Conclusion

Xanthomonas oryzae pv. oryzae (Xoo) causing Bacterial Blight is one of the most dangerous pathogen of rice plant as it exerts serious crop loss up to 50 % under severe epidemics. It is a vascular pathogen and enters the host via hydathodes and wounds and spreads through xylem vessels causing development of blight symptoms. Xoo deploys multiple virulent factors like EPS production, exoenzymes secreted by T2SS and effectors by TTSS, etc. to conquer the host plant. Traditionally, the control of BB disease has been carried out by various strategies such as using chemical antagonists, biological agents as well as biomolecules and breeding for resistance genes in the host varieties. However, with time, variants of pathogens resistant to the existing strategies arise. Employing a single compound or strategy leads to the emergence of such resistance faster than when a combination of them is used. Thus, an innovative combinatorial approach not only leads to an effective management of the disease but also carries a far lower risk of resistance development. Combination of new antibiotic along small molecule compounds, which attenuate virulence factors of pathogen could be one of such integrated approach to control BB. This study dealt with combinations of antibiotic with various small molecule chemical compounds for alleviation of the bacterial blight disease of rice caused by Xoo. One of the strategies of this work involved isolating and characterizing a potent antibiotic against Xoo from an antagonistic bacterial isolate. The other part of the work involved assessment of the pathogenesis of Xoo by small molecule chemical compounds. Further integrating the two approaches for studying their combined effect on Xoo.

Different plants of *Poaceae* family related to rice were selected for isolation of bacterial antagonist showing strong activity against Xoo BXO43. Isolate S2 exhibiting highest inhibitory activity amongst the screened isolates, was selected as a strong antagonist of Xoo BXO43. Its supernatant was found to contain the bioactive metabolite (antibiotic S2) and effectively controlled the disease symptoms of bacterial blight on leaves of susceptible rice plant, TN-1. The isolate was identified as *B. altitudinis* by sequence analysis of the genetic biomarkers 16S rRNA and *gyrB*. The antibiotic S2 was tested to be effective against other bacteria such as *S. aureus* ATCC 6538 P as well, while it was harmless to PGPR strains of *Pseudomonas* and *Bacillus*. Thus, antibiotic S2 was established as a safe and suitable biocontrol agent owing to its potency and antimicrobial spectrum. Also, it

did not show any antifungal activity as tested with few commonly found phytopathogens like *Rizoctonia* and *Fusarium* species.

The production of antibiotic S2 was induced in the presence of either of the biotic or abiotic stress as it was produced in a nutrient limiting conditions as well as in the presence of the pathogen. Among the various media tested for *in vitro* production of the antibiotic S2, a synthetic minimal medium, Sucrose Bushnell Haas (SBH) broth was found to be favourable. Notably, antibiotic production was not induced in rich media like Tryptic soy broth, Luria Bertani, etc. were used. Antibiotic activity was detected upon co-inoculation of Xoo BXO43 along with *B. altitudinis* S2 in PS broth. Antibiotic S2 was found to be polar in nature with liquid extraction analysis. Different carbon sources, temperatures and pH of the media were tested for optimum production of the antibiotic wherein sucrose was found to be the best carbon source; while 30 ^{o}C of incubation temperature and neutral pH were found to be ambient. Highest production of antibiotic S2 was noted at the stationary phase of growth of the isolate. A crude extract of the antibiotic S2 was found to efficiently control the disease symptoms on rice plant in the Kresek disease model. Antibiotic S2 was stable over a wide range of pH and temperatures and its activity was unaffected by various lytic enzymes viz. proteinase K, trypsin and amylase. This information led to understanding of suitability of the antibiotic S2 in wild and diverse field conditions.

Further purification and characterization of the antibiotic S2 was done using thin layer chromatography followed by direct bioautography wherein a single band exhibiting the antibiotic activity was obtained. Its non-proteinaceous nature was confirmed via observed resistance to proteolytic enzymes, and via Tricine SDS-PAGE. Semi-preparative HPLC and LC-ESI-MS/MS gave two peaks from the active fraction with m/z value of each below 1000. Further, amplification of type III polyketide synthesis genes *pks* and *pmt* coding for polyketide synthase and phospholipid methyltransferase, respectively confirmed the presence of genetic machinery for type III Polyketide Synthase in isolate *B. altitudinis* S2. Quantification and bactericidal nature of the antibiotic S2 was inferred from MIC, MBC, live-dead staining and time-kill assay. The MIC and MBC values of partially purified antibiotic S2 for Xoo BXO43 were 8 µg/ml and 16 µg/ml, respectively. Mode of action of antibiotic S2 was found to be protein inhibitory in nature as

elucidated by SEM and SDS-PAGE Thus, antibiotic S2 was characterized to be non-proteinaceous, potent bactericidal antibiotic and was found to be possibly acting as protein synthesis inhibitor.

In another aspect of this study, small molecule compounds that do not exhibit bactericidal activity but rather attenuate the virulence factors and disarm the pathogen of causing pathogenesis were studied. Certain phenolic acids which are also plant products and few synthetic compounds acibenzolar S methyl and indole acrylic acid were used at their sub-inhibitory concentrations to study their effect on different virulence factors of Xoo. XopQ is an effector secreted by TTSS and the expression of TTSS and effector genes is regulated by a common regulator HrpG/HrpH; hence promoter of *xopQ* gene was used for the construction of a transcriptional reporter system to study the effect of compounds on TTSS of Xoo. The results showed that caffeic acid, p-coumaric and ferulic acid downrgulated the expression of *xopQ* thus affecting TTSS, one of the most potent virulence factor of Xoo. Notably, no effect on other virulence factors like exoenzymes secreted by T2SS, motility, EPS production, etc. was observed. Moreover, the ability of these compounds to control the disease symptoms was verified using leaves of susceptible rice cultivar TN-1. The results indicated that cinnamic acid and its derivatives caffeic acid, ferulic acid were the most potent among the tested compounds. Thus, these small molecule compounds carry out the virulence attenuation of the Xoo by targeting its TTSS.

Integration of the two methods, i.e. combining antibiotic S2 and small molecule compounds was further studied. Combined effect of small molecule compounds and antibiotic S2 was tested by checkerboard assay and the results showed synergy between antibiotic S2 and caffeic acid in inhibiting the growth of Xoo BXO43. Small molecule compounds increase the efficacy of antibiotics by inhibiting the efflux pumps and are termed as efflux pump inhibitor (EPI). Synergy of caffeic acid with other conventional antibiotics like Streptomycin and Ofloxacin and reduction in efflux of EtBr, a common substrate of efflux pumps established caffeic acid as a potent EPI. Assessment of involvement of efflux pumps was carried out by transcription analysis of six representative genes of different efflux pumps using RT-PCR. Amongst the selected genes, *norM* gene was found to be upregulated nearly two-folds. The results strongly indicated that NorM efflux pump

is involved in the synergy between antibiotic S2 and caffeic acid. Thus, it is clear that caffeic acid improves the efficacy of antibiotic S2 by retaining it in the Xoo cells based on EtBr and Checkerboard assay, however to understand the exact mechanism of interaction of caffeic acid and efflux pump further investigation is needed. Thus, the combination of antibiotic S2 and small compound like caffeic acid can be used as an integration approach in controlling the pathogenesis of Xoo through multiple targets.