Research Article

Estimation of Diversity Among Various Isolates of *Xanthamonas Axonopodis* pv. *Cyamopsidis* Using Molecular Markers

G.L. Kakraliya[1]*, N.K. Khatri[2], M.K. Jat[3], S. Choudhary[3], and A.L. Yadav[3]

[1]Department of Plant Pathology, College of Agriculture, Bikaner
[2]Department of Plant Pathology, Agriculture Research Station, SKRAU, Bikaner-334006 (Rajasthan)
[3]Department of Plant Pathology, S.K.N. college of Agriculture, Jobner

Abstract

In the present study four primers used, had generated a significant information. The number and size of amplicons depended on primers and strains used. Though, most amplicons were smaller than 3kb in size but one of the amplification product was larger than 5kb. Our study showed that the regional *Xanthomonas axonopodis* pv. *cyamopsidis* population had a high degree of genetic diversity. In this study, strain Nokha was not clustered with other strains either in the phytogenetic cluster or the RAPD analysis. Nokha and Bikaner isolates belonging to equally distant places were most diverse one. In conclusion it can be said that the *Xanthomonas axonopodis* pv. *cyamopsidis* depicts a very high diversity in harsher climate of Rajasthan probably for its survival.

Keywords: Clusterbean, bacterial blight strains, diversity, molecular marker

***Correspondence**
Author: G.L. Kakraliya
Email: glkpp1234@gmail.com

Introduction

Clusterbean [*Cyamopsis tetragonoloba (*Linn.) Taub.] locally known as 'Guar' is a legume of Indian origin is attacked by more than 30 fungal, bacterial and viral pathogens. Among them bacterial blight caused by *Xanthomonas axonopodis* pv. *Cyamopsidis* [9], is the most important and wide spread disease. It appears all most every year and may cause considerable damage depending upon the variety and environment conditions. In case of heavy infection, it may cause 58-68 per cent losses in grain yield[1]. Thus there is an urgent need to search for economic and eco-friendly alternative strategies for the management of this disease. Recently greater emphasis on stable source of resistance and other useful control measures viz., biological, cultural and botanicals have been laid[2]. However their efficient employment would require information on genetic diversity present in the pathogen.

The traditionally applied techniques for diversity studies based on morphological and biochemical traits need tedious culture and analytical procedures. More recently following.

DNA fingerprinting is an ideal method and has been widely used for specific identification of many organisms, including bacteria. The PCR based random amplified polymorphic DNA (RAPD) method, one of the most successful methods of DNA fingerprinting, can be used to generate distinctively amplified patterns for different tested species.

RAPDs have been successfully used to study bacterial blight communities [3]. Looking to the possibility that RAPD markers can be successfully used for characterization of bacterial blight communities, present investigation was taken up to find out effect of annual plant species like clusterbean on bacterial diversity in arid regions of Rajasthan. An attempt has been made in the present study to determine the genetic diversity present in the *Xanthomonas axanopodis* pv. *Cyamopsidis* in a vide cluster bean grown region.

Materials and Methods

For the isolation of causal bacterium, young diseased portions/lesions were cut into small bits and rinse with spirit and immediately transferred into mercuric chloride solution (0.01%) for 15 seconds and were finally passed through 3-changes of sterile distilled water for surface sterilization. The bits were then transferred individually on flame sterilized, coded glass slide containing drop of sterilized water under aseptic conditions. The lesion were cut into 2 halves with heat sterilized razor blade and the slide was left for few minutes (2-5 min) to permit the diffusion of bacteria from tissue into the water drops. A loopful of suspension was streaked - quadrantly on previously sterilized

petriplates containing nutrient agar (NA) and potato sucrose peptone agar (PSPA) media with the help of sterilized inoculation needle. The inoculated plates were incubated at 27± 2°C for over night. The bacterial colonies appeared on the surface of the medium like straw yellow droplets. The single fresh and pure colony was picked up under a stereoscopic microscope, with sterilized inoculation needle and transferred on other petriplates having NA and PSPA medium. The process was repeated until all the colonies developed on the medium had similar cultural appearance. Such culturally identical single colony was then transferred on yeast glucose chalk agar storage medium slants, and maintained by preserving in a refrigerator for all future experiments, and making periodic transfers after every fortnight. The stock culture was kept in refrigerator after covering culture slants with sterilized mineral oil.

