1 Introduction and Review of Literature

1.1 Rhizobia

Centuries ago Greeks and Romans observed that intermittent legume cultivation with cereals led to increased fertility of the soil but it was Hellriegel and Wilfarth in 1888 who identified that the bacteria inside root nodules of the legumes converted the N_2 of the air to ammonia (Peter *et al.,* 1996) .Beijerinck in 1888 isolated these bacteria and called them *Bacillus radicicola* and Frank later in 1889 called them *Rhizobium* (Peter *et al.,* 1996). Rhizobia (singular: rhizobium), is the commonly used term to denote all the diazotrophic members of the classes alpha and beta proteobacteria which form symbiotic association with legumes. Within specialized organelles "nodules"– one of the most important and comprehensively studied interkingdom mutualism, they 'fix' the atmospheric nitrogen (N) into forms utilizable to the plants. Thought to have evolved over 500 million years ago — way before the angiosperms came into existence (Hirsch *et al.*, 2001) and longer before the legumes existed (Rivas *et al.,* 2009) rhizobia are environmental heterotrophs and can exist as free living bacteria in soil. However the nodulation of the legumes is believed to have evolved only 70 mya (Doyle, 2011). Rhizobia are currently classified into more 98 species across 13 genera (Weir, 2016) while a lot of new studies keep adding new species into the list. The legume-rhizobium symbiosis leads to fixation of about 21 x 10^9 kg N/year for the agriculturally cultivated legume crops alone and additionally up to 25 x 10^9 kg N per year (Herridge *et al.*, 2008). Thus, rhizobia are not only one of the most important unit of ecology and agriculture, they hold remarkable economic importance, too. They have mostly a very narrow host specificity wherein they nodulate a legumes of a single or limited number of species (Stacey, 2007). An exception being the species *Ensifer fredii* members of which nodulate hundreds of legumes (Stacey, 2007).

1.2 Process of Symbiotic Nitrogen fixation

Rhizobia induce the formation of the specialized structures called nodules on the host root where they differentiate into 'bacteroids' and carry out the Nitrogen fixation. This process of going from the saprophytic soil life style to N-fixing bacteroids is immensely intricate and requires signal exchanges and metabolic adjustments from both the participants (Schwember *et al.*, 2019). The process is presented in the below text in brief.

The Process of nodulation can be divided into stages of pre-infection and nodulation. A detailed account of these processes is given below and summarized in the illustration in the (Figure. 1.1).

1.2.1 Pre-infection stages of nodulation

1.2.1.1 Chemotaxis and attachment

The nodulation process begins with the colonization of rhizobia on the host root. A number of abiotic factors such as the soil acidity, water activity, temperature etc. (Turner *et al.*, 2013), and biotic factors such as the plant health, nodulation status and the existing microbiome determine the colonization efficiency of rhizobia (Tkacz *et al.*, 2015; Zgadzaj *et al.*, 2016). Thus the growth and survival of the rhizobia in the soil and in the rhizosphere partly determine the fitness of a rhizobium to nodulate the host. Rhizobia are attracted to the root hairs and move in the response to plant origin chemoattractants including flavonoids. Attraction is followed by rhizobial attachment to the surface of the root hair. This involves specific interaction between the lectins of the plant and glucomannans of the rhizobial origin or an interaction mediated by Ca^{2+}-dependent adhesins produced by some species of rhizobia. This, together with the extracellular fibrils present on the root-hairs, results into attachment of bacterial cells to the root hair tips. Post attachment to the root hairs, rhizobia aggregate and form a biofilm encased in EPS and cellulose, and is referred to as the Root hair cap (Smit *et al.*, 1987). (Figure 1.2) shows a micrograph depicting root hair caps captured in this study.

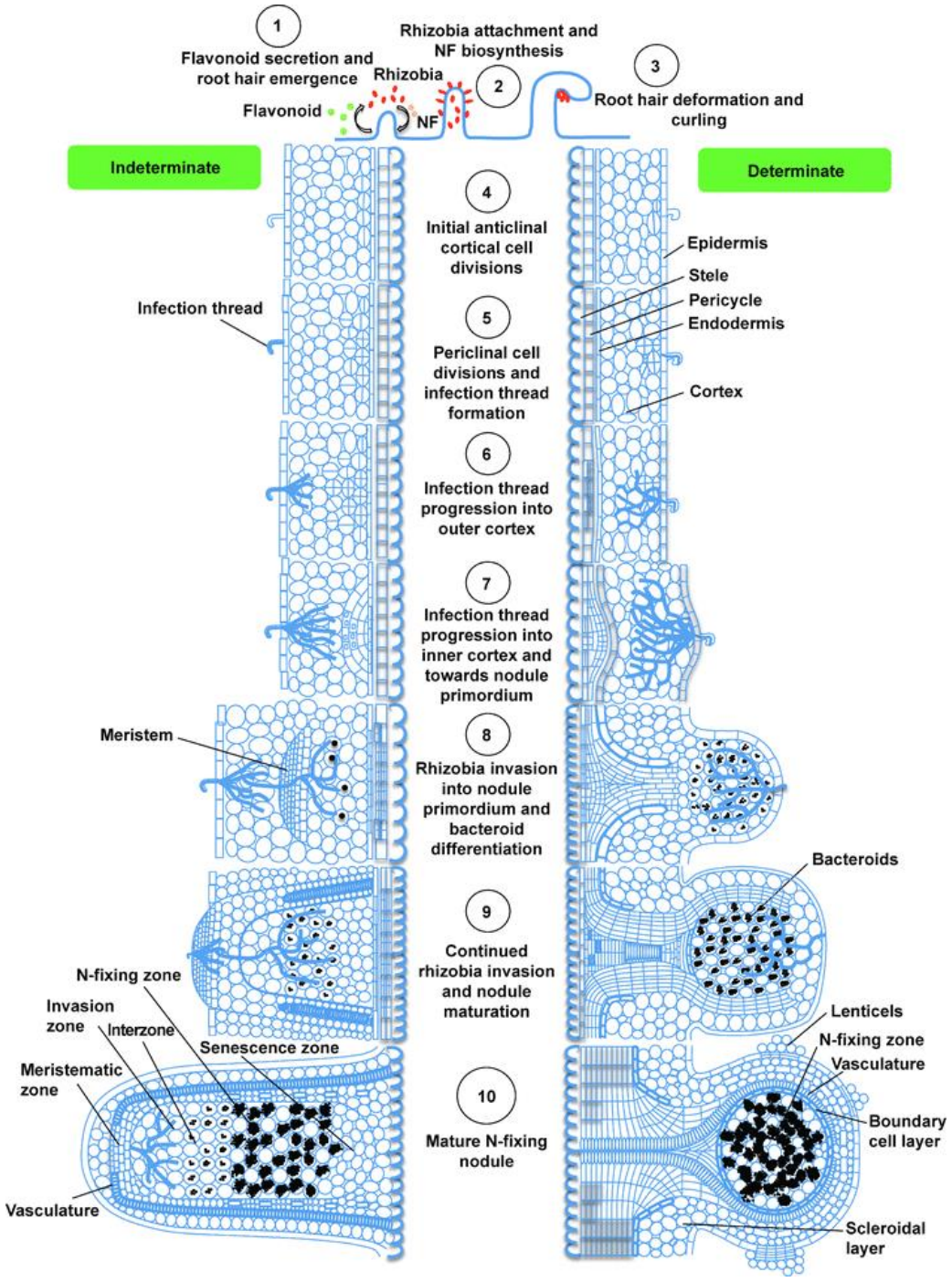

Figure 1.1 Process of Nodulation in Pea and Soybean. Developmental stages of nodules in pea (left; indeterminate) as well as soybean (right; determinate). The labels refer to the respective tissue types and zones of nodule tissue. [Source: Ferguson *et al.*, (2010)]

