Chapter 4:

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

4.1. Introduction

Many Gram-negative animal and plant pathogens recruit closely related secretion systems for causing virulence which makes them appealing targets (Felise et al., 2008; Hueck, 1998). It is hypothesized that targeting these virulence for antibacterial therapy will cause less evolutionary pressure for resistance development than conventional therapies, as these virulence factors are dispensable for bacterial growth (Kauppi et al., 2003). The agents which aims at virulence attenuation without affecting bacterial growth, has potential for novel drug development and are also important tools for understanding bacterial virulence (Duncan et al., 2012; Rasko & Sperandio, 2010; Shogren-Knaak et al., 2001).

Many assays have been developed for studying the virulence factors of the pathogens *in vitro*. Simple agar diffusion assays could be used for examining the secretion of various virulence exoenzymes. In these assays, substrates for these exoenzymes are used to make agar plates and their secretion is analyzed based on the zone of clearance produced in substrate-agar plate by their respective enzymes.

Many molecular tools have been developed to study the complex virulence factors like type III secretion system of bacterial pathogen *in vitro*. Since, these secretion system secrete out wide variety of effector molecules, which are difficult to assay by above mentioned physiological assay. A transcription fusion harbouring the promoter of any of the effector gene that is induced by the regulatory elements of the secretion system of the pathogen, can be used to carry out *in vitro* examination of such secretion systems of the pathogen. Promoter region of the gene is tethered upstream to a reporter gene. Many kind of reporter genes can be used which encode visually identifiable proteins like fluorescent compounds (GFP), luminescent compounds (luciferase); or genes that encode enzymes like β-galactosidase, β – glucuronidase, etc. which use chromogenic substrates. The expression of the reporter gene indirectly indicates the transcription and thus induction of the secretion system under study.

Like many Gram negative pathogens, Xoo possesses type III secretion system encoded by hypersensitive response and pathogenicity (*hrp*) gene clusters having approximately 24 genes (Cho et al., 2008). Hrp genes are grouped in two groups I and II in Gram negative phyto-pathogens, based on the differences in regulatory genes (Alfano & Collmer, 1997). In *Xanthomonas*, categorized in group

II, hrp genes are controlled by HrpG/HrpH regulators, which are the OmpR family response regulator and the AraC-type transcriptional activators, respectively (Wengelnik et al., 1996; Wengelnik & Bonas, 1996). Several genes regulated by HrpX have consensus sequence called Plant Inducible Promoter (PIP) box in their promoter region (Fenselau & Bonas, 1994). *xopQ* is a gene of an effector protein and has been reported to be under the control of hrpX in hrp inducible manner as reported in Xoo MAFF311018 strain (Furutani et al., 2006).

Since last decade libraries many 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 on phyto-pathogens like *Erwinia amylovora* (Khokhani et al., 2013), *Dickeya dadantii* (Yang et al., 2008), *Psuedomonas fluorescens* (Myszka et al., 2014), *Erwinia caratovora* (Truchado et al., 2012), *Psuedomonas syringe* (Felise et al., 2008) have been studied. These chemicals have mainly been reported as inhibitors of T3SS of the pathogens, along with other virulence factors employed by the pathogens for pathogenesis. Accordingly, in the processes involved in pathogenesis by *Pseudomonas aeruginosa*, salicylic acid has been reported to reduce adhesion, biofilm formation and pyocyanin production (Prithviraj et al., 2005); while salicylidene acylhydrazide derivatives have been shown to affect T3SS and flagellar based bacterial motility in *Salmonella enterica* (Negrea et al., 2007). Lee et al. (2013) has shown that Indole and its derivative affect multiple virulence factors of *S. aureus*. A thiazolidinone derivative was shown to affect TTSS and TIISS dependent functions in wide array of plant and animal pathogens (Felise et al., 2008). Although, these small compounds have been reported to suppress virulence of many pathogens, studies on attenuation of virulence factors of Xoo are sparse.

The present studies were therefore undertaken to screen potent anti-virulence small molecule compounds majorly phyto-chemicals against Xoo BXO43 strain and their effect on virulence factors involved in the pathogenesis in the host plant. Most of the compounds used in this study were plant phenolic compounds except acibenzolar S methyl, a thiadiazole derivative which has been reported to be effective against Xoo at field level, but not much is known about its specific effect on the pathogen. Effect of these compounds was examined on extracellular enzymes secreted via type II secretion system (TIISS), EPS production, motility

and on one of the reported effector XopQ of type III secretion system (TTSS). Further, the reduction in virulence by the selected compounds was also demonstrated in *in vitro* detached leaf assay by using rice leaves of susceptible cultivar Native Taichung-1 (TN-1).

4.2. Materials & Methods

4.2.1. Bacterial strains, plasmids and growth conditions

Xanthomonas oryzae pv. oryzae (Xoo) BXO43 strain was grown in PS medium and XOM2 medium. *Escherichia coli* DH5α was cultivated at 37 °C in Luria-Bertani (LB) medium. Antibiotic Ampicillin was added to media at final concentration of 100 µg/ml whenever required. The composition of PS medium and LB medium has been described in chapter 2 undersection 2.2.2.

4.2.1.1. XOM2 medium medium was used for xopQ-inducing conditions. Composition (g/l): D-(+) xylose, 1.8; mM L-methionine, 670; sodium L-(+) glutamate, 10 mM; KH2PO4, 14.7 mM; MnSO4, 40 mM; Fe(III)-EDTA, 240 mM and MgCl2, 5 mM. pH 6.5, the culture inoculated in XOM2 medium was incubated at 28 °C for induction of TTSS of Xoo (Tsuge et al., 2002).

4.2.1.2. Wheat bran (WB) medium This was used for exoenzyme production assay. Composition (g/l): wheat bran, 5.0 in Bushnell-Haas medium.

4.2.2. List of compounds

ferulic acid (4-hydroxy-3-methoxycinnamic acid), D-cinnamic acid, caffeic acid (3,4 -dihydroxycinnamic acid), 4-aminocinnamic acid, salicylic acid, trans p-coumaric acid, trans-3-indole acrylic acid, acibenzolar-S-methyl, ellagic acid were bought from Aldrich Sigma.

