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Optimization of surface sterilization and manipulation of *in vitro* conditions for reduced browning in pomegranate (*Punica granatum* L.) variety *Bhagava*

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Abstract
Success in commercially viable protocol for mass multiplication of plants using tissue culture begins with effective sterilization and reducing exudation from perennial fruit crops which makes the *in vitro* techniques more difficult. In the present study, nodal explants of pomegranate cv. *Bhagva* were established using different durations and concentrations of anti-microbial agents followed by treatments with anti-oxidants (citric and ascorbic acid), with and without illumination (light and dark) and presence or absence of activated charcoal in MS media for reducing browning. Among different treatments tested for sterilization, 300 mgl^{-1} bavistine (50% carbendazim) for eighteen mintues, 200 mgl^{-1} each of streptocycline and cefotaxime for eighteen and eight minutes, respectively were found to be most the effective treatments to obtain cent per cent axenic culture development. Incorporation of activated charcoal (0.25%) significantly reduced the browning and improved the establishment of cultures as compared to other anti-browning agents.

Keywords: Pomegranate, surface sterilization, anti-browning, phenolic exudation

Introduction
Micro-propagation technology is becoming increasingly popular now-a-days as alternative means of conventional and vegetative propagation techniques. Tissue culture techniques like shoot and nodal culture, meristem culture are used routinely for generating large number of clones in less time and virus free plantlets, respectively. Tissue culture protocols are available in most of the important plants including fruits, vegetables and ornamental plants. Pomegranate (*Punica granatum* L.) is one of the oldest known fruit trees of the tropics and sub-tropics, cultivated for its delicious edible fruits.

A number of *in vitro* studies have been carried out on pomegranate such as micropropagation through shoot proliferation, by means of nodal cultures (Naik *et al*., 1999 and 2000) [18, 19] somatic embryogenesis (Bhansali, 1990 [1]; Kanwar *et al*., 2010 [7]; Nataraja and Neelambika, 1996 [20]) synthetic seed production and utilization (Naik and Chand, 2006) [17] plant regeneration via shoot organogenesis either indirectly through callus cultures (Yang and Ludders, 1993) [29] or directly by adventitious organogenesis (Jaidka and Mehra, 1986[6]; Omura *et al*., 1987 [21]) in explants. Success in these techniques is depended upon (i) optimization of surface sterilization and (ii) *in vitro* response of starting material.

Surface sterilization of explants is one of the critical steps in developing plant tissue culture protocol. The living materials should not lose their biological activity and only contaminants should be removed during sterilization. The success of sterilization methods is depended upon the concentration and duration of anti-microbial agents (Oyebanji *et al*., 2009) [22]. Mercuric chloride is often used to overcome microbial contamination, however, it is considered as one of the most toxic elements for eco-system capable of causing major alterations in the tissues of both animals and humans (Lund *et al*., 1993 [10]; Mahboob *et al*., 2001 [12]). However, for horticultural plants due to hard tissues, use of mercuric chloride is indispensable as these tissues often have large microbial population on the surface.

Major problem in developing tissue culture protocol in pomegranate is the browning of media near the explants due to high phenolic exudation from the cut end of explants. Mass multiplication using nodal and shoot tip of pomegranate is generally the preferred method for large scale production of true to type clones in short time wherein browning is often

encountered using this propagation method. To overcome the *in vitro* growth inhibitory effects due to browning, use of adsorbing agents such as activated charcoal or polyvinylpyrrolidone (PVP), addition of anti-oxidants like citric acid, transfer of explants to fresh medium at frequent intervals or combinations of these methods has proven effective in controlling browning (Murkute *et al*. 2003, 2004 [15, 16]; Samir *et al*. 2009 [24]). The main aim of this research was to standardize both duration and concentration of anti-microbial agents for effective removal of contaminants and to investigate the effect of anti-oxidants and physical culture conditions for overcoming browning using nodal explants of pomegranate.

2. Materials and Methods

The study was conducted at Centre for Advanced Research in Plant Tissue Culture, Anand Agricultural University, Anand, Gujarat during the period from December, 2016 to February, 2016 to establish aseptic cultures with reduced browning in '*Bhagava*' cultivar maintained at Horticulture Farm, B. A. College of Agriculture, Anand Agricultural University, Anand, Gujarat, India.

2a. Explant collection and pre-treatment

Disease free healthy pomegranate '*Bhagava*' plants were sprayed three days with 1000 mgl^{-1} bavistin (50% carbendazim) before collection of nodal segments of about 2 to 3 cm long.

