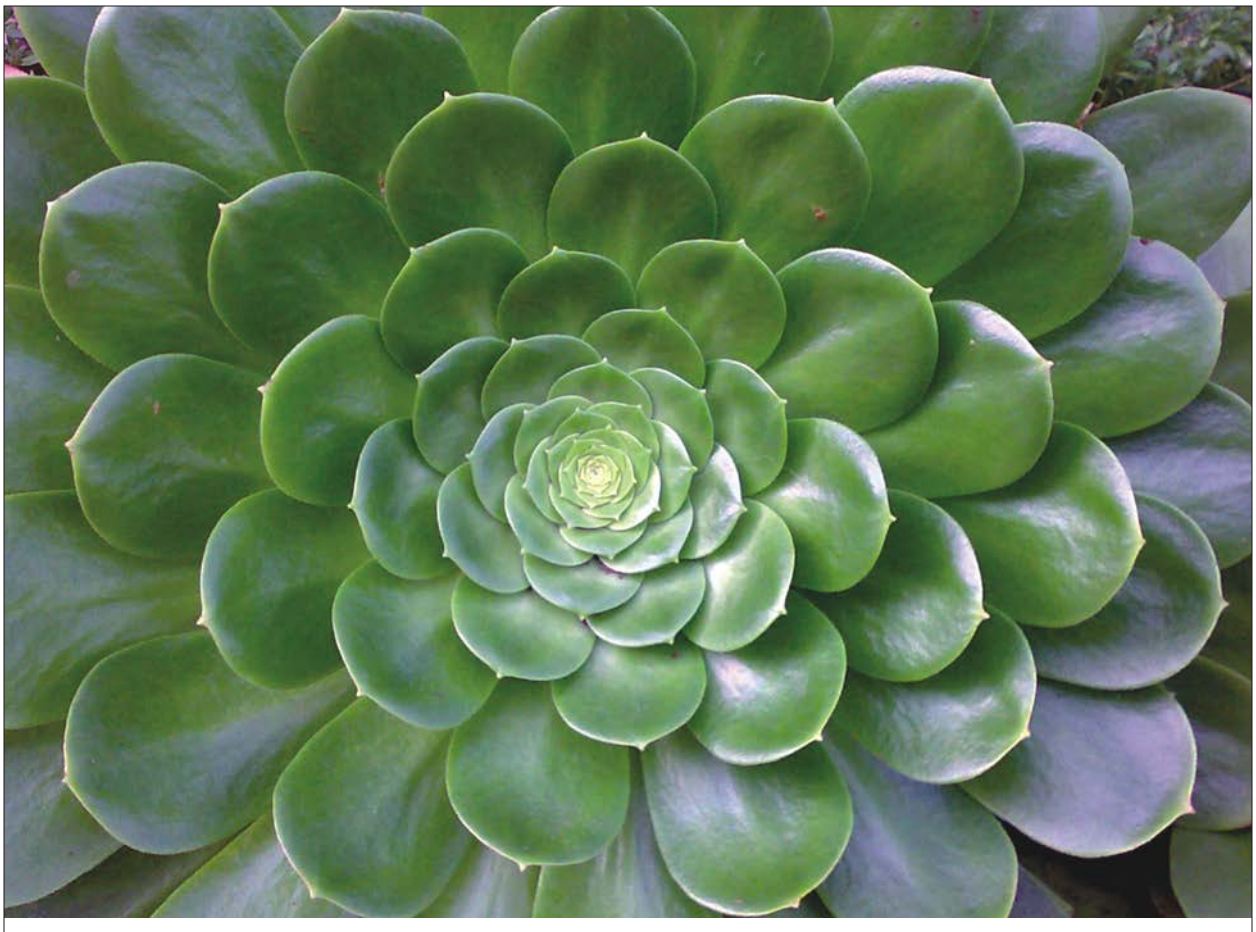

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Alkaloid Production and Isozymes Expression from Cell Suspension Culture of *Cereus peruvianus* Mill. (Cactaceae)

