# Characterization and molecular diversity of endophytic bacteria isolated from Chickpea (*Cicer arietinum*) nodules

By

**Ranjana Saini**

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*Thesis submitted to the Chaudhary Charan Singh Haryana Agricultural University, Hisar in the partial fulfillment of the requirements for the degree of*

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## CERTIFICATE – I

This is to certify that this thesis entitled **"Characterization and molecular diversity of endophytic bacteria isolated from Chickpea (*Cicer arietinum*) nodules"**, submitted for the degree of **Master of Science** in the subject of **Microbiology** to **Chaudhary Charan Singh Haryana Agricultural University, Hisar,** is a bonafide research work carried out by **Ms. Ranjana Saini,** Admn. no. **2009BS139M** under my supervision and guidance and no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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## CERTIFICATE - II

This is to certify that this thesis entitled, **"Characterization and molecular diversity of endophytic bacteria isolated from Chickpea (*Cicer arietinum*) nodules",** submitted by **Ms. Ranjana Saini,** Admn. No**. 2009BS139M** to **Chaudhary Charan Singh Haryana Agricultural University, Hisar**, in partial fulfillment of the requirements for the degree of **Master of Science** in the subject of **Microbiology**, has been approved by Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

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# CHAPTER –I

## INTRODUCTION

The chickpea (*Cicer arietinum*) (also garbanzo bean, Indian pea, ceci bean, Bengal gram) is an edible legume of the family Fabaceae, subfamily Faboideae. Chickpea, the world's third most important food legume, is currently grown on about 11 m ha, with 96% cultivation in the developing countries. Chickpea production has increased during the past 30 years from 7.3 mt (average of 1977-1979 triennium) to 8.4 mt (average of 2004-06 triennium) because of increase in productivity from 693 to 786 kg $ha^{-1}$ during this period. Chickpea is high in protein and one of the earliest cultivated legume. But still chickpea and its nodule bacteria need to be studied more carefully. Sufficient information generated from work on several temperate legumes is available to indicate the tremendous potential of adequate inoculation technology. Numerous studies to clarify the taxonomy and phylogeny of bacteria that are able to induce nodules on leguminous plants are now being carried out. This has led to the identification of 12 bacterial genera with 92 species able to nodulate different legumes. (Dudeja *et al.,* 2008; Weir, 2011).

Soil bacteria associated with plant roots that can exert beneficial effects on their hosts are designated as plant growth promoting rhizobacteria. Symbiotic interactions are the driving force in ecosystems; symbiosis ranges from parasitism to mutualism and includes everything in between. The fitness outcomes for plants differ accordingly: if a plant is highly susceptible to pathogens, its fitness is likely to be low in pathogen-rich environments; if a plant cooperates with mutualists, it is likely to thrive even in adverse environments. Bacteria, which colonize the interface between living plant roots and soil, namely the rhizosphere, are abundant symbiotic partners of plants. These so-called rhizobacteria are said to be plant growth promoting. Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host (Holliday, 1989; Schulz *et al.,* 2006). Although all of the approximately 300,000 plant species have been estimated to harbor one or more endophytes (Strobel *et al.,* 2004), few relationships between plants and these endophytes have been studied in detail; the legume-rhizobia symbiosis is an exception. Consequently, the opportunity to find new and beneficial endophytic microorganisms among the diversity of plants in different ecosystems is considerable.

Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants, ranging from woody tree species, such as oak and pear, to herbaceous crop plants such as sugar beet and maize. There may be two sources of endophytes: the surrounding environment and those inherited (Hallmann *et al.,* 1997). Soil is considered to be the major environmental source of bacteria found inside plants (Mahaffee *et al.,* 1997; Rasche *et al.,* 2006; Van Overbeek *et al.,* 2008; Long *et al.,* 2010) and it is thus not surprising that roots are usually the most heavily colonized plant organ (Hallmann *et al.,* 2006). Textbook examples of soil derived endophytes include vesicular arbuscular mycrorhiza (Brundrett, 2002) and nodule forming rhizobia (Slattery *et al.,* 2001). A critical stage for soil microbes to gain access to plants would be during germination and early development, becoming founders of the endophyte community of the adult plant and possibly its progeny.

Classical studies on the diversity of bacterial endophytes have focused on characterization of isolates obtained from internal tissues following disinfection of plant surfaces with sodium hypochlorite or similar agents (Miche *et al.,* 2001). The mutualistic interaction of legumes with rhizobia involves finely tuned recognition steps which ultimately lead to the production of root nodules in which the plants accommodate the bacteria (Oldroyd *et al.,* 2005). For other endophytic rhizobacteria, the processes of host-microbe signaling and colonization, and the mechanisms leading to mutual benefit are less-well characterized. The endophytic niche offers protection from the environment for those bacteria that can colonize and establish *in planta*. These bacteria generally colonize the intercellular spaces, and they have been isolated from all plant compartments including seeds (Posada *et al.,* 2005; Dudeja *et al.,* 2011a). As the bacteria can proliferate inside the plant tissue, they are likely to interact more closely with the host, face less competition for nutrients, and are more protected from adverse changes in the environment than those bacteria in the rhizosphere (Hurek *et al.,* 1998). Plants are quite resistant to microorganisms; few can overcome the multiple barriers which plants possess. Much of what is known about microbial entry into plants concerns pathogenic microorganisms, but many of the same mechanisms are likely employed by "harmless" endophytes as well. Microorganisms can penetrate into the plant via natural openings such as stomata, or wounds associated with traumatic damage. Microbes may also enter directly through the cuticle, epidermal cell walls and into epidermal cell or via middle lamella between two epidermal cell walls into the underlying intercellular spaces or into mesophyll cells. In order to penetrate these cells some microbes produce cell wall degrading enzymes including; cutinase, pectic enzymes, hemicellulase, cellulose, protease, and lignin-peroxidases to aid in entry into the plant (Moerschbacher, 2000).

Bacterial endophytes can accelerate seedling emergence, promote plant establishment under adverse conditions and enhance plant growth (Chanway, 1997; Bent *et al.,* 1998). Endophytic bacteria are believed to elicit plant growth promotion in one of two ways: either indirectly by helping plants acquire nutrients, e.g. via nitrogen fixation, phosphate solubilization (Wakelin *et al.,* 2004) or iron chelation (Costa *et al.,* 1994), by preventing pathogen infections via antifungal or antibacterial agents, by outcompeting pathogens for nutrients by siderophore production, or by establishing the plant's systemic resistance (van Loon *et al.,* 1998); or directly by producing phytohormones such as auxin or cytokinin (Madhaiyan *et al.,* 2006), or by producing the enzyme 1-aminocyclopropane-1-carboxylate deaminase, which lowers plant ethylene levels (Glick, 1995). In addition to these plant-growth-promoting traits, endophytic bacteria must also be compatible with host plants and able to colonize the tissues of the host plants without being recognized as pathogens (Rosenblueth *et al.,* 2006). A particular bacterium may affect plant growth and development using one or more of these mechanisms, and may use different ones at various times during the life cycle of the plant. While the mechanism of growth promotion remains unclear how consistently bacterial endophytes elicit responses in host and non-host plant species.

Endophytic bacteria have been isolated from different legume plants such as alfalfa, clover, soybean, *Argyrolobium uniflorum*, *Vicia*, *Oxytropis*, *Medicago*, *Melilotus*, *Onobrychis*, peanut and *Acacia.* Over 24 non-rhizobial and about 10 rhizobial genera have been isolated from legume tissues including *Agrobacterium*, *Bacillus*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Mycobacterium*, *Paenibacillus*, *Pseudomonas*, *Phyllobacterium, Ochrobactrum*, *Sphingomonas*, *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Burkholderia*, *Phyllobacterium* and *Devosia* (Lei *et al.,* 2008; Muresu *et al.,* 2008; Dudeja *et al.,* 2011a). Considering other parts of legume plants, endophytic bacteria were isolated from the foliage, tap roots and nodules of red clover plants (*Trifolium pratense* L.) (Sturz *et al.,* 1997; 1995; 1996; 2000). Thirty-one bacterial species of 14 different genera were recovered. Genera diversity and species number were greatest in foliage tissues. *Pantoea agglomerans* (59.6%) was the most frequent species recovered in foliage tissues, *Agrobacterium rhizogenes* in the tap root (49.2%) and *R. leguminosarum* bv. *phaseoli* and *Mesorhizobium loti* in the nodules (27.2% each). Clover root nodules were host to 12 bacterial species other than rhizobia, of which 8 were specific to this tissue. In root bacterization experiments, species of nodule bacteria promoted growth of red clover more often when applied in combination with *R. leguminosarum* bv. *trifolii* than when applied singly. However, *Bacillus megaterium*, *Bordetella avium* and *Curtobacterium luteum* consistently promoted growth either individually or in combination with rhizobia. Similarly, Zakhia *et al.,* (2006) described the

association of 14 bacterial genera with wild legume nodules in Tunisia. *Agrobacterium tumefaciens* strains were reported to be endophytic bacteria in the roots, stems, and root nodules of *Melilotus dentatus* and other legumes (Wang *et al.,* 2006; Mahdhi *et al.,* 2007).

Studies of several bacterial isolates recovered from surface sterilized root nodules of *Arachis hypogaea* L. (peanut) showed that these isolates belonged to gamma-proteobacteria, and included *Pseudomonas* spp., *Enterobacter* spp. and *klebsiella* spp. (Ibanez *et al.,* 2009). Similarly, thirty-nine endophytic bacterial strains isolated from the nodules of *Lespedeza* sp. were found to be belonging to alpha-proteobacteria, beta-proteobacteria, Actinobacteria, and Firmicutes phylum with nine different genera *Arthrobacter, Bacillus, Bradyrhizobium, Burkholderia, Dyella, Methylobacterium, Microbacterium, Rhizobium* and *Staphylococcus* (Palaniappan *et al.,* 2010). Various rhizobial and non rhizobial strains were isolated from root nodules of two widespread south eastern Australian tree legumes (*Acacia salicina*, *A. stenophylla*). This legume was nodulated primarily by *Bradyrhizobium*, while the results indicate significant associations with other root-nodule forming bacterial genera, including *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Burkholderia*, *Phyllobacterium* and *Devosia* (Hoque *et al.,* 2011).

Few studies have been published describing the molecular basis of the interactions between endophytic bacteria and plants. Adapting strategies that have been used to study bacterial gene expression in the rhizosphere and phyllosphere such as *in vivo* expression technology and recombination *in vivo* expression technology (Leveau *et al.,* 2001; Preston *et al.,* 2001; Zhang *et al.,* 2006) may provide an insight into genes that are required by bacteria to enter, compete, colonize the plant, suppress pathogens and generally survive within the plant. Molecular approaches for the isolation and characterization of bacterial endophytes and plant-associated bacteria and communities has been reviewed recently by Franks *et al.,* (2006) and particularly in legumes by Dudeja *et al.,* (2011a) but no such studies in chickpea nodules and particularly from Indian origin has been reported. Therefore, the present investigation was undertaken with the following objectives:

1. Characterization of chickpea nodule endophytes.
2. Molecular diversity of chickpea nodule endophytes.
3. Growth promotion of chickpea by endophytes.

## CHAPTER –II

## REVIEW OF LITERATURE

Bacteria are believed to have been the dominant life form on this earth for the first three billion years of the biosphere's history (DeLong *et al.,* 2001). Over this time, they have evolved *de novo* some of the genetic diversity present on Earth today in the form of countless different genes - a metagenome of knowledge on how to function in any given environment (Verstraete *et al.,* 2007). Microbes are successful life-forms is evidenced by their survival in every environment on this planet, prompting the Dutch microbiologist Martinus Beijerinck to famously pronounce, "Everything is everywhere, but the environment selects". As microbial and genetic technologies advance, researchers are increasingly appreciating the significance of this paradigm as the diversity and complexity of microbial ecosystems become more understood (O'Malley, 2008). In contrast to the vast diversity of microbes in the environment, eukaryotic hosts such as plants create a more restricted niche for microbes to inhabit (Bacon *et al.,* 2006). To gain access to such new habitats, some microbes established non-pathogenic relationships on plant surfaces and within plant bodies, evolving specialized processes to enhance their survival and to directly communicate with their hosts (Saikkonen *et al.,* 2004). Microbes (bacteria, archaea, and fungi) that inhabit the interior of the plant body without causing disease are known as endophytes (Rosenblueth *et al.,* 2006).

Major benefits for host plants partnering with endophytic microbes include enhanced nutrition and improved tolerance to biotic and abiotic stress (Rosenblueth *et al.,* 2006). Microbes are able to help wither, decompose or solubilise minerals in rock, or organic debris, making them available for plants. Other endophytes are able to biologically convert atmospheric nitrogen gas to fixed forms of nitrogen usable for their hosts. By colonizing plant surfaces and interior spaces, co-operative or commensal bacteria may competitively exclude disease-causing microbes from successful colonization. Finally, some endophytes have been shown to produce significant levels of secondary metabolites which help deter insect and mammalian herbivores (Clay, 1988). Bacteria inhabiting multicellular hosts exist as inter-related communities that can reflect and can be affected by the selective pressures imposed on them by their niche. Remarkably, a microbial community may possess many more genes than the host, extending its metabolic complexity and ecological fitness (Versalovic *et al.,* 2006).

Moving from the community to understanding an individual strain requires culturing of microbes to enable ecological and phenotypic study and evaluation of biotechnological potential (Bull, 2004a). Different microbes are likely to have different ecological functions within a plant associated community, and are also likely to have different colonization and survival dynamics (Strobel *et al.,* 2003; Bull, 2004b). Numerous studies have focused on characterizing the endophytic communities of plants (Rosenblueth *et al.,* 2006). Agriculturally, the plant genus *Cicer* is of particular interest as it includes chickpea or Bengal gram (*Cicer arietinum* L.), one of the world's most important crops. The genus *Cicer* comprises one cultivated species, the chickpea (*Cicer arietinum* L.) and 42 wild species (Vavilov, 1951).

Chickpea has been considered as a restrictive host for nodulation by rhizobia. However, recent studies have reported that several *Mesorhizobium* species may effectively nodulate chickpea (Dudeja *et al.,* 2009). With the purpose of investigating the evolutionary relationships between these different species with the ability of nodulating the same host, 21 Portuguese chickpea rhizobial isolates were analysed (Laranjo *et al.,* 2008). So, *Mesorhizobium* and other different species are known to nodulate chickpea. A large number of bacteria associated with nodules were ignored earlier but during the last decade the presence of these bacteria and their role in nutrient use efficiency and other beneficial characters have been reported. Therefore, in the present review, different microbes associated with nodules of legumes, their beneficial part being played is being discussed.

### 2.1 Diversity of endophytes in legume root nodules

The term "endophyte" was coined by Heinrich Anton de Bary in 1866 and is derived from the Greek words endon (within) and phyte (plant) (Bacon *et al.,* 2000). The term usually refers to non-pathogenic bacteria and fungi found in plant tissue, but sometimes is also used to refer to mycorrhizal fungi found in plant roots (Tadych *et al.,* 2009). Interestingly, it is believed that all plants are still host to at least one type of endophyte. There may be two sources of endophytes: the surrounding environment and those inherited (Hallmann *et al.,* 1997). Soil is considered to be the major environmental source of bacteria found inside plants (Mahaffee *et al.,* 1997; Rasche *et al.,* 2006; Van Overbeek *et al.,* 2008; Long *et al.,* 2010) and it is thus not surprising that roots are usually the most heavily colonized plant organ (Hallmann *et al.,* 2006). Soil derived endophytes include vesicular arbuscular mycrorhiza (Brundrett, 2002) and nodule forming rhizobia (Slattery *et al.,* 2001).

