CHAPTER 1

INTRODUCTION

1. Introduction

1.1. Nitrogen

Nitrogen is an essential element for all living organisms. It is required for synthesizing amino acids, proteins, nucleic acids and other nitrogen containing compounds necessary for life. It is a major component of amino acids, the building blocks of proteins. Nitrogen is also a component of energy transfer compounds, ATP (Adenosine triphosphate), nucleic acids such as DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid). It is also a component of chlorophyll, the compound by which plants carry out photosynthesis. Most of the air around us is composed of elemental nitrogen (N_2). However, most organisms cannot use this elemental nitrogen (Sprent & Sprent, 1990). Plants can only take up nitrogen in compound form such as ammonium (NH_4^+) and nitrate (NO_3^-) form. Plants get reactive nitrogen from the soil or water. Animals get their nitrogen from eating plants and other animals. Nitrogen has a strong triple bond between the two nitrogen atoms. It is difficult to separate the atmospheric nitrogen (N_2) and put it into usable compounds. The unavailability of fixed nitrogen is normally the limiting factor for plant growth and productivity (Vincent, 1970).

Nitrogen in the form of chemical fertilizer is a convenient way to supply nitrogen in the soil. However, its use is plagued by many problems. The cost of producing nitrogen fertilizer is high, that are not affordable to many small scale farmers. Urea, which is the most commonly used nitrogenous fertilizer, has now become a costly input for most of the farmers. In the last few decades there is a huge increase in the utilization of chemical fertilizers. This increased utilization of fertilizers results in drastic effects on environment such as water eutrophication, increased emissions of nitrogen oxides and soil acidification, which ultimately results

in hazardous effects to plants and animals (Olivares *et al*., 2013). When nitrogen fertilizers are applied to agricultural systems, the fertilizers are absorbed by plants or converted into various other forms such as nitrate or nitrite. Excess nitrate, which is not absorbed by the plants were carried away by rain water or irrigation reaching the ground water. Drinking water containing a high level of nitrate, may cause methemoglobinemia. The symptoms of which are gastrointestinal swelling, diarrhea, and protein digestion problems. Microbes that live in an infant's digestive tract can reduce nitrate to nitrite, causing conversion of hemoglobin into methemoglobin, which ultimately interferes with the oxygen-carrying ability of blood. Use of chemical fertilizers in agriculture is currently under debate due to environmental concern. Development and implementation of sustainable agriculture system, such as use of biofertilizers in agriculture is of major importance in alleviating environmental pollution (Lee & Song, 2007).

1.2. Nitrogen cycle

Nitrogen is present in the environment in a wide variety of chemical forms, including inorganic nitrogen gas, ammonium, nitrite, nitrate, nitrous oxide and nitric oxide. The processes of the nitrogen cycle transform nitrogen from one form to another. Many of those processes are carried out by microorganisms for harvesting energy. The nitrogen cycle involves five basic biological processes.

I. Nitrogen fixation: Nitrogen fixation is the conversion of atmospheric nitrogen (N_2) into reactive compounds such as ammonia (NH_4^+) and nitrate (NO_3^-).

II. Nitrification: Nitrification is the process by which ammonia is oxidized to nitrite ions (NO_2^-) and then to nitrate ions (NO_3^-).

III. Magnification: In this process, microbes break down nitrogenous wastes and organic matter found in animal waste and dead plants and convert it to inorganic ammonia (NH_4^+).

IV. Assimilation: Nitrates are the form of nitrogen assimilated by plants through the root hairs. Since heterotrophic organisms cannot absorb nitrogen as plants do, they depend on acquiring nitrogen-based compounds through the food.

V. Denitrification: Denitrification is the process by which nitrates are reduced to gaseous nitrogen (N2) and lost to the atmosphere. This process occurs by facultative anaerobes in anaerobic environments.