The experimental material for the present investigation consisted of six strains of isolates of *Xanthomonas axonopodis* pv. *cyamopsidis* which was collected from different locations e.g. Bikaner, Hisar, Durgapura (Jaipur), Udaipur, Jobner and Nokha.

RAPD analysis: RAPD analysis was conducted using purified DNA from each accessions, in order to determine genetic diversity among genotypes taken for study.

DNA isolation: Bacterial colonies were Stretched from peteriplates and dissolved in 1 ml $T_{10}E_1$ in appendrof. Centrifuged at 5000 rpm – 3 minutes, 40C. Added 800 -1000 µl (600C) DNA. Isolation buffer [2x CTAB DNA etraction buffer-100 mM Tris 20 mM EDTA, 1.4 M NaCl, 2% CTAB and 2 µl/ml (β-mercaptoethanol). To the isolation buffer 100 µl of 20% SDS was also added incubated for 1 hr at 60 ^{0}C with occasional mixing by genetle swirling in water bath. After removing from water bath one volume of chloroform: Isoamyl alcohol (24:1) was added and mixed by inversion for 15 minutes to ensure emulsification of the phase. It was then centrifuged at 15000 rpm for 10 minutes to recover aqueous phase in another tube. Ice cold 0.6 vol. of isopropanol was added to a aqueous phase to precipitate DNA. DNA-CTAB complex was precipitated as a fibrous network. It was lifted by Pasteur pipette and was transferred to washing solution. 20 ml of 70% alcohol was added to pellet and was kept for 20 minutes with gentle agitation. The pellet was collected by centrifugation at 10,000 rpm for 5 minutes at 20 ^{0}C. the tubes were inverted and drained on a paper towel. The pellet was dried over night after covering with parafilm with tiny holes. The pellet was re-dissolved in 100 µl of $T_{10}E_1$ buffer by keeping over night at room temperature.

Purification of DNA

RNA was removed by treating the sample with DNase free RNase. Protein including RNase was removed by treating with chloroform: Isoamyl alcohol (24 :1) treatment. Presence of single compact band at the corresponding band of λ phage DNA indicates high molecular weight of isolated DNA on 0.8% agarose gel.

The quantification of DNA was done by observing it at 260 nm and 280 nm wave lengths by using a spectrophotometer (UV visible from UNICAM). The quantified DNA was diluted to final concentration of 25 ng/µl in $T_{10}E_1$ buffer (10 mM Tris Cl, 1 mM EDTA, pH 8.0).

RAPD analysis

Random amplification of polymorphic DNA was done by using 10 primers of OPG series obtained from "OPERON TECHNOLOGIES" (Inc. Alameda, Calif). PCR reaction were performed in final volume of 25 µl containing 10 X Assay Buffer ("Bangalore Genei") 0.5 units of Taq./dilution DNA polymerase ("Bangalore Genei"), 200 µM each of dNTPs ("Bangalore Genei"), 10 pmols/reaction of random primer's (OPERON TECHNOLOGIES) and 50 ng of tempelate DNA. The PCR was performed in 'Biometra Thermocycler' using the following cycling parameters.

Cycle 1 : Denaturation (940C) 5 minutes. Primer annealing (370C) 1 minute. Primer Extension (720C) 2 minutes. Cycle 2-43 : Denaturation (940C) 1 minute. Primer annealing (370C) 1 minute. Primer Extension (720C) 2 minutes. Cycle 44: Denaturation (940C) 1 minute. Primer annealing (370C) 1 minute. Primer Extension (720C) 7minutes

Following the amplification, the PCR products were loaded on 1.2% Agarose gel (Merk) which was prepared in 1 x TBE buffer containing 0.5 µg/ml of the ethidium bromide. The Amplified products were electrophoresed for 4-5 hrs at 50 V with cooling. After separation the gel was viewed under UV transilluminator and photographed by digital camera.

