

## IN VITRO CONSERVATION AND LEAF ANATOMY OF DIFFERENT CHEMOTYPES OF *Lippia alba* (Mill.) N. E. BR

### CONSERVAÇÃO IN VITRO E ANATOMIA FOLIAR DE DIFERENTES QUIMIOTIPIOS DE *Lippia alba* (Mill.) N. E. BR

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**ABSTRACT:** The aim of this work was to develop an *in vitro* conservation protocol for *Lippia alba* that involved mineral oil and to study the leaf anatomy of *L. alba* genotypes in *in vitro* and *ex vitro* environments. *In vitro* conservation involved five genotypes; LA-13 and LA-57 (carvone chemotype); LA-22 (linalool chemotype); and LA-29 and LA-44 (citral chemotype). Each genotype was treated with mineral oil, with water, and a control at 18°C and 23°C. Survival (%), shoot height (cm) and leaf color were assessed at 180 and 270 days. The genotypes with the best *in vitro* conservation outcomes (LA-13 and LA-57) were used to study the anatomy of the leaves. Midrib thickness, leaf blade thickness, adaxial and abaxial cuticles and the number of glandular and tector trichomes were assessed. A temperature of 18°C was optimal for the conservation of genotypes of the carvone chemotypes. The explants were short and had green leaves. This was especially true for LA-57, whose height did not exceed 2.0 cm. At 270 days, the LA-13 and LA-57 genotypes continued to have green and viable leaves, especially LA-57, which had the lowest mean height. Field plants of the carvone chemotype had thicker midribs, leaf blades, and cuticles and had more trichomes. The LA-57 genotype had the most glandular and tector trichomes.

**KEYWORDS:** Verbenaceae. Slow Growth. Plant anatomy. Morphology.

## INTRODUCTION

*Lippia alba* (Mill.) N.E.Br. is a native South American plant that is used widely for medicinal purposes. The high chemical variability of its essential oils supports differentiation of the plants into several chemotypes and makes this species the object of studies that involve its antibacterial, antifungal and antiviral properties (GUPTA et al., 2001; HENNEBELLE et al., 2008). Such chemical diversity results from environmental factors and from the genotypic variation of these plants (TAVARES et al., 2005) and accounts for the growing interest in its conservation.

With the increased genetic erosion, it is necessary to prioritize studies regarding the conservation of germplasm of this species. *In vitro* conservation is one of the methods that can be used for germplasm conservation and involves maintaining the plants under specific protocols of decreased temperature and decreased light intensity and varying culture media that result in slow growth (SOUZA et al., 2009). The maintenance of genotypes under aseptic conditions, reduction of labor costs, optimization of the use of physical space, and ease of exchange of plant material are

some of the advantages of *in vitro* conservation (ENGELMANN, 2011).

The immersion of plant tissues in mineral oil, a method that was originally reported by Caplin (1959) and is still underutilized, is one of the many existing protocols for *in vitro* conservation. This method is important because it is inexpensive and does not require sophisticated equipment (SHARMA et al., 2012). There is no published study on the use of this method for *in vitro* conservation of *L. alba*.

The effectiveness of such conservation protocols requires a successful transition from an *in vitro* to an *ex vitro* environment. Knowledge of the anatomy of a species therefore becomes important because the vegetative organs of plants that are grown *in vitro* have poorly differentiated tissues and structures compared with plants that are grown in a greenhouse (Apóstolo et al. 2005; Dousseau et al. 2008). Knowledge of the leaf anatomy of medicinal plants is valuable for the correct identification of the species that are studied and is essential for the management and conservation of native medicinal species and for understanding relationships between these species and the environment (NASCIMENTO-SILVA; PAIVA 2007).

This study aimed to develop an *in vitro* conservation protocol for *L. alba* that used mineral oil and to analyze the leaf anatomy of its genotypes *in vitro* and *ex vitro* environments.

## MATERIAL AND METHODS

### Plant material and local

Five *L. alba* genotypes that belong to three chemotypes were used in this study: LA-13 and LA-57 (carvone chemotype), LA-22 (linalool chemotype), and LA-29 and LA-44 (citral chemotype). All of the plant material originated from the Active Germplasm Bank of the Federal University of Sergipe, which is located on the Research Farm "Campus Rural da UFS", in the municipality of São Cristóvão, Sergipe State, Brazil, 11°00' S latitude and 37°12' W longitude. The experiments were conducted in the Laboratory of Tissue Culture and Plant Breeding of the Department of Agronomic Engineering, Federal University of Sergipe, Brazil.

