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In vitro organogenesis in pomegranate (*Punica granatum* L.)

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Abstract
Shoot apex and nodal segment explants of pomegranate were placed on Murashige and Skoog Medium (MS medium, 1962) supplemented with different concentration of cytokinins and auxins alone or in combination for shoot bud induction. In shoot apex explants maximum shoot bud induction was observed on a medium containing 2.0 mg/l BAP with 90 percent frequency followed by 3.0 mg/l Kn+1.0 mg/l IAA with 70 percent frequency. Whereas, maximum shoot bud induction from nodal segment explant was observed on a medium containing 2.50 mg/l BAP with 90 percent frequency followed by 3.0 mg/l BAP+1.0 mg/l IAA with 70 percent frequency.

Keywords: Callus induction, Tissue culture, *In vitro*

Introduction
The Pomegranate (*Punica granatum* L.) is one of the oldest edible fruits also known as "fruit of paradise". The name Pomegranate is derived from two Latin words Pomum meaning apple and granatus meaning full of seeds. It belongs to the family Punicaceae, which comprises only one genus (Punica) and two species; P. granatum and P. protopunica (Samir, 2010) [20]. It is native to Persia and perhaps some surrounding areas. It was cultivated in ancient Egypt and early in Greece, Italy and Iraq. Later, it spread into Asian countries like Turkmenistan, Afghanistan, Iran, India, China, etc.), North Africa and Mediterranean Europe (Mel-garejo and Martínez 1992). In India, it is cultivated over 2.83 lakh ha with a production of 32.16 lakh tones and productivity of 11.07 tones/ha. Pomegranate is mainly cultivated in the states of Maharashtra, Gujarat, Karnataka, Tamil Nadu, Uttar Pradesh, Haryana, Andhra Pradesh and Rajasthan. (Anonymous). In Rajasthan, it is mainly grown in Jaipur, Ajmer, Alwar, Tonk, Sriganganagar, Pali, Kota, Jalore, Banswara, Sawai Madhopur, Bhilwara, Jhunjhunu, Bikaner and Sirohi districts in 12,000 ha area and acreage in Thar desert particularly Barmer, Jodhpur and Jaisalmer is increasing at faster rate. It is commercially cultivated for its delicious fruits. A fully matured fruit is highly nutritive and rich source of protein, fat, fiber, carbohydrate etc. The fruit are rich in Fe, Ca, and antioxidant component like phenol, pigments and tannins. Apart from its demand for fresh fruits and juice, the processed products like pomegranate wine, pomegranate tea and candy are also gaining importance in world trade. The pomegranate fruit juice is a good source of sugars, vitamin C, vitamin B, pantothenic acid, potassium, antioxidant polyphenols and a fair source of iron. In addition, the tree is also valued for its pharmaceutical properties. It is used for treating dyspepsia and considered beneficial in treating leprosy. The rind of the fruit and the bark of pomegranate tree are used as a traditional remedy against diarrhea, dysentery and intestinal parasites. Fruit rind, bark of stem and root are widely used for tannin production (Jindal *et al.* 2004) [5]. 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.*, 2009) [21]. 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) [9, 18], anthers (Naik *et al*., 1999) [12] or through embryogenesis from various seedling explants, petals and immature zygotic embryos (Kanwar *et al*., 2010) [18].

Materials and Methods

Present research work was done in Plant Micropropagation Lab, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner.

Explant collection

Pomegranate 'Bhagava' cultivar grown at Agricultural Research Station, Swami Keshwanand Rajasthan Agricultural University, Bikaner.

Explant isolation

Nodal segments and shoot apex of pomegranate 'Bhagava' cultivars were collected from mature trees.

Explant sterilization

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

Culture media

MS medium was 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 was added as 8.0 g/L for media solidification.

Plant growth regulators used in culture media

Different concentration of plant growth regulators were incorporated singly and in combinations in the MS Medium for direct shoot proliferation from shoot apex and nodal segments.

I. Plant growth regulators incorporated singly in the medium.

a. BAP/Kn: 0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, 2.25, 2.50, 2.75 and 3.0 mg/l

b. IAA/NAA: 0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, 2.25, 2.50, 2.75 and 3.0 mg/l

II. Plant growth regulators incorporated in combinations.

a. BAP (1.0 and 3.0 mg/I) + IAA/NAA (1.0 and 3.0 ml/I)

b. Kn (1.0 ml/l and 3.0 ml/l) + IAA/NAA (1.0 and 3.0 ml/l)

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.

