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Effect of inoculum age on kinetic of biomass formation and phenolic accumulations in *Larrea divaricata* Cav. cell suspension culture

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ABSTRACT

This work focuses on the analysis of the effect of inoculum age on the kinetic of biomass formation and phenolic accumulation in *Larrea divaricata* Cav. cell suspension culture. *L. divaricata* plant cell cultures (callus and cell suspension culture) successfully produce interesting phenolic compounds, being the main the lignan Nordihydroguaiaretic acid. The findings revealed that the inoculum age is an important factor on the kinetic biomass formation and phenolic compounds production in *L. divaricata* cell suspension culture. Four to seven month - inoculum age were suitable to establishment cell suspension culture, not only for biomass but also for the phenolic compounds production. On the contrary, when the age of the callus was over eighth month, no stimulation of phenolic compounds biosynthesis was observed.

Keywords: *Larrea divaricata*; plant cell cultures; phenolic compounds; NDGA; quercetin aglicon; *p*-coumaric acid; ferulic acid; sinapyl alcohol.

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Introduction

Larrea divaricata Cav. (Zygophyllaceae) is a native plant used in folk medicine to treat a variety of illnesses including infertility, rheumatism, arthritis, diabetes, gallbladder and kidney stones, pain and inflammation (Hieronymus, 1882, Ratera and Ratera, 1986; Barboza *et al.*, 2006; Goleniowski *et al.*, 2006). The species of *Larrea* presents abundant flavonoids (Mabry *et al*., 1975), with quercetine and kaempferol being considered to be anticancerigenic agents (Castrillo and Carrasco, 1987; Chabner, 1991; Croteau *et al.*, 2000). One of the main lignans nordihydroguaiaretic acid (NDGA), was isolated from *L. divaricata* in 1945 by Waller and Gisvol. It is considered a powerful inhibitor of lipoxygenases enzymes, which play an important role in cardiac diseases, asthma, arteriosclerosis and cancer (Wang *et al.*, 1991; Anesini *et al.*, 1999; Whitman *et al.*, 2002; Bongiovanni *et al.*, 2008, Qingqi Chen, 2009). This compound showed cytotoxic properties for a wide range of tumor-like cell lines, such as mammalian endothelial cells, cervical carcinoma and myeloid leukemia, among others (Sagar *et al.*, 1992; Madhavi and Das, 1994; Damtew 1997, Bongiovanni *et al*., 2008). This compound and its methyl derivatives have also *in vitro* inhibition against several viruses including HIV, herpes simplex I and II, and human papilloma, and Junin virus (Hwu *et al.*, 1998; Chen *et al.*, 1998; Craigo *et al.*, 2000; Konigheim *et al.*, 2005).

The medicinal use of this specie has led to the consideration of various alternatives for their metabolite production. Plant cell cultures are an excellent tool for screening and selecting plants with specific properties, or for manipulating the genetic material of plant cells. Moreover, with recent advances in biotechnology and biochemical engineering, there is a growing interest in using plant cell cultures to directly produce useful compounds from plant cells without having to cultivate crops (Verpoorte and Memelink 2002; Ramachandra and Ravishankar, 2002; Vanisree *et al.*, 2004; Ionkova, 2007).

The objective of the present study was to establishment *L. divaricata* callus culture and evaluates the effects of the inoculum age on the kinetic of biomass formation and metabolite accumulations in *L. divaricata* cell suspension culture.

Experimental

1. Plant material

Wild plants of *L. divaricata* Cav. were collected at Santa María de Punilla, "Sierras de Córdoba", located at 45 km north of Córdoba city, Argentina. A voucher specimen was deposited in the International Herbarium of the National University of Río Cuarto, Argentina (Accesses information RIOC 501).

2. Plant cell culture and culture conditions

Callus tissues of *L. divaricata* were established from wild leaves and were surface disinfested by immersion in 2% (w/v) copper sulphate solution for 15 min, followed by immersion in ethanol solution 70% (v/v) for 10 min, and sodium hypochlorite solution 1.5% (v/v) for 15 min, and finally rinsed three times with sterile distilled water. They were grown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2,4-dicholorophenoxyacetic acid (2,4-D)(2 mg. l^{-1}) and N_6-benzyladenine (BAP) (1 mg l^{-1}) (Palacio et al, 2006).

