Original Research Article

Effect of antioxidants, culture conditions and frequency of subculture to control phenols during *in vitro* culture of *Phalaenopsis* hybrid cultivar Oriana

Abstract

The present study was conducted to control phenols in the media during *in vitro* cultures using flower stalk explants of*Phalaenopsis* hybrid cultivar Oriana. MS medium + BAP (1.00 ppm) + NAA (0.50 ppm) + 3% sucrose (w/v) was used commonly for all the treatment. Results revealed that inclusion of 500 ppm citric acid in the media, rinsing the explants with citric acid 500 ppm for 20 minutes before inoculation, keeping culture in dark for 20 days and sub-culturing at 4, 6 and 8 days after inoculation were found to besignificantly reducing the browning intensity (very low browning) in medium. The explants remained green even after 4th week of culture with significantly minimum percentage of blackening of cultures (10.00%), minimum percentage of dead cultures (20.00%) and maximum percentage of cultures survived (80.00%).Inclusion of 500 ppm citric acid in the media, rinsing of explant with citric acid 500 ppm for 20 minutes before inoculation, incubating the culture in dark for 20 days and subculturing at an interval of 4, 6, and 8 day after inoculation in case of flower stalk explants were observed to control phenols in the media during *in vitro* cultures of *Phalaenopsis* hybrid cultivar Oriana.

Key words: *In vitro*, *Phalaenopsis*, phenols**,** Oriana.

Introduction

*Phalaenopsis*are highly priced in the floriculture industry due to their long shelf life and they can be used both as cut flowers and potted plants. Plantlets (keikis) are occasionally produced on their flower stalks and are traditionally propagated through division. Flowering takes at least two years in a greenhouse, and keiki separation can only be done after flowering, which is one of the major challenges in large scale multiplication of *Phalaenopsis*. Therefore, plant tissue culture technology is the only option for large scale multiplication of *Phalaenopsis* to feed into this industry. One of the primary problems found during the development of plantlets using micropropagation in *Phalaenopsis* was the secretion of phenols from the explants (Chugh, 2009). There are a few reports are available on controlling phenols from explants during *Phalaenopsis* micropropagation utilising flower stalks, but they are difficult to follow. To control phenols in the media during *in vitro* culture using flower stalk explants, a systematic investigation was carried out in *Phalaenopsis* hybrids cv. Oriana by manipulating adding antioxidants to the media, rinsing of antioxidants before inoculation and subculturing at different days of inoculation.

Materials and Methods

The experimentswere carried out at the Biotechnology-cum-Tissue Culture Centre, OUAT, Bhubaneswar, Odisha during 2017.

Plant material

After the flowers had withered, vegetative buds of the flower stalk were cut from *Phalaenopsis* hybrids cv. Oriana, which had been grown in a shade net house (50 percent shade) and brought to the laboratory with their cut ends soaked in distilled water. The nodal segments were cleaned in a liquid detergent solution (Labolene, Qualigen, India) for 10 minutes. Clean flower stalks were treated for 30 minutes with a 0.2 percent (w/v) Bavistin (BASF India Limited) solution, then washed three times with distilled water. The explants were surface sterilized with 0.1% $HgCl_2$ (w/v) for 3minutes. To eliminate residues of mercuric chloride, the explants were rinsed with sterile distilled water 2-3 times.Subsequently, the explants were treated with sodium hypochlorite 1% NaOCl (v/v) for 1 minute. They were washed thoroughly with sterile distilled water (2-3 times) to remove traces of sodium hypochlorite.

Culture media and conditions

With the help of a sterilized forceps and scalpel, the bracts on the node of the flower stalk were removed. Cut ends of nodal segments floral stalk (2.5-3cm) were inoculated in culture tubes containing MS (Murashige and Skoog, 1962) medium + BAP (1.00 ppm) + NAA (0.50 ppm) + 3% sucrose (w/v)medium. In a horizontal laminar airflow, these processes were carried out. In the culture room, the inoculated cultures were incubated under controlled conditions with fluorescent light of 3000 lux intensity. The culture room had a temperature of 25 ± 2^0C and a relative humidity of 70%. The photoperiod in the culture room was 16-8 (day-night) hours.

