World Applied Sciences Journal 19 (5): 674-687, 2012
ISSN 1818-4952;

DOI: 10.5829/idosi.wasj.2012.19.05.2770

In vitro Propagation of Apricot (*Prunus armeniaca* L.) and Assessment of Genetic Stability of Micropropagated Plants Using RAPD Analysis

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Abstract: A complete protocol for micropropagation of apricot (*Prunus armeniaca* L) locally known as cultivar El-Hamawey using shoot meristems was developed. Ethanol and NaOCl were applied in several combinations for disinfection. Meristem was isolated from actively growing plants in the spring and cultured on five kinds of basal media (MS, $^{1}/_{2}$MS, WP, B5 and Q&L) with plant growth regulators. The survival rate of meristem tips was 89.33%, average number of shoots/explant (3.98) and average shoot length/explants (1.85cm) on WP medium supplemented with 1.0 mg/L Zeatin, 0.1 mg/L IAA in the presence of 100 mg/l ascorbic acid and 150 mg/l citric acid of explants taken in spring compared to the other season. The highest average new number (5.37) of shoot multiplication was obtained with WP supplemented with 4 mg/l BA and 0.5 mg/l 2ip and the highest average length of shoots (3.24 cm) was cultured on WP obtained with 1mg/LBA and 0.5mg/L 2iP. The highest percent rooting (91%) of adventitious shoots, mean number of roots (7.64) and mean length of roots (3.43cm) were maintained for 7 days in the dark on MS medium obtained with 2 mg/L IBA and 0.5 mg/L NAA before transfer to a 16 hour photoperiod. In total, 73% of plantlets survived acclimatization to greenhouse. Random amplified polymorphic DNA (RAPD) markers were used to evaluate the genetic stability of micropropagated plants of *Prunus armeniaca* L cv. El-Hamawey. All RAPD profiles from micropropagated plants were monomorphic and similar to those of field grown control plants. No variation was detected within the micropropagated plants. The utilization of RAPD markers for the assessment of genetic stability of clonal materials and to certify genetic stability throughout the systems of micropropagation is discussed.

Key words: Apricot · *Prunus armeniaca* L. · El-Hamawey · *In vitro* propagation · RAPD

INTRODUCTION

Apricot (*Prunus armeniaca* L.) is the second most important species of the stone fruits, although far behind peach and very much geographically located by its peculiar ecological needs. During the last years the world production of apricot fruit has oscillated around 2.5 million tons and more than half of world production concentrated in the Mediterranean basin countries. Apricot is well suited for *in vitro* propagation because cuttings are difficult to root and scions are currently budded onto apricot seedlings [1]. Therefore, growing apricots on their own roots appears to be a logical undertaking. Most commercial production of micropropagated fruit trees has been focused on the production of rootstocks. Although grafting of scions onto seedling rootstocks is a common practice, the production of scions on their own roots has been limited. To our knowledge, apricot cultivars have never been commercially micropropagated and also apricot rootstocks [2]. The literature on *in vitro* culture of apricot however is very scanty compared to that on other *Prunus* species [3, 4, 5]. Most of the organisms that are encountered are of no particular importance to the plant *in vivo*, but result in contamination when cultured *in vitro* because bacteria and fungal spores will grow rapidly on the rich culture medium. Consequently, the first step in preparing a plant for tissue culture is to eliminate microorganisms. Contamination of apricot shoots is a serious problem in tissue culture since pathogens that are of no importance for mother trees can cause heavy losses and serious debasement of the plant material when transferred *in vitro*. Meristem culture is often useful for this purpose because most organisms are eliminated [6, 7]. Depending on meristem size, viruses also can be eliminated. Hence, they are often used as initial explants for

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large-scale micropropagation [8]. Recently, many fruit trees have been successfully propagated *in vitro*. Moreover, the establishment of culture methods is indispensable for the application of biotechnological techniques for cultivar improvement of apricot. Plant growth regulators are very important among determining conditions. RAPD has proven to be quite efficient in detecting genetic variations [9]. At present, RAPD marker has been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various plants [10]. A few papers have been published on micropropagation of apricot cultivars but they are limited to European-type cultivars [4, 5]. No information on micropropagation of apricot cv. El-Hamawey. El-Hamawey cultivar is more tolerance to drought and salinity than other cultivars. Here we describe a protocol containing different steps necessary to micropropagate apricot cultivar El-Hamawey. Also, the present study was undertaken to determine the genetic stability of the micropropagated plants of apricot cv. El-Hamawey by RAPD analysis.

MATERIALS AND METHODS

In vitro Propagation of Local Apricot Plant

Plant Material: Plant material used to introduce apricot cultivar El-Hamawey grown in El Wadi El-Gedeed region of Egypt, through shoot tips are obtained between October and April. Shoot tips are removed from adult apricot field-grown trees.

Explants Preparation and Sterilization: Shoot tip explants were taken from mature apricot trees cv. El-Hamawey and stored at 5°C. Explants were washed with tap water for two hours and were soaked in 100 mg/L ascorbic acid and 150 mg/L citric acid for one hour before surface sterilization followed by 10 min. immersion in 1% fungicide (Neotoxin) containing three drops of Tween 20/500 ml. Seven treatments were evaluated for the disinfection of the plant material: (a) One min. Immersion in 70% ethanol and then 10 min. immersion, with agitation, in 0.75% NaOCl containing three drops of Tween 20/500 ml. (b) One min. immersion in 70% ethanol and then 15 min. immersion, with agitation, in 0.75% NaOCl containing three drops of Tween 20/500 ml. (c) One min. immersion in 70% ethanol and then 20 min. immersion, with agitation, in 0.75% NaOCl containing three drops of Tween 20/500 ml. (d) Spray with 70% ethanol and then 10 min. immersion, with agitation, in 1.0% NaOCl containing three drops of Tween 20/500 ml. (e) One min. immersion in 70% ethanol and then 10 min. immersion, with agitation, in 1.0% NaOCl containing three drops of Tween 20/500 ml. (f) One min. Immersion in 70% ethanol and then 15 min. immersion, with agitation, in 1.0% NaOCl containing three drops of Tween 20/500 ml. (i) One min. immersion in 70% ethanol and then 20 min. immersion, with agitation, in 1.0% NaOCl containing three drops of Tween 20/500 ml. Finally shoot tips were rinsed three times with sterile water.

