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Growth regulators alter the development and metabolism of lemon balm seedlings cultured *in vitro*

Reguladores de crescimento promovem alterações no desenvolvimento e no metabolismo de plântulas de melissa cultivadas *in vitro*

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ABSTRACT - *Melissa officinalis* L., popularly known as lemon balm, is an aromatic plant widely used in medicine, cosmetics, and pharmaceutical industries for its essential oil rich in phenylpropanoids, terpenes, and phenolics. This study aimed to assess the effect of growth regulators on the development and physiological and biochemical metabolism of *M. officinalis* cultured *in vitro*. Seeds were inoculated in Murashige and Skoog medium and added with the regulators 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) according to six different treatments. After 90 days of culture, plants were evaluated for growth and biochemical and physiological parameters (flavonoids, anthocyanins, and chlorophyll). The balance between regulators interfered with plant growth, which increased in the presence of 0.2 mg L^{-1} BAP. In this treatment, the plants had greater growth with more leaves, and the biomass production of shoots and roots was higher than the control. Growth regulators did not influence nitrogen assimilation or flavonoid production; however, total chlorophyll and anthocyanin indexes were enhanced by treatment with BAP at concentrations ranging from 0.2 to 0.5 mg L^{-1}. Auxin treatment did not improve root production or growth but favored callus formation when combined with 0.5–3.0 mg L^{-1} BAP. The results indicate that high BAP concentrations (above 1.0 mg L^{-1}) should not be used in *in vitro* production of lemon balm.

Keywords: Anthocyanins. Chlorophyll. Lamiaceae. Micropropagation.

RESUMO - A espécie *Melissa officinalis* L. conhecida como erva cidreira é uma planta aromática muito utilizada na medicina, indústria de cosméticos e farmacêutica, em função do seu óleo essencial rico em fenilpropanóides, terpenos e fenólicos. Objetivou-se com esta pesquisa avaliar a ação dos reguladores de crescimento sobre o crescimento, processos fisiológicos e bioquímicos de plantas de *M. officinalis* cultivadas *in vitro*. Sementes foram inoculadas em meio de cultura Murashige e Skoog com os reguladores benzilaminopurina - BAP e ácido 1-naftalenoacético - ANA em seis diferentes tratamentos. Ao final de 90 dias foram avaliados: crescimento da planta, índices bioquímicos e fisiológicos sendo flavonoides, antocianinas e clorofila. O balanço entre os reguladores interferiu no crescimento da melissa que foi incrementado em 0,2 mg L^{-1} de BAP. Neste tratamento, as plantas tiveram maior crescimento com mais folhas como também a produção de biomassa das brotações e das raízes foi maior comparada ao controle. Os reguladores de crescimento não influenciaram na assimilação de nitrogênio e na produção de flavonoides, no entanto as clorofilas totais e as antocianinas foram incrementadas com a adição de BAP na faixa de 0,2 a 0,5 mg L^{-1}. A adição de auxinas não melhorou aspectos de produção e crescimento da raiz, mas favoreceu a formação de calos quando foi combinada com o BAP a 0,5 e 3,0 mg L^{-1}. Portanto, concentrações acima 1,0 mg L^{-1} não devem ser utilizadas no cultivo *in vitro* da melissa.

Palavras-chave: Antocianinas. Clorofilas. Lamiaceae. Micropropagação.

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INTRODUCTION

Melissa officinalis L. (Lamiaceae), popularly known as lemon balm, is native to western Asia and the eastern Mediterranean region (MORADKHANI et al., 2010). This aromatic plant finds application in the pharmaceutical, cosmetic, and food industries (SHAKERI; SAHEBKAR; JAVADI, 2016) due to its rich essential oil in phenylpropanoids, terpenoids, and antioxidant phenolics such as rosmarinic acid (LUZ et al., 2014; SHAKERI; SAHEBKAR; JAVADI, 2016). Lemon balm is also used in the preparation of herbal medicines with antimicrobial, antioxidant, and biocidal properties (MIRAJ; RAFIEIAN; KIANI, 2017; CARVALHO; DUARTE; FERREIRA, 2021) and soothing effects that help alleviate stress, anxiety, nervousness, and digestive problems (MORADKHANI et al., 2010).

