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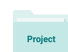
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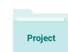
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Research Article

Identification and Characterization of New Resistance Sources against Sclerotinia Stem Rot (*Sclerotinia sclerotiorum)* in Oilseed *Brassica*

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Abstract

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is a big threat to the production of *Brassica* crop worldwide. In this study, a total of hundred rapeseed and mustard genotypes were screened for tolerance/resistance to Sclerotinia stem rot under artificial stem inoculation conditions in the sick plot. Out of the promising 100 genotypes, eight genotypes namely, EC 597317, EC 597328, RH 1222-28, DRMR-261, DRMR-360, DRMR-1493, DRMR-1034, DRMR 2035 were tolerant, whereas 3 genotypes including Rohini, EC 597314 and NRCYS5-2 were highly susceptible. Further, 42 polymorphic SSR (Simple Sequence Repeats) markers were used to evaluate genetic relationships among these eleven genotypes based on UPGMA dendrogram. In the cluster analysis, the genotypes were discriminated very well in two groups where *three* susceptible genotypes were grouped in cluster I while 8 tolerant genotypes fell together in cluster II thus differentiating tolerant and susceptible genotypes. The similarity coefficient ranged from 0.38 to 1.00 indicating the existence of wide diversity among these genotypes. These genotypes could be explored for the management of stem rot in Brassica production.

Key words: Oilseed Brassica, Sclerotinia, disease, tolerance, molecular markers

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Sclerotinia stem rot of oilseed Brassica, caused by the phytopathogenic fungus *Sclerotinia sclerotiorum*, is a major impediment in the cultivation of Brassica, where it causes an yield loss ranging from 5 to 100% (Zhao et al 2004; Saharan and Mehta 2008; Delourme *et al* 2011). Infection occurs on leaves, pods and stems at different stages of crop growth leading to 80% yield losses and significant reductions in oil content and quality (Aggarwal *et al* 1997; Sharma et al 2015). Identification and deployment of effective host resistance mechanism has been hindered by the occurrence of virulent pathotypes (Tiedemann *et al* 2011; Ge *et al* 2012) and significant genotype-strain interactions where host responses for individual genotypes are determined by the strain of the pathogen (Barbetti et al 2014). Mustard breeders have been paying attention to physiological (Dong et al 2008) and morphological (Li et al 2006) characters of host genotypes to develop resistant or tolerant genotypes against stem rot. Host resistance may be an effective, economical and ecofriendly approach to manage this disease (Zhao et al 2004). Variable responses of oilseed rape germplasm to inoculation with *S. sclerotiorum*, especially in field evaluations, are one of the main limitations for assessing resistance. Thus, an efficient, reliable and inexpensive screening method, which may lead to large-scale screening of the germplasm and cultivars for Sclerotinia stem rot resistance, is required to speed up the development of resistant genotypes. Among the various methods used for screening for resistance to Sclerotinia stem rot in oilseed rape includes cotyledon inoculation (Garg et al 2008), detached leaf inoculation (Bailey, 1987), cut petiole inoculation, oxalic acid assay and stem inoculation technique (Li et al 2006; Buchwaldt et al 2005).

On the other hand, the advancements and discovery of new techniques in genetics and cellular biology have been useful in the development of reliable and powerful DNA markers techniques based on polymerase chain reaction (PCR) for studying genetic differences in crop species (Mohammadi, 2002). Among various molecular markers, SSRs are considered as most powerful in genotype identification and genetic diversity estimation (Powell et al 1996). They have been used as a valuable tool for characterising germplasm in Brassica species (Halton et al 2002). Thus, keeping all these facts in view, the present study was designed to identify and screen various Brassica genotypes for Sclerotinia stem rot resistance and further characterize the tolerant genotypes for diversity analysis using SSR markers.

Materials and Methods

Germplasm. Seeds of hundred promising germplasms of *Brassica juncea, B. carinata, B. napus* and *B. rapa* from India (75), China (09) and Australia (16) were evaluated under Sclerotinia sick field.

