CATRINA (2012), 7 (1): 25 -31
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In vitro propagation of *Pistacia vera* L. rootstock

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ABSTRACT

The present study was carried out to investigate the influence of different types, combinations and concentrations of plant growth regulators added to Murashige and Skoog (MS) medium on establishment, shoot multiplication and rooting of pistachio (*Pistacia vera* L.) rootstock. An efficient and rapid method for micropropagation of *P. vera* was developed using stem node sections which were collected in the spring from adult trees grown in El-Maghara Experimental Station (Middle Sinai). The explants were cultured on MS medium supplemented with different concentrations of 6- benzyl adenin (BA) (0.0,0.5,1.0, and 1.5 mg/l) in combination with β-naphthalene acetic acid (NAA)(0.0, 0.01 and 0.05 mg /l). Obtained shoots from establishment stage were placed on medium supplemented with various types of cytokinins BA, kinetin (Kin) and Thiodiazuron (TDZ) each at different concentrations (0.5, 1.0, 1.5 and 2.0 mg/ l). The highest average number of shoots and shoot length (4.7 and 4.2 cm, respectively) were obtained when shoots were placed on MS medium supplemented with 1 mg/l BA. However, MS medium supplemented with TDZ at 0.5 mg /l raised the average shoot number to 3.8 and average length to 3.9 cm. Root initiation and growth was obtained on half strength MS medium supplemented with 2.0 mg/l, indol-3-butyric acid (IBA). IBA proved to be more effective for rooting than NAA. *In vitro* formed plantlets were left for a month in jars under lab conditions till forming a whole rooting system. Rooted pistachio plantlets were acclimatized successfully following the regular methods. Such a protocol will be helpful to expand the cultivation of pistachio in Sinai.

Key words: *In vitro*, Rootstock, *Pistacia vera*, micropropagation, Sinai.

INTRODUCTION

Pistachio tree (*Pistacia vera* L.) belongs to the family Anacardiaceae and includes at least 11 species. *P. vera* L. (cultivated pistachio) is by far the most economically important species in the genus. *P. vera* L. is the only species in this genus that is successfully grown in orchards. (Al- Saghir, 2010 a). It is a dioecious species cultivated widely in the Mediterranean regions of Europe, North Africa, Middle East, China and California (Onay *et al*., 2004). The leading countries producing pistachio are Iran, Turkey, U.S.A. and Syria (FAO, 2004). Pistachio Khin Juk (Khenjok pistachio tree), shrub or small deciduous nut tree are widely distributed in Egypt, North Iraq, Iran and Kasmir (Kawashty *et al*., 2000). Pistachio is adapted to a variety of soils and is probably more tolerant to alkaline and saline soils than most tree crops (Tous and Ferguson, 1996).

It is a drought and salt resistant species that can be grown without irrigation in conditions where other crops cannot be cultivated. So, with other few species, pistachio opens the marginal land to cultivation and provides significant income not only to the local breeders, but also to the country's economy (Ozden *et al*., 2008). Moreover, it thrives in hot, dry and desert like conditions. *P. vera* with reduced vigour, is the most used rootstock in the world. Most orchards in Iran, Turkey, Syria and Tunisia are planted using this rootstock (Sheibani, 1996). Development of pistachio plantations is limited by the absence of adequate nursery stock due to the difficulty of its propagation by conventional methods such as seedling, grafting and cutting, which may have a negative effect on health and are time and labour consuming (Holtz *et al*., 1995). However, methods available for *in vitro* propagation have the potential to provide high multiplication rates of plant resulting in short time gains (Durzan, 1988 and Hammatt and Grant, 1993). In El-Maghara Experimental Station (Middle Sinai) of Desert Research Center (DRC), few rootstocks were cultivated, from ACSAD Syria as a source of plants and these plants bloomed, fruit set occurred and yielded excellent fruits. *P. vera* produce edible nut seeds and considerable commercially importance. (Al-saghir, 2010 b).

