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GENETIC VARIABILITY AMONG ISOLATES OF *Xanthomonas citri* subsp. *citri* BASED ON rep-PCR

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ABSTRACT

Citrus canker caused by *Xanthomonas citri* subsp. *citri* is one of the most important disease of citrus in India. In the present study 23 isolates of *Xanthomonas citri* subsp. *citri* were collected from Varanasi and Jaunpur districts of Uttar Pradesh (India). Isolates showed similar biochemical characteristics whereas molecular analysis involving repetitive polymerase chain reaction [rep-PCR] (BOX-, ERIC- and REP-PCR) grouped these isolates into six clusters at 50% similarity coefficient, thereby revealing presence of variation in *X. citri* subsp. *citri* isolates.

Key words: Canker, citrus, *Xanthomonas*, rep-PCR

INTRODUCTION

Citrus is the 3rd most important fruit crop in the world. In India, the most important commercial citrus fruit crops are mandarine orange (*Citrus reticulata* Blanca), sweet orange (*C. sinensis* Asbeck), acid lime (*C. aurantiifolia* Swingle) and lemons (*C. limon* Burn) [Singh, 2001]. However, most of the commercial species/varieties are prone to various diseases, including citrus canker. Citrus canker, caused by *Xanthomonas citri* subsp. *citri* (syn. *Xanthomonas campestris* pv. *citri*), is one of the most virulent diseases of citrus throughout the world including Asia (Graham and Gottwald, 1991). Amongst citrus species grown in India, acid lime is more susceptible to this disease which inflicts 50-60% yield reduction (Das, 2001). The citrus canker pathogen produces symptoms ranging from pustules to necrotic lesions consisting of erumpent corky tissue surrounded by water-soaked tissues and yellow halo on leaves, stems and fruits, which in severe infection cause leaf abscission, twig dieback and premature fruit drop (Vauterin *et al*., 1991).

At least five forms or pathotypes of *X. citri* subsp. *citri* have been identified on the basis of host range, symptomatology, phage typing, DNA fingerprinting and geographical distribution and named as form A,B,C,D and E (Vauterin *et al*., 1995). Among these, pathotype A causes true or Asiatic citrus canker and infects most commercial citrus varieties (Stall and Civerolo, 1991). The identification and assessment of variability among pathotypes of *X. citri* subsp. *citri* through biochemical and molecular characterization is essential to devise effective disease management strategies (Khalid *et al.*, 2010). Various approaches have been used to assess variation in *X. citri* which include physiological and biochemical assays (Verniere *et al*., 1998), serological approaches (Stall and Civerolo, 1991), bacteriophage sensitivity (Verniere *et al*., 1998), fatty acid profile (Vauterin *et al*., 1991), plasmid DNA analysis (Gabriel, 1985), RFLP analysis (Hartung and

Civerolo, 1989) and genomic DNA fingerprinting using rep-PCR. Among these, PCR-based genomic fingerprinting based on repetitive sequences (rep-PCR) has effectively been used for the analysis of several bacterial species/strains (Louws *et al*., 1994) and for assessing genetic diversity (Cubero and Graham, 2002). Rep-PCR genomic fingerprinting involves the use of DNA primers complementary to highly conserved repetitive DNA sequences such as 35-40 bp repetitive extra-genic palindromic (REP) sequence, 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence and 154 bp BOX element present in multiple copies in the genome of most G -ve and several G +ve bacteria (Lupski and Weinstock, 1992). Rep-PCR has been used to separate patho-types of *X. citri* subsp. *citri* or to differentiate strains in the same pathotype. This technique allows the assessment of diversity of *X. citri* in certain geographical areas of the world (Cubero and Graham, 2002; Lee *et al*., 2008). In India not much work has been done on the assessment of variation in *X. citri* subsp. *citri* using rep-PCR technique, however it has been done using RAPD (Suryawanshi *et al*., 2012). Therefore, present study was taken up with the objective of assessment of variation in the isolates of *X. citri* subsp. *citri*, collected from Varanasi and Jaunpur districts of Uttar Pradesh (India), on the basis of their biochemical characteristics and rep-PCR technique.

