



# Photoperiod modulates growth, morphoanatomy, and linalool content in *Lippia alba* L. (Verbenaceae) cultured in vitro

Kamila Motta de Castro<sup>1</sup> · Diego Silva Batista<sup>2</sup> · Evandro Alexandre Fortini<sup>1</sup> · Tatiane Dulcineia Silva<sup>1</sup> · Sérgio Heitor Sousa Felipe<sup>1</sup> · Amanda Mendes Fernandes<sup>1</sup> · Raysa Mayara de Jesus Sousa<sup>1</sup> · Laís Stehling de Queiroz Nascimento<sup>3</sup> · Victória Rabelo Campos<sup>3</sup> · Richard Michael Grazul<sup>4</sup> · Lyderson Facio Viccini<sup>3</sup> · Wagner Campos Otoni<sup>1</sup>

Received: 17 June 2019 / Accepted: 8 August 2019 / Published online: 12 August 2019  
© Springer Nature B.V. 2019

## Abstract

Interactions between circadian clock regulation and metabolic responses are believed to explain the importance of rhythmic behavior in plant growth and survival. *Lippia alba* is an important species because of the medicinal properties of its essential oil extract. The objective of this work was to evaluate the effect of photoperiod on anatomy, growth, essential oil profile, and the expression of genes related to the synthesis of monoterpenes, sesquiterpenes, and the circadian clock in *L. alba* grown in vitro. The plants were cultured in vitro under different photoperiods (4, 8, 16, and 24 h of light) and irradiance of 41  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . After 40 days of culture, results showed that *L. alba* presented high physiological plasticity under different photoperiods, with improved performance when exposed to continuous light. The best growth; anatomical organization of the mesophyll, stem, roots, and bundles; amount of photosynthetic pigments; photosynthetic rate; and protein synthesis occurred under a photoperiod of 24 h. The biosynthesis of linalool, the major compound, was increased under the 24-h photoperiod, possibly due to reduced geraniol synthesis. These findings allow a better understanding of how photoperiod acts in the regulation of primary and secondary metabolism, and especially with regard to the composition of essential oils.

## Key message

Photoperiod modulates primary metabolism, growth, morphoanatomy, photosynthesis, and essential oil content in the medicinal plant *Lippia alba* cultured in vitro under 4, 8, 16 or 24 h of light.

**Keywords** Circadian rhythm · Geraniol synthase · Internal clock · Medicinal plant · Photosynthesis

---

Communicated by Mohammad Faisal.

✉ Wagner Campos Otoni  
wcotoni@gmail.com

<sup>1</sup> Departamento de Biologia Vegetal/BIOAGRO, Universidade Federal de Viçosa, Viçosa, MG, Brazil

<sup>2</sup> Programa de Pós-Graduação em Agricultura e Ambiente, Universidade Estadual do Maranhão, Cidade Universitária Paulo VI, Av. Lourenço Vieira da Silva, s/nº, São Luís, MA, Brazil

<sup>3</sup> Laboratório de Genética e Biotecnologia, Departamento de Biologia, Universidade Federal de Juiz de Fora, Juiz de Fora, MG, Brazil

<sup>4</sup> Departamento de Química, Núcleo Multifuncional de Pesquisas Químicas (NUPEQ), Universidade Federal de Juiz de Fora, Juiz de Fora, MG, Brazil

## Introduction

The photoperiod is established by the rotation of the Earth around its own axis and around the sun, and impacts significantly the metabolism of most living beings (Serrano-Bueno et al. 2017). In plants, several processes are controlled by photoperiod, like growth, flowering induction, cold tolerance and regulation of the circadian clock (Bordage et al. 2016; Barros et al. 2017; Shin et al. 2017; Maurya and Bhalerao 2017; Mahmud et al. 2018; Triozzi et al. 2018).

Interactions between the circadian clock, primary metabolism, and signaling are thought to explain the importance of rhythmic behavior for plant growth and survival (Harmer et al. 2000; Dodd et al. 2005; Shin et al. 2017). Despite a variety of reports involving the circadian regulation of primary metabolism pathways in plants, it remains to be

determined how rhythmic oscillators are influenced by metabolism (Dodd et al. 2005).

Several rhythmic genes are involved in regulating the endogenous clock in plants. In *Arabidopsis thaliana*, *CCA1*, *late elongated hypocotyl (LHY)*, *pseudo response regulator (PRR)*, and *timing of cab expression 1 (TOC1)* can be up- or down-regulated throughout the day and night (Ito et al. 2009; Huang et al. 2012; Haydon et al. 2013). *CCA1* and *LHY* (Alabadí et al. 2001) are expressed together with *PRR5*, 7, and 9 (Farré et al. 2005) in the morning only. Similarly, *TOC1* (Ito et al. 2009), *gigantea (GI)* (Park et al. 1999), *early flowering 3 and 4 (ELF3/4)*, and *luxar rhythm (LUX)* (Lu et al. 2009; Nusinow et al. 2011) transcripts become more numerous during the night period.

Some authors have sought a correlation between the plant endogenous clock and secondary metabolism. Barbosa et al. (2012) analyzed the influence of circadian rhythm on the composition of *Piper divaricatum* essential oils, but found no change in saffrole content among leaves collected at different times of the day. Thus, the regulation of genes involved in the biosynthesis of essential oils remains unknown. Some transcription factors were predicted to regulate the expression of genes or groups of genes in different plant species. For example, the synthesis of isoprenoids in *Populus* is regulated by the transcription factors *LHY* and *CCA1* (Loivamäki et al. 2007).

Medicinal and aromatic plants have been domesticated for thousands of years as a source of natural compounds with a broad range of pharmacological applications (Böhme et al. 2014; Wagas et al. 2016). The increasing interest in these compounds associated with technological advances has enabled large-scale production of secondary metabolites for pharmaceutical use (Wei et al. 2016).

Essential oils are bioactive compounds used by the pharmaceutical, cosmetic, food, and agrochemical industries. They are used in the treatment of cardiovascular diseases, bronchitis, cough, asthma, as well as stomach and intestinal disorders (Pascual et al. 2001; Lorenzi and Matos 2008; Saljoughian et al. 2018), whereby they provide analgesic, anti-inflammatory, anti-ulcerogenic, and anti-helminthic relief (Raut and Karuppaiyil 2014; Amin and Hosseinzadeh 2016). Furthermore, these compounds may also be used in controlling pests (Pavela and Govindarajan 2017; Benelli et al. 2018), neutralizing oxidizing agents (Proestos et al. 2014), and promoting food preservation (Peng and Li 2014; Szczepanski and Lipski 2014; Otoni et al. 2016; Pola et al. 2016).

*Lippia alba* has great cultural and economic importance, due to the medicinal properties of its essential oil extract (Pascual et al. 2001; Lorenzi and Matos 2008). A peculiarity of this genus is the chemical variability among its genotypes (chemotypes), with respect to the composition of essential oils and non-volatile compounds (Hennebelle et al. 2008).

Furthermore, *L. alba* is characterized by high phenotypic and genomic plasticity (Reis et al. 2014; Viccini et al. 2006, 2014).

The physiological plasticity of this genus may help respond to different abiotic factors, which may affect secondary metabolism, and particularly the profile of essential oils (Ragagnin et al. 2014; Batista et al. 2016, 2017a; Lazzarini et al. 2018). Among these factors, spectral variation of light (quality), irradiance (intensity), CO<sub>2</sub> concentration, and temperature seem to be particularly important (Morais 2009; Batista et al. 2016, 2017a; Lazzarini et al. 2018).

With the growing demand for essential oils in the pharmaceutical industry, *L. alba* is a relevant medicinal species, with emphasis on its capacity to modulate the profile of essential oils due to abiotic factors (Batista et al. 2016, 2017a). Such an insight will help establish strategies to optimize the production of essential oils in *L. alba*. Moreover, there have been no studies to date examining the influence of photoperiod on the metabolism and production of essential oils in this species, and new findings may be interesting even to extrapolate this relationship between photoperiod and secondary metabolism to other species. Hence, the objective of this work was to evaluate the effect of photoperiod on anatomy, growth, profile of essential oils, and expression of genes related to the synthesis of essential oils and circadian clock in *L. alba* cultured in vitro.

## Materials and methods

### Plant material

*Lippia alba* (BGEN-04) was obtained from the Germplasm Bank of the Department of Biology, Federal University of Juiz de Fora (UFJF, Juiz de Fora, MG, Brazil), and the propagules were granted by Embrapa Genetic Resources and Biotechnology (Cenargen, Brasília, DF, Brazil). The specimens were deposited in the Leopold Krieger Herbarium (Herbarium CESJ 48372, UFJF). The plants were propagated in vitro in MS medium (Murashige and Skoog 1962) supplemented with 30 g L<sup>-1</sup> sucrose, 100 mg L<sup>-1</sup> myo-inositol, and 6.5 g L<sup>-1</sup> Merck agar (Merck Millipore Corp., Darmstadt, Germany). The pH of the medium was adjusted to 5.7 ± 0.01. The medium was autoclaved at 120 °C and 108 kPa for 20 min at the Plant Tissue Culture Laboratory (LCT II), located at the Institute of Biotechnology Applied to Agriculture—BIOAGRO, Federal University of Viçosa (UFV).

### Effect of photoperiod

Eight nodal segments (~2 cm in length) were inoculated into glass flasks (600 mL capacity) containing 80 mL of MS

medium supplemented with 30 g L<sup>-1</sup> sucrose, 100 mg L<sup>-1</sup> myo-inositol, and 6.5 g L<sup>-1</sup> Merck agar. The pH of the medium was adjusted to 5.7 ± 0.01. The medium was autoclaved at 120 °C and 108 kPa for 20 min. Cultures were maintained in a growth room at 25 ± 2 °C and exposed to different photoperiods (4, 8, 16, and 24 h light) and constant irradiance of 41 μmol m<sup>-2</sup> s<sup>-1</sup> provided by two white LED bulbs (SMD 100, 18 W, Vilux®, Vitória, ES, Brazil). The flasks were sealed with rigid polypropylene lids with two vents (10 mm diameter) covered with 0.45 μm PTFE membranes (MilliSeal® AVS-045 Air Vent, Milipore Merck) allowing for a CO<sub>2</sub> exchange rate of 25 μL L<sup>-1</sup> s<sup>-1</sup> (Batista et al. 2017b).

