Summary of the work

The work of this thesis is divided in three Chapters dealing with (i) Isolation of pigeon pea rhizobia and characterization of their quorum sensing (QS) components and regulation, (ii) Engineering of QS system of standard lab strains of rhizobia to assess its effect on nodulation competitiveness, and (iii) Understanding the spatiotemporal colonization dynamics of rhizobia on host roots. The major findings are summarized below chapterwise.

Chapter 2: Elucidation of quorum sensing components and their role in regulation of symbiotically important traits in pigeon pea nodule bacteria

- Pigeon pea nodulating bacteria were isolated and screened for the host growth promotion and production of n-acyl homoserine lactone (AHL) indicative of occurrence of quorum sensing (QS) and three best strains were selected for further studies.
- The screened strains were identified to belong to the genus *Ensifer* and clustered near *E. terangae* when analysed using the obtained 16S rDNA and recA gene sequences.
- Identification of the AHLs produced by the three *Ensifer* strains using LC-MS/MS revealed production of diverse AHLs including long chain AHLs by the three strains and identification of two AHLs not hitherto reported in *Rhizobiaceae*.
- Degenerate primers were designed to specifically amplify the reported QS gene homologues *sinI. sinR, traI, traR* and *expR* in *Ensifer* using which all these gene homologues were detected in all the three strains. The sequencing results indicated that some of the gene sequences were as similar as 99%, while some others only matched 69% to the homologous sequences from other reported strains.
- The QS gene homologues were found to have the conserved domains with unique amino acid variation in the strains mapping to different domains.

- Regulation of phenotypes crucial to root colonization by QS in one of the strains (*Ensifer* sp. HP127) revealed that formation of biofilms and chemotaxis were reciprocally regulated by QS—high quorum favoring biofilm and low favoring the motility.
- The transcriptional regulation of symbiotically crucial genes in *Ensifer* sp. HP127 revealed that the QS circuit genes and the genes regulating root-attachment as well as production of exopolysaccharides were upregulated by AHLs induction (simulating high quorum) whereas genes regulating motility and sugar metabolism were downregulated.

Chapter 3: Engineering QS system of rhizobia to assess its effect on nodulation competitiveness.

- A homologous cloning of cinRI and sinRI (the top-of-hierarchy QS circuits) in *Rhizobium leguminosarum* 3841 and *Ensifer meliloti* 8530 was done in the broad host range Gram negative expression vectors of pBBR1MCS series.
- This cloning led to constitutive over-expression of the respective QS signaling in each of the strains as revealed by the dynamics of the AHL production.
- QS overexpression led to increased capability to attach the host root as well as that of forming biofilms (on polystyrene surface) and production of exopolysaccharides.
- Scanning electron microscopy of the colonies of Rlv3841 overexpressing *cinRI*, on the host roots when compared with those of the vector control strain revealed that the former were significantly denser and embedded in the thick secretory matrix.
- Gene expression analysis of Rlv3841(pCINRI) as compared to its VC revealed that the genes regulating motility were downregulated and that regulating glucomannan synthesis required for attachment to root was upregulated.

- Inoculation of the QS over-expressing strains on to the respective host plants (pea for Rlv3841 and alfalfa for Em8530) did not result in significant differences between the plant growth parameters except in case of Em8530(pSINRI) treatment which in comparison to the VC was found to increase the root and shoot weight..
- There was small increase in the nodulation competitiveness of Rlv3841(pCIRI) as compared to the WT strain when applied in the equal ratio. At the other ratios, there was no significant difference found in the nodulation competitiveness. In case of Em8530(pSINRI) competing with WT, there was no significant difference at any of the tested ratios.

Chapter 4: Spatiotemporal patterns of root colonization by Ensifer spp individually and competitively on host plant.

