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Zinc and methyl jasmonate modulate the growth and the volatile compounds of the 'Albahaca Dante' basil cultivated *in vitro*

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Abstract

The species *Ocimum basilicum* has many cultivars with different chemotypes. The basil cultivar 'Albahaca Dante' has great economic potential. Nevertheless, there is little data about the production of volatiles and the growth using elicitors. This study aims to evaluate growth, physiological disorders, enzymatic activity, biochemistry, stomatal analysis, and the volatile compounds of *Ocimum basilicum* L.' Albahaca Dante' cultivated *in vitro* under different concentrations of zinc sulfate ($ZnSO_4$) and methyl jasmonate (MeJa). The experiment was conducted in a completely randomized design (CRD). Five treatments were evaluated based on the combination of MeJa x Zinc Sulfate, using a Murashige and Skoog medium. The results demonstrate that MeJa reduced the formation of abnormal seedlings. Nevertheless, the growth and the number of leaves were not incremented compared in half without elicitors. The number of volatile compounds was lower in the treatment without elicitors and with 25µM $ZnSO_4$ + 1µMMeja. Methyl chavicol was the main compound in both treatments. In this case, the seedlings had smaller stomata with higher density. The seedlings that were developed under unfavorable conditions (75µM $ZnSO_4$ + 1µM MeJa and 75µM $ZnSO_4$ + 5µM MeJa) produced compounds such as Eugenol, Linalool, Methyleugenol, α-Bergamotene, and showed a reduction in the stomatal density, but larger size. The elicitors influenced the activity of antioxidant enzymes, except for 75µM $ZnSO_4$ + 1µM MeJa, which occasioned an acute decrease of all enzymes. The elicitors altered the volatile composition of this basil cultivar and its biochemical responses.

Keywords: Elicitors, Lamiaceae, Terpenoids, Phenylpropanoids.
Abreviations: APX_ascorbate peroxidase; BAP_Benzyl adenine; CAT_Catalase; CRD_Completely randomized design; $CuSO_4$_Copper sulfate; GC/MS_Gas chromatography/mass spectrometry; H_2O_2_Hydrogen peroxide; LED_Light emitter diodes; MeJa_Methyl jasmonate; MS_Medium Murashige and Skoog; NBI_Nitrogen balance index; NBT_Nitro blue tetrazolium; O_2•_Singlet oxygen; PCA_ Principal Component Analysis; RAI_Relative anthocyanins index; RCI_Relative chlorophyll index; RFI_Relative flavonoids index; ROS_Reactive oxygen species; SISVAR 5.6_Statistical Analysis; SOD_superoxide dismutase; Zn_zinc; $ZnSO_4$_zinc sulfate.

Introduction

Ocimum basilicum L. (Lamiaceae) is an essential source of essential oils and aromatic chemical compounds used in various products, such as perfumes, cosmetics, and traditional medicine (Trettel et al., 2018). It has verified antioxidant antitumoral, antimicrobial (Miyoshi et al., 2021), and biocide actions (Govindarajan et al., 2013). The species *O. basilicum* is a plant that has several cultivars, among them the 'Albahaca Dante'. The cultivation of this species is usually performed on rustic flowerbeds with few technologies to increase productivity and modulate the chemical composition of plants.

The compounds biosynthesis is influenced by climatic factors (Hussain et al., 2008) such as cultivation, diseases, and pests (Yaldiz et al., 2015). Together, these factors promote heterogeneity in the chemical composition, the absence of compound standardization, and predictability limitations (Alvarez, 2014). Thus, an alternative to increase the efficiency of basil cultivation would be using a technique of plant tissues cultivation with *in vitro* elicitation (Trettel et al., 2018; Comar et al., 2019). Elicitors induce the defense system of plants. When applied in the plant, they stimulate metabolic pathways responsible for producing compounds and enzymes that mitigate oxidative stress (Alvarez, 2014).

Methyl jasmonate (plant hormone) and Zinc sulfate (metal ion) can be used as elicitors for the induction of desirable metabolic pathways (Złotek et al., 2016; Comar et al., 2019). The jasmonic acid controls plant senescence, induces proteinases in response to damages or pathogens, and

increases the synthesis capacity of other compounds derived from lipids used in vegetal defense (Dar et al., 2015; Deuner et al., 2015). Zinc acts as an enzymatic cofactor, essential for the activity, regulation, and stabilization of protein structure, affecting the synthesis and conservation of auxins, hormones involved in plant growth (Tsonev and Cebola, 2012). This study evaluates the effect of methyl jasmonate and zinc in the elicitation of volatile compounds in the cultivation of 'Albahaca Dante'.

The tissues culture and the action of elicitors may favor the growth and the production of terpenoids and phenylpropanoids in plants (Alvarez, 2014). For this aim, each species requires the elicitor to be adjusted to the plant metabolism, and the specific compounds desired, factors that determine the type and concentration of elicitors (Trettel et al., 2017). This method permits fast plant growth and the production of secondary metabolites during the whole year regardless of seasonal and climatic conditions (Favorito et al., 2011).

Another critical aspect in elicitors application is verifying how they influence plant growth. Elicitors can enhance several factors of the plants, including the architecture and lignin deposition (Gallego et al., 2018). Thus, they tend to produce more vigorous plants. For instance, Trettel et al. (2018) verified that 25 mM $CuSO_4$ resulted in plants with no abnormalities and leaf abundance. Nevertheless, they may also provoke physiological disorders; hyperhydricity, abnormality, and oxidation are the more common (Górski et al., 2021). These disorders compromise the yield and the production of essential oil. In ideal quantities, elicitors influence the cleaning of reactive oxygen species (ROS) by antioxidant enzymes (Barbosa et al., 2014; Welz et al., 2020) and the induction of pathways of shikimic and mevalonic acids (Alvarez, 2014; Kwon et al., 2021), which are the main involved in the synthesis of basil compounds.

This study aims to evaluate growth, physiological disorders, enzymatic activity, biochemistry, stomatal analysis, and the volatile compounds of *Ocimum basilicum* L. 'Dante' cultivated *in vitro* under different concentrations of Zinc sulfate and Methyl Jasmonate.

