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Full Length Research Paper

Micropropagation of pomegranate (*Punica granatum* L.) 'Bhagava' cultivar from nodal explant

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Reliable and reproducible protocols to get healthy and well formed plants from nodal explants of the pomegranate (*Punica granatum* L) cv 'Bhagava' has been developed. Nodal segments were cultured on two different media at full strength Murashige and Skoog (MS) and Woody Plant Medium (WPM). The media was prepared as a basal medium supplemented with 0.2 to 2 mg/L 6-benzylaminopurine (BAP), 0.1 to 1 mg/L 1-naphthalacetic acid (NAA), 0.5 to 2.5 mg/L silver nitrate ($AgNO_3$) and 10 to 50 mg/L adenine sulphate for establishment stage. For proliferation stage, 0.1 to 0.5 mg/L BAP and NAA was tested. For rooting stage 0.0, 0.25 and 0.5 mg/L 3-Indolebutyric acid (IBA) and NAA on MS and WPM medium were tested. The nodal explants grown on MS medium containing 1.8 mg/L BAP, 0.9 mg/L NAA, 1 mg/L silver nitrate and 30 mg/L adenine sulphate had the highest proliferation rate (10 to 15 shoots/explants) in establishment stage. The same trend was found concerning the maximum leaves numbers (15 to 20 leaves/explants) on proliferation medium containing 0.4 mg/L BAP and 0.3 mg/L NAA. The plantlets grown on MS medium were found to have better survival compared to WPM medium. 0.5 mg/L NAA and 0.5 mg/L IBA showed equal rooting response in both the medium, whereas thick root formation was observed in the medium containing IBA.

Key words: Micropropagation, proliferation, rooting, *Punica granatum*.

INTRODUCTION

Pomegranate (*Punica granatum* L.) is generally known in a distinct family (Punicaceae), which comprises only one genus (*Punica*) and two species; *P. granatum* and *P. protopunica* (Samir, 2010). It is an economically important species of the tropical and subtropical regions of the world due to its delicious edible fruits and pharmaceutical and ornamental usage (Jayesh and Kumar, 2004). Pomegranate is considered native to Iran, Afghanistan and Southern Pakistan's Baluchistan region to the Himalayas in Northern India. It has been widely cultivated throughout drier parts of Southeast Asia, Malaysia, the East Indies tropical Africa and India (Raj and Kanwar, 2008). In India, it is found from Kanyakumari to Kashmir but is cultivated commercially only in Maharashtra. Small-scale plantations are also seen in Gujarat, Rajasthan, Karnataka, Tamil Nadu, Andhra Pradesh, Uttar Pradesh, Punjab and Haryana (Vineeta, 2010). The total area under pomegranate cultivation in India is 100,000 ha yielding 0.45 million tons of fruit per year.

The fruit juice is a good source of sugars, vitamin C, vitamin B, pantothenic acid, potassium, antioxidant polyphenols and a fair source of iron. Some parts of the pomegranate tree (leaves, immature fruits, fruit rind and flower buds) have been used traditionally for their medicinal properties and also for tanning of leather. Wild pomegranate is too acidic and of little value except as souring agent (Anardana). The double-flowered pomegranates (which do not bear fruits) are grown in parks and ornamental gardens for their beautiful red flowers (Raj and Kanwar, 2010).

Pomegranate is propagated vegetatively by the rooting of hard wood cuttings, although the establishment of new plants requires one year. Micropropagation in fruit tree would help in overcoming difficulties of vegetative propagation, producing true to-type plants and rapid and mass production of planting materials (Samir et al.,

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2009). Hence, several studies have been conducted on micropropagation of pomegranate trees over the past several years. Protocols have been developed for regeneration of *P. granatum* L. plantlets *in vitro* through either organogenesis from callus derived from leaf segments, cotyledons (Murkute et al., 2002; Raj and Kanwar, 2008; Kanwar et al., 2010), anthers (Naik et al., 1999) or through embryogenesis from various seedling explants, petals and immature zygotic embryos (Kanwar et al., 2010).