Experimental field site. The field experiment was conducted in *Sclerotinia*-infected sick plot during 2014-15 and 2015-16, post-rainy (October-April) season at the Experimental farm, ICAR-Directorate of Rapeseed-Mustard Research (ICAR-DRMR), Bharatpur (77°27'E, 27°12'N, 178.13 m above mean sea level), India. The germplasm was sown in randomized complete block design with two replications. Each replication comprised a single row of 3 m length with 30 cm row to row width and 10 cm plant to plant spacing using *B. juncea* cv. Rohini and NRCYS5-2 as susceptible check. All other recommended agronomic practices were followed during crop.

Inoculum. Single isolate of *S. sclerotiorum* (SR 08) was collected from infected *B. juncea* at experimental farm, Bharatpur. Single sclerotia of *S. sclerotiorum* (SR-08) was surface sterilised in 1% (v/v) sodium hypochlorite and 70% ethanol for 4 min followed by three washes in sterile distilled water for 1 min (Clarkson et al 2003). The sclerotium was cut in to half and placed on potato dextrose agar (PDA) under sterilized conditions. *S. sclerotiorum* was sub-cultured and maintained at 20±2 C on PDA under 12 h alternate fluorescent light in culture room.

Inoculation. Inoculum was mass multiplied in laboratory on autoclaved sorghum grains in glass jars. Each jar containing 250 g sorghum grains was inoculated with six mycelial bit (5 mm) of 5-days old *S. Sclerotiorum* culture and kept in BOD at 20±2 C for 7-days. Later, these were incorporated in to the soil prior to sowing. The test lines were also sprayed by automizer at 45 days after sowing with mycelial suspension of the same isolate The concentration of suspension was adjusted to an optical density of 1·8 at 600 nm with a spectrophotometer. Further, the plants were inoculated after 60 days of sowing on the stem with the pathogen growing on agar blocks in both the cropping season. Stem inoculation was carried out according to the method used by Buchwaldt *et al* (2005). A single 5 mm mycelial bit cut from *S. sclerotiorum* colony of 4-5 days old culture growing on potato dextrose agar was used to inoculate each plant. In each replication 5 plants per entry were inoculated on same day. The mycelial bit along with cotton swab soaked in sterilized distilled water was placed on a small piece of parafilm (5-7 cm). Mycelial bit touching the stem at 15 cm height was then secured by twisting the ends of the parafilm strip around the stem. Wet cotton swab maintained high humidity during the infection period (Sharma et al 2012). During the inoculation soil moisture was 11.8 per cent while maximum temp 22.4 *C,* minimum 5.4 *C* and 77.6% RH were prevailed. The mean weather condition during three weeks was 19.9 C (max temp), 6.4 C (min temp), 76.8% (RH) and 98.2% RH in crop canopy.

Disease assessment. Disease incidence was assessed by recording the size of stem lesion length (cm) and disease severity (%) 3 weeks after inoculation. This has been shown to be the most ideal time to demonstrate the host response to the pathogen (Buchwaldt et al 2005; Li et al 2007). Disease reaction were categorised with modification in Garg et al (2010) as three different classes based on their responses, namely tolerant, susceptible and highly susceptible with stem lesion lengths ranging from 0 to <3.0 (disease severity <10%) ; 3.0 to 10.0 cm (disease severity 10-20%); and >10.0 cm (disease severity >20%), respectively.

DNA extraction and genetic diversity analysis of genotypes. Genomic DNA was extracted from the true leaves of 11 genotypes (8 tolerant and 3 highly susceptible) using the method described by Murray and Thompson (1980). A total of forty two

polymorphic SSR markers were used for evaluating genetic variability and relatedness among these 11 Indian mustard genotypes using PCR (Table 3). The PCR reaction was performed in a 10 µl reaction mixture containing 1µL of 1X buffer, 200 µM of each dNTP, 0.4 µM of forward and reverse primer, 1U Taq polymerase and 1µL of template DNA (20ng/µl) in a 96-well Thermal Cycler (Eppendorf, Germany). The PCR protocol comprised of the initial denaturation at 94 C for 5.0 min followed by 38 cycles of 30s at 94 C for denaturation, 40s of 55 C for annealing and 30s at 72 C for primer extension. Final extension was carried out at 72 C for 7 min and stored at 4 C. The PCR products were electrophoretically separated on 3.5% agarose gel and visualized in a gel documentation system (IG/LHR, Syngene, UK).

