Chapter 5

Efficacy of quorum quenching based virulence attenuation of quorum sensing pathogen *in situ*

5.1 Introduction

Over 20 years of research into AHL-signalling in a wide variety of species, has revealed complex molecular mechanisms, with some systems regulating the production of several AHL molecules at the same time. Most of this work has been performed in test tubes in rich growth medium and relatively little is known about AHL signalling in more natural environments. Quorum sensing plays a dominant role in the process of infection by pathogens in animals, insects and plants. Solubility, diffusivity, stability and dispersion of AHLs are critical parameters for communication between cells to elicit a successful population level response. Variation in these parameters may result in concentrations of AHLs too low to trigger a response over the physical distance or the spatial scale between an AHL producing cell and an AHL perceiving cell (known as calling distance) in natural environments (Decho et al. 2011). Therefore, understanding about the spatial distribution of the population in environments such as on the roots of a host plant, can help in understanding the infection process of phytopathogens. Gantner et al. (2006) reported this calling distance to be < 10 µm for AHL signalling on tomato and wheat root surfaces between *Pseudomonas putida* cells. However, the same study showed that some AHLs were able to travel relatively long distances (up to 78 µm) and still induce quorum sensing.

In nature, AHL signalling and in turn quorum sensing can be affected by abiotic environmental factors, by members of the bacterial community such as AHL-degrading bacteria and by compounds which are AHL mimic or inhibitor produced by eukaryotic host (Boyer and Wisniewski-Dye 2009). The abiotic factors which contribute in degradation of AHL are pH, temperature and environmental conditions for the niche (Decho et al. 2009). Whereas biological mechanisms include hydrolyses by enzymes, usually produced by other bacteria. AHL inactivating microbes are indeed active in the environment and physiologically relevant concentrations of quorum signals are subject to a rapid biodegradation. According to Wang and Leadbetter (2005) it may take abiotic AHL degradation months to match the level of signal decay achieved in days by biological activity in species rich natural environments like soil. The way different biotic and abiotic factors affect quorum sensing, similarly quorum quenching activity is also affected by such factors in nature. These factors (pH, temperature and environmental conditions for the niche) are able to modulate the quorum quenching efficiency of the bacteria in natural environment (Uroz et al. 2009).

In order to communicate efficiently, the spatial structure of pathogen cells on the root of the host plant plays a key role. The distance travelled by an AHL molecule from the AHL

producing cell to the AHL perceiving cell (calling distance) in a population of the pathogen will determine its quorum sensing efficiency and its pathogenicity. In similar way for quorum quenching as well the spatial structure of quorum sensing pathogen and AHL degrading bacteria would play important role. A detailed understanding suggests that to disrupt quorum sensing a quorum quenching bacteria should colonise the roots in such a manner that it is able to interfere with the signalling between the pathogen cells. Taking into consideration the AHL calling distance and the spatial distribution of the pathogen and quorum quenching bacteria on the plant root are important parameters which will determine the efficiency of quorum quenching as biocontrol strategy.

The quorum sensing system of the opportunistic human pathogen *Pseudomonas aeruginosa* is well studied. It is established that AHL mediated quorum sensing plays a dominant role in the process of infection by *P. aeruginosa* in humans, animals, insects and plants. It contains two linked quorum sensing systems, the *las* and *rhl* systems which are responsible for the regulation of numerous genes involved in pathogenesis through two different signalling molecules. The AHL signals produced by *P. aeruginosa* are 3-oxo-C12-HSL and C4-HSL. They are generated by AHL synthases called LasI and RhlI and subsequently bind to the cognate receptors called LasR and RhlR, respectively. When the bacterial cell density reaches a particular threshold, the LasIR quorum sensing system is initiated. The 3-oxo-C12-HSL-LasR complex activates *rhlI* expression as well as LasR-controlled genes including *lasI*, the cognate signal synthase, which then leads to activation of the RhlIR system. Either or both the *las* and *rhl* systems activate the production of virulence factors such as elastase, alkaline protease, exotoxinA, rhamnolipids, pyocyanin, lectines, and super oxide dismutase (Smith and Iglewski 2003; Schuster et al. 2013; Darch et al. 2012).

The studies undertaken in this chapter were carried out to understand the efficacy of the virulence attenuation exercised by the quorum quenching strain *Lysinibacillus* sp. Gs50 on the pathogen. The quorum quenching strain would be effective in the environmental conditions favourable for its activity since it is enzymatic and the juxtaposition of the QQ strain and the pathogen will also have equal effect on the efficacy of the virulence attenuation. Two aspects studied here to address this were (1) influence of different environmental factors on quorum quenching activity of cells of *Lysinibacillus* sp.Gs50 and (2) understanding the importance of spatial structuring of quorum quenching bacteria (*Lysinibacillus* sp. Gs50) and quorum sensing pathogen (*P. aeruginosa* PAO1) when grown on plant roots. This latter study would also help understand the biology of a quorum quenching based virulence attenuation approach during actual infection.

5.2 Materials and Methods

5.2.1 Bacterial strains

Table 1 Bacterial strains and plasmids used for infection and CSLM

Plasmids/strains	Characteristics/use in this study	References
pMHLAS-*rfp*	Amp^R Gm^R; pMH391 carrying P*lasB-rfp* (ASV) P*lac-lasR*	Hentzer et al. 2002
P. aeruginosa PAO1 wild type	Mung bean infection assays	Laboratory stock from Dr. Diggle's Lab, University of Nottingham, UK
P. aeruginosa PAO1 *lasI⁻ rhlI⁻*	Mung bean infection assays	Cornforth et al. 2014
PAO1-*gfp* (pME6032::GFP)	Confocal Microscopy	Popat et al. 2012
PAO1 *lasI⁻ rhlI⁻* labelled with pMHLAS::*rfp*	Confocal Microscopy	This study
E. coli pSB1142	Biosensor used for detection of 3-oxo-C12-HSL	Winson et al. 1998
E. coli pSB536	Biosensor used for detection of C4-HSL	Winson et al. 1998
Lysinibacillus sp. Gs50	Bacterial isolate used for quorum quenching	Laboratory stock

Cultures were grown in Lysogeny Broth (LB) with appropriate antibiotics and were incubated at 30°C at 200 rpm. The *E. coli* pSB1142 and *E. coli* pSB536 were grown in LB broth containing 20µg/ml of Tetracycline and 50 µg/ml of Ampicillin respectively at 37°C /200 rmp, while *Lysinibacillus* sp. Gs50 was grown at 30°C at 200 rpm.

5.2.2 Influence of environmental factors on quorum quenching activity of *Lysinibacillus* sp. Gs50

5.2.2.1 Effect of temperature

Overnight grown *Lysinibacillus* sp. Gs50 was centrifuged at 7000 rpm for 5 min and the cell pellet was washed and resuspended in 500 µl of PBS. The reaction mixture contained 20 µl of cell suspension and 80 µl of 25µM C6-HSL which was incubated for 2 hours. The incubation was carried out for temperature range 5°C, 15°C, 20°C, 25°C, 30°C, 37°C, 45°C and 50°C. After incubation the supernatant of the reaction mixture was subjected to the agar diffusion bioassay (described in section 4.2.17) for the quantification of remaining C6-HSL in the reaction mixture.

5.2.2.2 Effect of pH

Overnight grown *Lysinibacillus* sp. Gs50 culture was centrifuged at 7000 rpm for 5 min and the cell pellet was washed and resuspended in 500 µl of PBS. The reaction mixture contained 20 µl of cell suspension and 80 µl of 25µM C6-HSL. The pH of individual reaction mixture was set to 3.6, 4.0, 4.6, 5.0, 5.6, 6.0, 6.6, 7.0, 7.6, 8.0, 8.6 and 9.0 and incubated for 2 hours. After incubation the supernatant of the reaction mixture was subjected to the agar diffusion bioassay (described in section 4.2.17) for the quantification of remaining C6-HSL in the reaction mixture.

5.2.2.3 Effect of C6-HSL concentration

Overnight grown *Lysinibacillus* sp. Gs50 was centrifuged at 7000 rpm for 5 min and the cell pellet was washed and resuspended in 500 µl of PBS. The reaction mixture contained 20 µl of cell suspension and 80 µl of C6-HSL having the concentration of 40, 60, 80, 100, 150, 200, 300, 500 and 700µM. After incubation the supernatant of the reaction mixture was subjected to the agar diffusion bioassay (described in section 4.2.17) for the quantification of remaining C6-HSL.

5.2.3 Efficacy of quorum quenching on mung bean roots

For these studies initially mung bean infection model for *P. aeruginosa* was established and further the importance of spatial structure of the pathogen and QQ strain in attenuation of quorum quenching was demonstrated in *in planta* studies.