Existing research is inconclusive, mainly concerning the regulators regarding their dose and type and the interaction with the genotype. Compared to conventional propagation, usually carried out through seeds or cuttings, plants obtained *in vitro* are more standardized, have better health, and can be produced at any time of the year (CHATTERJEE; GHOSH, 2020).

For *in vitro* plant production, it is necessary to define the optimum

composition of the culture medium. Currently, the Murashige and Skoog medium is the most common in micropropagation protocols (PHILLIPS; GARDA, 2019). However, salt, sugar, and vitamin concentrations may vary depending on the nutritional needs of the plant species (PHILLIPS; GARDA, 2019). Levels of plant growth regulators (PGRs), which play a key role in plant growth signaling and gene activation, may also vary (NEELAKANDAN; WANG, 2012; SMALL et al., 2018).

PGRs are compounds analogous to plant hormones that act integrated during plant development (NEELAKANDAN; WANG, 2012). The major classes of PGRs are auxins, which promote root induction and cell elongation, and cytokinins, which act on the expression of cell cycle genes, the multiplication of cells, the growth of shoots, and the development of the aerial part (SMALL et al., 2018). Through such mechanisms, PGRs contribute to aerial part sprouting and seedling multiplication (NEELAKANDAN; WANG, 2012). The optimal concentration of PGRs needs to be carefully defined because, when used in excess, these compounds cannot be effectively metabolized by plant cells and may lead to abnormalities during seedling development, such as callus formation, absence of specific organs, tissue oxidation and necrosis, hyperhydricity, and, in more severe cases, plant death (SMALL et al., 2018).

There are few studies on the use of PGRs in lemon balm production. A study found inconclusive evidence of the effect of PGRs on lemon balm development (MEFTAHIZADE; LOTFI; MORADKHANI, 2010). This situation is further complicated by the fact that there is an interaction between PGRs and plant genotype, often with favorable and undesirable responses observed within the same genus. In the case of lemon balm, the limited information available focuses on genotypes found in Europe and the Middle East (MEFTAHIZADE; LOTFI; MORADKHANI, 2010); information on cultivars used in Brazil is scarce (REIS et al., 2008).

Plants exposed to balanced levels of the appropriate PGR classes develop satisfactorily and vigorously without showing signs of stress (PHILLIPS; GARDA, 2019). Physiological homeostasis is also influenced by factors such as proper nutrition, gene expression, and metabolic and physiological processes (GREENWAY et al., 2012). Few studies have examined the relationship between PGRs and physiological homeostasis for lemon balm. It is expected that plants exposed to adequate PGR concentrations will have higher nitrogen and chlorophyll indexes, whereas plants under stress (that is, exposed to unbalanced concentrations of PGRs) will show higher production of secondary metabolites, such as anthocyanins and flavonoids, which have the ability to scavenge free radicals that degrade lipids, proteins, and nucleic acids (ALVAREZ, 2014; BARBOSA et al., 2014).

Given the observations mentioned above, this study aimed to assess the effect of PGRs on the growth and physiological and biochemical parameters of lemon balm cultured *in vitro.*

MATERIAL AND METHODS

Plant material

The experiment was conducted at the Laboratory of Plant Tissue Culture of Paranaense University (UNIPAR), Paraná, Brazil. Seeds of *M. officinalis* were acquired from TopSeed Garden® (seed lot no. 056204, 99% purity, 85% germination rate). Under a laminar flow hood, seeds were immersed in 70% alcohol for 2 min and then in 2% sodium hypochlorite for 20 min. Subsequently, the seeds were washed four times in sterilized deionized water to remove the sodium hypochlorite.

General experimental procedures

Seeds were inoculated in full-strength Murashige and Skoog medium (MURASHIGE; SKOOG, 1962) supplemented with 30 g L^{-1} sucrose and 6.5 g L^{-1} agar, pH 5.8. Two PGRs were used in this study: 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA). PGR concentrations were based on previous studies (MEFTAHIZADE; LOTFI; MORADKHANI, 2010; TRETTEL et al., 2019). Treatments are described in Table 1.

Table 1. Concentrations of plant growth regulators used for *in vitro* cultivation of *Melissa officinalis* L.

