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# Allelic Diversity in Apple Germplasm for Fruit Quality and Scab Resistance Using SSR Markers

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Authors AR, KMB and HUR designed the study. Authors AJ and ZAD performed the statistical analysis. Authors HUR and MAM wrote the protocol and the first draft of the manuscript. Authors HUR and AP performed the final draft. Authors KMB, AR and HUR managed the analyses of the study. Authors AR, HUR and AJ managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

The present investigation entitled "Allelic diversity in apple germplasm for fruit quality and scab resistance using SSR markers" was conducted in division of plant biotechnology, SKUAST-K, Shalimar during the year 2018. In the present study, four SSR markers namely Md-ACS-1, Md-ACO-1, ALO-7 and AM-19 were used for screening of 40 apple cultivars presently under cultivation in SKUAST-K for quality and scab resistance. The results revealed that out of 40 cultivars evaluated, 3 were found homozygous (ACS-1-2/2), 17 were heterozygous (ACS-1-1/2) and 10 were homozygous (ACS-1-1/1), while as no amplification of ACS-1 was found for 10 cultivars. Similarly, for Md-ACO-1, 3 cultivars were found homozygous, 35 as heterozygous and 2 did not show any amplification. As far as presence of $V_f$ gene conferring resistance against scab disease, ALO-7 amplified at 820bp and 570bp alleles, 570 bp fragment was observed in resistant genotypes and was absent in susceptible genotypes, while the 820 amplicon was observed in both resistant and susceptible genotypes. The markers AM19 led to the amplification of 520 bp fragment in few cultivars.

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*Keywords: SSR; fruit quality; scab resistance; apple cultivars; homozygous; heterozygous.*

## 1. INTRODUCTION

As a complement to morphological characterization, molecular markers have been developed and are currently being used for germplasm genotyping alone or as a complement to morphological characterization [1]. In case of apples, a high number of molecular markers have been described [2,3]. The allelic diversity of various genes involved in ethylene bio-synthesis and scab disease resistance have been reported by several workers [4].

Being a climacteric fruit, loss of firmness in apple seems to be physiologically related to ethylene. Ethylene biosynthetic pathway is controlled by two large gene families coding for 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO) [5]. Retention of desirable firmness after prolonged storage is one of the key requirements for apple cultivars to provide year-round high-quality apples to consumers [6]. Two allelic forms of Md-ACS1 were Md-ACS1-1 and Md-ACS1-2 [4]. The three allelic combinations, ACS1-1/1, 1-1/2 and 1-2/2, generally confer high, medium and low ethylene production, respectively [7,8]. Similarly, Md-ACO1 is primarily expressed in fruit tissues among the Md-ACO gene family members [9]. MdACO1 has been demonstrated to have a relatively minor, but clear and independent role in ethylene biosynthesis In general, there is a close relationship among ethylene biosynthesis in genotype, ethylene production and fruit storability or shelf-life [7,8]. The markers developed for ACS1 and ACO1 belong to the emerging category of molecular markers and termed as “functional” or “perfect” markers, which are derived from sequence variation of functionally analyzed genes. As their allelic effects are known and they are developed “within or very close to” genes of interest, these markers are ideal for selection of desired genotypes.

Apple scab also known as black spot, caused by *Venturia inaequalis* (Cke.) Wint. is one of the most serious diseases of apple reported from almost all apple producing countries and causes huge economic losses (up to 70% reduction). The availability of the apple genotypes showing resistance to apple scab is highly relevant for the fruit industry through their use in integrated production and facilitating the production of organic fruit. Additionally, these cultivars could be incorporated into appropriate breeding programs, commencing with investigations on the genetic basis of their resistance. For the effective resistance breeding process, it is necessary to identify and evaluate valuable sources of resistance within genetic resources [10]. Kashmir valley is known to host several hundred cultivars of apple, which constitute the bulk of diversity of apple germplasm in India and this germplasm is a potential source of important agronomic traits including scab resistance [11]. The present investigation is a part of assessment of the apple germplasm present at Horticulture farm, Division of Fruit Science, SKUAST, Shalimar to generate passport data information, to identify elite cultivars for disease resistance and other fruit quality.

