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Phenotypic and molecular screening for diseases resistance of apple cultivars and selections against apple scab (*Venturia inaequalis*)

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Abstract
Apple scab is one of the most widespread diseases of apple trees causing huge economic losses both quantitatively and qualitatively. The increased expenses associated with labor and fungicides justify the investigation of an alternative, effective, least expensive resistance approach to deal with this disease. In present study we evaluated different cultivars and selections against apple scab disease, using phenotypic and molecular approaches. Phenotypic screening using leaf detach method showed that, in certain cultivars and selections fungus did not proliferate macroscopically and the leaves remained alive, green and also showed hypersensitive response, which gives indication of resistance against apple scab. To validate the results of phenotypic screening, all twenty four cultivars were evaluated for the presence of *Vf* gene conferring resistance against apple scab disease using gene specific primers. Certain gene specific markers amplified only one fragment in resistant cultivars and hence confirmed the presence of *Vf* gene in these cultivars. Some amplified fragments in both susceptible and resistant lines, hence couldn't distinguish between resistant and susceptible ones. Present study confirmed the resistance both by phenotypic and molecular screening, hence can play an important role in designing future molecular breeding programme in apple for introgression of scab resistant genes.

Keywords: apple, screening, resistance, marker, breeding

1. Introduction
Apples (*Malus* × *domestica* Borkh.) are one of the most commonly consumed fruits in the world and commercially most important horticultural crop grown in temperate parts of the world (Ferree and Warrington, 2003) [10]. The quality of an apple depends on its external characteristics, such as color, size, and surface texture, and internal parameters, such as sweetness, acidity, firmness, tissue texture, ascorbic acid, and polyphenolic compounds. Apples are susceptible to more than seventy diseases caused by fungi, bacteria, viruses and phytoplasmas. Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cke.) Wint. (anamorph *Spilocaea pomi* Fr.), is major disease affecting apples, grown in temperate climates around the world. This disease can have a significant economic impact, as diseased fruit are not marketable. Complete crop loss is possible if steps are not taken in the orchard to reduce infection. The disease negatively affects fruit size and quality, due to blemishes and poor ripening. It is ranked number one disease in Apple in terms of yield loss, as it causes huge economic losses up to 70% in apple production (Mac Hardy, 1996) [20].

Managing apple scab requires the application of numerous fungicide treatments during the growing season that heavily affects the production costs and the environment. Public concerns over pesticide residues in food have generated a great deal of interest in reducing fungicide use on all food crops In some years control may reach 75% of total costs of apple protection against pests and diseases. The increased expenses associated with labor and fungicides alone justify the investigation of an alternative approach to deal with this disease. An alternative approach is the use of resistant varieties, which is most effective, least expensive and earliest for farmers to adopt. A large range of scab resistant cultivars have been released, most of them go back to the resistance derived from wild crab apple *Malus floribunda 821* called *Vf* and later renamed *Rvi6* (Bus *et al.,* 2009) [3]. This resistance gene has been widely utilized in apple breeding programme throughout the world for more than 40 years and has been incorporated into substantial number of apple cultivars (Crosby *et al.,* 1992) [6].

The *Vf* gene confers resistance to five out of seven known races of *V. inaequalis* and has held up quite well in the orchards for over 80 years. Many scab resistant cultivars have been bred in various countries (Crosby *et al*., 1992; MacHardy *et al*., 2001) [21, 6] majority of which contain *Rvi6*, previously known as *Vf* resistance gene. Efforts to produce durable scab resistant cultivars with market acceptability should be given priority in breeding programmes. In order to devise such programmes with success, apple genotypes have to be screened for scab resistance with fungal races present in particular region (Didelot *et al*., 2007, Le Van *et al*., 2011) [8, 18]. Hence, there is a need to search for scab resistant cultivars against different races for fungus. The aim of our study was to ascertain scab resistance in different cultivars and selections under *in vitro* conditions and by molecular approaches, to identify resistance against apple scab disease.

2. Material and Methods

2.1 Plant material

In our experiment, 24 apple cultivars and selections were used for phenotypic and molecular screening (Table 1). Among them two cultivars viz. Gala Redlum and Fuji Azitec are known susceptible cultivars.

