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Research Article

GENETIC DIVERSITY ASSESMENT AND MOLECULAR CHARACTERIZATION OF *XANTHOMONAS AXONOPODIS* PV. *PUNICAE*

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ABSTRACT

Pomegranate (*Punica granatum* L.) is a preferred nutritional fruit in tropical and sub-tropical regions of the world which belongs to family *Lyrthaceae*, having chromosome number 2n=16. All time outbreak of oily spot disease of pomegranate is caused by *Xanthomonas axonopodis* pv. *punicae* (*Xap*), is the major problem and limiting factor in Maharashtra state comparing alone accounts for loss of 80% out of total cultivated area. Keeping this in view, present study is envisaged towards genetic diversity, isolation, identification and characterization of *X. axonopodis* pv. *punicae* strains at molecular level from infected plant samples *by using* RAPD marker. Among the five primers, primer OPB 20 showed most significant by producing 32.75% polymorphism and would be exploited for further study of races identification and development of species specific SCAR marker.

Keywords: *Xanthomonas*, Pomegranate, *Lyrthaceae*, Disease, RAPD marker, SCAR marker

1. INTRODUCTION

Pomegranate (*Punica granatum* L.) is an important commercial fruit crop of India and other subtropical countries. Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* [1], is becoming a major threat to pomegranate production in Maharashtra as well as other pomegranate growing states of India. It causes yield loss to the extent of 80-90 %. Considering importance of this disease, study regarding assessment of genetic diversity and molecular marker development is accomplished.

A survey was conducted and 10 strains of *X. axonopodis* pv. *punicae* were collected and used for assessment of genetic diversity by using five RAPD primers. Whereas 10 strains of *X. axonopodis* pv. *punicae* were differentiated and distinguished into three major groups representing Solapur, Latur, Osmanabad and Parbhani region of South Maharastra, India.

However the genetic similarity within 10 strains of *X. axonopodis* pv. *punicae* was ranged between 47 to 79 % similarity. The combine data analysis by using unweighted pair group method with arithmetic mean (UPGMA) method also revealed, the three distinct group representing two of them were strains of *X. axonopodis* pv. *punicae*.

This study gives valuable information towards developing species specific primer for early detection of *X. axonopodis* pv. *punicae* using molecular biotechnology tools.

Pomegranate is a preferred nutritional fruit in tropical and sub-tropical regions of the world which belongs to family *Lyrthaceae*, having chromosome number 2n=16. It is native to Iran but extensively cultivated in Mediterranean regions especially in Spain, Morocco, Egypt and Afghanistan. Also, it is being cultivated in Burma, China, Japan, USA, USSR, Bulgaria and Southern Italy. India is the leading pomegranate producer which contributes nearly 50% of the world's production. Pomegranate is being cultivated on an area of 1.25 lakh ha in India with production of 8.20 lakh tonns. However the total world trade of pomegranate is around 1.00 to 1.12 lakh tonns, India ranks second with an annual export of 33,415 tonns after Iran (67,000 tonns) [2]. The Maharashtra state, alone occupies an area of 98,900 ha followed by Karnataka 13,200 ha. Andhra Pradesh 5,600 ha. and Gujarat 4,400 ha. Even though, Maharashtra state has largest area of 0.99 lakh ha., production and productivity are of 5.55 lakh tonns and 5.6 tonns per ha. respectively [3].

Pomegranate is good source of carbohydrates and minerals such as calcium, iron and sulphur. It is rich in vitamin C and citric acid, which is the predominant

organic acid in pomegranate [4]. The Glucose (5.46%) and fructose (6.14%) are the main source of sugars present in pomegranate. In disparity of cultivation of pomegranate, it being fascinated by many problems such as long dry spells, non-availability of suitable varieties, environmental vagaries, nutritional deficiencies etc. in addition biotic as well as abiotic constraints comprising pest, temperature, rainfall and wind etc. are becoming the major limiting factor of pomegranate cultivation. Even though 25 to 30 % of total cost of production is being spent on plant protection especially on pesticides, the biotic constraints could not be managed effectively. Among the diseases of pomegranate, the bacterial disease, popularly known as bacterial blight or oily spot caused by *X. axonopodis* pv. *punicae* is as a major production constraint [5-6].

