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Race-specific host-plant resistance against black rot (*Xanthomonas campestris* pv. *campestris*) in alien *Brassicas*

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ABSTRACT

Black rot disease (*Xanthomonas campestris* pv. *campestris* (Pam.) Dowson, *Xcc*) is one of the most devastating diseases in cauliflower worldwide. Potential reservoirs for black rot resistance in alien *Brassicas* (A and B genomes) were taken due to the availability of limited resistant germplasm for black rot disease in *Brassica oleracea* (C genome). Total 26 accessions of *Brassica* species were screened against *Xcc* race 1, 4, and 6 during three consecutive years, *viz.*, 2015, 2016, and 2017 under artificial inoculation conditions. One accession of *B. carinata*, and *B. napus*, two accessions of *B. juncea*, and all accessions of *B. nigra* were highly resistant against *Xcc* race 1, 4, and 6 except SRB-98 (moderate resistance). On the other hand, cauliflower varieties/lines *viz.*, Pusa Meghna, DC 41-5 and Pusa Sharad were highly susceptible against *Xcc* races 1, 4, and 6. Based on higher homoeology between the A and C sub-genome, a new resistant source BN-2-1 of *B. napus* was identified, which will be helpful to develop black rot resistant genetic stock with cauliflower through pre-breeding techniques such as *in vitro* embryo rescue and or somatic hybridization.

Keywords: Cauliflower, screening, *Brassica species,* black rot, resistance

INTRODUCTION

Black rot is the most important bacterial disease of crucifers caused by *Xanthomonas campestris* pv. *campestris* (Pam.) Dowson and most devastating disease in cauliflower. *Xanthomonas* bacterium infects the crop from nursery to seed production stage causing reduction in yield (10-50%) and quality under congenial environmental conditions (Singh *et al*., 10). Cauliflower is an important vegetable crop grown and consumed extensively worldwide and in India, it's now grown almost all the year round, but its production is limited due to black rot disease which takes a heavy toll of the crop thus causing a huge loss to the farmers. Managing this disease is very difficult as the bacterium spreads within and between fields by water splashes, wind, insects, machinery and irrigation. Chemical control is costly, time consuming, cumbersome besides causing health hazards and environmental pollution. Since Cole crops lack sufficient resistance sources, therefore emphasis have to be laid on search for more potent resistance sources for employing in the breeding programme. It is essentially crucial that plant population must be exposed to pathogen in such a way that resistant and susceptible plants can be distinguished without any ambivalence improving the efficacy of screening.

Majority accessions/lines of *B. oleracea* (C genome) group were reported highly susceptible (Raghvendra *et al.,* 6). In order to develop of a breeding programme, it is essential to know the variability of response of this pathogen present in the crucifer gene pool. Despite the fact that a large gene pool offers a great variability for disease resistance, it still remains largely under exploitation. To exclusively use germplasm resources for black rot resistance breeding, it will be useful to resolve the presence of *Xcc* resistance not just within cultivated *Brassica* vegetables, but also within related crucifer accessions.

Although, nine pathogenic races of *Xcc* have been identified worldwide (Tonu *et al*., 16), but race 1 and 4 are predominant. Recently, *Xcc* races 1, 4, and 6 were identified in India and, among this race 1 followed by race 4 dominated in most of the states (Singh *et al*., 11). Resistance to these two races (race 1 and 4) did not exist or was very rare, in contrast to common resistance to less important races (2, 3, and 6) in *B. oleracea* races 1, 4 and 6 of *Xcc* were identified and, among these races, race 1 followed by race 4 dominated most of the states of India (Taylor *et al*., 13).

The aim of this research was to evaluate *Brassica* species at the juvenile plant stage and quantify the presence of race specific *Xcc* resistance. This was urgently needed to unveil novel sources in *Brassica* species for race specific resistance to utilize in vegetable *Brassica* pre-breeding for introgression of durable resistance.

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MATERIALS AND METHODS

The *Brassica* germplasm used in present study were maintained as inbred lines at Division of Vegetable Science, Indian Agricultural Research Institute, New Delhi. In total 26 accessions/lines of five *Brassica* species (Table 1).

were evaluated using challenge inoculation technique under field conditions during three consecutive years from 2015 to 2017.

