Effect of different culture medium components on production of alkaloid in callus tissues of *Cereus peruvianus* (Cactaceae)

Luciano Kleber da Rocha[1], Arildo José Braz de Oliveira[2], Claudete Aparecida Mangolin[1] and Maria de Fátima Pires da Silva Machado[1*]

*[1]Departamento de Biologia Celular e Genética, Universidade Estadual de Maringá. [2]Departamento de Farmácia e Farmacologia, Universidade Etadual de Maringá, 87020-900, Maringá, Paraná, Brazil. *Author for correspondence. e-mail: mfpsmachado@uem.br*

ABSTRACT. The production of alkaloids from *Cereus peruvianus* callus cultured in medium supplemented with different tyrosine concentrations, different 2,4-D/kinetin levels, and with NaCl was investigated using long-term callus culture. α- and β-Esterase isozyme patterns were also analyzed to investigate the differential gene expression in callus subcultured with the different tyrosine concentrations. The greatest amounts of alkaloids were obtained from callus maintained in medium containing 200 mg/L of tyrosine. Tyrosine induced a differential gene expression for the synthesis of specific α and β-esterase isozymes. Two α-carboxylesterases (EC 3.1.1.1) and one α/β-arylesterase (EC 3.1.1.2) were induced in callus, one α/β-acetylesterase (EC 3.1.1.6) and two α-carboxylesterase were detected as more intensely stained while two α/β-carboxylesterases were absent or detected as more weakly stained bands after the addition of tyrosine as precursor.

Key words: alkaloids, cactus, callus tissue culture, columnar cactus, mandacaru, tyrosine precursor.

RESUMO. Efeito de diferentes componentes no meio de cultura na produção de alcalóides em tecidos de calos de *Cereus peruvianus* (Cactaceae). Tecidos de calos em cultura prolongada de *Cereus peruvianus* foram utilizados para investigar a produção de alcalóides pelos calos quando mantidos em meio de cultura suplementados com diferentes concentrações de tirosina, com diferentes níveis de 2,4-D/cinetina, e contendo NaCl. Foi analisado também, o padrão de isozimas α- e β-esterases, para investigar a expressão diferencial de genes nos calos subcultivados com as diferentes concentrações de tirosina. A maior quantidade de alcalóides foi obtida a partir de calos mantidos em meio contendo 200 mg/L de tirosina. A adição de tirosina no meio induziu uma expressão gênica diferencial para a síntese de isozimas α- e β-esterases. Duas α-carboxilesterases (EC 3.1.1.1) e uma α/β-arilesterase (EC 3.1.1.2) foram induzidas nos calos, uma α/β-acetilesterase (EC 3.1.1.6) e uma α-carboxilesterase foram detectadas como bandas mais intensamente coradas, enquanto duas α/β-carboxilesterases não foram detectadas ou foram fracamente coradas após a adição de tirosina como precursor no meio de cultura.

Palavras-chaves: alcalóides, cactos, cultura de tecidos de calos, cactos colunar, mandacaru, tirosina.

Introduction

Most investigations have used plant cell culture, with considerable advantage to living plants, for the production of secondary metabolites (Kutney, 1996; Sato *et al*., 2001). Cell culture is beneficial due to the fact that useful metabolites are obtained under a controlled environment, regardless of climatic changes and soil conditions. In addition, the products are free from microbe and insect contamination, and cloning of cell lines provides further optimization for end product yields. Living plants generally present various concentrations of the target compounds, which may depend on specific seasons during which plant collection is performed (Salmore and Hunter, 2001; Puricelli *et al*., 2002; Ralphs and Gardner, 2001, 2003). This is much more difficult if the desired plant species grow in geographically or politically inaccessible regions.

In the cactus *Cereus peruvianus*, comparison of total alkaloid production from plants with callus tissues indicated that larger amounts of alkaloids were obtained from long-term callus tissues. In their

turn, the latter had been cultured in original medium and from callus tissue cultured in medium containing tyrosine as a supplement (Oliveira and Machado, 2003). Based on these results, tyrosine in *C. peruvianus* callus tissue may be incorporated into the alkaloid pathway and act directly as a precursor of tyramine synthesis, which is the main alkaloid in callus tissue. Actually, tyrosine has also been considered a precursor for numerous secondary metabolites (Jensen, 1986; Croteau *et al*., 2000).

