Chapter 3

Characterization of the selected AHL degrading *Bacillus* isolates for attenuation of soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum*

3.1 Introduction

Pectobacteria cause a range of plant diseases like blackleg and soft rot in a wide range of crop and ornamental plants that include *P. atrosepticum* (*Pa*), *P. carotovorum* subsp. *carotovorum* (*Pcc*), and the recently characterised *P. wasabiae* and *P. carotovorum* subsp. *brasiliense*. Among these, *Pcc* has widest host range of all the soft rot bacteria. Potato being one of the economically important crops affected by *Pcc* in temperate regions, the epidemiology of potato soft rot is well studied (Davidsson et al. 2013). In soft rot, tuber tissue is macerated giving creamy consistency which turns black later in the presence of air, producing a foul smell when invaded by secondary organisms. In storage, the lesion can spread to the whole tuber and then to neighbouring tubers. In inadequately ventilated cool stores, rotting can spread to adjoining tubers as liquid from the rotting tubers percolates onto others, sometimes leading to massive rotting pockets in the stored tuber lot. Thus Soft rot of potato tubers caused by *Pcc* can result in extensive post-harvest losses, especially during storage (Czajkowski et al. 2011). In the field conditions the healthy plants can get infected by *Pcc* found near seed tuber in soil (De Boer et al. 1978; Helias et al. 2000). *Pcc* colonizes on potato roots and enters the stem and progeny tubers through the vascular system. Once in the stems, the pathogen do not necessarily cause stem rot (blackleg) but can survive in latent form (Czajkowski et al. 2010). The pathogen released by the rotten mother tuber is transmitted through soil water to contaminate neighbouring progeny tubers. Flying insect vectors or aerosols produced by rain can bring about long-distance transmission (Perombelon 1974).

Some plant pathogens such as *Pseudomonas syringae* require living host tissue as part of the infection process, during which they actively manipulate host defences. These kinds of pathogens have been termed ‘stealth’ pathogens due to their ability to manipulate plant defences as part of the infection process. In contrast to this group of pathogens, *Pcc* is termed as ‘brute force’ pathogen since it causes necrotrophic damage through physical attack on plant cell wall primarily due to production of cell wall degrading enzymes that cause the necrosis of host tissue. This co-ordinated and prolific production of virulence factors at high population density is regulated by a cell-cell communication mechanism known as quorum sensing (QS) preventing premature activation of plant defences and elevating the possibility of successful infection (Zhang and Dong 2004; Liu et al. 2008). The signalling molecules of QS are *N*-acyl homoserine lactones (AHLs) responsible for controlling the virulence factors

production which include plant cell wall degrading enzymes (PCWDE) pectate lyases, pectinase and cellulase. Quorum sensing also regulates harpin and other factors secreted by Type III secretion system, small number of virulence regulators, and the antibiotic carbapenem for the full virulence in *Pcc* (Jones et al. 1993; Chatterjee et al. 1995; Mattinen et al. 2004; Cui et al. 2005; McGowan et al. 2005; Pemberton et al. 2005). One such pytopathogenic strain of *Pcc* from eggplant (*Pcc*BR1) was earlier isolated and its epidemiology was thoroughly studied in our laboratory (Maisuria and Nerurkar 2013). It was found to cause soft rot in wide range of fruits and vegetables like *Beta vulgaris* (Sugar beet), *Trichosanthes cucumerina* (Snake gourd), *Brassica oleracea* (Cabbage), *Lpomoea batatas* (Sweet potato), *Pyrus communis* (Pear fruit), *Daucus carota* (Carrot), *Solanum melongena* (Brinjal), *Lagenaria siceraria* (Molina) Standl. (Bottle gourd), *Cucumis sativus* (Cucumber), *Solanum tuberosum* (Potato). This strain belongs to class II strains of *Pcc* and produces the quorum sensing signal molecule 3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) that regulates its PCWDE production to cause soft rot to different host plants. A thorough biochemical characterisation of its virulence factors viz. polygalturonase and pectate lyase has been carried out (Maisuria et al. 2010; Maisuria and Nerurkar 2012).

Quorum sensing, being a prerequisite for pathogenesis of *Pcc* disrupting its regulation could prevent the production of virulence factors which makes it an appropriate target for development of new phytoprotective agents (Barnard and Salmond 2007). The rhizosphere of plants, along with AHL producing pathogens like *Pcc,* also hosts AHL degrading bacteria (D'Angelo-Picard et al. 2005; Jafra et al. 2006; Chan et al. 2011). The degradation of AHLs inactivates the signal and leads to the disruption of QS which is termed as quorum quenching (QQ) (Uroz et al. 2009). The first QQ bacteria were identified from soil and belong to the Gram-positive *Bacillus* (Dong et al. 2000). Potato rhizosphere also showed the abundance of AHL degrading bacteria and they mainly belong to *Agrobacterium*, *Bacillus*, *Pseudomonas*, *Delftia*, *Ochrobactrum* and *Rhodococcus* genera (Jafra et al. 2006; Cirou et al. 2007). Use of AHL degrading bacteria for attenuation of soft rot on potato against *P. atrosepticum* (Smadja et al. 2004) or *P. carotovorum* has also been demonstrated (Molina et al. 2003; Uroz et al. 2003; Uroz et al. 2009). A distinct characteristic of this strategy is that it attenuates the production of virulence factors and does not kill the pathogen as other antimicrobial methods do (Faure and Dessaux 2007).

Commonly biocontrol strategy against *Pcetobacterium* spp. is antagonism by fluorescent pseudomonads and *Bacillus* strains for the control of diseases like blackleg and soft rot. The antagonistic properties exhibited by these strains were due to antibiotic synthesis, iron competition and induced systemic resistance in host (De Weger et al. 1986; Xu and Gross 1986; De Weger et al. 1995; Cronin et al. 1997; Sharga and Lyon 1998). In order to confirm them as biocontrol agents these strains possessing antagonistic activity against pathogen were evaluated exhaustively for their biocontrol attributes. Along with the *in vitro* antagonistic activity of the isolates, the focus of the studies was also on their ability to colonize root surfaces and the persistence of *Pseudomonas* strains on treated plants. Additionally, the influence of other abiotic factors like soil texture and pH on the expression of antagonistic activities was observed to establish the antagonist as biocontrol agent (Diallo et al. 2011). Similar studies testing the bacterial QQ mechanism as effective biocontrol approach are sparse in literature. AHL degraders and their molecular mechanism of AHL degradation are being identified with great interest (Lee et al. 2002; Park et al. 2003; Park et al. 2005; Park et al. 2006; Pan et al. 2008; Mei et al. 2010; Wang et al. 2012; Yin et al. 2010) but evaluation of the biocontrol attributes of the AHL degrading bacteria for controlling the soft rot caused by *Pcc* has received less attention.

Current study discusses the biocontrol properties of QQ bacteria such as adherence on seeds, colonisation ability and persistence of QQ isolates on root (mung bean model), broad host range which is susceptible to *Pcc* (Potato, Carrot and Cucumber) on which the QQ isolates can survive and exhibit the biocontrol potential. Additionally, studies showing absence of deleterious effect of the isolates on the host plant and the ability of the QQ isolates to control the disease pre-infection (preventive) and post-infection (curative) are included. The broad objective of the present study is to evaluate diverse biocontrol properties of QQ bacteria for the control of soft rot caused by *Pcc*BR1.

