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Growth and volatiles in the micropropagation of Santa Maria herb[1]

Crescimento e voláteis na micropropagação de Erva-de-Santa-Maria

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ABSTRACT - The aims of this study were to establish micropropagation for *Chenopodium ambrosioides* L. and analysis of volatile constituents *in vitro* as a function of different salt concentrations of MS medium and sucrose. The apical buds were established on MS medium added with 3% sucrose. The vegetative growth of nodal segments was tested in different salt concentrations of MS basic medium (0.25, 0.50, 1.00, 1.50, and 2.00) and of sucrose (5, 10, 20, 30, and 40 g L^{-1}). Nodal and apical segments were tested in vertical and horizontal orientations. Different volumes of culture medium (20; 35, 50, 65, and 80 mL) were examined in the growth of nodal segments. Plants were acclimatized in three different substrates. The apical segments collected from matrices plants were successful when established in MS medium. A multiplication rate of 8.5 was obtained with nodal and apical segments without using growth regulator. MS medium with its salt concentration reduced by half (0.50 MS) and sucrose at the concentration of 30 g L^{-1} provided better *in vitro* growth of nodal segments. Four major volatile compounds (α-terpinene, *p*-cymene, Z-ascaridole, and *E*-ascaridole) varied at different salt concentrations and only α-terpinene showed difference for sucrose concentrations. Apical segments in vertical orientation and nodal segments in horizontal with 50 mL of culture medium showed better responses for the *in vitro* cultivation of *C. ambrosioides* L.

Key words: *Chenopodium ambrosioides* L.. Medicinal plant. Tissue culture.

RESUMO - Objetivou-se com o presente trabalho estabelecer a micropropagação para *Chenopodium ambrosioides* L. e análise de constituintes voláteis *in vitro* em função de diferentes concentrações de sais do meio MS e de sacarose. As gemas apicais foram estabelecidas em meio MS acrescido de 3% de sacarose. O crescimento vegetativo dos segmentos nodais foi testado em diferentes concentrações de sais do meio básico MS (0,25; 0,50; 1,00; 1,50 e 2,00) e de sacarose (5; 10; 20; 30 e 40 g L^{-1}). Segmentos nodais e apicais foram testados nas orientações vertical e horizontal. Diferentes volumes de meio de cultura (20; 35; 50; 65 e 80 mL) foram examinados no crescimento de segmentos nodais. As plantas foram aclimatizadas em três diferentes substratos. Os segmentos apicais coletados de plantas matrizes obtiveram sucesso ao estabelecerem-se em meio MS. Uma taxa de multiplicação de 8,5 foi obtida com segmento nodal e apical sem a utilização de regulador de crescimento. O meio MS com sua concentração de sais reduzida pela metade (0,50 MS) e a sacarose na concentração de 30 g L^{-1} proporcionaram melhor crescimento *in vitro* de segmentos nodais. Quatro principais compostos voláteis (α-terpineno, p-cimeno, Z-ascaridol e *E*-ascaridol) variaram nas diferentes concentrações de sais e para as concentrações de sacarose, apenas o α-terpineno apresentou diferença. Segmentos apicais na orientação vertical e segmentos nodais na horizontal, com 50 mL de meio de cultura, apresentaram melhores respostas para o cultivo *in vitro* de *C. ambrosioides* L.

Palavras-chave: *Chenopodium ambrosioides* L.. Planta medicinal. Cultura de tecidos.

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INTRODUCTION

Chenopodium ambrosioides L. is a medicinal herb and spice popularly known worldwide, by names: Mexican tea, wormseed, "epazote", "mastruz" and "Erva-de-santa-Maria" (BLANCKAERT *et al.*, 2012). It is used as anthelmintic, anti-inflammatory, anti-Leishmania, antidiarrheal, among other (CYSNE *et al.*, 2016). The plant's anthelmintic property has been attributed to the presence of the monoterpene ascaridole, with few species showing capacity to produce it (SÁ; SOARES; RANDAU, 2015). Its therapeutic potential was recognized in such a way that the Brazilian Ministry of Health included it in the National List of Medicinal Plants of Interest to the SUS (Relação Nacional de Plantas Medicinais de Interesse ao SUS - RENISUS)(VERISSIMO *et al.*, 2011).

C. ambrosioides L. seeds present a high dormancy degree revealed by low germinative power (MARTINS; SILVA; JUNIOR, 2010). Medicinal plants are usually collected in their natural habitats and this practice harms the environment and can cause the extinction of species (DEBNATH; MALIK; BISEN, 2006). Thus, micropropagation represents a viable alternative for the rapid propagation of large numbers of clones in a small space, besides preserving germplasm and producing compounds of secondary metabolism.

According to Morais *et al*. (2012), micropropagation allows studying the accumulation of secondary metabolites and choosing of new species sources of bioactive compounds, improving the phytopharmaceutical production. Still according to these authors, the agricultural production of medicinal plants in pharmaceutical standards can be compromised by the heterogeneity of individuals due to the genetic and biochemical variability and difficulty of multiplication.

In the *in vitro* cultivation, a large amount of plant biomass can be obtained by using bioreactors and thus increasing the content of secondary metabolites of interest. When comparing *in vitro* and *in vivo* essential oil composition, Manan *et al*. (2016) observed that the amount of methyl chavicol of *Ocimum basilicum in vitro* (93.71%) exceeded the amount found in plants *in vivo* (66.29%). According to Mendes *et al*. (2013), the amount of monoterpene sabinene was higher in shoots of *Thymus caespititius* cultivated *in vitro* (40-45%) than in plants grown *in vivo* (18%).

In order to conduct *in vitro* studies, factors as the type and concentration of the culture medium must be studied and adjusted for each species, making available mineral elements essential for plant metabolism and the sucrose concentration suitable for energy supply and precursor carbons for the biosynthesis of structural and functional components (JO *et al*., 2009; PIVETTA *et al*., 2010; REIS *et al.*, 2009).

