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Standardization of *In vitro* Regeneration Protocol for Pomegranate (*Punica granatum* L.) var. Bhagwa Using Different Media Composition

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In the present study, MS media was found to be the best media among the three different media tried for the establishment of explants. Among the different periods of surface sterilization with 0.1 % $HgCl_2$, 3 min for shoot tip and 4 min for nodal segment were found to be appropriate for surface sterilization which recorded 85.50 per cent *in vitro* culture survival in shoot tips and 89.25 per cent *in vitro* culture survival in nodal segment. The significantly maximum establishment for shoot tips (12.00 %) and nodal segment (66.67%) was recorded on MS medium. MS media supplemented with 2.0 mg/l BAP and 20 mg/l AS was found suitable for maximum establishment in shoot tips (10.67 %) and nodal segments (66.67%). MS media supplemented with 2.5 mg/l BAP + 0.5 mg/l NAA + 2.5 g/l AC was found to be most efficient media for multiplication through nodal explants which produced more number of shoots per explant (11.30) with 62.53 shooting per cent. The ½ MS media supplemented with 1.5 mg/l IBA + 2.0 g/l AC was most suitable for rooting which recorded maximum number of roots/shoot (4.50), more length of roots (3.53 cm), maximum rooting percentage (40.63) and maximum survival of tissue culture plants (82.03 %).

Keywords: Pomegranate, *in vitro* regeneration, establishment, multiplication, rooting.

Pomegranate (*Punica granatum* L.) is an ancient favourite table fruit of tropical and sub-tropical regions of the world. It belongs to the family Punicaceae, sub-class, Rosidae believed to be native of the region between Iran and Northern India. Pomegranate, a sub-tropical plant is winter hardy drought tolerant and can even withstand desert conditions and therefore, has been rightly called as a plant with versatile adaptability to the varied climatic conditions (Sharma *et al.*, 2009).

Conventionally, pomegranate is propagated vegetatively by air layering of hardwood and softwood cuttings, but rate of multiplication and disease free plants material is a problem. Propagation by seed is not preferred because of the resulting variability in tree and fruit characters. Hence, micropropagation is an attractive alternative to conventional propagation techniques for rapid clonal production (Naik *et al.*, 2000; Kanwar *et al.*, 2004).

The micropropagation technique have been widely used for propagation of several plant species. *In vitro* propagation of pomegranate cultivar through axillary proliferation : from nodal explants of mature tree has been reported earlier by Singh *et al.* (2013), Kaji *et al.* (2013) Singh and Patel (2014). However, the limitations for the protocols was the cultivar and the location. Therefore, in the present study, research work was carried out with an objective to develop a regeneration protocol for pomegranate var.Bhagwa

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through high frequency shoot proliferation from shoot tips and nodal segments.

MATERIALS AND METHODS

The shoot tip and nodal segment explants of pomegranate var. Bhagwa and Mrudula were obtained from the plants indexed and maintained in polyhouse of State Level Biotechnology Centre, M.P.K.V., Rahuri. Borosilicate glassware's of BOROS1L brand were used for the experiments. For nutritional studies of different cultures, wide mouth jam bottles (6 cm diameter x 13 cm height) with autoclavable polypropylene caps were used. The test tubes of 25 mm x 150 mm size were also used.

The basal nutrient media used was MS medium, B5 and WPM. All the cultural operations including disinfection, surface sterilization of explants and subsequent sub-culturing were carried out in a laminar-air flow bench provided with an ultraviolet lamp and fluorescent tube. Double distilled water and surgical material were autoclaved at 1.06 kg/cm^2 pressure and 121°C for 20 minutes and kept in oven at 80-100 °C for 2-4 hours. Then these instruments were stored in dust proof room after cooling. The inoculated cultures were incubated at 25 ± 2°C in an air conditioned culture room. Photoperiod was maintained 16 hr (3000-3500 lux) supplied by cool white fluorescent tube lights daily followed by 8 hr of darkness as suggested by Conger (1981).

After taking the explants of optimum size from plant source, they were washed with detergent (Tween-20) solution and under running tap water 4-5 times followed by 3-4 washings with distilled water. Further operations were carried out in aseptic conditions. All the explants were surface sterilized with 0.1% $HgCl_2$ solution for 1-5 minutes and immediately rinsed with sterile distilled water 3-4 times to remove all the traces of $HgCl_2$. The explants were cut aseptically in laminar air flow cabinet to get explants of appropriate size and shape (2-3 cm long and 4-5 mm in thickness). The nodal segments and shoot tips were taken as explants with atleast one axillary bud.

