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Selection of Powdery Mildew Resistant Brassica Genotypes Based on Disease Indexing and Microsatellite Markers

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Powdery mildew disease of oilseed mustard caused by *Erysiphe cruciferarum* is a primary reason of yield reduction not only in India but also throughout the world. Identification and cultivation of resistant mustard genotypes against powdery mildew is the only way to overcome this challenge. In the present investigation, we targeted to screen 75 Brassica genotypes against powdery mildew based on disease indexing under field conditions and gene-specific molecular markers. Disease reaction on both the cotyledonary and true leaves was screened with using a modified 0-9 scale score as well as with nineteen disease linked microsatellite markers. In disease indexing under field conditions, genotypes *viz.*, L-4 and PC-5 were identified as immune, China and RP-9 were considered as highly resistant and GSC-7 and PC-6 were recognized as resistant whilst genotypes *i.e.*, RB-50, Pusa Bold, WRR-10 and GSL-1 were accredited as moderately resistant. Molecular markers based UPGMA dendrogram classified Rohini, WRR-22, PC-6, PusaBold, China, WRR-8, GSL-1, WRR-7, RH-749, L-4 and RB-50 as highly resistant mustard genotypes. In addition, disease linked marker cnu_m616 had the highest polymorphic information content (0.75) with greatest ability to differentiate resistant genotypes from susceptible genotypes, may be employed directly in mustard breeding programmes in future.

Keywords: Mustard; powdery mildew; disease indexing; gene-linked markers; pathogens.

1. INTRODUCTION

"The genus Brassica is one of the most spirited oil seed crops being cultivated in India with high grade of genetic diversity" [1-3]. "The Indian mustard, yellow sarson, browns arson, raya, and toria crops are all included in the rapeseed-mustard group" [4-6]. "It is self-pollinated crop with some extent of cross-pollination" [7-11]. "It is the most important oilseed crop of India having significant economic, nutritional, and industrial applications" [12-14]. "Despite having significant area under farming of oilseed crops (26.20 million hectare), India is a major importer of edible oil. India imports >Rs. 0.73 trillion worth of edible oil annually" [15]. "During the 2018–2019 growing season, the estimated global rapeseed-mustard area, production, and yield were 36.59 Mha, 72.37 Mt, and 1980 kgha^{-1}, respectively. India contributed 19.8 and 9.8%, respectively, to global acreage and production" [16].

Brassica species comprises between 38 and 46% oil [17]. The oilseed brassica characteristically comprises 27% oleic and linoleic acid, 4.7–13% linolenic acid, and 38–57% erucic acid, all of which have substantial nutritional worth and are crucial for human health [18,19]. Mustard crop is exploited in numerous submissions for instance paste, sauce, flavours, ingredients, and the most well-known spices [20,21].

Mustard is susceptible to disease attacks owing to the genetic homogeneity among all grown types [22]. "Among various biotic factors responsible for severe yield loss in mustard powdery mildew causes serious damage worldwide" [23,24]. "Powdery mildew disease caused by *Erysiphe cruciferarum* Opiz. ex. L. Junell counts yield loss up to 17 % in India" [25]. All the brassica cultivars currently cultivated in India are extremely vulnerable to powdery mildew and so far, no resistance resource has been reported [26]. For prevention of losses due to plant diseases, chemical compounds have been employed but abuse in their employment has led to the development of fungicide resistance in pathogens. Fungicides also have greater repercussion on environment and human health. Therefore, use of resistant varieties seems to be a better alternative. Host plant resistance (R) genes have been broadly deployed to develop disease-resistant cultivars in mustard breeding for diminishing yield losses owing to different pathogens. Information on the resistant resources from *B. juncea* against powdery mildew are lacking. However, related species such as *B. napus, B. carinata, B. japonica, B. alboglabra* [27,9], *B. oleracea, B. rapa,* and *B. napus* [28] have been reported as sources of resistance against powdery mildew. This information makes it necessary to identify sources of resistance against powdery mildew in *B. juncea* as well.

