

Volatile organic compound (VOC) profile and plantlet growth of *Aeollanthus suaveolens* under conventional and alternative membrane systems

Diene Xavier Araújo

UFLA: Universidade Federal de Lavras

Tainá Teixeira Rocha

UFLA: Universidade Federal de Lavras

Alexandre Alves de Carvalho

UFLA: Universidade Federal de Lavras

Ana Paula Ribeiro Medeiros

UFLA: Universidade Federal de Lavras

Fernanda Naiara Santos Ribeiro

UFLA: Universidade Federal de Lavras

Rafael Marlon Alves de Assis

UFLA: Universidade Federal de Lavras

Suzan Kelly Vilela Bertolucci

UFLA: Universidade Federal de Lavras

José Eduardo Brasil Pereira Pinto (✉ jeduardo@ufla.br)

UFLA: Universidade Federal de Lavras <https://orcid.org/0000-0002-1141-7907>

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Abstract

In conventional *in vitro* culture, plantlets are kept in closed containers to avoid contamination and drying of the explant. Ventilation inside the containers improves plant growth and affects secondary metabolism, as it modifies the microenvironmental conditions. Therefore, the objective of this study was to evaluate the effect of the use of ventilation systems on the growth characteristics and production of photosynthetic pigments and volatile organic compounds (VOCs) in *Aeollanthus suaveolens* Mart. ex Spreng. (Lamiaceae) cultured *in vitro*. Nodal segments containing one pair of leaves were cultured in a conventional system (NMS) and in natural ventilation systems with one (AMS1), two (AMS2) and four (AMS4) porous membranes. At 40 days, the plantlets were evaluated for growth, VOC concentration, and photosynthetic pigment production. The number of porous membranes used in the vial cap affected growth, photosynthetic pigments and VOCs. A higher number of porous membranes (AMS4) led to greater dry weight accumulation, increased production of photosynthetic pigments, and enhanced synthesis of (Z)- β -farnesene. Lower growth and fewer photosynthetic pigments, and increase linalool acetate synthesis were observed in the culture without the use of porous membranes (NMS). The leaf area of plantlets cultivated with the use of four membranes was 3.8 times greater than that of plantlets cultivated without the use of membranes. For the photoautotrophic cultivation of *A. suaveolens* *in vitro*, the use of natural ventilation with four membranes is recommended because it promotes better growth, increases the production of photosynthetic pigments and is superior to the conventional sealed system.

Introduction

The species *Aeollanthus suaveolens* Mart. ex Spreng. (Lamiaceae), an aromatic plant of African origin, was introduced in Brazil for medicinal purposes (Ferreira et al. 2017). In the Amazon region, it is popularly known as “*Catinga de mulata*” and is used in scent baths, in religious rituals, and in homemade perfumes (Harley 2012). In addition, it is used in folk medicine to combat fever, headache, skin, and eye disorders, and as a sedative and anticonvulsant (Simionatto et al. 2007). Studies have shown that crude extracts obtained from *A. suaveolens* have antibacterial activity (Veloso et al. 2020) and that the essential oil has potential neuroeffects when used as a nanoemulsion (Ferraz et al. 2020) and has antimicrobial activity (Ngo-Mback et al. 2019).

Tissue culture techniques are important biotechnological tools for the multiplication and analysis of medicinal plants (Siahsar et al. 2011). In conventional micropropagation, the culture container is usually sealed to prevent contamination and dehydration of the explants and culture medium (Schuelter et al. 2015). However, the cap or the type of seal can limit gas exchange between the *in vitro* and *ex vitro* environments, causing morphophysiological disorders (Lazzarini et al. 2019; Saldanha et al. 2012). The microenvironment formed inside the vial in conventional culture shows a high relative humidity, ethylene accumulation, and a low carbon dioxide (CO₂) concentration (Chen 2006; Schuelter et al. 2015). Another important characteristic of *in vitro* culture is the presence of sucrose in the culture medium, which is considered the main or only source of carbon and energy for plant growth (Xiao et al. 2011). According to

Kozai and Xiao (2008), the presence of sucrose in the culture medium increases the risk of microbial contamination.

Plantlets grown in conventional micropropagation systems may have anatomical and physiological abnormalities. Some of these problems include reduced photosynthetic activity, a low chlorophyll content, abnormal stomatal opening, lack of a leaf cuticle layer, and abnormal leaf parenchyma and xylem (Vahdati and Hassankhah 2014). These morphophysiological changes cause high plant mortality during acclimatization (Alvarez et al. 2012). The use of a natural ventilation system in *in vitro* culture is an alternative to reduce these problems.

The use of a natural ventilation system has great advantages for micropropagation. Such systems decrease relative humidity, increase aeration, and maintain adequate CO₂ concentrations to stimulate photosynthesis (Moreira et al. 2013; Saldanha et al. 2012). The use of natural ventilation systems in *in vitro* culture has shown positive responses in terms of the growth and accumulation of secondary metabolites in some species: *Plectranthus amboinicus* (Lour.) Spreng. (Lamiaceae) (Silva et al. 2017); *Lippia gracilis* Schauer (Verbenaceae) (Lazzarini et al. 2019); *Azadirachta indica* A. Juss. (Meliaceae) (Rodrigues et al. 2012); *Pfaffia glomerata* (Spreng.) Pedersen (Amaranthaceae) (Saldanha et al. 2012); and *Ananas comosus* L. Merr (Bromeliaceae) (Silva et al. 2014).

