DISCUSSION

5. DISCUSSION

The biology and biochemistry of N_2 fixation have been extensively studied on the various aspects in many leguminous plants, but studies on the taxonomy based on the numerical analysis is little studied (de Lajudie *et al.*, 1998; Young *et al.*, 2001). ***P. dulce, E. indica*** and ***S. grandiflora*** are tree legumes, distributed in two families. ***P. dulce*** belongs to Mimosaceae whereas ***E. indica*** and ***S. grandiflora*** are grouped together under Papilionaceae which is now called as Fabaceae.

5.1. Nodule characteristics

It is well known that the nodules are characteristically produced in most of the leguminous plants. These nodules are the external manifestation of the symbiotic association between the host legume plant and the endosymbiotic rhizobia. It has been well established that the nodules developed in the roots of the legume host showed nodule diversity in the shape, size, number and distribution which is often influenced by the host and the associated rhizobium (Gaur *et al.*, 1972; Corby, 1988). In the present study it was found that the three species of tree legumes showed two types of nodules, elongated palmately branched (***P. dulce***) and unbranched spherical (***E. indica and S. grandiflora***).

In this context, it would be appropriate to take into consideration of the classification proposed by Dart (1977) for discussion. He distinguished three basic types of nodules such as (a) elongate and cylindrical type as in clover and medic, (b) spherical as in soybean and (c) collar nodules as in Lupin. However, as discussed earlier, only two types of nodules namely elongate and spherical were found in the three host species studied.

The colour of the nodules is playing an important role in distinguishing "effective'' and "ineffective" nodules (Viands *et al.*, 1979; Allen and Allen 1981; and Subba Rao *et al.*, 1985). Subba Rao, (1980) reported the red coloured nodules in many legumes. Vest *et al.* (1973) reported the red colour of the nodules is due to the development of the red pigment called the leghaemoglobin. In the present investigation, yellowish brown or brown coloured effective nodules in ***P. dulce*** and fluffy white less effective nodules in ***E. indica and S. grandiflora*** were observed.

Leghaemoglobin in root nodules are soluble protein confined to the cytoplasm of the bacteriod containing cells and are synthesized by the host cells. They protect the nitorgenase against oxygen damage while showing efficient oxidative phosphorylation in bacteriods (Becana and Sprent, 1989). Leghaemoglobin found in legume root nodules have been correlated directly with the effective symbiotic N2 fixation. These proteins are concerned with the oxygen supply to bacteriods in nodule cells (Bergerson and Turner, 1980). Leghaemoglobin content of the nodules, in the present study, was more in the nodules of ***P. dulce*** than that of ***E. indica*** and ***S. grandiflora.***

Nitrogenase is the key enzyme responsible for the biological conversion of molecular nitrogen to ammonia. It is localized in the bacteriod fraction of the root nodules in legumes. When isolated bacteriods were incubated under limiting O2 concentration, nitrogenase activities were enhanced by the presence of leghaemoglobin (Bergerson and Turner, 1980. It has also been reported that the red colour of the nodule is due to the development of leghaemoglobin, which is responsible for the nitrogenase activity (Virtanen *et al.*, 1947; Vest *et al.*, 1973). In the present study, among the nodules of tree legumes tested, nodules of

P. dulce recorded higher level of nitrogenase activity (in terms of ethylene production) when compared to nodules of ***E. indica*** and ***S. grandiflora.***

From the foregoing discussion it is concluded that the effective yellowish brown nodules of ***P. dulce*** with high amount of leghaemoglobin recorded higher level of nitrogenase activity lends support the earlier findings that leghaemoglobin found in root nodules have been correlated directly with the effective symbiotic N_2 fixation (Bergerson and Turner, 1980). It has also been reported in soybean and pea that leghaemoglobin contents participate in more effective N_2 fixation by controlling O_2 transport to bacteriods (Uheda and Syono, 1982).

5.2. Bacterial Endosymbiont

One of the important aspects of the study on nodulation in leguminous members would be the identification of the bacterium, that effectively nodulate in host plant. The authentic identification of the bacterium can be made by using cultural tests and thereby confirming the bacterium belonging to the genus ***Rhizobium*** and not ***Agrobacterium***.

