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First ever probable oats (*Avena sativa*) QTLs mapped on chromosome 1 and 7 for pyrenophora leaf spot resistance in India

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ABSTRACT

An experiment was conducted during 2021–22 at Punjab Agricultural University, Ludhiana, Punjab for the development of linkage map and identification of QTLs for pyrenophora or Helminthosporium leaf spot resistance in oats (*Avena sativa* L.). A total of 96 F_2 plants were used which was derived from the cross EC/0007662 (resistant source) and EC/0131291 (susceptible source). Linkage map was constructed for genotypic data of 96 population lines with 24 polymorphic SSR markers using QTL cartographer. A total of 7 linkage groups (LGs) had been generated from this data. But out of 7 LGs, 5 LGs remained individually at 0 position (unlinked) i.e. belonged to different group each and LG1 and LG7 were grouped. First LG included ABAM232, ABAM493 and ABAM077 from 0 to 66.5 cM. Similarly, 7th LG had two marker position i.e. ABAM342 and ABAM425. Maximum distance between the two markers was found to be 33.4 cM (between the marker interval of ABAM232 and ABAM497. Further QTL analysis was done using cartographer with composite interval mapping to identify the QTLs associated with disease resistance by comparing the phenotypic data of F_2 population for disease and genotypic data of the population. The work reported here constitutes a major step toward identification of genetic regions responsible for disease resistance. However, the utility of QTL for marker assisted selection requires that QTLs are localized in a narrow region tightly linked with associated markers. The result of the experiment can be used for marker assisted breeding to transfer such genes identified as part of our research in order to reduce disease severity and yield losses in oats.

Keywords: Leaf spot, Linkage map, Oats, Pyrenophora, QTLs, Resistance

Winter cereal crop oats (*Avena sativa* L.), a member of the genus *Avena* and family Gramineae, originated in the Mediterranean region. This self-pollinating crop, with limited variation (Rana *et al.* 2019), includes *Avena sativa, A. nuda* and *A. byzsantina* as the only commercially grown species. Cultivated oats are allohexaploid with a chromosomal number of 2n = 6x = 42. It ranks sixth in global production after wheat, maize, sorghum, rice and barley (Hilli *et al.* 2021, 2022). The European Union leads in oat production, followed by Russia, Canada, the United States and Australia (Kumari *et al.* 2019). Oats are increasingly consumed as human food, particularly in breakfast cereals (Boshoff *et al.* 2019). In India, approximately 10.0 lac hectares are dedicated to growing fodder oats in which Uttar Pradesh contributes 34%, followed by Punjab (20%), Haryana (9%) and Madhya Pradesh (6%). Punjab alone cultivates oats on 1.0 lakh ha (Indiastat 2020)

In India, oats are primarily cultivated for forage, serving as a vital source of green fodder for livestock. Simultaneously, it is recognized as an excellent dual-purpose cereal crop meeting the needs of both humans and animals (Chaudhary *et al.* 2021). The dual-purpose aspect is determined by crucial parameters such as higher green fodder and grain yields from the same crop, allowing harvests for fodder at 65 to 70 days post-sowing and later for grains at maturity. Oats boast the highest concentration of soluble fibre beta-glucan per 100 g serving, ranging from 5.0 g (oat flour) to 7.2 g (oat bran) (Kianian *et al.* 2000, Amin 2014, Kapoor *et al.* 2022).

Focusing on threats to oat production, diseases and pests, predominantly foliar, pose challenges. Leaf spot diseases like *Drechslera avenae* leaf spot can cause substantial yield losses (10–40%) under favourable conditions (Zhou and Steffenson 2013). However, these diseases have not been detected in Punjab and India (Mohammed 2019). Molecular biology-based breeding strategies have significantly contributed to crop improvement, with DNA markers playing a major role in crop advancement. The ongoing work aims to identify and map quantitative trait loci (QTLs) responsible for resistance to Helminthosporium leaf blight (HLB), addressing critical challenges in oat cultivation.

