CHAPTER - II

REVIEW OF LITERATURE

Introduction

Rhizobia are a genetically diverse and physiologically heterogenous group of bacteria (Somasegaran and Hoben, 1994) and they are able to elicit nodule formation on legumes are called rhizobia (Denarie et al., 1996). Rhizobia comprises of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (Denarie et al., 1992) and *Sinorhizobium* (Denarie et al., 1996). Rhizobia are a ubiquitous part of the soil micro-flora in a free-living state in the rhizosphere of legumes (Allen and Allen, 1981 and Somasegaran and Hoben, 1994) until the point where nodulation becomes possible (Rendig and Taylor, 1989). The nature and properties of soil allows billions of organisms of coexist (Pepper and Upchurch, 1991). The ability of form symbiotic relationships with members of the plant family Fabaceae is a unique feature associated with bacteria belonging to the family *Rhizobiaceae* (Pepper and Upchurch, 1991).

The number of symbiotic relationships that can form between rhizobia and hosts is restricted and vice versa (Denarie et al., 1992). Rhizobia elicit on their host and the formation of nodules in which they fix nitrogen (Denarie et al., 1992; Denarie et al., 1996 and Prescott et al., 1996) and provide the plant with ammonia for growth (Rendig and Taylor, 1989).
Despite the widespread distribution of leguminous crops, many soils remain void of rhizobial strains (Brockwell et al., 1995).

Rhizobia are bacteria that selectively infect the roots of some legumes and have the following characteristics; gram negative, motile rod-shaped (approximately 0.5-0.9 µm in width and 1.2-3.0 µm in length) and heterotrophic (Pepper and Upchurch, 1991; Somasegaran and Hoben, 1994; Prescott et al., 1996). Root nodule bacteria generally grow under the following conditions 25-30°C (optimum) in the pH range of 6-7 (Somasegaran and Hoben, 1994). *Rhizobium* growth normally occurs under aerobic conditions. However, when fixing nitrogen, low levels of oxygen are required to protect the enzyme nitrogenase (Rending and Taylor, 1989) and hence, *Rhizobium* are able to grow in microaerophilic conditions (Somasegaran and Hoben, 1994).

Fujihara (2009) described that the rhizobia are of great importance for nitrogen acquisition through symbiotic nitrogen fixation in a wide variety of leguminous plants. These bacteria differ from most of other soil microorganisms by taking dual forms, *i.e.*, a free-living form in soils and a symbiotic form inside of host legumes. Therefore, they should have a versatile strategy for survival, whether inhabiting soils or root nodules formed through rhizobia-legume interactions. Rhizobia generally contain large amounts of the biogenic amine homospermidine, an analog of spermidine which is an essential cellular component in most living systems.
The external pH, salinity and a rapid change in osmolarity are thought to be significant environmental factors affecting the persistence of rhizobia. The regulation of homospermidine biosynthesis in response to environmental stress and its possible functional role in rhizobia. Legume root nodules, an alternative habitat of rhizobia, usually contain a variety of biogenic amines besides homospermidine and the occurrence of these amines is closely associated with rhizobial infections.

2.1 ISOLATION AND CHARACTERIZATION OF BLACKGRAM NODULE BACTERIA

Balasundaram and Subbarao (1974) reported the methods adopted for isolation screening and selecting efficient strains of *R. japonicum* and their performance was compared with exotic cultures in different agro climatic conditions.

Bromfield and Jones (1980) reported that many fast growing rhizobia produced acid from sugars when grown in culture medium, while slow growers produce alkaline metabolites. These are gram negative, non-spore forming rods containing poly P-hydroxy butyrate granules motile bipolar, sub polar and peritrichous flagella. Colonies are circular, semi translucent, raised mucilaginous, usually 2 to 4 mm in diameter within 3 to 5 days, on YEMA medium. Pronounced turbidity develops after 2 to 3 days in agitated broth.
Fuhrmann (1990) collected 860 isolates of *B. japonicum* from 18 locations and characterized serologically with an ELISA, morphologically by colony type on YEMA medium, using multilocus enzyme electrophoresis on 51 isolates of *Leguminosarum biovar. Phaseoli*, found great diversity between these isolates.

### 2.2 NITROGEN FIXING EFFICIENCY OF RHIZOBIUM ISOLATES.

Effectiveness of the symbiosis is measured either directly by determining the amount of nitrogen fixed (or) indirectly by measuring the plant dry weight. Hardy *et al.*, (1968) gave the methodology, characteristic and application of the sensitive ARA for measurement of N₂ fixation rate by nitrogenase preparations and bacteria cultures in the laboratory and by legumes and free-living bacteria in situ. This assay is based on the nitrogenase-catalysed reduction of C₂H₂ to C₂H₄ and quantitative measurement of C₂H₄ and quantitative measurement of C₂H₄ using a gas chromatograph with flame ionization detector.

Thiobodean and Jaworki (1975) used soyabean (*Glycine max* (L.) *Merril*) *ev.* in a pot experiment of assess acetylene reduction activity at different stages of the plant growth. From their study they concluded that the ARA was maximum between flowerings to early pod filling stage.

Burton (1981) listed the following characteristics as most desirable for the selection of effective strain. Ability to form N₂ fixing nodule over the range of environmental conditions, Competitiveness in nodule formation and
survival in the presence of other *Rhizobium*, prompt nodulation and good nitrogen fixation, persistence in the soil and good growth ability in the culture medium in the carrier and in the soil.

Phillips and De Jong (1984) and Streeter (1988) reported that poor nodulation also may be related the high soil No$_3^-$ N, however interactions between soil N, nodulation and N$_2$-Fixation in soybean are complex. Soil No$_3^-$ N concentration greater than 2mM No$_3$ (about 60kg N ha$^{-1}$) commonly prevent (or) delay nodule formation and nitrogen fixation.

Huang *et al.*, (1988) in their experiment with soybean concluded that nitrogenase activity was closely related with concentration of leghaemoglobin in the nodules. The reduction in the nodule leghaemoglobin content was the limiting factor for the reduced nitrogen fixation.

For *Rhizobium*, the only enzyme directly involved in the dinitrogen utilization pathway is nitrogenase, which is encoded by three genes (nif k, nif D, nif H) which have been cloned from many *Rhizobium* species by direct homology (Long, 1989).

Nitrogen fixing microorganisms will therefore is an important component of sustainable agriculture systems. Tauer (1989) stated that increasing the efficiency of legumes to fix N$_2$ might have an annual Vs benefit of $1067$ million while decreasing N-fertilization by 1547 thousand metric tons.
Maria et al. (1989) studied the nitrate reductase (NR) of selected uptake hydrogenase-positive (hup\(^{+}\)) and uptake hydrogenase-negative (hup\(^{-}\)) strains of *Brady rhizobium japonicum* both in free living cells and in symbiosis with soybean. It was noted that, cultured cells, nodules and bacteriodes of strains with hup\(^{-}\) genotype had higher rates of NR activity than hup\(^{+}\) genotype.

The normally grown plants of *Vicia faba* (Vidal et al., 1992) and *Phaseolus* sp. showed maximum nitrogenase activity at flowering stage and declined after pod filling. Nitrogenase activity was positively correlated with nodule number during this stage of both *Vicia* and *Phaseolus* plants.

Sung (1993) found difference in nitrogenase activity under water logged condition in soybean inoculated with *Brady rhizobium japonicum*. After four days of inoculation, the ARA was \(10^4\) umol C\(_2\) H\(_2\) g\(^{-1}\) dw h\(^{-1}\) and 80 µmol C\(_2\) H\(_2\) g\(^{-1}\) dw h\(^{-1}\) under water logged conditions.

Biological Nitrogen fixation is important from the point view of saving N- fertilizer and reducing the cost of production. In addition the use of nitrogen fixing legume crop will.

1. Reduce ground water pollution in comparison to cereal crops, which needs to be fertilized.
2. Enhance protein produced due to high content of ‘N’ in legume crops.
3. Contribute N to succeeding crops.
4. Build up soil fertility.
Narendra Kumar et al. (1996) reported the effectiveness and competitive ability of *Bradyrhizobium* Strains. The nodulation, plant biomass, N uptake and grain yields of inoculated plants were significantly higher in loamy soils than alluvial sandy soils.

Kirichenko and Malichenko (2000) reported the effect of legume lectins on symbiotic activity of nodule bacteria. The effect of lectins from soybean on the symbiotic activity of *B japonicum* strain was investigated. Preincubation with lectins isolated from the specific host plant resulted in both enhancement of nodulation and stimulation of the nitrogen fixing activity of fully developed nodules but the lectin isolated from the non specific host plant had no such effect.

Peoples and Herridge (2001) reported that atmospheric N\textsubscript{2} fixed symbiotically by the association between *Rhizobium* species and legumes represents a renewable source of N for agriculture. Values estimated for various legume crops and pasture crop species are offer impressive commonly falling in the range of 200 to 300 Kg of N ha\textsuperscript{-1} year\textsuperscript{-1}. Yield increases of crops planted after harvesting legumes are often equivalent to those expected from 30 to 80 Kg of fertilizer ha\textsuperscript{-1}.

