Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2311

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **4 (3):** 160-166 (2016)

International Journal of Pure & Applied Bioscience

Research Article

OPEN ACCESS

Studies on Molecular Variability Among *Xanthomonas axonopodis* pv. *punicae* Isolates Collected from Different Locations

Swaranjali Kishor Gadhe[1]*, Suresh Haribhau Antre[2], Bhausaheb Babanrao Ghorpade[3], Rishikesh Haribhau Autade[4] and Rushil Ramesh Mandlik[5]

[1,2]Department of Biochemistry and Molecular Biology, College of Agricultural Biotechnology, Loni, ITI Campus, Chandrapur Road, Tal. - Rahata, Dist- Ahmednagar 413736, (M.S.), India

[3,4,5]Department of Plant Biotechnology, College of Agricultural Biotechnology, Loni, ITI Campus, Chandrapur Road, Tal. - Rahata, Dist- Ahmednagar 413736, (M.S.), India

*Corresponding Author E-mail: gadheswaranjali@gmail.com

Received: 7.06.2016 | Revised: 15.06.2016 | Accepted: 19.06.2016

ABSTRACT

Bacterial blight is one of the most important disease of Pomegranate caused by bacterium Xanthomonas axonopodis pv. punicae (Xap). The disease is characterized by small, irregular, translucent, water soaked spots with light to dark necrotic centre surrounded by prominent yellow margin on leaves. The isolates were made from the symptomatic samples collected from five different locations viz. Loni, Astagaon, Talegaon, Sangamner and Rahuri belonging to Ahmednagar district of Maharashtra state, India.

The variability was studied among the five isolates of Xanthomonas axonopodis pv. punicae by using 10 RAPD primers, out of which 07 primers produces total 26 scorable bands with an average of 3.7 bands per primer. Out of 26 bands, 21 bands were found to be polymorphic and level of polymorphism was 80.76%. The Jaccard's similarity coefficient showed that the isolate Loni (Code Xap I) was found to have higher value of similarity coefficient 0.88 with Astagaon (Code Xap II), whereas Talegaon (Code Xap III) was found to have lower value of similarity coefficient 0.20 with Rahuri (Code Xap V). The cluster analysis based on similarity coefficients divides the five isolates into two major and two sub clusters. These results indicate a high level of genomic variability among the isolates even within the same geographical regions.

Key words : *Bacterial blight, Pomegranate, polymorphism, RAPD, Xanthomonas axonopodis pv. punicae.*

INTRODUCTION

Pomegranate (*Punica granatum L.*) belongs to the family *Lythraceae*, is one of the favorite table fruit grown in tropical and subtropical regions of the world. Pomegranate is good source of carbohydrates and minerals such as calcium, iron and sulphur. It is rich in vitamin C and citric acid is the predominant organic acid in pomegranate[1].

Cite this article: Gadhe, S.K., Antre, S.H., Ghorpade, B.B., Autade, R.H. and Mandlik, R.R., Studies on Molecular Variability Among *Xanthomonas axonopodis* pv. *punicae* Isolates Collected from Different Locations, *Int. J. Pure App. Biosci.* **4(3):** 160-166 (2016). doi: http://dx.doi.org/10.18782/2320-7051.2311

According to National Horticultural Board of India, the total area under cultivation of Pomegranate in India is 131.00 thousand ha and production is around 1346.00 thousand tons in 2013-14.

In Pomegranate plant, diseases can be found in various parts such as fruit, stem and leaves like bacterial blight, Anthracnose and wilt complex. Among which bacterial blight is the most severe disease of the Pomegranate, caused by bacterium *Xanthomonas axonopodis p*v. *punicae* (*Xap*) (Hingorani and Singh) Vauterin *et al*. Bacterial blight of Pomegranate was first reported in India from Delhi (1952)[2] and later from Bangalore (1959)[3]. It was noticed the epidemics of bacterial blight of pomegranate causing 60 to 80 % losses at Indian Institute of Horticultural Research (IIHR) experimental plots[4].

