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In vitro propagation of lemon verbena: a plant native of South America

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ABSTRACT. *In vitro* propagation increases the supply and commercialisation of products of interest. For this, optimising the growing conditions and the composition of the culture medium is crucial to benefit the full development of the plants. Thus, the objective was to evaluate the *in vitro* propagation of *Aloysia triphylla* on different culture media, with varying agar and sucrose concentrations. The experiment was conducted as a completely randomised design, 3×3×3 factorial scheme, with three culture media (MS, JADS and WPM), three sucrose concentrations (8, 10 and 12 g L^{-1}) and three agar concentrations (15, 30 and 45 g L^{-1}), with three replicates each and experimental units composed of one plant per replicate. After 25 days of cultivation, the fresh and dry mass of the plants, numbers of leaves, numbers of nodes, plant lengths, numbers of oxidised leaves, hyperhydricity and acclimatization percentages were evaluated. The WPM medium resulted in a reduced fresh mass, reflecting in the low hyperhydricity observed in the explants, and favoured the acclimatization of the plants. Thus, the WPM medium with sucrose (15 g L^{-1}) and agar (12 g L^{-1}) is recommended as the medium most suitable for the *in vitro* regeneration of *Aloysia triphylla*.

Keywords: *Aloysia triphylla*; Morphological disorders; Hyperhidricity; Acclimatization.

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Introduction

Brazil stands out in the production of essential oils alongside India, China and Indonesia, which are considered the world's largest producers (Paulus, Valmorbida, Toffoli, & Paulus, 2014). Essential oils are products of plant origin that are composed of low molecular weight compounds, mainly monoterpenes and sesquiterpenes, which are prescribed for a variety of health problems worldwide (Raut & Karuppayil, 2014; Bahramsoltani et al., 2018).

Aloysia triphylla (L'Herit) Britton (common name, lemon verbena) is a medicinal plant of the Verbenaceae family native to South America and grown in North Africa and Southern Europe, and it grows up to 3.0 meters in height (Zeppenfeld et al., 2014; Bahramsoltani et al., 2018). The species has several botanical synonyms, among them *Aloysia citriodora*, and it is popularly known as cidró, cidrão or erva-luísa. It is cultivated in southern Brazil, and it is adaptable to temperate climates (Rojas, Palacios, & Ronceros, 2012; Paulus, Valmorbida, Toffoli, Nava, & Paulus, 2013; Schmidt et al., 2017; Bahramsoltani et al., 2018).

The most important medicinal product of this plant is the essential oil, which contains neral, geraniol, limonene, cineol, β-caryophyllene and espatulenol, and it is responsible for a series of biological activities, such as antimicrobial, insecticidal, neurophysiological, anxiolytic, gastrointestinal, anti-inflammatory, cardiovascular and anticancer effects, among others (Gomes et al., 2009; Jimenez-Ferrer et al., 2017; Bahramsoltani et al., 2018). Specifically, this essential oil has a high potential to be used as an alternative to the use of synthetic antibiotics (Souza et al., 2017) as well as anaesthetics, and it is capable of inducing aversive behaviour in fish (Junior et al., 2018). In humans, current studies on the effectiveness of this plant are mainly related to antioxidant activity, with the improvement of the antioxidant capacity of blood cells, the induction of endogenous defence antioxidants and the reduction of oxidative damages (Bahramsoltani et al., 2018).

Several climatic factors directly affect the quality, efficacy and safety of the final product, and to avoid these problems, industries have been working to increase the quantity and quality of this raw material through the cultivation of medicinal plants at a large scale. For this, plant tissue culture techniques provide an alternative for *Aloysia triphylla* multiplication, allowing large-scale production of seedlings through *in vitro* propagation, which offers the phytosanitary quality of the material at a rapid rate and in a short period. In addition, the *in vitro* propagation of medicinal plants can provide uniform chemical compositions and high yields of essential oil; however, the relevant information about the species in question is still limited.

The optimisation of the cultural conditions as well as the composition of the culture medium becomes important, because when any of these factors are in disorder, it can lead to morphophysiological alterations that are detrimental to the development of the plant (Palma, Schuelter, Stefanello, & Fortes, 2011). These include hyperhydricity, a disorder that has as its main symptom the swelling of the shoots, which are light green in colour, with translucent leaves that have a glass-like appearance and are elongated, turgid and fragile (Vasconcelos, Tomas, Camara, & Willadino, 2012). This disorder can affect the regeneration and micropropagation of species (Liu et al., 2017). In addition, the same authors report that hyperhydricity has affected applications of *in vitro* culture in scientific research and industrial production.

Considering the great medicinal potential of *Aloysia triphylla*, and given the scarcity of information in the literature about *in vitro* cultivation, the present study is justified. Thus, the objective was to evaluate the potential of *in vitro* propagation of *A. triphylla* submitted to different culture media having different agar and sucrose concentrations, with the purpose of developing an efficient protocol for the rapid production of shoots in successive subcultures, since that is a fundamental step of this technology.

Material and methods

Plant material and culture conditions

We used nodal segments of *Aloysia triphylla* matrix plants as explants. These plants were grown in pots containing Carolina® commercial substrate and maintained in a protected environment (galvanised steel greenhouse), arranged in the east-west direction, with a semi-circular roof, 10 × 20 m and 3.0 m high, covered with 150 µm thick transparent low-density polyethylene film treated to resist ultraviolet radiation, with non-selective 87% transmittance.