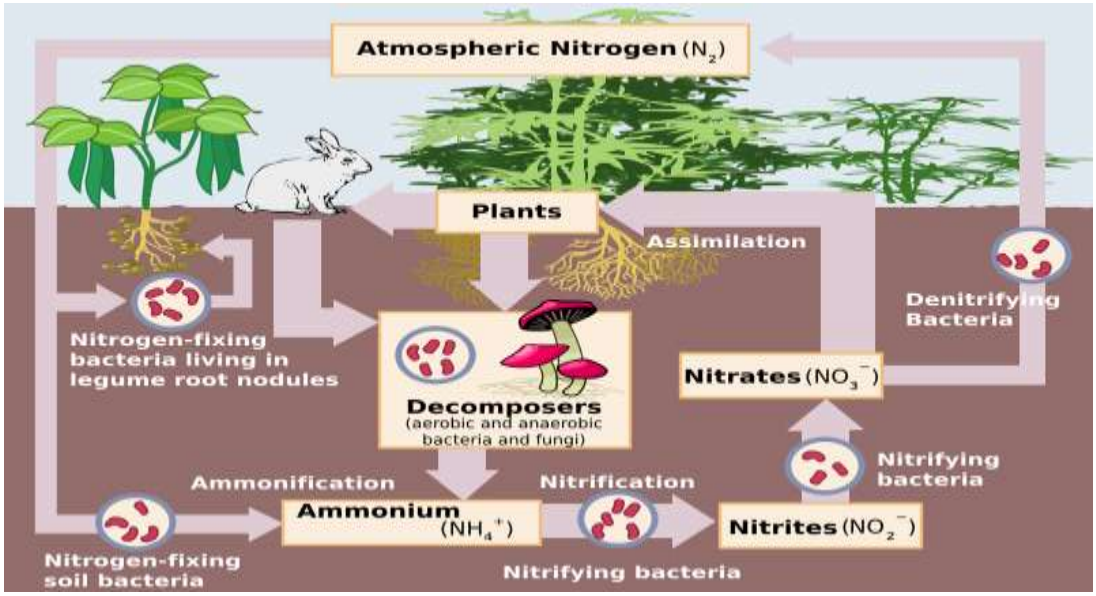

Figure 1: Nitrogen cycle
(Source:https://citysewn.files.wordpress.com/2010/02/nitrogencycle.png)

1.3. Nitrogen Fixation

Nitrogen fixation is a process in which nitrogen in the atmosphere is converted into usable form such as ammonium.The fixation process frees up the nitrogen atoms from their triply bonded diatomic form. Generally nitrogen fixation can take place in 3 different ways natural nitrogen fixation, industrial nitrogen fixation and biological nitrogen fixation (Peoples & Craswell, 1992). In natural nitrogen fixation some

natural events such as lightning, forest fires, *etc.* provides energy to fix a small amount of nitrogen into ammonia and nitrates. Lightning accounts for about 10% of the world's supply of fixed nitrogen (Sprent & Sprent, 1990). In industrial nitrogen fixation, nitrogen is fixed in the form of nitrogenous fertilizer in industries. Haber–Bosch is considered as the most common and efficient method of industrial nitrogen fixation. The Haber–Bosch process utilizes high pressures and high temperatures, for fixing nitrogen in the form of fertilizer. In this process natural gas is used as a hydrogen source and air as a nitrogen source. This process produces large amounts of nitrogen in the form of ammonia, which is mainly used for the purpose of agriculture (Galloway *et al*., 2008). The nitrogen fertilizers produced industrially are the main source of nitrogen inagricultural systems. However, it was found that the applied nitrogen fertilizer is not efficiently utilized by the plants and the un-utilized nitrogen fertilizer enters the ecosystem and causes environmental pollution.

1.4. Biological nitrogen fixation

Biological nitrogen fixation or BNF is the process that changes inert nitrogen to biologically useful ammonia with the help of microorganisms (Somasegaran & Hoben, 1994). The process of biological nitrogen fixation was carried out by an enzyme called nitrogenase. Microorganisms use these enzymes to break the triple bond of nitrogen and convert it to ammonium. BNF offers a better alternative over chemical fertilizers, besides supplying nitrogen to crop it maintains soil health and productivity. BNF contributes about 16% of total nitrogen supply in agricultural production (Ollivier *et al*., 2011). The overall process of BNF can be summarized in the following equation.

$$N_2 + 8\,H^+ + 8\,e^- \rightarrow 2\,NH_3 + H_2$$

The nitrogen fixing microorganisms obtain the energy by oxidizing organic molecules. Free living nitrogen fixing microbes obtain these organic molecules from other organisms. Photosynthetic microorganisms use sugars produced by photosynthesis for energy generation. Symbiotic nitrogen fixing microorganisms obtain these organic compounds from their host plant. BNF reduces the requirements for chemical fertilizers during the growth of plants and increases their productivity (Dilworth & Parker, 1969). Nitrogen fixed by BNF is the major sources of nitrogen for living organisms in ecosystems. The nitrogen fixing microbes involved in BNF belongs to 2 different categories; free living nitrogen fixers (fixes atmospheric nitrogen in free living condition) and symbiotic nitrogen fixers (fixes atmospheric nitrogen by forming symbiotic association with plants).