The disease symptoms can be initially found on stem part which gradually pervades to leaves and then to fruits. The disease is characterized by appearance of small, 2 to 5 mm diameter, irregular, prominent water soaked spots which later become necrotic with light to dark brown center on leaves. The dark brown to black spots also can be seen on stems, often leads to cracking and breaking of branches. The lesions on fruits appears as irregular, brown to black spots with Y-or-L shaped cracking or splitting of pericarp which reduces the marketability of fruits.

The bacterium were small rods, appeared singly, rarely in pairs, Gram negative, non-capsulated and no spore forming with monotrichous flagellation[5]. Earlier studies showed the molecular variability in different isolates of *Xap* [6-9].

The present investigation was carried out to understand molecular diversity in *Xanthomonas axonopodis* pv. *punicae* isolated from different parts of Ahmednagar district of Maharashtra state, India.

MATERIALS AND METHODS

Collection of diseased sample and isolation of pathogen :

The diseased leaves samples of Pomegranate showing typical symptoms of bacterial blight were collected for isolation of bacterium *Xanthomonas axonopodis* pv. *punicae* from five different locations *viz.* Loni, Astagaon, Talegaon, Sangamner and Rahuri belonging to Ahmednagar region of Maharashtra state during December 2014 - January 2015. The isolation of bacterium was carried out by tissue isolation method on Nutrient Sucrose Agar (NSA) medium at 25 to 28 ˚C for 3-5 days.

Biochemical characterization of the pathogen:

The identification of the pathogens causing oily spot in Pomegranate was determined by conducting studies on its morphological, biochemical and physiological features of the pathogen. Biochemical tests *viz.* Gram's reaction, Starch hydrolysis, Indole Production, Catalase test, KOH test, Gelatin liquefaction and Acid production etc. were carried out for biochemical confirmation of *Xanthomonas axonopodis* pv. *punicae* as per standard microbiological procedures.

Maintenance of bacterial culture :

The developed well separated typical, bright yellow, mucoid colonies on plate were further streaked onto the agar slants containing the NSA medium and incubated at 25 to 28 ˚C for 3 days. Then cultures were stored in the refrigerator at 4 ^{0}C, which served as a stock culture for further studies. The isolates were designated as per different locations *viz.* Loni (*Xap* I), Astagaon (*Xap* II), Talegaon (*Xap* III), Sangamner (*Xap* IV) and Rahuri (*Xap* V).

Molecular variability :

Extraction of total genomic DNA :

Single colony of each isolate incubated for 72 hours old NSA plate, was inoculated in 10 ml of nutrient broth. The flasks were kept for incubation at 28 ^{0}C for 72 hours with vigorous shaking at 120 rpm. About 1.5 ml aliquots of broth culture were taken in 2.0 ml eppendorf tubes and centrifuged at 13000 rpm for 5 minutes. The supernatant was poured off, 200 µl of lysis buffer was added to the tubes containing pellet and mixed well. Further 66 µl of 5 M NaCl was added and mixed well, later contents were centrifuged at 13000 rpm for 10 minutes. Supernatant (250µl) obtained was transferred to a new tube, to which 1 µl RNase (20mg/ml) was added, mixed well and incubated at 37 ^{0}C for 30 minutes. An equal volume of chloroform isoamyl alcohol was added, mixed gently by

inverting the tubes, centrifuged at 13000 rpm for 6 minutes. The upper aqueous phase was transferred to a clean tube to which 1.0 ml of cold 95 % ethanol was added and gently mixed. The tubes were then kept in deep freezer at -20 ^{0}C for overnight and then centrifuged at 13000 rpm for 6 minutes. Ethanol was poured off and DNA pellet was air dried for five minutes. Pellet was resuspended in 50 µl of 1X TE buffer then kept in the refrigerator at 4 ^{0}C for overnight and stored in deep freezer at -20^{0}C. 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.