After 40 days of culture (Fig. 1), the following parameters were analyzed: primary metabolites, growth, anatomy, photosynthesis, profile of essential oils, as well as expression of genes involved in mono- and sesquiterpenes synthesis and the endogenous clock. All samples were collected at 17 h.

### Development analysis

The following developmental characteristics were analyzed: fresh mass (g), dry mass (g), total shoot length (cm), total length of the largest root (cm), and leaf area (cm<sup>2</sup>). The latter was measured with ImageJ software (Schneider et al. 2012).

### Anatomical analysis

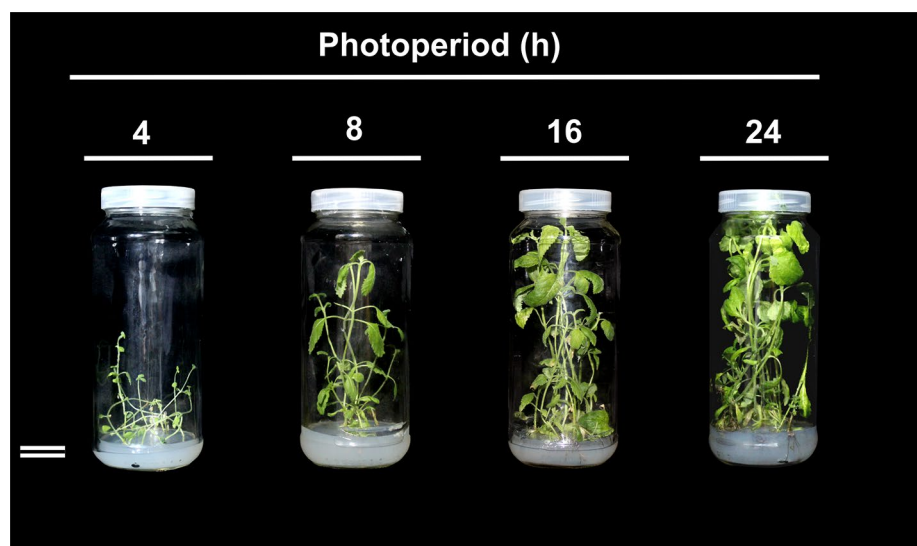
For an anatomical structural characterization, samples of the second pair of expanded leaves, stems of the median region of the second internode, and main roots of the median region were fixed in Karnovsky solution (Karnovsky 1965). After fixation, the samples were dehydrated in increasing ethanolic series (10, 20, 30, 40, 50, 60, 70, 80, 85, 90, and 95%) and included in acrylic resin (Historessin; Leica

Instruments, Jena, Germany). Cross-sections of 5-μm thickness were obtained on a rotary microtome (RM2155, Leica Microsystems Inc., Buffalo Grove, IL, USA) and stained with toluidine blue (pH 3.2) (O'Brien and McCully 1981). Slides were mounted on Permount® SP15-500 synthetic resin (Fisher Chemicals-Fisher Scientific, Springfield, NJ, USA). A light microscope (AX70 TRF; Olympus Optical, Tokyo, Japan) with the U-photo system, coupled to a digital photographic camera (Spot Insightcolour 3.2.0; Diagnostic Instruments Inc., Merrick, NY, USA) and microcomputer with Spot Basic image capture software were used to capture the images.

### In vitro photosynthetic rate

Gas exchange and quantification of in vitro photosynthetic rate were performed as proposed by Costa et al. (2014) with some modifications. An AQ-S151 infrared CO<sub>2</sub> analyzer (Qubit Systems, Kingston, ON, Canada) was used for measurements and data collection was performed using LoggerLite 1.8.1 software (Vernier Software & Technology, Beaverton, OR, USA). Reference CO<sub>2</sub> was calculated by inflowing air into an empty flask, pumped from the external environment at a constant air flow rate of 300 mL min<sup>-1</sup>, located within an illuminated chamber (white LED bulbs). The plants were maintained in the absence of light for a period of 8 h prior to analysis. Soon after measuring the reference CO<sub>2</sub>, the flasks containing the plants were coupled to the system and the CO<sub>2</sub> was calculated at the stabilization point. Gas exchanges were calculated by computing the difference between the reference CO<sub>2</sub> and the CO<sub>2</sub> of the plants exposed to atmospheric air. Air temperature and humidity in the flask were measured by a Spec sensor (Thermo Recorder RS-11, Takai Spec Corp., Aichi, Japan). The in vitro photosynthetic rate (A) was calculated by the following formula:

**Fig. 1** *Lippia alba* plants grown in vitro under different photoperiods (4, 8, 16, and 24 h) after 40 days of culture. Bar = 2 cm



$$A (\mu\text{mol m}^{-2} \text{s}^{-1}) = \frac{\Delta\text{CO}_2}{\text{MolFlow}}$$

where

$$\Delta\text{CO}_2 (\text{ppm}) = \text{Reference CO}_2 - \text{Analysis CO}_2$$

$$\text{Mol Flow} = \frac{\text{Air flow rate (L min}^{-1}\text{)}}{\left( \frac{(\text{Constant for perfect gases (22.4)} \times \text{Temperature (K)})}{60000} \right) \text{Leaf area (cm}^2\text{)}}}$$

### Photosynthetic pigments and quantification of carbohydrates, proteins, amino acids, and starch

Samples from the shoots were collected, frozen in liquid nitrogen, ground, and lyophilized. To determine the amount of photosynthetic pigments, approximately 10 mg of lyophilized tissues were used for extraction with acetone as described by Welburn (1994). For the determination of carbohydrates (starch, sucrose, glucose, and fructose) content, 25 mg of lyophilized tissues were used in the extraction with methanol and evaluated as described by Fernie et al. (2001). Quantification of protein and total amino acids was conducted as suggested by Cross et al. (2006).

### Fluorescence of chlorophyll

Electron transport rate (ETR), non-photochemical quenching (NPQ), effective PSII quantum yield ( $F_v/F_m$ ), and photochemical quenching (qP) were quantified in fully expanded *L. alba* leaves submitted to a light–dark cycle and using a MINI-PAM chlorophyll fluorometer (Walz, Effeltrich, Germany). The data for minimum fluorescence ( $F_0$ ), maximum fluorescence ( $F_m$ ), and variable fluorescence ( $F_v$ ) were calculated as proposed by Kooten and Snel (1990). ETR, NPQ, and qP were calculated according to Maxwell and Johnson (2000).

### Microextraction of essential oils

For microextraction of essential oils, ~300 mg of leaves were collected and stored at  $-18^\circ\text{C}$  in a test tube with a screw cap. After freezing, 1 mL hexane and 0.5 mL methanol were added to each sample. The samples were kept in an ultrasonic bath (Thornton-INPEC, Vinhedo, SP, Brazil) at 70 kHz and room temperature for 1 h. Subsequently, the supernatant was filtered through a sterile cotton wick. Resulting samples of 1  $\mu\text{L}$  of clear solution containing the extracted oils were analyzed by gas chromatography.

### Qualitative analysis of essential oils

The qualitative analysis of essential oils was carried out using a gas chromatographer coupled to a mass spectrometer (GCMS-QP2010 Plus; Shimadzu, Suzhou, China) and Rtx-5MS<sup>®</sup> column (Restek, Bellefonte, PA, USA) of 30 m  $\times$  0.25 mm. The oven temperature was started at  $70^\circ\text{C}$ , maintained for 3 min, followed by an increase of  $6^\circ\text{C min}^{-1}$  to  $300^\circ\text{C}$ . The injector was operated in split mode (1:10) at  $240^\circ\text{C}$ , and the interface and mass detector were operated at  $300^\circ\text{C}$ . Helium was used as the carrier gas, with a flow of  $1.53 \text{ mL min}^{-1}$ . A standard mixture of linear hydrocarbons ( $\text{C}_9\text{H}_{20}$ ,  $\text{C}_{10}\text{H}_{22}$ ;...  $\text{C}_{25}\text{H}_{52}$ , and  $\text{C}_{26}\text{H}_{54}$ ) was injected under the same conditions as the samples. Identification of the constituents was performed by comparing the obtained mass spectra with those in the NIST 9.0 database (correlation  $> 97\%$ ) and confirmed by their retention indices (Kováts Index), which were calculated for each constituent and compared to published data (Adams 1997).

### Extraction of mRNA, cDNA synthesis, and analysis by real-time PCR (RT-qPCR)

Total RNA was isolated from the leaves with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Invitrogen) following the manufacturer's recommendations. Quantification was performed with a NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized using reverse transcriptase (Ludwig Biotec, Bela Vista-Alvorada, RS, Brazil) and 800 ng of total RNA. RT-qPCR was performed on a CFX96 Touch<sup>™</sup> cyclor (Bio-Rad, Hercules, CA, USA). The total reaction volume was 10  $\mu\text{L}$ : 1  $\mu\text{L}$  cDNA, 3  $\mu\text{L}$  diethyl pyrocarbonate water, 2  $\mu\text{L}$  of 4  $\mu\text{M}$  forward and reverse primers, and 4  $\mu\text{L}$  SYBR-Green mix/Rox (Ludwig Biotec). Primers for *nerolidol/linalool synthase* (*LaNES/LIS*), *geraniol synthase* (*LaGES*), *timing of cab expression 1* (*LaTOC1*), *pseudo response regulator 5* (*LaPRR5*), *late elongated hypocotyl* (*LaLHY*), and the reference gene *alcohol dehydrogenase* (*LaADH*) were designed based on the de novo transcriptome sequence of *L. alba* (Souza et al. unpublished data).