Meristem tips were dissected from disinfected shoot tips. The meristem tip explants, composed of the apical dome and a few leaf primordia, were then excised and explanted. The explant size averaged from 0.5 to 1 mm tall. The explants were isolated under a binocular microscope on March 20. Thirty meristem isolated from shoot tip explants were used for each treatment. Meristem explants were placed on WP medium containing 1.0 mg/L Zeatin, 0.01 mg/L IAA, 3% sucrose and 2.5 g/l phytagel in the presence of 100 mg/l ascorbic acid and 150 mg/l citric acid. Survival rate, number of shoots and length of shoots per explants were evaluated one month later.

Screening of Basal Medium for Meristem Tip Culture: Five media were screened for meristem culture; MS [11], WP [12], half strength of MS medium, B5 [13] and Q&L macronutrients [14] basal salt medium. All media were supplemented with 1.0 mg/L Zeatin, 0.1 mg/L IAA (indole acetic acid), 3% sucrose, 100 mg/L myoinsitol and 2.5 g/l phytagel in the presence of 100 mg/L ascorbic acid and 150 mg/L citric acid and the pH was adjusted to 5.7±0.1. Medium was dispensed into 25 x 150 mm culture tubes, which were covered with permeable membrane caps and sterilized at 121°C for 20 min. fifteen explants were used for each medium. In all experiments, cultures were maintained at 26°C under a 16 hr-light/8 hr-dark with a light intensity of 2000-3000 lux from white fluorescent light. After 45 days of culture, survival rate and formation of shoots/explant and shoot length were recorded.

Effect of Seasonal Variations on the Survival Percentage: This experiment involved the study of the effect of seasonal variations on the survival percentage of the meristem tips explants of the apricot (*Prunus armeniaca* L.) cultivar El-Hamawey. This was achieved by using the best medium for meristem tip explants.

Effect of Different Concentrations of Growth Regulators on Shoot Proliferation: Sterilized meristem tips of apricot were cultured on WP medium containing 100 mg/L myoinsitol, 30 g/L sucrose and 2.5 g/L phytagel in the

presence of 100 mg/L ascorbic acid and 150 mg/L citric acid. These media supplemented with plant growth regulators at various concentrations: BA at 1.0- 2.0 mg/L, Zeatin at 0.5- 2.5 mg/L, 2iP at 0.5- 1.5 mg/L, TDZ at 1.0- 2.0 mg/L in combinations with IBA at 0.01-0.25 mg/L and IAA at 0.01-0.25 mg/L for shoot formation. In addition, the WP salts without growth regulators were used as a standard apricot proliferation medium.

Shoot Multiplication: In the present investigation attempts were made to standardize a reproducible protocol for *in vitro* clonal propagation of apricot (*Prunus armeniaca* L.) cultivar El-Hamawey. When required subcultures were carried out at regular intervals for the formation of large number of shoots from a single culture. Several plant growth regulators (PGRs) were added to the culture medium at various concentrations: N6 Benzyladenine (BA) alone or in combinations with, 2-isopentenyl adenine (2ip) and Thiadiazuron (TDZ) on WP medium for optimizing multiple shoots regeneration. Explants were subcultured every five weeks in glass jars containing 50 ml of WP medium with different concentrations of growth regulators. After five weeks, the number and length of new shoots were recorded.

Shoot Elongation: Clusters of shoots and small shoots were transferred to the WP medium supplemented with different concentrations (0.5, 1.0 and 1.5 mg/L) of Gibberellic acid (GA_3) alone or different concentrations (0.5, 0.75 and 1.0 mg/L) of 2-isopentenyl adenine (2ip) were incubated at the same conditions similar to those in the above experiments. In addition, the WP salts without growth regulators were used as a standard apricot elongation medium.

Rooting and Acclimatization: In the first experiment, shoots were culture on WP basal medium containing different types of auxins at different concentrations, IBA (3-indole-butyric acid at 1.0, 1.5 and 2 mg/L) alone or in combinations with NAA (Napthaleneacetic acid at 0.5 and 1.0 mg/L). The rooting percentage, number of roots and mean rooted shoots were recorded. After 30 day in culture on rooting medium. In the second experiment, shoots were cultured on full strength MS salts with 3% sucrose, 100 mg/L myoinsitol and 40 mg/L phloroglucinol (liquid medium), containing different concentrations of IBA (1.0, 1.5 and 2.0 mg/L) alone or in combinations with NAA at 0.5 and 1.0 mg/L in the dark for 7 days, After induction period, shoots were transferred to a hormone-free medium under a 16 h photoperiod for two weeks and then shoots were transferred to a rooting medium under a 16h for two weeks. After 35 days of culture on rooting medium, the effect of the different concentrations of auxins (IBA and NAA) on rooting percentage, number of roots and mean rooted shoots were evaluated in comparison to a hormone free medium. In all experiments 100 ml of media were dispensed in 500 ml vials. At least three replicates with 12 shoot explants each were used for each treatment. Shoot explants derived from elongation stage were at least 3-5 cm length, the basal callus was eliminated and several shallow longitudinal cuts were made at the base of each shoot in order to increase absorption of auxins. Cultures were incubated under light and temperature conditions similar to those in the above experiments. Plantlets produced from rooting stage were transferred to pots containing a mixture media of peat and sand (1:1); plastic pots enveloped in polyethylene bags were incubated under 3000 Lux light intensity derived from cool white fluorescent lamps for 16 hours photo period at 26±1°C in growth cabinets for 10 days and then transferred to greenhouse. The plastic that covered the tunnel was opened gradually from a few minutes a day until normal greenhouse conditions could be maintained without desiccation of the plants. The acclimatization period was 15-20 days, depending on external climatic conditions.

