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Characterization of *Xanthomonas* species causing bacterial leaf spot disease of pepper (*Capsicum annuum*) in India

G S RAMYASHREEDEVI[1], DINESH SINGH[2], ARPITA SRIVASTAVA[3], K K BISWAS[4] and A K GUPTA[5]

ICAR-Indian Agricultural Research Institute, New Delhi 110 012

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ABSTRACT

The present study was under taken to characterize and identify prominent species of *Xanthomonas* causing bacterial leaf spot disease on pepper and their genetic diversity in India. Thirty one isolates of Xanthomonads isolated from infected leaf and fruit samples of tomato, chilli and bell pepper from 5 different states of India, viz. Himachal Pradesh, Karnataka, Uttarakhand, Tamil Nadu and Haryana were characterized as translucent, yellow, raised colonies on YGCA medium, Gram negative, rod shaped, positive in starch hydrolysis, variation in pectin utilization. These isolates showed symptoms on chilli cv. Pusa Jwala and tomato cv. Pusa Ruby and they were grouped into pepper-tomato group (XCVPT), belonged to *Xanthomonas euvesicatoria.* Two sets of primers, i.e. Bs-XeF and Bs- XeR and Xeu 2.4 and Xeu 2.5 specific to *X. euvesicatoria* were used for further confirmation and the primers amplified DNA of all the isolates at 173bp and 208 bp respectively. Genetic diversity of 31 isolates of *X. euvesicatoria* along with four out group bacteria *P. fluorescence* DTPF-3, *X. campestris* pv. *campestris* Xcc-4, and Xcc-C23 and *B. subtilis* DTBS-5 was done by REP, ERIC and BOX- PCRs fingerprinting. Out of 12 DNA types, maximum isolates of *X. euvesicatoria* (12 isolates) belong to DNA type 2 isolated from tomato plants from different states of India and formed separated group isolated from pepper. High level of genetic diversity among the *X. euvasicatoria* isolates was observed. All out group bacteria were separated from *X. euvesicatoria* isolates.

Key words: Bacterial leaf spot, Genetic diversity, Pepper, Tomato, *Xanthomonas* sp.

Capsicum spp. are prone to various fungal, bacterial and viral diseases and among them bacterial leaf spot caused by *Xanthomonas* spp. is one of devastating diseases leading to significant crop losses up to 23–44% (Bashan *et al.* 1985) which once it spreads, is very difficult to manage especially in regions with warm and humid climate. In India, for the first time the disease was reported from Puna (Maharastra) in 1948; then it has also been reported from Kerala, Karnataka, Uttar Pradesh, Himachal Pradesh, Uttarakhand and Maharashtra (Chand *et al.* 1994).

Bacterial leaf spot of tomato and pepper caused by *X. campestris* pv. *vesicatoria* was proposed by Dye (1978). Later, the pathogen was categorized into four groups, i.e. A, B, C and D based on phenotypic and phylogenetic characters (Vauterin *et al.* 1995, Jones *et al.* 2004). Groups A and C are transferred into a new species–pathovar combination, *X. axonopodis* pv. *vesicatoria,* whereas group B, which showed clear distinctive features, was named as *X. vesicatoria* and three new species were proposed as *X. gardneri*, for group D strains, *X. euvesicatoria*, for group A and *X. perforans*, for the group C on the basis of DNA homology (Jones *et al.* 2000). The main host plants of *X. vesicatoria*, *X. euvesicatoria* and *X. gardneri* are both tomato and pepper, whereas *X. perforans* infects mainly tomato (Jones *et al.* 2004) but there is one report on affecting pepper (Schwartz *et al.* 2015). Kornev *et al.* (2007) have characterized and identified Xanthomonands causing bacterial leaf spot on tomato based on the physiological and biochemical characters and they identified as *X. vesicatoria* and *X. gardeneri*. They also reported that six carbon sources like starch, pectin, D-galactose, dextrin, cisaconitic acid, Cisacontic can be used to differentiate among four species. The occurrence of three species of *Xanthomonas*, i.e. *X. euvesicatoria*, *X. vesicatoria* and *X. perforans* on tomato and pepper causing bacterial leaf spot disease was reported from Taiwan (Lue *et al.* 2010) and characterized them based carbon sources utilization patterns using Biolog GN2 microplate (Lue *et al.* 2010, Stoyanova *et al.* 2014). They reported that the cluster analysis based on metabolic fingerprints of 27 substrates can distinguish between *X. vesicatoria, X. euvesicatoria* and *X.*