Results and Discussion

Total genomic DNA was isolated with the CTAB method described by [4] with some modification and treated with RNase to eliminate RNA. DNA concentration was measured by UV absorbance method and the quantity of DNA

ranged from 32 µg/ml to 108 µg/ml of culture (Table 1). The integrity of isolated DNA was verified by visualization of DNA on agarose gel (0.8%) with DNA standard uncut lambda DNA. The appearance of single band corresponding to the size of lambda uncut confirms high molecular size of DNA isolated. The quality of DNA was further determined for the presence of contaminants as the ratio of A_{260}/A_{280}, ranged from 1.73 to 2.00 which is indicative of good quality bacterial DNA and the ratio was almost consistent irrespective of the *Xanthomonas axonopodis* pv. *cyamopsidis* strains.

The total genomic DNA isolated using SDS supplemented (TAB method yielded satisfactory quantity, 32 µg/ml to 108 µg/ml from one petri plate) The DNA was of high molecular weight mostly visible in single band corresponding to λ DNA and A260/A280 ratio was also between 1.73 to 1.90 indicating contamination free preparation.

All the six strains of *X. axonopodis* pv. *cyamopsidis* were examined for random amplified polymorphic DNA (RAPD) genetic markers with 20 decamer primers (Operon Technology, USA) (Table 1) [5,6]. Primers of OPG series were screened taking two strains, only four primers giving consistent results were used for final amplification (OPG-3, OPG-4, OPG-5, OPG-19).

All the four primers were thus repeated at least twice to confirm the polymorphism[7]. This is because the stoichastic nature of primer annealing has been known to change the band and banding pattern of DNA amplification with RAPD. Reproducibility of the banding pattern has been found to change.

Finally, only those bands were considered as polymorphic, which did not amplify in accessing on repetition. The banding pattern generated by each primer was primer and strain dependent and varied from 7-13 at 370C annealing temperature. A total of 38 amplicons were obtained with 4 primers with an average of 9.5 bands per primer. All the 38 bands were found to be polymorphic and the level of polymorphism was 100 per cent (**Table 1**, **Figure 1**).

Identified markers were generated all of the isolates by various primers OPG-4 and OPG-5 generated maximum isolate amplicons 4 isolates (Table 2).

Table 1 List of primers and polymorphic amplicons generated

Primers	Sequence (5'-3')	Total No. of bands (a)	No. of polymorphic bands (b)	Polymorphism b/a x 100 (%)
OPG-3	GAGCCCTCCA	8	8	100
OPG-4	AGCGTGTCTG	7	7	100
OPG-5	CTGAGACGGA	13	13	100
OPG-19	GTCAGGGCAA	10	10	100
	Total	**38**	**38**	**100**

Table 2 Primer that gave diagnostic markers for individuals accessions or groups of strains

Primer	Strains	Marker size (bp)	Present (+) or absent (-)
OPG-3	Job	5148	+
	Nok	564	+
OPG-4	Udr	3514	+
	Dur	2351	+
	His	820	+
	Job	564	+
OPG-5	Dur	1620	+
	Nok	1400, 230, 110	+
	Bik	640, 400, 150	+
	His	310	+
OPG-19	Bik	1904	-
	Udr	1010	+

The Jaccard's pair wise similarity coefficient values estimated on the basis of RAPD patterns for six *Xanthomonas axonopodis* pv. *cyamopsidis* isolates revealed a high degree of diversity among the isolates (**Table 3**). The range of diversity was observed to be 57% between Job and Dur) to 92 % among Bik and Dur isolates while average being 79%. The highest similarity coefficient 0.43 between Job and Dur was followed by 0.40 among His and Udr isolates. However, least similar isolates were Bik and Dur (0.08) followed by Nok-His and Nok-Udr (0.09).

