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Assessment of the genetic diversity among oily spot *(Xanthomonas axonopodis pv. punicae)* pathogen of pomegranate by randomly amplified polymorphic DNA analysis

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Abstract: 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 in Maharashtra as well as other states of India. It causes yield losses up to the extent of 80 – 90 %. This study aims to the collection of 7 strains (*X. axonopodis)* and utilized for assessment of genetic diversity by using RAPD markers. Whereas 7 strains of *X. axonopodis* were differentiated and distinguished into seven major region representing Malegaon, Nampur, Satana, Khakurdi, Ramnagar, Rahuri and Nashik region. However the genetic similarity within 7strains of *X. axonopodis* was ranged between 72.72-100 %. Similarly the genetic relationship of strains *X. axonopodis* was also established and showed 72.72 %, 72.72 %, 72.72 % 92.30%, 94.11% and 100 % similarity with region of Malegaon, Nampur, Satana, Khakurdi, Ramnagar, Rahuri and Nashik region. Average polymorphism in strains of *X. axanopodis* based on RAPD analysis was 84.095 %. The combine data analysis by using UPGMA method also revealed the two distinct groups representing seven strains of *X. axonopodis* and in first group M1 and second group R4 and subgroup RH6, N2, NS7, S3, K5, these genotypes were present respectively. Considering predominance and economic loss caused by *X. axonopodis* pv. *punicae* in pomegranate, it is an essential to undertake preliminary work on characterization of this pathogen at the molecular level.

Keywords: Bacterial blight, Diversity, Oily spot pathogen, Pomegranate, RAPD

INTRODUCTION

Pomegranate (*Punica granatum* L.) is a favourite table fruit in tropical and sub-tropical regions of the world which belongs to family *Lyrthaceae*, having 2n=16 number of chromosome. The most popular varieties suitable for processing and table use of pomegranate are Ganesh, Mridula, Arakta, Bhagwa, Supar-Bhagwa. Total area for growing pomegranate is increasing worldwide mainly because of wider adaptability, stress tolerance, higher yield levels, excellent keeping quality and prices benefits in both domestic as well as export markets. India is the leading pomegranate producer which contributes nearly 50% of the world's production. Pomegranate is being cultivated on 63,000 hectares area of 5.00 lakh tonnes in India with production of 8.20 lakh tonnes. Incidence of oily spot disease of pomegranate (Teliya) caused by *Xanthomonas axonopodis* pv. *punicae* (*Xap*), is the major problem and limiting factor in Maharashtra state which alone accounts for loss of 80% of 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. The consumer preference for this fruit is because of its attractive, juicy, sweet, acidic and refreshing arils. Moreover nowadays there is a growing trend for quality fruits for table purpose as well as in processing industries for juice, syrup and wine. Among the diseases of pomegranate, the bacterial disease, also known as bacterial blight or oily spot caused by *X. axonopodis* (Hingorani and Mehta, 1952 and Vauterin *et al.*, 1995) is a major threat which severely affects production of this crop. The species designation of pomegranate blight bacterium has recently been changed from *X. campestris* pv. *punicae* to *X. axonopodis* based on gyrB sequence (Parkinson *et al.*, 2009). The bacterial blight disease caused by *X. axonopodis* is a major threat for pomegranate cultivation (Mondal and Sharma, 2009; Mondal and Singh, 2009; Petersen *et al.*, 2010; Mondal *et al.*, 2012). It is a serious disease in the states of Karnataka, Maharashtra and Andhra Pradesh and causes losses to the extent of 60 to 80% in India (Mondal and Mani, 2009).The disease symptoms appearing on all plant parts, initially small, irregular, grayish black, water soaked lesions were observed on leaves which enlarged with the progression of the dis-

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ease. Similarly blackish lesions were observed, on nodes of this stem later on resulting in canker formation with depressed growth. Disease symptoms were also reported prominent on developed fruit where the lesion enlarged coalesced and covered the entire fruit surface (Sharma, 2006; Sharma *et al.*, 2008).