Data scoring and analysis. Gel photographs were used and presence of amplified products was scored as '1' and its absence as '0'. Variable bands were used to score for polymorphism and binomial data matrix was generated. These data matrix were entered into NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System Programme) and the data was analyzed using Jaccard's similarity coefficients (Jaccard, 1908). A dendrogram was constructed using the unweighted pair-group method with Arithmetic average (UPGMA) in the NTSYS program (Sneath and Sokal, 1973).

Results and Discussion

Screening of genotypes. During the study, a total of 100 genotypes were screened for Sclerotinia stem rot tolerance including stem lesion, length and disease severity under artificial conditions. Further, the promising 8 tolerant and 3 highly susceptible genotypes were used for genetic diversity analysis with SSR markers. After one week of inoculation, necrotic and bleached lesions developed on the plants and later during 1-3 weeks after inoculation white cottony mycelia were observed on the surface of the plants inoculated with *S. sclerotiorum.*

Table 1. Evaluation and performance of Brassica genotypes against *Sclerotinia sclerotiorum* (SR 08) in the field under artificial conditions.

Lesion size	% Disease severity	Reaction	Genotypes
< 3 cm	<10%	Tolerant	EC 597317, EC 597328, RH 1222-28, DRMR-261, DRMR-360, DRMR-1493, DRMR-1034, DRMR 2035 **(08)**
3-10 cm	10-20%	Susceptible	EC 597312, EC 597315, EC 597316, EC 597320, EC 597321, EC 597325, EC 597331, EC 597333, EC 597334, EC 597340, EC 552576, EC 552578, EC 552579, RH 1134, RH 1223, NRCDR 601, Basanti, Bhawani **(18)**
>10cm	>20%	Highly Susceptible	Rohini, LES-49, PDZ-2, CS 8000-1-2-8, DRMR-2-11, 44S31, RH0923, DRMR-150-35, RH 1037, PRO-5111, RMWR-09-5, PDZ 1, DRMR-1153-12, RGN-337, RLC-3, CS-13000-3-1-1-4-2, CS-15000-1-2-2-2-1, RGN 332, NPJ 180, CS-1100-1-2-2-3, PBR 422, PRE-2010-8, DRMR IJ 13-38, Q-90009, RH 1053, RGN 330, 3IJ-3403, PRE-2010-19, PRL-2010-8, PBR-417, DRMR-1-5, PRE-2010-15, RAUDT-10-33, TH-1102, Bayer-0306, PYS-2010-3, RTM-1351, EC 597314, EC 597319, EC 597338, EC 597341, EC 597343, EC 597344, EC 552573, EC 552581, EC 552583, EC 552584, RH 1140, RH 1060, RH 1441, RH 1118, RH 1120, RH 1210, RH 1215, RH 1019, RH 1053, RH 1117, RH 0749, DRMRIJ 31, RH 406, NRCDR-2, NRCHB 506, DMH-1, CS 52, CS 54, NPJ-112, JD 6, RB 50, PM 26, LES 43, Navgold, TL 15, NRCYS5-2, YSH 401 **(74)**