[1]M Sc student (e mail: ramyasomashekaraih@gmail.com), [2]Principal Scientist (e mail: dinesh_iari@rediffmail.com), Division of Plant Patholgoy, [3]Senior Scientist (e mail: asrivastava45@gmail.com), Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi 110 012. [4]Principal Scientist (e mail: drkkbiswas@yahoo.co.in), [5]Principal Scientist (e mail: akguptampp@gmail.com), Department of Plant Pathology, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan 173 230

gardneri. They stated that simple characteristics like amylase activity and utilization of cis-aconitate cannot be used for differentiation of species of *Xanthomonas*. Aleksandrova *et al.* (2014) characterized *X. gardneri* isolates isolated from tomato plots of Bulgaria based on metabolic fingerprints obtained by using the BIOLO GTM GN2. Molecular characterization of these bacteria has also been done by various workers (Koenraadt *et al.* 2009, Moretti *et al.* 2009, Potnis *et al.* 2011). Koenraadt*et al.* (2009) developed four species specific primers for identification of all four species of xanthomonads associated with bacterial spot of pepper and tomato species based AFLP based approach. Moretti *et al*. (2009) developed the *X. euvesicatoria* specific primer from repetitive extra genic palindromic sequence of *X. euvesicatoria.* Araujo *et al*. (2012) further validated the species specific primers developed by Koenraadt *et al.* (2009) and developed a multiplex-PCR assay for simultaneous detection of all four species associated with bacterial leaf spot of pepper and tomato.

The assessment of the genetic variability of a bacterium can facilitate investigation on its taxonomy, epidemiology and detection. Genomic fingerprinting by PCR amplification, with primers specific to the highly conserved, repetitive elements such as the 35–40 bp repetitive extragenic palindromic (REP) sequence, the 124–127 bp enterobacterial repetitive intergenic consensus (ERIC) and the 154 bp BOX element, was used successfully to characterize a large number of bacteria and differentiate closely related strains of bacteria (Louws *et al.* 1994). Repetitive DNA polymerase chain reaction-based fingerprinting (rep-PCR) (Mulema *et al.* 2012, Rathour *et al.* 2016, Singh *et al.* 2016) is a rapid, low-cost, and reliable method that has been extensively used to assess the genetic diversity of *Ralstonia solanacearum* (Singh *et al.* 2014) and *X.campestris* pv. *campest is* (Singh *et al.* 2016).

Though the disease occurrence is reported from various places of India but all species of *Xanthomonas* among four species causing leaf spot are present India is not reported so far. The diversity of this bacterium prevalent in India is not known well. The present study was taken to know the status of *Xanthomonas* species infecting pepper and their genetic diversity using modern techniques to help breeders to develop resistant varieties of tomato and peppers against bacterial leaf spot disease.

MATERIALS AND METHODS

Bacterial leaf spot infected samples of tomato, capsicum and chilli were collected from Karnataka, Himachal Pradesh, Tamil Nadu, Haryana and Uttarakhand states of India (Table 1). From each field a leaf with typical BLS symptoms

Table 1 Isolates of *Xanthomonas euvesicatoria* isolated from different hosts and places and their cross infectivity test

XCV	Host crop	Place of collection	Cross infectivity test	
			Pathogenicity on Pusa Jwala	Pathogenicity on Pusa Ruby
XCVT-1	Tomato	Kandaghat, Solan, HP	+	+
XCVT-2	Tomato	Jadari, Solan, HP,	+	+
XCVP-3	Bell pepper	Judain, Solan, HP,	+	+
XCVT-4	Tomato	Kailer, Solan, HP,	+	+
XCVP-5	Bell pepper	Berti, Solan, HP,	+	+
XCVT-6	Tomato	Berti, Solan, HP,	+	+
XCVT-7	Tomato	Chatti, Solan, HP,	+	+
XCVP-8	Hot pepper (Chilli)	Berti, Solan, HP,	+	+
XCVP-9	Bell pepper	Kailer, Solan, HP,	+	+
XCVP-10	Bell pepper	Kailer, Solan, HP,	+	+
XCVT-11	Tomato	Darodenika, Solan, HP,	+	+
XCVT-12	Tomato	IIHR, Bangalore, Karnataka	+	+
XCVT-13	Tomato	IIHR, Bangalore, Karnataka	+	+
XCVT-14	Tomato	IIHR, Bangalore,Karnataka	+	+
XCVT-15	Tomato	IIHR, Bangalore, Karnataka	+	+
XCVT-16	Tomato	Nainital, Uttarakhand	+	+
XCVT-17	Tomato	Nainital, Uttarakhand	+	+
XCVT-18	Tomato	Nainital, Uttarakhand	+	+
XCVT-19	Tomato	Nainital, Uttarakhand	+	+
XCVT-20	Tomato	Nainital, Uttarakhand	+	+
XCVT-21	Tomato	Nainital, Uttarakhand	+	+
XCVP-22	Hot pepper	Nainital, Uttarakhand	+	+
XCVP-23	Hot pepper	Nainital, Uttarakhand	+	+
XCVP-24	Hot pepper	Nainital, Uttarakhand	+	+
XCVP-25	Hot pepper	Nainital, Uttarakhand	+	+
XCVP-26	Bell pepper	Hosur,Tamilnadu	+	+
XCVP-27	Bell pepper	Kullu, HP,	+	+
XCVT -28	Tomato	Kailer, HP,	+	+
XCVP 29	Bell pepper	Sonipat, Haryana	+	+
XCVP -30	Bell pepper	Kullu, HP,	+	+
XCVP 31	Bell pepper	Kullu, HP,	+	+