Callus tissues were grown at 25 (±2) ºC under a 16/8 h light/dark photoperiod by using cool-white fluorescent light (45 mol m^{-2} s^{-1}) and sub-cultured every two weeks. After a period of 4-subculture, established callus cultures were taken up for growth study and quantification of phenolic compounds.

Cell suspension cultures were initiated by transferring the calli to a standard liquid medium, using an inoculation rate of 1/10 w/v, and cultured on a rotary shaker (120 rpm) at the same temperature and day/night regime condition as described above for 15 days.

3. Analysis

3.1. Biomass determination

Cell from callus and suspension culture were subsequently collected to determine the biomass and phenolic compounds at defined times. Dry weight (DW) was determined from lyophilized fresh material, expressed as g of DW, and stored at -80 ºC until use.

The results presented in this paper have been summarized from three independent experiments. All determinations were performed in triplicate.

3.2. Extraction procedures and determination

The dried cell biomasses were homogenized, with mortar and pestle then pure ethanol was used to extract by maceration in a shaker at 120 rpm for

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24 h/dark at room temperature. Ethanol was evaporated and the concentrated extracts were re-dissolved in methanol (1 ml), filtered through a membrane filter (0.45 m pore size) and analysed by HPLC.

The assays were performed using a WaterTM 2690 HPLC system (Milenium software). Separations were carried out on a reversed phase column Phenomenex Luna 5µ C18 (2) 100Å (4.6 mm x 250 mm). The mobile phase consisted of solvent A (water / 1%phosphoric acid) / solvent B (acetonitrile / 1% phosphoric acid), in linear gradient at a flow of 1ml/min. The eluent was monitored with a multichannel photodiode array detector (Water 996). The metabolites were identified using the external standard method and quantificated from their calibration curves. All experiments were repeated three times and the quantitative determination was performed in triplicate and the results are presented as mean values ± SD.

3.3. Chemicals and standards

NDGA and quercetin aglicon were purchased from Sigma-Aldrich (St. Louis, MO, USA). The solvent used for the extraction of samples was analytical grade (ethanol and diethyl ether) while those used for the HPLC analysis were HPLC grade (acetonitrile, phosphoric acid, Milli Q distilled water and methanol). All were purchased from Merck Co.

Result and Discussion

The kinetic of biomass formation and the phenolic compounds accumulation in *L. divaricata* cell suspension culture are influenced by the effect of inoculum age.

Callus formation and phenolic compounds accumulation. For this purpose, we achieved a NDGA-Quercetin-producing *L. divaricata* callus culture line in MS media supplemented with 2,4-D and BA (2:1 mg/l) over a period of 18 months. The induced calli were friable, whitish yellow, showing a suitable biomass (Fig. 1).

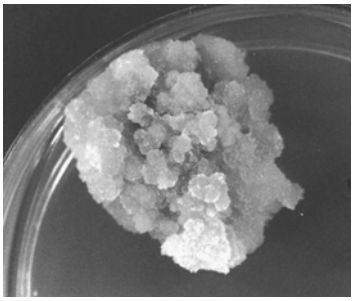

Figure1. Callus mass.

The presence of phenolic compounds was analyzed after three months of callus culture. High performance liquid chromatography analysis revealed that leave-derived callus culture produced two important antioxidants, the lignan NDGA and the flavonoid quercetin aglicon. The callus culture started to synthesize these products since the fourth month. The chromatographic analysis revealed a higher level of NDGA at the fourth month (28±0.9 ug/g DW) whereas quercetin aglicon was maximal accumulated at the seven month (12.9±0.8 ug/g DW) (Fig. 2).

