Scientia Agraria Paranaensis – Sci. Agrar. Parana.
ISSN: 1983-1471 – Online
DOI: https://doi.org/10.18188/sap.v21i4.30208

# ESTABLISHMENT AND GROWTH OF *Lippia gracilis* SCHAUER CULTIVATED UNDER DIFFERENT *in vitro* CONDITIONS

Luiz Eduardo Santos Lazzarini[1*], Fernanda Ventorim Pacheco[1], Alexsandro Carvalho Santiago[1], Adriane Duarte Coelho[1], Suzan Kelly Vilela Bertolucci[1], José Eduardo Brasil Pereira Pinto[1]

SAP 30208 Received: 03/05/2022 Accepted: 19/09/2022
Sci. Agrar. Parana., Marechal Cândido Rondon, v. 21, n. 4, oct./dec., p. 362-370, 2022

**ABSTRACT -** *Lippia gracilis* Schauer (Verbenaceae), popularly known as alecrim de tabuleiro‖ is used in folk medicine to treat colds, coughs, sinusitis, bronchitis, headache and externally skin conditions, burns, wounds, and ulcers. The tissue culture method of micropropagation has produced the greatest impact on plant production. It allows large scale plant multiplication within short periods of time using a limited physical space during any season of the year. The objective of this study was to evaluate the explant type, its position and the culture medium used for the establishment and *in vitro* culture of *Lippia gracilis*. For the establishment experiment, the treatments were: two types of explants (apical and nodal) taken from two cultivation environments (field and greenhouse), and inoculated in two types of culture medium (MS and MS/2). In order to verify the influence of the explant position, the treatments consisted of explants horizontally or vertically inoculated. For the evaluation of sucrose in the culture medium, the concentrations of 7.5; 15; 30 and 60 g $L^{-1}$ were tested, while for salt concentration, the treatments consisted of 4 different salt concentrations: ¼ MS; ½ MS; MS and 2MS. In all experiments, a completely randomized design was used. Half of the salt concentration and the use of explants from a greenhouse reduced the rates of contamination, oxidation, and necrosis in the *in vitro* establishment of *Lippia gracilis*. The sucrose concentration of 30 g $L^{-1}$ and ½ MS culture medium showed the best growth results for the species. Although the production of dry matter was reduced in this concentration of salts, the plantlets were more vigorous to proceed to acclimatization. Explants from a greenhouse inoculated horizontally in ½ MS medium supplemented with 30 g $L^{-1}$ of sucrose increase growth parameters and the multiplication rate in the *in vitro* propagation of *L. gracilis*.
**Keywords:** Verbenaceae, carbohydrates, culture medium, micropropagation, salts.

# *ESTABELECIMENTO E CRESCIMENTO DE Lippia gracilis SCHAUER CULTIVADA EM DIFERENTES CONDIÇÕES in vitro*

**RESUMO -** A *Lippia gracilis* Schauer (Verbenaceae), popularmente conhecida como alecrim de tabuleiro é utilizada na medicina popular para tratar resfriados, tosses, sinusite, bronquite, dor de cabeça e externamente afecções da pele, queimaduras, feridas e úlceras. O método de micropropagação por cultura de tecidos produzem maior impacto na produção vegetal. Permite a multiplicação de plantas em larga escala em curtos períodos de tempo usando um espaço físico limitado durante qualquer estação do ano. O objetivo desse estudo foi avaliar o tipo de explante, a posição e o meio de cultura utilizado para o estabelecimento *in vitro* da cultura *Lippia gracilis*. Para o experimento do estabelecimento, os tratamentos foram: dois tipos de explantes (apical e nodal), retirados de dois ambientes de cultivo (campo e casa de vegetação) e inoculados em dois tipos de meio de cultura (MS e MS/2). Metade da concentração de sais e o uso de explantes originados da casa de vegetação reduziram as taxas de contaminação, oxidação e necrose no estabelecimento *in vitro* da *Lippia gracilis*. Para verificar a influência da posição do explante, os tratamentos consistiram em explantes inoculados horizontalmente ou verticalmente. Para a avaliação do açúcar no meio de cultura, as concentrações de 7.5; 15; 30 e 60 g $L^{-1}$ foram testadas, enquanto para as concentrações de sal, os tratamentos consistiram em 4 diferentes concentrações de sais: ¼ MS; ½ MS; MS and 2MS. A concentração de açúcar de 30 g $L^{-1}$ e meio de cultura ½ MS apresentou os melhores resultados de crescimento para a espécie. Embora a produção de matéria seca foi reduzida nessa concentração de sais, as plântulas ficaram mais vigorosas para proceder a aclimatação. Portanto a inoculação de explantes na posição horizontal em meio de cultura ½ MS, suplementado com 30 g $L^{-1}$ de sacarose é recomendado para o cultivo *in vitro* de *L. gracilis*.
**Palavras-chaves:** Verbenaceae, carboidratos, meio de cultura, micropropagação, sais.

## INTRODUCTION

*Lippia gracilis Schauer* (Verbenaceae), popularly known in Brazil as “alecrim-do-sertão”, is a plant typical of the vegetation of the Northeastern semiarid (LORENZI and MATOS, 2008). The leaves of this species are rich in essential oil with antibacterial, molluscicidal, larvicidal, antinociceptive and anti-inflammatory activity (FERRAZ et al., 2013). In northeastern Brazil, this species is used in

[1]Universidade Federal de Lavras, Departamento de Agricultura, Lavras, MG, Brasil. Email: luizlazzarini@outlook.com. *Corresponding author.

folk medicine to treat colds, flu, bronchitis and cough (FELIX et al., 2021).

