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ARTICLE

Molecular and genome size analyses of somaclonal variation in apple rootstocks Malling 7 and Malling 9

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ABSTRACT The cultivated apple (*Malus domestica*) is important fruit crops cultivated in world. For production and breeding of high quality apple, inducing and enhancing new genetic diversity and suitable traits are necessary. In Iran, different local and imported apple genotypes are cultivated and common apple root stocks (Malling 7) M7 and (Malling 9) M9 are mostly used root stocks in the country. Therefore, we studied genetic diversity of M7 an M9 tissue culture regenerated plants produced by different treatments by using twenty ISSR markers. In total 51 randomly selected plants were studied for the occurrence of somaclonal variation in apple mother plants and tissue culture regenerated plants. Four different treatments were used for tissue culture. Genetic diversity parameters, genetic distance and polymorphism percentage were studied in regenerated plants. Variations in loci frequency and combination were checked by STRUCTURE and the presence of similar loci in the plants was studied by reticulation NJ tree. Genetic relationship versus distinctness was determined by principal coordinate analysis. The results showed the occurrence of genetic variation among mother plants and tissue culture regenerated plants of each subculture due to somaclonal variation. Significant difference in the genome size among some of the regenerated plants indicates that change in genetic structure of plants during tissue culture is also accompanied with quantitative change in DNA. However, degree of genetic variation differed among apple rootstocks and also among different treatments used.
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KEY WORDS

apple
ISSR
genome size
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Abbreviations

BAP: 6-benzyladenine; IBA: 3-indole butyric acid;
DKW medium: Driver and Kuniyuki medium; MS medium: Murashige and Skoog medium

Introduction

Apple (genus *Malus* Mill., family Rosaceae) is one of the most important fruit crops cultivated in temperate and subtropical climate (Coart et al. 2006). With the ever-growing requirements for environmental protection and food safety in the production of high quality apples, the modern apple breeding becomes more and more dependent on resistant gene resources and other genes responsible for suitable traits from the cultivated and wild genetic resources (Crosby et al. 1992).

The origin of apple (*Malus domestica* Borkh.) is believed to be Central Asia from where it has spread to the rest of the world (Harris et al. 2002). On the basis of morphological, molecular, and historical evidences, the wild Central Asian species *Malus sieversii* has previously been identified as the main contributor to the genome of the cultivated apple (*Malus domestica*) (Cornille et al. 2012).

Most of the apple cultivars are diploid (2n=2x=34), but some varieties are haploid, with 17 single chromosomes, and some others, especially crab apples, are polyploid trees ranging from triploid, tetraploid to hexaploid levels (Burford 1998; Forte et al. 2002). In fact, some apples have as many as eighty-five chromosomes and each of the genes on each of the chromosomes can have different alleles (alternative forms). A single seed may thus contain a lot of genetic variation that has accumulated down through the ancestral lines. For example, 'Gravenstein' apples are triploid with a chromosome number of 51 (3n=51). They are produced by the union of a diploid egg (2n=34) and a haploid pollen (n=17).

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This is accomplished by crossing a tetraploid plant (4n=68) with an ordinary diploid plant (2n=34). Because the triploid (3n) varieties are sterile, they must be propagated by grafting, where the scions of choice cultivars are grafted to hardy, pest-resistant root stalks (Burford 1998).

Production of new genetic variants in crop plants is one of the possible sources of obtaining elite genotypes to be used in hybridization and breeding programs. Plant tissue culture leading to somaclonal and developmental variation has been considered as one of the possible sources of inducing genetic variability in crop plants.

Somaclonal variation is the occurrence of genetic variants among the regenerated plants of a single subculture derived from *in vitro* procedures. During several subculturing developmental genetic changes occur among the regenerated plants (Isabel et al. 1993). It is also called tissue or culture-induced variation (Bordallo et al. 2004).

Variability induced in regenerated plants during tissue culture often arises as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells and is expected to generate stable plants carrying interesting heritable traits (Soniya et al. 2001). Four important variables influence the induced variability discussed: these are genotype, explant origin, cultivation period and the cultural condition in which the culture is made (Evans and Sharp 2000). Variations induced may be manifested as cytological abnormalities, frequent qualitative and quantitative phenotypic mutation, sequence change, gene activation and silencing (Sheidai et al. 2008).

Identification of possible somaclonal variants at an early stage of development is very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants (Soniya et al. 2001; Sheidai et al. 2010). Different strategies can be used to evaluate genetic structure in tissue culture regenerated plant clones, including cytogenetic (karyotype and flow cytometry) analysis and isoenzyme markers (Soniya et al. 2001), various molecular markers including RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragments Length Polymorphism), ISSR (Inter Simple Sequence Repeats) and SSR (Simple Sequence Repeats) (Soniya et al. 2001; Sheidai et al. 2010; Nybom and Schaal 1990; Watillon et al. 1991; Guilford et al. 1997; Zhou and Li 2000; Goulão and Oliveira 2001; Gupta et al. 2009; Ping et al. 2011).

Almost none of the available apple cultivars entirely meet the high demands of propagators, growers and the rapidly changing apple market. Moreover, improvement of one or a few characteristics in commonly cultivated apple varieties is impractical through sexual hybridization, since apple is a heterozygous crop with high self-incompatibility, a high level of heterozygosity, frequent polyploidy, inbreeding depression, long life cycle and large growth form (Viršek-Marn et al. 1999). Somaclonal variation is considered as an important alternative source of variation for selection of new valuable apple mutants, especially since it has been suggested that novel variants, which cannot be obtained by classical mutation breeding, can be raised through tissue culture (Viršek-Marn et al. 1999).

