Indian J. Hort. 68(3), September 2011: 307-311

In vitro culture establishment studies on pomegranate

Nripendra V. Singh*, S.K. Singh and V.B. Patel
Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi 110 012

ABSTRACT

***In vitro* studies were undertaken for culture establishment in pomegranate cv. G-137 using shoot tips and nodal segment collected from mature plants. Sealing of the cut ends of explants with pre-sterilized paraffin was found to minimize browning by preventing phenol exudation and giving high culture establishment (83.95%) as compared to control (17.55%). Fungicidal pre-treatment of explants with Bavistin® 0.1% (carbendazim) + Ridomil® 0.1% (Syngenta) for 1 h gave the highest culture establishment (80.60%) with nodal segment and lowest microbial contamination (40.60%), with shoot tip as explant. Interaction between explant, sterilant and duration of treatment revealed that highest culture establishment (81.00%) was observed with 3 min. surface sterilization with 0.1% $HgCl_2$. The maximum culture establishment was obtained with treatment 2.0 mg/l BAP + 0.1 mg/l NAA + 0.5 mg/l GA_3 which also gave the earliest bud sprouting (5.67 days) as compared to control.**

Key Words: Pomegranate, *In vitro* culture establishment, medium browning.

INTRODUCTION

Pomegranate is widely cultivated throughout India. It is mostly propagated by hardwood cuttings, though it is an inadequate approach for early spread of new genotypes. *In vitro* clonal propagation of pomegranate is advantageous and can be utilized as an alternative to fulfill the demand of good quality disease-free planting material in large number. In pomegranate, being a woody plant, *in vitro* culture establishment is greatly hindered due to browning of culture medium, surface contaminants and low frequency of shoot regeneration. Medium browning results due to exudation of phenolic compounds released from the cut end of the explants. The oxidized product of phenols, quinines are known to be highly reactive and inhibit enzyme activity leading to the death of explants (Hu and Wang, 5). The strategies commonly employed to combat the harmful effects of browning include the use of phenol adsorbing agents such as activated charcoal or polyvinylopyrrolidone, addition of antioxidants in the medium or agitation of explant in antioxidant solution, frequent transfer of explant to fresh medium (Llyod and McCown, 8), etc. Successful disinfection of explants is a pre-requisite for *in vitro* culture establishment and often involves different treatments, which vary with the type of explant and species. Culture establishment and days to shoot bud induction is significantly influenced by the crop species, explant type and also by growth regulators. Keeping the above problems in view in the present investigation some techniques were employed to overcome the harmful effect of media browning and *in vitro* shoot regeneration.

*corresponding author's present address : NRC on Pomegranate, Solapur; E-mail: nripendras72@gmail.com

MATERIALS AND METHODS

Studies were carried out at the Division of Fruits and Horticultural Technology, IARI, New Delhi. Two types of explant, *i.e.*, shoot tip and nodal segment were collected from healthy 10-year-old mature plant of cultivar G-137 growing in the Experimental Orchard. Explants were washed under running water for thirty minutes. Thereafter, three different methods were employed to minimize the problem of phenol exudation during culture establishment. This included agitation of explants in a solution of ascorbic acid (0.2%) and citric acid (0.4%) for 30 min., addition of activated charcoal (100 mg/l) in the culture medium and sealing of cut ends of explants with pre-sterilized paraffin was. Phenol exudation was estimated in the medium after one week of culture and percent establishment of explants was recorded. The alcoholic extraction of total phenol exudated in the culture medium was estimated by the procedure suggested by Singleton and Rossi Jr. (13).

In another set of experiment, the explants were pre-treated with different fungicides for different duration by agitation. The different solutions used were distilled water (control), 0.1% Bavistin (carbendazim) (1 h), 0.2% Bavistin (2 h), 0.1% Bavistin + 0.1% Ridomil (1 h) on a horizontal shaker at 120 rpm. In a third experiment, after pre-treatment with 0.1% Bavistin + 0.1% Ridomil for 1 h explant was surface-sterilized with ethanol (70%, v/v) for one min. followed by sterilization with different sterilants (0.1% $HgCl_2$ and 1.0% NaOCl) for different durations of exposure, *i.e.*, 1, 2, 3, 4, 5 min. Explants were then thoroughly rinsed with sterilized double-distilled water four to five times and air-dried on tissue paper cushion. After drying, the basal cut ends of explants were sealed with pre-sterilized molten paraffin wax using quick dip method were then cultured in test

tubes containing establishment medium. The sterilized explants were transferred on MS medium (Murashige and Skoog, 10) containing different combinations and concentrations of growth regulators to study their effect on culture establishment and shoot regeneration. The culture tubes were maintained in culture room at 25 ± 1°C temperature under a 16/8 h photoperiod of light and dark cycles. All the experiments were laid out in completely randomized design with seven replications comprising of 25 culture tubes for each treatments. The data were subjected to analysis of variance.