Consequently, this work was designed for micro-propagation of pomegranate (*P. granatum* L.) 'Bhagava' cultivar and to study the effect of medium type (Murashige and Skoog, and Woody plant medium) and growth regulators for establishment, multiplication and rooting of pomegranate (*P. granatum* L.). In the present communication, we reported for the first time, effect of adenine sulphate and silver nitrate (ethylene inhibitor) on micropropagation and overcome difficulties of vegetative propagation for producing true to-type plants for rapid and mass multiplication through micropropagation of the most popularly grown pomegranate cultivar 'Bhagava'.

MATERIALS AND METHODS

This work was done in Tissue Culture Laboratory, Biotechnology Division; Nirmal Seeds Pvt. Ltd. (India) during the period from August, 2009 to October, 2010 to establish micropropagation protocol of one main pomegranate 'Bhagava' cultivar grown in India.

Explant collection

The plant was authenticated and a voucher specimen has been deposited in the herbarium of the Kanishka Farm of Nirmal Seeds Pvt. Ltd. (Neri- Wadgaon, Tq- Bhadgaon Dist- Jalgaon).

Explant isolation

Nodal segments of about 2 to 3 cm long of pomegranate 'Bhagava' cultivars were collected from mature trees.

Explant sterilization

Isolated nodal segments were cleaned under running tap water for about 15 to 20 min. Anti-oxidant solution treatment was given to isolated nodal segments by soaking in antioxidant solution (150 mg/L ascorbic acid and 100 mg/L citric acid) for 20 min. each under laminar air flow hood and followed by three times rinsing in sterile distilled water. Nodal segments were further soaked in fungicide (M-45) solution (1 mg/L) for 45 min. and then again washed with sterile distilled water. Streptomycin solution (100 mg/L) treatment was also given to explants for 20 min. and then washed by sterile distilled water. Finally 1 g/L mercuric chloride solution for 10 min. was used to treat these explants followed by three times washes with sterile distilled water for complete sterilization of nodal explants.

Inoculation

Completely sterilized explants were inoculated on establishment media. After establishing transferred explants on proliferation media for growth, completely proliferated explants were then transferred to rooting media.

Culture media

Two different media; viz MS medium and WPM medium were tested for micropropagation of pomegranate cultivar 'Bhagava'. Media was prepared as a basal medium supplemented with organic acids and vitamins. Sucrose was added at 30.0 g/L and myoinositol at 0.1 g/L. The pH of the prepared media was adjusted between 5.6 to 5.8 and agar-agar (Hi-Media) was added as 8.0 g/L for media solidification. For establishment stage, BAP 0.2 to 2.0 mg/L, NAA 0.1 to 1.0 mg/L, silver nitrate 0.5 to 2.5 mg/L and adenine sulphate 10 to 50 mg/L were used while for proliferation stage, BAP 0.1 to 0.5 mg/L and NAA 0.1 to 0.5 mg/L were tested. Also, for rooting stage, two different auxins; IBA and NAA were tested at 0.0, 0.25 and 0.5 mg/L on MS and WPM medium at full strength.

RESULTS

Effect of BAP on regeneration of shoots

For the nodal explants grown on MS medium with different concentration of BAP (0.2 to 2 mg/L), the highest average growth response (99%) was recorded on MS medium containing BAP 1.8 mg/L, whereas 4 to 6 shoots per explants having highest shoot length (0.7 to 1.9 cm) was recorded at same concentration (Figure 1).

Effect of NAA on regeneration of shoots

Results for the nodal explants grown on MS medium with different concentration of NAA 0.1 to 1 mg/L indicated that the highest average growth response (97%) was recorded on MS medium containing 0.9 mg/L NAA, whereas two to three shoots per explants having highest shoot length (0.7 to 1.1 cm) was recorded at same concentration (Figure 2).

Effect of silver nitrate and adenine sulphate on regeneration of shoots

The nodal explants grown on MS medium with different concentration of silver nitrate (0.5 to 2.5 mg/L) and adenine sulphate (10 to 50 mg/L), three to six shoots per explants was recorded in the medium having 1 mg/L silver nitrate, while three to five shoots per explants were recorded on the medium containing 30 mg/L adenine sulphate (Figure 3). The data in Table 1 shows that the highest average growth response (96 and 97%) was recorded on MS medium containing silver nitrate and 30 mg/L adenine sulphate, respectively.