The fruits are particularly small, achene type and their seeds rarely germinate. Propagation is usually carried out via cutting of thinner branches (LORENZI and MATOS, 2008). However, micropropagation through plant tissue culture has been an indicated technique for the cultivation of this species. Micropropagation makes it possible to obtain a large number of individuals in a short period of time and the germplasm conservation, ensuring the maintenance of the biodiversity of endemic species (SANTOS et al., 2016).

In addition, the totipotence of plant cells allows them to be readily used for in vitro propagation or cell culture. Furthermore, when micropropagation is used for aromatic species, it allows the standardization of the plant material by the multiplication of clones with high content of essential oil and high levels of major constituents for the species (SILVA et al., 2017), allowing the propagation and production of plants on a commercial scale. However, for *in vitro* culture it is necessary to select a culture medium that best adapts to the explant, being necessary to determine the most appropriate concentration of the components. Even though the medium contains all the nutrients required by the plant, their concentrations can affect the plant's ability to extract nutrients and water, essential for the *in vitro* adaptation of the species (PINHAL et al., 2017).

In addition, woody species, such as *Lippia gracilis*, when cultivated *in vitro*, have high concentrations of phenolic compounds and leaf abscission of plant material, causing ethylene accumulation in tissues submitted to this environment, promoting oxidation that can lead to the death of plant material (SARTOR et al., 2013). Therefore, *in vitro* cultivation and establishment involves determining the type of explant and the culture medium that will allow the best adaptation of the explants to laboratory conditions and a posterior development in the field (ALBUQUERQUE et al., 2016).

Changes in the components of the culture medium and explant position have been reported to improve the development of several species grown *in vitro*. Ferreira et al (2020) evaluating different species of *Passiflora* spp., observed greater shoot length from nodal segments in the complete MS medium compared to apical segments. For *Uncaria guianensis*, the explant inoculation position (horizontal and vertical), influenced the average number of shoots elongated by original explants (PADUA et al., 2014). *Citrus limonia* Osbeck showed greater growth under high doses of sucrose *in vitro* cultivation (SCHMILDT et al., 2015). Whereas, for *Billbergia zebrina*, the best development of the roots occurred when grown in a medium with half the concentration of sucrose recommended for the MS medium (MARTINS et al., 2015). For *Mentha arvensis* there was a greater accumulation of dry matter when grown in MS medium supplemented with 30 g $L^{-1}$ of sucrose (OLIVEIRA et al., 2016). Thus, although there are standard protocols defined for *in vitro* culture, they are specific to each species.

Despite the great medicinal potential of the species *Lippia gracilis*, up to the present time, no concrete results have been found in terms of the efficiency of protocols for establishment, development, regeneration and *in vitro* conservation. Therefore, the objective of this research was to verify the *in vitro* cultivation of this species, under different types and position of explants, different concentrations of sugar and salts in the MS culture medium.

## MATERIAL AND METHODS

For *in vitro* establishment, apical and nodal segments of plants from greenhouse cultivation were used. These plants were obtained through vegetative propagation of apical cuttings (± 5 cm) collected from adult plants in the UFLA medicinal plant garden, rooted in a polypropylene tray and after 30 days replanted in plastic pots with a mixture of soil+sand in the 3:1 ratio (v/v), in greenhouse. Drastic pruning was carried out in the plants grown in a protected environment, followed by fertilization with ammonium sulfate. Also, field water capacity was maintained in order to stimulate regrowth. Two doses of fungicide and systemic bactericide (Kazumin) were applied seven and two days before the plant material for establishment was collected, in a dosage of 3 mL $L^{-1}$ of water (0.06 g of active ingredient), aiming to reduce contamination in the establishment phase.

In order to perform the establishment tests, apical and nodal segments were washed in running tap water for 30 min. and immersed in 50% v/v sodium hypochlorite solution (1.25% active chlorine), under constant agitation for 15 min. In aseptic laminar flow, the explants (± 1.0 cm) were inoculated in test tubes (150 x 25 mm), containing 15 mL of MS medium (MURASHIGE and SKOOG, 1962) complete and with half the concentration of the salts supplemented with 30 g $L^{-1}$ of sucrose, 6 g $L^{-1}$ agar, and pH 5.7 ± 0.1, adjusted with NaOH and HCl (0.1 and 0.5 N), before autoclaving (125°C, 25 min., 1.2 atm). After inoculation, the tubes were placed in a growth room with fluorescent lamps with a light intensity of 39 µmol $m^{-2}$ $s^{-1}$, photoperiod of 16 h of light and temperature of 25 ± 1°C. The explants were evaluated for the percentage of contamination, oxidation and necrosis in the different conditions evaluated, every 3 days until stabilization at 14 days.

After 35 days, the established plantlets were multiplied in flasks containing 40 mL of the MS medium with half the concentration of salts and kept under identical conditions of light and temperature. Nodal segments (1.0 cm) were inoculated in the vertical and horizontal positions, in test tubes containing 15 mL of the MS medium with half the concentration of the salts, supplemented with 30 g $L^{-1}$ of sucrose, 6 g $L^{-1}$ of agar, and pH 5.7 ± 0.1, adjusted with NaOH and HCl (0.1 and 0.5 N), before autoclaving (125°C, 25 min., 1.2 atm).

The experimental design used was the completely randomized with ten replicates per treatment. Each repetition was composed of three plants, totaling thirty plants analyzed per treatment. After 35 days, the growth

parameters for each treatment were evaluated. For growth, the plantlets obtained were evaluated for shoot length (cm), root length (cm), number of shoots, number of leaves, leaf dry matter, stem, root and total dry matter (mg). To obtain dry matter data, the leaves, roots and stems were placed separately in paper bags and dried in a forced air oven at 40°C, until weight stabilization, approximately 72 h. The weight was then determined using a semi-analytical digital scale.