RESUTS AND DISCUSSION

The phenol exudation was clearly visible as media browning in case of unsealed explants. High phenol was estimated in control, which was markedly reduced due to sealing of cut ends with wax (Table 1). Sealing the cut end of the explants effectively arrested medium browning and significantly higher culture establishment (83.95%) was observed compared to the explants agitated in antioxidant solution of ascorbic acid + citric acid (70.90%) and control (17.55%). Similar results were reported by Bhat and Chandel (1), and Mishra *et al.* (9), while working on *Dioscorea alata* and *Emblica officinalis*, respectively. Among the explants, nodal segment proved better than shoot tip as far as culture establishment was concerned. However, phenol exudation was higher in case of nodal segment since it was more mature and woody compared to shoot tip. The effect of sealing the cut edge with sterilized wax is shown in Fig. 1. Surface contaminants could be effectively eliminated by pre-treatment with fungicidal and bactericidal compounds. Among the different fungicidal treatments tried explant agitation in Bavistin 0.1% + Ridomil 0.1% for 1 h gave the highest culture establishment (80.60%) and lowest contamination (40.60%). Similar results were also obtained by Joshi *et al.* (6) who also suggested the efficacy of fungicides in

Table 1. Effect of different treatments on *in vitro* phenol exudation and culture establishment.

Treatment	Phenol exudation (µg/ml medium)			Culture establishment (%)		
	Shoot tip	Nodal segment	Mean	Shoot tip	Nodal segment	Mean
Control	22.87	28.74	25.80	19.70 (26.35)*	15.40 (23.11)*	17.55 (24.73)*
Ascorbic acid (0.2%) + citric acid (0.4%)	14.41	16.37	15.39	70.50 (57.10)	71.33 (57.61)	70.90 (57.35)
Activated charcoal (100 mg/l)	11.89	16.34	14.11	72.30 (58.24)	72.5 (58.37)	69.40 (56.42)
Wax sealing of cut edge	4.54	6.69	5.61	80.10 (63.51)	87.8 (69.56)	83.95 (66.34)
Mean	13.16	17.19		61.78 (51.18)	65.08 (53.73)	
CD at 5%						
Treatment (T)	= 3.67				= 7.84	
Explant type (E)	= 2.14				= 3.61	
T × E	= 5.45				= 9.86	

*Values in parenthesis are transformed values

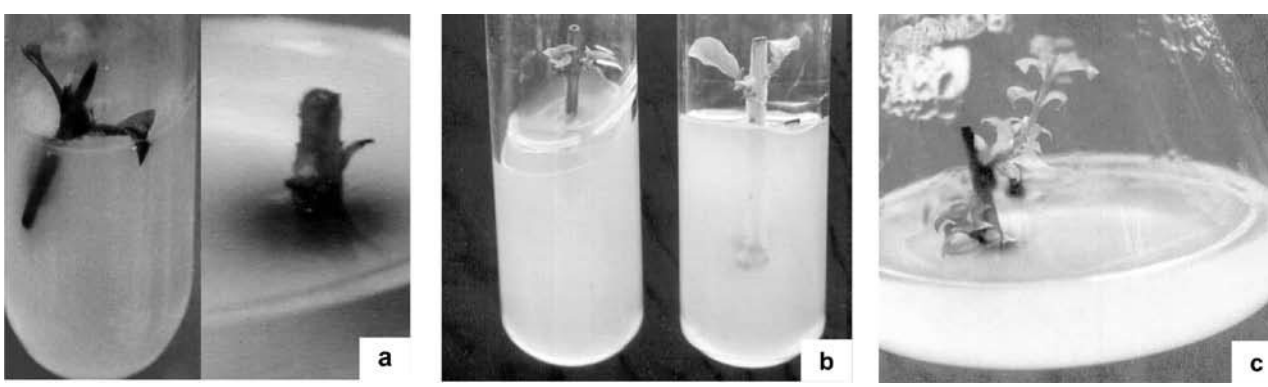

Fig. 1. *In vitro* culture initiation in pomegranate. (a) Explant without wax sealing (control ; (b) Wax sealed explant, and (c) shoot regeneration on established culture.

controlling microbial infection in culture establishment. However, contamination in shoot tip was significantly lower as compared to nodal segment with the same pre-treatment. All the pre-treatments gave significantly better response compared to control, where no culture establishment coupled with the highest contamination was noted (Table 2).

