In Vitro Analysis of Antimicrobial Susceptibility of *Xanthomonas axonopodis pv.punicae* and Comparative Study of Protein Profiling under Biotic Stress

Abstract

Bacterial blight of pomegranate caused by *X. axonopodis pv. Punicae (XAP)* assumed epidemic form and resulted in economic burden on farmers. In the current study the pathogen infected samples were collected and the isolated *XAP* was identity and confirmed through the morphological, biochemical characterization and Pathogenicity test. Bacterium was reisolated from infected plant to prove Koch's postulates. Efficacy of different chemicals and oils were tested by disc diffusion assay and turbidometrically. Bronopol 3000 ppm (25.6±1.6 mm) and Clove oil (18.0±0.7 mm) formed highest zone of inhibition Turbidometri showed the highest O.D. (0.908 nm) by Copper oxy chloride and Neem oil showed maximum inhibition of growth with O.D. (0.842 nm). Biotic stress (pathogen) induced protein response was studies by using SDS-PAGE method after protein extraction from *XAP*, healthy *P. granatum L.* and infected *P. granatum L.* The protein band pattern showed the unique band no. 2 (Mol.Wt.66000 Da) in infected *P. granatum L.* as compared to the banding pattern of *XAP* and healthy *P. granatum L.* The over expressed protein due to biotic stress could be useful as a marker for detection of the disease at the early stage and for control of the diseases after knowing the biochemical significance of the protein.

Keywords: Pomegranate, bacterial blight, *Xanthomonas axonopodis* pv. *punicae*, *In vitro* efficacy, Antimicrobial activity, SDS-PAGE.

Introduction

Pomegranate *(Punica granatum L.)* belongs to the family Lythraceae, is an ancient, medical fruit and cash crop of Maharashtra. I t is also grown in commercially in other state of india viz. Karnataka, Andhra Pradesh, Rajasthan, Gujarat, and Tamil Nadu (Manjula et al.,2009). India is one of the major producers of pomegranate in the world with average total production of 8 lakh tons per annum (Vauterin *et al.*, 1995).

Many diseases occurred on the pomegranate plant out of them bacterial disease caused by the *Xanthomonas axonopodis pv. punicae* (Xap) strongly damaged the pomegranate production, including large economic losses to the Indian growers (Mondal et al. 2009). Bacterial disease cause substantial loss to the productivity of major crop plants. Unlike fungal disease, bacterial disease not control effectively through chemical methods.

The symptoms of the bacterial blight were observed on the leaves stems and fruits as small (2 to 5 mm) in size, irregular, prominent water soaked spots, which later become black often leads to the breaking of branches and cracking of fruits, reducing to the market value of the fruit (Rangaswamy, 1962; Manjula and khan 2002).

It is gram negative, aerobic rods belonging to the family *Pseudomonodaceae.* The pathogen causes disease actively at high temperature (up to 32°C) and high rainfall and humidity and p^{H} at7.0. The pathogen causes destruction of seedlings and adult plants and thus results in yield reduction. The aim of the study was to screen *in vitro* a range of antimicrobial agents and their efficacy against Xap and protein profiling; in laboratory conditions.

Material and methods

Collection and isolation of disease sample:

The samples were collected from two different districts of Maharashtra viz. Ahmednagar and pune as per typical symptoms showed by plant externally. The fresh infected leaves samples were used for isolation employing tissue isolation method by using Nutrient Agar medium (Antre et.al. (2015)).

Purification and Maintenance of bacterial culture:

The respective bacterial cultures were maintained on NA medium at room temperature by adopting subsequent subculturing at periodical, regular intervals. Three days old cultures were used for further studies (Kanwar et.al. (1976)).

Identification of the pathogen:

The identification of the pathogen involved in causing of oily spot in pomegranate was determined by conducting studies on its morphological (Ryan *et.al.* (2011); Kishun R *et al.* (1993)) and biochemical (Duche *et.al.*(2015); Giri *et.al.*(2011)) features of the pathogen as per standard microbiological procedures.

