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In vitro Growth of Genovese Basil in Response to Different Concentrations of Salts and Interaction of Sucrose and Activated Carbon

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Abstract

Genovese basil has great economic potential; however, there is no established micropropagation protocol for this species. Therefore, this study aimed at assessing the *in vitro* growth of Genovese basil in response to different concentrations of salts in the Murashige and Skoog medium (MS) and interaction of sucrose and activated carbon. Two assays were conducted independently in an *in vitro* environment using the MS medium, regulators, agar, and five salt concentrations (0, 25, 50, 70 and 100%). In the second assay, two concentrations of sucrose (30 and 60 g L^{-1}) and three concentrations of activated carbon (0, 3.0, and 4.5 g L^{-1}) were tested.In addition, copper and zinc were quantified in the roots. The results showed that shoots were favored when the medium was at its full strength (100% salts), with seedlings forming more leaves.This result may be associated with a higher demand for nitrogen and because of the ionic balance between NH_4^+ and NO_3^-. High concentrations of salts affected the roots, but a reduction to 70% salt favored root development. Doubling the usual dose of sucrose (60 g L^{-1}) damaged the growth of the seedlings. Damage caused by osmotic and oxidative potentials, and by toxic compounds may be related to the observed results. The amount of copper and zinc in the root increased with increased concentrations of activated carbon in the medium. The presence of activated carbon reduced callus formation but did not mitigate the effects of increased sucrose concentration.

Keywords: *Ocimum basilicum*, Lamiaceae, micropropagation, medium strength, zinc, copper

1. Introduction

Basil (*Ocimum basilicum*) is a plant belonging to Lamiaceae which originates in India, Africa, and South Asia. Currently, basil is cultivated worldwide (Bertoli et al., 2013). According to Hussain et al. (2008), basil contains secondary metabolites. The principal constituent of the basil's essential oil is Linalool, followed byepi-α-cadinol, α-bergamotene, γ-cadinene, germacrene-D, and camphor. Basil is valued for its pharmaceutical properties in addition to its culinary and medicinal use. The essential oil in basil has antioxidant Hussain et al. (2008), antitumoral Kathirvel and Ravi (2012) and antimicrobial properties and is used for flavoring in food and in perfumes (Bais et al., 2002; Vieira et al., 2014).

Despite the importance in medicinal, pharmaceutical, and food industries, studies on basil cultivation and propagation methods are still inconclusive—in general, the unfamiliarity with the physiology and *in vitro* propagation of *Ocimum* genus. In addition, the lack of a micropropagation protocol slows down advancement, prevents advances in the production and technologies for these plants. Thus, obtaining quality seedlings is very difficult, this is because in conventional production methods the process of seedling production is delayed and the plants are exposed to biotic and abiotic factors (Ahmadian et al., 2013). Callus formation, adventitious root growth, and vitrification are events commonly observed *in vitro* cultivation, although they remain poorly understood.

Not having a micropropagation protocol prolongs the seedling production stage and thus increases the cost of production. The culture medium, where the seedling is produced, is the most expensive material used in the

micropropagation process (Kumar & Loh, 2012). In addition, the lack of a micropropagation protocol prevents the advancement of other research lines with tissue culture, such as breeding programs and *in vitro* production of secondary metabolites, which are important compounds for the pharmaceutical industry (Alvarez, 2014). Thus, to address the above situations requires that the micropropagation protocols are established.

Plant tissue culture is based on cellular totipotency and requires optimal conditions to regenerate a plant (George et al., 2008; Kumar & Loh, 2012). The most commonly used culture medium for *in vitro* culture is the MS medium (Murashige & Skoog, 1962), which must be adapted for each plant crop (Kumar & Loh, 2012) with appropriate salt concentrations (strength of the medium).Carbon sources, antioxidants, complex substances, and growth regulators are elements that make up the culture medium and can directly interfere with *in vitro* seedling development (George et al., 2008). Sucrose has been the most widely used carbon source in this process (Yaseen et al., 2013), as it is cheap and accepted by most plants. The activated carbon is the most used antioxidant (Thomas, 2008) because it promotes the adsorption of growth inhibitors and hence decreases the toxicity in the culture medium.

