Conclusion

The expert seldom gives an objective view;
They give their own view; objectively.

- Morarji Desai
The formation of nitrogen fixing nodules on the roots of leguminous plants is a highly complex interaction between Gram negative soil bacteria of the genus *Rhizobium* and a limited range of host plants. Although the nodule originates from the meristem, that is located in the inner cortex of the root, proliferation of these cells is not the first event in the whole process. The meristems are in fact induced by the microsymbionts from a distance [200,201].

The normal developmental steps involved in the establishment of symbiosis can be summarized as follows: attachment of the bacteria to the root surface, curling of root hair, formation of infection thread towards the nodule meristem, and proliferation of the bacteria transformed to bacteroids in the meristem. This has been well studied in fast growing *Rhizobium* sp.

Cow pea miscellany group of *Rhizobium* is a poorly characterized group of Rhizobia. They are generally relatively slow growing, have a broad host range, and nodulate tropical legumes. Very little is known about the signal transduction that takes place during the initial stages of *Rhizobium*-legume interaction in this group. Among fast growers which nodulate temperate legumes, flavonoids secreted by the plants are known to be the inducers [169]. The Rhizobia produce exopolysaccharide, lipopolysaccharide and lipochitino-oligosaccharide in response to these signals [33]. The genes encoding nodule formation are plasmid-borne in the fast growing *Rhizobium* sp., while they are chromosomally located in *Bradyrhizobium* sp. [115]. The objective of this study was to investigate the physiological and molecular mechanisms underlying the cow pea Rhizobial interaction with its host(s).

Three different isolates used in the present investigation were isolated from nodules of locally grown varieties of *Cajanus cajan* (pigeon pea or tuer) i.e. *Rhizobium* sp. S2 and *Rhizobium* sp. Sp23 and *Vigna catjang* (cow pea or chowli) i.e. *Rhizobium* sp. SK9. All three isolates were found to harbor a large plasmid when total DNA was extracted by Wheatcroft and Willams' methods.

The parent strains *Rhizobium* sp. S2 and SK9 were cured of the plasmid by repeated transfer in acridine orange. The plasmid-cured derivatives obtained were termed muc- and SK9.P respectively. Both the plasmid-cured derivatives showed same antibiotic sensitivity pattern as their parent suggesting absence of antibiotic markers on the plasmid. However, the plasmid-cured derivatives differ from their parent strain in producing negligible amount of EPS and lack of *ex planta* nitrogenase activity.

Root exudate has been shown to affect growth and proliferation of Rhizobia in soil [18]. Respective host root exudate showed a positive effect on the growth and EPS production of parental Rhizobial strains. *Rhizobium* sp. S2, SP23, SK9 showed a 4 to 6.5 fold increase in total EPS synthesis when grown in presence of their respective host root exudate, whereas the plasmid-cured derivatives showed only a 1.5 to 2 fold increase under similar conditions.

*Rhizobium* sp. S2 showed a positive chemotactic response towards root exudate and the components present therein. Studies with different Tn5 mutants showed two types of behavioral mutants, one sensing nutrients and the other sensing signal compounds. Bergman et al. [15] have shown existence of dual chemotaxis pathway in *Rhizobium meliloti* by studying similar behavioral mutants. Extrapolating these results to our preliminary observations, we conclude that a similar dual chemotaxis pathway may exist in *Rhizobium* sp. S2, one specific for sugars and amino acids, and the other for sensing signal compounds like naringenin.
The total EPS of Rhizobial cultures grown in presence and absence of host root exudate was tested for the presence of nod factors by Had bioassay. EPS from only the induced (+ root exudate) cells gave positive Had activity, suggesting that nod factors are produced only on induction with host root exudate, as seen among fast growers [46]. The plasmid-cured derivatives failed to synthesize any nod factors under similar growth conditions. The nod factors were partially purified by butanol-soluble, ethyl acetate-insoluble method of extraction. These fractions from the wild type also showed positive Had activity where as those from the plasmid-cured derivatives did not. This is suggestive of inability of the plasmid-cured derivatives to synthesize nod factors due to loss of plasmid.

