

Different types of explants and natural ventilation systems influence the accumulation of dry weight and of total phenolic compounds in *Aloysia gratissima* (Verbenaceae)

Diferentes tipos de explantes e sistemas de ventilação natural influenciam o acúmulo de massa seca e de compostos fenólicos totais em *Aloysia gratissima* (Verbenaceae)

Diferentes tipos de explantes y sistemas de ventilación natural influyen en la acumulación de masa seca y compuestos fenólicos totales en *Aloysia gratissima* (Verbenaceae)

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Abstract

The objective of this study was to evaluate the effect of the use of different natural ventilation systems on the growth and accumulation of total phenolic compounds in *Aloysia gratissima*. Nodal and apical segments with 1 pair of leaves were inoculated in flasks containing ½ MS culture medium without sucrose and supplemented with 0.1 mg L⁻¹ indolebutyric acid. The cultivation systems included a conventional system (NMS) and alternative membrane systems with 1 (AMS1), 2 (AMS2) and 4 (AMS4) low-cost membranes. After 30 days, the *in vitro* growth, leaf area, dry weight and accumulation of phenolic compounds were evaluated. Plantlets from apical segments showed superior results in all variables analyzed. The use of the NMS compromised plantlet growth. However, improved results were obtained with the use of porous membranes, with the best growth observed in AMS4. The leaf area of plantlets originating from apical segments was 3.03 times greater than that of plantlets from nodal segments. Plantlets in the NMS had the lowest values of leaf area, root length, number of leaves and dry weight. However, the use of membranes allowed higher growth. The AMS4 treatment increased the leaf dry weight accumulation by 6.13-fold compared to that obtained with the NMS treatment. The accumulation of total phenolic compounds increased with the use of greater numbers of porous membranes. The use of apical segments and lids with 4 porous membranes is recommended for

micropropagation of the species. The use of an alternative membrane system positively influences the accumulation of total phenolic compounds.

Keywords: Plant tissue culture; Secondary metabolites; Photoautotrophic; Micropropagation.

Resumo

Objetivou-se avaliar o uso de diferentes sistemas de ventilação natural no crescimento e no acúmulo de compostos fenólicos totais de plântulas de *Aloysia gratissima*. Segmentos nodais e apicais com um par de folhas foram inoculados em frascos contendo meio de cultivo ½ MS, sem sacarose e suplementado com 0,1 mg L⁻¹ de AIB. Os sistemas de cultivo foram: sistema convencional (SC) e sistema de ventilação natural com uma (SVN1), duas (SVN2) e quatro (SVN4) membranas de baixo custo. Após 30 dias, o crescimento *in vitro*, área foliar, matéria seca e o acúmulo de compostos fenólicos foram avaliados. Plântulas provenientes de segmentos apicais apresentaram resultados superiores em todas as variáveis analisadas. O uso do SC comprometeu o crescimento das plântulas. Entretanto, observou-se uma melhora nos resultados com o uso das membranas porosas, com melhores resultados de crescimento no SVN4. A área foliar de plântulas decorrentes de segmentos apicais foi 3,03 vezes superior às de segmentos nodais. Plântulas em SC apresentaram os menores valores de área foliar, comprimento de raízes, número de folhas e matéria seca. Entretanto, o uso das membranas permitiu maiores valores de crescimento. O uso do SVN4 aumentou o acúmulo de matéria seca das folhas em 6,13 vezes em relação ao SC. O acúmulo de compostos fenólicos totais aumentou com o uso das membranas porosas. É indicado o uso de segmentos apicais e tampas com 4 membranas porosas para a micropropagação da espécie. O uso do sistema de ventilação natural influencia positivamente o acúmulo de compostos fenólicos totais.

Palavras-chave: Cultura de tecidos vegetais; Metabólitos secundários; Micropropagação; Fotoautotrófica.