To evaluate the effect of different concentrations of sucrose on *in vitro* cultivation of *L. gracilis*, nodal segments (1.0 cm) of plantlets previously established *in vitro* were used and four different sucrose concentrations (7.5, 15, 30 and 60 g $L^{-1}$) in the MS medium were tested. A completely randomized design was used, with seven replications per treatment and three plants per repetition, totaling 21 plantlets analyzed per treatment. The explants were inoculated in test tubes (150 x 25 mm) containing 15 mL of MS medium with different concentrations of sucrose, 6 g $L^{-1}$ agar (Himedia®, type I) and pH 5.7 ± 0.1, adjusted with NaOH and HCl (0.1 and 0.5 N). Subsequently, they were autoclaved at 125°C for 25 min., at 1.2 atm. After inoculation, the explants were placed in a growth room with cold white fluorescent lamps (OSRAM®, Brazil) in a light intensity of 39 µmol $m^{-2}$ $s^{-1}$, photoperiod of 16 h of light and temperature of 25 ± 1°C. After 35 days, the plantlets obtained were evaluated for shoot length (cm), root length (cm), number of leaves, number of shoots, leaf dry matter, stem dry matter, root and total dry matter (mg). The dry matter was obtained in the same way as described for the analysis of the explant position.

Four different salt concentrations of the MS medium (2MS, MS, ½ MS, ¼ MS) were tested for in vitro culture of *L. gracilis*. Test tubes (150 x 25 mm) containing 15 mL of the culture medium were used, supplemented with 30 g $L^{-1}$ of sucrose, 6 g $L^{-1}$ of agar (Himedia®, type I), and pH 5, 7 ± 0.1, adjusted with NaOH and HCl (0.1 and 0.5 N). The test tubes with the culture media were autoclaved at 125°C for 25 min., at 1.2 atm. After inoculation, the tubes were placed in a growth room with cold white fluorescent lamps (OSRAM®, Brazil) with intensity of 39 µmol $m^{-2}$ $s^{-1}$, photoperiod of 16 h of light and temperature of 25 ± 1°C. The experimental design was completely randomized, with seven replications per treatment and three plants per repetition, totaling 21 plants analyzed per treatment. After 35 days, the plantlets obtained were evaluated for shoot length (cm), root length (cm), number of leaves, number of shoots, leaf dry matter, stem dry matter, root dry matter and total dry matter (mg). The dry matter was obtained in the same way as described in the previous items.

The data obtained were submitted to ANOVA by the F test ($p < 0.05$), using the software Statistica®, version 12 (trial version, StatSoft). After verifying the significance of the variables using the F test, the averages were compared using the Scott-Knott test at 5% probability.

## RESULTS AND DISCUSSION

The *in vitro* establishment of *Lippia gracilis* was affected by the explant type and culture medium (Table I). The apical segments had the lowest contamination percentages. However, they had the highest percentages of oxidation and necrosis. Different results were obtained in *Calathea crotalifera*, where nodal explants had lower contamination percentages when compared to apical explants (EFZUENI ROZALI et al., 2014).

The reduction in the oxidation percentages found in nodal explants, especially in the treatment with ½ MS (Table I) may be related to the low concentration of salts in the medium. Studies suggest that oxidation rates are dependent on several factors, including the type of explant and the concentration of salts in the medium (GOLLE et al., 2013). The type of explant may have a genetic influence on oxidation. Meanwhile, salt concentrations are related to the oxidative stress levels that the culture medium provides to the explant. Similar results were found by Marinho et al. (2011) testing different methods to reduce oxidation rates in the establishment of *Lippia gracilis*, including reduction in the concentration of salts in the culture medium.

**TABLE 1 -** Percentage of contamination, oxidized shoots and necrotic shoots in apical and nodal explants of *Lippia gracilis* cultivated *in vitro* under different concentrations of salts (MS and ½ MS).

| Culture medium | Explant | Contamination | Oxidized shoots | Necrotic shoots |
|---|---|---|---|---|
| | | (%) | | |
| MS | Apical | 29 | 35 | 18 |
| | Nodal | 37 | 26 | 5 |
| 1/2 MS | Apical | 37 | 21 | 8 |
| | Nodal | 67 | 0 | 8 |
| | Average | 43 | 28 | 10 |
| | CV(%) | 21.24 | 35.91 | 26.76 |

The high percentages of necrosis observed in apical explants, specially the explants grown under the MS medium, is related to the intense cellular activity provided by the type of meristem and the degree of oxidative stress caused by the salts. Apical meristems have reduced plasmodesmata connections and an intense rhythm of cell division, compared to axillary buds (FERREIRA et al., 2020). This may have caused intense cell death in the newly formed cells. It was not necessary to use growth regulators for the *in vitro* establishment of *Lippia gracilis*,

as the surviving plantlets developed and rooted normally without the addition of these regulators.

Regardless of the position of explant inoculation, *L. gracilis* plantlets were vigorous (Figure 1). However, those originating from the horizontally inoculated explants had the highest averages for the growth variables analyzed, with the exception of shoot length and root length (Table 1). It is observed that the explants in the horizontal presented more than twice the number of shoots in relation to the inoculated vertically, consequently increasing the rate of multiplication of the explants.

