Australian Journal of Crop Science AJCS

AJCS 13(07):1131-1140 (2019) ISSN:1835-2707
doi: 10.21475/ajcs.19.13.07.p1649

# *In vitro* organogenesis and growth of *Ocimum basilicum* 'Genovese' (basil) cultivated with growth regulators

**Jéssica Rezende Trettel[1], Andressa Bezerra Nascimento[2], Letícia Neris Barbosa[2], Hélida Mara Magalhães[3]**

**[1]DSc Student Postgraduate Programs in Biotechnology Applied to Agriculture - Paranaense University.**
**[2]Agronomist - Paranaense University.**
**[3]Postgraduate Programs in Biotechnology Applied to Agriculture - Paranaense University.**

***Corresponding author: helidamara@prof.unipar.br**

**Abstract**

Genovese basil is a medicinal plant broadly used in medicine, perfumery, and cooking. In this study, the *in vitro* organogenesis and growth of Genovese basil seedlings are evaluated through the effect of growth regulators applied in different doses. Eight treatments were performed combining different concentrations of auxins and cytokinins, plus one control. At the end of the trial, growth characteristics of the seedlings were measured, and anatomical sections of the leaves and stems of the basil were prepared. The results demonstrated the need for cytokinins to increase the number and length of shoots and the length of roots. The treatment with 0.2 $mgL^{-1}$ benzyl aminopurine (BAP) $mgL^{-1}$ demonstrated the best result. The combined action of auxins and cytokinins increased fresh and dry masses of roots (0.3 $mgL^{-1}$ BAP + 0.6 $mgL^{-1}$naphthaleneacetic acid [NAA]). Partial changes in the leaf architecture as well as a reduction in the thickness of the palisade and lacunar parenchyma were observed, especially in treatment T8 (0.4 $mgL^{-1}$ BAP+ 0.2 $mgL^{-1}$NAA). In addition, all treatments with regulators presented adventitious roots in the stem, originating from inner-stem cells or vascular tissues. The formation of callus at the base of the plants and the occurrence of abnormal seedlings indicate that Genovese basil was responsive to the action of the growth regulators, although other factors regarding the medium could influence such responses. The growth regulators also function in the expression of genes linked to the stretching process (auxins) and cell proliferation (cytokinin).

**Keywords**: *Ocimum basilicum* L.'Genovese'; growth regulators; Lamiaceae; leaf anatomy; micropropagation.
**Abbreviation:**ANOVA_ analysis of variance; BAP_ benzyl aminopurine;BN1_ medium BN1;CDKA_ gene family;$CuSO_4$_copper sulphate; DMR_ dry mass of roots; E2FC_ genes related to cell division; Eb_abaxial epidermal; Ed_adaxial epidermal; Ep_total leaf thickness; FEA_formaldehyde, ethanol, and acetic acid; FMR_fresh mass of roots; FMS_fresh mass of shoots; IAA_ Indole-3-acetic acid; IBA_ indolebutyric acid; KIN_6-furfurylaminopurine; LR_length of roots; LS_length of shoots; MPF_maturation-promoting factor; MS_ medium Murashige and Skoog; NAA_ naphthaleneacetic acid; NL_ number of leaves; NS_ number of shoots; PP_length of the palisade; PS_Cells spongy parenchyma; PVP_ polyvinylpyrrolidone film; WIND1_ transcription factors.

## Introduction

Lamiaceae is a family of plants that includes species of medicinal, perfumery, and culinary use (Miele et al., 2001; Bauer et al., 2008; Pavithra, 2014). This family represents over 46 genera found in Brazil (Flora do Brasil, 2020). Among them, *Ocimum* includes basil species originating from Africa, Asia, and Central and South America. Some authors mention 65–150 species of basil existing in the world (Makri and Kintzios, 2008). Seven species are cataloged in Brazil, 2 of which are native (Flora do Brasil, 2020). Genovese basil (*Ocimum basilicum* L.'Genovese'), known as sweet basil, presents large and sturdy leaves (Darrah, 1980). The major essential oil compounds of this species are linalool (19–38%) and eugenol (5.5–88.2%) (Miele et al., 2001; Labra et al., 2004). Eugenol has anesthetic, antioxidant, and anti-inflammatory properties (Pavithra, 2014), and linalool is used in perfumery (Bauer et al., 2008) and exhibits anti-microbial activity (Park et al., 2012).

The cultivation techniques of Genovese basil are not well known. There is a lack of information regarding micropropagation methods to produce quality seedlings for successful crops(George et al., 2008). In this context, tissue culture is a promising alternative for producing seedlings in a protected and aseptic environment, free of diseases, and with a high standard of quality (George et al., 2008). In addition, tissue culture is used in breeding programs, physiology studies, and the *in vitro* production of secondary metabolites. *Ocimum basilicum*L. is of great economic importance, primarily due to its essential oil, which is rich in terpenoids and phenylpropanoids. Tissue culture has been a useful alternative for producing *O. basilicum* L. essential oils (Manan et al., 2016; Złotek et al., 2016) with predictable compounds and homogeneous products.

Growing *in vitro* plants is a complex process, as numerous factors can potentially interfere with organogenesis (George et al., 2008). It is necessary to balance all constituents of the medium, which should be adjusted according to the plant

species and their respective genetics and physiologies (Perrot-Rechenmann, 2010). Growth regulators are capable of regulating gene expression and directing plant growth processes. Auxins and cytokinins are the primary growth regulators in this sense (Perrot-Rechenmann, 2010; Zürcher and Müller, 2016).

It is important that studies consider anatomical evaluation to understand morphological changes occurring during the *in vitro* cultivation. Not only do growth regulators provide successful plant regeneration, but they also indirectly favor acclimatization (Kumar and Rao, 2012). According to Chandra et al. (2010) acclimatization is a concern because of the considerable loss of seedlings that cannot adapt to autotrophic conditions. Therefore, anatomical studies help verify the normality of tissues and can indicate the appropriate treatments for the success of the *in vitro* cultivation(e.g. thicker cuticle, epidermis, and leaf blade) (Kumar and Rao, 2012).

With respect to *O. basilicum* L., studies on the action of growth regulators are still incipient and inconclusive. However, some Lamiaceae species have already shown promising results. According to Manan et al. (2016), 1.0 mg·$L^{-1}$benzyl aminopurine (BAP) induced the shoots of all *O. basilicum* plants tested (100%). Saha et al. (2014) observed that 0.5 $mgL^{-1}$ BAP + 0.3 $mgL^{-1}$naphthaleneacetic acid (NAA) resulted in a higher number of shoots and roots in *Ocimum canum* Sims. Meftahizade et al. (2010) also verified that 3.0 $mgL^{-1}$ BAP, when combined with 1.0 $mgL^{-1}$NAA, improved the occurrence of shoots and roots in *Melissa officinalis* L.

The results of studies examining Lamiaceae species have indicated that the type and concentration of growth regulators are determinants of the success of regeneration, anatomy, and morphology of the plants. Knowing this, it was expected that the growth regulators would influence the growth of *O. basilicum* L. 'Genovese', as well as its *in vitro* organogenesis. The aim of this study was to enable and accelerate the growth of relatively vigorous seedlings and to indicate treatments with higher possibilities of success during the acclimatization processes. The purpose of this work was to evaluate the *in vitro* development of Genovese basil seedlings subjected to treatments with different concentrations of growth regulators and to examine their morphological and anatomical characteristics.

