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Evaluation of apple cv. Jeromine raised on *in vitro* and conventionally propagated clonal apple rootstocks

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ABSTRACT

Apple is a significant cash crop that is cultivated in the temperate areas around the globe. In the past, seedling rootstocks were used for raising commercial apple orchards however, the increasing demand for apple has led to a rising interest in clonal rootstocks due to early maturity, good fruit quality, disease resistance, and wider temperature adaptability. Traditional propagation methods for rootstock multiplication are time-consuming and labor-intensive, which has prompted the adoption of micro-propagation as a viable alternative for large-scale production of uniform, disease-free, and high-quality plant material. *In vitro* propagation protocols for apple clonal rootstocks *viz*., M7, M9, MM106, MM111, and Merton793 have been developed but tissue culture propagated rootstocks are not widely accepted due to concerns regarding graft incompatibility and risk of inducing genetic variability, necessitating the evaluation of regenerated plants for successful commercial utilization. In the present study, no significant variability was observed at morpho-physiological level between tissue culture raised and conventionally propagated clonal rootstocks grafted with 'Jeromine'. To overcome the limitations of traditional phenotypic observation, further confirmation was done using RAPD and SCoT markers. Out of 33 RAPD primers, 18 primers were successful in DNA amplification resulting in 64 amplicons, out of which 58 were monomorphic. Nineteen out of 36 SCoT primers resulted in DNA amplification leading to 83 amplicons, out of which 36 were monomorphic. Overall, the apple cv. Jeromine trees raised on *in vitro* and conventionally propagated apple rootstocks grafted were significantly similar at morpho-physiological and molecular levels. These findings support the recommendation of tissue culture-raised clonal rootstocks to farmers for commercial apple cultivation, as they offer the potential for increased yields and improved profitability in high-density apple plantations.

Key words: *Malus* × *domestica* Borkh., PCR, rootstocks, RAPD, SCoT.

INTRODUCTION

Apple (*Malus* × *domestica* Borkh.) is a widely cultivated temperate fruit crop with significant economic value. In Himachal Pradesh, it occupies 48.83 percent of the total area under fruit crops contributing to 80.81 percent of the total fruit production and has emerged as the leading commercial fruit crop (https://www.nhb.gov.in/).

The successful cultivation of apple trees relies on the availability of healthy and high -quality plant material such as rootstocks and cultivars. In Himachal Pradesh, the majority of apple orchards are established using seedling rootstocks however, there is a growing demand for clonal rootstocks as they are genetically identical and ensure uniform trees with controlled growth, precocious bearing, disease and pest resistance, good quality and high fruit production (Chai *et al*., 1). Traditional plant propagation methods are no longer favoured since they are time-consuming, labour intensive, season-dependent, require significant land allocation with limited success rates. Micro-propagation offers a solution by enabling large-scale production of disease-free and uniform plants throughout the year, thereby ensuring a consistent supply of high-quality plant material (Thakur *et al*., 19). However, there is a potential risk of inciting genetic variability, specifically somaclonal variation among sub-clones derived from a single parental line in micro-propagation method hence, evaluating these variations in the regenerated plants is crucial for successful commercial utilization of this technology.

Traditionally, the identification of apple cultivars and rootstocks has relied on phenotypic observation and numerous researchers have focused on characterizing morphological and biochemical traits of apples over the past decade. However, this process is time-consuming due to the long juvenile period of the trees and highly influenced by environmental factors. To overcome these limitations, molecular markers have emerged as a valuable tool for

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establishing genetic similarity or dissimilarity amongst the population. Unlike phenotypic observations, DNA markers offer independence from environmental influences, are highly informative and reproducible in nature. Among DNA markers, RAPD and SCoT are dominant, user-friendly, and widely applicable in various plant breeding aspects. These markers have been applied in several crops to facilitate genetic variability analysis, molecular characterization, linkage mapping, DNA fingerprinting,gene tagging and establishing phylogenetic relationships among the cultivars (Modgil *et al*., 13; Gupta *et al*.,6; Thakur *et al*., 20; Sharma *et al*., 15; 16).

In vitro propagation protocols for apple clonal rootstocks *viz*., M7, M9, MM106, MM111, and Merton793 were developed in the Department of Biotechnology, Dr YS Parmar University of Horticulture and Forestry, Nauni, Solan, H.P. (India) for large-scale production of planting material (Sharma *et al*.,17; Kaushal *et al*.,10; Modgil *et al*., 12; Soni *et al*.,18; Modgil and Thakur, 11). However, tissue culture propagated rootstocks of apple are not preferred by the orchardists over their conventionally propagated counterparts due to inhibitions regarding graft incompatibility, fruit bearing and overall performance. Hence, this study was carried out to compare *in vitro* and conventionally propagated rootstocks grafted with 'Jeromine' for morphological, physiological, and molecular traits to ensure the acceptance of tissue culture raised planting material by the farmers.