3.2 Materials and method

3.2.1 Bacterial strains and culture conditions

Pectobacterium carotovorum subsp. *carotovorum* BR1 soft rot causing plant pathogen (Laboratory stock) and *Bacillus* isolates were grown and maintained on LB medium at 30°C under shaking condition.

3.2.2 *Pcc*BR1 growth inhibition assay

Overnight grown culture of *Pcc*BR1 was overlaid (100 µl) on LA in 1% soft agar and 48 hours grown *Bacillus* culture supernatants were added to the wells punched into these plates. 1% SDS was added to one of the wells as a positive control for zone of inhibition. The zone of inhibition was then observed for each isolate.

3.2.3 Effect of *Bacillus* isolates on potato host

Potato tubers purchased from local market, were gently washed with running tap water and surface sterilized in laminar hood by immersing sequentially in 0.01% mercury chloride, 70% ethanol and sterile distilled water (for a thorough rinse), followed by drying under laminar hood. Slices of uniform thickness (4-5 mm) were cut with a UV sterilized slicer and a single potato slice was placed in each sterile Petri dish with 3-4ml sterile water. 5 µl of overnight grown *Bacillus* isolates (~ 2.5 $\times 10^8$ CFU/ml) were injected at three points on each potato slice using sterile disposable syringes in aseptic condition. For negative control only LB was inoculated on a slice in the similar manner. The slices were placed in Petri dishes and further incubated at 30°C for 24 hours. Presence of maceration was observed as compared to the positive control.

3.2.4 *In vitro* co-culture assay

Overnight cultures of selected *Bacillus* isolates (10^8 CFU/ml) were used to initiate co-cultures with *Pcc*BR1 (10^8 CFU/ml) in LB medium and incubated at 30°C under shaking condition for 12 hours. Similarly, *Pcc*BR1 alone was inoculated as control to monitor its growth rate and 3-oxo-C6-HSL production. The CFU counts of the members of the co-cultures were recorded separately based on their distinct colony morphologies on LA plates. Samples were withdrawn after 12 hours for determination of the amount of 3-oxo-C6-HSL present in culture supernatant using the biosensor *C. violaceum* CV026. The supernatant from pure culture of *Pcc*BR1 was loaded into an 8 mm well cut into an LA plate overlaid with *C. violaceum* and the presence of 3-oxo-C6-HSL produced was demonstrated by occurrence of purple coloured zone surrounding the well. Similarly the supernatant from each co-culture was loaded into an 8 mm well cut into an LA plate overlaid with *C. violaceum* and the degradation of 3-oxo-C6-HSL produced by *Pcc*BR1 was determined by the reduction in purple-coloured zone around the well. All experiments were done in triplicate.

3.2.5 Effect of *Bacillus* isolates on virulence factor production

Selected *Bacillus* isolates (10^8 CFU/ml) were individually co-cultured with *Pcc*BR1 (10^8 CFU/ml) in LB and incubated at 30°C under shaking conditions for 24 hours (Maisuria and Nerurkar 2015). A pure culture of *Pcc*BR1 was used as a control. The activity of two major *Pcc*BR1 virulence factors, polygalacturonase (PG) and pectin lyase (PNL) was measured.

PG activity was determined by measuring reducing sugar released due to hydrolysis of polygalacturonic acid using dinitrosalicylic acid (DNS) reagent (Miller 1959). 100 µl of culture supernatant from either pure *Pcc*BR1 cultures or from co-cultures was added to 200 µl 0.5% (w/v) polygalacturonic acid (PGA) in 50mM Tris-Cl (pH 8.0) and the reactions were incubated at 40°C for 30 min. The absorbance of the reaction product (reducing sugar) was quantified at 540nm. D-galacturonic acid was used as a reducing sugar standard. One unit of enzyme activity was defined as the amount of enzyme required to release 1µmole of D-galacturonic acid per minute.

PNL activity was determined by measuring unsaturated oligosaccharides released as a result of the cleavage of polygalacturonic acid using a thiobarbituric acid (TBA) reagent (Nedjma et al. 2001). 100 µl of culture supernatant from either pure *Pcc*BR1 cultures or from co-cultures was added to 250 µl of 0.5% (w/v) Pectin in 50mM Tris-HCl buffer (pH 8.0) containing 5mM EDTA and the reactions were incubated at 40°C for 1 hour and the absorbance of the final reaction product measured at 550nm. One unit of PNL activity was defined as the amount of enzyme required to change absorbance by 0.01 O.D per hour. All experiments were done in triplicate.

3.2.6 *In vitro* soft rot attenuation assay on different hosts of *Pcc*BR1

The *in vitro* soft rot attenuation assay as described by Dong et al. (2004) was modified and performed on potato tuber slices. Potato tubers purchased from local market and slices were prepared according to section 3.2.3. Each potato slice was weighed under sterile conditions (using a sterile container) before inoculation. The *Bacillus* isolates and *Pcc*BR1 were cultivated overnight. *Bacillus* isolates were then sub-cultured and were allowed to attain ~ 2.5 $\times 10^8$ CFU/ml and *Pcc*BR1 was cultivated to attain ~2.8 $\times 10^6$ CFU/ml in LB broth at 30°C. Thereafter 50 µl of AHL degrading isolate was mixed with 50 µl of *Pcc*BR1. 5 µl from above mixture was injected at three points on each potato slice using sterile disposable syringes in

sterile condition. The slices were placed in Petri dishes and 3-4 ml of sterile distilled water was added into the plates and further incubated at 30 °C for 24 hours. The maceration area (in mm^2) was calculated using diameter of the macerated region measured by a foot ruler. Macerated tissue weight, additionally, was measured by scooping out the macerated region and weighing. Maceration (%) was calculated using the following formula

$$\text{Maceration (\%)} = \frac{\text{Weight of macerated tissue}}{\text{Weight of tissue before inoculation}} \times 100$$

*Pcc*BR1 is broad host range pathogen and it can infect carrot (*Daucus carota*) and cucumber (*Cucumis sativus*). Carrot and cucumber purchased from local market and slices were made according to 3.2.3. Slices of uniform thickness were inoculated in the center of the slice with the mixture of *Bacillus* isolates (~10^8 CFU/ml) and *Pcc*BR1 (~2.8 ×10^6 CFU/ml). The slices were placed in Petri dishes and incubated at 30 °C for 24 hours. Quantification of biocontrol parameters were done as mentioned above. Potato, carrot and cucumber slices inoculated with *Pcc*BR1 alone were positive control for maceration and slices inoculated with sterile LB were no treatment controls. Treatments consisted of three replicate slices in separate Petri plates. Experiments done with all host consisted three or five independent trials each having three replicates.