The aim of this study was to establish micropropagation for *C. ambrosioides* L. and to study the influence of different salt concentrations of MS medium and sucrose on the growth of species and their relationships with volatile constituents *in vitro*.

MATERIAL AND METHODS

General conditions of the experiments

In all experiments, autoclaving of the culture medium was performed at 120°C and 1 atm for 20 min. The tubes or flasks were kept in a growth room with photoperiod of 16 h of light, temperature of 25 ± 2 ºC and photon flux density of 32 μmol m^{-2} s^{-1} (PAR Photon Flux Sensor, ProCheck -Decagon devices); the pH of the culture medium was adjusted to 5.7 ± 0.1 before autoclaving and the culture medium was supplemented with 0.6% agar.

The growth variables shoot length (SL), number of shoots (NS) and leaves (NL), leaf (LDW), shoot (SDW), root (RDW), and total dry weight (TDW) were evaluated after 40 days. SL variable expresses the size of the main shoot measured from its base to the apical bud. The evaluation was performed with the aid of a digital caliper. LDW, SDW and RDW were measured on a precision scale after drying in a convection oven at 35 ºC for three days. TDW was obtained by the sum of RDW, SDW and LDW.

Establishment of explant

Wild plants from *C. ambrosioides* L. species located in the medicinal herb garden of UFLA were used as seed source. The exsiccate was deposited in the herbarium of the Department of Biology of this Institution under record 10137. The seeds were collected and sowed in a polystyrene tray of 128 cells, containing commercial Plantmax® substrate in order to obtain matrix plants. After 60 days, the germinated plants were transplanted into 3.6 L polyethylene pots, containing the same substrate and kept in a greenhouse with irrigation.

After 30 days, the apical segments were collected from these transplanted plants and washed in running water and neutral soap for 30 min. In a laminar flow cabinet, the explants were immersed in 70% ethyl alcohol for 30 s, followed by a 40% bleach solution for 10 min and finally washed five times in distilled and autoclaved water. At the end of the asepsis, the explants were inoculated into 120 test tubes containing 15 mL of MS culture medium (MURASHIGE; SKOOG, 1962) and 3% sucrose. The tubes were capped and sealed with

paraffin film and kept in the growth room. At 40 days, the percentage of contamination and plants established *in vitro* was evaluated.

Concentrations of salts, sucrose, and volume of culture medium

Nodal segments of, approximately, 2 cm length obtained from plants established *in vitro* were used as explants for all the three experiments. For each experiment, a completely randomized design (CRD) with four replications was used. The experimental unit was represented by three flasks containing three segments per replicate.

For the study of salt and sucrose concentrations of the MS medium, nodal segments were inoculated vertically in, 250 mL, flasks containing 35 mL of the medium at five different MS concentrations (2.00, 1.50, 1.00, 0.50, and 0.25) and five sucrose concentrations (5, 10, 20, 30, and 40 g L^{-1}). Nodal segments were inoculated vertically in flasks containing different solution volumes (20; 35, 50, 65, and 80 mL) of the culture medium.

Orientations of apical and nodal segments

Nodal and apical segments were inoculated in the vertical and horizontal orientations in the half of the MS salt concentration and supplemented with 30 g L^{-1} sucrose. The experimental design was completely randomized (CRD) in a 2x2 factorial design, being the explant type factor with two levels (apical and nodal) and the explant orientation factor (vertical and horizontal). Five replicates were used and the experimental unit consisted of three flasks, containing three segments per replicate.

Acclimatization of plants

After 40 days, the micropropagated plants were removed from the culture flasks, washed in running water, transferred to 53 cm^3 plastic tubes containing three types of substrates (ProVaso®, vermiculite, and sand) and kept in a greenhouse with irrigation. The used experimental design was the completely randomized (CRD) with seven replications. The experimental unit was represented by four tubes containing, one plant each. After 30 days, the percentage of surviving plants was evaluated.

Chemical analysis by headspace GC/MS

The leaves were collected from plants at 40 days of *in vitro* culture from experiments of different salt concentrations of MS and sucrose. Samples were dried in a convection oven at 35 °C. For analysis of the volatile constituents, 60 mg of dry leaves were used in triplicate.

The chemical analyses were performed by *headspace* -CG/MS, using an Agilent® 7890A gas chromatograph coupled to an Agilent® MSD 5975C mass selective detector. The operating conditions were established with the following parameters: sample incubation temperature of 110 °C for 30 min, syringe temperature at 120 °C. A HP-5MS fused silica capillary column (30 m long x 0.25 mm internal diameter x 0.25 μm film thickness) was used.

Volatile fraction constituents were identified by comparing their retention indices relative to the co-injection of a standard solution of n-alkanes (C8-C20, Sigma-Aldrich®, St. Louis, USA) and by comparing mass spectra from the database of NIST/EPA/NHI (NATIONAL INSTITUTE OF STANDARDS TECHNOLOGY, 2008) and of the literature (ADAMS, 2007).

Statistical analysis

Statistical analyses were performed using R software and the statistical package ExpDes (FERREIRA; CAVALCATI; NOGUEIRA, 2011), according to R DEVELOPMENT CORE TEAM (2012). The regression analysis for quantitative factors and the Tukey test, for qualitative factors were used for treatments whose averages showed significant differences at a 5% probability level.

RESULTS AND DISCUSSION

The establishment and asepsis of apical segments inoculated in complete MS medium, supplemented with 30 g L^{-1} sucrose were efficient with 98.3% survival. Apical segments inoculated in the MS medium had an approximate growth of 4 cm after 40 days, with a greenish staining.

The established plants were inoculated at different salt concentrations of MS medium and sucrose in order to study the influence of these factors on the growth of species and its relationships with the *in vitro* volatile constituents. Differences in herb growth were observed for the proposed treatments (Figure 1).