Results

Physiological disorders and nodal segments growth

No plant with hyperhydricity was observed in this experiment in all treatments. Regarding abnormal seedlings, the maximum value obtained was in T2 (1µM MeJa and 25µM $ZnSO_4$ - 32.5%) (Fig. 1A S1). Abnormality was more expressive in the first 30 days in all treatments. However, it was reversible during the *in vitro* cultivation. Lower occurrence of this disorder was obtained in T4 (5µM MeJa and 25µM $ZnSO_4$ -18%) and T5 (5µM MeJa and 25µM $ZnSO_4$ -14%) at 90 days (Fig. 1A S1). The mean values verified in T1, T2, and T3 treatments were similar at 90 days. They presented the highest percentage of abnormality, respectively, 23.5%, 24%, and 22.0%. The seedlings had twisted, rachitic leaves and stem etiolation in these treatments. In the dendrogram, T1 and T2 were put in the same group, indicating their similarity. T5 was isolated in a group on its own, while T3 and T4 were in the same group (Fig. 1A S1).

The average number of leaves of the cultivar *'Albahaca Dante'* remained similar in the first 30 days in all treatments. It reached higher values in T1 (without elicitors ~ 17) and T2 (~16) at the end of the experiment (Fig. 1B S1). After 30 days, the gain for this characteristic was 58.04 % and 53.25 % higher than in the second evaluation (60 days). The increase of leaves was less expressive from then on, with values of 8.19% and 11.0% for these same treatments at 90 days. This fact indicates that this cultivar can be kept for at least 60 days *in vitro* condition to guarantee better leaf production, a material whose peltate glands are used to synthesize essential oil. The lower final amount of leaves at 90 days was verified in T3 (1µM MeJa and 75µM $ZnSO_4$) and T4 (5µM MeJa and 5µM $ZnSO_4$). T1 was isolated in the dendrogram while two other groups were formed, one by T2 and T4 and the other by T3 and T5 (Fig. 1B S1).

Elicitors distinctly influenced the growth of the cultivar 'Albahaca Dante'. In general, the number of sprouts emitted was low independently of the elicitor used, being the maximum values verified in T1 (2.25) and T2 (2.13) (Table 2). The treatments strongly influenced the seedling size of the basil Dante. The use of elicitors diminished the length of sprouts and roots (Table 2). The control treatment presented the highest means for sprout length (108.009 mm) and was 9.6% higher than T2 (1µM MeJa and 25µM $ZnSO_4$ - 32.5%), the second better treatment, and 41.08% higher than T5, the worst treatment. Regarding the roots, the control had a 7.2% increase in size compared with T2, the second better treatment, and 24.78% compared with T5, which had the lower mean value for this characteristic (Table 2).

Nevertheless, the gain of sprouts mass was higher in T2 (5.634 g), which presented a 21% increase compared to the control and 70.62% to T5, the worst treatment (3.302 g). Concerning root dry mass, the results were similar between the control and T2, higher than the others. T2 presented an average dry matter/root increase of 59.9% and T1 of 54.83% compared with T5, which had the lowest mean (Table 2).

Stomatal morphometry

Stomata presented alterations in their conformation and amount due to elicitors. The cultivar 'Albahaca Dante' showed higher density in the adaxial surface of the leaves in the treatments T1 - control, T2, T3 (~9.0 mm²) and in the abaxial surface in T1 and T2. However, in this region of the leaf, the behavior was inverted, and there was a substantial reduction in this characteristic for T3 (1µM MeJa and 75µM $ZnSO_4$ - 0.53 mm²), T4 (5µM MeJa and 75µM $ZnSO_4$ - 1.88 mm²), and T5 (5µM MeJa and 75µM $ZnSO_4$ - 0.66 mm²) (Table 3 S1).

In all treatments, elicitors also influenced pore opening, higher in the abaxial surface of leaves. On this occasion, stomata remained more closed in T1-control (2.9 µm) and T5 (3.45 µm). In general, stomata were bigger, independently of the leaf surface evaluated, in the treatments T3, T2, and T4, in this order, which is evidenced by the width and length of guard and subsidiary cells. Stomata of the abaxial surface were slightly larger than those of the adaxial surface. T3 and T2 presented a width increase in guard cells of 50.39%, 48.02%, and in the subsidiary cells of 58.35%, 61.79%, compared with T1-control (Table 3 S1).

Volatile compounds of *Ocimum basilicum* 'Dante'

Table 4 lists the volatile chemical compounds induced by the treatments with MeJa and $ZnSO_4$. Thirteen compounds were identified in T1 (control), predominantly Methyl Chavicol (84.03%). In this treatment, Methyl chavicol content was higher than in the other treatments. More than 80% of the compounds were oxygenated monoterpenes in the control treatment (Fig. 2c). Plant treated with 1µM MeJa and 25µM

$ZnSO_4$ (T2) also presented 13 compounds, especially: Methyl chavicol (71.71%), Carbon dioxide (17.08%), *α*-Bergamotene (2.96%), and Linalool (2.73%) (Table 4 S2). T2 was composed essentially of oxygenated monoterpenes (77%) and hydrocarbon sesquiterpenes (2%) (Fig. 2c). Ten compounds were identified in T3, being the primary compounds carbon dioxide (90.51%) and *α*-Bergamotene (4.91%) (Table 4 S2). This treatment presented the higher relative area (%) of carbon dioxide. The compounds identified in this treatment were mainly hydrocarbon sesquiterpenes (5%) (Fig. 2c).

A higher number of compounds was observed in T4 and T5. Eighteen volatile compounds were identified in T4, the highest variety in this study. The primary compounds were carbon dioxide (57.37%), *α*-Bergamotene (11.35%), Eucalyptol (6.73%), Linalool (4.26%), and *β*-Cubebene (2.54%) (Table 4 S2). In T4, the main compounds identified were categorized as hydrocarbon sesquiterpenes (14%) and oxygenated monoterpenes (10%) (Fig. 2c). T5 presented 16 chemical compounds, especially carbon dioxide (72.22%), Methyleugenol (13.73%), Eucalyptol (2.86%), Eugenol (2.43%), Linalool (2.25%), and *α*-Bergamotene (2.27%). Methyleugenol and α-Bulnesene were the major compounds (Table 4 S2). In this treatment, the predominant compounds were oxygenated monoterpenes (3%), hydrocarbon sesquiterpenes (4%), and others (10%) (Fig. 2c).

PCA represented 99.25 % of the variance for the compounds and 98.71% for the chemical groups (Fig. 2a-b). Two compounds stood out, methyl chavicol and carbon dioxide, which were under direct action in the treatments T1, T2, and T3, T4 and T5, respectively (Fig. 2a). The PCA of the chemical grouping reinforced the previous analysis because methyl chavicol is an oxygenated monoterpene and its synthesis reduced drastically in the other treatments. This result also confirmed the higher carbon dioxide liberation in T3, T4, and T5 (Fig. 2b).