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Abstract: Cell suspension culture from friable callus tissue of *Cereus peruvianus* was established in a liquid medium, similar to that used for long-term callus culture. Its aim was to increase, in an alternative manner, alkaloid and enzymes production and to induce alkaloid release in the culture medium. Growth of callus-cell in suspension culture slightly increased, though maximum cell mass was detected after 42 days. Whereas total alkaloid production in cell suspension appeared in the early phase of the growth cycle, the second alkaloid flush appeared at the end of growth stage. In the growth medium, maximum accumulation of alkaloid also occurred after the period of maximum growth. Alkaloid production from cell suspension culture of *C. peruvianus* may be considered effective since the intracellular production showed that the level of alkaloids was three times higher in cell suspension than in callus tissues and extra-cellular accumulation of the alkaloid may be directly recovered from the culture medium. Suspension cell culture from callus can also be a source for enzyme extraction of the Est and ADH isozymes and may provide further possibilities in biotransformation reactions.

Key words: Cell suspension culture, cactus, alkaloids, esterase, alcohol dehydrogenase, isozymes.

Introduction

Yield increase of compounds and reduced cost of time and capital are the great goal of biotechnology. When compared to the main molecules found in plants, the plant's secondary compounds are usually low in abundance. Different strategies, including *in vitro* systems, have been extensively studied to improve the production of secondary plant compounds (Kutney, 1996; Boungard *et al*., 2001; Sato *et al*., 2001). *In vitro* cell culture is beneficial because useful metabolites are obtained under a controlled environment, regardless of climatic changes and soil conditions (Collin, 2001). Living plants are inconvenient because generally present different concentrations of the target compounds, which may depend on the specific time of the plant harvest (Salmore and Hunter, 2001; Puricelli *et al*., 2002; Ralphs and Gardner, 2001). Moreover, their exploitation may cause gradual genetic erosion.

Callus tissues culture of *Cereus peruvianus* have been used as source of alkaloids (Oliveira and Machado, 2003), polysaccharides (Machado *et al*., 2004) and fatty acids

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(Machado *et al*., 2006). Comparison with alkaloid production by *C. peruvianus* plants and by callus tissue has indicated that alkaloid levels were almost twice as high in callus tissues than in plant shoots (Oliveira and Machado, 2003). Tyramine and hordenine (N,N-dimethyltyramine) are the main alkaloids in the *C. peruvianus* species (Vries *et al*., 1971). Tyramine has shown important pharmacological applications. It has been reported that tyramine mimics insulin action in diabetic rats and in cell cultures (Bairras *et al*., 2003; Visentin *et al*., 2003; Subra *et al*., 2003). Whereas tyramine is also a neurotransmitter/neuromodulator that acts through G protein-coupled receptors in invertebrates (Roeder, 2004; Donini and Lange, 2004), hordenine has been reported to be an inhibitor of noradrenaline uptake (Barwell *et al*., 1989).

Tyramine and hordenine were obtained from callus tissues of *C. peruvianus* (Oliveira and Machado, 2003). However, callus culture develops special problems for downstream processing since cells should be collected and the product extracted. It would thus be beneficial to develop methods by which product excretion would be directly introduced into the growth medium (San Miguel-Chaves *et al*., 2003). When compared to callus culture, cell suspension recovers larger quantities of relatively homogeneous, undifferentiated cells from which compounds may be more easily isolated and makes possible the excretion of secondary products into the medium. Extra-cellular production avoids destruction of the biomass used for compound extraction since these may be directly recovered from the medium.

Most suspension culture is obtained by the transfer of friable callus lumps for agitated liquid medium of the same composition as that used for callus growth. In current study, suspension cell culture from *C. peruvianus* callus tissues provides an alternative method to increase alkaloid and enzymes production.

Materials and Methods

Callus tissues of *C. peruvianus* were induced from hypocotyls in MS medium (Murashige and Skoog, 1962) containing B5 vitamins (Gamborg *et al*., 1968), 0.8% agar, 3% sucrose, 4.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.0 mg L^{-1} N-(2-furanylmethyl)-1H-purine-6 amine (kinetin) and maintained at 32°C, during a 16 h photoperiod (15 m^{-2} sec^{-1} light intensity) (Oliveira *et al*., 1995). Long-term and non-regenerant callus tissues have been subcultured in fresh medium at 25 to 30 day intervals during 13 years in the Plant Tissue Culture and Electrophoresis Laboratory (State University of Maringa; Maringa, PR., Brazil).