Statistical Analysis: All experiments were arranged in completely randomized designed. Each treatment contained three replicates and all the best treatments were repeated twice. Significant differences among the various treatments were compared using Duncan's methods according to Snedecor and Cochran [15].

DNA Extraction: DNA was extracted from fresh leaves of micropropagated and field grown plants by the CTAB method [16]. Approximately, 200 mg of fresh leaf were ground to a powder in liquid nitrogen, using a mortar and pestle. The powder was transferred to a 25 ml sterile Falcon tube with 10 ml of CTAB buffer. The extraction buffer consisted of 2% (w/v) CTAB (cetyltrimethyl amonium bromide, Sigma), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 9.5 and 0.2% (v/v) *b*-mercaptoethanol. The homogenate was incubated at 65°C for 1h, extracted with an equal volume of chloroform and centrifuged at 10,000 rpm for 20 min. DNA was precipitated from the aqueous phase by mixing with 1/10 volume (ml) of 3 M sodium acetate and an equal volume of isopropanol. After centrifugation at 10,000 rpm for 10 min, the DNA pellet was washed with 70% ethanol; air dried and

Table 1: Effect of disinfection treatment on the survival rate, contamination rate and number of shoots/explant of apricot cv. El-Hamawey with meristem tip explants

Treatment with 70% ethanol	Time of immersion in NaOCl 0.75% (min)	Survival rate (%)	Contamination rate (%)	Number of shoots
1 min. immersion	10	91.76	34.33	1.1[b]
1 min. immersion	15	89.33	23.72	1.2[b]
1 min. immersion	20	80.34	13.53	2.1[a]
Treatment with 70% ethanol	Time of immersion in NaOCl 1.0% (min)	Survival rate (%)	Contamination rate (%)	Number of shoots
Spray	10	76.66	26.66	2.2[a]
1 min. immersion	10	78.33	18.17	1.4[b]
1 min. immersion	15	83.73	9.66	2.3[a]
1 min. immersion	20	37.64	5.33	0.6[c]

resuspended in 10 mM Tris pH 8.0, 0.1 mM EDTA buffers. DNA quantity was estimated spectrophotometrically by measuring absorbance 260 nm.

DNA Amplification: Fourteen arbitrary 10-base primers (Operon Technologies Inc., Alameda, California) were used for polymerase chain reaction (PCR) (Table 1), following the protocol of Williams *et al*. [9], with minor modifications. Amplification reactions were performed with 25 µl of 10 x assay buffer (Stratagene), 2.0 µl of 1.25 mM each of dNTP's (Pharmacia), 15 ng of the primer, 1 x Taq polymerase buffer, 0.5 units of Taq DNA polymerase (Genei, India), 2.5 mM $MgCl_2$ and 30 ng of genomic DNA. DNA amplification was performed in a Perkin Elmer Cetus 480 DNA Thermal Cycler (Perkin Elmer Ce tus, Norwalk, Conn, USA) programmed for 45 cycles as follows: 1st cycle of 3.5 min at 92°C, 1 min at 35°C, 2 min at 72°C; followed by 44 cycles each of 1 min at 92°C, 1 min at 35°C, 2 min at 72°C followed by one final extension cycle of 7min at 72°C. The amplification products were size separated by electrophoresis in 1.2% (w/v) agarose gels with 0.5 x TBE buffer, stained with ethidium bromide and photographed under UV light.

Amplified DNA Marker Scoring: Amplified DNA markers were scored as present or absent in each micropropagated plant. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored. The data were analyzed using locus-to-locus gel readings and the rates of *in vitro* DNA polymorphism were calculated and given as percentage of the total number of bands for the apricot clones.

RESULTS AND DISCUSSION

Surface Sterilization: Contamination is a serious problem in *in vitro* cultures. The first and foremost step in establishing *in vitro* cultures of any plant is to eliminate microorganisms. Shoot tips and axillary shoots are easier to propagate compared with other types of explants such as meristems [17, 18]. Meristem culture is often used to produce pathogen free plants from a systemically infected individual and is also recommended for establishing aseptic explants for micropropagation. In woody species and particularly in *Prunus*, the available reports on meristem tip culture are rather limited [8, 19]. The highest survival rate (83.73%) and low contamination rate (9.66%) of meristem tip explants was achieved by 1min. immersion in 70% ethanol and then 15 min immersion in 1.0% NaOCl containing three drops of Tween 20/500 ml compared to the other treatments of this experiment (Table 1). Also, the highest rate (2.3) of number of shoots was achieved by this treatment compared to the other treatments. Perez-Tornero *et al*. [8] have also applied successfully similar concentrations of disinfectants in other apricot cultivars. Also, these results can be considered consistent for *in vitro* establishment of *Prunus* and are in according with the survival rates of 27% to 93% obtained by Hammerschlag [20] in the *in vitro* establishment of 11 peach tree cultivars.

Screening of Basal Medium and Effect of Seasonal Variation on Meristem Culture: The results of screening for an optimal basal medium and effect of seasonal variation on meristem culture of *Prunus armeniaca* L. cultivar El-Hamawey are shown in Table 2. The survival rate of meristem tips was 89.87%, number of shoots (3.98) and average shoot length (1.85cm) on WP medium supplemented with 1.0 mg/L Zeatin, 0.1 mg/L IAA in the presence of 100 mg/l ascorbic acid and 150 mg/L citric acid of explants taken in spring, whereas survival rate of meristem tips was 85.23% on B5 medium, 73.38% on MS medium, 78.33% on 1/2MS and 81.23% on Q&L medium with the same growth regulators of explants taken in spring compared to the other season. WP medium was most promotive for number of shoot and shoot elongation, MS medium caused shoot necrosis and shoot vitrification after several subculture.