Three *Xcc* races 1, 4 and 6 were multiplied in Yeast Glucose Chalk Agar (YGCA) media at 25º C for 3 days. The culture was carefully scrapped from the media with sterilized slide. The scraped bacterial culture was mixed in 100 ml sterilized distilled water and mixed thoroughly by vortex and final concentration of 10^8-10^9 cfu/ml was made. One youngest leaf from ten plants of each accession was inoculated in three replications with *Xcc* race 1, 4 and 6 at 30 days after sowing in direct seed sown *Brassicas* and in cauliflower at 30 days after transplanting. The plant leaves were inoculated by clipping the secondary veins at the margins with small scissors dipped in the bacterial suspension. The inoculation was carried out at 10 points per leaf.

The mean monthly weather data for experimental period (November- December, 2015–2017) were collected from Division of Agriculture Physics, ICAR-IARI, New Delhi. The average mean temperature was 19.9°C, with minimum and maximum temperature ranging from 11.8 °C to 28.1°C, relative humidity from 44.40% to 88.78% and total rainfall 0.3 mm during the phenotyping period (November–December).

Besides, the experimental pots were watered frequently during the period of inoculation to maintain high humidity required for proper disease development. These agro meteorological conditions favoured disease establishment and development during the phenotyping period. The final disease reaction was recorded at 30 days after inoculation. The inoculated plants were assessed for disease reaction based on disease scores '0 to 9' and percentage of inoculated points in leaves showing symptoms were recorded as per scale given in Table 2 as suggested by Vicente *et al*. (17).

The total number of inoculated points and the number of points showing symptoms were recorded and the percentage of infected points were calculated and classified as PDI.

$$\text{PDI (Percent disease incidence)} = \frac{\text{Number of points showing symptoms}}{\text{Total number of inoculated points}} \times 100$$

The data of three years (2015, 2016 and 2017) were pooled and mean disease severity and percent disease incidence (PDI) were calculated and on the basis of this analysis the final disease reaction was determined according Table 3. Correlation analysis was carried out among three *Xcc* races 1, 4 and 6 for disease severity and PDI (Gomez and Gomez, 2).

RESULTS AND DISCUSSION

Cauliflower cultivation has been expended across the seasons and regions but its production suffers from many biotic and abiotic stress factors resulting in

Table 1. Alien *Brassica* germplasm/lines used for challenge inoculation for black rot disease.

Brassica species	Genomic constitution	Germplasm/Line/Accessions
Brassica juncea	AABB	Pusa Bold, Pusa Vijay, PVDH01
Brassica nigra	BB	IC560690, IC56072, EC 289661, SRB-98, IC-247
Brassica carinata	BBCC	NPC-9, NPC-17, IGC-01
Brassica napus	AACC	Zhang Shuang (China), ISN-530, ISN-114, BN-3, WFN(WF), ISN- 129, ISN530, BN-2-1, GSL-5, GSL-2, GSL-15 GSL-1, NDH
B. oleracea var. *botrytis*	CC	Pusa Meghna, DC 41-5 and Pusa Sharad

Table 2. Black rot disease phenotyping scale (0-9).

Score	Description
0	No symptoms
1	Small necrosis or chlorosis surrounding the infection point
3	Typical small V-shaped lesion with black veins
5	Typical lesion half way to the middle vein
7	Typical lesion progressing to the middle vein
9	Lesion reaching the middle vein

Table 3. Final disease reaction determination on the basis of disease severity and PDI.

Disease Reaction	Resistant	Moderate Resistant	Susceptible	Highly Susceptible
Severity	0 - 3	3 - 5	5 - 7	7 - 9
Incidence (%)	0 to < 25	25 to <50	50 to <75	75 to 100

yield and quality loss. Three *Xcc* races 1, 4 and 6 were identified and, among these races, race 1 followed by race 4 dominated most of the states of India causing huge loss in cauliflower production. New sources of disease resistance are of interest to *Brassica* breeders and are often sought in regions where the pathogen and host plant taxa have long been associated. Screening and validation of germplasm to identify race specific resistance source against disease is an essential step in resistance breeding programme.

