Journal of Applied Biological Sciences 7 (1): 47-51, 2013
ISSN: 1307-1130, E-ISSN: 2146-0108, www.nobel.gen.tr

In vitro Plant Regeneration from callus in semi dwarf cultivar of *Punica granatum* L. *'Nana'*

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Received: 24 August 2012
Accepted: 03 October 2012

Abstract

Leaf explants of a semi dwarf cultivar of pomegranate were placed on Murashige and Skoog [6] (MS) medium supplemented with various concentrations of 6-benzyl adenin (BA) and naphthalene acetic acid (NAA) for callus induction. Maximum callus induction was observed on a media containing 1 mg L^{-1} BA with 0 mg L^{-1} NAA. However, the highest callus growth was obtained on a medium containing 1 mg L^{-1} BA and 0.5 mg L^{-1} NAA.. Maximum shoot proliferation was observed when shoots were cultured on woody plant medium (WPM) supplemented with 2 mg L^{-1} BA. In this treatment, after 4 subcultures, 85 shoots were produced from one original explant. Among treatments used in rooting experiments, shoots cultured on WPM medium with 0-0.2 mg L^{-1} indol butyric acid (IBA) had the maximum root percentage (100%). Rooted plantlets were cultured in a soil mixture containing vermiculite (60%), perlite (30%) and coco peat (10%) v/v. After 2 months, 100% of plants survived and transferred to the greenhouse.

Keywords: Callus induction, pomegranate, Rooting, Shoots proliferation, Tissue culture

INTRODUCTION

Semi dwarf cultivar of pomegranates (*Punica granatum* L. '*Nana')* belongs to the Punicaceae family. It is a beautiful shrub with 50 to 100 cm height and produce small red and orange flowers and subsequently small pomegranate fruits. 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. [10]; Naik, et al. [8, 9]; Shao, et al. [12] and Terakami, et al. [13]. In this study an efficient system for callus induction, shoot regeneration, rooting and acclimation of semi dwarf cultivar of 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 semi dwarf cultivar of 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.

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 (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

All treatment containing BA produced callus. Calli were formed on the cut surfaces of leaves (Figure 1). Treatments containing 1 mg L^{-1} BA in combination with 0.0, 0.2 or 0.4 mg L^{-1} NAA had the maximum callus induction percentage (100%) (Table 1). Time needed to produce shoots in callus increased with increasing BA concentration, so in 5 mg L^{-1} BA callus could not produced any shoots after 50 days (table 3) .BA had a critical role in callus induction since no callus was formed when leaf explants were placed on MS medium without BA. Mean callus induction in treatments containing 1 mg L^{-1} BA was 85%, 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 0.5 mg L^{-1} NAA (Table 2). This treatment had the highest callus fresh weight (0.60 g), dry weight (0.06 g) and diameter (16.75 mm) and was significantly different (P<5%) with other treatments except a treatment containing 1 mg L^{-1} BA and 0.1 mg L^{-1} NAA (Table 2).

Shoot regeneration

The first shoot regenerated after 20 days in MS medium containing 1 mg L^{-1} BA with 0.1 mg L^{-1} NAA. At high level of BA, the time needed to produce shoot was longer so that in 4 mg L^{-1} BA, first shoot regenerated after about 60 days (Table 3).The maximum number of shoots were regenerated on MS medium containing 2 mg L^{-1} BA and 0.1 mg L^{-1} NAA (Figure 2). In higher concentrations, mean number of shoots was decreased in such a way that in medium containing 5 mg L^{-1} BA number of shoot was 0.0 in each callus pieces after 60 days. There was a significant difference between treatments containing MS medium supplemented with 2 mg L^{-1} BA with other treatments in relation to number of shoots produced on each callus (Table 3).

Shoot proliferation

Among treatments used for shoot proliferation, treatment containing WPM medium supplemented with 2 mg L^{-1} BA had maximum shoot proliferation. After 4 subsequent subcultures, total number of shoot produced in WPM medium containing 2 mg L^{-1} BA was 85. In average, the highest number of shoots produced in the first subculture was 5.1 followed by 4.3, 2.6 and 1.5 shoots per explants in second, third and forth subcultures, respectively (Figure 3).The highest shoot growth occurred in WPM medium supplemented with 1 mg L^{-1} BA (2.5 cm). Mean shoot growth in treatments containing BA was 2.08 cm (Table 4).

