

## DIRECT SHOOT REGENERATION FROM LEAF AND INTERNODE EXPLANTS OF *ALOYSIA POLYSTACHYA* [GRIS.] MOLD. (VERBENACEAE)

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(Received 20 September 2004; accepted 1 December 2005; editor M. A. O'Connell)

### SUMMARY

Adventitious bud regeneration from leaf and internode explants of *Aloysia polystachya* was achieved. Shoots from nodal segments grown *in vitro* were cut into pieces and used as sources of explants. Organogenesis was induced from both explants cultured on quarter-strength Murashige and Skoog (MS) semisolid medium (plus sucrose 5 g l<sup>-1</sup>) containing different combinations of 6-benzyladenine (BA) and  $\alpha$ -naphthaleneacetic acid (NAA) under 116  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD), 14-h photoperiod, and at a temperature of 27  $\pm$  2°C. The type of explant markedly influenced organogenesis and growth of the regenerated shoots. The regeneration frequencies were higher with leaf explants, while the number of shoots formed per responsive explant was greater with internode explants. However, the growth of regenerated shoots from internodes was seriously affected by vitrification. The number of shoots produced per responsive leaf explant increased from one to seven as the percentage of leaf explants producing shoots increased from 20 to more than 80%. NAA at 0.05  $\mu\text{M}$  in combination with BA at 0.5  $\mu\text{M}$  induced the highest regeneration rate (87  $\pm$  8.8%) after 20 d of culture, yielding 5.9  $\pm$  0.8 shoots per responsive leaf explant. Histological examination confirmed the occurrence of direct organogenesis. The regenerated shoots from the best induction treatment were transferred to a fresh medium without plant growth regulators for 30 d. Finally, the elongated shoots were rooted by pre-treatment in an aqueous solution of NAA at 500  $\mu\text{M}$  for 2 h and transferred to 1/4 MS. All plantlets raised *in vitro* were phenotypically normal and successfully hardened to *ex vitro* conditions. An experimental field plot with 2-yr-old *in vitro*-regenerated plants was established.

**Key words:** shoot bud differentiation; medicinal plant;  $\alpha$ -thujone.

### INTRODUCTION

*Aloysia polystachya* ('burrito'), which belongs to the family *Verbenaceae*, is a native medicinal semi-shrub largely distributed in Argentina (Botta, 1979). The leaves are used for medicinal purposes as a digestive and carminative (Martínez and Pochettino, 1992) due to the content of carvone and its precursor limonene as the main components of *Aloysia*'s essential oil (Fester et al., 1961; Bouwmeester et al., 1998; Cabanillas et al., 2003). *A. polystachya* also contains sabinene, pinene, and  $\alpha$ -thujone (Zumelzú et al., 2003). However, animal experiments have shown that  $\alpha$ -thujone is neurotoxic (Millet et al., 1981). Its convulsant actions in mice have been attributed to its antagonistic effects on  $\gamma$ -aminobutyric acid (GABA) receptor type A-mediated responses (Höld et al., 2000). In addition, Deiml et al. (2004) by means of kinetic modeling showed that  $\alpha$ -thujone reduced the cloned human 5-HT<sub>3</sub> receptor activity (which modulates the release of various neurotransmitters, e.g. catecholamines and GABA) by an effect on mechanisms involved in receptor desensitization, which depend on receptor subunit composition.

In order to expand cultivation of *A. polystachya*, the first step is the production of high quantities of genetically homogeneous plant material without  $\alpha$ -thujone oil. In this context, micropropagation would be useful for the multiplication of select clones. Plant production through nodal segments has been reported for *A. polystachya* (Sansberro and Mroginski, 1995), but the efficiency was low.

This paper describes the procedures used to induce direct regeneration of shoots and subsequently plantlet production from leaves and internodes of *A. polystachya*. To our knowledge, organogenesis of the genus *Aloysia* has not been reported to date.

### MATERIALS AND METHODS

**Plant material.** Two-year-old plants of *A. polystachya* (Gris) Mold., obtained by *in vitro* micropropagation from nodal segments as previously described (Sansberro and Mroginski, 1995), were grown in pots under greenhouse conditions.

