Synopsis of the Thesis
on

Inhibition and virulence attenuation studies of *Xanthomonas oryzae* pv. oryzae causing rice blight

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Introduction

Rice is the staple diet of one-half of world's population. Hence, detailed study regarding its diseases and methods to reduce the crop loss is required. Bacterial blight (BB) is one of the major diseases prevalent in tropical countries like India, Indonesia, and Philippines. Loss up to 50% has been reported under severe epidemics (Shanthi et al., 2010; Mew & Cruz, 2001). In India, it is a severe disease under irrigated and high nitrogen fertilizer input conditions. BB is one of the major bacterial diseases in rice, making it stand second in list of microbial pathogens that lead to loss of rice yield.

Bacterial blight is caused by a Gram negative bacteria *Xanthomonas oryzae* pv. oryzae (Xoo). Genus *Xanthomonas*, classified under γ-Proteobacteria and comprises of 27 species. These bacteria infect approximately 124 monocotyledonous and 268 dicotyledonous plants. These pathogens show a high level of host plant specificity and many of them exhibit tissue specificity, invading either the xylem elements (vascular pathogens) or the mesophyll tissue (mesophyllic pathogens) of the host (Ryan et al., 2011). This makes the bacteria of this genus economically important and interesting research model.

Xanthomonas oryzae pv. oryzae is a vascular pathogen and mainly infects the shoot part of the rice plant. It is a yellow pigmented, slime-producing, motile, rod shaped with a polar flagellum. It enters host via hydathodes (water pores) and even through wounds and cracks. It enters the plant by swimming, multiply in spaces of underlying epithem and then it spreads systemically through xylem vessels (Nini-Liu et al., 2006). Within a few days, bacterial cells and EPS produced in copious amount fill the xylem vessels and ooze out from hydathodes. Spots at the tips and margins of fully developed leaves are the typical symptoms of bacterial blight. The spots expand along the veins, merge, and become chlorotic and then necrotic, forming opaque, white to grey colored lesions that typically extend along the whole leaf tip. The disease can affect rice plants at any growth stages. Symptoms develop at tillering stage and peaks at flowering stage, while kresek develops in plants less than 21 days old and severely affect crop yield (Mew et al., 1993).

Many virulence factors of Xoo are involved in successful invasion of pathogen in host plant are xanthan gum, hydrolytic enzymes like xylanase, pectinases, cellulase, protease, etc. Enzymes like xylanase, esterase, etc. are secreted by Type II secretion system are virulence factors and show functional redundancy (Rajeshwari et al., 2005). Type III secretion system (T3SS) in many phytopathogens have been demonstrated to mediate secretion of virulence factors in the host. It presumably acts to prevent or inhibit a general resistance response or otherwise enhance the colonization of the plant by the bacteria. Mutants of T3SS genes of *Xanthomonas sp*. fail to cause virulence on plants (Wang et al., 2007). Mutation in *gum* genes lead to loss of EPS and virulence in *Xanthomonas oryzae* pv. oryzae (Dharmapuri & Sonti, 1999).

Different strategies employed to control Xoo:

Many approaches like biological, chemical and host resistance genes have been implemented for rice disease management and its control during endemic and epidemic conditions; however, all these strategies have their own limitations.

Based on repeated field trials, agrochemicals based on L- chloramphenicol, nickel-dimethyldithiocarbamate, dithianon and fentiazon were used at commercial levels. However, they were unreliable due to variable results on different pathogen population (Gnanamanickam et al., 1999). Bismerthiazol and Streptomycin have been used by farmers to control the disease; however, Xoo has started developing resistance to it (Xue, 2002; Xu et al., 2010). Many resistant varieties carrying multiple resistant genes are used as the most effective disease management strategy. However, various pathotypes of Xoo have overcome the deployed resistance genes (Lore et al., 2011).