### Seedling production and in vitro establishment

To obtain the explants, seedlings were produced by cutting and were planted in 8-liter pots with sand, soil and composted cattle manure (2:1:1 v/v/v) as the substrate. The seedlings were maintained in an agricultural greenhouse that had 50% black shade cloth, irrigation and intermittent misting. Nodal segments of these seedlings that had been disinfected were used as explants for *in vitro* establishment. To establish the plants, the plant material was immersed in running water (30 min), followed by an initial immersion in 70% ethanol (v/v; 30 sec) and a subsequent immersion in 1% mercuric chloride solution that was stirred (8 min). The nodal segments were then rinsed in distilled and autoclaved water. The explants were inoculated into test tubes that had 15 ml of MS (MURASHIGE; SKOOG, 1962) culture medium (Sigma-Aldrich, Sao Paulo, Brazil), that was supplemented with 0.7 g.L<sup>-1</sup> agar and 30 g.L<sup>-1</sup> sucrose, had a pH of 5.8 and had been autoclave-sterilized (121±1°C and 1.05 atm for 15 min). After the inoculation of the explants, the test tubes were transferred to a growth room that had a controlled temperature of 25±2°C and were exposed to 60 µmol.m<sup>-2</sup>.s<sup>-1</sup> light intensity with a 12-12 photoperiod.

### In vitro conservation

Nodal segments that were approximately 2.0 cm tall and had originated from plants that had already been established *in vitro* and were 60 days old were used as explants. The explants were

inoculated into test tubes (16x150 mm) with 15 ml of MS culture medium (MURASHIGE; SKOOG, 1962) that was supplemented with 0.7 g.L<sup>-1</sup> agar and 30 g.L<sup>-1</sup> sucrose. There were three experimental treatments. The first involved supplementation with 3.0 ml per test tube of mineral oil that had been autoclaved previously and cooled to 25°C. The mineral oil was placed in the tubes with a micropipette until it completely covered the explant. The second treatment involved supplementation with 3.0 ml of distilled water per test tube, until the water completely covered the explant. The third treatment was a control that involved unsupplemented culture medium only.

The experimental design was completely randomized in a 5 x 3 x 2 factorial arrangement, with six replicates. Each replicate consisted of five test tubes that each contained a nodal segment. There were five genotypes (LA-13, LA-22, LA-29, LA-44 and LA-57), three treatments (with mineral oil, with water and without mineral oil [control]), and two temperatures (18°C and 23°C). The material was maintained in a biochemical oxygen demand (BOD) chamber at the respective temperatures with a 12-12 photoperiod. The evaluated parameters were: survival (%), shoot height (on a rating scale on which 1 = less than or equal to 2.0 cm, 2 = 2.1 to 4.0 cm, 3 = 4.1 to 6.0 cm, 4 = 6.1 to 8.0 cm, 5 = 8.1 to 10.0 cm and 6 = equal to or greater than 10.1 cm) and leaf color (on a rating scale that was adapted from Lemos *et al.* (2002) on which 1 = green leaves, 2 = pale-green leaves, 3 = onset of leaf drying, 4 = between 30% and 50% dead leaves, 5 = more than 50% dead leaves and 6 = completely dead). Measurements were made at 180 and at 270 days.

### Leaf anatomy

The genotypes with the best outcomes from *in vitro* conservation with mineral oil were selected for the experiment that compared the leaf anatomy of the *in vitro* plants and acclimated and field plants that had originated from the Active Germplasm Bank of the Federal University of Sergipe. They plants were acclimated and maintained in a protected environment that had 50% black shade cloth and intermittent misting that ensured high humidity. Leaf anatomy was assessed by means of samples that were collected in the middle region of the third fully expanded leaf of plants that had been grown under the three test conditions. The samples were subsequently fixed and preserved in 70% ethanol. Cross sections of the middle region of the leaves were sterilized with sodium hypochlorite (1%

active chlorine) that was followed by triple rinsing in distilled water.

The material was then stained with safrablau (safranin solution [1%] and astra blue solution [1%] at a proportion of 7:3) and mounted on semi-permanent slides with glycerol water and a cover slip. The experimental design was completely randomized and consisted of 15 replicates of cross sections of each genotype and three growth conditions (in vitro, acclimated and field). The photomicrographs were taken with an optical microscope that was equipped with a Leica DM500 camera (Leica Microsystems Inc., Buffalo Grove, Illinois, U.S.A.) and were displayed on a computer screen by means of LAS EZ software (Leica Microsystems Inc.). The parameters that were measured were midrib thickness ( $\mu\text{m}$ ), leaf blade thickness ( $\mu\text{m}$ ), adaxial and abaxial cuticles ( $\mu\text{m}$ ) and the number of glandular and tector trichomes/ $\text{mm}^2$ . All anatomical measurements were performed using ImageTool image analysis software (University of Texas Health Science Center, San Antonio, U.S.A.). The images were calibrated with a microscope ruler that was photographed at the same magnification as the photomicrographs.

