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Gene mapping of the mustard aphid (*Lipaphis erysimi* (Kalt.) Hemiptera: Aphididae) linkages of resistance gene in canola genotypes associated with RAPD markers

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ABSTRACT

In this study, the resistance of selected canola genotypes to the mustard aphid *Lipaphis erysimi* (Kaltenbach) was investigated. Molecular characterization of plant resistance was conducted, and genetic variability was observed between the resistant (KS-75) and susceptible (Abaseen) genotypes of brassica. Three RAPD markers were identified that were associated with the resistance. This study was conducted to compare the genetic data between two genotypes (KS-75 resistant and Abaseen susceptible), with an average of 4.3 bands per primer. The amplification bands per primer ranged from 9 to polymorphic fragments of 8, with fragment sizes ranging from 450 to 1700 base pairs to 9 kilo base pairs (kbps). Amplification of the largest fragment in 9 kbps using four primers (A 1–12, B 1–12, K 1–12 and L 1–12) resulted in a mean 92 percent identity index between the first two resistant and susceptible genotypes. KS-75 resistant had the highest mean genetic diversity of 92 percent, while Abaseen susceptible had the lowest mean genetic diversity of 8 percent. Out of the three markers tested, two were found to be positively associated with aphid resistance, while one was negatively correlated. These markers can be used to help develop a pre-emptive strategy for aphid resistance in brassica species, without having to simultaneously select for yield and quality related traits.

1. Introduction

Improved seed oil and meal quality have facilitated oilseeds Brassicas in gaining the second largest oilseed crop position and a major crop for the raw material supply in biodiesel production (Abuyousuf et al., 2018). Researchers have developed *Brassica napus* L. and *Brassica rapa* L. for enhancing oil and other qualities. Another type of mustard crop species *Brassica juncea* L. is also an established throughout Pakistan, India and China as oilseed crop, as they contain higher percentage of erucic acid in the oil and elevated concentrations of glucosinolates in the cake. Mohammadi and Prasanna, (2003) have conducted research on genetic relationships using pedigree analysis and morphological characters/using molecular markers. Among all these techniques, molecular markers proved the best choice for calculating genetic diversity as they are not influenced by environmental factors. Recommended Polymerase chain reaction (PCR) methods are commonly used for the characterization of plants and animals genetic resources. Randomly amplified polymorphic DNA is perhaps the simplest method among numerous available molecular techniques recruited to measure constitution, change and progression of genetic material. Despite controversies on limitations and reproducibility of RAPD markers, they are still significantly used to tackle various objectives in molecular biology, like tagging a simple and dominant gene as well as to identify multiple chromosome intervals controlling a quantitative trait (Pan and Chen, 2010).

Insect pests and diseases are important factors responsible for yield reduction in canola crops. The leaves become curly and turn pale yellowish resulting in premature fall of the flowers. Yield losses that occur only due to the mustard aphid *Lipaphis erysimi* (Kaltenbach) (Homoptera: Aphididae) in crop account for approximately 50–75 % (Tolba, 2020). Aphids utilize their sensory organs in locating host plant (Johnson et al., 2010). After landing on a

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source, aphid utilizes its tactile for monitoring surface features and gustatory cues for short probes to sample host cell contents. Fahey (2012) described two types of host selection; generalists and specialists. Cabbage aphid (*Brevicoryne brassicae* Linnaeus.), pea aphid (*Acyrthosiphon pisum* Harris) and the mustard aphid (*Lipaphis erysimi* Kaltenbach) are specialists with a limited host range covering closely related species. Application of mustard crops field with heavy chemicals can cause mortality of natural enemies and also may cause environmental pollution (Mpumi et al., 2020).

Plant defenses against aphids are antixenosis, antibiosis and tolerance, utilized against aphids at multiple levels of interaction (Pandey, 2003). The plant surface serves initial obstacle to aphids attack in different form either by secreting secondary metabolites that are detrimental to insects or by releasing volatiles that are aversive in nature (Zhang et al., 2015). Further, surface waxes also add to plant-aphid interactions (Liu, 2014). Use of surface waxes on plant varieties that lack waxes like triticale posed deterrence effect against aphids and subsequently increased their mortality. Presence of plant trichomes can in turn manipulate aphid infestation provided with physical barrier for their movement (Livak and Schmittgen, 2001); however, glandular trichomes possess sugar esters and secondary metabolites (2- tridecanone) that have injurious effect on herbivore insects (Zhang et al., 2015) where several contains (E)–β-farnesene, commonly known as aphid alarm pheromone that triggers disturbance in aphid species (Saha, 2016).

Canola genotypes that can less express apoplastic ascorbate oxidase negatively affect the aphid fecundity in comparison to genotypes that overexpress this metabolite and therefore their reduction in canola crop pose detrimental effect on the target organism (Li et al., 1999). Thus, assessment of genetic diversity among *B. napus* and *B. juncea* accessions from present study will be wisely help in establishing a baseline for the development of canola varieties that are more acceptable to farmers with ultimate increase in good quality oils. The development of insecticide resistance in various species of insect pests has forced the plant protectnists to opt for an alternative strategy. Thus, the most durable pest control is through integrated pest management strategy with no or little adverse effect on environment, economy, natural enemies and health hazards (Ingle et al., 2020).

Keeping in view the importance of selected canola genotypes between resistant and susceptible (*B. napus*) were further screened against aphid (*L. erysimi*) infestation and relative molecular characterization green-house study. Thus, assessment of genetic diversity among *B. napus* accessions from present study will be helpful in base building for the development of canola genotypes more acceptable to farmers and ultimately share of good quality oil will be increased in local economy.

