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Factors affecting *in vitro* degree of browning and culture establishment of pomegranate

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The present study was conducted to identify the most suitable types of nodal explants and browning control treatment for *in vitro* regeneration of pomegranate. Murashige and Skoog (MS) medium containing 1.0 mg/L BAP + 0.5 mg/L NAA was used commonly for all the treatments tested. Result revealed that the intensity of browning was increased with increased position and the length of explants. Minimum browning intensity was observed in 1st nodal explants having 1.5 cm length. However, explants of 3rd node with 2.5 cm length registered higher establishment (68.5%) and growth of explants. Furthermore, the most effective browning control was observed in subculturing of nodal explants twice, at the first day and third day of inoculation, which also found better in establishment of explants followed by activated charcoal 200 mg/L into the medium. Maximum length of shoots (3.9 cm) was recorded in 1st position of node with 2.5 cm length of explants.

Key words: Nodal segments, position, antioxidants, browning, establishment.

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family Punicaceae. Pomegranate is widely grown in many tropical and subtropical countries, especially in moderate climate of meditation region (Salaheddin and Kader, 1984). Generally, cultivation of pomegranate is done by using vegetative propagated (hardwood cutting and air layering) plantlet for the field planting. However, the conventional propagation methods of pomegranate are not found suitable to provide large-scale of planting material at a time, as it is rather slow for multiplication of plants. Consequently, the availability of planting materials is restricted throughout the year. Tissue cultured plants are more advantageous than those by conventional propagation (Moore et al., 1991). Moreover, *in vitro* techniques are one of the reliable sources used for commercial plantlet production of pomegranate. *In vitro* propagation of woody plants is recalcitrant for growth because of browning problem at initial establishing stage of *in vitro* culture (Zaid, 1984; Pirttila et al., 2008; Krishna et al., 2008), due to leaching of phenolic substances and secondary metabolites from cut surface which hamper further morphogenesis response and rooting of explants (Aliyu, 2005). Explants and medium browning is a major problem in pomegranate due to the exudation of high

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amount of phenols, especially in mature explants (Naik and Chand, 2010).

Phenols are chemical compounds that embraces a wide range of plant substances which posses in common, an aromatic ring bearing one or more hydroxyl constituents (Onuoha et al., 2011). Various attempts has been made to multiply pomegranate by using tissue culture techniques through shoot tip and nodal segment explants of mature plant (Kantharajah et al., 1998; Singh and Khawale, 2006; Kanwar et al., 2009; Samir et al., 2010). However, the problem of browning and death of culture during *in vitro* propagation of pomegranate has been reported earlier by Sharon and Sinha (2000) and also Murkute et al. (2004).

In perennial fruit crops, establishment of explants requires special procedures to escape the problem that associated with exudation of polyphenol compounds from cut surface. Different attempts has been made to eliminate browning problem in woody plant species like pre-socking of explants in antioxidants solution, incorporation of oxidants into medium, incubation of culture in to dark period and frequent subculturing of explants (Ahmad et al., 2013). Exudation of phenols can also be reduced by sealing the cut ends of explants with liquid paraffin wax (Bhatt and Chandel, 1991; Singh et al., 2011). However, the effectiveness of these methods varies from species to species and physiological conditions of plant. Corduk and Aki (2011) reported that the addition of 1.0 g/L morpholine ethane sulfonic acid (MES) into MS medium significantly reduced browning in *Sideritis trojana*. Use of antioxidants and absorbents in browning control have been demonstrated by several workers in mango (Chandra et al., 2003), in pomegranate (Chaugule et al., 2007) and in pear (Poudyal et al., 2008). They have also noticed that keeping the culture continuously into dark period for 96 h reduced phenol extraction in pear. Pre-socking of apical and axillary buds in 0.5% polyvinylpyrrolidone (PVP) + 3% sucrose for 30 min was found effective for browning control in mango (Chavan et al., 2000). Production of phenolic compounds indirectly stimulated by various factors such as physiological condition, size and age of explants (Dineshbabu et al., 2002; Tian, 2008; Ahmad et al., 2013).

Therefore, the present investigation was carried out to study the effect of antioxidants, position and size of nodal segment explants on degree of browning and culture establishment of pomegranate cv. Ganesh.

