The Bioscan 8(3): 845-850, 2013
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

CHARACTERIZATION OF *XANTHOMONAS AXONOPODIS* PV. *PUNICAE* ISOLATES FROM WESTERN MAHARASHTRA AND THEIR SENSITIVITY TO CHEMICAL TREATMENTS

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KEYWORDS
Pomegranate
Bacterial blight
ISSR analysis

Received on :
20.02.2013

Accepted on :
07.06.2013

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ABSTRACT
Bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* is a major biotic constraint in peninsular India. Field survey was undertaken in the major pomegranate growing regions of Western Maharashtra, which revealed the high prevalence of bacterial blight incidence in Solapur, Sangli and Nashik districts. Four different isolates of this pathogen were obtained from the highly infected plant materials collected during the field survey. *X. axonopodis* pv. *punicae* was detected from infected plant material and its identity was confirmed by morphological, physiological, hypersensitive and pathogenicity tests. Nashik isolate was most virulent. On Inter Simple Sequence Repeat (ISSR) analysis they formed separate clusters with Akkalkot-Solapur isolate being most divergent, while Deola-Nashik and Sangamner-Ahmednagar isolates were most similar. Six chemical treatments showed complete control under *in vitro* conditions while rest varied in their response to isolates. Complete control in all four isolates was observed with Bordeaux mixture (1%); captan (0.25%) + Copper oxychloride (0.3%), captan (0.25%) + copper hydroxide (0.3%), bromopol (500 ppm) + copper oxychloride (0.3%), streptocycline (250 ppm) + copper hydroxide (0.3%), streptocycline (500 ppm) + copper hydroxide (0.3%) during *in vitro* study.

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family Lythraceae, is one of the favorite table fruit of tropical and subtropical regions. The fruit crop is native of Iran to the Himalayas in northern India and cultivated over the whole 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, Malaya, Myanmar, China, Japan, USA (California), East Indies and tropical America.

According to National Horticulture Board of India, India is the largest pomegranate producer (7.43 lakh tones in 2010-2011) in the world sharing about 36 per cent of the world's production and about 30 per cent of the international pomegranate trade by exporting 30,158 tonnes fruit in 2011-12 (Anonymous, 2012). Maharashtra State (area 82.0 thousand ha) is considered as pomegranate basket in India contributing about 66.2 per cent (4.92 lakh tonnes) of pomegranate production followed by Karnataka, Andhra Pradesh, Gujarat, Rajastan and Tamilnadu.

Pomegranate crop is prone to number of diseases among which bacterial leaf blight caused by *X. axonopodis* pv. *punicae* (Hingorani and Singh) Vauterin et al is a serious problem and threat to pomegranate production due to its high epidemic potential. The disease affects all the above ground plant parts including flower, leaves, twigs, stem, buds and fruits, but it is more destructive when fruits are infected. The disease is reported to cause 30-50 per cent losses on an average. However, under favourable environmental conditions 80-100 per cent losses are reported. All the commercial grown cultivars are susceptible to this disease. The disease spread easily in the orchard through plant to plant contact, rains, runoff water or spray water splashes, windblown rain splashes, person handling the plant, contaminated tools and insects. It has been seen that under favourable temperature and humid conditions, disease incidence increases after every shower (Anonymous, 2008).

Bacterial blight of pomegranate was first reported in India from Delhi in 1952 (Hingorani and Mehta, 1952) and later from Banglore (Karnataka) in 1959 (Hingorani and Singh, 1959). The disease was of minor importance until 1991, when it appeared in epidemic proportion at IIHR Experimental plot in Bangalore, resulting in 60-80 per cent yield losses (Chand and Kishun, 1991). Further the outbreak of disease was noticed in pomegranate growing areas of Karnataka causing severe losses both in terms of yield and quality. Incidences of bacterial blight disease were also reported in Western Maharashtra particularly from Solapur district and the disease was observed throughout the year on pomegranate trees (Dhandar et al., 2004). Today the disease has become a threat to pomegranate production in all the three major pomegranate growing states *viz.*, Maharashtra, Karnataka and Andhra Pradesh. On molecular and biochemical characterization of isolates, it was revealed that their variability was independent of geographical location (Giri *et al.*, 2011). Recently this disease has been reported even outside Indian subcontinent (Petersen *et al.*, 2010).