Many biocontrol strategies are proposed in protecting the rice against bacterial blight caused by Xoo. *Pseudomonas sp.*, *Bacillus subtilis*, *Lysobacter antibioticus* and other PGPR have been studied at field level and have been reported to control of bacterial leaf blight of rice caused by Xoo (Velusamy et al., 2006; Gnanamanickam et al., 1999; Udayashankar et al., 2011). Antibiotics produced by fungus *Phomopsis longicolla* S1B4 (Lim et al., 2010) and Sphaeropsidins and its various derivatives have been shown by *in vitro* assay to be antibacterial against Xoo (Evidente et al., 2011). An antibacterial peptide cecropin overexpressed in rice showed enhanced resistance against bacterial blight (Sharma et al., 2000).

Resistance development is an inevitable survival mechanism of bacteria. Hence, there is constant need of designing novel strategies and searching new compounds for the control of the disease. Antagonistic bacteria producing natural metabolites are considered as potential source of structurally diverse antimicrobial compounds. Additionally, using small molecule compounds as adjuncts to these antimicrobial compounds can be more effective measure to control the disease as they delay the resistance development in the pathogens.

Virulence attenuation targets and small molecule compounds:

Since the last decade, many libraries of natural and synthetic small molecules have been screened for their anti-virulence effect on animal as well as plant pathogens. Effective use of these compounds to control pathogenesis by phytopathogens including *Erwinia amylovora*, *Dickeya dadantii*, *Psuedomonas fluorescens*, *Erwinia carotovora*, *Psuedomonas syringae* (Duncan et al. 2012) has been extensively studied. These compounds have mainly been reported as inhibitors of T3SS of the pathogens as well as other virulence factors. For example, salicylic acid reduces the adhesion, biofilm formation and pyocyanin production which are involved in the pathogenesis processes of *Pseudomonas aeruginosa*; while salicylidene acylhydrazide derivatives have been shown to affect T3SS and flagellar based bacterial motility in *Salmonella enterica*. Recently, it has been reported that indole and its derivatives affect multiple virulence factors of *Staphylococcus aureus*. A thiazolidinone derivative was shown to affect T3SS and T2SS dependent functions in wide array of plant and animal pathogens.

Although these small molecule compounds have been reported to suppress virulence of many pathogens, studies on attenuation of virulence factors of Xoo are sparse.

Like many Gram negative pathogens, Xoo possesses T3SS encoded by hypersensitive response and pathogenicity (*hrp*) gene clusters having approximately 24 genes. These are the most important genes related to Xoo pathogenicity. Hrp genes are grouped in two hrp groups: group I and group II in Gram negative phytopathogens, based on the differences in regulatory genes. *Xanthomonas* has been categorized in group II, wherein hrp genes are regulated by hrpG and hrpX. In Xoo, many hrp genes have consensus sequence called Plant Inducible Promoter (PIP) box in their promoter region and their expression is controlled by HrpX, a transcriptional regulator (Furutani et al. 2006). In phytopathogens, hrp transcriptional regulators regulate and co-express many effector genes with the T3SS, which has also been established by Furutani et al. (2006) for effector gene xopQ in case of pathogen *Xanthomonas oryzae* pv. oryzae. Additionally, XopQ is a homolog of inosine-uridine nucleoside Nribohydrolase which is highly conserved effector protein among most xanthomonads and other phytopathogens (Adlung et al. 2016). It possesses a PIP box and a −10 box-like sequence and has been reported to be under the control of hrpX in hrp inducible manner as reported in XooMAFF311018 strain (Furutani et al. 2006, 2009). Therefore, the expression of xopQ can be correlated with the induction of the T3SS making it to be an appropriate target for the virulence attenuation studies.

Another use of small molecule compounds have been reported to overcome bacterial resistance as they could be potential efflux pump inhibitors (EPIs) that could restore antibiotic activity in resistant strains (Schmitz et al., 1998). Plants use antimicrobials to protect themselves from environmental stresses, but unlike fungi or bacteria activities are often weak and with a narrow spectrum. This poses the interesting question of how plants react towards increasing antimicrobial resistance. Some authors postulate that plants use an anti-MDR strategy to potentiate their antimicrobials. The combination of a resistance inhibitor with an antibiotic has already proven its efficacy with the clavulanic acid/amoxicillin association.

