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Standardization of sterilization protocol and control of polyphenol exudation in micropropagation studies of pomegranate (*Punica granatum* L.) cv. Bhagwa

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Abstract
The present investigation entitled "*In vitro* propagation studies in pomegranate (*Punica granatum* L.)" was carried out in which CRD was applied to find the best treatments for controlling polyphenol exudation, shooting media, rooting media and hardening potting mixtures. 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 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. 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. The polyphenol exudation recorded in MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 0.6 mg/l (T_6) on 6th, 8th, 10th and 12th 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.

Keywords: Sterilization, microbial contaminants, polyphenol exudation, pomegranate

Introduction
Pomegranate (*Punica granatum* L.) belongs to botanical family Punicaceae. The pomegranate tree is native to Iran and the most important growing regions are Egypt, China, Afghanistan, Pakistan, Bangladesh, Iran, Iraq, India, Burma and Saudi Arabia. It is commonly known as 'Anar' and grown in tropical and subtropical regions of the world. The fruit juice is a good source of sugars, vitamin C, vitamin B, pantothenic acid, potassium, antioxidant polyphenols, good source of iron, Containing polyphenols which inhibit estrogen synthesis and pomegranate seed oil was effective against proliferation of breast cancer cells. 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) [15]. Pomegranate is used since ancient times for itsfruit, ornamental, dietary and medicinal purposes hence tissue culture of pomegranate is a method to obtain more number of seedlings of same variety rapidly. Bhagwa is a variety of pomegranate which is a heavy yielder and possesses desirable fruit characters. This variety matures in 180-190 days with average yield of 30.38 kg fruits/tree which would be of great use for the farmers and this variety is recommended by Mahatma Phule Agriculture University, Rahuri for cultivation in Maharashtra. The tissue culture method can be utilized to substantially enhance the rate of multiplication. A million-fold increase per year in the rate of multiplication over conventional methods is not unrealistic1 (Murashige, 1974) [7].
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) [12, 14, 2, 5, 10].

Pomegranate is commercially propagated by stem cuttings (hardwood cuttings) or by layering to get true to type planting material. 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, micropropagation 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; Kanwar *et al*., 2010, anthers Soumendra Naik *et al*., 1999) [8, 4, 16] or through embryogenesis from seedling explants, petals and immature zygotic embryos (Kanwar *et al*., 2010) [4].

Consequently, this work was designed for *In vitro* multiplication of pomegranate (P. *granatum* L.) 'Bhagwa' cultivar and to study the effect of MS medium and growth regulators for establishment, multiplication and rooting of pomegranate (P. *granatum* L.). In the micropropagation of pomegranate the most common problems faced were microbial contamination and polyphennol exudation in the media. Very less reports are available in respect to micropropagation of pomegranate concerned with phenolic problems of pomegranate and surface-sterilization of explants. Hence in this study different approaches were tested to overcome the problems associated with surface sterilization and phenolic exudation.

Materials and Methods

The present work was done at COH, Venkataramannagudem and at Acharya Nagarjuna University, Guntur 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. 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.

Sterilization of equipment and glassware

The laminar air flow chamber was surface sterilized by thorough cleaning with spirit and the UV-light of laminar air-flow chamber was switched on for 30-40 minutes before the use. UV-light was turned off and airflow was kept on during the operation. All the equipment *ie.*, forceps, scalpels, scissors *etc.*, were dipped in spirit inside the laminar air-flow chamber and were frequently sterilized on the Bunsen burner flame during the operation.

All the glassware *viz*., pipettes, beakers, flasks, measuring cylinders, test tubes, petri dishes and culture bottles *etc*., used during the investigation were washed with laboratory detergent (teepol) followed by thorough washing with running tap water and then rinsed with double distilled water. The blade holders, forceps were wrapped in aluminium foil and glass beads were kept in culture bottles and wrapped with kiln wrap. All these equipment sterilized in autoclave at 121^0C for 20 minutes at 15 lb. /inch^2. All the culture vessels were dried in hot air oven at 60-70^0C for 24 hours.

Preparation of stock solutions and their storage

All the stock solutions required for the preparation of media were prepared by dissolving the required chemicals in double distilled water. Salts were dissolved by adding one compound at a time. Precipitation was usually avoided by preparing stock solutions of growth regulators such as auxins and cytokinins by dissolving in small volume of NaOH or HCl and then final volume was made by adding double distilled water in 1:1 ratio. All the stock solutions were kept in bottles with lids and stored at 4^0C.

Preparations of culture media and its sterilization

The media used for this investigation was Murashige and Skoog's (1962) [6] basal media. 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. The nutrient media 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 ^{0}C. 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 with addition of 1.0 N NaOH or 1.0 N HCl using a pH meter. Agar (0.8%) was added to the nutrient solution while boiling. The media is heated to melt the agar and continuous stirring was done during melting to prevent the agar from setting and burning. When the agar was

fully dissolved the medium was dispensed in to culture bottles measuring 25 mm diameter and 150 mm height or 200 ml culture bottle closed with polypropylene caps. The culture bottles with media were autoclaved at 121^{0}C for 20 minutes (15 lb. / inch^2). Autoclaved culture bottles and glassware with media were allowed to cool and solidify. The culture bottles were used for inoculation after four days of storage to ensure that the media was free from contamination before inoculation.