Management of bacterial blight of pomegranate is a major concern. This disease could not be effectively managed with

conventional antibiotics like streptocycline in field conditions. Thus the investigations were carried out on management of disease with other chemicals. The aim of the present study to find out bacterial blight incidence across pomegranate growing regions of Maharashtra; to isolate the *X. axonopodis* pv. *punicae*; to characterize them at molecular level for their uniqueness and test them for sensitivity to different chemical treatments.

MATERIALS AND METHODS

Field survey and collection of samples

Field survey was undertaken in major pomegranate growing regions of Maharashtra, India i.e. Solapur, Nashik, Sangli, and Ahmednagar districts, during the month of June to Nov. 2009 and Feb. to June 2010. During the field survey the randomly selected plants were inspected at the nursery stage and in orchard for incidence of bacterial blight. Distribution of bacterial blight of pomegranate was recorded in these districts of Western Maharashtra. Incidence of disease was also recorded in the different commercial varieties under natural conditions. Plants were diagnosed as infected on the basis of typical symptoms of bacterial blight, viz., yellow water soaked lesions at early stages and corky, dark oily spots at later stages of infection. The suspected plant parts were collected separately from the orchard, labeled and brought to the laboratory for the further studies.

Isolation and characterization of the pathogen

The bacteria were isolated from the infected leaves, small twigs and fruits of pomegranate collected from affected orchards of western Maharashtra. These tissues were washed, air dried, cut into small sections with sterilized razor blades and then disinfected with 0.1% $HgCl_2$ for about 1-1½ minute and washed thrice with sterile water to remove traces of $HgCl_2$. They were macerated with sterilized blade in a sterile petridish containing few drops of sterile distilled water in order to allow the bacteria to diffuse out. A loopful of suspension was then transferred with the help of sterilized bacteriological needle to sterilized petriplates filled with nutrient agar medium with sucrose (NAS) and incubated at $28 \pm 2°C$ for 24-72 hr. After 2-3 days, incubated plates were observed for the presence of typical pale yellow, glistening colonies which were transferred to the NAS slants and maintained on yeast extract glucose agar with charcoal slant for further studies.

Identification and characterization of the bacterial blight pathogen was carried out by subjecting the bacterial isolates to various biochemical tests, such as Gram staining, potassium hydroxide (KOH) solubility test, Kovac's oxidase test (Hilderbrand and Schroth, 1972) starch hydrolysis, Lipase activity and Arginine dehydrogenase test (Lelliot and Stead, 1987), gelatin hydrolysis, and catalase tests.

Pathogenicity test: Inoculation was undertaken to prove pathogenicity of four isolates. For this the healthy seedlings of pomegranate cultivar Bhagwa were obtained from the central nursery of M.P.K.V., Rahuri. A moist chamber was prepared in order to maintain humidity. The plants were kept in the humid chamber for about 2 to 3 days before inoculation. Veinlets from one set of plants were injected with 48 h old bacterial suspension prepared out of 48 h old bacterial culture with the help of hypodermal syringes. Some plant leaves were inoculated by injuring the leaves with sharp needle and by spraying the bacterial suspension on injured leaves. A set of healthy fruits were inoculated with bacterial suspension by using hypodermal syringes

The plants and fruits were kept for incubation in moist chamber. A set of control was maintained for each set which were sprayed with distilled water only. Warm and humid conditions were maintained in the chamber by spraying sterilized water daily in the morning and at evening time. The organism was reisolated from artificially inoculated leaves and fruits of pomegranate plants showing typical symptoms of disease. The reisolation was carried out on NAS medium and the growth of organism was observed for next 7-10 days.

