Chapter 5

Virulence attenuation of *Pcc*BR1 by quorum quenching phytochemical compounds

5.1 Introduction

Phytophenolic compounds have been widely shown to act as quorum sensing inhibitors (QSI) in potential biocontrol strategy against Pectobacteria. Polyphenols are secondary metabolites ubiquitously distributed in all higher plants, which have important roles as defense against plant pathogens. As regard to chemical structure, they comprise a wide variety of molecules with polyphenol structure which include terpenes, phenylpropanoids, benzoic acid derivatives and flavonoids. Most of these compounds show direct effects on LuxI-type synthases and/or LuxR-type receptor proteins. Terpenes (carvacrol and l-carvone), phenylpropanoids (cinnamaldehyde and eugenol) and flavonoids (including quercetin) are "ideal QS inhibitors" because of the following features: They (i) are extremely stable compounds that are resilient to degradation by host metabolism, a feature that permits for their transportation to the location of action; (ii) can infiltrate bacterial cells and mix and focus on proteins due to low molecular weight molecules; and (iii) act as QS activators that promptly interact with high-specificity phytochemicals. Two other mentioned groups of plant-derived compounds show different styles of action on AHL-dependent quorum sensing which do not mix with LuxI/LuxR proteins but disturb the QS-related intracellular regulatory pathways. These "non-specific" mechanisms were assessed for sulfur-containing compounds (ajoene and iberin), altering several phenotypic traits by reducing the regulatory sRNAs expression, and coumarins leading to QS operons repression by means of c-di-GMP metabolism reduction. Thus, these compounds do not meet specificity standard (iii), because they have a repressive effect on an assortment of QS systems, regardless of the type of the autoinducer and the signal reception (Deryabin et al., 2019).

It is reported that sub-lethal concentrations of carvacrol, a component of oregano essential oil, reduced motility and biofilm formation in staphylococci and *Salmonella* strains (Burt et al., 2014) . Eugenol is one of the major constituents of essential oils from clove, nutmeg, cinnamon, basil, and bay leaf. Studies have identified the anti-biofilm and QS inhibitory effects of eugenol against *Pseudomonas aeruginosa* from patients with catheter-associated urinary tract infections. (Rathinam et al., 2021). Aspirin (6 mg/ml) showed significant reduction of quorum sensing signals in *P. aeruginosa*, including expression of elastase, proteases, and pyocyanin without affecting bacterial viability (El-Mowafy et al., 2014). In another study, various plants

extracts were screened and identified for their QSI activity against *P. aeruginosa* and it was found out that three compounds trans-cinnamaldehyde, tannic acid and salicylic acid were found to be potential QS inhibiting compounds. Trans- cinnamaldehyde was found out to be most effective of the three with further studies (Chang et al., 2014). Carvacrol, a hydrophobic terpene component of oregano essential oil, has been proposed as a potential inhibitor of biofilm formation and other virulence factors like PCWDE and motility in *P. carotovorum* (Joshi et al., 2016).

This chapter covers the selection of phytochemicals used for quorum sensing inhibition against *Pcc*BR1, finding their potential sub-lethal concentrations against the phytopathogen and their effect on AHL produced by *Pcc*BR1. Further, the ability of the phytochemicals to reduce soft rot *in vitro* was checked. The effect of the phytochemicals on the virulence traits (Motility, PCWDEs, Biofilm formation) was observed and finally their ability to stop infection in storage conditions and *in planta* was demonstrated.

5.2 Materials and methods

5.2.1 Bacterial strains

Pectobacterium carotovorum subsp. *carotovorum* BR1 and *Pcc*BR1 pHC GFP:

*Pcc*BR1 has been described in chapter 3 section xxxxx. *Pcc*BR1 pHC GFP was a GFP strain of *Pcc*BR1 which has been made by inserting the plasmid pHC60 with GFP. As mentioned earlier *Pcc* used in the entire studies produces 3- oxo-hexanoyl homoserine lactone which regulates production of PCWDE which cause soft rot in various plant hosts. The cultures were grown in LB media at 30 °C under shaking condition.

Chromobacterium violaceum CV026:

Chromobacterium violaceum CV026 was the biosensor strain already described in chapter 3 section xxxxx. It was grown in LB with 30 µl/ml Kanamycin at 30 °C.

5.2.2 Determination of sub-lethal concentration of phyto-chemical compounds

Following compounds were used to assess their effect on the quorum sensing ability of *Pcc*BR1.

Table 5.1 List of phytochemical compounds used

Phyto-chemicalcompounds	Stock concentrations
Cinnamic acid	40mg/ml
Caffeic acid	10mg/ml
Vanillin	760mg/ml
Salicylic acid	70mg/ml
Ferulic acid	220mg/ml
Elagic acid	2mg/ml
Catechol	10mg/ml
Coumaric acid	22mg/ml
Acibenzolar-S- methyl	0.5mg/ml
4-Amino cinnamic acid	2.5mg/ml
Eugenol	0.1M
Carvacrol	0.1M

All compounds were purchased from Sigma-Aldrich (Sigma-Aldrich Co., MO, USA). Minimum Inhibitory Concentrations (MICs) of compounds were determined by the micro broth dilution method. *Pcc*BR1 was grown overnight in LB and normalized to 1 x 10^8 colony forming units (CFU/ml) with fresh liquid LB. 2X concentration of the stock solution of the phytochemical compounds was used to begin the 2-fold serial dilution in a 96 well microtitre plate. 10 µl of culture was inoculated into each well and the plate was incubated overnight at 30 °C. Sub-lethal concentration of the phytochemical compounds was selected by comparing the OD with the control *Pcc*BR1 with no phytochemicals in it on Tecan microplate reader. The concentration at which the growth of PccBR1 was slightly reduced was taken as sublethal concentration for that compound. Further, the effect of the sub-lethal concentrations of the several compounds was checked on the virulence traits of *Pcc*BR1 i.e motility, PCWDEs and biofilm formation.

5.2.3 Qualitative assay for the detection and degradation of AHL by phytochemicals

A qualitative assay was performed as per Joshi et al. (2016) to assess the effect of the phenolic compounds on the production of AHL (3-oxohexanoyl homoserine lactone), produced by *Pcc*BR1. *Pcc*BR1 was grown in LB media with and without the phenolic compound (sub lethal concentration) overnight at 30˚C. The reporter strain *Chromobacterium violaceum* CV026 was grown in sterile LB media accompanied

with Kanamycin (10 µg/mL). Then, CV026 and *Pcc*BR1 were streaked perpendicularly (in T-shape) on LA plates as shown in the Fig 5.1. A gap of approximately 2 mm was maintained between the reporter strain and the *Pcc*BR1 streak (Fig. 5.1). The plates were incubated overnight at 30°C and the intensity of violet colour exhibited by the reporter strain was assessed .