Resumen

El objetivo fue evaluar el uso de diferentes sistemas de ventilación natural en el crecimiento y la acumulación de compuestos fenólicos totales en plántulas de *Aloysia gratissima*. Se inocularon segmentos nodales y apicales con un par de hojas en frascos con medio ½ MS, sin sacarosa y suplementado con 0,1 mg L⁻¹ de AIB. Los sistemas de cultivo fueron: sistema convencional (SC) y sistema de ventilación natural con una (SVN1), dos (SVN2) y cuatro (SVN4) membranas de bajo costo. Después de 30 días, se evaluó el crecimiento *in vitro*, área foliar, materia seca y acumulación de compuestos fenólicos. Las plántulas de los segmentos apicales presentaron resultados superiores en todas las variables analizadas. El uso del SC afectó el crecimiento de las plántulas. Sin embargo, hubo una mejora en los resultados con el uso de membranas porosas, con mejores resultados de crecimiento en SVN4. El área foliar de las plántulas procedentes de los segmentos apicales fue 3,03 veces mayor que la de los segmentos nodales. Las plántulas en el SC presentaron los valores más bajos de área foliar, longitud de raíz, número de hojas y materia seca. Sin embargo, el uso de membranas porosas permitió obtener mayores valores de crecimiento. El uso de SVN4 incrementó la acumulación de materia seca foliar en 6,13 veces en comparación con SC. La acumulación de compuestos fenólicos totales aumentó con el uso de las membranas porosas. El uso de segmentos apicales y sombreros con 4 membranas porosas es indicado para la micropropagación de la especie. El uso del sistema de ventilación natural influye positivamente en la acumulación de compuestos fenólicos totales.

Palabras clave: Cultivo de tejidos vegetales; Metabolitos secundarios; Micropropagación; Fotoautotrófica.

1. Introduction

Aloysia gratissima (Gillies & Hook.) Tronc. (Verbenaceae) is a perennial shrub that can reach up to 3 m in height and has an irregular growth pattern, a fine stem and small, whitish flowers with an intense perfume (Moroni & O'leary, 2019). Known popularly as whitebrush, its leaves, flowers and stems are used in folk medicine to treat pain and respiratory problems (Santos et al., 2015). Its essential oil has antifungal (Silva et al., 2014), antibacterial (Souza & Wiest, 2007) and anesthetic (Benovit et al., 2015) activities.

The production of medicinal plants via micropropagation aims to obtain identical and pathogen-free plants to be used in the study of the secondary metabolism of the species or for the large-scale production of plantlets (Silva et al., 2017). However, the micropropagation of woody species is difficult to conduct because the plants have a low *in vitro* growth rate due to morphological and physiological disorders, such as oxidation of explants and malformed roots (Thorpe & Harry, 1990).

The use of alternative membrane systems (AMSs) has been shown to be promising in the optimization of the growth of medicinal plants *in vitro* (de Oliveira et al., 2021; Lazzarini et al., 2019; Rocha et al., 2022; Silva et al., 2016; Silva et al., 2017). The use of AMSs allows the entry of CO₂ into the microenvironment and improves photosynthetic capacity while maintaining asepsis and reducing the relative humidity and ethylene concentration in the flasks, enabling the production of

more vigorous plants with greater survival in the acclimatization period (Saldanha et al., 2012). In addition, photoautotrophic micropropagation enables cultivation in the absence of sucrose, reducing production costs and the chances of bacterial contamination (Iarema et al., 2012).

Recent studies have indicated an influence of AMSs on the *in vitro* growth and secondary metabolism of medicinal plants. The use of 1 and 2 porous membranes induced the rooting of *Plectranthus amboinicus* (Lour.) (Lamiaceae), while the use of 4 membranes favored the accumulation of carvacrol in this species (Silva et al., 2017). In a study with *Lippia gracilis* (Schauer) (Verbenaceae), the use of AMS resulted in greater dry weight accumulation, reductions in γ -terpinene and ρ -cymene metabolites and increases in thymol and carvacrol in plantlets grown for 35 days in a cultivation system containing 4 porous membranes (Lazzarini et al., 2019).

