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Does the water regime differentially modulate the responses to water stress in *Lippia alba* (Verbenaceae) genotypes with different ploidy levels?

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ABSTRACT

Lippia alba (Mill.) NE Br. ex Britton & P. Wilson is an important medicinal herb widely distributed in Latin America. However, the success of this crop depends on several biotic and abiotic environmental factors, being water availability the most limiting one. Therefore, the present paper aimed to investigate the responses of one tetraploid (tet) and two diploids (dip1 and dip2) genotypes of *L. alba* to drought stress by using irrigation suspension under 0 (control) or 15 (stressful factor) days. The physiological parameters water content, gas exchange, chlorophyll *a* fluorescence, photosynthetic pigments contents, and the qualitative profile of volatile organic compounds were evaluated by uni- and multivariate statistical analyses. Drought stress negatively affected the physiological processes in all *L. alba* specimens equally, regardless of the genotype. It was observed that photosynthesis was impaired due to stomatal limitation, which was triggered by low water availability. In contrast, the qualitative profile of volatile organic compounds was strongly determined by genotype without drought stress influence. Furthermore, gas exchange and chlorophyll *a* fluorescence were the most sensitive parameters to evaluate drought stress in *L. alba*. More specifically stomatal conductance is recommended to be evaluated in *L. alba* breeding programs focused on drought stress tolerance, given that its high contribution to photosynthesis regulation.

1. Introduction

Lippia alba (Mill.) NE Br. ex Britton & P. Wilson is an important medicinal herb widely distributed in Latin America (Hennebelle et al., 2008). It has a shrub growth habit and could reach up to 1.5 m with thin branches. Its leaves are elliptical with sawn edges and rich in trichomes, arranged in decussate phyllotaxis pattern and the flowers are small, pink, and arranged on capitula with short axes (Baracuhy et al., 2016; da Silva et al., 2018). The glandular trichomes present in the leaves store large quantities of essential oils that have antibacterial (da Silva et al., 2019), anti-inflammatory (Aziz et al., 2019), antiprotozoal (Raut and Karuppayil, 2014), and cytotoxic properties (García et al., 2017). In addition, its essential oils can be used as a repellent against stored grain insects (Peixoto et al., 2015), as a mosquito larvicide (Pavela, 2015), and has potential to be used in cosmetic products (Bravo et al., 2020). Thus, the production of the essential oils is the reason why *L. alba* is important

Abbreviations: A, net photosynthesis; *E*, transpiration; g_s, stomatal conductance; *Ci*, CO_2 internal content; WUE, water use efficiency; CE, RUBISCO carboxylation efficiency; F_v/F_m, maximum photochemical quantum yields of photosystem II; ϕPSII, effective photochemical quantum yield of photosystem II; NPQ, non-photochemical quenching; qP, photochemical quenching coefficient; ETR, electron transport rate; WC, water content; VOCP, volatile organic compounds profile; DIS, days of irrigation suspension.

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to the industry, to produce medicines or other products.

The extraction of essential oils is carried out from cultivated plants in agricultural ecosystems widely distributed in the American continent (Raut and Karuppayil, 2014; Bravo et al., 2020). However, the success of those crops depends on several biotic and abiotic environmental factors, being water availability the most limiting one (Dinneny, 2019). Water is closely related to growth and most physiological process in plants, including photosynthesis (Arbona et al., 2016; Lawson and Vialet-Chabrand, 2019). The assimilation of carbon in plants is totally dependent on the photosynthetic process which is strongly regulated by water availability since this environmental resource controls the stomatal conductance in leaves (Lawson and Vialet-Chabrand, 2019). At low water availability, stomatal closure prevents excessive water loss through transpiration but this also leads to low CO_2 availability in the mesophyll, since CO_2 uptake occurs via stomatal opening (Henry et al., 2019; Lawson and Vialet-Chabrand, 2019). At this point, a hypothetical plant is on a trade-off between growth and water loss (Henry et al., 2019). In addition, stress is here defined as an environmental factor that prevents a plant from reaching its full physiological potential, by reducing the rate of some physiological process (Lambers et al., 2019).

Water availability is an important environmental factor to be considered nowadays to crop production because the world is going through rapid and marked climate changes (Elliott et al., 2014). In this context, many places that have a historical high precipitation index are suddenly under drought conditions and this can lead to a crisis in crop production, endangering food security and other eco-social aspects dependent on plant resources (Iglesias et al., 2010; He et al., 2019). Thus, understanding how plants respond to drought is more important than ever for all humanity: if this can be understood, then new approaches can be created to try to overcome it. In addition, measures of variables related to water status, gas exchange, chlorophyll *a* fluorescence, and the level of photosynthetic pigments are relevant for demonstrating how plants respond to drought (Zegada-Lizarazu et al., 2015; dos Santos et al., 2019; Miao et al., 2020).

Besides water availability, genotypic factors are very important to determinate plant responses to drought, given that phenotypic traits depend on the interaction between environmental and genotypic factors. It is widely described in the literature that plants with a polyploid genome have different morpho- and physiological traits due to polyploidy, including responses to stressful conditions (Sattler et al., 2016). In *L. alba*, it is described that polyploidy is a factor that regulates the chemotype of plants: tetra- and diploid plants are citral chemotype, triploid ones are linalool chemotype and hexaploid ones are carvona chemotype, but less is known about how polyploidy modulates its physiology (Viccini et al., 2014; Lopes et al., 2020). Indeed, despite the advanced knowledge of the biology of polyploid plants, little is known about the relation between polyploidy and plant physiology (Soltis et al., 2016), especially the responses due to different water availability.

Within this context, three genotypes of *L. alba* with different ploidy levels were evaluated in terms of how they acclimate to drought stress, measuring traits related to photosynthesis and volatile organic compounds profile production. It was proposed to answer the following questions: a) Do *L. alba* genotypes respond differently to drought stress?, b) What modifications in those biological processes can be observed?, and c) What parameters are more sensitive to drought stress?

2. Material and methods

2.1. Plant material and experimental approach

Plant material was obtained from the germplasm collection at the Laboratory of Genetics and Biotechnology (Biology Department) located in the Federal University of Juiz de Fora (UFJF), Juiz de Fora, MG, Brazil. Three *L. alba* genotypes were used: dip1, dip2, and tet, corresponding, respectively, to diploid, diploid, and tetraploid chromosome numbers. Plants were propagated vegetatively using cuttings with three pairs of lateral buds immersed in a solution containing 0.29 mM Indole-3-butyric acid for 24 h. After that time, the material was transferred to 9 L pots containing a mix of soil:sand:cattle manure in a 3:2:1 (v/v/v) proportion, leaving one plant cutting per pot. Plants were grown in an open-sided greenhouse for 90 days, in field capacity. Adult plants with similar traits as height, number of branches, and leaves were then selected to be studied.