The MS medium has a macronutrient and micronutrient composition of 4.41 g L^{-1}. It is considered as a culture medium with high salt concentrations (MONFORT *et al.*, 2015). Thus, the MS medium in its original, lower and higher salt concentrations were tested. The analyzed explant growth variables showed significant differences in the salt concentrations of MS medium (Figure 2).

RDW declined linearly in increasing salt concentrations. For the other analyzed variables, significance for quadratic regression was verified and the maximum points were calculated for each function.

The estimated maximum shoot length was 61.84 mm at the concentration of 0.54 MS. The maximum number of leaves (24.27) and shoots (2.13) were obtained at concentrations of 0.90 and 0.88 MS, respectively. In relation to the dry weight, 22.13, 10.17, and 14.89 mg at the salt concentrations 1.05, 0.74, and 0.25 MS were verified for leaf, shoot, and root, respectively.

The lower salt concentration (0.25 MS) affected root growth. RDW decreased significantly from 0.25 MS to 2.00 MS. George, Hall and Klerk (2008) state that the addition of components to the culture medium, especially macronutrients and carbon sources, represents a considerable decrease in the osmotic potential of the medium. The effect of salt concentration of the medium can affect the availability of water to the roots (osmotic stress) and absorption of salts by the plant can reach a toxic level in the tissue (ionic stress). In general, the effect of salt stress on growth can reduce dry weight, leaf area, root length, shoot length, and senescence if the concentration is beyond the tolerated limit (TAIZ; ZEIGER, 2009).

Monfort *et al*. (2015) observed higher root length using low salt level (0.25 MS), which may be mainly due to the decrease in nitrogen level. The MS, 0.5 MS, and 0.25 MS media contain 60 mM, 30 mM, and 15 mM nitrogen, respectively. Also according to these authors, root growth is inhibited by $NH4^{+}$ and promoted by NO_3^{-}. A higher nitrate/ammonium ratio in medium with lower nitrogen concentration may have occurred to aid in rooting.

TDW of the plant was between half and the original salt concentration of the MS medium, reaching a maximum of 0.89 of the MS concentration, with a weight gain of 45.82 mg.

The reduction or gain in growth of the explant grown under salt conditions is a consequence of several physiological responses, including changes in ionic balance, water availability, mineral nutrition, photosynthetic efficiency, and carbon allocation and utilization. Qiu, Lu and Lu (2003) report that the reduction in growth observed in several species subjected to salt concentration is often associated with a decrease in photosynthetic capacity.

In relation to nutrient absorption, lower concentrations (0.25 MS) may limit some nutrient essential for plant metabolism (EPSTEIN; BLOOM, 2005). On the other hand, the excess of one ion reduces the absorption of another. According to Paula *et al*. (2015), the K absorption is inhibited when the Mg concentration is high (competitive inhibition). Moreira *et al*. (2003) observed that in the presence of high concentrations of Mg^{2+}, the absorption of Zn and Mn is impaired. According to these authors, the inhibition of Mg^{2+} on the absorption of Zn and Mn refers to the non-competitive type. Zinc is a very important element, since it is directly responsible for the synthesis of tryptophan, a precursor of auxin (indoleacetic acid), and indirectly for protein synthesis(VILLA *et al.*, 2009).

Regarding the volatile chemical composition, four constituents represented 92.27% of the total relative area of chromatographic peaks in the plant samples grown at different salt concentrations of MS medium. The constituents were α-terpinene (20.06%), *p*-cymene (16.65%), *Z*-ascaridole (45.81%), and *E*-ascaridole (9.75%), being that the indicated contents indicate the average of treatments. Cavalli *et al*. (2004) identified five constituents in the essential oil of *Chenopodium ambrosioides* L., characterized by *Z* -ascaridole (58.38%), *p*-cymene (16.2%), α-terpinene (9.7%), *E*-ascaridole (4.3%) and limonene (3.8%).

Figure 1 - Micropropagated plants of *C. ambrosioides* L. cultivated in: A- different salt concentrations of the MS medium and B- different concentrations of sucrose in the medium

