In vitro propagation of two Iranian commercial pomegranates (*Punica granatum* L.) cvs. ‘Malas Saveh’ and ‘Yusef Khani’

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Abstract An efficient in vitro propagation is described for *Punica granatum* L. using shoot tip and nodal explants. The influence of two basal medium, WPM and MS, and different plant growth regulators was investigated on micropropagation of the Iranian pomegranate cultivars, ‘Malas Saveh’ and ‘Yousef Khani’. For proliferation stage, media supplemented with different concentrations (2.3, 4.7, 9.2 and 18.4 μM) of kinetin along with 0.54 μM NAA was used. WPM proved to be more efficient medium compared to MS. The best concentrations of kinetin were 4.7 μM for ‘Malas Saveh’ and 9.2 μM for ‘Yousef Khani’, resulting in the highest number of shoots per explants, shoot length and leaf number. For both cultivars, half-strength WPM medium supplemented with 5.4 μM NAA was most effective for rooting of shoots. Rooted plantlets were successfully acclimatized and transferred into soil. The micropropagated plants were morphologically uniform and exhibited similar growth characteristics and vegetative morphology to the mother plants.

Keywords In vitro propagation · Plant growth regulator · Pomegranate

Abbreviations

WPM Woody Plant Medium
MS Murashige and Skoog
NAA Naphthalene Acetic Acid
IBA indole-3-butyric acid

BA N^6 -Benzyladenine
BAP 6-Benzylaminopurine
ZR Zeatinriboside
TDZ Thidiazuron

Introduction

Pomegranate (*Punica granatum* L.) is an economically important fruit tree of the tropical and subtropical regions of the world that is cultivated for its delicious fruits. In addition, the tree is also valued for its pharmaceutical properties and ornamental usage (Julie Jurenka 2008).

Pomegranate is considered native to Iran (Stover and Mercure 2007) and it has been widely cultivated in this country. The total area under pomegranate cultivation in Iran is about 70,000 ha and Iran, with an annual pomegranate production of about 700,000 tones, ranks first in pomegranate production in the world (Tehrani et al. 2010).

Pomegranate is conventionally propagated by hard wood and soft wood cuttings. However, this traditional propagation method does not ensure disease-free and healthy plants and it depends on the season. In addition, this method is a very time-consuming and labor-intensive process (Kanwar et al. 2010) and a large numbers of cuttings do not survive during plantation. Plant regeneration of pomegranate is also an essential step in the success of generation of transgenic lines and determines the effectiveness of a transformation protocol (Aldwinckle and Malnoy 2009). Hence, developing an efficient in vitro technique for the propagation of pomegranate is of great importance. During the past decade, tissue culture techniques have been widely used for the propagation of some important tropical and subtropical fruit trees (Agarwal et al. 2004; Tsukamoto et al. 2007; Perez-Tornero et al. 2010; Skiada et al. 2010).

Protocols for regeneration of *P. granatum* have been reported by a number of researchers (Naik et al. 1999;

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Naik et al. 2000; Shao et al. 2003; Chauhan and Kanwar 2012); however, these protocols display several problems related to establishment of plant materials and in vitro responsiveness (Chauhan and Kanwar 2012). The most commonly used protocols for organogenesis have employed seedling derived plant materials (Chauhan and Kanwar 2012), but this approach is not suitable for elite cultivars.

Therefore, this study was undertaken to determine requirements for in vitro establishment, multiplication and rooting of cultures, and to develop an efficient micropropagation protocol for ‘Malas Saveh’ and ‘Yousef Khani’, two Iranian leading pomegranate cultivars. Consequently, this work has been designed to study the effect of medium types MS (Murashige and Skoog 1962) and WPM (Lloyd and McCown 1980) and growth regulators, Kinetin, NAA and IBA; on establishment of explants, multiplication and rooting of pomegranate plantlets as a step for further studies of improvement and breeding. To the best of our knowledge, this is the first report on micropropagation of Iranian pomegranate cultivars.

Materials and methods

The experiments were carried out in the Tissue Culture Laboratory, Department of Horticultural Sciences, University of Bu-Ali Sina, during the period from 2010 to 2011. Two pomegranate cultivars ‘Malas Saveh’ and ‘Yousef Khani’, obtained from the Pomegranate Research Station in Saveh (Central Iran), were used. Explants were obtained from 2 years old plants grown in 10 l pots in the green house with normal daylight and a temperature range of 20–35 °C during night/day.