3.2.7 *In vitro* curative biocontrol assay for soft rot caused by *Pcc*BR1

The approach used by Molina et al. (2003) was followed to perform curative *in vitro* biocontrol assay. Potato tubers were purchased from local market, surfaced sterilized and sliced as mentioned in the section 3.2.6. Single potato slice was placed in each sterile Petri dish. Each potato slice was weighed under sterile conditions (using a sterile container) before inoculation. *Pcc*BR1 was grown for overnight and was sub-cultured to reach ~2.8 ×10^6 CFU/ml in LB broth at 30°C. Each potato slice was inoculated with 5 µl of *Pcc*BR1 in the centre using sterile disposable syringes in sterile condition. 3-4 ml of sterile distilled water was added to each Petri plate containing potato slice. *Pcc*BR1 inoculated slices were incubated at 30°C for 12 hours. *Bacillus* isolates were grown overnight and then sub-cultured to reach ~ 2.5 ×10^8 CFU/ml. After 12 hours of incubation of potato slices, 5 µl of *Bacillus* culture was inoculated at the same point where the *Pcc*BR1 was inoculated and again the slices were kept at 30°C for additional 24 hours of incubation. Inoculation of *Pcc*BR1 alone was done on one of the potato slice as positive control and only LB was inoculated on a slice in a similar way to act as a negative control. The maceration area (in mm^2) and maceration

(%) was calculated as mentioned in section 3.2.6. Five independent trials were carried out each trial contained three replicates.

3.2.8 *In vitro* preventive biocontrol assay for soft rot by *Pcc*BR1

To perform the preventive *in vitro* biocontrol assay too the approach used by Molina et al. (2003) was followed with slight modification. Potato slices were made and inoculated as per section 3.2.6. Each potato slice was inoculated with 5 µl of *Bacillus* in the centre using sterile disposable syringes in sterile condition. 3-4 ml of sterile distilled water was added to each Petri plate containing potato slice. These slices were incubated at 30°C for 12 hours. *Pcc*BR1 was grown overnight and was sub-cultured to reach ~2.8 $\times 10^6$ CFU/ml in LB broth at 30°C. After 12 hours of incubation of potato slices, 5 µl of *Pcc*BR1 culture was inoculated at the same point where the *Bacillus* isolates were inoculated and again the slices were kept at 30°C for more 24 hours of incubation. Inoculation of *Pcc*BR1 alone was done on one of the potato slice as positive control and only LB was inoculated on a slice in a similar way to act as a negative control. The maceration area (in mm^2) and maceration (%) was calculated as mentioned in section 3.2.6. Five independent trials were carried out each trial contained three replicates.

3.2.9 Colonization ability of *Bacillus* on mung bean roots

For this assay seeds of mung beans were surface sterilized in laminar hood by immersing sequentially in 0.01% mercury chloride, 70% ethanol and sterile distilled water (for a thorough rinse), followed by drying under laminar hood. The surface sterilized mung bean seeds were kept in the sterile Petri plate containing moist cotton and were kept in dark condition at room temperature for two day for germination. The *Bacillus* isolates were cultivated overnight and were then sub-cultured. They were allowed to reach ~ 2.5 $\times 10^8$ CFU/ml in 100 ml LB broth at 30°C. The cultures were centrifuged at 7000 rpm for 10 mins. The supernatant was discarded and the cell pellet of each isolate was resuspended in 10 ml PBS. In a sterile scintillation vial 2 ml aliquot of culture suspension of each isolate was taken and 3 germinated mung bean seedlings were placed. They were incubated at 30°C for 24 hours in culture suspension. Viable count of each isolate from the roots of germinated mung bean seedling was recorded as mentioned in the section on LA plates. Three trials were carried out for each experiment and each trial contained three replicates for each treatment.

3.2.10 *In planta* assay for biocontrol of spoilage of mung bean sprouts

Seeds of a susceptible variety of mung bean (*Vigna radiate*) were surface sterilized by immersing them sequentially for 1 min in 0.01% mercury chloride, 1min in 70% ethanol and then rinsing in sterile distilled water. This cycle was repeated three times. Sterilized healthy mung beans were kept in Petri plate containing moistened filter paper at room temperature for two days in dark conditions for obtaining germinated mung bean sprouts. The *Bacillus* isolates and *Pcc*BR1 were cultivated overnight. *Bacillus* isolates were then sub-cultured and were allowed to reach ~ 2.5 $\times 10^8$ CFU/ml and *Pcc*BR1 was cultivated to reach ~2.8 $\times 10^9$ CFU/ml in 100 ml LB broth at 30°C. The cultures were centrifuged at 7000 rpm for 10 mins. The supernatant was discarded and the cell pellet of each isolate was resuspended in 10 ml PBS. Culture suspension of each *Bacillus* isolate was mixed with equal volume of culture suspension of *Pcc*BR1 in a final volume of 2 ml in a sterile scintillation vial. In each vial 3 mung bean sprouts were added and incubated for at 30°C in the static condition for 24 hours. Sprouts were then transferred to glass tubes containing Murashige and Skoog (HiMedia pvt ltd) media. Murashige and Skoog medium was prepared by adding 76 mg/L of micronutrients, 4.29 g/L of macronutrients and 0.8 % agar (w/vol) (as per instructor's manual). The media was digested in microwave to make it homogenous and was dispensed in glass tubes. The glass tubes containing the media were autoclaved and were allowed to cool down. The sprouts were subjected to a natural day-night cycle for 7 days in gnotobiotic condition for their growth. Mung bean sprouts subjected only to *Pcc*BR1 culture suspension and similarly mung bean sprouts subjected to sterile PBS were taken as controls. Three trials were carried out for each experiment and each trial contained three replicates for each treatment.

3.2.11 Data analysis

The data on the effects of AHL degrading *Bacillus* on virulence factors production and control of soft rot caused by *Pcc*BR1 were analysed using a one way analysis of variance (ANOVA) and means were compared using Bonferroni's multiple comparisons test using Graphpad Prism software (version 6). Differences at $p < 0.05$ were considered significant.

3.3 Results

3.3.1 *Pcc*BR1 growth inhibition by *B. subtilis* Pls8

The biocontrol strategy explored in this study is not the more popular strategy of antagonism but targeted towards the quorum sensing system of *Pcc*BR1 termed quorum quenching such that it is unable to express the virulence factors. To eliminate false positives in further studies, the selected isolates were tested for their ability to inhibit the growth of *Pcc*BR1. *Pcc*BR1 was overlaid on LA in 1% soft agar and 48 hours grown culture supernatants of *Bacillus* isolates were added to the wells punched in these plates. Since most antagonistic substances, like antibiotics and surfactants, are secreted out by the cells during the stationary phase, 48 hours grown culture supernatants were used for this bioassay. Only *B. subtilis* Pls8 showed a zone of inhibition (Figure 11). Therefore it was excluded from further studies as any attenuation of virulence by it due to direct killing of the pathogen will interfere with and may not be because of quorum quenching.

Figure 1 *B. subtilis* Pls8 inhibiting the growth of *Pcc*BR1

8mm wells bored in LA plate contained 0.1% SDS solution, culture supernatant of *Lysinibacillus* sp. Gs50, culture supernatant of *B. subtilis* Pls8 and uninoculated LB consecutively in clockwise direction.

3.3.2 Deleterious effect of *B. aerius* Pls17 on potato

A very important property of a good biocontrol agent is that it should not harm the host in any way and should specifically target the pathogen. To verify this selected isolates were

inoculated on surface sterilized potato slices and only *B. aerius* Pls17 among the isolates showed maceration of the potato slice, hence was eliminated from further studies (Figure 12).

