DIEGO SILVA BATISTA

GROWTH PERFORMANCE AND ESSENTIAL OIL PRODUCTION IN *in vitro* GROWN *Lippia alba* PLANTLETS AS AFFECTED BY LIGHT QUALITY AND ELEVATED CO_2

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 17 de fevereiro de 2016.

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BIOGRAFIA

DIEGO SILVA BATISTA, filho de Maria de Fátima Silva Batista e Arnaldo Gomes Batista, nasceu em 10 de fevereiro de 1987, na cidade de Esperança, estado da Paraíba. Graduou-se em Agronomia em julho de 2010 pela Universidade Federal da Paraíba. Iniciou o mestrado em Fisiologia Vegetal em agosto de 2010 na Universidade Federal de Viçosa, tendo defendido tese em fevereiro de 2012. Iniciou o doutorado em Fisiologia Vegetal em março de 2012 na Universidade Federal de Viçosa, tendo defendido tese em fevereiro de 2016.

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RESUMO

BATISTA, Diego Silva, D.Sc., Universidade Federal de Viçosa, fevereiro de 2016. **Crescimento e produção de óleos essenciais de plantas de *Lippia alba* cultivadas *in vitro* influenciadas pela qualidade de luz e aumento de CO_2**. Orientador: Wagner Campos Otoni. Coorientador: Lyderson Facio Viccini.

Com o objetivo de avaliar o efeito da qualidade de luz na morfogênese e produção de óleos essenciais e do incremento de CO_2 na morfogênese, anatomia, perfil qualitativo de produção de óleos essenciais e expressão de genes relacionados à rota de síntese em três quimiotipos de *Lippia alba* (BGEN-01, BGEN-02 e BGEN-42), três experimentos foram conduzidos *in vitro*. No primeiro experimento, as plantas foram cultivadas em meio MS sem reguladores em três diferentes condições lumínicas: lâmpadas fluorescentes, LEDs brancas e LEDs azul/vermelho, após 40 dias a análise qualitativa de perfil de óleos essenciais e a análise quantitativa dos parâmetros morfogênicos: altura de planta, massa fresca e seca, clorofilas totais e carotenoides totais foram realizadas. No segundo experimento, o efeito das trocas gasosas no perfil de óleos essenciais foi avaliado comparando plantas crescidas *in vitro* 45 dias sob três diferentes taxas de troca de CO_2: 14, 21 e 25 $\mu L\, L^{-1}\, s^{-1}$ de CO_2. No terceiro experimento, além dos tratamentos de 14 e 25 $\mu L\, L^{-1}\, s^{-1}$ de CO_2, as plantas foram colocadas em caixas com ventilação forçada, perfazendo concentrações internas de 360 e 1000 $\mu L\ L^{-1}$ de CO_2, além disso um controle adicional com frascos vedados foi colocado nas caixas, totalizando cinco tratamentos. Após 45 dias, análises de perfil de óleos essenciais, histoquímica, de densidade estomática, morfogênicas e reação da polimerase em cadeia em tempo real (RT-qPCR) foram realizadas. A qualidade de luz influenciou significativamente o crescimento *in vitro* em *L. alba*. LEDs azul/vermelho induziram maiores massa fresca e massa seca nos quimiotipos BGEN-01 e BGEN-02 e menores em BGEN-42. Os teores de pigmentos fotossintéticos também foram maiores em plantas cultivadas em LEDs azul/vermelho em todos os quimiotipos. A análise multivariada permitiu identificar diferentes padrões de produção de óleos essenciais entre os tratamentos. A composição dos compostos voláteis variou entre as diferentes qualidades de luz e quimiotipos, essa variabilidade se deu principalmente devido às diferenças nas quantidades de eucaliptol e linalol. O enriquecimento com CO_2 aumentou os pesos fresco e seco, clorofilas totais e carotenoides em BGEN-01 e BGEN-02, e também aumentou a densidade estomática e os teores de lignina em todos os quimiotipos. A análise multivariada dos óleos

essenciais mostrou que estes variaram não só entre os diferentes quimiotipos, mas também dentro de BGEN-01 e BGEN-02 a produção qualitativa foi diferenciada nos tratamentos com renovação forçada de ar e enriquecimento com CO_2. A sintase do farnesil pirofosfato (FPPS) e a sintase do geranil pirofosfato (GPPS) não variaram seus níveis de expressão, exceto nos tratamentos com renovação forçada de ar (360 e 1000 µL L^{-1}) no BGEN-01, nos quais houve uma supra-regulação de *FPPS*. A *sintase do geraniol* (*GS*) foi supra-regulada em todas as condições de CO_2 para BGEN-02 e em 360 e 1000 µL L^{-1} de CO_2 para BGEN-01. A *sintase do nerolidol* (*NS*) foi supra-regulada somente em BGEN-01 sob as condições de 360 e 1000 µL L^{-1} de CO_2. Esses resultados fornecem um melhor entendimento da relação entre a qualidade de luz e o perfil de óleos essenciais e também de como o CO_2 regula a produção de metabólitos secundários, fornecendo subsídios para futuros estudos que permitirão uma produção direcionada de óleos essenciais com maiores interesses industrial e econômico.

ABSTRACT

BATISTA, Diego Silva, D.Sc., Universidade Federal de Viçosa, February, 2016. **Growth performance and essential oil production in *in vitro* grown *Lippia alba* plantlets as affected by light quality and elevated CO_2**. Adviser: Wagner Campos Otoni. Co-Adviser: Lyderson Facio Viccini.

Aiming to evaluate the effect of light quality in morphogenesis and essential oil production and CO_2 elevation on morphogenesis, anatomy, essential oils qualitative production and expression of genes related to the synthesis pathway in three chemotypes of *Lippia alba* (BGEN-01, BGEN-02 and BGEN-42), three experiments were conducted *in vitro*. In the first experiment, plants were grown in hormone-free MS medium at three lights conditions: fluorescent lamps, White LED bulbs and blue/red LEDs, after 40 days qualitative analysis of essential oils profile and quantitative analysis of the morphogenic parameters: plant length, fresh and dry weight, total chlorophyll and total carotenoids were performed. In the second experiment, the effects of gas exchange in essential oil profile were evaluated by comparing plants 45-days grown under three CO_2 exchange rates: 14, 21 and 25 $\mu L\, L^{-1}\, s^{-1}$ CO_2. In the third experiment, in addition to the previous 14 and 25 $\mu L\, L^{-1}\, s^{-1}$ CO_2 treatments, plants were put into a chamber with continuous forced air ventilation, leading to inner of 360 and 1000 $\mu L\ L^{-1}$ of CO_2, an additional control without allowing gas exchange was added inside the chambers, totaling five treatments. After 45 days of growth, analyses of essential oils profile, histochemical, stomatal density, morphogenic evaluation and real-time reverse transcription polymerase chain reaction (RT-qPCR) were performed. The light quality significantly influenced the *in vitro* growth of *L. alba*. Blue/red LEDs induced higher fresh and dry weigh in BGEN-01 and BGEN-02 chemotypes and the lower to BGEN-42. Photosynthetic pigments were also higher in plants grown under blue/red LEDs for all chemotypes. The multivariate analysis allowed to identify different patterns of essential oil production among the treatments. The composition of the volatile compounds ranged with the light quality and chemotypes, this variability is due mainly to differences in amounts of eucalyptol and linalool. The enrichment with CO_2 enhanced plant dry and fresh weight, total chlorophylls and carotenoids in BGEN-01 and BGEN-02, and also increased stomatal density and lignin content for all chemotypes. The multivariate analysis showed that essential oil profile varied not only among the different chemotypes, but also within BGEN-01 and BGEN-02 the

qualitative production were different in the treatments with forced air renovation and CO_2 enrichment. Farnesyl pyrophosphate synthase (FPPS) and geranyl pyrophosphate synthase (GPPS) did not vary, except for the treatments with forced air ventilation (360 and 1000 µL L^{-1}) in the BGEN-01, which had *FPPS* upregulated. *Geraniol synthase* (*GS*) was upregulated in all BGEN-02 treatments and to the BGEN-01 treatments with 360 and 1000 µL L^{-1} CO_2. *Nerolidol synthase* (*NS*) was upregulated only in the BGEN-01, at the treatments with 360 and 1000 µL L^{-1} CO_2. These findings provide a better understanding of the relation between light quality and essential oil profile and also of how CO_2 regulates secondary metabolites production, giving basis for further studies that can allow an oriented production of essential oils with greater economic and industrial interest.

INTRODUCTION

Many natural compounds present in plants, herbs and spices have shown biological activity, coming to be a source of antimicrobial agents against several pathogens. Among these compounds, stands out the essential oils, extracted from plants by different techniques. They are important in phytosanitary control and provide an opportunity for develop other techniques, which aim to reduce the negative effects of oxidants, free radicals and microorganisms that cause damage in the food industry (Pereira et al. 2008).

In recent decades, the search for natural products with biological activities that minimize the environmental and health impact caused by various synthetic substances increased. Accordingly, plants are an endless source of potentially active substances and although vegetables contain thousands of chemical constituents, the properties associated with them are especially related to secondary metabolites (Andrade et al. 2012).

Plants synthesize compounds, which are divided into two major groups: primary metabolites and secondary metabolites. The first includes carbohydrates, proteins, lipids, among others, which are classes of compounds originating from the primary metabolism, which are part of cellular activity and vital functions of virtually all living beings. The second group includes compounds originated from the secondary metabolism of low molecular weight, usually produced in small quantities, holders of very different chemical characteristics and sometimes complex, such as flavonoids, alkaloids, terpenoids, tannins, steroids and essential oils (Dewick 2002, Kardong et al. 2012).

For a long time, the secondary metabolites were considered as just excretory products of the plants. Currently, it is known that these compounds are directly involved in the defense mechanisms against herbivores and microorganisms, attraction of pollinators or seed disperser animals, protection against UV rays and other features related to the use of secondary metabolites by humans (Häkkinen et al. 2013, Kliebenstein 2013).

Essential oils are volatile and highly lipophilic compounds, with molecular weight less than 300 g mol^{-1}, which are usually physically isolated by a membrane from other plant components or tissue (Turek & Stintzing 2013). They can be classified into

two groups: those derived from acetyl-CoA, via isopentenyl pyrophosphate and dimethylallyl pyrophosphate formation, which produce the terpenes (monoterpenes, sesquiterpenes, diterpenes, among others); and those derived from the shikimate pathway, forming the phenylpropanoids. All the essential oil constituents are formed from these precursors (Chemat et al. 2013).

Essential oils are considered as promising natural ingredients for the food industry due to their antimicrobial effects (Burt 2004, Atarés et al. 2010, Böhme et al. 2014, Szczepanski & Lipski 2014, Llana-Ruiz-Rabello et al. 2015, Valdés et al. 2015). They can also be used as reducing agents and free radical scavengers in antioxidant (Amorati et al. 2013) and antimicrobial (Otoni et al. 2014a, Otoni et al. 2014b, Otoni et al. 2016) films or even applied directly as conservatives, since they can show a synergistic effect with structural food components (Hyldgaard et al. 2012, Böhme et al. 2014, Otoni et al. 2014c, Proestos et al. 2013, Peng & Li 2014).

The genus *Lippia* (Verbenaceae) is widely distributed in tropical regions as shrubs or small trees. Most species of *Lippia* are concentrated in Brazil, Paraguay and Argentina, with some endemic species found in Africa. The estimated number of species is about 200 (Pascual et al. 2001). In Brazil, there are two important biodiversity centers of *Lippia*, located in the mountains of Cadeia do Espinhaço, at Minas Gerais state, and in the Chapada Diamantina, at Bahia state. In both locations, some species, especially those used in folk medicine, are endangered (Viccini et al. 2006, Peixoto et al. 2006).