1.4.1. Nitrogen fixation by free living microbes

Free living nitrogen fixers live in the soil and fix significant levels of nitrogen without the direct interaction with other organisms (Bhattacharyya & Jha, 2012). These bacteria live close to the root such that the atmospheric nitrogen fixed by the bacteria is taken up by the plant. However, free living nitrogen fixing bacteria provide only a small amount of the fixed nitrogen that the host plant requires. Examples of this type of free living nitrogen fixing bacteria include species of *Azotobacter*, *Azospirillum*, *Bacillus*, *Paenibacillus* and *Klebsiella*. These free living nitrogen fixers find their own source of energy by oxidizing organic molecules released by other organisms or from decomposition. Because nitrogenase can be inhibited by oxygen, free-living organisms behave as anaerobes while fixing nitrogen.

1.4.2. Symbiotic nitrogen fixation

In symbiotic nitrogen fixation, microorganisms fix atmospheric nitrogen symbiotically by partnering with a host plant. The host plant provides carbon from

photosynthesis that are utilized by the nitrogen-fixing microorganism for the energy it needs for nitrogen fixation (Vincent, 1970). In exchange for these carbon sources, the microbe provides fixed nitrogen to the host plant for its growth. The most important nitrogen-fixing symbiotic associations are the relationships between legumes and rhizobia. Symbiotic nitrogen fixing rhizobia infect and establish symbiotic relationship with leguminous plants. The establishment of the symbiosis involves a complex interaction between host and symbiont resulting in the formation of the nodules wherein the rhizobia colonize as intracellular symbionts and fixes atmospheric nitrogen. In a previous study it was also reported that over 50% of the biological source of fixed nitrogen is obtained from the symbiosis of legume and rhizobia (Zahran, 1999). In rhizobia-legume symbiosis, legume provides shelter and food to rhizobia (dicarboxylic acids, malate and succinate as a carbon and energy source) and in return rhizobia supply ammonia to the plant for its growth.

1.5. Nodule endophytic bacteria

Nodule endophytic bacteria are those bacteria which are incapable of inducing nodules by themselves, but they coexist with nodulating rhizobia inside nodules without causing any harm to the host plant. Many previous reports have suggested the presence of endophytic bacteria in the root nodules of legume plants. (Hallmann *et al*., 1997; Pandey *et al*., 2005; Lei *et al*., 2008; Mhamdi *et al*., 2005). These endophytic bacteria can colonize different parts of the plant such as root, stem, nodule, etc. Till date it is not very clear how endophytic bacteria enter nodules. The endophytic bacteria find safe space inside the nodules to protect themselves from environmental stress and microbial competition. In return, these bacteria benefit the growth of the host plant by suppressing plant disease causing bacteria via competition for nutrients, production of phytohormones and by solubilizing mineral phosphates (Li *et al*., 2008).

The endophytic bacteria mainly belong to the genera *Agrobacterium*, *Bacillus*, *Paenibacillus, Pseudomonas, Burkholderia* and *Serratia*.

1.6. Rhizobia

Rhizobia a group of gram negative, rod shaped bacteria are one of the best examples of symbiotic nitrogen fixer. It forms nodules in the roots of leguminous plants and fixes atmospheric nitrogen (van Rhijn & Vanderleyden, 1995). Beijerinck (1888) was the first to isolate a bacteria from plant root nodules, which he named *Bacillus radicicola* (Beijerinck, 1888). In 1889, Frank renamed this bacterium as *Rhizobium leguminosarum* (Frank, 1889). The term "rhizobia" was originally used to name bacteria belonging to the genus *Rhizobium*. However, further research on rhizobial diversity revealed that only a small fraction of rhizobia belongs to this *Rhizobium* genus and many other genera were identified, such as *Azorhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium*. Earlier rhizobia were thought to belong exclusively to the alpha-proteobacteria, which also includes many species that are not legume symbionts. However, in 2001 Moulin *et al*. reported the isolation of rhizobia from the beta-proteobacteria class from root nodule of some wild legumes (Moulin *et al*., 2001). The rhizobia-legumes association is highly specific

1.6.1. General classification of *Rhizobium*

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Alphaproteobacteria
Order:	Rhizobiales
Family:	*Rhizobiaceae*
Genus:	*Rhizobium*

1.6.2. Characteristics of rhizobia

Rhizobia have the following characteristics; gram negative, rod-shaped (0.5 - 0.9 μm in width and 1.2 - 3.0 μm in length) and heterotrophic in nature (Somasegaran & Hoben, 1994). They do not form endospores and consist of 2 - 6 peritrichous flagella. Rhizobia generally grow at 25 - 30°C of temperature and in the pH range of 6 - 7 (Vincent, 1970). Rhizobia growth normally occurs under aerobic conditions. However, when fixing nitrogen, low levels of oxygen are required to protect the enzyme nitrogenase.

