Tropical and Subtropical Agroecosystems, 12 (2010): 283 - 288

Tropical and Subtropical Agroecosystems

EFFECT OF AUXIN AND CYTOKININ ON VINCRISTINE PRODUCTION BY CALLUS CULTURES OF *Catharanthus roseus* L. (APOCYNACEAE)

[EFECTO DE AUXINA Y CITOQUININA EN LA PRODUCCIÓN DE VINCRISTINA POR CULTIVOS DE CALLOS DE *Catharanthis roseus* L. (APOCYNACEAE)]

Chinnamadasamy Kalidass[1], Veerabahu Ramasamy Mohan[1*] and Arjunan Daniel[2]

[1]*Ethnopharmacology unit, Research Department of Botany*
V.O.Chidambaram College, Tuticorin-628008, Tamil Nadu, India.
e-mail: vrmohan_2005@yahoo.com
[2] *Institute of Forest Genetic Tree Breeding, Coimbatore, Tamil Nadu, India.*
**Corresponding author*

SUMMARY

Callus cultures of *Catharanthus roseus* L. were established to verify whether they produce vincristine as the intact plant. Different growth regulator combinations were applied to Murashige and Skoog (MS) medium to influence the level of production of vincristine. The effects of various combinations (0.5 µM to 3.0 µM) of auxin and cytokinin on the growth and accumulation of vincristine were investigated. MS medium supplemented with 2,4-Dichlorophenoxy acetic acid (2,4-D) 1.0 µM and 6-furfurylaminopurine (Kinetin) 1.0 µM was used to support the growth of callus cultures and the maximum amount of dry biomass (598.04 mg) was produced after seven weeks of culture. High performance liquid chromatographic (HPLC) analysis of methanol extracts from callus cultures of *C. roseus* revealed that the cultures produced vincristine. The concentrations of the growth regulators alpha-naphthalene acetic acid (NAA) and kinetin played a critical role in the production of vincristine.

Key words: Madagascar periwinkle; alkaloids; growth regulators.

RESUMEN

Se realizaron cultivo de callos de Catharanthus roseus L. para verificar su producción de vincristina. Se emplearon combinaciones auxina y citoquinina (0.5 µM a 3.0 µM) como reguladores de crecimiento para influir en la producción de vincristina. Se cultivo en medio Murashige y Skoog (MS) suplementado con 2,4-ácido diclorofenoxi acético (2,4-D) 1.0 µM y 6-furfurilaminopurina (Kinetin) 1.0 µM para soportar el crecimiento del callo y la producción máxima de biomasa seca (598 mg) se produjó a las siete semanas de cultivo. Análisis por HPLC de extractos metanólicos revelaron que los cultivos de *C. roseus* produjeron vincristina. Las concentraciones de los reguladores de crecimiento ácido acético alfa-naftaleno (NAA) y kinetin tuvieron un papel importante en la producción de vincristina.

Palabras clave: Alcaloides, reguladores de crecimiento; plantas medicinales.

INTRODUCTION

Catharanthus roseus L. (Apocynaceae) Madagascar Periwinkle is one of the most extensively investigated medicinal plants. It was found to contain a very large number of alkaloids, about 100 of which have been isolated so far (Verpoorte *et al*., 1997; Hughes and Shanks, 2002; Samuelsson, 1999). The root contains, the major alkaloids are ajmalicine and serpentine, which are used in the treatment of circulatory diseases (Lemmens *et al.,* 1999). The antitumor alkaloids are produced in trace amounts (0.0003% dry weight). The high prices of these anticancer products, ranging from $1 million to $3.5 million per kilogram, have led to a widespread research interest over the past 25 years in the development of alternative sources for the production of these compounds (Verpoorte *et al*., 1991). The importance of this plant is due to the presence of two bisindole antitumor alkaloids, vinblastine and vincristine. The vinblastine and vincristine can lower the number of white cells in blood. A high number of white cells in the blood indicate leukemia. So they act as anti-cancer drug. These alkaloids prevent mitosis in metaphase and they bind to tubulin, thus prevents the cell from making the spindles it needs to divide. The cellular pharmacology

and mechanism of action of vinca alkaloids (vincristine, vinblastine and vindesine) used in cancer chemotherapy have not been clearly established. Their intracellular binding to tubulin with subsequent dissolution of microtubules and arrest of cells in mitosis are considered necessary to mediate their cytotoxic action (Creasey, 1979). However, although these alkaloids have only minor structural differences and behave in the same way at the level of drug-tubulin interaction (Himes *et al.*, 1976; Owellen *et al.*, 1977), their toxicity and spectrum of clinical activity differ considerably.