Effect of NAA and IBA in MS medium on rooting

The MS medium containing 0.5 mg/L NAA and 0.5 mg/L

Figure 1. Plants showing the highest average growth response as recorded on MS medium containing 1.8 mg/L BAP.

Figure 2. Plants showing the highest average growth response as recorded on MS medium containing 0.9 mg/L NAA.

IBA showed the highest rooting response. The data in Table 2 shows that the highest average rooting response was recorded on MS medium containing 0.5 mg/L NAA (97%) and 0.5 mg/L IBA (97%). Both NAA and IBA therefore showed same rooting response (Figures 4 and 5). However, thick root formation was observed in medium containing 0.5 mg/L IBA. Root length of 0.3 to 3.4 cm was recorded on medium containing IBA, whereas 1.3 to 3.2 cm root length was recorded on medium containing NAA.

Figure 3. The highest average growth response as recorded on MS medium containing 1.0 mg/L silver nitrate and 30 mg/L adenine sulphate.

Effect of different media viz. MS and WPM medium for shoot regeneration

The data in Table 3 shows that the highest average growth and regeneration of 10 to 15 shoots per explants was recorded on MS medium compared to the six to eight shoots per explants on WPM medium containing 1.8 mg/L BAP, 0.9 mg/L NAA, 1 mg/L silver nitrate and 30 mg/L adenine sulphate (Figures 6 and 7).

Effect of BAP and NAA in proliferation medium on leaf induction

For the nodal explants grown on MS medium with concentration of 0.1 to 0.5mg/L of BAP and NAA, the data in Table 4 shows that the highest average maximum leaf number (15 to 20) was recorded on MS medium containing 0.4 mg/L BAP and 0.3 mg/L NAA. Similarly 98 and 96% explants showed maximum leaf formation in proliferation medium having same concentration of BAP and NAA, respectively (Figure 8).

DISCUSSION

We were successful in developing a protocol for the micropropagation of pomegranate (*P. granatum* L.)

Table 1. Effect of silver nitrate and adenine sulphate in MS medium on the rates of nodal explants regenerating shoots for establishment stage.

S/N	Medium	Concentration (mg/L)	Number of shoot/ explant	% of explants showing response	Shoot length (cm)
1	MS+ Silver nitrate	0.5	4 ± 1	87 ± 1	1.9 ± 0.9
2	MS+ Silver nitrate	1.0	6 ± 1	96 ± 1	2.5 ± 1.3
3	MS+ Silver nitrate	1.5	4 ± 1	83 ± 1	1.6 ± 0.5
4	MS+ Silver nitrate	2.0	3 ± 1	77 ± 1	0.9 ± 0.3
5	MS+ Silver nitrate	2.5	2 ± 1	69 ± 1	0.7 ± 0.1
6	MS+ Adenine sulphate	10	3 ± 1	75 ± 1	0.6 ± 0.2
7	MS+ Adenine sulphate	20	4 ± 1	95 ± 1	1.2 ± 0.6
8	MS+ Adenine sulphate	30	5 ± 1	97 ± 1	2.5 ± 0.8
9	MS+ Adenine sulphate	40	4 ± 1	86 ± 1	0.7 ± 0.3
10	MS+ Adenine sulphate	50	3 ± 1	72 ± 1	0.5 ± 0.1

Table 2. Effect of NAA and IBA in MS medium on rooting.

S/N	Medium	Concentration (mg/L)	Number of root/ explant	% of explants showing response	Root length (cm)
1	MS+ IBA	0.0	1 ± 1	20 ± 1	0.3± 0.2
2	MS+ IBA	0.25	4 ± 1	82 ± 1	1.5 ± 0.2
3	MS+ IBA	0.50	6 ± 1	97 ± 1	3.4 ± 0.5
4	MS+ NAA	0.0	1 ± 1	15 ± 1	1.3 ± 0.2
5	MS+ NAA	0.25	4 ± 1	80 ± 1	2.5 ± 0.2
6	MS+ NAA	0.50	6 ± 1	97 ± 1	3.2 ± 0.5

