QUORUM QUENCHING APPROACH FOR CONTROL OF PHYTOPATHOGEN *Pectobacterium carotovorum* subsp. *carotovorum*

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Guide

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INTRODUCTION

Origin of the proposal

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. However, increasing use of chemical inputs causes several negative effects, i.e., development of pathogen resistance to the applied agents and their non-target environmental impacts. Furthermore, the growing cost of pesticides and consumer demand for pesticide-free food has led to a search for substitutes for these products. There are also a number of diseases for which chemical solutions are few, ineffective, or nonexistent. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Compant et. al., 2005). The use of microorganisms that antagonize plant pathogens (biological control) is risk-free when it results in enhancement of resident antagonists against the pathogen. Moreover, the combination of such biological control agents (BCAs) with reduced levels of pesticides (integrated control) promotes a degree of disease suppression similar to that achieved with full pesticides treatment.

Quorum Sensing: Quorum sensing (QS) is a bacterial cell–cell communication process that involves the production, detection, and response to extracellular signaling molecules called autoinducers (AIs). AIs accumulate in the environment as the bacterial population density increases, and bacteria monitor this information to track changes in their cell numbers and collectively alter gene expression. QS controls genes that direct activities that are beneficial when performed by groups of bacteria acting in synchrony. Processes controlled by QS include bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor secretion. Despite differences in regulatory components and molecular mechanisms, all known QS systems depend on three basic principles. First, the members of the community produce AIs, which are the signaling molecules. At low cell density (LCD), AIs diffuse away, and, therefore, are present at concentrations below the threshold required for detection. At high cell density (HCD), the cumulative production of AIs leads to a local high concentration, enabling detection and response. Second, AIs are detected by receptors that exist in the cytoplasm or in the membrane. Third, in addition to activating expression of genes necessary for cooperative behaviors, detection of AIs results in activation of AI production. This feed-forward autoinduction loop presumably promotes synchrony in the population. Gram-positive and Gram-negative bacteria use different

types of QS systems. Gram-positive bacteria use peptides, called autoinducing peptides (AIPs), as signaling molecules. Once produced in the cell, AIPs are processed and secreted. When the extracellular concentration of the AIP is high, which occurs at HCD, it binds to a cognate membrane-bound two-component histidine kinase receptor. Usually, binding activates the receptor's kinase activity, it autophosphorylates, and passes phosphate to a cognate cytoplasmic response regulator. The phosphorylated response regulator activates transcription of the genes in the QS regulon. In some cases of Gram-positive bacterial QS, AIPs are transported back into the cell cytoplasm where they interact with transcription factors to modulate the transcription factor's activity and, in turn, modulate gene expression changes. Gram-negative bacteria communicate using small molecules as AIs. These are either acylhomoserine lactones (AHLs). AIs are produced in the cell and freely diffuse across the inner and outer membranes. When the concentration of AIs is sufficiently high, which occurs at HCD, they bind cytoplasmic receptors that are transcription factors. The AI-bound receptors regulate expression of the genes in the QS regulon (Li and Tian, 2012; Rutherford and Bassler, 2012)

Quorum Quenching Prevents Bacteria from Communicating: The term "quorum quenching" (QQ) was coined to describe all processes that interfere with QS. QQ strategies do not aim to kill bacteria or limit their growth. Rather, they affect the expression of a specific function. This is an important feature because these strategies exert a more limited selective pressure for microbial survival than biocide treatments. This is a valuable trait for the development of sustainable biocontrol or therapeutic procedures in the present context of rising antibiotic resistance. Three steps of the AHL based QS regulation mechanism could be targets for QQ procedures: 1) the production of signal molecules, 2) the signal molecule itself, and 3) sensing of the signal molecule by the cognate regulatory protein (Dong et al, 2005; Fetzner 2015). Actinobacteria are gram-positive bacteria, many of which are inhabitant of soil and have been reported to produce enzymes which breakdown AHL molecules. Several actinobacteria have the ability to colonize plant surfaces and thereby exclude plant pathogens either by competition or through inhibition by antibiotic production. Over the last decade, six actinobacterial genera: *Arthrobacter, Microbacterium, Mycobacterium, Nocardioides, Rhodococcus* and *Streptomyces* have been reported for their quorum quenching activity. Out of these reported most actinobacteria have a AHL lactonase, but *Rhodococcus* are