Molecular approaches for the isolation and characterization of bacterial endophytes and plant-associated bacteria and communities have been reviewed recently by (Franks *et al.,* 2006). Microbial communities inhabiting stems, roots and tubers of various varieties of plants were analysed by 16S rRNA gene-based techniques such as terminal restriction fragment

length polymorphism analysis, denaturing gradient gel electrophoresis as well as 16S rRNA gene cloning and sequencing. Five taxa exhibiting the most promising levels of colonization and an ability to persist were identified as *Cellulomonas*, *Clavibacter*, *Curtobacterium*, *Pseudomonas* and *Microbacterium* by 16S rRNA gene sequence, fatty acid and carbon source utilization analyses (Elvira-Recuenco *et al.,* 2000; Zinniel *et al.,* 2002).

Forty-two bacterial isolates from root nodules of *Argyrolobium uniflorum* growing in the arid areas of Tunisia were characterized by phenotypic features, RFLP, and sequencing of PCR-amplified 16S rRNA genes. The isolates were found to be phenotypically diverse. The majority of the isolates tolerated 3% NaCl and grew at temperatures up to 40 °C. Phylogenetically, the new isolates were grouped in the genera *Sinorhizobium* (27), *Rhizobium* (13), and *Agrobacterium* (2). Except for the two *Agrobacterium* isolates, all strains induced nodulation on *Argyrolobium uniflorum*, but the number of nodules and nitrogen fixation efficiency varied among them. *Sinorhizobium* sp. strains were the most effective symbionts (Mrabet *et al.,* 2006). Similarly, Zakhia *et al.,* (2006) described the association of 14 bacterial genera with wild legume nodules in Tunisia. *Agrobacterium tumefaciens* strains were reported to be endophytic bacteria in the roots, stems, and root nodules of *Melilotus dentatus* and other legumes (Wang *et al.,* 2006; Mahdhi, 2007).

The Qinghai-Tibet plateau is the highest place in the world, and the environment in that plateau is harsh for both animals and plants, with low temperature, low concentration of oxygen and high solar radiations. In a study, 61 root nodule isolates from *Vicia*, *Oxytropis*, *Medicago*, *Melilotus* and *Onobrychis* species grown in Qinghai-Tibet and a loess plateau were comparatively characterized. Based upon the results of numerical taxonomy, ARDRA, AFLP, DNA-DNA hybridization and 16S rDNA sequencing, the isolates were classified as *Rhizobium leguminosarum*, *Ensifer* (*Sinorhizobium*) *meliloti*, *Ensifer* (*Sinorhizobium*) *fredii*, *Mesorhizobium* sp., *Phyllobacterium* sp., *Stenotrophomonas* sp. and two non-symbiotic groups related to *Agrobacterium* and *Enterobacteriaceae*. The strains isolated from the Qinghai-Tibet plateau and from the loess plateau were intermixed in these species or groups. *Oxytropis* spp. and *Medicago archiducis-nicolai* grown on the Qinghai-Tibet plateau were recorded as new hosts for *R. leguminosarum*, as well as *Oxytropis glabra* and *Medicago lupulina* for *Ensifer* (*Sinorhizobium*) *fredii* (Kan *et al.,* 2007).

A total of 154 bacterial strains isolated from nodules of eighteen *Vicia* species mainly grown in the temperate Chinese provinces were characterized by ARDRA, ITS PCR–RFLP, BOX-PCR, sequencing of 16S rDNA, *nodC, nifH, atpD* and *gln*II, and nodulation tests. The results demonstrated that most of the *R. leguminosarum* strains were effective microsymbionts of the wild *Vicia* species, while genomic species related to *Rhizobium gallicum, Mesorhizobium huakuii, Ensifer meliloti* and *Bradyrhizobium* spp. were

symbiotic bacteria occasionally nodulating with *Vicia* species (Lei *et al.,* 2008). In addition, fourteen strains related to *Agrobacterium, Phyllobacterium, Ensifer, Shinella* and *R. tropici*, as well as 22 strains of *R leguminosarum* might be nodule endophytes without symbiotic genes.

Non-rhizobial endophytes from the surface sterilized root nodules of alfalfa (*Medicago sativa* L.) were isolated and characterized (Stajković *et al.,* 2009). Out of 15 endophytic nonrhizobial strains isolated, three isolates were gram positive and were identified as *Bacillus megaterium*, *Brevibacillus chosinensis* and *Microbacterium trichothecenolyticum*. None of these isolates was able to nodulate alfalfa when re-inoculated under sterilized conditions. Several bacterial isolates were recovered from surface-sterilized root nodules of *Arachis hypogaea* L. (peanut) growing in soils from Argentina (Ibanez *et al.,* 2009). The 16S rDNA sequences of seven fast-growing strains were obtained and the phylogenetic analysis showed that these isolates belonged to the gamma-proteobacteria and included *Pseudomonas* spp., *Enterobacter* spp., and *Klebsiella* spp. These strains were unable to induce nodule formation in *Arachis hypogaea* L. plants, but they enhanced plant yield. When the isolates were co-inoculated with an infective *Bradyrhizobium* strain, they were found as endopytes in the nodules and supported the idea that these isolates are opportunistic endophytes.

Similarly, 39 endophytic bacterial strains were isolated from the nodule of *Lespedeza* sp. grown in two different locations of South Korea (Palaniappan *et al.,* 2010). The strains were identified by using 16S rRNA gene sequence analysis as belonging to alpha-proteobacteria, beta-proteobacteria, Actinobacteria and Firmicutes phylum with nine different genera *Arthrobacter, Bacillus, Bradyrhizobium, Burkholderia, Dyella, Methylobacterium, Microbacterium, Rhizobium* and *Staphylococcus*.

Various rhizobial and non rhizobial strains were isolated from root nodules of two widespread south eastern Australian tree legumes (*Acacia salicina*, *A. stenophylla*). This legume was nodulated primarily by *Bradyrhizobium*, while the results indicate significant associations with other root-nodule forming bacterial genera, including *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Burkholderia*, *Phyllobacterium* and *Devosia* (Hoque *et al.,* 2011).

A total of 159 endophytic bacteria were isolated from surface-sterilized root nodules of wild perennial *Glycyrrhiza* legume growing on 40 sites in central and northwestern China. Amplified fragment length polymorphism, genomic fingerprinting and sequencing of partial 16S rRNA genes revealed that the collection mainly consisted of *Mesorhizobium*, *Rhizobium*, *Ensifer* (*Sinorhizobium)*, *Agrobacterium* and *Paenibacillus* species. Based on symbiotic properties with the legume hosts *Glycyrrhiza uralensis* and *Glycyrrhiza glabra*, the nodulating species were divided into true and sporadic symbionts (Li *et al.,* 2011).

Deng *et al.,* (2011) isolated 115 endophytic bacteria from root nodules of the wild legume *Sphaerophysa salsula* grown in two ecological regions of Loess Plateau in China. RFLP and sequencing of 16S rRNA gene and enterobacterial repetitive intergenic consensus-PCR revealed the genetic diversity and phylogeny of the strains. Fifty of the strains found were symbiotic bacteria belonging to eight putative species in the genera *Mesorhizobium, Rhizobium* and *Ensifer (Sinorhizobium)*, harboring similar *nifH* genes; *Mesorhizobium gobiense* was the main group and 65 strains were nonsymbiotic bacteria related to 17 species in the genera *Paracoccus*, *Sphingomonas*, *Inquilinus*, *Pseudomonas*, *Serratia*, *Mycobacterium*, *Nocardia*, *Streptomyces*, *Paenibacillus*, *Brevibacillus*, *Staphylococcus*, *Lysinibacillus* and *Bacillus*, which were universally coexistent with symbiotic bacteria in the nodules.

Countless endophytes are believed to still exist undiscovered or poorly understood in nature, and thus pose a huge potential source of novel mechanisms and genetics which may be co-opted for the improvement of plant agricultural practices. Another important aspect is the plant growth promotion by possessing beneficial traits that endophytes use to improve plant nutrient use efficiency and potential avenues of future research. As nitrogen and phosphorus are the most limiting nutrients for plant growth and endophytes increase their procurement and are used to improve plant nutrient use efficiency.

### 2.2 Presence of beneficial characters in nodule endophytes

Some endophytes appear to posses the ability to manipulate host plant metabolism to increase nutrient uptake and change nutrient homeostasis (Kaldorf *et al.,* 1998). Modification of the soil via exudates is an important way that roots may increase the availability of nutrients. Plants have been shown to secrete up to 40% of their fixed carbon through their root systems as amino acids, organic acids, sugars, phenolics, mucilage, proteins and an array of additional secondary metabolites which may aid in optimizing their rhizospheres chemically and microbially (Badri *et al.,* 2009). Endophytes also increase nutrient availability and nutrient use efficiency by various methods.

#### 2.2.1 Endophytes enhance root and plant growth by producing phytohormones

Endophytes improve nutrient use efficiency by modulation of root growth. There are two key root system traits which can increase nutrient use efficiency if they are enhanced: root branching and root hair production, the latter which can account for up to 70% of total root length. Though root development is genetically programmed, many endophytes are able to modulate root size and structure (Schulz, 2006). Specifically, plant growth promoting rhizobacteria and endophytes including *Gluconacetobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Herbaspirillum* and *Pseudomonas* species, have been observed to improve plant growth through stimulation of root development (Dobbelaere *et al.,* 2007).

There are different mechanisms by which endophytes modify root development, but most appear to produce or block phytohormones including auxin, ethylene, cytokinin and gibberellin.

Endophytes stimulate root growth through secretion of auxin within the plant (Spaepen *et al.,* 2007). Indole-3-acetic acid is the most common plant auxin and can affect almost every aspect of plant development including cell enlargement and division, tissue differentiation, and responses to light and gravity (Boerjan *et al.,* 1995; Taiz *et al.,* 1998). In addition to being produced by plants, indole-3-acetic acid is also produced by root associated bacteria such as *Enterobacter* spp., *Pseudomonas* spp., *Azospirillium* spp. or *Streptomyces* sp. (Gao *et al.,* 2010). Indole-3-acetic acid is known to stimulate the elongation of primary roots when applied at low concentrations, but at higher concentrations induces ethylene production and inhibits primary root elongation to reduce root depth, but simultaneously induces initiation of lateral and adventitious roots and root hairs, which serve to increase root surface area. These phenomena have been well illustrated in auxin resistant *Arabidopsis* mutants *axr1* and *axr2* which produce fewer lateral roots than the wild type, while in *Arabidopsis* auxin over-producing mutants, *rooty* and *superroot*, there is a dramatic increase in the formation of lateral roots and root hairs (Boerjan *et al.,* 1995; Taiz *et al.,* 1998). Microbial biosynthesis of auxin was initially discovered in *Agrobacterium* where it plays a role in gall formation and pathogenesis, but since then many non-pathogenic bacteria and fungi have been shown to synthesize auxin using up to six different genetic pathways (Spaepen *et al.,* 2007).

Out of the 65 bacterial endophytes isolated from stem, root and nodule of two soyabean varieties *Glycine max* & *Glycine soja*, *83%* were observed to produce indole-3-acetic acid (Hung *et al.,* 2007). Similarly, Khan *et al.,* (2009) reported that four strains isolated from sweet potato (*Ipomoea batatas* (L.) Lam.) produce indole-3-acetic acid, out of eleven different strains belonging to the genera, *Enterobacter, Rahnella, Rhodanobacter, Pseudomonas, Stenotrophomonas, Xanthomonas* and *Phyllobacterium*. Thirty-nine endophytic bacterial strains were isolated from the nodule of *Lespedeza* sp. grown in two different locations of South Korea. Most of the isolates showed multiple plant growth promoting activity including indole acetic acid production (Palaniappan *et al.,* 2010). Similarly, another endophyte isolated from *Sophora alopecuroides* root nodules belonging to *Bacillus cereus* was shown to produce indole-3-acetic acid (Zhao *et al.,* 2011).

Root growth promotion can also be through the reduction of the volatile plant hormone, ethylene. Ethylene that is synthesized by plants upon exposure to abiotic or biotic environmental stress is called stress ethylene and is thought to initiate many plant stress symptoms including senescence, chlorosis and organ abscission which reduce plant

productivity and survival (Glick *et al.,* 2007). Ethylene affects roots by inhibiting elongation, promoting lateral root growth and stimulating root hair formation. In this way, ethylene in roots antagonizes auxin function and reduces root surface area available for nutrient absorption. Rhizosphere-inhabiting bacteria can affect plant ethylene by secreting 1-aminocyclopropane-1-carboxylate deaminase which breaks down the ethylene precursor 1-aminocyclopropane-1-carboxylate into α-ketobutyrate and ammonia. Though 1-aminocyclopropane-1-carboxylate deaminase-expressing endophytes are very important when roots are producing high levels of ethylene, they may not confer much benefit to plants growing under ideal conditions (Bernard, 2005).

Other microbially produced phytohormones, including cytokinins and gibberellins, can alter growth but have not been widely reported as root specific growth promoting mechanisms by endophytes (Tanimoto, 2005). Gibberellins is involved in seed germination, seedling emergence, stem and leaf growth, floral induction, flower and fruit growth, and most importantly, promotion of root growth and root hair abundance (Bottini *et al.,* 2004). Cytokinins are known to stimulate cell division, trigger cell expansion, promote stomatal opening, stimulate shoot growth and decrease root growth. It has been reported as an exudate of many bacteria, but its importance as a mechanism of plant growth promotion by bacteria has only been demonstrated in a few examples (García de Salamone *et al.,* 2001).

### 2.2.2 Endophytes enhance plant growth by solubilizing insoluble phosphates

Some soil rhizosphere bacteria and fungi that can exist as endophytes within roots, are able to mineralize organic or insoluble forms of nitrogen and phosphorus. As it has rarely been shown that endophytes exit the root to directly affect the soil, it is not clear how such apparent phosphate solubilisation or organic compound degradation could occur. Phosphorus is major essential macronutrients for biological growth and development. Microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic phosphorus of soil and make it available to the plants. The ability of some microorganisms to convert insoluble phosphorus to an accessible form, like orthophosphate, is an important trait in an endophyte for increasing plant yields (Rodriguez *et al.,* 2006; Chen *et al.,* 2006). Legumes like alfalfa and clover show a high positive response to phosphorus supplementation (Gyaneshwar *et al.,* 2002), but most of the supplemented phosphorus become unavailable when it reacts with soil components. Many soil microorganisms are able to solubilise this unavailable phosphorus through their metabolic activities exudating organic acids, which directly dissolve the rock phosphate, or chelating calcium ions that release phosphorus to the solution. A total of 373 endophytic bacteria were isolated from leaves, stems and roots of two soybean cultivars, out of that, 49% were able to solubilise mineral phosphate (Kuklinsky-Sobral *et al.,* 2004). Five strains isolated from roots of sunflower (*Helianthus*

*annus*) plants grown under irrigation or drought conditions were found to be good phosphate solubilizers (Forchetti *et al.,* 2007). Phosphate solubilization activity was also observed by non-rhizobial endophytes isolated from the surface sterilized root nodules of alfalfa (*Medicago sativa* L.) (Stajkovic *et al.,* 2009). Around 62% of nodule endophytes of *Lespedeza* sp. grown in two different locations of South Korea were observed to be able to sulobilise phosphate (Palaniappan *et al.,* 2010). Recently, Guiñazú *et al.,* (2010) reported phosphate solubilizing *Pseudomonas* sp. and *Bacillus* sp. from the soil and their single and co-inoculation with *Ensifer* (*Sinorhizobium) meliloti* improved alfalfa growth better compared to *Ensifer* (*Sinorhizobium) meliloti* inoculation. Solubilization of inorganic phosphate was exhibited by *Pseudomonas putida* strain isolated from roots of wild plants, small cactus (*Mammillaria fraileana*) colonizing rocks in the southern Sonoran desert (Lopez *et al.,* 2011).