5.2.3.1 Quorum sensing pathogen *P. aeruginosa* and Mung bean infection model

5.2.3.1.1 Mung bean infection by *P. aeruginosa* PAO1

Mung bean seeds were soaked in 70% ethanol in a sterile flask for 30 seconds. The ethanol was discarded and seeds were soaked in 0.01% Mercury Chloride ($HgCl_2$) for 30 seconds. $HgCl_2$ was discarded and seeds were washed with sterile water. Washing with 70% ethanol and 0.01% Mercury Chloride was repeated for 3 times. In the last cycle seeds were washed thoroughly with sterile water. Surface sterilised seeds were aseptically transferred on the soft agar (0.8% w/v agar) plate for germination. Approximately 1.5 – 2 ml of sterile water was added to plates. The plates were incubated for 24 hours at 37°C under humidified condition for germination. Up to eight germinated seeds per group were used for further infection process. For seed bacterization, *P. aeruginosa* PAO1 and PAO1 *lasI rhlI* were inoculated into 100 ml LB from overnight grown culture and incubated at 30°C at 200 rpm till it achieved the cell density of ~10^9 CFU/ml. The log phase cultures were centrifuged at 7000 rmp for 10 mins and pellets were resuspended into 4 ml sterile PBS (pH: 7). Eight geminated mung bean seeds were added to this culture suspension. Equal numbers of seeds were incubated in 4 ml of PBS as uninfected control. All the seeds were incubated at 30°C for 24 hours at static condition. After the incubation three seeds from each treatment group were withdrawn and viable count form those sprouts was done to know the number of bacteria adhered to it. Seeds were added into sterile PBS and vortexed for 1 minute and an aliquot was plated onto a LB plate. After 24 hours of incubation at 30°C colonies were counted. For Plant growth 30 ml of 4.4 g/L Murashige-Skoog (Sigma Aldrich) containing 0.8% w/v agar (Oxoid) was dispensed into large glass tubes (30×200 mm,) and sterile conditions were maintained throughout the growth of the plants. The infected and the control un-infected mung beans (five seeds per group) sprouts were transferred aseptically onto the Murashige-Skoog media. The sprouts were allowed to grow in natural daylight cycle for 7-10 days in gnotobiotic conditions. After incubation the growth parameters of the plants was measured in in terms of root length, shoot length, mortality rate of sprouts and number of lateral roots. The roots from all the plants were resuspended in 3 ml PBS, vortexed vigorously for 1 minute and an aliquot from this was plated on LB plates. After 24 hours of incubation 30°C the colonies were counted. Six different trials having five replicates each were carried out.

5.2.3.1.2 Transformation of *P. aeruginosa* PAO1 *lasI⁻ rhlI⁻*

Plasmid pMHLAS-*rfp* from *E.coli* strains was purified using a GeneElute Plasmid miniprep kit (SIGMA) by following the manufacturer protocol. Transformation of *P. aeruginosa* PAO1 *lasI⁻ rhlI⁻* was performed by making competent cells using a modified procedure of Smith and Iglewski (1989). 1% (v/v) inoculum from an overnight grown *P. aeruginosa* PAO1 *lasI⁻ rhlI⁻* culture was added to sterile LB medium and grown at 37°C to an OD_{600} of 0.4 -0.5. The cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C and washed three times in sterile ice cold 300 mM (w/v) sucrose solution then the cells were resuspended in 200 µl of the sucrose solution and placed on ice for 30 min and then divided into 40 µl aliquots. The plasmid pMHLAS-*rfp* was electroporated using 0.2 mm electroporation cuvettes containing 40 µl of competent cells and 2 µl DNA. Electroporation pulse of 1.6 kV was administered using Biorad electroporation unit (BioRad Laboratories, Watford, UK), 960 µl aliquot of LB broth was added and incubated for 45 min at 37°C. 200 µl aliquot was plated onto LB agar plates containing Gentamicin (100µg/ml) and Carbenicillin (300µg/ml) and incubated at 37°C overnight to select for transformants. Carbenicillin was used as a counter selective agent since *P. aeruginosa* is sensitive to it and Gentamicin resistance is encoded in pMHLAS-*rfp*.

5.2.3.1.3 Role of quorum sensing in *P. aeruginosa* infection using CSLM

Mung bean seeds were surface sterilised and germinated sprouts were obtained (as described in the section 5.2.3.1.1). These germinated, healthy sprouts were transferred to Murashige-Skoog medium contained in glass tubes. Infection to healthy sprouts was carried out by adding 100 µl of culture suspensions of wild type *P. aeruginosa* PAO1-*gfp* and RFP labeled QS mutant *P. aeruginosa* PAO1 (*lasI⁻ rhlI⁻*) either alone or in mixtures to the plant growth medium. *In planta* experiments comprised of following groups (i) only *P. aeruginosa* PAO1producer strain treated (ii) only *P. aeruginosa* PAO1 sensor strain treated (iii) treated with sensor strain and synthetic 3-oxo-C12HSL and (iv) treated with the co-culture of producer and sensor strain (v) only PBS treated. Sprouts were allowed to develop in gnotobiotic conditions in natural day-night cycle. After seven days plants along with their roots were harvested and roots were cut with sterile scissors. They were fixed with 4% paraformaldehyde in PBS (w/v) for five minutes at room temperature. Root samples were then washed twice with 1-2 ml of PBS, embedded in 2% agarose and the root tips were cut into 0.5 cm fragments. The embedded root fragments were sectioned longitudinally into 10-20µm slices using a Leica VT1000s vibrotome. The root samples were stained with DAPI

(Sigma). The prepared root tips, in all the cases above were mounted on slides using ProLong® Gold Antifade (Thermo Fisher Scientific). The slides were kept at room temperature overnight in dark and then the root tips were imaged using confocal laser scanning microscope Ziess LSM 700. Images were taken at 63X magnification and 405nm, 488nm, 555nm lasers were used for detecting the DAPI, GFP and RFP respectively. All the images were analysed and processed using the ImageJ and LSM Browser software.

5.2.3.2 Quorum quenching of *P. aeruginosa* by *Lysinibacillus* sp. Gs50

5.2.3.2.1 *In vitro* AHL degradation assay with *Lysinibacillus* sp. Gs50

Lysinibacillus sp. Gs50 was grown overnight in 10 ml LB, centrifuged at 7000 rpm for 10 mins, and the pellet resuspended in 1 ml PBS (pH 7) was used for AHL degradation assay. 5µM C4-HSL and 5, 10, 20, 30, 40 and 50 µM 3-oxo-C12-HSL were added individually to 100µl cells from the resuspended pellet of *Lysinibacillus* GS50 and incubated for 24 hours at 30°C. After incubation the reaction mixture was centrifuged at 10,000 rpm for 10 minutes to eliminate settled bacterial cells. 100µl of the supernatant of the reaction mixture and100 µl log phase cultures of lux based biosensor *E.coli* (0.1 OD_{600}) was added into the 96 well microtiter plates and incubated at 37°C for one hour. After incubation bioluminescence was measured using a Tecan (Infinite F200 pro) plate reader. The amount of AHLs present in the well of microtiter plate was obtained from the ratio of Bioluminescence/OD at 600nm. The bioluminescence recorded from the reaction supernatant signifies the residual AHL after the treatment of AHL degrading *Lysinibacillus* sp. Gs50. Untreated C4-HSL and 3-oxo-C12 HSL of same concentrations were taken as controls to calculate the amount of AHL being degraded. The experiment was carried out in triplicates.

5.2.3.2.2 *In planta* quorum quenching assay of *P. aeruginosa* by *Lysinibacillus* sp. Gs50

Mung beans were surface sterilised and germinated using the same protocol as given in section 5.2.3.1.1. Culture suspension of wild type *Pseudomonas aeruginosa* PAO1 (~10^9 CFU/ml) and *Lysinibacillus* sp. Gs50 (~10^8 CFU/ml) were prepared by inoculating both the strains separately in 100 ml of LB and were grown at 30°C at 200 rpm till they reached desired cell density. The cultures were centrifuged at 7000rpm for 10 min and pellet was resuspended in 10 ml of sterile PBS. For infection of sprouts, a total 4 ml bacterial suspension was prepared by adding 2 ml of *Pseudomonas aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 culture suspensions prepared in PBS. The germinated mung beans were infected with this mixed bacterial suspension and the infection process was carried out as mentioned 5.2.3.1.1.

5.2.3.2.3 CSLM analysis quorum quenching of *P. aeruginosa* by *Lysinibacillus* sp. Gs50

Mung bean seeds were surface sterilised and germinated sprouts were obtained (as described in the section 5.2.3.1.1). These germinated, healthy sprouts were transferred to Murashige-Skoog medium contained in glass tubes. Infection to healthy sprouts was carried out by adding 100 µl of culture suspensions of wild type *P. aeruginosa* PAO1-*gfp* and *Lysinibacillus* sp. Gs50 either alone or in mixtures to the plant growth medium. Sprouts were allowed to develop in gnotobiotic conditions in natural day-night cycle. After seven days plants along with their roots were harvested and roots were cut with sterile scissors. The samples for CSLM were prepared as described in section 5.2.3.1.3. The slides were kept at room temperature overnight in dark and then the root tips were imaged using confocal laser scanning microscope Ziess LSM 700. Images were taken at 63X magnification and 405nm and 488nm lasers were used for detecting the DAPI and GFP respectively. All the images were analysed and processed using the ImageJ and LSM Browser software.

5.2.4 Data Analysis

The data were subjected to one way analysis of variance (ANOVA) and mean separation were performed using Bonferroni's multiple comparisons test using Graphpad Prism software (version 6). Differences at $p < 0.05$ were considered significant.

5.3 Results

5.3.1 Influence of environmental factors on quorum quenching activity of *Lysinibacillus* sp. Gs50

To understand the influence of commonly encountered soil factors on the quorum quenching activity of *Lysinibacillus* sp. Gs50 effect of various physiological parameters was studied.