Silva et al. (2017) verified that the presence of activated carbon in the culture medium prevented callus formation and promoted leaf sprouting in Red Rubin basil plants. The authors also demonstrated that high salt concentrations (> 75%) damaged the root system, although the complete MS mediumfavored shoot development. Fadel et al. (2010) reported that the use of half salt concentration doubled the rooting for *Mentha spicata* in comparison with the MS medium at its complete strength. However, results may vary according to the variety or cultivar of Lamiaceae; therefore, studies should be specifically targeted.

Thus, it is expected that the salt concentration of the MS medium would interfere with the physiology of plant growth, especially in the root system of Genovese basil. The interaction of activated carbon and sucrose may influence the balance between the development of roots and shoots, callus formation, and abnormal seedling occurrence (Thomas, 2008; Yaseen et al., 2013; Silva et al., 2017). In addition, the activated carbon can promote seedling growth but limits the uptake of micronutrients such as Zn and Cu, which are important enzymatic cofactors (Thomas, 2008; Marschner, 2011). Research has demonstrated that the growth standards differ depending on the species. Knowledge on the interaction effects of sucrose and activated carbon as well as their isolated effects, in addition to the best salt concentration in the MS medium for this species is lacking and this needs to be studied.

It is possible to formulate a mathematical model to assess the salt concentration from equidistant values. Equally, it is possible to estimate the growth of seedlings even without a complete evaluation of the concentration (Shanock et al., 2010). Such estimates can be performed for each variable of interest. Verifying the interaction of sucrose and activated carbon is useful to understand how efficient the activated carbon is in mitigating the effects of adsorption of compounds deleterious to seedling growth, mainly those from the Maillard reaction, without, however, prejudicing the absorption of micronutrients such as Zn and Cu (Thomas, 2008).

Therefore, this study aimed at assessing the effect of different salt concentrations, and activated carbon and sucrose and their interaction effect on the growth of Genovese basil cultivated *in vitro* in the MS medium.

2. Material and Methods

This study was carried out at the Laboratories of Molecular Biology and of Plant Tissue Culture in the Paranaense University (UNIPAR).

2.1 Plant Material Asepsis

Genovese basil seeds (Horticeres®) were used as propagating material and was acquired in the local retail market, bearing batch number 1400168, and were marked as 99% pure resulting in 91% germination. The seeds were sterilized in laminar flow and held for 2 min in 70% ethyl alcohol and then kept in a 2% sodium hypochlorite solution for 15 min under stirring. Subsequently, four successive washes were performed with deionized and autoclaved water. The same asepsis method was used in all assays.

2.2 Assay 1: Salt Concentrations

Five salt concentrations of MS medium (0, 25, 50, 70, and 100%) were tested. The MS media were supplemented with 30 g L^{-1} of sucrose, 0.2 mg L^{-1} of 1-Naphthaleneacetic acid (NAA), 0.1 mg L^{-1} of 6-Benzylaminopurine (BAP), 1 mg L^{-1} of Gibberellin 3 (GA_3), and 6.5 g L^{-1} of agar (Kasvi®) with pH adjusted to 5.8. The experimental arrangement was a completely randomized design with five repetitions and five vials per repetition, each vial containing four seeds.

2.3 Assay 2: Activated Carbon and Sucrose

This experiment was done in a factorial design (2 × 3) with two sucrose concentrations (30 and 60 g L^{-1}) and three activated carbon concentrations (0.0, 3.0, and 4.5 g L^{-1}), totaling six treatments. Agar (6.5 g L^{-1}), NAA (0.2 mg L^{-1}), and BAP (0.1 mg L^{-1}) were added to the MS medium and the pH was adjusted to 5.8. Each treatment consisted of five repetitions, with four plots per repetition and four seeds per plot. The basil's dry roots were obtained from the 85-day seedlings and submitted to Zn and Cu quantification according to the method proposed by Malavolta et al. (1987). All nutritional analyses were done in duplicate.

2.4 In vitro Conditions

The MS medium was closed in glass vials with clear plastic lids, sealed with polyvinyl pyrrolidone film (PVP) before autoclaving at 121 °C for 20 min. Then the vials were taken to the growth chamber and maintained at 25±2 °C under a 24 h photoperiod, with an irradiance of 70.2 μmol m^{-2} s^{-1} provided by 6400K cool white 10-20 W fluorescent lamps (Empalux®, Curitiba, Brazil).