### 2.2.3 Metabolite production by endophytes

Various types of secondary metabolites like antibiotics, organic acids, ammonia, enzymes and growth hormone (type of organic acids) are produced by the bacterial nodule endophytes. These metabolites beneficially affect the plant directly or indirectly. Ammonia fulfills the demand of nitrogen of plants and organic acids helps in solubilization of metals. A number of different scientists have reported metabolite production by endophytes. Hung *et al.,* (2007) reported that 33% of bacterial endophytes were producing pectinase enzyme, 51% of endophytes were producing cellulase enzyme isolated from soybean. Similarly, out of 91 bacterial isolates from roots of coastal sand dune plants, 23 were producing protease, 37 were producing pectinase, and 38 were producing chitinase (Dong-Sung *et al.,* 2007). Organic acid producing endophytic bacterial strains have been isolated by Forchetti *et al.,* (2007) from roots of sunflower. Three strains when grown in control medium produced jasmonic acid, 12-oxo-phytodienoic acid and abscisic acid. These three strains did not differ in amount of jasmonic acid or 12-oxo-phytodienoic acid produced, however abscisic acid content was higher than that of jasmonic acid and production of both abscisic acid and jasmonic acid increased under drought condition. Stajkovic *et al.,* (2009) observed that out of the selected five isolates among the 15 endophytic non-rhizobial strains isolated from surface sterilized root nodules of alfalfa (*Medicago sativa* L.), none of the tested strains could produce organic acids, but three strains were positive for ammonia production. Nimnoi *et al.,* (2010) isolated ten ammonia producing actionobacteria from healthy shoots and roots of *Aquilaria crassna* (eaglewood) and amount of ammonia ranged between 2 to 60 mg $ml^{-1}$. Similarly, in total 61.1% of endophytic bacteria were ammonia producers and 69.4% were acetoin producers, out of 36 selected bacterial isolates from five mangroves and two salt-marsh plant species (Gayathri *et al*, 2010).

A few non-hormone metabolites secreted by microbes appear to modify root architecture as well. Under low oxygen conditions, rhizobacteria emitting the volatile glucose metabolites, acetoin and 2,3 butanediol have been shown to stimulate *Arabidopsis* root growth (Ryu *et al.,* 2003). Another non-hormone metabolite important for plant growth promotion by bacteria is the antibiotic 2,4-diacetylphloroglucinol which is produced by *Pseudomonas fluorescens* isolates containing the *phlD* gene. This has been shown to inhibit primary root growth, while stimulating lateral root production in tomato seedlings through alteration of auxin signalling (Brazelton *et al.,* 2008).

**2.3 Beneficial effects of application of endophytic inoculants**

Applications of endophytes to agriculture have traditionally taken the form of soil or seed inoculation. Externally inhabiting endophytes have the ability to gain entry to their hosts, from the specialized signalling rhizobia and mycorrhiza use to form symbiosomes, to more basic crack entry such as that observed by *Klebsiella pneumoniae* 342 at lateral root junctions (Dong *et al.,* 2003). To be effective, inoculations should be properly planned and steps should first be taken to assess whether there are beneficial endophytes already present in the soil, how the microbes of interest might persist and compete, and whether these are able to promote improved mineral nutrition of the plant of interest under the expected growing conditions. Rhizobial inoculants for legumes are a well established and widely sold agricultural product and combining it with the effects of accessory microbes, like the phosphate solubilising soil fungus, *Penicillium bilaii*. A clear direction for the future application of endophytes to inoculants will be this kind of microbial synergism; for example, it was shown that mixing *Bacillus thuringiensis* KR1 (isolated from nodules on kudzu vines) with *Bradyrhizobium japonicum* SB1 yielded significantly greater nodule number and plant biomass, than inoculation with the *Bradyrhizobium* alone (Mishra *et al.,* 2009). Further, in All India Coordinated Research Project (AICRP) on chickpea MULLaRP and pigeon pea it have been shown that combined inoculation of rhizobia with phosphate solubilizing bacteria, plant growth promoting rhizobacteria, vesicular arbuscular mycorrhiza and *Trichoderma* enhance the nodulation and crop yield of the pulse crops (Dudeja *et al.,* 2011b).

A few other examples of endophyte inoculants exist in tropical countries where biofertilizers are viewed as economically attractive alternatives or supplements to chemical fertilizers (Uribe *et al.,* 2010). Endophytic strains of *Pseudomonas fluorescens* are sold in India by Mani Dharma Biotech which promotes root growth, while FOSFORINA is a strain of *P. fluorescens* distributed in Cuba to reduce the need for mineral phosphorus applications by solubilising phosphate in plant rhizospheres. BIOGRO is a biofertilizer mixture containing a strain of *P. fluorescens*, two bacilli and a soil yeast isolated from rice rhizospheres in Vietnam; they were selected for their ability to fix nitrogen, solubilise mineral phosphate, and

secrete antibiotic compounds. This blend has been shown to significantly increase grain and straw yields, total nitrogen uptake, and grain concentration of nitrogen: farmers using this product in Vietnam were able to reduce their nitrogen application by 43 kg $Nha^{-1}$ while increasing rice yields (Cong *et al.,* 2009). While there is much lab, greenhouse and field evidence that other endophytes can increase the nutrient use efficiency of commercially important crops like rice, corn, sugarcane and wheat, fully developed commercial products and inoculation practices have not been developed for a large number of endophytes.

Interactions in the rhizophere may have a pronounced effect on plant growth. Plant growth promoting rhizobacteria were isolated from bean (*Phaseolus vulgaris* L.) nodules. One of the strain was found to increase bean yield and growth when plant were co-inoculated with *Rhizobium leguminosarum* bv. *phaseoli* under nitrogen-free conditions. This helper strain was a gram-positive, spore-forming and rod-shaped bacterium. Biolog test and phylogenetic analysis of 16S rRNA gene hypervariant region sequences demonstrated the strain to be *Bacillus subtilis* (Lee *et al.,* 2005). Similarly, Stajković *et al.,* (2009) observed in alfalfa plants that co-inoculation of all non-rhizobial strains with *Ensifer* (*Sinorhizobium*) *meliloti* positively influenced nodule number, while shoot and root parameters were comparable to those of alfalfa plants inoculated with *Ensifer* (*Sinorhizobium*) *meliloti* alone. However, single inoculation with non-rhizobial strains caused significant increase in shoot and root parameters compared to uninoculated plants, indicating that non-rhizobial strains possess some plant growth promoting potential. Stajkovic *et al.,* (2011) examined two *Bacillus* and two *Pseudomonas* strains for their ability to promote common bean growth (*Phaseolus vulgaris* L.) when co-inoculated with *Rhizobium phaseoli*. Co-inoculation with *Rhizobium* and *Pseudomonas* sp. LG or *Bacillus* sp. Bx improved shoot dry weight, nitrogen and phosphorus contents in bean plants, compared to inoculation with *Rhizobium* alone. It was observed that *Pseudomonas* sp. LG promoted bean growth and particularly phosphorus uptake more efficiently than *Bacillus* sp. Bx.

To continue the development of endophytes for agricultural application, discovery of novel strains will be a fruitful endeavour which may result in new species that can be directly used in agriculture, or at least provide us with important additional information about how endophytes can influence and improve plant nutrient use efficiency. If every plant harbours at least one novel endophyte, then there are still about 300,000 undiscovered endophytes in the world! Wild plants may grow in nutrient poor environments enriched in endophytes that help their hosts to acquire nutrients or resist stresses not found in domesticated crops growing under optimized conditions. For example, plants have been shown to enrich their endophytic communities for hydrocarbon metabolizers when grown in petroleum contaminated soil (Siciliano *et al.,* 2001). One important future direction of endophyte research will be the

dissection, isolation, upregulation and genetic transfer of beneficial mechanisms from endophytes, to other microbes or even to plants. A large number of endophyte genomes are now available or are being sequenced (Krause *et al.,* 2006). As some endophytes live within plants in unculturable states, it may be important to undertake metagenomic approaches to acquire a more complete picture of the endophytic community; this is being done for rice in an ambitious project to sequence 100 Mb of endophyte DNA extracted from inside rice plants. Isolation of the genes involved in these mechanisms may allow for their pyramiding within endophytes or their transfer into plants for enhanced nutrient use efficiency. Transfer of 1-aminocyclopropane-1-carboxylate deaminase from *Enterobacter cloacae* into rhizospheric *Azospirrilum brasilensis* increased the root elongation potential of this strain in tomato and canola (Holguin *et al.,* 2001), suggesting that similar transgenic techniques may increase the root growth promoting ability of endophytic strains. This has been shown to be an effective technique in nodule forming rhizobia: 1-aminocyclopropane-1-carboxylate deaminase genes from *Ensifer* (*Sinorhizobium)* sp. BL3 were introduced into *Rhizobium* sp. strain TAL1145, increasing its 1-aminocyclopropane-1-carboxylate deaminase activity, resulting in nodules with greater number and sizes, and producing higher root mass on the tree legume *Leucaena leucocephala* (Tittabutr *et al.,* 2008). Despite much investment and some promising experiments, only one genetically modified endophyte has thus far been commercially released: strain RMBPC-2 of *Ensifer* (*Sinorhizobium) meliloti*, sold by the American company, Research Seeds Inc., has been modified with genes to enhance C4-dicarboxylic acid uptake and nitrogen fixation in symbiosis with alfalfa.

# CHAPTER –III

# MATERIALS AND METHODS

## 3.1 Chemicals

All the chemicals and reagents used during the course of study were of analytical grade obtained from BDH laboratories, Hi-Media laboratories Pvt. Ltd., E. Merck laboratories Pvt. Ltd., CDH laboratory reagents and Bengal chemicals and pharmaceuticals Ltd. Chemicals used for molecular studies were obtained from Bangalore Genei Pvt. Ltd., Hi-Media, Fermentas and Bioron. The primers were synthesized from Sigma Aldrich Pvt. Ltd., USA.

## 3.2 Instruments

The instruments used during the present research work were Microcentrifuge (Eppendorf, Germany); Horizontal Electrophoresis Apparatus (Tarsons, Kolkata, India); Mastercycler gradient (Eppendorf, Germany); Orbital Shaking Incubator (Remi Equipments Ltd., Mumbai, India); BOD incubator (Sew, India); Gel documentation System (DNR Bio-Imaging Systems, Mini Bis Pro, Israel); Water bath (Remi Equipments Ltd., Mumbai, India); refrigerator (Gem, India); autoclave (Narang Scientific Works Pvt. Ltd., New Delhi, India); deep freezer (Vestfrost, Blue star, India); pH-meter (Hanna Instruments, Mauritius); Microscope (Geytnor, India); UV-Vis Spectrophotometer (Elico SL -1159, Elico Ltd., Hyderabad, India); Camera (Sony cyber shot 8.1 megapixels); electronic balance (Afcoset, The Bombay Burmah Trading Corp. Ltd., India) and oven (Associated Scientific Technologies, Delhi, India).

## 3.3 Seeds

Seeds of chickpea (Cicer arietinum L.) var. HC-5 were obtained from Pulses Section of Department of Genetics and Plant Breeding, College of Agriculture, Chaudhary Charan Singh Haryana Agricultural University, Hisar.

## 3.4 Preparation of Media

All the media were prepared in distilled water and autoclaved at 15 lbs $in^{-2}$ pressure for 20 min. While preparing broth, agar-agar was omitted from the media. Compositions of various media used in the present investigation are given below:

### 3.4.1 Trypton soya agar medium (Andreote *et al.,* 2008)

| **Components** | **Quantity ($gL^{-1}$)** |
|---|---|
| Tryptone | 15.0 |
| Peptone | 5.0 |
| NaCl | 5.0 |
| Agar-agar | 20.0 |
| pH | 7.0 |

### 3.4.2 Peptone water broth tubes (Cappuccino *et al.,* 1992)

| **Components** | **Quantity ($gL^{-1}$)** |
|---|---|
| Peptone | 10 |
| NaCl | 5 |

### 3.4.3 Pikovskaya's medium (Pikovskaya, 1948)

| **Components** | **Quantity ($gL^{-1}$)** |
|---|---|
| Glucose | 10 |
| Yeast extract | 0.5 |
| $NH_4SO_4$ | 0.5 |
| NaCl | 0.2 |
| KCl | 0.2 |
| $MgSO_4.7H_2O$ | 0.1 |
| $FeSO_4.7H_2O$ | 0.005 |
| $MnSO_4.7H_2O$ | 0.01 |
| $Ca_3(PO_4)_2$ | 5.0 |
| Agar-agar | 15.0 |
| pH | 7.2 |

### 3.4.4 Methyl red-Voges Proskauer broth

| **Components** | **Quantity ($gL^{-1}$)** |
|---|---|
| Peptone | 5.0 |
| $K_2HPO_4$ | 5.0 |
| Glucose, 10% solution | 50 mL (Sterilized separately) |
| pH | 7.6 |

### 3.5 Solutions for molecular studies

#### 3.5.1 CTAB/NaCl solution:

NaCl (4.1 g) was dissolved in 80 mL of distilled water and 10 g of CTAB was added with continuous stirring and heating. Final volume was made to 100 mL and the solution was sterilized.

#### 3.5.2 5 M NaCl:

NaCl (29.22 g) was dissolved in 80 mL of distilled water. Final volume was made to 100 mL and the solution was sterilized.

#### 3.5.3 10% SDS:

Sodium dodecyl sulphate (100 g) was dissolved in 900 mL of distilled water with heating at 68˚C. By adding conc. HCl, pH was adjusted to 7.2 and final volume was made to 1000 mL.

#### 3.5.4 0.5 M EDTA (pH 8.0):

EDTA (186.1 g) was dissolved in 800 mL of distilled water using a magnetic stirrer. By adding NaOH pellets, pH was adjusted to 8.0. Final volume was made to 1000 mL and the solution was sterilized.

#### 3.5.5 1 M Tris (pH 8.0):

Tris base (121.1 g) was dissolved in 800 mL of distilled water. Solution was allowed to cool and then pH was adjusted to 8.0 by adding conc. HCl. Final volume was made to 1000 mL and the solution was sterilized.

#### 3.5.6 TE buffer:

To prepare TE buffer, 10 mL of 1M Tris (pH 8.0) and 2 mL of 0.5 M EDTA were added in 900 mL of distilled water and the final volume was made to 1000 mL.

#### 3.5.7 70% Ethanol:

To 70 mL of dehydrated ethanol, 30 mL of distilled water was added.

#### 3.5.8 24:1 Chloroform/Isoamyl alcohol:

Isoamyl alcohol (4 mL) was added in to 96 mL of chloroform.

#### 3.5.9 25:24:1 Phenol/Chloroform/ Isoamyl alcohol:

Equal volumes of tris saturated phenol and 24:1 chloroform/Isoamyl alcohol were mixed.

### 3.6 Electrophoresis buffer and materials

#### 3.6.1 10X TBE Buffer (Tris Borate EDTA):

TBE buffer was prepared by dissolving 108 g of tris base, 40 mL of 0.5 M EDTA (pH 8.0) and 55 g of boric acid in one litre of distilled water to make a concentrated stock (10X). It was diluted 10 times to work with (1X).

### 3.6.2 Agarose gel:

For PCR product, 1.5% (w/v) and for genomic DNA, 1% (w/v) agarose was prepared in 1X TBE buffer and used for gel preparations.

### 3.6.3 Gel loading dye (6X):

Gel loading dye was prepared by mixing 10 mM Tris-HCl, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60 mM EDTA, 60% glycerol.

### 3.6.4 Ethidium bromide:

Ethidium bromide (0.1 g) was added to 10 mL of distilled water and the solution was stirred on magnetic stirrer for several h. For staining 4 µL of stock solution was added to 100 mL of gel solution.

### 3.6.5 DNA molecular weight markers:

A 100 bp ladder was used as molecular weight marker for comparing PCR amplification product and for DNA, *Eco*R1 digest was used as marker. For each loading, 1 µL of the ladder mixed with 3 µL of loading dye was used.

## 3.7 Solutions for polymerase chain reaction (PCR)

### 3.7.1 10X Assay buffer:

The 10X assay buffer commercially provided contains 100 mM Tris-HCl (pH 8.8), 500 mM KCl, 15 mM $MgCl_2$.

### 3.7.2 Taq DNA polymerase:

Taq DNA polymerase was at a concentration of 3U $\mu L^{-1}$ in a storage buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1mM DTT, 0.5 % Tween 20 (v/v), 0.5% Igepal and 50% Glycerol (v/v).