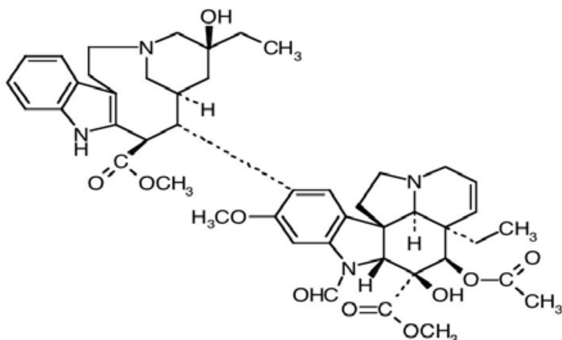

Figure 1. Vincristine (Jennifer *et al.*, 2006)

Plant cell and tissue culture have been suggested as a feasible technology for the production of many plant secondary metabolites. For example, ginsenoside from *Panax gingseng*; rosmarinic acid from *Coleus blumei*; shikonin from *Lithosermum erythrohizon*; diosgenin from *Dioscorea*; ubiquinone-10 from *Nicotiana tabacum*; berberin from *Coptis japonica* and podophyllotoxin from *Juniperus chinensis* accumulated at much higher levels in cultured cells than in intact plants (Misawa *et al.*,1985; Smith, 2002; Premjet *et al.*, 2002). Growth regulators 2, 4-D, NAA, BA and kinetin are frequently used to induce callus tissues in many plant species (Misawa, 1994). They were selected for initiation of callus cultures of *C. roseus*. Furthermore, MS medium supplemented with NAA, and kinetin were utilized by previous researchers for *in vitro* cultures of several other species (Catapan *et al.*, 2001; Catapan *et al.*, 2002; Liung and Lai, 2006; Kalidass and Mohan, 2009). The aim of this study is to develop conditions for callus cultures of *Catharathus roseus* L. by the manipulation of different combinations of plant growth regulators, with the aim of inducing vincristine production.

MATERIALS AND METHODS

Establishment of callus cultures

Leaf and stem segments from mature plants of *Catharathus roseus* L. were used in the present study. The explants were collected, washed thoroughly under running tap water for 15 min, treated in 1% Savlon for five min and were surface sterilized with 0.1% $HgCl_2$ for different lengths of time to ensure contamination-free culture. Thereafter the explants were washed three – four times with autoclaved distilled water to remove traces of $HgCl_2$ inside a laminar air flow cabinet. The surface sterilized shoots were cut into 1.0 - 1.5 cm long segments, each containing a single node. Explants of ca. 1 cm. were excised and individually transferred into 4-ounce glasses containing 20 ml of Murashige and Skoog (MS, 1962) culture medium, with 3 % (w/v) sucrose and differing combinations of NAA, 2, 4-D, BA and Kinetin. To test the effect of growth regulators, 32 treatments with factorial combinations of four levels of 2, 4-D, Kinetin, NAA and BA (0, 0.5, 1 and 2 µM) were designed. The pH value of the cultured medium was adjusted to 5.7 ± 0.1 prior to autoclaving (121°C, 15 min) and the medium was solidified with 6-8% agar. The culture conditions were maintained at 25 ± 1 °C with a 16 h photoperiod under a photon flux of 2,000-3,000 Lux, provided by fluorescent lamps. The explants were cultured for seven weeks and each treatment was repeated three times (Kalidass *et al.*, 2008). Callus cultures were subcultured every 30 days. The callus cultures of the third subculture were used to investigate the effect of 32 different combinations of growth regulators on growth and vincristine production. All combinations of growth regulators induced callus growth without an organogenesis response over 49 days of cultivation.

