dehydrated alcohol	: Bengal Chemicals and Pharmaceuticals, Ltd
Ferrous sulphate	: Ranbaxy Fine Chemicals Ltd, India

Glycerol	: Qualigens Fine Chemicals, India
Lactic acid	: Qualigens Fine Chemicals, India
Magnesium sulphate	: Ranbaxy Fine Chemicals Ltd, India
Manganese chloride	: Central Drug House Pvt, India
Mannitol	: Qualigens Fine Chemicals, India
Methyl red	: Glaxo Laboratories Ltd, India
Phenol	: Nike Chemical, India
p-nitrophenol indicator	: Qualigens Fine Chemicals, India
Potassium biphthalate	: Qualigens Fine Chemicals, India
Potassium chloride	: Qualigens Fine Chemicals, India
Potassium dihydrogen phosphate	: Merk Ltd, India
Potassium hydroxide	: SD fine-chem Ltd, India
Potassium nitrate	: Merk Ltd, India
Potassium sulphate	: Qualigens Fine Chemicals, India
Sodium bicarbonate	: Ranbaxy Fine Chemicals Ltd, India
Sodium chloride	: Ranbaxy Fine Chemicals Ltd, India
Sodium hydroxide	: Ranbaxy Fine Chemicals Ltd, India
Sodium hypochlorite	: Qualigens Fine Chemicals, India
Sodium molybdate	: Qualigens Fine Chemicals, India
Sodium sulphate	: Ranbaxy Fine Chemicals Ltd, India
Yeast extract	: Qualigens Fine Chemicals, India
Zinc sulphate	: Qualigens Fine Chemicals, India

Stains

Congo red	: Qualigens Fine Chemicals, India
Trypan blue	: Loba chemie indoaustranal Co. India

APPENDIX-IV

Table I. Analysis of Variance (ANOVA) of nodule number at different days after plantation

Soil	Day		Sum of Squares	df	Mean Square	F	Sig.
Sterilized	21	Between Groups	78.550	3	26.183	34.911	0.000*
		Within Groups	12.000	16	.750		
		Total	90.550	19			
	35	Between Groups	707.350	3	235.783	19.208	0.000*
		Within Groups	196.400	16	12.275		
		Total	903.750	19			
	50	Between Groups	2624.800	3	874.933	59.217	0.000*
		Within Groups	236.400	16	14.775		
		Total	2861.200	19			
	65	Between Groups	2800.550	3	933.517	79.280	0.000*
		Within Groups	188.400	16	11.775		
		Total	2988.950	19			
Un-sterilized	21	Between Groups	169.000	3	56.333	70.417	0.000*
		Within Groups	12.800	16	.800		
		Total	181.800	19			
	35	Between Groups	1409.200	3	469.733	16.821	0.000*
		Within Groups	446.800	16	27.925		
		Total	1856.000	19			
	50	Between Groups	1296.400	3	432.133	19.058	0.000*
		Within Groups	362.800	16	22.675		
		Total	1659.200	19			
	65	Between Groups	1136.550	3	378.850	7.787	0.002*
		Within Groups	778.400	16	48.650		
		Total	1914.950	19			

df = degree of freedom

* = significant difference between the treatments occurred at p<0.05

Table II. Analysis of Variance (ANOVA) of nodule fresh weight at different days after plantation

Soil	Day		Sum of Squares	df	Mean Square	F	Sig.
Sterilized	21	Between Groups	164.760	3	54.920	16.877	0.000*
		Within Groups	52.067	16	3.254		
		Total	216.828	19			
	35	Between Groups	33578.818	3	11192.939	122.528	0.000*
		Within Groups	1461.600	16	91.350		
		Total	35040.418	19			
	50	Between Groups	96138.150	3	32046.050	53.306	0.000*
		Within Groups	9618.800	16	601.175		
		Total	105756.950	19			
	65	Between Groups	146012.150	3	48670.717	28.894	0.000*
		Within Groups	26951.600	16	1684.475		
		Total	172963.8	19			
Un-sterilized	21	Between Groups	625.750	3	208.583	23.838	0.000*
		Within Groups	140.000	16	8.750		
		Total	765.750	19			
	35	Between Groups	23291.296	3	7763.765	35.719	0.000*
		Within Groups	3477.744	16	217.359		
		Total	26769.040	19			
	50	Between Groups	21684.292	3	7228.097	5.195	0.011*
		Within Groups	22263.128	16	1391.446		
		Total	43947.420	19			
	65	Between Groups	298822.151	3	99607.384	19.268	0.000*
		Within Groups	82713.211	16	5169.576		
		Total	381535.361	19			

df = degree of freedom

* = significant difference between the treatments occurred at p<0.05

Table III. Analysis of Variance (ANOVA) of nodule dry weight at different days after plantation

Soil	Day		Sum of Squares	df	Mean Square	F	Sig.
Sterilized	21	Between Groups	22.197	3	7.399	8.239	0.002*
		Within Groups	14.369	16	.898		
		Total	36.566	19			
	35	Between Groups	2352.600	3	784.200	16.928	0.000*
		Within Groups	741.200	16	46.325		
		Total	3093.800	19			
	50	Between Groups	4524.522	3	1508.174	18.771	0.000*
		Within Groups	1285.528	16	80.346		
		Total	5810.050	19			
	65	Between Groups	26159.600	3	8719.867	28.343	0.000*
		Within Groups	4922.400	16	307.650		
		Total	31082.000	19			
Un-sterilized	21	Between Groups	227.149	3	75.716	21.472	0.000*
		Within Groups	56.420	16	3.526		
		Total	283.569	19			
	35	Between Groups	2007.445	3	669.148	19.362	0.000*
		Within Groups	552.960	16	34.560		
		Total	2560.406	19			
	50	Between Groups	1246.753	3	415.584	4.424	0.019*
		Within Groups	1503.092	16	93.943		
		Total	2749.845	19			
	65	Between Groups	31217.350	3	10405.783	31.101	0.000*
		Within Groups	5353.200	16	334.575		
		Total	36570.550	19			

df = degree of freedom

* = significant difference between the treatments occurred at $p<0.05$

Table IV. Analysis of Variance (ANOVA) of root length at different days after plantation

Soil	Day		Sum of Squares	df	Mean Square	F	Sig.
Sterilized	21	Between Groups	343.450	3	114.483	16.857	0.000*
		Within Groups	108.660	16	6.791		
		Total	452.110	19			
	35	Between Groups	50.022	3	16.674	8.245	0.002*
		Within Groups	32.356	16	2.022		
		Total	82.378	19			
	50	Between Groups	119.729	3	39.910	8.189	0.002*
		Within Groups	77.976	16	4.873		
		Total	197.705	19			
	65	Between Groups	99.038	3	33.013	8.342	0.001*
		Within Groups	63.320	16	3.958		
		Total	162.358	19			
Un-sterilized	21	Between Groups	203.148	3	67.716	12.381	0.000*
		Within Groups	87.512	16	5.470		
		Total	290.660	19			
	35	Between Groups	466.237	3	155.413	9.828	0.001*
		Within Groups	253.000	16	15.813		
		Total	719.238	19			
	50	Between Groups	653.837	3	217.946	22.527	0.000*
		Within Groups	154.800	16	9.675		
		Total	808.637	19			
	65	Between Groups	408.770	3	136.257	16.365	0.000*
		Within Groups	133.220	16	8.326		
		Total	541.990	19			

df = degree of freedom

* = significant difference between the treatments occurred at $p<0.05$

Table V. Analysis of Variance (ANOVA) of root fresh weight at different days after plantation

Soil	Day		Sum of Squares	df	Mean Square	F	Sig.
Sterilized	21	Between Groups	1.251	3	.417	3.490	0.040*
		Within Groups	1.912	16	.120		
		Total	3.164	19			
	35	Between Groups	7.957	3	2.652	5.605	0.008*
		Within Groups	7.571	16	.473		
		Total	15.528	19			
	50	Between Groups	6.961	3	2.320	6.793	0.004*
		Within Groups	5.465	16	.342		
		Total	12.427	19			
	65	Between Groups	5.253	3	1.752	7.590	0.002*
		Within Groups	3.691	16	.231		
		Total	8.944	19			
Un-sterilized	21	Between Groups	.281	3	.094	16.863	0.000*
		Within Groups	.089	16	.006		
		Total	.370	19			
	35	Between Groups	.265	3	.088	24.715	0.000*
		Within Groups	.057	16	.004		
		Total	.322	19			
	50	Between Groups	1.081	3	.360	3.542	0.039*
		Within Groups	1.628	16	.102		
		Total	2.709	19			
	65	Between Groups	6.107	3	2.036	23.622	0.000*
		Within Groups	1.379	16	.086		
		Total	7.486	19			

df = degree of freedom

* = significant difference between the treatments occurred at $p<0.05$

Table VI. Analysis of Variance (ANOVA) of root dry weight at different days after plantation

Soil	Day		Sum of Squares	df	Mean Square	F	Sig.
Sterilized	21	Between Groups	.006	3	.002	.494	0.692
		Within Groups	.068	16	.004		
		Total	.074	19			
	35	Between Groups	.138	3	.046	2.410	0.105
		Within Groups	.306	16	.019		
		Total	.445	19			
	50	Between Groups	.220	3	.073	8.286	0.001*
		Within Groups	.142	16	.009		
		Total	.362	19			
	65	Between Groups	.566	3	.189	7.656	0.002*
		Within Groups	.394	16	.025		
		Total	.960	19			
Un-sterilized	21	Between Groups	.006	3	.002	4.764	0.015*
		Within Groups	.007	16	.000		
		Total	.012	19			
	35	Between Groups	.076	3	.025	10.719	0.000*
		Within Groups	.038	16	.002		
		Total	.114	19			
	50	Between Groups	.127	3	.042	4.718	0.015*
		Within Groups	.144	16	.009		
		Total	.271	19			
	65	Between Groups	.551	3	.184	20.464	0.000*
		Within Groups	.144	16	.009		
		Total	.695	19			

df = degree of freedom

* = significant difference between the treatments occurred at $p<0.05$

Table VII. Analysis of Variance (ANOVA) of shoot length at different days after plantation

Soil	Day		Sum of Squares	df	Mean Square	F	Sig.
Sterilized	21	Between Groups	98.894	3	32.965	10.307	0.001*
		Within Groups	51.172	16	3.198		
		Total	150.066	19			
	35	Between Groups	224.914	3	74.971	40.291	0.000*
		Within Groups	29.772	16	1.861		
		Total	254.686	19			
	50	Between Groups	544.274	3	181.425	17.840	0.000*
		Within Groups	162.712	16	10.170		
		Total	706.986	19			
	65	Between Groups	595.346	3	198.449	25.833	0.000*
		Within Groups	122.912	16	7.682		
		Total	718.258	19			
Un-sterilized	21	Between Groups	13.557	3	4.519	2.611	0.087
		Within Groups	27.692	16	1.731		
		Total	41.249	19			
	35	Between Groups	219.588	3	73.196	22.688	0.000*
		Within Groups	51.620	16	3.226		
		Total	271.208	19			
	50	Between Groups	872.500	3	290.833	82.389	0.000*
		Within Groups	56.480	16	3.530		
		Total	928.980	19			
	65	Between Groups	1009.901	3	336.634	134.037	0.000*
		Within Groups	40.184	16	2.512		
		Total	1050.085	19			

df = degree of freedom

* = significant difference between the treatments occurred at $p<0.05$

Table VIII. Analysis of Variance (ANOVA) of shoot fresh weight at different days after plantation

Soil	Day		Sum of Squares	df	Mean Square	F	Sig.
Sterilized	21	Between Groups	1.944	3	.648	4.122	0.024*
		Within Groups	2.516	16	.157		
		Total	4.461	19			
	35	Between Groups	9.999	3	3.333	3.306	0.047*
		Within Groups	16.133	16	1.008		
		Total	26.132	19			
	50	Between Groups	26.947	3	8.982	9.922	0.001*
		Within Groups	14.485	16	.905		
		Total	41.431	19			
	65	Between Groups	99.482	3	33.161	19.584	0.000*
		Within Groups	27.092	16	1.693		
		Total	126.574	19			
Un-sterilized	21	Between Groups	.703	3	.234	12.985	0.000*
		Within Groups	.289	16	.018		
		Total	.991	19			
	35	Between Groups	5.177	3	1.726	10.339	0.001*
		Within Groups	2.670	16	.167		
		Total	7.847	19			
	50	Between Groups	20.988	3	6.996	17.552	0.000*
		Within Groups	6.377	16	.399		
		Total	27.365	19			
	65	Between Groups	83.343	3	27.781	14.149	0.000*
		Within Groups	31.414	16	1.963		
		Total	114.757	19			

df = degree of freedom

* = significant difference between the treatments occurred at $p<0.05$

Table IX. Analysis of Variance (ANOVA) of shoot dry weight at different days after plantation