Thies et al. (2001) reported that inputs into terrestrial ecosystems of BNF from the symbiotic relationship between legumes and their rhizobial
amount at least 70 million tons of N per years. This enormous quantity will have to be augmented as the world’s population increases and as the natural resources and the supply fertilizer N supply was diminished. This objective will be achieved through the development of superior legume varieties and increased efficiency of the nitrogen fixing process itself better management of the symbiotic relationship between plants and bacteria.

Shivesh Sharma and Sharma (2002) reported that the inoculation with Rhizobium improved growth parameters of Black gram i.e. the inoculation significantly enhanced the plant ht (cm) and dry matter accumulation (g/plant) of blackgram as compared to uninoculated treatment at various stages of crop growth.

BNF accounts for approximately two-thirds of the nitrogen fixed globally, while the rest of the nitrogen is industrially synthesized by the Haber–Bosch process (Rubio and Ludden, 2008). Biological nitrogen fixation occurs, generally at mild temperatures, by nitrogen fixing microorganisms, which are widely distributed in nature (Raymond et al., 2004).

Nitrogen fixing organisms are generally categorized as (a) symbiotic N₂ fixing bacteria including members of the family rhizobiaceae which forms symbiosis with leguminous plants (e.g. Rhizobia) (Ahemad and Khan, 2012b) and non-leguminous trees (e.g. Frankia) and (b) non-symbiotic (free
living, associative and endophytes) nitrogen fixing forms such as cyanobacteria (Anabaena, Nostoc), *Azospirillum*, *Azotobacter*, *Gluconoacetobacter diazotrophicus* and *Azocar*us etc. (Bhattacharyya and Jha, 2012). However, non-symbiotic nitrogen fixing bacteria provide only a small amount of the fixed nitrogen that the bacterially-associated host plant requires (Glick, 2012).

### 2.3 INDOLE ACETIC ACID PRODUCTION BY RHIZOBIUM

Indole-3-acetic acid (IAA) - IAA is the foremost phytohormone that accelerates plant growth and development by improving root/shoot growth and seedling vigor. IAA is involved in cell division, differentiation and vascular bundle formation and an essential hormone for nodule formation. It has been estimated that 80 % of bacteria isolated from the rhizosphere can produce IAA (Patten and Glick, 1996). The salient ones are *A. caulinodans*, *B. japonicum*, *B. elkanii*, *M. loti*, *R. japonicum*, *R. leguminosarum*, *R. lupine*, *R. meliloti*, *R. phaseoli*, *R. trifolii* and *Sinorhizobium* spp. (Afzal and Bano 2008; Antoun et al., 1998; Biswas et al., 2000; Boiero et al., 2007; Chi et al., 2010; Chandra et al., 2007; Dazzo et al., 2005; Naidu et al., 2004; Senthilkumar et al., 2009; Yanni et al., 2001; Weyens et al., 2009).

IAA production in rhizobium takes place via Indole-3-pyruvic acid and indole-3-acetic aldehyde pathway. On inoculation of *R. leguminosarum* bv. viciae, 60-fold increase in IAA was observed in the nodules of vetch
roots (Camerini et al. 2008). One of the highest productions of IAA had been reported with the inoculation with *B. japonicum*-SB1 with *B. Thuringiensis*-KR1 (Mishra et al. 2009).

Co-inoculating *Pseudomonas* with *R. galegae* bv. orientalis had shown to produce IAA that had contributed to increases in nodule number, shoot and root growth and nitrogen content. Both environmental stress factors (acidic pH, osmotic and matrix stress and carbon limitation) and genetic factors (auxin biosynthesis genes and the mode of expression) were shown to influence the biosynthesis of IAA (Spaepen et al. 2007; Spaepen and Vanderleyden 2011).

2.4 EXOPOLYSACCHARIDE PRODUCTION (EPS) BY *RHIZOBIUM*

Bacterial Exopoly saccharide (EPS) influences many cellular functions including permeability of the cell to toxic substances. Adhesion to surface of other bacteria, and pathogenicity of diseases of animals and plants. In some rhizobia and bradyrhizobia, EPS binding proteins known to influence nodulation, host specificity. In many fast growing rhizobia, EPS production is required for nodule development. Several rhizobial polysaccharides where found to be effective stabilizing agents of most soils than either synthetic soil conditioner or the other reference compounds.
The exopolysaccharides, cellular polysaccharides and extracellular protein of *Rhizobium* have been identified as the root curling factor. (Yao and Vincent 1976).

Zevenhuizer (1971) found out that polysaccharides of *R.meliloti*, *R.Phaseoli* and *R.japonicum* were found to contain glucose, galactose, glucouronic acids, pyruvic acid, and acetylene in the ratio of 5:1:2:2:3.

The extracellular material obtained from the strains of *Rhizobium japonicum* produced different effects on the nodulating ability of soybean. Extracellular material was found to contain 3-9 percent of carbohydrates, 3-15 percent of RNA, and less than 1 percent protein, glucose, galactose, mannose, glucoamine and other two unidentified compound were the components of the carbohydrate moiety (Huber *et al.*, 1981).

A strong correlation existed between lectin binding and infectivity in the *R.trifoli- clover* symbiosis (Dazzo and Hubbel, 1975). Kennedy (1976) reported the presence of methyl ribose in the exopolysaccharides of the slow growing strain belonging to cowpea group.

Napoli Albershem (1980) observed correlation between the production of extracellular polysaccharides production resulted in reduced inflection and nodulation efficiency.
Abe et al., (1984) reported that the treatment of clover seedlings with as little as 2.5mg of cellular polysaccharides (or) exopolysaccharides fragments prior to concomitant increase in infection threads to 70-106%. Higher concentrations were not effective in promoting infection thread formation. Bacteria belonging to the genera *Rhizobium* and *Brady rhizobium* secrete copious amount of exopolysaccharides when they are cultured in vitro, several reports have indicated that, the exopolysaccharides may play an important role in the process by which these bacteria modulated (legumes and a close relationship between exoploysaccharides production and infectivity.

Muller et al. (1988) reported those symbiotic mutants of *Rhizobium meliloti* (Tn 5 mutagenesis) differed in exopolysaccharide production and in nodulation ability. Composition and function studies of extracellular polysaccharides of cowpea rhizobia revealed that polysaccharides were mixture of acidic exopolysaccharides and low molecular weight neutral glucons. The acidic polysaccharides contained glucose, fucose galactose, gluconic acid, mannose and fucose (Bindra and Modi 1989).

Kosenko et al., (1989) reported that lectin receptor ability of Kpopolysaccharide of active strain was twice as high as that of inactive ones. Further studies revealed that extracellular polysaccharides of the active and virulent strains manifested highest degree of affinity with the pea lectin and vice versa.
Connel et al. (1990) developed mutants of *Rhizobium* strain (CIAT 899) deficient in exopolysaccharide, which induced interveinal chlorosis in the first trifoliate leaf of their symbiotic host *Phaseolus vulgaris*.

EPS can be defined as polysaccharides found external to structural surface of the microbial cell and term can be applied to carbohydrate polymers of diverse composition and of different physical types (Sutherland, 1990).

*Rhizobium meliloti* SU 47 can produce two EPS, a succinoglycan (EPS I) and a galactoglucon (EPS II). Most physiological and genetical studies have been performed with derivatives of this strain, which under normal growth conditions produced only EPI (Glazebrook et al., 1990). Galactoglucon is produced under phosphate limitation conditions (Zhan et al., 1991).

Lagares et al. (1992) developed a Transposon Tn 5 induced mutant of *Rhizobium meliloti* (Rm 6963). Which was deficient in polysaccharide production, and caused delayed (or) reduced nodulation. Further analysis revealed that plants inoculated with this mutant were stunted in growth than control.

Breedveld et al. (1992) have fractioned a low molecular weight succinoglycan of *Rhizobium meliloti* repeat unit monomers, trimers, and tertramers. It was also detected that each class has a varying degree of anionic character.
Van Brussel et al. (1992) reported the possibility of induction of preinfection thread in the leguminous host plant by the addition of mitogenic lipopolysaccharides produced by *Rhizobium leguminosarum biovar viciae* in the absence of the symbiont.

Breedveld et al. (1993) developed Tn 5 mutants of *Rhizobium Leguminosarum* which were defective in the synthesis of extracellular polysaccharide, lipopolysaccharide, and capsular polysaccharide but synthesized and secreted significant amount of Cyclic beta (1-2 glucons).

The polysaccharides of *B.japonicum* is a bacteria product and not a plant product. Two types of polysaccharides are formed corresponding to the two DNA-homology groups of *B.japonicum*. The polysaccharide produced in nodules (termed NPS) is different in composition from that produced in culture (EPS) (Streeter and Salminen, 1993).