Treatments	BAP mg L^{-1}	NAA mg L^{-1}
1	0.0	0.0
2	0.2	0.0
3	0.5	0.0
4	0.5	0.5
5	1.0	0.0
6	3.0	0.5

After preparation, the medium was aliquoted in flasks and autoclaved at 121 °C for 20 min. Inoculation was performed in an aseptic chamber. Four seeds were placed in glass bottles containing 50 mL of culture medium. Then, bottles were closed with transparent plastic lids, sealed with polyvinyl chloride film, and incubated in a growth chamber for 90 days under a 24 h light photoperiod. Illumination was provided by LED lamps (Blumenau®, LED T8 10 W, 6,000 K,

100–240 V, 50/60 Hz, power factor ≥ 0.92, 25 ± 2 °C, 34.02 µmol m^{-2} s^{-1}) (WELZ et al. 2020).

Analytical procedures

Germination and physiological disorders

The percentages of germinated seeds (%), abnormal and oxidized seedlings (%), and hyperhydric plants (%) were determined after 30, 60, and 90 days of culture. Seeds were considered germinated when the radicle had at least 0.5 mm in length. Seedling oxidization and abnormalities were assessed visually according to criteria described by Górski, Gerotti and Magalhães (2021). Seedlings with hyperhydricity were evaluated based on visual criteria, being characteristic of this disorder, swollen leaves with water accumulation and light green color (TRETTEL et al., 2020).

Analysis of plant growth

On the 90th day of culture, plants were evaluated for leaf number, shoot length, root length, shoot dry weight, root dry weight, and callus dry weight. Length measurements were taken using a digital caliper. For dry weight determination, samples were oven-dried at 65 °C for three days.

Biochemical and physiological parameters

Total nitrogen, chlorophyll, flavonoid, and anthocyanin indexes were measured on fully expanded fresh leaves using an optical leaf-clip meter (Quick Start, DUALEX Scientific™, FORCE A®, Paris, France) according to the instructions of the manufacturer. Twelve replications were assessed per treatment.

Statistical analysis

The experiment followed a completely randomized design with six treatments of two PGRs in different concentrations. Forty replications were used per treatment, totaling 240 bottles containing four seeds each.

Data were tested for normality by the Shapiro–Wilk test (FERREIRA, 2011). When normality assumptions were met, means were subjected to analysis of variance (ANOVA) at $p \leq 0.05$ and compared by the Tukey test at $p \leq 0.05$ using Sisvar software version 5.6 (FERREIRA, 2011). Data on germination percentage and abnormal and oxidized seedlings did not follow a normal distribution and were plotted in Excel according to the three evaluation periods. Similarities between treatments were analyzed, as suggested by Trettel et al. (2020) and Górski, Gerotti and Magalhães (2021), through multivariate analysis of percentage data according to the evaluation period. Clustering was based on the Euclidean distance, and a dendrogram was constructed using Ward's method. These analyses were performed using Statistica software version 13.3 (STATSOFT, 2017).

RESULTS AND DISCUSSION

Germination and physiological disorders induced by PGRs

Lemon balm seeds had a mean germination rate of 23.64% after 30 days of culture; seed germination was slow and uneven. Seeds cultured in media containing 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA or 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA had germination percentages below the mean: 8.645% and 14.62%, respectively. After 90 days, the highest germination rate was observed in seeds treated with 1.0 mg L^{-1} BAP alone (33.541%), followed by control seeds (29.513%), representing an increase of 2.63% and 8.73%, respectively, concerning their germination percentages at 30 days. These findings demonstrate that germination percentages were low regardless of treatment, and most seeds germinated within the first 30 days of culture (Figure 1A). In the dendrogram, the two treatments that afforded the highest germination percentages were allocated to the same group. A second group was formed by the treatments 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA, whereas the 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA treatment was isolated from the others (Figure 1A).

Tissue oxidation was observed in newly sprouted seedlings, calluses, and fully developed seedlings. Treatments differed in the degree of tissue oxidation. Some seedlings overcame this disorder and emitted new shoots and roots, whereas others did not succeed and ultimately died. Two treatments caused expressive oxidation responses in seedlings: 0.5 mg L^{-1} BAP and 1.0 mg L^{-1} BAP. In the former, 10.83% of seedlings showed signs of oxidation, with this percentage decreasing to 4.33% after 90 days of culture, demonstrating the capacity of seedlings to regenerate (Figure 1B). In the 1.0 mg L^{-1} BAP treatment, the opposite was observed. At 60 days, 2.83% of seedlings showed signs of oxidation, and this value increased to 16.35% at 90 days, yielding the highest oxidation percentage observed during the experiment. This treatment in the dendrogram. Seedlings and calluses exposed to the other treatments had a low oxidation rate (less than 2.0%), particularly those treated with 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA, which did not show any oxidized calluses (Figure 1B). The remaining treatments were grouped in a second group, which showed the same response to PGRs and their concentrations (Figure 1B).