4.2.3. Effect of compounds on growth of Xoo for determination of sub inhibitory concentration

Xoo culture was inoculated to attain 0.1 OD at 600 nm in PS/XOMV2 broths containing individually different concentrations of the compounds mentioned in section 4.2.2 in a broad range of 250, 500 and 750 µM concentrations. The turbidity was monitored to quantify growth at 24 and 48 hrs using Synergy-4

multimode microplate reader (Tecan). Further based on these results, subinhibitory concentration of all the compounds was checked in the lower range of 20, 50, 100, 200 and 250 μM except caffeic and ellagic acid. Ellagic acid was eliminated since it was highly inhibitory at even lower concentrations. Caffeic acid was tested at a concentration range of 300, 400, 500 and 600 μM. Control was without supplementation of any compound. Finally the subinhibitory concentration of each of these compounds were determined by comparing the growth in terms of turbidity with that of control. Two trials with triplicates were conducted in each experiment.

4.2.4. Construction of reporter strain

4.2.4.1. Bioinformatic analysis and amplification of *xopQ* gene promoter: Search for the sequences identical to the promoter region of *xopQ* was carried out with the help of a reference type strain Xoo PXO99A, which is genetically closely related to Xoo BXO43. *xopQ* effector gene of Xoo PXO99A strain possessing gene id PXO_RS01415 in NCBI was used for designing primers of promoter region for Xoo BXO43. Precisely 400 base pair region upstream to start site of gene PXO_RS01415 was used for designing the primers. With the help of Primer3 software and NEB tool, HindIII site was added at 5' end of forward primer XopQF and no primer was added to reverse primer XopQR (Table 4.1) to facilitate directional cloning. 50 μL PCR mixture contained 10 mM Tris-HCl, 1.5 mM MgCl2, 200 μM each of dNTP, 100 ng primer, 2.5 U of Taq polymerase (Invitrogen) and 10-50 ng of genomic DNA template. Negative control contained all the components of PCR mixture but without the DNA template. PCR amplification was carried out in a thermocycler (Applied Biosystem) using the following conditions: 1 cycle of 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 45 secs at 62 °C, 45 secs at 72 °C; and one cycle of a final extension for 5 min at 72 °C. 400 bp sized PCR product were separated on a 1 % agarose gel in 1× TAE buffer, containing ethidium bromide (1 $\mu g.ml^{-1}$) and visualized and photographed using transmitted UV light at 295 nm (Fig. 4.3 b). PCR bands were eluted from the gel by gel elution method according to Sambrook & Russel protocol (2001). The amplicon were sequenced at AgriGenome Labs, India. The BLAST result confirmed the sequence matching to the corresponding region of reference strain Xoo PXO99A.

4.2.4.2. Cloning of promoter to construct transcriptional fusion: The amplified promoter region of effector gene xopQ was cloned in TA cloning vector (Fermentas kit) according to user's manual. It was digested with Hind-III and Kpn -I and ligated in pSS122, a low copy number plasmid that is stably maintained in *E. coli* and *Xanthomonas* (Ferluga & Venturi, 2009). The pSS122 vector was a gift kindly provided by Dr. Venturi from International Centre for Genetic Engineering and Biotechnology, Trieste, Italy. The pXopQ122 so obtained was transformed in electrocompetent cells of *E. coli* DH5α and subsequently into electrocompetent cells of Xoo BXO43 by electroporation using Genepulser (Biorad) using 0.1 cm gap cuvette at 2.2 kV/cm. The competent cells were prepared as per the protocol of Eppendorf. Positive transformants were selected from Ampicillin LB-agar plates. The confirmation of the clones was done by different sets of restriction digestion enzymes of the plasmid isolated from the putative clones. Empty vector pSS122 transformed in Xoo BXO43 was used as negative control. Reporter strain XooBXO43XOPQ (Table 4.1) so constructed was used to assay the promoter activity which was measured by β-Glucuronidase (GUS) assay.

Table 4.1: Strains, plasmids and oligonucleotides used in construction of transcription-reporter assay system in Xoo BXO43

Strains, plasmids or oligonucleotides	Characteristics or sequences	Reference or source
Strains		
E. coli DH5α	F j80dlacZDM15 D(lacZYA-argF)U169 endA1 deoR recA1 hsdR17(rKmK+) phoA supE44 lthi-l gyrA96 relA1	Lab collection
Xoo BXO43	Wild Type strain	Yoshitola et al., 1997
Xoo BXO43XOPQ	Xoo BXO43 transformed with pXopQ122	This study
Plasmids		
pSS122	Promoter probe vector, IncW, Apr Gmr	Ferluga & Venturi, 2009
pXopQ122	xopQ promoter cloned with HindIII-KpnI in pSS122	This study
Oligonucleotides		
xopQ F	5' CCCAAGCTTGCTGTGCCATCGCAGTCC3'	This study
xopQ R	5' GGGGACCTCTCATAGTCA 3'	This study

4.2.5. GUS reporter gene assay

Xoo BXO43 carrying pXopQ122 or pSS122 were grown in PS broth at 30 °C and 24 hrs and after saline wash, transferred in XOM2 media supplemented with different compounds and incubated at 30 ºC for 20 hrs. Cells were then centrifuged at 12000 rpm for 10 min and resuspended in 600 µl of GUS buffer (50 mM sodium phosphate, 1 mM EDTA and 14.3 mM 2-mercaptoethanol). 23 µl of 3 % Triton X-100 in GUS buffer and 23 µl of 3 % sodium lauryl sarcosinate in GUS buffer were added to the suspension and incubated at 30 °C for 10 min, and then 30 µl of 25 mM p-nitrophenyl-β-D-glucuronic acid (PNPG) was added as substrate. The reaction was stopped by adding 280 µl of 1 M Na_2CO_3 solution after sufficient yellow colour developed and readings were taken at 415 nm. One unit of GUS activity was defined as the amount of nano-moles of p-nitrophenol released per minute (Tsuge et al. 2002). Percent relative activity (% relative activity represents the relative promoter activity of *xopQ* and calculated as:

$$\%\, relative\ activity = \frac{activity\ units(XOM2\ with\ indicated\ compound)}{activity\ units\ (XOM2\ without\ compound)}\ x\ 100$$

