Conclusion

Quorum quenching strategies are effective biocontrol strategies because they do not aim to kill bacteria or limit bacterial growth but affect the expression of a specific function exerting a limited selective pressure for microbial survival than biocide treatment. The objective of work undertaken was to enrich and isolate soil borne Actinomycetota and screen them for their AHL degrading ability to assess them further as biocontrol agents. Soil Actinomycetota were envisaged as suitable biocontrol agents as they inhabit the soil where this approach would be operational. Total 79 isolates were isolated using strategies that selectively enriched Actinomycetota and screened for AHL degrading activity and post-screening 22 isolates were found out to be positive for the AHL degrading phenotype. This screening provided two uncommon Actinomycetota namely, *Glutamicibacter nicotianae* AI5a and *Rhodococcus pyridinivorans* AI4 which are not yet reported for their AHL degradation ability. Quorum quenching though is potentially effective method of biocontrol, the AHL degrader should have certain additional but desirable characteristics in order to be used in a sustainable biocontrol strategy against phytopathogens. In the present study the selected isolates, in addition to their AHL degrading capability, were evaluated for relevant biocontrol attributes. All bacterial species with AHL degrading ability are not potential biocontrol agents and it is necessary to negate any detrimental effect on the host before their use as biocontrol agents. The two isolated strain *G. nicotianae* AI5a and *R. pyridinivorans* AI4 along with the third isolate *R. erythropolis* CRD13.3C could degrade signalling molecule of *Pcc*BR1 and did not affect pathogen growth. These isolates also decreased the production of 3-oxo-C6-AHL produced by *Pcc*BR1 *in vitro*. *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C also reduced the PCWDEs (PNL, PL and PGA) *in vitro* which suggested the possible involvement of quorum quenching in decreasing the virulence enzyme production indicating their biocontrol potential against this phytopathogen. *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C could survive on potato and cucumber and in virulence bioassays showed significant attenuation of soft rot symptoms in the two hosts, implying that these isolates have potential as broad-range biocontrol agents. Further, the ability to control symptoms pre and during infection is a valuable trait in a biocontrol agent, and these three isolates demonstrated both successful preventive and attenuation biocontrol of *Pcc* soft rot. *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were able to degrade 5 different AHLs: C4-AHL, C6-

AHL, 3-oxo-C6-AHL, C8-AHL and 3-oxo-C8- AHL. This showed that the selected Actinomycetotal QQ isolates could degrade broad range of AHLs. This may widen the range of their usage as different phytopathogens produce different AHLs.

Though all three isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C showed QQ based biocontrol potential, *G. nicotianae* AI5a demonstrated maximum AHL degradation and biocontrol ability throughout the experiments conducted. Moreover, *Glutamicibacter* genus hasn't been reported for QQ based biocontrol potential, therefore potential identification of the mechanism of AHL degradation in *G. nicotianae* AI5a was of interest. Also, *G. nicotianae* AI5a was of special interest as organisms in this genus were previously regarded as members of the genus *Arthrobacter*, but the taxonomic status of these microorganisms was changed to genus *Glutamicibacter* so this is the first report of AHL degrading *Glutamicibacter* strain. Major QQ enzymatic mechanisms operate either through lactone hydrolysis carried out by AHL lactonases or amidohydrolysis carried out by AHL acylases. AHL lactonases hydrolyse the lactone ring of AHL, yielding the corresponding *N*-acyl homoserine which can be restored to *N*-acyl homoserine lactone at acidic pH. In accordance with this, the product of C6-AHL degradation after treatment with *G. nicotianae* AI5a could be restored at pH 2 after addition of HCl. *G. nicotianae* AI5a was able to grow on glucose as sole source of carbon in minimal media but not on AHL as sole source of carbon. This further indicated the enzyme in *G. nicotianae* AI5a being an AHL lactonase. Finally, upon conducting HPLC analysis it was observed that upon degradation by *G. nicotianae* AI5a the C6-AHL gave 2 peaks instead of the one observed in undegraded AHL. With these three experiments it was concluded that the QQ enzyme in *G. nicotianae* AI5a could be a putative AHL lactonase. Biochemical characterization of QQ enzyme of *G. nicotianae* AI5a revealed its optimum temperature and range at 37°C and 20°C to 37°C, respectively. The pH optimum and range was 8.5 and 6.5 to 9 respectively. Metal ions Mg^{2+} and Cu^{2+} a negative effect on the enzyme activity. Zn^{2+}, Ca^{2+} and Cd^{2+} increased the activity of the enzyme. The K_M for C6-HSL was 3 μM while the while the maximum enzyme velocity was 0.61 μM min^{-1}. Thus, the enzyme was found active in the physical conditions prevalent in the soil environment which is aa attribute that supports its biocontrol potential. *G. nicotianae* AI5a was able to degrade varying chain length AHLs suggesting its broad specificity. It was also observed that *G. nicotianae* AI5a

can perform quorum quenching in a wide range of pH, temperature and substrate concentration hence has potential to be used as biocontrol agent against quorum sensing plant pathogens.