MATERIALS AND METHODS

Leaves and fruits of citrus showing typical canker lesions were collected from Varanasi and Jaunpur districts of Uttar Pradesh (India) and named X1 to X23 (Table I). Bacterial isolation and purification was done as per Broadbent *et al*. (1992). Briefly, typical lesions sterilized in 70% alcohol were tweezed for the release of bacteria. A loopful of bacteria was then streaked on the plates containing sucrose peptone agar medium (SPA) [sucrose - 20 g; peptone - 5 g; K_2HPO_4 - 0.5 g; $MgSO_4.7H_2O$ - 0.25 g and agar - 20 g L^{-1} water]. The plates were incubated at 28°C for 72 hr. Single yellow, domed and mucoid colonies were selected and re-streaked twice to procure pure culture. Pure colonies were transferred to SPA slants and preserved at 4°C till use.

Pathogenicity studies were carried out on 9 month old seedling of kagzi lime (*C. aurantifolia*). For this, a suspension of approximately 1×10^8 viable cells mL^{-1} from 72 hr old culture was inoculation on the sterilized leaves of kagzi lime. The leaves were punctured by needles and bacterial suspension poured over them. Control leaves were treated with sterile distilled water only. Inoculated leaves were covered with moist freezer bags for 5 days and symptoms expression evaluated after 14 days (Broadbent *et al*., 1992).

All the 23 isolates of *X. citri* subsp. *citri* were assessed for various biochemical characters. Gram reaction was determined as per the standard staining procedure (Schaad, 1988). Catalase and gelatin liquefaction tests were conducted as per Dickey and Kelman (1988). For starch hydrolysis, the isolates were streaked on starch agar medium and incubated at 28°C for 72 hr. Starch and casein hydrolysis were assessed as per techniques described by Aneja (1996). Nitrate reduction, salt tolerance and arginine dihydrolase tests were conducted as per Fahy and Persley (1983).

Total genomic DNA of all 23 *X. citri* subsp. *citri* isolates were extracted using CTAB/NaCl (Wilson, 1987). One strain each of *X. campestris* pv. *campestris* (Xcc-168), *X. oryzae* pv. *oryzae* (Xoo-1) and *X. axonopodis* pv. *punicae* (Xap) were included in the study. PCR was performed using a thermocycler (Biorad C1000^{TM} thermal cycler). Genetic variation was analyzed by rep-PCR (BOX-, ERIC- and REP-PCR). ERIC-1R (ATGTAAGCTCCTGGGGATTCAC), ERIC-2 (AAAGT AAGTGACTGGGGTGAGCG), REP-1R (IIIICGICGICATCIGGC), REP-2 (ICGICTTAT TATCI GGCCAC) (Versalovic *et al*., 1994) and BOX-A1R (CTACGGCAAGGCGACGCTGACG) (Versalovic *et al*., 1994) primers (Invitrogen Biosciences) were used for rep-PCR fingerprinting. PCR was performed with a final volume of 25 µL containing 5 µL 5X PCR buffer, 1 µL of each primer, 2.5 mM $MgCl_2$, 2.5 mM dNTPs, 20 ng genomic DNA and 2.5 U *Taq* DNA polymerase.

PCR amplification was carried out in a thermocycler with following cycling conditions: initial denaturation at 95°C for 7 min; 30 cycles of denaturation at 94°C for 1 min; annealing at 45°C with REP-1R and REP-2, at 46°C with ERIC-1R and ERIC-2 and at 53°C with BOX-A1R; extension at 65°C for 8 min. with a final extension cycle for 15 min. PCR products were separated on 1.5% agarose gel in 1X TAE buffer at 100V for 6 hr. The gels were stained with ethidium bromide (2 µg mL^{-1}) and photographed using gel documentation system (Biorad, Geldoc^{TM} XR). A marker of molecular weight of 1 kb was used for molecular size reference of the amplified fragment of each isolate yielded from rep-PCR. The rep-PCR finger printings were analyzed on the basis of presence or absence of bands at a specific position (0 - absence; 1- presence). The data was calculated with the programme NTSYSpc 2.02e computer software and UPGMA alogrithum was used to perform the hierarchial clustering analysis to construct the dendogram.