Based on previous experiments of culture establishment (data not shown), the plant nodal were excised and kept in a sodium hypochlorite solution (1% active chlorine) and under laboratory conditions. Under laboratory the leaves were removed and the nodal segments retained in running water for one hour. In a laminar flow chamber, nodal segments of approximately 1.5 cm in length were disinfected in ethyl alcohol (70%) for 30 seconds, followed by sodium hypochlorite (0.8% active chlorine) for 15 minutes and five washes in autoclaved distilled water. After this procedure, the nodal segments were excised to 1.0 cm in length and then inoculated into test tubes (25 × 150 mm) each containing 10 mL of macronutrients, micronutrients and vitamins in the MS medium (Murashige & Skoog, 1962) supplemented with 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose and 1.0 mg L^{-1} 6-benzylaminopurine (BAP). The medium was solidified with 8 g L^{-1} agar and the hydrogen potential (pH) was adjusted to 5.8 ± 0.1, and the tubes were autoclaved at 120°C and 108 kPa for 15 minutes. The explants were maintained under grow room conditions for seven days in the dark and 18 days with a photoperiod of 16 hours of light, a temperature of 25 ± 2°C and a luminous intensity of 36 µmol m^{-2} s^{-1} from two fluorescent lamps (Luz do Dia Especial, 40W, Osram, Brazil).

In order to obtain explants amount necessary for the installation of the experiment, *Aloysia triphylla* shoots previously established *in vitro* were sub-cultured five times every 25 days in glass bottles (550 mL) containing 50 mL of the same culture medium and maintained under the same conditions as above.

Experimental design and treatments

The experiment was conducted in a completely randomised design (CRD), in a 3×3×3 factorial scheme with three replications, and the experimental unit was composed of one plant per replicate. The three culture media tested were MS (Murashige & Skoog, 1962), JADS (Correia, Gonçalves, Couto, & Ribeiro, 1995) and WPM (Lloyd & Mccown, 1980), and the three sucrose concentrations were 15, 30 and 45 g L^{-1}. The three agar concentrations were 8, 10 and 12 g L^{-1} (Table 1).

Table 1. Treatments (T1-T27) used for analysis of the variables in *Aloysia triphylla* plants submitted to different culture media, sucrose (suc) and agar concentrations (g L^{-1}).

Code	Treatments	Code	Treatments	Code	Treatments
T1	MS + 15 suc + 8 agar	T10	JADS + 15 suc + 8 agar	T19	WPM + 15 suc + 8 agar
T2	MS + 15 suc + 10 agar	T11	JADS + 15 suc + 10 agar	T20	WPM + 15 suc + 10 agar
T3	MS + 15 suc + 12 agar	T12	JADS + 15 suc + 12 agar	T21	WPM + 15 suc + 12 agar
T4	MS + 30 suc + 8 agar	T13	JADS + 30 suc + 8 agar	T22	WPM + 30 suc + 8 agar
T5	MS + 30 suc + 10 agar	T14	JADS + 30 suc + 10 agar	T23	WPM + 30 suc + 10 agar
T6	MS + 30 suc + 12 agar	T15	JADS + 30 suc + 12 agar	T24	WPM + 30 suc + 12 agar
T7	MS + 45 suc + 8 agar	T16	JADS + 45 suc + 8 agar	T25	WPM + 45 suc + 8 agar
T8	MS + 45 suc + 10 agar	T17	JADS + 45 suc + 10 agar	T26	WPM + 45 suc + 10 agar
T9	MS + 45 suc + 12 agar	T18	JADS + 45 suc + 12 agar	T27	WPM + 45 suc + 12 agar

Plants with a nodal segment and without adventitious roots were inoculated in 550 mL glass flasks containing 50 mL each of one of the different culture media, plus the different sucrose concentrations, 100 mg L^{-1} myo-inositol and 1.0 mg L^{-1} of BAP and solidified with different agar. concentrations. The pH was adjusted to 5.8 ± 0.1 and the vials were autoclaved at 120°C at 108 kPa for 15 minutes. The plants were maintained in a growth room with a photoperiod of 16 hours of light, a temperature of 25 ± 2°C and a luminous intensity of 36 µmol m^{-2} s^{-1} from two fluorescent lamps (Luz do Dia Especial, Osram, Brazil).

For the acclimatization evaluation, the plants were kept in 200 mL plastic pots containing Carolina® substrate and maintained under laboratory conditions. In this evaluation, four replications were used, and the experimental unit was composed of one plant per replicate.

After 25 days of cultivation, the variables fresh and dry mass of plants, number of leaves, number of nodes, plant length, number of oxidised leaves and hyperhydricity were evaluated. The percentage of acclimatization was evaluated after 15 days of *ex vitro* conditions. For the variable percentage of acclimatization, a CRD was used with 27 treatments (Table 1).