Biochemical and enzymatic activities of leaves

In the biochemical analysis of the leaves, the nitrogen balance index (NBI) was higher in T5 (73.073). It increased compared with T1 in 4.7%, with T2 in 15.98%, with T3 in 31.74%, and with T4 in 27.25%. The higher means for the total chlorophyll index were verified in the control (17.81) and T5 (17.53). The control treatment was 35.56% higher than T4 and 64.37% than T3. T5 was 33.27% higher than T4 and 61.6% than T3 (Table 5 S3). The mean values were constant in all treatments regarding flavonoids, presenting no difference between them. However, regarding anthocyanins, T3 showed the highest mean (0.131), which was 35.13% higher than the control, which had the lowest result (0.1113) (Table 5 S3).

A significant variation was perceived in the enzyme activity due to the concentrations of MeJa and Zinc. The three enzymes acted distinctly in the remotion of reactive oxygen species (ROS). The enzymatic activity of ascorbate peroxidase was increased in T5 (8.2 g^{-1} FW min^{-1}), with a value of 11.2 and 90 times higher than the control treatment and T2 (Fig. 3a S2). Catalase presented high activity in T2 and T4. Nevertheless, in control, T3, and T5, there was a drastic reduction in this enzyme activity (Fig. 3b S2).

The superoxide dismutase, which is the first in the defense line against ROS, demonstrated higher activity in the control T1 (120.0 U SOD mg^{-1} FW min^{-1}). T2 and T3 presented the lower activity, which was twice and thrice lower than the control (Fig. 3c S2). Therefore, it was verified that, in at least one treatment, one enzyme acted more significantly to mitigate ROS damages, except for T3, which always presented low activity regardless of the enzyme evaluated.

Discussion

Common problems observed during *in vitro* establishment phase of basil cultivars are callus formation, hyperhydricity, abnormality, tissue, and adventitious roots oxidation (Trettel et al. 2020, Górski et al., 2021). Thus, these problems should be monitored because elicitors can induce or mitigate their occurrence depending on the concentration. Among these phenomena, hyperhydricity was significant in the cultivars "Alfavaca Verde" (Trettel et al., 2020) and 'Grecco a Palla' (Górski et al., 2021) in the presence of auxins and cytokinins. This problem is characterized by alterations in the structure of seedlings and sprouts due to the excess water absorbed inside the vacuole or between cells (Liu et al., 2017).

In this study, no seedling with this condition was evaluated. We associate this fact with the genetic constitution of the cultivar 'Dante' and the lack of cytokinin addition in the medium. This physiological disorder is more often observed when basil is cultivated with high growth regulators concentration, especially cytokinins such as Benzyl adenine (BAP) and varies according to the cultivar used (Monfort et al., 2018). It is essential to evaluate the physiological disturbances of basil cultivars in the in vitro system (Górski et al., 2021).

Normal plants have expanded leaves, without bends or twisted downwards, and a standard root system (Trettel et al., 2020). This condition may be reversed during the cultivation cycle, but it depends on the interaction of elicitors with the genotype and other conditions of cultivation. All treatments presented high abnormality rates in the first 30 days that decreased during the experiment. Verma et al. (2016) demonstrated that different $ZnSO_4$ concentrations could not morphologically modify *in vitro* cultivated basil. According to Trettel et al. (2020), during *in vitro* establishment, several factors may influence the growth and development of seedlings, such as the constitution of the growth medium, pH, presence of growth regulators, type of explant used, among others. These factors are directly related to the emergence of abnormal seedlings. The lower rate of abnormality was verified in the treatments with higher MeJa concentration, which is already known by promoting the growth of vegetal tissues and being involved in the synthesis of primary metabolites (Yu et al., 2019). Moreover, this elicitors is a methyl ester of the plant hormone Jasmonic acid (Wasternack and Hause, 2019), which is related to processes of plant morphological and physiological formation (Mahmood et al., 2012).

The amount of leaves produced by the plant is another essential characteristic for medicinal plants because this is the central place for essential oil synthesis in Lamiaceae, which is produced in capitate and peltate trichomes (dos Santos and Rodrigues, 2019). Trettel et al. (2018) suggest a direct relationship between the number of leaves produced and oil yield. Hyperhydricity and oxidation compromise the oil yield because such phenomena are more common in leaves. In the case of oxidation, it can also occur in calli or cell cultures. Among the elicitors tested, only T4 induced a number of leaves similar to the control. In the others, this characteristic was below the control. This result diverges from other cultivars. For instance, 25 µM $CuSO_4$ doses in the cultivar 'Basilicão verde' resulted in seedlings with more and larger leaves (Trettel et al., 2018). However, 25 µM $ZnSO_4$ doses in the cultivar 'Manolo' increased by 50% the number

of leaves compared with the medium without elicitors (Comar et al., 2019). In basil, it has been observed that the plants were responsive to cytokinins for leave induction, especially BAP because the balance between auxins and cytokinins provides the gene expression of leaf differentiation and cell multiplication (Bar and Ori, 2014).

The higher growth in the sprouts and the root was perceived in the treatment without elicitors. Seedlings whose culture media was supplemented with elicitors, nutrients, or hormones tend to adjust their metabolism and deviate their metabolic pathways to produce secondary compounds, causing nutritional unbalance, affecting growth (Monfort et al., 2018). Górski et al. (2021) also described how the medium without plant growth regulators presented seedlings with higher growth than the treatments with plant growth regulators, while using nodal segments of the basil 'Grecco a palla'. The authors explain that the seedling took profit from the endogenous dosage of hormones to establish roots primarily (Górski et al., 2021).

Another aspect evidenced in this research is that in the higher $ZnSO_4$ concentration (75 µM) and lower MeJa concentration, the seedlings presented the worst performance for growth characteristics. This fact indicates that they were stresses beyond what can be endured by seedlings of the cultivar 'Albahaca Dante'. MeJa had a protective role because it can activate the defense mechanisms of plants (Dar et al., 2015; Yu et al., 2019).

The T1 (0 µM MeJa and 0 µM $ZnSO_4$) and T2 (1 µM MeJa and 25 µM $ZnSO_4$) had the better growth characteristics, besides having the higher stomatal density. This fact indicates that the plants were subject to more favorable development conditions in these treatments because high stomatal density and lower sizes are characteristic of normal physiological conditions with low stress (Barbieri et al., 2012).

