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Pathogenic variability and genetic diversity using BOX -PCR of *Xanthomonas campestris* pv *campestris* isolated from cole crops

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Xanthomonas campestris pv *campestris* (*Xcc*) (Pammel) Dowson causes black rot disease in crucifers, which is one of the most important diseases in India and occurs in all parts of the country including the states of West Bengal, Odisha, Maharashtra, Uttar Pradesh, Uttarakhand, Himachal Pradesh, Rajasthan, Delhi, Meghalaya and Jammu and Kashmir (Singh and Shri Dhar 2011). The disease is vascular disease and typical symptoms of the disease in the field are V-shaped chlorotic to necrotic lesions starting from leaf margins and with blackening of the veins and vascular tissues. *Xanthomonas campestris* possesses interspecies variations on the level of pathovars, haplotypes, serogroups and based on the interaction of various isolates of *X. campestris* pv *campestris* with different *Brassica* species, a total of seven pathogenic races of *X. campestris* pv *campestris* have been reported (Jensen *et al.* 2010). PCR-based DNA-fingerprinting is a fast, reliable and comparatively low cost method to study the genetic diversity of bacteria (Zaccardelli *et al.* 2007, Valverade *et al.* 2007), which is better than conventional methods of characterization. Several methods for assessment of genetic diversity of bacteria employing PCR with different primers homologous to repetitive sequences under three families of repetitive sequences (Rep) including repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences and BOX element were identified and their use as primers for PCR leads to selective amplification of distinct genomic regions including highly polymorphic intergenic regions of bacteria (Zaccardelli *et al.* 2007, Singh *et al.* 2011).

In India, study on genetic diversity and virulence pattern of *X. campestris* pv. *campestris* is lacking and the information needs to be generated. However, a little information on resistance against black rot is available in commercial lines of *Brassica* especially in cole crops. Knowledge of the existence of genetic variability in the pathogen population is important for plant breeding and consequently in crop improvement program. Thus, the variation within *X. campestris* pv. *campestris* must be determined in order to design effective control strategies, especially seed health tests. In the present study, we collected black rot infected cole crops from diverse agro-climatic regions of the country for assessing the genetic diversity by using BOX-PCR and pathogenic variability of *X. campestris* pv *campestris*.

One or two leaf samples, representing one or two plants from each field, were collected from cole crops growing states of India and isolated from cabbage Xcc- C 28 and Xcc-C124 (IIHR, Bangalore, Karnataka), Xcc-C117 (CS Azad University of Agricultural & Technology, Kanpur, UP), Xcc-C6 (IARI Farm, New Delhi), Xcc- C130 (Ranchi, Jharkhand), Xcc-C129 (Sonipat, Haryana), Xcc-C118 (CSKHPKV, Palampur, HP), and Xcc-C121 (AAU, Anand, Gujarat), from cauliflower Xcc-C132 (GKVK, UAS, Bangalore, Karnataka), Xcc-C23 & Xcc-C1(IARI Farm, New Delhi), Xcc-C125 & Xcc-C126 (Basht, Udham nagar, Jammu & Kashmir), Xcc-C127 (Cuttak, Odisha), Xcc-C115 (Jagatsighpur, Odisha), Xcc-C111& Xcc-C131 (BAU, Ranchi, Jharkhand), Xcc-C110 (Sonipat, Haryana), Xcc-C 128 (CSKHPKV, Palampur, HP), Xcc-C119 (ICAR Research Complex For NEH Region, Shilong, Meghalaya), Xcc-C116 (GBPUAT Ranichauri, Uttarakhand), and Xcc-C120 (GBPUAT Pantnagar, Uttarakhand), from broccoli Xcc-C4 (IARI Farm, New Delhi) and from knoll khol Xcc-C14 (Basht, Udham nagar, Jammu & Kashmir). A loopful of suspension was streaked on nutrient sucrose agar medium and incubated at 28ºC for 48 to 72 hr A typical *X. campestris* pv *campestris* colony (pale yellow, raised, mucoid) from each isolation plate was subcultured on YGCA slants (Schaad *et al.* 2001). The cultures were routinely

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grown on YGCA medium and maintained at –80°C with 20% glycerol for further study. Pathogenicity of 24 isolates of *X. campestris* pv *campestris* was tested on 50 days old plants of cauliflower cv Pusa Sharad as described earlier (Schaad *et al.* 2001).