MATERIALS AND METHODS

Four-year old apple trees of cv. Jeromine grafted on tissue culture and conventionally propagated clonal rootstocks *viz*. M7, M9, MM106, MM111, and Merton 793 in three replicates growing in the trial site of the Department of the Fruit Science, Dr. Yashwant Singh Parmar University of Horticulture & Forestry, Nauni, Solan (H.P.) were used as source of experimental plants.

Morphological traits *viz*., leaf area, inter-nodal length, tree height, first flower, <50% bloom, full bloom, leaf type, leaf shape, stomatal conductance, photosynthesis rate and transpiration rate were analyzed in all the apple trees raised on five clonal rootstocks. The leaf area was measured using a portable leaf area meter. (Model No. LI-3000C, LI-COR Biosciences Pvt Ltd.) and the values were expressed as average leaf area/leaf in square centimetre (cm^2). The inter-nodal length (cm) was measured using a normal standard scale (Camlin, India) in three replicates. Tree height (cm) was measured using an inch tape, extending from ground level to the highest point reached by the tree during its dormant period. The date of first flowering was recorded when 4-5 floral buds had opened. The stage when less than 50% and 75% flowers opened was considered as 50% and full bloom, respectively. The leaf types and shapes were evaluated visually and their particular gradation was noted based on the descriptor developed by the International Union for the Protection of New Varieties of Plants (UPOV). The photosynthesis rate, stomatal conductance and transpiration rate in all the experimental trees were estimated from leaves using IRGA (Infra-red Gas Analyzer, Model No. LI-6400XT, LI-COR Biosciences Pvt Ltd.) and the values were expressed in µmol/m^2/s.

Fresh apple leaves were used to extract total genomic DNA using the CTAB method. The DNA samples underwent treatment with RNase to eliminate any RNA contaminants. Quantity of isolated DNA was checked using a UV/VIS spectrophotometer (BioSpectrometer, Eppendorf) and quality was assessed on 0.8 percent agarose gel. Isolated DNA showed optimal quantity and quality for PCR amplification.

DNA amplification was conducted using a thermal cycler (Bio-RAD) using thirty- six SCoT and thirty-three RAPD primers with varying concentrations of (i) template DNA, (ii) Taq DNA polymerase (iii) dNTPs and (iv) Mg^{2+} ions to optimize the reaction mixture. PCR reactions were carried out in 15 µL aliquot consisting of 0.03 U/µLpertaining to Taq DNA polymerase, 0.25 mM dNTPs, 1X PCR buffer that includes 2.1 mM $MgCl_2$, 11.1 pmolof primer (Bangalore GeNei™) along with template DNA (80-100 ng/µL). DNA amplification was performed at 94°C for 3 minutes, followed by 50 cycles of denaturation (92°C for 45 seconds), annealing (varied with factors like Tm ± 5°Cof primer) for 1 minute, extension (72°C for 2 minutes) and final extension at 72°Cfor 12 minutes. After PCR amplification, The DNA products were examined using a 1.5% (w/v) agarose gel that had 3 µL of ethidium bromide per 100 mL.To each PCR tube, 2 µL of 6X loading dye was added, and 15 µL of the sample was loaded into each well, along with a DNA ladder to evaluate the size of the amplified products.The amplified products were visualized using a UV transilluminator and captured with a gel documentation system (Biovis).

Fragments ranging in size from 100 to 2500 bp were consistently reproducible, and DNA fragment profiles were recorded in a binary format, with '0' representing the absence of a band and '1' indicating the presence of a band in a Microsoft Excel spreadsheet.Software packages NTSYSpc-2.2 and R-Studio 4.2.1 were used to determine similarity and distance (dissimilarity) matrix of binary data using Jaccard's coefficient and Euclidean's distance method. Cluster analysis was conducted using the unweighted pair group method with arithmetic

average (UPGMA) (Rohlf, 14), and a dendrogram was created to categorize tissue culture-raised and conventionally propagated clonal rootstocks.

The data collected for various parameters were analyzed using randomized block designs (Cochran and Cox, 2). Statistical analysis of variance was applied to the randomized block design based on the mean values for each treatment.

RESULTS AND DISCUSSION

Selecting the appropriate rootstock is a crucial aspect of orchard management because it significantly affects the scion cultivars and, consequently, fruit production. The gene expression pattern in apple tree scions influenced by rootstocks leads to various changes in the trees, such as early fruiting, enhanced fruit quality, nutrient accumulation and disease resistance. Depending on the rootstock it is grafted onto, an apple cultivar may exhibit varying levels of morpho-physiological and molecular changes.