Incidence of oily spot disease of pomegranate (Teliya) caused by *X. axonopodis* pv. *punicae* (*Xap*), is the major obstacle and limiting factor in Maharashtra state comparing alone accounts for loss of 80% out of total cultivated area, particularly in Nasik, Solapur, Sangli and Ahmednagar districts. Bacterial blight infection results in appearance of water soaked oily spot symptoms on leaves, stems and fruits which consequently decreases fruit production and market value. Severity of incidence and losses varies among different isolates and influenced by existing climatic conditions and geographical distribution [7-10].

Earlier, the pomegranate farming in Maharashtra and Karnataka state were always in profit and yielded an earnings in the range between Rs. 60,000 to Rs. 1, 00,000 per ha. However, since 2002, it is in declining stage due to severe outbreak of oily spot disease. This could result in huge yield losses both in terms of quality and quantity due to the rapid built up of inoculums and wide spread of the pathogen of this disease. Thus disease is becoming more prevalent and predominant throughout all the season of the year.

Therefore, considering predominance and economic loss caused by *X. axonopodis* pv. *punicae* in pomegranate, it is an essential to undertake preliminary work on characterization and strain identification of this pathogen at molecular level.

2. MATERIAL AND METHODS

2.1. Collection, isolation and maintenance of bacterial strains

The oily spot infected samples of pomegranate were collected from Solapur, Latur, Osmanabad and Parbhani districts of South Maharashtra, India. The pathogen (*X. a.* pv. *punicae*) was isolated, purified by single colony method and maintained aseptically on Yeast Glucose Calcium carbonate Agar (YGCA) medium at 30°C for 3 to 4 days. These cultures were stored at frequently sub cultured and maintained in freeze at 4°C.

2.2. Culture media

Yeast Glucose Calcium carbonate Agar (YGCA-(Yeast extract 10 g, Glucose 20 g, Calcium carbonate 10 g, Agar agar 20 g, Sterile water 1Land pH- 7.0) medium was used as basal medium for the isolation, multiplication and maintenance of the culture of pathogen *X. axonopodis* pv. *punicae.*

Table 1: List of strains of *Xanthomonas axonopodis* pv. *punicae.*

Isolate Name	Sample Type	Location of Isolate
Xap-SB	Leaf	Brahamni (Latur)
Xap-SW	Fruit	Renapur (Latur)
Xap-NA1	Branch	NRC-Pomegranate, A1 (Solapur)
Xap-NKBD	Fruit	NRC-Pomegranate, KBD(Solapur)
Xap-SM	Fruit	Udgir (Latur)
Xap-SS	Fruit	Chakur (Latur)
Xap-LA1	Leaf	Latur
Xap-LA2	Leaf	Ausa (Latur)
Xap-PP	Fruit	Parbhani
Xap-AS	Leaf	Kalam (Osmanabad)

2.3. Genomic DNA isolation of *Xanthomonas axonopodis* pv. *punicae*

Overnight inoculated culture, TES buffer (pH 8.0)-50 mM Tris-HCl (pH 8.0), 50 mM disodium EDTA, 15 mM NaCl;, *Proteinase* K 20 mg/ml, 20% SDS, 3M Sodium acetate, Chloroform, Isoamyl alcohol, Saturated phenol, Chilled isopropanol, Absolute ethanol, 70 % ethanol, *RNase* (2.5 mg/ml), Agarose, 6X gel loading dye, Electrophoresis buffer, Ethidium bromide, Sterile water.

2.3.1. Confirmation of Xanthomonas by using PCR based method

Genomic DNA, 10X PCR buffer, MgCl2, dNTP mixture, Taq DNA Polymerase, Xanthomonas genus specific primer, Sterile water, Kb DNA ladder and 6X gel loading dye.

2.3.2. Collection and isolation of Xanthomonas strains from infected sample

The oily spot infected samples of pomegranate were collected from different pomegranate regions of

Maharashtra state *viz.,* Solapur, Latur, Osmanabad and Parbhani districts.

