REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Bacteria responsible for the formation of morphologically defined nodules on the roots of members of the family Leguminosae constitute the genus ***Rhizobium***. The common capacity of such symbiosis to reduce dinitrogen to ammonia and to incorporate this product into the nitrogen metabolic stream of the host plant gives the genus as a place of outstanding importance in natural ecosystems and agricultural production. The potential role of tree legumes in maintaining soil fertility and forest management is well recognized (Granhall, 1987; Odee, 1989. Zhang *et al.*, 1991; Turk *et al.*, 1993).

2.1. Nodulation status of tree legumes

Leguminosae are characterized by the development of root nodules which include three subfamilies now raised to the rank of families namely Fabaceae, Caesalpiniaceae and Mimosaceae (Hutchinson, 1964; Heywood, 1971; Allen and Allen, 1981). The perusal of the literature showed that not all the leguminous plants produced root nodules (Allen and Allen, 1981). Eventhough woody and tree species are represented in all the three subfamilies of Leguminosae the bulk of the tree species are found in Mimosoideae and rhizobia, isolated from tree species of Mimosoideae, were slow growing and hence assigned to the genus ***Bradyrhizobium*** (Allen and Allen, 1981).

Occurrence of the root nodules in different tree forms of Mimosoideae has been reported, which include species of ***Acacia, Albizia, Adenanthera*** (Banados, 1954; Grobbelaar *et al.*, 1964). ***Acacia, Calliandra, Entada, Inga, Leucaena, Mimosa, Pentaclethra, Pithecellobium, Zygia*** and ***Parkia*** (De Souza, 1966; Norris, 1969).

Grobbelaar and Clarke (1964) reported root nodules from 38 species belonged to ***Leucaena, Dichrostachys, Prosopis*** and ***Mimosa*** of Mimosoideae.

Grobbelaar *et al.* (1967) screened 246 species of plants for the occurrence of root nodules and found that 17 species belonged to Mimosoideae had root nodules. Among them, 11 species were belonged to ***Acacia***, two were of ***Mimosa*** and one each ***Albizia***, ***Desmanthus, Dichrostachys*** and ***Elephantorrhiza.***

It has been reported that among 69 species of Mimosoideae studied, 62 species had root nodules. Among them 33, 16, 4, 4, 3, 1 and 1 species belonged to ***Acacia, Albizia, Dichrostachys, Elephantorrhiza, Entada*** of Mimosoideae (Corby, 1974).

Granhall (1987) discussed that the tree species, selected for planting in the tropics to provide fuel wood, should be fast-growing. Management of plantations such as selection of rhizobia, mycorrhization may be carried out in nutritional requirements of N_2-fixing trees such as ***Leucaena leucocephala, Sesbania grandiflora, Gliricidia maculata*** and ***Erythrina indica.***

Sanginga (1988) reported that the root nodule isolates of ***S. grandiflora, S. punctata, S. rostrata*** and ***Leucaena leucocephala*** were fast growing acid producers, whereas the isolates of ***Tephrosia vogelii*** and ***Acacia albida*** were slow growing alkali producers.

Odee (1989) studied that ***Sesbania grandiflora*** and ***S. sesban*** effectively nodulated and grew well with their own isolates, while

Prosopis juliflora produced partially effective association only with the isolate from ***Calliandra calothyrus***. ***Acacia albida, A. mearnsii*** and ***Leucaena leucocephala*** produced effective or partially effective combinations with several isolates and ineffective ones (some not even nodulated) with others.

Sixty rhizobial strains isolated from the root nodules of ***Acacia senegal*** and ***Prosopis chilensis*** in the suddan were compared with 37 rhizobia isolated from woody legumes in other regions and with 25 representatives of recognized ***Rhizobium*** species by performing a numerical analysis of 115 phenotypic characteristics by Zhang *et al.* (1991).

Martinez *et al.* (1991) proposed a new name as ***Rhizobium tropici*** for the ***Rhizobium*** species of ***Phaseolus vulgaris*** and ***Leucaena*** spp. based on multilocus enzyme electrophoresis, DNA-DNA hybridization, a sequence analysis of 16S rRNA and an analysis of phenotypic characteristics.

Turk and Keyser (1992) reported that the tree legumes such as ***Gliricidia sepium, Calliandra calothrus*** and ***Leucaena leucocephala*** nodulated effectively with rhizobia isolated from each of the three genera. With a few exceptions, ***Sesbania grandiflora*** and ***Robinia pseudoacacia*** nodulated effectively only with rhizobial strains isolated from each genus respectively.

Rhizobium or ***Bradyrhizobium*** that nodulate tree legumes such as ***Acacia auriculiformis, A. mangium, A. mearnsii, Albizia lebbeck, A. saman, Calliandra calothyrus, Flemingia macrophylla, Gliricidia***

sepium, Leucaena diversifolia, L. leucocephala, Paraserianthes falcataria, Robinia Pseudoacacia, Sesbania grandiflora and ***S. sesban*** have been enumerated by Turk and Keyser (1993) by using the most probable number (MPN) plant infection assay.