The disease spreads from infected orchard to healthy one, through pomegranate cuttings. The disease become predominant and appearing in epiphytotic form during last five years in pomegranate growing states of South-West India, including Maharashtra and Karnataka. Nevertheless, the disease also spread rapidly to the states of Rajasthan, Gujarat, parts of Haryana and Uttar Pradesh due to the bulk movement of planting materials (cuttings) of most popular and bacterial blight susceptible cultivar, Bhagwa from Maharashtra (Kumar *et al*., 2009; Mondal and Sharma, 2009; Mondal *et al*., 2012).The control of plant pathogens relies on the ability to identify infecting species. There had been several studies regarding the use of different molecular markers for identifying the genetic diversity of different *Xanthomonas* species. RAPD (Randomly Amplified Polymorphic DNA) provided a simple, rapid, and reliable method to identify genetic variation. RAPD-PCR utilizing arbitrary oligonucleotides to prime DNA synthesis at low annealing temperature was performed to divulge genomic diversity (Versalovic and Lupski, 2002). Simliarly, (Abdo-Hasan *et al*., 2008) examined genetic diversity of *X. axonopodis malvacearum*, the causal agent of cotton angular leaf spot disease by RAPD fingerprint method. RAPD primer has identified 7 isolates with the majority having several unique bands. They concluded that, analysis of genomic DNA using RAPD was a suitable typing method which was fast, sensitive, and reliable for determining genetic relationships among isolates of *X. a*. pv. *Malvacearum.* By Considering the severity of this pathogen, it is essential to develop diagnostic primer in order to restrict the spread of the *X. axonopodis* pv. *punicae.* It would be useful to generate disease free planting material and also exploited to ensure the presence of this pathogen in to suspected planting materials. Therefore, keeping in view these facts, the present study was proposed on genetic diversity analysis and development of DNA based molecular markers for detection of oily spot pathogen of pomegranate.

MATERIALS AND METHODS

Collection, isolation and identification of *Xanthomonas* strains from infected sample: The investigation was undertaken to assess development of DNA based marker for identification *X. axonopodis* causing oily spot disease of pomegranate. This study was conducted at Department of Biochemistry & Molecular Biology, K. K. Wagh College of Agril Biotechnology, Nashik, affiliated to MPKV, Rahuri. The oily spot infected samples of pomegranate were collected from Malegaon, Nampur, Satana, Khakurdi, Ramnagar, Rahuri and Nashik region of Maharashtra state as the list given in Table-1. The pathogen *X. axonopodis* was isolated from leaves, fruits samples. Infected lesions was cut by using sterilized scalpel blade followed by sterilization with 0.1 % $HgCl_2$ and 70 % ethanol for 15 second, then thoroughly rinsed with sterile distilled water. The resulting suspension was streaked on yeast glucose calcium carbonate agar (YGCA) plates and incubated at 28°C in an incubator for 48-72 hr. Single colonies having circular, convex, mucoid, shiny and yellow morphological characteristics of *X. axonopodis* were picked by sterilized loop and purified cultures were obtained by streaking on fresh yeast glucose calcium carbonate agar (YGCA) medium. The bacterial isolates were subjected to conventional diagnostic tests using standard protocols for cultural, physiological, and pathogenic characterization as shown in Table-2 .

Genomic DNA extraction: Genomic DNA was isolated by method of Murray and Thompsons(1980) with slight modifications. The cells were pelleted at 10,000 rpm for 2 min. The cell pellet was lysed in 30 µl of 10 % SDS and incubated for 1 hr. at 37°C. To remove proteins and cell debris about 100 µl of 5 M NaCl were added and mixed again. Then Add 80 µl of CTAB/NaCl solution.The mixture was suspended at 12,000 rpm for 10 min. Remove aqueous, viscous supernatant to a fresh micro-centrifuge tube, Add an equal volume of phenol/chloroform/isoamyl alcohol, and spin in a microcentrifuge for 5 min. After transferring the supernatant to a fresh tube add 0.6 volume of isopropanol to precipitate the nucleic acid. Wash twice with the 70% ethanol and re-spin for 5 min at room temperature to re-pellet it. Carefully remove the supernatant and briefly dry the pellet and re-dissolve the pellet in 100 µl TE buffer.