The increase in the number of shoots of the nodal segments inoculated horizontally can be explained by the break in the apical dominance, which is induced by the meristem, inhibiting the translocation of auxin and leading to the stimulation of the growth of lateral shoots (ASSIS et al., 2012). According to Taiz et al. (2017) the transport of auxin in the plant is polar, and this behavior is important to control the inhibition of the development of axillary buds. Therefore, treatments that inhibit or reduce the movement of auxins in explants, stimulates the increase in the number of shoots per explant (ARAB et al., 2014), corroborating the data found. The greater number of leaves and dry matter in the explants inoculated horizontally is justified by the greater number of shoots existing in this treatment.


**FIGURE 1 -** Visual aspect of *Lippia gracilis* seedlings at 35 days, grown *in vitro* in MS medium, with vertical (a) and horizontal (b) explants.

Explants inoculated vertically showed higher shoot length than those inoculated horizontally. This may be due to the limitations imposed by the test tube, restricting the growth space, with an increase in the number of shoots in the horizontally inoculated explants (ASSIS et al., 2012). Horizontally inoculated explants showed a reduction in root length compared to vertically inoculated (Table 2). This reduction can be explained by the fact that in this treatment the plant tissues had a greater area of contact with the culture medium, thus increasing the area of absorption of nutrients, in relation to those inoculated vertically (GENG et al., 2016).

**TABLE 2 -** *In vitro* growth of *Lippia gracilis* grown in ½ MS medium for 35 days under different explant positions (horizontal and vertical) (SL: shoot length; RL: root length; NS: number of shoots; NL: number leaves; LDM: leaf dry matter; SDM: stem dry matter; RDM: root dry matter; TDM: total dry matter).

| Explant position | SL | RL | NS | NL | LDM | SDM | RDM | TDM |
|---|---|---|---|---|---|---|---|---|
| Horizontal | 3.24 b* | 1.85 b | 4.34 a | 32.90 a | 20.31 a | 10.26 a | 7.64 a | 38.20 a |
| Vertical | 4.68 a | 2.35 a | 2.00 b | 19.31 b | 16.63 b | 6.50 b | 8.28 a | 31.40 b |
| CV(%) | 10.61 | 12.45 | 8.91 | 10.12 | 19.45 | 15.78 | 17.24 | 23.79 |

*Averages followed by the same lowercase letter in the column belong to the same group by the Scott Knott test at the 5% probability level.

In addition to the culture conditions, the organogenic response *in vitro* is dependent on the species. Konate et al. (2013) found different results for *Vigna subterranea*, where explants inoculated vertically showed a higher number of shoots than those inoculated horizontally. For *Corylus avellana*, it presented better responses for the number of shoots and for the shoot length, when explants were inoculated horizontally, compared to those cultivated vertically (PRANDO et al., 2014). Similar results to the present study were observed for Hyptis marrubioides and Aloysia triphylla that had greater growth, greater dry matter production and higher number of shoots when the explants were inoculated horizontally (BOTREL et al., 2015; SILVA et al., 2017).

The different concentrations of sucrose promoted changes in the growth of *L. gracilis* plantlets (Figure 2). The treatment with the highest concentration of sucrose (60 g $L^{-1}$) showed the lowest survival rates (29%), lower growth, shoot length less than 0.5 cm and few roots (data not shown). According to George et al. (2008) sucrose not

only has a nutritious effect for plants, but also influences growth and development through osmotic properties. The increase in sucrose concentration in the culture medium may have caused an osmotic imbalance, leading to the death of the explant, or preventing adequate cell growth and organogenesis.

The different concentrations of sucrose in the culture medium promoted changes in the growth of *Lippia gracilis* (Table 3). The increase in the concentration of sucrose, up to 30 g, promoted an increase in the shoot and root length. Meanwhile, the number of leaves and shoots were not changed due to sucrose concentrations. Thus, the original sucrose concentration for the MS medium (30 g) provides greater length and balance for plantlets of *Lippia gracilis* grown *in vitro*.

These results show the importance of sucrose in the culture medium to provide energy for the development of shoots, since its reduction may have caused an energy deficiency for the adequate growth of explants in treatments in which there was a decrease in sugars in the medium (MONFORT et al., 2015). Lemes et al. (2016) also observed an increase in the shoot length of *Miltonia flavescens* grown *in vitro* under sucrose concentrations of 30 to 45 g. Oliveira et al. (2016) also found higher production of dry matter of *Mentha arvensis* when grown in MS medium with 30 g of sucrose.


**FIGURE 2 -** Visual aspect of *Lippia gracilis* plantlets at 35 days, *in vitro* growth, in MS medium under different concentrations of sucrose (7.5, 15, 30 and 60 g $L^{-1}$).

**TABLE 3 -** *In vitro* growth of *Lippia gracilis* grown in ½ MS medium for 35 days under different concentrations of sucrose (30, 15 and 7.5 g $L^{-1}$) (SL: shoot length; NL: number of leaves; RL: root length; NS: number of shoots; LDM: leaf dry matter; SDM: stem dry matter; RDM: root dry matter; TDM: total dry matter).

| Sucrose concentration (g $L^{-1}$) | SL | NL | RL | NS | LDM | SDM | RDM | TDM |
|---|---|---|---|---|---|---|---|---|
| 30 | 1.50 a* | 19.03 a | 0.47 a | 2.27 a | 30.16 a | 6.69 a | 2.43 a | 39.28 a |
| 15 | 1.21 b | 18.48 a | 0.20 b | 2.61 a | 20.65 b | 4.59 b | 0.29 b | 25.52 b |
| 7.5 | 0.91 c | 18.91 a | 0.05 c | 2.44 a | 8.45 c | 3.02 c | 0.00 b | 11.46 c |
| CV(%) | 25.34 | 15.68 | 23.34 | 19.54 | 27.95 | 21.30 | 26.37 | 35.35 |

*Averages followed by the same lowercase letter in the column belong to the same group by the Scott Knott test, at the 5% probability level.