Rationale:

Many approaches like biological, chemical and host resistance genes have helped in protecting the rice against bacterial blight caused by *Xanthomonas oryzae* pv. oryzae; all these strategies have their own limitations. Hence, there is a constant need of searching for different ways for the effective control of this disease. Biological and chemical control methods or combining biological and host resistance methods could be used for higher level of protection and sustainable rice yield.

Hence, current studies have focused on two eco-friendly methods- antimicrobial compound produced by the bacterial antagonist and the usage of small molecule compounds, which do not affect the survival of bacteria but attenuate the virulence factors of the pathogen, in controlling the pathogenesis on host plant. Accordingly, for inhibition and virulence attenuation studies of Xoo, the objectives designed were as follows:

Objectives:

I) Isolation of antagonistic bacteria against *Xanthomonas oryzae* pv. oryzae

II) Virulence attenuation studies with *Xanthomonas oryzae* pv. oryzae.

III) Characterization of antibacterial metabolite produced by the selected antagonistic bacterial isolate *Bacillus sp.* S2

I) Isolation of antagonistic bacteria against *Xanthomonas oryzae* pv. oryzae (Xoo) BXO43

Healthy rice plants and related grass varieties were collected from various places for isolation of antagonists against the test pathogen Xoo BXO43. Sample collection was carried out in monsoon season and samples were stored in cold conditions. Isolation of antagonistic bacteria against the test pathogen was done from rhizosphere, phyloplane and endophytes of the collected plant samples.

Isolation and Screening of bacterial antagonists for Xoo BXO43

Antagonists from rhizosphere and phyloplane were carried out by pour plate method where test pathogen Xoo BXO43 was seeded in top agar and incubated for 6 hrs. Sample was given saline wash for three times and various dilutions were spread on it. Isolates showing zone of inhibition were purified; and again were checked using agar plate assay. Endophytes were isolated in King's B medium after surface sterilization. Isolates with different morphology were picked after 24-48 hrs. Purified isolates were then checked against Xoo BX043. Total 90 isolates were tested, out of which 68 did not show any antagonism, 8 isolates showed moderate inhibition, while 14 showed strong inhibition against the test pathogen Xoo BXO43.

Inhibition of isolates showing strong antagonism was further quantified by Colony Zone by Colony size (Cz/Cs) ratio. Based on Cz/Cs ratio, 12 isolates could be grouped in two categories, one showing Cz/Cs ratio above 3.5 and another those showing Cz/Cs ration below 3.5. The former included 5 isolates: L1, L21, R2, S2 and N4. Isolate S3 showed a different kind of inhibition of growth of Xoo BXO43. It grew on PS agar plate covering the major part of the plate and effectively inhibiting growth of Xoo BXO43 and hence it was also selected for further studies along with above mentioned 5 isolates.

Identification of isolates

Morphological and biochemical traits of selected 6 isolates were carried out. Further, these isolates were identified based on 16S rRNA gene. 16S 27F and 16S 1541R universal primers were used for amplification of 16S rRNA gene. On this basis, two isolates were found to be *Bacillus subtilis,* one as *Bacillus altitudinis*, one as *Bacillus amyloliquifaciens,* while other two were identified as *Pseudomonas* sp. and *Paenibacillus sp*. Result of BLAST is showed in the table below:

Isolates	% Identity	Querry coverage	Nearest match (Accession no.)
S2	99%	99%	*Bacillus altitudinis* MRN16 MG062748.1
L21	100%	100%	*Bacillus subtilis* BAB-1710 KF535133.1
S3	99%	100%	*Paenibacillus alvei* AN5 JQ868768.1
L1	99%	100%	*Bacillus subtilis* VEB17 KF460571.1
N4	100%	99%	*Pseudomonas* sp 4-3 EF690395.1
R2	98%	100%	*Bacillus* zhangzhouensis *LNHL13* MG008662.1

Screening isolates for Plant Growth Promoting Rhizobactetia (PGPR) traits and controlling the disease symptoms

These isolates were studied for PGPR traits like Biofilm formation, Phosphate solubilization, Zinc solubilization, IAA production, Siderophore production and Nitrogen fixation. Isolates S2, R2 and L1 showed higher biofilm formation amongst the six isolates in *in vitro* assay using crystal violet staining method. Isolates N4 and S2 showed presence of all PGPR traits except siderophore production. Further, plant assay was carried out to examine effect of isolates on rice plant. Rice cultivar TN1 was used and all isolates except R2 showed positive effect on plant growth.