PCR based assay identification of *Xanthomonas* strains: The general PCR procedure has been described by Sambrook *et al.* (1989). The PCR amplification was performed with a thermal cycler (Master Cycler, Eppendorf,) in a 25 µl reaction mixture containing 1 µl of genomic DNA, 0.5 µmol l^{-1} of each primer, 0.25 mmol l^{-1} of each deoxynucleoside triphosphate, and 1 µl of reaction buffer (1.5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1}KCl, 10 mmol l^{-1}Tris-HCl, pH 8.8).The 7 strains of *X. axonopodis* was confirmed by using genus specific diagnostic primers:OPB-06 (5' GATCATAGAT 3'), OPB-19(5' TGCATGCAGC 3'), OPB-20(5' CGTCGTACAA 3') OPC-01(5' GCAGCATGCA 3'), OPC-02(5' GTCAGTGCAA 3') OPC-08(5' CAGTCAGTAA 3') by Gupta *et al.,* (2001). Genomic DNA of each isolates of *X. axonopodis* was added to the individual tubes containing the master mixture. The following cycling conditions were used.1 cycle of denaturation for 1 min at 94°C and 29 cycles consisting of denaturation at 94° C for 45 sec. annealing at 56°C for 45 sec., and extension at 72°C for 1 min, final extension at 72°C for 5min. The amplified DNA was detected by electrophoresis in 1% agarose gels (HiMedia, India) in 1× TAE buffer (40 mmol L-1 Tris-acetate, 1 mmol L-1 EDTA, and pH 8.0).

Table 1. Strains of *X. axonopodis* used in the study.

S. N.	Isolate name	Sample type	Location of isolate
1	*Xap*-MC	Fruit	Chandanpuri (Malegaon)
2	*Xap*-SN	Fruit	Nampur (Satana)
3	*Xap*-S	Fruit	Satana
4	*Xap*-MK	Fruit	Khakurdi(Malegaon)
5	*Xap*-MR	Fruit	Ramnagar (Malegaon)
6	*Xap*-AR	Fruit	Rahuri (Ahemednagar)
7	*Xap*-N	Leaf	Nashik

Table 2. Microbiological test for different strains of *X. axonopodis.*

S. N.	Test name	Bacterial organism in the fruit sample						
		Xap (MC)	Xap (SN)	Xap (S)	Xapb (MR)	Xap (MK)	Xap (AR)	Xap (N)
1	Gram Staining	+	+	+	+	+	+	+
2	Motility test	+	+	+	+	+	+	+

Above all tests were compared with Bergeys Manual of Bacteriology

Determination of genetic variability by using RAPD analysis: Genomic DNA from the different strains was used as a template for PCR fingerprinting using random primers *viz.*, OPB and OPC series (eurofins), namely OPB-06 (5' GATCATAGAT 3'), OPB 19(5' TGCATGCAGC 3') OPB 20(5' CGTCGTACAA 3'), OPC 01(5' GCAGCATGCA 3'), OPC-02 (5' GTCAGTGCAA 3')OPC-08 (5' CAGTCAGTAA 3'), for RAPD analysis of 7 strains of *X. axonopodis* described in table 1. Genomic DNA of each strain of *Xanthomonas* species was added to the individual tubes containing the master mixture. The reaction condition maintained are one cycle of 94°C for 4 min,followed by 39 cycles of 94°C for 1min (denaturing), 35°C for 1 min (annealing), and 72°C for 1.5 min (extension). A final extension was carried out at 72°C for 10 min. The PCR profiles were visualized by electrophoresis in 1.5% agarose gels and staining with ethidium bromide.

RESULTS AND DISCUSSION

A survey was conducted in year 2013-14 at pomegranate growing region of Maharashtra. A pathogen *X. axonopodis* 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 and the data have been utilized for the development of DNA based marker for identification of *X. axonopodis*.

Pathogenicity test: The 7 putative isolates of *X. axonopodis* were collected from different pomegranate growing regions of Maharashtra, isolated and purified by single spore method. The pathogenic potential of all 7 isolates was proved on the pomegranate cv. Bhagwa. Pure cultures of *X. axonopodis* were inoculated on pomegranate cv. Bhagwa, by using pinprick method. A known concentration of aqueous cell suspension of bacterial inoculums was pinpricked to the pomegranate leaves in healthy tree pot containing moistened absorbent paper. Control plant was inoculated by same way with sterile distilled water and maintained under humidity of 70- 80 %. The observations were recorded since 6 to 9 days after inoculation. Primarily, the symptoms appeared on leaves as irregular water soaked spots.