Soil	Day		Sum of Squares	df	Mean Square	F	Sig.
Sterilized	21	Between Groups	.079	3	.026	4.132	0.024*
		Within Groups	.102	16	.006		
		Total	.180	19			
	35	Between Groups	.270	3	.090	7.117	0.003*
		Within Groups	.203	16	.013		
		Total	.474	19			
	50	Between Groups	.995	3	.332	3.430	0.042*
		Within Groups	1.547	16	.097		
		Total	2.542	19			
	65	Between Groups	14.065	3	4.688	13.405	0.000*
		Within Groups	5.596	16	.350		
		Total	19.661	19			
Un-sterilized	21	Between Groups	19.661	3	.012	5.666	0.008*
		Within Groups	.036	16	.002		
		Total	.069	19			
	35	Between Groups	.556	3	.185	13.857	0.000*
		Within Groups	.214	16	.013		
		Total	.770	19			
	50	Between Groups	2.303	3	.768	14.423	0.000*
		Within Groups	.852	16	.053		
		Total	3.154	19			
	65	Between Groups	13.974	3	4.658	31.850	0.000*
		Within Groups	2.340	16	.146		
		Total	16.314	19			

df = degree of freedom

* = significant difference between the treatments occurred at $p<0.05$

APPENDIX-V

Table X. Percent increment in nodule number of soybean plant grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatment	21 days	% inc.	35 days	% inc.	50 days	% inc.	65 days	% inc.
Sterilized	Uninoculated control	0.40	-	6.6	-	9.6	-	10.40	-
	B. japonicum	2.6	550.0	19.6	196.96	30.4	216.66	34.4	230.76
	P. indica	1.8	350.0	14.6	121.21	23.6	145.83	28.8	176.92
	B. japonicum + *P. indica*	5.8	1350.0	22.2	236.36	41.2	329.16	42.6	309.61
Un-sterilized	Uninoculated control	1.6	-	10.0	-	20.0	-	25.4	-
	B. japonicum	3.0	87.5	21.8	118.0	32.8	64.0	38.0	49.60
	P. indica	5.8	262.5	27.8	178.0	36.6	83.0	40.4	59.05
	B. japonicum + *P. indica*	9.2	475.0	32.4	224.0	41.8	109.0	46.0	81.10

Table XI. Percent increment in nodule fresh weight of soybean plant grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatment	21 days	% inc.	35 days	% inc.	50 days	% inc.	65 days	% inc.
Sterilized	Uninoculated control	1.12	-	17.68	-	35.8	-	42.2	-
	B. japonicum	7.1	533.92	98.26	455.76	192.0	436.3	232.8	451.65
	P. indica	6.4	471.42	71.60	304.97	184.0	413.9	174.0	312.32
	B. japonicum + *P. indica*	8.8	685.71	129.3	631.33	208.0	481.0	266.0	530.33

Un-sterilized	**Uninoculated control**	9.2	-	36.0	-	111.36		358.22	-
	B. japonicum	20.8	126.08	78.2	117.22	163.84	47.12	631.60	76.31
	P. indica	21.6	134.78	88.2	145.0	191.54	72.0	640.80	78.88
	B. japonicum + ***P. indica***	23.4	154.34	132.0	266.66	192.46	72.82	648.04	80.90

Table XII. Percent increment in nodule dry weight of soybean plant grown in sterilized and unsterilized potted soils at different days after plantation

Soil	**Treatment**	**21 days**	**% inc.**	**35 days**	**% inc.**	**50 days**	**% inc.**	**65 days**	**% inc.**
Sterilized	**Uninoculated control**	0.54	-	6.40	-	8.58	-	17.60	-
	B. japonicum	3.02	459.25	26.80	318.75	39.20	356.87	78.0	343.18
	P. indica	2.42	348.14	22.0	243.75	35.80	317.24	65.40	271.59
	B. japonicum + ***P. indica***	3.20	492.59	36.40	468.75	49.20	473.42	119.0	576.13
Un-sterilized	**Uninoculated control**	4.82	-	12.60	-	31.98	-	84.6	-
	B. japonicum	10.02	107.88	30.12	139.04	42.90	34.14	108.0	27.65
	P. indica	12.26	154.35	33.0	161.90	49.24	53.97	140.0	65.48
	B. japonicum + ***P. indica***	13.70	184.23	39.74	215.39	52.74	64.91	190.0	124.58

Table XIII. Percent increment in root length of soybean plant grown in sterilized and unsterilized potted soils at different days after plantation

Soil	**Treatment**	**21 days**	**% inc.**	**35 days**	**% inc.**	**50 days**	**% inc.**	**65 days**	**% inc.**

Sterilized	**Uninoculated control**	41.30	-	46.42	-	48.52	-	49.10	-
	B. japonicum	31.20	24.45	42.0	9.52	41.60	14.26	43.10	12.21
	P. indica	31.90	22.76	44.48	4.17	45.10	7.04	47.0	4.27
	B. japonicum* + *P. indica	37 .40	9.44	44.80	3.48	45.12	7.00	47.70	2.85
Un-sterilized	**Uninoculated control**	18.62	-	26.70	-	27.10	-	30.50	-
	B. japonicum	23.74	27.49	30.60	14.60	34.10	25.83	37.12	21.70
	P. indica	26.24	40.92	33.70	26.21	38.30	41.32	38.66	26.75
	B. japonicum* + *P. indica	26.60	42.85	39.90	49.43	42.60	57.19	43.10	41.31

Table XIV. Percent increment in root fresh weight of soybean plant grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatment	21 days	% inc.	35 days	% inc.	50 days	% inc.	65 days	% inc.
Sterilized	**Uninoculated control**	1.08	–	2.01	–	2.22	–	2.90	–
	B. japonicum	1.19	10.18	2.32	15.42	2.56	15.31	3.51	21.03
	P. indica	1.32	22.22	2.52	25.37	2.98	34.23	4.05	39.66
	B. japonicum* + *P. indica	1.74	61.11	3.68	83.08	3.80	71.17	4.21	45.17
Un-sterilized	**Uninoculated control**	0.33	–	0.37	–	0.87	–	1.58	–
	B. japonicum	0.37	12.12	0.46	24.32	1.34	54.02	1.88	18.99
	P. indica	0.56	69.69	0.62	67.56	1.42	63.21	2.45	55.06
	B. japonicum* + *P. indica	0.60	81.81	0.65	75.67	1.44	65.51	3.02	91.14

Table XV. Percent increment in root dry weight of soybean plant grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatment	21 days	% inc.	35 days	% inc.	50 days	% inc.	65 days	% inc.
Sterilized	Uninoculated control	0.16	–	0.51	–	0.54	–	0.59	–
	B. japonicum	0.19	18.75	0.54	5.88	0.64	18.52	0.68	15.25
	P. indica	0.20	25.0	0.68	33.33	0.77	42.59	0.94	59.32
	B. japonicum + *P. indica*	0.21	31.25	0.71	39.22	0.80	48.15	0.99	67.79
Un-sterilized	Uninoculated control	0.07	–	0.13	–	0.30	–	0.54	–
	B. japonicum	0.09	28.57	0.18	38.46	0.44	46.67	0.64	18.52
	P. indica	0.10	42.86	0.25	92.31	0.49	63.33	0.89	64.81
	B. japonicum + *P. indica*	0.11	57.14	0.29	123.08	0.50	66.67	0.93	72.22

Table XVI. Percent increment in shoot length of soybean plant grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatment	21 days	% inc.	35 days	% inc.	50 days	% inc.	65 days	% inc.
Sterilized	Uninoculated control	14.70	–	20.60	–	31.50	–	32.10	–
	B. japonicum	15.96	8.57	23.20	12.62	38.40	21.90	40.60	26.48
	P. indica	18.30	24.49	27.10	31.55	42.84	36.0	43.80	36.45
	B. japonicum + *P. indica*	20.50	39.46	29.24	41.94	45.20	43.49	46.66	45.36
Un-	Uninoculated control	6.68	–	12.12	–	25.70	–	25.78	–
	B. japonicum	8.40	25.75	13.44	10.89	28.0	8.95	35.44	37.47

sterilized	***P. indica***	8.64	29.34	19.26	58.91	38.10	48.25	42.66	65.48
	B. japonicum* + *P. indica	8.66	29.64	19.42	60.23	41.40	61.09	43.46	68.58

Table XVII: Percent increment in shoot fresh weight of soybean plant grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatment	21 days	% inc.	35 days	% inc.	50 days	% inc.	65 days	% inc.
Sterilized	**Uninoculated control**	1.61	–	3.88	–	4.26	–	7.63	–
	B. japonicum	2.08	29.19	4.87	25.52	6.63	55.63	10.38	36.04
	P. indica	2.17	34.78	5.50	41.75	7.02	64.79	12.54	64.35
	B. japonicum* + *P. indica	2.48	54.04	5.69	46.65	7.08	66.19	13.40	75.62
Un-sterilized	**Uninoculated control**	0.56	–	1.05	–	2.67	–	7.29	–
	B. japonicum	0.87	55.36	1.23	17.14	4.15	55.43	9.38	28.67
	P. indica	1.0	78.57	1.90	80.95	5.17	93.63	11.80	61.87
	B. japonicum* + *P. indica	1.03	83.93	2.31	120.0	5.18	94.01	12.40	70.10

Table XVIII. Percent increment in shoot dry weight of soybean plant grown in sterilized and unsterilized potted soils at different days after plantation

Soil	Treatment	21 days	% inc.	35 days	% inc.	50 days	% inc.	65 days	% inc.
Sterilized	**Uninoculated control**	0.34	–	1.19	–	1.70	–	2.13	–
	B. japonicum	0.38	11.76	1.30	9.24	1.88	10.59	3.52	65.26

	P. indica	0.47	38.24	1.45	21.85	2.09	22.94	4.13	93.90
	B. japonicum* + *P. indica	0.49	44.12	1.48	24.37	2.29	34.71	4.23	98.59
Un-sterilized	**Uninoculated control**	0.11	–	0.37	–	0.72	–	1.76	–
	B. japonicum	0.16	45.45	0.43	16.22	1.10	52.78	2.90	64.77
	P. indica	0.20	81.82	0.65	75.68	1.26	74.99	3.61	105.11
	B. japonicum* + *P. indica	0.22	100.0	0.78	110.81	1.67	131.94	3.93	123.30

Table XIX. Percent increment in root NPK contents of soybean plant grown in sterilized and unsterilized potted soils on 50th day after plantation

Soil	Treatment	%N	% inc.	%P	% inc.	%K	% inc.
Sterilized	**Uninoculated control**	0.56	–	0.135	–	0.558	–
	B. japonicum	0.74	32.14	0.262	94.07	0.635	13.8
	P. indica	0.72	28.57	0.356	163.7	0.836	49.82
	B. japonicum* + *P. indica	0.81	44.64	0.453	235.56	1.150	106.09
Un-sterilized	**Uninoculated control**	1.40	–	0.067	–	0.458	–
	B. japonicum	1.58	12.86	0.074	10.45	0.593	29.48
	P. indica	1.90	35.71	0.096	43.28	0.723	57.86
	B. japonicum* + *P. indica	1.91	36.43	0.118	76.12	0.889	94.10

Table XX: Percent increment in shoot NPK contents of soybean plant grown in sterilized and unsterilized potted soils on 50th day after plantation