**Results**

***Growth at different times***

The results of the analysis of variance demonstrated the action of growth regulators and their concentrations on the development of basil seedlings at p (≤0.05). The three observations of the *in vitro* cultivation demonstrated that callus formation and abnormal seedlings occurred due to the interactions between the growth regulators and evaluation periods (Table 2). In general, the occurrence of calluses increased alongside cultivation and for the treatments with 0.3 BAP + 0.6 NAA $mgL^{-1}$(T5) and 1.0 BAP + 1.2 NAA $mgL^{-1}$(T6) (Table 2). Most of the calluses were friable; however, some had a blackened and frigid appearance. These calluses originated from the basal part of the seedlings, rarely occurring on those with more than one shoot (Fig. 1B), and they remained present even after seedling formation (Fig. 1D).

Similar behavior throughout the *in vitro* culture was observed for the percentage of abnormal seedlings. The occurrence of abnormality increased from period 2 (Table 2), with 56.89% of abnormal plants in treatment T5. The seedlings generally presented distended stems, with a few mall and twisted leaves (Fig. 1D). The opposite was observed in normal seedlings, which were vigorous and presented many leaves (Fig. 1E, F) and well-formed roots (Fig. 1G).

With respect to percent oxidation, no interaction effect between the growth regulators and evaluation periods was observed. Only the isolate effect of both factors was identified in this study. The highest occurrence of oxidation was observed in period 4 and at the end of the experiment (80 d after inoculation) (Table 3). The lowest percent oxidation was observed in treatment T8, followed by treatment T6. The highest percent oxidation was found in the control treatment, followed by treatments with 0.1 $mgL^{-1}$BAP (T2) and 0.2 $mgL^{-1}$ BAP + 0.6 NAA $mgL^{-1}$ (T7), being even higher on the evaluation periods 3 and 4, with 12.57% and 17.61%, respectively.

After 80d of inoculation, a higher number of leaves was observed primarily in the control and treatments T2, T7, and T8. Similar results were observed for the number of shoots (Table 4). However, results differed for shoot and root length. Only treatment T6 (1.0 $mgL^{-1}$ BAP + 1.2 $mgL^{-1}$NAA) limited the shoot development, while the other treatments presented similar results (means between 1.03–1.44 g) (Table 4). The growth regulator effect was more intense on roots, mainly for treatment T2 (0.2 BA $mgL^{-1}$), which caused an average increase of 23.7% in the volume of roots in comparison with treatments T3 and T6 (Fig. 1H). Treatments T3 and T6 presented the lowest observed averages (Table 4). Similar results were observed for the dry and fresh masses of shoots. The highest averages were observed in the control and treatment T3, and the lowest averages were observed in treatments T5 and T6. For roots, the highest averages were observed in treatment T5, followed by treatment T6 (Table 4).

***Anatomical analysis of leaves of O. basilicum***

The leaf blade of Genovese basil presented a uniseriate adaxial epidermis, rounded edges, and was covered by a thin cuticle with rectangular cells (Fig. 2 A, B). The surface was irregular, with cavities and tectorial and glandular trichomes. The palisade parenchyma presented juxtaposed cells (uniseriate), longitudinally elongated and with small intercellular spaces (Fig. 2 E, F). The spongy parenchyma presented two or four lays of cells, which were irregular octahedral in shape and had large intercellular spaces (Fig. 2 E, F). The abaxial epidermis presented a similar shape to the adaxial epidermis, however the thickness and size of the tissue were slightly smaller (Fig. 2D, E).

In the main vein, the epidermis was thicker, had a circular shape, and was larger in size when compared to the distal portions of the leaf blade (Fig. 2A). The cells of the parenchyma were large, rounded, and octahedral, with very thin cell walls and with small spaces between cells (Fig. 2C, D). The vascular tissue presented the xylem inward and phloem outward, being of the collateral bundle type. The metaxylem elements had thicker and rounded cell walls that formed layers of cells lined up toward the medulla (Fig. 2A,

D). The metaphloem presented smaller and more irregular cells (Fig. 2A, D).

Quantitative evaluations indicated the action of growth regulators on the development of leaf tissues. In general, the control and treatment T2 (0.2 BAP $mgL^{-1}$) presented the highest averages, while treatment T8 presented the lowest. The control and T2 presented 2.7 times greater thickness in the adaxial epidermis in comparison with treatment T8 (Table 5). However, a greater number of enlarged cells in the abaxial epidermis were found in treatment T6 (1.0 BAP + 1.2 NAA $mgL^{-1}$), followed by the control and treatmentT2. Regarding the palisade and spongy parenchyma, differences in size were considerably high. Cells of treatment T2 were 4.6 times higher than those of treatment T8. Treatment T2 also increased the total thickness of the leaves, in line with the increased cell size described above (Table 5).

***Anatomical analysis of stems of O. basilicum***

In general, *O. basilicum* L. 'Genovese' presented irregularly shaped stems with rounded edges (Fig. 3B) and a uniseriate epidermis (Fig. 3A). In sequence, the cortex presented large-rounded cells with thin cell walls (Fig. 3A). Collenchyma cells may also be noticed in the angular regions of the stem (Fig. 3B). Xylem cells were lined up to the medulla (center of the stem) (Fig. 3C). Small adventitious roots in the stem were a phenomenon observed in the *O. basilicum*L. 'Genovese' seedlings, especially in treatments with growth regulators (Fig. 1E, F). Roots were small (5–10 mm), thin, hairy, and present in all portions of the stem.

The cross section of the stem showed that these roots originated from the inner-stem cells, near to the vascular tissues (Fig. 3D). In this region there were cells with meristematic activities and intense mitosis. The cells had a dense cytoplasm and prominent nucleus. The roots were likely formed from inner-stem, phloem, or xylem cells, but the angulation of the cross-section did not allow a conclusive view (Fig. 3E, G). The adventitious roots penetrated the cortex of the stem and were outwardly projected (Fig. 3D, E).

The roots presented a cortex with cells of the fundamental parenchyma and vascular tissues connected to the medulla (Fig. 3E). In the distal region, fundamental meristematic cells were in a continuous process of cell division. Cells had bulky and dense nuclei (Fig. 3F). A thin hood (coif) covered the entire region to protect the root meristem (Fig. 3F).

**Discussion**

Growth regulators were important for the development of *O. basilicum*L. 'Genovese', but in some cases,they induced the formation of abnormal seedlings. It seems that the Lamiaceae family is prone to callus formation—sometimes even without growth regulators. Gogoi and Kumaria (2011) reported 100% callus formation in *Ocimum tenuiflorum* L. when cytokinins and auxins were present in the culture medium. Toma et al. (2004) observed that seedlings of *Hyssopus officinalis* L. cultivated in BN1 medium added to 1 $mgL^{-1}$ BAP + 0.5 $mgL^{-1}$NAA formed calluses, which remained present on the basal portion of the seedlings until the end of the cultivation.

Callus formation is regulated by several factors, including growth regulators. According to Ikeuchi et al., (2013) a balance between cytokinins and auxins in the culture medium can result in the formation of such cell masses. This can also happen via wounds in the plant tissue and epigenetic regulation, which affects the chromatin gene expression, thus modifying histones and the production of compounds, commonly phenols. For each of these factors, there is a group of genes and proteins regulating cell proliferation; cytokinins and auxins induce the LBD transcription factors, and the E2Fa regulates the cell replication processes. Once a super-expression of LBD factors occurs, there is callus formation.

