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CONTROL OF MEDIA BROWNING IN MICROPROPAGATION OF GUAVA (*PSIDIUM GUAJAVA* L.)

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Abstract

Guava (*Psidium guajava* L.) is a highly valuable fruit of the tropical regions of the world. This species faces browning or blackening of culture medium during *In vitro* culture due to leaching of phenolic, microbial contagion and tissue recalcitrance. A study therefore designed to evaluate the effects of antioxidants in reduction of phenolic exudation which hampers *In vitro* regeneration. The nodal explants of the plant were cultured on MS media after pre-soaking in antioxidant solutions of citric acid, ascorbic acid, poly vinyl pyrrolidine (PVP) and charcoal. After culturing explants, the amount of phenolic exude was determined periodically on spectrophotometer at 750 nm absorbance. Phenolic exudation from guava was significantly reduced in nodes treated with charcoal as compared to control and rest of the treatments. Moreover, guava nodes survival percentage was also significantly increased in charcoal treated nodes. It is concluded that pre-soaking in different antioxidants significantly reduced the media browning and thus micro-propagation of guava could be achieved on commercial basis.

Key words: Browning, Phenolics, Guava, Antioxidants, Micro-propagation, Spectrophotometry.

Introduction

Guava (*Psidium guajava* L.) is a member of Myrtaceae family which is known as the poor man's fruit of the tropical regions. This species is indigenous to the tropical America; however, it stretches from Mexico to Peru. At present, it is grown in tropical to subtropical countries of the world (Samson, 1986) including Pakistan (Loh & Rao, 1989; Zamir *et al.*, 2003). This is an open pollinated species that may be attributed to get evolve true to type new genotypes in existing plantation through seed (Mehmood *et al*., 2013, 2014). For acquiring the desired breeding materials, vegetative means of propagation is essential; however, asexual propagation is a big problem in guava (Rehman *et al*., 1991; Zamir *et al.,* 2003; Kareem *et al.,* 2013). Various traditional methods viz. aerial layering, budding, cuttings, grafting and inarching are employed in horticulture for guava perpetuation. The aerial layering is mostly suggested a successful tool in regenerating this species; yet it is too costly. Tissue culture technique provides a substitute method for the vegetative reproduction for a variety of species. Through this technique, various woody species such as like eucalyptus (Burger, 1987), fruits plants like Kiwi (Hassan *et al.,* 2000) and guava (Amin & Jaiswal, 1987; Jaiswal & Amin, 1992) and wild vegetable (Iftikhar *et al.*, 2015; Islam *et al*., 2015) have successfully propagated.

During *In vitro* propagation of guava, the explants cuttings become browning or blackening in culture medium, which eventually impedes morphogenetic activity (Gassman *et al.*, 1978). Browning of tissues is triggered by the corrosion of tannin, polyphenols and the development of quinone which are very much toxic to tissues. Several oxidases such as monophenol (tyrosinase), polyphenol (Cate-choloxidase) oxidize the hydroxyl group resulting in the formation of quinone and water (Loomis & Battaile, 1966). Plant tissue contains these substances in separate pools or compartments. During tissue integration, an oxidation process is initiated (Monaco *et al.*, 1977). It is possible to overcome this problem through the application of different antioxidants. These compounds were employed in apple (Jones *et al.*, 1979) and mango (Litz & Vijayakumar, 1982; Sharma and Singh, 2002). Nevertheless, browning of guava and walnut tissues was not overcome (Gassman *et al.*, 1978; Miller *et al.*, 1982). Therefore, these selective substances from seedling and adult bearing tree of guava were applied on the juvenile tissue with sterilization agents to study their performance against browning. The present investigation is an attempt to standardize protocol to minimize the lethal effect of phenolic compounds (secondary metabolites) for rapid and successful *In vitro* multiplication of guava on commercial basis.