In addition to the biocontrol experiments on vegetable slices, biocontrol potential of the isolates was explored for their ability to prevent systemic infection of *Pcc*BR1 through seeds, roots, whole vegetables on a range of host plants, vegetables and fruits. The idea behind this was to cover all facets of potential infection range of *Pcc*BR1 diseases like soft rot and blackleg in different ways. An *in planta* cucumber infection model for *Pcc*BR1 pathogenicity was developed. This model provided the results within 3 weeks. This allowed the experimentation to be done faster. Also, the cucumber model is suitable for tropical climates where growing potato plant (a common host for Pcc) would be difficult to grow as it prefers cooler climate. All three QQ isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 or *R. erythropolis* CRD13.3C prevented the spoilage of seeds at the germination stage and in *in planta* experiments while colonising and persisting on the roots of cucumber plant. While the tuber maceration studies were suitable for soft rot disease studies the cucumber model developed here were found suitable for black leg infection studies.

Potato is a natural host for phytopathogens belonging to *Pectobacteria* sp. causing blackleg in plant stage and soft rot in tuber stage during storage. A comprehensive study performed in winter season in greenhouse included three different ways to infect the potato plant with *Pcc*BR1: I) Soil inoculation II) Stem inoculation and III) Leaf and lateral stem inoculation. In soil inoculation studies, the growth parameters such as shoot and root length, number of leaves and percentage of diseased leaves and weight were low upon infection with *Pcc*BR1 but upon treatment with *G. nicotianae* AI5a a significant decline in blackleg symptoms was observed. When the stem was infected with the pathogen, Stem rot, a characteristic blackening of the stem base, or both were detected in 90% of the plants. Symptoms of infection below the ground were observed when stem was inoculated with *Pcc*BR1. This suggests that the pathogen moves downwards even after being infected at the site above the ground i.e. stem. Treatment with *G. nicotianae* AI5a improved the growth parameters and reduced to negligible symptoms of blackleg were observed. Lastly, when the plants were infected with *Pcc*BR1at the leaves and lateral stem severe dessication of leaves and stem was noted, which was improved by applying the biocontrol isolate *G. nicotianae* AI5a. A

wholesome picture of the ways of infection of *Pcc*BR1 *in planta* in potato is provided in Fig. 4.27.

Storage is another stage where infection can be caused by *Pcc*BR1 especially in vegetable like potato. Experiments were performed on various hosts under storage conditions. It was observed that the QQ isolate *G. nicotianae* AI5a was able to reduce the soft rot in vegetables such as potato, tomato, capsicum and brinjal in storage conditions. This further strengthens the possibility of using AHL degrading bacteria and *G. nicotianae* AI5a for the attenuation of quorum sensing mediated black leg and soft rot in different conditions of handling the vegetables post-harvest.

The quorum quenching approach is imperative in the soil conditions in the rhizosphere where the QQ bacterium is in juxtaposition to the phytopathogen. Essentially its main feature is reduction in virulence potential of the pathogen and not the elimination of the pathogen. In order to emphasise this aspects studies conducted here included a GFP marked *Pcc*BR1 so that their numbers could be monitored. For this purpose, *Pcc*BR1 was tagged with GFP using biparental mating by inserting the pHC60 plasmid containing the *gfp* gene in it. Specific 16S rRNA genes of *G. nicotianae* AI5a and *Pcc*BR1 pHC60 were transformed in *E. coli* DH5α using pTZ57R/T and pJET cloning vectors, respectively. Clones *E. coli* DH5α pTZ57R/TGn and *E. coli* DH5α pJETPcc were obtained. The plasmids from the clones were required to generate standard graphs for quantitative Real Time PCR which were used to quantify *G. nicotianae* AI5a and *Pcc*BR1 pHC60 in the experiments ahead.