Soil	Treatment	%N	% inc.	%P	% inc.	%K	% inc.
Sterilized	**Uninoculated control**	1.50	–	0.220	–	0.660	–
	B. japonicum	1.90	26.67	0.398	80.91	0.820	24.24
	P. indica	1.80	20.0	0.460	109.09	1.076	63.03
	B. japonicum* + *P. indica	2.45	63.33	0.493	124.09	1.510	128.79
Un-sterilized	**Uninoculated control**	2.15	–	0.103	–	0.622	–
	B. japonicum	2.46	14.42	0.106	2.91	0.806	29.58
	P. indica	2.51	16.74	0.117	13.59	0.840	35.05
	B. japonicum* + *P. indica	2.85	32.56	0.122	18.45	1.042	67.52

Table XXI: Percent increment in soil NPK contents of soybean plant grown in sterilized and unsterilized potted soils on 50th day after plantation

Soil	Treatment	%N	% inc.	P	% inc.	K	% inc.
Sterilized	**Uninoculated control**	0.04	–	10.80	–	76.50	–
	B. japonicum	0.07	75.0	14.80	37.04	92.40	20.78
	P. indica	0.06	50.0	16.80	55.56	105.90	38.43
	B. japonicum* + *P. indica	0.08	100.0	23.30	115.74	113.20	47.97
Unsterilized	**Uninoculated control**	0.05	–	18.10	–	91.20	–
	B. japonicum	0.09	80.0	33.0	82.32	98.6	8.11

	P. indica	0.11	120.0	42.90	137.02	113.2	24.12
	B. japonicum + ***P. indica***	0.12	140.0	57.80	219.34	120.60	32.24

Table XXII. Percent increment in soil NPK contents of soybean plant grown in sterilized and unsterilized potted soils after harvesting after maturity

Soil	Treatment	%N	% inc.	P	% inc.	K	% inc.
Sterilized	**Uninoculated control**	0.06	–	20.0	–	82.40	–
	B. japonicum	0.09	50.0	29.8	49.0	98.60	19.66
	P. indica	0.08	33.33	43.9	119.5	114.60	39.08
	B. japonicum + ***P. indica***	0.11	83.33	50.2	151.0	115.20	39.81
Unsterilized	**Uninoculated control**	0.08	–	23.10	–	98.60	–
	B. japonicum	0.10	25.0	35.80	54.98	105.90	7.40
	P. indica	0.13	62.50	48.90	111.69	122.0	23.73
	B. japonicum + ***P. indica***	0.15	87.50	62.80	171.86	126.60	28.40

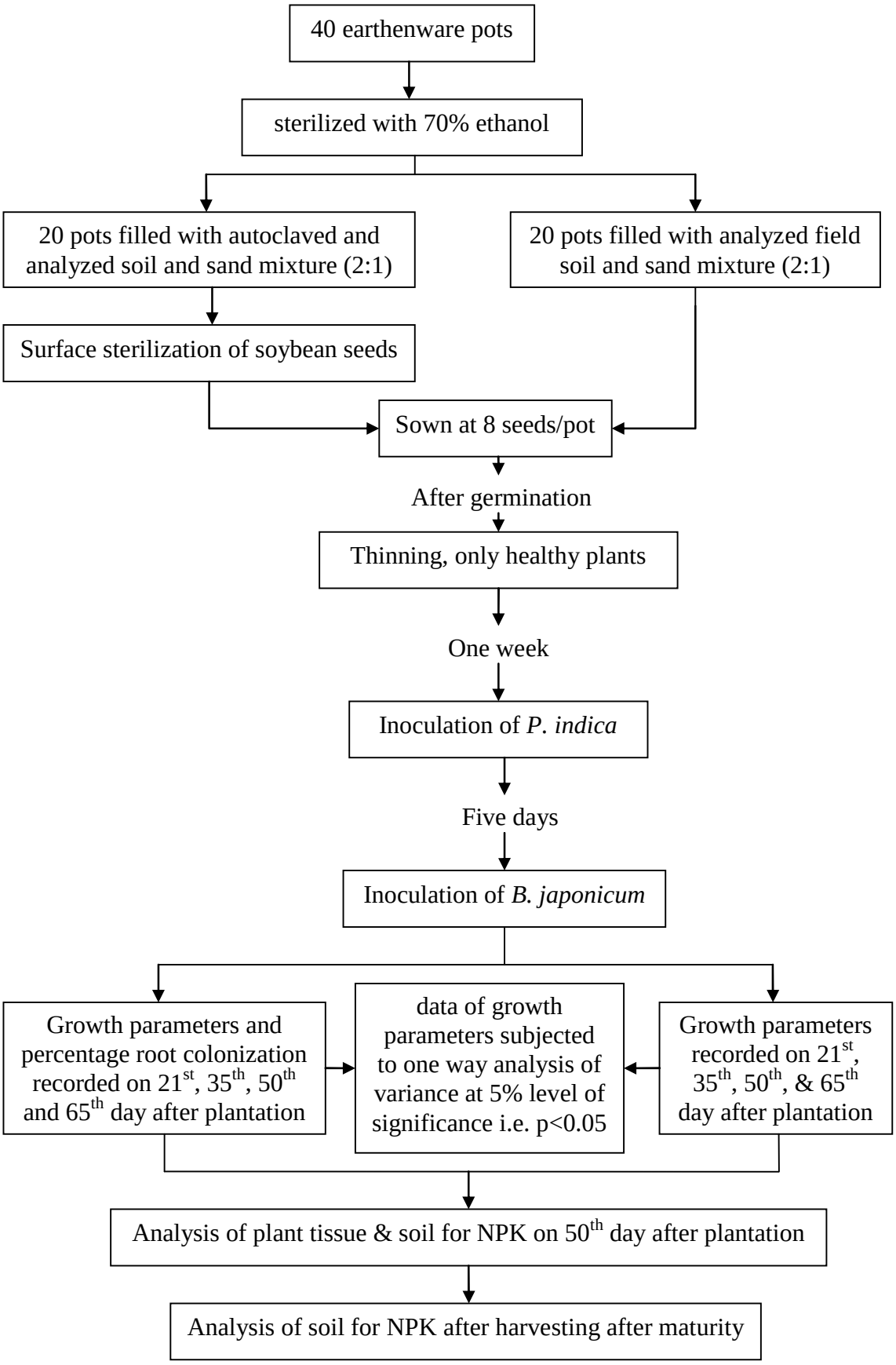

40 earthenware pots
sterilized with 70% ethanol
20 pots filled with autoclaved and analyzed soil and sand mixture (2:1)
20 pots filled with analyzed field soil and sand mixture (2:1)
Surface sterilization of soybean seeds
Sown at 8 seeds/pot
After germination
Thinning, only healthy plants
One week
Inoculation of P. indica
Five days
Inoculation of B. japonicum
Growth parameters and percentage root colonization recorded on 21st, 35th, 50th and 65th day after plantation
data of growth parameters subjected to one way analysis of variance at 5% level of significance i.e. p<0.05
Growth parameters recorded on 21st, 35th, 50th, & 65th day after plantation
Analysis of plant tissue & soil for NPK on 50th day after plantation
Analysis of soil for NPK after harvesting after maturity

Figure 2. Effect of microbial inoculants on nodule number of soybean grown in sterilized soil

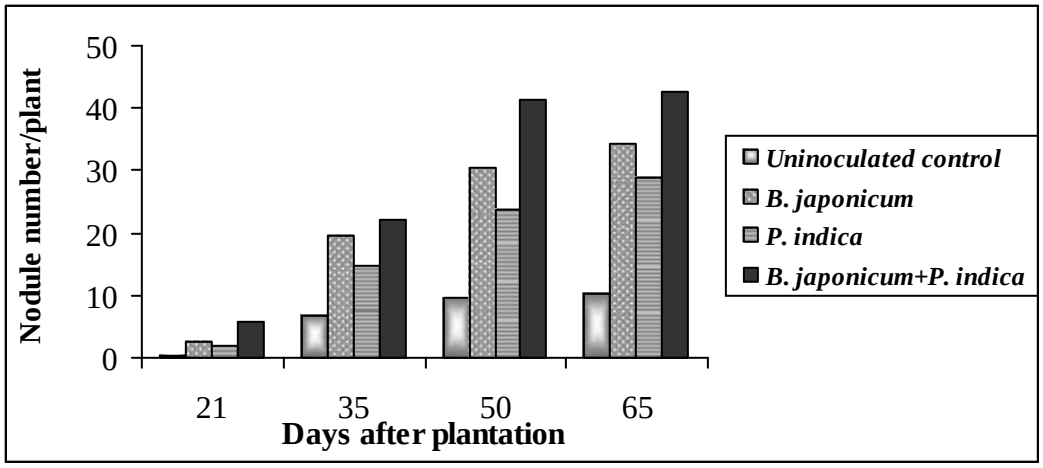


Figure 3. Effect of microbial inoculants on nodule number of soybean grown in unsterilized soil

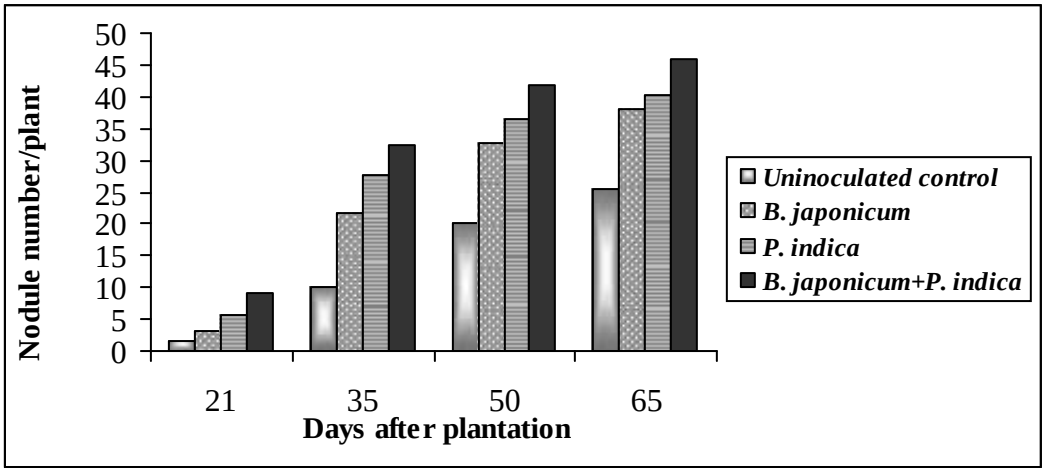


Figure 4. Effect of microbial inoculants on nodule fresh weight of soybean grown in sterilized soil

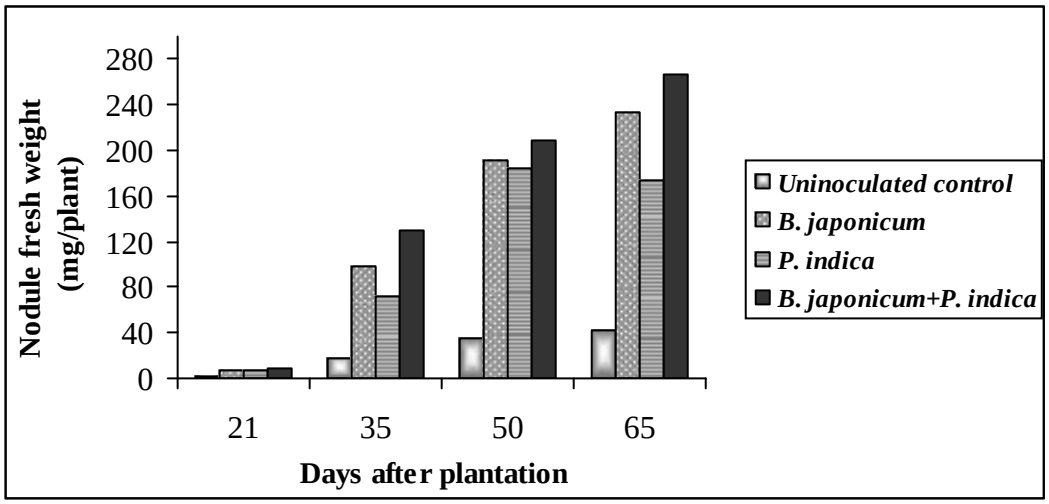


Figure 5. Effect of microbial inoculants on nodule fresh weight of soybean grown in unsterilized soil

