CONCLUSION
Conclusions:

About 65% of the biosphere's nitrogen is produced via the biological reduction of atmospheric nitrogen to ammonium, mediated to a large extent by legume-rhizobium-symbioses (Lodwig et al., 2003). It is for this reason that rhizobia is often amended into agricultural fields as a nitrogen biofertilizer for leguminous plants. The successful performance of rhizobial inoculant strains depends upon their capability to outcompete the indigenous soil bacteria, survive and propagate, and enter into effective symbiosis with the host plant. One of the important micronutrient for the survival of rhizobia in soil is iron which is present at around $10^{-18}$ M, much below the concentration required by soil microflora. As a result there is always iron stressed condition prevailing in the soil. Rhizobia, due to its poor survivability in this iron limiting soil environment, when applied as a bioinoculant, gets outcompeted by other rhizospheric microflora and hence most of the times, is ineffective in enhancing legume productivity (Miller and May 1991; Streeter, 1994). A study was initiated with rhizobacteria isolated from peanut and mung bean to understand the complex interactions taking place between different bacterial species in the soil, mainly due to the iron limitation which prevails, and mediated through siderophore production and utilization of the available siderophore in the soil. Understanding the basis of these interactions could be then extrapolated to predict the survival and functioning of the bacterial species in natural environment.

Several siderophore producing organisms were isolated from the rhizosphere of peanut and mung bean. Majority of these isolates belonged to the Enterobacteriaceae family and produced catechol type of siderophores. Few other fluorescent pseudomonads and fungi, producers of hydroxamate siderophores were also used in the study. The striking observation made during the study for two strains G9 and G6 was that the former could utilize maximum numbers of siderophores while the later failed to utilize most of the siderophores provided. The multiple siderophore cross-utilizing ability provided G9 with a growth advantage in the presence of siderophores it cross-utilized, under iron limiting laboratory conditions, in comparison to the growth of G6 isolate which failed to utilize the siderophores provided. In contrast when G9 was grown in the presence of G6 siderophore which it failed to cross-utilize, its growth was completely inhibited. It was
hypothesized that G9 siderophore was of a very low affinity and the growth inhibition caused was due to the formation of Fe$^{3+}$-G6 siderophore complexes which could not be chelated by G9 siderophore. A novel method was devised and was proved that the affinity of G6 siderophore for iron was about three orders of magnitude higher than that of G9 siderophore. All the above observations led to the conclusion that in conditions where a low affinity siderophore is produced, organisms evolve to utilize ferric siderophore complexes of various types from different organisms as represented by the isolate G9. This could be brought about either by use of low specificity receptor that recognizes heterologous siderophores (Crowley et al. 1991) or by possessing multiple siderophore receptors (Cornelis and Matthijs, 2002). Since isolate G9 does not show the presence of multiple OMPs (Fig. 4.3), the possibility that it could be synthesizing a relatively broad substrate specificity receptor cannot be denied.

Another rhizospheric isolate G11, was identified to be an efficient siderophore cross-utilizer, as it utilized relatively less number of catecholate siderophores, but was able to utilize hydroxamate siderophores produced by fluorescent pseudomonads and soil fungi. G11 was able to utilize the siderophore of PsB (peanut plant growth inhibitory Pseudomonas aeruginosa strain) and because PsB failed to utilize the siderophore of G11, under iron limited laboratory conditions, upon co-inoculation of both the organisms, G11 was able to outcompete PsB, by causing partial growth inhibition. Peanut seedlings when inoculated with the above organisms, PsB clearly inhibited plant growth when inoculated alone, but when co-inoculated with G11, its pathogenic effect was almost inhibited. G11 was therefore able to act as a biocontrol agent against the pathogen. To conclude, the plant growth promoting property of G11 can be attributed to its better siderophore production, and might be able to mobilize plant iron resources efficiently. Its ability to utilize siderophores produced by rhizospheric bacteria and fungi, can make iron acquisition easy for G11, and cause iron starvation to pathogenic organisms. The failure of other organisms tested to be unable to utilize siderophore of G11 may also be important in survivability of G11 and hence could support plant growth even in presence of antagonistic organisms.
The studies conducted so far, could conclude that, any organism having ability to cross utilize number of siderophores, will definitely have a growth advantage in the presence of these siderophores. In addition higher siderophore production, and possession of a siderophore with a relatively higher affinity can also increase the survival competence of the organism in soil.