An *in planta* mung bean model was used to check the potential ability of *G. nicotianae* AI5a against phytopathogen *Pcc*BR1 pHC60. This entire experiment was done under gnotobiotic conditions. Upon *Pcc*BR1 pHC60 infection during bacterization, the mung bean plant did not grow properly but upon treating *Pcc*BR1 pHC60 with *G. nicotianae* AI5a during bacterization, the plant growth appeared to be normal and better than the infected set. It was important to note that the copy number of *Pcc*BR1 pHC60 did not reduce upon treatment with *G. nicotianae* AI5a which was observed by qPCR. This is the notable trait of a QQ biocontrol isolate which is highlighted in this study. Furthermore, the fluorescence microscopic studies demonstrated that all large-celled tissues between bundles and toward the periphery were free from the pathogen.

Further delving into the quorum quenching strategies against phytopathogen *Pcc*BR1, phytochemicals which are integral part of plants were used to test their potential effect

on the virulence of *Pcc*BR1. The objective again was quorum quenching i.e. the phytochemical should reduce the virulence of *Pcc*BR1 but at the same time not kill the pathogen for it to be considered under quorum quenching strategy. It was important to find out the sub-lethal concentration against *Pcc*BR1 and their AHL degradation ability. Three phytochemicals Eugenol, Carvacrol and Salicylic acid at their sub-lethal concentrations were selected out of twelve phytochemicals for their ability of reduce QS based virulence of *Pcc*BR1. Eugenol, Carvacrol and Salicylic acid were able to contain the virulent traits of *Pcc*BR1 such as AHL production, motility, PCWDEs and biofilm formation ability at sub-lethal concentrations. The three phytochemicals were also effective in reducing soft rot symptoms in potato and cucumber host in an *in vitro* soft rot attenuation assay. Under storage conditions Eugenol, Carvacrol and Salicylic acid reduced the soft rot in potato host without killing *Pcc*BR1. An *in planta* experiment using mung bean as host under gnotobiotic conditions also saw Eugenol, Carvacrol and Salicylic acid reduce the pathogenicity of *Pcc*BR1 without reducing the amount of *Pcc*BR1 in the plant. The quantification of *Pcc*BR1 in storage and in planta experiment was done using qPCR. The investigations here with phytochemicals further expanded the repertoire of quorum quenching agents from bacteria to phytochemicals.

In conclusion, it is clearly demonstrated that while the selected isolates (*G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C) and phytochemicals (Eugenol, Carvacrol and Salicylic acid) along with strong AHL degradation ability have many preferred biocontrol attributes which can make them effective biocontrol agents. Notably, AHL degradation is necessary criterion but is not sufficient in developing quorum quenching based biocontrol agent. Further, Plant-associated microorganisms can be beneficial, deleterious or neutral to the plant and some beneficial bacteria (*Rhizobium* and *Pseudomonas*) depend on quorum sensing for their beneficial traits. Therefore, off target effects of quorum quenching strategy cannot be ruled out but despite that this strategy remains an effective one. The quorum quenching may inadvertently affect the AHL regulated beneficial bacteria. However, its use in storage conditions is notable and important. It can be argued that the impact of non-target effects would depend on the factors like specificity of the AHL degrading enzyme and proximity of AHL degrading bacteria and pathogen/beneficial bacteria. Therefore, the knowledge about the spatial distribution of the QS and QQ populations

involved in more complex multispecies natural environments like rhizosphere will help in establishing whether QQ has future biocontrol potential.

Future Perspectives

Extracting, isolating and translating the QQ enzyme of *G. nicotianae* AI5a into a practical working applicative form in the field is an interesting challenge which can be looked upon. Similarly, dosage and cocktail studies of phytochemicals in the field would be an interesting study. As for the phytochemicals, controlling the quantity in field is a challenge as many factors such as death of the plant to death of the pathogen may come into play in case the quantity of the phytochemicals is not measured at sub-lethal levels. However, the use of phytochemicals in minute quantities opens exciting possibilities. Converting the knowledge obtained by this study, the extensive effect of *G. nicotianae* AI5a on different hosts, stages of infection of *Pcc* and the current available literature into applicative form would be the future. A combination of QQ strategy with a cocktail of QQ enzyme of biocontrol isolates with QQ phytochemicals is an idea to look into. Also, augmentation with other compatible strategies should be more effective in biocontrol. Such studies comprising field applications of chemical and non-chemical biocontrol QQ strategies and an alternative to pesticides and insecticides against *Pcc* and other quorum sensing pathogens will prove the usefulness of quorum quenching based biocontrol approach.