Table 1: List of Apple Cultivars and Selections

S. No	Name of variety	S. No	Name of variety
1	Firdous	13	Gavin
2	Shireen	14	Prima
3	Selection no.1	15	Quirina
4	Selection no.2	16	Ec. Sel.276
5	Shalimar-1	17	Liberty
6	Selection no.3	18	Sir Prize
7	Selection no.4	19	Mac free
8	Selection no.5	20	Nova Easygro
9	Selection no.6	21	Bonza
10	Selection no.7	22	Priscilla
11	Selection no.8	23	Fuji Zhen Aztech
12	Selection no.9	24	Gala Redlum

2.2 Isolation and Preparation of inoculum

The inoculum was prepared from naturally infected samples collected from several places and cultivars in Kashmir Valley. Mono-conidial isolations were done using single spore isolation technique and inocula were produced as per Barbara *et al.* (2008) [1] and were adjusted to 5×10^5 conidia per milliliter. Evaluation of susceptibility to scab was done using a mixed conidial inoculum as described by Parisi *et al.* (1993) [23], in order to increase the pathogenic variability, inoculums from different isolates was mixed and used for inoculation (Yepes and Aldwinckle 1993) [29].

2.3 Leaf Detach Method

For leaf inoculations, young, succulent, actively growing leaves from all 24 apple cultivars and selections were collected and selected for inoculation. Leaves were washed with distilled water and their surfaces were air dried and placed randomly in glass petri plates containing moist Whatman filter paper. Leaves (2-3) per plate were inoculated with 20-30ul drops of inoculum per cultivar at random to adaxial leaf surface and each experiment was repeated at least 3 times. For each experiment three leaves in separate petri plate were inoculated with distilled water and were kept as control. After inoculation, all plates were incubated in a growth chamber at 19°C with a 16-h photoperiod from white fluorescent lamps at 40 µmol/mZs light intensity. Symptoms were assessed 14 days after inoculation (Yepes and Aldwinckle 1993, Chevalier, *et.al.,* 1991) [29, 5]. Leaf discs were prepared for light microscopy as described in Gessler and Stumm 1984 [13].

2.4 DNA Isolation

Total DNA was isolated from fresh young leaves (0.5 g) and extracted according to a method described by Doyle and Doyle (1990) [7]. The extract was further purified by RNaseA treatment using standard methods (Sambrook *et al.* 1989). The purified total DNA was quantified on 0.8 % agarose gel stained with ethidium bromide and the quality of the DNA was verified spectrophotometrically on NanoDrop (Thermo). DNA samples showing pure (A260/A280=1.80-2.00) and concentrated (≥500 $\mu g\cdot g^{-1}$) were further used for PCR amplification (Tartarini S 1999) [27].

2.5 PCR primers for gene specific markers

In total 10 pairs of molecular markers were used for study for the amplification of *Vf (Rvi6)* gene. The primer sequences for molecular markers used in present study are given in Table2.

Table 2: List of primers used for amplification of scab resistant genes

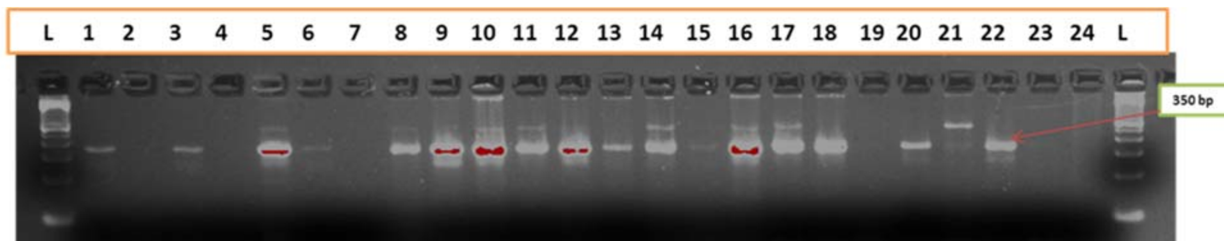
S.No.	Primer sequence (F+R)	Marker Name	References
1	5`- GTAAAGCAAGCACTTCAACG-3’ 5`- GTAAAATAGATGTGTGGGTAGC-3’	UI_{400}	Gianfranceschi *et.al.*,1996 [12]
2	5`-TGGAAGAGAGATCCAGAAAGTG-3’ 5`-CATCCCTCCACAAATGCC-3’	AL07	Khajuria *et al*., 2014 Patocchi *et al.,* 1999
3	5`-CGTAGAACGGAATTTGACAGTG-3’ 5`- GACAAAGGGCTTAAGTGCTCC-3’	AM19	Tartarini *et al.,* 1999 [27] Khajuria *et al*., 2014
4	5’-AATTCCGACTCTCATTGGGATTTCG-3’ 5’-TGGATTTTAGACTCTCATTGGGATTTCG-3’	*Vf1*	Boudichevskaia,2009 [2]
5	5’-GTTGTTTGTGATCATGTAACCGGCC-3’ 5’-ATTCGTTCCCCCCGAGATTAAGAGT-3’	*Vf2*	Boudichevskaia,2009 [2]
6	5’-CCTTTGACGCAGCTT-3’ 5’-CCTTGACGCATCTACG-3’	*Vm(Rvi5)*	Cheng *et.al.,* 1998 [4] Khajuria *et al*., 2014
7	5’-TCTCAACTTCTTGGACCTAAG-3’ 5’-GTGATATTTTGTGAACTGCCC-3’	*Vf2*ARD	Boudichevskaia,2009 [2]
8	5’-CCGTAGAACGGAATTTGACAGTG-3’ 5’-GACAAAGGGCTTAAGTGCTCC-3,	*Vf(Rvi6)* a	Tartarini *et al.,* 1999 [27] Gessler *et al.,* 2006 [14]
9	5’-GTAAAGCAAGCACTTCAACG-3’ 5’-GTAAAATAGATGTGTGGGGTAGC-3’	*Vf(Rvi6)* b	Tartarini *et al.,* 1999 [27] Gessler *et al.,* 2006 [14]

