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Genetic diversity of *Xanthomonas campestris* pv. *campestris* isolated from *Brassica* crops using RAPD and Rep-PCR

DINESH SINGH[1], PRIYANKA SINGH RATHAUR[2], ASHWARIYA SINGH[3] and RICHA RAGHUWANSHI[4]

Indian Agricultural Research Institute, New Delhi 110 012

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ABSTRACT

Xanthomonas campestris pv. *campestris* (Xcc*)* is causal agent of black rot disease in crucifers. Genetic diversity, 28 isolates of Xcc collected from cole crops (cauliflower, cabbage, broccoli and knol khol), turnip, brown and yellow mustard crops from northern region of India was performed by using rep-PCR fingerprinting (BOX, REP and ERIC) and random amplified polymorphic DNA (RAPD). Five DNA type groups were formed by using rep-PCR fingerprinting. In RAPD study, six primers showed monomorphic bands and remaining six primers (OPB-10, OPB-15, UBC-245, D-11, UBC-220 and OPA-11) exhibited polymorphic amplified product in all isolates of Xcc. Xcc-C281 and Xcc-C261 and Xcc-C268 were formed separate group as DNA types, 1, 2, 3, 4, 5 and 6 respectively. Genetic variability was found among the isolates of Xcc based on their hosts. They were categorized into eight groups at DNA typing at ~50% similarity coefficient and formed 8 DNA type groups at ~ 50% similarity coefficient. Eighteen isolates of Xcc were grouped under DNA type 7, whereas in DNA type 8 (4 isolates).

Key words: *Brassica*, Crucifers, Genetic diversity, Rep-PCR, RAPD, *X. campestris* pv. *campestris*

Black rot of crucifers caused by *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson is one of the most important bacterial diseases of crucifers worldwide. Economically, important host of *X. campestris* pv. *campestris* is *Brassica oleracea* (including cabbage, cauliflower, broccoli, Brussels sprouts, and kale), but it also attacks other cruciferous crops like oilseed crops, weeds, and ornamentals. In India the disease occurs across the country under different agro-climatic conditions (Singh *et al.* 2011). Classical methods (Schaad *et al.* 2001) and advanced molecular techniques especially those based on PCR are very powerful in diagnosis of bacterial diseases (Berg *et al.* 2006, Massimo *et al.* 2007, Singh and Shri Dhar 2011, Singh *et al.* 2014c). In PCR based technique, primers have been developed by using different conserved genes of bacteria, which is group specific and identify the bacteria at species or pathovar level (Berg *et al.* 2006, Vincell and Tisserat 2008, Singh and Shri Dhar 2011).

The assessment of the genetic variability of a microorganism 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. Repetitive DNA polymerase chain reaction-based fingerprinting (rep-PCR) (Singh *et al.* 2011, Mulema *et al.* 2012) is a rapid, low-cost, and reliable method that has been extensively used to assess the genetic diversity of Xcc strains. Random oligonucleotide primers are used RAPDs that have been used extensively as molecular markers in different plant pathogenic bacteria (Shikano and Taniguchi 2002, Singh *et al.* 2014b). RAPDs also have the advantage that no prior knowledge of the genome is necessary for successful application if results are reproducible.

Northern India has largest and most diverse species of cultivated crucifers in India. The genetic diversity of Xcc strains has not been studied in India on the basis of host. The objectives of this study were to use the morphological, biochemical, physiological and pathogenicity tests for identification of Indian Xcc strains, the causal agents of black rot disease of crucifers, and examine the genetic diversity of these strains using molecular techniques.

MATERIALS AND METHODS

Two leaf samples, representing two plants from each field, were collected from cabbage, cauliflower, broccoli

[1] Principal Scientist (Plant Pathology) (e mail: dinesh_iari@rediffmail.com), [2] Senior Research Fellow (Plant Pathology) (e mail: priyarathour26@gmail.com), Division of Pathology; [3] M Sc Student (e mail: aish.singh7@gmail.com), GLA University Mathura; [4] Assistant Professor (e mail: richabhu@yahoo.co.in), Department of Botany, Mahila Maha Vidyalya, Banaras Hindu University, Varanasi, Uttar Pradesh 221 005

and knol kohl, brown mustard, yellow mustard and turnip from Indian Agricultural Research Institute, New Delhi and Laxminagar area of Delhi, India (Table 1). A loopful of bacterial suspension was streaked on nutrient sucrose agar medium containing 23 g of nutrient agar, 20 g of sucrose and 5g of agar powder in 1 000 ml of water and incubated at 28°C for 48 hr (Raghavendra *et al.* 2012). A typical Xcc colony (pale yellow, raised, mucoid) from each plate was sub-cultured on YGCA (yeast extract, glucose, calcium carbonate and agar) slants containing 10 g of yeast extract, 10 g of D-glucose anhydrous, 20 g of calcium carbonate and 20 g of agar powder in 1 000 ml of water (Schaad *et al.* 2001). The cultures were routinely grown on YGCA medium and maintained at -80°C with 20% glycerol for further study.