A pre-test was carried out and the following periods of irrigation suspension (DIS) were tested to simulate drought stress: 0, 5, 10, or 15 days. Irrigation of pots was suspended on different days in such a way that the experimental time could finish on the same day, thus allowing the conduction of all measurements and material collection also on the same day. With these previous results of water content and gas exchange parameters (see supplementary material), it was clear that only 15 DIS were stressful to the plants. Subsequently, a second experiment was carried out in a similar way using only two DIS times: 0 (control) or 15 (stressful condition) days. The results of this experiment were analyzed and showed in this paper.

2.2. Environmental conditions

The experiment was carried out from 26 August 2017 to 12 September 2017 at UFJF's Experimental Station of Plant Growth (coordinates 21°46′48.9″S 43°22′25.6″W, and altitude of 970 m). According to Köppen's climate classification, the region presents a Cwa climate (mesotermic). Data about temperature and relative humidity were obtained from the National Institute of Meteorology website (INMET, 2019), originated from a monitoring station located at UFJF, and are shown in Fig. 1.

2.3. Physiological measurements

Gas exchange was measured in the middle third of the first fully expanded leaf, between 8:00 and 11:00 am. Net photosynthetic rate (*A*), stomatal conductance (g_s), transpiration rate (*E*), and CO_2 internal content (*Ci*) were obtained using an IRGA LI-6400XT, (LI-COR, US) portable gas exchange analyzer. Water use efficiency (WUE) and Rubisco carboxylation efficiency (CE) were obtained from A/g_s (Cernusak, 2020) and *A/Ci* (Zambrosi et al., 2016) ratios, respectively. The CO_2 concentration in the chamber was kept constant at 400 μmol mol^{-1}, the temperature was set to 25 °C, and the light intensity (photosynthetic photon flux density) was set to 1000 μmol m^{-2} s^{-1}. At the same leaf of each plant and also using IRGA LI-6400XT stationary state (F_s) and maximum (F_m') chlorophyll *a* fluorescence in light-acclimated leaves were determined.

After 30 min of acclimation to dark, additional chlorophyll *a* fluorescence parameters were determined at the same leaves used to gas exchange measurements. A portable fluorometer Handy PEA (Hansatech Instruments, UK) was used for measuring values of minimum (F_o) and maximum (F_m) fluorescence, and these values were used to calculate maximum photochemical quantum yields of photosystem II (F_v/F_m) as $F_v/F_m=(F_m-F_o)/F_m$ (Zambrosi et al., 2016). The effective photochemical quantum yield of photosystem II (ϕPSII) was calculated as $\phi PSII=(F_m'-F_s)/F_m'$ (Zambrosi et al., 2016). Non-photochemical quenching (NPQ) was calculated as $NPQ=(F_m/F_m')-1$ (Rohacek, 2002). The photochemical quenching coefficient (qP) was calculated as $qP=(F_m'-F_s)/(F_m'-F_o')$ (Rohacek, 2002). The variable quantum yield of PSII was used to calculate linear electron transport rate (ETR) as $ETR=(F_m'-F_s)/F_m'*PPFD*0.5*0.88$; where PPFD is the photosynthetic photon flux density (White and Critchley, 1999).

The plant material necessary for physiological analysis in the laboratory was collected shortly after the end of non-destructive measurements on each plant. The same leaves used on gas exchange and chlorophyll *a* fluorescence measurements were collected to determine chlorophyll *a*, chlorophyll *b*, and carotenoids contents (Lichtenthaler, 1987). Approximately 0.1 g fragments of a fresh leaf of each plant were

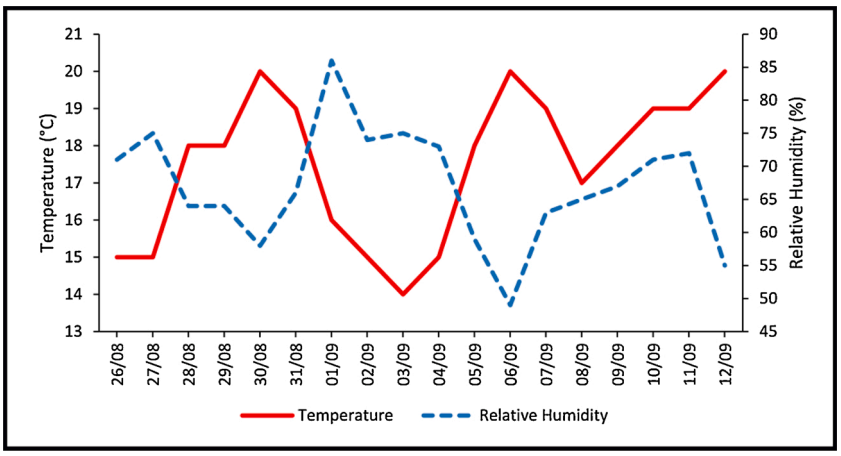

Fig. 1. Environmental conditions (temperature and relative humidity) during the experimental period.

held in darkness, weighed, and macerated in 80% acetone solution (v/v). The extract was filtered through filter paper and then completed to a final volume of 10 mL. Spectrophotometric measurements were performed using UV-1800 (Shimadzu, JP) spectrophotometer at 646, 663, and 470 nm, corresponding to absorptions peaks of chlorophyll *a*, chlorophyll *b*, and total carotenoids, respectively. From those results, total chlorophyll (chlorophyll *a*+chlorophyll *b*), and the ratios chlorophyll *a*/chlorophyll *b* and total chlorophyll/carotenoids were calculated. In addition, to express all content results, the dry mass was estimated using the expression dry mass=(1-water content)*weighed fresh mass.

One leaf (~300 mg) per plant was collected to conduct the water content (WC) determination assay. Three 0.89 cm^2 detached leaf discs were weighed on an analytical balance to obtain fresh mass weight (FM). Next, the leaf fragments were paper bagged and placed in a forced circulation oven at 65 °C until they reach a constant mass, obtaining the dry mass weight (DM). The WC was calculated as WC=(FM-DM)/(FM) and expressed in percentage (Morales et al., 2015).

2.4. Analysis of volatile organic compounds profile by gas chromatography and mass spectrometry (GC–MS)

All plant material necessary for chromatographic analysis was collected shortly after the end of non-destructive measurements on each plant and the analysis protocol was based on those described by Juliao et al. (2020) and Chaves et al. (2020) with modifications. One leaf (~300 mg and similar to those leaves used in physiological measurements) of each plant was collected to the determination of volatile organic compounds profile (VOCP). The material was macerated with 1.5 mL hexane and the samples were kept in an ultrasonic bath for 1 h at room temperature. Subsequently, the supernatant was filtered through a sterile cotton wick.