### 3.7.3 dNTP's mix:

To prepare 10 mM dNTP's mix, 1 µL of each 100 mM dNTPs viz. dATP, dCTP, dGTP and dTTP was added into 36 µL of distilled water.

### 3.7.4 Primers:

The forward and reverse primers used for 16S rDNA gene amplification were:

fD1 (5' AGAGTTTGATCCTGGCTCAG 3') (Lukow *et al.,* 2000)

rD1 (5' AAGGAGGTGATCCAGCCGCA 3') (Heuer *et al.,* 1997)

The stock solution of primer was prepared by reconstituting lyophilized primer in 100 µL sterilized millipore water and was stored at -20°C. As a working solution 100 ng $\mu L^{-1}$ concentration was used by dissolving 7.6 µL of above prepared stock in 92.4 µL of sterilized Millipore water.

## 3.8 Restriction endonucleases

Three restriction endonucleases were used for the Restriction Fragment Length Polymorphism (RFLP) of amplified 16S rDNA product.

**3.8.1** ***HaeIII***

5’ ——GGCC—— 3’
3’ ——CCGG—— 5’

**Source:** ***Haemophilus aegypius*****.**

**3.8.2** ***MspI***

5’——CCGG——3’
3’——GGCC——5’

**Source:** ***Moraxella*** **species.**

**3.8.3** ***HinfI***

5’——GANTC——3’
3’——ATNAG——5’

**Source:** ***Haemophilus influenzae*** **Rf**

**3.9 Solutions for total nitrogen contents in shoots of chickpea plant by Kjeldahl’s method (Bremner, 1965).**

**3.9.1 Digestion mixture:**

It was prepared by mixing $K_2SO_4$, $CuSO_4$ and $SeO_2$ in ratio of 10:4:1 (W/w).

**3.9.2 Boric acid indicator solution:**

In 700 mL of hot distilled water 20 gram of boric acid was dissolved. The cooled solution was transferred to one litre volumetric flask containing 20 mL of mixed indicator solution (prepared by dissolving 100 mg bromocresol green and 50 mg of methyl red in 100 mL of ethanol). After mixing the contents of the flask, the solution was diluted to 1000 mL with distilled water and mixed thoroughly.

**3.9.3 0.02N $H_2SO_4$:**

It is prepared by adding 0.546 mL of conc. $H_2SO_4$ in distilled water to make 1000 mL of final volume.

**3.9.4 40% NaOH solution:**

Dissolve 40 g of NaOH pellets dissolved in 100 mL of distilled water.

**METHODS**

### 3.10 Isolation of endophytic bacteria from chickpea nodules:

Chickpea plants being grown in Pulses Section, University Farm, Chaudhary Charan Singh Haryana Agricultural University, Hisar, were uprooted. Healthy nodules were removed separately from chickpea plants and were surface sterilized by using 0.2 % $HgCl_2$ and ethanol (Vincent, 1970). Thereafter, a cut was made in nodules with a sterilized knife in a sterilized petriplate and a loopful of nodule sap was streaked on TSA media plates. Simultaneously, from each batch, nodule samples were kept on TSA medium plates to ensure proper surface sterilization of nodule sample. The plates were incubated at 28+2˚C and growth was observed daily for 5-6 days. The endophytic bacterial isolates were picked up from the plates and were restreaked for purification purpose. Single endophytic bacterial clones were picked up from the plates and maintained on TSA medium slants. In case of growth around the nodules, all the isolates made from that batch were discarded, so as to ensure only endophytic bacteria are isolated. The slants were stored at 4˚C in a refrigerator for further studies.

### 3.11 Study of morphological characters of endophytic bacterial isolate

Different morphological characters like colony morphology, cell morphology, Gram's reaction and position of spores were studied for all the isolates

#### 3.11.1 Study of colony morphology

All the bacterial isolates were observed for colony morphology during the purification process. The colony morphology was studied on plates after streaking a loopful of isolated colony and colony colour, colony size, colony shape and gum production were observed.

#### 3.11.2 Study of cell shape, Gram's reaction and presence of spore

Gram's staining and spore staining was performed on all the bacterial isolates. A smear of isolated colonies was prepared and heat fixed and then, stained with Gram's stain and spore stain separately. Slides were observed under Geytnor microscope at 100x. Cell shape, cell size, Gram's reaction and presence of spore were observed and where ever necessary photographs were also taken.

### 3.12 Characterization of selected chickpea nodule endophytic bacterial isolates for beneficial traits:

Selected endophytic bacterial isolates were characterized for promotion of chickpea root growth, phosphate-solubilization, ammonia and organic acid production.

#### 3.12.1 Chickpea root growth promotion by nodule endophytic bacteria:

Plant growth promoters or auxins are produced by different bacteria which can also be assessed by using root growth promotion assay. Therefore, chickpea root growth promotion by the nodule endophytes was assessed.

To study root growth promotion in water agar plates, 1.5% and 1.2% water agar media was prepared by dissolving 15 and 12 g of agar-agar in 1000 mL of distilled water respectively and autoclaved. Then, the prepared media was poured into sterilized petriplates.

Healthy chickpea seeds were selected for growth promotion assay and sterilized with 0.2% $HgCl_2$ and ethanol and after 5-6 washing with sterilized distilled water seeds were transferred to 1.5% water agar plates. After 24-48 h when seeds began to germinate they were transferred to the freshly prepared 1.2% water agar plates in triplicates and inoculated with freshly grown nodule endophytes using 0.25 mL of the test culture per seedling. The plates were incubated at 28+2˚C for 7 days. Observations were taken for root growth and root length was measured in comparison to uninoculated control. Different isolates were placed in different categories on the basis of root length i.e. <5 cm; 5 to <10 cm, 10 to <15 and ≥15 cm, so as to have a better comparison among the different nodule endophytes.

### 3.12.2 Phosphate solubilization by nodule endophytic bacteria

Phosphate solubilizing activity of nodule endophytic bacteria was assessed on Pikovskaya's medium (Pikovskaya, 1948). All the isolated cultures were inoculated in flasks containing 25 mL TSA broth. The flasks were incubated at 28+2˚C in Orbital Shaking Incubator for 3 days. Log phase growing cells of each culture from the culture flasks were spotted on Pikovskaya's medium plates (Pikovskaya, 1948) with the help of an inoculating loop. These plates were incubated at 28+2˚C for 5-7 days. Formation of solubilization zone around the spotted colonies indicated P-solubilizing bacteria. On the basis of solubilization zone, different endophytes were categorized into different categories like low, moderate, high and very high to have a better comparison among the different nodule endophytes.

### 3.12.3 Ammonia production by nodule endophytic bacteria in peptone water

All the chickpea nodule endophytic bacterial isolates were screened for the production of ammonia in peptone water (Cappuccino *et al.,* 1992). Freshly grown cultures were inoculated in test tubes containing 10 mL of peptone water broth in each tube and incubated at 28±2°C for 4-5 days. Subsequently, Nessler's reagent (1 mL) was added in each tube. The development of colour from yellow to brownish orange was a positive test for ammonia and on the basis of intensity of colour developed on adding Nessler's reagent, the endophytes were categorized into different categories.

### 3.12.4 Determination of organic acid production by the nodule endophytic bacteria

Organic acid producing ability of all the bacterial isolates was determined by methyl red test (Sambrook *et al.,* 2001). The tubes of MR-VP broth were prepared and test cultures were inoculated. Tubes were incubated at 28±2°C for 5 days. Following incubation, 5-6 drops of methyl red indicator solution was added in each tube and the color obtained was observed. A positive reaction was indicated by appearance of bright red color. Yellow color indicated a negative reaction while a weekly positive test was indicated by orange color. Based on this type of observation, all the bacterial endophytes were ranked as very high, high, moderate, low and very low acid producers.

### 3.13 Isolation of Genomic DNA of chickpea nodule endophytic bacteria (Modified method of Ausubel *et al.,* 2001)

Genomic DNA was extracted from endophytes by using CTAB method. The selected 55 nodule endophytic bacterial isolates were grown in flasks containing 25 mL of TS medium on a rotary shaker at 28±2°C for 1-2 days. After the attainment of growth, 1.5 mL of each culture was transferred in sterilized eppendorf tube, centrifuged in microcentrifuge for 2 min at 9000 rpm or until a compact pellet was formed. Supernatant was discarded carefully and the pellet was resuspended in 570 µL of 1X TE buffer by repeated pipetting. To each of these tubes, 30 µL of 10% SDS was added. It was mixed thoroughly and incubated overnight at 37˚C. After incubation, 100 µL of 5M NaCl was added and mixed thoroughly. Then 80 µL of CTAB/NaCl solution was added in this mixture, mixed thoroughly and incubated for 10 min at 65˚C. Approximately, equal volume of chloroform/isoamyl alcohol (24:1) was added to it and spinned for 10 min at 10,000 rpm. Aqueous, viscous supernatant was transferred to fresh tubes and to each tube, equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added. The contents of the tubes were mixed thoroughly and centrifuged at 10,000 rpm for 10 min. Again the aqueous phase was transferred to fresh tubes and to each tube, an equal volume of isopropanol was added. The contents of tubes were mixed gently for several times and tubes were incubated in freezer at -4˚C for overnight. Next day, tubes were centrifuged at 10,000 rpm for 15 min and the supernatant was discarded carefully. The pellet was washed with 200 µL of 70% ethanol and centrifuged at 10,000 rpm for 5 min. The supernatant was discarded carefully and the tubes were air dried till there was no smell of ethanol. Then, the pellet was redissolved in 60 µL of sterilized distilled water by tapping followed by incubation at 65˚C for 10 min and again tapping was done. Finally, the DNA contained in water was stored at -20ºC in deep freezer.

### 3.14 Quantification of genomic DNA

The genomic DNA was diluted 10 times and quantified by measuring the absorbance at 260 nm and 280 nm. The amount of DNA was estimated using the relationship that O.D. of 1.0 corresponds to 50 µg $mL^{-1}$. The purity of DNA was assessed by measuring A260/A280 ratio; A260: A280 = 1.5 - 1.8 for pure DNA. Further, purity of DNA was also checked on 1% agarose gel and bands were observed. In case of faint or no bands, DNA extraction was repeated as described in section 3.13.

### 3.15 Polymerase Chain Reaction (PCR)

The purpose of a PCR is to amplify the particular segment of DNA so that, we can obtain multiple copies of that segment. In this study, PCR was used to amplify the 16S rDNA

genes of endophytic bacterial isolate. The PCR conditions were optimized to get maximum quantity of amplified product.

### 3.16 Amplification of 16S rDNA sequences (Lukow *et al.*, 2000)

Amplification of 16S rDNA sequences was carried out by polymerase chain reaction using a thermal cycler. The primers fD1 and rD1 enable the amplification of 16S rDNA sequences present in endophytic bacterial DNA. The amplification reaction was performed in 24.0 µL volume per reaction. The contents of reaction mixture are given below:

**PCR Reaction Mixture:**

| **Constituents** | **Volume (µL)** |
|---|---|
| dNTP's mix (10 mM) | 0.5 |
| Taq buffer (10x) with 1.5 mM $MgCl_2$ | 2.5 |
| Taq DNA polymerase (3U $\mu L^{-1}$) | 0.5 |
| fD1 (10 µM) | 1.0 |
| rD1 (10 µM) | 1.0 |
| Template DNA | 2.0 |
| SDW | 16.5 |
| Total Volume | 24.0 |

DNA amplification was carried out with the following PCR conditions:

1. Initial denaturation at 94˚C for 4 min.
2. Denaturation at 94˚C for 30 sec.
3. Annealing at 50˚C for 40 sec.
4. Extension at 72˚C for 90 sec.
5. Repeat steps 2 to 4 at least 40 times.
6. Final extension at 72˚C for 10 min.
7. Holding at 4˚C.

   Lid temperature was maintained at 105˚C.

### 3.17 Gel electrophoresis

Genomic DNA was resolved by using 1% agarose gel in 1X TBE buffer and 1.5% agarose gel was used for analysis of PCR amplified 16S rDNA products and for analysis of digested PCR products. Before casting the gel, 4 µL of EtBr was added per 100 mL of agarose solution. DNA (5 µL) was mixed with 3 µL of loading dye and loaded in the well, marker DNA was also loaded. The gel was run at 60 V. After 2.5-3 h, loaded gel was visualized under UV light in Gel Documentation system. *Eco*R1 digest was used as marker for extracted DNA. Similarly, DNA ladder of 100 bp was used as the marker DNA for 16S rDNA PCR products and digested PCR products.

### 3.18 Restriction fragment length polymorphism (RFLP) or Amplified ribosomal DNA restriction analysis (ARDRA)

Restriction fragment length polymorphism is a technique used for identification of individual based upon specific restriction pattern of DNA. In RFLP analysis, amplified DNA sample is cut with restriction enzyme and resulting fragments are separated by agarose gel eletrophoresis according to their molecular size. In this work, the amplified product of 16S rDNA was subjected to three restriction endonucleases: *HaeIII*, *MspI* & *HinfI* and was incubated under constant temperature 37˚C for 12 h in a thermal cycler.

**Digestion mixture for *HaeIII* consisted of:**

| | |
|---|---|
| Milli pore water | 9 µL |
| Taq buffer (2x) | 2 µL |
| Restriction endonuclease (3U $\mu L^{-1}$) | 1 µL |
| 16S rDNA amplification product | 6 µL |

**Digestion mixture for *MspI* consisted of:**

| | |
|---|---|
| Milli pore water | 9 µL |
| Y+/Tango buffer (2x) | 2 µL |
| Restriction endonuclease (3U $\mu L^{-1}$) | 1 µL |
| 16S rDNA amplification product | 6 µL |

**Digestion mixture for *HinfI* consisted of:**

| | |
|---|---|
| Milli pore water | 9 µL |
| Taq buffer (2x) | 2 µL |
| Restriction endonuclease (3U $\mu L^{-1}$) | 1 µL |
| 16S rDNA amplification product | 6 µL |

The digested product was resolved on 1.5 % agarose gel and the polymorphic patterns were recorded as described earlier in section 3.17.

#### 3.18.1 Scoring and data analysis:

The PCR was repeated 3 times for each isolate and only reproducible bands were scored. The size of each band was compared with the standard marker and the profiles of the isolates were made. Depending upon the presence or absence of a particular band, 0-1 matrix was prepared. Similarity matrices were constructed following SimQual Coefficient and were analyzed by UPGMA (unweighted pair grouping with mathemetic average) cluster analysis using NTSYS-pc program (Version 2.1: Exeter Software, Setauket, N.Y.) (Rohlf, 1998). Dendrograms were constructed from the genetic similarity between different nodule endophytic bacteria by the UPGMA.

### 13.19 Screening of nodule endophytes for promotion of plant growth and nitrogen fixation under pot culture conditions

The plant growth promoting and enhancing nitrogen fixation efficiency of selected 39 bacterial nodule endophytic isolates was assessed under pot culture conditions using chickpea as test host. Sandy loam soil was collected from dry land area of Chaudhary Charan Singh Haryana Agriculture University research farm. The soil analysis showed that soil was sandy loam soil with pH 8.6; organic carbon 0.23 kg $ha^{-1}$; electrical conductivity 0.53 $dSm^{-1}$; phosphorus 4 kg $ha^{-1}$; potassium 293 kg $ha^{-1}$ and available nitrogen as 133 kg $ha^{-1}$. Six to seven kg of soil was taken in earthern pots. Seeds of chickpea var. HC-5 were surface sterilized by using 0.2% mercuric chloride and alcohol (Vincent, 1970). Three replicates of each treatment were kept and in each pot uniform inoculation of *Mesorhizobium* strain CH1233 was done. All the seeds were inoculated with 3 mL of inoculum of bacterial root endophytic isolates. Five controls were also kept; one absolute control without any treatment, one only with *Mesorhizobium* inoculation and three inoculants of PSB (Strain PS36), PSB1 (AICRP) and *Piriformaspora indica* were used. After germination three plants in each pot were maintained. Pots were irrigated on alternate day or as and when required. After 60 days of growth, plants were uprooted and observation on nodule numbers, nodule, root & shoot dry weight and total shoot nitrogen content were recorded after drying the samples in oven at 80˚C till constant weight.

