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In vitro effects of regulators on growth and morphogenesis of *Ocimum basilicum* L. 'Alfavaca Green' stem apexes

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Abstract. Large-scale cultivation of contamination free plants requires a good standardization protocol and production methods. Basil is widely used for cosmetics, food and pharmaceutical industries as it is rich in many bioactive compounds. This present study aimed to evaluate the growth and *in vitro* anatomical aspects of apical buds of basil grown under different concentrations rowth regulators like: NAA (Naphthalenoacetic Acid), BAP (6-benzylaminopurine), and KIN (Kinetin). The *in vitro* establishment was evaluated every 20 days to calculate the, the percentage of plants with calluses, appearance of the roots, any abnormal seedlings, any oxidized seedlings, and the number of sprouts per plant. Growth, physiological, and morpho-anatomical evaluations were performed at 80 days. Basal callogenesis was observed when cytokinin's and auxins are used in combination. Auxin treatments caused hyperhydricity in the stems and leaves. Medium A2 (0.05 mg L^{-1} of NAA and 0.1 mg L^{-1} of BAP), and A3 (0.05 mg L^{-1} of NAA and 0.1 mg L^{-1} of KIN) resulted in the best development of basil plants, cultivar 'Alfavaca Green'. The A2 produced plants with greater numbers of leaves, an average bud length of 59.81 mm, and the best root properties. A2 and A1 have a higher percentage of hyperhydricity (83 and 67%). The A3 resulted in an acceptable number of leaves (range: 21–39), and this treatment produced the best shoot properties as well as fewer plants with hyperhydricity. In addition, the A3 treatment produced plants with a shoot length, high shoot fresh and dry mass (2.82 and 0.23 g), high chlorophyll index and leaf anatomy that was similar to the control. Excluding the control, the other treatments presented more than 90% of the explants with calluses in their bases.

Key words: callus, hyperhydricity, Lamiaceae, morphological processes, leaf tissues.

INTRODUCTION

The Lamiaceae family are valued for the pharmaceutical properties of their biologically active components and for their use as culinary seasonings (Makri & Kintzios, 2008; Carović-Stanko et al., 2010; Taie et al., 2010; Vieira et al., 2014). Basil (*Ocimum basilicum* L.) is a rich source of natural compounds such as monoterpenes, sesquiterpenes, phenylpropanoids, anthocyanins, and phenolic acids (Monfort et al., 2018; Trettel et al., 2018a).

The application of plant tissue culture techniques to medicinal plants improves phytopharmaceutical production and can increase plant biomass (Morais et al., 2012; Miralpeix, 2013). One of the advantages of *in vitro* culture is that growing conditions can be controlled (Vanisree et al., 2004). This enhances the predictability of production,

allows for rapid and efficient isolation of target compost, and improves the health and quality of the seedlings produced (Ahsan et al., 2013; Gonçalves & Romano, 2013). Thus, to establish a protocol, it is necessary to specify the basic requirements of the species or cultivar. In addition to the commonly used nutrients and sucrose in culture media, growth regulators are also essential for plant growth (George et al., 2008; Senhaji et al., 2019). Regulators are compounds similar to natural hormones that act on plant development through altering plant metabolism (Small & Degenhardt, 2018). The most commonly used regulators belong to the auxin and cytokine groups. Auxins mainly promote cell stretching and root formation (Perrot-Rechenmann 2010), whereas cytokines neutralize the auxins that cause apical dominance and promote cell multiplication and lateral growth of the plant (Perilli et al., 2010).

There is also a lack of information on how growth regulators influence the formation and differentiation of tissues in basil, especially leaf tissues (Trettel et al., 2019). The genus *Ocimum* is composed of 50–60 species (Makri & Kintzios, 2008) and has several cultivars, making it necessary to establish the concentrations and types of regulators needed for each cultivar (Monfort et al., 2018). Research has revealed that different regulators can impact organogenesis in various cultivars. For example, partial changes in leaf architecture and reductions in palisade and spongy parenchyma thickness were observed in *O. basilicum* 'Genovese' when 0.4 mg L^{-1} of BAP and 0.2 mg L^{-1} of NAA were applied simultaneously (Trettel et al., 2019).

Thus, it can be expected that combining growth regulators will influence the morphoanatomical characteristics of the leaves (Rout et al., 2000), and will particularly affect palisade and spongy parenchyma thickness (Stefanova et al., 2011). Thus, anatomical studies allow us to assess the normality of the tissues and to identify treatments with a higher likelihood of success, for example, treatments that produce plants with thicker cuticles, epidermises, and leaf limbs (Kumar & Rao, 2012). Given the above-mentioned facts, the present study aimed to evaluate the effects of different concentrations of auxin (naphthaleneacetic acid - NAA) and cytokine (6-benzylaminopurine - BAP, and kinetin - KIN) growth regulators on the *in vitro* establishment and leaf morphoanatomy of *Ocimum basilicum* L. 'Alfavaca Green'.

MATERIALS AND METHODS

Obtention of propagative material

The research was carried out at the Laboratory of Plant Tissue Culture at the *Universidade Paranaense* - UNIPAR. Seeds were used as initial propagating material. The cultivar used in this study was 'Alfavaca Green' (Feltrin®, Farroupilha, Brazil), and the batch number was 0061401530016050. Before the start of the experiment, seeds were immersed in distilled water and were oxygenated for 2 hours before being disinfected in a laminar flow cabinet. Seeds were immersed in 70% ethyl alcohol for 2 minutes, then transferred to 2% sodium hypochlorite solution and stirred for 15 minutes. Subsequently, four successive washes were performed with deionized and autoclaved water.