Various morphological and physiological characteristics of apple trees, specifically the leaf area, inter-nodal length, tree height, first flower occurrence, bloom stages, leaf type, leaf shape, photosynthesis, stomatal conductance, and transpiration rate were assessed in *in vitro* (TC) and conventionally (C) propagated M7, M9, MM106, MM111, and Merton793 rootstocks grafted with Jeromine (Table 1) with no significant variations. The first flower bloom in all the trees was observed within 2 to 7 days followed by 50% bloom within 3 to 9 days and full bloom within 5 to 12 days, in the month of April. Three leaf types, namely crenate, crenate to serrate, serrate (Fig. 1), five leaf shapes, including V-shaped, concave, flat with raised margins, flat, and convex (Fig. 2), with no variations in fruit quality, color and bearing were recorded in all the experimental trees (Fig. 4). Distinct morphological traits can be effectively utilized for characterizing and classifying various apple genotypes under similar environmental and soil conditions (Hayat *et al*., 7); however, these traits were not represented in the current study, as only a single cultivar, 'Jeromine,' was employed as a scion.

By studying the physiological traits, rootstocks that are better equipped to withstand challenging environmental conditions could be utilized for orchard management. The photosynthetic rate in all the experimental apple trees ranged from 6.64 µmol/m^2/s (trees on conventionally propagated M7) to 13.18 µmol/m^2/s (conventionally propagated MM111) and no significant variation was observed in trees raised on same rootstock, propagated through *in vitro* or conventional methods. Photosynthetic rate is a crucial factor which determines the plant's fruit production and selection of the rootstocks with high photosynthetic activity could help the farmers to achieve maximum tree growth and yield. Similarly, stomatal conductance and transpiration rate were also found similar in trees on *in-vitro* and conventionally propagated apple rootstocks. They reflect soil moisture levels and are closely related to a plant's reaction to environmental factors, including temperature and humidity (Fernandez *et al*., 4).

Morphological and physiological traits represent the expression of many genes and contain high information but are unreliable due to the strong

Table 1. Morpho-physiological characteristics of apple cv. Jeromine raised on tissue culture (TC) and conventionally (C) propagated clonal rootstocks.

Cultivar Jeromine grafted on rootstock	Leaf area (cm^2)	Inter-node length (cm)	Tree height (cm)	First flower Days	<50% bloom Days	Full bloom Days	Photosynthesis rate (µmol/m^2/s)	Stomatal conductance (µmol/m^2/s)	Transpiration rate (µmol/m^2/s)
M7TC	27.18	4.1	164.85	5	6	9	6.64	0.13	5.66
M7C	21.31	4.2	150.03	2	4	5	10.78	0.09	3.93
M9TC	30.11	3.6	134.45	4	7	10	11.33	0.15	5.86
M9C	25.69	3.5	146.47	5	6	10	9.85	0.10	4.24
MM106TC	21.94	3.7	154.43	4	5	10	8.52	0.16	6.19
MM106C	20.75	3.8	151.98	7	9	12	12.23	0.15	6.07
MM111TC	23.58	4.1	166.29	2	4	6	13.18	0.16	6.42
MM111C	22.65	4.4	186.94	3	4	7	11.50	0.17	6.63
Merton 793TC	23.29	4.2	182.52	2	3	8	12.00	0.20	7.54
Merton 793C	21.37	4.6	189.99	3	5	8	10.02	0.16	6.39
$C.D._{(0.01)}$	0.88	0.18	4.78	0.16	0.24	0.37	0.496	0.01	0.22
$S.E._{(m)}$	0.29	0.06	1.60	0.05	0.08	0.12	0.17	0.00	0.08

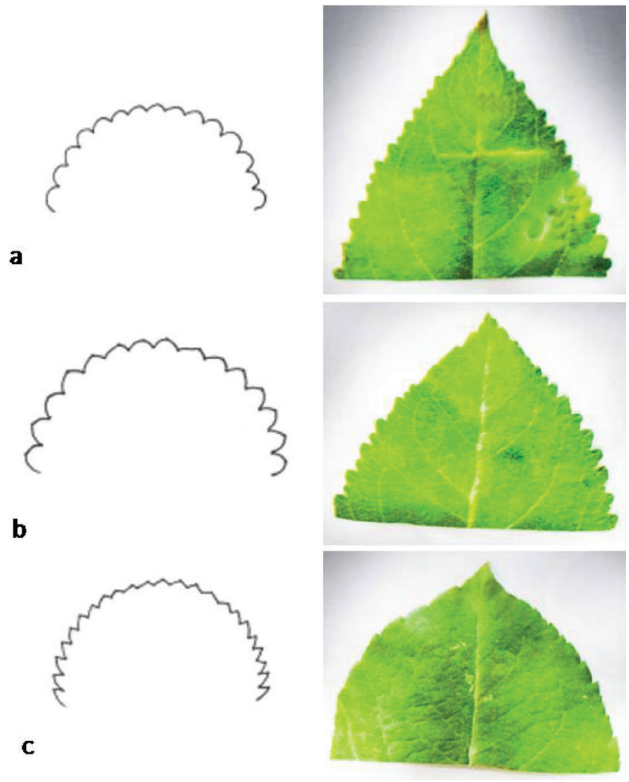

Fig. 1. Different leaf type *viz*., crenate (a); crenate to serrate (b) and serrate (c) observed in apple clonal rootstocks (MALUS_DOM, UPOV, 2021) (Color picture online) (MALUS_DOM, UPOV, 2021).