Material and Methods

The nodes (2-3 cm) of mature guava plant were cultured on MS basal medium in Plant Tissue Culture Cell, Institute of Horticultural Sciences, University of Agriculture, Faisalabad. Prior to culturing, selected nodes were exposed to running tap water for 30 minutes and then dipped separately in solution of ascorbic acid (250 mg/l), citric acid (300 mg/l), PVP (500 mg/l) and charcoal (1000 mg/l); each for 5 hours. The washed explants were brought to laminar air flow cabinet for surface sterilization treatment. The disinfected nodes were inoculated in test tubes were various size, vertically on MS medium. These inoculated nodes were then placed in growth chamber at 25±2°C under 16 hour's photoperiod. After regular intervals of 48 hours of culturing, three tubes were picked randomly to observe browning and stored at -80°C (Murashige & Skoog, 1962).

For the determination of total phenolic exudates, explants showing exudation were detached from culture

tubes and to each tube, 10 ml of 80% ethanol was supplemented and shaken systematically with a glass rod. The same were kept on a gyratory shaker at 100 rpm for half an hour, followed by centrifugation at 4000 rpm for further half an hour. The obtained supernatant was poured and filtered with Whatman's filter paper No. 42. Final volume of the filtrate was made to 20 ml with 80% ethanol and 2 ml of this extract was employed for determining phenolic compounds. In the alcoholic extract, 10ml of 1/10 diluted Folin-Ciocalteau stock reagent was added. After 10 minutes, 8 ml of 7.5% Na_2CO_3 solution was added and the reaction mixture was allowed to stand for 2 hours at room temperature for development of colour. The absorbance of colour was recorded by spectrophotometer at 765 nm. Then total phenol contents in the samples, in terms of gallic acid were calculated and expressed in µg/1 of fresh weight through calibration curve (Sharma, 1994).

Results and Discussion

Effect of antioxidants on visual media browning: The effect of different antioxidants on MS media browning of guava nodal culture is presented in Table 1. Irrespective of culturing hours, the samples treated with antioxidants had significantly reduced browning of MS media ($p<0.05$) than those of the control at 48, 96, 192 and 240 hours of culture for all the antioxidant treatments. Furthermore, the samples exhibited non-significant variations among the treatments ($p>0.05$) for different incubation period. The samples treated with ascorbic acid showed the least browning ($p<0.05$) at 336 hours among all the treatments. The control, PVP and ascorbic acid samples significantly increased browning ($p<0.05$) at 192 hours of nodal culturing. The observed browning of the samples treated with citric acid increased significantly ($p<0.05$) at 240 hours, while those treated with activated charcoal, at 288 hours. It means the treatment with antioxidants (activated charcoal, PVP, citric acid and ascorbic acid) reduced the browning of MS media and best results were observed at 336 hours of culturing. The results showed that after 48 hours of culturing, there was a non-significant difference in the media browning. The explant has reserved food and as uptake of nutrients starts with the passage of time, media browning increases gradually. In this study, observed media browning was remarkably reduced in the samples (especially at 336 hours of incubation) treated with ascorbic acid as compared to control. These findings are in agreement with those of Gassman *et al*. (1978), who reported that the use of various antioxidants is dependent to overcome browning of the guava and walnut explants. During the experiment, it was observed that at 75:50 mg/l dose of citric acid and ascorbic acid were somewhat effective in controlling browning of shoot tips as compared to nodal explants, which gave poor results. It was noticed that browning of the material was more intense in case of nodal explants, because these contained more phenolic compounds as compared to shoot tips.