On the other hand, it was demonstrated that stress was responsible for reducing stomata per area in basil leaves (Barbieri et al., 2012; Jensen et al., 2018). Our results support those data because seedlings in T3 (1 µM MeJa and 75 µM $ZnSO_4$) and T5 (5 µM MeJa and 75 µM $ZnSO_4$) that presented the lower means for growth also showed a reduction in stomatal density and increased size. Reduced stomatal density is probably associated with the fact that basil slowly accumulates molecules and inhibition signs of stress, such as abscisic acid and proline (Barbieri et al., 2012). The presence of larger stomata can be considered an attempt by the plant to capture more oxygen to compensate for density reduction (Agami et al., 2016). Furthermore, another evaluation that supports this thesis is that the carbon dioxide content detected in T3 and T5 was higher than other treatments. Studies demonstrate that gas accumulation in these tissues served as a signal and stimulated density change, stomata size, and produced more reactive oxygen species (Chater et al., 2015). This occurrence may be related to the responses verified in these treatments and the higher respiration rate, which must be analyzed in further studies.

The different treatments used in this study widely differed regarding the nature of the volatile chemical compounds of the essential oil. T1 (0 µM MeJa and 0 µM $ZnSO_4$) and T2 (1 µM MeJa and 25 µM $ZnSO_4$) promoted better plant growth development and produced high levels of methyl chavicol. While T4 (5 µM MeJa and 25 µM $ZnSO_4$) presented the predominance of eucalyptol, linalool, eugenol, and α-bergamotene, T5 (5 µM MeJa and 75 µM $ZnSO_4$) had more eucalyptol, eugenol, and methyl eugenol. Secondary metabolites of plants derived from phenylalanine ammonia-lyase, including phenylpropanoids, are described by their high antioxidative activity (Nazir et al., 2019).

Eugenol and methyl eugenol already presented higher antioxidative activity in basil than methyl chavicol (de Araújo Couto et al., 2019). These authors also state that the eugenol production in the essential oil contributed to its antioxidative activity. The addition of 25 µM $CuSO_4$ in the growth medium also presented a 98.92% increase of methyl eugenol and 31.64% of eugenol compared with the control, resulting in a 77.71% higher production of phenylpropanoids in the basil 'Basilicão Verde' (Trettel et al., 2017).

These results indicate a reason for the high eugenol and methyl eugenol contents, besides the higher number of compounds produced in T4 and T5. It may have provided a stress tolerance caused by the higher concentration of MeJa in these treatments, while its low concentration elevated the production of methyl chavicol. Similar results were found by Złotek et al. (2016), whereas the use of 1µM MeJa in the growth medium promoted a 40.69 % increase of methyl eugenol in the essential oil of *O. basilicum*, while 100µM provided 20.88% of linalool and 24.88% of eugenol. Similarly, the expansion of the monoterpenes and sesquiterpenes synthesis in T3, T4, and T5 may be related to a need for more antioxidative compounds, as these treatments showed the worst performances.

Two physiological indexes stood out: the lower accumulation of nitrogen and chlorophylls in the plants with lower growth (T3). These occurrences may partially explain what was observed in these plants under high zinc concentrations. Tsonev and Cebola (2012) suggest that, in excess, zinc can interfere in the absorption of other nutrients in plants, decline and inhibition of the elongation biomass and cell division, and cause withering and necrosis in old leaves. In the same treatment, a higher presence of anthocyanins was perceived, probably related to oxidative stress control. This phenomenon was demonstrated for *Salvia sclarea* L. where the excess of Zn (900 µM) significantly increased the synthesis of total phenols and anthocyanins in leaves. These last ones had an essential role in Zn detoxification and protection against oxidative stress (Dobrikova et al., 2021).

The main antioxidative enzymes suffered significant alterations by the treatments. In T3, all enzymes presented low activity. Plants have two control mechanisms for oxidative stress: the non-enzymatic performed by the secondary compounds and vitamins and the control by antioxidative enzymes (Barbosa et al., 2014). The result in T3 reinforced that, in this situation, the plant invested in non-enzymatic mechanisms. On the other hand, the enzyme catalase, considered a key enzyme, rose in T2 and T4, while SOD increased in T1 and APX in T5.

SOD is considered the first defense line against ROS and catalyzes the dismutation of two O_2• radicals - generating H_2O_2 and O_2. However, CAT converts two molecules of H_2O_2, in water and oxygen, or oxidates substrates, such as methanol, ethanol, formaldehyde, and formic acid. On the other hand, APX acts on the cycle ascorbate-glutathione, in which the H_2O_2 formed by the action of SOD is reduced. APX and CAT are the two most important enzymes among the detoxification components of H_2O_2 in plants (Barbosa et al., 2014).

Table 1. Elicitors methyl jasmonate, zinc sulfate, and their concentrations used in *Ocimum basilicum* 'Albahaca Dante'.

Treatments	MeJa (µM)	Zinc Sulfate (µM)
T1	0	0
T2	1	25
T3	1	75
T4	5	25
T5	5	75

Table 2. Growth analysis of *Ocimum basilicum* 'Albahaca Dante' cultivated *in vitro* regarding the different concentrations of methyl jasmonate and zinc sulfate.

Treatments	Number of sprouts	Sprout length (mm)	Root length (mm)
T1	2.25 ± 0.45^{a}	108.009± 10.5^{a}	104.026 ± 18.46^{a}
T2	2.13 ± 0.83^{a}	98.496 ± 13.88^{ab}	97.031 ± 30.916^{ab}
T3	1.57 ± 0.7^{b}	82.677 ± 17.66^{bc}	85.386 ± 22.97^{b}
T4	1.75 ± 0.577^{ab}	94.93 ± 17.001^{ab}	83.363 ± 15.133^{b}
T5	1.25 ± 0.447^{b}	76.555 ± 22.51^{c}	93.918 ± 14.227^{ab}
Treatments	Sprout fresh mass (g)	Root fresh mass (g)	Root dry mass (g)
T1	4.656 ± 0.81^{ab}	3.636 ± 1.56^{a}	0.336 ± 0.082^{a}
T2	5.634 ± 1.815^{a}	5.818 ± 2.702^{a}	0.347 ± 0.076^{a}
T3	3.795 ± 1.59^{bc}	3.45 ± 1.21^{b}	0.226 ± 0.087^{b}
T4	4.295 ± 0.723^{abc}	6.418 ± 1.061^{a}	0.290 ± 0.069^{ab}
T5	3.302 ± 1.017^{c}	6.424 ± 1.133^{b}	0.217 ± 0.057^{b}

*Means followed by the same letter in the column do not differ by the Tukey's test at 5%. T1) MeJa 0 µM and $ZnSO_4$ 0 µM; T2) MeJa 1 µM and $ZnSO_4$ 25 µM; T3) MeJa 1 µM and $ZnSO_4$ 75 µM; T4) MeJa 5 µM and $ZnSO_4$ 25 µM; T5) MeJa 5 µM and $ZnSO_4$ 75 µM.