- Two pigeon pea nodulating strains *Ensifer* spp. HP127 and HP113 isolated and characterized for their QS and plant growth promotion in Chapter 2 were employed to study their colonization dynamics.
- When the *Ensifer* spp. Hp127 and HP113 were applied on the root of pigeon pea growing in gnotobiotic conditions, they were found to colonize differentially, with the former covering greater length of the primary root as well as a better spread over to the secondary roots while the latter was seen to be restricted in its spread over primary as well as secondary roots.
- The strains when allowed to compete for colonization in conditions mimicking the wild conditions of the soil wherein one of the strains was coated on the seed while the other was mixed with the soil (at 10^4 or 10^6 cfu/g in separate sets). The two strains were swapped for the position as well. The colonization dynamics revealed that an area with abundance of one strain restricted the colonization of the other strain partially. This was true for both strains in both locations. In an interesting observation, *Ensifer* HP127 whenever present, led to better spread of the *HP113* as compared to when HP113 was alone.

- The strains were tested for the capability to form biofilm and EPS production wherein *Ensifer* sp. HP127 formed more biofilms (on polyester surface) and secreted greater exopolysaccharides as compared to *Ensifer* sp. HP113.
- Confocal laser scanning microcopy (CLSM) of both the strains revealed that HP127 colonized in denser biofilms whereas HP113 was found to colonize as sparse and discrete microcolonies.
- Scanning electron microscopy results corroborated the CLSM findings and revealed that the HP127 colonies are filled with copious amounts of secretions whereas the HP113 colonies almost completely lacked any matrix.
- A microfluidics time lapse imaging of association between Em8530 and alfalfa revealed that the former colonized the roots of the latter immediately after coming in the contact and that the crown proximal areas increased in the colonization by the Em8530 as compared to the rest of the areas of the root and by 20 h, strong biofilms were observed.

In conclusion,

The characterization of QS systems from *Ensifer* isolates nodulating pigeon pea revealed that these isolates possessed the canonical systems reported for *Ensifer* spp. nodulating other host plant with minor deviations. This is to the best of author's knowledge, the first characterization of QS of pigeon pea nodule bacteria. The deviations in the QS of these strains observed indicates that there may be an unexplored repertoire of diversity. Further studies of importance of QS in these strains can be done by mutagenic inactivation. In that regard, the level of conservation among species can be good scaffold to expand into the details of the novel isolate strains that can enhance the crop health. Knowledge of these differences along with the conserved patterns of QS regulation can herald the opportunitiy of generation of strains improved for their colonization of the host and

thus improved nodulation competitiveness. This can be of immense significance to the plants largely omitted from the molecular studies of their symbiotic association. QS is often regarded as “bacterial talking”; in this regard the on-the-root talks between rhizobia hold key to the effectiveness of their colonization and ultimately nodulation. Generation of “talkative-and-loud” mutants of two of such strains that constitutively overexpressed QS, resulted in them showing amplified phenotypes determining the symbiotic competence. This however did not yield significant betterment in competitive colonization in the experimental setup employed. This suggests that the complexity of the QS regulation in multi-tiered manner possibly maintains homeostasis which could be explored further. It is also surmised that a heterologous expression of QS from one species to another might be of interest for future studies this approach combined with more “controlled” comparisons with strains of varying nodulation competitiveness can reveal better insights into the scope of such genetic engineering. The colonization dynamics patterns of rhizobia on roots of the hosts revealed the importance of attachment, biofilm formation and production of mucous polysaccharides by pigeon pea rhizobia, in determining their colonization on the root when supplied as a coated organism. A significant greater occupation of the root may in turn restrict the nodulation by another competing rhizobial strains. A significant nodulation happens on the secondary roots which was also improved by the presence of these properties. Thus, the capabiltty of better attachment to the host root or that of secretion of mucous polysaccharides can be desirable in inoculant strains, alternatively, bearers of such properties may also be employed as ‘hlper’ strains. Microfluidics combined with time lapse confocal microscopy also revealed the importance of immediate interactions between host root and rhizobia and the further colonization.