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Minimizing medium browning during *in vitro* cultures of herbaceous peony (*Paeonia lactiflora* Pall.)

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ABSTRACT

One of the major problems associated with peony tissue culture is the browning of culture media, which invariably leads to death of explants. All the five types of explant used in the present study resulted in media browning, which was maximum with leaf and ovary segments, while minimum with petiole segments and underground buds. Incorporation of chemicals into the medium and incubation under different conditions had a significant influence on media browning. Polyvinyl pyrrolidone (5.0 g/l) proved best chemical for controlling browning, while antioxidants (citric acid and ascorbic acid) failed to suppress the problem. Incubation of explants in a refrigerator for 48 h at 4°C was found best for minimizing the media browning of all explants types. Growing of stock plants under low light intensity drastically reduced the leaf segment media browning to 8.69% compared to 83.25% as observed with explants grown under open field conditions. Harvesting of leaves at different stages of development significantly reduced the media browning from a maximum of 89.09% with fully-expanded pink leaves to a minimum of 0.91% with unexpanded inner green leaves of terminal bud.

Key words: Medium browning, herbaceous peony, antioxidants, polyvinly pyrrolidone, micropropagation.

INTRODUCTION

Peony is one of the most valuable ornamental plants highly demanded for garden decoration and grows best in temperate climates. It is one of the best cut flower with good vase-life and sweet fragrance. Planting material of herbaceous peony is scarce because its propagation coefficient is low in nature and the traditional propagation methods are low yielding and time consuming. Development of micropropagation methods for peonies is an alternative for their mass production but is constrained by many problems among which culture medium browning is a serious one.

Explants of some plant species exude phenolic substances or secondary metabolites from cut surfaces and turn the media brown, which is toxic to the explants. In normal tissue, no browning happens, because polyphenol oxidase (PPO) and phenolic compounds are separated by a membrane structure but browning results when these structures are broken. Polyphenolic compounds are present in vacuoles, while the PPO is located in the cytoplasm in normal cells and browning happens when compartmentation is broken by any means. Phenols released by the explants into the medium are not detrimental for their establishment and survival but their oxidative products produce harmful affects. Polyphenols are oxidized by air or polyphenoloxidases. Phenolic compounds upon oxidation get converted into stable compounds called quinones, which are highly reactive compounds that polymerize rapidly and form covalent bonds with proteins. Oxidized polyphenolic compounds inhibit enzyme activity and may result in the declined growth or lethal browning or death of the explants (Hu and Wang, 6).

Browning has been reported with all types of peony explants. Hansen *et al.* (4) observed the browning of culture medium to be a serious problem in the initial explant establishment and sub-culturing in herbaceous peony *in vitro* cultures. In this study, an attempt has been made to minimize medium browning in herbaceous peony through manipulation of physical environment and modification of the culture medium.

MATERIALS AND METHODS

Present investigations were carried out in the Biotechnology Laboratory of the Division of Pomology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar during 2006-2009. Five types of explants (leaf, stem, petiole, ovary and underground bud) were used to study the extent of browning in blank (control) and chemical supplemented Murashige and Skoog, 9 medium (MS). Explants were collected from mature stock plants and washed under running tap water to remove any adhering dirt and dust. Small sections of explants were surface sterilized under laminar hood with mercuric chloride (0.1%) for 10 min. and then treated with 70% ethyl alcohol for 10 sec. A few drops of Tween-20® were

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added to mercuric chloride solution for increasing its effectiveness. Explants were then washed five times with double distilled sterile water to remove traces of sterilants completely. Explants were prepared and inoculated in test tubes on half-strength Murashige and Skoog (9) medium blank or supplemented with different types of chemicals.

Various techniques were used for controlling the media browning. Chemicals like antioxidants (citric acid and ascorbic acid) and adsorbent (polyvinyl pyrrolidone) were incorporated into the medium before adjusting the pH at 5.8. Explants were then incubated under three different conditions (light, dark and refrigeration). Incubation under light was carried out by culturing the explants under 16/8 h light cycle in culture room at 24 ± 1°C. During dark incubation, test tubes with explants were kept in a cardboard box and incubated under culture room conditions at 24 ± 1°C. Refrigerated incubation was done by placing the inoculated test tubes in a refrigerator at 4°C. Following incubation under three different conditions for 48 h, all the cultures were kept in culture room under light at 24 ± 1°C for 48 h more and then data related to medium browning was recorded.