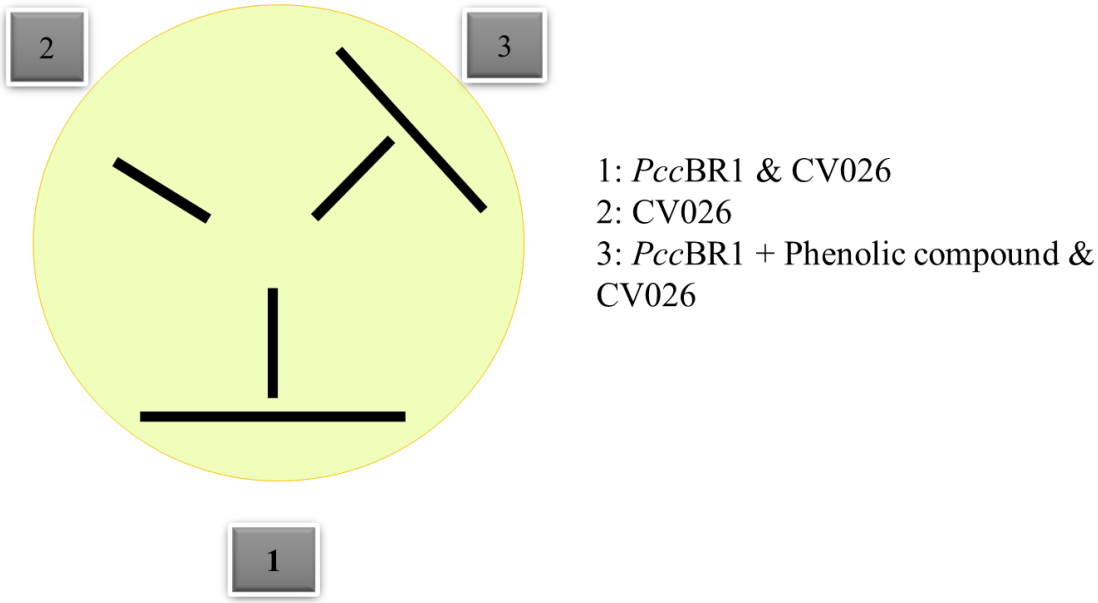

Figure 5.1 Schematic of Qualitative assay for the detection and degradation of AHL of *Pcc*BR1 by phytochemicals

5.2.4 *In vitro* soft rot assay

The *in vitro* soft rot attenuation assay as described by Dong et al., (2004) was modified and performed on potato and cucumber slices. Potato tubers and cucumber was purchased from local market and washed under tap water. Slices of equal thickness were cut with the knife. The slices were surface sterilized in the laminar hood by immersing sequentially in 1% sodium hypochlorite, 70% ethanol and autoclaved distilled water, followed by drying the slices on filter paper in laminar hood itself. These slices were then placed in autoclaved Petri plates having Whatman filter paper No. 1 for moisture retention. The Petri plates were weighed with and without cucumber and potato slices. Wells were bored on the potato slices for culture inoculation while sterile syringes were used for the same for cucumber slices. *Pcc*BR1 with and without phenolic compound was grown overnight at 30 °C and 5 µl of it was inoculated in the

well bored in potato while in case of cucumber a sterile syringe was used to inject the inoculum. 2-3 ml of sterile distilled water was added into the plates for providing moisture and the plates were then incubated at 30 °C for 24 h. The macerated tissue was scooped from the slices of potato and cucumber and weighed.

Maceration (%) was calculated using the following formula:

Maceration (%) = Weight of macerated tissue/Weight of tissue before maceration x 100

5.2.5 Effect of Phytochemical compounds on the motility of *Pcc*BR1

Motility assay was carried out in LB media with 0.3% agar. Phenolic compound was added into the media itself after autoclaving and dispensed into Petri plates. *Pcc*BR1 was grown in LB broth at 28°C overnight, centrifuged, washed and resuspended in saline. The bacterial suspension was adjusted to 10^8 cells/ml. 5 µl of this suspension was placed in the center of the plate and incubated at 28 °C for 2 days. The motility of *Pcc*BR1 was evaluated by measuring the diameter of the zone covered by bacteria (Talreja & Nerurkar, 2018).

5.3.6 Effect of Phytochemical compounds on the PCWDEs of *Pcc*BR1

Polygalacturonase activity was determined by measuring reducing sugar released as a result of hydrolysis of the polymer substrate i.e PGA using di-nitrosalicylic acid (DNSA) reagent (adapted and modified Miller, 1959). 250 µl of 0.5 % w/v substrate in 50 mM Tris-Cl pH 8.0 was incubated with 50 µl crude enzyme at 40 °C for 10 min. Reaction was stopped by adding 300 µl of DNSA reagent and boiled for 10 min. After cooling, volume was made up to 1.5 ml and absorbance at 540 nm was measured. The reducing sugar formed was quantified using D-galacturonic acid or D-xylose or D-glucose as a standard respectively. One Unit of enzyme activity was defined as the amount of enzyme required to release 1 µmole of respective reducing sugar per min at optimum conditions.

Pectin lyase (PNL) and polygalacturonate or pectate lyase (PL) activities were determined in crude pectinases by measuring unsaturated oligogalacturonides released as a result of the enzymatic cleavage of pectin and PGA respectively using TBA reagent (adapted and modified as per Nedjma et al., 2001). 250 µl of 0.5 % w/v substrate (pectin or PGA) in 50 mM Tris-Cl pH 8.0 was incubated with 50 µl of crude

enzyme at 50 °C for 30 min. Reaction was stopped by adding 0.5 ml of 1 M NaOH and incubating at 76 °C for 10 min. Addition of 0.6 ml of 1 M HCl followed by 0.5 ml of 40 mM TBA and incubation at 76 °C for 10 min developed a pink color, absorbance at 550 nm was measured. One unit of PNL or PL activity was defined as the amount of enzyme that caused a change in the absorbance of 0.01 per hour at optimum condition.

5.3.7 Effect of Phytochemical compounds on the biofilm formation of *Pcc*BR1

Microtiter plate assay was carried out for biofilm quantification. Biofilm formation was assayed by measuring the bacterial biomass adhered to microtiter wells. One mililitre of LB media was inoculated with 10^8 cells of *Pcc*BR1 along with non-inhibitory concentration of phenolic compounds in a 24-well polystyrene microtiter plate. The plate was incubated for a period of 72 hours at 30 °C under non-shaking. The wells were then rinsed five times with 1.5 ml of sterile PBS to remove any adhering planktonic cells. The biofilm was then stained with 1.5 ml of 1% crystal violet for 45 min, rinsed five times with 1.5 ml of water and destained with 70% ethanol for 15 min. Absorbance of the solution was measured at 595 nm (Srinandan et al., 2010).

5.3.8 Biocontrol of *Pcc*BR1 pHC60 soft rot on potato tubers by phytochemical compounds in storage conditions

Anti-virulence activity against soft rot pathogen *Pcc*BR1 pHC60 was checked in storage of potato tubers as per the method modified from Hadizadeh et al., (2019). Potato tubers were purchased from local market and washed by the tap water and with R.O. water thoroughly and then allow them to dry on filter paper. After air-drying for two hours, all the potatoes were weighed and labelled. *Pcc*BR1 pHC60 was cultivated overnight. *Pcc*BR1 pHC60 was sub-cultured to attain ~2.8×10^6 CFU/ml in LB broth at 30 °C. Two consecutive washes with PBS were given to the overnight grown culture which was then centrifuged and pellet was finally resuspended into 30 ml PBS. Inoculation was done aseptically by stabbing the vegetable to 3-5 cm deep with the help of sterile tooth picks after dipping it into the *Pcc*BR1 culture thrice.

In experiment control only *Pcc*BR1 pHC60 was inoculated by stabbing. While in (negative) control only the 3 phytochemicals were inoculated and one set kept as

overall negative control stabbed with PBS. In the experimental set 20 ml of *Pcc*BR1 pHC60 was mixed with 10 ml of biocontrol phytochemicals and then used for stabbing in the similar fashion as described in Chapter 4 Fig. 4.6. All the inoculated potato tubers were placed in plastic boxes which were sterilized and incubated at 30 ℃ for 10 days. The post infection bacterial load was quantified using quantitative Real Time PCR. Five replications of each set were used to check efficiency of eugenol, carvacrol and salicylic acid as biocontrol agents.

The results were observed and data was analysed by protocol shown by Hadizadeh et al.,(2019) as follows:

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 the phytochemicals 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 x 100)

Statistical analyses done by one way ANOVA and Holm-Sidak's multiple comparisons test. p value < 0.0001 (n=5). All comparisons done with *Pcc*BR1 pHC60 GFP.