Aloysia gratissima is a source of phenolic compounds. Zeni et al. (2013) studied the aqueous extract of *A. gratissima* in 2 seasons and reported values of 21.84 and 18.93 mg GAE/g leaf in leaves collected in autumn and winter, respectively. However, there are no reports in the literature on the micropropagation of this species and its accumulation of total phenolic compounds (TPC) under cultivation *in vitro*. Thus, the objective of this study was to evaluate the use of different AMSs with low-cost alternative membranes for the *in vitro* growth and accumulation of TPC in *A. gratissima* plantlets.

2. Methodology

2.1 General experimental conditions

Seeds of *Aloysia gratissima* (Gillies & Hook.) Tronc. belonging to the Medicinal Plants Garden of the Federal University of Lavras-Minas Gerais were collected and used for *in vitro* establishment. A voucher specimen of the species was deposited in the PAMG herbarium of the Agricultural Research Company of Minas Gerais (EPAMIG) in July 2019 under registration number 58693.

For the *in vitro* establishment of the species, the seeds were immersed in 70% alcohol for 30 seconds, followed by immersion in bleach (1.25% active chlorine) for 15 minutes. Subsequently, they were washed 5 times with autoclaved distilled water and inoculated in test tubes containing 12.5 mL of half-strength ($\frac{1}{2}$) MS culture medium (Murashige & Skoog, 1962) supplemented with 30 g L⁻¹ sucrose and 5.5 g L⁻¹ agar, and the pH was adjusted to 5.7 ± 0.1 before autoclaving (20 minutes at 121 °C). The tubes were maintained in a growth chamber (26 ± 1 °C and a photoperiod of 16 hours) under fluorescent light (39 $\mu\text{mol}^{-2} \text{s}^{-1}$). The plantlets were subculture every 50 days in $\frac{1}{2}$ MS medium supplemented with 0.1 mg L⁻¹ indole-butyric acid (IBA) until the plant material necessary for the experiment was obtained.

2.2 Growth in alternative membrane systems (AMSs)

Apical and nodal segments of approximately 1 cm with a pair of leaves were inoculated vertically in flasks containing 50 mL of $\frac{1}{2}$ MS culture medium (Murashige & Skoog, 1962) without sucrose and supplemented with 5.5 g L⁻¹ agar and 0.1 mg L⁻¹ IBA. Auxin was added to the culture medium because it was previously observed that explants in a conventional system failed to root, lost their leaves and died at approximately the second week of *in vitro* culture. The flasks had lids containing 0, 1, 2 or 4 low-cost porous membranes, prepared according to Saldanha et al. (2012).

After inoculation, the flasks were placed in a growth room under the same conditions used in the *in vitro* establishment phase. After 30 days, the shoot length (SL; cm), longest root length (LRL; cm), number of leaves (NL), number of shoots (NS), leaf, stem, root and total dry weight (LDW, SDW, RDW and TDW; mg) and total leaf area (TLA) were evaluated. The experimental design was a completely randomized design (CRD) in a 2x4 factorial arrangement, with 2 types of explants (apical and nodal) and 4 cultivation systems: 1 conventional system (NMS) and alternative membrane systems with 1 (AMS1), 2 (AMS2) or 4 membranes (AMS4). In total, 8 treatments were prepared with 3 replicates, 2 flasks per replicate and 2

plantlets per flask. To analyze TLA, 4 plantlets per treatment were used, and all leaves were measured using WinFOLIATM software and an EPSON PERFECTION V700 PHOTO scanner.

Four plantlets representative of each treatment were chosen for growth analysis, and the TLA of all leaves was evaluated from the obtained values. The other evaluated parameters included the leaf area ratio (LAR = TLA/TDW); specific leaf area (SLA = TLA/LDW); specific leaf weight (SLW = LDW/TLA) which is the inverse of SLA and gives an estimate of leaf thickness; and leaf weight ratio (LWR = LDW/LDW+SDW); these parameters were calculated according to Benincasa (2003).