During the investigation, crowns of herbaceous peony were dug out in early spring when the buds were underground. They were washed carefully and planted in plastic pots filled with sterilized medium (sand + soil + sheep manure in 1:1:1 ratio). Pots were watered and kept in culture room conditions at 24°C under 16/8 light cycle for forced growth of stock plants. Fully expanded small green leaves were collected and used as explants after surface sterilization with mercuric chloride (0.1%) for 10 min. Leaf segments were cultured on half strength MS blank medium under three different incubation conditions along with another set of leaf segments from field grown stock plants. This was done to ascertain the influence of forcing under low light conditions upon the medium browning.

Peony plant passes through different stages from sprouting to flowering. Explants at different stages were taken to see their effect on media browning. Leaves were collected at four developmental stages from the field-grown stock plants of herbaceous peony – H1 (expanded pink leaf), H2 (expanded green leaf), H3 (outer unexpanded green leaves of terminal bud) and H4 (inner unexpanded green leaves of terminal bud). Experiments were laid out under completely randomized design with three replications. Data obtained in the experiments was subjected to analysis of variance using Minitab statistical package. Mean comparison was performed using Least Significant Difference (LSD) method. Percent data were Arc sine, transformed.

RESULTS AND DISCUSSION

All the five explant types were used in the present study resulted in media browning and confirmed the earlier findings of Radtke (10), and Hansen *et al*. (4) in peony. Majority of the explants did not show any sign of growth kept on the same medium. Portion of the explant inserted in the medium turned black which resulted in reduced explant growth/death. Browning intensity varied with explant type. It was maximum with ovary and leaf segments and minimum with petiole segments and underground bud. Profuse browning in leaves and ovary segments may be attributed to the fact that leaves are more exposed to sun light and contain more plastids, which are active sites of phenol synthesis in plants. Ovary segments contained ovules, which got injured during explant preparation and leached out phenolic compounds into the medium, which further complicated browning problem. Lang and Luo (7) also reported different browning rates with different types of explants in herbaceous peony.

Main effect of chemicals upon the media browning was significant for all types of explants except ovary segments (Table 1). Both the antioxidants individually or in combination failed to control the medium browning. All types of explants recorded maximum browning in media supplemented with citric acid/ ascorbic acid and in control. Ascorbic acid even intensified the browning phenomenon. PVP (5.0 g/l) proved the best chemical for reducing the medium browning. Combination of PVP with antioxidants decreased its efficiency. The positive role of PVP in browning reduction is supported by Yang and Pei (11) who reported that PVP (0.05%) prevented browning effectively in peony and improved growth of micro plantlets.

Effect of incubation conditions on media browning was significant. All types of explants resulted in maximum media browning, when incubated under light at 24 ± 1°C. Media browning was lowered to some extent, when incubation of explants was carried out under dark at 24 ± 1°C. However, reduction in the browning was substantial, when explants were incubated under refrigerated conditions at 4°C for 48 h (Table 2). Light stimulates phenol production, while dark conditions had a reverse effect. Habib *et al*. (3) reported higher medium browning with shoot tips of herbaceous peony within 2–3 h when incubated under light. However, less than 15% of explants placed in the dark for 48 h developed browning. Light was implicated in increased browning and probably resulted in increased phenol production. He *et al*. (5) also mentioned that browning degree appeared worse under light compared to dark.

Low temperature is beneficial for controlling browning, while high temperature accelerates the

Table 1. Main effect of chemicals on medium browning (%) by different types explants of herbaceous peony cv. Sara Bernhardt.

Chemical	Explant type				
	Leaf	Stem	Petiole	Ovary	Underground bud
Citric acid (CA) 0.2 g/l	100.00 (89.09)	45.56 (42.15)	31.11 (33.14)	100.00 (89.09)	26.67 (30.69)
Ascorbic acid (AA) 0.2 g/l	100.00 (89.09)	56.67 (48.90)	34.44 (35.36)	100.00 (89.09)	35.56 (36.26)
Polyvinylpyrrolidone (PVP) 5 g/l	64.44 (57.01)	12.22 (19.19)	10.00 (17.39)	97.78 (85.19)	5.56 (10.64)
CA 0.1 g/l + AA 0.1 g/l	92.22 (78.75)	44.44 (41.64)	28.89 (32.16)	100.00 (89.09)	21.11 (27.13)
CA 0.1 g/l + PVP 2.5 g/l	70.00 (62.07)	18.89 (25.49)	12.22 (20.24)	98.89 (87.14)	13.33 (17.51)
AA 0.1 g/l + PVP 2.5 g/l	74.44 (66.50)	20.00 (26.07)	14.44 (22.04)	98.89 (87.14)	10.00 (15.13)
Control	98.89 (87.14)	46.67 (42.72)	32.22 (34.34)	100.00 (89.09)	27.77 (31.12)
CD (P = 0.05)	5.547	4.142	3.939	NS	3.781

Data in parenthesis are Arc sine transformed values of the original percentages.