The increase in the length of the roots obtained in treatments with a higher concentration of sucrose (30 g) may be related to the energetic function of sucrose *in vitro* cultivation. According to Monfort et al. (2015) the energy expenditure for growth and root formation in plantlets is high, thus a high amount of sucrose (30 g) provides an increase in the length of roots in plantlets grown *in vitro*. In addition, Barrales-López et al. (2015) found that the absence of sucrose in the culture medium promotes low rooting rates. Occimum selloi grown in MS medium with 30 g of sucrose also found an increase in root length (MONFORT et al., 2015).

Regarding the dry matter production, it was verified that there was an increase in the leaf, stem, root and total dry matter in plantlets grown under 30 g of sucrose (higher concentration). This increase in dry matter may be related to the energy function and supply of sucrose carbon skeletons *in vitro* cultivation (MONFORT et al., 2015). Sucrose is important, as it is the main source of carbohydrate for plants, since explants do not have the appropriate conditions to have high photosynthetic rates *in vitro* cultivation (ADELBERG et al., 2013). Therefore, for the *in vitro* cultivation of *L. gracilis* the sucrose concentration established for the MS culture medium (30 g) is the one that promotes the highest accumulation of dry matter. According to Longo et al. (2016) culture media that induce dry mass accumulation should be selected as they facilitate acclimatization to a nonsterile substrate, thus reducing the risks of death *in vivo* cultivation.

However, the effect of sucrose concentrations in the culture medium shows interspecific responses, achieving different responses. For *Allium sativum*, higher leaf dry matter production was observed in MS medium supplemented with 30 g of sucrose (LONGO et al., 2016). While, in *Ocimum selloi*, the largest accumulation of shoot dry matter was observed in plantlets grown *in vitro* with

15 g of sucrose and higher dry matter of the root when they were grown under 30 g of sucrose (MONFORT et al., 2015). For *Ananas comosus* the greatest accumulation of dry matter was observed when the plantlets were grown with 15 g of sucrose (CAMARGO et al., 2017).

The treatment with the concentration of salts twice of the MS medium (2MS), presented 25% survival, and plantlets with size less than 0.5 cm and without roots (data not shown). The lack or small length of roots in plantlets grown under high concentrations of salts may have promoted this increase in mortality, since the standard MS medium is already considered a cultivation medium that has high concentrations of salts for numerous species. In works carried out with the species *Eleutherococcus koreanum*, there was greater root growth when cultivated in medium MS with low levels of salts (1/4, 1/2 and 3/4 MS) compared to cultivations under high levels of salt, where the roots of plantlets grown in medium 2MS were shorter, thinner and less numerous compared to other concentrations (MANUHARA et al., 2015).

The different concentrations of salts in the culture medium also promoted changes in the growth of *Lippia gracilis* (Figure 3). Plantlets grown under MS medium with half the concentration of salts showed the highest averages for shoot length (Table 4). While the standard MS medium (MS) allowed a greater number of shoots and number of leaves. Similar results were also observed for other species grown *in vitro* (MONFORT et al., 2018; CARVALHO et al., 2018).


**FIGURE 3** - Visual aspect of *Lippia gracilis* plantlets at 35 days, grown *in vitro* in MS, in MS medium under different concentrations of salts (¼ MS; ½ MS, MS, 2MS).

The addition of macronutrients to the culture medium leads to a considerable decrease in the osmotic potential of the medium (GEORGE et al., 2008). The effect of the high salt concentration in the conventional MS and 2MS medium leads to an osmotic stress that affects the availability of water for plants. In addition, the absorption of this high level of salts by the roots can reach a level that is toxic in the tissues. The effect of this salt stress affects growth, reduces the production of dry matter, leaf area, length of roots and shoot length as observed in the present study (TAIZ et al., 2017).

**TABLE 4 -** *In vitro* growth of *Lippia gracilis* grown in MS medium for 30 days, under different concentrations of salts (MS, ½ MS and ¼ MS) (SL: shoot length; NL: number of leaves; RL: root length; NS: number of shoots; LDM: leaf dry matter; SDM: stem dry matter; RDM: root dry matter; TDM: total dry matter).

| Culture medium | SL | NL | RL | NS | LDM | SDM | RDM | TDM |
|---|---|---|---|---|---|---|---|---|
| ¼ MS | 3.08 b* | 13.72 b | 1.55 a | 1.72 b | 24.90 c | 8.20 c | 4.25 b | 37.35 c |
| ½ MS | 3.91 a | 17.97 a | 1.70 a | 1.87 b | 38.85 b | 10.18 b | 6.92 a | 55.93 b |
| MS | 1.70 c | 19.91 a | 0.82 b | 2.80 a | 56.56 a | 12.96 a | 2.73 c | 72.25 a |
| CV(%) | 24,18 | 18,91 | 21,87 | 20,34 | 25,59 | 19,03 | 28,36 | 31,34 |

*Averages followed by the same lowercase letter in the column belong to the same group by the Scott Knott test, at the 5% probability level.

The increase in the shoot length in the lowest concentrations of salts may be due to the reduction of osmotic stress in treatments ½ MS and ¼ MS in which the plantlet found more favorable conditions for its full development. Schmildt et al. (2015) studying *Citrus limonia* also observed a longer shoot length of plantlets grown *in vitro* in MS medium containing 66.7% of salts. *Vriesea incurvata* also showed better growth responses when grown in medium with diluted concentrations of macro and micronutrients (SASAMORI et al., 2016). For *American genipa*, ½ MS medium provided the best shoot

length in relation to the standard MS (ALMEIDA et al., 2013).