### Statistical analysis

Experiments were conducted following a completely randomized design; the experimental unit consisted of a culture flask containing eight plants. Statistical analyses were performed using Genes software version Windows/2004.2.1 (Cruz 2016). Data were submitted to analysis of variance (ANOVA) by the F test and the means were compared by the Scott & Knott test ( $P \leq 0.05$ ). RT-qPCR expression levels were calculated by the  $2^{-\Delta\Delta\text{Ct}}$  method proposed by Livak and Schmittgen (2001) with

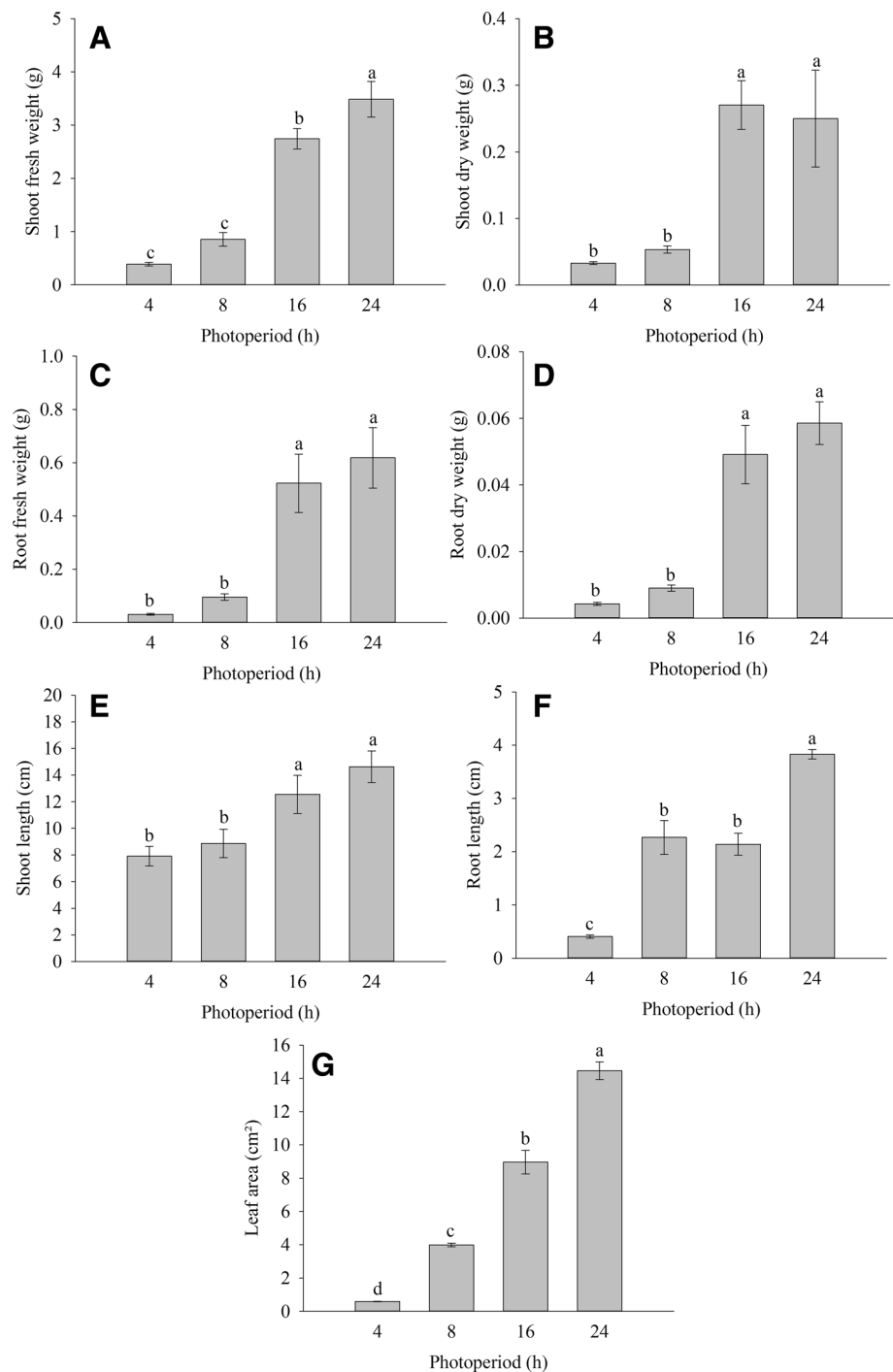
three biological replicates and at least three technical replicates. Means were compared by Dunnett's test ( $P \leq 0.05$ ). Pearson correlation analysis was performed for the *LaLHY*, *LaPRR5*, and *LaTOC1* gene expression data.

## Results

### Photoperiod interferes with growth of *L. alba* in vitro

Treatments with 16 and 24 h of light increased the values of all growth variables analyzed (Fig. 2a–g), with the 24-h light treatment resulting in the greatest shoot fresh weight

**Fig. 2** Growth variables of *Lippia alba* after 45 days of in vitro culture under different photoperiods (4, 8, 16, and 24 h). Means marked by the same letters do not differ according to the Scott & Knott test at a 5% probability. Data are presented as means ( $n=4$ ). Vertical bars represent the standard error





(Fig. 2a), root length (Fig. 2f), and leaf area (Fig. 2g). Therefore, the longer was the period of light exposure, the greater was the overall growth response.

### The morphoanatomy of vegetative organs in *L. alba* cultivated in vitro is influenced by photoperiod

In vitro culture of *L. alba* over different photoperiods promoted variations in the anatomy of stems, leaves, and roots (Fig. 3). In the stem, it was possible to observe a higher cellular organization with increasing photoperiod. Specifically, plants grown under daily 16- and 24-h light regimens had a more differentiated vascular tissue, with larger and more organized bundles (Fig. 3a, d, g, j).

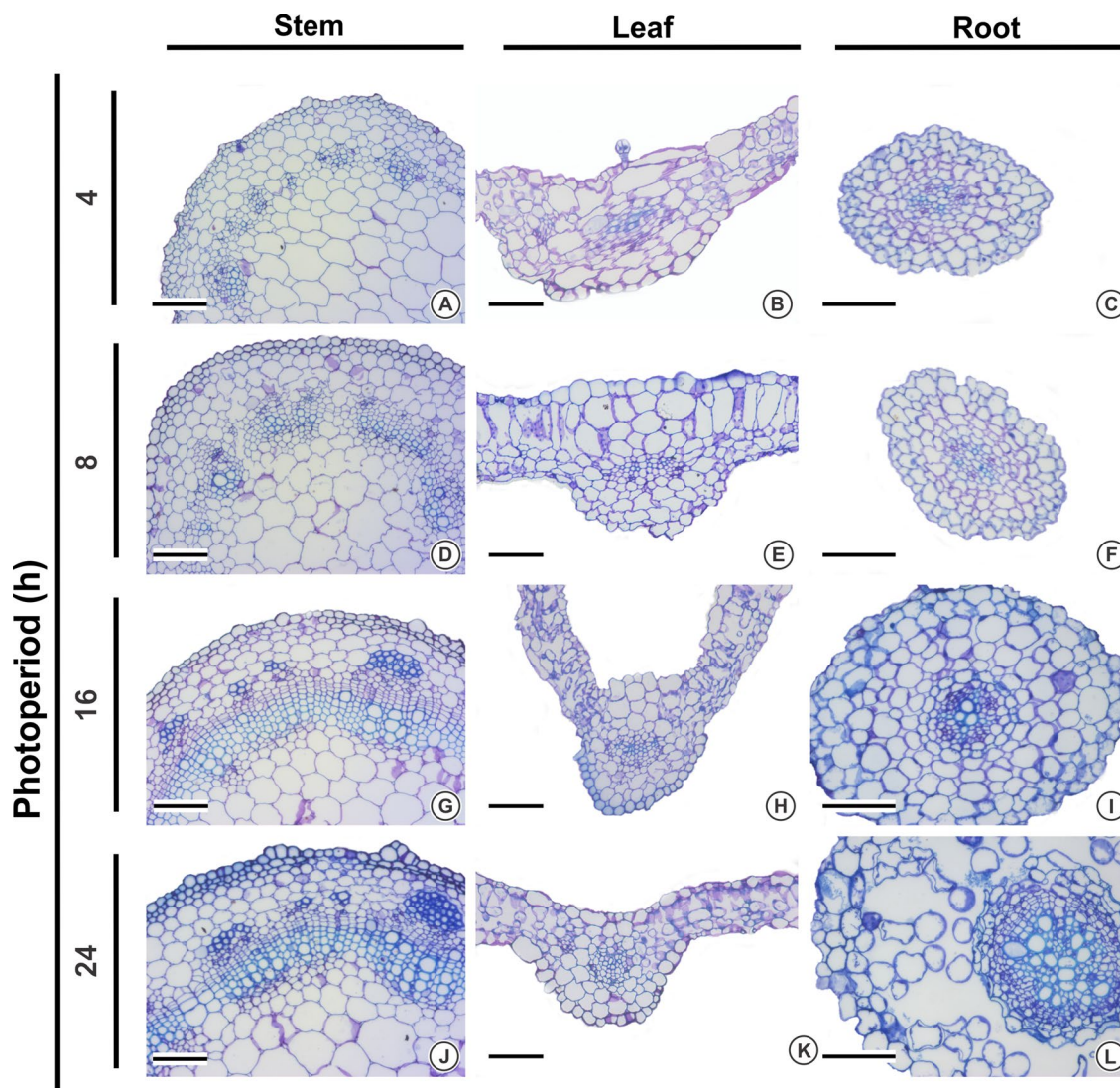
The central rib region of plants grown over photoperiods of 16 and 24 h presented greater mesophyll cell organization

and vascular bundles in comparison to those exposed to 4- and 8-h light regimens (Fig. 3b, e, h, k).