Environmental/Cultural conditions

After inoculation of explants in to the media, the culture bottles were closed with caps and wrapped up with wrapper and labelled. They were then kept in growth room at a temperature of 25 + 2 ^{0}C and illuminated for 16 hours of light at 3000 lux and 8 hours dark per day maintaining a relative humidity of 70 Percent.

Nodal explants excised from the field of mature plants of pomegranate var. Bhagwa were used in this experiment. The size of explants was 2.0 to 5.0 cm length and 1.0 to 3.0 mm thickness. The explants were inoculated in MS media supplemented with BAP (1.0 mg/l). Frequent sub-culturing of explants for 3-4 times was done in the same media and the first sub-culturing was done within 24 hours because exudation of phenols. Three explants were inoculated in each bottle.

In all the treatments, explants were treated with Tween-20 for 5 minutes followed by mercuric chloride 1000 mg/l for 15 minutes and bavistin 1500 mg/l for 30 minutes to eliminate fungal and bacterial contamination.

Each treatment, contained thirty culture bottles *i.e.*, ten bottles for each replication with one explant inoculated in the medium. The culture bottles were observed daily for contamination and polyphenol exudation. The Percent survival of different explants which were uncontaminated and were not affected by polyphenol exudation in each treatment was observed upto 12 days of inoculation.

Results and Discussion

Polyphenol exudation control from explants during callus formation using antioxidants

By using various antioxidants, antifungal and antibacterial treatments all treatment recorded 100 Percent browning and contamination during 2nd and 4th day after inoculation even with frequent subculturing. Therefore there is no influence of any treatment during this period.

Percent of polyphenol exudation at 2 days interval (2nd, 4th, 6th, 8th, 10th and 12th day) showed that lowest polyphenol exudation (17.50 Percent) recorded on 12th day in MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 0.6 mg/l (T_6) preceded by MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 0.8 mg/l (T_7) on the same day (26.66 Percent) while highest polyphenol exudation (100.00 Percent) was shown by 2nd and 4th day by all the treatments followed by 6th day in MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 1.0 mg/l (T_8) and 8th day MS medium + ascorbic acid 100 mg/l + silver nitrate 0.6 mg/l (T_2) (Both 97.50 Percent).

The polyphenol exudation recorded in MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 0.6 mg/l (T_6) on 6th, 8th, 10th and 12th 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) (Fig-1). Similar results were reported by Murkute *et al.* (2004) [9] by using adsorbents (activated charcoal at 1.0, 2.0 and 3.0 g/l) or antioxidants (ascorbic acid 50,100 and 150 mg/l and frequent subculturing after 1, 2 or 3 days of inoculation reduced the polyphenol exudation and resulted in the culture establishment.

Percent of microbial contaminated explants

The microbial contamination was highest (100.00 Percent) in MS medium (T_1) followed by MS medium + Activated charcoal 200 mg/l + Silver nitrate 0.6 mg/l (T_3) (10.82 Percent), lowest (3.33 Percent) in MS medium + ascorbic acid 100 mg/l + activated charcoal 200 mg/l + silver nitrate 0.6 mg/l (T_6) preceded by both in 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 4.99 Percent) (Table-2) (Fig-2). This was due to the presence of required amount of antioxidants and adsorbents which resulted in lowest phenolic exudation, browning and microbial contamination Similar results were reported by Wang *et al* (1994) [18] that addition of antioxidants like ascorbic acid and citric acid showed reduction in browning, contamination and favoured culture establishments. Addition of adsorbing agents, activated charcoal 2000mg/l resulted in lesser browning lesser microbial contamination and higher culture establishment (Guranna and Hoolageri, 2017) [3].

The culture medium turned brown within 2 to 4 days of inoculation of the explants due to secretion of secondary metabolites from the cut ends of explants which gradually spread into the surrounding medium inhibiting shoot multiplication. The browning of explants was avoided by repeated sub culturing on similar medium within 3 to 5 days. Secretion of secondary metabolites from the cut ends of explants can be overcome by repeated sub culturing (Murkute *et al*. 2004; Usharani *et al*. 2014) [10] and addition of antioxidants like ascorbic acid and citric acid (Naik *et al*. 2000) [11] in to the medium and addition of silver nitrate for better sterilisation by Kanwar *et al.* (2010) [15] in pomegranate and Tagelsir *et al*. (2016) [17] in guava.

Survival of explants

The maximum Percent survival (90.00 Percent) 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 Percent) was due to the presence of required amount of antioxidants and adsorbents which resulted in lowest phenolic exudation, browning and microbial contamination (Table-3) (Fig-2).

