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Original article

Light affects *Varronia curassavica* essential oil yield by increasing trichomes frequency

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ABSTRACT

Light can act on essential oil yield directly on synthesis of secondary metabolites, or indirectly on plant growth. *Varronia curassavica* Jacq., Boraginaceae, is a native medicinal species from Brazil known as "erva-baleeira", with anti-inflammatory activity related to its essential oil. Despite pharmacological evidences of this species and its economic importance for herbal medicine production, little is known about the effect of light on growth and essential oil production. This study aimed to analyze the influence of different irradiances on growth, frequency of trichomes, essential oil yield and composition of *V. curassavica*. The irradiance affected plant growth, but no significant alteration on leaf biomass was detected. The increase in essential oil content under higher irradiance reflected on essential oil yield, and is associated with higher frequency of glandular, globular trichomes. The essential oil composition, rich in caryophyllene derivatives was affected by irradiance, but α-humulene, the constituent of pharmaceutical interest, remained unchanged.

Introduction

Varronia curassavica Jacq. (sin. = *Cordia verbenacea* DC.), Boraginaceae, popularly known as "erva-baleeira", "catinga-de-barão", "maria-preta" and "maria-milagrosa" occurs naturally from Central to South America, and in Brazil is associated with the Atlantic Forest. It is a medicinal plant traditionally used to treat inflammation, ulcers, arthritis, and pain. Phytochemical studies revealed the presence of flavonoids, phenols and essential oils, responsible for its anti-inflammatory, antimicrobial and allergenic activities (Carvalho et al., 2004; Sertié et al., 2005; Ticli et al., 2005; Passos et al., 2007). The main components of *V. curassavica* essential oil are α-pinene (29.69%) and *trans*-caryophyllene (25.27%) other components in significant concentration are *allo*-aromadendreno (9.99%) and α-humulene (4.64%) (Carvalho et al., 2004).

The synthesis, storage and release of essential oils in plants occur in specialized secretory structures, such as oil cells, ducts, lysigenous and schizolysigenous cavities, or glandular trichomes according to its the botanical families (Simões and Spitzer, 2000). In *V. curassavica* the essential oil is secreted and stored in glandular globular trichomes present on the leaf surface (Ventrella and Marinho, 2008), whose frequency and development can be influenced by irradiance (Gomes et al., 2009; Costa et al., 2010).

Besides genetic characteristics, many environmental factors can lead to variations in secondary metabolites content. The monoterpenoids and sesquiterpenoids are metabolites in essential oils often subject to variations due to abiotic factors.

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The light intensity can change the essential oil production via activation of photosensitive enzymes involved in the mevalonic acid pathway, precursors of terpenes (Gobbo-Neto and Lopes, 2007). Thus, irradiance can influence directly the production of essential oil, or indirectly, through the increase of plant biomass (Pegoraro et al., 2010). Irradiance is essential for plant growth, since it affects the primary metabolism providing energy for photosynthesis and generating signals that regulate their development (Lima et al., 2010).

In 2004, the Agência Nacional de Vigilância Sanitária in Brazil, approved the registration of the first topic antiinflammatory made from the essential oil of the Brazilian plant, *V. curassavica*, whose active ingredient is α-humulene. Thus, increasing the need for research on culture and management of this species.

The aim of this study was to evaluate the effect of irradiance on *V. curassavica* growth, trichome frequency, and essential oil yield and composition.

Materials and methods

Plant material

Seedlings of *Varronia curassavica* Jacq., Boraginaceae, were obtained by seed propagation. A voucher specimen was deposited at the Universidade Estadual de Santa Cruz Herbarium under the accession number 13.895. A month after germination, the plants were transferred to pots containing 10 l of soil and subjected to four treatments of light irradiance (20, 50, 70 and 100% light-full sun) for 90 days. The full sun treatment corresponded to natural irradiance conditions in the Medicinal Plants Garden of Universidade Estadual de Santa Cruz, while the other treatments were obtained using black screens for cover. The photosynthetically active radiation (PAR) in each environment was measured with a quantum sensor BQM-SUN (Apogee, USA) for five days in one-hour intervals from 7 am to 5 pm. The maximum PAR was observed in full sun (2240 $\mu mol.m^{-2}.s^{-1}$) followed by the shaded treatments at 1610, 1120 and 574 $\mu mol.m^{-2}.s^{-1}$, which are 70, 50 and 20% of light, respectively.

