RESEARCH ARTICLE

Nitrogen in the defense system of *Annona emarginata* (Schltdl.) H. Rainer

Felipe Girotto Campos[1], **Maria Aparecida Ribeiro Vieira**[1], **Amanda Cristina Esteves Amaro**[2], **Iván delaCruz-Chacón**[3], **Marcia Ortiz Mayo Marques**[4], **Gisela Ferreira**[1], **Carmen Sílvia Fernandes Boaro**[1]*

1 Instituto de Biociências, UNESP: Universidade Estadual Paulista, Campus Botucatu, Departamento de Botânica, Botucatu, São Paulo, Brazil, **2** Faculdade de Ciências Agronômicas, UNESP: Universidade Estadual Paulista, Campus Botucatu, Departamento de Horticultura, Botucatu, São Paulo, Brazil, **3** Laboratorio de Fisiología y Química Vegetal, Instituto de Ciencias Biológicas, Universidad de Ciencias y Artes de Chiapas (UNICACH), Tuxtla Gutiérrez, Chiapas, Mexico, **4** Centro de Pesquisa de Recursos Genéticos Vegetais, Instituto Agronômico (IAC), Campinas, São Paulo, Brazil

* csfboaro@ibb.unesp.br
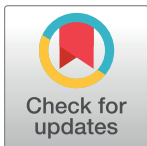
Abstract

The concentration of nitrogen can generate different strategies in plants in response to stress. In this study, we investigated how nitrogen concentration interferes with the defense system of *Annona emarginata*. Low concentrations of nitrogen increased the allocation of photosynthetic resources to carbon metabolism, resulting in an increase in the synthesis of volatile substances involved in signaling and defense that contributed to antioxidant enzymes in overcoming stress. The availability of nitrogen at 5.62 mM concentration might have helped to induce increased resistance in the plants because at this concentration, signaling substances and defense substances (monoterpenes and sesquiterpenes) were observed. Plants cultivated with the highest nitrate concentration displaced energy for the reduction of this ion, likely forming nitric oxide, a signaling molecule. This condition, together with the decrease in carbon skeletons, may have contributed to the lower synthesis of volatile substances of the specialized metabolism that are also involved with signaling. Varying the nitrogen in *Annona emarginata* cultivation revealed that depending on the concentration, volatile substances show higher or lower synthesis and participation in the system of signaling and defense in the plant. These results may suggest that volatile substances participate in resistance to pests and diseases, which is a necessary condition for *Annona emarginata* to be preferentially used as rootstock for *Annona* x *atemoya*.

Introduction

Plants require high amounts of nitrogen (N) because this element is involved in the biosynthesis of substances of the primary and specialized metabolisms [1]. In these metabolisms, substances formed during photosynthesis and nitrogen assimilation are converted into proteins, nucleic acids, lipids, chlorophyll, phenylpropanoids, flavonoids, terpenes, and alkaloids, which are important for adaptation to biotic and abiotic stresses [2].

Plants grown under low N levels do not express their complete genetic potential, which can interfere with their physiology [3]. N deficiency may cause altered allocation of assimilated carbon (C), leading to changes in the amounts of leaf starch, sucrose, and monosaccharides [4,5] and reductions in the amount and carboxylation activity of Rubisco. The ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme requires 30% N for its synthesis [6–8]. Thus, N deficiency can affect the biochemical phase of photosynthesis, altering the availability of carbon skeletons [9] used in the synthesis of carbohydrates and amino acids [4]. However, high N levels can also affect carbon metabolism, particularly when N is in the form of nitrate, which requires reducing agents for its assimilation, thereby competing with the assimilation of C [2,10].

The concentration and source of N are critical for a plant to develop effective defenses against pathogens. Nitrate may improve plant defenses through the signal molecule nitric oxide (NO) [11]. The enzymes peroxidase (POX), superoxide dismutase (SOD) and catalase (CAT) may indicate increased accumulation of reactive oxygen species (ROS), which signals growth processes, thereby accelerating the growth of plants [12]. *Catharanthus roseus* cultivated with nitrate showed increased activity of POX and SOD, and when the source was ammonium, CAT activity increased [13].

Increased activity of superoxide dismutase, peroxidase, and catalase control reactive oxygen species, which interfere with the maintenance of membrane integrity through lipid peroxidation. However, the presence of ROS acts on physiological processes such as growth and signaling for the production of defense molecules [14–16].

Reactive oxygen species not controlled by the enzymatic antioxidant system can trigger signals that intensify the production of molecules of the specialized metabolism, helping to neutralize and prevent structural damage at the membrane level [16–18].

Volatile substances are a mix of terpenes, phenolic molecules, and fatty acid derivatives, among others, which may vary in number and quantity when plants are subjected to climatic and nutritional changes and according to the phenological phase of the species at a given time [19]. Mono- and sesquiterpenes may be involved in plant defense pathways against biotic and abiotic stress [20,21] and aid the antioxidant enzymes in the neutralization of reactive species [22]. Fatty acid derivatives are also involved in signaling events of stress [23] and may be altered by variations in the concentration of nitrogen. Low nitrogen availability can signal stress, triggering the biosynthesis of substances involved in overcoming stress, such as fatty acid derivatives and jasmonic acid [1,23–25].

The incorporation of nitrogen and the biosynthesis of terpenes, including sesquiterpenes and monoterpenes, share the same reaction sites and are potential competitors for carbon skeletons and reducing agents [26]. Thus, variations in the concentration of nitrogen can result in changes in the availability of carbon skeletons and reducing agents that are used in the biosynthesis of hydrocarbons [4,27].

The biosynthesis of monoterpenes occurs via the 2-C-methylerythritol 4-phosphate pathway in plastids, particularly in the chloroplasts. Sesquiterpene biosynthesis occurs via the mevalonic acid pathway in the cytoplasm [28]. The biosynthesis of fatty acid derivatives also occurs in the plastids through the lipoxygenase (LOX) and hydroperoxide lyase (HPL) pathways [1].

The process of nitrate reduction to ammonia and incorporation requires much energy [4]. In this reduction, the conversion of nitrate to nitrite occurs in the cytoplasm by the action of the enzyme nitrate reductase. Subsequently, the nitrite is reduced to ammonia in the chloroplast by the enzyme nitrite reductase. NH_4 is incorporated by the enzymes glutamine synthetase and glutamate synthase into carbonic chains through reactions that require reducing agents generated during the acyclic photochemical phase of photosynthesis [7,27].

Thus, the availability of nitrogen can influence the dynamics of specialized metabolite expression and the release of volatile substances, an important condition in plant-animal relations [29]. Plants of *Zea mays* var. Delprim, which are preyed on by the beet armyworm, *Spodoptera exigua*, showed an increase in the release of volatiles as the nitrogen supply decreased [30]. The addition of nitrogen to cotton plants decreased the concentration of leaf volatiles, and the larvae of *S. exigua* and *Cotesia marginiventris* (Cresson) were not repelled [29].

The biosynthesis of these substances depends on the availability of carbon (C), nitrogen (N) and energy supplied by the primary metabolism. As a consequence, the availability of these building blocks greatly affects the concentration of a specialized metabolite, demonstrating a high degree of connectivity between the primary and specialized metabolisms [1].

Nitrate may interfere with primary metabolism, and whether availability is high or low will influence the specialized metabolism and defense system. Thus, adequate concentrations can cause the production of signaling molecules that allow plants to acclimatize and overcome stress. When availability is low, the targeting of resources to the production of specialized metabolites can also assist in plant defense.

Annona emarginata has physical and chemical characteristics that make it resistant to various pests and diseases and, due to its compatibility with the hybrid *Annona* x *atemoya*, which produces fruit with high commercial value, is used as its rootstock [31]. Volatile substances should be highlighted among the chemical characteristics that contribute to the strength of *A. emarginata* rootstock. The concentration of nitrogen can influence primary metabolism, such as hydrocarbon biosynthesis, which generates the raw material for the synthesis of volatile substances. Nitrogen concentration variation can help to show how this element contributes resistance to pests and diseases of *A. emarginata*.

In this study, we investigated how nitrogen concentration influences interferes with the defense system of *Annona emarginata*.

Materials and methods

Plant species and N concentrations

Seedlings of *Annona emarginata* (Schltdl.) H. Rainer variety 'terra-fria' were brought from São Bento do Sapucaí, São Paulo, Brazil, which is located at 45°44′11″ W, 22°41′18″ S and 874 m above sea level. The seedlings were grown in a paddy-fan greenhouse at 26 ± 4°C located at 48°24′35″ W, 22°49′10″ S and 800 m above sea level at the Instituto de Biociências, São Paulo State University, Campus Botucatu, São Paulo. The experiment was a factorial design with four N levels and five harvests.

The plants were grown in 6-L pots containing Hoagland & Arnon's nutrient solution n°. 1, using nitrate as the source of nitrogen [32] at 7.5 mM N, modified to provide N levels of 5.62, 3.75, and 1.87 mM (S1 Table). Plants were maintained until harvest at 150, 164, 172, 192, and 206 days after beginning treatment (DBT).

The temperature and relative humidity recorded in the greenhouse during the gas exchange evaluations are shown in S2 Table.

Gas exchange

Gas exchange was measured from 9:00 a.m. to 11:00 a.m. using a LI-6400 portable photosynthesis system with an infrared radiation analyzer for CO_2 and water vapor [Infrared Gas Analyzer (IRGA); Li-Cor Inc., Nebraska, USA]. The measurements, which were performed on the 2nd or 3rd leaves with completely expanded blades, were used to obtain estimates based on the difference between the air CO_2 and water vapor levels for the reference and those of the

sample. The levels of water vapor and CO_2 released and assimilated by the leaf stomata were then determined.

The CO_2 assimilation rate (A_{net}, μmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol water vapor m^{-2} s^{-1}), and stomatal conductance (g_s, mol m^{-2} s^{-1}) were determined. Water use efficiency [WUE, μmol CO_2 (mmol H_2O^{-1})] was calculated using the ratio between CO_2 assimilation and transpiration rates (A_{net}/E). The apparent carboxylation efficiency was calculated according to the ratio between the CO_2 assimilation rate and the intercellular CO_2 concentration of the leaf (A_{net}/C_i, mol m^{-2} s^{-1} Pa^{-1}) [33].

The photosynthetic potential was evaluated using CO_2 response curves (A_{net}/C_i curves) [34] at 192 DBT under a saturating irradiance of 1,200 μmol m^{-2} s^{-1} photons, in accordance with a previous experiment on this species. The curves were fitted according to the Sharkey model [35] after calculating the maximum carboxylation rate of ribulose-1,5-bisphosphate carboxylase/oxygenase (V_{cmax}, μmol CO_2 m^{-2} s^{-1}), the photosynthetic electron transport rate (J_{max}, mmol electrons m^{-2} s^{-1}), and the respiratory rate (Rd^*, μmol CO_2 m^{-2} s^{-1}).

Net assimilation and relative growth rate

The plants were separated into leaf blades, stems plus petioles, and roots. After determining the leaf area, leaf weight, and total dry weight, the physiological indices net assimilation rate (NAR) and relative growth rate (RGR) [36] were determined following Portes and Castro Jr. [37].

Nitrate reductase activity

For the measurement of nitrate reductase activity, leaves were kept on ice until the analysis, as described by Jaworski [38].

Leaf carbohydrate and total amino acid concentrations

The extraction of total soluble sugars was performed as described by Garcia *et al.* [39] with minor modifications, and starch extraction was conducted according to Clegg [40].

The procedure for determining the concentration of total soluble sugars was conducted according to Morris [41], that for starch as described by Yemm and Willis [42], that for reducing sugars as determined by Miller [43], that for sucrose as established by Passos [44] and that for free amino acids as described by Yemm and Cocking [45].

The activity of antioxidant enzymes and lipid peroxidation

The extraction of antioxidant enzymes was performed as described by Kar and Mishra [46]. The activities of the enzymes superoxide dismutase, EC 1.15.1.1, and catalase, EC 1.11.1.6, were determined by the method of Peixoto et al. [47]; the activity of peroxidase, EC 1.11.1.7, was established according to Teisseire and Guy [48]; and the total soluble proteins were quantified as described by Bradford [49].

Lipid peroxidation was determined according to Heath and Packer [50], according to Devi and Rama Prasad [51].

