South African Journal of Botany 151 (2022) 800–807

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Effects of sucrose concentration and membrane filter number on the growth and volatile compounds of micropropagated *Lippia rotundifolia* Cham. Plantlets

Bety Shiue de Hsie[a], Ana Izabela Sales Bueno[a], Alexandre Alves de Carvalho[a], Melvis Celeste Vilanculos Cossa[a], Rafael Marlon Alves de Assis[a], Priscila Pereira Botrel[b], Suzan Kelly Vilela Bertolucci[c], José Eduardo Brasil Pereira Pinto[a,*]

[a] *Laboratory of Plant Tissue Culture and Medicinal Plants, Department of Agriculture, Federal University of Lavras, Lavras 37200-900, Brazil*
[b] *Laboratory of Biotechnology and Plant Tissue Culture, Department of Agriculture, Federal Institute of Education, Science and Technology of Southern Minas Gerais, Muzambinho 37890-000, Brazil*
[c] *Laboratory of Phytochemistry and Medicinal Plants, Department of Agriculture, Federal University of Lavras, Lavras 37200-900, Brazil*

ARTICLE INFO

Article History:
Received 15 September 2022
Revised 29 October 2022
Accepted 6 November 2022
Available online xxx

Edited by: Dr P. Bhattacharyya

Keywords:
"Chá-de-pedestre"
Porous membranes
Sugar
Leaf area
Volatile compounds

ABSTRACT

Lippia rotundifolia Cham. is a medicinal species, native to Brazilian mountainous regions, with leaves rich in monoterpenes. The objective of this study was to evaluate the effect of different concentrations of sucrose in MS medium with a natural ventilation system using porous membranes on the growth and contents of volatile compounds in *L. rotundifolia* plantlets. The experimental design was completely randomized, with 12 treatments, consisting of a factorial system of four ventilation systems and three sucrose concentrations. The treatments were a system with no porous membrane (NMS) and three natural ventilation systems (AMS) containing one, two, and four porous membranes. The sucrose concentrations were 0, 15, and 30 g L^{-1}. The natural ventilation systems were superior to NMS. The natural ventilation system with two and four porous membranes and 15 g L^{-1} sucrose in MS medium led to higher *in vitro* growth rates in *L. rotundifolia*. AMS using manufactured porous membranes was found to be a more efficient *in vitro* cultivation method than NMS. The ventilation system with four porous membranes and a sucrose concentration of 15 g L^{-1} was the most efficient, since an increase in the photosynthetic rate and less evaporative water loss were observed in this ventilation system. Volatile organic content was influenced by ventilation systems and sucrose concentrations. Major compounds myrcene, limonene, myrcenone and ocimenone were identified. AMS4 and presence of sucrose (15 and 30 g L^{-1}) increased the myrcene and ocimenone content. The myrcenone content was favored by NMS without sucrose. Overall, the alternative membrane system with 15 g L^{-1} of sacarose can be recommended in the *in vitro* culture method of *L. rotundifolia*.

1. Introduction

Species of the genus *Lippia* have economic importance due to the wide use of their essential oils and to their medicinal properties (Pascual et al., 2001). The most characteristic medicinal properties of *Lippia* species are mainly antimicrobial, antifungal, repellent, and larvicidal (Oliveira et al., 2006; Santos et al., 2004; Bassole et al., 2003; Pascual et al., 2001). *Lippia rotundifolia* Cham. (Verbenaceae) is a species endemic to the Brazilian Cerrado, found in the Espinhaço mountain chain in the state of Minas Gerais, and is characterized by the presence of monoterpene-rich glandular trichomes in the leaves. The volatile compounds found in *L. rotundifolia* include limonene, myrcene, and myrcenal (Resende et al., 2015; Leitão et al., 2008). Agronomic studies on *L. rotundifolia* report a low seed germination rate, reaching a maximum of 40% when subjected to gibberellin (GA_3) concentrations. With conventional vegetative propagation methods, its rooting and plantlet survival percentages are low. Based on these agronomic data and a high risk of extinction due to extraction, further studies on *L. rotundifolia* are important (Salimena and Silva, 2009; Pimenta et al., 2007).

Micropropagation of medicinal plants has become widespread due to the possibility of producing a large number of homogeneous plants with high sanitary quality and the possibility of conserving the germplasm, ensuring the maintenance of biodiversity and assisting in genetic improvement (Morais et al., 2012; Rout et al., 2000). However, a widespread use of micropropagation is still limited mainly by the significant loss of plantlets *in vitro* due to microbial

* Corresponding author.
E-mail address: jeduardo@ufla.br (J.E.B.P. Pinto).

https://doi.org/10.1016/j.sajb.2022.11.007
0254-6299/

contamination, physiological and morphological disturbances, poor rooting, low transpiration rates, and low survival percentage in the *ex vitro* acclimatization phase (Núñez-Ramos et al., 2021; Hazarika, 2006; Makunga et al., 2006; Majada et al., 2002). Some of these problems are caused by the type of seal used in the culture vials. Plantlets developed by *in vitro* cultivation usually require a seal in the cultivation container to prevent contamination and dehydration of explants and media. However, the type of seal or cap may limit gas exchange between the *in vitro* and *ex vitro* environments, leading to morpho-physiological disorders that cause high plant mortality during acclimatization (Alvarez et al., 2012; Nguyen and Kozai, 2001).

In these systems, low transpiration and photosynthesis rates and restricted water and nutrient absorption are observed, which reduces the explant growth rate (Saldanha et al., 2012). In addition, the high relative humidity inside the cultivation vessel reduces the deposition of epicuticular waxes and the development of functional stomata, which can lead to losses during acclimatization (Chandra et al., 2010). To improve the ventilation of *in vitro* culture vials, different caps with membranes that modify the vial microenvironment can be used, resulting in greater gas exchange, reduced relative humidity, reduced ethylene concentration, increased plant transpiration, and increased water and nutrient absorption (Xiao et al., 2011; Kozai, 2010; Kozai and Kubota, 2001). Several different membrane types are commercially available, which promote gas exchange in *in vitro* test tubes; however, they are associated with a high maintenance cost. Saldanha et al. (2012) developed handcrafted membranes combining microporous tape and polytetrafluoroethylene (PTFE) as inexpensive alternative membranes that can be used to promote gas exchange and successful *in vitro* propagation. The objective of this study was to evaluate the effect of different concentrations of sucrose in MS medium with an alternative membrane system on the growth and contents of volatile compounds in *L. rotundifolia* plantlets.

2. Materials and methods

2.1. Establishment of explants

Voucher specimens were deposited in the PAMG Herbarium of the Agricultural Research Corporation of Minas Gerais (EPAMIG) under record number 58,027. Axillary buds were collected from plants approximately 3 months old, placed under running water for 30 min, and immersed under stirring in a bleach solution (1% active sodium hypochlorite) for 20 min. In a sterile laminar flow hood, the explants (± 1 cm) were washed four times in distilled and autoclaved water and inoculated in test tubes (25 × 150 mm) containing 15 mL of medium, closed with a plastic cap.