George *et al.* (1994) studied the genetic characterization of ***Rhizobium*** sp. strain TALI145 which nodulates ***Leucaena leucocephala, Phaseolus vulgaris*** and many tropical tree legumes.

Rhizobial strain of ***Leucaena leucocephala, Sesbania grandiflora, Acacia auriculiformis, Albizia lebbeck*** and ***Gliricidia maculata*** were studied by Keka-Sarkar *et al.* (1997) for the establishment of tree legumes to increase biomass production in uncultivated land by cost effective means.

Surange (1997) reported that ***Rhizobium*** strain isolated from ***Albizia lebbek*** survived at 50°C, while ***Rhizobium*** strains isolated from ***Sesbania formosa, Acacia farnesiana*** and ***Dalbergia sissoo*** were well adapted to grow on pH 12. All the ***Rhizobium*** strains tolerated salt concentrations upto 5 per cent.

Reddy *et al.* (2002) stated that, recent advances in plant molecular biology provide new tools to study specific genes and it can be expected that application of these techniques will shortly provide more knowledge in the area of ***Rhizobium*** – Taxonomy.

2.2. *Rhizobium* Taxonomy

The formation of nodules in the roots is an external manifestation of symbiotic association of a bacterium, ***Rhizobium***, with the roots of the

leguminous plants. As rightly pointed out by Fred *et al.* (1932), the credit for the first report of the root nodules goes to Fuchs, who first described the root nodules from ***Aphaca, Vicia faba*** and ***Trigonella foenum-graecum*** in the first edition of the book entitled "Historia stirpium commentarii insignes" published in 1542. The actual insight into the origin and the function of the root nodules in relation to the utilization of atmospheric nitrogen by leguminous plants was given and explained by Hellriegel in 1886. Nevertheless, the role of bacterium in the nitrogen utilization was not established until 1888. It was only in 1888, Beijerink, who first isolated the bacterium (***Rhizobium***) from the root nodules and cultured on the media in the laboratory. Since then, the attention of the microbiologists was centred around understanding the morphology of the bacterium, its distribution, ecology and its physiological and genetical relationship with the roots and nature, morphology and distribution of nodules in the roots of the host plants in relation to different habitats and environmental conditions; and the research works on these aspects have been reported and reviewed from time to time (Allen and Allen, 1936, 1947; Martin, 1948; Banadoss and Fernandez, 1954; Bowen, 1956; Masefield, 1955; Lange, 1959; De Souza, 1966).

Bacteria, responsible for the formation of morphologically defined nodules on the roots of members of the family Leguminosae, were assigned to the genus ***Rhizobium*** (Frank, 1889). But the nomenclature of the rhizobia, at present is in a state of flux, with a flood of proposals for new names.

Fred *et al.* (1932) pointed out that some fast growing rhizobia were closely related to ***Agrobacterium***. Graham (1964) proposed that the ***Agrobacterium*** could be merged with the ***Rhizobium*** because of its fast

growing nature. Mannetje (1967) reported that the genus ***Rhizobium*** need not split in the generic level and the ***Agrobacterium*** need not necessarily be merged with ***Rhizobium***. According to Moffett and Colwell (1968) ***Rhizobium*** is associated with ***Agrobacterium*** and ***Chromobacterium*** in the family Rhizobiaceae within the order Eubacterials. Jordan and Allen (1974) reported that ***Rhizobium*** and ***Agrobacterium*** are the two genera of the family Rhizobiaceae in the eighth edition of Bergey's manual of Determinative Bacteriology.

However, two distinct groups of rhizobia, slow growing and fast growing on laboratory media were recognized as early as 1932 by Fred *et al.* These two distinct groups were based on the proposal of Mannetje (1967) and on the desire to allow more information to accumulate which could permit the separation of the two groups into two different genera such as slow growing non acid producing root nodule bacteria in a genus ***Bradyrhizobium*** and all the fast growing acid producing nodule bacteria in a genus ***Rhizobium*** (Jordan and Allen, 1974; Jordan, 1982, 1984; Kreig and Holt, 1984).

Dreyfus *et al.* (1988) recognized ***Azorhizobium*** as a new genus for the rhizobia nodulating ***Sesbania rostrata***. Based on the modern molecular data such as sequences of the small subunit ribosomal RNA (SSU or 16S r RNA) rhizobia are subdivided into three genera namely ***Rhizobium, Bradyrhizobium*** and ***Azorhizobium***. Chen *et al.* (1988) created a new genus ***Sinorhizobium*** to include ***Rhizobium fredii*** and related soybean rhizobia.

