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COLD STRESS-INDUCED CALLOGENESIS FROM ISOLATED ANTHERS OF *CATHARANTHUS ROSEUS* (L.) G. DON

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ABSTRACT

Androgenic haploid production is a key tool for the production of homozygous plants within a short duration. In present study, the effect of cold stress as a pretreatment on anther culture of *Catharanthus roseus* (L.) G.Don was taken under consideration for callus induction. Cold pretreatment of buds at 8°C for 10 days and subsequent incubation on MS media in the dark was found to be best i.e. 59.99 ± 3.33%, for calli production. 2, 4 - D as a potent inducer of calli was taken at 10 mgL^{-1} and BAP in 2 mgL^{-1}, at a constant concentration, where the varying concentration of NAA at 0.5 mgL^{-1} was best suitable for callus production. Anther culture on Bioera anther culture teaching kit showed less potential for callus production at the same set of condition as compared to the optimized MS media.

KEY WORDS: 2,4- D, callogenesis, *Catharanthus roseus*, Cold stress, Pretreatment.

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INTRODUCTION

Catharanthus roseus (L.) G.Don or periwinkle is a tropical and subtropical plant originates from Madagascar and belongs to the family Apocynaceae. Androgenesis results in homozygous progeny from a heterozygous parent in a single generation and provides excellent material for research, plant breeding and plant transformation [1]. Production of haploids and dihaploid plants has been useful in providing access to recessive genes and for biotechnological manipulations [2]. Plants can be regenerated either via androgenesis direct embryogenesis from isolated microspores (the direct way) or via indirect embryogenesis and organogenesis from dedifferentiated callus tissues originated from meiocyte containing anthers (the indirect way). The induction of microspores to sporophytic instead of the gametophytic pathway is strongly influenced by some kind of stress treatment of the anthers before culture. The response to chilling or heat treatment is also genotype dependent. However, a temperature shock has been reported to improve the androgenetic response in many plant species [3, 4]. The first investigation on *in vitro* differentiation and regeneration was undertaken by Dhruva et al. [5] and observed the callus initiation and differentiation into roots. Hirata et al. [6] showed that growth and morphological differentiation such as the formation and development of shoots were affected by phytohormones and that these effects resulted in the changes in the level of alkaloids in culture. Murray et al. [7] reported higher levels of 2, 4-D or 2, 4, 5 - T increased callus production. Saradamani et al. [8] reported that callus induction was found to be best in 2, 4 - D. Because of the presence of the therapeutically valuable cytotoxic alkaloids vinblastine and vincristine etc. this plant has become one of the major fields of interest in modern plant cell biotechnology. The low yields of these dimericindole alkaloids (approx. 0.0005%) and the subsequent high price of them were major motives to study the possibilities for the production of these alkaloids by cell and tissue cultures [9]. The present investigation describes the essentiality of pretreatment and response of anthers towards cold temperature stress for callus induction at a high concentration of 2, 4 - D along with other two growth regulators combination.

MATERIALS AND METHODS

DONOR PLANT

Flower buds having anther at early to late uninucleate stage of development were taken as explants for studying callogenesis in *Catharanthus roseus* (L.) G. Don. A selection of buds could be done on the basis of morphology, which can be correlated with microspore developmental stage, as shown in figure 1. The Plant grown in Botanical Garden at Department of Botany, Sant Gadge Baba Amravati University, Amravati having GPS location N 20°56.364', E 077°48.052' was taken as donor plant. In order to determine the anther development stage, the content of 1 anther from each flower bud was smeared in acetocarmine.

PRETREATMENT OF ANTHER

Excised floral buds having anther at early to late uninucleate stage of development were kept in polythene bags and faced to following cold pretreatments

A. 8°C for 5 days; and
B. 8°C for 10 days

ANTHER CULTURE

Pretreated floral buds then subjected to surface sterilization prior to inoculation on nutrient media. During surface sterilization, firstly floral buds were washed for 1 minute in double distilled water then single washed in 0.1% $HgCl_2$ for 2 minutes. After that, 3 successive washes in double distilled water were given for 2 minutes. Later, anthers were excised from buds for inoculation.