## 2. MATERIALS AND METHODS

In this study DNA was extracted from fresh and young leaves of 40 apple cultivars using CTAB method [12]. After melting of DNA by thawing, 200 µl of RNase A (10 µl/ml) was added to each sample mixed properly and incubated at 37ºC for 1 hour in water bath. The samples were gently mixed by inverting for 10 minutes and centrifuged at 13,000 rpm for 10 minutes after mixing phenol:chloroform (1:1). Supernatant was collected and equal volumes of chloroform and isoamyl alcohol (24:1) was added and centrifuged at 13,000 rpm for 10 minutes. Supernatant was collected in fresh 2.0 ml centrifuge tubes. 0.1 volume of 3M sodium acetate (pH 8.0) and double volume of chilled ethanol was added. The tubes were centrifuged at 7,000 rpm for 5 minutes to get the DNA pellet. Supernatant was discarded and pellet was washed with 1ml of 70% ethanol for 10 minutes and centrifuged at 1,000 rpm for 5 minutes. Ethanol was discarded and pellet was dried at room temperature for 1 hour. The pellet containing DNA was dissolved in 50 µl of TE buffer and stored at 4ºC.

The concentration and purity of DNA was checked by agarose gel electrophoresis. DNA samples were photographed using photo gel documentation system. Quality of DNA samples was judged based on whether DNA formed a single high molecular weight band (good quality) or a smear (degraded or poor quality).

A mixture of 20 µl of various PCR reagents, based on the stock and final concentration of different components was prepared as outlined in Tables 1 and 2.

Genotyping of the apple varieties was carried out using gene specific markers which are given in Table 3.

## 3. RESULTS AND DISCUSSION

### 3.1 Marker Analysis for Fruit Firmness

The markers Md-ACS1 and Md-ACO1 (see Table 3) were used for screening of 40 apple genotypes. Two allelic forms of both Md-ACS1 and Md-ACO1 were typically amplified as Md-ACS1-1, Md-ACS1-2 and Md-ACO1-1, Md-ACO1-2. Out of the 40 genotypes evaluated, 3 were found homozygous (ACS1-2/2) *viz.,* Gala Mast, Gala Redlum and Fuji Zhen Aztech, 17 were heterozygous (ACS1-1/2) *viz.,* Super Chief, Braeburn, Shalimar Apple-1, Red Velox, Granny Smith, Golden Delicious Reinders, Mollies Delicious, ASP-69, Firdous, Starkrimson, Ambri, American Apirouge, ASP-12, ASP-10, Oregon Spur, Irish Peach and Wiltons Star and 10 were homozygous (ACS1-1/1) *viz.,* ASP-1, Lal Ambri, Sunhari, Shireen, ASP-3, Akbar, Cox's Orange Pippin, Maharaji, Benoni and Red Gold.. Amplification was not observed in the remaining genotypes (Fig. 1).

For Md-ACO1, 3 genotypes were found homozygous Md-ACO1-1/1 *viz.,* Shalimar Apple-1, Red Velox, Oregon Spur, 35 were heterozygous Md-ACO1-1/2 *viz.,* Super Chief, Braeburn, ASP-1, Lal Ambri, Granny Smith, Golden Delicious Reinders, Sunhari, Gala Redlum, Mollies Delicious, ASP-69, Gala Mast, Firdous, Starkrimson, Fuji Zhen Aztec, Ambri, American Apirouge, Shireen, ASP-10, ASP-4, ASP-3, Akbar, Shalimar-Apple 2, Silver Spur, Golden Delicious, Red delicious, Maharaji, Cox's Orange Pippin, June eating, Lord Lambourne, Red Gold, Benoni, Yellow Newton, Scarlet Siberean, Irish Peach and Wiltons Star and none was homozygous Md-ACO1-2/2 (Table 4 and Fig. 1).