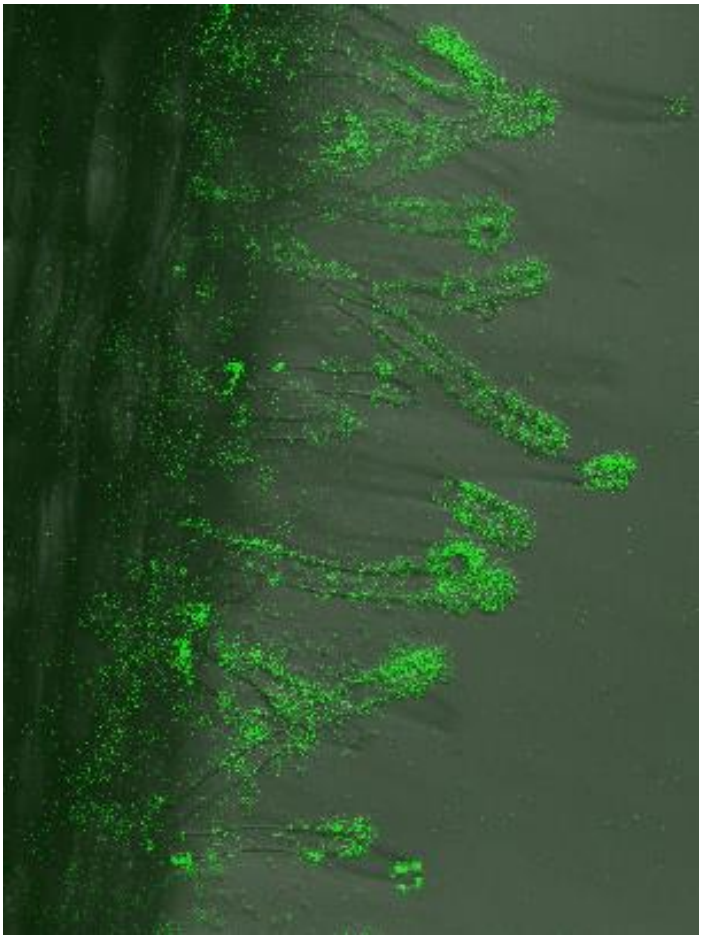

Figure 1.2 Root hair cap in *Ensifer meliloti*. Cells expressing eGFP can be seen to "cap" the tips of root hairs- the lateral projections from the vertical primary root. The root shown is of *Medicago sativa* being grown in a microfluidic chamber. (Source: This work; Chapter 4)

Mutants not able to establish cap structures and thus incapable of stable attachment have been found to be unaffected in the nodulation *per se* (Finnie *et al.*, 1997) but are believed to be affected in the competitive nodulation as is the case in the soil (Poole *et al.*, 2018). This idea is reinforced by the fact that rhizobial mutants with greater attachment capabilities led to an improvement of nodulation competitiveness (Mongiardini *et al.*, 2009). Chemotaxis over the root and the motility to reach to the site of receptive root hairs is one of the most important processes of colonization and has been linked with the ability of the rhizobia to competitively nodulate the host in several studies (Miller *et al.*, 2007; Bernabéu-

Roda *et al.*, 2015; Calatrava-Morales *et al.*, 2017; Liu *et al.*, 2017;) . In laboratory experiments, rhizobia when inoculated over the growing root, have been seen to colonize over the root tip within minutes of inoculation (Turgeon and Bauer, 1982). The rhizobial biofilm on the root hair increases the local cell densities to manifold and thus an efficient chemical dialogue takes place at even small concentrations of the mediators (Miller *et al.*, 2000). Rhizobia on the root cap sense the flavonoids with the NodD receptor and in response produce lipochitooligosaccharides popularly known as 'Nod factors'. These nod factors are sensed by the host legume and result in the activation of SYM signaling pathways in them. Nod factors recognition is host-restricted and determine the host-rhizobium specific pairing that is the hallmark of this mutualistic association (Strain *Ensifer* NGR234 is an exception and it produces more than hundred different types of nod factors leading to the promiscuous nodulation that it is popular for) (Poole *et al.*, 2018). Activation of SYM pathway results into positive feedback loop between and flavonoid and nod factors and ultimately culminates into cytosolic changes resulting in the deformations of root hair (such as curling).

1.2.1.2 Root hair deformations

Root hairs are essentially the extensions of epidermal cells of the legume root. Root hairs of the infection zone exhibit altered growth behavior, called root hair deformation or root hair curling. This is essentially the reorientation of the growth of root tip present in the susceptible zone of the root which is normally polarized (straight). The deformations can be classified as either adjoining, intertwining, spatulation, branching, bending or curling. The deformed root hair allows the entry of rhizobial cells (although rhizobia are also capable of entering legume tissue via cracks on the root as well). Occurrence of a particular type of deformation has been believed to depend on the stage of root hair development while coming in contact with the rhizobium; on the other hand it has also been reported that rhizobia only initiate the nodulation from newly emergent root hairs and the root hairs present prior to contact with the rhizobia are not receptive for formation of infection thread (Turgeon *et al.*, 1982). Root hair deformation is an immediate effect of local nod

factor signaling by rhizobia present at the tip. This is has been also validated by addition of purified nod factors leading to root hair deformations and formation of hollow infection threads (Heidstra *et al.*, 1997). The molecular events triggered by the nod factors are perceived by LysM type receptors on the root hairs, leading to root hair deformations and involve increased intracellular Ca^{2+} levels, Ca2+spiking response, and rapid changes of the cytoskeleton mediated by actin filaments (Lei *et al.*, 2015). A transcriptomic analysis of a single cell of root hair in the presence of *Bradyrhizobium japonicum* revealed the differential regulation of close to two thousand genes including those coding for transcription factors and signaling molecules, transporters, enzymes of energy metabolism etc. (Libault *et al.*, 2010). With the root hair deformations allowing entry to the rhizobia, formation of infection thread commences.

1.2.1.3 Formation of infection thread

Most rhizobia enter the host tissues by formation of a tube-like invasion starting from the root hair surface to the cortex of the root tissue, called the infection thread. Infection threads typically begin at the site of rhizobia entrapment at the root hair curl. Subsequently there is local degradation of the mucigel material of plant cell walls mediated by the enzymes of plant origin itself (Wood and Newcomb, 1989; Mateos *et al.*, 2001). The plasma membrane then invaginates inwards and a new cell wall begins to form around it resulting in the initiation of the infection thread formation in the epidermal cells. Rhizobia in these tubules proliferate and make their way further in; nod factors production is known to be highly upregulated in the rhizobia inside the infection threads (Walker and Downie, 2000; Krishnan, 2002). Cytoskeleton rearrangements drive the advancement of the transcellular infection threads whereas the intercellular infection threads progress relatively minimally. Bacterial cell division inside the infection tube is essential for its advancement, these bacteria are reported to utilize amino acids from the plants for their growth supplied to them across the tube barrier (Lodwig and Poole, 2003).

1.2.2 Nodulation

Parallel to the infection thread progression, the cortical cells dedifferentiate and undergo mitotic cell divisions to form the nodule primordium. Ultimately, the cells of this initial primordium will give rise to a persistent meristem that will maintain a population of actively dividing until nodule senesces. The infection threads targeting towards the cortical cells is regulated by these cell divisions and by the action of the phytohormone cytokinin (Jiménez-Zurdo *et al.*, 2000; Mathesius *et al.*, 2000). The progressing infection threads grow past the actively dividing cells of the nodule primordium in indeterminate nodules (for e.g. in *Cajanus cajan* variety BDN 2 and *Medicago sativa*) (Foucher and Kondorosi, 2000); in case of determinate legumes (such as *Lotus japonicas)*, however, the nodules do not have a persistent meristem and all cells in the interior of the nodule proliferate, differentiate and senesce in a synchrony (Mergaert *et al.*, 2006).

