Chapter 4

In planta greenhouse and storage biocontrol studies with Quorum Quenching biocontrol isolate *Glutamicibacter nicotianae* Al5a against *Pectobacterium carotovorum* subsp. *carotovorum*

4.1 Introduction

Pectobacterium carotovorum has many subspecies with certain host specificities along with broad host range, out of which *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*BR1) has widest known host range (Maisuria & Nerurkar, 2013). Most widely used model for *in vitro* pathogenicity or biocontrol studies is potato (Dong et al., 2004). A lot of studies have been done on potato plant model, but it is a temperate crop and requires more time to grow. Since host range of *Pcc*BR1 is wide and its pathogenicity varies temporally, a plant model as per suitability to a region is required.

Potato is the world's number one non-grain food commodity and 75% of the total production are being harvested in densely populated developing countries such as China and India. In India, 73% potatoes are consumed in different forms such as cooked, roasted, french-fries, chipped etc. Cooking often reduces mineral and vitamin constituents. Potatoes are semi perishable because they contain about 80% water and 20% dry matter. The optimum temperature for storing ware potatoes is 4 °C -7 °C and for seed potatoes 2 °C -5 °C but such temperatures are hardly managed in India due to logistical issues. More than 90% of the Indian potato production happens during the winter season and quite a significant portion (45-50%) is stored and consumed over the entire year. Temperature, humidity, CO_2 and air movement are the most important factors during storage. In India, the storage losses in potato storages account for 3-10% of the stored product in the form of rotting, cold injury, weight loss, sprouting, etc. However, the losses may increase up to 38-45% due to defective cold storage and poor management (Chourasia and Goswami, 2007a, 2007b, 2007c).

In studies with potato (*Solanum tuberosum* L.) plant model cultivated with hydroponics, the N-AHL degrading bacterial isolates associated with potato plants were *Rhodococcus* sp. when treated with gamma caprolactone. Of which, *R. erythropolis* exhibited best biocontrol activity against *Pectobacterium carotovorum* subsp. *atrosepticum* (Cirou, et al., 2011).In four autonomous greenhouse assays potato plants were manually infected with pathogenic *Dickeya dianthicola* and exposed to a mixture of three biocontrol bacteria, viz., *Pseudomonas putida* PA14H7 and *Pseudomonas fluorescens* PA3G8 and PA4C2. This treatment constantly reduced the amount of blackleg symptoms as well as the spread of *D. dianthicola* to the tuber offspring (des Essarts et al., 2016).

A study carried out earlier in our laboratory on biocontrol of *Pcc*BR1 in *in planta* mung bean model showed that the *Bacillus* isolates were able to prevent the spoilage of beans at the germination and later stages in experiments while colonizing and persisting on the roots of mung bean (Garge and Nerurkar, 2017). In order to have a suitable infection model to suit the tropical climate for *Pcc* infection development of a cucumber *in planta* infection model for phytopathogen *Pcc*BR1 was carried out for assessing the biocontrol ability of quorum quenching Actinomycetotal isolates against *Pcc*BR1. Further greenhouse studies were carried out on the biocontrol by quorum quenching isolate *G. nicotianae* AI5a of *Pcc*BR1 on potato plants.

4.2 Materials and Methods

4.2.1 Bacterial strains and conditions

Pectobacterium carotovorum subsp. *carotovorum* BR1:

*Pcc*BR1 is a lab isolate which was used as pathogenic strain against which biocontrol was to be achieved (Maisuria & Nerurkar, 2013). It is quorum sensing isolate which produces 3- oxo-hexanoyl homoserine lactone which regulates production of different virulence factors like pectin lyase, pectate lyase, polygalacturonase etc. which cause soft rot in various plant hosts. The culture was grown in LB media at 30°C under shaking condition.

Chromobacterium violaceum CV026:

CV026 is a mini-Tn5-mutant of the wild type strain that lacks cviI encoded AHL synthase and thus cannot synthesise AHL aa a result it can only produce violacein in response to externally supplied AHL signal molecules. Exposure of this strain to exogenous AHLs (AHLs having acyl chains of C4 to C8 in length) that are able to interact with CviR, results in rapid production of a visually clear purple pigmentation (McClean et al., 1997; Steindler et al., 2007). It was grown in LB with 30 µl/ml Kanamycin at 30 °C.

E. coli S17-1 λpir pHC60:

E. coli S17-1 λpir strain contains the pir gene (λpir). This strain has chromosomally integrated conjugal transfer functions (RP4 transfer functions), so when it is used as specific host strain into which the transposon vector DNA is transformed, the transfer occurs by biparental mating (a helper strain is not necessary). The genotype of the strain is pro, res^- hsdR17 (rK^- mK^+) $recA^-$ with an integrated RP4-2-Tc:Mu-Km::Tn7,

Tp^r. The pHC60 plasmid is a spontaneous mutant of plasmid pHC41 carrying a constitutively expressed green fluorescent protein (gfp-S65T) gene and bears an in planta stabilization fragment RK2

Glutamicibacter nicotianae AI5a:

G. nicotianae AI5a was an AHL degrading Actinomycetotal strain isolated and identified in this study. The culture was grown in RMA media at 30°C under shaking condition (Chapter 2 section 2.2.3).

Escherichia coli DH5α

E. coli DH5α with genotype F− endA1 glnV44 thi1 recA1 relA1 gyrA96 deoR nupGΦ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK− mK+), λ– was grown in LB media at 37 °C under shaking condition.

4.2.2 Media

Murashige and Skoog Media

Table 4.1 Composition of Murashige and Skoog (MS) Media

Constituents	grams/L
MACROELEMENTS	
Ammonium nitrate	1650.000
Calcium chloride	332.200
Magnesium sulphate	180.690
Potassium nitrate	1900.000
Potassium phosphate monobasic	170.000
MICROELEMENTS	
Boric acid	6.200
Cobalt chloride hexahydrate	0.025
Copper sulphate pentahydrate	0.025
EDTA disodium salt dihydrate	37.300
Ferrous sulphate heptahydrate	27.800
Manganese sulphate monohydrate	16.900
Molybdic acid (sodium salt)	0.213
Potassium Iodide	0.830
Zinc sulphate heptahydrate	8.600

4.2.3 Development of Cucumber plant infection model for blackleg caused by *Pcc*BR1

The cucumber variety used for *in planta* biocontrol assay of *Pcc*BR1 by quorum quenching Actinomycetota (*G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R.*

erythropolis CRD13.3C) was *Cucumis sativus* var. sharmili obtained from local market. Cucumber seeds were surface sterilized by sequential washing with 1% sodium hypochlorite, 70% ethanol for 1 min each, and final rinsing with autoclaved distilled water. The seeds were kept for germination on 0.8% water agar media at 30 °C for 3 days in dark. Overnight grown *Pcc*BR1, *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were cultivated in 100 ml LB, and were allowed to grow upto 10^8 CFU/ml at 30˚C. The cultures were centrifuged and subsequent 2-3 PBS (pH 7.0) washes were given. Resuspension was done in 15 ml PBS (pH 7.0). In separate experiments, the germinated seeds were then incubated for 4 h with PBS, bacterized with only cultures of *G. nicotianae* AI5a, *R. pyridinivorans* AI4, *R. erythropolis* CRD13.3C and *Pcc*BR1, and co-cultures of *G. nicotianae* AI5a, *R. pyridinivorans* AI4, *R. erythropolis* CRD13.3C with *Pcc*BR1. These seeds were then planted in pots with autoclaved garden soil and were kept in greenhouse with temperature maintained at 30 ℃ and humidity at 70% for 3 weeks. After 3 weeks, the root lengths, shoot lengths, wet weights were taken while the dry weights were taken after a week. The number of trials (N) and number of replicates (n) for *in planta* biocontrol assay were 6 and 15, respectively.

4.2.4 Biocontrol of *Pcc*BR1 potato black leg by Actinomycetota in greenhouse assay

4.2.4.1 Potato tuber germination

The Potato tubers were used for *in planta* biocontrol assay of *Pcc*BR1 by quorum quenching Actinomycetota *G. nicotianae* AI5a were purchased from the local market. While selecting the potatoes it was made sure that the potatoes had no injury at the time of buying. Potatoes were washed with tap water and then Reverse Osmosis purified water thoroughly. The potatoes were kept for germination on tray with filter paper at the bottom at 28 ℃ -30 ℃ for 15 days. Autoclaved distilled water was sprinkled twice a week on to promote germination and maintain moisture content.

4.2.4.2 Experimental setup

Uv sterilized Poly Grow Bags (20 cm(L) x 20 cm(W) x 35 cm(H)) were used. Soil used in pots was purchased from the local plant nursery. Soil was filtered using a sieve and pebbles, leaves and roots were removed from the soil to attain fine uniform texture.

The soil was autoclaved thrice with a successive overnight incubation period. The soil was cooled down after third incubation and mixed with *Trichoderma* powder (0.002%) to avoid fungal infection and 1% cocopeat. Properly germinated potatoes were placed in the soil and kept in the Greenhouse with temperature maintained at 30 °C and humidity at 70% for further observation (Fig. 4.1). Overnight grown *Pcc*BR1, *G. nicotianae* AI5a were sub-cultured in 100 ml LB, and were allowed to grow upto 10^8 CFU/ml at 30 °C. The cultures were centrifuged and subsequent 3 PBS (pH 7.0) washes were given. Final resuspension in 100 ml PBS (pH 7.0) was used as inoculum.

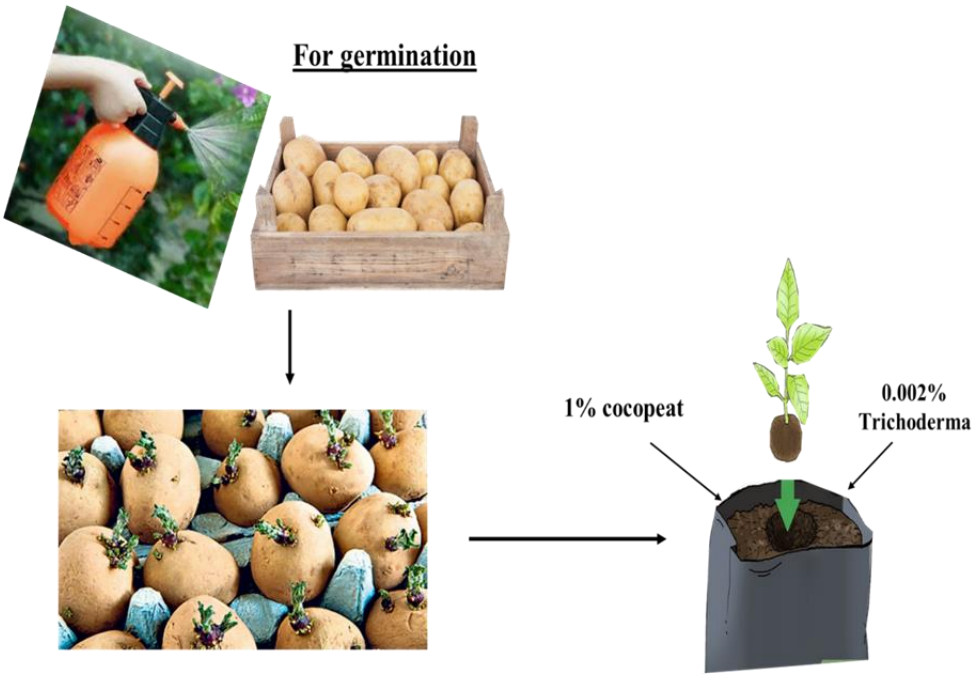


Figure 4.1 Process of setting up the Potato *in planta* experiments

4.2.4.3 Experimental setups

Three different sets of experimental setups were conducted using different inoculation approaches:

I. Soil inoculation

II. Stem inoculation

III. Leaf and lateral stem inoculation

I. Soil inoculation

In this technique, the soil was inoculated by directly mixing the Actinomycetotal inoculum prepared into the soil. Soil inoculations were carried out according to the sets respectively as shown in Table 4.2

After the soil was inoculated, the poly grow bags were kept in greenhouse with temperature maintained at 30 °C and humidity at 70% for 8 weeks. Watering with autoclaved R.O water done thrice a week. After 8 weeks, shoot length, number of leaves, percentage of diseased leaves and wet weight were taken, and dry weights were taken after another week. The number of replicates (n) for *in planta* biocontrol assay were 5 per set (Fig. 4.1).

Table 4.2 Greenhouse experimental setup of soil inoculation of germinated potato tubers with *G. nicotianae* AI5a

Sets	Inoculation into Soil
Control	Autoclaved distilled water
Negative control	*G. nicotianae* AI5a
Positive control	*Pcc*BR1
Experimental	*Pcc*BR1 + *G. nicotianae* AI5a

II. Stem inoculation

The potatoes were planted in the soil contained in poly grow bags and were grown for 3 weeks. After 3 weeks when the plants were approximately 27 to 30 cm high and stolons were already formed, the potato stems were inoculated using a syringe of 1 ml. Out of 20 plants, 5 plants were inoculated with the *Pcc*BR1, 5 plants were used for the water-inoculated control, 5 for negative control *G. nicotianae* AI5a and 5 for experimental set i.e *G. nicotianae* AI5a + *Pcc*BR1. Stem was inoculated 15 cm above the ground level with 1000 µl of the *Pcc*BR1 suspension in water at 10^8 CFU/ml or 1000 µl of sterile D/W water (in case of control). The stem was stabbed halfway with a syringe needle at an angle of 45° and subsequently wrapped with parafilm to prevent drying and leakage of bacteria along the stem surface to the soil. The inoculation was done at three different places in a potato plant. To minimise the risk of soil contamination with the *Pcc*BR1 released from diseased (rotten) stems, the soil surface was covered with plastic film. Plants were watered from the bottom of the bags (Fig. 5.2).

III. Leaf and lateral stem inoculation

The potatoes were planted in the soil contained in the poly bags and were grown for 3 weeks. After 3 weeks when the plants were approximately 27 to 30 cm high and stolons were already formed, the potato stems were inoculated using a syringe of 1 ml. For

each plant, leaves were inoculated by abrasion with 1 ml of water suspension containing *Pcc*BR1 at 10^8 CFU/ml. Control plants were inoculated with sterile distilled water. Both axial and abaxial leaf surfaces were softly brushed with the culture suspensions for 25 sec. In total, 20 plants were used. Five for each set. Lateral Stem was inoculated with 1 ml of the *Pcc*BR1 in water at 10^8 CFU/ml or 1 ml of sterile D/W water (control). A needle was stabbed halfway into the lateral stem at an angle of 45° and subsequently wrapped with parafilm to prevent drying and leakage of bacteria along the stem surface to the soil. Direct soil contamination with the bacteria from inoculated leaves and lateral stem was prevented by covering the soil surface with plastic film. Plants were watered from the bottom of the bags using a garden pump water sprayer in the holes of the polybags (Fig. 5.2).

Statistical analysis for this experiment was done using One Way ANOVA and Holm-Sidak's multiple comparisons test to measure efficiency of *in planta* biocontrol of *Pcc*BR1 by *G. nicotianae* AI5a and $p < 0.0001$.

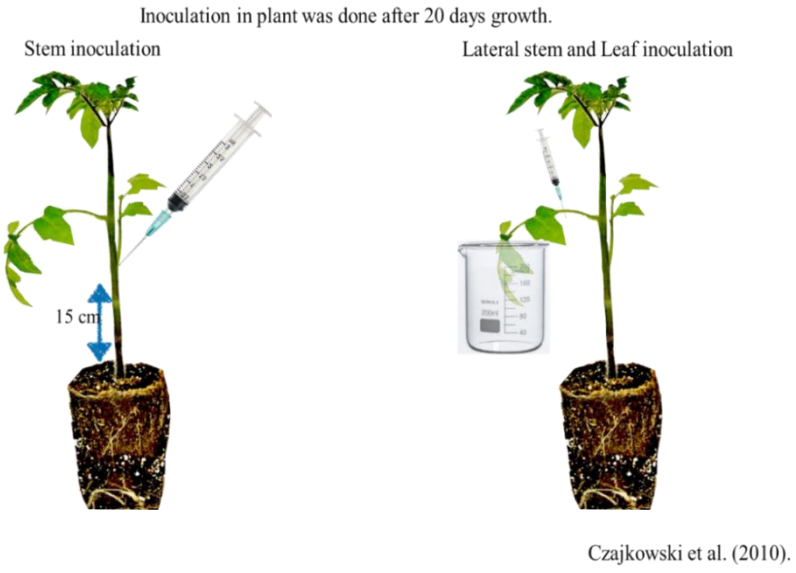


Figure 4.2 Procedure for Stem inoculation and Leaf and lateral stem inoculation with *Pcc*BR1

4.2.5 Biparental mating for GFP tagging of *Pcc*BR1

E. coli S17-1 λpir strain harbors plasmid pHC60 (Fig. 4.3) which constitutively expresses GFP, was used as a donor strain and *Pcc*BR1 as the recipient strain. The donor strain was grown in LB media in the presence of Tetracycline (10 µg/ml) while

the recipient strain was also grown in LB media but without antibiotic Tetracycline and both the strains were incubated overnight at 37 °C and 30 °C. The donor and recipient were added in a ratio of 0.5:1 onto a filter membrane of pore size 0.22 microns placed on LA plate and incubated at 37 °C for 24 h. The filter membrane was then dissolved in saline, dilutions were performed and aliquots were spread onto LA + Tetracycline plates and incubated at 30 °C (Flores-Félix et al., 2015; Ayuso-Calles et al., 2020)

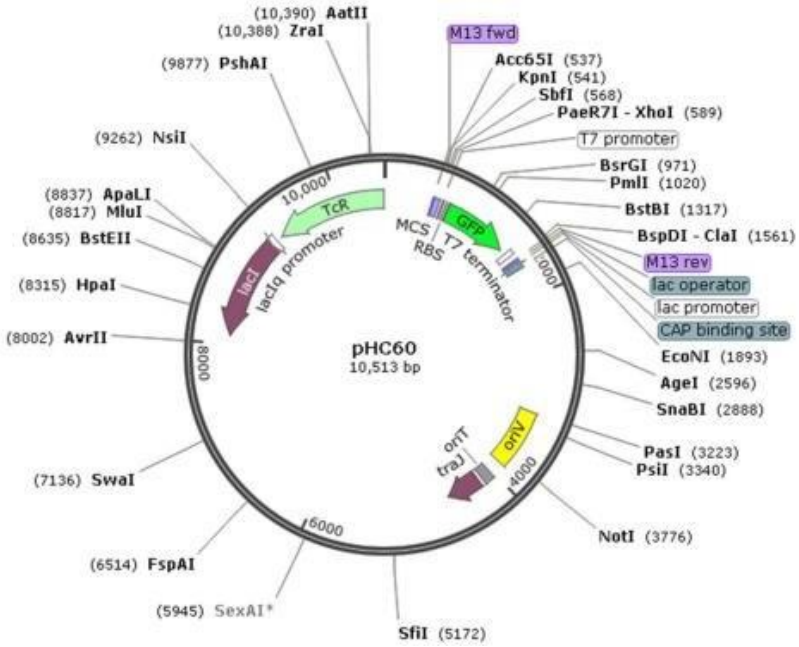


Figure 4.3 Map of plasmid pHC60

4.2.6 Co-culture assay

The coculture assay was performed to check whether the transconjugant obtained after biparental mating is *Pcc*BR1 and if it can produce AHL. *C. violaceum* CV026 a biosensor strain that is employed widely for QS studies was used. 1% overnight grown cultures of transconjugant and *C. violaceum* CV026 were inoculated into LB tubes and incubated for 48 h at 30 °C for the tube assay. A similar assay was carried out on plate where the transconjugant and *C. violaceum* CV026 were mixed and then streaked on LA plate.