4.2.6. Cellulase & Pectinase assay

0.2 OD at 600 nm of Xoo BXO43 culture was inoculated in wheat bran (WB) medium incubated for 48 hrs in shaking conditions. Growth was monitored in terms of turbidity (OD at 600 nm) and colony forming unit (CFU/ml). For Cellulase plate assay, 13 mm wells were punched in 0.5 % CMC agarose medium and filter sterilized supernatant equivalent to 0.2 OD was loaded in the wells and the plates were incubated at 30 ºC for 24 hrs. Similarly, for pectinase assay, 13 mm wells bored in 0.1 % pectin agarose plates were loaded with filter sterilized supernatant and incubated at 30 ºC for 24 hrs. To visualize zone of clearance by cellulase, the CMC plates were flooded with 0.5 % Congo red for 5 min and extra stain was removed with repeated washes of 3 % NaCl solution where clear zones in red background were visualized. For pectinase, pectin plates were flooded with 1 % cetyl peridinium chloride for 45 min and translucent zones were visualised in opaque background (Chatterjee et al., 1995).

4.2.7. EPS isolation and estimation

Xoo BXO43 was grown in PS broth supplemented with and without compound where the latter was used as control. Cells were centrifuged at 1000 rpm for 10 min and supernatant was filtered using 2 micron size nylon filters. EPS was isolated adding 3 % KCl and precipitated with 2 volumes of chilled ethanol for 16 hrs in -20 ^{o}C (Xu et al., 2015). This was centrifuged at 13000 rpm for 15 min and quantitated by the colorimetric method for estimation of pentoses and hexoses by Phenol-sulfuric acid method (Dubois et al., 1956).

4.2.8. Motility Agar assay

Xoo BXO43 was grown in PS broth and adjusted to 10^9 cells/ml, centrifuged at 7,000 rpm, washed and resuspended in 5 µl volume of sterile water. The cell suspension was placed at centre of the soft agar PS medium containing 0.3 % agar (swim plates) and incubated for 2 days at 28 °C and swimming motility was evaluated by measuring the diameter of the zone covered by bacteria (Tremaroli et al. 2010; Robleto et al. 2003).

4.2.9. Detached leaf assay

Effect of chemicals on disease symptoms of bacterial blight caused by pathogen Xoo BXO43 was studied by detached leaf assay. The protocol has been described in Chapter 2 section 2.2.10.

4.2.10. Statistical analysis

Effect of small molecule compounds, on virulence factors of Xoo BXO43 as well as on disease symptoms using detached leaf assay, was statistically analyzed by one-way analysis of variance (ANOVA) using software Graphpad Prism version 6.0. Differences among means were compared by Bonferroni's multiple comparisons test. $p < 0.05$ was considered as significant.

4.3. Results

4.3.1. Determination of sub-inhibitory concentration of the small molecule compounds

In order to study the effect of the selected compounds on virulence of Xoo BXO43, it is important to determine the sub-inhibitory concentration of each of these compounds, where the growth of the pathogen is not affected. The effect of different concentrations of the compounds were monitored in terms of growth of Xoo BXO43, which was measured spectrophotometrically at 600 nm at 48 hrs. Initially broad range of concentrations i.e. 750, 500, and 250 µM for the all the compounds were selected (Fig.4.1 (a)). Further, a suitable lower range was selected for those compounds for which the subinhibitory concentration was not determined with the earlier broad range. From the figures 4.1 b & c, the optimized concentrations of the selected compounds were determined to be 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), amino cinnamic acid (100 µM) and acibenzolar S methyl (100 µM).

4.3.2. Growth response of Xoo BXO43 to sub-inhibitory concentration of the selected compounds

In order to study the effect of the selected compounds on virulence determinants of Xoo BXO43, the growth response of Xoo BXO43 was monitored at the selected sub-inhibitory concentration of each of these compounds at 24 and 48 hrs in two media viz. PS broth a nutritionally rich medium as well as in XOM2 medium, which is reported to induce T3SS (Tsuge et al., 2002). The optimized concentrations of the selected compounds in XOM2 media was same as in PS broth (Fig. 4.2a), it was observed that none of the compounds affected the growth of Xoo BXO43 in PS broth as the OD was found to increase at 24 and 48 hrs. Also, the growth of Xoo BXO43 was monitored in XOM2 medium (Fig. 4.2b) and none of the compounds except indole acrylic acid showed any inhibitory effect. Hence, indole acrylic acid was excluded from further studies.

