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Oil production at different stages of leaf development in *Lippia alba*

Diego Pandeló,[1] Talita D. Melo,[1] Júnya L. Singulani,[1] Fernanda A. F. Guedes,[1] Marco A. Machado,[2] Cíntia M. Coelho,[1] Lyderson F. Viccini,[1] Marcelo O. Santos*[,1]

[1]Departamento de Biologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Campus Martelos, Brazil,
[2]Embrapa Gado de Leite, Brazil.

Abstract: The aim of this work was to analyze terpene oil production and terpene synthases (TPS) gene expression from leaves at different developmental stages of different chemotypes of *Lippia alba* (Mill.) N.E. Br. ex Britton & P. Wilson, Verbenaceae. Hydro-distilled essential oil were used for chemical analysis and gene expression of three monoterpene synthase genes called *LaTPS12, LaTPS23* and *LaTPS25* were used for analyses of gene expression associated to oil production. The putative genes were associated to TPS-b gene class. Semi-quantitative PCR and quantitative PCR (qPCR) analysis were used to investigate the expression profile of those three putative genes in different leaf stages and different chemotypes. Additionally, total oil production and gene expression of putative *TPS genes* cloned from *L. alba* chemotype linalool were evaluated at different stages of leaf development. The expression level of those three genes was higher when the highest oil production was observed, mainly in young leaves at the fourth nodal segment for all evaluated chemotypes. Total oil production was higher at leaves that had unopened trichomes. We also observed that the 1mM of MeJA treatment increased the gene expression in all chemotypes after 24 h elicitation.

Article

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Introduction

Plants have a wide variety of terpene compounds that may produce diverse biological responses with differential expression mediated by developmental and stress-related programs response synthesized by terpene synthase (TPS) (Tholl, 2006). *Lippia alba* (Mill.) N.E. Br. ex Britton & P. Wilson, Verbenaceae, occupies an outstanding position among medicinal plants because of many therapeutic actions due to terpenic compounds (Hennebelle et al., 2008). The essential oil from *L. alba* has additional aromatic properties that could make the specie an excellent source of monoterpenes such as geraniol, limonene, linalool and others. Some advantages of *L. alba* includes the vegetative propagation (Biase & Costa, 2003) and the simplicity of methods used for oil isolation. Some chemotypes yields a dextrogen form of linalool, which is the main compound of essential oil used in industry, with almost 100% of purity (Siani et al., 2002). Tepernes are synthesized by terpene synthases (TPS) a class of enzymes that carry a motif LQLYEASFLL that seems to be part of the active site of those enzymes (Crowell et al., 2002), and two other conserved domains DDXXD/E and the "NSE/DTE" motif (N/D)DXX(S/T)XXXE that are involved in magnesium binding during fixation of pyrophosphate substrate (Christianson, 2006). Molecular biology and genetic improvement of such enzyme and its genes are useful tools to improve the quality and quantity of essential oil (Siani et al., 2002).

The wealth of terpenic compounds in *L. alba* make relevant the analyses of TPS genes expression to improve the quality and quantity of the essential oil. Thus, the objective of the present study was to evaluate the oil production and TPS gene expression during different leaf developmental stages among different chemotypes of *L. alba.*

Material and Methods

The total oil was extracted from fresh leaves of three different chemotypes of *Lippia alba* (Mill.) N.E. Br. ex Britton & P. Wilson, Verbenaceae, citral (CESJ 29423), carvone (CESJ 29420) and linalool (CESJ 29422) (Tavares et al., 2005) from three leaf positions: i) the second nodal segment; ii) the fourth nodal segment; and iii) the eighth nodal segment. The essential oil was extracted by hydro-distillation (Clevenger apparatus) during 2 h. The oil from different chemotypes was harvested and injected at GC/

MS eluted in 200 µL of *n*-hexane (HPLC grade) followed by thin layer chromatography (TLC) T-6520 silica gel polyester plate. One microliter of each sample was injected in gas chromatograph (Shimadzu GCMS-QP2010 Plus) with auto injector (AOC-5000) coupled to the Rtx-5MS capillary column (30 m long, 0.25 mm internal diameter, 0.25 µm film thickness) (Supelco DB 5). The programmed temperature began at 50 °C and increased by 4 ºC/min up to 220 °C (5 min). Injector and detector temperatures were 200 and 220 °C, respectively. Helium gas was used as carrier. The area of the GC peak was used for quantitative determination. Kovats index (KI) was used for essential oil identification by comparing their mass spectra with NIST98 (National Institute of Standards and Technology, Gaithersburg) mass spectral database and by comparing their GC retention indexes (RI), related to the retention time of a series of n-alkanes with linear interpolation, with those of literature data (Adams, 2007).