2. Materials and methods

2.1. Plant material

The research work was evaluated in the glass-house condition, during, 2018 and 2019. The study was designed by using CRD with ten replications. Ten various brassica genotypes represented from brassica species included *B. napus* and *B. juncea*, varieties, viz. Rainbow, KS-75, Dunkled, Shiralee, Abaseen, Hoyla-401, Oscar and Zahoor belong to *B. napus* while Omega and Raya Anmol belong to *B. juncea*, obtained from the Institutes of Biotechnological and Genetics Engineering, The University of Agriculture, Peshawar, Pakistan (IBGE).

The seeds of two canola genotypes were selected from previous finding of two years experiments data like screening of different genotypes of canola in the field, on the basis of minimum and maximum aphids per plant and some laboratory experiments like antixenosis, antibiosis and tolerance (Muhammad and Khan, 2022). Then seed of selected resistant and susceptible two (02) canola genotypes were sown in cup container. The plants were grown in 10 cm height and 16 cm circular growing cup container filled with the textural class of the soil was silty, clay, loam and having alkaline in reaction and calcareous in nature grown culture and 02 seed were cultivated/mutant. Susceptible canola plants (for raising aphid colony), when required, were substituted with a new plant and old plants were carefully destroyed to avoid cross contamination. When enough colonies of aphids started to establish, the *L. erysimi* were carefully introduced to fresh clean plants. Before transferring aphids to new plants, careful observations were made to check and identify the presence of mummified forms. The crops were sown in a growth chamber at 21 ± 1 °C, 50–70 % RH, with 10:14 LD photoperiod rigs.

2.2. Genotyping

The selected genotypes 'Abaseen' is characterized as susceptible and KS-75 as resistant to aphids in the previous experiments (Muhammad and Khan, 2022). Six (06) plants of these genotypes were grown in green-house and used for detecting genetic diversity and identification of alleles associated with resistance gene. Young leaves were taken from plants at four-six leaves stage put in 2 ml an Eppendorf vials, quickly frozen in aqueous nitrogen, and kept in a −20 °C refrigerator till further processing. The frozen tissue was ground to fine powder using a mortar and pestle method. The DNA was isolated utilizing a CTAB procedure (Saghai-Maroof *et al.*, 1984). Polymerase Chain Reaction was utilized to test the crop genotypes.

Table 1
RAPD primers with corresponding bands scored polymorphic bands observed in 02*B. napus* accessions.

Primer name	Sequence	Amplified fragments	Polymorphic fragments	Fragment size (bp)
1OPK-10	GTGCAACGTG	7	7	600–1850
2OPK-12	TGGCCCTCAC	6	5	450–2500
3OPK-15	CTCCTGCCAA	5	5	550–1859
4OPL-05	ACGCAGGCAC	6	3	600–1600
5OPL-11	ACGATGAGCC	8	7	600–2050
6OPL-19	GAGTGGTGG	5	4	575–1650
7OPM-12	GGGACGTGG	5	5	500–1200
8OPM-13	GGTGGTCAAG	4	3	400–3000
9OPM-20	AGGTCTTGGG	6	5	300–1500
10OPN-04	GACCGACCCA	6	5	250–1500
11OPN-06	GAGACGCACA	6	6	300–1450
12OPN-07	CAGCCCAGAG	9	8	450–1700
13OPN-18	GGTGAGGTCA	7	7	500–2400
14OPO-09	TCCCACGCAA	5	5	800–1600
15OPO-12	CAGTGCTGTG	7	6	350–1670
Total		92	81	

The Primers for Random Amplify Polymorphic RAPD marker were used for identify and selected single copy markers in variable canola genotypes for facilitating marker-assisted selection in breeding. Primer sequences used for generating a RAPD marker profile of the canola genotypes are given in Table 1.

The amplification reactions were set up 50 µl solutions contained 0.2 µM of every primers, 0.2 µM of every dNTP, 5X Ampliqon reactions buffers with a 15 µM, MgC-l2, and 1U of Ampliqon, DNA polymerases. For amplifications, heat cycle was 1cycles at 95 °C for three minutes, 35 cycle at 95 °C for thirty seconds, 60 to 55 °C for one minutes and 72 °C for 1.3 min and then 1cycle at 72 °C for five minutes after that by cooled to 4 °C till to running is stop manual. The specially designed primers used for every genes. The PCR materials were separates on one percent agarose-gel, discolored with ethidium-bromide and visually look at UV lights. The ethidium bromide-stained DNA separated through agarose gel was digitally photographed and further analyzed (Lopez et al., 2008).

2.3. Statistical analysis

The data were arranged and statistically analyzed by using analysis of variance (STATISTIX 8.1 package). Alleles were identified by obtaining a profile of the gel picture through ImageJ software and visual observations with 95 % confidence interval (%CI). Association of a marker band with phenotype was considered when $p < 0.05$. The F-value was calculated at the probability level ($p < 0.05$). The significant data were identified by calculating LSD (Steel et al., 2004).

