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Evaluation of the *in vitro* behaviour of *Aloysia citriodora* Palau: Histological and chemical study

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INTRODUCTION

Plants of the Verbenaceae family call the attention of researchers not only due to their high botanical diversity and wide distribution, but also because of their varied applications. The infusion or decoction of the leaves of *Aloysia citriodora* ('cedrón') is used in popular medicine as antispasmodic, antibacterial, antimycotic, expectorant and stomachal. The essence of 'Lemon verbena' is one of the most expensive and rare in the market of essential oils. Aerial parts contain 0.2 to 1% essential oil, its major components being: limonene, citral, geraniol, sesquiterpenes, verbenone, aldehydes and ketones (Lamaison and Petitjean, 1993; Muñoz, 1996). Stashenko *et al*. (2003), when making the whole analysis of volatile secondary metabolite 'cedron' composition, determined that the major chemical family is that of oxygenated monoterpenes, of which 60% account for citral. The pharmacological action of this species is mainly related to the essential oil. This species is official in the National Pharmacopeia Argentina 6th ed. (1978), and is included in the Argentinean Alimentary Code (Código Alimentario Argentino, 1969). Since it is difficult to obtain seeds owing to the argentinean climate, usual multiplication procedure is vegetative (Muñoz, 1996). The intensive multiplication by *in vitro* culture techniques is a powerful tool for micropropagation and genetic improvement of the species. Hence, the aim of the present work was to develop a methodology for micropropagation of *A. citriodora*, to carry out histoanatomic studies, and to analyse chemically its essential oils.

METHODOLOGY

Uninodal cuttings of wild plants with 2-3 axillary buds were used. Explant disinfection was carried out by submerging them for 15 minutes in commercial sodium hypochlorite (1.5% active chlorine) with the addition of Tween 20, and washed 3 times with sterile distilled water. They were cultured in Murashige and Skoog (1962) basal medium, 2 and 4 times diluted (MS ½ and MS ¼), supplemented with 3% sucrose, 0.8% agar, and different concentrations of benzyladenine (BA) and naphthalene acetic acid (NAA): 0:0 (medium 0), 0.1:0.1 (medium A), 0.5:0.1 (medium B), 0.1:0.5 (medium C) and 0.5:0.5 (medium D) mg/l. The pH was adjusted to 5.6. Culture media were sterilized by autoclaving at 121 °C, and incubation was carried out in a climatized chamber at 25 ± 2 °C with a 16 h photoperiod, and 60 $\mu mol/m^2.s$ irradiance. A totally randomized statistical design was applied, and the number of repetitions was of 25 replications for each treatment. After 28 days culture, the percentage of nodal segments that had response, and the average shoot number per nodal segment were determined. For the histological study, samples were taken at 0, 2, 6, 9 and 13 days of culture, were fixed in Craft, and included in paraffin, cuts being carried out at 10 µm, which were stained with safranine-fast green. For the determination of essential oils an extraction was made by hydrodistillation using 5 g leaves obtained at field and *in vitro*, submerged in water for 3 h. Oil was separated from water using sulfuric ether as organic solvent. Gas chromatography was carried out with a Perkin-Elmer equipment, composed of a GC Autosystem, equipped with an injector split (30:1), coupled to an MS Turbomass (GC-MS). The consulted database was NIST. For separation of mixtures a SE 30 nonpolar column of 25 m x 0.22 mm internal diameter was used. Programmed oven temperature was from 80 °C (1 min) up to 250 °C @ 6°C/min. Helium was the carrier gas with a lineal velocity of 1 mm/min. The injected volume of the extracts was 2 µl. Mass spectra were obtained by electron impact with a 70 eV energy.

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RESULTS AND DISCUSSION

Uninodal segments showed 1 to 3 axillary buds at inoculation time. Nodal segment percentage that had response in different media at 28 days culture is shown in Table 1. In half-diluted MS medium, explants showed the best response. The average number of shoots per nodal segment was higher in MS ½ without regulators, and in this treatment a maximum of 6 shoots/explant was obtained, which indicated that in this culture the number of shoots/knot was increased with regard to the natural growth of this species.

Nodal segments showed calli formation in the cut surface, except for those that remained in MS½ without regulators, which was the only medium without formation of basal callus. The 76% explants rooted in MS ½ without regulators after 28 days of culture. In *A. citriodora*, the capacity to form sprouts and roots was not directly related with the occurrence of growth regulators, since plant regeneration took place in their absence. According to Evans *et al*. (1981), it is frequent to find species in which the best responses for obtaining plants from buds is achieved with the use of culture media only composed of minerals, vitamins and sucrose, and lacking growth regulators.

Table 1. Percentage of nodal segments that had response at 28 days culture in **MS ½** and **MS ¼** media with different BA and NAA concentrations.

BA:NAA	MS ½	MS ¼
0	100	80
A	100	72
B	80	68
C	96	84
D	94	76

BA: benzyladenine; **NAA**: naphthalene acetic acid.

It was corroborated through histological studies that root differentiation in the medium without regulators took place from internal cortical parenchymatic cells, next to stem phloem, while in MS ½ with regulators, roots originated from a basal callus.

Thirty one volatile secondary metabolites were isolated from wild and *in vitro* plants. As reported by Stashenko *et al*. (2003), main peaks accounted for neral and geranial (t_R < 18), followed in importance by *iso*-caryophyllene (t_R = 11.76) with 3.1% in wild plant, and 8.5% in the *in vitro* plant.

CONCLUSIONS

Nodal segments are good explants for *in vitro* multiplication of *A. citriodora*. The half-diluted MS medium without growth regulators stimulates the highest sprouting from axillary buds, and rooting of the regenerated sprouts. The content of essential oils present in wild plants is different from those in *in vitro*-cultured plants, the major constituents of both being neral and geranial.

Note: This study was presented at the 'I Reunión de Biotecnología aplicada a plantas medicinales y aromáticas' (First Biotechnology Meeting on Medicinal and Aromatic Plants), Córdoba, Argentina, 2006.

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