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and Delmira da Costa Silva

Full Length Research Paper

Different levels of irradiance and plant regulators modify the leaf structure and essential oil production of *Lippia origanoides* H.B.K (Verbenaceae)

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The quality and quantity of secondary metabolites produced by plants can be influenced by biotic and abiotic factors, since the biochemical and physiological processes that coordinate the synthesis of these compounds are influenced by these. Plant regulators can also interfere with plant growth and terpene biosynthesis in aromatic species, which is why they are widely used in agriculture. *Lippia origanoides* H.B.K is an aromatic shrub that has glandular trichomes on the leaf, which secrete and accumulate essential oil used in the production of culinary seasonings and in the cosmetic and pharmaceutical industry. The objective of this research was to evaluate the effects of plant regulators associated with different levels of light availability in *L. origanoides* exposed to levels of 100, 50, 35 and 25% of irradiance. The increase in irradiance in plants treated with plant regulator caused changes in the structure and leaf dry biomass, density of glandular trichomes and essential oil content in the 100% full sun treatment. The treatments did not influence the chemical profile of the essential oil, which presented thymol as the major component. However, the application of bioregulators in full sun increased the production of essential oil demonstrating an excellent option for increasing the production.

Keywords: Aromatic plant, shading, biorregulador vegetable, leaf anatomy.

INTRODUCTION

Plants are important sources of bioactive substances with high demand and interest in exploration for new drug discovery (Newman and Cragg, 2016). Aromatic plants the quantity and concentration of secondary metabolites produced suffer interference from environmental factors, which may influence the course of physiological processes by changing the plant metabolism (Bourgaud

et al., 2001; Rezaie et al., 2020).

Acclimation related plasticity in situations differentiated luminous intensity availability can change photosynthetic apparatus, in order to increase the ability of carbon assimilation and promote growth (Alvarenga et al., 2005). Growth is changed according to the availability of light radiation (Miralles et al., 2011) and in aromatic plants can

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significantly alter the production of essential oil (Fernandes et al., 2013).

Vegetable regulators, synthetic substances similar to plant hormones influence the response of plants to different environmental conditions, interfering in the morphology, physiology, growth and production (Shukla and Faoroqi, 1990). The use of plant regulators is a practice widely used agricultural (Khan et al., 2020). Seeking the development of new agricultural management techniques vegetable regulators have been used for the purpose of increasing production and improving the quality of the oil. The responses to the effects of these plant regulators on essential oil production have been highly variable between aromatic species.

Lippia origanoides H.B.K are aromatic shrubs native to Central America and the northern region of South America. They are raw materials for the production of culinary seasonings and essential oils enriched in phenylpropanoids that are useful in cosmetic and pharmaceutical industries (Hernandes et al., 2017; Ortiz et al., 2017; Stashenko et al., 2018). In the ontogeny of the vegetable organs, phytohormones as the cytokinins have key role in the regulation of cell division and the formation of the photosynthetic apparatus (Zubo et al., 2008). This phytohormones may still suffer influence of physiological responses of plant interfered in the accumulation of monoterpenes are typical of essential oil (Cappelari et al., 2019).

The aim of this study was to determine the effects of a biostimulant made up of 90 mg, 50 mg of kinetin indole butyric acid and gibberellic acid 50 mg per litre of the product, with the availability of light radiation on biomass in foliar morphology, anatomy and in the production of essential oil in *L. origanoides*.

MATERIALS AND METHODS

Plant material and growing conditions

The experiment was conducted on the campus of the Universidade Estadual de Santa Cruz (UESC), Ilhéus, Bahia, Brazil (14°47'00"S, 39°02'00"W). Meteorological data were obtained through the Meteorological Database of the National Institute of Meteorology (INMET), presenting temperature averages of 26°C, 41 mm of precipitation and 86% of relative humidity.

Plants obtained by vegetative propagation from cuttings, were transplanted to pots containing substrate ratio 4:1 (soil: sand). In the luminous radiation treatments were used black plastic screens of type 'sombrite', set out in individual frames of wood, constituting individual environments for each level of light, under field conditions. The values of photosynthetically active radiation (PAR) were obtained at intervals of 1 h of 07:00 am to 06:00 pm through a quantum sensor BQM-SUN (Apogee Instruments, Inc., USA) in full sun and in every environment.

Three applications were held biostimulant made up of 90 mg of kinetin (Kt), 50 mg of indole butyric acid (IBA) and 50 mg of gibberellic acid (GA₃) per liter of product. The sprays were carried out by foliar route at 30, 50 and 70 days after transplanting. The treatments were constituted by plants treated and not treated with plant regulators, submitted to different levels of light. These being:

full sun + biostimulant (T1), full sun without biostimulant (T2), 50% of full sun + biostimulant (T3), 50% of full sun without biostimulant (T4), 35% of full sun + biostimulant (T5), 35% of full sun without biostimulant (T6), 25% of full sun + biostimulant (T7), 25% of full sun without the biostimulant (T8).