**Table 1. Stock and final concentration of different components used in PCR**

| S. no. | Component | Stock concentration | Final concentration | Volume (µl) |
|---|---|---|---|---|
| 1. | Water | - | | 10.2 |
| 2. | PCR buffer | 10X* | 1X | 2.0 |
| 3. | MgCl2 | 25 mM | 1.5 mM | 1.2 |
| 4. | dNTPs | 100 mM | 2 mM | 0.4 |
| 5. | Primer Forward | 100 µM | 10 µM | 2.0 |
| 6. | Primer Reverse | 100 µM | 10 µM | 2.0 |
| 7. | *Taq* Polymerase | 5U/5 µl | 1 Unit | 0.2 |
| 8 | DNA template | 50 ng/µl | 100 ng/µl | 2.0 |
| Total | | | | 20.0 |

**10X PCR buffer: 200 mM Tris-HCl (pH 8.3), 500 mM KCl*

**Table 2. Temperature profile in PCR**

| S. no. | Step | Temperature (°C) | Time (minutes) | No. of cycles |
|---|---|---|---|---|
| 1. | Initial Denaturation | 94.0 | 5 | 1 |
| 2. | Denaturation | 94.0 | 1 | |
| 3. | Annealing | 58-63 | 1 | |
| 4. | Elongation (Extension) | 72.0 | 2 | 35 |
| 5. | Final Extension | 72.0 | 7 | |
| 6. | Hold | 4.0 | | |

**Table 3. Markers for detection of scab and fruit quality traits**

| S. no. | Trait | Markers | References |
|---|---|---|---|
| 1 | Fruit storability | Md-ACS1, Md-ACO1 | Zhu and Barritt [12] |
| 2 | Scab | AL-07, AM-19 | Tartarini et al. [13] |