The pathogen *X. axonopodis* pv. *punicae* was isolated from leaves, fruits and flowers samples. Each of infected lesions was cut into small pieces sequential by sterilized scalpel blade followed by sterilization through 0.1% $HgCl_2$ and 70% ethanol for 15 second. The trace of surface sterilizing agent was removed by three times rinsing with sterile distilled water. Further tissue was chopped or diced in a drop of sterile distilled water. The resulting suspension was streaked on Yeast Glucose Calcium carbonate Agar (YGCA) plates. The culture plates were incubated at 28°C in an incubator for 48-72 h. and observed for bacterial growth.

Single colonies having circular, convex, mucoid, shiny and yellow morphological characteristics of *X. axonopodis* pv. *punicae* were picked by sterilized loop and purified cultures were obtained by streaking on fresh Yeast Glucose Calcium carbonate Agar (YGCA) medium.

2.3.3. Preservation and maintenance of Xanthomonas strains

For maintaining the purity of sample collected, a small amount of the suspension was streaked on YGCA plates with pH 7 to get distinct single colony. The plates were incubated at 28°C for 48 h. Bacterial colonies from these plates were used for further sub-culturing, genomic DNA isolation and morphological tests etc. The pure cultures were frequently subcultured and maintained on YGCA plate at 4°C.

2.4. Pathogenicity test

Pathogenicity of the strains of *X. axonopodis* pv. *punicae* was tested by pinprick method [11]. In this method the strains of *X. axonopodis* pv. *punicae* were inoculated on the detached leaves of pomegranate. Inoculated leaves of pomegranate were provided > 90% humidity and maintained at 28+2°C. The infected leaves were observed for the appearance of bacterial blight symptoms. The pathogen *X. axonopodis* pv. *punicae* was re-isolated from infected leaves and compared with original culture for proving Koch postulate.

2.5. Assessment of genetic variability among the isolates of *X. axonopodis* pv. *punicae* using RAPD marker

2.5.1. Extraction of genomic DNA

The extraction of genomic DNA from isolated of *X. axonopodis* pv. *punicae* (Xap) was carried out by following a protocol described by Adachi and Takashi [12] with some modifications. The 3 ml culture of *X. axonopodis* pv. *punicae* was used to centrifuge for 5 min at 7,000 rpm. The pellet of isolates of *X. axonopodis* pv. *punicae* were washed with 0.7% NaCl and TEN (Tris HCl 50 mM, pH-7.5, EDTA 10 mM, pH-8.0, and NaCl 150 mM) buffer, vortexed and centrifuged at 7,000 rpm for 5 min. The bacterial cells were resuspended in 500 µl of proteinase K solution (150 µg /µl in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) and kept at 56°C for 15 min, followed by incubation at 80°C for 15 min to denature the proteinase K, further suspension was centrifuged for 5 min at 13000 rpm. The supernatant was transferred to a new microfuge tube and treated with RNase A (100 µg/ ml) for 30 min at 37°C. Further the reaction mixture was subjected for DNA precipitation extraction with phenol:chloroform:isoamyl alcohol (25:24:1) followed by addition of 0.1 volume of ammonium acetate and 2 volumes of chilled ethanol. The DNA pellet was washed with 70% ethanol and resuspended in 50 µl sterile water. The DNA samples were quantified by using Spectrophotometer at 260 nm wavelength. Similarly the quality of DNA was assessed by separating on 0.8 % agarose gel.

2.5.2. Determination of genetic variability by using RAPD analysis

2.5.2.1. PCR reaction

The PCR protocol for RAPD reaction was optimized with various PCR components and thermal cycler programme.

Random primers *viz.,* OPB-06, OPB-07, OPB-08, OPB-10, OPB-20 (Operon Technologies, USA) were used for RAPD analysis of 15 strains of *X. axonopodis* pv. *punicae* and four other *Xanthomonas* species described in Table 3. Master mixture (24.0 µl) containing all the above reactants, except template DNA were dispensed in autoclaved PCR tubes (0.2 ml). Genomic DNA of each strain of *Xanthomonas* species was added to the individual tubes containing the master mixture. The contents of each tube were mixed by tapping with fingers followed by a brief spin to collect contents at the bottom of the tube. The tubes were placed in Thermocycler and subjected to PCR according to the protocol adopted below (Table 5).