For virulence analysis, seeds of oilseed cruciferous crops were obtained from Division of Genetics, while the seeds of cole crops were taken from Division of Vegetable Science, IARI, New Delhi the experiment was conducted at Division of Plant Pathology, IARI, New Delhi. During November 2010 – March 2011. All the isolates of *X. campestris* pv *campestris* were grown on YGCA at 28°C for 48 hr, then the bacterial growth was scraped from the Petri plates and the inoculum maintained at 10^8 to 10^9 cfu/ml by pouring 10 ml of sterile distilled water. Leaves were inoculated by giving a small cut, near the margins, with scissor 35 days after sowing of oilseed crops, turnip and radish and 25 days after transplanting of cole crops. Totally 10 cuts were given to each leaf by dipping the scissor in the bacterial suspension as described Singh *et al.* (2011). The three young leaves on each plant were inoculated. The disease incidence and severity of symptoms were assessed after 15 and 30 days of inoculation. The total number of inoculated points and number of points showing disease symptoms were recorded and the percentage of infected points were calculated to obtain disease incidence data. The severity of symptoms was assessed by using 0-9 scale based on the relative lesion as described by Singh *et al.* (2011). Virulence analysis was calculated based on how many cultivars of crucifers were infected by *X. campestris* pv *campestris* isolates showed susceptible and resistant reaction, 0-3 rating is considered as resistant reaction (R) and, the rating above 3 is susceptible reaction (S). The data generated on disease incidence and disease severity were statistically analysed.

The total genomic DNA of bacterium was extracted with guanidium thiocyanate as described by Pitcher *et al.* (1989). The purity and quantity of DNA samples were confirmed through OD values recorded at 260 and 280 nm by nano spectrophotometer. Genetic diversity of 24 isolates of *X. campestris* pv *campestris* was studied by using BOX primer (BOXA1R – 5-‘CTACGGCAAGGCGACGC TGACG-3’) as described by Schaad *et al.* 2001. 25 µl of reaction mixture containing 5.0 µl of 5X PCR buffer, 2.0 µl of 25 mM $MgCl_2$, 0.2 µl of BSA, 2.5 µl of DMSO, 1.25 µl of 25 mM dNTPs, 1.0 µl of primer, 1 unit Taq polymerase and 1.0 µl of 20 ng DNA was used. Reactions were run for 35 cycles and each consisting of 30s at 94^0C, 30s at 92^0C, 1 min at 53^0C for BOX-PCR, 8 min at 65^0C with initial incubation of 2 min at 95^0C and final extension of 15 min at 65^0C. PCR products were analysed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and photographed using the gel documentation system (BIO-RAD, GEL DOCTM XR+). The digital image of each gel was analyzed. The normalized data generated from REP and BOX fingerprinting profiles were used 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.

Pathogenic variability

The pathogenic pattern was studied for the 24 isolates of *X. campestris* pv *campestris* (Table 1) isolated from cauliflower, cabbage, broccoli and knoll khol from diverse agro-climatic regions of India by artificial inoculation on to the 27 cruciferous cultivars including four genera of crucifereae family, viz. *Brassica* spp (7 species of *Brassica* as *B. campestris, B. carinata, B. juncea, B. napus, B. nigra, B. oleracea* and *B. rapa*)), *Eruca sativa, Sinapsis alba* and *Raphanus sativus* under field conditions. All 24 isolates of *X. campestris* pv *campestris* were divided in to three groups viz. Group I, Group II and Group III based on pathogenic reaction on crucifer cultivars. 20 strains of *X. campestris* pv *campestris* grouped into Group I, and all cultivars showed susceptible reaction except cultivars of *B. juncea* against these strains. Xcc-C120 is the only member of Group II, which showed resistant reaction on Pusa Ageti of cabbage and PTWG of turnip, in addition to the cultivars of *B. juncea*. Whereas, Xcc-C118, Xcc-C110 and Xcc-C1 of Group III showed resistant reaction to *B. juncea* and PTWG cultivar of turnip. The disease incidence was increased significantly in due course time in all the cultivars of crucifers in due course of time after inoculation and 100% disease incidence was found most of the cultivars caused by these isolates of *X. campestris* pv *campestris* except cultivars of *B. juncea,* viz. Pusa Bold, Varuna, Pusa Mustard 21 and Pusa Vijay, which was lesser than other cultivars of crucifer. Black rot disease severity in cultivars varied from 0.29 ± 0.10 - 6.40 ± 0.43, 0.23 - 6.60 and 0.26 ± 0.06 – 6.48 ± 0.27 out of 9 scale in Group I, Group II and Group III after 30 days of inoculation respectively (Table 1). Maximum disease severity (6.60) was found in cultivar BSH 9 (brown sarson) caused by group II Xcc- C120 followed by Pusa Gold (yellow sarson) (6.48 ± 0.27) caused by group III isolates of *X. campestris* pv. *campestris*. All the isolates of *X. campestris* pv *campestris* showed highly virulent in respective of their location and able to cause black rot disease in all the cultivars. The black rot symptoms appeared within six days in susceptible cultivars, while symptoms development was delayed >20 days in resistant cultivars of *B. juncea* like Pusa Bold, Varuna, Pusa Mustard 21 and Pusa Vijay. Most of the cultivars of variety *B. oleracea* (cole crops) showed susceptible to highly susceptible reaction except Pusa Ageti, which showed resistant against isolate Xcc- C120 belonging to Group II.