#### 3.19.1 Total nitrogen estimation in plant sample by Kjeldahl's method:

Dried plant samples were ground and 200 mg of plant sample was taken in the digestion tube. To each tube 1 g of digestion mixture and 10 mL of sulfuric acid was added and kept overnight at room temperature. There was one blank without plant sample. It was then digested on Kjeldatherm digester (Gerhardt, Germany) till bluish green colour appeared in the tube. After cooling it to room temperature the contents with the washings were transferred in a 25 mL volumetric flask and 10 mL of the diluted sample was steam distilled by adding excess of 40% NaOH using Parnus Wagner distillation apparatus till dull brown colour appeared. Ten mL of boric acid indicator solution was taken in 100 mL Erlenmeyer flask that was marked to indicate a volume of 50 mL and flask was placed under the condenser of steam distillation apparatus. Distillation was started by closing stopcock on the steam bypass tube of distillation apparatus. When the distillate reached to 50 mL mark of receiver flask, opening the stopcock on steam bypass tube stopped the distillation and the end of the condenser was rinsed. The contents were titrated with 0.02 N $H_2SO_4$. The colour changed at the end point was from greenish blue to permanent faint pink.

**Percentage N was calculated as follows:**

1 mL of 0.02N $H_2SO_4$ used = 0.28 mg nitrogen

$$\%N = \frac{0.28\ (S-B)\ X\ 100}{\text{Weight of plant sample in mg}}$$

Where,

S = mL of N/50 $H_2SO_4$ used for sample

B = mL of N/50 $H_2SO_4$ used for blank

Total nitrogen uptake by shoot = % nitrogen × shoot weight

# CHAPTER –IV

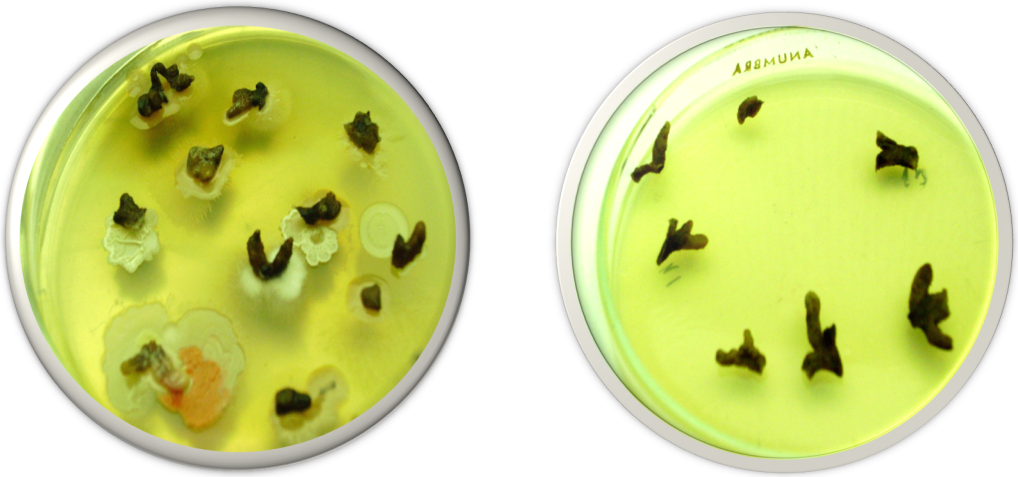
# RESULTS

Plants are constantly involved in interactions with a wide range of bacteria. These plant associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes) and the inside of plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots, nodules and seeds of various plant species. Endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth, nitrogen fixation and induction of resistance to plant pathogens. Rhizobia are perhaps the best known beneficial plant-associated bacteria because of the importance of the nitrogen fixation that occurs during the *Rhizobium*-legume symbiosis. However, endophytic bacteria have been isolated from roots and noduls of legume plants such as alfalfa, clover and pea. Bacteria of several genera have been isolated from legume tissues, including *Aerobacter, Aeromonas, Agrobacterium, Bacillus, Chryseomonas, Curtobacterium, Enterobacter, Erwinia, Flavimonas, Pseudomonas* and *Sphingomonas*. However, rarely any report is available from Indian subcontinent regarding the isolation of endophytes from nodules of legumes. Therefore, the present investigation was undertaken to isolate, characterize and to know the molecular diversity of endophytic bacteria from chickpea nodules. The results obtained in the present investigation are presented below:

## 4.1 Isolation of bacterial nodule endophytes from chickpea

In the present study a total of 76 endophytic bacteria were isolated from the nodules of chickpea plants being raised in Pulses Section of the Department of Genetics and Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar. To ensure that only endophytic bacteria are isolated from the nodules, care of nodule serilization was taken. Nodules were first sterilized with 0.1% mercuric chloride and were kept on TSA media plate, after a few days contamination was observed around the nodules. Then, the nodules were serilised with 0.2% mercuric chloride and this time no contamination was observed even upto 20 days (Fig. 4.1). Endophytic bacterial isolates were then purified (Fig. 4.2) and maintained on TSA media slants and further studies were carried out. CNE (chickpea nodule endophyte) prefix was given to all the cultures during the nomenclature. The different endophytes were

named on the basis of isolates made from different nodules like CNE2 to CNE1044 or isolates from the same node like CNE7 and CNE7-1.



**A** **B**

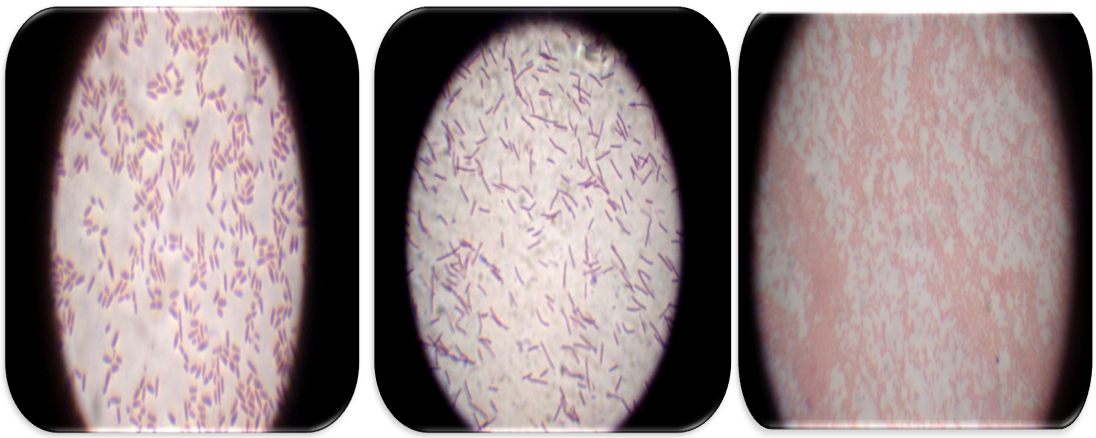
**Fig. 4.1: Microbial growth around chickpea nodules after sterilization of nodules with 0.1% mercuric chloride (A) and with 0.2% mercuric chloride (B)**


**Fig. 4.2: Purified endophytic bacteria from chickpea nodules**

**3.2**

**Characterization of chickpea nodule endophytic bacterial isolates for morphological characters**

All the nodule endophytic bacterial isolates were used to study the different morphological characters. During the purification of isolates, colony morphology was studied and simultaneously, gram staining was done by making smears from isolated colonies and shape and size of the cell was also monitored. Spore staining was also done to know the presence and position of spores. A huge variation was seen in the colour of the colonies (Fig. 4.2). Colouration of the colony was transparent, white, pale white, off white, creamy white, cream, dull cream, pinkish cream, Orangish cream, brownish cream, pale, yellow, light brown, brown, dull brown, silver brown, pale brown, pinkish brown, very light pink, pink, red. Colony morphology of chickpea nodule endophytes varied from gummy to non gummy; flat to raised; irregular, uneven & rhizoid to round; colony size was minute, small & medium to large. Cell size also varied from very small, small, medium & long to very long; cell shapes were variable from cocci to rods. Out of 76 bacterial isolates from chickpea nodules, 71 isolates were gram positive and five were gram negative (Fig. 4.3). Among the gram positive bacterial isolates 61 were spore formers and position of the spores varied from central to terminal (Table 4.1)



**A** **B** **C**

**Fig. 4.3: Gram's staining of bacterial nodule endophytes showing gram positive (A and B) and gram negative (C) bacteria**

### 3.3 Characterization of chickpea nodule endophytic bacterial isolates for beneficial traits

All the endophytes from chickpea nodules were screened for the presence of beneficial characters like root growth promotion, phosphate solubilization, ammonia and organic acid production.

**Table 4.1: Morphological characters of bacterial endophytes from chickpea nodules**

| Sr. no. | Bacterial isolates | Colony morphology | Gram's staining | Spores |
|---|---|---|---|---|
| **1.** | **CNE2** | Transparent, gummy, small, round, medium rods | Gram +ve | Absent |
| **2.** | **CNE3** | Cream, non gummy, medium, round & raised, medium rods | Gram +ve | Present |
| **3.** | **CNE4** | White, gummy, medium, round, long rods | Gram +ve | Present |
| **4.** | **CNE6** | Cream, gummy, medium, round & flat, small rods | Gram +ve | Present |
| **5.** | **CNE7** | Orangish cream, gummy, medium, round & flat, long rods | Gram +ve | Present |
| **6.** | **CNE7-1** | Dull cream, gummy, medium, round, very small rods | Gram +ve | Present |
| **7.** | **CNE10** | Creamy white, gummy, small, round & raised, small rods | Gram +ve | Present |
| **8.** | **CNE12** | Light brown, gummy, large, round, very long rods | Gram +ve | Absent |
| **9.** | **CNE12-1** | Cream, gummy, large, round, long rods | Gram +ve | Present |
| **10.** | **CNE12-2** | White, gummy, large, uneven, small cocci | Gram -ve | Absent |
| **11.** | **CNE16** | Cream, gummy, medium, round, long rods | Gram +ve | Present |
| **12.** | **CNE16-1** | Light brown, gummy, large, irregular & raised, small rods | Gram +ve | Present |
| **13.** | **CNE18** | Pale white, non gummy, large, uneven & raised, small rods | Gram +ve | Present |
| **14.** | **CNE20** | Pinkish cream, gummy, large, irregular & raised, long rods | Gram +ve | Present |
| **15.** | **CNE21** | Creamy white, gummy, medium, round & raised, small rods | Gram +ve | Present |
| **16.** | **CNE27** | Pinkish cream, gummy, medium, uneven & raised, medium rods | Gram +ve | Present |
| **17.** | **CNE32** | Cream, gummy, medium, round & raised, small rods | Gram +ve | Present |
| **18.** | **CNE37** | Brown, gummy, small, round, small rods | Gram +ve | Present |
| **19.** | **CNE42** | Red, gummy, medium, round, cocci | Gram +ve | Absent |
| **20.** | **CNE44** | Light brown, non gummy, large, round & raised, medium rods | Gram +ve | Present |
| **21.** | **CNE45** | Yellow, gummy, small, round, cocci, | Gram +ve | Absent |
| **22.** | **CNE45-1** | Silver brown, gummy, large, round, cocci | Gram +ve | Absent |
| **23.** | **CNE48** | White, non gummy, large, rhizoid & raised, medium rods | Gram +ve | Present |
| **24.** | **CNE51** | Cream, gummy, small, round, small cocci | Gram –ve | Absent |
| **25.** | **CNE53** | Cream, gummy, medium, round, medium rods | Gram +ve | Present |
| **26.** | **CNE58** | Dull brown, gummy, medium, round and raised, small rods | Gram +ve | Present |

| | | | | |
|---|---|---|---|---|
| **27.** | **CNE58-1** | Silver brown, gummy, medium, round & raised, small rods | Gram +ve | Present |
| **28.** | **CNE64** | Cream, non gummy, large, uneven, small rods | Gram +ve | Present |
| **29.** | **CNE76** | Pale, non gummy, large, round & raised, very small rods | Gram +ve | Present |
| **30.** | **CNE77** | Off white, slight gummy, large, round, small cocci | Gram –ve | Absent |
| **31.** | **CNE78** | White, non gummy, medium, uneven & raised, very small rods | Gram +ve | Present |
| **32.** | **CNE79** | Pale, gummy, medium, round, long rods | Gram +ve | Present |
| **33.** | **CNE80** | Cream, non gummy, large, rhizoid & raised, small cocci | Gram +ve | Present |
| **34.** | **CNE81** | Pale brown, non gummy, large, uneven & flat, long rods | Gram +ve | Present |
| **35.** | **CNE82** | White, non gummy, small, rhizoid & raised, medium rods | Gram +ve | Present |
| **36.** | **CNE82-1** | Yellow, slight gummy, small, round, medium rods | Gram +ve | Present |
| **37.** | **CNE82-2** | White, gummy, medium, round, small rods | Gram +ve | Present |
| **38.** | **CNE202** | Cream, gummy, small, round, small rods | Gram +ve | Present |
| **39.** | **CNE202-1** | Pink, gummy, medium, round, small rods | Gram +ve | Present |
| **40.** | **CNE203** | Brownish cream, gummy, medium, round & raised, small rods | Gram +ve | Present |
| **41.** | **CNE206** | Cream, gummy, large, round & flat, small rods | Gram +ve | Present |
| **42.** | **CNE206-1** | Cream, gummy, medium, round & raised, small & long rods | Gram +ve | Present |
| **43.** | **CNE206-2** | Pink, gummy, medium, round, medium rods | Gram +ve | Present |
| **44.** | **CNE207** | Cream, gummy, medium, round & raised, medium rods | Gram +ve | Present |
| **45.** | **CNE208** | Cream, gummy, small, round, very small rods | Gram +ve | Present |
| **46.** | **CNE209** | Pale, gummy, large, round, large cocci | Gram -ve | Absent |
| **47.** | **CNE210** | White, non gummy, large, uneven & raised, long rods | Gram +ve | Present |
| **48.** | **CNE211** | Pink, non gummy, small, rhizoid, rods | Gram +ve | Present |
| **49.** | **CNE212** | Cream, gummy, medium, round & raised, medium rods | Gram +ve | Present |
| **50.** | **CNE213** | Light brown, gummy, medium, round, medium rods | Gram +ve | Present |
| **51.** | **CNE215** | Pinkish brown, gummy, medium, uneven & raised, rods | Gram +ve | Present |
| **52.** | **CNE216** | Cream, gummy, medium, uneven, rods | Gram +ve | Present |
| **53.** | **CNE217** | White, gummy, medium, round, rods | Gram +ve | Present |
| **54.** | **CNE217-1** | Yellow, gummy, minute, round, cocci | Gram -ve | Absent |
| **55.** | **CNE281** | Brownish cream, slightly gummy, small, round, small rods | Gram +ve | Absent |

| | | | | |
|---|---|---|---|---|
| **56.** | **CNE283** | Cream, gummy, small, round, medium rods | Gram +ve | Present |
| **57.** | **CNE284** | Pinkish cream, non gummy, large, round & raised, small cocci | Gram +ve | Present |
| **58.** | **CNE285** | Cream, gummy, medium, round, small rods | Gram +ve | Present |
| **59.** | **CNE286** | Light brown, gummy, medium, round, small rods | Gram +ve | Present |
| **60.** | **CNE287** | White, gummy, small, round & raised, very long rods | Gram +ve | Present |
| **61.** | **CNE287-1** | Yellow, gummy, small, round, rods | Gram +ve | Present |
| **62.** | **CNE288** | Cream, slight gummy, medium, uneven & raised, very long rods | Gram +ve | Present |
| **63.** | **CNE288-1** | Yellow, gummy, small, round, medium rods | Gram +ve | Present |
| **64.** | **CNE288-2** | Brown, gummy, large, round & raised, long rods | Gram +ve | Present |
| **65.** | **CNE289** | Light brown, non gummy, large, uneven & raised, medium rods | Gram +ve | Present |
| **66.** | **CNE292** | Pink, non gummy, large, uneven & raised, rods | Gram +ve | Present |
| **67.** | **CNE292-1** | Very light pink, non gummy, large, uneven, small rods | Gram +ve | Present |
| **68.** | **CNE293** | Cream, gummy, medium, round, small rods | Gram +ve | Present |
| **69.** | **CNE294** | Pink, slight gummy, medium, round & raised, small rods | Gram +ve | Present |
| **70.** | **CNE297** | Pink, gummy, medium, round, medium rods | Gram +ve | Present |
| **71.** | **CNE297-1** | White, gummy, medium, round & raised, very small rods | Gram +ve | Present |
| **72.** | **CNE299** | Pale white, gummy, medium, round, small rods | Gram +ve | Present |
| **73.** | **CNE1036** | White, gummy, medium, round, small rods | Gram +ve | Present |
| **74.** | **CNE1040** | Cream, gummy, medium, round & raised, small rods | Gram +ve | Present |
| **75.** | **CNE1042** | Pale white, non gummy medium, round & raised, small rods | Gram +ve | Present |
| **76.** | **CNE1044** | Cream, gummy, medium, round, small rods | Gram +ve | Present |

### 76.31 Chickpea root growth promotion by chickpea nodule endophytes

The production of the phytohormone can be determined qualitatively and quantitatively by chemical methods. However, alternatively root growth promotion assay is also a good parameter to find out whether these isolates are producing phytohormones or not. A large number of microorganisms are known to produce phytohormones like auxins, gibberellins and cytokinins. These hormones promote the early growth of root. Therefore, root growth promotion by these bacterial endophytes on water agar plates was studied after inoculation of chickpea seedlings with the bacterial isolates. After 5 to 7 days of incubation these seedlings were observed for root lengths (Fig. 4.4). On the basis of root length, growth

promotion was categorized into different categories <5 cm, 5 to <10 cm, 10 to <15 cm and ≥15 cm. Root growth promotion by different nodule endophytes showed that out of 39 bacterial nodule endophytes, a total of 74.3% were found root growth promoter (Table 4.2). Chickpea nodule endophytes CNE215, CNE210, CNE284, CNE1036 and CNE1040 showed good root growth promotion of chickpea.