The ability of the four isolates: L21, S2, S3 and N4 to suppress BB in rice leaves containing Xoo BXO43 were examined by detached leaf assay. Isolates S2 and S3 showed maximum resistance as compared to other four isolates. However, leaves treated with S3 isolate also showed hypersensitivity response like symptoms. Based on these results, isolate S2 was chosen for further studies.

Conclusively, totally 90 isolates from different plants of Poaceae family were isolated and screened for their antagonistic effect. From Cs/Cz and detached leaf assay, isolate S2 identified as *Bacillus sp.* was selected as the best bacterial antagonist amongst the screened isolates.

II) Virulence attenuation studies with *Xanthomonas oryzae* pv. oryzae.

For studying the effect of small molecule compounds on various virulence factors of Xoo BXO43, the compounds used included: salicylic acid, cinnamic acid and its derivatives ferulic acid, caffeic acid, aminocinnamic acid, p-coumaric acid and other small molecule compounds like ellagic acid, acibenzolar S methyl and indole acrylic acid. Diverse natural or synthetic small molecule compounds have been shown to cause attenuation of virulence of different pathogens (Felise *et* al., 2008). Though T3SS has been reported as conserved over wide variety of Gram negative bacteria, phytochemicals screened for T3SS blockers have been shown to have different effect on different pathogens, at varying concentrations in the literature. Hence, it was essential to optimize the concentration of the chemical compounds

used in this study. Growth response of Xoo BXO43 to these compounds was studied in two media viz. a nutritionally rich medium, PS broth as well as in T3SS inducing XOM2 medium (Tsuge *et* al., 2002). The sub-inhibitory concentration of compounds 200 µM (ferulic acid), 300 µM (caffiec acid), 200 µM (cinnamic acid), 20 µM (salicylic acid), 40 µM (indoleacrylic acid), 250 µM (p-coumaric acid), 100 µM (aminocinnamic acid) and 100 µM (acibenzolar S methyl) did not affect the growth of Xoo BXO43 in PS broth when monitored at 24 and 48 h and therefore, were used for virulence attenuation studies.

Transcriptional reporter system of the effector gene is commonly used for studying the effect of chemicals on T3SS. In *Xanthomonas oryzae* pv. oryzae, as reported by Furutani *et* al., (2006, 2009), XopQ is a unique effector secreted via T3SS which shares homology with reported effectors and possesses PIP box and -10 like sequence in its promoter region. Hence, it was selected to study the effect of small molecule compounds on T3SS of Xoo BXO43. A Transcriptional reporter system developed by Furutani and workers to identify the effectors of T3SS in *Xanthomonas* was adopted to determine the effect of selected compounds on T3SS of Xoo BXO43, where their effect on promoter activity of XopQ, an effector secreted by T3SS was determined. Primers were designed using the promoter region of xopQ gene from the reported genome sequence of Xoo PXO99A strain. pXopQ122, a transcriptional reporter gene construct was prepared by cloning the promoter region of xopQ gene in the plasmid pSS122 harbouring promoterless β-glucuronidase gene so that the latter was regulated by the effector protein xopQ promoter. The positive transformant XooBXO43XOPQ obtained was used as reporter strain, where it was grown in XOM2 medium supplemented with sub-inhibitory concentration of the selected compounds and the promoter activity of xopQ was measured in terms of GUS units. Effect of sub-inhibitory concentration of the selected compounds on the promoter activity of xopQ showed that the p-coumaric acid and acibenzolar S methyl reduced the activity by 40-48%; while aminocinnamic acid and salicylic acid reduced the activity by 20-30%. Cinnamic acid and its derivatives caffiec acid and ferulic acid were found to be the most effective and reduced the GUS activity by ≥ 50%, consequently indicating its effect on promoter activity of xopQ gene.

