Chapter 5:

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

5.1. Introduction

Many antibiotics are used in control of plant diseases like blasts, bacterial blight, soft rot of flowers and seeds (McManus et al., 2002; Goodman, 1959). The resistance mechanism owing to overuse of antibiotics have led to emergence of new variants of pathogens. It is predicted that by 2050 post-antibiotic era serious threats will be caused by the infectious disease, which currently are treatable (Spellberg et al., 2008). Hence, there is constant need for search of new antibiotics and more importantly, new strategies which can combat the infectious diseases caused by pathogens. Amongst such new approaches are controlling the resistance mechanisms by implementing new non-antibiotic compounds. Many such compounds belonging to different classes of natural, synthetic and semi synthetic compounds are being screened exhaustively for their application as an alternative to conventionally used antibiotics or to be used in combination with the conventionally used antibiotics. Bacteria can show resistance by target modification, by inactivating the antibiotic, via efflux pump or plasmidic pump (Wagner & Ulrich-Merzenich, 2009; Sheldon, 2005). Bacterial efflux pumps are one of the potential targets to combat the resistance that appears against the antibiotics employed against various pathogens.

It has been demonstrated in many bacteria that efflux pumps are capable of extruding many different and structurally varying antibiotics; and thus impart resistance against such antibiotics (Bohnert & Kern, 2005; Viveiros et al., 2005; Dridi et al., 2004; Pumbwe et al., 2004). Different classes of antibiotics and their mode of action with few of their representative examples has been tabulated in Table 5.1. Amongst them, many antibiotics have been reported to be the substrate of different efflux pumps (Shriram et al., 2018). Inhibition of such efflux pumps by Efflux Pumps Inhibitors (EPIs) retains the antibiotic in the pathogen cells resultantly decrease the MIC (minimum inhibitory concentration) of antibiotics and thus increase its efficacy (Lomovskaya & Watkins, 2001). Consequently, using such EPIs along with antibiotics delay the emergence of resistant variants and thus increase the antibiotic life. When the antibiotics are used in combination with EPIs showing decreased MIC, the two are said to be in synergy. However, the synergy effect is rather difficult to define. The most demonstrative method called the

‘‘isobole method’’ of Berenbaum (1989) is used experimentally for the calculating the synergy effects.

An isobole is understood to be a line or curve between points of the same effect and it can be created with the help of a checkerboard assay. A checkerboard assay is the most common method practiced using a microtitre plate. In the microtitre plate, two compounds are serially diluted perpendicular to each other. For example, for 2 compounds A and B, a series of dilution of compound A would be plated in all columns while that of compound B in all rows such that each well will have different combination of concentration of both the compounds, as depicted in Fig. 5.1(a). One row or column would contain same concentration of one compound. Synergism can be calculated mathematically by dividing the inhibitory concentration of compound A in presence of compound B by the MIC of compound A from well containing serial dilution of only compound A. The Fractional Inhibitory Concentration (FIC) index is calculated by adding the FICs of both the compounds. An FIC index of ≤ 0.5 indicates synergy, a value between 0.5 < x < 1 indicates no interaction and a value ≥4.0 indicates antagonism (Kalan & Wright, 2011).