Growth measurement

Growth was measured as the dry weight (DW) of callus cultures grown for 50 days by harvesting the biomass, followed by lyophilization.

Extraction and determination of vincristine

Harvested cultures were immediately frozen at 80°C. Each of 32 lyophilized extracts from treated tissue samples (500 mg) was ground to a powder, followed by extraction with methanol for 24 hour. The extracts were then concentrated under vacuum and re-dissolved in a small amount of the same solvent before separation using High performance liquid chromatographic (HPLC). The vincristine content of each sample was determined using HPLC performed on a reversed-phased column (C18 Supelco ODS Hypersil, 4.6 x 250mm, 5 µm) in an Agilent 1100 liquid chromatograph with a diode array (UV detection at 280 nm) by isocratic elution with $MeOH/H_2O$ (50:50 v/v) as a mobile phase. The flow rate was set at 0.5 ml/min and the oven temperature was set to 40°C. The injection volume was 20µl. the methanolic extract of vincristine was identified by comparing its retention time with that of a reference sample of vincristine. The Thermo Separations Products Spectra system HPLC system used included a P4000 pump unit, an AS3000 auto sampler, and a UV2000 detector. For the

detection of vincristine, a protocol was adapted from the literature with UV detection at 280 nm (Tikhomiroff and Jolicoeur, 2002). Statistical analyses were performed with ANOVA. The Duncan multiple range test (DMRT) was used to compare means, with a significant level of 5% (SPSS Software for windows release 16.0 SPSS Inc., Chicago IL, USA). Data presented in tables correspond to the mean values of three replicates.

RESULTS AND DISCUSSION

Establishment of callus cultures of *C. roseus*

Callus cultures were initiated from young stems and leaves of C. *roseus* obtained from the well grown plants found in the Herbal garden, V.O.Chidambaram College, Tuticorin, Tamil Nadu, India. The overall response to plant regulators in stem segments was superior, while leaf explants resulted in poor callus induction. Friable greenish-yellow or beige callus was successfully induced from wound sites in the young stem explants at a culture time in the range of 5-10 days.

Effect of growth regulators on callus cultures growth

The production of secondary metabolites in callus cultures is controlled by environmental factors and by plant material. These factors are medium component, pH, temperature. Plant growth regulator such as auxins and cytokinins has shown the remarkable affects on growth and differentiation and thus metabolism of cultured cells (Zenk *et al.,* 1977; Brown, 1990). In order to obtain callus biomass and vincristine in high concentration, therefore, experiments were carried out varying of growth regulators of the medium. The effects of various concentrations of auxins (2, 4-D, NAA) and cytokinins (BA, Kinetin) on the growth of callus cultures derived from stem segment explants are presented in Figures 2 and 3. Five concentrations of NAA, 2, 4-D, BA and kinetin (0, 0.5, 1, 2, 3 µM) were varied in 32 combinations. All treatments were established with a fresh weight of 0.5 g of callus cultures. The stem-derived calluses showed a steady growth with a maximum up to seven weeks and decreased by the 60th day. Calluses contained water content approximately 90% of fresh weight. Figure. 2 shows that increasing level of BA resulted in a high cell dry biomass. In contrast, NAA in all concentrations had less effect in enhancing cell biomass. The highest percentage of cell dry weight was observed in combination of BA (2.0 µM) and NAA (2.0 µM). Figure.3 illustrates the dry mass of callus cultures grown in MS medium with different concentrations of 2, 4-D and Kinetin. Calluses cultured in medium without growth regulator showed the least dry biomass (34.56 mg). The maximum dry weight (598.04 mg) was observed for cultures containing 2, 4-D (1.0 µM) and kinetin (1.0 µM). The interaction of 2, 4-D and kinetin had a significant effect on cell dry weight. Among single growth regulators, kinetin alone increased cell biomass at the higher concentration (2.00 µM). These results demonstrated that, of the cytokinins supported growth of callus and provided higher biomass. However, of the two auxins, 2, 4-D had more effect on growth of callus when compared to the NAA.