2.5 Periodic Analysis

During the course of the experiments, evaluations were performed in three distinct periods. In the first assay, observations were done at 38, 59, and 85 days after inoculation. In the second assay, observations were done at 59, 73, and 80 days after inoculation. Data on callus formation (C%), abnormal seedlings (AB%), and contamination (CT%) were collected. The contamination criterion was the presence of fungus and bacteria. Seedlings were considered abnormal based on the criteria described by MAPA (2009).

2.6 Final Evaluation

The first assay was concluded at 85 days after inoculation and the second at 80 days after inoculation, considering the size of seedlings reported in assays undertaken previously (Trettel et al., 2018). In the final evaluation, the following characteristics of the Genovese basilwere analyzed: callus formation (C%), number of leaves (NL), formation of shoots (S%), length of shoots (LS), length of roots (LR), fresh mass of shoots and roots (FMSR), fresh mass of roots (FMR), Fresh mass of shoot (FMS), dry mass of shoots (DMS), and dry mass of roots (DMR).First, the LS and LR were measured with a digital caliper and FMSR and FMR were measured using an analytical balance. Then the material was placed in an oven at 65 °C for three days to obtain the DMR.

2.7 Statistics and Data Analysis

In the first assay, data from the three seedling stages were submitted to analysis of variance ($p \leq 0.05$) and the means compared by the Tukey test ($p \leq 0.05$). Data from the final evaluation were submitted to analysis of variance ($p \leq 0.05$) and the means compared by polynomial regression ($p \leq 0.05$). In both analyzes, SISVAR software version 5.6 was used (Ferreira, 2011).

In the second assay, data collected during *in vitro* cultivation were analyzed in a 3 × 2 × 3 factorial design, with three periods of evaluations, two concentrations of sucrose, and three concentrations of activated carbon. At the end of the experiment, the amount of Cu and Zn and the other parameters were evaluated in response to the interaction of sucrose and activated carbon and the isolated action of each. All analyzes were undertaken using SISVAR software version 5.6 (Ferreira, 2011). A p-value less than 0.05 was considered statistically significant for all the tests.

3. Results

3.1 Assay 1: Salt Concentrations

Of the characteristics of basil that were evaluated during the *in vitro* culture, it was found that the C% did not respond to the treatments and presented an average occurrence of 36.57%. Abnormal seedlings were present more in the first two evaluations and decreased by approximately 17% at the end of the assay. Tissue oxidation was greater at 59 days of cultivation, decreasing by the end of the assay (Table 1).

Table 1. Formation of calluses, abnormal seedlings and oxidation in 3 different periods

Period (days)	Calluses (%)	Abnormal (%)	Oxidation (%)
38	$36.63^{\pm 5.12a}$	$39.59^{\pm 3,38a}$	$22.23^{\pm 4.05b}$
59	$37.07^{\pm 3.73a}$	$39.33^{\pm 3.36a}$	$52.03^{\pm 5.35a}$
85	$36.02^{\pm 9.3a}$	$21.96^{\pm 3.02b}$	$38.89^{\pm 9.35a}$

Note. * Means followed by the same letter in the column do not differ by Tukey test ($p \leq 0.05$).

With respect to the other characteristics evaluated after 85 days of inoculation, no response of the DMSR was observed to the salt concentrations. However, there was a significant increase in the NL with increased salt concentrations. In the MS medium without salts, only two leaves were observed, totaling approximately sixteen leaves at the end of the experiment (85 days) (Figure 1A). The LS responded positively up to 72% salts, decreasing at increased concentrations (Figure 1B). However, the final FMS presented a linear behavior up to 1.6 g FMS (Figure 1C). It was observed that the seedlings were lower in number and with more leaves.

The roots became longer as the concentration of salts increased. The LR varied by approximately 140 mm within the concentration range applied to the medium. However, the roots became thinner at higher concentrations compared to lower concentrations (Figure 1D). The opposite was observed for FMR, which dropped with more than 69.76% salts.

