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In vitro propagation studies in pomegranate (*Punica granatum* L.) using nodal explant

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Abstract
The present study experiments were carried out to develop reliable protocols for producing healthy and well-formed plants from nodal explants of pomegranate (*Punica granatum* L.) cv 'Bhagwa'. In standardization of the process for controlling polyphenol exudation from explants during callus formation using antioxidants, MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 0.6 mg/l was the best media with less polyphenol exudation (74.86 per cent), lowest microbial contamination (3.33 per cent) and highest survival percentage of explants (90.00 per cent). It was observed and concluded that MS media when supplemented with ADS 15mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l had given maximum shoot proliferation (59.99 per cent), number of shoots (0.99), number of leaves (2.88) along with highest number of nodes (1.43) and intermodal length (0.68 cm). Among auxins used for rooting it was observed and concluded that when MS media supplemented with IBA 0.6 mg/l had given maximum number of roots developed per shoot (0.53), per cent of shoots giving roots (91.65) and number of new leaves (4.49). Among all potting mixture tested, it was observed that maximum survival (83.33 per cent) of plants from nodal explants were found on medium containing red soil: cocopeat: vermicompost (1:2:1).

Keywords: Establishment, multiplication, nodal explant, pomegranate, micro propagation

Introduction
Pomegranate (*Punica granatum* L.) is an important and ancient table fruit represents a distinct family 'punicaceae' which comprises only one genus (*Punica*) and two species *P. granatum* and *P. protopunica* (Samir, 2010) [19]. The name *Punica granatum* is derived from *pomum (*apple) and granatum (grainy) or seeded apple. It is commonly known as 'Anar' and grown in tropical and subtropical regions of the world. It is exploited for nutritional value of its fruits, medicinal properties of different parts of the tree and for ornamental purpose as it bears bright red flowers (Naovi *et al*. 1991; Sepulveda *et al*. 2000; Jayesh and Kumar, 2004; Johanningsmeier and Harris, 2011) [13, 18, 6, 7]. The double flowered pomegranate plants (which do not bear fruits) are grown in parks and ornamental gardens for their beautiful red flowers (Raj and Kanwar, 2010) [16].
The fruit is a good source of sugars and minerals. The seeds and fleshy pulp are dried and used as a condiment. The bark of the stem and roots contain certain alkaloids belonging to pyridine group which are used in preparation of medicine for diarrhea and dysentery.
In India, it is found from Kanyakumari to Kashmir but it is commercially cultivated only in Maharashtra, Gujarat, Rajasthan, Karnataka, Tamil Nadu, Andhra Pradesh, Telangana State, Uttar Pradesh, Punjab and Haryana. Formerly, India used to import pomegranate fruits from Afghanistan and West Pakistan, but since last decade, India exporting mainly to Gulf and SAARC (South Asian Association for Regional Cooperation) countries. In India, the Deccan Plateau is having ideal climatic conditions for quality fruit production throughout the year (Chandra *et al.*, 2006 and 2008; Chandra and Meshram, 2010) [3-5]. Maharashtra is the largest producer occupying 2/3rd of total area in the country followed by Karnataka, Andhra Pradesh, Gujarat and Rajasthan (Jayesh and Kumar, 2004) [6].
The area under pomegranate is increasing worldwide because of its hardy nature, wider adaptability, drought tolerance, higher yield levels, excellent keeping quality and remunerative prices in domestic as well as export markets. It thrives well in dry tropics, sub tropics and comes up very well in soils of low fertility status, adding to that it is salt tolerant too. Because of its ability to grow well in slightly saline soils, it is considered as a saline tolerant plant (Patil and Waghmare, 1983; Rao and Khandelwal, 2001; Arsey *et al*., 2002 Levin, 2006; Marathe *et*