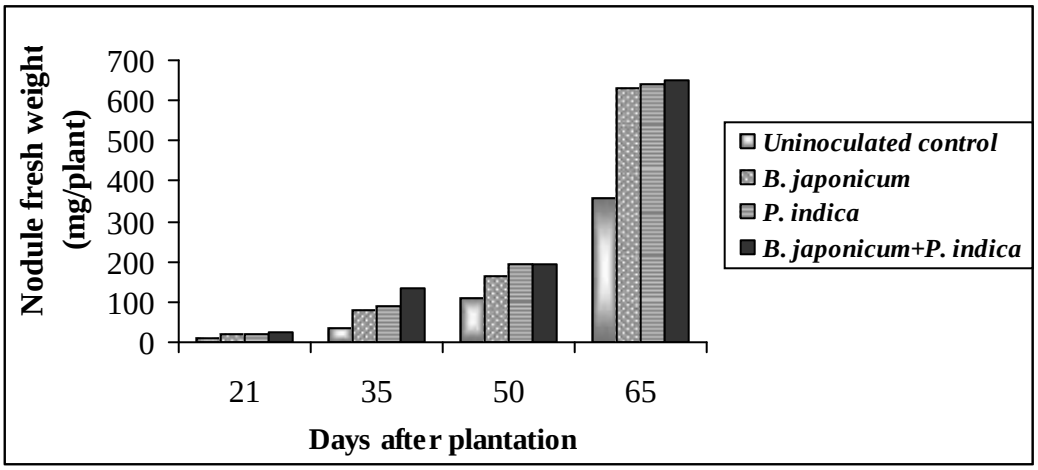


Figure 6. Effect of microbial inoculants on nodule dry weight of soybean grown in sterilized soil

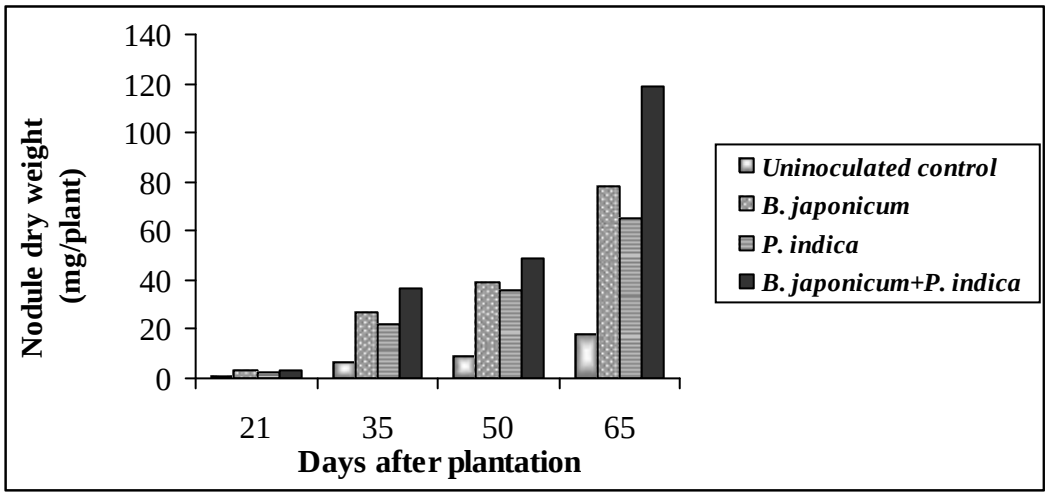


Figure 7. Effect of microbial inoculants on nodule dry weight of soybean grown in unsterilized soil

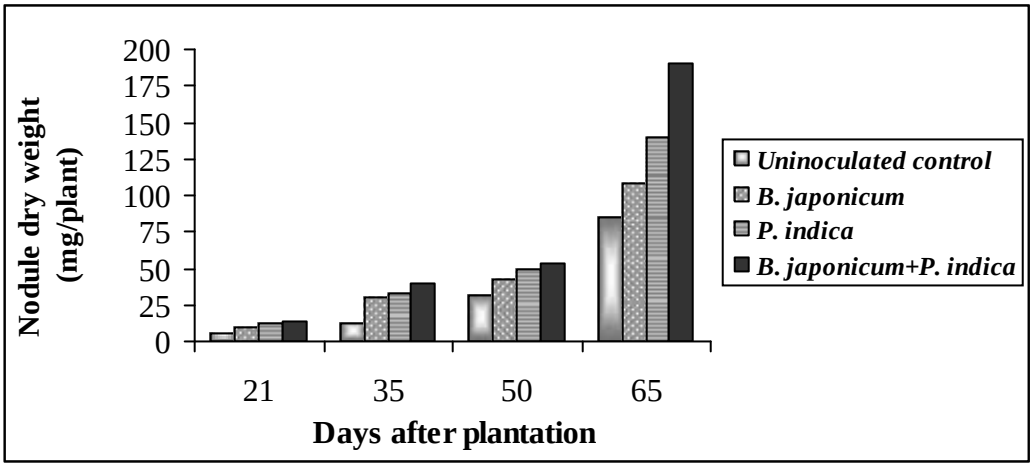


Figure 8. Effect of microbial inoculants on root length of soybean grown in sterilized soil

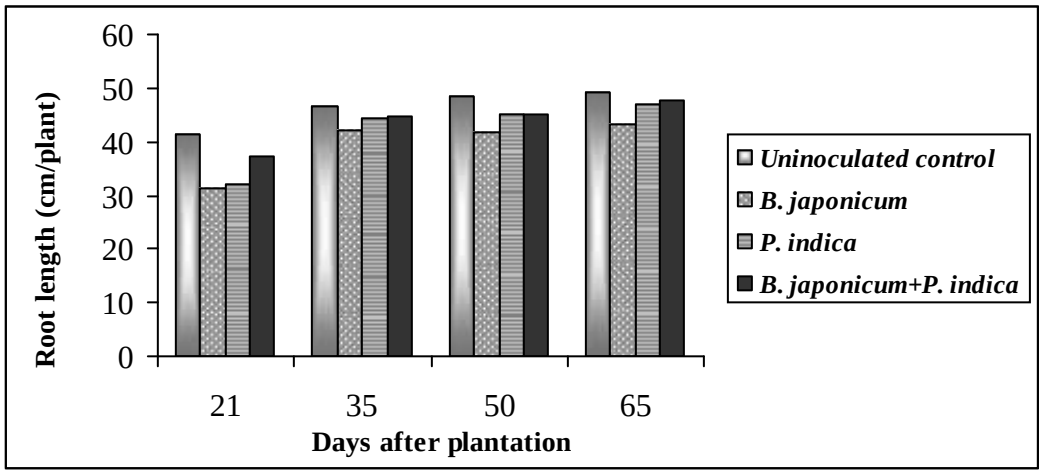


Figure 9. Effect of microbial inoculants on root length of soybean grown in unsterilized soil

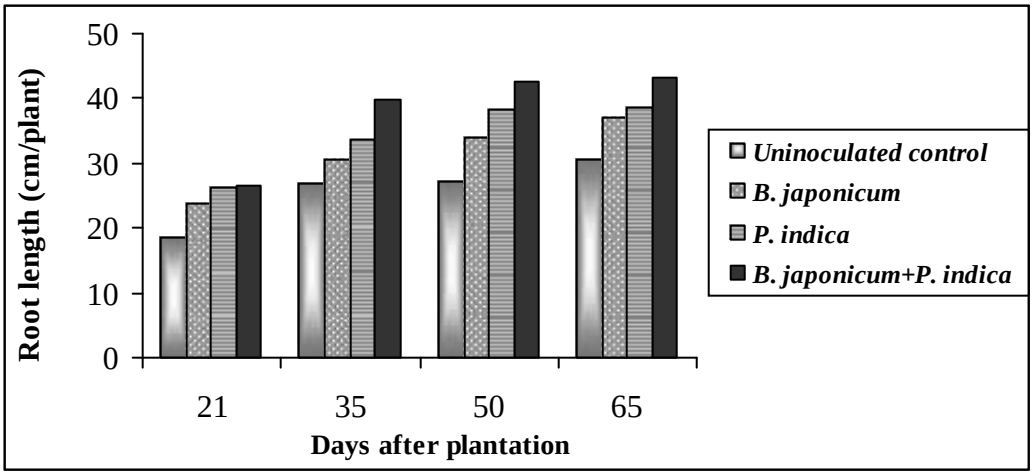


Figure 10. Effect of microbial inoculants on root fresh weight of soybean grown in sterilized soil

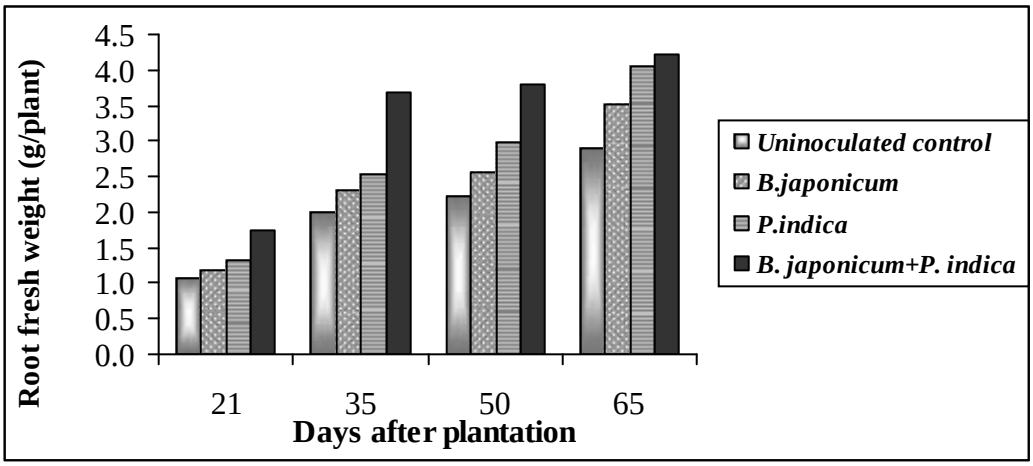


Figure 11. Effect of microbial inoculants on root fresh weight of soybean grown in unsterilized soil

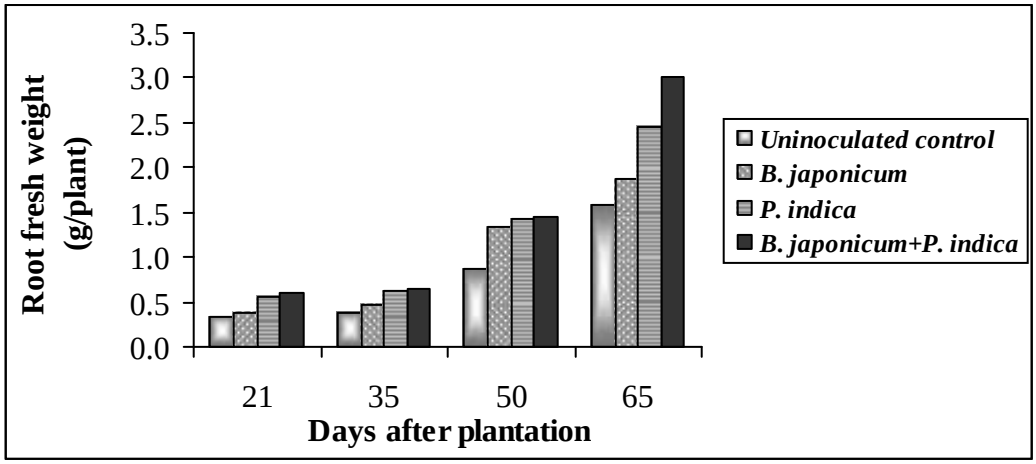


Figure 12. Effect of microbial inoculants on root dry weight of soybean grown in sterilized soil