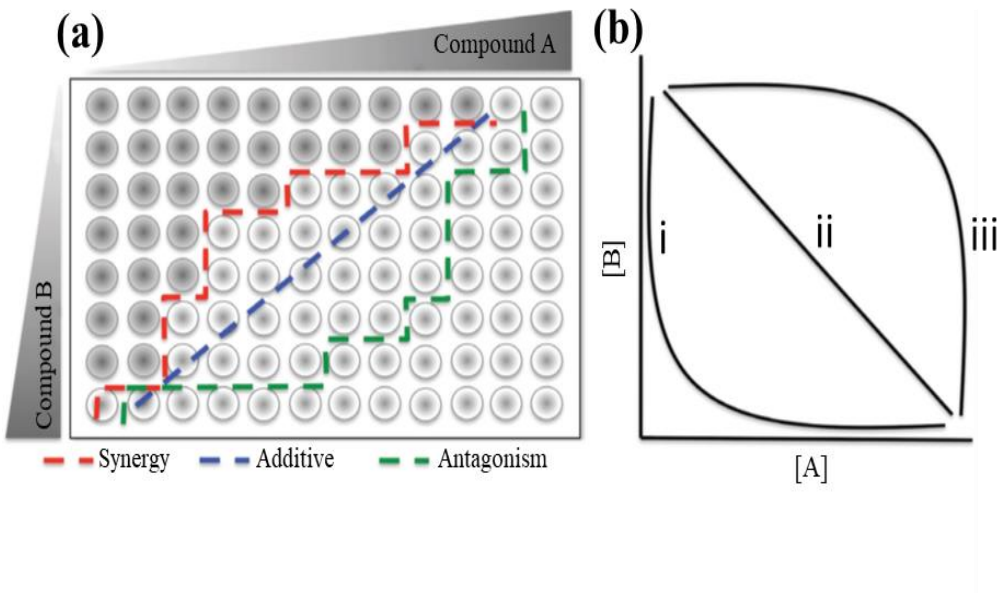

Fig. 5 1: Checkerboard assay for calculation of FIC index of two compounds.
(a) Schematic diagram showing the concentration gradient of the two compounds. Each well has different concentration of each compound giving unique combination; (b) Isobologram created by scoring the growth of the organism showing different associations i) synergism ii) additive iii) antagonism. (Adapted from Kalan & Wright, 2011).

Table 5.1: Antibiotics groups based on their mode of action and chemical nature

Class/group	Mode of action	Representative antibiotic
Penicillin	Inhibits cell wall synthesis	Penicillin G, ampicillin
Polyene (polyketides)	Inhibits 30S subunit	Tetracyclin
Cephalosporins/ β-lactamase inhibitors	Inhibits cell wall synthesis	Cephapririn, cetazolin, cetepime
Fluoroquinolones	DNA synthesis inhibitors	Ciprofloxacin, ofloxacin, norfloxacin
Lincomycins (macrolides)	Protein synthesis inhibitors (50 S subunit)	Erythromycin, clarithromycin, clindamycin
sulfonamides	Folic acid synthesis inhibitors	Sulfisoxazole, sulfamethoxazole
Glycopeptides	Cell wall inhibitors	Vancomycin
Aminoglycosides	Protein synthesis inhibitors (30 S subunit)	gentamycin
Carbapenems	Cell wall synthesis inhibitor	Cilastatin, doripenem
Rifampicin	RNA synthesis inhibitor	rifampicin

Different methods have been used for screening of potential EPIs for Gram negative and Gram positive bacteria. Chequerboard assay, as discussed above, has been most commonly used to identify agents which can exhibit synergy when used in combination with antibiotics and can be potential EPIs (Tegos et al., 2011; Markham et al., 1999). The serial dilution of substrate of the pump as well as the putative pump inhibitor is carried out and dispensed in the micro-titre plate perpendicular to each other. The scoring is done on the basis of the growth of the test organism in the similar way as is done for MIC determination (Odds et al., 2003).

The modulation assay is the modified chequerboard assay and is an easy method to identify the potential EPIs. A preliminary assay to study inhibitory activity using crude extract should be done to avoid false positive results. Usually 4-fold lower MIC value is chosen and is kept constant, while the antibiotic which is the substrate of the efflux pumps is serially diluted in the wells of microtitre plate and results are scored similar to chequerboard assay (Gibbons et al., 2003). Accumulation of substrate is also used for EPI identification. Various substrates like Berberine (Tegos et al., 2002), EtBr (Li et al., 2003), Novobiocin (Baranova et

al., 2002) have been used in screening potential EPI. The accumulation assay can be performed in various ways. The efflux inhibitor is added in midway of a time-course assay. Another way is to run two separate assays in presence and absence of the test compound. An increase in substrate accumulation in the presence of an inhibitor indicates the compound to be an EPI (Smith et al., 2007). EtBr has been reported to be a substrate of efflux pumps of different classes. Hence, the efflux of EtBr can be measured fluorimetrically. The principle of EtBr efflux inhibition assay is based on the fact that EtBr is naturally effluxed out of bacterial cells as they are toxic. The presence of EPIs in the test sample will cause retention of EtBr which can be measured fluorimetrically. Hence, the difference in the fluorescence due to EtBr retention in the presence and absence of test compound will indicate the presence of EPI (Kaatz et al., 2000). Other substrates like Acriflavine or Pyronin Y can also be used for similar efflux assays (Mitchell et al., 1999). Berberine is an antibacterial alkaloid and intercalates in DNA (Jennings & Ridler, 1983). Hence, it is used for screening the potential EPIs. The scoring is done on the basis of growth difference of the test bacteria in the presence and absence of the compound. Growth in organism in the presence of Berberine only while less or no growth in the presence of the extract along with Berberine indicates the presence of an efflux pump inhibitor in the extract (Stermitz et al., 2000; Tegos et al., 2002).