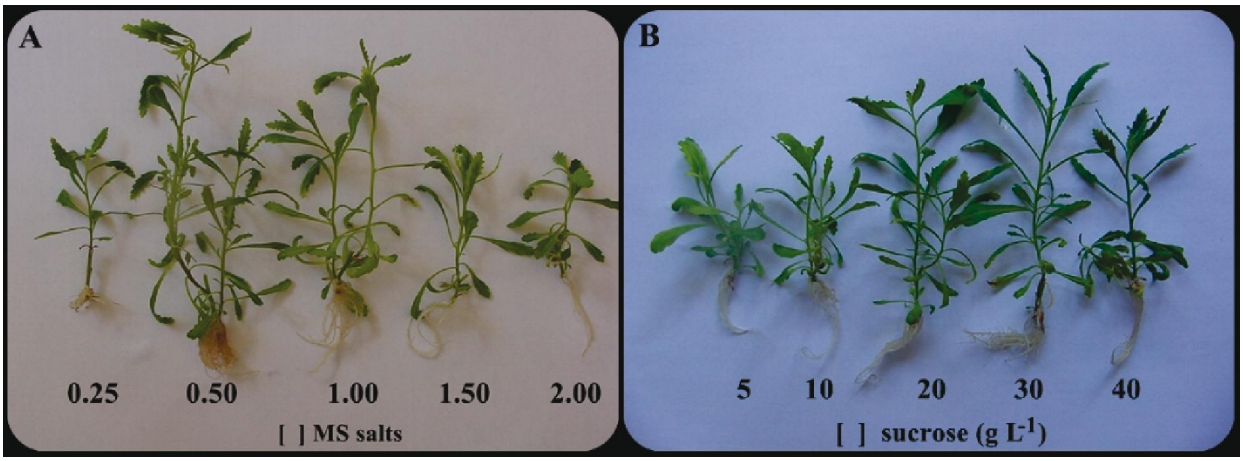
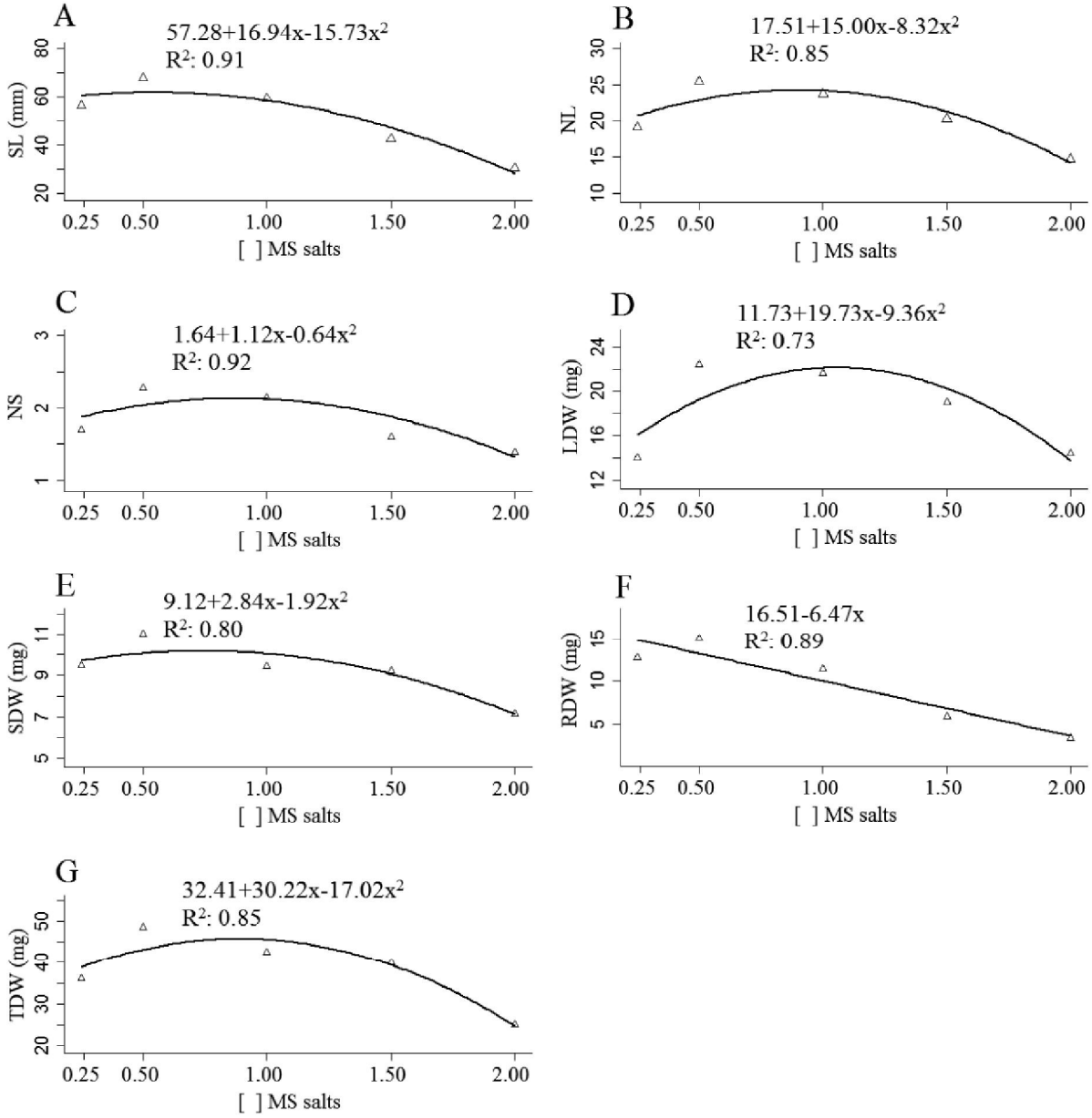


Figure 2 - Effect of different salt concentrations of MS medium in: A- shoot length (SL), B- number of leaves (NL), C- number of shoots (NS), D- leaf dry weeight (LDW), E- shoot dry weight (SDW), F- root dry weight (RDW), and G- total dry weight (TDW) of *C. ambrosioides* L plants cultivated *in vitro*

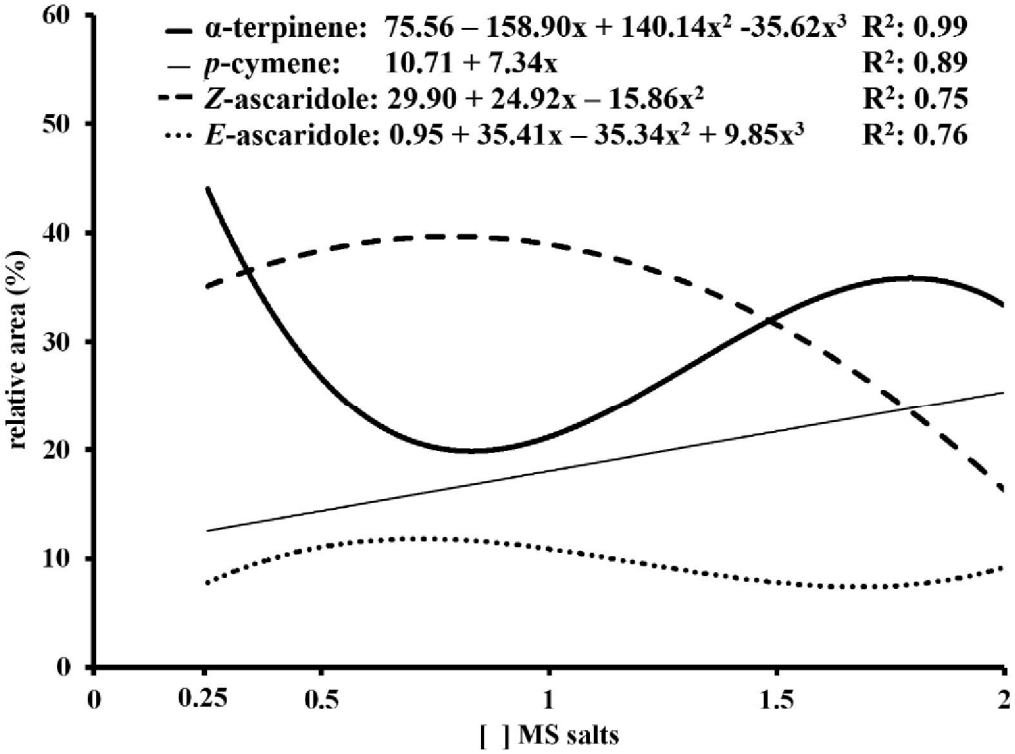


Comparing the volatile chemical composition of the cultivated plant in the normal MS medium and the other concentrations, there were significant differences among the relative areas of the constituent peaks (Figure 3).