The culture medium used for establishment was Murashige & Skoog (MS) medium (Murashige and Skoog, 1962), free of growth regulators, supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar (Sigma-Aldrich®), pH 5.6 ± 0.1, and autoclaved (for 15 min at 121°C). After inoculation, the tubes were kept in a growth room under cold-white fluorescent lamps with a light intensity of 39 μmol m^{-2} s^{-1}, a 16-h photoperiod, and a temperature of 25 ± 2°C.

2.2. Growth analysis

Nodal segments (± 1 cm), with one pair of leaves, from plantlets pre-established *in vitro* were cultivated in semisolid MS medium without the addition of growth regulators. The explants were inoculated into 250-mL glass vials containing 50 mL of MS medium and kept in a growth room. The experimental design was completely randomized, with 12 treatments, consisting of a factorial system of four ventilation systems and three sucrose concentrations in the MS medium. The treatments were a nonporous membrane system (NMS) and three alternative membrane systems (AMS) containing one, two, and four porous membranes. The sucrose concentrations used were 0, 15, and 30 g L^{-1}. Five replicates were used, each replicate consisting of four plantlets, totaling 20 plantlets analyzed per treatment. The membranes were prepared according to Saldanha et al. (2012). In short, the holes of flask caps (10 mm diameter) were covered with membrane filters developed using one layer of polytetrafluoroethylene film (Amanco®) and three layers of microporous tape (Cremer®). The growth data were collected after 45 days. Shoot height (SH), stem dry weight (SDW), leaf dry weight (LDW), root dry weight (RDW), and total dry weight (TDW) were evaluated. The dry weight parameters were determined by drying the plant material in a forced-air oven at 36 ± 2°C for approximately 48 hours to constant weight. To determine the leaf area, nine plantlets from each treatment were measured with WinFOLIA™ software (Regent Instruments, Inc.). From each of the plantlets, all leaves were removed and placed on an Epson Perfection V700 photo scanner. The variables analyzed were calculated according to Benicasa (2004), and the total leaf area (TLA), leaf area ratio (LAR = TLA/TDW), specific leaf area (SLA = TLA/LDW), specific leaf weight (SLW = LDW/TLA), and leaf weight ratio (LWR = LDW/TDW) of the plantlets were evaluated.

2.3. Quantification of photosynthetic pigments

To measure photosynthetic pigments, fresh, fully expanded leaves were taken starting on the third node 45 days after inoculation. The extraction was performed as described by Arnon (1949), using 0.10 g of fresh matter homogenized in 80% acetone, followed by reading in a spectrophotometer at wavelengths 470, 646.8, and 663.2 nm for chlorophyll *a* and *b* and carotenoids, respectively. The quantification of these photosynthetic pigments and of the chlorophyll *a:b* ratio, total chlorophyll, and carotenoids were performed according to the method of Lichtenthaler and Buschmann (2001).

2.4. Analysis of volatile compounds by headspace gas chromatography/mass spectrometry (GC/MS)

For the analysis of volatile compounds, dehydrated leaves of *L. rotundifolia* from each treatment were used. Individual 100-mg-leaf-dry-weight samples in triplicate were added to 20-mL-headspace vials sealed with silicone/PTFE caps until analysis.

The static headspace technique was used for the extraction of the volatile fraction of *L. rotundifolia*. For this purpose, the automatic headspace extractor/sampler Combi PAL Autosampler System (CTC Analytics AG, Switzerland) coupled to the GC/MS system was used. The extraction parameters applied were sample incubation temperature 110°C, incubation time 30 min, syringe temperature 120°C, and 500 μL of the vapor phase injected automatically. The volatile fraction was analyzed in an Agilent® 7890A gas chromatography system coupled to an Agilent® MSD 5975C mass selective detector (Agilent Technologies, California, USA) operated by electron impact ionization at 70 eV in scanning mode, with a mass acquisition range of 40-400 m/z at a speed of 1.0 scan/s. An HP-5 MS fused-silica capillary column (30 m length × 0.25 mm internal diameter × 0.25 μm film thickness) (California, USA) was used. Helium gas was used as the carrier gas at a flow rate of 1.0 mL/min. The injector and mass spectrometer transfer line temperatures were kept at 230°C and 240°C, respectively. The initial oven temperature was 60°C, followed by a temperature ramp of 3°C/min up to 230°C, followed by a ramp of 10°C/min up to 250°C, and holding for 1 minute. The injection was performed in split mode at an injection ratio of 1:20. The concentrations of the constituents present in the volatile fraction were expressed as the percentage normalized area of the chromatographic peaks.

The volatile fraction constituents were identified by comparing their linear retention indices relative to the coinjection of a standard solution of *n*-alkanes (C_9-C_{18}, Sigma-Aldrich®, St. Louis, USA) and by comparing the mass spectra from the NIST library and from Adams (2017). The retention index was calculated using the equation

proposed by van Den Dool and Kratz (1963), and for the attributions, the retention indices of Adams (2017) were consulted.

2.5. Statistical analysis

The data were subjected to analysis of variance, and the means were compared using the Duncan test at 5% probability. These analyses were performed using Sisvar® software (Ferreira, 2011). Principal component analysis (PCA) was used to study the influence of different concentrations of sucrose and alternative membrane systems on the volatile constituents of *L. rotundifolia*. The PCA was performed in Statistica® version 13.4 (StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Dry weight of different plantlet parts

The number of porous membranes and the sucrose concentrations in the medium significantly affected the growth and development of *L. rotundifolia in vitro* (Table 1; Fig. 1). The gain in biometric properties (SDW, LDW, RDW, TDW) with the membrane system (AMS4) supplemented with 15 g of sucrose was higher than that with the other treatments; this group had a TDW of 72.42 mg. The explant growth in the medium supplemented with 30 g of sucrose and with four porous membranes (AMS4) was lower than in the AMS4 treatment with 15 g of sucrose in the medium. In this case, sucrose supplementation inhibited dry weight accumulation in the plantlets. The explants grown in the medium without sucrose with AMS2 or AMS4 showed TDW accumulations of 37.33 and 48.51 mg per plantlet, respectively (Table 1). The TDW of the explants grown in the NMS treatment without sucrose was 16.82 mg per plantlet, and the TDW in the AMS4 treatment without sucrose was 48.51 mg per plantlet, a 2.88-fold higher dry weight gain. For cultivation without sucrose, little root development was observed in the treatments. However, when using AMS2 and AMS4, there was root system development (Fig. 1). Thus, the alternative membrane system without sucrose stimulated the growth of *L. rotundifolia in vitro*. Lazzarini et al. (2019), working with *Lippia gracilis*, also observed better growth parameters with AMS4. According to Saldanha et al. (2012), *Pfaffia glomerata* plantlets grown in vials with different AMSs have shown higher shoots and root dry weight accumulation, indicating the importance of gas exchange to *in vitro* morphogenesis. Similar results were also found in *Acacia mangium* plantlets grown under CO_2-enriched growth conditions, which showed a four-fold-higher performance than plantlets micropropagated under conventional conditions (Kozai and Kubota, 2001). Choi et al. (2004) also reported that *Platycodon grandiflorum* plantlets grown with sucrose (4.5%) and with filter membranes had higher dry weight and chlorophyll content than those grown without using filter membranes.