an exception, which have been reported to have an unusually high number of AHL-inactivating lactonases, as well as AHL-acylases and AHL- oxido-reductases. On the other hand, *Arthrobacter* have been reported to produce Ahl- inactivating lactonases (Park et al, 2003; Cirou et al., 2012; Polkade et al, 2016).

The mechanisms that are involved could be either of abiotic or biotic origins.

Enzymatic Quorum quenching- The enzymatic degradation of the AHL molecules is a biotic factor that occurs in a very broad range of organisms.

Quorum quenching enzymes:

(1) AHL lactonase (Lactone hydrolysis)

AHL-lactonases induce the hydrolysis of the homoserine lactone ring of the AHLs; this leads to the generation of acyl homoserine. (Fig 7). This hydrolysis is identical to the pH-mediated lactonolysis, and as such, the reaction can be reversed by acidification of the medium. The first demonstration of AHL-lactonase activity was obtained in *Bacillus* sp. This lactonase hydrolyzes a large range of AHLs, from C4- to C14-HSL, with or without a substitution at the C3 position. This enzymatic activity has been identified in both Gram-positive and -negative bacteria (Park et al, 2003; Garge and Nerurkar, 2016).

(2) AHL acylase (Amide bond hydrolysis)

AHL acylases catalyze the complete and irreversible degradation of the AHLs through the hydrolysis of their amide bond; this releases homoserine lactone and the corresponding fatty acid. It was first demonstrated in *Variovorax paradoxus*. All these AHL-acylases degrade long-chain AHLs more efficiently than short-chain forms.

(3) AHL oxidoreductase (Modification of the acyl chain)

AHL oxidase and reductase: In contrast to the previous QQ enzymes, these activities do not lead to the degradation of the AHL molecules into QS-inactive molecules. Instead, they catalyze a modification of the chemical structure of the signal. This is nevertheless important, because this modification might affect the specificity and recognition of the AHL signal, and therefore interfere with QS-regulated functions. (Chen et al, 2013)

Non-Enzymatic Quorum quenching- The potential biocontrol strategy that has been adopted against *Pcc*BR1 is that of quorum sensing Inhibition (QSI) using phytophenolic compounds. Polyphenols are secondary metabolites ubiquitously distributed in all higher plants, which have important roles as defence 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 characteristics: (i) highly stable compounds that are resistant to degradation by host metabolism, a factor that allows for their transport to the site of action; (ii) low molecular weight molecules that can penetrate bacterial cells and interact with target proteins; and (iii) high-specificity phytochemicals that directly interact with QS activators. Two other referred groups of plant-derived compounds show otherwise modes of action on AHL-dependent quorum sensing which do not interfere with LuxI/LuxR proteins but affected the QS-related intracellular regulatory pathways. These "non-specific" mechanisms were evaluated for sulphur-containing compounds (ajoene and iberin), changing numerous phenotypic traits by lowering 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 criterion (iii), because they have an inhibitory effect on a variety of QS systems, regardless of the nature of the autoinductor 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. 2017).