2b. Explant sterilization

Excised nodal segments were thoroughly washed under running tap water for about 5 to 10 min followed by washing with distilled water and 1 to 2 drops of surfactant, Tween-20.

2c. Surface sterilization

Different antimicrobial agents of varying combinations of duration and concentration were tested for establishing axenic explant cultures. Explants were treated with anti-microbial agents streptocycline, cefotaxime, bavistin (50% carbendazim) in different concentrations (200 and 300 mgl^{-1}) and durations (10 to 20 mins). The explants were treated finally with mercuric chloride (1000 mgl^{-1}) for different durations (1 to 5 mins) after the above anti-microbial treatments.

2d. Control of browning

The following experiments were conducted for reducing the browning from the cut end of nodal segments of pomegranate.

(A) Antioxidant treatments

Explants were soaked in different combinations of anti-oxidant chemicals like ascorbic acid and citric acid (100 and 200 mgl^{-1}) for 10 and 15 min. each under laminar air flow hood followed by three times rinsing in sterile distilled water.

(B) Physical conditions (light and dark)

The established axenic cultures were kept under dark for first three days followed by exposure to light (300 $\mu Molm^{-2}s^{-1}$) for 16 hours and 8 hours dark at 25 ± 2°C.

(C) Activated charcoal

Explants were inoculated on MS media (Murashige and Skoog, 1962) [14] with and without activated charcoal (0.25%).

2e. Statistical analysis

Complete randomized design was used in all the combinations with 20 tubes per treatment with five tubes each representing four replications per treatment. Parameters observed for surface sterilization included bacterial and fungal, total contamination, browning of explants and are expressed in percentage. The parameters recorded for control of browning experiment were browning intensity (partial, full or green explants), days to sprouting and sprouting percentage. Standard errors of the means were computed whenever appropriate.

3. Results and Discussion

3a. Sterilization Treatments

Naturally growing plants have a diverse group of micro-organisms on their surface which is the major source of *in vitro* contaminants observed during the development of tissue culture protocol. The duration of the anti-microbial agents have a significant effect on explants, wherein the lower concentration resulted in higher contamination percentage while higher concentration resulted in loss of explants viability. The explants exposed to 300 mgl^{-1} bavistin (50% carbendazim) for eighteen mins, 200 mgl^{-1} streptocycline and cefotaxime for 18 and 8 minutes respectively, followed by 1000 mgl^{-1} mercuric chloride gave 93.33% axenic cultures with least contamination percentage of 6.67 for fungal contaminants, among all the treatment combinations. The earliest visible bacterial and fungal contaminations appeared on 4^{th} and 10^{th} days after culture for the treatment involving 200 mgl^{-1} bavistin (50% carbendazim) for ten mins, 200 mgl^{-1} streptocycline for ten mins and 1000 mgl^{-1} mercuric chloride for six mins. (Table 1). The browning intensity in explants was also found to be highest in the treatments exposed to mercuric chloride for more than three minutes. Exposing explants to anti-microbial agents (bavistin, streptocycline, cefotaxime, mercuric chloride) was found to be effective for the disinfection of explants (Bhat, 1990 [2]; Chaudhary, 1989 [3]; Machado *et al*., 1991 [11]).

3b. Effects of antioxidants on explants browning

In vitro growth parameters like number of tubes giving sprout, loss of viability due to partial, full brown and green explants and browning intensity were observed with different treatments of anti-oxidants, culturing explants in dark and light conditions and presence or absence of activated charcoal (Table 2). The explants pretreated with 100 mgl^{-1} citric acid and ascorbic acid showed least browning intensity with 0.10 ± 0.06 sprout development between 9 to 15 days of culture (Table 2). The treatment was found to be effective only for days to sprouting with earliest sprout appeared on 9 days after culture, however, the number of new sprouts after 21 days of culture was found to be the least as compared to charcoal based medium. (Fig. 1)

Similar results with ascorbic and citric acid were observed in overcoming explants browning of bird of paradise by Ziv and Halaevy (1983) [30]. However, the efficiency of treatment in inducing the sprouting was found to be less which is not desirable and hence the pretreatment was found in-effective in inducing sprouting. The duration of the pretreatment was found to be effective in controlling browning intensity and was inversely related with increase in duration of pretreatment. Browning at the cut ends of nodal explants was observed in 48 hours of pretreatment mainly due to reduction in pH of pre-soaking solution by the antioxidant. The cut region of explants secreted polyphenols which upon oxidation

caused browning of media, however, pretreatment with the antioxidants reduced the pH due to which the oxidation of polyphenols ceases (Khan, 1977) [8]. Similar in-effective results using anti-oxidants treatments in reducing nodal explant browning in pomegranate were reported by Murkute and co-workers (2002) [16].