The root length was also greater in plantlets grown under lower concentrations of salts. There are reports in the literature that indicate that woody species are more responsive to *in vitro* cultivation when there is a reduction in macronutrients in the environment (SCHMILDT et al., 2015; LENCINA et al., 2018). As observed for *L. gracillis* in this study, with an improvement in root growth parameters with reduced concentration of the MS medium.

The increase in root length at low salt concentrations can also demonstrate a species strategy in situations of low nutrient availability. According to Araújo et al. (2012) species subjected to nutrient deficiency increased the translocation of photoassimilates to the roots, leading to an increase in the root system in relation to the aerial part. In addition, *Lippia gracilis* is a species that grows naturally in soils with low nutrient availability, such as the Brazilian semi-arid soil (LORENZI and MATOS, 2008).

The leaf, stem and total dry matter were higher in the concentrations of salts of the standard MS medium (MS), reducing in treatments with lower concentrations (Table 4). Distinctive results were observed in *Ocimum selloi*, which showed no significant difference for dry matter production when grown in MS, ½ MS and ¼ MS (Monfort et al., 2015). Manuhara et al. (2015) found a greater accumulation of dry matter of *Eleutherococcus koreanum* cultivated in medium ½ MS. Carvalho et al. (2018) reported a higher accumulation of total dry matter in *Chenopodium ambrosioides* when grown in MS with 89% of the salt concentration of the MS medium.

The root dry matter was higher at lower salt concentrations (Table 4). This result corroborates with the root length observed in that study. Consequently, it can be affirmed that the species has a response strategy to soils with low nutrient availability. Additionally, this lower root growth may be related to a high concentration of salts. George et al. (2008) suggests that a media with high concentrations of salts, such as the MS medium, often inhibit the formation of roots, suggesting that if the *in vitro* cultivation aims the plantlet rooting, a more diluted media should be preferred.

This may also be related to the decrease of nitrogen levels in the culture medium, as normally $NH_4^+$ inhibits root growth and $NO_3^-$ stimulates growth. So, the reduction of the concentration of the culture medium may have promoted a higher nitrate / ammonium ratio and stimulated root development (MONFORT et al., 2015). These authors also reported that nitrogen is important for aerial part differentiation, which may be the reason for the MS medium to present the best results for leaf, stem and total dry matter. However, in *in vitro* cultivation, the small size and the dry matter production of the roots, observed in the highest concentrations of salts, can impair the acclimatization and establishment of the species. Better plantlet root development leads to faster acclimatization and better field survival rate (SILVEIRA et al., 2013). Therefore, in future works it would be necessary to verify the acclimatization and the establishment of these plantlets grown in these concentrations of salts.

## CONCLUSION

The origin of the protected environment explant and the use of ½ MS medium were efficient to reduce the levels of contamination, oxidation and necrosis in the *in vitro* establishment of *Lippia gracilis*.

The horizontal inoculation of *L. gracilis* explants provides a higher multiplication rate and number of shoots.

The concentration of sucrose and salts is critical for the *in vitro* cultivation of *L. gracilis*, as it significantly affects the growth and development of the species.

Explants from a greenhouse inoculated horizontally in ½ MS medium supplemented with 30 g $L^{-1}$ of sucrose increase growth parameters and the multiplication rate in the *in vitro* propagation of *L. gracilis*.

## ACKNOWLEDGEMENTS

The authors wish to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for granting scholarships and for their financial support.

## REFERENCES

ADELBERG, J.; DRIESSE, T.; HALLORAN, S.; BRIDGES, W.C. Relationships between nutrients and plant density in liquid media during micropropagation and acclimatization of turmeric. **In Vitro Cellular & Developmental Biology-Plant**, v.49, p.724-736. 2013.

ALBUQUERQUE, M.M.S.; BRITO, A.L.; LIMA, A.P.P.S.; ALVIM, B.F.M.; SANTANA J.R.F. *In vitro* establishment of *Comanthera curralensis*, "sempre viva" native of Chapada Diamantina - Bahia. **Ciência Rural**, v.46, n.6, p.991-995, 2016.

ALMEIDA, C.S.; LÉDO, A.S.; ARAÚJO, A.G.; SILVA, A.V.C.; SILVA JUNIOR, J.F.; SANTOS, J.E.; RIBEIRO, M.M.J.; NETA, J.L.V. Efeito do meio de cultura na germinação *in vitro* jenipapeiro. **Scientia Plena**, v.9, n.10, p.1-6, 2013.

ARAB, M.M.; YADOLLAHI, A.; SHOJAEIVAN, A.; SHOKRI, S.; GHOJAH, S.M. Effects of nutrient media, different cytokinin types and their concentrations on *in vitro* multiplication of G×N15 (hybrid of almond×peach) vegetative rootstock. **Journal of Genetic Engineering and Biotechnology**, v.12, n.2, p.81-87, 2014.

ARAÚJO, J.L.; FAQUIN V.; VIEIRA, N.M.B.; OLIVEIRA, M.V.C.; SOARES, A.A.; RODRIGUES, C.R.; MESQUITA, A.C. Crescimento e produção do arroz sob diferentes proporções de nitrato e amônio. **Revista Brasileira de Ciência do Solo**, v.36, [s.n.], p.921-930, 2012.

ASSIS, K.C.; PEREIRA, F.D.; ALBERTO, P.S.; SILVA, F.G.; SILVA, J.W.; MENEZES, C.C.E. *In vitro* cultivation of *Anacardium othonianum* Rizz.: effects of growth regulator, explant orientation and lighting. **African Journal of Biotechnology**, v.11, n.7, p.1559-1566, 2012.