2.6 PCR Amplification
DNA from an individual plant of each apple cultivar and selection were screened with 10 pairs of gene specific primers primers (Table 2). Amplifications were performed in a volume of 20µL containing 25-50 ng genomic DNA, 1x PCR buffer (20 mM Tris- Cl pH 8.4, 50mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.0 unit of Taq DNA polymerase. The following PCR conditions were used: Initial denaturation at 94 °C for 5 min was followed by 35 cycles at 94 °C for 1 min, 58–63 °C for 1 min and 72 °C for 2 min. The final extension was carried out at 72 °C for 7 min. Amplifications were performed in a gradient thermal cycler (Make TAKARA, Japan). PCR reactions were repeated two times to ensure the reproducibility of amplification profiles. The amplified fragments were resolved in 2 % metaphore agarose gel in a 0.5X TBE buffer. The gels were stained with ethidium bromide (0.5µg/ml) and visualized under UV light.

3. Results and Discussion
3.1 Phenotypic Screening of Detached Leaves
On cultivars like Shireen, Firdous, Shalimar 1, Gavin, Ec.Sel.276, Liberty, Nova Easy Gro, Prima, Bonza, Priscilla and selections S1, 2, 4, 5, 6,7,8,9, the fungus did not proliferate macroscopically and the leaves remained alive and green and gave the indication of resistance against apple scab(Fig. la), as did the non-inoculated control leaves. A hypersensitive response (Fig.1b) was also observed on detached leaves of the most of cultivars like Shalimar 1, Shireen, Freedom, Liberty, and Prima etc. Hypersentive response was also confirmed on resistant varieties after inoculation on leaves by Yepes and Aldwinckle 1993 [29] and Dar *et al.,* 2015. Detached leaves could be used also to study host-parasite interactions, characterization of scab-resistant variants and test scab inhibitory compounds on detached leaves of susceptible and resistant apple cultivars ((Yepes and Aldwinckle 1993) [29]. In hypersensitive response plants inhibit the pathogen directly by releasing Pathogenesis related (PR) proteins, including chitinase, β-1,3-glucanase and cysteine-like protease, to prevent colonization (Gau *et al*., 2004) [15] On susceptible cultivars Gala Redlum and Fuji Zhen Aztech, the fungus grew abundantly on the surface of the leaf. Visible symptoms developed faster on susceptible cultivars like Gala Redlum and Fuji Zhen Aztec under *in vitro* conditions. Sporulation and macroscopic symptoms were observed after 20-30 days on detached leaves. Macroscopic symptoms on detached leaves of susceptible cultivars after innoculation developed initial chlorosis 3 weeks after inoculation, followed by necrosis after 5 weeks (Fig.1c). Collapse of the epidermal cells was observed using light microscopy 48-72 h after inoculation in susceptible cultivars like (Fig. ld.). This argument was supported by microscopic observations of circular 5-mm leaf punches of the 'detached leaves assay', successful infections and sporulation observed in Golden delicious cultivars (Liebhard, R. *et al*., 2003) [19].

