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Differential Tolerance of Primary Metabolism of *Annona emarginata* (Schltdl.) H. Rainer to Water Stress Modulates Alkaloid Production

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Abstract: *Annona emarginata* produces alkaloids of ecological and pharmacological interest and is tolerant to water and biotic stress, so it is used as rootstock for other Annonaceae fruits. There are few reports in the literature on how contrasting water stress impacts the production of specialized metabolites in Annonaceae and how primary metabolism adjusts to support such production. The objective of this investigation was to evaluate how drought and flooding stress affect alkaloid concentration and the primary metabolism of young *A. emarginata* plants. Three water levels (flooding, field capacity, and drought) were studied at two moments (stress and recovery). Variables analyzed were gas exchange levels, chlorophyll *a* fluorescence, leaf sugars, total alkaloid content, alkaloid profile, and Liriodenine concentration. The photosynthetic metabolism of *A. emarginata* was affected by water stress, with plants having a greater ability to adapt to drought conditions than to flooding. During the drought, a reduction in photosynthetic efficiency with subsequent recovery, higher starch and trehalose concentrations in leaves, and total alkaloids in roots (480 $\mu g.g^{-1}$) were observed. Under flooding, there was a reduction in photochemical efficiency during stress, indicating damage to the photosynthetic apparatus, without reversal during the recovery period, as well as a higher concentration of total sugars, reducing sugars, sucrose, glucose, and fructose in leaves, and Liriodenine in roots (100 $\mu g.g^{-1}$), with a lower concentration of total alkaloids (90 $\mu g.g^{-1}$). It could be concluded that there is differential tolerance of *A. emarginata* to water stress, inducing the modulation of alkaloid production, while drought promotes a higher concentration of total alkaloids and flooding leads to an increase in the Liriodenine concentration.

Keywords: Annonaceae; Liriodenine; photosynthesis; leaf sugars; flooding; drought

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1. Introduction

The Annonaceae family has medical and economic importance and significantly contributes to the diversity of tree species in neotropical and tropical forests [1,2]. *Annona emarginata* (Schltdl.) H. Rainer is a species native to the South American continent [3–5] and is known as having two morphotypes, "terra fria" and "mirim" [6]. In Brazil, it has a prominent presence in the Atlantic Forest, a biome that has enormous water availability [3,4]. In the economic sphere, *A. emarginata* is commonly utilized as a rootstock for the large-scale production of atemoya seedlings (*Annona atemoya* Mabb) [7], as it is tolerant to pathogens that attack plant roots and stems (*Phytophthora nicotianeae* var. parasítica, *Pythium* spp.,

Rhizoctonia solani) [8–10]. The species also presents adaptations to different humidity and temperature conditions [8] and tolerance to dry soils [11]. In addition, there are personal reports from field producers that the species survives for months under excess water conditions in areas subject to flooding.

Annonaceae presents specialized metabolites of ecological and pharmacological importance like alkaloids, acetogenins, and volatile terpenes [12–15]. Alkaloids assembly the group of nitrogenous metabolites with the mayor diversification [16]. In this context, Liriodenine is the most important alkaloid, considered a chemotaxonomic marker [17–20], with antibacterial [17], antiprotozoal [21,22], cytotoxic [12,23], and antifungal activities [24,25], in addition to being a defense molecule against phytopathogens [26]. In addition, *Annona emarginata* is capable of producing alkaloids that are stored in roots, stems, and leaves, such as Liriodenine [6,19].

The biosynthesis of these metabolites depends on several environmental conditions like temperature, light and water regime. [27–29] and is related to primary metabolism, given that the precursors of the specialized metabolism come from the primary metabolism [19,28,30]. Thus, climate changes that cause irregularities in rainfall distribution and volume affect agricultural production, including in the *Annona* cultivation regions [24,25], which can impact the production of specialized metabolites such as alkaloids. Water restriction directly affects ecological and agricultural systems, given that plant reactions to this type of stress vary according to their intensity and duration [31,32]. On the other hand, flooding imposes restrictions on the cultivation of many species due to the lack of adaptations to saturated soils with low oxygen availability to the root system [33].