Lippia alba (Mill.) N. E. Brown, popularly known as bushy matgrass, bushy lippia, hierba negra and pitiona, is commonly employed in leaf infusion in the treatment of colds, bronchitis, cough, asthma as well as stomach and intestinal disorders (Allen 2007, Lorenzi & Matos 2008). In the Northeast of Brazil, the citral-limonene-carvone chemotype (melissa) is more common and mainly used in the treatment of abdominal pain (Pinto et al. 2006). Also are reported for this species analgesic, antiulcerogenic, antimicrobial, anti-inflammatory, anthelmintic, antioxidant, gastroprotective and cytostatic properties. These effects are attributed to the essential oils produced by this species (Pascual et al. 2001, Carvalho et al. 2003, David et al. 2007, Fontenelle et al. 2007, Singulani et al. 2012, Carmona et al. 2013).

Singulani et al. (2012) made the first report of the chemical composition and antioxidant activity of sixteen species of *Lippia*. Chemical analysis revealed various

terpenoids which are common to the genus. Furthermore, compounds of essential oils obtained from leaves showed high reducing activity, suggesting the potential of these plants as a natural source of strong antioxidants substances that can be used as a natural food additive and pharmaceutical industries.

The chemotype seems to be a more effective factor for separating different treatments in essential oil profiles. Differences among chemotypes are correlated with DNA content of plants, whereas the ploidy levels vary as the constituents of essential oils vary as well. The majority of species of *Lippia* have a haploid chromosome number from 10 to 14. Few species have a higher chromosome number, which suggests the occurrence of polyploidy. *Lippia alba* shows a large variation in genome size, with five distinct chromosome numbers as 2n = 30, 38, 45, 60, 90 (Reis et al. 2014, Viccini et al. 2006, 2014).

Atmospheric carbon dioxide (CO_2) is essential for plant growth and global food production. The concentration of atmospheric CO_2 increased by 22 % between 1960 and 2007, and climate models predict that it can reach increase from 381 $\mu L\,L^{-1}$ (present days) to 550 $\mu L\,L^{-1}$ by the next decades and may even doubling by the end of this century (IPCC 2007, Misra & Chen 2015).

High concentrations of CO_2 may alter secondary metabolite accumulation pattern in plants. On the other hand, some environmental factors such as light, temperature, soil, culture medium and nutrients can interact with CO_2 levels and result in a non-predictable behavior (Sharafzadeh & Ordookhani 2011).

Knowing that the secondary metabolites are highly influenced by environmental conditions, some authors have reported the effects of photoautotrophic *in vitro* culture on the production of these metabolites (Badr et al. 2011, Ghasemzadeh & Jaafar 2011, Iarema et al. 2012, Mohamed & Ibrahim 2012, Supaibulwattana et al. 2012, Saldanha et al. 2014).

Light actively participates in several metabolic pathways in plants, being the *in vitro* tissue culture a useful tool to produce compounds of interest. With changes in the light quality, the *in vitro* development and some metabolic pathways in several species can be manipulated. The spectral quality also affects leaf anatomy, seeming to have greater effects during the leaf-blade expansion process, showing the high level of physiological plasticity (Saebo et al. 1995, Schuerger et al. 1997, Sims & Pearcy 1992, Sáez et al. 2013).

In *in vitro* plant tissue culture, the use of light-emitting diodes (LEDs) shows several advantages over traditional forms of lighting, usually with fluorescent lamps. Characteristics of the LEDs such small size, durability, long lifetime, and the efficiency to achieve the light demand for some particular points of the light spectrum make them more suitable for plant culture. In addition, these feature also favor the use of LEDs in assays in which is desired to study the effect of the quality or quantity of light *in vitro* (Massa et al. 2008, Gupta & Jatothu 2013).

The objective of this work was to examine the effects of light quality and different levels of CO_2 in the morphogenesis *in vitro*, in physiology and production of essential oils in three chemotypes of *Lippia alba*, using techniques of chemistry, anatomy and molecular biology.

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CHAPTER 1

Light quality affects *in vitro* growth and essential oil profile in *Lippia alba* (Verbenaceae)

Abstract

The influence of light quality in the morphogenesis and essential oil production in three chemotypes of *Lippia alba* (BGEN-01, BGEN-02 and BGEN-42) grown *in vitro* was evaluated. The treatments were: fluorescent lamps, White LED bulbs and blue/red LEDs, with the same irradiance. After 40 days of culture in hormone-free MS medium, qualitative analysis of essential oils profile and quantitative analysis of the growth parameters: plant length, fresh and dry weight, total chlorophyll and total carotenoids were performed. The light quality influenced the *in vitro* growth of *L. alba*. Blue/red LEDs induced higher fresh and dry weigh in BGEN-01 and BGEN-02 chemotypes and the lower to BGEN-42. Photosynthetic pigments were also higher in plants grown under blue/red LEDs for all chemotypes. Multivariate analysis allowed the identification of different patterns of essential oil production among the treatments. The composition of the volatile compounds ranged with the light quality and chemotypes, this variability is due mainly to differences in amounts of eucalyptol and linalool. Thus, the knowledge of this relation between light quality and essential oil profile give basis for further studies at genetic level that may elucidate how this regulation works, thereby enabling an oriented production of compounds of interest.

Keywords: LEDs, lemongrass, light spectrum, medicinal plant.

Introduction

Lippia alba (Mill.) N. E. Brown (Verbenaceae) is a small shrub native from South America. It is a species that produces essential oils that have medicinal properties among them analgesic, anti-inflammatory, anticonvulsivant, antifungal and myorelaxant (Aguiar et al. 2008, Hennebelle et al. 2008, Carmona et al. 2013, Oliveira et al. 2014).

The production of essential oil in *L. alba* occurs at the different stages of leaf development and shows different chemotypes named after the major component such as the monoterpenes linalool, citral and carvone (Pandeló et al. 2012).

Recent studies indicated that different portions of the visible region of electromagnetic radiation affect several metabolic pathways in plants. Blue light, red, far-red and even the green light play specific roles at the plant morphogenesis and its regulation (Golovatskaya & Karnachuk 2015, Wang et al. 2015, Zienkiewicz et al. 2015). Thus, *in vitro* culture is a helpful tool to better understand how this relationship works, since it is easier to manipulate light quality, irradiance and photoperiod (Sáez et al. 2013).

Some studies show that with changes in light quality the *in vitro* development as such some metabolic pathways in several species can be manipulated. The spectral quality also affects leaf anatomy, seeming to have greater effects during the leaf-blade expansion process, showing the high level of physiological plasticity (Saebo et al. 1995, Schuerger et al. 1997, Sims & Pearcy 1992).

The use of LED lamps in plant tissue culture seems to be more advantageous than the classic fluorescent ones, since they can be more efficient to achieve the light demand for some particular points of the light spectrum (Gupta & Jatothu 2013). Because of this, in recent years LEDs have been largely used as light source in growth chambers and bioreactors to improve *in vitro* plant development (Yeh & Chung 2009). In addition, the possibility of LED panels with controlled spectral peaks can provide specific spectral patterns leading to desired physiological responses (Gupta & Jatothu 2013).

Plants of chrysanthemum (*Dendranthema grandiflorum* Kitam ‘Cheonsu’) grown *in vitro* under red and far-red LEDs resulted in the highest stem elongation, however the blue component (450 nm) displays an influence and interact with the red and far-red components (Kin et al. 2004, Kurilčik et al. 2008).

A radiation mixture of blue, red, or far-red light allowed greater elongation of internodes in salvia (*Salvia splendens* cv. Red Vista) and marigold (*Tagetes erecta* L. cv. Orange Boy) (Heo et al. 2002). In strawberry, the best growth rate of plantlets was achieved with the balance of 70% red LEDs and 30% blue LEDs (Nhut et al. 2003).

The spectral quality of light also interferes in secondary metabolism. Changes in light quality and quantity in yarrow (*Achillea millefolium* L.) lead to a variation in number, content and profile of volatile constituents, both quantitatively and qualitatively (Alvarenga et al. 2015). The accumulation of the total anthocyanin was enhanced in *Perilla frutescens* var. acuta Kudo with an 8:1:1 mixture of red, blue and white LEDs (Park et al. 2013).

Despite previous studies pointed out the influence of light quality in the plant physiology and morphology, certain effects are difficult to quantify, since they are species-specific, which means that the spectral-dependent plant responses should be analyzed for each species (Massa et al. 2008, Poudel et al. 2008).

In reviewing the literature, no data was found on the association between the influence of light quality on growth and essential oil production in gender *Lippia*. Here we describe how the quality of the light spectrum affects growth, and hence the qualitative production of essential oils of three *Lippia alba* chemotypes.

Materials and methods

Plant material

Three chemotypes of *Lippia alba* (BGEN-01, BGEN-02 and BGEN-42) were obtained from the Department of Biology, Federal University of Juiz de Fora (UFJF, Juiz de Fora, MG, Brazil) and from from Embrapa Genetic Resources and Biotechnology (Cenargen, Brasília, DF, Brazil). *In vitro* plantlets were subcultivated on a monthly basis in a MS-based medium devoid of growth regulators, in the Laboratory of Plant Tissue Culture, at the Institute of Applied Biotechnology for Agriculture (BIOAGRO, Federal University of Viçosa, MG, Brazil).

Light quality effect

Hypocotyl segments with 1 cm of length were transferred to 500 mL glass flasks closed with polypropylene lids with two 10 mm-orifices each covered with a 0.45-μm-diameter pore membranes (PTFE; MilliSeal® AVS-045 Air Vent, Tokyo, Japan). Explants were inoculated into a culture medium comprised of MS salts and vitamins (Murashige & Skoog 1962), 30 g L^{-1} of sucrose, 100 mg L^{-1} of myo-inositol, and 6.5 g L^{-1} of agar Merck™. The pH of the medium was adjusted to 5.7 ± 0.01, and the medium was autoclaved at 120°C and 108 kPa for 20 minutes. These flasks were kept in growth room environment at 25 °C and a 16-h photoperiod.

In order to compare the light quality, three different light sources were tested for each chemotype: 2 fluorescent lamps (HO Sylvania T12, 110W, São Paulo, Brazil), 2 White LED bulbs (SMD 100, 18W, Vilux®, Vitória, ES, Brazil) and 2 blue/red (LabPAR LL-HR/DB-480, 11.6 W) (LabLumens, São Paulo, Brazil), totalizing nine treatments (Table 1). The irradiance was standardized at 41 μmol m^{-2} s^{-1} via a light meter (LI-250A, LI-COR Inc., USA) and the absorption spectra were recorded over a wavelength range of 200 to 800 nm with an spectroradiometer and Ocean Optics Spectra-Suite data acquisition software system (Ocean Optics, Dunedin, FL) (Fig. 1).

After 40 days of cultivation qualitative analysis of essential oils profile and quantitative analysis of the growth parameters (plant length, fresh and dry weight, total chlorophyll and total carotenoids) were performed.

Table 1. Treatments used in the light experiment.