The hexanic extracts containing the volatile compounds to determinate the VOCP were analyzed using a gas chromatographer coupled to a mass spectrometer GCMS-QP2010 Plus (Shimadzu, CN) using an Rtx-5MS column (Restek, US) of 30 m ×0.25 mm. The oven temperature was started at 70 °C, maintained for 3 min, followed by an increase of 6 °C min^{-1} to 300 °C. The injector was operated in split mode (1:10) at 240 °C, and the interface and mass detector were operated at 300 °C. Helium was used as the carrier gas, with a flow of 1.53 mL min^{-1}. A standard mixture of linear hydrocarbons (C_9H_{20}, $C_{10}H_{22}$: … $C_{25}H_{52}$, and $C_{26}H_{54}$) was injected under the same conditions used for samples. The identification of constituents was performed by comparing the obtained mass spectra with those in NIST 9.0 database (correlation > 97 %) and confirmed by their retention indices (van Den Dool and Dec. Kratz, 1963), which were calculated for each constituent and compared with published data (Adams, 2005). Results were expressed using the normalization procedure, calculating the percentage of each peak area in relation to the total area of peaks under interests.

2.5. Experimental design and statistical analyses

The experiment was conducted in a completely randomized blocks design using a factorial scheme 2 (DIS times) x 3 (genotypes) with 3 replicates per treatment, totalizing 18 plants. All statistical analyses were performed using software R (R Development Core Team, 2009) and a probability of 5% (*p-value* ≤0.05) was considered. The experimental results were submitted to Two-way Analysis of Variance (Two-way Anova) and data normality was verified by the Shapiro-Wilk test. Subsequently, the results were compared by the Scott-Knott test (using the *ScottKnott* package) and the plots were made using the *ggplot2* package. Also, a linear model between *A* and g_s was developed. Integrative data analyses through principal component analysis (PCA) (using the *factoextra* package) and correlation network analysis (using the *qgrapgh* package with Pearson's correlation method) were also performed based on measures of physiological and VOCP, and physiological variables, respectively. The Fruchterman-Reingold algorithm was used in correlation network analysis, see Epskamp et al. (2012) for further information. The main physiological variables of the first two PCA principal components were correlated with VOCP using Pearson's correlation method.

3. Results

3.1. Physiological parameters

In statistical analysis about physiological traits, only DIS was a significant factor, meaning that differences between the genotypes were not a factor which influenced the response to drought stress in *L. alba*. Also, no interaction between these two factors was detected by Two-way Anova. Thus, the plants under 15 DIS were drought-stressed, because they presented decreased values of physiological traits when compared with the control plants. In addition, no block effects were found, meaning that the experimental design was efficient. Water deficiency conditions promoted significant and expressive changes in physiological parameters (Figs. 2–5). Reductions were observed in WC (15%), *A* (~4 times), g_s, (~8 times), *E* (~5 times), *Ci* (21%), CE (~3 times), F_v/F_m (5%), ϕPSII (50%), ETR (50%), qP (47%), chlorophyll *a* (36%), chlorophyll b (34%), total carotenoids (25%), total chlorophyll (35%), and chlorophyll/carotenoids ratio (14%) in stressed plants. On the other hand, WUE increased ~1.6 times, and NPQ 80%. Chlorophyll *a*/chlorophyll *b* ratio was not affected by drought stress. As shown in Fig. 6, the linear model between *A* and g_s was highly explanatory.

The correlation network analysis based on the physiological

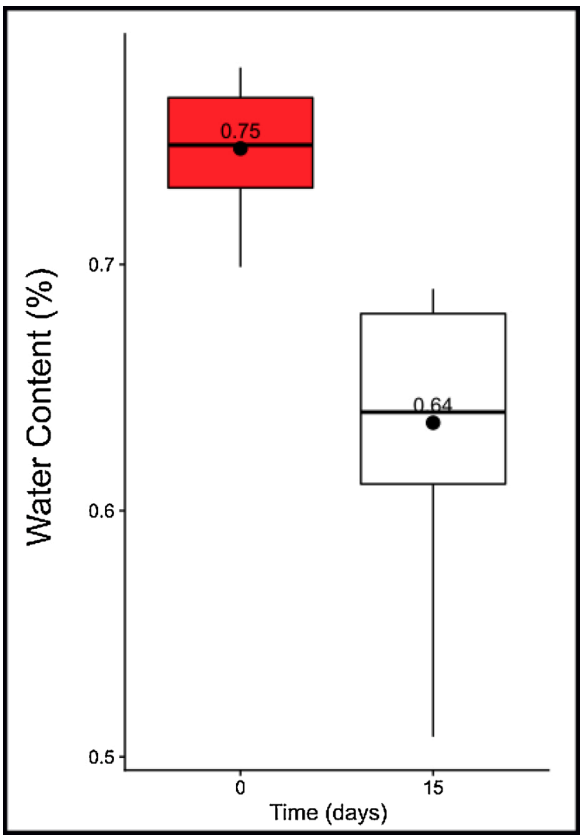

Fig. 2. Water content in three *Lippia alba* genotypes under two water regimes. Only water regime was a significant factor by Anova. Treatments with the same box color do not differ from each other, Scott-Knott test (*p-value*≤0.05). The points inside the boxes represent the mean values.

measures showed some interesting results, as the two main variables clusters which were formed (Fig. 7). The first cluster was composed of parameters related to photosynthetic pigments and F_v/F_m. Differently, the second cluster was composed of parameters related to gas exchange, the ones related to chlorophyll *a* fluorescence (F_v/F_m was an exception), and WC. Furthermore, NPQ was in opposition with all parameters included in the second cluster. At the cluster level, the first and the second clusters were related between them too, but not so strongly as inside of each one. It was also observed that Chlorophyll *a*/Chlorophyll *b* ratio, WUE, and *Ci* did not strongly cluster with any other parameters but it is clear that they were more proximal and correlated to the second cluster than to the first cluster. In addition, WUE was strongly and negatively correlated to *Ci*, while this one was weakly and positively correlated to g_s.

Using PCA, 79.4% of data total variance was explained. Regarding parameters clustering, they followed very similar trends as found in the correlation network analysis (Fig. 8.A). Principal component 1 (PC 1) and Principal component 2 (PC 2) explained 60% and 19.4% of the total variance, respectively. The parameters which composed the PC 1 were related to gas exchange and chlorophyll *a* fluorescence, and *A*, g_s, ETR, and ϕPSII were the most explanatory. On the other hand, PC 2 was mainly composed of parameters related to photosynthetic pigments and chlorophyll *b* content was the most explanatory. As seen in Fig. 8.B, the plants clustered according to their water regime and no genotype influence was noticed.