The name ***Mesorhizobium*** was put forwarded by Lindstrom *et al.* (1995) for a group of species within fast growing rhizobia and it was

described as a new genus by Jarvis *et al.* (1992). It formed on intermediate group between typical "fast growers" (***Rhizobium***) and "slow growers" (***Bradyrhizobium***). Young and Haukka (1996) while discussing the phylogeny and taxonomy of rhizobia, recognized six genera for which he has given names of the five genera as ***Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium*** and ***Mesorhizobium***. However, he has mentioned the name as unnamed genus to include ***R. galegae***.

Based on the polyphasic research, using various molecular biological data, de Lajudie *et al.* (1998) created a new genus ***Allorhizobium*** which includes ***R. galegae, A. vitis*** and the isolates of ***N. natans***. Recent listing showed that rhizobia comprise six genera ***Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium, Azorhizobium*** and ***Allorhizobium*** (de Lajudie *et al.*, 1998).

Later, comparison of the sequence of the 16S rRNA genes (Williems and Collins, 1993; Yanagi and Yamasato, 1993; Sawada *et al.*, 1993) revealed four phylogenetic sub lineages on the ***Agrobacterium, Rhizobium*** branch, (i) a first sublineage contains ***Agrobacterium*** bv. 1. ***Agrobacterium rubi*** and ***Agrobacterium vitis***; ***Rhizobium galegae*** and the recently proposed new species. ***R. giardinii*** (Amerger *et al.*, 1977) also belong to this sub lineage but have somewhat separate positions. (ii) a second phylogenetic sublineage contains ***R. leguminosarum*** (type species of ***Rhizobium***), ***R. tropici, R. etli, Agrobacterium*** bv 2 and a recently proposed new species ***R. gallicum***. (iii) a third sublineage, sufficiently different to diverse separate genus status for which the name ***Sinorhizobium*** was given which contains ***S. meliloti, S. fredii, S. xingiangenes, S. terangue, S. rhizobium saheli*** (de Lajudie *et al.*, 1994) and ***S. medicae*** (Rome *et al.*, 1996) (iv) the fourth sub lineage

consists of species recently transferred in the new genus ***Mesorhizobium*** (Jarvis *et al.*, 1997) namely ***M. loti, M. luakuli, M. ciceri, M. tianshanense, M. trediterraneum*** and ***M. flurifarium*** (de Lajudie *et al.*, 1998).

2.3. Cross inoculation studies

A group consists of plants among which the rhizobia are interchangeable mutually in terms of nodule production, and this group becomes basis for the taxonomy of rhizobia and the recognition of infection specificity led to the "cross inoculation group" concept (Fred *et al.*, 1932; Vincent, 1974). ***Rhizobium*** species have been defined in terms of plant cross inoculation groups (Jordan and Allen, 1974). Although, speciation of ***Rhizobium*** is based on the cross inoculation grouping suggested by the classical studies of Fred *et al.* (1932) which has been widely adapted as a workable and practical method to differentiate rhizobia. Attempts have been made from time to time to differentiate species on the basis of growth reaction on defined substrate, morphological, ecological, physiological characters and DNA base composition (Graham, 1964; Mannetje, 1967; Vincent, 1970).

Three bivoars occur (trifolii, phaseoli and viceae) in the species of ***R. leguminosarum***. It causes formation of root nodules on some, but not necessarily all, species of ***Pisum*** (field pea), ***Lathyrus*** (pea), ***Vicia*** (vetch), ***Lens*** (lentil), temperate species of ***Phaseolus vulgaris*** (kidney bean), ***P. angustifolius*** (bean), ***P. multiflorus*** (scarlet runnel) and ***Trifolium*** (clover) (Frank, 1889).

According to Fred *et al.* (1932), cross inoculation group is classified into seven groups such as ***R. leguminosarum*** (pea group);

R. phaseoli (bean group); ***R. trifolii*** (clover group); ***R. meliloti*** (alfalfa group); ***R. lupini*** (lupini group); ***R. japonicum*** (soybean group) and ***Rhizobium*** spp. (cow pea group). They also reported that the groundnut and green gram fall under the same cross inoculation group (cowpea group).

The cowpea type rhizobia which nodulates many tropical legumes, is culturally slow growing, symbiotically promiscuous and represents an ancestral type of ***Rhizobium*** common to the tropical legumes (Norris, 1956).

Graham (1964), after studying more than 100 characteristics of rhizobia, classified them into three genera and species such as ***R. leguminosarum*** (combining the farmer ***R. trifolii, R. leguminosarum*** and ***R. phaseoli***) ***R. meliloti*** and ***Phytomyxa japonicum*** (combining the former ***R. lupini*** and ***R. japonicum***).