Table 2: PCR components used for genetic diversity analysis of strains *X. axonopodis* pv. *punicae*

PCR Components	Required Concentration	Volume/reaction
PCR Buffer (10X)	1X	2.5 µl
$MgCl_2$ (25 mM)	1.5 mM	1.5 µl
dNTP mix. (10 mM)	200 µM	2.0 µl
Primer (10 µM)	0.4 µM	1.0 µl
Taq DNA Polymerase (3 U/µl)	1.25 U	0.5 µl
Template DNA	30 ng	1.0 µl
Nuclease free water		16.5 µl
Total		**25 µl**

Table 3: Standardized PCR protocols for amplification of DNA

Steps	Temperature		Time
Initial Denaturation	94°C		4 min
Denaturation	94°C	39 cycles	1 min
Annealing	35°C		1 min
Primer Extension	72°C		1.5 min
Final extension	72°C		10 min
Hold	4°C		Forever

2.5.2.2. Resolution of amplified product

The amplified RAPD PCR product was separated on 1.2 % agarose gel, stained with ethidium bromide and visualized under Gel Documentation System (Alpha Innotech, USA). The polymorphism was detected by comparing RAPD fingerprinting pattern of all *X. axonopodis* pv. *punicae* strains and four other species of *Xanthomonas*.

2.5.2.3. Scoring and data analysis

RAPD markers across the 18 isolates were scored for their presence '1' or absence '0' of bands for each primer. By comparing the banding patterns of isolates for a specific primer, isolates-specific bands were identified and faint or unclear bands were not considered. The binary data so generated were used for diversity analysis. The data were entered into binary matrix and subsequently analyzed using NTSYS pc version 2.10. Coefficients of similarity were calculated by using Jaccard's similarity coefficient by SIMQUAL function and cluster analysis was performed by agglomerative technique using the UPGMA method by SAHN clustering function of NTSYS-pc. Relationships between the 10 isolates of *X. axonopodis* pv. *punicae* were graphically represented in the form of dendrograms. The cophenetic correlation analysis was carried out using the COPH function of NTSYS-pc.

In this method the dendrogram and similarity matrix were correlated to find the goodness-of-fit of the dendrogram constructed based on the similarity coefficients. The matrix comparison was carried out using the MXCOMP function in the NTSYS pc version 2.02*i*.

2.5.2.4. Polymorphism percentage

The polymorphic percentage of the obtained bands was calculated by using the following formula,

$$\text{Polymorphism\%} = \frac{\text{No. of polymorphic bands}}{\text{Total bands}} \text{ X } 100$$

3. RESULTS AND DISSCUSION

Oily spot (bacterial blight) disease caused by *Xanthomonas axonopodis* pv. *punicae is* most destructive disease of Pomegranate (*Punica granatum* L.) causes qualitative as well as quantitative losses. The losses estimated were, 60 -70 % in the country [13].

A survey was conducted in year 2013-14 at pomegranate growing region of Maharashtra. A pathogen *X. axonopodis* pv. *punicae* (Xap) was purified from infected fruits and leaf sample and confirmed by proving their pathogenicity and PCR based by genus specific primer. The molecular variability was assessed by RAPD analysis. Result of this investigation is compiled, statistically interpreted and presented.