Effect of different antioxidants to control phenolsin the media

Antioxidants like, citric acid (100, 200, 300, 400 and 500 ppm), ascorbic acid (100, 200, 300, 400 and 500 ppm), Poly Vinyl Pyrrolidone (100, 200, 300, 400 and 500 ppm) and cysteine HCl (100, 200, 300, 400 and 500 ppm) (Table 1) were added toculture medium (MS medium (Murashige and Skoog, 1962) + BAP (1.00 ppm) + NAA (0.50 ppm) + 3% sucroseto reduce exudation of phenolscausing blackening in themedia.

Effect of different methods to control phenolsin the media

After sterilization of explant with 0.1% $HgCl_2$ (w/v) for 3minutesfollowed by 1% NaOCl for 1minute, they were treated with freshly prepared solutions of either PVP (250 and 500 ppm), ascorbic acid (250 and 500 ppm) or citric acid (250 and 500 ppm) for 20 minutes.

The explants treated with the antioxidants were kept on an autoclaved filter paper in sterilized petridishes and air dried to remove excess moisture to avoid contamination just before inoculation and then inoculated on MS medium (Murashige and Skoog, 1962) + BAP (1.00 ppm) + NAA (0.50 ppm) + 3% sucrose each in single test tube containing the sterilized media and another treatment *i.e*., keeping culture in dark after inoculation for 20 days (Table 2) to reduce phenols blackening in the mediawere done.

Effect of frequency of subculture to control phenols in the media

To reduce phenolic exudation and blackening in the media, sub-culturing was done at different intervals (days)*i.e*., 4, 6, 8, 10, 12, 14, 16, 18 and 20 days (Table 3).

Statistical analysis

Experiments were carried out usingacompletely randomized design as per Gomez and Gomez (1984).Treatments were repeated three times, each treatment consisted of 20 cultures. The browning intensity in mediumwas assessed visually during the second, third and fourth weeks of cultureusing+++++ intense browning, ++++ high browning, +++ moderate browning, ++low browning, + very low browning.

The effects of different treatments were quantified as the mean percentage of blackening, percentage of survival and percentage of dead cultures. Data obtained in percentage were subjected to angular transformation before analysis. The data were analyzed statistically to find out standard error.

Results and Discussion

The data pertaining to response of inclusion of antioxidants to control phenols in the media are represented in Table 1. The blackening of the media due to phenolic exudation was reduced with increase in the concentration of antioxidants (citric acid, ascorbic acid, poly vinyl pyrrolidone and cysteine HCl). Very low browning of the medium was recorded with inclusion of 500ppm of citric acid at the end of 4 weeks of culture. In this treatment, the explants remained green with minimum percentage of blackened cultures (10.00%) and minimum percentage of dead cultures (20.00%). It also resulted in the highest percentage of surviving cultures (80.00%). This treatment was followed by ascorbic acid (500 ppm), which recorded very low browning intensity of the medium at 2^{nd} week of culture, explants appeared green at 2^{nd} week of culture, low browning intensity of the medium at 4^{th} week of culture, minimum percentage of blackened cultures (31.67%), minimum percentage of dead cultures (25.00%) and highest percentage of surviving cultures (75.00%).

Similar finding were reported byBhaskar and Rajeevan (2003) whoobserved that addition of antioxidants (citric acid, ascorbic acid, PVP and cysteine HCl) to the media reduced browning.After 8 weeks of culture, citric acid (100 and 200 ppm) resulted in the blackening of 100% of the cultures, while citric acid at 500 ppm, resulted in cultures without blackening of media.