Metabolites produced by plants have immense potential in controlling the diseases caused by microbes. Many class of compounds like quinines, alkaloids, lectins, polypeptides, flavones, flavonoids, flavonols, coumarin, terpenoids, essential oils and tannins are being studied for their potential to be used as alternative agents in controlling various infectious diseases and their mode of action (Chandra et al., 2017). Phenolic compounds are secondary metabolites produced by plants and have been reported as EPI (Efflux pump inhibitor) using many Gram negative and Gram positive pathogens like *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, *Staphylococcus aureus*, *Bacillus cereus* (Maisuria et al., 2015; Lechner et al., 2008, Belofsky et al., 2004).

The studies covered in this chapter are mainly for synergy of small molecule compounds with the antibiotic S2 produced *B. altitudinis* S2 using checkerboard assay. Further, synergy of the selected compound with different commercially available antibiotics was examined. The representative antibiotics of different

classes of antibiotic Streptomycin, Ofloxacin, Tetracyclin and Gentamycin, have been tabulated in Table 5.1. Verification of these compounds as EPIs was carried out by EtBr efflux assay. To identify the kind of efflux pump involved, amongst the many efflux pumps found in Xoo genome, relative expression of representative genes of various efflux pumps was attempted.

5.2. Materials & Methods

5.2.1. MIC of antibiotics

The concentration of small molecules used were subinhibitory to the MIC values: cinnamic acid (400 µM), ferulic acid (400 µM), caffeic acid (600 µM), aminocinnamic acid (200 µM), p-coumaric acid (500 µM), salicylic acid (40 µM), trans acrylic acid (80 µM), acibenzolar S methyl (200 µM) as determined in chapter 4 section 4.3.1. The MIC of the partially purified antibiotic S2 from *B. altitudinis* S2 was as per the value determined in chapter 3 section 3.3.8. MIC of other antibiotics used like Streptomycin, Gentamycin, Ofloxacin, Tetracycline was also determined in the same manner as antibiotic S2 where Xoo BXO43 was grown in PS broth and adjusted to 0.1 OD_{600} equivalent to 10^6 CFU/ml suspension. Dilutions of the antibiotics were prepared with sterile distilled water and then equal amount of PS medium was added in all the wells. The wells were then inoculated with the Xoo BXO43. The well with no bacterial suspension was the negative control and the well without any antibiotic was the positive control for the growth of the bacteria. The microtitre plates were then incubated in shaking condition at 30 ^oC at 120 rpm for 16 hrs. The growth was noted visually and the well showing no visible growth as compared to the positive control was considered as the MIC of that antibiotic for that bacterial strain.

5.2.2. Checkerboard microdilution assay

The checkerboard microdilution assay (Odds, 2003; Rand et al., 1993) was used for studying the synergy between antibiotics and the small molecule compounds *in vitro*. Two-fold serial dilutions of the compound and the antibiotic S2 were prepared using sterile distilled water. The small molecule compounds concentration below its MIC was chosen. The serial dilutions were then added into

96 well plates (Corning, Fisher Scientific, India), one compound in column pattern and the other in row pattern to achieve combinations having different concentrations of each of the compound; and equal amount of PS medium was added in all the wells. Each well was subsequently inoculated with 0.1 OD_{600} (10^6 CFU/ml) of the bacterial cell suspension and incubated in shaking condition at 120 rpm at 30 ^oC for 16 hrs. Fractional Inhibitory Concentration (FIC) Index for the two compounds was calculated using the formula:

$$FIC_{\text{compound 1}} = MIC_{\text{(1 compound in combination)}} / MIC_{\text{(1 compound used alone)}}$$

$$FICI = FIC_{\text{compound 1}} + FIC_{\text{compound 2}}$$

Interpretation of the results:

FICI of ≤ 0.5 indicates synergy between the two compounds under study; $0.5 < FICI \leq 1$ indicates no interaction between the compounds; FICI of >4 indicates the two compounds rather show antagonism.

5.2.3. EtBr efflux assay

To evaluate the effect of the phenolic compounds on the inhibition of the efflux pump mechanism of the bacteria, an EtBr efflux assay was carried out (Kaatz et al., 2000). Prior to this experiment effect of different concentrations (10, 5, 4 and 2 µg/ml) EtBr on cell viability was checked where 4 µg/ml did not compromise the viability of Xoo BXO43 cells. A system of 150 µl containing 0.1 OD_{600nm} cells from mid-log phase Xoo BXO43 culture grown in PSA medium in opaque wall 96 well plate (Corning, Fisher Scientific, India) , 4 µg/ml EtBr and the small molecule compounds, at its subinhibitory Minimum effective concentration (MEC) values as obtained in section 5.3.1 were prepared. Control well contained only Xoo BXO43 and EtBr in same concentration and the plates were incubated for 15 min (30 ^oC, 120 rpm). The fluorescence readings were taken at every 3 min intervals for 30 min at room temperature using Microplate reader (Synergy HT, Biotek) to monitor the EtBr efflux from the cells at excitation λ of 530nm and emission λ of 600nm. The efflux activity was indicated by low fluorescence intensity in the cells. The cells exhibit florescence due to intercalation of EtBr in DNA. and show reduction in fluorescence intensity due to efflux of EtBr over time. The loss of fluorescence is reduced if the small molecules act as EPI reflecting an interference in EtBr efflux.