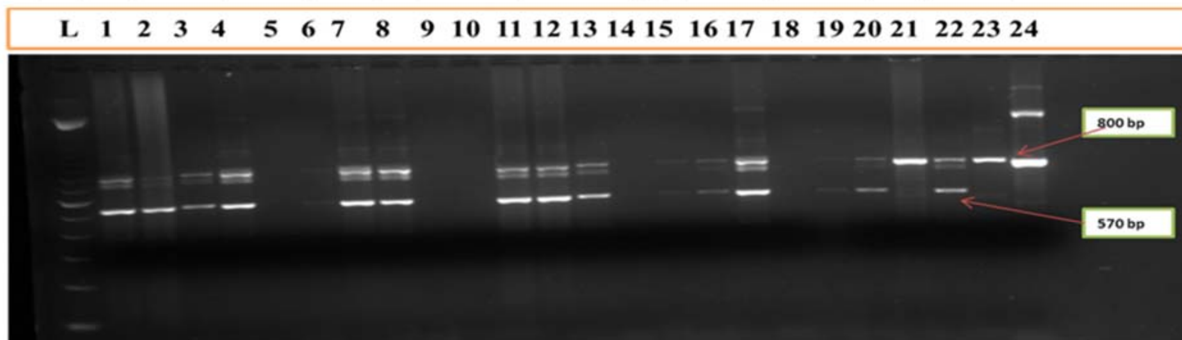
Fig 1: a: leaves are live and freen after inocution, b: hypersensitive response, c: necrosis on susceptible leaves, d: collapse of episemal cells after inoculation

3.2 Molecular Screening
To confirm the results of phenotypic screening, all twenty four cultivars were evaluated for the presence of *Vf* gene conferring resistance against apple scab disease. Marker UI_{400} (*vf*) amplified a specific fragment of 350 bp (Fig. 2a) in only resistant cultivars Firdous, Prima, Liberty, Gavin, Nova easy gro, Priscella, Ec. Sel.276, Sir prize and selections S1,3, 5,6,7,8,9 and was absent in susceptible cultivars Gala Redlum and Fuji Zhen Aztech. Since the marker amplified specific band in resistant cultivars only, therefore it can be used to distinguish between scab susceptible and resistant cultivars and selections. Primers for the marker AL07 (*Vf*) amplified two fragments 800bp (Fig. 2b) and 570 bp, but 570 bp was observed in resistant cultivars like Shireen, Firdous Shalimar-1, Gavin, Liberty, Nova easy gro, Priscella, Selections 1, 2,4,5,8,9 and was absent in susceptible cultivars like Gala Redlum and Fuji Zhen Aztech (Fig. 1b). Primer for the marker AM19 led to amplication of 550bp (Fig.2c) in resistant cultivars Shireen, Firdous, Gavin, Ec.Sel.276, Liberty, Nova Easygro, Bonza, Priscilla and selections S1, 2, 4, 5, 6,7,8,9, and absent in susceptible cultivars Gala Redlum and Fuji Zhen Aztech (Fig.1c). Marker *Vf(Rvi6)*a resulted in amplication of a 550 bp (Fig. 2d) in resistant cultivars like Shireen, Firdous, Gavin, Ec.Sel.276, Liberty, Nova Easygro, Bonza, Priscella and selections S1, 2, 4, 5, 6,7,8,9, and absent in susceptible cultivars Gala Redlum and Fuji Zhen Aztech. Another marker of *Vf(Rvi6)*b amplified fragment of 450 bp (Fig. 2e) in resistant cultivars and selections, no amplification was observed in susceptible cultivars Gala Redlum and Fuji Zhen Aztech, hence can be used for distinguishing resistant and susceptible cultivars at molecular level (Fig.1d). Marker *Vf2*ARD amplified fragment of 570 bp (Fig. 2f) in resistant cultivars and selections, no amplification was observed in susceptible cultivars Gala Redlum and Fuji Zhen Aztech, hence can be used for distinguishing resistant and susceptible cultivars at molecular level. Marker *Vf2* amplified 700bp (Fig. 2g) fragment in resistant cultivars and selections like Shalimar 1, Prime, Liberty, Sirprize and no fragment was observed in susceptible varieties, Gala Redlum and Fuji Zhen Aztech. Marker *Vm (Rvi5)* and *Vf1* amplified 850bp (Fig. 2h) and 650 bp (Fig.2i) amplicon respectively in both susceptible and resistant lines, hence these markers cannot be used to distinguish between resistant and susceptible ones.