### Statistical analysis

The in vitro conservation and leaf anatomy results were subjected to Analysis of variance using Sisvar<sup>®</sup> software (<http://www.dex.ufla.br/~danielff/software.htm>) (FERREIRA, 2011). Means, when significant, were clustered using Tukey's test at a 5% level of significance.

## RESULTS

### In vitro conservation at 180 days

The 18°C temperature was effective in conserving the five *L. alba* genotypes as measured by the survival, color and height of the explants. The 23°C temperature was unsuitable for explant survival, with mean values below 24% for all three parameters and no significant differences between the mineral oil treatment and the water and control treatments (Table 1). Explants that were immersed in mineral oil and maintained at 18°C had a survival rate that was greater than 86% at 180 days, and there were no significant differences in survival among the genotypes.

The survival rates of the water-immersed and control explants were lower than that of explants that were immersed in mineral oil, except for the LA-57 genotype, which had means greater than 83% under all three treatments (Table 1). In terms of leaf color at 18°C, the treatment with mineral oil differed from the control treatment and from the water-immersion treatment. LA-13 and LA-57, which both belong to the carvone chemotype, stood out among the genotypes that were immersed in mineral oil by having means that corresponded to the green leaves category (1.2 and 1.1, respectively). The other genotypes had means that corresponded to the 30-to-50% dead leaves category.

**Table 1.** Survival (%) of *Lippia alba* genotypes at 180 days at different temperatures and with treatments.

Treatments	Genotypes				
	LA-13	LA-22	LA-29	LA-44	LA-57
-----18°C-----					
With mineral oil	100.0 <sup>aAa</sup>	93.3 <sup>aAa</sup>	86.7 <sup>aAa</sup>	86.7 <sup>aAa</sup>	100.0 <sup>aAa</sup>
With water	50.0 <sup>bBa</sup>	40.0 <sup>bBb</sup>	50.0 <sup>bBa</sup>	20.0 <sup>bBa</sup>	86.7 <sup>aAa</sup>
Control	46.7 <sup>bBCa</sup>	36.7 <sup>bBCb</sup>	56.7 <sup>bABa</sup>	23.0 <sup>bCa</sup>	83.3 <sup>aAa</sup>
-----23°C-----					
With mineral oil	16.7 <sup>aAβ</sup>	13.3 <sup>aAa</sup>	20.0 <sup>aAβ</sup>	3.3 <sup>aAβ</sup>	23.3 <sup>aAaβ</sup>
With water	10.0 <sup>aAβ</sup>	13.3 <sup>aAa</sup>	6.7 <sup>aAβ</sup>	6.7 <sup>aAβ</sup>	13.3 <sup>aAaβ</sup>
Control	6.7 <sup>aAβ</sup>	10.0 <sup>aAa</sup>	13.3 <sup>aAβ</sup>	10.0 <sup>aAβ</sup>	16.7 <sup>aAaβ</sup>
CV (%)	50.00				

Means that are followed by the same lower case letters in the columns of the same temperature, upper case letters in rows, and Greek letters between temperatures in columns and the same treatments with mineral oil, showed no significant differences according to Tukey's test ( $p>0.05\%$ ).

All of the treatments were in categories of more than 50% dead leaves at 23°C, and there were no differences among genotypes (Table 2). All genotypes, with the exception of LA-22, that were

treated with mineral oil at 18°C had mean plant heights that were lower than 3.0, which corresponds to growth ranging from 2.1 to 4.0 cm (Table 3). However, as is shown in Table 2, the LA-29 and

LA-44 genotypes, which both belong to the citral chemotype, had means for the color parameter that were greater than 4.5, which corresponds to the category of 30 to 50% dead leaves, which is undesirable for in vitro conservation. The genotype LA-22 (linalool chemotype) grew taller than 10.0 cm, and LA-13, LA-29, LA-44 and LA-57 were the shortest, as mentioned above.

The 23°C temperature accelerated explant growth in all three treatments, except for the LA-29 and LA-44 genotypes, which showed high mean

values of the color parameter but low mean heights in the mineral oil treatment. Thus, for 180 days of conservation at 18°C, the mineral oil treatment of genotypes LA-13 and LA-57 (carvone chemotype) had the best results in terms of the three parameters that were analyzed. There was 100% survival of these two genotypes, their color means, which were 1.2 for LA-13 and 1.1 for LA-57, corresponded to green leaves, and they grew to approximately 4.0 cm in height.

**Table 2.** Color (scores 1 to 6)\* of *Lippia alba* genotypes at 180 days at different temperatures and with different treatments.