The strains were also subjected to the hypersensitive reaction in tobacco (*Nicotiana tabacum*) plants (Carlton *et al*., 1998). Each test was conducted with four replicates and repeated twice.

ISSR analysis for studying distinctiveness of isolates

Genomic DNA was isolated from 48 h nutrient broth culture of all four bacterial isolates using 2% Sarkosyl/protease digestion method (Chimote, 2000). DNA concentration and integrity was checked both with spectrophotometer (Nano drop ND 1000) by taking OD 260/280 and gel electrophoresis along with lambda DNA. For ISSR (Inter Simple Sequence Repeat) analysis of genomic DNA amplification was carried out on a thermal cycler (Eppendorf, Master Cycler Gradient, Germany). Initial denaturation at 95°C for 5 min, was followed by 40 cycles of denaturation step at 95°C for 30 second, annealing temperature (different for different primer) for 30 seconds and primer extension at 72°C for 30 second. A final extension at 72°C for 10 min was given at the end of programme. Banding pattern were recorded as binary data and analyzed by NTSYSpc software (Rohlf, 1998) to generate a tree (dendrogram). Principal Co-ordinate analysis (PCO) was also performed to develop 2-D scatter plot.

In vitro evaluation against different chemical treatments

In order to study the effect of different chemicals under *in vitro* conditions mention different chemicals below with different concentrations were used and tested against the bacterium. The basic view was to evaluate the best chemical for the control of organism and to study the response of different isolates to different chemicals at various concentrations (Table 3).

'Poison Food Technique' recommended by Nene and Thapliyal (1979) was followed for this purpose. The NAS media was made poisonous by adding chemical in media prior to pouring into petri plates. Bacterial suspension was prepared from 48 h old culture grown on NAS media by adding few drops of sterile distilled water. Five mm diameter discs of filter paper were prepared by punching machine and discs were sterilized and dipped in bacterial suspension and placed in sterilized petridish (containing NAS with chemicals) with the help of sterilised forcep. An adequate control was maintained without any chemical. The petridish were then incubated in

BOD incubator at 28 ± 2°C temperature for 48 h in order to know about growth inhibition. Measurement of bacterium growth from the periphery of treated and untreated discs were taken after 48 h and per cent growth inhibition was calculated as per procedure given by following Vincent (1947).

$$I = \frac{(C - T)}{C} \times 100$$

Where, I = Inhibition of bacteria in per cent C = Growth of bacteria (mm) in control treatment T = Growth of bacteria (mm) in chemical treatments. By measuring inhibition percentage to different chemicals the variation among the four bacterial isolates were studied.

RESULTS

Field survey and collection of sample

Bacterial blight of pomegranate was studied in 4 major pomegranate growing districts of Western Maharashtra i.e. Nashik, Solapur, Sangli and Ahmednagar districts. The results of surveys as given in Table 1 indicated that the infection of bacterial blight disease was observed mostly in Solapur district mainly in Sangola, Pandharpur, Mohol, Akkalkot talukas. In Nashik district, high disease prevalence was observed in both Deola and Satana taluka. In Sangli district the disease incidence was higher in Jat talukas. Pomegranate orchards in Sangamner and Rahata talukas of Ahmednagar district were also observed to be affected by this disease. Incidence of disease was observed in all the commercial varieties like Bhagawa, Ganesh, Mridula and Nandini Ratna under natural conditions. Prevalence per cent of bacterial blight disease was maximum in Sangli district (88%) followed by Nashik (80%) and Solapur districts (79%). Severity of disease on tree was maximum in Nashik district (32%) followed by Sangli (21%) and Solapur (18.90%) districts (Table 1).

