3 Engineering quorum sensing systems of rhizobia to assess its effect on their nodulation competitiveness.

3.1 Introduction

Quorum sensing (QS), the mechanism of regulation of gene expression in bacteria in response to their cell-density is found in several gram positive and gram negative species and often plays important role in regulating interaction with a higher host (Boyer and Wisniewski-Dyé, 2009). Section 1.6 gives a detailed account of the general idea of QS in Gram negative bacteria. This chapter deals with engineering quorum sensing in two rhizobial species– *Rhizobium leguminosarum* strain 3841 (Rlv3841) and *Ensifer meliloti* strain 8530 (Em8530). Both rhizobial strains are well-studied laboratory strains and have their quorum sensing elucidated in detail (Calatrava-Morales *et al.*, 2018). Rlv3841 is known to nodulate peas, lentils and vetches (Rodelas *et al.*, 1999), whereas Em8530 nodulates alfalfa.

Rlv3841 possesses multiple QS circuits including the *cinRI* which is at the top of the regulatory hierarchy, as well as *rhiRI* and *raiRI* (González and Marketon, 2003). In addition to these complete circuits, the QS operation in Rlv also involves functions of cinS – a 67-amino acid protein, as well as ExpR and BisR – solo luxR family transcriptional regulators (Frederix *et al.*, 2014). Quorum sensing in *E. meliloti* 8530 consists of *sinRI* and *traRI* circuits and *expR* solo regulator (Gurich and González, 2009). The *cinI* and *sinI* are the top-of-the-hierarchy AHL synthases in each strain. They both produce long chain AHLs which are bound by *cinR* and *sinR* respectively which leads to feed forward activation loop of each of the operon (Calatrava-Morales *et al.*, 2018). Both the systems regulate phenotypes that can determine the outcome and efficiency of symbiotic associations with the respective hosts such as attachment, biofilm formation, secretion of exopolysaccharides etc. (Sourjik *et al.*, 2000; Sanchez-Contreras *et al.*, 2007; Gurich and González, 2009; Frederix *et al.*, 2011). This feature of QS-mediated regulation of phenotypes that

determine the efficacy and success of rhizobium-host symbiosis is shared among most if not all rhizobial strains characterized for QS. This property makes QS-regulation an interesting target to engineer in order to improve the symbiotic efficacy of rhizobial strains.

Even though the strains share many common features in their QS systems, they vary in certain details. The *cinI* is only known to produce one long chain AHL (3-OH-C14:1-HSL), *sinI*, on the other hand, is known to produce at least 7-8 different long chain AHLs (details given in Section 1.6.3). Although the *expR* genes are present in both the strains and appear to be orthologues, they have markedly different operation in the two strains (Frederix *et al.*, 2014). In Em8530 ExpR depends on the sinI-made AHLs and binds to hundreds of promoters itself (Zatakia *et al.*, 2014), but ExpR in Rlv3841 is believed to not require CinI-made AHLs for its activity, and is known to regulate a relatively smaller number of targets in conjunction with CinS (Frederix *et al.*, 2014).

This chapter deals with homologous cloning and overexpression of the master-quorum sensing regulatory circuits i.e. *cinRI* of *Rhizobium leguminosarum* strain 3841 (Rlv3841), and of *sinRI* in *Ensifer meliloti* strain 8530 (Em8530). Using various *in vitro* assays, scanning electron microscopy, and analysis of gene expression with q-RT PCR, the two QS overexpression strains were tested for the modulations in symbiotically important phenotypes and were found to differently respond to the engineering in terms of attachment.

3.2 Materials and Methods

3.2.1 Bacterial strains and culture conditions

Bacterial strains, plasmids and PCR primers used in this Chapter are listed in Table 3.1. *Rhizobium leguminosarum* bv viceae strain 3841 (referred to as Rlv or Rlv3841 hereafter) and *Ensifer meliloti* strain 8530 (referred to as Em8530 hereafter) were routinely cultivated in TY broth containing Tryptone (0.6 g/l), Yeast extract (0.3 g/l) and calcium chloride (3 mM) added with 100 μg/ml streptomycin in both cases

unless mentioned otherwise. *Escherichia coli* strains were grown in Luria Bertani broth. Any of the *E. coli* or rhizobial strains bearing pBBR1MCS2 or pBBR1MCS5 were grown in respective media containing Kanamycin (30 μg/ml) or Gentamycin (40 μg/ml) respectively.

Name	Relevant characteristics	Source
Bacterial strains		
Rhizobium leguminosarum bv viceae strain 3841 (Rlv3841)	Nodulates peas, vetches, lentils; standard laboratory strain with genome sequenced and QS systems characterized	(Johnston and Behringer, 1975)
Ensifer meliloti 8530 (Em8530)	Nodulates alfalfa, standard laboratory strain with genome sequenced and QS systems characterized; $expR^+$ version of Em1021 (In Em1021, the expR ORF is interrupted by insertion)	Gift from Prof. Juan Gonzalez, UT-Dallas; Described in Pellock *et al.* (2002)
Rlv3841-pCINRI	Rlv3841 containing cloned copy of *cinR*I regulon in pBBR1MCS2	This study
Em8530-pSINRI	Em8530 containing a cloned copy of *sinR*I regulon in pBBR1MCS5	
Rlv3841-pBBR1MCS2 (Rlv3841 VC)	Rlv3841 containing the empty vector pBBR1MCS2	This study
Em8530-pBBR1MCS5 (Em8530 VC)	Em8530 containing the empty vector pBBR1MCS5	
E. coli DH5α	Standard cloning strain	(Sambrook and Russel, 2001)
E.coli ET12567	dam^- ; dcm^- strain for cloning of DNA with methylation sensitive restriction site	Kind gift from Dr. J. Pohnerkar, Dept. of Biochemistry,

		MSU Baroda; Described in (MacNeil *et al.*, 1992); ATCC® BAA-525™
Plasmids		
pBBR1MCS2	Broad host range Gram negative bacterial cloning and expression vector; Kan^R	(Kovach *et al.*, 1995)
pBBR1MCS5	Broad host range Gram negative bacterial cloning and expression vector; Gen^R	Kind gift from prof. Maravić-Vlahoviček; (Kovach *et al.*, 1995)
pJET1.2	Cloning vector blunted with EcoRV; Amp^R; eco471 lethal gene	Thermo Scientific (USA)
pJET-cinRI	cinRI from Rlv3841 cloned in pJET1.2	This study
pJET-sinRI	sinRI from Em8530 cloned in pJET1.2	
pCINRI	CinRI locus of Rlv3841 cloned in pBBR1MCS2	
pSINRI	sinRI locus of Em8530 cloned in pBBR1MSC5	
Primers (*5→3' ; All designed in this study*)		
Primers used for cloning of QS circuits		
cinRI Fwd	*agg*GGATCCTGGAGG*gcaa*ATGATTGAGAACACCTACAGCG ♦	
cinRI Rev	*gtc*CTGCAGTCATGCTGCCATCTCCAG	
sinRI Fwd	*agt*GGATCCGGAGGC*acggc*ATGGCTAATCAACAGGCTGTCCTC ♦	
sinRI Rev	*act*TCTA**GATC**AGGCGGCGCGTGCCGTTTCAAGCG	
Primers used for qRT-PCR		
flaA Fwd	AGAACCTGAACAACACGCAGAA	
flaA rev	GGCGGTGTAAGCAGTGTCG	
cheY2 Fwd	TCGCTCGCGGAGAAAATCAA	
cheY2 Rev	TCACCAGCCGATGTGATCTG	
mcpD Fwd	AGGCTTATTCGCTCTACCGC	
mcpD Rev	CTAAGGCTCTTCGCCAGGTC	

PraR Fwd	CGACACCGGAACATTGAGGA
PraR Rev	TCCATGTTGGCAGCCGTATT
gmsA Fwd	CGGCGAACCACGGGATATAG
gmsA Rev	TGCCGACACCTACACCATTC
motA Fwd	CTTGCCGGAATCCTTCACCA
motA Rev	TAGTGACTTGCGGCTGTGTC

Table 3.1 Bacterial stains and plasmids used in this study.

♦ Italicised and lower case letters in the primer sequences indicate nucleotides added to facilitate cleavage close to end (5') or to act as a spacer (between the RBS and start site). Start site is indicated in larger point size. Regions with continuous underline indicate restriction site whereas regions with dashed underline indicate RBS. The boldface nucleotide in sinRI Rev indicate the methylation sensitive site. The start codons are shown in bigger typefaces.

3.2.2 Cloning of *cinRI* and *sinRI* regulons from Rlv3841 and Em8530 respectively

3.2.2.1 PCR amplification of cinRI regulon from Rlv3841 and sinRI from Em8530

CinRI locus of Rlv3841 (Genbank accession no. AM236080.1) consists of *cinR* and *cinI* separated by 158 nucleotides. The primers were designed to amplify the entire sequence including the intervening nucleotides giving rise to an amplicon of 1.55 kb. cinRI was amplified from the isolated genomic DNA of Rlv3841 and *sinRI*, the amplification was done from the genomic DNA from Em8530. The primers were designed using Em1021 genome (Accession no. NC_003047.1) sequence as the reference, the strain differing from Em8530 only with respect to an insertion in *expR* ORF (Pellock *et al.*, 2002). The primers targeted the beginning of the *sinR* gene and amplified up to the end of *sinI* gene along with the 156 nucleotides in between, thus giving rise to an amplicon of 1.53 kb. The amplified products of each of the regulons were confirmed for the size by running them on agarose gel and for accuracy by DNA sequencing. The genomic DNA of respective strains was isolated using CTAB method (Wilson, 2001). PCR amplification was performed as follows.