Treatment	Chemotype	Light source
BGEN-01:B/R	BGEN-01	Blue/red LEDs
BGEN-01:F	BGEN-01	Fluorescent lamps
BGEN-01:W	BGEN-01	White LEDs
BGEN-02: B/R	BGEN-02	Blue/red LEDs
BGEN-02:F	BGEN-02	Fluorescent lamps
BGEN-02:W	BGEN-02	White LEDs
BGEN-42: B/R	BGEN-42	Blue/red LEDs
BGEN-42:F	BGEN-42	Fluorescent lamps
BGEN-42:W	BGEN-42	White LEDs

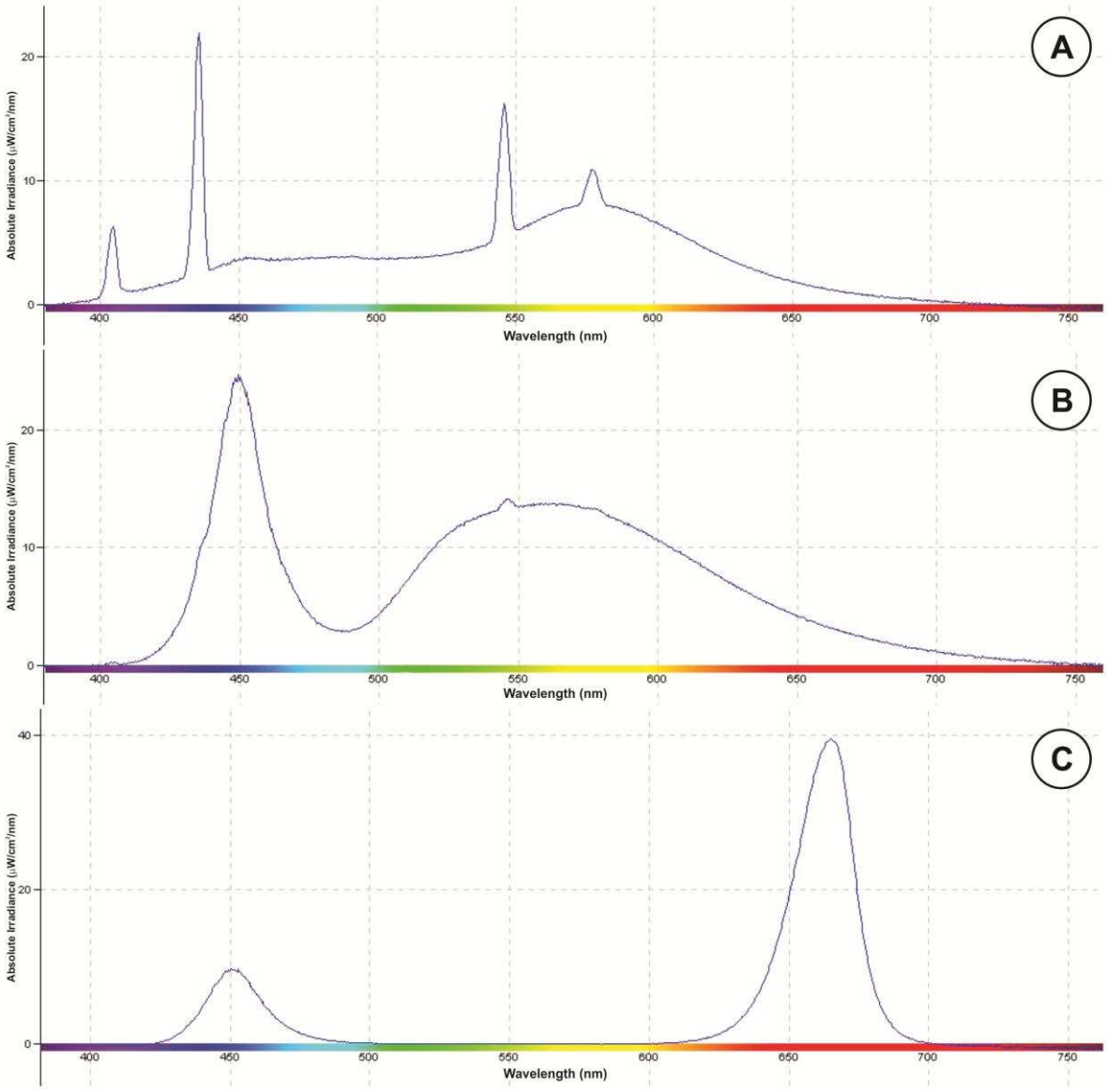

Figure 1. Light spectral of three different light conditions used in the experiment. **A** - Two fluorescent lamps (HO Sylvania T12, 110W); **B** - Two white LEDs (Philips LED, 15W); **C** - Two blue/red LEDs (LabPAR LL-HR/DB-480, 11.6W).

Extraction of the essential oils

Fresh leaves and stems were added to a round bottom flask with 500 mL of capacity. The method used was hydrodistillation for 2 hours using a modified Clevenger apparatus. After this, the hidrolact was collected and centrifuged at 1100 g for 5 min. The essential oil was removed with a Pasteur pipette, packed in glass bottle wrapped with aluminum foil and stored under refrigeration (Agência Nacional de Vigilância Sanitária 2000).

Qualitative analyses of the essential oils

Qualitative analyses were carried out in a Shimadzu CG-17A gas chromatograph coupled to a QP 5000 mass spectrometer (GC-MS), under the following operational conditions: DB5 fused silica capillary column, using helium as a carrier gas; the injector temperature was 250°C; the column temperature was held at 50 °C for 2 min, followed by an increase of 4 °C min^{-1}, until achieving 200 °C; the initial column pressure was 100.2 kPa; and the split ratio was 1:10. The injected sample volume was 1 μL (dichloromethane 1% solution). Under the same conditions of the samples, a series of hydrocarbons was injected (C_9H_{20} ... $C_{26}H_{54}$) (Mjos et al. 2006). The chromatograms obtained were compared with the library Wiley 229 and the Kovats retention indices were calculated for all constituents. The identification of the oils was carried out based on the comparison of its retention indexes with the ones in the literature (Adams 2007).

Statistical analysis

The experiments were carried out according to a factorial scheme 3x3 (3 chemotypes and 3 light conditions), with 12 replicates, being each replicate composed by one flask with 8 plantlets. Regarding to growth and physiological parameters, data were submitted to analysis of variance by test F, followed by Scott & Knott's test at a significance level of 5%.

The essential oil data were subjected to canonical discriminant analysis (CDA), with the study factor consisted of combinations of chemotypes and light qualities. A biplot graph was generated to assess multivariate differences between treatments and examination of the interrelations among the variables and the treatments in a two-

dimensional plane. The Candisc Package (Friendly & Fox 2013) in the software R (R Core Team 2014) was used to the CDA.

Results

There was no significant effect of the light qualities assayed on plant length, which differed just among chemotypes. BGEN-02 was higher than others in all evaluated light conditions (Table 2).

The highest mass accumulation rates in BGEN-01 and BGEN-02 were achieved with blue/red LEDs, whereas for BGEN-42 the opposite was observed: higher mass accumulation with fluorescents bulbs and white LEDs (Table 2).

The contents of chlorophyll and carotenoids were higher in BGEN-42, which was superior to the others chemotypes in all light conditions analyzed. Further, comparing within BGEN-42 treatments, the highest levels of photosynthetic pigments were obtained with blue/red LEDs (Table 2).

Table 2. Growth and physiological parameters of *Lippia alba* in vitro cultures as affected by light conditions, after 40 days of cultivation.

PLANT LENGHT (cm)			
Light condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
Blue/red LED	12.73 Ba	14.73 Aa	12.88 Ba
Fluorescent lamps	13.73 Ba	16.72 Aa	11.73 Ba
White LED	13.32 Ba	15.82 Aa	12.10 Ba
FRESH WEIGHT (g)			
Light condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
Blue/red LED	3.76 Ba	4.08 Aa	2.21 Cb
Fluorescent lamps	2.41 Bb	2.78 Bb	3.66 Aa
White LED	2.67 Bb	3.01 Bb	3.62 Aa
DRY WEIGHT (g)			
Light condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
Blue/red LED	0.384 Ba	0.415 Aa	0.188 Cb
Fluorescent lamps	0.201 Bc	0.299 Ab	0.301 Aa
White LED	0.283 Bb	0.395 Aa	0.293 Ba
TOTAL CLOROPHYLL (µg/cm^2)			
Light condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
Blue/red LED	36.31 Ba	39.00 Ba	62.12 Aa
Fluorescent lamps	32.15 Bb	30.04 Bb	44.93 Ab
White LED	27.46 Bb	31.89 Bb	46.24 Ab
TOTAL CAROTENOIDS (µg/cm^2)			
Light condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
Blue/red LED	4.21 Ca	5.12 Ba	6.35 Aa
Fluorescent lamps	2.59 Cb	4.07 Bb	5.87 Ab
White LED	2.46 Cb	4.44 Bb	5.94 Ab

Means indicated by the same uppercase letters at horizontal and lowercase at vertical are not significantly different as assessed by the Scott & Knott's test at 5% probability level.

By means of CDA analysis, different groups were formed separating the treatments (Figure 2). With the first canonical variable was just possible to retain nearly 85% of the information of variability between treatments. This variability is due practically to differences in amounts of eucalyptol and linalool. These two were negatively correlated with nerolidol, carvone and alpha-bisabolol (Table 3).

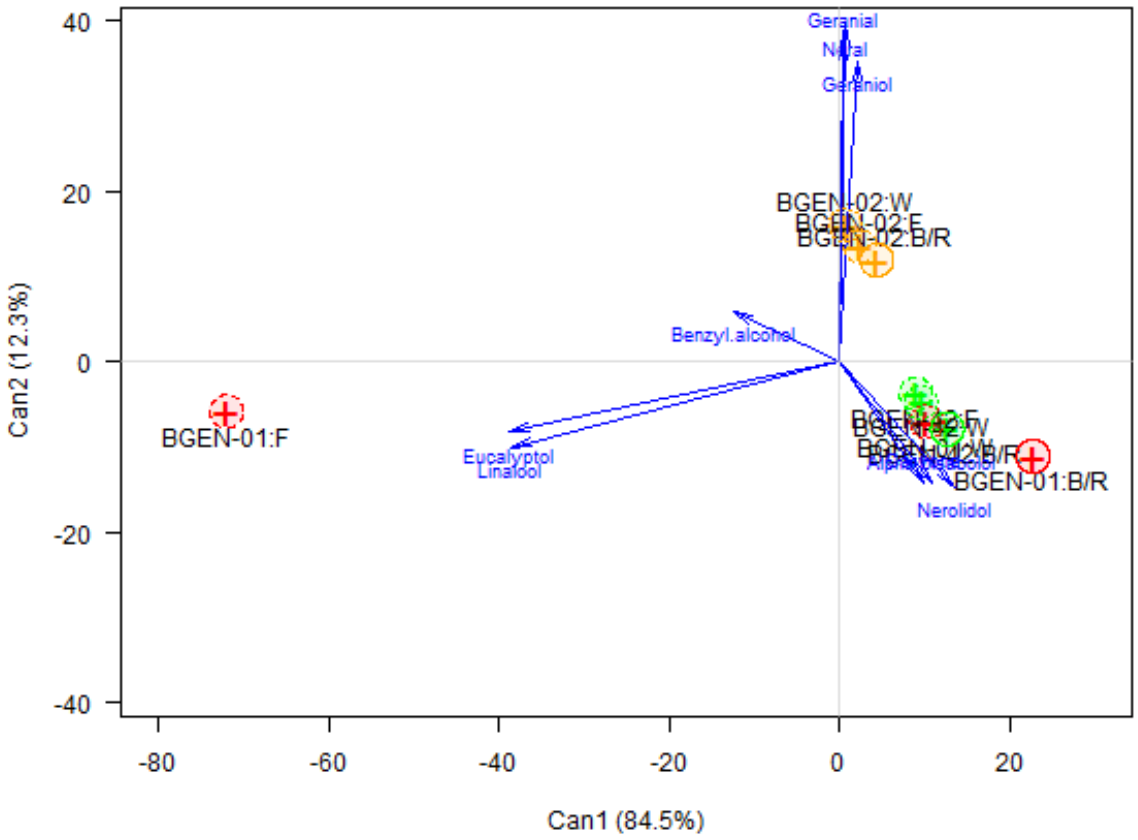

Figure 2. Biplot containing the mean scores showing 95% confidence ellipses for the treatments (Chemotype: Light quality) and original variables on the first two canonical variables.

Table 3. Loadings of original variables in the first two canonical variables for the treatments (Chemotype: Light quality).

	Can 1	**Can 2**
Eucalyptol	-0.96	-0.20
Linalool	-0.95	-0.25
Neral	0.02	0.98
Geranial	0.01	0.93
Alpha-Bisabolol	0.27	-0.35
Carvone	0.24	-0.35
Nerolidol	0.33	-0.36
Benzyl alcohol	-0.31	0.15
Geraniol	0.05	0.87

BGEN-01/F was the treatment with the higher means of eucalyptol and linalool and the lowest mean of carvone.