Bacterial polysaccharides are necessary for a functional *Rhizobium* legume symbiosis. Exopolysaccharides (EPS) Lipopolysaccharides (LPS) capsular polysaccharides and cyclic p (1-2) glucon play essential roles in the formation of the infection thread and in nodule development. (Kannenberg and Brewin 1994).

Javierllor et al. (1998) reported the exopolysaccharide production by *Rhizobium meliloti* is influenced by salt. The halotolerant strain *Rhizobium meliloti* EF BI modifies the production of EPS in response to salt. This
Bacterium grown in the presence of 0.3M NaCl showed decrease in mucoidy and when grown in salt supplemented liquid medium this organism produced 40 per cent less exopolysaccharides.

A very large number of microorganisms including *Rhizobium* sp. (Bergmaier *et al.*, 2003; Xiao *et al.*, 2004; El-Tayeb and Khodair, 2006) produce variety of exo-polysaccharide (EPS) possessing remarkably high moisture holding capacity and serve to maintain minimum moisture in their immediate environment.

EPS protects the producing organism from desiccation and serves as a potential energy reserve as it can be catabolized under nutrient deficient conditions. Microbial EPS have been commercialized as possible future industrial commodities for food and in agriculture for the encapsulation of somatic embryoid, which offer a greater feasibility for precise delivery of plant growth regulators, fungicides and pesticides (Mathur and Mathur, 2001).

Influence of culture conditions on polysaccharide production are reported for various organisms (Bergmaier *et al.*, 2003; Xiao *et al.* 2004; El-Tayeb and Khodair, 2006 ; Yeh and Chen, 2004). It has been reported that the use of sugar components e.g. sucrose, dextrose, mannitol etc. as a sole source of carbon yields more EPS than cell biomass (Pace and Righelta, 1980).
Minerals and growth factors are also known to regulate EPS yield (Mathur and Mathur, 2001).

Sayyed et al., (2011) reported that, Under optimized conditions \textit{Rhizobium} sp. inoculated in modified YEMB and incubated at 28 °C for 6 days at 120 rpm, yielded 2.60 g L\(^{-1}\) of EPS. Increase in seed germination and overall growth and vigor in groundnut and wheat and effective root colonization reflected the potential of \textit{Rhizobium} sp. as efficient bioinoculant for sustainable agriculture.

Marczak et al., (2013) stated that, the synthesis of rhizobial EPS has been best studied in two species, namely \textit{Sinorhizobium meliloti} and \textit{Rhizobium leguminosarum}.

Castellane et al., (2014) reported that the \textit{Rhizobium tropici}, has the ability to synthesize and secrete extracellular polysaccharides (EPS). Rhizobial isolates and \textit{R. tropici} mutants that produced higher levels of EPS than the wild type strain SEMIA4080.

2.5 INTRINSIC ANTIBIOTIC RESISTANCE OF RHIZOBIA

To study the nodule occupancy by inoculated markers are needed for cross verification serological methods such as the fluorescent antibody technique and enzyme linked immunosorbant assay have been used widely, the identification of limited number of strains. Strains recognition in the laboratory could be acheived using morphological character like effective
(or) ineffective nodulation, root swelling etc, but these were not reliable for
the strain identification. Using antibiotic resistance is one of the appropriate
methods used commonly for easy identification of the introduced rhizobia
into the soil.

Effectiveness was lost frequently in colonies resistant to kanomycin
and polymycin while streptomycin resistant strains where stable in
effectiveness (Schwinghamer, 1964).

Law and strijdom (1989) compared the Rhizobium strain in
commercial inoculants. Streptomycin resistant mutants did not differ from
the commercial (sensitive to streptomycin) strains in their activity to fix
nitrogen. Competition for nodulation and activity to grow in the rhizosphere.

The lupine nodule bacteria Rhizobium lupini acquired resistance to
streptomycin when the antibiotic concentration in the media was gradually
increased. The acquired property was a well retained in the medium with out
streptomycin (kantseljaruk et al., 1978)

Balasubramanian and prabakaran (1979) developed 1000 ppm
streptomycin resistant mutant from an efficient parent strain Rhizobium
sp.(ak-6 cowpea groups) in vitro growth, polysaccharide production and
competence for modulation in both sterilized as well as normal soil were
better with str$^+$ mutant.
Josey et al., (1979) identified rhizobial strains using a novel method of intrinsic antibiotic resistance as a stable property, which enhanced the identification of rhizobia isolated from root nodules of inoculated Pisum sativum.

Multiple drug resistance is a common attribute of bacterial pathogens of human and domestic animals. Cole and Elkan (1979) first surveyed this type of resistance pattern in 48 strains of B. japoricum and found over 60 percent of the strains were resistant to chloramyrphenol polymyxin-B and erythromycin and 47 percent were resistant to neomycin and penicillin G.

The TG and NEg strains showed poor saprophytic competence compared to native rhizobia even through the parent strains were efficient (Ramanathan 1979)

Beyond and Josey (1980) found that R. phaseoli (strain 1234) was resistant to high concentration of streptomycin (200 µg ml)

Antibiotic concentration gradient was used to characterize several cultures of R. phaseoil and Rhizobium sp. based on the intrinsic resistance (Bromfield et al., 1986). Kremer and Paterson (1982) investigated the intrinsic antibiotic resistance pattern among Rhizobium and Brady rhizobium strains of different species and found that the identifying strains in the fields collected nodules to provide stand and inoculant size for application.
Bromfield and Jones (1985) studied the relationship between cryptic plasmid and rifampicin resistance, in trifluencing nodulesion and competitiveness in _Buzobium meliloti_ and reported that presence of altered DNA polymerase was responsible for rifampicin resistance character in the isolates.

Dakora and Felix (1985) used intrinsic antibiotic resistance to characterise 83 isolates from nodules of cowpea and field bean. Finger print patterns of isolates revealed considerable heterogeneity amongst the population, whereas slow growing rhizobia from _vigna unguiculata_ nodules were generally more resistant to the concentration of antibiotics used.

Garg _et al._ (1985) reported that only 165 among 200 rhizobial isolates were resistant to one (or) more antibiotics at a concentration of 5µg ml\(^{-1}\). The intrinsic antibiotic resistance of fast and slow growing rhizobia was extremely high against nalidixic acid (400µg ml\(^{-1}\)) and penicillin (200µg ml\(^{-1}\)).

Dadarwal _et al._ (1987) studied the intrinsic antibiotic resistance and effect of curing treatment on plasmid pattern, loss of antibiotic resistance and symbiotic characters slow and fast growing _Rhizobium_ strains nodulating greengram, Wackgram, cowpea, pigeonpea, mothbean and clusterbean. Most of the slow growers where resistance to tetracycline, viomycin, polymycin and rifampicin (50µg ml\(^{-1}\)) and fast grower showed resistance to kanomycin, ampicillin, chloramphenicol and streptomycin (750µg ml\(^{-1}\)) when these strains were cured by alcidine orange (25µg ml\(^{-1}\)) (or) ethidiumbromide (40µg ml\(^{-1}\))
continuously along with incubation at 40°C yielding clones sensitive to 2.3 antibiotics and ineffective nodulation in strains of greengram.

Majority of 50 _Brady rhizobium japonicum_ exhibited resistance to tetracycline (100 µg ml⁻¹). Chlormaphenicol (100 µg ml⁻¹) two strains had high levels of resistance to ampicillin (250 µg ml⁻¹) streptomycin (500 µg ml⁻¹) where as six strains were very sensitive to several antibiotics (Borges _et al._, 1990)

Date and Hurse (1992) reported spontaneous resistance to rifampini (30 µg) and streptomycin (500 µg) in strains of _BradyRhizobium_ which had similar growth and rhizosphere colorizing abilitities, but these were not equally effective in N₂ fixation in _Desmordium intortum_ cv. green leaf.

Roughley _et al._ (1992) observed that out of 38 isolates of root nodule bacteria of Malaysian soil, 13 isolates were resistant to streptomycin (600 µg m⁻¹) and Spectinomycin (1000 µg m⁻¹).

Rhizobial isolates obtained from 10 wild legumes of the Sal forest eco system in the subtropical north western Himalayas were evaluate for intrinsic antibiotic resistance using different concentration of six antibiotics (Streptomycin, Gentamycin, Rifampicin Chloramphenicol, Nieomycin and Tetracycline) Results suggested that (i) IAR patterns were both strains specific and Antibiotic specific, (ii) IAR techniques was sensitive enough to discriminate between rhizobial isolates of the same legume inhabiting
different habitats. (iii) IAR patterns of the fast growing rhizobia were dissimilar from those of slow growing isolates. (Subramanian and babu, 1993)

Germell and Roughly (1993) developed a method for studying the population dynamic of rifampicin resistant rhizobia even when they were less numbers compared with naturalized strains in soil.