The treatment of explants with different sterilants for different duration (Tables 3 & 4) revealed that 4 min. duration of treatment gave the lowest contamination (12.32%). The highest culture establishment irrespective of the explant type and sterilant was recorded with 3 min. duration (77.25%), which was highly significant over the control. It was found the explants were killed when treatment duration was increased beyond 4 min. for both 0.1% $HgCl_2$ and 1.0% NaOCl. Similar results were earlier reported by Habib *et al.* (4) in mulberry. Irrespective of treatment duration and explant type, $HgCl_2$ gave significantly lower microbial contamination along with the highest culture establishment. Shoot tip gave better results, which was significantly better over nodal segment. Out of two explants nodal segment recorded slightly higher culture establishment.

Interaction between duration of treatment and explant type revealed that the treatment with 0.1% $HgCl_2$ for 3 min. recorded the highest culture establishment (78.17%). Interaction between duration and sterilant as shown in Table 3, suggested that 0.1% $HgCl_2$ treatment

Table 2. Effect of different pretreatments on *in vitro* culture establishment in pomegranate.

Treatment	Explant	Culture establishment (%)	Contamination (%)
Control (distilled water) for 1 h	ST	0.00 (0.00)*	100.00 (90.00)
	NS	2.17 (8.53)	94.00 (75.82)
Bavistin 0.1% for 1 h	ST	40.33 (39.54)	95.80 (78.17)
	NS	61.87 (51.83)	91.40 (72.95)
Bavistin 0.1% for 2 h	ST	60.07 (50.83)	89.60 (71.19)
	NS	67.50 (55.24)	80.20 (63.58)
Bavistin 0.1% + Ridomil 0.1% for 1 h	ST	75.23 (6.13)	40.60 (39.58)
	NS	80.60 (63.87)	62.50 (52.24)
CD at 5%		6.09	6.76

*Values in parenthesis are transformed values, ST = Shoot tip, NS = Nodal segment.

Table 3. Effect of explant type, surface sterilant and duration of treatment on *in vitro* culture contamination.

Treatment (min.)	Contamination (%)								
	Shoot tip			Nodal segment			D × S		
	$HgCl_2$	NaOCl	Mean	$HgCl_2$	NaOCl	Mean	Pooled mean	$HgCl_2$	NaOCl
1	47.66 (43.62)	53.33 (46.69)	50.05 (45.29)	55.00 (47.87)	59.33 (50.36)	57.16 (49.08)	53.61 (47.12)	51.33 (45.75)	56.33 (48.62)
2	13.33 (21.39)	19.00 (25.84)	16.16 (23.66)	31.00 (33.83)	33.00 (35.06)	23.00 (34.45)	19.58 (26.21)	22.1 (28.04)	26.00 (30.66)
3	10.33 (18.72)	11.33 (19.64)	10.83 (19.19)	14.00 (21.97)	16.00 (23.58)	15.00 (22.79)	15.92 (21.05)	12.17 (20.36)	13.67 (21.64)
4	11.00 (19.37)	12.00 (20.27)	11.05 (19.82)	12.00 (20.27)	12.66 (20.79)	12.32 (20.53)	11.69 (19.19)	11.50 (19.82)	12.33 (20.53)
5	0.00** (0.00)	0.00** (0.00)	0.00** (0.00)	0.00** (0.00)	0.00** (0.00)	0.00** (0.00)	0.00** (0.00)	0.00** (0.00)	0.00** (0.00)
	16.47 (23.89)	19.13 (25.92)	17.75 (24.88)	22.40 (28.29)	24.19 (29.40)	23.29 (28.79)	20.52 (26.92)	19.43 (26.13)	21.67 (27.69)
CD at 5%					Duration of treatment (D)				= 2.12
Explant (E)			= 1.34		D × E				= 3.00
Sterilent (S)			= 1.34		D × S				= 3.00
E × S			= 1.90		E × S × D				= 4.24

*Values in parenthesis are transformed values

**Complete explant necrosis

Table 4. Effect of explant type, surface sterilant and duration of treatment on *in vitro* culture establishment.