2.3 Analysis of total phenolic compounds (TPC)

The effect of cultivation system on the accumulation of TPC was evaluated. For this purpose, extracts prepared from dried leaves of *A. gratissima* pulverized with a mortar and pestle were used. Thirty milligrams of *A. gratissima* powder was weighed directly in microtubes. An aliquot of 1 mL of EtOH:H₂O (3:1) was added to the microtubes. Subsequently, the tubes were vortexed for 30 seconds and subjected to sonication extraction for 15 minutes at room temperature, followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was collected for analysis. The quantification of TPC was performed according to the Folin-Ciocalteu method with modifications (Singleton & Rossi, 1965). Fifty microliters of extract, 500 µL of Folin-Ciocalteu ethanol solution (10%) and 500 µL of Na₂CO₃ (7.0%) were added to 2 mL microtubes. The samples were incubated for 2 hours in the dark at room temperature. After this period, 300 µL was added to a 96-well microplate, and the absorbance was read at 760 nm. The calibration curve consisted of a gallic acid standard (Sigma-AldrichR®, ≥ 98%) in the range of 0.0078 to 0.25 mg m L⁻¹, and the corresponding formula was $y = 6.6918x + 0.2450$ ($R^2 = 0.9947$). All treatments were evaluated in triplicate.

2.4 Statistical analysis

The data were subjected to analysis of variance (ANOVA), and the means of the treatments were compared by the Scott-Knott test at 5% probability using R Development Core Team software.