3.2. VOCP

On average, 96.21% of chemical compounds found in VOCP analysis were identified in each sample, totalizing 15 chemical constituents, and results are presented in Table 1. Only genotype was a significant factor in statistical analysis about VOCP, meaning that dehydration time was not a factor which influenced qualitatively the volatile compounds production in *L. alba*. No interaction between these two factors was also detected by Two-way Anova. Furthermore, no block effects were found, meaning that the experimental design was efficient.

All plants presented citral (neral+geranial) chemotype, regardless of genotype or dehydration time. The sum of total neral and geranial represented more than 70% of chemical constituents in all plants. Despite this, VOCP constituents showed specific patterns for each genotype as shown in Fig. 9. In VOCP PCA, 68.2% of data total variance was explained by PC 1 and PC 2. In addition, it was no possible to separate sabinene from its isomer β-thujene in the chromatograms. As can be seen in the scatter plot (Fig. 9.B) and following Two-way Anova results, DIS did not modulate VOCP and genotype was the only factor capable of doing it. Another result is that genotypes dip1 and dip2 (diploids) were more proximal between them than tet (tetraploid). In addition, it is important to highlight that tet produced more limonene, germacrene D, g-terpinene, elemol, and zingiberene than diploid genotypes (Fig. 9.A). On the other hand, diploid genotypes produced more caryophyllene oxide, β-myrcene, and β-ocimene than tet. About differences found between dip1 and dip2 genotypes, the first one produced more caryophyllene oxide, humulene, linalool, and β-ocimene, and the second one produced more selenene (Table 1). All identified chemical constituents were classified as mono- or sesquiterpenoids. Thus, all genotypes presented the same chemotype but different VOCPs.

The correlations between the main physiological variables of PC 1 and PC 2 (g_s and Chlorophyll *b*, respectively) and volatile compounds shown in Table 2 demonstrated that significant correlations occurred only between g_s and geranial and also between g_s and sabinene+β-thujene.

4. Discussion

It is widely described in the literature that polyploid genotypes are more vigorous than their diploid relatives in several aspects, including response to stresses (Sattler et al., 2016). Due to the larger number of gene copies in the nucleus, polyploid cells are bigger than diploid cells and it is called "gigas" effect. Often, the "gigas" effect leads to an enlargement of plant organs and this is related to vigor increase (Sattler et al., 2016; Doyle and Coate, 2019). Although, polyploid plants are not always more vigorous under stressful conditions than their diploid relatives, as observed in the *L. alba* genotypes here described. Thus, the effects of polyploidy are species-specific (Sattler et al., 2016). Furthermore, no pattern relating leaf morphology and polyploids in *L. alba* was observed by Viccini et al. (2014). Given that a) leaves were the plant organ used in the experiment here described, and b) plant physiology is closely related to plant morphology, it may explain why no different responses to drought stress at the same ploidy level (comparing genotypes dip1 with dip2) or different ploidy levels (comparing genotype tet with dip1 or dip2) were observed.

Photosynthetic and water status parameters are widely used as markers to evaluate plant performance under drought conditions (Zhou et al., 2019). In an integrative way, drought diminishes water content in plant cells, leading to a physiological adjustment across all plant tissues and it impacts directly on photosynthesis (Geilfus and Geilfus, 2019). Indeed, it was observed decreasing values of WC under drought conditions (Fig. 2), which is a common result as reported by Wu et al. (2016); Cheng et al. (2018), and Alvarenga et al. (2011) in *Dendrobium moniliforme* (L.) Sw., *Scutellaria baicalensis* Georgi (SBG), and *Lippia sidoides* Cham., respectively. Thus, leaf hydration is totally dependent on water availability in a directly proportional relationship, and to try to maintain WC adequate under drought stress, plants can use some physiological adjustment strategies to overcome that (Geilfus and Geilfus, 2019). The lowest *A* observed in stressed plants was followed by also lowest g_s, *E,* and *Ci* in the same treatment (Fig. 3). It is a flow: if stomata are more

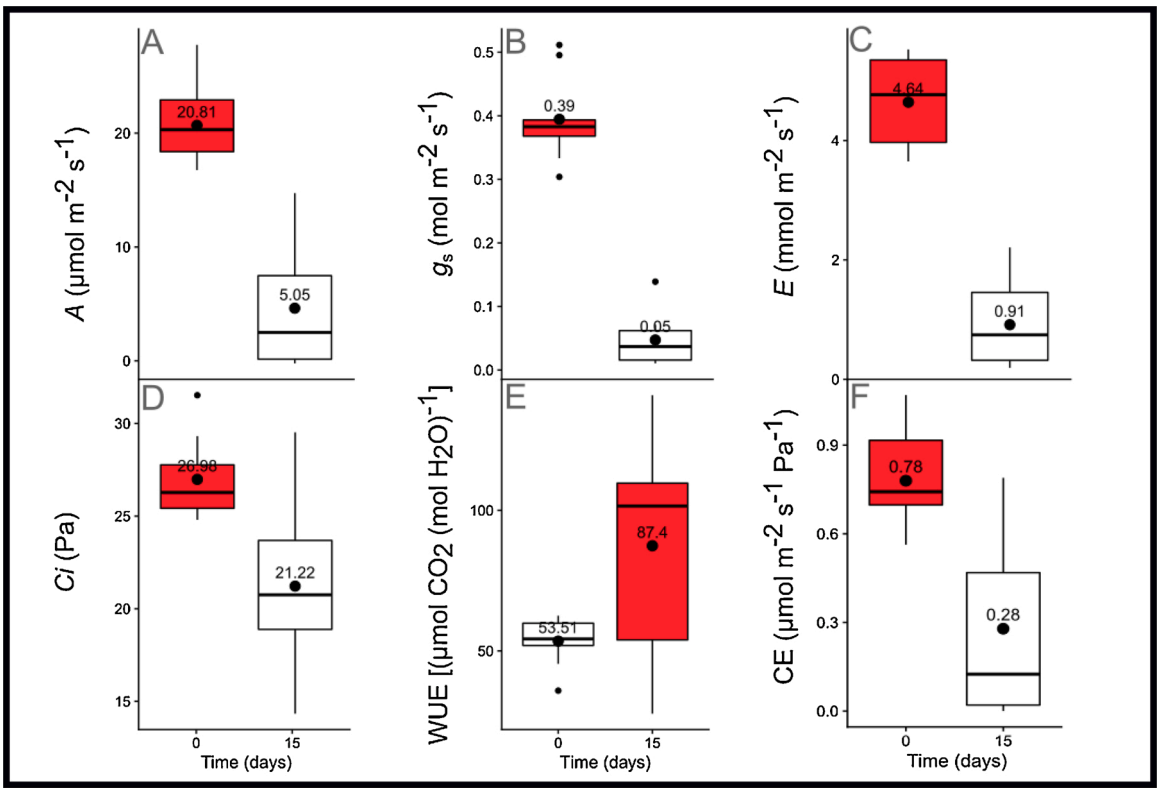

Fig. 3. Net photosynthesis (A), stomatal conductance (B), transpiration (C), CO_2 internal content (D), water use efficiency (E), and RUBISCO carboxylase efficiency (F) in three *Lippia alba* genotypes under two water regimes. Only water regime was a significant factor by Anova. Treatments with the same box color do not differ from each other, Scott-Knott test (*p-value*≤0.05). The points inside the boxes represent the mean values and those outside represent outliers.