DNA-based genetic markers are being used more frequently lately for quick cultivar identification, cultivar fingerprinting, and cultivar diversity analysis [29-37]. "Due to their high abundance, reproducibility, simplicity, co-dominant inheritance, higher rate of

polymorphism, and wider genome coverage, SSRs have emerged as the top choice among different types of DNA-based markers for plant breeders and biotechnologists to assess genetic diversity and cultivar selection" [38-48].

In this context, brassica genotype (s) resistant to powdery mildew needs to be introduced so that major yield loss due to this disease could be minimized and productivity improved without affecting quantity and quality of oil. Therefore, the present investigation was undertaken to screen resistant mustard genotype (s) against powdery mildew disease based on disease indexing and disease -linked microsatellite markers.

2. MATERIALS AND METHODS

2.1 Plant Material

Present study was commenced on a total of 75 mustard genotypes including RB-50, Pusa Bold, Varuna, Rohini, Kranti, RH-725, Maya, Vardan, Vasundhara, Swarn Jyoti, Pusa Jagannath, PusaJaiKisan, Albeli, Sej-2, Shraddha, DMH1, L-4, JMWR-908-1, RGN-73, NRC-HB-101, NRC-HB-506, RVM-3, RH-749, NRCDR-2, DRMRIJ-31, China, GSL-1, GSC-7, PC-5, PC-6, RP-9, Kiran, JTC-1, JM-1, JM-2, JM-3, RVM-1, RVM-2, PM-25, PM-26, PM-27, PM-28, PM-30, Pusa Vijay, JMM-927, JMM-991, RMM-10-01-01, RMM-12-01-18, RMM-12-03-18, WRR-5, WRR-6, WRR-7, WRR-8, WRR-10, WRR-11, WRR-12, WRR-13, WRR-14, WRR-15, WRR-16, WRR-17, WRR-18, WRR-19 , WRR-20, WRR-21, WRR-22, WRR-25, WRR-26, WRR-27, WRR-28, WRR-29, WRR-30, WRR-31 and WRR-32 having different reaction against powdery mildew disease, acquired from All India Coordinated Research Project on Rapeseed and Mustard, Zonal Agricultural Research Station, Morena, Rajmata Vijayaraje Scindia Krishi Vishwa Vidyalaya (RVSKVV), Gwalior, M.P., India. All the genotypes were grown in a randomized block design with three replications in *Rabi* 2021 at the experimental field of the Department of Genetics & Plant Breeding, College of Agriculture, RVSKVV, Gwalior, India. Each genotype was planted in a plot of one row of 2-meter length with an arrangement of 30 cm apart between rows and 15 cm plant to plant. The crop was enchanted with protective irrigations and recommended packages of practices right through the growing season. Five arbitrarily chosen plants from each treatment were marked for observation. Molecular work was conducted at Department of Plant Molecular Biology & Biotechnology, College of Agriculture, RVSKVV, Gwalior.

2.2 Disease Assessment

The observations and screening were carried out as per the 0-9 rating [49] scale suggested by AICRP on Rapeseed and Mustard, Bharatpur at cotyledonary as well as true leaf stage. The extent of disease was rated as indicated in a List 1.

2.3 Severity of Disease

To calculate the percent disease index (PDI) of powdery mildew at cotyledonary stage, ten cotyledonary leaves from each replication of an accession were taken at 15 days after sowing. Leaves are evaluated individually by giving (0-9) disease rating (Table 1). At true leaf stage, average disease index on true leaves were registered at 60, 75 and 90 days after sowing in each replication by using 0-9 disease rating scale. Fifteen leaves were randomly collected from each accession and scored; disease index was calculated separately for each accession. Disease intensity was recorded at the maturity stage as per the scale. The PDI was calculated by the formula.