Also the second canonical variable displays that BGEN-01/F and BGEN-01/W showed different behavior: BGEN-01:F accumulated more benzyl alcohol, but less

saputenol than BGEN-01:W. BGEN-02:B/R and BGEN-02/W were similar between them, with values close to the overall mean. BGEN-02/F, BGEN-42:B/R, BGEN-42/F and BGEN-42/W showed the lowest means of eucalyptol and linalool and the highest mean of carvone.

Discussion

We found that light quality leads to different growth responses among the chemotypes in *Lippia alba*. This genotypic variation within the same species was also demonstrated by Kurepin et al. (2015), in which two ecotypes of *Stellaria longipes* respond differently to low red to far-red ratio light signaling.

The higher contents of mass and photosynthetic pigments in plants grown under blue/red LEDs support the fact that there is a greater use of light in these regions of the visible spectrum. Higher photosynthetic rates can be achieved when the leaf is illuminated with light in the red region (600-680 nm) than others (Kalaji et al. 2014). This light color stimulates PSII activity (Zienkiewicz et al. 2015). And also wavelengths in the blue range (around 480 nm) result in the strongest preferential excitation of PSII (Hogewoning et al. 2012). Conversely, for BGEN-42 the blue/red LEDs have not increased the plant biomass, which can be explained by the high rate of leaf abscission occurred at the plants of this chemotype growing under blue and red light only. Hoffman et al. (2015) also reported that red and blue LEDs can modify the timing for some stress responses, such as leaf yellowing and chilling injury.

The best growth under blue and red light was also demonstrated by Lin et al. (2011), wherein blue LEDs or red and blue LEDs could significantly promote the production of shoots of *Dendrobium officinale* and increase the dry matter and the accumulation of shoot dry matter *in vitro*. Blue light during growth is qualitatively required for normal photosynthetic functioning and quantitatively mediates leaf responses resembling those to irradiance intensity (Hogewoning et al. 2012).

Photosynthetic pigments were higher with blue/red LEDs, which is in line with the results of biomass accumulation. It is generally acknowledged that decrease in photosynthetic pigments is related to reduction in leaf photosynthesis and consequently lead to poor photosynthetic performance (Soussi et al. 1998, Braun et al. 2006). Similar results were observed for *Dendranthema grandiflorum*, in which net photosynthetic rate was consistent with plantlet growth, showing highest rate in plantlets grown under red and blue LEDs (Kin et al. 2004).

In this study, the different light qualities induced to changes in patterns of essential oils in *L. alba*, even changing the major constituents. In yarrow (*Achillea millefolium*), the amount and composition of the volatile compounds ranged with the

intensity and quality of light (Alvarenga et al. 2015). Yu et al. (2005) reported that the best accumulation of ginsenoside, the most important active components in *Panax ginseng* roots, was optimum in the cultures grown under fluorescent light, emphasizing how the responses of secondary metabolites to light source are species-dependent.

However, the chemotype seems to be a more effective factor for separating different treatments in essential oil profiles. Viccini et al. (2014) demonstrated that these differences among chemotypes are correlated with DNA content of plants, since BGEN-01, BGEN-02 and BGEN-42 are respectively triploid, diploid and hexaploid; and accessions with varied ploidy levels show different profiles of essential oils as well as distinct major component.

The alteration of the light quality in the cultivation of *Lippia alba* leads to changes in the synthesis route and, consequently, the qualitative pattern of essential oils. The deeper understanding of how this regulation works at genetic or epigenetic level can give basis for an oriented-based production of essential oils of greater economic and industrial interest. In this way, further studies will be needed to elucidate these routes.

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CHAPTER 2

Elevated CO_2 improves growth, modifies anatomy, and modulates essential oil qualitative production and gene expression in *Lippia alba* (Verbenaceae)

Abstract

Carbon dioxide (CO_2) concentrations have grown in recent decades and will continue to raise during this century, and this increased CO_2 atmosphere affects plant physiology and development. Aiming to evaluate the effect of CO_2 elevation on growth, anatomy, essential oils qualitative production and expression of genes related to biosynthesis of these compounds, three chemotypes of *Lippia alba* (BGEN-01, BGEN-02 and BGEN-42) were cultivated *in vitro*. Firstly, we focused on the effects of gas exchange in essential oil profile by comparing three CO_2 exchange rates: 14, 21 and 25 $\mu L\, L^{-1}\, s^{-1}$ CO_2. Following, in addition to the previous 14 and 25 $\mu L\, L^{-1}\, s^{-1}$ CO_2 treatments, plants were placed into a chamber with continuous forced air ventilation, leading to inner of 360 and 1000 $\mu L\ L^{-1}$ of CO_2; an additional control without allowing gas exchange was added inside the chambers, totaling five treatments with 6 replicates. After 45 days of growth, analyses of essential oils profile, histochemical, stomatal density, growth evaluation and transcript analysis were performed. The enrichment with CO_2 enhanced plant dry and fresh weight, total chlorophylls and carotenoids in BGEN-01 and BGEN-02, and also increased stomatal density and lignin content for all chemotypes. The multivariate analysis showed that essential oil profile varied not only among the different chemotypes, but also within BGEN-01 and BGEN-02 the qualitative production were different in the treatments with forced air renovation and CO_2 enrichment. Regarding the gene expression analyses, *farnesyl pyrophosphate synthase* (*FPPS*) and *geranyl pyrophosphate synthase* (*GPPS*) did not vary, except for the treatments with forced air ventilation (360 and 1000 $\mu L\ L^{-1}$) in the BGEN-01, which had *FPPS* upregulated. *Geraniol synthase* (*GS*) was upregulated in all BGEN-02 treatments and to the BGEN-01 treatments with 360 and 1000 $\mu L\ L^{-1}$ CO_2. *Nerolidol synthase* (*NS*) was upregulated only in the BGEN-01, at the treatments with 360 and 1000 $\mu L\ L^{-1}$ CO_2. These findings provide a better understanding of how CO_2 regulates secondary metabolites production, giving basis to clarify the pathway regulation, further enabling the targeted production of essential oils with greater economic and industrial interest.

Keywords: Gas exchange, geraniol synthase, nerolidol synthase, RT-qPCR.

Introduction

According to the latest IPCC report, current carbon dioxide (CO_2) concentration has increased from about 280 $\mu L\,L^{-1}$ at the start of the industrial revolution (ca. 1750) to over 400 $\mu L\,L^{-1}$ at present and it is expected to exceed 550 $\mu L\,L^{-1}$ by 2050 (IPCC 2007). This CO_2 enrichment of the atmosphere leads to changes in plant physiology such as growth, development, and metabolism (Klaiber et al. 2013, Oehme et al. 2013). In addition, it has been shown that elevated levels of CO_2 also alter expression of the genes involved in regulating several physiological processes, such as leaf senescence, carbon accumulation, flowering, redox control and transport (Li et al. 2008, Springer et al. 2008, Kontunen-Soppela et al. 2010, May et al. 2013).

Plants have been largely used as sources of secondary metabolites. Many natural compounds present in plants show biological activity and act as antimicrobial agents. Among these compounds are the essential oils, which are extracted from plants by different techniques. The essential oils are used in pest control and are involved with the development of technologies to reduce the negative effects of oxidants, radicals and microorganisms (Pereira et al. 2008). They are considered as promising natural ingredients for the food industry due to their antimicrobial effects (Burt 2004, Atarés et al. 2010, Böhme et al. 2014, Szczepanski & Lipski 2014, Llana-Ruiz-Rabello et al. 2015, Valdés et al. 2015). They can also be used as reducing agents and free radical scavengers in antioxidant and antimicrobial films or even applied directly as conservatives, since they can show a synergistic effect with structural components of the foods (Hyldgaard et al. 2012, Amorati et al. 2013, Böhme et al. 2014, Otoni et al. 2014a, Otoni et al. 2014b, Otoni et al. 2014c, Proestos et al. 2013, Peng & Li 2014, Otoni et al. 2016).

Essential oils are volatile and highly lipophilic compounds, with molecular weight less than 300 g mol^{-1}, which can be physically separated from other plant components into storage organelles or membranes (Turek & Stintzing 2013). They can be classified into two groups: those derived from acetyl-CoA, forming the isopentenyl pyrophosphate and dimethylallyl pyrophosphate, that originate the terpenes (monoterpenes, sesquiterpenes, diterpenes) and those derived from the shikimate pathway forming the phenylpropanoids. From these precursors all the constituents present in the essential oils are formed, which can have in its structure groups like alcohol, ethers, aldol, ketone, among others (Chemat et al. 2013).

The storage of essential oils normally occurs in specialized structures such as trichomes, which are specialized epidermal cells capable of synthesizing, storing, and secrete large quantities of specialized metabolites (Werker 2000, Schilmiller et al. 2008).

The genus *Lippia* (Verbenaceae) is widely distributed in tropical regions as herbs, shrubs or small trees. Most species of *Lippia* is concentrated in Brazil, Paraguay and Argentina, with some endemic species found in Africa. It is estimated a total of 200 species (Pascual et al. 2001).

The extracts from *Lippia alba* have antiulcerogenic, antimicrobial, anti-inflammatory, anti-helminth, antioxidants, cytostatic and gastroprotective properties, which are attributed to essential oils produced by this species (Pascual et al. 2001, Camurça-Vasconcelos et al. 2007, David et al. 2007, Fontenelle et al. 2007, Aguiar et al. 2008, Singulani et al. 2012). A remarkable feature in *Lippia alba* is the chemical variability among different genotypes of this species, called chemotypes, which vary both in composition of essential oil and composition of non-volatile compounds (Hennebelle et al. 2008). Viccini et al. (2014) demonstrated that this chemical variation can be correlated with the DNA content, since different ploidy levels showed varied patterns of essential oils.

Despite the functions, mechanisms of synthesis and regulation of secondary metabolites are as yet unclear, it is known that they have a direct relation to environmental conditions. Changes in climatic conditions modify the type and intensity of abiotic stresses imposed on the plant, altering not only the growth and yield, but also secondary metabolite production (Kang et al. 2013, Rodziewicz et al. 2014, Gandhi et al. 2015).

The differences between *ex vitro* and *in vitro* environment such as air composition, supply of nutrients and light conditions may lead to distinct physiological and developmental responses. Due to these differences, *in vitro* culture conditions cause major disruptions in cellular metabolism, which can reduce the capacity of survival in the acclimatization process (Badr et al. 2015, Ncube et al. 2015).

CO_2 concentration inside the culture vessels is reduced, which limits photosynthesis. Thus, in traditional *in vitro* propagation methods, sucrose is provided as a carbon source to sustain plant growth and development (Kozai 2010, Xiao et al. 2011). On the other hand, in *in vitro* photoautotrophy the plants can be propagated with reduction or even suppression of the carbohydrate from the media increasing gas exchange between culture flasks and the external environment. The regime of higher CO_2 concentration improves the transition from *in vitro* to *ex vitro* and increases plant quality and survival. Moreover, there are advantages in removing

carbohydrates from the culture media, such as the prevention of rapid growth of microorganisms, reducing the costs and increasing plant survival during acclimatization (Mosaleeyanon et al. 2004, Kozai 2010, Xiao et al. 2011, Pérez-Jiménez et al. 2015).

Knowing that secondary metabolites are influenced by environmental conditions, some authors have reported the effect of *in vitro* photoautotrophy on the production of these metabolites (Badr et al. 2011, Ghasemzadeh & Jaafar 2011, Iarema et al. 2012, Mohamed & Ibrahim 2012, Supaibulwattana et al. 2012, Saldanha et al. 2013, 2014).