When rhizobia reach the inner plant cortex- the target tissue, they have to be internalized by a cortical cell and should establish a niche within that cell. Each rhizobial cell is endocytosed by a target cortical cell in an individual and unwalled membrane compartment that originating from the infection thread. This entire unit, consisting of the endocytic membrane and an individual bacterium is referred to as the symbiosome. The rhizobial cells in the symbiosome subsequently differentiate into a form called bacteroids. In case of indeterminate type nodules, the individual symbiosomes further divide concomitant with the bacteroids and thus mostly lead to individual bacteroids within a symbiosome whereas in the determinate type nodules, the bacteroids divide in the symbiosomes and form a mass of cells (Prell and Poole, 2006). The endoreduplication of genomes of indeterminate bacteroids occurs subsequently, possibly allowing for the higher metabolic rate in order to sponsor nitrogen fixation. Rhizobia which have completed the differentiation into bacteroids and are now anoxically enclosed in the symbiosomes, express nitrogenase and start to fix the atmospheric Nitrogen. The process of N-fixation is energy intensive and is believed to be fueled by the polyhydroxybutyrate granules that are accumulated by invading cells (Willis and Walker, 1998), however the mutants of synthesizing or degrading PHB have been found to be unaffected in the

symbiotic function, indicating availability of alternate carbon sources (Aneja *et al.*, 2005a) . The fixed nitrogen is assimilated in the form of ammonium and is diffused off to the plant tissue which exports it to the xylem vessels in the form of the amino acid aspargine (Poole *et al.*, 2018).

Nodule senescence and autoregulation of nodulation

Nodule senescence is the last stage of the process of nodulation; it is a regulated phenomenon of tissue degeneration of the developed nodules. The nodules formed by rhizobia unable to fix N_2 undergo senescence sooner due to the plant sanctions. Additionally, perturbations such as defoliation, darkening, drought, or available nitrogen leads to inhibition of N_2 fixation as well. The senescence process occurs differently in the determinate and indeterminate nodules; while in the degradation of cells start from the center of the nodules, in the latter the signs of senescence appear first in the inner cortex cells proximal to the root first and then progress towards the center. The senescence initiation is marked by the decreased nitrogenase activity (Patriarca *et al.*, 2004; El Msehli *et al.*, 2019). One of the hallmarks of nodule senescence is the perturbed balance between the generation of free radical species and their neutralization by the antioxidants such as glutathione and ascorbic acid from plants (Becana *et al.*, 2010). This is accompanied by an increases concentration of H_2O_2 in the cytoplasm of the plant cells and an upregulation of cysteine proteases (Pierre *et al.*, 2014; El Msehli *et al.*, 2019).

While production of nodules is advantageous to the plants, excessive nodulation may be detrimental since it consumes the fixed carbon by the plant (Suzaki and Nishida, 2019). Thus, once a threshold number of nodules are made on the host, the plant inhibits further nodulation by a conserved, long-distance (between shoot and root) signaling mechanism called autoregulation of nodulation (AON) (Ferguson *et al.*, 2019). AON is initiated during the development of the nodule by the synthesis of a root-origin signal peptide called ‘Q’. This peptide travels to the shoot via xylem and activates local signaling pathways leading to the production of a compound called SDI (Shoot derived inhibitor) which travels back to the root via phloem and acts as a signal to inhibit the further nodulation. Details of the downstream effectors

of this signal are not fully clear so far (Ferguson *et al.*, 2019). Other than these, phytohormones such as jasmonic acid, cyokinin and ethylene are also involved in regulating the number of nodules. Nodulation, in addition of this is also inhibited in the presence of available nitrogen, acidic soils and under stresses such as low water or light availability, extreme temperatures, and osmotic stress owing to high soil salinity etc (Ferguson *et al.*, 2010). In a recent research, Sorroche *et al.* (2019) discovered a mechanism wherein the endosymbiotic *Ensifer* reduced the susceptibility of the host *Medicago* to be nodulated further- this presents new insights into the autoregulation of nodulation and the results extend the earlier observations by Zgadzaj *et al.* (2016) of endosymbiotic rhizobia governing the microbiome of the host root.

A lot of research through decades has described the process of nodulation in various legume hosts in great depths. With the availability of increasing volumes of genomic data and advancements in the technology, however, this area of research yields newer insights continuously. This is best exemplified by a recent stellar work from Ren *et al.*, 2019 in which they described a novel mechanism of communication between the rhizobia and the host wherein bacterial tRNA fragments mediate the expression of genes of the host. This is the work of remarkable importance and present the first case of transkingdom regulation by the use of tRNA fragments.

1.3 Host root colonization by rhizobia

Rhizosphere being remarkably nutrient-richer than the bulk soil, there exists a very high selection pressure among particular microorganisms for its colonization (Philippot *et al.*, 2013). The colonization process is bidirectional — plants "select" the microbes it hosts, and the microbes contribute to the plant health- such bacteria are known as plant growth promoting rhizobacteria (PGPR). One of the most important and deeply-studied plant–PGPR interactions is the mutualism between legumes and rhizobia. A successful nodulation event, thus requires rhizobial survival in soil followed by the root colonization and subsequent competition to finally gain the entry to the plant. Most of rhizobial research has been focused on

individual specific aspects of the host-rhizobium interaction and do not take into account rhizobia as a member of soil community as well as the effect of competition they face in saprophytic, associative and symbiotic lifestyle and determinants of the switches. A significant knowledge, thus, is available on the signaling and regulatory components of nodulation process (described in Section 1.2) but a detailed understanding of principles driving the attachment on and colonization of host root are not understood (Rivilla *et al.*, 2017). Some of the early studies have collectively revealed the broader nature of the early rhizobium–host-root interaction. Calvert *et al.*, (1984) in revealed that the initial point of contact between the host root and rhizobium affects the distribution of nodules. This can have a remarkable significance to the use of rhizobia as inoculants. This idea is corroborated by the work of Bogino *et al.*, (2011) wherein they report a higher relative nodule occupancy on peanut root by an inoculant strain of *Bradyrhizobium* when supplied in-furrow as opposed to seed-coating. In line with these studies, biological crowding has also been suggested to reduce root colonization and nodulation by rhizobia (Li and Alexander, 1986). Wadisirisuk *et al.*, (1989) revealed that there is a significant effect of initial placement of rhizobium on the host root on its colonization and that the motility did not result significantly in their distribution. Many other studies have examined various factors affecting root-colonization such as percolating water, the edaphic factors, activity of nematodes etc (Caetano-Anollés *et al.*, 1992; Benizri *et al.*, 2001; Lopez-Garcia *et al.*, 2002; Knox *et al.*, 2004) . Some of the features of rhizobial host root colonization are interestingly elementary, for e.g. rhizobial distribution over the lengths of the root is significantly greater via percolation of water as compared to the active motility (Vlassak and Vanderleyden, 1997; Lopez-Garcia *et al.*, 2002). Some of these principles regulating colonization and distribution are shared by many rhizospheric microbes (Benizri *et al.*, 2001; Tecon and Or, 2017) while many are unique to rhizobia (Poole *et al.*, 2018). These studies reveal a broader idea of the colonization process and effect of individual or a few parameters and lack the details and resolution possible by advanced techniques. Additionally, most colonization studies use in-vitro systems such as hydroponics or agar based media or vermiculite to study the

distribution of rhizobia on the roots failing to accommodate the chemical, physical and biological conditions faced by rhizobia in the soil. With the advent of microfluidics and advanced imaging techniques, the host-bacterial interactions can be studied at high spatiotemporal resolution (Rusconi *et al.*, 2014; Aufrecht *et al.*, 2018); recently (Massalha *et al.*, 2017) devised a dynamic coupling of microfluidics and high-res confocal microscopy to visualize and study bacterial colonization of plant root in real time. Thus, assessing the attachment and colonization of host root in a variety of rhizobial strains under soil conditions can not only fill the gap in the knowledge of initial events of rhizobium-legume symbiosis but can also lead to development of better biofertilizer solutions and tackle the issue of nodulation competitiveness.