Somaclonal variation analyses have been performed in different apple cultivars and rootstocks by various molecular approaches. For example, Viršek-Marn and coworkers (1999) studied molecular (RAPD) and morphological variation among the regenerated plants of Golden Delicious Bovey and Goldspur cultivars, which were obtained by adventitious shoot regeneration from apple leaves.

Butiuc-Keul et al. (2010) studied the expression of several enzymatic systems in apple (*Malus domestica* Borkh., cvs. Florina, Romus3, Romus4, Colmar, Rebra, Goldrush and Idared) plants grown *in vitro* in comparison with the *in vivo* donor plants and reported difference in pattern of izoesterases among them.

Donovan and coworkers (2012) studied somaclonal variation in apple cultivar Greensleeves and obtained 270 somaclones, regenerated from leaf discs. These plants showed increased root number per rooted shoot with subsequent subculturing. Lane et al. (1982), obtained compact (dwarf) apple trees by tissue culture, while, Gupta et al. (2009) studied the genetic fidelity of micropropagated apple rootstock plants, EMLA 111, using RAPD markers. The regenerated plants showed 0.92 to 1.00 genetic similarities to the mother plant. To our knowledge there has been no report on somaclonal variation of the apple cultivars/root stocks available in Iran and this is the first report on the subject.

Material and Methods

Tissue culture

Apple rootstocks Malling 7 (M7) and Malling 9 (M9) were used to obtain tissue culture regenerated plants. Actively growing shoots of the year were collected in spring from plants pots in greenhouse. One centimeter of internode sample cut and cultured after sterilization. For sterilization, single nodes were taken from M7 and M9 rootstocks and then ddH_2O, NaOCl based on present protocols were used (Ciccotti et al. 2008). The DKW medium (Driver and Kuniyuki, 1984) and MS medium (Murashige and Skoog 1962) containing 30 g/l sucrose, 7.0 g/l agar and hormones were used for shooting and branching explants production. Four different treatments were used in tissue culture as follows:

MS medium + BAP (2 mg/l) hormone+ IBA (0.1 mg/l) hormone,

2- MS medium + 2ip (2 mg/l) hormone + IBA (0.1 mg/l)

hormone,

3- DKW medium + BAP (2 mg/l) hormone + IBA (0.1 mg/l) hormone

and 4- DKW medium + 2ip (2 mg/l) hormone+ IBA (0.1 mg/l) hormone

(These four treatments are indicated as T1-T4 throughout the paper).

The MS media used with 2 mg/l BA and 0.1 mgl^{-1} IBA (SB) and 2 mgl^{-1} 2-ip and 0.1 mgl^{-1} IBA (SP), also DKW used with 2 mgl^{-1} BA and 0.1 mgl^{-1} IBA (DB) and 2 mgl^{-1} 2-ip and 0.1 mgl^{-1} IBA (DP), (IBA concentration was equal in all of media culture). All media were adjusted to pH 5.8 and were sterilized by autoclaving at 121 °C for 20 min. Five to six samples were placed into glass jars and maintained at 25±2 °C under a 16/8 h light photoperiodic under a light intensity 3000 lux in a germinator. After 30 days, all samples were transferred to fresh medium and processed till second subculture.

DNA extraction and ISSR assay

Three to five fresh leaves of each genotype were pooled and used for DNA extraction. The total genomic DNA was extracted using the CTAB method by Murray and Thompson (1980) with modification described by De la Rosa and coworkers (2002). Quality of extracted DNA was examined by running on 0.8% agarose gel.

ISSR assay

Twenty ISSR primers including UBC807, 810, 811, 823, 834, 849 designed by University of British Columbia and (CA)9GT, (GA)9T, (GA)9A, (GA)9C, (CA)7AT, (CA)7AC, (GT)7TG, (GT)7CA, CAG(GA)7, GCT(GA)7, (AGC)5GA, (AGC)5GG, (AGC)5GT, (AGC)5GC as well as three hetero-ISSR primers were used. PCR reactions were performed in a 25 µl volume containing 10 mM Tris-HCl buffer (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer, 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). Amplification reactions were performed in thermocycler (Techne, Germany) with the following program: 5 min initial denaturation step at 94 °C, then 30 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C. The reaction was completed by final extension step of 7 min at 72 °C. Amplification products were separated on a 2% agarose gel visualized by ethidium bromide staining. Fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Flow cytometry

The genome size in mother plants and tissue culture regenerated plants were determined by flow cytometry. For each plant 3 readings were obtained. The nuclear suspensions were prepared from small amount of mature fresh leaf tissue together with an equal weight of mature leaf tissue of the external standard. The external standard used in for *Malus* was *Allium cepa* cultivar Ailsa Craig (2C = 33.55 pg).

One-step protocol was used for the preparation of nuclear suspension. Leaves were chopped with a single-used sharp scalpel in the presence of 400 µl nuclei isolation buffer in a plastic petri dish at room temperature. For nuclear staining, 1600 µl DNA fluorochorome or DAPI (2,6-diamidino-2-phenylindole) was added and suspensions were filtered through a 50 µm nylon mesh into labeled sample tubes. Stained nuclear suspensions were analyzed with a Partec Flow Cytometer (Partec, Germany). The flow cytometric statistics, such as coefficient of variation (CV), mode, mean and the number of cells counted in each sample, are showed in the histograms obtained.