3. Results

3.1. Genetic diversity of RAPD primer OPK-10 in canola genotypes

The amplification pattern of genomic DNA of six plants from each genotype of *Brassica napus* through RAPD primer OPK-10 is presented in Fig. 1A. A PCR profile was obtained for each plant through this primer. The banding profile from the gel was obtained through ImageJ and banding pattern of the susceptible and resistant genotypes were compared (Fig. 1B). The banding pattern revealed amplification of a total of eight (08) bands ranging in size from 600 to 3500 bp, designated as OPK10.1 to OPK10.8. It can be further inferred from the banding pattern that a total of six alleles were detected in the susceptible Abaseen (OPK10.1, 10.2, 10.4, 10.5, 10.6 and 10.8) and 7 alleles were detected in the resistant KS-75 genotype (OPK10.1, 10.2, 10.3, 10.5, 10.7, 10.8). Furthermore, minimum of 4 alleles were amplified in plant 10 (KS-75), whereas maximum 7 alleles were amplified in six plant (1 Abaseen

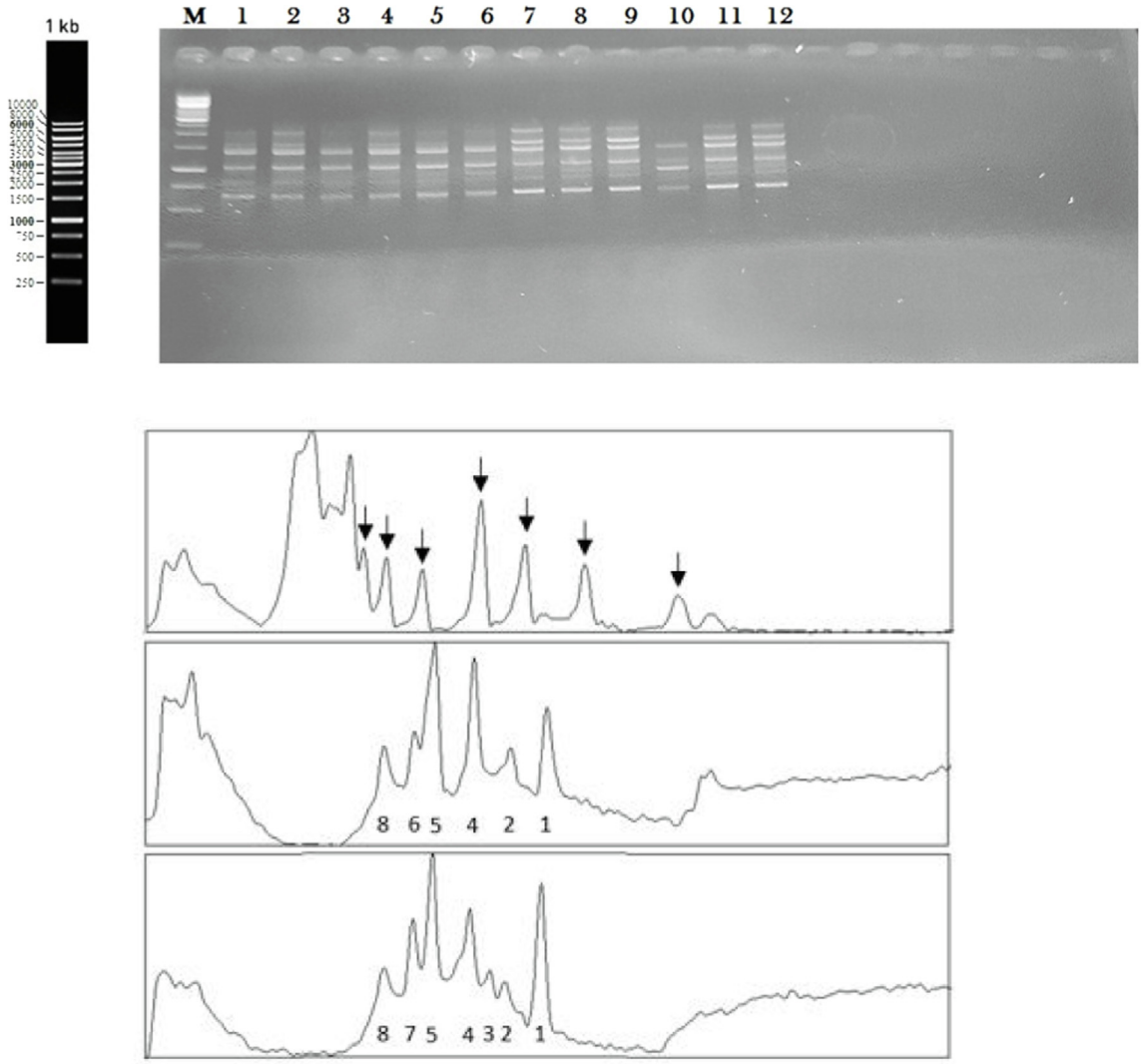

Fig. 1. A,B PCR amplification profile of 02 Canola genotypes (A) and densitogram showing banding pattern obtained through ImageJ Software (B) using RAPD primer OPK-10. M = 1 Kb Molecular weight marker.

and 5 KS-75). Furthermore, allele OPK10.3 (~900 bp) and OPK10.7 (~2400 bp) were amplified only in the resistant KS-75 plants and allele OPK10.4 (~1050 bp) was amplified only in the susceptible Abaseen plants and only these alleles were used for association of bands with resistance. All the other alleles were monomorphic and expressed in all the plants of susceptible or resistant genotypes used for amplification.

Frequencies and association of bands distinctly amplified in either the susceptible or resistant genotype is presented in Table 2. Data indicated that the alleles OPK10.3 and OPK10.7 were expressed at higher frequencies in the plants from resistant KS-75 (f = 0.66 and 0.80, respectively) and allele OPK10.4 was expressed in higher frequency in the susceptible Abaseen genotype (f = 0.50). It can further be deduced from the data that presence of OPK10.3 and OPK10.4 alleles increased the chances of resistance (OR = 4.00 and 25.00, 95 %CI = 0.36–44.11 and 1.20–520.76, respectively) whereas OPK10.4 is associated with susceptibility (OR = 0.10, 95 % CI = 0.01–1.54) (Table 2). However, the association of OPK10.7 with resistance was statistically significant (p = 0.038) and the association of OPK10.3 and OPK10.4 was not significant statistically (p > 0.05).