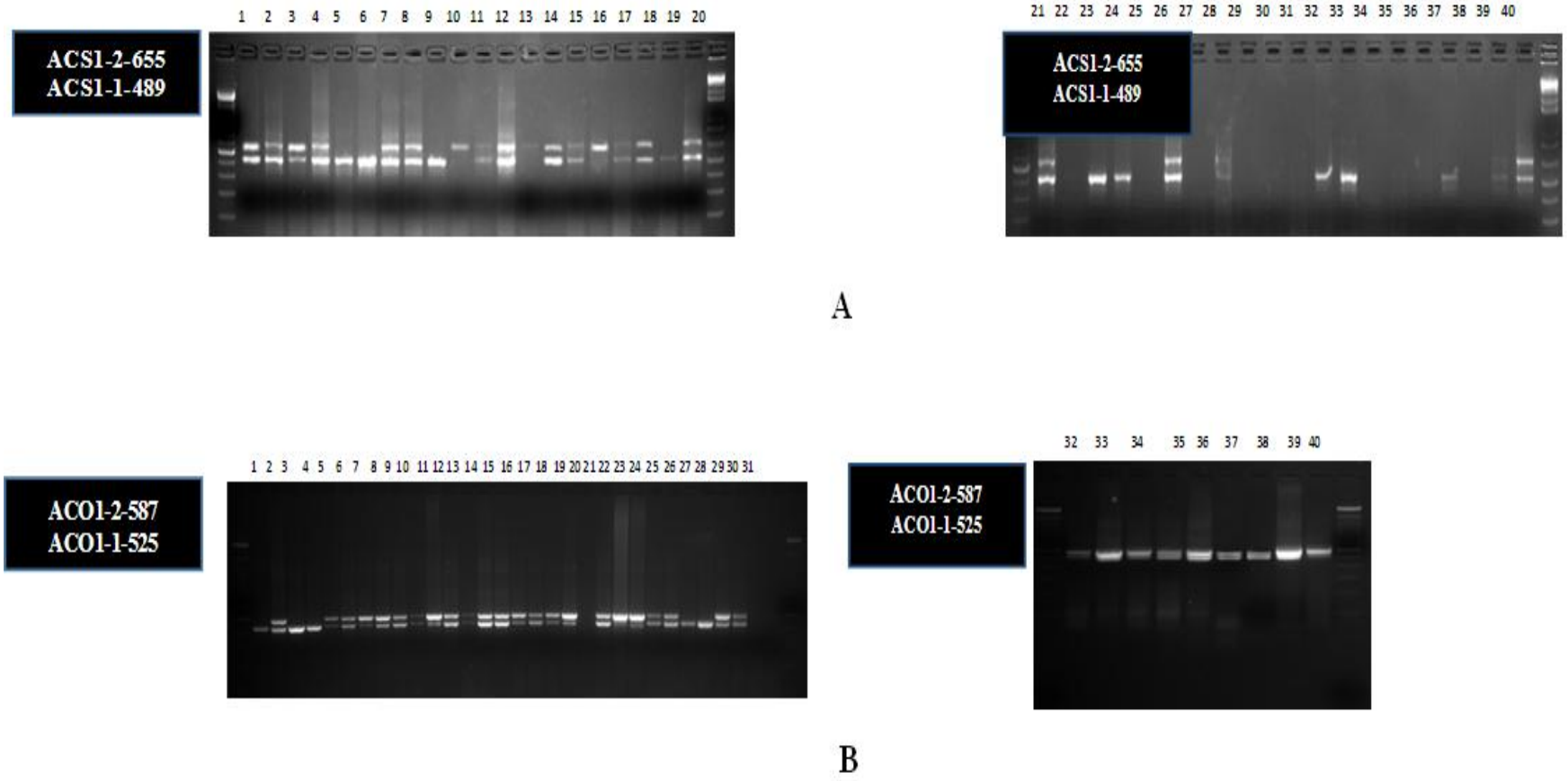


**Fig. 1. Gel electrophoresis profile of markers md-acs1 (a) and md-aco1 (b)**

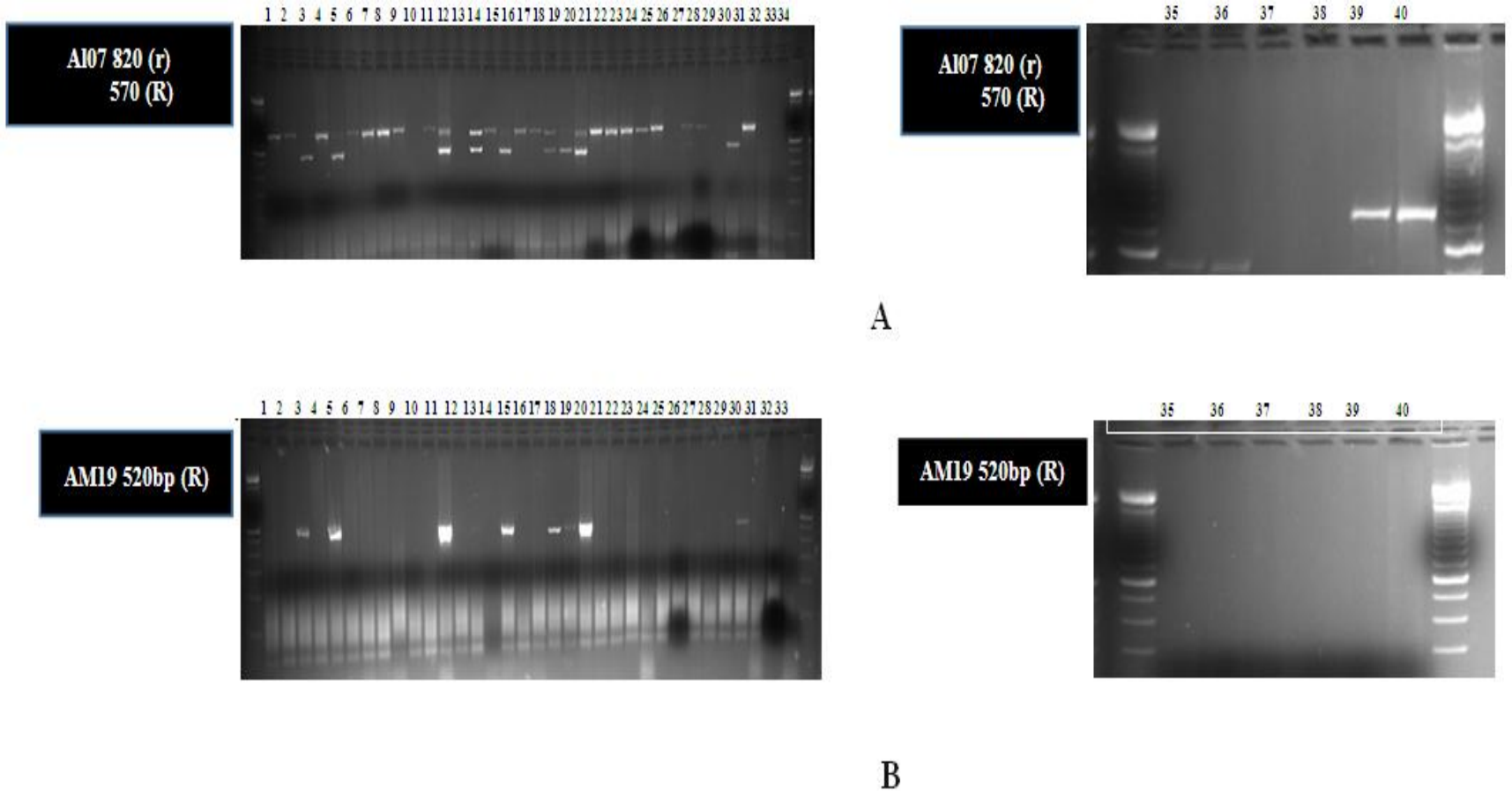


**Fig. 2. Gel electrophoresis profile for markers al07 (a) and am19 (b)**

**Table 4. Marker based genotyping of apple germplasm for Scab resistance and fruit quality traits**

| S. no. | Genotypes | ALO7 | AM19 | S. no. | Genotypes | ACO1 | ACS1 |
|---|---|---|---|---|---|---|---|
| 1. | Super chief | rr | - | 1. | Super chief | 1/2 | 1/2 |
| 2. | Braeburn | rr | - | 2. | Braeburn | 1/2 | 1/2 |
| 3. | Shalimar Apple-1 | Rr | R | 3. | Shalimar Apple-1 | 1/1 | 1/2 |
| 4. | Red Velox | rr | - | 4. | Red Velox | 1/1 | 1/2 |
| 5. | ASP-1 | Rr | R | 5. | ASP-1 | 1/2 | 1/1 |
| 6. | Lal Ambri | rr | - | 6. | Lal Ambri | 1/2 | 1/1 |
| 7. | Granny Smith | rr | - | 7. | Granny Smith | 1/2 | 1/2 |
| 8. | Golden Delicious Reinders | rr | - | 8. | Golden Delicious Reinders | 1/2 | 1/2 |
| 9. | Sunhari | rr | - | 9. | Sunhari | 1/2 | 1/1 |
| 10. | Gala Redlum | - | - | 10. | Gala Redlum | 1/2 | 2/2 |
| 11. | Mollies delicious | rr | - | 11. | Mollies delicious | 1/2 | 1/2 |
| 12. | ASP-69 | Rr | R | 12. | ASP-69 | 1/2 | 1/2 |
| 13. | Gala Mast | - | - | 13. | Gala Mast | 1/2 | 2/2 |
| 14. | Firdous | Rr | R | 14. | Firdous | 1/2 | 1/2 |