Production and induction media for alkaloids have been well characterized in other plant species in terms of high sugar concentrations and different ammonium/nitrate ratios (Mérillon *et al*., 1986; Schripsema and Verpoorte, 1992). Concentration and carbon source, basal medium components, as well as concentration and type of growth regulator, pH, temperature, light intensity, photoperiod, and stress conditions are additional factors that may increase productivity of cultured plant cells (Moreno *et al*., 1995; Kodja *et al*., 1989; Zhao *et al*., 2001). Cytokinins have also been reported to stimulate alkaloid synthesis by cultures from different plant cell tissue (Rhodes *et al*., 1986; Teramoto and Komamine, 1988; Arvy *et al*., 1994; Hara *et al*., 1994).

In the present study, we investigated the production of alkaloids from callus tissue cultured in medium supplemented with different tyrosine concentrations, at different 2,4-D/kinetin levels, and in medium supplemented with sodium chloride. Long-term callus tissue growing in original medium served as a comparative control. α- and β-Esterase isozyme patterns were also analyzed to investigate the differential gene expression in callus tissues subcultured in medium containing different tyrosine concentrations. The addition of tyrosine in the culture medium may change the expression of esterase genes.

Material and methods

Callus culture

Callus tissues of *C. peruvianus* were obtained from six hypocotyls which were cut and used as explants for callus induction in MS medium (Murashige and Skoog, 1962) containing B5 vitamins (Gamborg *et al*., 1968), 0.8% agar, 3% sucrose, 4.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.0 mg/L N-(2-furanylmethyl)-1H-purine-6 amine (kinetin), or 4.0 mg/L 2,4-D and 8.0 mg/L kinetin, and mantained at 32°C under a 16 h photoperiod (15 $m^{-2} \cdot sec^{-1}$ light intensity). Non-regenerant callus tissues were subcultured in fresh medium at 25 to 30 day intervals (Oliveira *et al*., 1995).

Callus tissues from long-term cultures (13-years-old) induced in medium containing 4.0 mg/L 2,4-D and 4.0 mg/L kinetin were transferred to petri dishes (9 callus pieces/dishes) containing fresh medium supplemented with 50, 100, 150, and 200 mg/L tyrosine. So that the effect of different 2,4-D/kinetin levels could be assessed, callus tissues were transferred to petri dishes containing fresh medium with 2.0 mg/L 2,4-D and 4.0 mg/L kinetin, and 1.0 mg/L 2,4-D and 4.0 mg/L kinetin. Further, callus tissues were transferred to petri dishes containing fresh medium with NaCl 50 mM so that salt stress could be analysed. Callus tissues from long-term cultures, transferred to the original fresh medium (4.0 mg/L 2,4-D and 4.0 mg/L kinetin) without tyrosine and salt, were used as control to verify the effect of different culture media supplements on production of alkaloid in callus tissues of *C. peruvianus*. The three experiments were done in triplicate.

Extraction and isolation of alkaloids extracts

Callus tissues of *C. peruvianus* were lyophilized separately. The freeze-dried material (1 g) was ground and refluxed for 1 hour with a solution of 2% acetic acid prepared with 70% ethanol (50 mL, v/v). After filtration, ethanol was evaporated under reduced pressure to produce an acid aqueous solution. The solution was alkalinized with NH_4OH to reach pH 8.0-9.0, and extracted three times with the same volume of CH_2Cl_2. Extract was subsequently evaporated under reduced pressure and the residue was dissolved in 10 mL of ethanol.

Identification of alkaloids

Alkaloids tyramine and hordenine were isolated and identified by analyzing their physical and spectral properties (Oliveira and Machado, 2003). Alkaloids in the ethanol extracts were identified by thin-layer chromatography, silica gel G60 saturated with 0.1 N KOH, using $CHCl_3$:Methanol (95:5, v/v) as eluent and ninhydrin spray was used as the detecting reagent, by comparison with authentic standards of tyramine (R_f = 0.12) and hordenine (R_f = 0.66).