Later on these spots appeared on the leaves with yellow 'halos' on axial 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 *X. axonopodis* was done by several researchers. However, the isolation of such slow growing *X. axonopodis* 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.* Besides this, other medium have been tried by several researchers for isolation of this pathogen *viz*., yeast dextrose chalk agar medium (Jyoti *et al.*, 2005) and nutrient agar medium (Manjula and Khan, 2003). The YGCA media was found best selective medium for isolation of pathogen *X. axonopodis.* Similarly, the pathogenic potential of isolates of *X. axonopodis* was proved by several researchers by using Celite powder method (Raut *et al.*, 1993), Pin point method (Kanwar, 1976), Pin prick method (Dhutraj and Soryawansbi, 2010) and syringe inoculation method. Whereas Pinprick method was found more efficient method for completion of pathogenicity test of this pathogen The average sizes of amplicons generated by all RAPD primer were found in the range between 25 bp to 10 kb. The RAPD-PCR amplification result showed that 6 RAPD primers generated total 74 bands, out of these, 66 bands were found polymorphic with an average of 12.33 bands per primers among 7 strains of *Xanthomonas*. The primer OPC-01, OPC-08, OPC-02 and OPB-19 were more found informative as they could generate total number of 11, 11, 13 and 14 bands, respectively. The primer OPB-06 has generated highest number of bands i.e. 17. RAPD PCR fingerprint profile of primer OPC-02 and OPB-06 enabled to differentiate 7 strains of *X. axonopodis* depending upon their origin. These primers also