Isolation and characterization of the isolates

Four isolates showing typical characters of *X. axonopodis* pv. *punicae* with yellow mucoid shining colonies were obtained on NSA medium. These isolates were obtained from samples collected from Deola–Nashik; Pandharpur-Solapur Akkalkot–Solapur and Sangamner–Ahmednagar. The isolates of *X. axonopodis* pv. *punicae* were purified and used for further studies. The isolated bacteria tested positive for KOH test and negative for gram staining indicating the gram negative nature of the bacteria. The bacteria were positive for oxidase test, catalase test, starch hydrolysis, gelatin liquefaction and lipid hydrolysis; however they tested negative in Arginine dehydrogenase test.

Pathogenicity test

In their pathogenicity test, plants of pomegranate variety 'Bhagawa' inoculated with all four isolates showed symptoms

Table 1. Status of bacterial blight in four major pomegranate growing districts of Western Maharashtra

S. No.	District	Tehsil	Variety	Average prevalence %	Average severity on tree (%)
1.	Solapur	Pandharpur, Mohol, Akkalkot, Sangola	Bhagwa, Ganesh, Nandini Ratna	79.0	18.9
2.	Sangli	Jat, Atpadi	Mrudula	88.0	21.0
3.	Ahmednagar	Sangamner, Rahata	Bhagwa	20.0	5.0
4.	Nashik	Deola, Satana	Bhagwa	80.0	32.0

Table 2 Pathogenic virulence variability among different isolates

S.No.	Isolate	Period required from inoculation (days)			
		Full development of symptoms on leaves (Incubation period)	For yellowing of leaves	For blackening of spots/ symptoms	For drooping of leaves
1.	Deola –Nashik	4	6	8	14
2.	Pandharpur- Solapur	6	8	10	15
3.	Akkalkot – Solapur	6	8	9	14
4.	Sangamner - Ahmednagar	5	7	9	15

Table 3: Details of ISSR analysis of *X. axonopodis* pv. *punicae*

S. No.	Name	Primer Sequence	T_{ann}	Bands	PB	MB	UB	% P
1	ISSR 8	CACACACACACACAGC	52°C	8	4	0	4	50
2	ISSR 12	GTGTGTGTGTGTGTTG	48°C	6	0	2	4	0
3	ISSR 13	GTGTGTGTGTGTGTGTCA	55°C	-	-	-	-	-
4	ISSR 811	GAGAGAGAGAGAGAGAC	50°C	11	5	0	6	45.45
5	ISSR 816	CACACACACACACACAT	54°C	5	3	2	0	60
6	ISSR 817	CACACACACACACACAA	54°C	8	0	1	7	0
7	ISSR 820	GTGTGTGTGTGTGTGTC	54°C	4	1	3	0	25
8	ISSR 827	ACACACACACACACACACG	55°C	9	5	2	2	55.55
9	ISSR 834 100)	AGAGAAAAAAGYT	45°C	-	-	-	-	-
10	ISSR 841	GAGAGAGAGAGAGAGAYC	46°C	5	1	1	3	20
11	ISSR 857(101)	ACACACACACACACYC	46°C	10	3	1	6	30
12	UBC 8932800	AGCAGCAGCAGCGT	51°C	6	2	1	3	33.33
13	UBC 8932801	AGCAGCAGCAGCAT	51°C	6	0	5	1	0
14	UBC 8932814	CGAGAGAGAGAGAGA	44°C	4	1	3	0	25

T_{ann} = Annealing Temp; PB = Polymorphic bands; MB = Monomorphic bands; UB = Unique bands; %P = % polymorphism

Table 4. *In vitro* evaluation of *X. axonopodis* pv. *punicae* isolates against different chemical treatments (Percent Inhibition)