Mishra and Bhattacharyya (1994) studied the inhibitory effect of Amoxycillin, Ampicillin, Cloaxicillin, Norbactin, Oxytetracycline and Tetracycline at a concentration of 50, 100, 250, 500, 1000 ppm on *Rhizobium leguminosarum* and inferred that rhizobial strains showed sensitive at higher dose for few antibiotics while at lower dose. *Rhizobium* sp. was found to be more tolerant to different antibiotics.

Gopalakrishnan *et al.*, (2015) reported that the Production of antibiotics such as 2,4-diacyetyl phloroglucinol (DAPG), Kanosamine, Phenazine-1-Carboxylic acid, Pyoluteorin, Neomycin A, pyrrolnitrin, Pyocyanin and Viscosinamide. Among them, DAPG is important since it has a broad spectrum antibacterial, antifungal and antihelminthic activity.

### 2.6 SIDEROPHORE PRODUCTION

Iron containing protein figures prominently in the nitrogen fixing symbiotic bacteria (i.e. *Azorhizobium*, *Rhizobium*, and *Bradyrhizobium* and their respective plant hosts for the synthesis of iron containing compounds
like nitrogenase, leghaemoglobin, ferridoxin, hydrogenase and cytochromes, symbiotic bacteria must require an adequate supply of iron. Availability of iron is reduced to precipitation, forming oxyhydroxide polymers of Fe(OH)₃. Therefore to compete successfully for iron, organism has evolved specific, high affinity iron, organism has evolved specific, high affinity mechanism to acquire iron. In symbiotic bacteria, these systems are composed to ferric specific ligands (siderophores) and their cognate membrane receptors (Verma and Long 1983, Neilands, 1988).

Modi et al. (1985) reported that cowpea *Rhizobium* RA-1 produced catechol like siderophore at the rate of 6.2 mg l⁻¹ of culture fiterate and further indicated that Glycine and threonine were detected in the siderophore. Maximum siderophore production was observed at 36h of growth in cowpea *Rhizobium* RA-1.

Over production of siderophore occurs in bacteria and fungi under acute iron starvation. Siderophores dissolve the complex form of ferric ion chelated in the highly insoluble oxyhydroxides. Neilands (1988) reported that in *E.coli* iron transport was regulated by a repressor protein, which bound to the ferrous ion.

Patel et al. (1988) inferred that *R.leguminosarum* IARI-102 produced 2, 3 dihydroxy benzoic acid (phenolate siderophore) threonine and hydroxamic acids were not detected.
Skoruspska et al. (1988) observed the Rhizobium trifolii produced 2, 3 dihydroxybenzoic acid and threonine, which were accumulated during stationary growth phase in iron deficient media. Presence of FeCl₂ in media suppressed the production of siderophore.

Cowpea Rhizobium RA-1 produced catechol like siderophore, which decreased with increase in the concentration of molybdenum (above 1mM) and presence of iron increased the molybdenum uptake but 2,3-dihydroxy benzoic acid did not show any increase in the uptake there by confirming that entire siderophore molecule was required for the transport of molybdenum (Urvashi Patel et al. 1988).

Buyer and Sikora (1990) developed a monochoral antibodies to ferric pseudobactin (siderophore of Pseudomonas putida Bio) to determine the concentration and localization of siderophore in the rhizosphere.

Gajendran and Mahandevan (1990) inferred that Rhizobium sp. utilized catechol upto 10mM as sole carbon source (synthetic medium) and survived for 9 months in soil containing catechol and further inferred that in the presence of organic acids and sugars catechol was cometabolized.

Guerinot (1991) studied the uptake and metabolism of iron in Rhizobium legume symbiosis and detected that production and utilization of siderophore affected rhizosphere and in bulk soil. Gill et al (1991) developed 1021 defective in siderophore production to study their role in
legume symbiosis and reported that siderophores increased the ability to fix nitrogen and resulted in an increase in plant growth.

Studies on differential siderophore utilization and iron uptake by soil and rhizosphere bacteria used ferrioxamine B as the sole Fe source in Fe deficient medium, while about 12, 10, 2 and more than 1 per cent respectively (TNSK2 SK3 and WCS S58) were able to use ferric chrome and pseudobactions (Jurkevitch et al., 1992)

Introduction of transposon Tn5 (K m⁻¹) in to a siderophore production Chinese Rhizobium fredii resulted in mutants of over producing siderophore. Nodule occupancy in greenhouse experiment by these tow mutants were 3 and 4 per cent compared to 19 per cent by wild strain proving that over production of siderophore resulted in less competitive strains, (Manjunatha et al., 1992).

Rajkumar et al., (2010) revealed that in the aerobic environment, iron occurs principally as Fe³⁺ and is likely to form insoluble hydroxides and oxyhydroxides, thus making it generally inaccessible to both plants and microorganisms.

Khan et al., (2009) reported that commonly, bacteria acquire iron by the secretion of low-molecular mass iron chelators referred to as siderophores which have high association constants for complexing iron. Most of the siderophores are water-soluble and can be divided into
extracellular siderophores and intracellular siderophores. Generally, rhizobacteria differs regarding the siderophore cross-utilizing ability; some are proficient in using siderophores of the same genus (homologous siderophores) while others could utilize those produced by other rhizobacteria of different genera (heterologous siderophores).

In both Gram-negative and Gram-positive rhizobacteria, iron (Fe3+) in Fe3+ siderophore complex on bacterial membrane is reduced to Fe2+ which is further released into the cell from the siderophore via a gating mechanism linking the inner and outer membranes. During this reduction process, the siderophore may be destroyed/recycled (Rajkumar et al., 2010). Thus, siderophores act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation (Indiragandhi et al., 2008).

Not only iron, siderophores also form stable complexes with other heavy metals that are of environmental concern, such as Al, Cd, Cu, Ga, In, Pb and Zn, as well as with radionuclides including U and Np (Neubauer et al., 2000; Kiss and Farkas, 1998). Binding of the siderophore to a metal increases the soluble metal concentration (Rajkumar et al., 2010).

Hence, bacterial siderophores help to alleviate the stresses imposed on plants by high soil levels of heavy metals. Plants assimilate iron from bacterial siderophores by means of different mechanisms, for instance,
chelate and release of iron, the direct uptake of siderophore-Fe complexes, or by a ligand exchange reaction (Schmidt, 1999). Numerous studies of the plant growth promotion vis-a’-vis siderophore-mediated Fe-uptake as a result of siderophore producing rhizobacterial inoculations have been reported (Rajkumar et al., 2010).

Crowley and Kraemer (2007) revealed a siderophore mediated iron transport system in oat plants and inferred that siderophores produced by rhizosphere microorganisms deliver iron to oat, which has mechanisms for using Fe-siderophore complexes under iron-limited conditions. Similarly, the Fe-pyoverdine complex synthesized by Pseudomonas fluorescens C7 was taken up by Arabidopsis thaliana plants, leading to an increase of iron inside plant tissues and to improved plant growth (Vansuyt et al., 2007).

Sharma et al. (2003) assessed the role of the siderophore-producing Pseudomonas strain GRP3 on iron nutrition of Vigna radiata. After 45 days, the plants showed a decline in chlorotic symptoms and iron, chlorophyll a and chlorophyll b content increased in strain GRP3 inoculated plants compared to control.

2.7 MUTAGENESIS AND MUTANTS OF RHIZOBIUM

The use of spontaneous (or) induced mutation appears to be more generally applicable to strains improvement because it may be used with any strains without compatibility restriction. Mutation could be used to add
desirable character to an already effective inoculant strain. (Bergersen et al., 1971).

The frequency at which spontaneous mutation occurs is usually too low to enable mutants to be obtained. Mutagenic technique increases mutation rates. The successful isolation of mutants having a non-selective phenotype depends upon highly induced mutation frequency. Beringer (1973) pointed out at not all species (or) even strains of microorganisms are equally sensitive to mutagenic treatments and the choice of mutagen can be determined by the susceptibility of this strain. The efficacies of different mutagens for *Rhizobium* sp. have been reported by many workers, (Raina and Modi 1969; Beringer 1973; Meade and Singer, 1977).

Robert Maier and Winstonbrill (1969) reported that a strain of *Rhizobium japonicum* was mutagenized using chemical mutagen N-methyl-N nitro-N nitrosoguanidine (NTG) By a rapid effective assay, the mutants were screened in soybean plants. It was found that two mutant strains nodulated earlier than the wild type, in addition, more root nodules were reported than that of wild type. Similarly, using NTG, effective and non-effective mutants were derived from the *Rhizobium* (Williams, 1983).

Walton and Moseley (1981) compared the efficacy of a variety of common mutagens like ethyl methane sulphonate (EMS), Methyl methane sulphonate (MMS), decarboxyl mitocin-c, nitrous acid, N-methyl-N-nitro-
N-nitroso guanidine (MNNG), gamma irradiation and ultraviolet irradiation to induce mutations on *R. trifolii* and concluded that mutagenesis with MNNG yield more frequency in mutations and comparatively the frequency as that of Tn5 mutagenesis.