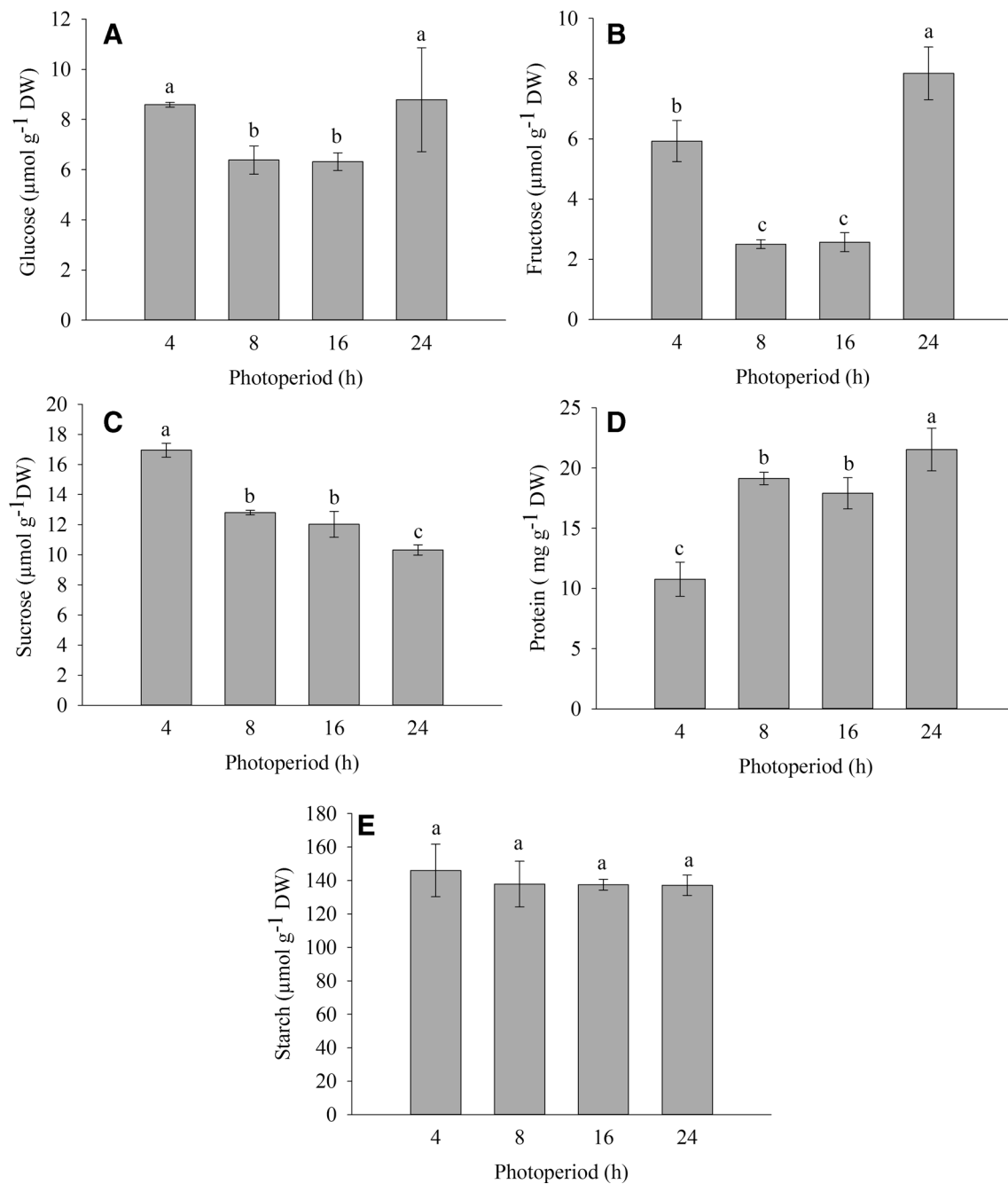
The cellular organization and thickness of the roots increased along with longer photoperiods, with plants exposed to light for 24 h having more spaced cells in the cortex (Fig. 3c, f, i, l). Furthermore, the vascular bundle was more organized and of greater caliber in relation to other treatments.

### Photoperiod influences primary metabolism in *L. alba* cultured in vitro

Metabolites involved in osmoregulation, such as glucose and fructose, increased during the 24-h treatment, although the glucose level did not differ from 4 h (Fig. 4a,



**Fig. 3** Cross-sections of stems (a, d, g, j), leaf blades (b, e, h, k), and roots (c, f, i, l) of *Lippia alba* after 45 days of in vitro culture under different photoperiods (4, 8, 16, and 24 h) and stained with toluidine blue. Bars = 20  $\mu$ m



**Fig. 4** Sugar, protein, and starch contents in leaves of *Lippia alba* after 45 days of in vitro culture under different photoperiods (4, 8, 16, and 24 h). **a** Glucose; **b** fructose; **c** sucrose; **d** protein; and **e** starch.

Equal letters denote no difference according to the Scott & Knott test at a 5% probability. Data are presented as means ( $n=3$ ). Vertical bars represent the standard error

b); whereas sucrose levels decreased as the photoperiod increased (Fig. 4c).

Protein content increased with an increasing photoperiod (Fig. 4d). However, there was no difference in starch accumulation among the treatments (Fig. 4e).

### The profile of essential oils in *L. alba* cultured in vitro is altered by photoperiod

Photoperiod altered the composition profile of essential oils in *L. alba*. The 4-h treatment was limited by the low amount

of plant material in the samples and, thus, no essential oil components could be detected. However, plants submitted to uninterrupted light conditions (24-h photoperiod) showed a significant increase in linalool content when compared to the 8- and 16-h photoperiods. The levels of eucalyptol and germacrene did not differ significantly among treatments (Fig. 5).

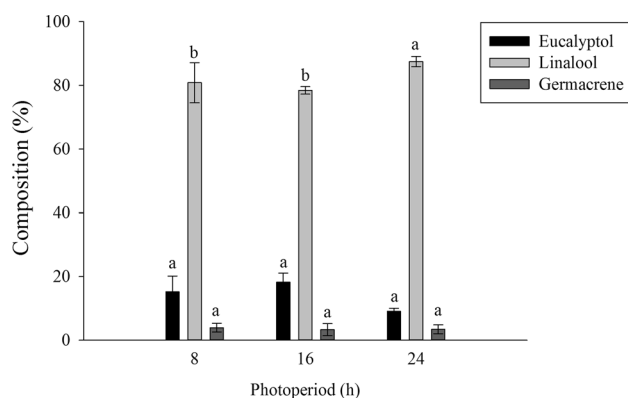
### A shorter photoperiod reduces photosynthetic capacity and alters chlorophyll *a* fluorescence in *L. alba* grown in vitro

In vitro photosynthesis of *L. alba* was reduced in plants cultivated for the shortest photoperiod; they exhibited a significant decrease in photosynthetic rates compared to those cultivated under 8, 16, and 24 h of light (Fig. 6).

Fluorescence measurements revealed that photoperiod was able to influence NPQ and ETR in the electron transport chain of *L. alba* plants. Plants grown over a 4-h photoperiod exhibited a three-fold NPQ induction compared to plants grown over a 24-h photoperiod. None of the treatments, however, showed significant differences in relation to *Fv/Fm* and *qP* (Fig. 6).

### Photoperiod alters photosynthetic pigment levels in *L. alba*

Chlorophyll *a* content was highest with the 24-h photoperiod (Fig. 7a); whereas chlorophyll *b* was highest with the 8-h light treatment, followed in equal measure by 16- and 24-h photoperiods, and finally the 4-h treatment (Fig. 7b). The chlorophyll *alb* ratio was highest for the 4-h photoperiod, followed by 16- and 24-h light treatments, and eventually the 8-h photoperiod (Fig. 7c). Overall, the highest levels of



**Fig. 5** Effect of photoperiod on the composition profile of essential oils in *Lippia alba* after 45 days of culture in vitro. Means were compared by the Scott & Knott test at a 5% probability. Data are presented as means ( $n=3$ ). Vertical bars represent the standard error

total chlorophyll and carotenoids were observed with the 8-, 16-, and 24-h light treatments (Fig. 7d, e).

### Photoperiod reduces geranyl synthase expression, but does not alter expression levels of *LaNES/LIS* and circadian clock genes in *L. alba*

Expression of *LaGES* was significantly down-regulated following a 24-h photoperiod compared to control and other treatments; whereas relative expression of *LaNES/LIS* showed no significant difference between treatments (Fig. 8a, b).

Regarding circadian clock genes, the relative expression of *LaLHY*, *LaPRR5*, and *LaTOC1* was not affected by light treatments (Fig. 8c–e). As for *LaLHY* and *LaPRR5*, a positive and significant correlation was detected between their expression levels and length of photoperiod (Fig. 8f).