It was observed that when wheat bran- a crude plant derived raw material, was used in the medium both cellulase and pectinase activities were detected in significant amount in cellulase and pectinase plate assays. Wheat bran medium was amended with subinhibitory concentration of the selected compounds, there was no substantial difference in the production of these extracellular enzymes as compared to that in unamended medium. Additionally, we used this medium for studying the effect of compounds on T3SS of Xoo BXO43, and it was found that wheat bran which supported the secretion of enzymes like cellulase and pectinase by Xoo, could also be used as T3SS induction medium in lieu of synthetic media as described in the literature.

Motility and EPS production are chronologically related in the pathogenesis in many bacteria. Motility has been reported to be important virulence factor in foliar pathogens. Xoo being systemic pathogen enters the host through hydathodes where motility plays an important role. EPS produced by Xoo BXO43 in PS broth without supplementing any compounds was

500 ± 100 µg of glucose equivalents/10^9 cells. Acibenzolar S methyl and aminocinnamic acid showed decrease in EPS production, while ferulic acid and cinnamic acid induced EPS production. The differences in EPS production were not statistically significant. Further, when effect of the sub-inhibitory concentration of the compounds was examined on swimming motility of Xoo BXO43, acibenzolar S methyl was found to reduce swimming motility significantly, while the phenolic compounds did not show any substantial effect. Xoo BXO43 demonstrated swimming motility with a diameter of about 16 mm at 30°C after 48 h in 0.3% PS agar medium. With acibenzolar S methyl treatment, the motility was found to be 10 mm which is 40% lower as compared to the control. Compounds like ferulic acid and caffiec acid showed reduced motility with 12.7 mm and 12.3 mm diameter, respectively, while rest of the compounds did not show any substantial reduction in diameter

After studying the effect of selected compounds on various virulence factors, detached leaf assay was carried out to examine their effect on disease symptoms on host. Negative control wherein only saline was infiltrated in the leaves showed no lesions, while leaves infiltrated with Xoo BXO43 gave lesions covering around 76.0 ± 19.0 percent of the leaf area. Lesion formation was reduced to 16 ± 6.0 and 24 ± 2.5 percent of leaf area when leaves were treated with ferulic acid and p-coumaric acid, respectively, giving highest reduction in the disease symptom development. With treatment of caffiec acid and cinnamic acid, lesions measured were 23.0 ± 11.0 and 31.0 ± 5.0 percent of leaf area, respectively. Lesions on leaves treated with aminocinnamic acid and acibenzolar S methyl covered 66.0 ± 18.0 and 52.0 ± 17.0 percent of leaf area, respectively.

Conclusively, phenolic compounds like cinnamic acid and its derivatives p-coumaric acid, ferulic acid and caffiec acid showed inhibitory effect on T3SS, an important viulence determinant of Xoo, as measured by reduction in promoter activity of xopQ gene, while other virulence factors were not affected. When leaves of susceptible plant variety TN-1 were treated with the compounds like cinnamic acid and its derivatives ferulic acid, caffiec acid and p-coumaric acid significant reduction in lesion formation was observed it can be deduced that probably these compounds reduce pathogenesis on rice by specifically attenuating T3SS of Xoo. Compound acibenzolar S methyl showed reduction on multiple virulence determinants of Xoo BXO43.

III) Characterization of antibacterial metabolite of the selected antagonistic bacterial isolate.