In certain rhizobium plant combinations auxotrophy in the bacterial partner is associated with specific blocks in symbiosis. One metabolic group, which is apparently important, is purines, particularly adenine. In *R. leguminosarum* purine a auxotrophs lost the ability to nodulate pea plant and all their prototrophic revertants were Nod+. An auxotroph with a double growth requirement (adenine) was found to be defective in nodule development. (pain,1989). Scherrer and Danarie (1971) isolated three adenine and one uracil requiring mutants of *R. meliloti*. Adeuine metabolism in *Rhizobium* is of great interest since it plays important role not only in nutric acid synthesis but also in energy metabolism. Polysaccharides synthesis and possibly in production of plant growth regulator compounds such as cytokinins. (Verma and Sharon Long 1983).

Singh *et al.*, (1992) have reported that strains were derived from *Rhizobium leguminosarum* by nitrosoguanidine and transposon mutagenesis. He also reported that with in a concentration range of 100-500 ug/ml nitrosoguanidine had no lethal effect on *R. trifoli*. But produced a significant number of mutants.
Casse et al., (1979) improved Brady rhizobium japonicum strain with NTG and screened on soyabean seedlings. He reported that the mutants showed greater ability to reduce acetylene (bio-assay for nitrogen fixation) than that of their wild type parents. Nuti et al. (1990) have reported that an effective mutant strain of Rhizobium leguminosarum was defective in symbiotic iron acquisition. The strain was isolated after nitrosoguaridine mutagenesis. They concluded that a particular DNA insert eneroding an element of iron acquisition system in essential for symbiotic nitrogen fixation.

Pankhurst et al. (1995) have reported that three symbiotic pea mutants were derived by using chemical mutagens. Grafting studies confirmed that the mutation were due to different genes. The nodulation was controlled by the factors originating in the stem and roots. The super nodulation factor was localized to cytochrome.

Sagan and Due (1995) reported that using Ethyl Methane Sulphonate, mutants were derived from Pisum sativum rhizobia. He also reported that two new genes viz. Sym 28 and Sym 29 were involved in regulation of nodulating in pea.

The chemical mutagen EMS was most effective mutagen in inducing auxotrophy in Rhizobium trifolii. But the frequency of reversion was reported to be high in the derived mutants (Jones and Bromfield, 1981).
Berinerger (1974) isolated various auxotrophic mutants of cowpea rhizobia by using NTG (n-methyl-N nitro-N-nitroquanadine) chemical mutagen.

Kohli et al. (1977) have reported that the mutagenic effect of ethyl methane sulphonate (EMS) on Bradyrhizobium japonicum. They isolated various auxotrophic mutants of histidine, glycine, arginine, threonine and aspartic acid. The frequency of spontaneous reversion of the mutants were low and thus showed that EMS was a potent mutagen for producing stable mutants.

Similarly using ethyl methane sulphonate auxotrophic mutants of various amino acids were derived from Rhizobium meliloti (Jones and Bromfield, 1981).

Deirdre and Baltz (1981) have reported about the efficiency of common mutagens such as N-methyl-N' Nitrosoguanidine (MNMG), nitrous acid, EMS, o.v irration in producing mutation on Rhizobium trifolii. Among them, N-methyl-N-Nitro-N-Nitrosoguanidine (MNMG) was effective in producing eight auxotrophs of different amino acids. Likewise, transposon mutagenesis with TN5 yielded the same frequency and range of auxotrophs as did MNMG.

In comparison between chemical and irradiational mutagenic effects on R.leguminosarum, irradiation yielded morphological mutants which had higher nodulation efficiency, while chemical mutagenesis yielded auxotrophic
mutants which were superior to parent strain in nodulation but less efficient than the radiation mutants (Khanuja and Archrasumas 1993).

Hadley et al. (1983) have reported that the physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon TN 5 mutagenesis. Two classes of symbiotic mutants were isolated. Four of the twenty formed no nodules at all and sixteen formed nodules which failed to fix nitrogen.

Sherman et al. (1984) have isolated different auxotrophic mutants of various amino acids from *Rhizobium japonicum*. The glutamate auxotrophs were studied in detail and it showed an altered expression of nitrogenase activity in free living condition.

Four Listerine requiring auxotroph of *Bradyrhizobium japonicum* strains USDA 122 were isolated by Sadowsky et al., (1986) two of these were nodulating and other two were non-nodulating.

Khanuja and Archrasumas (1993) examined a variety of common mutagens such as NTG, EMS, nitrous acid and Ultraviolet irradiation on Cowpea rhizobia. The percentage of auxotrophy was high by ultraviolet irradiation followed by EMS and NTG respectively. Majority of the mutants were auxotrophs of aminoacids and methonine. Symbiotically defective autotrophic mutants of *Rhizobium fredii* was induced by transposon mutagenesis. Fourteen auxotrophs were derived. All the auxotrophs induced
nodulation on soyabean, but Symbiotic effectiveness of each mutant was different.

Aind and Modi (1989) have reported that twenty three amino acid auxotrophs of *Bradyrhizobium japonicum* were derived by either nitrous acid (or) TN5 induced mutagenesis; they also reported that three of the eleven tryptophan dependent auxotrophs formed nodules. The other Trp”, Pro” and His” mutants formed only abortive nodules. The doubly auxotrophic strain TA5A5 did not form any nodule like structure.

William and Phillips (1990) reported that a protroph revertant (TA 11 NOD⁺) of a nodulation defective tryptophan auxotrophs of *Bradyrhizobium japonicum* showed higher nodulation, and enhanced nitrogen fixation than that of normally nodulating wild type strain *Bradyrhizobium japonicum* showed higher nodulation, and enhanced nitrogen fixation than that of normally nodulating wild type strain *Brady rhizobium japonicum* 1-110 ARS (strain-I-110 ARS & 2 as the parent of auxotrophic mutant TA-11).

Malek and Kowalski (1990) reported that two auxotrophic mutants were isolated by treating *Rmeliloti* cells with EMS. They have characterized the mutants as base requiring ones and these two mutuants formed nodules in the host plant, but the nodules were found to be ineffective and did not contain the bacteroids.
2.8 LEGHAEMOGLOBIN CONTENT OF NODULES

Subba Rao (1977) reported the natural relationship among bacteriodes leghaemoglobin and d-nitrogen content of Egyptian clover (*Trifolium alexandrium* and Gram Cicer Arietinum) at different age levels. The relationship among the three factors was found to be positive and significant in both the plants.

Thakkar *et al.*, (1973) studied the indole extracts different for their absorption spectra, leghaemoglobin and iron content. The leghaemoglobin content varied from 7.54 to 13.08 mg g\(^{-1}\) fresh weight and from 39.08 to 71.15 mg g\(^{-1}\) on dry weight basis. The specific activity varied from 0.34 to 0.95 the highest being of the leghaemoglobin of moth bean, the iron content varied from 3.25 to 4.15 of dry weight of nodules.

Broughton and Dilworth (1971) reported that leghaemoglobin is a haemoprotein found in legume root nodules and the presence has been correlated directly with the effectiveness in nitrogen fixation. Leghaemoglobin function in facilitating oxygen diffusion at relatively Low tension.

Thakkar and Vyas (1975) reported that the leghaemoglobin of daincha gave absorption maxima at 415 nm 530 nm and 574 run. But the pyridine homochromogen of daincha and soybean leghaemoglobin have similar chemical nature.
Huang et al. (1988) have shown the quality of leghaemoglobin is regulated by bacterial strain and is related to oxidation reduction conditions required by the *Rhizobium* Cowpea miscellany Rhizobia and not related to effectively beyond certain level. Uptake hydrogenase in *Rhizobium* and nodule leghaemoglobin in Cowpea miscellany host.

Kumar (1988) stated that leghaemoglobin content of nodules was directly correlated with total nitrogen fixation and was increased with rhizobial inoculation.

Prasad (1983) reported that root exudates were found to chemotactic. Nodulation and leghaemoglobin were found to be most range afic.

Gopal and Prasad (1992) reported that graded levels of potassium increased the laemoglobin content of nodules of horsegram plants raised with *Rhizobium* ailation. The Leghaemoglobin content increase was maximum (80.16 % over trol) at 50 kg of K₂O ha⁻¹.

### 2.9 CHEMOTACTIC ACTIVITY OF LEGUME ROOT EXUDATES

Plant root exudates interact with the root nodule bacteria in different ways. Some of these compounds are known to induce nodulation (nod) genes in *Rhizobium*. Chemotaxis of bacteria was reported by several workers (Kush and Daderwal, 1978). Gitte et al. (1978) noticed chemotaxis of *Rhizobium* towards root exudates.
Extracts and Exudates from nodulated root systems were different from Biose in system without *Rhizobium* nodules. (Hale and Moore, 1979) Soby and Aguilar *et al.*, (1988) reported that chemotaxis and mobility have been found to make important contributions to the symbiotic interactions of *Rhizobium* with its host.