Analysis and identification of the volatile substances

Dried leaf blades were used for the extraction of volatiles in plants subjected to different concentrations of nitrogen to determine the volatile substance profile.

For this purpose, the leaves were dried in an air-flow oven at 40°C. The volatiles were captured by headspace solid-phase microextraction mode with an SPME Fiber Assembly [75 μm of carboxen/polydimethylsiloxane (CAR/PDMS)] for use with a manual holder (Supelco).

Leaves (250 mg of dry mass) were placed in a glass vial (10 mL) with distilled water (5 mL), and the vial was sealed. The mixture was heated in a water bath at 90°C for 1 h. After this period, the fiber was exposed to the headspace for 15 min. The volatiles were immediately desorbed at 220°C, separated and detected using gas chromatograph coupled to a mass spectrometer (GC-MS-Shimadzu, QP-5000) GC-MS.

The chemical composition of the volatile substances extracted from the leaves was determined by GC-MS (Shimadzu, QP-5000) with electron impact (70 eV); detector at 230°C; a fused silica capillary column, DB-5 (30 m × 0.25 mm × 0.25 μm); helium as the carrier gas (flow 1.0 mL min^{-1}); a temperature program of 60°C (2 min) and then 60–240°C at 3°C min^{-1}; a split ratio of 1/20; and a flow rate of 1 mL min^{-1}. The identification of substances was performed by comparison of their mass spectra with the GC-MS system database (Nist. 62 Libr.), literature [52] and retention indices (RI). To obtain the RI of the substances, a mixture of *n*-alkanes (C_9-C_{24}; Sigma Aldrich 99%) was employed and analyzed under the same operating conditions as those of the samples, and the Van den Dool & Kratz equation was used [53].

All procedures were conducted from 9:00 a.m. to 11:00 a.m. with completely expanded leaves.

Statistical analyses

The data were subjected to analysis of variance (ANOVA), and means were compared using Tukey's test at the 5% probability level; regression analysis was also performed. Using the SAS statistical software package 9.2 (SAS Institute Inc., Cary, NC), Levene's test was used to test the homogeneity of variances in the treatments [54].

The effects of nitrogen on available volatiles were analyzed in relation to the number of molecules in the profile (profile density) in general or based on the group to which the substances belonged (monoterpenes, sesquiterpenes, and fatty acid derivatives) and in relation to the relative abundances of these substances. The profile of the volatiles was also expressed as the phytochemistry diversity index using the Shannon index (H).

The diversity index of the total volatiles (signaling and defense substances (mono- and sesquiterpenes)) was calculated using the following formula: H = −Σ (pi ln pi), where pi = the proportion of substance i in relation to the total volatile substances (relative abundance of the substance i) calculated by ni/N, where ni = the relative percentage of substance i and N = the sum of the percentage of total substances.

The diversity indices (H index) and the number and relative percentages of substances were subjected to ANOVA, and the means were compared using Tukey's test at the probability level of 5%. Using SigmaPlot 12.0 (Systat Software, Inc. SigmaPlot for Windows), Levene's test was applied to test the homogeneity of variances across treatments.

Principal component analysis (PCA), Pearson's correlation coefficient and hierarchical cluster analysis (HCA), and similarity and Pearson's coefficient (UPGMA) were performed with the relative percentages for substance identification at the different nitrogen concentrations and evaluation time points, and a different hierarchical cluster analysis (Ward's algorithm, index of dissimilarity of Euclidian distance) was performed using the values of the chemical diversity at the four concentrations of nitrogen and evaluation time points by the software XLSTAT (2017).

Results

Gas exchange

The plants subjected to intermediate N levels (5.62 and 3.75 mM) showed a high CO_2 assimilation rate (A_{net}), stomatal conductance (g_s), transpiration rate (E), and carboxylation efficiency

(A_{net}/C_i) throughout the experimental period (Fig 1). These plants also showed a high maximum carboxylation velocity (V_{cmax}) and respiratory rate (R_d^*) at 192 DBT (Table 1). For the plants grown with 7.5 mM N, the values of V_{cmax}, J, and R_d^* were high (Fig 1 and Table 1).

Net assimilation and relative growth rates

The net assimilation and relative growth rates decreased over time in the plants grown with 1.87, 3.75, and 5.62 mM N but increased in those grown with 7.5 mM N (Fig 2). The leaf-specific weight varied slightly among the different treatments and increased from 192 DBT onward in the plants grown with 7.5 mM N (Fig 2).

Nitrate reductase activity

The activity of nitrate reductase increased over time in plants grown with 1.87 mM N but with less activity ($p \leq 0.05$) at 164 and 192 DBT (Fig 3).

The plants grown with 3.75 and 7.5 mM N showed an increase in nitrate reductase enzyme activity until 164 DBT (Fig 3), whereas the plants grown with 5.62 mM N showed an increase in nitrate reductase activity over time, with increased activity at 192 and 206 DBT ($p \leq 0.05$; Fig 3).

Leaf total amino acid concentration

Overall, for the plants grown with 1.87 mM N, the amino acid content decreased over time (Fig 4), whereas the plants grown with 3.75, 5.62, and 7.5 mM N showed an increase at 192 DBT ($p \leq 0.05$).

Leaf carbohydrate concentration

Generally, the plants grown with 1.87 mM N presented the highest total soluble sugar concentration ($p \leq 0.05$), which decreased over time, and showed high and constant values of starch and reducing sugars (Fig 5).

The plants grown with 3.75 mM N presented low concentrations of total and reducing sugars at 164 and 178 DBT ($p \leq 0.05$). The starch concentration increased over time but with reduced concentrations at 150 and 178 DBT ($p \leq 0.05$; Fig 5). The plants grown with 7.5 mM N showed low accumulation of total and reducing sugars at 164 and 178 DBT ($p \leq 0.05$) and a constant concentration of starch (Fig 5).

Although the carbohydrate concentration of plants grown with 5.62 mM N was relatively stable over time (Fig 5), the starch concentration decreased at 178 DBT (Fig 5).

The sucrose concentration remained constant over time and did not differ among the treatments (Fig 5).

The activity of antioxidant enzymes and lipid peroxidation

In general, SOD activity increased at 192 DBT in all plants, regardless of the nitrate level. The highest activity was in the plants grown with 7.5 mM N, compared with the activity in those grown with 5.62 mM N ($p \leq 0.05$; Fig 6).

The plants grown with 1.87 mM N showed the highest POX activity at 192 DBT ($p \leq 0.05$; Fig 6).

CAT activity was the highest in plants grown with 1.87 mM N at 192 DBT and the lowest in plants grown with 5.62 mM N ($p \leq 0.05$; Fig 6). Lipid peroxidation did not change with the different nitrate levels ($p \leq 0.05$; Fig 6).
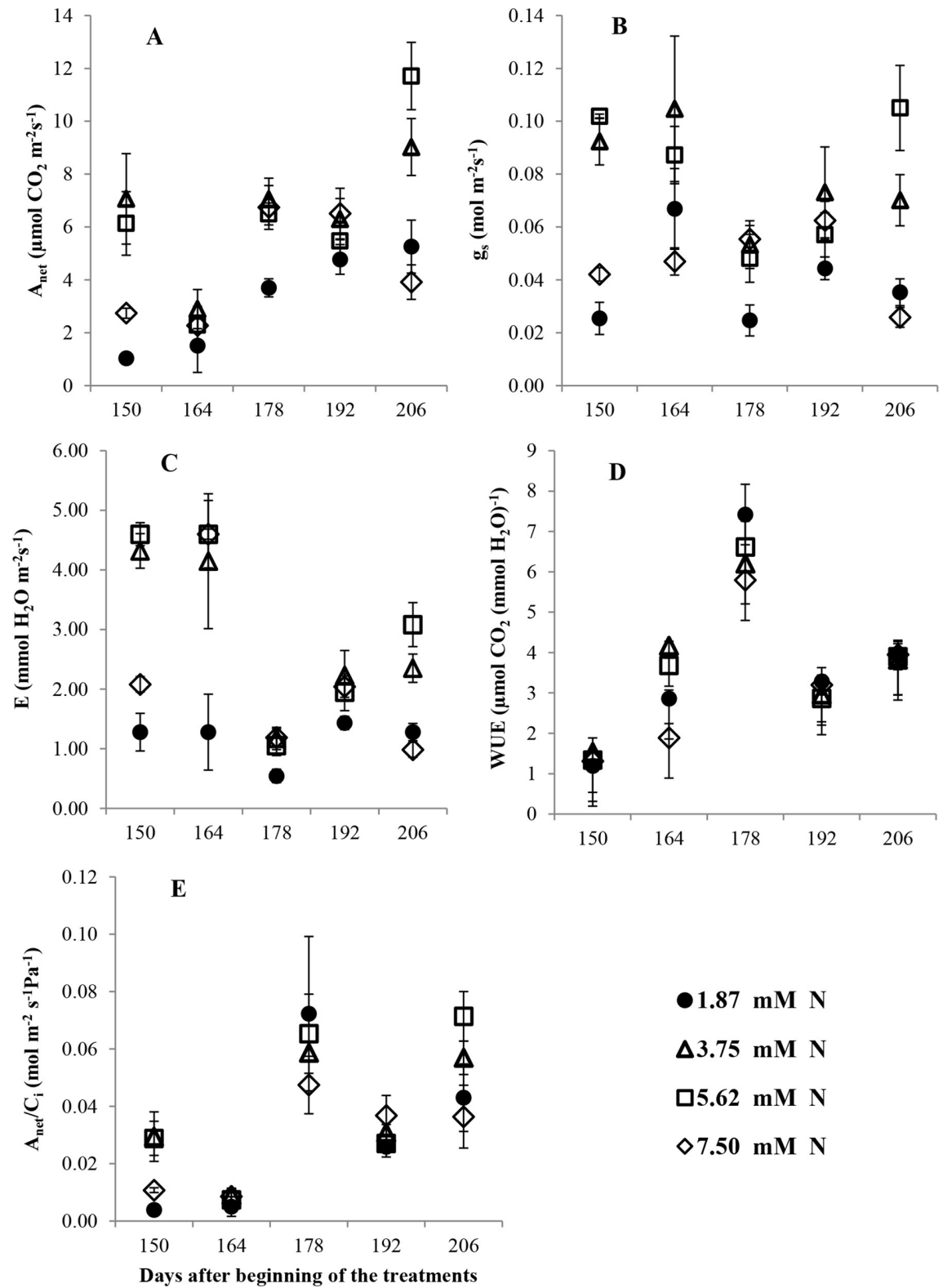
Fig 1. (A) Net assimilation rate (A_{net}, µmol CO_2 m^{-2} s^{-1}); (B) stomatal conductance (g_s, mol m^{-2} s^{-1}); (C) transpiration (E, mmol water vapor m^{-2} s^{-1}); (D) water-use efficiency [*WUE*, µmol CO_2 (mmol H_2O^{-1})]; and (E) apparent carboxylation efficiency (A_{net}/C_i, mol m^{-2} s^{-1} Pa^{-1}) in *Annona emarginata* subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment (DBT). Data are presented as the mean ± SE ($n = 4$).

Table 1. Photosynthetic potential of *Annona emarginata* grown under different nitrogen concentrations at 192 days after beginning treatment.

Nitrogen Concentration			
	V_{cmax}	J	Rd^*
1.87 mM	74.00 ± 15.52 b	98.33 ± 19.50	2.57 ± 0.99 b
3.75 mM	103.67 ± 22.01 ab	85.67 ± 11.85	2.93 ± 0.71 b
5.62 mM	129.67 ± 17.50 a	106.33 ± 9.50	4.96 ± 0.12 a
7.50 mM	134.67 ± 1.53 a	102.33 ± 0.58	5.19 ± 0.72 a

Data are presented as the mean ± SE ($n = 3$).

https://doi.org/10.1371/journal.pone.0217930.t001

Analysis and identification of volatile substances

Forty-eight substances were identified in the profile of volatiles from the leaves of *A. emarginata* cultivated at varying nitrogen concentrations (Table 2). Volatiles were classified as signaling and defense substances (mono- and sesquiterpenes). Signaling substances were the volatile substances with the lowest molecular mass detected in the leaves of *A. emarginata*. The relative abundances of these volatiles were low, but they increased when the nitrate concentration decreased (F = 18.117, $p \leq 0.001$). In general, in the high-nitrate treatments (7.5 and 5.62 mM N), the sum of the values was close to 0.5 and 1.5%, respectively, and in the low-nitrate treatments (1.87 and 3.75 mM N), these leaf volatiles reached 3% relative abundance (Table 2).