5.3.1.1 Influence of different temperature

The aim of this study was to observe if *Lysinibacillus* sp. Gs50 is able to perform quorum quenching at various temperatures. *Lysinibacillus* sp. Gs50 showed C6-HSL degradation between the temperatures 20°C to 37°C wherein the maximum activity was achieved at 37°C (Figure 32). The activity was completely lost temperatures below 20°C and above 37°C.

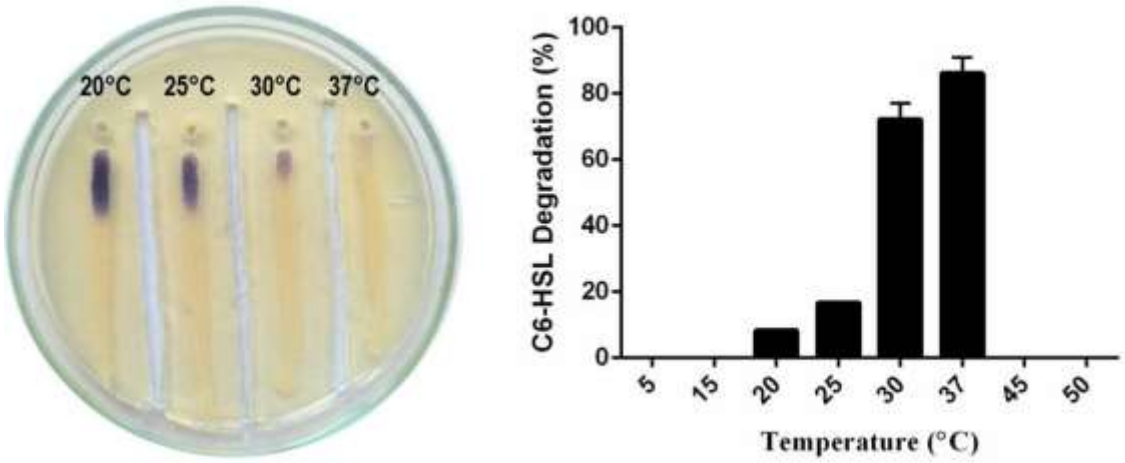


Figure 1 Influence of temperature on quorum quenching activity of *Lysinibacillus* sp. Gs50

5.3.1.2 Influence of different pH

To observe if *Lysinibacillus* sp. Gs50 can perform quorum quenching at various pH the assay was conducted. *Lysinibacillus* sp. Gs50 showed C6-HSL degradation between pH 3.6 to pH 8.0 (Figure 33). Unlike temperature range, *Lysinibacillus* sp. Gs50 was able to perform quorum quenching at all the pH in acidic as well as in basic conditions.

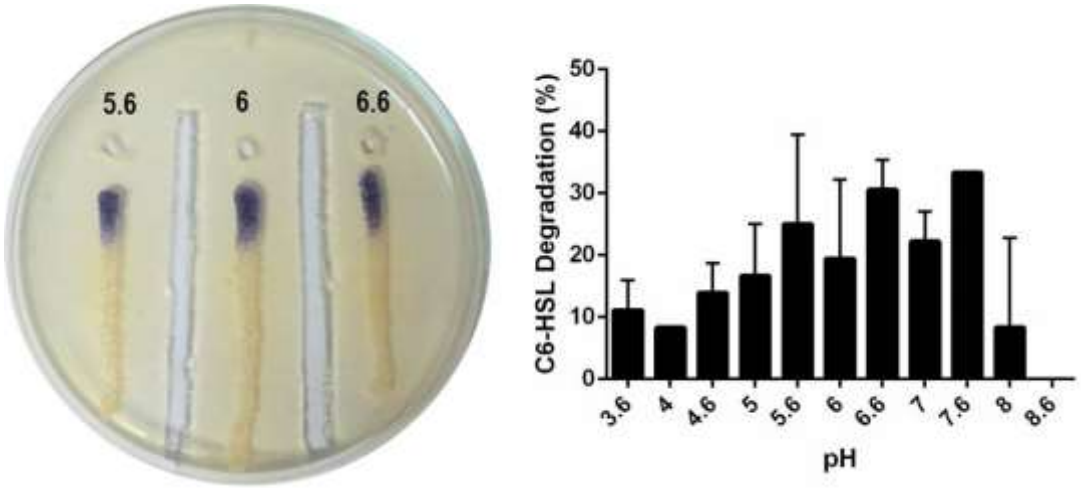


Figure 2 Influence of pH on quorum quenching activity of *Lysinibacillus* sp. Gs50

5.3.1.3 Influence substrate concentration on quorum quenching activity of *Lysinibacillus* sp. Gs50

The aim of this study was to evaluate the efficiency of quorum quenching activity of *Lysinibacillus* sp. Gs50 with increase in concentration of C6-HSL. *Lysinibacillus* sp. Gs50 showed varying percentage (%) of degradation of all different concentrations in 2 hours of incubation as shown in Figure 34.

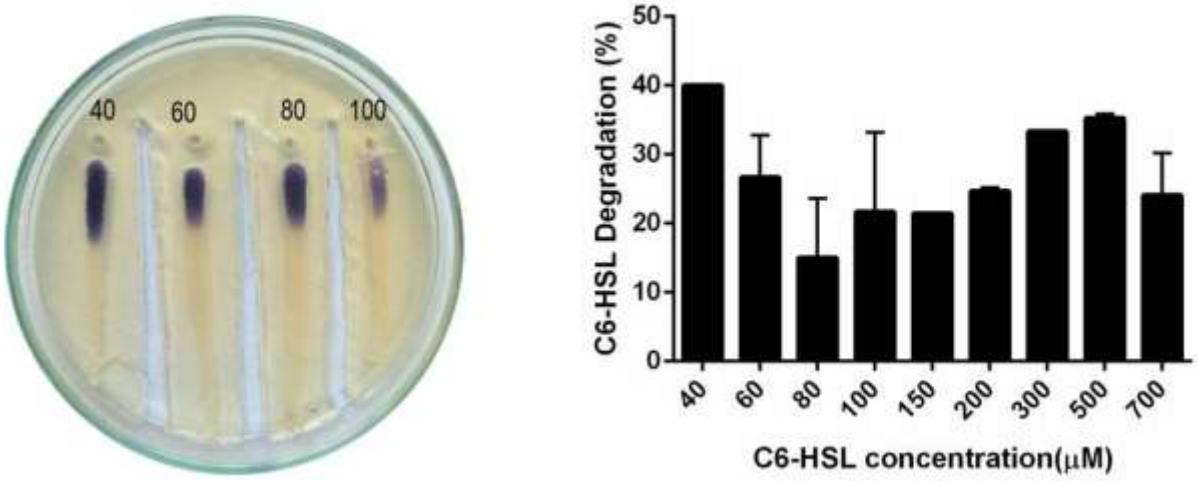


Figure 3 Influence of substrate concentration on quorum quenching activity of *Lysinibacillus* sp. Gs50

5.3.2 Efficacy of quorum quenching by *Lysinibacillus* sp. Gs50 in virulence attenuation of QS pathogen

In addition to the effect of environmental factors the spatial distribution of the QQ bacteria and the pathogen will determine the importance of where the quorum quenching bacteria should be in order to break down pathogen produced AHLs. The distance between the quorum quenching strain and pathogen is likely to be a crucial determinant in deciding whether the virulence is attenuated and therefore the quorum quenching is effective. In order to study the *in situ* spatial distribution of the quorum quenching *Lysinibacillus* sp. Gs50 and the pathogen *Pseudomonas aeruginosa* PAO1 on mung bean root, initially the infection model of *P. aeruginosa* PAO1 in host mung bean plant was established.

5.3.2.1 Mung bean infection model of *P. aeruginosa* PAO1

Germinated mung bean sprouts incubated with the culture suspensions of *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 (*lasI*$^{-}$ *rhlI*$^{-}$) achieved substantial adhesion of bacterial cells onto the seeds to the order of 1.92×10^{8} and 1.80×10^{6} CFU/ml respectively (Table 10). Subsequently, the cell count from roots of the plants 10 days post inoculation demonstrated that both the PAO1 strains were viable and colonisation at CFU/ml of 10^{5}-10^{6} on the roots throughout the growth period of plants as shown in Table 10 was maintained.

Table 2 Viable count of *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 (*lasΓ rhlΓ*) on mung bean sprouts and roots

	Initial CFU/ml	CFU/ml adhered to sprouts	CFU/mg of roots 10 days post inoculation
P.aeruginosa PAO1	$1.16\pm0.65\times10^{9}$	$1.92\pm1.64\times10^{8}$	$1.6\pm0.6\times10^{6}$
P. aeruginosa PAO1 (*lasΓ rhlΓ*)	$1.36\pm0.68\times10^{9}$	$1.80\pm1.65\times10^{6}$	$3.5\pm1.4\times10^{5}$

P. aeruginosa PAO1 wild type was not only viable on mung bean plant roots but it also caused severe infection to the host plants. Mortality rate of the sprouts treated with *P. aeruginosa* PAO1 was compared to *P. aeruginosa* PAO1 (*lasΓ rhlΓ*) treated sprouts in each trial of infection and it was found that the mortality rate was high for wildtype *P. aeruginosa* PAO1 treated sprouts (Table 11) where out of six trials it showed 40 % or more mortality rate in four trials and in the trial showing no mortality the plants were unhealthy. On the other hand, the QS mutant *P. aeruginosa* PAO1 (*lasΓ rhlΓ*) did not show any mortality. The mortality rate was defined as the percentage of sprouts that died from the total number of sprouts that were treated with the culture suspension.