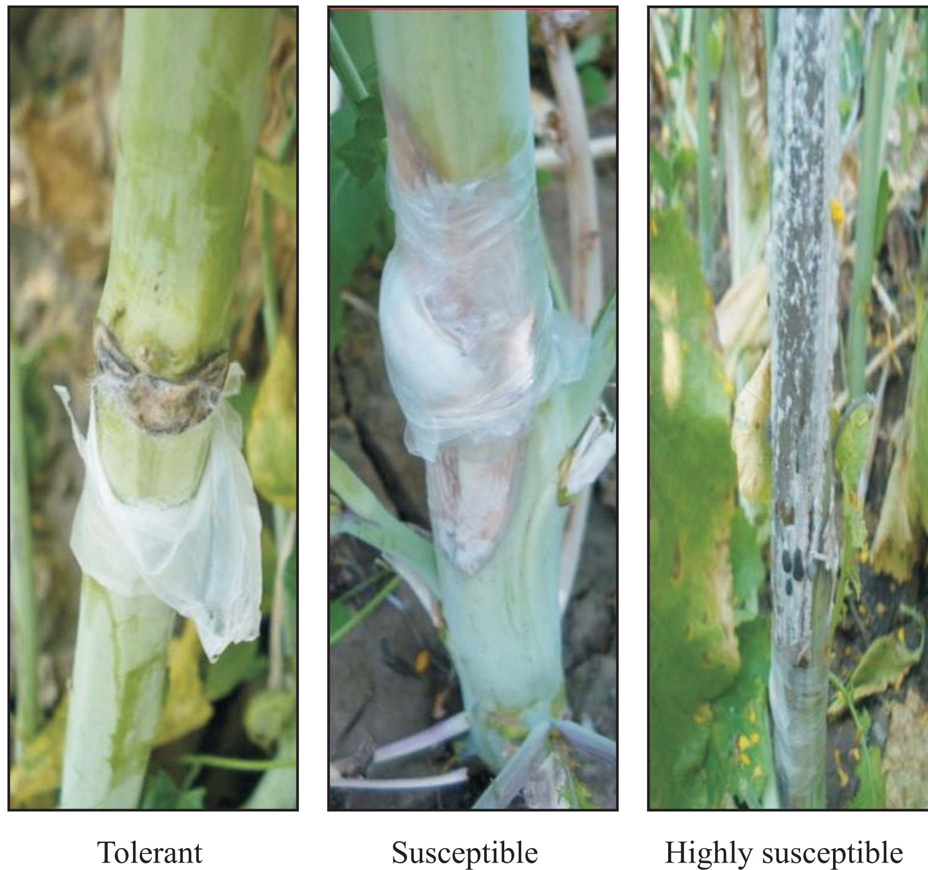

Figure 1. Sclerotinia disease reaction after stem inoculation

Sclerotia were also observed, mainly inside but also on the outside of infected stems. Stem lesion length was measured at 3 weeks after inoculation and there were significant differences among genotypes in relation to stem lesion length and per cent disease severity. Scoring of disease was done based on stem lesion length and disease severity (Sharma et al 2012). Eight genotypes having small lesion size (<3cm) and <10% disease severity were considered as tolerant. These include EC 597317, EC 597328, RH 1222-28, DRMR-261, DRMR-360, DRMR-1493, DRMR-1034, DRMR 2035 genotypes. Among them, RH1222-28 and DRMR 2035 were most tolerant and did not show any lesion on the stem (lesion not developed) compared to EC 597317, EC 597328 and DRMR-261 which were having small lesions on the stem (0.4 to 0.8 cm). Eighteen genotypes were considered as susceptible (3-10 cm lesion length and 10-20% disease severity) while the remaining 74 genotypes were scored as highly susceptible (>10 cm stem lesion length and >20% disease severity) (Fig 1). Rohini, EC 597314 and NRCYS5-2 were the three most susceptible genotypes (Table 1 and Fig. 2). There were substantial differences between the genotypes in relation to the range in mean lesion size across the genotypes. The genotypes which have a narrow variation in the range for resistance will be more reliable. In recent years, there have been a number of studies highlighting the complex nature of resistance in oilseed Brassicas to this pathogen. Li et al (2007) found that the variable impact of the time of stem inoculation on disease level depended upon the time of disease assessment and that could be reduced to an insignificant level if the assessment was delayed until 3 weeks post-inoculation and as such the different maturities of these populations would not have influenced their expression of host reaction to the pathogen. Significant interactions of *S. sclerotiorum* isolate were observed across Brassica diverse germplasm in various studies (Ge et al 2012; Uloth et al 2013 a, b).

Table 2. Summary of similarity matrix among 11 Brassica genotypes.

Genotypes	EC 597314	EC 597317	EC 597328	DRMR-360	DRMR-1493	DRMR-1034	RH 1222-28	DRMR 2035	DRMR-261	Rohini	NRCYS 5-2
EC 597314	1.000										
EC 597317	0.625	1.000									
EC 597328	0.562	0.812	1.000								
DRMR-360	0.437	0.812	0.750	1.000							
DRMR1493	0.500	0.750	0.812	0.937	1.000						
DRMR1034	0.562	0.687	0.750	0.625	0.687	1.000					
RH1222-28	0.437	0.813	0.750	1.000	0.937	0.625	1.000				
DRMR 2035	0.687	0.812	0.625	0.750	0.687	0.625	0.750	1.000			
DRMR261	0.500	0.625	0.687	0.562	0.500	0.4375	0.562	0.687	1.000		
Rohini	0.687	0.562	0.500	0.375	0.437	0.500	0.3750	0.625	0.437	1.000	
NRCYS5-2	1.000	0.625	0.562	0.437	0.500	0.562	0.437	0.687	0.500	0.687	1.000