Sr. No.	Name of chemical	Deola -Nashik	Pandharpur-Solapur	Akkalkot-Solapur	Sangamner-Ahmednagar
1.	Bourdex mixture (1%)	100	100	100	100
2.	Copper oxychloride $Cu_2(OH)_3Cl$	95.2	89.43	96.59	76.53
3.	Copper hydroxide $Cu(OH)_2$	91.9	85.37	75	83.68
4.	Captan	87.1	84.56	100	88.75
5.	Captan + $Cu_2(OH)_3Cl$	100	100	100	100
6.	Captan + $Cu(OH)_2$	100	100	100	100
7.	Bromopol 250 ppm	87.1	100	94.32	88.75
8.	Bromopol 500 ppm	91.9	100	98.86	100
9.	Bromopol 250 ppm + $Cu_2(OH)_3Cl$	91.9	100	96.91	89.63
10.	Bromopol 500 ppm + $Cu_2(OH)_3Cl$	100	100	100	100
11.	Bromopol 250 ppm + $Cu(OH)_2$	97.6	100	100	100
12.	Bromopol 500 ppm + $Cu(OH)_2$	98.4	100	100	100
13.	Streptocycline 250 ppm	74.2	99.19	63.64	58.56
14.	Streptocycline 500 ppm	91.9	100	88.64	85.71
15.	Streptocycline 250 ppm + $Cu_2(OH)_3Cl$	93.6	100	96.6	86.73
16.	Streptocycline 500 ppm + $Cu_2(OH)_3Cl$	100	100	97.73	100
17.	Streptocycline 250 ppm + $Cu(OH)_2$	100	100	100	100
18.	Streptocycline 500 ppm + $Cu(OH)_2$	100	100	100	100

typical of bacterial blight disease when incubated under glasshouse conditions. Control plants inoculated with distilled water did not show any symptoms. Each isolate required different induction period for development of water soaking reaction on leaves of pomegranate plants (Table 2). The isolate from Nashik produced watersoaking reaction on the 4th day and while isolate from Ahmednagar district produced water soaking reaction on 5th day after infiltration of bacterial suspension into veinlets of mature leaves. Both isolates from Solapur district produced water-soaking symptoms on 6th day. More or less similar period was taken for dropping of leaves in all cases. Necrotic, hypersensitive symptoms were observed in tobacco plants within 24 h of infiltrated with bacterial cells, where as sterile distilled water infiltrated leaf regions did not show any change in the leaf color, which served as control.