At the end of the experiment six plants were harvested to evaluate dry biomass of roots (R), stems (S), leaves (L), total plant (T), leaf area (LA) and leaf mass per area (LMA). The dry biomass was obtained using an oven with air ventilation at 70°C, until constant weight and leaf area was measured, using an electronic leaf area meter, model LI-3100 (Li-Cor, Inc. Lincoln, Nebraska, USA).

Isolation and chemical analysis of essential oils

Leaves of four plants were oven-dried at 40°C with air ventilation for essential oil extraction by hydrodistillation using a Clevenger apparatus. The collected hydrolate was submitted to liquid-liquid partition chromatography with dichloromethane, the organic fractions were separated and dried with anhydrous sodium sulfate, and further concentrated. The mean concentrations of essential oils were calculated as weight of oil (g) per 100 g of dry leaf biomass, and essential oil yield per plant ($g\ plant^{-1}$).

Gas chromatography analyses were performed using a Varian Saturno 3800 gas chromatograph equipped with a flame ionization detector (FID) and a Varian VF-5ms fused silica capillary column (30 m × 0.25 mm; 0.25 µm film thickness). The carrier gas was helium at a flow rate of 1.0 $ml.min^{-1}$. The injector and detector temperatures were 250 and 280°C, respectively. The column oven temperature started at 60°C and increased by 6°C min^{-1} up to 280°C, where it was maintained for 5 min. The sample (1 µl) diluted in 10% ethyl acetate was injected in the split mode (1:10). The concentration of the essential oil constituents was calculated from the peak area in relation to the total area of the sample. The qualitative analysis was performed in a Varian 2000 mass spectrometer, using the 70 eV electronic impact method, scan mass range of 40-450 *m/z* at a sampling rate of 1.2 scan/s, with the transfer line temperatures at 280°C, manifold at 120°C, and trap at 240°C equipped with a VF-rms capillary column (30 m × 0.25 mm × 0.25 µm). The injector temperature was 250°C; the carrier gas, the column temperature and the injection flow were operated under similar GC-FID conditions with an electron impact of 70 eV. Chemical constituents were identified by comparison with the library system (NIST08), literature and Kovats retention index (Adams, 2007).

Trichomes distribution

Fully expanded leaves were collected from the fourth node from the apex to the base of the plant. Segments of the median portion leaf were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 6.9, dehydrated in crescent ethanol series and dried at critical point (CPD 030, Bal-Tec, Balzers, Liechtenstein), and gold coated using a sputter coater apparatus (SCD 050, Bal-tec, Balzers, Liechtenstein). After that, the samples were examined using a scanning electron microscope (JEOL JSM-6390LV). Four replicates were carried out and six observation fields were randomly selected, totalling 24 observation fields per treatment. The trichome frequency was performed in the regions between the leaf veins of both leaf surfaces.

Statistical analysis

All data were subjected to analysis of variance considering the completely randomized design to test the difference between treatments on each variable. Plant growth characteristics, the content and essential oil yield and trichomes frequency were subjected to regression analysis. The mean concentration of the essential constituents oil was compared with Tukey's test (5% significance) using the statistical program SISVAR (Ferreira, 2011).

Results and discussion

The biomass accumulation of different organs of *Varronia curassavica* Jacq., Boraginaceae, presented different responses to the irradiance levels (Fig. 1). Total dry biomass showed a quadratic fit, with maximum production of 193.96 g at 54% of irradiance. While the stem dry biomass decreased linearly with

irradiance, the root dry biomass followed a quadratic fit, with higher production (101.73 g) at irradiance of 75%. Although the production of leaves was not affected by higher irradiance (Fig. 1A), the leaf area was reduced severely, accompanied by an increase of LMA (Fig. 1B).