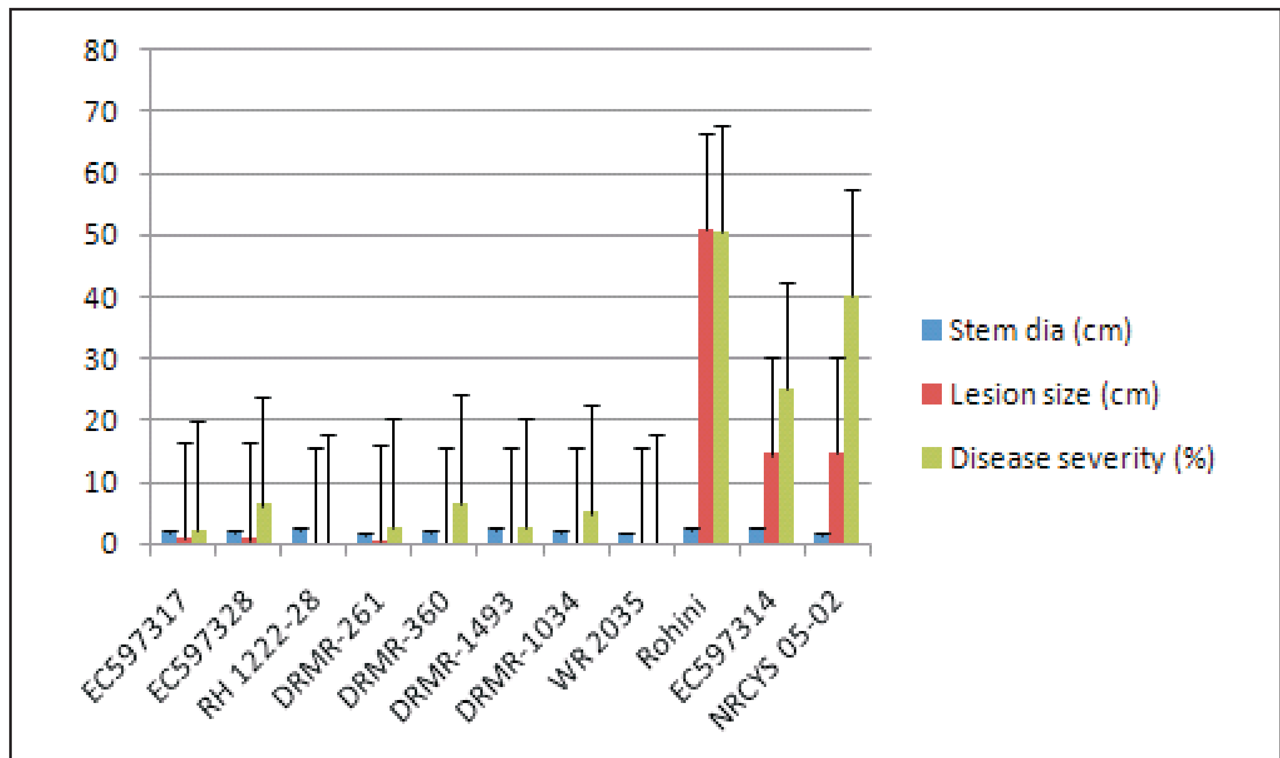

Figure 2. Correlation between lesion size and disease severity among eleven genotypes

Genetic diversity among the genotypes. A total of forty two polymorphic SSR markers used for evaluating genetic diversity among the 11 Indian mustard genotypes gave good amplification and the product size varied from 70-380 bp (Fig.3). Diversity analysis with the eleven genotypes (eight tolerant and 3 highly susceptible) indicated the presence of two distinct groups (Fig.4). The cluster analysis evidently discriminated the 11 genotypes into tolerant and susceptible. The similarity coefficients varied from 0.38 to 1.0 thus revealing the presence of maximum diversity between these genotypes (Table 2). The highest value for genetic similarity (100%) was between EC 597314 and NRCYS5-2; DRMR-360 and RH 1222-28 followed by DRMR-360 and DRMR-1493 along with DRMR-1493 and RH 1222-28. Genotype EC 597317 had a high similarity of 81.25% with DRMR-360, RH1222-28 and DRMR 2035. Similarly, EC 597328 was found to be similar to DRMR-1493 at a similarity of 81.25%. Rohini exhibited a least similarity of 37.5% both with

Table 3. Details of SSR (Simple Sequence Repeat) primers used for molecular characterization among 11 genotypes.