4.2.7 PCR with specific 16S rRNA gene primers of *Pcc*BR1

An additional confirmation of the transconjugant would be by performing PCR using specific 16S rRNA primers of *Pcc*BR1. The sequence of the forward primer was

5’:GCCCCCTGGACAAAAACAGA:3’ and
5’:TTAACCTTGCGGCCGTACTC:3’.

The gene was amplified using Taq polymerase and genomic DNA of *Pcc*BR1 as template. The PCR was carried out in thermal cycler for 35 cycles having initial denaturation at 94 °C for 5 min followed by each cycle having denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s, elongation at 72 °C for 45 s and a final elongation at 72 °C for 10 min.

4.2.8 Plasmid vectors used for cloning

pTZ57R/T cloning vector

The pTZ57R/T cloning vector is linearized and ddT tailed for direct use in cloning of PCR products, generated with *Taq, Tth, Tfl* or other DNA polymerases or polymerase mixtures, which add extra adenines to the ends of PCR products. pTZ57R/T cloning vector (Fig. 4.4) was used to clone 16S rRNA gene specific to *G. nicotinae* AI5a in *E. coli* DH5α. The construct was called as pTZ57R/TGn. The cloning was done using InsTAclone PCR Cloning Kit (Thermo Scientific)(Mortazavi et al., 2014).

pJET1.2/blunt cloning vector

The pJET1.2/blunt cloning vector (Fig. 4.5) was linearized with Eco32I (EcoRV). pJET1.2/blunt is a linearized cloning vector, which allows inserts from 6 bp to 10 kb. The 5'-ends of the vector comprise of phosphoryl groups, so that phosphorylation of the PCR primers is not necessary. Blunt-end PCR products produced by proofreading DNA polymerases can be ligated right to the vector. PCR products with 3’-dA projections produced using *Taq* DNA polymerase or other non-proofreading thermostable DNA polymerase are exposed for 5 min with a exclusive thermostable DNA blunting enzyme before ligation. The cloning was performed using Clone JET Cloning Kit (Thermo Scientific). The pJET cloning vector was employed to clone 16S rRNA gene specific to *Pcc*BR1 in *E. coli* DH5α (Kit, 2019). The construct was called as pJETPcc.

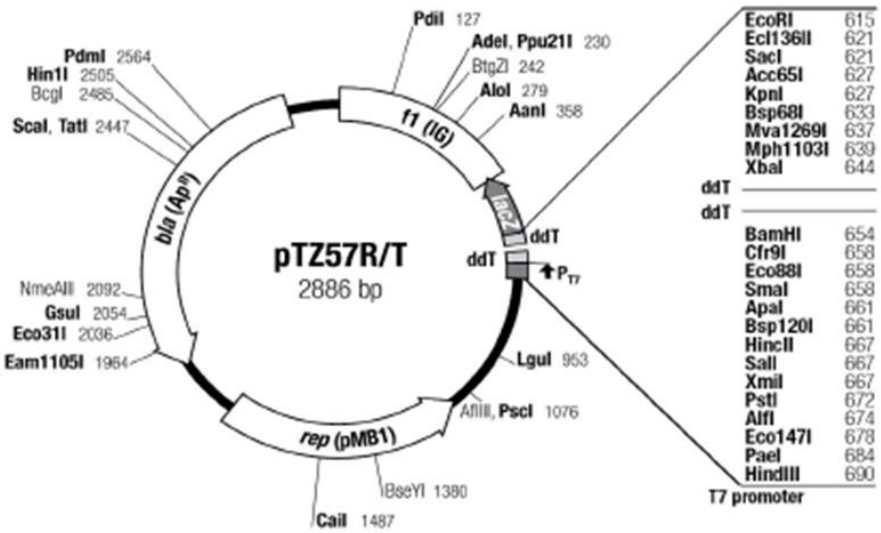


Figure 4.4 Gene map of pTZ57R/T cloning vector

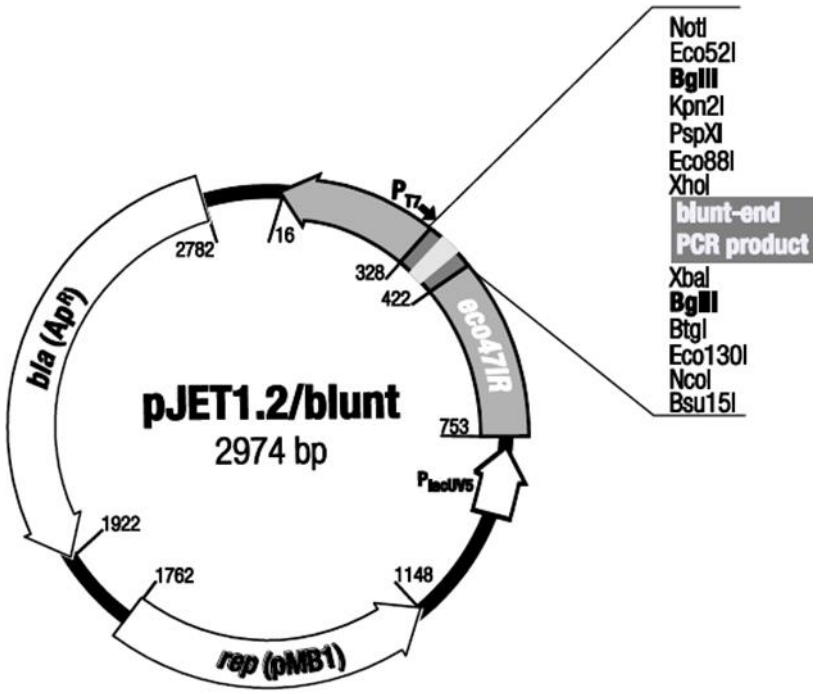


Figure 4.5 Gene map of pJET1.2/blunt cloning vector

4.2.9 Genomic DNA isolation of bacterial isolates *Pcc*BR1 and *Gn*AI5a

Genomic DNA was isolated using CTAB/NaCl method. Isolates were inoculated in 5 ml liquid media (LB) and kept at appropriate growth conditions for 16-18 h. After

incubation, 1.5 ml of culture was taken in a microcentrifuge tube and centrifuged for 10 min at 10,000 rpm and the supernatant was discarded. Pellet was resuspended in 300 µl TES buffer, then 20 µl of lysozyme was added and incubated at 37 °C for 1 h. After incubation 20 µl of 10% SDS and 4 µl of proteinase K was added and incubated further for 1 hour at 55 °C. To the mixture 67 µl of 5 M NaCl was added and mixed thoroughly. Further 44 µl of CTAB/NaCl solution was added, mixed and incubated for 10 min at 65 °C. To the mixture equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and centrifuged at 12,000 rpm for 20 min. The supernatant was transferred into a fresh tube, equal volume of chloroform was added and centrifuged at 12,000 rpm for 20 min and the aqueous layer was transferred into a fresh tube. 0.6^{th} volume of isopropanol was added and kept for precipitation at RT for 1 h. DNA was extracted with 0.6^{th} volume of isopropanol, and was centrifuged to obtain precipitate. This precipitate was washed with 70 % ethanol and all the ethanol was allowed to evaporate. DNA was dissolved in 30µl TE buffer and treated with 1µl of RNase and kept at 65 °C for 10 min (Wilson, 2001).

Agarose Gel Electrophoresis was performed to check for the presence of DNA. The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue) and subjected to electrophoresis through 0.8% agarose (containing 0.5 µg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer, pH 8.0. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator (Alpha Imager HP).

4.2.10 Amplification of specific 16S rRNA genes of *Gn*AI5a and *Pcc*BR1

Amplification of specific 16S rRNA gene of *Gn*AI5a

Specific primers for *G. nicotianae* AI5a 16S rRNA gene were designed using NCBI primer designing tool Primer-BLAST (Thornton et al., 2011; Ye et al., 2012) and amplification of the 16S rRNA gene for *G. nicotianae* AI5a was carried out using the following primers;

forward primer **AI5aF2: 5' AAGTCGAACGATGAAGCCCA 3'** and

reverse primer **AI5aR2: 5' ACCGATAAATCTTTCCACCCCG 3'.**

The gene was amplified using *Taq* polymerase and genomic DNA of *Gn*AI5a as template. PCR was carried out in thermal cycler for 35 cycles having initial

denaturation at 94 °C for 5 min followed by each cycle having denaturation at 94 °C for 45 s, annealing at 63 °C for 45 s, elongation at 72 °C for 1 min and a final elongation at 72 °C for 10 min.

Amplification of specific 16S rRNA gene of *Pcc*BR1

Specific primers for *Pcc*BR1 16S rRNA gene were designed using NCBI primer designing tool Primer-BLAST (Thornton et al., 2011; Ye et al., 2012) and amplification of the 16S rRNA gene for *Pcc*BR1 was carried out using the following primers;

forward primer **PccF1: 5' GCCCCCTGGACAAAAACAGA 3'** and

reverse primer **PccR1: 5' TTAACCTTGCGGCCGTACTC 3'.**

The gene was amplified using *Taq* polymerase and genomic DNA of *Pcc*BR1 as template. PCR was carried out in thermal cycler for 35 cycles having initial denaturation at 94 °C for 5 min followed by each cycle having denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s, elongation at 72 °C for 1 min and a final elongation at 72 °C for 10 min.

PCR products were purified for further experiments using absolute ethanol.

4.2.11 Ligation

4.2.11.1 Ligation system for *G. nicotianae* AI5a

Ligation reaction of final volume 10 µl was set up having the following components: 1µl pTZ57R/T Plasmid, 2µl 5X Ligation Buffer, 1.5 µl purified PCR Product, 0.4µl T4 Ligase and 5.1 µl Milli Q. Ligation reaction was incubated at 22 °C for 1 h.

4.2.11.2 Ligation system for *Pcc*BR1

pJET1.2/blunt cloning vector was utilized. Blunt-end PCR products produced by proofreading DNA polymerases can be ligated right to the vector. PCR products with 3'-dA projections produced using *Taq* DNA polymerase or other non-proofreading thermostable DNA polymerase are treated for 5 min with a exclusive thermostable DNA blunting enzyme before ligation. Ligation was performed in two steps.

- **Step 1**: Blunting reaction on ice:

Blunting reaction of final volume 9 µl was set up on ice having the following components: 5µl 5X Blunting Buffer, 0.5 µl purified PCR Product, 0.5µl DNA blunting enzyme and 3µl MilliQ water. The reaction mixture was incubated at 70 °C for 5 min and cooled on ice.

- **Step 2:** Ligation reaction on ice:

0.5 µl of pJET vector and 0.5 µl T4 DNA Ligase was added to the blunting reaction mixture and incubated at 22 °C for 30 min. (Kit, 2019)

4.2.12 *E. coli* DH5α competent cell preparation and transformation

4.2.12.1 *E. coli* DH5α competent cell preparation

E. coli DH5α culture was inoculated in 50 ml LB and incubated overnight at 37 °C. 500 µl of overnight grown culture was transferred into 50 ml LB and incubated at 37 °C for 2-3 h till the $O.D_{600}$ reached 0.6. Culture was transferred into ice-cold Oakridge tubes of volume ~45-50 ml and centrifuged at 4100 rpm for 10 min at 4 °C. Supernatant was discarded and pellet resuspended in 30 ml of ice cold $MgCl_2$-$CaCl_2$ solution and centrifuged at 4100 rpm for 10 min at 4 °C. Supernatant was discarded, 2 ml of 1 M $CaCl_2$ was added and the mixture was stored at 4 °C overnight.

4.2.12.2 Transformation

Transformation of the pTZ57R/T clones pTZ57R/TGn and pJET clones pJETPcc having the specific 16S rRNA gene insert of *Gn*AI5a and *Pcc*BR1 as a result of ligation was used to transform the competent *E. coli* DH5α cells using heat shock method. This consists of inserting the ligation product into bacteria. 200 µl of competent cells in 1.5 ml chilled microcentrifuge tube was mixed with 5 µl of ligation mixture and incubated in ice for 30 min. After incubation in ice, the mixture was placed at 42 °C for 45 s (heat shock) and then placed back in ice. 800 µl of LB media was added and incubated at 37 °C for 45 min. 100 µl of the mixture was spread on LA plates having 100 µl Ampicillin and 40 µl of X-Gal and IPTG for pTZ57R/T clones for Blue/White screening and LA plates having 100 µl Ampicillin for pJET clones. Remaining mixture was centrifuged at 5000 rpm for 5 min, supernatant discarded, 100 µl of LB media was added to the pellet and spread on LA plates having 100 µl Ampicillin and 40 µl of X-Gal and IPTG. Plates were incubated at 37 °C for 16-18 h (Russell and Sambrook, 2001)

4.2.13 Plasmid preparation and clone confirmation

4.2.13.1 Plasmid preparation from *E. coli* DH5α(pTZ57R/TGn) and *E. coli* DH5α(pJETPcc) transformants for clone confirmations

Plasmid preparation from recombinant *E. coli* DH5α(pTZ57R/TGn) and recombinant *E. coli* DH5α(pJETPcc) respectively was done using alkaline lysis method. 1.5 ml of overnight grown culture was taken in a microcentrifuge tube and centrifuged at maximum speed for 30 s at 4 °C. Supernatant was discarded and the dry pellet was resuspended in 100 µl of ALS-I solution by vigorous shaking. 200 µl of ALS-II was added and the contents were mixed by inverting the tubes. To the mixture 150 µl of ALS-III solution was added, centrifuged at maximum speed for 5 min at 4 °C and the supernatant was transferred into a new tube. Equal volume of phenol: chloroform was added to the solution, centrifuged at maximum speed for 2 min at 4 °C. Nucleic acid was precipitated by adding 2X ethanol and the precipitated nucleic acid was centrifuged at maximum speed for 5 min at 4 °C. DNA was recovered by adding 1 ml of 70% ethanol and centrifuged at 4 °C. DNA was dissolved in 50 µl of TE Buffer.

Agarose Gel Electrophoresis was performed to check for the presence of plasmid DNA. The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue) and subjected to electrophoresis through 0.8% agarose (containing 0.5µg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer, pH 8.0. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator (Alpha Imager HP) (Russell and Sambrook, 2001).

4.2.13.2 PCR for recombinant *E. coli* DH5α(pTZ57R/TGn) clone confirmation with *Gn*AI5a 16S rRNA gene specific primers

PCR was set up using *Gn*AI5a F2/R2 16S rRNA gene specific primers designed for *G. nicotianae* AI5a. The gene was amplified using *Taq* polymerase and plasmid DNA as template. PCR was carried out in thermal cycler for 35 cycles having initial denaturation at 94 °C for 5 min followed by each cycle having denaturation at 94 °C for 45 s, annealing at 63 °C for 45 s, elongation at 72 °C for 1 min and a final elongation at 72 °C for 10 min.

4.2.13.3 Restriction enzyme double digestion of isolated plasmid pTZ57R/TGn from recombinant *E. coli* DH5α(pTZ57R/TGn) for clone confirmation

Plasmid DNA (0.75 µl) was used for RE double digestion (RE DD). For RE DD and insert release, a combination of two REs flanking the insert i.e., EcoR1 and HindIII were chosen. Reaction mixture with a final volume of 30 µl having the following components: 1 µl Eco R1 RE, 1 µl of Hind III RE, 3 µl of 10X Buffer, 0.75 µl plasmid DNA and 24.25 µl of Milli Q was setup. The reaction mixture was incubated at 37 °C for 3 h.

Agarose Gel Electrophoresis was performed to check for the presence of plasmid DNA and release. The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue) and subjected to electrophoresis through 2% agarose (containing 0.5µg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer, pH 8.0. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator (Alpha Imager HP).

4.2.13.4 PCR for recombinant *E. coli* DH5α(pJETPcc) clone confirmation with *Pcc*BR1 16S rRNA gene specific primers

PCR was set up using Pcc 16S rRNA gene specific primers designed for *Pcc*BR1. The gene was amplified using *Taq* polymerase and plasmid DNA as template. PCR was carried out in thermal cycler for 35 cycles having initial denaturation at 94 °C for 5 min followed by each cycle having denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s, elongation at 72 °C for 1 min and a final elongation at 72 °C for 10 min.

4.2.13.5 Restriction enzyme digestion (RE D) of isolated plasmid pJETPcc from *E. coli* DH5α recombinant for clone confirmation using BglII RE

Plasmid DNA (1 µl) was used for RE D. For RE D and insert release, BglII RE, which cuts the plasmid at two different sites was chosen. Reaction mixture with a final volume of 31 µl having the following components: 1 µl BglII, 3 µl of 10X Buffer, 1 µl plasmid DNA and 26 µl of Milli Q was setup. The reaction mixture was incubated at 37 °C for 3 h.

Agarose Gel Electrophoresis was performed to check for the presence of plasmid DNA and release. The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue) and subjected to electrophoresis through **2%**

agarose (containing 0.5µg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer, pH 8.0. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator (Alpha Imager HP).

4.2.14 Standardization of quantitative Real Time PCR using recombinant *E. coli* DH5α strains

4.2.14.1 Standardization of quantitative real time PCR using recombinant *E. coli* DH5α(pTZ57R/TGn)

Plasmid DNA having 16S rRNA gene specific to *Glutamicibacter nicotianae* AI5a from recombinant *E. coli* DH5α(pTZ57R/TGn) was serially diluted and copy number was determined for each dilution. qRT PCR system of final volume 10 µl was set up using plasmid DNA of different dilutions. qRT PCR system had the following components: 5µl of Master mix, 0.1 µl Rox, 0.2 µl AI5aF2, 0.2 µl AI5aR2, 2 µl plasmid DNA and 2.5 µl Milli Q.