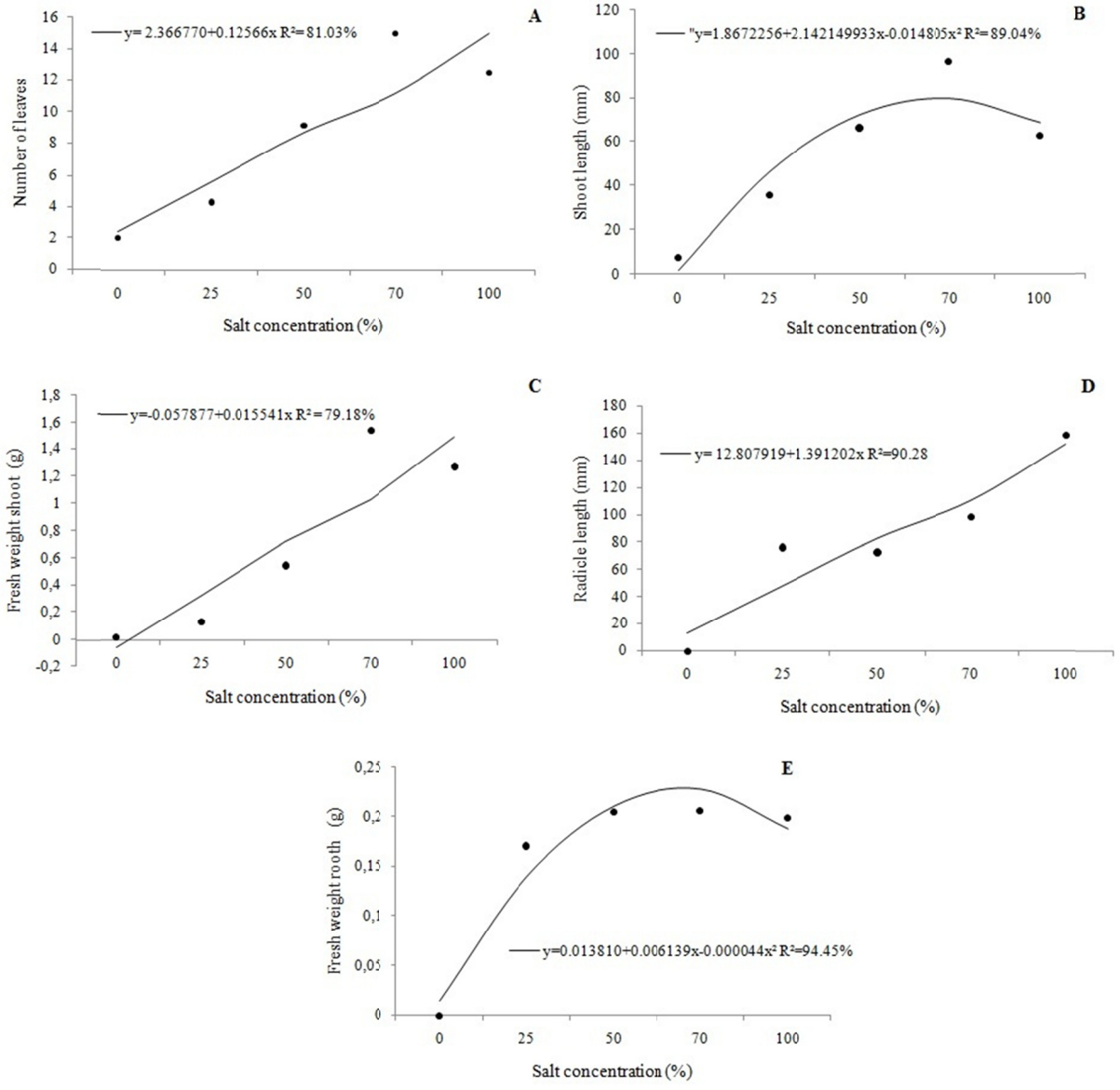

Figure 1. Final evaluation of *in vitro* Genovese basil submitted to different salt concentrations. a) total number of leaves, b) length of shoots, c) fresh mass of shoots, d) length of roots and, e) fresh mass of roots

3.2 Assay 2: Sucrose and Activated Carbon

No interaction effect was observed for the evaluation period on the basil's characteristics. The 85-day observations indicated two situations. First, the LR, FMS, DMS, and DMR responded to the interaction of

sucrose and activated carbon (Table 2). Second, the NL and LS responded to the isolated action of sucrose while C%, NL, LS, and FMR responded to the isolated effect of activated carbon (Table 4).