Delay and Rassal (1965) propsed three species based on DNA-base composition, such as ***R. leguminosarum, R. meliloti*** and ***R. japonicum***. Mannetje (1967) reported only two species, based on a different method of numerical taxonomy, such as ***R. japonicum*** and ***R. leguminosarum*** under one genus of ***Rhizobium***.

Delay (1968) reexamined ***Rhizobium*** and ***Agrobacterium***, the two closely related genera and clubbed them under one genus ***Rhizobium*** with five species such as ***R. leguminosarum, R. meliloti, R. rhizogenes*** (the former ***Agrobacterium rhizogenes***), ***R. radiobacter*** (the former ***A. tumefaciens, A. radiobacter, A. rubi***) and ***R. japonicum***.

One of the significant Indian work (Gaur *et al.*, 1973) in the classification of ***Rhizobium*** has been the finding that rhizobia from ***Cicer arietinum*** have no relationship with existing cross inoculation groups and information gathered to date justifies the placement of cicer rhizobia in separate cross inoculation group.

Rewari *et al.* (1978) reported that the difference in nodulation was due to strains of rhizobia host cultivars and various environmental factors. Fast growing, acid producing Lotus rhizobia are clearly distinguishable from ***R. leguminosarum*** and ***R. meliloti*** (Pankhurst and Biggs, 1980; Crow *et al.*, 1981).

The species of ***Rhizobium*** including both root and stem nodules in ***S. rostrata*** has been designated as ***Azorhizobium caulinodans*** (Dreyfus and Dommergues, 1981; Ndoye and Dreyfus, 1988).

The relationship between ***R. loti*** and ***R. meliloti*** is more distant than that between ***R. meliloti*** and ***Agrobacterium cluster***. ***R. loti*** species normally causes formation of root nodules on some, but not all the following hosts; ***Lotus corniculatus*** (birdsfoot trefoil), ***Lotus tenuis*** (slender birdsfoot trefoil), ***Lupinus desiflorus*** (Lupine), ***Anthyllis vulneraria*** (kidney vetch), ***Ornithopus sativus*** (serradella), ***Cicer arietinum*** (chick pea), ***Leucaena leucocephala*** (lead tree) and ***Mimosa*** (Jarvis *et al.*, 1982).

Bradyrhizobium japonicum belonged to ***Bradyrhizobium*** normally causes the formation of root nodules on species of ***Glycine*** (soybean) and on ***Macroptilium atropurpureum*** (Jordan, 1982).

Work on cross inoculation testing of rhizobial strains with different hosts has been in progress in India from time to time showed that rhizobia from nodules of pea (***Pisum sativum***), lentil (***Lens culinaris***), Lakerne (***Medicago sativa***) foenum-greek (***Trigonella foenum-graecum***), soybean (***Glycine max***) and kidney bean (***Phaseolus vulgaris***) could be placed into definite species as defined by Fred *et al.* (1932) whereas those from cluster bean (***Cyanopsis tetragonoloba***), cowpea (***Vigna unguiculata***), green gram (***Vigna radiata***), black gram (***Vigna mungo***), red gram (***Cajanus cajan***), groundnut (***Arachis hypogaea***), moth bean (***Vigna aconitifolia***), species of ***Sesbania, Desmodium, Crotalaria, Clitoria, Mimosa*** and ***Acacia*** could be clubbed under ***Rhizobium*** spp. (cowpea group) (Dadarwal *et al.*, 1982; Basak and Goyal, 1975).

Sinorhizobium meliloti forms a nitrogen-fixing symbiosis with plants of the genera ***Medicago, Melilotus*** and ***Trigonella*** including the crop alfalfa (Schulte and Kondorosi, 1998).

2.4. Characterization of *Rhizobium*

Many characteristics are used in classifying and identifying microorganisms. This section briefly reviews some of the most taxonomically important properties. For the sake of clarity characteristics have been divided into classical and molecular. Classical approaches to taxonomy such as morphological, ecological, biochemical, biomolecule estimations and genetic characteristics have been employed in microbial taxonomy for many years (Prescott, 1996). They are quite useful in routine identification and may provide phylogenetic information as well.

It has been well established that the nodules developed in the roots of the plants showed considerable variation in the shape, size, number and

distribution which is often influenced by the species of the plant and the associated organism (Gaur *et al.*, 1972; Corby, 1988). The shape of the nodules developed is constant with reference to a particular species of plant which has been used as a diagnostic feature in the classification of nodules (Dart, 1977; Corby, 1988). It has been reported that the red colour of the nodules responsible for the nitrogenase activity (Virtanen *et al.*, 1947; Vest *et al.*, 1973). The leghaemoglobin content and the extent of bacterial tissue in nodules have direct correlation with the amount of nitrogen fixed by legumes (Bergersen and Briggs, 1958; Bergersen and Turner, 1980). Udeha and Syono (1982) reported in soybean and pea that leghaemoglobin components participate in more effective nitrogen fixation by controlling oxygen transport to bacteriods. Yoshioka and Maruyama (1991) stated that the leghaemoglobin content was not affected by the nitrate addition. The leghaemoglobin of the milk vetch nodules was separated into 5 components by PAGE and their relative amounts varied with the growth.