Table 2. Main effect of incubation conditions on media browning (%) by different types explants of herbaceous peony cv. Sara Bernhardt.

Incubation condition	Explant type				
	Leaf	Stem	Petiole	Ovary	Underground bud
Light	99.04 (87.87)	44.28 (41.26)	31.91 (33.72)	100.00 (89.09)	30.00 (32.56)
Dark	93.33 (79.71	39.04 (37.90)	24.28 (28.69)	100.00 (89.09)	20.47 (25.63)
Refrigeration	64.76 (59.41	21.42 (26.34)	13.81 (21.01)	98.10 (85.75)	9.52 (14.02)
CD (P = 0.05)	3.63	2.71	2.57	2.38	2.47

Data in parenthesis are Arc sine transformed values of the original percentages

phenomenon. Lang and Luo (7) reported increased browning rate at higher temperature in peony tissue culture. During refrigerated incubation in the present study, explants were incubated in a refrigerator at 4°C under complete darkness. Both these conditions – darkness and low temperature are beneficial for browning reduction. Hence, minimum medium browning was recorded under refrigerated incubation.

Interaction effect between chemicals and incubation conditions for each explant was recorded significant except ovary segments. Minimum medium browning was observed from leaf, stem petiole and underground buds, when explants were inoculated in medium supplemented with 5.0 g/l PVP and incubated under refrigerated conditions for 48 h (Table 3). Incubation of explants in PVP supplemented medium under refrigerated conditions proved best method for minimizing medium browning in herbaceous peony. Both these factors had a substantial interactive influence upon reduction of medium browning.

Trying to minimize phenolic oxidation *in vitro* by using different chemicals ignore the fact that the activity of many enzymes systems involved in the synthesis and oxidation of phenolic compounds are light-induced (Davies, 2). Various *in vivo* manipulations have been tried to ameliorate the internal phenol content of stock plants for minimizing browning problem. In the present study, forcing of stock plants proved

Table 3. Explant-wise interaction effects of chemicals and incubations condition upon medium browning (%) of different explants of herbaceous peony (*Paeonia lactiflora* Pall.) cv. Sara Bernhardt.

Chemical	Leaf			Stem			Petiole			Ovary			Underground bud		
	Light	Dark	Ref.	Light	Dark	Ref.	Light	Dark	Ref.	Light	Dark	Ref.	Light	Dark	Ref.
Citric acid (CA)	100.00	100.00	100.00	60.000	53.333	23.333	46.667	33.333	13.333	100.00	100.00	100.00	36.667	26.667	16.667
0.2 g/l	(89.09)	(89.09)	(89.09)	(50.76)	(46.92)	(28.78)	(43.07)	(35.21)	(21.14)	(89.09)	(89.09)	(89.09)	(37.22)	(30.99)	(23.85)
Ascorbic acid (AA)	100.00	100.00	100.00	66.667	60.000	43.333	50.000	36.667	16.667	100.00	100.00	100.00	50.000	36.667	20.000
0.2 g/l	(89.09)	(89.09)	(89.09)	(54.78)	(50.76)	(41.15)	(45.00)	(37.22)	(23.85)	(89.09)	(89.09)	(89.09)	(45.00)	(37.22)	(26.56)
Polyvinylpyrrolidone	93.333	80.000	20.00	16.667	13.333	6.667	13.333	10.000	6.667	100.00	100.00	93.33	10.000	6.667	0.000
(PVP) 5 g/l	(80.54)	(63.93)	(26.56)	(23.85)	(21.14)	(12.59)	(21.14)	(18.43)	(12.59)	(89.09)	(89.09)	(77.40)	(18.43)	(12.59)	(0.90)
CA 0.1 g/l +	100.00	96.977	80.000	53.333	50.000	30.000	36.667	30.000	20.000	100.00	100.00	100.00	23.333	23.333	16.667
AA 0.1 g/l	(89.09)	(83.25)	(63.93)	(46.92)	(45.00)	(33.00)	(37.22)	(33.21)	(26.07)	(89.09)	(89.09)	(89.09)	(28.78)	(28.78)	(23.85)
CA 0.1 g/l +	100.00	83.333	26.677	23.333	20.000	13.333	16.667	10.000	10.000	100.00	100.00	96.67	30.000	10.000	0.000
PVP 2.5 g/l	(89.09)	(66.14)	(30.99)	(28.78)	(26.56)	(21.14)	(23.85)	(18.43)	(18.43)	(89.09)	(89.09)	(83.25)	(33.21)	(18.43)	(0.90)
AA 0.1 g/l +	100.00	93.333	30.000	26.667	20.000	13.333	20.000	13.333	10.000	100.00	100.00	96.67	20.000	10.000	0.000
PVP 2.5 g/l	(89.09)	(77.40)	(33.00)	(30.99)	(26.07)	(21.14)	(26.56)	(21.14)	(18.43)	(89.09)	(89.09)	(83.25)	(26.07)	(18.43)	(0.90)
Control	100.000	100.00	96.677	63.333	56.667	20.000	40.000	36.667	20.000	100.00	100.00	100.00	40.000	30.000	13.333
	(89.09)	(89.09)	(83.25)	(52.77)	(48.84)	(26.56)	(39.23)	(37.22)	(26.56)	(89.09)	(89.09)	(89.09)	(39.23)	(33.00)	(21.14)
CD (P = 0.05)		9.608			7.174			6.823			NS			6.549	