Figure 2: *Rhizobium trifolii* (Source: http://commtechlab.msu.edu/sites/dlc-me/zoo/zdrr0101.jpg; Image courtesy: Dazzo, Frank)

Rhizobia utilize simple carbohydrates and amino compounds as a source of energy. The number of native rhizobia in any soil can be very diverse, including many species and several distinct strains within one species. Several factors affect the number of rhizobia. These include vegetation, cropping history and soil conditions. The population of rhizobia in the soil is also influenced by rainfall. They generally prefer to live in soils that are moist but not waterlogged. Soil temperature and acidity are also other important conditions.

1.6.3. Mechanism of root nodule formation by rhizobia

The symbiotic relationship between the legume and rhizobia is formed as a result of specific signal exchange between the two partners. In the first step of

noduleformation legume plants secretes secondary metabolites, such as flavonoids. Rhizobia living in the rhizosphere sense the flavonoids secreted by the plant roots and get attracted towards the legume plants (Perret *et al*., 2000). nodD protein act as a receptor of the signal, it recognizes the flavonoids and acts as an activator of expression of the rhizobial nodulation genes *nodA, nodB, nodC*. These three nodulation genes codes for acyl transferase, chitin oligosaccharide deacetylase and N-acetylglucosaminyl transferase enzymes which catalyze the production of the backbone of the nod factors. When the nod factor is sensed by the root of legume plants, a number of morphological changes happen: cell division is triggered in the root to create the nodule, and the hair growth is redirected to wind around the bacteria multiple times until it fully encapsulates the bacteria (Jones *et al*., 2009). Rhizobia attach to the plant root by a two-step attachment process. At first rhizobia bind loosely as single cell to the root hair surface. In the next step, additional rhizobia accumulate at the site of adhesion. The bacteria then enter the developing nodule through infection thread, which grows through the root hair into the epidermis cell and onwards into the root cortex. In the next step a plant-derived membrane surround the bacteria. The bacteria finally differentiate into bacteroids that fix nitrogen. Nodules can beseen 15 - 20 days after planting, depending on legume species and germination conditions. When nodules are young they are usually white or gray inside. A mature and functional nodule become pink colored, indicating nitrogen fixation has started. The pink or red color of nodule is caused by leghemoglobin that controls oxygen flow to the bacteria.

1.6.4. Mechanism of nitrogen fixation by rhizobia

The process of nitrogen fixation is carried out by a complex enzyme, the nitrogenase (Berman-Frank *et al*., 2003). In nodules of legumes, nitrogenase synthesis

normally follows very shortly after bacteria are released from infection threads. Based on the metal cofactor three different nitrogen fixing systems has been identified Mo-nitrogenase, V-nitrogenase and Fe-nitrogenase (Smith, 1999). Structurally, nitrogen fixing system varies among different bacterial genera. The Mo-nitrogenase is the most common nitrogen fixing system and is found in a wide variety of bacteria. Nitrogenase enzyme uses high temperatures and high pressures to convert nitrogen gas and a hydrogen source into ammonia. Mo-nitrogenase is a complex of 2 easily separable proteins designated as molybdenum-iron (MoFe) protein or component I and iron (Fe) protein or component II. The iron protein has 2 Mg-ATP binding site and as ATP binds to these sites, the potential of electron at the (4Fe-4S) cluster is reduced, allowing iron protein to donate electrons to the molybdenum-iron protein. The molybdenum-iron protein is a tetramer ($\alpha 2\beta 2$). It contains 2 atoms of Mo and 24 - 32 atoms of Fe and S per molecule. The role of molybdenum-iron protein is to transfer electrons to N_2 and H+. The process by which electrons are sequentially delivered to the Mo-Fe protein and subsequently to substrate involves two interlocking cycles called the Fe protein cycle and the Mo-Fe protein cycle. The Fe protein cycle involves oxidizing and reducing the Fe protein's (4Fe-4S) cluster between two redox states as it sequentially delivers electrons to the MoFe protein and is re-reduced by other electron transfer proteins. The Mo-Fe protein cycle involves the progressive reduction of the Mo-Fe protein, which ultimately leads to N_2 binding and reduction.

The assimilation of nitrogen fixed in the bacteroids is exported through the inner and outer membrane of the microbe. The nitrogenase enzyme complex is rapidly and irreversibly denatured by oxygen. Thus the enzyme is functional only in low oxygen environments. The important contributor to solving the oxygen dilemma is

leghemoglobin an oxygen binding protein found within nodule infected cells. This plant protein, which is very similar to animal hemoglobin gives nodules their pink color. Leghemoglobin facilitates the diffusion of available oxygen though the plant cell cytoplasm to bacterial cells at concentrations which allow oxidative phosphorylation to occur without inactivation of nitrogenase activity.