Table 3 Mortality rates in different mung bean infection plant trials in *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 (*lasI⁻ rhlI⁻*) treated sprouts.

	Mortality Rate (%)					
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6
P. aeruginosa PAO1	50%	20%	40%	40%	100%	n.m.
P. aeruginosa PAO1 (*lasI⁻ rhlI⁻*)	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.

There were six sprouts/trial. n.m. is no mortality.

The remaining sprouts which survived after *P. aeruginosa* PAO1 treatment showed retarded growth. The plant growth parameters such as root length, shoot length, number of lateral roots, number of leaves of plants, and chlorophyll content of the leaves are the parameters used to quantitatively assess the damage due to disease. In this infection model, three parameters were taken into consideration to score the infection of PAO1 which were root length, shoot length and number of lateral roots. The root and shoot length of *P. aeruginosa* PAO1 treated plants was significantly reduced to 0.9±0.6 cm and 2.3±1.1 cm as compared to *P. aeruginosa* PAO1 (*lasI⁻ rhlI⁻*) treated plants and PBS treated healthy plant controls which was 2.1±0.5 cm and 4.4±0.9 cm and 3.5±0.7 cm and 5.9±0.6 cm respectively (Figure 35 (a), (b)). Also, the root development of plants treated with the wild type strain *P. aeruginosa* PAO1 was impaired as number of lateral roots developed were reduced to 0.8±1.0 compared to 3.7±2.0 of *P. aeruginosa* PAO1 (*lasI⁻ rhlI⁻*) treated plants and 6.9±2.1 of PBS treated healthy plant controls (Figure 35 (c)). Sprouts treated with the QS mutant *P. aeruginosa* PAO1 (*lasI⁻ rhlI⁻*) developed into healthy plants after 10 days. However, the values for *P. aeruginosa* PAO1 (*lasI⁻ rhlI⁻*) treated plant growth parameters (root length, shoot length and no. of lateral root branches) were intermediate, between the PBS treated plants and *P. aeruginosa* PAO1 wild type treated plants. This implies that QS is involved in *P. aeruginosa* virulence in mung bean plant infections.

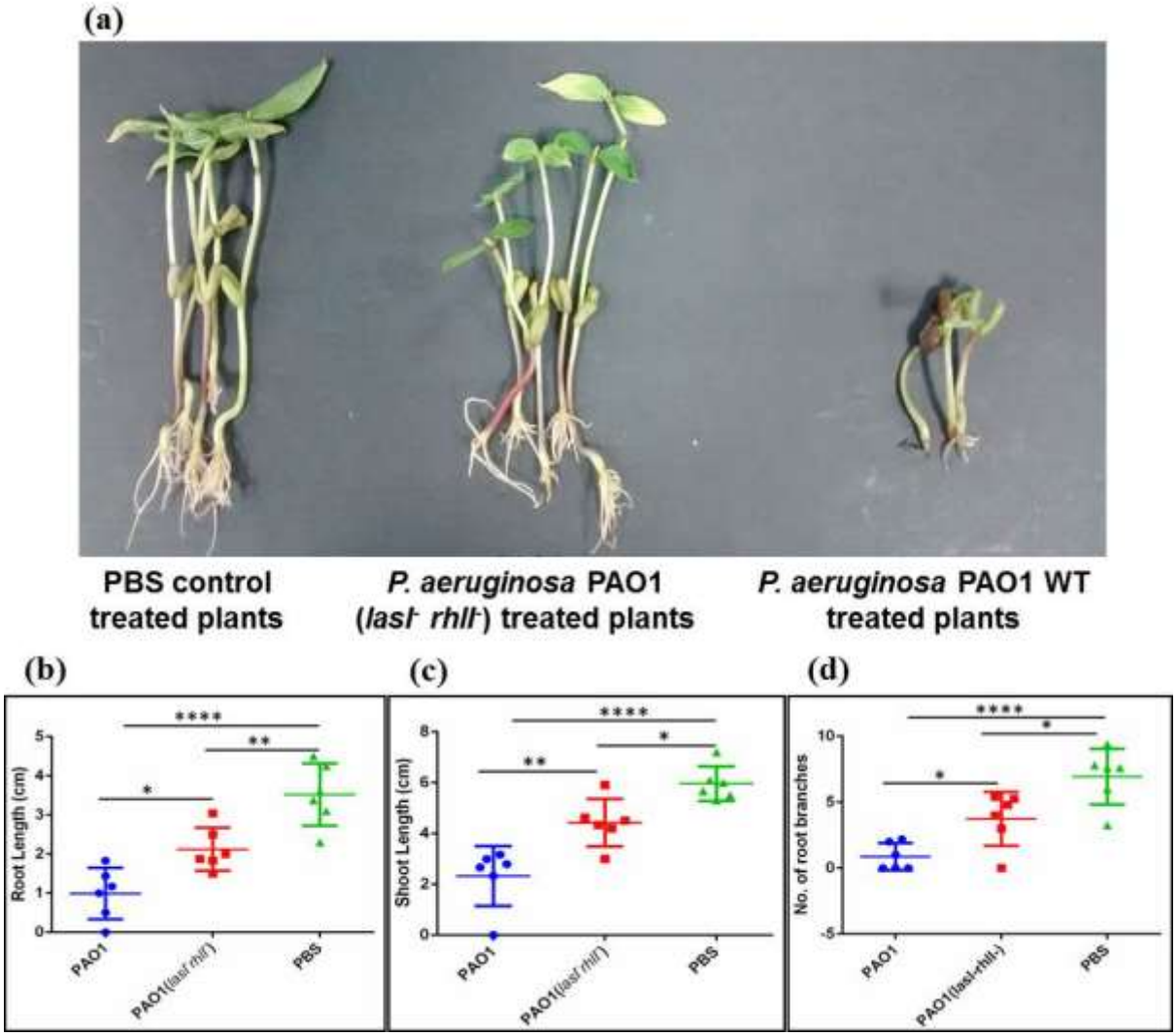


Figure 4 Comparison of *P. aeruginosa* PAO1 wild type and *P. aeruginosa* PAO1 (*lasI- rhlI-*) treated mung bean plant growth parameters

(a) Heatlth of the plants treated with *P. aeruginosa* PAO1, *P. aeruginosa* PAO1 (*lasI- rhlI-*) and PBS **(b)** Root length **(c)** Shoot length and **(d)** Number of lateral roots. Values represent the mean of six trials. Bars indicate standard deviation of the mean. Each trial has five replicates. Statistical analysis was done using one-way ANOVA Bonferroni's multiple comparisons test (*= $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$).

5.3.2.2 Role of quorum sensing in infection of *P. aeruginosa*

To further investigate the role of QS in *P. aeruginosa* PAO1 infection, their root colonisation using CSLM was examined. For this, GFP labelled *P. aeruginosa* PAO1-*gfp* (pME6032::GFP) herein after called as producer strain and the QS mutant PAO1 *lasI- rhlI-* was labeled with pMHLAS::*rfp* herein after called as sensor strain were used. The expression of RFP in the QS mutant sensor strain was AHL inducible hence in presence of either C4-HSL and/or 3-oxo-C12HSL, RFP was expressed.