The objective of this study was to evaluate the effects of natural ventilation systems on the growth, production of photosynthetic pigments, and volatile organic compounds (VOCs) in *A. suaveolens* plantlets grown *in vitro*.

Materials And Methods

In vitro establishment of plant material

Voucher specimens are deposited in the herbarium of EMBRAPA/Eastern Amazon in Belém, Pará, Brazil, under record number IAN 184689. The *A. suaveolens* seeds obtained from mother plants grown in a greenhouse were used in this study. The seeds were treated with sodium hypochlorite (1% active chlorine) for 5 min under constant stirring. Subsequently, the seeds were washed with autoclaved distilled water three times in a laminar flow hood. The seeds were then inoculated in test tubes containing 15 mL of MS medium (Murashige and Skoog

1962) supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar, and the pH was adjusted to 5.7 ± 0.1 before autoclaving at 121°C at 0.1 atm for 20 min. The tubes were kept in a growth room under cold-white fluorescent lamps (OSRAM®, Brazil) with an intensity of $39 \mu\text{mol m}^{-2} \text{ s}^{-1}$, 16-h photoperiod and temperature of $25 \pm 1^\circ\text{C}$. After 30 days, the grown plantlets were multiplied in vials containing 40 mL of MS medium.

Alternative membrane system (AMS)

Nodal segments ($\pm 1 \text{ cm}$) with one pair of leaves were cultured *in vitro* in a conventional sealed system (no-membrane system - NMS) or alternative membrane systems (AMSs). For NMS, the vials were sealed with polypropylene rigid closure, as conventional systems. In the AMSs, holes of 10 mm in diameter were made in the caps of the vials and then covered with membranes. Porous membranes were prepared using one layer of polytetrafluoroethylene film (Amanco®) and three layers of microporous tape (Cremer®), similar to manufactured by Saldanha et al. (2012) (Fig. 1). The explants were removed from plantlets established *in vitro* and inoculated in 250-mL vials containing 45 mL of MS medium without sucrose supplemented with 5.5 g L^{-1} agar (Himedia®, type I), and the pH was adjusted to 5.7 ± 0.1 before autoclaving at 121°C at 0.1 atm for 20 min. After inoculation, the tubes were placed in a growth room under cold-white fluorescent

lamps (OSRAM®, Brazil) with an intensity of $39 \mu\text{mol m}^{-2} \text{s}^{-1}$, 16-h photoperiod and temperature of $25 \pm 1^\circ\text{C}$. The natural ventilation systems were established by placing polytetrafluoroethylene (PTFE) membranes on the caps of the culture vials. The membranes were mounted according to Saldanha et al. (2012).

The experiment was conducted in a completely randomized design totaling four treatments: NMS and AMSs with one (AMS1), two (AMS2) and four (AMS4) porous membranes. Each treatment had five replicates, with four vials per replicate, and three explants per vial.

After 40 days, the plantlets were evaluated for the shoot number (SN), leaf number (LN), root number (RN), shoot length (SL, cm), length of longest root (LLR, cm), total leaf area (TLA, cm^2), leaf dry weight (LDW, mg), stem dry weight (SDW, mg), root dry weight (RDW, mg), total dry weight (TDW, mg), concentration of photosynthetic pigments, and presence of VOCs. To determine the dry weight, the leaves, stems and roots were placed in paper bags and dried in a forced air oven at 35°C to constant weight. TLA was measured in WinFOLIA™ software using an Epson Perfection V700 photo scanner to scan all leaves present in five plantlets from each treatment. The leaf area ratio (LAR = TLA/TDW), specific leaf area (SLA = TLA/LDW), leaf weight ratio (LWR = $\text{LDW}/(\text{LDW} + \text{SDW})$), and specific leaf weight (SLW = LDW/TLA) were calculated according to Benincasa (2003). In addition, a ratio was established

between the parameters obtained for AMS4 and NMS (AMS4/NMS).

Quantification of photosynthetic pigments

The extraction of the pigments chlorophyll *a* and *b*, total chlorophyll (*a* + *b*) and carotenoids was performed according to the method described by Costa et al. (2014a) with modifications to the amount of plant material and the use of dimethyl sulfoxide (DMSO) not saturated with CaCO_3 as a solvent. A leaf fresh weight of 50 mg was used without the midrib. Next, the plant material was incubated in 10 mL of DMSO in Falcon tubes wrapped in aluminum foil and placed in an oven at 65°C for 24 hours. After this period, 3-mL aliquots of the solutions were transferred to a 3-cm³ quartz cuvette to perform spectral absorbance readings in a Tecan Infinite M200 PRO microplate reader operated with the software I-control® version 3.37 at 480, 649 and 665 nm wavelengths for carotenoids and chlorophyll *b* and *a*, respectively. The blank contained DMSO only. All readings were performed in triplicate.

From the readings obtained, the concentrations of chlorophyll *a* and *b* and carotenoids were determined using the equations proposed by Wellburn (1994): chlorophyll *a* = $(12.47 \times H_{665}) - (3.62 \times H_{649})$; chlorophyll *b* = $(25.06 \times A_{649}) - (6.5 \times A_{665})$; and carotenoids: $(1000 \times A_{480} - 1.29 \times C_a - 53.78 \times C_b)/220$. Total chlorophyll was calculated by

summing the results of the equations for chlorophyll *a* and *b*, with the results expressed in mg/g.

Analysis of volatile compounds by headspace GC/MS

For the analysis of VOCs, dry leaves of *A. suaveolens* from each treatment were used. Individual 90-mg aliquots (leaf dry weight) were added in triplicate to 20-mL headspace vials and sealed with silicone/PTFE caps until analysis.