The compounds were shown in greater relative area percentage for salt concentrations of MS medium in 1.79 MS (α-terpinene), 2.00 MS (*p*-cymene) 0.79 MS (*Z*-ascaridole), and 0.71 MS (*E*-ascaridole). It can also be observed that the decrease in α-terpinene content resulted in increases in ascaridole (*Z*) and its isomer (*E*), as well as the opposite. This relationship occurred because terpinene is a precursor of ascaridole biosynthesis (DEMBITSKYA; SHKROBB; HANUSA, 2008). For the monoterpenes Z and *E* -ascaridol, the maximum content was observed in the concentration of culture medium that provided the greatest growth of nodal segments (between 0.50 and 1.00 MS).

Studies on the factors that affect the volatile composition of *C. ambrosioides* L. are important to obtain plants with higher accumulation of the compounds

Figure 3 - Relative area (%) of chromatographic peaks of the main constituents of the volatile fraction of *C. ambrosioides* L. grown *in vitro* in different salt concentrations of MS medium



of interest. The variability in the content of major and/ or active constituents is one of the main difficulties of developing herbal medicines with action reproducibility (SÁ; SOARES; RANDAU, 2015).

The culture medium should be supplemented with carbohydrates due to the low photosynthetic activity and irradiation and to the limited gas exchange. It was observed that plants showed maximum growth up to a sucrose concentration limit, followed by a reduction in the evaluated parameters, except for SDW and NS, where they occurred linearly (Figure 4).

Plants showed maximum growth (65.38 mm) at the concentration of 24.25 g L^{-1} sucrose, followed by a reduction. The highest LDW estimated was 30.92 mg at the concentration of 33.75 g L^{-1}. The most efficient concentration of sucrose for RDW accumulation was 34 g L^{-1} for a gain of 10.97 mg. The maximum sucrose concentration for TDW accumulation was 29.88 g L^{-1} with, a gain of 47.72 mg by regression curve analysis (Figure 4).

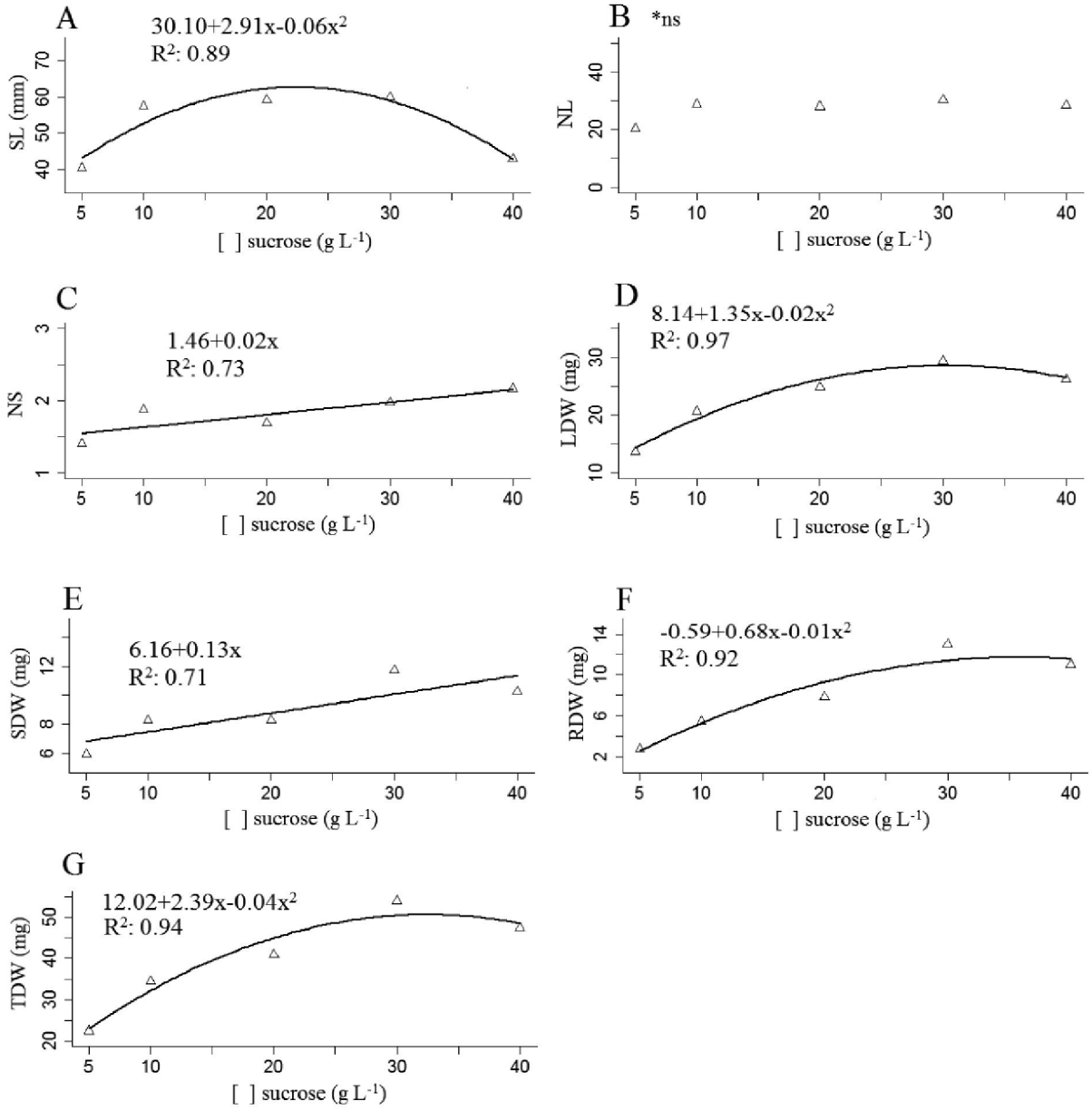
Monfort *et al.* (2015) studied the effect of interactions between three MS variations (MS, 0.5 MS, and 0.25 MS) and three sucrose concentrations (0, 15, and 30 g L^{-1}) on the growth of *in vitro* nodal segments of *Ocimum selloi*. According to these researchers, there was no effect of the MS medium on the length and weight of shoots in the absence of sucrose. This result demonstrated the importance of sucrose in the basic medium in order to provide energy for shoot growth.