ISSR analysis of *X. axonopodis* pv. *punicae*

On ISSR analysis of the *X. axonopodis* pv. *punicae* isolates

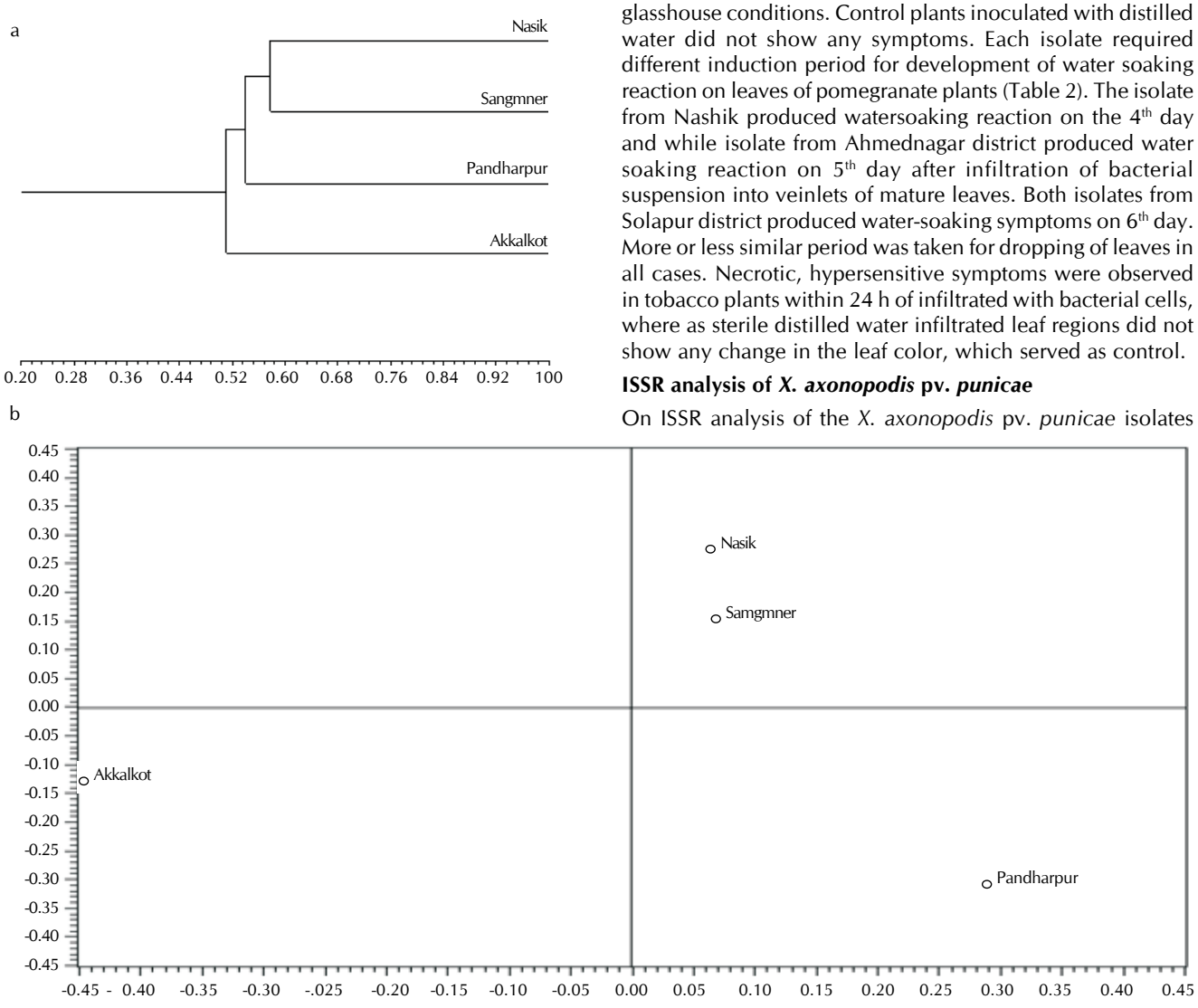

Figure1: ISSR analysis derived dendrogram (a) and 2D PCO scatter plot (b)

with 14 primers, only 12 primers yielded amplification. They amplified a total of 82 bands with size ranging from 449 to 2052 bp (Table 3). Out of them, 36 bands were unique to specific isolate, 23 were monomorphic with the rest being polymorphic amongst them. According to ISSR based-tree and 2D PCO scatter plot the isolates Deola-Nashik and Sangamner-Ahmednagar were closely placed with each other as compared to Pandharpur-Solapur isolate (Figure 1a/b). While isolate from Akkalkot-Solapur region was distinct from remaining three isolates.

In vitro evaluation of *X. axonopodis* pv. *punicae* against chemical treatments

All the four isolates behaved differently in their sensitivity to different chemicals (Table 4). Pandharpur-Solapur isolate showed almost complete control (over 99% inhibition) in 15 treatments as against only 7 treatments in Deola-Nashik isolate. Complete inhibition of all four isolates was observed with six treatments viz. Bordeaux mixture (1%); captan (0.25%) + Copper oxychloride (0.3%); captan (0.25%) + copper hydroxide (0.3%); bromopol (500ppm) + copper oxychloride (0.3%); streptocycline (250ppm) + copper hydroxide (0.3%); streptocycline (500ppm) + copper hydroxide (0.3%). Therefore, these chemicals can be used alternatively against the bacterium under field conditions. In case of streptocycline 250ppm, most variable response of isolates was observed, ranging from 58.56% inhibition in Sangamner isolate to 99.19% in Pandharpur-Solapur isolate.