1.7. Genes involved in nodulation

For the establishment of an effective symbiosis between legume and rhizobia, 2 major bacterial symbiosis genes are required, nodulation and nitrogen fixation genes. Nodulation genes encode enzymes responsible for the biosynthesis and secretion of nod factors. Nod factors are lipochitooligosaccharides that interact with the plant flavonoids. The process of nodulation requires the coordinated expression of severalnodulation genes. In addition, distinct rhizobia carry different combinations of nodulation genes that were probably acquired by the microorganisms during the course of evolution. It is generally believed that natural events of lateral gene transfer and duplication of nodulation genes have contributed to the evolution and spread of nodulation ability throughout several genera of bacteria (Moulin *et al*., 2004). Nod factor synthesis depends on the expression of nodulation (*nod*) genes, comprising the *nod, nol* and *noe* genes. In many previous studies the presence of several nod genes which are directly or indirectly related to nodulation has been reported. In general the nod genes are divided into 2 classes: common and host specific. Common nod genes (*nodABC*) are found in all rhizobia. In all rhizobia species the *nodABC* genes are essential for the synthesis of the core lipochitooligosaccharides:*nodC* synthesis the chito-oligosaccharide and *nodB* removes N-acetyl groups from the sugar at its non-reducing end, *nodA* finally transfers a fatty acid from an acyl carrier protein (Cooper, 2007). In addition, many previous studies reported the presence *nodD* gene

in rhizobia. *nodD* gene encodes NodD protein which, is a positive transcription regulator and is thought to interact with flavonoid molecule and subsequently activates other nod genes. Host specific nod genes varied from species to species. For example, in *Rhizobium meliloti* the nod gene consists of *nodEFG*, *nodQP*, *nodH* genes (Horvath *et al*., 1994) and in *Rhizobium leguminosarum* nod gene consists of *nodFEL, nodMNT, nodO* genes (Surin *et al*., 1989). These genes areresponsible for the modification of chito-oligosaccharide (Kiss *et al*., 1998).

1.8. Genes involved in nitrogen fixation

Two major classes of genes are involved in the process of symbiotic nitrogen fixation, these are *nif* genes and *fix* genes. The *nif* genes are found in both free living nitrogen fixing bacteria and in symbiotic bacteria associated with various plants. The structural *nif* genes which encodes nitrogenase are identical and function in an almost similar manner in all the nitrogen fixing microbes (Ruvkun & Ausubel, 1980). A majority of the *nif* genes are plasmid borne in the rhizobia, but are located on chromosome in some microorganisms (*Bradyrhizobium*). Nitrogen fixation in symbiotic microbes and free living microbes is catalyzed by an enzyme complex known as nitrogenase which is encoded by *nifDK* and *nifH* genes (Kim & Rees, 1994). Nitrogenase is a complex enzyme and it consists of 2 subunits, a molybdenum-iron containing protein (MoFe), subunit I and an iron (Fe) containing protein, subunit II. In addition, a FeMo cofactor (FeMo-Coo) is also required for the activation of MoFe protein. The MoFe protein subunit is encoded by *nifK* and *nifD* and Fe protein subunit is encoded by the *nifH* gene. The organization of *nif* genes in microorganisms vary tremendously. For example, in *Klebsiella pneumonia*, at least 20 *nif* genes are organized in about 8 operons. These 20 *nif* genes includes *nifA, nifB,nifD, nifE, nifF, nifH, nifJ, nifK,nifL, nifM, nifN, nifQ,nifS, nifT, nifU, nifV, nifW, nifX, nifY* and *nifZ*

(Dean and Jacobson, 1992). In most systems, the regulation of all *nif* genes are controlled by *nifA* (a positive activator of transcription) and *nifL* (the negative regulator). The *nifH* gene is the most often used marker for the molecular analysis of nitrogen fixing bacteria. In addition to *nif* genes, some other microbial genes are also involved in symbiotic nitrogen fixation, these collectively referred to as *fix* genes. Environmentally, the expression of *nif* gene is regulated by both oxygen and nitrogen levels (Merrick & Edwards, 1995). Generally nitrogenase is inactivated by oxygen and hence environmental oxygen is one of the major regulatory factors in nitrogen fixation. In the case of strict anaerobes, oxygen regulation has less importance since the anaerobes can only survive in an oxygen free environment.

1.9. Environmental factors affecting symbiosis

The growth and activity of legume plants are controlled by several environmental factors. In rhizobia-legume symbiosis the process of nitrogen fixation depends on the functional state of the legume plant and environmental conditions supporting the rhizobia (Hungria & Vargas, 2000). Rhizobia usually vary in their tolerance to different environmental factors. The major environmental factors affecting symbiosis and nitrogen fixation are salinity, unfavorable soil pH, temperature extremes, insufficient soil moisture, plant diseases, *etc*. A given stress may also have more than one effect e.g., salinity may act as a water stress or may affect nodule metabolism directly. Populations of rhizobia vary in their tolerance to major environmental factors.