Table 2: Effect of different medium and seasonal variations on the survival rate, formation of shoot perexplant and shoot length of apricot meristems after 10 weeks culture

	Survival rate (%)			Number of shoots			Average shoot length (cm)		
Basal medium	A	W	S	A	W	S	A	W	S
MS	69.46	66.67	73.38	1.43^{c}	0.89^{d}	1.98^{bc}	1.14^{cd}	0.94^{d}	1.29^{c}
$^{1}/_{2}$ MS	73.33	70.54	78.33	1.72^{c}	0.78^{d}	1.34^{c}	0.92^{d}	0.83^{e}	1.04^{d}
WP	86.43	78.56	89.87	2.98^{ab}	1.43^{c}	3.98^{a}	1.67^{b}	1.42^{bc}	1.85^{a}
B5	82.34	72.33	85.23	2.03^{b}	1.33^{c}	2.12^{b}	1.36^{c}	1.16^{cd}	1.55^{bc}
Q&L	80.33	71.34	81.23	1.81^{bc}	0.83^{d}	$1.93b^{c}$	1.23^{c}	1.19^{cd}	1.32^{c}

*A= autumn, W= winter, S= spring

Generally, the optimum medium for the survival rate and shoot growth were achieved with WP medium. Snir [4] reported that the axillary buds in 'Canino' apricot were successfully established *in vitro* on a medium consisting of Knop's solution. Sugiure *et al*. [21] reported that $^{1}/_{2}$ MS or WP medium was suitable for the culture of Japanese persimmon. The reason full strength MS was not being successful for culturing apricot apices might be attributed to high concentration of nitrogen and/or high total salts. Nitrogen concentration of WP, $^{1}/_{2}$ MS, B5 media or Knop's solution is less than that of MS medium, therefore the nitrogen level in the last one may have been excessive. Explant material was axillary buds from one year old branches collected from mature plants in the field in February. Fourth basal media were compared: MS, LF, QL and WPM containing 0.1 mg/L GA_3 and 0.01 mg/L IBA of apricot cultivars 'Sulina', 'Carmela', 'NJA 19', 'Favorit' and 'Mamaia' [22].

It can be concluded that spring was optimum time for explant collection, which represents the active growing season at which new growing shoots were collected. These results are agreed with those obtained by Tao *et al*. [23], Das and Mitra [24] who found that explants collected from new shoots in the summer exhibited the highest survival percentage compared to explants collected from late period of the growing season.

Effect of Different Cytokinins and Their Concentrations on Shoot Proliferation: The meristem tip explants were inoculated on WP medium with different combinations of BA at 0.0- 2.0 mg/L, Zeatin at 0.0- 2.5 mg/L, 2iP at 0.0-1.5 mg/L, TDZ at 0.0-2.0 mg/L in combinations with IBA at 0.01-0.25 mg/L and IAA at 0.01-0.25 mg/L. The effective best results were obtained from the combinations given in the Table 3, after few days the explants swell and turn green and produce shoots within eight weeks. The results in the Table 3 indicated that survival percentage reached 89.33% by using 1.0 mg/L Zeatin in combination with 0.1 mg/L IAA, followed by the next combination of 1.5 mg/L TDZ + 0.1 mg/L IAA in the presence of 150 mg/L citric acid + 100 mg/L Ascorbic acid (Fig. 1) as compared to the other treatments which having less which ranged from (66.34-80.66 %). The average number of shoot proliferation on 1.0 mg/L Zeatin and 0.1mg/L IAA in the presence of 100 mg/L ascorbic acid and 150 mg/L citric acid was reached the highest value 3.98, followed by the next combination of 1.5 TDZ and 0.1 IAA was reached 3.80 as compared to the average number of shoot proliferation of the other treatments, ranged from (1.27-3.02). However average shoot length treated with 1.0 mg/L Zeatin + 0.1mg/L IAA in the presence of 100 mg/L ascorbic acid and 150 mg/L citric acid was the best treatment (1.85cm) as compared to other treatments. The poor response of shoot initiation ability was noticed in other concentrations and different combinations of the growth regulators. These results are agreement with those obtain by Pérez-Tornero and Burgos [25] studied factors affecting *in vitro* propagation of several apricot cultivars with WP medium were contained between 1.78µM and 3.11 µM BA with different concentrations of IBA. The most widespread method to establish plant material *in vitro* is by use of stem nodal segments. In our experiments with apricot, we found it almost impossible to establish plant material by culturing axillary shoots, mainly because of high contamination, not only after the culture was initiated (83% as average of introduced shoots). Similar results were obtained by Yldrm *et al*. [26] studied that several factors affecting *in vitro* culture, rapid proliferation, rooting and acclimatization of the apricot cultivar Hachaliloglu. The problem of contamination in plant tissue culture has been largely reviewed by Leifert and Waites [27] and when contaminants are persistent, meristem culture, used to obtain virus-free *Prunus* plant material [28], is recommended also as a method to establish aseptic explants for micropropagation [29].

Shoot Multiplication of Apricot cv. El-Hamawey: Multiplication is a rapid in crease of organs which can ultimately give rise to plant. This increase is achieved by

Table 3: The best concentration of different growth regulators for shoot formation from meristem tips of apricot cv. El-Hamawey after 10 weeks culture.