The inoculated anthers were kept in 2 different culture conditions i.e.

A. Temperature 25 ± 1°C; Relative Humidity 50 – 60% and 16 hours light period followed by 8 hours dark period
B. Temperature 25 ± 1°C; Relative Humidity 50 – 60% and complete 24 hours dark period.

NUTRIENT MEDIA SELECTION

A. Anthers were inoculated on Himedia's MS media, PT021 [10], with $CaCl_2$ and Vitamins without Sucrose and Agar. Sucrose and Agar were added in the concentration of 30gmL^{-1} and 8gmL^{-1} respectively. For studying callus induction as an effect of cold temperature stress on anther culture, concentration of 2, 4 - D was kept constant and relatively high in two combinations of experimental sets as 10 mgL^{-1} (D_{10}) while NAA and BAP was kept at 0.5 mgL^{-1}($N_{1/2}$) and 2 mgL^{-1} (B_2) respectively for one set and for another set both were kept at 2 mgL^{-1} (N_2) concentration.

B. For the same pretreatment another readymade media was utilized for culture viz. Bioera anther culture teaching kit BTK 160911.

Controlled sets (without any cold pretreatment) were also run while working with same media and growth regulator combinations.

DATA ANALYSIS

All experiments were repeated thrice and the data taken was subjected to ANOVA at 95% confidence interval through Graphpad Prism version 6.

RESULTS AND DISCUSSION

Anthers at early to late uninucleate stage of development were selected for inoculation after a cold pre-treatment. The effect of cold pre-treatment and growth regulators were significant ($p < 0.05$) for anther callus induction. Also, it was confirmed that interaction effect of both factors was significant at 95% confidence level. The earliest response of anther was noted in 15 days in 5 days cold pre-treated culture for both the growth regulator combination maintained in complete dark (table 1) and also in both 5 and 10 days cold pre-treated culture at NAA concentration of 2 mgL^{-1}, where other two growth regulators are at constant concentration, exposed to 16 hours light period, as shown in table 2. The entire callus generated (except *)