When wounds were induced in *Arabidopsis*, the WIND1 transcription factors were identified as the major regulators of the response. In Genovese basil, cytokinins and auxins were likely the principal regulators of the process. In Lamiaceae, calluses might effectively produce secondary metabolites of industrial interest, as species in this family commonly produce terpenes and phenylpropanoids (Labra et al., 2004). According to Sahraroo et al. (2014), sections of *Satureja khuzestanica* shoots treated with 1.0 $mgL^{-1}$indole3-butyric acid (IBA) + 1.0 $mgL^{-1}$(6-furfurylaminopurine) (KIN) produced 7.5% of rosmarinic acid per dry weight of callus. Other studies reported approximately 1.2% of rosmarinic acid for other Lamiaceae species (Zgorka and Glowniak, 2001).

The presence of abnormal seedlings can be explained by the presence of regulators, as well as by the lack or failure of some other compound in the culture medium, since it happened in all treatments. The protocol of *in vitro* cultivation is still in the adaptation process for this species. Therefore, there is a need to create a balance in the culture medium to avoid abnormalities. Trials performed in our laboratory demonstrated that culture media enriched with 25 µm copper sulphate ($CuSO_4$) can reduce abnormality in basil plants (Trettel et al., 2017). The oxidation at the end of the cultivation (80 d after inoculation) likely occurred due to the depletion of the culture medium.

In general, treatments with BAP (T2 and T8) favored shoot development, and treatments with NAA (T5 and T6) favored root development. Growth regulators were important for the development of Lamiaceae seedlings; the results of the present study are largely satisfactory. The number and length of *Dracocepha lumkotschyi* Boiss. shoots increased with 5.0 $mgL^{-1}$ BAP + 0.2 $mgL^{-1}$NAA in the culture medium. A significant increase in the number of shoots (11.4) was also observed for *Ocimum americanum* L. after addition of 0.5 $mgL^{-1}$BAP + 0.5 $mgL^{-1}$Indole-3-acetic acid (IAA) to the culture medium. On the other hand, an increased number of calluses were verified in treatments with 2.4-dichlorophenoxyacetic acid (2.4-D), applied to the same species (Sharma et al., 2014). Other cytokinins such as KIN, at concentrations close to 0.5 $mgL^{-1}$, were also efficient in the multiplication and growth of shoots in Lamiaceae plants (Irina, 2008; Gopal et al., 2014; Zeinali et al., 2014).

The action of cytokinins on cell division depends on the responsiveness of the tissue and the flow of cytokinins to aerial parts of the plant. The synthesis of this compound primarily occurs on the root apical meristem, and its metabolism occurs in this tissue (Zürcher and Müller, 2016). This partially explains the performance of the two best treatments with respect to the shoot growth of Genovese basil.

**Table 1.** Composition of the treatments with the BAP (benzyl aminopurine) and NAA (naphthaleneacetic acid).

| Treatment | BAP ($mgL^{-1}$) | NAA ($mgL^{-1}$) |
|---|---|---|
| T1 | 0.0 | 0.0 |
| T2 | 0.1 | 0.0 |
| T3 | 0.0 | 0.2 |
| T4 | 0.1 | 0.2 |
| T5 | 0.3 | 0.6 |
| T6 | 1.0 | 1.2 |
| T7 | 0.2 | 0.1 |
| T8 | 0.4 | 0.2 |

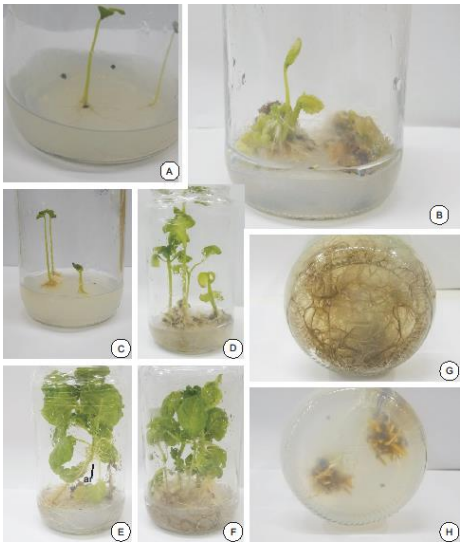


**Fig 1.** *In vitro Ocimum basilicum* 'genovese' seedlings submitted to treatments with growth regulators. Initial growth (A), occurrence of callus and abnormal plants (B), abnormal development of seedlings (C), Small, sickle-shaped and chlorotic leaves (D), well-formed roots (G), seedlings with adventitious roots (E, D), malformed roots (H).Adventitious root (ar).

**Table 2.** Percentage of calli and abnormal seedlings verified in *Ocimum basilicum*L. 'genovese' as function of different concentrations of auxins and cytokinins per periods of evaluation.

| Treatment | CALLI (%) | | | |
|---|---|---|---|---|
| | Period 1 | Period 2 | Period 3 | Period 4 |
| T1 | $0.00±0.00^{Ba*}$ | $9.29±5.05^{Ea}$ | $10.00±0.80^{Ea}$ | $6.67±0.80^{Ca}$ |
| T2 | $7.56±3.57^{Bb}$ | $21.14±3.68^{Da}$ | $22.27±3.65^{Da}$ | $13.10±7.25^{Cb}$ |
| T3 | $28.94±5.58^{Ab}$ | $60.98±16.78^{Ba}$ | $51.67±12.66^{Ca}$ | $59.87±10.55^{Ba}$ |
| T4 | $19.79±3.74^{Ab}$ | $70.51±14.38^{Ba}$ | $68.43±3.16^{Ba}$ | $67.00±16.15^{Ba}$ |
| T5 | $15.86±4.69^{Ab}$ | $100.00±0.00^{Aa}$ | $100.00±0.00^{Aa}$ | $100.00±0.00^{Aa}$ |
| T6 | $22.17±3.42^{Ab}$ | $100.00±0.00^{Aa}$ | $100.00±0.00^{Aa}$ | $100.00±0.00^{Aa}$ |
| T7 | $17.79±1.61^{Ac}$ | $44.93±18.69^{Cb}$ | $72.00±22.65^{Ba}$ | $70.67±22.53^{Ba}$ |
| T8 | $3.08±1.0^{Bd}$ | $63.51±14.46^{Bc}$ | $96.92±4.21^{Aa}$ | $88.45±11.74^{Ab}$ |
| Treat | **ABNORMAL SEEDLINGS (%)** | | | |
| | Period 1 | Period 2 | Period 3 | Period 4 |
| T1 | $9.47±2.94^{Cb*}$ | $10.00±1.99^{Bb}$ | $10.00±2.36^{Bb}$ | $36.28±29.43^{Aa}$ |
| T2 | $19.69±5.37^{Ca}$ | $11.91±1.74^{Ba}$ | $21.08±2.32^{Ba}$ | $31.21±8.20^{Aa}$ |
| T3 | $35.07±5.05^{Ba}$ | $21.96±5.55^{Ba}$ | $34.57±8.11^{Aa}$ | $41.54±10.42^{Aa}$ |
| T4 | $35.61±13.35^{Ba}$ | $23.55±5.09^{Ba}$ | $28.37±2.00^{Aa}$ | $36.24±4.2^{Aa}$ |
| T5 | $74.87±16.69^{Aa}$ | $44.10±7.20^{Ab}$ | $39.43±8.70^{Ab}$ | $56.89±9.16^{Ab}$ |
| T6 | $86.62±11.11^{Aa}$ | $18.14±5.44^{Bb}$ | $12.97±1.66^{Bb}$ | $33.86±9.40^{Ab}$ |
| T7 | $66.02±24.29^{Aa}$ | $37.07±4.94^{Ab}$ | $32.67±9.02^{Ab}$ | $42.67±7.23^{Ab}$ |
| T8 | $78.09±12.70^{Aa}$ | $40.96±7.87^{Ab}$ | $35.63±9.59^{Ab}$ | $47.97±7.38^{Ab}$ |

*Means followed by the same capital letter in the column and lowercase in the row do not differ by the Tukey test at 5%. T1= control; T2= 0.1 $mgL^{-1}$ BAP; T3 = 0.3 $mgL^{-1}$NAA; T4= 0.1 $mgL^{-1}$ BAPand 0.2 $mgL^{-1}$NAA; T5= 0.3 $mL^{-1}$ BAPand 0.6 $mgL^{-1}$NAA; T6=1.0 $mgL^{-1}$ BAPand 1.2 $mgL^{-1}$NAA; T7=0.2 $mgL^{-1}$ BAPand 0.1 $mgL^{-1}$NAA; T8=04 $mgL^{-1}$ BAPand 0.2 $mgL^{-1}$NAA.

