

Callus Induction and Plant Regeneration in *Punica granatum* L. 'Nana' from Leaf Explants

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ABSTRACT

In this investigation, leaf explants of a local cultivar of dwarf pomegranate were placed on Murashige and Skoog (1962) (MS) medium supplemented with various concentrations of 6-benzyl adenin (BA) and naphthalene acetic acid (NAA) for callus induction. After 40 days, maximum callus induction was observed on a media containing 1 mg L⁻¹ BA and 0.2 to 0.4 mg L⁻¹ NAA. However, the highest callus growth was obtained on a medium containing 1 mg L⁻¹ BA and 1 mg L⁻¹ NAA. The highest number of shoots (7 shoots per explants) was obtained by transferring the calli to the media containing 5 mg L⁻¹ BA with 0.1 mg L⁻¹ NAA. Maximum shoot proliferation was observed when shoots were cultured on woody plant medium (WPM) supplemented with 5 mg L⁻¹ kinetin (Kin). In this treatment, after 4 subcultures, 36 shoots were produced from one original explant. Among treatments used in rooting experiments, shoots cultured on WPM medium containing 0.2 mg L⁻¹ indol butyric acid (IBA) had the maximum root percentage (100%) and good root growth (2.06 cm mean length and 2 roots in each explants). Rooted plantlets were cultured in a soil mixture containing vermiculite (60%), perlite (30%) and coco peat (10%) v/v. After 2 months, 80% of plants survived and transferred to the greenhouse.

Key words: Callus induction, Dwarf pomegranate, Rooting, Shoots proliferation, Tissue culture

INTRODUCTION

Dwarf pomegranates (*Punica granatum* L. 'Nana') belong to the Punicaceae family. It is a beautiful shrub with 30 to 50 cm height and produce attractive small red and orange flowers and subsequently small pomegranate fruits. Traditionally, this plant is propagated by seeds resulting in a highly variable progeny. Cutting is another method for propagation but it is not efficient because of being hard-to-root and consequently a low number of new plants can be obtained from a mother plant. An efficient *in vitro* regeneration protocol has not yet been developed for ornamental pomegranate.

Although tissue cultures of edible pomegranate via shoot organogenesis, somatic embryogenesis, and enhanced auxiliary bud proliferation have been reported by Omura, et al. (1987); Naik, et al. (1999, 2000); Shao, et al. (2003) and Terakami, et al. (2007). In this study an efficient system for callus induction, regeneration, rooting and acclimation of dwarf pomegranate is reported. This protocol could be used for propagation, distinguishing drought and salt tolerant cultivars and gene transfer in this plant.

MATERIALS AND METHODS

A one year-old local cultivar of dwarf pomegranate (*Punica granatum* L. 'Nana') was used in this study. Plants were grown in the greenhouse of the Department of Horticultural Science, Shiraz University, Iran. Leaves excised from the mother plant were washed with running tap water and surface sterilized with 10% sodium hypochlorite for 7 minutes. They were then washed 3 times with sterile distilled water and used in following experiments.

Callus induction and growth

Leaf segments (5×10 mm length) were placed on 5 cm petridishes containing MS medium supplemented with various concentrations of BA (0, 1, 2, 3, 4, 5 mg L⁻¹) and NAA (0, 0.1, 0.2, 0.3, 0.4, 0.5 mg L⁻¹) for callus induction. Treatments were maintained for 4 weeks under a dark condition and percentage of callus induction was recorded. In a separate experiment, callus segments (about 5×5 mm) cultured on MS medium supplemented with various concentrations of BA (0-0.5, 1 mg L⁻¹) and NAA (0-0.5, 1 mg L⁻¹) for callus growth. All treatments were maintained for 4 weeks under a dark condition and callus fresh and dry weights and diameters were measured.

Shoot regeneration and proliferation

Small pieces of callus (about 5–8 mm) were cultured on MS medium supplemented with various concentrations of BA (1, 2, 3, 4, 5 mg L⁻¹) and NAA (0, 0.1, 0.2, 0.3, 0.4, 0.5 mg L⁻¹) for shoot induction. Treatments were subcultured on the same medium after 4 weeks, number of shoots and times needed to regenerate shoots in each treatment were recorded. Regenerated shoots were cut to small pieces (about 2 cm in length with two accompanying buds) and cultured on WPM medium supplemented with various concentrations of BA and Kin (0, 1, 2, 3, 4, 5 mg L⁻¹) for shoot proliferation. Treatments were subcultured after 4 weeks on the same medium and length and total number of shoots were recorded after 4 repeated subcultures. All treatments maintained under a 16- hour photoperiod with 1500 lux light intensity emitted from cool-white fluorescent lamps.