Pathogenicity test:

Veinlets of some leaves were injected with 48 h old bacterial suspension with the help of sterile Dispo Van syringe. Some plant leaves were inoculated by injuring the leaves with sharp needle and by cotton swabbing the bacterial suspension on injured leaves. The plant was kept for incubation. After 10 – 12 days observations made for typical symptoms of bacterial blight on leaves, the organism was reisolated from artificially inoculated leaves of Pomegranate plants showing typical symptoms of disease. The re-isolation was carried out on NAS medium and the growth of organism was observed for next 2-3 days (Popovic *et al.* (2013); Abdo-Hasan *et al*. (2008)).

In vitro efficacy of different chemicals and plant derived oils against Xap by using paper disc and turbidiometric method:

In vitro efficacy of different chemicals and oils was checked against *Xanthomonas axonopodis pv. Punicae* by using paper disc and turbidiometric method (Copper oxy chloride: 2500ppm, 1000ppm, Bronopol: 3000ppm, 1000ppm, Bordeaux mixture: 3000ppm, 1000ppm, Bactomycin: 1200ppm, 1000ppm, Clove oil: 100%, Nilgiri oil: 100%, Neem oil: 100%). (Kizil et al.(2005), Pawar et al. (2014)).

Isolation of protein from healthy and infected pomegranate leaves:

Kumar et al. (2015) standardized the protein isolation protocol from plant leaves.

Isolation of protein from *Xanthomonas axonopodis pv. punicae*:

Bioera's teaching kit (2017) described the protein extraction from bacteria and clear result was observed.

1 Day I:

i) The LB plates were inoculated with loopful stock culture of *Xanthomonas axonopodis*

ii) The paltes were incubated overnight at 37°C.

1 Day II:

iii) A single colony was picked from the LB plate and inoculated into 5ml LB Broth.

iv) The LB broth was incubated at 37°C on a shaker overnight.

2 Day III:

v) The overnight incubated1ml ofculture was inoculatedinto 50ml of LB Broth (In a 500ml conical flask).

vi) The culture flask was incubated at 37°C on the shaker, until cells reach mid log phase of growth that is A600 is 0.5 (approximately 4-5 hours).

vii) 5ml of the culture from step 6 was transferred into test tube.

viii) The 3ml (1.5ml at a time) of culture was centrifuged in 1.5ml vials at 8000 rpm for 10 minutes. The supernatant was discarded at each time.

i) The cell pellets was resuspend in 100µl of cell lysis buffer.

ii) 25µl of protein loading dye was added to suspension and mixed gently.

iii) The samples was boiled in water bath for 20 minutes.

iv) The samples was centrifuged at 6000 to 8000 rpm for 10 minutes.

v) The 50µl protein marker and 35µl of sample supernatant was loaded on to SDS-PAGE.

vi) The SDS-PAGE was runat 50 volts until bromophenol blue reaches near the bottom of separation gel.

vii) Stained the gel with protein staining solution.

Results and discussion:

Collection and isolation of diseased sample:

Bacterial blight infected samples were collected from Ahmednagar and Pune districts of Maharashtra, associated bacterium *Xanthomonas axonopodis pv.punicae* was isolated. Purified culture was maintained on NSA slants and coded as Xap I, Xap II respectively. (Bradbury et al. (1970)).

Identification of the pathogen:

Morphological and biochemical characteristic viz., shape, colony colour, Gram reaction, starch hydrolysis, gelatein liquefaction, indole production, acid and gas production, KOH, catalase test were performed and confirmed to identity the bacterium as *Xanthomonas axonopodis* pv. *punicae* and following table 1 showed the morphological and biochemical test. (Gamangatti et al. (2013),Bhardwaj et al. (2014)).

Pathogenicity test:

Pathogenicity test of isolates was confirmed by injecting bacterial solution to disease free pomegranate plant. Occurrence symptoms of bacterial blight symptoms on plant leaf confirmed pathogenicity test of isolates (Fig. 1). Bacterium was reisolated from infected plant to prove Koch's postulates. (Yenjerappa et al. (2009)).

In vitro efficacy of different chemicals and plant derived oils against Xap by using paper disc method:

In order to assess the efficacy of chemicals and oils against two isolates of *Xanthomonas axonopodis pv. Punicae* an experiment was conducted and the evaluation was made by paper disc method. The data presented in (Table 2) revealed the significant differences among the different treatment. In the chemical treatment

Bronopol were significantly superior in inhibiting the growth of bacteria (18.6±0.7 mm, 25.6±1.6 mm) against *Xap.* Among the oils Clove oil showed maximum zone of inhibition 18.3±0.7 mm (Fig. 2). (Meerts and Fadavi et al. (2009)).