Pectobacterium carotovorum* subsp. *carotovorum

Pectobacterium carotovorum subsp. *carotovorum* (previously *Erwinia carotovora*) is a bacterium of the family Enterobacteriaceae. *P. carotovorum* subsp. *carotovorum* is a plant pathogen with a diverse host range, including many agriculturally and scientifically important plant species such as carrot, potato, tomato, leafy greens. *P. carotovorum* subsp. *carotovorum* causes general tissue maceration, termed soft rot disease, through the production of plant cell wall degrading enzymes, like cellulases and pectinases which hydrolyze the cellulose and pectin between the individual plant cells. This causes the cells to separate, a disease termed bacterial soft rot. *P. carotovorum* subsp. *carotovorum* uses the mechanism of Quorum sensing to carry out its pathogenicity. The QS machinery of *P. carotovorum* subsp. *carotovorum* also produces an antibiotic along with the virulent enzymes, which has been hypothesized

to have functions to fend off competitor bacteria that attempt to invade the plant by taking advantage of the wound produced by the *P. carotovorum* subsp. *carotovorum* enzymes. In *P. carotovorum* subsp. *carotovorum,* production of virulence factors such as pectinolytic and cellulolytic enzymes and harpins is positively controlled by AHLs. In *Pectobacterium carotovorum* subsp. *carotovorum,* inhibition of AHL synthesis or degradation of the AHLs produced results in the absence of the expression of the QS-regulated genes and consequently in a decrease of the virulence symptoms on potato plants (Miller and Bassler, 2001; Cirou et al., 2012).

RATIONALE

With the emergence of antibiotic-resistant and pesticide resistant strains of bacteria, the available options for treating bacterial infections have become very limited, and the search for a novel general antibacterial therapy has become important. Actinobacteria are inhabitants of soil, endophytic in nature and are reported to have AHL degrading enzymes. AHL degrading Actinobacteria in the soil can be used as biocontrol agents against *Pectobacterium carotovorum* subsp. *carotovorum*. At the same time, phytochemicals such as Eugenol, Carvacrol and Salicylic acid are reported to reduce the virulence of *Pectobacterium carotovorum* subsp. *carotovorum* via quorum quenching. These phytochemicals are naturally occurring plant compounds and can be used against the pathogenicity of *Pectobacterium carotovorum* subsp. *carotovorum*. This quorum quenching approach by AHL-degrading bacteria and phytochemical compounds using enzymatic and non-enzymatic strategies, respectively is a potential means of efficiently attenuating the virulence of *Pcc*. In this perspective the study is proposed taking into consideration the paucity of work on this aspect at national level.

OBJECTIVES:

1. **Isolation, screening and identification of quorum quenching soil actinobacteria and Characterization of quorum quenching of the selected actinobacterial strain(s)**
2. **Storage and Greenhouse biocontrol studies against soft rot causing plant pathogen Pectobacterium carotovorum subsp. carotovorum with selected actinobacterial strain(s)**
3. **Selection and effect of non-enzymatic quorum quenching phytochemical compounds on virulence of Pectobacterium carotovorum subsp. Carotovorum in biocontrol studies.**

Work done till 01-07-2020

1. Isolation, screening and identification of quorum quenching soil actinobacteria and Characterization of quorum quenching of the selected actinobacterial strain(s)

Isolation of Actinobacteria from soil and plant samples:

Two isolation strategies were adopted to effectively isolate actinobacteria- Acetonitrile enrichment technique and Heat Shock treatment. Acetonitrile enrichment for selectively enrich and isolate Actinobacteria, more specifically *Rhodococcus sp.* as shown by Langdahl, et al (1996) and also adopted by Borisova (2011) for isolation of *Rhodococcus*. Heat shock method was also used as described by Borisova (2011) for isolation of Actinobacteria by reducing number of other heat intolerant bacteria. Addition of cycloheximide in the medium helped to minimise fungal contamination. Using Acetonitrile enrichment 17 isolates were obtained whereas by heat shock method 62 isolates were obtained. During isolation it was observed that the shifting the isolates from minimal defined media like M3 or DEA to a rich medium like RMA, isolates produced EPS and pigmentation. This can be attributed to nutrient rich composition of RMA. Combining both the strategies 79 isolates were obtained which were then screened for their AHL degrading ability.