Various studies have been conducted to characterize

Table 1 Pathogenic groups of *X. campestris* pv *campestris* isolates with cruciferous cultivars after 30 days of inoculation under field conditions in 2010-11.

Name of cultivars	Disease incidence (%)			Disease severity (0-9 scale)			Pathogenic reaction with cruciferous cultivars		
	Group I	Group II*	Group III	Group I	Group II*	Group III	Group I	Group II	Group III
Brassica juncea (Indian mustard-Pusa Bold)	29.16 ± 13.50	33.33	16.66 ± 5.77	0.44 ± 0.26	0.26	0.30 ± 0.14	R	R	R
Brassica juncea (Indian mustard-Varuna)	22.50 ± 15.55	20.00	17.77 ± 10.18	0.37 ± 0.27	0.50	0.26 ± 0.06	R	R	R
Brassica juncea (Indian mustard-Pusa Mustard 21)	23.50 ± 9.88	26.66	15.55 ± 6.93	0.311 ± 0.12	1.06	0.38 ± 0.30	R	R	R
Brassica juncea (Indian mustard-Pusa Vijay)	19.83 ± 7.83	20.00	17.77 ± 5.09	0.29 ± 0.10	0.23	0.36 ± 0.10	R	R	R
B. napus (Rapeseed-GSL-1)	100 ± 0	100	100 ± 0	6.01 ± 0.37	5.60	5.93 ± 0.46	S	S	S
B. napus (Rapeseed-GSL-2)	100 ± 0	100	100 ± 0	5.86 ± 0.36	4.66	6.15 ± 0.54	S	S	S
B. carinata (Ethiopian mustard-IGC-01)	100 ± 0	100	100 ± 0	4.38 ± 0.41	4.13	4.57 ± 0.25	S	S	S
B. carinata (Ethiopian mustard-NPC-9)	100 ± 0	100	100 ± 0	5.08 ± 0.57	5.93	5.24 ± 0.03	S	S	S
B. campestris L. var. toria (Toria-TL-15)	100 ± 0	100	100 ± 0	5.24 ± 0.45	5.26	5.75 ± 0.25	S	S	S
B. campestris L. var. toria (Toria-Pt-303	100 ± 0	100	100 ± 0	6.00 ± 0.36	5.40	6.31 ± 0.03	S	S	S
B. rapa subsp. *Sarson* (Brown sarson-BSH-9)	100 ± 0	100	100 ± 0	6.39 ± 0.24	6.60	6.26 ± 0.34	S	S	S
B. rapa subsp. *Sarson* (Brown sarson-Pusa Kalyani	100 ± 0	100	100 ± 0	6.36 ± 0.32	6.26	6.24 ± 0.24	S	S	S
B. rapa var *trilocularis* (Yellow sarson- PBT-37)	100 ± 0	100	100 ± 0	6.27 ± 0.32	5.80	6.46 ± 0.06	S	S	S
B. rapa var *trilocularis* (Yellow sarson- Pusa Gold)	100 ± 0	100	100 ± 0	6.40 ± 0.43	6.40	6.48 ± 0.27	S	S	S
Sinapus alba (IC-390162)	100 ± 0	100	100 ± 0	5.19 ± 0.48	4.26	5.62 ± 0.74	S	S	S
Eruca sativa (Taramira- RTM-314)	100 ± 0	100	100 ± 0	6.25 ± 0.25	6.13	6.06 ± 0.29	S	S	S
Eruca sativa (Taramira- T-27)	100 ± 0	100	100 ± 0	5.90 ± 0.38	5.66	5.71 ± 0.23	S	S	S
B. oleracea var. *capitata* (Cabbage- Pusa Ageti)	100 ± 0	100	100 ± 0	3.00 ± 0.33	3.86	3.15 ± 0.23	S	R	S
B. oleracea var. *botrytis* (Cauliflower- CCM5)	100 ± 0	100	100 ± 0	5.86 ± 0.57	5.46	5.44 ± 0.40	S	S	S
B. oleracea var. *botrytis* (Cauliflower- Pusa Agrajyoti)	100 ± 0	100	100 ± 0	5.91 ± 0.46	6.46	6.37 ± 0.30	S	S	S
B. oleracea var. *gongylodes* (Knoll khol- Palam Tenderknol)	100 ± 0	100	100 ± 0	5.39 ± 0.51	4.60	5.20 ± 0.37	S	S	S
B. oleracea var. *italica* (Broccoli- Palam Vicnita)	100 ± 0	100	100 ± 0	5.13 ± 0.37	4.66	5.26 ± 0.13	S	S	S
Brassica rapa var. *rapa* (Turnip- PTWG)	100 ± 0	100	100 ± 0	4.99 ± 0.60	2.60	3.00 ± 0.55	S	R	R
Brassica rapa var. *rapa* (Turnip- Pusa Swarnima)	100 ± 0	100	100 ± 0	5.23 ± 0.44	4.86	5.24 ± 0.43	S	S	S
Brassica rapa var. rapa (Turnip- Pusa Swesi)	100 ± 0	100	100 ± 0	5.87 ± 0.60	5.40	5.22 ± 0.32	S	S	S
Raphanus sativus (Radish- Pusa Mrudula)	100 ± 0	100	100 ± 0	4.88 ± 0.57	3.93	5.57 ± 0.32	S	S	S
Raphanus sativus (Radish- Pusa Chetaki)	100 ± 0	100	100 ± 0	4.86 ± 0.60	4.00	4.06 ± 0.69	S	S	S