***In vitro* efficacy of different chemicals and plant derived oils against Xap by using turbidiometric method:**

Efficacy different chemicals and oils was tested turbidometrically. After 24 hours of incubation list O.D. 0.713 nm was exhibited by Bronopol(3000 ppm), followed by Copper oxy chloride (3000 ppm) with O.D. 0.908 nm and Bordeaux (3000 ppm) 0.834 nm respectively whereas Bronopol (1000 ppm) with O.D 0.219 nm showed minimum inhibition of growth and Among the oils tested Neem oil showed maximum inhibition of growth with O.D. 0.842 nm, followed by Clove oil with O.D. 0.810 nm, Nilgiri oil showed minimum inhibition of growth among oils showed in following table 3.

Protein isolation from Healthy pomegranate leaves, infected pomegranate leaves and *Xap I, Xap* II Bacteria:

Data analysis of Healthy pomegranate leaves, infected pomegranate leaves and Bacteria:

Following data analysis table shows the actual position of bands on the gel as compared to the standard marker on the basis of molecular weights provided by BioEra's Teaching Kit.

. The protein banding pattern showed the unique band no. 2 (Mol.Wt.66000 Da) in infected *P. granatum L.* as compared to the banding pattern of *X. axonopodis pv.punicae and healthy P. granatum L.* (Fig. 3) (Walker J.M. (2005)).

Conclusion:

In the current study the pathogen infected samples identified as infected with *XAP* was through morphological, biochemical characterization and Pathogenicity test. Efficacy of different chemicals and oils were tested by disc diffusion assay and turbidometrically. Bronopol 3000 ppm (25.6±1.6 mm) formed highest zone of inhibitionand Clove oil formed highest zone of inhibition (18.0±0.7 mm). Turbidometri showed the highest O.D. (0.908 nm)

by Copper oxy chloride and Neem oil showed maximum inhibition of growth with O.D. (0.842 nm).

Biotic stress (pathogen) induced protein response was studies by using SDS-PAGE method after protein extraction from *XAP*, healthy *P. granatum L.* and infected *P.granatum L.* The protein banding pattern showed the unique band no. 2 (Mol.Wt.66000 Da) in infected *P. granatum L.* as compared to the banding pattern of *X. axonopodis pv.punicae and healthy P. granatum L.*The overexpressed protein due to biotic stress could be useful as a marker for detection of the disease at the early stage and for control of the diseases after knowing the biochemical significance of the protein.

Table1. Morphological and Biochemical characteristics of *Xanthomonas axonopodis* pv. *punicae* isolates.

Sr. No.	**Morphological and Biochemical test**	***Xap I***	***Xap II***
1.	Colony color	Yellow	Yellow
2.	Gram's reaction	-ve	-ve
3.	Starch hydrolysis	+++	++
4.	Indole production	++	+
5.	Catalase test	+++	+++
6.	KOH test	++	+
7.	Gelatin liquefaction	++	++
8.	Acid production	++	+

- Negative, + Positive, Varying degree of + ve reaction (+ poor, ++ moderate, +++ strong)

Table 2 revealed the significant differences among the different treatment by paper disc method.

Sr. No.	Treatment	Concentration	Zone of inhibition produced by Xap I	Zone of inhibition produced by Xap II
T_0	Control(+ve) water	10µl	00	00
T_1	Control(-ve) Ampicillin	15µl	17.9±0.6	27.7±0.7
T_2	Bronopol	1000 ppm	10.8±0.4	18.6±0.7
T_3	Bordeaux	1000 ppm	10.2±0.2	9.3±0.1
T_4	COC	1000 ppm	12.1±0.3	11.9±1.5
T_5	Bactomycin	1000 ppm	10.1±0.3	13.2±0.9
T_6	Bronopol	3000 ppm	11.6±0.4	25.6±1.6
T_7	Bordeaux	3000 ppm	9.7±0.7	8.1±.02
T_8	COC	2500 ppm	7.1±1.0	9.2±0.4
T_9	Bactomycin	1200 ppm	11.2±0.4	15.3±0.2
T_{10}	Clove oil	100%	15.9±0.9	18.3±0.7
T_{11}	Neem oil	100%	13.4±1.6	7.3±1.1
T_{12}	Nilgiri oil	100%	8.3±0.9	10.6±0.4