AHL degradation assay:

Primary screening of the isolates was based on the ability of isolates to degrade AHL. Microtiter plate assay for AHL degradation was used with *C. violaceum* CV026 as biosensor strain. Out of 79 isolates screened for AHL degradation 22 isolates were observed to be good AHL degraders.

16S rRNA gene sequencing and identification of isolates:

Out of the 22 AHL degrading isolates, 7 isolates showed consistent AHL degradation activity. These isolates were selected for 16S rRNA gene sequencing and their identification. Out of the 7 isolates, 2 isolates AI5a and AI4 were obtained by acetonitrile enrichment while 5 isolates AC2, DFS1, DFS2, CL4, DL3 were isolated using heat shock treatment. Genomic DNA isolated from these 7 isolates was used as template for 16S rRNA gene amplification using PCR. Primers used for amplification were 27F and 1541R which gave amplicon of around 1500 base pair length. Partial

sequencing of these 7 isolates was carried out using 27F primer and sequence analysis was done using NCBI BLAST and bacterial 16S EzBioCloud database (Yoon et al, 2017).

Results of 16S rRNA sequencing:

Based on NCBI BLAST and EzBioCloud database, 2 isolates AI5a and AI4 were identified to be Actinobacteria. Based on partial 16S rRNA gene sequencing 2 Actinobacterial isolates were identified. To confirm that their identity complete 16S rRNA gene sequencing was carried out and the sequences were analysed using NCBI BLAST and EzBioCloud. Using genomic DNA of isolates AI5a and AI4, amplification of the 16S rRNA gene was done using 27F and 1541R primers. For sequencing 4 primers were used- 27F, 926F, 1107R and 1541R. Reverse complementary sequences of the sequences obtained using 1107R and 1541R were generated and all 4 sequences were aligned to obtained a single full-length sequence of around 1500 base pairs. This alignment and reverse complementary sequence generation was carried out using Chromas Pro software. Using NCBI BLAST and EzBioCloud analysis, the identity of the 2 Actinobacterial isolates was confirmed. Isolate AI5a showed 100% similarity to *Glutamicibacter nicotianae* with 100% completeness of sequence whereas isolate AI4 showed 100% similarity to *Rhodococcus pyridinivorans* with 97% completeness of sequence.

Biochemical characteristics:

After performing various biochemical tests, it was observed that *R. pyridinivorans* AI4 and *G. nicotianae* AI5a were sugar non-fermenting though they could utilise glucose, lactose, maltose, sucrose. Both organisms did not produce indole (indole negative), high amounts of mixed acids (methyl red negative) or acetoin (VP negative). Being aerobic organisms, both the isolates showed effervescence in presence of H_2O_2 (catalase positive). *G. nicotianae* AI5a was able to utilise citrate as carbon source (Citrate positive). *R. pyridinivorans* AI4 was able to reduce nitrate. Both organisms utilised pyridine as sole carbon source but even after 12 days of incubation neither isolate was able to produce pigments. Both the isolates were unable to solubilise phosphate and grow on nitrogen free Jenson's Agar medium indicating that they possess nitrogen fixation ability.

Growth inhibition of *Pcc*BR1 by actinobacterial isolates:

Quorum quenching (QQ) biocontrol strategy only interferes with the QS signalling molecule that is 3-oxo-C6-AHL in case of *Pcc*BR1, and did not interfere with the growth of pathogen that lowers the chances of acquiring resistance by the pathogen. Attenuation of virulence due to direct killing of pathogen was not be due to disruption of QS. Growth inhibition assay was performed by overlaying *Pcc*BR1 (10^8 CFU/ml) on LA plates, and then adding supernatants of *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD into the wells bored in LA plates. *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD did not inhibit the growth of *Pcc* hence, were selected for further biocontrol studies.