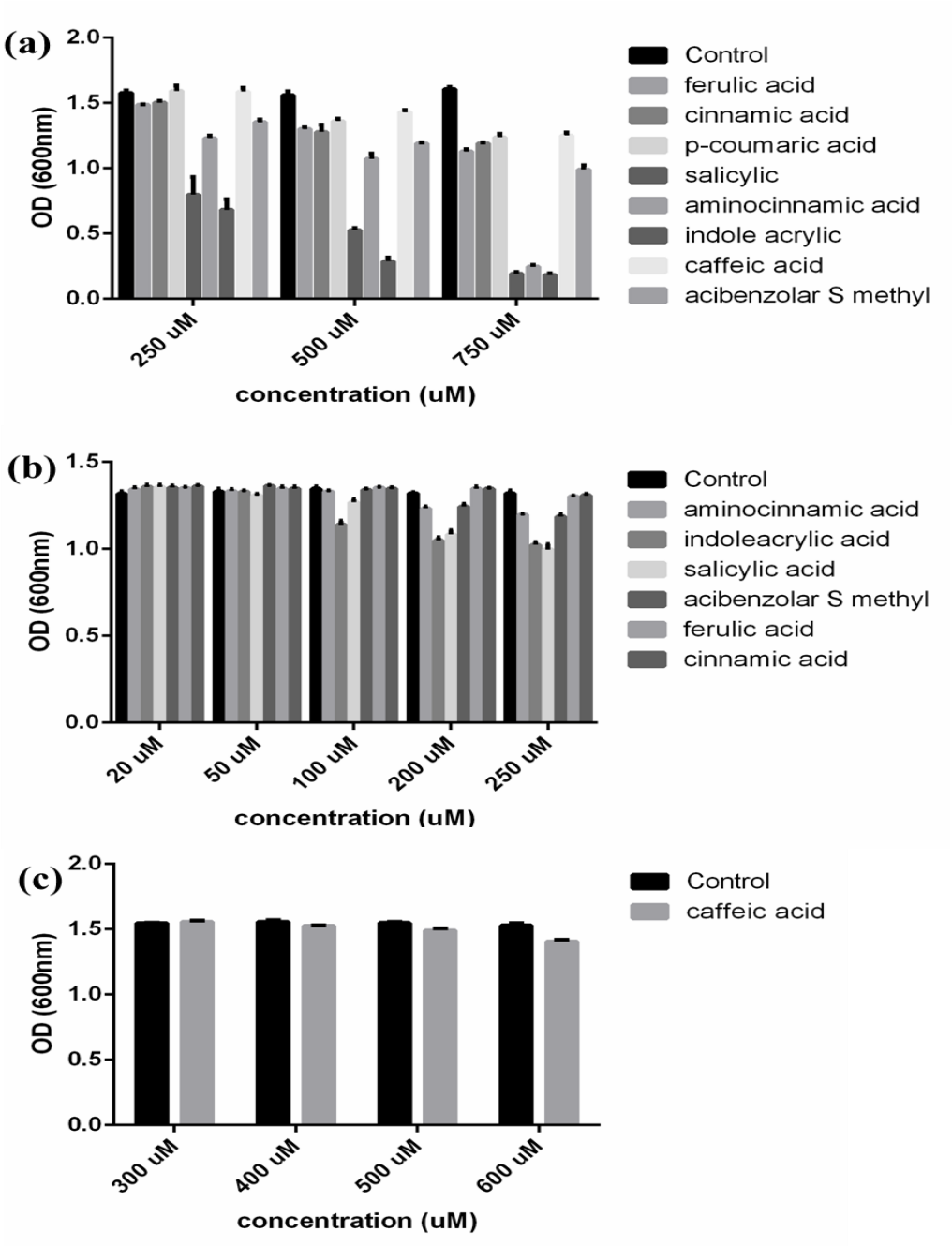

Fig. 4.1: Standardization of sub-inhibitory concentration of compounds based on the growth of Xoo BXO43 in PS broth

(a) at three different concentrations 250 µM, 500 µM and 750 µM to cover broad range concentrations. (b) standardization using small range of compound concentration based (c) standardization of caffeic acid using narrow concentration range. Bars are mean with standard deviation of two independent experiments each with triplicates.

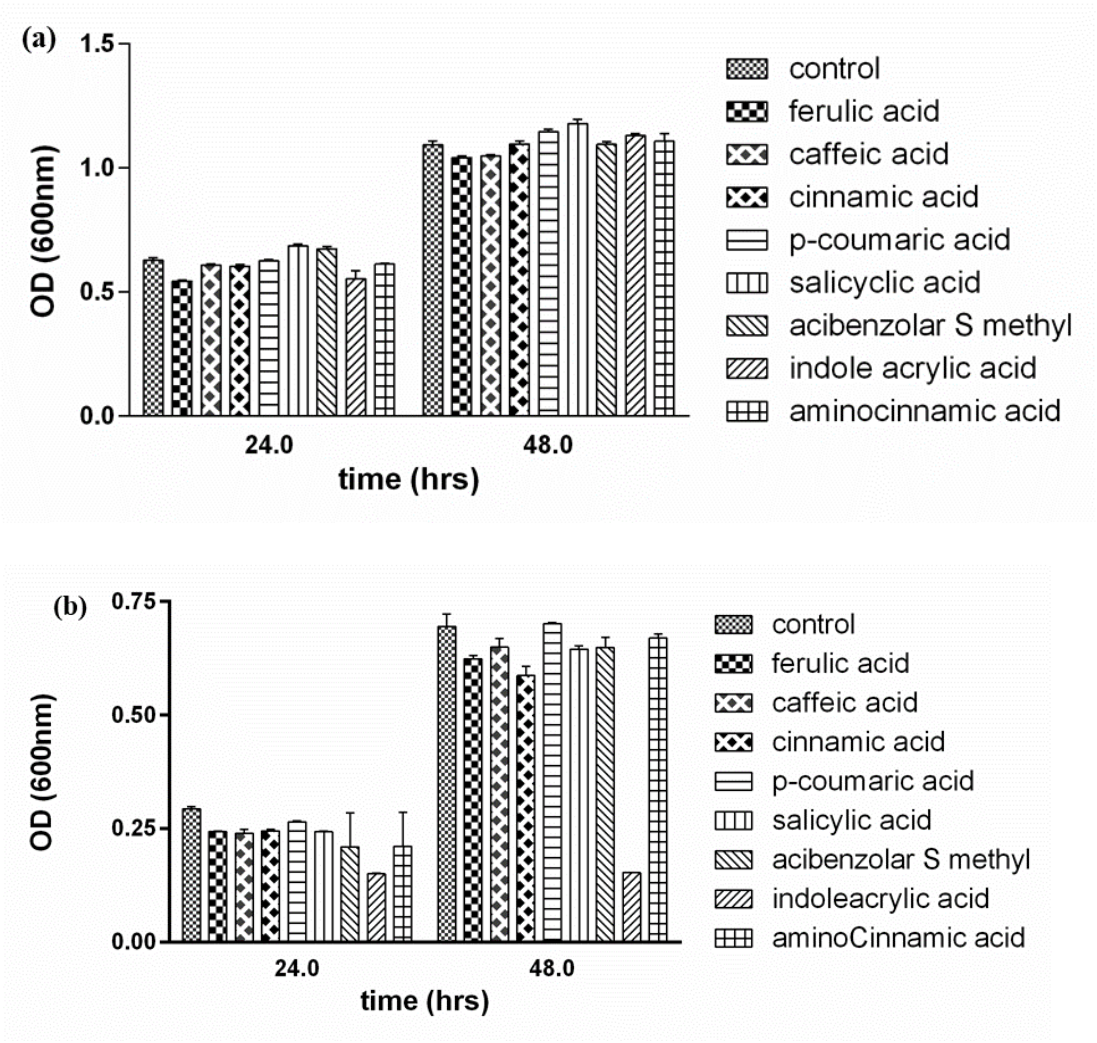

Fig. 4. 2: Effect of subinhibitory concentration of compounds on growth of Xoo BXO43

Growth in (a) PS broth and (b) XOM2. Bars are mean with standard deviation of two independent experiments each with triplicates

4.3.3. Effect of the selected compounds on T3SS of Xoo BXO43

A Transcriptional reporter system developed by Furutani and workers to identify the effectors of TTSS in *Xanthomonas* was adopted to determine the effect of selected compounds on TTSS of Xoo BXO43, where their effect on promoter activity of XopQ, an effector secreted by TTSS was determined. Primers were designed using the promoter region of xopQ gene from the reported genome sequence of XooPXO99A 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 (Fig. 4.3 a).