Growth measurements

The growth variables were evaluated in five plants per treatment, at the end of the experiment, totaling 40 plants. Number of leaves (NL) was quantification; the dry biomass of root (RDB), stems (SDB), leaves (LDB) and total biomass (TB) were obtained by drying at 75°C in a circulating air oven until constant biomass was reached. Leaf area (LA) was obtained using an LI-3100 Area Meter (Li-Cor) and 30 millimeter ruler was used to determine the height of the plants (HP) and stem diameter (SD), just above ground level, with aid of digital caliper.

Production and chemical composition of essential oil

The essential oil was extracted from the dried leaves by the hydrodistillation using a Clevenger apparatus, with all dry biomass of the plant in 1500 mL of distilled water for 90 min, with five repetitions for treatment. The essential oil was separated from the high grade using dichloromethane and then dried with anhydrous sodium sulphate (in excess) and concentrated. The content was determined based on the volume extracted per 100 g dry biomass leaf (% w/v). Essential oil production was obtained by total biomass of oil extracted from the leaves of each treatment (g plant⁻¹).

The chemical composition of essential oil was analyzed performed by gas chromatography using Varian Saturn 3800 (Varian Palo Alto, CA) equipped with a flame ionization detector (GC-FID) and VF-5ms capillary column (30 m × 0.25 mm × 0.25 µm film thickness), using helium as the carrier gas at a flow rate of 1.0 mL min⁻¹. The injector and detector temperatures were 250 and 280°C, respectively. The column temperature program began at 60°C, increased by 8°C min⁻¹ to 240°C for 5 min. One milliliter of a 10% solution of oil in chloroform was injected in the 1:10 split mode. The concentration of the chemical constituents was calculated through the area of their respective peaks in relation with the total area of all the constituents of the sample. Qualitative analysis of the oils was performed out using a mass spectrometer (Agilent 5975C) triple quadrupole and ZB-624 capillary column (20 m × 0.18 mm × 1.00 µm). The injector temperature was 250°C. The column temperature program began at 50°C, increased by 8°C min⁻¹ to 240°C for 10 min. The operation mode were electronic impact at 70 eV, using helium as gas the carrier, with electronic injection of 0.1 µL. The chemical constituents were identified by computer comparing with the apparatus library and literature, and linear retention indices of Kováts (IK) were calculated by injecting a series of *n*-alkanes (C₈-C₂₆) under the same chromatographic conditions as used for the samples.

Leaf anatomy

Fragments of leaves third node, with three repetitions per treatment, were fixed in 2.5% glutaraldehyde (v/v), embedded in resin, and transverse cross-sections 5 µm thick were obtained with a microtome, stained with Toluidine Blue O and imaged under light microscopy Leica, model ICC50HD. Were measurements thickness of the adaxial (EDA) and abaxial (EBA) surface of the epidermis, palisade (PP) and spongy parenchyma (SP) and mesophyll using the *Leica application suite* software V3.

For analysis of surfaces fragments of leaves fixed in 2.5%

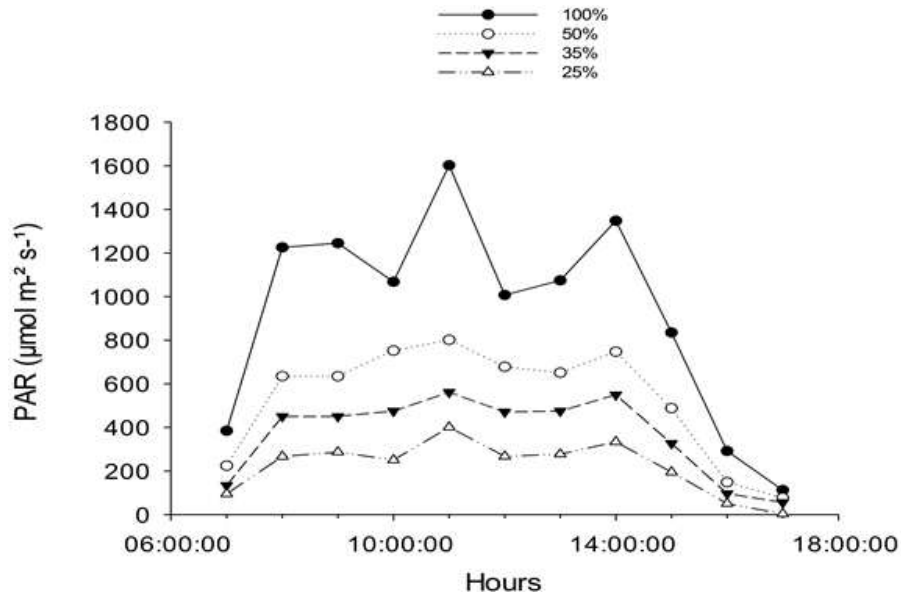


Figure 1. Diurnal course of photosynthetically active radiation (PAR) in $\mu\text{mol m}^{-2} \text{s}^{-1}$, on T1 and T2 (full sun – 100%) and in different environments of light radiation attenuation. Ilhéus, Bahia, Brazil, averages six days of occasional measurements during the experimental period (March-may 2013). Source: Authors

glutaraldehyde (v/v), were dehydrated in ethanol series, covered with gold and observed in scanning electron microscope Quanta 250 model. Density of trichomes and stomata were determined quantified the number of structures for mm^2 .