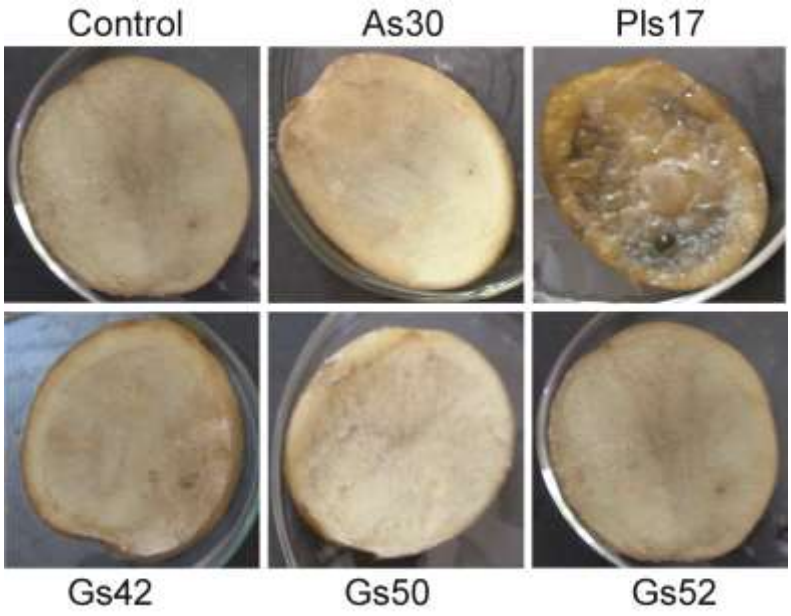


Figure 2 *B. aerius* Pls17 showing maceration on potato host tissue

Upper Panel (left to right): Potato slices inoculated with LB control, *B. firmus* As30, *B. aerius* Pls17 and Lower Panel (left to right): *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52

3.3.3 Effect of selected *Bacillus* isolates on 3-oxo-C6HSL accumulation and growth of *Pcc*BR1

After eliminating the isolates which were either inhibiting the growth of pathogen or were harmful to the host plant, remaining isolates namely *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 were individually co-cultured with the soft rot causing phytopathogen *Pcc*BR1 to confirm that they specifically degrade the 3-oxo-C6-HSL produced by *Pcc*BR1 without altering the growth of *Pcc*BR1. As shown in Figure 13 (a) the purple zone is due to violacein pigment production which is proportionate to 3-oxo-C6HSL produced by *Pcc*BR1whereas the purple zone decreased when *Pcc*BR1 was co-cultured with individual isolates indicating the decrease in 3-oxoC6HSL produced by *Pcc*BR1. *Lysinibacillus* sp. Gs50 completely degraded 3-oxo-C6HSL produced by *Pcc*BR1 hence there was no purple pigmentation produced by *C. violaceum* CV026 around the well containing the supernatant from co-culture sample of *Lysinibacillus* sp. Gs50 and *Pcc*BR1. Similar result was obtained for *B. subtilis* Gs42. *B. thuringiensis* Gs52 partially degraded the 3-oxo-C6HSL produced by *Pcc*BR1 hence the supernatant form the co-culture sample of *B. thuringiensis* Gs52 and *Pcc*BR1 resulted in development of purple colour around the well. As

the purple zone produced around the well containing the culture supernatant from *B. firmus* As30 and *Pcc*BR1 co-culture sample was prominent indicating that *B. firmus* As30 degraded negligible amount of 3-oxo-C6HSL produced by *Pcc*BR1. On the contrary the growth of *Pcc*BR1 was recorded 10^9 CFU/ml when inoculated alone and in all co-cultured samples as well. This implies growth of *Pcc*BR1 was not inhibited by the co-cultured isolates during the 12 hour period (Figure 13 (b)).

(a)

(b)

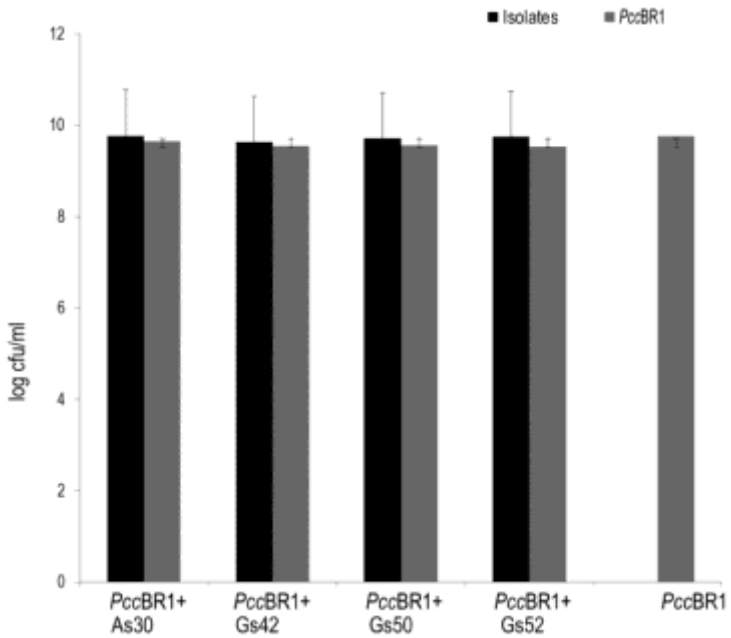


Figure 3 Influence of *Bacillus* isolates on 3-oxo-C6HSL accumulation and growth of *Pcc*BR1 in co-culture assays

(a) Purple pigmentation by *C. violaceum* CV026 due to 3-oxo-C6HSL produced by *Pcc*BR1 and reduction or disappearance of purple zone due to 3-oxo-C6HSL degradation by *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 **(b)** Growth of *Pcc*BR1 when inoculated alone and in co-culture with *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp.Gs50 and *B. thuringiensis* Gs52 after 12 hours. Values represent the mean of three replications. Bars indicate standard deviation of the mean

3.3.4 Effect of *Bacillus* isolates on virulence enzymes production

Plant cell wall degrading enzymes (PCWDE) produced by *Pcc* play a major role in pathogenesis of soft rot. The virulence enzymes polygalacturonase (PG) and pectin lyase (PNL) are two of these PCWDE required for infection to the host plant and are under tight regulation of quorum sensing. *Pcc*BR1 when grown alone for 24 hours, 2.2±0.1 U/ml of polygalacturonase activity was detected. PG activity from the co-culture sample of *B. thuringiensis* Gs52 and *Pcc*BR1 was 0.3±0.2 U/ml, indicating *B. thuringiensis* Gs52 showed maximum reduction in PG activity. While other isolates *B. firmus* As30, *B. subtilis* Gs42 and *Lysinibacillus* sp. Gs50 decreased the PG activity to 0.5±0.1, 0.8±0.0 and 0.7±0.2 U/ml respectively (Figure 14 (a)).

*Pcc*BR1 grown alone for 24 hours was found to produce 20.5±2.3 U/ml PNL activity. PNL activity was not detected in the supernatant form the co-culture sample of *B. subtilis* Gs42 and *Pcc*BR1. While the isolates *B. firmus* As30, *Lysinibacillus* sp.Gs50 and *B. thuringiensis* Gs52 decreased the PNL activity of *Pcc*BR1 appreciably to 5.9±6.1, 2.0±3.4 and 12.9±.2 U/ml respectively (Figure 14 (b)).