Treatment (min.)	Culture establishment (%)								
	Shoot tip			Nodal segment			D × S		
	$HgCl_2$	NaOCl	Mean	$HgCl_2$	NaOCl	Mean	Pooled mean	$HgCl_2$	NaOCl
1	39.66	35.66	37.66	28.00	23.66	37.33	37.49	38.33	36.17
	(39.00)	(36.63)	(37.82)	(31.95)	(37.23)	(37.64)	(37.76)	(38.53)	(36.93)
2	74.00	73.00	73.50	69.66	66.00	67.83	70.67	71.83	69.50
	(59.34)	(58.69)	(59.02)	(56.54)	(54.33)	(55.43)	(57.17)	(57.92)	(56.48)
3	80.00	77.00	78.50	76.33	75.66	76.00	77.25	78.17	76.33
	(63.44)	(61.34)	(62.37)	(60.87)	(60.40)	(60.67)	(61.48)	(62.10)	(60.87)
4	27.66	29.66	28.36	43.22	51.33	47.16	37.76	35.33	40.50
	(31.69)	(32.96)	(32.14)	(58.82)	(51.33)	(43.34)	(37.88)	(36.45)	(39.52)
5	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)
	44.26	43.06	43.66	45.40	45.93	45.66	44.66	44.33	44.50
	(41.67)	(40.98)	(41.32)	(42.36)	(42.65)	(45.66)	(41.96)	(42.02)	(41.84)

CD at 5%		Duration of treatment (D)	= 3.21
Explant (E) =	= 2.03	D × E	= 4.53
Sterilent (S) =	= 2.03	D × S	= 4.53
E × S	= 2.89	E × S × D	= 6.41

*Values in parenthesis are transformed values

**Complete explant necrosis

Table 5. Effect of growth regulators on *in vitro* culture establishment and shoot regeneration in pomegranate.

Treatment (mg/l)	Culture establishment (%)			Days for shoot bud induction		
	Shoot tip	Nodal segment	Mean	Shoot tip	Nodal segment	Mean
MS (control)	12.33 (20.53)*	14.00 (21.97)*	13.17 (21.22)*	17.67	16.67	17.17
MS + 1.0 BAP	39.00 (38.65)	43.33 (41.15)	41.17 (39.87)	13.00	12.33	12.67
MS + 2.0 BAP	42.67 (40.74)	46.33 (42.88)	44.50 (41.84)	11.67	11.33	11.50
MS + 1.0 BAP + 0.1 NAA	66.33 (54.51)	69.33 (56.35)	67.83 (55.43)	8.33	7.33	7.83
MS + 1.0 BAP + 0.5 NAA	68.33 (55.73)	70.00 (56.79)	69.17 (56.23)	8.67	7.67	8.17
MS + 2.0 BAP + 0.1 NAA	72.33 (58.24)	74.00 (59.34)	73.17 (58.76)	6.67	6.33	6.50
MS + 2.0 BAP + 0.5 NAA	70.67 (57.17)	73.33 (58.89)	72.00 (58.05)	7.30	7.00	7.17
MS + 1.0 BAP + 0.1 NAA + 0.5 GA_3	77.33 (61.55)	78.00 (62.03)	77.67 (61.75)	6.33	6.00	6.17
MS + 2.0 BAP + 0.1 NAA + 0.5 GA_3	80.00 (63.44)	82.00 (64.90)	81.00 (64.16)	5.70	5.67	5.67
Mean	58.78 (50.01)	61.48 (51.59)		9.41	9.00	
CD at 5 %						
Explant type (E)		= 2.12			= 0.38	
Treatment (T)		= 4.51			= 0.80	
E × T		= 6.37			= 1.14	