The static headspace technique was used in the extraction of VOCs from *A. suaveolens*. For this purpose, a Combi PAL Autosampler System automatic headspace extractor/sampler (CTC Analytics AG, Switzerland) coupled to the gas chromatography/mass spectrometry (GC/MS) system was used. The extraction parameters applied were as follows: sample incubation temperature of 110°C for 30 min, syringe temperature of 120°C, gas-phase automatic injection and 500 µL injection volume. The volatile fraction was analyzed in an Agilent® 7890A GC system coupled to an Agilent® MSD 5975C mass selective detector (Agilent Technologies, California, USA) operated with electron impact ionization at 70 eV in scanning mode, a mass acquisition range of 40–400 m/z and a speed of 1.0 scan/s. An HP-5MS fused silica capillary column (30 m length x 0.25 mm internal diameter x 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 to 230°C, followed by a ramp of 10°C/min to 250°C, and a hold for 1 min. 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 percent normalized area of the chromatographic peaks.

The volatile fraction constituents were identified by comparing their linear retention indices relative to those of a coinjected standard solution of *n*-alkanes (C₈-C₂₀, Sigma-Aldrich®, St. Louis, USA) and by comparing the mass spectra with those from the NIST library and from Adams (2007). The retention index was calculated using the equation proposed by van den Dool and Kratz (1963), and for the attributions, the retention indices in Adams (2007) were consulted.

Statistical analysis

The data were subjected to analysis of variance by the F-test, and the means were compared by the Scott-Knott test at 5% probability. The statistical program SISVAR® (Ferreira 2011) was used for data processing. Principal component analysis (PCA) was used to study the influence of different AMSs on the volatile constituents and growth parameters of *A. suaveolens*. PCA was performed in Statistica® version 13.4 (StatSoft, Tulsa, OK, USA).

Results And Discussion

Growth analysis

The number of porous membranes influenced the growth of *A. suaveolens* cultured *in vitro* in MS medium without sucrose supplementation (photoautotrophic). Plantlets grown in the AMSs showed better growth variable responses than those grown in NMS. AMS4 had the highest means for all growth variables analyzed. Conversely, plantlets grown in NMS showed the lowest values for all evaluated parameters (Table 1, Fig. 2). The SL and LLR values obtained for AMS4 were 2.7-fold and 1.8-fold greater, respectively, than those of plantlets grown in NMS (Table 1). The plantlets cultured with ventilation were much more vigorous (Fig. 1).

Table 1

In vitro growth of *Aeollanthus suaveolens* grown for 40 days in natural ventilation systems with one (AMS1), two (AMS2) and four (AMS4) porous membranes and in a conventional system (NMS). SN: shoot number; SL: shoot length; LN: leaf number; RN: root number; LLR: length of the longest root; LDW: leaf dry weight; SDW: shoot dry weight; RDW: root dry weight; TDW: total dry weight. AMS4/NMS: ratio between the values for AMS4 and NMS. CV: coefficient of variation.

Culture system						
Variables	NMS	AMS1	AMS2	AMS4	AMS4/NMS	CV%
SN	1.12 ± 0.13c	1.59 ± 0.28b	1.67 ± 0.30b	2.01 ± 0.04a	1.79	13.56
SL (cm)	1.75 ± 0.28c	3.08 ± 0.11b	4.58 ± 0.58a	4.69 ± 0.48a	2.7	11.55
LN	6.94 ± 0.37c	9.18 ± 0.53b	10.78 ± 0.76a	11.16 ± 0.64a	0.14	6.21
RN	4.90 ± 0.11c	5.57 ± 0.32b	5.60 ± 0.39b	6.59 ± 0.47a	1.3	6.17
LLR (cm)	4.92 ± 0.20c	8.48 ± 0.49b	8.50 ± 0.27b	9.14 ± 0.63a	1.8	5.58
Means (± standard deviation) in the same row followed by the same letter do not differ significantly according to the Scott-Knott test at the 5% probability level.						

The AMS4/NMS ratio showed that there was a gain in dry weight of between 4.5 and 6.1 times in AMS4 compared to that in NMS. This finding may be related to the photoautotrophic conditions promoted by the greater number of porous membranes, which allowed greater nutrient absorption due to greater evapotranspiration of the culture medium and greater gas exchange (Fig. 1). The poor growth of the plantlets grown in NMS is explained by the absence of sucrose in the culture medium and low gas exchange. According to Kozai (2010), the supplementation of a carbon source in the culture medium is important because it performs several essential functions, providing energy for plant metabolism.

Plantlets grown with membranes showed higher dry weight accumulation, indicating the importance of gas exchange for *in vitro* morphogenesis in this species. This result may be related to the increased photosynthetic rate caused by the greater availability of CO₂ in AMSs than in NMS. According to Arigita et al. (2010), gas exchange between the external and internal environment in a natural ventilation system

favors the effective absorption of nutrients from the culture medium by the plantlets, increasing their growth.

Several studies have reported that increased gas exchange in the vials used for *in vitro* propagation of plants favors plant growth and development. In the study by Saldanha et al. (2012), it was found that AMSs improved the *in vitro* growth of *Pfaffia glomerata* (Spreng.) Pedersen. AMSs with one and two membranes optimized the *in vitro* growth of apical and nodal segments of *Plectranthus amboinicus* (Lour.) Spreng (Silva et al. 2017). An AMS was also beneficial for the *in vitro* propagation of *Azadirachta indica* A. Juss (Rodrigues et al. 2012). Lazzarini et al. (2019) also reported that an AMS with four porous membranes led to better growth and increased leaf area in *Lippia gracilis*.