**Explant sources.** Explants consisting of 3–4 cm long stem segments containing three axillary buds were cultured. The explants were collected from young non-lignified branches, surface sterilized with 70% ethanol for 1 min, immersed in commercial bleach containing 1.2% NaOCl and 0.1% Triton<sup>®</sup> for 15 min, and thoroughly washed with sterile-distilled water. They were aseptically cultured for 30 d in 40 ml glass tubes containing 10 ml of semi-solid (agar Sigma A-1296, 0.65%) quarter-strength Murashige and

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Skoog (MS) (1962) medium (1/4 MS, both minerals and organics compounds were four times reduced), supplemented with 5 g l<sup>-1</sup> sucrose, pH 5.8, and incubated in a growth room at 27 ± 2°C with 14-h photoperiod (116 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux density (PPFD), from white fluorescent lights), as previously stated (Sansberro and Mroginski, 1995).

**Induction of shoots and roots.** Two sources of explants were used for the regeneration experiments: (1) expanded leaves, and (2) internode segments from shoots of 4-wk-old *in vitro*-established nodal segments. Each leaf or internode ranging from node numbers 3–5 from the apex was divided by two or three cuts transversely to the mid-rib and cultured in 11 ml glass tubes containing 3 ml basal medium of identical composition, but supplemented with naphthaleneacetic acid (NAA: 0, 0.5, 2.5, or 5 μM) and 6-benzylaminopurine (BA: 0.5, 2.5, or 5 μM) for 30 d. In order to stimulate *in vitro* rooting, regenerative leaves from the best induction medium were subcultured in 1/4 MS without plant growth regulators for the same period. Finally, the elongated shoots were rooted by pre-treatment in an aqueous solution of NAA or indolebutyric acid (IBA: 50 or 500 μM) for 2 h and then transferred to 1/4 MS lacking plant growth regulators. All cultures were incubated under the same physical conditions as described above.

**Acclimatization.** The plantlets obtained *in vitro* were carefully washed under running water and set in 200 ml pots filled with a mixture of sterile soil and sand [1:1 (v/v)] plus 0.5 g of controlled release micro-fertilizer (Osmocote®: N, P, K; 18, 5, 9; 180-d release), and covered with transparent polyethylene which was subsequently lifted to reduce humidity. They were grown for 6–8 wk under day/night air temperature of 25–27/20–22°C and substrate temperature of 22–25°C; 14-h photoperiod was kept using eight cool-white fluorescent lamps (40 W) set at 1.8 m over the plants, which provided 120 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD in the wavelength range of 400–700 nm.

**Statistical analysis.** Each treatment consisted of 10 explants, and the experiment was repeated three times. The explants were arranged randomly on the shelves in the growth room. The results presented are the means of the replications with the standard error (±SE). The number of shoots is presented as the mean number of shoots regenerated per morphogenic explant. Regeneration rate is expressed as the average percentage of leaves/internodes that differentiated shoots over total number of leaves/internodes. Data were subjected to analysis of variance (ANOVA) (GraphPad Software, San Diego, CA) following Tukey's multiple comparison test. To assess statistical significance, a probability level of 0.05 was chosen.

**Histological analysis.** A histology observation was performed according to González and Cristóbal (1997). Samples of cultured explants were fixed in a formalin:ethanol:acetic acid (FAA) solution and dehydrated with Biopur® series. Transverse and longitudinal serial sections 8–10 μm thick were stained with safranin (C.I.50240)-Astra blue and mounted in Canada balsam. The photomicrographs were taken with an Olympus CH30 photomicroscope with a Sony ExwaveHAD camera. Samples were taken 1, 7, 12, and 18 d after the beginning of the induction phase.

**Gas chromatography.** The essential oil composition of leaves from micropropagated plants was analyzed by capillary gas chromatography on a Hewlett-Packard 5840 GC (HP-5 column, 30 m long, i.d. 0.53 mm, film thickness 0.88 μm) connected with a flame ionization detector. Identification was carried out by comparison of retention times and relative intensities of characteristic ions. Measurements were done in triplicate using three different extracts.

## RESULTS AND DISCUSSION

Adventitious bud regeneration was possible in all treatments tested using either leaf or internode explants (Table 1). The type of explant markedly influenced shoot organogenesis and growth of the regenerated shoots. In general, the regeneration frequencies were higher with leaf explants. The number of shoots formed per regenerative explant was greater with internodes than leaves (Table 2); however, the growth of regenerated shoots was affected by vitrification. This aberrant growth form causes significant loss to the micropropagation system because of the poor survival rate of plantlets when they are transferred from tissue culture to the *ex vitro* environment during the rustification phase (Gribble et al., 2003). For this reason, the use of internodes as the source of explants was abandoned.