Rooting experiments

Regenerated shoots were cut to small pieces (about 2 cm in length with two accompanying buds) and the bottom of shoots were inserted in various concentrations of IBA solution (0, 500, 1000 and 1500 mg L⁻¹) for 5 seconds and then cultured on MS/2 and WPM media. Shoots were also placed on WPM medium supplemented with

various concentrations of IBA (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg L⁻¹) for one week and then transferred to hormone free WPM medium. Cultures were kept for 6 weeks under a 16- hour photoperiod with 1500 lux light intensity emitted from cool-white fluorescent lamps and the percentages, numbers and lengths of roots were recorded.

All experiments were conducted in a completely randomized design with four replications. In all experiments, five explants were cultured in each culture vessel. All media contained 8 g L⁻¹ agar, 30 g L⁻¹ sucrose and pH was adjusted to 5.7. Means were separated by LSD test using the MSTAT-C statistical program.

RESULTS

Leaf explants in all treatment containing BA initiated callus about 15 days after culture. Calli were formed on the cut surfaces of leaves (Figure. 1). Among all treatments, those containing 1 mg L⁻¹ BA in combination with 0.2 or 0.4 mg L⁻¹ NAA had the maximum callus induction percentage (100%) (Table 1). BA had a critical role in callus induction since no callus was formed when leaf explants were placed on MS medium without BA. Adding BA to medium increased percentage of callus induction while further increase in BA levels decreased this trait. Mean callus induction in treatments containing 1 mg L⁻¹ BA was 78.89%, which showed significant differences ($P<5\%$) with other levels of BA. Callus produced in media with low levels of BA was friable and greenish yellow in color but at the highest level of BA callus produced was green and hard and almost ready to initiate shoots. Maximum callus growth was obtained in MS medium supplemented with 1 mg L⁻¹ BA and 1 mg L⁻¹ NAA (Table 2). This treatment had the highest callus fresh weight (0.61 g), dry weight (0.068 g) and diameter (16.16 mm) and was significantly different ($P<5\%$) with other treatments except a treatment containing 1 mg L⁻¹ BA and 0.5 mg L⁻¹ NAA (Table 2).



Figure 1. Green calli initiated from leaf explants of dwarf pomegranate culture on MS medium containing 1 mg L⁻¹ BA with 0.2 mg L⁻¹ NAA.

Table 1. Effects of various concentrations of BA and NAA on callus induction percentages of dwarf pomegranate after 30 days.

NAA (mg L ⁻¹)	BA (mg L ⁻¹)					
	0	1	2	3	4	5
0	0	33.43 fghijkl*	98.03 a	50 defghi	50 defghi	28.33 mn
0.1	0	56.67 bcdefgh	66.66 abcdef	46.67 efghijk	46.67 efghijk	28.33 mn
0.2	0	100 a	76.42 abcd	70 abcdef	70 abcdef	21.67 n
0.3	0	90 ab	85 abc	66.66 abcdef	66.66 abcdef	30 klmn
0.4	0	100 a	53.33 cdefghi	80 abcd	80 abcd	36.67 hijklm
0.5	0	83.33 abcd	74.81 abcde	83.33 abc	83.33 abc	38.33 hijklm

*Value with different letters are significantly different at 5% level of probability using LSD test.

Table 2. Effects of BA and NAA on callus growth characters of dwarf pomegranate

BA (mg L ⁻¹)	NAA (mg L ⁻¹)	Fresh weight (g)	Dry weight (g)	Diameter (mm)
0	0	0.225 d*	0.025 e	10.75 b
0	0.5	0.313 cd	0.035 de	11.67 b
0	1	0.30 cd	0.035 de	11.66 b
0.5	0	0.366 bc	0.048 cd	12.08 b
0.5	0.5	0.345 bcd	0.038 de	12.80 b
0.5	1	0.423 bc	0.046 cd	13.50 ab
1	0	0.461 b	0.053 bc	12.58 b
1	0.5	0.605 a	0.065 ab	15.75 a
1	1	0.611 a	0.068 a	16.16 a

*Values with different letters (in column) are significantly different at 5% level of probability using LSD test.