shows pale yellow to white coloration and all were rough and hard. The highest percentage of callus induction i.e. 59.99 ± 3.33 was observed in culture maintained in complete dark (table 1, Figure 2). This result agrees with the previous result [11] where 70% callus induction was observed in Japonica and Japonica × Indica hybrids incubated in the dark. Callus induction rate was found to be zero in control (without cold pre-treatment; table 1 – 4). This finding coincides with the results obtained by Ayed et al. [12] while studying on durum wheat. The observed zero callus induction rate confirms the importance of pretreatments in anther culture experiments. Likewise, Sopory and Munshi [2] stated that temperature is the most important factor that influences the pollen callus/embryo induction. Reddy et al. [13] concluded that pretreatment of anthers by low and elevated temperatures had a stimulatory effect for callus induction in Rice. The present study revealed that, buds treated at 8°C for 10 days gives best callus induction percentage whereas the lowest percentage of callus induction for the same experimental set was observed in buds treated for 5 days at the same temperature as shown in table 1. Callus induction rate observed in table1 was found to be the best amongst all other three experimental sets (table 2 – 4). From the all other cultures, the lowest rate of callus production was observed in buds pretreated at 8°C for 10 days but in 16 hour light period. In these experiments, cultures on optimized MS media gives better results as compared to Bioera anther culture teaching kit. Among the cultures on Bioera anther culture teaching kit maximum calli induction was observed on 10 days cold pretreatment of buds and incubated in dark and minimum was observed on 5 days cold pretreatment and incubated in 16 hours light period (table 3 – 4). After compared both culturing media better results were obtained in the dark period, table 1 and 3 and comparatively low callus was obtained in 16 hours light period, table 2 and 4. Such finding justifies the need of dark period for incubation as mentioned previously. The present investigation is in agreement with studies conducted by many researchers on incubation of anther and pollen culture in complete dark period depending on the time of response [14, 15, 12]. Like present study importance of cold temperature pretreatment is also deduce by Herath et al. [11], where Cold pre-treatment at 8°C for 14 days gave the best performance in callus induction. Orbert et al. [16] have reported that the induction of callus from cultured anthers of flax was highest after cold pretreatment for 7 days at 8°C. From a few reports present on androgenic callogenesis in *Catharanthus roseus*, Kim et al. [17] also obtained over 50% of calli in anther culture of *C. roseus*. But, unlike present investigation, stress/pretreatment of buds prior to culture and incubation in complete dark period till calli development was not taken into account which assumes to be primary elements in androgenic plant regeneration [18, 19, 20, 21]. According to Pechan and Smykal [22], application of cold pretreatment has become an essential measure to increase the efficiency of androgenesis in many species. According to the report on wheat by Tomar and Punia [23]; Qin et al. [24]; Xing et al. [25] increase in the concentration of 2, 4-D in culture media produced good callus. Fazeli-nasab et al. [26] have used MS medium supplemented with 10 mg L^{-1} 2,4- D and 30 gmL^{-1} sucrose. As per Perera et al. [14] NAA in combination with 2,4-D promoted callus/embryo formation. Considering such callus enhancement effect of 2,4-D, it was taken in the concentration of 10 mg L^{-1} with a constant combination of BAP 2 mgL^{-1} and concentration of NAA varies as 0.5 and 2 mg L^{-1} in experiments conducted on MS media. Results revealed that maximum calli were present in NAA concentration at 0.5 mg L^{-1} maintained in dark (table 1, figure 2) and the minimum were present in 2 mg L^{-1} maintained in light/dark (table 2) at constant concentration of 2,4-D and BAP. In contrast, Peng and Wolyn [27] reported that the presence of 2,4- D with NAA resulted in the lowest callus induction in *Asparagus officinalis* whereas no callus production was observed with only 2,4- D. Caroline et al.[28] reported profuse growth of fragile callus in long term callus culture with 2.0 mg/l 2,4 – D and 5.0 mg/l BAP in *Bryonopsis laciniosa*. Whereas, unlike present report, Suthar and Shah [29] showed highest callus development from hypocotyls explants of *Capsicum annum* at a relative low concentration of 2,4-D i.e. 0.1 µM/L 2, 4-D + 0.1 µM/L BAP.

Table 1

Anther culture of C. roseus on MS media supplemented with growth regulators maintained in Dark

Sr. No.	Explant Number	Pretreatment	Growth Regulators	Days to callus induction	Total callus induced	% callus induced
1	30	Control	$D_{10}N_{1/2}B_2$	-	-	-
	30		$D_{10}N_2B_2$	-	-	-
2	30	5 days	$D_{10}N_{1/2}B_2$	15	7.33±0.58	24.44±1.92
	30		$D_{10}N_2B_2$	15	7.67±1.15	25.55±3.85
3	30	10 days	$D_{10}N_{1/2}B_2$	36	18.00±1*	59.99±3.33
	30		$D_{10}N_2B_2$	36	12.67±2.51	42.22±8.39

Out of all, only 1 callus was observed bright white in color

This experimental set was repeated three times. Mean within columns are significant at P<0.05.

Table 2

Anther culture of C. roseus on MS media supplemented with growth regulators maintained in Light/ Dark

Sr. No.	Explant Number	Pretreatment	Growth Regulators	Days to callus induction	Total callus induced	% callus induced
1	30	Control	$D_{10}N_{1/2}B_2$	-	-	-
	30		$D_{10}N_2B_2$	-	-	-
2	30	5 days	$D_{10}N_{1/2}B_2$	36	11.33±1.52	37.78±5.09
	30		$D_{10}N_2B_2$	15	8.67±0.58	28.89±1.92
3	30	10 days	$D_{10}N_{1/2}B_2$	36	5.33±1.52	17.78±5.09
	30		$D_{10}N_2B_2$	15	0.33±0.57	1.11±1.92

The experimental set was repeated three times. Mean within columns are significant at P<0.05.