The data were submitted to analysis of variance and the means were compared by the Tukey test at 5% of significance. All statistical analyses were performed using the statistical program SISVAR (Ferreira, 2011). For the non-parametric hyperhydricity variable, scores of 1 to 3 (1 = without hyperhydricity, 2 = 50% of hyperhydricity and 3 = 100% of hyperhydricity) were given, and it was tested by the Kruskal-Wallis test at 5% significance, in all 27 treatments.

Results

By the analysis of variance F test at 5% of significance, there was no difference for the dry mass of plants and number of leaves. The fresh mass of plants showed a significant difference for the factors culture media × sucrose concentrations and sucrose concentrations × agar concentrations. For the number of nodes, there was significant difference only for sucrose concentrations. For plant length, there was significance for the type of culture medium and the sucrose concentrations as well as for sucrose concentrations × agar concentrations. For the number of oxidised leaves, there was a significant difference for the triple interaction of factors (culture media, sucrose and agar).

When compared to culture media × sucrose concentrations, the fresh mass of plant had a significant difference only for the MS culture medium, for which concentrations of 30 and 15 g L^{-1} were similar (0.207 and 0.241 g, respectively) and compared to the addition of 45 g L^{-1} (0.156 g). Comparing sucrose concentrations × culture media, there was a significant difference only at the 15 and 30 g L^{-1} concentration, in which the MS medium was superior to JADS and WPM (Figure 1A). For the sucrose concentrations × agar concentrations, there was a significant difference for 10 g L^{-1} agar associated with 15 and 30 g L^{-1} sucrose (0.204 and 0.192 g, respectively), which was greater than that of 45 g L^{-1} (0.134 g). For 12 g L^{-1} agar, 30g L^{-1} sucrose was higher when compared to the other sucrose concentrations (0.186 g). In contrast, for agar concentrations × sucrose concentrations, 15 g L^{-1} associated with 8 and 10 g L^{-1} of agar were higher (0.180 and 0.204 g, respectively). The concentration of 45 g L^{-1} of sucrose with 8 g L^{-1} of agar was superior to that of all others (0.186 g) (Figure 1B).