Results

Shoot Bud Induction

The shoot apex were started to break bud within 14-21 days of inoculation at all the levels of BAP (0.25 – 3.0 mg/l). Maximum number of shoot bud (1.43) induction was observed at 2.0 mg/l BAP with 90 percent frequency (Table 1 and Fig.1). Whereas, the minimum number of shoot buds (1.02) were observed at 3.0 mg/l BAP with 50 percent frequency. Increasing concentration of BAP up to 3.0 mg/l promoted shoot bud induction. However, increasing concentration of BAP (> 3.0 mg/l) reduced the shoot bud induction progressively. All the level of BAP induced callus at the base of shoot apex explants except lower and higher levels of BAP. Medium callusing were observed at 2.0 - 2.25 mg/l BAP with 60 percent frequency and other levels induced only low callus.

When nodal segment explants were inoculated on MS medium at different levels of BAP (0.25 – 3.0 mg/l), induction of shoot bud was observed within 2.5 - 3 weeks of incubation at all the levels. Maximum number of shoot bud induction (1.42) was observed at 2.50 mg/l BAP with 90 percent frequency (Table 1 and Fig. 2). Increasing level of BAP increased shoot bud induction up to 3.0 mg/l. However, higher level of BAP showed inhibitory effect on shoot bud induction. As like shoot apex, nodal segment also induced medium callus at 2.0 mg/l BAP. Both lower (0.25– 1.25 mg/l) and higher (2.75 - 3.0 mg/l) levels of BAP were insufficient to induced callus in nodal segment explants. Frequency of callus induction was 30-60 percent at different level of BAP in nodal segment explant.

Shoot apex explants responded to combined Kn (1.0 and 3.0 mg/l) and IAA (1.0 and 3.0 mg/l) treatments through shoot bud induction within 18 – 20 days of inoculation with 60-80 percent frequency. Highest shoot bud induction (1.30) was observed at 3.0 mg/l Kn + 1.0 mg/l IAA. Minimum shoot bud induction (1.10) was observed at 1.0 mg/l Kn + 1.0 mg/l IAA and 1.0 mg/l Kn + 3.0 mg/l IAA (Table 2 and Fig 3).

BAP (1.0 and 3.0 mg/l) in combination with IAA (1.0 and 3.0 mg/l) induced only shoot buds in nodal segment explants within 18 – 20 days of inoculation with 60-80 percent frequency at all the levels of combinations. Highest shoot bud (1.30) was observed at 3.0 mg/l BAP + 1.0 mg/l IAA and 3.0 BAP + 4.5 mg/l IAA. Minimum shoot bud (1.10) was observed at 1.0 mg/l BAP + 3.0 mg/l IAA. Replacement of NAA with IAA also responded similarly by inducing more shoot bud break at lower levels of IAA (1.0 mg/l) with both levels of BAP (1.0 and 3.0 mg/l) in shoot apex and nodal segment explants (Table 2 and Fig 4).

Discussion

We were successful in developing the micropropagation of pomegranate (*P. granatum* L.) 'Bhagava' cultivar using MS media. MS medium proved to produce best vegetative growth characteristics. However in contrast to our findings Samir *et al.* (2009) [21] 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) [12, 9]. 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 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) [11], who observed similar result with cotyledon tissue of pomegranate.

NAA and BAP combinations were rewarding in many fruit tree species (Zimmerman and Swartz, 1994) [26]. For the shoot regeneration, cytokinin is effective when used in combination with an auxin (Nike *et al*., 1999) [12]. Synthesis and activities of auxin, cytokinins and ethylene are thought to be closely related (Klee and Romano, 1994) [7]. Ramesh *et al*. (2006) [19] also reported that the addition of adenine sulphate (60 mg/L) along with other growth regulators was the most effective in inducing shoot multiplication. 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) [16, 8, 24, 1, 13, 11, 25]. 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) [10]. 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. BAP and NAA alone or in combination within MS medium to induce high frequency adventitious shoot regeneration.

Fig 1: Shoot bud induction in shoot apex explant on MS medium supplemented with 2.0 mg/l BAP.

Fig 2: Shoot bud in ductionin nodal segment explant on MS medium supplemented with 2.5 mg/l BAP.

Fig 3: Shoot bud induction in shoot apex explant on MS medium supplemented with 3.0 mg/l Kn and 1.0 mg/l IAA.

Fig 4: Shoot bud induction in nodal segment explant on MS medium supplemented with 3.0 mg/l BAP and 1.0 mg/l IAA.

Table 1: Morphogenetic effect of various concentration of cytokinin (BAP) supplemented singly in the MS medium on different explants of Pomegranate.