MATERIALS AND METHODS

An experiment was conducted during 2021–22 at Punjab Agricultural University, Ludhiana, Punjab for

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the development of linkage map and identification of QTLs for pyrenophora or Helminthosporium leaf spot resistance. Experimental material includes 167 germplasm lines selected from NBPGR, New Delhi mostly of them having origin from USA and Sweden. These Germplasm lines belonged to different species, viz. *sativa, sterilis, abyssinica, eriantha, longiglumis, brevis, nuda chinensis* and *sativa* hulless. Average rainfall of the area was 700 mm and the weather was favourable during the entire crop growth. Materials were evaluated in randomized block design (RBD) in single row having 2 m length with row-to-row distance of 30 cm. Recommended package of practices were maintained. Further the isolation of pathogen**,** disease screening by artificial inoculation, identification of resistant and susceptible sources and development of mapping populations have already been discussed by Hilli *et al.* (2022). This article includes development of linkage maps and QTL mapping.

DNA extraction and chemicals preparation: For preparation of 1 litre of CTAB buffer, 20 g of cetrimide, 12.114 g of Tris base, 81.9 g of NaCl and 7.445 g of EDTA was added to a vessel and double distilled water was added up to 1 litre. It was then allowed to dissolve by keeping it on hot plate and after all the salts got dissolved it was then autoclaved. Before using, B-Mercaptoethanol and polyvinyl pyrrolidone was added to the CTAB. Then its *p*H was checked and adjusted to 8.0.

The DNA extraction involved crushing plant tissues with liquid nitrogen to create fine leaf powder. Cetyl trimethyl ammonium bromide (CTAB) was added to the powder, followed by incubation in a water bath and addition of chloroform:isomyl alcohol. After centrifugation, the supernatant was mixed with chilled isopropanol, leading to DNA pellet formation upon centrifugation. Washing with ethanol and drying resulted in visible DNA pellets. Finally, TE buffer was added to create a DNA suspension, and the samples were stored at -20ºC.

Assessment of quantity and quality of DNA: DNA quantification was performed using agarose gel electrophoresis. A mixture of 0.8 g agarose and 100 ml 0.5X TBE buffer was prepared. The 10X loading dye, composed of 0.4% Bromophenol blue, 0.4% Xylene cyanol, and 50% glycerol, was diluted to 6X. It was then added to DNA in an 8:2 ratio (8 µl dye and 2 µl DNA). The DNA samples were visualized under UV light using a photo-gel documentation system. Quantification and quality assessment of the mapping population were determined based on band intensity, and appropriate dilutions were made accordingly.

Selection of SSR primers and their dilution: For the survey of polymorphism present between the parents, 220 SSR (microsatellite) molecular markers were screened. The list of the markers used for parental polymorphism survey is given in Table 1. The polymorphic markers were then analysed on DNA of 96 F_2 population. Each primer was dissolved in 100 µl of 1X Tris EDTA (TE) buffer and diluted further with deionized water to the working concentration of 20 µM. The primers were diluted as per following formula:

$$\mu M \text{ of oligo in } 100\ \mu l \text{ of solution } \frac{OD}{10}$$

In vitro DNA amplification through polymerase chain reaction (PCR): *In vitro* amplification of the DNA was performed in Eppendorf master cycler by using specific SSR primers by using the protocol given by Oliver *et al.* (2010). The analysis of PCR was performed using the reaction vol. of 20 µl which contained the template DNA, forward and reverse primers, $MgCl_2$, dNTPs, *Taq* polymerase and PCR buffer.

Scoring of SSR alleles: For every SSR marker used in survey, each allele which was amplified was numbered as 1, 2, 3, and so on. The bands which were amplified properly scored as 1 (band present) and 0 (band absent). There were also some bands which were difficult to score or there was no amplification, those all bands were scored as missing. PIC value or polymorphic information content value of the primer provides us with an estimation of the discriminatory power of a locus, by involving the number of alleles which were expressed and also relative frequencies for those amplified alleles was calculated as:

$$PIC_i = 2f_i\ (1\text{-}f_i)$$

Table 1 Polymorphic markers identified between two parental lines (EC/0131291 and EC/0007662)

Primer	Primer size (bp)	Annealing temperature (ºC)	Amplification size (bp)
AM15	41	53	230
AM17	36	51	180
AM41	39	49	195
AM44	40	56	195
HVM20	38	55	120
L39777	39	55	290
ABAM155	40	57	260
ABAM1015	42	55	340
ABAM219	40	55	300
ABAM354	40	53	210
ABAM493	40	53	360
ABAM425	40	53	210
ABAM232	40	53	305
ABAM467	40	54	420
ABAM577	40	57	330
ABAM814	40	55	200
ABAM 077	40	55	310
AM46	39	53	310
ABAM457	40	55	170
ABAM342	40	53	310
ABAM485	40	55	210
ABAM095	40	55	195
ABAM093	40	53	250

where PIC_i, Polymorphic information content for locus i; f_i, Frequency of an amplified allele; $1\text{-}f_i$, frequency of non-amplified allele.