After the disinfection process, groups of four seeds were inoculated into flasks containing 50 mL of culture medium. The MS medium (Murashige & Skoog, 1962) was used at its full strength, and was supplemented with 30 g L^{-1} sucrose and 6.5 g L^{-1} agar. The pH was adjusted to 5.8. After the medium was prepared, flasks were autoclaved for

20 minutes at 120 °C. After seeds were inoculated, the flasks were kept in a growth chamber at 25 ± 2 °C with a light intensity of 72.0 μmol m^{-2} s^{-1}, which was maintained using LED lamps (Blumenau® Porto Alegre, Brazil; LED T8 10W 6.000K, 100-240 V, -50/60 Hz, power factor: ≥ 0.92; High PF). The plants were kept in the growth chamber for 80 days under a 24 h d^{-1} photoperiod.

Implementation and establishment *in vitro*

Seedlings free of abnormalities and oxidation were selected, and stem apexes of approximately 1.5 cm with two buds were obtained. The baseline medium used for all treatments consisted of full-strength MS medium supplemented with 30.0 g L^{-1} sucrose and 6.5 g L^{-1} agar; the pH of the medium was adjusted to 5.8. Combinations of NAA (Naphthaleneacetic Acid), BAP (6-benzylaminopurine), and KIN (Kinetin; Sigma Aldrich® Hamburg, Germany) growth regulators were added according to Table 1.

Table 1. Treatments used to growth of *Ocimum basilicum* 'Alfavaca Green' with different growth regulators NAA (Naphthaleneacetic acid) BAP (6-benzylaminopurine) and KIN (Kinetin)

Treatments	NAA	BAP	KIN
	(mg L^{-1})		
A1	0.0	0.0	0.0
A2	0.05	0.1	0.0
A3	0.05	0.0	0.1
A4	0.2	0.4	0.0
A5	0.2	0.0	0.4
A6	1.0	2.0	0.0
A7	1.0	0.0	2.0
A8	0.5	0.0	1.0
A9	0.2	5.0	0.0

Regulator concentrations in the treatments were based on those suggested by Stefanova et al. (2011), in addition to on tests previously performed. Fifty milliliters of culture medium was added to each 350 mL flask. One apex was inoculated per flask; then flasks were kept in a growth chamber for 80 days under the conditions previously cited.

Evaluation during *in vitro* establishment

Two evaluations were performed during *in vitro* cultivation. The first evaluation was performed 20 days after explant inoculation and the second at 40 days post-inoculation. In these evaluations, the percentage of plants with calluses, the appearance of the roots, and any abnormal or oxidized seedlings were recorded. Evaluations were performed in six replications, with five flasks per replication.

Physiological and growth evaluation

Number of leaves (NL), sprout length (SPL), root length (RTL), sprout fresh mass (SFM), root fresh mass (RFM), callus fresh mass (CFM), sprout dry mass (SDM), root dry mass (RDM), callus dry mass (CDM), and chlorophyll index (CLI) were analyzed after 80 days. Any hyperhydricity or adventitious roots were also recorded. The measurements were obtained with a digital caliper, and the samples were kept in an oven at 65 °C for three days before being weighed on a precision scale to obtain the dry mass. Total chlorophyll index was determined based on the middle third of the plants, and a Chlorophyll meter ClorofiLOG® CFL 1030 was used to obtain these measurements, according to the manufacturer's instructions (Falker® Porto Alegre, Brazil).

Anatomical and morphometric evaluation of the leaves

After 80 days, three replicates from each treatment were selected for anatomical evaluation. The leaves were fixed in formalin solution, acetic acid, and ethyl alcohol (FAA50) for 24 hours and were stored in 70% alcohol. They were subsequently dehydrated in succession with butyl (50%, 70%, 85%, 95%, and 100%) according to the methodology of Johansen (1940), and were then incorporated into paraplast (Kraus & Arduim, 1997).

The region from the petiole to the midpoint of the leaf limb was chosen for cutting. Cross-sections of 10 μm in thickness were obtained using the Leica (RM2125 RT) hand rotating microtome (Biosystems® Wetzlar, Germany). Afterwards, sections were transferred to glass slides and were deparaffinized. They were then dehydrated in a decreasing ethanol series (90%, 80%, 70%, and 50%) before being stained in a mixture of astrablue and safranin (9:1 v/v) (Bukatsch, 1972) modified to a concentration of 0.5%, according to the methodology of Antoniazzi et al., (2016). Sections were then washed in water and were again passed through an increasing ethanol series (50%, 70%, 80%, and 90%). Glass varnish was used for coverslip fixation (Paiva, 2006).

Photographs were taken at 40× magnification under a microscope (Olympus BX-60® Tokyo, Japan) with a camera attached. Motic Images Plus 3.0 software was used for anatomical evaluations (Motic, 2016). The measurements were taken from each photo: adaxial epidermis (AE), palisade parenchyma (PP), spongy parenchyma (SP), abaxial epidermis (XE), and the distance between the inferior and superior epidermis (EE). The thickness of these regions was obtained in three different images and from each tissue ten measurements were taken.

Experimental design and statistical analyses

The experiment was carried out with a completely randomized design to produce nine treatments, six replications, and five flasks per replication containing one stem apex each. Callus formation, appearance of the roots, and any abnormal or oxidized seedlings were recorded at two evaluation times (at 20 and 40 days) in a 2×9 factorial scheme, and nine treatments were evaluated. Leaf growth, physiological data, and morphometric data obtained in the final evaluation were submitted to analysis of variance One-way ($p \leq 0.05$) and were compared using Tukey tests ($p \leq 0.05$) on Sisvar 5.6 software (Ferreira, 2011).

The data obtained for the presence and absence of hyperhydricity and adventitious roots were noted down as binary annotations. The number 1 signified the presence of the characteristic and 0 signified its absence. Data were converted to a dissimilarity index using the Jaccard Tanimoto formula, and data were transformed to $d = (1\text{-}r)\times100$ with 100 replicates using the *http://genomes.urv.cat/UPGMA/* platform. Data were exported to the Statistica 13.3 software (Statsoft, 2017), and the hierarchical clustering technique was used to interconnect the samples by associations. The software generated a dendrogram within which similar samples were grouped (Moita Neto & Moita 1998). Euclidean distance was used to measure the similarity between the centroids of each isolate, and Ward's method was adopted for grouping. The results of the analysis are presented in graphic form (dendrogram).