Alkaloids analysis

Alkaloid concentration was determined by an ultraviolet-spectrophotometer (Varian Carey-1E). UV detection wavelength was 274 nm and tyramine (1 mg/mL, Sigma-Aldrich Co., St. louis, MO) was used as standard, since tyramine and hordenine were

the alkaloids previously identified from *C. peruvianus* (Oliveira and Machado, 2003).

Results and discussion

Comparison of total alkaloids production from *C. peruvianus* callus tissues indicated that larger amounts of alkaloids were obtained from callus tissues maintained in medium containing 200 mg/L of tyrosine as supplement (Table 1). Since tyrosine is the precursor of tyramine, supplementation of *C. peruvianus* callus culture with tyrosine resulted in increased yields of tyramine. The induction of alkaloid production in cell culture has been primarily attributed to a remarkable increase in the activity of specific methyltransferase (Hara *et al*., 1994).

Table 1. Alkaloid production from callus tissue of *Cereus peruvianus* cultured under original conditions (MS medium and absence of tyramine) and in MS medium containing 50, 100, 150, 200 mg/L tyrosine, and containing proportions 4/4, 2/4, 1/4 of 2,4-D/ KIN, and 4/4 2,4-D/ KIN proportion and 50 mM NaCl.

	Total alkaloid production (mg/g of dry wt)
Tyrosine mg/L	
Zero	2.623 ± 0.679 [a]
50	2.160 ± 1.079 [a]
100	2.820 ± 0.463 [a]
150	2.158 ± 1.456 [a]
200	3.116 ± 0.418 [b]
2,4-D / Kin proportion	
4/4	2.623 ± 0.679 [a]
2/4	2.480 ± 0.337 [a]
¼	1.470 ± 0.636 [b]
4/4 + 50 mM NaCl	2.110 ± 1.183 [a]

± Standard deviation for triplication experiments for each treatment. Mean following by the same letter are non significant by Turkey test (SAS System copyright 1999-2000 by SAS Institue Inc., cory, NC, USA)]

The continuous exposure of *C. peruvianus* callus tissues (30 days) in medium containing tyrosine induced differential gene expression for the synthesis of specific α- and β-esterase isozymes. Esterase isozyme patterns in long-term callus culture (Figure 1) showed that two α-carboxylesterases (EC 3.1.1.1; EST-6 and EST-7 isozymes) and one α/β-arylesterase (EC 3.1.1.2; EST-8 isozyme) are induced in callus which had been cultured with tyrosine; one α/β-acetylesterase (EC 3.1.1.6; EST-2 isozyme) and two α-carboxylesterases (EST-3 and EST-12 isozymes), detected as more intensely stained, indicated an enhanced expression of this esterases, while two α/β-carboxylesterases (EST-10 and EST-11 isozymes) were absent or detected as more weakly stained bands (Figure 1). Tyrosine, incorporated into the alkaloid pathway of callus tissues of *C. peruvianus,* acts directly as a precursor of tyramine synthesis or as precursor of other alkaloids, an aromatic domain of suberized tissues, lignins and lignans (Croteau *et al*., 2000) in which intermediate compounds containing acetil groups such as ρ–coumaroyl-CoA may be substrate to acetylesterases.