***In vitro* co-culture assay:**

The quorum quenching isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD did not inhibit the growth of *Pcc*BR1. Co-culture assay was performed to check their ability to degrade the AHL (3-oxo-C6- AHL) produced by *Pcc*BR1. *G. nicotianae* AI5a (10^8 CFU/ml), *R. pyridinivorans* AI4 (10^8 CFU/ml) and *R. erythropolis* CRD (10^8 CFU/ml) were co-cultured with *Pcc*BR1 (10^6 CFU/ml) for 36 hrs, and the aliquots were taken at every 12 hrs starting from 0 hr to 36 hrs of the co-culture. The supernatant of the co-culture added to wells bored on the CV026 (biosensor strain) overlaid LA plates. AHL produced by *Pcc*BR1 were used by CV026 to give purple colored zone around the wells with *Pcc*BR1 (control) and co-culture supernatants. No purple colored zone was seen around the wells with only *G. nicotianae* AI5a and *Pcc*BR1 co-culture, while the zone was much reduced in case of *R. pyridinivorans* AI4 and *R. erythropolis* CRD and *Pcc*BR1 co-culture as compared with *Pcc*BR1 (control). This was due to degradation of AHL produced by *Pcc*BR1 by *G. nicotianae* AI5a), *R. pyridinivorans* AI4 and *R. erythropolis* CRD. Thus *R. pyridinivorans* AI4 was able to directly affect QS circuit of *Pcc*BR1and therefore affect the virulence factor production by *Pcc*BR1.

Effect of actinobacterial isolates on Plant cell wall degrading enzymes (PCWDEs) by *Pcc*BR1:

Plant cell wall degrading enzymes (PCWDE) are the major virulence factors produced by quorum sensing in *Pcc*BR1. PCWDE produced by *Pcc*BR1 involved in soft rot development are majorly pectinases namely, pectin lyase (PNL), pectate lyase (PL) and

polygalacturonase (PGAse) (Maisuria and Nerurkar, 2012). Since their production is quorum sensing mediated, effect of quorum quenching *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C was checked by co-culturing them with *Pcc*BR1. The co-culture was performed for 48 hrs and, aliquots were assayed for enzyme activity of PNL, PL and PGAse every 12 hrs starting form 0 hr to 48 hrs. The enzyme activity of PNL was found to be the higher compared to PGAse and PL in *Pcc*BR1 control amongst the three.

On the whole, it is evident that *in vitro G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD were able to significantly reduce *Pcc*BR1 virulence factor production. Reduction in virulence factor production was found to be best in case of *G. nicotianae* AI5a.

In vitro soft rot reduction on host vegetables by selected actinobacterial isolates:

In preventive assay for cucumber, the *Pectobacterium carotovorum* subsp. *carotovorum* BR1 infected slices showed 74% maceration but when treated by *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C for 12 h prior to infection with *Pcc*BR1, the maceration percentage reduced to 6.1%, 2.3% and 15.3%, respectively. In attenuation assay for cucumber, the *Pcc*BR1 infected slices showed 99% maceration, but when simultaneously infected with *Pcc*BR1 and treated with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C, the maceration percentage reduced to 0%, 3.1% and 11.8% after 24 h, respectively.

In preventive assay for potato, the *Pcc*BR1 infected slices showed 89.7% maceration but when treated with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C for 12 h and then infected with *Pcc*BR1 the maceration percentage reduced to 2%, 5.5% and 22.6%, respectively. In attenuation assay for potato, the *Pcc*BR1 infected slices showed 91.2% maceration but when infected by *Pcc*BR1 and treated by *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C the maceration percentage reduced to 2.7%, 11.8% and 26.5%, respectively.

Biochemical characterization of the QQ enzyme of *G. nicotianae* AI5a:

Effect of temperature on QQ enzyme activity

The QQ enzyme of *G. nicotianae* AI5a could exhibit C6-AHL degrading activity in the temperature range of 20 °C to 37 °C for 25μM AHL concentration. For a higher concentration of 50 μM, a bell-shaped curve was obtained in the activity of the enzyme

where maximum enzyme activity was seen at 37 °C. Thereafter the activity declined in both cases at temperature 45 °C possibly due of enzyme inactivation.