RESULTS

Evaluation during *in vitro* establishment

The control treatment had the lowest percentage of plants with calluses during the whole period of *in vitro* establishment. During the second evaluation period, plants subjected to experimental treatments showed callus formation, and the percentage of plants with calluses ranged from 96.67 to 100% (Fig. 1). Although calluses were present in all treatments, this did not limit seedling growth. Sprouting occurred first, and was then followed by root growth. Approximately 17.90% of cultivated seedlings took root within the first 20 days. Between the first and second evaluation periods, there was a 15.89% increase in seedling rooting (Fig. 1).

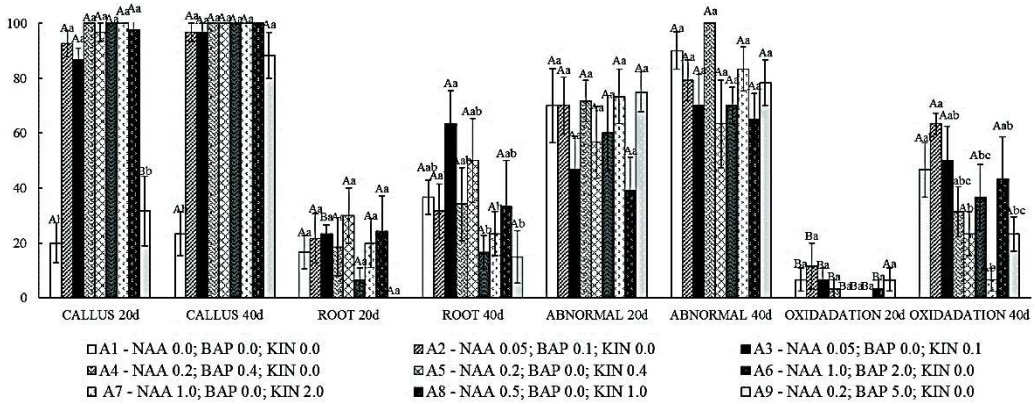

Figure 1. Percentage of abnormal and oxidized seedlings, and formation of callus and roots, at 20 and 40 days after *in vitro* establishment of the stem apexes of *Ocimum basilicum* 'Alfavaca Green'.

*Tukey test ($p \leq 0.05$) Equal letters do not differ from each other. Uppercase letters compare the same treatment between evaluation times. Lowercase letters compare the different treatments within the same evaluation time.

Regarding the presence of abnormal plants, it was observed that 77.68% of the seedlings showed abnormalities at 40 days post-inoculation. There were no significant differences ($p \leq 0.05$) in this trait among treatments or between the evaluation times. However, 90 and 100% of the seedlings were abnormal under the A1 and A4 treatments, respectively (Fig. 1). Among the visualized characteristics, the most common abnormalities were hyperhydricity, adventitious roots, and twisted sickle-shaped leaves (Fig. 2).

Oxidation was higher at 40 days than at 20 days post-inoculation; treatments A2 and A7 had the highest and lowest percentages of oxidation at 63.33% and 6.67%, respectively (Fig. 1). On average, the experimental treatments generated low shoot formation per seedling compared to the control, with an average of 0.61 shoots per plant at 20 days, and 0.84 shoots per plant at 40 days.

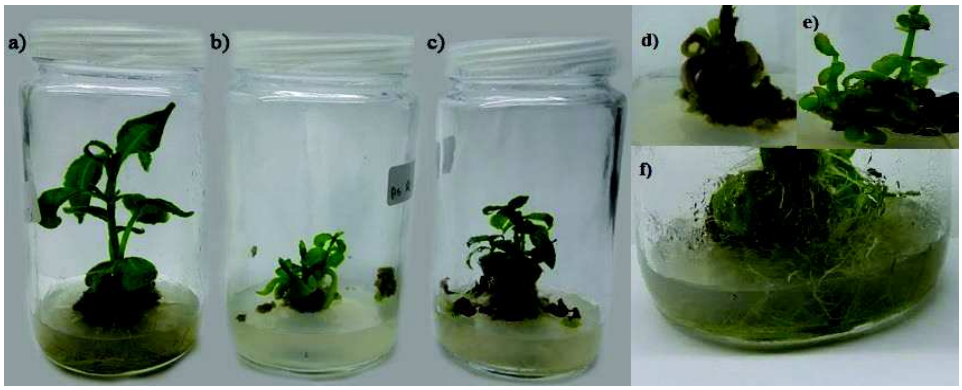

Figure 2. Abnormal seedlings of *Ocimum basilicum* 'Alfavaca Green' at 80 days after *in vitro* establishment of stem apexes: a) seedling with twisted leaves; b) Seedling with hyperhydricity; c) Seedling with adventitious roots; d) callus; e) hyperhydricity symptoms; f) adventitious rooting.

Evaluation of physiological and growth characteristics

After 80 days of cultivation, analysis of the physiological and growth characteristics was performed for all treatments (Table 2). Treatments A1, A2, and A3 produced plants with the highest numbers of leaves. Among them, the A2 treatment generated an average of 21.84 leaves per seedling, and this was followed by A3 and A1 (21.39 and 18.72 leaves per seedling, respectively). The highest results for SPL were observed under the A2 treatment (59.81 mm), followed by A3 (56.25 mm) and by A1 (48.35 mm). There were no significant differences among these results ($p \leq 0.05$). The average shoot length of treatments A2, A3, and A1 was 55.69% higher than the average shoot length under the A7 treatment, which produced the smallest SPL (24.28 mm) (Table 2).

Regarding root system formation, it was observed that the A2 produced a higher average RTL (71.08 mm), which was 63.11 and 23.39% higher than the averages of treatments A6 and A9, with the lowest values of root length respectively. The fresh and dry masses of roots under the A2 were 3.24 g and 0.17 g, respectively, which represented 12 and 8.5 times greater fresh and dry masses of roots compared to the A4 (0.26 g and 0.02, respectively) (Table 2). In general, the A3 medium was better in terms of characteristics related to shoot biomass, with averages of 2.32 and 0.23 g for fresh and dry shoot mass, respectively. No callus formation was observed in the control treatment (A1). Treatments A2 and A3 had low fresh and dry masses of calluses (A2 = 1.79 and 0.12 g; A3 = 1.48 and 0.09 g, respectively). The A3 medium produced the highest average CLI (41.89), which was higher than the averages of A1 and A2 (21.43 and 21.39, respectively) (Table 2).