Random primers and PCR amplification :

Molecular variability was detected among five isolates of *Xanthomonas axonopodis* pv. *punicae* using RAPD marker. Ten random primers (GeNei^{TM} Bangalore) were selected for genetic diversity study. The PCR amplification for RAPD analysis was performed with certain modifications[10]. The reaction mixture was containing 2.5 µl 10x assay buffer with 17.5 mM $MgCl_2$, 1.0 µl dNTPs mix (2.5 mM each), 1.0 µl Primer (5 pM/µl), 1.0 µl Template DNA (25 ng/µl), 14.30 µl Sterile distilled water, 0.2 µl *Taq* DNA polymerase (5.0 U/µl). The PCR amplifications were performed using Thermal Cycler (Eppendorf, Master cycler gradient, Germany). Initial denaturation of 94°C for 10 min followed by 40 cycles at 94°C for 1 min, annealing at 37°C for 1 min, primer extension at 72°C for 2 min and a final extension at 72°C for 10 min was given at the end of 40th cycle. All amplified DNA products were resolved by electrophoresis on agarose gel (1.5%) in TAE (1X) buffer at 60 volts for 2 h stained with Ethidium bromide (0.5 µg /ml). The gel was visualized through a U.V. transilluminator and images were taken by using Gel documentation system (BioEra^{TM} Pune).

Scoring of amplified fragments :

The amplified profiles for all the primers were compared with each other and bands of DNA fragments were scored as '1' for presence and '0' for absence, generating '0' and '1' matrix. % polymorphism was calculated by using the formula, Number of polymorphic bands / Total number of bands x 100.

Pair-wise genetic similarities between isolates were estimated by Jaccard's similarity coefficient. Clustering was done using the symmetric matrix of similarity coefficient and cluster obtained based on Unweighted Pair Group Arithmetic Mean (UPGAM) using Sequential Agglomerative Hierarical Nested (SAHN) cluster analysis of NTSYS-PC version 2.0 [11].

RESULTS AND DISCUSSION

Isolation and biochemical characterization of the pathogen :

Five isolates showing typical characters of *X. axonopodis* pv. *punicae* with yellow mucoid shining colonies were obtained on NSA medium. The isolates of *X. axonopodis* pv. *punicae* were purified and used for further studies. The isolated bacteria were shown negative reaction for gram staining. The bacteria were positive for Starch hydrolysis, Indole Production, Catalase test, KOH test, Gelatin liquefaction, Acid production test with varying degree of reaction (Table 1.).

Molecular variability :

The detail profile of DNA bands of different primers for five isolates of *X. axonopodis* pv. *punicae* is given (Table 2). Total 10 RAPD primers were used to detect the genetic diversity, out of them 07 primers exhibited good amplification with scorable bands. A total of 26 DNA bands were detected by 07 primers, of which, 21 bands were found polymorphic **(**Plate 1 and Plate 2). Among these primers, OPG-01, OPF-09, OPA-02 and OPC-11 were shown 100 % polymorphism. Remaining OPA-16, OPA-11 and OPH-01 were shown 80%, 75%, 66.67% polymorphism respectively. The results of RAPD analysis revealed that a total of 80.76% polymorphism was found between the isolates, indicating molecular variability among the *X. axonopodis* pv. *punicae* isolates.

Information on the banding pattern for all the primers were used to determine genetic distance between the isolates and to construct a dendrogram by using unweighted pair group method (UPGMA)[8]. Based on the Jaccard's similarity coefficient a genetic similarity matrix was constructed to assess the genetic relatedness among the five isolates. The similarity coefficient value ranged from 0.20 to 0.88 across

five isolates indicating high degree of genetic variation. This ultimately means high range of genetic diversity among the isolates studied. The highest genetic similarity coefficient to an extent of 0.88 was recorded between *Xap* I and *Xap* II isolates followed by 0.72 between *Xap* II and *Xap* III isolates. Least genetic similarity coefficient 0.20 was observed in between *Xap* III and *Xap* V.