The irradiance is a key factor to plant growth and development to provide energy for photosynthesis, generate signals that regulate development and interfere with translocation of assimilates among different plant organs. The investment in root production by *V. curassavica* under higher irradiance, also verified by Paulilo et al. (2010) in the same conditions, favors greater water and nutrients absorption and increases the ability to withstand higher rates of transpiration and photosynthesis. On the other hand, *V. curassavica* plants grown under shaded conditions had a greater leaf area as an acclimation strategy to increase the surface for light capture, while the reduced leaf area of plants grown in environments with higher light intensity can be related to reduce water loss trough evapotranspiration. The less surface area is in contact with wind or heat, the less amount of water is lost. The leaf area reduction associated with no change on leaf biomass of *V. curassavica* resulted on the increase of leaf mass per area. LMA variation is mainly related to differences in leaf density, which result from variations of the degree of sclerification of the lamina. From an ecological viewpoint, a greater leaf mass per area can be interpreted as a greater allocation of resources to support and defense functions (Castro-Díez et al., 2000).

Essential oil content and yield of *V. curassavica* were sensitive to irradiance with a rising linear behavior, with the highest values found in plants grown under higher irradiance (Fig. 2).

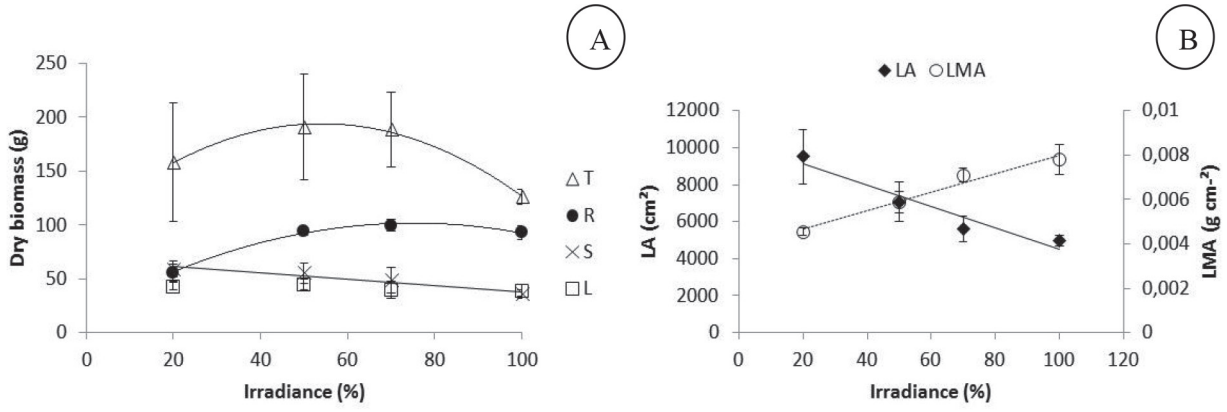

Figure 1 – Dry biomass of *Varronia curassavica* plants grown under different irradiance intensities. Total dry biomass (T) $\hat{y}$ = -0.031661*×2 +3.418059*× +101.717582* (R^2 = 0.99), root dry biomass (R) $\hat{y}$ = -0.014961**×^{2}+2.246223**× +17.424869 (R^2 = 0.99), stem dry biomass (S) $\hat{y}$ = -0.0291**× +67.2407** (R^2 = 0.93), leaf dry biomass (L) $\hat{y}$ = 45.553. B. Leaf area of *Varronia curassavica* plants grown under different irradiance intensities (LA) $\hat{y}$ = -57.6277**× +10259** (R^2 = 0.91) and Leaf mass per area of *Varronia curassavica* plants grown under different irradiance intensities (LMA) $\hat{y}$ = 0.000042**× +0.003817 (R^2 = 0.97). * Significant at $p \leq 0.05$, ** Significant at $p \leq 0.01$. Bars indicate the mean standard error (n = 6).