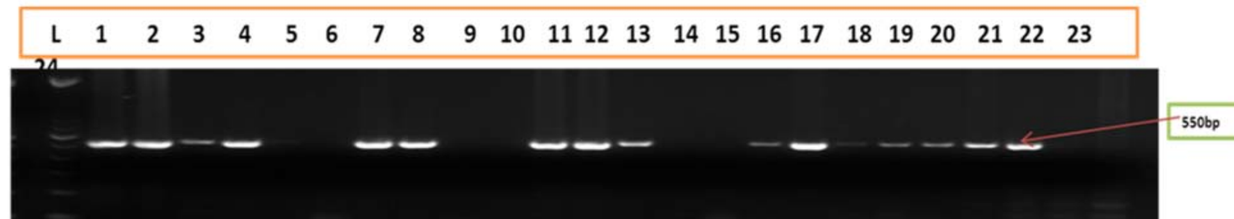
UI400

Fig:2a:-L-Ladder, 1-Firdous,**2**-Shireen,**3**-Sel.1,**4**-Sel.2,**5**-Shalimar1,6-Sel.3,**7**-Sel.4, **8**-Sel.5,**9**-Sel.6,**10**-Sel.7,**11**-Sel.8,**12**-Sel.9,**13**-gavin,**14**-Prima,**15**-Quirina,**16**-Sel.10,**17**-Liberty,**18**-Sirprize,**19**-Macfree,**20**-Nova easy gro,**21**-Bonza,**22**-Priscilla,**23**-Fuji Azitech,**24**-Redlum Gala.



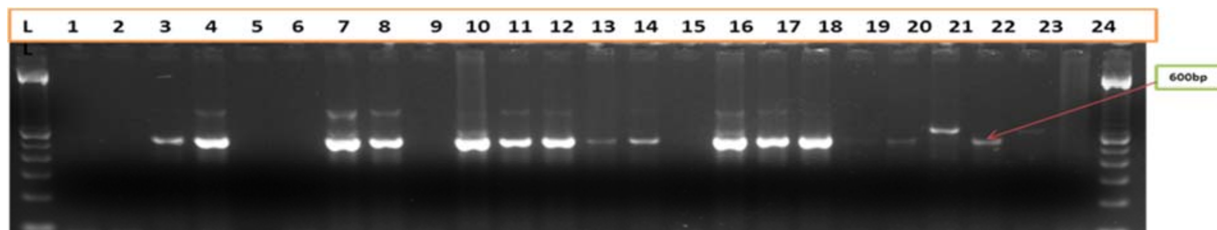
AL07

Fig:2b-L-Ladder, 1-Firdous,**2**-Shireen,**3**-Sel.1,**4**-Sel.2,**5**-Shalimar1,6-Sel.3,**7**-Sel.4, **8**-Sel.5,**9**-Sel.6,**10**-Sel.7,**11**-Sel.8,**12**-Sel.9,**13**-gavin,**14**-Prima,**15**-Quirina,**16**-Sel.10,**17**-Liberty,**18**-Sirprize,**19**-Macfree,**20**-Nova easy gro,**21**-Bonza,**22**-Priscilla,**23**-Fuji Azitech,**24**-Redlum Gala.