4.2.14.2 Standardization of quantitative real time PCR using recombinant *E. coli* DH5α(pJETPcc)

Plasmid DNA having 16S rRNA gene specific to *Pcc*BR1 from recombinant *E. coli* DH5α(pJETPcc) was serially diluted and copy number was determined for each dilution. qRT PCR system of final volume 10 µl was set up using plasmid DNA of different dilutions. qRT PCR system had the following components: 5 µl of Master mix, 0.1 µl Rox, 0.2 µl PccF1, 0.2 µl PccR1, 2 µl plasmid DNA and 2.5 µl Milli Q.

4.2.15 Storage studies

4.2.15.1 Biocontrol of *Pcc*BR1 soft rot on vegetables and fruit by *G. nicotianae* AI5a in storage conditions

Anti-virulence activity against soft rot pathogen *Pcc*BR1 pHC60 GFP was checked in storage of different vegetables and fruit, method was modified from (Hadizadeh et al., 2019). The protocol was performed on potato, tomato, capsicum and brinjal. All fruit and vegetables were purchased from local market were washed with the tap water first, then with R.O. water and then allowed to dry on sterile filter paper. After air-drying for two hours, all the vegetables were weighed and labelled. *G. nicotianae* AI5a and *Pcc*BR1 pHC60 GFP were cultivated overnight. The QQ isolate was then sub-cultured

and was allowed to attain ~ 2.5 ×10^6 CFU/ml and *Pcc*BR1 pHC60 GFP was cultivated to attain ~2.8 × 10^6 CFU/ml in LB broth at 30°C. Two consecutive washes with PBS were given to both overnight grown cultures, they were centrifuged, resuspended into 30 ml PBS and the pellets were dissolved to ensure the even distribution. Aseptically, Inoculation was done by stabbing the vegetable to 3-5 cm deep with the help of sterile tooth picks after dipping it into the culture thrice.

In experiment control only *Pcc*BR1 pHC60 GFP was inoculated by stabbing. While in (negative) control only *G. nicotianae* AI5a was inoculated and one set kept as overall negative control stabbed with PBS. In the experimental set 20 ml of *Pcc*BR1 pHC60 GFP mix with 20 ml of biocontrol and then used for stabbing in the similar fashion (Fig. 4.6). All the inoculated vegetables and fruit were placed in plastic boxes which were sterilized and incubated at 30 ℃ for 3-10 days depending on the vegetable/fruit. The post infection bacterial load was quantified using quantitative Real Time PCR for potato. Five replications of each vegetable and fruit were used to check efficiency of biocontrol strain.

The results were observed for all vegetables and fruits and data was analysed by protocol shown by (Hadizadeh et al., 2019).

i) Disease severity was estimated by using the percentage weight loss after removal of rotting tissues. The change between the initial weight before treatment and the weight after discarding the infected tissues was divided by the initial weight and multiplied by 100.

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

ii) The soft rot disease severity was assessed based on visual assessment of maceration area of individual tubers using a scale of 0 to 5, where 0 = no rot, for negative control to 5 = complete rot.

In this method, the efficacy of biocontrol agent to suppress soft rot development was expressed as a decrease in disease severity based on the percentage of weight loss and was determined using the formula described by (Hadizadeh et al., 2019).

Percentage of disease reduction (PDR) = (disease severity/loss (by weight) in control - disease severity/loss (by weight) in treatment) / disease severity in control × 100).

Statistical analyses done by one way ANOVA and Holm-Sidak's multiple comparisons test. p value <0.0001. All comparisons done with *Pcc*BR1 pHC60 GFP.

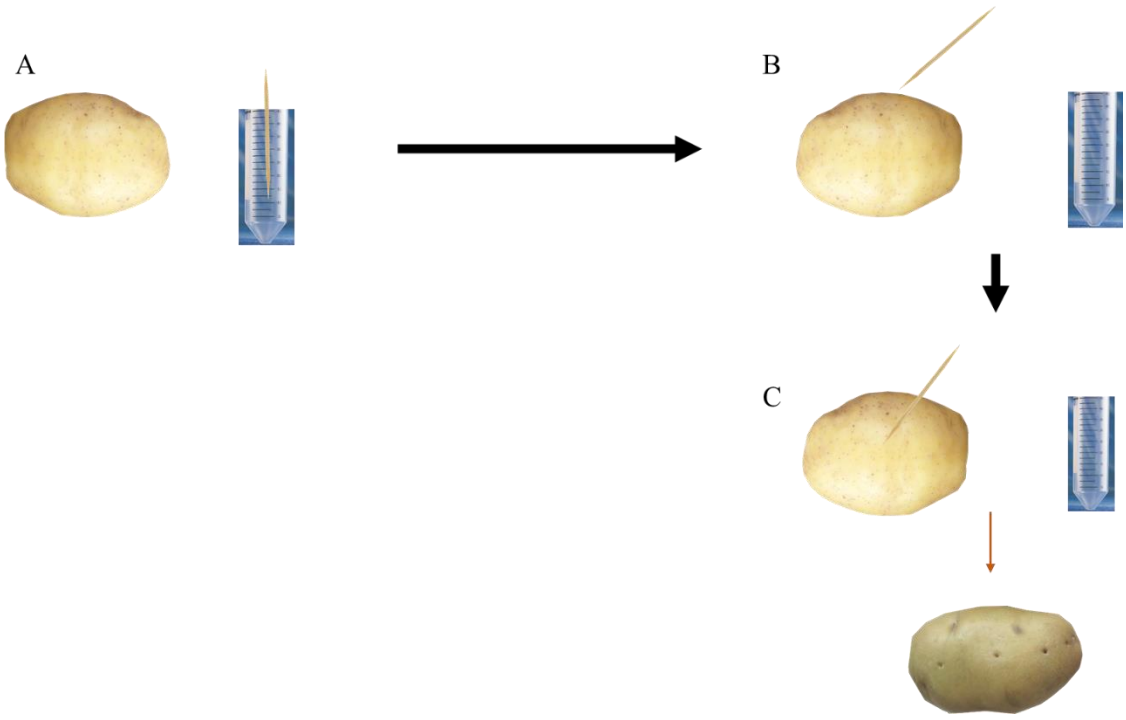


Figure 4.6 Experimental setup of storage experiments. (A) sterile toothpick dip in sorval containing inoculum. (B) and (C) stab the potato/respective vegetable shown with red arrow.

4.2.16 *In planta* assay for effect of biocontrol isolate *G. nicotianae* AI5a on the colonization of *Pcc*BR1 pHC60 GFP in mung bean

Mung bean (*Vigna radiata*) seeds of a susceptible variety of were surface sterilized by immersing them successively for 1 min in 0.1% sodium hypochlorite, 1 min in 70% ethanol and then rinsing in autoclaved distilled water. This cycle was repeated three times. Surface sterilised seeds were aseptically transferred on the soft agar (0.8% w/v agar) plate for germination. Approximately 1–1.5 ml of sterile water was added to plates. The plates were incubated for 24 h at room temperature under humid condition for germination. For seed bacterization, *Pcc*BR1 pHC60 GFP and *G. nicotianae* AI5a were inoculated into 100 ml LB from overnight grown culture and incubated at 30 °C at 140 rpm till they achieved the cell density of ~10^6 CFU/ml. The log phase cultures were centrifuged at 8000 rpm for 10 min and pellets were resuspended into 10 ml

sterile PBS (pH: 7). Culture suspension of each *G. nicotianae* AI5a was mixed with equal volume of culture suspension of *Pcc*BR1 pHC60 GFP in a final volume of 4 ml in a sterile scintillation vial for the experimental set. Five geminated mung bean seeds were added to this culture suspension. Equal number of seeds were incubated in 4 ml of PBS as uninfected control, 4 ml of *Pcc*BR1 pHC60 GFP as infection control and 4 ml of *G. nicotianae* AI5a as positive control to make sure it does not infect the plant on its own. All the seeds were incubated at 30 °C for 3 h 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. For Plant growth 30 ml of 4.4 g/L Murashige-Skoog (HiMedia) containing 0.8% w/v agar (HiMedia) was dispensed into large sugar tubes (30×200 mm,) and sterile conditions were maintained throughout the growth of the plants. The infected and the control mung beans (six seeds per set) sprouts were transferred aseptically onto the Murashige-Skoog media. The sprouts were allowed to grow in natural daylight cycle for 9-10 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 final bacterial load (calculated by quantitative Real Time PCR). The roots from all the plants were resuspended in 3 ml PBS, vortexed vigorously for 1 min and an aliquot from this used for quantification via Real Time PCR.

After ten 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. The root tips were cut into 0.5 cm fragments. The embedded root fragments were sectioned transversally into 10-20 μm slices. The prepared root tips, in all the cases above were mounted on slides, fixed and covered with a coverslip. The root tips were imaged using fluorescent microscope Ziess LSM 700. Images were taken at 4 X and 60X magnification while 488 nm lasers were used for detecting the GFP. All the images were analysed and processed using LSM Browser software. Overall, two different trials having three replicates each were carried out for this entire experiment.

4.3 Results and Discussion

4.3.1 Effect of quorum quenching isolates on *in planta* cucumber infection model

Disease progression was compared between seeds bacterized with only *Pcc*BR1 and seeds bacterized with a mixture of *Pcc*BR1 and QQ isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 or *R. erythropolis* CRD13.3C. The root lengths of plants bacterized with a mixture of *Pcc*BR1 and *G. nicotianae* AI5a, *R. pyridinivorans* AI4 or *R. erythropolis* CRD13.3C were 5.5, 4.2 and 3.5- fold higher respectively, than the plants bacterized with only *Pcc*BR1 (Figs. 4.7 and 4.8A). The shoot lengths of seeds bacterized with a combination of *Pcc*BR1 and *G. nicotianae* AI5a, *R. pyridinivorans* AI4 or *R. erythropolis* CRD13.3C were 5.4, 4.6 and 4.4- fold higher respectively, than the seeds bacterized with only *Pcc*BR1 (Figs. 4.7 and 4.8B). Plant weights (wet and dry) showed a similar result (Fig. 4.8C, D). When treated with *G. nicotianae* AI5a the root, shoot length and the wet, dry weight of the cucumber plant was significantly higher than in the *Pcc*BR1 infected plants. Cucumber seeds incubated with PBS i.e experimental control showed normal growth whereas seeds bacterized individually with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 or *R. erythropolis* CRD13.3C (i.e., no pathogen) i.e. positive control too showed normal growth without any deleterious effect as expected (Fig. 4.7 and 4.8).

Potato has been usually the most frequently used host plant model to study blackleg (Czajkowski et al., 2011b). *Pectobacterium carotovorum* subsp. *carotovorum* BR1 has a wide host range, infecting not only potato but vegetables like cucumber, carrot, brinjal etc (Maisuria and Nerurkar, 2013). Blackleg is a bacterial disease that occurs from the seed and its occurrence is worldwide. The bacterial pathogen characteristically originates from the seed and infects the developing sprouts (Ma et al., 2018). This blackleg disease impacts the stems of maturing plants and varies from tuber soft rot that impacts tubers in the soil and in storage. This has been widely reported usually in potato, but *Pcc* having a wide host range is also able to cause blackleg in Cucumber too (Czajkowski et al., 2015a). Based on these observations the cucumber infection model was conceived and developed to study the *Pcc* blackleg and its biocontrol. Cucumber was susceptible to blackleg caused by *Pcc*BR1 but not much literature is available with respect to *in planta* studies regarding cucumber. Cucumber plant *Pcc* infection model was developed which facilitated the *in planta* assays in

quicker time and with the added benefit that the it occupied less area. Cucumber plant required 2 weeks to grow while potato plant takes up to 12 weeks to grow. Typical symptoms of blackleg stunted plant growth, diseased leaves and decaying of lower stem portion were observed in *Pcc* infected plants (Duarte et al., 2004). The potential of three Actinomycetotal biocontrol isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C against the pathogenicity of *Pcc*BR1 checked *in planta* showed reduction in blackleg symptoms demonstrating their potential as biocontrol anti-virulence agents against *Pcc.*

4.3.2 Biocontrol of *Pcc*BR1 by quorum quenching *G. nicotianae* AI5a on potato *in planta* model

Pcc infection is prevalent in field, in transit and post-harvest condition. Biocontrol of *Pcc*BR1 infection by selected quorum quenching Actinomycetotal isolate *G. nicotianae* AI5a is advantageous as it resides in soil. Throughout this study *G. nicotianae* AI5a showed better biocontrol ability in comparison with the other two Actinomycetotal strains *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C. The potato *in planta* studies require large space and more time compared to studies on cucumber or mung bean, therefore in the studies ahead *G. nicotianae* AI5a was selected for further studies against phytopathogen *Pcc*BR1. In further biocontrol study Potato *in planta* model was used. The susceptibility of potato to *Pcc*BR1 was ensured prior to application of biocontrol agents. Then the studies conducted in following three ways:

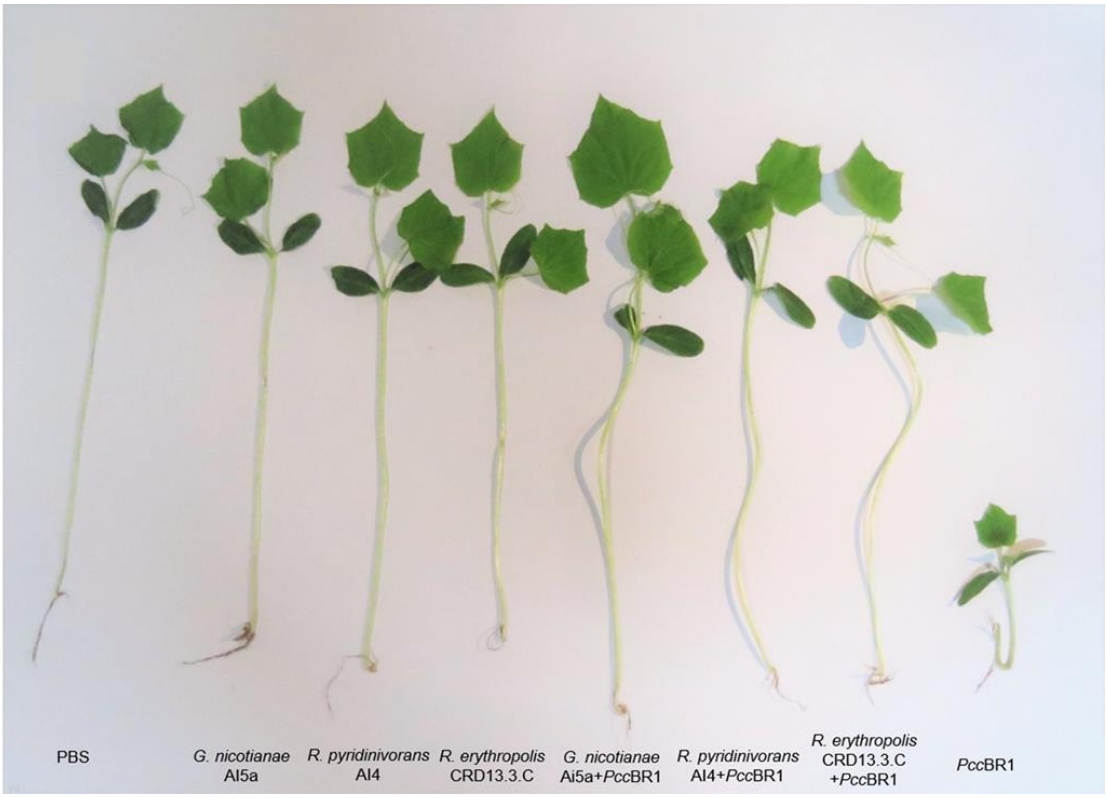


Figure 4.7 *Pcc*BR1 in planta infection model of cucumber and biocontrol effect of Actinomycetotal isolates.

I) Soil inoculation II) Stem inoculation and III) Leaf and lateral stem inoculation provided following results.

I) Soil inoculation

Plants grown in soil infected with *Pcc*BR1 showed stunted growth with reduced growth parameters (shoot length, number of leaves, percentage of diseased leaves, wet weight and dry weight) as compared to autoclaved distilled water controls. The plants treated with *G. nicotianae* AI5a with *Pcc*BR1 had growth parameters shoot length, number of leaves, percentage of diseased leaves, wet weight and dry weight at 108.2 cm, 115 leaves, 8 % diseased leaves, 48.5 g and 15.14 g respectively, while the same parameters for *Pcc*BR1 infected plants were 17.6 cm, 16 leaves, 99 % diseased leaved, 9.3 g and 2.2 g, respectively. The parameters of the *G. nicotianae* AI5a treated set were as good as untreated plants (Fig. 4.9). The plants inoculated with only *G. nicotianae* AI5a, showed growth similar to controls and did not show any negative effect (Fig. 4.9). After measuring wet and dry weight of whole plant after removing tuber there is significant reduction in weight in case of *Pcc*BR1 infected plant as compared to controls and experimental due to its stunted growth.

II) Stem inoculation

Inoculation of potato plant stem was performed 3 weeks after planting, when the plants were approx. 28 to 30 cm tall and stolons were by this time established. After 15 days of stem inoculation, the first symptoms started to develop in inoculated the plants. At 30 days post infection, all the stem-inoculated plants showed disease and chlorosis of leaves and migrated downward to infect the mother tuber causing soft rot and destroying the mother tuber and 50% diseased leaves were observed at the end of experiment. Stem rot, a characteristic blackening of the stem base, or both were detected in 90% of the plants. Symptoms of infection below the ground when stem was inoculated with *Pcc*BR1 shows the maceration of mother tuber in which stem was inoculated with *Pcc*BR1. Stem treated with mixed cultures (1:1) *G. nicotianae* AI5a with *Pcc*BR1 show normal mother tuber compared with control and *Pcc*BR1(Fig 4.10a).

Sections of stems taken from the inoculation point (inoculation point marked with an arrow). Browning and blackening of the vascular and pith tissue of stems detected above and below the inoculation point. In stems of *Pcc*BR1 inoculated plants, a necrosis of vascular tissue was found, resulting in light- and dark-brown lesions. Control plants (sterile Mili Q inoculated) were free of disease symptoms. Plant inoculated with *Pcc*BR1 and *G. nicotianae* AI5a, shows the inhibition of disease symptoms while stem inoculated with *G. nicotianae* AI5a is as good as control (Fig. 4.10).