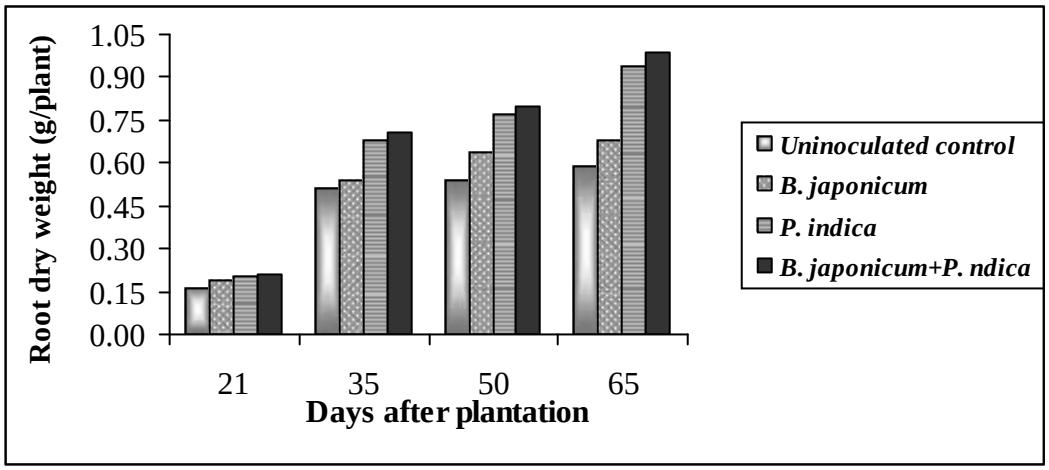


Figure 13. Effect of microbial inoculants on root dry weight of soybean grown in unsterilized soil

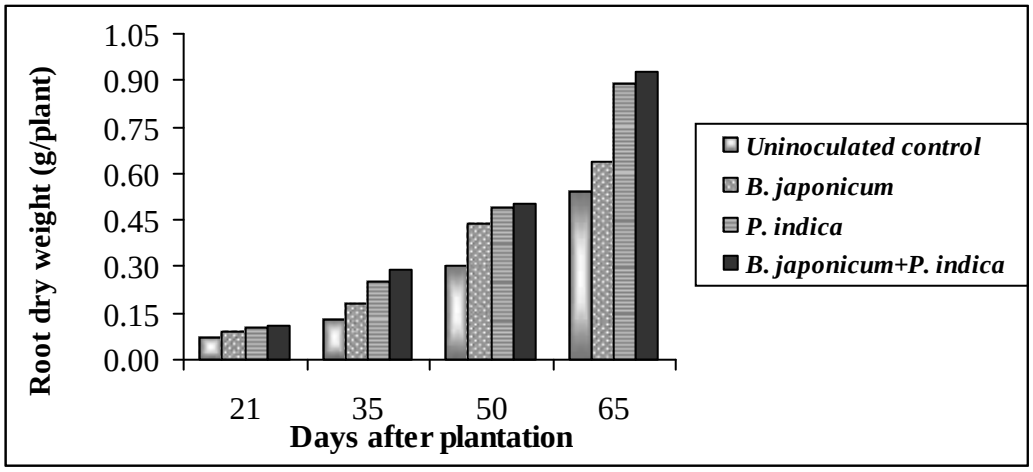


Figure 14. Effect of microbial inoculants on shoot length of soybean grown in sterilized soil

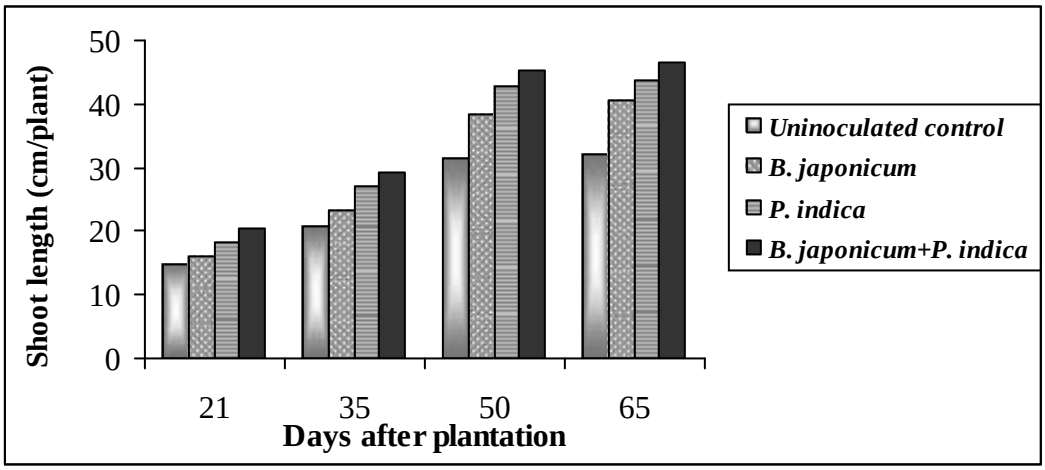


Figure 15. Effect of microbial inoculants on shoot length of soybean grown in unsterilized soil

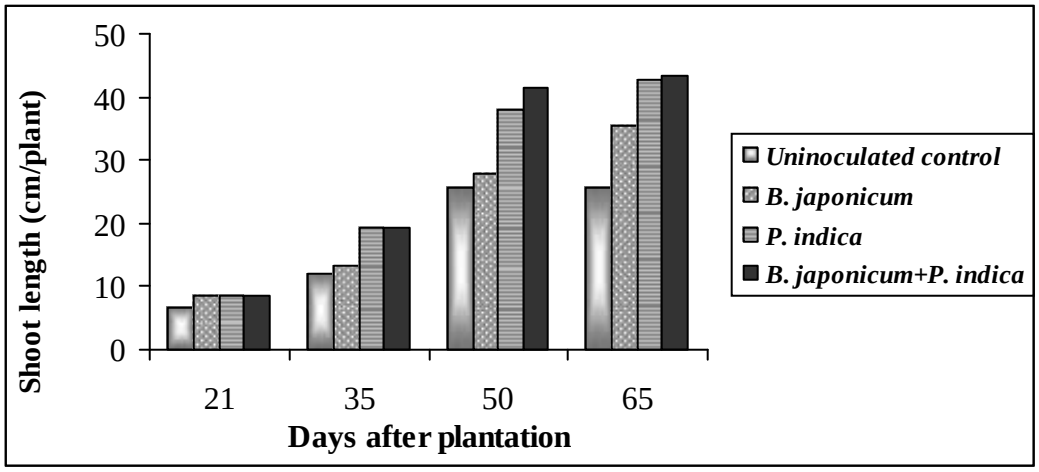


Figure 16. Effect of microbial inoculants on shoot fresh weight of soybean grown in sterilized soil

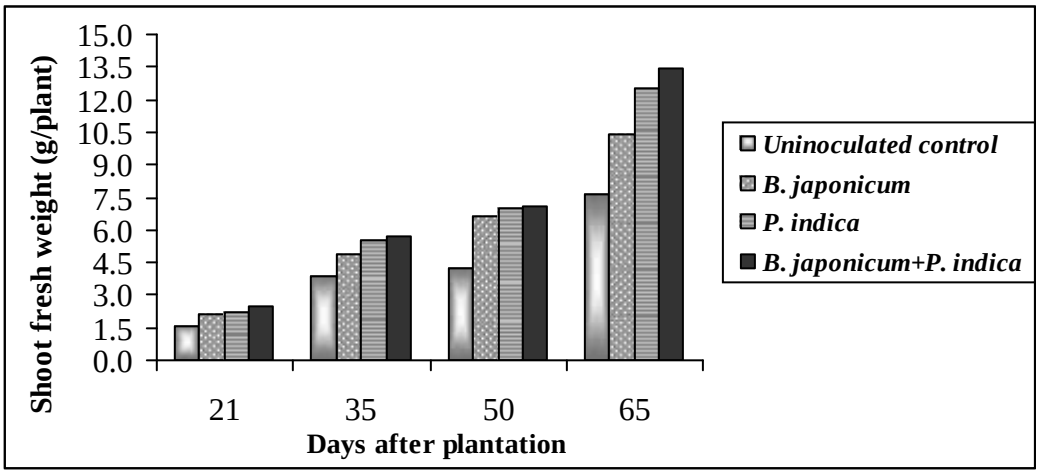


Figure 17. Effect of microbial inoculants on shoot fresh weight of soybean grown in unsterilized soil

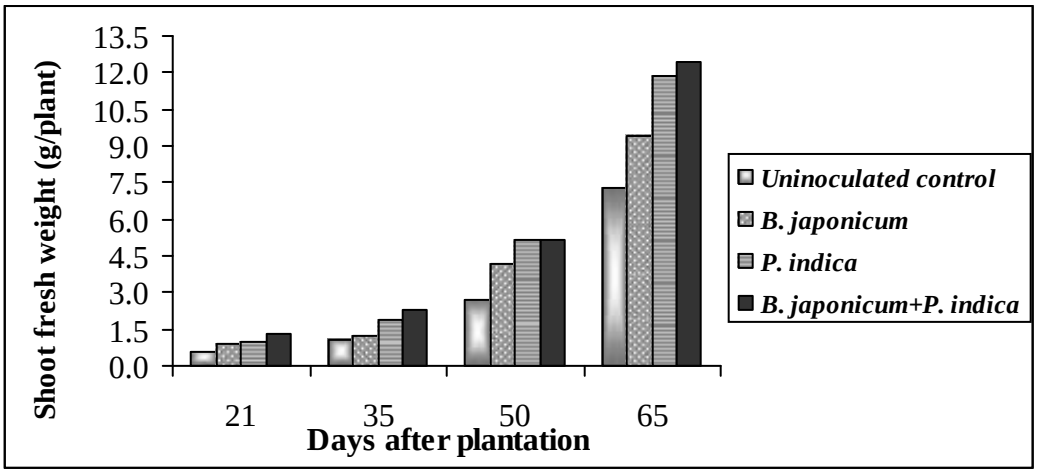


Figure 18. Effect of microbial inoculants on shoot dry weight of soybean grown in sterilized soil

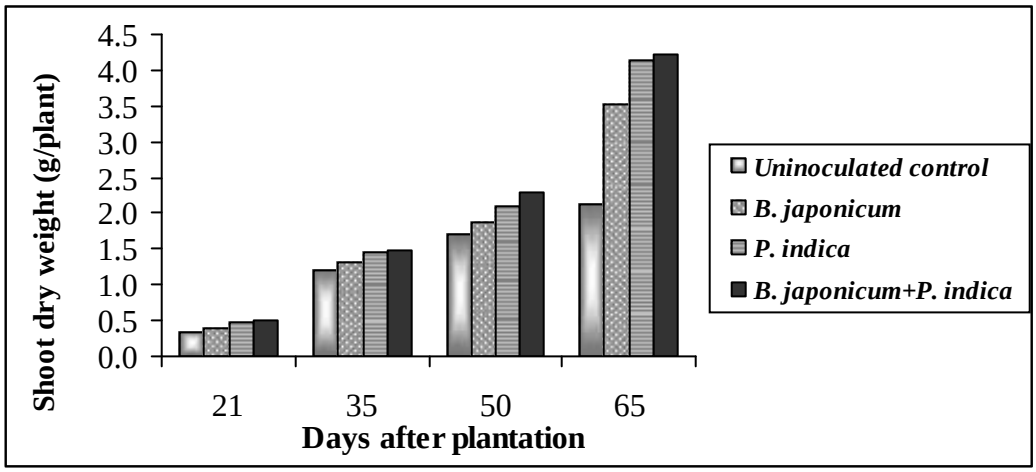


Figure 19. Effect of microbial inoculants on shoot dry weight of soybean grown in unsterilized soil

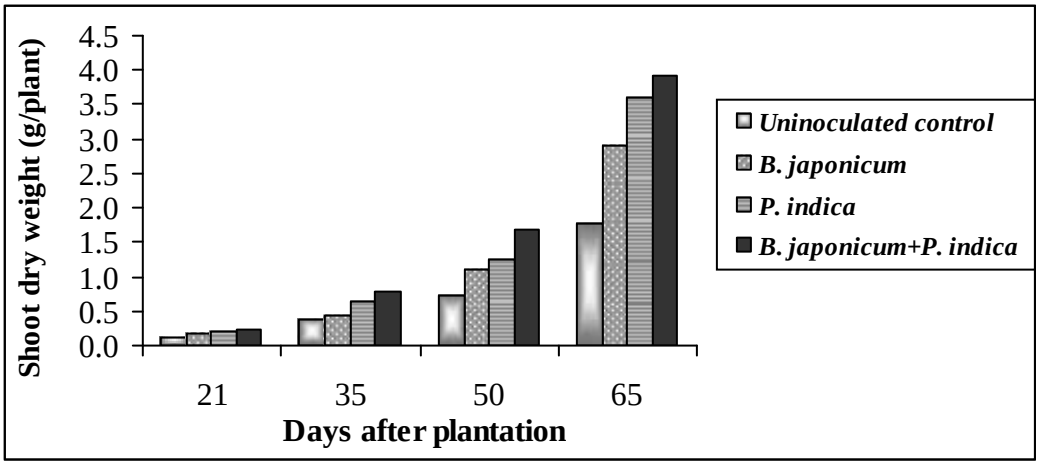


Figure 20. Effect of microbial inoculants on root and shoot nitrogen content of soybean grown in sterilized soil on 50 days