Graham and Parker, (1964), and Jordan, (1984), surveyed many strains of rhizobia and used many cultural tests, pointed out that the rhizobia typically show slow growth on peptone glucose agar, form little or no H_2S, from bismuth sulphite and give no precipitate in glycerol phosphate agar. In the present study, the bacteria isolated from the nodules of three different leguminous tree species grew poorly on peptone glucose agar, but grew well on YEMA in the form of white or watery colonies within two days, which clearly indicated that the isolated bacterium could possibly be the fast growing ***Rhizobium***. They produced no H_2S from bismuth sulphite and gave no precipitate in glycerophosphate

agar indicating the presence of ***Rhizobium***. In citrate utilization the ***Rhizobium*** behaved like the ***Agrobacterium*** by utilizing citrate.

According to Vincent (1974) the rhizobia can use a wide range of carbohydrates. In the present study, the isolates obtained from leguminous tree species used most form of carbohydrates except mannitol, lactose, mannose, salicin and sorbitol. It is very difficult to distinguish ***Rhizobium*** from ***Agrobacterium*** on the basis of the utilization of carbohydrates as carbon source (Jordan, 1984; de Lajudie *et al.*, 1998). However, ***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 (11) in which ***Agrobacterium*** can grow, and not able to utilize (Hofer, 1941; Hahn, 1966 and Kleczkowska *et al.*, 1968). In the present investigation, the isolates of ***P. dulce, E. indica and S. grandiflora*** failed to absorb Congo red from YEMA medium containing Congo red, able to grow in Hofer's alkaline broth with high pH (11) and did not utilize lactose from the medium. Moreover the isolates grew well in temperature ranging from 26° to 30° ± 2°C which is conducive for ***Rhizobium***.

Based on the above discussion, it can be concluded that isolates of ***P. dulce, E. indica*** and ***S. grandiflora*** as ***Rhizobium***, there by lending support to the earlier investigations (Tan & Broughton, 1981; Dreyfus *et al.*, 1986; Alazard *et al.*, 1988, Ranganathan and Sundaram, 1992, Subba Rao *et al.*, 1985).

5.3. Cross inoculation studies

The association of strains into groups based on host specificity is called cross inoculation group which has provided a useful classification

system for rhizobial strains. It is a convenient and workable method of classifying root nodule bacteria into species (Fred *et al.*, 1932 ; Jordan and Allen, 1974).

Species of ***Rhizobium*** are usually separated on the basis of cross inoculation and grouping as suggested by Fred *et al.*, (1932). The basis for cross inoculation and grouping are mainly depending on the ability of the isolates of ***Rhizobium*** to form nodules on roots of a limited species of legumes. Based on this principle, the rhizobia that can form nodules on roots of certain legumes have been collectively taken as a species. Fred *et al.*, (1932) recognised seven species of ***Rhizobium***, but gave no species name to the rhizobia producing nodules on plants of the so called "cow pea group'' and identified them as ***Rhizobium*** spp.

The cross inoculation study carried out in the present investigation showed that ***Rhizobium*** of ***P. dulce, E. indica*** and ***S. grandiflora*** was able to produce nodules on ***V. mungo, V. uniguiculata, V. radiata, Arachis hypogaea*** and ***Cicer arietinum*** which belong to the "cow pea group''. Hence the species name could be identified as ***Rhizobium*** spp. This finding lends support to the earlier reports by Tan and Broughton (1981), Ranganathan and Sundaram (1992) and Subba Rao *et al.*, (1992).

R. leguminosoram contain three biovars (Trifolii, Phaseoli and Viceae) causes formation of root nodules on some, but not necessarily all, species of ***Pisum***, ***Lathyrus***, ***Vicia***, ***Lens***, temperate species of ***Phaseolus*** such as ***P. vulgaris, P. multiflorus***, ***P. angustifolius*** and ***Trifolium*** (Frank, 1889). ***R. meliloti*** normally causes formation of root nodules on some but not all species of ***Melilotus, Medicago*** and ***Trigonella***. ***R. loti*** normally causes formation of root nodules on some, but not all the following hosts ***Lotus corniculatus, Lotus tenuis, Lupinus densiflorus***,

Anthyllis vulneraria and ***Leucaena leucocephala*** (Jarvis *et al.*, 1982). ***Bradyrhizobium japonicum*** belonged to ***Bradyrhizobium*** genus normally causes the formation of root nodules on species of ***Glycine*** (Jordan, 1982).