Treatments	Genotypes				
	LA-13	LA-22	LA-29	LA-44	LA-57
-----18°C-----					
With mineral oil	1.2 <sup>aAα</sup>	4.6 <sup>aBα</sup>	4.6 <sup>aBα</sup>	4.8 <sup>aBα</sup>	1.1 <sup>aAα</sup>
With water	5.1 <sup>bBα</sup>	5.4 <sup>bBCα</sup>	5.5 <sup>bBCα</sup>	5.8 <sup>bCα</sup>	4.3 <sup>bAα</sup>
Control	5.1 <sup>bBα</sup>	5.4 <sup>bBCα</sup>	5.2 <sup>bBCα</sup>	5.7 <sup>bCα</sup>	4.1 <sup>bAα</sup>
-----23°C-----					
With mineral oil	5.8 <sup>aAβ</sup>	5.9 <sup>aAβ</sup>	5.8 <sup>aAβ</sup>	5.0 <sup>aAβ</sup>	5.8 <sup>aAβ</sup>
With water	5.9 <sup>aAβ</sup>	5.7 <sup>aAα</sup>	5.9 <sup>aAβ</sup>	5.8 <sup>aAα</sup>	5.9 <sup>aAβ</sup>
Control	5.9 <sup>aAβ</sup>	5.9 <sup>aAβ</sup>	5.8 <sup>aAβ</sup>	5.9 <sup>aAα</sup>	5.7 <sup>aAβ</sup>
CV (%)	6.91				

Means that are followed by the same lower case letters in columns of the same temperature, upper case letters in rows, and Greek letters between temperatures in columns and the same treatments with mineral oil, showed no significant differences according to Tukey's test ( $p>0.05\%$ ); \*Rating scale: 1 = green leaves; 2 = pale-green leaves; 3 = onset of leaf drying; 4 = from 30% to 50% dead leaves; 5 = more than 50% dead leaves; 6 = completely dead.

**Table 3.** Height (score 1 to 6)\* of *Lippia alba* genotypes at 180 days at different temperatures and with different treatments.

Treatments	Genotypes				
	LA-13	LA-22	LA-29	LA-44	LA-57
-----18°C-----					
With mineral oil	2.3 <sup>aCα</sup>	6.0 <sup>aDα</sup>	1.2 <sup>aBβ</sup>	1.1 <sup>aAα</sup>	1.2 <sup>aABα</sup>
With water	6.0 <sup>bAα</sup>	6.0 <sup>aAα</sup>	6.0 <sup>bAα</sup>	6.0 <sup>bAα</sup>	6.0 <sup>bAα</sup>
Control	6.0 <sup>bAα</sup>	6.0 <sup>aAα</sup>	6.0 <sup>bAα</sup>	6.0 <sup>bAα</sup>	6.0 <sup>bAα</sup>
-----23°C-----					
With mineral oil	6.0 <sup>aBβ</sup>	6.0 <sup>aBα</sup>	1.0 <sup>aAα</sup>	1.0 <sup>aAα</sup>	6.0 <sup>aBβ</sup>
With water	6.0 <sup>aAα</sup>	6.0 <sup>aAα</sup>	6.0 <sup>bAα</sup>	6.0 <sup>bAα</sup>	6.0 <sup>aAα</sup>
Control	6.0 <sup>aAα</sup>	6.0 <sup>aAα</sup>	6.0 <sup>bAα</sup>	6.0 <sup>bAα</sup>	6.0 <sup>aAα</sup>
CV (%)	1.46				

Means that are followed by the same lower case letters in columns of the same temperature, upper case letters in rows, and Greek letters between temperatures in columns and the same treatments with mineral oil showed no significant differences according to Tukey's test ( $p>0.05\%$ ).

### In vitro conservation at 270 days

For 270 days of conservation at 18°C, the LA-13 and LA-57 genotypes that were immersed in mineral oil showed mean survival rates that were greater than 96%, and there was no significant difference between them in survival (Table 4). Both genotypes had color means that corresponded to green and viable leaves (Figure 1A and 1B).

The two genotypes did differ significantly in height. LA-57 had a height category mean of 2.5, which corresponds to a height of 2.1 to 4.0 cm, and LA-13 had height category mean of 4.3, which corresponds to a height of 6.1 to 8.0 cm (Table 4). The water-immersion and control treatments resulted in low survival rates but high values for color and height (Table 4). At 270 days of conservation, both the LA-13 and LA-57 genotypes resumed their growth

