academicJournals

Vol. 12(20), pp. 2863-2868, 15 May, 2013
DOI: 10.5897/AJB2013.11962
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

In vitro mass multiplication of pomegranate from cotyledonary nodal explants cv. *Ganesh*

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Accepted 22 April, 2013

For surface sterilization of explants, treatment involving $HgCl_2$ (0.1 %) for 3 min gave better sterilization of cotyledonary nodal explants. The maximum percentage establishment of cotyledonary node explants was observed on Murashige and Skoog (MS) medium + 1.0 mg/l 6-benzylaminopurine (BAP) + 0.5 mg/l naphthaleneacetic acid (NAA). However, MS medium fortified with 1.0 mg/l BAP + 1.0 mg/l kinetin + 200 mg/l activated charcoal exhibited maximum multiplication rate for the first two subcultures. The maximum frequency of multiple shoots in cotyledonary explants (86.33 %) was observed on treatment MS + 1.0 mg/l BAP + 1.0 mg/l kinetin + 200 mg/l activated charcoal. *In vitro* rooting of regenerated shoot was found in half strength MS medium supplemented with 0.5 mg/l NAA + 200 mg/l activated charcoal, which recorded the maximum number of root/shoot (4.17) and root length (3.87 cm). *In vitro* grown plantlets having 5 to 6 cm length of shoot were transferred to vermicompost + soil (1:1v/v) media kept in net house, which showed better survival of plantlet (85.50%) within 11.75 days.

Key words: Establishment, multiplication, rooting, acclimatization, *Punica granatum*.

INTRODUCTION

Pomegranate *Punica granatum* L. belongs to the family Punicaceae which comprises only one genus (*Punica*) and only two species, *P. granatum* and *P. proptopunica.* This is popularly known as anar, Dalima in India and is one of the oldest favourite table fruits of tropical and sub tropical regions of the world (Pekmezci and Erkan, 2003). Pomegranate has great adaptability to saline soil and drought condition (Sepulveda et al*.,* 2000). The fruit are rich in Fe, Ca, and antioxidant component like phenol, pigments and tannins. India is the second largest producer of pomegranate with a production of 8.07 lakh tons. Pomegranate is mainly cultivated in the states of Maharashtra, Gujarat, Karnataka, Tamilnadu, Uttar Pradesh, Haryana, and Andhra Pradesh (Anonymous, 2009). To get true to type planting material, pomegranate is commercially propagated by stem cuttings (Hardwood cutting) or by air layering. However, it has several limitations like low success, very slow propagation methods and new plants require one year for establishment. This results in non- availability of plantlets throughout the year. Micro propagation technique is being exploited for many fruit crops. Reliable and efficient regeneration *in vitro* through stimulation of axillary bud proliferation from nodal segment explant and apical buds or through organogenesis or embryogenesis directly from various explants or through callus have been already

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Abbreviations: MS, Murashige and Skoog medium; **B5,** Gamborg medium; **WPM,** Woody plant medium; **NAA,** naphthaleneacetic acid; **BAP,** 6-benzylaminopurine; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **Kin,** kinetin; **AC,** activated charcoal.

Table 1. Effect of different media on the establishment and growth of cotyledonary nodal explants of pomegranate cv. Ganesh

Media	Explant establishment (%)	Days to establishment	Length of shoot (cm)	No. of internodes
MS	75.57	10.43	3.06	2.86
B5	52.86	12.29	1.19	1.07
WPM	33.43	16.86	0.91	1.00
S.Em. ±	0.31	0.26	0.07	0.02
CD at 5%	0.94	0.77	0.21	0.08

demonstrated in a number of woody species (Agrawal, 2004). *In vitro* propagation of pomegranate *Punica granatum* has been reported by several workers using different explants shoot tip and nodal explants (Murkute et al., 2004; Singh and Khawale 2006; Chaugule et al., 2007; Samir et al., 2009; Singh et al., 2011) and cotyledonary nodal explants (Murkute et al., 2002; Raj and Kanwar, 2010; Kanwar et al., 2010). However, till date there are hardly any reports described in details of different media and serial sub culturing process using cotyledonary nodal explants in pomegranate. Therefore, the main objective of the present study was to develop an *in vitro* protocol for the large-scale multiplication of pomegranate *P. granatum* using cotyledonary nodal explants.