1.9.1. Soil salinity

An increase in the salinity of agriculture soils or water used for irrigation decreases the growth of plants which in turn reduces the productivity of crop plants. It is a serious threat to agriculture. Increasing salt concentrations have a negative effect

on soil microbial populations as a result of direct toxicity as well as through osmotic stress. The rhizobia-legume symbiosis and nodule formation on legumes are sensitive to salt. The effects of salt stress on nodulation and nitrogen fixation of legumes have been examined in several studies (Hussain *et al*., 2002). The reduction of nitrogen fixing activity by salt stress is usually attributed to a reduction in the production of cytosolic protein, specifically leghemoglobin (Delgado *et al*., 1994). The salt induced distortions in nodule structure could also be reasons for the decline in the nitrogen fixation rate by legumes. Reduction in photosynthetic activity might also affect nitrogen fixation by legumes under salt stress.

Many species of bacteria adapt to saline conditions of soil by the intracellular accumulation of low molecular weight organic solutes known as osmolytes (Csonka & Hanson, 1991). The accumulation of osmolytes is thought to control the dehydration effect of low water activity in the medium without interfering macromolecular structure or function. Successful rhizobia-legume symbiosis under salt stress requires the selection of salt tolerant rhizobia from the indigenous population.

1.9.2. Soil acidity

Soil acidity is a significant problem facing by the farmers in many areas of the world. Soil acidity results in decrease productivity of legume plants. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they perform symbiotic nitrogen fixation. Legumes and rhizobia exhibit varied responses to acidity. Strains of a given species vary widely in certain cases in their pH tolerance. The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0 (Jordan, 1984), and relatively few rhizobia grow well at pH less than 5.0. In earlier studies it was reported that the fast growing strains of rhizobia such as

Mesorhizobium, *Rhizobium* have generally been considered less tolerant to acidic pH when compared with slow growing strains of rhizobia like *Bradyrhizobium* (Graham *et al*., 1994). Although some strains of the fast growing rhizobia *e.g*., *Rhizobium loti* and *Rhizobium tropici*, are highly acid tolerant. Differences in lipopolysaccharide composition, proton exclusion and extrusion and synthesis of acid shock proteins have been associated with the growth of cells at acidic pH (Zahran, 1999). The composition and structure of the outer membrane could also be a factor in pH tolerance (Graham *et al*., 1994).

It appears that the pH sensitive stage in nodulation occurs early in the infection process and attachment of rhizobia to root hairs is one of the stages affected by acidic conditions in soils. In addition, low pH can affect the production and excretion of nodulation factors in some strains of rhizobia. Rhizobia appear to be varying in their symbiotic efficiency under acidic conditions. The number of nodules, the nitrogenase activity, the nodule ultrastructure and the weights of nodules were affected at a low pH (<4.5). These additional stresses affect the growth of rhizobia. In the last few decades different strategies have been adopted to solve the problem of soil acidity for better rhizobia-legume symbiosis. One of these strategies includes selection of optimal combination of rhizobial inoculums and the legume genotype for acidic soils.

1.10. Plant growth promoting properties of rhizobia

Rhizobia are an important member of PGPR and many previous studies reported the plant growth promoting abilities of rhizobia. Plant growth promoting rhizobacteria (PGPR), are the bacteria that can enhance plant growth by a wide variety of mechanisms. They are the natural resource which colonizes roots of plants and stimulates growth and yield directly and indirectly (Figueiredo *et al*., 2011).

Direct promotion of growth by PGPR includes fixation of nitrogen, production of plant hormones such as auxins, cytokinins, gibberellins and through the solubilization of minerals like phosphorus. Indirect plant growth promotion includes production of HCN, antibiotics, siderophores that decrease or prevent the deleterious effects of plant pathogenic microorganisms (Bhattacharyya & Jha, 2012). There are a number of mechanisms used by plant for growth promoting bacteria, a few of them are discussed below.

1.10.1. Phytohormone production

PGPR usually produce phytohormones such as auxins, cytokinins, gibberellins and ethylene which can affect the growth and development of plants. Among plant growth regulators, Indole Acetic Acid (IAA) is the most common natural auxin found in plants (Datta & Basu, 2000). In a previous work it was reported that about 80 % of rhizobacteria can synthesize IAA and act in conjunction with endogenous IAA in plant to stimulate cell proliferation (Patten & Glick, 1996). IAA affects plant cell division, extension and differentiation, stimulates seed germination. IAA also increases the rate of development of xylem and initiates lateral and adventitious root formation. IAA production by rhizobiain both symbiotic as well as in free living states has been reported in many previous works. There are indications of more IAA synthesis by rhizobia when associated with host plant as compared to IAA produced in the free living state (Glick & Glick, 2012).