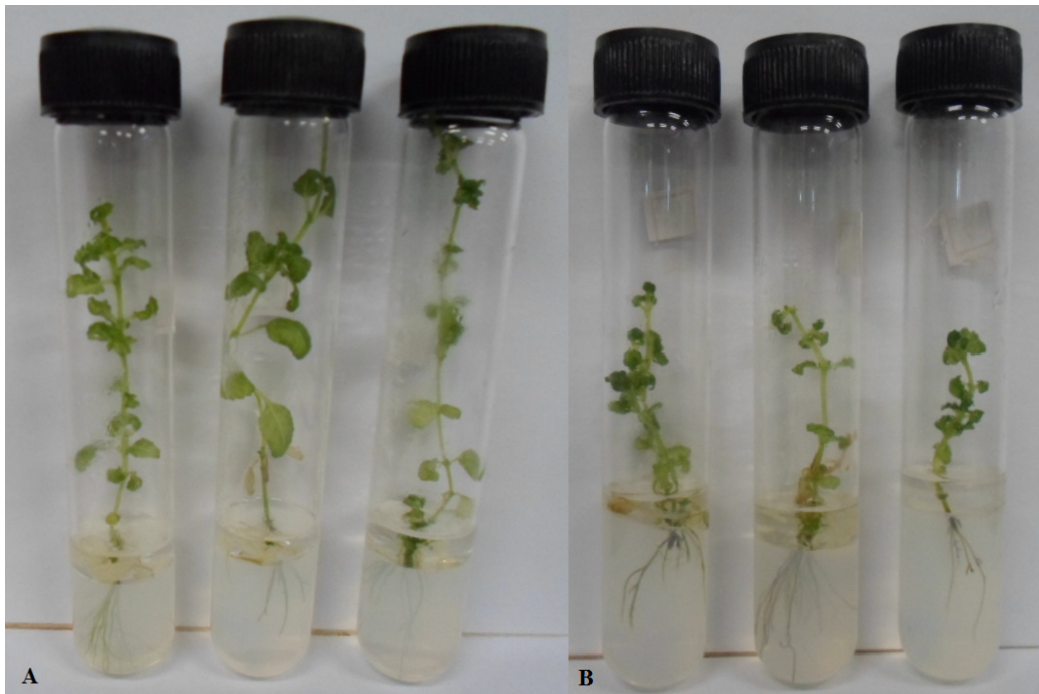
when placed under normal conditions of in vitro multiplication, which is critical for the success of the method because the plants must have sufficient

vigor when they are transferred to the propagation medium (Figure 2A and 2B).

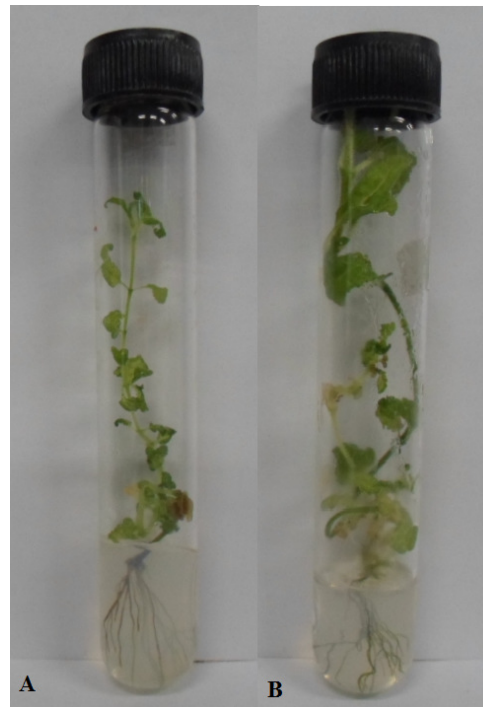
**Table 4.** Survival (%), color and height of *Lippia alba* genotypes at 270 days at 18°C.

Treatments	Genotypes	
	LA-13	LA-57
	----- Survival (%) -----	
With mineral oil	96.7 <sup>aA</sup>	100.0 <sup>aA</sup>
With water	10.0 <sup>aB</sup>	3.3 <sup>aB</sup>
Control	10.0 <sup>aB</sup>	0.0 <sup>aB</sup>
CV (%)	35.44	
	----- Color (score 1 to 6)* -----	
With mineral oil	1.9 <sup>bA</sup>	1.4 <sup>aA</sup>
With water	5.9 <sup>aB</sup>	5.9 <sup>aB</sup>
Control	5.9 <sup>aB</sup>	6.0 <sup>aB</sup>
CV (%)	5.88	
	----- Height (score 1 to 6)** -----	
With mineral oil	4.3 <sup>bA</sup>	2.5 <sup>aA</sup>
With water	6.0 <sup>aB</sup>	6.0 <sup>aB</sup>
Control	6.0 <sup>aB</sup>	6.0 <sup>aB</sup>
CV (%)	7.94	

Means that are followed by the same lower case letters in rows (between genotypes) and upper case letters in columns (between treatments with mineral oil), showed no significant differences according to Tukey's test ( $p > 0.05\%$ ); \*Rating scale: 1 = green leaves; 2 = pale-green leaves; 3 = early leaf drying; 4 = from 30 to 50% dead leaves; 5 = more than 50% dead leaves; 6 = completely dead; \*\* Rating scale: 1 = less than or equal to 2.0 cm, 2 = 2.1 to 4.0 cm, 3 = 4.1 to 6.0 cm, 4 = 6.1 to 8.0 cm, 5 = 8.1 to 10.0 cm and 6 = equal to or greater than 10.1 cm.