The dendrogram constructed by UPGMA for RAPD analysis shows that isolates can be grouped into two major clusters *viz.* A and B (Fig.1). Cluster A divided into two sub clusters namely cluster A1 and cluster A2. Sub cluster A1 shows three isolates, *Xap* I (Loni), *Xap* II (Astagaon) and *Xap* III (Talegaon). Out of these, *Xap* I and *Xap* II shows 0.88 similarity coefficient, whereas *Xap* III shows 0.68 similarity coefficient with *Xap* I and *Xap* II. Sub cluster A2 having single isolate *Xap* IV (Sangamner) showing 0.53 similarity coefficient. The cluster B distinct from cluster A containing single isolate *Xap* V (Rahuri) shows 0.38 similarity coefficient value. The RAPD profiles generated using single decamer primers in polymerase chain reaction was studied to detect the diversity in *Xanthomonas campestris* pv. *campestris* population prevalent in the region[12]. The genetic variability studied in Maharashtra and Delhi strains of pomegranate bacterial blight pathogen through ERIC-PCR. The results indicated a high level of genomic variability among the isolates even within the same geographical regions[6-8] and same in *Xanthomonas citri* subsp. *citri* strains isolated from different provinces of Iran[13]. The ISSR analysis of *Xanthomonas axonopodis* pv. *punicae* isolated from different locations formed the separate clusters with different isolates showing diversity[9].

Table 1. Biochemical characteristics of *Xanthomonas axonopodis* pv. *punicae* isolates

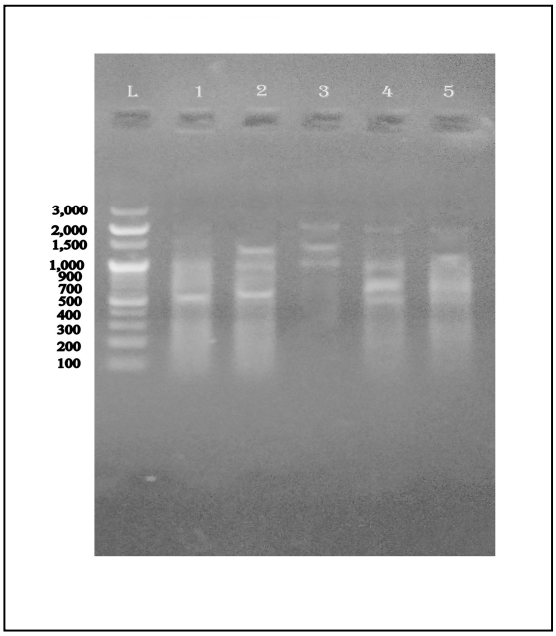
Sr. No.	Biochemical Tests	*Xap* I	*Xap* II	*Xap* III	*Xap* IV	*Xap* V
1.	Gram's reaction	-ve	-ve	-ve	-ve	-ve
2.	Starch hydrolysis	+	++	+++	++	+
3.	Indole production	+	+	++	+	+++
4.	Catalase test	++	+	+++	+++	++
5.	KOH test	++	++	++	+	+++
6.	Gelatin liquefaction	++	+++	+	+	++
7.	Acid production	++	+	++	++	+++

- negative
+ positive

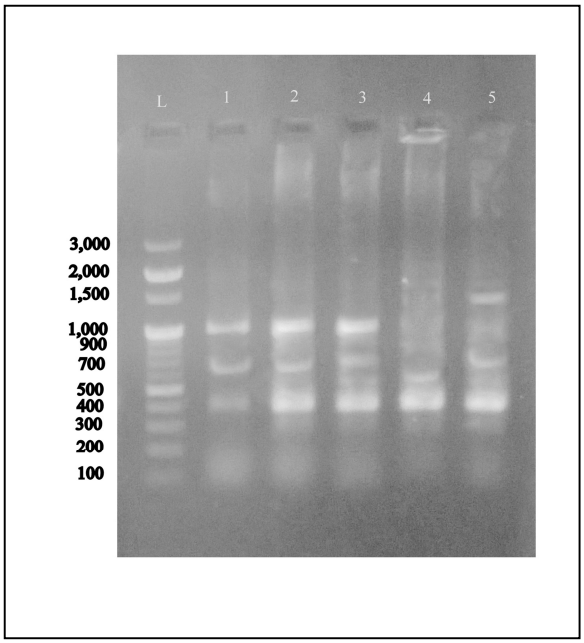
Varying degree of + ve reaction
: + poor
: ++ moderate
: +++ strong