Relative host specificity in these strains was evident from the Had activity elicited by the culture supernatant of cultures induced with host root exudate and non-host root exudate. Further the butanol-soluble, ethyl acetate-insoluble fractions were extracted from culture supernatant of *Rhizobium* sp. S2, SP23 and SK9 grown in presence of heterologous host root exudate. These fractions were screened for their ability to elicit a positive Had activity on pigeon pea and cow pea root hair. A positive Had activity was obtained only in case of *Rhizobium* sp. SP23 grown in presence of non-origin host, cow pea. *Rhizobium* sp. S2 and SK9 did not elicit any Had activity with heterologous host. Thus, *Rhizobium* sp. S2 and SK9 were host specific whereas *Rhizobium* sp. SP23 was relatively broad host range. Neither of the nod metabolites synthesized in response to one host gave any Had activity on another host suggesting a host specific nature of the nod factors of pigeon pea and cow pea probably due to structural differences.

It is well established that the *nodABC* are the first common *nod* genes to be transcribed on induction with host signals [109] and these genes are structurally conserved and functionally interchangeable between different species of Rhizobia [76]. PIJ1477 containing *nodABC* genes under its own promoter, with *lacZ* fused to *nodC* was transferred via conjugation into the parent and plasmid-cured derivatives. Increased levels of β-galactosidase were observed in the parent strains under induced (+RE) conditions as compared to basal or uninduced (-RE) conditions. Similar experiments were carried out with other cow pea *Rhizobium* sp. isolates. The results paralleled the Had activity and nodulation ability of the strains. The plasmid-cured derivative SK9.P\(^{-}\) failed to show any increase in β-galactosidase activity, which reflected its inability to infect its host. An interesting observation was the increase in β-galactosidase activity of the transconjugant obtained from muc\(^{-}\), under induced conditions. The level was higher than that observed with parent *Rhizobium* sp. S2. Two inference can be drawn from these observations:

1) The *nodD* gene may be present on the chromosome in muc\(^{-}\) which accounts for the presence of β-galactosidase activity on induction even in absence of plasmid.

2) The *nodD* gene is not activated by heterologous host flavonoid and therefore *Rhizobium* sp. S2 and SK9 are not able to cross-infect.

This reporter gene system can also be used to determine the host range of any given field isolate in this group of Rhizobia [157].

Colony blot and dot blot hybridization analysis of the wild-type and plasmid-cured derivatives, and the purified plasmid and chromosome was carried out using *nod* (*nodABC*), *nif* (*nifKDH*) and EPS (*psS*) genes from *R. leguminosarum*. The hybridization studies showed presence of these determinants on the plasmid with no copy on the chromosome. This also explains the nod\(^{+}\), nif\(^{-}\), EPS\(^{-}\) phenotype of the plasmid-cured derivatives. The large plasmid of *Rhizobium* sp. S2 and SK9 were subjected to restriction
digestion and southern hybridization was carried out using the same above probes. The positive EcoR1 fragments were fished out and subcloned into pSUP2021. Random Tn5 mutants were derived using pGS9 as the transposon donor and screened for nod, nif phenotypes. The plasmids from these mutants were isolated and in-gel hybridization was carried out. The results showed that the Tn5 mutants had their respective characteristics because of structural disruption of the gene(s) and not loss of it.

The 3.8 kb fragment from *Rhizobium* sp. S2 which gave a positive hybridization with the *psb* gene probe was subcloned in pML122, a broad host-range expression vector and mobilized into *Rhizobium* sp. S2.19, a nod− nif− Tn5 mutant of parent *Rhizobium* sp. S2. The transconjugant was screened for phenotypic reversion of exopolysaccharide production. The transconjugant of the Tn5 mutant, S2.19 showed production of EPS which bound to cellufluor white, suggesting restoration of ability to produce acidic EPS. Thus, the 3.8 kb fragment from the large plasmid of *Rhizobium* sp. S2 encodes for acidic polysaccharide determinant.

In order to characterize the nod metabolite synthesized by the parent strains under induced conditions, experiments were carried out by metabolic radiolabeling using 14C-acetate as substrate. The incorporated metabolites were detected by phosphoimaging or autoradiography. *Rhizobium* sp. S2 showed the presence of three new bands on reverse-phase TLC and *Rhizobium* sp. SP23 showed presence of two new metabolites on induction with naringenin. On autoradiography of a similar fraction from *Rhizobium* sp. SP23 induced with non-origin host cow pea root exudate, there were four distinct bands, which were hydrophilic as compared to the naringenin-induced bands. Multidimensional TLC also showed production of amphiphilic to hydrophobic nod metabolites produced by *Rhizobium* sp. S2 and SP23 on induction with naringenin, whereas a more hydrophilic metabolite by *Rhizobium* sp. SK9 and SP23 on induction with cow pea root exudate. Thus, the nature of the nod metabolites synthesized in response to two different hosts is different.