The suspension culture of a cell line was initiated from the long-term cultures on a liquid medium similar to that for callus culture, excluding the agar. Medium was adjusted to pH 5.8 and then sterilized by autoclaving at 121°C, for 20 min. The suspension culture was maintained in 250 mL Erlenmeyer flasks containing 100 mL of liquid medium and 1g (fresh weight) of friable callus tissue. The mixture was stirred on a rotation shaker (120 rpm and 28±2°C).

Cell growth was determined by measuring the increase of the culture's dry cell weight. The biomass in the culture was thus separated from the liquid by filtration under vacuum and then lyophilized to obtain the dry cell weight.

Extraction and Isolation of Alkaloids

The freeze-dried material (Table 1) was ground and refluxed for 1h with a solution 0.33% acetic acid prepared in 70% ethanol (50 mL, v/v). After filtration, ethanol was evaporated under reduced

Table 1: Growth (dry wt., mg mg^{-1}) of *Cereus peruvianus* cell over a period of 56 days in suspension and intra- (mg mg^{-1} of dry wt.) and extra-cellular (mg mL^{-1}) alkaloid production

		Alkaloid production		
Day	Cell weight	Intracellular	Extra cellular	Total
0	43.78	--	--	--
7	46.2	0.4442	0.6035	1.0479
14	50.2	0.7898	0.5651	1.3549
21	57.4	0.2224	0.4938	0.7162
28	61.4	0.4140	0.5230	0.9294
35	65.4	0.6038	0.5389	1.1430
42	70.8	0.5771	0.3738	0.9509
49	66.2	1.0259	1.2714	2.2973
56	54.6	1.1142	0.4417	1.5560

pressure to produce an acid aqueous solution. The solution was alkalinized with NaOH 0.5 M 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 HCl 0.1 N.

Identification of Alkaloids

Alkaloids tyramine and hordenine were isolated and identified by their physical and spectral properties (Oliveira and Machado, 2003). Alkaloids in the dichlorometane 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, whereas ninhydrin spray was used as chromogenic 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 ultraviolet-visible (UV-VIS) spectrophotometer (Varian Cary-1E). UV detection wavelength was 274 nm; tyramine hydrochloride (1 mg mL^{-1}, Sigma-Aldrich Co., St. Louis, MO) was used as standard, since tyramine and hordenine, the alkaloids previously identified from *C. peruvianus* (Oliveira and Machado, 2003), presented the same chromophore group in UV spectrum.

Electrophoresis for Enzyme Analysis

Electrophoresis evaluations were carried out on samples consisting of cells in suspension. Cells (200 mg) were homogenized with a glass rod in an Eppendorf microcentrifuge tube using 20 µL of 1.0 M phosphate buffer, pH 7.0, containing 5% PVP-40, 1.0 mM EDTA, 0.5% β-mercaptoethanol, 0.5% triton X-100 and 10% glycerol solution. After homogenization, the samples were centrifuged at 25,000 rpm for 30 min at 4°C in a Sorval 3K-30 centrifuge and the supernatant (35 µL) was used for each sample.

For esterase isozymes (Est; EC 3.1.1.) analysis was used polyacrylamide gels (12%) prepared with 0.375 M Tris-HCl, pH 8.8, as buffer (Ceron *et al*., 1992). Electrophoresis was performed for 5 h at 4°C, at a constant voltage of 200V. Running buffer was 0.1 M Tris-glycine, pH 8.3. Staining techniques of Johnson *et al*. (1966) and Steiner and Johnson (1973), modified by Ceron *et al*. (1992), identified the esterases. The gels were soaked for 30 min in 50 mL 0.1 M sodium phosphate, pH 6.2, at room temperature and esterase activity was visualized by placing the gels for 1 h in a staining solution prepared with 50 mL of sodium phosphate solution, 30 mg of β-naphthyl acetate, 40 mg of α-naphthyl acetate, 60 mg of Fast Blue RR salt and 5 mL of N-propanol.