3.2. Genetic diversity of RAPD primer OPL-05 in susceptible and resistant brassica genotypes

The amplification pattern of genomic DNA of six plants from each genotype of Brassica through RAPD primer OPL-05 is presented in Fig. 2A. A PCR profile was obtained for each plant through this primer. The banding profile from the gel obtained through ImageJ and banding pattern of the susceptible and resistant genotype were compared (Fig. 2B) The banding pattern revealed amplification of a total of seven (07) bands ranging in size from 830 to 2800 bp. The banding pattern hinted towards the presence of six alleles in the susceptible Abaseen and whereas seven alleles were identified in the resistant KS-75 genotype. Moreover, at least six alleles were amplified from the studied two genotypes six each from Abaseen and KS-75. In the 5 plants of all KS-75 genotype, a maximum of 7 alleles were amplified, with allele 1 (~830 bp) only being present in the resistant KS-75 plant. This allele was the only one used to associate bands with resistance, while all the other alleles were monomorphic and expressed in all the plants used for amplification.

The Table 3 presents the frequencies and associations of bands that are amplified differently in either the susceptible or resistant genotype. The data suggests that the allele OPL05.1 was more commonly found in the resistant KS-75 plant (f = 0.83). Additionally, the data indicates that the presence of OPL05.1 significantly increased the chances of resistance in brassica (p = 0.024, OR = 21.00, 95 %CI = 1.50–293.27).

3.3. Genetic diversity of RAPD primer OPN-04 in susceptible and resistant brassica genotypes

The banding pattern of the susceptible and resistant genotypes of Brassica, obtained through Image J after PCR amplification of genomic DNA from six plants of each genotype using RAPD primer OPN-04, is presented in Fig. 3B for comparison. The banding pattern showed that seven bands of sizes ranging from 700 to 1400 bp were amplified. This pattern indicated that six alleles were detected in the susceptible Abaseen plants and four alleles were detected in the resistant KS-75 genotype. At least three alleles were amplified in three plants (KS-75), while the maximum of six alleles were amplified in five plants (Abaseen). The susceptible Abaseen plants showed higher frequencies of alleles 3 (~1000 bp) and six (~1400 bp), and these were the only alleles used to associate bands with resistance. All the other alleles were monomorphic and present in all the plants used for amplification.

Table 4 shows the frequencies and associations of bands that were amplified to a greater degree in either the susceptible or resistant genotype. The alleles OPN04.3 and OPNO4.6 were found to be more prevalent in the susceptible Abaseen plants (f = 0.83 both) than in KS-75 (f = 0.17 and f = 5.0, respectively). The data suggests that OPN04.3 had a significant effect on reducing the resistance of brassica (OR = 0.40, 95 %CI = 0.002–0.833, p = 0.038). Additionally, OPN04.6 was associated with a decrease in resistance, although this was not statistically significant (OR = 0.200, 95 %CI = 0.014–2.911, p > 0.05).

3.4. Genetic diversity of RAPD primer OPM-12 in susceptible and resistant brassica genotypes

The amplification of genomic DNA from six plants of each genotype of Brassica using RAPD primer OPM-12 is presented in Fig. 4A. PCR profiles were obtained for each plant and the banding pattern from the gel was analyzed using ImageJ. The banding pattern of the susceptible and resistant genotypes were compared (Fig. 4B), revealing a total of eleven (11) bands ranging in size from 600 to 2000 bp. The banding pattern suggests that 10 alleles were present in both the susceptible Abaseen and resistant KS-75 genotypes, with a minimum of 10 alleles amplified in 3 plants of each genotype and a maximum of 11 alleles amplified in 3 plants of each genotype (i.e. Abaseen and KS-75). There was no difference in the alleles between the susceptible and resistant genotypes, so they cannot be linked to resistance.

3.5. Genetic diversity of RAPD primer OPO-12 in susceptible and resistant canola genotypes

Amplification pattern of genomic DNA of six plants from each genotype of Brassica through RAPD primer OPO-12 is presented in Fig. 5A. The PCR profile of each plant was obtained using the primer, and the banding pattern of the susceptible and resistant genotypes were compared by analyzing the gel with Image J (Fig. 5B). The banding pattern revealed amplification of a total of eleven (11) bands ranging in size from 600 to 2000 bp. It can be further inferred from the banding pattern that a total of 10 alleles were monomorphic and detected in the susceptible Abaseen and resistant KS-75 genotype. For both Abaseen and KS-75, a minimum of

Table 2
Allele frequencies and association analysis of bands distinctively expressed in the susceptible (Abaseen) and resistant (KS-75) brassica genotypes.