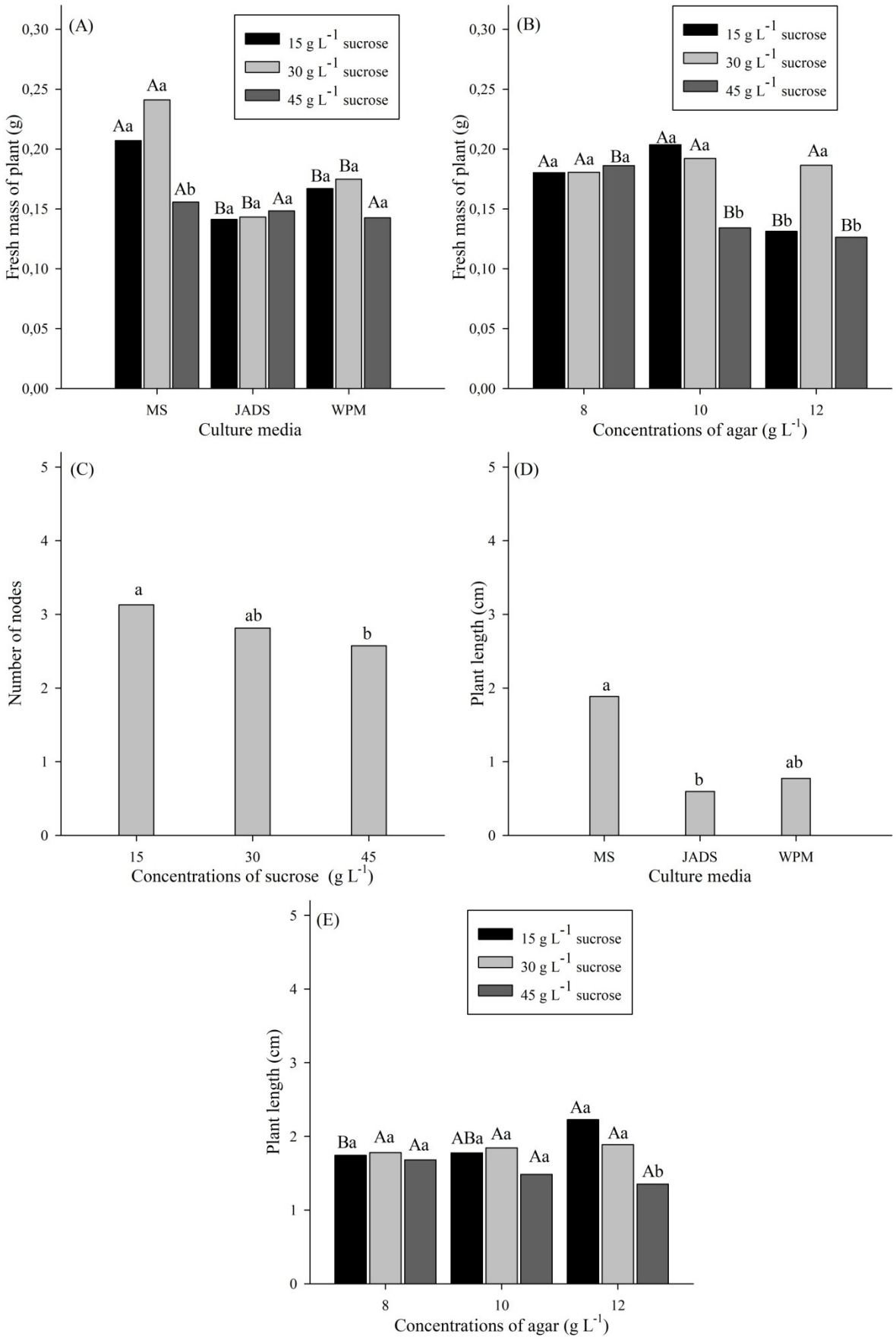

Figure 1. Fresh mass (A, B), number of nodes (C) and plant length (D, E) of *Aloysia triphylla* plants grown in different culture media and sucrose and agar concentrations. A: Equal lowercase letters for the same culture medium and uppercase letters for the same agar concentration do not differ by Tukey test (p<0.05). B and E: Equal lowercase letters for the same concentration of agar and uppercase letters for the same sucrose concentration do not differ from each other by the Tukey test (p<0.05).

For the number of nodes, the concentration of 15 g L^{-1} sucrose (3.129) was higher only when compared to 45 g L^{-1} (2.574) (Figure 1C). The plant lengths were higher when submitted to MS medium (1,887 cm), presenting a significant difference when compared to JADS medium (1,597 cm) (Figure 1D). For the combined factors sucrose concentrations × agar concentrations, there was a significant difference only for 12 g L^{-1}, with which 15 and 30 g L^{-1} (2.228 and 1.889 cm, respectively) were higher than when compared to 45 g L^{-1} of sucrose (1.352 cm) (Figure 1E).

When comparing sucrose concentrations at culture medium levels with agar concentrations, there was a significant difference only with the addition of 45 g L^{-1} of sucrose, in which the JADS medium plus 12 g L^{-1} of agar had a higher number of oxidised leaves (5.50) when compared to concentrations of 8 and 10 g L^{-1} (2.5 and 2.67, respectively).

For the culture medium within each level of agar and sucrose concentrations, the JADS culture medium (5.50) was superior only when compared to MS (1.67) at the concentrations of 45 g L^{-1} sucrose and 12 g L^{-1} agar. For agar concentrations within culture medium levels × sucrose concentrations, JADS supplemented with 45 g L^{-1} sucrose (5.50) was superior to that of 15 and 30 g L^{-1} (1.5 and 2.0, respectively) (Figure 2).

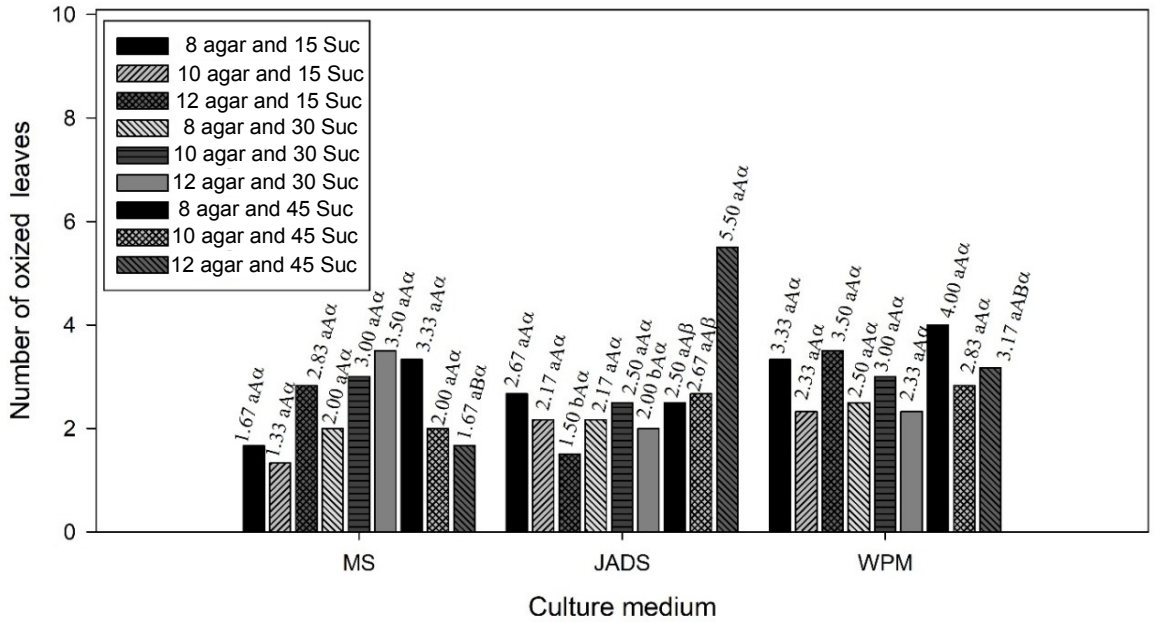

Figure 2. Variable number of oxidized leaves of *Aloysia triphylla* plants submitted to different culture media, sucrose and agar concentrations. *Equal lowercase letters at agar concentrations within each level of culture medium and sucrose concentrations; Equal capital letters in culture medium within each level of sucrose and agar concentrations; Equal greek letters at sucrose concentrations within each level of culture media and agar concentrations do not differ from each other by the Tukey test (p<0.05).

