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PLANT PATHOLOGY

Studies on *Xanthomonas axonopodis* pv. *punicae,* Causing Bacterial Blight of Pomegranate in Punjab

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ABSTRACT

Bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae,* have economic importance to reduced crop yield in India. Farm survey was undertaken in the pomegranate research block, Fruit Research Station, Jallowal- Lesriwal Jalandhar during 2015. It was revealed that the maximum disease incidence (91%), severity (14.41%) and fruit cracking (12%) was found in cv. Ganesh. Twenty isolates of *Xanthomonas axonopodis* pv. *punicae* showed different virulent reaction with respect to incubation period, fruit cracking and drooping. It was observed that 9 isolates *viz*. Xap4, Xap5, Xap6, Xap9, Xap12, Xap14, Xap15, Xap16 and Xap17 were highly virulent on Ganesh cultivar. The optimum growth of bacterium was observed at 30 °C while 5 and 50 °C temperatures did not show any growth on Kings B Medium. All the five chemical treatments showed control of bacterium under *in vitro* conditions. However, the combination of Blitox (2500ppm) and Kocide (2500ppm) with Cristocycline (100ppm) showed maximum inhibition zone of 53 and 52 mm respectively followed by streptomycin (50.12 mm) and Cristocycline (50mm) with 500 ppm concentration. While Bordeaux mixture with concentration (1%) showed 45 mm inhibition.

Highlights

- The pathogen *Xanthomonas axonopodis* pv. *punicae* isolated and characterized. Find the favorable temperature for their growth and antimicrobial agent's under *in vitro* condition.

Keywords: *Xanthomonas axonopodis* pv. *punicae, Bacterial blight, antibacterial agent and pomegranate*

Pomegranate (*Punica granatum* L.) is cultivated worldwide with dominancy in Mediterranean countries like Spain, Morocco, Egypt, Iran, Afghanistan and Baluchistan since ancient times. It is widely cultivated in India and the drier part of southeast Asia besides China, Japan, USA (California), East Indies and tropical America (Raghuwanshi *et al.* 2013). Tropical and sub-tropical regions of the Indian sub-continent are the prominent areas under cultivation of this fruit crop. India plays a leading role in pomegranate production with contribution of nearly 50% of global pool. The total area under pomegranate is 132 thousand hectare with annual production of 1357 thousand MT in 2014-2015 (Anon 2015). Pomegranate production is constrained by a number of abiotic and biotic factors such as unfavorable climate, nutritional imbalance, diseases caused by fungi and bacteria. Among the biotic factors, the diseases caused by fungi and bacteria are economically more important because they cause heavy yield losses. Bacterial blight of pomegranate is a major hurdle in quality production and high yield in the crop causing up to 60-80% losses (Mondal and Sharma 2009; Kumar *et al*. 2009). The disease has also been recorded in Pakistan and has been reported to caused severe losses (Akhtar and Bhatti 1992).

The disease was first reported from Delhi in 1952 (Hingorani and Mehta 1952). The pathogen infects all the aerial parts of the plant and in severs conditions, fruits show deep cracking coupled with splitting of pericarp, thereby reducing the marketability of the fruits. In the last few years, the disease appeared in epiphytotic form in the major pomegranate growing states of India including Maharashtra and Andhra Pradesh (Mondal and Sharma 2009). In Punjab, this disease invariably appears every year in the pomegranate orchards causing significant yield and quality loss. The disease is caused by *Xanthomonas axonopodis* pv. *punicae*, a gram-negative rod shaped bacterium that produces light yellow, circular, convex, and smooth colonies. Bacterial cells are capable of surviving in soil for >120 days and also survive in fallen leaves during the off-season. High temperatures and low humidity or both favor disease development.

Fungicide and antibiotic solutions inhibit the growth of bacterium under *in vitro* and *in vivo* condition (Dekker 1963; Desai *et al.* 1967; Thirumlachar 1968; Rangarajan and Chakravarti 1969). Streptocycline alone and in combination with copper checks the development of *Xanthomonas axonopodis* pv. *punicae* (Raju *et al.* 2011; Ravikumar *et al*. 2011; Lokesh *et al*. 2013). Realizing the importance of this disease, the present studies were initiated to isolate, identify and characterize the pathogen with respect to its aggressiveness and also to evaluate different antibacterials in vitro against this pathogen.