The nod factor nature of the nod metabolites was confirmed by their susceptibility to exochitinase and lysozyme. Similar establishment of nod factor nature has been carried out in case of nod factors produced by *Rhizobium* sp. NGR234 by Price and Carlson [147].

Large-scale extraction of the metabolites to purify the nod factors for structural analysis was attempted; however, the quantity of the purified fraction obtained was very low to do so. The nod metabolites produced by *Rhizobium* sp. S2 in response to naringenin gave two peaks at retention time 44 and 50 min. at approximately 50% acetonitrile concentration on a preparative C18 RP-HPLC. This reconfirmed the amphiphilic nature of the naringenin induced nod factors. The HPLC analysis of *Rhizobium* sp. SP23 induced with naringenin gave similar results. Interestingly, the HPLC chromatograms of *Rhizobium* sp. SK9 and SP23 grown in presence of cow pea root exudate were also similar. However, the nod factor peaks had a lower retention time on the column (40-47 min.) suggesting a more hydrophilic nature of the nod factors produced. These results are in agreement with the observations on the radioautographs. The purified peaks from *Rhizobium* sp. S2 gave positive Had activity.

The role of cell surface components is well established in *Rhizobium*-legume symbiosis [62,91]. The effect of host root exudate on EPS, LPS and protein profiles was therefore studied.

The acidic and neutral polysaccharide fractions of the wild-type and the plasmid-cured derivatives were found to increase on induction with host root exudate. However,
the increase in production was maximum in case of the wild-type on induction with respective host root exudate. On the other hand, there was only a marginal increase in case of plasmid-cured derivatives. On analysis of the acidic polysaccharide fraction on an anion exchange chromatography, presence of highly negatively charged EPS was detected on induction with naringenin in case of *Rhizobium* sp. SP23. On comparison of the EPS produced by *Rhizobium* sp. S2 and muc, the plasmid-cured derivative was unable to synthesize the highly anionic fraction produced by the parent; the overall synthesis of the acidic polysaccharide was also low. The requirement of the acidic polysaccharide during symbiosis, especially during infection and growth in the infection thread has been well-established [63,108,132].

Differences in the LPS-PAGE profiles of the three *Rhizobium* sp. isolates grown under various growth conditions were studied. *de novo* synthesis of LPS in the LPS1 banding region was observed on induction with host root exudate. Induction with non-host root exudate did not elicit any response in *Rhizobium* sp. S2 and SK9. However, there was production of a slow migrating LPS 1 band in case of *Rhizobium* sp. SP23 grown in presence of heterologous host cow pea root exudate. No change in the LPS-PAGE pattern was observed in the plasmid-cured derivatives muc and SK9 in absence or presence of homologous or heterologous host root exudate. Root exudate has been shown to bring about *de novo* synthesis of LPS 1 with modification in the M, and O-antigen component of LPS 1 [158]. *de novo* synthesis of a high molecular weight band in the LPS 1 banding region was observed when the wild-type Rhizobial isolates were grown at low oxygen concentration and on succinate as sole carbon source. The banding pattern was found to be similar to that found in bacteroids of these isolates.

*Rhizobium* sp. showed distinctive differences in the protein profiles of whole cell and the individual cell fractions. There was host specific *de novo* synthesis of membrane proteins on induction with host root exudates suggesting their role in signal transduction.

Thus, the major functions involved in symbiosis viz., nod, nif, EPS are plasmid-borne. There exists a dual chemotactic pathway to detect the presence of host. The relative broad host specificity reported to be present in this group of Rhizobia is strain-specific. The host specificity could be due to the nature of nod metabolites produced in response to a given host. The *nodABC-lacZ* reporter gene system can be used to determine the host range of any given field isolate and the method has the advantage of being relatively faster as compared to the conventional nodulation process. Changes in surface components like EPS, LPS and proteins are seen to be induced by presence of host. The present investigation thus brings us to conclude in general, that there is a shift in metabolic and physiological machinery for life from free-living to an endosymbiont.