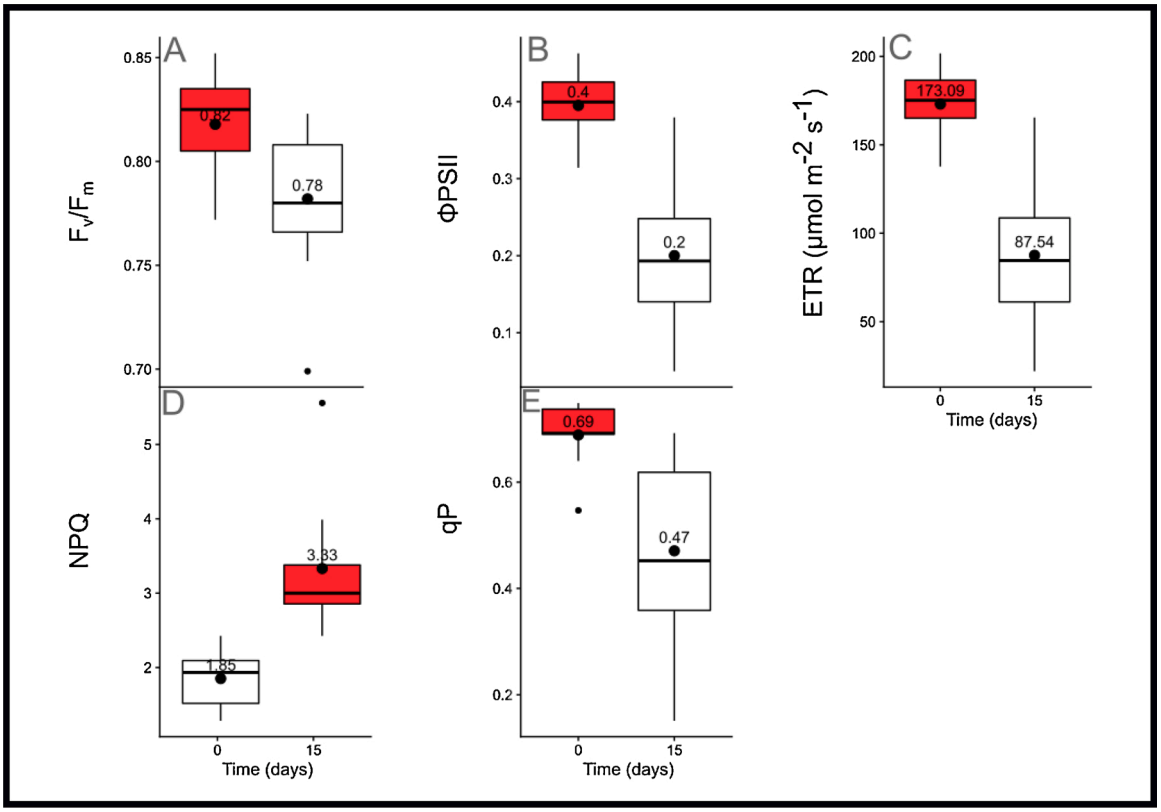

Fig. 4. Potential quantum yield of PSII (A), effective quantum yield (B), electron transport rate (C), non-photochemical quenching (D), and photochemical quenching (E) in three *Lippia alba* genotypes under two water regimes. Only water regime was a significant factor by Anova. Treatments with the same box color do not differ from each other, Scott-Knott test (*p-value*≤0.05). The points inside the boxes represent the mean values and those outside represent outliers.

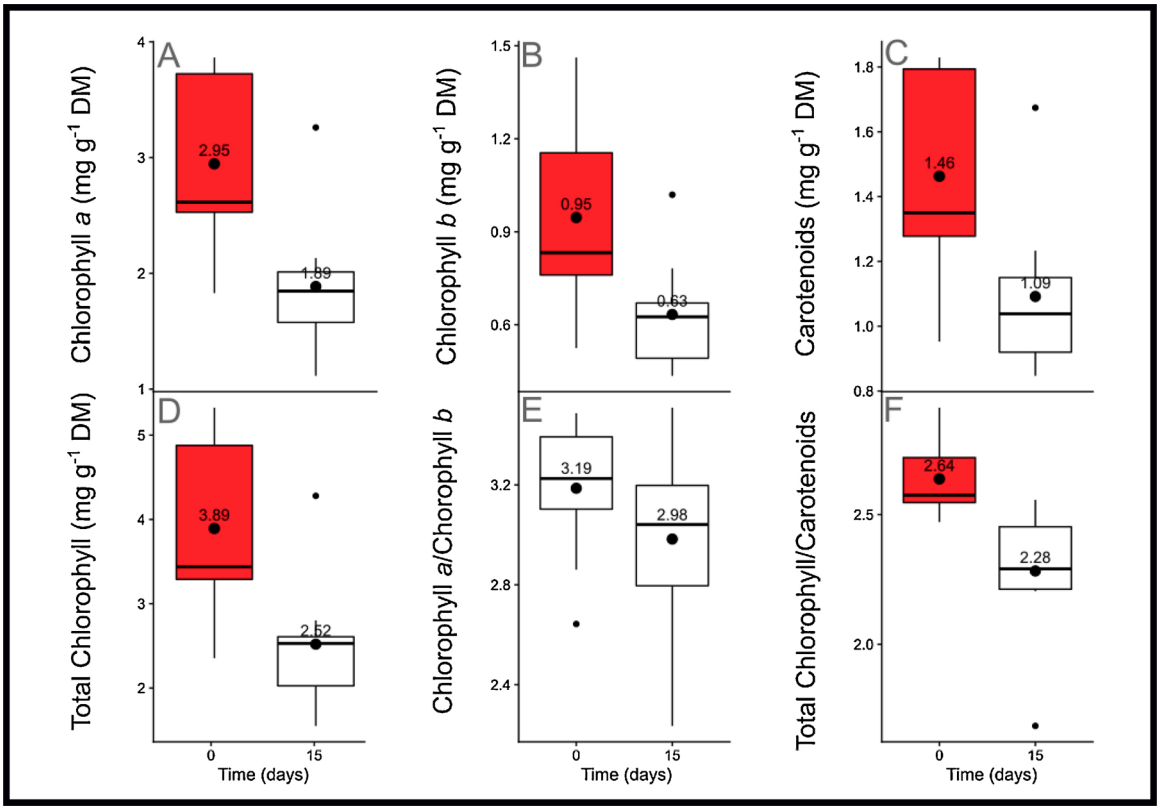

Fig. 5. Contents of chlorophyll *a* (A), chlorophyll *b* (B), carotenoids (C) and total chlorophyll (D), and the ratios chlorophyll *a*/chlorophyll *b* (D), and total chlorophyll/total carotenoids (E) in three *Lippia alba* genotypes under two water regimes. Only water regime was a significant factor by Anova. Treatments with the same box color do not differ from each other, Scott-Knott test (*p-value*≤0.05). The points inside the boxes represent the mean values and those outside represent outliers.