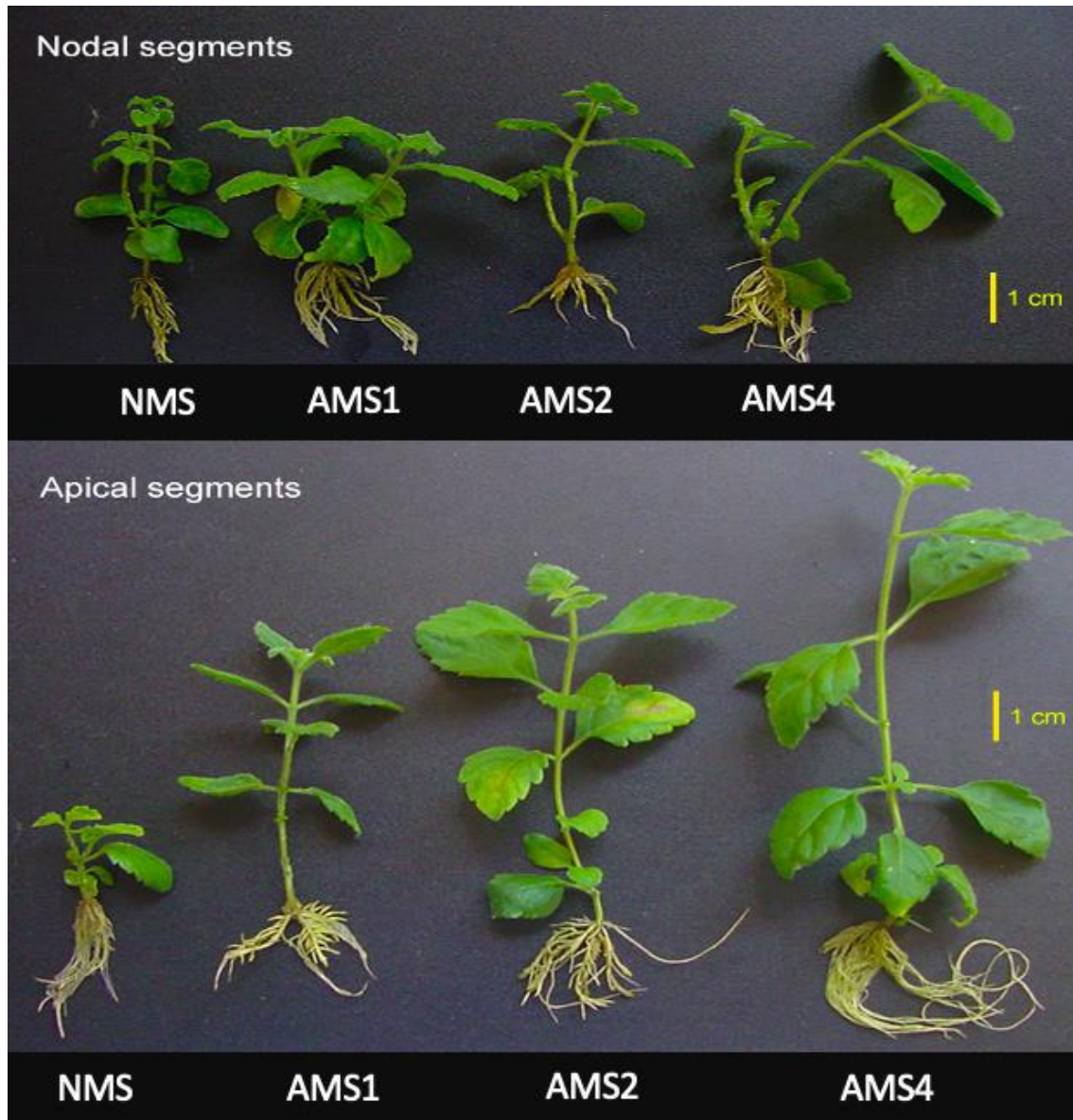
3. Results e Discussion

3.1 *In vitro* growth

The different cultivation systems and types of explants used affected the growth and development of *A. gratissima* plantlets grown *in vitro* (Figure 1 and Table 1). SL, LRL and NL were higher in treatments with apical segments (Table 1). A more developed root system was observed when using apical segments and AMS4 (Figure 1). The use of NMS (control) compromised plantlet growth, leading to lower growth values for all variables analyzed, probably due to the low concentration of CO₂ and the accumulation of ethylene in the flasks.

Better *in vitro* growth was observed with an increased number of membranes (Table 1). The use of porous membranes allows the entry of CO₂ into the culture flasks, reducing the accumulation of ethylene; stimulating the photosynthesis, growth and development of the cultivated plantlets; favoring the absorption of nutrients due to greater transpiration; and allowing vigorous plantlets to be obtained (Saldanha et al., 2012).

Figure 1 - *Aloysia gratissima* plantlets from nodal and apical segments cultured for 30 days in different cultivation systems: a conventional system (NMS) and systems with 1 porous membrane (AMS1), 2 membranes (AMS2) and 4 membranes (AMS4).



Source: Authors (2022).

Table 1 - Shoot length (SL), largest root length (LRL), number of leaves (NL), total leaf area (TLA), leaf dry weight (LDW), stem dry weight (SDW), root dry weight (RDW) and total dry weight (TDW) of *Aloysia gratissima* plantlets derived from nodal and apical segments cultured *in vitro* in different cultivation systems: a conventional system (NMS) and systems with 1 porous membrane (AMS1), 2 membranes (AMS2) and 4 membranes (AMS4) at 30 days.