Experimental design

The experimental was conducted following a completely randomized design, in split-plot array with two conditions: Plants treated and not treated with plant regulators, submitted to different levels of light. These being: Full sun + biostimulant (T1), full sun without biostimulant (T2), 50% of full sun + biostimulant (T3), 50% of full sun without biostimulant (T4), 35% of full sun + biostimulant (T5), 35% of full sun without biostimulant (T6), 25% of full sun + biostimulant (T7), 25% of full sun without the biostimulant (T8), and five replications per treatment, ($n = 40$ plants). The data were examined by analysis of variance (ANOVA, F -test). For the treatment averages, polynomial regression models were adjusted using Sisvar 5.3 (Ferreira, 2010). Tukey tests were used to assess the average values within qualitative treatments. Significance was defined as $P \leq 0.05$.

RESULTS AND DISCUSSION

Photosynthetically active radiation (PAR) daily average for the environment to full sun (T1 and T2) and other treatments presented maximum values of 1600, 800, 561 and $400 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively to 11:00 h (Figure 1).

L. origanoides cultivated under different light levels and application of biostimulant presented changes in growth. The average values dry biomass of root and aerial part suffered influence only of light radiation levels. Plants

exposed to full sun condition accumulated more biomass in root and those cultivated to 25% of full sun presented increased in dry biomass of stem (Figure 2A and B).

Plant species grown at different intensities and quality of the incident light spectrum may undergo changes related to biomass production, biometric characteristics and metabolite production (Kunz et al., 2020), which occur as result of changes in carbon allocation to the different organs (Valladares and Niinemets, 2008).

Dry biomass of plants that received the application of growth regulators to 100% of full sun was about 40% larger than plants that received 25% of full sun (Figure 3A to B).

The levels and proportions of hormones in the composition of growth regulators influence the efficiency of these in the plant physiology, also as in carbon allocation (Castro and Vieira, 2001; Andrade et al., 2018), which may explain the influence only of the observed light radiation availability and production of biomass and essential oil. The ability to grow rapidly when shaded is a mechanism of acclimatization of the plants with a strategy to escape shading, can be attributed to a greater investment in cell elongation (Osunkoya and Ash, 1991; Moraes Neto et al., 2000).

Number of leaves increased linearly with increasing light levels, however, leaf area, plant height and stem diameter decreased linearly (Figure 4A to D).

The increase in leaf area with add to shading is one ajuste to increase the photosynthetic surface and ensure greater use of low light intensities (Lambers et al., 2008; Liu et al., 2019). According to Passos (1997), the higher

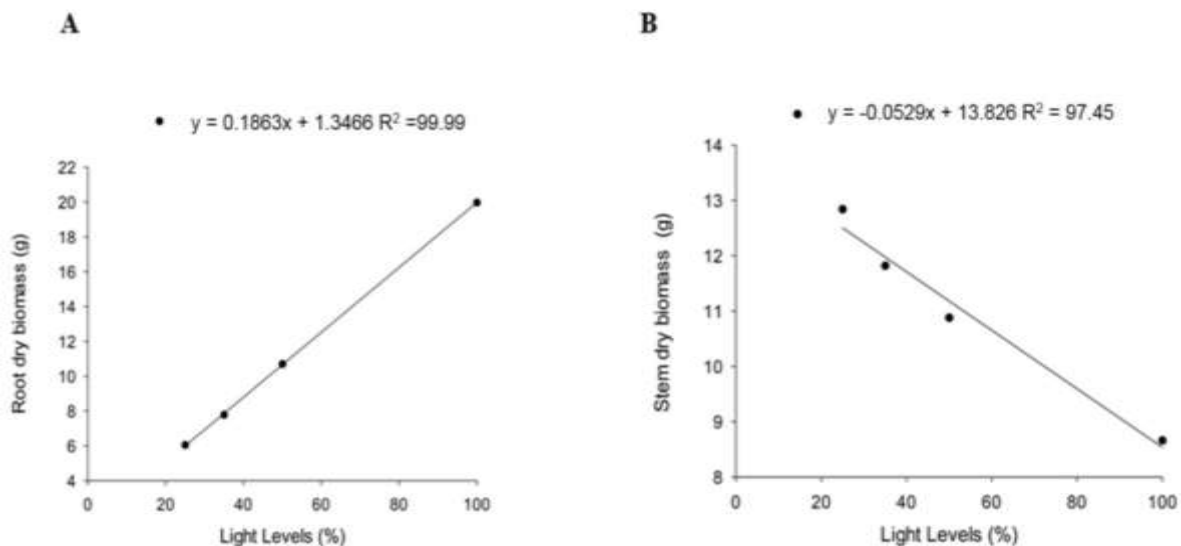


Figure 2. Plant growth variables of *L. origanoides* grown at different light levels. Root dry biomass (A); Stem dry biomass (B).