Concentration of PGRs (mg/L)	Survival (%)	Average number of shoots/explant	Average shoot length (cm)
0.0 BA + 0.0 IBA	0.00	0.00^{e}	0.00^{e}
1.0 BA + 0.01 IBA	74.43	2.78^{b}	1.24^{c}
1.5 BA + 0.01 IBA	80.66	3.02^{ab}	1.38^{b}
1.5 BA + 0.05 IBA	76.78	2.88^{b}	1.45^{b}
2.0 BA + 0.25 IBA	66.84	1.68^{c}	0.89^{e}
0.5 Zeatin + 0.05 IBA	69.78	2.94^{b}	1.44^{b}
1.0 Zeatin + 0.1 IBA	72.34	2.74^{b}	1.32^{bc}
0.5 2iP + 0.05 IBA	66.34	1.69^{c}	1.73^{a}
1.0 TDZ + 0.1 IBA	78.64	2.91^{b}	1.13^{c}
1.5 TDZ + 0.1 IBA	77.76	2.79^{b}	1.02^{d}
1.0 BA + 0.1 IAA	69.33	1.54^{cd}	1.17^{c}
1.5 BA + 0.1 IAA	71.36	2.62^{b}	1.03^{d}
1.0 Zeatin + 0.1 IAA	89.33	3.98^{a}	1.85^{a}
1.0 2iP + 0.05 IAA	77.23	2.66^{b}	1.58^{b}
1.0 2iP + 0.1 IAA	73.34	1.27^{d}	1.63^{ab}
1.5 TDZ + 0.1 IAA	84.67	3.80^{a}	1.53^{b}
2.0 TDZ + 0.25 IAA	79.46	2.78^{b}	1.49^{b}

Table 4: Shoot multiplication of apricot cv. El-Hamawey cultured in tissue culture jars for five weeks on WP medium supplemented with different concentrations of BA alone or in combination with 2ip and TDZ using shoots produced from meristem tips

	Increase in	
Concentration of PGRs (mg/L)	Mean number of new shoots	Mean length of shoots (cm)
2.0 BA	3.08^{e}	2.03^{b}
3.0 BA	3.78^{c}	1.98^{c}
4.0 BA	4.49^{b}	1.87^{c}
1.0 BA + 0.5 2iP	3.76^{c}	3.24^{a}
2.0 BA + 0.5 2iP	3.95^{c}	2.82^{b}
3.0 BA + 0.5 2iP	4.78^{b}	2.56^{b}
4.0 BA + 0.5 2iP	5.37^{a}	1.94^{c}
2.0 BA + 0.5 TDZ	3.56^{c}	1.88^{c}
3.0 BA + 0.5 TDZ	3.84^{c}	1.76^{c}
3.0 BA + 1.0 TDZ	4.36^{b}	1.64^{c}
4.0 BA + 0.5 TDZ	3.34^{c}	1.03^{d}
4.0 BA + 1.0 TDZ	4.88^{b}	1.37^{c}

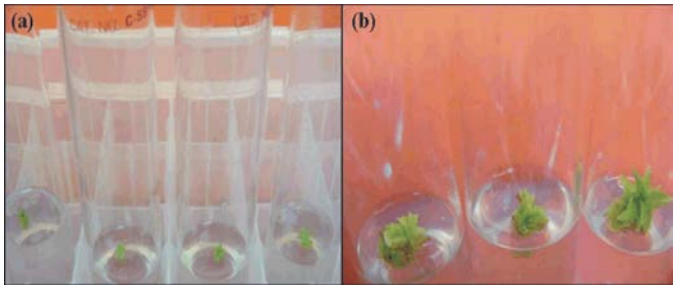

Fig. 1: Plantlet regeneration; (A) the shoot growth from meristem tip of apricot on WP medium supplemented with 1mg/l zeatin and 0.1mg/l IAA after 8 weeks. (B) After 10 weeks

enhancing axillary shoot initiation [30]. This stage is repeated at regular intervals to produce large- scale shoot multiplication to be commercially useful [31]. In the present study, the used different concentrations of benzyl adenine BA alone or in combination with Δ^2-isopentenyl adenine (2ip) and Thiadiazuron (TDZ) for multiplication of shoots produced from meristem tips. Data presented in Table 4 indicated that the maximum increase in the mean number of new shoots 5.37of *Prunus armeniaca* L. cultivar El-Hamawey obtained on WP medium containing 4 mg/L BA + 0.5 mg/L 2ip (Fig. 2b). Followed by the next combination of 4 mg/L BA and 1mg/L TDZ was reached

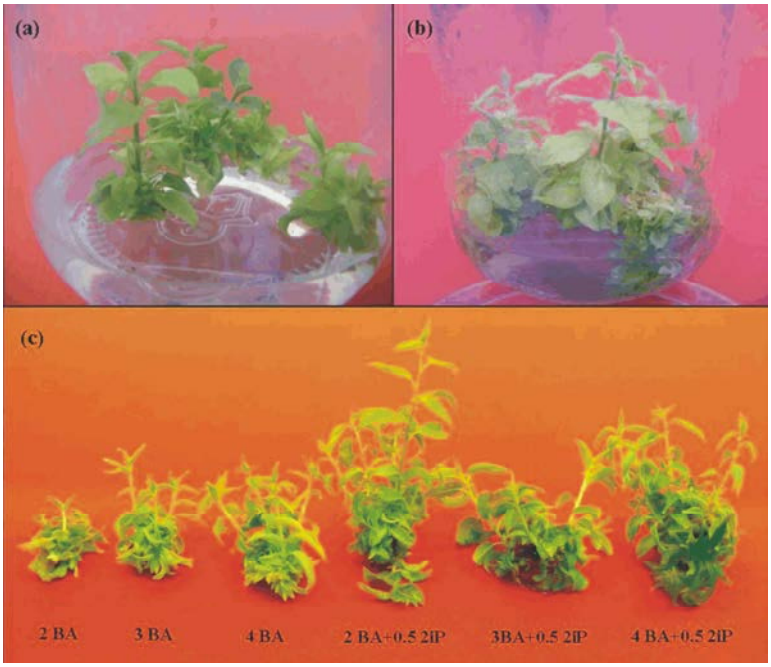

Fig. 2: Effect of different cytokinins and their concentrations on shoot multiplication of apricot cv. El-Hamawey; (a) WP medium supplemented with 4 mg/l BA and 1 mg/l TDZ. (b) WP medium supplemented with 4 mg/l BA and 0.5 mg/l 2iP. (c) Effect of different concentrations of BA alone or in combinations with 2iP on shoot multiplication

4.88 (Fig. 2a) which were significantly higher than the other treatments. However, the maximum increase in the mean length of shoots 3.24 cm obtain on WP medium containing 1mg/L BA + 0.5 mg/L 2ip, which was the best treatments compared the other treatments.