Plant material and explant preparation

Shoot tip and nodal segments (0.5–1.5 cm long) were excised from 2 years old plants grown in a greenhouse. Following removing the leaves, explants were washed with running tap water for 30 min, then were disinfected for 10 min in a 0.1 w/v calcium hypochlorite solution with 2–3 drops Tween 20 and rinsed three times in autoclaved distilled water. The sterilized explants (two explants/jar) were vertically cultured on induction medium.

Culture media and culture conditions

Two different media; MS and WPM were used. Sucrose was added at 30.0 g l⁻¹ and myo-inositol at 0.1 g l⁻¹. The pH of the prepared media was then adjusted to 5.6–5.8 with 0.1 N NaOH, before adding 6 g l⁻¹ agar.

For proliferation stage, kinetin was employed at 0, 2.3, 4.7, 9.2 and 18.4 µM along with 0.54 µM NAA (0.54 µM

NAA was chosen based on the preliminary experiments). Media were then poured in 200 ml jars; media volume for each jar was 25 ml. The jars containing media were autoclaved at 121 °C and 1.5 kg cm⁻² pressure for 15 min and left to be air-cooled for media solidification. Explants cultured upright with the basal end of the node inserted a few millimeters into the culture medium. Explants were incubated in the dark for the first 7 day and then cultures were grown at 25±1 °C with white light (40 µmol m⁻² s⁻¹) and a 16 h photoperiod. Each cytokinin treatment consisted of seven replicate jars with two explants in each and repeated twice. After 28 day, shoot tips originating from explants were transferred to fresh media of the same kind and grown under above photoperiod condition. Subcultures were initiated by cutting shoots into nodal sections. Sprouted buds were further grown on the same media.

In vitro rooting of shoots

Shoots with 2–4 cm long developed in vitro were excised and cultured in half-strength WPM medium containing 50 mg l⁻¹ myo-inositol, 15 g l⁻¹ sucrose and 3 g l⁻¹ agar. The medium was further supplemented with 0, 2.5, 4.9 and 9.8 µM IBA or 0, 2.7, 5.4 and 10.8 µM NAA. After 8–10 day, the rooted shoots were transferred to an auxin-free half-strength medium for further elongation of the roots. Each auxin treatment consisted of seven replicate jars with one shoot in each. After 4 weeks in culture, rooting percentage, number and length of roots per rooted shoot were evaluated.

Acclimatization of regenerated plantlets

Well rooted shoots were removed from the culture medium. The roots were washed gently with tap water to remove agar, and then transferred to small plastic pots containing autoclaved cocopeat-perlite mixture. The pots were covered with polyethylene bags to maintain high humidity and kept at 25±1 °C in artificial light (50 µmol m⁻² s⁻¹) provided by white fluorescent tubes for 3–4 weeks. To harden the plants, polyethylene bags opened gradually, from a few min a day until normal conditions. Plants were then transferred to larger pots (18 cm diameter) containing garden soil (soil: compost, 1:1); kept under shade for another 2 weeks and then transferred to direct sunlight condition. The survival rate was examined 40 day after transfer.

Data analysis and measurements

For proliferation stage, the experiment was conducted under completely randomized design (CRD) with two cultivars,

two types of culture media (MS and WPM) and five concentrations of kinetin. A completely randomized design (CRD) with two cultivars and eight concentrations of auxin (IBA and NAA) was used for rooting experiments. The data were analyzed using SAS Version 9.1. Significant differences were assessed using Duncan's multiple range test at $P < 0.05$.

The percentage of shoot proliferation, shoot number, length of shoots, node number, leaf number, root number, length of roots and rooting percentage were recorded. Data included only new shoots longer than 0.5 cm.

For each cultivar, the relationship between Kinetin concentrations and shoot number, shoot length, node number and leaf number was best described by a second-order polynomial.

Results

The multi-factorial trial allowed a thorough statistical analysis, including the estimation of main factors and interaction effects (Table 1) with respect to the parameters investigated. Number of nodes was significantly influenced by cultivar ($P = 0.0006$), "Malas Saveh" had more nodes per plantlet in comparison with "Yousef Khani". However, cultivars showed no significant difference regarding percentage of shoot proliferation, number of shoots, length of shoots and number of leaves (Table 1).

Culture media

Culture media significantly affected the percentage of shoot proliferation ($P = 0.0243$), length of shoots ($P < 0.0001$) and the number of leaves ($P = 0.0370$), but it had no considerable influence on the number of nodes and shoots (Table 1). There was no significant interaction between cultivars and media, and both cultivars similarly responded to MS and WPM media (Table 1). The plantlets produced in WPM medium were apparently more vigorous, with shoots being longer, which is an important feature in micropropagation. MS was inferior, compared to WPM, in terms of the percentage of shoot proliferation, length of shoots, and number of leaves produced in this medium (Table 1) and the morphological appearance of the shoots. Therefore, WPM medium was subsequently used in rooting studies.