Table 1: Effects of antimicrobial agents on appearance, colony size, growth and fungal and bacterial contamination in *Punica granatum* (L.).

S. No.	Sterilization Agents	Concentration (mgl^{-1})	Duration of treatment (mins)	Contamination (%)	Day of appearance contamination			Average colony size *		Average growth of colony**		Browning of explants (%)
				Bacterial	Fungal	Bacterial	Fungal	Bacterial	Fungal	Bacterial	Fungal	
1	Bavistin Streptocycline $HgCl_2$	200 200 1000	10 10 6	93.33	93.33	4	10	+++++	+++++	+++++	+++++	100.00
2	Bavistin Streptocycline $HgCl_2$	200 200 1000	15 15 7	93.33	86.67	4	10	+++++	+++++	+++++	+++++	100.00
3	Bavistin Streptocycline $HgCl_2$	200 200 1000	20 15 10	86.67	86.67	5	12	+++++	+++++	+++++	+++++	100.00
4	Bavistin Streptocycline $HgCl_2$	200 200 1000	20 20 8	80.00	93.33	4	13	++++	+++++	++++	+++++	100.00
5	Bavistin Streptocycline $HgCl_2$	200 200 1000	15 15 3	80.00	86.67	6	13	++++	++++	++++	++++	12.50
6	Bavistin Streptocycline $HgCl_2$	200 200 1000	18 18 4	73.33	80.00	6	14	+++	++++	+++	++++	20.80
7	Bavistin Streptocycline $HgCl_2$	200 200 1000	21 21 5	66.67	73.33	6	14	+++	+++	+++	+++	25.00
8	Bavistin Streptocycline $HgCl_2$	300 200 1000	15 15 3	53.33	46.67	7	15	+++	++	+++	++	12.50
9	Bavistin Streptocycline $HgCl_2$	300 200 1000	18 18 4	40.00	40.00	7	16	++	++	++	++	53.33
10	Bavistin Streptocycline $HgCl_2$	300 200 1000	20 20 5	33.33	46.67	7	16	++	++	++	++	60.00
11	Bavistin Streptocycline Cefotaxime $HgCl_2$	300 200 200 1000	18 18 8 4	20.00	26.67	9	18	++	++	++	++	20.00
12	Bavistin Streptocycline Cefotaxime $HgCl_2$	300 200 200 1000	18 18 8 2	6.67	13.33	9	18	+	+	+	+	13.33

Note: * +++++ (largest colony) to + (smallest colony) ** +++++ (highest growth) to + (lowest growth)

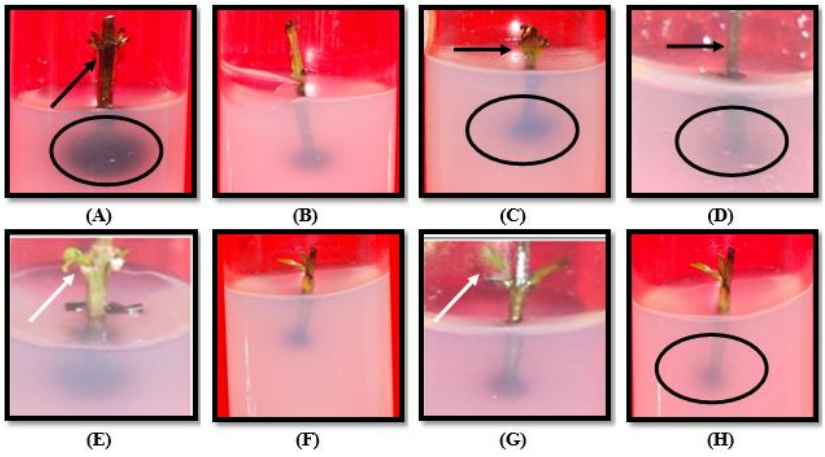

Fig 1: Effect of antioxidants (ascorbic acid and citric acid) on nodal explants of pomegranate var. *Bhagava*. Explants pretreated with citric acid ($100mgl^{-1}$) (A), citric acid ($200mgl^{-1}$) (B), ascorbic acid ($100mgl^{-1}$) (C), ascorbic acid ($200mgl^{-1}$) (D), citric acid ($100mgl^{-1}$) + ascorbic acid ($100mgl^{-1}$) (E), citric acid ($100mgl^{-1}$) + ascorbic acid ($200mgl^{-1}$) (F), citric acid ($200mgl^{-1}$) + ascorbic acid ($100mgl^{-1}$) (G) and citric acid ($200mgl^{-1}$) + ascorbic acid ($200mgl^{-1}$) (H) (black arrows indicate browning of explants, white arrows indicate sprouting and black circle represents browning due to exudation).