1.4 Rhizobia as biofertilizers

Currently 2.4 billion people rely on chemical fertilizers to meet the protein demand and the predictions of population rise indicate that there will be a requirement of additional 15M tons of protein nitrogen over the next 50 years (Gonzàlez-Andrés and James, 2016). Thus, in order to cope up with the increasing demand of food in the developing world there is an increasing demand of chemical N fertilizers (Rubio-Canalejas *et al.*, 2016). Even though the chemical N-fertilizers give reliable boost to the legume crop yield in a cost effective manner, and can potentially do away the requirement of regular rotation with legumes while growing cereals, the solution not only lacks sustainability but also has disastrous ecological outcomes such as damage to the land and severe threats to biodiversity (Selma *et al.*, 2010). On the contrary, the organic nitrogen fixed by rhizobia becomes slowly available to the plants and thus has a long term benefit (Sessitsch *et al.*, 2002), while not affecting the biogeocycling of Nitrogen. Additionally rhizobia are also known to possess other Plant growth promoting (PGP) characteristics such as increasing the availability of nutrients (for eg, by producing organic acids to solubilize otherwise unavailable phosphate), induce the growth of host biomass through the production of phytohormones such as Indole acetic acids. Rhizobial strains have also been reported to exert biocontrol of pathogenic bacteria and fungi on the host roots. Thus,

rhizobia are not only capable of fixing nitrogen in the symbiotic association with the legume host but can also benefit the host in several other ways (García-Fraile *et al.*, 2012).

Rhizobia have also been applied to cereals as PGPR for some of the above mentioned reasons. In various reports rhizobia have been isolated from either the rhizosphere or the endophytic compartment of the cereal plants such as maize and rice (Zaim *et al.*, 2017). Since flavonoids are one of the most important determinants of the interaction between rhizobia and legumes and that they are also produced by many of the non-legume roots as well, they may possibly drive the rhizobial colonization of cereal crops (Cesco *et al.*, 2010).

Despite the remarkable benefits of rhizobial inoculation, their viability, application and persistence in the field is challenged by at least majorly three factors (i) low quality inoculant with suboptimal viability; (ii) failure to tolerate the physical and chemical conditions in the soil (iii) inability strains of to compete with native rhizobia to occupy the nodules on host (Cummings, 2005). The first two issues are addressable with the help of novel inoculum preparation techniques and selection of local varieties of rhizobia. The last issue, , requires a thorough understanding of the factors that govern the ability of a strain to nodulate in competition with other strains also proficient at nodulating the same plant and applicable solutions to the problem.

1.5 Nodulation competitiveness

The ability to elicit greater nodulation on the host in the presence of other rhizobial strains is termed as Nodulation competitiveness (Toro, 1996). The competition to nodulate a host can exist between members of different species of the same genus such as nodulation of soybean by *Bradyrhizobium japonicum* or *B. elkani* or that between the rhizobia of different genera nodulating a promiscuous host such as nodulation of *Phaseolus vulgaris* by any of the 25 different species belonging to the genera *Ensifer, Rhizobium, Mesorhizobium*, *Bradyrhizobium* and *Bulkholderia*. However, more commonly in the agricultural setup, the competition for nodulating the host occurs between different strains of the same species (Yates *et al.*, 2011).

There is a high selection pressure in the soil for occupation of rhizosphere and nodulation owing to the high fitness advantage this confers to the nodule occupants, however, there is little or no selection for nitrogen fixation capability. This leads to a high presence of locally adapted strains that are highly potent for survival on and nodulation of the host but are often ineffective for the nitrogen fixation.

Using elite rhizobial strains proficient at nitrogen fixation as an inoculant has been a standard practice in agriculture. However, these attempts have been largely unsuccessful to the extent of having as low as only 5% nodule occupation by the introduced strain. In fact, the autochthonous strains in the soil are so proficient at nodulation that even a small number of them can significantly reduce the nodule representation of the inoculant given the number of viable inoculants that can practically be supplied over seeds (Thies *et al.*, 1991, Parke, 1991).

While the issue of nodulation competitiveness remains unsolved, engineering rhizobial strains for augmentation of competitiveness has been considered as a plausible approach. As reviewed by Savka *et al.*, (2002), following are some of the promising approaches to achieve the same. 1. Promote the fitness and multiplication of inoculant and reduce the limit the abundance of indigenous strains, 2. Engineering the exudation by host, and 3. Interfering in the signaling between the host and among rhizobia in the rhizosphere such as Quorum sensing.

Rhizobia lead a saprophytic lifestyle in the bulk soil or rhizosphere among numerous and diverse other bacterial species. Subject to an effective survival in these, and following a complex molecular dialogue they engage into the mutualistic association with the legume host. Thus, conceivably the success and effectiveness of the nodulation depends collectively on a variety of biotic as well as abiotic factors. Rhizobial traits determining the nodulation competitiveness are listed below.

Rhizobial traits affecting nodulation competitiveness are described below.

1.5.1 Motility and chemotaxis

Ability to sense various chemical cues and elicit movement towards or away from them and a general capability of motility over different kinds of surfaces are essential traits in rhizobia. They are of importance not only for the saprophytic lifestyle in the soil but are also required for growth and spread in the rhizosphere. Additionally, they have been characterized to play a significant role in the process of transitioning from the free living stage to the symbiotic nodulation. These processes help in recognizing and responding to chemical signals esp. from their host plants. Flagellar motility has been found to be controlled by nutriotinal status of the cell and the effect varies in magnitude across strains (Wei and Bauer, 1998). Thus a difference in motility may account for difference in competitiveness in part. In a recent report in *B. japonicum*, nodulation competitiveness was found to be greater with in the strain with higher expression of flagellar proteins as opposed to the one with lower flagellar proteins (Liu *et al.*, 2015). Earlier, in *B. japonicum* motility was shown to be correlated with competitive nodulation when the rhizobia were inoculated via in-furrow method indicating that the positioning and motility of rhizobia are central to the nodule occupancy (López-García *et al.*, 2009). Motility in *Sinorhizobium meliloti* was also shown to be important for competitive nodulation (but not for N-fixation) recently (Calatrava-Morales *et al.*, 2017b). Miller *et al.*, 2007 demonstrated in *Rhizobium leguminosarum* the role of chemotactic motility and root colonization in determining the competitive nodule occupancy. Flagellar motility and root colonization were implicated in determining nodulation competitiveness in *Mesorhizobium loti*- nodulating *Lotus japonicus* as well as in *Burkholderia phymatum* nodulating various legumes as well (Zheng *et al.*, 2015; Lardi *et al.*, 2017). Thus, it is apparent that motility and chemotaxis are central to the symbiotic interaction among rhizobia across different genera.

1.5.2 Secretory and surface components

Rhizobia produce and secrete numerous metabolites, including diverse polysaccharides, of which several have been found to be crucial for symbiotic interaction with the host. They contribute not only to adhesion and surface colonization but also act as a physical barrier in defense (Sorroche *et al.*, 2018).

More importantly, they have been widely reported to be responsible for the recognition and signaling during various stages of nodulation starting from the initial root attachment stage to the progression of infection threads (Janczarek, 2011). These secretory polysaccharides can be classified as Exopolysaccharides, lipopolysaccharides, cyclic β-glucans and capsular polysaccharides. (Fraysse *et al.*, 2003) Several mutants of strains belonging to *Bradyrhizobium* as well as *Rhizobium* rendered incapable of producing exopolysaccharides have been characterized in detail and even though most of them show normal physiology and displayed no defect in nodulation *per se,* they were all affected in their competitive nodulation (Archana, 2010). Production of some classes of exopolysaccharide in members of *Ensifer* as well as *Rhizobium* has been reported to be regulated by quorum sensing which is conceivable given their symbiotic importance and since they are long polysaccharides and thus incur large metabolic cost (Calatrava-Morales *et al.*, 2018).

