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Somaclonal Variation in Micro-propagated Strawberry Detected at the Molecular Level

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ABSTRACT

Meristem tips of three Strawberry cultivars, namely Chandler, SweetCharlie and Gaviota were excised and cultured for 5 weeks on the starting medium. Subculture was carried out for five weeks on shooting medium, and finally shoots transferred to the rooting medium. The obtained plantlets were acclimated under greenhouse condition in medium consisting of peat moss and sand (2:1). DNA extracted from *in vitro*-derived plantlets and standard propagated plants were analyzed by RAPD-PCR to detect possible drift in genetic stability of micro-propagated plants. Most of the obtained bands from *in vitro*-derived plantlets in all primers used with the three cultivars were found to be present in the fingerprints of standard propagated plants, demonstrating no variation in the pattern obtained with DNAs from the two sources of strawberry plants. It is concluded that mass propagation via meristem tip culture is reliable in producing genetically similar plants to the mother ones.

Key Words: *Fragaria x ananassa;* Meristem tip; Micropropagation; Somaclonal variation; RAPD-PCR

INTRODUCTION

One of the important goals of the agricultural policy in Egypt is to increase the acreage of strawberry to meet the demand of local fresh market, processing and export. Importing mother plants is expensive. Healthy stocks used for propagation through conventional methods are not available. The *in vitro* culture of meristem has been successful in the mass propagation of strawberry plants in some countries. It was estimated that several millions of plants can be produced within a year from a few mother plants by tissue culture technique (Boxus, 1983). This technique is useful in case of the introduction of new cultivars. Moreover, the storage of tissue cultured propagules requires less space than traditional runner plant and the *in vitro* storage can be initiated at any time during the production cycle (Swartz *et al*., 1981). Another advantage of micro-propagation is the elimination of pest and pathogen stress during the production cycle, assuming that the initial stock plant is free of diseases. Therefore tissue culture technique was applied to evaluate its feasibility for a wider use. Several authors have dealt with this subject from several aspects. Boxus (1983) reported that each m^2 of growing area of strawberry can produce 40000 plantlets $year^{-1}$, but Abramenko (1983) has cultured apical meristem of strawberry and found that from one meristem 3000 shoot primordial were produced in 6 months and half of these developed into normal plants. These plants were vigorous and after transplanting in the soil some produced up to 500 new runner plants and found that new runner plants were 10 times that produced by conventional material. The production program suggested by Vit *et al*. (1983) for commercial planting material is based on the selection of economically important clones; freeing these of virus by a combination of heat therapy and meristem culture. Tissue culture aimed particularly at the rapid propagation of breeding material as briefly described with reference to strawberry by (Zimmerman, 1981).

However, through micro-propagation of strawberries (*Fragaria x ananassa*, Duch.), several morphological abnormalities were detected as somaclonal variation. Changes include leaf color variants and dwarf plants, among others (Swartz *et al*., 1981; Sansavini *et al*., 1990; Irkaeva & Matveena, 1997; Morozova, 2003). Callus-derived "Redcoat" strawberry plants differ significantly from standard runner plant for several vegetative characteristics (Nehra *et al*., 1992). These variants pose a problem for production of uniform, true-to-type plants. Nehra *et al*. (1994) found that two cultivars of strawberry responded differently to various forms of *in vitro* propagation and in both cases variants were found in callus-derived plantlets, but not those derived from meristems or via direct leaf regeneration. Some studies have shown that modified characteristics are epigenetic and disappear over time (Koruza & Jeleska, 1993). Numerous authors have reported that genetic changes including insertions, deletions, point mutations and rearrangements occur during tissue culture (Kane *et al*., 1992), but few of the phenotypic symptoms found are heritable (Karp, 1995; Kumar *et al*., 1999). Most somaclonal variations occur in plants regenerated from cultures that have undergone a differentiation phase. Temporary variations may be due to methylation changes in the DNA. Methylation pattern changes have been detected in suspension cultures of soybean [*Glycine max* (L.) Merr.] and maize (*Zea mays* L.) callus culture regenerates (Kaeppler & Phillips, 1993). The objective of this

investigation was to compare between standard propagated and *in vitro*-derived plantlets as detected by RAPD molecular markers in three strawberry cultivars.