DNA amounts are measured in picograms (pg) and the status of nuclei is described in terms of 'C' values (Doležel et al. 2007). One pg of DNA represents 978 mega base pairs (Mbp). The amount of nuclear DNA of each sample was calculated based on the values of the G1 peak means (Doležel and Bartoš 2005) as follows:

[Sample 2C peak mean position/Standard 2C peak mean position] ×Standard 2C DNA amount = Sample 2C DNA (pg) amount

ANOVA test was performed to show difference in *2C*-value content among the plants studied.

Molecular data analyses

We screened ISSR bands in the first and second subcultures separately among regenerated plants obtained from each treatment and subsequent statistical analyses were performed in these data.

ISSR bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). To assess the genetic diversity, different genetic parameters including the number of common alleles and their frequency, the number of specific alleles, Shannon's Information Index, observed heterozygosity, expected heterozygosity, and unbiased expected heterozygosity (Freeland et al. 2010) were determined for each subculture and each treatment used.

The relationship of genotypes was determined by Nei and Li genetic distance (Podani, 2000) followed by NJ (Neighbor Joining) tree construction by using DARwin ver. 5 (2000). Moreover, relationship of genotypes versus distinctness was tested by Principal Coordinate Analysis plot (PCoA) performed after 999 permutation by GeneAlex ver. 6 (2006).

The Bayesian model-based clustering was used to elucidate the genetic structure among genotypes using STRUCTURE ver. 2.3 (Pritchard et al. 2000). The program structure implements a model-based clustering method for inferring

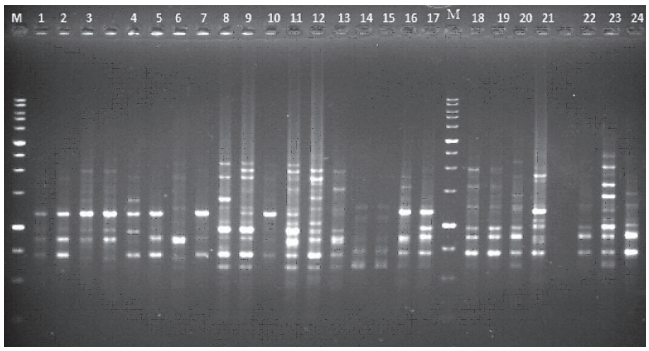


Figure 1. ISSR band profiles produced by $(CA)_7GT$ primer on M7 regenerated plants in four treatments and two subcultures. M: 100 bp ladder. Lanes (L)1-3: first subculture of T1; L4-6: first subculture of T2; L7-8: first subculture of T3; L10-12: first subculture of T4; L13-15: second subculture of T1; L16-18: second subculture of T2; L19-21: second subculture of T3; L22-24: second subculture of T4.

population structure using genotype data consisting of unlinked markers. The model applied in the analysis assumes the existence of *K* clusters. Applications of this model include demonstrating the presence of population structure, assigning individuals to populations or jointly to two or more populations if their genotypes indicate that they are admixed. We took advantage of an admixture ancestry model under the correlated allele frequency model. The Markov chain Monte Carlo simulation was run 20 times for each value of *K* for 10^6 iterations after a burn-in period of 10^5. All other parameters were set at their default values. The proportional membership of each cluster was estimated for each individual and each population. Finally we studied the possible gene exchange of populations by reticulation and networking by DARwin ver. 5 (2000).

In order to reveal genetic difference among regenerated plants of different treatments, Analysis of Molecular Variance (AMOVA) was performed by GENEALEX ver. 6. (2006). The genetic variance was partitioned into among populations and within populations.

Results

ISSR analysis

Malling 7 (M7)

Out of the 20 ISSR primers used alone and in combinations, 10 primers produced amplicons (Fig. 1). The size of the detected bands (amplicons) ranged from 300 bp to 1950 bp. In the first subculture, the highest number of polymorphic bands (9) was obtained with primer (AGC)5GT followed by primer UBC-811 (8). Similarly the highest number of monomorphic bands was produced by primers UBC-807, (GA)9C and mixed primer (3 bands).

In the second subculture, the highest number of total and polymorphic bands (12 and 11, respectively) occurred with primer UBC-811, followed by (AGC)5GG (9-9 bands). The genetic diversity parameters are presented in Table 1. The highest values of Shannon's Information Index (I) and Expected Heterozygosity occurred in regenerated plants of T1 (0.233 and 0.156, respectively), followed by T2 in the first subculture (0.147 and 0.097, respectively). However, in the second subculture, the highest values of these parameters occurred in T2 (0.291 and 0.197, respectively) and T4 (0.214 and 0.145, respectively).

AMOVA test showed significant genetic difference among mother plants and regenerated plants ($p<0.01$) in both subcultures. The results showed that in the first subculture, 42% of total variation occurs due to among populations (treatments) and 58% due to within populations variation, while in the second subculture, 24% of total variation is due to among populations and 76% due to within population.