Allele	Allele Frequency		Odd Ratio	95 % CI	*p*-value
	Abaseen	***KS-75***			
OPK10.3	0.33	0.66	4.00	0.36–44.11	0.258 ^{ns}
OPK10.4	0.50	0.17	0.10	0.01–1.54	0.099 ^{ns}
OPK10.7	0.17	0.83	25.00	1.20–520.76	0.038*

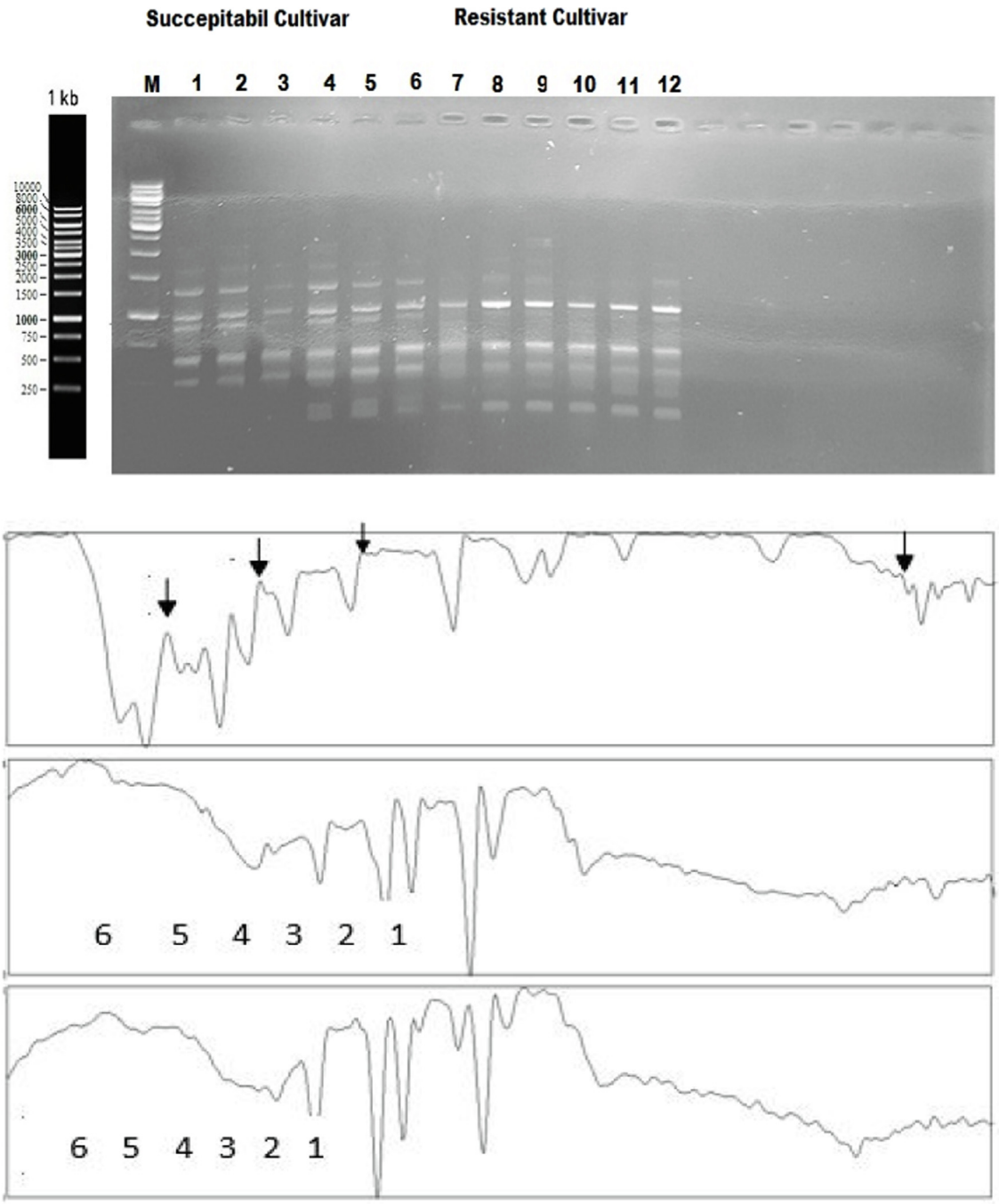

Fig. 2. A,B PCR amplification profile of 02 Canola genotypes (A) and densitogram showing banding pattern obtained through ImageJ Software (B) using RAPD primer OPL-05. M = 1 Kb Molecular weight marker.

Table 3
Allele frequencies and association analysis of bands of OPL-05 distinctively expressed in the susceptible (Abaseen) and resistant (KS-75) brassica genotypes.

Allele	Allele Frequency		Odd Ratio	95 % CI	*p*-value
	Abaseen	***KS-75***			
OPL05.3	0.00	0.83	21.00	1.50–293.27	0.024*

10 alleles were amplified in 3 plants, and a maximum of 11 alleles were also amplified in 3 plants. All the alleles were monomorphic or amplified in the same amount in both the susceptible and resistant genotypes, so they cannot be linked to resistance.

4. Discussion

The use of DNA markers is widespread for the identification of DNA polymorphism (Gupta and Varshney, 2000). Different molec-