Each 50 µL PCR reaction performed in a thermocycler system contained 200 µM of dNTP mix, 1.0 µM of each primer, 1.5 mM $MgCl_2$, PCR buffer, 1.5 U of *Pfu* DNA polymerase and 20 ng of genomic DNA template. Negative controls consisted of an equal volume of nuclease-free water in place of the DNA template. PCR conditions used were as follows: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 52°C (for CinRI) or at 80°C (for sinRI) for 100s and extension at 72°C for 1 min with a final extension of 72°C for 10 min. The primers used for cloning are enlisted in Table 3.1 with annotations. In addition to the complementary sequence to the ORF starting with 'ATG', the primers contained additional sequences including spacers and restriction sites and the Ribosome binding site. RBS in case of Rlv3841 was used as suggested by Ma *et al.,* (2002) and for Em8530, as suggested by Schroeder *et al.,* (2005). The gel-purified amplicons were used for cloning in pJET1.2 for intermediate cloning.

3.2.2.2 Blunt end cloning in pJET1.2

Since the amplification was performed with *pfu* polymerase, the resultant fragments could be used directly for the blunt end ligation and cloning. This was achieved using the cloneJET cloning kit (Thermo,USA). The vector map of pJET1.2 is given in Figure 3.1. The ligation procedure was as per the kit-manufacturer's instruction as given below:

Component	Volume
2X Reaction Buffer	10 µl
Gel-purified PCR amplicon	Eq. to 0.15 pmol ends
pJET1.2/blunt Cloning Vector (50 ng/µl)	1 µl (0.05 pmol ends)
Water, nuclease-free	up to 19 µl
T4 DNA Ligase	1 µl
Total volume	*20 µl*

The ligation system was set up in in ice and incubated at 20°C for 5 min for the ligation to complete followed by transformation of ligation mixture in *E. coli* DH5α.

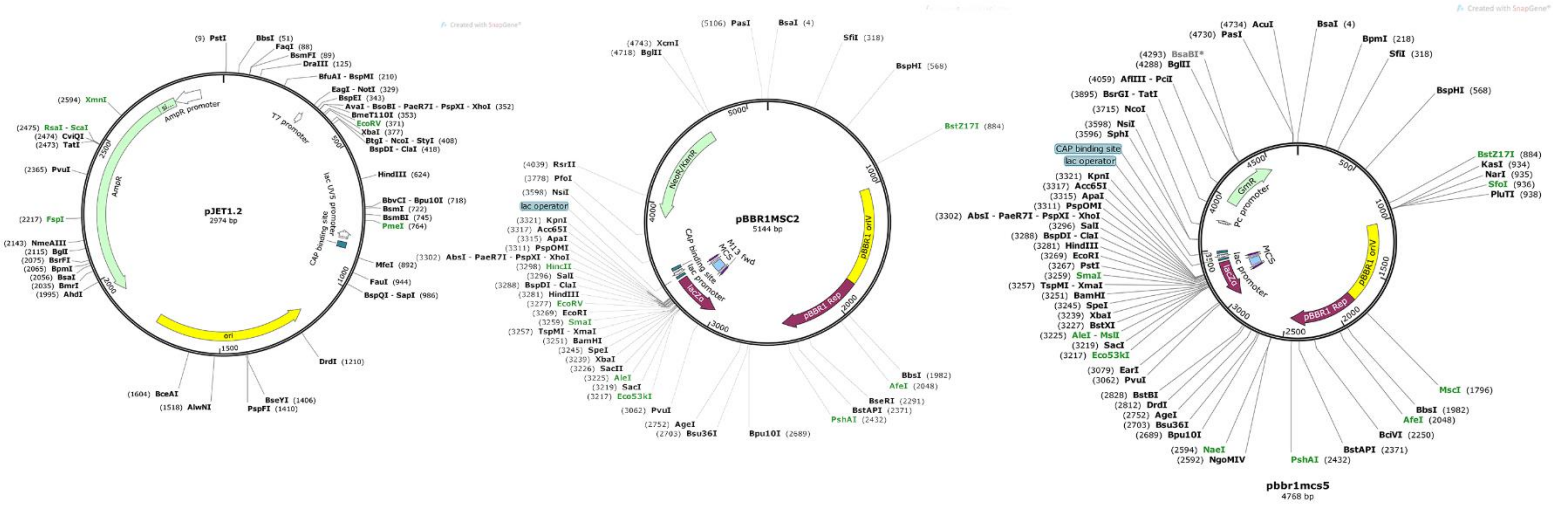

Figure 3.1 The plasmid vecors used in the study. pJET1.2 (Amp^R)is a blunt end cloning vector, pBBR1MSCS2 (Kan^R) and pBBR1MSCS5 (Gen^R) are broad host range expression vectors for Gram negative bacteria.

3.2.2.3 Transformation of pJET-cinRI/pJET-sinRI in E.coli, confirmation of clones and sub-cloning in pBBR1MCS2 and pBBR1MSC5 respectively:

Plasmid transformation of pJET-cinRI was done in *E. coli* DH5α was whereas pJET-sinRI was transformed in *E. coli ET12567* (*dam*$^-$; in order to prevent the context-generated methylation site that was rendering XbaI site resistant to cut) and was performed using the modified version of the $CaCl_2$ induced chemical transformation of DNA as per Sambrook and Russell (2001). Inoculated from an overnight culture, *E. coli* was grown to an OD_{600} of 0.4-0.6 at 37ºC in LB broth. At this point, the cells were chilled for about 10 minutes in ice and centrifuged at 3000 rpm for 10 minutes at 4ºC. the pellet washed with 0.05M CaCl2 with half of the original culture volume followed by a centrifugation as earlier. The pellet was gently resuspneded in half the original culture volume of chilled 0.1M $CaCl_2$ and incubated at 4ºC for 45 min to 1h. The pellet was then slow-centrifuged as earlier and resuspended in chilled 0.1M $CaCl_2$. This resuspension solution contained 20% glycerol in addition to the $CaCl_2$ if the competent cells were to be stored at -80 ºC for a future use.

Transformation was done according to the standard heat shock method (Sabrook and Russel, 2001). The transformed colonies obtained after overnight incubation of the ampicillin containing plates at 37°C were then subjected to plasmid DNA isolation. Clones were confirmed by restriction digestion analysis. The RE sites in the primers for cinRI (BamHI: fwd and PstI: rev) were designed for cloning in another vector and thus were not in the correct orientation for cloning in pBBR1MCS2, and thus, flanking sites XhoI and HindIII of pJETcinRI clones, were used to sub-clone the fragment further in pBBR1MCS2. The clone with pJET-cinRI in the appropriate orientation (XhoI—BamHI—PstI—HindIII) (as shown in Figure 3.3) was required for this and was screened for by single digestion the clone with PstI (1.9kb fragment release from the vector indicates the correct orientation and ~370kb release from the vector indicates the opposite and undesired orientation).

3.2.3 Electroporation of plasmid constructs in rhizobium hosts

The resultant constructs pCINRI and pSINRI were transferred to Rlv3841 and Em8530 respectively using electroporation. The electroporation of rhizobia with these plasmids was performed as described by Garg *et al.*, (1999) with following details. Five hundred milliliters of TY broth was inoculated with desired rhizobial culture up to the OD_{600} of 0.8. The cells were then given successive washes with 10% glycerol as described in the cited reference and the finally resuspended in 500 µl of 10% glycerol. These "electrocompetent" cells were used immediately or preserved at -80ºC for up to 3 months. The electroporation was performed with Gene Pulser Xcell electroporation system (BIO-RAD) using 2 mm gap cuvettes. Forty microliters aliquote of electrocompetent cells mixed with 1 µg of the prepared plasmid were incubated in ice for 15 min. This was added to the chilled cuvettes which were then maintained in ice until pulsed. A pulse of 2000 V, 25 µF and at 200 Ω set resistance was given to the cells followed by immediate recovery by the addition of 1ml TY broth at room temperature (outgrowth). This resuspension in TY broth was incubated in slow shaking at 30ºC for 3 h after which the cells were plated on TY agar plate containing respective antibiotic. The resultant clones were confirmed by plasmid isolation and restriction digestion similar to the above section.

3.2.4 Measurement of production kinetics of n-Acyl homoserine lactones

Exogenous expression of quorum sensing would result in both, greater and earlier production of n-acyl homoserine lactones (AHLs) as compared to the wildtype. In order to test this, Rlv3841(pCINRI) and was tested for its AHL production kinetics as follows. It was inoculated in a 5ml TY broth tube from a single colony. Following about 20h of incubation, 50µl of this suspension was washed with 0.85% saline and used to inoculate fresh TY broth in a 250 ml Erlenmeyer flask. The vector control was grown the same way. One milliliter of culture was withdrawn at regular intervals from the flasks, their optical density at 600nm was measured, and 10µl the culture supernatant from it was plated on the *A. tumefaciens* AHL bioassay plate

(prepared as described in section 2.2.7). Following a 12h incubation of the bioassay, the diameter of the zones were noted. The difference in the zone diameter gave indication of the extent of production of AHLs at a given OD_{600}.