Defense substance monoterpenes were the second most abundant class of volatile substances in the leaves of *A. emarginata*. The abundance of monoterpenes varied with nitrate concentration and time of evaluation (F = 16,308, $p \leq 0.001$). The highest nitrate content (7.5 mM N) resulted in a low relative percentage of monoterpenes of 6.27, 7.25 and 4.61% at 164, 192 and 206 days after beginning treatment, respectively. The lowest concentration of nitrate (1.87 mM N) showed an increase in monoterpenes to 21.57 and 16.39% at 150 and 206 DBT (Table 2).

Defense substance sesquiterpenes contained the most substances (up to 31) and were the most abundant class (approximately 80%). The density of the sesquiterpene class was also influenced by nitrate concentration and time of evaluation (F = 22.670, p ≤ 0.001; Table 2).
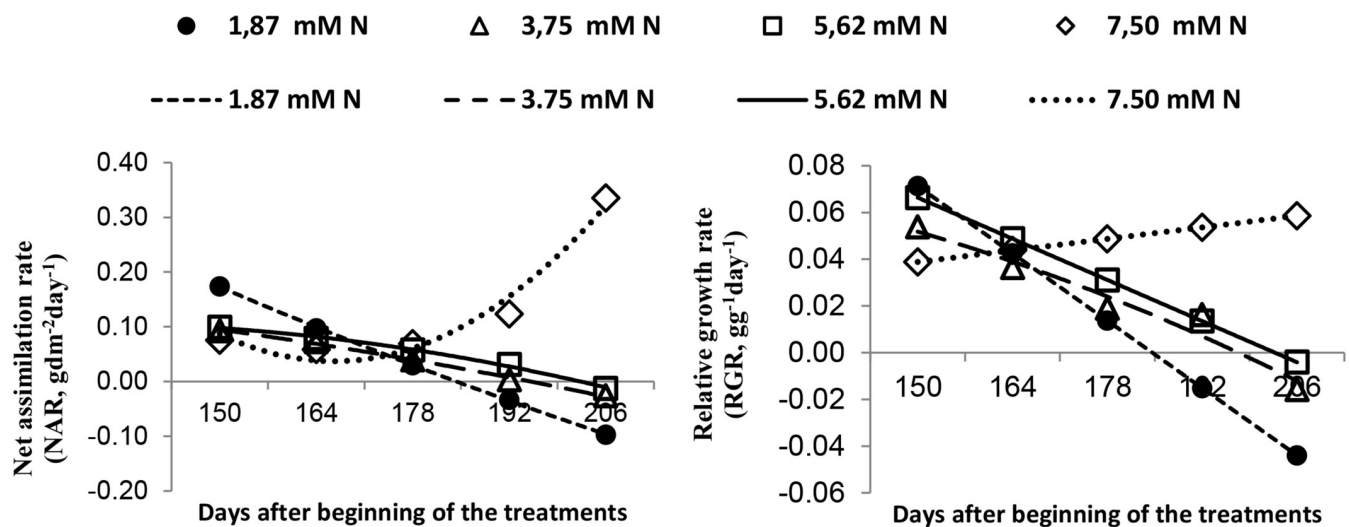

Fig 2. (A) Net assimilation rates (NAR, dm^2 g); (B) relative growth rates (RGR, g g^{-1} day^{-1}); and (C) leaf-specific weight (LSW, dm^2 g^{-1}) of *Annona emarginata* subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment (DBT). S1 Fig.

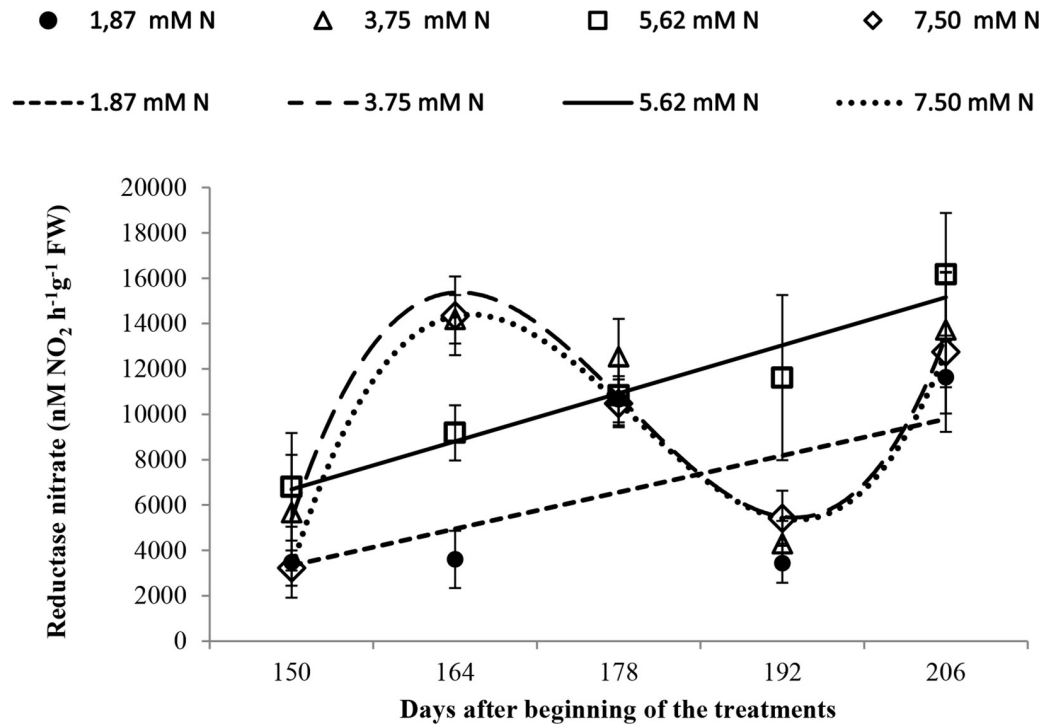https://doi.org/10.1371/journal.pone.0217930.g002

Fig 3. Nitrate reductase activity in *Annona emarginata* leaves subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment (DBT). Data are presented as the mean ± SE (*n* = 4). S2 Fig.

https://doi.org/10.1371/journal.pone.0217930.g003

The most frequent and abundant sesquiterpenes were *trans*-caryophyllene, bicyclogermacrene and γ-gurjunene (Table 2 and Fig 7).

The diversity indices (H) for *A. emarginata* with different concentrations of nitrate confirmed that the species produced greater phytochemical diversity under low concentrations of nitrate and at later evaluation times. The volatile substance indices were high in the treatments with low nitrate (3.75 and 1.87 mM), particularly at the last evaluation. At 206 days, the low nitrate treatments were 16 and 18% more diverse (H: 2.66 and 2.71, respectively) than the treatment with the highest concentration of nitrate (H: 2.3; Table 3).

The principal component analysis (PCA) of volatile substances of *A. emarginata* grown at different concentrations of nitrate and evaluated at different times after beginning the treatments explained 95.61% of the chemical variation. Three primary substances were responsible for discriminating among nitrate concentrations and time points: *trans*-caryophyllene, γ-gurjunene, and bicyclogermacrene (Fig 8).

The grouping analysis of the primary volatile substances in *A. emarginata* resulted in the formation of three clusters. Cluster I grouped all nitrate concentrations at 164 DBT with other time points. Cluster II constituted the three lowest nitrate concentrations at the first evaluation time point. Cluster III grouped all the concentrations and the three latest evaluation times (Figs 8 and 9). Twenty-five primary substances were responsible for these groupings and are shown in Fig 7.

Discussion

The use of nitrate as a source of nitrogen variation in *A. emarginata* caused changes in the leaf volatile profile as a consequence of changes observed in the primary metabolism, which contributed to the species' defense system.
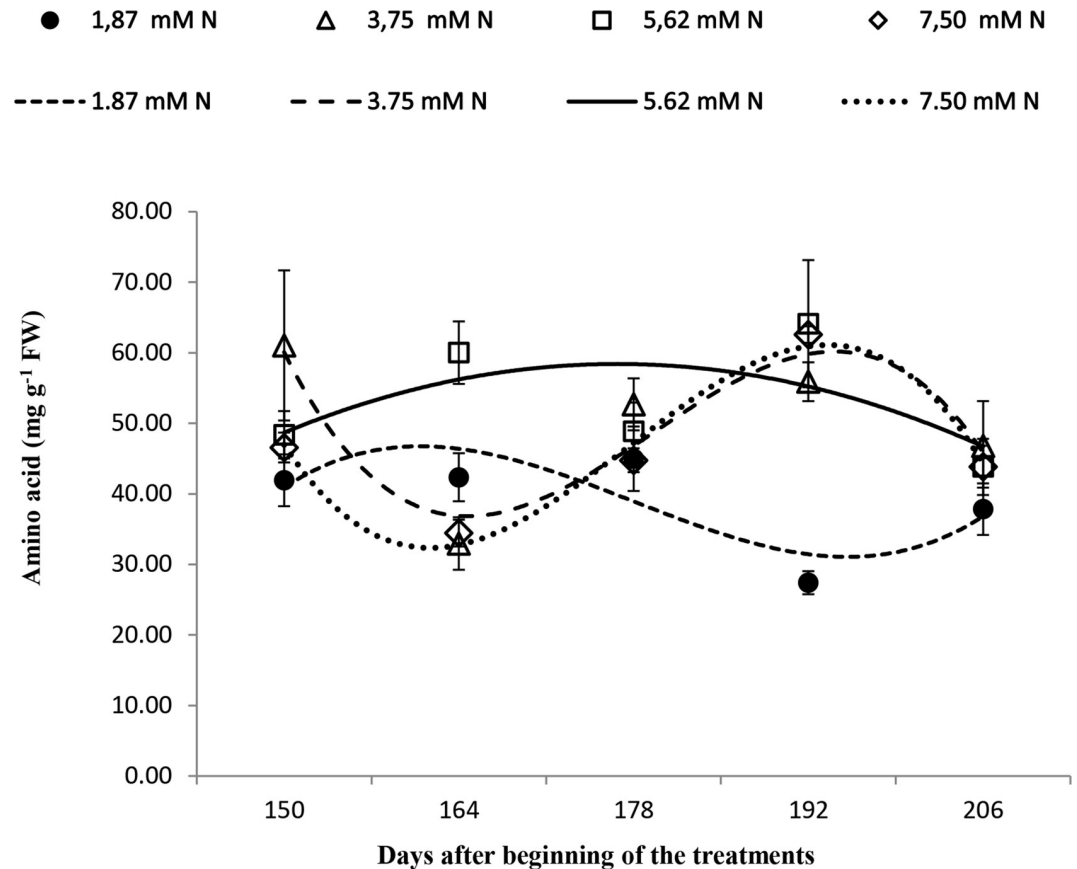
Fig 4. Leaf amino acid concentration of *Annona emarginata* subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment (DBT). Data are presented as the mean ± SE (n = 4). S3 Fig.

The high activity of nitrate reductase observed in *A. emarginata* plants grown with 5.62 mM N with their photosynthetic potential determined by the A_{net}/C_i curve at 192 DBT helped to explain the high respiratory rate (Rd *), which might require nicotinamide adenine dinucleotide (NADH+H$^+$) for nitrate reduction [55–58]. The plants cultivated at 5.62 mM N, presented constant activity of the antioxidant enzymes SOD, POX, and CAT and volatile signaling (n-nonanal, 6-methyl-5-hepten-2-one, hexyl acetate) and defense (mono- and sesquiterpenes) substances, whose action in the control of lipid peroxidation may have prevented membrane damage and allowed photosynthetic acclimatization. This would be revealed by the rapid increase in carbohydrate and amino acid concentrations as a consequence of the efficient use of reducing agents and carbon skeletons in the metabolism of carbon and nitrogen in *A. emarginata*. Thus, the availability of nitrogen at 5.62 mM N improved the resistance of *A. emarginata* because at this concentration, signaling substances were observed, with an increased number of monoterpenes and a high relative percentage of sesquiterpenes, which resulted in a high diversity index (H) at two time points. The volatile substances associated with the activity of the antioxidant enzymes might be related to the ability of nitrate to induce defense pathways of *A. emarginata* [11]. A concentration equal to 5.62 mM N can allow *A. emarginata* to be resistant to pests and diseases, which is the rootstock most common for *Annona x atemoya*.