MATERIALS AND METHODS

Explant preparation and surface sterilization

Two weeks old shoots having at least five nodes each were collected from 4 to 5 year old mature plant of pomegranate cv. Ganesh from Horticulture Experimental Farm, Navsari Agricultural University, Navsari, Gujarat. Shoots were washed thoroughly under running tap water for 30 min and leaves were removed leaving the petiole. Sterilization of explants were carried out by keeping in a solution of 0.2% Bavistin (Carbendazim 50% WP) and 0.05% Streptomycin for an hour. Shoots were treated with 10% solution of Teepol for 10 min. All traces of Teepol were removed by repeated washing in double glass distilled water. Pre-sterilized shoots having at least 5 nodes each at different positions (5 levels) viz. 1st, 2nd, 3rd, 4th and 5th from the apex to the base, cut and separated into different size (5 levels) viz. 1.5, 2.0, 2.5, 3.0 and 3.5 cm of each position. Further sterilization procedure was carried out in the laminar air flow hood, using 0.1% mercury chloride ($HgCl_2$) for 5 min. The explants were then rinsed at least thrice with autoclaved double distilled water.

Culture media and culture condition

MS (Murashige and Skoog, 1962) was used as basal medium for the experiment. Analytical grade chemicals, obtained from Hi Media Laboratories (India) were used for media preparation. Screw caps glass bottles (250 ml) were used as culture vessels. The medium was supplemented with 3.0% sucrose and solidified with 0.8% (w/v) agar. The pH of medium was adjusted to 5.8 prior to addition of agar and then medium was autoclaved at 121°C on 15 lb/in^2 for 20 min. Cultures were incubated in a culture room at a temperature of 26 ± 2°C with relative humidity at 55 ± 5% in the 16/8 h light/dark photoperiod at 3000 lux.

Effect of explants position and size

Sterilized nodal segments were inoculated into MS medium fortified with 6-benzylaminopurin (BAP) 1.0 mg/L + Naphthalene acetic acid (NAA) 0.5 mg/L. Total 25 treatment combinations (size of nodal segments 5 levels with each position of node 5 levels) were tested. 2 to 3 explants were inoculated in each 250 ml glass bottles having 40 ml medium. Treatments were replicated three times with 100 explants in each replication. Observations were recorded after one week of culture. Subculturing of explants was conducted at two week intervals.

Effect of antioxidants and subculturing of explants

Effect of antioxidants on browning intensity and frequent subculturing of explants was tested using 2.5 cm nodal segment explants. Different antioxidants viz. activated charcoal (3 levels) 100, 200 and 300 mg/L, polyvinylpyrrolidone (PVP), 3 levels viz. 5, 10 and 15 mg/L, ascorbic acid (3 levels) 50, 100 and 150 mg/L and citric acid (3 levels) 20, 40 and 60 mg/L were added into MS medium with 1.0 mg/L BAP + 0.5 mg/L NAA. Subculturing of explants was conducted at first days after inoculation (DAI), second DAI, first and third DAI.

Statistical analysis

Experiments were carried out using a factorial completely randomized design (CRD). Treatments were repeated at least three times, each treatment consisted of 4 explants and the mean separation was conducted according to least significant differences (LSD) at 5% level. The surface browning of tissue was evaluated visually at every transfer using scores ranging from 1 to 5 (0: no browning, +: very low browning, ++: low browning, +++: moderate browning, ++++: high browning and +++++: intense browning.

Table 1. Effect of position and size of nodal segments explants on browning intensity in pomegranate cv. Ganesh.

Position of node (N)	Size of node (L)				
	1.5 cm	2.0 cm	2.5 cm	3.0 cm	3.5 cm
1st	+	++	+++	+++	+++
2nd	++	+++	+++	++++	++++
3rd	+++	+++	+++	++++	++++
4th	+++	++++	++++	+++++	+++++
5th	+++++	+++++	+++++	+++++	+++++

+++++ = Intense browning, ++++ = High browning, +++ = Moderate browning, ++ = Low browning, + = Very low Brown.