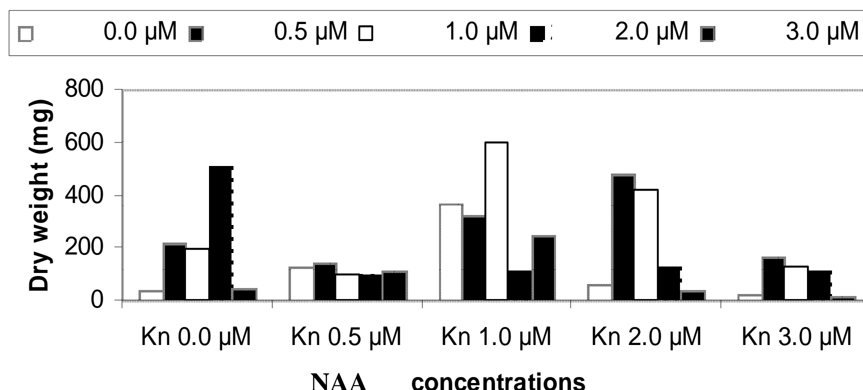

Figure 2. Effect of different combinations of NAA and BA on the growth of callus cultures of *C. roseus.*

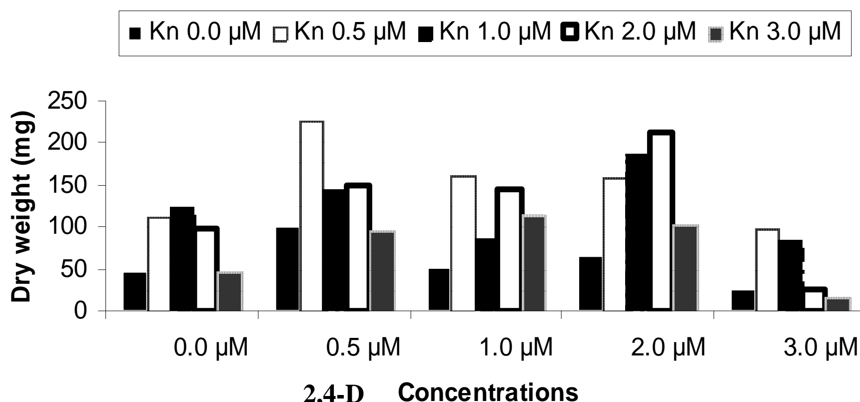

Figure 3. Effect of different combinations of 2,4-D and kinetin on the growth of callus cultures of *C. roseus*.

The effect of growth regulators on vincristine production

HPLC analysis was used to defect the presence of vincristine. Data presented in tables 1 and 2 demonstrate the effects of growth regulators on the content of vincristine. It was found that NAA, BA, 2, 4-D and KN have a significant effect on vincristine production in callus cultures of *C. roseus*. The accumulation of vincristine was observed for each of the combination of NAA and BA as well as 2,4-D and KN. Vincristine content were varied from 0 to 20.38 mg/g DW. Callus grown in MS medium without growth regulator produced vincristine in amount of 0.034 – 0.045 mg/g DW. Among single growth regulators, increasing in the level of 2, 4-D stimulated accumulation of vincristine while NAA performed contrast results. The callus gave low response in product accumulation to BA and KN. Though the combinations NAA and BA were less effective then combinations of 2,4-D and KN in promoting callus growth. They caused a marked increase in vincristine production. The greatest vincristine production (20.38 mg/g DW) resulted from the presence of 1.0 µM of NAA and 0.5 µM of BA, as compared to other combinations. We defect vincristine in callus grows on medium 2, 4-D and combination of 2, 4-D + KN of growth regulators that produced the maximum dry weight. In early reports exist on the effects of plant growth regulators on secondary metabolism of *in vitro* cultures, and in certain cases they enhance the isoquinoline alkaloid production in several plant species, for example, berberine in *Coptis japonica* (Nakagawa *et al*., 1986; Ikuta and Itokawa, 1989), and *Papaver somniferum* (Tyler *et al.,* 1989).