**Figure 1.** *Lippia alba* plants of the carvone chemotype that were immersed in mineral oil for 270 days, LA-13(A) and LA-57 (B).



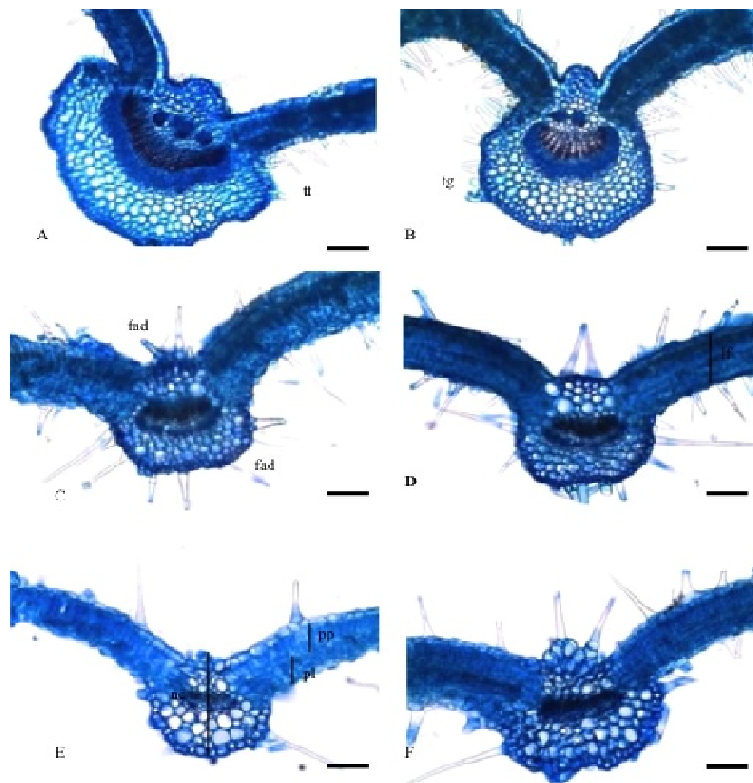
**Figure 2.** The resumption of plant growth of *Lippia alba* (carvone chemotype) after 40 days of immersion in mineral oil, LA-13(A) and LA-57(B).

Both LA-13 and LA-57 were viable as measured by the parameters that represent survival, color and leaf size. LA-57 had the lowest height values in the mineral oil treatment (Table 4). Thus, conservation by immersion in mineral oil after the

#### **Leaf anatomy of the LA-13 and LA-57 genotypes**

The mesophyll of *L. alba* is characterized by palisade parenchyma (pp) that is in contact with the adaxial side (ads) and by spongy parenchyma (sp) on the abaxial side (abs; Figure 3). There was a significant difference in midrib (mr) thickness between environments. As hypothesized, plants that had already been established in field conditions had thicker midribs than acclimated plants. Among the field plants, the LA-13 genotype had significantly thicker midribs than the LA-57 genotype (Table 5). Acclimated and field plants had thicker leaf blades than plants that were maintained in vitro. LA-13 had the thickest leaf blades (Table 5). There was a significant difference in the number of glandular trichomes among environments for both genotypes. The leaves of field plants, especially those of the LA-57 genotype, had more trichomes. LA-57 had 8.6 glandular trichomes per mm<sup>2</sup> (Table 5). There were no significant differences in the number of tector trichomes among the in vitro, acclimated and field environments for the LA-13 genotype, and there was no significant difference between the in

270-day period was more effective for the carvone chemotype (LA-13 and LA-57). Because of the promising results with LA-13 and LA-57 when they were immersed in mineral oil, these two genotypes were selected for the examination of leaf anatomy, vitro and field environments for the LA-57 genotype (Table 5). Both genotypes had thicker cuticles on the adaxial side under field conditions. There was no significant difference between acclimated and field conditions in the cuticle thickness on the adaxial side of leaves of plants of the LA-57 genotype (Table 6). The leaf cuticle could not be measured for the plants that were grown in vitro.



**Figure 3.** Photomicrographs of cross sections of the leaf structure of the carvone phenotype of *Lippia alba*, that originated from the field: LA-13(A) and LA-57(B); had been acclimated: LA-13(C) and LA-57( D); or grown in vitro: LA-13(E) and LA-57(F); lb: leaf blade; mr: midrib; tt: tector trichome; gt: glandular trichome; pp: palisade parenchyma; sp: spongy parenchyma; ads: adaxial side; abs: abaxial side. Scale bars: 50 μm.

**Table 5.** Midrib thickness (mr), leaf blade thickness (lb), the number of glandular trichomes (gt) and the number of tector trichomes (tt) of the LA-13 and LA-57 genotypes of *Lippia alba*, in three environments.

