Summary

Xanthomonas oryzae pv. oryzae (Xoo) is a devastating pathogen of rice and incurs heavy loss of the crop yield by causing the diseased called bacterial blight. A number of approaches to control the onset and progression of the Xoo infection have been developed involving breeding for resistant genotypes as well as application of biological and chemical compounds that either kill the pathogen or check its pathogenesis. However, progressively, new pathovars of Xoo emerge by developing resistance to the deployed methods. Hence, there is a constant need to search for novel agents and approaches to control this phytopathogen. Hence, two approaches with different mechanisms have been take up in the current study, with strategy of integrating the two as an effective measure/method for the control of Xoo. Thus, one aspect of the work dealt with isolating and characterizing a bacterial antagonist of Xoo isolated from the field and further elucidation of its mechanism of antagonism. The other aspect of the work focused on screening small molecule compounds effective against the virulence determinants of the pathogen. Further, a combination of the two strategies viz. inhibition and virulence attenuation to combat Xoo pathogenesis via multiple targets was tested. The results of the studies are summarised below. While they were individually effective in controlling the disease symptoms in rice, their combination conferred a synergistic effect on inhibition of Xoo

Chapter 2

Isolation, screening and characterization of bacterial antagonists against *Xanthomonas oryzae* pv. oryzae BXO43

- Plants from different members of the family *Poaceae* viz. *Oryzae sativa* (rice), *Pennisetum purpureum* (napier grass), *Eleusine coracana* (finger millet), *Echinochloa colona* (jungle rice), *Eleusine indica* (indian goosegrass) and *Cenchrus ciliaris* (buffel grass) from the rice field were collected as source of isolation.
- Different plant parts like rhizosphere, phylloplane and root tissue as well as aerial parts of stem, leaves of the collected plant samples were used for isolation of potential antagonists.
- Total 90 bacterial isolates were screened for their antagonistic activity against Xoo BXO43. Fourteen isolates showed wide zones of clearance, eight isolates

showed moderate zones of clearance, whereas 68 isolates did not show any zone of clearance against the test organism Xoo BXO43.

- To select the bacteria showing strong antagonism against Xoo BXO43, fourteen isolates exhibiting wide zone of clearance were further quantified on the basis of ratio of Zone of clearance/Colony size (Cz /Cs). Based on Cz /Cs ratio, five isolates designated as: L1, L21, R2, S2 and N4 with Cz /Cs ratio above 3.5, were selected for further characterization. Additionally, from the two isolates namely GL1 and S3, demonstrated spreading all over the plate inhibiting the growth of Xoo BXO43, S3 was also selected for further characterization.
- Six of the above isolates were identified on the basis of their morphological, biochemical and 16S rRNA gene sequencing. Their identification and GenBank accession nos. are as follows: *Bacillus altitudinis* S2 (KU697351), *Bacillus safensis* R2(KU697354), *Pseudomonas* sp. N4 (KU697353), *Paenibacillus* sp. S3 (KU697352) and *Bacillus subtilis* L1 (KU697356) and *Bacillus subtilis* L21 (KU697355) .
- Results of effect of these six isolates on growth of rice plant using susceptible rice cultivar TN-1 by *in planta* assay indicated the isolate R2, identified as *Bacillus safensis* exhibited negative effect on the growth of rice plant; and hence was not used in further studies. Among the two *B. subtilis* strains, strain L21 was selected as it gave better Cz/Cs ratio. Remaining four isolates were further screened for their ability to control BB on rice leaves by detached leaf assay.
- Detached leaf assay for examining the ability of the selected four isolates to control disease symptoms on susceptible rice cultivar TN-1 showed isolates *Bacillus altitudinis* S2, *Paenibacillus* sp. S3 and *Bacillus subtilis* L21 were able to reduce the % DLA (percentage of diseased leaf area) to 13 %, 20 % and 18 % as compared to 70 ±35 % DLA of the leaves treated with only pathogen Xoo BXO43. *Bacillus altitudinis* S2 reduced it maximally by 87 % indicating its potential of being a strong anatagonist against Xoo.
- Colonization ability on root of the rice cultivar TN-1 by these four isolates indicated that their population increased to 10-100 folds as observed on 15^{th} day after the inoculation, which was observed to decrease by 30^{th} day. Isolate *Pseudomonas sp.* N4 was not detected on 30^{th} day of inoculation.