In case of susceptible genotypes, the yellowish spot enlarged assuming 'V' shape developed from margin to the centre of the leaves. This resulted in collapsing of tissue within chlorotic lesions and darkening of veins extending from the lesion. The increase in severity of disease was due to yellowish growth which increased towards mid rib of the leaves. As the disease progressed, the chlorosis became more pronounced and exhibited black veins symptom. Resistant accessions generally did not show any symptoms at 10 (Days After Inoculation) DAI. The resistant genotypes exhibited individualized restricted chlorotic region around the hydathodes and even the chlorotic spot increased up to 2- 3 mm at 30 DAI. The Average disease severity and disease incidence of three years of *Brassica* accessions against *Xcc* race 1, 4 and 6 are given in Table 4 and illustrated in Fig. 1 &2. The correlation analysis

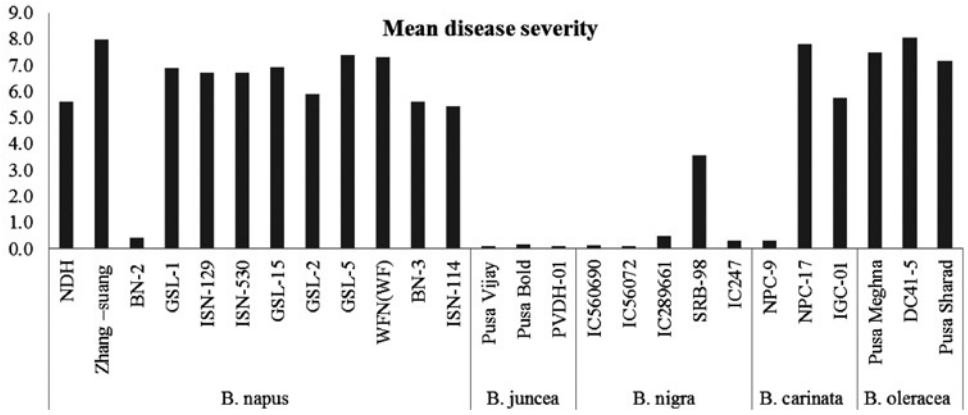

Fig. 1. Black rot mean disease severity (0-9) (y-axis) against *Xcc* race 1, 4 & 6 of different accessions/ varieties (x-axis) of alien *Brassica* species.

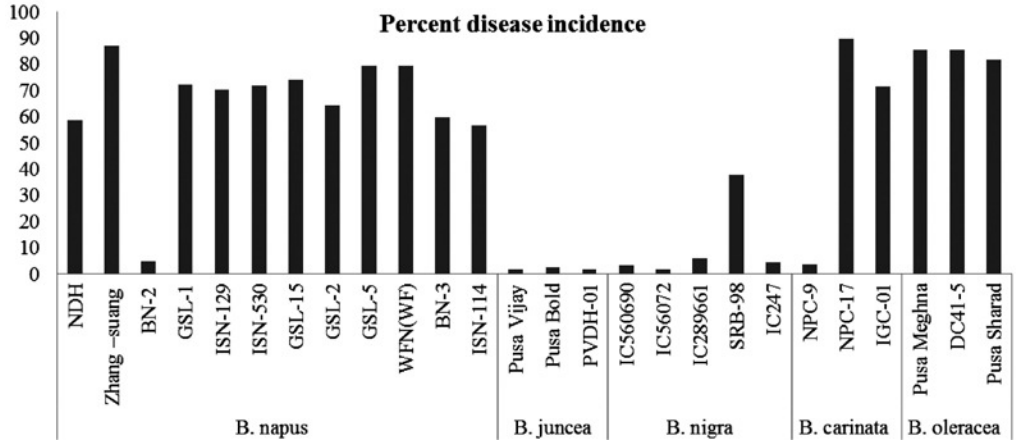

Fig. 2. Black rot disease mean incidence (%) (y-axis) against *Xcc* race 1, 4 & 6 of different accessions/ varieties (x-axis) of alien *Brassica* species.