It has been reported that rhizobia are characteristically short to medium, gram negative rods (0.5 – 0.9 μm wide x 1 – 3 μm long) and older cells are likely to contain one to several prominent highly refractile granules of poly-β-hydroxy butyrate (PHB) (Fred *et al.*, 1932; Vincent *et al.*, 1962).

Rhizobium can easily be distinguished from ***Agrobacterium*** by not absorbing congo red from YEMA containing congo red, unable to grow in Hofer's alkaline broth with high pH in which ***Agrobacterium*** can grow, not able to utilize lactose from the medium, which ***Agrobacterium*** can utilize (Hofer, 1941).

Rhizobia characteristically produce colourless white or cream coloured, translucent elevated colonies on a yeast extract mannitol agar (Graham and Parker, 1964; Vincent, 1970). Graham and Parker (1964) surveyed many strains of rhizobia and they pointed out that the rhizobia typically show slow growth on peptone glucose agar, no H_2S from bismuth sulphite and give no precipitate in glycerophosphate agar. Ranjit Singh *et al.* (1967) stated that all the isolates showed variation in their ability to utilize different carbon sources.

Vincent (1970) reported that the colonies of fast growers are often convexly elevated and may have a "pearl-like" appearance when viewed from the under side. Others are relatively flat; some tend to flow along the inoculation line. Slow growers likely to have mean generation time of 6 – 8 hours, yielding small colonies ($\leq$ 1 mm) after 7-10 days; generally colourless, white or cream coloured, rarely pink, gumless abundant than in the fast growers, dense and sticky.

The slow growing, non-acid producing rhizobia have been considered the ancestral forms of rhizobia since they are associated with primitive tropical legumes growing in alkaline environment (Vincent, 1970).

Vincent (1970) also reported that abundant water soluble gum (exopolysaccharide) is a characteristic of the fast growing rhizobia.

Lim (1970) observed that ***Rhizobium*** from different legumes showed good growth in asparagin, glutamin and inhibited by hydroxylamine, nitrate and hydrazine.

Dullart (1970) reported that ***Rhizobium*** produces auxins, mainly indole acetic acid (IAA) indole carboxylic acid in culture from tryptophan.

Bergerson (1971) reported that glucose, galactose, fructose, arabinose, xylose are reasonably well used by fast and most slow growers, rhamnose, lactose, trehalose and raffinose are poorly utilized by the slow growers.

The fast growers can use a wide range of sugars, sugar alcohols and organic acids, slow growing rhizobia are more restricted, glucose, galactose, fructose, xylose and mannitol were utilized by most strains, sucrose, trehalose and dulcitol were utilized least (Doudoroff, 1974).

Doudoroff (1974) observed that ***Rhizobium*** utilizing a wide range of carbohydrates and salts of organic acids as carbon sources without gas formation. Cellulose and starch are not utilized, produce an acidic reaction in mineral salts containing mannitol or other carbohydrates. Growth on carbohydrate media is usually accompanied by copious extracellular polysaccharide slime.

Rhizobia are gram negative rods (500 – 900 nm x 1200 – 1300 nm) motile when young, often with prominent non-staining regions due to highly refractive polymeric β-hydroxy butyrate (PHB) granules (which is an energy source) as reported by Vincent *et al.* (1977). Brewin (1991) studied the role of extracellular polysaccharides in legume-rhizobium symbiosis and the characterization and symbiotic importance of acidic extracellular polysaccharides of ***Rhizobium*** sp. strain GRH_2 isolated from ***Acacia*** nodules were studied by Lopez-Lara *et al.* (1993). Povolo *et al.*

(1994) reported that the mutants of ***Rhizobium meliloti*** unable to synthesize poly-beta-hydroxybutyrate.

Many properties are ecological in nature since they affect the relation of microorganism to their environment. Often they are taxonomically valuable because even very closely related microorganisms can differ considerably with respect to ecological characteristics.

Rhizobia showed tolerance to wide range of pH (6.0 to 9.0) Madhok (1935). Soil pH appeared to be an important determinant of N_2 fixing capacity among field populations of ***R. leguminosarum*** bv. ***trifolii.*** Ineffectiveness was found to be more common in soil with low pH (Jones and Burrow, 1969).

Bradyrhizobium strains grow well at pH 4.5. Over 30 per cent of the strain grow at pH 4 and few as low as pH 3.5. Optimum pH for the growth of ***Rhizobium*** ranges from 6.0 to 7.0. Growth usually doesn't occur above pH 9.0 (Doudoroff, 1974).