(irregular water soaked chlorotic necrotic spot surrounded by yellow halo) was sampled from each of 10 well separated plants. Samples were dried in between sheets of paper at room temperature before isolation. The causal bacteria were isolated from infected leaf samples of tomato and pepper by using standard procedure described by Schaad *et al.* (2001). A typical colony from each isolation plate was sub cultured on YGCA slants. Isolates were stored at -80° C. Cultures of out group bacteria such as *X. campestris* pv. *campestris* Xcc-C 4, Xcc-C23*, Pseudomonas fluorescense* DTPF-3 and *Bacillus subtilis* DTBS-5 were obtained from Division of Plant Pathology, ICAR- IARI, New Delhi and used under this study.

Isolates isolated from tomato were tested for pathogenicity on 4 weeks-old plants of tomato cv. Pusa Ruby and isolates from pepper (bell pepper and chilli) were tested on chilli cv. Pusa Jwala. For testing cross infectivity isolates from pepper were tested on tomato cv. Pusa Ruby and isolates isolated tomato were tested on chilli cv. Pusa Jwala. Culture for inoculation was prepared by multiplying on YGCA media at 28°C for 48 h before inoculation. Bacterial growth was scraped from the plates and suspended in 10 ml of sterile tap water to produce a turbid suspension (10^8 to 10^9 CFU/ml). Plants were inoculated 4 weeks after sowing. On the lower side of the leaves wounds were made with the help of sterilized pin dipped in the bacterial inoculum. Then a small piece of sterile cotton wool soaked in sterilized water is placed on the inoculated area and covered with the polythene cover sprinkled with water for about 24 hours (Dhutraj *et al.* 2010)

Morphological character like colony on YGCA medium, KOH test and gram staining and biochemical characterization such as production of hydrogen sulfide from cysteine, oxidase test, catalase test, starch hydrolysis test, pectin utilization test of isolated bacteria were done using standard methods as described by Schaad *et al.* 2001.

Genomic DNA of 31 isolates of *Xanthomonas* spp. and four out group bacteria *X. campestris* pv. *campestris, fluorescense* and *B. subtilis* were extracted by CTAB method (Murray and Thompson 1980).

Five isolates XCVP-5 (capsicum, H.P.), XCVT-13 (tomato, Karnataka), XCVP-12 (chilli, Karnataka), XCVP-3 (capsicum, HP) and XCVT-15 (tomato, Karnataka) of *X. euvesicatoria* were selected for this study. Amplification of 16 S rRNA geneof size 709 bp was done by using set of universal primer (UNI_OL5 :5`-GTGTAGCGGT GAA ATGCG-3`) and UNI_OR (5` ACGGGCGGTGTGTACAA-3`) (Wattiau *et al.* 2001). A master mixed of 25 µl was prepared containing 5X PCR buffer 5.0 µl, 25 mM $MgCl_2$ 2.0 µl, 10 m MdNTPs 0.2 µl, F primer 0.2 µl, R primer 0.2 µl, 1 U Taq polymerase 0.25 µl, 20ng DNA template 1.0 µl and Nuclease free water 16.25 µl. PCR reactions, running of gel and gel photography using the gel documentation system (BIO-RAD, GEL DOCTM XR+ with image Lab^{TM} software)were carried outas described by Singh *et al.* 2016. A50 µl of the PCR unpurified product was sent for sequencing. Sequence of amplified fragment was blasted in NCBI nucleotide BLAST. First sequence of 10 sequences showing similarity with the query sequence was retrieved from NCBI data base and phylogenetic tree was constructed using MEGA 4 software.

All the 31 isolates were tested with *X. vesicatoria* specific Bs-XvF and Bs-XvR primer set; *X. euvesicatoria* specific Bs-XeF and Bs-XeR primer set; *X. perforans* specific Bs-XpF and Bs-XpR primer set and *X. gardeneri* specific Bs-XgF and Bs-XgR (Koenraadt *et al.* 2009) (Table 2). A 25 µl of master mix containing 5X PCR buffer 5.0 µl, 25 mM $MgCl_2$ 2.5 µl, 10 mM NTPs, 0.5 µl, F primer1.0 µl, R

Table 2 Primer used under this study for characterization of *Xanthomonas* spp.