Data in parenthesis are Arc sine transformed values of the original percentages.

Table 4. Influence of explant harvesting stage and incubation conditions on medium browning (%) using leaf segments in herbaceous peony cv. Sarah Bernhardt.

Stage of harvesting	Incubation condition			
	Light	Dark	Refrigerated	Mean
H1 = Expanded pink leaf	100.00 (89.09)	100.00 (89.09)	100.00 (89.09)	100.00 (89.09)
H2 = Expanded green leaf	100.00 (89.09)	100.00 (89.09)	96.67 (83.25)	98.89 (87.14)
H3 = Unexpanded outer green leaf	43.33 (41.15)	40.00 (39.23)	20.00 (26.56)	34.44 (35.65)
H4 = Unexpanded inner green leaf	0.00 (0.91)	0.00 (0.91)	0.00 (0.91)	0.00 (0.91)
Mean	60.83 (55.06)	60.00 (54.58)	54.16 (49.95)	
CD (P = 0.05) Stage of harvesting	= 2.998			
Incubation conditions	= 2.597			
Interaction	= 5.194			

Data in parenthesis are Arc sine transformed values of the original percentages.

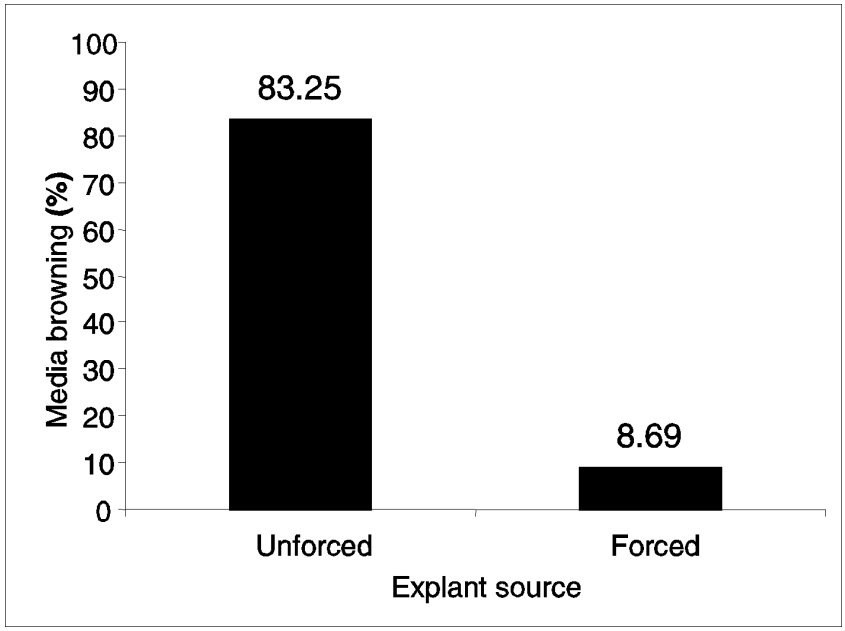

Fig. 1. Influence of explant source on medium browning with leaf segments of herbaceous peony.