It is known that many soil fungi produce hydroxamate siderophores, and that hydroxamate siderophores are stably present in the soils in large concentrations (Powell et al., 1980). Among these hydroxamates, the ferrichrome type siderophores constitute a major fraction, and they are present in the soil at concentrations as high as 78 nM (Powell et al., 1983).

*Pseudomonas* sp. are well known for their rhizospheric stability, which is attributed to the diverse ferri-siderophore uptake systems that they possess (Cornelis and Matthijs, 2002). Our analysis of their complete genomes revealed the presence of 45 TonB dependent siderophore receptors in the genome of *P. fluorescens*, 31 in *P. putida* and 36 in *P. aeruginosa*. In contrast to this, a visible scarcity of TonB dependent siderophore receptors became evident on doing a complete genome scan of a few members of rhizobiales; 3 were present in *R. etli*, 3 in *Mesorhizobium* sp. BNC1, 2 in *Mesorhizobium loti*, 2 in *Ag. tumefaciens* and 2 in *S. meliloti* This led us to think that increasing the number of outer membrane siderophore receptors could make the rhizobial strains more efficient with respect to iron acquisition, and hence colonizing the rhizosphere. FegA, ferrichrome receptor gene from *Bradyrhizobium japonicum* 61A152 was chosen to be cloned and expressed in the rhizobial strains with the assumption that the large pool of ferrichrome siderophore if made available would enable the organism to effectively acquire iron and as a result would successfully colonise the rhizosphere, leading to enhanced nodulation ability as its direct consequence. *fegA* was amplified from *B. japonicum* 61A152, cloned in pUCPM18, the construct named pFJ, and transferred by conjugation to *Rhizobium* sp. ST1 and *Mesorhizobium* sp. GN25. The parent strains were initially non-utilizers of ferrichrome, but the transconjugants (TCs) GN25pFJ4, GN25pFJ9, ST1pFJ8 and ST1pFJ12 were able to utilize it indicating the successful expression of FegA into these rhizobial strains. The ferrichrome up take ability conferred
upon the TCs a significant growth stimulation in the presence of pure ferrichrome as well as when coinoculated with the FC producing strain of *U. maydis* under iron limiting laboratory conditions. SDS-PAGE analysis of Outer Membrane (OM) proteins revealed the presence of a 79 kDa protein on the outer membrane of the TCs which was absent in the parent GN25, implying the expression of the FegA protein.

Pot culture studies carried out with peanut plants inoculated with *fegA* bearing transconjugants showed marked increase in shoot weight, nodule number and chlorophyll content of leaves compared to plants inoculated with the parent strain under both autoclaved and un-autoclaved soil conditions. The positive effect of *fegA* transconjugant inoculation on plant growth was more pronounced when FC producing *U. maydis* was coinoculated in the systems.

The nodule occupancy of transconjugants on pigeon pea plants increased to 67% and 65% as compared to 39% by the parent strain, which further increased to 79% and 82% respectively, upon *U. maydis* coinoculation. This could be imparted to the increased survival of these strains in the soil condition. The studies thus support the hypothesis that presence of specific siderophore receptor gene would lead to increase in iron acquisition and hence survival competence of the organism in the rhizosphere.

However, recombinant plasmids are usually very unstable in rhizobia. Moreover, in this case, plasmid containing *fegA* contains the *mob* region, which would facilitate its transfer into some pathogenic organism that could cause serious consequences, hence the integration of the *fegA* gene onto the chromosome of the organism is essential for field applications.