Shoot regeneration

The first shoot regenerated after 32 days in MS medium containing 5 mg L⁻¹ BA with 0 mg L⁻¹ NAA. At low level of BA, the time needed to produce shoot was longer so that in 1 mg L⁻¹ BA, first shoot regenerated after about 60 days (Table 3). The maximum number of shoots were regenerated on MS medium containing 5 mg L⁻¹ BA and 0.1 mg L⁻¹ NAA (Figure 2). In lower concentrations, mean number of shoots was decreased in such a way that in medium containing 1 mg L⁻¹ BA average number of shoot was 0.83 in each callus pieces after 60 days. There was a significant difference between treatment containing MS medium supplemented with 5 mg L⁻¹ BA with other treatments in relation to number of shoots produced on each callus and the time needed for shoot regeneration (Table 3, 4).

Table 3. Effects of various concentrations of BA and NAA on time (days) of shoot regeneration in callus of dwarf pomegranate.

NAA (mg L ⁻¹)	BA (mg L ⁻¹)				
	1	2	3	4	5
0	66.3 a*	56 c	46.3 fgh	46 fghi	41.6 k
0.1	63 b	55 cd	45.6 fghi	45.6 fghi	42 jk
0.2	63.3 ab	52 de	45 fghij	47.3 fg	42.3 jk
0.3	63.3 ab	51.6 e	46.3 fgh	46.6 fg	43 ijk
0.4	62.6 b	51.3 e	45 fghij	44.6 fghijk	43.3 hijk
0.5	61.3 b	51.6 e	45 fghij	44.6 fghijk	43.6 ghijk

*Values with different letters are significantly different at 5% level of probability using LSD test.

Table 4. Effects of various concentrations of BA and NAA on number of shoot regeneration in callus of dwarf pomegranate.

NAA (mg L ⁻¹)	BA (mg L ⁻¹)				
	1	2	3	4	5
0	0.83 j*	1.25 gij	2.5 fg	4 cd	7 a
0.1	0.93 ij	1.43 hij	2.91 ef	4 cd	7.33 a
0.2	1.06 hij	1.41 hij	3 ef	4.41 bc	7.16 a
0.3	1.33 hij	1.66 hi	3.08 ef	4.82 b	7.16 a
0.4	1.23 hi	1.56 hij	3.25 def	4.75 bc	6.83 a
0.5	1.66 hi	1.83 gh	3.33 de	5 b	6.83 a

*Values with different letters are significantly different at 5% level of probability using LSD test.

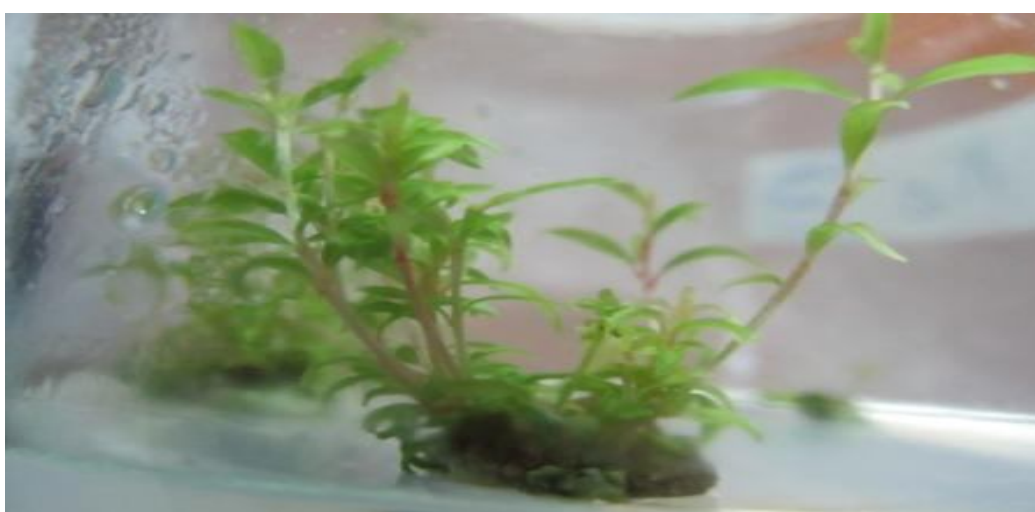


Figure 2. Shoots regenerated in MS medium containing 5 mg L⁻¹ BA with 0 mg L⁻¹ NAA.