Effect of growth regulators

Shoots were obtained on medium with or without kinetin within 1–2 weeks; however, shoots failed elongate on medium without kinetin and shoot buds elongation was observed

only on media containing Kinetin (Table 1). Multiple shoots were obtained from explants cultured on media supplemented with Kinetin in different concentrations (2.3, 4.7, 9.2 and 18.4 μM) along with 0.54 μM NAA (Fig. 1a, b and c). Summary of the regression analysis for shoot proliferation responses as affected by different Kinetin concentrations is presented in Fig. 2. With increasing Kinetin concentration in the medium all growth indices improved; however, there were significant interactions between cultivars and Kinetin concentrations for the percentage of shoot proliferation, number of shoots, length of shoots and number of leaves. "Malas Saveh" and "Yousef Khani" showed better in vitro proliferation and produced more vigorous shoots with 4.7 and 9.2 μM Kinetin, respectively (Table 1).

Hyperhydricity appeared in plantlets cultured in other combinations, especially when high concentration of the cytokinin was used (data not shown). For "Yousef Khani", the supplementation of media with 9.2 μM Kinetin and 0.54 μM NAA seemed to favor proliferation, producing vigorous plantlets with the highest leaf number, shoot number and length of shoot without callus (Table 1).

In vitro rooting of shoots and acclimatization of plantlets

WPM medium devoid of NAA or IBA failed to induce roots and root induction was encouraged by addition of either NAA or IBA to medium (Table 2). Cultivars showed no significant differences in terms of rooting percentage, and number and length of roots. In addition, kind and concentration of auxins, and cultivars had no interaction for rooting indices. For both cultivars, half-strength WPM medium supplemented with 5.4 μM NAA or 4.9 μM IBA were most effective on rooting percentage and root number of shoots; however, length of roots was more increased when NAA was used (Table 2; Fig. 1d and e).

Rooted plantlets of both cultivars were successfully acclimatized and established in soil (Fig. 1f) with 80 % survival frequency. All the established plants were apparently uniform and did not show any visually detectable variation.

Discussion

The major problems generally faced in culturing tissues from fruit trees are contamination, recalcitrance of adult trees, hyper hydration, and browning or blackening of the culture medium and/or the explants due to leaching of phenolics which may be phytotoxic and cause necrosis of the explants (Bhojwani and Razdan 1996; Krishna and Singh 2007). In the present study, shoot tip and nodal segments obtained from two year old plants were used as explants. Better plant regeneration from node of young trees was also reported in *Oroxylum* (Dalal and

Table 1 Effect of different concentrations of Kinetin and culture medium on in vitro shoot proliferation of the pomegranate cultivars, ‘Malas Saveh’ and ‘Yousef Khani’.