Table 3 Efficacy of chemicals and oils against *Xanthomonas axonopodis pv punicae* by turbidometric method:

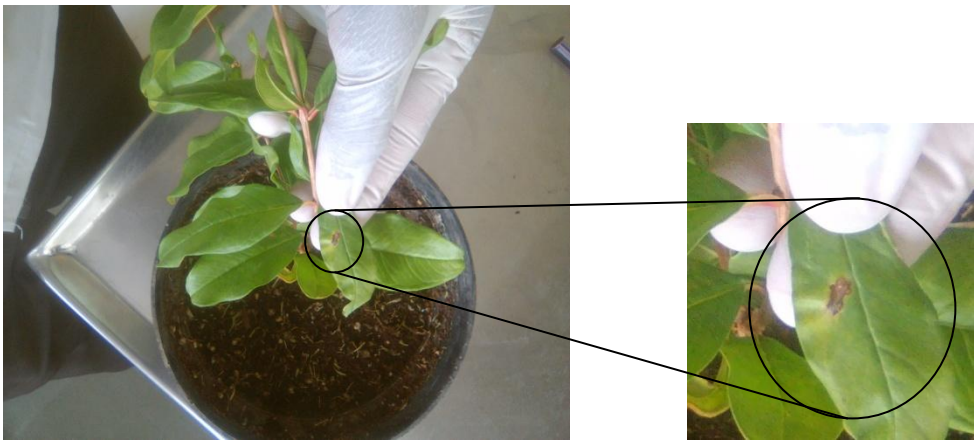
Sr.No.	Treatment	Concentration	O.D. of culture	O.D. after 24 hrs at 620 nm
T_0	Control(+ve)Nutrient Broth	1 ml	0.998	00
T_1	Control (-ve) antibiotic	500 µl		0.324
T_2	Bronopol	1000 ppm		0.219
T_3	Bordeaux	1000 ppm		0.337
T_4	COC	1000 ppm		0.410
T_5	Bronopol	3000 ppm		0.713
T_6	Bordeaux	3000 ppm		0.834
T_7	COC	3000 ppm		0.908
T_8	Clove oil	100%		0.810

T_9	Neem oil	100%		0.842
T_{10}	Nilgiri oil	100%		0.392

Table4. Data analysis of Healthy pomegranate leaves, infected pomegranate leaves and bacteria

Sr. No.	**Standard Marker (Band no)**	**Molecular weight**	**Healthy plant (Band no)**	**Infected plant (Band no)**	***Xap I* proteins (Band no)**	***Xap II* proteins (Band no)**
1.	1	97,400	Absent	Absent	Absent	Absent
2.	2	66,000	Absent	**Present**	Absent	Absent
3.	3	43,000	Present	Present	Absent	Absent
4.	4	29,000	Present	Present	Absent	Absent
5.	5	20,100	Present	Present	Present	Present
6.	6	14,300	Present	Present	Present	Present

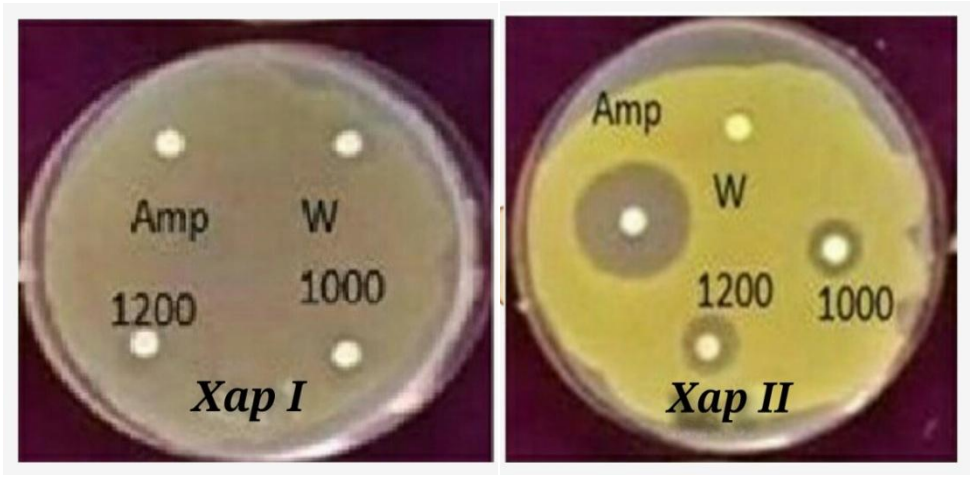
Fig. 1 (a) Symptoms of bacterial blight observed after 10-12 days of inoculation (b) Reisolated *Xanthomonas axonopodis pv. punicae* after pathogenicity test