3.2.5 Root attachment assay

In order to assess the difference caused (if any) due to the QS over- expression on the tendency of the strain to attach to the specific host root, Rlv3841(pCINRI) and Em8530(pSINRI) as were compared with the empty vector control strains for their colonization. Seeds germination and surface sterilization was performed as detailed in the Section 2.2.3. The germinated seeds were incubated for 4h with a suspension of ~10^8 cfu/ml either Rlv3841(pCINRI) or Em8530(pSINRI), or their respective vector controls as the control set for the short term experiment, at the end of which the coated seedlings were assessed for the bacterial counts per. A long term colonization experiment was set as a hydroponics setup as wherein the seedlings coated with respective rhizobia were transferred to the jars containing Hoagland's Nitrogen free minimal medium (see section 4.2.9 for composition) and incubated for 7d in 12h dark-light conditions at 30 ºC and the bacterial counts were recorded after 7d. At the end of the respective colonization period, 1 cm portions of the root from proximal area of the roots were excised and immersed in 1 ml PBS (0.0666 M $Na_2HPO_4•7H_2O$ + 0.0334 M $NaH_2PO_4•H_2O$) and vortexed for 10s to remove the uncolonised and loosely attached cells. The root piece was carefully transferred in the fresh vial containing 200 µl PBS and subjected to a 10s pulse in a sonic bath that dislodged the colonized cells and brought them in the suspension which was then serially diluted and plated on respective antibiotic containing TY agar plate. A similarly prepared and treated set immediately after adding the bacterial culture to the roots but without any incubation was also used for counting the immediate and nonspecifically bound cells. The 1 cm pieces were completely dried in the heating oven at 50ºC, followed by which they were weighed on a sensitive weighing scale. The dry weight was used to normalize the counts across samples.

3.2.6 Biofilm formation assay

The ability of the rhizobia to attach and form biofilm on the solid surfaces was quantified as suggested by Fujishige *et al.*, (2006). Respective rhizobial culture was allowed to grow to OD_{600} of 1.0 in Rhizobium Defined Medium (RDM; composition at the end of this text) containing 2% sucrose. They were then washed and resuspended in the same medium to 0.2 OD_{600} and added at a final volume of 200 µl per well to a 96-well polystyrene plate. Triplicates or quadruplets were taken for each sample. Some wells were added with uninoculated RDM—this served as a blank at the end of the experiment. The plates were closed with their lid and sealed with parafilm, and incubated at 28ºC in static conditions. At the end 48h, the plate was briefly shaken in a Microtiter Plate reader (Tecan InfinitePro 2000) and OD_{595} was read to assess the planktonic growth. The bacterial suspension in the wells was then removed, the wells washed and air-dried, and the biofilms were stained with 0.01% crystal violet for 20 min. Excess dye was washed away with three changes of sterile water. The dye that stained the biofilm was then solubilized with 95% ethanol, and the amount of dye was quantified by measuring the absorbance at 570 nm. The values of OD_{570} indicate the magnitude of formation of biofilm.

3.2.7 Exopolysaccharide production assay

The rhizobial strains were inoculated and grown in Tryptone yeast extract broth as well as Yeast extract mannitol broth (in g/L; mannitol 10.0, yeast extract 1.0, $MgSO_4.7H_2O$ 0.2, NaCl 0.1, K_2HPO_4 0.5) upto OD_{600} of each of the culture reached 1.5. The cultures were then harvested at 10,000rpm for 15 min on centrifuge. The cell mass was dried overnight followed by measurement of dry biomass weight. The supernatant was added with double the volume of isopropanol and precipitation of EPS was allowed for 45 min at room temperature. The precipitated EPS was then harvested by centrifugation at 12,000rpm for 15 min. The tube was sealed with porous paper and the pellet was allowed to dry in hot air oven overnight. The dried EPS was suspended in 2ml of distilled water and the quantification was carried out by estimation of reducing sugars by dinitrosalicylic acid (DNSA) assay. One milliliter of DNSA reagent was added to 1ml of EPS suspension and was heated at

100ºC for 15 minutes. The solution was cooled and 8ml of distilled water was added to make a 10ml system. The absorbance of the system was measured at OD_{540}. The OD values were used in the linear regression equation obtained by standard curve of DNSA prepared using range of glucose concentrations. Final concentration of EPS was expressed as ratio of reducing sugars per µg of dry biomass.

3.2.8 Scanning electron microscopy

The 7d old, Rlv3841 and vector control treated roots were subjected to scanning electron microscopy (SEM) to image their attachment pattern and their surface properties. The sample preparation for SEM analysis was as follows. After the requisite incubation, a root fragment was excised from the from the relatively closer to the crown region of the root and immersed in and washed with 50 mM phosphate buffer saline pH 7.2 (PBS) prior to incubation in 2.5% v/v glutaraldehyde (diluted with PBS) for 30 min. The fragment was then washed once with PBS followed by a successive passage in 10%, 25%, 50%, 75% v/v ethanol in order to slowly dehydrate the tissue. Finally, it was immersed in the absolute ethanol and stored at -20 ºC until imaging. Just prior to the imaging, the tissue was briefly air- and infrared- dried followed by mounting on the metal stub using adhesive carbon tape and sputter-coated in vacuum with platinum using the coating device (JEOL-JFC-1600). The samples were examined at 10 kV under the scanning electron microscope (JEOL, JSM-7600F-FEG-SEM). Coating and microscopy was performed at Sophisticated Analytical Instrument Facility, IIT-Bombay, India.

3.2.9 Real-time quantitative reverse transcription PCR

The qRT-PCR analysis was performed for Rlv3841(pCINRI) for the genes *flaA, cheY2, mcpD, praR, gmsA* and *motA* with Rlv3841 VC as the control. and the primers for all these are mentioned in Table 3.1 The cultivation of both the strains, isolation of total RNA, synthesis of first strand cDNA and qRT-PCR were performed as mention in section 2.2.15.

3.2.10 Determination of host-growth parameters

The seeds of garden pea (variety GDF-1, procured from Pulse research station, Sardarkrushinagar Dantiwada Agricultural University, Banaskantha, Gujarat, India) and alfalfa (variety AL-3, procured from Forage Research Station, Anand Agricultural University, Anand, India) were surface sterilized and germinated. Germinated seeds of garden pea were coated with the slurry of the culture of Rlv3841(pCINRI) or WT Rlv3841 (control), whereas the germinated seeds of alfalfa were coated with Em8530(pSINRI) or WT Em8530. The cell density of the coat culture was adjusted to 10^8 cfu/ml before coating. The coated seeds were sown in pots containing 3kg sterilized soil; 3-4 seeds were sown per pot. The seed sterilization, germination, coating and seed sowing was followed as described in section 2.2.3. The pots were maintained at constant 12h dark-light and green house conditions. At the end of 6 weeks, the plants were uprooted and the dry and wet weight, lengths of the root and shoot were measured.

3.2.11 Determination of nodulation competitiveness

The seeds of pea and alfalfa were surface sterilized and germinated as described earlier. The germinated seeds were coated with the mixtures of Rlv3841(pCINRI)–RLv-WT or with those of Em8530(pSINRI)–Em8530 WT at the following ratios. 90:10, 70:30, 50:50, 30:70. 10:90 are the ratios that were taken; the numbers denote the percent of each of the strain in the mixture, the number on the left of colons, as a norm, is the representation of WT, while the one at the right denotes percentage of the transformant. In order to achieve this, the respective strains were grown to an OD_{600} of 0.8 (the cfu/ml of each WT-transformant pair at are identical at same OD_{600}). These cultures were then centrifuged at 5000 rpm for 5 min and the pellets were once washed with 0.85% saline. The resultant suspensions were then mixed at appropriate volumes to create a 20ml slurry of mixtures that contained each of the strain at desired ratio. The total cfu at this suspension strength was ~10^8 cfu/ml. The seeds are suspended in the slurry for 4h with intermittent swirls and sown at the end, as described earlier. The plants were maintained under same conditions

and watered with equal amount of water and were grown for six weeks. Plants were carefully uprooted then and the total number of nodules on each of the plants were counted. Each of the nodule was then placed in an individual 0.5ml microcentrifuge tube containing 50µl of sterile 0.85% saline and crushed with pair of forceps. Ten microliters each of the resultant suspension was plated on TY + streptomycin (TYS) as well as TY + streptomycin + kanamycin (TYSK) plate in case of pea nodules and on TY + streptomycin as well as TY + streptomycin + gentamycin (TYSG) plate. A nodule that gave rise to colony on only TYS plate was considered as a colony made by the WT strain whereas the one that grew colonies on both was considered as a nodules made by the transformant strain. The number of nodules by each strain per plant were counted and plotted

3.3 Results

3.3.1 Overexpression of cinRI and sinRI in Rlv3841 and Em8530 respectively

Using the standard molecular biological tools, the respective bicistronic coding regions for the QS systems of Rlv3841 and Em8530 viz. cinRI and sinRI respectively were PCR amplified and cloned in broad host range expression vector and subsequently transferred in the homologous parent host using electroporation as described in the section 3.2.3. Figure 3.2 shows the restriction digestion patterns of clones of the intermediate constructs pJETcinRI, pJET-sinRI as well that of the constucts of pCINRI and pSINRI. The pJET1.2-cinRI single digestion with pstI released ~1.9 kb fragment confirming the orientation of insertion in pJET. In this orientation, the cinRi could be excised with HIndIII and xhoI and inserted in pBBR1MCS2 in the correct orientation. pJET-cinRI also gave insert release on BglII digestion the sites of which flank the insertion site of the vector; and finally the construct also gave an insert release on BamHI and PstI double digestion. Double digestion with BamHI and PstI could also release the product from pCINRI thus confirming the cloning. and the cinRI fragment and an additional ~400 kb fragment of the vector; There was no desired orientation for sinRI in its pJET

clones, since the primer borne sites (BamHI: fwd and XbaI:rev) are in the correct orientation for the final expression vector–pBBR1MSC5. Confirmation of pJET-sinRI clones and pSINRI clones was thus done using BamHI and XbaI double digestion of the respective plasmids transformed in *E. coli* ET12567 (Figure 3.2). Figure 3.3 shows the plasmid maps of the final constructs of each of the pJET- and pBBR- clones. Homologous cloning ensures that the QS, in these strains is overexpressed and is constitutive and free of cell-density mediated regulation.