Vincent (1977) reported the pH range for ***R. leguminosarum*** is 4.5 to 9.5 and ***R. meliloli*** being the most alkali tolerant species.

R. meliloti was most tolerant of high pH, 91 per cent strains growing at pH 9.5; 9 per cent at pH 4.5. ***R. leguminosarum, R. trifoli, R. phaseoli*** groups were more tolerant (65 per cent) at pH 4.5 and less tolerant to alkaline reaction (19 per cent at pH 9.0). The slow growing strains were even more tolerant to acid (35 per cent at pH 4.0; 84 per cent at pH 4.5) and less tolerant to alkalie 3 per cent at pH 9) (Vincent, 1977).

Pandler and Khalan (1978) while studying the effect of pH on rhizobial growth stated that none of the rhizobial isolates grew at pH 3 and showed good growth at pH levels ranging from 6.5 to pH 8.0. Beyond 8.5 was not lethal, however it did not support the growth.

Evans *et al.* (1980) stated that low pH has been found to inhibit nodulation by ***R. leguminosarum***, ***R. phaseoli*** and ***Bradyrhizobium*** spp. Cooper *et al.* (1985) found that low soil pH exerts a significant influence on survival of native rhizobial populations. They also stated that ***Rhizobium*** strains vary in their acid tolerance and usually slow growing ***Bradyrhizobium*** are more acid tolerant than the fast growing rhizobia.

R. meliloti was the most tolerant (36.5 to 42.5°C) being 8°C higher on the average than those of ***R. leguminosarum*** and ***R. trifolii*** (31.0-38.0°C). The collection from tropical legumes (cowpea miscellany) ranged from the lowest to the highest (30.0°-42.0°C) (Graham, 1964).

Graham and Parker (1964) stated that the ability to grow at 39°C is a property likely to distinguish ***R. meliloti*** from other fast growing rhizobia. Burton (1967) also said that the ***Rhizobium*** spp. grow best in the range of 30.0 to 32.0°C. Vincent (1970) studied the temperature range, which is 40-42.5°C; however, growth at 4°C is rare, and only ***R. meliloli*** can grow at 42.5°C. The temperature maximum for ***R. leguminosarum*** is 38°C. Roughly and Dart (1970) said that the low temperature delay root-hair infection and nodule formation. Rhizobia are lower range mesophiles with optimal temperatures in the range of 20.0-30.0°C. They are relatively intolerant to high temperature, and they lose viability quite rapidly at 40°C (Vincent,1974).

Growth of ***Azorhizobium caulinodans*** occurs from 12.0-43.0°C. Equally good growth was observed between pH 5.5 and 7.8 (Doudorff and Palleroni, 1974). All strains of ***Agrobacterium*** grow between 20 and 28°C and can not grow above 30°C (Skinner *et al.*, 1977).

Trinick (1982) suggested that the optimum temperature for nodulation and N_2 fixation by host rhizobia ranges between 25.0-30°C. Low temperature delays root hair infection.

The maximum temperature for growth of ***Bradyrhizobium*** ranges from 30.0-42.0°C, with most strains failing to grow above 39.0°C (Cooper *et al.*, 1982).

Hofer (1941) reported that ***Bradyrhizobium*** fail to grow in media containing 2 per cent NaCl. The concentrations of NaCl as high as 2 per cent and as low as 0.2 per cent were lethal to most fast and slow growing ***Rhizobium,*** Graham and Parker (1964). Rhizobia are more tolerant towards salt than its host legume and therefore survives in saline soils. ***R. meliloti*** was more tolerant than its alfalfa host; nodulation was more sensitive than either of the component of the symbiosis (Subba Rao *et al.*, 1974).

Populations of naturalized rhizobia as well as introduced rhizobia have been shown to differ in their tolerance to these major environmental factors, which affect the persistence and survival of individual species in the soil. Neeru-Bala *et al.* (1990) reported that ***Rhizobium*** isolated from tree legumes (***Acacia, Albizia, Dalbargia, Desmodium, Gliricidia, Leucaena, Pithecellobium, Prosopis*** and ***Sesbania***) were found to be highly tolerant to salinity (9.0-12 ds/m).

The most problematic environments for rhizobia are marginal lands with low rainfall, extremes of temperature, acid soils of low nutrient status and poor water-holding capacity (Bottomley, 1992). Hashem *et al.* (1998) reported that ***Rhizobium*** strains isolated from root nodules of ***Laucaena*** trees grew at 42°C and tolerant to 7.3 per cent NaCl.

Some of the most powerful approaches to taxonomy are through the study of proteins, plasmids, fatty acids and nucleic acids. They are either direct gene products or the genes themselves, so comparisons of proteins, plasmids and fatty acids yield considerable information about true relatedness.