Species specific primer	Sequence of the primer (5 3')	Fragment size	Tm value (ºC)	Region amplified
Bs-XvF	CCA TGT GCC GTT GAA ATACTT G	138 bp	56	AFLP
Bs-XvR	ACA AGAGATGTT GCT ATG ATTTGC			
Bs-XeF	CAT GAA GAACTC GGC GTA TCG	173 bp	56	AFLP
Bs-XeR	GTC GGA CAT AGT GGA CAC TAC			
Bs-XpF	GTC GTG TTG ATG GAG CGT TC	196 bp	56	AFLP
Bs-XpR	GTGCGAGTCAATTATCAGAAT GTG G			
Bs-XgF	TCA GTG CTT AGT TCC TCA TTG TC	154 bp	56	AFLP
Bs-XgR	TGA CCG ATA AAG ACT GCG AAA G			
Xeu2.4	CTG GGA AAC TCA TTC GCA GT	208 bp		REP
Xeu2.5	TTG TGG CGC TCT TAT TTC CT			
BOXA1R	CTACggCAAggCgACgCTgACg	BOX-PCR Multiple bands		
REP1R	IIIICgICgICATCIggC	REP-PCR multiple bands		
REP21	ICgICTTATCIggCCTAC			
ERIC1R	ATgTAAgCTCCTggggATTC	ERIC-PCRmultiple bands		
ERIC2	AAgTAAgTgACTggggTgAgCg			

primer 1.0 µl, Taq polymerase 0.25 µl, 20ng DNA template 1.0 µl and Nuclease free water 15 µl was prepared. PCR reactions were carried out with initial denaturation step at 94°C for 5 minutes, annealing temperature about 56°C for 1 min followed 30 cycles each consisting of 30 seconds at 94°C, 72°C for 1 minute and final extension of 7 min at 72^0C. PCR amplified fragments were separated on 1.5% agarose gel in electrophoresis unit using 1X TAE buffer as described earlier.

For all samples, amplification of REP region of size 208 bp, specific to *X. euvesicatoria*, was checked with primers Xeu2.4 and Xeu2.5 was done as described by Moretti *et al.* (2009). Gelelectrophoresis of PCR product and photography were done as described earlier.

DNA extraction and rep-PCR amplification was done for all pathogenic isolates. For each isolates infecting tomato and pepper along with, out group bacteria such *P. fluorescence* DTPF-3, *X. campestris* pv. *csmpestris* Xcc-C4 and Xcc-C23 and *B. subtilis* DTBS-5, rep-PCR was carried out by using ERIC, REP and BOX primers (Singh *et al* (2011). REP, ERIC and BOX-PCR amplifications were carried out in a final volume of 25 µl containing as described by Singh *et al* (2011). Gelelectrophoresis of PCR product was done as described earlier. The normalized data generated from ERIC, REP and BOX fingerprinting profiles were combined for generating similarity matrix by using SIMQUAL module for the NTSYSpc 2.02e. The similarity matrix thus generated was used for cluster analysis by unweighted pair group method of arithmetic average (UPGMA) using sequential, agglomerative, hierarchical, nested clustering module of NTSYSpc. The output data were graphically presented as a phylogenetic tree and also prepared similarity matrix table.

RESULTS AND DISCUSSION

Morphological and general biochemical tests

Bacteria were isolated from infected leaf sample of tomato, capsicum (bell pepper) and chilli (hot pepper) on YGCA medium, yielded the typical *Xanthomonas* colonies within 72h at 28ºC in incubator. The bacterial colonies were yellow, mucoid, translucent, and raised on YGCA medium as earlier reported by Schaad *et al.* (2001).Total 31 isolates of Xanthomonads were isolated from tomato (17 isolates), chilli (5 isolates) and bell pepper (capsicum) (9 isolates) belonging to different places such as Himachal Pradesh followed Uttarakhand, Karnataka, Haryana and Tamil Nadu (Table 1). They were rod shaped, gram negative, KOH test positive, oxidase negative, catalase positive. They hydrolysed gelatine and produced hydrogen sulphide from casein. All the isolates of *X. euvesicatoria,* showed positive in starch hydrolysis by making clear zone on the medium after 72 h of inoculation (Table 4). *X. euvesicatoria*isolates formed clear zone by utilizing pectin varied from 1.0 to 1.2 cm diameter after 72 h. Among them only 67% of the isolates of *X. euvesicatoria* showed positive in pectin utilization and most of them isolated from tomato (Table 4). According to Jones *et al.* (2000), four species associated with BLS of tomato and pepper have following phenotypic characters, as *X. vesicatoria* and *X. perforans* hydrolyse starch and utilize pectin whereas; *X. euvesicatoria* and *X. gardeneri* are negative in starch hydrolysis and pectin utilization. The similar results were also reported by Stoyanova *et al.* (2014) and they found that *X. euvesicatoria* isolates varied in ability to utilize starch and pectin. Based on these results, thus we can conclude that simple biochemical tests like starch hydrolysis and pectin utilization tests cannot be used to differentiate four species of *Xanthomonas* associated with BLS of pepper and tomato, i.e. *X. euvesicatoria, X. vesicatoria, X. perforans* and *X. gardneri*.