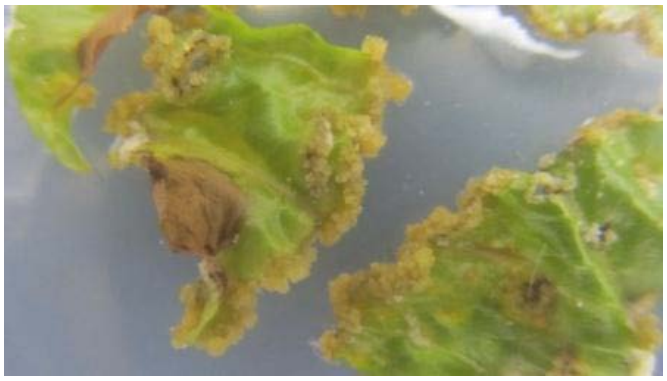

Figure 1. Green calli initiated from leaf explants of semi dwarf pomegranate culture on MS medium containing 1 mg L^{-1} BA.

Figure 2. Shoots regenerated in MS medium containing 2 mg L^{-1} BA with 0 mg L^{-1} NAA

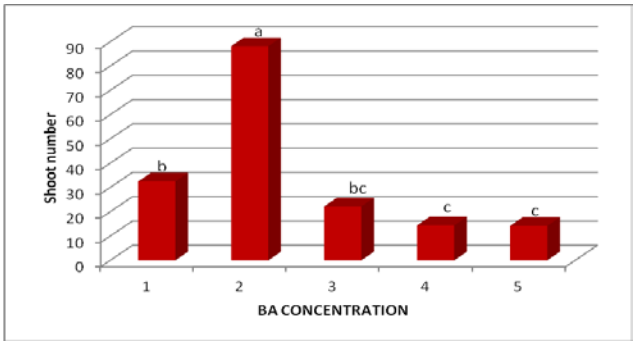

Figure 3. Number of shoots produced from 1 explant in varius concentration of BA after 4 sub culture.

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 0.0 to 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 5). Rooted plantlets were transferred to 15 -cm pots containing 60% vermiculite, 30% perlit and 10% cocopeat v/v and were grown under greenhouse conditions. Plant transferred to pots showed good development and high survival rates (100%) after 2 months (Data not shown). Plants grew successfully into normal mature plants in greenhouse .

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

NAA (mg L^{-1})	BA (mg L^{-1})					
	0	1	2	3	4	5
0	0	100a*	91.67ab	76.62abcd	60 defgh	36.67h
0.1	0	74.90abcdefh	85 abcd	75 abcdef	55 efgh	43.33gh
0.2	0	100 a	100a	89.03abc	61.67 cdefgh	51.67efgh
0.3	0	77.19 abcde	51.67 efgh	58.33defghf	73.33 abcdef	60 defgh
0.4	0	100 a	47.07fgh	63.33cdefgh	78.33 bcde	70 bcdef
0.5	0	73.33 abcdef	64.34bcdefgh	78.33abcde	80 abcde	73.33abcdef

*Values 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 semi dwarf pomegranate

BA (mg L^{-1})	NAA(mg L^{-1})	Fresh weight (g)	Dry weight (g)	Diameter (mm)
0	0	0.23 d*	0.02 d	11.75 b
0	0.5	0.30cd	0.03 cd	11.67 b
0	1	0.30 cd	0.03 cd	12.66 b
0.5	0	0.35bc	0.03 cd	12.98 b
0.5	0.5	0.35 bc	0.03 cd	12.80 b
0.5	1	0.42 bc	0.04 cd	13.50 ab
1	0	0.46 b	0.05 b	13.58 b
1	0.5	0.60 a	0.06 a	16.75 a
1	1	0.55 a	0.06 a	16.16 a

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

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

NAA (mg L^{-1})	BA (mg L^{-1})				
	1	2	3	4	5
0	25.3 a*	25 a	46.3 fgh	50 fghi	0
0.1	20 a	25 a	45.6 fghi	55.6 fghi	0
0.2	27.3 ab	25a	45 fghij	57.3 fg	0
0.3	28.3 ab	30.6 e	46.3 fgh	56.6 fg	0
0.4	30.6 b	32.3 e	45 fghij	64.6 fghijk	0
0.5	35.3 b	35.6 e	45 fghij	64.6 fghijk	0

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

Table 4. Effects of BA concentrations on shoots proliferation of semi dwarf cultivar of pomegranate.

Treatments(mg L^{-1})	Shoot number	Shoot length(cm)
BA 1	46.32 b*	2.5 a
BA 2	85.2 a	2.01 b
BA 3	22.23 bc	2.02 b
BA 4	14.4 bc	2.10 bc
BA 5	14.26 c	1.77 c

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

Table 5. Treatments used for rooting of regenerated shoots of semi dwarf cultivar of pomegranate.