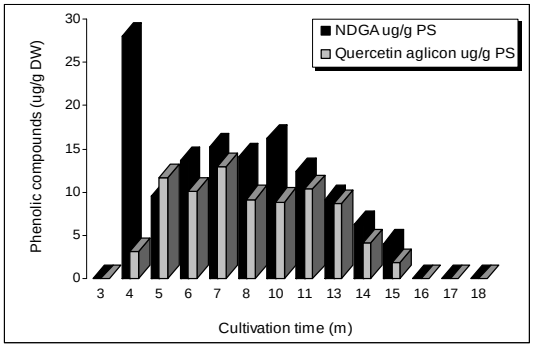

Figure 2. Kinetics of phenolic compound production (µg/g DW) by *L. divaricata* callus cultures, cultivated on MS medium containing 2,4-D and BA (2:1 mg/l). Values are the mean of three independent experiments.

The phenolic compounds accumulation decreased progressively with the time of culture, and gradually loses their biosynthetic capacity (Fig. 2). NDGA-Quercetin levels dropped over eleventh month up to non-detectable level at sixteen month of culture. The cause of these changes in the biosynthetic capacity can be explained by a result of genetic variability in the cultures, which in long term, as a result of selective pressure imposed by subculturing (Wichers *et al.*, 1990, Martienssen and Colot, 2001; Verpoorte *et al.*, 2002; Georgiev *et al.*, 2006, Kolewe *et al.*, 2008).

Cell suspension culture. Cell suspension cultures in MS medium containing 2,4-D and BA (2:1 mg/l) exhibited biomass formation with a lag growth phase of approximately 5 and 7 days for fresh and dry weight, respectively, followed by an exponential phase up day 9 and with a subsequent stationary phase until the end of the experiment (15 days) (Fig.3.a).

This study clearly showed that these homogeneous cell cultures had the ability to

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produce four interesting phenolic compounds: the lignan NDGA, and phenylpropanoid pathway intermediates: *p*-coumaric acid (3-(4-hydroxyphenyl)-2-propenoic acid), ferulic acid ((E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid) and sinapyl alcohol (4-hydroxy-3,5-dimethoxycinnamyl alcohol) (Fig. 3.b). The kinetics of phenolic compounds production by cell cultures are presented in Fig.3.b. NDGA content began to increase rapidly during the early lag growth phase, reaching a maximum value at day 4 (136.22±11.32 ug/g DW). During the exponential growth phase, the NDGA yield gradually decreased until the stationary phase, when it remained quite constant. In contrast, sinapyl alcohol began to accumulate in the late lag growth phase of cell culture, the highest production (70.22±9.82 ug/g DW) being reached at day 5. Contents of *p*-coumaric acid and ferulic acid were determined for the first time in the exponential growth phase. The maximal level of *p*-coumaric acid (47.43±9.01 ug/g DW) was reached at day 7 of culture, and of ferulic acid (61.12±11.26 ug/g DW and 57.12±7.41ug/g DW) at days 7 and 11 (Fig.3.b).

Our results shown that the four to seven month - inoculum age were appropriated to establishment *L. divaricata* cell suspension culture, not only for biomass production but also for the phenolic compounds production. No stimulation of phenolic compounds biosynthesis was observed when the age of the callus was over eighth month, thus the kinetics of biomass formation presented different patterns (Fig.3.c-g).

Conclusion

Thus, from the present studies, it is concluded that *L. divaricata* plant cell cultures (callus and cell suspension culture) were appropriately established.

L. divaricata callus culture had the ability to produce two interesting compounds, the lignan NDGA and the flavonoid quercetin aglicon, while the callus-derived cell suspension culture produced NDGA and the *novo* three phenylpropanoid pathway intermediates, *p*-coumaric acid, ferulic acid, sinapyl alcohol, but no quercetin aglicon.

The present study confirmed that the age of the cells in the inoculum had influence on metabolite production and determines the way the culture grows. Over eighth month no stimulation of phenolic compounds biosynthesis was observed.

Note: Part of this study was presented at the 'II Reunión de Biotecnología aplicada a plantas medicinales y aromáticas' (Second Biotechnology Meeting on Medicinal and Aromatic Plants), Córdoba, Argentina, 2009.