3.1. Collection, isolation and maintenance of isolates of *Xanthomonas axonopodis* pv. *punicae*

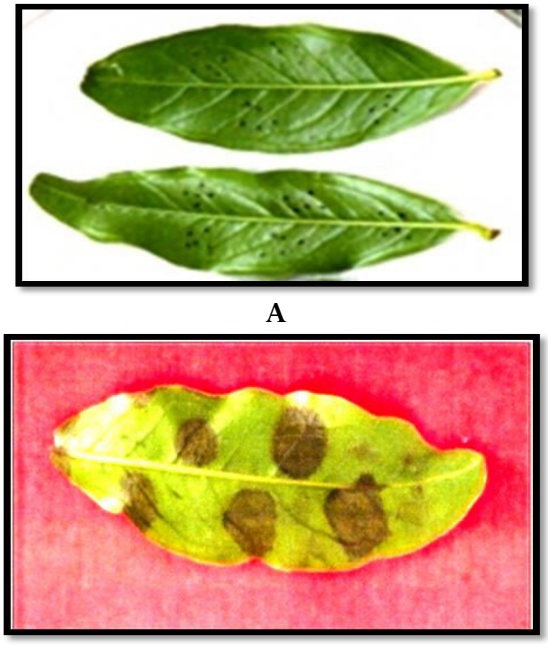
The oily spot infected samples of pomegranate were collected from Solapur, Latur, Osmanabad and Parbhani districts of Maharashtra. The isolation of oily spot pathogens was carried out on YGCA medium. Pathogen was isolated from infected leaves, fruit and flower parts collected from various pomegranate growing regions of

Maharashtra .These cultures were stored at 4°C and frequently sub-cultured.

Fig. 1: *Xanthomonas axonopodis* pv. *punicae*

3.2. Pathogenicity test

The 10 recognized isolates of *X. axonopodis* pv. *punicae* were collected from different pomegranate growing regions of Maharashtra, isolated and purified by single spore method. The pathogenic potential of all 10 isolates were proved on the pomegranate cv. Ganesh. Pure cultures of *Xanthomonas axonopodis* pv. *punicae* were inoculated on pomegranate cv. Ganesh, by using pinprick method (Fig 2.) [11]. A known concentration of aqueous cell suspension of bacterial inoculums was pinpricked to the pomegranate leaves placed in petriplates containing moistened absorbant paper. Control plant was inoculated by same way with sterile distilled water and maintained under humidity of 70- 80 %.



A

B

Fig. 2: Infected samples

The observations were recorded since 6 to 9 days after inoculation. Primarily, the symptoms appeared on leaves as irregular water soaked spots. Latter on these spots appeared on the leaves with yellow 'halos' on abxial side through rough texture. Further re-isolation of pathogen form infected leaves through single colony method was accomplished and compared with original culture.

The isolation, purification and pathogenicity test of *Xanthomonas* was done by several researchers. However, the isolation of such slow growing *Xanthomonas* pathogen is usually difficult due to the masking effect of fast growing, yellow pigmenting bacteria. Various types of media have been used for isolation. Among which yeast glucose chalk agar (YGCA), a selective medium was found suitable for isolation of *X. axonopodis* pv. *punicae*. Besides this, other medium have been tried by several researchers for isolation of this pathogen *viz*., yeast dextrose chalk agar medium [14] and nutrient agar medium [15]. The YGCA media was found best selective medium for isolation of pathogen *X. axonopodis* pv. *punicae*.

Similarly, the pathogenic potential isolates of *X. axonopodis* pv. *punicae* was proved by several researchers by using Celite powder method [16], Pin point method [17], Pin prick method [13] and syringe inoculation method [18]. Whereas, Pinprick method was found more efficient for completion of pathogenicity test of this pathogen.

3.3. DNA extraction

The 10 strains of *X. axonopodis* pv. *punicae* and four other species of *Xanthomonas* as described in Table 1, were grown overnight at 28°C on nutrient broth. Bacterial cells were harvested by centrifugation of liquid culture and pellet was subjected for enzymatic digestion by *proteinase*-K and SDS lysis. The *proteinase*-K and SDS method described by Adachi and Takashi [12], was found suitable and further employed with some modification.

The purity of DNA was checked by spectroscopic analysis at OD 260-280 nm. The OD 260:280 ratio was 1.8 indicating that DNA was sufficiently pure and free from contamination.

The good quality of DNA was assessed, by resolving on 0.8% agarose gel. Similarly, the quantification of DNA was done by comparing DNA samples with known amount of DNA i.e. lambda DNA. The concentration of DNA of all *Xanthomonas* strains was found in the range between 70-100 ng /µl. These DNA sample were

diluted with sterile water and used in molecular characterization study.

3.4. Standardization of PCR protocol

The PCR protocol described by Williams *et al.,* [19] was used with some modifications by using genus specific diagnostic primer for confirmation of 10 strains of *X. axonopodis* pv. *punicae*.