Some abnormalities were observed at the end of the experiment. Among them, the most recurrent were hyperhydricity and adventitious roots. The results of these analyses are shown in Fig. 3. In general, the first group (A9, A8, A6, A4, and A3 - Fig. 3, a) contained treatments with a higher concentration of the NAA regulator, except for treatment A3. When comparing the dendrogram with the percentage graph (Fig. 3, b), it can be observed that the treatments grouped at the left extremity, A9 and A8, are those with lower percentages of hyperhydricity (56 and 61% - Fig. 3, b) compared to the right extremity (A2 and A1), which had higher percentages of hyperhydricity (83 and 67% - Fig. 3, b).

Table 2. Number of leaves, sprout and root length, fresh and dry mass of sprout, root and callus, and chlorophyll index evaluated at 80 days after *in vitro* cultivation of *Ocimum basilicum* 'Alfavaca Green' stem apexes

Treat	NL	SPL (mm)	RTL (mm)	SFM (g)	RFM (g)	CFM (g)	SDM (g)	RDM (g)	CDM (g)	CLI
A1	18.72 ± 2.69^{ab}	48.35 ± 10.44^{a}	51.00 ± 19.21^{ab}	2.13 ± 0.56^{abc}	1.21 ± 1.05^{ab}	0.00 ± 0.00^{d}	0.16 ± 0.04^{abc}	0.06 ± 0.07^{ab}	0.00 ± 0.00^{e}	21.43 ± 13.30^{ab}
A2	21.84 ± 5.21^{a}	59.81 ± 10.43^{a}	71.08 ± 13.61^{a}	2.82 ± 0.77^{ab}	3.24 ± 2.20^{a}	1.79 ± 1.01^{dc}	0.19 ± 0.08^{ab}	0.17 ± 0.11^{a}	0.12 ± 0.06^{edc}	21.39 ± 7.61^{ab}
A3	21.39 ± 3.43^{ab}	56.25 ± 15.03^{a}	59.90 ± 15.32^{ab}	2.32 ± 0.79^{abc}	2.25 ± 1.43^{ab}	1.48 ± 0.86^{d}	0.23 ± 0.08^{a}	0.12 ± 0.07^{ab}	0.09 ± 0.05^{edc}	41.89 ± 9.11^{a}
A4	13.06 ± 3.40$^{c\ b}$	25.21 ± 9.85$^{c\ b}$	37.74 ± 18.45^{ab}	0.95 ± 0.30^{c}	0.26 ± 0.21^{b}	2.02 ± 1.41^{dc}	0.09 ± 0.03^{cb}	0.02 ± 0.02^{b}	0.33 ± 0.22^{abc}	9.64 ± 2.51^{b}
A5	14.78 ± 3.47^{abc}	42.58 ± 10.46^{abc}	71.58 ± 15.21^{a}	1.86 ± 0.81^{abc}	2.82 ± 1.49^{ab}	2.82 ± 0.85^{dc}	0.18 ± 0.09^{abc}	0.14 ± 0.08^{ab}	0.14 ± 0.09^{edcb}	25.46 ± 9.63^{ab}
A6	13.14 ± 5.66$^{c\ b}$	25.20 ± 12.84$^{c\ b}$	26.22 ± 26.84^{b}	1.43 ± 0.77^{cb}	0.92 ± 0.98^{ab}	9.95 ± 4.57^{a}	0.08 ± 0.04^{cb}	0.03 ± 0.05^{ab}	0.50 ± 0.31^{a}	13.47 ± 3.63^{b}
A7	9.67 ± 3.63^{c}	24.28 ± 11.19^{c}	72.11 ± 36.50^{a}	1.03 ± 0.44^{cb}	1.03 ± 0.98^{ab}	7.12 ± 1.01^{ab}	0.06 ± 0.02^{c}	0.03 ± 0.03^{ab}	0.41 ± 0.12^{ab}	12.09 ± 7.54^{b}
A8	15.31 ± 6.19^{abc}	46.21 ± 15.18$^{b\ a}$	63.71 ± 23.45^{ab}	3.25 ± 2.19^{a}	2.78 ± 3.05^{ab}	5.12 ± 1.57^{cb}	0.11 ± 0.09^{cb}	0.12 ± 0.13^{ab}	0.28 ± 0.06^{dabc}	17.60 ± 4.40^{b}
A9	18.56 ± 4.87^{ab}	25.16 ± 1.70$^{c\ b}$	26.02 ± 29.62^{b}	2.43 ± 0.78^{abc}	0.67 ± 0.75^{ab}	0.86 ± 1.01^{d}	0.15 ± 0.06^{abc}	0.04 ± 0.05^{ab}	0.05 ± 0.04^{ed}	19.22 ± 6.20^{b}

*Tukey test ($p \leq 0.05$). Equal letters do not differ from each other in the column. NL – number of leaves; SPL – sprout length; RTL – root length; SFM – sprout fresh mass; RFM – root fresh mass; CFM – callus fresh mass; SDM – sprout dry mass; RDM – root dry mass; CDM – callus dry mass; CLI – chlorophyll index. A1 (control) without growth regulators; A2 – 0.05, 0.1 and 0 mg L^{-1}; A3 – 0.05.0 and 0.1 mg L^{-1}; A4 – 0.2, 0.4 and 0.0 mg L^{-1}; A5 – 0.2, 0.0 and 0.4 mg L^{-1}; A6 – 1.0, 2.0 and 0.0 mg L^{-1}; A7 – 1.0, 0.0 and 2.0 mg L^{-1}; A8 – 0.5, 0.0 and 1.0 mg L^{-1}; A9 – 0.2, 5.0 and 0.0 mg L^{-1} of NAA, BAP and KIN, respectively.