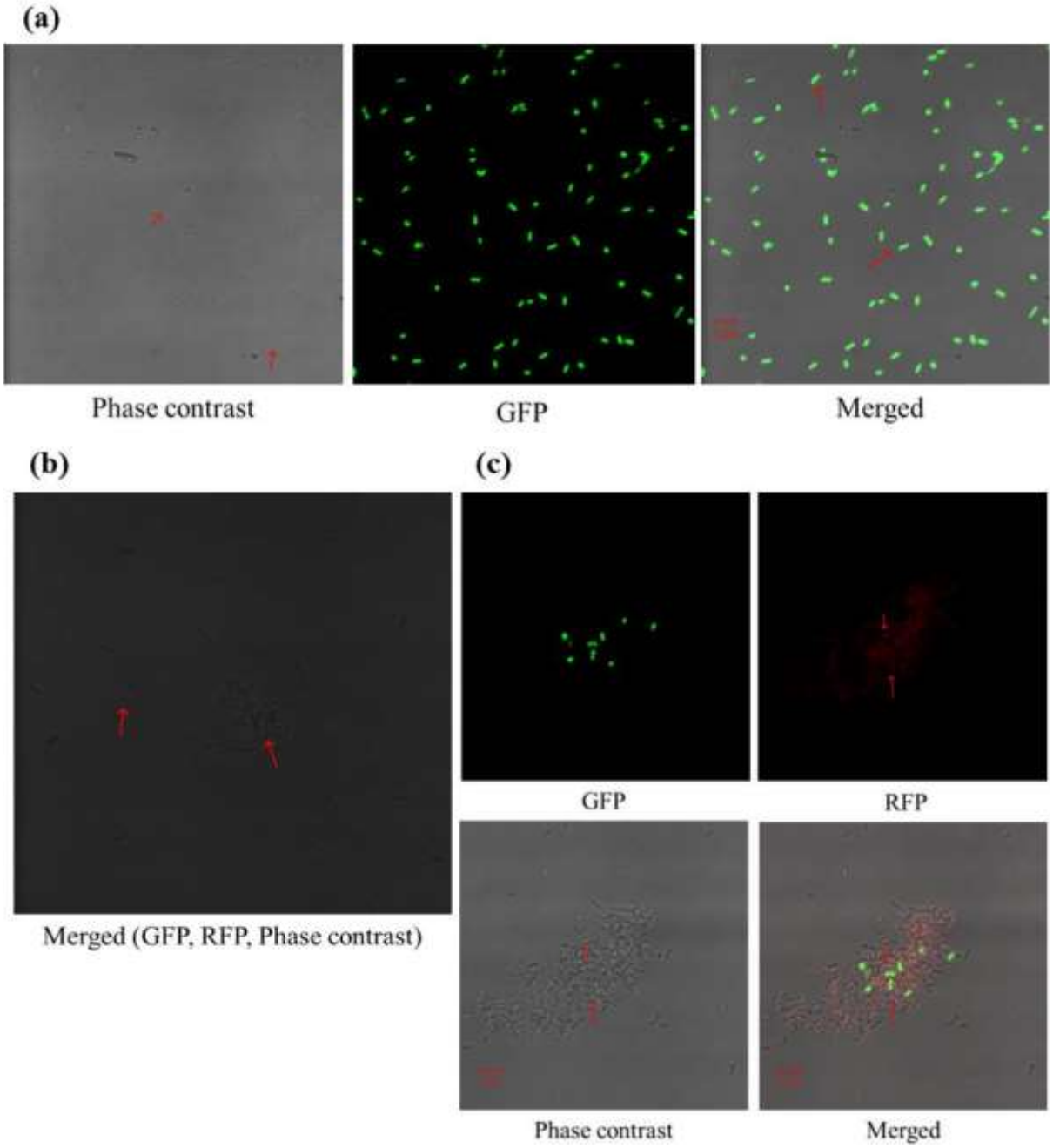


Figure 5 CSLM images of validation of *P. aeruginosa* PAO1 strains

(a) Producer cells grown alone and resuspended in PBS observed under phase contrast and imaged at 488 nm for green fluorescence **(b)** Sensor strain cells grown alone and resuspended in PBS observed under phase contrast and imaged at 555 nm for red fluorescence **(c)** Producer and sensor cells co-cultured and resuspended in PBS and imaged at 488 and 555nm

For validation, cultures of (i) AHL producer strain (ii) AHL sensor strain and (iii) co-cultured producer and sensor strainswere analysed under CSLM. *P. aeruginosa* PAO1 producer strain grown alone and having constitutively expressing GFP showed green fluorescence (Figure 36 (a)), whereas the co-cultured producer and sensor cells showed both green and red fluorescence (Figure 36 (b)). Sensor strain grown alone failed to show any red fluorescence

(Figure 36 (c)). Thus, after confirming the plasmids and strains *in vitro*, *in planta* experiments for understanding the interaction of them on plant roots were undertaken.

Active QS (AHL diffusion) was studied on the root samples of producer-sensor co-culture treated plants referred to as group (iv). As observed by CSLM *P. aeruginosa* PAO1 strains in all the groups were evenly distributed at the root tip and root hairs while on other areas of root they aligned in big heterogeneous clusters or strings along the root cell wall.

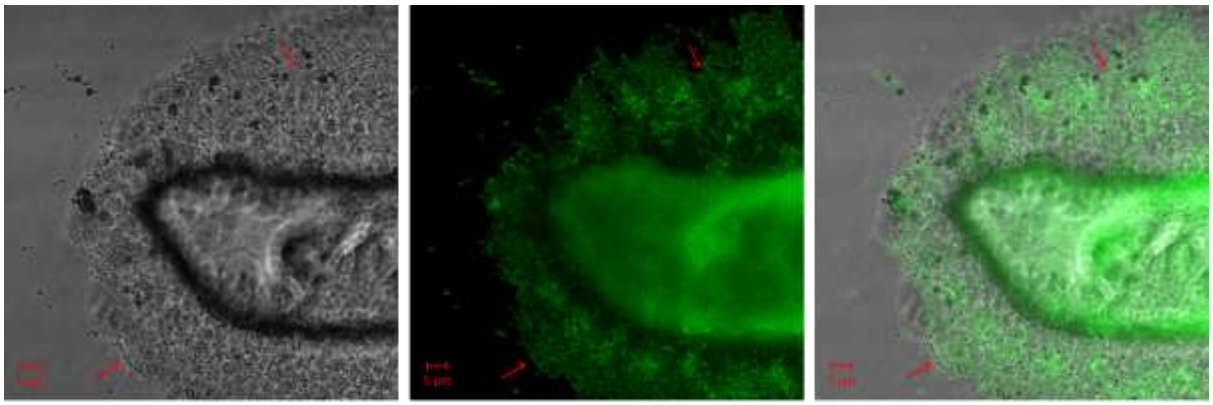
The roots infected with *P. aeruginosa* PAO1 producer strain alone (group i) showed only green fluorescent rod shaped cells all over the root in high density. Dense aggregates of bacteria on the root surface were also evident in this case (Figure 37 (a)).

In case of the group (ii) plants *P. aeruginosa* PAO1 sensor strain failed to show any red fluorescence on roots when inoculated alone (as AHL was absent), although they were present as seen in the DAPI stained images (Figure 37 (b)).

Also, the group (iii) plants treated with sensor strain along with synthetic 3-oxo-C12-HSL showed red fluorescent rod shaped cells all over the root upon imaging (Figure 37 (c)). This showed that synthetic AHL was available to the sensor strain for as long as seven days which diffused through the medium and reached the sensor cells which were colonizing the roots.

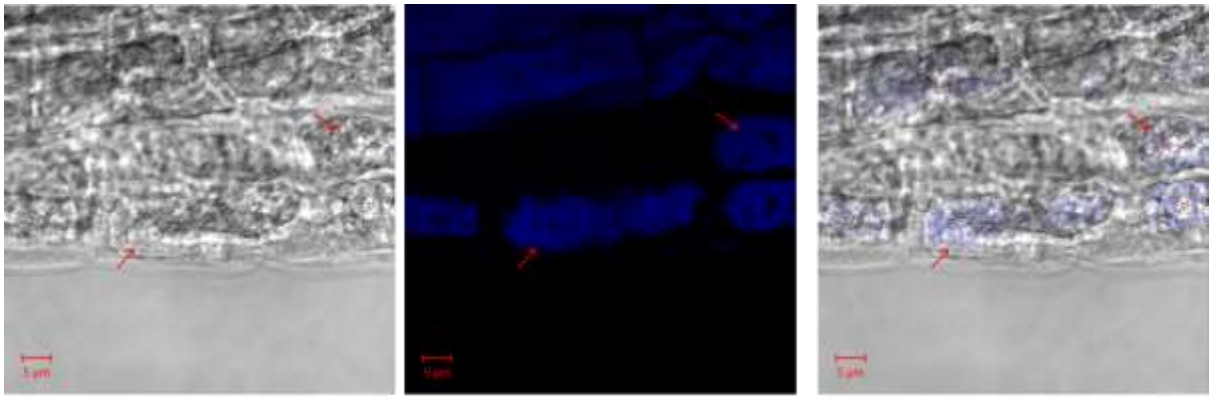
In the group (iv) plants where the AHL producing strain (PAO1-*gfp*) was co-inoculated with the AHL sensor strain PAO1 (*lasI*$^{-}$ *rhlI*$^{-}$ pMHLAS::*rfp*), the AHL producing strain activated the sensor strain as it showed red fluorescence (Figure 37 (d)). Interestingly it was observed that AHL producing strain and sensor strain were colonizing the root in microcolonies of mixed population of two strains or in other words they were co-localized. *P. aeruginosa* PAO1-producer cell was surrounded by multiple sensor red fluorescent *P. aeruginosa* PAO1 sensor cells. At the same time, the sensor strain was not necessarily always seen to associate with the AHL producer strain. Thus the co-localization was not very close in all the cases, as in one of the samples the sensor cell was imaged as far as ~ 40µm away from an AHL producing cell. Also there were DAPI stained cells of sensor strain which had failed to encounter any AHL produced by the PAO1 producer cells (Figure 37 (d)). This suggests the importance of the spatial structure of AHL producer cells and AHL receiving cells for successful quorum sensing.