List 1. Description of extent of disease

0	Immune (I)	No lesion
1	Highly resistant (HR)	Non-sporulating pinpoint size or small brown necrotic spots, less than 5% leaf area covered by lesion.
3	Resistant (R)	Small roundish slightly sporulating larger brown necrotic spots, about 1-2 mm in diameter with a distinct margin or yellow halo, 5-10% leaf area covered by lesions
5	Moderately resistant (MR)	Moderately sporulating, non-coalescing larger brown spots about, 2-4 mm in diameter with a distinct margin or yellow halo, 11-25% leaf area covered by the spots
7	Susceptible (S)	Moderately sporulating, coalescing larger brown spots about, 4-5 mm in diameter, 26-50 % leaf area covered by the lesions
9	Highly susceptible (HS)	Profusely sporulating, rapidly coalescing brown to black spot measuring more than 6 mm diameter without margin covering more than 50% leaf area

$$Disease index(\%) = \frac{\text{Sum of all numerical ratings}}{\text{Number of leaves examined} \times \text{Maximum Grade (9)}} \times 100$$

2.4 SSR Marker Analysis

2.4.1 Isolation of genomic DNA

The genomic DNA of mustard genotypes was extracted using the CTAB method [50] slightly modified by Tiwari et al. [51] from 2-3 fresh young leaves. DNA was further purified by RNase digestion followed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1) and ethanol precipitation.

2.4.2 Polymerase chain reaction (PCR) amplification

A total of 19 powdery mildew resistance linked microsatellite markers as suggested by Gong et al. [52] were used to find out polymorphism in selected mustard genotypes (Table 2). The SSR primers were synthesized by Eurofins Genomics India Pvt. Ltd. The PCR was performed in 10 µl reaction mixture comprising of 1X PCR buffer, 0.1 U *Taq* DNA polymerase, 1 µl dNTP (1 mM), 0.5 µl of forward and reverse primers each (10 pM) and 2 µl (10 ng/µl) of genomic DNA in a thermocycler (Bio-Rad, U.S.A.). The PCR profile started with 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 20 sec, annealing at 52-58 °C (depending on primer) for 20 sec and extension at 72 °C for 90 sec. A final extension at 72 °C for 10 min was carried out. The PCR products were resolved on 3% agarose gel at 65V for 2-3 h and documented using Gel Documentation System (Syngene, USA).

2.5 Data Analysis

Scoring of each polymorphic SSR marker was done with the help of Gel Analyzer V19.1 software. Diversity parameters *viz.*, gene diversity, allele number, major allele frequency and polymorphism information content (PIC) were calculated using Power Marker v3.25 software [53]. Neis genetic distance based UPGMA tree was constructed with the help of power marker v3.25 and visualized using MEGA 6.0 software [54].

Table 1. Disease intensity scale for calculation of the percentage disease index

S. No.	Rating Scale For leaf	Leaf area covered (%)	Disease Reaction
1	0	0	Immune
2	1	<5%	Highly Resistant (HR)
3	3	5-10%	Resistant (R)
4	5	11-25%	Moderately Resistant (MR)
5	7	26-50%	Susceptible (S)
6	9	>50%	Highly Susceptible (HS)

Table 2. Details of 19 Powdery mildew disease linked microsatellite markers used in present investigation Gong et al. [54]