Nei's genetic distance (Table 2) showed the highest genetic difference between mother plants and T2 regenerated plants (0.599) followed by T1 regenerated plants (0.579), in the first subculture, while the lowest value of genetic difference occurred between T3 and T4 regenerated plants. Similar analysis performed in the second subculture (Table 2), showed the highest genetic difference between mother plant and T3 (0.596) and the lowest genetic difference between T2 and T4 plants (0.10).

Details of genetic relationship among mother plant and tissue culture regenerated plants are presented in Figures 2-5. NJ tree of the first subculture plants is presented in Figure 2A. The mother plant is well separated from regenerated plants due to its molecular difference. Among regenerated plants, one of the plants from T1 is much different from the others and stands far from the others and forms a single cluster. The other plants are distributed in 3 major clusters. The tree in general shows genetic similarity between regenerated plants of T1 and T2, and between plants of T3 and T4. However, the plants studied in a single treatment are not all placed in a single cluster close to each other and show genetic differences. Genetic distinctness of all regenerated plants from mother plant is well documented in PCoA plot of ISSR data (Fig. 3A).

The analyses of 12 randomly selected plants of second subculture (Figs. 2B and 3B) produced almost similar results to the first subculture. They show separation of mother plant from tissue culture regenerated plants, and also show presence of genetic diversity among plants of each treatment as they are placed in different clusters/groups. However, the pattern of distribution of plants in the PCoA plot of the second subculture indicates wider distribution of these plants showing

Table 1. Genetic diversity parameters in M7 plants.

	Group	Na	Ne	I	He	UHe	P%
M7	Mother plant	0.651	1	0	0	0	0
subculture1	T1	1.186	1.265	0.233	0.156	0.187	41.86
	T2	1.07	1.156	0.147	0.097	0.116	27.91
	T3	0.93	1.119	0.097	0.066	0.08	16.28
	T4	0.884	1.109	0.086	0.059	0.071	13.95
	Total	0.944	1.13	0.112	0.076	0.091	20
M7	Mother plant	0.706	1	0	0	0	0
subculture2	T1	1.176	1.215	0.187	0.126	0.151	33.33
	T2	1.431	1.348	0.291	0.197	0.225	52.94
	T3	1.118	1.256	0.203	0.141	0.169	33.33
	T4	1.255	1.252	0.214	0.145	0.174	7.25
	Total	1.137	1.214	1.179	0.122	0.144	25.34
M9	Mother plant	0.617	1.000	0.000	0.000	0.000	0
subculture1	T1	0.783	1.094	0.081	0.055	0.074	13.33
	T2	0.917	1.096	0.094	0.061	0.073	18.33
	T3	0.883	1.117	0.092	0.064	0.077	15
	T4	0.867	1.133	0.103	0.072	0.086	16.67
	Total	0.813	1.088	0.074	0.050	0.062	12.66
M9	Mother plant	0.620	1.000	0.000	0.000	0.000	0
subculture2	T1	1.190	1.320	0.250	0.174	0.209	45.51
	T2	1.228	1.286	0.237	0.162	0.194	40.51
	T3	1.089	1.210	0.176	0.120	0.144	30.38
	T4	0.873	1.125	0.107	0.073	0.098	17.72
	Total	1.000	1.188	0.154	0.106	0.129	26.82

T1: MS medium supplemented with BAP (2 mg/l) and IBA (0.1 mg/l); T2: MS medium supplemented with 2ip (2 mg/l) and IBA (0.1 mg/l); T3: DKW medium supplemented with BAP (2 mg/l) and IBA (0.1 mg/l); T4: DKW medium supplemented with 2ip (2 mg/l) and IBA (0.1 mg/l). Na: number of different allele; Ne: number of effective allele; I= Shannon index; He: expected heterozygosity; UHe: unbiased expected heterozygosity; P%: percentage of polymorphism.

a higher degree of genetic variation among plants in this subculture, compared to the first subculture.

Genetic variations among plants of each treatment are well supported by STRUCTURE analysis (Fig. 6A). For example, in the first subculture, the first plant in T1 (first tissue culture treatment) shows molecular difference with the other 2 plants of the same treatment. This plant differs from the others, both in the frequency of loci (having a higher proportion of loci colored) and also in lacking the loci (yellow color), which is present in other plants. This plant shows similarity in its loci to the mother plant, although differs in the proportions of these similar loci. In case of plants from T2, T3 and T4 (tissue culture treatments 2-4), two of the plants are more alike in loci content and frequency and 1 plant differs from these two, also supporting NJ tree result.

The presence of common bands in the tissue culture regenerated plants was observed in reticulation/network tree (Fig. 2A). Some loci of the parental plant are present only in 5 regenerated plants, while some of its loci are lacking in some other plants. However, some loci from T1 plant occur in some other regenerated plants (dashed lines). Similar analyses in second subculture show that plants of the T2 are having similar ISSR loci but differ in their frequency (Fig. 6B). The same statement is true for other plants in other treatments too, with plant No. 7 differing more from the other plants in T2, and plant No. 9 differing from other plants in T3. Reticulation tree of the second subculture (Fig. 2B) also shows the occurrence of similar loci in T1, T2 and T4 plants.

Malling 9 (M9)

In the first subculture, the highest values of Shannon's Information Index (I) and Expected Heterozygosity occurred in regenerated plants of T4 (0.110 and 0.072, respectively), while, in the second subculture, the highest values of these parameters occurred in T1 (0.250 and 0.174, respectively).