Table 3

Anther culture of C. roseus on Bioera anther culture teaching kit maintained in Dark

Sr. No.	Explant No.	Pretreatment	Days to Callus induction	Total Callus induced	% Callus induced
1	25	Control	--	-	-
2	25	5 days	36	4.33±1.52	17.33±6.11
3	25	10days	36	7.33±2.08	26.67±8.32

The experimental set was repeated three times. Mean within columns are significant at P<0.05.

Table 4

Anther culture of C. roseus on Bioera anther culture teaching kit maintained in Light/ Dark

Sr. No.	Explant No.	Pretreatment	Days to Callus induction	Total Callus induced	% Callus induced
1	25	Control	--	-	-
2	25	5 days	40	1.33±0.58	5.33±2.30
3	25	10 days	35	4.00±1	16.0±4

The experimental set was repeated three times. Mean within columns are significant at P<0.05.

Figure 1

Morphological variation in floral bud with respect to pollen developmental stage (A: Meiotic bud; B: Pollen tetrad stage; C-F: Inoculation bud; G-L: Mature pollen)

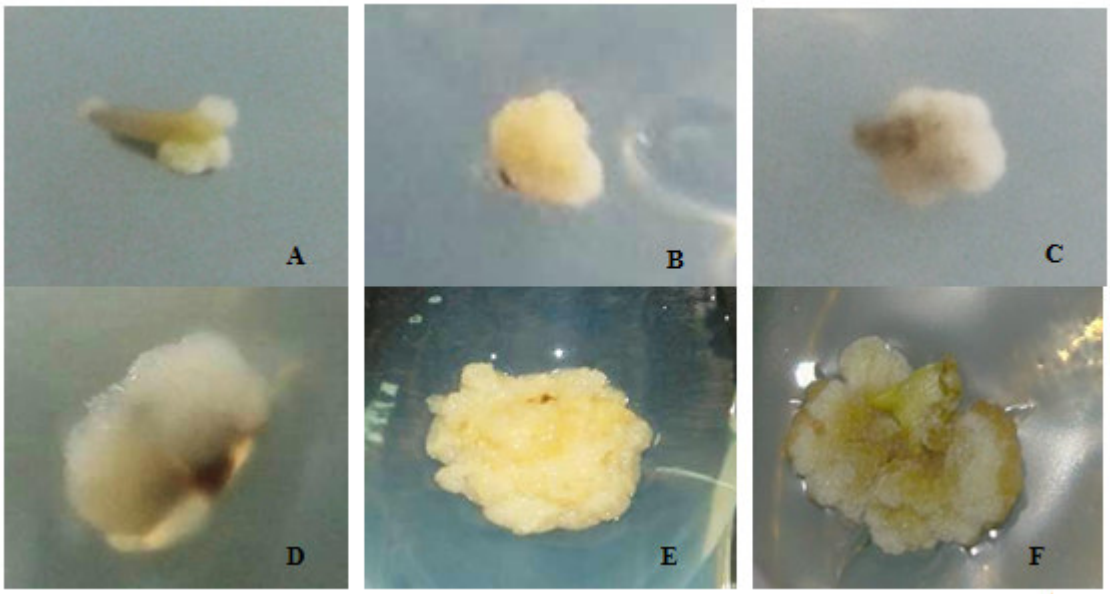

Figure 2
Successive stages in androgenic callogenesis in Catharanthus roseus (L.) G.Don. in cold preated buds at 8^0C for 10 days cultured on MS media $D_{10}N_{1/2}B_2$ maintained in the dark.