5.3.9 *In planta* assay for effect of phytochemical compounds on the colonization of *Pcc*BR1 pHC60 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 was inoculated into 100 ml LB from overnight grown culture and incubated at 30 °C at 140 rpm till it 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 *Pcc*BR1 pHC60 was mixed with equal volume of Eugenol, Carvacrol and Salicylic acid respectively, in a final volume of 4 ml in a sterile scintillation vial for the experimental set. Five geminated mung bean seeds were added to *Pcc*BR1 culture suspension. Equal numbers of seeds were incubated in 4 ml of PBS as uninfected control, 4 ml of *Pcc*BR1 pHC60 as infection control and 4 ml of eugenol, carvacrol and salicylic acid respectively as positive control to make sure they do not infect the plant on their 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 glass 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 is 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.

5.3 Results and Discussion

5.3.1 Determination of sub-lethal concentration of phytochemical compounds on *Pcc*BR1

Polyphenols and phytochemicals are secondary metabolites ubiquitously distributed in all higher plants, which have important roles as defense against plant pathogens. The antimicrobial activity of polyphenols and phytochemicals occurring in vegetable foods and medicinal plants has been extensively investigated against a wide range of microorganisms (Daglia et al., 2011) , however the quorum quenching activity has been sparsely studied. Growth of *Pcc*BR1 at different concentrations of the phytochemicals has been studied in the present study. *Pcc*BR1 without any phytochemicals was used as control. The sub-lethal concentrations of the phytochemicals was determined by comparing the growth of *Pcc*BR1 with the phytochemicals to that of the control *Pcc*BR1 without any phytochemicals (Fig. 5.2). The the concentration of the phytochemical at which the growth of *Pcc*BR1 was slightly lower to the growth of the control was taken as sublethal concentration of that compound. The sub-lethal concentrations of different phytophenolic compounds tested are mentioned in Table 5.2.

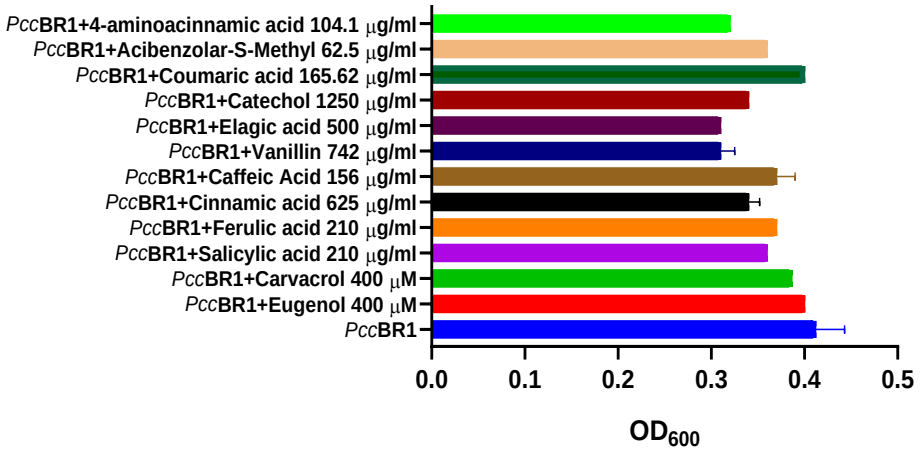

Figure 5.2 Growth of *Pcc*BR1 at different sublethal concentrations of different phytophenolic compounds

Table 5.2 Sub-lethal concentrations of phytochemical compounds obtained

Compounds	Sub-lethal concentration
Cinnamic acid	625 µg/ml
Caffeic acid	156 µg/ml
Vanillin	742 µg/ml
Salicylic acid	210 µg/ml
Ferulic acid	210 µg/ml
Elagic acid	500 µg/ml
Catechol	1250 µg/ml
Coumaric acid	165.62 µg/ml
Acibenzolar-S-Methyl	62.5 µg/ml
4-Amino cinnamic acid	104.1 µg/ml
Eugenol	0.4 µg/ml
Carvacrol	0.4 µg/ml

6.3.2 Effect of phytophenolic compounds on the production of quorum sensing molecule in *Pcc*BR1

AHL is the known signaling molecule of the QS system in *Pectobacterium* spp. that regulates the virulence traits viz. the synthesis of PCWDEs, biofilm formation and motility. The phytophenolic compounds Eugenol and Carvacrol at 250 µM concentration were found to reduce the production of AHL in *P. aroidearum* PC1 and *P. carotovorum* subsp. *brasiliense* Pcb1692 which was observed using the sensor strain CV026 (Joshi et al., 2016). In order to check for the same, the phytophenolic compounds listed in Table 5.2 were analyzed for their ability to disrupt the QS mechanism in *Pcc*BR1 and it was observed that Eugenol (400 µM), Carvacrol (400 µM) and Salicylic acid (210 µg/ml) inhibited AHL as no purple pigment (violacein) was detected when tested in the bioassay with the biosensor strain *Chromobacterium violaceum* CV026 (Fig. 5.3). Other compounds did not show reduction in purple pigment violacein as compared to the control. While Catechol and Acibenzolar-S-Methyl showed AHL inhibition but at the same time also reduced the growth of *Pcc*BR1 and thus were not considered in further experiments.

Three non-lethal concentrations of Eugenol and Carvacrol were experimented with- 200, 300 and 400 µM. Inhibition of AHL of *Pcc*BR1 was only observed at 400 µM and that concentration is used in further experiments. Out of 12 phytophenolic compounds tested, Eugenol, Carvacrol and Salicylic acid showed AHL inhibition of *Pcc*BR1 and were selected for further studies (Fig. 5.4).