PGR treatment also influenced the occurrence of abnormalities. Some examples include a lack of roots, lack of shoots, etiolated stems, and stunted leaves. The rate of abnormalities remained below 5% in most treatments during the 90 days of incubation. All explants grown in the presence of 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA were converted to calluses. After 60 days of culture, the highest percentage of abnormal seedlings was observed in the 0.5 mg L^{-1} BAP treatment. After 90 days, the rate decreased again to below 5% (Figure 1C). The results indicated that, regardless of treatment, a period of at least 60

days was necessary for culture stabilization. Dendrogram analysis identified one group comprising the control and plants treated with 0.2 mg L^{-1} BAP and 0.5 mg L^{-1} BAP. The other treatments were isolated from each other (Figure 1C).

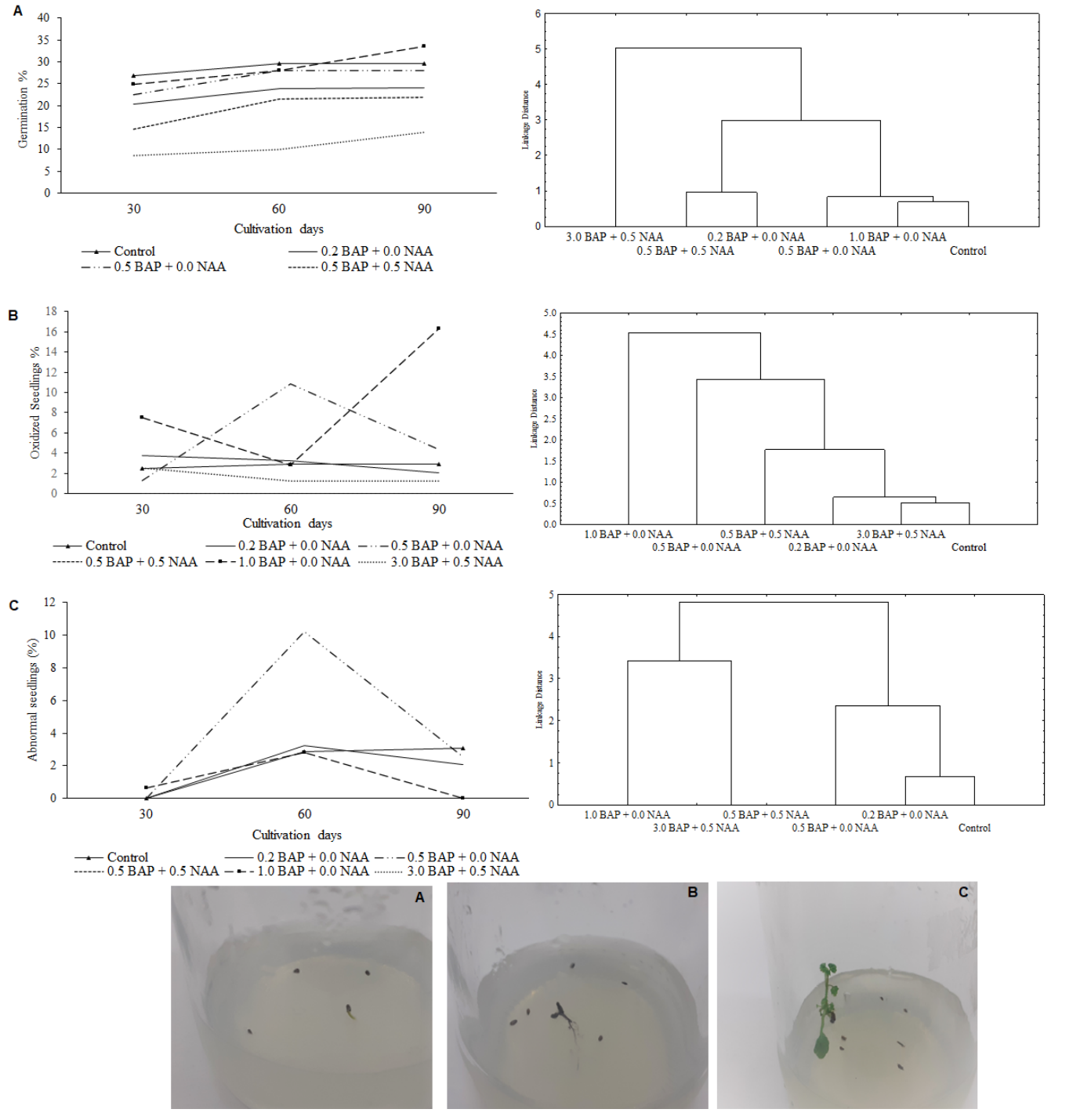


Figure 1. Dissimilarity dendrograms and percentages of germinated (A), oxidized (B), and abnormal (C) seedlings of *Melissa officinalis* L. cultured *in vitro* in the presence of different concentrations of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA). Evaluations were performed 30, 60, and 90 days after seed inoculation.

Physiological disorders such as oxidation and tissue abnormalities are common in plants grown *in vitro*. Oxidation is one of the major problems affecting seedling establishment. It is worth emphasizing that necrotic tissues do not regenerate; therefore, complete recovery is only possible if oxidative damage is overcome by plant defense mechanisms

(SZECHYŃSKA-HEBDA et al., 2007; PRUDENTE; SOUZA; PAIVA, 2020). Although their use may be necessary to promote plant growth and development, PGRs have been cited as inducers of tissue oxidation when used at unbalanced concentrations in plants of the family Lamiaceae (TRETTEL et al., 2019). After excluding explants that turned into calluses, it was observed that the highest BAP concentration tested (1.0 mg L^{-1}), excluding the treatment in which there was the formation of calluses (3.0 mg L^{-1}), promoted greater