very effective in controlling browning phenomenon in peony. Reduction in the media browning was substantial, when explants were collected from forced stock plants compared to explants collected from field grown stock plants where, browning was maximum (Fig. 1). This may be attributed to low synthesis of phenols by plants growing under low light conditions. Dalal *et al*. (1) reported that forcing treatment resulted in a pronounced reduction of endogenous levels of total phenols in explants of grapevine prior to culture, which was associated with substantial reduction of media browning and high *in vitro* explant survival. He *et al*. (5) reported higher browning rate of petioles of tree peony, when explants were collected from stock plants exposed to high light intensity than those grown under shady cover. Marks and Sally (8) also reported that phenolic oxidation was minimized or even eliminated when cultures of many woody plant species were initiated *in vitro* following collection of explants from stock plants grown under darkness

in field conditions or those exposed to 1% field irradiance.

Controlling the medium browning in peony without use of chemicals is highly desirable as many researchers have observed some negative effects of antioxidants and phenol adsorbents upon the explant growth in different crops. Explants collected from the same plant at four different developmental stages had a significant effect upon media browning (Table 4). Maximum browning was observed with explants collected at H_1 (fully expanded pink leaf) and H_2 (fully expanded green leaf) stages. Pink leaves contain more anthocyanin (water soluble phenolic compound) content, which probably got easily leached out when these leaves were used as explants and put into the medium. Pink leaves turn green on exposure to sunlight after 10 to 15 days. Sunlight has been found good for phenol synthesis in plants. Plant parts exposed to more sun light have more phenolic content than those which get lesser sunlight. Thus, higher browning was observed with green leaf explant also. Reduction in the browning was very substantial, when explants were collected at H_4 (unexpanded inner light green leaf) stage and incubated under refrigerated conditions. Inner leaves were light green in colour and had no exposure to direct sunlight, thus resulting in minimum browning. Lower browning of inner leaves may also be attributed to their younger or juvenile nature. Results obtained in the present investigation are supported by He *et al.* (5), who reported different browning rates, when explants were sampled at different stages of maturity.

During the present investigation, we succeeded in controlling the media browning in herbaceous peony with or without the use of chemicals. These techniques can be tried for increasing the explant establishment and survival not only in peony but also in other plant species, where browning is a problem.

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REFERENCES

1. Dalal, M.A., Sharma, B.B. and Rao, M.S. 1992. Studies on stock plant treatment and initiation culture mode in control of oxidative browning in *in vitro* cultures of grapevine. *Scientia Hort.* **51**: 35-41.

2. Davies, M.E. 1972. Polyphenol synthesis in cell suspension cultures of Paul's Scarlet. *Planta*, **104**: 50-56.

3. Habib, A., Donnelly, D.J. and D'Aoust, L. 2001. Micropropagation of herbaceous peony. *Amer. Peony Soc. Bull.* **319**: 19-24.

4. Hansen, C., Stephens, L. and Zhang, H. 1995. *In* vitro propagation of fern-leaf peony. *Amer. Peony Soc. Bull*. **296**: 7-10.

5. He, S.L., Chen, X.L., Chen, L., Kong, D.Z. and Ren, N.H. 2005. The preliminary researches on browning of leaf stalk in tissue culture of *Paeonia suffruticosa*. *Henan Sci*. **23**: 47-50.

6. Hu, C.Y. and Wang, P.J. 1983. Meristem, shoot tip and bud cultures. **In**: *Handbook of Plant Tissue Culture*, Vol. I. D.A. Evans, W.R. Sharp, P.V. Ammirato and Y. Yamada (Eds.), MacMillan, pp. 177-227.

7. Lang, Y.T. and Luo, X.F. 2007. The research on callus induction and browning prevention of peony. *J. Henan Forest Sci. Technol*. **27**: 4-6.

8. Marks, T.R. and Sally, E.S. 1990. Reduced phenolic oxidation at culture initiation *in vitro* following the exposure of field grown stock plants to darkness or low levels of irradiance. *J. Hort. Sci.* **65**: 103-11.

9. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. **15**: 473-97.

10. Radtke, G.W. 1983. Tissue culture of herbaceous peonies. *Amer. Peony Soc. Bull*. **246**: 19-23.

11. Yang, H.C. and Pei, D.L. 2006. Study on embryo culture of peony (*Paeonia* L.) seed. *Agric. Sci. Guangxi*, **37**: 108-10.

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