The general knowledge of the plant responses to CO_2 alterations has improved greatly in the recent years (Walker et al. 2015). Mainly due to advances in methodologies for CO_2 experiments associate with the development of modern tools for gene expression and metabolomics studies and recent progress in using stable isotope labeling, high resolution MS, and flux analysis that have a great potential to improve the secondary metabolism analysis in plants (Schilmiller et al. 2012, Misra & Chen 2015). Nevertheless, there is a need for further development, together with detection techniques of the secondary compounds, but also gene expression analysis to better understand how the increase of CO_2 affects the transcriptional level.

The objective of this study was to evaluate the effect of gas exchange and CO_2 enrichment on *in vitro* morphogenesis, anatomy, qualitative production of essential oils as well as its effect in expression of genes related to the synthesis of essential oil in three chemotypes of *Lippia alba*.

Materials and methods

Plant material

Three chemotypes of *Lippia alba* (BGEN-01, BGEN-02 and BGEN-42) were obtained from the Department of Biology, Federal University of Juiz de Fora (UFJF, Juiz de Fora, MG, Brazil) and from Embrapa Genetic Resources and Biotechnology (Cenargen, Brasília, DF, Brazil). *In vitro* plantlets were subcultivated on a monthly basis in a MS-based medium devoid of growth regulators, in the Laboratory of Plant Tissue Culture, at the Institute of Applied Biotechnology for Agriculture (BIOAGRO, Universidade Federal de Viçosa, MG, Brazil).

Gas exchange effect

Hypocotyl segments with 1 cm of length were transferred to glass flasks containing 60 mL of Murashige and Skoog basal salt solutions (Murashige & Skoog 1962), 30 g L^{-1} of sucrose, 100 mg L^{-1} of myo-inositol, and 6.5 g L^{-1} of agar Merck™. The pH of the media was adjusted to 5.7 ± 0.01, and the media was autoclaved at 120°C and 108kPa for 20 min. These flasks were kept in growth room environment at 25 °C for a 16-h photoperiod under 41µmol m^{-2} s^{-1} irradiance from two white LED bulbs (SMD 100, 18W, Vilux®, Vitória, ES, Brazil) at 25 ± 1°C.

Three types of flask sealing systems were used: polypropylene lids (PL) without membranes (14 µL L^{-1} s^{-1} CO_2 exchange rate (CO_2ER)); PL with one 0.45-µm-pore size membranes (PTFE; MilliSeal® AVS-045 Air Vent, Tokyo, Japan) (21 µL L^{-1} s^{-1} CO_2ER); and PL with two membranes (25 µL L^{-1} s^{-1} CO_2ER). After 45 days of cultivation, analyses of essential oils profile were performed.

CO_2 enrichment

Nodal segments (average 10 mm in length) without leaves and with two axillary buds were cultivated in 500 mL glass flasks closed with PL with two membranes. Explants were inoculated into MS media in the same culture conditions as described in the previous item. The flasks were placed into two 41×26×60-cm, 4-mm-thick acrylic box with forced ventilation: one with the atmospheric concentration of CO_2 (360 µL L^{-1}) and the other with external input of CO_2 (1000 µL L^{-1}), following the model described by Saldanha et al. (2013).

One additional treatment with flasks closed with PL without membranes was added into the box as a control (CTRL 0M). Additionally, two control treatments of each chemotype were kept outside the box: closed with PL without membranes and closed with PL with two membranes (CTRL 0M* and CTRL 2M*). After 45 days of cultivation, extraction and analyses of essential oils profile, histochemical analysis, stomatal density, growth evaluation and expression analysis were performed.

Extraction of the essential oils

The extraction of essential oils was performed at the Laboratory of Organic Chemistry - Essential Oils, Department of Chemistry, Federal University of Lavras. Fresh leaves and stems were added to a round bottom flask with 500 mL of capacity. The method used was hydrodistillation for 2 h using a modified Clevenger apparatus. After this, the hidrolact was collected and centrifuged at 1100 *g* for 5 min. The essential oil was removed with a Pasteur pipette, packed in glass bottle wrapped with aluminum foil and stored under refrigeration (Agência Nacional de Vigilância Sanitária 2010).

Qualitative analyses of the essential oils

Qualitative analyses were carried out in a Shimadzu CG-17A gas chromatograph coupled to a QP 5000 mass spectrometer (GC-MS), under the following operational conditions: DB5 fused silica capillary column, using helium as a carrier gas; the injector temperature was 250 °C; the column temperature was held at 50 °C for 2 min, followed by an increase of 4 °C min^{-1}, until achieving 200 °C; the initial column pressure was 100,2kPa; and the split ratio was 1:10. The injected sample volume was 1 μL (dichloromethane 1% solution). Under the same conditions of the samples, a series of hydrocarbons was injected (C_9H_{20} ... $C_{26}H_{54}$) (Mjos et al. 2006).The chromatograms obtained were compared with the library Wiley 229 and the Kovats retention indices were calculated for all constituents. The identification of the oils was carried out based on the comparison of its retention indexes with the ones in the literature (Adams 2007).

Histochemical analyses and stomatal density evaluation

Samples were collected from the middle third of the stem. Cross sections were prepared using a table microtome (LPC, Rolemberg & Bohering, Retail and Import Ltda.,

Belo Horizonte, Brazil) and stained with phloroglucinol (Johansen 1940) to show lignin contents and Nadi reagent (David & Carde 1964) demonstrating the presence of essential oils.

Whole leaves (second and third fully expanded leaves from the shoot tip) were processed by diaphanization with 10% w/v sodium hydroxide, bleached with 10% v/v sodium hypochlorite, stained with 0.001% w/v basic alcoholic fuchsin, and mounted in glycerinated gelatin. Slides were sealed with clear nail polish. Images of both abaxial and adaxial epidermis surfaces of each leaf were captured with an Olympus AX70TRF microscope (Olympus Optical, Tokyo, Japan) with a U-Photo Camera System (Spot Insight Color 3.2.0, Diagnostic Instruments Inc., USA). Stomatal density was calculated using the software ANATI QUANTI (Aguiar et al. 2007).

RNA extraction and cDNA synthesis

TRI Reagent® (Sigma-Aldrich, St. Louis, Missouri, United States) was used to isolate the RNA from the samples. Quantifications and determination of RNA purity was performed with a NanoDrop Spectrophotometer 2000C (Thermo Scientific NanoDrop Technologies, Wilmington, Delaware, United States). To avoid nuclear DNA, 10 µg of RNA was suspended in 50 µL water with 1 µL DNase I (Thermo Scientific NanoDrop Technologies, Wilmington, Delaware, United States), 5µL of buffer and heated to 37 °C for 30 min. 450 µL water was added and the samples purified with 500 µL phenol/chloroform/isoamyl alcohol (v/v, 25:24:1), 1 mL chloroform was added and supernatant collected, RNA samples were precipitated using 800 µL 100 % ethanol and 40 µL 3 M sodium acetate (pH 5.2) at -20 °C for 60 min. A wash with 70 % ethanol (v/v) was performed and RNA pellet was resuspended in 30 µL of DEPC-treated distilled water. The RNA quantity and quality was determined using a Nanodrop 2000C (Thermo Scientific, Wilmington, DE).

A Super Script™ III, First-Strand Synthesis System Kit (Invitrogen®, Carlsbad, California, United States) was used for the reverse transcription reaction. Reactions were done by mixing 1 µg of RNA with nuclease free water, 1 µL of oligo$(dT)_{20}$ (50µM) and 1µL 10mM dNTP Mix, and the resulting solution was incubated for 5 min at 65 °C and then placed on ice for at 2 min. 0.1 M DTT, 40 units RNase Out, and 200 units of SuperScript III enzyme were then added. The resulting solution was incubated at 50 °C for 60 min, and finally at 70 °C for 15 min.

Real time RT-PCR analysis

Real-time RT-PCR was performed on a StepOnePlus™ real time PCR system (Applied Biosystems). Primers of two genes of essential oil synthesis: *geraniol synthase* (*GS*) and *nerolidol synthase* (*NS*); two intermediaries of the route: *farnesyl pyrophosphate synthase* (*FPPS*) and *geranyl pyrophosphate synthase* (*GPPS*); and a constitutive gene: *alcohol dehydrogenase* (*ADH*) were used.

All the RT-PCR samples were performed using qPCR-SYBR-Green mix/Rox (Ludwig Biotec®, Alvorada, Brazil). *GS*, *NS*, *FPPS* and *GPPS* gene expression profiles were normalized to the alcohol dehydrogenases (ADH) levels and calculated using the ΔΔCt ($2^{-\Delta\Delta Ct}$) levels.

Statistical analysis

The experiments were carried out following a factorial scheme 3x3 to the gas exchange effect (chemotypes x gas exchange) with 12 replicates and 3x5 to the CO_2 enrichment (chemotypes x levels of CO_2), with 6 replicates, being each replicate composed by one flask with 8 plantlets. The growth parameters data were submitted to analysis of variance by test F, followed by Scott & Knott's test at 5% significance level.

The data on essential oils were subjected to canonical discriminant analysis (CDA), with the study factor consisted of combinations of chemotypes and level of CO_2. A biplot graph was built for inspection of multivariate differences between treatments and examination of the interrelations among the variables and the treatments in two-dimensional plane. The Candisc package (Friendly & Fox 2013) in the software R (R Core Team 2014) was applied to the CDA.

Regarding RT-PCR analysis, the calculations for the values were made using the comparative threshold cycle (ΔΔCt) method, as proposed by Yuan et al. (2006), of 3 biological replicates and the means were compared using confidence interval by the t test at ($P \leq 0.05$).

Results

The CO_2 enrichment was effective to increase plant length and plant mass accumulation, for both 360 and 1000 $\mu L\ L^{-1}$ CO_2 treatments, comprising the highest means. The control without CO_2 incorporation (outside the box) and capped with lids with two fluoropore membranes showed intermediate values for these characteristics, however higher than the controls lacking membranes. The latter presented the lowest means in growth features and did not differ between each other, evidencing that the use of lids without membranes was effective to suppress the CO_2 enrichment (Table 1).

For the photosynthetic pigments, chemotypes performed differently. In that respect, BGEN-42, 360 $\mu L\ L^{-1}$ CO_2 and CTRL 2M* reached the highest means, whereas BGEN-02 had the higher chlorophyll and carotenoids contents at 1000 $\mu L\ L^{-1}$ CO_2. For BGEN-01 only the CTRL 0M was lower than the others, which did not differ between them (Table 1).

CO_2 enrichment also induces an increase in the stomata density. However, there was no difference in stomata density, regardless the concentrations of 360 $\mu L\ L^{-1}$ CO_2 and 1000 $\mu L\ L^{-1}$ CO_2 (Table 1).

Table 1. Growth parameters of *Lippia alba* evaluated after 40 days of cultivation.