AMOVA test showed significant genetic difference among mother plants and regenerated plants ($p<0.01$) in both subcultures. The results showed that in the first subculture 55% of total variation occurred between populations (treatments) and 45% within populations, while in the second subculture 22% of total variation occurred between the populations and 78% within the populations.

Nei's genetic distance determined for mother plants and

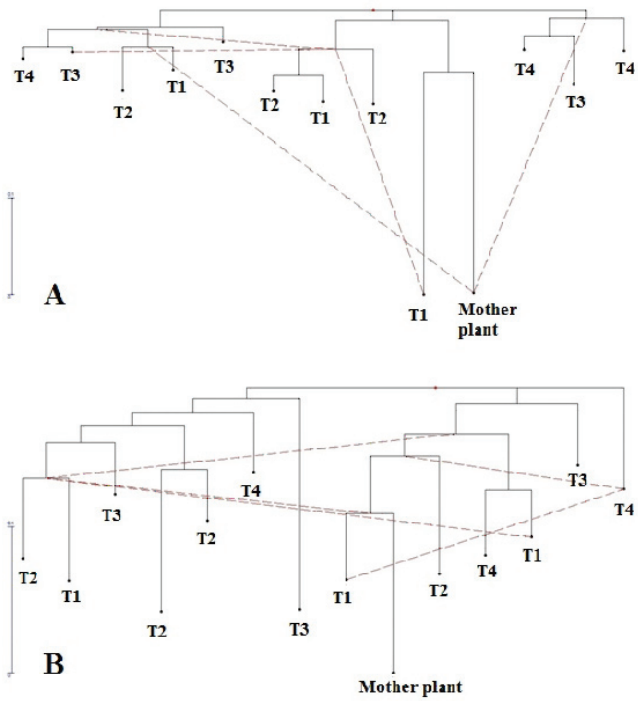


Figure 2. NJ tree and reticulation of M7 plants in first subculture (A) and second subculture (B). Abbreviations T1-T4 are according to Table 1.

regenerated plants (Table 2) showed the highest genetic difference between mother plants and T4 regenerated plants in the first subculture (0.427), while the lowest value of genetic difference occurred between T3 and T4 regenerated plants. The highest and lowest values of this parameter in the second subculture occurred between mother plant and T4 and between T1 and T2 (0.299 and 0.084, respectively).

NJ tree and PCoA plot of the first and second subculture plants are presented in Figs. 4 and 5. In all analyses, the mother plant is well separated from regenerated plants. The regenerated plants show more genetic affinity towards each other but some of the plants inside each treatment differ from the others and are placed in different clusters.

STRUCTURE analysis (Fig. 6C and D) revealed genetic variations between plants of each treatment in both subcultures. These plots also show genetic difference of the mother plants from tissue culture regenerated plants and indicate change in frequency of loci among somaclones obtained.

Reticulated NJ tree (Fig. 4A) displays more common loci between mother plant and tissue culture regenerated plants of the first subculture, while common loci are of less occurrence among the second subculture plants (Fig. 4B) indicating the presence of more extensive genetic changes in these plants. These results are well supported by PCoA plots of both subcultures in M9 plants (Fig. 5). Moreover, PCoA plot (Fig. 5A) shows that the mother plant is well separated from its tissue culture regenerated plants in the first subculture and that tissue culture plants are placed close to each other and show genetic similarity. However, PCoA plot of the second subculture plants (Fig. 5B) shows less distance between mother plant and its tissue culture regenerated plants, but more distance is present among tissue culture plants indicating presence of a higher genetic variation in the second subculture.

Table 2. Pairwise population matrix of Nei genetic distance among mother plants and regenerated plants. Abbreviations T1-T4 are according to Table 1.

		Mother plant	T1	T2	T3	T4
M7	Mother plant	0				
subculture1	T1	0.579	0			
	T2	0.599	0.103	0		
	T3	0.528	0.189	0.063	0	
	T4	0.435	0.169	0.086	0.045	0
M7	Mother plant	0				
subculture2	T1	0.284	0			
	T2	0.403	0.142	0		
	T3	0.596	0.186	0.136	0	
	T4	0.435	0.132	0.104	0.117	0
M9	Mother plant	0				
subculture1	T1	0.377	0			
	T2	0.397	0.086	0		
	T3	0.397	0.123	0.103	0	
	T4	0.427	0.095	0.084	0.037	0
M9	Mother plant	0				
subculture2	T1	0.270	0			
	T2	0.290	0.084	0		
	T3	0.327	0.144	0.114	0	
	T4	0.299	0.201	0.152	0.155	0