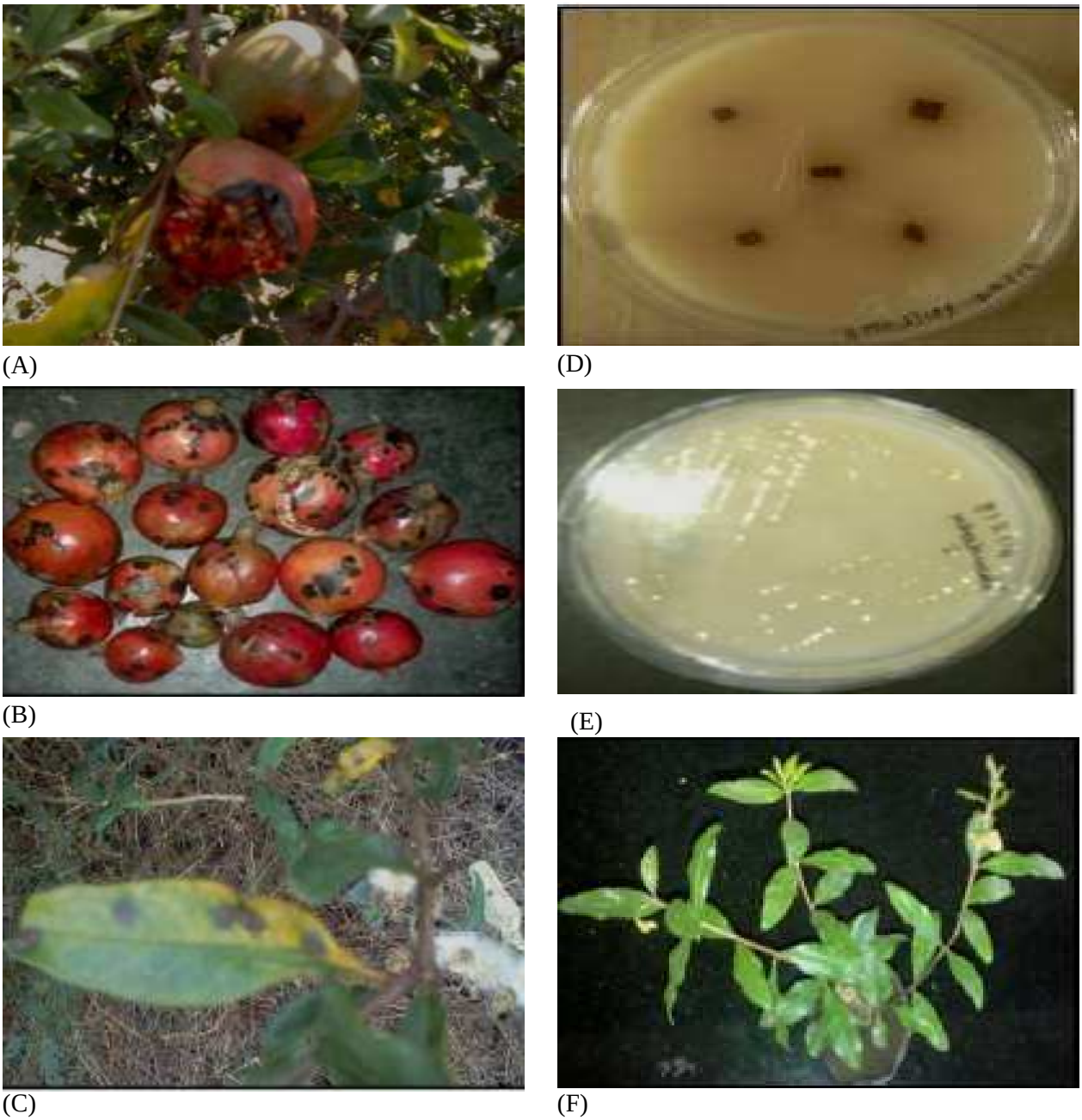

Fig. 1. *A- Khakurdi region infected fruits B- Nampur region infected fruits C- Nasik region infected leaf D- Nasik region isolation of pathogen E Nampur region -isolation of pure pathogen F Pot showing healthy plant before inoculation.*

categorized 7 strains of *X. axonopodis* specific to their origin.

Similarly, the comparative study of 7 strains of *X. axonopodis* based on RAPD analysis established their genetic relationship with each other. The data obtained by RAPD markers was analyzed by NTSYS pc2.02i and dendrogram was depicted by using Jaccard's similarity coefficient. Dendrogram generated based on UPGMA analysis of RAPD data grouped all these isolates in to two major clusters. These clusters were formed on the basis of genus as well as species level. Thus dendrogram depicted on RAPD data could assess genetic diversity and relationship existed among 7 strains of *X. axonopodis*. RAPD analysis is accepted to be a proven tool to distinguish variability between organisms or even between different strains of same organisms.

Abdo-Hasan (2008) also examined genetic diversity of *X. axonopodis* pv. *Malvacearum* , the causal agent of cotton angular leaf spot disease and identified seven islates with several unique bands by RAPD fingerprint method. Similarly during this course of investigation, inter species as well as intra-species variability on genetic ground was assessed by using 6 RAPD primers. The primer OPB-06has generated a common amplicons of size 0.5-1.4 kb in all 7 *Xanthomonas* strains could be used as diagnostic marker for identification of