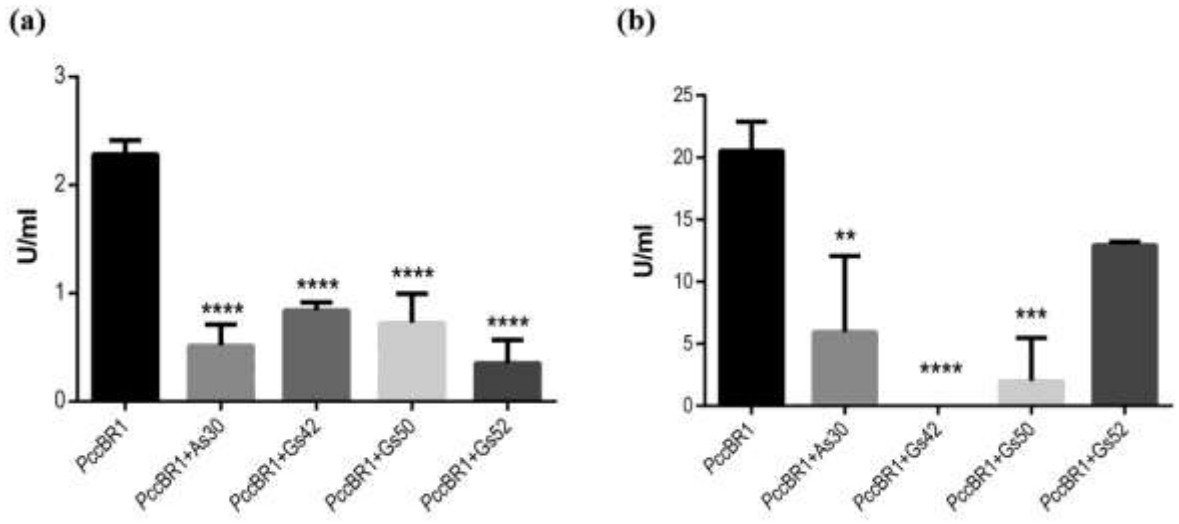


Figure 4 Effect of AHL degrading *Bacillus* isolates on virulence enzymes production by *Pcc*BR1

(a) Polygalacturonase activity from the culture supernatant of either from *Pcc*BR1 or co-cultured with *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 after 24 hours of growth **(b)** Pectin lyase activity from the culture supernatant of either from *Pcc*BR1 or co-cultured with *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 after 24 hours of growth. Values represent the mean of three replications. Bars indicate standard deviation of the mean. Statistical analysis was done using one-way ANOVA Bonferroni's multiple comparisons test (**** = $p < 0.0001$, ** = $p < 0.01$)

PG and PNL activities detected in co-culture supernatant did not have any contribution from *Bacillus* isolates, since all four isolates showed complete absence of PG and PNL activities. Moreover, PG and PNL activities were not detected at 3, 6 and 12 hours of intervals from the co-cultured samples. This clearly shows that when *Pcc*BR1 was co-cultured with AHL degrading *Bacillus* isolates, the enzyme activities of both the virulence determinant enzymes PG and PNL in the supernatant from all combinations were found to decrease. Summing up the results of section 3.3.3 and 3.3.4, it could be concluded that the selected AHL degrading isolates interrupted quorum sensing signalling of soft rot causing *Pcc*BR1 in co-culture, and as observed such signal interference resulted in drastic attenuation of *Pcc*BR1 virulence factor production.

3.3.5 Attenuation of soft rot caused by *Pcc*BR1 on different host plants

To investigate the possibility of using AHL degrading bacteria to control AHL signals mediated bacterial infections on plant host, selected AHL degrading isolates *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 were used to control the development of soft rot infection caused by *Pcc*BR1 on different vegetable host plant (potato, carrot and cucumber). *Pcc*BR1 when inoculated alone caused severe potato tissue maceration resulting in 26.28 ±7.25 mm^2 maceration area and 6.94±3.14 macerated tissue (%) per site of inoculation on each potato slice. Similarly when slices of carrot and cucumber were inoculated with pathogen alone maceration obtained was 70.62±16.35 mm^2, 10.57±1.16 % and 457.3±51.81 mm^2, 23.99±3.58% respectively single site per slice (Figure 15 (a), Panel A) However, when potato slices were co-inoculated with AHL degrading isolates and *Pcc*BR1, the soft rot symptom of maceration was significantly attenuated compared to *Pcc*BR1 inoculated alone (Figure 15 (a), (b)) wherein *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 were able to decrease the maceration area to 7.24±0.40 and 6.66±2.64 mm^2 and the % of macerated tissue to 2.61±0.61 and 3.27±1.39 respectively. When carrot slices were co-inoculated with AHL degrading isolates with *Pcc*BR1significantly less maceration was observed in all cases (Figure 15 (a), (c)) but maximum reduction in maceration was achieved by *Lysinibacillus* sp. Gs50 (10.94±5.33 mm^2, 2.4±0.17%) and *B. thuringiensis* Gs52 (12.98±4.24 mm^2, 1.9±0.51%). On the slices of cucumber *Lysinibacillus* sp. Gs50 showed maximum ability to attenuate soft rot. Co-inoculation of *Lysinibacillus* sp. Gs50 with *Pcc*BR1 resulted in only 72.85±89.87 mm^2, 3.6±1.73% maceration of slice (Figure 15 (a), (d)).