PLANT LENGHT (cm)			
CO_2 condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
1000 µL L^{-1} CO_2	15.01 Aa	16.54 Ab	8.16 Ba
360 µL L^{-1} CO_2	14.63 Ba	17.84 Aa	9.15 Ca
CTRL 0M*	8.60 Ac	9.56 Ac	6.73 Bb
CTRL 2M*	11.74 Bb	14.98 Ab	9.48 Ca
CTRL 0M	9.63 Ac	10.87 Ac	3.52 Bc
FRESH WEIGHT (g)			
CO_2 condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
1000 µL L^{-1} CO_2	3.29 Aa	3.55 Aa	2.37 Bc
360 µL L^{-1} CO_2	2.99 Ab	2.80 Ab	2.62 Ab
CTRL 0M*	1.38 Bc	1.86 Ac	1.78 Ad
CTRL 2M*	2.60 Cb	3.04 Bb	3.43 Aa
CTRL 0M	1.21 Bc	1.98 Ac	1.85 Ad
DRY WEIGHT (g)			
CO_2 condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
1000 µL L^{-1} CO_2	0.35 Aa	0.35 Aa	0.16 Bb
360 µL L^{-1} CO_2	0.28 Ba	0.41 Aa	0.28 Ba
CTRL 0M*	0.03 Bb	0.12 Ab	0.15 Ab
CTRL 2M*	0.26 Ba	0.43 Aa	0.28 Ba
CTRL 0M	0.09 Bb	0.19 Ab	0.20 Ab
TOTAL CLOROPHYLL (µg/cm^2)			
CO_2 condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
1000 µL L^{-1} CO_2	15.30 Ba	22.44 Aa	13.99 Bc
360 µL L^{-1} CO_2	11.76 Ba	13.79 Bb	19.86 Aa
CTRL 0M*	12.96 Ba	16.16 Ab	12.31 Bc
CTRL 2M*	11.58 Ba	12.94 Bb	18.27 Ab
CTRL 0M	4.55 Bb	0.39Cc	12.91 Ac
TOTAL CAROTENOIDS (µg/cm^2)			
CO_2 condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
1000 µL L^{-1} CO_2	27.18 Ba	42.98 Aa	25.23 Bb
360 µL L^{-1} CO_2	21.79 Ba	26.71 Bb	38.63 Aa
CTRL 0M*	22.65 Aa	28.87 Ab	25.47 Ab
CTRL 2M*	19.59 Ba	24.94 Bb	35.63 Aa
CTRL 0M	6.78 Bb	0.22Cc	25.14 Ab
STOMATAL DENSITY (stomata mm^{-2})			
CO_2 condition	**Chemotype**		
	BGEN-01	**BGEN-02**	**BGEN-42**
1000 µL L^{-1} CO_2	18.73 Aa	19.38 Aa	22.37 Aa
360 µL L^{-1} CO_2	17.72 Aa	20.83 Aa	22.47 Aa
CTRL 0M*	12.83 Ab	12.44 Ab	15.34 Ab
CTRL 2M*	13.34 Bb	15.25 Bb	19.50 Aa
CTRL 0M	14.24 Ab	10.24 Bb	11.99 Bb

Means indicated by the same uppercase letters at horizontal and lowercase at vertical are not significantly different as assessed by the Scott & Knott's test at 5% probability level.

The histochemical test with phloroglucinol showed a clear difference between the chemotypes, where BGEN-02 showed higher lignin content than the others. Regarding the different levels of CO_2 it was observed a thicker layer of xylem at the treatment of 1000 $\mu L\ L^{-1}$ for all chemotypes (Figure 1 - M, N, O), although this difference was more prominent in BGEN-01 and BGEN-42. Among the control treatments, CTRL 2M* showed more vascular tissue differentiation than the controls without membrane.

The Nadi reaction highlighted the essential oils stained in blue, being enclosed into capitated glandular trichomes (Figure 2).

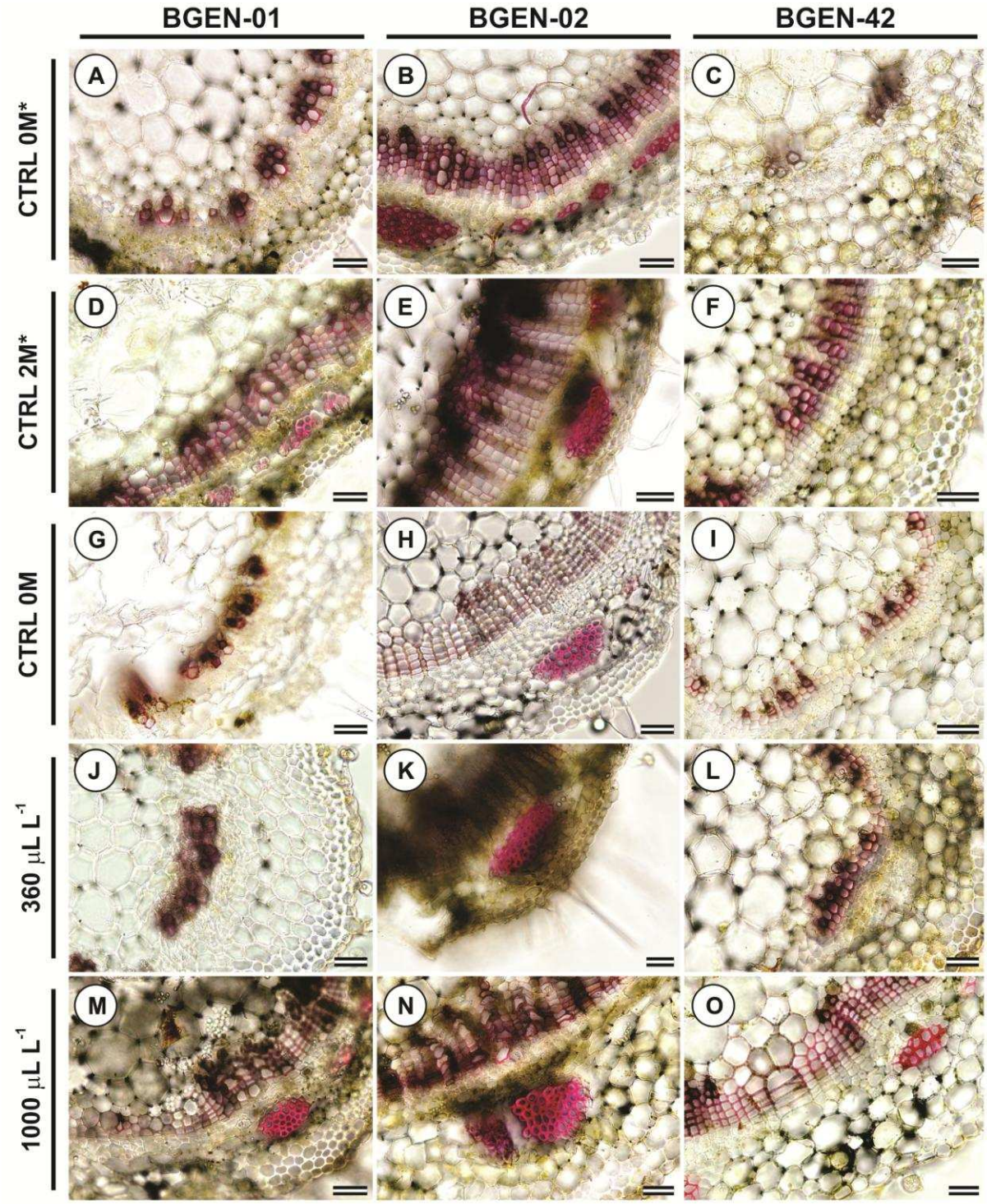

Figure 1. Cross-sections from stems submitted to the phloroglucinol test of three chemotypes of *Lippia alba* plantlets (BGEN-01, BGEN-02 and BGEN-42) after 45 days growth *in vitro* at five different CO_2 conditions: **A**, **B**, **C**: CTRL 0M* - Control without membranes without forced ventilation; **D**, **E**, **F**: CTRL 2M* - control with two membranes without forced ventilation; **G**, **H**, **I**: CTRL 0M - control without membranes inside forced ventilation box; **J**, **K**, **L**: 360 µL L^{-1}; **M**, **N**, **O**: 1000 µL L^{-1}. Bar = 50µm.

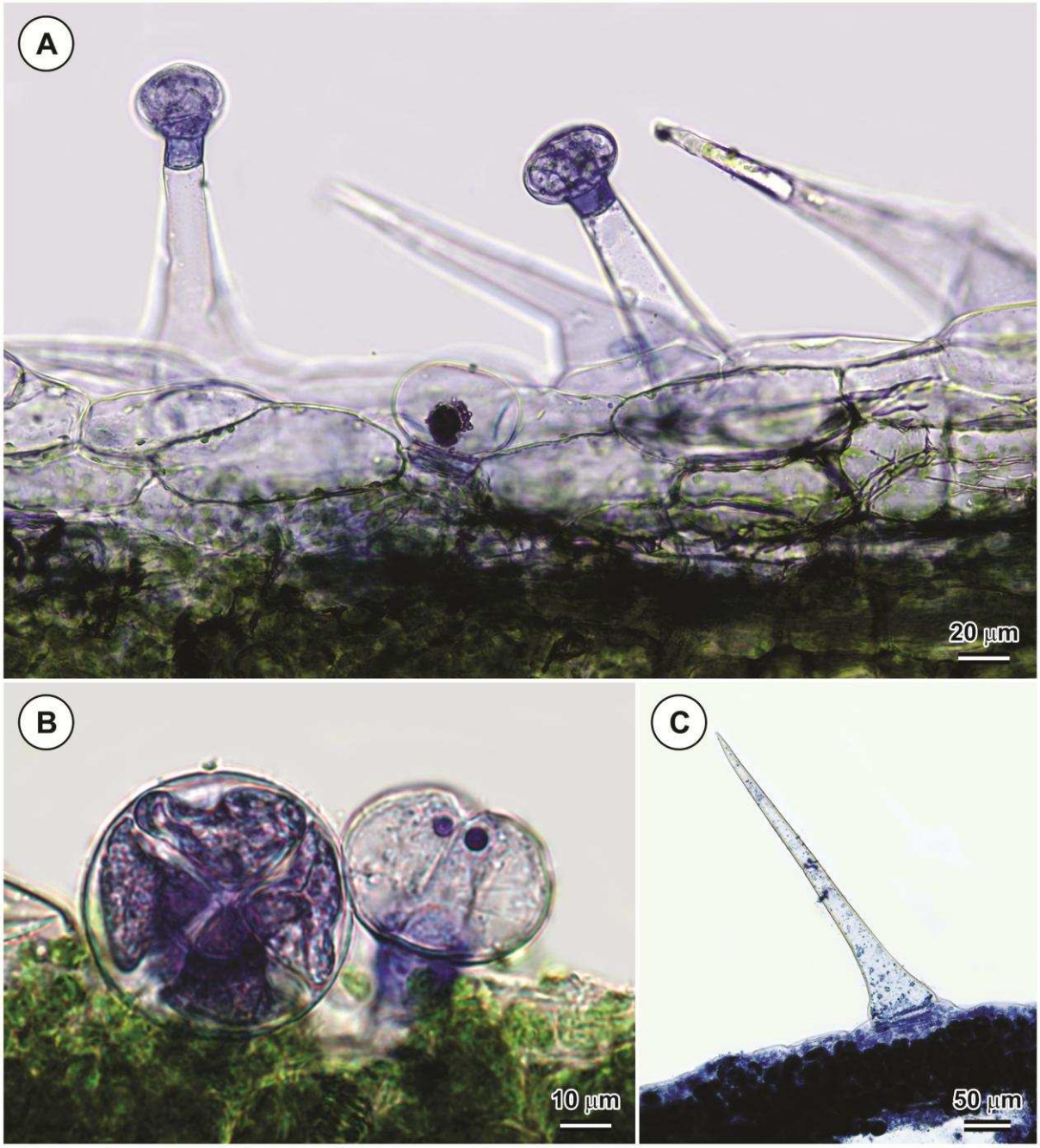

Figure 2. Cross sections from leaf of *Lippia alba* after 45 days growth *in vitro* stained with Nadi reagent. **A**: Glandular and tector trichomes on the adaxial leaf surface. **B**: Detail of glandular trichomes highlighting the presence of essential oils inside. **C**: Detail of a tector trichome on the adaxial surface.

In respect to the essential oil profile in the gas exchange experiment, with the first canonical variable was just possible to retain nearly 45% of the variability information among treatments. The palmitic acid was the main responsible for this variability. The second canonical variable also allows us to separate treatments, with almost 25% of the variability, which is due mainly to differences in amounts of pentacosane, limonene, carvone, tetrahydromyrcenol and geranial. The latter two were negatively correlated with the others.

There were no significant effects of the interaction, nor isolated effects of chemotype and membrane, but the combinations between them (Figure 3).