Effect of antioxidants on phenolic exudation: Generally, the total phenolic exudates in guava nodal culture decreased by presoaking of explants with antioxidants, but increased with culturing duration (Table 2). The differences among the treatments were non-significant ($p>0.05$) at all durations, with few exceptions at 48 and 336 hours. The activated charcoal, PVP, citric acid and ascorbic acid samples exhibited significantly lower total phenolic exudates ($p<0.05$) at all the durations of nodal culturing than those in control. The control samples, as well as those treated with activated charcoal and PVP exhibited significantly higher values of total phenolic exudates ($p<0.05$) with increasing culturing duration, except control samples at 36 hours. Citric acid and ascorbic acid treated samples, however, showed significant increase in total phenolic exudates ($p<0.05$) after 96 hours of culturing, with an exception for ascorbic acid at 192 hours. The restricted phenolic exudation of treated explants, in fact indicates an increase in survival rate at selected culturing durations. Maximum phenolic exudation (555.16±8.94 µg/1) was quantified in control treatment while minimum values (331.51±4.83 µg/1) were recorded in samples treated with activated charcoal (Table 2).

Time factor and phenolic exudation: A variety of methods like etiolation of stock plants, pre-treatments of explants by means of liquid shaker culture, addition of medium with antioxidants and selection of suitable explants were tested to prevent browning in explant culture. However, no strategy was found successful due to strong browning reaction in mango explant culture (Krishna & Singh, 2007). A positive relationship was established between age of explant and phenolic exudation in tissue culture of cotton. In the present study, shoot tips of explant suffered die back; however, at 1st, 2nd and 3rd nodal position were not affected by phenolic exudation. This clearly depicts that these compounds upsurge with the age in shoots due to environmental strain (Ozyigit, 2008). The results of current study are in the line with those of Krishna & Singh (2007).

Effect of antioxidants on size of explants: The size of explants, pre-treated with antioxidants showed significant increase at 96 hours, especially in case of citric acid and ascorbic acid treated guava nodal cultures (Table 3). All the treatments exhibited significant increases ($p<0.05$) in size of explants at 192 hours. Furthermore, the ascorbic acid and PVP treated samples showed significant increase in size of explants at 288 and 336 hours, respectively. However, no clear-cut trend was observed in case of explant size for any of the treatment. So, soaking of guava nodes with activated charcoal, PVP, citric acid and ascorbic acid had no remarkable effect on the size of explants. The occurrence of little variation in explant size, advocated a similar behavior of browning that exhibited in the samples treated with different antioxidants. The intensity of browning differs among species and varieties, tissues and development phase of organ, age of tissue and medium. The occurrence of browning may be attributed to oxidized phenolic compounds (Lagrimini, 1992).

Table 1. Effect of different antioxidants on observed media browning in guava tissue culture.

Culture duration (hrs.)	Control	Activated charcoal	PVP	Citric acid	Ascorbic acid
48	1.34 ± 0.58^{Ac}	0.34 ± 0.58^{Bc}	0 ± 0.00^{Be}	0 ± 0.00^{Bd}	0 ± 0.00^{Bd}
96	1.67 ± 0.58^{Ac}	0.67 ± 0.58^{Bc}	0.34 ± 0.58^{Bde}	0.67 ± 0.58^{Bcd}	0.67 ± 0.58^{Bcd}
144	2.00 ± 1.00^{Abc}	0.67 ± 0.58^{Bc}	0.67 ± 0.58^{Bcde}	1.00 ± 0^{ABcd}	1.01 ± 1.00^{ABbcd}
192	3.00 ± 1.00^{Aab}	1.34 ± 0.58^{Bbc}	1.34 ± 0.58^{Bbcd}	1.01 ± 1.00^{Bcd}	1.67 ± 0.58^{Babc}
240	3.34 ± 0.58^{Aa}	1.67 ± 0.58^{Babc}	1.67 ± 0.58^{Bbc}	2.00 ± 1.00^{Bbc}	2.00 ± 1.00^{Bab}
288	3.67 ± 0.58^{Aa}	2.34 ± 0.58^{Bab}	2.34 ± 0.58^{Bab}	3.00 ± 1.00^{ABab}	2.00 ± 0^{Bab}
336	4.00 ± 0.00^{Aa}	2.67 ± 1.16^{BCa}	3.00 ± 1.00^{ABCa}	3.67 ± 0.58^{Aba}	2.34 ± 0.58^{Ca}

Values are Mean± SD (n=3). In rows, capital superscript letters ABC represent significant differences (p<0.05) among Treatments, whereas in columns, small superscript letters abc represent significant differences (p<0.05) with Time (hours), (Duncan multiple range test)

Table 2. Effect of different antioxidants on phenolic exudation in guava.