It's a rich source of energy, The nuts contain many health benefiting nutrients, minerals, anti-oxidants and vitamins that are essential for optimum health. Just a hand full of pistachio a day provides enough recommended levels of phenols, anti-oxidants, minerals, vitamins and proteins (Tous and Ferguson, 1996). Many efforts focused on establishing *in vitro* propagation procedures for *P. vera* and several other *Pistacia* species (Barghchi and Alderson,1989; Picchioni and Davies, 1990). Abousalim (1991) reported that nodal segment explants are the best explants for *P. vera* micropropagation. However, (Ramon and Elisabet 1995; Onay, 2000; Tilkat *et al*., 2005 and 2009) use shoot tip cultures of pistachio species as explants which improve shoot multiplication rates. Cetiner *et al*. (1997) reported that during *in vitro* propagation of *Pistachio* species, some problems, such as browning, death of shoot tips, vitrification, viriation and callus production,were seen. This study aims to establish a protocol for *in vitro* propagation of *P.vera* rootstock as well as to determine the most optimum conditions for establishment, multiplication, rooting and acclimatization using different growth regulators. The rootstock plantlets produced resulting from *in vitro* propagation could be recultured in El-Maghara or under the same climatic condition in Egypt.

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MATERIALS AND METHODS

Plant material and sterilization

Actively growing shoots of pistachio (*Pistacia vera* L.) rootstock were collected in the spring (April and May) from the mature field-grown trees in El-Maghara Experimental Station. After explants collection and excision, they were soaked in antioxidant solution (100 mg/l ascorbic acid and 150 mg/l citric acid) for 2 hrs to overcome phenol exudation. Stem node sections (1.0 – 2.0 cm) were washed with soap under running tap water for about 1h, dipped in 70% (v/v) ethanol for 2 min, followed by surface sterilization for 20 min by manual agitation in 2.5% sodium hypochlorite solution containing few drops of Tween–20. The stem node sections were then disinfected, using 0.1% (w/v) mercuric chloride for 5 min. Thereafter, the explants were rinsed 4 times with sterile distilled water (to remove traces of the sterilant), and rinsed 2 times with the sterile antioxidant solution.

Nodal explants were trimmed using a sterile blade in a mixture of ascorbic acid and citric acid solution (100 mg/l, each) and blotted on a sterile filter paper before implanting on the media.

Culture media and conditions

The culture medium used in the present work was Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, ascorbic acid and citric acid; 150 mg/l each and 8 g/l Difco Bacto agar. Different plant growth regulators (PGRs); cytokinins {benzyl adenine (BA), Kinetin (Kin), and Thiodiazuron (TDZ)} and auxins {Indole butyric acid (IBA) and naphthalene acetic acid (NAA)}, at different concentrations and combinations were added individually to the medium to determine PGR_s requirements for bud sprouting, multiple shoot induction and rooting. The pH of the medium was adjusted to 5.7$\pm$ 0.1 before autoclaving. Fifteen ml of medium were dispensed into 150 x 25 mm tissue culture tubes for the establishment stage, and 30 ml in large jars for the multiplication and rooting stages.

The containers containing culture media were autoclaved at 121 ^{o}C for 20 min, under a pressure of 1.2 Kg/Cm^2. Cultures were incubated at 25$\pm$2^{o}C with a 16 hrs photoperiod using cool white fluorescent lamps (FL 40 T9 D/ 38, Toshiba). Data were scored after 8 weeks of culture.

Culture establishment

Solidified MS basal medium supplemented with various concentrations of BA (0.0, 0.5, 1.0 and 1.5 mg/l) in combination with NAA (0.01 and 0.05 mg/l), was used for culture establishment. Explants were transferred to a fresh medium every week to overcome the negative effect of the phenolic exudation. The percentage of survival of explants and the percentage of explants forming growth, in addition to average shoot length (cm) were measured after 8 weeks of culture.

Shoot multiplication

MS basal medium supplemented with different concentrations (0.5, 1.0, 1.5 and 2.0 mg/l) of various cytokinins (BA, Kin, and TDZ) were individually tested for the multiplication of shoots. The average number of shoots per explant and shoot length (cm) were recorded after 6 weeks of culture.