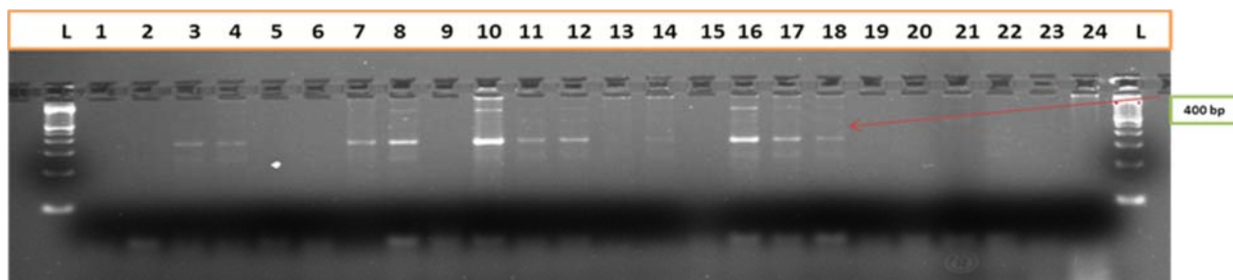
AM19

Fig:2c:-L-Ladder, 1-Firdous,**2**-Shireen,**3**-Sel.1,**4**-Sel.2,**5**-Shalimar1,6-Sel.3,**7**-Sel.4, **8**-Sel.5,**9**-Sel.6,**10**-Sel.7,**11**-Sel.8,**12**-Sel.9,**13**-gavin,**14**-Prima,**15**-Quirina,**16**-Sel.10,**17**-Liberty,**18**-Sirprize,**19**-Macfree,**20**-Nova easy gro,**21**-Bonza,**22**-Priscilla,**23**-Fuji Azitech,**24**-Redlum Gala.



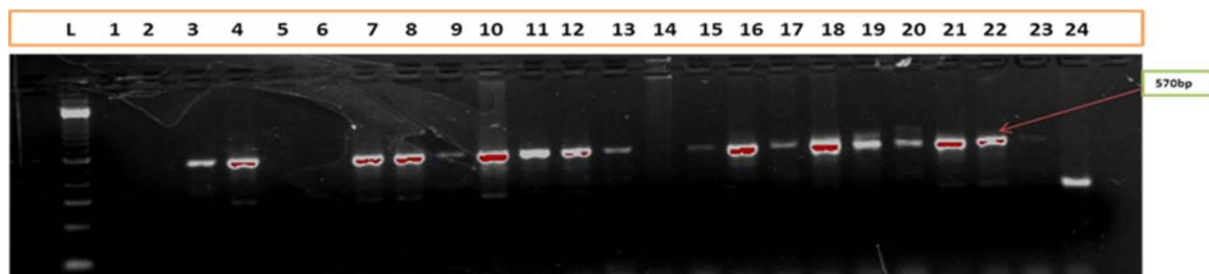
VfRvi6a

Fig:2d:-L-Ladder,1-Firdous,**2**-Shireen,**3**-Sel.1,**4**-Sel.2,**5**-Shalimar1,6-Sel.3,**7**-Sel.4, **8**-Sel.5,**9**-Sel.6,**10**-Sel.7,**11**-Sel.8,**12**-Sel.9,**13**-gavin,**14**-Prima,**15**-Quirina,**16**-Sel.10,**17**-Liberty,**18**-Sirprize,**19**-Macfree,**20**-Nova easy gro,**21**-Bonza,**22**-Priscilla,**23**-Fuji Azitech,**24**-Redlum Gala.



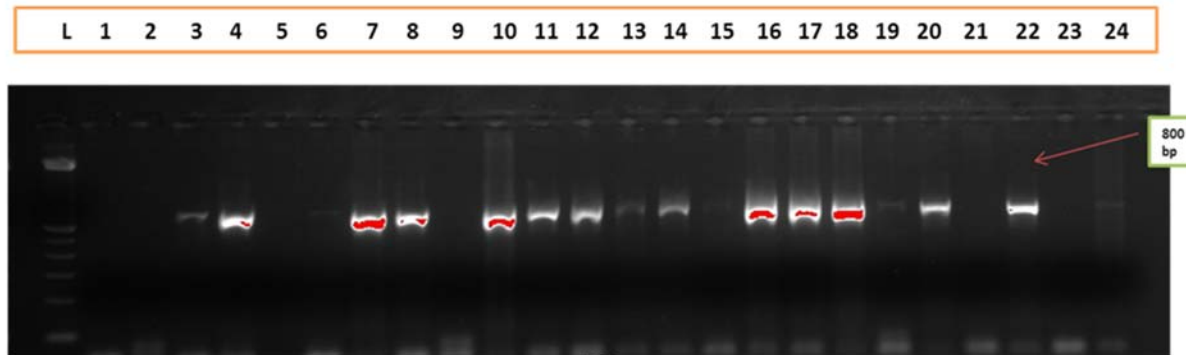
VfRvi6 b

Fig:2e:-L-Ladder, 1-Firdous,**2**-Shireen,**3**-Sel.1,**4**-Sel.2,**5**-Shalimar1,6-Sel.3,**7**-Sel.4, **8**-Sel.5,**9**-Sel.6,**10**-Sel.7,**11**-Sel.8,**12**-Sel.9,**13**-gavin,**14**-Prima,**15**-Quirina,**16**-Sel.10,**17**-Liberty,**18**-Sirprize,**19**-Macfree,**20**-Nova easy gro,**21**-Bonza,**22**-Priscilla,**23**-Fuji Azitech,**24**-Redlum Gala.