Lipopolysaccharides are one of the most crucial parts of Gram negative bacterial outer membrane. In rhizobia, however, they play crucial roles during several stages of infection including suppressing host defenses, facilitating bacterial entry in the root hairs, infection thread formation and progression, and finally, differentiation into bacteroids (Sorroche *et al.,* 2018). In addition to adhesion to root surfaces, LPS has been implicated in the formation of biofilms by various rhizobia including in *Bradyrhizobium* (Lee *et al.,* 2010) and *Rhizobium* (Vanderlinde *et al.,* 2009). Frederix *et al.,* 2014 later found that biofilm formation in *R. leguminosarum* is regulated by quorum sensing and directly correlated with the nodulation competitiveness. LPS mutants of *Ensifer meliloti* show a severe effect on nodulation depending on the host; while the mutants failed to completely nodulate *Medicago trancatula,* they formed many and small nodules on *M. sativa* but were severely compromised in their nodulation competitiveness as compared to the wild type (Niehaus *et al.,* 1998).

Cyclic β-glucans production has also been reported to be crucial to interaction with the legume host and nodulation in multiple rhizobia genera. In *B. japonicum* it was

found to be crucial for survival from the plant defense response during the nodulation (Mithöfer *et al.*, 2001) whereas in *E. meliloti*-alfalfa symbiosis it was shown to be crucial for the formation of infection threads (Jones *et al.*, 2007).

These reports collectively indicate that the cell-surface molecules are crucial mediators and determinants of effective symbiosis with the respective hosts in multiple rhizobial genera.

1.5.3 Survival and metabolic fitness

Flanking a nodulation event rhizobia may have to spend a long time living a saprophytic life in the bulk soil. Bulk soil is a poor source of nutrients and has a high selection pressure (Archana, 2010). Additionally, rhizobia also colonize the rhizosphere of the legume root which is rich and variable in the available nutrient sources significantly from that in the bulk soil. Studies have correlated the ability to utilize different carbon sources such as sugars and amino acids as well as the ability to synthesize storage compounds such as rhizopines with the nodulation competitiveness of rhizobial strains (Prell *et al.*, 2006). In a study by (Aneja *et al.*, 2005b), the mutant *Ensifer* strain incapable of synthesizing polyhydroxybutyrate (PHB) — a C-storage form, was reported to be compromised for the nodulation competitiveness whereas the mutant incapable of utilizing the PHB was only significantly affected in nodulation competitiveness when starved of carbon cyclically. Wielbo *et al.* (2007) found that the ability to catabolize diverse carbon sources was more consistent in rhizobial strains with relatively greater nodulation competitiveness. This correlation was particularly strong for utilization of organic and amino acids as compared to that of the sugars. Dicarboxylic acids are preferably utilized by rhizobia in the rhizosphere (Iyer *et al.*, 2016); these two facts combined indicate that the effect of catabolism of carbon sources on nodulation competitiveness is exerted strongly in the rhizosphere as compared to the bulk soil. Proline is also one of the exudates of the host root and is catabolized by rhizobia when colonizing roots (Toro, 1996; Webb *et al.*, 2014) and rhizobia with impaired ability to catabolize proline have been known to be affected in colonization of the root and competitive nodulation (Jimenez-Zurdo *et al.*, 1995; Liu *et al.*, 2017).

Similar dependence in *Bradyrhizobia* has also been tested in case of stachydrine and homoserine in the root exudates of soybean (Liu *et al.*, 2017). A recent study, with a battery of rhizobial biosensors, detected a diversity of carbon sources in the rhizosphere and nodules dynamically changing spatiotemporally and depended on the N-fixation status of the rhizobia (Pini *et al.*, 2017).

In addition to these, several other factors such as resistant to salinity, acid and drought stress (Roumiantseva and Muntyan, 2015), susceptibility to or production of rhizobiocins such as trifolitoxin (Archana, 2010) have also been implicated in the contribution to nodulation competitiveness.

These findings indicate that rhizobial survival and fitness in the soil as well in the rhizosphere strongly determine the likelihood of their nodulation.

1.6 Quorum sensing

Quorum-sensing (QS) is a mechanism of regulation of gene expression in bacteria as a response to their population density (Miller and Bassler, 2001). The process involves production, accumulation, release and uptake and sensing of one or more signaling molecules known as *autoinducers*. QS was first discovered in *Vibro fischerii* — symbiont of giant squid where it controls the production of light allowing the host to mask its shadow and protect itself from the predators (Nealson *et al.*, 1970). The genes regulating this are *luxI* and *luxR*, the former codes for the protein that synthesizes a soluble signaling molecules while the latter codes for the sensor/response regulator protein. QS has been characterized in several strains across many genera of Gram positive as well as Gram negative bacteria thereafter where they regulate diverse processes ranging from attachment, motility, biofilm formation, virulence to conjugal transfer of plasmids, nodulation and bioluminescence (West *et al.*, 2012).

An interesting alternative explanation of autoinducer mediated signaling in bacteria has been discussed by Redfield (2002) suggesting that process is intended as a means of diffusion sensing. Diffusion sensing may allow bacteria to assess the external milieu of the cells and may prevent loss of metabolically expensive public

goods such as complex exopolysaccharides or virulence factor proteins. On the other hand, the concept of 'efficiency sensing' considers both the QS and the diffusion sensing and presents an integrated view of the phenomenon and suggests that the autoinducer production and sensing may serve dual purpose of coordinated actions of a population and sensing of mass-transfer properties surrounding the bacterial colony (Hense *et al.*, 2007). Several bacteria including *V. cholarae, V. harveyi, Streptococcus pneumoniae, Bacillus cereus* and many more employ a different QS system mediated by AI-2 and regulate a wide range of phenotypes including motility, biofilm formation, attachment and production of virulence factors. This system is not discussed here but is reviewed well by Pereira *et al.*, (2013).

Below is a short description of QS in Gram positive and Gram negative bacteria followed by a review of QS in rhizobia and its significance.

1.6.1 Quorum sensing in Gram positive bacteria

Gram positive bacteria employ secretory oligopeptides as autoinducers and membrane bound histidine kinase receptors as sensors. The peptide signals are often cleaved from a precursor peptide and are subsequently conjugated to lactone ring or similar structures. These are secreted from the cells via dedicated transporter proteins and accumulate as the population rises. These autoinducers are sensed by one of the members of a two component system– the membrane bound cognate receptor that is specific for each peptide and the strain that produces these autoinducers. The sensor is often a histidine kinase and binding of the ligand triggers a series of downstream phosphorylation events in the cytosol leading to the phosphorylation and activation of the transcription factor(s) that govern the expression of genes under the QS regulation (Miller *et al.*, 2001).

One of the examples of this is *Agr* quorum sensing system of *Staphylococcus aureus*. The autoinducer peptide (AIP) is coded by *agrD* and the *AgrC-AgrA* two component signaling pair mediates the sensing and response regulation respectively. Another component of this system *AgrB* is responsible for the addition of thiolactone ring to the peptide. This process mediates the switch between

attachment/colonization and production of virulence factors— the two processes occurring at low and high cell densities respectively. Other examples of Gram positive bacteria performing QS include members of *Streptococcus*, *Bacillus*, *Enterococcus* etc (Sturme *et al.*, 2002).

1.6.2 Quorum sensing in Gram Negative bacteria

QS has been identified in several species of Gram negative bacteria and almost all of them are homologous to the QS of *Vibrio fischerii*- the organism in which the QS was first characterized. These typical QS circuits encompass homologues of two *V. fischeri* proteins — *LuxI* and *LuxR*. The LuxI-homologues code for synthases responsible for production of one or more specific n-acyl homoserine lactone molecule(s) (AHL) which act as the autoinducer. AHL concentrations increase with increasing population density. The *LuxR*-homologue proteins bind the cognate AHL at the critical threshold concentration of an AHL subsequently activating target gene transcription (Parsek and Greenberg, 2000). A general scheme of QS in Gram negative bacteria is shown in Figure 1.3.