**Control** **CNE215**

**Fig. 4.4: Chickpea root growth promotion by chickpea nodule endophytic bacteria in water agar plates**

| Categorization based on root length (cm) | Bacterial nodule endophytes | | | | | Total number of isolates promoting root growth |
|---|---|---|---|---|---|---|
| ≥ 15 | **CNE215** | - | - | - | - | 1 |
| 10 to < 15 | **CNE210** | **CNE284** | **CNE1036** | **CNE1040** | - | 4 |
| 5 to < 10 | CNE16-1 | CNE18 | CNE20 | CNE27 | CNE32 | 24 |
| | CNE42 | CNE48 | CNE53 | CNE58 | CNE77 | |
| | CNE79 | CNE81 | CNE82 | CNE82-1 | CNE207 | |
| | CNE209 | CNE212 | CNE216 | CNE217 | CNE289 | |
| | CNE292-1 | CNE293 | CNE294 | CNE299 | - | |
| ≤ 5 | CNE4 | CNE6 | CNE16 | CNE45 | CNE80 | 10 |
| | CNE202 | CNE213 | CNE286 | CNE287 | CNE288 | |
| **Root length of control < 5 cm** | | | | | | |

**Table 4.2: Chickpea root growth promotion by bacterial endophytes from chickpea nodules using water agar plate method**

### 76.32 Phosphate solubilization by chickpea nodule endophytes

Phosphate solubilization is an important character of the endophytes to make the availability of phosphorus to plants. Therefore, all the bacterial nodule endophytes were screened for the presence of phosphate solubilization activity on Pikovskaya's medium (Fig. 4.5). Depending upon the quantity of phosphate solubilization the isolates were categorized as very high, high, moderate and low phosphate solubilizers according to the solubilization zone so as to compare the different isolates (Table 4.3). Phosphate solubilization test showed that overall 73.7% of nodule endophytic bacterial isolates were phosphate solubilizers. The chickpea nodule endophytes placed in the very high category of phosphate solubilization were CNE215, CNE216, CNE286, CNE1036 and CNE1040.

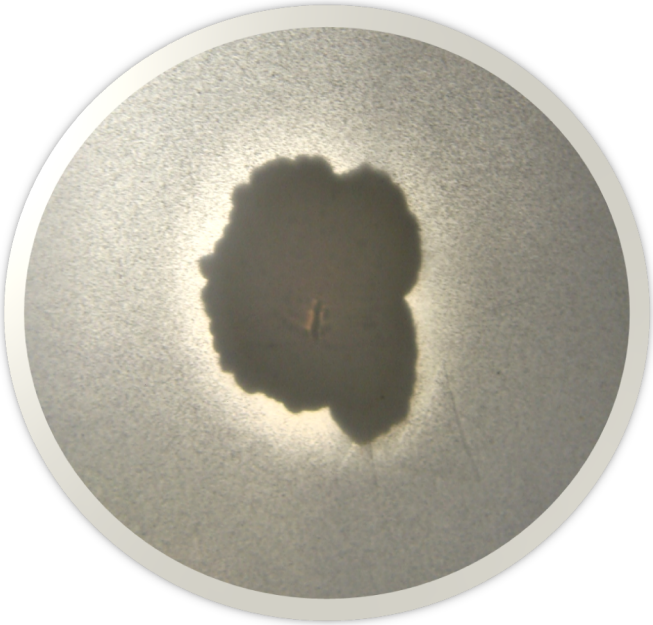


**Fig. 4.5: Phosphate solubilization by chickpea nodule endophytic bacteria**

**Table 4.3: Phosphate solubilization by bacterial endophytes from chickpea nodules**

| Categorization on the basis of size of zone formation | Bacterial nodule endophytes | | | | | Percent phosphate solubilizers |
|---|---|---|---|---|---|---|
| Very High | **CNE215** | **CNE216** | **CNE286** | **CNE1036** | **CNE1040** | 6.5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| High | CNE10 | CNE12 | CNE20 | CNE51 | CNE53 | 25 |
| | CNE58 | CNE58-1 | CNE64 | CNE77 | CNE81 | |
| | CNE208 | CNE210 | CNE212 | CNE217-1 | CNE287 | |
| | CNE288 | CNE289 | CNE292 | CNE292-1 | - | |
| Moderate | CNE2 | CNE12-1 | CNE6 | CNE16-1 | CNE21 | 25 |
| | CNE27 | CNE32 | CNE42 | CNE48 | CNE79 | |
| | CNE82 | CNE203 | CNE284 | CNE285 | CNE287-1 | |
| | CNE288-3 | CNE293 | CNE1042 | CNE1044 | - | |
| Low | CNE4 | CNE12-2 | CNE16 | CNE18 | CNE44 | 17.1 |
| | CNE45 | CNE76 | CNE78 | CNE80 | CNE206 | |
| | CNE206-2 | CNE294 | CNE297 | - | - | |
| Total bacterial nodule endophytes | | | | | | 73.6 |

### 76.33 Ammonia production by chickpea nodule endophytes

Ammonia is an important metabolite produced by the endophytes as it fulfills the requirement of nitrogen for the plants. So, all the bacterial endophytes isolated from chickpea nodules were screened for ammonia production activity in peptone water by adding Nessler's reagent. To compare the different isolates visually they were categorized into five categories viz. very high, high, moderate, low and very low producers of ammonia on the basis of colour (yellow to dark orange) produced after adding Nessler's reagent (Fig. 4.6). Yellow coloured tubes shows a negative result indicating that ammonia is not produced by the bacterial isolate and orange colour shows the production of high amount of ammonia. Results showed that a total of 80.3% bacterial nodule endophytes were ammonia producers. Chickpea nodule isolates CNE76, CNE80, CNE202-1, CNE206-2 and CNE215 were among the high ammonia producers (Table 4.4).

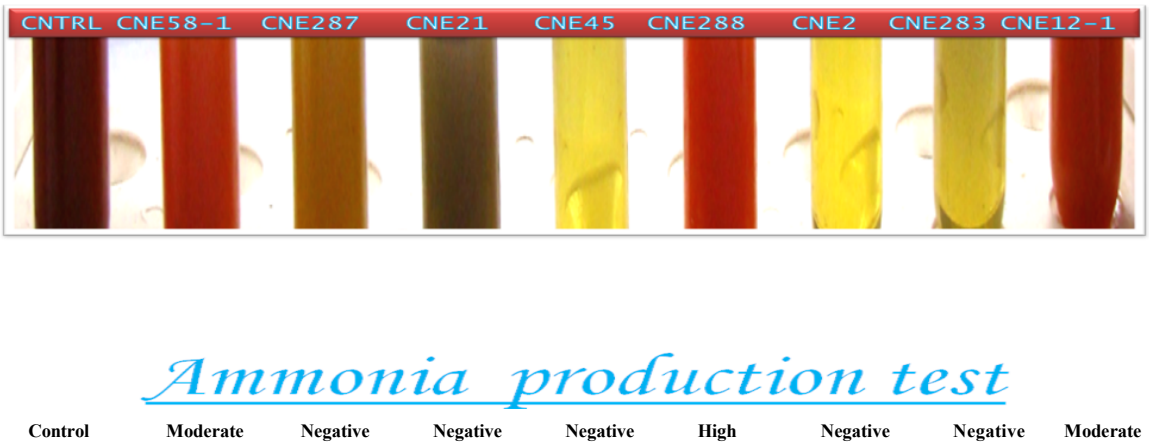


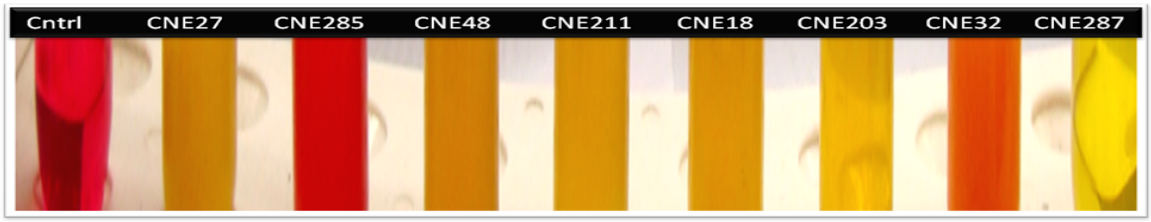

**Fig. 4.6: Ammonia production by bacterial endophytes from chickpea nodules and their categorization**

**Table 4.4: Ammonia production by bacterial endophytes from chickpea nodules**

| Categorization based on colour intensity | Bacterial nodule endophytes | | | | | Percent ammonia producers |
|---|---|---|---|---|---|---|
| Very High | **CNE 76** | **CNE80** | **CNE202-1** | **CNE206-2** | **CNE215** | 6.5 |
| High | CNE42 | CNE77 | CNE288 | CNE288-2 | - | 5.2 |
| Moderate | CNE6 | CNE10 | CNE12-1 | CNE12-2 | CNE27 | 22.4 |
| | CNE48 | CNE58-1 | CNE64 | CNE79 | CNE82 | |
| | CNE203 | CNE208 | CNE212 | CNE284 | CNE286 | |
| | CNE1036 | CNE1040 | - | - | - | |
| Low | CNE7 | CNE16 | CNE16-1 | CNE18 | CNE20 | 30.2 |
| | CNE32 | CNE37 | CNE44 | CNE58 | CNE78 | |
| | CNE81 | CNE206 | CNE210 | CNE216 | CNE217 | |
| | CNE285 | CNE287 | CNE287-1 | CNE289 | CNE292 | |
| | CNE294 | CNE297 | CNE1042 | - | - | |
| Very Low | CNE3 | CNE4 | CNE12 | CNE53 | CNE206-1 | 15.8 |
| | CNE209 | CNE213 | CNE281 | CNE292-1 | CNE293 | |
| | CNE299 | CNE1044 | - | - | - | |
| Total bacterial nodule endophytes | | | | | | 80.3 |

### 4.3.4 Organic acid production by chickpea nodule endophytes

A number of organic acids are produced by the bacterial endophytes like indole-3-acetic acid, gibberelic acid, jasmonic acid and abscisic acid etc. These organic acids support plant growth by solubilization of minerals and chelation of metals and making the availability of nutrients. Therefore, all the bacterial endophytes were screened for organic acid production by using methyl red dye and depending upon the quantity of organic acid produced these isolates were categorized as very high, high, moderate, low and very low producers of organic acid according to the intensity of colour produced (orange to dark red) so as to compare the different isolates (Fig. 4.7). This test showed that only 18.4% of bacterial nodule endophytes were found to produce organic acid (Table 4.5) and chickpea nodule endophytes CNE12-1, CNE12-2, CNE79, CNE215, CNE217 and CNE285 were among the very high organic acid producers.




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Control** | **Negative** | **Very high** | **Negative** | **Negative** | **Negative** | **Negative** | **Moderate** | **Negative** |


| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Very high | Very high | Negative | Very high | Negative | Negative | High | Negative | Low |

**Fig. 4.7: Organic acid production by bacterial endophytes from chickpea nodules and their categorization**

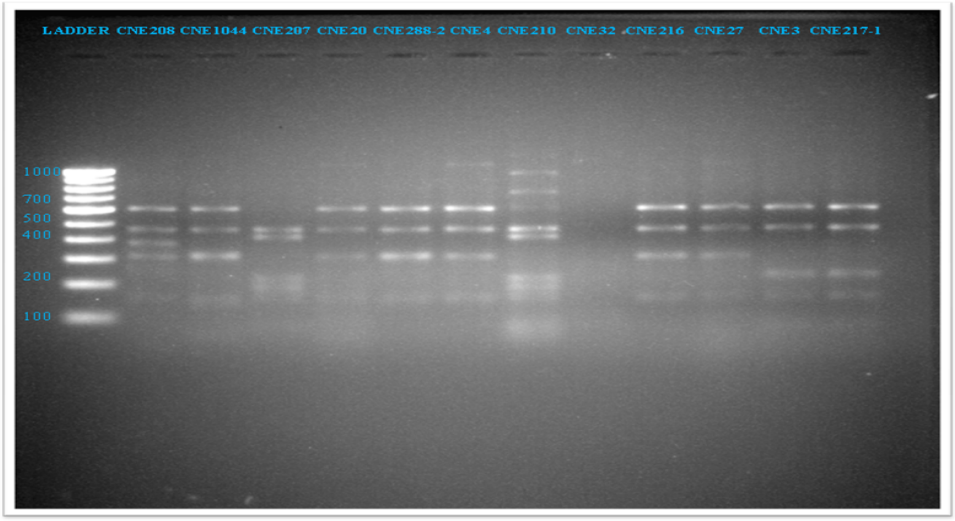
**Table 4.5: Organic acid production by bacterial endophytes from chickpea nodules**

| Categorization based on colour intensity | Bacterial nodule endophytes | | | | | Percent organic acid producers |
|---|---|---|---|---|---|---|
| Very high | **CNE12-1** | **CNE12-2** | **CNE79** | **CNE215** | **CNE217** | 7.8 |
| | **CNE285** | - | - | - | - | |
| High | CNE7 | CNE1040 | - | - | - | 2.6 |
| Moderate | CNE32 | CNE48 | CNE51 | - | - | 3.9 |
| Low | CNE216 | CNE1036 | - | - | - | 2.6 |
| Very Low | CNE1042 | - | - | - | - | 1.3 |
| Total bacterial nodule endophytes | | | | | | 18.4 |

## 76.4 Molecular diversity of chickpea nodule endophytic bacteria

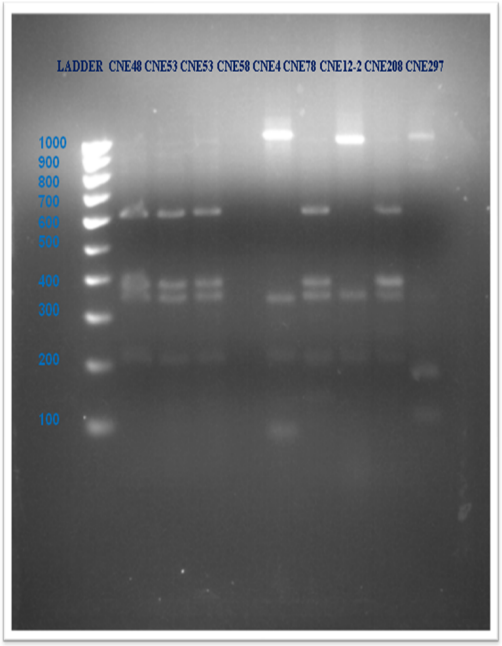
Since, it is interesting to know whether the bacterial endophytes isolated from nodules of chickpea are the same or different, whether the host, host tissue or soil population or environmental conditions determines the endophytic population inside the plant tissues. Therefore, the molecular diversity of the selected 55 chickpea nodule endophytes was determined. DNA was extracted and 16S rDNA was amplified followed by RFLP using three restriction endonucleases. The banding pattern generated by different restriction endonucleases as assessed in gel is shown in fig. 4.8 a, b and c. Each amplification and RFLP was repeated three times, till uniform banding pattern was observed. Analysis of results showed wide diversity among the chickpea nodule endophytes in CCS HAU farm. In major cluster I, 14 isolates and in cluster II, 28 isolates were present, considering cluster I as one biotype or genotype of bacterial endophytes and cluster II as another biotype, likewise 13 biotypes of chickpea nodule endophytes were present at 80% level of similarity coefficient. CNE biotype I was present in 26% of the nodules while CNE biotype II in 15% of the nodules. Major cluster I included isolates CNE3, CNE215, CNE286 CNE1040, CNE1036, etc. and cluster II included isolates CNE6, CNE216, CNE284, CNE212, CNE210 etc.