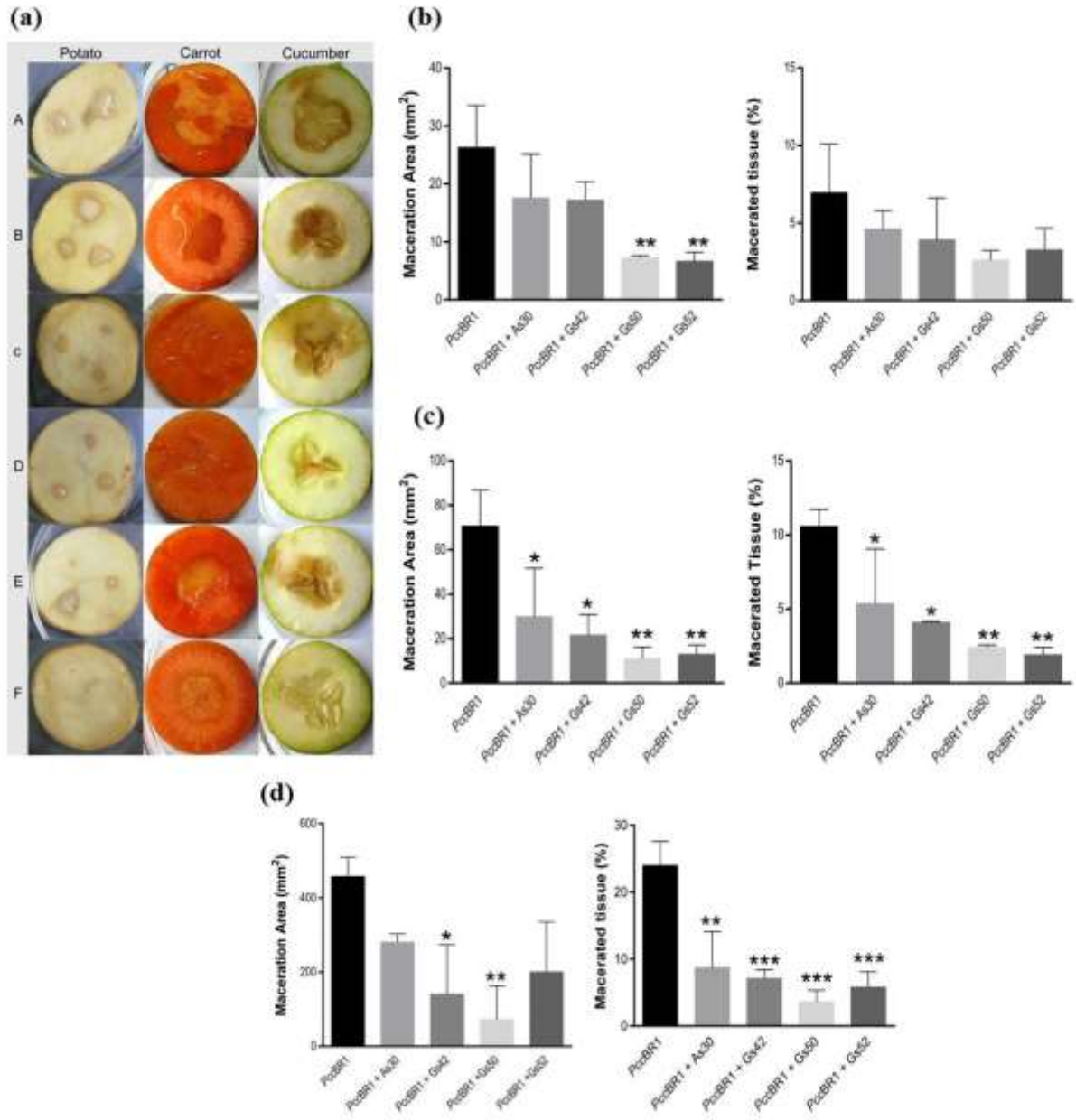


Figure 5 *In vitro* soft rot attenuation assay on different host of *Pcc*BR1

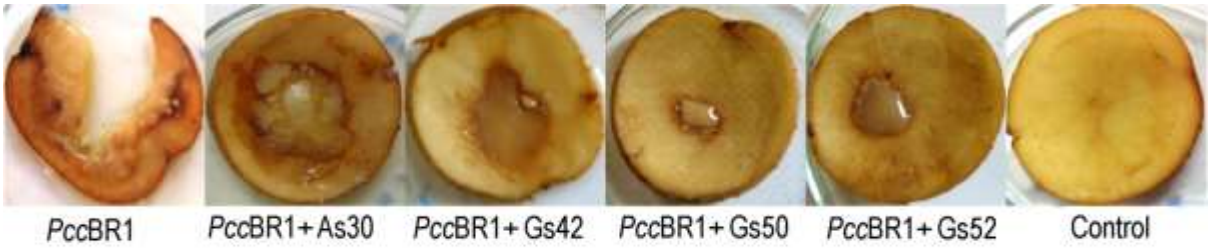
(a) *In vitro* soft rot attenuation assay on potato, carrot and cucumber respectively. Panel A is *Pcc*BR1 inoculated alone on each host. Panel B is *Pcc*BR1 + *B. firmus* As30. Panel C is *Pcc*BR1 + *B. subtilis* Gs42. Panel D is *Pcc*BR1 + *Lysinibacillus* sp. Gs50. Panel E is *Pcc*BR1 + *B. thuringiensis* Gs52. Panel F is PBS inoculated on each host as control **(b)** Quantification of potato tissue maceration. **(c)** Quantification of carrot tissue maceration **(d)** Quantification of cucumber tissue maceration. Values represent the mean of three trials. Each trial has three replicates. Bars indicate standard deviation of the mean. Statistical analysis was done using one-way ANOVA Bonferroni's multiple comparisons test (*= $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

3.3.6 Curative biocontrol of soft rot caused by *Pcc*BR1

In curative biocontrol, when the AHL degraders were applied 12 hours after *Pcc*BR1 substantial decrease was observed in disease symptoms than in treatment with *Pcc*BR1 alone. The *Pcc*BR1 alone inoculated potato slice showed maceration of 163.26±29.44 mm^2 area and

42.62±11.51% macerated tissue whereas the potato slices that were inoculated with AHL degraders along with *Pcc*BR1, especially *Lysinibacillus* sp. Gs50 showed decrease in the maceration to 41.19±43.10 mm^2 and 6.11±2.98 % of macerated tissue (Figure 16 (a), (b)). Whereas *B. firmus* As30, reduced the maceration to 62.46±38.84 mm^2 and 10.34±4.89 %; *B. subtilis* Gs42 reduced the maceration to 65.67±24.46 mm^2 and 11.95±3.09 %; *B. thuringiensis* Gs52 reduced the maceration to 56.27±45.66 mm^2 and 7.81±1.77 significantly. These results indicate that if the AHL degrading *Bacillus* isolates are applied on already infected host, they have the potential to stop further spread of the disease which is a desirable attribute of a biocontrol agent.

(a)



(b)

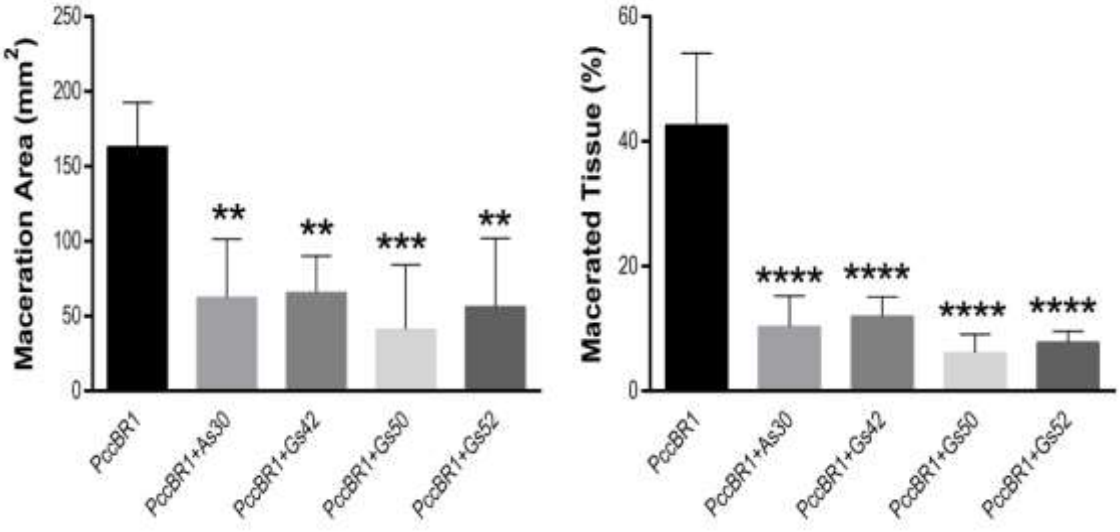


Figure 6 *In vitro* curative biocontrol of potato soft rot caused by *Pcc*BR1

(a) Curative biocontrol assay where *Pcc*BR1 was inoculated on potato slices 12 hours before the inoculation of *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 **(b)** Quantification of potato tissue maceration for curative biocontrol. Values represent the mean of three trials. Each trial has three replicates. Bars indicate standard deviation of the mean. Statistical analysis was done using one-way ANOVA Bonferroni's multiple comparisons test (*= $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

3.3.7 Preventive biocontrol of soft rot caused by *Pcc*BR1

In *in vitro* preventive biocontrol assay, when the AHL degrading *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 were applied 12 hours before the pathogen *Pcc*BR1 rot was almost completely prevented. The potato slice which was inoculated only with *Pcc*BR1 showed maceration symptoms but all other potato slices which were co-inoculated with *Pcc*BR1 and AHL degraders *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 showed no maceration symptoms (Figure 17). These results indicate that prior treatment of AHL degrading *Bacillus* isolates on the susceptible host can prevent the infection of *Pcc*BR1.