The present study showed that ***Rhizobium*** of ***P. dulce***, ***E. indica*** and ***S. grandiflora*** were not able to produce nodules on inoculated seedlings of ***Pisum sativum*** (pea group) ***Phaseoulus vulgarsis*** (bean group), ***Trifolium repens*** (clover group), ***Trigonella foenum-graecum*** (alfalfa group), and ***Glycin max*** (soybean group) which revealed that ***Rhizobium*** of above mentioned tree species would not belong to above mentioned cross inoculation groups. This finding lends support to the earlier reports of Fred *et al.*, 1932. Rhizobia from nodules of ***Vigna mungo***, ***V. radiata***, ***V. unguiculata***, ***Cicer arietinum***, ***Cajanus cajan***, ***Vigna aconitifolia***, ***Arachis hypogaea*** and ***Cyamopsis tetragonoloba*** could be clubbed under ***Rhizobium*** spp. (Fred *et al.*, 1932). In the present study, among the seven cross inoculation groups tested, the isolates of ***P.dulce, E. indica*** and ***S. grandiflora*** formed nodules only with plants belonging to cow pea group and not with others. This suggested that the three isolates of leguminous tree species belonging to ***Rhizobium*** spp. cow pea group.

5.4. Numerical Taxonomy

Classification of bacteria is complicated by the microscopic structures and unlike other higher organisms, bacteria are all single cell organism that show very little distinguishing details. There are hundreds of different species of bacteria that have exactly the same appearance. So morphological criteria must be supplemented with other types of molecular characteristics to distinguish between apparently identical organisms.

In ***Rhizobium*** taxonomy, six species are currently designated on the basis of cross inoculation test leaving a large number of strains to form a group called cowpea "miscellany''. Taxonomy of this group is uncertain and is designated as ***Rhizobium*** spp. (Fred *et al.*, 1932).

The root nodulating rhizobia were earlier classified mainly on their host cross inoculation specificity. The integrity of the traditional cross inoculation group classification has long been questioned and is now in general disputed. This is largely because of the increasing recognization of high levels of rhizobial specificity within groups of legumes, once regarded as symbiotically homogeneous, (Thies *et al.* 1991) and because of the anomalies in which the same host has the capacity to form nodules with strains of both ***Rhizobium*** and ***Bradyrhizobium***. Since the above system of classification had several anomalies, recently using numerical taxonomy involving several number of distinguishing characters including protein, plasmid, fatty acid profile, DNA-DNA hybridization, DNA sequencing and 16S rRNA sequencing (de Lajudie *et al.*, 1992).

Electrophoresis of proteins is perhaps the most useful and widely applied to distinguish between closely related species (Cooke and Draper, 1986; Gaw ford, 1990). Electrophoretic variation in proteins has been increasingly used in assessing genetic relatedness and in establishing evolutionary relationship within species complex (Ladizinsky and Hymowitz, 1979). In the present study, the isolates P_1 and P_3 of ***P. dulce*** had identical protein profiles showing 100% similarity. Similarly S_2 and S_3 of ***S. grandiflora*** also exhibited indistinguishable protein electrophoregram with 100% similarity. The above results indicated that they (P_1 and P_3 ; S_2 and S_3) constitute two different homogeneous clusters with most likely high internal DNA-DNA homologies, in each cluster.

Similar results were also observed by Dreyfus *et al.*, (1988). While studying the protein pattern of four stem nodulating strain of ***Azorhizobium***, they concluded that the strains, had almost identical electrophoregram indicating that the strains constitute a very homogeneous cluster. The isolates E_1 and P_2 showed 76.6% similarity with each other and form a third cluster whereas E_2, E_3 and S_1 showed below 60% similarly with each other as well as with other groups. So they were treated as three different groups. From the above discussion, based on the protein profile, it is concluded that totally six different clusters were recognized and showing over all similarity level at 13 per cent.

Plasmids are undoubtedly important in taxonomy because they are present in most bacterial genera and carry genes coding for phenotypic traits. Because, plasmids could have a significant effect on clssification, if they carried the gene for a character of major importance in the classification scheme (Prescott *et al.* 1996). In the genus ***Rhizobium***, in its species and strains, harbour plasmids of different sizes. The number and size of the plasmids vary among different isolates (Lang, 1989; Denarie *et al.* 1992). Thurman *et al.*, (1985), reported the presence of 2 to 10 plasmids in ***R. trifolii. R. meliloti*** contains a large megaplasimd with one or more cryptic plasmids (Bromfield *et al.*, 1984). ***R. leguminosarum*** contains six plasmids while ***R. loti*** carrying single plasmid (Brom *et al.*, 1992) In the present investigation, the electrophoregram of nine ***Rhizobium*** isolates from ***P. dulce, E. indica*** and ***S. grandiflora*** showed the occurance of single plasmid in each isolate. The size of the plasmids ranged from 603 to 1353bp (Fig.26). Based on the size of the plasmids, the isolates were grouped into four clusters (P_1, P_3 and E_3; P_2,, E_2 and S_1; S_2 and S_3 and E_1).