## Discussion

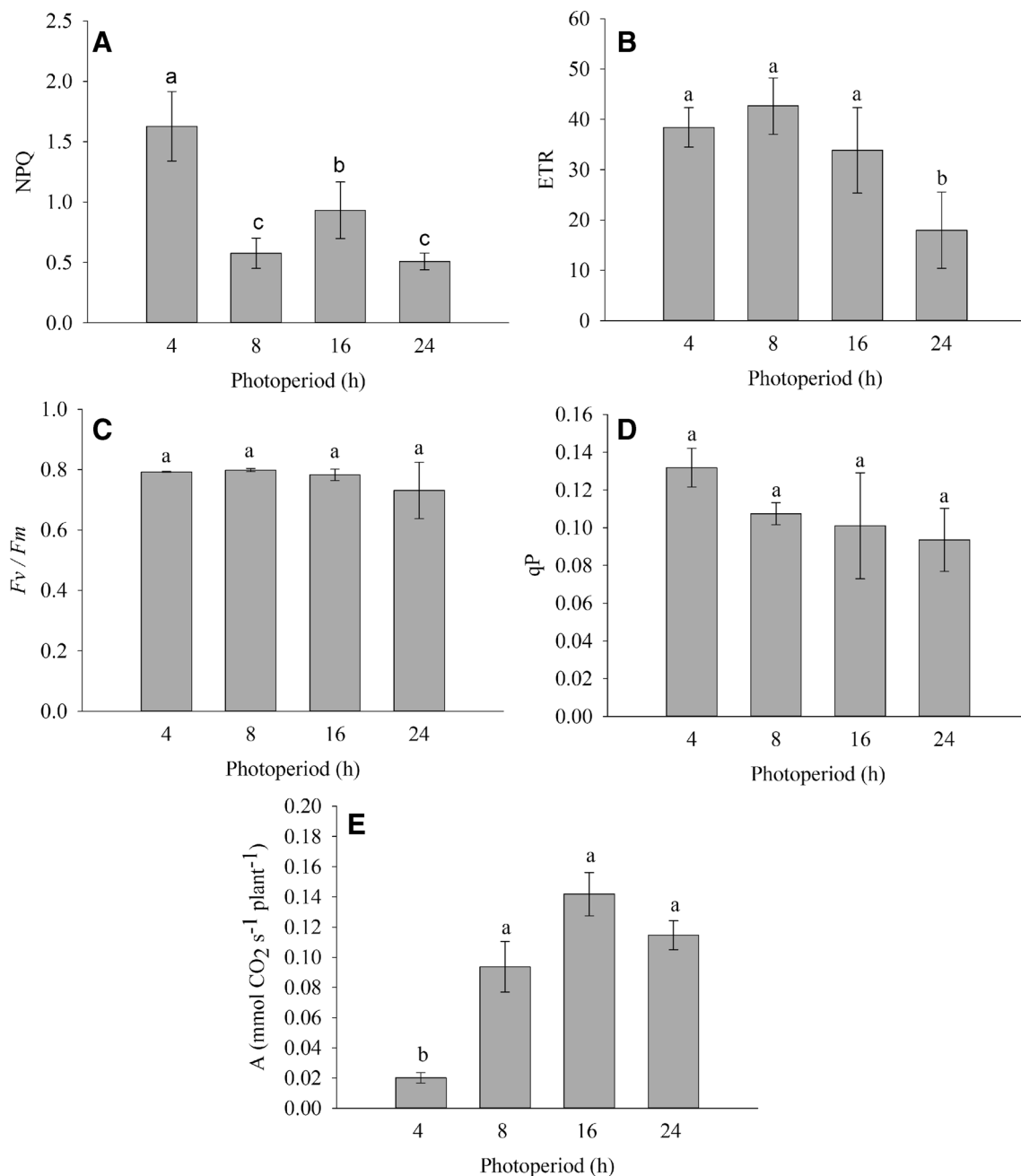
Plant growth and development are influenced by several abiotic factors, which influence also the biosynthesis of essential oils (Ragagnin et al. 2014; Batista et al. 2016, 2017a; Lazzarini et al. 2018). Several studies have investigated the impact of photoperiod on different physiological processes in plants (Bordage et al. 2016; Barros et al. 2017; Maurya and Bhalerao 2017; Mahmud et al. 2018; Song et al. 2018; Wang et al. 2018). This is the first study to investigate the effect of photoperiod on the profile of essential oils and physiological responses in *L. alba*.

Exposure to 16- and 24-h photoperiods resulted in an increase in both shoot and root dry mass, as well as increased stem and root length. Accordingly, increased exposure to light may have a positive influence on *L. alba* root growth, as observed by Mahmud et al. (2018) when evaluating the effect of photoperiod on the growth and development of roots in vines. Baerenfaller et al. (2015) described also a larger average leaf areas in plants developed under long photoperiods (16 h).

In contrast, exposure to short photoperiods resulted in stunted leaf area development, which may explain the worse photosynthetic performance and lower concentration of photosynthetic pigments and photoprotectors (carotenoids) in plants subjected to a 4-h photoperiod. Fr chet te et al. (2016) observed a decline in the net assimilation of photosynthetic  $\text{CO}_2$  in *Pinus strobus* under short-day conditions, suggesting the importance of photoperiod in the regulation of photosynthesis.

Changes in the concentrations of photosynthetic pigments were dependent on the daily dose of light, as exemplified by reduced contents of *a*, *b*, and total chlorophylls but increased chlorophyll *alb* ratio in plants subjected to



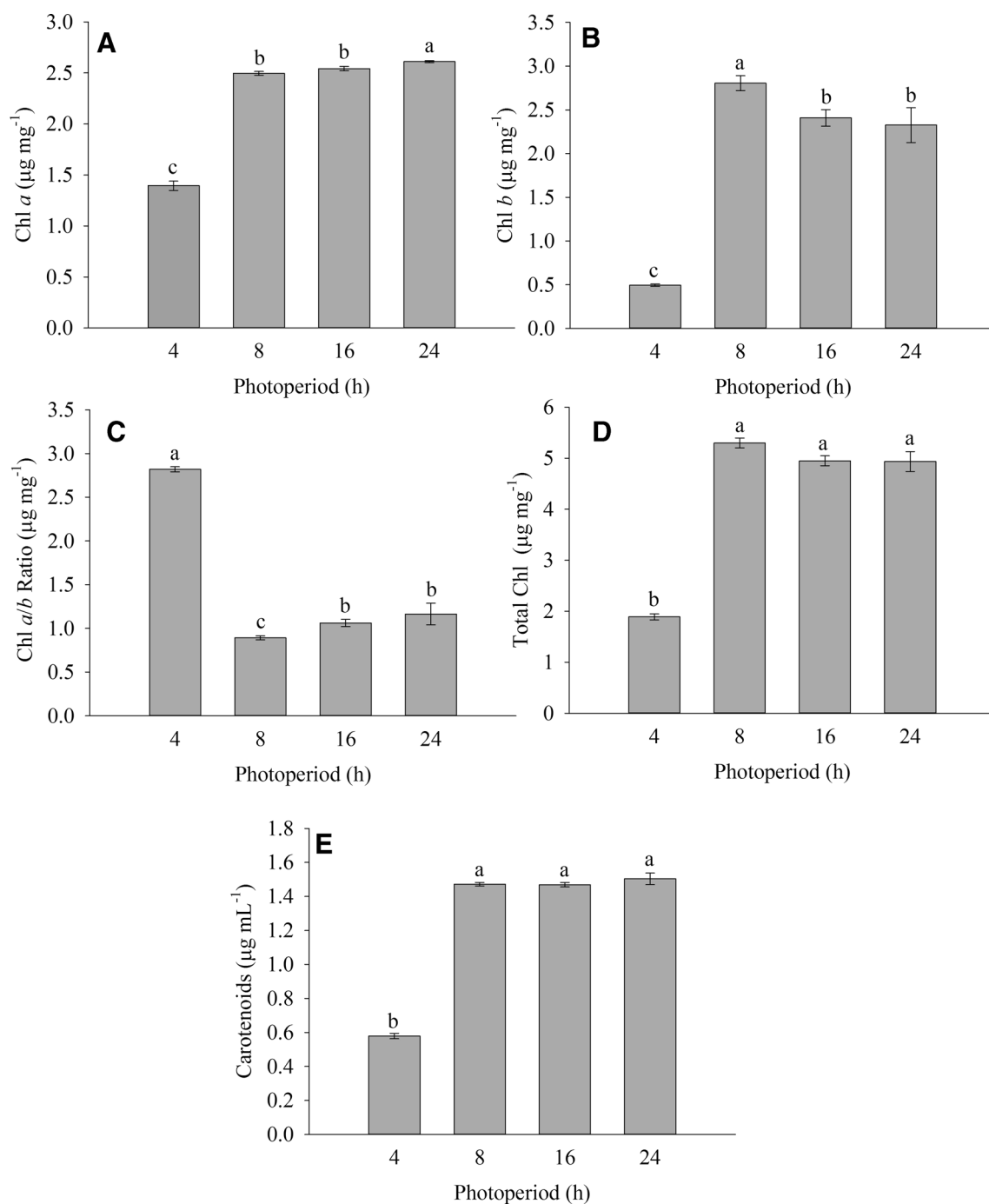


**Fig. 6** Photosynthetic variables of *Lippia alba* after 45 days of in vitro culture under different photoperiods (4, 8, 16, and 24 h). **a** NPQ; **b** ETR; **c**  $F_v/F_m$ ; **d** qp; and **e** photosynthetic rate. Means were compared by the Scott & Knott test at a 5% probability. Data are pre-

sented as means ( $n=4$ ). Vertical bars represent the mean standard error. NPQ, non-photochemical quenching; ETR, electron transport rate;  $F_v/F_m$ , effective PSII quantum yield; qp, photochemical quenching

a 4-h photoperiod. Similar results were obtained by Vogg et al. (1998) in *Pinus sylvestris* L., whose chlorophyll *a*, *b*, and total contents were found to be relatively lower on short days (9 h) compared to long days. Here, a short photoperiod (4 h) increased NPQ compared to plants exposed to light for 24 h. This result suggests that the input of photosynthetic energy may have been beyond what the plant could exploit and, therefore, the plants might have

strategically acclimatized themselves by increasing the fraction of absorbed energy, dissipating it into heat (Bilger and Bjorkman 1990; Maxwell and Johnson 2000). Thus, there is likely a physiological plasticity at low light exposure, and neither  $F_v/F_m$  nor photochemical dissipation differed significantly relative to other treatments. Although ETR was lower in the uninterrupted photoperiod compared to other treatments, this did not have a negative impact



**Fig. 7** Chlorophyll and carotenoid contents in *Lippia alba* after 45 days of in vitro culture under different photoperiods (4, 8, 16, and 24 h). Means were compared by the Scott & Knott test at 5% prob-