The literature is abundant regarding relationships between primary and specialized metabolisms under water stress. However, in *Annona*, studies are generally aimed at evaluating the effect of hydric stress on plant production, especially drought [34,35] or secondary metabolites [18,36]. Thus, there are no reports on how drought and flooding stress affect the primary metabolism and production of alkaloids in *Annona emarginata*. Therefore, this work aims to evaluate how drought and flooding affect alkaloid production (total alkaloids, Liriodenine, and profile) and how primary metabolism (gas exchange, chlorophyll *a* fluorescence, and quantification of sugars) in young *Annona emarginata* plants adapt to support or explain the production of these metabolites.

2. Materials and Methods

2.1. Plant Material

Young *Annona emarginata* (Schltdl.) H. Rainer plants, "araticum de terra-fria" morphotype, were obtained from the Seedling Production Center of São Bento do Sapucaí, CATI (Technical and Integrated Assistance Coordination), municipality of São Bento do Sapucaí—São Paulo.

Seedlings were produced with seeds obtained from ripe fruits from a collection of *Annona emarginata* matrices, used by CATI as rootstock for atemoya production. Sowing took place in April 2019 in sand beds and transplanting in June of the respective year using 2-liter plastic bags with commercial substrate (Carolina Soil®—composed of Sphagnum peat, expanded perlite, and vermiculite expanded and toasted rice husk).

To start the experiment, young plants (3 months old and with 10–15 mature leaves) were acclimatized for 15 days in the greenhouse of the Department of Forestry Science, Soil, and Environment of the Faculty of Agricultural Sciences, Unesp—Botucatu (coordinates 22°51′ latitude S and 48°26′ longitude W).

2.2. Experimental Design

The experiment was carried out in July 2019, with average temperatures in Botucatu-São Paulo varying between 12 and 23 °C and relative humidity between 10 and 30% [37].

The experimental design was in randomized blocks in a 3 × 2 factorial scheme, with three replicates of 19 plants per treatment. Treatments consisted of water levels (flooding, field capacity, and drought) and two moments (stress and recovery), totaling 342 plants.

In the period before the beginning of the experiment, all plants were watered until the beginning of water percolation, then allowed to drain. As soon as the drainage stopped, indicating that the soil was at field capacity (control group), seedlings were individually weighed, and from that point, drought and flooding conditions were implemented.

In plants submitted to drought, irrigation was completely removed, and for those submitted to flooding, plants were kept constantly in trays with substrate covered in water. At the same time, to maintain field capacity, seedlings were weighed, and water was replenished daily. These treatments were maintained for thirty days. After this period, plants subjected to flooding and drought returned to field capacity for another 17 days in order to recover from water stress conditions. Plants at field capacity were maintained under this condition until the end of the experiment.

The response of the primary metabolism of *A. emarginata* to variations in water regimes was evaluated considering gas exchange, chlorophyll *a* fluorescence, and quantification of sugars, while the amount of total alkaloids, Liriodenine, and the presence of eleven other alkaloids were the specialized metabolism variables.

2.3. Gas Exchange

Gas exchange levels were weekly evaluated, with three plants from each replicate per treatment (totaling 27 plants) at each evaluation moment (stress and recovery), on the 2nd or 3rd completely expanded leaf from 09:00 a.m. to 11:00 a.m., with the aid of a CO_2 gas analyzer and water vapor by infrared radiation (Infra-Red Gas Analyzer, model GFS-3000—Heinz Walz GmbH, Effeltrich, Germany) with a saturating light of 800 m^{-2} s^{-1} determined using a light curve [38].

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 (*gs*, mmol m^{-2} s^{-1}) were determined. Instantaneous water use efficiency [WUE, µmol CO_2 (mmol H_2O^{-1})] was calculated using the ratio between assimilated CO_2 and transpiration rate (A_{net}/E). The apparent carboxylation efficiency was calculated according to the ratio between the CO_2 assimilation rate and the intercellular leaf CO_2 concentration (A_{net}/Ci, µmol m^{-2} s^{-1} Pa^{-1}).