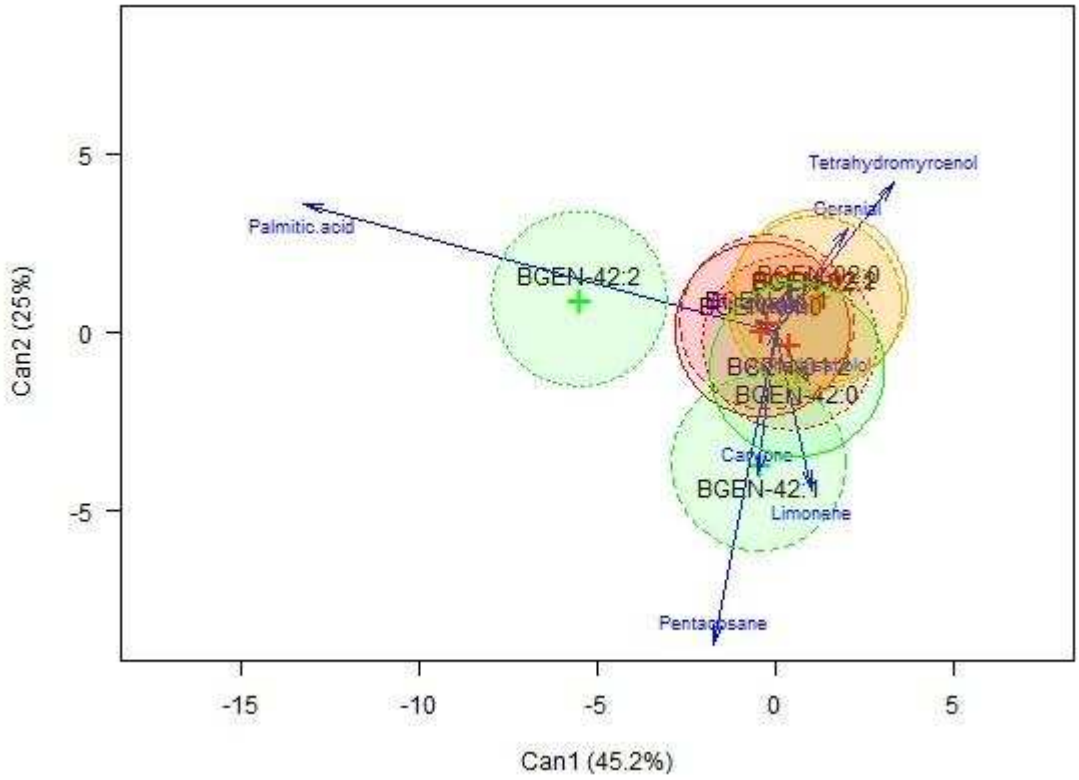

Figure 3. Biplot containing the mean scores showing 95% confidence ellipses of treatments (Chemotype: Gas exchange) and loadings of original variables on the first two canonical variables.

The essential oil profile under the CO_2 enrichment experiment, with the first canonical variable was just possible to retain nearly 48% of the variability information among treatments. Neral, thymol, geraniol, geranial, carvone and carvacrol were the main responsible for this variability. The second canonical retained almost 32% of the variability and also allowed to discriminate treatments, especially the different chemotypes. Nerolidol, linalool, viridiflorol and eucalyptol were the main responsible for this variation. The treatments with 1000 µL L^{-1} significantly differed from the controls in the BGEN-01 and BGEN-02 chemotypes (Figure 4).

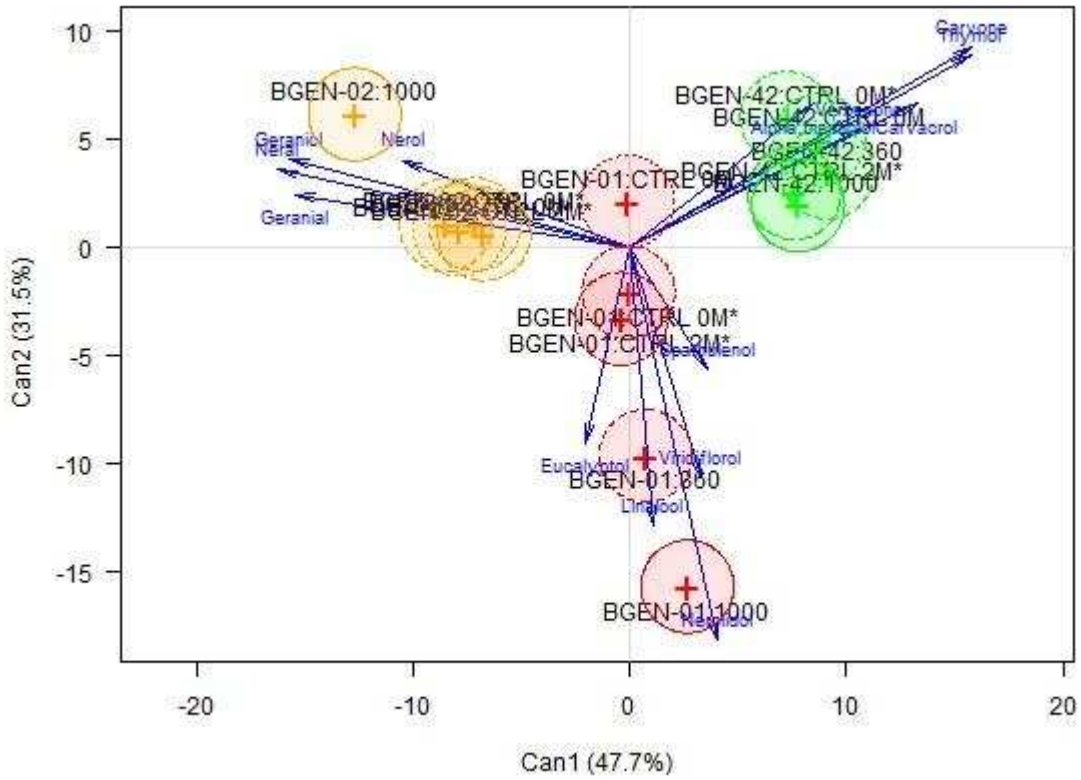

Figure 4. Biplot containing the mean scores showing 95% confidence ellipses of treatments (Chemotype: Levels of CO_2) and loadings of original variables on the first two canonical variables.

We monitored the expression of the genes related to the sesquiterpenes and monoterpenes synthesis pathway. A summary of these pathways with the main metabolites and enzymes is shown in the figure 5.

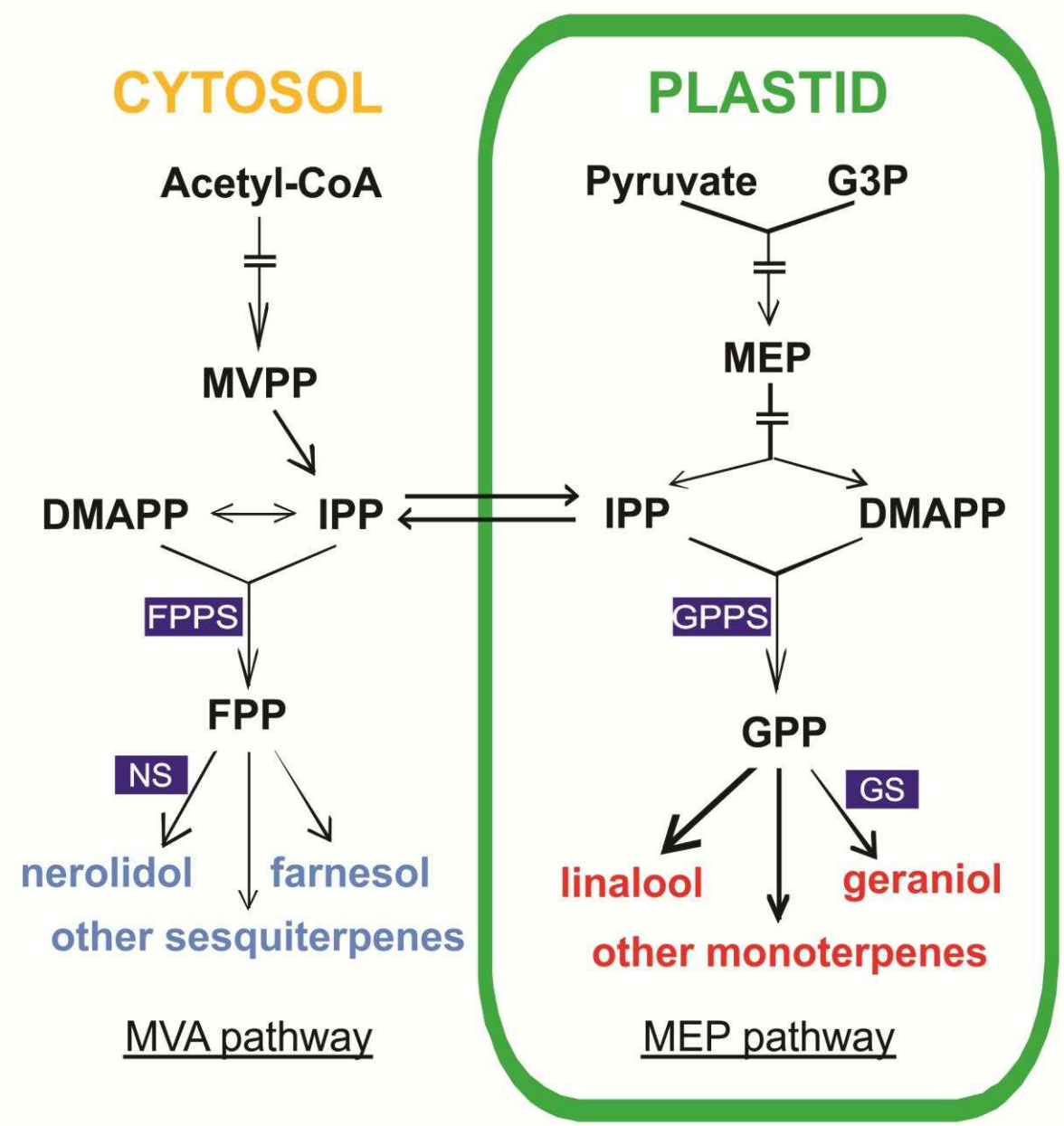

Figure 5. Summarized biosynthetic pathway of monoterpenes and sesquiterpenes. The methyl erythritol phosphate (MEP) pathway and the mevalonic acid (MVA) pathway occurs in the plastid and cytosol, respectively. The following enzymes and metabolites are shown: glyceraldehyde 3-phosphate (G3P), 2-C-methyl-D-erythritol 4-phosphate (MEP), isopentenyl pyrophosphate (IPP), dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate synthase (*GPPS*), geranyl pyrophosphate (GPP), mevalonate-5-pyrophosphate (MVPP), farnesyl pyrophosphate synthase (*FPPS*), farnesyl pyrophosphate (FPP), geraniol synthase (*GS*) and nerolidol synthase (*NS*). Enzymes highlighted in blue boxes were targeted for expression analysis.

FPPS and *GPPS* did not vary, except for the treatments with forced air ventilation (360 and 1000 µL L^{-1}) in the BGEN-01, which had upregulation related to the control to the *FPPS* gene (Figure 6).