MATERIALS AND METHODS

This investigation was carried out at the Plant Biotechnology Department, National Research Center, Dokki, Giza, Egypt.

Source of plant materials. Two kind of plant materials of Chandler, Sweet Charlie and Gaviota strawberry cultivars were used in this investigation as the following:

1- Micro-propagated mother plants (MP) produced in *in vitro*.

2- Frigo plants of strawberry propagated by traditional methods (TM) in the greenhouse.

Strawberry micro-propagation was performed using the four stages procedures:

A. Initiation stage. Cultures were initiated from shoot tip consisting of meristem plus 2 or 3 leaf primordia, which were dissected from stolon of greenhouse-grown plants. The tips were sterilized prior to dissection by immersion in 50% Clorox solution (commercial bleach) for 15 min followed by four washes in sterile distilled water. They were then cultured in 15 x 2.5 cm, test tubes each amended with 10 mL of culture medium (Table I) and closed with aluminum foil.

B. Rapid multiplication stage. Meristem-derived plantlets were multiplied by sequential monthly subculture into fresh medium in 300 mL jar each with 30 mL of culture medium (Table I) and closed with plastic caps. At this stage of culture, the shoots proliferated rapidly in all varieties within one month. Four subcultures were then conducted.

C. Rooting stage. Obtained shoots were divided aseptically and subsequently rooted by placing (10 shoots/jar) in 30 mL of culture medium (Table I) contained in 300 mL jar. After three weeks rooted plantlets were obtained. All cultures were maintained in a growth room at 25 ± 1ºC with 16 h light daily (1000 lux).

D. Acclimatization stage. All rooted plantlets were removed from cultural jar and washed thoroughly with tap water to remove the remains of agar from the root system, then transplanted into plastic pots (6 cm) containing a soil mixture of 2 peat moss: 1 sand (v/v). The pots were enclosed in polyethylene bags to minimize moisture loss. The bags are opened gradually for conditioning to greenhouse environment.

Traditionally propagated Frigo plants were also allowed to grow in plastic pots (6 cm) in the greenhouse under the same conditions of those produced by micro-propagation.

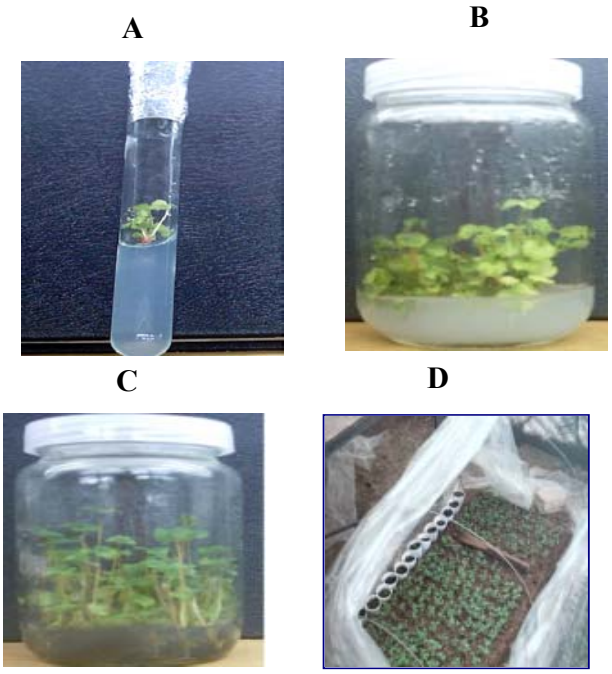
Genomic DNA extraction. Genomic DNA was isolated on a mini-prep scale as mentioned by Murray and Thompson (1980). Small pieces (0.5 g) of leaf tissue of *in vitro*-derived plantlets (MP) and cold stored (TM) plants were frozen in liquid nitrogen in Eppendorf tubes and homogenized in 500 µL of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-HCl, pH 8.0, 0.1 M ß-Mercatoethanol). The extract was incubated at 60ºC for 20 min. To this 500 µL of phenol: chloroform: isoamyl alcohol (24: 24: 1) were added and mixed by vortexing for 30 sec followed by centrifugation at 10,000 *g* for 5 min at room temperature. The aqueous phase was transferred to another tube. This was once again extracted with 500 µL of chloroform: isoamyl alcohol (24: 1) in Eppendorf tube. To the aqueous phase, 0.6 volume of isopropanol were added, precipitated the genomic DNA and spooled the fibrous genomic DNA. Genomic DNA was then washed three times with 70% ethanol, dried in vacuum, dissolved in TE containing 10 mg mL^{-1} RNase and incubated at 37ºC for 30 min. followed by extraction with phenol: chloroform: isoamyl alcohol and the aqueous phase was transferred to a fresh tube. There-after the genomic DNA was precipitated