MATERIALS AND METHODS

Preparation of explants

For explants, the source seeds were collected from fully ripe fruits of 'Ganesh' cultivar of pomegranate *Punica granatum* L. The extracted seeds were dipped into 0.1% H_2O_2 (hydrogen peroxide) solution for 5 min for completely removing the juicy testa of seeds and kept under running tap water for 20 min, triple washed with sterilized water. The seeds were sterilized with 0.1% $HgCl_2$ (mercuric chloride) for 3 min and rinsed thrice with autoclaved distilled water. Seed coat was removed aseptically and embryo along with the cotyledons was inoculated on double distilled water without using any nutrient media into the test tube. Germination was initiated within two to three days of inoculation. The germinated seeds were kept in 16/8 h light/dark period having 3000 lx light intensity at 26 ± 2°C for four to five weeks and the cotyledonary nodal part of the axenic seedlings were used as explants. The cotyledonary nods were excised and inoculated in to 250 ml screw capped glass bottle. Initially three different media MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968) and WPM (Lloyd and McCown, 1981) were tested for culture establishment. Media were supplemented with 1.0 mg/l benzylaminopurine (BAP) + 0.5 mg/l naphthaleneacetic acid (NAA). Sucrose was added at 30.0 g/l and media were autoclaved at 121°C and 15 lb/in^2 for 20 min. The pH of media was adjusted to 5.6 to 5.8 prior to autoclaving. Cultures were incubated for four weeks. Best medium found in culture establishment was further used for multiplication. The cultures were maintained as per the conditions described earlier for seed germination process.

Shoot multiplication

For multiplication, 1.5 to 2.0 cm long raised plantlets from cotyledonary explants were aseptically isolated and transferred to MS medium supplemented with various concentration of growth substances BAP (0.5, 1.0, 1.5 and 2.0 mg/l) alone and in combination with NAA (0.5mg/l) and kinetin (0.5 and1.0 mg/l) along with the 200 mg/l activated charcoal (AC) in various treatment combinations. Subculture was made on the same medium after three weeks of culture.

In vitro rooting and acclimatization

After four weeks of subculture, individual shoots were transferred onto the rooting medium. For rooting stage MS (full and half strength) and White media were tested with different concentrations of NAA (0.1, 0.2, 0.5 and 0.8 mg/l) with 200 mg/l activate charcoal. The nutrient medium was gently removed and washed thoroughly in tap water ensuring that all agar particles were completely removed without damaging the roots. For acclimatization different media like, vermicompost, leaf mould, and soil were tested alone and also in combination with vermicompost: soil (1:1v/v) and vermicompost: leaf mould: soil (1:1:1v/v) in plastic pots. *In vitro* rooted plants were placed in a culture room at 26±2°C. The rooted plantlets were then, dipped in 0.05% bavistin (carbendazim 50 per cent WP) and planted in plastic pots. They were covered with a plastic cup continuously for six to seven days and kept in an air conditioned room. The cover was gradually removed after seven days, initially for 3 h followed by 6 h and 12 h in next three days. The cover was removed during night and lights put-off for next three days. Subsequently, the period of keeping the plantlets without any cover was gradually increased and after 15 days they were brought outside the room in shade. Within next 10 days by gradually exposing them to sun, they were acclimatized to natural environment.

Statistical analysis

All the experiments were setup in the completely randomized design and repeated three times, each treatment consisted of 50 explants and the means separation were done according to least significant differences (LSD) at 5% level.