(a)



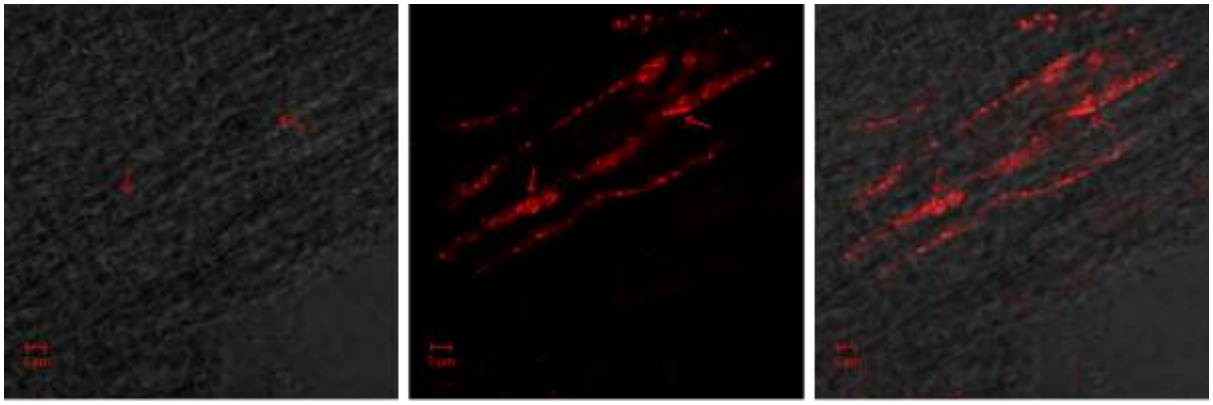
Phase contrast GFP Merged

(b)



Phase contrast DAPI Merged

(c)



Phase contrast RFP Merged

(d)

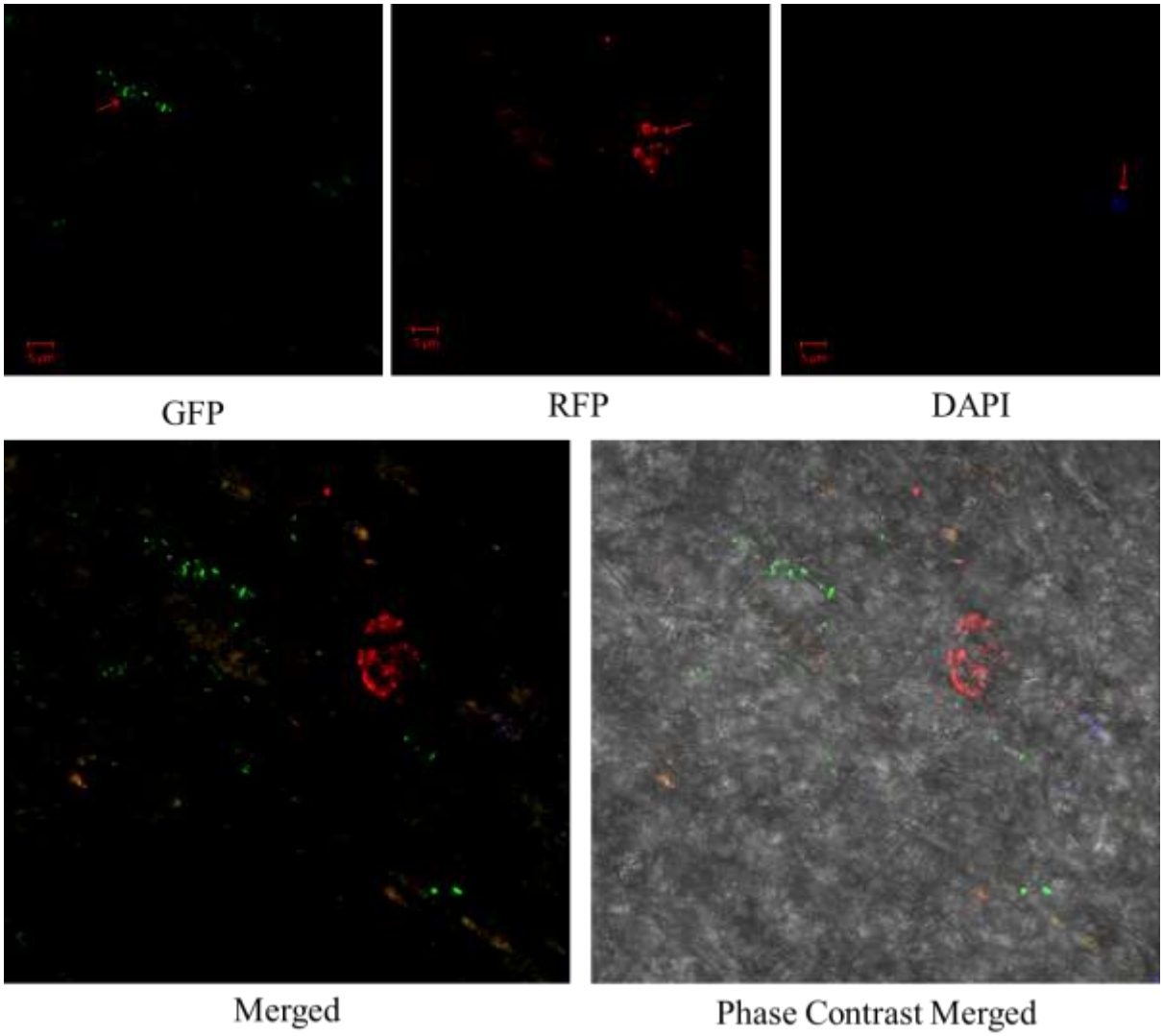


Figure 6 CSLM images of different plant root samples for visualizing active QS on mung bean roots

(a) Group (i) - image of plant root inoculated with wild type *P. aeruginosa* PAO1producer strain. The root imaged at 488nm which shows green fluorescent PAO1 cells (indicated by arrow in GFP and in merged micrograph) on and around root tip **(b)** Group (ii) - image of plant root inoculated only with sensor *P. aeruginosa* PAO1 strain stained with DAPI and imaged at 405 and 555nm. Blue colored sensor *P. aeruginosa* PAO1 cells (indicated by arrow in DAPI and in merged micrographs) on root but no red fluorescence detected due to absence of AHL. **(c)** Group (iii) - image of plant root inoculated with sensor *P. aeruginosa* PAO1 strain when synthetic 3-oxo-C12HSL is provided in plant growth medium. The root imaged at 555nm and red fluorescence showed of *P. aeruginosa* sensor PAO1 cells on root (indicated by arrow in RFP and in merged micrograph) due to AHL provided in the medium **(d)** Group (iv) - image of plant root co-inoculated with *P. aeruginosa* PAO1producer and PAO1 sensor strain. The root imaged at 405, 488 and 555nm shows green fluorescent cells of *P. aeruginosa* PAO1producer (indicated by arrow in GFP and visible in merged micrograph) red fluorescent cells of sensor *P. aeruginosa* PAO1 strain which have encountered AHL produced by *P. aeruginosa* PAO1 producer (indicated by arrow in RFP and visible in merged micrograph) and only DAPI stained blue cells (visible in merged), the cells of sensor strains which failed to receive any AHL produced by PAO1-*gfp* on mung bean plant root. A PBS control plants were also imged to normalize the noise in CSLM.

5.3.3 Quorum quenching of *P.aeruginosa* PAO1 by *Lysinibacillus* sp. Gs50

5.3.3.1 *In vitro* AHL degradation by *Lysinibacillus* sp. Gs50

Lysinibacillus sp. Gs50 has been shown to degrade different AHLs like hexanoyl homoserine lactone (C6-HSL), 3-oxo-hexanoyl homoserine lactone (3-Oxo-C6HSL) and octanoyl homoserine lactone (C8-HSL) using the biosensor *C. violaceum* CV026. *P. aeruginosa* produces two QS signalling molecules C4-HSL and 3-oxo-C12-HSL to regulate some of its virulence factor production (Schipper et al. 2009). *In vitro* AHL degradation assay with synthetic C4-HSL and 3-oxo-C12-HSL by *Lysinibacillus* sp. Gs50 was carried out to establish the quorum quenching activity of *Lysinibacillus* sp. Gs50 on these two AHLs. For this *Lysinibacillus* sp. Gs50 cell pellet was incubated with 5 µM of C4-HSL and after the end of the reaction, the remaining C4-HSL was detected using the biosensor strain *E. coli* pSB536. From the ratio of Bioluminescence/O.D. of 600nm for untreated 5 µM of C4-HSL and *Lysinibacillus* sp. Gs50 treated 5 µM C4-HSL, it could be observed that approximately 29% of 5 µM C4-HSL was degraded by *Lysinibacillus* sp. Gs50 compared to untreated 5 µM of C4-HSL (Figure 38 (a)). In contrast, 83% of 5µM of 3-oxo-C12-HSL was degraded by *Lysinibacillus* sp. Gs50 compared to untreated 3-oxo-C12-HSL control (Figure 38 (b)). As *Lysinibacillus* sp. Gs50 showed better efficiency in degradation of 3-oxo-C12-HSL, a similar AHL degradation assay was carried out with different concentration of 3-oxo-C12-HSL starting from 10 µM to 50 µM. As shown in Figure 38 (b) *Lysinibacillus* sp. Gs50 degraded all the concentrations of 10 µM, 20 µM, 30 µM, 40 µM and 50 µM 3-oxo-C12-HSL with high efficiency as compared to respective untreated controls suggesting the degradation of 3-oxo-C12-HSL by *Lysinibacillus* sp. Gs50.