The background fluorescence of the medium was subtracted from all measurements using the reading of the negative control containing only EtBr. The assay was performed three times independently.

5.2.4. Design of primers for efflux pump genes from Xoo database using bioinformatics and validation

Representative efflux pump genes from different families of the efflux pumps in bacteria were selected. Accordingly following genes *norM* (MATE), *pmrA* (MAR), *emrA* (SMR), *acrD* and *mexC* (RND) and *tetP* were selected from the published genome sequences of Xoo KACC 10331 and Xoo PXO99A and database DOOR (Database of Prokaryotic Operons).Primers for the genes were designed and verification was carried out by PCR. Oligonucleotide primers designed using Primer design of NCBI are listed in Table 5.2.

Table 5.2: Primers designed for different efflux pump genes for qRT-PCR

Efflux pump gene	Referred locus from Xoo PXO99A genome	Primer sequence (5' – 3')	Amplicon size (bps)
norM	PXO_04042	CGCTGATGTTCGTGTTCCTG TGAGGTAGCGCATGCAAAAG	150
pmrA	PXO_01157	AGGTAGGTGCTCAACGATGC AGGTGGACTTCAGCCATGTG	187
emrD	PXO_04271	TGCTCTATGCATGGCGACTG GATGCGGTCGTCGATTTTGG	182
acrD	PXO_03981	TGTCGTCGATGGTCTGCATC ATTCGATCTCGGACGTGGAC	189
mexC	PXO_04839	TGCGGTATTGGTGGATGTGG AAGGTGGCATGAGAACTGGC	198
tetP	PXO_03308	TGCTGGGATACACGTAGTGC CGGCTTGGCCTTGTTGAATG	200
gyrB	PXO_03480	AACGGCACTTACGACTCCAG CCATCGACCAGGATTTTCGC	197

The gene verification of the designed primers was done using genomic DNA of Xoo BXO43 by PCR to get the expected size amplicons. DNA isolation of the isolates was carried out according to the protocol of Sambrook & Russel (2001). PCR was carried out in thermo cycler (AB system) using 10 μL PCR mixture

containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 200 µM each of dNTP, 100 ng primer, 2.5 U of Taq polymerase (Invitrogen) and 10 ng of genomic DNA template. The PCR cycle conditions were: Initial denaturation at 95 ^oC for 5 min, 35 cycles of 15 secs at 95 ^oC, annealing for 15 secs at 58 ^oC ,extension for 60 secs at 72 ^oC and final extension for 7 min at 72 ^oC.

5.2.5. RNA extraction, cDNA synthesis and comparative qRT-PCR

Xoo BXO43 was grown in PS broth to give mid-log phase growth of OD_{600} 0.5 -0.7 at 30 ^oC for 20 rpm. Different experiments were set up where 0.2 OD_{600} of the mid-log phase was used in 2ml system containing antibiotic and phenolic compound (caffeic acid was selected) combined as well as individually. Total RNA was extracted using TriZol kit method (TaqaRa, India) from each experiment. RNA concentration was quantified by measuring the absorbance of the sample at 260 and 280 nm, and 300 ng of RNA was used for cDNA synthesis using the high capacity cDNA reverse transcription kit (Virso kit). Expression of target genes was quantified using quantitative real time PCR (qRT-PCR) using the cDNA as template obtained from the RNA. qRT-PCR was performed using thermal cycler (one plus, Applied Biosystems) using SYBR GREEN PCR master mix (Applied Biosystems, India). Conditions for qRT-PCR were as follows: initial denaturation at 95 ^oC for 5 min, and 45 cycles of 15 secs at 95 ^oC and annealing for 15 secs at 58 ^oC and extension for 30 secs at 72 ^oC. Results were analysed with SDS software, version 2.2 (Applied Biosystems). Melting curve analysis was carried out to verify absence of any contamination. Data was normalized to the endogenous reference gene *gyr*B. The threshold cycle method ($2^{-\Delta\Delta T}$) was used to analyse changes in gene expression in the experimental sample relative to the control (cells grown under the same conditions without antibiotic and phenolic compound treatment) (Livak & Schmittgen, 2001). For each sample of cells, qRT PCR was performed in triplicate and the entire experiment was repeated twice with RNA samples extracted from all experiments setup.