| 15. | Starkrimson | rr | - | 15. | Starkrimson | 1/2 | 1/2 |
| 16. | ASP-3 | Rr | R | 16. | Fuji Zhen Aztec | 1/2 | 2/2 |
| 17. | Ambri | rr | - | 17. | Ambri | 1/2 | 1/2 |
| 18. | American Apirouge | rr | - | 18. | American Apirouge | 1/2 | 1/2 |
| 19. | Shireen | Rr | R | 19. | Shireen | 1/2 | 1/1 |
| 20. | ASP-12 | Rr | R | 20. | ASP-12 | - | 1/2 |
| 21. | ASP-10 | Rr | R | 21. | ASP-10 | 1/2 | 1/2 |
| 22. | ASP-4 | rr | - | 22. | ASP-4 | 1/2 | - |
| 23 | Fuji Zhen Aztec | rr | - | 23 | ASP-3 | 1/2 | 1/1 |
| 24. | Akbar | rr | - | 24. | Akbar | 1/2 | 1/1 |
| 25. | Shalimar Apple-2 | rr | - | 25. | Shalimar Apple-2 | 1/2 | - |
| 26. | Silver Spur | rr | - | 26. | Silver Spur | 1/2 | - |
| 27. | Oregon Spur | - | - | 27. | Oregon Spur | 1/1 | 1/2 |
| 28. | Golden Delicious | Rr | - | 28. | Golden Delicious | 1/2 | - |
| 29. | Red Delicious | rr | - | 29. | Red Delicious | 1/2 | - |
| 30. | Chamure | - | - | 30. | Chamure | - | - |
| 31. | Maharaji | Rr | R | 31. | Maharaji | 1/2 | 1/1 |
| 32. | Cox's Orange Pippin | rr | - | 32. | Cox's Orange Pippin | 1/2 | 1/1 |
| 33. | June Eating | Rr | - | 33. | June Eating | 1/2 | - |
| 34. | Lord Lambourne | rr | - | 34. | Lord Lambourne | 1/2 | - |
| 35. | Red Gold | RR | - | 35. | Red Gold | 1/2 | 1/1 |