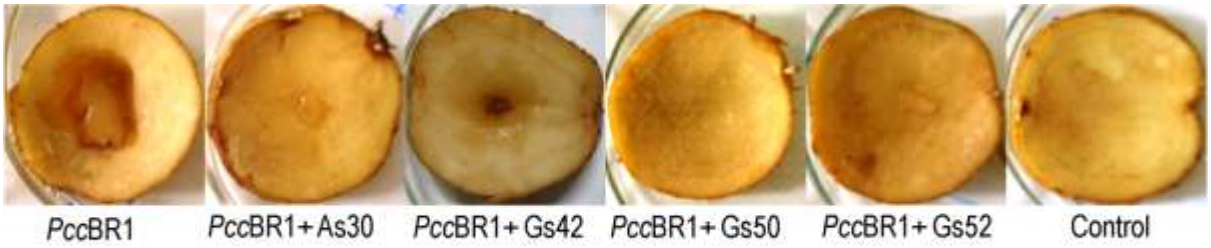


Figure 7 *In vitro* preventive biocontrol assay

B. firmus As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 were inoculated 12 hours before the inoculation of *Pcc*BR1

3.3.8 Root colonisation ability of *Bacillus* isolates

Root colonisation ability and persistence on the root are desirable characteristics of biocontrol agents. These properties were evaluated for *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 on the seeds and roots of mung bean. The healthy seedlings of mung bean were coated with ~10^8 CFU/ml of *Bacillus* isolates and viable count after 24 hours was recorded before planting them into Murashige and Skoog medium. After seven days post inoculation when the seedlings developed into plants again the viable count form the roots were recorded for each isolate (Table 6). All four isolate *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 showed ~10^6 CFU/ml adhesion on seedling and ~10^8 CFU/ml root colonisation which demonstrated root colonisation and good persistence on the roots of mung bean.

Table 1 Root colonisation ability of *Bacillus* isolates on mung bean

	Initial CFU/ml	CFU/ml adhered	CFU/ml on roots 7

		on seedlings	days post inoculation
B. firmus As30	$5.54\pm3.9\times10^{8}$	$2.01\pm1.64\times10^{6}$	$3.1\pm0.6\times10^{8}$
B. subtilis Gs42	$3.67\pm3.48\times10^{8}$	$2.37\pm1.65\times10^{6}$	$3.7\pm1.4\times10^{8}$
Lysinibacillus sp.Gs50	$4.73\pm3.83\times10^{8}$	$2.5\pm1.45\times10^{6}$	$5.5\pm2.4\times10^{8}$
B. thuringiensis Gs52	$1.40\pm2.3\times10^{8}$	$1.03\pm1.75\times10^{6}$	$2.6\pm5.4\times10^{8}$

3.3.9 Biocontrol of spoilage of bean sprouts caused by *Pcc*BR1 on susceptible variety of mung bean (*Vigna radiata*)

The studies were carried out with the mung bean sprouts where they were either treated with the pathogen alone *Pcc*BR1 (10^{9} CFU/ml) or in a mixture with one of the *Bacillus* isolates (~10^{8} CFU/ml). As shown in Figure 18 the sprout that was treated with *Pcc*BR1 showed retarded growth at seedling stage after seven days whereas the sprouts those were treated along with *Bacillus* isolates resulted in the growth of healthy seedling. The growth of these seedlings was similar to the sprout that was treated with PBS as control. The PBS treated seedling had root length 16.66± 5.77 mm, shoot length 87± 6.08 mm, root weight 73.33± 30.55 mg and shoot weight 143.33± 35.11 mg. In this assay AHL degrading isolate *Lysinibacillus* sp. Gs50 showed better ability of biocontrol of spoilage of bean sprouts caused by *Pcc*BR1. The plant which was treated with the mixture of this isolate and pathogen showed healthy growth similar to PBS treated seedlings having the root length 13.33± 10.40 mm, shoot length 98.33± 35.47 mm, root weight 70± 45.82 mg and shoot weight 153.33± 40.41 mg (Table 7).

Figure 8 *In planta* assay for biocontrol of spoilage of bean sprouts caused by *Pcc*BR1 on susceptible variety of mung bean (*Vigna radiata*)

Different treatments given were **A** - with the culture suspension of *B. thuringiensis* Gs52 and *Pcc*BR1. **B** - with culture suspension of *B. firmus* As30 and *Pcc*BR1. **C** - with culture suspension of *Lysinibacillus* sp. Gs50 and *Pcc*BR1. **D** - with culture suspension of *B. subtilis* Gs42 and *Pcc*BR1. **E** - only with *Pcc*BR1 culture suspension. **F** - with PBS as control

Table 2 *In planta* biocontrol of spoilage of bean sprouts caused by *Pcc*BR1 using AHL degrading *Bacillus* isolates

	Root Length[a] (mm)	**Shoot Length[a] (mm)**	**Root Weight[a] (mg)**	**Shoot Weight[a] (mg)**
*Pcc*BR1	No growth	No growth	No growth	No growth
*Pcc*BR1+ *B. firmus* As30	10±5	26.66±16.07	23.43±11.37	80±40
*Pcc*BR1+ *B. subtilis* Gs42	6.66±2.88	23.33±10.4	46.66±15.27	96.66±23.09
*Pcc*BR1+ *Lysinibacillus* sp. Gs50	13.33±10.4	98.33±35.47	70±45.82	153.33±40.41
*Pcc*BR1+ *B. thuringiensis* Gs52	5.66±5.13	67.33±20.03	59.33±27.59	156.66±40.41
PBS Control	16.66±5.77	87±6.08	73.33±30.55	143.33±35.11

[a] Three trials were carried out for each experiment and each trial contained three replicates for each treatment. Values represent the standard deviation of the mean of three trials.