Caetano-Anolles *et al.*, (1988) found out both *Rhizobium* and *Brady rhizobium* are attracted by amino acids, dicarboxylic acids and sugars present in the root exudates. Rhizobia have been reported to be attracted by very low concentrations of excreted compounds like flavanoids that may have low nutritional value (Aguilar *et al*, 1988).

Soybean plant root exudates contains sugars like sucrose, galactose, arabinose, the organic acids like malic acid, malonic acid and citric acid and the amino acids like arginine, Histidine, Threonine, tyrosine, and Valine. (Barbour *et al.*, 1991).

Napoles *et al.* (1998) reported the chemotaxis of *B. japonicum* towards organic acids and soybean seed exudates. Soybean exudates were strong bacterial chemoattractant and the effect decreased when dilution increased. Organic acids showed weak effects on the micro symbiont attraction.

Snchal Pondya *et al.* (1999) found out the chemotaxis of *Rhizobium* towards *Cajuns cajan* root exudates and its major components. The
chemotactic response of *Rhizobium* sp a show growing *Cajanus cajan* isolate towards, its host root exudates was examined, two classes of mutants, one non-chemotactic towards nutrients (amino acids and sugars) and signal compounds like flavonoids and other mutant non-chemotactic. Towards amino acids and sugars but positive towards naringenin.

Taramenon and Rao (1999) pigeon pea exudates not only increased the nitrogenase activity and exopolysaccharide synthesis of the microsymbiont but also induced it, to synthesize the root hair deformation factor (Nod factor).

### 2.10 NITROGEN TRANSPORT IN LEGUMES - UREIDES AND UREIDE ASSIMILATORY ENZYMES

Legume plants have adopted different strategies for assimilating and transporting the fixed nitrogen. In tropical legumes, ureides (allantoin and allantoic acid) were synthesized from products of N fixation, (*viz.*, purine synthesis and degradation).

Allantoin and allantoic acid were the major forms of organic nitrogen translocated from the nodules and ureides were stored and assimilated mainly in the shoot. (New comb *et al.*, 1981; Ohyama and Kumazawa 1981).

Shantz (1959) suggested a path way for ureide synthesis in plants involving the four enzymes namely Xanthine Oxidase, Uricase, allantoinase and allantoicase in the aerobic oxidation of purines.
The following pathway explains the various enzymes involved in the purine catabolism.

**PRODUCTS OF PURINE CATABOLISM**

- Adenine
  - Hypoxanthine
    - Guanine
    - Xanthine
      - Xanthine Oxidase
      - Uric acid
        - Uricase
        - Allantion
          - Allantoinase
          - Allantioic acid
            - Allantiocase
              - Urea + glycoxylate
                - Urease
                  - Ammonia + Co₂
Luthra et al (1983) investigated the developmental changes of ureides and the activities of ureide assimilating enzymes with allantoinase and urease in the leaves and different parts.

The results showed that, the Urease activity in all the organs with maximum activity at 60 DAS in leaves followed by roots and nodules. The presence of substantial amount of Urease at 120 days in both pod walls and seeds showed that Ureids were metabolised to urea and further to ammonia and CO$_2$.

Matsumoto et al (1977) in A-62-1 soybean cultivar (nodulating type) observed a positive correlation between nodule weight and Ureide concentration in stem, roots and leaves at different stages of the plant growth and the concentration of Ureides peaked at 9 weeks after growing. The nodulating soybean variety A-62-1 accumulated more Ureides in stem and roots than non modulating type A-62-2 at flowering to early pod filling stage.

In case of cultivars coloona, the relative abundance Ureides was 95 per cent at 29-39 DAS, 81 per cent at 47-53 DAS and 80 per cent at 61-65 DAS. Similar pattern of Ureides decrease in average also found in Xylem sap of mung bean Kl-klilezek grown incidential conditions (Pate et al., 1980).
Mcclure *et al* (1980) reported that Xylem sap Ureide analysis are a convenient means to account for N$_2$ derived by biological nitrogen fixation. This experiment with Soybean cultivar Daris under greenhouse conditions showed that, acetylene treatment to inhibit N$_2$ - fixation drastically reduced Ureide content in Xylem sap. This was clear identification that Xylem sap Ureides could be as an indicator of N$_2$ fixation.

Herridge (1982) reported a linear relationship between nodulated roots and shoot axis with respect to their Ureide concentration. There was an inverse relationship between nitrate and Ureide contents in the different plant parts in both nodulating and non nodulating Soybean cultivars. The nitrogenase activity was negatively correlated with NO$_3$ content of Soybean var. Bragg peaked at 60 DAS which was positively correlated with the concentration Ureides.

The Ureides, allantoin, and allantoic acid are the major nitrogenase solutes in Xylem exudate in tropical legumes, which are entirely dependent upon symbiotic N$_2$ fixation; the plants which are less symbiotically dependent contain proportionally lower concentrations of Ureides (Herridge 1984). The Ureide concentration was maximum at R2-R3 stages in all cultivars and gave a positive correlation with a grain yield of Soybean cultivars (Sarkar *et al*., 1991).
Castro and Acuna (1992) in green house trails determined the Ureide content at three growth stages in leaves and stems of Soybean and bean. In both Soybean and bean, the Ureide content was higher in stem and decreased with an increasing age.

Fue et al., (1995) screened eight wild cultivars of Soybean for their nitrogenase activity and Ureide content at different stages of growth. The content in the stem was positively correlated with the nitrogenase activity.

Avline et al (1995) proposed that Ureides assay method has been found to be suitable for Soybean to assess nitrogen fixation in wide range of genotypes. Their results confirmed that the relative abundance of Ureides was closely related to the derived from symbiotic N2 fixation at vegetative and reproductive stages.

2.11 STRESS CONDITIONS

Hamida and Shaddad (2010) suggested that several environmental factors adversely affect plant growth and development and final yield performance of a crop. Drought, salinity, nutrient in balances (including mineral toxicities and deficiencies) and extremes of temperature are among the major environmental constraints to crop productivity worldwide. Development of crop plants with stress tolerance, however, requires, among others, knowledge of the physiological mechanisms and genetic controls of the contributing traits at different plant developmental stages. In the past 2
decades, biotechnology research has provided considerable insights into the mechanism of biotic stress tolerance in plants at the molecular level. Furthermore different abiotic stress factors may provoke osmotic stress, oxidative stress and protein denaturation in plants, which lead to similar cellular adaptive responses such as accumulation of compatible solutes, induction of stress protein and acceleration of reactive oxygen species scavenging systems. To improved plant tolerance to salinity injury through either chemical treatments or biofertilizers treatments (Asymbiotic nitrogen-fixing bacteria, symbiotic nitrogen-fixing bacteria and Mycorrhiza) or enhanced a process used naturally by plants to minimize the movement of Na+ to the shoot, using genetic modification to amplify the process.

Ali et al. (2009) evaluated that the effect of salt, pH and temperature on the growth of rhizobia isolated from *Leucaena leucocephala*, *Tephrosia purpurea* and *Crotalaria medicaginea* grown in arid and semiarid regions of Rajasthan with a view to screen out stress tolerant isolates. A total of 27 isolates have been used for screening their stress tolerating ability with contrast to environmental abiotic soil conditions commonly prevailing in arid and semi-arid regions of Rajasthan. All the isolates were phenotypically and biochemically characterized followed by their plant assay test in growth pouches and pot experiment under controlled environmental conditions. Growth of pure rhizobial isolates on Yeast Extract Mannitol (YEM) medium having variable range of pH (4-10) and different concentrations of NaCl
(0.01-4.5%) were recorded at 540 nm using UV-Vis spectrophotometer after incubation at 28± 2°C for 2 days. The stress tolerant traits of these rhizobia are of potential value from the point of view of biofertilization of legume seedlings during of forestation of degraded areas in arid and semiarid tropics of Rajasthan (Ali et al., 2009).

Hung et al. (2005) reported that the root-nodulating bacteria were isolated and characterized from 7 native shrubby legumes and measured growth rates in various media, colony morphology and tolerances to extremes of temperature, salt and pH. Among the 83 isolates that were screened, the majority were fast-growing rhizobia, 28 strains tolerated high concentration of salt (45% NaCl) and grew well between temperatures of 37 and 45°C. The majority of the strains also tolerated extreme pH in their medium from 3.5 to 12. All strains formed nitrogen fixing nodules and the highest activity was detected in the legume Hedysarum crinita L. PCR restriction fragment length polymorphism (PCR-RFLP) and sequencing of the small subunit ribosomal RNAs revealed that the majority of the isolates belonged to the genera Rhizobium, Bradyrhizobium and Agrobacterium.