RESULTS AND DISCUSSION

Establishment of explants

The results presented in Table 1 reveal that medium type

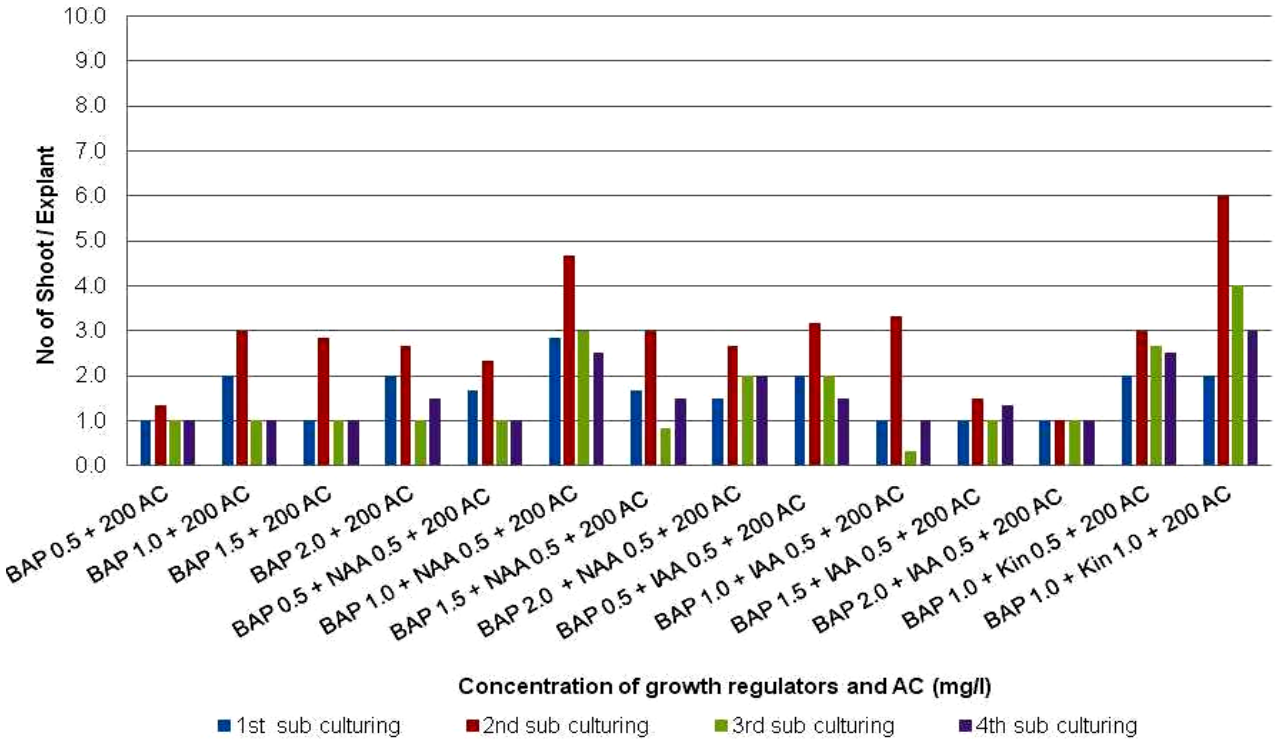

Figure 1. Effect of serial sub culturing and growth regulators on the multiplication of cotelydonary nodal explants of pomegranate cv. Ganesh.

affected significantly the establishment of explants. Maximum establishment (75.57%) was achieved on treatment MS medium supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA (Figure 2A) followed by B5 medium supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA and WPM medium supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA. The minimum time for initiation of shoot (10.43 days) was taken by the treatment involving MS medium supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA. This treatment also induced significantly higher number of shoots and internodes/explants followed by treatment B5 and WPM. Several workers including Murkute et al. (2004) and Singh and Khawale (2006) used MS medium and achieved success for establishment of explants and growth in pomegranate. However, Samir et al. (2009) reported the WPM is best for culture establishment and shoot growth in Manfalouty and Nab El-Gamal cultivars of pomegranate. Medium formulation displayed a strong effect on the growth of shoot like length of shoot and length of internodes. The multiplication rate of the MS medium was superior as compared to the WPM and B5 medium.

Frequency of shoot regeneration and multiplication

In order to study the multiplication rate, trials were conducted with treatments involving different levels of BAP, NAA and kinetin. The results for the 14 treatments tested are shown in Table 2. The sub culturing was done at three weeks interval. Shoots of cultures cut into a nodal segments (1.5-2.0 cm) were used for the multiplication. The treatment MS fortified with 1.0 mg/l BAP with 1.0 mg/l kinetin + 200 mg/l activated charcoal was found significantly superior for producing high fre-quency of multiple shoots and highest length of shoot in culture from cotyledonary nodal explants (Figure 2B). Cytokinin which could be used to release apical dominance in the lateral buds. The dominant buds of vegetative apex are stimulated to grow and elongated into presence of cytokinin and also produce new axes. For the shoot proliferation BAP is more effective when used in combination with auxin Singh and Khawale (2006). Similarly, Naik et al. (2000) also reported frequency of shoot multiplication in BA and kinetin from cotyledonary nodal explant of pomegranate.

Effect of serial sub culturing on shoot regeneration

The result for shoot multiplication with serial sub culturing of cotyledonary nodal explants is shown in (Figure 1). Regeneration of multiple shoots at the rate of 6 folds was observed in MS + 1.0 mg/l BAP + 1.0 mg/l kinetin + 200