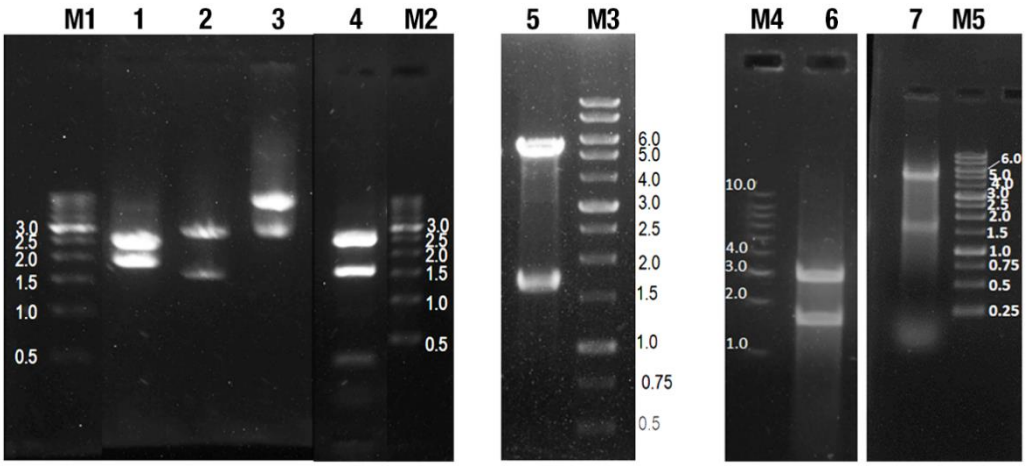

Figure 3.2 Restriction digestion patterns for confirmation of pJET- and pBBR1MCS2/5 clones of cinRI and sinRI. The gel legends are as follows. M1: DNA size marker for wells 1-3; M2: DNA size marker for Well 4; M3: DNA size marker for Well 5; M4: DNA size marker for Well 6; M5: DNA size marker for Well 7; Well 1: PstI digested pJET1.2-cinRI; Well 2: BglII digested pJET1.2-cinRI; Well 3: Undigested pJET1.2-cinRI; Well 4:) BamHI and PstI digested pJET1.2-cinRI; Well 5: pCINRI digested with BamHI and PstI; Well 6: BamHI and XbaI digested pJET1.2-sinRI; Well 7: BamHI and XbaI digested pSINRI. The numbers next to DNA bands of the size markers indicate their size in Kb.

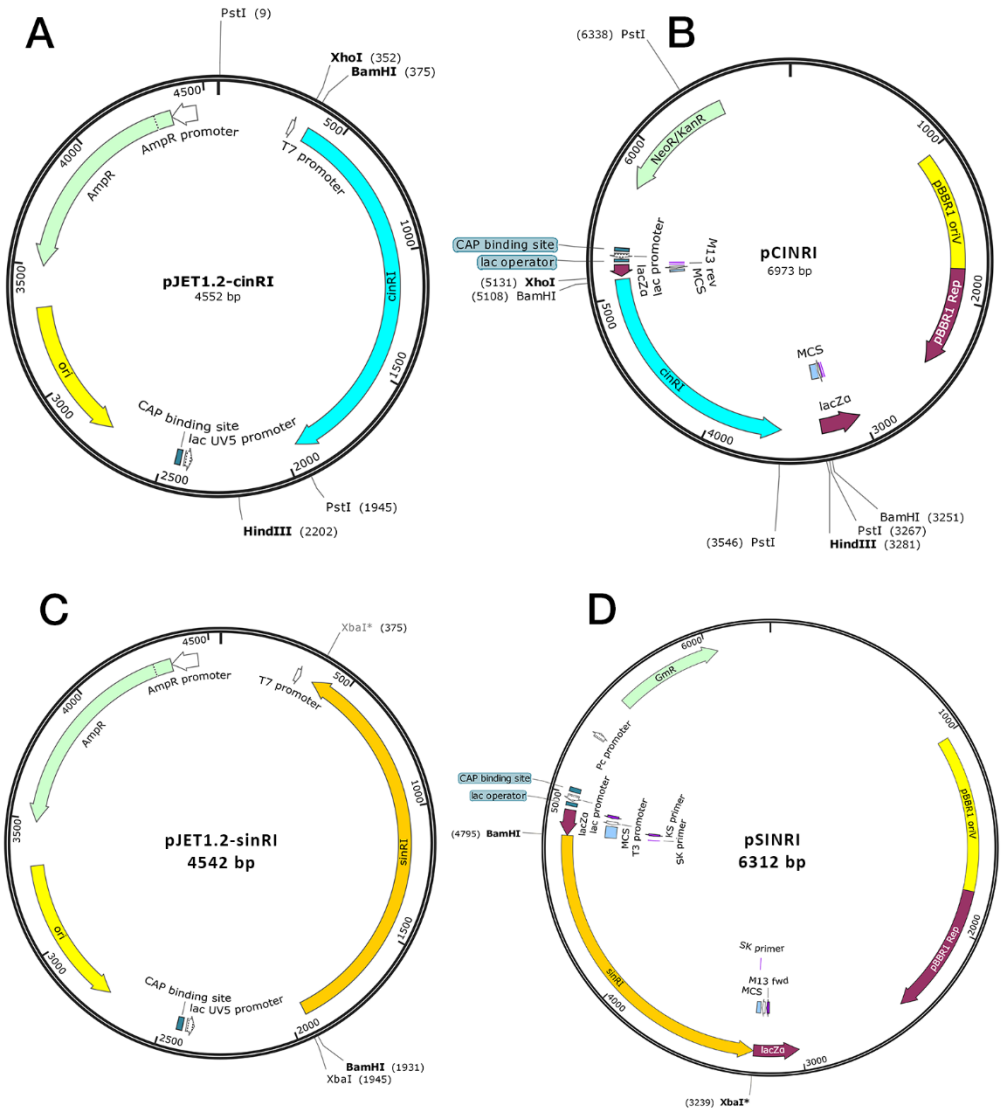

Figure 3.3 Plasmid maps of the constructs of cinRI and sinRI in cloning and expression vectors. (A) pJET1.2-cinRI —*cinRI* amplified product of Rlv3841 cloned in the blunt end cloning site of pJET1.2 (B) pCINRI — *cinRI* subcloned from pJET1.2-cinRI using XhoI and HindIII and cloned in the same sites of pBBR1MCS2; BamHI and PstI are part of the forward and reverse primer (shown at 5108 and 3546th base in the image). (C) pJET1.2-sinRI — sinRI amplified product of Em8530 cloned in the blunt end cloning site of the pJET1.2; (D) pSINRI — sinRI subcloned from pJET1.2-sinRI using BamHI and XbaI and cloned in the same sites of pBBR1MCS5. The * in C and D next to XbaI denotes the methylation sensitivity of XbaI site of the reverse primer if cloned in the dam^{+} dcm^{+} host.

3.3.2 AHL production kinetics of the QS overexpression strains

Rlv3841 transformants with only the empty vector pBBR1MCS2 as well as one harbouring pCINRI were tested at different stages of growth, starting from the lag phase to late log phase for AHL production as described in Section 3.2.4. The Rlv3841(pCINRI) was found to start secreting AHLs since the lag phase of the growth (OD_{600}=0.03) as seen from the blue zones of detected AHLs on bioassay plates (Figure 3.4A). At that stage of the growth, the vector control did not produce detectable quantities of AHLs. Further, the amount of AHL production was found to gradually increase in case of vector control whereas AHLs accumulated rapidly in case of the Rlv3841(pCINRI). The zone diameters have been measured and plotted against the OD_{600} of growth in the bottom panel of Figure 3.4B. Similarly, four different growth stages were assessed in case of Em8530(pSINRI) for the amount of AHL production at each stage. Similar pattern of AHL production was found in Em8530(pSINRI) as well. The results of pSINRI are also indicated in the Figure 3.4A and B.

3.3.3 Effect of QS overexpression on attachment of rhizobia to the host root

Rlv3841(pCINRI) and Em8530(pSINRI) were tested for their ability to attach to the respective host root. As shown in Figure 3.5, Rlv3841(pCINRI) was found to adhere greater to the germinated pea root at the 4 h interval as compared to the vector control strains. Similarly, the QS overexpression strain of Em8530 was also found to colonize significantly better as compared to its respective vector control strain. The difference between the colonization after a week of hydroponic growth, a much starker difference was observed between the QS overexpression strain and the vector control. Interestingly, this effect was seen in case of both the strains- the Rlv3841(pCINRI) as well as Em8530(pSINRI). The QS overexpressing strains of both species were found to be at least one order of magnitude more abundant on the roots than their respective empty vector controls.