S.N.	Marker	Sequence (5'-3')	Tm (^{0}C)	Range of amplified product (bp)
1	cnu_m583a	TTGTAGAGAGAGAGAGAGGGCA CCTTCAAAAGAAAGGAGGGG	55	200-250
2	cnu_m584a	CGGAAAGAACACATTGAAAGAA AGCATCAAAGAAAAAGGCGA	55	200-250
3	cnu_m593a	TAAGGCAAATTGTTGGGCAT CCATCTCTCCTTGTCCTCCA	55	150-220
4	cnu_m596a	AAAAACGAACCTCTTTGGGG CTGACGAAGGCTCCAATTTC	55	200-260
5	cnu_m597a	TTGAACCCACGAAAACTTCC AGAAGGGAGAGAGGTCAGGC	55	250-280
6	cnu_m600a	TCATTTTGCTATCGTCGGTTC TGCAGAGATTCCCAAATCAA	55	230-260
7	nia_m043a	CCATTCGAGGTGGTCGTAAA AGAAAACGGACCTCGATTCA	55	250-300
8	nia_m053a	AAAATCTCGCTTCTGCGCTT TCCCTCACCCTGTGCAATAG	55	350-380
9	Ni2AO1	TGCTGCTACAGACAGTGTTGG AAAGGCTACACACTCATGAAACC	55	200-250
10	Ni2AO7	GGAACCCAACAAGTGAGTCC AGAGCTTGAGACACATAACACC	55	170-250
11	A12	ACGATGGGTTCTTCTTGTCG CAAGAAACTTTCGAGGAACCC	55	100-130
12	B02	CGCTGCAATTATACGAAAGC CCTCATGCTCTCCAAAGACC	55	70-90
13	B03	ACTTCTTGCCCTCCTCACC AAATACTCACTGCAATACCCAGG	55	200-230
14	B07	AGAGATTCAAACCGAGTGCC GGGGCTAGCTTCATCATCC	55	170-190
15	DO3	CGTATGTGAAAAATAAATGG TTGAGCTTGAGATCATCCCC	55	200-250
16	D10	GATGCCCCAAATCTGTTACG CAATTCGTGAAAAATAGCCG	55	200-250
17	D12	GAGATGAGGATTTGCTTTTGC ACAGTATGAGAGAGAGAGAGAGAG	55	150-200
18	E05	CTCGTCTCAGGGATTATGTCG CAGACAGAGGATAGACCGAACC	55	130-200
19	E07	GAGCGAGTCGATTACTTTTGC GAATGGATTTCCGATGATGG	55	120-170
20	F01	CGTATGTAGAGAGAGAGAGAGAGAGAG AGAACCGTTGAGGTGCTGTC	55	200-250

Contd...