Table 3 List of *Xanthomonas* spp., whose 16S rRNA genes sequences were used in the study

Bacteria name	NCBI accession number	Crop	Country of isolation	Source
Xanthomonas axonopodispv. citri	HQ875740	Citrus	Thailand	NCBI
X. campestris pv. *viticola*	JQ513818	Grape	Brazil	NCBI
X. citri subsp. *citri*	CP009016	Citrus	China	NCBI
X. fragariae	HQ223083	Strawberry	Australian	NCBI
X. oryzae pv. *oryzae*	AB680146	Rice	Japan	NCBI
X. euvesicatoria	KU315004	Tomato	Ethiopia	NCBI
X. euvesicatoria	KU315003	Tomato	Ethiopia	NCBI

Pathogenicity and cross infectivity

For pathogenicity test, all isolates of Xanthomonads isolated from tomato and pepper (chilli and bell pepper) were inoculated on 4 weeks old tomato cv. Pusa Ruby and chilli cv. Pusa Jwala plants by pin prick method on abaxial surface of leaf. All the isolates produced typical disease symptoms was initiated after 5 days and typical symptoms as necrotic lesions surrounded by yellow halo within 2 weeks of inoculation as previously described by Dhutraj *et al.* (2010) produced typical BLS symptom at site of infection i. e. necrotic lesions surrounded by yellow hallow. Based on the pathogenicity test, they can be confirmed that all 31 isolates were *Xanthomonas* associated with BLS. In cross infectivity test, isolates from chilli and bell pepper were tested on tomato cv. Pusa Ruby and isolates from tomato were tested on chilli cv. Pusa Jwala and they produced typical symptoms of leaf spot on the both pepper and tomato plants irrespective of host plant they were isolated. All the isolates isolated from tomato infected chilli cv. Pusa Jwala and all the isolated from capsicum and chilli produced typical leaf spot symptoms of BLS on tomato cv. Pusa Ruby within 5 days of inoculation (Table 2) and they are grouped under pepper-tomato group (XCVPT) according to the grouping done by Chand *et al.* (1994). Based on pathogenicity and cross infectivity, these isolates were confirmed to be

Table 4 Starch hydrolysis, pectin utilization, molecular characterization and DNA typing of *X. euvesicatoria* isolated from tomato and pepper plants

Strains	Starch hydrolysis test		Pectin utilization test		Molecular characterization			
	After 72 h	Zone size(cm)	After 72 h	Zone size (cm)	Amplification of 16S rRNA	AFLP based primer Bs XeF& Bs-XeR	Rep based primer Xeu2.4&Xeu2.5	DNA types
XCVT-1	+	1.4	+	1.0	+	+	+	1
XCVT-2	+	1.6	+	1.1	+	+	+	1
XCVP-3	+	0.5	-	0	+	+	+	6
XCVT-4	+	1.4	+	1.2	+	+	+	1
XCVP-5	+	0.4	-	0	+	+	+	9
XCVT-6	+	1.4	+	1.0	+	+	+	2
XCVT-7	+	1.3	+	1.0	+	+	+	2
XCVP-8	+	1.7	+	1.0	+	+	+	8
XCVP-9	+	0.5	-	0	+	+	+	6
XCVP-10	+	1.3	+	1.0	+	+	+	2
XCVT-11	+	1.6	+	1.1	+	+	+	2
XCVT-12	+	1.5	+	1.0	+	+	+	2
XCVT-13	+	1.3	+	1.0	+	+	+	3
XCVT-14	+	1.4	+	1.1	+	+	+	8
XCVT-15	+	1.6	+	1.2	+	+	+	2
XCVT-16	+	1.6	+	1.0	+	+	+	2
XCVT-17	+	1.4	+	1.0	+	+	+	2
XCVT-18	+	1.6	+	1.0	+	+	+	2
XCVT-19	+	1.5	+	1.1	+	+	+	2
XCVT-20	+	1.6	+	1.1	+	+	+	2
XCVT-21	+	0.2	-	0	+	+	+	2
XCVP-22	+	0.3	-	0	+	+	+	7
XCVP-23	+	0.5	-	0	+	+	+	7
XCVP-24	+	0.4	-	0	+	+	+	7
XCVP-25	+	0.2	-	0	+	+	+	7
XCVP-26	+	0.3	-	0	+	+	+	7
XCVP-27	+	1.4	+	1.0	+	+	+	5
XCVT -28	+	0.2	-	0	+	+	+	7
XCVP 29	+	0.2	-	0	+	+	+	7
XCVP -30	+	1.5	+	1.0	+	+	+	5
XCVP 31	+	1.4	+	1.1	+	+	+	4

Xanthomonas euvesicatoria infecting tomato and pepper under group XCVPT.