For the environmental conditions of the present study, it was observed that the low sucrose concentration (5 g L^{-1}) culminated in lower results for all variables with significant differences. In the *in vitro* culture, sucrose acts as a source of energy and provides carbons that will be used during respiration and are precursors for the biosynthesis of structural and functional components, such as oligosaccharides, amino acids and other molecules necessary for growth (TORRES; CALDAS; BUSO, 1998).

The highest concentration of this carbohydrate (40 g L^{-1}) did not interfere in SDW and NS. However, there was inhibition of explant growth in the other parameters.

It is observed a decrease in the weight gain by the behavior of the regression curve in concentrations above 30 g L^{-1} of sucrose. According to Desjardins, Dubuc and Badr (2009) and Villa *et al*. (2006), excess sucrose may be harmful because the presence of carbohydrate in the culture medium inhibits the synthesis of chlorophyll and increases the osmotic potential of the medium. Therefore,

Figure 4 - Effect of different sucrose concentrations on: A- shoot length (SL), B- number of leaves (NL), C- number of shoots (NS), D- leaf dry weight (LDW), E- shoot dry weight (SDW), F- root dry weight (RDW), and G- total dry weight (TDW) of *C. ambrosioides* L. plants cultivated *in vitro*. *[ns] Not significant by F test



it reduces the photosynthetic capacity of the crops, even being essential for growth.

Similarly to the experiment with the salt concentration of the MS medium, the presence of the same four volatile constituents (α-terpinene, *p*-cymene, *Z*-ascaridole, and *E*-ascaridole) were observed. However, different concentrations of sucrose did not significantly influence the content of *p*-cymene, *Z*-ascaridole, and *E*-ascaridole. In sucrose additions, only the α-terpinene content increased linearly (Figure 5).

Figure 6A shows the growth of plants resulting from the inoculation of nodal and apical segments in the vertical and horizontal orientations. In Figure 6B, nodal segments were inoculated vertically in flasks containing different solution volumes of medium (20; 35, 50, 65, and 80 mL).

For NS and NL, there was not significant interaction between orientation and explant type. In the vertical orientation, there was higher leaf production per plant and NS was not statistically influenced, as well as

Figure 5 - Relative area (%) of chromatographic peaks of the main constituents of the volatile fraction of *C. ambrosioides* L. grown *in vitro* at different sucrose concentrations. *ns Not significant by F test

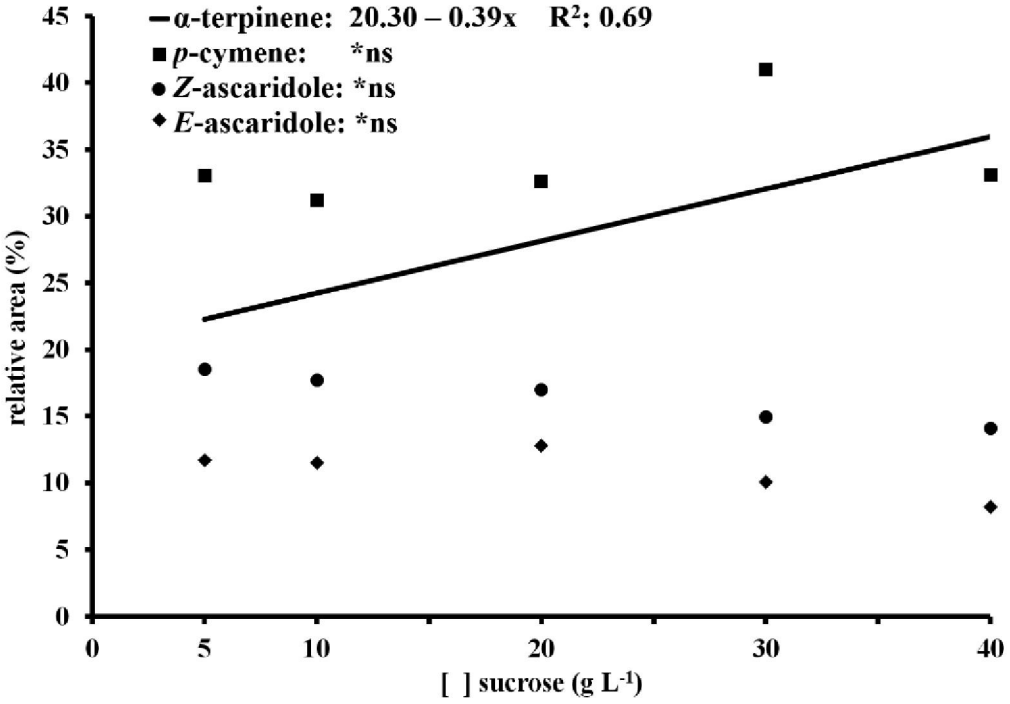
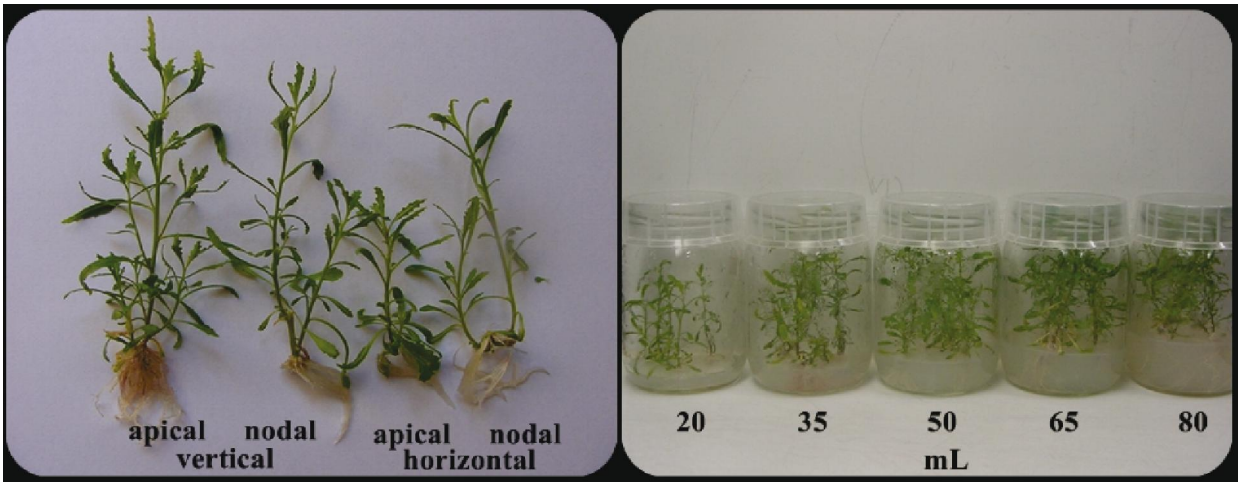