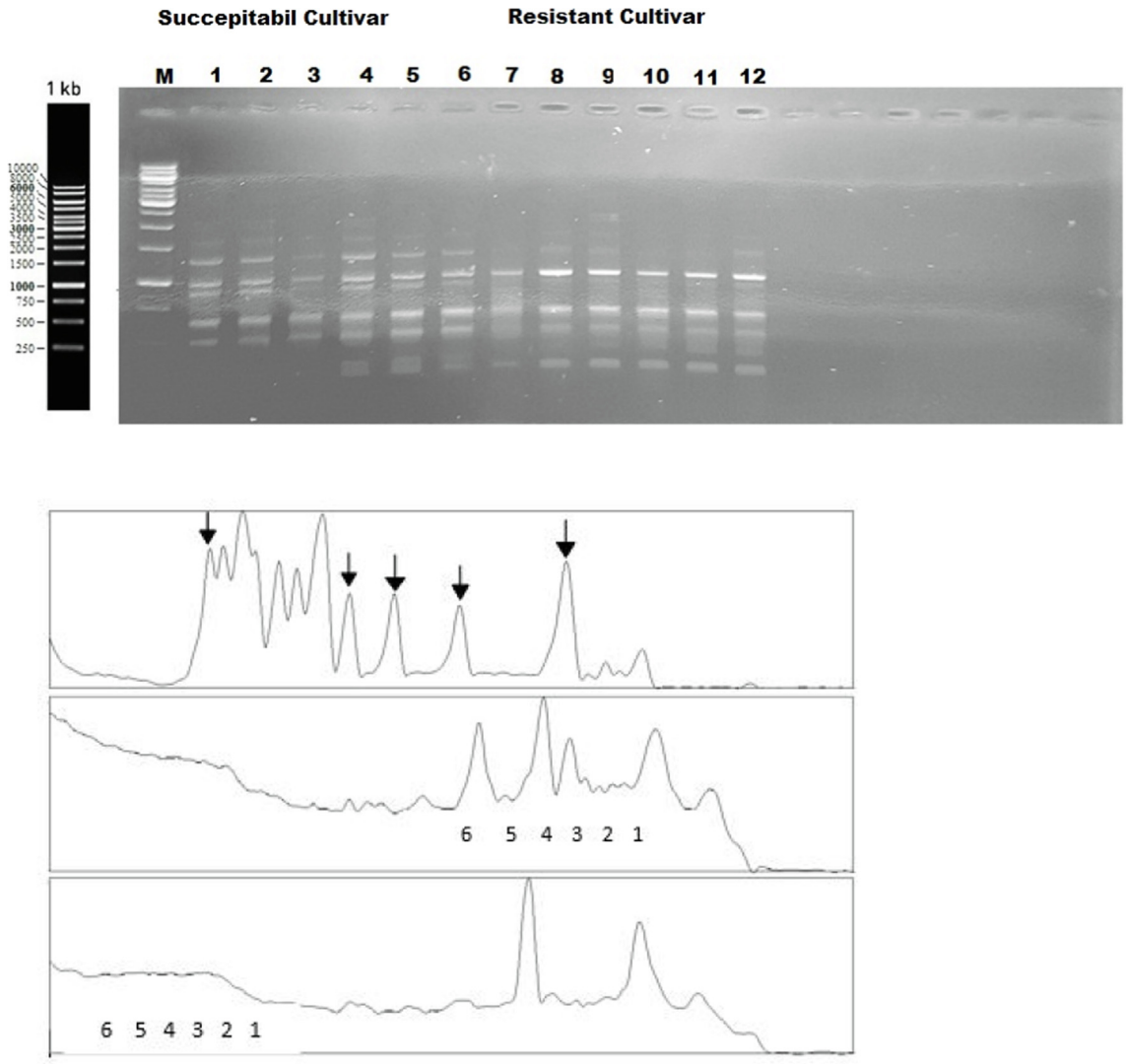

Fig. 3. A,B PCR amplification profile of 02 Canola genotypes (A) and densitogram showing banding pattern obtained through ImageJ Software (B) using RAPD primer OPN-04. M = 1 Kb Molecular weight marker.

Table 4
Allele frequencies and association analysis of bands of OPL-05 distinctively expressed in the susceptible (Abaseen) and resistant (KS-75) brassica genotypes.

Allele	Allele Frequency		Odd Ratio	95 % CI	*p*-value
	Abaseen	***KS-75***			
OPN04.3	0.83	0.17	0.040	0.002–0.833	0.038*
OPN04-6	0.83	0.50	0.200	0.014–2.911	0.239 ^{ns}

ular markers have been employed in experiments to ascertain the genetic relationship between various plants (AlQurainy, 2007; Lopez et al., 2008). Agarwal and Shrivastava (2008) and El-Mouei et al. (2011) have reported that randomization of DNA (RAPD), fragmentation of lengthened polymorphisms (AFLPs), single nucleotide polymorphisms (SNP), cleaved amplified polymorphisms sequences (CAP) and simple sequences repeated (SSR) are utilized to measure variation and diversity at molecular levels. Marker assisted selection is a useful tool for breeders to cost-effectively identify desirable genotypes at an earlier stage of the breeding process. For it to be effective, there must be a connection between the markers and the genes that control a particular trait. The RAPD markers are a widely used tool for genetic characterization of living organisms with limited knowledge of their DNA sequence, and are also an important tool for genetic mapping. Additionally, RAPD markers linked to resistance R-genes have been identified in various plant species (Liu *et al*., 2005). In Abbas et al., 2009, utilized RAPD markers to genetically characterize Brassica genotypes. Qun et al. (2009) observed amplifications of DNA fragments ranging from 300 to 1800 base pairs in *B. napus*, which were used to differentiate between different plant species. Shengwu et al. (2003) found that there was substantial genetic variation between Chinese and European accessions of rapeseed due to the difference in gene pools.