TABLE 1

EFFECTS OF NAA AND BA ON DIRECT SHOOT ORGANOGENESIS FROM LEAF AND INTERNODE SEGMENTS OF *A. POLYSTACHYA*

PGRs (μM)		Percentage of explants producing shoots	
NAA	BA	Leaves	Internodes
0	0.5	50 ± 10.0 abc <sup>z,y</sup>	13 ± 3.3 a
	2.5	27 ± 12.0 a	13 ± 3.3 a
	5	17 ± 6.7 a	20 ± 10.0 a
0.05	0.5	87 ± 8.8 c	34 ± 7.2 a
	2.5	57 ± 3.3 abc	33 ± 3.3 a
	5	20 ± 0 a	33 ± 6.7 a
0.5	0.5	73 ± 6.7 bc	27 ± 8.8 a
	2.5	70 ± 10.0 bc	17 ± 6.7 a
	5	27 ± 12.0 a	20 ± 11.5 a
5	0.5	40 ± 5.7 ab	10 ± 5.8 a
	2.5	40 ± 5.7 ab	10 ± 5.8 a
	5	37 ± 3.3 ab	3 ± 3.0 a
Analysis of variance			
<i>F</i> value		8.1	2.3
<i>P</i> value		<0.0001	0.0439

<sup>z</sup> Values are mean ± SE of three independent experiments.

<sup>y</sup> Means in each column followed by different letters are different according to Tukey's multiple comparison test (*P* ≤ 0.05).

TABLE 2

EFFECTS OF NAA AND BA ON ADVENTITIOUS SHOOT PROLIFERATION FROM *A. POLYSTACHYA* EXPLANTS

PGRs (μM)		Mean number of regenerated shoots per responsive explant	
NAA	BA	Leaves	Internodes
0	0.5	4.8 ± 1.1 abc <sup>z,y</sup>	3.7 ± 1.2 ab
	2.5	1.7 ± 0.2 ab	4.3 ± 0.9 ab
	5	2.1 ± 0.7 ab	3.9 ± 1.1 ab
0.05	0.5	5.9 ± 0.8 bc	8.0 ± 0.6 b
	2.5	2.5 ± 0.4 ab	5.8 ± 0.4 ab
	5	3.3 ± 0.2 abc	7.7 ± 0.3 b
0.5	0.5	7.3 ± 1.1 c	5.2 ± 0.6 ab
	2.5	5.5 ± 1.7 bc	6.0 ± 2.1 ab
	5	1.2 ± 0.2 a	5.5 ± 1.2 ab
5	0.5	3.1 ± 0.6 abc	1.2 ± 0.2 a
	2.5	3.2 ± 0.8 abc	2.7 ± 0.2 ab
	5	2.3 ± 0.5 ab	4.0 ± 1 ab
Analysis of variance			
<i>F</i> value		5.0	3.1
<i>P</i> value		0.0006	0.0124

<sup>z</sup> Values are mean ± SE of three independent experiments.

<sup>y</sup> Means in each column followed by different letters are different according to Tukey's multiple comparison test (*P* ≤ 0.05).

A high cytokinin to auxin ratio usually results in shoot formation (Krikorian, 1995). In *Scaevola aemula*, the addition of BA to the medium has been reported to induce shoots from leaf explants (Bhalla and Sweeney, 1999). Later, Wang and Bhalla (2004) reported that the inclusion of higher concentrations of NAA in the medium inhibited the production of shoots and roots from the same explants. In the case of *A. polystachya*, our results showed that a low concentration of NAA and BA (0.05 and 0.5 μM, respectively) is needed for the highest

regeneration rate ( $87 \pm 8.8\%$ ). Although the regeneration rate was not significantly different with  $0.5 \mu\text{M}$  NAA plus  $0.5 \mu\text{M}$  BA, this treatment was discarded due to proliferation of callus. The length of the induction phase is also important. All plant-growth regulators tested for a long period had a negative effect that resulted in delayed shoot proliferation with browning of the explants and regenerated shoots. During the incubation period, leaves excised from *in vitro* shoots doubled their size and adventitious buds appeared within 10 d of culture initiation for all treatments tested. The number of