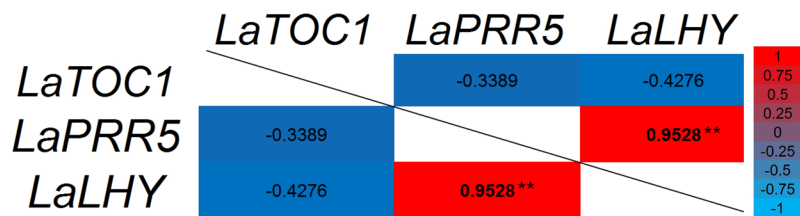
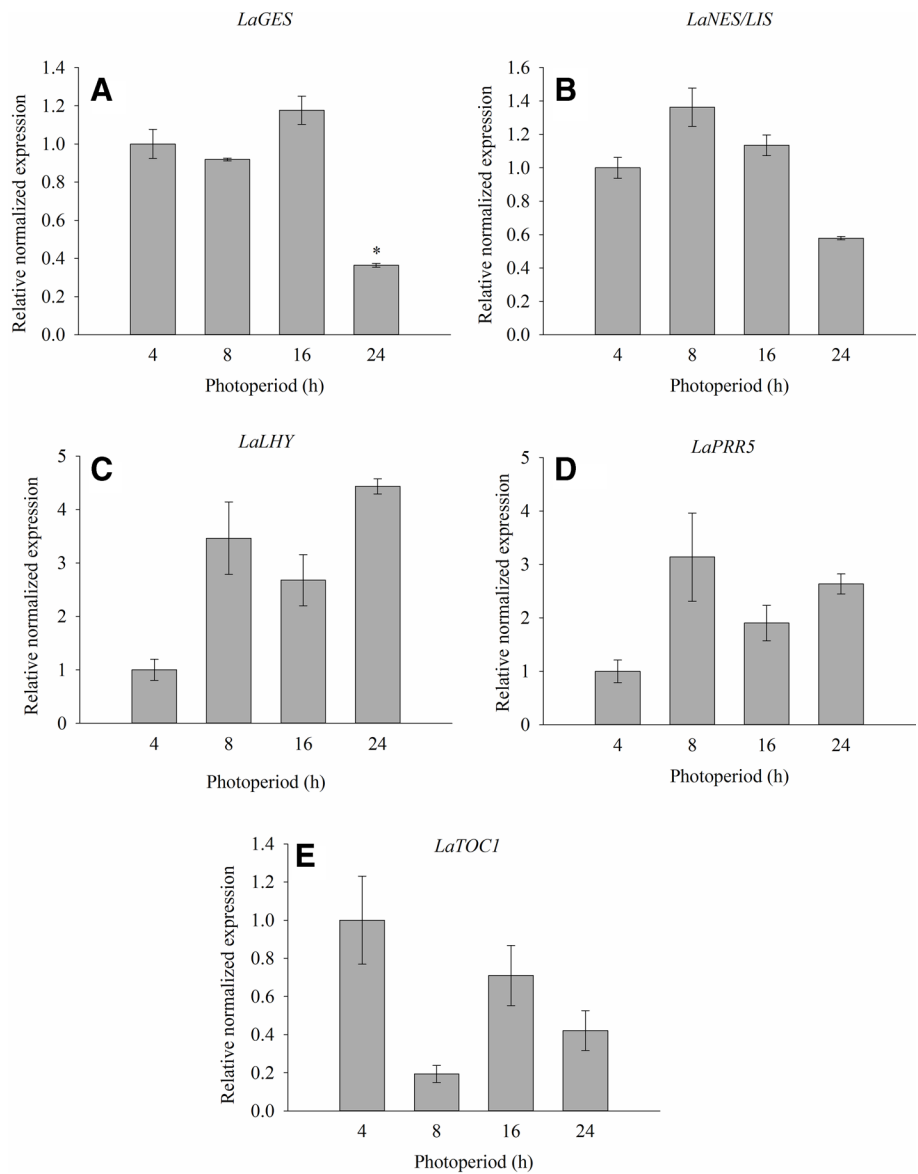
ability. Data are presented as means (n=6). Vertical bars represent the mean error. Chl a, chlorophyll a; Chl b, chlorophyll b

on the physiological processes of the plants analyzed in this study. Shibaeva and Markovskaya (2013) reported a smaller ETR in cucumber (*Cucumis sativus* L.) exposed to a 24-h photoperiod, thus emphasizing the importance of photoperiod in agricultural production. Overall, in spite of decreased photosynthesis, leaf area, photosynthetic

pigments, and carotenoids, our results indicate that *L. alba* possesses great adaptability to different photoperiods.

During the 4-h photoperiod, there was a reduction in protein content, indicating that photoperiod may be regulating protein synthesis of *L. alba* in vitro. Fr chet te et al. (2016) reported that a reduced protein level during lower

**Fig. 8** Gene expression levels in *Lippia alba* after 45 days of in vitro culture under different photoperiods (4, 8, 16, and 24 h) and Pearson correlation analysis. *LaGES*, geraniol synthase; *LaNES/LIS*, neralidol/linalool synthase; *LaTOC1*, timing of cab expression 1; *LaLHY*, late elongated hypocotyl; *LaPRR5*, pseudo response regulator 5. Expression is relative to alcohol dehydrogenase (*LaADH*). Data are presented as means (n=3) and are compared using Dunnett’s test at 5% probability



photoperiods might arise in response to photosynthetic limitation. This plasticity in protein levels under different photoperiods may allow for a faster growth during long photoperiods (Baerenfaller et al. 2015). Glucose and sucrose were generally higher during photoperiods of 4 h, but lower under 24-h light treatment. These findings are similar to those reported by Mengin et al. (2017), who noted that sucrose regulation during short photoperiods restricted growth and caused the export of carbon during the day. Here, the various

treatments did not alter the levels of starch in *L. alba* in vitro, as opposed to other studies, whereby shorter photoperiod led to an increase in starch accumulation (Hädrich et al. 2012; Mengin et al. 2017). Hence, duration of the photoperiod in *L. alba* in vitro has a great influence on primary metabolism, with sugar and protein contents being important for controlling growth and development.

The in vitro culture of *L. alba* under different photoperiods affected morphogenesis, showing that longer

photoperiods (16 and 24 h) improved structuring of the mesophyll, stem, and roots, and differentiation of vascular tissues, resulting in more organized and larger bundles. These results are consistent with those of Castro et al. (2005), who reported that vascular bundle size and arrangement were smaller under short-day photoperiods (8 h).

Shorter photoperiods did not influence the profile of essential oils as much as did uninterrupted light (24 h), demonstrating that photoperiod has a significant effect on the profile of essential oils of *L. alba*. Long days and elevated light intensity have been reported before to activate genes related to anthocyanin biosynthesis and promoting its accumulation in the leaves of *Malus* spp. (Lu et al. 2009), as well as flavonoids, anthocyanins, and catechins in *Ipomoea batatas* (L.) Lam. (Carvalho et al. 2010).

Here, linalool content was increased under the 24-h light photoperiod compared to the 8-, 16-, and 24-h periods. This increase was not followed by the expression of *LaNES/LIS*. However, expression levels of *LaGES* were reduced. Possibly, the increased synthesis of linalool was related to a lower expression of *LaGES*, which in turn hampered the synthesis of geraniol, another monoterpene. Thus, plant metabolism may be directing the precursor of the geranyl pyrophosphate (GPP) route to the synthesis of linalool, causing a significant increase in its levels. Viccini et al. (2014) reported that the main components of essential oils in *L. alba* were related to ploidy level; here, diploids, triploids, and tetraploids of all accessions were capable of producing linalool and citral. However, triploids are inefficient in catalyzing this additional biotransformation to citral, and thus predominantly generate linalool. Such inability may explain the observed reduction in *LaGES* expression as the accession used in this study was a triploid. In addition, it is probable that the 24-h photoperiod caused some oxidative damage in plant tissues, which resulted in the aforementioned variation in linalool levels. Furthermore, several studies have reported that monoterpenes are effective in protecting plants against stress (thermal or oxidative); their levels become increased under these circumstances to allow for reactive oxygen species removal and protection of photosynthetic systems (Chen et al. 2009; Zuo et al. 2017).

The relative expression of circadian clock genes (*LaLHY*, *LaPRR5*, and *LaTOC1*) was not affected by any of the treatments. Even though studies show that *LHY* expression is repressed by *PRR5* and *TOC1*, photoperiod treatments did not alter the expression of clock genes in *L. alba* (Nakamichi et al. 2010; Gendron et al. 2012; Huang et al. 2012). Instead, we report here the co-expression of *LaLHY* and *LaPRR5*.

The present results indicate that photoperiod modulates primary and secondary metabolisms in *L. alba* plants cultured in vitro. The uninterrupted photoperiod treatment (24 h) provided greater growth; better anatomical organization of the mesophyll, stem, roots, and bundles; increased

photosynthetic pigment levels, higher photosynthetic rate; and greater synthesis of total proteins. Batista et al. (2016) and Hsie et al. (2019) already demonstrated that *Lippia* alters its metabolism under different qualities of light, now we found that this physiological plasticity also is triggered by the photoperiod, with better performance during long days, which can be advantageous in the tropical regions. In addition, the biosynthesis of linalool, a major component of the *L. alba* chemotype used in this study, was characterized by reduced expression of *LaGES*. These results will allow a better understanding of how photoperiod affects the regulation of primary and secondary metabolism, which is of great importance given the species' value for the pharmacological industry.

**Acknowledgements** The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, DF, Brazil; grants 432412/2016-6 and 313740/2017-8 to LFV), and Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior, CAPES, DF, Brazil; Finance Code 001), for financial support. Dr. Roberto Fontes Vieira (Embrapa Recursos Genéticos e Biotecnologia—Embrapa/Cenargen, Brasília, DF, Brazil) is also acknowledged for providing the *Lippia alba* accession. We would like to thank Editage ([www.editage.com](http://www.editage.com)) for English language editing.