1.10.2. Phosphate solubilization

Phosphorus is a key element in the nutrition of plants, next to nitrogen. It plays an important role in many metabolic processes in the plant, including photosynthesis, energy transfer, signal transduction and respiration (Khan *et al*., 2010). It is largely available in soils in both organic and inorganic forms. Plants are unable to utilize

phosphate because the maximum amount of phosphate present in soil is in insoluble and precipitated form. PGPR employ different methods to make use of unavailable forms of phosphorus and help in making phosphorus available for plants to absorb. In the last few decades, phosphate solubilizing microorganisms which are included in the genera *Bacillus*, *Beijerinckia*, *Burkholderia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* have attracted the attention of agriculturists as soil inoculums to improve plant growth and yield. The main phosphate solubilization mechanisms employed by PGPR include: release of mineral dissolving compounds, e.g. organic acid anions, proton hydroxyl ions, CO_2, liberation of extracellular enzymes and the release of phosphate during substrate degradation (Sharma *et al*., 2013).

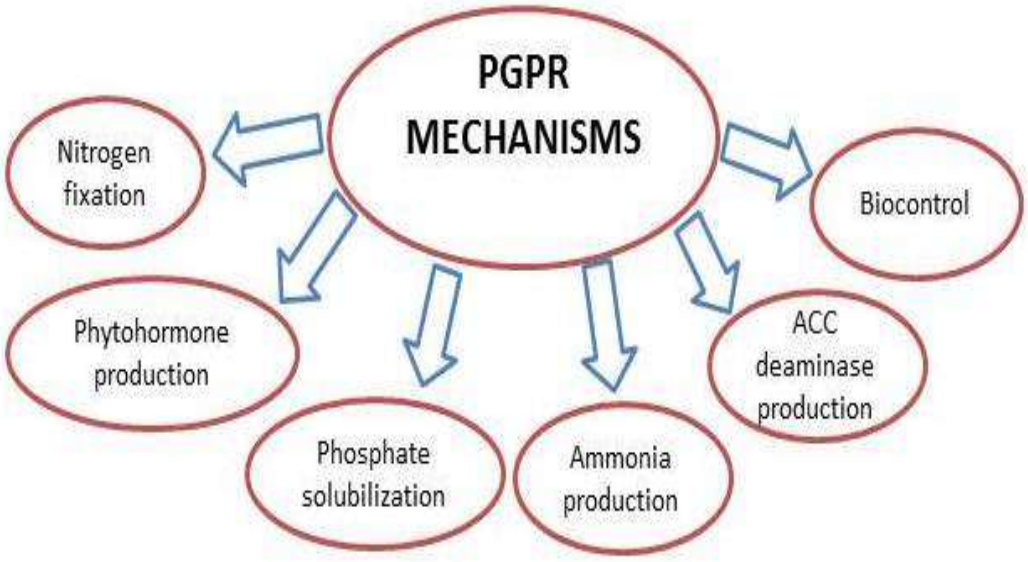

Figure 3: Diagram showing different mode of action of PGPR

1.10.3. Ammonia production

Ammonia production is a common trait associated with nitrogen fixing free living and symbiotic bacteria. It is an important biochemical reaction of biological nitrogen fixation which occurs mainly through symbiotic association of nitrogen fixing microorganisms with legumes that converts atmospheric elemental nitrogen

into ammonia. A range of PGPR can produce ammonia and significantly increase the vegetative growth and grain yield of plants (Kennedy *et al*., 2004).

1.10.4. 1-aminocyclopropane-1-carboxylate (ACC) deaminase production

Ethylene is an essential plant metabolite required for the normal growth and development of plants. This plant growth hormone is produced endogenously by plants and is also produced by different biotic and abiotic processes in soils. Ethylene has a wide range of biological activities and can affect plant growth and development in different ways, including inhibiting root elongation, promoting fruit ripening, promoting flower wilting, promoting leaf abscission, etc. The high concentration of ethylene may lead to reduced crop performance. The enzyme 1-aminocyclopropane-1 carboxylic acid (ACC) is required for ethylene production. Many previous reports suggested that PGPR which secretes the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, facilitate plant growth and development by decreasing ethylene levels (Nadeem *et al*., 2007).

1.10.5. In-direct mechanisms

The application of environmental friendly microorganisms to control plant diseases is an agriculturally significant approach. The major indirect mechanism of plant growth promotion in rhizobia is through acting as biocontrol agents (Glick & Glick, 2012). Rhizobia can suppress the growth of various phytopathogens in a variety of ways like competing for nutrients and space, niche exclusion, induced systemic resistance and antifungal metabolite production. Many rhizobacteria have been reported to produce antifungal metabolites like, HCN, phenazines, pyrrolnitrin and tensin (Bhattacharyya & Jha, 2012). Interaction of rhizobia with the plant roots can result in plant resistance against some pathogenic bacteria, fungi, and viruses. This phenomenon is called induced systemic resistance.