Culture duration (hrs.)	Total Ph enolic Exudation (µg/1)				
	Control	Activated charcoal	PVP	Citric acid	Ascorbic acid
48	124.49 ± 5.25^{Af}	69.27 ± 5.08^{Cg}	54.03 ± 4.98^{Dg}	81.78 ± 11.46^{Be}	58.96 ± 3.04^{CDe}
96	203.34 ± 20.82^{Ae}	108.60 ± 5.63^{Bf}	123.79 ± 24.92^{Bf}	118.54 ± 7.91^{Be}	109.84 ± 4.3^{Bde}
144	294.00 ± 13.53^{Ad}	184.61 ± 18.85^{Be}	184.70 ± 8.12^{Be}	219.26 ± 35.31^{Bd}	170.82 ± 35.37^{Bd}
192	328.67 ± 25.8^{Ac}	266.30 ± 21.63^{Bd}	236.18 ± 8.86^{Bd}	261.34 ± 24.10^{Bcd}	248.22 ± 6.88^{Bc}
240	415.25 ± 11.55^{Ab}	288.58 ± 6.74^{Bc}	284.68 ± 18.85^{Bc}	301.67 ± 32.28^{Bbc}	320.92 ± 59.37^{Bb}
288	530.71 ± 16.91^{Aa}	315.13 ± 8^{Bb}	346.80 ± 6.70^{Bb}	331.07 ± 49.45^{Bb}	336.41 ± 4.74^{Bb}
336	555.16 ± 8.94^{Aa}	331.51 ± 4.83^{Ca}	431.01 ± 6.82^{Ba}	497.63 ± 62.96^{ABa}	425.00 ± 61.62^{BCa}

Values are Mean± SD (n=3). In rows, capital superscript letters ^{ABC} represent significant differences (p<0.05) among Treatments, whereas in columns, small superscript letters ^{abc} represent significant differences (p<0.05) with Time (hours), (Duncan multiple range test)

Table 3. Effect of different antioxidants on observed browning under various size of explants in guava.

Culture duration (hrs.)	Size (cm) of explants				
	Control	Activated charcoal	PVP	Citric acid	Ascorbic acid
48	2.28 ± 0.19^{a}	2.18 ± 0.08	2.18 ± 0.13	2.39 ± 0.16^{a}	2.27 ± 0.08
96	2.05 ± 0.16^{Ca}	2.08 ± 0.08^{C}	2.17 ± 0.24^{BC}	2.49 ± 0.24^{Aa}	2.36 ± 0.10^{AB}
144	2.12 ± 0.15^{Aba}	1.96 ± 0.31^{B}	2.26 ± 0.05^{A}	2.36 ± 0.08^{Aab}	2.29 ± 0.06^{A}
192	1.47 ± 0.55^{Cb}	2.67 ± 0.60^{A}	2.06 ± 0.14^{B}	2.23 ± 0.09^{ABab}	2.26 ± 0.08^{AB}
240	2.34 ± 0.13^{Aa}	2.23 ± 0.12^{AB}	2.08 ± 0.2^{B}	2.30 ± 0.18^{ABab}	2.26 ± 0.15^{AB}
288	2.07 ± 0.21^{Ba}	2.50 ± 0.15^{A}	1.94 ± 0.21^{B}	2.07 ± 0.17^{Bb}	2.34 ± 0.12^{A}
336	2.2 ± 0.09^{Ba}	2.28 ± 0.09^{AB}	2.39 ± 0.08^{A}	2.25 ± 0.10^{Bab}	2.18 ± 0.09^{B}

*Values are Mean ± SD (n=3). In rows, capital superscript letters ABC represent significant differences (p<0.05) among Treatments, whereas in columns, small superscript letters abc represent significant differences (p<0.05) with Time (hours), (Duncan multiple range test)

Table 4. Effect of different antioxidants on survival% of explants in guava.