Serraj and Gyamfi (2004) suggested that the inclusion of a legume in a cropping system does not always ensure the attainment of optimal levels of symbiotic nitrogen fixation (SNF) in the field. Several environmental factors including drought, temperature and soil nutrient status are known to dramatically affected process at molecular/functional level and thus play a
part in determining the actual amount of nitrogen fixed by a given legume in
the field. Natural occurrence of *Sinorhizobium meliloti* nodulate *Medicago
sativa* and *Rhizobium leguminosarum* bv. *trifolii* nodulate and *Trifolium
alexandrinum* L. were examined in soils of 125 locations. *S. Meliloti*
occurred in almost all the soils, while *R. leguminosarum* bv. *Trifolii*
occurred in a very few soils. The reasons of the difference in their
occurrence were attributed to the variations in the occurrence or cultivation
of their respective host legumes and also the variation in the ability of these
nodule bacteria to survive under extremes of the environment. Inoculation of
these two legumes with their respective nodule bacteria improved the forage
yield; and the response was more on *Trifolium alexandrinum* than on
*Medicago sativa* (Gaur et al., 2002).

Wild legumes (herb or tree) are widely distributed in arid regions and
actively contribute to soil fertility in these environments. The N$_2$-fixing
activity and tolerance to drastic conditions may be higher in wild legumes
than in crop legumes. The wild legumes in arid zones harbour diverse and
promiscuous rhizobia in their root-nodules. Specificity existed only in few
rhizobia from wild legumes, however, the majority of them are with wide
host range. Based on phenotypic characteristics and molecular techniques,
the root-nodule bacteria that was isolated from wild legumes and been
classified in to 4 genera (*Rhizobium*, *Bradyrhizobium*,*Mesorhizobium* and
*Sinorhizobium*). The rhizobia of wild legumes in arid zones, exhibit higher
tolerance to the prevailing adverse conditions, e.g. salt stress, temperatures and desiccation. These rhizobia may be used to inoculate wild, as well as, crop legumes, cultivated in reclaimed desert lands. Recent reports indicated that the wild-legume rhizobia formed successful symbioses with some grain legumes. Rhizobia have specific traits that can be transferred to other rhizobia through genetic engineering tools or used to produce industrially important compounds. Therefore, these bacteria are very important from both economic and environmental points of view (Zahran, 2001).

2.12 SALT STRESS ON THE LEGUMINOUS PLANTS

Predeepa and Ravindran (2010) observed that the *Rhizobium* legume symbiosis is one of the most well-established symbiotic nitrogen fixing system for agronomic studies. Salt-tolerant rhizobial strains or salt-tolerant cultivar does not necessarily promise a salt-tolerant symbiotic system, as the symbiotic system is more sensitive to salt stress than the bacterium and/or the plant. Salt tolerance of the symbiotic system decreased by 1 ds/m, and also that there is a gradual shift in the spatial distribution of the nodules from the primary roots to the secondary roots under increased salt levels and is time-dependent. *Arachis hypogaea* L. is considered to be one of the most important crop have high nutritive value and a source of edible oil and tested the effects of different salt levels on mineral nutrient partitioning and morphological characteristics of plant. Three concentrations of salt solution including 50, 100 and 200 mM NaCl and the control were used in irrigation.
The leaf relative water content (LRWC) provoked by the salinity in nutrient solution decreased from 85.08 to 79.70 per cent. Salt stress reduced significantly the plant height, the number of leaves, dry weight of roots, the dry weight of stems, plant and dry weight of leaves and also K+, Mg2+, Ca2+, P, N, K+/Na+ and Ca+/Na+ uptake of peanut plant organs were significantly reduced with increasing salinity (Desire et al., 2010).

Dardanelli et al. (2009) studied that the effects of saline and osmotic stress on four peanut rhizobia, plant growth and symbiotic N2-fixation in Arachis hypogaea were studied. Abiotic stress was applied by adding 100 mM NaCl. At the rhizobial level, Bradyrhizobium ATCC 10317 and TAL 1000 showed stronger tolerance to stress than TAL 1371 and SEMIA 6144. The effect of salinity on the bacterium – plant association was studied by using the variety Blanco manfredi M68. In the absence of stresses, all the strains induced a significantly higher number of nodules on the roots, although TAL 1371 and SEMIA 6144 were more effective. Both stresses affected the interaction process, while TAL1371 was the best partner.

Taffouo et al. (2009) found that the effects of NaCl concentrations on physiological behaviour of organs of five leguminous plants. Plants were submitted to 5 levels of salt stress at the roots (0, 50, 100, 150 and 200 mM of NaCl). NaCl had an understanding effect on growth of stems and seed germination of species. The reduction of stems growth rate were not significant in P. adenanthus whereas in M. poggei and V. Unguiculata this
inhibition was observed just when nutritive solutions were enriched with 200 mM. The lipid contents were reduced in all the species under salt stress, whereas proteins and proline contents in the leaves were substantially increased in tolerant species of *M. poggei*, *P. adenanthus* and *V. unguiculata*. Proteins and leaf proline contents were negatively affected by salt concentrations to *G. max* and *P. vulgaris*. Seed germination, proteins, proline contents could be used as physiological criteria of early selection for salt tolerant leguminous plants. Effect of low temperature, salinity, nutrient level and photoperiod has been studied on 3 varieties of *Pisum sativam var.*, P-48, meteor and AM-inoculated with *Rhizobium* strain PS-1. The plants were grown at 5, 10, 15, 20 and 25°C. The number of viable cells was counted at each temperature by most probable number technique. The number of viable cells were constant at 5 and 10°C but increased between 15-30°C. Nodule formation was not observed at 5, 10 and 25°C at 15°C the nodules were less in number, whereas at 20°C best nodulation was observed. The pea plants were also subjected to different salinity levels i.e., 0, 1, 2, 3, 4, 5, 10 and 15 dsm-1. High number of nodules was formed at 0 and 1 dsm-1 whereas at 2 and 3 dsm-1 the nodule number was reduced and yet at higher salinity levels no nodules were formed. Pea plants nodulated best at 12 hours photoperiod and 1/6th concentration of Hoagland’s nutrient solutions. The mature nodules developed at different temperatures and salinity levels (Naeem *et al.*, 2008).
Gaballah and Gomaa (2005) investigated the impact of *Rhizobium* inoculation and/or sodium benzoate application on performance of two contrasting faba bean varieties i.e., Giza Blanka (salt tolerant) and Giza 634 (salt sensitive) grown in sandy soil under two levels of salinity (3000 and 6000 ppm). High salinity (6000 ppm) greatly reduced nodules formation in both varieties. *Rhizobium* inoculation reduced the inhibitory effect of salinity and plants were able to survive better. *Rhizobium* inoculation increased plant leaf area, reduced MDA content in plant leaves and did not show a significant effect on SOD activity in plant roots, but the interaction between *Rhizobium* inoculation and sodium benzoate resulted in a significant increase in SOD enzyme activity plant roots.

Bouhmouch *et al.* (2005) studied that the effect of salt stress on the *Rhizobium*-common bean symbiosis. The comparison of the behaviour of five cultivars of *Phaseolus vulgaris* differing in seed colour, growing on nitrates and different concentrations of NaCl, showed genotypic variation with respect to salt tolerance. *R. tropici* strain RP163 and *R. Giardinii* strain RP161. Their relative growth was moderately decreased at 250 mM NaCl, but they were able to grow at a low rate in the presence of 342 mM NaCl. Their viability at the minimal inhibitory concentration was slightly affected. In the absence of salinity, the strains induced a significantly higher number of nodules on the roots of the cultivar, SMV 29-21 compared to those of Coco Blanc. In the presence of salinity, Coco Blanc was more severely
affected when associated with RP163 than with RP161. Salinity affected the
nodulation development more than it affected the infection steps. Neither of
the 2 strains was able to nodulate SMV 29-21 under saline conditions, in
spite of the fact that this was considered the most salt-tolerant variety.
Common bean plants inoculated with salt-tolerant *Rhizobium tropici* wild-
type strain CIAT899 formed a more active symbiosis than did its decreased
salt-tolerance (DST) mutant derivatives (HB8, HB10, HB12) or almost
ineffective (HB8, HB13) modules (Fixd) under non-saline conditions. The
DST mutant formed nodules that accumulated more proline than did the
wild-type nodules, while soluble sugars were accumulated mainly in
ineffective nodules. The salt stress affected the plant growth, nitrogen
fixation and the activities of the antioxidant defense enzymes of nodules.
Mutant nodules showed lower antioxidant enzymes activities than wild-type
nodules (Tejera *et al.*, 2004).

Anthraper and DuBois (2003) studied that the effect of varying NaCl
concentrations on growth, N2 fixation and percentage of total tissue nitrogen
in different organs in *L. leucocephala*. Seeds were germinated and grown for
either 0, 7, 14, 21 or 28 week with either deionized water (control),
0.000625 mol/L, 0.0125mol/L, 0.025 mol/L, 0.05 mol/L or 0.1 mol/L NaCl
in addition to the fertilizer every 2 week. Growth was measured as plant
height, nodule number and mass, dry tissue mass. N2 fixation was measured
by the acetylene reduction assay. Percentage of tissue nitrogen was
determined using Kjeldahl analysis. In younger plants (7 weeks treatment), major fluctuations in NaCl tolerance were observed in the different plant organs. As plant matured (14 and 21 week treatment) NaCl concentrations of 0.025 mol/L and higher caused the greatest reduction in growth and tissue nitrogen. The NaCl concentrations of 0.025 mol/L and greater caused a major decrease growth, N2 fixation and percentage of tissue nitrogen in *L. leucocephala* plants.