5.3. Results

5.3.1. Minimum Inhibitory concentrations and modulating activities of small molecule compounds

MIC value of small molecule compounds were determined in chapter 4 (section 4.3.1). MEC of the compounds hence will be the sub-inhibitory concentrations at which these molecules do no exhibit any growth inhibitory effect on the test organisms. Checkerboard assay was used to determine the MEC, synergy between the small molecules and antibiotics and consequently FIC (Fig.5.1). Only caffeic acid gave the FIC value below 0.5 from all the tested compounds. At MEC of 13.5 mg/l it showed 4 fold decrease in MIC of antibiotic S2 against Xoo BXO43 where the FIC index was calculated to be 0.325 (Table 5.3). Other compounds cinnamic acid, acibenzolar S methyl and indole acrylic showed 2 -4 fold decrease in MIC of antibiotic S2; however, their FIC index was found to be in the range of 0.75 to 1.75. While amino cinnamic acid, ferulic acid, p-coumaric acid and salicylic acid did not have any effect on the MIC of antibiotic S2.

Table. 5.3: Effect of small molecule compounds on the MIC of antibiotic S2 Without against Xoo BXO43

Compound	MIC (mg/l)	MEC (mg/l)	MIC of antibiotic S2 (mg/l)		Fold reduction in MIC of antibiotic	FIC index
			With compound	Without compound		
cinnamic acid	60	30	8	4	2	1.0
caffeic acid	108	13.5	8	2	4	0.375
acibenzolar S methyl	42	21	8	2	4	0.75
salicylic acid	40	30	8	8	1	1.75
ferulic acid	76	45	8	8	1	1.59
amino cinnamic acid	40	30	8	8	1	1.75
p-coumaric acid	82	41	8	8	1	1.5
indole acrylic acid	16	8	8	4	2	1.0

MIC of antibiotic S2 for Xoo BXO43 was 8 µg/ml.

5.3.2. Effect of Caffeic acid on MIC of other antibiotics

Other commercially available antibiotics of different representative class were tested for reduction in MIC by using caffeic acid at its MEC (concentration that reduced the MIC of antibiotic S2 to 4 fold). As shown in Table 5.4, MIC of antibiotics Streptomycin and Ofloxacin were reduced by 4 fold, whereas that of Tetracyclin by 2 fold. Synergy of caffeic acid with Streptomycin and Ofloxacin was inferred as per the FIC index calculated as given in section 5.2.2. In case of Tetracyclin MIC of Tetracyclin was reduced by 2 fold but the FIC value was slightly above 0.5 indicating weak synergy. Gentamycin exhibited no synergy as the FIC value observed was 1.125 (Table 5.4).

Table 5.4: Effect of caffeic acid on MIC of other antibiotics against Xoo BXO43

Antibiotic	MEC of caffeic acid (mg/l)	MIC of antibiotic (mg/l)		Fold reduction in MIC of antibiotic	FIC index
		Without caffeic acid	With caffeic acid		
Streptomycin	13.5	125	31.3	4	0.375
Ofloxacin		667	166	4	0.375
Tetracyclin		133	53.5	2.5	0.527
Gentamycin		53.3	53.5	1	1.125

5.3.3. Evaluation of inhibition in EtBr efflux by small molecule compounds

The ability of Xoo BXO43 to accumulate EtBr was assessed by plating it on PSA plates having different concentration of EtBr where the culture exhibited florescence on exposure to ultraviolet light (Fig.5.2).

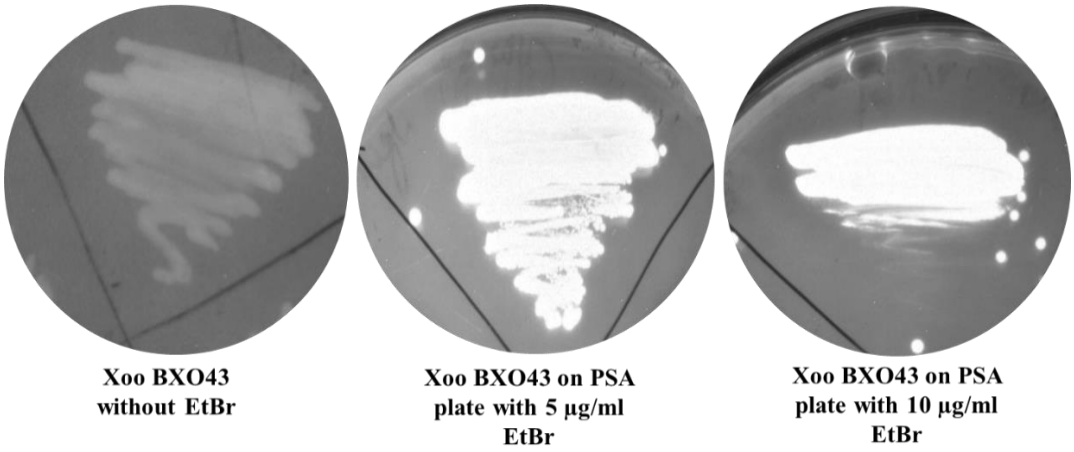

Fig. 5 2: Fluorescence of Xoo BXO43 on PSA plates containing without and with 5 and 10 µg/ml EtBr