The POX and CAT in *A. emarginata* grown with 3.75 mM N showed low activity, indicating that the increase in the relative percentage of the signaling substances optimized signaling
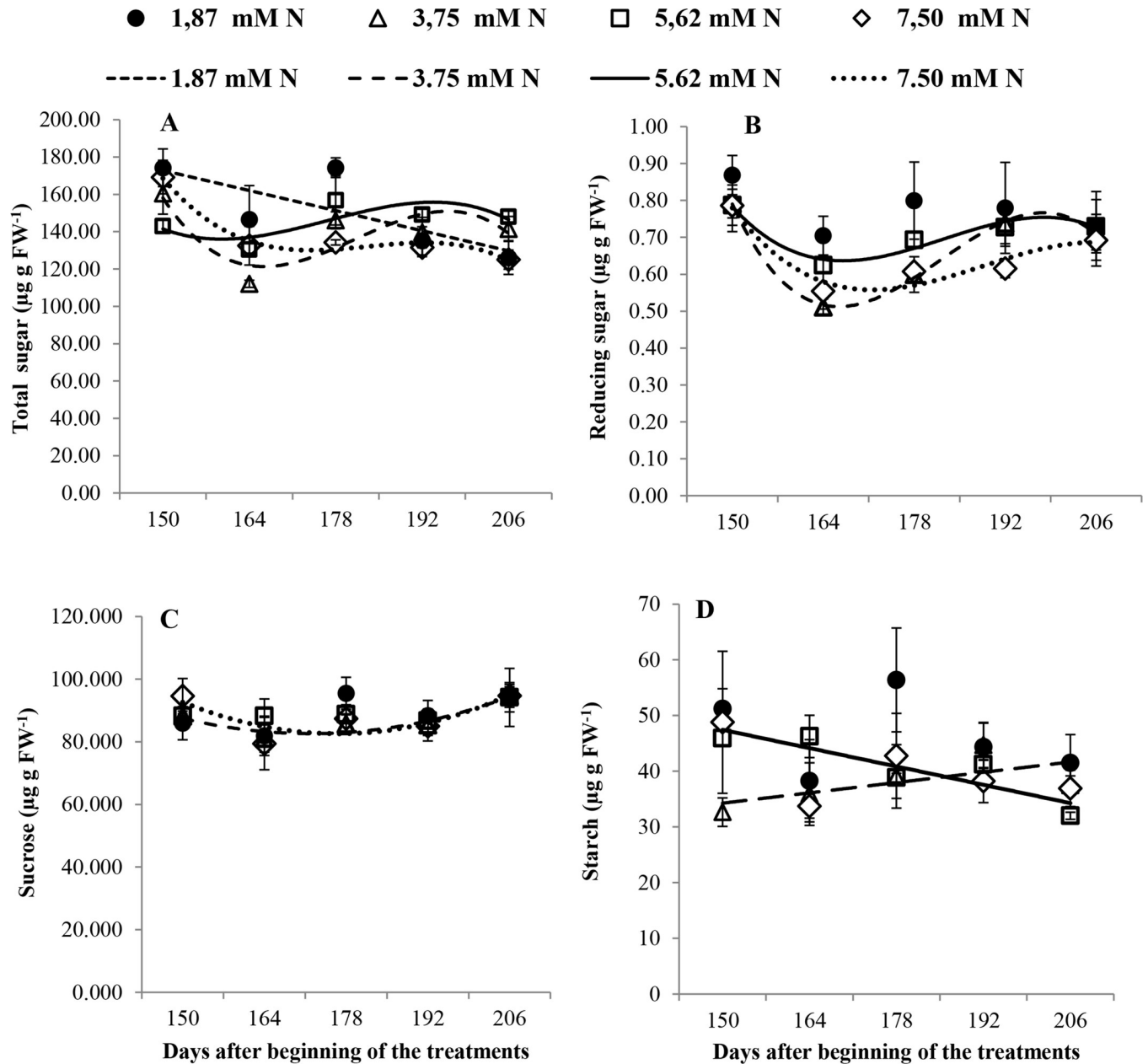
Fig 5. (A) Total sugar (µg g FW^{-1}); (B) reducing sugar (µg g FW^{-1}); (C) sucrose (µg g FW^{-1}); and (D) starch (µg g FW^{-1}) in *Annona emarginata* subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment (DBT). Data are presented as the mean ± SE (n = 4). **S4 Fig**.

and the response to stress [23]. Mono- and sesquiterpenes in these plants might have aided the antioxidant enzymes in the neutralization of reactive oxygen species, maintaining stable lipid peroxidation levels [20,59,60].

The plants grown with 1.87 mM N presented low nitrate reductase activity, resulting in the lowest free amino acid accumulation, high carbohydrate accumulation observed in the leaves until 178 DBT, and accelerated growth, as shown by the increase in the slopes of the NAR and RGR lines. These outcomes might be the result of the highest presence of ROS of all concentrations, as indicated by the high activity of antioxidant enzymes, particularly POX and CAT, at
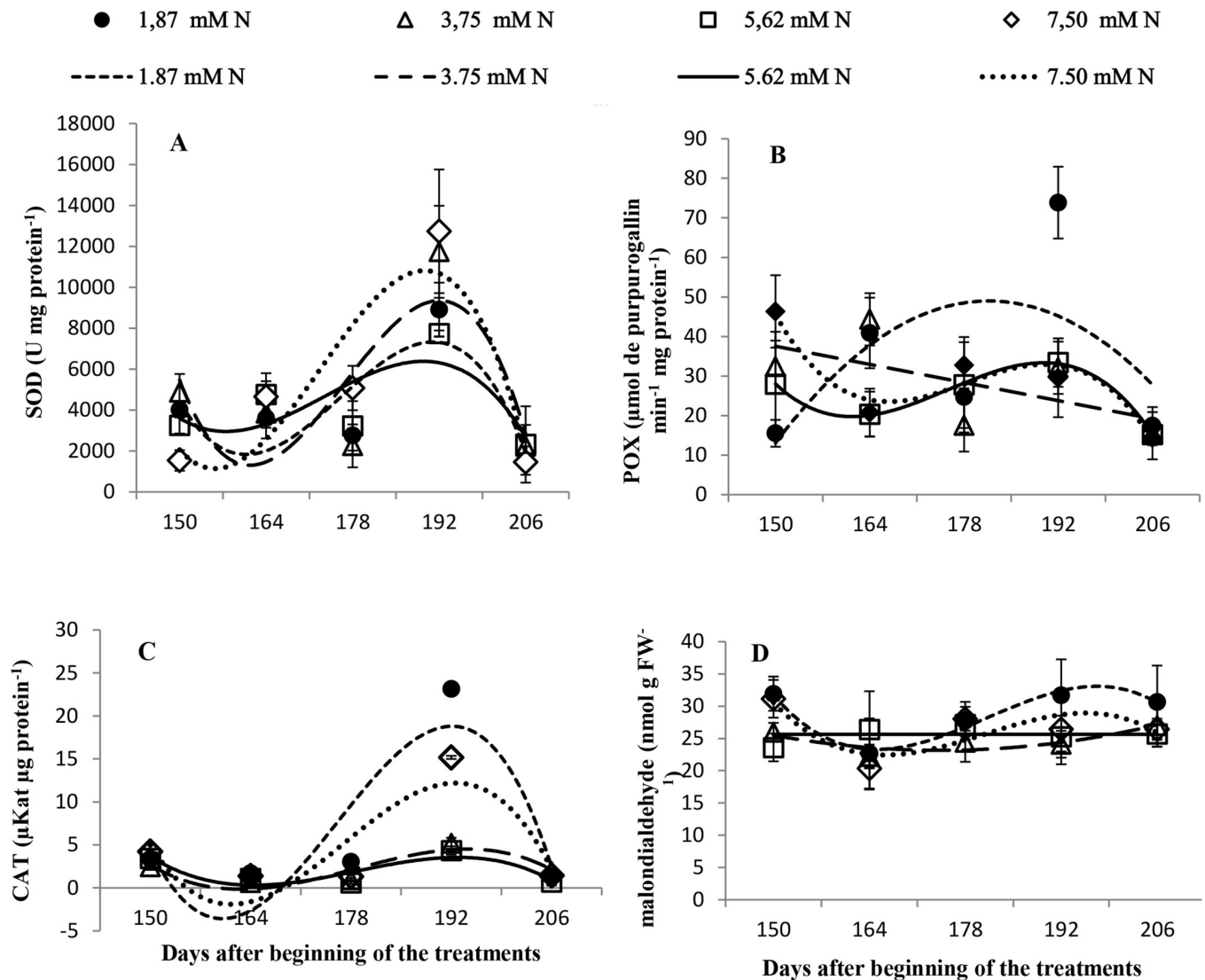
Fig 6. (A) Lipid peroxidation (MDA, malondialdehyde nmol g^{-1} of FW); (B) superoxide dismutase (SOD, U mg^{-1} protein); (C) peroxidase (POX, μmol of purpurogallin min^{-1} mg^{-1} protein); and (D) catalase (CAT, μKat μg^{-1} protein) of *Annona emarginata* subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment (DBT). S5 Fig.

https://doi.org/10.1371/journal.pone.0217930.g006

192 DBT. ROS are powerful signaling molecules involved in plant growth control [12] that prevent plants from vegetating and accelerating their growth [61,62].

The low growth triggered by the low concentration of nitrogen contributed to the targeting of reducing agents and carbon skeletons to increase the volatile substances and carbohydrates, in particular, starch. The proportions of monoterpenes and signaling substances increased, as shown by the diversity index (H), contributing to the defense of *A. emarginata* to reverse stress (66) once levels of lipid peroxidation remained stable.

A high nitrate supply (7.5 mM N) can increase cell nitrite concentrations in *A. emarginata*, which induce nitrate reductase to reduce nitrite to nitric oxide using NADPH+H$^+$ as an electron donor [63–65]. Nitric oxide affects several physiological processes in plants, including stomatal closure, which might explain the low CO_2 assimilation observed. Additionally, the high nitrate reductase activity detected at 164 DBT led to increased amino acid accumulation and

Table 2. Mean relative percentages and number of volatiles of *A. emarginata* subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment (DBT).