*Standard deviation did not calculate since the group contain single isolate.

Group I = Xcc-C124, Xcc-C28, Xcc-C132, Xcc-C117, Xcc-C23, Xcc-C4, Xcc-C6, Xcc-C125, Xcc-C14, Xcc-C126, Xcc-C127, Xcc-C115, Xcc-C130, Xcc-C111, Xcc-C131, Xcc-C129, Xcc-C128, Xcc-C118, Xcc-C119, Xcc-C116, Xcc-C121; Group II- Xcc-C120; Group III- Xcc-C118, Xcc-C110, Xcc-C1.

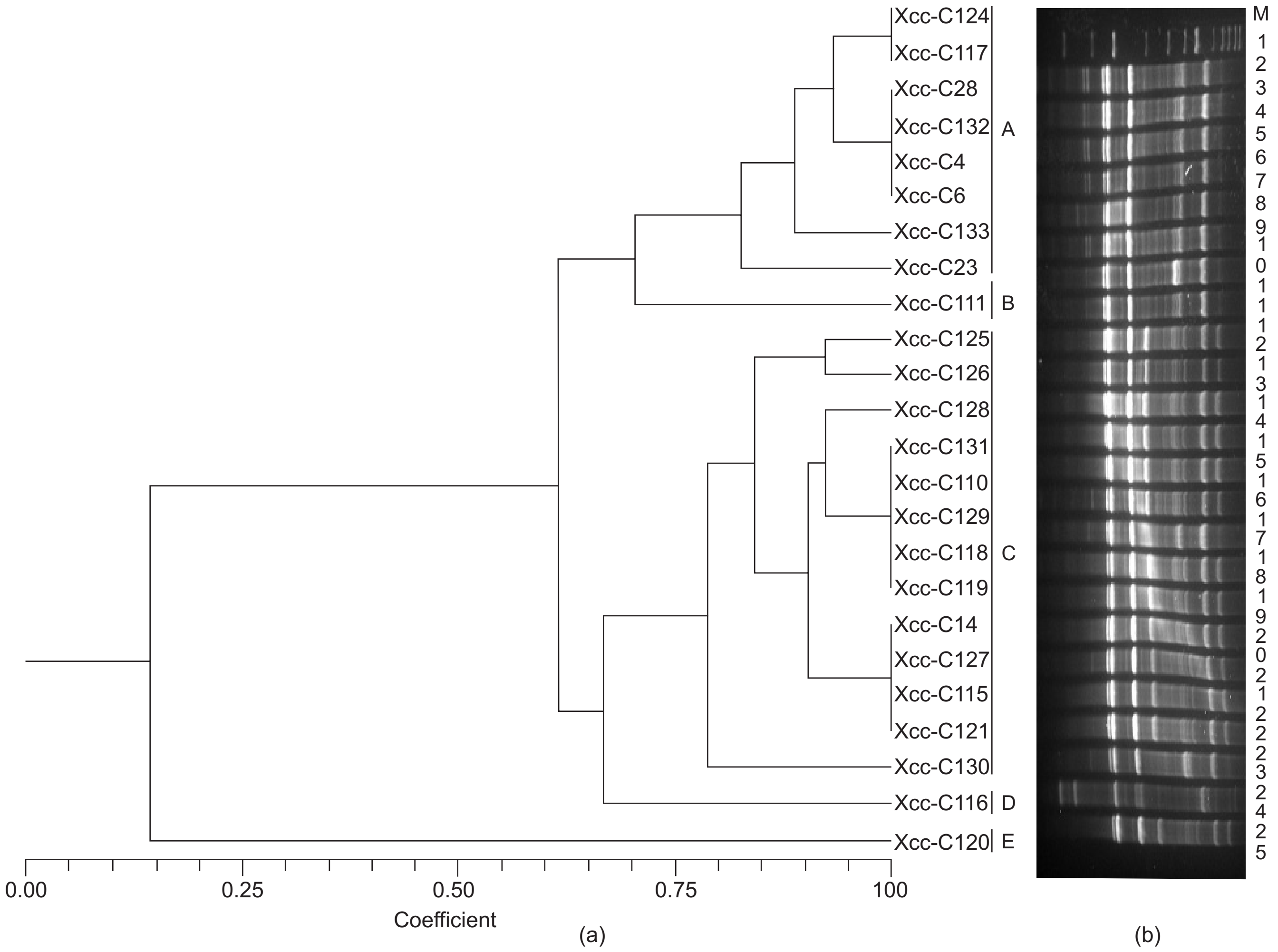

Fig. 1. (a) Cluster analysis of the BOX- PCR fingerprint patterns generated from purified genomic DNA of 24 isolates of X. *campestris* pv *campestris*. Unweighted paired group mathematical average (UPGMA) dendrogram were generated using Pearson's correlation of coefficient from hole patterns of individual gel tracks. (b) Box-PCR generated fingerprinting of 24 isolates of *X campestris* pv. *campestris* isolated from cole crops from different agro-climatic region of India. lane M= 1kb ladder, lanes 1-3: Xcc-C124, Xcc-C28, Xcc-C132(Karnataka), 4: Xcc-C117 (U.P.), 5-8 : Xcc-C23, Xcc-C4, Xcc-C6, Xcc-C1 (Delhi), 9-11 : Xcc-C125, Xcc-C14, Xcc-C126 (Jammu), 12-13 : Xcc-C127, Xcc-C115 (Odisha), 14-15 : Xcc-C130, Xcc-C111, Xcc-C131 (Jharkhand), 16-18: Xcc-C110, Xcc-C129(Haryana), 19-20: Xcc-C128, Xcc-C118 (H.P.), 21: Xcc-C119 (Meghalaya), 22-23: Xcc-C116, Xcc-C120 (Uttarakhand), 24: Xcc-C121 (Gujarat), 25: Negative control.

pathogenic variability and also race characterization of *X. campestris* pv *campestris* in other countries (Jensen *et al.* 2010.). However, in this case, we could not distinct race of *X. campestris* pv *campestris* but the sufficient pathogenic variability was found in this pathogenic bacteria. Isolates Xcc-C124, Xcc-C6, Xcc-C125, Xcc-C111, Xcc-C131, Xcc-C130 and Xcc-C127 were highly virulent, which were isolated from across the country and no clear cut distinction in pathogenic variation among these isolates were recorded.