al., 2009) [14, 15, 2, 9, 12]. The edible portion (arils) is about 45-61 per cent of total fruit weight which includes about 60-85 per cent juice and 15-25 per cent seeds; peel forms 33-40 per cent of the fruit. The fruit juice is a good source of sugars, vitamin C, vitamin B, pantothenic acid, potassium, antioxidant polyphenols and a fair source of iron. Due to its immense potential for health benefits, pomegranate has achieved the title of "super –food". It can be consumed as a raw fruit or juice. It is having a great history of nutritional value. The fruit has a wide consumer preference for its attractive colored, juicy, sweet, acidic refreshing arils. There is a growing demand for good quality fruits both for fresh use and processed juice, syrup and wine. The entire seed is consumed raw though the watery aril is the desired part. The taste differs depending on the subspecies of pomegranate and its ripeness. About 100g arils provides 72.0 Kcal of energy, 1.0 g of protein, 16.6 g carbohydrate, 10 mg sodium, 379 mg potassium, 13.0 mg calcium, 12.0 mg magnesium, 0.7 mg Iron, 0.17 mg copper, 0.3 mg niacin and 7.0 mg vitamin C.

To get true to type planting material, pomegranate is commercially propagated by stem cuttings (hardwood cuttings) or by layering. However it has several limitations like low success, very few propagation methods and new plants require one year for establishment (Anon, 1982) [1]. This results in non-availability of plantlets throughout the year. In recent years, 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 explants and apical buds or through organogenesis or embryogenesis directly from various explants or through callus have been already demonstrated in a number of woody species. 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, cotyledon (Murkute *et al*., 2002; Raj and Kawar, 2012; Kanwar *et al*., 2010, anthers Soumendra Naik *et al*., 1999) [11, 16, 17] or through embryogenesis from seedling explants, petals and immature zygotic embryos (Kanwar *et al*., 2010) [16].

However, till date there are hardly any reports described in detail about different media and serial sub culturing process using nodal explant in pomegranate. Consequently, this work was designed for evolving a micropropagation technique for pomegranate (*P. granatum* L), var. 'Bhagwa' using nodal explants and to study the effect of growth regulators for establishment, multiplication, rooting, hardening and acclimatization.

Materials and Methods

The work was done at COH, Venkataramannagudem and at Acharya Nagarjuna University, Guntur during 2015-17 to establish microprapagation protocol of pomegranate. In the present investigation total five experiments were carried out in which CRD was applied to find the best treatments for controlling polyphenol exudation, shooting media, rooting media and hardening potting mixtures.

Explant collection and preparation

The explants were collected from third node of the shoot apex, early in the morning during the spring season in the months of February and March. The type of explant used for the culture was nodal segment with axillary bud, collected during February and March. The explants were collected in the morning, third node from the shoot tip. The explants were collected from healthy plants which were authenticated. The size of explants was 2.0 to 5.0 cm in length, 1.0 to 3.0 mm in thickness and were excised with the help of sterilized scalpel and scissors. The freshly collected explants were washed with running tap water for 30 minutes, treated with an aqueous solution of teepol for 5 minutes and again washed 5-6 times thoroughly with distilled water.

Sterilization of explants

The explants were surface sterilized to eliminate bacteria and fungal spores present on their surface. Isolated nodal explants were first washed thoroughly under running tap water for two minutes followed by rinsing with distilled water in order to remove dust and extraneous material. Then the material was rinsed in Tween-20 for 5 minutes, followed by Mercuric chloride 1000 mg/l for 15 min and bavistin 1500 mg/l for 30 min to eliminate the fungal and bacterial contamination. Finally the explants were thoroughly washed with double distilled water for 5-6 times.

Culture media

The media used for this investigation was Murashige and Skoog's (1962) [10] basal media (Table 1). The MS media was supplemented with different concentrations of alpha-naphthalene acetic acid (NAA), indole-3-butyric-acid (IBA), 6-benzyl aminopurine (BAP) and kinetin and their combinations. MS medium consisted of inorganic nutrients, carbon source and vitamins. The stock solutions were prepared for macronutrients, micronutrients, iron and vitamins by dissolving them in distilled water to the volume required. The stocks were stored in sterilized amber coloured bottles by keeping them in refrigerator at 4^0C. While preparing the media, appropriate amount of stock solutions were pipetted out, mixed and final volume of the media was made after dissolving sucrose (30g/l) in triple distilled water. The required concentrations of growth regulators as per the treatment were added and the volume was made up. The pH of the media was adjusted to 5.8 and Agar (0.8%) was added for media solidification. For establishment stage NAA 0.0 to 2.0 mg/l, BAP 0.0 to 2.0 mg/l, ADS 15mg/l and kinetin 0.1mg/l. For rooting stage, NAA 0.2 to 0.6 mg/l and IBA 0.2 to 0.6 mg/l were tested. Whereas for ***ex vitro*** media composition for hardening and acclimatization of *ex agar* pomegranate plants red soil, coco peat, vermiculite and vermicompost were tested with different combinations.