adventitious buds slightly increased during the following days. The highest regeneration rate from leaf explants grown in  $0.05 \mu\text{M}$  NAA and  $0.5 \mu\text{M}$  BA occurred during the first 10 d, and the number of buds formed increased until the 20th day of incubation. Concurrently, the frequency of adventitious shoots dying increased as the induction phase proceeded, ranging from  $5 \pm 2.8\%$  (day 20) to  $33 \pm 4.4\%$  (day 40).

Histological examination provided morphological details for understanding the process of organogenesis from *A. polystachya*

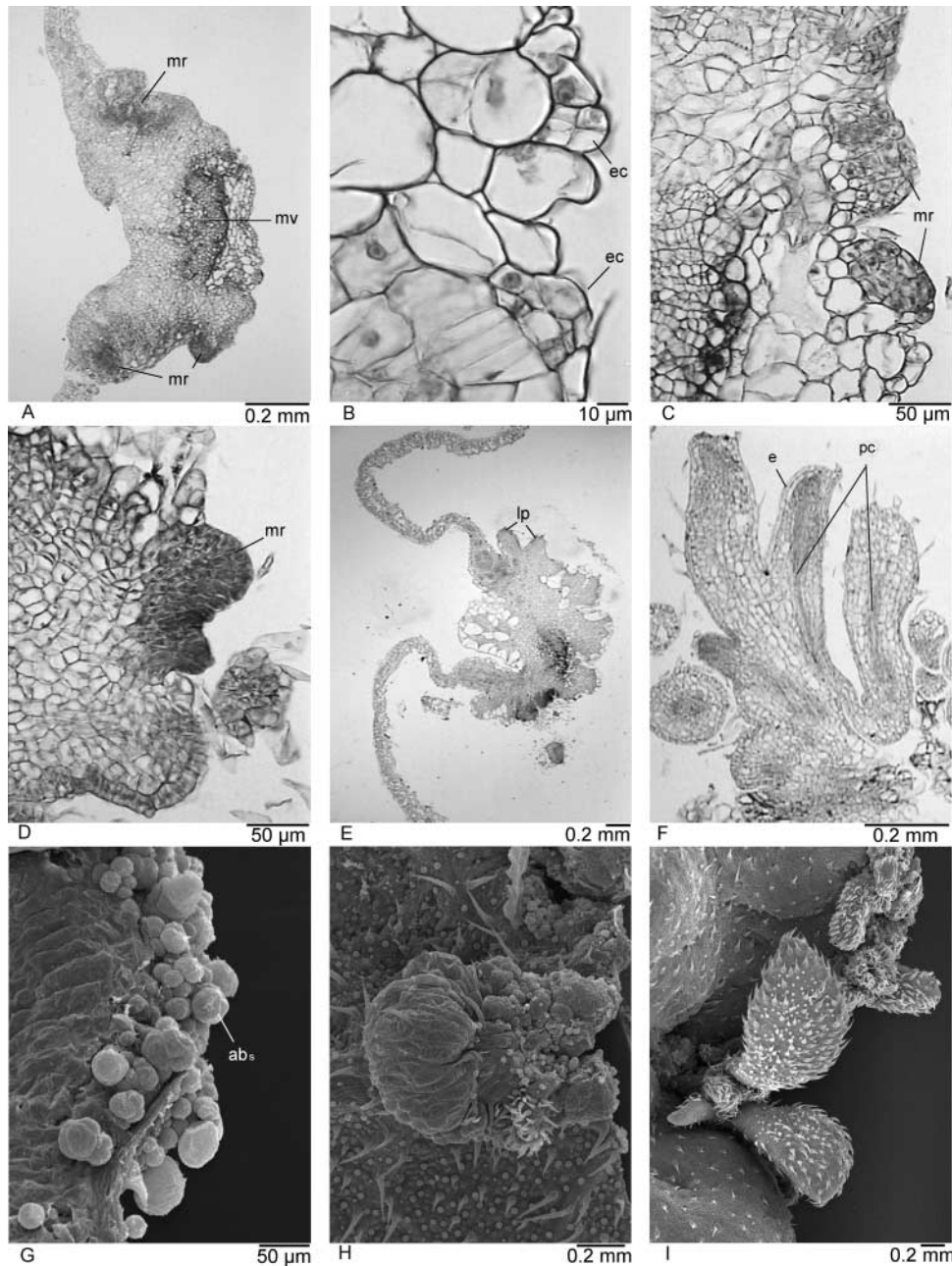


FIG. 1. Direct organogenesis from leaf explant of *A. polystachya*. A, B, Section of regenerating leaf explant showing meristemoid region with smaller cells with dense cytoplasm, small vacuoles, and a prominent nucleus situated in the center of the cell. C–E, Sections showing the initiation of the adventitious buds. F, Longitudinal section of a well-developed bud. G–I, Photographs from electron microscopy showing the successive steps of sprouting buds. ab, adventitious bud; e, epidermis; ec, epidermic cells; lp, leaf primordia; mr, meristemoid region; mv, midvein; pc, procambium.