By the results of the Kruskal Wallis test (p<0.05) for the hyperhydricity variable, when comparing all the treatments (Table 1), the most influential factor was the culture medium. This is since the WPM medium (T19 to T27), independent of the concentration of sucrose and agar used in its composition, had the lowest values for this variable, with a maximum score of 6. The JADS medium (T10 to T18) showed a low occurrence of hyperhydricity for treatments with 12 g L^{-1} of agar, with a minimum score of 6 (T12, T15 and T18) and a maximum of 12 (T10, T11, T13, T14, T16 and T17). In contrast, the MS medium (T1 to T9) showed the highest occurrence of hyperhydricity, with T1 and T2 having the highest scores (18). Only T6 had a minimum score of 6 (Table 1, Figure 3A).

For the percentage of acclimatization, the most influential factor was the culture medium, as the WPM medium, with the treatments T21 (WPM + 15 sucrose + 8 agar), T22 (WPM + 30 sucrose + 8 agar), T23 (WPM + 30 sucrose + 10 agar) and T25 (WPM + 45 sucrose + 8 agar) were superior for the acclimatization of plants (100, 100, 100 and 75%, respectively). They differed from T2 (MS + 15 sucrose + 10 agar), with 25% survival, and it was higher when compared to the other treatments, which did not survive when submitted to *ex vitro* conditions (Table 1, Figure 3B).

The treatments that presented high acclimatization and low hyperhydricity were those with WPM medium, being proved with the production of more vigorous and developed plants (Figure 4), except for T24, T26 and T27, which comprised a high sucrose or agar concentration, with apparent yellowing and oxidation of plants.

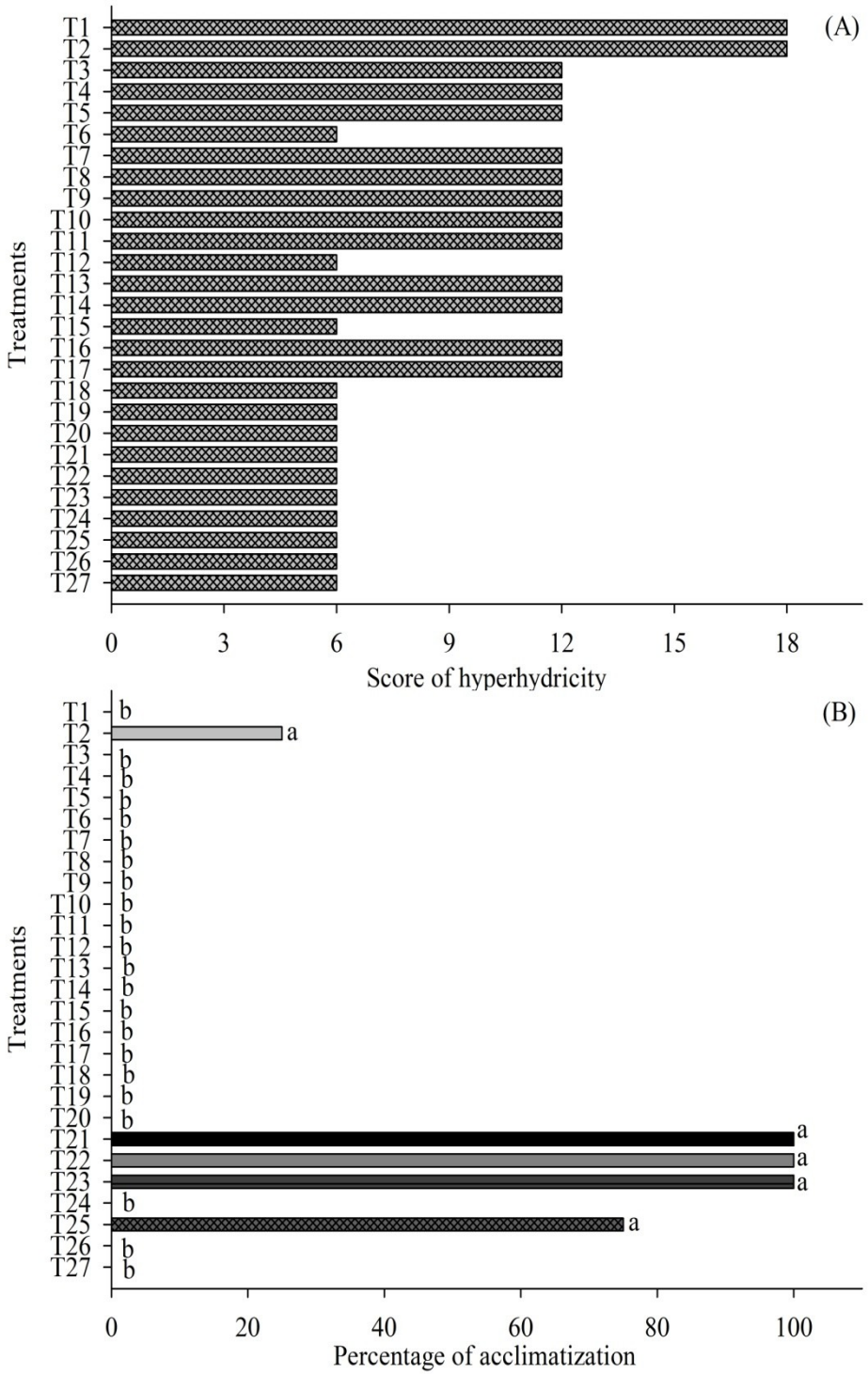

Figure 3. Hyperhydricity (A) and percentage of acclimatization (B) of *Aloysia triphylla* plants grown in different culture media and sucrose and agar concentrations. (B) Equal letters do not differ by Tukey test at 5% significance.