LuxI family AHL synthases use S-adenosylmethionine (SAM) and an intermediate of fatty acid biosynthesis, acyl-acyl carrier protein to synthesize AHLs. Different *LuxI* homolog enzymes have different specificities for the fatty acyl chains and generate different acyl-HSLs that vary in the chain length of the acyl group, degree of saturation and the substitutions on them; general structure of AHLs is shown in Figure 1.4.

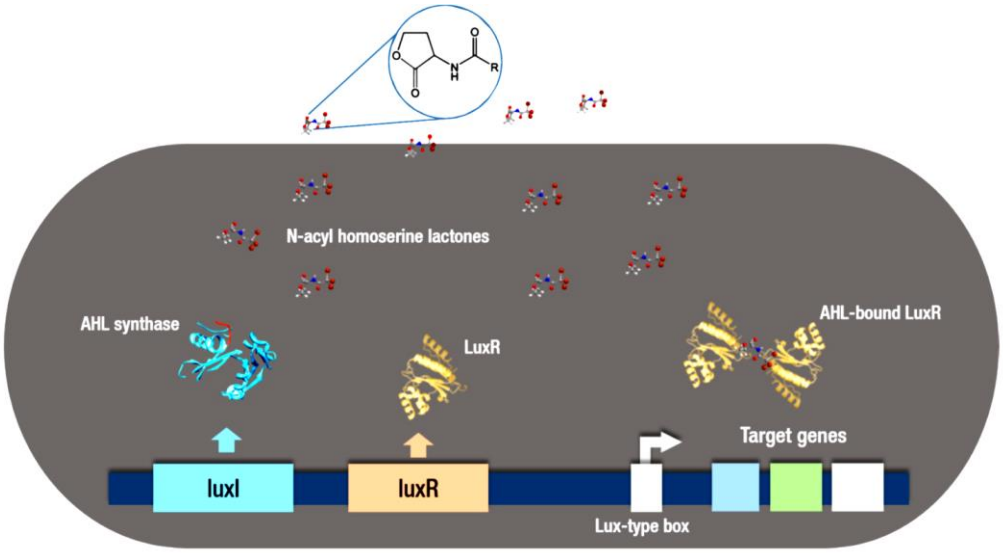

Figure 1.3 Typical quorum sensing circuit of Gram negative bacteria. LuxI homologues code for AHL synthase which produce AHLs. These AHLs are bound by lux-R family response regulator proteins which in turn modulate the expression of downstream effector genes including the lux operon itself and may also regulate operons of any other QS systems if present.

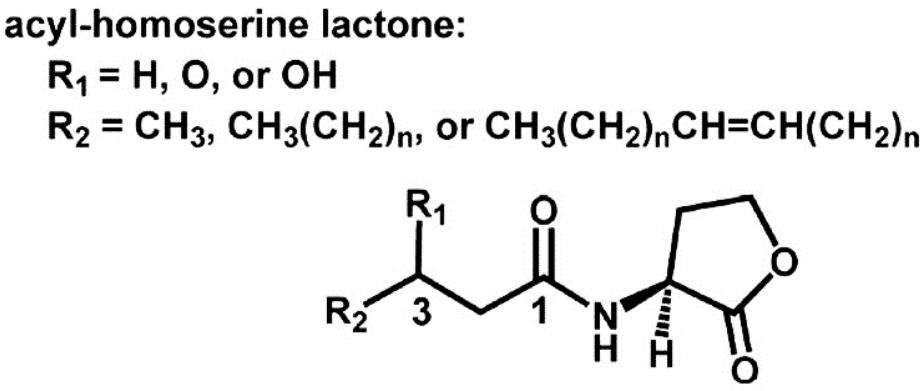

Figure 1.4 General structure of acyl homoserine lactones. The homoserine lactone ring is fused to the acyl chain. The variations in the structure arise due to the length, degree of unsaturation in the fatty acyl chain and the substitutions at the third carbon. The chain length (n) ranges from 4 to 18.

For e.g. *RhlI* of *Pseudomonas aeruginosa* primarily synthesizes N-butyryl-homoserine lactone (C4-HSL), and whereas *LasI* of the same organism catalyzes synthesis of N-(3-oxododecanoyl)-HSL (3-O-C12-HSL). Short-chain AHLs such as C4-HSL diffuse freely in and out of the cell membrane whereas 3-O-C12-HSL partitions in the cells, presumably in the cell membrane. The 3-O-C12-HSL molecules can diffuse into the surrounding but the export is enhanced by the activity of efflux systems such as *mexAB-oprM*, and perhaps more (Whitehead *et al.*, 2001). The population increase gradually increases the intracellular concentration of AHLs above the threshold where they can be sensed and exert the downstream effect. The specific cytosolic receptors for AHL autoinducers are members of *LuxR* family of transcriptional regulators. They have been proposed to contain two domains, the C-terminal DNA-binding, and the N-terminal AHL-binding domain (Papenfort and Bassler, 2016).

QS has been reported in more than 25 species of Gram negative bacteria, it is however commonly found in those that associate with plant or animal hosts. While the QS operation remains more or less similar to the *Vibrio* paradigm, there exists a great deal of diversity in the QS among the bacteria. A general scheme of QS in Rhizobia is described below.

1.6.3 Quorum sensing in Rhizobia

Rhizobia lead a saprophytic lifestyle in the soil and following a specific and complex molecular signaling with a legume host, colonize its root and exert nodule formation on it. Growth and survival in all the phases of life cycle as well the transitions depend on a lot biotic and environmental parameters and the success in any of these thus requires a coordinated execution of specific physiological activities. Many of these tasks are regulated by QS in rhizobia. Most rhizobia follow the quintessential *LuxRI* type AHL mediated QS with several conserved aspects of regulation as well as significant underlying differences. Table 1.1 details the QS systems in representative rhizobial strains. It is noteworthy that rhizobia have multiple QS circuits and solo regulatory genetic elements and they produce several

different AHLs. These QS can be seen to be regulating nodulation or phenotypes that affect the efficiency thereof.

Rhizobial strain	AHLs produced	Gene circuits	Phenotypes regulated
Rhizobium leguminosarum bv viceae strain 3841	3-OH-C14:1-HSL, C6-HSL, C7-HSL, 3-oxo-C8-HSL, C8-HSL	*cinI/cinR/cinS*, *raiI/raiR*, *rhiI/rhiR*, *trai/traR*, *bisR* *expR*,	Nodulation, Growth inhibition, EPS processing, Biofilm formation, Plasmid transfer
Ensifer meliloti strain Rm41	C12-HSL, C14-HSL, 3-oxo-C14-HSL, C16-HSL, 3-oxo-C16-HSL, C16:1-HSL, 3-oxo-C16:1-HSL, C18-HSL, 3-oxo-C8-HSL	*sinI/sinR*, *expR* *traI/traR*	EPS production, surface motility chemotactic motility, growth, nodulation, and Plasmid transfer
Mesorhizobium loti Strain NZP2213	3-oxo-C6-HSL, C8-HSL, C10-HSL C12-HSL	*mrlI2*, *mrlI1*	Nodulation
Bradyrhizobium japonicum multiple peanut nodulating strains	C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL	unknown	Biofilm formation, cell aggregation, Motility

Table 1.1 Quorum sensing systems in rhizobia.

1.6.3.1 Quorum sensing in Rhizobium leguminosarum

Rhizobium leguminosarum (Rlv) nodulates several hosts including peas, lentils, vetches etc. R. leguminosarum bv. viceae strain 3841 is the most characterized strain of the genes for its QS and serves as a reference in order to understand the mechanism and regulation of QS in Rlv. A schematic of QS systems is given in Figure 1.5. Four different *LuxI* family AHL synthases viz. *CinI, RaiI, RhiI* and *TraI* as well as their cognate *LuxR* homologues viz. *CinR, RhiR, RaiR,* and *TraR* respectively have been characterized in Rlv3841. Additionally, BisR and ExpR are solo LuxR type regulators are also part of its QS. It produces several long as well as short chain AHLs, some of which bear substitutions at the third carbon of the acyl chain (see Table 1.1 for the list).