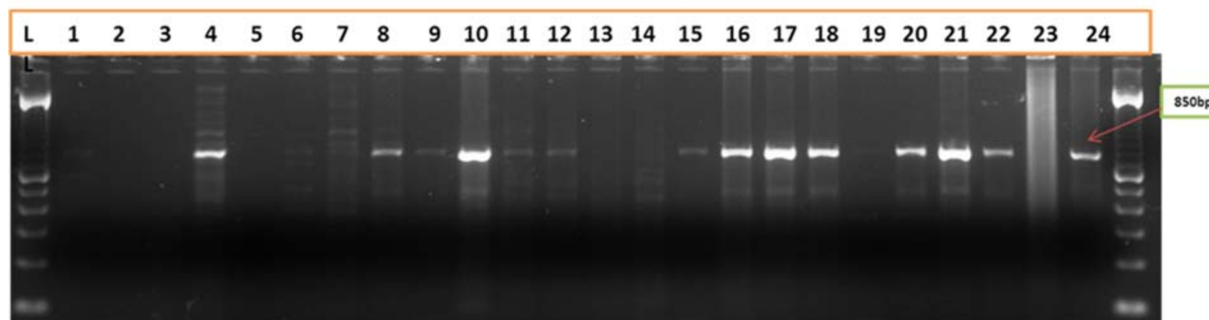
Vf2ARD

Fig:2f:-L-Ladder, **1**-Firdous,**2**-Shireen,**3**-Sel.1,**4**-Sel.2,**5**-Shalimar1,6-Sel.3,**7**-Sel.4, **8**-Sel.5,**9**-Sel.6,**10**-Sel.7,**11**-Sel.8,**12**-Sel.9,**13**-gavin,**14**-Prima,**15**-Quirina,**16**-Sel.10,**17**-Liberty,**18**-Sirprize,**19**-Macfree,**20**-Nova easy gro,**21**-Bonza,**22**-Priscilla,**23**-Fuji Azitech,**24**-Redlum Gala.



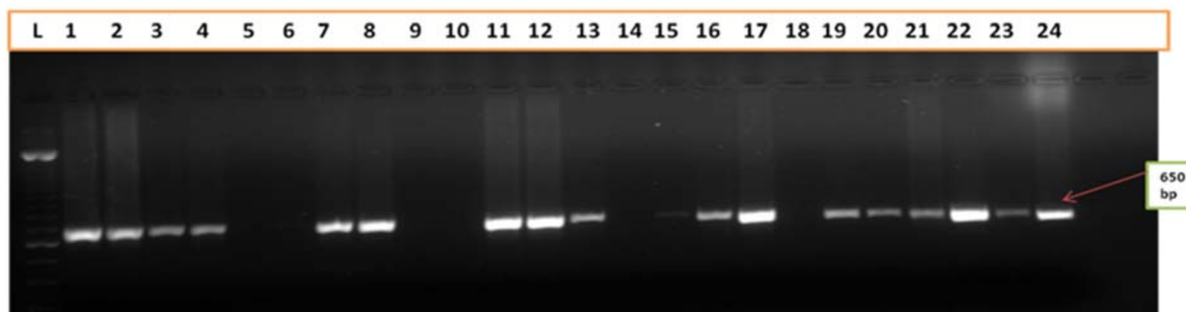
Vf2

Fig:2g;-L-Ladder, **1**-Firdous,**2**-Shireen,**3**-Sel.1,**4**-Sel.2,**5**-Shalimar1,6-Sel.3,**7**-Sel.4, **8**-Sel.5,**9**-Sel.6,**10**-Sel.7,**11**-Sel.8,**12**-Sel.9,**13**-gavin,**14**-Prima,**15**-Quirina,**16**-Sel.10,**17**-Liberty,**18**-Sirprize,**19**-Macfree,**20**-Nova easy gro,**21**-Bonza,**22**-Priscilla,**23**-Fuji Azitech,**24**-Redlum Gala.