Regarding TLA, the AMS4 treatment was superior to all other cultivation systems (Table 2). It is believed that the AMS4 treatment favored greater entry of CO₂ and, consequently, greater photosynthetic capacity, leading to greater plantlet growth. According to Souza et al. (2014), the higher the leaf area is, the higher the photosynthetic rate of the plants will be. The LAR, SLA and LWR were higher in NMS and AM1.

Table 2

Total leaf area (TLA), leaf area ratio (LAR), specific leaf area (SLA), leaf weight ratio (LWR) and specific leaf weight (SLW) of *Aeollanthus suaveolens* grown *in vitro* at 40 days in different cultivation systems (a conventional system (NMS) and natural ventilation systems (AMSs) with 1, 2 and 4 porous membranes).
CV: coefficient of variation.

Culture system	TLA (cm ²)	LAR (cm ² /mg)	SLA (cm ² /mg)	LWR (mg/mg)	SLW (mg/cm ²)
NMS	5.03 ± 0.83d	0.55 ± 0.11a	0.88 ± 0.18a	0.85 ± 0.01a	1.14 ± 0.33b
AMS1	12.00 ± 1.21c	0.49 ± 0.07a	0.86 ± 0.12a	0.83 ± 0.02a	1.17 ± 0.17b
AMS2	15.08 ± 2.19b	0.38 ± 0.07b	0.67 ± 0.13b	0.82 ± 0.02b	1.50 ± 0.39a
AMS4	19.06 ± 2.67a	0.41 ± 0.05b	0.73 ± 0.08b	0.81 ± 0.01b	1.36 ± 0.14a
CV (%)	14.68	16.57	16.76	2.01	4.97
Means (± standard deviation) in the same row followed by the same letter do not differ significantly according to the Scott-Knott test at the 5% probability level.					

LAR is the ratio between the leaf area responsible for the interception of light energy and CO₂ and the TDW of the plant. LAR declines as the plant grows due to self-shading, with a tendency to decrease the useful leaf area (Benincasa 2003). The plantlets grown in the NMS treatment had a higher LAR due to lower TDW accumulation, and those grown in the AMS4 treatment had a lower LAR, suggesting that the plantlets grown in the AMS4 treatment were more efficient in dry weight production. Lazzarini et al. (2019) worked with explants with and without leaves and observed a lower LAR in explants with leaves, which were more efficient in dry weight accumulation. The same was observed by Souza et al. (2014)

and Chagas et al. (2013) in *Rosmarinus officinalis* L. and *Mentha arvensis* L., respectively, in a greenhouse.

SLA is expressed as the ratio between TLA and LDW. It is considered an important factor from a physiological standpoint because it describes the allocation of leaf biomass per unit area (Scalon et al. 2017). Plantlets cultured in the NMS and AMS1 treatments had a higher SLA, indicating that a larger leaf area was needed to accumulate dry weight. The SLA was lower with a higher number of membranes (AMS2 and AMS4), indicating that the plantlets were more efficient in biomass allocation. According to Barreiro et al. (2006), decreases in SLA indicate an increase in leaf thickness resulting from the increase in the number and size of plant cells. SLW is also indicative of leaf thickness (Benincasa 2003). The *A. suaveolens* plantlets cultivated with greater aeration (AMS2 and AMS4) had a higher SLW, which indicates that they had a greater leaf thickness (Table 2).

LWR represents the fraction of dry weight that is not exported from the leaves to other plant parts. The NMS and AMS1 treatments showed a small difference in LWR from the other ventilation systems (Table 2). Thus, it can be inferred that the export of dry weight from the leaf to other parts of the plantlet was balanced and that under lower ventilation, export was lower. Lazzarini et al. (2019) reported no difference in LWR between ventilation systems, except when the presence or absence of leaves on the initial explant differed.

In young plantlets, which consist mostly of leaves, the LWR values are high and decrease over time because other plant parts emerge and grow from the material exported from the leaves. LWR is thus an important growth index for plants whose leaves are of economic interest (Costa et al. 2014b).

Quantification of photosynthetic pigments

The increase in gas exchange increased the production of photosynthetic pigments in *A. suaveolens* plantlets (Table 3). The AMS2 and AMS4 treatments showed the maximum accumulation of chlorophyll *a*, total chlorophylls, and carotenoids. AMS1 had the highest concentration of chlorophyll *b*, and the other treatments (NMS, AMS2, and AMS4) did not differ significantly from each other. The photosynthetic activity of plantlets may vary according to genotype and cultivation conditions (light intensity, sucrose addition, etc.). *In vitro* culture may result in some species having weak photosynthetic activity and others having higher photosynthetic activity.

Table 3

Concentration of photosynthetic pigments of *Aeollanthus suaveolens* plantlets grown *in vitro* for 40 days in different culture systems: a conventional system (NMS) and natural ventilation systems with one (AMS1), two (AMS2) and four (AMS4) porous membranes. CV: coefficient of variation; FW: fresh weight.