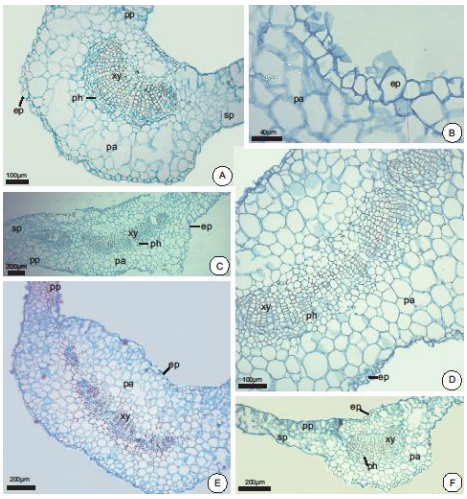


**Fig 2.** Transversal section of leaves of *in vitro Ocimum basilicum*'genovese' submitted to treatments with growth regulators. Leaf obtained from the control treatment (A) epidermis of the control treatment (B), leaf originated from seedlings treated with 0.1 $mgL^{-1}$ BAP + 0.2 $mgL^{-1}$NAA T4 (C and D), leaf originated from seedlings treated with 0.3 $mgL^{-1}$ BAP + 0.6 $mgL^{-1}$NAA T5 (E), leaf originated from seedlings treated with 0.4 $mgL^{-1}$ BAP + 0.2 $mgL^{-1}$NAA T8 (F). Epidermis (ep), Parenchyma (pa), Spongy parenchyma (sp), Palisade parenchyma (pp), Phloem (ph), xylem (xy).

**Table 3.** Single effect of periods and different concentrations of auxins and cytokinins on the oxidation of *Ocimum basilicum* L. 'genovese'

| **Periods** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **1** | **2** | **3** | **4** | | |
| Oxidation (%) | | | 0.00±0.00$^{b}$ | 3.83±10.47$^{b}$ | 12.57±16.80$^{a}$ | 17.61±16.16$^{a}$ | | |
| **Regulators** | | | | | | | | |
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| Oxidation (%) | 14.97±24.28$^{a}$ | 11.45±10.45$^{ab}$ | 9.37±17.05 ab | 7.55±12.53 ab | 8.33±13.37 ab | 3.81±7.30 ab | 10.42±14.99$^{ab}$ | 2.09±4.40 b |

*Means followed by the same capital letter in the column and lowercase in the row do not differ by the Tukey test at 5%. T1= control; T2= 0.1 $mgL^{-1}$ BAP; T3 = 0.3 $mgL^{-1}$ NAA; T4= 0.1 $mgL^{-1}$ BAP and 0.2 $mgL^{-1}$NAA; T5= 0.3 $mL^{-1}$ BAP and 0.6 $mgL^{-1}$NAA; T6=1.0 $mgL^{-1}$ BAP and 1.2 $mgL^{-1}$NAA; T7=0.2 $mgL^{-1}$ BAP and 0.1 $mgL^{-1}$NAA; T8=04 $mgL^{-1}$ BAP and 0.2 $mgL^{-1}$NAA

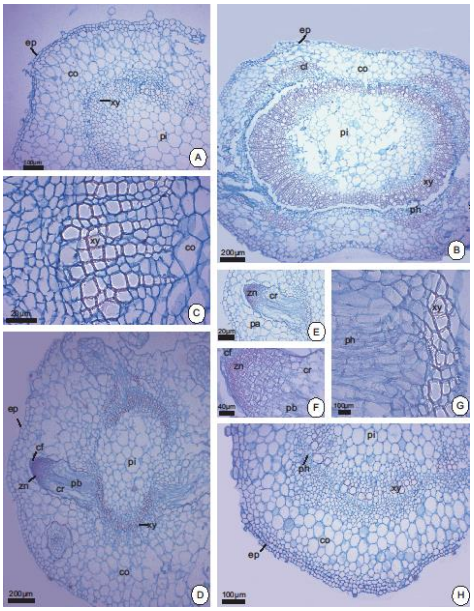


**Fig 3.** Transversal section of stems of *in vitro Ocimum basilicum*'genovese' seedlings submitted to treatments with growth regulators.Control (A, B), 0.1 $mgL^{-1}$ BAP + 0.2 $mgL^{-1}$NAA T4 (C), 3 $mgL^{-1}$ BAP + 0.6 $mgL^{-1}$NAA T5 (D, E, F, G) and0.4 $mgL^{-1}$ BAP + 0.2 $mgL^{-1}$NAA T8 (H). Coif (cf), stem cortex (co), root cortex (cr), epidermis (ep), fundamental meristem (gm), parenchyma (pa), procambium (pb) medulla (pi), Phloem (ph), xylem (xy), meristematic zone of adventitious root growth (zn).

**Table 4.**Number of leaves (NL), number of shoots (NS), length of shoots (LS), length of roots (LR), fresh mass of shoots (FMS), fresh mass of roots (FMR) dry mass of shoots (DMS) and dry mass of roots (DMR) of *Ocimum basilicum*L. 'genovese'as function of different concentrations of auxins and cytokinins.

| Treatment | NL | NS | LS (mm) | LR (mm) | FMS (g) | FMR (g) | DMS (g) | DMR (g) |
|---|---|---|---|---|---|---|---|---|
| T1 | 13.80±1.39$^{a}$ | 1.28±0.21$^{ab}$ | 76.39±9.11$^{a}$ | 48.90±1.79$^{abc}$ | 1.58±0.26$^{a}$ | 1.80±0.32$^{ab}$ | 0.14± 0.04$^{a}$ | 0.07±0.02$^{c}$ |
| T2 | 11.63±2.47$^{abc}$ | 1.42± 0.72$^{a}$ | 76.06±9.98$^{a}$ | 60.22±7.62$^{a}$ | 1.55±0.14$^{ab}$ | 1.08± 0.35$^{b}$ | 0.11±0.01$^{ab}$ | 0.07±0.02$^{c}$ |
| T3 | 8.66±1.46$^{bcd}$ | 1.03±0.33$^{ab}$ | 79.88±3.93$^{a}$ | 29.16±9.34$^{d}$ | 1.66±0.28$^{a}$ | 1.55±0.39$^{ab}$ | 0.10±0.02$^{ab}$ | 0.12±0.03$^{bc}$ |
| T4 | 9.99±2.21$^{abc}$ | 1.04±0.34$^{ab}$ | 69.81±9.37$^{a}$ | 32.05±7.21$^{cd}$ | 1.23±0.27$^{ab}$ | 1.86±0.35$^{ab}$ | 0.08±0.02$^{bcd}$ | 0.13±0.02$^{ab}$ |
| T5 | 7.21±2.68$^{cd}$ | 1.01±0.28$^{ab}$ | 48.87±3.97$^{ab}$ | 36.86±8.03$^{bcd}$ | 0.65±0.33$^{bc}$ | 3.05±0.38$^{a}$ | 0.03±0.003$^{cd}$ | 0.18±0.02$^{a}$ |
| T6 | 4.58± 2.05$^{d}$ | 0.59±0.32$^{b}$ | 19.58±3.89$^{b}$ | 29.47±6.11$^{d}$ | 0.29±0.07$^{c}$ | 2.59±0.32$^{ab}$ | 0.02±0.01$^{d}$ | 0.17±0.03$^{a}$ |
| T7 | 12.36±3.25$^{ab}$ | 1.45±0.26$^{a}$ | 72.25±7.28$^{a}$ | 54.41±14.44$^{ab}$ | 1.49±0.51$^{ab}$ | 2.00±0.33$^{ab}$ | 0.10±0.04$^{ab}$ | 0.13±0.02$^{ab}$ |
| T8 | 13.00±1.86$^{ab}$ | 1.44±0.35$^{a}$ | 73.28±10.31$^{a}$ | 43.78±7.31$^{abcd}$ | 1.60±0.37$^{a}$ | 2.05±0.41$^{ab}$ | 0.09±0.03$^{bc}$ | 0.12±0.02$^{b}$ |