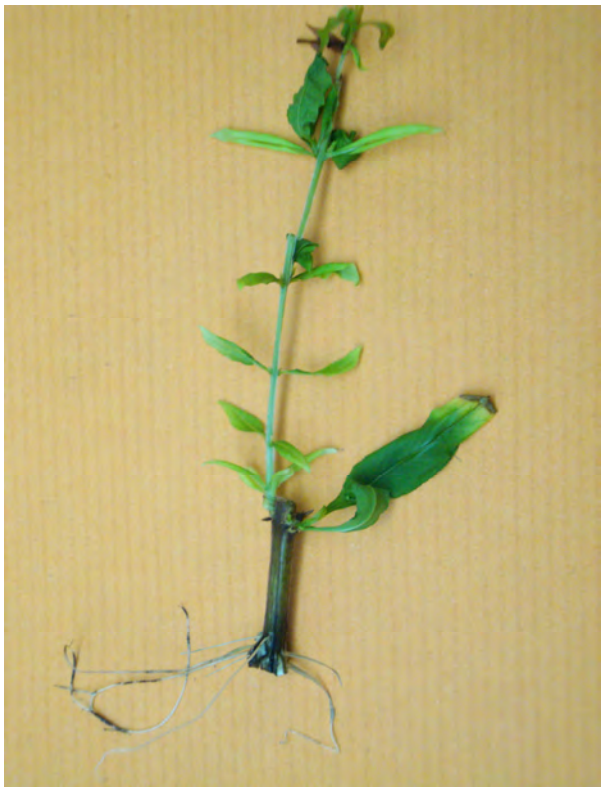

Figure 4. MS medium containing 0.5 mg/L NAA showing the highest rooting response.

Figure 5. MS medium containing IBA 0.5 mg/L shows highest rooting response and thick root formation.

Table 3. Effect of BAP, NAA, silver nitrate and adenine sulphate in MS medium and Woody Plant Medium on the rates of nodal explants regenerating number of shoots per explant.

S/N	Medium	Number of shoot/ explant				Number of shoot/ explant
		BAP (1.8 mg/L)	NAA (0.9 mg/L)	Silver nitrate (1 mg/L)	Adenine sulphate (30 mg/L)	BAP + NAA + $AgNO_3$ + Adenine sulphate
1	MS Medium	5 ± 1	3 ± 1	6 ± 1	5 ± 1	12 ± 3
2	WPM Medium	3 ± 1	2 ± 1	4 ± 1	2 ± 1	7 ± 1

Figure 6. MS medium containing BAP, NAA, silver nitrate and adenine sulphate show highest shoot regeneration.

Figure 7. WPM containing BAP, NAA, silver nitrate and adenine sulphate showing lower shoot regeneration as compared to MS medium.

'Bhagava' cultivar using two different media combination MS and WPM. MS medium proved to produce best vegetative growth characteristics compared to WPM medium. However in contrast to our findings Samir et al. (2009) found that WPM is best for vegetative growth compared to MS and NN medium.

Micropropagation establishment from field grown plants is a very critical process as it is met with an array of different problems such as microbial contamination, phenol exudation, etc. Phenol secretion from the cut ends of explants leads to browning of the medium and reduces development of explants. To avoid and minimize this problem, various workers tried the use of fungicide (M - 45), streptomycin, mercuric chloride and antioxidant solution (ascorbic acid and citric acid) etc. and such compounds have been suggested to control phenol exudation (Naik et al., 1999, Murkute et al., 2002). We were successful in developing a protocol for surface sterilization by using fungicide (M - 45), streptomycin, and mercuric chloride. 85 to 90% reduction in contamination was observed using this treatment. We also used antioxidant solution (ascorbic acid and citric acid; 150 mg/L and 100 mg/L respectively) to reduce the phenol secretion and transferred the explants to fresh medium.

NAA and BAP combinations were rewarding in many fruit tree species (Zimmerman and Swartz, 1994). For the shoot regeneration, cytokinin is effective when used in combination with an auxin (Nike et al., 1999). Synthesis and activities of auxin, cytokinins and ethylene are thought to be closely related (Klee and Romano, 1994). Ramesh et al. (2006) also reported that the addition of adenine sulphate (60 mg/L) along with other growth regulators was the most effective in inducing shoot multiplication. It is also well documented that silver nitrate (Nike et al., 2003), as well as adenine sulphate promotes shoot multiplication (Shrivastava and Banerjee, 2008). Our report also support that the presence of silver nitrate show remarkable improvement in shoot multiplication rate of pomegranate. Silver nitrate alone at concentration of 1

Table 4. Effect of BAP and NAA in MS medium on the rates of nodal explants regenerating maximum leaves for proliferation stage.