Most plants in MS culture medium showed hyperhydricity and greater yellowing and oxidation symptoms (Figure 3A and 5). The same was observed when the plants were submitted to JADS culture medium, in which the elevation of sucrose or agar concentrations, the plants remained hyperhydric, yellow leaves and apparent oxidation (Figure 6).

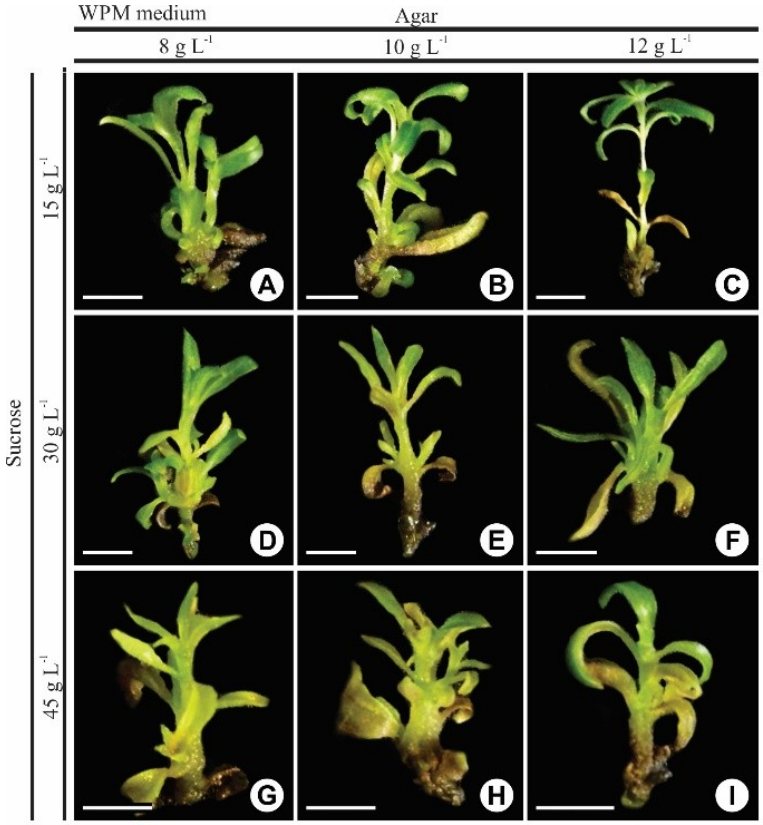

Figure 4. *In vitro* propagation of *Aloysia triphylla* in WPM medium supplemented with different sucrose and agar concentrations. T19 - 15 sucrose + 8 agar (A); T20 - 15 sucrose + 10 agar (B); T21 - 15 sucrose + 12 agar (C); T22 - 30 sucrose + 8 agar (D); T23 - 30 sucrose + 10 agar (E); T24 - 30 sucrose + 12 agar (F); T25 - 45 sucrose + 8 agar (G); T26 - 45 sucrose + 10 agar (H); T27 - 45 sucrose + 8 agar (I). Bars: 1 cm.