The data regarding response of different methods to control phenols in the media are represented in Table 2. When the explants were rinsed with different concentration of antioxidants (citric acid, ascorbic acid and poly vinyl pyrrolidone) before inoculation for 20 minutes, phenolic blackening of the media got reduced and the rate was found to increase with duration of culture. After 4 weeks of culture, the explants rinsed with citric acid 500 ppm and incubated in dark for 20 days recorded very low browning of the medium at 2^{nd}, 3^{rd} and 4^{th} week of culture. The explants appeared green at 2^{nd}, 3^{rd} and 4^{th} week of culture with minimum percentage of blackened cultures (10.00%), minimum percentage of dead cultures (20.00%) and maximum percentage of surviving cultures (80.00%). This treatment was followed by rinsing the explants with ascorbic acid, 500 ppm which resulted in very low browning intensity of the medium at 2^{nd} week of culture; the explants appeared green at 2^{nd} week of culture, low browning intensity of the medium at 3^{rd} and 4^{th} week of culture, minimum percentage of blackened cultures (31.67%), minimum percentage of dead cultures (26.67%) and highest percentage of surviving cultures (73.33%). Different methods have been tried to reduce phenolic blackening of the medium, in different plants, in the past. Incubating the cultures in the dark was found to be the most effective approach for reducing phenolic blackening of the media during *in vitro* culture of *Phalaenopsis* hybrid using inflorescence stalk nodes according to Bhaskar and Rajeevan (2003).In another study, when explants of bird of paradise (*Strelitziareginae*)were soaked in citric acid and ascorbic acid for 12 hours,lethal browning was slightly reduced, while for 24 hours immersion, it was considerably reducedaccording to Ziv & Halevy (1983).The percentage of explants that survived was correlated to browning; lower the browning, the higher the percentage of explants that survived.

The data regarding response of subculturing at different intervals (days) after inoculation to control phenols in the media are presented in Table 3. It was observed that, the blackening of the media increased with increase in interval of subculture. The explants sub-cultured at 4^{th}, 6^{th} and 8^{th} day after inoculation exhibited very low browning intensity of the medium after 4 weeks of culture. The explants appeared green with minimum percentage of

blackened cultures (10.00%), minimum percentage of dead cultures (20.00%) and highest percentage of surviving cultures (80.00%). This treatment was followed by sub-culturing at 10^{th} day after inoculation which recorded low browning intensity of the medium after 4^{th} week of culture, the explants appeared slightly green after 4^{th} week of culture with minimum percentage of blackened cultures (31.67%), minimum percentage of dead cultures (26.67%) and highest percentage of surviving cultures (73.33%).Previously, sub-culturing at different intervals to reduce phenolic blackening in the mediahas been done in different plants. Exudation of polyphenols, results in browning and necrosis of the medium. Subculturing of explants twice, once after the first day and second after third day of inoculation was the most efficient way toovercome this problem. Browning or blackening occurs as a result of phenolic substances exudate from the cut surface of explants and oxidized due to the preoxidases, polyphenols (Onuoha*et al*., 2011).As a result, frequent sub-culturing helps in changing the position of explants, resulting in increased browning at first and no browning subsequently according to Murkute *et al*. (2004) and Mishra *et al.* (2007).

Conclusion

Inclusion of 500 ppm citric acid in the media, rinsing of explant with citric acid 500 ppm for 20 minutes before inoculation, incubating the culture in dark for 20 days, and subculturing at an interval of 4, 6, and 8 day after inoculation in case of flower stalk explants were observed to be more effective in reducing the browning intensity of the media. The explants appeared green at 2^{nd}, 3^{rd} and 4^{th} week of culture with minimum percentage of blackening of cultures, minimum percentage of dead cultures and maximum percentage of surviving cultures.

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Table 1: Effect of different antioxidants to control phenols in the media during *in vitro* cultures using flower stalk explants of*Phalaenopsis* hybrid cultivar Oriana