Contd... Table 3

S.N.	Marker	Sequence (5'-3')	Tm (^{0}C)	Range of amplified product (bp)
21	F04	TTTCTTCTTAACCATCGGCG		
		TCTTCCTCTGCTTCTGGTGC	55	100-150
22	Ni3-B07	GGAGAAGAGGAAGAAGAAGCC		
		CGACTTCTAGAGGAACCCCC	55	100-200
23	C05	TTTCGTGCTTTGGTGTGAAG		
		TCCCCAAATCGAACCATAAG	55	200-250
24	C08	CCCTAACACGGTGTCAACAG		
		GGCAGAATCATCGAGAGGTC	55	300-400
25	Ni3-D03	ACCGGAGACGAAACTACCG		
		CCTCTTCGACGTTTTTGGTG	55	150-220
26	Ni3-F01	AGCCGCTAAAGAGAAGGTCC		
		CGCTTTCAAGCTCTCTCCC	55	150-200
27	Ni3-G07	CACTCTCTCCGCCATTTTTC		
		CTTGAAGCGTTAAAGCCGAC	55	200-250
28	A04	ATGTGGTCTTTCCCAGTTGC		
		CATCCTCTGCTTTAGTGGGC	55	220-270
29	A09	AAAGGGCGAAGAAGCAGC		
		TTTCTTCCATTTGACCGACC	55	100-170
30	C09	AGCATCAATCTTTTGCTCTGC		
		TGCACACAAACTCCTTCTCC	55	200-260
31	G02	TTGGTGTCAGAAACAACG		
		ACACACGACGGATCTCTGC	55	200-260
32	G09A	CTCGAGGCGCGTTTTACC		
		CTCAATCGCATGCATAATCG	55	100-150
33	G10	AGACTGAAATATTTGGGACC		
		CGTTCTTCAACTTGTTCATCATC	55	175-250
34	G11	GCACCATCGAAAAAGGTCC		
		CTGGGTATGGTTGGAACAGG	55	175-200
35	H01	TGAAAACACACACACACACACAC		
		AACCCTGATGTTGAAGGTCG	55	100-150
36	H09	AGAGATTCAAACCGAGTGCC		
		GGGGCTAGCTTCATCATCC	55	200-250
37	Ni2A07	GGAACCCAACAAGTGAGTCC		
		AGAGCTTGAGACACATAACACC	55	200-250
38	Ni2A12	ACGATGGGTTCTTCTTGTCG		
		CAAGAAACTTTCGAGGAACCC	54	80-130
39	Ni2B07	AGAGATTCAAACCGAGTGCC		
		GGGGCTAGCTTCATCATCC	55	200-230
40	Ni2D10	GATGCCCCAAATCTGTTACG		
		CAATTCGTGAAAAATAGCCG	52	170-200
41	BrgMS732	GCGCCGACGAAACAATTA		
		ATGCTCGTGCCCACAAAA	50	300-350
42	BrgMS399	TTTGAATAGTGTGTACCCGCAC		
		CGAGACTGGGACCTGATACTTC	55	320-370