Molecular characterization of X. euvesicatoria 16 S rRNA based characterization

Out of 31 isolates of *X. euvesicatoria***,** 5 isolates such as XCVP-5 (Bell pepper, HP), XCVT-13 (Tomato, Karnataka), XCVT-12 (Tomato, Karnataka), XCVP-3 (Bell pepper, HP) and XCVT-15 (Tomato, Karnataka) were taken for characterization of pathogen by using partial nucleotide sequence of 16 S rRNA. Out of 5 isolates of *X. euvesicatoria*, 4 isolates (XCVT-13, XCVP-12, XCVP-3 and XCVT-15) showed very close relationship and grouped along with *X. euvesicatoria* having 96-99% sequence similarity. These isolates were also very close to *X. axonopodis* pv. *citri, X. campestris* pv. *viticola, X. citri* subsp. *citri* and *X. fragaria*. Isolate XCVP-5, which was isolated from bell pepper from Solan, HP formed separate cluster (Fig 1). Although it showed sequence similarity about 96-98% with other isolates of *X. euvesicatoria* XCVT-13, XCVP-12, XCVP-3 and XCVT-15 (Table 3). From the phylogenetic tree, it was cleared that four species of *Xanthomonas* causing BLS could not be differentiated clearly and this finding was also supported by Hauben *et al.* (1997), because of their close sequence similarity of 16S rRNA among them. They reported that sequence analysis of 16S rRNA, only slight difference in 16S rRNA sequence was found between many Xanthomonads.

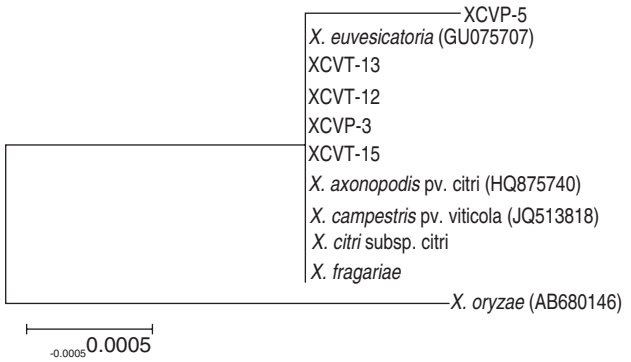

Fig 1 Evolutionary tree based on 16 S rRNA partial nucleotide sequence of 5 isolates of *X. euvesicatoria* along with 6 strains of different species of *Xanthomonas* using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.00388698 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. There were a total of 596 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Identification of the species associated with BLS using species specific marker

For further confirmation of *X. euvesicatoria* formerly called *X. campestris* pv. *vesicatoria* and *X. axonopodis* pv. *vesicatoria*, all the 31 isolates of Xanthomonands were tested by using species specific primers, as for *X. vesicatoria,* Bs-XvF and Bs-XvR primers; for *X. euvesicatoria*, Bs-XeF and Bs-XeR primers; for *X. perforans*, Bs-XpF and Bs-XpR primers and for *X. Gardeneri,*Bs-XeF and Bs-XgR for amplification at 138 bp, 154 bp, 173 bp and 197 bp respectively (Koenraadt *et al.* 2009). Protocols for all the four primer sets were standardized. However amplification was not observed in any of the isolates of test bacteria with *X. vesicatoria* specific Bs-XvF and Bs-XvR primes; *X. perforans* Bs-XpF and Bs-XpR primers and *X. gardeneri* specific Bs-XgF and Bs-XgR primers which were repeated two times. Amplification was observed at 173 bp with all isolates with *X. euvesicatoria* specific Bs-XeF and Bs-XeR primers (Fig 2a). For further confiramation of *X. euvesicatoria* isolates, PCR amplification was tested with another set of primers Xeu2.4 Xeu2.5 specific to *X. euvesicatoria* (Moretti *et al.* 2009). All the isolates were amplified at 208 bp and proved that all 31 isolates isolated from tomato, chilli and bell pepper belonged to *X. euvesicatoria*.

Genetic diversity

Genetic diversity of 31 isolates *X. euvesicatoria* along with four out group bacteria such as *X. campestris* pv. *campestris* (Xcc-C4 and Xcc-C23), *P. fluorescense* DTPF-3 and *B. subtilis* DTBS-5 were studied by using rep-PCR, ERIC-PCR and BOX-PCR primers. Amplification profile of each isolates of *X. euvesicatoria* varied in all three methods of PCRs as 2 to 19 amplicons were obtained in ERIC-PCR, 3 to13 in REP and 8 to 17 in BOX-PCR. Computer assisted analysis of combined BOX, ERIC and REP-PCR fingerprinting data showed a very high level of genetic diversity among the *X. euvesicatoria* isolates (Fig 3). The fingerprinting pattern of 31 isolates of *X. euvesicatoria* and 4 out group bacteria such as *P. fluorescens* DTPF-3, *X. campestris* pv. *campestris* Xcc-4, Xcc-C23 and *B. subtilis* DTBS-5. Total 12 DNA types of 35 isolates of bacteria were observed based phylogenetic analysis of 75% similarity co-efficient. Out of 12 DNA types, 3 isolates belong to DNA type 1 (XCVT-1, XCVT-2,