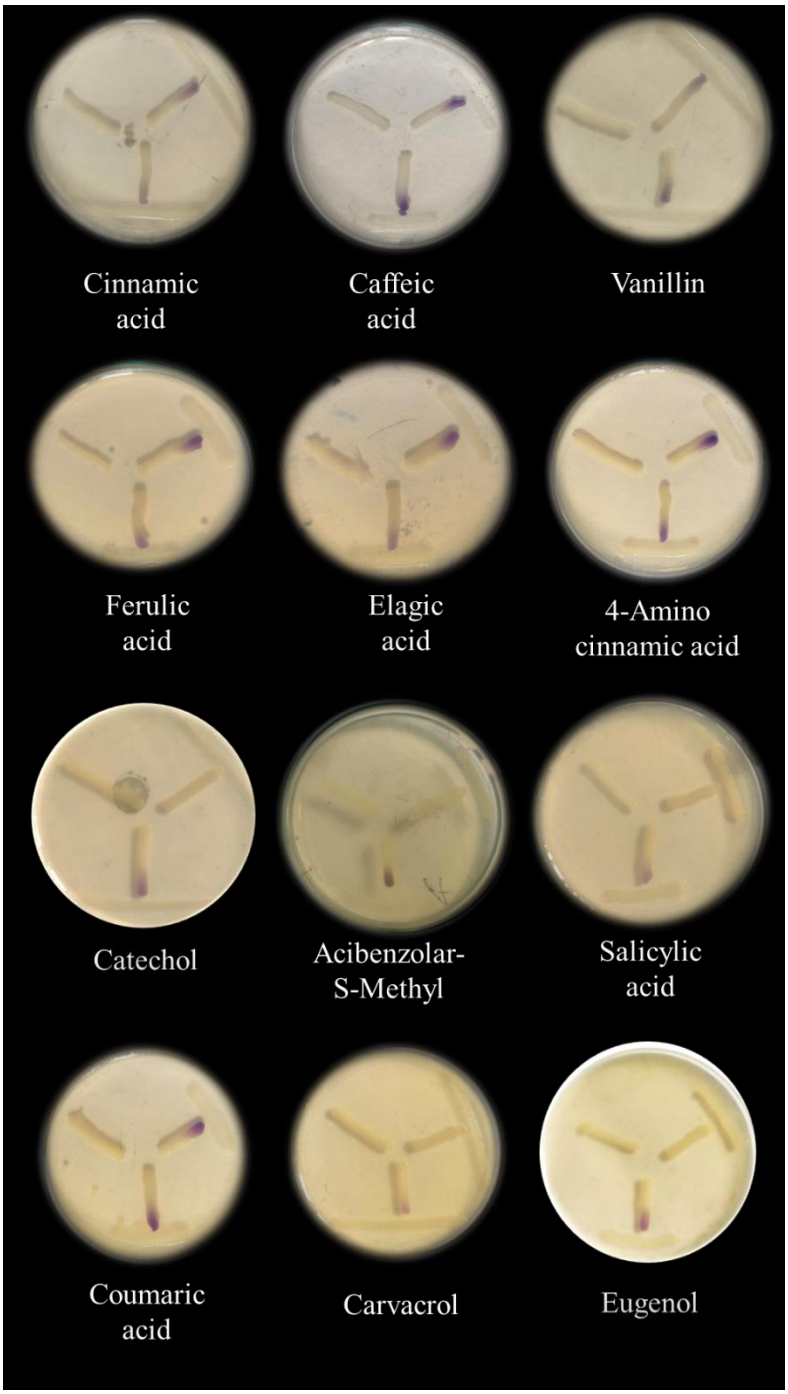

Figure 5.3 Effect of phytochemical compounds on production of AHL by *Pcc*BR1

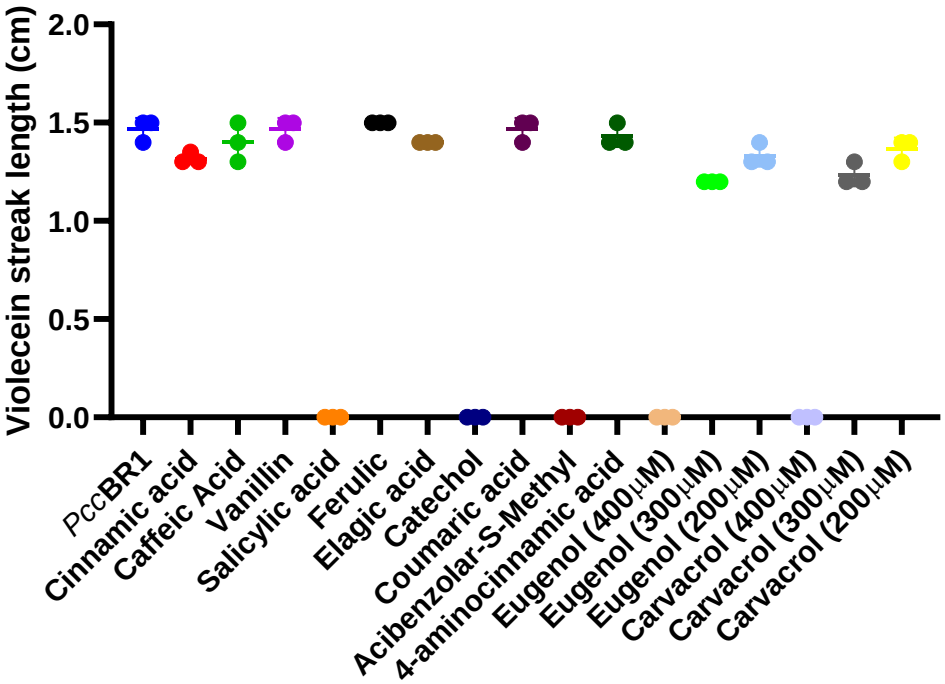

Figure 5.4 Analysis of AHL production by *Pcc*BR1 in the presence of phytophenolic compounds.

5.3.3 *In vitro* biocontrol assay for soft rot caused by *Pcc*BR1 in Potato and Cucumber

Pectobacteria are aggressive necrotrophs that harbor a large arsenal of plant cell wall-degrading enzymes as their primary virulence determinants are regulated by quorum sensing. These enzymes together with additional virulence factors are employed to macerate the host tissue and promote host cell death to provide nutrients for the pathogens (Davidsson et al., 2013). Since Eugenol, Carvacrol and Salicylic acid were inhibiting the production of the quorum sensing signaling molecule AHL, their effect on maceration of plant tissue would be interesting to observe. The biocontrol studies were done *in vitro* on cucumber because of its easy susceptibility to soft rot, and potato which is the most susceptible crop to soft rot. (Bhat et al., 2010; Pérombelon, 2002). *Pcc*BR1 when inoculated alone caused severe potato tissue maceration resulting in 77.73 % macerated tissue per site of inoculation on each potato slice (Fig. 5.7(A)). Similarly, when slices of cucumber were inoculated with pathogen alone maceration obtained was 99 % per slice (Fig. 5.7(B)). However, when the phytophenolic compounds (Eugenol, Carvacrol and Salicylic acid) were added along with *Pcc*BR1

the soft rot symptom of maceration in potato slices were significantly reduced to 10%, no maceration, 15% in Eugenol (400 µM), Carvacrol (400 µM) and Salicylic acid (210 µg/ml), respectively (Fig 5.5 and 5.7). Similar effects of the phytophenolic compounds were observed in case of cucumber slices (Fig. 5.6 and 5.7).The results of this assay agree with that reported by Joshi et al., (2015b; 2016). They reported that there is very less or no maceration in potato slices at the inoculation sites in the presence of Salicylic acid, Eugenol (5 mM) and Carvacrol (3 mM).

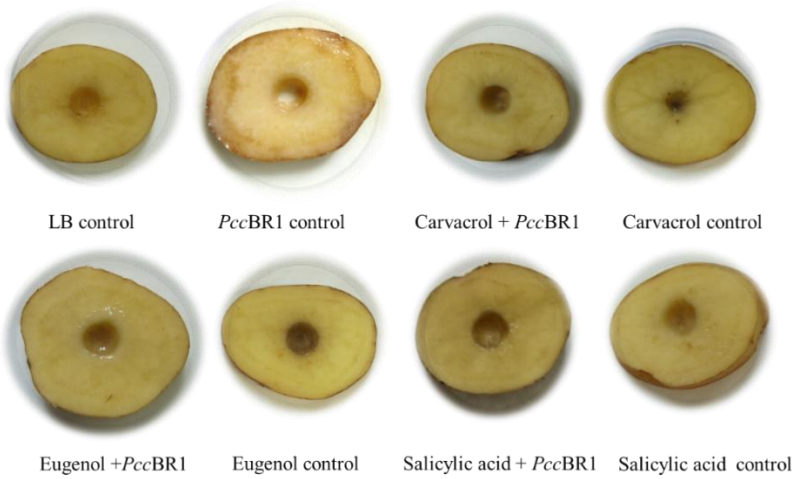

Figure 5.5 *In vitro* biocontrol assay for soft rot caused by *Pcc*BR1 on potato and biocontrol by phytochemicals