*Values in parenthesis are transformed values

for 4 min. gave the lowest contamination (11.50%). The three way interaction between explant, sterilant and duration of treatment revealed the minimum contamination for shoot tip treated with the 0.1% $HgCl_2$, interaction between explant, sterilant and duration of treatment reveled that highest culture establishment (80.00%) was observed with 0.1% $HgCl_2$ for 3 min. Culture establishment and days to shoot bud induction were significantly influenced by crop species, explant type and also by growth regulators. Surface sterilization with 0.1% $HgCl_2$ was also found very effective in reducing the contamination *in vitro* by Ranjan *et al*. (12). It was evident from data that culture establishment was higher in nodal segment compared to shoot tip. Maximum culture establishment (81.00%) was obtained in treatment 2.0 mg/l BAP + 0.1 mg/l NAA + 0.5 mg/l GA_3, which was significant as compared to control and other treatment combinations irrespective of explant type, similar results were obtained by Chaugule *et al*. (2), where 78.94% of cotyledon explants of pomegranate formed callus on MS medium containing 0.4 mg/l NAA and 0.1 mg/l BAP. The maximum culture establishment (82.00%) and earliest bud sprouting (5.67 days) from nodal segment were obtained with the treatment 2.0 mg/l BAP + 0.1 mg/l NAA + 0.5 mg/l GA_3 and was significantly better as compared to control (hormone-free MS medium). Recently, Kanwar *et al*. (7) cultured cotyledonary explants, excised from *in vitro* germinated seedlings. They found solid MS medium supplemented with 21 µM NAA and 9 µM BA gave over 80% callusing and shoot organogenesis was obtained on MS medium supplemented with 8 µM BA, 6 µM NAA, and 6 µM gibberellic acid (GA_3). Culture establishment and days to shoot bud induction in both the explant types, *i.e.*, nodal segment and shoot tip were improved by addition of 0.5 mg/l GA_3 in MS medium supplemented with 2.0 mg/l BAP and 0.1 mg/l NAA (Table 5). Similar results were reported using shoot using shoot tip by Drazete (3) and nodal segment by Naik *et al.* (11).

REFERENCES

1. Bhat, S.R. and Chandel, K.P.S. 1991. A novel technique to overcome browning in tissue culture. *Plant Cell Rep.* **10**: 358-61.

2. Chaugule, R.R., More, T.A., Patil, R.S. and Kamble, A.B. 2005. Callus culture for rapid regeneration of pomegranate. *J. Maharashtra Agric. Univ*. **30**: 85-86.

3. Drazeta, L. 1997. Pomegrante (*Punica granatum* L.) propagation by *in vitro* method of tissue culture. Rev. Res. *Work Faculty of Agric. Belgrade*, **42**: 49-59.

4. Habib, A., Ali, M.R., Amin, M.N. and Rahman, M.M. 2003. Clonal propagation of white mulberry (*Morus alba* L.) using *in vitro* technique. *J. Biol. Sci.* **3**: 1181-87.

5. Hu, C.Y. and Wang, P.J. 1983. **In**: Evans, D., Sharp, D.R., Ammirato, P.V. and Yamada. Y. (Eds.). *Handbook of Plant Cell Culture*, Vol. 1, MacMillan, New York, pp. 177-217.

6. Joshi, M.K., Singh, V.S. and Garg, G.K. 1988. Use of *Brassica* callus culture to induce sporulation in *Alternaria brassicae* (Berk.) Sacc. *Plant Cell Tissue Organ Cult.* **14**: 59-62.

7. Kanwar, K., Joseph, J. and Deepika, R. 2010. Comparison of *in vitro* regeneration pathways in *Punica granatum* L. *Plant Cell Tissue Organ Cult.* **100**: 199-207.

8. Llyod, O.B. and McCown, B.H. 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot tip culture. *Comb. Proc. Int. Plant Prop. Soc*. **30**: 421-27.

9. Mishra, M., Saxena, R.P., Pathak, R.K. and Srivastava, A.K. 1999. Studies on micropropagation of *aonla* (*Emblica officinalis* Gaertn.). *Progr. Hort.* **31**: 116-22.

10. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plant.* **15**: 473-97.

11. Naik, S.K., Pattnaik, S. and Chand, P.K. 2000. High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (*Punica granatum* L.). *Scientia Hort.* **85**: 261-70.

12. Ranjan, J.K., Singh, S.K., Chakrabarti, A.K. and Pragya. 2009. *In vitro* shoot regeneration from cotyledonary leaf explant in chilli and bio-hardening of plantlets. *Indian J. Hort*. **67**: 43-49.

13. Singleton, V.L. and Rossi Jr., J.A. 1965. Colorimetry of total phenol with phosphomolybdic-phospho-tungstic acid reagents. *American J. Enol. Vitic.* **16**: 144-58.

Received: May, 2008; Revised: February, 2011; Accepted : July, 2011