Figure 6 - Micropropagated plants of *C. ambrosioides* L. after 40 days, cultivated in: A- different orientations of nodal and apical segments and B- different volumes of medium (mL)



the explant type (apical and nodal) for these variables. However, the interaction was significant for SL, SDW, RDW, and LDW (Table 1).

It is observed that, in absolute values, the apical segment inoculated in the culture medium in the vertical position obtained greater growth gain, which not occurred in the horizontal position (Table 1). In these segments, the highest growth may be due to apical dominance. It is known that auxin is synthesized at the shoot apical and transported towards the base, giving greater development. This transport is called polar auxin transport. Therefore, it does not occur when the apical segment is inoculated in the horizontal position.

In relation to nodal segments, the horizontal position favored the growth in relation to apical segments. According to Erig and Schuch (2002), obtaining a higher

Table 1 - Shoot length, shoot, leaf and root dry weight of apical buds and nodal segments of *C. ambrosioides* L. grown *in vitro* in vertical and horizontal orientations

Variables	Types	Orientation	
		Vertical	Horizontal
Shoot length (mm)	Apical	57.75 Aa	39.23 Bb
	Nodal	51.34 Aa	50.10 Aa
Shoot dry weight (mg)	Apical	10.74 Aa	7.80 Ab
	Nodal	8.50 Ba	16.04 Aa
Leaf dry weight (mg)	Apical	21.36 Aa	15.28 Ba
	Nodal	15.56 Ab	16.78 Aa
Root dry weight (mg)	Apical	12.42 Aa	7.84 Ba
	Nodal	8.54 Ab	9.96 Aa
Total dry weight (mg)	Apical	44.52 Aa	30.92 Bb
	Nodal	32.66 Bb	42.78 Aa

Averages followed by the same capital letter on the line and lowercase on the column do not differ significantly among themselves by Tukey test at 5% significance

number of shoots with the nodal segment in the horizontal orientation is mainly due to the breakdown of apical dominance, which in this orientation may have inhibited the auxin polar translocation, with a higher sprouting in nodal segments.

The response to plant growth depends on the genotype. Papafotiou and Martini (2009), studied leaf explants of *Zamioculcas zamiifolia* and observed that the horizontal explant orientation provided less development compared to the vertical orientation. However, Botrel *et al.* (2015) observed in *Hyptis marrubioides* and Silva *et al.* (2017) in *Aloysia triphylla*, higher dry weight gain in the horizontal position.

Nodal segments were inoculated vertically in flasks containing different solution volumes (20; 35, 50, 65, and 80 mL) of the 0.5 MS culture medium, supplemented with 30 g L^{-1} sucrose. The studied response variables showed significant differences. SL showed cubic behavior and the other quadratic behavior (Figure 7).

The maximum values for SL (62.48 mm), NL (43.82), and NS (5.03), occurred in volumes 42.25, 64.31, and 60.34 mL, respectively. The most efficient volume for accumulation of LDW (28.86 mg), SDW (22.11 mg), and RDW (28.57 mg) was observed at 60.53, 60.06, and 63.02 mL, respectively.