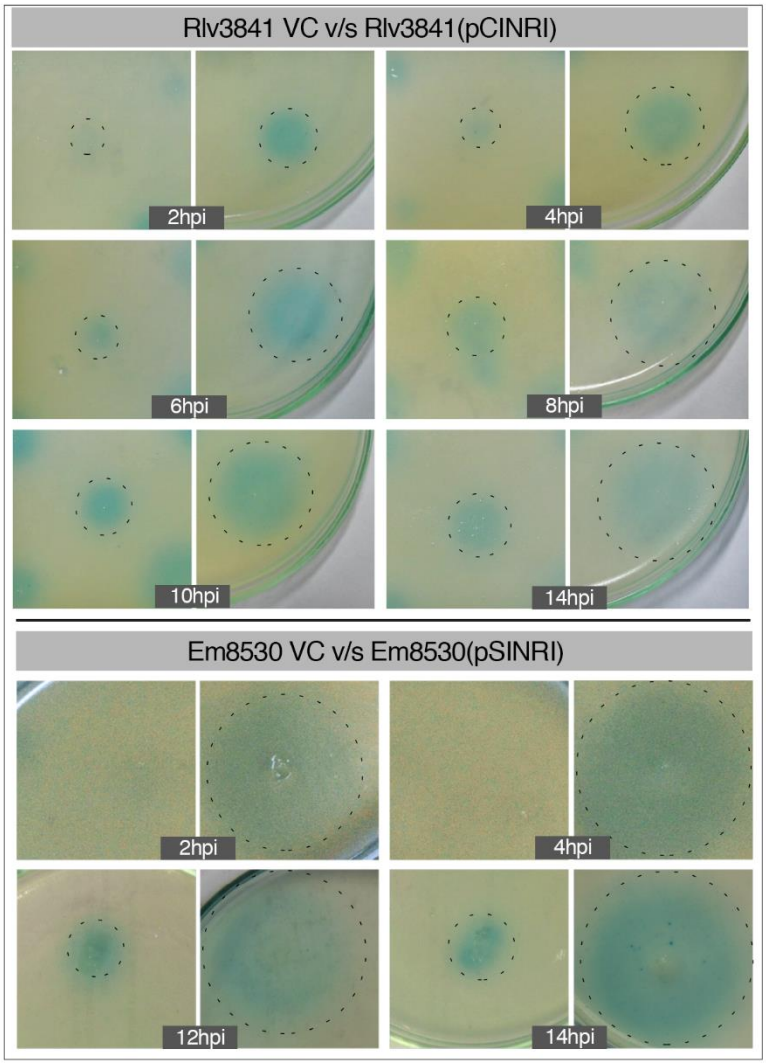

Figure 3.4A AHL production kinetics of QS overexpressing rhizobia (Bioassay pictures). Blue zones indicate the detected AHLs on the *Agrobacterium* AHL bioassay plates. The tile-pairs are labelled with the time of drawing sample post inoculation. The zones have been circled with a dotted line for clarity. The graph at the bottom shows the values of the zone diameters against the OD_{600} of the drawn sample for both the rhizobial species.

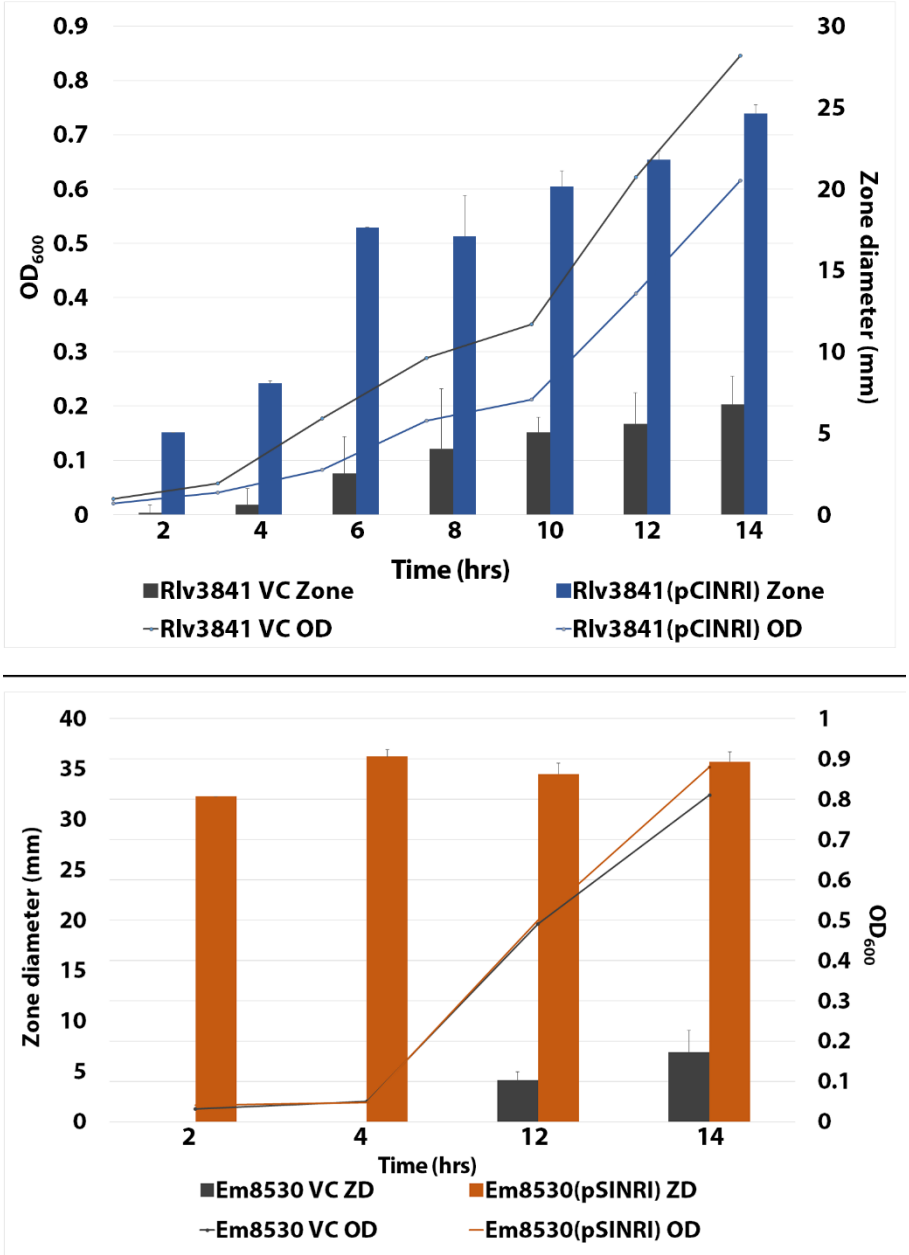

Figure 3.4B. AHL production kinetics of QS overexpressing rhizobia (plots). The top plot is for the Rlv3841 vector control (VC) and Rlv3841(pCINRI) while the bottom is for Em8530 VC and Em8530(pSINRI) pair. In both the graphs, the X-axis indicates the hpi of drawing the sample for AHL measurement. The zone diameters produced by respective vector control (VC) and the overexpression strain on the AHL bioassay plates are plotted as vertical bars and scaled to the one of the two axis. The OD_{600} at each of the retrieval is plotted as a line and is scaled on to the second Y-axis.

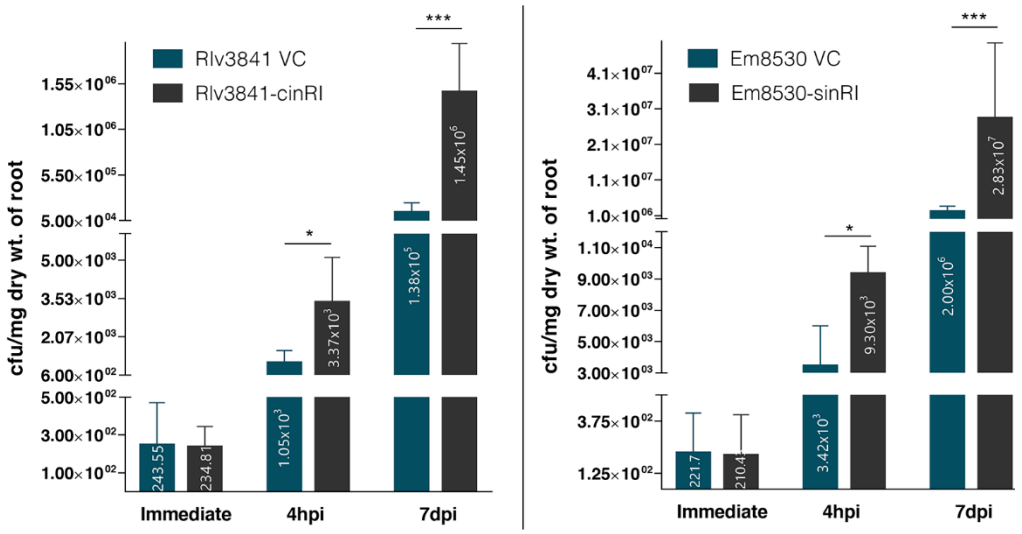

Figure 3.5 Counts of cfu recovered from pea roots coated with Rlv-3841-cinRI or Rlv3841 vector control, and alfalfa roots coated with Em8530(pSINRI) or Em8530 vector control. The Y axis shows the cfu normalized by the dry weight of the root fragment from which the counts are done. The values in the bar represents the mean cfu/g dry weight of the root. 4hpi indicates counts of the roots coated for 4 hours prior to recovery and counting whereas 7dpi indicates the set where the plant roots were coated with organisms and incubated in hydroponics for 7 days prior to recovery and count. *p≤0.05; ***p≤0.001; n=4

3.3.4 Effect of homologous QS overexpression on biofilm formation

Biofilm formation is one of the most crucial properties of rhizobia affecting quality of association with the host root. Rlv3841(pCINRI) and Em8530(pSINRI), when assessed for their ability to form biofilm on polystyrene surface were found to form greater amount of biofilm as compared to their respective controls(Figure 3.6) While there is a substantial difference between the biofilm formation case of Rlv transfromants between the vector control and the overexpressing counterpart, the difference was not statistically significant in case of Em8530 (VC) and Em8530(pSINRI).