The second group generally consisted of the treatments with lower concentrations of BAP and KIN. Thus, it was observed that the A3 treatment was situated between the two large groups. It can be inferred that the treatment A3 was associated with the first group due to the low percentage of hyperhydricity it produced, and with the second group due to the low concentration of NAA and KIN in its composition (Fig. 3, a).

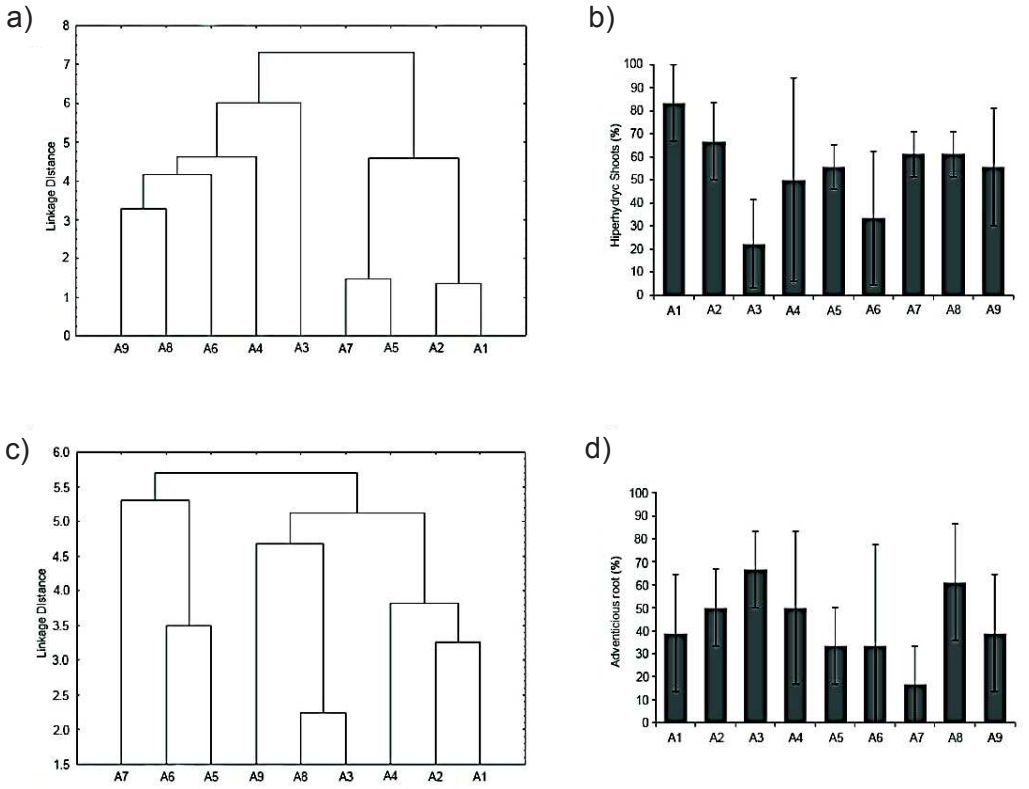

Figure 3. Dendrogram and percentage of *Ocimum basilicum* 'Alfavaca Green' seedlings with hyperhydricity and adventitious roots grown at different concentrations of growth regulators. a) Dendrogram referring to hyperhydricity; b) Percentage of the presence of hyperhydricity; c) Dendrogram referring to the presence of adventitious roots; d) Percentage of presence of adventitious roots.

A1 (control) without growth regulators; A2 – 0.05, 0.1 and 0 mg L^{-1}; A3 – 0.05.0 and 0.1 mg L^{-1}; A4 – 0.2, 0.4 and 0.0 mg L^{-1}; A5 – 0.2, 0.0 and 0.4 mg L^{-1}; A6 – 1.0, 2.0 and 0.0 mg L^{-1}; A7 – 1.0, 0.0 and 2.0 mg L^{-1}; A8 – 0.5, 0.0 and 1.0 mg L^{-1}; A9 – 0.2, 5.0 and 0.0 mg L^{-1} of NAA, BAP and KIN, respectively.

The dendrogram of the presence of adventitious roots were grouped into two main clusters (Fig. 3, c). The first cluster contains treatments A6, A5, and A7, and the second cluster grouped the treatments into two subgroups containing A9, A8 and A3, and A4, A2, and A1, respectively. Treatments A9, A8, and A3 had the presence of KIN in common, and produced the highest percentages of adventitious roots (100, 61, and 67%, respectively) (Fig. 3, d). Both treatments A2 and A4 produced plants 50% of plants with adventitious roots (Fig. 3, d), and also contained intermediate concentrations of BAP.

Anatomy and morphometry of basil leaves

Normal, twisted, and hyperhydric leaves were observed under the experimental treatments (Fig. 4). Leaf tissue morphometry differed among the treatments tested (Table 3). As shown in Table 3, the A2 medium presented the greatest average adaxial epidermis (AE) thickness, with 19.51 µm, followed by A9, which produced an average AE thickness of 18.44 µm. The A9 treatment produced the greatest abaxial epidermis (XE) thickness (18.08 µm). The thickness of the palisade parenchyma (PP) was greater under treatments A7 and A8; the average of both was 65% greater than that of A4, which had the lowest mean PP thickness (55 µm). Treatment A8 also produced the highest average spongy parenchyma (SP) thickness (492.68 µm), and this was 80.15 and 75.50% higher than the A1 and A3 treatments respectively, which had the lowest average PL thicknesses at 97.77 and 120.68 µm, respectively. A8 also produced plants with the greatest distance between the inferior and superior epidermis (DE), with an average of 659.45 µm, which was 74.22 and 70.13% higher than the A1 and A4 treatments respectively.