The Xoo BXO43 showed fluorescence at both the concentration of 5 µg/ml and 10 µg/ml. Then the small molecule compounds were assessed for their potential as EPIs using EtBr efflux assay. Compounds like caffeic acid and acibenzolar S methyl displayed ability to prevent efflux of EtBr indicated by decreased loss of fluorescence intensity over time as measured in Xoo BXO43 cells as compared to the control wells lacking any treatment. Other compounds like indole acrylic acid, p-coumaric acid and cinnamic acid displayed similar loss of fluorescence intensity as that of the control over time. Ferulic acid treatment showed higher loss in fluorescence intensity as compared to untreated control (Fig. 5.3).

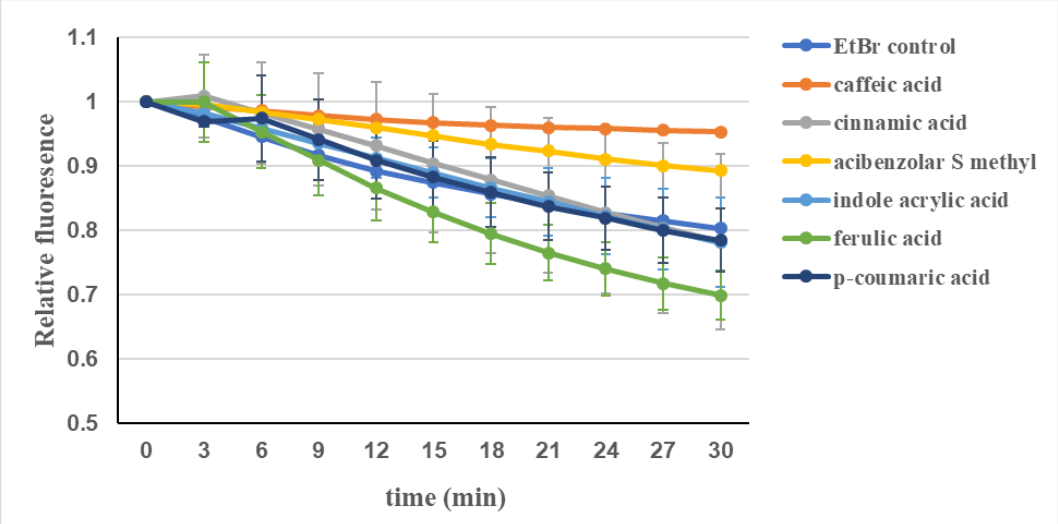

Fig. 5 3: Effect of small molecule compounds on the ethidium bromide efflux from Xoo BXO43 cells

5.3.4. Effect of caffeic acid on expression of efflux pump genes of XooBXO43

To assess the involvement of efflux pump in the synergism between caffeic acid and antibiotic S2, the transcriptional analysis of various genes representative of the efflux pumps present in Xoo BXO43 were evaluated. qRT-PCR was carried out for analysing of differential expression of genes *norM, emrD, tetP, pmrA, acrD* and *mexC* as described in section 5.2.5. The fold change in case of norM gene was found to increase by 1.1 and 1.5 folds when only antibiotic S2 or caffeic acid treatment was given, while 1.8 fold increase was observed with antibiotic S2 combined with caffeic acid. Other genes like *emrD, tetP, mexC* and *acrD* were found to be downregulated in most of the cases. 0.4 and 0.8 fold decrease was found in case of *emrD* with treatment of antibiotic S2 and caffeic acid, respectively and 0.8 fold decrease when treatment of antibiotic S2 along with caffeic acid was given. 0.67, 0.43 and 0.42 fold decrease was observed in case of gene *tetP* in case of treatment with antibiotic S2, caffeic acid and the combination, respectively. Similar results with *acrD* and *mexC* were found with fold change in the range of 0.67 -0.83 and 0.81 -0.98, respectively. Apart from *norM*, only *pmrA* gene showed slight increase in expression of about 1.14 fold in case of treatment with caffeic acid. No statistically significant fold change was observed in case of all the genes studied (Fig. 5.4)