Culture duration (hrs.)	Survival (%) of explants				
	Control	Activated charcoal	PVP	Citric acid	Ascorbic acid
48	100.00 ± 0^{a}	100.00 ± 0^{a}	100.00 ± 0^{a}	100.00 ± 0^{a}	100.00 ± 0^{a}
96	66.67 ± 14.44^{Bab}	91.67 ± 14.44^{Aa}	100.00 ± 0^{Aa}	83.34 ± 14.44^{ABab}	91.67 ± 14.44^{Aab}
144	58.34 ± 14.44^{b}	83.34 ± 28.87^{ab}	75.00 ± 25.00^{ab}	75.00 ± 0^{ab}	83.34 ± 14.44^{ab}
192	50.00 ± 25.00^{b}	66.67 ± 14.44^{abc}	58.34 ± 14.44^{b}	58.34 ± 14.44^{bc}	58.34 ± 14.44^{bc}
240	33.34 ± 38.19^{bc}	50.00 ± 0^{bc}	50.00 ± 0^{b}	33.34 ± 14.44^{cd}	58.34 ± 14.44^{bc}
288	8.34 ± 14.44^{c}	50.00 ± 25.00^{bc}	50.00 ± 25.00^{b}	25.01 ± 25.00^{d}	41.67 ± 38.19^{c}
336	0 ± 0^{Bc}	33.34 ± 28.87^{Ac}	16.67 ± 14.44^{ABc}	8.34 ± 14.44^{ABd}	25.01 ± 25.00^{ABc}

Values are Mean ±SD (n=3). In rows, capital superscript letters ABC represent significant differences (p<0.05) among Treatments, whereas in columns, small superscript letters abc represent significant differences (p<0.05) with Time (hours), (Duncan multiple range test)

Effect of different antioxidants on survival (%) of explants: Effect of different antioxidants on survival of guava nodes is presented in Table 4. The effect of pre-soaking of explants with antioxidants increased survival percentage at all the culturing durations, though with few exceptions, the differences were non-significant ($p>0.05$). The samples pre-treated with activated charcoal, PVP and ascorbic acid exhibited significant increase ($p<0.05$) in survival percentage at 144 hours of incubation. Furthermore, the samples pre-treated with activated charcoal and ascorbic acid exhibited significantly highest ($p<0.05$) survival percentage (83.34% ach). On the other hand, the survival percentage of explants decreased with increasing culture duration for all the treatments. In case of control samples, treatment durations of 144 and 288 hours resulted in significant decrease ($p<0.05$) of survival percentage. After 192 hours treatment duration, there was significant decrease ($p<0.05$) in survival percentage for PVP, citric acid and ascorbic acid, and 240 hours for activated charcoal (Table 4).

Ziv & Halevy (1983) reported that lethal browning was slightly decreased in bird of paradise (*Strelitzia reginae*) when explants were soaked in citric acid and ascorbic acid for 12 hours, while at 24 hours immersion, it was considerably reduced. Survival percentage was correlated to the browning; lower the browning, higher will be survival percentage of explants. Roussos & Pontikis (2001) correlated the *In vitro* survival of explants with media browning and reported differences in survival of explants collected from glasshouse developed seedlings and field-grown stock plants that signify variations between their physiological ages. The use of ontogenic young or juvenile tissues has long been in use to get better organogenesis (Nas *et al*., 2003; Kibbler *et al*., 2004).

Conclusion

From the results, it is concluded that the media supplemented with the antioxidants significantly controlled the lethal browning that led in efficient micro-propagation of guava on commercial basis.

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