MATERIALS AND METHODS

Isolation and identification of the pathogen

Infected samples were collected from two locations *viz.* Pomegranate Research Block, Fruit Research Station, Jallowal- Lesriwal Jalandhar and Punjab Agricultural University Ludhiana, Punjab in the month of June, 2015. Disease incidence was recorded by counting the number of infected fruits per plant. For disease severity, 20 fruits were selected randomly and scoring was done with help of 1-6 scale (Anon. 2006). The peel tissues were washed, air dried, cut into small sections with sterilized razor blade and then disinfected with 75% ethanol solution for one minute followed by three washings with sterilized distilled water. After surface sterilization, the peel was cut and put in sterile water on a glass slide and the bacterial ooze was streaked on to King's B medium (HiMedia™) (Bray and Thorpe 1954). Agar plates were incubated at 28 °C for 2 days. Single isolated colonies of the bacterium were further purified, preserved in silica gel and multiplied on the King's B medium, whenever necessary. Twenty strains of the bacterial blight pathogen were isolated from two different locations. Out of those ten (Xap1- Xap10) were isolated Fruit Research Station, Jallowal-Lesriwal and other ten (Xap11- Xap20) were isolated from Punjab Agricultural University, Ludhiana.

Pathogenicity and aggressiveness

Pathogenicity tests were performed on fruits of 10 year old pomegranate plants cv. Ganesh in June 2015. Inoculum was prepared by growing all the isolates in liquid King's B media for 24 hrs at 28 °C and bacterial concentration was adjusted to approximately 10^8-10^9 cells/ml. Full sized pomegranate fruits were inoculated with individual isolates of *Xanthomonas axonopodis* pv. *punicae* using the method described by Raghuwanshi *et al.* (2013). The fruits inoculated with sterile water only served as control. After inoculation, the plants were frequently irrigated to keep up high humidity and soil moisture which is vital for bacterial blight development. Initial disease symptoms were observed after 24 hrs of inoculation and final observations were recorded after 7 days. Disease severity was recorded 1-6 scale, (Table 1), (Anon. 2006).

Table 1: Disease severity scale of bacterial blight of pomrgranate

Grade	Per cent Infection on fruit
0	0.00
1	Up to 1
2	> 1-10
3	> 10-20
4	>20-40
5	> 40-70
6	>70-100

The pathogen was re-isolated from the infected inoculated fruits and reconfirmed as *Xanthomonas axonopodis* pv. *punicae.*

In vitro assessment of growth of *Xanthomonas axonopodis* pv. *punicae* at different temperature

Isolated bacterium culture (Xap-16) were incubated on different temperatures. Initially prepared Peptone Sugar Broth (PSB) medium composed of peptone 5 gm and sucrose 10gm per liter broth medium was used. Bacterial culture was then inoculated in broth culture in test tube and incubated for 24 hrs at 28°C. After 24 hrs, 100ul broth culture (diluted 10^{-4} times) was spread on petriplates containing King's B media with help of spreader and incubated at different temperatures *viz*. 5 , 10, 15, 20, 25, 30, 35, 40, 45 and 50°C for 48 hrs.

In vitro efficacy of different antibacterials against *X. axonopodis* pv. *punicae*

The antibacterial test was carried out by agar disc diffusion method (Jorgensen *et al.* 1999). Twenty four hrs old actively growing culture of *X. axonopodis* pv. *punicae* in liquid broth was inoculated onto King's B Agar plates using spreading plate method. Different concentrations of test antibacterials such as Blitox, Kocide, Cristocycline, Streptocycline, Blitox+ Streptocycline, Kocide+ Streptocycline and Bordeaux mixture were prepared in 100ml Erlenmeyer flasks. Circular (100mm) discs of Whatman filter paper no.1 were impregnated for 5 minutes. The inoculated King's B Agar plates were overlain with individual impregnated discs of different antibacterial. The plates were kept at 4 °C for 4 hrs so that chemically from the discs is diffused through the plates. All the concentrations were replicated three times with control (disc impregnated with sterilized water). The petridishs were then incubated for 48 hrs. Zones of inhibition with respect to different chemicals were measured with help of scale (Moosdeen *et al.* 1998).