CONCLUSION

The present study concludes that the response of another culture in *C. roseus* mainly depends on the cold temperature pretreatment along with photoperiod duration and to some extent on growth regulators concentration. The percentage range of callus induction varied from 1.11±1.92 to 59.99±3.33. Best calli production i.e. 59.99 ± 3.33 % was observed in MS media with NAA 0.5 mg L^{-1}, BAP 2 mg L^{-1} and 2,4-D 10 mg L^{-1} in cold pretreated buds at 8°C for 10 days and cultured in the dark. Whereas least calli production i.e. 1.11 ± 1.92 % was observed in MS media with NAA 2 mg L^{-1}, BAP 2 mg L^{-1} and 2,4-D 10 mg L^{-1} in cold pretreated buds at 8°C for 10 days and cultured in the light/dark. Further studies are important on optimization of different pretreatment, growth regulators and media composition for the development of an efficient protocol for anther derived callus production in *C. roseus*.

REFERENCES

1. Wang M., Sandra V.B., Bert V.D. Insights into a key developmental switch and its importance for efficient plant breeding. Plant Physiology, 124: 523–530, (2000).
2. Sopory S.K., Munshi M. Anther culture. In: Jain S.M., Sopory S.K., Veilleux R.E. In vitro Haploid Production in Higher Plants, vol. I, Kluwer Academic Publishers, Dordrecht, The Netherlands, 145-176, (1996).
3. Xie J.H., Gao M.W., Liang Z.Q., Shu Q.Y., Cheng X.Y. Xue Q.Z. The effect of cool pretreatment on the isolated microspore culture and the free amino acid change of anthers in japonica rice (*Oryza sativa* L.). J. Plant Physiol. 151: 79–82, (1997).
4. Kiviharju E., Pehu E. The effect of cold and heat pretreatments on anther culture response of *Avena sativa* and *A. sterilis*. Plant Cell Tiss. Org. Cult. 54: 97–104, (1998).
5. Dhruva B., Ramakrishnan T. Vaidyanthan C.S. Studies in *Catharanthus roseus* callus culture, callus initiation and differentiation. Current Science 46 (11): 364 - 365, (1977).
6. Hirata K., Miyamoto K., Miura Y. *Catharanthus roseus* L. (Periwinkle): Production of Vindoline and Catharanthine in multiple shoot cultures. In: Bajaj Y.P.S. (eds.), *Biotechnology in Agriculture and Forestry*, *Vol.4: Medicinal and Aromatic plants I,* Springer- Verlag, Berlin Heidelberg, 46 - 55, (1994).
7. Murray W.N., James W.H., Thomas A.D., Kirby J.D. Long-duration, high frequency plant regeneration from cereal tissue cultures. Planta 157: 385-391, (1983).
8. Saradamani N., Muralimohan S., Sudhakar R., Pola S.V., Dora V.S.N. *In vitro* morphogenesis in cultivated varieties of *Sorghum bicolor* (L.) Moench. Plant Cell Biotechnology and Molecular Biology 4: 43-48, (2003).
9. Heijden R.V.D., Verpoorte R., Hensj G., Hoopen T. Cell and tissue cultures of *Catharanthus roseus* (L.) G. Don: a literature survey. Plant Cell, Tissue and Organ Culture 18: 231-280, (1989).
10. Murashige T., Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-97, (1962).
11. Herath H.M.I., Bandara D.C., Samarajeewa P.K., Wijesundara D.S.A. Effect of low temperature pre-treatment on anther culture in selected indica, japonica rice varieties and their inter sub-specific hybrids. Cey. J. Sci. (Bio. Sci.) 38 (1): 11-16, (2009).
12. Ayed O.S., Buyser J.De., Picard E., Trifa Y., Amara H.S. Effect of pre-treatment on isolated

microspores culture ability in durum wheat (*Triticum turgidum subsp.Durum Desf.)*. Journal of Plant Breeding and Crop Science 2(2): 030-038, (2010).