Stem inoculated with only *Glutamicibacter nicotianae* AI5a, show normal shoot lengths and percentage diseased leaves as compared to sterile miliQ. Stem treated with mixed cultures (1:1) of *Glutamicibacter nicotianae* AI5a with *Pcc*BR1 show normal shoot lengths, percentage diseased leaves as compared to *Pcc*BR1 infected plant (Fig. 4.10 c and d).

III) Lateral Stem and leaf inoculation

In leaf and lateral stem inoculated plants, plants inoculated with *Pcc*BR1 showed the very first symptoms appeared at 7 days post infection; all inoculated leaves showed chlorosis, disease, and the potato plant dried up completely and was dead at the end of 8 weeks. In the following 2 weeks, these symptoms were also started developing in

adjacent leaves (Fig. 4.11). Leaf and lateral stem treated with biocontrol and *Pcc*BR1 did not show any symptoms and the growth of plants in all parameters as good as un-inoculated control and also new tuber arises, while there was no negative effect seen on plants which were inoculated with *G. nicotianae* AI5a.

Symptom observed on leaves and lateral stem inoculated with *Pcc*BR1 after 60 days showed maceration, rotting, and necrosis of leaf tissue in plants with drying of stem can be observed (Fig. 4.11b), whereas leaves and lateral stem of sterile miliQ and *G. nicotianae* AI5a inoculated control plants showed no chlorosis or necrosis and lateral stem was healthy. The set in which *Pcc*BR1 infected plant treated with *G. nicotianae* AI5a showed only chlorosis and slight necrosis due to mechanical damage.

Leaves and lateral stem inoculated potato plants with mixed cultures (1:1) of *Glutamicibacter nicotianae* AI5a, with *Pcc*BR1 and *G. nicotianae* AI5a control show normal growth and also new healthy tuber arise from mother tuber as compared to *Pcc*BR1 infected plant. *Pcc*BR1 inoculated plant showed complete maceration of mother tuber and completely dried up and died (Fig 4.11a).

Stem inoculated with only *Glutamicibacter nicotianae* AI5a, show normal shoot lengths and percentage diseased leaves as compared to sterile miliQ. Stem treated with mixed cultures (1:1) of *Glutamicibacter nicotianae* AI5a, with *Pcc*BR1 show normal shoot lengths, percentage diseased leaves as compared to *Pcc*BR1 infected plant (Fig. 4.11c and d).

Literature states that *Dickeya* spp. could translocate downward in the vascular system via xylem vessel and colonize to the underground plant parts including roots and tubers (Czajkowski & Jafra, 2009). A reverse (downward) transport in xylem vessels via degradation of xylem vessel followed by colonization of the xylem elements (Nelson & Dickey, 1970)and only when xylem sap is exposed to negative hydraulic pressure due to minimal water uptake from roots and decreased leaf evaporation can water travel from leaves to roots in xylem. *E. amylovora* was retrieved from the roots of apple plant owing to negative hydraulic pressure of the plant sap in xylem vessels post stem inoculation (Bogs et al., 1998).

This study showed that inoculation of *Pcc*BR1 in stem's vascular system can result in downward translocation and colonization to the tuber and its roots. Stem inoculation caused distinctive blackleg symptoms in the bulk of *Pcc*BR1 infected plants. On the other hand, the plant which was treated with bio-control isolate *G. nicotianae* AI5a

prevented the translocation of *Pcc*BR1 and also inhibited the stem rot or black leg development.

The results suggested that the plants that were inoculated through soil drenching in the absence of any root injury required month to develop symptoms, while those inoculated at stem/lateral stem and leaf showed disease symptoms within a few days. Stem infection during cultivation practices can results in infected progeny due to the internal movement of *Pcc*BR1 to underground plant parts but the biocontrol isolate *G. nicotianae* AI5a significantly inhibited the infection in the plant which was due to phytopathogen *Pcc*BR1 and allowed the normal growth of the plant. The conditions in the experiment were highly favorable for disease development. For *Pcc*BR1, high bacteria density which led to high virulence were used. Also, the high relative humidity, high temperature and closed maintained environment in green house favored the disease development.

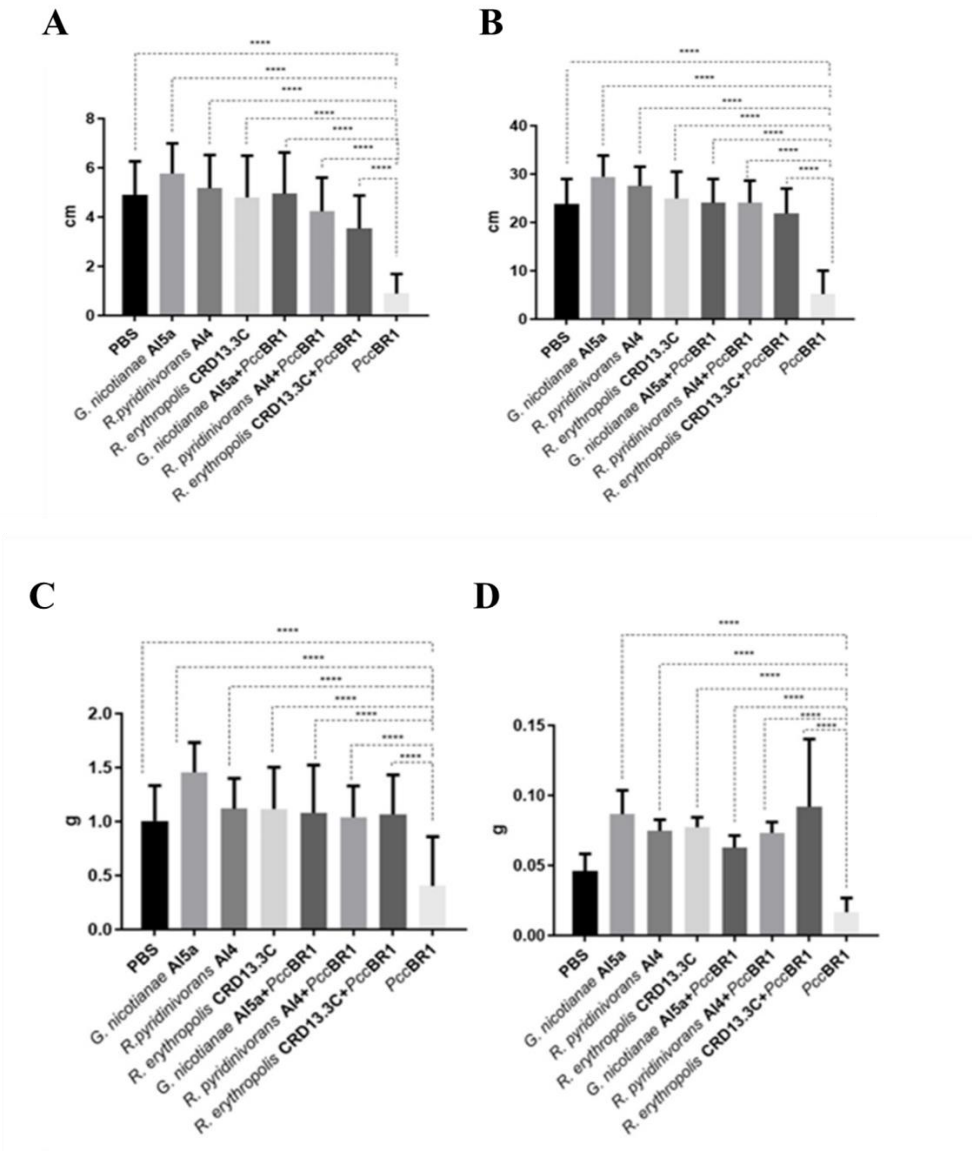


Figure 4.8 *Pcc*BR1 *in planta* infection model of cucumber and biocontrol effect of Actinomycetotal isolates showing growth parameters

(A) Root length of cucumber plants; (B) Shoot length of cucumber plants; (C) Wet weight of cucumber plants; (D) Dry weight of cucumber plants.
**** indicates P < 0.0001, per one-way ANOVA with Holm-Sidak's multiple comparisons test (n =15)

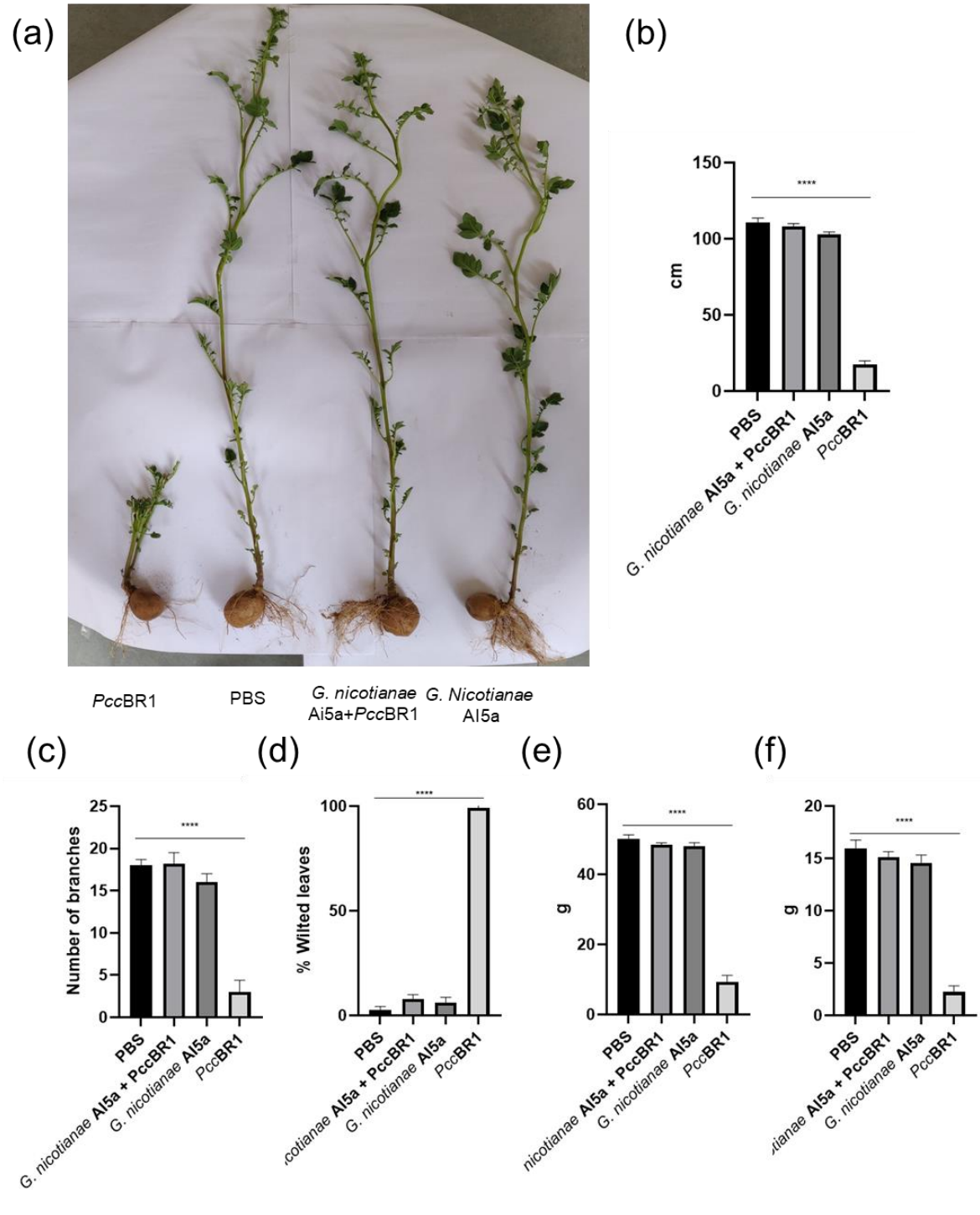


Figure 4.9 Soil inoculation (a) Soil inoculation greenhouse experiments showing the plant after 8 weeks (b) Shoot length (c) Number of leaves (d) Percentage of diseased leaves (e) Wet weight (f) Dry weight

**** indicates P < 0.0001, per one-way ANOVA with Holm-Sidak's multiple comparisons test (n =5)

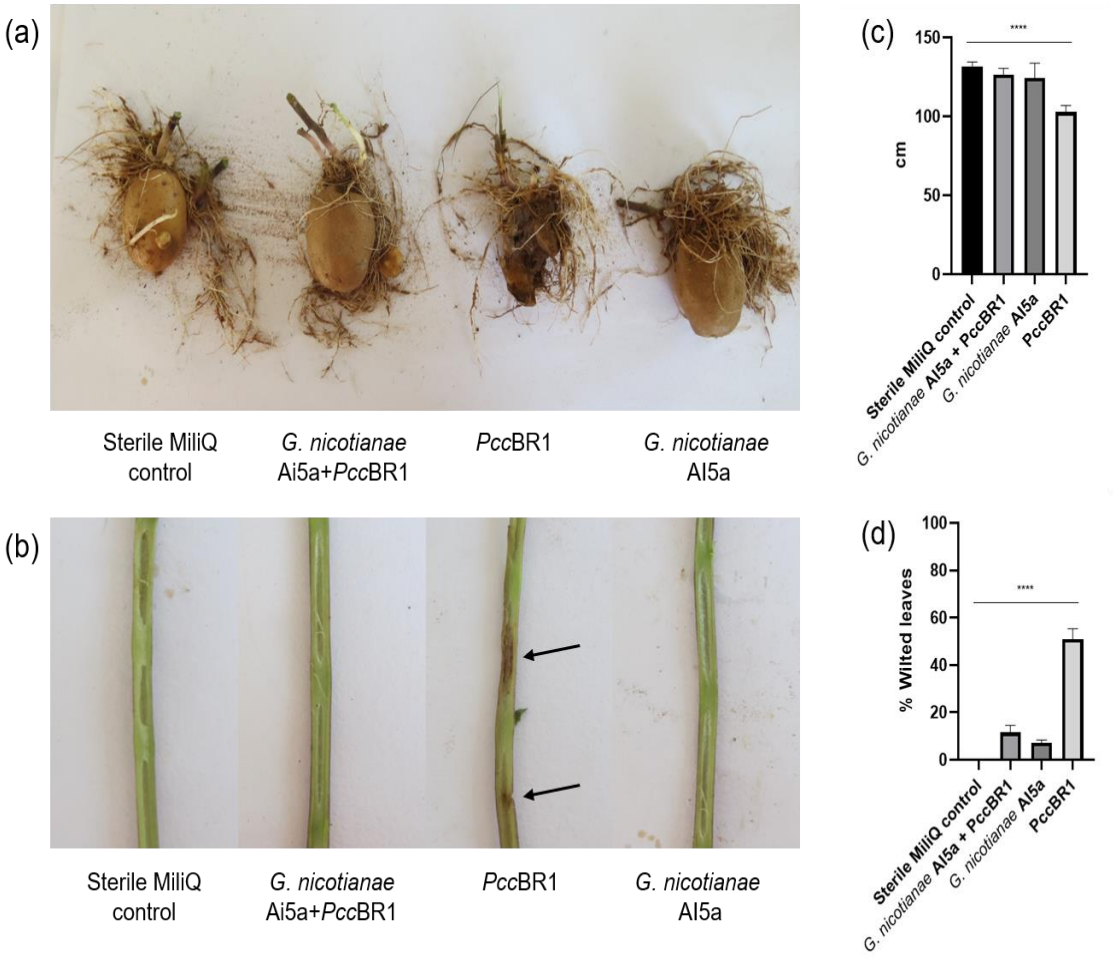


Figure 4.10 Stem inoculation (a) Effect on mother potato observed after 8 weeks of stem inoculation (b) LS of potato stem showing the effect of infection post 8 weeks (c) Shoot length (d) Percentage of diseased leaves

**** indicates P < 0.0001, per one-way ANOVA with Holm-Sidak's multiple comparisons test (n =5)

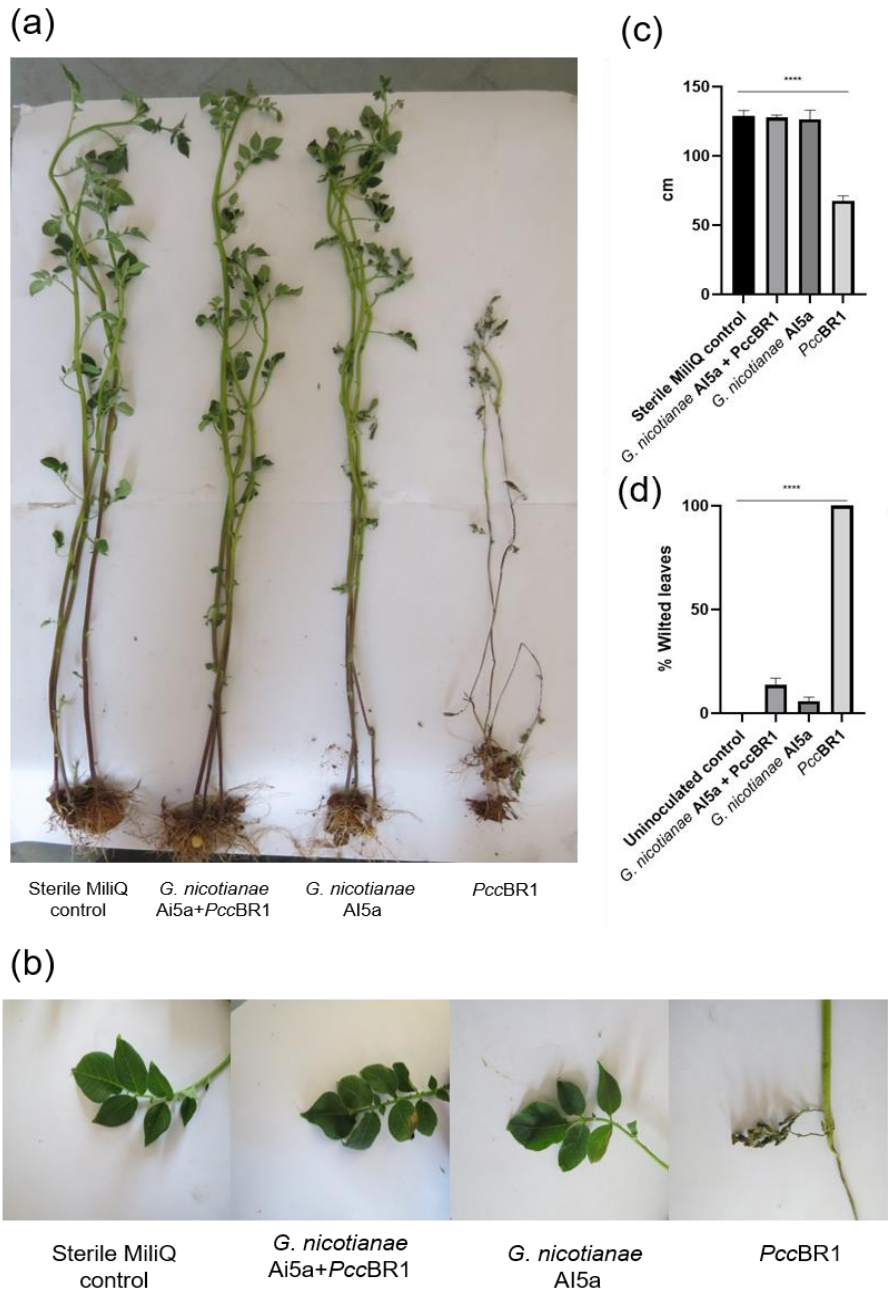


Figure 4.11 Lateral stem and leaf inoculation (a) Whole plant comparative representative images (b) Leaves images (c) Shoot length (d) Percentage diseased leaves