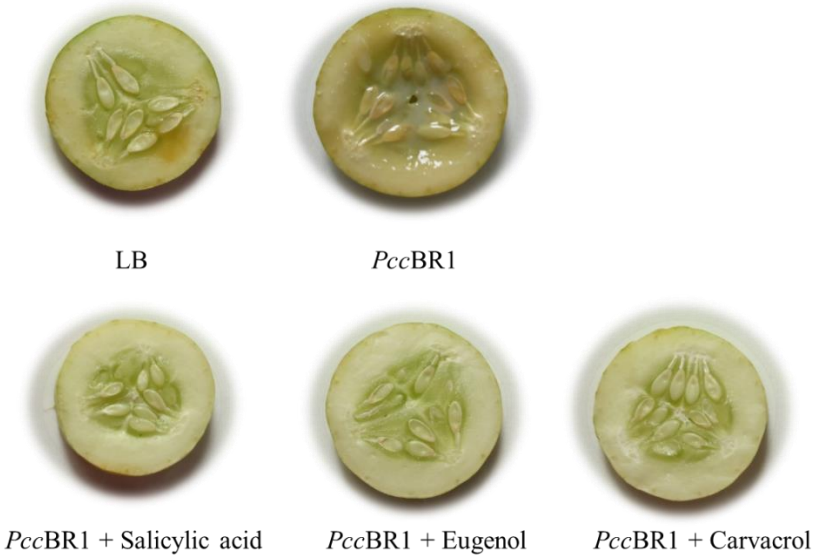

Figure 5.6 *In vitro* biocontrol assay for soft rot caused by *Pcc*BR1 on cucumber and biocontrol by phytochemicals

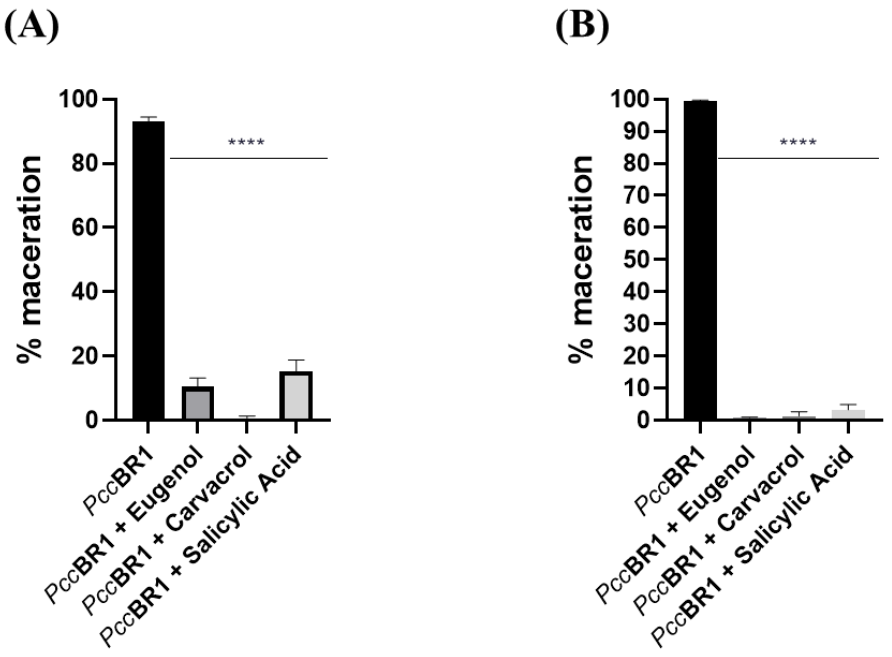

Figure 5.7 Quantification of maceration in *in vitro* biocontrol assay for soft rot caused by *Pcc*BR1 (A) Potato (B) Cucumber

5.3.4 Effect of Phytophenolic compounds on motility of *Pcc*BR1

The effects of the phytophenolic compounds on the motility of *Pcc*BR1 are represented in Fig. 5.8. It can be observed that diameter of *Pcc*BR1 colony in the soft agar plate was 18 mm which got reduced to 12.5 mm in case of Eugenol (400 µM), 10.5 mm for Carvacrol (400 µM) and 11 mm for Salicylic acid (210 µg/ml). It has been reported that motility is an important virulence factor of many plant-pathogenic bacteria, including *Pectobacterium* and has been correlated with the capacity of the microorganism to colonize the host surfaces (Duan et al., 2013; Hossain et al., 2005). Joshi et al., (2015a) have reported reduced motility of *Pectobacterium carotovorum* (*Pc*11) in presence of salicylic acid. In another study by Gutierrez-Pacheco et al. (2018) it is reported that 0.66 mM of Carvacrol had considerably reduced the motility of *P. carotovorum* from 54.7 mm in control to 6.5 mm in presence of Carvacrol. Another study carried out by Sivaranjani et al., (2016) have elucidated that the expression of some virulence factors including motility are regulated by AHL dependent QS mechanism in *Pectobacterium* spp.

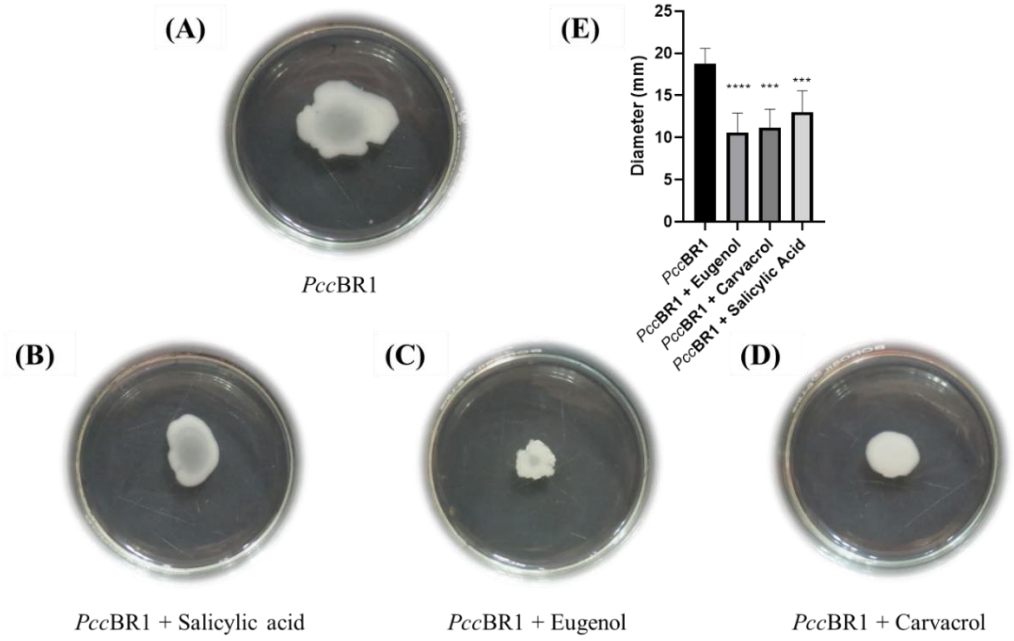

Figure 5.8 (A,B,C,D) Effect of phytophenolic compounds on motility of *Pcc*BR1 (E) Quantification of motility

5.3.5 Effect of phytophenolic compounds on PCWDEs production by *Pcc*BR1

Exoenzymes are major virulence determinants of Pectobacteria. These enzymes are secreted to the extracellular space through the type II secretion system (Davidsson et al., 2013; Moleleki et al., 2017) To understand the role of the phytophenolic compounds on the production of different exoenzymes by *Pcc*BR1 quantitative assays were done for the following enzymes: Polygalacturonase (PGA), Pectin lyase (PNL) and polygalacturonate or pectate lyase (PL).

The assay was carried out for 48 hours, every 12 hours aliquots were taken and enzyme activity was measured using cell supernatant. All the enzymes showed a decline in enzyme production with time in presence of phytochemicals and by 48 hours their activity was negligible. In all the assays a similar trend was observed wherein up to 36 hours the enzyme activity increased then declined and was negligible at 48 hours. Also, the enzyme activity of Pectin lyase (PNL) was more in *Pcc*BR1 compared to Pectate lyase (PL) and Polygalacturonase (PGA) (Fig. 5.9).