IBA pretreatment (mg L^{-1})	Culture media	Root (%)	Root no.	Root length (cm)
0	WPM	100 a*	2.25 cde	3.68 a
500	WPM	90.25 ab	2.11 cd	1.71 abcd
1000	WPM	90 ab	3.03 ab	1.7 abcd
1500	WPM	85.ab	2.65 bc	1.50 bcd
0	½ MS	87.25 ab	1.37 ef	2.81 ab
500	½ MS	32.25 d	3.73 a	1.5 bcd
1000	½ MS	44.25 c	1.8 e	1.6 bcd
1500	½ MS	43.75 c	2.9 ab	0.8 d
-	WPM+0.1IBA(mg L^{-1})	100. a	1 f	3.57 a
-	WPM+0.2 IBA(mg L^{-1})	100 a	2.62 bc	3.06 ab
-	WPM+0.3IBA(mg L^{-1})	95.05 ab	2.17 cd	1.85 abc
-	WPM+0.4IBA(mg L^{-1})	70 bc	1.20 f	1.96 abc
-	WPM+0.5IBA(mg L^{-1})	75.50 b	1.39 ef	1.48 bcd

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

DISCUSSION

The results of this investigation showed that leaf explant was an excellent source for plant multiplication in semi dwarf cultivar of *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 semi dwarf cultivar of pomegranate might need moderate cytokinin levels for callus induction. These results are in agreement with those of Omura, et al. [10] 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. [7] 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. [4] 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 semi dwarf pomegranate. Our results are in agreement with many reports such as Omura, et al. [10] 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. [4] 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 [7] 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 [2] 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.

Omura, et al. [10] 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 [9] 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. Rashed, et al. [11] 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. [1] 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. These results were in agreements with our results in relation to shoot proliferation which 2 mgL^{-1}BA produced higher number of shoots. Browning of the culture medium due to phenolic compounds was a common problem in tissue culture of pomegranate cultivars (Naik, et al., [8]). In the present study, the browning problem was overcome by repeatedly transferring the explants to a new fresh medium. In present study, we found that rooting on WPM medium containing low level of IBA was the best treatment for rooting of semi dwarf cultivar of

pomegranates. IBA was reported to have favored root induction in pomegranate (Naik, et al., [8]), sweet orange (Khalil, et al., [5]) and in *Citrus limonia* Osbeck (Jajoo, [3], Omura, et al., [10]) reported optimum rooting of dwarf pomegranate was obtained in MS/2 medium supplemented with .01 to 0.1 µM 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 semi dwarf cultivar of pomegranate.

REFERENCES

[1] Haripyaree H, Guneshwor K, Sunitibala H, Damayanti M. 2011. *In vitro* propagation of *Citrus megaloxycarpa*. Envir. Exper. Biol. 9: 129–132.

[2] Jaidka K, Mehra PN.1986. Morphogenesis in *Punica granatum* L. (pomegranate). Canad. J. Bot. 64:1644–1653.

[3] Jajoo A. 2010. *In vitro* propagation of *Citrus limonia* Osbeck through nucellar embryo culture. Curr. Res. J. Bio. Sci. 2: 6-8.

[4] Kanwar K, Jommy J, Deepika R. 2010. Comparison of *in vitro* regeneration pathways in Punica *granatum* L. Plant CellTiss. Organ Cult. 100:199–207.

[5] Khalil SA, Zamir R, Ahmad N, Sajid M, Fazal H, KhanMA, Seema N, Alam R. 2011. *In vitro* regeneration of plantlets from unpollinated ovary culture in sweet orange (Citrus sinensis L. Osbeck). Affric. J. Biotechnol. 10: 15130-15134.

[6] Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant.15:473-497.

[7] Murkute A, Patil S, Patil BN, Mayakumari MS. 2002. Micropropagation in pomegranate, callus induction and differentiation. South Indian Hort. 50:49–55.

[8] Naik S, Pattnaik S, Chand P. 1999. In vitro propagation of pomegranate 'Ganesh' through auxiliary shoot proliferation from nodal segment of mature tree. Sci. Hort. 79:175-183.

[9] Naik S, Pattnaik S, Chand P. 2000. High frequency auxiliary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate. Sci. Hort. 79:175-183.

[10] Omura M, Matsuta N, Moriguchi T, Kuzaki I, Sanada T. 1987. Establishment of tissue culture methods in dwarf pomegranate (*Punica granatum* L. var. 'Nana') and application for the induction of variants. Bull. Fruit Tree Res. 14:17-44.

[11] Rashid M, Mumtazkhan M, Fatima B, Abbasi M, hahid A. 2005. *In vitro* regeneration and multiple shoots induction in Citrus reticulata Blanco. Int. J. Agric. Biol. 3:414-416.

[12] Shao J, Chen C, Deng X. 2003. *In vitro* induction of tetraploid in pomegranate (*Punica granatum* L.). Plant Cell Tiss. Organ Cult. 3:241-246.

[13] Terakami S, Matsuta N, Yamamoto T, Sugaya S, Gemma H, Soejima J. 2007. Agrobacterium-mediated transformation of the dwarf pomegranate (*Punica granatum* L. var.' Nana'). Plant Cell Rep. 26:1243–1251.