*Means followed by the same capital letter in the column and lowercase in the row do not differ by the Tukey test at 5%. T1= control; T2= 0.1 mgL$^{-1}$ BAP; T3 = 0.3 mgL$^{-1}$ NAA; T4= 0.1 mgL$^{-1}$ BAP and 0.2 mgL$^{-1}$NAA; T5= 0.3 mL$^{-1}$ BAP and 0.6 mgL$^{-1}$NAA; T6=1.0 mgL$^{-1}$ BAP and 1.2 mgL$^{-1}$NAA; T7=0.2 mgL$^{-1}$ BAP and 0.1 mgL$^{-1}$NAA; T8=04 mgL$^{-1}$ BAP and 0.2 mgL$^{-1}$NAA

**Table 5.** Adaxial epidermis, abaxial epidermis, palisade parenchyma, spongy parenchyma and total thickness of *Ocimum basilicum*L. 'genovese'leaves as function of different concentrations of auxins and cytokinins.

| Treatment | Adaxial epidermis(µm) | Abaxial epidermis (µm) | Palisade parenchyma(µm) | Spongy parenchyma(µm) | Total thickness (µm) |
|---|---|---|---|---|---|
| T1 | 16.04±1.97$^{a}$ | 13.70±3.07$^{ab}$ | 65.90±20.63$^{bc}$ | 152.76±24.01$^{ab}$ | 233.35±18.52$^{ab}$ |
| T2 | 15.24±0.89$^{a}$ | 12.18±1.54$^{ab}$ | 133.66±41.66$^{a}$ | 182.24±14.45$^{a}$ | 327.45±17.15$^{a}$ |
| T3 | 12.02±0.57$^{ab}$ | 9.95±2.59$^{ab}$ | 87.50±10.16$^{abc}$ | 107.95±37.49$^{b}$ | 195.83±33.27$^{b}$ |
| T4 | 8.47±1.83$^{bc}$ | 10.32±1.72$^{ab}$ | 67.95±26.06$^{bc}$ | 129.57±24.90$^{ab}$ | 201.95±10.86$^{ab}$ |
| T5 | 8.46±1.37$^{bc}$ | 7.79±0.84$^{b}$ | 58.39±14.51$^{bc}$ | 110.02±12.09$^{b}$ | 171.66±10.71$^{bc}$ |
| T6 | 15.30±4.46$^{a}$ | 18.02±7.24$^{a}$ | 98.02±21.37$^{ab}$ | 157.56±29.37$^{ab}$ | 279.08±35.04$^{ab}$ |
| T7 | 8.90±0.24$^{bc}$ | 9.60±2.03$^{ab}$ | 59.98±5.58$^{bc}$ | 147.13±15.73$^{ab}$ | 216.35±14.53$^{ab}$ |
| T8 | 5.96±0.46$^{c}$ | 6.98±1.37$^{b}$ | 28.36±8.51$^{c}$ | 39.64±6.21$^{c}$ | 68.60±16.93$^{c}$ |

*Means followed by the same capital letter in the column and lowercase in the row do not differ by the Tukey test at 5%. T1= control; T2= 0.1 mgL$^{-1}$ BAP; T3 = 0.3 mgL$^{-1}$ NAA; T4= 0.1 mgL$^{-1}$ BAP and 0.2 mgL$^{-1}$NAA; T5= 0.3 mL$^{-1}$ BAP and 0.6 mgL$^{-1}$NAA; T6=1.0 mgL$^{-1}$ BAP and 1.2 mgL$^{-1}$NAA; T7=0.2 mgL$^{-1}$ BAP and 0.1 mgL$^{-1}$NAA; T8=04 mgL$^{-1}$ BAP and 0.2 mgL$^{-1}$NAA

Root formation is a process comprising several endogenous and exogenous factors. According to Zhang et al. (2017), the process can be divided into three phases: 1) root development induction by molecular and biochemical mechanisms, 2) radicle initiation with anatomical changes, and 3) protrusion of the radicle. Studies with *Arabidopsis* have demonstrated that auxins act mainly in the CDKA gene family, which is directly related to root formation, and in the E2FC genes, related to cell division. Like cytokinins, factors such as translocation, responsiveness, and enzymatic actions have influence in the exogenous action of auxins.

Saha et al. (2016) reported that the number and length of *Ocimum tenuiflorum* L. roots increased with 3 $mgL^{-1}$ NAA + 0.5 $mgL^{-1}$ BAP. Zarki and Elmtili (2012) observed 100% rooting in *Mentha pulegium* L. treated with indole butyric acid (IBA). After rooting, new amounts of auxins are needed in low concentrations for meristem cell division. Dissimilarly, cytokinins are needed to differentiate root tissues—mainly the vascular tissues (Zürcher and Müller, 2016).

***Morphological and anatomical analysis of leaves and stems of O. basilicum***

The leaf anatomy observed in this study was consistent with that of other reports for the *Ocimum* genus (Furlan et al., 2012; Fernandes et al., 2014) and other Lamiaceae species (Baran et al., 2008; Baran and Özdemir, 2009; Yetişen, 2014).The presence of growth regulators partially altered the architecture of Genovese basil leaves; this finding was verified in a morphometric change of tissues according to each treatment applied to basil seedlings, consistent with previous results (see Kosar and Mahmoud, 2012 and Stefanova et al.,2011).

The treatments with 0.2 BA $mgL^{-1}$ (T2) and 0.4 BAP + 0.2 NAA $mgL^{-1}$ (T8) favored shoot development but not leaf morphometry. The highest means in all tissues were observed in the treatment T2, while a substantial decrease in the thickness of tissues was observed in treatment T8. The seedlings of treatment T8 likely experienced greater difficulty during acclimatization, as they were subjected to stressful events such as loss of water or mechanical damage (Kumar and Rao, 2012).