Table 2. DNA banding profile and percent polymorphism observed in primers

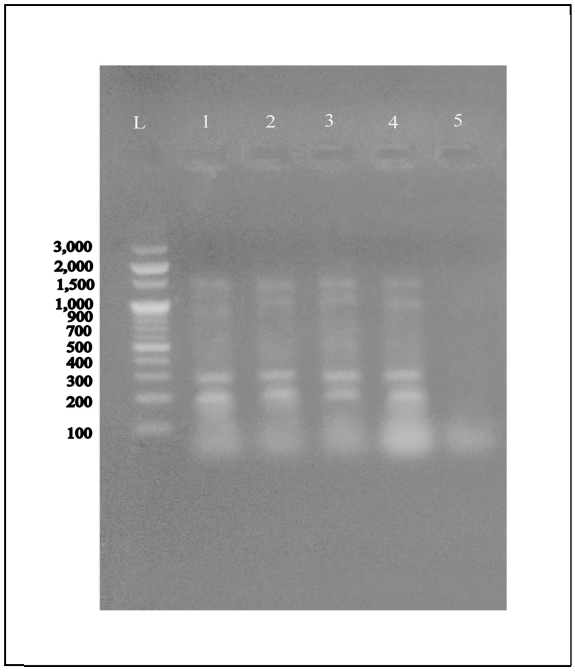
Sr. No.	Primer Code	Primer sequence (5' – 3')	Total bands	Polymorphic bands	% Polymorphism
1.	OPG-01	CTACGGAGGA	4	4	100
2.	OPA-16	AGCCAGCGAA	5	4	80
3.	OPF-09	CCAAGCTTCC	4	4	100
4.	OPA-02	TGCCGAGCTG	4	4	100
5.	OPA-11	CAATCGCCGT	4	3	75
6.	OPC-11	AAAGCTGCGG	2	2	100
7.	OPH-01	GGTCGGAGAA	3	2	66.67
	Total		**26**	**21**	**80.76**