Two micrograms of total RNA was used for the first strand cDNA synthesis. Reverse transcription reaction was done using oligo (dT) anchored primer and the enzyme MuMLV (Invitrogen, Carlsbad, CA, USA) in accordance to the manufacturer instructions. Genes were amplified by Master Cycle Gradient thermocycler (Eppendorf, Westbury, USA), using GoTaq DNA polymerase (2.5U) (Promega, Madison, USA); $MgCl_2$ (5 mM); dNTP mix (0.4 mM); forward primer (4 µM); reverse primer (4 µM) and DNA (200 ng) in a 25 µL total reaction volume. Each reaction was carried out during 3 min at 94 °C; 34x (94 °C 1 min; 54 °C 1 min and 72 °C 1 min) followed by final extension with 10 min at 72 °C. PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, USA) and sequenced by ABI Prism 7300 Sequence Detection System (Applied Biosystems, USA). Leafs from I, II and III position from each chemotypes were evaluated by RT-PCR and qPCR analysis and the 18S rRNA gene was choose as control (Santos et al., 2005). The qPCR reactions were carried out using the kit iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, USA), 50 ng of cDNA and 400 nM of specific primer. The conditions of amplification was 95 ºC during 3 min; 40 cycles of denaturation at 95 ºC during 15 s; and annealing and extension at 60 ºC during 60 s. After forty amplification cycles, the samples were subjected to dissociation curve analysis in order to validate the absence of non-specific products and dimmer formation. The samples were heated from 60 ºC up to 94 ºC, increasing 1 ºC at each 30 s.

Each amplification reaction was carried out in ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, USA) in separated wells using SYBR Green Dye. The results were analyzed using Relative Expression Software Tool (REST ©), v. 2.0.

Results and Discussion

The analyses of gas chromatography coupled to a mass spectrometer were carried out and the major components of the three evaluated chemotypes and the nature of main terpene composition (Figure 1). The evaluation of total oil showed that high production was concentrated at the fourth leaf nodal segment in all evaluated chemotypes. Leaves at the first analyzed stage of development do not produced oil (Table 1).

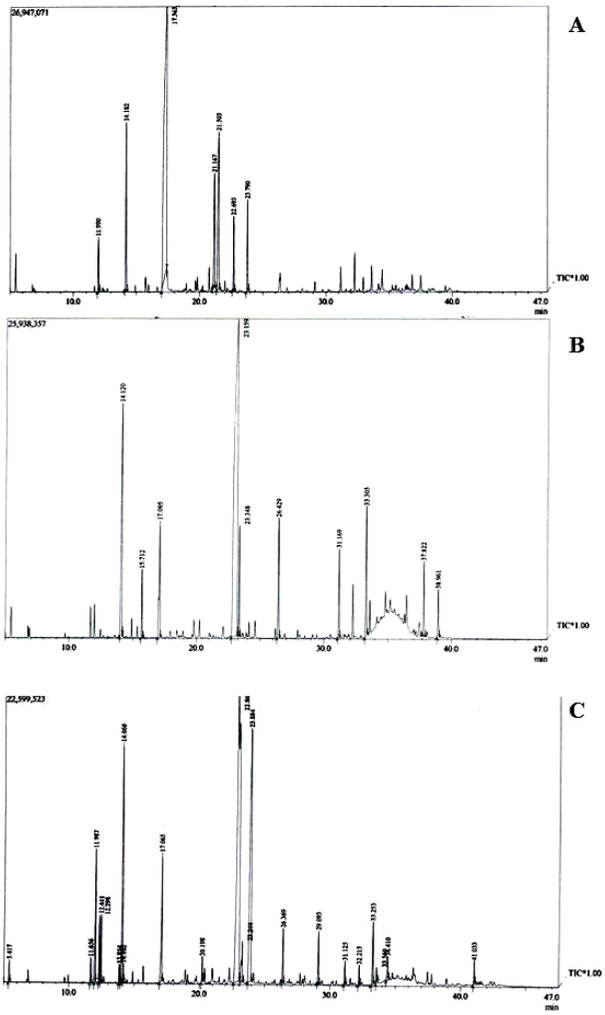

Figure 1. Chromatogram of essential oil analysis from chemotypes of *Lippia alba*: a) linalool; b) carvone and c) citral (neral/geranial).

According to *E-values* of blast-x algorithm, three different putative genes encoding terpene synthases were designated *LaTPS12* (GQ279096), *LaTPS23* (GQ279097) and *LaTPS25* (GQ279098). The three sequences were highly similar to several other monoterpene synthases, mainly to limonene synthase of different plant species. *LaTPS12* showed an *E-value* ranging from 3 e-47 to 3 e-37; *LaTPS23* showed an *E-value* ranging from 2 e-45 to 5 e-37; and *LaTPS25* showed an *E-value* ranging from 4 e-53 to 2 e-06, but *LaTPS25* also showed similarities to

linalool synthase.