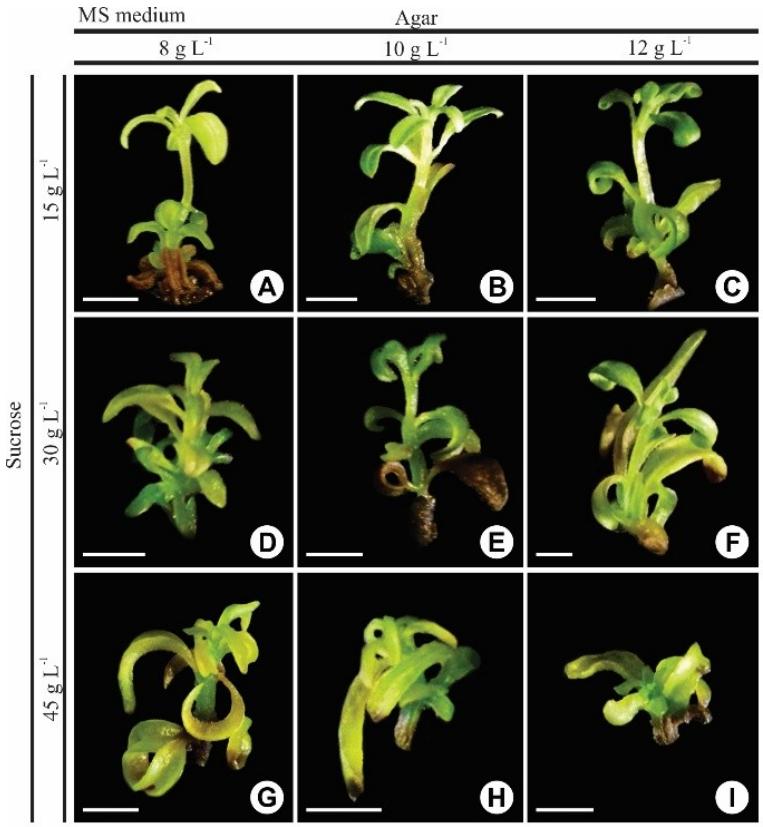

Figure 5. *In vitro* propagation of *Aloysia triphylla* in MS medium supplemented with different sucrose and agar concentrations. T1 - 15 sucrose + 8 agar (A); T2 - 15 sucrose + 10 agar (B); T3 - 15 sucrose + 12 agar (C); T4 - 30 sucrose + 8 agar (D); T5 - 30 sucrose + 10 agar (E); T6 - 30 sucrose + 12 agar (F); T7 - 45 sucrose + 8 agar (G); T8 - 45 sucrose + 10 agar (H); T9 - 45 sucrose + 12 agar (I). Bars: 1 cm.

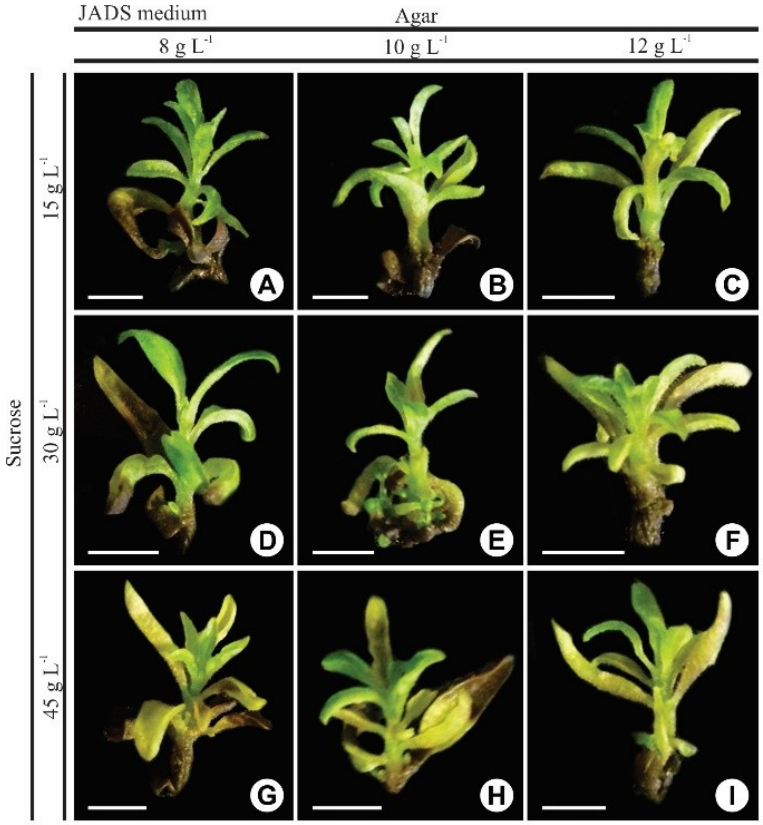

Figure 6. *In vitro* propagation of *Aloysia triphylla* in JADS medium supplemented with different sucrose and agar concentrations. T10 - 15 sucrose + 8 agar (A); T11 - 15 sucrose + 10 agar (B); T12 - 15 sucrose + 12 agar (C); T13 - 30 sucrose + 8 agar (D); T14 - 30 sucrose + 10 agar (E); T15 - 30 sucrose + 12 agar (F); T16 - 45 sucrose + 8 agar (G); T17 - 45 sucrose + 10 agar (H); T18 - 45 sucrose + 12 agar (I). Bars: 1 cm.

Discussion

This is the first report of *in vitro* propagation of *Aloysia triphylla* with different types of culture media and sucrose and agar concentrations, that evaluated growth parameters, as well as hyperhydricity and the influence of these factors on the acclimatization of plants and the difficulty in acclimatising them. Other works have reported the difficulty of *in vitro* propagation of plants of the Verbenaceae family, as is the case of *Lippia alba*, in which the authors reported a high mortality rate of the explants due to oxidation (Tavares, Leitão, Reinert, & Lage, 2013).

Perennial plants are rich in substances derived from their secondary metabolisms, which play important roles in the survival of these species. Among these, phenolic compounds and precursors of lignin synthesis may oxidise, which may be linked to the regulation and growth processes, i.e. depending on the endogenous concentration of auxin in the tissues. This may increase the oxidation of plants, which is one of the main problems that negatively affects the *in vitro* cultural conditions (Freitas, Oliveira, Dombroski, Câmara, & Silva Neto, 2009; Tavares et al., 2013).

In this work, there was the influence of hyperhydricity in the plants produced, especially when kept in MS medium, which resulted in the yellowing and oxidation of the plants with consequent reduction of survival during the acclimatization stage. The specificity of explants, culture medium, plant growth regulators, environment and culture conditions showed some effects on the incidences of hyperhydricity (Liu et al., 2017; Isah, 2019). Hyperhydricity occurs because of the passive diffusion of water into the tissues or an active phenomenon related to a disturbance in the metabolic process of the plant (Vasconcelos et al., 2012). The constitution of the medium has a prominent role in relation to the growth patterns of cells and tissues, which can be modified according to the need of the culture and the requirement of the type of explant (Rodrigues, Penoni, Soares, & Pasqual, 2013). These complex factors directly result in the difficulty of prevention and control of hyperhydricity (Liu et al., 2017).