S. No.	Marker Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
1	BoGMS0570	TACAATCTTCTTCGCTGCT	AAACCTGAAACTCCCTCAA
2	BoGMS0811	GCACTGTCAAATCACTCAAA	ACTTCTCCCAATCTCTGTCTC
3	BoGMS0826	GCAATGATGAAGTTAGGAGAA	AACCAGCGAGAGAGAGGT
4	BoGMS0369	AGGACAGCATCGGTATGA	CTGGATAGTTTCTCTTTCTTGG
5	BoGMS0687	GACAACACAACAGACGCA	GCATTTCCCATTACTTCCA
6	BoGMS0977	TTTGTCTTCCTTCCTCTAAAC	CAGCAATAACCATCTCCTCA
7	BoGMS0812	GCTGGCACATAGTTGTAATG	CTCATCTCCTCTGCTGGA
8	BoGMS0112	ACTGGTCCGAATAGGTGAG	CGATAAATGGTTCAATGTCC
9	BoGMS1166	TAATGGAAACGCACCAAG	AAACAATCTTAGCAGAGGAGG
10	cnu_m073	TGGCATTGACAGAGCTAGTA	TTTATTTAGTTTCATACCCT
11	cnu_m046	GCTAAAGGTTTAGTCCAAATAGGATTC	GCAAAATGATGCCCCATAAA
12	cnu_m098	TGCGACCCAAGTAGGTGAAAC	TGTCTCTCGCTCATTCATCCAA
13	cnu_m616	TCGGTACGAAACTCAATGTTC	GCGTCAGTCTGAACAAAACG
14	cnu_m617	GGAATGCTGCTGGAAGAATC	TTCAATTTGTCCCTGCATTC
15	nia_m105	GACGAAGGAGCGTATGAAAA	AATGCAATCTCAACAAAGGT
16	nia_m110	GACAGTGGTTGAAAAAGCCA	CATTCAGTGAGGTTTCACCA
17	nia_m113	CAAAAAGTTGCGGTCAATCT	CCTCCAAAGCTCAATCACTG
18	nia_m138	GTTTTAAATGCCGCGTTG	GGGATCAAGAGATGTGGGA
19	MF-2	GGTTCCGTCGTTCCCATCGC	CATAATAATTAGATAAATCTGTTCC

3. RESULTS AND DISCUSSION

3.1 Field Screening

The field screening against powdery mildew was done based on disease intensity in the scale of 0-9. Since the growth of *E. cruciferarum* is superficial, disease symptoms can be observed easily by visual inspection. Powdery mildew first appeared on the upper surface in the lower most (oldest) leaves as small (1 to 2 cm diameter), scattered, white almost circular colonies which coalesced as the colonies grew further, eventually covering the entire leaf surface, stem, primary, secondary branches, and siliquae, progressively (Fig. 1).

The *Brassica* genotypes were classified based on categorizations of reactions against powdery mildew (Table 3). For powdery mildew infestation, genotypes *viz*., L-4 and PC-5 were found to be immune, whilst China and RP-9 genotypes investigated to be highly resistant, whereas genotypes namely GSC-7 and PC-6 considered to be resistant whilst genotypes *i.e*., RB-50, Pusa Bold, WRR-10 and GSL-1 were found to be moderately resistant. However, rest of the genotypes were exhibited their susceptibility to disease (Fig. 1, Table 3). According to our findings, genotypes Varuna, Kranti, Maya, Albeli, Shraddha, DHM-1, WR-908-1, RGN-73, NRC HB-101, NRC DR-2, DRMR IJ-31, Kiran, JTC-1, JM-2, RVM-1, PM-25, PM-26, PM-27, PM-30, RMM-12-01-18, RMM-12-03-18, WRR-5, WRR-6, WRR-7, WRR-8, WRR-9, WRR-11, WRR-12, WRR-13, WRR-14, WRR-15, WRR-16, WRR-17, WRR-18, WRR-19, WRR-20, WRR-21, WRR-22, WRR-25, WRR-26, WRR-27, WRR-28, WRR-29, WRR-30, WRR-31, WRR-32 and Shraddha were considered susceptible whilist genotypes including Rohini, RH-725, Vardan, Vasundhara, Swarn Jyoti, Pusa Jagannath, Pusa Jay Kisan, Sej-2, NRC-HB-506, RVM-3, RH-749, JM-3, PM-28, Pusa Vijay, JMM-927, JMM-991 and RMM-10-01-01 were considered as highly susceptible.