	Nitrogen concentration (mM)																			
	7.5					5.62					3.75					1.87				
	150	164	178	192	206	150	164	178	192	206	150	164	178	192	206	150	164	178	192	206
Signaling substances																				
Heptanal	0bB	0bB	0bC	0.25aA	0bC	0aB	0aB	0aC	0aB	0aC	0bB	0bB	0.34 aB	0.27aA	0.28aA	0.39aA	0.35aA	0.43aA	0cB	0.19bB
n-nonanal	0bC	0bB	0bC	0.26aB	0bB	0.40bB	0.81aA	0.65 abB	0.18 cB	0.47aA	0.51 bAB	0.67 abA	0.49 bB	0.72aA	0.64 abA	0.62bA	0.69bA	1.15 aA	0.29cB	0.45 bcA
n-decanal	0aB	0aB	0aC	0aB	0aB	0aB	0aB	0aC	0aB	0aB	0bB	0bB	0.24aB	0.25aA	0.17aA	0.18bA	0.23bA	0.87aA	0.28bA	0.25bA
6-methyl-5-hepten-2-one	0aB	0aB	0aC	0aB	0aC	0cB	0.27abA	0.31aB	0.00cB	0.17bB	0.55aA	0cB	0.33 bB	0.25bA	0.30bA	0cB	0.29bA	0.52 aA	0.35bA	0.30bA
hexyl acetate	0bC	0bB	0.46 aAB	0bB	0bC	0.27aB	0.41 aA	0.25 abB	0.09bB	0.27aB	0.12 bBC	0.32aA	0.46aA	0.32aA	0.48aA	0.59aA	0.29bA	0.30 bB	0.25bA	0.43 abAB
%	0	0	0.46	0.51	0	0.67	1.48	1.21	0.26	0.91	1.17	0.98	1.86	1.8	1.86	1.77	1.84	3.26	1.16	1.61
N°	0	0	1	2	0	2	3	3	2	3	3	2	5	5	5	4	5	5	4	5
Defense substances Monoterpene																				
α-tujene	12.78aA	3.80bA	10.21 aA	4.29 bBC	2.40bB	7.75 abB	5.15bA	1.96cC	9.18aA	7.62 abA	7.57 aB	6.24aA	6.57 aB	6.17aB	1.83bB	11.88 aA	4.44cA	5.54 bcB	3.07cC	8.18bA
α-pinene	3.63aA	1.46bA	3.73aA	1.60bA	0.99bA	2.96aA	2.68aA	1.86aB	2.40aA	2.42aA	2.15aA	2.10aA	1.82aB	2.67aA	1.78aA	3.94aA	1.17bA	1.06bB	1.30bA	2.44 abA
Sabinense	0bC	0bC	0bB	0bB	0.89 aB	1.41 abB	1.96aA	1.04bA	1.52 abA	1.68 abA	1.45 aB	1.22 aAB	1.17 aA	1.53aA	0.77aB	2.52aA	0.90bB	1.06 bA	0.77 bAB	1.72 abA
β-pinene	0.74bA	0.69bA	1.75aA	0.66bA	0.33cA	0.23 abB	0bB	0bB	0.22bB	0.39aA	0aB	0.32aB	0.27aB	0.25aB	0.35aA	0.29abB	0bB	0bB	0.45 aA	0.19abA
Mircene	0cC	0.33bB	0.69aA	0.40bB	0cC	0.27bB	0.39bB	0.68aA	0.88aA	0.82aB	0bC	0.69aA	0.72aA	0.67aA	0.75a	0.74bA	0.67bA	0.65 bA	0.54bB	1.42aA
δ-3-carene	0aB	0aC	0aB	0aC	0aC	0bB	0bC	0bB	0.38aA	0.35aB	0cB	0.34bA	0.43aA	0.26bB	0cC	0.20bA	0.24bB	0cB	0.26bB	0.55aA
o-cimene	0bB	0bB	0bC	0.22aA	0bC	0bB	0bB	0.26aB	0.16abA	0.26aB	0.50aA	0.41abA	0.32abB	0.20bA	0.40 abB	0.67aA	0bB	0.54 aA	0.29bA	0.70aA
Limonene	0aC	0aC	0aC	0.10aC	0aC	0.24aB	0bC	0.42aA	0.35aB	0.40aB	0cC	0.63aA	0.39bAB	0.67aA	0.41bB	0.46bA	0.31bB	0.41 bA	0.66aA	0.80aA
1,8-cineol	0aA	0aB	0a	0a	0a	0aA	0aB	0aA	0aB	0aB	0cA	0.25bA	0cA	0.25bA	0.90aA	0aA	0aB	0aA	0aB	0aB
Trans-β-ocimene	0aA	0aA	0aA	0aB	0aB	0aA	0aA	0aA	0aB	0aB	0bA	0bA	0bA	0bB	0.18aA	0bA	0bA	0bA	0.18aA	0bB
γ-terpinene	0aB	0aC	0aC	0aB	0aB	0bB	0bC	0.22aB	0.31aA	0.17aA	0cB	0.39aA	0.43aA	0cB	0.17bA	0.65aA	0.21bB	0.23 bB	0.22 bAB	0.19bA
terpinen-4-ol	0aB	0aA	0aC	0aB	0aB	0bB	0bA	0.13aB	0bB	0bB	0cB	0cA	0.29aA	0.19bA	0cB	0.25aA	0bA	0bC	0bB	0.22aA
%	17.15	6.27	16.37	7.25	4.61	12.85	10.17	6.56	15.38	13.71	11.67	12.58	12.39	12.84	7.52	21.57	7.93	9.48	7.72	16.39
N°	3	4	4	6	4	6	4	8	9	8	4	10	10	10	10	10	7	7	10	10
Defense substances Sesquiterpene																				
δ-elemene	0cA	0.98bA	0.32cA	2.55aA	0.55 bcB	0.12aA	0.31 aB	0.51aA	0.39aB	0.55aB	0.30bA	0.68 bAB	0.34bA	0.34bB	1.89aA	0.29aA	0.10aB	0.38aA	0.26aB	0.27aB
α-cubebene	0cB	0cB	0.51abA	0.62aA	0.39bA	0.31bA	0cB	0.57aA	0.34bB	0.33bA	0.24aA	0.30aA	0.26 aB	0.37aB	0bB	0.19aA	0.28aA	0.28 aB	0bC	0.32aA
Cyclosativene	0aB	0aB	0aC	0aB	0aB	0bB	0bB	0.25aA	0bB	0bB	0cB	0.09bA	0cC	0.17abA	0.24aA	0.15bA	0cB	0.09bcB	0cB	0.29aA
Longicyclene	0aA	0aB	0aA	0aA	0aB	0aA	0aB	0aA	0aA	0aB	0bA	0.09aA	0bA	0bA	0bB	0bA	0bB	0bA	0bA	0.19aA
α-copaene	0.89bB	1.51bBC	2.77aA	1.38 bAB	2.67aB	2.32bA	1.62 bcB	3.44aA	1.65bcA	0.95cC	1.37cB	2.88aA	0.86cB	1.08cAB	5.83aA	1.79aAB	0.68bC	1.29abB	0.80bB	1.52aC
β-bourbonene	0.98bcA	0.40cAB	0.70cC	1.49bB	3.69aA	0.89cA	0.24dAB	2.74aA	2.30abA	1.94bC	1.23bA	0.46cAB	0.40cC	1.20bBC	1.98aC	0.33aB	0.90bcA	1.45bB	0.65cdC	2.74aB
β-cubebene	0.82bAB	0.81bC	2.22aA	1.20 bAB	1.26bA	0.94 bAB	0.21 cB	1.62aB	1.47abA	1.35 abA	1.28aA	0bB	0.48bC	0.76abB	1.17aA	0.48bB	1.09aA	1.51aB	0bC	1.43aA

(Continued)

Table 2. (Continued)

β-elemene	7.63aA	7.56 aA	1.37cA	1.09cB	4.48bB	7.85aA	0.85 cC	0.84cA	4.57 bAB	5.34 bAB	3.34 bB	2.04 bcBC	1.05 cA	1.72B	7.26aA	0.98cC	3.69bB	0.68 cA	4.84 abA	6.78aA
Ciperene	0cC	0.80aA	1.11aA	0.42bA	0cB	1.15aA	0.55bA	0.70bB	0cB	0.71bA	0.90aAB	0.70abA	0.37bC	0.68abA	0.07bB	0.67aB	0.47aA	0.60aB	0.39aA	0bB
cis-caryophyllene	1.95aC	0.63bA	0bA	0bA	0bA	5.47aA	0.56 bA	0bA	0.35 bA	0bA	5.44 aA	0.27bA	0.13 bA	0.19bA	0bA	3.57aB	0.22bA	0bA	0bA	0bA
α-gurjunene	0aB	0aB	0aB	0aB	0aB	0aA	0aA	0aA	0aA	0aB	0aA	0aA	0aA	0aA	0aB	0bA	0bA	0bA	0bA	0.15aA
trans-caryophyllene	34.26aB	19.84bcB	21.36 bB	12.22 cB	25.47 bA	26.70 aC	28.69 aA	15.17 bB	24.78 aA	10.53 bB	43.62 aA	30.48 bA	29.83 bA	9.58cB	17.23 cB	38.93 aAB	24.21 bAB	21.45 bB	24.74 bA	12.05 cB
β-copaene	0.55bAB	0.43 aA	0.48aB	0.69aA	0.65aB	0.32bB	0.52 bA	0.85aA	0.76 abA	0.95aA	0.60 abA	0.49 abA	0.43 bB	0.75aA	0.67 abB	0.34bB	0.47bA	0.53 bB	0.40bB	0.82 aAB
β-gurjunene	0cA	0.34bA	0cC	0.39 abA	0.51aA	0cA	0cB	0.45aA	0.27 bAB	0.47aA	0bA	0.27aA	0.16 abB	0.27 aAB	0bC	0bA	0.39aA	0.28 aB	0.23aB	0.30aB
aromadendrene	0.77aA	0.56 aA	0.79 aAB	1.13aA	0.61aA	0.87aA	0.59 aA	0.42aB	0.50 aB	0.84aA	0.51 aA	0.39aA	0.51 aAB	0.67aB	0.50aA	0.67 abA	0.73 abA	2.24 aA	0.48bB	0.35bA
α-humulene	3.33aA	2.12bA	2.19bA	1.84bA	2.36 abAB	3.75aA	3.65 aA	3.07 abA	2.45 bcA	1.54cB	4.04 aA	3.57 abAB	2.94 bA	1.77cA	3.05bA	3.28aA	2.57 abB	2.15 bA	2.51 abA	1.69bB
allo-aromadendrene	1.26aB	1.53aA	1.59aA	1.29aA	0.69bA	1.82aA	1.06 bAB	0.95bcB	1.30bA	0.54cA	0.67 aC	0.93aC	1.09 aB	0.66aB	0.66aA	1.30aB	1.39aB	0.39 bC	0.47bB	0.65bA
cis-cadine-1 (6),4 diene	0cB	0.63bB	0.92aB	1.06aA	0.71bA	0.09cB	0cC	1.22aA	0.58 bB	0.74bA	0.70aA	0.78 aAB	0.45 bC	0.44 bBC	0.72aA	0cB	0.83aA	0.38 bC	0.37bC	0.81aA
cis-muurole-4 (14),5 diene	0aA	0aA	0aA	0aB	0aC	0cA	0cA	0cA	0.17 bA	0.37aA	0bA	0bA	0bA	0.21aA	0.22aB	0bA	0bA	0bA	0bB	0.33aA
γ-gurjunene	2.28cB	2.90 cC	22.55 aA	21.53 aA	11.27 bB	1.04 cdB	1.46 cC	21.07 aA	13.82 bB	13.92 bB	7.65 bcA	11.91 abB	6.44 bcB	6.12cC	16.09 aAB	1.27cB	16.75 aA	12.19 bB	6.03cC	19.73 aA
trans-muurole-4 (14),5 diene	0dB	0.38bA	0dB	0.52aA	0.29 cB	0.09cA	0dB	0.48aA	0.27 bC	0.42aA	0aB	0aB	0aB	0aD	0aC	0cB	0cB	0cB	0.34bB	0aC
bicyclogermacrene	15.08 aA	16.07 aB	19.61 aA	22.12aAB	16.83aA	4.34cB	15.34bB	19.48abA	24.86aA	22.14abA	6.81bB	16.85aB	17.20aA	20.38aAB	15.76aA	9.24cAB	27.29aA	17.93bA	15.22bcB	15.15bcA
trans-β-guaiene	0.94aA	0.89 abA	0.75abA	0.63bB	0.73 abB	0.70 abAB	0.61bB	0.89 abA	0.	0.63bB	0.79aAB	0.69 aAB	0.69aA	0.80aB	0.70aB	0.66bB	0.46bB	0.64bA	1.18aA	1.46 aA
germacrene A	1.90abAB	2.41aB	0.67bA	1.17bB	2.72 aA	1.95aA	0.55bC	0.38bA	1.14 abB	1.83 aA	0.73bB	0.66bB	0.72bA	0.66bB	2.06aA	2.13bA	3.75aA	0.08cA	2.52bA	2.52 bA
γ-cadinene	1.10aA	1.11aA	0.40bB	0.93aA	0bB	0.51bB	0.44bB	1.18aA	0.66bA	1.19a	0.95aA	0.69aB	0.71aB	0.96aA	1.04a	0.44cB	0.62 bcB	1.54aA	0.65 bcA	0.95b
δ-cadinene	1.31 bcA	1.46bA	0.77cB	2.37aA	0.70cC	1.47 abA	1.39 abA	1.98aA	0.96bB	1.51 abB	1.40cA	1.13cA	1.04cB	2.27bA	3.56aA	1.63aA	1.05 abA	0.93bB	1.20 abB	1.02 abBC
α-cadinene	0aA	0aA	0aB	0aA	0aB	0aB	0aB	0.22aA	0bA	0bB	0bA	0bA	0bB	0bA	0.14aA	0aA	0aA	0aB	0aA	0aB
germacrene B	0.12 bAB	0.16bA	0bA	0.99aA	0.44 abB	0.60 abA	0.29bA	0bA	0.28 bBC	1.10 aA	0bB	0.23bA	0.27bA	0.82 bAB	1.55aA	0.28 aAB	0aA	0aA	0.22aC	0.28aB
Spathulenol	0bB	0bB	0bB	0bB	0.54 aA	0.28 aAB	0.30aA	0.44aA	0.25aA	0bC	0.44aA	0.30aA	0bB	0.31aA	0bC	0.20 abB	0.22aA	0bB	0.18 abAB	0.27aB
caryophyllene oxide	0.82aA	0.35 bcB	0dB	0.06 cdB	0.48 bA	0.50bB	1.17aA	0.51bA	0.24 bAB	0.50bA	0.51aAB	0.31 abB	0.39 abA	0.43 abA	0.20bA	0.16aC	0.24aB	0.21aAB	0.26 aAB	0.33 aA
epi-α-cadinol	0bB	0.35aA	0bA	0bB	0bB	0aB	0aB	0aA	0aB	0aB	0.39aA	0cB	0cA	0.25bA	0cB	0.43bA	0cB	0.08cA	0.25bA	0.34abA
%	775.95	664.18	881.05	777.67	777.98	664.01	558.74	779.38	885.26	770.32	883.87	777.95	666.76	553.78	882.55	669.35	888.77	667.25	664.14	772.69
N°	18	24	19	23	22	24	20	25	25	24	23	25	23	27	23	24	24	23	23	27
N° TOTAL	21	28	24	31	26	32	27	36	36	35	30	37	38	42	38	38	36	35	37	42