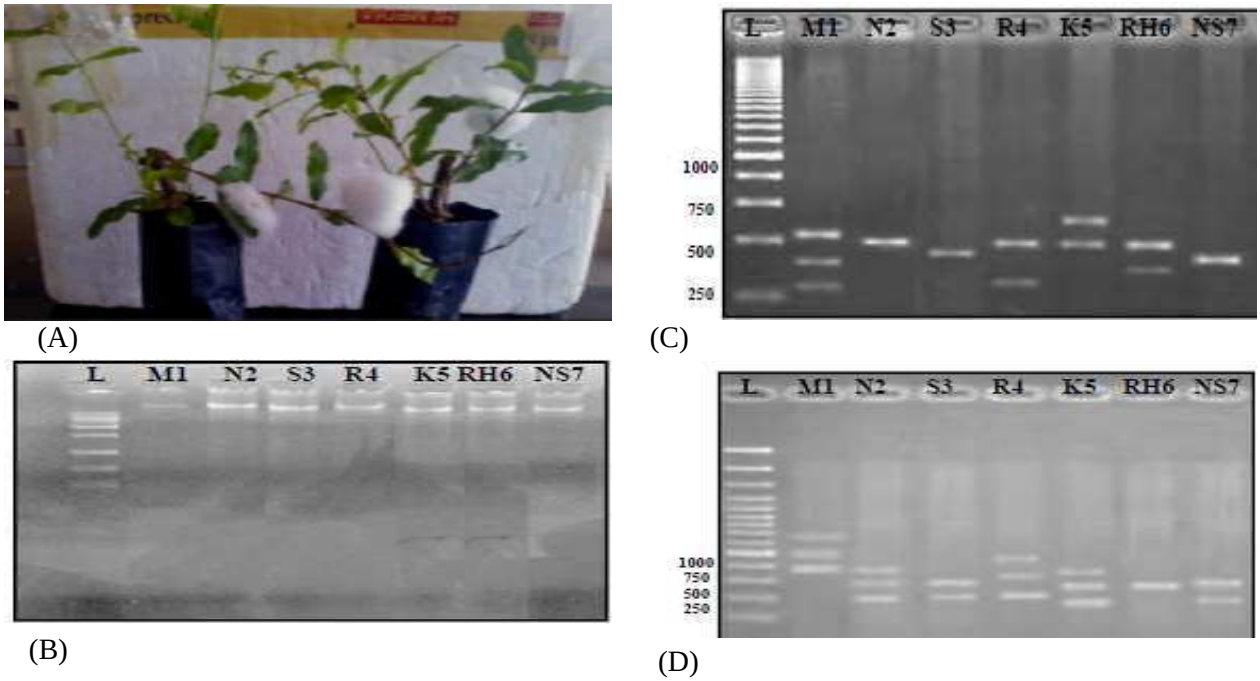

Fig. 2: ***A****- Infected plant pot 6 - 9 days post inoculation* ***B****- Genomic DNA isolation from different isolates of Xanthomonas species. L: (1Kb ladder)* ***C****- RAPD analysis of different.*

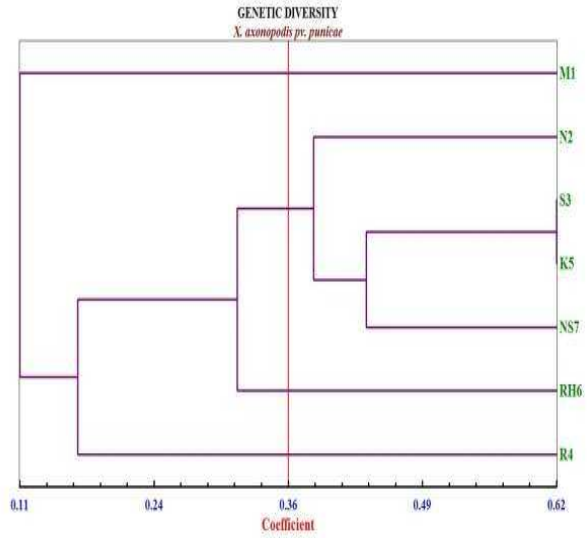

Fig. 3: *Dendrogram of seven strains of Xanthomonas axonopodis pv. punicae.*

genus *Xanthomonas* Primer OPC-02 and OPB-06 could separate Xap strains as per their geographical origin. The fragment commonly shared by isolates from specific region would be exploited for development of region specific diagnostic marker for *X. axonopodis.* Manulis *et al.* (1994) developed a species-specific primer for detection of *X. compesteis pv. Pelargonii* by RAPD based marker. Similarly, primer OPB-06 has also generated a unique amplicons as per geographical origin of isolates. An attempt was made by Mondal *et al.,* (2012) for development of a polymerase chain reaction (PCR) based detection of blight pathogen of pomegranate by using primers designed on gyrB gene. In a similar study by Sabin et al (2012) genetic diversity among seven isolates of *Xanthomonas* representing four species by using RAPD and ISSR PCR-based techniques. Similar type of work was carried out by Pavel and Edivanio et al.,(2013) for the development of a PCR based method for specific detection of *X. pv. vesicatoria*, a causal agent of bacterial spot of tomato and pepper.

Combined data analysis: The dendrogram generated based on UPGMA analysis of RAPD primer data grouped, all *Xanthomonas* isolates in to two major clusters and Genetic Diversity 0.11 up to 0.62. Cluster I comprised of seven strain of *X. axonopodis* in that cluster Malegaon region showing (0.11) distinct similarity with each other 7 strain. Cluster II comprised that Ramnagar strain of Xap together showing distinct or divers similarity with each other. The cluster II showed isolate of Satana and Khakurdi was showing similar or identical 0.62 with each other. The cluster II comprised a lone strain of Nampur, Rahuri and Nashik which was very distant similarity. The percent polymorphism and genetic similarity showed by 7 strains of *X. axonopodis* were graphically discussed and represented. A genetic similarity matrix was constructed using the similarity of coefficient. The highest value for similarity index was obtained for Khakurdi and Satana (0.62) and Ramnagar with Satana and Khakurdi having lowest similarity 0.00.

Conclusion

The present study on the pomegranate growing region of Maharashtra particularly Malegaon, Nampur, Sa-

tana, Khakurdi, Ramnagar, Rahuri and Nashik revealed that, RAPD primer was found efficient and suitable genetic typing method for discrimination of strains of *X. axonopodis*. There might be existence of four major strains of *X. axonopodis* representing from Malegaon region, Satana region and third strain was found from Rahuri and Nashik region. Existence of variations among the strains of these regions might exist due to their genetic evolution, prevalence to the respective region, mutational changes and climatic conditions etc.

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