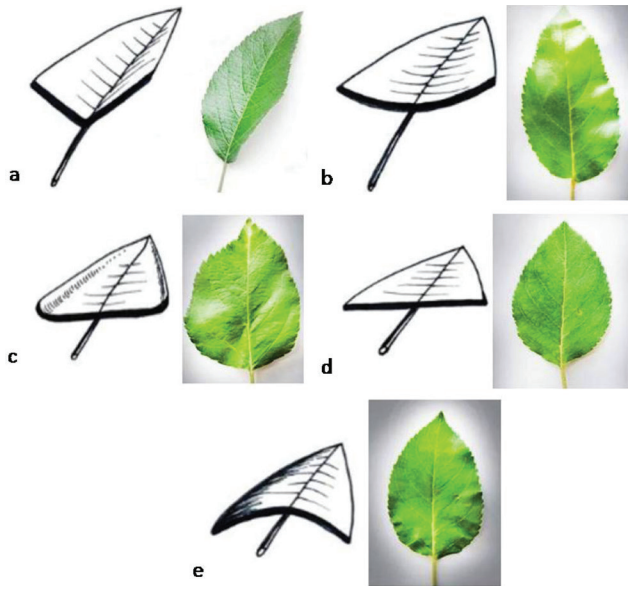

Fig. 2. Different leaf shape *viz*., V-shaped (a); concave (b); flat with raised margins (c); flat (d) and convex (e) observed in apple clonal rootstocks (MALUS_DOM, UPOV, 2021).

influence of the environment. Molecular markers offer a more precise and informative approach for assessing genetic variation (Sharma *et al*., 16). The genetic integrity of micro-propagated plants holds significant practical and economic implications especially in commercial crops. In this study, an evaluation of molecular fingerprinting profiles of apple trees raised on clonal rootstocks produced through tissue culture and conventional methods were performed with RAPD and SCoT markers. These DNA markers provide higher information content and enable the detection of any genetic variations that may be present in tissue culture-derived plants. Among the various DNA markers available, RAPD has been widely utilized for studying clonal integrity, identifying genetic similarities, and detecting somaclonal variations. They have proven to be cost-effective for genetic marker identification and have facilitated large-scale fingerprinting studies. However, in recent years, several novel and promising marker techniques have emerged. One technique is Start Codon Targeted (SCoT) polymorphism, which uses dominant and reproducible markers derived from short conserved regions flanking the ATG translation start (or initiation) codon in plant genes (Collard and Mackill, 3). Hence, in this study, RAPD and SCoT markers were employed to assess the similarity/variability present in five apple clonal rootstocks obtained through tissue culture and conventional methods, grafted with 'Jeromine'.

A total of 33 RAPD primers were employed to analyze experimental apple trees, out of which only 17 primers amplified the genomic DNA in all samples successfully. The amplified products displayed a size range of 100 to 2000 base pairs (bp), averaging 3.6 bands per primer, which resulted in a total of 64 amplified bands. Among the primers used, fourteen primers (OPA 01, OPA 02, OPA 08, OPA 10, OPA 11, OPA 12, OPA 13, OPA 16, OPB 18, OPC 12, OPC 20, OPF 08, OPG 08, and OPJ 10) were found to be monomorphic, indicating no genetic variation among the experimental trees, while only four primers (OPB 01, OPG 01, OPG 10, and OPJ 04) demonstrated polymorphism (Fig. 3; Table 2).The generated variability among tissue culture raised and conventionally propagated rootstocks might be due to somaclonal variations as a result of gene mutation or changes in epigenetic markers (Thakur *et al*., 20).