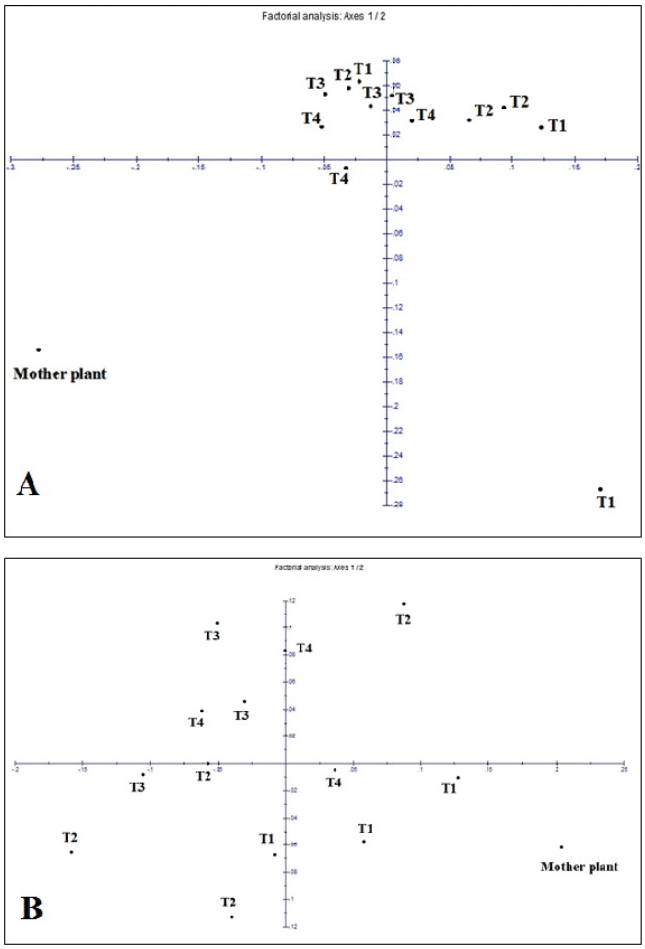


Figure 3. PCoA plot of M7 first (A) and second (B) subculture plants. Abbreviations T1-T4 are according to Table 1.

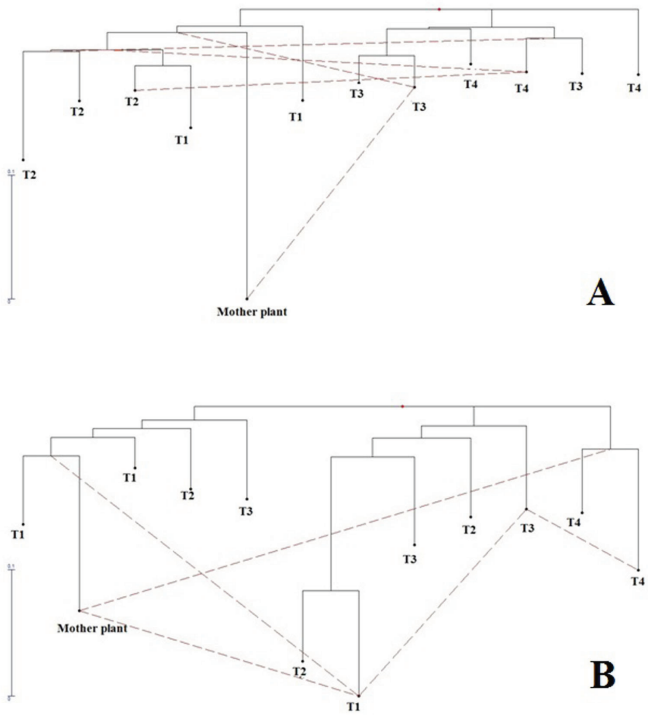
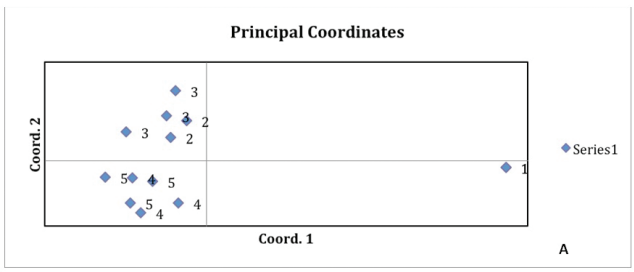


Figure 4. NJ tree and reticulation of M9 plants in first subculture (A) and second subculture (B). Abbreviations T1-T4 are according to Table 1.



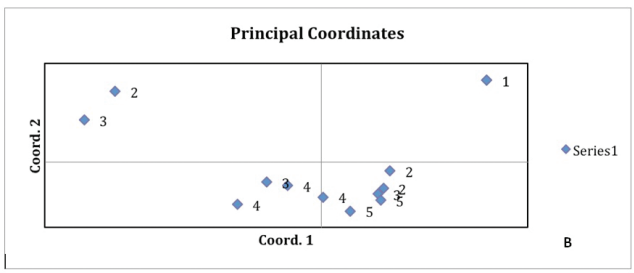


Figure 5. PCoA plots of M9 first (A) and second (B) subculture plants. 1: mother plant; 2-5: treatments 1-4.

Genome size

The ANOVA test suggested significant differences in genome sizes of the investigated plants: it varied between 1.35-4.12 picogram (Fig. 7). The genome size results revealed differences between both the mother plants (M7 and M9), and the tissue culture regenerated plants, as well as between plants of each subculture. Comparison of three readings in genome size for each plant shows significant differences in some cases, *e.g.*, tissue culture plants of the first subculture showed significantly higher mean value of genome size compared to their mother plant M7 ($p<0.05$), while tissue culture plants of the second subculture did not differ in genome size from their mother plant M7. No significant difference was observed in the genome size of M9 mother plant and the mean value of all its tissue culture regenerated plants. However, if we compare M9 with regenerated plants, we observe significant increase in the genome size of some tissue culture plants (No. 12 and 16) (Fig. 7).