Our findings are in accordance with a recent study conducted by Nanjundan et al. [26] in which “lineages resistant source to *E. cruciferarum* have been identified. A total of 1,020 Indian mustard accessions were evaluated against *E. cruciferarum* PMN isolate, at Wellington, The Nilgiris, Tamil Nadu, India under natural hot spot conditions. The study identified one accession *viz*., RDV 29 with complete resistance against *E. cruciferarum* PMN isolate for the first time, which was consistent in five independent evaluations. Genetic investigation of F_1, F_2 and backcross populations acquired from the cross RDV29 (highly resistant) x RSEJ775 (highly susceptible) for two seasons showed that the resistance is administrated by two genes through gene dosage and semi-dominant effect”. However, according to Khalik [55]) the *B. juncea* plant's physical characteristics, such as the numbers of non-glandular trichomes on plants, may also be connected to the resistance to powdery mildew. A larger density of non-glandular trichomes may increase the risk of fungal infections because they are able to catch airborne particles like fungus spores [56]. Unless they have an anti-fungal hydrolase, non-glandular trichomes are good homes for pathogens [57]. This hydrolase prevents or lessens fungal infection by hydrolyzing fungi's cell walls.

Powdery mildew infection risk and disease severity have both increased in India as a result of the widespread usage of one or more highly sensitive cultivars of *Brassica* species. The successful management of powdery mildew of brassica in India is possible through deployment of cultivar resistance. Genetic engineering is a promising technique to fight infection since host resistance is a significant factor in the spread of disease. *B. juncea* transgenic lines with BjNPR1 have shown promising results in terms of increased *E. cruciferarum* resistance in Australia [58].

3.2 Molecular Screening

The field screening does not reflect the comprehensive screening of the genotypes because of the expression of many morphological traits is masked *via* various environmental factors [59-61]. The genotypes having similar disease symptoms may exhibit differences at DNA level [39]. Therefore, in the present investigation, powdery mildew linked microsatellite markers were also employed for the screening brassica genotypes at molecular level. In the present study, 19 powdery mildew linked microsatellite markers were deployed for screening of 75 brassica genotypes. Among employed 19 microsatellite markers, only five markers displayed consistent polymorphism among brassica genotypes and were selected for further assessment.

Table 3. Categorizations of reactions of *Brassica* genotypes against powdery mildew disease

Severity (%)	Disease reaction	Number of Brassica genotypes			Categorized Brassica genotypes based on pooled
		R1	R2	R3	
0	Immune	2	2	2	L-4, PC-5
<5	Highly Resistant	2	2	2	CHINA, RP-9
5.0-10	Resistant	2	2	2	GSC-7, PC-6
10.1-25	Moderately Resistant	4	4	4	RB-50, Pusa Bold, WRR-10, GSL-1
25.1-50	Susceptible	47	47	47	Varuna, Kranti, Maya, Albeli, Shraddha, DMH 1, JMWR-908-1, RGN-73, NRC-HB-101, NRC DR-2, DRMR IJ-31, KIRAN, JTC-1, JM-2, RVM-1, PM-25, PM-26, PM-27, PM-30, RMM-12-01-18, R M M -12-03-18, WRR-5, WRR-6, WRR-7, WRR-8, WRR-9, W R R -11, WRR-12, WRR-13, WRR-14, WRR-15, WRR-16, WRR-17, WRR-18, WRR-19, WRR-20, WRR-21, WRR-22, WRR-25, WRR-26, WRR-27, WRR-28, WRR-29, WRR-30, WRR-31, WRR-32, Shraddha
>50.1	Highly Susceptible	18	18	18	Rohini, RH- 725, Vardan, Vasundhara, Swarn Jyoti, Pusa Jagannath, Pusa JaiKisan, Sej-2, , NRC-HB- 506, RVM-3, RH-749, JM-3, PM-28, Pusa Vijay,JMM-927, JMM-991, RMM-10-01-01