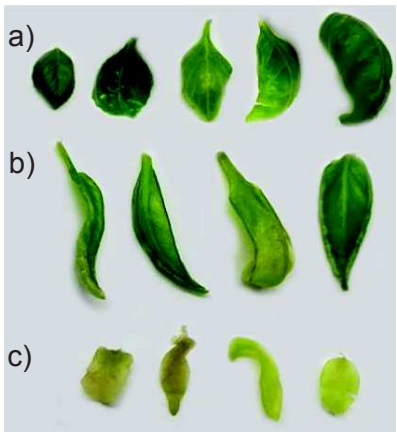

Figure 4. Leaves of *Ocimum basilicum* 'Alfavaca Green' at 80 days of *in vitro* cultivation of stem apexes at different concentrations of growth regulators: a) Normal leaves; b) twisted and sickle shaped leaves; c) leaves with hyperhydricity.

Table 3. Leaf morphometry of *Ocimum basilicum* 'Alfavaca Green' grown at different concentrations of regulators NAA, BAP, and KIN

TRAT	AE (µm)	XE (µm)	PP (µm)	SP (µm)	EE (µm)
A1	8.80 ± 3.79^{b}	9.65 ± 3.72^{b}	66.54 ± 37.64^{bc}	97.77 ± 31.76^{d}	160.95 ± 66.59^{f}
A2	19.51 ± 3.87^{a}	15.62 ± 3.83^{ab}	111.71 ± 23.23^{abc}	394.22 ± 4.56^{b}	520.73 ± 9.33^{b}
A3	14.13 ± 1.16^{ab}	12.35 ± 1.61^{ab}	74.85 ± 3.09^{bc}	120.68 ± 16.57^{d}	227.85 ± 35.55^{ef}
A4	9.72 ± 0.72^{b}	13.36 ± 4.04^{ab}	55.00 ± 9.03^{c}	130.03 ± 32.07^{cd}	196.92 ± 42.41^{f}
A5	8.33 ± 0.74^{b}	9.68 ± 1.30^{b}	78.67 ± 10.37^{bc}	185.93 ± 26.01^{cd}	277.98 ± 45.12^{def}
A6	11.01 ± 1.75^{b}	9.94 ± 0.90^{b}	124.38 ± 41.01^{ab}	187.93 ± 32.86^{cd}	396.14 ± 35.66^{bcd}
A7	11.46 ± 1.34^{b}	12.36 ± 3.11^{ab}	152.01 ± 8.65^{a}	323.20 ± 37.10^{b}	458.40 ± 44.95^{bc}
A8	13.65 ± 0.55^{ab}	15.59 ± 1.52^{ab}	166.31 ± 15.76^{a}	492.68 ± 61.75^{a}	659.45 ± 65.47^{a}
A9	18.44 ± 2.78^{a}	18.08 ± 0.57^{a}	115.14 ± 21.08^{abc}	221.90 ± 21.29^{c}	351.72 ± 38.32^{cde}

*Tukey test ($p \leq 0.05$). Equal letters do not differ from each other in the column.

AE – adaxial epidermis; XE – abaxial epidermis; PP – palisade parenchyma; SP – spongy parenchyma; DE – distance between the inferior and superior epidermis.

A1 (control) without growth regulators; A2 – 0.05, 0.1 and 0 mg L^{-1}; A3 – 0.05.0 and 0.1 mg L^{-1}; A4 – 0.2, 0.4 and 0.0 mg L^{-1}; A5 – 0.2, 0.0 and 0.4 mg L^{-1}; A6 – 1.0, 2.0 and 0.0 mg L^{-1}; A7 – 1.0, 0.0 and 2.0 mg L^{-1}; A8 – 0.5, 0.0 and 1.0 mg L^{-1}; A9 – 0.2, 5.0 and 0.0 mg L^{-1} of NAA, BAP and KIN, respectively.

Morphological observations showed that *O. basilicum* 'Alfavaca Green' leaves had an epidermis with rectangular and oval cells at the apexes and a thin cuticle layer (Fig. 5, a, b, f). Next, a layer of juxtaposed palisade parenchyma was observed, with few cell spaces present (Fig. 5, b). The spongy parenchyma occupied four layers of the leaf limb, with irregular to rounded cells that varied in size (Fig. 5, c). These cells were larger and had large spaces between them in the midrib. The conductive vessels were formed by metaxylem vessel elements that had a thicker rounded cell wall and formed layers of cells oriented towards the collateral bundle (Fig. 5, e).

Differences in the distribution of cells in the parenchyma were also observed between treatments. In treatment A1 (Fig. 5, a), palisade parenchyma (PP) with smaller cells distributed in two layers were observed, which also occurred under treatments A2, A7, and A8 (Fig. 5, b, c, d). The other treatments produced plants with PP in a single layer and with longer cells. In all treatments, the extension of the spongy parenchyma was formed in two or more layers and was greater than the extension of the PP.