BARRALES-LÓPEZ, A.; ROBLEDO-PAZ, A.; TREJO, C.; ESPITIA-RANGEL, E.; RODRÍGUEZ, J.L. Improved *in vitro* rooting and acclimatization of *Capsicum chinense* Jacq. Plantlets. ***In Vitro* Cellular & Developmental Biology Plant**, v.51, n.3, p.274-283, 2015.

BOTREL, P.P.; RODRIGUES, M.A.; BERTOLUCCI, S.V.; LIMA, A.F.; ALVARENGA, I.C.A.; PINTO, J.E.B.P. Factors affecting *in vitro* propagation and chromatographic analysis of compounds in *Hyptis marrubioides* EPL., a threatened medicinal plant. **Acta Horticulturae**, v.1083, [s.n.], p.319-325, 2015.

CAMARGO, S.; SOUZA, A.; SOMMER, L.; MOREIRA, M.; SCHUCH, M. Propagação *in vitro* de romãzeira (*Punica granatum* Linn.). **Revista Brasileira de Plantas Medicinais,** v.19, n.3, p.332-342. 2017.

CARVALHO, A.A.D.; BERTOLUCCI, S.K.V.; SILVA, S.T.; PINTO, J.E.B.P. Growth and volatiles in the micropropagation of Santa Maria herb. **Revista Ciência Agronômica**, v.49, n.4, p.624-635, 2018.

EFZUENI ROZALI, S.; RASHID, K.A.; MAT TAHA, R. Micropropagation of an exotic ornamental plant, *Calathea crotalifera*, for production of high quality plantlets. **The Scientific World Journal**, v.2014, [s.n.], p.1-12, 2014.

FELIX, S.F.; RODRIGUES, A.M.; RODRIGUES, A.L.M.; FREITAS, J.C.C.; ALVES, D.R.; SILVA, A.A.; MORAIS, S.M. Chemical composition, larvicidal activity, and enzyme inhibition of the essential oil of *Lippia grata* Schauer from the Caatinga Biome against dengue vectors. **Pharmaceuticals**, v.14, n.3, p.250-266, 2021.

FERREIRA, L.V.; TANIGUCHI, M.; BARRETO, C.F.; DA SILVA, T.B.; ANTUNES, L.E.C.; DUTRA, L.F. Indução de brotos *in vitro* em maracujazeiro doce brs mel do cerrado. **Brazilian Journal of Development**, v.6, n.3, p.9644-9652, 2020.

FERRAZ, R.P.C.; BOMFIM, D.S.; CARVALHO, N.C.; SOARES, M.B.P.; SILVA, T.B.; MACHADO, W.J.; PRATA, A.P.N.; COSTA, E.V.; MORAES, V.R.S.; NOGUEIRA, P.C.L.; BEZERRA, D.P. Cytotoxic effect of leaf essential oil of *Lippia gracilis* Schauer (Verbenaceae). **Phytomedicine**, v.20, [s.n.], p.615-621, 2013.

GENG, F.; MORAN, R.; DAY, M.; HALTEMAN, W.; ZHANG, D. Increasing *in vitro* shoot elongation and proliferation of 'G. 30' and 'G. 41' apple by chilling explants and plant growth regulators. **HortScience**, v.51, n.7, p.899-904, 2016.

GEORGE, E.F.; HALL, M.A.; KLERK, G.J. **The components of plant tissue culture media II: organic additions, osmotic and pH effects, and support systems.** In: GEORGE, E.F.; HALL, M.A.; KLERK, G.J.D. (Eds.). Plant Propagation by Tissue Culture. Basingstoke, p.115-173, 2008.

GOLLE, D.P.; REINIGER, L.R.S.; BELLÉ, R.A.; CURTI, A.R. Desinfestação superficial de explantes isolados de ramos semilenhosos e herbáceos de *Eugenia involucrata* DC.(Myrtaceae). **Cerne**, v.19, n.1, p.77-82, 2013.

KONATE, S.; KONE, M.; KOUAKOU, H.T.; KOUADIO, J.Y.; ZOUZOU, M. *Callus* induction and proliferation from cotyledon explants in *Bambara* groundnut. **African Crop Science Journal**, v.21, n.3, p.255-263, 2013.

LEMES, C.S.R.; SORGATO, J.C.; SOARES, J.S.; ROSA, Y.B.C.J. Meios de cultivo e sacarose no crescimento inicial *in vitro* de *Miltonia flavescens*. **Ciência Rural**, v.46, [s.n.], p.499-505, 2016.

LENCINA, K.H.; BISOGNIN, D.A.; PIMENTEL, N.; KIELSE, P.; MELLO, U.S.D. Produtividade de microcepas de grápia (*Apuleia leiocarpa*) mantidas *in vitro*. **Ciência Florestal**, v.28, n.1, p.150-159, 2018.

LONGO, A.E.D.O.E.; SIQUEIRA, W.J.; PASSOS, I.R.D.S.; SCOTT, M.D.S.; AZEVEDO FILHO, J.A. Micropropagação e bulbificação *in vitro* de alho (*Allium sativum* L.). **Plant Cell Culture & Micropropagation**, v.8, n.1-2, p.18-26, 2016.

LORENZI, H.; MATOS, F.J.A. **Plantas medicinais no Brasil:** nativas e exóticas. 2nd ed., São Paulo: Instituto Plantarum. 2008. 576p.