The frequency can be calculated as the proportion between the number of alleles amplified at every locus and total number of genotypes. The average PIC value of the total number of loci for the one primer gives the PIC value for that specific primer,

Construction of linkage map using QTL cartographer: A linkage map was created using QTL Cartographer software. Markers were assigned to linkage groups through pairwise analysis, setting a LOD score range of 1.0 to 3.0 and a maximum recombination frequency of 0.3. Map distances were calculated using the Kosambi mapping function. The marker order within linkage groups was determined through multipoint analysis with the 'Auto Order' command, and the final order was confirmed using the 'Ripple' command.

CIM (Composite Interval Mapping) for identification of QTL by using QTL Cartographer 2.5: QTL Cartographer software was utilized to assess marker-trait associations using phenotypic and genotypic data. Significance levels were determined based on *P*-values: *($0.01<P<0.05$), **($0.001<P<0.01$), and ***($P<0.001$). Single marker analysis preceded composite interval mapping for more accurate QTL detection, combining interval mapping with forward and backward regression analysis. To establish significance, a threshold LOD score was calculated using 1,000 permutations at 5% level.

RESULTS AND DISCUSSION

Phenotypic evaluation of population for disease resistance: Genotypes, resistant and susceptible to leaf spot disease were identified in a population developed by Hilli *et al.* (2022). The population exhibited a continuous disease reaction distribution. EC/0007662 (22% DSI) was the most resistant parent, while EC/0131291 was the most susceptible (78% DSI). Transgressive segregation was observed in individuals with resistance values exceeding the susceptible parent's range. Molecular analysis was conducted on the population derived from these parents.

Genotyping of population (PIC values, polymorphic markers): Parents EC/0007662 and EC/0131291 were assessed for parental polymorphism using 220 SSR markers (Table 1, Fig. 1). Among them, 24 markers were found to be polymorphic. These 24 markers were utilized for genotyping the mapping population, and their polymorphic information content is detailed in Table 2. Fig. 2 illustrates the run of polymorphic markers on the developed population.

Construction of linkage map: A linkage map was created for 96 population lines using 24 polymorphic SSR markers through QTL Cartographer (LOD score 3.0, recombination fraction 0.3) (Fig. 3). Chi-squared tests identified markers deviating from Mendelian segregation

Fig. 1 Banding pattern between two parental lines using SSR marker.

Table 2 Polymorphic information content of various marker over two parents tested

Primer	Count 0	Count 1	Freq count 0 (P)	Freq count 1 (Q)	P^2	Q^2	$P^2 + Q^2$	PIC $1-(P^2+Q^2)$
AM3	2	0	1	0	1	0	1	0
AM4	2	0	1	0	1	0	1	0
AM7	2	0	1	0	1	0	1	0
AM8	2	0	1	0	1	0	1	0
AM15	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AM17	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AM18	2	0	1	0	1	0	1	0
AM21	2	0	1	0	1	0	1	0
AM24	2	0	1	0	1	0	1	0
AM25	2	0	1	0	1	0	1	0
AM37	2	0	1	0	1	0	1	0
AM38	2	0	1	0	1	0	1	0
AM39	2	0	1	0	1	0	1	0
AM40	2	0	1	0	1	0	1	0
AM41	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AM44	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AM45	2	0	1	0	1	0	1	0
AM46	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AM50	2	0	1	0	1	0	1	0
AM54	2	0	1	0	1	0	1	0
HVM4	1	1	0.5	0.5	0.25	0.25	0.5	0.5
HVM 20	1	1	0.5	0.5	0.25	0.25	0.5	0.5
HVM 62	2	0	1	0	1	0	1	0
L39777	1	1	0.5	0.5	0.25	0.25	0.5	0.5
Z48431	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB-AM-059	2	0	1	0	1	0	1	0
AB-AM-065	2	0	1	0	1	0	1	0
AB-AM-068	2	0	1	0	1	0	1	0
AB-AM-074	2	0	1	0	1	0	1	0
AB-AM-076	2	0	1	0	1	0	1	0
AB-AM-077	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB-AM-093	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB-AM-095	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB-AM-130	2	0	1	0	1	0	1	0
AB-AM-155	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB-AM-202	2	0	1	0	1	0	1	0
AB-AM-219	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB-AM-223	2	0	1	0	1	0	1	0
AB-AM-226	2	0	1	0	1	0	1	0
AB-AM-227	2	0	1	0	1	0	1	0
AB-AM-232	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB-AM-242	2	0	1	0	1	0	1	0
AB-AM-258	2	0	1	0	1	0	1	0
AB-AM-259	2	0	1	0	1	0	1	0