Rooting and acclimatization

When the explants reached 3-4 cm in length, they were transferred to a half- strength MS medium (½ MS) supplemented with different concentrations of (0.0, 1.0 and 2.0 mg/l of each) auxins (IBA and NAA) for *in vitro* root induction. The percentage of rooting, average number of roots per explants and average root length (cm) were recorded for each explants. Rooted plantlets were removed from the medium, gently rinsed in running tap water to remove the adhering media before transferring into plastic pots containing a sterilized mixture of peat moss and sand (2:1, v:v). Pots were kept in a greenhouse (28$\pm$2^{o}C, 75-80% relative humidity). The potted plantlets were irrigated with ½ MS medium and initially covered with plastic bags which were gradually removed within 8 weeks to complete the acclimatization process. Benlate fungal disinfectant solution 1.0 g/l was sprayed on the plants to prevent fungal contamination.

Statistical analysis

Analysis of variance (ANOVA) followed by Duncan's multiple range test were performed to analyze the obtained data, at least 20 replicates were raised for each treatment. The differences among means for all treatments were tested for significance at 5% level. Means followed by the same letter are not significantly different at $P \leq 0.05$ (Duncan, 1955) as described by Snedecor and Cochran (1967).

Results and Discussion

The major problems facing culturing tissue from mature trees are contamination, recalcitrance of adult trees, hyperhydration, and browning or blackening of the culture medium and explants due to leaching of phenols which may be phototoxic and cause necrosis of the explants (Bhojwani and Razdan, 1996).

In vitro shoot multiplication

In the present study, stem node sections obtained from 6-week old shoots of adult trees were used as explants. Bud break occurred 45 days after planting the nodal explants on MS medium supplemented with different concentrations of NAA and BA. Data in Table (1) and Figure (1A) indicated that shoots were raised on all tested media containing PGRs. The highest value of survival percentage (90%), growth to survival (94%), with the highest average shoot length (4.6 cm) were obtained on MS medium containing 1.0 mg/l BA supplemented with 0.05 mg/l NAA. It was noticed that the lowest concentration of NAA (0.05 mg/l) gave the

Table (1): Effect of MS medium and growth regulators (BA and NAA) on the *in vitro* propagation of *Pistasia vera* L. rootstocks.

Concentration (mg/l)		% survived explants	% of explants forming growth	Average shoot length (cm)
BA	**NAA**			
0.0	0.00	20 f	00.0 f	00.0 c
0.5	0.01	60 e	66.7 e	2.9 b
1.0	0.01	70 d	76.9 d	3.2 a
1.5	0.01	75 c	80.0 c	3.7 a
0.5	0.05	70 d	77.8d	3.9 b
1.0	0.05	90 a	94.1 a	4.6 a
1.5	0.05	85 b	92.9 b	4.1 a

Table (2): Effect of different types, combinations and concentrations of cytokinins on *in vitro* shoot multiplication of *Pistasia vera* L. rootstock.

Concentration (mg/l)			Average number of shoots per explants	Average shoot length (cm)
BA	Kin	TDZ		
0.0	0.0	0.0	0.0g	0.0g
0.5	0.0	0.0	1.9e	2.8c
1.0	0.0	0.0	4.7a	4.2a
1.5	0.0	0.0	3.4b	3.6b
2.0	0.0	0.0	2.0e	3.0c
0.0	0.5	0.0	1.6f	1.3e
0.0	1.0	0.0	2.0e	1.6e
0.0	1.5	0.0	2.4d	2.2a
0.0	2.0	0.0	3.0c	1.9d
0.0	0.0	0.5	3.8b	3.9a
0.0	0.0	1.0	2.7d	2.8c
0.0	0.0	1.5	2.0e	1.6e
0.0	0.0	2.0	2.2e	2.0d

highest results (90, 94.1 and 4.6) with respect to survival and growth percentages and average shoot length. There was no sign of growth when explants were cultured in the medium without PGRs. This indicated that the addition of cytokinins had a positive and stimulating effect because their uptake, transport, and metabolism differ between varieties and they can interact with endogenous cytokinins of explants (Strand *et al*., 1997; Vanstaden *et al*., 2008). Since, cytokinins (especially BA) are N6 – substituted adenines with growth regulation activities in plants that promote cell division and cell expansion in plant tissue culture (Mc Gaw and Burch, 1995).