Explant	Cultivation system	SL (cm)	LRL (cm)	NL	TLA (cm ²)	LDW (mg)	SDW (mg)	RDW (mg)	TDW (mg)
Nodal	NMS	2.37 e	1.97 c	6.50 c	2.66 e	6.37 e	1.46 d	1.40 f	9.23 e
	AMS1	3.17 d	1.30 c	6.67 c	2.78 e	7.19 e	1.23 d	2.97 e	11.39 e
	AMS2	3.30 c	2.18 c	6.67 c	2.86 e	11.40 d	1.24 d	4.71 d	17.69 d
	AMS4	3.97 c	3.42 b	8.00 b	10.30 c	19.88 c	3.62 c	5.72 c	29.22 c
Apical	NMS	2.17 e	2.03 c	4.00 d	2.82 e	2.68 f	1.37 d	1.60 f	5.65 f
	AMS1	4.20 c	3.20 b	7.33 c	4.92 d	13.58 d	2.53 d	4.85 d	20.97 d
	AMS2	5.50 b	3.30 b	8.33 b	17.77 b	23.99 b	4.67 b	6.73 b	35.39 b
	AMS4	7.70 a	4.65 a	9.67 a	27.75 a	35.65 a	7.23 a	8.77 a	51.65 a
Average for explants									
	Nodal	3.20 b	2.22 b	6.96 b	4.39 b	11.21 b	1.89 b	3.70 b	16.88 b
	Apical	4.89 a	3.30 a	7.33 a	13.32 a	18.98 a	3.95 a	5.48 a	28.41 a
Average for cultivation system									
	NMS	2.27 d	2.00 c	5.25 c	2.24 d	4.53 d	1.41 c	1.50 d	7.44 d
	AMS1	3.68 c	2.25 c	7.00 b	3.85 c	10.39 c	1.88 c	3.91 c	16.18 c
	AMS2	4.40 b	2.74 b	7.50 b	10.30 b	17.70 b	2.96 b	5.72 b	26.54 b
	AMS4	5.83 a	4.03 a	8.83 a	19.03 a	27.77 a	5.42 a	7.25 a	40.43 a
SD (%)		16.29	15.17	8.45	7.96	11.61	19.00	12.02	8.61

Means followed by the same letter do not differ statistically from each other by the Scott-Knott test at the 5% probability level. Sources: Authors (2022).

The best type of explant for micropropagation varies with species and therefore needs to be tested. For example, *Morus alba* had a higher number of shoots in nodal segments than in apical segments (Anis et al., 2003). However, much of the literature indicates that apical segments are the most efficient in micropropagation. *Coleus blumei* had better growth *in vitro* with the use of apical segments than nodal segments (Rani et al., 2006). A similar result was obtained for *Alternanthera sessilis* L. (Amaranthaceae), in which the explants that developed better were also apical segments (Edward et al., 2011). For *Eugenia involucrata*, the best explants were apical segments because they required ½ MS medium to obtain the same results as nodal segments in full-strength MS medium, which corresponds to reagent savings and increased practicality (Golle et al., 2012). One of the explanations for the greater efficiency of apical segments than nodal segments is the faster development of new plantlets, as occurred in the genus *Rubus* (Wu et al., 2009). When there is a significant effect of the position of the explant on characteristics of interest, the explant type must be standardized to avoid estimation errors in experiments (Pereira et al., 2005).

Gas exchange in micropropagation has been shown to be an effective means of improving plantlet quality. Silveira et al. (2019) observed that natural ventilation resulted in increases of 448.53% in the number of leaves, 85.64% in chlorophyll a content and 74.90% in chlorophyll b content in micropropagated *Eugenia dysenterica* DC. (Myrtaceae). Gas exchange in tissue culture decreases contamination of the culture medium (Xiao et al., 2011) and helps during the acclimatization period by favoring anatomical and physiological characteristics of resistance to the external environment (Martins et al., 2015; Wu & Lin, 2013). In addition, the control of excess water prevents hyperhydricity, or vitrification, of the explant (Kozai et al., 1997). Another problem that is minimized is the accumulation of ethylene gas, which causes harmful physiological changes to plantlets (Kozai & Kubota, 2001). Lazzarini et al. (2019) reported that an alternative system with 4 membranes and the type of explant significantly influenced the growth and volatile fraction of *Lippia gracilis*. Rocha et al. (2022) also reported that the

natural ventilation with four membranes increase plantlet dry weight, photosynthetic pigments and phenolic compounds of *Lippia dulcis*. Alternative membrane systems allow more aeration, positively influencing the plantlets quality and vigor of *M. arvensis* and *M. viridis* ((de Oliveira et al., 2021).

The use of apical segments led to a 3.03-fold increase in TLA (Table 1). A larger TLA is directly related to higher TDW, since the plant is able to intercept more photons for the production of photoassimilates via photosynthesis (Magalhães, 1979). Plantlets grown in AMS4 had the highest mean TLA value (19.03 cm²), which was higher than those of all other treatments. Similar results were observed by Ševčíková et al. (2019) when studying the micropropagation of tobacco (*Nicotiana tabacum*): the TLA of plantlets grown in an AMS was 6.5-fold greater than that of plantlets grown in a conventional system.