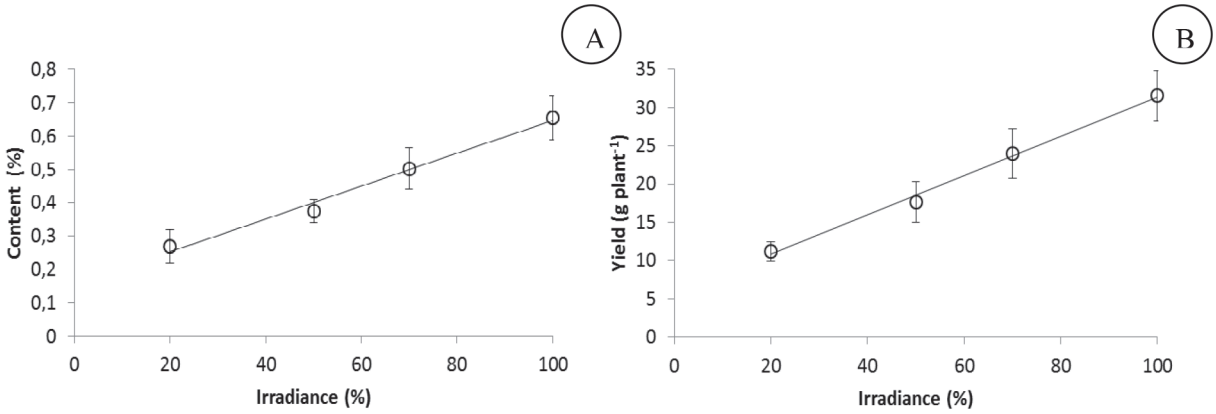

Figure 2 – A. Essential oil content $\hat{y}$ = -0.005162**× +0.16091 (R^2 = 0.93); B. Essential oil yield $\hat{y}$ = -0.260044**× +5.2423** (R^2 = 0.98) of *Varronia curassavica* plants grown under different irradiance intensities. **Significant at $p \leq 0.01$, * Significant at $p \leq 0.05$. Bars indicate the mean standard error (n = 4).

The secondary metabolites represent a chemical interface between the plants and their environment; therefore, their synthesis is often affected by environmental conditions (Kutchan, 2001). Irradiance acts on the activation of biosynthetic pathways of essential oils, which are dependent on supply of carbon backbone obtained by photosynthetic processes, which are generally higher in enlightened environments (Schuh et al., 1997). Similarly to *V. curassavica*, *Ocimum basilicum* (Chang et al., 2008) and *Lippia citriodora* (Gomes et al., 2009) grown at different light intensities, also showed higher essential oil content when were grown under full sunlight. As the essential oil yield is a direct result of essential oil content and plant biomass (Costa et al., 2010), and no change in the leaf dry biomass production of *V. curassavica* was observed, the highest essential oil yield observed under high irradiance, can be attributed to the higher essential oil content found in these conditions.

The irradiance affected the essential oil composition of *V. curassavica* eliciting a variation of 26, 26, 32 and 34 identified components at 20, 50, 70 and 100% of irradiance, respectively (Table 1). At lower irradiance the essential oil presented a more complex mixture with only 76.35% of the constituents identified. Some oil compositional fluctuations were evident under different light treatments. A large amount of sesquiterpene derivatives ranging from 53.02% to 74.06% was observed at the greatest amount in higher irradiance. Seven derivatives of caryophyllene, which are substances with recognized anti-inflammatory action (Sertié et al., 2005; Passos et al., 2007), were identified. The monoterpenes, α- and β-pinene were found in greater quantities in the intermediate irradiance values. There was an increase in the amount of oxygenated monoterpenes with the increase of light incidence, unlike sesquiterpenoic-oxygenated substances. Other sesquiterpenes present in significant amounts were derived from aromadendrene, germacrene, δ-cadinene, (Z)-α-santalol and acarenone.

The chemical composition of the essential oils can be modified under different conditions of light, temperature, water and nutritional levels (Lima et al., 2003). The synthesis of these metabolites in some plants may be affected by irradiation, as the biosynthesis of isoprene, its basic constituent, is dependent on the irradiance (Schuh et al., 1997). In general, the composition of the essential oils is very sensitive and can suffer numerous reactions under the influence of various factors (Simões and Spitzer, 2000). Caryophyllene oxide and *trans*-caryophyllene are the major components of *V. curassavica* essential oil suggesting a new chemotype different from that described by Carvalho et al. (2004), whose main components were α-pinene (29.69%) and *trans*-caryophyllene (25.27%). While the highest values of caryophyllene oxide were found in less illuminated treatments (20 and 50%), the opposite was described for *trans*-caryophyllene, possibly related to the oxidation of *trans*-caryophyllene as a result of light stress suffered by plants in these environments. The variation of α-pinene content with higher production in conditions of intermediate irradiance (50 and 70% light) and small in the other treatments, demonstrates that this component is also influenced by irradiance. The constituent of pharmaceutical interest, α-humulene, remain unchanged, nevertheless, its relative concentration, the ratio between concentration of component with the amount of essential oil produced, increased in plants grown under full sunlight.