- Isolate *B. altitudinis* S2 gave highest Cz/Cs ratio and good protection against the BB symptoms as observed by detached leaf assay. Hence, it was selected for further studies.
- Next, the cell free supernatant (CFS) of *B. altitudinis* S2 was tested to check lesion formation by detached leaf assay and it was able to significantly reduce % DLA on TN-1 rice leaves from 48 ±10 % by Xoo to 15 ±1 % by CFS of *B. altitudinis* S2 . This clearly indicated that the *B. altitudinis* S2 produced the extracellular antibacterial metabolite.
- Another identification biomarker *gyrB* gene was used for taxonomic validation of isolate S2. The Restriction digestion pattern of the *gyrB* amplicon was found similar to *B. altitudinis* 41KF2b validating it as *B. altitudinis*. *B. altitudinis* S2 has been deposited in National Centre for Microbial Resource (NCMR) of National Centre for Cell Science (NCCS) Pune and its accession no. is MCC 3404.
- Antimicrobial activity of *B. altitudinis* S2 as tested with other Gram negative and Gram positive bacteria showed activity against *S. aureus* but not against other bacteria like *Escherichia coli*, *Salmonella typhi*, *S. paratyphi* A, *S. paratyphi* B, *Klebsiella pneumonia*, *Shigella dysenteriae*, *Bacillus subtilis* and *Pseudomonas fluorescens* CHA0. It did not show any antifungal activity against the tested fungi. Thus, the *B. altitudinis* S2 demonstrated extracellular antibacterial activity against *S. aureus* ATCC 6538 P in addition to Xoo. Further studies were carried out with *B. altitudinis* S2.

Chapter 3

Production, extraction and characterization of bioactive metabolite (antibiotic S2) produced by *Bacillus altitudinis* S2

- Different media like Luria Bertani (LB) broth, PS (Peptone Sucrose) broth, TSB (Tryptic Soy) broth and SBH (Sucrose Bushnell Haas) broth were tested as the media for antibiotic production by *Bacillus altitudinis* S2.
- Only synthetic minimal medium i.e. SBH broth was found to support the production of antibiotic S2, while no antibiotic production was detected in rich media like LB, TSB. *B. altitudinis* S2 produced antibiotic S2 when Xoo BXO43 was co-inoculated in complex media like PS broth. This indicated

antibiotic S2 production was induced in the presence of biotic or abiotic stress conditions.

- In order to extract the antibiotic S2 from the CFS, amongst the organic solvents, polar organic solvents like Isopropanol, Ethanol and Methanol were found to extract antibiotic from the medium, while other less polar organic solvents like Butanol, Chloroform, Ethyl Acetate, Hexane, etc. were unable to extract antibiotic S2.
- Sucrose was found to be the most effective carbon source for antibiotic S2 production and about 426.67 AU /ml of antibiotic S2 from 1.15 ± 0.07 OD_{600} biomass was produced in medium containing 2 % of Sucrose (SBH).
- Optimum temperature and pH were 30 ^{o}C and 7 -7.5, respectively for antibiotic S2 production.
- Time-course production assay showed production of antibiotic S2 was optimum when stationary phase is achieved producing 6823 AU/ml at 192 hrs and beyond.
- Kresek disease model used for studying the efficacy of antibiotic S2 showed 60 AU/ml of antibiotic S2 was significantly able to protect the rice cultivar TN-1 plant from disease symptoms.
- Stability assay showed that antibiotic S2 was stable over wide range of temperature of 40 -65 ^{o}C, but lost about 27 % activity when exposed to 121 ^{o}C for 15 min. It was stable over wide range of pH 2 -9 and to various treatments of enzymes like proteinase K, Trypsin and β-amylase.
- TLC helped to resolve the antibiotic S2 at Rf 0.92, while bioautography on TLC plate demonstrated the inhibitory activity against both the test organisms Xoo BXO43 and *S. aureus* at this same Rf value.
- Absorption maxima of the antibiotic S2 obtained from preparative TLC was found to be in the UV region at 225-227 nm.
- Semi preparative HPLC and ESI-MS/MS showed two peaks of m/z value 365 and 573.2131 of $[M + H]^{+}$ ions at RT 1.081 and 10.018 min, respectively.
- In MS^2 of peak of m/z 573.2167, 113.948 ion was found to be most abundant when 43.39 % of collision energy is applied. Notably, 573.216 is multiple of 113.94 indicating probably the parent ion is made of repeat units of ions of m/z 113.94.

- Type III polyketide operon codes for two enzymes namely, Polyketide Synthase (PKS) and Phospholipid methyltransferase (PMT). Primers were designed for genes *pks* and *pmt*, using the type strain *B. altitudinis* 41KF2b and whole genome sequences of other *B. altitudinis* strains obtained from NCBI. The amplicons of expected sizes of 598 and 283 bps of PKS and PMT genes, respectively indicated the presence of genes for type III polyketide antibiotic in *B. altitudinis* S2.
- MIC and MBC of antibiotic S2 for Xoo BXO43 were found to be 8 µg/ml and 16 µg/ml. Live-dead staining of test organisms, Xoo BXO43 and *S. aureus* showed intact dead cells when treated with antibiotic S2 at MBC and further confirmed by time-kill assay of Xoo BXO43. These assays implied the bactericidal nature of the antibiotic.
- SEM results of sensitive organisms Xoo BXO43 and *S. aureus* indicated no effect of antibiotic S2 on surface morphology, while effect on total protein of Xoo BXO43 indicated decrease in total protein production upon treatment with increasing concentration of antibiotic S2 as observed by SDS-PAGE. The results show that probably antibiotic S2 affects the protein synthesis of the cells.