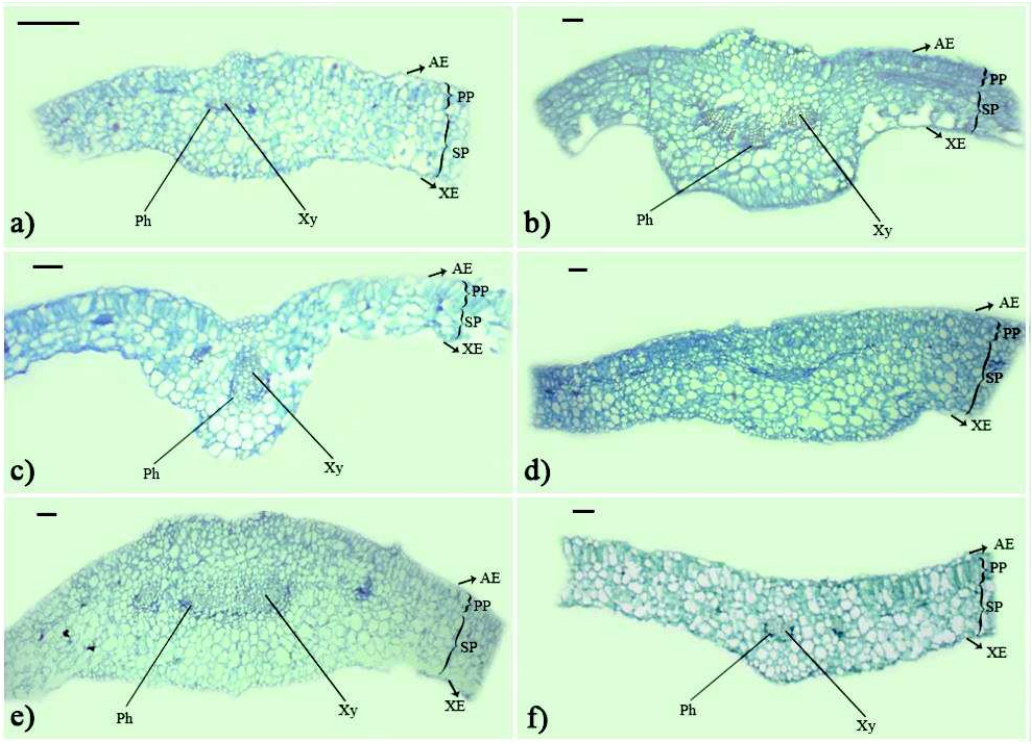

Figure 5. Cross section of *Ocimum basilicum* 'Alfavaca Green' leaves at 80 days of *in vitro* cultivation of stem apexes at different concentrations of growth regulators.

A1 (control) without growth regulators; A2 – 0.05, 0.1 and 0 mg L^{-1}; A3 – 0.05.0 and 0.1 mg L^{-1}; A4 – 0.2, 0.4 and 0.0 mg L^{-1}; A5 – 0.2, 0.0 and 0.4 mg L^{-1}; A6 – 1.0, 2.0 and 0.0 mg L^{-1}; A7 – 1.0, 0.0 and 2.0 mg L^{-1}; A8 – 0.5, 0.0 and 1.0 mg L^{-1}; A9 – 0.2, 5.0 and 0.0 mg L^{-1} of NAA, BAP and KIN, respectively. AE – adaxial epidermis; PP – palisade parenchyma; SP – spongy parenchyma; XE abaxial epidermis; Xy – Xylem; Ph – Phloem (bar = 100µm).

DISCUSSION

Evaluation during *in vitro* establishment

Transformations during the organogenesis process of *in vitro* cultivated plants have been studied in many plants of commercial interest (George et al., 2008). For medicinal plants, there has been a lack of studies using this information to advance the technologies used in micropropagation (Alvarez, 2014). These plants, when subjected to *in vitro* conditions, have responded in numerous ways including adventitious root formation, hyperhydricity, callus formation, oxidation, and abnormal seedlings. These observations are variable throughout *in vitro* culture (Toma et al., 2004; Stefanova et al., 2011; Kosar & Mahmoud, 2012).

During the first evaluation period, the experimental treatments containing different concentrations of regulators produced plants with calluses at the base of the inoculated apex. In a study by Ikeuchi et al. (2013), some mechanisms behind the formation of these structures were clarified. Among the mechanisms mentioned, it was indicated that some hormones, mainly cytokines and auxins, signal and regulate the expression of transcription factors responsible for callus formation. Most studies involving micropropagation of *Ocimum* plants have shown that a small number of cytokines and auxins cause callus formation when used together (Gogoi & Kumaria, 2011; Asghari et al., 2012; Monfort et al., 2018). If regulators are excluded from the medium there is hardly any callus formation, highlighting their crucial role in this process. This sensitivity of *O. basilicum* to callus formation during *in vitro* culture has also been reported as common for other cultivars (Monfort et al., 2018), and can also be caused by other characteristics of the culture medium, such as medium concentration (Silva et al., 2017), antioxidants and sugars (Trettel et al., 2018b).

There was an increase in oxidation during the *in vitro* process, which may also be associated with callus formation. Only the treatments containing the highest concentrations of cytokines and the lowest concentrations of auxins had low percentages of oxidized plants, with the opposite being observed for the treatments containing the lowest concentrations of regulators. The most likely reason for this is that oxidation occurred due to compounds and free radicals being released by the calluses (Silva et al., 2017). Seedlings that grew under higher stress conditions (higher concentrations of regulators) may have increased their secondary compound production. In particular, plants may have increased their phenolic compound production (Monfort et al., 2018), which can neutralize free radicals and toxic compounds (Stashenko et al., 2004). Another hypothesis is that oxidation was caused by increases in antioxidant enzymes such as polyphenol oxidase, peroxidase, catalase, and superoxide dismutase (Stashenko et al., 2004). Although oxidation occurred in many plants, it was not severe enough to cause plant death, which reinforces the idea that the biochemical stress mechanisms of *O. basilicum* 'Alfavaca Green' seedlings combatted oxidative effects.

Evaluation of physiological and growth characteristics

Growth regulator concentration strongly altered the developmental pattern and organogenesis of *O. basilicum* 'Alfavaca Green' seedlings, and this alteration has also been observed in other *O. basilicum* cultivars (Asghari et al., 2012; Manan et al., 2016; Monfort et al., 2018; Trettel et al., 2019). This demonstrates that the culture medium

with same formulation should only be used as a basis for initial studies of micropropagation protocols.

O. basilicum 'Alfavaca Green' seedlings that showed the best growth were those subjected to low concentrations of both auxin and cytokine regulators (A2 and A3 treatments), which demonstrates that, although necessary in higher concentrations, regulators promote seedling anomalies. The occurrence of abnormalities is closely linked to the amount of regulator that promotes signal cascades by binding to membrane receptors and to the amount of regulator that binds to intracellular factors such as enzymes, which clear excess regulators (Neelakandan & Wang, 2012). The types of auxin and cytokine used also influences the occurrence of abnormalities because, the particular types of these regulators seem to be standardized according to the plant genotype in the cell membrane (Zaidi et al., 2006; Perrot-Rechenmann, 2010). Therefore, the A2 and A3 experimental treatments resulted in greater numbers of leaves per plant, taller plants, and greater seedling biomass.