Stefanova et al. (2011) reported that *Lamium album* L. seedlings treated with 0.2 $mgL^{-1}$ BAP +0.5 $mgL^{-1}$ IBA presented better leaf structure. However, the combination of these two regulators, but with a lower dose of IBA (0.05 $mgL^{-1}$), promoted a very strong, negative effect on leaf histogenesis; this was especially prominent in palisade parenchyma cells, which became shorter and had greater cell spaces, atypical for this tissue type. Toma et al. (2004) reported that morphological and anatomical differences were found in *Hyssopus officinalis* Linn. leaves; however, when this species was treated with 1 $mgL^{-1}$ BAP, the callus formation was reduced, and the morphometric characteristics of leaves remained unaffected. Plants subjected to a medium enriched with BN1 1 $mgL^{-1}$ BAP + 0.5 $mgL^{-1}$NAA exhibited a decrease in the size of palisade parenchyma cells, while the size of cells of the spongy parenchyma remained normal.

Similarly, a shortening of cells of the palisade and lacunar parenchyma was observed, which greatly contributed to the results observed for treatment T8. The growth regulators likely increased the cell size or amount of cell layers present in the leaf tissue. The cell size is related to the cell elongation capacity (Perrot-Rechenmann, 2010), while the amount of cell layers is related to cell division (Zürcher and Müller, 2016). The mechanism in which the auxins promote cell elongation is well known. The PIN proteins that transport this regulator across cell membranes (polar auxin transport) promote cell elongation in addition to tissue maturation (Barbosa et al., 2014). The genes that coordinate this process, as well as the morphogenic and biochemical responses, are largely elucidated for *Arabidopsis*. However, it is difficult to explain these mechanisms in plants that are not genetically sequenced, such as *O. basilicum* L. The first hypothesis for this result is that the gene expression for the PIN proteins was discontinuous. Without the ideal number of regulators, the stimuli of expansive proteins and other factors (responsive to auxins) were not sufficient, leading to thinner cells (Perrot-Rechenmann, 2010), primarily in the parenchyma in *O. basilicum* L.

The palisade parenchyma hypothesis is likely more acceptable. Other studies examining Lamiaceae—especially the *Ocimum* genus—have indicated that this tissue has one or two cell layers in the leaf(Martins, 2002; Toma et al.,2004; Furlan et al.,2012). In contrast, spongy parenchyma is formed by up to five cell layers that are irregularly overlapped (Baran and Özdemir, 2009). With this, the aspect of cell proliferation deserves attention in future studies.

Therefore, the second hypothesis is related to the cell proliferation, which is stimulated by cytokinins (Zürcher and Müller, 2016). This process is linked to cell activation by cyclins. When bound with the dependent kinases, cyclinsform the maturation-promoting factor (MPF). MPFs activate other proteins through phosphorylation. These phosphorylated proteins, in turn, are bound with AHP1-5, which are capable of activating DNA genes to start the cell proliferation process (Sugiyama, 1999; Tank and Thaker, 2011). If cytokinins become ineffective, cell division does not proceed. The reasons for this are complex and numerous. In addition to the amount and availability of cytokinin-responsive cells, there might be other factors related to the metabolism of the regulator influencing its presence and action. With respect to stem anatomy, the angular shape and collenchyma disposition at the angular extremities, verified for *O. basilicum* 'genovese', was also reported for other Lamiaceae species (Duarte and Lopes, 2007; Baran and Özdemir, 2009; Kosar and Mahmoud, 2012). This information can help taxonomists identify Lamiaceae species. In addition, *O. basilicum* L. 'Genovese' presented a layer of epidermis on the stem, corroborating the findings of Duarte and Lopes (2007) and Baran et al. (2008). The organization in series of the xylem, toward the medulla, was verified in *Plectranthus neochilus* Schltr. (Duarte and Lopes, 2007). The formation of adventitious roots in the stem occurred in all treatments containing growth regulators. This indicates that Genovese basil was responsive to cytokinins and auxins, especially the cells close to the medulla and conducting tissues. It was observed that plants treated with cytokinins alone also formed adventitious roots in the stem. The cytokinins likely interacted with endogenous auxins, produced by Genovese basil seedlings, and this small amount of auxin was enough to create the stimulus (Falasca et al., 2004).

Similar results were described for *in vitro Arabidopsis* treated with kinetin (6-furfurylaminopurine) (KIN) and

indole3-butyric acid (IBA). The authors hypothesized that, although the importance of auxins in inducing rooting is recognized, cytokinins might enhance auxin response (Falasca et al., 2004). The addition of $CaCl_2$ in the culture medium at concentrations ranging from 0.3 mM to 3.0 mM positively interacted with the hormones, increasing adventitious rooting in that species. In the present study, we believe that the interaction between hormones, combined with the species responsiveness, and likely with the presence of calcium in the medium induced the observed responses. Similar results were observed in *Rosa multiflora.* Thunb (Collet, 1985), *Camellia japonica* L. (Samartin et al., 1986), and *Arabidopsis* (Falasca et al., 2004). These authors also reported adventitious roots resulting from inner-stem cells, as occurred in *O. basilicum* L. 'Genovese'. This process was also reported by several studies examining woody plants propagated by cuttings and treated with auxins (Hatzilazarou et al., 2006; Amissah et al., 2008). These research results reveled that *O. basilicum* L. 'genovese' is responsive to the action of regulators and, changes in its growth and morphogenesis depend on the doses of the regulators. An increased number of calluses was verified 40 d after seed inoculation in treatments with 1.2 $mgL^{-1}$NAA. The presence of regulators did not increase the number of leaves, which would be important for essential oil extraction. The number and length of shots and the length of roots were favored in the treatment 0.2 BAP $mgL^{-1}$ (T2). Fresh and dry masses were increased in the treatment 0.3 mg $L^{-1}$ BAP + 0.6 mg $L^{-1}$NAA (T5). Regarding the morphological changes, the regulators partially altered the architecture of leaves to be small, twisted, and sickle-shaped. With respect to anatomical changes, the regulators shortened the palisade and spongy parenchyma, primarily in treatment T8 (0.4 mg $L^{-1}$ BAP + 0.2 mg $L^{-1}$NAA). The stem was rectangular, had a uniseriate epidermis, and xylem with cells lined up toward the medulla. Adventitious roots grew from the stem in all treatments with regulators. These roots likely originated from the inner-stem cells (medulla) or water-conducting vessels (xylem and phloem). The results of the present study support most of our hypotheses, with few exceptions regarding leaf number and shoot fresh and dry mass, which were not influenced by the growth regulators. It is worth noting that high doses of regulators are not always necessary to alter plant morphogenesis. This process is complex and depends on the plant responsiveness (genotype), interaction of regulators with the medium components, and then the regulator uptake by seedlings. Additionally, varying doses can result in similar responses. Is it related to the activation of alternative metabolic pathways? What other regulating factors could influence the process of morphogenesis? This study revealed interesting and promising results for *O. basilicum* L. 'genovese'. However, further investigation is necessary to understand the influence of other culture medium components in the verified responses.

## Materials and methods

### *Plant material and seed asepsis*

Tests were conducted at the Molecular Biology and Plant Culture Tissue laboratories of the Paranaense University (UNIPAR). Italian genovese basil seeds (Horticeres®), known as sweet basil, were purchased from a local retailer (batch number 14000168). After selection, seeds were treated with 70 % ethanol for 2 min and immersed into 2 % sodium hypochlorite solution for 15 min, with agitation. Seeds were then rinsed four consecutive times using sterile distilled water.