To establish the genetic relationships among different apple cultivars, a comparative analysis was conducted. The Jaccard coefficient was used to assess pairwise genetic distances, and a dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The dendrogram effectively clustered the samples into two main groups,

namely cluster A and cluster B. Cluster A comprised apple trees propagated through tissue culture as well as conventionally propagated MM111, Merton793, and M9, whereas cluster B included MM106 and M7. These relationships have been visually depicted in Fig. 7a. The highest genetic similarity (94%) was observed between 'Jeromine' trees grafted on rootstock M9 propagated *in vitro* and through conventional means. The genetic similarity values ranged from 0.53 to 0.94, indicating a reasonable level of genetic resemblance within the trees grafted on the same rootstocks, irrespective of their propagation method. Previous studies have extensively utilized RAPD markers to assess genetic stability in *in vitro* propagated clonal apple rootstocks, MM106, M7 (Modgil *et al*., 13) and EMLA111 (Gupta *et al*., 6). However, it is important to note that the reproducibility of RAPD results cannot be guaranteed, as they are affected by factors such as the quantity of the template, amplification profile, and primer annealing temperature requiring an additional marker system to validate the findings. Hence, SCoT markers were further employed to obtain more reliable and reproducible results.

In comparison to RAPD markers, SCoT markers exhibit higher reproducibility, possess dominant characteristics and are known for their extensive polymorphism as well as resolving power. In this study, 36 SCoT primers were employed to analyze experimental apple trees, resulting in successful amplification of genomic DNA by 19 primers. On an average, 4.4 bands per primer were observed with a total of 87 amplicons ranging from 100 to 2500 base pairs in size. Out of these amplicons, 35 were found to be monomorphic, while 52 exhibited polymorphism. Primer S34 displayed the highest count of 11 scorable bands whereas, primers S2, S6, S13, and S22 exclusively generated monomorphic bands. Overall, the SCoT primers exhibited a 52.48% monomorphism rate and had an average Polymorphic Information Content (PIC) value of 0.094 (Fig. 4; Table 3), which determines the efficiency of these primers for fingerprinting the genotypes. The differences in the banding profile of apple trees were most likely due to rootstock effects. It is also possible that some of the molecular differences observed in trees grafted on conventionally and *in vitro* raised rootstocks was due to somaclonal variations (Jensen *et al*., 8) as mentioned in RAPD studies.

To analyze the genetic distance between apple trees grafted using *in vitro* and conventionally propagated clonal rootstocks, Jaccard's coefficient was utilized for pairwise comparison. The resulting

Table 2. Data obtained from RAPD primers in the study.

Primer Code	Sequence (5'-3')	Melting temperature (ºC)	Amplified bands	Monomorphic bands	Monomorphism (%)	PIC	Amplified product size range (bp)
OPA 01	CAGGCCCTTC	38.2	4	4	100.00	0.123	500-800
OPA 02	TGCCGAGCTG	34.0	4	4	100.00	0.134	500-1000
OPA 08	GTGACGTAGG	32.0	4	4	100.00	0.105	500-800
OPA 10	GTGATCGCAG	32.0	4	4	100.00	0.127	300-800
OPA 11	CAATCGCCGT	32.0	3	3	100.00	0.105	500-1000
OPA 12	TCGGCGATAG	32.0	1	1	100.00	0.207	500-700
OPA 13	CAGCACCCAC	34.0	6	6	100.00	0.142	650-1700
OPA 16	AGCCAGCGAA	32.0	5	5	100.00	0.101	350-1200
OPB 01	GTTTCGCTCC	32.0	3	1	33.33	0.112	500-1000
OPB 18	CCACAGCAGT	32.0	4	4	100.00	0.058	600-2000
OPC 12	TGTCATCCCC	32.0	2	2	100.00	0.105	200-1200
OPC 20	ACTTCGCCAC	32.0	3	3	100.00	0.184	350-800
OPF 08	GGGATATCGG	32.0	6	6	100.00	0.066	400-1000
OPG 01	CTACGGAGGA	28.9	4	3	75.00	0.062	350-900
OPG 08	TCACGTCCAC	28.9	1	1	100.00	0.070	100-100
OPG 10	AGGGCCGTCT	41.3	4	3	75.00	0.042	150-1000
OPJ 04	CCGAACACGG	41.0	3	1	33.33	0.082	250-500