RESULTS AND DISCUSSION

Browning intensity

Minimum browning intensity was observed in the 1st position of nodal explants having 1.5 cm length. The intensity of browning was observed very high in 4th and 5th node. It was greatly increased with increasing the position and size of nodal segments (Table 1). Moreover, the browning intensity in 1st position of node was low to moderate in all the size of nodes. Browning of explants was noticed first at the cut end then gradually diffused into the medium. Exudation of phenols in 4th and 5th position of node was started within a minute of explants inoculated into medium (Figure 1A). It was noticed that the 1st and 2nd nodes having 1.5 to 2.0 cm length showed very low to moderate browning in the medium. However, the establishment percent was also recorded less. Small size explants exudates less phenols (Kaushal et al., 2005) as they are soft and succulent in nature and succumbs easily due to toxic effect of sterilents (Pati et al., 2008). On the other side, 3rd, 4th and 5th position of node showed moderate to intense browning in all (1.5 to 3.5 cm) length of nodes. This might be due to the synthesis of polyphenols more in older age node as compared to new aged node. Older explants exhibited more browning than younger ones (George and Sherrington, 1984). Gitonga et al. (2010) reported low browning intensity in 1st, 2nd and 3rd nodes of macadamia nut shoot. The intensity of browning was correlated with size and position of node. Ozyigit (2008) observed positive relationship between age of explant and phenolic exudation in tissue culture of cotton.

Culture establishment

Maximum establishment (68.5%) was recorded in 3rd nodal explants having 2.5 cm length followed by 1st node with 2.5 cm length (Table 2). Maximum length of shoot (3.9 cm) was induced in 1st position of node followed by in 2nd and 3rd nodes with 2.5 cm length of explants (Table 3). However, minimum establishment was observed in 5th node having 3.5 cm length. Moreover, the establishment of 3rd nodal explants significantly increased due to increase in the length of node up to 2.5 cm. Maldonado et al. (2000) also reported the better culture establishment in 3rd and 4th nodal position of *Annona muricata* L. Pati et al. (2008) observed that the upper node (1st to 5th) did not survive in culture medium, whereas, 11th to 15th nodal segment were found better for establishment in Bael cv. CISHB1. Similarly, Douglas (1984) found that the 4th to 7th internodal explants of papulus species was better for *in vitro* shoot regeneration. Moreover, the increasing trend in establishment of nodal explants was observed up to moderate intensity of browning. Thereafter, the establishment decreased. Decreasing trends in establishment with increasing in size of explants was also reported by Muralikrishna (1988) in pomegranate and Gitonga et al. (2010) in macadamia nut. Establishment and growth of explants was significantly influenced by position and size of nodal explants. Variability in establishment and growth of internodes might be due to the difference in the regeneration potential of different nodes. Regeneration potential of different explants is attributed by the physiological state, age and cellular differentiation among the constituent cells (Murashige, 1974; Laxmi et al., 2013). Moreover, stem internodes contained adequate level of cytokinins for adventitious shoot production (Douglas, 1984). In the present experiment 3rd node having 2.5 cm length was found as best explants for maximum establishment and growth (Figure 1D). This could be due to the less exudation of phenol and endogenous auxin, and cytokinin level in the constituent cells.

Effect of antioxidants and serial subculturing

The data regarding response of antioxidants and frequent subculturing of explants on browning intensity and culture