The lowercase letters indicate the differences in the time of evaluation (DBT) for each nitrogen concentration (mM N), and the uppercase letters indicate the differences in the concentrations of nitrogen at the evaluation time. The means were compared using Tukey's test, with the probability level of 5%.
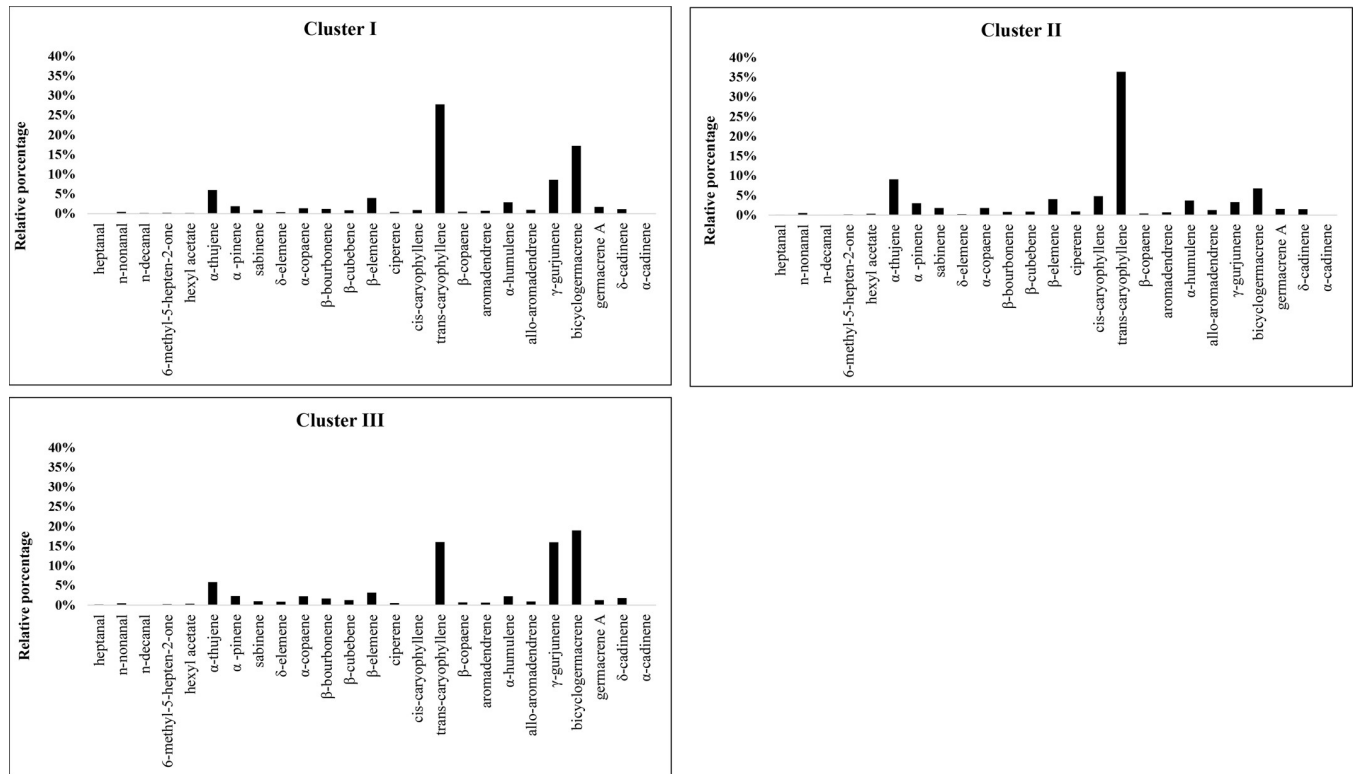
Fig 7. Means of the 25 most important volatile substances in Clusters I, II, and III of *A. emarginata*.

reduced levels of reducing sugar at 192 DBT, suggesting competition for reducing agents and carbon skeletons [4] because the resources produced were for amino acid synthesis. This is the most likely explanation for the increased physiological indices (NAR and RGR) shown by the plants grown at the highest N concentration, resulting in increased leaf yield [61,62].

Table 3. Diversity indices of the volatile substances (H index) of *A. emarginata* subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment.

Nitrogen (mM)	Days after beginning of the treatments					
	150	164	178	192	206	
7.50	2.14 ± 0.03 Dc	2.41 ± 0.07 Aa	2.25 ± 0.04 Cb	2.38 ± 0.05 ABb	2.30 ±0.01 BCc	F = 23.09; P<0.01
5.62	2.48 ± 0.04 Ba	2.12 ± 0.05 Dc	2.50 ± 0.05 Ba	2.37 ± 0.03 Cb	2.61 ± 0.04 Ab	F = 74.35; P<0.01
3.75	2.22 ± 0.06 Cc	2.34 ± 0.05 Bab	2.25 ± 0.07 BCb	2.71 ± 0.03 Aa	2.66 ± 0.02 Aa	F = 85.24; P<0.01
1.87	2.31 ± 0.08 Bb	2.28 ± 0.03 Bb	2.40 ± 0.1 Ba	2.38 ± 0.01 Bb	2.71 ± 0.01 Aa	F = 30.32; P<0.01
	F = 24.63; P<0.01	F = 23.83; P<0.01	F = 11.94; P<0.01	F = 109.1; P<0.01	F = 236.2; P<0.01	

The diversity indices (H index) were subjected to analysis of variance, and the means were compared using Tukey's test, with the probability level of 5%.

Capital letters show the differences in the row, and small letters show the differences in the columns.
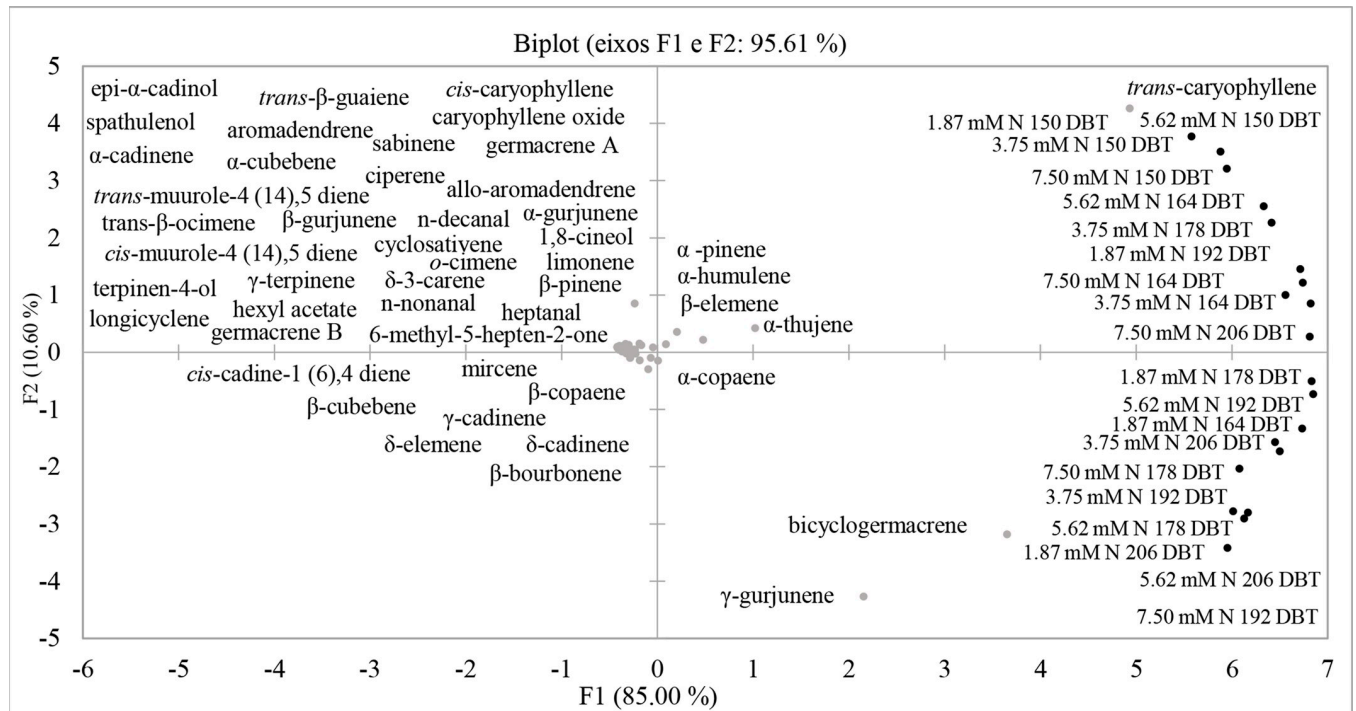
Fig 8. PCA of the total volatile substances with relative percentages of *A. emarginata* subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment (DBT).