tissue oxidation. According to Szechyńska-Hebda et al. (2007), cytokinins can trigger oxidative damage because they enhance the production of reactive oxygen species capable of attacking cell membranes, lipids, nucleic acids, and proteins. Another possible explanation for the oxidizing effects of PGRs was given by Small et al. (2018), who suggested that excess phytohormones that are not efficiently metabolized by cells have a toxic effect on cell membranes, components, and metabolism. The oxidation rates observed for lemon balm in the current study were similar to those reported for other Lamiaceae species in studies using the same PGRs at similar concentrations (TRETTEL et al., 2019; GÓRSKI; GEROTTI; MAGALHÃES, 2021).

Another disorder commonly found in plants from this family is the development of abnormal seedlings. PGRs act directly on the expression of various developmental genes such as baby boom (*BBM1*), cyclin-dependent kinase (*CDK*), enhancer of shoot regeneration (*ESR*), and knotted 1 (*KN1*). These genes control the process of cell division and the organization of plant tissues, mainly the meristems (NEELAKANDAN; WANG, 2012). The prevalence of abnormal seedlings in lemon balm was low (less than 5%), indicating that the concentrations used in the experiment were adequate. In other species of this family, higher abnormality rates were observed with doses of 0.3–0.4 mg L^{-1} BAP and 0.2–0.6 mg L^{-1} NAA (TRETTEL et al., 2019; WELZ et al., 2020).

Hyperhydricity, common in some genera of Lamiaceae, such as *Ocimum* (GÓRSKI; GEROTTI; MAGALHÃES, 2021), was not observed in the present experiment. The disorder may cause significant changes in seedlings, especially in leaves. Affected leaves acquire a swollen, brittle appearance and become light green and dysfunctional (VASCONCELOS et al., 2012). The disorder was observed in basil, lavender, and sage treated with excess BAP, although the exact cause of hyperhydricity remains unknown. Some reports indicated that BAP possibly causes cell hypertrophy in plants (ZUZARTE et al., 2010; GÓRSKI; GEROTTI; MAGALHÃES, 2021; JAN et al., 2021).