Effect of pH on QQ enzyme activity

The QQ enzyme could exhibit C6-AHL degrading activity in the pH range from 5 to 9 at 25 μM AHL concentration. The activity declined slightly as the pH was raised to 9. The potential interference of non-enzymatic pH-dependent lactone hydrolysis was precluded by analysis of the controls in which same amount of C6-AHL was incubated in the reaction system of different pH without the QQ culture. The enzyme appeared unstable at pH 6.5 and 9, hence the activity declined.

Effect of metal ions on QQ enzyme activity

Various metal ions Mg^{2+} and Cu^{2+} decreased the activity of enzyme. On the other hand, $Zn^{2+,}$ $Ca2^{+}$ and Cd^{2+} increased the activity of the enzyme as compared to untreated. Maximum activity was seen in the presence of Ca^{2+} cations. Whereas, Cu^{2+} completely inhibited the enzyme activity suggesting it interferes and deactivates the enzyme. We can conclude that Ca^{2+} aids the AHL degradation by *G. nicotianae* AI5a the most, followed by Zn^{2+} , Ca^{2+} and Cd^{2+} ions.

Determination of Vmax and Km

AHL degradation kinetics of the QQ enzyme was determined by plotting velocity versus substrate concentration. The Km was calculated by fitting the data to the Michaelis-Menten equation. The enzyme showed Km value of 3 μM for C6-AHL at pH 8.5 and 37 °C while the maximum enzyme velocity was 0.61 μM min-1.

AHL utilization and restoration assay:

G. nicotianae AI5a failed to grow in the BH minimal medium supplemented with AHL as carbon source but good growth was observed when supplemented with glucose. In preliminary observation, AHL restoration assay it was observed that AHL degraded by *G. nicotianae* AI5a showed reappearance of purple colour upon treatment with HCL indicating restoration of the lactone ring of AHL.

HPLC analysis of AHL degradation products:

In HPLC analysis C6-AHL control showed a peak of 0.1 AU at 20 min retention time. In C6-AHL degraded by *G. nicotianae* AI5a, a decrease in the C6-AHL peak of 0.06 AU at 20 min retention time and a new peak of 0.1 AU of the presumably degraded product was observed at 15 min retention time.

2. Storage and Greenhouse biocontrol studies against soft rot causing plant pathogen Pectobacterium carotovorum subsp. carotovorum with selected actinobacterial strain(s)

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

On performing Biparental mating between *E. coli* S17-1 λpir and *Pcc*BR1 two transconjugant colonies were obtained which were then sub-cultured onto LA plate containing tetracycline (10 μg/ml) as selective marker. The colonies on the plates were then observed under a UV transilluminator where they showed green fluorescence. The transconjugant colonies obtained were co-cultured with biosensor strain CV026, where purple colour was produced due to AHL production. Confirmation of the transconjugants as *Pcc* was done by running PCR using specific 16S primers of *Pcc*BR1. The transconjugants gave amplification while *E. coli* S17-1 λpir did not because the primers that were used had been specifically designed for *Pcc*BR1. Finally, the virulence capacity of the transconjugant was also evaluated where it was seen that it had the same potential as that of *Pcc*BR1 in causing soft rot on potato tuber.

Biocontrol of *Pcc*BR1 soft rot on vegetables by *G. nicotianae* AI5a in storage condition:

Storage experiments were performed with different vegetables where the vegetables are infected with *Pcc*BR1 when in storage. Further, they were treated with *G. nicotianae* AI5a to check the biocontrol ability. The results observed for each vegetable were-

Potato: *Pcc*BR1 inoculated potato tubers showed 57.78% maceration of the tissue but when treated with biocontrol *G. nicotianae* AI5a, percentage maceration reduced to 6.51% from 57.78% when compared to *Pcc*BR1 control and LB control.