Well-documented genetic diversity in *B. napus* and *B. oleracea* is limited (Astarini et al., 2004). Genotypes of rapeseed and vegetables from the Brassicaceae family are selected for economic, health and quality traits. Host factors are a key part of the quantitative

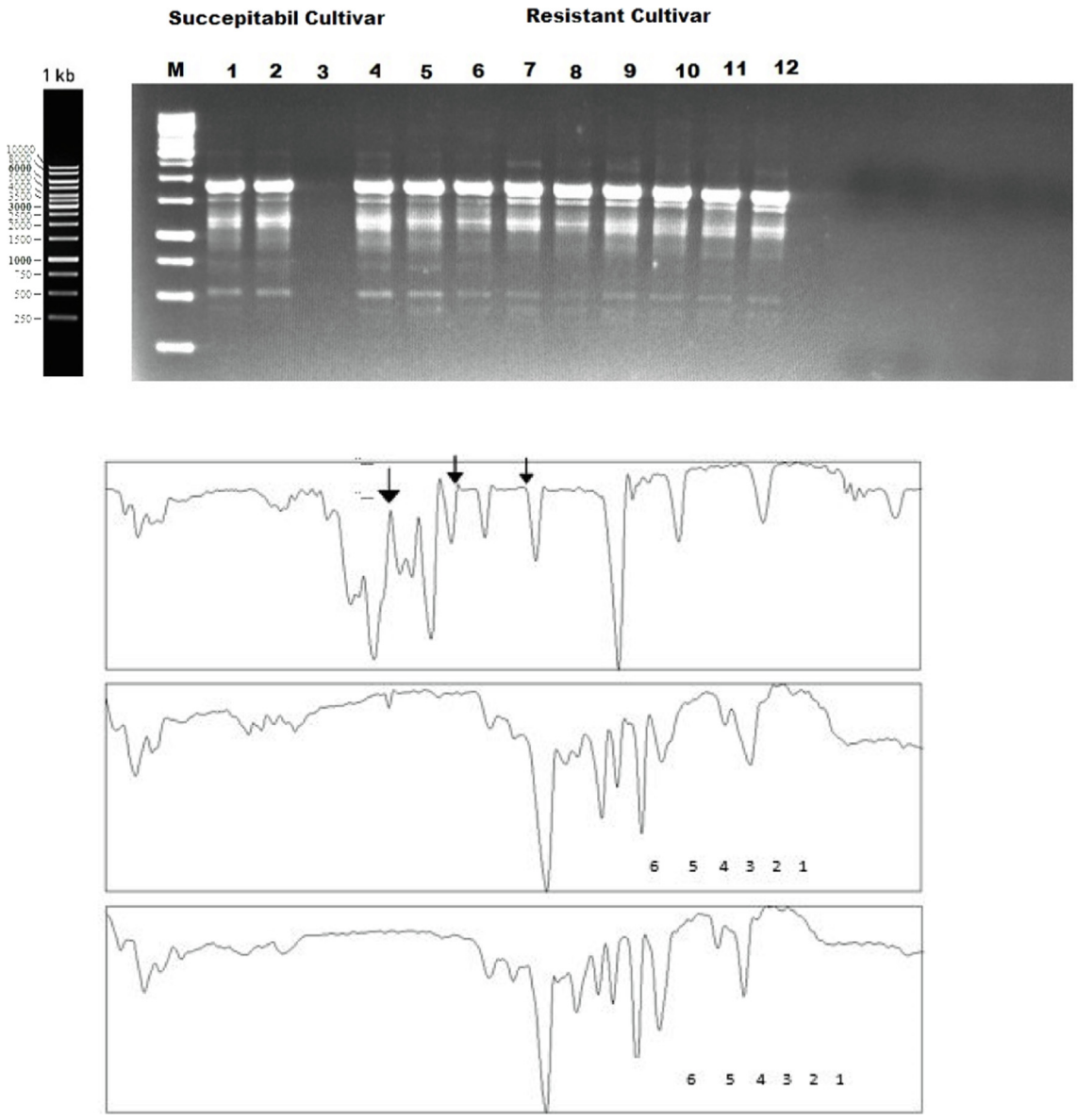

Fig. 4. A,B PCR amplification profile of 02 Canola genotypes (A) and densitogram showing banding pattern obtained through ImageJ Software (B) using RAPD primer OPM-12. M = 1 Kb Molecular weight marker.

resistance mechanisms of aphids in brassica. In this study, RAPD markers were used to identify and develop a marker for selection of aphid resistant plants in MAS program. This was done by amplifying five RAPD markers that developed consistent banding patterns in two canola genotypes, one resistant (KS-75) and one susceptible (Abaseen) to aphid attack. The identification of markers associated with disease resistance genes will help in the development of resistant genotypes. Despite the fact that a deep relationship between rapeseed genotypes from Pakistan and Spain had been documented by Tekelwold and Becker (2006) using RAPD markers, the limited number of plants used and the low reliability of the markers necessitates the identification of DNA sequences and the development of Sequence Characterized Amplified Region (SCAR) markers for application in breeding brassica for aphid resistance. Research has demonstrated the potential of using RAPD fragments to identify genotypes that are resistant to insect pests and diseases in Brassica. Several studies have already employed RAPD markers to address various pathological issues in Brassica (Amer et al., 2009).