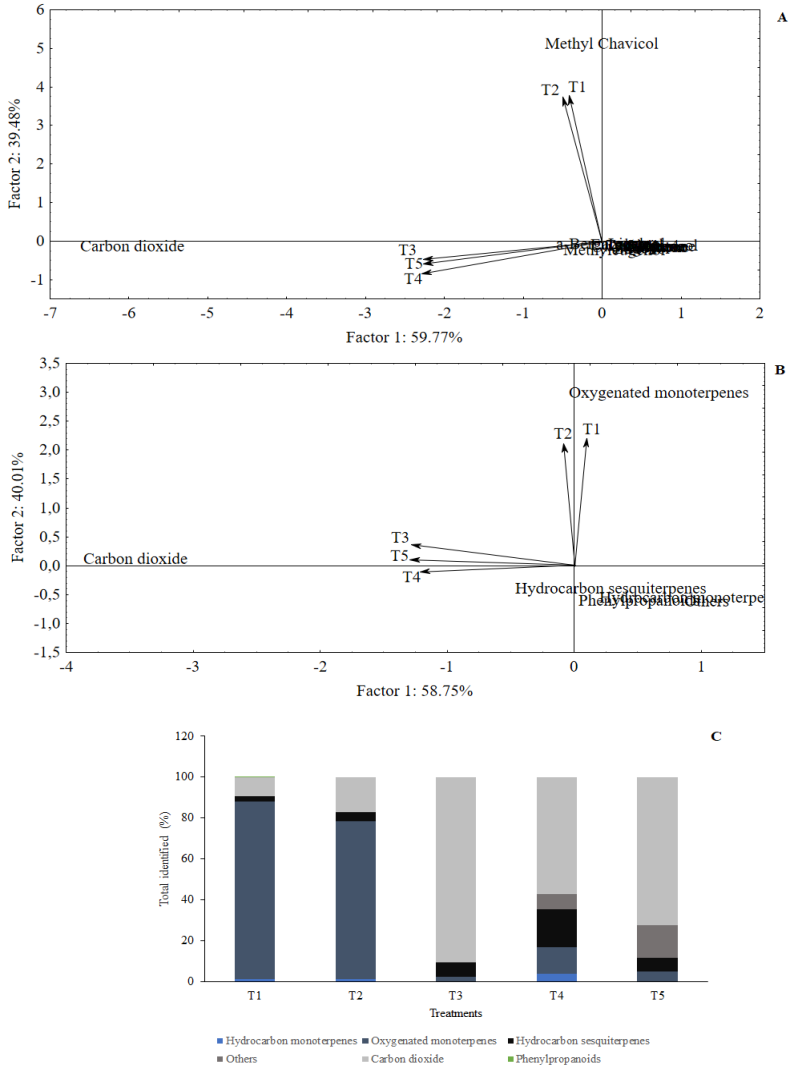

Fig 2. Principal components analysis of the chemical composition (A) and chemical groups (B), and the percentage of the chemical groups identified in *Ocimum basilicum* 'Albahaca Dante' leaves cultivated *in vitro* regarding the different concentrations of methyl jasmonate and zinc sulfate. T1) MeJa 0 µM and $ZnSO_4$ 0 µM; T2) MeJa 1 µM and $ZnSO_4$ 25 µM; T3) MeJa 1 µM and $ZnSO_4$ 75 µM; T4) MeJa 5 µM and $ZnSO_4$ 25 µM; T5) MeJa 5 µM and $ZnSO_4$ 75 µM.

Table 3. Stomatal morphometry of the adaxial and abaxial surfaces of *Ocimum basilicum* 'Albahaca Dante' cultivated *in vitro* regarding the different concentrations of methyl jasmonate and zinc sulfate.

Treatments	Adaxial surface	Abaxial surface	Adaxial surface	Abaxial surface
	Density (mm²)		Pore opening (µm)	
T1	9.18 ± 1.93^{a}	14.89 ± 5.64^{b}	2.77 ± 0.79^{ab}	2.9 ± 2.17^{b}
T2	9.12 ± 1.87^{a}	20.35 ± 4.73^{a}	2.99 ± 0.83^{a}	4.59 ± 1.82^{a}
T3	9.31 ± 1.86^{a}	0.53 ± 0.14^{c}	2.94 ± 0.96^{a}	4.75 ± 1.89^{a}
T4	6.68 ± 0.85^{b}	1.88 ± 0.55^{c}	2.47 ± 0.5^{ab}	4.28 ± 1.63^{ab}
T5	6.56 ± 1.00^{b}	0.66 ± 0.12^{c}	2.18 ± 0.45^{b}	3.45 ± 1.11^{ab}
	Guard cell (µm)		Guard cell (µm)	
	Length	Length	Width	Width
T1	17.62 ± 2.17^{b}	13.89 ± 8.7^{c}	9.14 ± 1.64^{b}	6.31 ± 4.35^{b}
T2	20.31 ± 3.35^{a}	22.07 ± 3.42^{ab}	9.74 ± 1.5^{ab}	12.14 ± 2.29^{a}
T3	19.21 ± 1.87^{ab}	25.32 ± 3.22^{a}	10.72 ± 1.5^{a}	12.72 ± 2.54^{a}
T4	19.9 ± 2.41^{a}	19.79 ± 4.52^{ab}	9.00 ± 1.4^{b}	11.97 ± 2.19^{ab}
T5	19.93 ± 2.24^{a}	16.34 ± 2.77^{b}	9.07 ± 0.92^{b}	10.03 ± 1.67^{ab}
	Subsidiary cell (µm)		Ratio DL/DE guard cell	
T1	30.21 ± 1.8^{ab}	22.11 ± 13.28^{c}	2.95 ± 0.5^{a}	3.33 ± 0.31^{a}
T2	32.66 ± 4.09^{a}	35.78 ± 3.65^{a}	2.88 ± 0.37^{a}	3.48 ± 0.3^{a}
T3	29.81 ± 2.56^{b}	37.89 ± 4.48^{a}	2.8 ± 0.37^{b}	2.91 ± 0.42^{a}
T4	29.79 ± 3.05^{b}	33.22 ± 4.9^{ab}	2.66 ± 0.28^{ab}	3.19 ± 0.46^{a}
T5	30.21 ± 2.48^{ab}	28.19 ± 3.29^{bc}	2.78 ± 0.27^{a}	3.43 ± 0.23^{a}

*Means ± standard deviation followed by the same letter in the column do not differ by the Tukey test at 5%. T1) MeJa 0 µM and $ZnSO_4$ 0 µM; T2) MeJa 1 µM and $ZnSO_4$ 25 µM; T3) MeJa 1 µM and $ZnSO_4$ 75 µM; T4) MeJa 5 µM and $ZnSO_4$ 25 µM; T5) MeJa 5 µM and $ZnSO_4$ 75 µM.