13. Reddy V.S., Leelawathi S., Sen S.K. Influence of genotype and culture medium on microspore callus induction and green plant regeneration in anthers of *Oryza sativa.* Physiol. Plant. 63: 309–314, (1985).
14. Perera P.I.P., Yakandawala D.M.D., Hocher V., Verdeil, Weerakoon L.K. Effect of growth regulators on microspore embryogenesis in coconut anthers. Plant Cell Tiss. Org. Cult. 96: 171–180, (2009).
15. Tahir S.M., Victor K., Abdulkadir S. The Effect of 2, 4-Dichlorophenoxy Acetic Acid (2, 4-D) Concentration on Callus Induction in Sugarcane *(Saccharum officinarum)*. Nigerian Journal of Basic and Applied Science 19 (2): 213-217, (2011).
16. Obert B., Dedieova B., Hricova A., Samaj J., Pretova A. Flax anther culture: effect of genotype, cold treatment and media. Plant Cell Tiss. Org. Cult. 79 (2): 233–238, (2005).
17. Kim S.W., Song N.H., Jung K.H., Kwak S.S., Liu J.R. High frequency plant regeneration from anther-derived cell suspension cultures via somatic embryogenesis in *Catharanthus roseus*. Plant Cell Rep, 13: 319-322, (1994).
18. Hu T., Kasha KJ. Cytological study of pretreatments used to improve isolated microspore cultures of wheat (*Triticum aestivum L.*) c.v. chris. Genome 42: 432-441, (1999).
19. Touraev A., Tashpulatov A., Indrianto A., Barinova I., Katholnigg H., Akimcheva S., Ribarits A., Voronin V., Zhexsembekova M., Heberle- Bors E. Fundamental aspects of microspore embryogenesis. In: *Biotechnological approaches for utilization of gametic cells*. Cost 824 final meeting, Bled, Slovenia, 205-214, (2000).
20. Liu W., Zheng M.Y., Konzak C.F. Improving green plant production via isolated microspore culture in bread wheat (*Triticum aestivum L.*). Plant Cell Rep. 20: 821-824, (2002).
21. Zheng M.Y., Liu W., Weng Y., Polle E., Konzak C. F. Production of doubled haploids in Wheat (*Triticum aestivum*L.) through microspore embryogenesis triggered by inducer chemicals. In: M. Maluszynski et al. (eds.), *Doubled Haploid production in crop plants*, 83 – 94, (2003).
22. Pechan P.M and Smykal P, Androgenesis: affecting the fate of the male gametophyte. Physiol. Plant, 111:1-8, (2001).
23. Tomar PC and Punia MS. Callus induction and efficient plant regeneration in wheat (*Triticum aestivum* L.). Annals of Agri-Bio-Research. 8(1): 1-4, (2003).
24. Qin C, Dong Z, Lin W. Deng and Tang L. Effect of exogenous Plant growth regulators on *in vitro* regeration of Cotyledonary explants in Pepper. Not. Bot. Hort. Agrobot. Cluji, 33 (1): 25 - 32, (2005).
25. Xing Z.Y., Yuan Y.H., Wang L.F., Zheng L.P. Regenerating Plants from in vitro culture of *Erigeron breviscapus* leaves. African Journal of Biotechnology 9(26): 4022-4024, (2010).
26. Fazeli-nasab B., Omidi M., Amiritokaldani M. Callus induction and plant regeneration of wheat mature embryos under abscisic acid treatment. International Journal of Agriculture and Crop Sciences 4 (1): 17-23, (2012).
27. Peng M., Wolyn D.J. Improved callus formation and plant regeneration for shed microspore culture in asparagus (*Asparagus officinalis* L.). Plant Cell Rep 18: 954–958, (1999).
28. Caroline V.J.E., Madhavi S., Mallaiah B. *In Vitro* Callogenesis in *Bryonopsis laciniosa* (L). Naud. International Journal of Pharma and Biosciences 3 (2): 703-718, (2012).
29. Suthar R.S., Shah K.R. Optimization of callus and cell suspension culture of *Capsicum annum* L. International Journal of Pharma and Biosciences 6 (4): 664-671, (2015).