PGRs had different effects according to plant organs. Treatment with 0.2 mg L^{-1} BAP provided the largest increase in the number of leaves, affording a 65.28% increase compared with the control and an 85.05% increase compared with 0.5 mg L^{-1} BAP treatment (Table 2). Although the number of leaves was high in the 0.2 mg L^{-1} BAP group, leaves were smaller and more rounded than control leaves. Root and shoot growth did not differ between the control and treatments without NAA (0.2 and 1.0 mg L^{-1} BAP). BAP treatments provided the highest root and aerial part growth, with a mean gain of 70.82% in shoots and 79.61% in roots compared with the worst treatment (0.5 mg L^{-1} BAP) (Table 2).

Table 2. Number of leaves, shoot length, root length, shoot dry weight, root dry weight, and callus dry weight of seedlings of *Melissa officinalis* L. cultured *in vitro* treated with different concentrations of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA).

Treatments	LN	SL (mm)	RL (mm)
Control	60.6±23.64 b	126.08±21.87 a	116.24±29.76 a
0.2 BAP +0.0 NAA	174.3± 59.41a	130.53±15.64 a	127.04±23.39 a
0.5 BAP +0.0 NAA	22.6± 13.81c	37.65± 30.9b	26.07± 24.43b
0.5 BAP +0.5 NAA	0.0± 0.0d	0.0± 0.0c	0.0± 0.0b
1.0 BAP +0.0 NAA	62.6±35.20 b	128.98± 14.17a	140.53± 41.98a
3.0 BAP +0.5 NAA	0.0±0.0 d	0.0± 0.0c	0.0± 0.0b
CV%	29.53	17.83	20.16
Treatments	SDW (g)	RDW (g)	CDW (g)
Control	1.074±0.04 c	0.058± 0.01ab	0.0± 0.0b
0.2 BAP +0.0 NAA	1.111± 0.08b	0.142± 0.02ab	0.0± 0.0b
0.5 BAP +0.0 NAA	1.098± 0.05bc	0.039± 0.03ab	0.0± 0.0b
0.5 BAP +0.5 NAA	0.0± 0.0d	0.0± 0.0b	0.048± 0.01a
1.0 BAP +0.0 NAA	1.116± a	0.165± 0.02a	0.0± 0.0b
3.0 BAP +0.5 NAA	0.0± d	0.0± 0.0b	0.033± 0.02a
CV%	1.89	5.14	2.24

*Means in the same column followed by the same letter are not significantly different by the Tukey test ($p \leq 0.05$). Leaf number (LN), shoot length (SL), root length (RL), shoot dry weight (SDW), root dry weight (RDW), and callus dry weight (CDW).

The optimal concentrations of PGRs in culture media for various plant species remain unknown. For the Lamiaceae family, few studies have focused on this aspect, with some efforts on plants of the genus *Ocimum* (MONFORT et al., 2018; TRETTEL et al., 2019), *Mentha* (ASMAR et al., 2011; MORAIS; ASMAR; LUZ, 2014), and, to a lesser extent, *Melissa* (MEFTAHIZADE; LOTFI; MORADKHANI, 2010).

The effect of BAP on leaf emergence has been observed in basil (MONFORT et al., 2018), mint (SANTORO et al., 2013), and lavender (MACHADO; SILVA; BIASI, 2011). The correct balance between auxins and cytokinins in leaf buds ensures leaf emergence and development, whereas high concentrations of endogenous auxins lead to bud dormancy (ALONI et al., 2005). The addition of cytokinins induces the synthesis of protein kinases closely linked to cell multiplication (NEELAKANDAN; WANG, 2012). In the current study, lemon balm leaf tissues were highly responsive to BAP.

The highest shoot (1.098 g) and root (0.165 g) weights were afforded by treatment with 1.0 mg L^{-1} BAP. The treatment 0.2 mg L^{-1} BAP provided the second-best result in shoot weight, whereas root weight did not differ between the remaining treatments. Callus formation was observed in all explants treated with 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA or 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA, revealing that adding auxins was harmful to the organogenesis of leaves and roots. Calluses had a light green color and were friable. Callus dry weight did not differ between 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA treatments (Table 2).

The importance of cytokinins for biomass production is evident, which may be explained by the ability of these molecules to induce genes linked to cell multiplication (NEELAKANDAN; WANG, 2012). The findings corroborate Reis et al. (2008), who observed greater bud formation and acclimatization efficiency in lemon balm treated with 1.0 mg L^{-1} BAP. However, plant tissues were sensitive to high cytokinin concentrations and the presence of auxins (NAA), leading to callus formation. Such sensitivity has been reported in other plants of the family Lamiaceae; calluses may be triggered by exposure of plants to moderately high concentrations of PGRs (MONFORT et al., 2018; TRETTEL et al., 2019). Callus formation was not observed in plants not exposed to PGRs (MEFTAHIZADE; LOTFI; MORADKHANI, 2010; ASGHARI et al., 2012; MONFORT et al., 2018; TRETTEL et al., 2019).