MANUHARA, Y.S.W.; KRISTANTI, A.N.; UTAMI, E.S.W.; YACHYA, A. Effect of sucrose and potassium nitrate on biomass and saponin content of Talinum paniculatum Gaertn. hairy root in balloon-type bubble bioreactor. **Asian Pacific Journal of Tropical Biomedicine**, v.5, n.12, p.1027-1032, 2015.

MARINHO, M.J.M.; ALBUQUERQUE, C.C.; MORAIS, M.B.; SOUZA, M.C.G.; SILVA, K.M.B. Estabelecimento de protocolo para micropropagação de *Lippia gracilis* Schauer. **Revista Brasileira de Plantas Medicinais**, v.13, n.2, p.246-252, 2011.

MARTINS, J.P.R.; VERDOODT, V.; PASQUAL, M.; PROFT., M. Impacts of photoautotrophic and photomixotrophic conditions on *in vitro* propagated *Billbergia zebrina* (Bromeliaceae). **Plant Cell Tissue and Organ Culture**, v.123, n.1, p.121-132, 2015.

MONFORT, L.E.F.; BERTOLUCCI, S.K.V.; LIMA, A.F.; CARVALHO, A.A.; MOHAMMED, A.; BLANK, A.F.; PINTO, J.E.B.P. Effects of plant growth regulators, different culture media and strength MS on production of volatile fraction composition in shoot cultures of *Ocimum basilicum*. **Industrial Crops and Products**, v.116, [s.n.], p.231-239, 2018.

MONFORT, L.E.F.; PINTO, J.E.B.P.; BERTOLUCCI, S.K.V.; ROSSI, Z.T.T.; LIMA, A.F.; SILVA, S.T.; SILVA, G.M.D. Micropropagação e germinação de sementes *in vitro* de atroveran. **Revista Ceres**, v.62, n.2, p.215-223, 2015.

MURASHIGE, T.; SKOOG, F.A. Revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, v.15, [s.n.], p.473-497, 1962.

OLIVEIRA, T.; PACHECO, F.V.; ALVARENGA, I.C.A.; BERTOLUCCI, S.K.V.; PINTO, J.E.B.P. Crescimento *in vitro* de *Mentha arvensis* L. cultivada com diferentes concentrações de sacarose. **Revista Brasileira de Plantas Medicinais**, v.18, n.3, p.685-690, 2016.

PADUA, M.S.; PAIVA, L.V.; PIRES, M.F.; SILVA, L.G.T.; CASTRO, E.M.; STEIN, V.C. Influence of culture medium and age of zygotic embryos on *in vitro* germination of *Elaeis guineensis* Jacq. **African Journal of Biotechnology**, v.13, n.14, p.1515-1523, 2014.

PINHAL, H.F.; COSTA, A.E.; CARNEIRO, P.A.P.; ASMAR, S.A.; MELO, B.; LUZ, J.M.Q. Concentration of ms medium and cutting of seeds on *in vitro* establishment of baruzeiro (*Dipteryx alata* Vog.). **Bioscience Journal**, v.33, n.2, p.306-313, 2017.

PRANDO, M.S.; CHIAVAZZA, P.; FAGGIO, A.; CONTESSA, C. Effect of coconut water and growth regulator supplements on *in vitro* propagation of *Corylus avellana* L. **Scientia Horticulturae**, v.171, p.91-94, 2014.

SANTOS, C.P.; PINTO, J.A.O.; SANTOS, C.A.; CRUZ, E.M.O.; ARRIGONI-BLANK, M.F.; ANDRADE, T.M.; SANTOS, D.A.; ALVES, P.B.; BLANK, A.F. Harvest time and geographical origin affect the essential oil of *Lippia gracilis* Schauer. **Industrial Crops and Products**, v.79, [s.n.], p.205-210, 2016.

SARTOR, F.R.; ZANOTTI, R.F.; PÔSSA, K.F.; PILON, A.M.; FUKUSHIMA, C.H. Diferentes meios de cultura e antioxidantes no estabelecimento *in vitro* do jacarandá da Bahia. **Bioscience Journal**, v.29, n.2, p.408-411, 2013.

SASAMORI, M.H.; ENDRES JÚNIOR, D.; DROSTE, A. Baixas concentrações de macronutrientes beneficiam a propagação *in vitro* de *Vriesea incurvata* (Bromeliaceae), uma espécie endêmica da Floresta Atlântica, Brasil. **Rodriguésia**, v.67, n.4, p.1071-1081, 2016.

SCHMILDT, O.; SCHMILDT, E.R.; OLIVEIRA, M.J.V. Sais e sacarose na germinação *in vitro* de limoeiro 'cravo'. **Nucleus**, v.12, n.1, p.215-222, 2015.

SILVA, G.M.; ALVARENGA, J.P.; BERTOLUCCI, S.K.V.; TAVARES, M.C.; RODRIGUES, M.A.; PINTO, J.E.B.P. Effect of chemical and physical factors *in vitro* propagation and volatile fraction analysis of *Aloysia triphylla* (L'Herit) Britton. **Acta Horticulturae**, v.1155, [s.n.], p.309-316, 2017.

SILVEIRA, D.G.; VIDAL, Á.M.; LEDO, C.A.D.S.; SANTANA, J.R.F.D.; SOUZA, F.V.D. Aspectos morfofisiológicos na pré-aclimatização *in vitro* e aclimatização de plantas de caroá. **Revista Ciência Agronômica**, v.44, [s.n.], p.544-553, 2013.

TAIZ, L.; ZEIGER, E.; MOLLER, I.M.; MURPHY, A. **Fisiologia vegetal e desenvolvimento vegetal.** 6ª ed. Porto Alegre: Editora Artmed. 2017. 858p.