Chapter 4

Studies on virulence attenuation of *Xanthomonas oryzae* pv. oryzae by small molecule compounds

- The sub-inhibitory concentrations of various compounds were determined to study the effect of the small molecules on virulence factors of Xoo BXO43. The values obtained were as follows: ferulic acid (200 µM), caffeic acid (300 µM), cinnamic acid (200 µM), salicylic acid (20 µM), indole acrylic acid (40 µM), p-coumaric acid (250 µM), aminocinnamic acid (100 µM) and acibenzolar S methyl (100 µM).
- Growth of Xoo BXO43 in presence of small molecules at their sub-inhibitory concentrations in PS broth and XOM2, a TTSS-inducing medium was studied. It was unaffected in the presence of all the compounds tested except indole acrylic acid. Indole acrylic acid hence was not included in further study.

- A transcriptional reporter plasmid, pXopQ122 was constructed using promoter region of one of the effector gene XopQ of Xoo, to study the effect of small molecule compounds on TTSS of Xoo BXO43.
- The plasmid pXopQ122 was transformed into Xoo BXO43 to give XooBXO43XOPQ as a reporter strain. The promoter activity of xopQ was measured in terms of GUS units using this reporter strain. Cinnamic acid and its derivatives, ferulic acid and caffeic acid reduced GUS activity by 50 % and more and thus indicating its profound effect on expression from XopQ gene promoter.
- p-coumaric acid and acibenzolar S methyl reduced the activity by 40 -48 %, while very less effect was observed with amino cinnamic acid and salicylic acid which reduced the activity only by 20 -30 %.
- Effect of small molecule compounds on exoenzymes cellulase and pectinase production was determined using wheat bran supplemented media and no substantial difference in the production of exoenzymes was observed.
- Effect of phenolic compounds on other virulence factors like EPS production and motility was negligible. Nevertheless, Acibenzolar S methyl decreased the motility of Xoo BXO43 to 10 mm which is 40 % as compared to control.
- Slight decrease in motility was observed in case of ferulic acid and caffeic acid which was measured as 12.7 mm and 12.3 mm diameter, respectively as compared to 16 ±1 mm of control.
- In detached leaf assay, the cinnamic acid and its derivatives ferulic acid, caffeic acid and p-coumaric acid showed more than 50 % reduction in lesion formation by Xoo BXO43 on rice leaves.

Chapter 5

Studies on effect of combination of antibiotic S2 and small molecule compounds on *Xanthomonas oryzae* pv. oryzae

- Checkerboard assay was used to study the potentiating effect of small molecule compounds on antibiotic S2 on Xoo BXO43. Synergy was observed between antibiotic S2 and caffeic acid as indicated by FIC index of 0.375, while no synergy with acibenzolar S methyl was found as per FIC index which was measured to be 0.75; similarly with other

small molecule compounds it was > 1 indicating no synergy. Hence, only caffeic acid displayed synergy with antibiotic S2.

- Other conventional antibiotics were used for examining synergy between caffeic acid and antibiotics. Streptomycin and Ofloxacin showed synergy with caffeic acid as indicated by FIC index 0.375, while mild synergy with Tetracycline as observed by 0.527 and no synergy with Gentamycin as its FIC index was calculated to be 1.125.
- EtBr efflux assay was carried out to examine the potential of small molecule compounds as efflux pump inhibitors (EPI) and results indicated that caffeic acid was most effective among the compounds tested, in reducing the EtBr efflux as compared to that observed in control wells.
- To elucidate if the efflux pumps are involved the mechanism of synergy exhibited between antibiotic S2 and caffeic acid, genes for efflux pumps were searched from the genome of Xoo from NCBI. Six different efflux pumps were found and primers were designed to study the expression of the representative genes of the six efflux pumps.
- Results of RT-PCR showed nearly two fold (1.8) increase in the expression of *norM* gene, while no or insignificant difference in the expression of other genes was observed indicating *norM* is involved in some way in synergy exhibited by the two compounds, antibiotic S2 and caffeic acid. The difference in the expression of *norM* with caffeic acid treatment hinted at a possible association and further experiments may reveal the exact mechanism of the action of caffeic acid mediated antibiotic retention.