After 45 days of cultivation, the volume of culture medium in the vials decreased considerably with AMS2 and AMS4 (Fig. 1). This is because AMS1 had an area of 0.79 cm^2, AMS2 had 1.58 cm^2, and AMS4 had 3.16 cm^2. The use of porous membranes allows greater evapotranspiration, thus causing water loss in the culture medium. A natural ventilation system reduces the relative humidity and ethylene inside the vial, thus promoting greater transpiration and mineral absorption by plants *in vitro*, leading to greater growth (Erig and Schuch, 2005; Kozai and Kubota, 2001).

When analyzing only the influence of sucrose on the *in vitro* cultivation of *L. rotundifolia*, the treatments with 15 and 30 g L^{-1} sucrose had higher SH, SDW, LDW, RDW, and TDW values (Table 1). The SDW in the treatments with 15 and 30 g L^{-1} sucrose (11.85 and 11.13 mg $plant^{-1}$) were almost double those obtained with 0 g L^{-1} sucrose (6.74 mg $plant^{-1}$). The RDW in the treatment with 15 g L^{-1} sucrose was 7.87 mg $plant^{-1}$, which was more than double that in the 0 g L^{-1} treatment (3.58 mg $plant^{-1}$), while that in the 30 g L^{-1} treatment was almost triple that in the 0 g L^{-1} treatment (10.02 mg $plant^{-1}$). The TDW in the treatments with 15 and 30 g L^{-1} sucrose (51.67 and 54.28 mg $plant^{-1}$, respectively) were higher than 164 to 172%, respectively, compared to the treatment with 0 g L^{-1} sucrose

Table 1
Effect of the alternative membrane system (AMS) and sucrose concentration on the growth and development of *Lippia rotundifolia* cultured *in vitro* for 45 days.

Cultivation system	Sucrose (g L^{-1})	SH (cm)	SDW (mg)	LDW (mg)	RDW (mg)	TDW (mg)	Chlorophyll (mg g^{-1} FW)			Chl *a:b*	Carotenoids (mg g^{-1} FW)
							a	*b*	Total		
NMS[b]	0	4.40 f[a]	4.37 g	10.90 h	2.00 e	16.82 g	0.62 f	0.21 f	0.80 h	2.97 e	0.15 f
	15	6.81 ab	8.69 ef	20.51 fg	4.59 d	33.79 f	0.94 d	0.31 c	1.13 e-g	3.10 bc	0.22 c
	30	5.36 cd	9.61 de	24.64 ef	6.13 d	40.38 fe	0.87 d	0.29 c	1.20 d-f	3.21 a	0.20 d
AMS1	0	4.14 f	5.02 g	16.05 g	2.26 e	23.33 g	0.45 g	0.15 g	0.68 h	2.94 e	0.12 g
	15	5.93 b-d	9.80 de	27.26 e	5.89 d	42.95 de	0.96 d	0.31 c	1.26 de	3.08 bc	0.23 c
	30	6.10 b-d	11.54 cd	29.56 de	9.64 cb	50.74 c	1.09 c	0.35 b	1.44 c	3.09 bc	0.23 c
AMS2	0	5.22 de	7.65 f	25.40 ef	4.84 d	37.33 fe	0.85 de	0.26 de	1.06 fg	2.99 de	0.19 e
	15	7.54 a	13.72 ab	34.59 cd	9.24 c	57.54 b	1.42 b	0.52 a	1.99 a	3.08 bc	0.23 c
	30	5.66 cd	13.01 bc	38.52 bc	10.59 cb	62.11 b	1.46 ab	0.49 a	1.69 a	3.10 b	0.27 b
AMS4	0	6.29 bc	9.91 de	33.38 cd	5.22 d	48.51 cd	0.75 e	0.25 e	1.01 g	3.01 c-e	0.14 f
	15	6.76 ab	15.21 a	45.43 a	11.79 ab	72.42 a	1.56 a	0.51 a	2.08 a	3.06 b-d	0.32 a
	30	4.04 f	10.35 de	39.84 b	13.73 a	63.91 b	1.41 b	0.36 b	1.33 cd	2.97 e	0.24 c
Means for cultivation systems											
NMS		5.52 b	7.56 c	18.68 d	4.24 d	30.33d	0.81 b	0.27 c	1.04 d	3.09 a	0.19 b
AMS1		5.39 b	8.79 b	24.29 c	5.93 c	39.01 c	0.83 b	0.27 c	1.13 c	3.04 b	0.20 b
AMS2		6.14 a	11.46 a	32.83 b	8.22 b	52.33 b	1.24 a	0.42 a	1.58 a	3.06 ab	0.23 a
AMS4		5.70 ab	11.82 a	39.55 a	10.24 a	61.61 a	1.23 a	0.37 b	1.45 b	3.01 b	0.23 a
Means for sucrose concentrations											
0		5.01 b	6.74 b	21.43 b	3.58 c	31.50 b	0.67 b	0.22 c	0.89 c	2.98 b	0.15 c
15		6.76 a	11.85 a	31.95 a	7.87 b	51.67 a	1.21 a	0.41 a	1.60 a	3.08 a	0.25 a
30		5.29 b	11.13 a	33.14 a	10.02 a	54.28 a	1.21 a	0.37 b	1.41 b	3.09 a	0.23 b
Source of variation											
Cultivation system × Sucrose		***	***	ns	ns	*	***	***	***	***	***
Cultivation system		*	***	***	***	***	***	***	***	*	***
Sucrose		***	***	***	***	***	***	***	***	***	***

Significant effect: $^{*}p \leq 0.05$ $^{**}p \leq 0.01$ $^{***}p \leq 0.001$; *ns* not significant.
[a] Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.
[b] NMS: no-membrane system; AMS1, -2, -4: alternative membrane system with 1, 2, and 4 filters; SH: shoot height; SDW, LDW, RDW, TDW: shoot, leaf, root, and total dry weight.

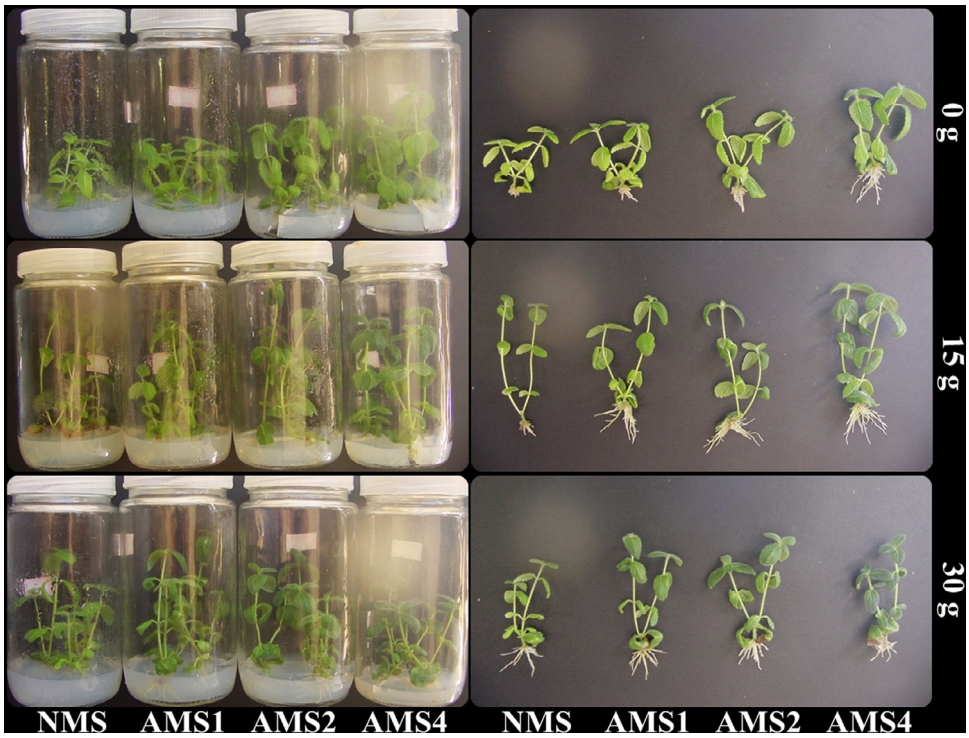

Fig. 1. *Lippia rotundifolia* plantlets grown under different alternative membrane systems, supplemented or not with sucrose, after 45 days.