Table I. Chemical composition of culture media of strawberry micro-propagation in MS medium Murashige and Skooge(1962)

Additions	Initiation stage	Shooting stage	Rooting stage
BA (mg/ml)	0.1	0.5	0.0
IBA (mg /ml)	0.0	0.0	1.0
Sucrose (g /l)	30.0	30.0	30.0
Agar (g /l)	7.0	7.0	7.0

MS = Murashigo and Skoog (1962). BA = 6-Benzyladenine. IBA = Indol butyric acid.
-The pH of the medium was adjusted before autoclaving to 5.7
-All media sterilized by autoclaving at 121ºC /20 min.

Fig. 1. Stages involved in large-scale micro-propagation of strawberry, (A) Initial plantlets from meristem tip, (B) Multiplication stage, (C) Rooting stage, (D) Acclimatization



by adding 0.3 M sodium acetate, pH 5.2 (final concentration) and 2.5 vol of ethanol and collected by centrifugation at 10,000 x g for 20 min at 4ºC. The pellet was washed with 70% ethanol, vacuum dried and dissolved in TE.

PCR condition. Seven random oligonucleotide (10 mer) primers (Operon technologies Inc., Alameda, California) were designed for use in RAPD analysis. The primers are OPK01 (5′ TGC CGA GCT G 3′), OPK02 (5′ GTG AGG CGT C 3′), OPK03 (5′ CCC TAC CGA C 3′), OPK04 (5′ TCG TTC CGC A 3′), OPK05 (5′ CAC CTT TCC C 3′), OPK06 (5′ GAG GGA AGA G 3′) and OPK07 (5′ CCA CAG CAG T 3′).

The PCR reactions were carried out in 50 µL volumes containing 100 ng of genomic DNA, 1.0 µM of each primer, 200 µM of dATP, dDTP, dCTP, dGTP, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin. The Taq DNA polymerase concentration was 1.5 units per assay. PCR reaction was conducted using a Perkin Elmer 2400 (Germany) thermocycler programmed for 45 cycles as follows: 94ºC/5 min (1 cycle), 94ºC/30 sec, 36ºC/30 sec, 72ºC/2 min (45 cycle) and 72ºC/7 min (1 cycle), then held at 4ºC. The amplification products of PCR were size-separated by gel electrophoresis in 1% agrose gels with 1 x TBE buffer using a Pharmacia G N. 100 submarine gel electrophoresis apparatus and stained with ethidium bromide and visualized with UV transilluminator and photographed. A 100 bp DNA ladder (Promega) was used as a standard with molecular sizes of 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp. DICE computer package was used for the analysis of RAPD-PCR products (Yang & Quiros, 1993).

RESULTS AND DISCUSSION

Strawberry micro-propagation. Our observation indicated that the strawberry cultivars differed in mass of shoots in every subculture. Generally, the average number of shoots/jar depended on the number of the subculture and cultivars. This result confirms the findings of Boxus (1974). All shoots formed roots after transferring to the rooting medium with differences in number of roots and root length, depending on the cultivar. Therefore it might be concluded that this rooting medium would be suitable for root formation in case of these three cultivars. These results confirm the finding of Popov and Trushechken (1972) and Abramenko (1983), whose indicated the importance of auxins for root formation. Meristem-derived rooted plantlets were planted in medium consisting of peat moss + sand (2:1), where most of the plantlets survived in the greenhouse.