Source: Authors

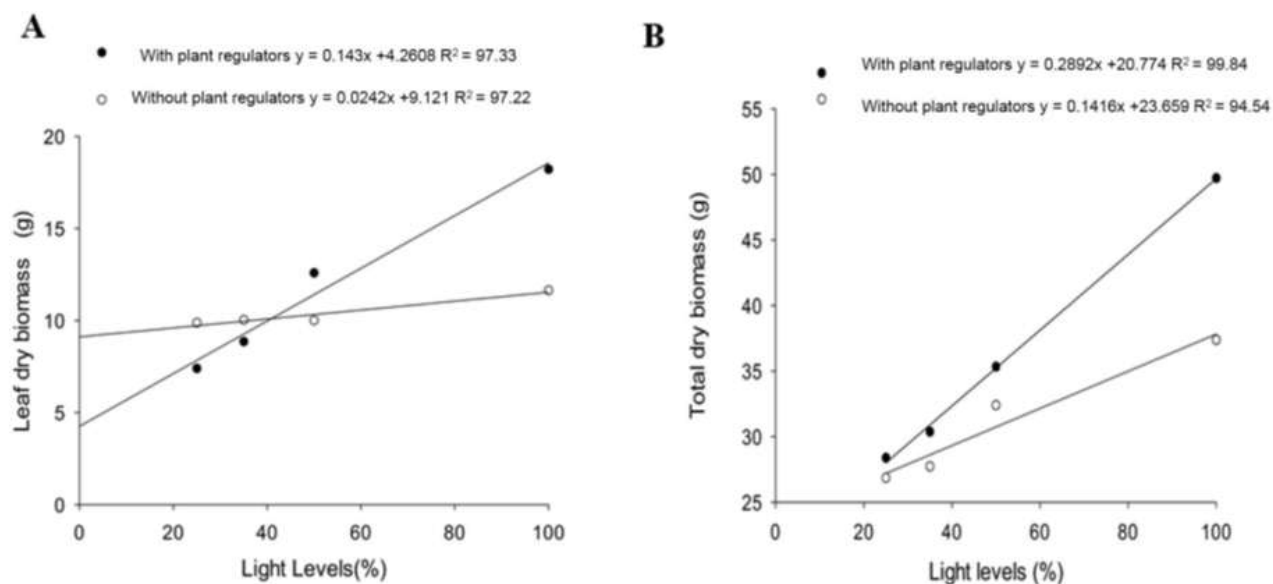


Figure 3. Plant growth variables of *L. origanoides* grown at different light levels and plant regulators. Leaf dry biomass (A); Total dry biomass (B).

Source: Authors

the number, the bigger the leaf CO_2 assimilation and consequently greater production of essential oil. *O. basilicum* plants treated with growth regulators increased the total number of leaves (Shedeed et al., 1990), differed from results observed in this study where there was no significant variation in the number of leaves in plants treated with growth regulators.

Due its composition (90 mg Kt, 50 mg IBA and 50 mg

GA_3 per liter of the product) the biostimulant used can increased growth and development plant by stimulating cell division and increase the uptake of water and nutrients by plants, resulting in increment total dry biomass (Vieira and Castro, 2004). The increased dry biomass of leaves is thus related the elongation of the palisade parenchyma cells when exposed to full sun with application of the biostimulant. According to Romero et