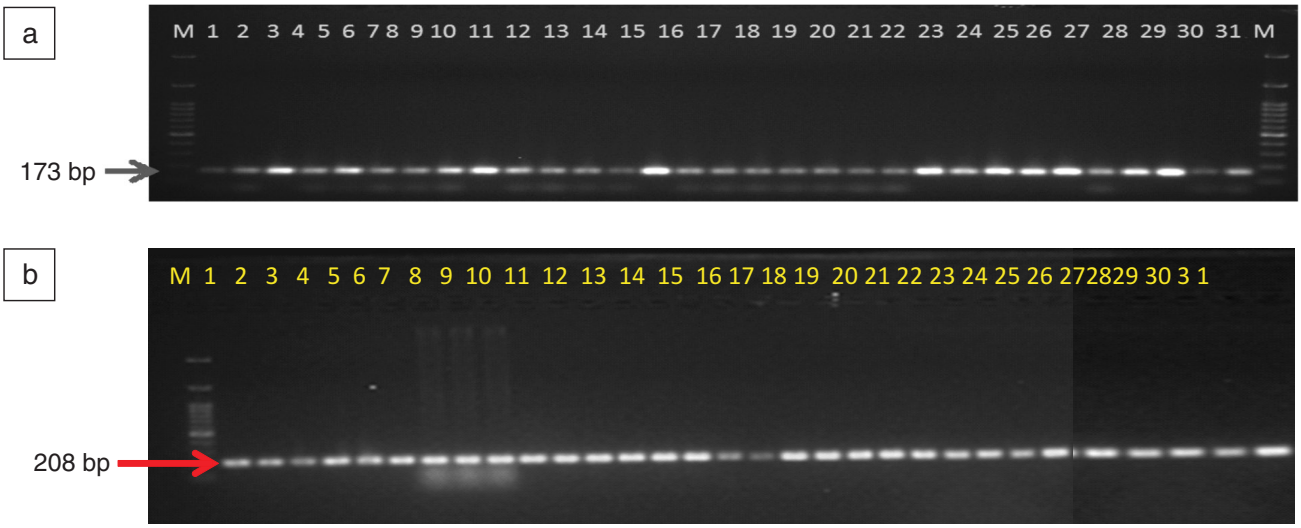

Fig 2. PCR amplification of *X. euvesicatoria* strains isolated from tomato, bell pepper and chilli at 173 bp and 208 bp amplifying *X. euvesicatoria* specific two sets of primers (a) Bs-XeF and Bs-XeR and (b) Xeu2.4 and Xeu2.5. Lane M: 100 bp DNA ladder, lanes 1-7: Tomato isolates from Himachal Pradesh, 8- 15: pepper isolates from Himachal Pradesh , 16-19: tomato isolates from Karnataka, 20-25: tomato isolates from Uttarakhand, 25-29: pepper isolates from Himachal Pradesh, 29 : isolate from Tamil Nadu, 31 : isolate from Haryana.