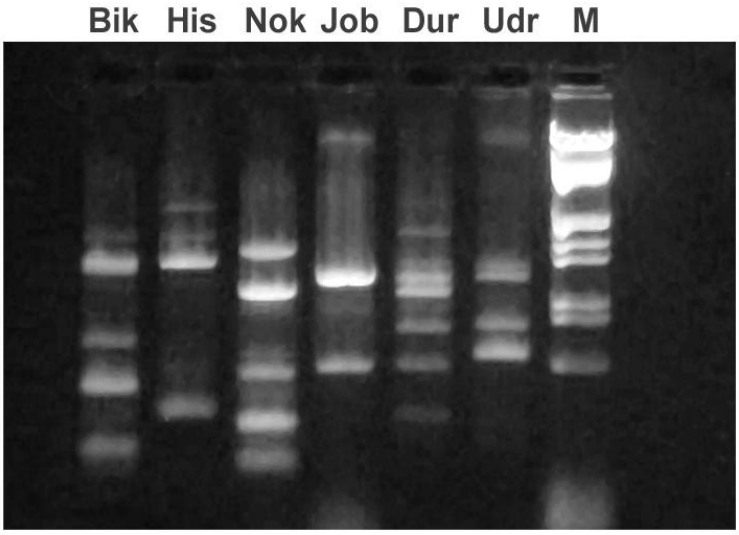

Figure 1 RAPD profile generated by primer OPG-5 in six *Xanthomonas axonopodis* pv. *Cyamopsidis* strains

Table 3 Jaccard's similarity coefficient among six isolates of *Xanthomonas axonopodis* pv. *cyamopsidis* based on RAPD

Isolates	Bik	His	Nok	Job	Dur	UDR
Bik	1.00					
His	0.19	1.00				
Nok	0.11	0.09	1.00			
Job	0.17	0.28	0.17	1.00		
Dur	0.08	0.29	0.13	0.43	1.00	
Udr	0.21	0.40	0.09	0.25	0.26	1.00

Genetic similarity estimates (Jaccard's coefficient) were further used to group the isolates using UPGMA (Un-weighted Pair Group Method for Arithmetic Average) algorithm. The genetic relation ships (clusters) so obtained have been presented in the form of dendrogram (**Figure 2**). The isolates could not form obvious groupings. However, four isolates (His, Udr, Dur and Job) obtained from relatively higher rainfall regions were comparatively more related than the two isolates (Bik and Nok) belonging to dryer regions. Nevertheless, similarity within group of four isolates was much less (~28%). The isolates from Nokha and Bikaner were most diverse.

The number and size of amplicons depended on primers and strains used. Though, most amplicons were smaller than 3kb in size one of the amplification product was larger than 5kb. RAPD marker system has been indicated to amplify fragments smaller than 3 kb by various authors [8] and are known to depend on primer and species used [9].

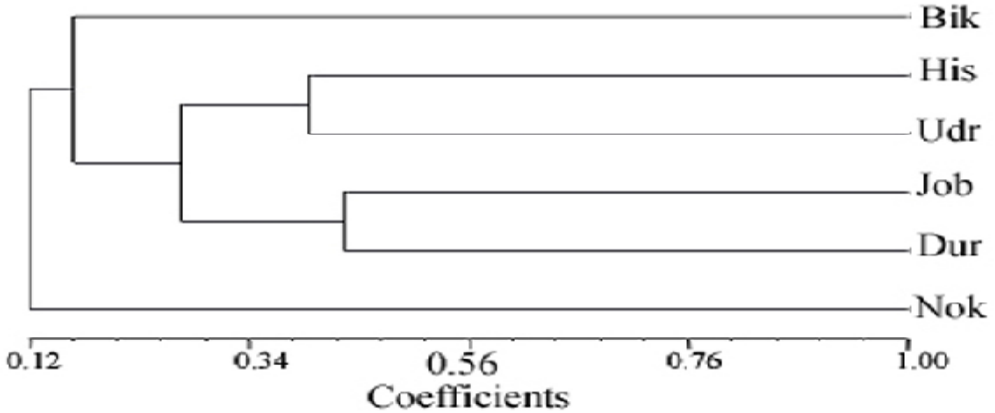

Figure 2 Dendogram generated on six Xanthomonas axonopodis pv. Cyamopsidis strain using UPGMA cluster analysis based on Jaccard's similarity coefficients for RAPD data