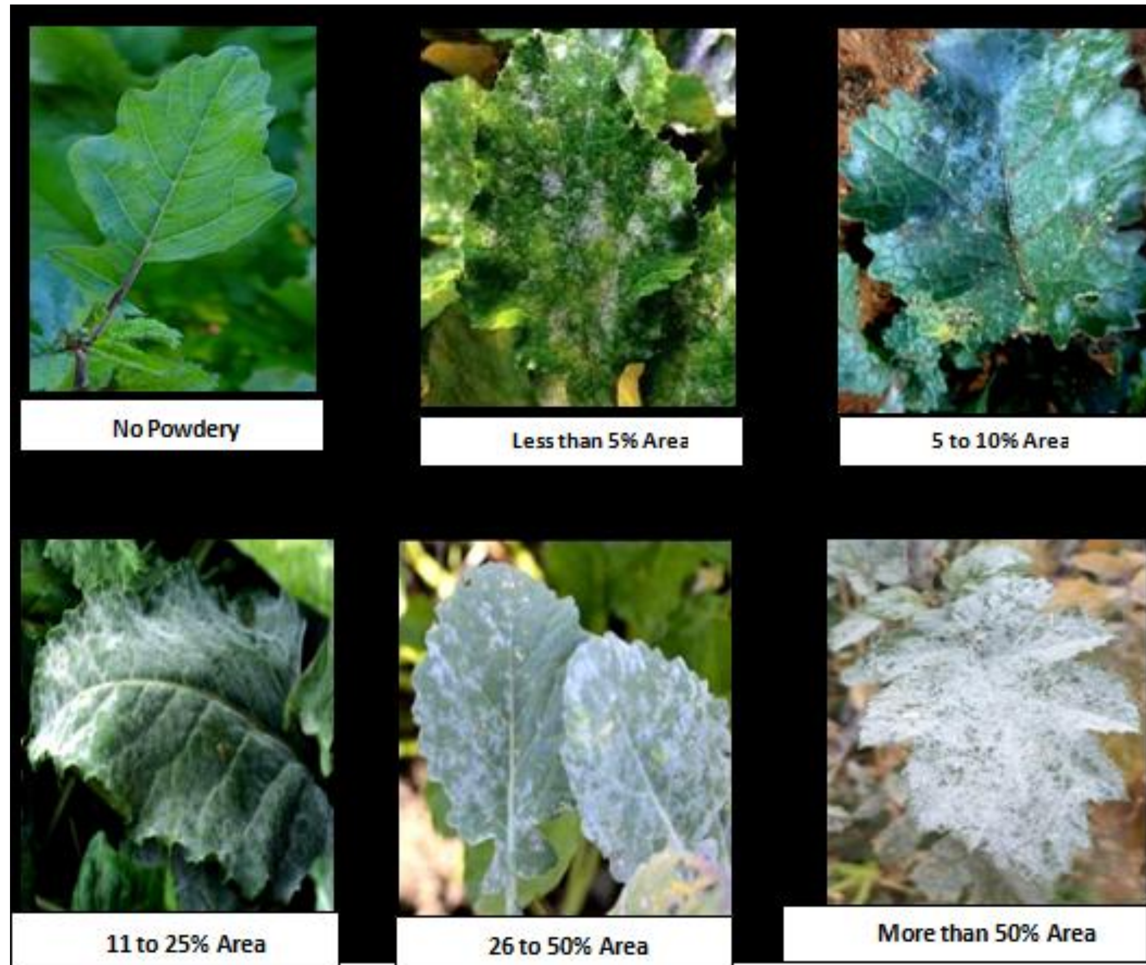

Fig. 1. Categorizations of reactions of Powdery Mildew (*Erysiphe cruciferarum*) in *Brassica* genotypes