Table 4. Chemical composition of the essential oil obtained from leaves of *Ocimum basilicum* 'Albahaca Dante' cultivated *in vitro* regarding the different concentrations of methyl jasmonate and zinc sulfate.

Peak	TR	[a]Compound	[b]IRlit	T1	T2	T3	T4	T5	Identification methods
				Relative area (%)					
1	1.398	Carbon dioxide		8.94	17.08	90.51	57.37	72.22	a. b. c
2	7.410	*α*-Pinene	932	0.37	0.38	t	1.04	t	a. b. c
3	8.734	Sabinene	969	-	-	-	1.66	-	a. b. c
4	9.274	*β*-Pinene	974	0.36	0.41	-	0.39	t	a. b. c
5	10.565	Eucalyptol	1026	1.43	1.88	1.41	6.73	2.86	a. b. c
6	11.222	*β*-Ocimene	1044	0.26	-	-	-	-	a. b. c
7	11.543	*γ*-Terpinene	1054	0.39	0.50	t	0.80	t	a. b. c
8	13.036	Linalool	1095	0.56	2.73	1.04	4.26	2.25	a. b. c
9	15.714	Terpinen-4-ol	1174	0.48	0.76	t	1.75	t	a. b. c
10	16.814	Methyl Chavicol	1195	84.03	71.71	-	-	-	a. b. c
11	19.421	Bornyl acetate	1287	0.26	-	-	-	-	a. b. c
12	21.823	Eugenol	1356	-	-	-	3.42	2.43	a. b. c
13	22.882	*β*-Cubebene	1387	0.70	0.77	1.24	2.54	1.3	a. b. c
14	23.392	*β*-Elemen	1389	-	0.34	-	0.97	0.82	a. b. c
15	24.273	Methyl eugenol	1403	-	-	-	3.83	13.73	a. b. c
16	24.790	*α*-Bergamotene	1432	2.00	2.96	4.91	11.35	2.27	a. b. c
17	25.644	Humulene	1436	0.20	t	-	0.58	0.49	a. b. c
18	25.658	*γ*-Elemene	1434	-	-	t	0.68	-	a. b. c
19	25.760	cis-*β*-Farnesene	1440	-	-	-	0.59	t	a. b. c
20	26.397	*α*-Bulnesene	1509	-	-	-	0.52	0.7	a. b. c
21	23.934	*δ*-Cadinene	1522	-	0.44	0.86	1.49	0.84	a. b. c
		Total identified		99.98	99.96	99.97	99.97	99.91	
		Hydrocarbon monoterpenes		1.38	1.29	-	3.89	-	
		Oxygenated monoterpenes		86.5	77.08	2.45	12.74	5.11	
		Hydrocarbon sesquiterpenes		2.9	4.51	7.01	18.72	6.42	
		Phenylpropanoids		-	-	-	7.25	16.16	
		Carbon dioxide		8.94	17.08	90.51	57.37	72.22	
		Others		0.26	-	-	-	-	

[a]Compounds listed in the elution order of the column HP-5; [b]IRlit: Retention index calculated using a homologous series of n-alkanes C9 – C30 in the column HP-5; [c]IR: Identification based on the mass spectra using NIST 11.0 library; Relative area (%): Percentage of the area occupied by the compounds in the chromatogram; t = traces of compounds in the sample; (-): absence of compounds in the sample; TR: time of retention; T1) MeJa 0 µM and $ZnSO_4$ 0 µM; T2) MeJa 1 µM and $ZnSO_4$ 25 µM; T3) MeJa 1 µM and $ZnSO_4$ 75 µM; T4) MeJa 5 µM and $ZnSO_4$ 25 µM; T5) MeJa 5 µM and $ZnSO_4$ 75 µM.

Table 5. Physiological and biochemical analyses of *Ocimum basilicum* 'Albahaca Dante' leaves cultivated *in vitro* regarding the different concentrations of methyl jasmonate and zinc sulfate. Nitrogen balance index (NBI), total chlorophyll index (Chl), total flavonoids (fla), and total anthocyanins (Anth).

Treatments	NBI	Chl	Fla	Anth
T1	69.791 ± 19.891^{ab}	17.817 ± 5.701^{a}	0.2443 ± 0.059^{a}	0.1113 ± 0.037^{b}
T2	63.0 ± 25.604^{ab}	15.156 ± 5.935^{ab}	0.251 ± 0.073^{a}	0.125 ± 0.042^{ab}
T3	55.465 ± 21.766^{b}	10.839 ± 4.517^{c}	0.235 ± 0.041^{a}	0.15 ± 0.039^{a}
T4	57.421 ± 20.278^{ab}	13.143 ± 3.412^{bc}	0.213 ± 0.053^{a}	0.131 ± 0.033^{ab}
T5	73.073 ± 18.581^{a}	17.56 ± 3.121^{a}	0.219 ± 0.038^{a}	0.122 ± 0.037^{ab}

*Means followed by the same letter in the column do not differ from each other by the Tukey's test at 5%. T1) MeJa 0 µM and $ZnSO_4$ 0 µM; T2) MeJa 1 µM and $ZnSO_4$ 25 µM; T3) MeJa 1 µM and $ZnSO_4$ 75 µM; T4) MeJa 5 µM and $ZnSO_4$ 25 µM; T5) MeJa 5 µM and $ZnSO_4$ 75 µM.