The type of explant and the cultivation system used also affected dry weight accumulation in *A. gratissima* plantlets (Table 1). Explants cultivated under AMS4 showed a mean LDW of 27.77 mg per plantlet, 6.13-fold higher than the LDW of plantlets grown in NMS (4.53 mg/plantlet). The use of apical segments increased LDW by 59% compared to that obtained with the use of nodal segments. There was a 5.43-fold increase in TDW when using AMS4 (40.43 mg/plantlet) compared to that obtained with NMS (7.44 mg/plantlet). The type of explant affects the amount of biomass and varies with species. Apical segments produced less dry weight than nodal segments in the species *Pfaffia glomerata* (Spreng.) (Amaranthaceae) (Nicoloso & Erig, 2002). In contrast, nodal segments of apple trees produced more dry weight (Pereira & Fortes, 2001). In potato, the highest biomass was observed in nodal segment treatment due to the greater NS (Pereira et al., 2005). In *A. gratissima*, the highest biomass and LRL were observed for apical segments (Table 1), which may have provided greater absorption of nutrients from the culture medium. In addition, the greater TLA and NL (Table 1) may have favored photosynthetic bioaccumulation.

Various parameters were used to evaluate plantlet growth and to infer plantlet behavior *in vitro* (Table 2). SLA relates TLA with LDW and is therefore a morphological and anatomical parameter. In accordance with increasing CO₂ concentration, SLA increased with an increase in the number of porous membranes in the flask. Ziska et al. (2008) observed a progressive increase in the SLA of *Papaver setigerum* with increasing CO₂ concentration. Lazzarini et al. (2019) reported no difference in SLA in *Lippia gracilis*. Benicasa (2003) reported that if we consider weight as an expression of leaf volume, the inverse of SLA, which is known as SLW, indicates leaf thickness. The *A. gratissima* plantlets showed greater thickness when grown at a lower concentration of CO₂ (Table 2). According to Benicasa (2003), LAR is a morphophysiological component because it is the ratio of the area available for the interception of CO₂ and light to TDW. *A. gratissima* plantlets exhibited a greater LAR with an increase in the number of porous membranes in the lid. It can be inferred that the plantlets grown with a greater number of filters in the lid needed a larger area to produce 1 g of dry weight. Thus, the plantlets achieved a higher dry weight for all organs (leaf, stem, and root). Lazzarini et al. (2019) observed that the lower the LAR was, the higher the efficiency of plant dry weight production was. LWR is the weight of dry weight retained in the leaves and the weight of dry weight stored in the whole plant; in this case, LWR was defined as LDW + SDW. Plantlets grown in the AMS treatments had a higher LWR, indicating that a greater fraction of dry weight accumulated in the leaves under these conditions than under cultivation without the use of porous membrane (NMS).

Table 2 - Plantlet growth analysis of *Aloysia gratissima* grown *in vitro* for 30 days under different alternative membrane systems: a conventional system (NMS) and systems with 1 porous membrane (AMS1), 2 membranes (AMS2) and 4 membranes (AMS4). Total leaf area (TLA), leaf area ratio (LAR), specific leaf area (SLA), leaf weight ratio (LWR) and specific leaf weight (SLW).