It was observed that BA was the best growth regulator for the induction of adventitious buds. Number of the buds increased with the concentration of BA, but high concentrations of BA (3-4mg/L) resulted in the expansion and translucency of explants and inhibited the buds' elongation, whereas low concentrations of BA led to normal development (Fig. 2c). These results are agreed with those obtained by Pérez-Tornero *et al*. [32] were used to determine the optimal BA level of apricot. Five different concentrations of BA, 0.88, 1.78, 2.66, 3.55 and 4.44 µM were tested. The best results were obtained with concentrations of BA close to 4.44 µM. Also, Yldrm, *et al*. [26] found that BA levels were tested for their effects on shoot proliferation and 1.0 mg/L of BA was the most suitable dose for promoting shoot multiplication cultures. The greatest number of shoots (3.42) was obtained using 2.0 mg/L of BA; this was significantly greater than that of the control, but there was no significant difference in the mean obtained with 1 mg/L of BA.

Shoot Proliferation from Meristems During Successive Subculture: In order to evaluate the reliability of micropropagation over time, meristems from established cultures were subcultured successively for seven culture periods (Table 5). Although considerable variation existed, an overall average of five shoots per meristem was obtained. Considering an initial plating of 100 meristems, 312,500 shoots could be produced in five successive culture periods at this multiplication rate. This rate of increase is commercially acceptable to produce plants for grafting and micropropagation.

Shoot Elongation: Shoots were subcultured on selection elongation medium as described in the material and methods. WP medium supplemented with different concentrations 0.5 and 1.0 mg/l of Gibberellic acid (GA_3) alone or 0.5, 0.75 and 1.0 of 2-isopentenyl adenine (2ip) were evaluated. The data indicated that the best results for shoot elongation (6.4 cm) were observed with WP medium containing 0.5 mg/l 2ip of *Prunus armeniaca* L. cultivar El-Hamawey compared to the other treatments (Fig. 3 and 4). The nodal explants of *Aegle marmelos* were transferred into the MS medium supplemented with 0.5 mg BA/l with different concentrations of either kinetin (KN) or GA3 or in combinations have shown healthy shoots with expanded shoot length [33].

Table 5: Shoot multiplication from meristems of during successive subculture[y]

Subculture period[z]	Average number of shoots/meristem	Average shoot length (cm)
1st	2.87[c]	1.89[b]
2nd	4.98[ab]	1.74[c]
3rd	5.37[ab]	1.93[b]
4th	5.59[a]	1.67[c]
5th	5.89[a]	1.98[b]
6th	4.23[b]	2.16[ab]
7th	4.08[bc]	2.43[a]

[z]Subculture on medium was WP with 4 mg/L BAP and 0.5mg/L 2iP

[y]Each culture period was five weeks

Fig. 3: Elongation shoot; (a) the shoot elongation of apricot on WP medium supplemented with 0.5 mg/l 2iP. (b) WP medium supplemented with 1mg/l GA3

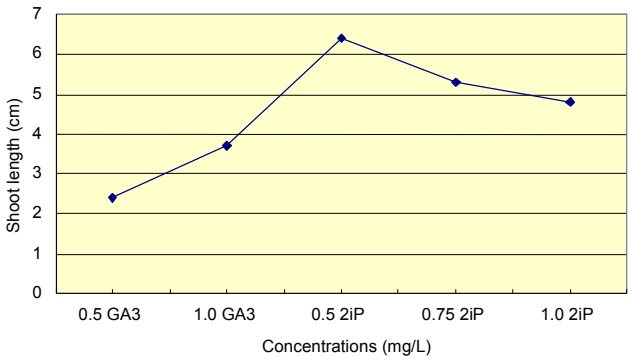

Fig. 4: Effect of different growth regulators and their concentrations on shoot elongation of apricot cv. El-Hamawey

Rooting of the Multiple Shoots: Roots formation in tissue culture can be induced by exogenous auxins such as IBA, NAA and IAA and their interaction with endogenous auxins which cannot be sufficiently synthesized by many tissues and small organs isolated *in vitro* and they are only required at an early stage to emerge new formed roots [34, 35]. The regenerated shoots reached a height of 3-5 cm; the individual shoots were carefully excised and transferred to different rooting media for apricot rooting. Data in Table 6 shows the effect of different concentrations of IBA alone or in combination with NAA and different media on rooting of apricot shoots after five weeks. In this experiment, the highest percentage of shoots forming roots (91%) the greatest mean number of roots per shoot (7.64) and the best mean length of roots (3.43cm) was obtained liquid MS medium with 2.0 mg/L IBA, 0.5 mg/L NAA and 40 mg/L phloroglucinol when shoots were maintained in the dark for 7 days before transfer a 16-h photoperiod (Fig. 5 and 6). Poor induction of roots at the base was observed when the rooting

Table 6: The efficiency of shoots forming roots for apricot cv. El-Hamawey after growing on MS and WP medium supplemented with different auxin concentrations

Media and Concentration of PGRs (mg/L)	% of shoot forming roots	Mean number of roots/explant	Mean length of roots (cm)
MS+1.0 mg/LIBA	20	4.23^{e}	1.33^{e}
MS+1.5 mg/LIBA	56	3.87^{d}	2.46^{b}
MS+2.0 mg/LIBA	76	4.86^{c}	2.25^{b}
MS+1 mg/LIBA+0.5 mg/LNAA	43	5.33^{b}	2.67^{b}
MS+2 mg/LIBA+0.5 mg/LNAA	91	7.64^{a}	3.43^{a}
WP +1.0 mg/LIBA	30	3.34^{d}	1.68^{c}
WP +1.5 mg/LIBA	55	3.68^{d}	1.26^{c}
WP+1 mg/LIBA+0.5 mg/LNAA	47	4.56^{c}	2.73^{b}
WP+2 mg/LIBA+0.5 mg/LNAA	82	5.36^{b}	3.26^{a}