Molecular analysis. In our study, two kinds of plant materials were used, *in vitro*-derived plantlets and cold-stored plants derived via traditional propagation by runners. Genomic DNAs of both materials were extracted and compared by RAPD-PCR, using random oligonucleotide primers. Of the seven primers, three (OPK01, OPK02 & OPK03) successfully yielded 111 bands across the cultivars tested. The other primers were not as efficient as the three mentioned primers in generating successive PCR products. Size ranged from 175 - 780 bp depending on the primers and cultivars. For computer analysis to detect the pair-wise differences between the two materials, intensive bands were considered as present (1), while weak or absent bands were considered as absent (0). Table II and Fig. 2 show the reaction of primers OPK01, OPK02 and OPK03 with the two different materials in the cultivar Sweet Charlie. All three primers yielded 41 different bands with size ranged from 180 to 761 bp. Five bands out of them were polymorphic. Their sizes were 720 bp of OPK01, 650 and 371 bp of OPK02 and 761 and 720 bp of OPK03. The percentage of polymorphism was 12.2% in average across the three primers.

In case of cultivar 'Chandler' primers, OPK01, OPK02 and OPK03 produced 29 different DNA bands with sizes ranged from 386 to 761 bp. Out of them three were polymorphic. Their sizes were 690 bp of OPK01 and 686 and 620 bp of OPK03, but OPK02 produce no polymorphic bands (Table III & Fig. 2).

On the other hand, the succeeded primers (OPK01, OPK02 & OPK03) in case of cultivar Gaviota produced 41 different DNA bands ranged in size from 175 to 780 bp. Out of them, five were polymorphic, their sizes were 660 bp of OPK01, 510,500and 400 bp of OPK02 and 350 bp of

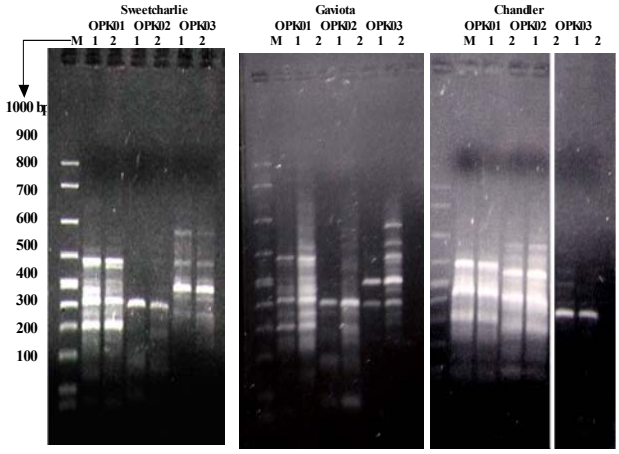
Table II. Amplified fragments obtained from the DNAs of *in vitro*-derived plantlets and traditionally propagated plantlets of strawberry cv. Sweet Charlie via RAPD-PCR

Primers	Size of bands (bp)	Source of plants	
		In vitro plantlets	Traditionally propagated plantlets
OPK01	720	0	1
	684	1	1
	670	1	1
	600	1	1
	584	1	1
	557	1	1
	450	1	1
	409	1	1
	371	1	1
Total		8	9
OPK02	650	0	1
	500	1	1
	450	1	1
	409	1	1
	371	0	1
	298	1	1
	180	1	1
Total		5	7
OPK03	761	0	1
	720	0	1
	700	1	1
	600	1	1
	531	1	1
	486	1	1
	371	1	1
Total		5	7

Table III. Amplified fragments obtained from the DNAs of *in vitro*-derived plantlets and Traditionally propagated plantlets of strawberry cv. Chandler via RAPD-PCR