VmRvi5

Fig:2h:-L-Ladder, **1**-Firdous,**2**-Shireen,**3**-Sel.1,**4**-Sel.2,**5**-Shalimar1,6-Sel.3,**7**-Sel.4, **8**-Sel.5,**9**-Sel.6,**10**-Sel.7,**11**-Sel.8,**12**-Sel.9,**13**-gavin,**14**-Prima,**15**-Quirina,**16**-Sel.10,**17**-Liberty,**18**-Sirprize,**19**-Macfree,**20**-Nova easy gro,**21**-Bonza,**22**-Priscilla,**23**-Fuji Azitech,**24**-Redlum Gala.



Vf1

Fig:2i:- L-Ladder, **1**-Firdous,**2**-Shireen,**3**-Sel.1,**4**-Sel.2,**5**-Shalimar1,6-Sel.3,**7**-Sel.4,**8**-Sel.5,**9**-Sel.6,**10**-Sel.7,**11**-Sel.8,**12**-Sel.9,**13**-gavin,**14**-Prima,**15**-Quirina,**16**-Sel.10,**17**-Liberty,**18**-Sirprize,**19**-Macfree,**20**-Nova easy gro,**21**-Bonza,**22**-Priscilla,**23**-Fuji Azitech,**24**-Redlum Gala.

The use of marker-assisted selection is an excellent instrument for identification of resistance genes and creation of resistance cultivars. The molecular marker AM19, vfrvi6a&b, *Vf2*, *Vf2*ARD proved to be highly useful because of its ability to distinguish resistant and susceptible cultivars on the basis of presence or absence of single band on gel. Hence these markers seem to be highly specific in detecting presence of *Vf (Rvi6)* gene in apple cultivars. However, due to dominant nature of the marker AM19, it cannot distinguish homozygous and heterozygous cultivars containing *Vf* (Rvi6) gene, similar results were confirmed by Khajuria *et.al.,* 2015. Marker AL07 produced two bands of 820 bp bank represents

the recessive *vf* allele and the latter the dominant allele *Vf*. Similarly results have also been reported by Khajuria *et al.*, 2014, hence, this marker can be used for identification of homozygous and heterozygous genotypes in different cultivars. Interestingly, the 820 bp amplicon was observed in both resistant and susceptible cultivars, while the fragment of 570 bp amplicon was amplified only in resistant cultivars as these results are in concurrence with Suat *et al*., 2013. Using this codominant marker, genotype of various cultivars was established. The marker $U1_{400}$ was able to distinguish resistant cultivar, namely, Shireen, Firdous, Prima, Liberty, Gavin, Nova Easy Gro, Priscella, Ec. Sel.276, Sir prize and selections S1,3, 5,6,7,8,9 and was absent in susceptible cultivars Gala Radium and Fuji Zhen Aztech. Thus, our results confirm the earlier reports that *Vf* (Rvi6) based resistance can be detected using these specific molecular markers. Some markers like *Vf1* and *Vm(Rvi5)* were not able to distinguish between resistant and susceptible cultivars and hence cannot be used to distinguish between susceptible and resistant as were also confirmed earlier by Khajuria *et al.,* 2014. These resistant cultivars and selections can be exploited easily to bred scab resistance cultivars using the aid of marker assisted backcrossing. These genes can be combined together provided comprehensive breeding programme will be initiated and such programmes have already started in many countries (Gleichauf *et al*., 2009, Flachowsky *et al*., 2011) [16, 11]. To draw conclusions for the durability of resistances in the orchard and to propose new breeding strategies, the best planting strategy is to mix varieties with resistance from different genetic sources (Fischer *et al*., 1994).

4. Conclusion
Evaluation of different cultivars and selections using phenotypic and molecular approaches, for identification of resistance genes against apple scab disease confirmed the resistance in certain cultivars and selections using gene specific markers. The gene specific primers identified in present study can be directly used for screening large apple germplasm in short period of time for developing marker assisted resistant varieties against apple scab. Present study provides the sufficient evidence of resistance phenomenon carried by scab resistant apple cultivars like Firdous, Shireen, Shalimar-1 etc as revealed by phenotypic and molecular screening. Designing of future breeding programmes based on present study are evident and hybridization programmes between resistant and susceptible cultivars can be taken up for development of high quality disease resistant apple cultivars by introgression of scab resistant genes into commercial cultivars like Oregon spur, Gala Redlum, Starkrimson, Silver spur, Red chief, Fuji zhen Azitech etc in Kashmir valley.

5. Acknowledgements
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