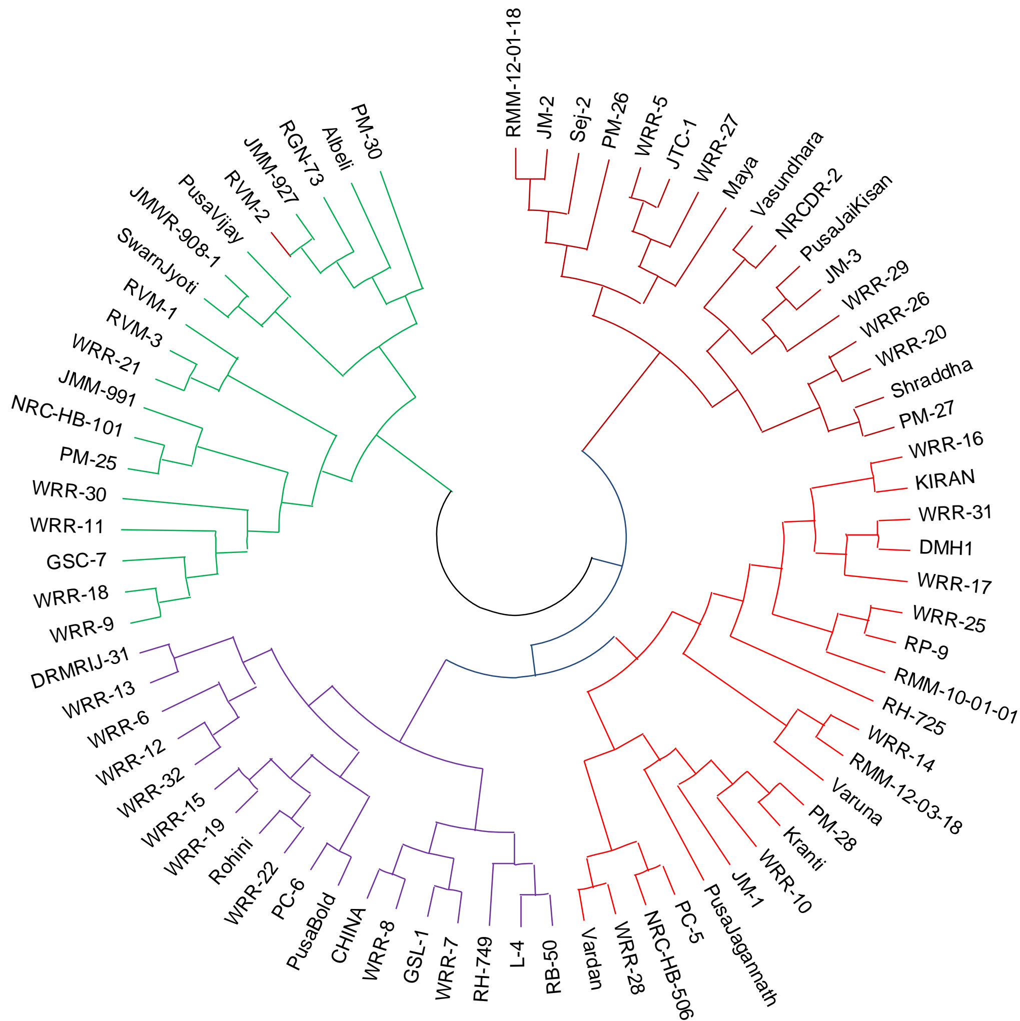

Fig. 2. Neis genetic distance based UPGMA tree of 75 mustard genotypes based on disease linked polymorphic microsatellite markers

Table 4. Details of 5 disease linked microsatellite markers exhibited consistent polymorphism among 75 mustard genotypes

S. No.	Marker	Allele No	Major Allele Frequency	Gene Diversity	PIC
1	cnu_m616	5	0.266	0.785	0.750
2	nia_m105	5	0.293	0.759	0.717
3	MF-7	5	0.333	0.731	0.682
4	MF-4	5	0.4	0.711	0.662
5	CCMP6	5	0.36	0.761	0.724
Mean		**5**	**0.330**	**0.749**	**0.707**
SD		**0**	**0.052**	**0.028**	**0.035**

SD- Standard Deviation, PIC- Polymorphic Information Content

Total 25 alleles were identified with 5.0 average allele per locus (Table 4). The genetic diversity ranged between 0.711 to 0.785 with a mean worth of 0.749. The maximum genetic diversity was investigated for the marker cnu_m616 and minimum for MF-4. The major allele frequency varied between 0.266 to 0.40 with an average value of 0.330. Maximum major allele frequency

shown by marker MF-4 and minimum by the marker cnu_m616. Whereas PIC values arrayed between 0.662 to 0.750 with a mean worth of 0.707. Maximum PIC value was evidenced for the markers cnu_m616 and minimum for MF-4 respectively.