When shoots, excised from established cultures, they were cultured on MS medium supplemented with a cytokinin to induce shoot multiplication. Multiplication was seen with all tested cytokinins (BA, Kin and TDZ). Table (2) and figure (1B) showed that the highest average number of shoots per explant was 4.7 and the highest average length of shoot reached 4.2 cm on MS medium containing 1.0 mg/l BA.

These results are in harmony with those obtained by Sureyya and Emine (2007), they found that the best shoot multiplication of *P. vera* were obtained when using 1.0 mg/l BA. In addation, Durkovic (2006), Mansseri-Lamrioui *et al*. (2009) and Akila *et al*. (2011), reported that high activity of the *in vitro* growth was observed in the presence of 1.0 mg/l BA for adult plant material of *Prunus avium* L. This finding also agrees with Adiyaman *et al.* (2007) they found that better shoot multiplication of Kiwifruit were obtained with BA treatment rather than kinetin treatments. Ruzic and Vujovic (2008) reported that BA was better for the proliferation phase of Cherry. From the observations in Table (2) it was found that increasing the concentration of BA decreased the average number and length of shoots. These results are parallel with Akbas *et al*. (2009) who worked on *Amygdalus communis* L. and noticed that the smallest percentage of sprouting and development of the shoots are due to strong concentrations of BA, which inhibit them. Concerning Kin and TDZ, they gave lower results in comparison with BA. Comparing the cytokinins, the shoots cultured on MS medium supplemented with 0.5 mg/l TDZ gave an average number of shoots of 3.8 and average shoot length of 3.9. In this respect, Huetteman and Preece (1993) and Ruzic *et al*. (2011), reported that TDZ gave a better response. Low concentration of TDZ are needed to stimulate axillary shoot proliferation in many woody trees.

It is a known fact that TDZ stimulates endogenous biosynthesis of cytokinins, which brings about an increase in the level of naturally occurring cytokinins, and it is likely to have a common site of action with them. These results agreed with those obtained by Sureyya and Emine (2007), who found that the best cytokinin for *P. vera* L. is BA. Kadota *et al*. (2001) and

Kadota and Niimi (2003) with *Pyrus pyrifolia* where they suggested that BA displayed more noticeable effect than TDZ and Kin, and also BA is more suitable for shoot multiplication of pear than phenyl urea derivatives. It was previously reported that BA could be used succ-essfully to induce shoot multiplication in *Prunus* sp. (Pruski *et al*., 2005). Generally, BA was more effective on shoot bud formation than Kin and TDZ in pistachio, indicating cytokinin specificity for shoot induction in its tissue.

Rooting and acclimatization

Rooting of proliferated shoots of *P. vera* was achieved on half-strength MS medium within 8 weeks of supplementation with auxins. From the data presented in Table (3) and Figure (1C), it is clear that IBA was significantly more efficient than NAA for root formation. The highest percentage of rooting (75%) was recorded on the medium supplemented with 2.0 mg/l IBA with an average number of roots of 11 and an average length of 4.05 cm. Also, the second best rooting percentage was 65% with average root number and length of 7 and 2.7 cm, respectively, n a half-strength MS medium containning 1 mg/l IBA. While, the least rooting percentages were 35 and 45% attained by using half-strength MS medium supplemented with 1.0 and 2.0 mg/l NAA, respectively. On the other hand, half strength MS medium without auxins (control) failed to induce rooting.

In general, to encourage root formation, IBA proved to be better than NAA. In this respect, IBA was used as a growth regulator for rooting of Benellic *et al.* (2001), Tanimoto (2005) and Ansar *et al.* (2009) have proved that IBA is the most effective auxin in olive as compared to NAA. In addition, Pan and Zhao (1994) proved that IBA is commonly used to promote root initiation *in vitro* Nissen and Sutter (1990) and Hausman (2003) have shown that in tissue culture media, IBA oxidized slowly (10%), its slow movement and delayed degradeation may be the primary reason of its better performance as compared to NAA. IBA may also enhance rooting *via* increased internal free IBA or may synergistically modify the action of the endogenous synthesis of IAA (Krieken *et al*., 1993).