| 36. | Benoni | RR | - | 36. | Benoni | 1/2 | 1/1 |
| 37. | Yellow Newton | - | - | 37. | Yellow Newton | 1/2 | - |
| 38. | Scarlet Siberean | - | - | 38. | Scarlet Siberean | 1/2 | - |
| 39. | Irish Peach | rr | - | 39. | Irish Peach | 1/2 | 1/2 |
| 40. | Wiltons Star | rr | - | 40. | Wiltons Star | 1/2 | 1/2 |

*rr : Homozygousrecessive Rr : heterozygous R : Dominant*
*1/1 & 2/2 : Homozygous 1/2 : Heterozygous*

### 3.2 Molecular Screening of Apple Genotypes for Scab Resistance Using *Vf* Gene Specific Markers

Forty genotypes of apple were screened for the presence of *Vf* gene conferring resistance against apple scab disease. To confirm the presence of *Vf* gene, two gene specific markers were used. The marker AL07 (*Vf*) (see Table 3) amplified at 820 bp and 570 bp alleles, the 570 bp fragment was observed in resistant genotypes Shalimar Apple-1, ASP-1, ASP-69, Firdous, ASP-3, Shireen, ASP-12, ASP-10, Golden Delicious, Maharaji, June Eating, Red Gold, Benoni and was absent in susceptible genotypes, while the 820 amplicon was observed in both resistant and susceptible genotypes. The markers AM19 led to the amplification of 520 bp fragment in genotypes Shalimar Apple-1, ASP-1, ASP-69, Firdous, ASP-3 Shireen, ASP-12, ASP-10, Maharaji (Table 4 and Fig. 2).