The surface sterilized and aseptically cut explants were finally placed on media in laminar air flow cabinet. The bottles containing medium prepared as per various treatments, were unplugged by holding them over spirit lamp and inoculations were performed by placing explants on the surface of the medium with the help of flame sterilized long forceps and replacing the cap of the bottle. During inoculation the explants were properly positioned on the media and were gently pressed with forceps to secure their firm contact with the media.The culture bottles/test tubes after inoculation were kept in culture room at 25 ± 2°C temperature with photoperiod of 16 hr light and 8 hr dark in culture room. Sub-culturing of explants was done regularly at three weeks interval on the fresh medium as per the treatments.

In vitro grown plantlets were removed from culture bottles, washed with distilled water to remove agar medium sticking to roots. The plantlets were dipped into 0.5 % bavistin for 5 minutes and then planted in plastic pro-trays containing cocopeat for primary hardening. These platlets were grown under polyhouse condition at 70 % humidity and 25-27°C temperature for 30 days. The plantlets were then shifed to shade net house for secondary hardening. The plantlets were planted in polythene bags containing different potting mixture like soil, FYM and vermicompost alone and also with different combinations . They were maintained at 50 per cent light intensity by judicious application of water and soluble fertilizers during next 45 days. The hardened plantlets were then exposed to the direct sunlight to get acclimitized for the natural environmental conditions. The count was made for survival per cent of tissue culture plantlets.

RESULTS AND DISCUSSION

The significantly maximum *in vitro* culture survival was observed 85.50 per cent in shoot tips for the treatment where in the explants were exposed to the 0.1 % $HgCl_2$ for 3 minutes. Whereas the treatment T_4 (4 min) and T_5 (5 min) showed complete necrosis of shoot tip explants. The maximum *in vitro* culture survival (89.25 %) was recorded in nodal segment explants, where the explants were exposed to sterilant for 4 minutes. Similarly, complete necrosis of nodal segment explants was observed in the explants which were exposed to the 5 minutes for 0.1 % $HgCl_2$ treatment.(Table 1) Findings of the present studies are in line with those reported by Singh *et al.* (2013). They found surface sterilization of explants,

involving $HgCl_2$ (0.1 %) for 3 min as better sterilization treatment for cotyledonary nodal explants.

The results pertaining to the effect of different nutrient medium was found to be significant for the shoot tip and nodal segments cultures. The significantly maximum establishment for shoot tips (12.00 %) and nodal segment (66.67%) was recorded on MS medium which is superior over the rest of treatments viz. B5 and WPM. This media also recorded least number of days for establishment in shoot tips (7.33) and nodal segment (7.17) respectively. The maximum days for establishment for shoot tips (26.17) and nodal segment (23.17) was observed in B5 nutrient medium (Table 2). Similar results were also recorded by Zhang and Stoltz (1991), Singh *et al.* (2011) and Patil *et al*. (2011).

MS media supplemented with 2.0 mg/l BAP and 20 mg/l AS (M7) recorded significantly maximum establishment of shoot tips (10.67%) and nodal segments (66.67 %). This media also recorded least number of days for establishment in shoot tips (7.40) and nodal segment (7.30). On an average MS media supplemented with 2.0 mg/l BAP and 20 mg/l AS was found to be better for shoot tip establishment and also it required less number of days for establishment of cultures (Table 3). Similar results were also recorded by Singh *et al.* (2013) where they reported maximum percentage of establishment of cotyledonary nodal explants on Murashige and Skoog (MS) medium fortified with

Table 1. Effect of explants type and duration of treatment with $HgCl_2$ (0.1 %) on *in vitro* culture survival

Treat. No.	Chemical	Time (min)	*In vitro* culture survival (%)	
			Shoot tip	Nodal segment
T_1	0.1 % $HgCl_2$	1	52.50(46.43)	59.25(50.33)
T_2	0.1 % $HgCl_2$	2	76.00(60.67)	80.50(63.82)
T_3	0.1 % $HgCl_2$	3	85.50(67.63)	80.00(56.64)
T_4	0.1 % $HgCl_2$	4	0.00**	89.25(70.89)
T_5	0.1 % $HgCl_2$	5	0.00**(00)	0.00**(00)
S.E. ±	0.275	6.01		
C.D. at 5 %	0.829	18.13		

** Complete explants necrosis.
Figures in the parentheses indicates arcsine transformed values.
Medium used: MS + 2 mg BAP/l + 20 mg AS/l

Table 2. Effect of different media on *in vitro* culture establishment from shoot tips and nodal segment explants of pomegranate var. Bhagwa