Tavares et al., (2013) also reported a high frequency of death by oxidation of the *Lippia alba* explants when conducted in MS culture medium, and this was due to the sub-bush habit of the species. This had also

been proven by Freitas et al. (2009), who observed oxidation of the explants of *Aloysia virgata*, another Verbenaceae.

In *in vitro* multiplication of *Zantedeschia aethiopica*, when evaluating the culture medium MS plus different sucrose concentrations (0; 15; 30; 45 and 60 g L^{-1}), Ribeiro, Pasqual, Silva, and Rodrigues (2008) observed superior results for fresh shoot mass when the plants were grown in media with 30 g L^{-1} of sucrose. Similar results were also observed by de Freitas, Nogueira, & Praxedes (2016); these authors cultivated *Justicia pectoralis* in MS medium and observed greater shoot lengths when compared to the WPM medium. The results of this research also demonstrated that plant lengths were higher when *A. triphylla* was maintained on MS medium when compared to WPM, although lengths did not differ significantly (Figure 1D).

The number of nodes of *A. triphylla* was higher in the medium containing 15 g L^{-1} of sucrose. Mohamed and Alsadon (2010), when cultivating *Solanum tuberosum,* also observed that the reduction of the carbohydrate source to the culture medium favoured an increase in the number of nodes. These authors reported that the plants kept in media with 20 g L^{-1} of sucrose had high values of chlorophyll, which favoured an increase in the number of nodes. In addition, the reduction in sucrose levels added to the culture medium may be beneficial under large-scale *in vitro* propagation conditions, as it reduces production costs (Jeong & Sivanesan, 2018).

In the present work, the superior results for the fresh mass of the plants when the cultures remained in MS medium, in fact, reflected in exaggerated accumulation of water in the vegetal cells, characterising the physiological disorder called hyperhydricity. This abnormal accumulation of water inside plant tissues and cells gives a characteristic translucent appearance to the plant (Vasconcelos et al., 2012), which can progress to the loss of tissue regeneration capacity (Barbosa et al., 2013). This physiological disorder can affect up to 60% of the micropropagated shoots (Palma et al., 2011), generating elongated and thick diameter buds and internodes shorter than normal as well as translucent, elongated and/or wrinkled, coiled, and brittle thick leaves (Figures 4-6).

For species of the genus *Prunus*, Radmann, Bianchi, Souza, Fachinello, and Oliveira (2009) observed higher rates of hyperhydricity in explants maintained in MS medium when compared to WPM. These effects were related to the higher total nitrogen concentration (nitrate and ammonium forms) of MS medium when compared to WPM, (Nepomuceno, Fonseca, Silva, Oliveira, & Santana, 2014; Radmann et al., 2009), which increases the saline concentration of the medium, directly influencing the osmotic potential. Isah (2019) observed that *Caladium bicolor* explants were characterised by brownish-white, succulent and translucent appearance with fragile, leathery whitish-brown leaves that were shorter and smaller. These symptoms may result in numerous limitations to the survival of *ex vitro* cultures; reinforcing the need to adapt the conditions of *in vitro* culture in order to overcome this problem (Oliveira, Xavier, Lopes, Takahashi, & Otoni, 2016).

The concentration of agar in the culture medium is a decisive factor for the *in vitro* culture, because when considered at high levels, this reagent can affect the availability and diffusion of the other constituents. However, it can act as an osmoregulator (Lencina, Bisognin, Kielse, Pimentel, & Fleig, 2014), thus reducing water availability in the growing medium and helping to reduce the hyperhydricity of plants. Both the quality and the quantity of the agar must be considered, since these factors affect the physical and chemical characteristics of the culture medium and, consequently, the adequate development of the explants (Rezende, Pasqual, Carvalho, Pereira, & Villa Villa, 2008).

WPM medium proves to be more efficient in the development of *Coffea* sp. than MS medium. Various formulations of culture media are being employed, but what differs between them is the concentration of salts in their composition, and some species may exhibit morphophysiological disorders, caused by *in vitro* culture conditions or due to the physiological conditions of the species. This makes the importance of the experimental determination of the nutrient medium for each type of explant, species or cultivar clear (Nepomuceno et al., 2014). Furthermore, there is no pre-defined specific culture media formulation for a particular genus, species, hybrid or clone, and the difficulty lies in finding successful results with the different combinations of the medium and culture conditions (Miyata, Villa, & Pasqual, 2014).

Conclusion

The results allow us to conclude that the composition of the culture medium significantly interferes with the osmotic potential, which causes physiological disorders in plants, and thus compromises the *in vitro* regeneration of *Aloysia triphylla*. Thus, the WPM medium with concentrations of 15 g L^{-1} of sucrose and 12 g L^{-1} of agar is recommended as the most suitable for the regeneration of plants, because it provides less occurrence of hyperhydricity and a higher percentage of acclimatization of plants, in addition to reducing costs with the *in vitro* propagation of *A. triphylla*.

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