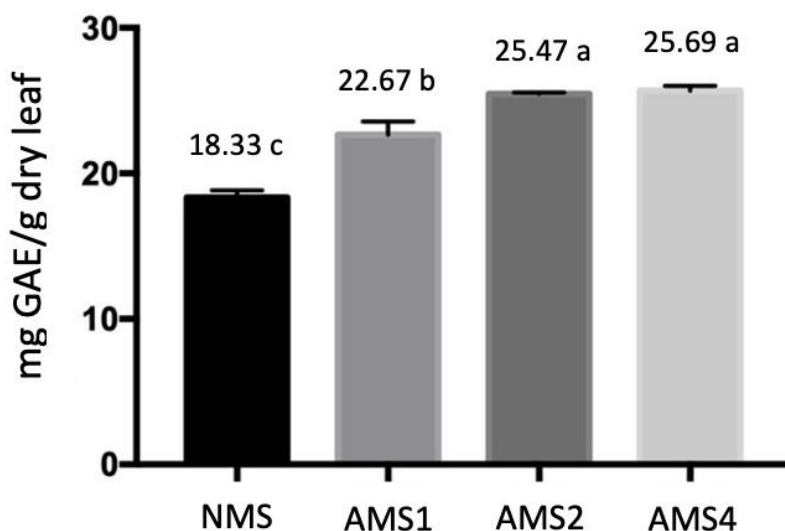
Cultivation system	TLA cm ²	LAR cm ² /mg	SLA cm ² /mg	LWR mg/mg	SLW mg/cm ²
Average for cultivation system					
NMS	2.24 d	0.301	0.494	0.763	2.022
AMS1	3.85 c	0.238	0.370	0.847	2.699
AMS2	10.30 b	0.388	0.582	0.857	1.718
AMS4	19.03 a	0.472	0.685	0.837	1.459

Means followed by the same letter do not differ statistically from each other by the Scott-Knott test at the 5% probability level.
Sources: Authors (2022).

3.2 Analysis of total phenolic compounds (TPC)

The use of AMSs positively influenced the accumulation of TPC in micropropagated *A. gratissima* plantlets (Figure 2). Plantlets grown under NMS had the lowest TPC values (18.33 mg GAE/g leaves), while the best results were observed using AMS2 (25.47 mg GAE/g leaves) and AMS4 (25.69 mg GAE/g leaves).

Figure 2 - Total phenolic compounds (TPC) of *Aloysia gratissima* plantlets cultured *in vitro* from nodal and apical segments in different cultivation systems: a conventional system (NMS) and systems with 1 porous membrane (AMS1), 2 membranes (AMS2) and 4 membranes (AMS4) at 30 days. Means followed by the same letter do not differ statistically from each other by the Scott-Knott test at the 5% probability level.



Source: Authors (2022).

Similar results were found by Ayuso et al. (2019), who reported high values of phenolic compounds in *Eryngium viviparum* (J. Gay) (Apiaceae) plantlets that showed good growth and development *in vitro*. The results observed may be related to the decrease in the volume of culture medium due to the higher evaporation observed in the AMS2 and AMS4 treatments, which may have affected the secondary metabolism of the plantlets, probably due to the stress caused. Silva et al. (2017) observed that a low moisture content and high gas exchange due to the use of a natural ventilation system were responsible for the activation of metabolic processes in *Plectranthus amboinicus* (Lour.) (Lamiaceae), where the plant responded with the activation of carvacrol biosynthesis.

The complexity of phenolic compounds hinders their artificial production, and biotechnological manipulation of plant tissue culture conditions is a safe alternative to increase their production (Dias et al., 2016). Gas exchange and the reduction of sugar in the culture medium force the plantlets to develop a photomixotrophic metabolism, which stimulates the production of phenolic compounds (Ríos-Ríos et al., 2019). This increase in the production of phenolic compounds is supported by the physiological improvements resulting from photomixotrophic propagation, such as reduced stress signaling caused by excess sugar (Badr et al., 2011; Horacio & Martinez-Noel, 2013) and reduced inhibition of photosynthesis (Couto et al., 2014).

4. Conclusion

The type of explant interferes with the growth and development of micropropagated *A. gratissima* plantlets. The type of micropropagation system (with or without ventilation) and the number of membranes used to perform gas exchange affect the dry weight and total phenolic compounds contents of the plantlets. For the micropropagation of *A. gratissima*, the use of apical segments and lids with 4 porous membranes is indicated. Future research should be conducted to analyze whether

membrane type and number affect the chemical compounds of the essential oil.

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