Two clones, *LaTPS12* and *LaTPS23*, were 445 pb long and encodes 148 aminoacids, whereas *LaTPS25* was 395 pb encoding a putative protein with 122 aminoacids. All sequences showed the motifs DDXXD and NSE/DTE (N,D)D(L,I,V)X(S,T)XXXE, both highly conserved in other terpene synthases, which apparently works as a metal ion cofactor and catalysis site (Crowell et al., 2002) in the C-terminal domain of these enzymes. Terpene synthase class of enzymes is classified from a to g (Dudareva et al., 2003) and a phylogenetic tree was generated by the neighbor-joining method with 1,000 repetitions containing all enzyme class and we observed that the three putative TPS clone genes belongs to *TPS*-b monoterpene class.

In *L. alba* most of the production of essential oil takes place in leaf trichome (Bolzani et al., 1999) and it was verified by semi-quantitative RT-PCR that unexpanded leaves at the fourth nodal segment (FNS) showed strongly expression followed by leaves situated in the second nodal segment (SNS) and leaves in a riper stage situated in the eighth node (ENS), respectively (Figure 2) for all genes and chemotypes evaluated. *LaTPS12, LaTPS23* and *LaTPS25* had already initiated in unexpanded leaves and continue up to their complete expansion. The higher expression transcription occurred in leaves situated in the fourth nodal segment and practically disappeared in riper stage leaves at the eighth nodal segment. Riper leaves situated around the eighth nodal segment showed little synthesis of mRNA and lower oil production. At this stage most of the leaf trichomes were collapsed as observed for *Mentha* (Sharma et al., 2003) and *Lippia scaberrima* (Combrink et al., 2007). In *Magnolia grandiflora*, it was also observed a similar pattern of expression of three different *TPS* genes coding for two monoterpene and one sesquiterpene synthase (Lee & Chappell, 2008). The younger leaves showed higher expression compared to riper leafs for all analyzed genes.

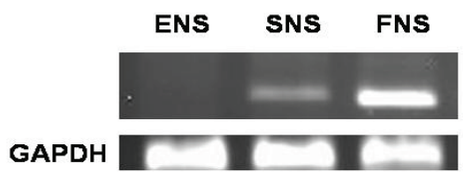

Figure 2. Expression of the genes *LaTPS12, LaTPS23* and *LaTPS25* in different leaf stages of three chemotypes. ENS: Leaf of eight nodal segment; SNS: leaf of second nodal segment; FNS: leaf of fourth nodal segment.

Comparing linalool and carvone chemotypes, *LaTPS12* and *LaTPS23* gene expression by qPCR analysis showed that they were down-regulated in linalool chemotype with a relative expression ratio of 0.351 and 0.339, respectively and *LaTPS25* gene was up regulated in the linalool chemotype showing around 16.42 more transcripts (Figure 3a).

The analysis between the chemotypes linalool and citral showed differential expression between *LaTPS12* and *LaTPS25* genes. The first was down-regulated whose ratio of relative expression was 0.248 and the second was up-regulated in linalool type, with relative expression ratio of 6.496. The gene *LaTPS23* did not show significant difference between these chemotypes (P (H1) =0.950) (Figure 3b).

The comparison between the chemotypes carvona and citral resulted in similar relative expression between the samples of all the genes and its expression was not statistically different between the analyzed chemotypes (Figure 3c). The values of P (H1) were 0.575 for *LaTPS1*2; 0.878 for *LaTPS23*; and 0.740 for *LaTPS25*.