Results and Discussion

Controlling polyphenol exudation from explants during callus formation using antioxidants

The polyphenol exudation recorded lowest in MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 0.6 mg/l (T_6) on 6^{th}, 8^{th}, 10^{th} and 12^{th} day due to presence of antioxidants and adsorbents in required quantities, whereas, the exudation of polyphenols was recorded highest in MS medium (T_1) without any antioxidants and adsorbents(Table-1).

Survival of explants

The maximum per cent survival (90.00 per cent) of explants was recorded in MS medium + ascorbic acid 100 mg/l +

activated charcoal 200 mg/l + silver nitrate 0.6 mg/l (T_6) followed by MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 0.8 mg/l (T_7) and MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 1.0 mg/l (T_8) (both 85.00 per cent) was due to the presence of required amount of antioxidants and adsorbents which resulted in lowest phenolic exudation, browning and microbial contamination.

Table 1: Per cent of explants showing polyphenol exudation at 2 days interval (at 2, 4, 6, 8, 10 and 12 days)

S. No.	Treatments	Number of days						Mean
		2 days	4 days	6 days	8 days	10 days	12 days	
1	T_1- MS medium	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)
2	T_2- MS medium + Ascorbic acid 100 mg/l + Silver nitrate 0.6 mg/l	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	97.50 (83.62)	85.83 (67.98)	55.83 (48.39)	89.86 (72.50)
3	T_3- MS medium + Activated charcoal 200 mg/l + Silver nitrate 0.6 mg/l	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	95.83 (78.37)	84.99 (67.31)	55.00 (47.89)	89.30 (70.90)
4	T_4- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.2 mg/l	100.00 (90.00)	100.00 (90.00)	98.33 (87.74)	94.16 (76.39)	74.83 (60.38)	43.33 (41.12)	85.10 (65.66)
5	T_5- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.4 mg/l	100.00 (90.00)	100.00 (90.00)	96.66 (81.00)	90.83 (72.55)	67.49 (55.28)	32.50 (34.72)	81.25 (60.90)
6	T_6- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.6 mg/l	100.00 (90.00)	100.00 (90.00)	94.99 (78.77)	85.00 (67.24)	51.66 (45.95)	17.5 (24.17)	74.86 (54.03)
7	T_7- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.8 mg/l	100.00 (90.00)	100.00 (90.00)	94.99 (78.77)	94.16 (76.39)	67.49 (55.26)	26.66 (30.46)	80.55 (60.23)
8	T_8- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 1.0 mg/l	100.00 (90.00)	100.00 (90.00)	97.50 (83.62)	91.66 (73.42)	76.66 (61.19)	28.33 (31.43)	85.40 (62.42)
	Mean	100.00 (90.00)	100.00 (90.00)	97.80 (84.61)	93.64 (77.25)	76.11 (62.92)	44.89 (43.52)	
	S.Em.+	0	0	2.78	1.89	1.87	3.11	
	CD (P=0.05)	0	0	8.1	5.52	5.46	9.07	

Media composition for optimum shoot proliferation

It is observed and concluded that MS media when supplemented with ADS 15mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l had given highest maximum shoot proliferation, number of shoots, number of leaves along with highest number of nodes and intermodal length and survival per cent, followed by MS media supplemented with ADS 15mg/l + NAA 1.0 mg/l + BAP 2.0 mg/l (Table-2) (Fig 1).