Four different types of trichomes were found on the leaf surface. While the globular and reniform glandular trichomes are present on both leaf surfaces, non-glandular trichomes vary according to their position on the leaf: short non-glandular trichomes are present only on the adaxial surface, and long non-glandular trichomes were observed only on the abaxial leaf surface of *V. curassavica* (Fig. 3). The frequency of different types of trichomes was higher with increasing irradiance, especially the glandular globular trichomes that showed the most intense response (Fig. 4 A e B).

The different types of trichomes observed in *V. curassavica* already described by Ventrella and Marinho (2008), were found in all treatments in distinct manner. The increased non glandular trichome frequency with high irradiation could be possibly related with reduced photoinhibition and heat loading (Skelton et al., 2012). Something similar occurs with reniform trichomes as its phenolics and flavonoids secretion (Ventrella and Marinho, 2008) also contribute to a most effective protection against radiation (Karabourniotis et al., 1998; Tattini et al., 2005). The glandular globular trichomes, responsible for the synthesis and storage of essential oils in *V. curassavica* (Ventrella and Marinho, 2008), showed the most marked response to increasing irradiance. The increased frequency of globular trichomes in *V. curassavica* leaves to acclimate at higher irradiances reflected directly in the essential oil content of the plant. As the number of trichomes is fixed in the early stages of leaf differentiation (Ascensão and Pais, 1987; Werker et al., 1993), it is clear that their frequency is higher in leaves owing a much reduced leaf area induced by the high irradiance. The yield is a function of the essential oil content by the biomass weight produced by the plant. Since the leaves constitute the main organ involved in essential oil biogenesis and accumulation, and leaf biomass was not affected by irradiance, the highest essential oil yield of *V. curassavica* is associated with the higher glandular globular trichomes frequency.

The data obtained showed that irradiance is a key factor for growth and essential oil production of *V. curassavica*. The highest essential oil yield observed at higher irradiances is associated with the enhanced content, since there was no change in leaf biomass production under the same condition. The variation of the essential oil content occurred as a direct response to an increase of globular glandular trichomes frequency due the reduced leaf area of the plant at higher irradiances.

Authors' contributions

EVRSF (MSc student) conducted the experiments, collected the data for analysis, ran the laboratory work, analyzed of the data and drafted the paper. RAO contributed to chromatographic

Table 1
Relative percentage of essential oil constituents of *Varronia curassavica* plants grown under different irradiances.