Table 4. Artificial screening of different accessions/ varieties of *Brassica* species against black rot disease (*Xcc* race 1, 4 & 6) during three consecutive years

Genotype	*Xcc* race 1			*Xcc* race 4			*Xcc* race 6		
	DS	PDI	Reaction*	DS	PDI	Reaction*	DS	PDI	Reaction*
Brassica napus (AACC)									
NDH	3.10	33.33	MR	6.90	70.40	S	6.87	72.30	S
Zhang –Shuang (China)	7.96	84.56	HS	8.50	90.40	HS	7.48	86.30	HS
BN-2-1	0.30	3.66	HR	0.50	6.66	HR	0.39	3.66	HR
GSL-1	6.60	66.30	S	7.25	77.00	HS	6.82	73.50	S
ISN-129	6.97	70.40	S	6.76	74.60	S	6.40	65.70	S
ISN-530	6.44	68.60	S	6.38	70.70	S	7.27	76.80	HS
GSL-15	6.70	69.70	S	7.28	78.80	HS	6.80	73.50	S
GSL-2	5.46	61.33	S	5.91	64.66	S	6.35	67.33	S
GSL-5	7.93	84.56	HS	7.36	80.46	HS	6.80	73.65	HS
WFN(WF)	8.19	93.40	HS	7.30	76.66	HS	6.41	67.80	S
BN-3	5.53	58.30	S	5.35	58.90	S	5.94	62.20	S
ISN-114	5.18	53.66	S	5.78	61.20	S	5.38	55.33	S
Range	0.30-8.19	3.66-93.40		0.50-8.50	6.66-90.40		0.39-7.48	3.66-86.30	
Brassica juncea (AABB)									
Pusa Vijay	0.09	1.34	HR	0.10	2.50	HR	0.05	1.50	HR
Pusa Bold	0.15	2.66	HR	0.13	2.90	HR	0.18	1.56	HR
PVDH-01	0.05	1.31	HR	0.10	2.5	HR	0.04	1.06	HR
Range	0.05-0.15	1.31-2.66		0.10-0.13	2.5-2.9		0.04-0.18	1.06-1.56	
Brassica nigra (BB)									
IC560690	0.14	3.67	HR	0.11	2.66	HR	0.13	3.50	HR
IC56072	0.11	2.66	HR	0.07	1.30	HR	0.09	1.60	HR
IC289661	0.52	6.60	HR	0.44	5.66	HR	0.48	5.66	HR
SRB-98	3.25	35.67	MR	3.83	40.50	MR	3.54	37.00	MR
IC247	0.18	3.33	HR	0.43	5.67	HR	0.31	3.66	HR
Range	0.11-3.25	2.66-35.67		0.07-3.83	1.30-40.50		0.09-3.54	1.60-37	
Brassica carinata (BBCC)									
NPC-9	0.27	3.33	HR	0.50	5.50	HR	0.13	2.50	HR
NPC-17	7.56	83.00	HS	8.13	96.66	HS	7.68	89.33	HS
IGC-01	5.92	72.00	S	5.74	71.66	S	5.55	71.33	S
Range	0.27-7.56	3.33-83		0.5-8.13	5.5-96.66		0.13-7.68	2.5-89.33	
B. oleracea var. *botrytis* (CC)									
Pusa Meghna	7.48	85.40	HS	7.50	87.60	HS	7.51	83.50	HS
DC41-5	7.38	76.87	HS	8.28	89.67	HS	8.46	90.40	HS
Pusa Sharad	7.10	76.23	HS	7.05	82.36	HS	7.31	86.46	HS
Range	7.10-7.48	76.23-85.40		7.05-8.28	82.36-89.67		7.31-8.46	83.5-90.40	

*HR= Highly Resistant, MR= Moderately Resistant, R= Resistant, S= Susceptible, HS= Highly Susceptible. (Average pooled data of three consecutive years *viz*., 2015, 2016 and 2017 under artificial inoculation conditions)

between black rot *Xcc* races (1, 4 and 6) is given in Table 5. The disease severity and PDI values for *Xcc* race 1 had significantly positive correlation with *Xcc* race 4 and 6 and *Xcc* race 4 and 6 were also correlated to each other in *Brassica napus* and *Brassica nigra.* In *Brassica carinata,* disease severity and PDI values for *Xcc* race 6 were significantly positive correlated with *Xcc* race 1 and 4. Over all it is concluded that disease reaction either susceptible or resistant for *Xcc* race 1, 4 and 6 remained constant in disease reaction.