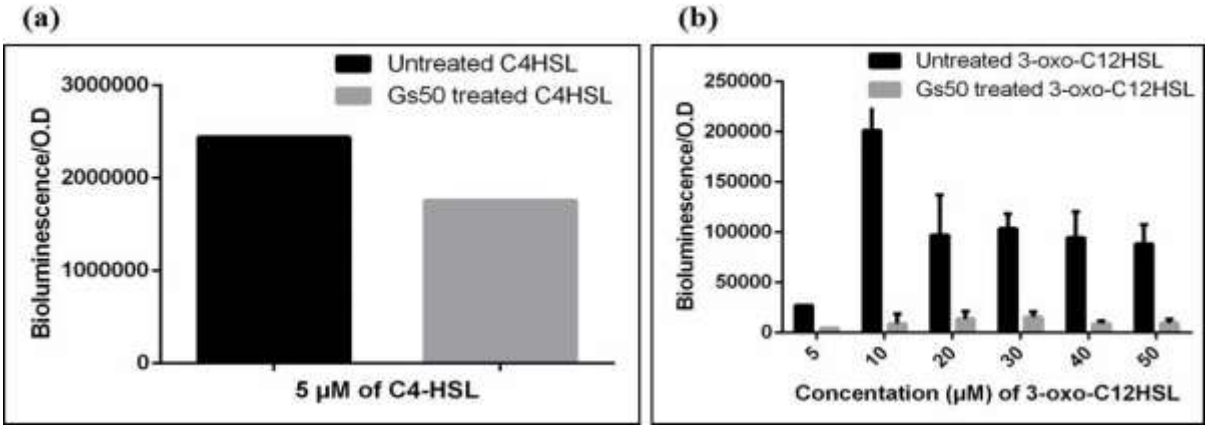


Figure 7 *In vitro* degradation of C4-HSL and 3-oxo-C12HSL by *Lysinibacillus* sp. Gs50

(a) Degradation of 5 µM of C4-HSL **(b)** Degradation of different concentration of 3-oxo-C12HSL. Values represent the mean of three trials. Bars indicate standard deviation of the mean. Each trial has three replicates.

5.3.3.2 *In planta* virulence attenuation of *P. aeruginosa* PAO1 by quorum quenching *Lysinibacillus* sp. Gs50

Next, *in planta* experiments were set up to understand the interactions between QS (pathogen) and QQ bacterial populations on plant roots when they were co-inoculated. ~10^9 CFU/ml of the pathogen *P. aeruginosa* PAO1 and ~10^8 CFU/ml of the biocontrol agent *Lysinibacillus* sp. Gs50 used for coating the seedlings were maintained at 10^5 and 10^6 CFU/ml after 10 days of inoculation demonstrated that neither had any bactericidal effects on each other i.e. they were not antagonistic to each other (Table 12). Moreover, the bacterial counts recovered from the sprouts and the roots 10 days post inoculation after treatment with both the pathogen and biocontrol agent showed similar viable count indicating that they had successfully colonized the sprouts and co-existed on the roots (Table 12).

Table 4 Viable count of *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 on mung bean sprouts and roots during the course of infection

	Initial CFU/ml	CFU/ml adhered on sprouts		CFU/mg of roots 10 days post inoculation	
		Individual control	Co-cultured	Individual control	Co-cultured
P. aeruginosa PAO1	$1.16\pm0.65\times10^9$	$1.92\pm1.64\times10^8$	$3.57\pm4.85\times10^8$	$1.3\pm0.3\times10^6$	$2.1\pm0.8\times10^5$
Lysinibacillus sp. Gs50	$4.10\pm2.42\times10^8$	$2.03\pm1.16\times10^7$	$1.24\pm1.49\times10^8$	$1.62\pm1.19\times10^7$	$1.72\pm1.32\times10^6$

The *P. aeruginosa* PAO1 wild type strain causes mortality of the mung bean sprouts and role of QS in causing the mortality was clearly observed. Mortality rate analysis showed there was a significant decrease in the mortality rate from 33 and 66 % in two trials in pathogen inoculated to no mortality in co-inoculated sprouts (Table 13). The pathogen infected plants showing no mortality were sickly in appearance. Therefore it can be inferred that the QQ ability of *Lysinibcaillus* sp. Gs50 was able to inhibit the detrimental effects of *P. aeruginosa* PAO1 on to the mung bean sprouts and allowed them to further develop into the plants.

Table 5 Mortality rate of sprouts treated with *P. aeruginosa* PAO1 alone and co-inoculated with *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50

	Mortality Rate (%)		
	Trial 1	Trial 2	Trail 3
P. aeruginosa PAO1 alone	33%	66%	n.m.
P. aeruginosa PAO1+*Lysinibacillus* sp. Gs50	n.m.	n.m.	n.m.

There were three seedlings/trial. n.m. is no mortality detected

The effect of QQ by *Lysinibacillus* sp. Gs50 was visible on the plants which were co-inoculated with *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50. The plants were healthy compared to plants infected with *P. aeruginosa* PAO1. Root length, shoot length and number of lateral roots of plants was significantly higher in plants co-cultured with *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 (2.4±0.3 cm, 3.6±0.6 cm and 5.1±1.1) compared to only *P. aeruginosa* PAO1 treated ones (0.6±0.5 cm, 1.3±0.7 cm and 0.6±0.5) (Figure 39 (a, b and c)). Here, the biocontrol potential of the AHL lactonase producing *Lysinibacillus* sp. Gs50 was tested against *Pseudomonas aeruginosa* PAO1 and was demonstrated to be as effective as against *Pcc*BR1 (as discussed in chapter 3).

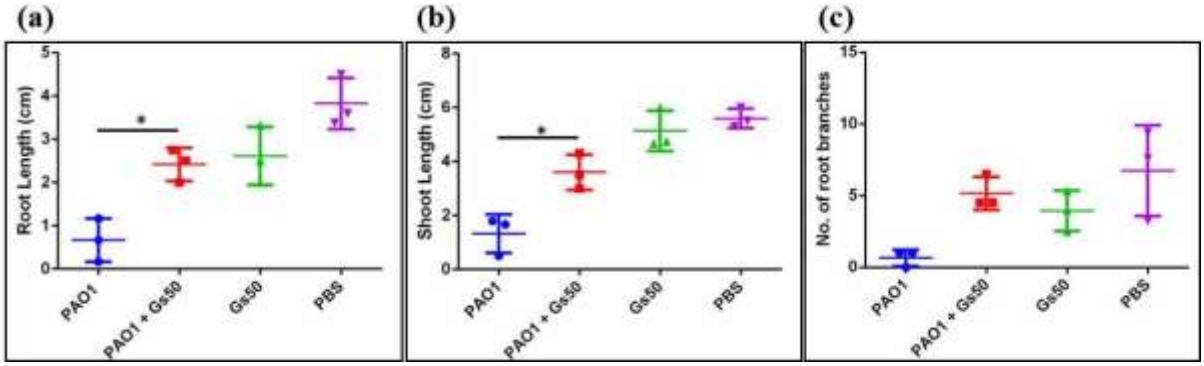


Figure 8 *In planta* virulence attenuation of *P. aeruginosa* PAO1 by quorum quenching *Lysinibacillus* sp. Gs50

(a) Root length **(b)** Shoot length **(c)** number of lateral roots of the plants treated with culture suspension of *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50. Values represent the mean of three trials. Bars indicate standard deviation of the mean. Each trial has three replicates. Statistical analysis was done using one-way ANOVA Bonferroni's multiple comparisons test (*= $p < 0.05$). The variance of the mean for other columns in all three graphs is nonsignificant

5.3.3.3 Spatial distribution of quorum sensing pathogen *P. aeruginosa* PAO1 and quorum quenching *Lysinibacillus* sp. Gs50

To understand the spatial distribution of two bacterial populations the visualization was done by confocal microscopy.