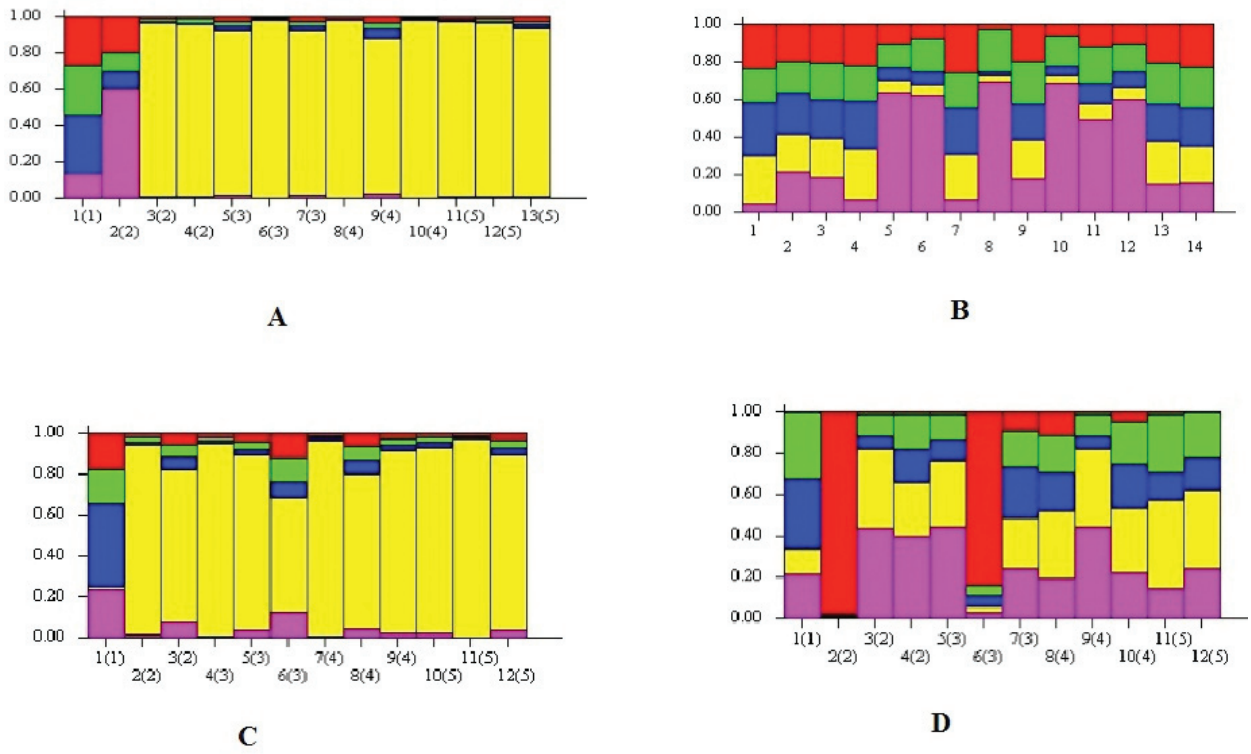


Figure 6. STRUCTURE plots of M7 and M9 first (A) and second (B) subcultures. A: M7 first subculture; B: M7 second subculture; C: M9 first subculture; D: M9 second subculture. No. 1: mother plant; No. 2-4: first treatment; No. 5-7: second treatment; No. 8-10: third treatment; No. 11-13: forth treatment plants.

Discussion

An efficient regeneration system and sufficient genetic variation among regenerates are prerequisites for the use of somaclonal variation for apple breeding (Viršek-Marn et al. 1999). Furthermore, for exploitation of somaclonal variation, knowledge about the amount of variation to be expected from the application of different in vitro techniques is very important.

The presence of ISSR polymorphic bands in the apple tissue culture regenerated plants indicates genetic polymorphism in these genotypes. Genetic and morphological variations among plants of single subculture indicated the occurrence of somaclonal variation. Moreover, the occurrence of specific bands/loci only in some of the regenerated plants illustrates the occurrence of unique insertion/deletion in DNA material of these genotypes, which may be used in planning apple hybridization.

There are reports on genetic fidelity of micropropagated apple plants, *e.g.*, Pathak and Dhawan (2010) used a total of 24 ISSR primers to assess the genetic stability of micropropagated plants regenerated through axillary buds of clonal apple (*Malus domestica* Borkh.) rootstock MM111 after 22 passages. They obtained 147 amplification products with an average of 10 bands per primer. A homogenous amplification profile was observed for all the micropropagated plants and no variation was observed.

Similarly, Gupta et al. (2009) studied genetic diversity among axillary bud and shoot apices regenerated plants of EMLA 111 (East Malling Long Ashton), a clonal rootstock of apple (*Malus pumila* Mill.) via RAPD analysis. The similarity matrix based on Jaccard's coefficient revealed that pairwise value between the mother plant and its tissue cultured plants ranged from 0.93 to 1.00. Among tissue cultured plants it was 0.92 to 1.00, thus indicated a high degree of genetic fidelity. However, there are some other studies showing the occurrence of induced genetic variation through tissue culture in apple cultivars. For example, Modgil et al. (2012) identified apple Malling 7 somaclones among regenerated plants obtained from leaf explants, which were resistant to white root rot disease. In their previous report (Modgil et al. 2005), they described genetic polymorphism (revealed by RAPD analysis) among axillary bud regenerated plants of apple rootstock MM106.