**Author contributions** KMC, DSB and WCO conceived and designed the experiments; KMC, TDS, EAF and SHSF performed the experiments, collected and analyzed the data; KMC, TDS, EAF, SHSF and RMJS performed the anatomical, physiological and biochemical analyses; LSQN, VRC and RMG carried out the microextraction and qualitative analysis of essential oils; DSB performed the gene expression analysis by RT-qPCR; KMC and DSB performed the statistical analysis; KMC, DSB, AMF, RMG, LFV and WCO contributed to the interpretation of the research and to the writing of the paper. All authors read and approved the manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

## References

- Adams RP (1997) Identification of essential oil components by gas chromatography/mass spectroscopy. *J Am Soc Mass Spectrom* 6:671–672
- Alabadí D, Oyama T, Yanovsky MJ, Harmon FG, Más P, Kay SA (2001) Reciprocal regulation between *TOC1* and *LHY/CCA1* within the *Arabidopsis* circadian clock. *Science* 293:880–883. <https://doi.org/10.1126/science.1061320>
- Amin B, Hosseinzadeh H (2016) Black cummin (*Nigella sativa*) and its active constituent, thymoquinone: an overview on the analgesic and anti-inflammatory effects. *Planta Med* 82:8–16. <https://doi.org/10.1055/s-0035-1557838>
- Baerenfaller K, Massonnet C, Hennig L, Russenberger D, Sulpice R, Walsh S, Stitt M, Granier C, Gruißem W (2015) A long photoperiod relaxes energy management in *Arabidopsis* leaf six. *Curr Plant Biol* 2:34–45. <https://doi.org/10.1016/j.cpb.2015.07.001>
- Barbosa QP, da Câmara CA, Ramos CS, Nascimento DC, Lima-Filho JV, Guimarães EF (2012) Chemical composition, circadian

- rhythm and antibacterial activity of essential oils of *Piper divaricatum*: a new source of saffrole. *Quím Nova* 35:1806–1808. <https://doi.org/10.1590/S0100-40422012000900019>
- Barros PM, Cherian S, Costa M, Sapeta H, Saibo NJM, Oliveira MM (2017) The identification of almond *GIGANTEA* gene and its expression under cold stress, variable photoperiod, and seasonal dormancy. *Biol Plant* 61:631–640. <https://doi.org/10.1007/s10535-017-0711-1>
- Batista DS, Castro KM, Silva AR, Teixeira ML, Sales TA, Soares LI, Cardoso MG, Santos MO, Viccini LF, Otoni WC (2016) Light quality affects in vitro growth and essential oil profile in *Lippia alba* (Verbenaceae). *In Vitro Cell Dev Biol-Plant* 52:276–282. <https://doi.org/10.1007/s11627-016-9761-x>
- Batista DS, Castro KM, Koehler AD, Porto BN, Silva AR, Souza VC, Teixeira ML, Cardoso MG, Santos MO, Viccini LF, Otoni WC (2017a) Elevated CO<sub>2</sub> improves growth, modifies anatomy, and modulates essential oil qualitative production and gene expression in *Lippia alba* (Verbenaceae). *Plant Cell Tiss Organ Cult* 128:357–368. <https://doi.org/10.1007/s11240-016-1115-1>
- Batista DS, Dias LLC, Rêgo MMD, Saldanha CW, Otoni WC (2017b) Flask sealing on in vitro seed germination and morphogenesis of two types of ornamental pepper explants. *Ciênc Rural* 47:1–6. <https://doi.org/10.1590/0103-8478cr20150245>
- Benelli G, Pavela R, Giordani C, Casettari L, Curzi G, Cappellacci L, Petrelli R, Maggi F (2018) Acute and sub-lethal toxicity of eight essential oils of commercial interest against the filariasis mosquito *Culex quinquefasciatus* and the housefly *Musca domestica*. *Ind Crops Prod* 112:668–680. <https://doi.org/10.1016/j.indcrop.2017.12.062>
- Bilger W, Bjorkman O (1990) Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. *Photosynth Res* 25:173–185. <https://doi.org/10.1007/BF00033159>
- Böhme K, Barros-Velázquez J, Calo-Mata P, Aubourg SP (2014) Antibacterial, antiviral and antifungal activity of essential oils: mechanisms and applications. *Antimicrobial compounds*. In: Villa T, Veiga-Crespo P (eds) *Antimicrobial compounds*. Springer, Berlin, pp 51–81
- Bordage S, Sullivan S, Laird J, Millar AJ, Nimmo HG (2016) Organ specificity in the plant circadian system is explained by different light inputs to the shoot and root clocks. *New Phytol* 212:136–149. <https://doi.org/10.1111/nph.14024>
- Castro EM, Pinto JEBP, Melo HCD, Soares AM, Alvarenga AA, Lima Júnior EC (2005) Aspectos anatômicos e fisiológicos de plantas de guaco submetidas a diferentes fotoperíodos. *Hortic Bras* 23:846–850. <https://doi.org/10.1590/S0102-05362005000300031>
- Carvalho IS, Cavaco T, Carvalho LM, Duque P (2010) Effect of photoperiod on flavonoid pathway activity in sweet potato (*Ipomoea batatas* (L.) Lam.) leaves. *Food Chem* 118:384–390. <https://doi.org/10.1016/j.foodchem.2009.05.005>
- Chen JW, Bai KD, Cao KF (2009) Inhibition of monoterpene biosynthesis accelerates oxidative stress and leads to enhancement of antioxidant defenses in leaves of rubber tree (*Hevea brasiliensis*). *Acta Physiol Plant* 31:95. <https://doi.org/10.1007/s11738-008-0205-z>
- Costa AC, Rosa M, Megguer CA, Silva FG, Pereira FG, Otoni WC (2014) A reliable methodology for assessing the in vitro photosynthetic competence of two Brazilian savanna species: *Hyptis marruboides* and *Hancornia speciosa*. *Plant Cell Tiss Organ Cult* 117(3):443–454. <https://doi.org/10.1007/s11240-014-0455-y>
- Cross JM, Von Korff M, Altmann T, Bartzetko L, Sulpice R, Gibon Y, Palacios N, Stitt M (2006) Variation of enzyme activities and metabolite levels in 24 *Arabidopsis* accessions growing in carbon-limited conditions. *Plant Physiol* 142:1574–1588. <https://doi.org/10.1104/pp.106.086629>
- Cruz CD (2016) Genes software-extended and integrated with the R, Matlab and Selegen. *Acta Sci Agron* 38:547–552. <https://doi.org/10.4025/actasciagron.v38i4.32629>
- Dodd AN, Salathia N, Hall A, Kévei E, Tóth R, Nagy F, Hibberd JM, Millar AJ, Webb AAR (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* 309:630–633. <https://doi.org/10.1126/science.1115581>
- Farré EM, Harmer SL, Harmon FG, Yanovsky MJ, Kay SA (2005) Overlapping and distinct roles of PRR7 and PRR9 in the *Arabidopsis* circadian clock. *Curr Biol* 15:47–54
- Fernie AR, Roscher A, Ratcliffe RG, Kruger NJ (2001) Fructose 2,6-bisphosphate activates pyrophosphate: fructose-6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. *Planta* 212:250–263. <https://doi.org/10.1007/s004250000386>
- Fréchette E, Chang CYY, Ensminger I (2016) Photoperiod and temperature constraints on the relationship between the photochemical reflectance index and the light use efficiency of photosynthesis in *Pinus strobus*. *Tree Physiol* 36:311–324. <https://doi.org/10.1093/treephys/tpv143>
- Gendron JM, Pruneda-Paz JL, Doherty CJ, Gross AM, Kang SE, Kay SA (2012) *Arabidopsis* circadian clock protein, *TOC1*, is a DNA-binding transcription factor. *Proc Natl Acad Sci* 109:3167–3172. <https://doi.org/10.1073/pnas.1200355109>
- Hädrich N, Hendriks JH, Kötting O, Arrivault S, Feil R, Zeeman SC, Gibon Y, Schulze WX, Stitt M, Lunn JE (2012) Mutagenesis of cysteine 81 prevents dimerization of the APS1 subunit of ADP-glucose pyrophosphorylase and alters diurnal starch turnover in *Arabidopsis thaliana* leaves. *Plant J* 70:231–242. <https://doi.org/10.1111/j.1365-313X.2011.04860.x>
- Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* 290:2110–2113. <https://doi.org/10.1126/science.290.5499.2110>
- Haydon MJ, Hearn TJ, Bell LJ, Hannah MA, Webb AA (2013) Metabolic regulation of circadian clocks. *Semin Cell Dev Biol* 24:414–421. <https://doi.org/10.1016/j.semcdb.2013.03.007>
- Hennebelle T, Sahpaz S, Joseph H, Bailleul F (2008) Ethnopharmacology of *Lippia alba*. *J Ethnopharmacol* 116:211–222. <https://doi.org/10.1016/j.jep.2007.11.044>
- Hsie BS, Bueno AIS, Bertolucci SKV, Carvalho AA, Cunha SHB, Martins ER, Pinto JEBP (2019) Study of the influence of wavelengths and intensities of LEDs on the growth, photosynthetic pigment, and volatile compounds production of *Lippia rotundifolia* Cham in vitro. *J Photochem Photobiol B* 198:111577. <https://doi.org/10.1016/j.jphotobiol.2019.111577>
- Huang W, Pérez-García P, Pokhilko A, Millar AJ, Antoshechkin I, Riechmann JL, Mas P (2012) Mapping the core of the *Arabidopsis* circadian clock defines the network structure of the oscillator. *Science* 336:75–79. <https://doi.org/10.1126/science.1219075>
- Ito S, Kawamura H, Niwa Y, Nakamichi N, Yamashino T, Mizuno T (2009) A genetic study of the *Arabidopsis* circadian clock with reference to the *timing of cab expression 1* (*TOC1*) gene. *Plant Cell Physiol* 50:290–303. <https://doi.org/10.1093/pcp/pcn198>
- Karnovsky MJ (1965) A formaldehyde glutaraldehyde fixative of high osmolality for use in electron microscopy. *J Cell Biol* 27:137
- Kooten O, Snel JFH (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynth Res* 25(3):147–150
- Lazzarini LES, Bertolucci SKV, Pacheco FV, Santos J, Silva ST, Carvalho AA, Pinto JEBP (2018) Quality and intensity of light affect *Lippia gracilis* Schauer plant growth and volatile compounds in vitro. *Plant Cell Tiss Organ Cult* 135:367–379. <https://doi.org/10.1007/s11240-018-1470-1>
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔCT</sup> method. *Methods* 25:402–408. <https://doi.org/10.1006/meth.2001.1262>