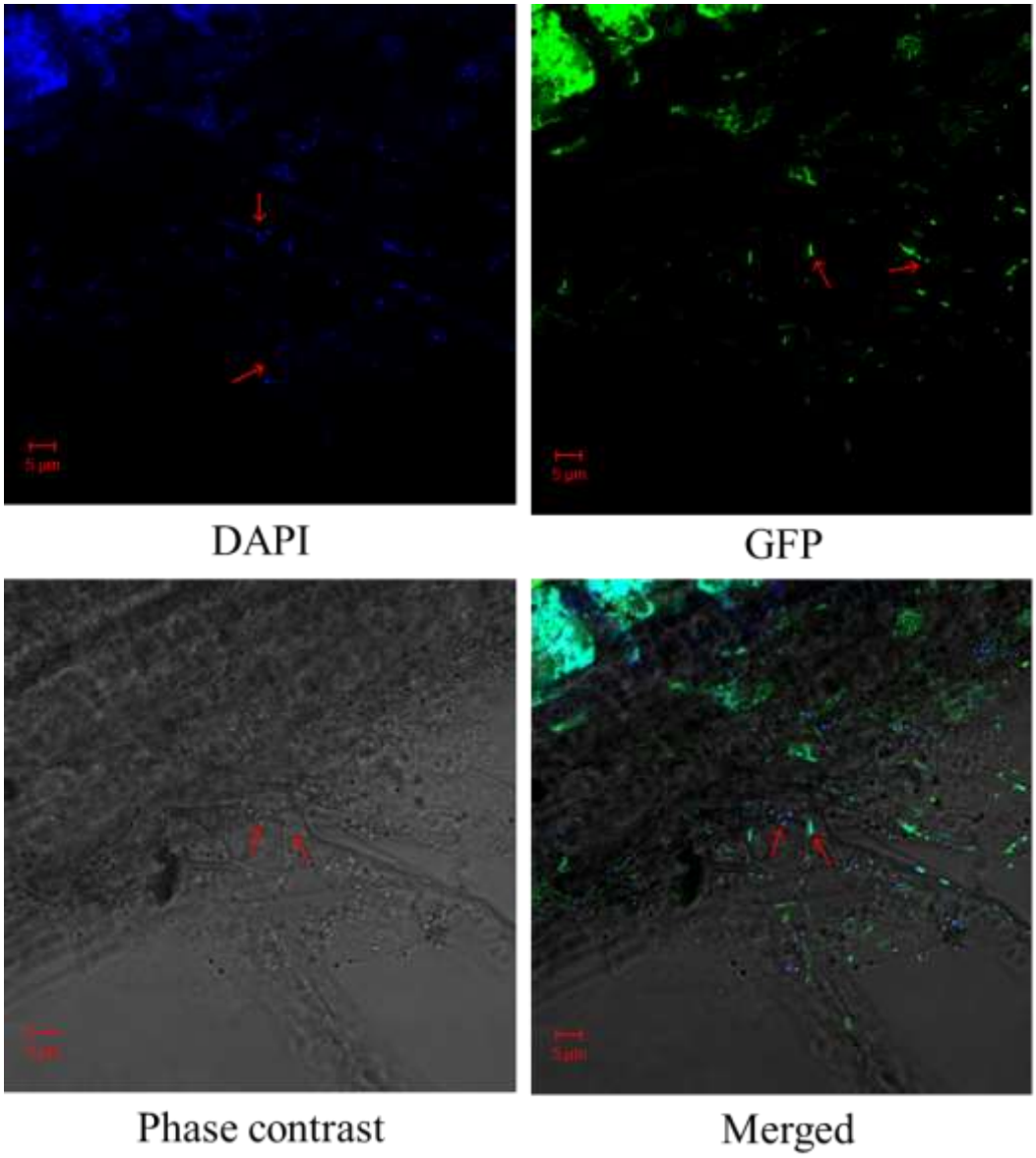


Figure 9 CSLM micrograph of *P. aeruginosa* PAO1-gfp and *Lysinibacillus* sp. Gs50 co-inoculated plant root

The sample was imaged at 405nm for DAPI showing blue *Lysinibacillus* sp. Gs50 and *P. aeruginosa* PAO1 cells (indicated by arrow in DAPI stained panel), 488nm for GFP showing *P. aeruginosa* PAO1-*gfp* only (indicated by arrow in GFP stained panel) and the merged image showing co-localized blue *Lysinibacillus* sp. Gs50 and blue-green *P.aeruginosa* PAO1 cells (indicated by arrows in merged)

Root tips/root hair of only *P. aeruginosa* PAO1-*gfp* treated plants showed that surface of root was completely covered by green fluorescent small rod shaped cells (As shown in above

section Figure 37 (a)). The images show that both *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 were equally efficient in colonizing the mung bean roots. The image of root co-inoculated with *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 showed that the root was massively covered with two different types of bacterial population. One population of smaller rod shaped cells having blue-green fluorescence was of PAO1 and other population of bigger rod shaped cells and stained only blue (DAPI) was of *Lysinibacillus* sp. Gs50 (Figure 40). Structures resembling spores of *Lysinibacillus* were also observed. Both these populations were co-localized throughout the root surface and their spatial distribution was homogeneous. *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 did not show any niche specificity in our studies and were not found in separate microcolonies of their own rather there were bacterial cell clusters having both the type of cells in close vicinity of each other.

5.4 Discussion

Study of the effect of different abiotic parameter on the biocontrol mechanism is crucial for optimising any biocontrol strategy. Physico-chemical parameters like pH, temperature and substrate concentration are important factors which can affect the biocontrol mechanism of biocontrol strains as they dectate the flora of soil. *Lysinibacillus* sp. Gs50 has previously been established as an efficient quorum quenching isolate therefore it was necessary to evaluate the effects of parameters like pH, temperature and substrate concentration on quorum quenching activity of *Lysinibacillus* sp. Gs50 for its further application as biocontrol agent. It is clear from the results that *Lysinibacillus* sp. Gs50 can perform quorum quenching in a wide range of pH, temperature and substrate concentration hence has potential to be used as biocontrol agent against quorum sensing plant pathogens. In addition to these parameters the efficacy of quorum quenching mechanism would also depend on the spatial structure of pathogen and QQ strain on the roots of the plants. The QQ strain Lysinibacillus sp. Gs50 was found to co-localise with the pathogen in our studies which further supports this biocontrol stategy as an effective means to attenuate the virulence of AHL regulated phytopathogens.

Given that *P. aeruginosa* is a natural soil inhabitant and a plant pathogen, a simple plant model to study mechanism of pathogenesis in this organism was developed. Mung bean sprouts over the more popular *Arabidopsis* model was chosen. The overall understanding of the *Arabidopsis* model suggests that it takes 4–6 weeks to grow the plants and then almost 5 days post inoculation to observe the symptoms with a lot of stringent conditions for growth of

the plant (Rahme et al., 1995; Walker et al., 2004). The mung bean model takes maximum 12 days for observation of symptoms which include 1 day to obtain germinated mung bean seedlings, 1 day for infecting the germinated seeds of mung bean with *P. aeruginosa* PAO1 cells and then 7-10 days to achieve the symptoms. The role of *las* and *rhl* quorum sensing systems in *P. aeruginosa* PAO1 pathogenesis was investigated by using a quorum sensing mutant. As was observed there was no mortality caused by *P. aeruginosa* PAO1 (*lasI⁻ rhlI⁻*) mutant and the health of the plants were significantly scored higher than *P. aeruginosa* PAO1 infected plants. This suggests the importance of *las* and *rhl* QS systems in the expression of virulence factors (protease, elastase, alkaline phosphatase) of *P. aeruginosa* PAO1 which are responsible for degradation of the plant tissues and cause the death/infection of the plant. Interestingly, comparison of the health of untreated plants (PBS treated control plants) and *P. aeruginosa* PAO1 (*lasI⁻ rhlI⁻*) treated plants shows, that the latter were not as healthy and the difference observed in the parameters like root length, shoot length and number of lateral roots is significant. It was observed that the loss of *las* and *rhl* QS systems significantly decreased the virulence levels, however the infection is not completely absent (Figure 41 a, b, c) which suggests that there are other factors responsible for *P. aeruginosa* PAO1 pathogenesis in the plant which are not regulated by *las* and *rhl* QS systems. Further, the wildtype *P. aeruginosa* PAO1 was actively carrying quorum sensing was confirmed by confocal images of roots which were co-inoculated with populations of *P. aeruginosa* PAO1 wildtype and QS mutant *P. aeruginosa* PAO1 (*lasI⁻ rhlI⁻*) pMHLAS-*rfp* where the latter prominently showed the induction of red fluorescence suggesting that the 3-oxo-C12HSL produced by *P. aeruginosa* PAO1 wildtype was able to diffuse and was perceived by QS mutant *P. aeruginosa* PAO1 (*lasI⁻ rhlI⁻*) leading to RFP expression. The effective concentration of 3-oxo-C12-HSL in plant roots can be speculated to be a minimum of 8 nM as the sensitivity of the sensor plasmid suggests.

Having developed the *P. aeruginosa* PAO1-mung bean infection model and further demonstrated the involvement of QS in regulation of *P. aeruginosa* PAO1 pathogenesis, next focus was to evaluate the QQ based biocontrol potential of *Lysinibacillus* sp. Gs50 against *P. aeruginosa* PAO1. The good health of the plants co-inoculated with *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 was evidence that 3-oxo-C12 HSL was degraded by *Lysinibacillus* sp. Gs50 *in planta*. This suggested that degradation of QS molecules caused the attenuation of virulence of *P. aeruginosa* PAO1. In spite of low *in vitro* degradation of C4-HSL as compared to 3-oxo-C12 HSL shown by *Lysinibacillus* sp. Gs50, good health of the plants co-inoculated with *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 can be explained by the fact

that *las* and *rhl* QS systems function hierarchically in *P. aeruginosa* PAO1. The degradation of 3-oxo-C12 HSL by *Lysinibacillus* sp. Gs50 *in planta* could result in decreased levels of C4-HSL as well, which as a consequence could have exerted a cumulative effect on down regulating both QS systems. This confirmed that the QQ activity of *Lysinibacillus* sp. Gs50 is effective against *Pectobacterium carotovorum* as well as *P. aeruginosa* and significantly attenuate their infection to the host plant. The co-localization of *Lysinibacillus* sp. Gs50 with *P. aeruginosa* PAO1 on mung bean roots has suggested that there are interactions taking place between QQ and QS populations in natural environments like root surface. These findings throw more light on the biocontrol mechanism exerted by QQ *Lysinibacillus* sp. Gs50 considering the fact that the enzyme AHL lactonase is expressed intracellularly (as confirmed in chapter 4) and the AHLs that are produced by *P. aeruginosa* PAO1 are freely diffusible in and out of the cell. The co-localization of these two populations makes the availability of the AHLs (substrate) to the AHL lactonase (enzyme) easier for AHL degradation (interference in QS or QQ) as shown in the mung bean model. The fact that the plants that were co-inoculated with QS and QQ bacteria showed better plant growth strengthens the role of QQ in attenuating the pathogenesis. The knowledge about the spatial distribution of the QS and QQ populations involved in more complex multispecies natural environments will help in establishing whether QQ has future therapeutic potential.