Environment	mr (μm)		lb (μm)		gt per mm <sup>2</sup>		tt per mm <sup>2</sup>	
	LA-13	LA-57	LA-13	LA-57	LA-13	LA-57	LA-13	LA-57
IV	223.6 <sup>aC</sup>	272.4 <sup>aC</sup>	100.6 <sup>aB</sup>	97.4 <sup>aB</sup>	2.4 <sup>bB</sup>	4.9 <sup>aB</sup>	9.0 <sup>bA</sup>	16.3 <sup>aA</sup>
AC	355.5 <sup>aB</sup>	363.8 <sup>aB</sup>	195.1 <sup>aA</sup>	156.9 <sup>bA</sup>	1.3 <sup>aB</sup>	1.3 <sup>aC</sup>	12.1 <sup>bA</sup>	10.6 <sup>bB</sup>
FL	672.5 <sup>aA</sup>	582.6 <sup>bA</sup>	208.1 <sup>aA</sup>	165.6 <sup>bA</sup>	6.5 <sup>bA</sup>	8.6 <sup>aA</sup>	11.6 <sup>bA</sup>	18.9 <sup>aA</sup>
CV (%)	19.64		18.47		38.55		32.34	

IV: In vitro; AC: Acclimated; FL: Field; Means that are followed by the same lower case letters in rows (between genotypes) and upper case letters in columns (among environments) did not differ significantly according to Tukey's test (p>0.05%).

**Table 6.** Cuticle thickness of the adaxial (cad) and abaxial (cab) side of the LA-13 and LA-15 genotypes of *Lippia alba*, in acclimated and field environments.

Environments	cad (μm)		cab (μm)	
	LA-13	LA-57	LA-13	LA-57
AC	14.8 <sup>bB</sup>	17.2 <sup>aA</sup>	12.8 <sup>aB</sup>	11.4 <sup>aB</sup>
FL	17.8 <sup>aA</sup>	17.3 <sup>aA</sup>	16.0 <sup>aA</sup>	16.2 <sup>aA</sup>
CV (%)	17.85		21.38	

AC: Acclimated; FL: Field; Means that are followed by the same lowercase letters in rows (between genotypes) and upper case letters in columns (between environments) did not differ significantly according to Tukey's test (p>0.05%).

## DISCUSSION

The results showed that immersing explants in mineral oil was effective in the in vitro conservation of *L. alba* genotypes that belong to the carvone chemotype. The effectiveness was shown for a 270-day period, and met the key conservation goals of reducing growth without affecting plant viability, reducing labor costs and maintaining the genetic material, which allows having longer time periods between subcultures or even extending them indefinitely (MOOSIKAPALA; TE-CHATO, 2010). It is noteworthy that genotypes responded differently to immersion in mineral oil for 180 days, although the responses were similar within chemotypes. Conservation of plants of the carvone chemotype (LA-13 and LA-57) in mineral oil at 18°C proved to be effective as measured by the three analyzed parameters. Plants of the citral chemotype (LA-29 and LA-44) had lower mean heights, but had an undesirable color (Table 2). Plants of the linalool chemotype (LA-22) had the highest values of color and height. These differences demonstrate the considerable plasticity that exists among *L. alba* genotypes.

Physically, mineral oil is an oily, colorless and chemically inert liquid, which makes it an optimal environment for conservation. Lack of oxygen under the mineral oil layer may account for the slow growth of explants because their growth is controlled by the supply of oxygen to tissues (MATHUR et al., 1991). In contrast, plant growth was not reduced in the control and water-immersed treatments because oxygen was available to the explants.

There are still few published studies in which mineral oil was used in in vitro conservation. An in vitro conservation protocol that included immersion in mineral oil was recently tested in genotypes of *Bacopa monnieri* (SHARMA et al., 2012), which was successful for a period of 6 to 24 months. Plants that were covered in mineral oil exhibited 80% survival compared with 66% in controls.

It is important to emphasize that plants with low color scores, i.e., plants that are greener, are preferable for in vitro conservation, even if they are somewhat taller than desired, because senescence is not desirable for conservation under a slow growth regime, and plants must be viable to resume growth when required (SANTOS et al., 2012).

Chemical analyses showed that the LA-57 genotype of *L. alba* has a higher concentration of carvone in its essential oil (77.2%) than the LA-13 genotype (39.6%). Carvone is an important

compound because it is widely used in the pharmaceutical and perfume industries in various products, including toothpaste, antiseptic mouthwash, bread, pickles, condiments and beverages. Carvone also has antiseptic, bactericidal and antifungal activity (ANTUNES, 2005).

The leaves of the in vitro plants had reduced mesophyll and palisade and spongy parenchyma differentiation, and smaller intercellular spaces, which explains their lower values of midrib and leaf blade thickness compared with the leaves of the ex vitro plants. A similar result was found in the leaves of ex vitro *Tabebuia roseo alba* ('ipê-branco') plants, which had thicker leaf blades and midribs than the leaves of plants that were grown in vitro (ABBADE et al., 2009).