Components	KI		20%	50%	70%	100%
	Exp	Lit	Relative percentage (%)			
α-pinene	933	939	1.07 ± 0.11[d]	11.72 ± 0.15[b]	14.39 ± 2.36[a]	5.33 ± 1.45[c]
β-pinene	987	983	—	0.39 ± 0.55[b]	1.24 ± 1.50[a]	—
eucalyptol	1040	1033	—	1.13 ± 0.08[b]	1.00 ± 0.05[b]	2.37 ± 0.20[a]
cis-thujone	1103	1102	1.13 ± 0.01[b]	—	2.09 ± 0.04[a]	—
camphenol	1111	1109	—	—	1.11 ± 0.11[a]	0.60 ± 0.52[b]
α-campholenal	1133	1125	—	—	0.31 ± 0.53[b]	1.23 ± 0.13[a]
trans-verbenol	1150	1144	0.41 ± 0.59[c]	—	1.27 ± 0.19[a]	1.01 ± 0.02[b]
cis-β-terpineol	1154	1146	—	—	—	2.50 ± 0.30[a]
verbenone	1210	1204	—	—	—	1.35 ± 0.02[a]
trans-carveol	1220	1217	—	—	—	0.66 ± 0.58[a]
isobornyl acetate	1289	1285	0.91 ± 0.03[c]	1.26 ± 0.04[b]	1.31 ± 0.11[b]	1.98 ± 0.13[a]
cis-4-thujanol acetate	1304	1291	—	—	1.58 ± 0.11[b]	3.48 ± 0.33[a]
neo-verbenol acetate	1315	1318	—	—	1.13 ± 0.10[b]	2.24 ± 0.28[a]
neo-iso-verbenol acetate	1326	1328	—	—	0.26 ± 0.44[b]	1.09 ± 0.17[a]
δ-elemene	1334	1339	1.00 ± 0.01[c]	—	1.37 ± 0.06[b]	1.53 ± 0.06[a]
verbenol acetate	1346	1340	2.74 ± 0.29[a]	0.82 ± 0.01[c]	—	0.96 ± 0.04[b]
citronellyl acetate	1351	1354	—	0.87 ± 0.04[c]	1.57 ± 0.37[a]	1.20 ± 0.07[b]
β-elemene	1393	1391	—	1.16 ± 0.06[b]	—	1.85 ± 0.17[a]
1.7-di-*epi*-α-cedrene	1404	1397	1.00 ± 0.08[a]	0.43 ± 0.62[b]	—	1.00 ± 0.06[a]
α-cedrene	1417	1409	1.44 ± 0.05[a]	0.90 ± 0.07[b]	1.36 ± 0.02[a]	1.41 ± 0.06[a]
trans-caryophyllene	1424	1418	11.39 ± 0.25[c]	7.69 ± 0.14[d]	14.74 ± 0.37[a]	14.37 ± 0.42[b]
aromadendrene	1427	1439	0.45 ± 0.65[c]	6.84 ± 0.70[a]	—	2.98 ± 0.18[b]
allo-aromadendrene	1457	1461	2.02 ± 0.02[b]	1.43 ± 0.04[d]	1.79 ± 0.01[c]	2.16 ± 0.23[a]
α-humulene	1464	1462	1.36 ± 0.06[b]	1.24 ± 0.48[c]	1.14 ± 0.05[d]	1.58 ± 0.45[a]
9-*epi*-*E*-caryophyllene	1468	1467	1.13 ± 0.11[c]	3.93 ± 0.08[b]	0.64 ± 0.56[d]	6.03 ± 0.31[a]
γ-curcumene	1484	1480	—	—	0.64 ± 0.56[a]	—
germacrene D	1487	1482	—	0.82 ± 0.10[a]	—	—
epizonarene	1501	1497	1.43 ± 0.01[a]	1.29 ± 0.07[b]	0.55 ± 0.48[c]	0.27 ± 0.47[d]
germacrene A	1508	1503	2.77 ± 0.37[a]	2.14 ± 0.11[c]	1.92 ± 0.06[d]	2.36 ± 0.15[b]
δ-cadinene	1523	1524	3.67 ± 0.28[b]	4.11 ± 0.06[a]	2.51 ± 0.20[c]	2.55 ± 0.30[c]
spathulenol	1582	1576	1.28 ± 0.12[c]	3.64 ± 0.24[a]	0.93 ± 0.60d	2.43 ± 0.06[b]
caryophyllene oxide	1593	1581	23.12 ± 1.15[a]	22.67 ± 1.84[b]	15.02 ± 6.46[c]	15.77 ± 0.91[d]
humulene epoxide ii	1621	1606	4.97 ± 0.68[a]	3.80 ± 0.33[b]	3.49 ± 0.25[c]	3.14 ± 0.19[d]
valerianol	1655	1655	—	0.47 ± 0.67[a]	—	—
14-hydroxy-9-*epi*-(*E*)-caryophyllene	1667	1662	1.69 ± 0.60[a]	0.50 ± 0.72[c]	0.76 ± 1.32[b]	0.28 ± 0.50[d]
(Ar)-turmerone	1670	1664	—	—	1.01 ± 0.93[a]	—
β-bisabolol	1678	1671	1.98 ± 0.20[a]	—	1.42 ± 0.55[b]	1.24 ± 0.16[c]
Z-α-santalol	1683	1678	1.76 ± 0.12[b]	2.00 ± 0.09[a]	1.03 ± 0.07[c]	0.61 ± 0.53[d]
acarenone	1687	1685	2.80 ± 1.29[a]	1.55 ± 0.85[c]	1.45 ± 0.88[d]	1.74 ± 0.40[b]
14-hydroxy-α-humuleno	1710	1709	1.10 ± 0.27[a]	—	—	—
iso-longifolol	1732	1726	1.93 ± 0.57[a]	1.02 ± 0.26[c]	1.25 ± 0.53[b]	0.76 ± 0.66[d]
pentadecanol	1775	1778	1.80 ± 0.22[a]	—	1.83 ± 0.51[a]	—
Total identified (%)			76.35 ± 0.26	83.82 ± 0.30	82.11 ± 0.60	90.04 ± 0.21
Monoterpens			1.07 ± 0.11	12.11 ± 0.20	15.63 ± 0.43	5.31 ± 1.45
Monoterpenes oxygenated			5.19 ± 5.19	4.08 ± 0.02	11.63 ± 0.14	20.67 ± 1.14
Sesquiterpenes			27.66 ± 0.16	31.98 ± 0.19	26.66 ± 0.20	38.09 ± 0.13
Sesquiterpenes oxygenated			40.63 ± 0.34	35.65 ± 0.40	26.36 ± 1.16	25.97 ± 0.20
Aliphatic alcohol			1.80 ± 0.22[a]	—	1.83 ± 0.51[a]	—