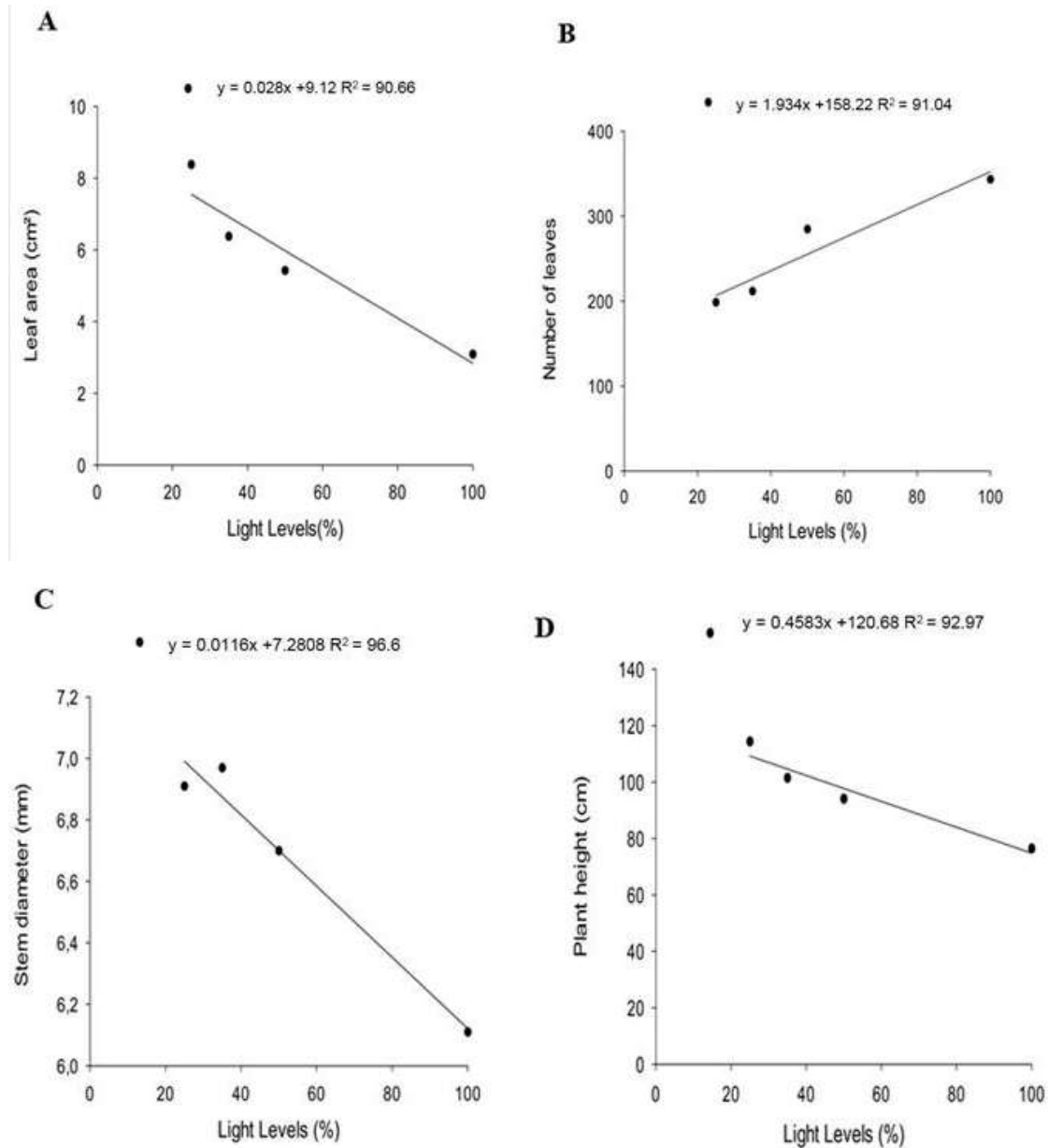


Figure 4. Plant growth variables of *L. origanoides* at different light levels. Leaf area (A), Number leaf (B), Stem diameter (C) and Plant height (D).

Source: Authors

al. (2002), this occurs by increased cell divisions and variation in the pattern of cell expansion. The application of GA₃ increased the dry weight of leaves from *Cymbopogon jwarancusa* (Ansari et al., 1988), dry biomass of leaves and consequently total dry biomass of *Citrus amblycarpa* (Leonel et al., 1994) and increased fresh (85.85%) and dry biomass (82.84%) in Basil (Shedeed et al., 1990). In the *Ocimum basilicum* treated with kinetine was verified the largest total dry biomass values when compared to plants that did not receive this plant hormone (Barreiro et al., 2006).

The essential oil production in leaves was significantly affected by the interaction between biostimulant and light levels. In plants treated with the plant regulator at full sun condition the essential oil production was significantly superior of to the other treatments ($R^2 = 99.89$) (Figure 5A). Only light levels showed significant effects on the essential oil content of *L. origanoides*. Plants grown in full sun presented highest essential oil content (0.93%), a positive linear trend in relation to the increase in the availability of light radiation, whereas plants subjected to 25% of full sun reached 0.71% (Figure 5B). The highest

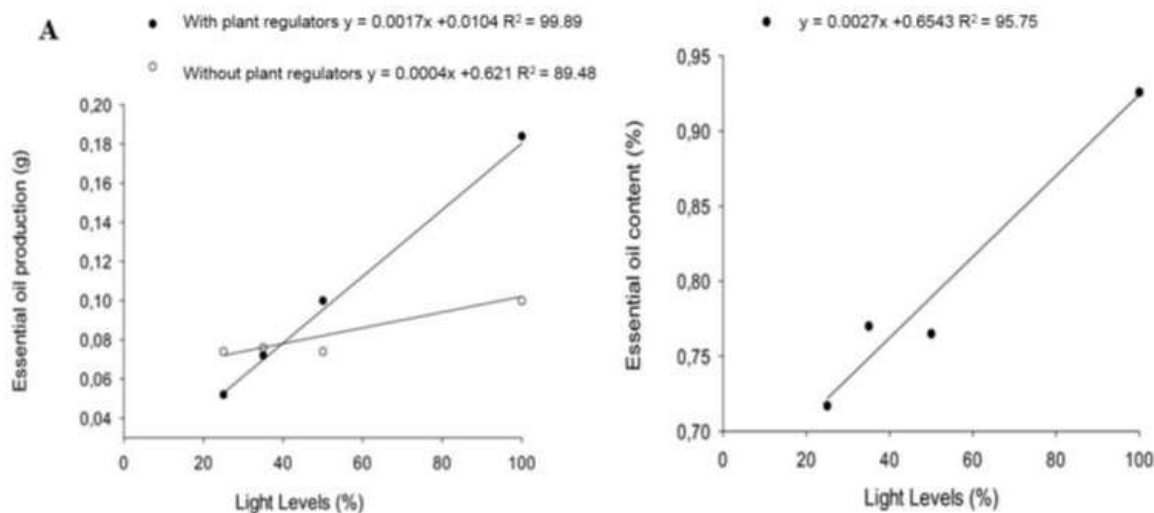


Figure 5. Production (g) (A) and content (%) (B) of essential oil in dried leaves of plants of *L. origanoides*, grown at different light levels with application of biostimulant.
Source: Authors