S/N	Medium	Concentration (mg/L)	Number of leaves/ explants	% of explants showing response	Shoot length (cm)
1	MS+ BAP	0.1	11 ± 1	35 ± 1	0.6 ± 0.1
2	MS+ BAP	0.2	14 ± 1	40 ± 1	0.9 ± 0.3
3	MS+ BAP	0.3	15 ± 1	85 ± 1	1.6 ± 0.8
4	MS+ BAP	0.4	19 ± 1	98 ± 1	2.0 ± 0.6
5	MS+ BAP	0.5	17 ± 1	82 ± 1	1.4 ± 0.2
6	MS+ NAA	0.1	12 ± 1	57 ± 1	1.0 ± 0.2
7	MS+ NAA	0.2	15 ± 1	77 ± 1	1.1 ± 0.4
8	MS+ NAA	0.3	18 ± 1	96 ± 1	1.5 ± 1.0
9	MS+ NAA	0.4	16 ± 1	87 ± 1	1.5 ± 0.9
10	MS+ NAA	0.5	14 ± 1	89 ± 1	1.2 ± 0.6

Figure 8. MS medium containing BAP and NAA showing maximum leaf numbers (15 to 20) at proliferation stage.

mg/L produced average shoot multiplication number of three to six shoots per explants after two week of inoculation. This is also in agreement with Nike et al. (2003), who observed similar result with cotyledon tissue of pomegranate. The average height of shoots varied in the range of 2.5 to 3 cm (Table 1). On the other hand adenine sulphate alone showed the multiplication rate of three to five shoots per explants in 10 to 50 mg/L, respectively. Maximum numbers of shoots were found at the optimum concentration of adenine sulphate (30 mg/L). In general, the combination of silver nitrite and adenine sulphate gave significantly higher multiplication rate. The highest shoot multiplication per explant was recorded in MS medium supplemented with silver nitrite (1 mg/L) in combination with adenine sulphate (30mg/L) (Table 1). 97% explants showed shooting response to combination of silver nitrate and adenine sulphate.

In this study, we used BAP (cytokinin), NAA (auxin), silver nitrate (ethylene inhibitor), and adenine sulphate (amino acid) for shoot regeneration. The highest number of shoot per explants was observed on MS medium containing 1.8 mg/L BAP, 0.9 mg/L NAA, 1.0 mg/L silver nitrate and 30 mg/L adenine sulphate with sucrose (30%) in comparison with WPM medium. In both WPM and MS medium when different levels of alone BAP and NAA were tried, WPM medium showed poor proliferation response compared to MS medium. Same trend was found concerning the maximum number of leaves on proliferation medium containing 0.4 mg/L BAP and 0.3 mg/L NAA. The plantlets grown on MS medium were found to have a better survival compared to WPM medium.

It has been reported by several researchers that NAA induced rooting in *P. granatum* L. (Omura et al., 1987; Mahishni et al., 1991; Yang and Ludders, 1993; Amin et al., 1999; Naik et al., 2000; Naik and Chand, 2003; Zhu et al., 2003). Contrary to this findings, rooting in regenerated shoots from cotyledon derived callus cultures of *P. granatum* L. cv. 'Ganesh' was observed in half strength MS medium supplemented with IBA by Murkute et al. (2004). In this experiment, when proliferated shoots were subjected to *in vitro* rooting and shoot elongation in MS medium containing IBA and NAA at 0.5 mg/L respectively with 30% sucrose, same rooting response was observed in both NAA and IBA containing medium. However, thick root formation was observed in media containing 0.5 mg/L IBA.

We therefore succeeded in the development of an efficient protocol by using plant growth regulators in combination with silver nitrate and adenine sulphate for mass scale micropropagation of pomegranate (*P. granatum* L.*)* 'Bhagava' cultivar. Hence, it is expected that this protocol can be used for constant supply of high valued pomegranate (*P. granatum* L.*)* 'Bhagava' cultivar tissue culture plants and also to demonstrate the role of silver nitrate and adenine sulphate alone or in combination with BAP and NAA in MS medium to induce high frequency adventitious shoot regeneration.

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