Roots were damaged by the combined action of sucrose and activated carbon. The average growth of roots at 60 g L^{-1} sucrose was 2.5 times greater than at 3.0 g L^{-1} activated carbon was also included. Similar results were also observed at 30 g L^{-1} sucrose. The difference in root length in response to sucrose concentration was small; therefore, doubling the sugar concentration is not justifiable, as the production cost increase without significant benefits.

The response of DMS and FMS were like the previously described results. The DMS did not respond only to treatments with 30 g L^{-1} sucrose and 3.0 g L^{-1}activated carbon (Table 2). The DMR did not show gains in biomass with activated carbon combined with sucrose in the medium. Biomass gains were only observed with the usual sucrose concentration (Table 2).

Table 2. Interaction effect of activated carbon and sucrose on Genovese basil's length of roots (LR), fresh mass of shoots (FMS),dry mass of shoots (DMS) and dry mass of roots (DMR)at 85 days after in vitro inoculation

Activated carbon (g L^{-1})	LR (mm)		FMS (g)		DMS (g)		DMR (g)	
	30	60	30	60	30	60	30	60
	---------- Sucrose (g L^{-1}) ----------							
0.0	47.22 ±6.1aA	50.16 ±7.8aA	1.1543 ±0.34aA	1.039 ±0.08aA	0.1234 ±0.02aA	0.1404 ±0.03aA	0.1004 ±0.01aA	0.0030 ±0.0001bA
3.0	39.90 ±6.2aAB	19.53 ±4.4bB	0.0837 ±0.03aB	0.0149 ±0.005aB	0.1423 ±0.03aA	0.0100 ±0.001bB	0.0148 ±0.005aB	0.0026 ±0.0002aA
4.5	25.40 ±4.33aB	32.20 ±5.5aB	0.0841 ±0.001aB	0.1446 ±0.04aB	0.1333 ±0.02aA	0.1759 ±0.06aA	0.0939 ±0.01aA	0.0200 ±0.005bA

Note. * Means followed by the same lowercase letter in the line and capital letter in the column do not differ by the Tukey test ($p \leq 0.05$).

Other characteristics of basil that responded to the isolated actions of activated carbon and sucrose revealed that double sucrose concentration (60 g L^{-1}) prevented the increased NL and LS (Table 3). The activated carbon reduced C% significantly, but did not affect the NL, DMS, FMS, and LR. These characteristics presented a little increase with 4.5 g L^{-1} activated carbon, but were not greater than the average values observed in the control treatment (Table 4).

Table 3. Isolated effect of sucrose on Genovese basil's number of leaves (NL) and length of shoots (LS) at 85 days after in vitro inoculation

Sucrose (g L^{-1})	NF	CBR (mm)
30	10.82±2.58a	77.71±9.07a
60	8.42±1.31b	44.80±19.55b

Note. * Means followed by the same letter in the column do not differ by Tukey test ($p \leq 0.05$).

With respect to micronutrient uptake in roots, the amount of Zn in roots increased with the use of activated carbon combined with 30 g L^{-1} sucrose (Table 5). However, the amount of Zn expressively reduced in the roots with 3.0 g L^{-1} activated carbon combined with 60 g L^{-1} sucrose in comparison with the control and the highest activated carbon concentration (Table 5).

Similar results were observed for the uptake of Cu by the roots when the medium had 30 g L^{-1} sucrose. The amount of Cu doubled in the roots with both concentrations of activated carbon (Table 5). The average values of Cu increased with the highest sucrose concentration (60 g L^{-1}), mainly when compared to the control treatment and to both concentrations of activated carbon. The highest average value was 19.5 mg Kg^{-1} (with 3.0 g L^{-1} activated carbon) followed by 11.5 mg Kg^{-1} (with 4.5 g L^{-1} activated carbon). It is probable that there is a relationship between the values of micronutrients, especially between Cu and sucrose concentration.

Table 4. Isolated effect of activated carbon on Genovese basil's callus formation (C%), number of leaves (NL), length of shoots (LS), and fresh mass of roots (FMR)at 85 days after in vitro inoculation

Activated carbon (g L^{-1})	C (%)	NL	LS (mm)	FMR (g)
0.0	42.05±8.83a	12.40±1.2a	80.94±13.05a	1.0969±0.23a
3.0	1.17±b	5.85±1.6b	38.64±7.7b	0.0493±0.02b
4.5	5.49±b	10.72±1.01a	64.17±6.42a	0.1143±0.009b

Note. * Means followed by the same letter in the column do not differ by Tukey test ($p \leq 0.05$).