Research has revealed that glucosinolates have a minimum level of variability in many plant species, including *Brassica napus* and *Brassica juncea* (Rabbani *et al*., 1998). Additionally, glucosinolates have been found to be present in varying amounts and to be effective in deterring insect pests in *B. juncea* and *B. napus* germplasm. Recent studies have revealed that genetic material contains low to moderate levels of glucosinolates, and this genetic base is further weakened due to the preferential selection of brassica for yield, yield components, and seed quality traits. KS-75 has been identified as aphid-resistant in these and other studies. The findings of Thiruvengadam et al. (2015) and Shah et al. (2015) both support the notion that aphids are vulnerable to yellow color and that L. erysimi is more attracted to yellow than other tested colors (red, white and green). Our results are in agreement with these studies. Our results are consistent with Kumar et al. (2011), which found that *L. erysimi* preferred the excised leaves of Brassica species. This is further supported by our second and third antixenosis experiments, which showed that L. erysimi preferred the excised leaves of canola and that the susceptible genotype Abaseen

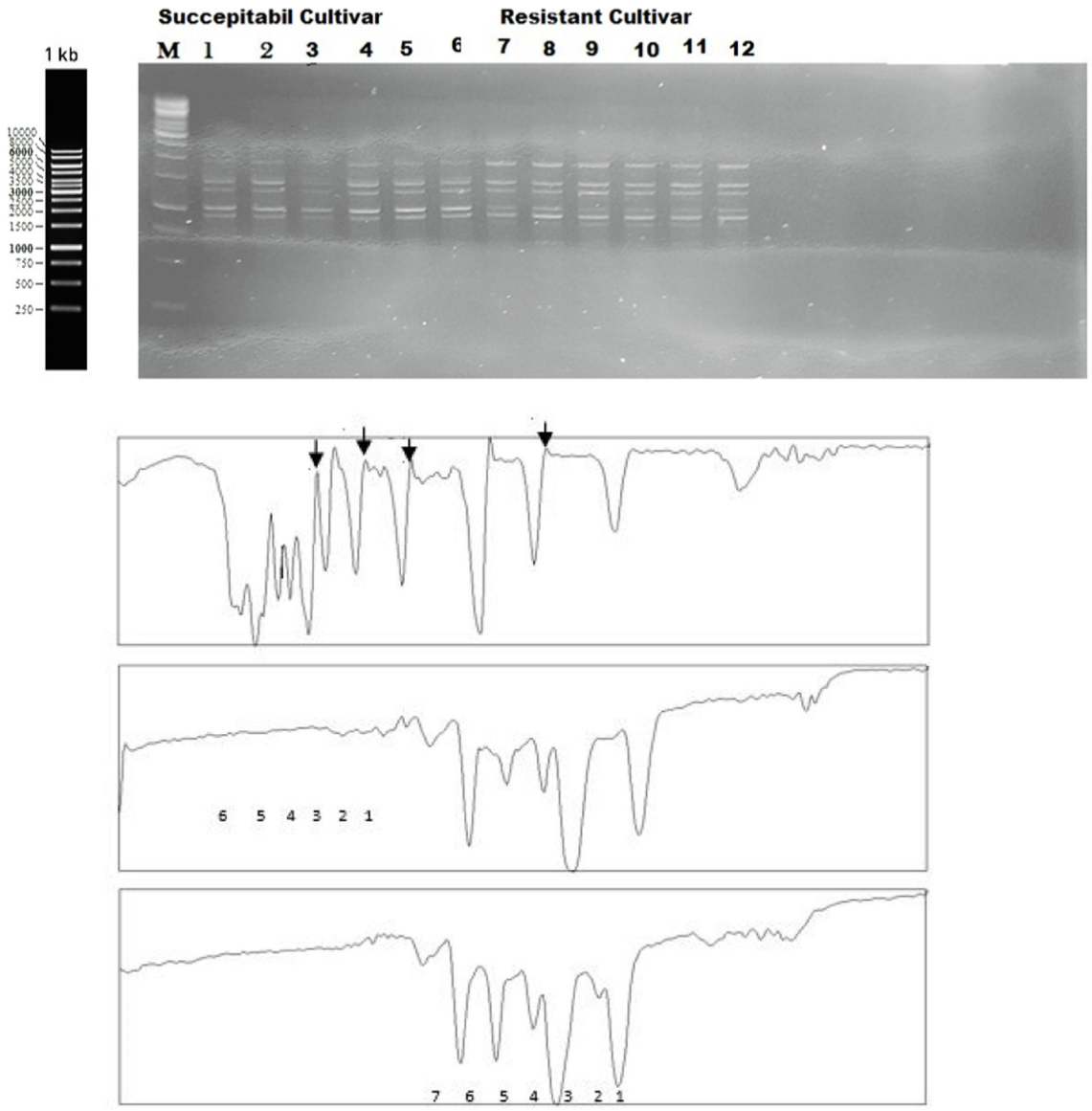

Fig. 5. A,B PCR amplification profile of 02 Canola genotypes (A) and densitogram showing banding pattern obtained through ImageJ Software (B) using RAPD primer OPO-12. M = 1 Kb Molecular weight marker.

attracted the most aphids compared to KS-75. Genetic recombination for improvement of glucosinolates in *B. napus*, a species susceptible to aphids attack, is essential for sustainable and integrated management, which is supported by the observation of genotype KS-75 with dark green color and sparse trichomes (Muhammad and Khan, 2022; Kishor et al., 2019). Varshney *et al.* (2004) utilized data from various molecular markers to create co-dominant and more dependable CAPS or SCAR markers. Additionally, many of the molecular markers associated with aphid resistance in brassica have been documented in other species.

5. Conclusion and recommendtions

5.1. Conclusion

It is determined that knowledge of the genetic diversity in canola *B. napus* varieties will be beneficial for making the most of the existing genetic resources to create canola genotypes. By introducing new genetic material from international sources, the genetic diversity of *B. napus* breeding genotypes could be increased. RAPD, a simpler and faster method, could be used to compare the genetic relatedness and the differences between resistant and susceptible accessions.

5.2. Recommendations

This study found a strong and meaningful relationship between 03 RAPD markers and aphid resistance in brassica. Two of the markers were associated with increased aphid resistance, while the other was associated with decreased aphid resistance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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