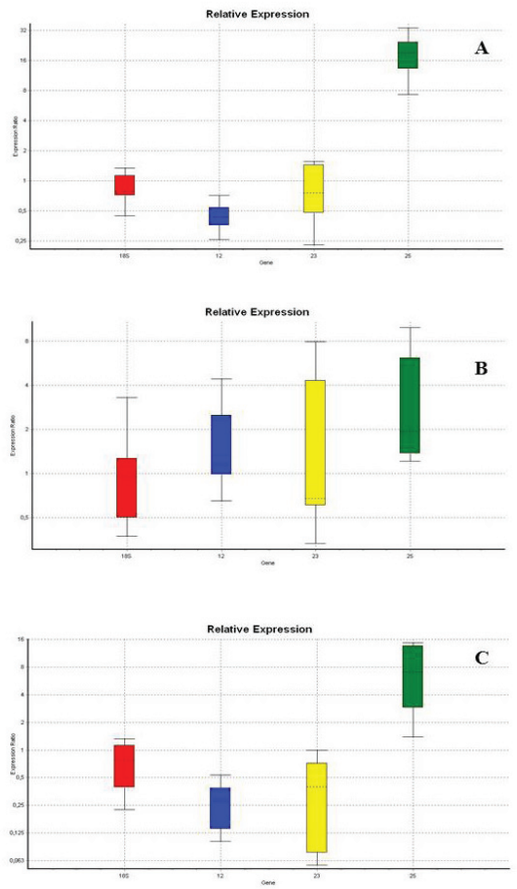

Figure 3. Relative expression of TPS between chemotypes of *L. alba* a) linalool and carvone; b) linalool and citral; c) carvone and citral. Red (18S); Blue *LaTPS12*; yellow *LaTPS22*; green *LaTPS25.*

The *LaTPS25* that showed highly similarity to linalool enzymes also showed higher expression in the chemotype linalool compared to the chemotypes carvone and citral. External biotic and abiotic factors affect total secondary metabolite production, such as tissue damage,

Table 1. Main composition of hydro-distilled essential oil from the chemotypes linalool, carvone and citral of *Lippia alba* and total oil production g/g dry weight leaves in different developmental stages.

Nome	*L. alba* 1 (citral)		*L. alba* 2 (carvone)		*L. alb*a 3 (linalool)	
	R.T.	%	R.T.	%	R.T	%
mushroom alcohol	11.987	5.36	***	***	11.990	2.70
5-hepten-2-one, 6-methyl-	12.298	2.23	***	***	***	***
myrcene	12.441	2.23	***	***	***	***
limonene	14.060	11.89	14.120	14.22	***	***
eucalyptol	***	***	***	***	14.182	10.14
linalol	17.065	5.54	17.095	6.12	17.363	57.71
neral	22.866	44.72	***	***	22.693	3.73
(*S*)-(+)-carvone	***	***	23.159	60.07	***	***
piperitone	***	***	23.348	4.03	***	***
geranial	23.884	23.10	***	***	23.790	4.89
germacrene D	***	***	31.169	3.71	***	***
elemol	33.253	2.57	33.305	6.06	***	***
Total oil production SNS	N.D.		N.D.		N.D.	
Total oil production FNS	0.47%		0.66%		0.36%	
Total oil production ENS	0.15%		0.21%		0.12%	

N.D.: not detected; (SNS: second nodal segment; FNS: Fourth nodal segment; ENS: eighth nodal segment); RT: retention time.

attack by pathogens and herbivores, drought, and excessive exposure to ultraviolet rays can induce the production of several defense substances, such as polyphenol oxidase. Under these conditions, different classes of secondary metabolites are produced as phenolic compounds, alkaloids and terpenic compounds (Bennett & Wallsgrove, 1994; Kessler & Baldwin, 2002). The leaves of *L. alba* were collected at the same time and under the same conditions from a greenhouse to avoid environmental influence and the results are a consequence of genetic variation among the chemotypes. These results are in accordance to previous studies with these chemotypes of *L. alba* that when cultivated at different conditions did not affected the composition of essential oil from leaves (Tavares et al., 2005) showing the same composition of main components of terpenes. Similar studies by our group showed that the quality of essential oil was not affected by dry or wet season in *Lippia* and *Lantana* species reinforcing genetic control of essential oil production (Silva et al., 2010). The addition of kinetin, an artificial cytokinin type of plant growth regulator in culture medium increased terpene production (Tavares et al., 2004). Increasing activity of FPP, the central precursor of terpenes, reduced the cytokinin level production since they share the same methabolic pathway (Masferrer et al., 2002). In *Ocimum basilicum,* the spraying of methyl jasmonate induced the production of several secondary metabolites, including linalool and other terpenoids (Zhigang et al., 2007). To test if elicitors may affect the putative TPS gene expression we also evaluated the expression of those cloned genes under MeJA treatment and we observed an increase of 2-3 times on transcription levels when treated with 1 mM methyl jasmonate solution of. It was observed that developmental stage such as flowering and vegetative growth affect the total oil production but not the quality of oil in leaves of *L. alba* (Siani et al., 2002; Tavares et al., 2005), reinforcing the suggestion of genetic control of oil production.

Thus, the selection of genotypes showing higher linalool gene expression and oil production can help the development an elite cultivar for oil production. Moreover, the results of TPS gene expression may contribute to better understand how to increase the essential oil production, mainly linalool. It also helps to plan future strategies regarding *L. alba* breeding programs using biotechnological applications.

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***Correspondence**

Marcelo O. Santos
Departamento de Biologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Campus Martelos, MG, Brazil
marcelo.santos@ufjf.edu.br
Tel. +55 32 2102 3206
Fax: +55 32 2102 3220