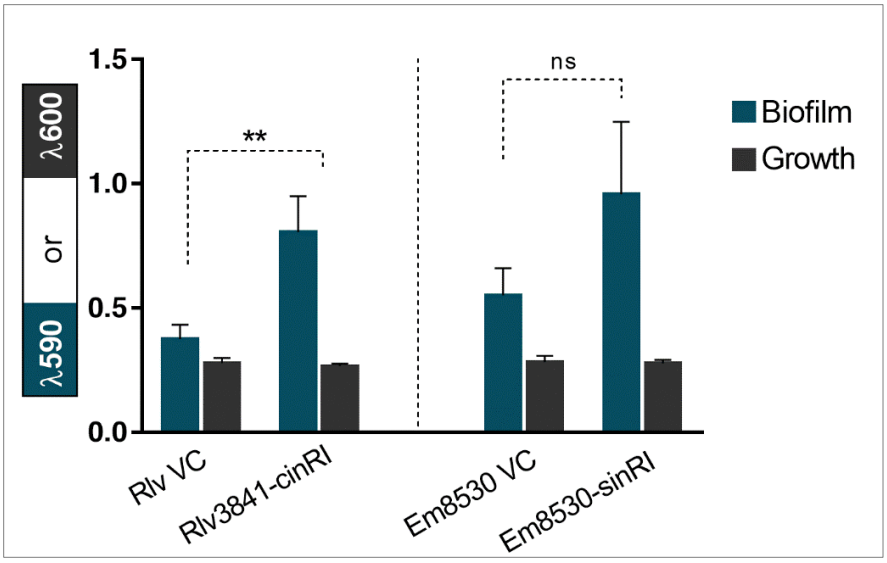

Figure 3.6 Effect of QS overexpression on biofilm formation abilities of Rlv3841 and Em8530. VC indicates vector control of respective rhizobial strain. The Y-axis indicates absorbance. The absorbance at 600nm is the turbidometric assessment of planktonic growth measured prior to staining for biofilm; absorbance at 590nm is by crystal violet strained biofilms and is proportional to the amount of biofilm formed. **p≤0.01; n=3

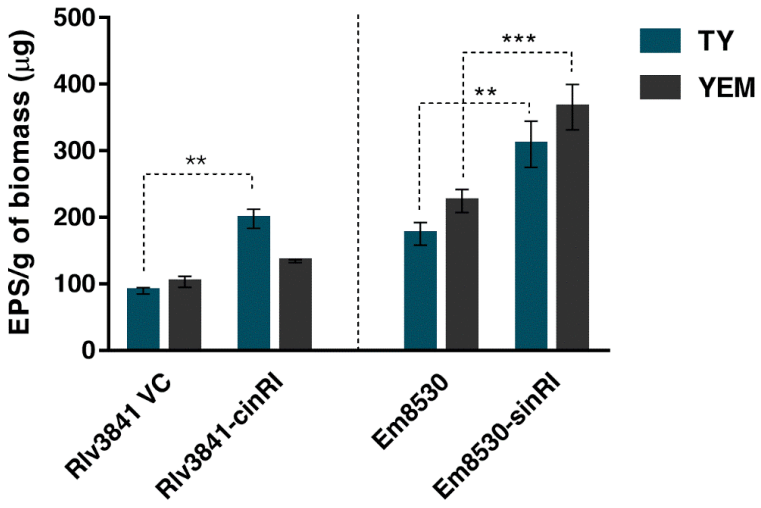

Figure 3.7 Exopolysaccharide production by QS overexpression strains. Y-axis denotes the total exopolysaccharides (EPS) produced by rhizobial strain normalized by their biomass. VC denotes vector control of respective strain. **p≤0.05; n=3

3.3.5 Effect of homologous QS overexpression on Exopolysaccharide production

The amount of exopolysaccharides produced upon on homologous over-expression of cinRI and sinRI in Rlv3841 and Em8530 respectively when quantitated showed an elevated production case of both of the QS overexpression clones when cultivated in TY broth (Figure 3.7). The difference between EPS production in overexpression strains and the respective control was smaller when they were cultivated in YEM broth; in case of Rlv3841 it was statistically insignificant.

3.3.6 Effect of QS overexpression of cinRI on the colony architecture of Rlv3841 on the pea root

At the end of seven days of hydroponic growth, the pea plants with roots coated with the vector control or the QS overexpression strain were removed and the fragments imaged under scanning electron microscope as detailed in section 3.2.8. The micrographs captured at 5000X, 10000X and 15000X are depicted in Figure 3.8. Cells of Rlv3841(pCINRI) were found to be surrounded by significantly greater quantity of exopolysaccharides as compared to the vector control. Both the strains were seen to form biofilms on the root surface, however, the biofilms formed by Rlv3841(pCINRI) were found to be denser with cells and also had greater and denser biofilm matrix as compared to that in the biofilms formed by vector control. The imaging revealed that QS overexpression causes significant differences to the microscopic patters of colonization of Rlv3841 on the pea root.

3.3.7 Gene expression analysis in Rlv3841(pCINRI)

The expression analysis of genes regulating motility and attachment was performed using qRT-PCR. The fold change values have been plotted in the Figure 3.9. The analysis revealed a downregulation of motility and chemotaxis related genes i.e. *flaA* and *cheY2* and an upregulation in the expression of *gmsA*- whose product is required for the glucomannan synthesis affecting attachment to the root. There no significant difference in the expression of the *motA*- another motility regulator and *praR*- a repressor protein regulating several symbiotically important phenotypes. There was a >3000 fold upregulation in the expression of *mcpD* as well.

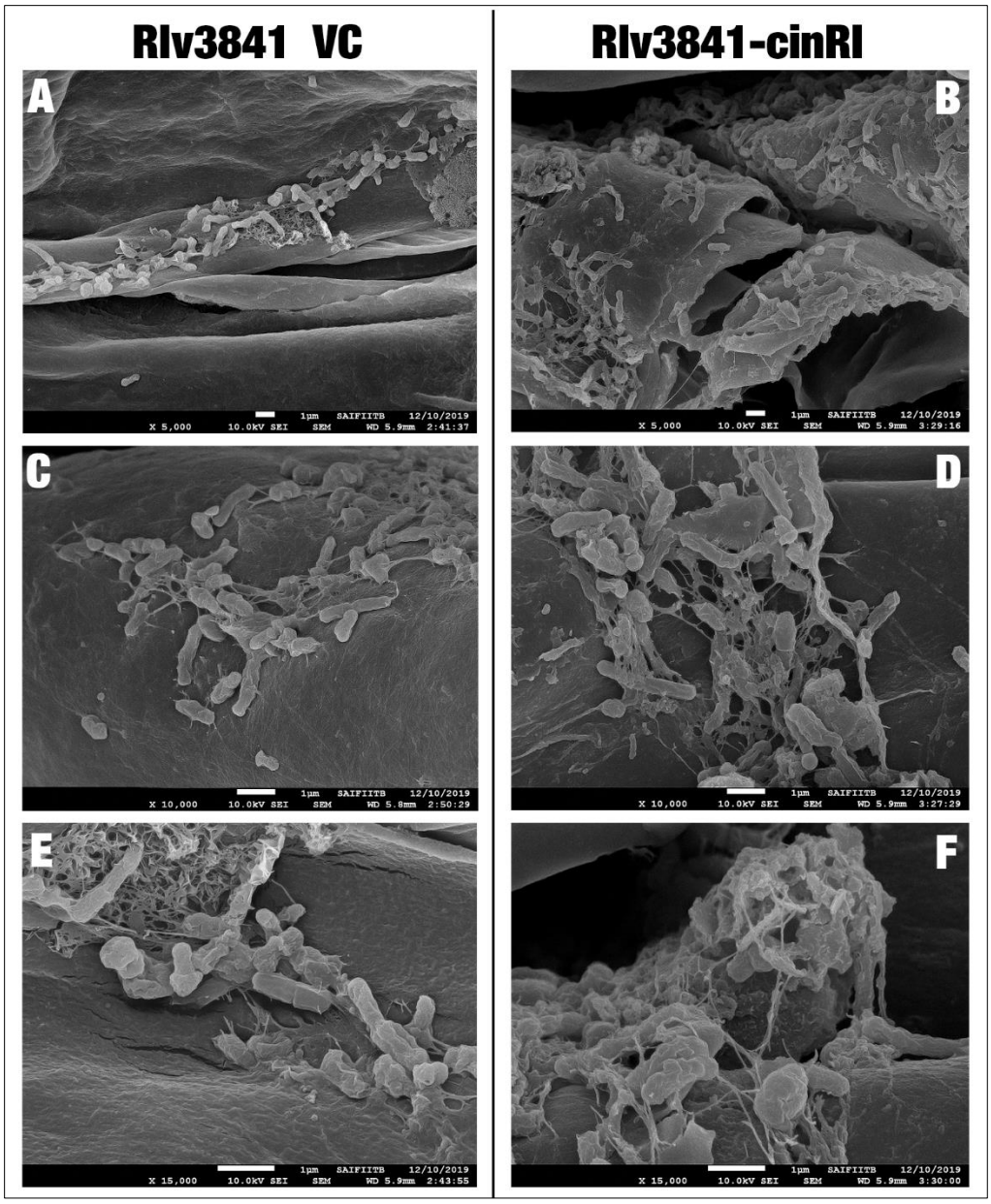

Figure 3.8 Scanning electron micrographs of Rlv3841 with homologous *cinRI* overexpression and the vector control. A, C and E are the micrographs of vector control of Rlv3841; B,D and F are of the Rlv3841(pCINRI). The bar in each image indicates 1 μm.