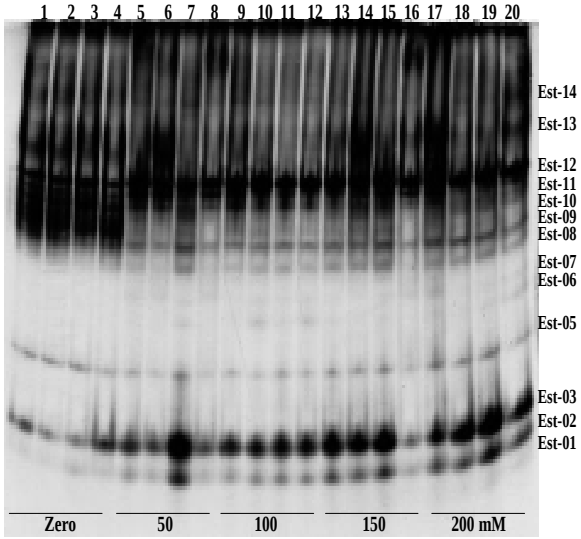

Figure 1. α- and β-esterases detected in long-term callus tissue culture of *Cereus peruvianus* subcultured to original conditions (absence of tyrosine; lanes 1-4), and in medium containing 50 (lanes 5-8), 100 (lanes 9-12), 150 (lanes13-16), and 200 mg/L (lanes 17-20) of tyrosine. The EST-6 and EST-7 α-carboxylesterases, and EST-8 α/β-arylesterase are induced in callus cultured with tyrosine; EST-2 α/β-acetylesterase and EST-3 and EST-12 α-carboxylesterases are detected as more intensely stained, and EST-10 and EST-11 α/β-carboxylesterases were absent or detected as more weakly stained bands.

PAGE and inhibition tests for biochemical characterization of esterases from shoot of plants, young callus tissue (recently induced: 1 - 12 months-old), and long-term callus cultures (13 years-old), showed a total of twenty three α-, β-, and α/β-acetyl, carboxyl, and arylesterases (unpublished results). Unchanged esterase pattern in most of a hundred calli have been detected in long-term callus cultures maintained in their original culture conditions. After a hundred subculture cycles (13 years with periodic subculture at 30-days-interval), callus showed the esterase pattern represented by samples 1-4 in Figure 1. A stable and repeated growth of the callus under standard conditions has also been observed. There are, therefore, indications that the callus tissue cultures of *C. peruvianus* form a stabilized cell line, which, according to Bougard *et al*. (2001), could be defined as such when growth parameters were repeated during three consecutive subculture cycles in stable culture conditions.

The production of alkaloid in callus culture growing under standard conditions has been

stimulated by lowering the proportion of 2,4-D:KIN (Di Cosmo and Towers, 1984; Robins *et al*., 1986; Rhodes *et al*., 1986) or by additions of salt in culture medium, however, in *C. peruvianus* lower alkaloid production was detected in callus maintained with 1:4 proportion of 2,4-D:KIN (Table 1). This is due to the fact that cytokinins not only regulate plant growth and development, but may influence differently the secondary metabolism in different higher plants (reviewed by Hara *et al*., 1994).

Although the esterase isozyme pattern cannot be directly associated to higher or lower levels of alkaloids, in the long-term callus tissue culture, specific genes may increase the alkaloid content in some callus, whereas suppression of endogenous gene activity may severely decrease the alkaloid content in other callus tissues after addition of precursor or specific elicitors. So that, to the tissue culture system of *C. peruvianus* becomes economically viable, the selection of variant cell lines with increased biosynthetic abilities for alkaloid production in medium containing additional supplements is of paramount importance. Despite difficulties for inducing a higher production of alkaloid in callus tissue, it is a more appropriate method than to employ living plants for obtaining higher alkaloid production.

References

ARVY, M.P. *et al*. 2,4-D and alkaloid accumulation in periwinkle cell suspension. *Biochimie*, Paris, v. 76, p. 410-416, 1994.

BOUNGARD, F. *et al*. Production of plant secondary metabolites: a historical perspective. *Plant Sci.*, Limenick, v. 161, p. 839-851, 2001.

CROTEAU, R. *et al*. Natural products (secondary metabolites). In: BUCHANAN, B. *et al* (Ed.), *Biochemistry & Molecular Biology of Plants*, American Society of Plant Physiology, 2000. p. 1250-1317.

DICOSMO, F.; TOWERS, G.H.N. Stress and secondary metabolism in cultured plant cells. In: TIMMERMANN, B.N. *et al*. (Ed.), *Phytochemical adaptations to stress*. New York: Plenum Press, 1984. p. 97-175.

GAMBORG, O.l. *et al*. Nutrient requirements of suspension cultures of soybean root cell. *Exp. Cell Res*., Orlando, v. 50, p. 151-158, 1968.

HARA, M. *et al*. Induction of a specific methyltransferase activity regulating berberine biosynthesis by cytokinin in *Thalictrum minus* cell cultures. *Phytochemistry*, Oxford, v. 36, p. 327-332, 1994.

JENSEN, R.A. The Shikimic Acid Pathway In: CONN E.E. (Ed.), *Recent Advances in Phytochemistry*. New York: Plenum Press, p.1-57, 1986.