RESULTS AND DISCUSSION

In all 23 *Xanthomonas citri* subsp. *citri* isolates were obtained from Kagzi lime leaves and fruits (Table 1). All the isolates formed circular, convex, mucoid and yellow colonies with smooth margin on sucrose peptone agar (SPA) medium. All the isolates formed typical canker symptoms on the leaves 2-3 week after inoculation. On leaves, lesions appeared first on the lower leaf surface and then on the upper surface. Lesions later merged gradually and formed erumpent callus-like tissue with water-soaked margin. Broad-bent *et al*. (1992) and Mohammadi *et al*. (2001) have reported similar symptoms on susceptible hosts. All the isolates were Gram -ve, catalase +ve, unable to reduce nitrate, arginine dihydrolase -ve, hydrolysed tween 80, starch hydrolysis +ve, gelatine liquefaction +ve and milk proteolysis +ve. On the basis of biochemical characters, the bacterial isolates were identified as *Xanthomonas citri* subsp. *citri* (Vauterin *et al.*, 1995; 1998).

Table 1: Amplicons generated in *X. citri* subsp. *citri* isolates and other *Xanthomonas* spp. by using BOX-, ERIC- and REP-PCR primers for fingerprinting

Isolates	Place of collection	No. of amplicons obtained		
		BOX-PCR	ERIC-PCR	REP-PCR
X1	Horticulture farm, BHU,	8	10	14
X2	Tarapur, Varanasi	10	10	14
X3	Tikari	10	10	14
X4	Bhagwanpur*	11	11	14
X5	Chandapur	14	16	4
X6	Ramna	14	16	4
X7	Chittpur	14	15	3
X8	Tariya	13	13	11
X9	Maruadih	16	17	5
X10	Bhaghutipur	13	17	18
X11	Muradev	16	16	18
X12	Chitaipur*	13	14	18
X13	Baraipur	19	17	16
X14	DafiL	19	20	5
X15	Babatpur	9	18	6
X16	Jaunpur	20	20	16
X17	Sandaha, Varanasi	20	17	4
X18	Lamhi, Varanasi	12	13	4
X19	Murdaha, Varanasi	14	15	7
X20	Kachhariya, Varanasi	21	18	7
X21	Narottampur, Varanasi	22	22	3
X22	Tarapur, Varanasi	11	11	3
X23	Newriya, Varanasi	10	6	6
X. campestris pv. *campestris* (Xcc-168)		19	8	2
X.oryzae pv. *oryzae* (Xoo-1)		19	17	5
X. axonopodis pv. *punicae* (Xap)		19	11	1
Total number of amplicons		36	45	37
Range of amplicons		8-22	6-22	1-18

*The affected plant parts taken were leaf, except for Bhagwanpur and Chitaipur wherefrom fruits were taken

Different fingerprints of isolates were generated using rep-PCR. Amplified DNA fragments ranged between 10 and 300 kb and revealed a high level of genetic diversity among the