Electrophoresis condition and the reaction mixture for the detection of alcohol dehydrogenase isozymes (ADH; EC 1.1.1.1) of *C. peruvianus* tissues in starch gels were reported by Mangolin *et al.* (1994).

Results and Discussion

Table 1 show the weight of the dry cell mass of *C. peruvianus* callus-cell in suspension culture over a period of 56 days and the corresponding growth. Growth increased slightly, though the maximal cell mass was detected on the 42nd day.

Production of alkaloid was not directly growth related (Fig. 1 and Table 1). There is evidence that total alkaloid production in *C. peruvianus* cell suspension appeared in the early phase of the growth cycle, but the higher alkaloid production appeared at the end of growth stage (after 42 days). Maximum accumulation of the alkaloid in the growth medium occurred too after the period of maximum growth.

Bougaud *et al.* (2001) explained why the alkaloid production is not directly related to cellular growth. According to these authors, the lack of secondary metabolite production during the early stages of growth may be explained by carbon allocation mainly distributed for primary metabolism (building of cell structures and respiration) during which growth is active. On the other hand, when growth ceases, carbon is no longer needed in large quantities for primary metabolism and secondary compounds are more actively synthesized.

Alkaloid production from cell suspension culture of *C. peruvianus* may be considered efficient since it is higher (about 2% yield; Table 1) than alkaloid production from callus tissue (about 0.6% yield; Oliveira and Machado, 2003). Thus, suspension cell culture from *C. peruvianus* callus tissues provides an alternative to increase alkaloid production. In fact, intra-cellular production

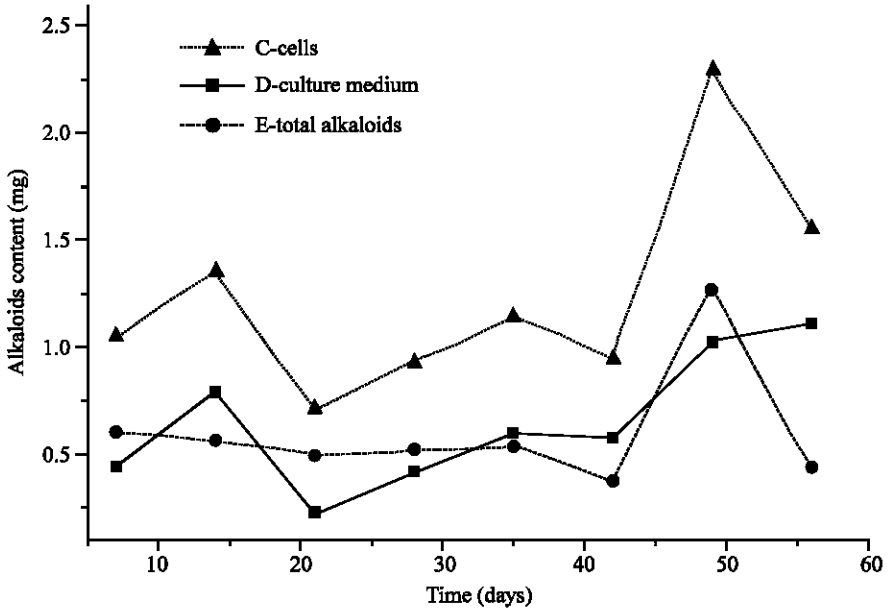

Fig. 1: Intracellular alkaloid production by *Cereus peruvianus* cell in suspension and accumulation of alkaloid in growth medium