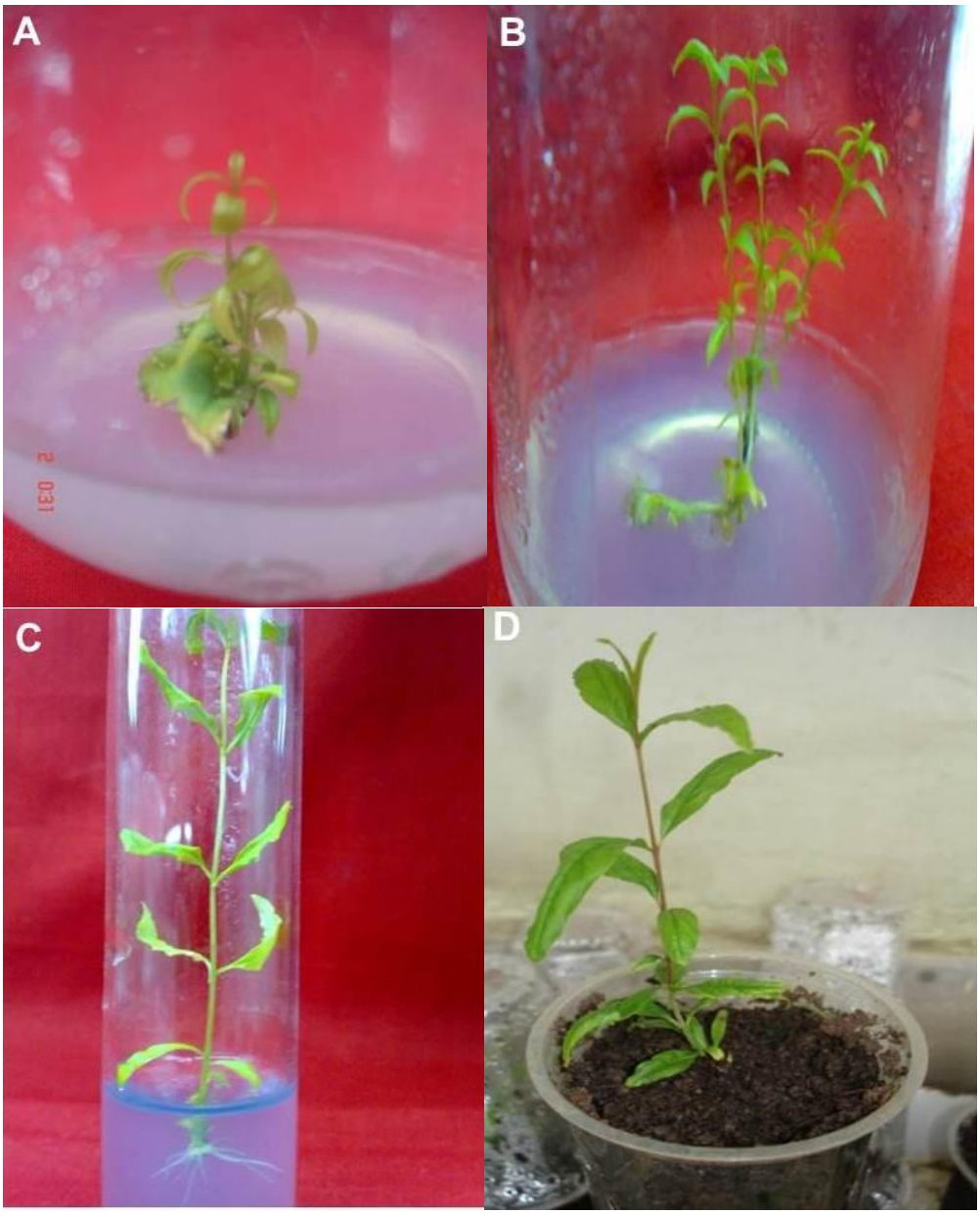

Figure 2. A. Establishment of explants on MS medium + 1.0 mg/l BAP + 0.5 mg/l NAA, **B.** Shoot multi[placation on MS medium + 1.0 mg/l BAP + 1.0 mg/l kinetin + 200 mg/l activated charcoal, **C.** *In vitro* rooting on half MS medium + 0.5 mg/l NAA + 200 mg/l activated charcoal, **D.** Hardening of plantlet in potting mixture of vermicompost + soil (1:1 v/v).

mg/l activated charcoal up to the second subculture, thereafter it declined. Decreasing rate of shoot multiplication is might be due to the endogenous hormonal levels of explant. In general the effect of sub culturing on multiplication is varies from species to species. The declined trend in shoot multiplication is highly dependent on hormonal composition of medium and incubation period Vujovic et al. (2012). Declining rate of shoot multiplication during serial sub culturing on medium of constant hormonal composition was also reported by Hamad and Taha (2008) in pineapple (Ananas comosus), Patel (2008) in papaya (Carica papaya) and Shinde (2008) in grape (Vitis vinifera).

Effect of NAA and strength of medium on rooting response

The data of *in vitro* rooting on full and half strength of MS

Table 2. Effect of plant growth regulators on frequency of shoot multiplication of cotyledonary nodal explants of pomegranate cv. Ganesh.