Shoot proliferation

Among treatments used for shoot proliferation, treatment containing WPM medium supplemented with 5 mg L⁻¹ Kin had maximum shoot proliferation. After 4 subsequent subcultures, total number of shoot produced in WPM medium containing 5 mg L⁻¹ Kin was 36. In average, the highest number of shoots produced in the first subculture was 4.1 followed by 3.35, 1.64 and 1.55 shoots per explants in second, third and fourth subcultures, respectively. Increase in Kin levels enhanced the number of shoots produced in each explants (Table 5). Using 1 mg L⁻¹ Kin, only 8 shoots produced from each explants and shoots proliferation gradually increased and reached to 36 in 5 mg L⁻¹ Kin. BA had different effects, highest number of shoots (26.6 shoots per explants) resulted from a low level of BA (WPM medium containing 1 mgL⁻¹ BA). Increase in BA level decreased number of shoots produced so that in 5 mg L⁻¹ BA only 12 shoots produced after 4 subcultures. The highest shoot growth occurred in WPM medium supplemented with 1 mg L⁻¹ Kin (3.05 cm). There was a significant difference between BA and Kin in relation to shoot growth. Generally, all treatments containing Kin had better growth than treatments containing BA (Table 5). Mean shoot growth in treatments containing Kin was 2.79 cm and in treatments containing BA was 1.95 cm with significant differences (P<5%) of probability (Data not shown).

Table 5. Effects of BA and Kin on shoot proliferation of dwarf pomegranate.

Treatments(mg L ⁻¹)	Shoot number	Shoot length(cm)
BA 1	26.63 ab*	2.05 d
BA 2	15.99 cdef	1.91 d
BA 3	20.23 bcd	1.92 d
BA 4	23.19 bc	2.10 cd
BA 5	12.23 def	1.77 d
Kinetin 1	8.12 f	3.05 a
Kinetin 2	10.38 ef	2.37 bcd
Kinetin 3	19.16 bcde	2.99 ab
Kinetin 4	19.40 bcde	2.8 ab
Kinetin 5	36.17 a	2.73 abc

*Values with different letters (in column) are significantly different at 5% level of probability using LSD test.

Rooting

Preliminary experiments on rooting showed that in vitro root growth and development were accelerated by the reduced MS salt concentrations. Among treatments, WPM medium containing 0.2 mg L⁻¹ IBA was the best treatment. Rooting percentage was 100% and explants had a suitable root growth and root number (Table 6). Rooted plantlets were transferred to 15 -cm pots containing 60% vermiculite, 30% perlite and 10% cocopeat v/v and were grown under greenhouse conditions. Plant transferred to pots showed good development and high survival rates (80%) after 2 months (Data not shown). Plants grew successfully into normal mature plants in greenhouse (Fig. 3).



Figure 3. Dwarf pomegranates growth in greenhouse condition after 2 months

Table 6. Treatments used for rooting of regenerated shoots of dwarf pomegranate. Values with different letters (in column) are significantly different at 5% level of

IBA pretreatment (mg L ⁻¹)	Culture media	Root (%)	Root no.	Root length (cm)
0	WPM	75 abc*	2.25 cde	2.68 a
500	WPM	83.25 ab	2.11 cd	1.71 abcd
1000	WPM	46 cde	3.03 ab	1.36 bcd
1500	WPM	81.25 ab	2.65 bc	1.50 bcd
0	½ MS	37.25 e	1.37 ef	1.81 abc
500	½ MS	32.25 e	3.73 a	1.42 bcd
1000	½ MS	44.25 de	1.8 de	1.05 cd
1500	½ MS	43.75 de	2.9 abc	0.8 d
-	WPM+0.1IBA(mg L ⁻¹)	67.50 abcd	1 f	1.57 bcd
-	WPM+0.2 IBA(mg L ⁻¹)	100 a	2.62 bc	2.06 ab
-	WPM+0.3IBA(mg L ⁻¹)	95.05 a	2.17 cde	1.85 abc
-	WPM+0.4IBA(mg L ⁻¹)	50 cde	1.20 f	1.96 abc
-	WPM+0.5IBA(mg L ⁻¹)	53.50 bcde	1.39 ef	1.48 bcd

probability using LSD test.