Contd.

Table 2 Polymorphic information content of various marker over two parents tested

Primer	Count 0	Count 1	Freq count 0 (P)	Freq count 1 (Q)	P^2	Q^2	$P^2 + Q^2$	PIC $1-(P^2+Q^2)$
AB-AM-269	2	0	1	0	1	0	1	0
AB-AM-275	2	0	1	0	1	0	1	0
AB-AM-283	2	0	1	0	1	0	1	0
AB-AM-285	2	0	1	0	1	0	1	0
AB-AM-290	2	0	1	0	1	0	1	0
AB-AM-323	2	0	1	0	1	0	1	0
AB-AM-324	2	0	1	0	1	0	1	0
AB_AM_342	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB_AM_349	2	0	1	0	1	0	1	0
AB_AM_354	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB_AM_399	2	0	1	0	1	0	1	0
AB_AM_425	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB_AM_431	2	0	1	0	1	0	1	0
AB_AM_451	2	0	1	0	1	0	1	0
AB_AM_453	2	0	1	0	1	0	1	0
AB_AM_457	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB_AM_467	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB_AM_469	2	0	1	0	1	0	1	0
AB_AM_483	2	0	1	0	1	0	1	0
AB_AM_485	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB_AM_488	2	0	1	0	1	0	1	0
AB_AM_491	2	0	1	0	1	0	1	0
AB_AM_493	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB_AM_553	2	0	1	0	1	0	1	0
AB_AM_576	2	0	1	0	1	0	1	0
AB_AM_577	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB_AM_692	2	0	1	0	1	0	1	0
AB_AM_709	2	0	1	0	1	0	1	0
AB_AM_805	2	0	1	0	1	0	1	0
AB_AM_814	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB_AM_824	2	0	1	0	1	0	1	0
AB_AM_825	2	0	1	0	1	0	1	0
AB_AM_839	2	0	1	0	1	0	1	0
AB_AM_842	2	0	1	0	1	0	1	0
AB_AM_844	2	0	1	0	1	0	1	0
AB_AM_846	2	0	1	0	1	0	1	0
AB_AM_847	2	0	1	0	1	0	1	0
AB_AM_878	2	0	1	0	1	0	1	0
AB_AM_915	2	0	1	0	1	0	1	0
AB_AM_956	2	0	1	0	1	0	1	0
AB_AM 1013	2	0	1	0	1	0	1	0
AB_AM 1015	1	1	0.5	0.5	0.25	0.25	0.5	0.5
AB-AM-269	2	0	1	0	1	0	1	0
AB-AM-259	2	0	1	0	1	0	1	0