Electrophoretic variation in proteins has been increasingly used in assessing genetic relatedness and in establishing evolutionary relationships within species complexes and to estimate gene frequencies in natural populations (Ladizinsky and Hymowitz, 1979).

Roberts *et al.* (1980) reported that Electrophoretic mobilities of whole cell proteins have been used to differentiate among isolates within the same sero group. Protein variability have been used extensively to detect patterns and levels of genetic diversity within species (Ladizinsky, 1983; Loveless and Hamrick, 1984). Kersters (1985) reported that bacteria with almost identical protein patterns possess a high genome similarity. Identical protein gel electrophoregrams indicating that they constitute a very homogeneous cluster with most likely high internal DNA-DNA homologies (Kersters, 1985). Electrophoresis of proteins is perhaps the most useful and widely applied to distinguish between closely related species (Cooke and Draper, 1986; Grawford, 1990).

Strains of ***Azospirillum halopraeferens*** were isolated from Kallar grass and were shown to be homogeneous using phenotypic tests and protein gel electrophoresis (Reinhold *et al.,* 1985).

Brougton *et al.* (1987) reported that electrophoretic mobilities of whole cell proteins was used to differentiate among the isolates within the same serogroup.

In the genus ***Sesbania***, stem-nodulating strains, constitute one species because they have almost identical protein electrophoregrams, which show high levels of DNA bindings and constitute a phenotypically narrow cluster (Dreyfus *et al.,* 1988).

Shatters *et al.* (1993) reported that purified protein of ***R. meliloti*** had related subunits of 46.5 and 49 KDa and a native molecular mass of 355 KDa indicating the native enzyme was an Octamer.

The presence of nif genes on large plasmid is a characteristic of plant-associated bacteria such as ***Rhizobium*** spp. and lignin degrading strain Lignobacter K_{17} (Casse-Boucher *et al.,* 1979). Masterson *et al.* (1982) investigated the plasmid profiles of fast growing soybean nodulating strains and found that the location of nif genes is homologous to the structured nif O and nif H genes in ***Klebsiella pneumoniae***. Sadowsky and Bohwol (1983) reported that megaplasmids play an important role in infectiveness of fast growing soybean nodule strains. Electrophoresis of ***R. meliloti*** GR4 and ***R. loti*** NZP 2037, containing plasmids of known molecular weight, allowed visualization of the corresponding plasmid bands.

Brewin *et al.* (1983) investigated the role of symbiotic plasmids to the nodualation competitiveness of ***R. leguminosarum*** strains. Some of ***R. meliloti*** strains contain one or more cryptic plasmids in addition to the large megaplasmids, harbouring the symbiotic genes (Bromfield, 1985). Thurman *et al.* (1985) demonstrated that ***R. trifolii*** isolates could possess between 2 and 10 plasmids and there was a negative correlation between plasmid number and symbiotic effectiveness but high plasmid numbers conferred fast growth rates in pure culture. In ***R. loti*** strain, the presence of cryptic plasmid decreased both symbiotic effectiveness and nodulation competitiveness and this was reported by Pankhrust *et al.* (1986). On the other hand Sanjuan and Olivares (1989) reported that a cryptic plasmid of ***R. meliloti*** GR_4, PR me GR4b increased nodualation competitiveness.

Sharma and Lakshminarayana (1990) studied the physical characterization of plasmids of cowpea ***Rhizobium*** and reported that plasmids of ***Rhizobium*** ***(Cajanus)*** were curable by high temperature giving rise to non-nodulating mutants while plasmids of ***Rhizobium*** (***Cyamopsis*** and ***Cicer***) were cured without the loss of nodulating ability indicating the chromosomal location of nodulation genes.

Many ***Rhizobium*** strains harbour plasmids and the genes affecting nodualation (nod genes), nitrogen fixation (nif and fix genes), polysaccharide production (exo and LPS genes) and other cellular functions are located on these plasmid (Denarie *et al.*, 1992).

Brom *et al.* (1992) also employed positive selection scheme to isolate plasmid cured derivatives of ***R. leguminosarum*** bv. ***Phaseoli*** strain CFN42, which contains six plasmids. Moenne-Coccoz and Weaver (1995) investigated the role of plasmids in colonization of clover

rhizosphere by ***R. leguminosarum*** bv. ***trifolii*** strain W142 which contains 4 plasmids. Delgado *et al.* (1995) reported that plasmids of ***R. leguminosarum*** showed 4 apparently contrascribed open reading frames Cyc H, Cyc J, Cyc K and Cyc L.

Fatty acid profile is considered very important and is even used for taxonomic species identification purposes in bacteria (Cummins and Harris, 1956; Abel *et al.*, 1963). Whole cell fatty acid profiles of strains grown under standardized conditions have been used for identification and classification of many bacteria (Abel *et al.*, 1963; Moss, 1981; Jackwood *et al.,* 1985 and Moore *et al.*, 1987).