DISCUSSION

The results of this investigation showed that leaf explant was an excellent source for plant multiplication in *Punica granatum* L. 'Nana'. Placing leaf pieces on MS medium supplemented with various concentrations of BA and NAA showed that BA alone supplemented to MS medium was sufficient for callus induction in leaf explants. This result indicating that leaf tissue of dwarf pomegranate might need moderate cytokinin levels for callus induction. These results are in agreement with those of Omura, et al.

(1987) who obtained high frequency of callus induction in leaf segment of dwarf pomegranate 'IZ 1' in 5 μM BA and other type of cytokinins. Murkute, et al. (2002) reported the highest callus induction (76.4%) and proliferation from cotyledonary explants of pomegranate 'Ganesh' when explants were cultured on MS basal medium supplemented with 4.44 μM BA and 2.69 μM NAA. Kanwar, et al. (2010) induced callus in wild pomegranate when cotyledonary explants were placed on MS medium containing 21 μM NAA and 9 μM BA and 80% of explants developed callus. In our results NAA was not necessary for callus induction; this may be related to adequate endogenous auxin content in leaf tissue of dwarf pomegranate. We obtained high frequency of shoot regeneration (7 shoot per explants) on MS medium with high level of BA and low level of NAA (BA 5 mg L^{-1} and NAA 0 mg L^{-1}). Our results are in agreement with many reports such as Omura, et al. (1987) in dwarf pomegranate who reported maximum shoot regeneration and shoot growth occurred in moderate level of BA (2 μM) and low level of NAA (0.1 μM). Also Kanwar et al. (2010) in wild pomegranate obtained high frequency of regenerated shoots from callus (54.67%) using MS medium supplemented with 8 μM BA, 6 μM NAA and 6 μM GA3. Murkute, et al. (2010) reported a 62.4% shoot bud differentiation frequency in cotyledonary explants of pomegranate on MS medium containing 1 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. Jaidka and Mehra (1986) also reported the highest frequency (80%) of shoot differentiation from the same explants. In these reports BA and NAA were necessary for best shoot regeneration but in our study only BA was necessary for shoot regeneration. Addition of NAA was not highly effective; only at low levels of BA; increase in NAA level enhanced the number of shoots produced. These differences in results may be related to difference in cultivars and callus sources. Browning of the culture medium due to phenolic compounds was a common problem in tissue culture of pomegranate cultivars (Naik, et al., 1998). In the present study, the browning problem was overcome by repeatedly transferring the explants to a new fresh medium.

Comparison between Kin and BA on shoot proliferation showed that Kin was more effective than BA; also Kin in high level and BA in low level had maximum effects on shoot proliferation of dwarf pomegranate. Omura, et al. (1987) reported 5 μM of BA was more effective than same concentration of Kin, 2ip and zeatin on shoot proliferation of dwarf pomegranate (98% bud formation and 4.2 shoot per explants). Naik, et al. (2000) reported BA was more effective than Kin for shoot proliferation in 'Ganesh' using MS medium supplemented with 9 μM BA (9.8 shoots per explants). In all of these reports, BA and Kin increased shoot proliferation but in this study Kin was more effective than BA and produced higher number of shoots. These differences may be related to type of medium used in experiments and genotypic differences between cultivars. There are several reports in relation to positive effects of Kin on shoot proliferation. Rashed, et al. (2005) reported that shoot tip explants of *Citrus aurantifolia* Swingle and *Citrus sinensis* L. cultured on MS media supplemented with 1 mg L^{-1} of BAP and 1.5 mg L^{-1} of Kin showed the highest shoot induction percentage. Haripyaree, et al. (2010) reported that the maximum number of shoots was induced on MS medium containing 1 mg L^{-1} BA with 0.50 mg L^{-1} Kin in *Citrus megaloxycarpa* Lush.

In present study, we found that rooting on WPM medium containing low level of IBA was the best treatment for rooting of dwarf pomegranates. IBA was reported to have favored root induction in pomegranate (Naik, et al., 1998), sweet orange (Khalil, et al.,

2011) and in *Citrus limonia* Osbeck (Jajoo, 2010, Omura, et al., 1987) reported optimum rooting of dwarf pomegranate was obtained in MS/2 medium supplemented with .01 to 0.1 μ M NAA. This method of propagation from leaf explants and further shoot proliferation and plantlet formation in 4- month duration could be alternatively used instead of conventional methods of propagation of dwarf pomegranate.

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