leaves. Leaf explants cultured to induce direct organogenesis showed that meristemoid regions were apparent through the first week of culture, and they originated by periclinal divisions of epidermal cells (Fig. 1A). Meristematic cells were found to be much smaller than the surrounding cells, and meristematic cells had a dense cytoplasm, small vacuoles, and a conspicuous nucleus (Fig. 1B). During organogenesis, the development of a meristemoid region resulted in a meristematic zone emerging from the leaf surface that produced shoots (Fig. 1C–E). Completely developed shoots were closely connected with the explants (Fig. 1F). The development of each meristem resulted in stem and leaves continuing their growth by

means of successive divisions in diverse planes and enlargement of the resulting cells (Figs. 1G–I and 2A).

The *in vitro* rooting of elongated shoots of *A. polystachya* (Fig. 2B) may be stimulated without plant growth regulators, as reported by Sansberro and Mroginski (1995), but the results of our experiment showed that pre-treatment of the elongated shoots with either NAA or IBA at 500  $\mu\text{M}$  increased this morphogenic process (Fig. 3). Although the differences between both plant growth regulators in regard to the rooting rate and the number of primary roots per rooted shoot (3–4) were not significant, the use of NAA allowed  $85 \pm 8.7\%$  of primary roots to produce secondary roots in contrast