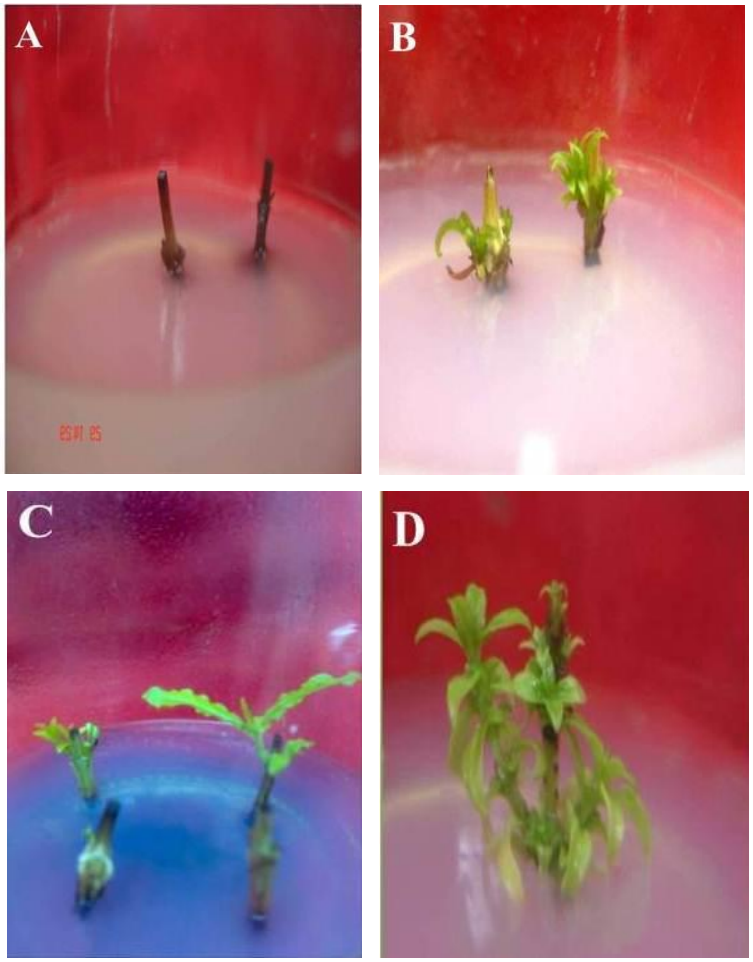

Figure 1. (A) Browning in nodal explants (B Explants after three subcultures at first and third DAI (Days after inoculation) (C) Establishment of 3rd node having 2.5 cm length of explant on 200 mg/l AC (activated charcoal) (D) Growth of 3rd nodal explants having 2.5 cm length after four weeks of culture.

Table 2. Effect of position and size of nodal segment explants on establishment of pomegranate cv. Ganesh.

Position of node (N)	Size of node (L)					Mean (N)
	1.5 cm	2.0 cm	2.5 cm	3.0 cm	3.5 cm	
1st	32.2	39.7	59.1	42.5	37.7	42.24
2nd	27.4	41.3	49.0	37.7	32.2	37.52
3rd	27.6	43.4	68.5	37.5	31.2	41.62
4th	26.5	24.3	25.8	23.7	22.1	24.48
5th	19.11	17.09	16.33	15.04	13.43	16.20
Mean (L)	26.56	33.16	43.75	31.29	27.33	-

S. Em± N = 0.16, L= 0.16, N × L= 0.37. CD at 5% N= 0.47, L= 0.47, N × L= 1.06. N= position of node L= Size of node.

establishment are presented in Table 4. Minimum browning intensity in explant and medium was observed in subculturing treatment at first and third DAI (Figure 1B). Among the different antioxidants, activated charcoal

Table 3. Effect of position and size of nodal segment explants on shoot growth of pomegranate cv. Ganesh.

Position of node (N)	Size of node (L)					Mean (N)
	1.5 cm	2.0 cm	2.5 cm	3.0 cm	3.5 cm	
1st	2.27	2.00	3.97	2.00	1.85	2.42
2nd	1.00	1.96	3.22	2.00	1.87	2.01
3rd	1.75	2.00	3.00	1.25	1.00	1.80
4th	1.65	1.00	0.73	0.84	0.56	0.95
5th	1.07	0.81	0.45	0.39	0.10	0.56
Mean (L)	1.55	1.55	2.27	1.29	1.07	-

S. Em ± N= 0.02, L= 0.02, N × L= 0.05. CD at 5%, N= 0.73, L= 0.73, N × L= 0.16. N = position of node L= Size of node.

Table 4. Effect of antioxidants on *in vitro* degree of browning and culture establishment of pomegranate cv. Ganesh.

Treatments	Browning intensity in medium	Appearance of explants	Cultural establishment (%)
Activated charcoal (mg/L)			
100	++++	Necrotic	12.20 (20.43)*
200	++	Green	41.20 (39.93)
300	+++	Slightly green	19.80 (26.42)
Citric acid (mg/L)			
20	+++	Necrotic	9.60 (18.04)
40	++++	Necrotic	10.20 (18.61)
100	++++	Necrotic	11.40 (19.73)
Ascorbic acid (mg/L)			
50	++++	Necrotic	9.00 (17.45)
100	++++	Necrotic	10.20 (18.62)
150	++++	Necrotic	11.20 (19.54
PVP (mg/L)			
5	++++	Necrotic	9.20 (17.64)
10	++++	Necrotic	10.80 (19.18)
15	+++	Slightly green	11.40 (19.72
Subculturing (DAI)			
One (DAI)	+++	Slightly green	24.40 (29.58)
Two (DAI)	++	Green	37.00 (37.46)
First and third (DAI)	+	Green	60.00 (50.77)
S.Em. ±	-	-	0.35
CD at 5%	-	-	1.01