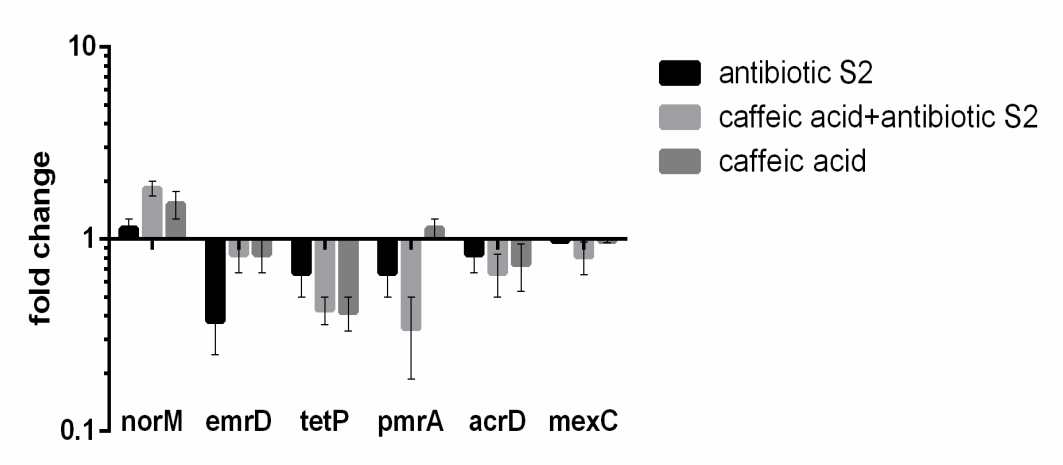

Fig. 5 4: Effect of caffeic acid on expression of different efflux pump genes of Xoo BXO43.

All genes were normalized using housekeeping gene *gyrB* relative to the expression of the respective normalized genes in the untreated control. The experiment was conducted twice in triplicates. The statistical significance was tested by one-way ANOVA Bonferroni's multiple comparison tests

5.4. Discussion

Gram negative bacteria possess thick outer hydrophobic membrane which act like barrier and restricts entry to many compounds especially those hydrophilic in nature, e.g., macrolides like Erythromycin (Nikaido, 1994; Poole, 2002). Various mechanisms like target modification, antibiotic inactivation, efflux through efflux pump mechanisms have been demonstrated for resistance. A single pump can bring about resistance to wide array of diverse compounds. Blocking agents for such efflux pumps can resolve the resistance mechanism to certain extend. Many fflux pump inhibitors have been demonstrated to act as blocking agents to the efflux pumps and resultantly bring about decrease in resistance to many antibiotics (Rao et al., 2018; Stavri et al., 2006).

Many phytopathogens use efflux pumps for entry in plants or for secreting out their virulence factors in plants. In *Erwinia chrysanthemi,* it was demonstrated that pathogenesis was affected in different hosts mutations in two RND-type and three MFS type efflux systems (Valecillos et al., 2006). Xanthomonadin pigment is transported by a putative RND-type transporter in *Xanthomonas oryzae* (Goel et al., 2002). IfeAB, RND-type transporter, aids in infection of *Agrobacterium tumefaciens* by rendering resistance to isoflavanoids secreted by plant (Palumbo et al., 1998). AlbF, is an MFS transporter, involved in self-protection in an Albicidin producing *Xanthomonas albilineans* strain (Bostock et al., 2006). MexAB-OprM efflux system in *P. aeruginosa* was demonstrated to export virulence factors and invade the plant tissue (Hirakata et al., 2002).

Naturally occurring as well as synthetic compounds have been found to act as EPIs (Rao et al., 2018; Kourtesi et al., 2013; Stavri et al., 2007). As defence response, plants produce a diverse array of compounds including phenolic compounds that contain polyphenol categories such as flavonoids, tannins, lignins, phenolic acids, quinones, flavones, flavonols and coumarins produced by plants which protect plant by various mechanisms. Many compounds elicit the host plant defence system which cause resistance to many pathogens and protects the plant (Wink, 2003; Hammerschmidt, 1999). Some chemicals also act on pathogens by causing its attenuation or inhibition and thus protect the plant. Many small molecule compounds of plant origin have been demonstrated to act in synergy and increase the efficacy of many inhibitory compounds majorly antibiotics against pathogens.

For example, Piperine present in black pepper (*Piper nigrum*) and long pepper (*Piper longum*) was demonstrated as potent EPI by showing its synergy with Ciprofloxacin against *S. aureus* by *in vitro* studies (Khan et al., 2006). Similarly, many polyphenolics and essential oils have been reported to reverse the bacterial resistance to antibiotics (Hemaiswarya et al., 2008). A series of plant phenolic compounds have been shown to act as EPI by EtBr efflux assay (Lechner et al., 2007).