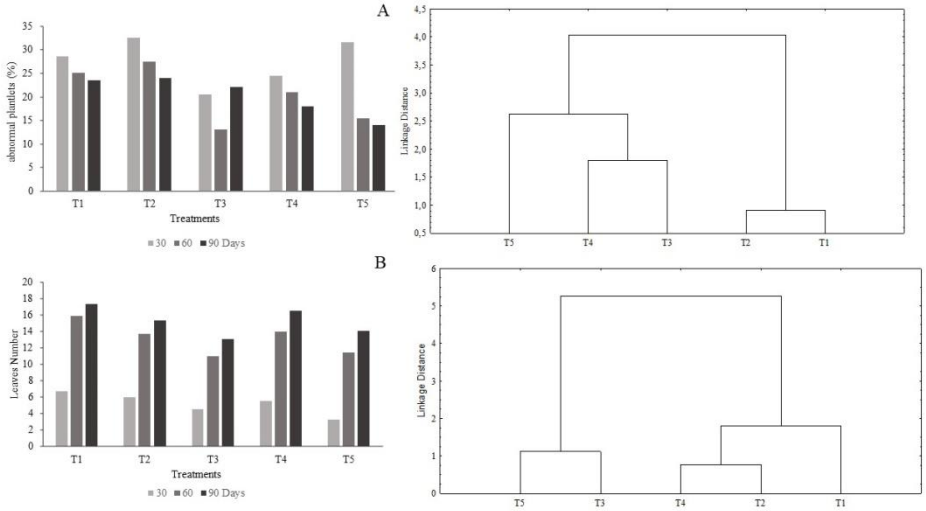

Fig 1. Percentage of abnormal seedlings, dendrogram (A), and average number of leaves (B) of *Ocimum basilicum* 'Albahaca Dante' cultivated *in vitro* regarding the different concentrations of methyl jasmonate and zinc sulfate. T1) MeJa 0 µM and $ZnSO_4$ 0 µM; T2) MeJa 1 µM and $ZnSO_4$ 25 µM; T3) MeJa 1 µM and $ZnSO_4$ 75 µM; T4) MeJa 5 µM and $ZnSO_4$ 25 µM; T5) MeJa 5 µM and $ZnSO_4$ 75 µM.

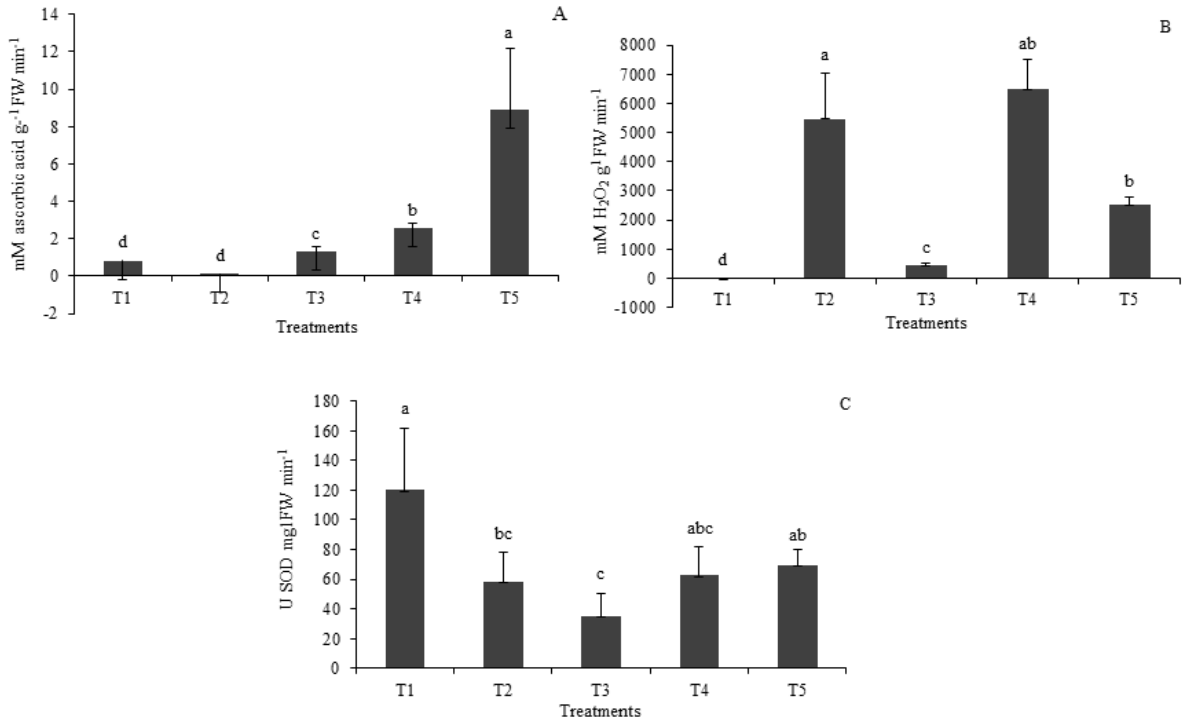

Fig 3. Enzymatic activity of ascorbate peroxidase (APX - A), catalase (CAT - B), and superoxide dismutase (SOD - C) of *Ocimum basilicum* 'Albahaca Dante' leaves cultivated *in vitro* regarding different concentrations of methyl jasmonate and zinc sulfate
T1) MeJa 0 µM and $ZnSO_4$ 0 µM; T2) MeJa 1 µM and $ZnSO_4$ 25 µM; T3) MeJa 1 µM and $ZnSO_4$ 75 µM; T4) MeJa 5 µM and $ZnSO_4$ 25 µM; T5) MeJa 5 µM and $ZnSO_4$ 75 µM.

Material and Methods

The vegetal material and sterilisation

The experiments were conducted in the Vegetal Tissues and Molecular Biology laboratories of the Universidade Paranaense. Seeds of the cultivar *Ocimum basilicum* L. 'Albahaca Dante' (Feltrin seeds, Farroupilha-RS, Brasil®) lot n. 7158/20, 96% germination percentage, were inoculated. The seeds were sterilized according to (Trettel et al., 2018).

Four seeds of *O. basilicum* L., 'Albahaca Dante' were inoculated in glass flasks of 350 mL with an MS medium (Murashige and Skoog, 1962) in its complete concentration, totaling 200 flasks per treatment, which were supplemented with 30 g L^{-1} sucrose and 6.5 g L^{-1} agar with pH adjusted for 5.8 (Trettel et al., 2018). They were kept in a growth room with a 24-hour photoperiod provided by light emitter diodes (LED) lamps Blumenau®, LED T8 10W 6.000K, 100-240V-50/60Hz, power factor: ≥ 0.92 (High FP), at a temperature of 25ºC± 2 ºC, and luminous intensity of 72.02 µmol $m^{-2} \cdot s^{-1}$ (Trettel et al., 2020). After 90 days of *in vitro* cultivation, the seedlings were used as explant donors.

Nodal segments cultivation

The explants nodal segments with an average of 1 cm and two leafless buds were inoculated in an MS medium (Murashige and Skoog, 1962) as previously described. The concentrations of elicitors used are presented in Table 1 and based on Złotek et al. (2016) and Comar et al. (2019).

Growth analysis

After the experiment implantation, evaluations of the physiological disorders were made at 30, 60, and 90 days of *in vitro* cultivation. In each period, they were measured visually regarding hyperhydricity, abnormal plants, and the number of leaves. These disorders were based according to (Górski et al., 2021).