The typical isolates of Xcc were tested for their pathogenicity on cauliflower plants. Thirty days old seedling of cauliflower cv. Pusa Sharad was grown in the field at Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi in 2014. The 48-hr-old culture of Xcc grown on NSA medium contain 4.36×10^9 cfu/ml was inoculated by leaf clipping method on the three youngest leaves of each plant as described by Singh *et al.* (2011).

The isolates of Xcc were grown in nutrient broth medium for 24 h at 28°C. The total genomic DNA of bacterium was extracted with CTAB method (Murray and Thompson 1980). Molecular characterization of Xcc isolates using *hrp* gene based PCR technique was done and amplified at 769 bp as described by Singh *et al.* 2014a)

Repetitive sequence-based PCR(rep-PCR) was performed under the BOX-PCR, ERIC (Enterobacterial Repetitive Intergenic Consensus) PCR and REP (Repetitive Extragenic Palindromic) PCR conditions, using the BOX primer: BOXA1R (52 CTACGGCAAGGCGACGCTGACG-3), ERIC primers: ERIC-1R (52 ATGTAAGCTCCTGGGGATTCAC-3) and ERIC-2 (5-AAGTAAGT GACTGGGGTGAGCG-3), REP primers: REP1R-I (5-IIIICGICGICATCIGGC-3) and REP2-I (5-ICGICTTATCIGGCCTAC-3). For REP, ERIC and BOX -PCR amplifications were carried out in a final volume of

Table 1 List of isolates of *Xanthomonas campestris* pv. *campestris* isolated from *Brassica* species crops from different locations of Delhi in 2014

Isolates	Host	Location of collection	Patho-genicity	*Hrp* gene based PCR	DNA typing at ~ 50% similarity coefficient		
					Rep-PCR	RAPD	Rep + RAPD
Xcc-C260	Brown mustard (*B. rapa* subsp. *dichotoma)*	IARI, Delhi	+	+	2	2	4
Xcc-C261	Turnip (*B. rapa* subsp. *rapa*)	IARI, Delhi	+	+	2	4	5
Xcc-C262	Cabbage (*B. oleracea* var. *capitata*)	IARI, Delhi	+	+	2	6	7
Xcc-C263	Broccoli (*B. oleracea* var. *italica*)	IARI , Delhi	+	+	2	6	7
Xcc -C264	Broccoli (*B. oleracea* var. *italica*)	IARI , Delhi	+	+	2	6	7
Xcc-C265	Broccoli (*B. oleracea* var. *italica*)	IARI , Delhi	+	+	2	6	7
Xcc-C266	Yellow mustard (*B. rapa* subsp. *trichularis)*	IARI, Delhi	+	+	2	6	7
Xcc-C267	Yellow mustard (*B. rapa* subsp. *trichularis)*	IARI, Delhi	+	+	2	6	7
Xcc-C268	Yellow mustard (*B. rapa* subsp. *trichularis)*	IARI, Delhi	+	+	2	3	6
Xcc-C269	Turnip (*B. rapa* subsp. *rapa*)	IARI, Delhi	+	+	3	4	8
Xcc-C270	Brown mustard (*B. rapa* subsp. *dichotoma)*	IARI, Delhi	+	+	2	7	6
Xcc-C271	Brown mustard (*B. rapa* subsp. *dichotoma)*	IARI, Delhi	+	+	3	4	8
Xcc-C272	Brown mustard (*B. rapa* subsp. *dichotoma)*	IARI, Delhi	+	+	2	5	7
Xcc-C273	Knol Khol (*B. oleracea* var. *gongylodes*)	Laxminagar, Delhi	+	+	2	4	7
Xcc-C274	Knol Khol (*B. oleracea* var. *gongylodes*)	Laxminagar, Delhi	+	+	2	6	7
Xcc-C275	Knol Khol (*B. oleracea* var. *gongylodes*)	Laxminagar, Delhi	+	+	2	7	7
Xcc-C276	Knol Khol (*B. oleracea* var. *italica*)	Laxminagar, Delhi	+	+	4	6	3
Xcc-C277	Cabbage (*B. oleracea* var. *capitata*)	Laxminagar, Delhi	+	+	2	6	7
Xcc-C278	Cabbage (*B. oleracea* var. *capitata*)	Laxminagar, Delhi	+	+	2	6	7
Xcc-C279	Cabbage (*B. oleracea* var. *capitata*)	Laxminagar, Delhi	+	+	5	6	7
Xcc-C280	Brocolli (*B. oleracea* var. *italica*)	Laxminagar, Delhi	+	+	2	8	7
Xcc-C281	Brocolli (*B. oleracea* var. *italica*)	Laxminagar, Delhi	+	+	2	5	2
Xcc-C282	Brocolli (*B. oleracea* var. *italica*)	Laxminagar, Delhi	+	+	2	6	7
Xcc-C283	Cauliflower (*B. oleracea* var. *botrytis*)	Laxminagar, Delhi	+	+	2	6	7
Xcc-C284	Cauliflower *B. oleracea* var. *botrytis*)	Laxminagar, Delhi	+	+	3	6	8
Xcc-C285	Cauliflower (*B. oleracea* var. *botrytis*)	Laxminagar, Delhi	+	+	3	6	8
Xcc-C286	Brown mustard (*B. rapa* subsp. *dichotoma)*	Laxminagar, Delhi	+	+	1	8	1
Xcc-C287	cauliflower (*B. oleracea* var. *botrytis*)	Laxminagar, Delhi	+	+	2	6	7