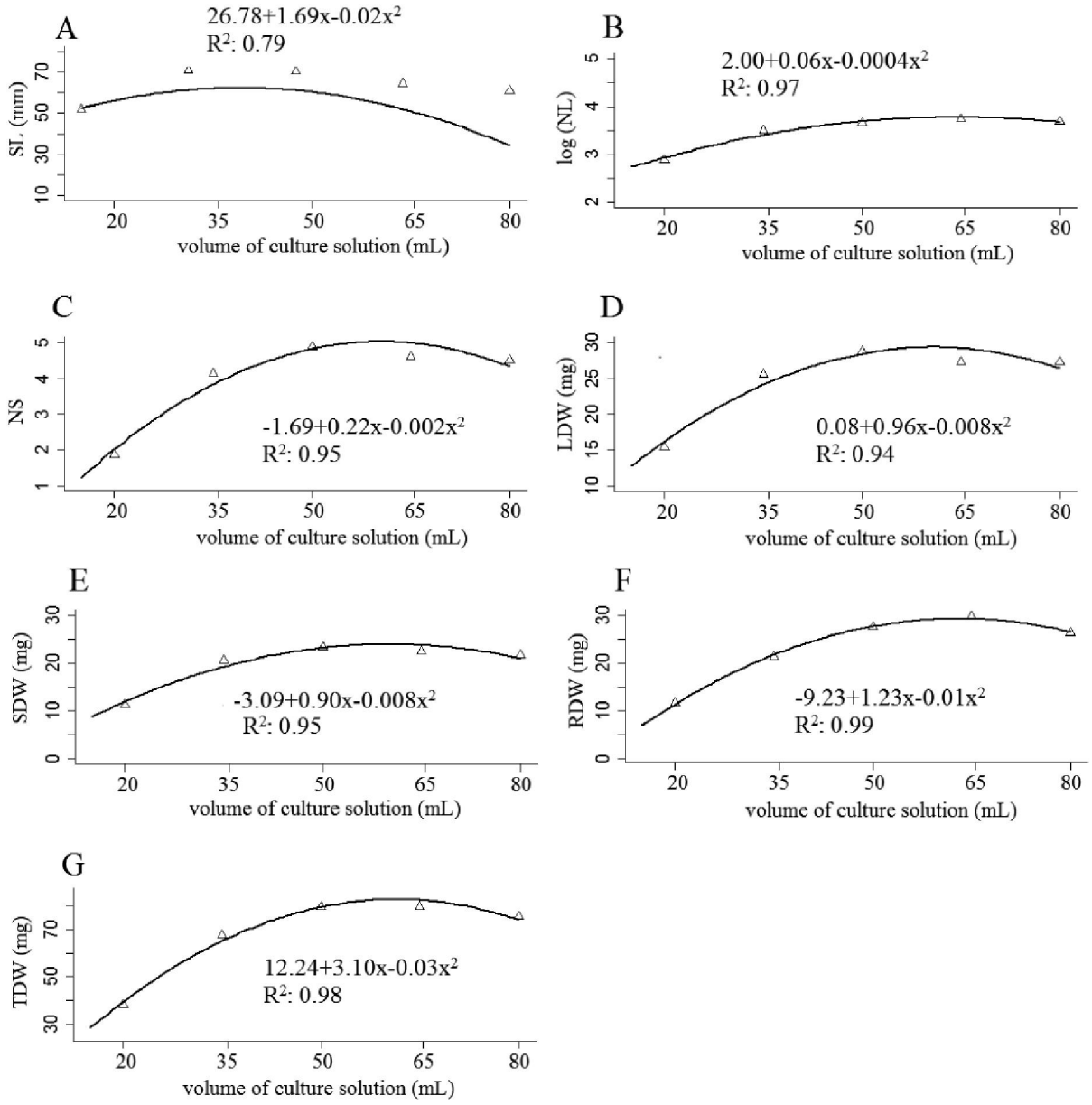
The type of vial and the amount of used culture medium are variables that have received little attention, although they directly affect the surface area of the culture medium-atmosphere interface, the depth and the volume of air over the culture medium, being that the use of ideal container for each species can optimize its production speed (MORAES *et al.*, 2010). Pereira *et al.* (2006) and Reis *et al.* (2007) studied the proliferation of 'curauá' *in vitro* (*Ananas erectifolius*) and the micropropagation of lemon balm (*Melissa officinalis*), respectively, using growing volumes of culture medium. These researchers observed that the increase in volume (30-40 mL) positively affected the growth of micropropagated plants.

The economic feasibility study was not performed. However, it can be observed generically that volumes of culture medium higher than 50 mL did not provide increasing increments in the explant growth. It is also worth noting that, when multiplying this species, either by apical or nodal segments, there will be no great differences in the number of plants that will be obtained between the volumes of 50 and 80 mL based on NS (apical buds) and NL (axillary buds) from both treatments. However, it will use 1.6 x more of culture medium, 80 mL when compared to 50 mL, in order to obtain the same multiplication rate.

The multiplication rate through the apical and nodal segments presented an average of 8.50, reflecting a satisfactory methodology of micropropagation. It is also emphasized the non-use of growth regulators. The most important is to obtain a satisfactory multiplication rate with the minimum variation among explants, the culture medium being one of the variables that can be used for this study (REIS *et al.*, 2008).

Nodal and apical culture is considered as ideal to reduce the risks of genetic irregularity. Since cellular dedifferentiation is not necessary, there is a lower probability of regeneration of plants with somaclonal

Figure 7 - A- shoot length (SL), B-log of number of leaves (NL)*, C- number of shoots (NS), D- leaf dry weight (LDW), E- shoot dry weight (SDW), and F- root dry weight (RDW) of *C. ambrosioides* L. grown *in vitro* in different volumes of culture solution. *Variable transformed by log (Y)



variation. The plants of the species were acclimatized in sand, vermiculite and ProVaso® substrate with a survival percentage of 100%.

CONCLUSIONS

1. Apical segments of *C. ambrosioides* L. matrix plants can be established *in vitro* with MS medium and 3% sucrose;
2. For better growth and multiplication of nodal segments, the explants should be inoculated in the MS culture medium, with half the original salt concentration and 3% sucrose;
3. Nodal segments can be inoculated in vertical or horizontal orientations. However, the horizontal position allows greater TDW. The apical segments should be inoculated vertically. For greater multiplication efficiency, it is recommended to use 50 mL of culture medium;

4. The percentage of relative area of the major volatiles constituents (α-terpinene, *p*-cymene, *Z*-ascaridole, and *E* -ascaridole) is influenced by the different salt concentrations of the MS medium. The different concentrations of sucrose influence only the α-terpinene content.

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