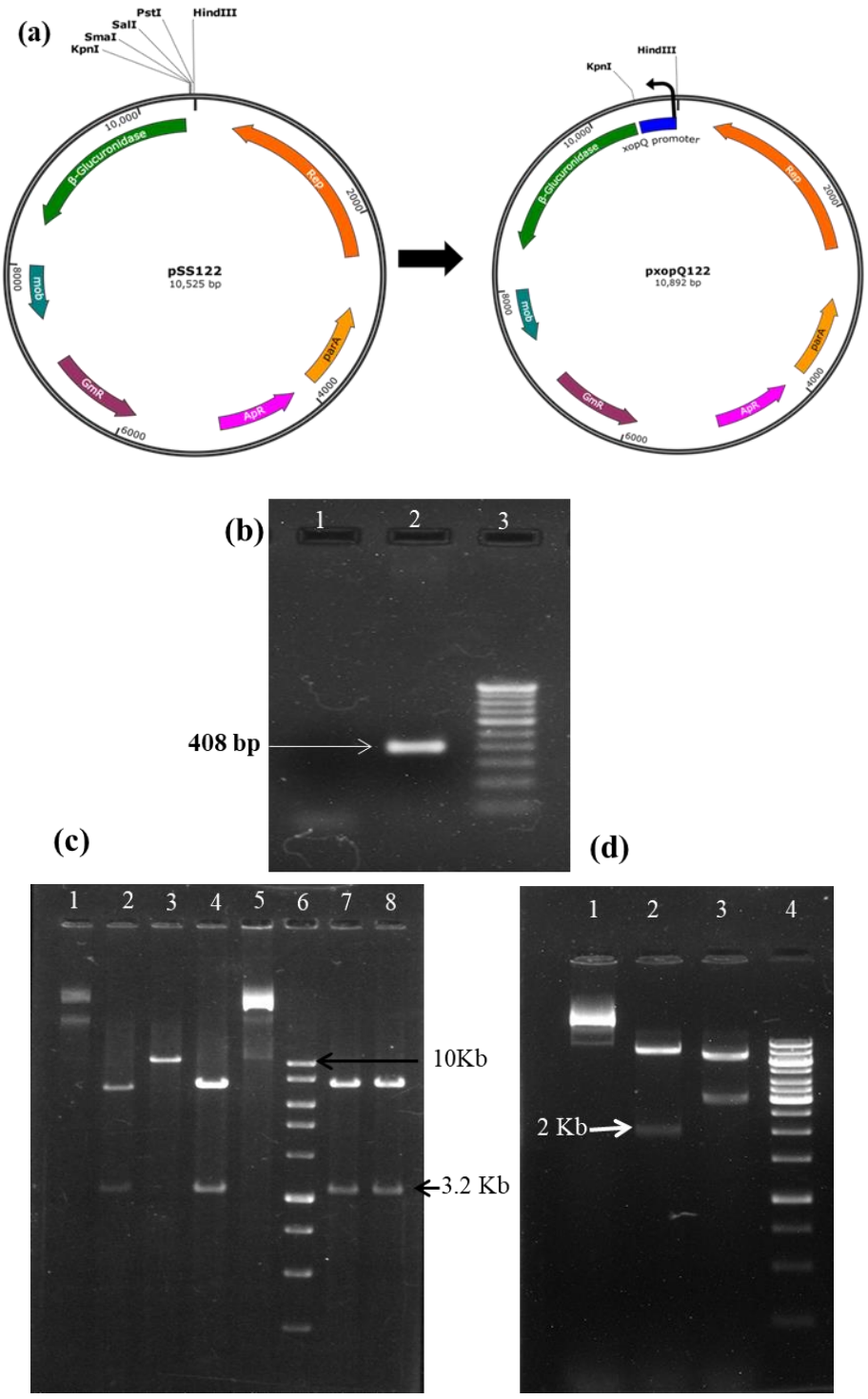

Fig. 4.3: Construction of transcriptional reporter vector

(a) schematic diagram of the broad host range vector pSS122 and the clone of promoter of *xopQ*, (b) PCR amplicon of promoter region of *xopQ* gene (Lane 1- no template control, Lane 2 – amplicon of 408 bp size, Lane 3 – l00 bp StepUp ladder); (c) clone confirmation by RE digestion (Lane 1- undigested empty vector pSS122, Lane 2- Bgl II digest of pSS122, Lane 3 – EcoRI digest of pSS122 (10.5kb), Lane 4 – Bgl II of pxopQ122 clone C2, Lane 5 – Undigested pXop122 clone C2, Lane 6 - 10 Kbp Marker, Lane 7 - Bgl II of pxopQ122 clone C5, Lane 8 – Bgl II of pxopQ122 C10); (d) clone confirmation by RE digestion (Lane 1- undigested pxopQ122 C2, Lane 2 – EcoRl digest of pxopQ122 C2, Lane 3 – Bgl II digest of pxopQ122 C2, Lane 4 – 10 kbp Marker)

The positive clone was confirmed by different restriction enzymes (Fig. 4.3 c & d). 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. 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 caffeic acid and ferulic acid were found to be most effective and reduced the GUS activity by 50 % and more, thereby indicating its effect on expression from the xopQ gene promoter (Table 4.2).