At the end of the 90 days, the number of leaves, number of sprouts, sprout length, root length, sprout fresh mass, root fresh mass, and root dry mass were evaluated according to (Trettel et al. 2018; Górski et al., 2021).

Stomata analysis

Stomata counting was conducted following the methodology described by Segatto et al. (2004). The following variables were measured in the adaxial and abaxial surfaces of leaves: stomatal density (mm^2), stomatal opening (µm), longitudinal diameter of the guard cells (µm), equatorial diameter (µm), and the ratio between the longitudinal and equatorial diameter of the guard cell.

Plant material images were obtained with an optical microscope (Olympus BX-60®, Tokyo, Japan) (10x and 40x lens) through the software Motic Images Plus 2.0 (Hong Kong). Stomatal cell counting in each area (scale of 100 µm) was made in 10 random leaf areas. These analyses were triplicated.

Chemical characterization of volatile compounds

The chemical compounds were obtained by the headspace technique (GCMS-QP2010 - Ultra Shimadzu headspace gas chromatograph-mass spectrometer) in which 2.0 g of fresh basil leaves after 90 days of cultivation were introduced in vials (glass flask, type 'vial') of 20 mL. The leaves were incubated for 20 min at 150°C and agitated at 550 rpm (1 min of cycle one, 10 s of cycle off), and the syringe temperature was adjusted to 100°C (Agilent CTC PAL Control). The volatile compounds were characterized with a gas chromatograph (model Agilent 7890B) attached to a mass spectrometer (Agilent 5977A MSD) GC-MS, equipped with the column HP-5MS UI Agilent with the 5% phase of phenylmethyl siloxane (30.0 m x 250 µmd.i. x 0.25 µm of film thickness), with an automatic injector (CTC PAL Control).

For the adequate separation of analytes in the system CG-EM, 1.5 mL of the volatile phase was injected in the column using the injection mode Split in the ratio 1:50 with the following oven conditions: initial temperature of 40°C followed by an increment of 4°C min^{-1} until 300 °C. Helium (He, purity 99.99%) was used as the carrier gas, and its flow rate was 1 mL min^{-1} at the constant pressure of 80 kPa. The injector and transference line temperatures were 260 and 280 °C. The MS detection system was made in the "scan" mode with a mass/load ratio of 40 – 550 m/z and a solvent delay of 3 min. The ionization was made by an electronic impact of 70 eV, with an ionization source temperature of 230 °C and a quadrupole of 150°C. Data collection was made through the program MassHunter, and the individual compounds were identified, comparing their mass specters with the libraries NIST 11 and WILEY 275 (http://www.sisweb.com/software/ms/wiley.htm). Mass Spectral Libraries (NIST 20 and Wiley Libraries) Identify unknowns from EI (GC/MS), and MS/MS spectra. (www.sisweb.com).

Physiological and biochemical evaluation

Nitrogen balance index (NBI), relative chlorophyll index (RCI), relative flavonoids index (RFI), and relative anthocyanins index (RAI) were measured from fresh leaves and obtained with the tool DUALEX SCIENTIFIC+TM model Quick Start (FORCE A®, France, Paris) following the manufacturer's instructions. Six replications were evaluated per treatment in the first expanded leaves.

Determination of enzymatic activity: superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT)

To obtain the enzymatic extract, 200 mg of fresh leaf tissue were macerated in liquid nitrogen, and the extract was obtained following Bonacina et al. (2017). The activity of the enzyme superoxide dismutase (SOD) (EC 1.15.1.1) was based on the enzyme capacity to inhibit the photoreduction of nitro blue tetrazolium (NBT) (Giannopolitis and Ries, 1977). The readings were conducted at 560 nm with a temperature of 28 °C per 10 min in triplicates.

The determination of the ascorbate peroxidase (APX) (EC 1.11.1.11) was based on Nakano and Asada (1981), and the activity was determined by the degradation of H_2O_2 in a 1-minute interval at 290 nm. Enzymatic activity was quantified using the molar extinction coefficient of 2.8 mM^{-1} cm^{-1}, expressed in mmol ascorbic acid g^{-1} MF min^{-1}.

Catalase (CAT) (EC 1.11.1.6) was determined following the method proposed by Havir and McHale (1987), based on the degradation of H_2O_2 in a 1-minute interval at 260nm. The enzymatic activity was quantified using the molar extinction coefficient of 36 M^{-1} cm^{-1} (Anderson et al., 1995) expressed in mmol H_2O_2 g^{-1} MF min^{-1}.

All enzymes were evaluated with the ELISA Spectra Max Plus 384® spectrophotometer (Molecular Devices, San Jose, CA, USA), using microplates of 96 wells with a flat bottom. The absorbance was registered using a spectrophotometer (UV-VIS Spectra Max Plus) with SoftMax Pro 6.5.1.

Experimental design and statistical analysis

The experiment was conducted in a completely randomized design (CRD). Five treatments were evaluated based on the combination of MeJa x Zinc Sulfate; each treatment had four repetitions of 50 flasks, which was shown in Table 1. The principal components analysis was used for the chemical composition and groups data. However, dendrograms of Euclidean distance were made for the number of abnormal leaves and seedlings (%), using Ward's method. Both analyses were carried out in the software Statistica 13.3 (Statsoft, 2017).

Growth, enzymatic, biochemical, and physiological activities data were first submitted to the Shapiro Wilk normality test. Then, the data were submitted to the analysis of variance at p≤ (0.05), and the means were compared by the Tukey test (p≤0.05) using SISVAR 5.6 (Ferreira, 2011).

Conclusion

Plants of the 'Albahaca Dante' basil cultivar developed well even in a medium with no addition of elicitors or lower concentrations of zinc and MeJa. Under such conditions, the content of the compounds identified was lower, and methyl chavicol predominated. Stomata density was higher and their size smaller, indicating better conditions for the growth of this cultivar. MeJa presented an essential role in the defense mechanism of plants against oxidative damage, favoring compounds increment, especially monoterpenes and sesquiterpenes, and activating antioxidant enzymatic mechanisms. The higher amount of Zn associated with the lower concentration of MeJa resulted in smaller plants with smaller stomata and lower density. There was a tendency for higher carbon dioxide formation, indicating an increased respiration rate. In this situation, the plant invested in the production of non-enzymatic mechanisms, such as anthocyanins, and there was a drastic reduction in the activity of antioxidant enzymes.

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Conflict of interest

The authors declare that they have no conflict of interest.

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