Treat. No.	Anti oxidants	Browning intensity in medium*						Percentage of cultures*		
		2nd week of culture	Appearance of explant (2nd week)	3rd week of culture	Appearance of explant (3rdweek)	4th week of culture	Appearance of explant (4th week)	Blackened	Surviving	Dead
T_0	Control	+++++	Necrotic	+++++	Necrotic	+++++	Necrotic	100.00 (90.00)	0.00 (0.001)	100.00 (90.00)
Citric acid (ppm)										
T_1	100	++++	Necrotic	+++++	Necrotic	+++++	Necrotic	90.00 (71.57)	90.00 (71.57)	10.00 (18.43)
T_2	200	++	Slightly Green	+++	Slightly Green	++++	Necrotic	78.33 (62.26)	90.00 (71.57)	10.00 (18.43)
T_3	300	+	Green	++	Slightly Green	+++	Slightly Green	73.33 (58.91)	88.33 (70.03)	11.67 (19.97)
T_4	400	+	Green	++	Slightly Green	++	Slightly Green	33.33 (35.26)	83.33 (65.91)	16.67 (24.09)
T_5	500	+	Green	+	Green	+	Green	10.00 (18.43)	80.00 (63.43)	20.00 (26.57)
Ascorbic acid (ppm)										
T_6	100	++++	Necrotic	+++++	Necrotic	+++++	Necrotic	91.67 (73.22)	88.33 (70.03)	11.67 (19.97)
T_7	200	+++	Slightly Green	++++	Necrotic	+++++	Necrotic	83.33 (65.91)	88.33 (70.03)	11.67 (19.97)
T_8	300	++	Slightly Green	+++	Slightly Green	++++	Necrotic	75.00 (60.00)	81.67 (64.65)	18.33 (25.35)
T_9	400	+	Green	++	Slightly Green	+++	Slightly Green	40.00 (39.23)	78.33 (62.26)	21.67 (27.74)
T_{10}	500	+	Green	++	Slightly Green	++	Slightly Green	31.67 (34.24)	75.00 (60.00)	25.00 (30.00)
Poly Vinyl Pyrrolidone (ppm)										

T_{11}	100	++++	Necrotic	+++++	Necrotic	+++++	Necrotic	93.33 (75.04)	86.67 (68.58)	13.33 (21.42)
T_{12}	200	+++	Slightly Green	++++	Necrotic	+++++	Necrotic	85.00 (67.21)	86.67 (68.58)	13.33 (21.42)
T_{13}	300	+++	Slightly Green	++++	Necrotic	+++++	Necrotic	78.33 (62.26)	80.00 (63.43)	20.00 (26.57)
T_{14}	400	++	Slightly Green	++	Slightly Green	++++	Necrotic	46.67 (43.09)	73.33 (58.91)	26.67 (31.09)
T_{15}	500	+	Green	++	Slightly Green	++	Slightly Green	40.00 (39.23)	68.33 (55.76)	31.67 (34.24)
Cysteine-HCl (ppm)										
T_{16}	100	++++	Necrotic	+++++	Necrotic	+++++	Necrotic	95.00 (77.08)	83.33 (65.91)	16.67 (24.09)
T_{17}	200	++++	Necrotic	++++	Necrotic	+++++	Necrotic	86.67 (68.58)	83.33 (65.91)	16.67 (24.09)
T_{18}	300	++++	Necrotic	++++	Necrotic	+++++	Necrotic	81.67 (64.65)	76.67 (61.12)	23.33 (28.88)
T_{19}	400	+++	Slightly Green	+++	Slightly Green	+++++	Necrotic	50.00 (45.00)	71.67 (57.84)	28.33 (32.16)
T_{20}	500	++	Slightly Green	+++	Slightly Green	+++	Slightly Green	45.00 (42.13)	60.00 (50.77)	40.00 (39.23)
SE(m) ±								1.56	1.04	1.22
C.D. at 5 %								4.46	2.98	3.47

*20 cultures per treatment; repeated thrice

Culture medium – MS(Murashige and Skoog, 1962) medium + BAP (1.00 ppm) + NAA (0.50 ppm) + 3% sucrose (w/v)

Number of (+) sign indicate the extent of browning

Browning intensity - +++++ Intense browning, ++++ High browning, +++ Moderate Browning, ++Low browning, + Very low browning

Table 2: Effect of different methods used to control phenols in the media during *in vitro* cultures of flower stalk explants of *Phalaenopsis* hybrid cultivar Oriana