The PCR amplification reaction was optimized by varying concentration of PCR components. Amplification reaction was carried out in 25 µl reaction mixtures containing 30ng of genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM primers and 1.25 U of *Taq* DNA polymerase (Table 2). PCR amplification was performed in master cycler gradient, Eppendrof PCR, and the program consisted of an initial denaturing step at 94°C for 4 min, followed by 30 cycles of 1 min. at 94°C, 1 min. at 56°C and 1.5 min. at 72°C, and a final extension was set at 72°C for 10 min. Further, PCR products finally hold at 4°C. PCR amplified product was separated by electrophoresis on 1.2 % agarose gel stained with Ethidium bromide and visualized under gel documentation System.

3.5. RAPD fingerprint analysis

The RAPD fingerprint of all 10 *Xanthomonas* isolates were generated using five random primers namely OPB-6, OPB-7, OPB-8, OPB-10 and OPB-20 (Fig.3 to Fig. 7). Based on the banding profile scoring was done and data was presented in Table 4.

Table 4: List of RAPD primers used and the number of total polymorphic band produced in *Xanthomonas* strains

Primer	Sequence (5'-3')	Total No. of Bands	Total No. of Polymorphic Bands	Total No. of Monomorphic Bands	Polymorphic Percentage (%)
OPB-06	GATCATAGAT	89	12	4	20.68
OPB-07	CAGTACATGA	35	6	3	10.34
OPB-08	CACAGTGTGC	65	12	4	20.68
OPB-10	CAGTGATCAA	60	9	2	15.51
OPB-20	CGTCGTACAA	105	19	3	32.75
	TOTAL	354	58	16	100

Average polymorphism in strains of *X. axonopodis* pv. *punicae* based on RAPD analysis was 73.76%.

The average sizes of amplicons generated by all RAPD primer were found in the range between 100 bp to 6.0 kb. The RAPD-PCR amplification result showed that 5 RAPD primers generated total 354 bands, out of these, 58 bands were found polymorphic and 16 bands were reported as monomorphic with an average of 70.8 bands per primers among 10 strains of *Xanthomonas*. The primer OPB-06, OPB-20 and OPB-10 were more found informative as they could generate total number of 12, 12 and 14 bands respectively. The primer OPB-07 has generated lowest number of bands *i.e* 35. The primer OPB-20 has produced higher number of amplicons i.e. 105, followed by OPB-06 which has produced 89 amplicons. While the primer OPB-07 has produced minimum number of amplicons *i.e.* 35.

Overall, all 5 primers were generated total 354 amplicons with an average of 70.8 amplicons per primers. Out of 354 amplicons, 58 amplicons were found polymorphic, they showed 73.76 % polymorphism. Similarly, out of the total amplicons, 16 amplicons were found monomorphic.

They showed 4.5 % monomorphism and the average number of monomorphic amplicons per primer.

The dendrogram was dissipated through UPGMA cluster analysis by using software NTSYSpc 3.2. The dendrogram analysis based on RAPD fingerprint profile of five primers showed two major clusters. Cluster I comprised two sub cluster showing 47 percent similarity. The first sub cluster comprised four stains such as Xap-SB, Xap-NA, Xap-SM and Xap-PP. These strains were further divided into two groups at 65 percent similarity. The strain Xap-SB, and Xap-NA having 77 percent similarity were in one group. And remaining two strains Xap-SM and Xap-PP were comprised into another group at 77 percent similarity. In IInd major cluster Xap-AS was out grouped at 66 percent similarity from Xap-SS, Xap-LA1 and Xap-LA2.

The two strains i.e. Xap-SS and Xap-LA1 showing maximum similarity of 79 percent though they were from different region and remains in a single cluster.

The overall dendrogram result shows there is presence of three species of *X. axonopodis* pv. punicae in Maharashtra, India.