(31.50 mg plant^{-1}). The use of sucrose in the culture medium as a source of carbon is typical for plant micropropagation, especially in the establishment and proliferation stages of the primary culture under heterotrophic and photomixotropic conditions (Cha-um et al., 2011; Cui et al., 2010; Pawlicki and Welander, 1995).

Greater root system development in medium supplemented with sucrose (20–45 g L^{-1}) has also been found in *Simmondsia chinensis* (Link) Schneider (Mills et al., 2009) and apple rootstocks (Yaseen et al., 2009). Nicoloso et al. (2003) compared carbon sources and observed that sucrose at a concentration of 30 g L^{-1} was the best source of carbohydrates for SH, number of shoots, and total number of nodal segments per plant in *P. glomerata*. In *Mellissa officinalis* L., higher mean shoot length has been obtained when grown in medium containing 30 g L^{-1} sucrose (Ribeiro et al., 2007). Núñez-Ramos et al. (2021) also reported that *Caesalpinia spinosa* (tara) plantlets grown in vials with porous membranes produced higher dry weight, higher concentrations of photosynthetic pigments, and more developed leaves.

3.2. Analysis of plantlet growth in vitro

Growth analysis was performed to evaluate the effect of growth environment changes with the use of natural ventilation in the caps. The analysis of growth parameters allows us to infer the contribution of different physiological plant processes (Benicasa, 2004). The TLA was significantly affected by the number of porous membranes and the concentration of sucrose used in the culture vials. Cultivation with AMS4 without sucrose supplementation (22.50 cm^2) and with 15 g L^{-1} (20.37 cm^2) resulted in larger TLAs (Table 2). This increase in TLA may have been caused by greater CO_2 exchange, leading to a greater photosynthetic capacity, since CO_2 is the main substrate for the synthesis of photoassimilates used in plant growth. According to Souza et al. (2014), the larger the leaf area is, the higher the photosynthetic rate of the plants is. However, supplementation with 30 g L^{-1} sucrose and the use of AMS4 inhibited TLA (Table 2). The activity of the carbon-fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the leaves of certain species grown *in vitro* is significantly impaired by the addition of exogenous sucrose to the culture medium (Grout, 1988), and according to Langford and Wainwright (1987), CO_2 uptake can be increased by gradually reducing the sucrose concentration in successive subcultures.

The membrane systems and sucrose concentrations in the medium affected the SLW (=LDW/TLA) (Table 2). The use of AMS4 increased the SLM. Considering weight as an anatomical component, as it is related to the internal thickness (number and/or size of the cells of the leaf mesophyll) and the surface as a morphological component, the leaf thickness can be determined. Thus, SLW is a parameter for estimating leaf thickness, a higher SLW value indicating less biomass export to other parts of the plant (Silva et al., 2007). Based on this, there was greater leaf thickening in the AMS4 system, which is a mechanism to minimize water loss by tissues and improve plantlet acclimatization.

LAR expresses the useful area of the plant for photosynthesis and is a morpho-physiological component. It is the ratio between the TLA responsible for the capture of light energy and CO_2 and the TDW of the plant (Benicasa, 2004). In this study, the light energy was the same in all treatments, and the number of membranes was changed (1, 2 and 4 filters per flask cap) to allow the gas exchange within the culture vial. In the NMS and AMS1 treatments, in which gas exchange was lower, LAR was higher than in the AMS2 and AMS4 treatments (Table 2). These findings suggest that a vial with no membrane (NMS) or with one membrane (AMS1) needs a larger leaf area to produce dry mass. This is because the higher the concentration of CO_2 available, the higher the photosynthetic rate and the higher the biomass production will be. Iarema et al. (2012) observed higher gas exchange per hour in vials sealed with a higher number of porous membranes. The use of these membranes allows greater ventilation in the culture vials and adequate concentration of CO_2, thus increasing photosynthesis and growth (Kitaya et al., 2005).

SLA is the ratio between leaf area and leaf dry weight. According to Benicasa et al. (2004), it is an important factor from a physiological standpoint because it describes the allocation of leaf biomass per unit area. NMS had the highest SLA (0.72 cm^{-2} mg^{-1}) (Table 2), showing that the vial in an NMS system needs a larger leaf area to produce dry

Table 2
Effect of the alternative membrane system (AMS) and sucrose concentration on growth parameters of *Lippia rotundifolia* cultured *in vitro* for 45 days.

Cultivation system	Sucrose (g L^{-1})	TLA (cm^2)	SLW (mg cm^{-2})	LAR (cm^2 mg^{-1})	SLA (cm^2 mg^{-1})	LWR
NMS[b]	0	8.59 d	1.24 e	0.53ab	0.83 a	0.65bc
	15	16.13bc	1.31 e	0.47 b	0.77ab	0.61cd
	30	13.28 c	1.92cd	0.33cd	0.54cd	0.61cd
AMS1	0	14.45bc	1.25 e	0.56 a	0.81 a	0.69 a
	15	15.79bc	1.75 d	0.37 c	0.58 c	0.63cd
	30	15.64bc	2.07bc	0.29 d	0.49cd	0.59 d
AMS2	0	17.37 b	1.45 e	0.47 b	0.70 b	0.67ab
	15	15.37bc	2.29 b	0.26 de	0.44 d	0.60 d
	30	17.20 b	2.24 b	0.28 d	0.46 d	0.62cd
AMS4	0	22.50 a	1.44 e	0.49 b	0.71 b	0.68 a
	15	20.37 a	2.21 b	0.28 d	0.46 d	0.62cd
	30	13.34 c	3.12 a	0.21 e	0.33 e	0.65bc
Means for cultivation systems						
NMS	12.66 c	1.49 d	0.45a	0.72 a	0.62 b	
AMS1	15.29 b	1.69 c	0.40 b	0.62 b	0.64 ab	
AMS2	16.64 b	1.99 b	0.34 c	0.54 c	0.63 ab	
AMS4	18.74 a	2.26 a	0.33 c	0.50 c	0.65 ab	
Means for sucrose concentrations						
0	15.60ab	1.35 c	0.51 a	0.76 a	0.67 a	
15	16.87 a	1.90 b	0.35 b	0.56 b	0.61 b	
30	14.86 b	2.34 a	0.28 c	0.45 c	0.62 b	
Source of variation						
Cultivation system × Sucrose	***	***	**	**	ns	
Cultivation system	***	***	***	***	*	
Sucrose	**	***	***	***	***	

Significant effect: *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$; *ns* not significant.
[a]Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.
[b] NMS: no-membrane system; AMS1, -2, -4: alternative membrane system with 1, 2, and 4 filters; TLA: total leaf area; SLW: specific leaf weight; LAR: leaf area ratio; SLA: specific leaf area; LWR: leaf weight ratio

mass. The higher SLA values show that the plants were affected by the smaller number of porous membranes in the cap. At the same time, the export of material from the leaves was also hampered under low gas exchange (NMS). Analyzing LAR and SLA indicated that plantlets grown in an NMS culture vial behaved similarly to plants grown *in vivo* under low light intensity. Under low-irradiance conditions, plants invest a relatively higher proportion of photoassimilates and other resources in increasing leaf area (did not occur), presenting higher SLA (occurred), and leaves with lower mass density (occurred). Generally, these changes aim to increase the capture of incident light, increasing the photosynthetic efficiency of the plant.