Schenk and Werner (1988) conducted an experiment in which 14 strains of ***Azospirillum*** and analysed their cellular fatty acids content and also demonstrated that the genus could be subdivided based on the fatty acid content. Fatty acid profile is used as a tool for classification of ***Rhizobium*** and ***Agrobacterium*** for Martinez-Romera (1994).

Tighe *et al.* (2000) studied the phenotypic relationships of ***Agrobacterium, Bradyrhizobium, Mesorhizobium, Rhizobium*** and ***Sinorhizobium*** based on the analysis of cellular fatty acids.

Numerical Taxonomic studies

The taxonomic criteria and powerful method used currently in the classification of the family Rhizobiaceae include numerical taxonomy based on protein patterns, plasmid profile, fatty acid profile, guanine plus cytosine content, deoxyribonucleic acid (DNA) homology, serology, phage typing, composition of extracellular polysaccharides and plant infection.

Based on numerical taxonomic studies, slow growing, non-acid producing root nodule bacteria of leguminous plants should be separated from fast growing, acid producing strains and placed in a new genus, ***Bradyrhizobium*** (Jordan, 1982).

Fast growing ***Lotus rhizobia*** were reviewed by Jarvis *et al.* (1982) and they proposed a new species ***Rhizobium loti*** based on numerical taxonomic studies. Based on the numerical analysis, Wedlock *et al.* (1986) proposed a new name as ***R. fredii***, which is taxonomically distinct from other known species in the genera ***Rhizobium*** and ***Bradyrhizobium***.

Jarvis *et al.* (1986) studied the intra and intergeneric similarities between ribosomal ribonucleic acid cistrons of ***Rhizobium*** and ***Bradyrhizobium*** species and some related bacteria based on numerical taxonomic analysis.

Based on numerical analysis Chen *et al.* (1998) proposed a new genus ***Sinorhizobium*** gen. nov. from fast growing soybean rhizobia. Dreyfus *et al.* (1988) studied stem nodulating nitrogen-fixing bacterium isolated from ***Sesbania rostrata*** and based on numerical taxonomic study proposed a new species ***Azorhizobium caulinodans***.

Based on the numerical taxonomic studies such as electrophoresis of whole cell protein, DNA G+C content data and DNA-DNA hybridization data from the root nodule isolates of ***Astragalus sinicus*** Chen *et al.* (1991) proposed a new name as ***Rhizobium haukuii*** sp. nov.

Noel *et al.* (1994) studied the strains isolated from chickpea (***Cicer arietinum***) based on the numerical analysis he proposed the new species

Rhizobium ciceri. Amerger *et al.* (1997) studied the ***Rhizobium*** from ***Phaseolus vulgaris*** and proposed a new name as ***Rhizobium gallicum***, based on the numerical taxonomic studies.

The new species ***Allorhizobium undicola*** was proposed by de Lajudie *et al* (1998) for the group of nodule isolates from ***Neptunia natans***, based on polyphasic taxonomy. Based on the numerical analysis Young *et al.* (2001) proposed new combinations such as ***Rhizobium radiobacter***, ***R. rhizogenes***, ***R. rubi***, ***R. undicola*** and ***R. vitis*** from ***Rhizobium*** and ***Bradyrhizobium.***

Biological nitrogen fixation by symbiotic association of legume with microorganism is economically more sound and environmentally more acceptable in agriculture (Halliday, 1982).

Sundara Rao (1971) reported that the rhizobia inoculated seedling of groundnut (***A. hypogaea***) showed significant increases in growth and yield than the uninoculated. Thakur and Hossain (1976); Rahman *et al.* (1982); Bishnoi and Dutt (1983); Fakir *et al.* (1988) also stated that Rhizobia inoculated soybean seedling showed significant growth than the uninoculated.

Rewari (1978) reported that the ***Rhizobium*** inoculation in ***V. mungo, V. radiata*** and ***A. hypoaea*** resulted in an increase by 58, 51 and 38 per cent respectively.

Significant positive effect of rhizobia inoculation on various growth and yield parameters were also observed by Hoque *et al.* (1980) in

black soybean and in ***Stylosanthes*** by Subramanya and Gopala Gowda (1994).

Significant increases in growth and yield due to the rhizobial inoculation have also been reported in pigeon pea (***Cajanus cajan***), Chick pea (***Cicer arietinum***) and cowpea (***V. unguiculata***) by Tilak *et al.* (1984) and groundnut (***A. hypogaea)*** by Sundara Rao (1971).

A significant increase in nodule biomass in inoculated soybean over the uninoculated was recorded by Hossain and Alexander (1986), Subramanya and Gopala Gowda (1994) and Bhuiyan *et al.* (1995).

Surendragopal and Shivappashetty (1996) reported that ***S. grandiflora*** rhizobia inoculated rice showed significant yield than the uninoculated.