In *B. napus* (AC genome) disease incidence and severity for *Xcc* race 1 ranged among the accessions from 3.66 to 93.40%, 0.30 to 8.19, respectively. The genotype WFN (WF) was found highly susceptible against *Xcc* race1. Disease incidence and severity for *Xcc* race 4 ranged among the accessions from 6.66 to 90.40%, 0.50 to 8.50, respectively. The genotype Zhang Shuang (China)was found highly susceptible against *Xcc* race 4. Disease incidence and severity for *Xcc* race 6 ranged among the accessions from 3.66 to 86.30%, 0.39 to 7.48, respectively. The genotype Zhang Shuang (China) was found highly susceptible against *Xcc* race 6. Out of twelve *B. napus* accessions, only one genotype (BN-2-1) was found highly resistant against all *Xcc* races (1,4 & 6) which exhibited small necrosis or chlorosis surrounding the infection point at 5th day after inoculation, but further progress of pathogen was stopped probably due to hypersensitive reaction. One accession NDH exhibited moderate resistance for *Xcc* race 1 while susceptible for *Xcc* race 4 & 6. The remaining accessions of *Brassica napus* under study were found to be susceptible and highly susceptible due to increase in disease incidence and severity. These accessions revealed typical lesion half way to the middle vein (susceptible) and progressing to the middle vein and later reached the middle vein and classified as very susceptible. Some of the leaves of very susceptible genotypes were rotten due to heavy infestation of pathogen. However, in previous findings demonstrated that resistance to *Xcc* race 4 (but not *Xcc* race 1) was most common in *B. napus (*AC genome) and *B. rapa* (A genome) which advocates an A genome origin (Taylor *et al.,* 13). In this experiment, we could identify host plant resistance against all three *Xcc* races (1, 4 & 6) in BN-2-1.

Seventy-six accessions belonging to four *B. napus* groups were screened for resistance to both pre dominant *Xcc* races (1 and 4) by Lema *et al.* (5) and found the strain race 1 was more virulent than race 4 on tested accessions. No race-specific resistance was found to race 1. Most genotypes were susceptible except Russian kale, from the pabularia group, which showed few resistant plants and some accessions with partial resistance. Griffiths *et al*. (3) identified incomplete resistance in two accessions of *B. napus* (PI 469733 and PI 469828).

In *B. juncea* (AB genome) was the most resistant species, showing either strong resistance to races 1 and 4 or quantitative resistance to all races (Griffiths *et al.,* 3). Range of disease severity and disease incidence in different accessions of *B. juncea* were very narrow for three *Xcc* races (1, 4 & 6). Disease incidence (%) and severity were observed very low in Pusa Vijaya against *Xcc* race 1 (1.34 %, 0.09), *Xcc* race 4 (2.50%, 0.10), *Xcc* race 6 and (1.56 %, 0.05),

Table 5. Correlation analysis between black rot races for disease severity and PDI.

Species	Races	Disease severity		PDI	
		Xcc race 1	*Xcc* race 4	*Xcc* race 1	*Xcc* race 4
Overall	*Xcc* race 4	0.968**		0.969**	
	Xcc race 6	0.958**	0.992**	0.956**	0.994**
B. napus	*Xcc* race 4	0.854**		0.862**	
	Xcc race 6	0.798**	0.957**	0.794**	0.971**
B. nigra	*Xcc* race 4	0.996**		0.996**	
	Xcc race 6	0.999**	0.999**	0.999**	0.998**
B. juncea	*Xcc* race 4	0.918^{NS}		1.000**	
	Xcc race 6	0.941^{NS}	0.998*	0.608^{NS}	0.592^{NS}
B. carinata	*Xcc* race 4	0.995^{NS}		0.990^{NS}	
	Xcc race 6	0.998*	0.999*	0.998*	0.997*
B. oleracea	*Xcc* race 4	0.595^{NS}		0.303^{NS}	
	Xcc race 6	0.417^{NS}	0.979^{NS}	0.785^{NS}	0.352^{NS}