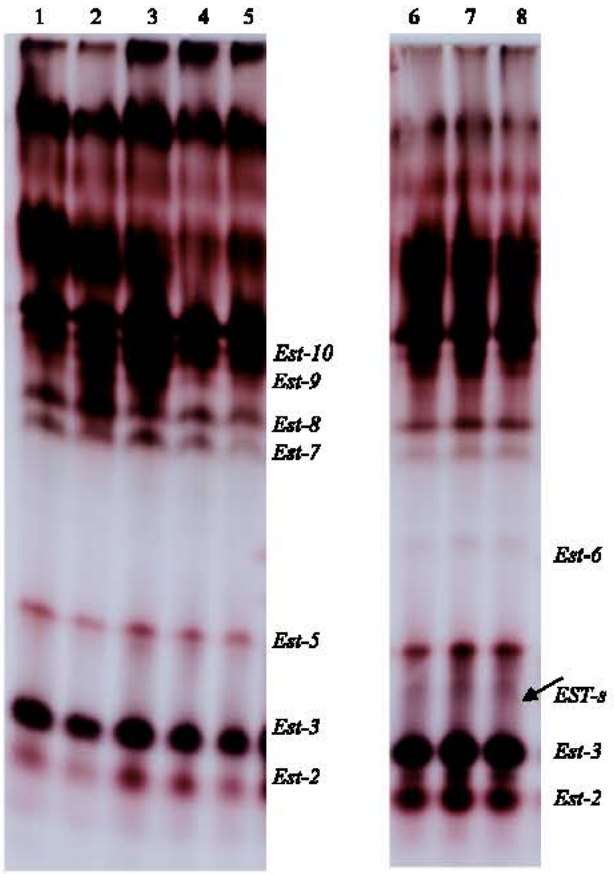

Fig. 2: α-and β-Esterase isozymes from callus (lanes 1-5) and suspension cell (lanes 6-8) of *Cereus peruvianus* in polyacrylamide gel showing specific esterase (Est-s) induced in suspension cells (arrow) and a increased expression of the isoesterases produced by *Est-2*, *Est-3*, *Est-5* and *Est-8* loci

showed that alkaloid level was three times higher in cell suspension than in callus tissues. Additionally, extra-cellular accumulation of the alkaloid may be directly recovered from the culture medium.

Extra-cellular production of alkaloid is an important positive aspect in the present study; extra-cellular accumulation of products is relevant in industrial processes involving bioreactor systems. It seems that there is an increase in intra-cellular concentration of alkaloid preceding further release into the medium. However, alkaloid concentration in the medium (μg mL^{-1}) was not in all cases lower than the concentration in the cells (μg mL^{-1}) (Table 1). It may be possible that, through long-term adaptation of *C. peruvianus* cell suspension into an optimal medium, higher alkaloid releases could be produced into the medium. The long-term adaptation of cell suspension culture to the optimal medium may produce changes in the alkaloid's quantitative and qualitative rates (Rhodes *et al*., 1986; Robins *et al*. 1986). Subsequent long-term adaptation to lower growth regulators medium has led to an increase in accumulation and in the relative proportion of alkaloids. Other strategies that can be used to induce the alkaloid releaser is the cell immobilized system.

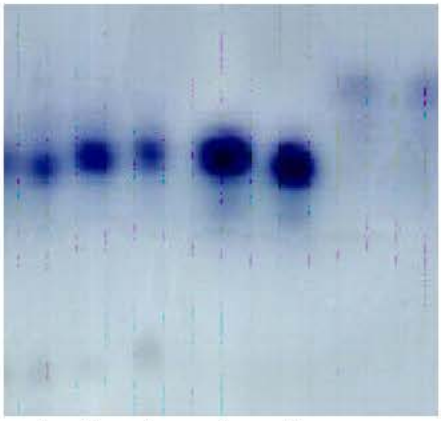

Fig. 3: Alcohol dehydrogenase isozymes (ADH) from callus (samples 1-3) and suspension cell (samples 4-5) of *Cereus peruvianus* detected in starch gel electrophoresis showing the increased expression of the ADH-3 isozyme (product of the *Adh-2* locus; Mangolin *et al*., 1994)

Higher levels of alkaloid from *C. peruvianus* cell-suspension culture may also be obtained by the addition of tyrosine in culture medium. In fact, tyramine content increased by 11% in callus tissues under original conditions when compared with control callus tissues to which tiramine was added in solid culture medium (Oliveira and Machado, 2003). The feeding of the culture with amino acids and its synthetic analogous (precursors of phenylpthylomins) can go to induction of different alkaloids from tyramine group.