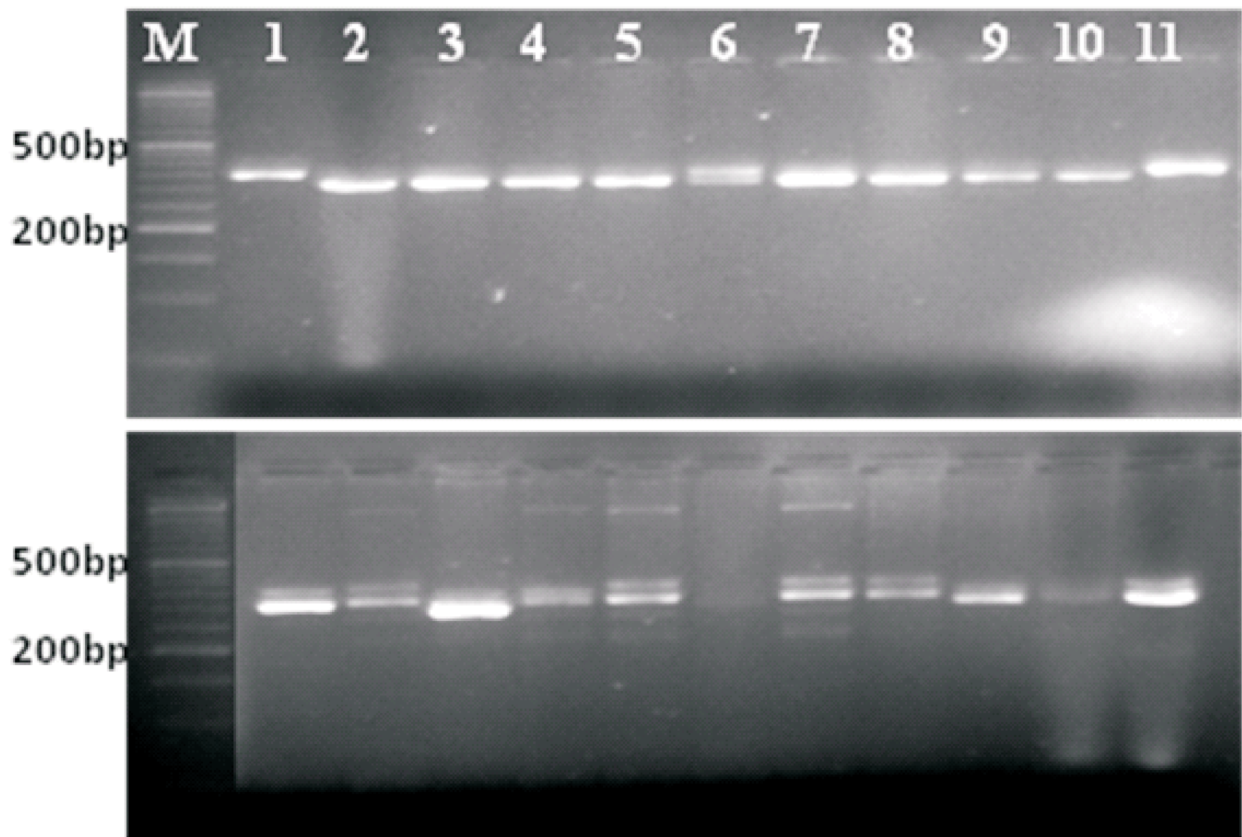

Figure 3. Agarose gels showing amplification profiles of genotypes using the primer cnu_m579a and cnu_m600a.

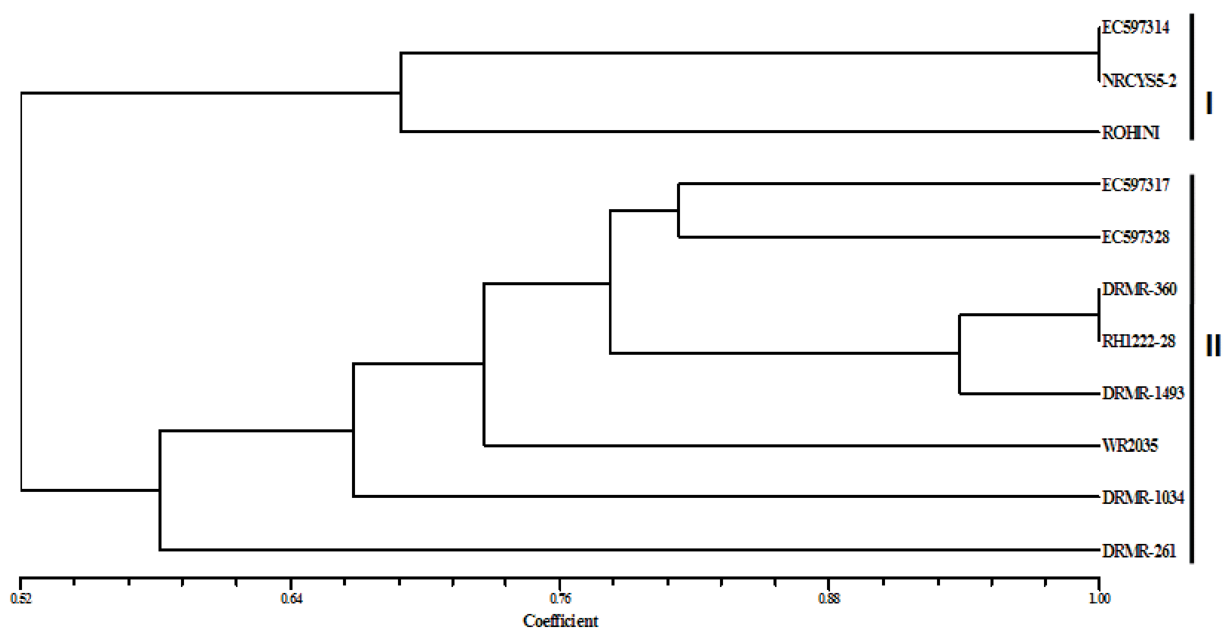

Figure 4. UPGMA dendrogram showing genetic relationships among eleven genotypes based on SSR markers.

RH1222-28 and DRMR-360. Cluster I comprised of 3 highly susceptible genotypes viz. Rohini, EC 597314 and NRCYS5-2 where EC 597314 and NRCYS5 exhibited highest similarity (100%) while Rohini and NRCYS5-2 showed similarity of 68.75%. Eight tolerant genotypes namely EC 597317, EC 597328, DRMR-360, DRMR-1493, DRMR-1034, RH 1222-28, DRMR 2035, DRMR-261 were present together in cluster II. Here, DRMR-360 and RH 1222-28 (100%) showed highest while DRMR-1034 and DRMR-261 (43.75%) were grouped at least similarity (Fig. 4).

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