**** indicates P < 0.0001, per one-way ANOVA with Holm-Sidak's multiple comparisons test (n =5)

4.3.3 Construction of GFP marked *Pcc*BR1 strain by Biparental mating

On performing Biparental mating between *Pcc*BR1 and *E. coli* S17- 1 λpir, two transconjugants were obtained which were then sub-cultured onto LA plate containing Tetracycline (10 µg/ml) which acts as a selective marker. The sub-cultured colonies when observed under UV transilluminator, showed fluorescence. In order to confirm that the colonies obtained were *Pcc*BR1 and not *E. coli* S17- 1 λpir, several test specific for *Pcc*BR1 were carried out. Firstly, in the co-culture assay carried out in LB tube and on LA plates where transconjugants and *E. coli* S17-1 λpir (donor strain) were co-cultured with the biosensor strain *C. violaceum* CV026 and incubated at 30 °C and 37 °C respectively the transconjugant *Pcc*BR1 pHC60 GFP did not grow at 37 °C, similar to *Pcc*BR1. Purple coloured ring was obtained in case of transconjugants due to biosensor strain while no such ring was obtained in case of *E. coli* S17-1 λpir. Similarly, only the transconjugants obtained on LA plates showed purple pigmentation thus confirming that the transconjugant *Pcc*BR1 pHC60 GFP can produce AHL (Fig. 4.12 and Table. 4.3) (Krzyzanowska et al., 2012), the molecule required to produce purple pigmentation in *C. violaceum* CV026 (McClean et al., 1997). *E. coli* does not produce AHL on its own, hence it does not show pigmentation with *C. violaceum* CV026 (Mayer et al., 2015). Further confirmation of the transconjugants was done by running PCR using specific 16S rRNA primers of *Pcc*BR1. Fig. 4.13 shows that the transconjugant *Pcc*BR1 pHC60 GFP gave amplification while *E. coli* S17-1 λpir did not because the primers that were used had been specifically designed for *Pcc*BR1. Hence, this suggests that the transconjugant *Pcc*BR1 pHC60 GFP obtained was *Pcc*BR1 with the pHC60 gene in it and its tagging with GFP was successful (Fig. 4.13). The virulence capacity of the transconjugant *Pcc*BR1 pHC60 GFP was also evaluated. It had the same potential as that of *Pcc*BR1 in causing soft rot on potato tuber slice where it had macerated the entire slice of potato just like *Pcc*BR1 (Fig. 4.14A).

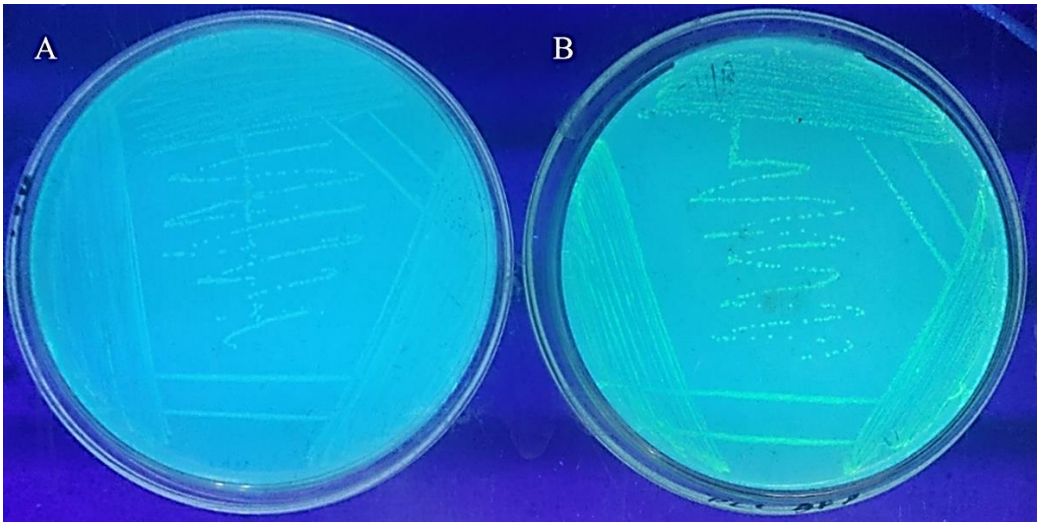
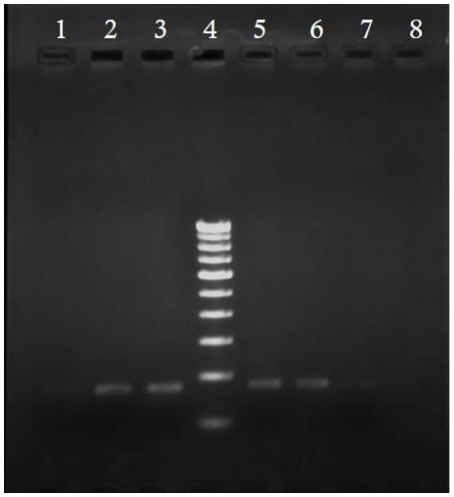


Figure 4.12 (A) *Pcc*BR1 (B) GFP tagged *Pcc*BR1 under transilluminator

Table 4.3 Results of co-culture assay using biosensor strain *C. violaceum* CV026

Tube Assay	
Culture	**Purple ring**
*Pcc*BR1	+
*Pcc*BR1 pHC60 GFP	+
E. coli S17-1 λpir pHC60	-
Plate Assay	
Culture	**Purple pigmentation**
*Pcc*BR1	+
*Pcc*BR1 pHC60 GFP	+
E. coli S17-1 λpir pHC60	-



1 - *E.coli* S17-1 λpir pHC60
2 - *Pcc*BR1 pHC60 gfp
3 - *Pcc*BR1 pHC60 gfp
4 -100bp ladder
5 - *Pcc*BR1
6 - *Pcc*BR1
7 - *E.coli* S17-1 λpir pHC60
8- No DNA control

Figure 4.13 PCR for confirmation of *Pcc*BR1 pHC60 GFP

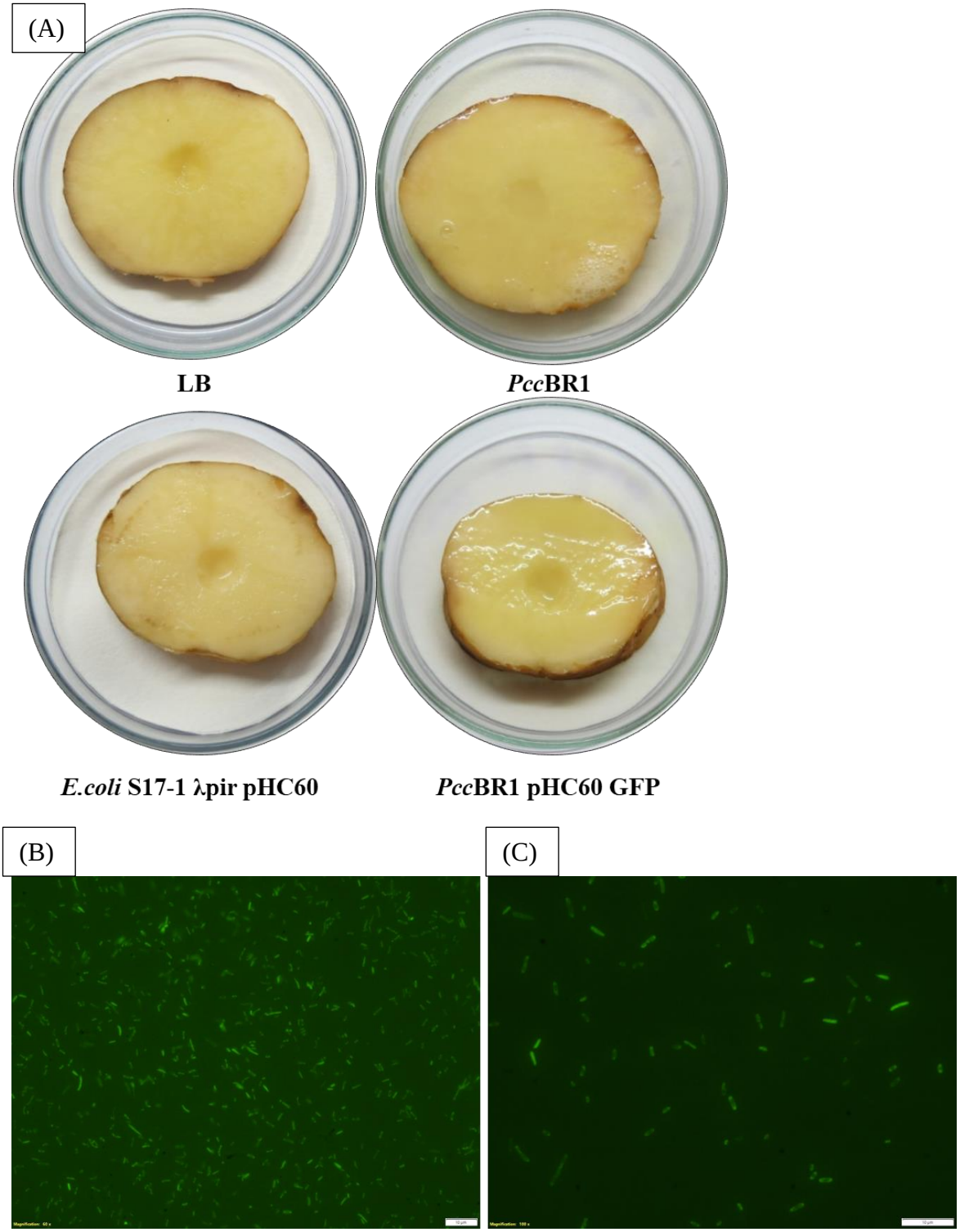


Figure 4.14 (A) Confirmation of *Pcc*BR1 pHC60 GFP for its virulence on potato.Fluorescence microgrpahs of *Pcc*BR1 pHC60 GFP at (B) 60X and (C) 100X magnification at 508 nm

4.3.4 Genomic DNA isolation of bacterial cultures, *Pcc*BR1 pHC60 GFP and *Gn*AI5a

Genomic DNA of the bacterial cultures *Pcc*BR1 pHC60 GFP and *Gn*AI5a was isolated from the overnight grown cultures using CTAB/NaCl. gDNA band was observed near the upper part of the gel as large gDNA fragments migrate slower than smaller DNA fragments. Single band of gDNA indicated isolation of intact chromosomal DNA. RNase treatment was found efficient in eliminating RNA contamination in samples as seen on gels after electrophoresis. Isolated gDNA from the cultures *Pcc*BR1 pHC60 GFP and *Gn*AI5a was used as template in PCR reactions (Fig. 4.15).

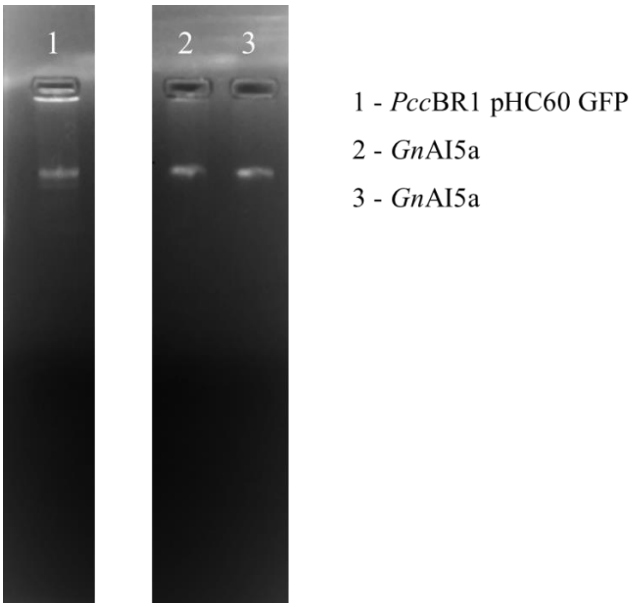


Figure 4.15 Agarose gel (0.8%) images of gDNA isolation from *Pcc*BR1 pHC60 GFP and *Gn*AI5a

4.3.5 Amplification of specific 16S rRNA gene by PCR

4.3.5.1 Amplification of specific 16S rRNA gene of *G. nicotianae* AI5a

Amplification of 16S rRNA gene using 16S rRNA gene specific primers and isolated gDNA as template was done for *Gn*AI5a. Observation of electrophoresed gels loaded with samples showed successful amplification only for 16S rRNA gene of *Gn*AI5a with amplicon size of ~160bp on 2% Agarose gel. Whereas, no amplification was found in case of *Pcc*BR1. (Negative control) (Fig. 4.16).

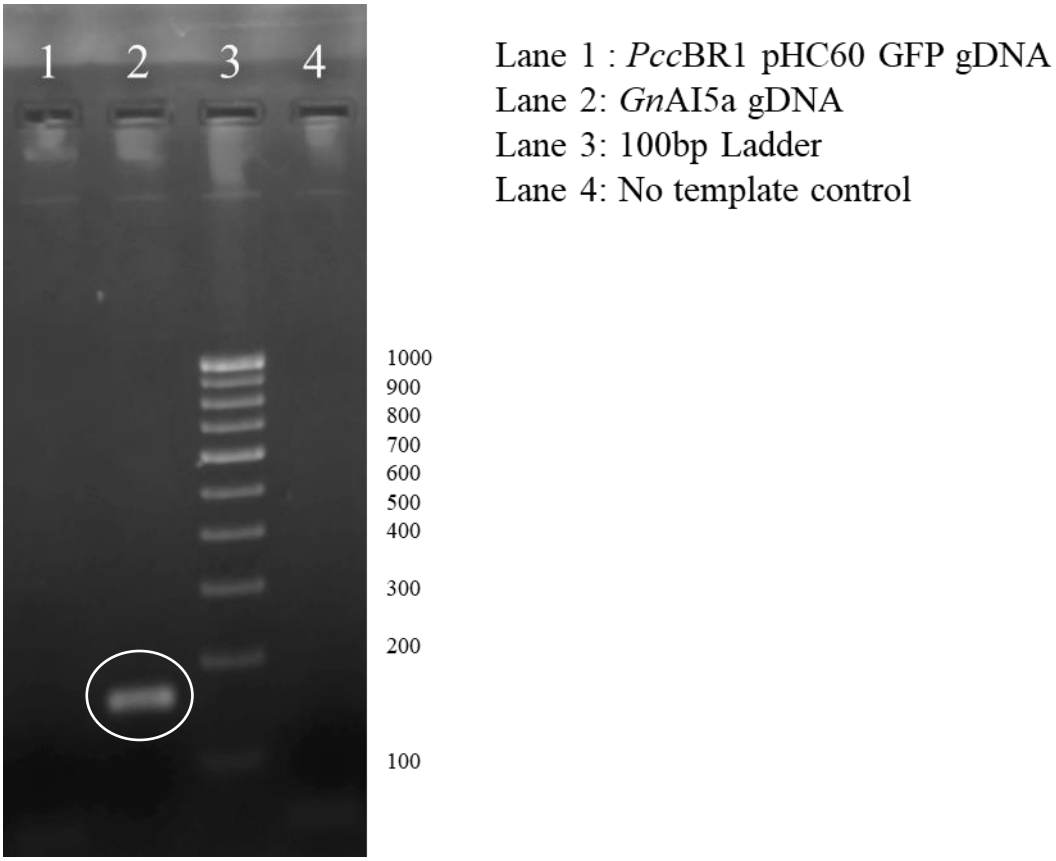


Figure 4.16 Amplification of specific 16S rRNA gene of *Gn*AI5a by PCR

4.3.5.2 Amplification of specific 16S rRNA gene of *Pcc*BR1 pHC60 GFP by PCR

Amplification of 16S rRNA gene using 16S rRNA gene specific primers and isolated gDNA as template was done for *Pcc*BR1. Observation of electrophoresed gels loaded with samples showed successful amplification for 16S rRNA gene of *Pcc*BR1 pHC60 GFP with amplicon size of ~180bp on 2% Agarose gel (Fig. 4.17).

4.3.6 Transformation

4.3.6.1 Transformation of *E. coli* DH5α using construct pTZ57R/TGn

Ligation of pTZ57R/T cloning vector and the 16S rRNA gene of *Gn*AI5a was carried out, the construct pTZ57R/TGn was obtained. *E. coli* DH5α competent cells were prepared. Transformation was carried out using heat shock method.

The transformed colonies were grown on plates after the addition of X-gal and IPTG. Cells transformed with non-recombinant plasmids show blue colonies while the cells transformed with vectors containing recombinant DNA show white colonies after incubation (Lu, 2003).

Post incubation, white colonies obtained on **LA + Ampicillin + X-Gal/IPTG** plates were selected and taken for further clone confirmation.

