Influence of Type of Explant, Salt Composition of Basal Medium and Cytokinins on Micropropagation of Pomegranate (*Punica granatum* 'Khadouri')

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Abstract

A study was carried out for micropropagation of pomegranate (*Punica granatum* 'Khadouri'). Explants, shoot tips and single nodes from mature trees, were surface sterilized and cultured on full strength Murashige and Skoog (MS) and woody plant medium (WPM) supplemented with benzyl adenine (BA) at 4.5 µM. To test the effect of cytokinin type and concentrations, BA, Kinetin (Kn) and Zeatin (Ze) were used at a concentration ranging from 2.23 to 13.5 µM. Results indicated that single node explants responded better than shoot tips based on number of growing shoots, number of leaf per explant and shoot length. Woody plant medium was superior to MS medium in shoot number and shoot length. Type and concentration of cytokinin significantly influenced shoot proliferation, BA and kinetin achieved the highest shoot proliferation as compared to zeatine. The maximum number of shoots per explant (2.9) was developed on medium containing 9.0 µM BA. Rooting of microshoots was achieved either in vitro using NAA at 1.0 µM or ex vitro using 500 mg/L quick dip. The latter procedure gave higher rooting percentage (90%) and produced well-grown and acclimatized plantlets in soil:peat moss mix (1:1 v/v).

INTRODUCTION

Pomegranate (*Punica granatum* L.) is a member of the family *Punicaceae*, which comprises only one genus (*Punica*) and two species; *P. granatum* and *P. protopunica* (Popenoe, 1974). It is an economically important species of the tropical and subtropical regions of the world. Pomegranate is grown in North African countries where the climate is suitable for its cultivation. Micropropagation in fruit trees would help in overcoming difficulties of vegetative propagation, producing true-to-type plants and mass production of planting materials (El-Agamy et al., 2009). Several studies have been published on micropropagation of pomegranate trees over the last two decades and protocols have been developed for micropropagation through organogenesis using different types of explants such as single nodes (Naik et al., 1999, 2000; Patil et al., 2011) and shoot tips (El-Agamy et al., 2009) or through somatic embryogenesis (Sharon et al., 2011). Growth regulators such as benzyladenine (BA), kinetin (Kn), zeatine riboside (ZR) or thidiazuron (TDZ) were used to enhance axillary branching with variable success rates (Patil et al., 2011; El-Agamy et al., 2009; Naik et al., 2000). Basal salts media have also been studied including woody plant medium (WPM) (Lloyd and McCown, 1980), and Murashige and Skoog medium (MS) (Murashige and Skoog, 1962).

Pomegranate is among several fruit trees cultured in Libya where the climate is favorable for its production. The 'Khadouri' cultivar probably is considered a local one and is spread widely in agricultural areas around Tripoli. In recent years, the tree was plagued with apple stem borer which resulted in devastation of a huge number of trees with other fruit trees. In an attempt to mass propagate this important tree, the present study was carried out to study factors that affect the response of pomegranate explants in culture such as explant type, cytokinin type and concentration and type of basal medium for micropropagation of cultivar 'Khadouri' through axillary shoot proliferation.

MATERIALS AND METHODS

Explant Preparation and Culture

Growing shoots of mature trees of 'Khadouri' pomegranate cultivar were collected from a private farm 30 km east of Tripoli and brought to the laboratory on the same day. Shoots were washed under running tap water for 30 min and cut into shoot tips and single nodes of about 2-3 cm, and soaked in an antioxidant solution of ascorbic acid at 150 mg/L for 20 min under the laminar air flow cabinet and surface sterilized using 1.75% (available chlorine) solution of Clorox, followed by three times rinses in sterile double distilled water. For explant establishment, growth regulators-free MS medium was used, and explants were cultured for one week. The first experiment tested the effect of explant type; single nodes and shoot tips, and type of medium; MS and WPM basal media supplemented with BA at 4.5 µM for four weeks. The second experiment tested the effect of type and concentration of cytokinins; BA, kinetin (Kn) or zeatin (Ze) at 0.0, 2.23, 4.5, 9.0 and 13.5 µM using single node explants cultured onto WPM for four weeks. Cultures were incubated at 25°C at 16/8 h of light and dark regime for 4 weeks. Data were collected on number of growing shoots, total number of leaves per shoot and shoot length.