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Figure 3. Kinetics of biomass formation (fresh and dry weight) (g) and phenolic compound production (μg/g DW) by *L. divaricata* cell cultures from different inoculum age: a-b) 4, 5 and 7 months; c) 8, d) 10, e) 11, f) 13 and h) 14 month

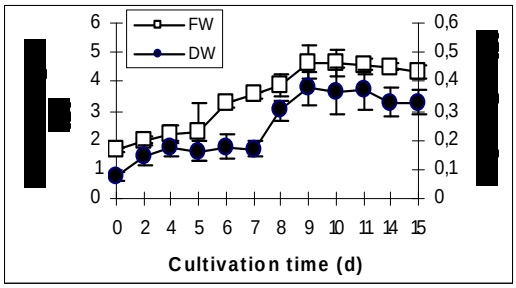
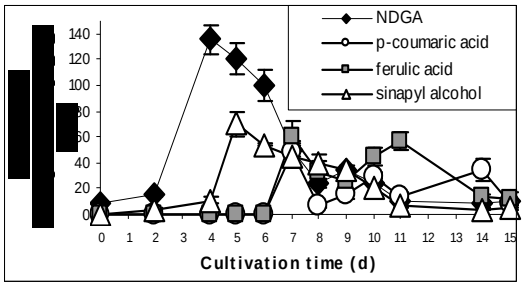


Fig.3.a



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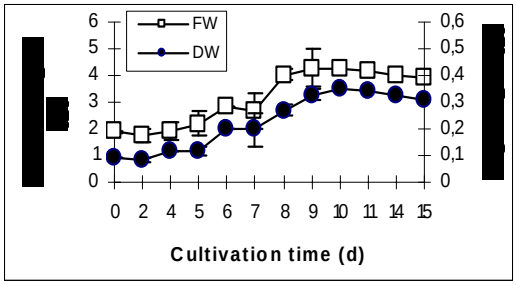

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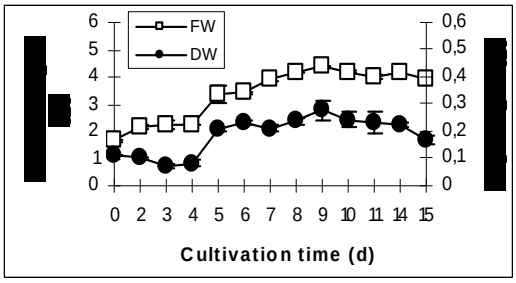

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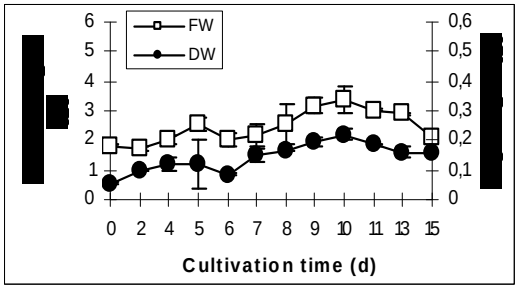

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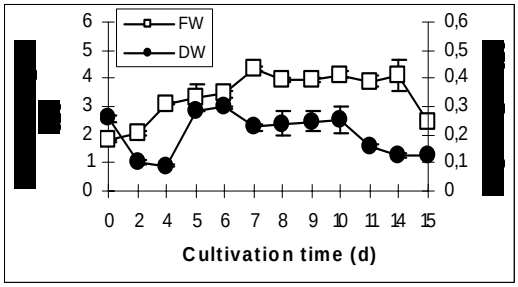

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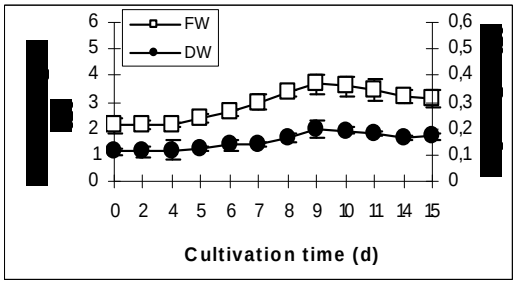

Fig.3.g