LWR is an important parameter when studying plant performance, as it is a physiological parameter that expresses the fraction of dry mass not exported from the leaves to the rest of the plant (Benicasa, 2004). The sucrose concentration influenced the LWR: The 0 g L^{-1} sucrose concentration showed higher LWR values (Table 2). The increased LWR in the treatment that did not receive sucrose (0.67) indicated that less material was transferred from the leaves to other plant parts; the same occurred with the vial with the highest number of membranes, which had an LWR value of 0.65.

3.3. Pigment analysis

The chlorophyll content was affected by the number of porous membranes and the concentration of sucrose in the culture medium. When using four membranes (AMS4) and medium supplemented with 15 g L^{-1} sucrose, higher concentrations of chlorophyll *a* and *b* and total chlorophyll were observed (1.56, 0.51, and 2.08 mg g^{-1} FW, respectively). However, when using two membranes (AMS2), the highest chlorophyll *a*, chlorophyll *b*, and total chlorophyll (1.46, 0.49, and 1.69 mg g^{-1} FW, respectively) were observed in the medium supplemented with 30 g L^{-1} sucrose (Table 1). Hassankhah et al. (2014) also reported that 15 g L^{-1} sucrose and the use of caps with ventilation was the best condition for chlorophyll content in *Juglans regia* (Persian walnut). All chlorophyll *a:b* ratio values were very close to 3.0, which according to Lichtenthaler and Buschmann (2001) is considered a normal value for C3 plants. In general, when photosynthetic pigments were evaluated, natural ventilation with two and four porous membranes led to higher values. These values corroborate the higher leaf dry biomass of the plantlets, demonstrating that the natural ventilation system efficiently increased gas exchange in *L. rotundifolia* plantlets grown *in vitro*.

The chlorophyll content of plants plays an important role in light absorption during photosynthesis (Zhang et al., 2009). Similar results were also found in tomatoes, where naturally ventilated vials with sucrose concentrations of 10 and 20 g L^{-1} had higher chlorophyll *b* (Mohamed and Alsadon, 2010). It can be inferred that a higher available CO_2 concentration in the vial and sucrose supplementation lead to an increase in gas exchange. Increased gas exchange may cause an increase in the biosynthesis of pigments in the leaves of plants grown *in vitro* (Saldanha et al., 2012; Ivanova and Van Staden, 2010; Mohamed and Alsadon, 2010).

The highest carotenoid content was observed in the AMS4 treatment with 15 g L^{-1} sucrose (0.32 mg g^{-1} FW). When analyzing sucrose only, this concentration also led to a higher carotenoid content (0.25 mg g^{-1} FW). The highest carotenoid contents were observed in the AMS2 and AMS4 systems, the treatments that also yielded the highest chlorophyll concentrations (Table 2).

3.4. Chemical analysis of volatile compounds

Chemical analysis by headspace GC/MS detected different contents of volatile compounds in the different treatments. In the analysis of volatile organic compounds from the leaves of *L. rotundifolia*, 10 chemical compounds with total contents above 97.56% were identified (Table 3). Five major compounds were identified, ranging from 91 to 95.88% of the total chemical composition. These compounds were myrcene, limonene, myrcenone, ocimenone, and pentadecane.

The different combinations of natural ventilation systems and sucrose concentrations yielded different levels of the major chemical constituents of the volatile fraction of *L. rotundifolia*. The highest myrcene content (18.43%) was detected in AMS4 with 30 g L^{-1} sucrose, of

Table 3
Contents (%) of the constituents of *Lippia rotundifolia* grown under different sucrose concentrations in medium with no membrane or an alternative membrane system.

		Without sucrose				15 g sucrose				30 g sucrose			
RI[a]	Compound	NMS	AMS1	AMS2	AMS4	NMS	AMS1	AMS2	AMS4	NMS	AMS1	AMS2	AMS4
990	Myrcene	14.16	16.22	14.85	16.07	14.89	15.30	15.14	16.64	17.23	14.68	17.05	18.43
1018	α-Terpinene	1.01	1.71	1.04	1.07	1.02	1.08	1.15	1.04	1.17	1.11	1.15	1.04
1025	Limonene	8.73	11.70	11.67	12.19	11.43	11.25	10.12	10.82	13.25	9.16	11.06	10.02
1100	Linalool	-	1.09	0.97	1.01	-	0.84	0.85	-	0.83	0.75	-	-
1145	Myrcenol	0.63	0.98	0.81	1.23	-	0.66	0.81	1.20	0.50	0.64	1.02	1.73
1148	Myrcenone	56.43	42.84	49.53	45.21	52.34	52.05	53.14	46.51	50.96	56.24	48.38	47.14
1230	Ocimenone	9.63	13.86	11.75	13.69	11.62	13.03	11.75	15.30	9.81	10.08	13.59	14.96
1300	Tridecane	1.47	2.77	1.97	2.02	1.97	0.68	1.57	1.51	1.12	0.93	1.27	-
1414	β-Caryophyllene	1.09	1.74	1.44	1.29	1.07	0.81	0.99	1.53	0.97	0.81	1.17	1.27
1500	Pentadecane	6.80	6.38	5.39	6.20	5.36	4.23	4.01	5.40	3.00	3.16	3.91	4.04
TOTAL		99.95	99.29	99.42	99.98	99.70	99.95	99.53	99.95	98.84	97.56	98.60	98.63

[a] RI = retention index

limonene (13.25%) in NMS with 30 g L^{-1} sucrose, of myrcenone (56.43%) in NMS without sucrose, of ocimenone (15.30%) in AMS4 with 15 g L^{-1} sucrose, and of pentadecane (6.80%) in NMS without sucrose (Table 3). In general, the myrcene content increased with increasing sucrose concentration regardless of the membrane group; myrcenone decreased with the use of AMS4 and increased with NMS regardless of the sucrose concentration; and pentadecane decreased with increasing sucrose concentration regardless of the type of membrane used. Limonene accumulation in NMS increased (8.73, 11.43, and 13.25%) with increasing sucrose concentration in the medium, whereas in AMS1 and AMS4, limonene decreased as sucrose increased.