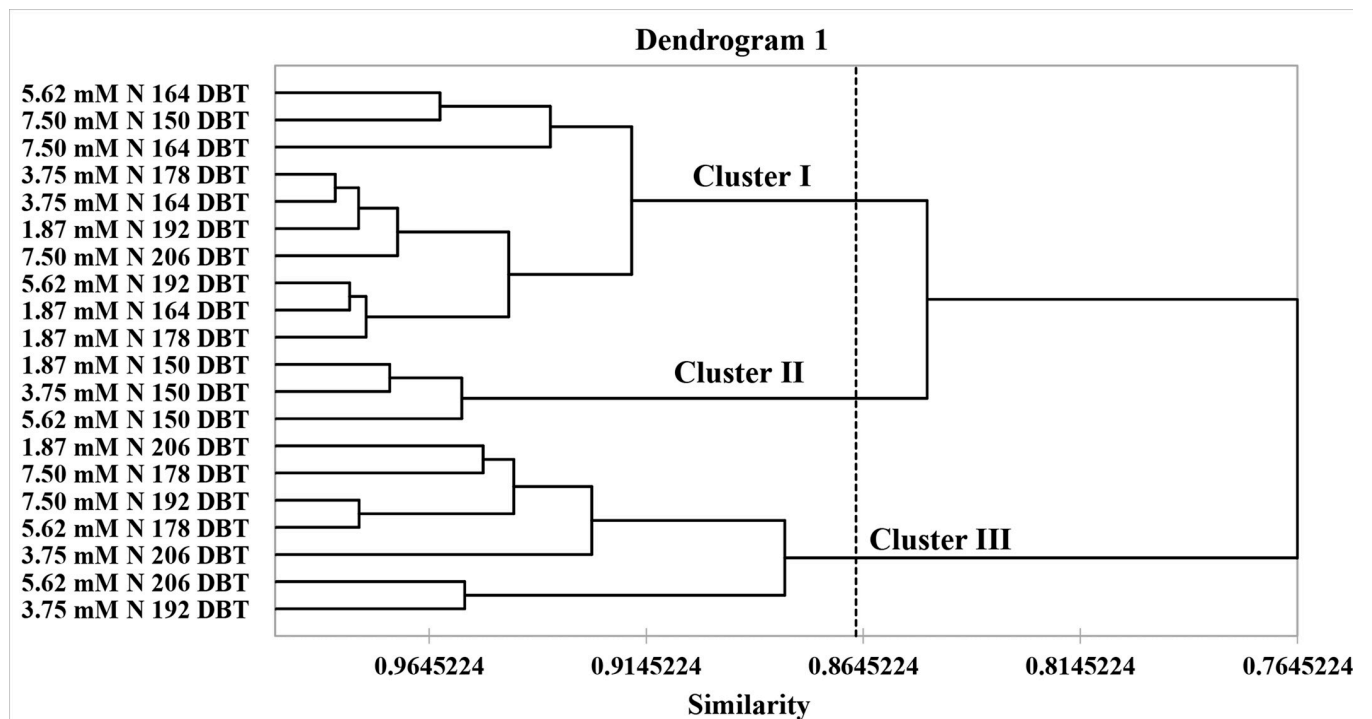https://doi.org/10.1371/journal.pone.0217930.g008

Fig 9. Dendrogram 1 of the volatile substances with relative percentages of *A. emarginata* subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment (DBT).

https://doi.org/10.1371/journal.pone.0217930.g009

The concentration of 7.5 mM N promoted an increase in the concentration of nitric oxide, a molecule that also acts on stress signaling [11] that, together with a decrease in carbon skeletons, may have contributed to the lower synthesis of volatile substances of the specialized metabolism involved with signaling. The low percentage of defense substance, monoterpenes, could be explained by the targeting of carbon skeletons and reducing agents for the reduction and incorporation of nitrate, once that monoterpene synthesis occur in the chloroplast, sharing the same resources [28,66,67]. The high percentage of sesquiterpenes may have aided the antioxidant enzymes in controlling lipid peroxidation.

The cultivation of *Annona emarginata* revealed *trans*-caryophyllene, bicyclogermacrene, and γ-gurjunene, synthesized on the same route [68]. These substances have bactericidal activity [31,69,70] and may contribute to the defense of the species. We found a higher relative percentage of *trans*-caryophyllene when the plants are younger (150 DBT) and a higher relative percentage of bicyclogermacrene and γ-gurjunene when the plants are older (206 DBT). It appears that *A. emarginata* directs the resources for the synthesis of these substances according to the stage of development. These results are confirmed in the dendrogram where we observed the separation of the plants cultivated with different nitrogen levels in two clusters.

Varying the nitrogen levels in *Annona emarginata* cultivation revealed that depending on the concentration, volatile substances show higher or lower synthesis and participation in the system of signaling and defense in the plant. These results may suggest that of volatile substances participate in resistance to pests and diseases, which is a necessary condition for *Annona emarginata* to be preferentially used as rootstock for *Annona* x *atemoya*.

Supporting information

S1 Table. Treatments applied to plants of *A. emarginata*, submitted in Hoagland and Arnon's nutrient solution n˚1 containing macronutrients, with different nitrogen concentrations, micronutrients and iron-EDTA solution.
(DOCX)

S2 Table. Temperature, relative humidity, and photosynthetically active photon flow density during evaluations of *Annona emarginata* subjected to 1.87, 3.75, 5.62 and 7.5 mM N at 150, 164, 178 and 206 days after beginning treatment (DBT).
(DOCX)

S1 Fig. Nitrate reductase activity in *Annona emarginata* leaves grown using different nitrogen concentrations. Data are presented as the mean ± SE (n = 4). The means were compared using the Tukey's test, with a probability level of 5%.
(DOCX)

S2 Fig. Leaf amino acid concentration of *Annona emarginata* grown using different nitrogen concentrations. Data are presented as the mean ± SE (n = 4). The means were compared using Tukey's test, with a probability level of 5%.
(DOCX)

S3 Fig. (A) Total sugar (µg g FW−1), (B) reducing sugar (µg g FW−1), (C) sucrose (µg g FW−1), (D) starch (µg g FW−1) in *Annona emarginata* grown under different nitrogen concentrations. Data are presented as the mean ± SE (n = 4). The means were compared using Tukey's test, with a probability level of 5.
(DOCX)

S4 Fig. (A) Lipid peroxidation (MDA, malondialdehyde nmol g−1 of FW) (B) Superoxide dismutase (SOD, U mg−1 protein); (C) peroxidase (POX, µmol of purpurogallin min−1 mg−1

protein); and (D) catalase (CAT, μKat μg−1 protein) of *Annona emarginata* grown under different nitrogen concentrations. The means were compared using Tukey's test, with a probability level of 5.
(DOCX)

S5 Fig. (A) Net assimilation rates (NAR, dm^2 g); (B) relative growth rates (RGR, $gg^{-1}day^{-1}$) and (C) leaf-specific weight (LSW, dm^2 g^{-1}) of *Annona emarginata* grown under different nitrogen concentrations.
(DOCX)

Author Contributions

Conceptualization: Felipe Girotto Campos, Carmen Sílvia Fernandes Boaro.

Data curation: Felipe Girotto Campos, Maria Aparecida Ribeiro Vieira, Amanda Cristina Esteves Amaro, Iván delaCruz-Chacón, Marcia Ortiz Mayo Marques.

Formal analysis: Felipe Girotto Campos, Maria Aparecida Ribeiro Vieira, Amanda Cristina Esteves Amaro, Iván delaCruz-Chacón.

Investigation: Felipe Girotto Campos, Amanda Cristina Esteves Amaro.

Methodology: Felipe Girotto Campos, Maria Aparecida Ribeiro Vieira, Amanda Cristina Esteves Amaro, Iván delaCruz-Chacón, Marcia Ortiz Mayo Marques, Gisela Ferreira.

Software: Maria Aparecida Ribeiro Vieira.

Supervision: Iván delaCruz-Chacón, Marcia Ortiz Mayo Marques, Gisela Ferreira, Carmen Sílvia Fernandes Boaro.

Validation: Marcia Ortiz Mayo Marques, Carmen Sílvia Fernandes Boaro.

Writing – original draft: Felipe Girotto Campos, Maria Aparecida Ribeiro Vieira, Amanda Cristina Esteves Amaro, Gisela Ferreira, Carmen Sílvia Fernandes Boaro.

Writing – review & editing: Felipe Girotto Campos, Maria Aparecida Ribeiro Vieira, Iván delaCruz-Chacón, Marcia Ortiz Mayo Marques, Carmen Sílvia Fernandes Boaro.

References

1. Dudareva N, Klempien A, Muhlemann JK, Kaplan I. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytol. 2013; 198: 16–32. https://doi.org/10.1111/nph.12145 PMID: 23383981

2. Stitt M, Sulpice R, Keurentjes J. Metabolic networks: how to identify key components in the regulation of metabolism and growth. Plant Physiol. 2010; 152: 428–444. https://doi.org/10.1104/pp.109.150821 PMID: 20018593

3. Lopes Cruz J, Ferreira Coelho E, Pelacani CR, Coelho Filho MA, Tosta Dias A, Taluana Dos Santos M. Crescimento e partição de matéria seca e de carbono no mamoeiro em resposta à nutrição nitrogenada. Bragantia. 2004; 63: 351–361. https://doi.org/10.1590/S0006-87052004000300005

4. Nunes-Nesi A, Fernie AR, Stitt M. Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. Mol Plant. 2010; 3: 973–996. https://doi.org/10.1093/mp/ssq049 PMID: 20926550

5. Peng Y, Li C, Fritschi FB. Diurnal dynamics of maize leaf photosynthesis and carbohydrate concentrations in response to differential N availability. Environ Exp Bot. Elsevier B.V.; 2014; 99: 18–27. https://doi.org/10.1016/j.envexpbot.2013.10.013

6. Lawlor DW, Kontturi M, Young a. T. Photosynthesis by flag leaves of wheat in relation to protein, ribulose Bisphosphate carboxylase activity and nitrogen supply. J Exp Bot. 1989; 40: 43–52. https://doi.org/10.1093/jxb/40.1.43

7. Hawkesford M, Horst W, Kichey T, Lambers H, Schjoerring J, Møller IS, et al. Functions of Macronutrients [Internet]. Marschner's Mineral Nutrition of Higher Plants. Elsevier Ltd; 2012. https://doi.org/10.1016/B978-0-12-384905-2.00006–6

8. Walker AP, Beckerman AP, Gu L, Kattge J, Cernusak L a., Domingues TF, et al. The relationship of leaf photosynthetic traits—V_{cmax} and J_{max}—to leaf nitrogen, leaf phosphorus, and specific leaf area: a meta-analysis and modeling study. Ecol Evol. 2014; 4: 3218–3235. https://doi.org/10.1002/ece3.1173 PMID: 25473475

9. Cruz JL, Alves A a. C, LeCain DR, Ellis DD, Morgan J a. Effect of elevated CO2 concentration and nitrate: ammonium ratios on gas exchange and growth of cassava (Manihot esculenta Crantz). Plant Soil. 2014; 374: 33–43. https://doi.org/10.1007/s11104-013-1869-8

10. Bloom AJ, Burger M, Rubio Asensio JS, Cousins AB. Carbon dioxide enrichment inhibits nitrate assimilation in wheat and Arabidopsis. Science. 2010; 328: 899–903. https://doi.org/10.1126/science.1186440 PMID: 20466933

11. Mur LAJ, Simpson C, Kumari A, Gupta AK, Gupta KJ. Moving nitrogen to the centre of plant defence against pathogens. Ann Bot. 2016; mcw179. https://doi.org/10.1093/aob/mcw179

12. Foyer CH, Shigeoka S. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol. 2011; 155: 93–100. https://doi.org/10.1104/pp.110.166181 PMID: 21045124

13. Misra N, Gupta AK. Effect of salinity and different nitrogen sources on the activity of antioxidant enzymes and indole alkaloid content in Catharanthus roseus seedlings. J Plant Physiol. 2006; 163: 11–18. https://doi.org/10.1016/j.jplph.2005.02.011 PMID: 16360799

14. Selmar D, Kleinwächter M. Influencing the product quality by deliberately applying drought stress during the cultivation of medicinal plants. Ind Crops Prod. Elsevier B.V.; 2013; 42: 558–566. https://doi.org/10.1016/j.indcrop.2012.06.020

15. Bogeski I, Kappl R, Kummerow C, Gulaboski R, Hoth M, Niemeyer BA. Cell Calcium Redox regulation of calcium ion channels: Chemical and physiological aspects. 2011; 50: 407–423. https://doi.org/10.1016/j.ceca.2011.07.006 PMID: 21930299

16. Takshak S, Agrawal SB. Defense potential of secondary metabolites in medicinal plants under UV-B stress. J Photochem Photobiol B Biol. 2019; 193: 51–88. https://doi.org/10.1016/j.jphotobiol.2019.02.002 PMID: 30818154

17. Loreto F, Schnitzler J-P. Abiotic stresses and induced BVOCs. Trends Plant Sci. 2010; 15: 154–66. https://doi.org/10.1016/j.tplants.2009.12.006 PMID: 20133178

18. Caser M, Chitarra W, D'Angiolillo F, Perrone I, Demasi S, Lovisolo C, et al. Drought stress adaptation modulates plant secondary metabolite production in Salvia dolomitica Codd. Ind Crops Prod. 2019; 129: 85–96. https://doi.org/10.1016/j.indcrop.2018.11.068

19. Kumari S, Pundhir S, Priya P, Jeena G, Punetha A, Chawla K, et al. EssOilDB: A database of essential oils reflecting terpene composition and variability in the plant kingdom. Database. 2014; 2014: 1–12. https://doi.org/10.1093/database/bau120 PMID: 25534749