Genetic diversity

Data on DNA fingerprinting was generated from genomic DNA extracted from all the 24 strains of *X. campestris* pv *campestris.* DNA fragments of 400 bp to 7 kb were amplified in BOX- PCR, which revealed a high level of genetic diversity among these isolates. Fig 1b reveals that maximum 15 fragments of amplicons were found in five isolates of *X. campestris* pv *campestris,* viz. Xcc-C28, Xcc-C132, Xcc-C4, Xcc-C6 and Xcc-C130 isolated from cabbage (IIHR, Bangalore, Karnataka), cauliflower (UAS, GKVK, Bangalore, Karnataka), broccoli and cabbage (IARI Farm, New Delhi), and cabbage (Ranchi, Jharkhand) respectively.

The fingerprinting data generated by using BOX-PCR with 24 isolates of *X. campestris* pv *campestris* showed polymorphic bands. The fingerprinting data were analysed and these isolates clustered into five groups at 75% similarity coefficient and designated as Group A, B, C, D and E. Out of 24, 13 isolates clustered in Group C (Xcc-C125, Xcc-

C126, Xcc-C128, Xcc-C131, Xcc-C110, Xcc-C129, Xcc-C118, Xcc-C119, Xcc-C14, Xcc-C123, Xcc-C115, Xcc-C121 and Xcc-C130) followed by 8 isolates in Group A. These strains were isolated from different agro- climatic condition and different hosts of cole crop. Strains Xcc-C111 and Xcc-C120 isolated from cauliflower from Jharkhand and Uttarakhand states respectively formed the separate cluster. However, the Xcc-C120 had differentiated at 50% and >75% similarity coefficient, whereas the Xcc-C111 and Xcc- C116 differentiated only >75% similarity coefficient. It indicates that genetic diversity of *X. campestris* pv *campestris* isolates was not affected by agro-climatic conditions and cultivars of cole crops. It may be due to mutation in DNA sequence of individual bacterium. No extensive analysis of genome of the strains belonging to *X. campestris* pv *campestris* from different geographical area of India has been performed earlier. BOX-PCR primer has also been used in the study of genetic diversity in previous studies on *X. campestris* pv *campestris* (Singh *et al*. 2011). The results indicate that clusters formed seem to be more related to the races than to the host or geographical origin of the isolates (Vicente *et al.* 2006). These findings are in contrast to the results reported by Tsygankova *et al.* (2004) that geographical origin was a major factor for *X. campestris* pv *campestris* genetic diversity.

The findings of the present study indicate that virulence pattern and genetic variability exist in the strains of *X. campestris* pv *campestris*. Disease incidence was mostly uniform in all the crucifer cultivars tested except on *B. juncea,* which showed resistant to all tested isolates of *X. campestris* pv *campestris*. These cultivars can be used as source of resistance in breeding program for development of resistant variety against black rot pathogen. High genetic diversity is existing among different Indian strains of *X. campestris* pv *campestris*. There is no correlation between definite pattern of virulence and their genetic variability exists in the strains. However, Xcc-C120, which was isolated from cauliflower (Pantnagar, Uttarakhand) was found different from other isolates of *X. campestris* pv *campestris* in both pathogenically as well as genetically.

SUMMARY

Black rot disease of cole crops caused by *Xanthomonas campestris* pv *campestris* (Xcc) is a very serious across the country. Twenty four isolates of *X. campestris* pv *campestris* were collected from cauliflower, cabbage, broccoli and knoll khol from different agro-climatic regions of India to study the pathogenic analysis and genetic diversity. These isolates were tested on 27 cultivars of crucifers including seven species of *Brassica, Sinapsis alba, Eruca sativa* and *Raphanus sativus* for studying pathogenic variability under field conditions at IARI, New Delhi during November to March 2010-11. 24 isolates of *X. campestris* pv *campestris* were grouped into three groups and maximum 20 isolates was kept under group I, while 1 isolate in group II and 3 isolates in group III. Black rot disease severity in crucifer cultivars varied from 0.29 ± 0.10 - 6.40 ± 0.43, 0.23 ± 6.60 and 0.26 ± 0.06 – 6.48 ± 0.27 out of 9 scale in group I, group II and group III respectively. Pusa Bold, Varuna, Pusa Mustard- 21 and Pusa Vijay of *B. juncea* showed resistant against all the isolates of *X. campestris* pv *campestris.* Genetic diversity of these isolates was studied using BOX-PCR, which indicated the existence of wide range of genetic diversity among the isolates. These isolates clustered into five Groups at 75% similarity coefficient and designated as Group A, B, C, D and E. In the present study, genetic diversity and virulence patterns of *X. campestris* pv. *campestris* isolates were established.

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