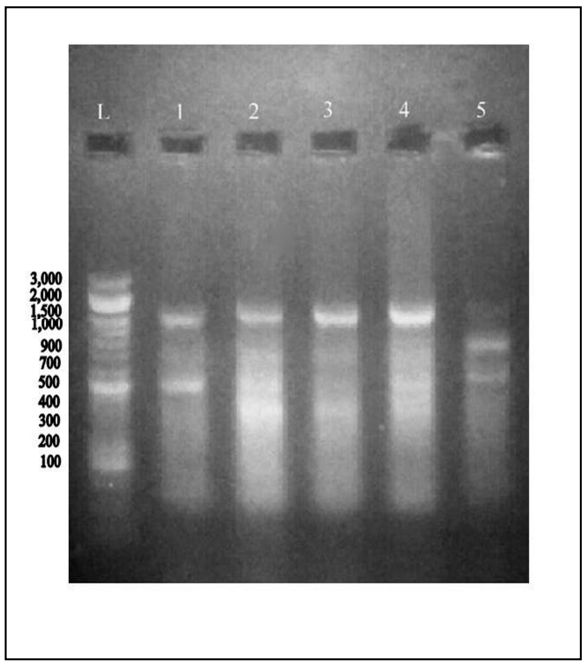
OPG-01



OPA-16

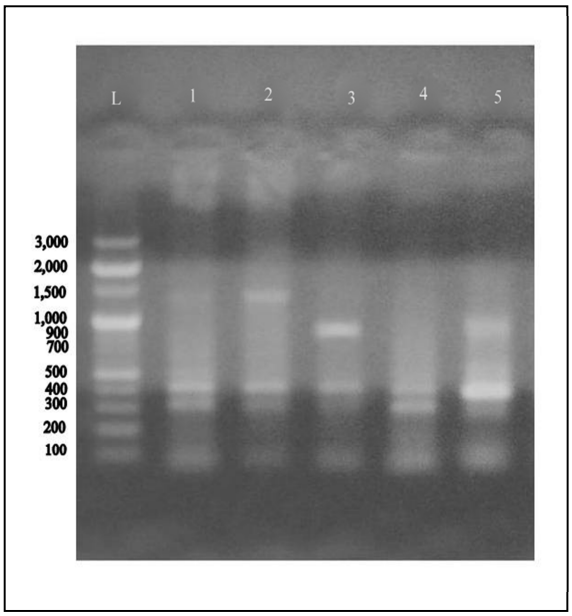


OPF-09

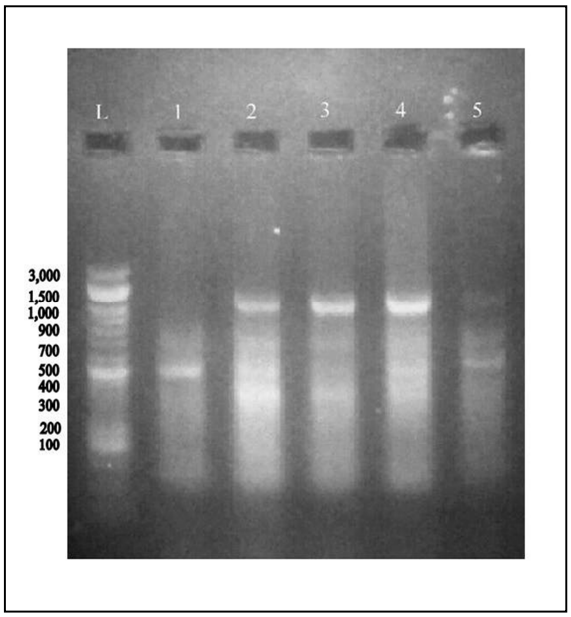


OPA-02

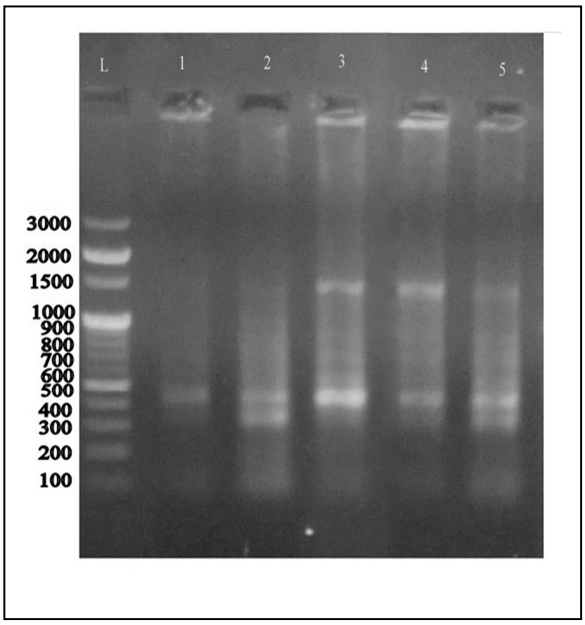
Plate 1. Banding pattern of five isolates of *X. axonopodis* pv.*punicae* by RAPD method



OPA-11



OPC-11



OPH-01

L : 1 kb Ladder

1 : *Xap* I (Loni)

2 : *Xap* II (Astagaon)

3 : *Xap* III (Talegaon)

4 : *Xap* IV (Sangamner)

5 : *Xap* V (Rahuri)

Plate 2. Banding pattern of five isolates of *X. axonopodis* pv.*punicae* by RAPD method