1.11. Description of the study area

Northeast region of India is one of the biodiversity hot spots and it harbors numerous types of flora and fauna. Assam is one of the eight states of northeast India. It lies at 89^0 42' to 96^0 E longitude and 24^0 8' to 28^0 2'N latitude. Assam has a geographical area of 78,438 km^2 and is bounded to the north by Bhutan and Arunachal Pradesh, to the east by Nagaland and Manipur, to the south by Mizoram and Tripura, and to the west by Bangladesh, Meghalaya and West Bengal. Assam is broadly classified into 3 main regions, the Brahmaputra river valley in the north, the Barak river valley in the south and the hill areas between Meghalaya, Nagaland and Manipur in the south-central parts of the state. At present, one-third of Assam was covered with various types of forests which include tropical evergreen and deciduous forests, broad-leaved hill forests, pine jungles and swamp forests, besides grasslands. In addition Assam is well known for 2 UNESCO (United Nations Educational, Scientific and Cultural Organization) world heritage sites, the Kaziranga National Park, on the banks of the Brahmaputra River, and the Manas Wildlife Sanctuary, near the border with Bhutan.

1.12. Diversity studies of rhizobia from Assam

The soil of Brahmaputra Valley and Barak Valley are alluvial and very fertile. But the soil in the North Cachar Hills is less fertile and consists of red soil. With the "Tropical Monsoon Rainforest Climate", Assam is temperate and experiences heavy rainfall and high humidity. Assam is well known for its highly diverse flora and fauna and most of the geographical area of Assam is covered by dense forest. Several leguminous plants were reported from Assam which can form an effective symbiosis

with different strains of rhizobia. However, work related to rhizobial diversity study from the legumes of Assam is very limited. Deka & Azad, (2006) isolated and characterized rhizobia from common pulses cowpea, green gram, black gram, pigeon pea, soybean and groundnut (Deka & Azad, 2006). Based on phenotypic and biochemical features they confirmed the isolates as strains of *Bradyrhizobium*. In 2010, Choudhury *et al*. isolated Rhizobium from the lower Brahmaputra valley region of Assam and studied the effect of pH on their growth (Choudhury *et al*., 2010). Kesari *et al*. (2013) isolated a novel rhizobial strain *Rhizobium pongamiae* sp. from root nodules of *Pongamia pinnata*, a biodiesel crop growing in North Guwahati, Assam, India (Kesari *et al*., 2013). In recent past, Deb *et al*. (2015) reported the isolation of rhizobia from root nodules of *Cajanus cajan* from different sites of Barak Valley, Assam and investigated their plant growth promoting properties (Deb *et al*., 2015).

1.13. Significance of the study

Rhizobia are considered as one of the most valuable natural resources and in the last few decades a lot of research has been carried out for isolation and selection of environment friendly rhizobia from different geographical regions of the world (Sadowsky *et al*., 1983). In addition to nodulation ability on plant roots, a number of different physiological, morphological and biochemical qualities were assessed for species differentiation of rhizobia. In earlier days due to lack of concern and unavailability of appropriate methods only two rhizobial genera (*Rhizobium* and *Bradyrhizobium*) were described in the first edition of Bergey's manual of systematic bacteriology (Jordan, 1984). However, the invention of advanced molecular techniques provided a breakthrough in the classification of rhizobia and also helped to evaluate rhizobia diversity in different environments.

Grain legumes are often cultivated as alternate crops with rice in different locations in India (George *et al*., 1988). Native rhizobial strains are well adapted to stress conditions and help in the reclamation of marginal lands as they enrich soil through symbiotic nitrogen fixation.

The aim of the present study is the isolation and characterization of indigenous rhizobia associated with root nodules of few selected leguminous plants from different locations of Assam and to assess their diversity based on different phenotypic and genotypic methods. The study also aims to assess the plant growth promoting, stress tolerant and nodulation ability of the isolates in order to select potential isolate which can be used as effective inoculants/biofertilizer for their respective host plants.

1.14. Objectives of the study

I. To isolate rhizobial strains from selected leguminous plants of Assam.

II. To perform primary screening of the isolates by employing different phenotypic and confirmatory tests.

III. To perform biochemical, plant growth promoting ability, acidity and salinity tolerance test with the isolates.

IV. To characterize the isolated strains by performing PCR amplification and analysis of 16S rDNA, *nifH* and *nodC* genes.