Primary and secondary pathways are intimately interconnected. And, sucrose plays an important role in plant metabolism as the major end product of many compounds including secondary metabolites. Beyond the sucrose being the most used carbohydrate source in plant tissue culture as a key component for seedling growth and development, it provides the carbon backbone for the biosynthesis of specialized metabolites, such as volatiles compounds (Pandey et al., 2022; Sheshadri et al., 2022). The type and the concentration of carbon sources can affect the morphogenetic potential and secondary metabolites in micropropagation plantlets (Yaseen et al., 2013). Therefore, natural ventilation systems and sucrose concentrations can influence the volatile compounds concentration of *L. rotundifolia*. Stojičić et al. (2022) reported that the type and the concentration of carbohydrates in culture media, significantly affected the composition of the volatile organic compounds in *Clinopodium pulegium*.

Other studies have also shown a variation in the number, concentrations, and profile of volatile compounds under the influence of ventilation. Lazzarini et al. (2019) reported in *L. gracilis* that the highest carvacrol and thymol contents were observed in plantlets grown in vials with four porous membranes. However, Silva et al. (2017) observed that *Plectranthus amboinicus* grown with one or two porous membranes had higher dry weight and carvacrol content. Greater ventilation inside the vial may increase plantlet growth and affect secondary metabolism with greater CO_2 availability. Zhu et al. (2015) reported that a higher CO_2 concentration led to an increase in artemisinin in *Artemisia annua*.

We next used principal component analysis (PCA) to evaluate the effect of the interactions between the ventilation system and the sucrose concentration on the evaluated parameters so that more information could be extracted from the results. PCA of the correlation matrix of the variables volatile organic compound contents, photosynthetic pigment contents, dry weight, TLA, and shoot length explained 75.9% of the total variation.

Correlations were observed between the AMS4/15 g L^{-1} sucrose treatment and ocimenone, myrcene, photosynthetic pigments, TLA, SH, and dry weight production (Fig. 2). With greater ventilation, there

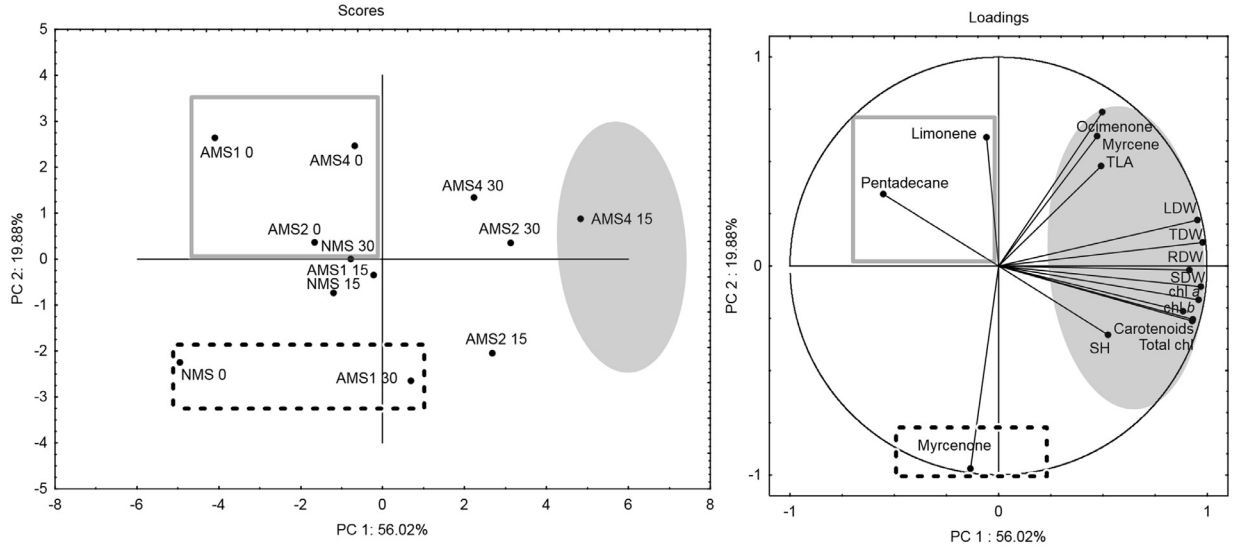

Fig. 2. Scores and loadings of the principal components analysis (PCA) in the correlation matrix built using data for photosynthetic pigments, shoot height, total leaf area, dry weight measures, and major compounds depending on the membrane system and sucrose concentration of *Lippia rotundifolia*.

was greater chlorophyll synthesis, resulting in higher dry weight gain and ocimenone and myrcene contents. The NMS treatment was negatively correlated with dry weight production and the other evaluated growth parameters. This analysis provides extra and important information about the results. The PCA shows that there was a marked difference between the number of filter membranes used in the cap over the plants and the volatile compounds of *L. rotundifolia*. The plantlets grown in the vials sealed with one, two, or four filter membranes without sucrose addition, or with no filter membrane but with 30 g L^{-1} sucrose, had a greater tendency to synthesize limonene and pentadecane. Conversely, the greatest synthesis of myrcenone occurred in the NMS+sucrose groups and the AMS1/30 g L^{-1} sucrose group.

Based on all the results obtained in this study, it is clear that porous membranes promote gas exchange, as observed mainly from the higher biometric data, growth parameters, and pigment contents. In addition, sucrose is an important source of carbon and energy, and its initial concentration may affect parameters such as the growth and yield of secondary metabolites (Cui et al., 2010).

4. Conclusions

The use of a natural ventilation system composed of manufactured porous membranes was found to be a more efficient *in vitro* cultivation method than NMS. The ventilation system AMS4 with a sucrose concentration of 15 g L^{-1} was the most efficient, as these plantlets had the highest photosynthetic rate and dry weight content. In general, the contents of volatile organic compounds varied differently with the use of the porous membrane and sucrose concentration in the culture medium.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

CRediT authorship contribution statement

Bety Shiue de Hsie: Visualization, Writing – review & editing. **Ana Izabela Sales Bueno:** Visualization. **Alexandre Alves de Carvalho:** Formal analysis. **Melvis Celeste Vilanculos Cossa:** Visualization. **Rafael Marlon Alves de Assis:** Writing – original draft. **Priscila Pereira Botrel:** Visualization. **Suzan Kelly Vilela Bertolucci:** Writing – review & editing. **José Eduardo Brasil Pereira Pinto:** Conceptualization, Visualization, Writing – review & editing.

Acknowledgments

The authors would like to thank the National Council for Scientifc and Technological Development (CNPq - Conselho Nacional de Desenvolvimento Científco e Tecnológico), the Coordination for the Improvement of Higher Education Personnel (CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and the Minas Gerais State Research Foundation (FAPEMIG - Fundação de Pesquisa do Estado de Minas Gerais) for fnancial support (scholarships and research grants).

References

Adams, R.P., 2017. Identification of Essential oil Components by Gas Chromatography/ Mass Spectrometry. 5 Online ed. Texensis Publishing.

Alvarez, C., Sáez, P., Sáez, K., Sánchez-Olate, M., Ríos, D., 2012. Effects of light and ventilation on physiological parameters during *in vitro* acclimatization of *Gevuina avellana*. Plant Cell Tissue Organ Cult. 110, 93–101. https://doi.org/10.1007/s11240-012-0133-x.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24, 1. https://doi.org/10.1104/pp.24.1.1.