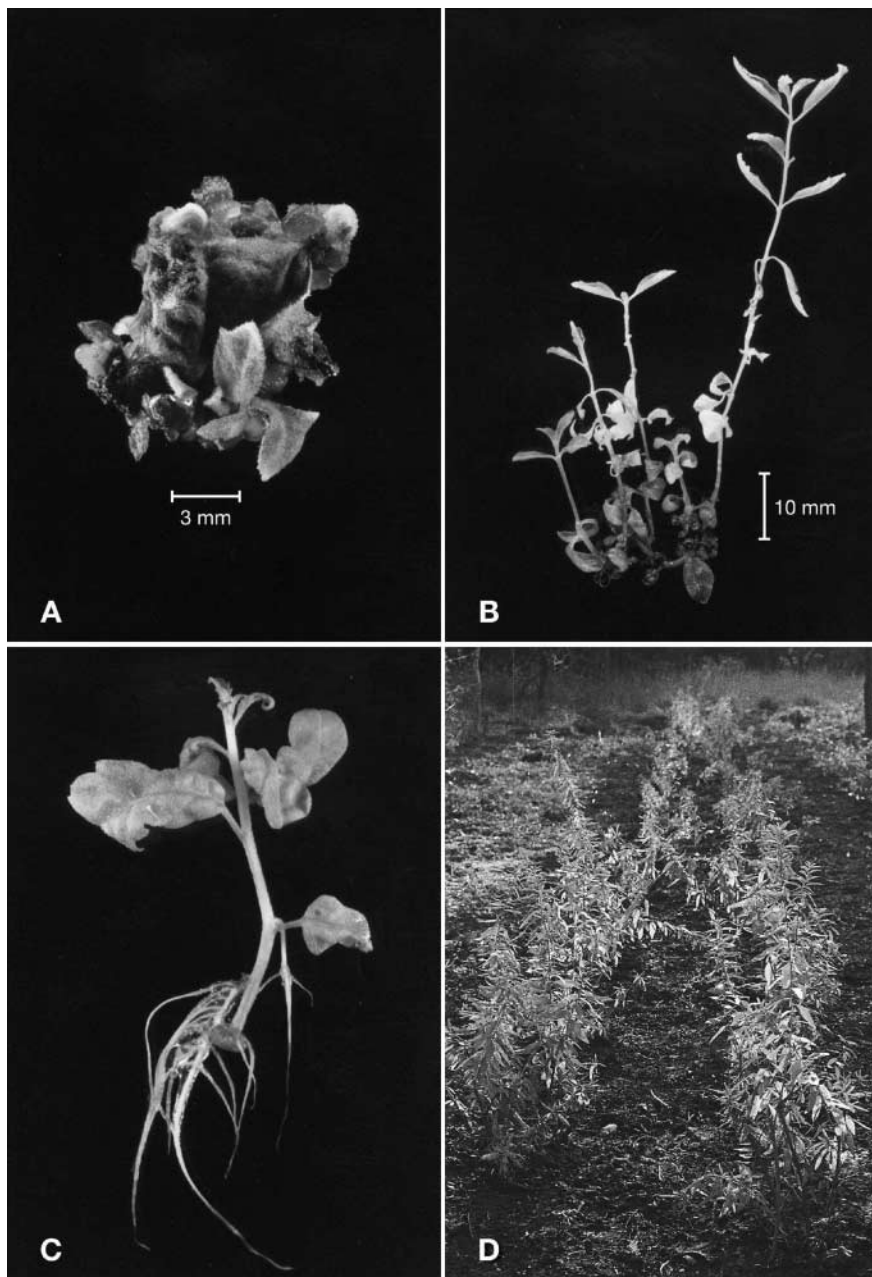


FIG. 2. Direct organogenesis from leaf explant and plantlet production in *A. polystachya*. A, Leaf with adventitious buds. B, Growth and development of shoots after transfer to a basal medium without hormones. C, *In vitro* rooting of the elongated shoots. D, Two-year-old plantlets growing in the field during the winter.

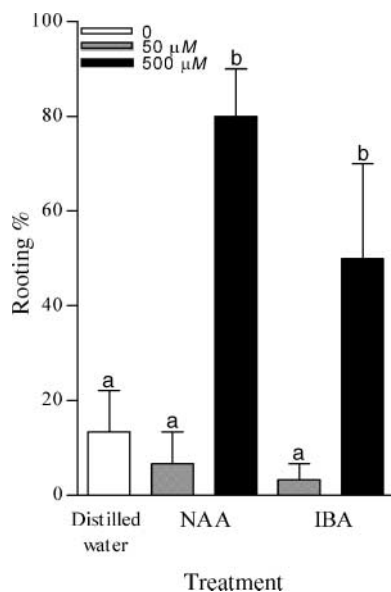


FIG. 3. Effect of NAA and IBA on *in vitro* rooting of elongated shoots. Values are mean  $\pm$  SE of three independent experiments. Bars followed by different letters are significantly different at a 5% level of significance (Tukey's multiple comparison test).

to IBA, where only  $18 \pm 8.9\%$  of primary roots developed secondary roots. The rooting process occurs without callus formation (Fig. 2C). More than 90% of the acclimatized plantlets were successfully established in soil (Fig. 2D).

When the essential oil composition of the leaves from micropropagated plants was analyzed by gas chromatography, the main component of the essential oil was found to be carvone (data not shown). Also,  $\alpha$ -thujone was not detected in ethanolic nor hexane extracts (data not shown).

In conclusion, our study provides a technique for efficient plantlet production for selected clones of *A. polystachya*. The technique described here for direct shoot organogenesis from leaf explants and subsequent plantlet regeneration facilitates the rapid propagation of this important medicinal plant species. It will also be useful in cryopreservation and genetic studies aimed at improving the essential oil composition of its extracts.

#### ACKNOWLEDGMENTS

The authors are grateful to Universidad Nacional del Nordeste (UNNE), Consejo Nacional de Investigaciones Científicas y Técnicas

(CONICET) and Establecimiento Las Marías for their financial support. They extend their deep appreciation to Alder Vásquez and Aldo Goytia for preparing the illustrations and Prof. Aliné Godoy Prats for English language checking. The authors also thank the anonymous reviewers for their critical comments.

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