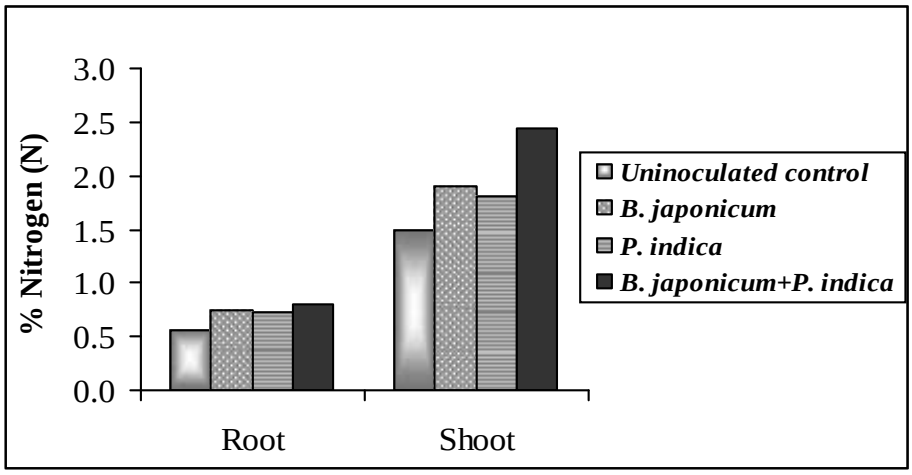


Figure 21. Effect of microbial inoculants on root and shoot phosphorus content of soybean grown in sterilized soil on 50 days

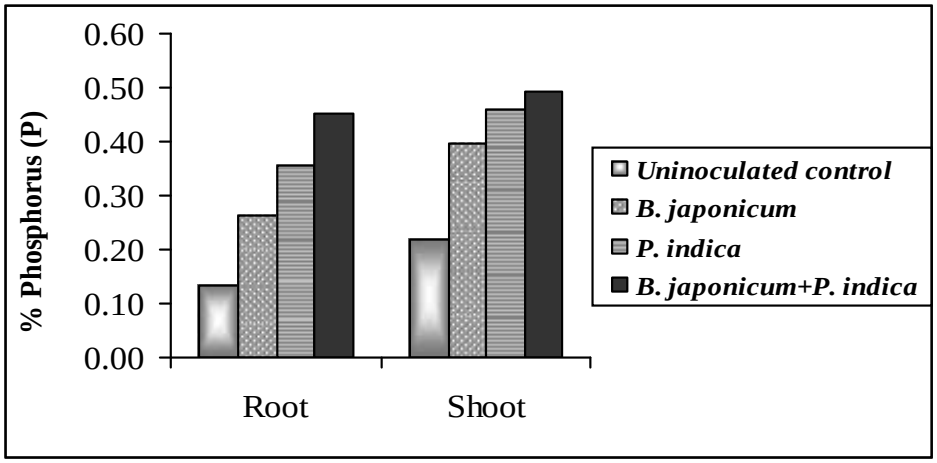


Figure 22. Effect of microbial inoculants on root and shoot potassium content of soybean grown in sterilized soil on 50 days

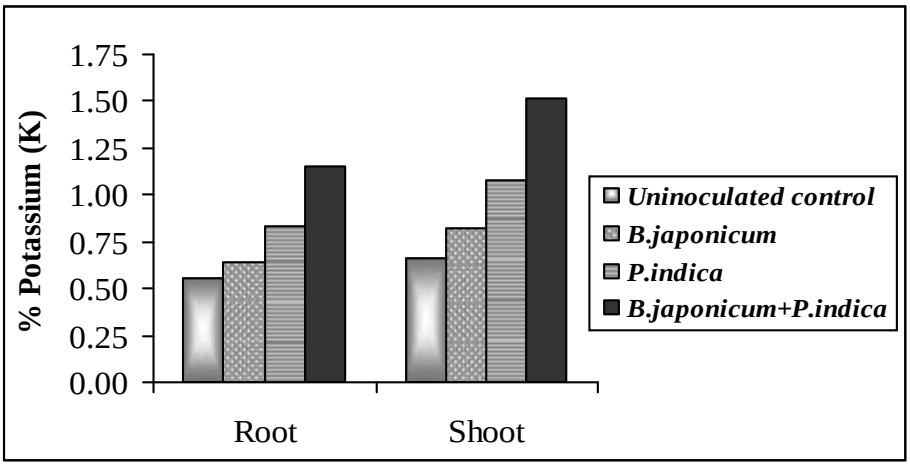


Figure 23. Effect of microbial inoculants on root and shoot nitrogen content of soybean grown in unsterilized soil on 50 days

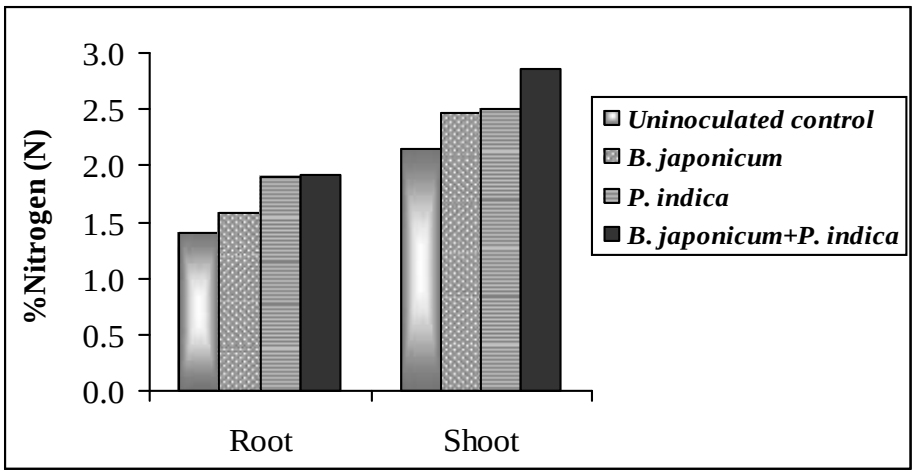


Figure 24. Effect of microbial inoculants on root and shoot phosphorus content of soybean grown in unsterilized soil on 50 days

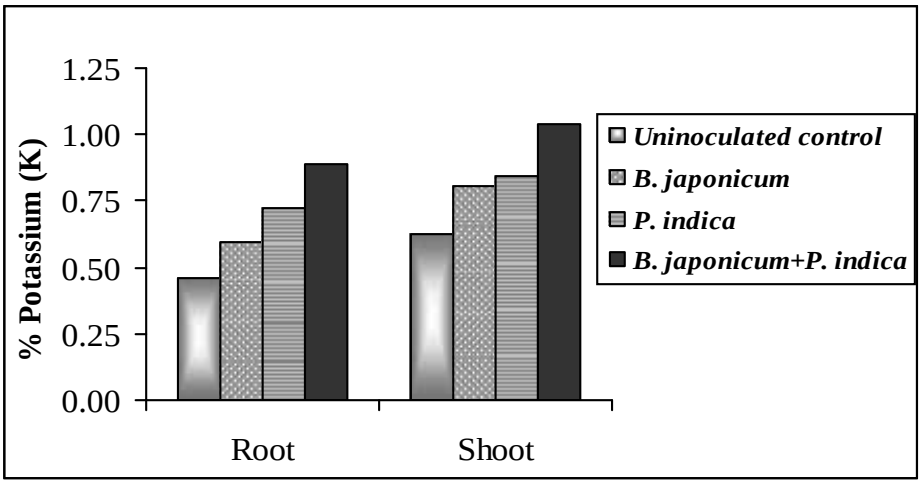


Figure 25. Effect of microbial inoculants on root and shoot potassium content of soybean grown in unsterilized soil on 50 days

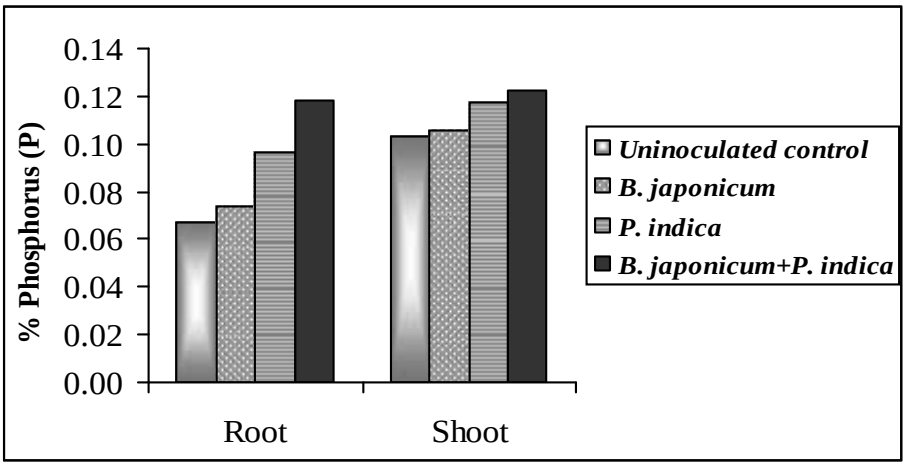


Figure 26. Effect of microbial inoculants on soil nitrogen content of soybean grown in sterilized soil

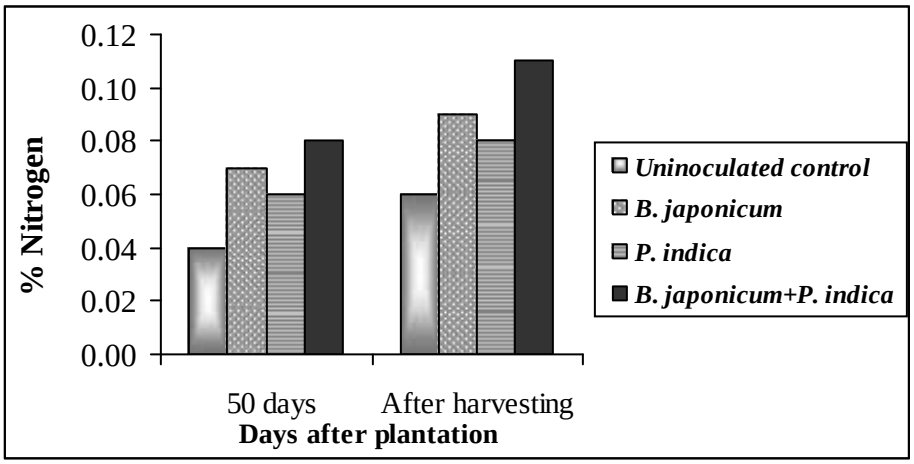


Figure 27. Effect of microbial inoculants on soil phosphorus content of soybean grown in sterilized soil

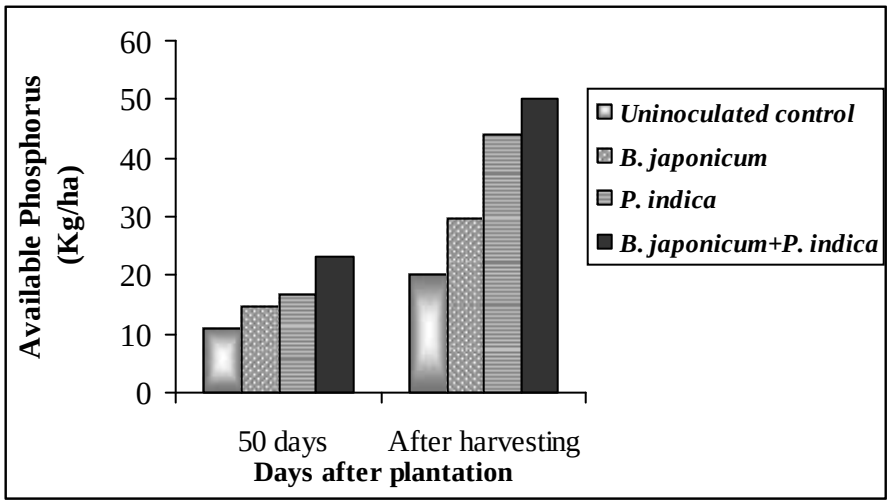


Figure 28. Effect of microbial inoculants on soil potassium content of soybean grown in sterilized soil