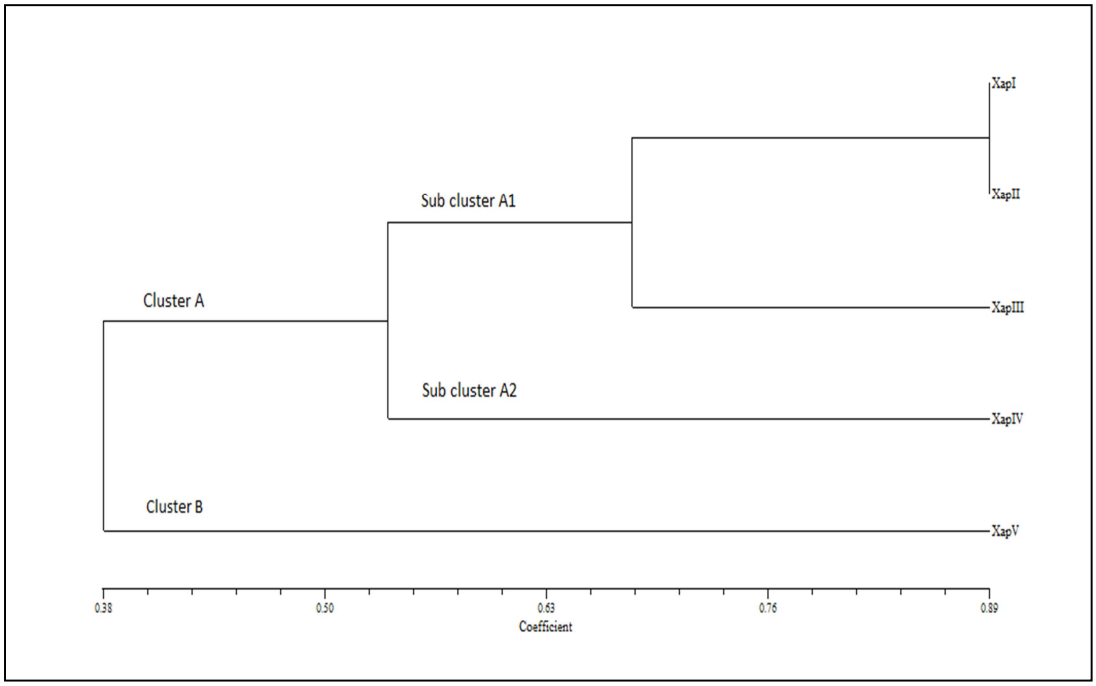


Fig. 1. Dendrogram based on RAPD analysis of five isolates of *X. axonopodis* pv. *punicae*

CONCLUSION

On the basis of the present study, it could be concluded that the population of bacterial blight pathogen *X. axonopodis* pv. *punicae* in Ahmednagar are genetically heterogeneous and showed high level of genetic variability within a same geographical regions from RAPD profiles.

ACKNOWLEDGMENT

The authors would like to thanks Principal and HOD of Biochemistry and Molecular Biology, College of Agricultural Biotechnology, Loni (M.S.) for providing necessary facilities for research work.

REFERENCES

1. Malhotra, N. K., Khajuria, H. N. and Jawanda., *Punjab Horti. J.*, **23 :** 158(1983).
2. Hingorani, M. K. and Mehta, P. P., *Indian Phytopathol.*, **5:** 55-56 (1952).
3. Hingorani, M. K. and Singh, N. J., *Indian J. Agric. Sci.*, **29 :** 45-48 (1959).
4. Chand, R. and Kishun, R., *Indian Phytopath.*, **44 :** 370-371 (1991).
5. Manjula, C. P., M.Sc. (Agri.) Thesis, Univ. Agric. Sci., Bangalore, Karnataka (India) (2002).
6. Mondal, K.K. and Mani, C**.,** *Curr. Microbiol*. **59:** 616- 620 (2009).
7. Yenjerappa, S. T., Ph. D. Thesis, Univ. Agri. Sci. Dharwad (India) (2009).
8. Giri, M. S., Prashanthi, S. K., Kulkarni, S., Benagi, V. I. and Hedge, Y. R., *Indian Phytopath.* **64 (1):** 2-6 (2011).
9. Raghuwanshi, K. S., Hujare, B. A., Chimote, V. P. and Borkar, S. G., *The Bioscan* **8 (3) :** 845-850 (2013).
10. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingry, S. V., *NUC. Acid. Res*., **12 :** 6531-6535 (1990).
11. Rohlf, F.J., NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, Version 2.2. Exeter Software. Setauket, New York. (2000).
12. Siraree, A., Yogesh, K., Negi and Kumar, J., *Nation. Symp. On Crop Surveillance : Disease Forecasting and Management*, IARI, New Delhi (India), February 19-21, pp. 48 (2004).
13. Rezaei, M K., Shams-Bakhsh M. and Alizadeh A., *J.Pl. Prot.Res.***52 (1) :** 1-9 2012).