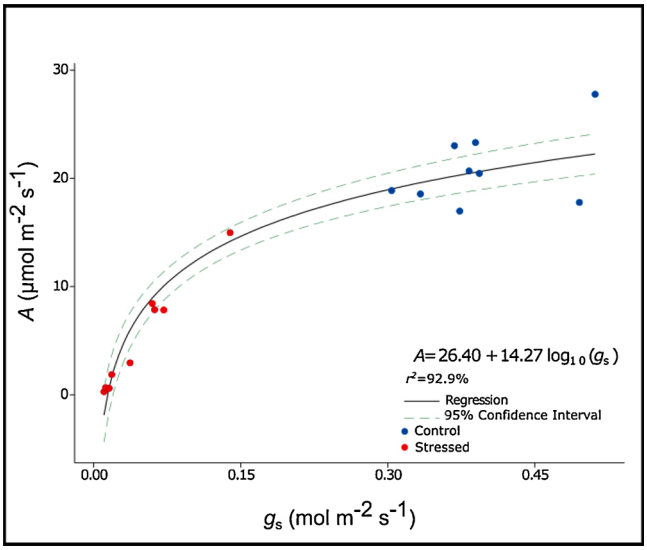

Fig. 6. Linear model between net photosynthesis (*A*) and stomatal conductance (g_s) in *Lippia alba* under two water regimes.

closed (↓g_s) then a plant will lose less water by *E*, but less CO_2 uptake will occur and lower *Ci* values will be observed. Since CO_2 is an essential molecule to the photosynthetic process, lower *A* will occur (Zhou et al., 2019). In addition, WUE increase under drought conditions is a common plant trait, given that *A* and g_s must be high and low as possible, respectively, to optimize carboxylation and water loss due to stomatal opening during carbon uptake (Cernusak, 2020). Also, CE was affected by photosynthesis limitation and some consequences occurred. If CE is diminished, the electron transport flow is inhibited due to chloroplast electron transport chain saturation, and ETR and ϕPSII values decrease (Baker and Oxborough, 2004). Then, a redox imbalance will occur along with reactive oxygen species accumulation and consequent damage to the photosynthetic apparatus, especially on D1 proteins present in photosystem II (Nishiyama et al., 2006). Thus, F_v/F_m and qP will decrease and NPQ will increase as a protection mechanism to minimize damages to photosystem II (dos Santos et al., 2019). This physiological flow related to chlorophyll *a* fluorescence was observed in the stressed plants results in comparison with the control plants (Fig. 4). Although drought depressed F_v/F_m, the mean value under drought was greater than 0.75, the acceptable down limit of this parameter to healthy plants (Bolhar-Nordenkampf et al., 1989). Thus, F_v/F_m decrease was a secondary photosynthesis limiting factor, occurred due to damage in the photosynthetic apparatus associated with stomatal closure and the redox imbalance that it causes.

Given that damage to the photosynthetic apparatus was detected, photosynthetic pigments contents were also affected. Chlorophylls and carotenoids are sensitive to reactive oxygen species attacks and it is common that both decrease under drought stress (Jaleel et al., 2009). Compared with the control plants, the stressed plants presented lower contents of all photosynthetic pigments (Fig. 5). As chlorophyll *a* and *b* contents diminished at similar proportions in the stressed plants, no imbalance in chlorophyll *a*/chlorophyll *b* ratio was observed due to drought stress. Differently, total chlorophyll/total carotenoids ratio was affected by drought stress and lower values were found in the stressed plants, since carotenoids reduced less than chlorophylls, suggesting that carotenoids content was less affected by reactive oxygen species attacks than the chlorophylls contents.

Correlation network analysis (Fig. 7) and PCA (Fig. 8), showed some interesting results. About variables, two main clusters were formed, one clustering gas exchange and chlorophyll *a* fluorescence parameters, and

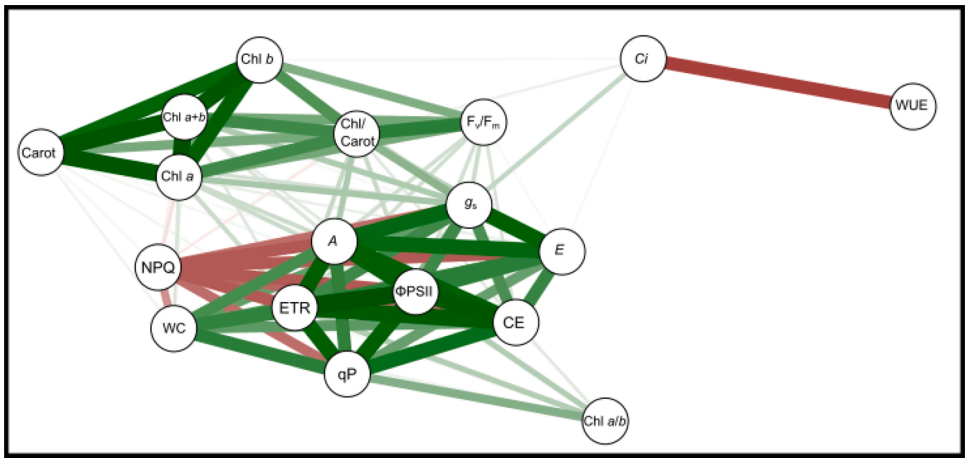

Fig. 7. Correlation network analysis of variables measured in three *Lippia alba* genotypes under two water regimes. Green and red lines represent positive and negative correlations, respectively. Line width is proportional to the correlation strength. Only significant correlations are shown (*p-value*≤0.05). *A*, net photosynthesis; *E*, transpiration; g_s, stomatal conductance; *Ci*, CO_2 internal content; WUE, water use efficiency; CE, RUBISCO carboxylation efficiency; F_v/F_m, maximum photochemical quantum yields of photosystem II; ϕPSII, effective photochemical quantum yield of photosystem II; NPQ, non-photochemical quenching; qP, photochemical quenching; ETR, electron transport rate; WC, water content; Chl *a*, chlorophyll *a*; Chl b, chlorophyll *b*; Chl a + b, total chlorophyll; Carot, carotenoids; Chl/Carot, total chlorophyll/total carotenoids; Chl a/b, chlorophyll *a*/chlorophyll *b*.