CinI, *CinR* and *ExpR* of Rlv are orthologous to the SinI, SinR and ExpR, respectively, of the alfalfa nodulating *Ensifer meliloti* Rm41 (discussed in the next section). CinRI system is placed at the top of the hierarchy of QS regulatory cascade and controls the expression of the rest of the QS systems as well as acts as regulates many of the rhizobial physiological aspects itself. CinI is responsible for the synthesis of 3-OH-C14:1-HSL which is bound by *CinR*, which in turn regulates *cinI* expression. C6-HSL is produced by *RhiI* and binds to *RhiR* leading to induction of expression of *rhiI* and the *rhiABC* operon. *RaiI* synthesizes 3-OH-C8-HSL and *RaiR* in conjuction, regulates the expression of *raiI*. One of the unique aspects of QS operation in Rlv is that the regulation relies on presence of the antirepressor: *CinS*. *CinS*, in cell density dependent manner is co-transcribed with *cinI*. cinS is responsible for the activation of the *Rhi* and *Rai* systems. Another important regulator in the QS cascade is *PraR*— a transcriptional regulator which represses the expression of *raiR* and *rhiR*. cinS has been found to bind *PraR* and act as the anti-repressor independent of AHLs thus leading to the activation of *raiR* and *raiR* promoters and its expression. However the compete activation of Rai and Rhi requires repression of praR transcription mediated by *ExpR*-AHL.

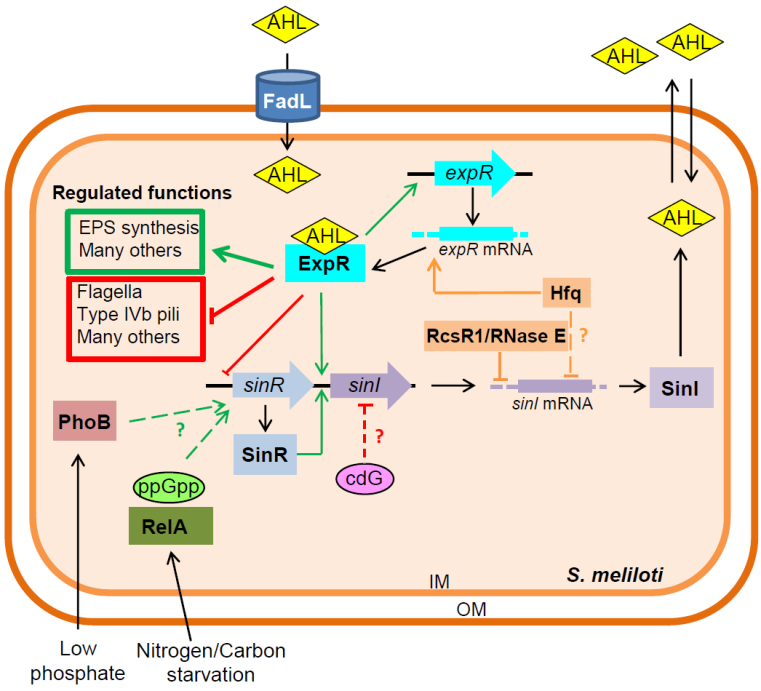

Figure 1.5 Scheme of quorum sensing in *R. leguminosarum* 3841. The strain produces multiple AHL autoinducers and hierarchically operating gene circuits, CinS is co-transcried with cinRI and inhibits the (repressing) activity of PraR which governs the other two QS and most of the symbiotically important phenotypes. [Source: Calatrava-Morales *et al.* (2018)]

1.6.3.2 Quorum sensing in Ensifer meliloti

Mutualism between *Ensifer meliloti* (previously known as *Sinorhizobium meliloti*) and its host *Medicago sativa* (alfalfa) is one of the models used to study plant-rhizobia interactions, nodulation and the regulatory events. Molecular events contributing to successful establishment of this mutualism include chemotactic motility, production of secretion of exopolysaccharide (EPS), biofilm formation, switching the carbon source for growth etc. Most of these processes are regulated as a matter of the cell density riven quorum sensing. QS genes of the strain *E. meliloti* 8530 encompasses sinRI circuit and the solo regulator expR. Em8530 and Em1021 are identical except for the insertional activation of the *expR* in the latter manifesting into disability to produce some glucomannons of EPS. Some strains

such as *E. meliloti* Rm41 also possess the traRI circuit. The strains produce a number of short and long chain AHLs listed in Table 1.1. Similar to *cinRI* of Rlv3841, *sinRI* of *E. meliloti* constitute the top-of-the-hierarchy QS. However, unlike with the former, the *SinR* does not carry out major downstream regulation. *ExpR* — the solo regulator binds to the long chain AHLs synthesized by *SinI* and regulates hundreds of promoters including the "autoinduction" of sinI gene expression (Gurich and González, 2009). *ExpR* also represses the transcription of *sinR* at high cell density leading to negative feedback regulation of *sinI*. The overall scheme of QS regulation is outlined in Figure 1.6. One of the interesting aspects of regulatory aspects of *E. meliloti* is the dependence of the QS-regulated promotor to AHL concentration. It was found that the promoters of genes promoting attachment (EPS production) are activated lower concentrations of AHLs than that for the inhibitions of the promoters that regulate flagellar motility. This feature allows the cells to temporal segregation of regulation of mutually opposite phenomena (Charoenpanich *et al.,* 2013). In addition to *sinR* and *expR*, several other *luxR*-type response regulator proteins have been reported in the genome of Em1021. While most them are only annotated as that and no functional information is available for them, VisN/VisR — two of these have been characterized to be regulating flagellar motility under the regulatory network of *sinI* made AHLs and *expR* activity (Sourjik *et al.,* 2000). Another LuxR type solo regulator identified in Em1021 has been named as *nesR* and is believed to regulate adaptation to environmental stresses and nodulation competitiveness (A. V. Patankar and Gonzalez, 2009). The long chain AHLs of *E. meliloti* are internalized by a transporter called *FadL* which is homologous to a fatty acid uptake transporter in *E. coli* (Krol and Becker, 2014). The AHLs are similarly taken up using fatty acyl transporters in other rhizobia including *Rhizobium* and *Mesorhizobium* as well owing to the production of long chain AHLs as a common trait (Calatrava-Morales *et al.,* 2018). In addition to the cell density dependent regulation, the QS also takes input of other signals such as nutritional status of the cell (McIntosh *et al.,* 2009). This regulatory scheme mediates several processes (Listed in Table1.1) important not only for the survival

and growth of *E. meliloti* in the free-living, associative and symbiotic life style but have also evolved to allow the effective transition among them.