The leaves of plants of the carvone-limonene chemotype of *L. alba* had a simple epidermis that was coated with a relatively thick cuticle, stomata with large substomatal chambers on both sides, and mesophyll that consisted of one or two layers of palisade parenchyma and three or four layers of spongy parenchyma (SANTOS et al., 2004).

The light intensity that was used for the in vitro cultures in the present study was much lower than the light intensity of the greenhouse and field conditions. The transplanted seedlings therefore experienced greater light intensity, which caused higher photosynthetic activity. Structural changes in the leaf blade can therefore be attributed to the new environmental conditions (Fermino-Jr and Scherwinski 2009). Leaves that grow under high light intensity, including the leaves of field plants, are known to have thicker leaf blades (SCHULZE; MULLER-HOHENSTEIN 2005; DOUSSEAU et al., 2008; LAMBERS et al., 2008).

Cuticle development was significantly smaller in the leaves of plants that were grown in vitro compared with the leaves of plants that were grown ex vitro. Indeed, cuticle development in the leaves of the in vitro plants was too small to be measured. The small size might have been due to differences in light intensity and atmospheric humidity between the in vitro and ex vitro environments. Light intensity is greater for acclimated plants and even greater for field plants, and atmospheric air humidity varies with the season for acclimated and field plants (Lamhanedi et al. 2003). Cuticle thickening is ecologically related to reduction of water loss, especially in leaves that are exposed directly to sunlight. The plants show increased resistance in environments that have a water deficit because the deficit results in greater conservation of water in the plant, which prevents



evaporation (PALLARDY; RHOADS, 1993; RISTIC; JENKS, 2002).

Glandular and tector trichomes were found on both sides of the mesophyll of all of the genotypes. Trichomes are epidermal appendages that can be found in various plant organs, including stems, flowers, roots and leaves (DICKINSON, 2000). The secretion of essential oils has been associated with the presence of trichomes in the genus *Lippia* (Nunes et al. 2000; Hassan et al. 2001). The availability of incident light might affect the density of glandular trichomes, which would result in an increase in the number of trichomes in plants that are grown in full sun (COSTA et al., 2010).

The tector trichomes of *L. alba* are multicellular and uniseriate. They contain two basal cells, one to three intermediate cells and one tapered cell at the end. Various functions have been attributed to these trichomes in plants, including temperature regulation, protection against herbivory, light reflection, reduction of water loss and serving as a mechanical barrier to various external factors (WAGNER et al., 2004).

No relevant anatomical variations were found within each of the chemotypes in this study.

Santos et al. (2004) examining the carvone-limonene chemotype of *L. alba*, reported a similar observation.

## CONCLUSIONS

*In vitro* conservation of *L. alba* genotypes of the carvone chemotype by immersion in mineral oil is feasible for 270 days and is feasible for 180 days for the other chemotypes.

Leaves of the carvone chemotype that were conserved for 270 days showed plasticity that was responsive to differences in vitro and ex vitro environments.

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**RESUMO:** O objetivo deste estudo foi desenvolver um protocolo de conservação *in vitro* com óleo mineral e estudar a anatomia foliar de genótipos de *L. alba* nos ambientes *in vitro* e *ex vitro*. A conservação *in vitro* constou de cinco genótipos LA-13 e LA-57 (quimiotipo carvona); LA-22 (quimiotipo linalol); LA-29 e LA-44 (quimiotipo citral), três tratamentos (com óleo mineral, com água e controle) e duas temperaturas 18 e 23°C. Aos 180 e 270 dias, avaliou-se a sobrevivência (%), altura da parte aérea (cm) e coloração das folhas. Os genótipos com os melhores resultados da conservação *in vitro* (LA-13 e LA-57) foram utilizados para estudos de anatomia. Avaliou-se espessura da nervura central, espessura do limbo foliar, cutículas adaxial e abaxial e quantidade de tricomas glandulares e tectores. A temperatura a 18°C foi a ideal para a conservação dos genótipos do quimiotipo carvona, com folhas verdes e baixos valores para altura, destacando-se LA-57 cuja altura não ultrapassou 2,0 cm. Aos 270 dias LA-13 e LA-57 permaneceram com folhas verdes e viáveis, com destaque para LA-57 com menores médias de altura. A anatomia do quimiotipo carvona mostrou que as plantas de campo apresentaram maiores espessuras da nervura, limbo e cutículas, e maiores quantidades de tricomas. O genótipo LA-57 obteve a maior quantidade de tricomas glandulares e tectores.

**PALAVRAS-CHAVE:** Verbenaceae. Crescimento Lento. Anatomia Vegetal. Morfologia.

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