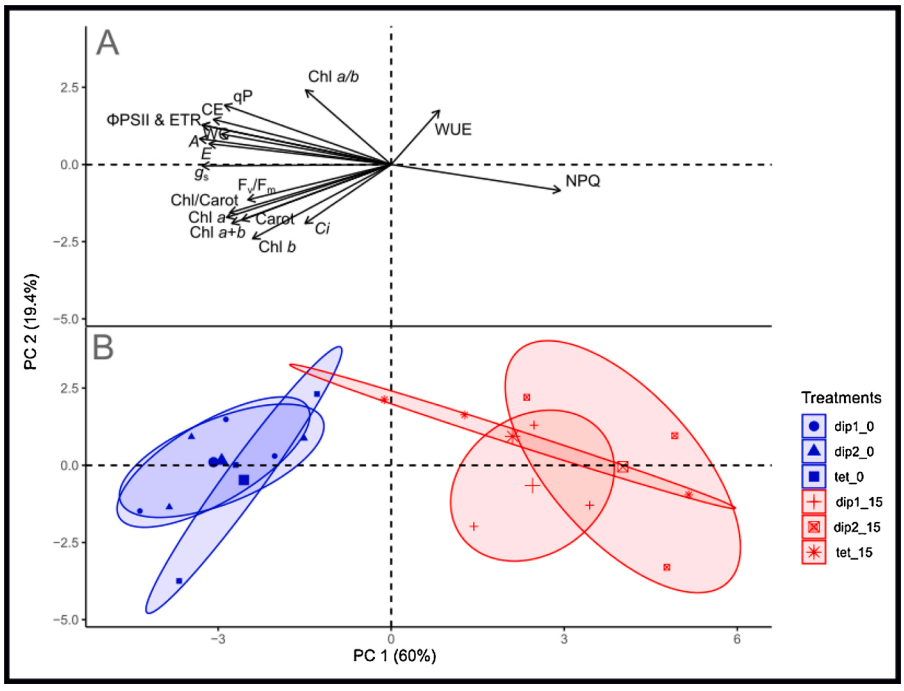

Fig. 8. Biplot showing variables (A) and individuals (B) of physiological variables measured in three *Lippia alba* genotypes under two water regimes. Treatments ID: "genotype"_"days of irrigation suspension". *A*, net photosynthesis; *E*, transpiration; g_s, stomatal conductance; *Ci*, CO_2 internal content; WUE, water use efficiency; CE, RUBISCO carboxylation efficiency; F_v/F_m, maximum photochemical quantum yields of photosystem II; ϕPSII, effective photochemical quantum yield of photosystem II; NPQ, non-photochemical quenching; qP, photochemical quenching; ETR, electron transport rate; WC, water content; Chl a, chlorophyll *a*; Chl b, chlorophyll *b*; Chl a + b, total chlorophyll; Carot, carotenoids; Chl/Carot, total chlorophyll/total carotenoids; Chl a/b, chlorophyll *a*/chlorophyll *b*.

another grouping photosynthetic parameters. Indeed, gas exchange and chlorophyll *a* fluorescence parameters are totally correlated to all photosynthetic processes, covering aspects from CO_2 diffusion (g_s) to its carboxylation (CE) and passing by light absorption (qP and NPQ) and electron flux (ETR) in chloroplasts. On the other hand, the photosynthetic pigments here analyzed demonstrated how their contents were affected by drought stress, which will probably affect photosynthesis but with no physiological flow perspective, contrary to what can be seen on the first parameters cluster. Also, PCA demonstrated in a multivariate scale how the different genotypes responded to drought stress. Those results suggested the same verified on univariate analysis: genotypic variations were not a significant factor in *L. alba* plants in terms of their physiological acclimation to drought stress.

The linear model where *A* was plotted as a dependent variable of g_s showed a logarithmic curve (Fig. 6). Indeed, that kind of relationship is a common plant aspect (Cernusak, 2020) and demonstrated that the stressed plants had more control over its stomatal dynamics than the control plants, increasing WUE. A very similar result was found by Singh and Raja Reddy (2011) studying the regulation of gas exchange parameters in cowpea (*Vigna unguiculata* (L.) Walp.) under drought. Thus, since g_s a) was the parameter that was more affected by drought, as shown by Anova (reduced ~8 times, see Fig. 3A), b) showed a tight correlation with *A*, as shown by PCA and correlation network analysis (Figs. 7 and 8), and c) highly explained *A* variance, as shown by the fitted linear model (Fig. 6), it was clear that g_s was the major limitation to *A* under drought conditions in *L. alba*, thus demonstrating a stomatal limitation of the process.

Secondary metabolites as mono- and sesquiterpenoids are involved in plant interactions with the environment and they are important in the acclimation process to stressful conditions (Caser et al., 2019; Alinejad et al., 2020). Then, it is reasonable to think that drought stress could modulate VOCP in *L. alba*, but it did not happen (Fig. 9 and Table 1). Similar results were found for Verbenaceae by Alvarenga et al. (2011); de O Cruz et al. (2014), and Prochnow et al. (2017) studying the effects of drought stress in *L. sidoides*, *Lippia gracilis* Schauer, and *Aloysia triphylla* (L'Herit.) Britton, respectively. Alves et al. (2018) also did not find changes in the essential oil profile of *L. alba* cultivated under different colored shading nets and nitrogen doses. Batista et al. (2017)

Table 1
Contents of volatile organic compounds in three *Lippia alba* genotypes under two water regimes. Only genotype was a significant factor by Anova. Means followed by the same letter in the rows do not differ from each other, Scott-Knott test (p-value≤0.05).

Compound	Content in tet (%)	Content in dip1 (%)	Content in dip2 (%)
Caryophyllene oxide	0.00b	4.98a	1.60b
p-Cymene	1.64a	2.57a	0.96a
Elemol	0.68a	0.00b	0.00b
Geranial	55.25a	52.34a	53.82a
Germacrene D	6.38a	1.54b	3.47b
g-Terpinene	5.48a	2.83b	0.92b
Humulene	0.08b	0.50a	0.21b
Limonene	5.51a	0.03b	0.06b
Linalool	0.54b	0.74a	0.37b
Neral	22.53a	21.12a	21.51a
Selenene	0.27b	0.61b	1.44a
Zingiberene	0.55a	0.00b	0.00b
β-Myrcene	0.16b	6.08a	6.16a
β-Ocimene	0.15c	1.77a	1.02b
Sabinene+β-Thujene	0.43a	0.57a	0.45a
Total	99.65	95.68	91.99

observed that elevated CO_2 *in vitro* modulates some volatile organic compounds production in *L. alba*, but with no changes in chemotype, and similar results were found by de de Castro et al. (2020) studying how osmotic stress influences essential oil production in *L. alba* grown *in vitro*. Viccini et al. (2014) demonstrated that genotype (in terms of ploidy level) is a factor that determines chemotype in *L. alba*. Thus, the results observed and found in the literature suggested that volatile compounds production in *L. alba* is strongly regulated by genetic factors instead of environmental conditions, contrary to what was observed in the physiological traits. Furthermore, PCA (Fig. 9) showed that diploid genotypes were more proximal between them than the tetraploid genotype. That result suggested that even in polyploidy levels with the same chemotype clusters can be formed due to minor constituents of VOCP, but more evidence is needed to confirm this hypothesis.