Fig. 5: Root formation of apricot cv. El-Hamawey on MS medium supplemented with 2.0 mg/L IBA, 0.5 mg/L NAA and 40 mg/L phloroglucinol (liquid medium)

Fig. 6: Root formation of apricot cv. El-Hamawey on MS medium supplemented with 2.0 mg/L IBA, 0.5 mg/L NAA and 40 mg/L phloroglucinol (solid medium)

medium supplemented various concentrations of IBA alone. The few reports on *in vitro* propagation of apricot indicate that good rooting competence is achieved early, with some species for which limited rooting was obtained [26]. In apricot low concentrations of auxins have been used to promote rooting without necrosis [36]. In several species, the utilization of high concentrations of IBA or NAA produced calluses and abnormal roots, which affected the survival of explants during the acclimatization [37, 38]. Low concentrations of auxins did not produce good results with apricot cv. 'Canino', which required an induction period in the dark [32].

Acclimatization of Rooted Plantlets: Plants produced from rooting stage which were containing 5cm, then rinsed once with water and then transfer into pots containing equal parts of peat and sand, then incubated under transparent plastic bags on 16h photo period at

Table 7: List of primers, their sequences, number and size of the amplified fragments generated by random amplified polymorphic DNA (RAPD) primers

Primer code	Sequence 5'----- 3'	Number of scorable bands per primer	Total number of bands amplified	Polymorphism (%)	Range of amplification (bp)
OPE C-02	5`- GTGAGGCGTC- 3`	7	63	15	400-2000
OPE C-05	5`- GATGACCGCC- 3`	6	46	17	350-2000
OPE M-15	5`- GACCTACCAC-3`	6	35	54	500-2000
OPE Q-12	5`- AGTAGGGCAC-3`	9	72	0	250-2000
OPE K-10	5`- GTGCAACGTG-3`	8	50	28	400-2500

Fig. 7: Adventitious shoots acclimatized to the greenhouse of apricot cv. El-Hamawey; (a) 4 weeks after potting (b) 20 weeks after potting

26°C for 2 weeks before transfer to a green house, after 4 weeks from transfer into green house, they were repotted into sterile soil consists equal parts of peat and sand (v/v) (Fig. 7a). *In vitro* plantlets were actively growing during the acclimatization process and no stress symptoms were observed after transfer to the greenhouse and transplanting to larger pots. After two months, plantlet size ranged from 25 to 35cm in height. In total, 73% of the potted apricot plantlets survived acclimatization and were kept in the greenhouse for five months before an over wintering period (Fig. 7b).

PCR Analysis: The use of RAPD in this study was able to differentiate between the mother plant and product of the various subcultures in apricot. Out of the fourteen randomly selected primers, five primers were able to generate highly specific amplification profiles. The identification of the specific primers can be useful for future studies in this apricot. Variations observed in this study on the total number of RAPD bands as well as the number of specific bands among the mother plants and different sub-cultures indicate minimal genetic differences of the different subculture when compared to the mother plants. Optimization of RAPD protocols and selection of primers ensured that the RAPD profiles were reproducible. Among the five random primers (Table 7) used for the initial screening, nine failed to amplify any bands, while the other five primers provided clear and scoreable amplification products in all the treatments (Figures 8, 9).

A total of 14 RAPD primers were used for initial screening with apricot plants but only five RAPD primers gave clear and reproducible bands. The numbers of scorable bands for each RAPD primer varied from 6 with OPE C-05 and OPE M-15 to 9 with OPE K-12 (Table 7). The five RAPD primers produced 266 distinct and scorable bands, with all samples. Each primer generated a unique set of amplification products ranging in size from 250 bp (OPE Q-12) to 2,500 bp (OPE K-10). No polymorphism was detected during the RAPD analysis of *in vitro*-raised clones with OPE Q-12 primer. Using the primer OPE C-5, one polymorphic band with a molecular mass 1000 bp in subculture 5 was present (Fig. 8), with the primer OPE K-10, two polymorphic bands with molecular mass 400 and 2500 bp were absent in subcultures 1 to 7. Also, using the primer OPE M-15, four polymorphic bands with molecular mass 1100, 1150, 1900 and 2000 bp in this subcultures were absent (Fig. 9). With the primer OPE C-2, four polymorphic bands with molecular mass 480 bp in subculture 4, 600bp in subculture 2 to 8, 1100bp in subculture 5 and 2000bp in subculture 6 were absent (Fig. 9). However, no polymorphic with the primer OPE Q-12. Figure 10 is the dendrogram based on RAPD data. This study provides the first information on the molecular basis of polymorphism detected as RAPD markers in micropropagated plants of *Prunus armeniaca* L. cultivar El-Hamawey. In our study, the amplified products exhibited monomorphisms among all the *in vitro* plants and were similar to those from mother plants.