Table 4.2: Effect of compounds at sub-inhibitory concentration on promoter activity in Xoo BXO43XOPQ harbouring reporter plasmid and grown in XOM2 medium

Compounds	Average Gus Activity Units ±SD [a]	% relative activity
Control	22.13 ±0.84	-
DMSO control ferulic acid	22.15 ±0.79 11.20 ±0.54*	50.65
caffeic acid	10.45 ±1.63*	47.25
p-coumaric acid	11.61 ± 0.57*	52.50
amino cinnamic acid	17.58 ± 2.10	79.48
acibenzolar S methyl	13.52 ± 3.19*	61.13
cinnamic acid	8.05 ±0.31*	36.41
salicylic acid	15.82 ±0.39	71.54
BXO43XOPQ in wheat bran medium	9.28 ±0.11	
E.coli pXopQ122 in Wheat bran medium	ND[†]	

[†]ND is no activity was detected when GUS activity was measured from E.coli transformant harbouring pXopQ122 was grown in WB medium similar to Xoo BXO43XOPQ transformant.

[a]GUS activity was measured at 20 hrs of bacterial growth in XOM2 medium supplemented with sub-inhibitory concentration of the indicated compounds. The Values are representative of three experiments and each having triplicates. * represent the statistically significant difference in GUS activity measured of bacteria grown in the presence of indicated compound as compared that with no compound at $p < 0.05$ according to one-way ANOVA Bonferroni's multiple comparison tests.

4.3.4. Influence of the selected compounds on exoenzyme production by Xoo BXO43

Various cell wall degrading enzymes, typically Cellulase, Xylanase and

Pectinase are involved in virulence of Xoo. In case of Xoo BXO43, cellulase and pectinase activities were below the detectable limit by plate assay, when cultures were grown in XOM2 medium; but when wheat bran a crude plant derived raw material was used in the medium, both cellulase and pectinase activities were detected (Fig. 4.4).

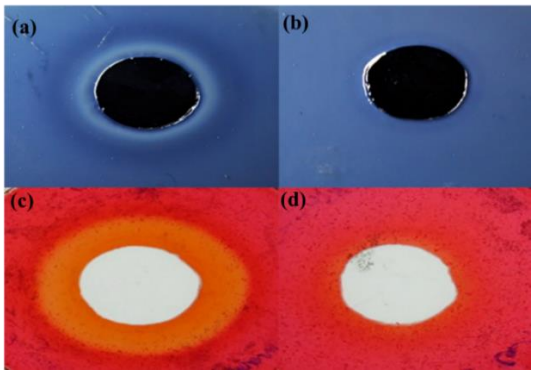

Fig. 4.4: Detection of Cellulase and Pectinase secreted by Xoo BXO43.
Xoo BXO43 culture was grown in WB in the presence of small molecule compounds for 48 hrs.(a) Zone of clearance due to pectinase (b) control for Pectinase (c) zone of clearance due to cellulase (d) control for cellulase, when plates were flooded with CPC and congo red for Pectinase and Cellulase, respectively.

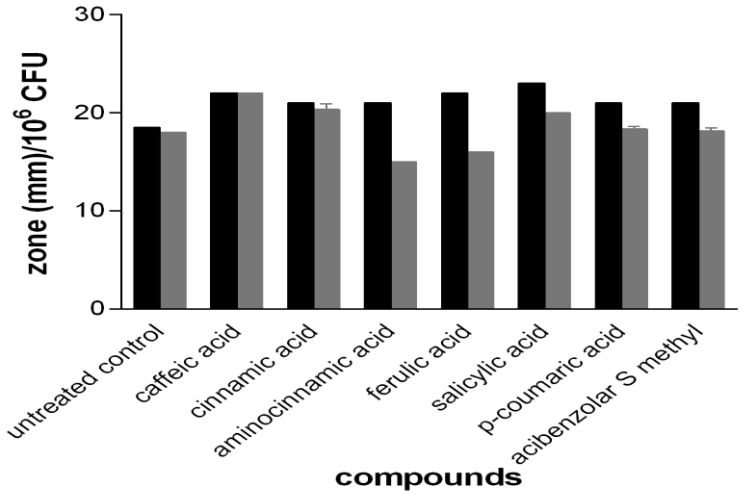

Fig. 4.5: Effect of small molecules on production of exoenzymes
Production of cellulase (black bars) and pectinase (gray bars) by Xoo BXO43 grown in wheat bran medium when amended with the selected compounds. Experiments were done in three trials, each having triplicates. Each bar represents the mean of three experiments and its standard deviation

Wheat bran medium was amended with sub-inhibitory concentration of the selected compounds, there was no substantial difference in the production of these extracellular enzymes as compared to that in unamended medium (Fig. 4.5).

Notably it was found that wheat bran which supported the secretion of enzymes like cellulase and pectinase by Xoo could also be used as TTSS induction medium in lieu of synthetic media as found in reports (Table 4.2).

4.3.5. Influence of the selected compounds on Extracellular Polysaccharide (EPS) production and swimming motility by Xoo BXO43

EPS produced by Xoo BXO43 in PS broth without supplementing any compounds was 500 ±100 µg of glucose/10^9 cells. Acibenzolar S methyl and aminocinnamic acid showed decrease in EPS production while ferulic acid and cinnamic acid induced EPS production. However, the differences were not statistically significant (Fig. 4.6).

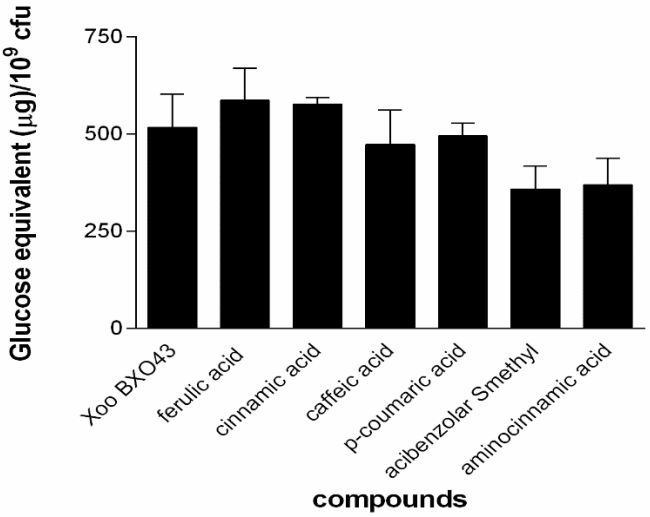

Fig. 4.6: EPS production by Xoo BXO43 in terms of glucose equivalents.
Each data point is average of triplicate and its standard deviation. Three trials were performed giving similar results. Statistical analysis revealed no statistically significant differences ($p \geq 0.05$) according to one-way ANOVA Bonferroni's multiple comparison tests