Bacterial isolate CNE6, CNE27, CNE212 and CNE216 showed 100% similarity. Likewise, CNE288 and CNE292-1 also showed 100% similarity between them.



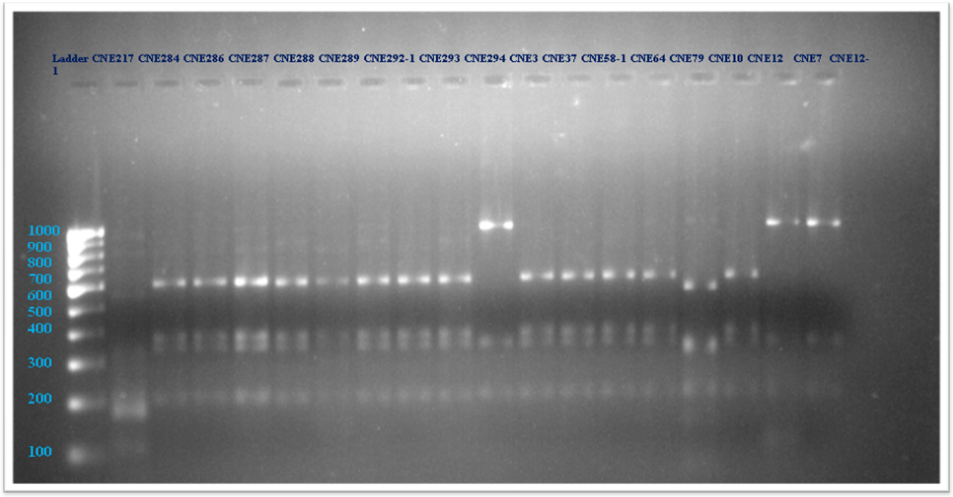

RFLP of 16S rDNA product with HaeIII

**Fig. 4.8a:** **Gel showing banding pattern of RFLP of amplified 16S rDNA sequence of different chickpea nodule endophytic bacteria by *HaeIII* restriction endonuclease.**




*Gel showing banding pattern of RFLP of amplified 16S rDNA sequence of different Chickpea nodule endophytic bacteria by HinfI restriction endonuclease*

**Fig 4.8b:** **Gel showing banding pattern of RFLP of amplified 16S rDNA sequence of different chickpea nodule endophytic bacteria by *MspI* restriction endonuclease.**




RFLP of 16S rDNA PCR product with HinfI

**Fig. 4.8c:** **Gel showing banding pattern of RFLP of amplified 16S rDNA sequence of different chickpea nodule endophytic bacteria by *HinfI* restriction endonuclease.**

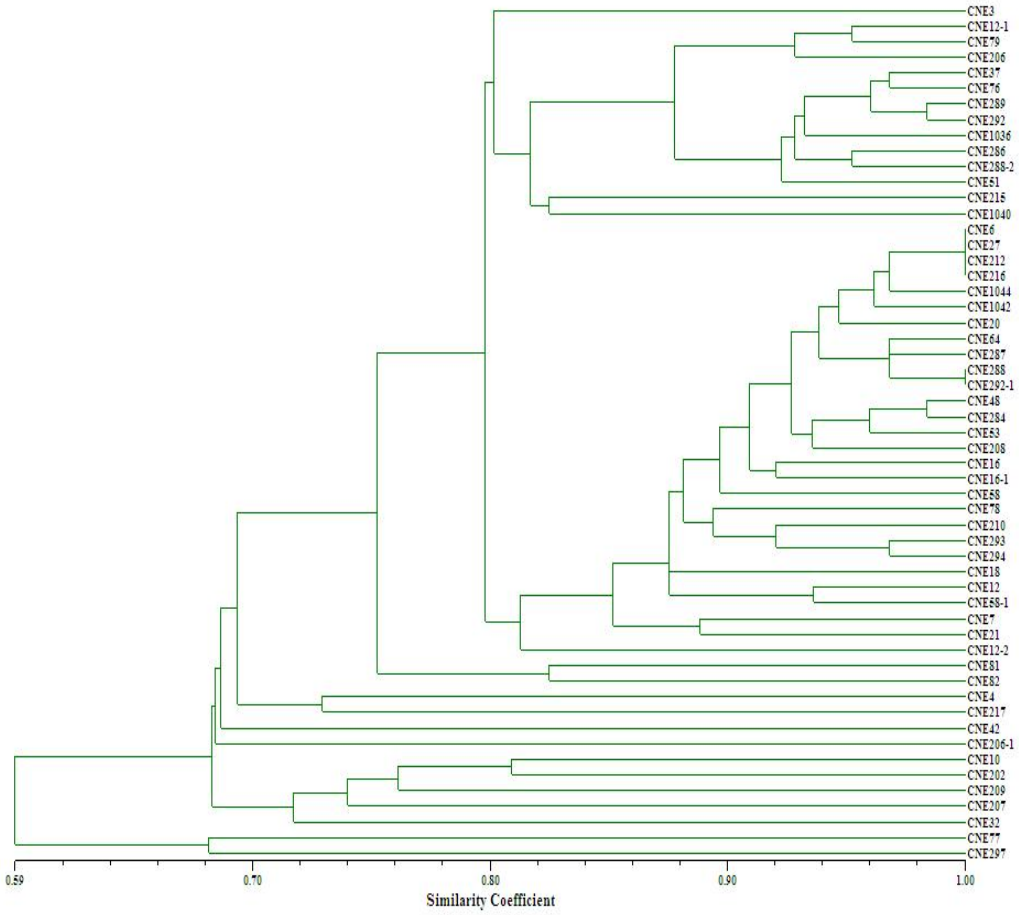


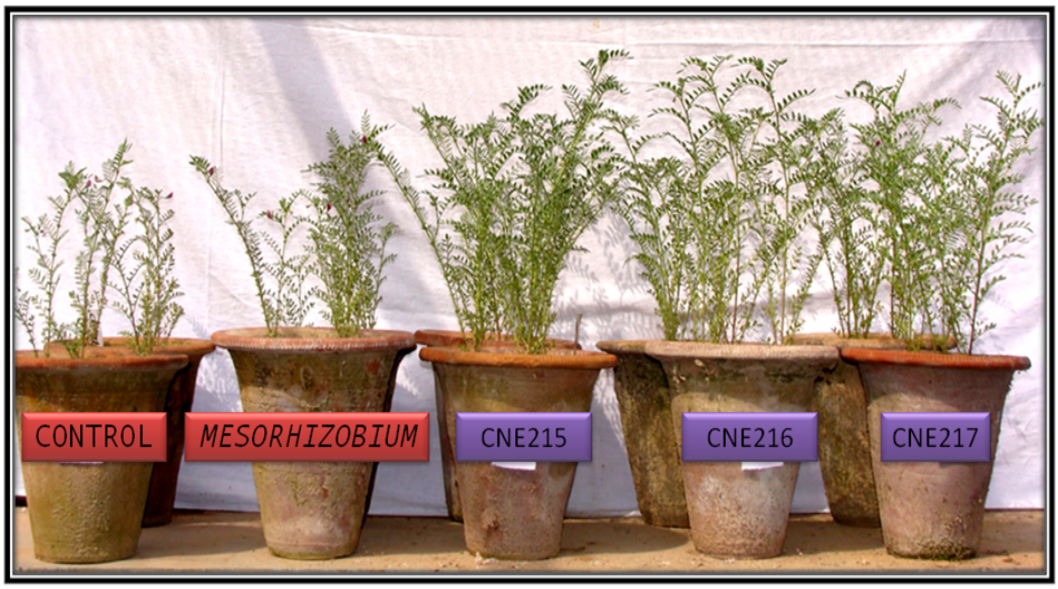

**Fig. 4.9: UPGMA (unweighted pair grouping with mathematic average) dendrogram showing similarity between RFLP of PCR amplified 16S rDNA of bacterial endophytes from chickpea nodules**

## 4.5 Promotion of plant growth and nitrogen fixation of chickpea by bacterial nodule endophytes

Since, it is presumed that endophytes in the plant tissue are also having a symbiotic relationship with plant. Plant is providing shelter and nutrients to the endophytes and in turn endophytes are benefiting the plants in one or another way. To find out whether these

endophytes are helping the nodule forming bacteria in better plant growth and nitrogen fixation or not, an experiment under pot culture conditions was conducted using chickpea as test crop (Fig. 4.10). Five controls were kept out of which one was absolute control. All the selected endophytes were used as inoculants along with uniform inoculation of *Mesorhizobium* strain CH1233. Different observations like nodulation, nodule, root and shoot dry weight and total shoot nitrogen content were determined after uprooting the plants at 60 days of growth. The results showed that there was significant increase in nodule number, nodule dry weight, shoot dry weight and total shoot nitrogen when chickpea seeds were inoculated with endophytes along with *Mesorhizobium* than *Mesorhizobium* alone. All the chickpea nodule endophytes were promoting growth of plants in combination with *Mesorhizobium*. Even visible growth promotion in pots could be observed (Fig. 4.11). Observations showed that nodulation in absolute control was 11 nodules, whereas in other controls was from 14-20 nodules plant$^{-1}$ (Table 4.6). But when inoculated with endophytes from chickpea nodules, nodulation ranged from 13 to 88 nodules plant$^{-1}$. Highest nodulation was in plants inoculated with CNE215 followed by CNE217, CNE216 and CNE1036. There was a significant increase in root dry weight which ranged from 115 to 637 mg plant$^{-1}$ and nodule dry weight which ranged from 8 to 81 mg plant$^{-1}$ as compared to controls which ranged from 137 to 208 mg plant$^{-1}$ and 14 to 38 mg plant$^{-1}$ respectively. Shoot dry weight of absolute control was 561 mg plant$^{-1}$, whereas in other controls it ranged from 562 to 863 mg plant$^{-1}$. But when inoculated with endophytes from chickpea nodules, shoot dry weight ranged from 512 to 1532 mg plant$^{-1}$. Highest shoot dry weight was observed in plants inoculated with CNE1036 followed by CNE217 and CNE215. Similarly, total shoot nitrogen contents of the absolute control was 1.29 mg plant$^{-1}$, whereas in other controls it ranged from 1.43 to 3.08 mg plant$^{-1}$. After inoculation with endophytes from chickpea nodules, total shoot nitrogen contents ranged from 1.52 to 8.15 mg plant$^{-1}$, showing a significant increase in total shoot nitrogen contents. Highest total shoot nitrogen contents were in plants inoculated with CNE1036 followed by CNE215 and CNE217. The increase in nodule number, nodule, root and shoot dry weight and total shoot nitrogen contents were statistically significant over the absolute control.

**Fig. 4.10:** **Chickpea crop raised in pots after inoculation with bacterial nodule endophytes**




*A view of differences in the shoot length*

**Fig. 4.11:** **Promotion of chickpea growth under pot culture conditions after inoculation with chickpea nodule endophytes.**

**Table 4.6: Promotion of chickpea growth and nitrogen fixation under pot culture conditions after inoculation with nodule endophytes**

| Sr. No. | Treatments | Nodule number (Plant$^{-1}$) | Nodule dry weight (mg Plant$^{-1}$) | Root dry weight (mg Plant$^{-1}$) | Shoot dry weight (mg Plant$^{-1}$) | Total shoot nitrogen contents (mg Plant$^{-1}$) |
|---|---|---|---|---|---|---|
| 1. | Control | 11 | 14 | 137 | 561 | 1.29 |
| 2. | *Mesorhizobium*(CH1233) | 14 | 17 | 140 | 665 | 1.43 |
| 3. | PSB (PS36) | 17 | 17 | 133 | 562 | 2.01 |
| 4. | PSB1 | 19 | 30 | 206 | 631 | 2.16 |
| 5. | *Piriformaspora indica* | 20 | 38 | 208 | 863 | 3.08 |
| 6. | *Mesorhizobium* + CNE4 | 29 | 21 | 219 | 573 | 1.72 |
| 7. | *Mesorhizobium* + CNE6 | 16 | 19 | 115 | 705 | 2.14 |
| 8. | *Mesorhizobium* + CNE16 | 13 | 10 | 120 | 656 | 1.87 |
| 9. | *Mesorhizobium* + CNE16-1 | 23 | 10 | 174 | 684 | 2.43 |
| 10. | *Mesorhizobium* + CNE18 | 18 | 16 | 159 | 668 | 1.69 |
| 11. | *Mesorhizobium* + CNE20 | 20 | 15 | 315 | 757 | 2.45 |
| 12. | *Mesorhizobium* + CNE27 | 22 | 14 | 262 | 595 | 1.84 |
| 13. | *Mesorhizobium* + CNE32 | 24 | 19 | 243 | 512 | 1.57 |
| 14. | *Mesorhizobium* + CNE42 | 43 | 35 | 291 | 610 | 2.08 |
| 15. | *Mesorhizobium* + CNE45 | 26 | 31 | 353 | 599 | 1.85 |
| 16. | *Mesorhizobium* + CNE48 | 19 | 17 | 224 | 815 | 2.48 |
| 17. | *Mesorhizobium* + CNE53 | 20 | 35 | 405 | 594 | 1.64 |
| 18. | *Mesorhizobium* + CNE58 | 34 | 33 | 284 | 762 | 2.10 |
| 19. | *Mesorhizobium* + CNE77 | 38 | 37 | 296 | 797 | 2.11 |
| 20. | *Mesorhizobium* + CNE79 | 24 | 31 | 320 | 659 | 1.75 |
| 21. | *Mesorhizobium* + CNE80 | 25 | 27 | 300 | 602 | 2.06 |
| 22. | *Mesorhizobium* + CNE81 | 26 | 35 | 397 | 798 | 2.84 |
| 23. | *Mesorhizobium* + CNE82 | 20 | 20 | 271 | 669 | 3.02 |
| 24. | *Mesorhizobium* + CNE82-1 | 26 | 21 | 393 | 887 | 2.79 |
| 25. | *Mesorhizobium* + CNE202 | 37 | 42 | 479 | 690 | 1.72 |
| 26. | *Mesorhizobium* + CNE207 | 41 | 44 | **588** | 645 | 1.91 |
| 27. | *Mesorhizobium* + CNE209 | 34 | 32 | **549** | 661 | 1.60 |
| 28. | *Mesorhizobium* + CNE210 | 45 | 56 | 478 | 966 | 2.25 |
| 29. | *Mesorhizobium* + CNE212 | 43 | 51 | **637** | 603 | 1.48 |
| 30. | *Mesorhizobium* + CNE213 | 34 | 41 | 273 | 705 | 1.82 |
| 31. | *Mesorhizobium* + CNE215 | **88** | **81** | 345 | **1361** | **4.67** |
| 32. | *Mesorhizobium* + CNE216 | **55** | 70 | 453 | 1038 | 3.23 |
| 33. | *Mesorhizobium* + CNE217 | **57** | **81** | 535 | **1393** | **4.62** |
| 34. | *Mesorhizobium* + CNE284 | 51 | 53 | 492 | 1179 | 4.18 |
| 35. | *Mesorhizobium* + CNE286 | 13 | 19 | 175 | 514 | 1.63 |
| 36. | *Mesorhizobium* + CNE287 | 31 | 47 | 260 | 521 | 2.08 |
| 37. | *Mesorhizobium* + CNE288 | 27 | 48 | 132 | 450 | 2.39 |
| 38. | *Mesorhizobium* + CNE289 | 40 | 48 | 356 | 977 | 2.91 |
| 39. | *Mesorhizobium* + CNE292-1 | 31 | 32 | 410 | 1023 | 3.72 |
| 40. | *Mesorhizobium* + CNE293 | 17 | 20 | 384 | 759 | 1.52 |
| 41. | *Mesorhizobium* + CNE294 | 24 | 15 | 403 | 940 | 3.24 |
| 42. | *Mesorhizobium* + CNE299 | 12 | 8 | 117 | 611 | 1.56 |
| 43. | *Mesorhizobium* + CNE1036 | 54 | **76** | 496 | **1532** | **8.15** |

| | | | | | | |
|---|---|---|---|---|---|---|
| 44. | *Mesorhizobium* + CNE1040 | 49 | 51 | 422 | 938 | 3.32 |
| 45. | **SE(m)** | **6.8** | **9.0** | **73.0** | **146.2** | **0.54** |
| 46. | **C.D. at 5%** | **19.1** | **25.3** | **205.3** | **412.0** | **1.5** |