Rooting

For rooting of microshoots, two methods were used; in vitro rooting was achieved using the auxins; indole butyric acid (IBA) and naphthalene acetic acid (NAA) at 0.0, 0.5, 1.0 and 2.0 µM on ½ strength WPM, or ex vitro by using either ofauxins at 500 mg/L quick dip for 30 s and cultured on sterile growing medium (peat moss:sand at 1:1 v/v). Data were collected on % rooting, number of roots per plantlets and root length after 4 weeks of culture.

Statistical Analysis

The experiment effects of (a): explant type and medium; (b) type and concentration of cytokinins, (c) auxin type and concentration (in vivo rooting) were set up as split-plot design using 5 replicates in a completely randomized design. Means separation was done using DMRT (Duncan, 1955) at 5% level.

RESULTS AND DISCUSSION

Explants which were cultured on media containing BA at 4.5 µM indicated that single nodes explants had significantly higher axillary shoot growth than shoot tips and other studied parameters (Table 1). Results on number of shoots per explant obtained in this experiment was lower than that reported by Patil et al. (2011) in which they used single node explants cultured on MS medium amended with silver nitrate (1.0 mg/L silver nitrate and 30 mg/L adenine sulphate) and supplemented with BAP and NAA in multiplication stage.

Results shown in Table 2 indicate that WPM significantly had a higher number of growing shoots and shoot length than MS medium. A few published reports on pomegranate micropropagation have used both kinds of media with variable success rates. Naik et al. (1999) used MS medium and obtained high proliferation rate in 'Ganesh' in India, while El-Agamy et al. (2009) used WPM for 'Manfalouty' and 'Nab El-gamal' pomegranate cultivars.

Among cytokinins tested, the best results obtained were on medium supplemented with BA, resulting in the production of 2.1 shoots/explant and 13.0 leaf per shoot (Table 3) followed by kinetin, and zeatine which was the least effective in this study. Among cytokinins concentration (main effect), 9.0 µM in general achieved the highest proliferation rate in all the studied parameters. Several authors have used cytokinins alone (Naik et al., 1999, 2000) or in combination with auxins (El-Agamy et al., 2009) for micropropagation. Results of the present study were in agreement with those reported by El-Agamy (2009) regarding the effect of cytokinins such as BA on number of shoots, number of leaves and shoot length. Naik et al. (1999, 2000) found that 9.0 µM BA was

optimal and gave the best result in micropropagation of 'Ganesh' pomegranate in which maximum number of shoots were developed on a medium containing 2.0 mg/L (9.0 µM).

Addition of an auxin to the medium was essential for root induction in microshoots. The main effect of auxins has shown that there was no statistical difference in rooting % between NAA and IBA, though NAA had slightly higher rooting% (Table 4). Root formation appeared approximately 14 days of culture on ½ WPM medium supplemented with NAA and IBA. NAA significantly had higher number of roots per plantlets (4.9) and root length (3.3 cm). The main effect of concentration indicated that no rooting occurred in control. Although there was no statistical difference among concentrations tested, 1.0 µM produced the highest rooting %, and led to highest number of roots per plantlet (5.1) and root length (4.7 cm). Many authors have used either ½ MS with 1.0 mg/L IBA (Naik et al., 1999), or ½ WPM supplied with 0.54 µM NAA (Naik et al., 2000) or ½ WPM supplied with 0.25 mg/L IBA (El-Agamy et al., 2009).