A) Characterization of antimicrobial compound produced by *Bacillus sp.* S2

Production of bioactive metabolite by S2 was carried out in PSA, 1/4th strength PSA and M9 broth and extraction was carried out using organic solvents having different polarity: Hexane, Ethyl acetate, n-butanol, Chloroform, Isopropanol, Ethanol and methanol. Extraction was successful from PSA-agar co-cultured with Xoo BXO43 and Sucrose-Bushnell Hass (SBH) agar plate using methanol indicating the polar nature of the metabolite. This also

indicates that bioactive metabolite production is inducible and is produced when bacterium is subjected to any biological or nutritional stress. The efficiency of bioactive metabolite extracted from S2, to control BB in rice leaves was examined by detached leaf assay.

Time-course production of antimicrobial compound and growth of isolate *Bacillus sp.* S2 in SBH broth was examined. Antimicrobial compound production was detected after 48 h of incubation and assay was carried till 7 days which gave maximum production of the bioactive compound. The production of antibiotic was carried out using agar diffusion well assay using sensitive strain Xoo BXO43 and the maximum zone of clearance of 40mm was observed. Cell free supernatant was lyophilized and further bioactive metabolite was extracted using isopropanol.

Effect of temperature and pH was examined on the stability of crude active metabolite extracted. Extract was incubated at different temperatures in water bath at 40°C–65°C for 2 h and activity was examined by agar diffusion well assay. It was found to be tolerant from 40°C–65°C temperature, while 28% activity loss was observed when autoclaved at 121°C for 15 minutes. It was found to be tolerant to acidic and alkaline pH ranging from pH 2-9. Sensitivity of bioactive compound to degradative enzymes was tested. The compound retained its antimicrobial activity even after treatment with Proteinase K, Trypsin and β-amylase, as tested by agar diffusion well assay.

TLC and bioautography on TLC was carried out using sensitive bacterial strains Xoo BXO43 and *S. aureus*. Scanning Electron Microscopy of two bacterial strains treated with bioactive metabolite produced by *Bacillus sp.* S2 showed distorted cell surface of the two test bacteria indicating its effect on both Gram negative as well as Gram positive bacteria.

Further experiments like MS/MS and FTIR will be performed, which will give details about the constituting components of the metabolite. This will help in identification of the antibiotic.

B) Effect of Combination of small-molecule compounds with the antimicrobial compound of Bacillus sp. S2 and Streptomycin on Xoo

The small molecule compounds cinnamic acid and its derivatives ferulic acid, p-coumaric acid, caffiec acid and other compound acibenzolar S methyl, which were found as effective virulence attenuating compounds were studied for their synergistic effect. Also, additional small molecule compound studied was indole acrylic acid. Pure antibiotic streptomycin to which Xoo is sensitive and the partially purified antimicrobial compound produced by *Bacillus sp.* S2 were the two antibiotics used in the study. The synergy between the small molecule compounds and the antimicrobial agents was studied by microdilution assay. MIC of the individual compounds and in combinations was done by microdilution assay. The Fractional Inhibitory Concentration Index (FICI) for each combination was calculated by using the following formula:

$$FIC_{component\ 1} = MIC_{component1,in\ combination} / MIC_{component1,alone}$$

$$FICI = FIC_{component\ 1} + FIC_{component\ 2}$$

The FICIs were interpreted as follows: FICI of ≤0.5 (synergy); 0.5 <FICI ≤1 (no interaction/indifference); FICI of >4 (antagonism).

Acibenzolar S methyl and indole acrylic acid gave 4- fold increase in sensitivity of Xoo to streptomycin. As FIC index (FICI) found was < 0.5, which indicates synergy between the two compounds used. Further, they were studied for their synergistic effect with partially purified bioactive metabolite produced by *Bacillus sp.* S2. Similarly, FIC index was found to be < 0.5.

Conclusively, the bioactive metabolite produced by the antagonist *Bacillus sp*. S2 was produced when the bacteria was grown in synthetic medium like Sucrose-Bushnell Hass medium. It was extracted by isopropanol and it was found stable over long range of pH and temperature and did not lose activity upon treatment with any of the degradative enzymes used in the study. With the small molecule compounds like acibenzolar S methyl and indole acrylic acid, it showed synergy in killing the pathogen Xoo BXO43.

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