CONCLUSION

Callus culture of *C. roseus* can produce a considerable amount of vincristine under the standard culture condition. The optimum dosage of NAA and BA enhances the alkaloid production. Considering such a high content (maximum 20.38 mg/g) of vincristine in callus cultures and various important biological activities of vincristine, if desired, for further pharmacological exploitation through the production of vincristine using the improved culture.

Table 1. The effect of different concentration of NAA and BA on vincristine accumulation of *C. roseus* callus derived from stem cultured on MS medium.

NAA µM	BA µM	Vincristine (mg/g DW)
0.0	0.0	0.045[bcd]
0.5	0.0	2.346[bc]
1.0	0.0	1.316[a]
2.0	0.0	0.85[cde]
0.0	0.5	0.57[de]
0.5	0.5	0.976[bcde]
1.0	0.5	20.38
2.0	0.5	0.9267[bcde]
0.0	1.0	2.053[bc]
0.5	1.0	2.50[b]
1.0	1.0	2.103[bcd]
2.0	1.0	1.67[bcd]
0.0	2.0	0.663[de]
0.5	2.0	1.343[bcde]
1.0	2.0	0.6433[de]
2.0	2.0	2.39[abc]

Means in the same column with different letter(s) different significantly according to Duncan multiple range test ($P<0.05$)

REFERENCES

Brown, T.J., 1990. The Initiation and Mentainance of callus cultures, in Methods in Molecular Biology, vol.6, Plant cell and Tissue culture (Jeffrey, W.P. and John, M.W., Ed.). The Human Press, New Jersey, pp: 57 – 63.

Table 2. The effect of different concentration of 2,4-D and KN on vincristine accumulation of *C. roseus* callus derived from stem cultured on MS medium.

2,4-D μM	KN μM	Vincristine (mg/g DW)
0.0	0.0	0.034[de]
0.5	0.0	2.093[bcd]
1.0	0.0	1.76[bcd]
2.0	0.0	1.486[bcde]
0.0	0.5	2.060[bcd]
0.5	0.5	1.036[bcde]
1.0	0.5	0.303[ef]
2.0	0.5	NS
0.0	1.0	0.166[ef]
0.5	1.0	NS
1.0	1.0	NS
2.0	1.0	0.346[ef]
0.0	2.0	0.332[ef]
0.5	2.0	1.65[abcd]
1.0	2.0	9.31[a]
2.0	2.0	0.163[ef]

Means in the same column with different letter(s) different significantly according to Duncan multiple range test ($P<0.05$) NS - Not significant.

Catapan, E., Marcio, L., Silva, B., Moreno, F.N., Viana, A.M. 2002. Micropropagation, callus and root culture of *Phyllanthus urinaria* (Euphorbiaceae). Plant Cell Tissue and Organ Culture. 70: 301 – 309.

Catapan, E., Otuki, M.F., Viana, A.M. 2001. *In vitro* culture of *Phyllanthus stipulantus* (Euphorbiaceae). Revista Brasileria Botanica. 24: 25 – 34.

Creasey, W.A. 1979. The vinca alkaloids. In Antibiotics. Vol. 5, ed. Hahn, F.E., pp. 414-438. New York: Springer-Verlag.

Himes, R.H., Kersey, R.N., Heller-Bettinger, J. Samson, F.E. 1976. Action of the Vinca alkaloids vincristine, vinblastine, and desacetyl vinblastine amide on microtubules *in vitro*. Cancer Research, 36: 3798-3802.

Hughes, E.H., Shanks, J.V. 2002. Metabolic engineering of plants for alkaloid production. Metabolic Engineering. 4: 41–48.

Ikuta A., Itokawa H. 1989. In: Bajaj, Y.P.S. (eds) Biotechnology in Agriculture and Forestry. Vol. 7. Springer-Verlag, Berlin, pp 110–128.

Dennison, J.B., Kulanthaivel, P., Barbuch, R.J., Renbarger, J.L., Ehlhardt, W.J., Hall, S.D. 2006. Selective metabolism of vincristine in vitro by CYP3A5, Drug Metabolism and Disposition Fast Forward, 34: 1317 – 1327.