Table 1. Percentage of the constituents of essential oil in plants of *L. origanoides* grown at different light levels and dealt with biostimulant.

Component	Treatments									
	IK*	IK*(Lit)	T1	T2	T3	T4	T5	T6	T7	T8
	Percentage relative (%)									
α -tujene	932	931	0.6	0.3	0.3	0.3	0.4	0.3	0.5	0.3
canfene	951	953	0.4	0.3	-	0.3	0.3	0.3	-	-
(Z)-4-tujanol	1087	1290	1.1	1.0	1.1	1.0	1.0	1.1	1.2	0.9
p -cimen-7-ol	1290	1287	9.0	8.7	9.1	9.0	9.7	9.5	9.3	9.8
thymol	1292	1290	80.1	81.2	79.6	80.9	80.7	80.7	80.7	80.4
acetate de thymol	1355	1343	0.6	0.5	0.7	0.7	0.5	0.4	-	-
(E)-caryophyllene	1424	1417	2.4	1.8	3.0	2.3	2.6	2.4	1.6	2.3
δ -cadinene	1522	1524	2.2	1.9	2.5	2.1	2.0	2.2	2.2	2.2
14-hidroxi-9-epi- β -caryophyllene	1658	1664	0.5	0.5	0.6	0.4	0.4	0.4	0.5	0.4
Total identified (%)			96.9	96.2	96.9	97	97.6	97.3	96	96.3

Ik * = index de Kovats experiment; IK (Lit) = index de Kovats of literature.
Source: Authors

essential oil content occurred under of full sun with biostimulant application conditions (Figure 5B).

The effects of plant regulators on essential oil production through leaf spraying are variable; one of the most common responses is the influence on the production of essential oil, through increased biomass production (Shukla and Farooqi, 1990; Prins et al., 2010), which was observed in this study. Cytokinins can stimulate the global accumulation of typical monoterpenes of the essential oil (El-keltawi and Croteau, 1987; Ivanova et al., 2014). In *Mentha piperita*, application of growth regulators increased the production of biomass and

essential oil (Çoban and Baydar, 2016). The condition of full sun is more stressful (low relative humidity, higher temperature and incidence of winds) and can raise the production of essential oil (Tavares et al., 2005).

The different light conditions associated to application of the biostimulant no affected on the chemical profile of the essential oil. Nine chemical components were identified in the essential oil; totaling about 97% of the chemical composition of the essential oil; thymol was the major component (Table 1).

The significant changes in the chemical composition of the essential oil in *O. basilicum* resultent of the decrease

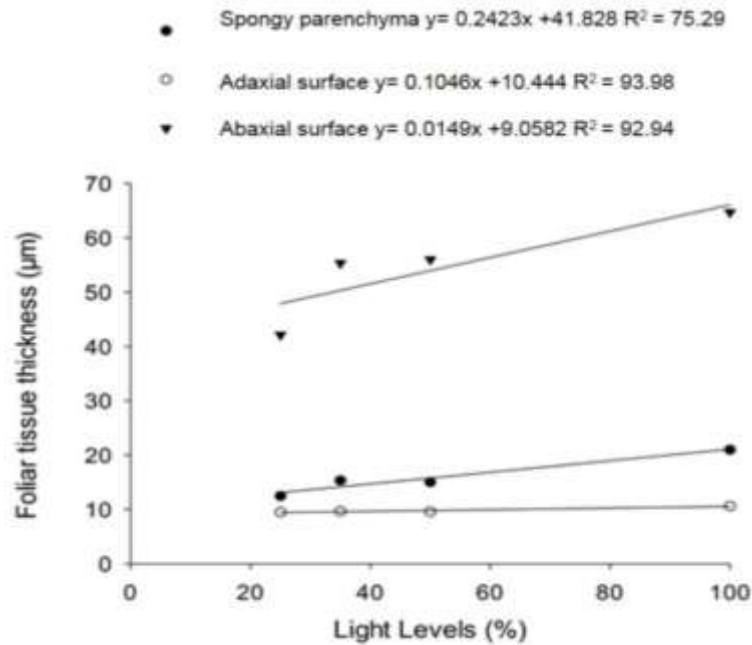


Figure 6. Foliar tissue thickness (µm): Abaxial surface, Adaxial surface; Spongy parenchyma in leaves of *L. origanoides* under different light levels. Source: Authors

of luminous radiation were attributed for Chang et al. (2008) as a result of a strategy of acclimatization to this condition, where they are usually checked higher transpiration rates by increased incidence of wind and solar radiation and, consequently, higher water requirement and other environmental agents. The analyses of leaf cross sections revealed significant differences in leaf anatomical characteristics. The thickness of the adaxial and abaxial surface of epidermis and spongy parenchyma were influenced only by irradiance treatments (Figure 6).