BAP (mg/l)	NAA (mg/l)	IAA (mg/l)	Kinetin (mg/l)	Shoot/explant %)
0.5	-	-	-	13
1	-	-	-	11.33
1.5	-	-	-	14.33
2	-	-	-	20
0.5	0.5	-	-	24.67
1	0.5	-	-	76.33
1.5	0.5	-	-	23
2	0.5	-	-	42.67
0.5	-	0.5	-	18.67
1	-	0.5	-	24.67
1.5	-	0.5	-	22
2	-	0.5	-	25
1	-	-	0.5	59
1	-	-	1	86.33
S.Em.±			0.56	
CD 5%			1.63	

Table 3. Effect of NAA and strength of medium on the rooting response in pomegranate cv. Ganesh

Medium strength	NAA + AC (mg/l)	Rooting (%)	Days to root initiation	Length of root (cm)	No. of root/shoot	Length of shoot (cm)
MS ½	0.1+200	63.07	12.43	3.07	3.4	4.4
	0.2+200	45.73	14.07	2.57	1.67	2.37
	0.5+200	68.8	10.5	3.87	4.17	6
	0.8+200	50.73	12.67	2	2.27	2.27
MS full	0.1+200	32.33	15.37	1.5	2.6	2.43
	0.2+200	20.33	13.33	2.43	2	2
	0.5+200	34.4	12.2	1.83	2.93	2.53
	0.8+200	23.67	15.97	2.43	2.5	2.13
White medium	0.1+200	48.33	14.87	1.2	3.1	2.3
	0.2+200	22.33	14.84	1.7	2	2.07
	0.5+200	52.83	11	2.87	3.47	4.07
	0.8+200	28.4	14.67	2.63	3.23	2.5
S.E.M. ±		0.5	0.33	0.07	0.11	0.07
CD at 5%		1.47	0.97	0.22	0.34	0.21

medium and white medium supplemented with different levels of NAA is shown in Table 3. The length of root, number of roots/shoot and length of shoot were significantly influenced by the treatments. Half strength MS supplemented with 0.5 mg/l NAA and 200 mg/l activated charcoal took significantly minimum number of days (10.50 days) to initiate roots than other treatments. Similarly, higher length of root (3.87 cm), number of roots/shoot (4.17) and length of shoot (6.0 cm) were found in the same treatment (Figure 2C) followed by MS half + 0.1 mg/l NAA + 200 mg/l. It was apparently seen that rooting of shoots on half MS medium was better in respect to all the rooting parameters than that was observed on ¼ MS medium, full MS medium and white medium. The present finding is supported by those of Murkute et al. (2004), Chaugule et al. (2007) and Singh and Khawale (2006) in pomegranate. Addition of activated charcoal in the rooting medium was found to improve rooting. The positive response of activated

Table 4. Effect of different potting mixtures on acclimatization of pomegranate cv. Ganesh.

Potting mixture	Survival of plantlet (%)	Days to sprouting	Length of shoot (cm)
Vermicompost	46.25	18.00	4.50
Leaf mould	0.00	0.00	0.00
Soil	37.50	15.50	5.75
Vermicompost + soil (1:1 v/v)	85.50	11.75	7.75
Vermicompost + leaf mould + soil(1:1:1 v/v)	57.25	18.00	5.00
S.Em. ±	0.61	0.31	0.20
CD at 5%	1.82	0.93	0.61

charcoal on rooting may be attributed to the known fact that activated charcoal absorbed phenolic compounds and extra concentration of growth hormones from the medium, thus reducing their inhibitory effect on rooting responses.

Effect of potting mixture on hardening of plantlets

The maximum survival (85.5%) of plantlets was found in vermicompost + soil (1:1v/v) followed by Vermicompost + leaf mould + soil (1:1:1 v/v) (Table 4). The potting mixtures used in the present investigation helped in giving better grip for the roots and ample aeration. Vermicompost + soil (1:1 v/v) mixture was found best hardening medium, when it kept into the net house (Figure 2D). Which gave maximum survival per cent of *in vitro* raised plantlets of pomegranate. These findings are supported by earlier workers those of Murkute et al. (2004) and Joseph (2005) in pomegranate.

Conclusion

The present investigation on '*in vitro* mass multiplication of propagation of pomegranate cv. Ganesh' through cotyledonary nodal explants has clearly demonstrated its potentiality for rapid clonal propagation. It estimated that using the present protocol of *in vitro* propagation, large number of plantlets can be produced in a year starting from single cotyledonary nodal explants.

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