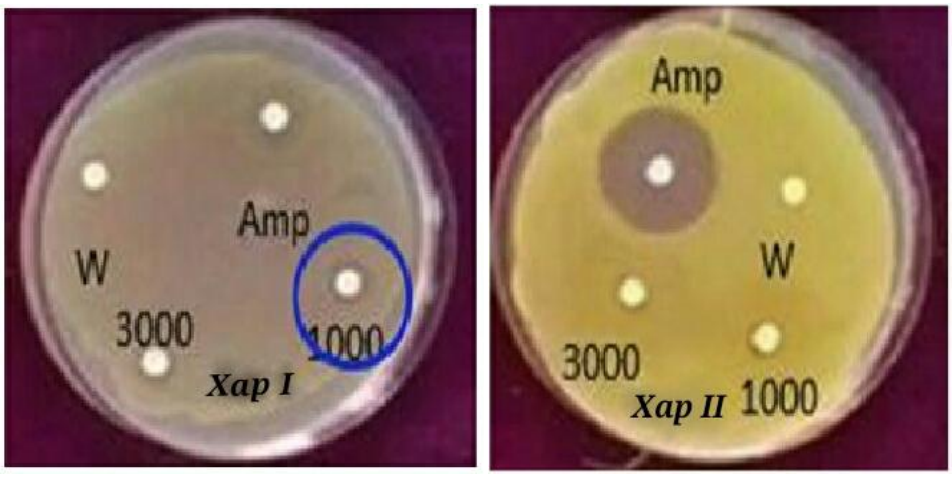
(a)

(b)

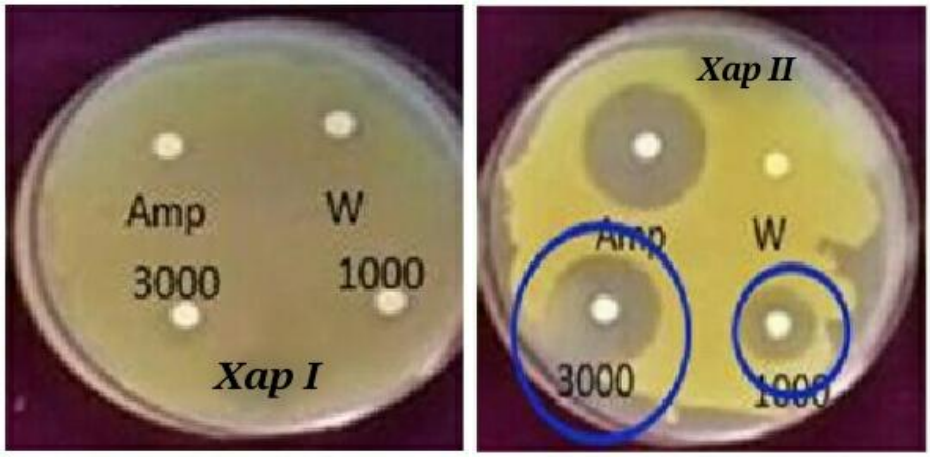
Fig. 2 Efficacy of different chemicals and oils against *Xanthomonas axonopodis pv. Punicae* by Paper disc method.