We tested certain small molecule compounds which included plant derived phenolic acids like caffeic acid, cinnamic acid, ferulic acid, salicylic acid, aminocinnamic acid, p-coumaric acid and certain synthetic compounds like acibenzolar S methyl and indole acrylic acid for their potential as EPI. The MEC of these compounds was determined from their sub-inhibitory concentrations, since it is proposed that ideally EPIs should not exhibit any growth inhibitory activity (Wagner & Ulrich-Merzenich, 2009; Bhardwaj et al., 2012). Amongst these tested compounds, caffeic acid was found to decrease the MIC of the antibiotic S2 giving FIC index below 0.5 indicating synergy between the antibiotic and the small molecule compound i.e. caffeic acid at its MEC. Further, caffeic acid was tested with other commercial antibiotics. Reduction in MIC and FIC index values with Streptomycin and Ofloxacin confirmed the synergism of this potent phytochemical phenolic acid.

Further, confirmation of these compounds as potent EPIs was carried out using EtBr efflux assay, wherein efflux of EtBr was measured. Ethidium bromide (EtBr) is a substrate for many efflux systems in organisms including *S. aureus* and mycobacteria (Khan et al., 2006; Stavri et al., 2007; Kumar et al., 2008). It can be detected using fluorimetric techniques as it binds to DNA and give fluorescence. From EtBr efflux assay, caffeic acid and acibenzolar S methyl were found potent in decreasing efflux of EtBr. From checkerboard assay and EtBr efflux assay, caffeic acid was found to increase the efficacy of antibiotic S2 and probably by affecting the efflux pump of Xoo BXO43.

Recently, caffeic acid have been reported to improve efficacy of various pathogens. In *S. aureus* it improved efficacy of different antibiotics like Erythromycin, Clindamycin, and Cefoxitin (Kepa et al., 2018). In *K. pneumoniae* KpE-8, synergy of caffeic acid and Ciprofloxacin was demonstrated (Dey et al.,

2016). In *S. aureus* 1199B, caffeic acid along with other phenolic acids were shown to act as potent agents that can cause reversal of resistance, by acting on efflux pump (dos Santos et al. 2018). Lima et al. (2016), showed synergy of caffeic acid in association with antibiotics against *P. aeruginosa, E. coli* and *S. aureus*.

Further, to investigate whether caffeic acid is acting as EPI, if at all, in increasing the efficacy of antibiotic S2; genes for efflux pumps were searched in Xoo BXO43 genome database. Gene clusters of many of the class of efflux pumps were found in Xoo databases. The qRT-PCR primers were designed and their gene validation was confirmed by PCR as all the primers gave amplification of expected size. Further, qRT-PCR was carried out to examine the effect of caffeic acid on transcription of these genes. Amongst the genes tested, expression of *norM* gene was observed to increase nearly two folds (1.8 fold) when the cells were exposed to caffeic acid and antibiotic S2 in combination, implying some kind of interaction between them. In phytopathogens, NorM have been speculated to be involved in protecting pathogen against and in *E. amylovora* it was demonstrated to render resistance against the antibiotic produced by an epiphyte *P. agglomerans* (Burse et al., 2004). Also, many recent reports have shown caffeic acid and other phenolic acid compounds as EPI of NorM efflux pump, for instance ferulic acid (Sundaramoorthy et al., 2018), caffeic acid and gallic acid (dos Santos et al. 2018). These reports support our hypothesis of caffeic acid as EPI of NorM, although further studies are needed to understand its mechanism. Interestingly, many strategies have been proposed for small molecule compounds to act as EPI The reviews by Rao et al., (2018) and Shriram et al., (2018) have very comprehensively compiled various mechanisms by which different EPIs can act on the efflux pumps. These mechanisms could be alteration at regulation level by acting at transcription of the genes involved, diminishing the motive forces by disturbing the proton gradient required for efflux by the pumps, by interfering the assembly of proteins involved in efflux pump structure, trapping of the EPIs and inactivating the pumps by competitive or non-competitive binding.

In conclusion, caffeic acid showed best results in synergy with antibiotic S2. It showed 2-4 fold reduction in MICs of other antibiotics demonstrating synergistic association when used with antibiotics. Further, EtBr efflux assay confirmed caffeic acid as a potent EPI. Increase in expression of only *norM* gene

and not of any other efflux pump genes found in genome of Xoo BXO43, when subjected to treatment of antibiotic S2, caffeic acid and both combined. This indicated strong probability of NorM efflux pump as a possible target of caffeic acid. Further studies like docking of the compound with this efflux pump can be useful to understand the details of the mechanism by which possibly it could be a target of caffeic acid.