KODJA, H. *et al*. Stimulation of indole alkaloid accumulation in suspension cell cultures of Catharanthus roseus (G. Don.) by cytokinin. *Comples Rendus de l´Academie des Sciences Paris*, Paris, v. 309, p. 453-458, 1989.

KUTNEY, J.P. Plant cell culture combined with chemistry – a powerful route to complex natural products. *Pure Appl. Chem*., Oxford, v. 68, p. 2087-2080, 1996.

MÉRILLON, I.M. *et al*. Indole alkaloid accumulation and thyptophan decarboxylase activity in cells cultures in three different media. *Plant Cell Rep*., Berlin, v. 5, p. 25-26, 1986.

MORENO, P.R.H., Van der Heijden, R., Verpoorte, R. Cell and tissue culture of *Catharanthus roseus*: a literature survey II, updating from 1988 to 1993. *Plant Cell Tissue Organ Cult.*, Dordrecht, v. 42, p. 1-25, 1995.

MURASHIGE, T.; SKOOG, F.A. A revised medium for rapid growth and biossays with tobacco tissue culture. *Physiol. Plant*., Copenhagen, v. 15, p. 474-479, 1962.

OLIVEIRA, A.J.B.; MACHADO, M.F.P.S. Alkaloid production by callous tissue cultures of *Cereus peruvianus* (Cactaceae). *Appl. Biochem. Biotechnol*., Clipton, v. 104, p. 149-155, 2003.

OLIVEIRA, S. A. *et al*. *In vitro* propagation of *Cereus peruvianus* Mill. (Cactaceae). *In Vitro Cell. Dev. Biol. Plant*., v. 31, p. 47-50, 1995.

PURICELLI, l. *et al*. In vivo and in vitro production of alkaloids by Haplophyllum patavinum. *Nat. Prod. Lett*., Basingstoke, v. 16, p. 95-100, 2002.

RALPHS, M.H.; GARDNER, D.R. Alkaloid levels in duncecap (*Delphinium occidentale*) and tall larkspur (*D. barbeyi*) grown in reciprocal gardens: separating genetic from environmental influence. *Biochem. Syst. Ecol.*, Kidlington, v. 29, p. 117-124, 2001.

RALPHS, M.H., GARDNER, D.R. Distribution of norditerpene alkaloids in tall larkspur plant parts through the growing season. *J. Chem. Ecol*., New York, v. 29, p. 2013-2021, 2003.

RHODES, M.J.C. *et al*. Cell suspension cultures of Cinchona ledgeriana; II. The effect of a range of auxin and cytokinins on the production of quinoline alkaloids. *Planta Med.*, Stuttgart, v. 52, p. 226-229, 1986.

ROBINS, R.J. *et al*. Cell-suspension cultures of cinchona-ledgeriana. 1. Growth and quinoline alkaloid production. *Planta Med.*, Stuttgart, v. 52, p. 220-226, 1986.

SALMORE, A.K., HUNTER, M.D. Elevational trends in defense chemistry, vegetation, and reproduction in *Sanguinaria canadensis*. *J. Chem. Ecol.*, New York, v. 27, p. 1713-1727, 2001.

SATO, F. *et al*. Metabolic engineering of plant alkaloid biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.*, Washington, DC., v. 98, p. 367-372, 2001.

SCHRIPSEMA, J.; VERPOORTER, R. Search for factors involved in indole alkaloid production in cell suspension cultures of *Tabernaemontana divaricana*. *Planta Med.*, Stuttgart, v. 58, p. 245-249, 1992.

TERAMOTO, S.; KOMAMINE, A. In: BAJAJ, Y.P.S. (Ed.), *Biotechnology in Agriculture and Forestry* 4. Berlin: Springer-Verlag, 1988, p. 209-224.

ZHAO, J. *et al*. Effects of stress factors, bioregulators, and synthetic precursors on indole alkaloid production in compact callus clusters cultures of *Catharanthus roseus*. *Appl. Microbol. Biotechnol.*, Berlin, v. 55, p. 693-698, 2001.

Received on June 25, 2004.
Accepted on February 18, 2005.