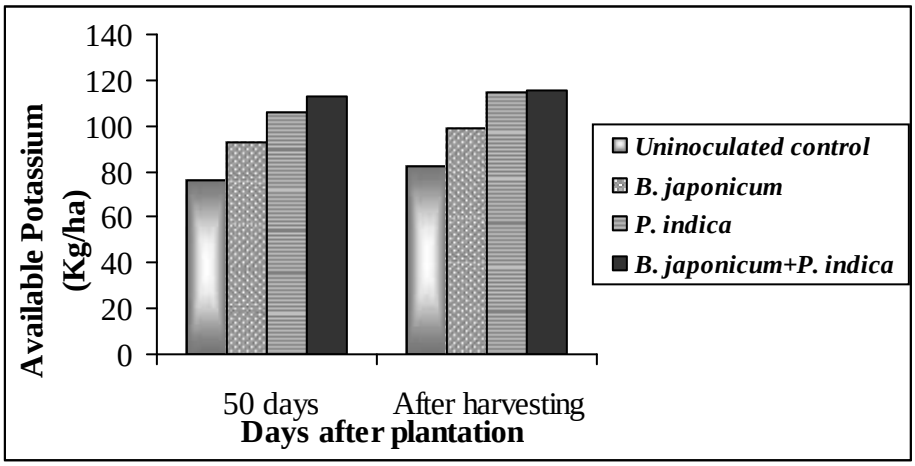


Figure 29. Effect of microbial inoculants on soil nitrogen content of soybean grown in unsterilized soil

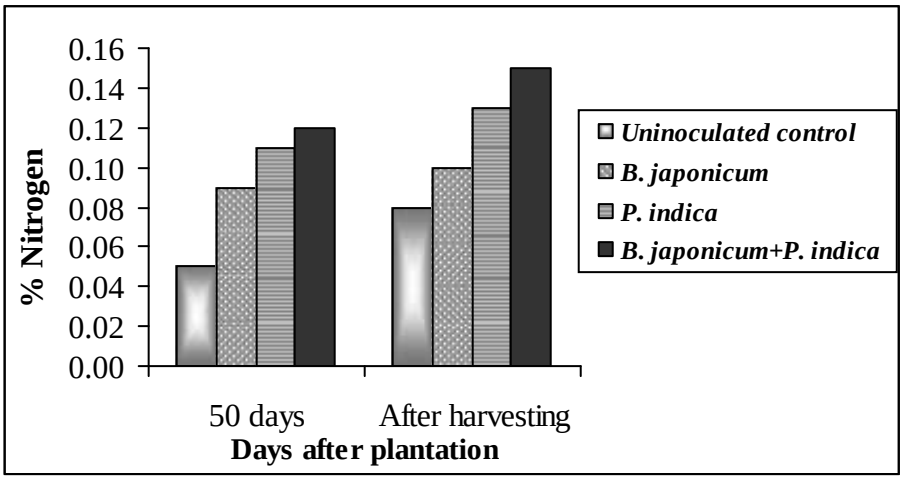


Figure 30. Effect of microbial inoculants on soil phosphorus content of soybean grown in unsterilized soil

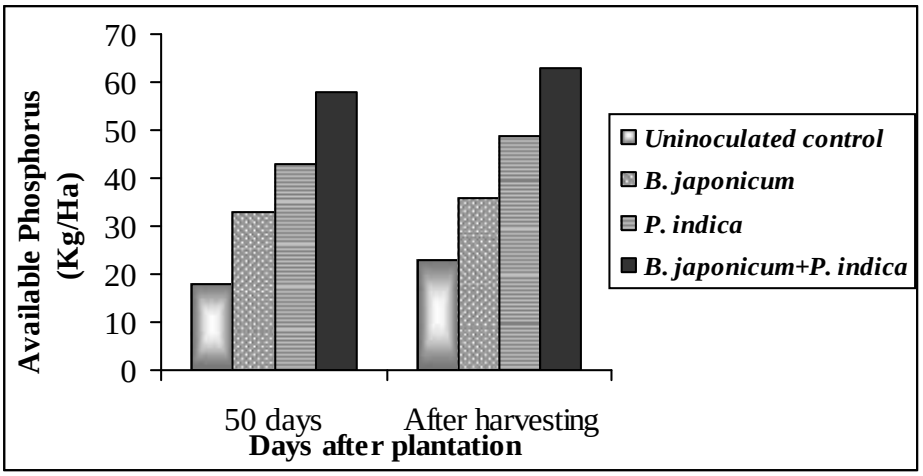


Figure 31. Effect of microbial inoculants on soil potassium content of soybean grown in unsterilized soil

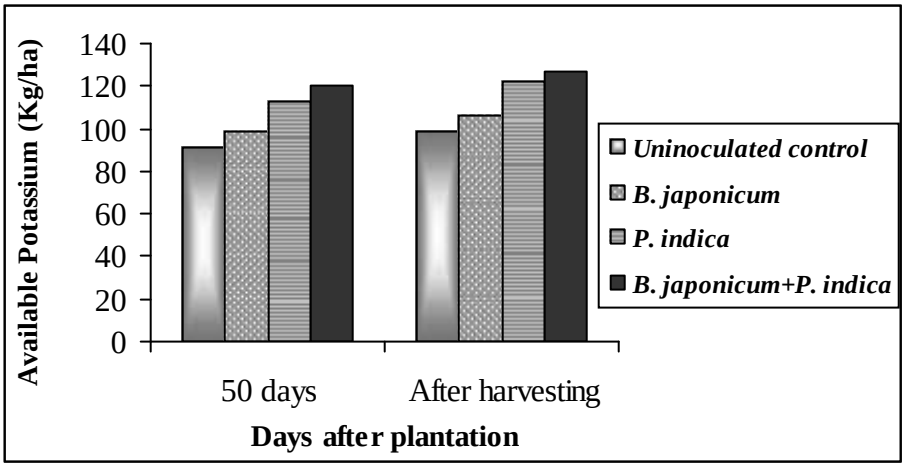
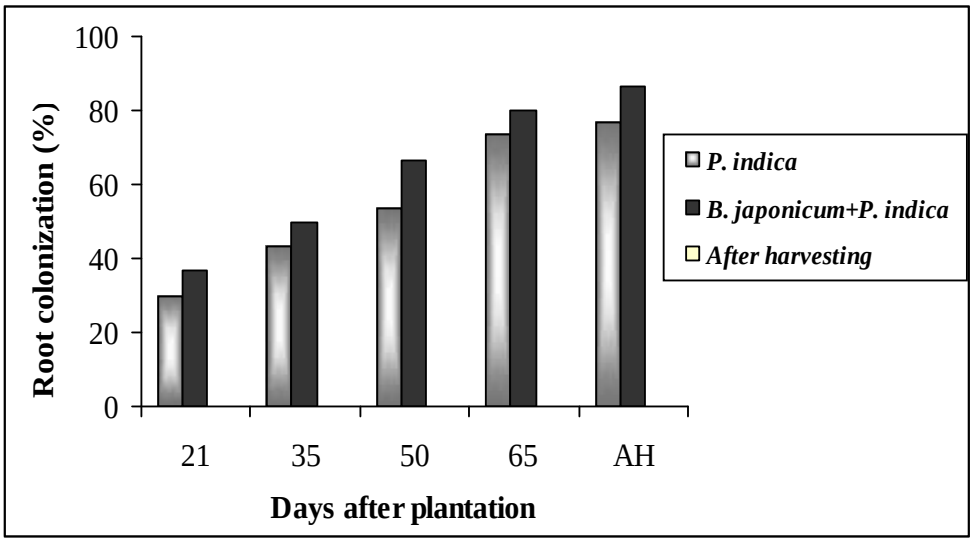
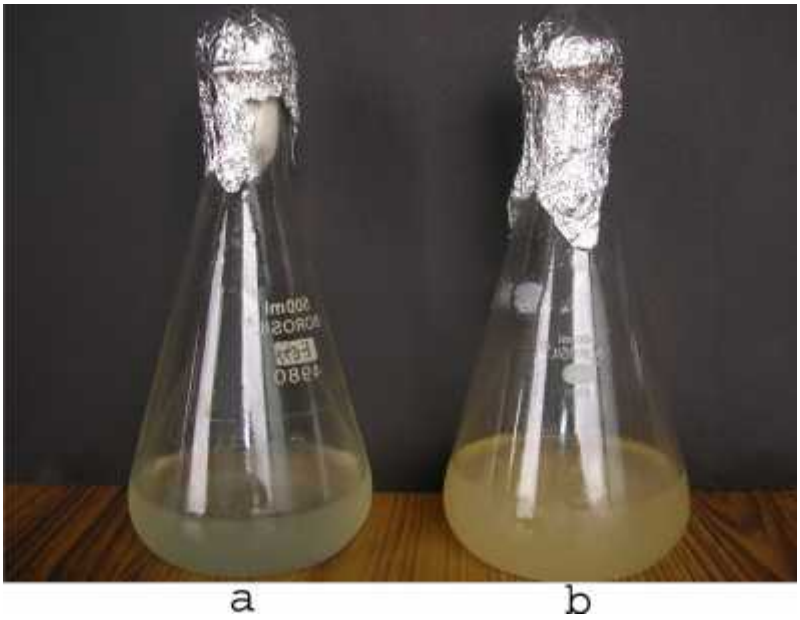


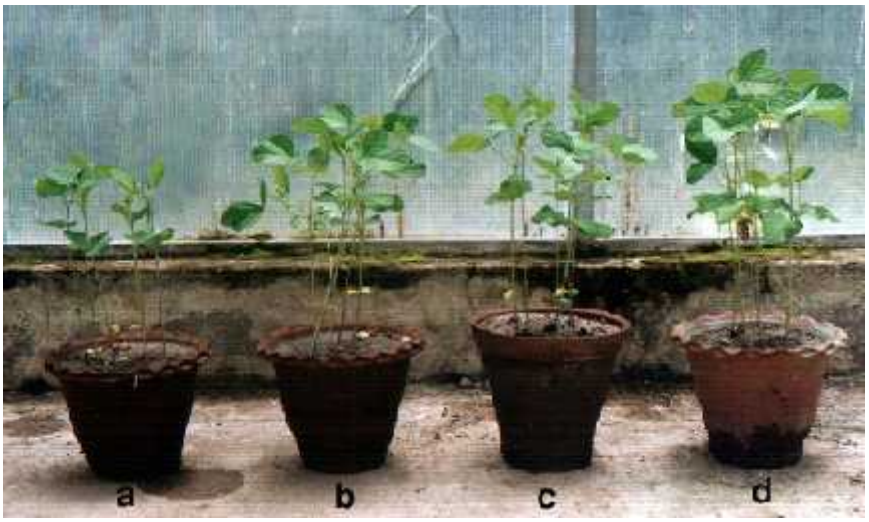
Figure 32. Percent root colonization by *P. indica* on soybean grown in sterilized soil at different days after plantation


100
80
60
40
20
0
Root colonization (%)
21
35
50
65
AH
Days after plantation
P. indica
B. japonicum+P. indica
After harvesting