Effects	Shoot proliferation (%)	Number of Shoots	Length of Shoots (cm)	Number of Nodes	Number of Leaves
Cultivar (C)					
‘Malas Saveh’	49.99±32.35	2.27±1.33	2.11±1.29	3.00±1.72 a	6.90±3.67
‘Yousef Khani’	49.99±32.47	1.8±1.18	2.13±1.31	2.72±1.55 b	6.98±3.76
Medium (M)					
MS	48.56±31.94 a	2.08±1.30	2.09±1.28 b	2.87±1.64	6.84±3.76 b
WPM	51.42±32.81 b	1.98±1.26	2.15±1.31 a	2.85±1.66	6.97±3.66 a
Kinetin (μM)					
K1(0)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00 d	0.00±0.00
K2 (2.3)	53.56±9.46	1.46±0.50	1.99±0.07	2.42±0.50 c	7.25±0.64
K3 (4.7)	79.16±9.04	2.57±0.57	3.33±0.60	4.10±0.49 a	9.92±1.01
K4 (9.2)	78.56±7.92	2.89±0.56	3.36±0.65	4.28±0.53 a	9.67±1.18
K5 (18.4)	38.68±7.92	3.25±0.58	1.92±0.08	3.50±0.57 b	7.60±0.49
C×K					
‘Malas Saveh’×K1	0.00±0.00 e	0.00±0.00 h	0.00±0.00 g	0.00±0.00	0.00±0.00 f
‘Malas Saveh’×K2	52.56±9.64 c	1.78±0.42 f	2.01±0.07 d	2.50±0.51	7.14±0.66 e
‘Malas Saveh’×K3	96.42±7.09 a	2.85±0.36 dc	3.92±0.08 b	4.50±0.51	10.71±0.46 a
‘Malas Saveh’×K4	60.71±8.28 b	3.21±0.42 ab	2.73±0.10 c	4.35±0.49	8.64±0.49 c
‘Malas Saveh’×K5	39.28±8.28 d	3.50±0.51 a	1.90±0.08 f	3.64±0.49	7.50±0.51 de
‘Yousef Khani’×K1	0.00±0.00 e	0.00±0.00 h	0.00±0.00 g	0.00±0.00	0.00±0.00 f
‘Yousef Khani’×K2	53.56±9.64 c	1.14±0.36 g	1.97±0.06 de	2.35±0.49	7.35±0.63 de
‘Yousef Khani’×K3	61.90±7.81 b	2.28±0.61 e	2.75±0.10 c	3.85±0.36	9.14±0.77 b
‘Yousef Khani’×K4	97.42±7.09 a	2.57±0.51 de	4.00±0.08 a	4.07±0.47	10.71±0.61 a
‘Yousef Khani’×K5	38.09±7.81 d	3.00±0.55 bc	1.93±0.08 ef	3.35±0.63	7.71±0.46 d
P-value					
Cultivar (C)	0.9870	<0.0001	0.1368	0.0006	0.0370
Medium (M)	0.0243	0.1805	<0.0001	0.8538	0.0260
Kinetin (K)	<.0001	<0.0001	<0.0001	<0.0001	<0.0001
C×M	0.9999	0.8477	0.7302	0.0992	0.1469
C×K	<.0001	0.0367	<0.0001	0.2389	<0.0001
M×K	0.4335	0.7897	0.0934	0.9355	0.5952
C×M×K	0.9853	0.9714	0.6511	0.5826	0.5599

Mean values (± SD) followed by similar letters under different treatments within the same column are not significantly different from each other at $P \leq 0.05$ (Duncan’s multiple range test)

Rai 2004), *Boswellia ovalifoliolata* (Chandrasekhar et al. 2005), *Pterocarpus santalinus* (Rajeswari and Paliwal 2006), *Stereospermum personatum* (Shukla et al. 2008). Browning of explants due to exudation of phenolics has been reported as a serious problem in establishing axenic cultures of tree species (Krishna and Singh 2007). However, this problem was not observed in this study where cultures were initiated from young explants. This is probably because young plants synthesize no or low amounts of phenolic substances (Rai et al. 2009).

Variation in regeneration ability among pomegranate cultivars has been previously reported (Naik and Chand 2011). In the present study, proliferation of ‘Malas Saveh’

was slightly better, compared to ‘Yousef Khani’. This variation may be resulted from the different requirements of each cultivar for growth regulators. Moreover, differences in stimulation of shoot elongation may be related to differences in macronutrients (Roubelakis-Angelakis and Zivanovitch 1991).

The use of plant growth regulators in pomegranate tissue culture is of fundamental importance. Many studies have been conducted in order to identify the optimal composition of plant growth regulators, for shoot and root differentiation as well as plant regeneration in pomegranate (Naik et al. 2000; Shao et al. 2003; Terakami et al. 2007; El-Agamy et al. 2009). The inclusion of cytokinins in the media is

Fig. 1 **a** shoot development from nodal segments of pomegranate cultivar ‘Malas Saveh’ after 10 day of culture on WPM medium containing $4.7\ \mu\text{M}$ Kinetin + $0.54\ \mu\text{M}$ NAA, **b** development of multiple shoots after 20 day of culture on the same medium, **c** elongation of shoots after 30 day of culture on the same medium, **d** root development in shoot cultured on half strength WPM medium containing $5.4\ \mu\text{M}$ NAA after 3 weeks of culture. **e** a well-rooted plantlet ready for acclimatization. **f** a healthy and well-hardened plant

generally considered necessary for proliferation of apical and axillary buds (Naik and Chand 2011); However, when only Kinetin was used in auxin free media shoot proliferation and elongation were decreased about 30 % for both cultivars (data not shown).