Among the PCWDEs (PNL, PL and PGA), the PNL activity of *Pcc*BR1 was found out to be 37.62 enzyme units (EU) and 41.3 EU at 24 and 48 h, respectively, which was highest amongst the three PCWDEs. The PNL activity was significantly reduced by

all three phytochemicals. At 24 h when co-cultivated with Eugenol, Carvacrol and Salicylic acid separately, *Pcc*BR1 showed reduced PNL activity of 11.26, 13.48 and 12.17 EU, respectively. At 48 h when co-cultivated with Eugenol, Carvacrol and Salicylic acid separately, *Pcc*BR1 showed reduced PNL activity of 0.81, 1.62 and 2.11 EU, respectively (Fig. 5.9A).

*Pcc*BR1 produced less PGA and PL when compared to PNL. PGA activity by *Pcc*BR1 at 48 h was 19.73 EU which was reduced to 1.32, 1.33 and 1.9 EU by co-culture with Eugenol, Carvacrol and Salicylic acid, respectively (Fig. 5.9B). PL activity by *Pcc*BR1 at 48 h was 20.1 EU which reduced to 1.36, 1.31 and 2.7 EU by co-culture with Eugenol, Carvacrol and Salicylic acid, respectively (Fig. 5.9C).

Overall, the three phytophenolic compounds must be inhibiting the QS mechanism due to which the activity of these enzymes was decreasing. Joshi, et al., (2016) have also reported a decline in the enzyme activity of exoenzymes produced by Pectobacteria. It was observed that Eugenol and Carvacrol completely blocked the activity of Peh in strain *Pectobacterium carotovorum* subsp. *brasiliense* Pcb1692 and reduced the activity of Pectate lyase to 30 % in presence of Eugenol and 50 % in case of Carvacrol.

5.3.6 Effect of phytochemicals on biofilm formation ability of *Pcc*BR1

Biofilm formation ability is a recognized trait in terms of its importance for successful infection of any bacteria including *Pectobacterium*. It is one of the important virulence traits that is under the control of Quorum sensing. It is represented as the absorbance of crystal violet dye bound to biofilm cells. Therefore, more absorbance indicates more biofilm and vice versa.

Before observing the effect of these phytophenolic compounds on biofilm formation; their effect on the viability of *Pcc*BR1 was analyzed for 72 h. It is very clear from Fig. 5.10 that these compounds do not affect the cell viability of *Pcc*BR1.

On exposure of *Pcc*BR1 to phytophenolic compounds i.e Eugenol (400 µM), Carvacrol (400 µM) and Salicylic acid (210 µg/ml) a significant reduction in biofilm formation was observed as compared to only *Pcc*BR1. In comparison to *Pcc*BR1 the biofilm formation was reduced to 31.8% for Eugenol, 24.2 % for Carvacrol and 37.5 % for Salicylic acid (Fig. 5.10). No such reduction was observed in the presence of ethanol, used as carrier of

these volatiles, and the LB media control treatment. Similar results were reported by (Joshi et al., 2015a) wherein for both the strains, *Pectobacterium aroidearum* PC1 and *Pectobacterium carotovorum* subsp. *brasiliense* Pcb1692 a significant (p <0.05) reduction

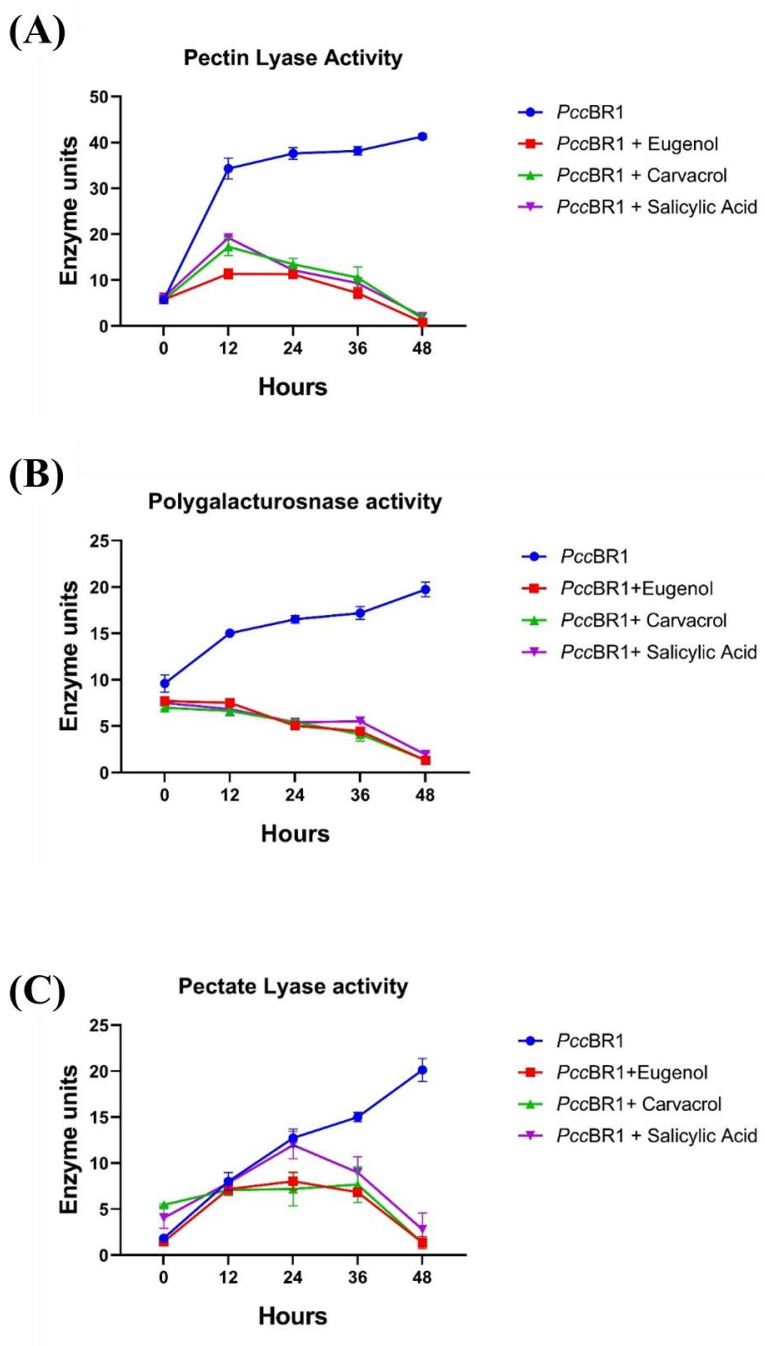

Figure 5.9 Effect of phytochemical compounds on the activities of exoenzymes: (A) Pectin lyase, (B) Polygalacturonase and (C) Pectate lyase

in biofilm formation was observed at 250 µM of carvacrol and eugenol. The biofilm ability of PC1 was reduced to 19% in presence of Eugenol and 9.6% with carvacrol and in case of Pcb1692 it got reduced to 7.6% in presence of Eugenol and 3.8% for carvacrol. Burt et al. (2014) also reported that carvacrol (at subinhibitory concentrations, < 0.5 mM) inhibited the biofilm formation of *Chromobacterium violaceum*, *Salmonella Typhimurium and Staphylococcus aureus*; whereas, (Tapia-Rodriguez et al., 2017) observed that this compound (at 3.9 and 0.7 mM) caused a reduction of pyocyanin and violacein, virulence factors regulated by QS.