"PIC value defines the discriminatory power of a marker and is the representative of allelic diversity and frequency among the genotypes" [7,8,14,31-37,40]. The occurrence of multiple bands from the same locus could be explained by the presence of cryptic site upstream, downstream and between the primer binding sites. Our study was found in accordance with previous study in which gene diversity ranged from 0.20 to 0.70 for markers Ni2F11 and BrgMS713 respectively, with an average value of 0.53 per marker [36]. Genetic diversity of *Brassica* spp. genotypes was studied by Baghel et al. [7] using SSR markers in which highest PIC value (0.6851) was reported for the marker Ol10-CO 5. In other investigation, Sharma et al. [36]) reported "higher PIC values than the average PIC value (0.44) in the reference set of genotypes using highly polymorphic 48 SSR markers that can be used in trait mapping studies in mustard".

The phylogenetic tree classified genotypes Rohini, WRR-22, PC-6, Pusa Bold, China, WRR-8, GSL-1, WRR-7, RH-749, L-4 and RB-50 in a separate group (Fig. 2). Grouping of these genotypes in the separate group may be due the amplification of similar alleles with all the polymorphic markers. Genotypes Rohini, WRR-22, PC-6, Pusa Bold, China, WRR-8, GSL-1, WRR-7, RH-749, L-4 and RB-50 were already exhibiting moderately resistance to immune disease reaction based on disease screening. Our result is an accordance with investigation of Ghosh et al. [62] in which the UPGMA based dendrogram resulted in two major clusters. Cluster I contain a wild type genotype B1D (*Sinapis alba*), genetically dissimilar from all other varieties. Cluster II divided into two sub-clusters cluster IIA and cluster IIB. The phylogenetic tree of *Brassica* spp. genotypes was also constructed by Baghel et al. [7] using SSR markers and divided 48 genotypes into three major groups, the first main cluster contained 17 genotypes, the second one had 24 genotypes and the third core group included 7 genotypes. Sharma et al. [36] also reported 59 leafy mustard accessions grouped into two distinct groups based on UNJ dendrogram. Genotype IC597868 formed a separate cluster *i.e.*, cluster I while, cluster II was comprising of 58 mustard genotypes. Cluster II was further sub divided into two subclusters IIA and IIB. Subcluster IIA consisted of three accessions including IC597901, IC597886 and 5IC597949, whilst the rest of the 55 accessions grouped into subcluster IIB.

According to the field screening results, genotypes that showed resistance against powdery mildew disease having possibilities to have resistant gene in these genotypes and power of polymorphic markers to separate them accordingly. Results indicate the linkage of polymorphic markers with powdery mildew resistance in *Brassica species.* However, to confirm their association it is important to perform further study using mapping populations. According to the Brassica Microsatellite Information Exchange (BMIE), there are still not enough SSR markers for brassica that have been mapped completely. The density of the current genetic maps would increase due to the polymorphic SSRs, and breeders would benefit from having a source of polymorphic DNA markers for high-throughput mapping, marker-assisted selection, and improvement of *Brassica species.*

4. CONCLUSION

Powdery mildew disease of oilseed mustard is one of the primary causes of yield penalty not only in India but also throughout the world. Most of the mustard cultivars currently grown in India are extremely vulnerable to powdery mildew and so far, no resistance resource has been reported. Disease indexing classified mustard genotypes L-4, PC-5 as immune against *Erysiphe cruciferarum*. Disease linked microsatellite markers showing consistent polymorphism among 75 mustard genotypes identified Rohini, WRR-22, PC-6, Pusa Bold, China, WRR-8, GSL-1, WRR-7, RH-749, L-4 and RB-50 genotypes as highly resistant genotypes and confirmed the results obtained from disease indexing. In addition, disease linked marker cnu_m616 having greatest aptitude to distinguish resistant genotype (s) from susceptible genotypes, could be employed straight in mustard breeding programms.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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