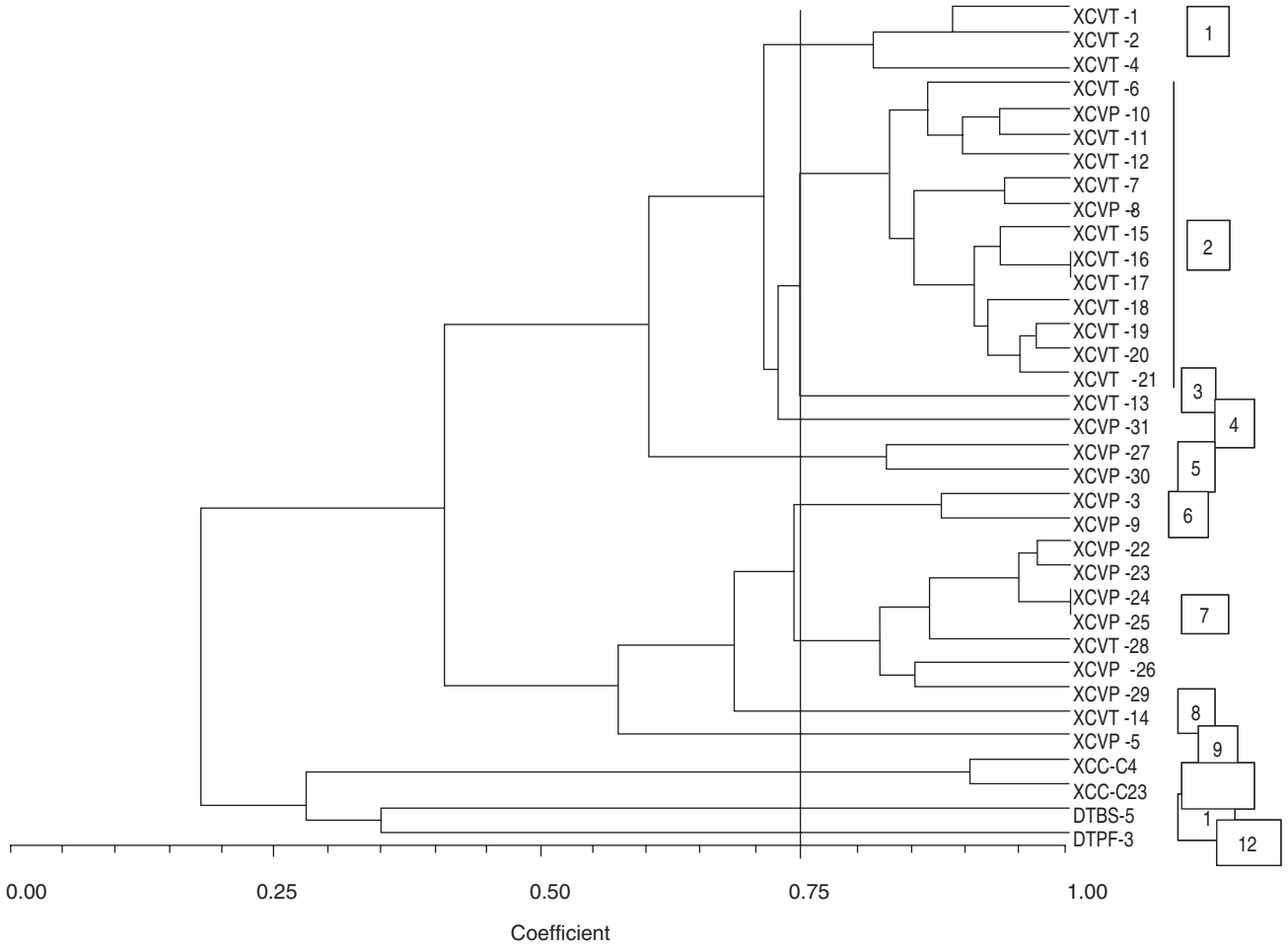

Fig 3 Phylogenetic analysis of 31 isolates of *Xanthomonas euvesicutariaa* long with isolates at 75 % similarity co-efficient by REP, ERIC and BOX PCR unweighted paired group mathematical average (UPGMA) dendrograms were generated using Jaccard's similarity coefficient. The significant of each branch indicated by the bootstrap percentage calculated for 1000 subsets (only values greater than 50 % are shown). The minimum similarity coefficient of all strains of *X. euvesicutaria, X. campestris* pv. *campestris* (Xcc-C4 and Xcc-C23), *B. subtilis* (DTBS-5), *P fluorescens* (DTPF-3) was used to define distinct groups which are labeled numerically.

XCVT- 4). Maximum 13 isolates of *X. euvesicatoria* belonged to DNA type 2(XCVT-6, XCVP-10, XCVT-11, XCVT-12, XCVT-7, XCVP-8,XCVT-15, XCVT-16, XCVT-17, XCVT-18, XCVT-19, XCVT-20, XCVT-21) isolated from tomato and pepper from different places of India. It was observed that when combined the fingerprinting data of all three primers, i.e. BOX, ERIC and REP, could not distinguish the isolates based on their geographical origin and their hosts.

These primer sets are highly conserved repetitive sequences which showed different banding pattern and *X. euvesicatoria* isolates collected from different hosts, i.e. tomato, chilli and bell pepper (capsicum) and also from different places of India. The analysis clearly reveals the potentiality of Rep-PCR to cluster the isolates of *X. euvesicatoria* based on their host and pathogenic behaviour of the bacteria. In present study, BOX and ERIC-PCR primers are able to separate majority of isolates collected from tomato and pepper, whereas REP-PCR primers did not able to separate *X. euvesicatoria* isolates based on their hosts. It indicates that genetic variability is also depending on tools used for the study. Similar result was as earlier reported by Vicente *et al.* (2006) and Singh *et al.* (2016) in *X. campestris* pv. *campestris*. These findings was contradictory to the result of Tsygonkova *et al.* (2004) in *X. campestris,* where they indicated that geographical origin was a major factor for bacterial genetic diversity. It proves that Indian isolate of *X. euvesicatoria* have wide genetic diversity but there are no effects of climatic conditions. This further confirms the utility of Rep-PCR for differentiation of closely related strains of bacteria and the potential usefulness for studying bacteria evolution in specific ecological area (Singh *et al.* 2016). However, this technique is not used for routine identification of bacteria.

The present study clearly indicates that the isolates isolated from tomato and pepper belong to *Xanthomonas euvesicatoria* infecting both the crops with high genetic diversity in India.

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