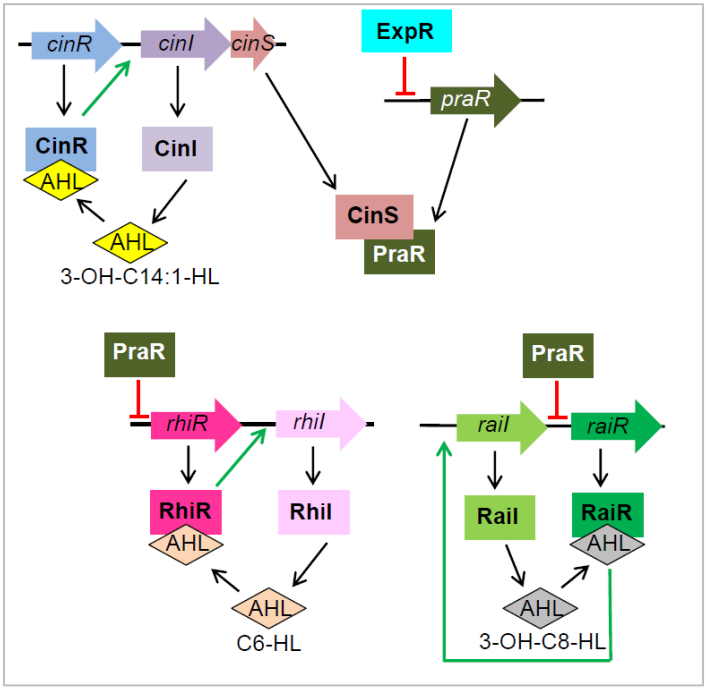

Figure 1.6 Quorum sensing regulatory network in *E. meliloti* 8530. *SinI* produces most of the AHLs. expR binds sinI-made AHLs and governs most of the symbiotically important processes. The long chain AHLs are taken up in the cells by the transporter FadL. PhoB is a member of phosphate sensing machinery of the cell and together with ppGpp as well as cyclic diguanylate monophosphate input nutritional status of the cell to the cell-density dependent QS circuit. [Source: Calatrava-Morales *et al.* (2018)]

1.6.4 Quorum sensing and Nodulation competitiveness

Rhizobia living in the natural conditions are often encountered with varying environments ranging from nutrition and water deficient soils to rich and attractive rhizosphere and thus survival and adaptiveness confers the competitive advantage over the "better" strains. Most of the cellular processes required for such adaption or switching require to be synced to the population or require sharing of public goods. In this way, the cell-density dependent regulation of gene expression – quorum sensing, has stood out to an effective regulatory mechanisms using the basic components i.e signal producer, the signal, and the response regulator

achieving governance of the extensive number of biological processes under diverse environmental conditions (Patankar and González, 2009). This scope of regulation is further broadened by employment of multiple hierarchically-operating QS gene circuits, presence of solo receptors and multiple AHL signaling molecules.

Several studies across different genera have outlined the involvement of quorum sensing mediated processes in determination of nodulation competitiveness. Quorum sensing in *R. leguminosarum* is operated under the master regulation of cinRI and is one of the downstream regulators of its effects is *PraR* (see Section 1.6.3.1). Recently it was shown to act as a repressor of biofilm formation and exopolysaccharide production; further, it's mutants exhibited greater attachment to host roots and improved nodulation competitiveness (Frederix *et al.*, 2014). Cell aggregation in *Bradyrhizobium japonicum* regulates initial stages of biofilm formation and alters the nodulation competitiveness in *B. japonicum*, and was identified to be under the regulation of QS (Nievas *et al.*, 2012). In a transcriptomic study to investigate QS-regulation in *Ensifer fredii* NGR234, Krysciak *et al.* (2014) identified motility, attachment and sugar catabolism related genes –known to determine nodulation competitiveness, to be regulated by QS. *E. meliloti* has the similar QS regulation and its involvement in determination of nodulation competitiveness has been established via several studies (Marketon *et al.*, 2003; Patankar and Gonzalez, 2009; Sugawara and Sadowsky, 2014; Calatrava-Morales *et al.*, 2017; Gosai *et al.*, 2020;). *ExpR*, the QS regulatory protein that mediates most of the symbiotically important phenotypes and affects QS in *E. meliloti* was found to be rapidly inactivated in several lab strains but not in field isolates indicating the advantage QS confers in the wild conditions including the ability to colonize the roots effectively. Chemotaxis — also regulated by QS in most rhizobia, governs the attraction to and colonization on the host root in many genera of rhizobia and the mutants have been shown to have impairments in competitive nodulation (Scharf *et al.*, 2016). Recently AHL mediated communication between two different rhizobial species was found to affect the colonization and nodulation capability of one of the strains (Miao *et al.*, 2018). Thus, AHL mediated QS in

almost all studied rhizobial species regulates phenotypes that are crucial to the life cycle of rhizobia and determines their fitness and competitive nodulation.

1.7 Pigeon pea

Pigeon pea (*Cajanus cajan*), a perennial legume of the Family *Fabacae*, is cultivated in more than 20 countries and on close to 7 million hectares of land (Saxena *et al.*, 2010; Chanda Venkata *et al.*, 2018). It has been is traditionally cultivated as an annual legume crop in semi-arid regions of the world and is the fifth largest consumed food legume worldwide as well the sixth most important crop of the world (Graham and Vance, 2003; Varshney *et al.*, 2012). The dry seeds of pigeon pea contain close to 25% protein and thus highly nutritious for the human consumption (Saxena *et al.*, 2010); it has also been referred to as the poor man's meat (Gates, 2014). Additionally the aerial parts have also been used as a protein source in fodder. It is a part of staple diet in Indian subcontinent holds promising outcomes for the agriculture and nutrition in the countries of global south (Varshney *et al.*, 2012). Owing to the biotic as well as abiotic stresses, and the fact that it is cultivated in semi-arid, and risk-prone environments, there exists a huge gap between the possible yield (2,500 Kg/ha) and obtained yields by farmers (736-866 kg/ha) (Varshney *et al.*, 2012). Despite its significance, pigeon pea has remained one of the neglected crops and is yet to benefit from the advanced research. Pigeon pea genome was only recently sequenced and is hoped to bring the biotechnological inputs to the conventional breeding based improvement programs (Varshney *et al.*, 2012). Pigeon nodulated by multiple genera of rhizobia, notably, *Bradyrhizobium, Rhizobium* and *Ensifer* (Fossou *et al.*, 2016). Herridge *et al.* (2008) reported that pigeon pea obtains almost 65% of the total nitrogen from symbiotic nitrogen fixation with rhizobia making them a crucial subject of investigation. However, most studies involving rhizobia of pigeon pea have not investigated the molecular details underlying their mutualistic association. Thus, development of effective bioinoculants for pigeon pea will be immensely valuable which can only be achieved by expanding our knowledge on the principles guiding interaction between the pigeon pea and rhizobial nodulating it.

Rationale and scope of this work

Biological fixation of nitrogen from atmosphere – its largest reservoir, in the bioavailable forms is a process restricted to only to prokaryotes. Rhizobia, carry this process out in a mutualistic association with legumes and thus not only contribute to the bioecological homeostasis but also bridge the agricultural economics and human/animal nutrition across the world. Rhizobial strains capable of fixing abundant amounts of nitrogen are used for inoculation of soils used for cultivating legumes in which no or ineffective native rhizobia are present. However, this application has been challenged by the failure of the inoculated strains to nodulate. This has been attributed to several reasons such as lack of the bioinoculants' ability to survive and function in the soil environment due to its non-nativeness while the well-adapted autochthonous strains– adept for root colonization and nodulation–have a competitive advantage over the introduced strains. The native bacteria are also often ineffective in fixing nitrogen. This problem, termed as nodulation competitiveness, is an important area of rhizobial research. Several studies have however revealed that this superiority of native strains or failure of the introduced strain is a manifestation of numerous metabolic or physiological differences between them. A clear understanding of this aspect holds a great promise for their use as effective biofertilizers. Several processes of rhizobia known to affect the colonization and nodulation competitiveness are regulated by Quorum sensing (QS)—the cell density-coordinated regulation of gene expression. However there are vast differences in the underlying mechanism of QS as well as the phenotypes regulated by QS among different rhizobia. Thus in line with these facts, this study was aimed at investigating nodulation efficacy by multiple approaches. Rhizobia nodulating pigeon pea-one of the most important of legumes of the global south and especially India have not been characterized for their QS systems and for understanding their role in nodulation. Part of the study addressed that. This study further aimed at engineering the QS in well-studied rhizobia in order to explore its effect on the nodulation

competitiveness. And finally, the work explores how rhizobia colonize the host roots and its implication on the nodulation competitiveness.

In order to achieve the above, the work has been divided into the following objectives.

1. *To characterize quorum sensing (QS) in rhizobia associated with pigeon pea nodules.*
2. *Engineer QS system of rhizobia and assess its effect on nodulation competitiveness.*
3. *To study root colonization dynamics of selected rhizobial strains as bioinoculants on pigeon pea plants*