20. Machado F, Dias MC, Pinho PG de, Araújo AM, Pinto D, Silva A, et al. Photosynthetic performance and volatile organic compounds profile in Eucalyptus globulus after UVB radiation. Environ Exp Bot. Elsevier; 2017; 140: 141–149. https://doi.org/10.1016/j.envexpbot.2017.05.008

21. Farzadfar S, Zarinkamar F, Hojati M. Magnesium and manganese affect photosynthesis, essential oil composition and phenolic compounds of Tanacetum parthenium. Plant Physiol Biochem. 2017; https://doi.org/10.1016/j.plaphy.2017.01.002 PMID: 28092849

22. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem. 2010; 48: 909–930. https://doi.org/10.1016/j.plaphy.2010.08.016 PMID: 20870416

23. Weber H. Fatty acid-derived signals in plants. Trends Plant Sci. 2002; 7: 217–224. https://doi.org/10.1016/S1360-1385(02)02250-1 PMID: 11992827

24. Pare PW, Tumlinson JH. Update on plant-insect interactions plant volatiles as a defense against insect herbivores. Plant Physiol. 1999; 121: 325–331. PMID: 10517823

25. Kachroo A, Kachroo P. Fatty Acid–Derived Signals in Plant Defense. Annu Rev Phytopathol. 2009; 47: 153–176. https://doi.org/10.1146/annurev-phyto-080508-081820 PMID: 19400642

26. Melis A. Photosynthesis-to-fuels: from sunlight to hydrogen, isoprene, and botryococcene production. Energy Environ Sci. 2012; 5: 5531–5539. https://doi.org/10.1039/C1EE02514G

27. Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann Bot. 2010; 105: 1141–57. https://doi.org/10.1093/aob/mcq028 PMID: 20299346

28. Nagegowda D a. Plant volatile terpenoid metabolism: Biosynthetic genes, transcriptional regulation and subcellular compartmentation. FEBS Lett. Federation of European Biochemical Societies; 2010; 584: 2965–2973. https://doi.org/10.1016/j.febslet.2010.05.045 PMID: 20553718

29. Chen Y, Schmelz EA, Wäckers F, Ruberson JR. Cotton Plant, Gossypium hirsutum L., Defense in Response to Nitrogen Fertilization. https://doi.org/10.1007/s10886-008-9560-x

30. Schmelz E a Alborn HT, Engelberth J Tumlinson JH. Nitrogen deficiency increases volicitin-induced volatile emission, jasmonic acid accumulation, and ethylene sensitivity in maize. Plant Physiol. 2003; 133: 295–306. https://doi.org/10.1104/pp.103.024174 PMID: 12970495

31. Campos FG, Vieira MAR, Baron D, Marques MOM, Ferreira G, Boaro CSF. Profile of Essential Oils From the Leaves of Annona Grafted. J Agric Sci. 2019; 11: 210–216. https://doi.org/10.5539/jas.v11n2p210

32. Hoagland DR, Arnon DI. The water-culture method for growing plants without soil. Calif Agric Exp Stn Circ. 1950; 347: 1–32. citeulike-article-id:9455435

33. Zhang S, Li Q, Ma K, Chen L. Temperature-Dependent Gas Exchange and Stomatal/Non-Stomatal Limitation to CO_2 Assimilation of Quercus Liaotungensis under Midday High Irradiance. Photosynth Res. 2001; 39: 383–388.

34. Ribeiro RV, Machado EC, Santos MG, Oliveira RF. Seasonal and diurnal changes in photosynthetic limitation of young sweet orange trees. Environ Exp Bot. 2009; 66: 203–211. https://doi.org/10.1016/j.envexpbot.2009.03.011

35. Sharkey TD, Bernacchi CJ, Farquhar GD, Singsaas EL. Fitting photosynthetic carbon dioxide response curves for C_3 leaves. Plant Cell Environ. 2007; 30: 1035–1040. https://doi.org/10.1111/j.1365-3040.2007.01710.x PMID: 17661745

36. Radford P. Growth Analysis Formulae—Their Use and Abuse. Crop Sci. 1967; 7: 171–175.

37. Portes T de A, De Castro LGJ. Analise de Crescimento de Plantas: um programa computacional auxiliar. Rev Bras Fisiol Veg. 1991; 3: 53–56.

38. Jaworski EG. Nitrate reductase assay in intact plant tissues. Biochem Biophys Res Commun. 1971; 43: 1274–1279. https://doi.org/10.1016/S0006-291X(71)80010-4 PMID: 5106073

39. Garcia IS, Souza A, Barbedo CJ, Dietrich SMC, Figueiredo-Ribeiro RCL. Changes in soluble carbohydrates during storage of Caesalpinia echinata LAM. (Brazilwood) seeds, an endangered leguminous tree from the Brazilian Atlantic Forest. Brazilian J Biol. Instituto Internacional de Ecologia; 2006; 66: 739–745. https://doi.org/10.1590/S1519-69842006000400018

40. Clegg KM. The application of the Anthrone reagent to the estimation of starch in cereals. J Sci Food Agric. 1956; 7: 40–44.

41. Morris DL. Quantitative Determination of Carbohydrates with Dreywood ' s Anthrone Reagent. Science (80-). 1948; 107: 254–255. https://doi.org/10.1126/science.107.2775.254 PMID: 17814729

42. Yemm EW, Willis AJ. The estomation of carboydrates in plant extracts by anthrone. Biochem J. 1954; 57: 508–514. https://doi.org/10.1042/bj0570508 PMID: 13181867

43. Miller GL. Use of Dinitrosalicyic Acid Reagent for Determination of Reducing Sugar. Anal Chem. 1959; 31: 426–428.

44. Passos LP. Métodos Analíticos e Laboratoriais em Fisiologia Vegetal. 1996. p. 223.

45. Yemm EC, Cocking EW. The determination of amino acids with Ninhydrin. Analyst. 1955; 80: 209–213.

46. Kar M, Mishra D. Catalase, Peroxidase, and Polyphenoloxidase Activities during Rice Leaf Senescence. Plant Physiol. 1976; 57: 315–319. https://doi.org/10.1104/pp.57.2.315 PMID: 16659474

47. Peixoto HPP, Cambraia J, Sant AR, Mosquim R, Moreira MA. Aluminum Effects on Lipid Peroxidation and on the Activities of Enzymes of Oxidative Metabolism in Sorghum 1. Rev Bras Fisiol Veg. 1999; 11: 137–143.

48. Teisseire H., Guy V. Copper-induced changes in antioxidant enzymes activities in fronds of duckweed (Lemna minor). Plant Sci. 2000; 153: 65–72.

49. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248–254. https://doi.org/10.1016/0003-2697(76)90527-3 PMID: 942051

50. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts. Arch Biochem Biophys. 1968; 125: 189–198. https://doi.org/10.1016/0003-9861(68)90654-1 PMID: 5655425

51. Devi SR, Prasad MN V. Copper toxicity in Ceratophyllum demersum L. (Coontail), a free floating macrophyte: Response of antioxidant enzymes and antioxidants. Plant Sci. 1998; 138: 157–165. https://doi.org/10.1016/S0168-9452(98)00161-7

52. Adams RP. Identification of essential oils components by gas chromatography/ mass spectroscopy. 4th ed. Stream C, editor. Allured Publishing Corporation; 2007.

53. Van Den Dool H, Kratz D. A generalization of the relation index system including liner temperature programmed gas-liquid partition chromatography. J Chromatogr A. 1963; 11: 463–467.

54. Zar JH. Biostatistical Analysis. 5 ᵃ. Upper Saddle River, NJ: Pearson Prentice Hall; 2010.

55. Shen T-C, Funkhouser EA, Guerrero MG. NADH- and NAD(P)H-Nitrate Reductases. Plant physiol. 1976; 58: 292–294. Available: http://www.plantphysiol.org/content/58/3/292.full.pdf https://doi.org/10.1104/pp.58.3.292 PMID: 16659665

56. Ahmad A, Abdin MZ. NADH: Nitrate reductase and NAD(P)H: Nitrate reductase activities in mustard seedlings. Plant Sci. 1999; 143: 1–8. https://doi.org/10.1016/S0168-9452(99)00016-3

57. Sadat Taghavi T, Babalar M. The effect of nitrate and plant size on nitrate uptake and in vitro nitrate reductase activity in strawberry (Fragaria?? ananassa cv. Selva). Sci Hortic (Amsterdam). 2007; 112: 393–398. https://doi.org/10.1016/j.scienta.2007.01.002

58. Geiger M, Geiger M, Haake V, Haake V, Ludewig F, Ludewig F, et al. The nitrate and ammonium nitrate supply have a major in uence on the response of photosynthesis, carbon metabolism, nitrogen metabolism and growth to elevated carbon dioxide in tobacco. Science (80-). 1999; 1177–1199.

59. Loreto F, Pinelli P, Manes F, Kollist H. Impact of ozone on monoterpene emissions and evidence for an isoprene-like antioxidant action of monoterpenes emitted by Quercus ilex leaves. Tree Physiol. 2004; 24: 361–367. https://doi.org/10.1093/treephys/24.4.361 PMID: 14757575

60. Palmer-Young EC, Veit D, Gershenzon J, Schuman MC. The sesquiterpenes(E)-β-farnesene and (E)-α-bergamotene quench ozone but fail to protect the wild tobacco Nicotiana attenuata from ozone, UVB, and drought stresses. PLoS One. 2015; 10: 1–22. https://doi.org/10.1371/journal.pone.0127296 PMID: 26030663

61. May A, Suguino E, Martins A, Pinheiro MQ. Produção de biomassa e óleo essencial de Mentha citrata em função do manejo cultural e doses de nitrogênio. Rev Bras Ciências Agrárias—Brazilian J Agric Sci. 2010; 5: 370–375. https://doi.org/10.5039/agraria.v5i3a809

62. Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales M a., Begoña B, Rios JJ, Romero L, et al. Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. Environ Exp Bot. 2011; 72: 167–173. https://doi.org/10.1016/j.envexpbot.2011.03.005

63. Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, et al. Nitric oxide, stomatal closure, and abiotic stress. J Exp Bot. 2008; 59: 165–176. https://doi.org/10.1093/jxb/erm293 PMID: 18332225

64. Rosales EP, Iannone MF, Groppa MD, Benavides MP. Nitric oxide inhibits nitrate reductase activity in wheat leaves. Plant Physiol Biochem. Elsevier Masson SAS; 2011; 49: 124–130. https://doi.org/10.1016/j.plaphy.2010.10.009 PMID: 21093280

65. Mendel RR, Kruse T. Cell biology of molybdenum in plants and humans. Biochim Biophys Acta—Mol Cell Res. Elsevier B.V.; 2012; 1823: 1568–1579. https://doi.org/10.1016/j.bbamcr.2012.02.007 PMID: 22370186

66. Spinelli F, Cellini A, Marchetti L, Mudigere K, Piovene C. Emission and Function of Volatile Organic Compounds in Response to Abiotic Stress. Abiotic Stress Plants—Mech Adapt. 2011; https://doi.org/10.5772/24155

67. Rosenstiel TN, Ebbets AL, Khatri WC, Fall R, Monson RK. Induction of Poplar Leaf Nitrate Reductase: A Test of Extrachloroplastic Control of Isoprene Emission Rate. Plant Biol. 2004; 6: 12–21. https://doi.org/10.1055/s-2003-44722 PMID: 15095130

68. Keszei A, Brubaker CL, Carter R, Köllner T, Degenhardt J, Foley WJ. Functional and evolutionary relationships between terpene synthases from Australian Myrtaceae. Phytochemistry. Elsevier Ltd; 2010; 71: 844–852. https://doi.org/10.1016/j.phytochem.2010.03.013 PMID: 20399476

69. Perigo CV, Torres RB, Bernacci LC, Guimarães EF, Haber LL, Facanali R, et al. The chemical composition and antibacterial activity of eleven Piper species from distinct rainforest areas in Southeastern Brazil. Ind Crops Prod. Elsevier B.V.; 2016; 94: 528–539. https://doi.org/10.1016/j.indcrop.2016.09.028

70. Campos FG, Vieira MAR, Santos AA Dos, Jorge LG, Marques MOM, Boaro CSF. Chemical Diversity of Volatiles From Parents, Rootstock and Atemoya Hybrid. J Agric Sci. 2019; 11: 271. https://doi.org/10.5539/jas.v11n4p271