The time course for intra-and extra-cellular alkaloid accumulation may be obtained by transference of higher callus tissues quantities for liquid medium. Preliminary researches have shown that transference of 5 g to 100 mL of medium reduced the maximal period for cellular growth to 28 days (Oliveira *et al*., 2005).

Besides alkaloid production other positive aspects for *C. peruvianus* cell-suspension culture is that specific Est-s isozyme was detected in cell suspension (Fig. 2, arrow) and carboxylesterases (EC 3.1.1.1) produced by *Est-2*, *Est-3* and *Est-8* loci and arylesterase (EC 3.1.1.2) p reduced by *Est-5* locus, were detected as most intensely stained band, from 5 days after transfer of callus cells to suspension culture (Fig. 2). Cell suspension cultured for 5, 10, 15 and 21 days also showed ADH-3 being more stronger stained (Fig. 3). The more intensely stained Est and ADH isozymes are an indication of differential gene expression and quantitative differences of isozymes production in callus-cell in suspension culture. Thus, suspension cell culture from callus tissues of *C. peruvianus* may be a source for esterase and alcohol dehydrogenase enzymes extraction and/or that can will be used in biotransformation reactions using induced enzymes. Suspension cell of *C. peruvianus* have been used for biocatalysis in the selective interconversion of functional groups such as carbonyl reduction and ester hydrolysis (unpublished results).

Callus-cell in suspension culture of *C. peruvianus* is beneficial because useful metabolites and enzymes are obtained under a controlled environment, free from microbe and insect contamination, regardless of climatic changes and soil conditions and the cell-suspension may be subcultured and indefinitely propagated on the optimized medium, substituting the use of adult plants. Cloning of

C. peruvianus cell lines may provide further optimization for end product yield and is a suitable source for industrial procedures of extraction because the same protocol can be quickly and easily standardized by using genetically uniform material.

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References

Bairras, C., C. Ferrand and C. Atgie, 2003. Effect of tyramine, a dietary amine, on glycerol and lactate release by isolated adipocytes from old rats. J. Physiol. Biochem., 59: 161-167.

Barwell, C.J., A.N. Basma, M.A. Lafi and L.D. Leake, 1989 . Determination of hordenine by monoamine oxidase and its action on vasa deferentia of rat. J. Pharm. Pharmacol., 41: 121-423.

Boungard, F., A. Gravot, S. Milesi and E. Gontier, 2001. Production of plant secondary metabolites: A historical perspective. Plant Sci., 161: 839-851.

Ceron. C.R., J.R. Santos and H.E.M. Campos Bicudo, 1992. The use of gelatin to dry cellophane wound slab gels in an embroidering hoop. Brazil. J. Genet., 15: 201-203.

Collin, H.A., 2001. Secondary product formation in plant tissue cultures. Plant Growth Regul., 34: 119-134.

Donini, A. and A.B. Lange, 2004. Evidence for a possible neurotransmitter/neuromodulator role of tyramine on the locust oviducts. J. Insect Physiol., 50: 351-361.

Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cell. Exp. Cell Res., 50: 151-158.

Johnson, F.M., C.G. Kanapi, R.H. Richardson, M.R. Wheeler and W.S. Stone, 1966. An operational classification of *Drosophila* esterases for species comparison. Univ. Texas Publ., 6615: 517-532.

Kutney, J.P., 1996. Plant cell culture combined with chemistry-A powerful route to complex natural products. Pure Applied Chem., 68: 2087-2080.

Machado, F.A.P.S.A., A.J.B. Oliveira, C.A. Mangolin, L. Gobbi-Filho and M.F.P.S. Machado, 2004. Polysaccharide production from callus cultures of Cereus peruvianus Mill. (Cactaceae). Crop Breed. Applied Biotechnol., 4: 127-134.

Machado, F.A.P.S.A., M. Capelasso, A.J.B. Oliveria, R.A.C. Gonçalves, M.L.M. Zamuner, C.A. Mangolin and M.F.P.S. Machado, 2006. Fatty acids production from plants and callus of *Cereus peruvianus* Mill. (Cactaceae). J. Plant Sci., (In press).