Kalidass, C., Mohan, V.R. 2009. *In vitro* Rapid clonal propagation of *Phyllanthus urinaria* Linn. (Euphorbiaceae)- a medicinal plant. Researcher, 1(4): 56 – 61.

Kalidass. C., Manickam, V.S., Glory, M. 2008. *In vitro* Micropropagtion of *Leptadenia reticulata* (Retz.) Wight. and Arn. (Asclepidaceae), Indian Journals of Multidisciplinary Research, 4: 221 – 225.

Lemmens, R.H., Bunyapraphatsara, M.J., Padua de L.S., N. 1999. Plant Resources of South-East Asia No 12(1) Medicinal and Poisonous Plants 1. Prosea Foundation, Bogor, Indonesia. pp: 386-387.

Liung, O.P., Lai, C.H. 2006. *In vitro* plant regeneration, flowering and fruiting of *Phyllanthus urinaria* (Euphorbiaceae). International Journal of Botany. 2: 409 – 414.

Misawa, M. 1994. Plant Tissue Culture: An Alternative for Production of useful metabolites (FAO Agricultural services Bulletin), Bio International Inc, Toronto, Canada, pp: 18 -19.

Misawa, M., Hayashi, M., Takayama, S. 1985. Accumulation of Antineoplastic Agents by Plant Tissue Cultures. In: Neumann K.H., Primary and Secondary Metabolism of Plant Cell Cultures, Springer-Verlag, Berlin Heidelberg. pp: 235-246.

Murashige, T, Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plantarum 15: 473–479

Nakagawa, K., Fukui, H., Tabata, M. 1986. Hormonal regulation of berberine production in cell suspension cultures of *Thalictrum minus*. Plant Cell Report. 5: 69–71.

Owellen, R.J., Donigian, D.W., Hartke, C.A., Hains, F.O. 1977. Correlation of biologic data with physicochemical properties among the vinca alkaloids and their congeners. Biochemistry. Pharmacology. 26: 1213-1219.

Premjet, D., Itoh, K., Tachibana, S. 2002. Stimulation of the production of Podophyllotoxin by biogenetic precursors and elicitor in *Juniperus chinensis* stem-derived callus cultures, Pakistan Journal of Biological Science. 5: 131-316.

Samuelsson, G. 1999. Drugs of natural origin, 4 ed. Swedish Pharmaceutical Press; p. 487.

Smith, M., 2002. An *in vitro* approach to investigate medicinal chemical synthesis by three herbal plants. Plant Cell Tissue Organ Culture. 70: 105-111.

Tyler, R.T., Eilert, U., Rijnders, C.O.M., Roewer, I.A., McNabb, D.K., Kurz, W.G.W. 1989. Studies on benzophenanthridine alkaloid production in elicited cell cultures of *Papaver somniferum*. In: Kurz, W.G.W. (ed.). Primary and Secondary Metabolism of Plant Cell Cultures II. Springer-Verlag, Berlin, pp. 200–207.

Verpoorte, R., van der Heijden R., Moreno, P.R.H. 1997. Biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus* cells. In: Cordell G.A. (ed). Alkaloids, vol. 49. Academic Press; p. 221.

Verpoorte, R., van der Heijden, R., Van Gulik, W.M., Ten Hoopen, H.J.G. 1991. Plant biotechnology for the production of alkaloids present status and prospects. In the Alkaloids; Brossi, A. (ed). Academic Press San Diego, vol. 40. pp 1 – 187.

Tikhomiroff, C., Jolicoeur, M., 2002. Screening of *Catharanthus roseus* secondary metabolites by high-performance liquid chromatography. Journal of Chromatography. A 955, 87–93.

Zenk, M.H., El-Shagi, H., Arens, H., Stockigt, J., Weiler, E.W., Dues, B. 1977. Formation of indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus.* In: Barz, W., Reinhard, E., Zenk, M.H. (eds.). Plant Tissue Culture and its Biotechnological Applications. Springer, Berlin, pp. 27–44.

Submitted June 11, 2009 – Accepted October 26, 2009
Revised received November 09, 2009