Table 2: Per cent proliferation at 20th, 40th and 60th day

S. No.	Treatments	Number of days			Mean
		20 days	40 days	60 days	
1	T_1- MS medium + ADS 15 mg/l + NAA 0.0 mg/l + BAP 0.0 mg/l	0.00 (0.00)	3.33 (6.14)	10.00 (18.43)	4.44 (8.19)
2	T_2- MS medium + ADS 15 mg/l + NAA 0.0 mg/l + BAP 1.0 mg/l	3.33 (6.14)	16.66 (23.85)	23.33 (28.78)	14.44 (19.59)
3	T_3- MS medium + ADS 15 mg/l + NAA 0.0 mg/l + BAP 2.0 mg/l	6.66 (12.28)	20.00 (26.56)	36.66 (37.22)	21.10 (25.36)
4	T_4- MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 0.0 mg/l	6.66 (12.28)	10.00 (18.43)	16.66 (23.85)	11.10 (18.19)
5	T_5- MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 1.0 mg/l	33.33 (35.00)	43.33 (41.07)	53.33 (46.92)	43.33 (41.00)
6	T_6- MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 2.0 mg/l	36.66 (37.22)	46.66 (43.07)	70.00 (56.99)	51.10 (45.76)
7	T_7- MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 0.0 mg/l	26.66 (30.29)	43.33 (41.15)	46.66 (43.07)	38.88 (38.17)
8	T_8-MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 1.0 mg/l	30.00 (33.00)	50.00 (45.00)	63.33 (52.77)	47.77 (43.59)
9	T_9- MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l	36.66 (37.22)	66.66 (54.78)	76.66 (61.21)	59.99 (51.07)
	Mean	19.99 (22.60)	33.33 (33.33)	44.07 (41.02)	
	S.Em.+	4.6	2.83	2.26	
	CD (P = 0.05)	13.68	8.4	6.72	

Effect of NAA and IBA on *in vitro* rooting

It could be concluded that MS media when supplemented with IBA 0.6 mg/l had given maximum number of roots per shoot, maximum root length, highest number of shoots producing roots and more number of new leaves followed by MS media supplemented with 0.6 mg/l NAA (Table-3) (Fig-2).

Effect of NAA and IBA on shoot growth

The shoot length was highest (5.66 cm) in MS medium + NAA 0.6 mg/l (T_4) followed by MS medium + IBA 0.6 mg/l (T_7) (5.16 cm), while lowest shoot length was observed in MS medium (T_1) on (both 0.00 cm) preceded by MS medium + NAA 0.2 mg/l (T_2) (0.76 cm).

Effect of NAA and IBA on leaf growth: It could be concluded that MS media when supplemented with IBA 0.6 mg/l had given maximum number of roots per shoot, highest number of shoots producing roots and more number of new leaves followed by MS media supplemented with 0.6 mg/l NAA.

Effect of *ex vitro* media composition for hardening and acclimatization of *ex agar* pomegranate plants

The number of newly formed leaves were highest (31.33) was recorded by cocopeat: vermiculite: vermicompost (1:1:1) (T_4) followed by cocopeat: vermiculite: vermicompost (2:1:1) (T_6) (29.96), whereas, lowest number of newly formed leaves were observed in red soil: vermicompost (1:1) (T_1) (5.10) preceded by red soil: vermicompost (1:1) (T_1) (9.76) at 30th day and cocopeat: vermicompost (1:1) (T_2) (10.16) (Table-4) (Fig-3).

Table 3: Length of roots in cm (at 20th day and 30th day)

S. No.	Treatments	Days		Mean
		20 days	30 days	
1	T_1- MS medium	0.00	0.00	0.00
2	T_2- MS medium + NAA 0.2 mg/l	0.60	1.00	0.80
3	T_3- MS medium + NAA 0.4 mg/l	0.50	1.30	0.90
4	T_4- MS medium + NAA 0.6 mg/l	2.46	2.86	2.66
5	T_5- MS medium + IBA 0.2 mg/l	0.00	0.23	0.12
6	T_6- MS medium + IBA 0.4 mg/l	2.13	2.56	2.35
7	T_7- MS medium + IBA 0.6 mg/l	1.90	2.70	2.30
	Mean	1.08	1.52	
	S.Em +	0.31	0.33	
	CD (P = 0.05)	0.95	0.99	

Table 4: Number of newly formed leaves (at 20th and 30th day)