The groupings made by NJ tree and PCoA plot of the

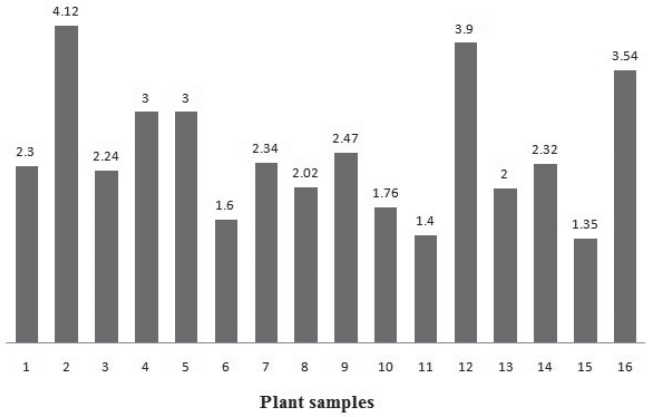


Figure 7. Genome size (pg) in apple plants studied. 1: M7 mother plant; 2-6: M7 first subculture plants; 7-8: M7 second subculture; 9: M9 mother plant; 10-12: M9 first subculture plants; 13-16: M9 second subculture plants.

subcultures 1 and 2 indicate the genetic changes among the mother plant and tissue culture regenerated plants. This is further supported by the occurrence of new ISSR bands/loci in the regenerated plants, which were not observed in parental plants.

The differences revealed by STRUCTURE analysis among plants of each treatment, suggest that genetic variations could occur even among individual plants of each subculture. This shows that genetic changes emerging during tissue culture cannot be predicted. This means that mutations could take place which enrich the existing diversity and gene pool of the apple plant to be used in further breeding.

In many somaclonal studies, performed on different plant species including cotton (Sheidai et al. 2008), olive (Peyvandi et al. 2010; Farahani et al. 2011), and banana (Sheidai et al. 2008), there are reports that genetic diversity among regenerated plants increase by an increase in the number of subcultures. The reticulated NJ tree obtained in the present investigation also supports such idea, as more common loci were observed among mother plant and tissue culture regenerated plants of the first subculture, while much lower number of similar loci was observed among the second subculture plants therefore, higher degree of genetic changes has occurred in these plants. This conclusion is also supported by the higher values of Shannon information index and expected heterozygosity observed in the regenerated plants of second subculture in both M7 and M7 genotypes.

The detected differences between the genome sizes of the regenerated plants suggest that changes in genetic structure of plants during tissue culture are also accompanied with quantitative changes of their DNA. The range of genome size we obtained here varied from 1.35 pg to 4.12 pg. Such variations have also been reported in cultivated apple plants. Tatiana et al. (2005) studied nuclear DNA content in *Malus* species and cultivated apples (*Malus domestica* Borkh.) by flow cytometry and reported a range of 1.45 pg for *Malus fusca* (diploid) to 2.57 pg for *Malus ioensis* (triploid). They also noticed a variation of the genome size among diploids (1.45-1.68 pg) and among triploids (2.37-2.57 pg). They concluded that given the complexity of the apple genome and its suggested allopolyploid origin, their study confirms the results of earlier reports that polyploids can easily withstand the loss of a certain amount of DNA, and that there is a slight tendency towards diminished haploid nuclear DNA content with increased polyploidy.

The reports on genome size change during somaclonal variation vary in different plant species. For example, Zonneveld and Pollock (2012) studied somaclonal variation in 124 selected cultivars of Hosta Tratt. (Hostaceae) by flow cytometry. They compared the measured 2C-values and ploidy levels of regenerated and the parent plants, and reported occasional changes from diploid to tetraploid level during tissue-culture propagation; both fully tetraploids and periclinal chimeras, such as partial tetraploids were obtained. Moreover, they noticed that nuclear DNA of some diploids increased with incomplete chromosome sets resulting in fully aneuploid plants. Finally, they concluded that the incident of chromosome losses or gains is an important source of new Hosta cultivars.

Similarly, Jin and coworkers (2008) investigated molecular (RAPD and SSR), cytogenetic and genome size variation in 67 regenerated cotton plants and observed that 2,4-D and kinetin hormone combination could induce relatively high somaclonal variation. Chromosome number counting and flow cytometry analysis revealed that the number of chromosomes and ploidy levels were nearly stable in all regenerated plants except two regenerated plantlets (lost 4 and 5 chromosomes, respectively) which meant that cytological changes were not correlated with the frequency of RAPD and SSR polymorphisms. This result also might mean that the cell lines with variation of chromosome numbers were difficult to regenerate plants. However, Purwantoro et al. (1999), while studying somaclonal variation in Easter lily (*Lilium longiflorum* Thunb.), although reported molecular variation (RAPD) among regenerated plants, they did not see any karyotype changes in them. Leal et al. (2006) also investigated genome size of somatic embryo-derived plants obtained from another culture in *Vitis vinifera* cultivars and found that only one among the 41 analyzed plants (2.4%) presented somaclonal variation (tetraploidy); the other plants were diploid and no significant difference occurred in the nuclear DNA content of these plants.

Somaclonal variation has been found to be associated with various types of genetic changes, such as numerical and structural aberrations of chromosomes, deamplification and amplification of genes, single gene mutations, activation of

transposable elements, and hypo- and hypermethylation of DNA (Purwantoro et al. 1999). Our present study revealed that somaclonal variation in apple rootstocks M7 and M9 are associated with molecular (qualitative) and genome size (quantitative) changes, therefore, such variations may be used in the breeding of this important tree.

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