Not identified (—); KI, Kovats Index.
Means followed by the same letter in a row, are not significantly different ($p > 0.05$) by Tukey's test at 5% (n = 3).

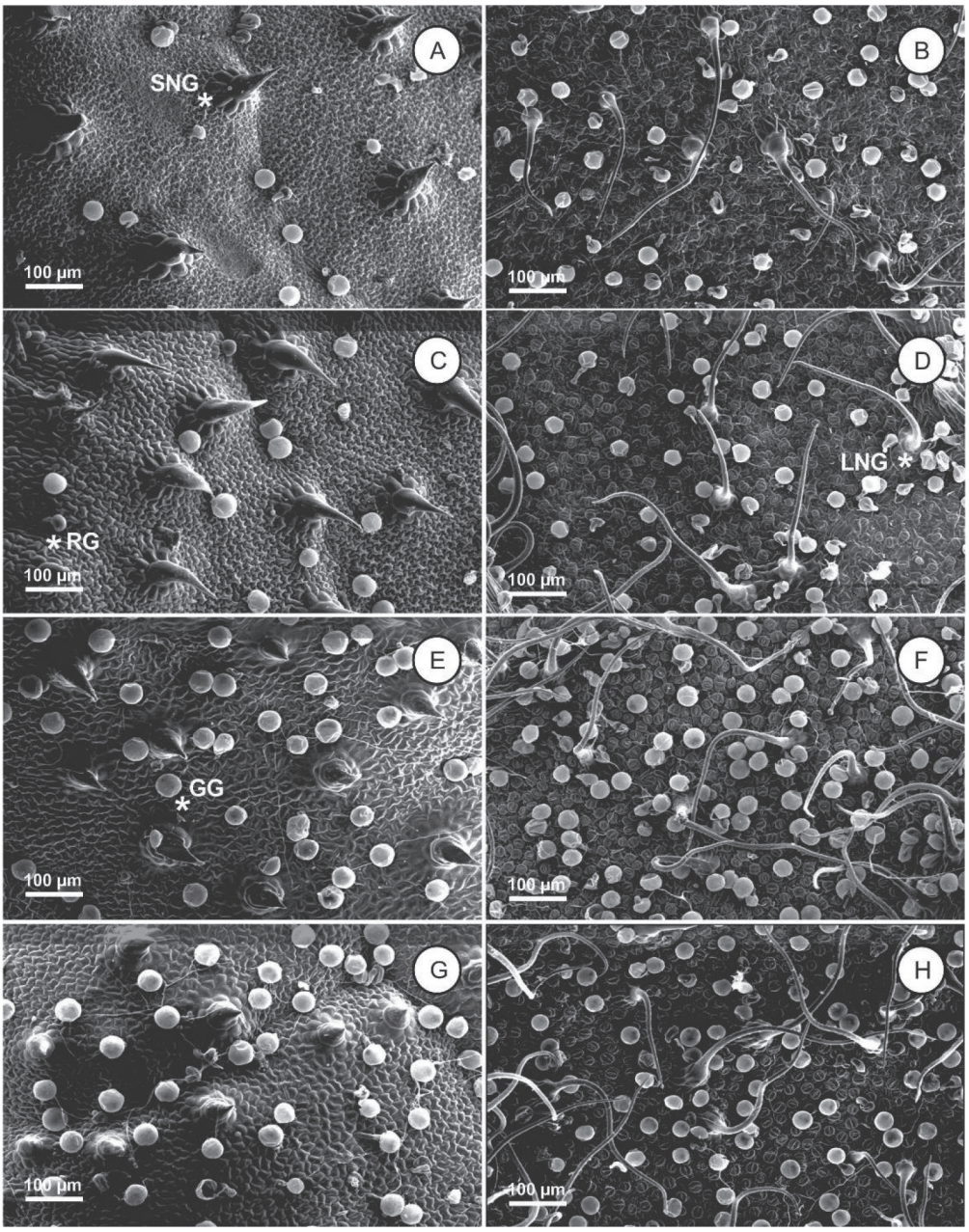

Figure 3 – Scanning electron micrographs of adaxial (A, C, E, G) and abaxial (B, D, F, H) leaf surface of *Varronia curassavica* plants grown under different irradiance intensities. A and B. 20%; C and D. 50%; E and F. 70%; G and H. 100%. SNG: short non-glandular trichome; LNG: long non-glandular trichome; GG: globular glandular trichome; RG: reniform glandular trichome. Bar = 100 µm.

analysis. LCBC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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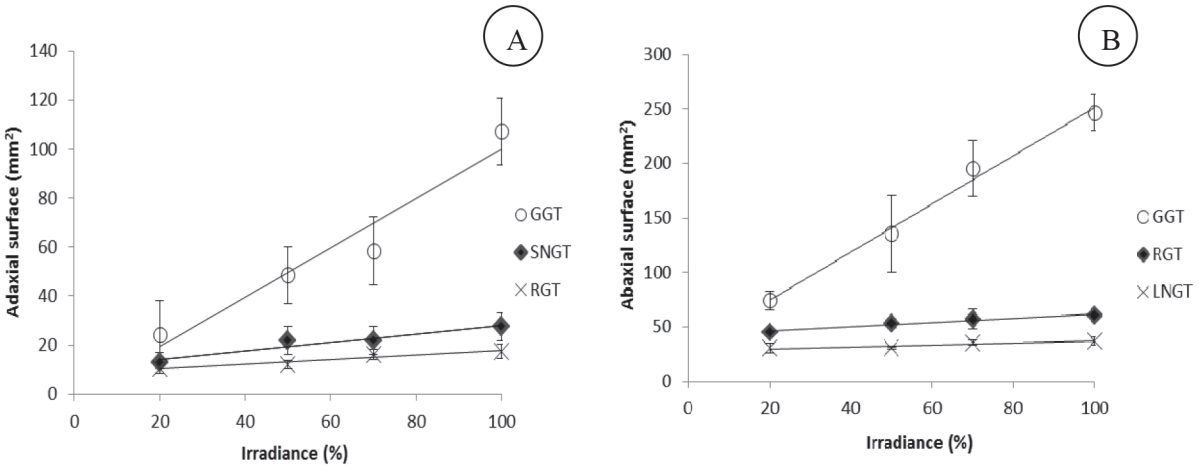

Figure 4 – Trichomes frequency on the adaxial (A) leaf surface of Varronia curassavica plants grown under different irradiance intensities. Glandular globular trichome (GGT) $\hat{y} = 1.0034^{**}\times - 0.5423^{**}$ ($R^2 = 0.94$), short non-glandular trichome (SNGT) $\hat{y} = 0.17060^{**}\times + 11.059^{*}$ ($R^2 = 0.86$) and reniform globular trichome (RGT) $\hat{y} = 0.09280^{**}\times + 8.4758^{**}$ ($R^2 = 0.92$). (B) Trichomes frequency on the abaxial leaf surface of Varronia curassavica plants grown under different irradiances. Globular glandular trichome (GGT) $\hat{y} = 2.2001^{**}\times + 30.9981^{**}$ ($R^2 = 0.99$), reniform glandular trichome (RGT) $\hat{y} = 0.1929^{**}\times + 42.802^{**}$ ($R^2 = 0.96$), long non-glandular trichome (LNGT) $\hat{y} = 0.09399^{**}\times + 28.082^{**}$ ($R^2 = 0.83$). *Significant at $p \leq 0.05$. **Significant at $p \leq 0.01$. Bars indicate the mean standard error (n = 24).

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