Table 5. Interaction effect of activated carbon and sucrose on the amount of Zinc and (Zn) and Copper (Cu) in Genovese basil's roots at 85 days after in vitro inoculation

Activated carbon (g L^{-1})	Root			
	Zn (mg Kg^{-1})		Cu (mg Kg^{-1})	
	30	60	30	60
	---------- Sucrose (g L^{-1}) ----------			
0.0	154.50±3.53aB	122.00±5.65bA	10.75±0.35aB	8.00±1.41B
3.0	179.00±1.41aA	53.00±4.24bC	20.50±0.70aA	19.50±0.70aA
4.5	189.25±1.06aA	108.75±1.76bB	20.55±0.35aA	11.50±0.70bB

Note. * Means followed by the same lowercase letter in the line and capital letter in the column do not differ by the Tukey test ($p \leq 0.05$).

4. Discussion

4.1 Salt Concentration in the MS Medium

The MS medium contains macro and micronutrients, particularly a high amount of NH_4^+ and NO_3^- (George et al., 2008). However, adjustments in the concentration of salts have been the focus of many studies to improve the growth of plants as the composition and concentration of salts in the MS medium causes different growth responses of shoots and roots. These aspects were verified in the Genovese basil in this study.

The increased salt concentration increased the number of leaves and the final biomass of shoot. This increase was probably due to the availability and uptake of nitrogen, which was metabolized and incorporated in the leaf and stem biomasses. The balance between NH_4^+ and NO_3^- in the medium favored the nitrogen use by seedlings (George et al., 2008). Grimes and Hodges (1990) demonstrated in rice that the balance between NH_4^+ and NO_3^- at 80:20 ratio causes an increase in seedling height (three times more) in comparison with the75:25 ratio, in which short shoots were reported.

Nitrogen is a constituent of amino acids, nitrogenous bases, and proteins; therefore, it is essential for plants, especially for shoots. The results of our study on the *in vitro* growth of basil has demonstrated that Lamiaceae species developed better at salt concentrations above 80% or in the medium's complete strength. Silva et al. (2017) have verified that the MS medium at its complete strength doubled the Red Rubin basil's biomass. Monfort et al. (2018) have reported a substantial increase in the number of leaves, dry and fresh masses of shoots and roots of *O. basilicum* in comparison with other culture media such as WPM, which contains less nitrogen, and half the MS strength (½MS).

On the other hand, roots that were longer, thinner, and of lower mass were observed when salt concentrations were above 75%. This response is probably because the roots are the first to be in direct contact with the medium and, given the nature of the tissue, suffer damages in the cell membranes or are damaged due to oxidation (Barbosa et al., 2014). This was reported by Fadel et al. (2010) for *Mentha spicata* L. and by Kumaraswamy and Anuradha (2010) for *Pogostemon cablin*, both cultivated in the MS medium (100%).

In addition, the synergism and antagonism between cations and anions interfere in the nutrient uptake by the roots. Synergism occurs when the presence of an ion increases the uptake of another ion, benefiting the plant development. Antagonism is the opposite (Malvi, 2011). For example, the excess of K makes the Ca, S, and P uptake difficult (Malvi, 2011; Silva & Trevisam, 2015). Possibly one of these factors or a combination of them interfered in the observed responses of Genovese basil. The various characteristics of the basil that were analyzed in this study responded to the salt concentrations in the MS medium, and this response was dependent on the time of evaluation. The callus formation did not differ during the cultivation, presenting values close to

36%. The occurrence of callus during *in vitro* cultivation has been reported for many Lamiacea especies (Meftahizade et al., 2010; Bakhtiar et al., 2016; Monfort et al., 2018), demonstrating a type of responsiveness to the medium.

It is probable that the callus formation was a result of the presence of cytokinin in the medium (Silva et al., 2017), since regulators were added to all treatments. Some researchers have observed a trend in callus formation in the presence of cytokinin, isolated or combined with auxin in the culture medium (Monfort et al., 2018). The cytokinin class of growth hormones is responsible for cell multiplication and, in *Arabidopsis*, it functions in the expression of several transcription factors such as *ESR1*, *ESR2*, *OBP1*, in addition to cyclins, and *CDKs* (Ikeuchi et al., 2013).