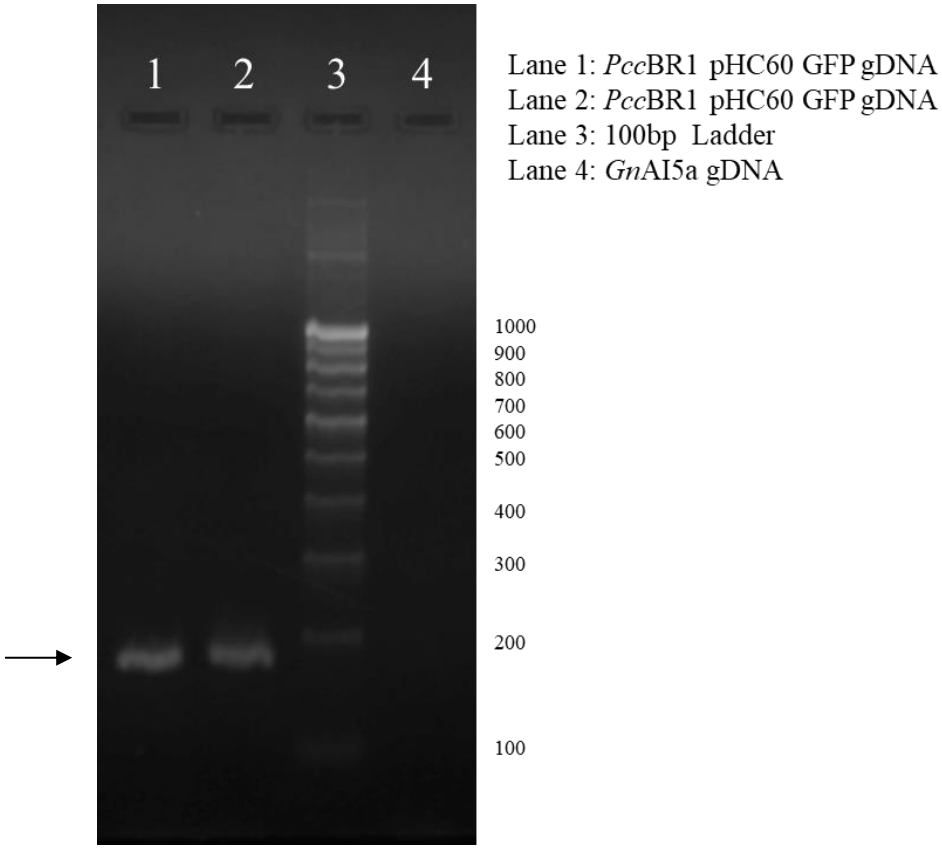


Figure 4.17 Amplification of specific 16S rRNA gene of *Pcc*BR1 pHC60 GFP by PCR

4.3.6.2 Transformation of *E. coli* DH5α using pJET plasmid using pJETPcc

Ligation of pJET plasmid and the 16S rRNA gene of *Pcc*BR1 was carried out. The construct pJETPcc was obtained. *E. coli* DH5α competent cells were prepared. Transformation was carried out using heat shock method.

pJET cloning vector contains a lethal gene that is interrupted by ligation of a DNA insert into the cloning site. Therefore, the cells with merely recombinant plasmids are able to proliferate, eradicating the requirement for costly blue/white screening (Kit, 2019).

Post incubation, colonies obtained on **LA + Ampicillin** plates are considered transformants and were taken for further clone confirmation.

4.3.7 Plasmid preparation from recombinant *E. coli* DH5α(pTZ57R/TGn) and recombinant *E. coli* DH5α(pJETPcc) for clone confirmation

Plasmid isolation from *E. coli* DH5α recombinants was carried out using alkaline lysis method. Isolated undigested plasmid from transformed *E. coli* DH5α *(*pTZ57R/TGn) and *E. coli* DH5α pJETPcc for *Gn*AI5a and *Pcc*BR1 pHC60 GFP respectively, showed banding pattern as expected when electrophoresed on 0.8% agarose gel containing 0.5 µg/ml ethidium bromide (Fig. 4.18). Plasmid isolation by alkaline lysis method was efficient. RNAse treatment was given to the samples before running on the agarose gel to remove any RNA contamination. Further experiments were done to confirm the cloned plasmid confirming the presence of the specific 16S rRNA gene for *Gn*AI5a in pTZ57R/T vector plasmid and specific 16S rRNA gene for *Pcc*BR1 pHC60 GFP in pJET vector plasmid.

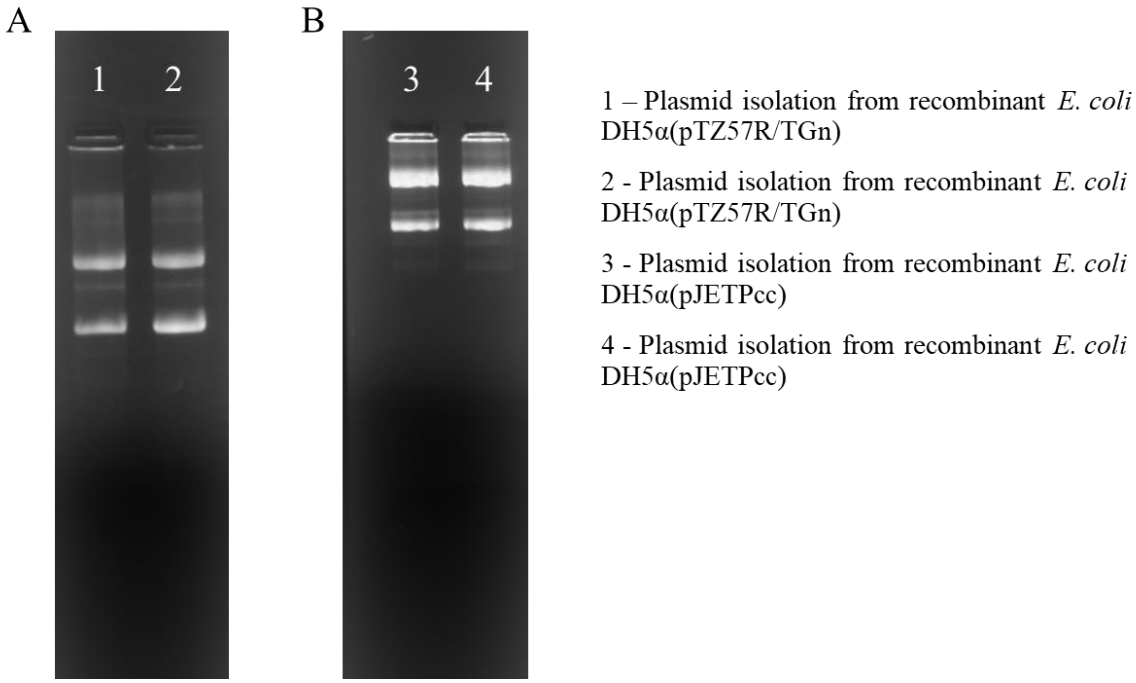


Figure 4.18 Extracted plasmids from (A) recombinant *E. coli* DH5α(pTZ57R/TGn) (B) recombinant *E. coli* DH5α(pJETPcc)

4.3.8 Clone confirmation

4.3.8.1 PCR for specific 16S rRNA gene for *Gn*AI5a clone confirmation (Primary confirmation)

PCR reaction was set up to amplify specific 16S rRNA gene of *Gn*AI5a using 16S rRNA gene specific primers for *Gn*AI5a and isolated plasmid DNA from the cloned construct pTZ57R/TGn plasmid as template. Observation of electrophoresed gels (2%) loaded with samples showed successful amplification for 16S rRNA gene of *Gn*AI5a with amplicon size of ~160 bp, hence providing primary confirmation of the presence of *E. coli* DH5α clone having 16S rRNA gene of *Gn*AI5a (Fig. 4.19).

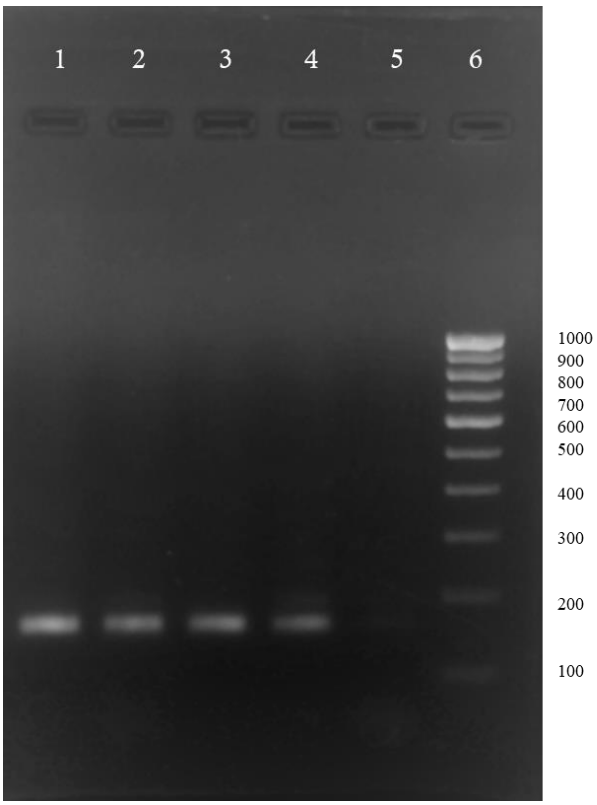


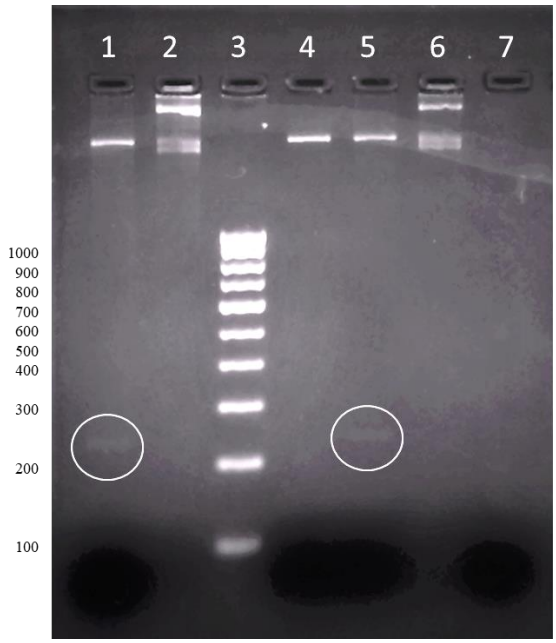
Figure 4.19 PCR for specific 16S gene for *Gn*AI5a for clone confirmation

4.3.8.2 Restriction enzyme double digestion (RE DD) of isolated plasmid for specific 16S rRNA gene for *Gn*AI5a for clone confirmation

Plasmid isolated from recombinant *E. coli* DH5α pTZ57R/TGn was subjected to restriction enzyme double digestion with EcoR1 and HindIII REs, flanking on either side of the insert site (Fig. 4.4).

The cloned construct pTZ57R/TGn with specific 16S rRNA gene of *Gn*AI5a upon double digestion with EcoR1 and HindIII gave a fragment release of approximately

~230 bp size and the vector backbone of ~2900 bp which confirmed the of cloning of specific 16S rRNA gene of *Gn*AI5a into plasmid vector pTZ57R/T (Fig. 4.20).



Sample

Lane 1: Cloned construct pTZ57R/TGn digested with EcoR1 and HindIII

Lane 2: Undigested cloned construct pTZ57R/TGn

Lane 3: 100 bp Ladder

Lane 4 : pTZ57R/T plasmid vector without insert digested with EcoR1 and HindIII

Lane 5: Cloned construct pTZ57R/TGn digested with EcoR1 and HindIII

Lane 6: Undigested cloned construct pTZ57R/TGn

Figure 4.20 Restriction enzyme double digestion for specific 16S rRNA gene for *Gn*AI5a for clone confirmation

4.3.8.3 PCR for specific 16S rRNA gene for *Pcc*BR1 pHC60 clone confirmation (Primary confirmation)

PCR reaction was set up to amplify specific 16S rRNA gene of *Pcc*BR1 pHC60 using 16S rRNA gene specific primers for *Pcc*BR1 pHC60 and isolated plasmid DNA from the cloned construct pJETPcc was used as template. Observation of electrophoresed gels (2%) loaded with samples showed successful amplification for 16S rRNA gene of *Pcc*BR1 pHC60 with amplicon size of ~180 bp, hence providing primary confirmation of *E. coli* DH5α recombinant pJETPcc-1 having 16S rRNA gene of *Pcc*BR1 pHC60 (Fig. 4. 21).

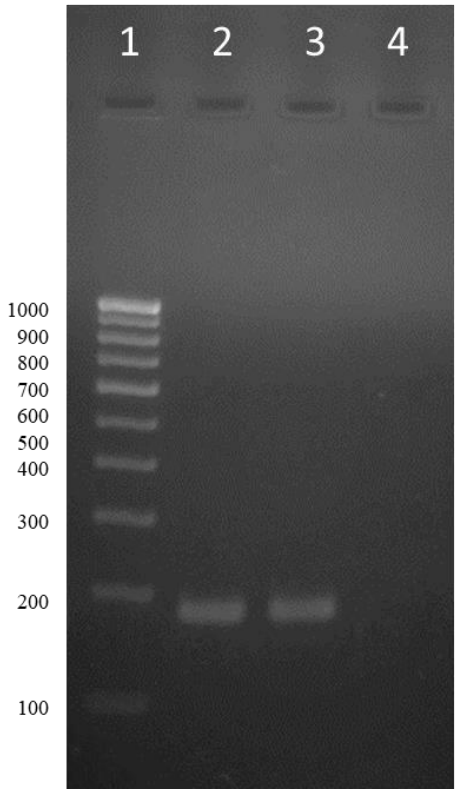


Figure 4.21 PCR for specific 16S rRNA gene for *Pcc*BR1 pHC60 for clone confirmation

4.3.8.4 Restriction enzyme digestion (RE D) of isolated plasmid for specific 16S rRNA gene for *Pcc*BR1 pHC60 GFP for clone confirmation

BglII has one restriction site flanking on either side of the insert site in pJET plasmid. BglII can alone be used for a RE digestion in this case. Construct pJETPcc isolated form recombinant *E. coli* DH5α pJETPcc was subjected to restriction enzyme double digestion with BglII RE, having one restriction site, flanking on either side of the insert site (Fig. 4.5).

The cloned construct pJETPcc upon double digestion with BglII gave a fragment release of the expected size and the vector backbone of ~2900 bp which gave us confirmation of cloning of specific 16S rRNA gene of *Pcc*BR1 pHC60 into plasmid vector pJET (Fig. 4.22).

After confirmation of clone with PCR and RE Digestion both clones were further used for standardization of quantitative Real Time PCR.

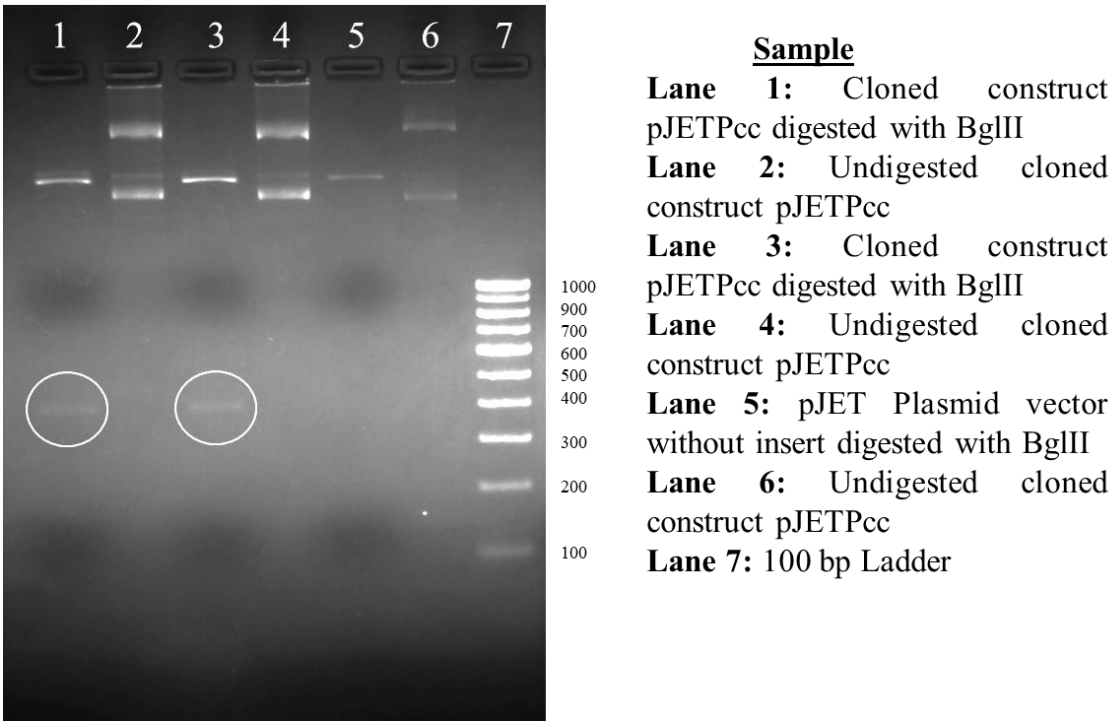


Figure 4.22 Restriction enzyme digestion for specific 16S rRNA gene for *Pcc*BR1 pHC60 for clone confirmation

4.3.9 Standardization of quantitative Real Time PCR using recombinant *E. coli* DH5α strains

4.3.9.1 Standardization of quantitative Real Time PCR using recombinant *E. coli* DH5α(pTZ57R/TGn)

Plasmid DNA from cloned recombinant *E. coli* DH5α**(**pTZ57R/TGn) was serially diluted and copy number was determined for each dilution. The copy number of the undiluted plasmid from recombinant *E. coli* DH5α**(**pTZ57R/TGn) was 10^{13} which was determined using URI Genomics & Sequencing Center copy number calculator (URI Genomics & Sequencing Center Copy - Google Scholar, 2004; Zozaya-Hinchliffe et al., 2008; Hahn et al., 2017) based on the concentration of plasmid DNA observed on the Nano Spec. qPCR system was set up using the plasmid DNA of different dilutions as mentioned earlier and their respective Ct values were determined. Graph of copy number v/s Ct value was plotted and a standard graph is observed. As the copy number of the plasmid decreased the Ct value increased. A difference of approximately 3 Ct values is observed between each copy number (dilution) to construct a proper standard graph (Fig. 4.23).