Like protein and plasmid profiles, fatty acid profile is also considered very important and is even used for taxonomic species identification purposes in bacteria (Cummins and Harris, 1956; Abel *et al.*, 1963). A study in which 14 strains of ***Azospirillum*** were analysed for their cellular fatty acid content and also demonstrated that the genus could be subdivided based on the fatty acid content (Schenk and Werner, 1988). The present finding revealed that the isolates of P_1 and P_3 have the same cellular fatty acid profile but differs characteristically from those of all other isolates of ***Rhizobium*** spp. similarly S_2 and S_3 of ***S. grandiflora*** showed identical fatty acid profile which differed from that of P_1 and P_3. Each of these two clusters showed almost 100 per cent similarity with their isolates. The remaining isolates did not show much similarity with each other though the isolates E_2 and E_1 showed less similarity level at 42.6 per cent forming a third cluster. The isolates E_3 of ***E. indica*** shared a high level similarity at 82.2 per cent with the cluster S_2 and S_3. This showed that even though E_3 belonged to another host species (***E. indica***), it had certain fatty acids which shared homology with the cluster S_2 and S_3 and formed a single cluster. Thus the isolates from three different leguminous tree species formed six distinct groups such as P_1 and P_3 ; S_2 and S_3 ; E_1 and E_3; E_3, S_1 and P_1. All the nine isolates showed the overall similarity level at 9.3 per cent.

Analysis of the results obtained for the nine rhizobial isolates was done according to Adonsonian principles of Bacterial taxonomy for the purpose of computer Analysis. The computer calculated values (r) ranged from 4.4 per cent to 99.7 per cent. Those values were indicated in the dendrogram as vertical lines at the appropriate value, linking the isolate stem. All the strains examined were linked at 4.4 percent similarity level. The nine isolates formed six groups such as S_2 and S_3

(99.7%), P_1 and P_2 (99.6%), E_1 and E_2 (29.7%), and E_3, S_1 and P_2 formed three separate groups. While studying the numerical taxonomy of ***Corynebacterium pyogenes***, Roberts (1968) suggested that the isolates joined in the dendrogram at the values of 88 per cent were closely related indicating as species, where as isolates linked at 55 per cent were only distantly related. Atlas (1995), proposed that the strains which have more than 90 per cent similarity co-efficient are treated as a single species, while other more distinct groups are classified as separate species and perhaps even genera. Zhang *et al.*, (1991) reported 60 rhizobial strains formed 19 clusters with 72.5 per cent similarity as the boundary level for species. However these values (similarity co-efficient) for a species may vary depending on the organism being studied and the taxonomist applying them.

In the present investigation, the values (similarity co-efficient) above 90 per cent were treated as a species where as the levels below 90 per cent were termed as distantly related. In the present study, isolates P_1 and P_3 of ***P. dulce,*** which were joined in the dendrogram at values of 99.7 per cent, were obviously closely related to form one cluster, which could be treated as a species. Similarly, the isolates S_2 and S_3 of ***S. grandiflora*** were linked at 99.6 per cent, forming another cluster treated as another species. Since these two clusters formed similarity only at 7 per cent level, they showed distant relationship with each other. Among the remaining isolates, E_1 and E_2 formed similarity at 29.7 per cent level. Since it showed very low level of similarity, (below 90 per cent) the isolates could not be treated as single major group. The isolates P_2 and S_1 showed similarity with other clusters only below 10 percent level, so they could not be included any of the major groups and hence they were treated as two separate groups. Though, E_3 formed a similarity at 80.3 per cent

level with the cluster S_2 and S_3, it could not be included along with S_2 and S_3 because the similarity level was less than 90 per cent.

The results of the various dendrograms (Fig.27c,28c,and 29c) of protein, plasmid and fatty acid profile also clearly indicated the formation of two major clusters, such as P_1 and P_3 and S_2 and S_3.