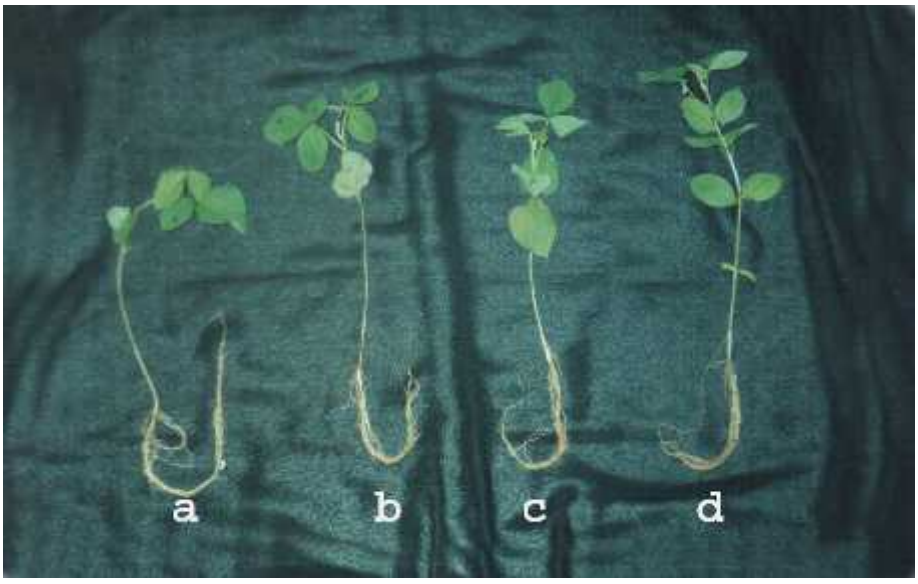
Photograph 1. 7 days old culture of *P. indica* on Potato Dextrose Agar



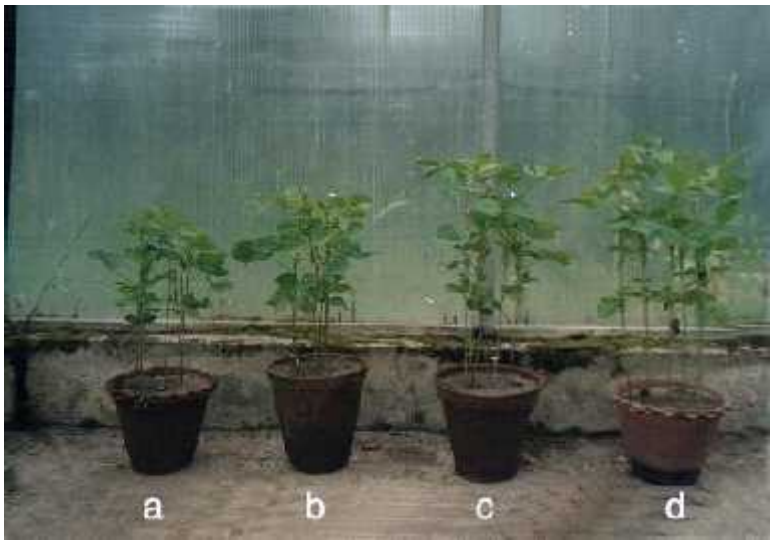
Photograph 2. 8 days old culture of *B. japonicum* on Yeast extract Mannitol Broth .a, control; b, *B. japonicum*



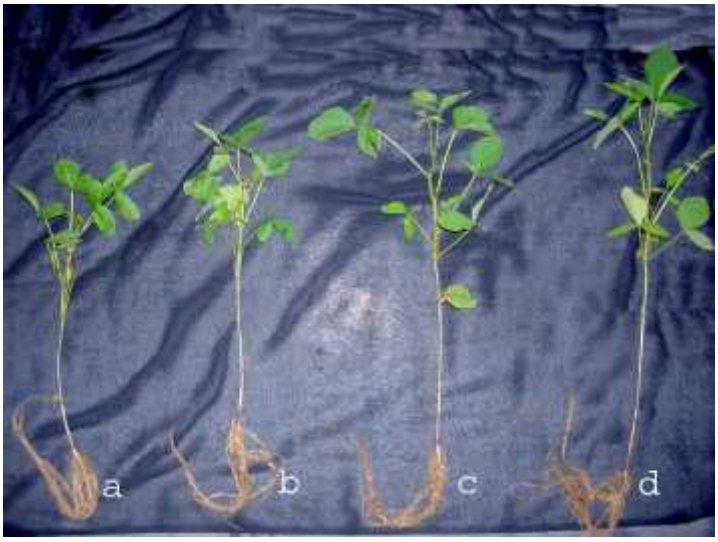
Photograph 3. Effect of microbial inoculants on soybean grown in sterilized soil on 35th day after plantation. Plants inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*



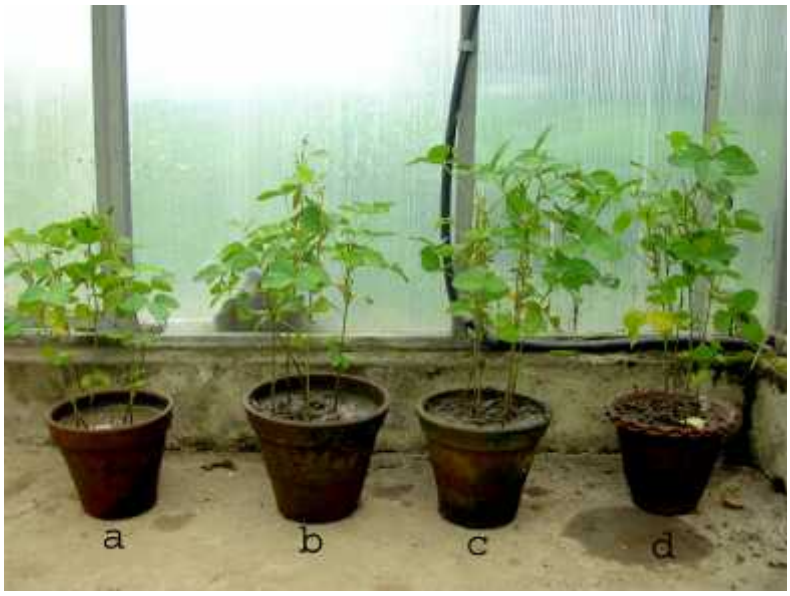
Photograph 4. Effect of microbial inoculants on soybean grown in sterilized soil on 35th day after plantation. Plant inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*



Photograph 5. Effect of microbial inoculants on soybean grown in sterilized soil on 50th day after plantation. Plants inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*

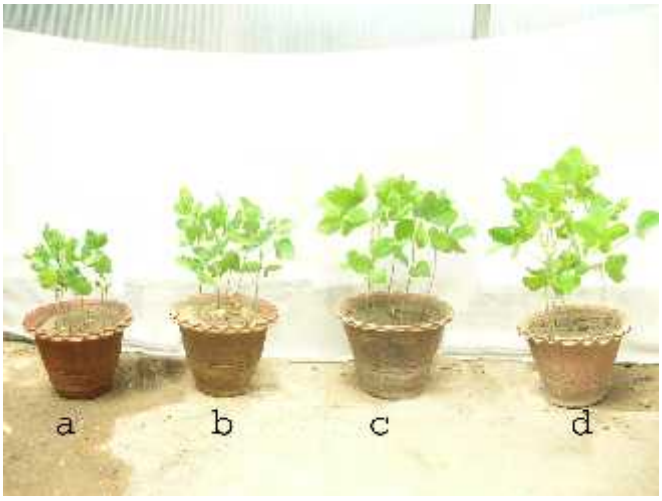


Photograph 6. Effect of microbial inoculants on soybean grown in sterilized soil on 50th day after plantation. Plant inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*

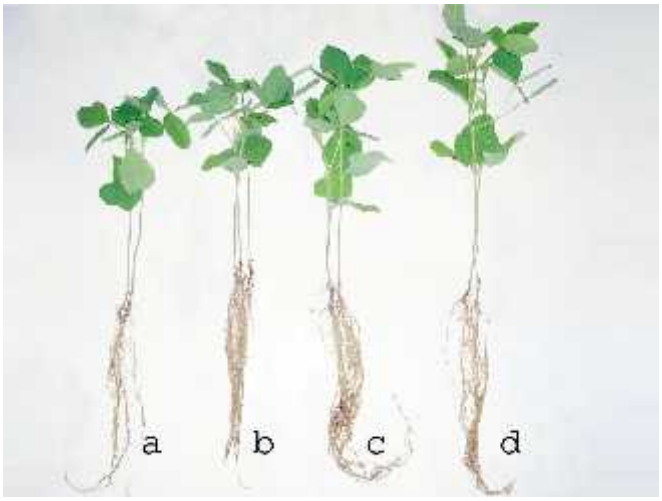


Photograph 7. Effect of microbial inoculants on soybean grown in sterilized soil on 65th day after plantation. Plants inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*

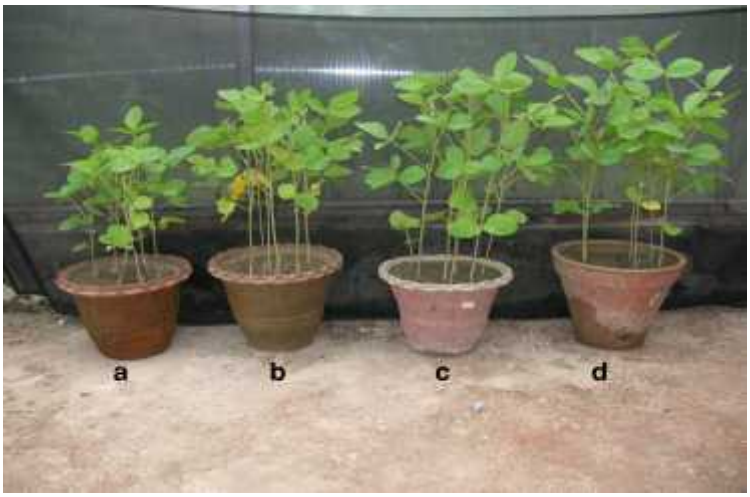
Photograph 8. Effect of microbial inoculants on soybean grown in sterilized soil on 65th day after plantation. Plant inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*



Photograph 9. Effect of microbial inoculants on soybean grown in unsterilized soil on 35th day after plantation. Plants inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*

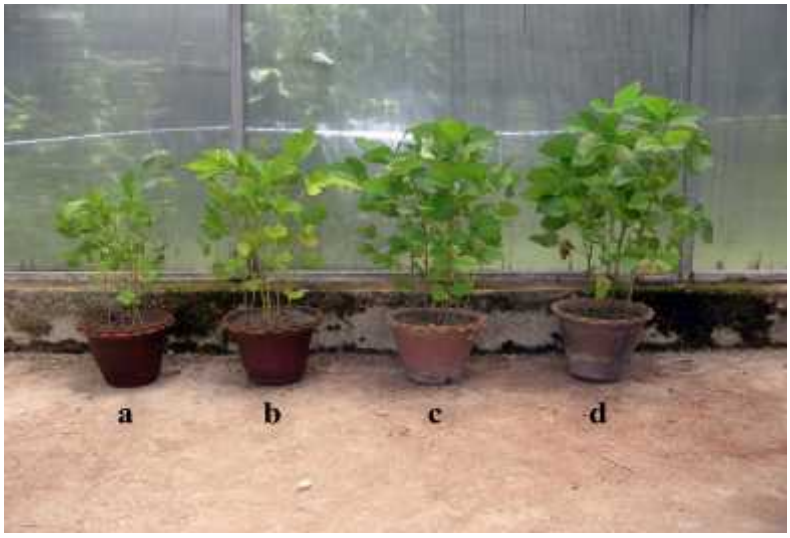


Photograph 10. Effect of microbial inoculants on soybean grown in unsterilized soil on 35th day after plantation. Plant inoculated with a, control; b, *B. japonicum;* c, *P. indica*; d, *B. japonicum+P. indica*



Photograph 11. Effect of microbial inoculants on soybean grown in unsterilized soil on 50th day after plantation. Plants inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*

Photograph 12. Effect of microbial inoculants on soybean grown in unsterilized soil on 50th day after plantation. Plant inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*



Photograph 13. Effect of microbial inoculants on soybean grown in unsterilized soil on 65th day after plantation. Plants inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*

Photograph 14. Effect of microbial inoculants on soybean grown in unsterilized soil on 65th day after plantation. Plant inoculated with a, control; b, *B. japonicum*; c, *P. indica*; d, *B. japonicum+P. indica*

Photograph 15. Spores of *P. indica* on soybean roots under light microscope (100X)