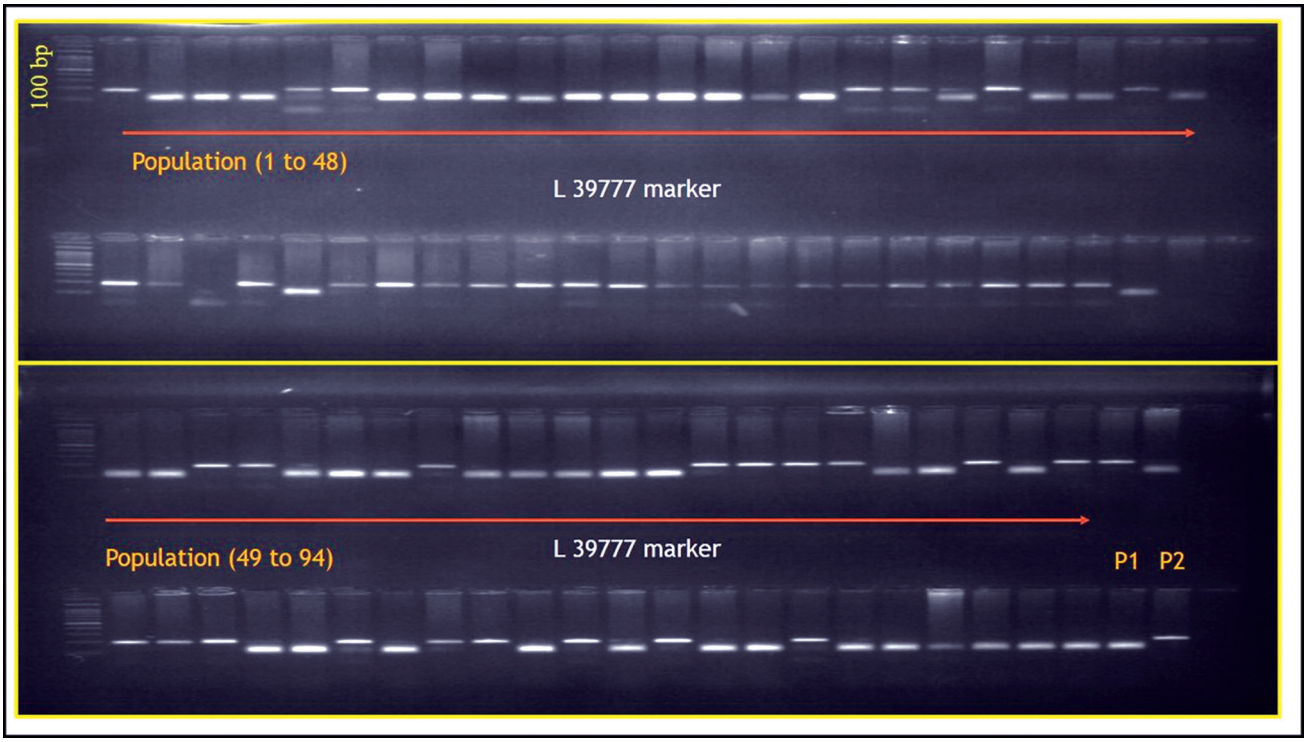

Fig. 2 Segregation pattern of L39777 marker over the population.

(P<0.05). Seven linkage groups were formed, with LG1 and LG7 subsequently grouped.

In the first linkage group (LG), ABAM232, ABAM493, and ABAM077 spanned 0 to 66.5 cM, while the 7th LG included ABAM342 and ABAM425 (Oliver *et al.* 2010). The maximum distance between markers was 33.4 cM (ABAM232 to ABAM497 interval). Five markers were on the linkage group, and the remaining markers were unlinked due to weak linkages between loci and markers.

QTL identification for leaf spot disease: QTL Cartographer, employing Composite Interval Mapping (CIM), identified disease resistance QTLs in the F_2 population. Markers ABAM232 and ABAM493 on chromosome 1, as well as ABAM425 and ABAM342 on chromosome 7, showed significant linkage probabilities. Another study by Kianian *et al.* (2000) used SIM and CIM to identify β-glucan QTLs in hexaploid oat. In barley, 3 QTLs for malt β-glucan were found on chromosome 2H (Han *et al.* 2018). Additionally, Marcotuli *et al.* (2016) identified 7 β-glucan QTLs in wheat using GWAS on chromosomes 1A, 2A (2), 2B, 5B, and 7A (2).

Marker locus falling on different chromosomal regions: Out of total 220 SSR markers used, 24 markers were found to be polymorphic and these 24 markers were falling on 17 different chromosomes i.e. markers lied on chromosome 1, 2,3,4,6,7,9,10,11,12,13,14,15,16,17,19 and 20. Maximum of 3 markers were falling on chromosome 1 and 7 respectively. ABAM077, ABAM232 and ABAM493 were falling on first chromosome and similarly ABAM219, ABAM342 and ABAM425 on chromosome 7. Rest of the markers position is depicted in Table 3.