DISCUSSION

In the present study, field survey was undertaken in the major pomegranate growing region of Western Maharashtra and the study revealed that the bacterial blight disease prevails in 20.0-88.0% orchards. The incidence of the disease was more in Sangli district followed by Nashik and Solapur districts. Incidence of bacterial blight disease of pomegranate was observed mainly in Sangola, Pandharpur, Mohol, Akkalkot from Solapur district. Severe incidence of the disease was also observed in Jat (Sangli district) and Deola (Nashik district). Bacterial blight of pomegranate was found to be highly destructive, wide spread disease and a threat to pomegranate production due to its high epidemic potential. Similar kind of work was carried out by Dhandar et al., (2002), they surveyed the pomegranate orchards in Chickmahood, Kadlas, Jadhavadi and Sangola in Solapur district of Maharashtra and observed bacterial blight incidence in all surveyed pomegranate orchards.

We confirmed the casual agent of bacterial blight of pomegranate as *Xanthomonas axonopodis* pv. *punicae* with all the four isolates producing typical symptoms during their pathogenicity test although at different incubation periods. The isolate from Nashik district was most aggressive while the isolates from Solapur districts were least aggressive. Further microbial and biochemical characterization confirmed them to be *X. axonopodis*. Bacterium *X. axonopodis* pv. *punicae* was reported as a causal agent of bacterial blight disease of pomegranate and its pathogenicity was demonstrated by Hingorani and Singh (1959) in India. Dhandar *et al.*, (2002) and Kale *et al*., (2012) isolated and proved the pathogenicity of this bacterium. Kale *et al*., (2012) further reported that restriction digestion of plasmids confirmed that this bacterium has megaplasmid of size around 200kbp typical of genus *Xanthomonas*.

ISSR analysis confirmed the uniqueness of all four isolates as they formed separate clusters. Akkalkot-Solapur isolate was the most divergent, while Deola-Nashik and Sangamner-Ahmednagar isolates were most similar. No correlation was observed between virulence of isolates and banding pattern. Mondal and Mani, (2009) reported relationship between ERIC-PCR generated fingerprints with pathogenic variability in *X. campestris* pv. *punicae (i.e. X. axonopodis* pv. *punicae*). High genetic variability was observed among strains of *X. axonopodis* pv. *punicae* on RAPD analysis (Giri *et al*., 2011).

Six chemical treatments showed complete control under *in vitro* conditions while rest varied in their response to each isolate. Streptocycline 250 ppm showed least effectiveness against isolates from Nashik, Sangamner and Akkalkot region while it was effective against Pandharpur isolates, while Captan was little less effective against isolate from Pandharpur region but it was very effective against remaining three isolates. The study indicated that selection of fungicide and antibiotics for mitigating the disease is very important because the efficacy of chemicals varies from location to location. Besides streptocyclyine, other chemicals like Bordeaux mixture, captan, bromopol were observed effective against the bacterium alone or in combination with copper hydroxide or copper oxychloride. Alternative use of these chemicals will help to avoid development of resistance in bacterium. Pesticide resistance is developed at a particular location and hence it is suggested to use alternative pesticides. Earlier, Ravikumar *et al*. (2011) reported that spray of streptocycline 500ppm +copper oxychloride 200ppm was effective against bacterial blight of pomegranate. Similarly combination of streptocycline (100-300 ppm) with copper oxychloride (0.3%) was reported 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* sp.

Hence the present work suggests that the periodic field survey will be necessary to understand the progression of blight disease. Alternative chemical control measures suggested from the *in vitro* studies need to be further evaluated under field conditions. Evaluation of more isolates collected from varying location and at different durations should also be conducted. Future studies are needed to understand the mode of infection, its ecological behavior and efficient control strategies under field conditions.

ACKNOWLEDGEMENT

The authors are thankful to the authorities of the Mahatma Phule Krishi Vidyapeeth, Rahuri for providing facilities to undertake this work.

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