Culture system	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Total chlorophyll	Carotenoids
	mg g ⁻¹ FW			
NMS	0.15 ± 0.03c	0.05 ± 0.01b	0.20 ± 0.04b	0.04 ± 0.01b
AMS1	0.23 ± 0.03b	0.11 ± 0.06a	0.33 ± 0.03a	0.04 ± 0.02b
AMS2	0.28 ± 0.02a	0.08 ± 0.01b	0.36 ± 0.01a	0.07 ± 0.01a
AMS4	0.29 ± 0.02a	0.09 ± 0.00b	0.38 ± 0.02a	0.08 ± 0.00a
CV%	10.84	18.37	8.12	21.64
Means (± standard deviation) in the same row followed by the same letter do not differ significantly according to the Scott-Knott test at the 5% probability level.				

A. suaveolens was found to be a species with high photosynthetic activity. Other studies also reported increased photosynthetic activity with ventilation inside the vials for *Plectranthus amboinicus* (Lour.) Spreng (Silva et al. 2017); *Pfaffia glomerata* (Spreng.) Pedersen (Saldanha et al. 2012); *Gevuina avellana* (Alvarez et al. 2012); *Pfaffia glomerata* (Iarema et al. 2012); and *Juglans regia* L. (Hassankhah et al. 2014). According to Saldanha et al. (2014), the increase in CO₂ concentration inside the vials during *in vitro* growth leads to high photosynthetic rates in plants grown under photoautotrophic conditions. However, other species, such as *Lippia gracilis* (Lazzarini et al. 2019), *Musa cavendishii* (Schoh et al. 1989), and *Prunus avium* (Righetti et al. 1990) showed weak photosynthetic activity. Lazzarini et al. (2019) reported that photosynthetic pigments decreased with the increase in the number of porous membranes in the culture vial. The CO₂ content in the culture vial and the light intensity are the limiting factors of photosynthesis in plantlets grown *in vitro* (Kozai 2010; Kozai et al. 1990).

AMSs maintain an adequate CO₂ concentration within the culture vial to stimulate photosynthesis (Saldanha et al. 2012). According to Zobayed et al. (2001), it is important for air exchange to occur between the environment and the vial so that plantlets grown *in vitro* develop the ability to build their photosynthetic apparatus and grow independent of exogenous carbon sources.

Effect of the number of membranes on VOCs by headspace GC/MS

The number of porous membranes affected the VOC concentrations of *A. suaveolens* grown *in vitro* (Table 4). Headspace GC/MS analysis identified 18 compounds with a total content above 96%. Five major compounds were identified, and their sum ranged from 88.38 to 90.52%. The compounds were linalool (9.78 to 11.10%), linalool acetate (26.64 to 32.31%), α-santalene (6.57 to 8.25%), (Z)-β-farnesene

(38.65 to 42.05%), and massoia lactone (2.01 to 2.44%). Regardless of the treatment, the VOCs identified were grouped into four main classes: hydrocarbon monoterpenes, oxygenated monoterpenes, hydrocarbon sesquiterpenes, and lactones (Table 4). Oxygenated monoterpenes tended to decrease with an increase in the number of membranes, and the opposite was observed for hydrocarbon sesquiterpenes.

Table 4

Chemical composition of the essential oil from *Aeollanthus suaveolens* grown in different membrane systems. NMS: no-membrane system, AMS: alternative membrane system with 1, 2 or 4 filter membranes.

RI ^a	Compounds	Content (%)			
		NMS	AMS1	AMS2	AMS4
Total hydrocarbon monoterpenes		1.97	2.54	2.30	2.47
929	α-Pinene	0.41 ± 0.03a	0.48 ± 0.03a	0.44 ± 0.03a	0.53 ± 0.12a
942	Camphene	0.47 ± 0.05a	0.56 ± 0.04a	0.52 ± 0.03a	0.63 ± 0.13a
971	β-Pinene	0.26 ± 0.03a	0.30 ± 0.03a	0.23 ± 0.02a	0.18 ± 0.12a
990	Myrcene	0.21 ± 0.02a	0.28 ± 0.03a	0.28 ± 0.08a	0.25 ± 0.04a
1024	Limonene	0.17 ± 0.01c	0.22 ± 0.02a	0.19 ± 0.01b	0.22 ± 0.02a
1036	(Z)-β-Ocimene	-	0.13 ± 0.01a	0.11 ± 0b	0.13 ± 0.01a
1046	(E)-β-Ocimene	0.45 ± 0.09b	0.57 ± 0.04a	0.53 ± 0.05a	0.53 ± 0.09a
Total oxygenated monoterpenes		42.44	39.60	36.58	37.03
1026	1,8-Cineole	0.18 ± 0.06a	0.2 ± 0.02a	0.15 ± 0.03a	0.19 ± 0.07a
1102	Linalool	9.78 ± 2.21b	11.1 ± 1.53a	9.48 ± 0.72b	10.01 ± 2.22b
1258	Linalool acetate	32.31 ± 0.11a	28.14 ± 0.24b	26.95 ± 1.67b	26.64 ± 3.83b
1282	Isobornyl acetate	0.17 ± 0.01a	0.16 ± 0.00a	-	0.19 ± 0.04a
Total hydrocarbon sesquiterpenes		51.14	52.59	55.69	55.60
1410	cis-α-Bergamotene	1.43 ± 0.22b	1.79 ± 0.09a	1.87 ± 0.04a	1.7 ± 0.16a
1415	α-Santalene	6.57 ± 0.12b	8.06 ± 0.42a	8.25 ± 0.24a	7.8 ± 0.73a
1431	trans-α-Bergamotene	3.07 ± 0.02b	3.76 ± 0.18a	3.84 ± 0.18a	3.72 ± 0.37a
1453	(E)-β-Farnesene	0.28 ± 0.02a	0.33 ± 0.02a	0.30 ± 0.05a	0.33 ± 0.05a
1459	(Z)-β-Farnesene	39.79 ± 2.99b	38.65 ± 2.2b	41.43 ± 2.33a	42.05 ± 4.13a
Total lactones		2.07	2.44	2.27	2.01
1476	Massoia lactone	2.07 ± 0.01b	2.44 ± 0.08a	2.27 ± 0.5b	2.01 ± 0.46b
Total others		0.11	0.11	-	-

^aRI: linear retention index calculated in relation to the *n*-alkane series (C₈-C₂₀) on an HP-5MS column in the elution order. Means ($\pm$ standard deviation) in the same row followed by the same letter do not differ significantly according to the Scott-Knott test at the 5% probability level.