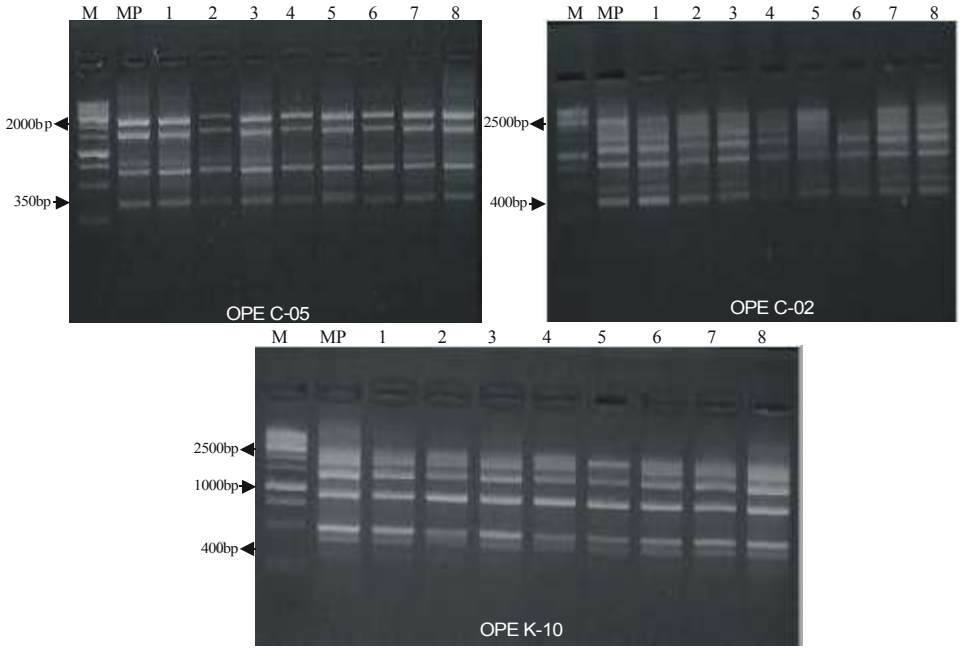

Fig. 8: RAPD profiles generated by primers OPE C-02, OPE C-05 and OPE K-10. Lane MP the field grown mother plant. Lanes 1 to 8 different subcultures of micropropagated apricot cv. El-Hamawey plants corresponding to the first subculture up to eight subcultures. Arrow indicates the size of the fragments as compared with markers

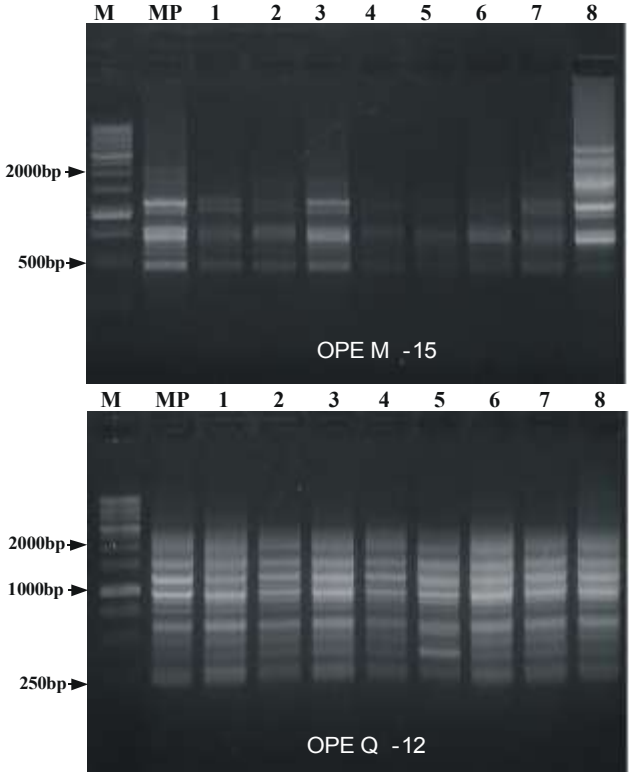

Fig. 9: RAPD profiles generated by primers OPE M-15 and OPE Q-12. Lane MP the field grown mother plant. Lanes 1 to 8 different subcultures of micropropagated apricot cv. El-Hamawey plants corresponding to the first subculture up to eight subcultures. Arrow indicates the size of the fragments as compared with markers

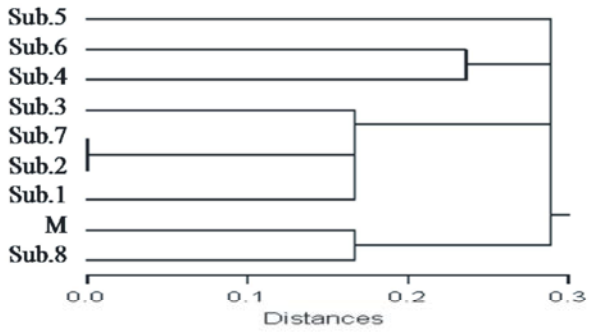

Fig. 10: Dendrogram demonstrating the relationships among the eight apricot subcultures and mother plant based on data recorded from polymorphism of RAPD markers. M: mother plants and Sub.1-8: subculture from one to eight

RAPD variation has been reported in many studies. For example, reports have indicated the occurrence of somaclonal variation in micropropagated banana plants raised from meristem culture [39]. The absence of genetic variation using RAPD has been reported in micropropagated shoots of *Pinus thunbergii* by Goto *et al*. [40], *in vitro*-regenerated turmeric by Salvi *et al*. [41] and *in vitro*-raised bulblets of *Lilium* [42]. The genetic stability of apricot (*Prunus armeniaca* L.) shown in our study is in agreement with those obtained by Angel *et al*. [43], who found no RAPD fingerprint variation when cassava (*Manihot esculenta*) plants derived from *in vitro* stored apical meristems. These findings support the fact that a meristem-based micropropagation system is much more stable genetically than those in which regeneration occurs via the callus phase. Plants regenerated from adventitious buds around axillary buds or from other well developed meristematic tissue showed the lowest tendency for genetic variation [44]. Even plants derived from organized meristems are not always genetically true to the type in many crops [45]. Hence, it becomes imperative to regularly check the genetic purity of the micropropagated plants in order to produce clonally uniform progeny while using different techniques of micropropagation.

In conclusion, our results demonstrate that RAPD analysis can be applied to assess the genetic fidelity of plants derived *in vitro* on an industrial scale as part of crop improvement programs. This method might be useful for monitoring the stability of *in vitro* germplasm collections and cryopreserved material

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