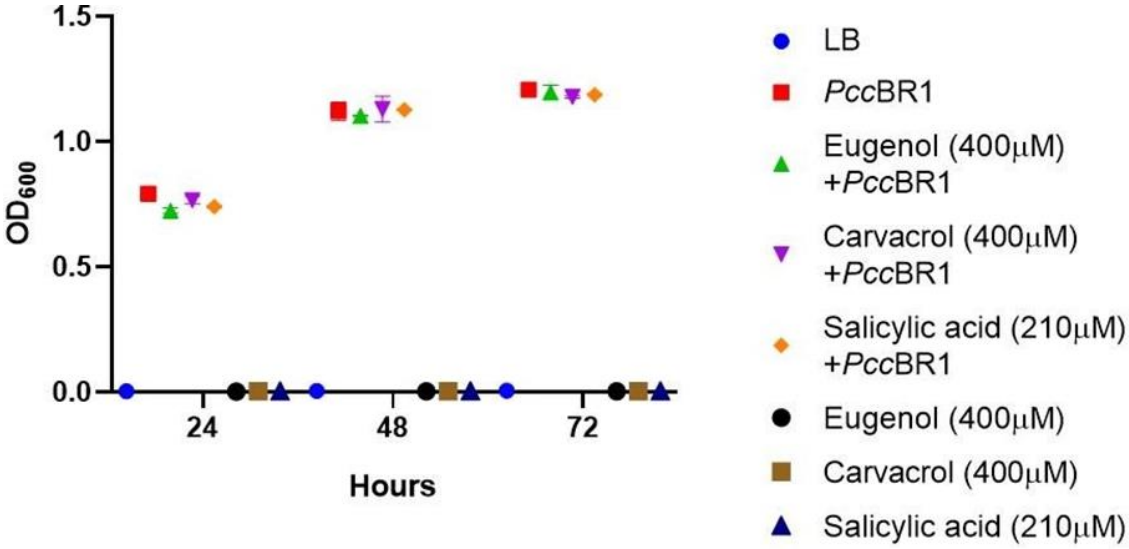

Figure 5. 10 Effect of sub-lethal concentrations of Eugenol, Carvacrol and Salicylic acid on growth of *Pcc*BR1

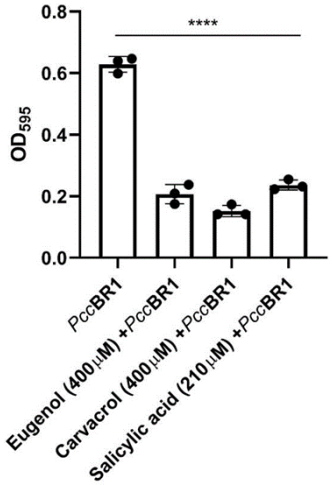

Figure 5.11 Effect of Eugenol, Carvacrol and Salicylic acid on biofilm formation ability of *Pcc*BR1.

5.3.7 Biocontrol ability of phytochemicals against soft rot causing phytopathogen *Pcc*BR1 in storage conditions

Potato is the vegetable that is stored for longest duration. In the present study, potato tubers treated with Eugenol, Carvacrol and Salicylic acid showed significant reduction in soft rot, as compared to *Pcc*BR1 positive control. The phytochemicals prevent the PCWDEs formation by *Pcc*BR1 to regulate virulence factor (PCWDEs) production as shown earlier. 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. The PDR (percentage disease reduction) was also calculated as shown in Table. 5.3. No symptoms were observed on the negative controls (potatoes only inoculated with the three phytochemicals. The present work was carried out using the method developed earlier for biocontrol of soft rot at room temperature storage condition for a wide range of hosts.

Quantification of the presence of the *Pcc*BR1 was also done by quantitative real time PCR studies have demonstrated using biocontrol bacteria against phytopathogens belonging to the *Pectobacteria* family but not many studies are available where in storage experiments are done using phytochemicals.

*Pcc*BR1 inoculated potato tubers showed 59.1% maceration of the tissue but when treated with biocontrol Eugenol, Carvacrol and Salicylic acid percentage maceration reduced to 2.2%, 3.35% and 5.1% respectively from 59.1% when compared to *Pcc*BR1 control (Fig. 5.11B).

Quantification of bacteria in potato tissue was done using quantitative Real Time PCR (Hadizadeh et al., 2019)(Fig. 5.11C). *Pcc*BR1 16S rRNA gene copy numbers were determined from the Ct values obtained on the standard plot by using *Pcc*BR1 specific 16S rRNA gene primers PccF1/R1. The Ct values obtained were converted to copies/µl using the Standard graph (Chapter 4 Fig. 4.24) (Ren et al., 2014).

The copy number at the time of inoculation for *Pcc*BR1 was 7.17×10^6 copies/µl.

After 10 days in storage, *Pcc*BR1 16S rRNA gene copy number was 1.77×10^6 copies/µl but when treated with Eugenol, Carvacrol and Salicylic acid the copy number of *Pcc*BR1 16S rRNA gene reduced to 1.64×10^6, 1.66×10^5 and 1.6×10^5 copies/µl, respectively. The copy number of *Pcc*BR1 16S rRNA gene did not decrease significantly upon adding the phytochemicals at sub-lethal concentrations but they were able to reduce soft rot and overall pathogenicity of *Pcc*BR1 on potato tubers.

There was no *Pcc*BR1 infection found induced or external upon injury to the potato tubers treated with just the phytochemicals. Further visual and statistical analysis was done using the protocol shown by (Hadizadeh et al., 2019). Potential combination of enzymatic and non-enzymatic QQ approach can be looked into. Formulations of its combined effect can be stuidied.

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). The maximum Percent Disease reduction (PDR) was observed in Eugenol treated set at 48.99% among the three phytochemicals (Table. 5.3).

Visual assessment of maceration

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. 5.4 shows the data where the set infected with *Pcc*BR1 shows complete rot while the infected sets treated with phytochemicals showed reduced rotting.

Table 5.3 Percentage Disease Reduction (PDR) of Potato after storage (Phytochemicals)

Set	Percent disease reduction (PDR)	Number of days
Eugenol + *Pcc*BR1	48.99%	10 days
Carvacrol + *Pcc*BR1	46.65%	
Salicylic acid + *Pcc*BR1	40.47%	