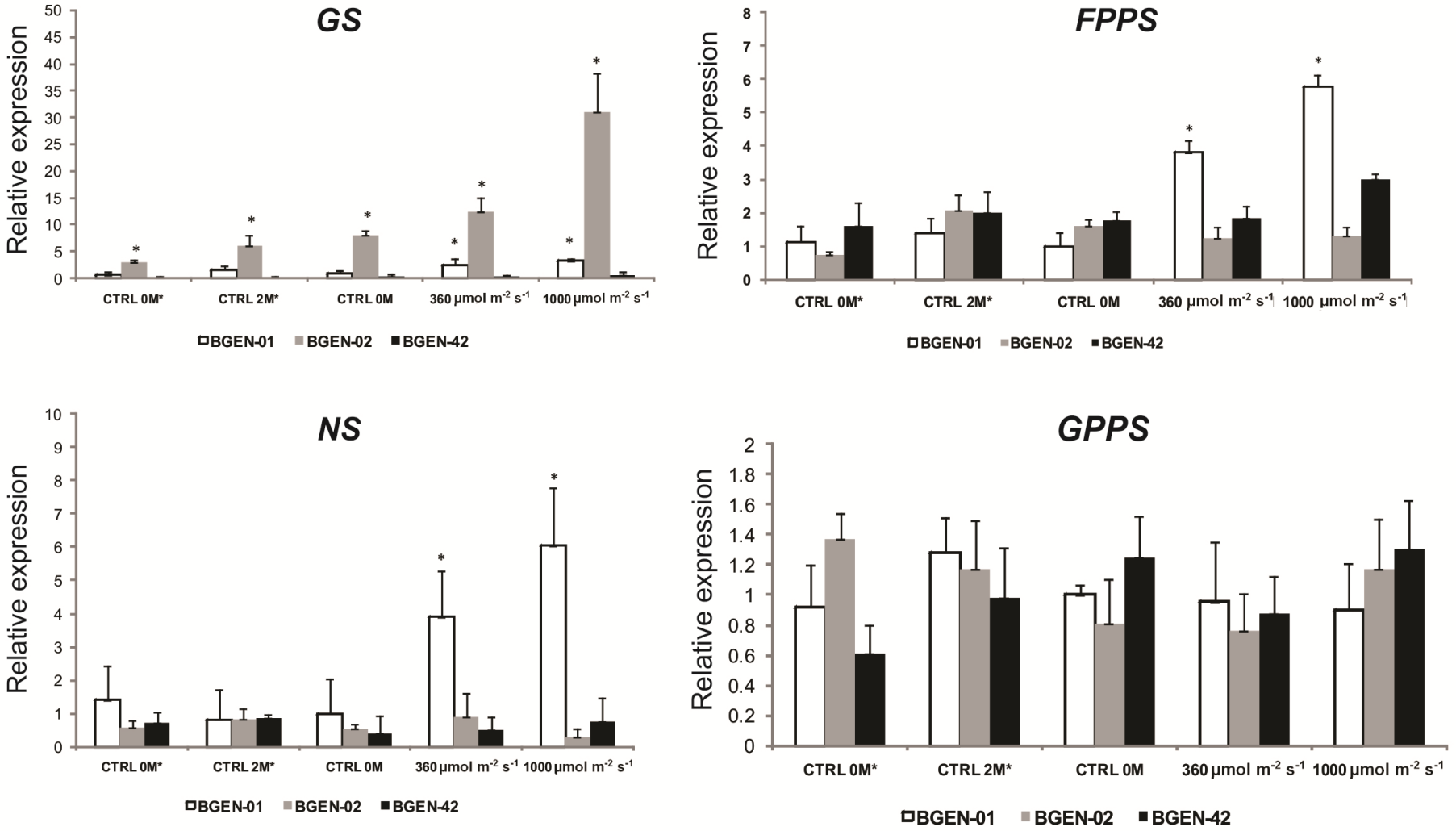

Figure 6. Real-time reverse transcription-polymerase chain reaction (RT-PCR) for *Lippia alba* genes after 45 days of cultivation *in vitro*. Relative gene expression of three chemotypes (BGEN-01, BGEN-02 and BGEN-42) at five different CO_2 conditions: Control without membranes without forced ventilation – CTRL 0M*; control with two membranes without forced ventilation – CTRL 2M*; control without membranes inside forced ventilation box – CTRL 0M; 360 µL L^{-1} and 1000 µL L^{-1}. Gene expression relative to alcohol dehydrogenase. Data presented as mean ± standard deviation (n = 3), *p<0.05.

GS showed upregulation related to the control in all BGEN-02 treatments, with a nearly 30-fold increase of expression at BGEN-02/1000, and also showed upregulation to the BGEN-01 treatments with forced air ventilation (360 and 1000 µL L^{-1}). *NS* gene had significantly increased expression only for the two treatments with forced ventilation, with 4-fold higher expression at the BGEN-01/360 and 6-fold higher expression at the BGEN-01/1000 (Figure 6).

Discussion

Currently, the atmospheric concentration of CO_2 continues to rise, standing now at 400 $\mu L\, L^{-1}$ and possibly reaching 1000 $\mu L\, L^{-1}$ by the end of this century. This rising in the atmosphere is the main responsible for global warming (IPCC, 2007). Increased CO_2 levels also induce to changes at plant growth, development, and metabolism (Klaiber et al. 2013, Oehme et al. 2013). Moreover, elevated CO_2 levels can modify gene expression patterns, which ultimately regulate physiological responses of plants (Kontunen-Soppela et al. 2010, May et al. 2013). To our knowledge, this is the first study to demonstrate the relation between essential oils profile and the genes related to its expression and route of synthesis. Besides, these experiments were coupled with histochemical and growth analysis.

Overall *L. alba* growth and photosynthetic pigments were increased with greater CO_2 concentrations being consistent with previous results for crop species like rice (*Oryza sativa* L.), wheat (*Triticuma estivum*) and tomato (*Solanum lycopersicum* Mill) (Yang et al. 2013, Mamatha et al. 2014, Bencze et al. 2014). The present study reinforced the idea that increasing CO_2 in *in vitro* culture systems induce to biomass gains and photosynthetic efficiency in plants, as demonstrated by Saldanha et al. (2013, 2014) in Brazilian ginseng (*Pfaffia glomerata*), Mosaleeyanon et al. (2004) in rain tree (*Samanea saman*), Dáder et al. (2015) in bell pepper (*Capsicum annuum*), Pérez-Jiménez et al. (2015) in artichoke (*Cynara scolymus*) and Wang et al. (2015) in okinawan spinach (*Gynura bicolor*).

High levels of CO_2 increase stomatal density in the chemotypes of *Lippia alba*. This characteristic is highly species-dependent, showing that different species have different strategies for controlling gas exchange. Most of the more recently derived angiosperms and even some gymnosperms as *Lepidozamia peroffskyana* and *Nageianagi* respond to environmental carbon dioxide modifications changing the stomatal conductance (g_s), usually reducing the g_s in response to an increase in the atmospheric concentration of CO_2. On the other hand, other species like *Solanum lycopersicum* and *Hordeum vulgare* show the same behavior that we found in *L. alba*, increasing the stomatal density with the rising of CO_2 (Woodward 1987, McAdam & Brodribb 2012, Haworth et al. 2013). Saldanha et al. (2013) found the opposite behavior

in *Pfaffia glomerata*, with plants grown under CO_2 enrichment reducing stomatal density compared to the control.

The increased lignin content at higher CO_2 concentrations evidences a greater mobilization of photoassimilates to the differentiation of vascular tissues. These results are consistent with the findings of AbdElgawad et al. (2014) that lignin levels increase under elevated and extreme CO_2 concentrations. Accordingly, in poplars (*Populus tremula x alba*) elevated CO_2 concentration (800 $\mu L\,L^{-1}$) provided carbon supply to the stem and lignin synthesis was enhanced, leading to increased lignin content (Richet et al. 2012).

The gas exchange experiment as well as the controls without forced air renovation at the CO_2 levels experiment (CTRL 0M, CTRL 0M* and CTRL 2M*) did not show variation in the essential oil profile as dramatically as the changes observed with the treatments with 360 and 1000 $\mu L\,L^{-1}$ CO_2. This suggests that CO_2 is the fraction of the atmospheric air that more effectively influences in gene expression and oils composition.

The forced ventilation and especially the CO_2 enrichment is effective to significantly alter the profile of essential oils in two of the three chemotypes analyzed (BGEN-01 and BGEN-02). This variation by increasing the levels of CO_2 was demonstrated before with an increase of proanthocyanidins and flavonoids in leaves of birch (*Betula pendula*) (Lavola & Julkunen-Tiitto 1994), of flavonoids and phenolics contents in oil palm (*Elaeis guineensis*) (Ibrahim & Jaafar 2012) and in bread wheat (*Triticum aestivum*) the flavonoid contents also increased in response to elevated CO_2 (Levine et al. 2008).

The higher content of geraniol in BGEN-01/360 and BGEN-01/1000 follows the increase of *GS* expression in these treatments compared to the BGEN-01 controls. In BGEN-02, geraniol also increased occurrence with 360 and 1000 $\mu L\,L^{-1}$ CO_2, being the latter the higher value, which is also in accordance with the *GS* expression. Interestingly, the expression analyses of a *GPPS* clone of *Picea abies* (*PaGDPS1*) indicated that *PaGDPS1* and GS were coexpressed in plastids, cytosol and mitochondria, being the geraniol and geraniol-derived production increased with the boosting of geranyl pyrophosphate (GPP) biosynthesis (Dong et al. 2016). *GPPS* expression level remained constant in all treatments, showing that cellular levels of this enzyme remains steady, probably because the regulation of its activity occurs through

the exchange between different subcellular compartments. *GPPS* can be transported from mitochondria to plastids and from cytosol to mitochondria, while there is no transport of *GPPS* from the cytosol to the plastids (Dong et al. 2016).

BGEN-01 shows a significant increase of nerolidol 360 and 1000 $\mu L\, L^{-1}$ CO_2, in agreement with the observed to the *NS* expression in these treatments. The increased production of sesquiterpenes (e.g. nerolidol) instead of monoterpenes in BGEN-01/360 and mostly in BGEN-01/1000, follows the increased expression of *FPPS*, which is the precursor of the sesquiterpenes in the cytosol (Dewick 2002). The presence of a sesquiterpenes in a significant trend only at forced ventilation and increased CO_2 suggests that the plant metabolism can recognize the lack of CO_2 as a stress that triggers the favoring production of monoterpenes instead of sesquiterpenes. As demonstrated by Atkinson & Arey (2003), monoterpenes are more reactive to damaging free radicals and their levels can be increased in stress situations to enhance plant defense.

This study revealed a positive relation between sesquiterpene production and biomass accumulation, and conversely, the treatments with less fresh and dry weight are those with the highest proportions of monoterpenes. Since the monoterpenes are synthesized in plastids, its production can compete with photosynthesis and consequently, mass accumulation and growth (Aharoni et al. 2003). Martin et al. (2012) demonstrated that the plastid pathway is likely the main source of IPP for monoterpenes biosynthesis in Gewürztraminer berries (*Vitis vinifera* L.).

In conclusion, our results highlight that enrichment with CO_2 enhances *in vitro* performance of *Lippia alba*, by increasing growth characteristics, photosynthetic pigments, stomatal density and lignin content. Elevated CO_2 also leads to qualitative modifications in the essential oil production. These changes are accompanied by alteration in expression of genes related to the biosynthetic pathway of monoterpenes and sesquiterpenes and their intermediates. These findings will allow a better understanding of how the CO_2 acts regulating the secondary metabolites production, whose knowledge of its production physiology is of high economic and industrial interest.

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CONCLUSIONS

The following conclusions can be drawn from the present study:

- The alteration of the light quality in the cultivation of *Lippia alba* alter the synthesis and the pattern of essential oils.

- CO_2 enrichment enhances *in vitro* morphogenesis of *L. alba*, increasing growth characteristics, photosynthetic pigments, stomatal density and lignin content.

- Elevated CO_2 also leads to qualitative modifications in the essential oil production. These changes are accompanied by alteration in expression of the genes related to the synthesis of monoterpenes and sesquiterpenes and its intermediates.

Together with recent transcriptome studies on the genus unraveling several genes of stratetegic interest and importance, overall the present study strengthens the idea that the physical environment, here represented by light spectral conditions and CO_2-enriched atmosphere, exerts a significant effect upon *in vitro* growth performance. These findings provide a better understanding of the relation between light quality and high-CO_2 and regulation of secondary metabolites production, thus broadening the foundations for further studies that can allow an oriented-based production of essential oils with greater economic and industrial interest. This understanding should also help to improve predictions of the impact of CO_2 raising atmospheres upon growth and secondary metabolism in medicinal plants.