Molecular screening was performed in all forty genotypes for the presence of *Vf* gene conferring resistance against apple scab disease using *Vf* gene specific primers, AL07 and AM19. Primer for

the marker AL07 amplified two fragments 820 bp and 570 bp, the former 820 bp band represents the recessive *vf* allele and the latter the dominant allele *Vf.* Hence, this marker can be used for the identification of homozygous and heterozygous genotypes. Interestingly, the 820 amplicon was observed in both resistant and susceptible genotypes, while the fragment of 570 bp amplicon was amplified only in resistant genotypes. Primers for the marker AM19 being dominant in nature, led to the amplification of one band in resistant genotypes Shalimar Apple-1, ASP-1, ASP-69, Firdous, ASP-3 Shireen, ASP-12, ASP-10 and Maharaji. The molecular marker AM19 proved to be highly useful because of its ability to distinguish resistant and susceptible genotypes on the basis of presence or absence of single band on gel. Using this marker only one band of 520 bp was amplified in nine resistant cultivars. Therefore this marker seems to be highly specific in detecting presence of *Vf* (Rvi6) gene in apple genotypes However, due to the dominant nature of this marker, it cannot distinguish homozygous and heterozygous genotypes containing *Vf* (Rvi6) gene. This observation is in agreement with the findings of Patrascu et al. [14]. Similar results were reported by Khajuria et al. [11] by screening apple germplasm of North-western Himalayas.

Ethylene regulates several physiological processes related to fruit ripening including changes in skin colour, flesh texture and synthesis of aromatic flavor compounds [15]. Ethylene biosynthesis during apple fruit ripening is generally regarded as a primary factor leading to softening. Apple is a climacteric fruit and ripening is characterized by an ethylene burst accompanied by an increase in respiration. The role of ethylene in apple ripening has been studied using ethylene action inhibitors and transgenic approaches. Fruit ethylene production genotypes for Md-ACS1 and Md-ACO1 were determined for 40 apple genotypes. ACS1 had a much greater influence on fruit firmness than ACO1. The association between ACS1 and ACO1 allelotypes and observed firmness phenotypes at harvest supports the practical utilization of both ACS1 and ACO1 functional markers for selecting the progeny at the seedling stage with low ethylene production, firm fruit and long storage potential. Two alleles for each gene are commonly found in cultivated apple. Earlier studies showed that cultivars homozygous for the ACS1-2 allele produce less ethylene and have firmer fruit than ACS1-1/2 and ACS1-1/1 genotypes. ACO1 plays a minor role compared to ACS1, with homozygous ACO1-1 having lower ethylene production**.** The results of the current study revealed low ethylene production genotypes for both Md-ACS1 and Md-ACO-1 in cultivars Fuji Zhen Aztec, Gala Mast, Gala Redlum and Shalimar Apple-1, Oregon Spur and Red Velox. Most of the genotypes under study had intermediate firmness with heterozygous genotypes for both Md-ACS1and Md-ACO-1 i.e. Md-ACS1-1/2 and Md-ACO1-1/2. In previous research, King et al. [16] also reported that the preponderance of heterozygotes was common for many apple traits. Zhu and Barritt [12] reported similar results for Md-ACS1and Md-ACO1 in 60 apple cultivars. The data reported in the present study is important for marker assisted selection of progeny for breeding low ethylene producing apple cultivars for better storability and improved consumer acceptance.

## 4. CONCLUSION

To sum up, apple cultivars under study possess a considerable allelic diversity for fruit quality and scab resistance using dominant (AM19) and co-dominant (AL07, ACO1 and ACS1) markers. Out of 40 cultivars evaluated 3 were homozygous ACS1-2/2 with highly firm fruits Most of the cultivars under study had intermediate firmness with heterozygous for both Md-ACS1and Md-ACO-1 i.e. Md-ACS1-1/2 and Md-ACO1-1/2. The results obtained provide sufficient evidence of resistance phenomenon carried by apple cultivars like Firdous, ASP-3 Shireen, Shalimar Apple-1, ASP-1, ASP-69, ASP-12, ASP-10 and Maharaji. The information of this research could be very valuable for determination of the most different cultivars to be used as parents for mapping populations as well as in hybrid breeding programs especially for improved storability and shelf life and scab resistance. Besides this study will help a lot in screening large apple germplasm in short period of time for developing marker assisted resistant varieties against apple scab.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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