Treat. No.	Nutrient Medium	Days for establishment		Establishment (%)	
		ST	NS	ST	NS
T_1	MS	7.33	7.17	12.00 (20.22)*	66.67 (54.74)*
T_2	WPM	20.17	19.67	4.33 (11.85)*	15.16 (23.60)*
T_3	B5	26.17	23.17	3.33 (10.27)*	8.67 (17.05)*
S.E. +	0.32	0.32	0.88	0.57	
C.D. at 5 %	0.95	0.95	2.65	1.73	

*Figures in the parentheses indicates arcsine transformed values.
MS: Murashige and Skoog (1962),
WPM: Woody plant medium (Lloyd and McCown, 1981)
B5 : Gamberg *et al.* (1968),ST: Shoot tips, NS: Nodal segment

1.0 mg/l 6-benzylaminopurine (BAP) + 0.5 mg/l naphthalene acetic acid (NAA).

The MS media supplemented with 2.5 mg/l BAP + 0.5 mg/l NAA + 2.5 g/AC (M8) recorded maximum number of shoots (11.30) per explants and more shooting percentage (62.53 %) whereas the media M9 (1.0 mg/l BAP + 0.5 mg/l IAA + 1.0 g/l AC) recorded significantly more length of shoots (5.10 cm) (Table 4).

Similar results were also recorded by Patil *et al.* (2011). Nodal explants when 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 - 15 shoots/ explants) and maximum number of leaves (15 - 20 leaves/ explants). Soukhak *et al*. (2011) also recorded 8.2 and 7.9 shoots per explant on MS liquid and agar medium supplemented with 13 ìM BA and 5.5 ìM NAA respectively. Kaji *et al.* (2013) reported highest number of nodes, shoot length and leaf number on MS media supplemented with 9.2 µM Kinetin. Singh *et al.* (2013) also observed

Table 3. Effect of MS media composition on *In vitro* culture establishment from shoot tips and nodal segment explants of pomegranate var. Bhagwa

Tr. No.	Details of medium	Establishment (%)		Days for establishment	
		ST	NS	ST	NS
M_1	MS + 1.0 mg/l BAP	2.00 (8.13)*	22.00 (29.95)*	18.30	16.53
M_2	MS + 1.5 mg/l BAP	2.00 (8.13)	22.00 (29.91)	17.30	17.00
M_3	MS + 2.0 mg/l BAP	4.00 (11.28)	26.00 (30.64)	16.87	14.90
M_4	MS + 2.5 mg/l BAP	4.00 (11.54)	30.00 (33.16)	16.23	16.57
M_5	MS + 1.0 mg/l BAP + 20 mg/l AS	4.67 (12.42)	30.00 (33.20)	14.60	11.17
M_6	MS + 1.5 mg/l BAP + 20 mg/l AS	4.67 (12.42)	45.33 (42.17)	14.33	9.60
M_7	MS + 2.0 mg/l BAP + 20 mg/l AS	10.67 (19.04)	66.67 (54.75)	7.40	7.30
M_8	MS + 2.5 mg/l BAP + 20 mg/l AS	8.00 (16.34)	63.33 (52.74)	8.50	8.67
M_9	MS + 1.0 mg/l BAP + 30 mg/l AS	3.33 (10.40)	30.00 (33.20)	13.83	12.00
M_{10}	MS + 1.5 mg/l BAP + 30 mg/l AS	3.33 (10.40)	27.33 (31.40)	14.57	11.30
M_{11}	MS + 2.0 mg/l BAP + 30 mg/l AS	8.00 (16.34)	60.00 (50.78)	8.73	10.23
M_{12}	MS + 2.5 mg/l BAP + 30 mg/l AS	6.00 (14.05)	56.00 (48.45)	8.80	10.70
M_{13}	MS + 1.0 mg/l BAP + 40 mg/l AS	6.67 (14.93)	48.67 (44.23)	10.57	11.00
M_{14}	MS + 1.5 mg/l BAP + 40 mg/l AS	6.67 (14.93)	47.33 (43.47)	12.97	11.27
M_{15}	MS + 2.0 mg/l BAP + 40 mg/l AS	7.33 (15.68)	42.67 (40.78)	14.37	12.43
M_{16}	MS + 2.5 mg/l BAP + 40 mg/l AS	8.00 (16.34)	53.33 (46.91)	13.57	11.86
S.E. +	0.80	1.99	0.51	0.26	
C.D. at 5 %		2.30	5.74	1.47	0.75

* Figures in the parentheses indicates arcsine transformed values.

when MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l kinetin + 200 mg/l activated charcoal exhibited maximum multiplication rate. Singh and Patel (2014) reported the maximum proliferation of shoot (78.25 %), number of shoots (3.75) per explants and shoot length (3.06 cm) on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l kinetin with 40 mg/l adenine sulphate.