RI ^a	Compounds	Content (%)			
		NMS	AMS1	AMS2	AMS4
977	1-Octen-3-ol	0.11 ± 0.00a	0.11 ± 0.00a	-	-
Total		97.73	97.28	96.84	97.11
^a RI: linear retention index calculated in relation to the <i>n</i> -alkane series (C ₈ -C ₂₀) on an HP-5MS column in the elution order. Means (± standard deviation) in the same row followed by the same letter do not differ significantly according to the Scott-Knott test at the 5% probability level.					

PCA was performed to understand the relationship among VOCs, dry weight accumulation and photosynthetic pigments and how these parameters vary according to the different number of porous membranes in the caps of the culture vials. For the analysis of variations in composition and treatment, 17 compounds and four treatments were subjected to PCA. The two principal components (PCs) together explained 95.44% of the total variance, with the first principal component (PC1) explaining 78.53% and the second principal component (PC2) explaining 16.91% (Fig. 3). The score plot revealed three distinct clusters (Fig. 3). The results indicated that *A. suaveolens* grown with greater ventilation inside the vial (AMS4 and AMS2) had greater synthesis of (*Z*)- β -farnesene and that samples grown with lower ventilation had greater synthesis of linalool acetate. In AMS1, there was greater synthesis of linalool and massoia lactone. There was also a strong correlation between the AMS4 treatment and all evaluated parameters, including photosynthetic pigments, dry weight production, TLA, NL and LLR and SL. The number, content and profile of VOCs and dry weight production in *Lippia gracilis* grown *in vitro* also varied according to different natural ventilation systems (Lazzarini et al. 2019). Silva et al. (2017) reported that the highest carvacrol content and growth parameters of *Plectranthus amboinicus* were obtained when the plantlets were cultivated with one or two porous membranes. Araújo et al. (2021) showed also that in *Aeollanthus suaveolens* the accumulation of VOCs was influenced by photon flux density and spectral quality.

PCA also confirmed that (*Z*)- β -farnesene were negatively correlated with linalool acetate, linalool and massoia lactone. In addition, all growth parameters studied showed a negative correlation with lower ventilation. In the present study, the number of membranes showed a positive and significant correlation with all studied dry weight production and VOC characteristics of *A. suaveolens*. Thus, improved ventilation with alternative membranes may produce better-quality plantlets.

Conclusion

The micropropagation system under natural ventilation with four membranes promotes better *in vitro* growth of *Aeollanthus suaveolens*, increases the production of photosynthetic pigments, and is superior to the conventional sealed system. The use of different ventilation systems led to differentiation in the contents of the main VOCs. For the *in vitro* culture of *A. suaveolens*, it is recommended that explants be cultivated with greater ventilation in the culture vial.

Abbreviations

VOCs Volatile organic compounds

AMS1 Alternative membrane system using lids with one membrane

AMS2 Alternative membrane system using lids with two membranes

AMS4 Alternative membrane system using lids with four membranes

NMS No membrane system

SN Shoot number

LN Leaf number

RN Root number

SL Shoot length

LLR Length of longest root

TLA Total leaf area

LDW Leaf dry weight

SDW Stem dry weight

RDW Root dry weight

TDW Total dry weight

Declarations

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Author Contributions

All authors contributed substantially to the work reported. Diene Xavier Araújo: writing of the manuscript, investigation. Tainá Teixeira Rocha: investigation. Alexandre Alves de Carvalho: data analysis, graphic

design. Ana Paula Ribeiro Medeiros: investigation. Fernanda Naiara Santos Ribeiro: investigation. Rafael Marlon Alves de Assis: investigation. Suzan Kelly Vilela Bertolucci: concept of the study, chemical analyses. José Eduardo Brasil Pereira Pinto: concept of the study, writing of the manuscript, project administration.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on a reasonable request.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