The best growth was observed in the treatments with low concentrations of NAA. Auxins are a group of growth hormones naturally present in plants, and they are mainly found in the apical meristem (Rademacher, 2015). However, the use of a cytokines was necessary to increase the number of leaves per plant and to improve biomass production, as in some cases the absence of this regulator resulted in no sprouts forming (Asghari et al., 2012). In this study, a concentration of 0.5 mg L^{-1} BAP was ideal to optimize these characteristics. Other *O. basilicum* L. cultivars have been shown to limit their growth patterns, such as shoot, and root formation, in response to BAP concentrations above 2.0 mg L^{-1}. For example, in *O. basilicum* 'Maria Bonita', the use of 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA resulted in lower leaf production, sprout production, and biomass (Monfort et al., 2018). In *O. basilicum* 'Sweet Thai', 1.0 mg L^{-1} BAP improved all analyzed characteristics (Manan et al., 2016).

Abnormal characteristics were also observed. The most frequently observed of these characteristics were hyperhydricity, adventitious roots, and twisted leaves. Several factors can induce hyperhydricity including pH, explant type, culture medium composition, light intensity, humidity, and ventilation (Liu et al., 2017). Because of this, studies are needed to verify which of these factors are involved in the induction of hyperhydricity in the 'Alfavaca Green' basil cultivar. Toma et al., (2004), which examined the impact of cytokines and auxins on the growth of nodal segments in *Hyssopus officinalis,* also observed plants with a vitreous appearance. The authors demonstrated that, in addition to callus formation, the seedlings demonstrated different degrees of hyperhydricity in the stems and leaves. George et al. (2008) claimed that the occurrence of hyperhydricity can cause damage and make the acclimatization process more difficult for micro-propagated plants, and therefore should be avoided.

A study by Klerk (1996) showed that agreed that auxin application can cause adventitious root formation. As in the present experiment, the presence of KIN in the medium resulted in the highest percentage of adventitious roots. The high number of plants with twisted leaves may also be related to copper deficiency in the *in vitro* culture of *O. basilicum.* The same study showed that a lower number of abnormal seedlings occurred when 25 μM $CuSO_4$ was added to the culture medium (Trettel et al., 2018a). One of the symptoms of copper deficiency is the presence of twisted, wilted, sickle-shaped leaves (Stepien & Wojtkowiak, 2016). This metal induces the production of phenylpropanoids, which are precursors of lignin (Moura et al., 2010), and in some cases

it can act on the activity of the laccase enzyme which participates in lignin formation (Moura et al., 2010; Liu, 2012).

Evaluations of Leaf Anatomy

Regulators impact cell structure through stretching, induction of cell division, and tissue maturation (Neelakandan & Wang, 2012). In cell stretching, the role of auxins is widely recognized, as they can act on nonpolar transport. In this way, auxins are transported from cell to cell via PIN proteins, as a function of the pH difference between the membranes and between the cell walls (Perrot-Rechenmann, 2010). The result is the activation of proteins called 'expansins', which loosen the cell wall and allow cell elongation. In *O. basilicum* 'Alfavaca Green', the A2 and A9 treatments produced planta with the thickest epidermises due to the low concentration of NAA and its action on cell elongation. Similar results were obtained by Trettel et al. (2019) in *O. basilicum* 'Genovese', and by Stefanova et al. (2011) in *Lamium album* L.

It is likely that the interaction between the highest concentration of NAA and the highest concentration of cytokine (BAP or KIN) caused increases in palisade and spongy parenchyma thickness. This may be due to the role that cytokines play in the cell multiplication process through the induction of cyclin-dependent kinase (CDK) transcription factors, which regulate the cell cycle (Neelakandan & Wang, 2012; Xie et al., 2018). This signaling process is a result of the interactions among regulator types, regulator doses, and regulator synergisms. In turn, these processes are closely linked to the plant genotype. The observed increases in tissue thickness are mirrored by those of Toma et al. (2004), in which *Hyssopus officinalis* L. seedlings cultivated with 0.5 mg L^{-1} indole-3-acetic acid (IAA) and 1.0 mg L^{-1} BAP displayed increased parenchymal thickness. Trettel et al. (2019) found that 0.1 mg L^{-1} of BAP produced *O. basilicum* 'Genovese' plants with increased palisade and spongy parenchyma thickness.

The total leaf limb thickness impacts the structure of the leaf interior, which is formed by the palisade and spongy parenchyma (Trettel et al., 2019). The formation of spongy parenchyma may be due to the use of KIN in the medium. Additionally, the greater spongy parenchyma thickness observed may be related to the formation of leaves with hyperhydricity. Treatment A3 had intermediate morphometry, which was close to normality. These results are similar to those found by Toma et al. (2004) in *Hyssopus officinalis* L. when 1.0 mg L^{-1} indole-3-butyric acid (IBA) was used as a source of auxin. The plants grown under the A3 treatment displayed a normal structure with a spongy parenchyma (SP) layer under the adaxial epidermis (AE), which suggests that this medium is most favorable to the development of basil.

CONCLUSIONS

Thus, the A3 treatment (0.05 mg L^{-1} NAA and 0.1 mg L^{-1} KIN) containing low concentrations of NAA and KIN is best suited for *in vitro* cultivation of *O. basilicum* 'Alfavaca Green', due to the observed increases in number of leaves, height, and sprout and root biomass. Additionally, a fewer plants with hyperhydricity and calluses were observed under this treatment. Our initial hypothesis regarding the effects of regulators on leaf tissues was confirmed; the epidermis was elongated under low auxin concentrations, and at higher concentrations there was an increase in cell multiplication.

Hyperhydricity is very common in the Lamiaceae family, especially in the *Ocimum* genus. Future research must seek to identify additional factors that could be associated with this disorder in order to reduce the problem. This is important for both the production of seedlings from *in vitro* culture and for obtaining leaves as a raw material for oil extraction, because this disorder promotes considerable yield losses and increased costs.

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