Bassole, I.H.N., Ouattara, A.S., Nebie, R., Ouattara, C.A.T., Kabore, Z.I., Traore, S.A., 2003. Chemical composition and antibacterial activities of the essential oils of *Lippia chevalieri* and *Lippia multiflora* from Burkina Faso. Phytochemistry 62, 209–212. https://doi.org/10.1016/S0031-9422(02)00477-6.

Benicasa, M., 2004. Plant Growth Analysis (Basic Notions). FUNEP, Jaboticabal.

Cha-um, S., Chanseetis, C., Chintakovid, W., Pichakum, A., Supaibulwatana, K., 2011. Promoting root induction and growth of *in vitro* macadamia (*Macadamia tetraphylla* L. 'Keaau') plantlets using CO_2-enriched photoautotrophic conditions. Plant Cell Tissue Organ Cult. 106, 435. https://doi.org/10.1007/s11240-011-9940-8.

Chandra, S., Bandopadhyay, R., Kumar, V., Chandra, R., 2010. Acclimatization of tissue cultured plantlets: from laboratory to land. Biotechnol. Lett. 32, 1199–1205. https://doi.org/10.1007/s10529-010-0290-0.

Choi, S.R., Kim, M.J., Eun, J.S., Ahn, M.S., Lim, H.C., Ryu, J., You, D.H., 2004. Effects of membrane filter and sucrose concentrations on the growth of balloon flower (*Platycodon grandiflorum* A. DC.) plantlets *in vitro*. J. Plant Biotechnol. 31, 209–217. https://doi.org/10.5010/JPB.2004.31.3.209.

Cui, X.H., Murthy, H.N., Wu, C.H., Paek, K.Y., 2010. Sucrose-induced osmotic stress affects biomass, metabolite, and antioxidant levels in root suspension cultures of *Hypericum perforatum* L. Plant Cell Tissue Organ Cult. 103, 7–14. https://doi.org/10.1007/s11240-010-9747-z.

Erig, A.C., Schuch, M.W., 2005. Photoautotrophic micropropagation and use of natural light. Cienc. Rural. 35, 961–965.

Ferreira, D.F., 2011. Sisvar: a computer statistical analysis system. Cienc. Agrotec. 35, 1039–1042. https://doi.org/10.1590/S1413-70542011000600001.

Grout, B.W.W., 1988. Photosynthesis of regenerated plantlets *in vitro* and the stresses of transplanting. Acta Hortic. 230, 129–136.

Hassankhah, A., Vahdati, K., Lotfi, M., Mirmasoumi, M., Preece, J., Assareh, M.H., 2014. Effects of ventilation and sucrose concentrations on the growth and plantlet anatomy of micropropagated *Persian walnut* plants. Int. J. Hortic. Sci. Technol. 1, 111–120.

Hazarika, B.N., 2006. Morpho-physiological disorders in *in vitro* culture of plants. Sci. Hortic. 108, 105–120. https://doi.org/10.1016/j.scienta.2006.01.038.

Iarema, L., da Cruz, A.C.F., Saldanha, C.W., Dias, L.L.C., Vieira, R.F., de Oliveira, E.J., Otoni, W.C., 2012. Photoautotrophic propagation of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]. Plant Cell Tissue Organ Cult. 110, 227–238. https://doi.org/10.1007/s11240-012-0145-6.

Ivanova, M., Van Staden, J., 2010. Natural ventilation effectively reduces hyperhydricity in shoot cultures of *Aloe polyphylla* Schönland ex Pillans. Plant Growth Regul. 60, 143–150. https://doi.org/10.1007/s10725-009-9430-8.

Kitaya, Y., Ohmura, Y., Kubota, C., Kozai, T., 2005. Manipulation of the culture environment on *in vitro* air movement and its impact on plantlets photosynthesis. Plant Cell Tissue Organ Cult. 83, 251–257. https://doi.org/10.1007/s11240-005-6839-2.

Kozai, T., 2010. Photoautotrophic micropropagation - environmental control for promoting photosynthesis. Propag. Ornam. Plants 10, 188–204.

Kozai, T., Kubota, C., 2001. Developing a photoautotrophic micropropagation system for woody plants. J. Plant Res. 114, 525–537. https://doi.org/10.1007/pl00014020.

Langford, P., Wainwright, H., 1987. Effects of sucrose concentration on the photosynthetic ability of rose shoots *in vitro*. Ann. Bot. 60, 633–640.

Lazzarini, L.E.S., Bertolucci, S.K.V., de Carvalho, A.A., Santiago, A.C., Pacheco, F.V., Yucesan, B., Pinto, J.E.B.P., 2019. Explant type and natural ventilation systems influence growth and content of carvacrol and thymol of *Lippia gracilis* Schauer. Plant Cell Tissue Organ Cult. 137, 33–43. https://doi.org/10.1007/s11240-018-01548-5.

Leitão, S.G., Oliveira, D.R.d., Sülsen, V., Martino, V., Barbosa, Y.G., Bizzo, H.R., Lopes, D., Viccini, L.F., Salimena, F.R.G., Peixoto, P.H.P., Leitão, G.G., 2008. Analysis of the chemical composition of the essential oils extracted from *Lippia lacunosa* Mart. & Schauer and *Lippia rotundifolia* Cham. (Verbenaceae) by gas chromatography and gas chromatography-mass spectrometry. J. Braz. Chem. Soc. 19, 1388–1393.

Lichtenthaler, H.K., Buschmann, C., 2001. Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. Curr. Protoc. Food Anal. Chem..

Majada, J.P., Fal, M.A., Tadeo, F., Sánchez-Tamés, R., 2002. Effects of natural ventilation on leaf ultrastructure of *Dianthus caryophyllus* L. cultured *in vitro*. *In Vitro* Cell. Dev. Biol. 38, 272–278. https://doi.org/10.1079/IVP2001271.

Makunga, N.P., Jäger, A.K., van Staden, J., 2006. Improved *in vitro* rooting and hyperhydricity in regenerating tissues of *Thapsia garganica* L. Plant Cell Tissue Organ Cult. 86, 77–86. https://doi.org/10.1007/s11240-006-9100-8.

Mills, D., Yanqing, Z., Benzioni, A., 2009. Effect of substrate, medium composition, irradiance and ventilation on jojoba plantlets at the rooting stage of micropropagation. Sci. Hortic. 121, 113–118. https://doi.org/10.1016/j.scienta.2009.01.021.

Mohamed, M.A.H., Alsadon, A.A., 2010. Influence of ventilation and sucrose on growth and leaf anatomy of micropropagated potato plantlets. Sci. Hortic. 123, 295–300. https://doi.org/10.1016/j.scienta.2009.09.014.

Morais, T.P., Luz, J.M.Q., Silva, S.M., Resende, R.F., Silva, A.S., 2012. Applications of tissue culture in medicinal plants. Rev. Bras. Plantas Med. 14, 110–121. https://doi.org/10.1590/S1516-05722012000100016.

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.

Nguyen, Q.T., Kozai, T., 2001. Growth of *in vitro* banana (*Musa* spp.) shoots under photomixotrophic and photoautotrophic conditions. *In Vitro* Cell. Dev. Biol. 37, 824. https://doi.org/10.1007/s11627-001-0137-4.