Concentration (mg/l)	Shoot apex explant					Nodal segment explant				
	Mean number of days taken for shoot bud break	Number of shoot buds /explant	Response (%)	Callus induction		Mean number of days taken for shoot bud break	Number of shoot buds /explant	Response (%)	Callus induction	
				Appearance	Frequency (%)				Appearance	Frequency (%)
0.25	21.30	1.10#±0.11	30	-	-	20.31	1.02#±0.16	40	-	-
0.50	20.14	1.12#±0.12	35	-	-	19.12	1.03±0.16	40	-	-
0.75	19.15	1.11#±0.14	45	+	-	18.30	1.12±0.16	45	-	-
1.0	14.60	1.12#±0.13	50	+	10	17.60	1.13±0.16	50	-	-
1.25	17.80	1.14± 0.15	60	+	10	15.70	1.13±0.16	60	-	-
1.50	18.10	1.16± 0.13	70	+	10	16.40	1.12±0.15	70	+	30
1.75	18.60	1.16± 0.12	70	+	20	16.90	1.30±0.13	70	+	40
2.0	18.80	1. 43± 0.18	90	++	60	17.70	1.32±0.10	80	++	60
2.25	18.50	1.32± 0.17	80	++	50	17.30	1.32±0.13	80	+	50
2.50	18.50	1.31± 0.10	80	+	40	17.10	1. 42±0.12	90	+	40
2.75	18.40	1.12± 0.12	60	+	10	17.50	1.13±0.10	60	-	-
3.0	18.50	1.02±0.14	50	-	-	18.40	1.02±0.17	50	-	-

(++)= Medium callus,(+)= Low callus,(-)= No response and (#)= Transformed value

Table 2: Morphogenetic effect of various concentration of cytokinin (BAP/Kn) and auxin (NAA/IAA) added in combination in the MS medium on different explants *viz.*, nodal segment and shoot apex.

Concentration (mg/l)	Nodal segment			Shoot apex		
	Mean number of days taken for shoot bud break	Number of shoot buds /explant	Response (%)	Mean number of days taken for shoot bud break	Number of shoot buds /explant	Response (%)
BAP	NAA (1.0 mg/l)					
1.0	20.10	1.10#±0.15	60	18.90	1.12#±0.14	70
3.0	19.90	1.12±0.16	70	19.80	1.30±0.15	80
BAP	NAA (3.0 mg/l)					
1.0	19.00	1.02±0.14	50	19.60	1.10±0.16	60
3.0	18.60	1.10±0.12	60	20.00	1.12±0.14	70
Kn	NAA (1.0 mg/l)					
1.0	19.90	1.12± 0.13	70	19.40	1.02±0.14	50
3.0	18.70	1.30±0.15	80	19.80	1.12±0.16	70
Kn	NAA (3.0 mg/l)					
1.0	19.60	1.10±0.15	60	18.50	1.02±0.15	50
3.0	19.70	1.30±0.16	80	19.20	1.12±0.13	70
BAP	IAA (1.0 mg/l)					
1.0	19.70	1.12±0.17	70	19.80	1.10±0.14	60
3.0	19.70	1.30±0.14	80	19.70	1.12±0.13	70
BAP	IAA (3.0 mg/l)					
1.0	18.50	1.10±0.15	60	18.50	1.02±0.15	50
3.0	19.00	1.30±0.13	80	18.70	1.10±0.14	60
Kn	IAA (1.0 mg/l)					
1.0	19.40	1.10±0.16	60	19.60	1.10±0.15	60
3.0	19.80	1.12±0.15	70	19.10	1.30±0.16	80
Kn	IAA (3.0 mg/l)					
1.0	18.50	1.02±0.14	50	18.60	1.10±0.14	60
3.0	18.40	1.10±0.13	60	19.40	1.12±0.13	70

(#) = Transformed value

References

1. Amin MN, Islam MN, Azad MAK. Regeneration of plantlets *in vitro* from the seedling explants of pomegranate (Punica granatum L.). Plant Tissue Cult. 1999;9(1):53-61.
2. Anonymous. Indian Horticulture Database, National Horticulture Board, Gurgaon, Haryana. 2015-16.
3. In: Vasil IK, Thorpe TA (eds). Plant Cell Tissue Culture. Kluwer Academic Publishers, Dordrecht, pp. 457-474.
4. Jayesh KC, Kumar R. Crossability in pomegranate (Punica granatum L.). Indian J. Horticulture. 2004;61:3.
5. Jindal KK, Sharma RC. Recent Trends in Horticulture in the Himalayas. Indus Publishing Company, New Delhi; c2004.
6. Kanwar K, Joseph J, Raj D. Comparison of *in vitro* regeneration pathways in Punica granatum L. Plant Cell, Tissue Organ Culture. 2010;100(2):199-207.
7. Klee HJ, Romano CP, Binns A. The roles of phytohormones in development as studied in transgenic plants. Critical reviews in plant sciences. 1994 Jan 1;13(4):311-24.
8. Mahishni DM, Muralikishna A, Shivashankar G, Kulkarni RS. Shoot tip culture method for rapid clonal propagation of pomegranate (Punica granatum L.). In:

Horticulture New Technologies and Applications. Proc. Int. Seminar on New Frontiers in Horticulture. Indo-American Hybrid Seeds. Bangalore, 1991, 215-217.
9. Murkute AA, Patil S, Patil BN, Kumari M. Micropropagation in pomegranate, callus induction and differentiation. South Indian Hort. 2002;50(1-3):49-55.
10. Murkute AA, Patil S, Singh SK. *In vitro* regeneration in pomegranate cv. Ganesh from mature trees. Indian J. Hort. 2004;61(3):206-208.
11. Naik SK, Chand PK. Silver nitrate and aminoethoxyvinylglycine promote *in vitro* adventitious shoot regeneration of pomegranate (Punica granatum L.). J. Plant Physiol. 2003;160:423-430.
12. Naik SK, Pattnaik S, Chand PK. *In vitro* propagation of pomegranate (Punica granatum L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. Scientia Horticulturae. 1999 Feb 26;79(3-4):175-83.
13. Naik SK, Pattnaik S, Chand PK. High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (Punica granatum L.). Scientia Horticulturae. 2000 Aug 28;85(4):261-70.
14. Native Indian Biodiesel Tree from Cotyledonary Node. Int. J. Biotechnol. Biochem. 6(4):555-560.
15. Shrivastava S, Banerjee M. *In vitro* clonal propagation of physic nut (Jatropha curcas L.): Influence of additives. Int. J. Integr. Biol. 2008;3(1):73-7.
16. Omura M, Matsuta N, Moriguchi T, Kozaki I. Adventitious shoot and plantlet formation from cultured pomegranate leaf explants. HortScience. 1987 Feb 1;22(1):133-4.
17. Raj D, Kanwar K. Efficient *in vitro* shoot multiplication and root induction enhanced by rejuvenation of microshoots in Punica granatum cv. Kandhari Kabuli. National Seminar on Physiological and Biotechnological Approaches to Improve Plant Productivity, CCSHAU, Hisar, India, 2008, 24.
18. Raj D, Kanwar K. *In vitro* regeneration of (Punica granatum) L. Plants from different juvenile explants. J. Fruit Ornamental Plant Res. 2010;18(1):5-22.
19. Ramesh M, Saravanakumar RM, Pandain SK. Benzyl amino purine and adenine sulphate induced multiple shoot and root induction from nodal explants of Brahmi (Bacopa monnieri L). Penn. Nat. Prod. Rad. 2006;5:44-51.
20. Samir Z. *In vitro* Salt and Drought Tolerance of Manfalouty and Nab El-Gamal Pomegranate Cultivars. Australian. Basic Appl. Sci. 2010;4(6):1076-1082.
21. Samir Z, El-Agamy, Rafat AA, Mostafa Mokhtar M, Shaaban Marwa T, El-Mahdy. *In vitro* Propagation of Manfalouty and Nab El- gamal Pomegranate Cultivars Research. J. Agric. Biol. Sci. 2009;5(6):1169-1175.
22. Shrivastava S, Banerjee M. *In vitro* clonal propagation of physic nut (Jatropha curcas L.): Influence of additives. Int. J. Integr. Biol. 2008;3(1):73-7.
23. Vineeta Shrivastava, Kant T. Micropropagation of Pongamia pinnata (L.). Pierre-a native Indian biodiesel tree from cotyledonary node. Int J Biotechnol Biochem. 2010 Oct 1;6(4):555-60.
24. Yang ZH, Ludders P. Organogenesis of Punica granatum L. var. Nana. Angewandte Botanik. 1993;67(5-6):151-156.
25. Zhu LW, Zhang SM, Song FS, Gong XM, Fang WJ, Sun J, *et al.* Regeneration system of pomegranate by *in vitro* culture. Acta. Hort. Sinica. 2003;430(2):207-208.
26. Zimmerman RH, Swartz HJ. *In vitro* culture of temperate fruits. Plant cell and tissue culture. 1994:457-74.