Table 5: Similarity matrix of 10 isolates of *X. axonopodis*.pv.*punicae* based on five RAPD primers

	Xap-SB	Xap-SW	Xap-NA1	Xap-NKBD	Xap-SM	Xap-SS	Xap-LA1	Xap-LA2	Xap-PP	Xap-AS
Xap-SB	1.00									
Xap-SW	0.58	1.00								
Xap-NA1	0.77	0.58	1.00							
XapNKBD	0.63	0.71	0.71	1.00						
Xap-SM	0.50	0.65	0.69	0.52	1.00					
Xap-SS	0.60	0.52	0.56	0.42	0.60	1.00				
Xap-LA1	0.65	0.50	0.69	0.52	0.54	0.79	1.00			
Xap-LA2	0.60	0.63	0.59	0.54	0.56	0.69	0.79	1.00		
Xap-PP	0.65	0.61	0.69	0.63	0.77	0.56	0.58	0.51	1.00	
Xap-AS	0.51	0.51	0.56	0.46	0.60	0.58	0.71	0.65	0.60	1.00

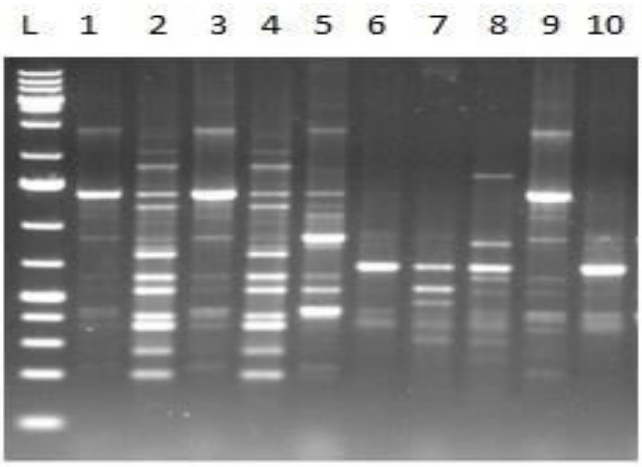


Figure 3

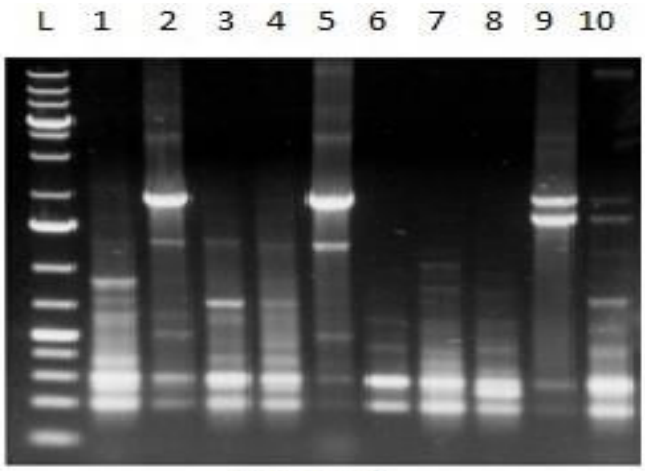


Figure 5

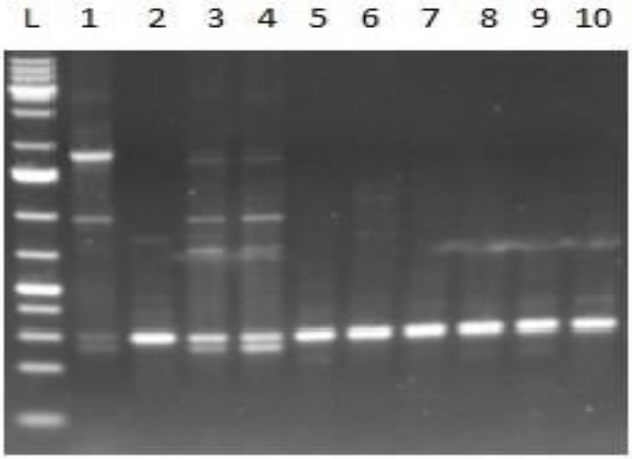


Figure 4

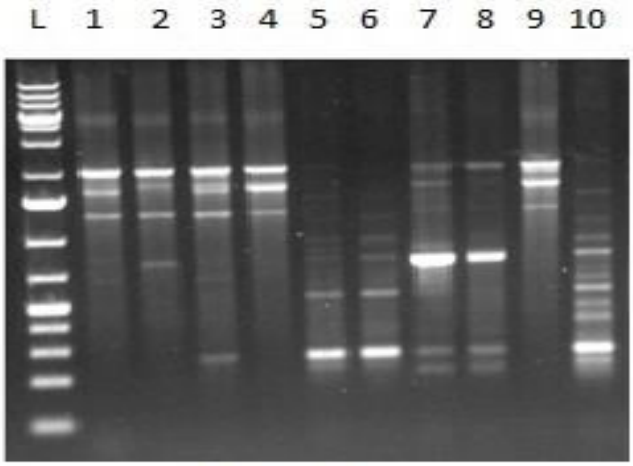


Figure 6

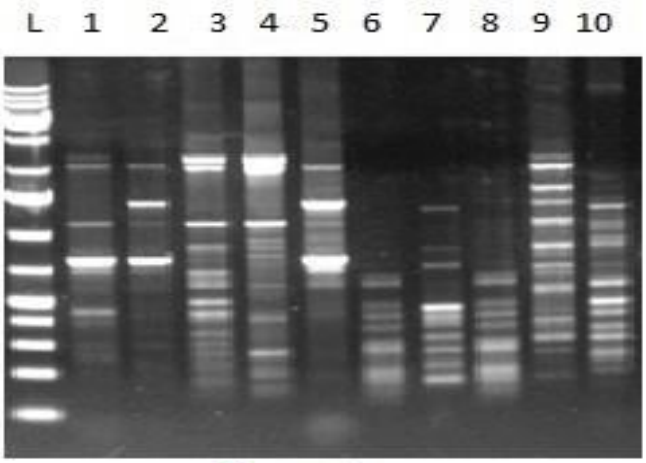


Figure 7

Fig. 3-7: RAPD fingerprint profile of 10 strains of *Xanthomonas* by using primer OPB-06, OPB-07, OPB-08, OPB-10 and OPB-20, L-l Kb Ladder, 1- Xap-SB, 2- Xap-SW, 3- Xap-NA1, 4- Xap-NKBD, 5- Xap-SM, 6- Xap-SS, 7- Xap-LA1, 8- Xap-LA2, 9- Xap-PP and 10- Xap-AS

3.6. Outcome

The bacterial disease, popularly known as bacterial blight or oily spot of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* severely affect the production of pomegranate in Maharashtra. The present investigation of *X. axonopodis* pv. *punicae* strains characterization leads to following outcome:

1. The genetic diversity among 10 starins *X. axonopodis* pv. *punicae* could add supporting data towards identification of races of this species at Latur, Osmanabad, Solapur and Parbhani districts of Maharashtra, India.
2. The DNA fingerprinting pattern generated by selected 10 strains collected from Maharashtra region having genetic variation and unable to separate as per their geographical location.