NS = non-significant, *, ** significant @ 0.01 and 0.05 probability respectively.

respectively. Some plants of Pusa Bold and Pusa Vijay were recorded symptomless resistant. These two *B. juncea* genotypes could be used to develop durable resistance against black rot. These findings are in line with those of previous studies (Griffiths *et al*., 3). Tonguc and Griffiths (14) explored *B. juncea* resistant accessions (PI 633077, PI 633078) to transfer black rot resistance in broccoli employing interspecific hybridization.

In *B. nigra* (B genome), disease incidence varied from 2.66 to 35.67%, while disease severity ranged 0.11 to 3.25 against *Xcc* race 1. Accession IC 56072 was observed with minimum disease incidence and severity, while some plant leaves were also observed with symptomless resistance against all three *Xcc* races (1, 4 & 6). Accessions EC289661, IC-247, IC-560690 and IC56072 were found highly resistant, whereas SRB-98 was found moderate resistant against all three *Xcc* races (1, 4 & 6). Other accessions such as EC 289661, Sangam, IC-247, IC-560690, and IC56072 showed resistance against *Xcc* race 1. In another previous study, *B. nigra* was also reported as highly resistant source against *Xcc* disease reaction (Griffith *et al*., 3). Most of the cultivars of *B. nigra* were highly resistant to races 1, 4 and 5, but susceptible to races 0 and 2 (Ignatov *et al*., 4). Recently, accession St 461 of *B. nigra* was employed for transferring strong *Xcc* race 1 resistance into susceptible *B. rapa* attempting embryo rescue techniques (Sheng *et al*., 9).

In *B. carinata* (BC genome) harbours valuable resistance/tolerance traits for biotic and abiotic stress viz. resistance to black leg, black rot and tolerance to aluminium, salinity, heat and drought (Enjalbert *et al*., 1). Disease incidence and severity for *Xcc* race 1 ranged among the accessions from 3.33 to 83%, 0.27 to 7.56, respectively. Disease incidence and severity for *Xcc* race 4 ranged among the accessions from 5.50 to 96.66 %, 0.50 to 8.13, respectively. Disease incidence and severity for *Xcc* race 6 ranged among the accessions from 2.50 to 89.33%, 0.13 to 7.68, respectively.

The genotype NPC-9 was found highly resistant against all *Xcc* races (1, 4 and 6) which exhibited small necrosis or chlorosis surrounding the infection point at 5th days after inoculation, but further progress of pathogen was stopped probably due to hypersensitive reaction. The accessions *viz.*, IGC-01 and NPC-17 were observed susceptible and highly susceptible, respectively. Accessions of *B. carinata* recorded wide variations (complete resistance to full susceptibility) in disease reaction against black rot pathogen races *Xcc* 1, 4 and 6. Both extreme accessions could be used for searching novel gene(s) controlling resistance loci/locus of black rot and development of mapping population to locate the gene (s). Griffiths *et al*., (3) reported resistance in five accessions of *B. carinata* out of 63 evaluated (PI 193460, PI193959, PI194254, PI 280230, PI 633077) determined by repeated symptomless responses after inoculation. Tonguc and Griffith (15) evaluated fifty-four accessions of *B. carinata* (BBCC) for black rot resistance and only two accessions A 19182 and A 19183 found free from any disease symptoms when inoculated with *Xcc* for all the plants tested.