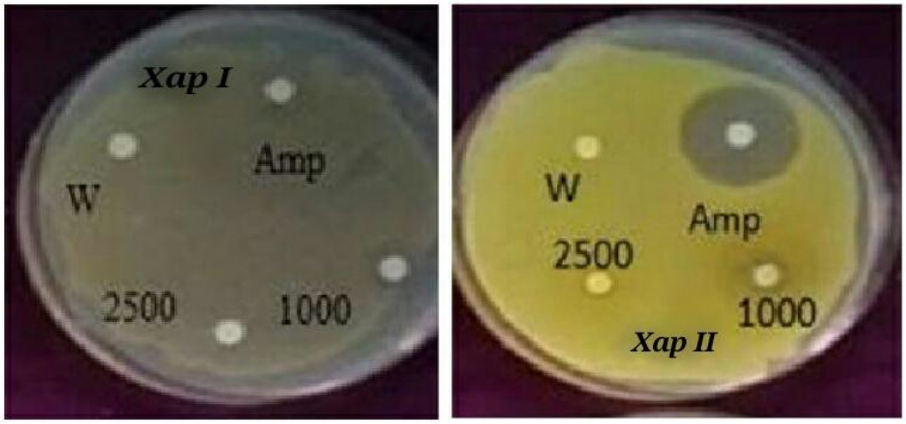
a) Bactomycin



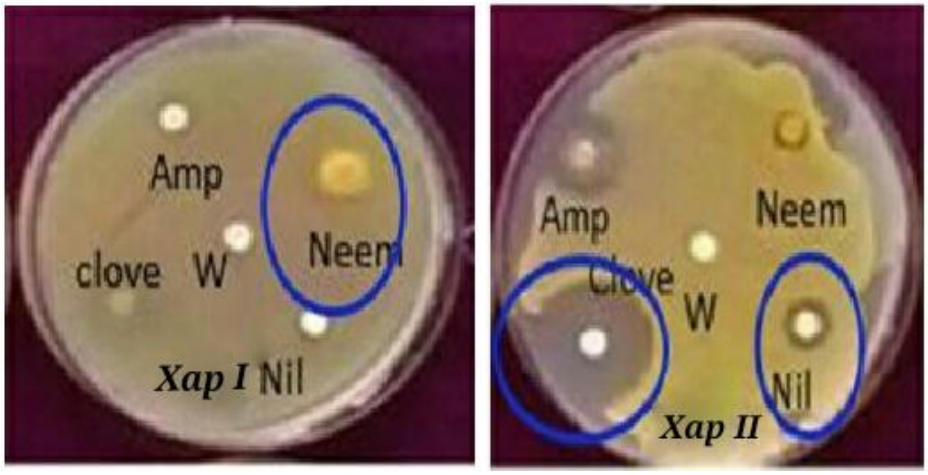
b) Bordeaux



c) Bronopol

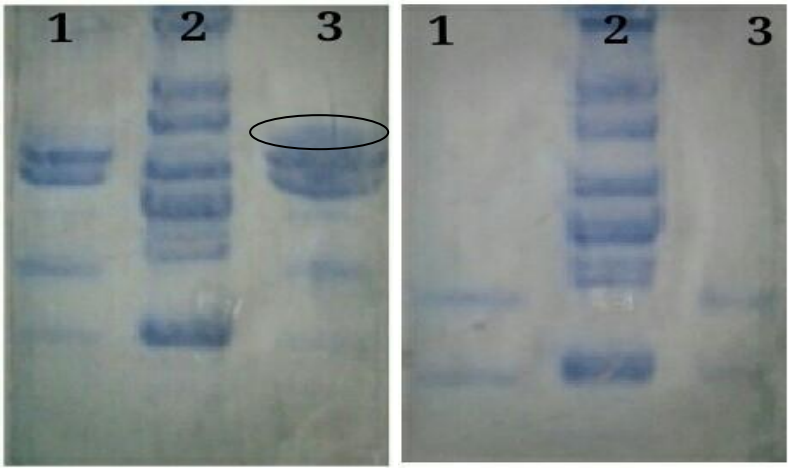


d) Copper oxychloride



e) Oils

Fig 3(a) Isolated proteins of 1) Healthy, 2) Marker, 3) Infected leaves (b) 1) *Xap I,* 2) Marker and 3) *XapII* Bacteria *(Xanthomonas axonopodis pv.punicae)*.



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