Tomato: *Pcc*BR1 inoculated tomato showed 43.23% maceration of the tissue but when treated with biocontrol *G. nicotianae* AI5a, percentage maceration reduced to 9.01% from 43.23% when compared to *Pcc*BR1 control and LB control.

Capsicum: *Pcc*BR1 inoculated capsicum showed 49.20% maceration of the tissue but

when treated with biocontrol *G. nicotianae* AI5a, percentage maceration reduced to 6.17% from 49.20% when compared to *Pcc*BR1 control and LB control.

Brinjal: *Pcc*BR1 inoculated brinjal showed 83% maceration of the tissue but when treated with biocontrol *G. nicotianae* AI5a, percentage maceration reduced to 15% from 83% when compared to *Pcc*BR1 control and LB control.

Ladyfinger: *Pcc*BR1 inoculated lady finger showed 50% maceration of the tissue but when treated with biocontrol *G. nicotianae* AI5a, percentage maceration reduced to 1% from 50% when compared to *Pcc*BR1 control and LB control.

Biocontrol of *Pcc*BR1 soft rot on vegetables by *G. nicotianae* AI5a in greenhouse conditions:

I) Development of *in planta* infection model of cucumber and effect of actinobacterial biocontrol isolates on *Pcc*BR1:

The results obtained in a newly developed infection model of cucumber in our laboratory were compared between the seeds bacterized with *Pcc*BR1 and seeds bacterized with a combination of *Pcc*BR1 and biocontrol strains *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C. The root length of plants bacterized with a combination of *Pcc*BR1 and *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C was 5.5, 4.2 and 3.5-fold higher respectively, than the plants bacterized with only *Pcc*BR1. The shoot length of seeds bacterized with a combination of *Pcc*BR1 and *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C was 5.4, 4.6 and 4.4-fold higher respectively, than the seeds bacterized with only *Pcc*BR1. The blackleg symptoms were found to be reduced by these quorum quenching isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C.

II) Biocontrol of *Pcc*BR1 by quorum quenching actinobacterial isolates on potato *in planta* model:

The susceptibility of the potato to *Pcc*BR1 was checked prior to application of biocontrol agents. The studies were conducted in following three ways- 1) Soil inoculation 2) Stem inoculation and 3) Leaf and lateral stem inoculation.

The plants grown in **Soil inoculated** with *Pcc*BR1 showed stunted growth with reduced growth parameters (Shoot length, no. of leaves, no. of branches, wet weight and dry

weight) as compared to controls where no *Pcc*BR1 was present. The plants treated with *G. nicotianae* AI5a with *Pcc*BR1 had growth parameters (root length, shoot length, wet weight and dry weight) near to normal comparable to uninoculated control

Inoculation of potato **plant stem** was performed 3 weeks after planting, when the plants were approx. 27 to 30 cm high and stolons were already formed. After 15 days of stem inoculation, the first symptoms started to develop in the infected plants. At 30 days post infection (DPI), all the stem-inoculated plants showed wilting and chlorosis of leaves and migrated downward to infect the mother tuber causing soft rot and destroying the mother tuber. 50% wilting of leaves was observed at the end of experiment. Stem rot, a typical blackening of the stem base, along with the wilting was observed in 90% of the plants. The trans-sections of stems of symptomatic plants showed degradation of vascular tissue and pith above and below the inoculation point, resulting in a hollowing of stems and browning of the internal stem tissue of 95% of tested plants.

In **leaf and lateral stem** inoculated plants, plants that were infected with *Pcc*BR1 showed the very first symptoms at 7 DPI (day post infection); all inoculated leaves showed chlorosis, wilting, and the potato plant dried up completely and was dead at the end of 2 months. In the following 2 weeks, these symptoms were also started developing in adjacent leaves**.** Leaf and lateral stem treated with a combination of actinobacterial isolates and *Pcc*BR1 did not show any symptoms. The biocontrol isolate *G. nicotianae* AI5a significantly inhibited the infection in the plant in all three types of infection due to plant pathogen *Pcc*BR1 and allow the normal growth of the plant.