RESULTS AND DISCUSSION

Bacterial blight is endemic to pomegranate orchard in Punjab. The disease incidence on cultivar Ganesh, a highly popular variety was observed to be very high. At fruit Research Station Jallowal, more than 90% of the plants showed symptops of this disease with mean disease severity of 14.41% (Table 2).

It was followed by Mridula and Jyoti varities where disease incidence was observed to be 90% and 85% respectively. Consequently fruit cracking was also observed to be high (10-12%) in these three varieties. In contrast disease incidence and severity of bacterial blight was low on Bhagwa and Ruby varieties. Fruit cracking was also observed to be low (2-3%) in these two (Table 2).

Table 2: Disease incidence, severity and fruit cracking of bacterial blight of pomegranate at Fruit Research Station, Jallowal- Lesriwal Jalandhar

Cultivars	DI%	DS%	Fruit cracking%
Ruby	72	7.6	3
Ganesh	91	14.41	12
Jyoti	85	13.12	10
Mridula	90	13.92	11
Bhagwa	70	6.2	2

Isolation and characterization of the pathogen

A total of twent strains were isolated from infected fruit samples. All the twenty isolates showing typical characters of *X. axonopodis* pv. *punicae* with yellow mucoid shining colonies obtained on Kings' B medium (Plate 1A). Bacteria isolated from symptomatic pomegranate plants were characterized as Gram negative rods.

Pathogenicity and aggressiveness of different strains of *X. axonopodis* pv. *punicae*

All the twenty isolates of *X. axonopodis* pv. *punicae* were inoculated individually on cv. Ganesh. Nine isolates *viz.* Xap4, Xap5, Xap6, Xap9, Xap12, Xap14, Xap15, Xap16 and Xap17 required only four days for expression of symptoms. The data pertaining to different disease variable such as fruit cracking, fruit drooping along with temporal development of disease was subjected to cluster analysis and presenting in Fig. 1; Table 3. It was observed that water soaked symptoms started appearing at the point of inoculation after 24 hrs in all the isolates (Plate 1C). However, variation were observed with respect to temporal development and expression of symptoms. Fruits inoculated with these nine isolates showed cracking within seven days of inoculation and drooping within 15 days (Plant 1D). These isolates were categorized as highly virulent (HV) with mean disease score of 6 (Table 3; Figure 1). Furthermore 8 isolates *viz*. Xap7, Xap8 Xap10, Xap11 Xap13, Xap18, Xap 19 and Xap20 showed incubation period of 6 days with fruit cracking and drooping singnificantely developed to 18-29 days. In group I three isolates *viz*. Xap1, Xap2 and Xap3

were observed to be less virulent (LV) with mean disease score 3.16. These isolates required more time for symptom development (7-8 days). However no fruit cracking and drooping were observed in these isolates. Similar observations were also reported by Raghuwanshi *et al.* (2013), wherein they observed that all the tested isolates of *X. axonopodis* pv. *punicae* showed water soaked lesion on leaves and fruits within a week on cv. Bhagwa. Similarly, *Petersen* (2010) recorded virulent reaction of two isolates *viz*. BD014–1 and BD014–2 in South Africa on pomegranate plant.