Commercial varieties/line of cauliflower namely Pusa Meghna, DC41-5 (early group), Pusa Sharad (mid group) were observed highly susceptible with high disease severity and incidence. Disease incidence and disease severity respectively ranged from 76.87 to 85.40 %, 7.10 to 7.48 for *Xcc* race 1, 82.36 to 89.60 %, 7.5 to 8.28 for*Xcc*4 and 83.50 to 90.40%, 7.51 to 8.46 for *Xcc* race 6. Typical V shape symptoms exhibited at 7 DAI and the yellowish spot enlarged, assuming 'V' shape developed from margin to the centre of the leaves. Disease severity and incidence progressed rapidly by collapsing of tissue within chlorotic lesions and black darkening of veins and reached up to mid veins. Cauliflower varieties namely Pusa Himjyoti, Pusa Sharad, Pusa Shukti and Palam Uphar were reported susceptible, while BR-161 was resistant during *in vitro* evaluation against *Xcc* race 1 (Saha *et al.*, 7). Sources of resistance to black rot disease in. *B. olearcea* group (C genome) were available in limited germplasm accessions. Saha *et al*., (8) identified most of the accessions of Cauliflower were susceptible to *Xcc* race 1 and 4, except accessions BR-207, BR-1, BR-202-2 and AL-15 to *Xcc* race 1.

Resistance to *Xcc* race 4 (but not *Xcc* race 1) was most common in *B. rapa* (A genome) and *B. napus (*AC genome), which suggested its A genome origin. Alien *Brassica* specie viz*., B. nigra* (B genome), *B. carinata* (BC genome) and *B. juncea* (AB genome) unveiled resistance against *Xcc* race 1, race 4, race 6, thus, hypothesizing probable B genome origin. This study revealed that all the genotypes showed variable disease severity symptoms. Within stipulated time period, rapid spread of the disease in artificial inoculation was due to congenial conditions for pathogen multiplication under optimum field conditions. This was, especially true for genotypes, which inherently do not have mechanism to restrict pathogen. The disease progress was less in resistant genotypes as compared to moderately resistant and susceptible genotypes.

In conclusion, BN-2-1 of *Brassica napus*, Pusa Vijaya, Pusa Bold of *B. juncea,* NPC-9 of *B. carinata,* five accessions namely EC 289661, IC-247, IC-560690, IC56072 of *B. nigra*, were found to be

resistant based on three consecutive years (2015, 2016, 2017) screening against *Xcc* race 1, 4 & 6.

The present cauliflower genomic data and phylogenetic analysis confirmed that the *Brassica* species with the B genome, such as *B. nigra*, are more ancient than those of *Brassica* species with the A or C genome. In *Brassica* species, the diploid species *B. nigra* with the B genome diverged from the A and C genomes approximately 9.1–13.4 million years ago (Mya). Then, the other two diploid species, namely, *B. oleracea* (CC genome) and *B. rapa* (AA genome), diverged ~8.5 Mya.The C genome of the *B. oleracea* ancestral species diverged into the C subgenome of allotetraploid *B. napus* and the C genome of diploid varieties, including *B. oleracea*. (Sun *et al*., 12)

Homoeology between C genome of *B. oleracea* and A genome *B. napus* is high as compared to that of other species *B. rapa* (AA) and *B. juncea* (AABB). Hence, identified new resistant source BN-2-1 of *B. napus* as compare to other alien *Brassicas* will be useful to introgress *Xcc* resistance into *B. oleracea.* It may be exploited through somatic and sexual hybridization with *B. oleracea*. It is feasible as recombination may occur between homeologus chromosomes of A and C genome, respectively as prerequisite for introgression and further backcrossing are necessary to create genotypes with 18 chromosomes of C genome and additional resistance to *Xcc.* It will reduce the worldwide limitations on production caused by this pathogen in this specie. The broad-spectrum resistance from *Brassica* species on its own or in combination with strong race-specific resistance can contribute to the long-term control of the disease.

AUTHORS' CONTRIBUTION

Conceptualization of research (BBS and PK), Designing of the experiments (BBS, PK); Contribution of *Xcc* races culture and maintenance (DS); Execution of field experiments and data collection (BBS and DS). Analysis of data and interpretation (BBS, SS and BST); Preparation of the manuscript (BBS, SS, PK DS and BST)

DECLARATION

The authors declare no conflict of interest.

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