Primers	Size of bands (bp)	Source of plants	
		In vitro plantlets	Traditionally propagated plantlets
OPK01	695	1	1
	690	0	1
	620	1	1
	600	1	1
	530	1	1
	400	1	1
Total		5	6
OPK02	761	1	1
	750	1	1
	650	1	1
	620	1	1
	590	1	1
	386	1	1
Total		6	6
OPK03	720	1	1
	686	1	0
	620	1	0
	600	1	1
Total		4	2

Fig. 2. RAPD-PCR polymorphism of DNA three cultivars of strawberry using OPK01, OPK02 and OPK03. 1- *In vitro*-derived plantlets., 2- Traditionally propagated plantlets., M- 1kb DNA ladder (Promega)



OPK03 (Table IV & Fig. 2), with 12.2% polymorphism. Table V summarizes the relevant results of the three. Furthermore most of the obtained bands from *in vitro*-derived plantlets in primers used with the three cultivars are confirmed to be present in the fingerprints of cold storage (TM) plants and the percentage of polymorphism of 12.2, 10.3 and 12.2 in Sweet Charlie, Chandler and Gaviota, respectively. It means in all cases, that the RAPD fingerprints produced with different primers were almost identical. The results of the present finding are in agreement with Castiglione *et al*. (1993), who found no RAPD fingerprint variation when different plants of the same clone

Table IV. Amplified fragments obtained from the DNAs of *in vitro*-derived plantlets and Traditionally propagated plantlets of strawberry cv. Gaviota via RAPD-PCR

Primers	Size of bands (bp)	Source of plants	
		In vitro plantlets	Traditionally propagated plantlets
OPK01	683	1	1
	660	0	1
	505	1	1
	480	1	1
	420	1	1
	350	1	1
	300	1	1
	250	1	1
	175	1	1
Total		8	9
OPK02	510	1	0
	500	0	1
	400	0	1
	300	1	1
	200	1	1
Total		3	4
OPK03	780	1	1
	750	1	1
	730	1	1
	700	1	1
	610	1	1
	600	1	1
	560	1	1
	500	1	1
	350	0	1
Total		8	9

of *Populus* species. Among the various methods developed to micro-propagate plants, enhanced axially branching culture has become the most important propagation method. This method is especially advantageous, because it is simple and the propagation rate is relatively high (Pierik, 1991). More importantly, it is generally considered to be an *in vitro* culture system with low risk of genetic instability (Pierik, 1991; Schoofs, 1992), because the organized mersitems are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions (Shenoy & Vasil, 1992). Notwithstanding this consideration, there are numerous reports on the incidence of somaclonal variation among micro-propagated plants. For example, reports have indicated the occurrence of somaclonal variation in micro-propagated bananas and plantains (Schoofs, 1992) raised through meristem culture. Various kinds of leaf chlorosis coupled with multiple apexing and dwarfing in strawberry (Martinelli, 1992) more jagged and pubescent leaves in grapevine (Grenan, 1992), spininess and albino strips in pineapple (Moore & Dewald, 1992)

In conclusion, the present investigation indicated that mass propagation via tissue cultures produces clones genetically similar to the mother plants. It also conclude that RAPD approach is convenient, fast and reproducible to detect the presence of genetic variation associated with tissue culture of strawberry.

Table V. Primers with arbitrary sequence tested for their effectiveness in the RAPD-PCR analysis that produced polymorphic bands of strawberry

Cultivars	Primer	Number of bands in the gel		Size of the plymorphic bands (bp)	% of polymorphism
		Total	Polymorphic		
Sweet- Charlie	OPK01	17	1	720	5.9
	OPK02	12	2	650,371	16.7
	OPK03	12	2	761,720	16.7
					12.2
Chandler	OPK01	11	1	690	9.1
	OPK02	12	0	-	0.0
	OPK03	6	2	686,620	33.3
					10.3
Gaviota	OPK01	17	1	660	5.9
	OPK02	7	3	400 ,500,510	42.9
	OPK03	17	1	350	5.9
					12.2

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