*Figure in parentheses are arcsine transformed value. Browning intensity - ++++ Intense browning, ++++ High browning, +++ Moderate Browning, ++Low browning, + Very low Brown.

200 mg/L was found better in reducing of medium and explants browning (Figure 1C). However, addition of 300 mg/L activated charcoal into medium adversely affected culture establishment and shoot growth. Citric acid and

ascorbic acid did not show any effect in browning control. Whereas, PVP 15 mg/L reduced explants browning to some extent. Maximum culture establishment (60.0%) was recorded in frequent subculturing of explants at first and third DAI followed by in 200 mg/L activated charcoal (41.2%). Similarly, the appearance of the explants was green in all the subculturing treatments. The results are coincident with the findings of Murkute et al. (2004) and Singh and Khawale (2006).

The presence of phenolic compounds in explant tissues is a serious problem for *in vitro* culture establishment (Compton and Preece, 1986). These phenolic substances exudate from the cut surface of explants and oxidized due to the preoxideses, polyphenols or air (Onuoha et al., 2011) resulting in the medium turning brown and death of the explants (Aliyu, 2005). Addition of the antioxidants into culture medium is quite effective for controlling medium browning, as it removes the quinines formed in the medium.

Several studies have reported the use of antioxidants in browning control in perennial fruit plants (Khattak et al., 1994; Vasar et al., 2003; Birmeta and Welander, 2004; Zamir et al., 2004; Patil et al., 2011). Whereas, in the present study, ascorbic acid and citric acid was ineffective in control of browning. In contrast with our results, Patil et al. (2011) found best results in browning control with 150 mg/L ascorbic acid and 100 mg/L citric acid in pomegranate. Similarly, PVP was also found less effective in browning control. Tyagi et al. (1981) and Prajapati et al. (2003) effectively controlled explant browning with PVP when added into medium. The effectiveness of different antioxidants in control of browning is varying among plants and species. This could be due to the specificity of these chemicals to certain plant and species. The specificity of PVP in browning control was also reported by Vaugh and Duke (1984). Further, addition of activated charcoal 300 mg/L reduced the growth of explants. It might be due to the absorption of nutrients from medium. Activated charcoal is a strong phenol adsorbent (Zhou et al., 2010) that reduces phenolic browning in explants. It absorbs not only toxic substances and phenols (Fernando et al., 2010) but also the higher amount of growth regulators and nutrients in medium. The most effective browning control measure was subculturing of explants twice, at first day and third day of inoculation of explants. Frequent transfer of explants within the same medium or into fresh medium fairly prevents *in vitro* browning of explants (Kotomory and Murashige, 1965; Block and Lankes, 1996).

Frequent transfer of explants into fresh medium seals cut end of the explants that stopped leaching of phenols (Ahmad et al., 2013). These results are in parallel to those of Muralikrishna (1988), Singh and Khawale (2006) and Singh et al. (2011). They claimed that the subsequent transfer of explants on fresh medium resulted in complete disappearance of browning in nodal segment explants of mature plants in pomegranate.

Conclusion

Position of node in the shoots of mother plant and node size has great influences on the *in vitro* degree of browning. Among all the node positions, 3rd node with 1.5 to 2.5 cm length showed higher establishment and growth of explants with less browning intensity. Furthermore, the most effective browning control measure was subculturing of explants twice, first and third day of inoculation. Addition of 200 mg/L activated charcoal into the medium was found quite effective to minimize browning problem in nodal segment of mature explants.

Conflict of Interests

The authors have not declared any conflict of interest.

Abbreviations

MS, Murashige and Skoog medium; **NAA,** naphthalene acetic acid; **BAP,** 6-benzylaminopurine; **AC** activated charcoal; **PVP,** polyvinylpyrrolidone; **DAI,** days after inoculation.

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