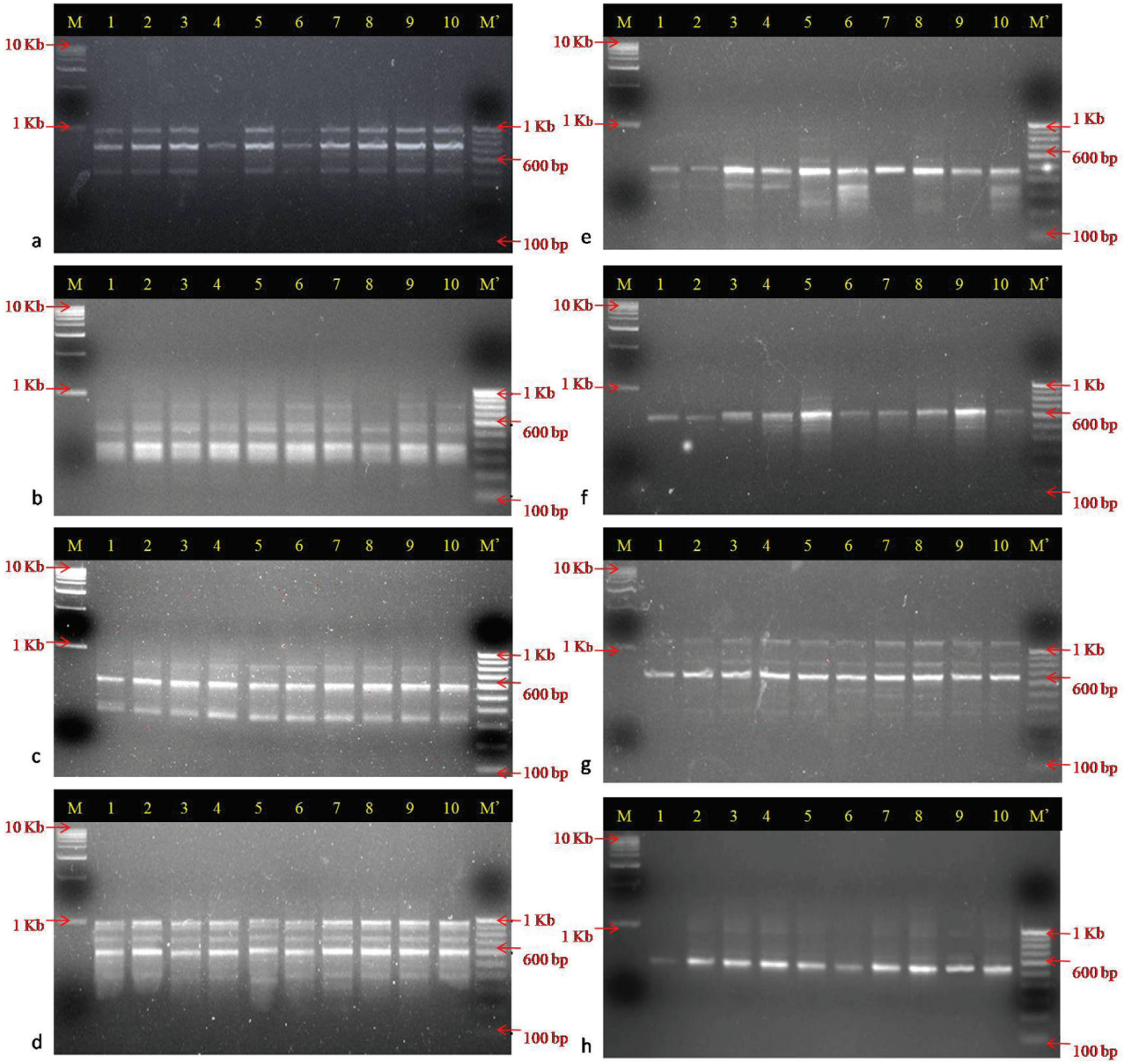

Fig. 3. RAPD banding profile generated using primers OPA-02 (a); OPA-10 (b); OPA-16 (c); OPB-01 (d); OPC-20 (e); OPF-08 (f); OPJ-04 (g) and OPJ-10 (h).

dendrogram exhibited two main clusters, namely cluster A and cluster B. Cluster A consisted of trees raised on tissue culture and conventionally propagated M9 rootstock, while cluster B included M7, MM111, Merton793, and MM106, with similarity matrix ranging from 0.32 to 0.84. The highest similarity (84%) was observed between the trees raised via tissue culture and conventionally propagated MM111, as illustrated in the Fig. 5. The study showed that SCoT primers were able to effectively distinguish between trees grafted on various rootstocks by analyzing the presence or absence of specific amplified bands. Additionally, SCoT analysis revealed that trees on similar rootstock, displayed similar banding patterns irrespective of their propagation strategy. Similar applications of SCoT markers were observed in genetic fidelity and diversity assessment of plum, *Malus* sp., *Prunus sibirica*, rose, and grapes. These findings highlight the applicability of SCoT markers to detect even minor genetic variations, making them valuable for species analysis and identification (Collard and Mackill, 3; Thakur *et al*., 20).