S. No.	Treatments	Days		Mean
		20 days	30 days	
1	T_1- Red soil: vermicompost (1:1)	5.10 (2.36)	9.76 (3.19)	7.43 (2.77)
2	T_2-Cocopeat: vermicompost (1:1)	10.16 (3.26)	18.20 (4.31)	14.18 (3.78)
3	T_3-Vermiculite: vermicompost (1:1)	7.16 (2.76)	18.33 (4.32)	12.74 (3.54)
4	T_4-Cocopeat: vermiculite: vermicompost (1:1:1)	12.06 (3.53)	31.33 (5.58)	21.69 (4.55)
5	T_5- Cocopeat: vermiculite: vermicompost (1:2:1)	8.13 (2.91)	21.16 (4.65)	14.64 (3.78)
6	T_6- Cocopeat: vermiculite: vermicompost (2:1:1)	9.50 (3.15)	26.96 (5.23)	18.23 (4.19)
7	T_7- Red soil: cocopeat: vermicompost (1:1:1)	7.93 (2.87)	17.50 (4.21)	12.71 (3.54)
8	T_8- Red soil: cocopeat: vermicompost (1:2:1)	9.43 (3.13)	19.16 (4.40)	14.29 (3.76)
9	T_9- Red soil: cocopeat: vermicompost (2:1:1)	6.20 (2.58)	14.03 (3.80)	10.11 (3.19)
	Mean	8.40 (2.95)	19.60 (4.41)	
	S.Em.+	0.18	0.27	
	CD (P = 0.05)	0.54	0.81	

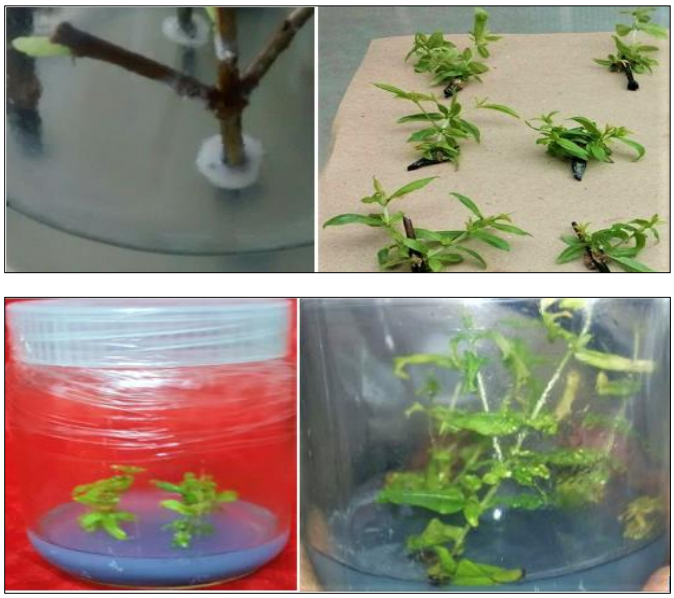

Fig 1: Shoot development and multishoots ready for subculturing

Fig 2: Primary hardened rooted plantlets of pomegranate cv. Bhagwa in sterilized cocopeat

Fig 3: Secondary hardened plantlets of pomegranate cv. Bhagwa in A) Red soil: cocopeat: vermicompost (2:1:1) (T_8) B) Red soil: cocopeat: vermicompost (1:2:1) (T_8) C) Fully hardened pomegranate plants ready for plating

Conclusions

The present investigation revealed that the process of polyphenol exudation from explants during callus formation, could be controlled by using antioxidants, MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 0.6 mg/l followed by MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 0.8 mg/l, which were the best media in controlling the phenol exudation, decreasing the microbial contamination and obtain highest survival per cent of explants.

It was observed that MS media when supplemented with ADS 15mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l had given highest maximum shoot proliferation, number of shoots, number of leaves along with highest number of nodes and intermodal length and survival per cent, followed by MS media supplemented with ADS 15mg/l + NAA 1.0 mg/l + BAP 2.0 mg/l.

It could be concluded that MS media when supplemented with IBA 0.6 mg/l had given maximum number of roots per shoot, highest number of shoots producing roots and more number of new leaves followed by MS media supplemented with 0.6 mg/l NAA.

Considering the survival per cent of primary hardened plants, shoot length and newly formed leaves, it was concluded that red soil: cocopeat: vermicompost (1:2:1) was the best followed by red soil: cocopeat: vermicompost (1:1:1) among the potting mixtures tested.

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