Even though VOCP was not modulated by the water regime, g_s was correlated to the contents of geranial and sabinene+β-thujene in direct and inverse proportions, respectively (Table 2). Geranial, sabinene, and β-thujene are monoterpenoids derived from geranyl diphosphate, being geranial an oxygenated monoterpenoid (Mochizuki et al., 2020). Since sabinene and β-thujene have a similar molecular structure, this feature prevented the separation of both in the analyzed chromatograms. As described in the literature, sabinene production is stimulated during water stress events in sage (*Salvia officinalis*L.) (Radwan et al., 2017). Similarly, β-thujene production is stimulated in drought-tolerant thymus (*Thymus vulgaris* L.) plants during periods of low water availability (Mahdavi et al., 2020). In agreement with the results found in *L. alba*, Said-Al Ahl et al. (2009) also found that water stress decreased geranial content in lemon balm (*Melissa officinalis* L.). In fact, terpenoids can act as antioxidant compounds, playing an important role against reactive oxygen species in stressful conditions to plants (Mahdavi et al., 2020). Thus, the relationship between sabinene+β-thujene and geranial with

Table 2
Correlations between stomatal conductance (g_s), chlorophyll *b* content, and volatile organic compounds in three *Lippia alba* genotypes under two water regimes. Outside the parentheses, the *r-value* is represented, and inside the parentheses, the *p-value* is represented.

Compound	g_s	Chlorophyll *b*
Caryophyllene oxide	−0.24 (0.34)	0.37 (0.13)
p-Cymene	−0.29 (0.24)	−0.02 (0.93)
Elemol	0.09 (0.72)	−0.07 (0.77)
Geranial	0.48 (0.04)	0.08 (0.76)
Germacrene D	0.12 (0.63)	−0.35 (0.16)
g-Terpinene	0.01 (0.96)	0.06 (0.81)
Humulene	−0.13 (0.61)	0.22 (0.39)
Limonene	0.08 (0.76)	0.01 (0.97)
Linalool	−0.12 (0.64)	0.10 (0.68)
Neral	−0.29 (0.24)	−0.04 (0.86)
Selenene	0.26 (0.30)	−0.17 (0.50)
Zingiberene	−0.17 (0.50)	−0.17 (0.49)
β-Myrcene	−0.15 (0.54)	0.03 (0.90)
β-Ocimene	0.12 (0.64)	0.23 (0.35)
Sabinene+β-Thujene	−0.49 (0.04)	−0.01 (0.98)

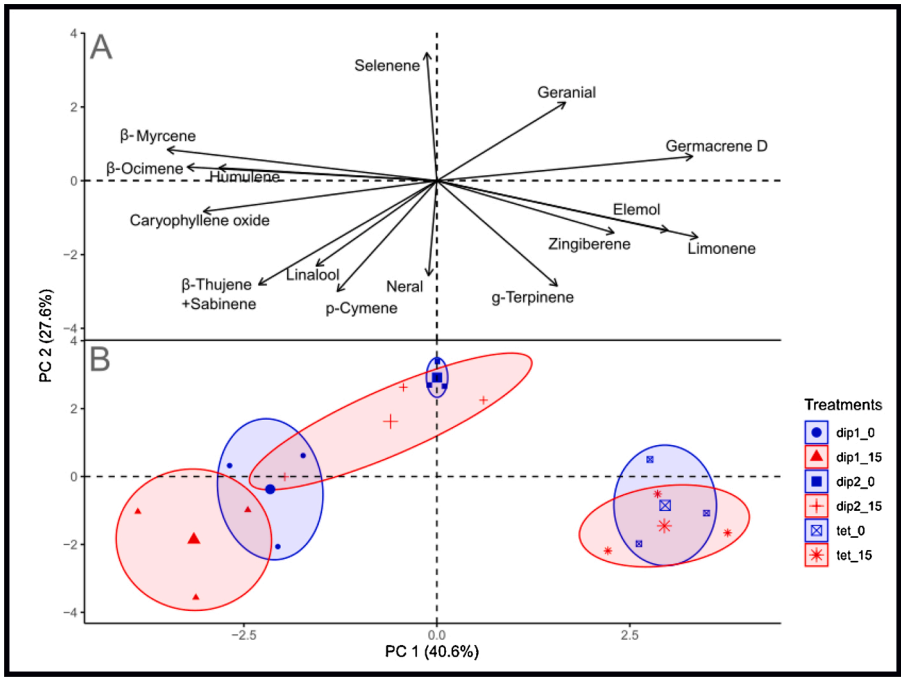

Fig. 9. Biplot showing variables (A) and individuals (B) of volatile compounds measured in three *Lippia alba* genotypes under two water regimes. Treatments ID: "genotype"_"days of irrigation suspension".

water stress needs to be further clarified in order to understand how drought stress modulates their production and also within a perspective of using them as markers of plant sensitivity or tolerance to drought.

Briefly, the results indicated that a) drought stress negatively affected physiological traits related to WC, gas exchange, chlorophyll *a* fluorescence, photosynthetic pigments, and b) the response to that stressful condition was independent of *L. alba* genotype, even comparing diploid with tetraploids genotypes. But the same did not occur to the VOCP, which was strongly determined by genotype free of drought stress influence. Also, gas exchange and chlorophyll *a* fluorescence parameters were the most sensitive to drought stress. More specifically g_s is recommended to be evaluated in *L. alba* breeding programs focused on drought stress tolerance, given that *A* was highly regulated by stomatal limitation.

CRediT authorship contribution statement

Jober Condé Evangelista Freitas: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization, Project administration. **Cristiano Ferrara de Resende:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision. **Vinícius Sacramento Pacheco:** Conceptualization, Methodology, Resources, Writing - review & editing. **Richard Michael Grazul:** Methodology, Investigation, Resources, Writing - review & editing. **Leandro Elias Morais:** Conceptualization, Writing - review & editing. **Leônidas Paixão Passos:** Conceptualization, Writing - review & editing, Supervision. **Paulo Henrique Pereira Peixoto:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2020.113137.

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