[a] DNA type was identified by DNA fingerprint analysis based on this study and previously reported by Horita *et al*. (2005, 2010)

25 µl containing as described by Schaad *et al.* (2001). Gel electrophoresis of PCR product was done as described by Singh *et al.* (2011).

The bacterial RAPD fingerprints were obtained using the procedure of Williams *et al.* with minor modifications such as annealing temperature and PCR conditions. Several different 10-mer oligonucleotide primers were tested. The twelve primers, viz. UBC-211 (5'-GAAGCGCGAT-3'UBC-232 (5'-CGGTGACATC-3'), OPA-11 (5'-TGGACCGGTG-3'; Rezaei *et al.* (2011), D-11 (5'-AGCGCCATTG-3'; Manulis *et al.* 1994) ,UBC-241 (5'-GCCCGACGCG-3'), UBC-245 (5'-CGCGTGCCAG-3'), UBS-295 (5'-CGCGTTCCTG-3'; Pooler *et al.* 1996), OPB-10 (5'-CTGCTGGGAC-3'), OPB-15 (5'-GGAGGGTGTT-3'; Berthier and Stanislav (1999), OPC-02 (5'-GTGAGGCGTC-3'), P-27 (5'-GGCAGGCTGT-3'), P-49 (5'-GAAACGGGTG-3'; Khood and Fakim (2004), were chosen on the basis of their capability to produce polymorphic bands in a preliminary evaluation, and reproducibility for RAPD fingerprinting. Final volume of 25 µl containing 2.5µl of 10 × PCR buffer, 2 µl of 25 mM $MgCl_2$, 0.8 µl of 10 mM dNTPs, 1.0 µl of 10 pmol of each primer, 1.5 U of Taq polymerase and 1.0 µl of 50 ng DNA template was used. PCR amplification was performed in a thermal cycler (BIO-RAD, C1000^{TM} thermal cycler) in the following cycles initial denaturation at 94°C for 5 min, was followed by 40 cycles of 94°C for 1 min, annealing at 35°C with P-27,P-49, D-11,OPC-02 and 220, 37°C with UBC-220, UBC-232 and OPA11, 36°C with OPB-10 and OPB-15 and 48°C with UBC-241, UBC-245 and UBC-295 for 1 min and extension at 72°C for 2 min with the final extension cycle for 10 min. All of the strains listed in were subjected to PCR amplification with each of these twelve primers. Amplified PCR fragments were separated on 1.5% agarose gel using 1X TAE (1X TAE prepared from a 50X TAE buffer stock solution. After the running of gel, the gel was photographed using the gel documentation system (BIO-RAD, GEL DOCTM XR+ with image Lab^{TM} software).

The normalized data generated from Rep-PCR (BOX, ERIC-, REP-PCR) and RAPD fingerprinting profiles were used either separately or combined together 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 2.02e. The output data were graphically presented as a phylogenetic tree.