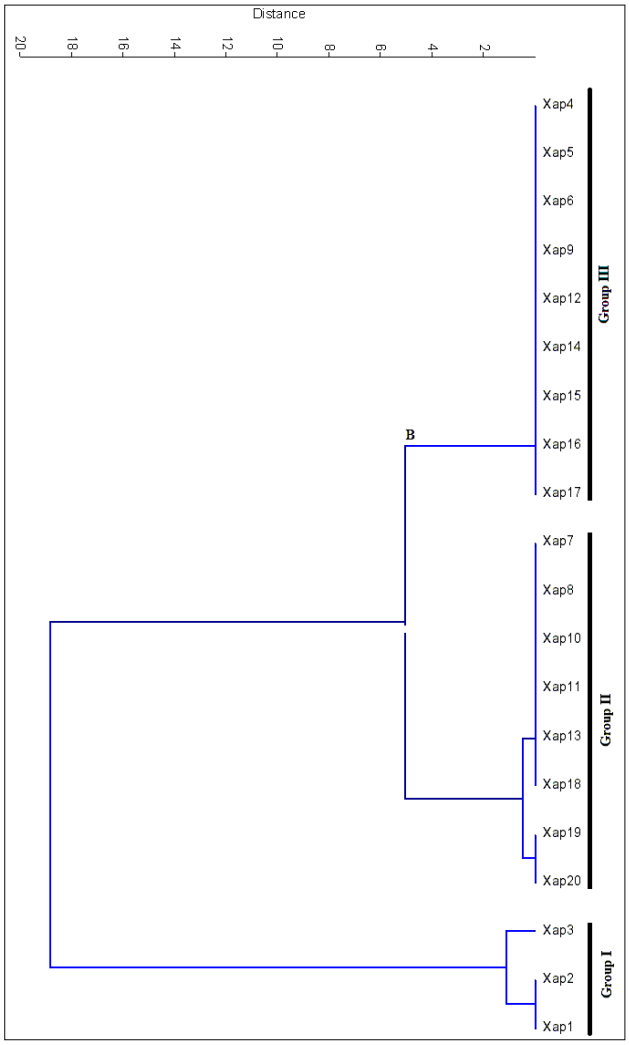

Fig. 1: Clustering of 20 isolates of *X. axonopodis* pv. *punicae* on the basis of their reaction on Ganesh cultivar

In vitro growth assessment of *Xanthomonas axonopodis* pv. *punicae* at different temperature

Temperature is an important factor for growth, reproduction and survival of bacterium. The results of *in vitro* growth assessment of *X. axonopodis* pv. *punicae* (Xap-16) was shown in Figure-2. The bacterium showed optimum growth at 30 °C (10.99 log cfu per ml) followed by 35, 40, 45, 25, 20, 15 and 10 °C while it did not show any growth at 5,and 50°C temperature. Raghuwanshi *et al.* (2013) also used 282 temperature for growth and pathogenicity test of *X. axonopodis* pv. *punicae.* Similarl findings were also reported by other authors (Hingorani and Mehta 1952; Bhat and Patel 1954; Chopade *et al.* 2014).

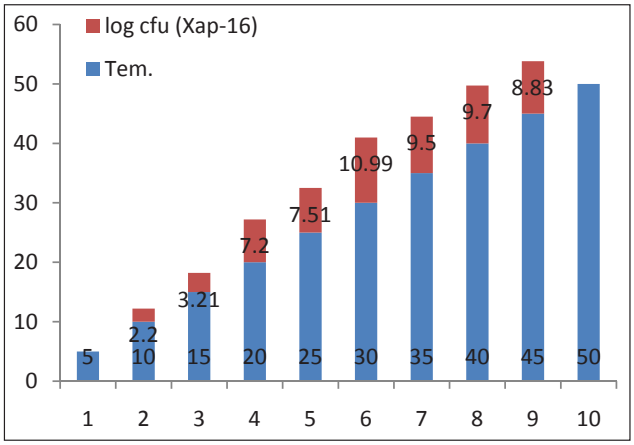

Fig. 2: *In vitro* effect of different temperature on *X. axonopodis* pv. *punicae*

In vitro efficacy of different chemicals against *X. axonopodis* pv. *punicae*

Five chemicals and their combination were tested *in vitro* for their antibacterial activity. All the antibacterial showed inhibitory action against the test isolate Xap 16. Microorganisms that are resistant to an antibiotic will not show a zone of inhibition (growing right up to the disk itself) or display a relatively small zone (Hudzicki 2009). It was observed that all the tested chemicals and antibiotics showed inhibition zone with different concentration (Plate 1C). Cupper based antibacterial chemicals such as Blitox, Kocide and Bordeaux mixture showed significant *in vitro* inhibitory activity against the pathogen either results to control the pathogen with antibiotic combinations or alone at the highest tested concentration (Table 4). Bordeux mixture produced 45 mm inhibition zone followed by Blitox (25.6 mm) and Kocide (21.3mm) at 1000ppm.