Further, effect of these compounds was examined on swimming motility of Xoo BXO43. Acibenzolar S methyl reduced swimming motility significantly, while the phenolic compounds did not show any substantial effect on motility. Xoo BXO43 demonstrated swimming motility with a diameter of about 16 mm after 48 hrs at 30 ^oC in 0.3 % PSA agar plate. With acibenzolar S methyl treatment, the measured motility was 10 mm diameter which is 40 % lower as compared to the control. Compounds like ferulic acid and caffeic acid showed reduced motility with

12.7 mm and 12.3 mm diameter respectively, while other chemicals did not show any substantial reduction in diameter (Fig. 4.7 and Table 4.3).

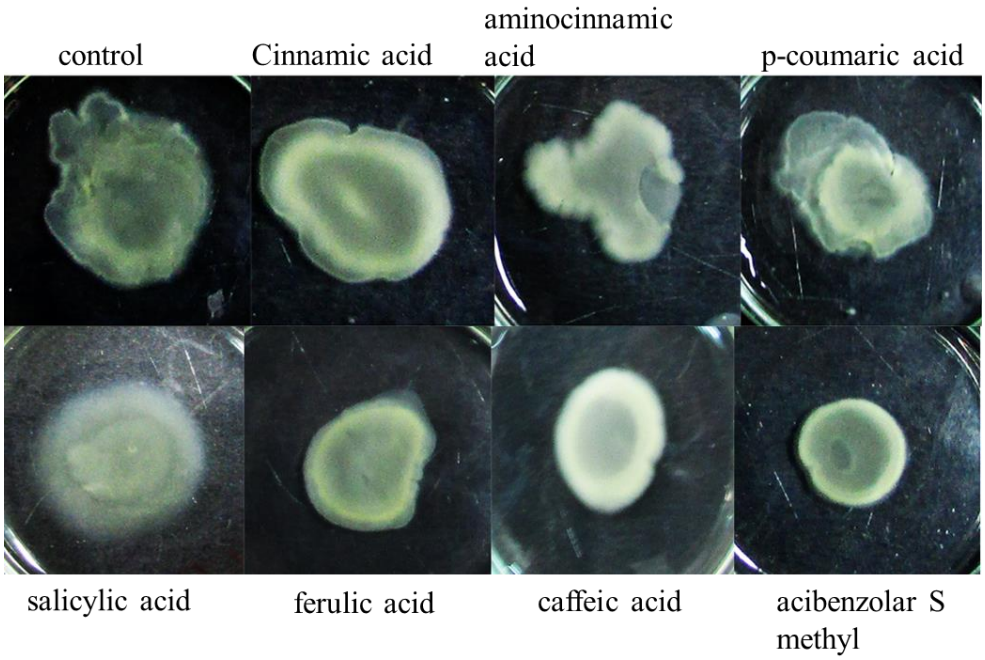

Fig. 4.7: Motility assay of Xoo BXO43
detection of swimming motility on 0.3 % soft agar in the presence of small molecule compounds. PS plates supplemented with indicated compounds after 48 hrs at 30 ^{o}C

Table 4.3: Quantification of swimming motility in terms of diameter covered by Xoo BXO43 in the presence of chemicals.

Chemical	Diameter of motility zone(mm)
Control	16 ±1.0
acibenzolar S methyl	10.3 ±0.6*
aminocinnamic acid	15.7 ±0.6
cinnamic acid	15.0 ±1.0
indoleacrylic acid	16.7 ±0.6
ferulic acid	12.7 ±0.6
caffeic acid	12.3 ±0.6
salicylic acid	15.8 ±0.6

The values are representative of mean and the SD of two independent experiments, each experiment was performed in triplicates. * indicates significant difference as compared to the untreated control at $P < 0.05$ according to one-way ANOVA Bonferroni's multiple comparison tests

4.3.6. Influence of selected compounds on lesion formation by detached leaf assay

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. Cinnamic acid and its derivatives ferulic acid, caffiec acid and p-coumaric acid showed more than 50 % reduction in lesions caused by Xoo BXO43 on rice leaves. While compounds like aminocinnamic acid and acibenzolar S methyl did not show significant reduction in lesions (Fig. 4.8).

(a)

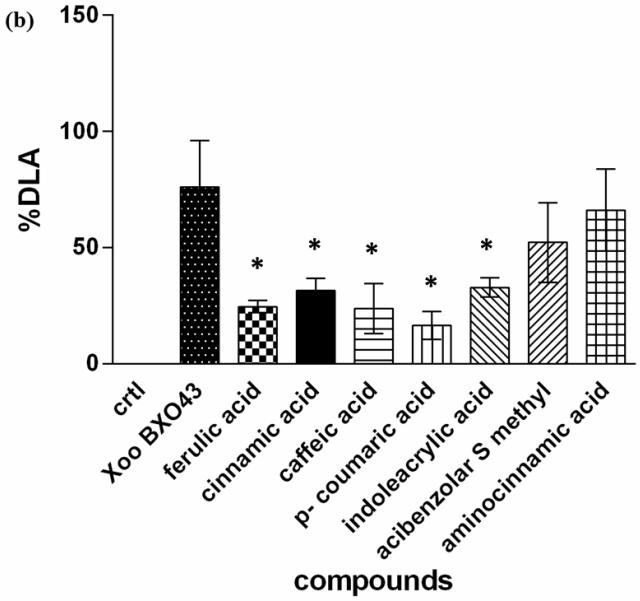

Fig. 4.8: Detached leaf assay to study the effect of selected compounds on pathogenesis by Xoo BXO43.
Representative images for the detached leaf assay. (b) Quantification of the reduction in lesion area by the selected compounds in terms of % Diseased Leaf Area (% DLA). Each bar is mean of 6 leaves (2 × 3 replicates) and its standard deviation.* represents the significant difference as compared to no compound treatment at $p < 0.05$ according to one-way ANOVA Bonferroni's multiple comparison tests