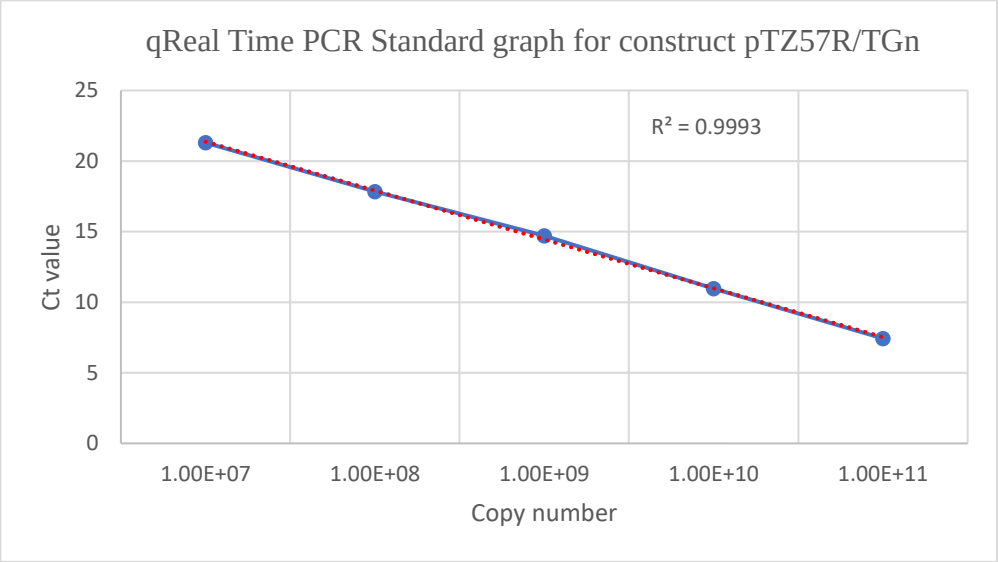


Figure 4.23 qPCR Standard graph for construct pTZ57R/TGn

4.3.9.2 Standardization of quantitative RT PCR using recombinant *E. coli* DH5α(pJETPcc)

Plasmid DNA from cloned recombinant *E. coli* DH5α**(**pJETPcc) was serially diluted and copy number was determined for each dilution. The copy number of the undiluted plasmid from recombinant *E. coli* DH5α**(**pJETPcc) was 10^{12} which was determined using URI Genomics & Sequencing Center Copy Number calculator (URI Genomics & Sequencing Center Copy - Google Scholar, 2004; Zozaya-Hinchliffe et al., 2008; Hahn et al., 2017) based on the concentration of plasmid DNA observed on the Nano Spec. qPCR system was set up using the plasmid DNA of different dilutions and their respective Ct values were determined. Graph of copy number v/s Ct value was plotted and a standard graph is observed. As the copy number of the plasmid decreased the Ct value increased. A difference of approximately 3 Ct values is observed between each copy number (dilution) to construct a proper standard graph. This Standardization of quantitative Real Time PCR further allows us to perform quantitative analysis of the specific bacteria in unknown quantities (Fig. 4.24).

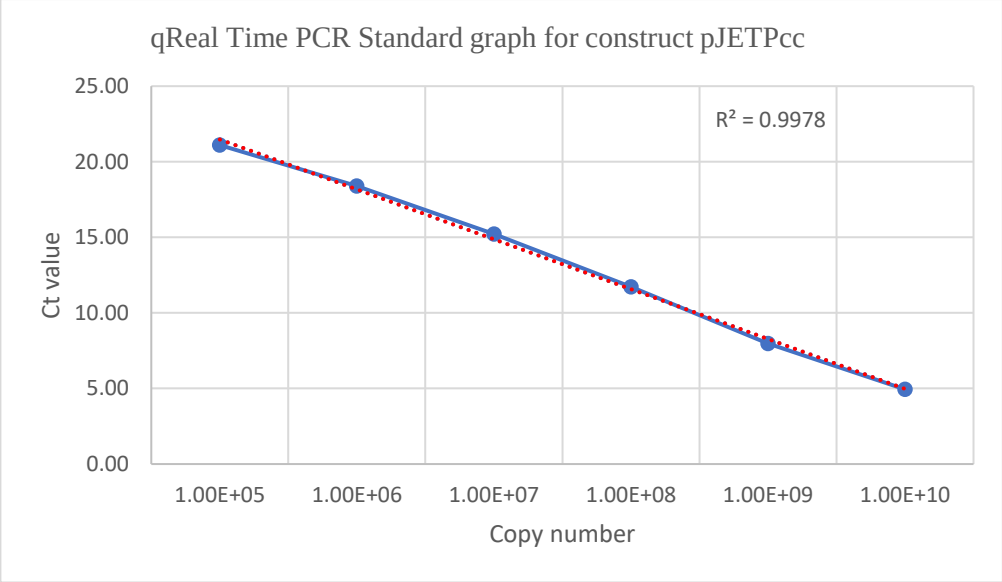


Figure 4.24 qRT PCR Standard graph for construct pJETPcc

4.3.10 Biocontrol ability of *G. nicotianae* AI5a against soft rot causing phytopathogen *Pcc*BR1 in storage conditions

Soft rot in potato and in other vegetables and fruit cause a great problem, which is difficult to control causing significant economic loss. Biocontrol could be one of the effective and non-harmful methods in controlling the soft rot development in storage condition. To evaluate the effectiveness of the biocontrol bacteria to protect wounds on vegetables and fruit against *Pcc*BR1 as might happen after harvest and grading, experiments were conducted using different vegetables and fruit. Different vegetables showed the difference in susceptibility to virulent bacteria *Pcc*BR1 and its inhibition by *G. nicotianae* AI5a (Fig. 4.25). Similar studies conducted by (Hadizadeh et al., 2019) showed that *Serratia plymuthica* A30 acts as a biocontrol against *Dickeya solani* strains and reduces the soft rot development by 93.2% in potato in storage condition at lower temperature. In the present study, all the vegetables and fruit treated with *G. nicotianae* AI5a showed significant reduction in maceration, as compared to *Pcc*BR1 positive control. Biocontrol isolate prevents the PCWDEs formation by *Pcc*BR1 by degrading the 3-oxo-C6-AHL produced by it to regulate virulence factor (PCWDEs) production. In this experiment, the percentage of lost weight and the visual ratings of treated vegetables and fruit with biocontrol agent were significantly lower than in the vegetables treated with the pathogen alone and PDR (percentage disease reduction) was also calculated as shown in Table. 5.3. Anti-virulence activity of *G. nicotianae*

AI5a delayed and reduced the incidence of soft rot on the different vegetables and a fruit in storage condition. No symptoms were observed on the negative controls. The present work was carried out to develop methods for biocontrol of soft rot at room temperature storage condition for a wide range of hosts. Quantification of the presence of the two bacteria was also done by quantitative real time PCR in case of Potato (Hadizadeh et al., 2019).

In similar studies it was found that the antagonist *Bacillus amyloliquefaciens subsp. plantarum* strain BGP20 caused severe decay in the wounds of potatoes, but only caused slight discoloration in the wounds of green peppers and Chinese cabbages (Zhao et al., 2013)**.** So, this antagonist can't be used as biocontrol for a wide range of host as it is harmful to the host whereas present studies with biocontrol agent *G. nicotianae* AI5a did not show any negative effect on the host and act as a potential biocontrol agent for a range of hosts against *Pcc*BR1 (Fig. 4.25).

Storage studies with different hosts

*Pcc*BR1 inoculated potato tubers showed 59.98% maceration of the tissue but when treated with biocontrol *G. nicotianae* AI5a, percentage maceration reduced to 6.51%. The potatoes were infected, treated in different experimental sets and were kept for a period of 10 days (Fig. 4.25A and B). Similar storage experiments were done using Tomato, Capsicum and Brinjal as hosts. Tomato and Capsicum were kept for 3 days of incubation. The percentage maceration for *Pcc*BR1 infected Tomato and Capsicum were 43.23% and 50.83% maceration, respectively. The percentage maceration in Tomato and Capsicum for *Pcc*BR1 treated with *G. nicotianae* AI5a reduced to 9.1% and 6.17% maceration, respectively (Fig. 4.25C, D, E and F). Brinjalin storage were kept for 7 days and the percent maceration upon treatment with *G. nicotianae* AI5a reduced to 14.6% maceration from 60.1% maceration for the *Pcc*BR1 infected control set (Fig. 4.25G and H).

Percent Disease Reduction (PDR)

Efficacy of biocontrol to suppress soft rot development was expressed as a decrease in disease severity based on the percentage of weight loss and was determined using the formula described by (Hadizadeh et al., 2019). Maximum Percent Disease reduction (PDR) was observed in brinjal at 79.02% (Table. 4.4).

Visual assessment

The soft rot disease severity was assessed based on visual assessment of maceration area of all the vegetables and a fruit individually using a scale of **0 to 5, where 0 = no rot, for negative control to 5 = complete rot** (Table. 4.5).

Table 4.4 Percentage disease reduction (PDR)

Vegetable	Percent disease reduction (PDR)	Number of days
Potato	55.03%	10 days
Tomato	41.23%	3 days
Capsicum	45.5%	3 days
Brinjal	79.2%	7 days

Table 4.5 Visual assessment

Set	Sample	Scale value
Potato		
(i)	*G. nicotianae* AI5a	**0**
(ii)	*G. nicotianae* AI5a + *Pcc*BR1	**1**
(iii)	*Pcc*BR1	**5**
(iv)	Uninoculated control	**0**
Tomato		
(i)	*G. nicotianae* AI5a	**0**
(ii)	*G. nicotianae* AI5a + *Pcc*BR1	**1**
(iii)	*Pcc*BR1	**5**
(iv)	Uninoculated control	**0**
Capsicum		
(i)	*G. nicotianae* AI5a	**0**
(ii)	*G. nicotianae* AI5a + *Pcc*BR1	**1**
(iii)	*Pcc*BR1	**5**
(iv)	Uninoculated control	**0**
Brinjal		
(i)	*G. nicotianae* AI5a	**0**
(ii)	*G. nicotianae* AI5a + *Pcc*BR1	**1**
(iii)	*Pcc*BR1	**5**
(iv)	Uninoculated control	**0**

Quantification of *G. nicotianae* AI5a and *Pcc*BR1 in potato storage studies using qPCR

G. nicotianae AI5a 16S rRNA gene copy number was determined from the Ct values obtained on the standard plot by using *G. nicotianae* AI5a specific 16S rRNA gene primers AI5aF2/R2 and *Pcc*BR1 16S rRNA gene copy number was determined from the Ct values obtained on the standard plot by using *Pcc*BR1 specific 16S rRNA gene primers PccF1/R1. The bacterial quantification in terms of gene copy number by qPCR was obtained as Ct values which were converted to copies/µl using the standard graphs depicted in Fig 4.23 and 4.24 (Ren et al., 2014).

The copy numbers of *G. nicotianae* AI5a and *Pcc*BR1 16S rRNA genes determined at the time of inoculation were 6.57 X 10^6 copies/µl and 7.17 X 10^6 copies/µl, respectively. Notably, after 10 days in storage, *Pcc*BR1 specific 16S rRNA gene copy number was maintained at 1.77 X 10^6 copies/µl and as expected when treated with *G. nicotianae* AI5a the copy number was unchanged (1.6 X 10^6 copies/µl). In the same set where *Pcc*BR1 was treated with *G. nicotianae* AI5a the copy number of *G. nicotianae* AI5a specific 16S rRNA gene increased to 5 X 10^9 copies/µl. The potatoes treated with only *G. nicotianae* AI5a (no pathogen), the copy number of *G. nicotianae* AI5a increased to 6 X 10^9 copies/µl (Fig. 4.26). This agrees with the concept of attenuation of pathogen's virulence by quorum quenching since *G. nicotianae* AI5a did not kill *Pcc*BR1 but reduced its pathogenicity. Further visual and statistical analysis of this data was done using the statistical tools given by Hadizadeh et al., (2019). Despite potential off-target effects, this remains a effective strategy. More insights and research into potential off target effects could be done.

Fig. 4.27 illustrates and summarizes the pathogenicity of *Pcc*BR1 *in planta* which is termed as the black leg disease and soft rot disease on grown potato tubers under storage conditions. Potato blackleg caused due to *Pectobacterium* species is a slimy, wet, black rot lesion spreading from the rotting mother tuber up the stems, especially under wet conditions. When conditions are dry, symptoms are stunting, yellowing, wilting and desiccation of stems and leaves. Tuber soft rot is initiated at lenticels, the stolon end and⁄or in wounds under wet conditions. The lesion can spread to the whole tuber and thus over to tubers nearby in storage. Tuber tissue is macerated to a creamy mush which turns black on exposure to air, releasing a foul odour when attacked by secondary organisms. When seed tubers start rotting in the field before emergence,

blanking occurs. In ineffectually ventilated cool stores, rotting can spread to touching tubers as liquid from the rotting tubers infects onto others, usually steering to huge rotting pockets in the stored tuber batch (Czajkowski et al., 2011b).In this chapter appreciable attenuation of both these manifestations of *Pcc* infection has been acheived using quorum quenching strain *G. nicotianae* AI5a.

4.3.11 Effect of *G. nicotianae* AI5a on the colonization of *Pcc*BR1 pHC60 in mung bean

Mung bean (*Vigna radiata* L.) seeds were surface sterilized, germinated and bacterized with *Pcc*BR1 pHC60 and a mixture of *Pcc*BR1 pHC60 and *G. nicotianae* AI5a. Fig. 4.28 shows the seedlings grown in MS media post bacterization. Initial count of bacteria during bacterization for *G. nicotianae* AI5a and *Pcc*BR1 pHC60 were 6.57 X 10^6 copies/µl and 7.17 X 10^6 copies/µl, respectively.After 10 days, the average shoot length and root length of the *Pcc*BR1 pHC60 infected set was 1.96 cm and 0.7 cm, respectively while the set in which *Pcc*BR1 pHC60 was treated with *G. nicotianae* AI5a 15.18 cm and 3.46 cm, respectively which was a significant difference. *G. nicotianae* AI5a negated the pathogenicity of *Pcc*BR1 pHC60. *G. nicotianae* AI5a itself did not have any harmful effect on the plant and the *G. nicotianae* treated plants were equally healthy (Fig. 4.29 A and B).

Whilw quantifying the bacteria by qPCR it was observed that the copy number of *Pcc*BR1 pHC60 specific 16S rRNA gene was 8.57 X 10^5 copies/µl, while the copy number of *Pcc*BR1 pHC60 specific 16S rRNA gene in the set treated with *G. nicotianae* AI5a was 7.6 X 10^5 copies/µl. The copy number of *G. nicotianae* AI5a specific 16S rRNA gene increased in the set with and without *Pcc*BR1 pHC60 which was 1.93 X 10^8 copies/µl and 2.16 X 10^8 copies/µl respectively (Fig. 4.29 C and D). *G. nicotianae* AI5a reduced the pathogenicity of *Pcc*BR1 pHC60 without reducing its number *in vitro*, *in planta* or under storage conditions.

Further, a Transverse section (T.S) of the mung bean root to check any possible colonization of *Pcc*BR1 pHC60 inside the root showed that the bacterial masses were confined to the vicinity of the cells (Fig 4.30A) .Fig. 4.30 (B) shows *Pcc*BR1 pHC60 at 60X magnification in phase contrast micrograph. All large-celled tissues between

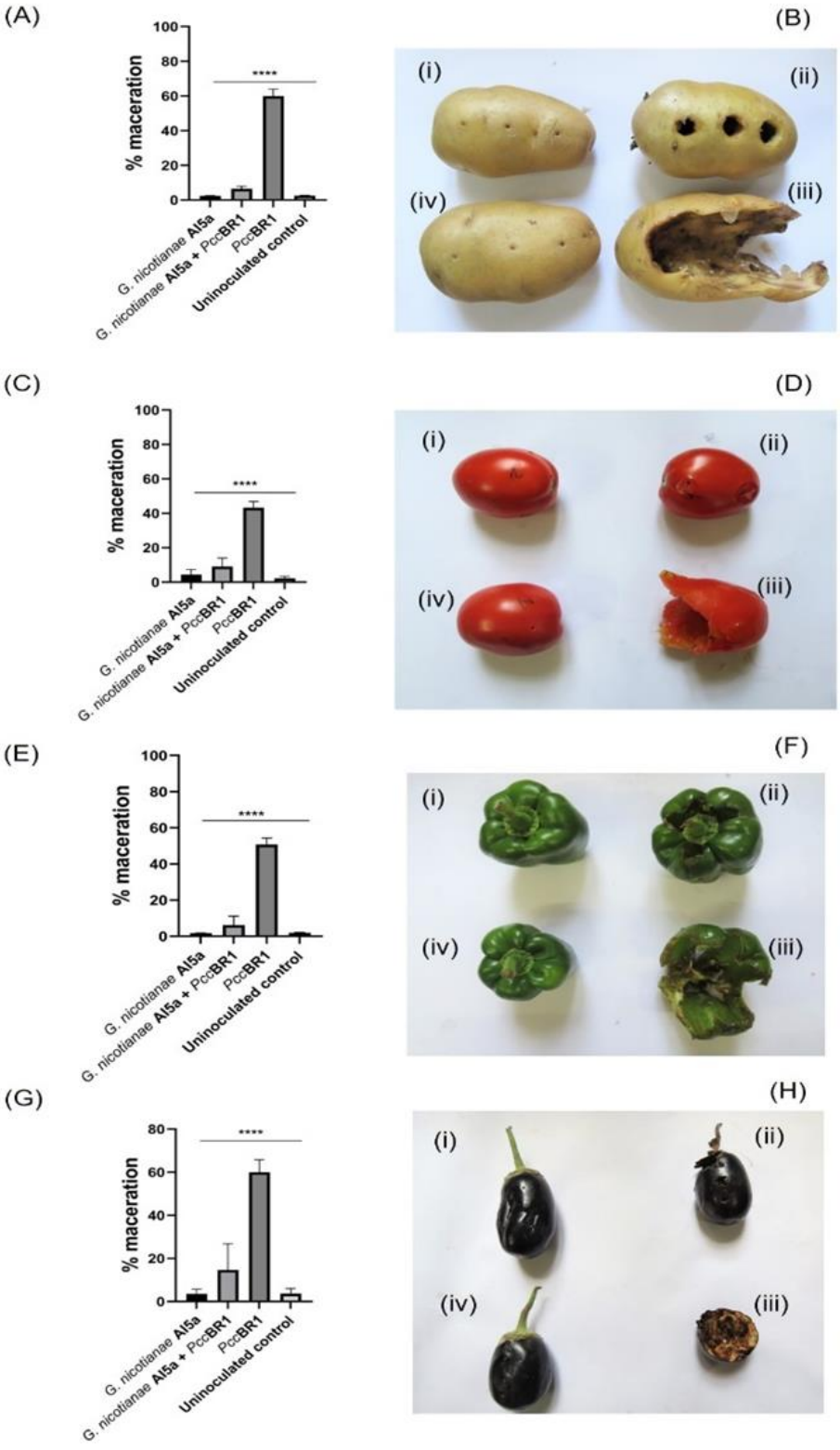


Figure 4.25 Storage experiments showing attenuation of *Pcc* induced soft rot symptoms by *G. nicotianae* AI5a with Potato, Tomato, Capsicum and Brinjal and as host and their Percent Maceration data

Set (i) *G. nicotianae* AI5a (ii) *G. nicotianae* AI5a + *Pcc*BR1 (iii) *Pcc*BR1 (iv) Uninoculated control

**** indicates P < 0.0001, per one-way ANOVA with Holm-Sidak's multiple comparisons test (n =5)

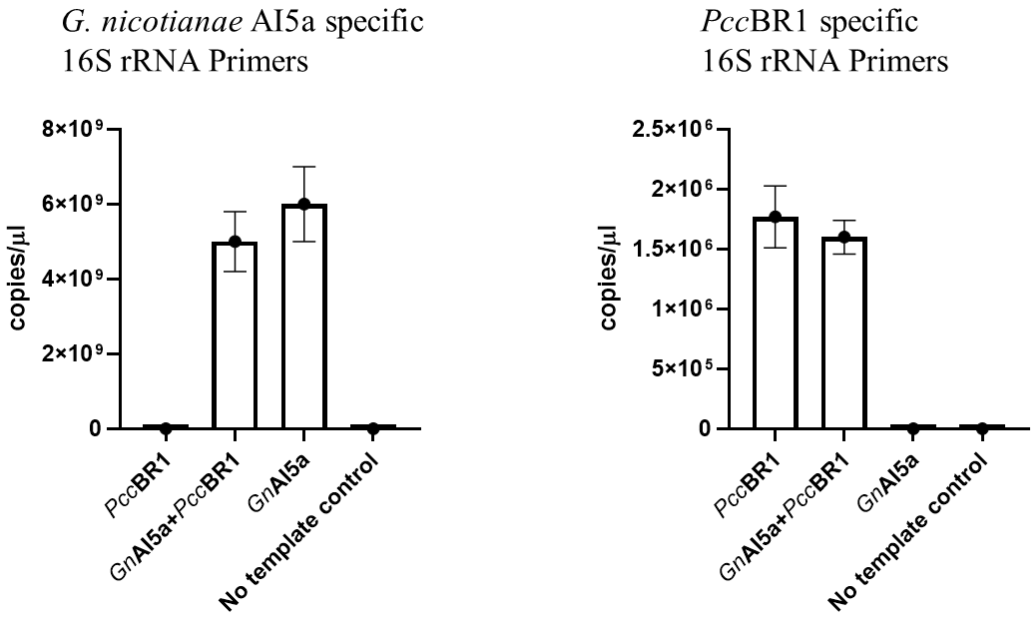


Figure 4.26 Quantitative real time PCR data using *G. nicotianae* AI5a specific 16S rRNA Primers and *Pcc*BR1 specific 16S rRNA Primers for Potato storage experiment.

bundles and toward the periphery were free from bacteria. (Fig 4.30A and C). The phloem and most of the xylem are also bacteria free (Fig 4.30 C).