However, antibacterial activity of Blitox and Kocide increased when combine with 100 ppm cristocycline with 53mm and 52 mm inhibition zone respectively.

Table 3: Grouping of 20 isolates of *Xanthomonas axonopodis* pv. *punicae* on the basis of virulence parameter on Ganesh cultivars

Group	Name of Isolates	No. of Isolates	Mean of			Disease score		Remark
			Incubation period (days)	Fruit cracking (days)	Fruit dropping (days)	Mean	Range	
I	Xap1, Xap2 and Xap3	3	7.6	0	0	3.16	3-3.5	Less Virulent
IIA	Xap7, Xap8 Xap10, Xap11 Xap13, Xap18, Xap 19 and Xap20	8	6	10	18	4.12	4-4.5	Moderately Virulent
IIB	Xap4, Xap5, Xap6, Xap9, Xap12, Xap14, Xap15, Xap16 and Xap17	9	4	7	15	6	6	Highly Virulent

Table 4: *In vitro* efficacy of different chemicals against *X. axonopodis* pv. *punicae*

Chemicals	Inhibition zone (mm)								
	Concentration in ppm								
	20	50	100	200	500	1000	2000	2500	Control
Blitox	0	0	2.67 (±0.66)	3.1 (±0.52)	13.33 (±1.66)	25.67 (±0.66)	30.33 (±0.88)	40 (±1.52)	0
Kocide	0	0	2 (±0.32)	2.56 (±0.09)	15.33 (±0.34)	21.33 (±0.33)	31 (±1.11)	38 (±0.33)	0
Cristocycline	13.33 (±1.6)	21 (±0.57)	30.33 (±0..33)	40 (±1.2)	50 (±1.22)	NT	NT	NT	0
Streptocycline	16.33 (±0.88)	30.67 (±0.66)	36 (±1.01)	38.2 (±1.3)	50.12 (±1.52)	NT	NT	NT	0
Blitox + streptocycline	NT	NT	NT	NT	NT	32.12 (±1.22)	40 (±1.23)	53 (±1.8)	0
Kocide + streptocycline	NT	NT	NT	NT	NT	28.11 (±1.3)	39 (±1.6)	52 (±1.92)	0
Bordeaux mixture	NT	NT	NT	NT	NT	45 (±1.6)	NT	NT	0

Figures in parentheses are standard deviations from mean of three replications, NT - concentrations not tested.

Both the antibiotics brands showed similar inhibition zones 50 mm at 500ppm concentration. Earlier, copper based fungicides have been reported to be highly effective against varies bacterial plant pathogen. Different workers have used these fungicides in combination with antibiotics for field application to manage bacterial disease. Ravikumar *et al*. (2011) reported that spray of combination of streptocycline +copper oxychloride was effective against bacterial blight of pomegranate. Similarly combination of streptocycline (100-300 ppm) with copper oxychloride (0.3%) was observed to be effective against *X. citri* (Kale *et al*. 1994) and *X. axonopodis* pv. *malvacearum* (Pathak and Godika 2006). Desai *et al*. (1967) and Raj and Moniz (1967) had reported effectiveness of streptocycline against *Xanthomonas oryzae*. Similar findings were also reported for other plant pathogenic bacteria (Dekker 1963; Desai *et al.* 1967; Thirumlachar 1968; Rangarajan and Chakravarti 1969; Raju *et al.* 2011; Lokesh *et al.* 2013; Ravikumar *et al*. (2011). These studies can help to explore the effective chemicals under field condition to manage *Xanthomonas axonopodis* pv. *punicae.*

CONCLUSION

The present study suggests that different isolates of *Xanthomonas axonopodis* pv. *punicae* are differentially aggressive on cv. Ganesh cultivar while some are highly aggressive/virulent, the others are moderate or less virulent. The pathogen is requiring warm temperature for optimum growth. Antibacterial

combination found effective *in vitro* will be further evaluated in field for the management of this highly devastating disease of pomegranate in Punjab.

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