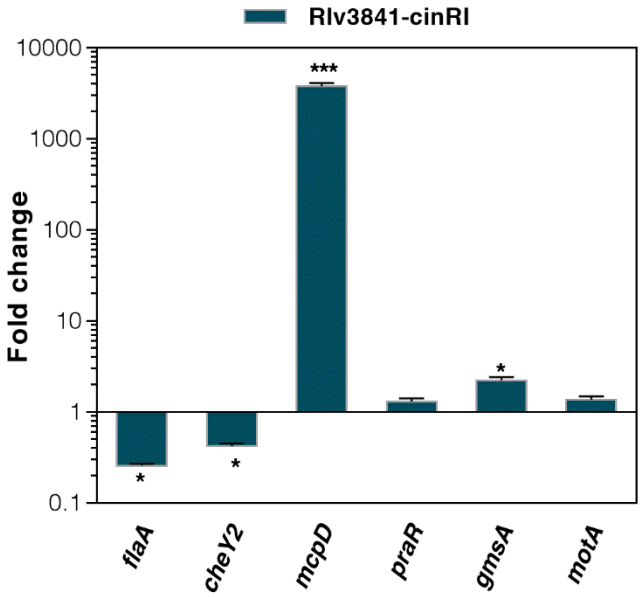

Figure 3.9 Gene expression analysis of candidate genes regulating symbiotically important processes. Y-axis shows the mean fold change value in the expression of respective gene in Rlv3841(pCINRI) as compared to the vector control which is represented by the baseline. Only the fold change values ≥ 2 or ≤ 0.5 mean differentially expression. Error bars = Standard error of means. * p≤0.05, *** p≤0.001, n=3.

3.3.8 Effect of inoculation QS overexpressing transformants on the plant growth parameters

The effects inoculation of Rlv3841(pCINRI) and Em8530(pSINRI) on the *Pisum sativum* (garden pea) and *Medicago sativa* (alfalfa) respectively on the root and shoot weight as well as height are summarized in the Figure 3.10. Inoculation of alfalfa with Em8530(pSINRI) was found to have significantly increased root weight as compared to the vector control. The rest of the parameters for both the pairs were not significantly different between the respective control and test pair. There was a significant difference between the uninoculated and either one of the inoculation in all the parameters of the growth in case of both the pairs.

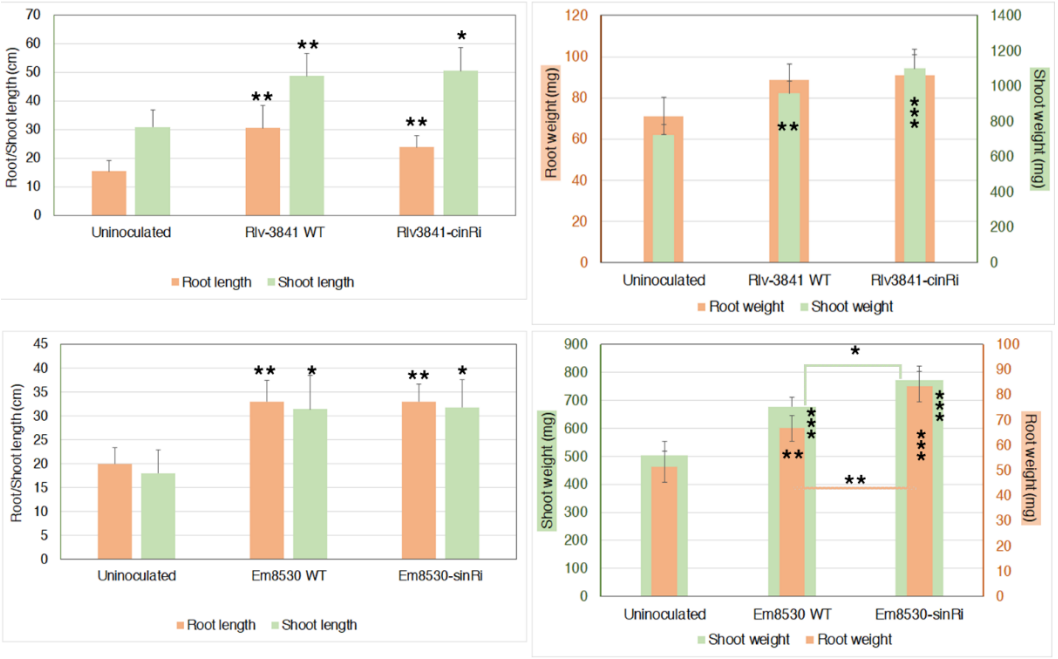

Figure 3.10: Effect of inoculation of Rlv3841(pCINRI) and Em8530(pSINRI) on host growth as compared to the WT. The * on bars indicate the significance of their comparison with the uninoculated control. Connecting lines indicate comparison between indicated groups. *p≤0.05; **p≤0.01; ***p≤0.001; n=3

3.3.9 Effect of QS overexpression on the nodulation competitiveness

The total number of nodules formed did not differ significantly in the wild type or in the overexpression strain of any of the pairs in independent inoculations. Total number of nodules formed by Rlv3841 WT and Rlv3841(pCINRI) on pea roots in gnotobiotic conditions at the end of six weeks were 28.75±7.36 and 34.33±6.68 respectively whereas the same for Em8530 WT and Em8530(pSINRI) were 21.25±7.18 and 23.25±5.19 respectively. Percentage of nodules occupied by respective strain when different ratios of WT–overexpression-strain were mixed in different proportions is shown in Figure 3.11. In case of Rlv-pea nodulation competitiveness test, the percent nodulation was overall higher for Rlv3841(pCINRI) at most ratios tested, however the statistically significant difference was observed only when the two strains were applied at equal amounts.

In case of *E. meliloti*-alfalfa nodulation competitiveness assay, there was no significant difference between the percent nodule occupancy between the Em8530 WT and Em8530-cinRI strains at any of the ratios of the ratios tested.

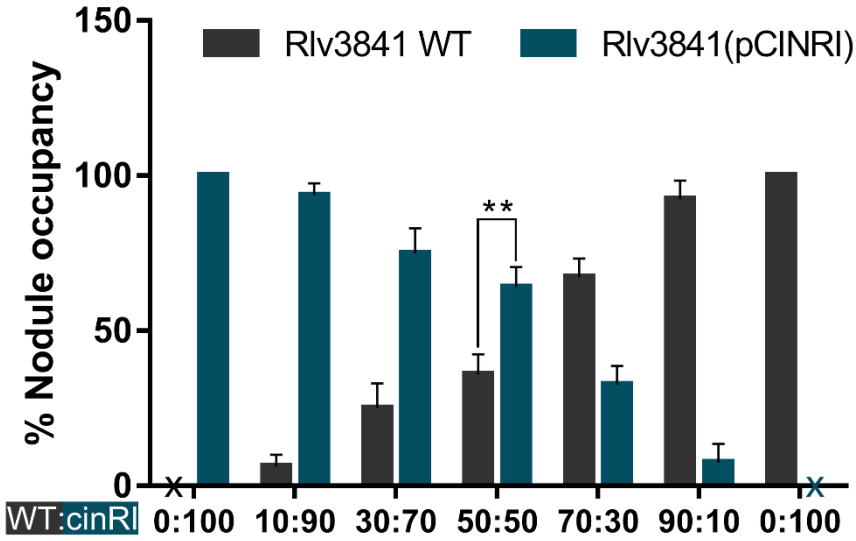

Figure 3.11 Relative competitive nodulation by QS overexpression strains as compared to the respective wildtype. The X-axis denotes the %ratio of Rlv WT:Rlv3841(pCINRI) in each coating mixture. And Y-axis denotes the % of nodules formed by the strains when present at respective % representation in the coating mixture. X indicates zero nodulation because of the absence of the particular strain. * indicates significance as compared to the WT. **$p \leq 0.05$; n=3

3.4 Discussion

Quorum sensing (QS) facilitates the lifestyle switches that rhizobia undergo while encountering the drastic changes in chemical composition to qualitative and quantitative nutritional properties of the microenvironment they transition in by modulating the physiological responses collectively in response to the cell density. It is thus conceivable that the QS regulation plays a determining role in the efficiency of symbiotic interaction and so has been reported for many species of rhizobia (Cooper, 2007; Jitacksorn and Sadowsky, 2008; Patankar and González, 2009). Another common property of QS in rhizobia is that most of them have multiple luxI-luxR circuits and that they often have a functional hierarchy wherein one of the QS regulates the functions of the other systems (González and Marketon, 2003). The *cinI-cinR* (*cinRI*) circuit of *Rhizobium leguminosarum* bv viceae strain 3841 (Rlv3841) is one of the three QS systems in the strain and has been reported to regulate discrete phenotypes that determine the symbiotic interaction with host (Sanchez-Contreras *et al.*, 2007; Frederix *et al.*, 2011). Similarly *sinI-sinR* (*sinRI*) is the top regulatory circuit in *Ensifer meliloti* 8530 which governs other circuit and orphan luxR mediator and is associated with the modulation of symbiotically important processes (Gurich and González, 2009).