In case of *Pcc*BR1 pHC60 the colonization inside the root was seen to be sparse. The phytopathogen causes its infection extracellularly. The fact that it remains on the surface could be making it more amenable to quorum quenching biocontrol mechanism.

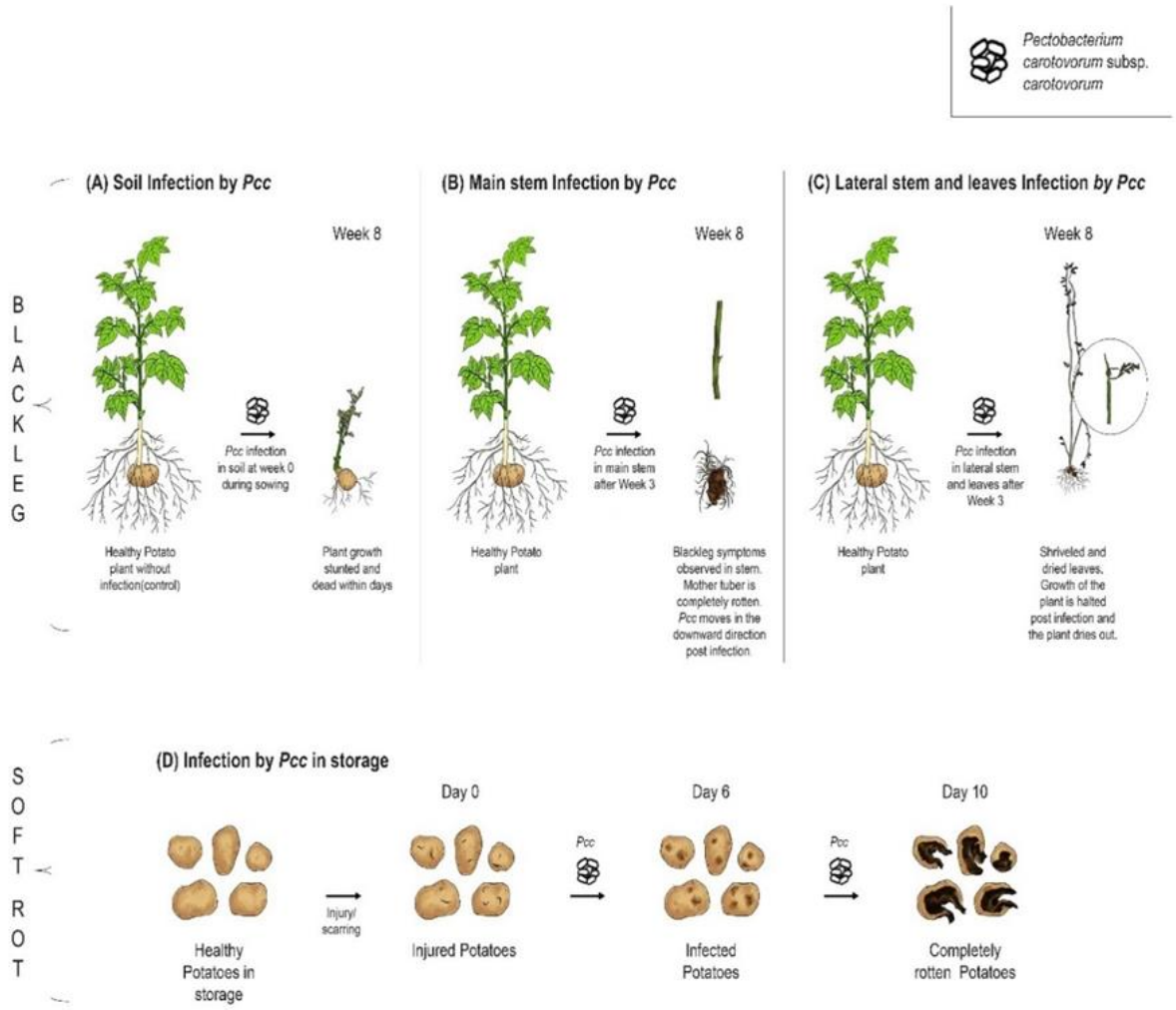


Figure 4.27 Summary of *Pcc*BR1 infection in potato *in planta* model (A,B and C) and storage (D).

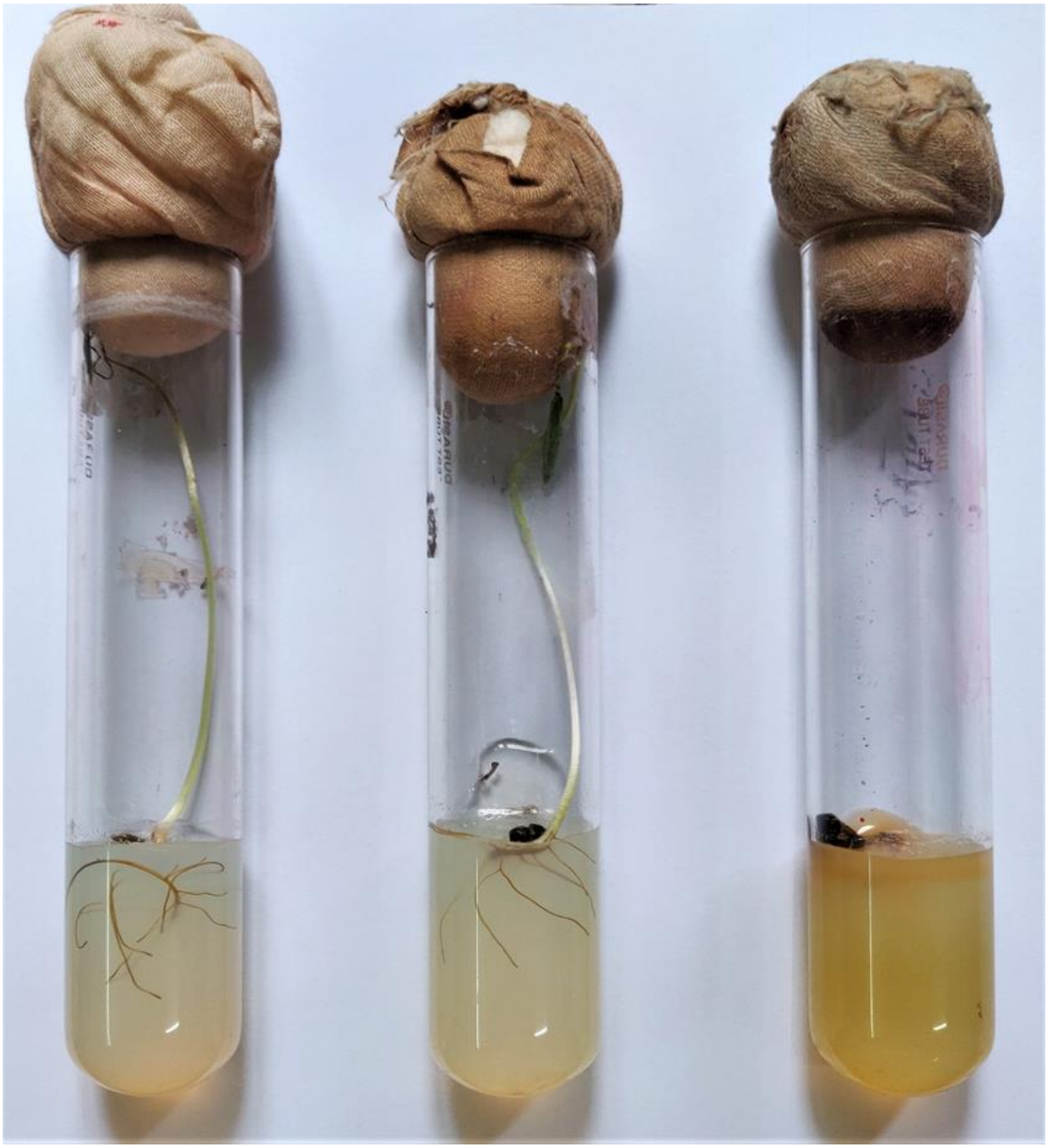


Figure 4.28 Image of Mung bean assay (A) *Pcc*BR1 pHC60 GFP + *G. nicotianae* AI5a (B) *G. nicotianae* AI5a (C) *Pcc*BR1 pHC60 GFP

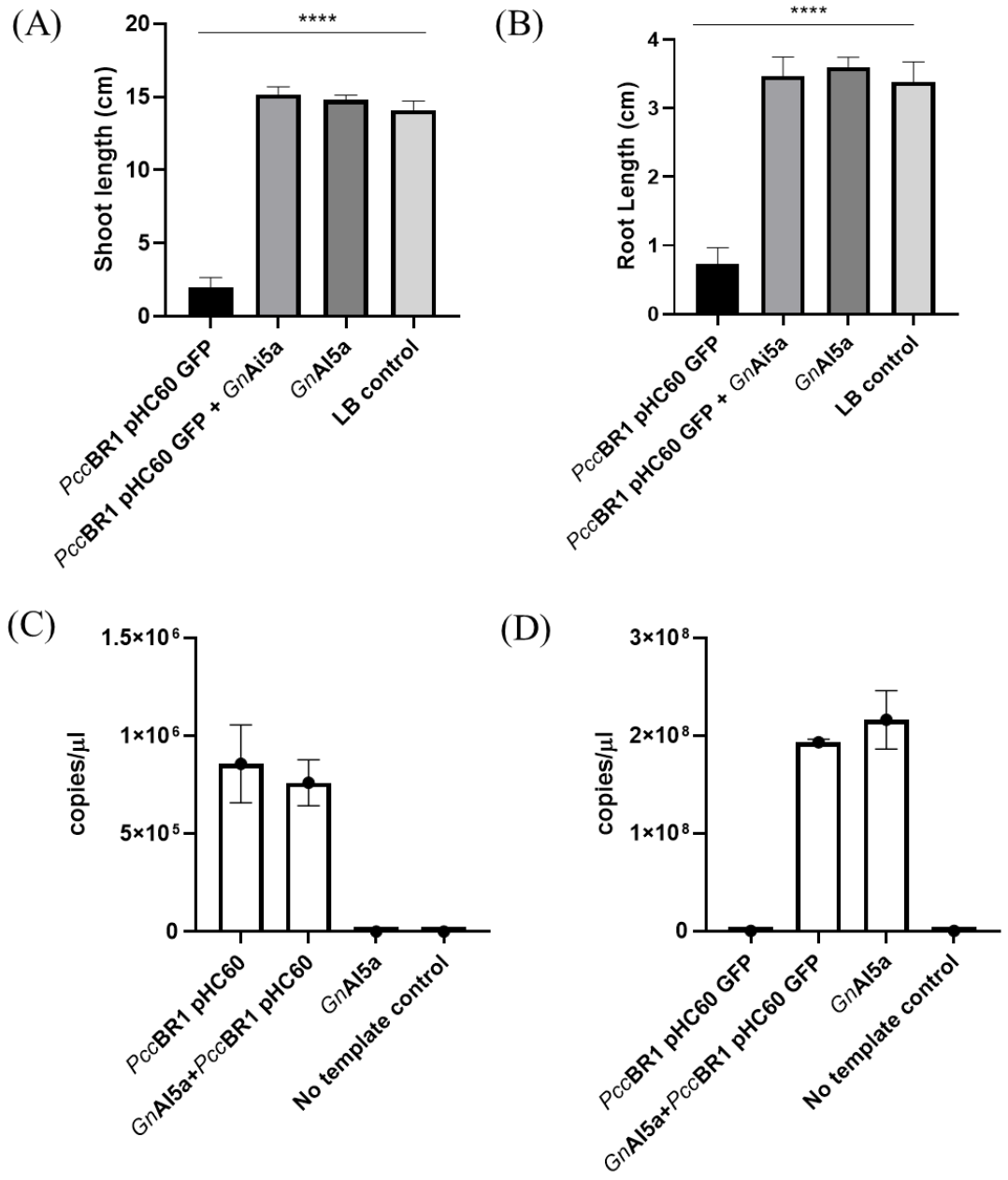


Figure 4.29 (A) Shoot length in Mung bean assay (n=6) (B) Root length in Mung bean assay (n=6) (C) qPCR analysis of *Pcc*BR1 pHC60 16S rRNA gene using specific primers for it (n=3) (D) qPCR analysis of *G. nicotianae* AI5a 16S rRNA gene using specific primers for it (n=3)

**** indicates P < 0.0001, per one-way ANOVA with Holm-Sidak's multiple comparisons test.

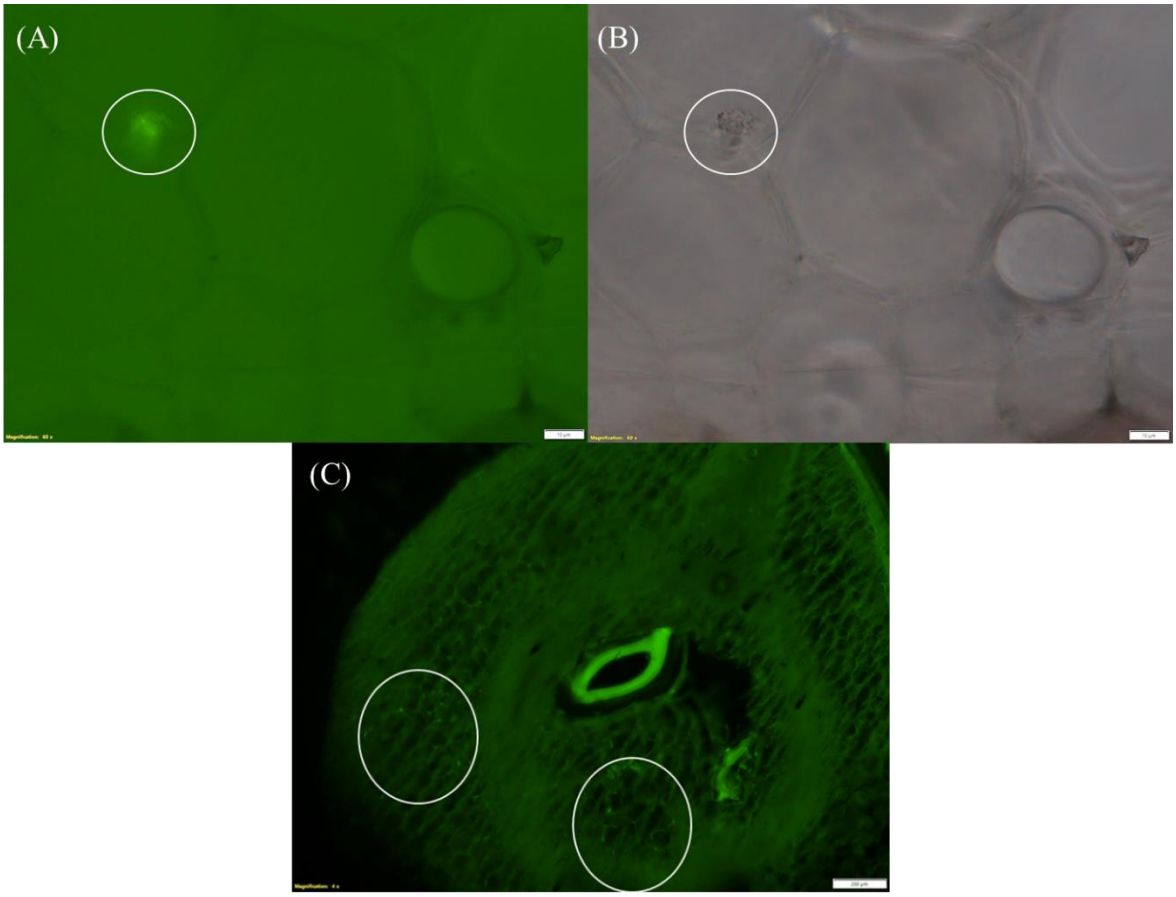


Figure 4.30 60X (A) and 4X (C) magnification images of TS of Mung bean root Fluorescence microscopy B) 60X Phase contrast microscopy.