Opposite effects on plant development were observed with treatment with 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA or 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA. Meftahizade, Lotfi and Moradkhani (2010) found that treatment of lemon balm with 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA promoted shoot growth and reduced callus occurrence. In this case, such effects were attributed to genotype. Within a given species, the response of plants to PGRs may vary according to variety and cultivar. We highlight that a large range of BAP concentrations (0.2–3.0 mg L^{-1}) was used because of the interaction between culture medium, genotype, and PGRs and that differences may be observed at the cultivar or variety level.

Effects of PGRs on physiological and biochemical characteristics

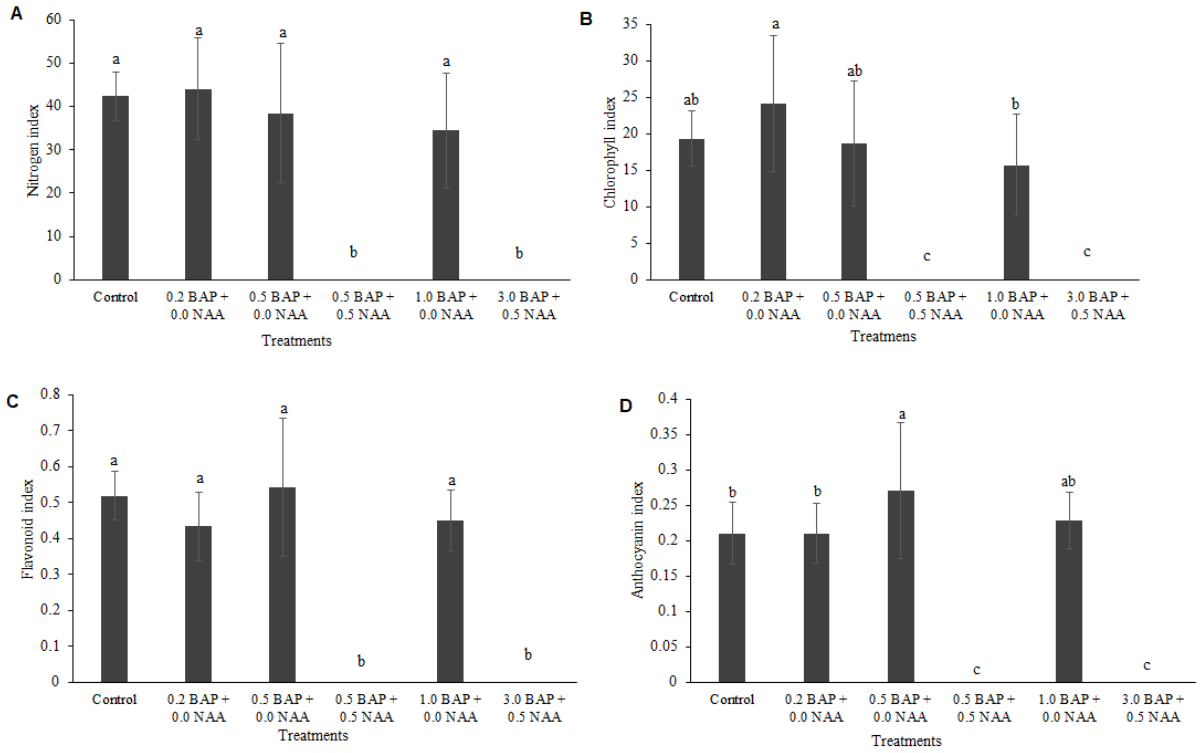
Physiological and biochemical assays were modified according to treatments, depending on their influence on the organogenesis of lemon balm plants. The nitrogen balance index of leaves did not differ between treatments that did not induce callus formation, with a mean of 39.77 (Figure 2A). Total chlorophyll index was highest in the 0.2 mg L^{-1} BAP group (24.11), being 34.89% higher than in the 1.0 mg L^{-1} BAP group. Control and 0.5 mg L^{-1} BAP treatments did not differ from each other (Figure 2B). The flavonoid index was also not influenced by PGR treatment, with a mean of 0.486 (Figure 2C). Anthocyanin index was highest in the 0.5 mg L^{-1} BAP treatment (0.0957), with an increase of 41.0% compared with the control and 0.2 mg L^{-1} BAP (Figure 2D).