The greatest thickening of the epidermis in sun leaves minimizes the effect of high irradiation under the sensitive tissues of the mesophyll (Markesteyn et al., 2007). The direct contact of the different faces of the epidermis with the environment, it may cause changes due to various environmental factors, mainly the luminosity, favoring the even distribution of light in the interior of the leaf (Volgelmann; Martin, 1993). The palisade parenchyma increased with the treatments with application of the biostimulant associated with the increased availability of luminous radiation resulting from the elongation of the cells that tissue, since there was no change in the number of layers of the even (Figure 7).

To optimize the absorption of photosynthetically active radiation, the leaves of shade in general have smaller thickness of the mesophyll (Gorton et al., 1999; Brodersen et al., 2008), which results from the difference in the pattern of cell division in sun and shade leaves (Yano and Terashima, 2004). The periclinal and anticlinal

divisions occur at the same time in sun leaves, while in shade leaves the periclinal division happens later. The cytokinins are hormones associated with the growth and development of plants, participating in the control of cell division (Nishimura et al., 2004), while the gibberellins are involved with regulation and foliar expansion by stimulating elongation and division (Scavroni et al., 2006). These being two of the three hormones present in plant regulator used, associated with the cell divisions.

The different levels of light influenced the density of the glandular and tectores trichomes in both surfaces of the leaf of *L. origanoides*. The density of tectores and glandular trichomes presented a quadratic adjustment in the surface abaxial with increased light radiation (Figure 8A to B). Glandular trichomes presented the highest mean values of density for both surfaces of the leaf with increased light radiation with application of biostimulant (Figure 8A). Tector trichome presented a maximum mean value of 43.18 mm^{-2} in the adaxial surface and 129.56 mm^{-2} in the abaxial surface to light levels of 88 and 100% full sun with application of biostimulant, respectively (Figure 8B). Stomatal density also showed an interaction between treatments applied with significant difference for the interaction of the factors: Light levels \times biostimulant application (Figure 8C).

The density of glandular trichome may vary according to leaf age, species and environmental conditions. Gomes et al. (2009) found that the density of glandular trichomes in the full sun, 70 and 50% of the full sun did not show significant differences among the species *Lippia*

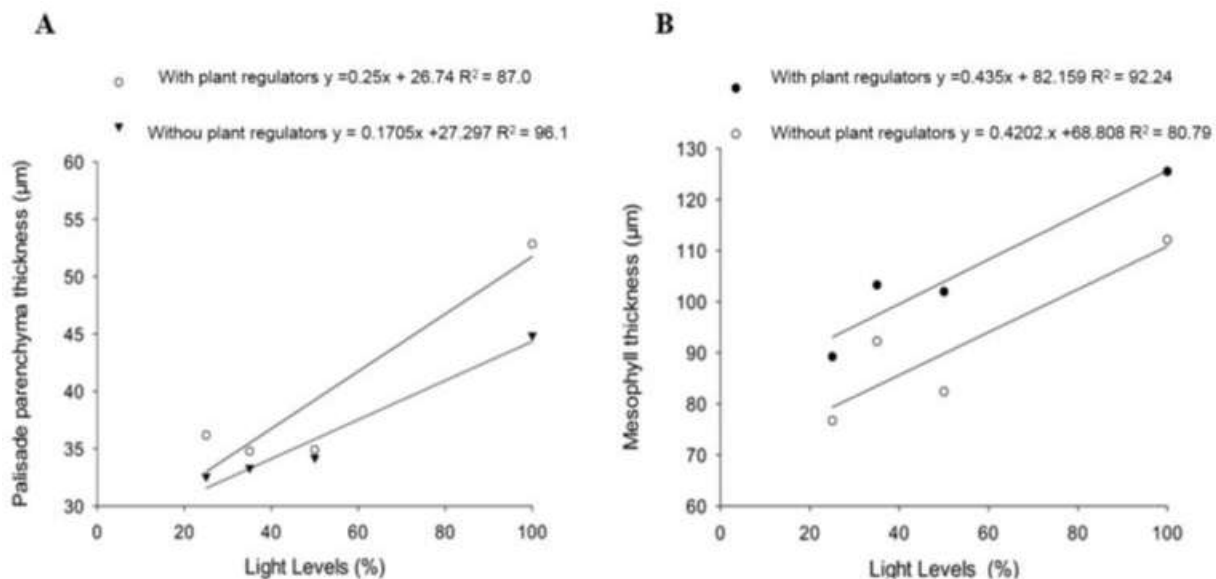


Figure 7. Leaf tissue thickness (μm): Palisade Parenchyma (A) and mesophyll (B), with plant regulators and without plant regulators under different light levels.
Source: Authors