3c. Effects of light and dark on explants browning

The explants incubated under normal cultural conditions with optimum light (300 µMolm^{-2}s^{-1}) for 16 hrs. showed higher intensity of browning compared to explants cultured under dark. Intensity of browning was recorded highest with explants exposed to normal *in vitro* growth conditions. However, the explants kept under dark conditions showed less browning intensity, *in vitro* response in terms of sprout development and days to sprout was less as compared to explants which received normal light. The explants remained green and viable under dark after 21 days of subculture and gave an average of 0.65 ± 0.13 sprouts with days to sprouting ranging from 11 to 17 days after culture (Table 2) (Fig. 2).

Table 2: Effect of anti-oxidants (ascorbic and citric acid), illumination (light and dark) and activated charcoal (with and without) in controlling browning of nodal explants of pomegranate, variety *Bhagava.*

Treatment details	Number of explants after 21 days of *in vitro* culture			Browning intensity	Number of tubes with sprout	Days to sprouting
	Partial brown	Full brown	Green			
Antioxidant treatment						
Control	0	20	0	+++	0.00 ± 0.00	0
Citric acid (100 mgl^{-1})	2	18	0	+++	0.00 ± 0.00	0
Citric acid (200 mgl^{-1})	3	16	1	+++	0.00 ± 0.00	0
Ascorbic acid (100 mgl^{-1})	3	17	0	+++	0.00 ± 0.00	0
Ascorbic acid (200 mgl^{-1})	3	15	2	+++	0.00 ± 0.00	0
Citric acid (100 mgl^{-1}) + Ascorbic acid (100 mgl^{-1})	9	9	3	++	0.10 ± 0.06	10-14
Citric acid (100 mgl^{-1}) + Ascorbic acid (200 mgl^{-1})	11	6	3	++	0.00 ± 0.00	0
Citric acid (200 mgl^{-1}) + Ascorbic acid (100 mgl^{-1})	11	7	2	+	0.30 ± 0.13	11-20
Citric acid (200 mgl^{-1}) + Ascorbic acid (200 mgl^{-1})	10	5	5	+	0.60 ± 0.14	9-15
Illumination						
Light	9	3	8	++	0.50 ± 0.13	11-18
Dark	6	4	10	+	0.65 ± 0.13	11-17
Activated charcoal						
With	0	0	20	-	0.90 ± 0.06	13-15
Without	8	3	9	++	0.55 ± 0.10	16-19

Note:
+++ High browning intensity with full brown explants
++ Moderate browning with partial green explants
+ Low browning and green explants
No browning after inoculation and green explants

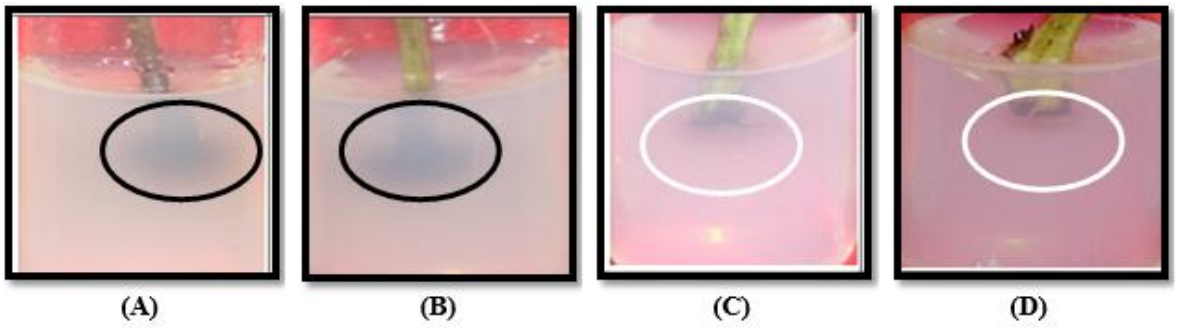

Fig 2: Effect of illumination (light and dark) on nodal explants of pomegranate var. *Bhagava*. Explants exposed to (A and B) Light (3000 lux, 16 hrs.) (C and D) Dark (black and white circle represents browning due to exudation)