According to the initial hypothesis, it was expected a greater influence of PGRs on the physiological and biochemical characteristics (secondary metabolites) of explants. Treatments influenced only chlorophylls and anthocyanins. The fact that the nitrogen balance index was similar between treatments indicates that nitrogen absorption was not impaired, even in the case of stunted seedlings or callus formation. However, the total chlorophyll index was higher with 0.2 mg L^{-1} BAP treatment, which increased the number of leaves.

Lamiaceae plants have been shown to have enhanced the development of shoots and leaves in full-strength Murashige and Skoog medium containing balanced concentrations of PGRs (WELZ et al., 2020). Photoreceptor cells in leaves, which contain chlorophyll, are preponderant for photosynthesis (STIRBET et al., 2018). Although photosynthesis is minimal when plants are grown *in vitro*, leaves develop and have a large volume of photosynthetic cells. When plants are acclimatized, that is, removed from *in vitro* culture and transplanted into a substrate, leaves must be functional to ensure the survival and growth of plants (PHILLIPS; GARDA, 2019).

Regarding biochemical activity, total flavonoids, the largest class of phenolics in plants (ALVAREZ, 2014), were not influenced by treatments. The class is divided into subgroups: isoflavones, flavonols, flavones, and anthocyanins (LATTANZIO, 2013; ALVAREZ, 2014). The results showed that treatments influenced phenolic subgroup contents; for instance, anthocyanin content was highest in the group treated with 0.5 mg L^{-1} BAP. It is possible that PGRs influenced other classes of flavonoids that were not analyzed here.

Flavonoids are part of the secondary metabolism of plants. They participate in numerous plant defense processes stimulated by abiotic stresses, such as changes in temperature, humidity, and salinity, and biotic stresses, including pests and pathogens (MATKOWSKI, 2008). Some components of this class exhibit antioxidant activity, eliminating free radicals, which are harmful to proteins, lipids, and nucleic acids (GILL; TUTEJA, 2010; BARBOSA et al., 2014).



*Different letters indicate significant differences by Tukey's test ($p \leq 0.05$).

Figure 2. Nitrogen balance index (NBI), flavonoid index, chlorophyll index, and anthocyanin index of fresh leaves of seedlings of *Melissa officinalis* L. cultured *in vitro* and treated with different concentrations of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA).

Initially, it was believed that plants exposed to the most stressful conditions would have a higher content of anthocyanins and flavonoids, but this hypothesis was not confirmed. It is possible that because plants had higher anthocyanin and flavonoid indexes, they had a better chance of fighting the effects of oxidative stress. Other biochemical mechanisms, such as free radical scavenging enzymes and other classes of compounds, might have contributed to mitigating the effects of oxidative stress in all treatment groups, even in plants with limited development (FERRARI et al., 2019).

Throughout evolution, plants acquired a set of mechanisms to fight free radicals, such as enzymatic defenses, phenolic compounds (MATKOWSKI, 2008), and vitamins C and E (RANI, 2017). These molecules act together or alone and alternate during plant development (FERRARI et al., 2019). There are few studies on antioxidative mechanisms in lemon balm plants grown *in vitro*. However, in *Ocimum* spp., it is known that these mechanisms are efficient, allowing good development (WELZ et al., 2020). Such findings reinforce that good development depends partially on the ability of plants to neutralize stress effects. More research is needed to elucidate the effects of PGRs on the metabolic routes of defense compounds; some studies have demonstrated the effectiveness of PGRs in enhancing the production of defense molecules (MONFORT et al., 2018).

CONCLUSIONS

It is recommended to use cytokinins for *in vitro* production of lemon balm, as BAP at concentrations of 0.2 and 1.0 mg L^{-1} stimulates leaf formation, growth, and biomass gain. However, NAA may result in callus formation when combined with BAP concentrations greater than 0.5 mg L^{-1}. Chlorophyll and flavonoid indexes increased after 90 days of culture using 0.2 or 0.5 mg L^{-1} BAP.

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