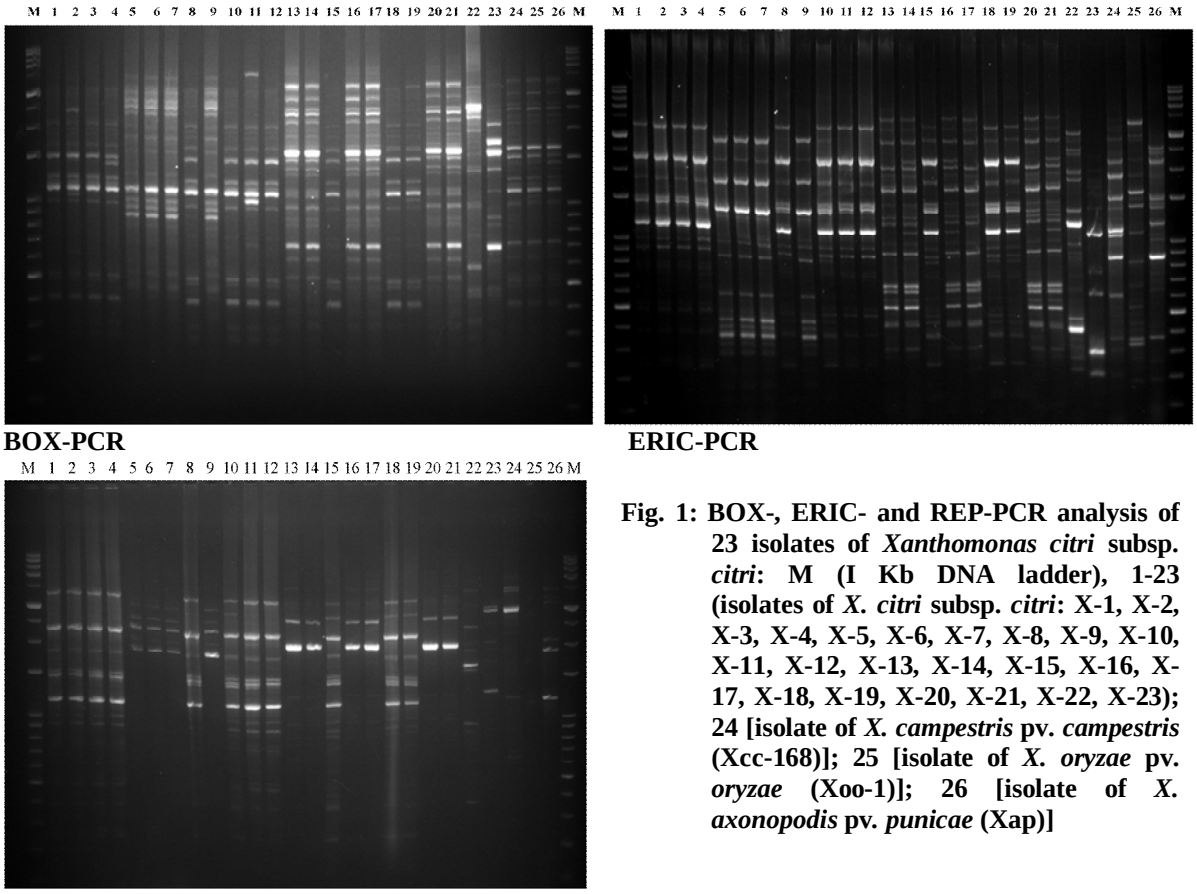

Fig. 1: BOX-, ERIC- and REP-PCR analysis of 23 isolates of *Xanthomonas citri* subsp. *citri*: M (I Kb DNA ladder), 1-23 (isolates of *X. citri* subsp. *citri*: X-1, X-2, X-3, X-4, X-5, X-6, X-7, X-8, X-9, X-10, X-11, X-12, X-13, X-14, X-15, X-16, X-17, X-18, X-19, X-20, X-21, X-22, X-23); 24 [isolate of *X. campestris* pv. *campestris* (Xcc-168)]; 25 [isolate of *X. oryzae* pv. *oryzae* (Xoo-1)]; 26 [isolate of *X. axonopodis* pv. *punicae* (Xap)]