### *In vitro conditions*

The *in vitro* condition was created in clear glass flasks. To each flask was added 50mL MS medium (Murashige and Skoog, 1962) enriched with 30 g $L^{-1}$ sucrose, 6.5 g $L^{-1}$ agar, and varying concentrations of BAP and NAA (Table 1), at a pH of 5.8. The medium was then transferred to 350 ml flasks and autoclaved at 121°C for 20 min. After inoculating four seeds per flask, the flasks were closed with clear plastic lids, sealed with polyvinylpyrrolidone film (PVP), and moved to the growth chamber. All treatments were subjected to the same conditions and were maintained in the growth chamber for 80 d at 25±2 °C under a 24 h photoperiod with an irradiance of 70.2 µmol $m^{-2}s^{-1}$provided by 20 W cool white fluorescent lamps (Empalux®, Curitiba, Brazil).

### *Evaluations during in vitro cultivation*

During the *in vitro* establishment, evaluations were performed during three distinct periods (40, 60, and 80 d after seed inoculation). These periods were defined considering the changes in morphology and size of the seedlings. Data on percent germination, callus formation, abnormal seedlings, and percent oxidation were collected. The contamination criteria were the presence of fungus and bacteria. Seedlings were considered abnormal when they presented physical deformities, twisted shoots, hyalines, deformed cotyledons, necrosis, or deformed leaves.

### *Final evaluation*

The test was concluded at 80 d after inoculation considering the size of the seedlings. During this period, the seedlings presented well-formed roots and expanded leaves, the maximum size for the *in vitro* establishment. The following variables were analyzed: number of leaves (NL), number of shoots (NS), length of shoots (LS), length of roots (LR), fresh mass of shoots (FMS), fresh mass of roots (FMR), and dry mass of roots (DMR). The lengths of shoots and roots were obtained with a digital caliper. Samples were kept in an oven at 65 °C for 3 d to obtain the dry mass of roots.

### *Experimental design and statistical analysis*

The experimental design was completely randomized with eight treatments and five repetitions. For each repetition, four Erlenmeyer flasks were used. All variables were previously analyzed for normality using the Shapiro-Wilk test at 5 %. In the case of a non-normal distribution, data were submitted to the Kruskal-Wallis test at 5 %. Data collected during *in vitro* cultivation were analyzed in a 3×8 factorial design, with 3 periods of evaluation (40, 60, and 80 d after inoculation) and 8 concentrations of BAP and NAA. The variables analyzed at 80 d were verified only for the action of the growth regulators. All data were statistically analyzed using one-way analysis of variance (ANOVA) at 5 %. Tukey's test was made by at least significant differences at the 5 %

probability level ($p \leq 0.05$). All statistical analyses were conducted using the statistical software SISVAR 5.6 (Ferreira, 2011).

***Histological and anatomical analysis***

For anatomical analysis, fragments of callus and shoots of each treatment were collected 80 d after inoculation. Leaves were randomly collected from the middle of the seedlings. The plant materials were then fixed in FEA (formaldehyde, 70 % ethanol, and acetic acid 1:1:18, v/v) for 24 h and dehydrated with increasing ethanol concentrations: 50 % and 60 % for 2h, and 70 % until preparation of the microscope slides (Johansen, 1940).

For permanent slides, the material was dehydrated in butyl series (Johansen, 1940) and embedded in paraplast (Kraus and Arduin, 1997) in an oven set to 60 °C. Sections (10 µm thick) were made with a rotatory microtome (Model Leica) at the Histopathology Laboratory of UNIPAR. The histological sections were submersed in a water-bath (45°C) and immediately removed with a glass slide. The slides containing plant material were immersed in water-bath with butyl acetate at 45 °C to remove the paraplast surplus. For the total paraplast removal, samples were dehydrated in ethylic series. The plant sections were then stained with safrablau solution—a blend of astra blue and safranin 9:1 (v/v) modified to 0.5 % (Bukatsch, 1972).The stained lamina was subsequently fixed with colorless stained glass acrilex and a coverslip (Paiva, 2006). The mounted slides were used to measure the adaxial epidermal (Ed) and abaxial epidermal (Eb) cells, length of the palisade (PP) and spongy (PS) parenchyma, and total leaf thickness (Ep). The variables were taken from cross-section images captured using a digital camera coupled with the Nikon Eclipse E100 light microscope with Motic Images Plus 3.0. The means of the variables were calculated using three repetitions (slides) with 10 measurements for each plant section. The means of each tissue were taken from the images and submitted to analysis of variance (ANOVA) and compared by the Scott-Knott test at p (≤0.05). All analyses were performed using SISVAR (Ferreira, 2011).

**Conclusion**

Callus formation increased 40 days after cultivation of plants treated with 1.2 mg. $L^{-1}$ NAA. The number and length of root buds improved with 0.2 mg. $L^{-1}$BAP. Higher values of fresh and dry root mass were verified for 0.3 mg. $L^{-1}$ BAP and 0.6 mg. $L^{-1}$NAA. The palisade and spongy parenchyma of plants shortened mainly in the 0.4 mg. $L^{-1}$ BAP and 0.2 mg. $L^{-1}$ NAA treatments.

**Acknowledgments**

We would like to thankthe *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) and *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) for funding this work. And *Universidade Paranaense* (UNIPAR) for financial support.

**References**

Amissah JN, Paolillo DJ, Bassuk N(2008)Adventitious root formation in stem cuttings of *Quercus bicolor* and *Quercus macrocarpa* and its relationship to stem anatomy. J Am SocHortic Sci. 133:479-486.

Baran P, Özdemir C (2009) The morphological and anatomical properties of *Lamium lycium* (Lamiaceae), endemic to Turkey. Nord J Bot. 27:388-396.

Baran P, Özdemir C, Aktas K (2008) The morphological and anatomical properties of *Salvia argentea* L. (Lamiaceae) in Turkey. Res J Agr Biol Sci. 4:725-733.

Barbosa IC, Zourelidou M, Willige BC, Weller B, Schwechheimer C (2014) D6 protein kinase activates auxin transport-dependent growth and pin-formed phosphorylation at the plasma membrane. Dev Cell. 29:674-685.

Bauer K, Garbe D, Surburg H (2008) Common fragrance and flavor materials: preparation, properties and uses, 1rd edn. Weinheim, New York.

Bukatsch F (1972) Benerkemgemzeirdoppelfar being sastrablau-safranina. Mikrokosmos. 61:255.

Chandra S, Bandopadhyay R, Kumar V, Chandra R (2010) Acclimatization of tissue cultured plantlets: from laboratory to land. Biotechnol Lett. 32:1199-1205.

Collet GF (1985) Enracinement amélioré lors de la production *in vitro* de rosiers. Rev Suisse ViticArboricHortic.17:259-263.

Darrah HH (1980) The cultivated basils,1rd edn. Buckeye Printing Company, Florida.

Duarte MR, Lopes JF (2007) Stem and leaf anatomy of *Plectranthus neochilus* Schltr. Lamiaceae. Rev Bras Farm. 17:549-556.

Falasca G, Zaghi D, Possent, M, Altamura MM (2004) Adventitious root formation in *Arabidopsis thaliana* thin cell layers. Plant Cell Rep. 23:17-25.

Fernandes VF, Bezerra LA, Mielke MS, Silva DC, Costa LCB (2014) Leaf anatomy and ultrastructure of *Ocimum gratissimum* under different light radiation levels. Ciência Rural. 44: 1037-1042.

Ferreira DF (2011) A computer statistical analysis system. Ciência e Agrot.359:1039-1042.

Flora do Brasil (2020) Lamiaceae em construção. Jardim Botânico do Rio de Janeiro. <http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB8276>.Acess in: 05 Jun. 2017

Furlan MR, Aoyama EM, Indriunas A, Mauro C (2012) Leaf anatomy of *Ocimum basilicum* L."Genovese" (Lamiaceae). Rev Fitos Elet. 7: 231-235.