The ½ MS media supplemented with 1.5 mg/l IBA + 2.0 g/l AC (M6) recorded the least number of days (26.77) to root initiation, maximum number of roots per shoot (4.50), more length of

Table 4. Effect of MS media composition on multiplication from nodal explants

Treat. No.	Details of medium	No. of shoots/ explant	Length of shoots (cm)	Shooting (%)
M_1	MS + 1.0 mg/l BAP + 1.0 g/l AC	1.63(1.46)*	3.37	30.60(33.58)**
M_2	MS + 1.5 mg/l BAP + 1.5 g/l AC	2.60(1.76)	3.37	28.67(32.37)
M_3	MS + 2.0 mg/l BAP + 2.0 g/l AC	4.50(2.23)	3.33	25.77(30.50)
M_4	MS + 2.5 mg/l BAP + 2.5 g/l AC	4.70(2.28)	3.06	23.83(29.22)
M_5	MS + 1.0 mg/l BAP + 0.5 mg/l NAA + 1.0 g/l AC	7.60(2.84)	3.93	50.87(45.39)
M_6	MS + 1.5 mg/l BAP + 0.5 mg/l NAA + 1.5 g/l AC	9.33(3.13)	4.13	57.97(49.58)
M_7	MS + 2.0 mg/l BAP + 0.5 mg/l NAA + 2.0 g/l AC	10.00(3.24)	4.23	60.20(50.88)
M_8	MS + 2.5 mg/l BAP + 0.5 mg/l NAA + 2.5 g/l AC	11.30(3.43)	3.73	62.53(52.25)
M_9	MS + 1.0 mg/l BAP + 0.5 mg/l IAA + 1.0 g/l AC	6.40(2.62)	5.10	42.20(40.51)
M_{10}	MS + 1.5 mg/l BAP + 0.5 mg/l IAA + 1.5 g/l AC	7.23(2.78)	4.90	50.17(45.09)
M_{11}	MS + 2.0 mg/l BAP + 0.5 mg/l IAA + 2.0 g/l AC	7.37(2.80)	4.47	51.33(45.76)
M_{12}	MS + 2.5 mg/l BAP + 0.5 mg/l IAA + 2.5 g/l AC	9.13(3.10)	4.00	54.17(47.39)
M_{13}	MS + 1.0 mg/l BAP + 0.5 mg/l Kin. + 1.0 g/l AC	6.70(2.68)	4.10	46.80(43.16)
M_{14}	MS + 1.5 mg/l BAP + 0.5 mg/l Kin. + 1.5 g/l AC	6.80(2.70)	3.90	44.77(41.99)
M_{15}	MS + 2.0 mg/l BAP + 0.5 mg/l Kin. + 2.0 g/l AC	6.93(2.71)	4.63	48.00(43.85)
M_{16}	MS + 2.5 mg/l BAP + 0.5 mg/l Kin. + 2.5 g/l AC	7.47(2.82)	3.83	49.10(44.48)
S.E. +	0.18	0.15	0.23	
C.D. at 5 %		0.51	0.42	0.66

* Figures in the parentheses indicates square root transformed values.
** Figures in the parentheses indicates arcsine transformed values.

Table 5. Effect of MS media composition on the rooting response of tissue culture plants