The variability noted among apple trees grown on different rootstocks may result from the transfer

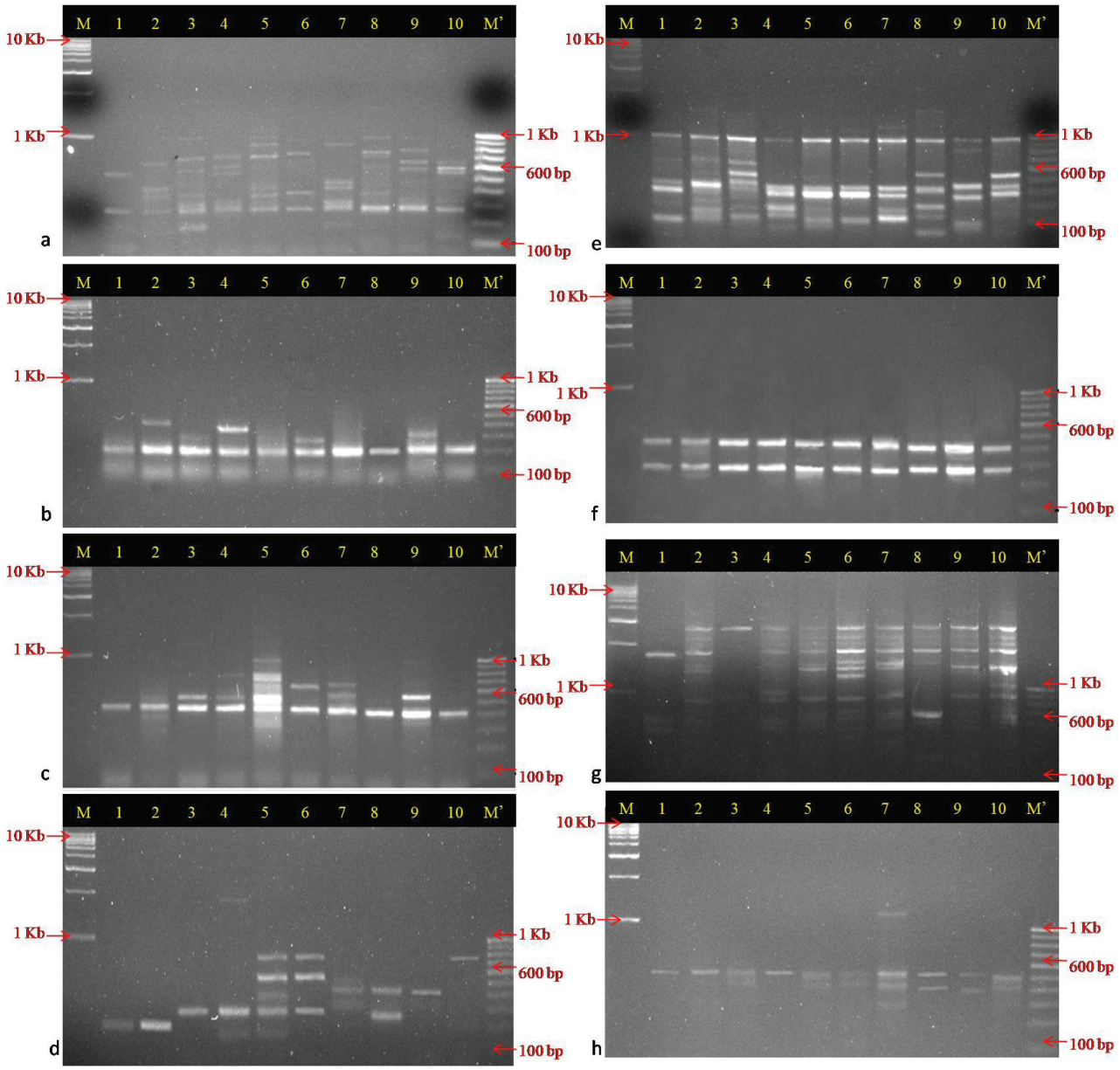

Fig. 4. SCoT banding profile generated using primers S4 (a); S5 (b); S7 (c); S10 (d); S16 (e); S30 (f); S34 (g) and S35 (h)

of genetic material across the graft junction from the stock to the scion, which can cause variations in hormonal signaling, gene expression, protein turnover, synthesis and accumulation of metabolites, RNA silencing, as well as water and ion uptake and transport in the grafted trees. At molecular level, mRNA transfer across the graft unions from the rootstocks to scion and vice versa alters chromatin structure and transcriptional reprogramming. Epigenetic changes can modulate DNA structure by histone modifications and DNA methylation (Kapazoglou *et al*., 9). In apple, the miRNA participates in long distance signaling from vascular tissue and modulates the expression of mRNA targets. Moreover, grafting led to the up-regulation of genes associated with hormonal signaling and metabolic processes (Guo *et al*., 5).

Apple trees of cv. Jeromine raised on tissue culture and conventionally propagated apple rootstocks were significantly similar at morphological, physiological and molecular levels. By utilizing tissue culture-raised clonal rootstocks, high-density apple plantations can be raised leading to higher productivity and improved efficiency in terms of land utilization as well as labor management with ensured consistent

Table 3. Data obtained from SCoT primers in the study.