In an ex vitro experiment, the same rooting percentage was observed (90%) in NAA and IBA containing medium (Table 5). NAA significantly led to a higher number of roots per plantlets (8.4) and root length (9.2 cm). Ex vitro rooting generally gave higher rooting than in vitro rooting method. Ex vitro rooting has been applied in many fruit trees including blueberry (Isutsa et al., 1994), apples (Jin et al., 2008) and passion fruits (Isutsa, 2004), and to the best of our knowledge, this is the first report on ex vitro rooting in 'Khadouri' pomegranate. The present study has shown that micropropagation of 'Khadouri' pomegranate is feasible using axillary bud branching though future work is needed to refine or improve multiplication rate. Figure 1 shows growth of single nodes, rooting and fully acclimatized plantlets.

CONCLUSIONS

A protocol was developed for micropropagation of pomegranate cultivar 'Khadouri' using single node explants cultured onto WPM supplemented with BA at 9.0 M. For rooting of microshoots, ex vitro method was suitable using NAA at 500 mg/L quick dip.

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Tables

Table 1. Influence of explant type on number of shoots and total number of leaves per shoot and shoot length in 'Khadouri' after 4 weeks in culture.

Explant type	Mean no. of shoots	Total number of leaves	Mean length of shoots (cm)
Single node	2.7a[x]	11.0a	2.0a
Shoot tip	1.0b	2.7b	1.3b

[x] Means in columns followed by the same letter are not significantly different using DMRT at 5% level.

Table 2. Influence of culture medium on number of shoots and total number of leaves per shoot and shoot length in 'Khadouri' after 4 weeks in culture.

Medium	Mean no. of shoots	Total number of leaves	Mean length of shoots (cm)
WPM	2.2a[x]	11.3a	2.3a
MS	1.5b	5.6b	1.0b

[x] Means in columns followed by the same letter are not significantly different using DMRT at 5% level.

Table 3. Influence of type and concentration of cytokinins on number of shoots, total number of leaves per shoot and shoot length in 'Khadouri' after 4 weeks in culture.

Variable	Mean no. of shoots	Total number of leaves	Mean length of shoots (cm)
Cytokinins			
BA	2.1a	13.0a	3.0a
Kn	2.0b	12.7b	2.2a
Ze	1.6b	9.6b	1.8b
Concentration (µM)			
0.0	1.0d	7.6d	1.2d
2.23	1.8c	11.1c	2.1c
4.5	2.2b	13.2b	2.5b
9.0	2.9a	15.6a	3.7a
13.5	1.6c	11.1c	2.2b

[x] Means in columns (for each main effect) followed by the same letter are not significantly different using DMRT at 5% level.

Table 4. Influence of type and concentration of NAA and IBA on % rooting, number of roots per plant and root length in 'Khadouri' after 4 weeks in culture (in vitro rooting).

Variable	Rooting of roots (%)	Mean number of roots (cm)	Mean length
Auxin			
NAA	78a	4.9a	3.3a
IBA	72a	3.7b	2.5b
Concentration (µM)			
0.0	0.0b	0.0c	0.0c
0.5	72a	4.2b	2.2b
1.0	90a	5.1a	4.7a
2.0	63a	3.9b	1.9b

[x] Means in columns (for each main effect) followed by the same letter are not significantly different using DMRT at 5% level.

Table 5. Influence of NAA and IBA on % rooting, number of roots per plantlets and root length in 'Khadouri' after 4 weeks in culture (ex vitro rooting).

Auxin	Rooting of roots (%)	Mean number per plantlet	Mean length of roots (cm)
NAA	90a	8.4a	9.2a
IBA	90a	5.4b	7.0b

[x] Means in columns (for each main effect) followed by the same letter are not significantly different using DMRT at 5% level.

Figures

Fig. 1. Growth of single nodes (upper left), in vivo rooting (upper right), ex vitro rooting (lower left) and fully acclimatized plants (lower right) of ‘Khadouri’.