

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 be significantly 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. Phenolic exudation induced poor regeneration potential in *Phalaenopsis* tissue culture, according to Tanaka and Sakanishi [1977]. Tissues with relatively high phenolic compounds are difficult to culture. There are a few reports 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 experiments were 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 3 minutes. 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.

Comment [LNL1]: Why was used HgCl_2 – is so toxic to explants? In our lab we use only alcohol and 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 $25 \pm 2^\circ\text{C}$ and a relative humidity of 70%. The photoperiod in the culture room was 16-8 (day-night) hour cycle.

Comment [LNL2]: Which was the light irradiance?

Effect of different antioxidants to control phenols in 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 to culture medium (MS medium (Murashige and Skoog, 1962) + BAP (1.00 ppm) + NAA (0.50 ppm) + 3% sucrose) to reduce exudation of phenols causing blackening in the media.

Effect of different methods to control phenols in the media

After sterilization of explant with 0.1% HgCl_2 (w/v) for 3 minutes followed by 1% NaOCl for 1 minute, 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 media were 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 using a completely randomized design as per Gomez and Gomez (1984). Treatments were repeated three times, each treatment consisted of 20 cultures. The browning intensity in medium was assessed visually during the second, third and fourth weeks of culture using the following criteria.

Browning intensity	Symbol
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

Comment [LNL3]: Was each experiment performed in isolation?

Comment [LNL4]: How were the oxidation colors checked? A very subjective criterion was used. You could insert a photo showing the differences about the colors.

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 2nd week of culture, explants appeared green at 2nd week of culture, low browning intensity of the medium at 4th 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 findings were reported by Bhaskar and Rajeevan (2003) who observed 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 2nd, 3rd and 4th week of culture. The explants appeared green at 2nd, 3rd and 4th 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 2nd week of culture; the explants appeared green at 2nd week of culture, low browning intensity of the medium at 3rd and 4th 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 (*Strelitzia reginae*) were soaked in citric acid and ascorbic acid for 12 hours, lethal browning was slightly reduced, while for 24 hours immersion, it was considerably reduced according to Ziv & Halevy (1983). The percentage of explants that

Comment [LNL5]: I think that your results can be different if the authors do not use HgCl₂ to disinfect the explants

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 4th, 6th and 8th 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 10th day after inoculation which recorded low browning intensity of the medium after 4th week of culture, the explants appeared slightly green after 4th 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 media has 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 to overcome this problem. Browning or blackening occurs as a result of cells being damaged and releasing quinines, followed by mixing of cellular contents such as enzymes and their substrates. 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 2nd, 3rd and 4th week of culture with minimum percentage of blackening of cultures, minimum percentage of dead cultures and maximum percentage of surviving cultures.

References

Gomez KA and Gomez AA. Completely Randomized Design. In: Statistical procedures for agricultural research 3rd edn, John Wiley and Sons, Singapore. 1984; 680.

- Bhaskar, J. and Rajeevan. Controlling phenolic blackening in *in vitro* cultures of *Phalaenopsis*, In: National symposium on recent advances in Indian floriculture, Kerala Agriculture University, Vellanikara, Trichur, November. 2003; 12: 59.
- Mishra DS, Tiwari JP and Shant Lal. *In vitro* cloning of guava (*Psidium guajava* L.) cv. Pant Prabhat. *Acta Hortic.* 2007; 33: 127-132.
- Murashige T and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Plant Physiol.* 1962; 15: 473- 497.
- Murkute M, Patil S and Singh SK. *In vitro* regeneration in pomegranate cv. Ganesh from mature plant. *Indian J. Hortic.* 2004; 61(3):206-208.
- Tanaka M and Sakanishi Y. Clonal propagation of *Phalaenopsis* by leaf culture. *American Orchid Society Bulletin.* 1977; 46: 733- 737.
- Ziv M and Halevy AH. Control of oxidative browning and *in vitro* propagation of *Strelitzia reginae*. *Hortic. Sci.* 1983; 18(4): 434-436.

The references are so old – I suggest insert new ones.

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*		
		2 nd week of culture	Appearance of explant (2 nd week)	3 rd week of culture	Appearance of explant (3 rd week)	4 th week of culture	Appearance of explant (4 th 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*		
		2 nd week of culture	Appearance of explant (2 nd week)	3 rd week of culture	Appearance of explant (3 rd week)	4 th week of culture	Appearance of explant (4 th 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 before inoculation 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.	Treatm ent details	Browning intensity in medium*						Percentage of cultures*		
		2 nd week of culture	Appeara nce of explant (1 st week)	3 rd week of culture	Appeara nce of explant (5 th week)	4 th week of culture	Appearance of explant (8 th 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

UNDER PEER REVIEW