Mangolin, C.A., A.J. Prioli and M.F.P.S. Machado, 1994. Alcohol dehydrogenase (ADH; EC 1.1.1.1) isozymes as markers at 2,4-dichlorophenoxyacetic acid x kinetin combination in callus cultures of *Cereus peruvianus* (Cactaceae). Biochem. Genet., 32: 191-195.

Murashige, T. and F.A. Skoog, 1962. A revised medium for rapid growth and biossays with tobacco tissue culture. Physiol. Plant, 15: 474-479.

Oliveira, A.J.B. and M.F.P.S. Machado, 2003. Alkaloid production by callus tissue cultures of *Cereus peruvianus* (Cactaceae). Applied Biochem. Biotechnol., 104: 149-155.

Oliveira, A.J.B., RA.C. Gonçalves, M.F.P.S. Machado, F.A.P.S.A. Machado, M. Capelasso and C.A. Mangolin, 2005. Screening of ADH and esterases isozymes in suspension cell culture of *Cereus peruvianus* (Cactaceae). In: 11th Brazilian Meeting on Organic Synthesis, pp: 116.

Oliveira, S.A., M.F.P.S. Machado, A.J. Prioli and C.A. Mangolin, 1995. *In vitro* propagation of *Cereus peruvianus* Mill. (Cactaceae). *In Vitro* Cell. Dev. Biol. Plant, 31: 47-50.

Puricelli, L., G. Innocenti, G. Delle Monache, R. Caniato, R. Filippini and E.M. Cappelletti, 2002. *In vivo* and *in vitro* production of alkaloids by *Haplophyllum patavinum*. Nat. Prod. Lett., 16: 95-100.

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

Rhodes, M.J.C., J. Payne and R.J. Robins, 1986. Cell suspension cultures of *Cinchona ledgeriana*; II. The effect of a range of auxin and cytokinins on the production of quinoline alkaloids. Planta Med., 52: 226-229.

Robins, R.J., J. Payne and M.J.C. Rhodes, 1986. Cell suspension cultures of *Cinchona ledgeriana*; I. Growth and quinoline alkaloid production. Planta Medica, 52: 220-226.

Roeder, T., 2004. Tyramine and octopamine: Modulation at different levels. Annu. Rev. Entomol., 50: 116-121.

Salmore, A.K. and M.D. Hunter, 2001. Elevational trends in defense chemistry, vegetation and reproduction in *Sanguinaria canadensis*. J. Chem. Ecol., 27: 1713-1727.

San Miguel-Chaves, R., M. Soto-Hernandez, A.C. Ramos-Valdivia and G. Kito, 2003. Production of alkaloids by *in vitro* culture of *Erythrina americana* Miller. Biotechnol. Lett., 25: 1055-1059.

Sato, F., T. Hashimoto, T. Hachiya, K. Tamura, K.B. Choi, T. Morishige, H. Fujimoto and Y. Yamada, 2001. Metabolic engineering of plant alkaloid biosynthesis. Proc. Natl. Acad. Sci. USA, 98: 367-372.

Steiner, W.W.M. and W.E. Johnson, 1973. Techniques for electrophoresis of Hawaiian *Drosophila* US-IBP. Island. Ecosyst. Tech. Rep., 30: 1-21.

Subra, C., E. Fontana, V. Visentin, X. Testar and C. Carpene, 2003. Tyramine and benzylamine partially but selectively mimic insulin action on adipose differentiation in 3T3-L1 cells. J. Physiol. Biochem., 59: 209-216.

Visentin, V., P. Marq, S. Bour, C. Subra, D. Prevot, N. Morin, P. Valet, M.C. Monje, F. Nepveu and C. Carpene, 2003. Effect of prolongated treatment with tyramine on glucose tolerance in streptozotocin-induced diabetic rats. J. Physiol. Biochem., 59: 225-232.

Vries, J.X., P. Moyna and V. Diaz, 1971. Alkaloids Cactos Uruguay. Revista Latinoamericana de Química, 3: 21-23.