# CHAPTER –V

## DISCUSSION

Endophytic bacteria are those bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host (Holliday, 1989; Schulz *et al.,* 2006). Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants, ranging from woody tree species, such as oak and pear, to herbaceous crop plants such as sugar beet and maize. There may be two sources of endophytes: the surrounding environment and those inherited (Hallmann *et al.,* 1997). Plant-associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes), and the inside of plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant, and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots, nodules and seeds of various plant species (McInroy *et al.,* 1995; Kobayashi *et al.,* 2000; Dudeja *et al.,*2011a). Some endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth (Sturz *et al.,* 1997), nitrogen fixation (Kirchhof *et al.,* 1997; Stoltzfus *et al.,* 1997; Reinhold-Hurek *et al.,* 1998), and induction of resistance to plant pathogens (Chen *et al., 1995*; Liu *et al.,* 1995; Sturz *et al.,* 1996). Recent studies have shown the potential role of endophytic bacteria in plant growth promotion and phytopathogen control (Sessitsch *et al.,* 2004; Berg *et al.,* 2005; Cao *et al.,* 2005, Mishra *et al.,* 2006). Thus, these endophytes have many beneficial characters for plant growth promotion and to improve soil health. During the last one decade, nodule endophytes from legume have generated a lot of interest. But Indian subcontinent is lacking behind in such studies. Therefore, the present investigation was planned to characterise nodule endophytes from chickpea, an important food legume of India.

A total of 76 endophytic bacterial isolates from surface sterilized nodules of chickpea were isolated. Similarly, elsewhere large number of bacterial nodule endophytes from different legume crops like 154 from *Vicia* species (Lei *et al.,* 2008); 29 from *Prosopis strombulifera* (Sgroy *et al.,* 2009); 39 from *Lespedeza* sp. (Palaniappan *et al.,* 2010); 28 from

*Sophora alopecuroides* (Zhao *et al.,* 2011) and from nodules of other legumes (Dudeja *et al., 2011a*) have been successfully isolated.

Different morphological characters of all the endophytes were assessed. Colony colour showed a huge variation transparent, white, pale white, off white, creamy white, cream, dull cream, pinkish cream, orangish cream, brownish cream, pale, yellow, light brown, brown, dull brown, silver brown, pale brown, pinkish brown, very light pink and pink to red. Colony size of different isolates varied from small to large and colony shape varied from round to rhizoid. Similarly, Palaniappan *et al.,* (2010) observed a large variation in morphology, colour and size in the isolates from *Lespedeza* sp. A large variation in colony morphology like colour, shape and size was also observed by Hung *et al.,* (2007) in different isolates from soyabean nodules. A greater percentage of gram positive bacteria in chickpea nodules was found, overall 93.4% of the isolates were gram positive while 6.5% were gram negative. In contrary to this Palaniappan *et al.,* (2010) isolated 84.6% of gram negative and 15.4% gram positive bacteria from *Lespedeza* sp. Similarly, earlier workers have reported a predominance of gram negative bacteria in the tissues of various plants (Stoltzfus *et al.,* 1997; Elbeltagy *et al.,* 2000). In northern India, actually temperature range is very high -2 to 47˚C and under these temperature conditions it is quite possible that gram positive bacteria and particularly spore formers are the better survivors. Therefore, more number of gram positive spore formers was observed in chickpea nodules. More number of gram positive isolates in *Glycine max* tissues and gram negative in *Glycine soja* were reported by Hung *et al.,*(2007) indicating that apart from environmental conditions, host is also an important factor in determining the population of gram positive and gram negative bacteria in legumes.

To select better strains for use as inoculants, all bacterial nodule endophytes were screened for the presence of beneficial traits. Chickpea root growth promotion assay showed that 74.3% of nodule endophytic bacteria were promoting chickpea root growth, indicating that majority of the isolates are secreting some auxins or gibberellins which is promoting the root growth. Many researchers have reported that a good producer of auxins or gibberellins is also a good growth promoter (Khan *et al*., 2009; Sgroy *et al.,* 2009 and Panchal *et al*., 2011) and enhanced plant growth (Camerini *et al*., 2008). All strains collected from *Lespedeza* sp. root nodules showed either indole-3-acetic acid or 1-aminocyclopropane-1-carboxylate deaminase production or both (Palaniappan *et al.,* 2010). Similarly, another endophyte isolated from *Sophora alopecuroides* root nodules belonging to *Bacillus cereus* was shown to be able to produce indole-3-acetic acid (Zhao *et al.,* 2011).

Further, phosphate solubilization helps plants for easy uptake of phosphate from soil and in the present study 73.7% of nodule endophytic isolates were solubilizing phosphate.

Similarly, phosphate solubilization activity was also observed by endophytes isolated from alfalfa (*Medicago sativa* L.), *Prosopis strombulifera*, *Lespedeza* sp. and *Mammillaria fraileana* and total percentage of phosphate solubilizers ranged from 0 to 62% (Stajkovic *et al.,* 2009; Sgroy *et al.,* 2009; Palaniappan *et al.,* 2010; Lopez *et al.,* 2011).

Ammonia production is another important beneficial character exhibited by the endophytes and other rhizospheric organisms. Among the 76 isolates, a total of 80.3% of bacterial nodule endophytes from chickpea were ammonia producer. How ammonia production is helping the plants is not known but probably is acting as nitrogen source. *Stajkovic et al.,* (2009) observed that among the selected isolates from the nodules of alfalfa, three strains were producing ammonia. Another beneficial trait is organic acid production. Organic acids play a very important role in chelation and mineralization and many phytohormones are also found in the form of organic acid which are known to be produced by endophytic bacteria (Forchetti *et al.,* 2007). Only 18.4% of total nodule endophytic bacteria were producing organic acids. Similarly, other workers elsewhere reported the production of organic acids by endophytes from different crops (Vetrivelkalai *et al.,* 2010; Sgroy *et al.,* 2009). In contrast to this, none of the isolates were observed to produce organic acid among the selected isolates from the nodules of alfalfa (Stajkovic *et al.,* 2009).

Endophytic bacteria are found in every plant known and in all the tissues of plants. Different types of bacteria, either tissue specific or non specific has been isolated from plants. Inspite of these differences these endophytes performs similar or different functions in all the tissues of plants. To have a better understanding of bacterial nodule enophytes molecular diversity of the chickpea nodule endophytic isolates was assessed. All the selected 55 nodule endophytic bacteria from chickpea upon RFLP analysis of PCR amplified 16S rDNA showed that these isolates belonged to 13 genotypes. Similar results were reported in *Glycine max.* and *Glycine soja* with 17 and 15 genotypes of endophytes respectively (Hung *et al.,* 2007). Lei *et al.,* (2008) identified 9 putative species among 154 bacterial strains isolated from root nodules of wild *Vicia* species grown in China. Similarly, endophytic bacterial isolates from *Lespedeza* sp. belonged to nine genera (Palaniappan *et al.,* 2010). Root nodules of leguminous plants were found to host large population of endophytic bacteria of diverse genera and species which are unrelated to rhizobial symbiotic nitrogen fixing bacteria (de Lajudie *et al.,* 1999; Zakhia *et al.,* 2006, Kan *et al.,* 2007; Mureus *et al.,* 2008; Li *et al.,* 2008; Dudeja *et al., 2011a*). Stajkovic *et al.,* (2009) identified the alfalfa nodule enodophytes that belonged to three different genera *Bacillus*, *Microbacterium* and *Brevibacillus*. *Bacillus* species comprise one of the most common soil bacteria and they are frequently isolated from the rhizospheres of plants, as well as from different plant tissues. The occurrence of *Bacillus*

species as nodule endophytes has been reported for soybean (Bai *et al.,* 2002), red clover (Sturz *et al.,* 1997), pigeon pea (*Cajanus cajan*) (Rajendran *et al.,* 2008), Kudzu (*Pueraria thunbergiana*) (Selavkumar *et al.,* 2008), *Calycotome villosa* (Zakhia *et al.,* 2006) and different wild legumes (Mureus *et al.,* 2008). *Microbacterium* and *Brevibacillus* species were also isolated from different tissues and plant nodules (Zakhia *et al.,* 2006; Sturz *et al.,* 1997).

Endophytes are also known to enhance plant growth promotion and nitrogen fixation in legumes when used as inoculants. All the endophytic bacterial isolates were inoculated together with *Mesorhizobium* in chickpea and showed enhanced plant growth, nodulation and nitrogen fixing parameters in chickpea particularly endophytic bacterial isolates in combination with *Mesorhizobium* than *Mesorhizobium* alone. However, in contrast to this, Stajkovic *et al.,* (2009) reported that in alfalfa co-inoculation of all non-rhizobial strains with *Ensifer* (*Sinorhizobium*) *meliloti* positively influenced nodule number but there was no significant effect on other growth parameters with respect to inoculation with *Ensifer* (*Sinorhizobium*) *meliloti* alone. Bacterization experiments in red clover showed that bacterial endophytes promoted growth more often when applied in combination with *R. leguminosarum* bv. *trifolii* than when applied singly (Sturz *et al.,* 1996). Similarly, Stajkovic *et al.,* (2011) reported that co-inoculation with *Rhizobium* and *Pseudomonas* sp. LG or *Bacillus* sp. Bx improved shoot dry weight, nitrogen and phosphorus contents in bean plants, compared to inoculation with *Rhizobium* alone. It was observed that *Pseudomonas* sp. LG promoted bean growth and particularly phosphorus uptake more efficiently than *Bacillus* sp. Bx.

The present study indicates that the bacterial endophytes isolated from nodules of chickpea showed the presence of beneficial characteristics under laboratory conditions as well as these endophytes were performing better under pot culture conditions. The best root growth promotion and phosphate solubilization was exhibited by CNE215. Best ammonia producer and organic acid producer was CNE215 whereas CNE217 was only best organic acid producer. Isolates CNE1036, CNE215 and CNE217 enhanced nitrogen fixing parameters like shoot dry weight and total shoot nitrogen contents in chickpea. These isolates showed better results under laboratory as well as pot culture conditions, so such nodule endophytic isolates of chickpea needs to be further confirmed under field conditions to select best inoculants for better crop production in agriculture.

## CHAPTER –VI

## SUMMARY AND CONCLUSION

- Chickpea plants were collected from the fields of Chaudhary Charan Singh Haryana Agricultural University, Hisar farm to isolate endophytic bacteria from the nodules.
- A total of 76 endophytic bacterial isolates were isolated from the nodules of chickpea plants.
- Studies of different morphological characters showed that colony morphology varied from gummy to non gummy, round to rhizoid, minute to large with varied colours. Majority of the isolates, 71 were gram positive and five were gram negative. Among the gram positive bacterial isolates, 61 were spore formers having central to terminal spores.
- The chickpea nodule endophytes were screened for the presence of beneficial characters.
    - Chickpea root growth promotion assay showed a total of 74.3% nodule endophytic bacteria were root growth promoter and isolate CNE215 was the best root growth promoter.
    - Out of 76 isolates, 73.7% nodule endophytic bacteria were phosphate solubilizers and CNE215 was the best phosphate solubilizer.
    - A total of 80.3% chickpea nodule endophytes were producing ammonia and CNE215 was the highest ammonia producer.
    - A very less number of chickpea nodule endophytes i.e. 18.4% were organic acid producers and isolates CNE217 & CNE215 were the highest organic acid producers.
- Genomic DNA of 55 nodule endophytic bacterial isolates was extracted, amplified with 16S rDNA primers and results of RFLP with three restriction endonucleases viz., *HaeIII, MspI* and *HinfI* showed wide diversity among the chickpea nodule endophytes and atleast 13 biotypes of bacterial endophytes were present in chickpea nodules in HAU farm.
- Inoculation of nodule endophytes promoted the plant growth and enhanced nitrogen fixing parameters of chickpea and most effective isolates were CNE1036, CNE215 and CNE217.
- The effectivity of isolates CNE1036 and CNE215 should be further confirmed under field conditions so as to select the best strain for use as inoculant.

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# ABSTRACT

| | | |
|---|---|---|
| Title of thesis | : | **Characterization and molecular diversity of endophytic bacteria isolated from Chickpea (*Cicer arietinum*) nodules.** |
| Name of the degree holder | : | **Ranjana Saini** |
| Admission number | : | **2009BS139M** |
| Title of degree | : | **Master of Science** |
| Name of discipline | : | Microbiology |
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| Year of award of degree | : | **2011** |
| Major subject | : | **Microbiology** |
| Total number of pages in thesis | : | **47 + ix** |
| Number of words in the abstract | : | **211 approx.** |

**Key words:** Endophytic bacteria, Chickpea, Molecular diversity, Beneficial traits, Nodules, Legumes

Endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth, nitrogen fixation and induction of resistance to plant pathogens. Elsewhere, endophytic bacteria have been isolated from roots and nodules of several legume plants. In the present investigation, a total of 76 endophytic bacterial isolates were isolated from the nodules of chickpea plants. Gram positive isolates were more than the Gram negative isolates. Among the Gram positive bacterial isolates, 61 were spore formers having central to terminal spores. A total of 74.3% endophytic bacteria in chickpea root growth promotion assay showed growth promotion and 73.7% were phosphate solubilizer. A total of 80.3% endophytic bacteria were producing ammonia while only 18.4% were organic acid producers. A total of 55 isolates were selected for molecular diversity studies. 16S rDNA was amplified and RFLP with three restriction endonucleases viz*., HaeIII, MspI and HinfI* showed wide diversity among the chickpea nodule endophytes and atleast 13 biotypes of bacterial endophytes were present in chickpea nodules. Inoculation of nodule endophytes promoted the plant growth and enhanced nitrogen fixing parameters of chickpea and most effective isolates were CNE1036, CNE215 and CNE217. The effectivity of isolate CNE1036 and CNE215 should be further confirmed under field conditions so as to select best inoculant strain.

**MAJOR ADVISOR** **DEGREE HOLDER**

**HEAD OF THE DEPARTMENT**

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