RESULTS AND DISCUSSION

Characterization of bacteria

Twenty eight isolates of *X. campestris* pv. *campestris* (Xcc) were collected from northern region of India**.** All the isolates were isolated from cole crops (cauliflower, cabbage, broccoli and knol khol), turnip and mustard from two location, i.e. IARI farm and Laxminagar areas of Delhi (Table 1). These isolates of Xcc produced yellow, translucent, raised, mucoid colonies on the NSA medium. They were gram negative, rod shaped, aerobic and monotrichous flagellum. Pathogenicity of all the isolates was tested on cauliflower (*B. oleracea* var. *botrytis*) cv. Pusa Sharad by artificial inoculation and all the isolates of Xcc produced typical black rot symptoms as yellow or dead tissue at the margin of infected leaves, similar to tip burn, frequently progress into a V-shaped and blackening of vein within 15 days after inoculation (Table 1). These isolates were Xcc on the basis of morphological and biochemical and pathogenicity tests (Table 1). Molecular marker based on *hrp* gene sequence amplified at 769 bp in the isolates.. It has been established that the symptoms are dependent on environmental condition, particular host genotype, or specific genes (Chen *et al*. 1994, Ignatov 1999). PCR based molecular technique with specific region of genome *hrp* (Berg *et al.* 2005), which is to highly conserved enable to differentiation of the pathovars (Singh *et al.* 2014a).

Genetic diversity by Rep-PCR

Data on DNA fingerprinting was generated from genomic DNA extracted from all the 28 isolates of Xcc. DNA fragments of 300 bp to 6 kb were amplified in BOX-PCR and 400 bp to 8 kb in ERIC-PCR and 300 bp to 6 kb REP-PCR and revealed a high level of genetic diversity among the isolates of *X. campestris* pv. *campestris*. Maximum number of amplicon were found in BOX–PCR (29 amplicon) and 27 amplicon in ERIC-PCR followed by 18 amplicon in REP–PCR. Amplification in each isolate of Xcc varied in all the methods of PCR as amplicon 6-10 in BOX-PCR, 8-14 in ERIC PCR, 3-9 in REP-PCR. In combined analysis rep-PCR including BOX, ERIC and REP-PCR all isolates clustered into five groups, i.e. DNA type 1–5 based on ~50 similarity coefficient and 21 isolates of Xcc have DNA type 1 followed by 4 isolates in DNA type 2 (Table 1). At 75% similarity coefficient, they were further grouped into twelve subgroups, viz. A, B, C, D, E, F, G, H, I, J, K and L. Fourteen isolates falls in subgroup A (Xcc-C260, Xcc-C265, Xcc-C262, Xcc-C287, Xcc-C263, Xcc-C264 Xcc-C266, Xcc-C267 Xcc-C273, Xcc-C275, Xcc-C277, Xcc-C278, Xcc-C282 and Xcc-C284). Isolates Xcc-C286, Xcc-C279 and Xcc-C276 were grouped into DNA types 1, 4 and 5 respectively. Although, these isolates were collected at Laxminagar, Delhi from different crops like brown mustard, cabbage and knol khol. For genomic variability, rep-PCR fingerprinting using primer sets (BOX, REP and ERIC) are highly conserved repetitive sequences, showed different banding pattern among Xcc isolates of different *Brassica* hosts in northern region in India. The results indicated that eight clusters of Xcc related to the geographical origin as earlier reported by Vicente *et al*. (2006). These findings contrast with results reported by Massomo *et al.* (2003) and Tsygankova *et al.* (2004) and indicated that geographical origin was a major factor for Xcc genetic diversity. It proves that Indian isolates of Xcc have wide range of genetic diversity within same

Table 2 Analysis of RAPD primers with genomic DNA of 28 isolates of *X. campestris* pv. *campestris* collected from different hosts of *Brassica* species