Tr. No.	Details of medium	Days to root initiation	Number of root/ shoot	Length of roots (cm)	Rooting (%)
M_1	½ MS + 0.5 mg/l NAA + 2.0 g/l AC	35.83	2.13(1.62)*	1.47	22.10(28.04)**
M_2	½ MS + 1.0 mg/l NAA + 2.0 g/l AC	38.30	2.17(1.63)	1.57	24.47(29.64)
M_3	½ MS + 1.5 mg/l NAA + 2.0 g/l AC	36.80	2.23(1.65)	1.73	28.83(32.47)
M_4	½ MS + 0.5 mg/l IBA + 2.0 g/l AC	30.10	3.33(1.95)	3.07	35.30(3645)
M_5	½ MS + 1.0 mg/l IBA + 2.0 g/l AC	29.50	3.50(2.00)	3.03	38.53(38.37)
M_6	½ MS + 1.5 mg/l IBA + 2.0 g/l AC	26.77	4.50(2.23)	3.53	40.63(39.60)
M_7	½ MS + 0.5 mg/l NAA + 0.5 mg/l IBA + 2.0 g/l AC	38.93	3.10(1.89)	2.67	32.37(34.67)
M_8	½ MS + 1.0 mg/l NAA + 0.5 mg/l IBA + 2.0 g/l AC	36.90	3.17(1.91)	3.00	32.90(35.00)
M_9	½ MS + 1.5 mg/l NAA + 0.5 mg/l IBA + 2.0 g/l AC	34.97	4.00(2.12)	3.07	33.50(35.36)
M_{10}	½ MS + 1.0 mg/l IBA + 0.5 mg/l NAA + 2.0 g/l AC	28.07	3.00(1.87)	2.57	33.47(35.34)
M_{11}	½ MS + 1.5 mg/l IBA + 0.5 mg/l NAA + 2.0 g/l AC	29.03	4.17(2.16)	2.60	35.07(36.31)
S.E. +	0.26	3.56	0.14	0.15	
C.D. at 5 %		0.75	0.10	0.40	0.44

* Figures in the parentheses indicates square root transformed values.
** Figures in the parentheses indicates arcsine transformed values.

Table 6. Effect of potting mixture on hardening and survival

Treatment	Potting mixture	Survival of plantlet (%)
T_1	Soil	10.00
T_2	FYM	37.50
T_3	Vermicompost	48.20
T_4	Soil + FYM (1:1 v/v)	65.25
T_5	Soil + Vermicompost (1:1 v/v)	69.50
T_6	Soil + FYM + Vermicompost (1:1:1 v/v)	80.75
	S.E. ±	0.62
	CD at 5 %	1.85

Table 7. Regeneration protocol standardized for pomegranate cultivar Bhagwa

S. No.	Particulars	Best Treatment
1.	Explant	Nodal segement
2.	Surface sterilization of explants	0.1 % $HgCl_2$ for 4 min
3.	Media	Full strength MS
4.	Establishment media	2.0 mg/l BAP + 20 mg/AS
5.	Multiplication media	2.5 mg/l + BAP + 0.5 mg/l NAA + 2.5 g/l AC
6.	Rooting media	½ MS 1.5 mg/l IBA + 2.0 g/l AC
7.	Potting mixture for hardening	Soil : Vermicompost (1:1 v/v)

roots (3.53 cm), maximum rooting per cent (40.63) and maximum survival of tissue culture plants (82.03) (Table5).Similar results were also reported by Naik *et al.* (1999) , Patil *et al.* (2011) and Soukhak *et al.* (2011). The results are also confirmative with the results reported by Singh *et al.* (2013) where the 1/2 MS medium supplemented with 0.5 mg/l NAA + 200 mg/l activated charcoal, recorded the maximum number of roots/shoot (4.17) and root length (3.87 cm).

The significantly maximum survival (80.75 %) of plantets was recorded in potting mixture containing Soil + FYM + Vermicompost followed by Soil + Vermicompost (69.50 %) and Soil + FYM (65.25 %).The least survival of plantlets (10 %) was recorded in potting mixture containing only soil (Table 6). Bonyanpour and Morteza (2013) also reported similar results. They also reported when plantlets were cultured in a soil mixture containing vermiculite (60 %), perlite (30 %) and cocopeat (10 % v/v), 80 per cent of plants survived and transferred to the green house after two months of period.

In the present investigations results are obtained to develop an *in vitro* plant regeneration protocol for pomegranate as depicted in the following table,

Thus, the protocol suggested above for direct regeneration from nodal segment explant in *Punica granatum* L. cv. Bhagwa, is the ideal method for maintaining and propagating pomegranate cultivar Bhagwa. However, further refinement is required before this culture method can be applied to different commercial cultivars.

CONCLUSIONS

As regard to explants, nodal segment was found to be most effective as compared to shoot tips for *in vitro* regeneration in pomegranate. Surface sterilization treatment with 0.1 % $HgCl_2$ for shoot tips with 3 minutes duration and 4 minutes duration for nodal segment was effective. Among the different basal media viz. MS, B5, and WPM, MS media was found most effective for establishment of pomegranate explants. MS media supplemented with 2.0 mg/l BAP + 20 mg/l AS was most effective for explant establishment of pomegranate cultivar Bhagwa. MS media supplemented with 2.5 mg/l BAP + 0.5 mg/l NAA + 2.5 g/l. AC was found best for multiplication of pomegranate cultivar Bhagwa. MS media supplemented with 1.5 mg/l IBA + 2.0 gm/l. AC was found best for rooting in pomegranate.

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