Abnormal seedlings were observed in all periods of evaluation but the numbers decreased towards the end of the cultivation. No statistically significant difference in response was observed between the different salt concentrations. The prominent characteristics of the abnormal seedlings were thin stems, sickle-shaped leaves, or absence of leaves. The results resemble that observed for Red Rub in basil that was also grown in the MS medium at different salt concentrations (Silva et al., 2017). Tissue oxidation was high mainly at 59 days after inoculation and was reversed towards the end of the *in vitro* cultivation. Oxidation occurred only in some parts of the plant tissue, demonstrating seedling resilience in recovering from damage.

A tissue can be rusty for many reasons such as presence of toxic compounds, free radicals, and physiological disturbance (Cassells & Cury, 2001). However, plants have defense mechanisms such as antioxidant substances, phenolic compounds, vitamins C and E, carotenoids (Silva et al., 2010), ROS's free radical scavengers (Barbosa et al., 2014), and an increase of proline and soluble sugars (Kozminska et al., 2017) to fight or mitigate damages of oxidation. Future studies could analyze the mechanisms that mitigate these damages in Genovese basil seedlings.

4.2 Interaction of Sucrose and Activated Carbon

Sucrose has been the principal source of carbon in plant tissue culture because it is cheap and easy to purchase. The concentration of 30 g L^{-1} is usually appropriate to prepare a MS medium according to Murashige and Skoog (1962). However, the carbon demand varies according to the plant material, and in many cases, sucrose can overcome costs of *in vitro* production and bring benefits to the micropropagation process. A substantial growth was observed in the seedling's shoots of *Curcuma* genus subjected to 60 g L^{-1} sucrose (Ferrari et al., 2016).

To avoid problems with seedling development becauseof the presence of phenolic compounds, flavonoids, quinones, and melanoidins from the Maillard reaction (Dong et al., 2016), activated carbon was added to the MS medium to remove the toxic substances (Thomas, 2008). It was expected that the doubled concentration of sucrose would benefit the shoot and root growth of Genovese basil and that the activated carbon would be efficient in preventing any negative effects of the toxic substances. However, our results did not confirm this hypothesis. The mean values for number of leaves, shoot and root lengths, and fresh and dry masses of both roots and shoots were similar between treatments with different sucrose concentrations implying that there was no gain from doubling the usual sucrose concentration. In addition, the activated carbon was not efficient in stopping damages mainly in the lowest concentration of sucrose.

To a certain extent, the negative effect of doubling the usual sucrose concentration in Genovese basil can be explained by the difference in osmotic potentials. Most likely, the osmotic potential is higher in the medium with doubled concentration. As a result, water flows into the root and newer and smaller roots will collapse because they do not yet have the structure to support the increased inflow of water (Mudgal et al., 2010). In addition, if the Maillard reaction occurs, the oxidation of lipids or sugars will create highly reactive intermediate carbonyl compounds (Dong et al., 2016). Reactive compounds can cause damage primarily to cell membranes and the cell respiration process (Cassells & Cury, 2001).

A similar result was observed for *Mentha piperita* (Sujana & Naidu, 2011) and for Red Rub in basil (Silva et al., 2017). In both these studies, the MS medium with 60 g L^{-1} sucrose did not favor shoot growth in seedlings in comparison with the usual concentration (30 g L^{-1}). In *P. cablin*, the reduction from 3% to 2% sugar concentration increased the fresh mass shoots to 67% (Swamy et al., 2010), indicating different responses according to the genotype.

Callus formation was strongly affected by the presence of activated carbon in the medium, as they reduced dramatically in comparison with the control. This result was also found in other studies with Lamiaceae (Silvia et al., 2017). Probably, there is an influence of factors related to callus formation, mainly growth regulators and activators of developmental genes. As previously discussed, callus formation in *Ocimum* is probably associated

with the presence of growth regulators in the culture medium (Bakhtiar et al., 2016). It is not clear yet the way in which activated carbon promotes the growth of seedlings. It seems simplistic to relate this event only to the retention ability of growth regulators in the presence of activated carbon (Thomas, 2008).