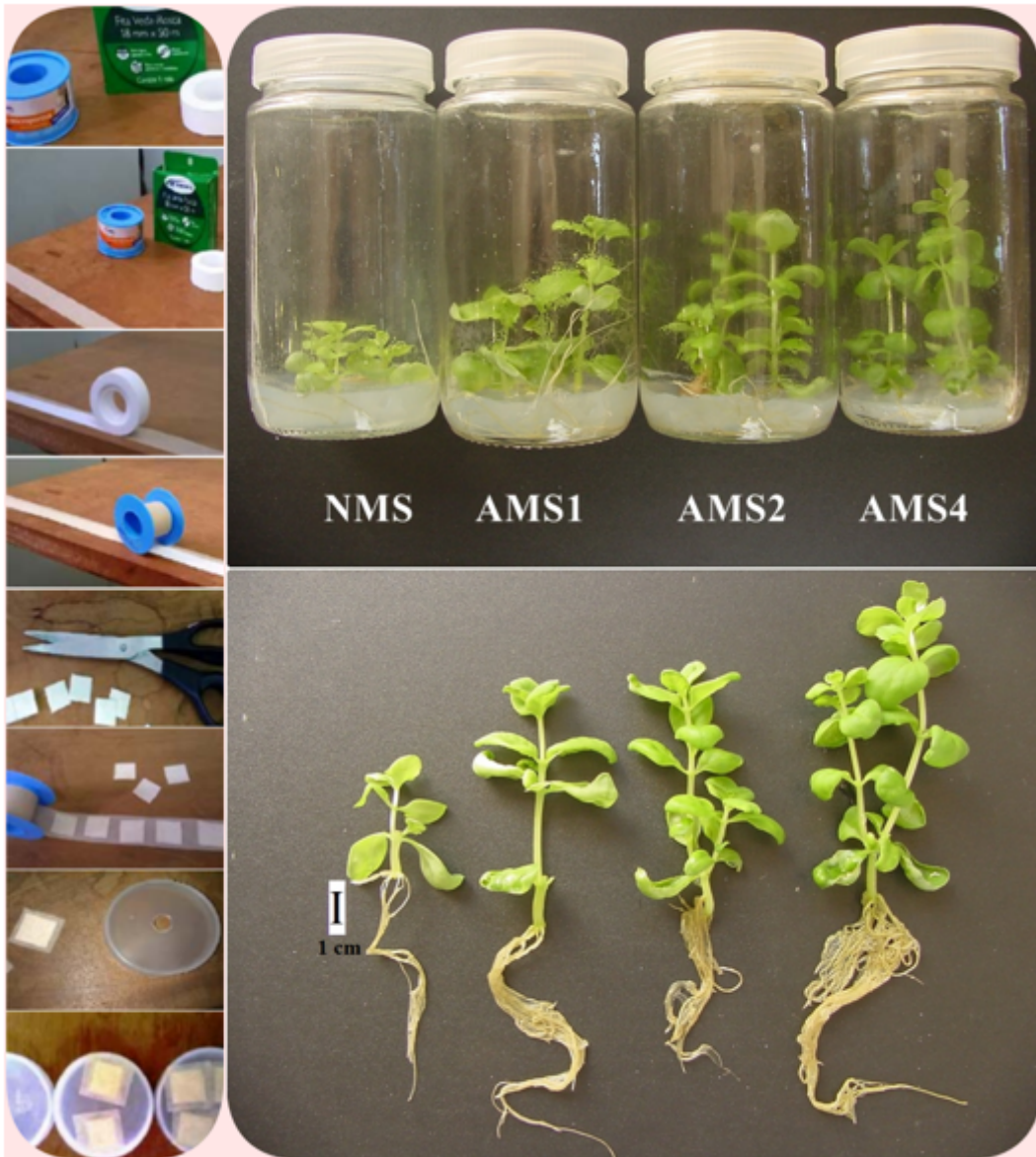


Figure 1

Scheme of the vials with used membranes and *Aeollanthus suaveolens* plantlets grown in MS medium without sucrose addition in a conventional system (NMS) and in natural ventilation systems with one (AMS1), two (AMS2) and four (AMS4) porous membranes.