Treat. No.	Treatment details	Browning intensity in medium*						Percentage of cultures*		
		2nd week of culture	Appearance of explant (2nd week)	3rd week of culture	Appearance of explant (3rd week)	4th week of culture	Appearance of explant (4th week)	Blackened	Surviving	Dead
T_0	Control	+++++	Necrotic	+++++	Necrotic	+++++	Necrotic	100.00 (90.00)	0.00 (0.00)	100.00 (90.00)
T_1	Rinsing the explant with PVP 250 ppm before inoculation for 20 min.	+++	Slightly Green	++++	Necrotic	+++++	Necrotic	83.33 (65.91)	78.33 (62.26)	21.67 (27.74)
T_2	Rinsing the explant with PVP 500 ppm before inoculation for 20 min.	+	Green	++	Slightly Green	+++	Slightly Green	40.00 (39.23)	66.67 (54.74)	33.33 (35.26)
T_3	Rinsing the explant with ascorbic acid 250 ppm before inoculation for 20 min.	+++	Slightly Green	++++	Necrotic	+++++	Necrotic	81.67 (64.65)	80.00 (63.43)	20.00 (26.57)
T_4	Rinsing the explant with ascorbic acid 500 ppm before inoculation for 20 min.	+	Green	++	Slightly Green	++	Slightly Green	31.67 (64.65)	73.33 (58.91)	26.67 (31.09)
T_5	Rinsing the explant with citric acid 250 ppm beforeinoculation for 20 min.	++	Slightly Green	+++	Slightly Green	++++	Necrotic	78.33 (62.26)	86.67 (68.58)	13.33 (21.42)
T_6	Rinsing the explant with citric acid 500 ppm before inoculation for 20 min.	+	Green	+	Green	+	Green	10.00 (18.43)	80.00 (63.43)	20.00 (26.57)
T_7	Keeping culture in dark after inoculation for 20 days	+	Green	+	Green	+	Green	10.00 (18.43)	80.00 (63.43)	20.00 (26.57)

SE(m) ±	0.83	1.25	1.34
C.D. at 5 %	2.49	3.75	4.00

*20 cultures per treatment; repeated thrice
Culture medium – MS(Murashige and Skoog, 1962) medium + BAP (1.00 ppm) + NAA (0.50 ppm) + 3% sucrose (w/v)
Number of (+) sign indicate the extent of browning
Browning intensity - +++++ Intense browning, ++++ High browning, +++ Moderate Browning, ++Low browning, + Very low browning

Table 3:Effect of frequency of subculture to control phenols in the media during *in vitro* culture using flower stalk of *Phalaenopsis* hybrid cultivar Oriana

Treat. No.	Treatment details	Browning intensity in medium*						Percentage of cultures*		
		2nd week of culture	Appearance of explant (1st week)	3rd week of culture	Appearance of explant (5th week)	4th week of culture	Appearance of explant (8th week)	Blackened	Surviving	Dead
T_0	Control	+++++	Necrotic	+++++	Necrotic	+++++	Necrotic	100.00 (90.00)	0.00 (0.00)	100.00 (90.00)
T_1	4 DAI	+	Green	+	Green	+	Green	10.00 (18.43)	80.00 (63.43)	20.00 (26.57)
T_2	6 DAI	+	Green	+	Green	+	Green	10.00 (18.43)	80.00 (63.43)	20.00 (26.57)
T_3	8 DAI	+	Green	+	Green	+	Green	10.00 (18.43)	80.00 (63.43)	20.00 (26.57)
T_4	10 DAI	++	Slightly Green	++	Slightly Green	++	Slightly Green	31.67 (34.24)	73.33 (58.91)	26.67 (31.09)
T_5	12 DAI	++	Slightly Green	++	Slightly Green	+++	Slightly Green	40.00 (39.23)	71.67 (57.84)	28.33 (32.16)
T_6	14 DAI	++	Slightly Green	+++	Slightly Green	+++	Slightly Green	51.67 (45.96)	68.33 (55.76)	31.67 (34.24)
T_7	16 DAI	+++	Slightly Green	+++	Slightly Green	++++	Necrotic	73.33 (58.91)	56.67 (48.83)	43.33 (41.17)
T_8	18 DAI	+++	Slightly Green	++++	Necrotic	+++++	Necrotic	81.67 (64.65)	50.00 (45.00)	50.00 (45.00)
T_9	20 DAI	++++	Necrotic	+++++	Necrotic	+++++	Necrotic	90.00 (71.57)	41.67 (40.20)	58.33 (49.80)
							SE(m) ±	1.13	0.72	0.89
							C.D. at 5 %	3.34	2.13	2.63