Table 3 Chromosomal location of different markers

Marker	Chromosome location
L39777	17
AB-AM-077	1
AB-AM-093	6
AB-AM-095	6
AB-AM-155	20
AB-AM-219	7
AB-AM-232	1
AM15	2
AM17	3
AM41	14
AM44	16
AM50	12
HVM4	4
HVM 20	15
AB_AM_342	7
AB_AM_354	20
AB_AM_425	7
AB_AM_485	4
AB_AM_457	9
AB_AM_467	10
AB_AM_493	1
AB_AM_577	11
ABAM1015	19
Z48431	13

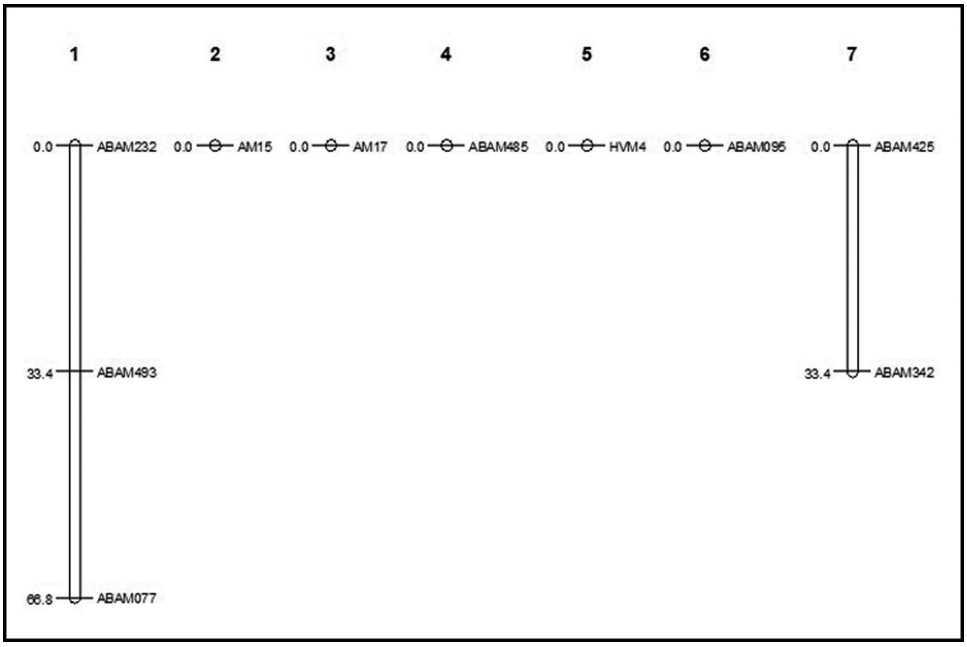

Fig. 3 Linkage map developed by QTL cartographer software.

Percent of variance explained (PVE %): QTL analysis using QTL Cartographer revealed significant associations between SSR markers and disease resistance. Chromosome 7 markers, ABAM425 and ABAM342, showed the highest percent of variance explained (PVE) at 36.43 and 0.71%, respectively. Similarly, on chromosome 1, ABAM493 and ABAM077 displayed PVE ranging from 0.018 to 23.94%. Linkage mapping of 96 population lines identified seven groups, with LG1 (ABAM232, ABAM493, ABAM077) spanning 0-66.5 cM and LG7 (ABAM342, ABAM425) at a maximum distance of 33.4 cM. Composite interval mapping (CIM) further identified QTLs associated with disease resistance on chromosomes 1 and 7. These findings provide valuable insights for genetic studies and marker-assisted selection in leaf spot disease.

The study acknowledges limitations in the number of individuals and markers, potentially impacting the detection of minor QTLs and leading to an overestimation of major QTL effects. Despite this, the practical approach utilized, with marker loci exhibiting expected segregation ratios, facilitated QTL identification for leaf spot disease. Future studies could benefit from increased sample size and marker density to uncover more major and minor QTLs. While the work represents a significant step in identifying genetic regions for disease resistance, further fine mapping is essential for pinpointing genes linked to resistance. The results hold potential for marker-assisted breeding (MAB) to transfer identified genes and mitigate disease severity and yield losses.

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