George EF, Hall MA, Klerk G (2008) Plant propagation by tissue culture: the background, 3rd edn. Springer, Dordrecht.

Gogoi K, Kumaria S (2011) Callus-mediated plantlet regeneration of *Ocimum tenuiflorum* L. using axillary buds as explants. Int J Plant. Sci. 2:1-5.

Gopal GV, Repalle S, Talluri VR, Ronda SR, Allu PR (2014)*In vitro* propagation and GC-MS Studies of *Ocimum basilicum* Linn. var. pilosum (Willd.) Benth. Br Biotechnol J. 4:96-107.

Hatzilazarou SP, Syros TD, Yupsanis TA, Bosabalidis AM, Economou AS (2006)Peroxidases, lignin and anatomy during *in vitro* and *ex vitro* rooting of gardenia (*Gardenia jasminoides* Ellis) microshoots. J Plant Phys. 163:827-836.

Ikeuchi M, Sugimoto K, Iwase A (2013) Plant callus: mechanisms of induction and repression. The Plant Cell. 25:3159-3173.

Irina G (2008) Effect of different plant hormones on *Salvia officinalis* cultivated *in vitro*. Int J Botany. 4:430-436.

Johansen DA (1940) Plant microtechnique,1rd edn.Mc Graw Hill, New York.

Kosar M, Mahmoud O (2012) Trichomes and regeneration by direct organogenesis of medicinal plant *Dracocephalum kotschyi* L. Using Shoot Tips (Lamiaceae). J Crop Sci Biotechnol. 15: 251-257.

Kraus JE, Arduim M (1997) Manual básico de métodos em morfologia vegetal, 1rd edn.EDUR, Seropédica.

Kumar K, Rao IU (2012) Morphophysiologicals problems in acclimatization of micropropagated plants in: *ex vitro* conditions-a review. J Ornam Hortl Plants. 2:271-283.

Labra M, Miele M, Ledda B, Grassi F, Mazzei M, Sala F (2004) Morphological characterization, essential oil composition and DNA genotyping of *Ocimumbasilicum* L. cultivars. Plant Sci. 167:725-731.

Makri O, Kintzios S (2008) *Ocimum* sp. (basil): Botany, cultivation, pharmaceutical properties, and biotechnology. J Herbs Spices Med Plants. 13:123-150.

Manan AA, Taha RM, Mubarak EE, Elias H (2016) *In vitro* flowering, glandular trichomes ultrastructure, and essential oil accumulation in micropropagated *Ocimum basilicum*. In vitro Cell Dev Biol Plant. 52:303-314.

Martins MBG (2002) Optical microscopy studies and scanning electron microscopy in leaf the *Menthaspicata* and *Menthaspicata* x *suaveolens* (Lamiaceae). Bragantia. 61:205-218.

Meftahizade H, Moradkhani H, Naseri B, Lofti M, Naseri A (2010)Improved *in vitro* culture and micropropagation of different *Melissa officinalis* L. genotypes. J Med Plant Res. 4:240-244.

Miele M, Dondero R, Ciarallo G, Mazzei M (2001) Methyl eugenol in *Ocimum basilicum* L. Cv. genovese gigante. J Agric Food Chem. 49:517-521.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant.15:473-497.

Paiva JGA (2006) Verniz vitral incolor 500®: a mounting medium alternative and economically viable. Acta Bot Bras. 20:257-264.

Park SN, Lim YK, Freire MO, Cho E, Jin D, Kook JK (2012) Antimicrobial effect of linalool and *α*-terpineol against periodontopathic and cariogenic bacteria. Anaerobe. 18:369-372.

Pavithra B (2014) Eugenol-A Review.J Pharm Pharm Sci. 6:153-154.

Perrot-Rechenmann C (2010) Cellular responses to auxin: division versus expansion. CSH Perspect Biol. 2:a001446.

Saha S, Dey T, Adhikari S, Mukhopadhyay S, Sengupta C, Ghosh P (2016) Effects of plant growth regulators on efficient plant regeneration efficiency and genetic stability analysis from two *Ocimum tenuiflorum* L. morphotypes. Rend Lincei Sci Fis Nat. 27:609-628.

Saha S, Roy S, Sengupta C, Ghosh P (2014) Micropropagation and analysis of genetic stability in regenerated plantlets of *Ocimum canum* Sims. Indian J Plant Physiol. 19:174-183.

Sahraroo A, Babalar M, Mirjalili MH, Moghaddam MRF, Ebrahimi SN (2014) *In-vitro* callus induction and rosmarinic acid quantification in callus culture of *Satureja khuzistanica* Jamzad (Lamiaceae). Iran J Pharm Res. 13:1447-1456.

Samartin A, Vieitez AM, Vieitez E (1986) Rooting of tissue cultured camellias. Hort Sci. 61:113-120.

Sharma NK, Choudhary RC, Kumar M (2014) Efficient and rapid *in vitro* regeneration of *Ocimum americanum* L. using nodal segments. Med Plants Int J Phyt Rel Ind. 6:62-67.

Stefanova MA, Koleva DP, Ganeva TG, Dimitrova MA (2011)Effect of plant growth regulators on the regeneration of *in vitro*-propagated *Lamium album* L. plants. J Pharm Res. 4:1982-1985.

Sugiyama M (1999) Organogenesis *in vitro*. Curr Opin Plant Biol. 2:61-64.

Tank JG, Thaker VS (2011) Cyclin dependent kinases and their role in regulation of plant cell cycle. Biol Plantarum. 55:201-212.

Toma I, Toma C, Ghiorghita G (2004) Histo-anatomy and *in vitro* morphogenesis in *Hyssopus officinalis* L. (Lamiaceae). Acta Bot Croat. 63:59-68.

Trettel JR, Gazim ZC, Gonçalves JE, Stracieri J, Magalhães HM (2017)Volatile essential oil chemical composition of basil (*Ocimum basilicum* L.'Green') cultivated in a greenhouse and micropropagated on a culture medium containing copper sulfate. In Vitro Cell Dev Biol Plant. 53:1-10.

Yetişen K (2014) Morphological and anatomical study of the endemic species *Phlomis monocephala* (Lamiaceae). Phytol Balc. 20:49-55.

Zarki KBL, Elmtil N (2012) Micropropagation of *Menthapulegium* L. through high-frequency shoots-tip and nodal explants culture.Moroccan J Biol.12:39-50.

Zeinali S, Otroshy M, Sadrarhami A, Moradi K, Mokhtari A (2014) Influence of plant growth regulators on *in vitro* culture and regeneration of *Dracocephalum kotschyi*. Int J AgricInnov. 3:108-111.

Zgorka G, Glowniak K (2001) Variation of free phenolic acids in medicinal plants belonging to the Lamiaceae family. J Pharm Biomed Anal.26:79-87.

Zhang W, Fan J, Tan Q, Zhao M, Zhou T, Cao F (2017) The effects of exogenous hormones on rooting process and the activities of key enzymes of *Malus hupehensis* stem cuttings. PLoS ONE. 12:e0172320.

Złotek U, Michalak-Majewska M, Szymanowska U (2016) Effect of jasmonic acid elicitation on the yield, chemical composition, and antioxidant and anti-inflammatory properties of essential oil of lettuce leaf basil (*Ocimumbasilicum* L.). Food Chemistry. 213:1-7.

Zürcher E, Müller B (2016) Cytokinin synthesis, signaling, and function-advances and new insights. Int Rev Cell Mol Biol. 324:1-38.