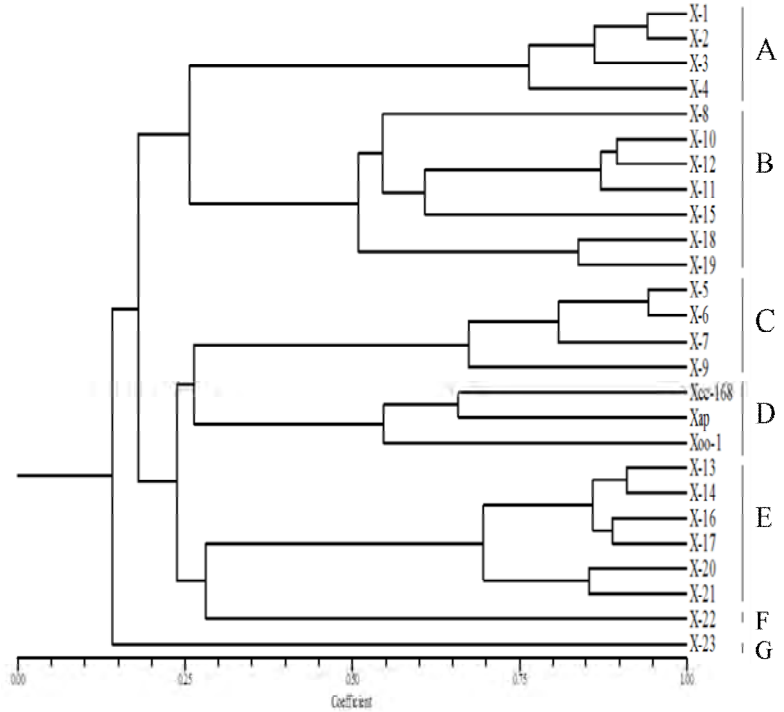

Fig. 2: Combined dendrogram showing the relationship between 23 isolates of *X. citri* subsp. *citri* by UPGMA using NTSYS software

isolates of *X. citri* subsp. *citri* [BOX-PCR, ERIC-PCR and REP-PCR (Fig. 1)]. A maximum of 45 amplicons were found in ERIC-PCR, followed by 37 amplicons in REP-PCR and 36 amplicons in BOX-PCR. Range of amplicons produced varied in all the 3 methods of PCR as 8-22 in BOX-PCR, 6-22 in ERIC-PCR and 1-18 in REP-PCR. In BOX-PCR a maximum of 22 fragments of amplicons were produced in isolate X-21; in ERIC-PCR 22 fragments of amplicons were produced in the same isolate i.e. X-21, while in REP-PCR 18 fragments of amplicons were produced in isolate X-10 and X-11 (Table 1). Based on minimum of 50% similarity coefficient and by analyzing

combined dendrogram of BOX-, ERIC- and REP-PCR a total of 7 clusters were obtained for all the *X. citri* subsp. *citri* isolates and other test bacteria (Fig. 2). The usefulness of rep-PCR for characterization of plant pathogenic bacteria and identification of strains and pathovars that were not distinguishable by traditional methods has previously been demonstrated (Louws *et al*., 1995; Pooler *et al*., 1996). Genetic diversity studies in *X. citri* subsp. *citri* using rep-PCR have also been performed by various workers (Cubero and Graham, 2002; Rezaei *et al*., 2012) and their findings also confirmed the potential of rep-PCR to differentiate among the strains of *Xanthomonas citri* subsp. *citri*. ERIC primers resulted in production of maximum number of amplicons and thus more efficiently differentiating the isolates. This has been reported in studies on genetic diversity in *Xanthomonas campestris* pv. *campestris* (Jensen *et al*., 2010; Singh *et al*., 2011), *Xanthomonas campestris* pv. *vesicatoria* (Trindade *et al*., 2005), *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas phaseoli* pv. *fuscans* (Lopez *et al*., 2006) in which they found ERIC primers to be the best in revealing genetic diversity in the tested bacterium.

Conclusion: The present study revealed the presence of variation in the isolates of *Xanthomonas citri* subsp. *citri* in Varanasi and Jaunpur districts of Uttar Pradesh (India). This study also shows the potential of rep-PCR as an efficient technique for evaluating the diversity of *Xanthomonas citri* subsp. *citri* from a particular geographical area. Prior knowledge of diverse population of citrus canker bacterium will certainly help in devising effective management practices for the disease.

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