- Loivamäki M, Louis S, Cinege G, Zimmer I, Fischbach RJ, Schnitzler JP (2007) Circadian rhythms of isoprene biosynthesis in grey poplar leaves. *Plant Physiol* 143:540–551. <https://doi.org/10.1104/pp.106.092759>
- Lorenzi H, Matos FJA (2008) Plantas medicinais no Brasil—nativas e exóticas. Instituto Plantarum de Estudos da Flora, Nova Odessa, p 512p
- Lu SX, Knowles SM, Andronis C, Ong MS, Tobin EM (2009) Circadian clock associated1 and late elongated hypocotyl function synergistically in the circadian clock of *Arabidopsis*. *Plant Physiol* 150:834–843. <https://doi.org/10.1104/pp.108.133272>
- Mahmud KP, Holzapfel BP, Guisard Y, Smith JP, Nielsen S, Rogiers SY (2018) Circadian regulation of grapevine root and shoot growth and their modulation by photoperiod and temperature. *J Plant Physiol* 222:86–93. <https://doi.org/10.1016/j.jplph.2018.01.006>
- Maurya JP, Bhalerao RP (2017) Photoperiod and temperature-mediated control of growth cessation and dormancy in trees: a molecular perspective. *Ann Bot* 120:351–360. <https://doi.org/10.1093/aob/mcx061>
- Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. *J Exp Bot* 51:659–668. <https://doi.org/10.1093/jexbot/51.345.659>
- Mengin V, Pyl ET, Moraes TA, Sulpice R, Krohn N, Encke B, Stitt M (2017) Photosynthate partitioning to starch in *Arabidopsis thaliana* is insensitive to light intensity but sensitive to photoperiod due to a restriction on growth in the light in short photoperiods. *Plant Cell Environ* 40:2608–2627. <https://doi.org/10.1111/pce.13000>
- Morais LAS (2009) Influência dos fatores abióticos na composição química dos óleos essenciais. *Hortic Bras* 27:50–63. [http://www.abhorticultura.com.br/eventos/trabalhos/ev\\_3/P\\_4\\_Pales tra\\_Resumo\\_Lilia\\_Ap.pdf](http://www.abhorticultura.com.br/eventos/trabalhos/ev_3/P_4_Pales tra_Resumo_Lilia_Ap.pdf). Accessed 10 Jan 2019
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15:473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Nakamichi N, Kiba T, Henriques R, Mizuno T, Chua NH, Sakakibara H (2010) Pseudo-response regulators 9, 7, and 5 are transcriptional repressors in the *Arabidopsis* circadian clock. *Plant Cell* 22:594–605. <https://doi.org/10.1105/tpc.109.072892>
- Nusinow DA, Helfer A, Hamilton EE, King JJ, Imaizumi T, Schultz TF, Kay SA (2011) The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. *Nature* 475:398. <https://doi.org/10.1038/nature10182>
- O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Melbourne, Termarcarphi Pty. Ltd, <https://doi.org/10.2307/1219725>
- Otoni CG, Espitia PJ, Avena-Bustillos RJ, McHugh TH (2016) Trends in antimicrobial food packaging systems: emitting sachets and absorbent pads. *Food Res Int* 83:60–73. <https://doi.org/10.1016/j.foodres.2016.02.018>
- Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, Kim HJ, Kay SA, Nam HG (1999) Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis* *GIGANTEA* gene. *Science* 285:1579–1582. <https://doi.org/10.1126/science.285.5433.1579>
- Pascual ME, Slowing K, Carretero E, Sánchez Mata D, Villar A (2001) *Lippia*: traditional uses, chemistry and pharmacology: a review. *J Ethnopharmacol* 76:201–214. [https://doi.org/10.1016/S0378-8741\(01\)00234-3](https://doi.org/10.1016/S0378-8741(01)00234-3)
- Pavela R, Govindarajan M (2017) The essential oil from *Zanthoxylum monophyllum* a potential mosquito larvicide with low toxicity to the non-target fish *Gambusia affinis*. *J Pest Sci* 90:369–378. <https://doi.org/10.1007/s10340-016-0763-6>
- Peng Y, Li Y (2014) Combined effects of two kinds of essential oils on physical, mechanical and structural properties of chitosan films. *Food Hydrocoll* 36:287–293. <https://doi.org/10.1016/j.foodhyd.2013.10.013>
- Pola CC, Medeiros EA, Pereira OL, Souza VG, Otoni CG, Camilloto GP, Soares NF (2016) Cellulose acetate active films incorporated with oregano (*Origanum vulgare*) essential oil and organophilic montmorillonite clay control the growth of phytopathogenic fungi. *Food Pack Shelf Life* 9:69–78. <https://doi.org/10.1016/j.fpsl.2016.07.001>
- Proestos C, Lytoudi K, Mavromelanidou OK, Zoumpoulakis P, Sinanoglou VJ (2014) Antioxidant capacity of selected plant extracts and their essential oils. *Antioxidants* 2:11–22. <https://doi.org/10.3390/antiox2010011>
- Ragagnin RCG, Albuquerque CC, Oliveira FFM, Santos RG, Gurgel EP, Diniz JC, Viana FA (2014) Effect of salt stress on the growth of *Lippia gracilis* Schauer and on the quality of its essential oil. *Acta Bot Brasilica* 28:346–351. <https://doi.org/10.1590/0102-33062014abb3369>
- Raut JS, Karuppaiyl SM (2014) A status review on the medicinal properties of essential oils. *Ind Crops Prod* 62:250–264. <https://doi.org/10.1016/j.indcrop.2014.05.055>
- Reis AC, Sousa SM, Vale AA, Pierre PMO, Franco AL, Campos JMS, Vieira RF, Viccini LF (2014) *Lippia alba* (Mill.) NE Br. (Verbenaceae): a new tropical autopolyploid complex? *Am J Bot* 101:1002–1012. <https://doi.org/10.3732/ajb.1400149>
- Saljoughian S, Shahin R, Alaa El-Din AB, Ralf G, Alireza O, Nooshin N, Amin MK (2018) The effects of food essential oils on cardiovascular diseases: a review. *Crit Rev Food Sci Nutr* 58:1688–1705. <https://doi.org/10.1080/10408398.2017.1279121>
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671–675. <https://doi.org/10.1038/nmeth.2089>
- Serrano-Bueno G, Romero-Campero FJ, Lucas-Reina E, Romero JM, Valverde F (2017) Evolution of photoperiod sensing in plants and algae. *Curr Opin Plant Biol* 37:10–17. <https://doi.org/10.1016/j.pbi.2017.03.007>
- Shibaeva TG, Markovskaya EF (2013) Growth and development of cucumber *Cucumis sativus* L. in the prereproductive period under long photoperiods. *Russ J Dev Biol* 44:278–285. <https://doi.org/10.1134/S1062360413020082>
- Shin J, Sánchez-Villarreal A, Davis AM, Du SX, Berendzen KW, Koncz C, Ding Z, Li C, Davis SJ (2017) The metabolic sensor AKIN10 modulates the *Arabidopsis* circadian clock in a light-dependent manner. *Plant Cell Environ* 40:997–1008. <https://doi.org/10.1111/pce.12903>
- Song YH, Kubota A, Kwon MS, Covington MF, Lee N, Taagen ER, Laboy Cintrón D, Hwang DY, Akiyama R, Hodge SK, Huang H, Nguyen NH, Nusinow DA, Millar AJ, Shimizu KK, Imaizumi T (2018) Molecular basis of flowering under natural long-day conditions in *Arabidopsis*. *Nat Plants* 4:824–835. <https://doi.org/10.1038/s41477-018-0253-3>
- Szczepanski S, Lipski A (2014) Essential oils show specific inhibiting effects on bacterial biofilm formation. *Food Control* 36:224–229. <https://doi.org/10.1016/j.foodcont.2013.08.023>
- Triozzi PM, Ramos-Sánchez JM, Hernández-Verdeja T, Moreno-Cortés A, Allona I, Perales M (2018) Photoperiodic regulation of shoot apical growth in poplar. *Front Plant Sci* 9:1030. <https://doi.org/10.3389/fpls.2018.01030>
- Viccini LF, Pierre PMO, Praça MM, Souza-Costa DC, Costa Romanel E, Sousa SM, Peixoto PHP, Salimena FRG (2006) Chromosome numbers in the genus *Lippia* (Verbenaceae). *Plant System Evol* 256:171–178. <https://doi.org/10.1007/s00606-005-0351-3>
- Viccini LF, Silveira RS, Vale AA, Campos JMS, Reis AC, Santos MO, Campos VR, Carpanez AG, Grazul RM (2014) Citral and linalool content has been correlated to DNA content in *Lippia alba* (Mill.) NE Brown (Verbenaceae). *Ind Crops Prod* 59:14–19. <https://doi.org/10.1016/j.indcrop.2014.04.028>

- Vogg G, Heim R, Hansen J, Schäfer C, Beck E (1998) Frost hardening and photosynthetic performance of Scots pine (*Pinus sylvestris* L.) needles. I. Seasonal changes in the photosynthetic apparatus and its function. *Planta* 204:193–200. <https://doi.org/10.1007/s004250050246>
- Wagas KK, ErumD Tanveer A, HammadI Bushra M (2016) Evaluation of *Ajuga bracteosa* for antioxidant, anti-inflammatory, analgesic, antidepressant and anticoagulant activities. *BMC Complement Altern Med* 16:375. <https://doi.org/10.1186/s12906-016-1363-y>
- Wang L, Han F, Zheng HQ (2018) Photoperiod-controlling guttation and growth of rice seedlings under microgravity on board Chinese spacelab TG-2. *Microgravity Sci Technol* 30:839–847. <https://doi.org/10.1007/s12217-018-9644-3>
- Wei Y, Qian-liang M, Bing L, Khalid R, Cheng-Jian Z, Ting H, Lu-ping Q (2016) Medicinal plant cell suspension cultures: pharmaceutical applications and high-yielding strategies for the desired secondary metabolites. *Crit Rev Biotechnol* 36:215–232. <https://doi.org/10.3109/07388551.2014.923986>
- Welburn AR (1994) The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *J Plant Physiol* 144:307–313. [https://doi.org/10.1016/S0176-1617\(11\)81192-2](https://doi.org/10.1016/S0176-1617(11)81192-2)
- Zuo Z, Wang B, Ying B, Zhou L, Zhang R (2017) Monoterpene emissions contribute to thermotolerance in *Cinnamomum camphora*. *Trees* 31:1759–1771. <https://doi.org/10.1007/s00468-017-1582-y>

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.