3. Selection and effect of non-enzymatic quorum quenching phytochemical compounds on virulence of *Pectobacterium carotovorum* subsp. *carotovorum* in biocontrol studies.

Polyphenols are secondary metabolites ubiquitously distributed in all higher plants, which have important roles as defense against plant pathogens. The antimicrobial activity of polyphenols occurring in vegetable foods and medicinal plants has been extensively investigated against a wide range of microorganisms. (Daglia 2012). Sub-lethal concentration of each phytophenolic compounds was determined by serial dilution method and further experiments were conducted with reference to that value for each compound.

Effect of phytophenolic compounds on the production of AHL in *Pcc*BR1:

The phytophenolic compounds Eugenol and Carvacrol 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 *Chromobacterium violaceum* CV026 (Joshi et al. 2016). In order to check the same, the phytophenolic compounds Eugenol, Carvacrol and Salicylic acid were analysed for their ability to disrupt the QS mechanism in *Pcc*BR1 and it was found that Eugenol (0.4 µg/ml), Carvacrol (0.4 µg/ml) and Salicylic acid (210 µg/ml) inhibited the production of AHL as no purple pigment (violacein) was observed when tested in the bioassay.

***In vitro* pathogenicity assay for soft rot caused by *Pcc*BR1:**

For *in vitro* soft rot pathogenicity assay for soft rot caused by *Pcc*BR1 was performed. *Pcc*BR1 when inoculated alone caused severe potato tissue maceration resulting in 77.73 % macerated tissue per site of inoculation on each potato slice. Similarly, when slices of cucumber were inoculated with pathogen alone maceration obtained was 99 % per slice. However, when phenolic compounds (Eugenol, Carvacrol and Salicylic acid) were applied along with *Pcc*BR1 the soft rot symptom of maceration in potato slices were significantly reduced to 10%, no maceration, 15% in Eugenol (0.4 µg/ml), Carvacrol (0.4 µg/ml) and Salicylic acid (210 µg/ml) respectively. Similar effects of the phytophenolic compounds were observed in case of cucumber slices.

Effect of Phytophenolic compounds on the virulence traits of *Pcc*BR1:

Biofilm Formation

In microtitre plate assay on exposure of biofilm formed *Pcc*BR1 to phytophenolic compounds i.e Eugenol (0.4 µg/ml), Carvacrol (0.4 µg/ml) and Salicylic acid (210 µg/ml) a significant reduction in biofilm formation was observed as compared to the biofilm of 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.

Effect of Phytophenolic compounds on motility of *Pcc*BR1

The diameter of *Pcc*BR1 colony on the soft agar plate was 18 mm which got reduced to 12.5mm Eugenol (0.4 µg/ml), 10.5 mm for Carvacrol (0.4 µg/ml) and 11 mm for Salicylic acid (210 µg/ml). Thus, motility reduction was found to be maximum in case of Carvacrol.

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

The activity of Plant Cell Wall Degrading Enzymes pectin lyase, pectate lyase and polygalacturonase produced by *Pcc*BR1 was observed. Polygalacturonase activity was determined by measuring reducing sugar released as a result of hydrolysis of the polymer substrate i.e PGA using di-nitro salicylic acid (DNSA) reagent (adapted and modified, Miller, 1959). Pectin lyase (PNL) and 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). The assay was carried out for 48 hrs, every 12 hrs aliquots were taken and enzyme activity was measured using cell supernatant. All the enzymes showed a decline in production with time and by 48 hrs their activity was negligible. In all the assays a similar trend was observed wherein up to 36 hrs the enzyme activity was increasing and then it declined and was nearing zero at 48 hrs. Also, the enzyme activity of Pectin lyase was more as compared to Pectate lyase and Polygalacturonase. Overall, it is evident that the phenolic compounds inhibit the QS mechanism due to which the activity of these enzymes was reduced.

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