3. Genetic similarity among these strain would help in study of evaluation of races of *X. axonopodis* pv. *punicae.*
4. The primer OPB 20 was found most significant by producing 32.75% polymorphism and would be exploited for further study of races identification and development of species specific SCAR marker.
5. Information of pathogen diversity can be used in characterizing, developing and screening of resistant genotypes.

4. CONCLUSION

Pomegranate (*Punica granatum* L.) is an important fruit crop of India and other subtropical countries. Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae*, is becoming a major threat to pomegranate production in Maharashtra as well as other pomegranate growing states of India. It causes yield loss to the extent of 80 – 90 %. Considering importance of this disease, study regarding assessment of genetic of diversity and molecular marker development is accomplished.

A survey was conducted and 10 strains of *X. axonopodis* pv. *punicae* were collected and used for assessment of genetic diversity by using five RAPD primers. Whereas 10 strains of *X. axonopodis* pv. *punicae* were differentiated and distinguished into three major groups representing Solapur, Latur, Osmanabad and Parbhani districts of Maharashtra, India.

However the genetic similarity within 10 strains of *X. axonopodis* pv. *punicae* was ranged between 47 to 79 % similarity The combine data analysis by using UPGMA method also revealed, the three distinct group representing two of them were of strains of *X. axonopodis* pv. *punicae*.

This study revealed the valuable information towards developing species specific primer for early detection of *X. axonopodis* pv. *punicae* using molecular biotechnology tools.

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