Primer Code	Sequence (5'-3')	Melting temperature (ºC)	Amplified bands	Monomorphic bands	Monomorphism (%)	PIC	Amplified product size range (bp)
S1	CAACAATGGCTACCACCA	53.69	8	3	37.50	0.148	150-2000
S2	CAACAATGGCTACCACCC	55.97	1	1	100.00	0.037	550-550
S3	CAACAATGGCTACCACCG	55.97	5	2	40.00	0.122	350-1200
S4	CAACAATGGCTACCACCT	53.69	7	2	28.57	0.164	200-1000
S5	CAACAATGGCTACCACGA	53.69	3	1	33.00	0.055	100-400
S6	CAACAATGGCTACCACGC	55.97	1	1	100.00	0.037	900-900
S7	CAACAATGGCTACCACGG	55.97	5	1	20.00	0.079	400-1000
S10	CAACAATGGCTACCAGCC	55.97	5	1	20.00	0.076	200-800
S13	ACGACATGGCGACCATCG	58.24	1	1	100.00	0.037	600-600
S16	ACCATGGCTACCACCGAC	58.24	7	3	42.86	0.185	100-1000
S19	ACCATGGCTACCACCGGC	60.52	3	2	66.67	0.093	100-600
S20	ACCATGGCTACCACCGCG	60.52	4	1	25.00	0.089	300-700
S22	AACCATGGCTACCACCAC	55.97	2	2	100.00	0.051	200-300
S27	ACCATGGCTACCACCGTG	58.24	7	2	28.57	0.062	400-1000
S28	CCATGGCTACCACCGCCA	60.52	6	3	50.00	0.148	400-2000
S30	CCATGGCTACCACCGGCG	60.52	3	2	66.67	0.076	300-400
S32	CCATGGCTACCACCGCAC	60.52	2	1	50.00	0.044	500-600
S34	ACCATGGCTACCACCGCA	58.24	11	6	54.55	0.225	500-2500
S35	CATGGCTACCACCGGCCC	62.80	3	1	33.33	0.069	350-1000

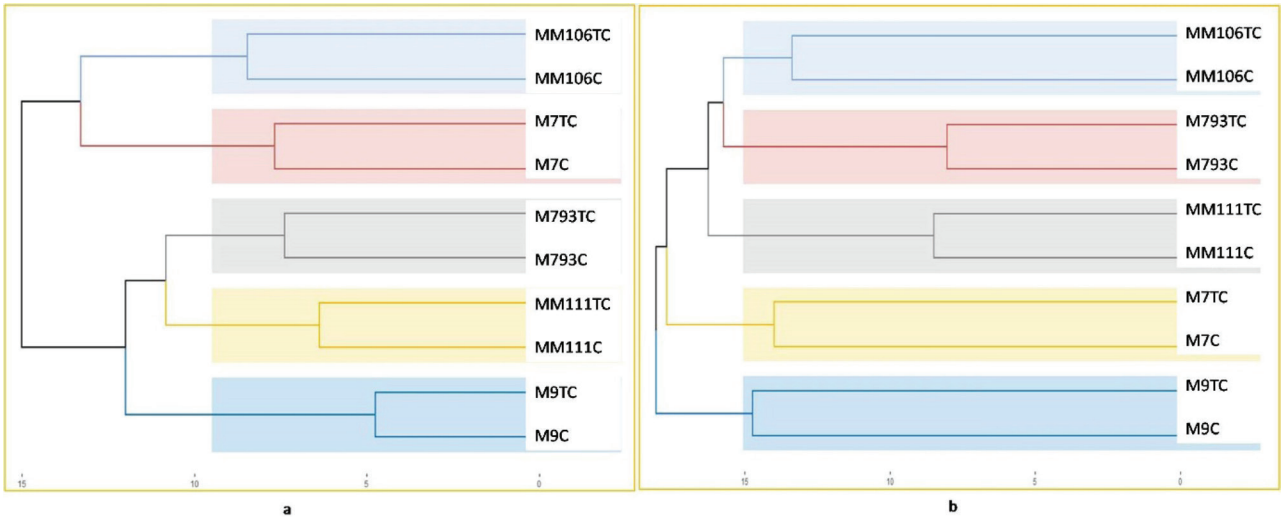

Fig. 5. RAPD (a) and SCoT (b) marker derived dendrogram based on Jaccard's similarity coefficient.

results, since these rootstocks are genetically uniform and have been shown to be similar to conventionally propagated rootstocks. Hence, tissue culture-raised clonal apple rootstocks can be recommended to the farmers for raising high-density apple plantations for commercial gains.

AUTHORS' CONTRIBUTION

Conceptualization of research (MT); Designing of the experiments (MT and PV); Contribution of experimental materials (MM and DPS); Execution of field/lab experiments (APS and MSR); Analysis

of data and interpretation (APS, MSR and PS); Preparation of the manuscript (MT, PS).

DECLARATION

The authors declare that there is no conflict of interest.

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