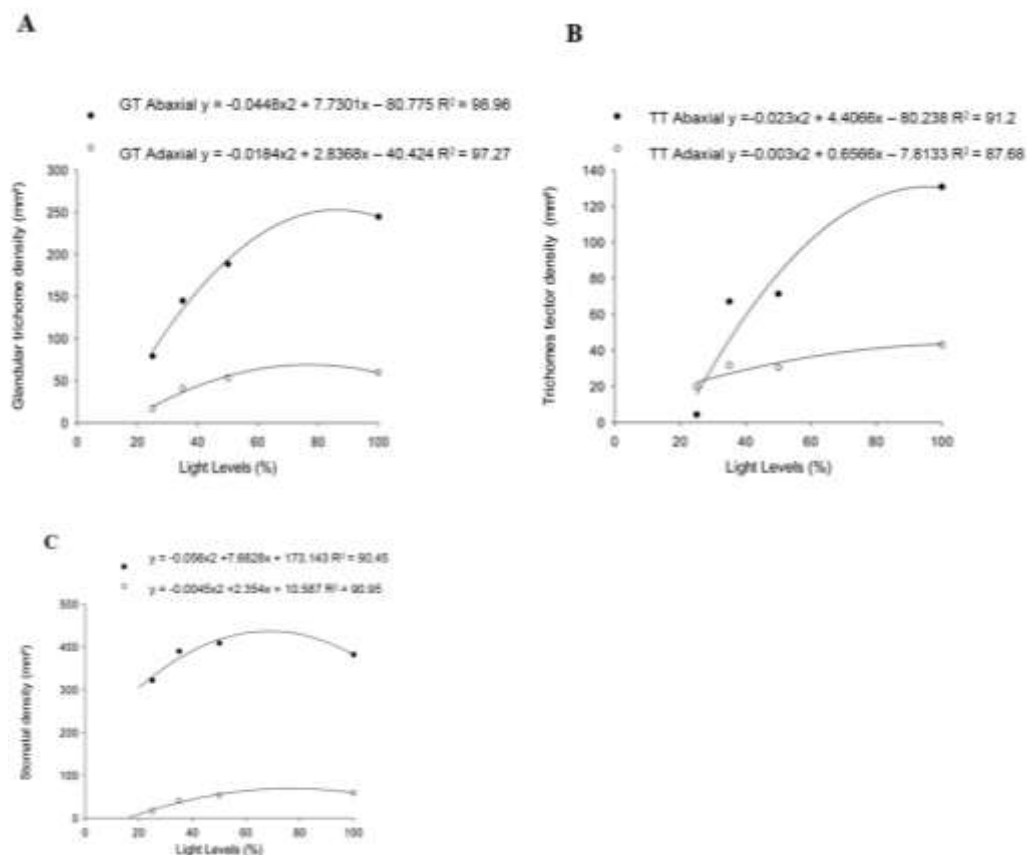


Figure 8. Density: Glandular trichome (GT abaxial and adaxial surface mm^2) (A), Tector trichome (TT abaxial and adaxial surface mm^2) (B); Stomatal (mm^2) on both sides of leaf *L. organoides*, grown at different light levels with application of biostimulant (C).
Source: Authors

citriodora. However, in plants of *O. gratissimum* presented higher density of tectonic trichomes when kept in full sun (Martins et al., 2008). The increase in the density of trichomes is a strategy of acclimatization to the reduction in the rate of perspiration, increased reflection of light, which decreases the temperature of the leaf (Gomes et al., 2009). As a result of the increased density of glandular trichomes, a production of essential oils has been increased because it is secretly stored in the glandular trichomes. It was observed an increase in density of glandular trichomes with application of plant regulators in *Mentha arvensis* (Bose et al., 2013), and not available in the present study.

The stomatal density could be also related to the photosynthetic capacity of the leaves, because the greater the number of stomata mm^{-2} , lower resistance to diffusion of gases on the sheet. Increased stomatal density is usually connected with a higher stomatal conductance (Justo et al., 2005). In this work treated plants the 25% of full sun showed lower stomatal density, which may contribute to lower growth variables such as dry biomass and foliar biomass total fresh leaves under shade (Lima Junior, 2006). Increased leaf area of shaded plants without applying the biostimulant may explain the lower density of stomata observed. In *Arabidopsis thaliana* the increased availability of luminous radiation promoted increased stomatal density of leaves showing be mechanisms responsible for stomatal density dependent adjustment of light (Shuluter et al., 2003). Gondim et al. (2008) studying plants of *Colocasia esculenta* (taro), found that the stomatal density decreases with reduced brightness.

Conclusion

L. organoides demonstrated structural and metabolic plasticity to the effects of applying a biostimulant made up of three plant growth regulators associated with different levels of light radiation. Significant effect occurred biostimulant and light levels in the production of essential oil, showing the interaction of these treatments. The cultivation in 100% of full sun with biostimulant application significantly increased the production of biomass and essential oil keeping the chemical profile.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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