Nicoloso, F.T., Erig, A.C., Russowski, D., Martins, C.F., 2003. Effect of carbohydrate concentration and source on growth of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen] *in vitro* cultured plants. Cienc. Agrotec. 27, 84–90. https://doi.org/10.1590/S1413-70542003000100010.

Núñez-Ramos, J.E., Quiala, E., Posada, L., Mestanza, S., Sarmiento, L., Daniels, D., Arroyo, C.R., Naranjo, B., Vizuete, K., Noceda, C., Gómez-Kosky, R., 2021. Morphological and physiological responses of tara (*Caesalpinia spinosa* (Mol.) O. Kuntz)

microshoots to ventilation and sucrose treatments. *In Vitro* Cell. Dev. Biol. 57, 1–14. https://doi.org/10.1007/s11627-020-10104-w.

Oliveira, D.R., Leitão, G.G., Santos, S.S., Bizzo, H.R., Lopes, D., Alviano, C.S., Alviano, D.S., Leitão, S.G., 2006. Ethnopharmacological study of two *Lippia* species from Oriximiná, Brazil. J. Ethnopharmacol. 108, 103–108. https://doi.org/10.1016/j.jep.2006.04.018.

Pandey, D.K., Konjengbam, M., Ghorai, M., Dwivedi, P., Roy, D., Kant, N., Gangaprasad, A., Dey, A., 2022. Biotechnology for micropropagation and camptothecin production in *Ophiorrhiza* sp. Appl. Microbiol. Biotechnol. 106, 3851–3877. https://doi.org/10.1007/s00253-022-11941-y.

Pascual, M.E., 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.

Pawlicki, N., Welander, M., 1995. Influence of carbohydrate source, auxin concentration and time of exposure on adventitious rooting of the apple rootstock Jork 9. Plant Sci. 106, 167–176. https://doi.org/10.1016/0168-9452(95)04074-5.

Pimenta, M.R., Fernandes, L.S., Pereira, U.J., Garcia, L.S., Leal, S.R., Leitão, S.G., Salimena, F.R.G., Viccini, L.F., Peixoto, P.H.P., 2007. Flowering, germination and cuttings in species of *Lippia* L. (Verbenaceae) - Portuguese. Braz. J. Bot. 30, 211–220.

Resende, C.F.d., Bianchetti, R.E., Oliveira, A.M.S.d., Braga, V.F., Peixoto, P.H.P., 2015. *In vitro* propagation and acclimatization of *Lippia rotundifolia*, an endemic species of Brazilian Campos Rupestres. Rev. Cienc. Agron. 46, 582–589.

Ribeiro, M.V., Lima, C.S.M., de Magalhães Bandeira, J., Rubin, S., Benitez, L.C., Peters, J.A., Braga, E.J.B., 2007. Sucrose concentrations and seal types in the *in vitro* cultivation of *Melissa officinalis* L. Rev. Bras. Biocienc. 5, 843–845.

Rout, G.R., Samantaray, S., Das, P., 2000. *In vitro* manipulation and propagation of medicinal plants. Biotechnol. Adv. 18, 91–120. https://doi.org/10.1016/S0734-9750(99)00026-9.

Saldanha, C.W., Otoni, C.G., de Azevedo, J.L.F., Dias, L.L.C., do Rêgo, M.M., Otoni, W.C., 2012. A low-cost alternative membrane system that promotes growth in nodal cultures of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]. Plant Cell Tissue Organ Cult. 110, 413–422. https://doi.org/10.1007/s11240-012-0162-5.

Salimena, F.R.G., Silva, T.R.S., 2009. Flora of grão-mogol, minas gerais: verbenaceae. Bot. Bull. 27, 119–120.

Santos, M.R.A.d., Innecco, R., Soares, A.A., 2004. Anatomical characterization of secretory structures and essential oil production of *Lippia alba* (Mill.) NE Br. as a function of harvest time in dry and rainy seasons. Rev. Cienc. Agron. 35, 377–383.

Sheshadri, S.A., Nishanth, M.J., Simon, B., 2022. Melatonin influences terpenoid indole alkaloids biosynthesis and 5′ upstream-mediated regulation of cell wall invertase in *Catharanthus roseus*. J. Plant Growth Regul.. https://doi.org/10.1007/s00344-022-10705-2.

Silva, B.M.d.S.e., Lima, J.D., Dantas, V.A.V., Moraes, W.d.S., Sabonaro, D.Z., 2007. Effect of light on the growth of *Hymenaea parvifolia* Huber seedlings. Rev. Arvore 31, 1019–1026.

Silva, S.T., Bertolucci, S.K.V., da Cunha, S.H.B., Lazzarini, L.E.S., Tavares, M.C., Pinto, J.E.B.P., 2017. Effect of light and natural ventilation systems on the growth parameters and carvacrol content in the *in vitro* cultures of *Plectranthus amboinicus* (Lour.) Spreng. Plant Cell Tissue Organ Cult. 129, 501–510. https://doi.org/10.1007/s11240-017-1195-6.

Souza, G.S.d., Silva, Santos, Oliveira, Jd, Santos Neto, U.C.d., Santos, R.B.d., A.R.d., 2014. Vegetative growth and essential oil production of rosemary plants grown under colored screens. Biosci. J. 30.

Stojičić, D., Tošić, S., Stojanović, G., Zlatković, B., Jovanović, S., Budimir, S., Uzelac, B., 2022. Volatile organic compound composition and glandular trichome characteristics of *in vitro* propagated *Clinopodium pulegium* (Rochel) Brauchler: effect of carbon source. Plants 11, 198.

van Den Dool, H., Dec. Kratz, P., 1963. A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. J. Chromatogr. A 11, 463–471. https://doi.org/10.1016/S0021-9673(01)80947-X.

Xiao, Y., Niu, G., Kozai, T., 2011. Development and application of photoautotrophic micropropagation plant system. Plant Cell Tissue Organ Cult. 105, 149–158. https://doi.org/10.1007/s11240-010-9863-9.

Yaseen, M., Ahmad, T., Sablok, G., Standardi, A., Hafiz, I.A., 2013. Review: role of carbon sources for in *vitro* plant growth and development. Mol. Biol. Rep. 40, 2837–2849. https://doi.org/10.1007/s11033-012-2299-z.

Yaseen, M., Ahmed, T., Abbasi, N.A., Hafiz, I.A., 2009. *In vitro* shoot proliferation competence of apple rootstocks M. 9 and M. 26 on different carbon sources. Pak. J. Bot. 41, 1781–1795.

Zhang, M., Zhao, D., Ma, Z., Li, X., Xiao, Y., 2009. Growth and photosynthetic capability of *Momordica grosvenori* plantlets grown photoautotrophically in response to light intensity. HortScience 44, 757–763.

Zhu, C., Zeng, Q., McMichael, A., Ebi, K.L., Ni, K., Khan, A.S., Zhu, J., Liu, G., Zhang, X., Cheng, L., Ziska, L.H., 2015. Historical and experimental evidence for enhanced concentration of artemesinin, a global anti-malarial treatment, with recent and projected increases in atmospheric carbon dioxide. Clim. Change 132, 295–306. https://doi.org/10.1007/s10584-015-1421-3.