From the foregoing discussion, it is concluded that, of the nine isolates, the isolates P_1 and P_3, and S_2 and S_3 clustures are qualified to elevate to the level of new species while the other isolates might be treated as miscellany under ***Rhizobium*** spp. cowpea group. It is further suggested that, the studies such as DNA-DNA hybridization DNA sequencing, 16S rRNA analysis have to be done inorder to confirm the present findings.

5.5. *Rhizobium* as Biofertilizer

Biological nitrogen fixation by symbiotic association of legume with microorganism is economically more sound and environmentally more acceptable than nitrogen fertilizer used in agriculture (Halliday, 1982). Kalisch and Hoflich, (1988) reported that rhizobial inoculation increased the shoot and root nitrogen content by 4-11 per cent over un inoculated plants.

This present study also showed that a significant positive effect of ***Rhizobium*** spp. (***P. dulce, E. indica***, and ***S. grandiflora***) inoculation on height of the plant, nodule number, root biomass, shoot biomass, total number of seeds and seed biomass in inoculated plants of ***V. mungo, V. radiata*** and ***A. hypogaea*** than the uninoculated plants, (Table 12,13, and 14). A similar and significant report has already been made in soybean (Rahman *et al.*, 1982 and Fakir *et al.*, (1988), Brack soybean (

Hoque *et al.*, 1980) and in Stylosanthes (Subramanya and Gopala Gowda, 1994).

A significant increase in nodule biomass in inoculated soybean over the uninoculated was recorded by Hosain and Alexander (1986), Subramanya and Gopala Gowda (1994) and Bhuiyan *et al.*, (1995). In the present investigation also, a significant increase in nodule biomass, in ***Rhizobium*** spp. inoculated leguminous crops over the uninoculated was observed. Among the three rhizobia inoculated, ***Rhizobium*** of ***P. dulce*** inoculated seedling of ***V. mungo***, produced highest nodule biomass when compared to ***Rhizobium*** of ***E. indica*** and ***S. grandiflora*** inoculated ***V. mungo*** seedlings. Similar results were obtained in the inoculated seedlings of ***V. radiata*** and ***A. hypogaea.*** This showed that the response of ***V. mungo*** to ***Rhizobium*** spp. of ***P. dulce*** was very high followed by ***Rhizobium*** of ***E. indica, S. grandiflora*** inoculated ***V. mungo.*** Similar results were also observed in ***V. radiata*** and ***A. hypogaea.***

Legumes inoculated with ***Rhizobium*** spp. (***P.dulce, E. indica*** and ***S. grandiflora)*** showed significantly higher root and shoot biomass over uninocualted. Hogue *et al.*, (1980) reported that inoculation with effective ***Rhizobium*** strain increased only 40% of shoot biomass in soybean over uninocualted. However, they have recorded 72% higher root biomass over the uninoculated soybean.

Among the inoculated plants, the highest yield was recorded in ***Rhizobium*** of P. ***dulce*** inoculated seedling of ***V. mungo*** (Table-12) followed by the ***Rhizobium*** of ***E. indica*** and ***S. grandiflora*** inoculated ***V. radiata*** and ***A. hypogaea*** over the uninoculated. A similar observation

was also made by Thakur and Hossain (1976) and Bishnoi and Dutt, (1983) in soybean.

Rhizobium inoculation significantly increased the yield (seed number and seed biomass) over uninoculated in all the three legumes studied. Among the ***Rhizobium***, of three tree legume species, ***Rhizobium*** of ***P. dulce,*** produced more effect on ***V. mungo*** followed by ***Rhizobium*** of ***E. indica*** and ***S. grandiflora*** of inoculated seedlings of ***V. mungo*** similar results were observed in ***V. radiata*** and ***A. hypogaea*** inoculated seedlings.

The three inoculated plants produced higher grain yield over un inoculated. Increase in grain yield due to ***Rhizobium*** inoculation was also reported by Thakur and Hossain, (1976) and Bishnoi and Dutt (1983) in soybean.

The results of the present study on the growth and yield parameters in ***V. mungo, V. radiata*** and ***A. hypogaea*** due to the inoculation with ***Rhizobium spp.*** (***P. dulce, E. indica*** and ***S. grandiflora***) were statistically significant. Such significant increase in growth and yield due to the rhizobial inoculation have also been reported in ***Cajanus cajan, Cicer arietinum*** and ***V. unguiculata*** and ***A. hypogaea*** (Subba Rao, 1971).

From the above discussion, it is concluded that the ***Rhizobium*** spp. of ***P. dulce,*** was more effective as biofertilizer and it can be recommended for the improvement of crop yield.