3.4 Discussion

Several pathogenic Gram-negative bacteria including those of the genera *Agrobacterium, Brucella, Burkholderia,, Enterobacter, Pectobacterium, Pseudomonas, Ralstonia, Serratia, Vibrio* and *Yersinia* utilise quorum sensing for the regulation of their virulence related phenotypes. This has made quorum sensing an emerging target for the application of quorum quenching biocontrol agents of phytopathogens. Quorum quenching strategies do not aim to kill the pathogen but affect the expression of a specific function regulated by quorum sensing therefore they theoretically exert a lesser selective pressure for resistance than biocide treatment. This makes quorum quenching a valuable strategy for the development of a sustainable biocontrol agent in the present context of rising antibiotic resistance (Faure and Dessaux 2007; Uroz et al. 2009). In this perspective, potential AHL degraders were isolated from plant root surfaces and soil. Pre-treatment to obtain sporulating bacteria was incorporated as Lee et al. (2002) suggested that AHL degrading ability is wide spread in *Bacillus* sp. Isolates were screened for their AHL degrading ability and few efficient AHL degraders were selected. Four such AHL degrading isolates (*B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52) could degrade signalling molecule (3-oxo-C6-HSL) of *Pcc*BR1 and did not affect pathogen growth. Moreover, *Bacillus* isolates could decrease the production of PG and PNL *in vitro* which suggests the possible involvement of quorum quenching in decreasing the virulence enzyme production indicating their biocontrol potential against this phytopathogen.

Quorum quenching though is potentially effective method of biocontrol, the AHL degrader should have certain additional but desirable characteristics in order to be used in a sustainable biocontrol strategy against phytopathogens. The attributes mentioned below are essential characteristics of an ideal biocontrol agent, viz, rapid growth rate and colonisation in different host-associated environments, lack of harm to the host, ability to use diverse substrates as nutrients, capability to survive and to produce the metabolite responsible for biocontrol while under stress, the absence of any non-target effects, ability to exhibit biocontrol pre- and/or post-infection (Santoyo et al. 2012). Some independently done studies have evaluated few of the biocontrol attributes of the AHL degraders. Dong et al. (2004) first reported the biocontrol potential of AHL degrading *B. thuringiensis in planta* and demonstrated significant decrease in the incidence of *E. carotovora* infection and symptom development. Molina et al. (2003) evaluated different biocontrol properties of *Pseudomonas*

fluorescens P3 expressing *aiiA* (AHL lactonase) gene by demonstrating i*n planta* reduction of potato soft rot caused by *E. carotovora* and crown gall of tomato caused by *Agrobacterium tumefaciens*. Moreover, the authors showed the curative and preventive biocontrol potential of *aiiA* expressing *Pseudomonas fluorescens* P3. Similarly, Qian et al. (2009) showed that the AHL lactonase expressing OH11A strain caused a strong reduction of *Pectobacterium* virulence on hosts like Chinese cabbage and cactus.

In the present study isolates, in addition to their AHL degrading capability, were evaluated for relevant biocontrol attributes such as those discussed above. Antibiotic producing *Pseudomonas fluorescens* could protect crops from root pathogen but this strain inhibited root development in tomato seedlings and could not be used as successful biocontrol agent (Brazelton et al. 2008). Similarly, in our studies isolate Pls17 was eliminated from biocontrol experiments due to potato tissue maceration, a detrimental effect on host. Maceration exhibited by the isolates might be due to the secretion of some tissue degrading enzyme(s) which are deleterious to the host. This observation suggests that all bacterial species with AHL degrading ability are not potential biocontrol agents and it is necessary to negate any detrimental effect on the host by the biocontrol agents. Like many phytopathogens, *Pcc*BR1 has a broad host range and can infect plants belonging to several different families, e.g. Apiaceae (carrot), Solanaceae (potato, eggplant) and Cucurbitaceae (cucumber, sank guard). Our isolates (*B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52) could survive on potato, carrot and cucumber and in virulence bioassays showed significant attenuation of soft rot symptoms in all of these hosts, implying that these isolates have potential as broad-range biocontrol agents. Further, the ability to control symptoms pre and post infection is a valuable trait in a biocontrol agent, and these isolates demonstrated both successful preventive and curative biocontrol of *Pcc*.

In addition to the biocontrol experiments on vegetable slices, biocontrol potential of the isolates was explored for their ability to prevent systemic infection of *Pcc*BR1 through seeds and roots. A susceptible variety of mung bean was used as a model because *Pcc* is known to cause spoilage of beans during their germination phase and can stop the growth of the plant by using its quorum sensing mechanism bacterial spoilage of bean sprouts is characterized by soft rot, in which maceration of plant tissue by bacterial enzymes results in collapse of the cell wall structure (Rasch et al. 2005). The experiments with mung bean sprouts also suggest this as a good model to study *Pcc* soft rot and its biocontrol in *in planta* assays. Gross (1988)

demonstrated that selected biocontrol *Pseudomonas* strains were able to colonize potato roots, but were ineffective in *Pectobacterium atrosepticum* disease suppression as the biocontrol strain failed to exhibit biocontrol property. Unlike the above observation all four isolates were able to prevent the spoilage of beans at the germination stage and in *in planta* experiments while colonising and persisting on the roots of mung bean. This further strengthens the possibility of using AHL degrading bacteria for the biocontrol of quorum sensing mediated soft rot at pre and post-germination stage of plant growth.

Plant-associated microorganisms can be beneficial, deleterious or neutral to the plant (Raaijmakers et al. 2009) and some beneficial bacteria (*Rhizobium* and *Pseudomonas*) depend on quorum sensing for their beneficial traits (Loh et al. 2002). Quorum quenching biocontrol strategy like other antimicrobial approach also suffers with the limitation of affecting the non-target bacteria in addition to the pathogen (target). This is demonstrated by Molina et al. (2003) wherein the AHL degrading biocontrol strain (*P. fluorescens* P3/pME6863 expressing the *aiiA* gene of the soil bacterium *Bacillus* sp. A24) decreased the quorum sensing regulated phenazine (antibiotic) production by *P. chlororaphis* PCL1391. Quorum quenching approach of utilizing AHL degrading bacteria for the biocontrol may result in the interference of quorum sensing of beneficial bacteria as well but it can be argued that the impact of non-target effects would depend on the factors like specificity of the AHL degrading enzyme and proximity of AHL degrading biocontrol agent and pathogen/beneficial bacteria. Cirou et al. (2012) have cautioned that the use of QQ biocontrol agents should be carefully evaluated. Nevertheless, this approach could be effective biocontrol in storage or pre harvest condition after the tubers have matured where such interactions are insignificant. It may also be noted that attention should be paid to the debate on bacteria evolving resistance against quorum sensing disruption.

It is clearly demonstrated here that while some isolates along with strong AHL degradation ability (e.g. *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52) have many preferred biocontrol attributes which can make them effective biocontrol agents while others (e.g. *B. subtilis* Pls8, *B. aerius* Pls17) do not. Notably, AHL degradation is necessary criterion but is not sufficient in developing quorum quenching based biocontrol agent. Evaluation of biocontrol properties is a prerequisite before the isolate is put to use in the field. In the present study the results reveal that the selected quorum quenching isolates possess promising biocontrol potential which needs to be tested in field conditions to assess

them as biocontrol agents. Though all four isolates *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 showed QQ based biocontrol potential, *Lysinibacillus* sp. Gs50 demonstrated remarkable AHL degradation and biocontrol ability. Moreover, *Lysinibacillus* genus is not yet reported for QQ based biocontrol potential, identification of the mechanism of AHL degradation in *Lysinibacillus* sp. Gs50 is of interest and therefore was taken for further studies.