This chapter involved homologous cloning of these circuits in the respective hosts and assessed their effects on the traits that aid the colonization of the root and nodulation competitiveness. pBBR1MCS- series vectors that are used in this study for the homologous cloning are broad host range (Kovach *et al.*, 1995) and provide stable and constitutive expression in rhizobia including *R. leguminosarum* and *E. meliloti* (Gage *et al.*, 1996; Wisniewski-Dyé *et al.*, 2002; Zatakia *et al.*, 2014). AHL production in the overexpression-strains was made independent of the cell-density dependence and was induced several folds. Using the AHL biosensor *Agrobacterium tumefaciens* NTL4 pZLR4 (Cha *et al.*, 1998) for this detection is very helpful because of its greater sensitivity especially for long chain AHLs (Abbamondi *et al.*, 2016) which are reportedly synthesized by CinI and SinI (González and Marketon, 2003). Attachment to the host root has been regarded as

one of the most important determinants of the success of the nodulation (Lopez-Garcia *et al.*, 2002; Wheatley and Poole, 2018). This has also been emphasized for the rhizobia that may be introduced over the seeds as a biofertilizer (Parke, 1991). When assessed for attachment after a short and a long term, both the QS overexpression strains were found to have an increased attachment as compared to the control strains particularly at 7dpi. The primary attachment of the root by rhizobium is known to be relatively weak and reversible whereas the secondary attachment is generally strong and may involve a embedding in the matrix (Wheatley and Poole, 2018). One of the important processes for sustaining the strong and non-reversible secondary attachment between the root and rhizobia is the secretion of exopolysaccharides (Wheatley and Poole, 2018). This study also found an increased secretion of exopolysaccharides (EPS) in both the QS overexpression strains. Exopolysaccharide production or processing is controlled by QS in both the strains (Marketon *et al.*, 2003; Calatrava-Morales *et al.*, 2018). Williams *et al.*, (2008) have seen that the mutants of *R. leguminosarum* incapable of production of EPS were severely impaired in the attachment to pea root. In *Ensifer* spp. EPS production has been implicated in the successful colonization of the host root (Arnold *et al.*, 2018; Acosta-Jurado *et al.*, 2020; Gosai *et al.*, 2020). *In R. leguminosarum* 3841, QS is known to alter the integrity of an acidic EPS (Frederix *et al.*, 2014) but details of if the production/secretion is regulated by QS are not clear. Recently, Rachwał *et al.*, (2015) found that *rosR* transcriptional regulator regulates the EPS production in *R. leguminosarum* bv trifoli. Whether *rosR* is dependent on QS in *R. leguminosarum* is not clear, its homologue *mucR* in *E. meliloti* which also regulates EPS production is known to be under the QS control (Hoang *et al.*, 2004; Bahlawane *et al.*, 2008; Mueller and Gonzalez, 2011). In a recent study, binding site for a QS mediator was discovered upstream to *rosR* ORF in *R. leguminosarum* bv. trifolii (Rachwał *et al.*, 2017). Role of Exopolysaccharides may vary among the species of rhizobia as does the regulation, for instance, in *E. meliloti* EPS is an important part of the biofilm (Rinaudi-Marron and González, 2015),while Santaella *et al.*, (2008) found that in the strain of *Rhizobium* they tested, EPS did not contribute significantly to the biofilm formation but the mutants

were still impaired in the root colonization. The presentis study found an increase in the biofilm formation capabilities of Rlv3841 upon on overexpression of QS. Biofilm formation plays a crucial role in the secondary strong attachment with the host root (Wheatley and Poole, 2018). Rhizobial biofilms are made up of diverse components including cellulosic fibers, specific attachment proteins and other glycoconjugates (Rinaudi and Giordano, 2010; Wheatley and Poole, 2018). Frederix *et al.*, (2014) observed that mutants of *praR*– a transcriptional repressor, form significantly greater biofilms. They revealed that *cinS* which is co-transcribed with *cinI* binds PraR and removes the suppression mediated by it. The observed increase in biofilm in Rlv3841(pCINRI) in this study could be attributed to the removal of praR mediated repression of biofilm formation. In case of *E. meliloti*, the slight increase in the biofilm formation can be due to the expR mediated upregulation of biofilm formation as observed by (Amaya-Gómez *et al.*, 2015). Interestingly, rap proteins, which are responsible for the attachment on the root hair and initiation of formation root cap (Wheatley and Poole, 2018)are also known to be under the control of QS in Rlv3841 and an increased expression of Rap proteins has been correlated with increased attachment to host root in *R. leguminisarum* (Frederix *et al.*, 2014) and *R. etli* (Mongiardini *et al.*, 2009). A collective effect of the increased attachment as well as elevated EPS secretion may have manifested into the colony architecture observed in the study (Figure 3.8) wherein Rlv3841(pCINRI) was seen to make denser colonies which are more covered in the mucous extracellular matrix as compared to the WT.

Gene expression analysis of the Rlv3841(pCINRI) revealed that motility and chemotaxis related genes were downregulated in it as compared to the vector control. The gene *flaA* is a component of flagellar apparatus and *cheY2* is a homologue of chemotaxis regulatory protein with the same name in *E. meliloti* and is believed to function by interacting directly with the flagellar motor. In *R. leuguminosarum*, they were found to be modulated positively by *rosR* in a study by Rachwał *et al.*, (2015). Tambalo *et al.*, (2010) similarly found a *visN/R* mediated upregulation of motility mediated by a protein named Rem. The *rosR* homologue *mucR* and the *VisN*/R homologues in *E. meliloti* are negatively regulated by QS.

This is consistent with the finding of this study. This current study also found an upregulation of *gmsA* on QS overexpression in Rlv3841. The gene product of *gmsA* is required for glucomannan synthesis in *R. leguminosarum* and mutants are incapable of attachment to the root (Tambalo *et al.*, 2010; Frederix *et al.*, 2014). *gmsA* was found to be transcriptionally repressed by *praR* in Rlv3841 indicating that at the higher quorum, when the repression is relieved, the cells will produces the glucomannan aiding the attachment to the root (Frederix *et al.*, 2014). This scheme of regulation resembles that in the *E. meliloti* wherein, at the higher cell densities (believed to be achieved in rhizosphere or on root) attachment is promoted and the motility is repressed (Hoang *et al.*, 2008; Charoenpanich *et al.*, 2013). *mcpD* is also a chemotaxis regulator, however present outside the *visN*/R cluster of regulation but regulated by *Rem* and *VisN*/R in the similarly (Tambalo *et al.*, 2010).The extreme upregulation of *mcpD* cannot be explained and should be investigated further. Although not significant, the *praR*-upregulated of transcription could be result of the repression it exerts on its own transcription, i.e. in the presence of active QS (say in the overexpression strain), the *praR* can be removed from the activity and thus the auto-repression taken off, its transcription can be upregulated (Frederix *et al.*, 2014).

In spite of the differences in the overexpressing strains in their attachement and biofilm phenotypes, there was no significant difference in the number of nodules that were formed in gnotobiotic conditions by any of the QS overexpressing strains as compared to their respective wildtypes. Marketon *et al.*, (2002) found that *sinI* or *sinR* mutants had only a small difference in the total number of nodules formed as compared to the WT in *E. meliloti* 1021, whereas in a study by Lithgow *et al.*, (2000) found no significant difference in the nodulation by cinI or cinR mutations. In a recent study in *R. etli, cinI* and *cinR* mutants showed little and/or contradicting effect on the total nodule numbers as compared to the WT (Zheng *et al.*, 2015). The absence of significant difference in the host growth observed in this study between the QS overexpression strain- and WT- inoculated plants can be attributed to the similarity in the number of nodules, while all the parameters in both cases were significantly improved as compared to the uninoculated plants. Interestingly,

Em8530(pSINRI) inoculation on alfalfa resulted in significant increase in the root weight as compared to inoculation with WT Em8530. The nodulation competitiveness of Em8530 did not significantly improve on the overexpression of sinRI, however, there was small but significant increase in the nodulation vigour of Rlv3841 on overexpression of cinRI at one of the ratios tested.

Homologous cloning of QS circuit resulted a favourable situation for both the QS overexpression strains, including the increased attachment, biofilm formation and the dense and EPS-rich colonies on the host roots. However, these successes were inadequate to manifest into a scalable difference in the nodulation competitiveness. Some of the possible reasons for the same are listed below.

A. Some of the phenotypes determining the nodulation competitiveness and controlled by QS are also regulated by some of the non-rhizobial agents for e.g. the root exudates. For instance, the upregulation of EPS by root exudates or low phosphates in case of *R. leguminosarum* bv trifoli, (Janczarek and Skorupska, 2011); Phosphate mediated (McIntosh *et al.*, 2009) or falavanoid–mediated (Spini *et al.*, 2016) QS modulation in *E. meliloti.* These effects may either negate the QS regulation of root colonization or equilibrate the apparent outcome between the WT and QS overexpressing strain leading to diminution of the apparent difference in nodulation competitiveness measured.

B. While the QS overexpression leads to an early and amplified onset of symbiotically important functions, it is important to consider that WT also develops microcolonies on root leading to high quorum and thus the comparison between two may not reflect an accurate and significant difference in the nodulation competitiveness in the overexpression strain. This is of greater impact when the said symbiotically crucial phenotype may be triggered at relatively low AHL concentrations as observed by Charoenpanich *et al.* (2013) in *Ensifer*.

C. One of the caveats of having a QS overexpression strain and a WT together on the root is that there is a strong possibility that the WT will benefit from the higher amount AHLs secreted by the overexpression strain. This is very likely given that

the AHLs are shared and meant as a community goods, and that the different strains sharing a niche may cross-utilize compatible AHLs. This not only narrows the window of quantitative difference in the nodulation competitiveness between the two, but also in theory, may impart a fitness benefit on the WT.

While the latter two issues (B and C) are inevitable when comparing the WT and QS overexpression strain on the root over a long term experiment, a different strain (preferably QS^{-}) can be deployed to compare individually with the WT and QS overexpressing and then compare the differences with each other to see how they fare. For the issue C, a comparison between QS mutant (incapable to sense the signal) and overexpressing strain may possibly show a greater difference in the nodulation competitiveness than the former. However, if the WT reaching quorum (issue B) still remains in this approach. One of the attractive option is to compare the WT and the QS++ strain against the cohort of wild strains such as in the unsterile soil.

It can be concluded from this study that Quorum sensing overexpression in each of the tested rhizobial strain lead to the promotion of the processes favoring better colonization of the host root. However, these effects could not be reflected in the increased competitive nodule occupancy by the QS overexpression strain as compared to the WT. This is likely to be the result of the multifactorial regulation of the QS and its regulated processes especially in the rhizosphere and the shared nature of AHLs. The QS overexpression strains if tested with different QS mutants and with other wild type strains with varying nodulation competitiveness can reveal more resolute details of the improvement in nodulation competitiveness as a consequence of QS overexpression.