As the microbial contamination was lowest, the Percent of survival of explants was highest. Patil *et al.* 2011 [13] also reported similar results, where MS medium + ascorbic acid 150 mg/l and 100 mg/l citric acid showed lowest polyphenol exudation and highest survival of explants. However the minimum Percent of survival of explants (0.00 Percent) was recorded in MS medium (T_1) preceded by MS medium + activated charcoal 200 mg/l + silver nitrate 0.6 mg/l (T_3) (70.00 Percent).

Table 1: Percent 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)	
	SEm. +	0	0	2.78	1.89	1.87	3.11	
	CD (P=0.05)	0	0	8.1	5.52	5.46	9.07	

Table 2: Percent of microbial contaminated explants

S. No.	Treatments	No. of explants inoculated	Percent of contamination
1	T_1- MS medium	30	100.00 (90.00)
2	T_2- MS medium + Ascorbic acid 100 mg/l + Silver nitrate 0.6 mg/l	30	9.16 (17.56)
3	T_3- MS medium + Activated charcoal 200 mg/l + Silver nitrate 0.6 mg/l	30	10.82 (19.17)
4	T_4- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.2 mg/l	30	7.48 (15.45)
5	T_5- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.4 mg/l	30	5.82 (13.84)
6	T_6- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.6 mg/l	30	3.33 (8.99)
7	T_7- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.8 mg/l	30	4.99 (12.73)
8	T_8- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 1.0 mg/l	30	4.99 (12.73)
Mean			18.23 (23.80)
S.Em. +			1.62
CD (P = 0.05)			4.72

Table 3: Percent survival of explants

S. No.	Treatments	No. of explants inoculated	Percent explants survived
1	T_1- MS medium	30	0.00 (0.00)
2	T_2- MS medium + Ascorbic acid 100 mg/l + Silver nitrate 0.6 mg/l	30	72.50 (58.45)
3	T_3- MS medium + Activated charcoal 200 mg/l + Silver nitrate 0.6 mg/l	30	70.00 (56.78)
4	T_4- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.2 mg/l	30	77.50 (62.30)
5	T_5- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.4 mg/l	30	82.50 (65.46)
6	T_6- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.6 mg/l	30	90.00 (74.14)
7	T_7- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 0.8 mg/l	30	85.00 (67.50)
8	T_8- MS medium + Ascorbic acid 100 mg/l + Activated charcoal 200 mg/l + Silver nitrate 1.0 mg/l	30	85.00 (67.50)
Mean			70.31 (56.51)
S.Em. +			2.91
CD (P = 0.05)			8.51

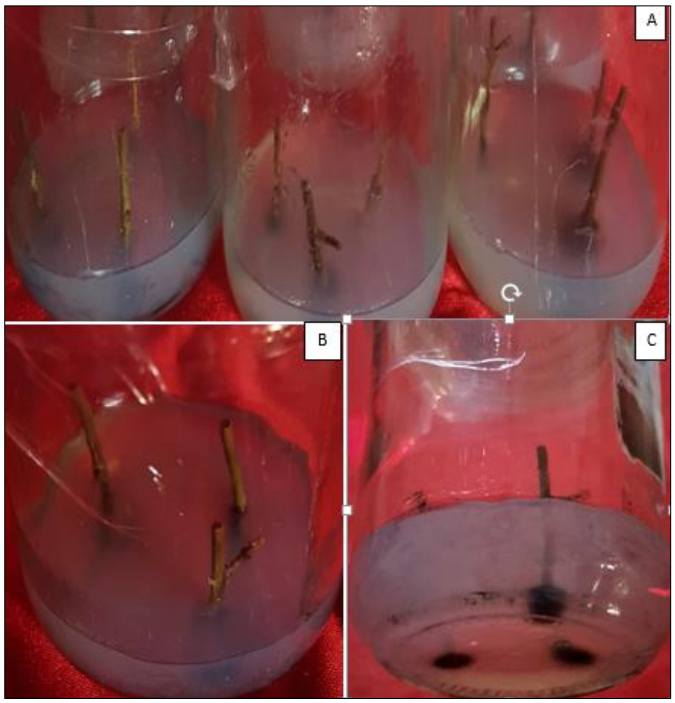

Fig 1: a) Inoculation of explants in culture bottles b) Explants in the media immediately after inoculation c) Explants in the media two days after inoculation

Fig 2: A) Fungal contamination after two days of inoculation of explant B) Fungal contamination after four days of inoculation of explant C) Bacterial contamination after inoculation of explant

Fig 3: Primary hardened rooted plantlets of pomegranate cv. Bhagwa in sterilized Cocopeat

Fig 4: 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) 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 Percent of explants. However, the frequent sub culturing of explants, at an interval of 24 h, was the only solution to completely overcome browning in most of the woody explants.

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