Differences in internal growth regulators contents of each genotype has been suggested to be the reason for different in vitro response of several plant species (Looney et al. 1998), and variability between pomegranate cultivars in micropropagation may be attributed to that difference. BA, BAP, Kinetin, ZR and TDZ have been the most commonly used plant growth regulators either alone or in combination with a low concentration of auxin (Naik and Chand 2011). Supplementing auxins in the culture medium is necessary for some cultivars, while others can be proliferated without this growth regulator (Naik and Chand 2011). The first is presumably the case for ‘Malas Saveh’ and ‘Yousef Khani’ as shoot proliferation was favored, in both cultivars, in the presence of NAA. According to some researches when using auxin is not obligatory the explants function as active centers of auxin biosynthesis (Ibáñez et al. 2005), which did not occur for our cultivars.

Media compositions have a key role in morphogenesis; MS in full salt strength was the most commonly used basal medium for pomegranate micropropagation, but half strength MS (1/2 MS), WPM and B5 (Gamborg et al. 1968) showed to be useful in some cultivars (Naik and Chand 2011). The fact that lower concentrations of Kinetin and NAA were demanded in WPM, compared to MS, supports the hypothesis that this medium is more suitable for ‘Malas Saveh’ and ‘Yousef Khani’.

Rooting of shoots derived from in vitro cultures is a prerequisite to provide the physiological establishment in soil. NAA or IBA has been the most commonly used auxin for promoting rooting of in vitro regenerated shoots of pomegranate. The type of auxin seems to strongly affect the rooting of explants (Heloir et al. 1997). It has been reported that NAA, especially in high concentrations, caused severe toxicity problems in explants (De Klerk et al. 1997). Similar results were obtained in this study with both auxins, NAA and IBA. In pomegranate, MS and WPM, mostly in half-strength, have been attempted with varying success (Naik and Chand 2011). In the present study, half-strength WPM medium was efficient enough for root induction. Low salt concentrations in medium are known to enhance rooting of shoots in several plant species (Rai et al. 2010; Thimmappaiah et al. 2007).

After 40 day of hardening, no survival problems were exhibited for both cultivars. The survival rate paralleled the physiological appearance of plants after auxin treatment, which enforces the suggestion that the concentration and the type of rooting growth regulator highly affect the plants after acclimatization (Kristiansen 1992). Morphology of plants at the end of the in vitro rooting is closely dependent on the *ex vitro* survival of plantlets (Chee et al. 1984).

Results obtained in this study are considered satisfactory for providing a potential commercial micropropagation protocol for ‘Malas Saveh’ and ‘Yousef Khani’, ensuring proper conditions for the in vitro maintenance of these two leading Iranian pomegranate cultivars, with unique characteristics. In vitro propagation of pomegranate cultivars has been reported earlier (Naik and Chand 2011). However, the limitations of

Fig. 2 Effects of Kinetin concentrations on shoot number, shoot length, node number and leaf number in Malas Saveh and Yousef Khani. Actual values (solid rhombuses and squares), the fitted by the

second order polynomial (solid lines), and the fitted regression model of shoot number, shoot length, node number and leaf number for each cultivar

previous protocols are browning of the culture medium followed by necrosis of the explants which influenced the establishment of shoot cultures, while this problem was negligible

in the present study. This method may also be used to select and clone these two superior cultivars, and this protocol could also be applied for pharmaceutical purposes.

Table 2 Effect of different concentrations of IBA and NAA on rooting of shoots of 'Malas Saveh' and 'Yousef Khani'

Effects	Rooting (%)	Root number	Root length (cm)
Cultivar (C)			
'Malas Saveh'	46.72±18.09	3.50±2.44	4.14±2.73
'Yousef Khani'	46.42±17.16	3.53±2.41	4.14±2.73
Auxin (μM)			
IBA (0)	0.00±0.00 d	0.00±0.00 d	0.00±0.00 g
IBA (2.5)	35.71±6.05 c	2.92±0.69 c	3.88±0.13 f
IBA (4.9)	95.23±7.81 a	6.35±0.49 a	7.07±0.12 b
IBA (9.8)	52.38±6.04 b	4.35±0.49 b	4.89±0.15 d
NAA (0)	0.00±0.00 d	0.00±0.00 d	0.00±0.00 g
NAA (2.7)	39.28±8.28 c	3.21±0.47 c	4.10±0.08 e
NAA (5.4)	96.42±7.09 a	6.64±0.49 a	7.82±0.12 a
NAA (10.8)	53.57±7.09 b	4.64±0.49 b	5.37±0.11 c
P-value			
Cultivar (C)	0.8052	0.6920	0.7308
Auxin (A)	<0.0001	<0.0001	<0.0001
C×A	0.9845	0.9717	0.5216

Mean values (± SD) followed by the similar letters under different treatments within a column are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test)

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