4.4. Discussion

Virulence of Xoo is attributed to many factors like EPS, motility, hydrolytic enzymes like cellulase and pectinase secreted via the T2SS and like many Gram negative pathogen, the T3SS to deliver the effector proteins in the host cell to further the pathogenesis. 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, phyto-chemicals screened for T3SS blockers have shown different effect on different pathogens, at varying concentrations (Khokhani et al., 2013; Yang et al., 2008; Nordfelth et al., 2005). Hence, it is important to optimize the concentration of the chemical compounds to be used for studying their effect on the virulence of the phytopathogen Xoo BXO43. Phenolic compounds reported in the literature were selected to study their effect on virulence determinants of Xoo BXO43; and acibenzolar S methyl, a sparsely studied synthetic derivative of thiadiazole was also included. These compounds have been studied for controlling Xoo pathogenesis but not much has been reported about its effect on virulence factors of Xoo. A transcriptional reporter gene system in viable bacteria is commonly used for studying T3SS (Marshall & Finlay, 2014). It is possible to monitor the T3SS specific transcription with the wild type carrying a construct where the virulence gene promoter is flanked upstream to the reporter gene. To screen the small molecule compounds, a similar system having reporter gene β-Glucuronidase enzyme under the control of promoter of an effector protein XopQ reported to be secreted by T3SS and is hrpX dependent (Furutani et al., 2004) was constructed. Based on the reporter gene product β-Glucuronidase enzyme activity, cinnamic acid and its derivative caffeic acid showed higher inhibition of *xopQ* gene promoter activity among the compounds used in present study. Other cinnamic acid derivatives like ferulic acid and p-coumaric acid and thiazolidone derivative, acibenzolar S methyl also showed significant reduction whereas amino cinnamic acid and salicylic acid did not show significant change on *xopQ* promoter activity as compared to control. Similar studies targeting the TTSS wherein HR response was studied by Fan and co-workers (2017) showed inhibitory effect of such phenolic compounds and their derivatives on another effector of TTSS viz. hpa1 using Xoo PXO99A strain. Their results too showed significant attenuation of HR response without affecting the growth and survival of Xoo. Small molecule

compounds have been reported to affect other virulence factors apart from TTSS system of other pathogens (Felise et al., 2008; Prithviraj et al, 2005). Hence, it was interesting to investigate whether the effect of these compounds is specific to TTSS or it affected other virulence factors of Xoo as well.

Many virulence factors are secreted by TIISS system such as cell wall degrading enzymes (CWDE). In Xoo, cellulase, cellobiosidase, xylanase, pectinase have been reported to be involved in virulence of Xoo (Tayi et al., 2016; Jha et al., 2007). Some of these exoenzymes like Cysteine Protease homolog, harbour imperfect PIP box and possibly could be under the regulation of HrpX (Furutani et al., 2004); hence the effect of these small molecule compounds on exoenzyme activity was studied. Different investigation reports have shown varying results regarding enzyme activities using luxuriant medium like PS broth or minimal media like MM9, XOM2, etc. (Rai et al., 2012; Tsushiya et al., 1982). However, no detectable level of activity using PS and XOM2 media was obtained in the assay used in current studies. Similar results have been reported for Xoo BXO43 by Ray and workers (2000). Interestingly, detectable level of cell wall degrading exoenzyme like cellulase and pectinase activity was observed when media containing wheat bran (WB) as a major component, was used. Since it is a natural plant material containing cellulose and hemicellulose, it is likely that these exoenzymes which are secreted by bacteria to degrade plant cell wall material were produced in higher amount when such source is used in medium. Small molecule compounds used in the present study did not affect the secretion of either of the exoenzyme secreted by Xoo BXO43. WB medium was also tested for induction of TTSS of Xoo BXO43. Interestingly, xopQ promoter activity was detected in this natural medium though lesser than that found in XOM2 medium. WB medium, thus, could be used as TTSS induction medium apart from the synthetic medium XOM2 cited in literature.

Xoo being systemic pathogen enter host through hydathodes. Motility has been reported to be important virulence factor in foliar pathogens (Beattie & Lindow, 1995). The compounds were further studied for their effect on motility of Xoo. Swimming and swarming type of motility has been reported to be carried out by flagella (Henrichsen, 1972). Certain inhibitors of TTSS have been reported to show different effect on motility of *Salmonella enterica* (Felise et al., 2008; Negrea

et al., 2007). Effect on motility of Xoo by small molecules has not yet been studied earlier. Acibenzolar S methyl reduced the motility to 40 %, while ferulic acid and caffeic acid, members of phenolic compounds showed modest reduction in motility whereas other compounds used in this study did not affect the motility. In the course of pathogenesis, bacteria loses motility and synthesises EPS that results in biofilm formation (Xu et al. 2015). Since the compounds like acibenzolar S methyl, ferulic acid and caffeic acid showed reduction in the motility of Xoo BXO43; effect of these chemicals on EPS production, an important virulent determinant of Xoo, was studied. No substantial effect on EPS production was observed. Lastly, in virulence assay by detached leaf method compounds like cinnamic acid and its derivatives ferulic acid, p-coumaric acid and caffeic acid were able to reduce the lesions on rice leaves by more than 50 %, while other compounds like amino cinnamic acid and 20 µM of acibenzolar S methyl did not show any significant reduction in lesions.

In conclusion, phenolic compounds like cinnamic acid and its derivatives p-coumaric acid, ferulic acid and caffeic acid showed inhibitory effect on TTSS an important virulence determinant of Xoo, as measured by reduction in promoter activity of *xopQ* gene. While they did not affect other virulence determinants of this pathogen. This is in agreement with the reports by other investigators and it can be deduced that probably these compounds reduce pathogenesis on rice by specifically attenuating TTSS of Xoo (Fan et al; 2017). While the synthetic compound acibenzolar S methyl showed reduction on multiple virulent determinants of Xoo BXO43 indicating its indirect effect on the TTSS and TIISS. These findings show potential in future development of anti-virulent strategies to control the disease and also help in basic understanding of the pathogen physiology.