Browning intensity was found to be significantly decreased in the absence of light which suggest inhibition by polyphenol oxidase (PPO) activity under dark conditions. PPO activity is required for oxidation of phenolic compounds leading to browning of explants (Hutcheson *et al*., 1980 [5] and Sommer *et al*., 1994 [26]). PPO is involved in catalyzing the reaction between phenolic derivatives and oxygen producing ortho-diquinones. These diquinones react in a highly non-specific manner with proteins and other cellular components leading to formation of dark pigments known as melanin (Stevens and Davelaar 1996 [27]; Thygesen *et al*. 1994 [28])

3d. Effects of activated charcoal on explant browning

Among three treatments, explants incubated on MS medium containing activated charcoal was found to be most effective in controlling browning. The explants showed no visible browning having green color which indicates 100% viability. Maximum number of sprouting, 0.90 ± 0.06 with almost all cultures gave uniform values for days to sprouting which ranged from 13 to 15 days after culture (Table 2, Fig.3). Similar results were reported by Sharada *et al*., (2003) [25] and Prajapati *et al*., (2003) [23] wherein phenolic exudation from cut region of explants was prevented using activated charcoal in the culture medium of *Celastrus paniculatus* and *C. orchioides*. Explants of eucalyptus showed better growth with reduced phenolic secretion in the medium supplemented with activated charcoal (Dibax *et al*., 2005) [4].

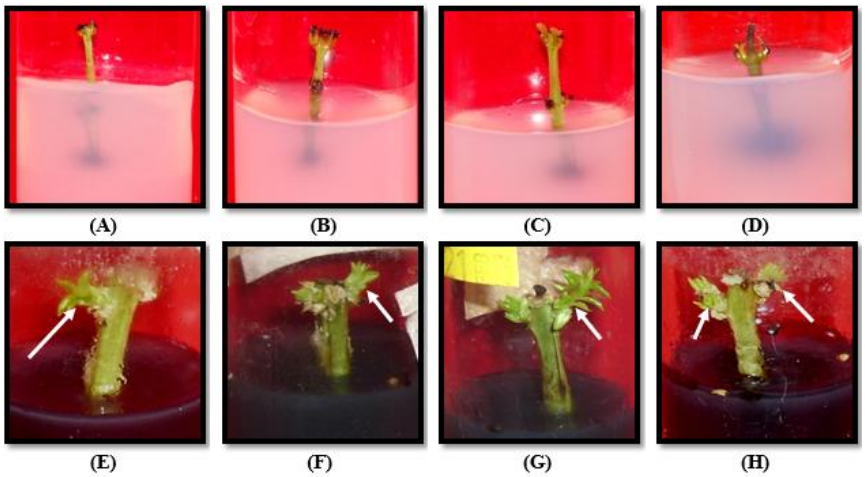

Fig 3: Effect of activated charcoal incorporation in MS media on nodal explants of pomegranate variety *Bhagava*. Explants exposed to media (A to D) without activated charcoal (D to H) with activated charcoal (0.25%) (White arrows represents sprouting of axillary buds).

The most preferred method to overcome browning due to phenolic secretion is to transfer explants to fresh medium. However, this method has several disadvantages like increase in cost of plantlet production, time consuming and increasing number of subculture. Incorporation of phenolic adsorbents like charcoal or polyvinylpyrrolidone (PVP) can overcome this problem. Activated charcoal makes complex with the phenolic secretion and thus inhibit the PPO and peroxidase activities leading to reduced browning. Dark environment created by activated charcoal may also play an important role in reducing browning as light can increase the activity of enzymes involved in phenol oxidation. Mensuali – Sodi and co-workers (1993) [13] reported overcoming of browning and enhanced shoot growth using activated charcoal in *in vitro* cultures of *Strelitzia reginae* and *Anemone ornoari*. Similar results were also reported by Linington, (1991) [9] wherein ascorbic acid and PVP was found to be less effective in controlling browning than activated charcoal in *Dipterocarpus alatus* and *D. intricatus*.

4. Conclusion

In the present study, comparative study on pomegranate nodal axenic culture development and reducing browning was attempted using antimicrobial agents with different duration and concentrations, anti-oxidants, with and without illumination and presence or absence of activated charcoal in MS media. The cent-percent axenic cultures of nodal explants was successfully established using 300 mgl^{-1} bavistin (50% carbendazim) for eighteen minutes 200 mgl^{-1} streptocycline and cefotaxime for 18 and 8 minutes, respectively. The pretreatment with anti-oxidants like ascorbic and citric acid was found to be effective in early sprouting while explants cultured on activated charcoal gave good sprouting response with least browning among all the treatments. The results suggested that pre-treatment with ascorbic and citric acid followed by culturing on activated charcoal containing MS media could be used for mass multiplication of pomegranate using nodal explants.

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