Dash and Panda (2001) presented that the NaCl salt stress induced changes in growth and enzyme activities in black gram seeds during germination. A decrease in germination percentage, root length, shoot length and fresh mass was noticed with an increase in NaCl concentration. With the increase in NaCl concentration and duration of stress proline content increased and catalase, peroxidase and polyphenol oxidase activities decreased. Strain Ch-191 of *M. Ciceri* was grown with different NaCl concentrations. Protein and lipopolysaccharide patterns were determined by electrophoresis. The strain Ch-191 tolerated up to 200mm 011-1 NaCl, although highest salt dosages limited its growth and induced changes in the slowest band and appearance of an intermediate mobility band. The accumulation of proline in response to salt stress surpassed that of glutamate. The protein profile showed major alterations at salinity levels which inhibited growth. The alterations in the LPS profile and accumulation of compatible solutes were evident from the lowest levels, suggesting that
these changes may constitute adaptive responses to salt, allowing normal
growth. The selection and characterization of salt tolerant strains, which also
show efficient symbiotic (Soussi et al., 2001).

The rhizobia from the alkaline soil showed significantly higher salt
tolerance than those isolated from neutral soil. *Rhizobium* sp. NBRI0102
_Sesbania* and *Rhizobium* sp. NBRI2502 _Sesbania_ tolerated yeast extract
mannitol broth (YEB) containing 10 and 28 per cent salt (NaCl, wt/vol) for
up to 18h of incubation at 30°C. Growth of *Rhizobium* sp. NBRI0102
_sesbania_ and *Rhizobium* sp. NBRI2505 _Sesbania_ at pH 7.11, and 12 was
identical, except for a lag period of about 10 h in the growth of *Rhizobium*
sp. NBRI0102 _Sesbania_ at pH 11 and 12, as compared with pH 7. *Rhizobium*
sp. NBRI0102 _sesbania_ and *Rhizobium* so. NBRI2505 _sesbania_ survived at
50°C and 65°C, in YEB at pH 7 (Kulkarni et al., 2000).

A commercial cultivar *Vicia faba* L.var. was inoculated with salt-
tolerant *Rhizobium leguminosarum* biovar, Viciae strain GRA 19 in solution
culture with different salt concentrations (0,50,75 and 100 m moles1\textsuperscript{-1} NaCl)
added immediately at the time of inoculation. *Rhizobium leguminosarum*
strain GRA 19 formed an infective and effective symbiosis with *faba* bean
under saline and nonsaline conditions. Salinity significantly decreased shoot
and root dry weight, nodule weight and mean nodule weight. Roots were
more sensitive to salinity than was plant growth. Analyses of ammonium
assimilating enzymes in the nodule showed that glutamine synthase, and that
it limits ammonium assimilation under saline stress (Cordovilla et al., 1999).

Singly and doubly - labelled antibiotic resistant mutants of 250 mg/ml of streptomycin and spectinomycin were isolated from chickpea Rhizobium 2-ICAR-UNK-Ch-191 (Ch191). All mutants exhibited similar characteristics to the wild-parent type in their response to nodulation and tolerance of salinity and temperature. Salinity (1.0ds/m) decreased root and shoot dry weight, total nodule number and nodule weight. Inoculated plants accumulated more N compared to N fertilized plants. The Rhizobium is more salt-tolerant than the cultivar and Rhizobium strain Ch191 is effectively fixing N in saline and non-saline conditions (Elsheikh, 1992).

Zahran (1991) suggested that the Rhizobium-legume symbiosis in arid ecosystem is particularly important for locations where the area of saline soils is increasing and becoming a threat to plant productivity. Legumes, which are usually present in arid ecosystems, may be adapted to fix more N\textsubscript{2} under saline conditions than legumes grown in other habitats. Legumes are known to be either sensitive or moderately resistant to salinity. The salt sensitivity can be attributed to toxic ion accumulations in different plant tissues, which disturb some enzyme activities. Among the basic selection criteria for salt-tolerant legumes and rhizobia are genetic variability within species with respect to salt-tolerant legumes and rhizobia are genetic variability within species with respect to salt tolerance, correlation between accumulations of organic solutes and salt tolerance and good relationships
between ion distribution and compartmentation and structural adaptations in
the legumes. Salt stress reduces the nodulation of legumes by inhibiting the
very early symbiotic events. Levels of salinity that inhibit the symbiosis
between legumes and rhizobia are different from those that inhibit the
growth of the individual symbionts. The poor symbiotic performance of
some legumes under saline environments include rhizobial colonization and
invasion of the rhizosphere, root-hair infection and the formation of
effective salt-tolerant nodules.

2.13 EFFECTS OF SALT CONCENTRATIONS ON GROWTH OF
RHIZOBIUM

Rhizobacteria, being soil microorganisms are confronted with
fluctuating osmotic pressures of the rhizosphere. *Rhizobium* is an important
microbe because of its impact and interaction with the host plant. Changes in
salt concentration affect the growth and functioning of *Rhizobium*. The
effects of varying salt concentrations from 0.2 M to 0.00625 M are reported.
*Rhizobium* is capable of osmoadaptation; it can tolerate high salt
concentrations, the growth tends to be inversely proportional to salt
concentration. In other worlds, growth decreases with increasing salt
concentration Rhizobial growth is more abundant at lower salt
concentrations ranging from 0.00625 M to 0.0125 M (Rafiq, 2007).

Mensah *et al.* (2006) found that the effects of salt concentrations
verifying from 0.005 M to 0.0200 M NaCl and a pH ranging from pH 3 on
growth of *Rhizobium* as well as the cowpea associated with the *Rhizobium*. The *Rhizobium* species from cowpea were capable of osmoadaptation and were found to tolerate a relatively high salt concentration of up to 0.200 M NaCl. The population count was inversely proportional to the salt concentration with high growth (30-31.6 x 10^4 cfu/ml) at lower concentration of 0.005-0.010 M and low growth (7.1-19.2 x 10^4 cfu/mL) at higher salt concentrations of 0.050-0.300 M. The optimal pH range for the growth of the *Rhizobium* sp. was pH 6-7 while lower or higher pH values recorded lower population counts. Low yield observed for the cowpea at higher salinity and low pH. To improve the yield of cowpea in a saline soil with low pH, it is essential to reduce the soil pH to a range of 6-8 and desalinate to enhance the growth of the cowpea as well as the *Rhizobium* sp. associated with it. The halotolerant strain *Rhizobium meliloti* EFBI modifies the production of extracellular polysaccharides response to salt EFBI colonies grown in the presence of 0.3M NaCl show a decrease in mucoidy and in salt-supplemented liquid medium this organism produces 40 per cent less exopolysaccharides. Transposon-induced mutant that, when grown in the absence of salt, had a colony morphology similar to the colony morphology of the wild type grown in the presence of salt. Calcoflour fluorescence, proton nuclear magnetic resonance spectroscopy and genetic analysis of the mutant indicated that galactoglucan, which is not produced under normal conditions by other *R. meliloti* strains, is produced by strain
EFBI and that production of this compound decreases when the organism is grown in the presence of salt (Lloret et al., 1998).

### 2.14 YIELD COMPONENTS

Sujata Bhati et al., (1998) vigorous mutants were also observed from 0.8 % EMS treated seeds of *Vegan mungo*. These mutants were taller and had more and larger root nodules, higher number of pods plant$^{-1}$ and increased yield plant$^{-1}$. The mutants showed 1.1 to 1.9% higher protein content, without any significant change in aminoacid composition and trypsin inhibitor activity.

Khanuja and Archna Suman (1993) reported that a transposon Tn 10 vehicle was developed using a self transmissible (Tra$^+$) plasmid pRK 2013, this plasmid can be employed to cause independent insertion mutations in rhizobia by Tn 10 transposition. Through this mutation technique *Vigna Mungo* crop can be improved the yield and protein content of the mutant.

Gautam and Mittal (1999) studied the effect of mild and chronic doses of gamma-rays on nodulation and nitrogen fixation in Mi generation of irradiated common bean Cv HUR-9. Gamma rays at 5, 10 and 15 KR were found to be stimulatory dry weight, leaf area, nitrogen content, and grain yield per plant significantly.

Okereke et al. (2000) conducted two field experiments at Akwa, Nigeria to assess the competitivenesess of foreign Bradyrhizobia infecting the
promiscuous soybean cultivar (TGX G36-021). Seeds were nocculted with antibiotic mutants of the Bradyrhizobia strains before sowing after land preparation. Nodule number significantly increased and showed great variability of 84 days after sowing probability due to difference in the ability of inoculant Bradyrhizobia form nodules with the soybean cultivar TGX 536-020.