The adsorption of Cu and Zn by activated carbon was not observed in any of the studied conditions, confirming earlier reports (Thomas, 2008; Awoyale et al., 2013). However, Silva et al. (2017) observed that the level of Zn in the leaves of Red Rubin basil decreased with the increased concentration of activated carbon in the medium, corroborating some observations of Thomas (2008). Therefore, the above indicates that the seedling's micronutrient assimilation in the presence of activated carbon can vary with the analyzed plant part (shoots and roots), and this can be further affected by the other constituents of the medium, in this case, sucrose concentration.

The presence of micronutrients primarily in the roots is probably due to two reasons. The first reason is the immobilization mechanisms, which results in insufficient concentrations in the culture medium. According to Trettel et al. (2018), seedlings of *O. basilicum* develop better with 25 μM $CuSO_4$, showing a higher number of leaves and lower number of abnormal seedlings in comparison with the control. In addition, the authors observed higher level of Cu in roots than in shoots. According to Llorens et al. (2000), plants can initiate an immobilization mechanism that accumulates Cu in the roots.

The second possible reason is the interaction of sucrose and micronutrients, mainly Cu. According to Dugas & Bartel (2008), sucrose has a function in the miR-398 microRNA regulation of CSD1 and CSD2, two Cu-dependent superoxide dismutase (SOD) enzymes. The authors verified that an increased concentration of sucrose (from 1% to 3%) caused a reduction in the levels of CSD1 and CSD2 and consequently, a decrease in the growth of *Arabidopsis* seedlings. In this study, higher concentrations of sucrose decreased the number of leaves and biomasses of roots and shoots. In addition, the level of micronutrients decreased with increased concentrations of sucrose. Therefore, future studies should analyze whether lower levels of micronutrients in higher concentration of sucrose would disturb the development of seedlings and if sucrose could affect the SOD enzyme activity that removes free radicals in stressful situations (Barbosa et al., 2014). The accumulation of these substances can cause damages in the cell membranes, reduce cell respiratory capacity, destroy cell organelles, and prejudice the cell multiplication process, thus damaging the plant growth (Barbosa et al., 2014).

This research revealed that the Genovese basil is responsive to salt concentrations in the culture medium. The root system developed better in salt concentrations lower than 70%, but the opposite occurred for shoots. The Genovese basil seedlings were also responsive to the interaction of sucrose and activated carbon. Doubling the usual sucrose concentration did not favor plant growth, while adding activated carbon to the medium was not efficient in stopping toxic substances, even in the highest concentrations. In addition, the Cu and Zn adsorption by roots was not affected by the presence of activated carbon in the medium.

Therefore, for this species to produce more biomass, it is suggested that the MS medium at its full strength (100% salts) is used to develop more sprouts. It would be of greater value to produce more leaves to extract essential oils as these oils are stored in the secretary glands of leaves. But if the objective is to acclimatize seedlings, it would be better to develop roots by reducing the salt concentration in the culture medium. Initially we considered that 60 g L^{-1} sucrose plus activated carbon would increase both shoot and root production, but thisdid not occur. Besides, it was verified that Zn and Cu increased in the roots, despite the reports on their adsorption by activated carbon.

Among the evaluated characteristics, abnormal seedlings and tissue oxidation were the major problems. The verified abnormality is probably the result of the concentration of the growth regulator or other medium constituent, indicating that the medium should be better balanced. Tissue oxidation could be a result of seedling abnormality. Inducing callus formation was not the objective of this study. We believe that the occurrence of calluses is associated with the responsiveness of Lamiaceae to a medium enriched with regulators, rather than the treatments themselves. Future research should be conducted to test these hypotheses.

5. Conclusion

The MS medium at its full strength (100% salts) favors the Genovese basil's seedling shoot production.

The reduction of the salt concentration to 70% favors the development of the root system of Genovese basil.

The occurrence of abnormal seedlings and tissue oxidation decreases throughout the *in vitro* cultivation regardless of the salt concentration applied in the medium.

The usual sucrose concentration (30 g L^{-1}) is recommended.

The concentration of 3.0 g L^{-1} activated carbon can dramatically decrease callus formation.

Zn and Cu were not retained in the medium with the presence of activated carbon. These micronutrients were found in great amounts in the roots with the highest concentrations of activated carbon.

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