On the other hand, NAA is not destroyed by auxin oxidase (Smulders *et al*., 1990), and its stable (Deklerk *et al*., 1999) presence in the tissue in free form might block out growth of the root initials. Rooted shoots exhibited a high ability to acclimatize (80%) in greenhouse having light intensity of 4000-10000 lux, 90-95% humidity with temperature ranging from 26-28^{o} C.(Sutter et al., 1992) (Fig. 1D).

In conclusion, a reproducible and efficient micro propagation protocol has been developed using stem segment explants of a selected plant of *p. vera*. High multiplication rate with uniform growth and length of shoots has been achieved. The shoots rooted *in vitro* and 80% of plantlets successfully acclimatized in greenhouse. The process developed can be applied to a large selection of *P. vera* rootstocks on a greater scale.

Table(3): Effect of half-strength MS medium supplemented with auxins (IBA and NAA) on the *in vitro* rooting of (*Pistasia vera* L.) rootstock.

Concentration of auxins (mg/l)		Rooting percentage (%)	Average number of roots/explant	Average root length (cm)
IBA	NAA			
0.0	0.0	0	0 d	0.00d
1.0	0.0	65	7 b	2.70b
2.0	0.0	75	11 a	4.05a
0.0	1.0	35	5 c	1.76c
0.0	2.0	45	7 b	2.10c

(A) (B) (C) (D)

Figure 1: Clonal propagation of *Pistacia vera* L. from stem segments.
A. Establishment of stem segments of *P. vera* on MS medium + 1 mg/l BA + 0.05 mg/l NAA.
B. Multiple shoots of *P. vera* on MS medium with 1 mg/l BA.
C. Rooting of shoots of *P. vera* on MS medium with 1 mg/l IBA.
D. Acclimatized shoots of of *P. vera*.

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Received July14, 2012
Accepted September 16, 2012

الإكثار المعملى لأصل نبات الفستق *Pistacia vera* L.

مهدية فريد جبر وصباح أنور حسانين

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١ ش متحف المطرية – القاهرة

الملخـــص العربـــي

يهدف البحث إلى إكثار أصل نبات الفستق معملياً ودراسة تأثير أنواع وتركيزات مختلفة من منظمات النمو النباتية المضافة إلى بيئة موراشيجى وسكوج (MS) على مراحل الإكثار المختلفة.

تم التوصل إلى طريقة فعالة للإكثار الدقيق لنبات الفستق وذلك بإستخدام العقد الساقية والتى تم جمعها فى الربيع من أشجار بالغة ونامية فى محطة تجارب المغارة (وسط سيناء) التابعة لمركز بحوث الصحراء. تم زراعة المنفصلات النباتية على بيئة MS المضاف إليها تركيزات مختلفة من البنزايل أدينين (BA) (٠,٥ - ١ – ١.٥ ملليجرام/لتر) فى تراكيب مع بيتا نفثالين حمض الخليك (NAA) بتركيزات (٠,٠١ - ٠,٥ ملليجرام/لتر) بالإضافة إلى بيئة مقارنة خالية من منظمات النمو النباتية. وقد تم زراعة الأفرع الناتجة من المرحلة البادئة على بيئة مضاف إليها أنواع مختلفة من السيتوكينينات BA و كينيتين (Kin) وثيوديازيرون (TDZ) بتركيزات مختلفة (٠,٥ ، ١ ، ١.٥ و ٢ ملليجرام / لتر) لكل منهم.

وتم الحصول على أعلى متوسط لعدد وطول الأفرع (٤.٧ و ٤.٢ سم، على التوالى) وذلك عند الزراعة على بيئة MS المضاف إليها ١ ملليجرام/لتر من BA ولقد وجد أن أندول حمض البيوتريك (IBA) أكثر فاعلية على تجذير أفرع النبات من نفثالين حمض الخليك (NAA). وقد تم الحصول على أعلى نسبة تجذير للأفرع على نصف قوة بيئة MS المضاف إليها ٢ ملليجرام/ لتر IBA.

تركت النبتات الناتجة معملياً لمدة شهر تحت ظروف المعمل حتى تكون مجموع جذرى كامل ثم نقلت إلى الصوبة وتمت الاقلمة بنجاح. يمكن تطبيق هذا البروتوكول لنشر زراعة نبات الفستق تحت ظروف سيناء.