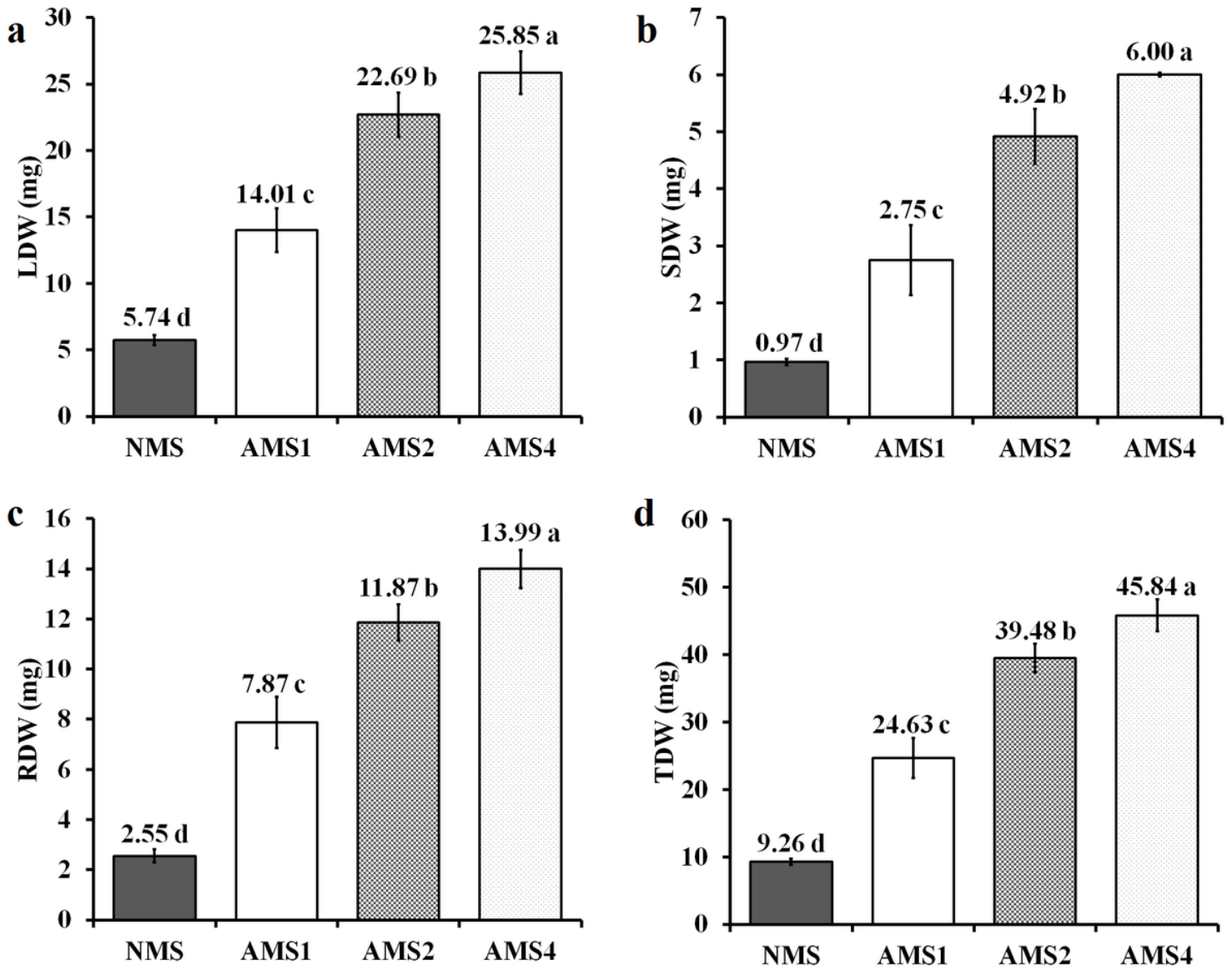


Figure 2

Dry weight of *Aeollanthus suaveolens* plantlets grown in MS medium without sucrose addition in a conventional system (NMS) and in natural ventilation systems with one (AMS1), two (AMS2) and four (AMS4) porous membranes. Leaf dry weight (LDW), shoot dry weight (SDW), root dry weight (RDW), total dry weight (TDW). The means ($\pm$ standard deviation) followed by the same letters do not differ by the Scott-Knott Test ($p < 0.05$).

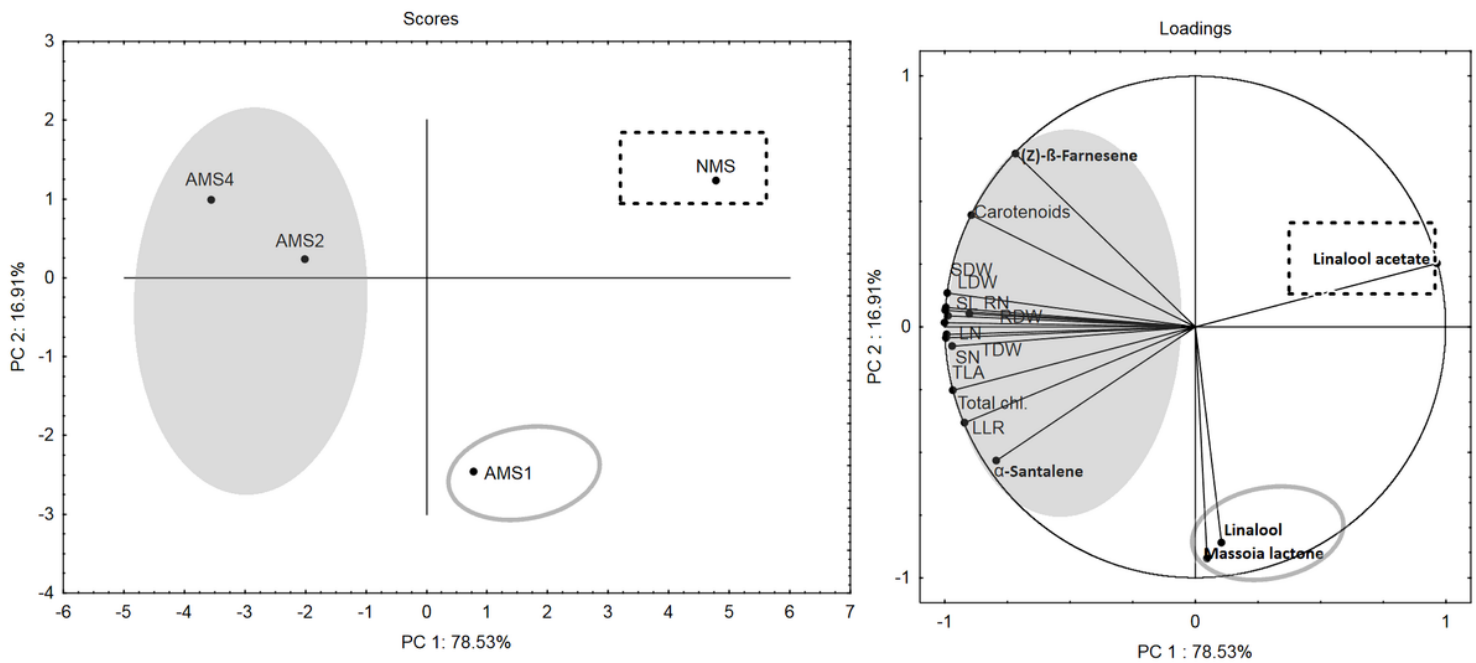


Figure 3

Scores and loadings of the principal component analysis (PCA) of the matrix of correlations based on data for the major compounds and growth parameters of *Aeollanthus suaveolens* Mart. ex Spreng. under different membrane systems. NMS: no-membrane system, AMS: alternative membrane system with 1, 2 or 4 filter membranes.