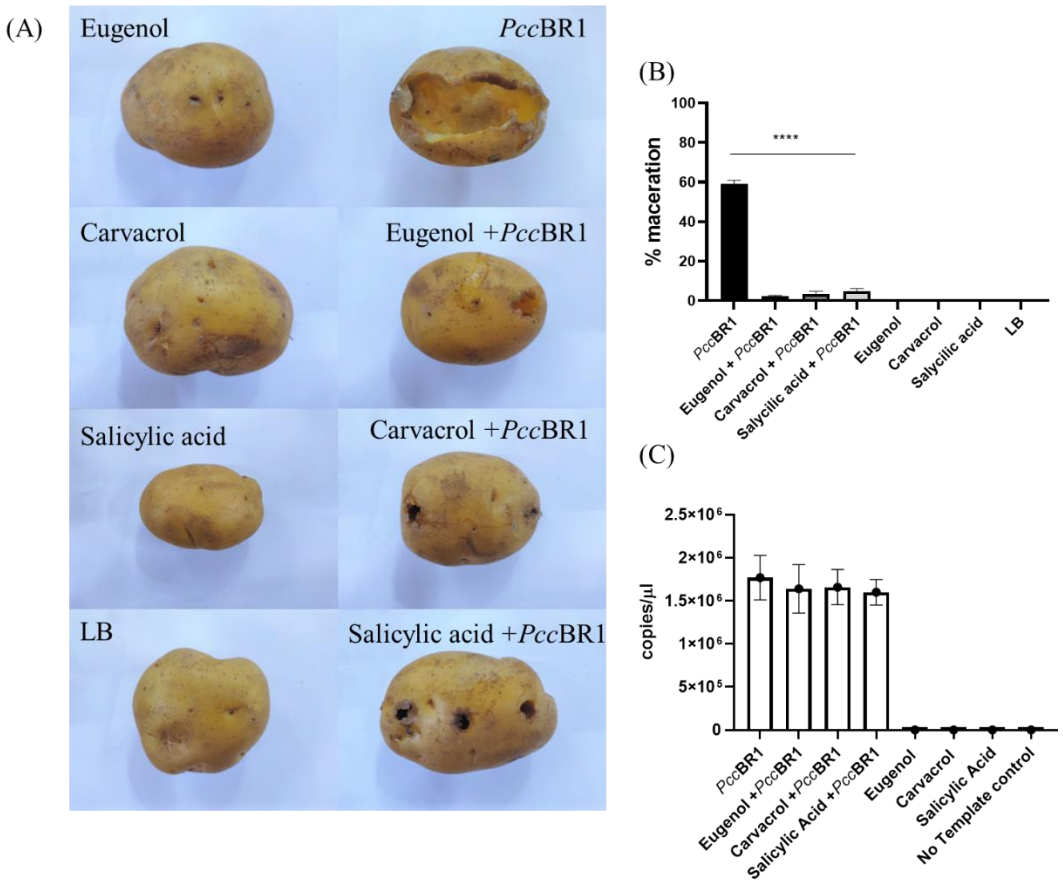

Figure 5.12 (A) Images demonstrating after 10 days storage of potato tubers with phytochemicals against *Pcc*BR1 (B) Percent Maceration data (C) Quantitative real time PCR data and *Pcc*BR1 specific 16S rRNA gene primers for Potato storage experiment.

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

Table 5.4 Visual assessment of Potato after storage (Phytochemicals)

Set	**Scale value**
Eugenol + *Pcc*BR1	**1**
Carvacrol + *Pcc*BR1	**1**
Salicylic acid + *Pcc*BR1	**1**
*Pcc*BR1	**5**
Eugenol	**0**
Carvacrol	**0**
Salicylic acid	**0**
Uninoculated control	**0**

5.3.8 Effect of phytochemicals 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 as control and a mixture of *Pcc*BR1 pHC60 and Eugenol, Carvacrol and Salicylic acid in separate experimental sets. The seeds planted in sterile MS media in tubes and grown in sterile conditions at 25°C for 10 days are shown in Fig. 5.12. After 10 days, the average shoot length and root length of the *Pcc*BR1 pHC60 set was 1.90 cm and 0.71 cm, respectively while the set in which *Pcc*BR1 pHC60 was treated with eugenol, carvacrol and salicylic acid the shoot length was 15.05 cm, 14.53 cm and 14.41 cm respectively and root length was 3.53 cm, 3.55 cm and 3.28 cm respectively, which was a significant difference. The three phytochemicals negated the pathogenicity of *Pcc*BR1 pHC60. Eugenol, Carvacrol and Salicylic acid themselves did not have any harmful effect on the plant (Fig. 5.13 A and B).

*Pcc*BR1 pHC60 was measured by the standard obtained by using *Pcc*BR1 specific 16S rRNA gene primers PccF1/R1. The Ct values obtained were converted to copies/µl using the Ct values obtained by the Standard graph (Chapter 4 Fig. 4.24) (Ren et al., 2014). Quantifying the bacteria by quantitative Real Time PCR we observed that *Pcc*BR1 pHC60 16S rRNA gene copy number was 8 X 10^5 copies/µl, while *Pcc*BR1 pHC60 GFP treated with Eugenol, Carvacrol and Salicylic acid the 16S rRNA gene copy numbers were 5.5 X 10^5, 3.1 X 10^5 and 2.8 X 10^5 copies/µl, respectively. Initial copy number of 16S rRNA gene of *Pcc*BR1 pHC60 during bacterization was 7.17 X 10^6 copies/µl. There was an overall one-fold reduction in *Pcc*BR1 pHC60 16S rRNA gene copy number when compared to the initial count during bacterization in the overall experiment, Notably, the copy number of *Pcc*BR1 pHC60 16S rRNA gene did not reduce and was maintained at in comparison to the range of 2.8-5 x 10^5 copies/ µl in the experimental sets, even upon treatment by Eugenol, Carvacrol and Salicylic acid (Fig. 5.13C).This agrees with the quorum quenching approach where the counts of the bacteria remains unaffected while the virulence of the pathogen is attenuated.

Figure 5.13 Representative image of Mung bean assay with *Pcc*BR1 and phytochemicals

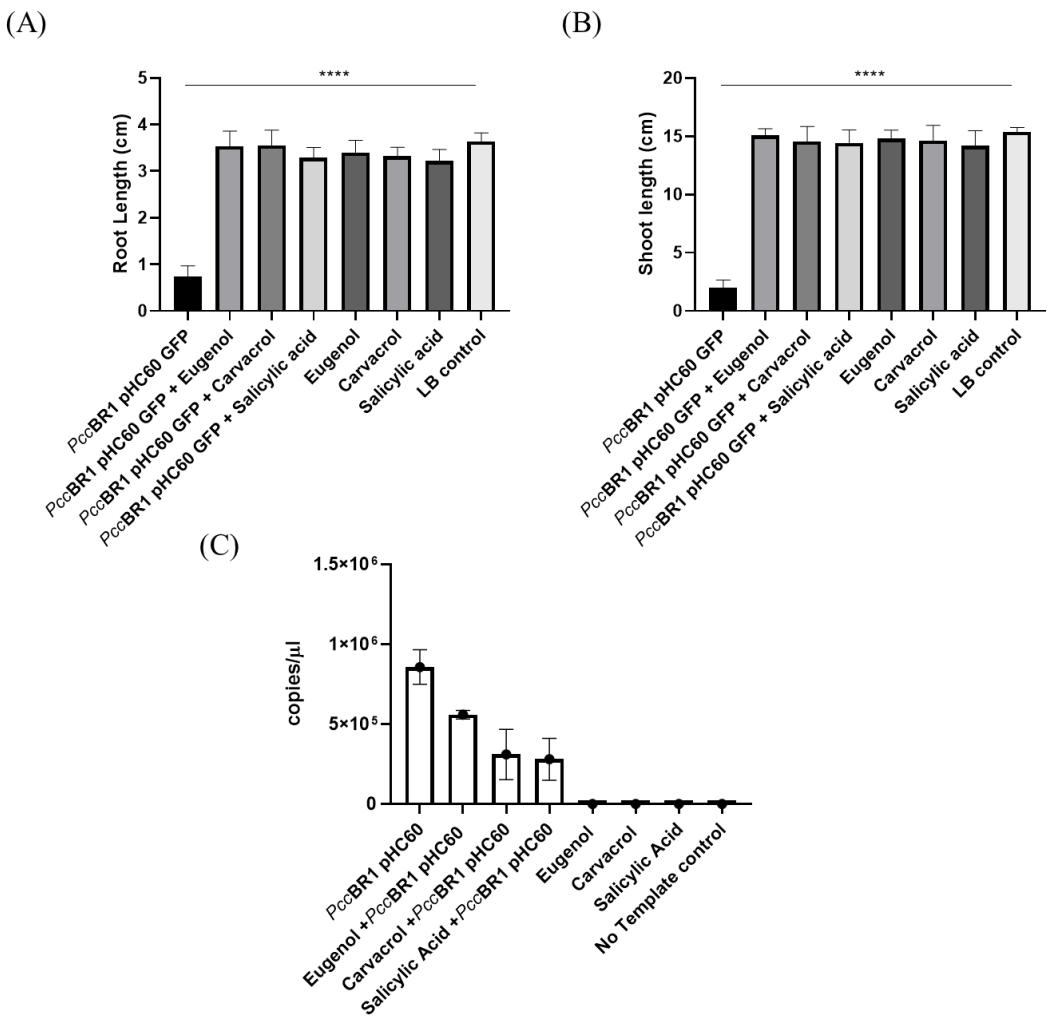

Figure 5.14 (A) Root length in Mung bean assay (n=6) (B) Shoot length in Mung bean assay (n=6) (C) qPCR analysis of *Pcc*BR1 pHC60 16S rRNA gene using specific primers for it (n=3)

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