RAPD Primer	Isolates of *Xanthomonas campestris* pv *campestris*																												Total bands	Polymorphic bands	Monomorphic bands	Amplified bands
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28				
OPB-10	5	1	2	1	2	2	1	1	4	3	2	2	2	2	6	4	5	5	7	1	1	2	3	3	3	3	1	5	79	7		7
OPB-15	3	4	3	3	3	3	3	3	4	3	3	4	5	3	2	4	5	4	4	3	6	2	2	4	5	5	4	4	101	7		7
P-49	6	2	1	1	1	1	1	1	6	2	5	1	3	3	1	3	5	3	4	4	2	2	2	2	2	1	1	1	67	9	1	10
P-27	3	2	1	1	1	1	1	2	6	3	2	1	2	2	1	3	6	3	3	1	3	1	2	2	2	2	3	2	62	8	1	9
UBC-241	4	9	8	5	2	6	2	7	7	7	10	9	7	8	8	7	6	6	5	8	5	2	7	6	8	8	5	6	178	13	1	14
UBC-245	4	5	6	7	7	6	7	9	2	4	4	5	4	4	5	3	3	5	5	6	5	2	6	2	5	5	6	5	137	11		11
D-11	3	4	2	1	2	1	2	1	3	2	1	2	2	4	2	4	3	3	3	2	2	1	3	3	4	2	1	3	66	15		15
UBC-220	2	3	2	2	3	4	4	3	2	4	1	2	2	2	3	2	2	4	3	2	3	2	3	3	3	1	1	1	69	9		9
OPA-11	3	1	3	2	2	1	2	3	3	3	1	2	4	5	2	2	3	4	2	2	1	6	2	1	3	2	3	3	71	13		13
UBC-295	5	5	7	2	3	3	5	7	6	3	2	4	4	5	4	4	4	7	4	5	2	2	1	3	2	2	1	2	104	11	2	13
UBC-232	2	2	2	2	2	2	2	2	4	4	2	4	6	3	3	3	2	3	5	4	3	2	2	2	3	3	1	2	77	10	1	11
OPC-02	3	4	3	3	3	3	3	3	4	3	3	2	1	2	4	5	6	2	3	7	8	4	3	2	1	1	2	1	89	10	1	11
Total	44	44	43	34	36	39	40	50	60	51	47	50	55	57	56	60	67	67	67	65	62	50	59	57	66	61	56	63	1100			130

geographical area and within the cole crops. In this study, genetic variability among Xcc isolates was found either on the same host or different *Brassica* hosts in the same locality.

Genetic diversity by RAPD

Genetic variation of 28 isolates of Xcc was evaluated using 12 polymorphic RAPD primers. In general, a total of 1100 scorable bands were observed and polymorphic in Xcc. The size of fragments ranged from 200 bp to 2.0 kb. The number of fragments produced by various primers ranged from 1-10 to with an average of 3.26 fragments per primer. The total highest 178 bands and lowest 62 bands were obtained with primers UBC-241 and P-27 respectively. Out of twelve primers, six primers (P-49, P-27, UBC-241, UBC-295, UBC-232 and OPC-02) showed monomorphic bands and remaining six primers (OPB-10, OPB-15, UBC-245, D-11, UBC-220 and OPA-11) exhibited polymorphic amplified product of all isolates of Xcc. Primer UBC- 295 produced two monomorphic bands in all the isolates (Table 2). The all 28 isolates of Xcc clustered into eight groups as DNA typing 1-8 at ~50% similarity coefficient (Table 1) and out of 28 isolates, 16 isolates of Xcc clustered under DNA type 6 Xcc-C262, Xcc-C263, Xcc-C264, Xcc-C265, Xcc-C266, Xcc-C267, Xcc-C274, Xcc-C276, Xcc-C277, Xcc-C278, Xcc-C279, Xcc-C282, Xcc-C283, Xcc-C284, Xcc-C284, Xcc-C285 and Xcc-C287) and five isolates under DNA type 4. Xcc-C281, Xcc-C268 and Xcc-C260 formed separate group and come under DNA types 1, 2 and 3 respectively. Twelve random oligonucleotide primers are used RAPDs that have been used extensively as molecular markers (Shikano and Taniguchi 2002). Primers UBC-241 and UBC-245 produced more polymorphic bands in Xcc isolates and suited for genetic diversity.

Dendrogram prepared using combined data of rep-PCR and RAPD for cluster analysis, reveals that eight clusters were formed as DNA typing 1–8. Maximum 18 isolates of Xcc were grouped under DNA type 7 followed by 4 isolates under DNA type 8 at ~50% similarity coefficient. Remaining 6 isolates of Xcc isolated from brown mustard (Xcc-C286, Xcc-C 260), knol khol (Xcc-C276), broccoli (Xcc-C281), turnip (Xcc-C261) and yellow mustard (Xcc-C268) were formed separate group as DNA types, 1, 2, 3, 4, 5 and 6 respectively (Table 1). In this study, we combined rep-PCR and RAPD in alone or combination, out of 28 isolates of Xcc, 8 DNA types were formed by using RAPD, 5 DNA types in Rep-PCR and 8 DNA types in RAPD + Rep-PCR at 50 % similarity coefficient. It indicates that more reliable data on genetic diversity is obtained as earlier used by Rouhrazi and Khodakaramian (2014) and is also depending on which tools were used for the study.

The data obtained from combination of rep-PCR and RAPD fingerprinting showed that genetic variability was found same geographical area and they in spite of their brassica hosts and no correlation was found between DNA typing of isolate of Xcc and location specificity.

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