The diverse origin of isolates from various places was reflected in very high diversity (average being 79%) obtained in the form of Jaccard's similarity coefficients. Though a high level of diversity among isolates belonging to different regions have been reported such a high diversity level has not been reported in *Xanthomonas* spp [10]. Job and Dur, the isolates collected from near by locations i.e. Jobner and Durgapura respectively though showed more closeness it was not the general trend followed in the present study. Nok and Bik isolates belonging to equally distant places were most diverse one. Overall, clustering was not conspicuous as within group diversity was more than between group diversity as evident by dendrogram. However, little similarity between isolates belonging to places having better rainfall (His, Udr) was evident. Nevertheless as stated earlier Nok and Bik isolates belonging to drier regions of Rajasthan (Nokha and Bikaner respectively) were most diverse ones. In conclusion it can be said that the *Xanthomonas axonopodis* pv. *cyamopsidis* depicts a very high diversity in harsher climate of Rajasthan probably for its survival.

References

[1] Gandhi, S.K. and Chand, J.N. 1985. Yield losses in guar due to bacterial blight caused by X. campestris pv. cyamopsidis. Indian Phytopath. 38 (3) : 516-519.

[2] Gupta, D.K. 1977. Evaluation of antibiotics and fungicides against bacterial blight of guar (Cyamopsis tetragonoloba (L) Taub) Veg. Sci. 4 : 25-27.

[3] Patel, M.K., Dhande, G.W. and Kulkarni, Y.S. 1953. Bacterial leaf spot of clusterbean. Curr. Sci. 22 : 183.

[4] Ausubel, F.M., Brent, R., Kigdom, R.E., Moore, D.D., Smith, J.A., Sidemon, S.G. and Strutid, K. 1992. Current protocols in molecular biology. Greene Publishing Associates, Wiley Interscience, New York.

[5] Clerc, A., Manceau, C. and Nesme, X. 1998. Comparison of randomly amplified polymorphic DNA with amplified length polymorphism to assess genetic diversity and genetic relatedness within genospecies. Microbiology 64 : 1180-1187.

[6] Louws, F.J., Rademaker, J.L.W. and de Bruijn, F.J. 1999. The three DS of PCR based genomonic analysis of phytobacteria, diversity detection and disease diagnosis. Annu. Rev. Phytopatho. 37 : 81-125.

[7] Fani, R., Damiani, G., Diserio, C., Gaillari, E., Friioni, A. and Bazzicalupo, M 1993. Use of random amplified polymorphic DNA (RAPD) for generating specific DNA probes for microorganisms. Mol. Ecol. 2 : 243-250.

[8] Kaur, B., Purkayastha, S., Dilbaghi, N. and Chaudhary, A. 2005. Characterization of Xanthomonas axonopodis pv. cyamopsidis, the bacterial blight pathogen of clusterbean using PCR-based molecular markers. J. Phytopathol. 153 : 470-478.

[9] Permaul, K., Pillay, D. and Pillay, B. 1996. Random amplified polymorphic DNA (RAPD) analsysis shows intraspecies differences among Xanthomonas albilineans strains. Cell Appl. Microbio. 23 : 307-311.

[10] Manulis, S., Valinsky, L., Lichter, A. and Gabriel, D.W. 1994. Sensitive and specific detection of Xanthomonas campestris pv. pelargonii with DNA primers and probes DNA analysis. Appl. Environ. Microbiol. 60 : 4094-4099.

Publication History
Received 08th Mar 2017
Revised 20th Mar 2017
Accepted 20th Mar 2017
Online 30th Mar 2017