2.4. Chlorophyll a Fluorescence

Chlorophyll *a* fluorescence was performed weekly from 09:00 a.m. to 11:00 a.m. using a fluorometer (LED-Array/PAM-Module3055-FL) on 27 plants (3 replicates with 3 plants per treatment) at each evaluation moment (stress × recovery). Leaves were acclimated for a period of 30 min in the dark, covered with aluminum foil, and then a pulse of actinic light of 4500 µmol $m^{-2}s^{-1}$ was applied to obtain Fm (maximum dark-adapted fluorescence). Subsequently, leaves were adapted to light, and the actinic light pulse was applied again to obtain Fm′ (maximum light-adapted fluorescence) [39]. In addition to maximum light- and dark-adapted fluorescence, Fo (minimum dark-adapted fluorescence) and Fo′ (minimum light-adapted fluorescence) values were also obtained. For this purpose, in the dark-adapted state (when all PSII centers are open), a light pulse is emitted. This light has an intensity low enough to induce electron transport from PSII but high enough to generate a minimum chlorophyll fluorescence value (Fo). Fo measurement and its light-adapted equivalent Fo′ are critical for fluorescence analysis. For an accurate Fo′ determination, it is necessary to use the far-red wavelength (FR) to stimulate PSI, thus extracting electrons from PSII, which ensures that quinone A (QA) remains fully oxidized during measurements [39].

Using Fm, Fo, Fm′, and Fo′, the maximum quantum yield (Fv/Fm), potential quantum efficiency (Fv′/Fm′) [40], effective quantum yield (*F*PSII) [41], photochemical quenching (qL) [42], non-photochemical quenching (NPQ) [43], and electron transport rate (ETR) were calculated, considering that 84% of light is absorbed by chlorophyll, with 50% of photons activating chlorophyll from photosystem II and 50% from photosystem I, energy from photosystem II that cannot be dissipated (*Ex*), and energy dissipated in the form of heat (D) [44].

2.5. Qualitative and Quantitative Analysis of Leaf Sugars

For the extraction of leaf carbohydrates, 36 samples were used, consisting of 6 replicates of 3 plants per treatment (flooding, field capacity, and drought) at two collection moments: when stress was established (18 samples) and after the recovery period (18 samples).

The extraction of total soluble sugars was carried out as proposed by Garcia et al. [45], as well as starch extraction according to Clegg [46]. The procedure to determine the concentration of total soluble sugars was performed according to Morris [47], for starch, the procedure was described by Yemm and Folkes [48], for reducing sugars, the procedure was determined by Miller [49], and for sucrose, the procedure was established by Passos [50].

Subsequently, 1 mL of total soluble sugars was separated for purification in columns containing Dowex cation and anion exchange resins eluted with 10 volumes of deionized water. The purified material had its pH neutralized with ammonium hydroxide and concentrated until complete drying in a freeze dryer. Samples were then resuspended in 5 mL of deionized water and analyzed in anion exchange liquid chromatography with pulsed amperometric detection (HPAEDPAD) (Colluna Dionex CarboPacTM PA-100, 4 × 250 mm) with a sodium hydroxide elution gradient (625 mM), ultrapure water (Milli Q), and sodium acetate (0.5 M). From standards, it was possible to identify the following sugars: arabinose, fructose, glucose, and trehalose.

2.6. Extraction of Total Alkaloids

The extraction of total alkaloids was carried out using leaf and root material from 60 samples, of which 30 corresponded to the stress moment (5 replicates per treatment, totaling 15 leaf samples and 15 root samples) and 30 to the recovery moment (5 replicates per treatment, 15 leaf samples and 15 root samples). The material was stored in an oven with forced air circulation at 30 °C for ten days, then crushed to obtain ± 5 g of dry mass for each replicate. Alkaloid extraction was carried out using the selective acid-base method, and extracts were stored in the dark [18].

2.7. Qualitative and Quantitative Analyses of Total Alkaloids and Liriodenine

To determine the total alkaloid content, the 60 alkaloid extracts were re-solubilized with $CHCl_3$, and their absorbance was determined by UV-Vis spectrophotometry (single-beam—model UV-M51—BEL Engineering®, Monza, Italy) at 254 nm using Liriodenine isolated from *Annnona mucosa* as reported in Sousa et al. [19], as a standard for elaborating the standard curve ($y = 0.0881x - 0.0112$, $R^2 = 0.9949$). The Liriodenine alkaloid was quantified using an ultra-high-performance liquid chromatograph with a UV-Vis detector (UHPLC—Thermo Fisher Scientific®, Waltham, MA, USA). The column was of reverse phase C18 (150 × 4.6 mm and 5 µm particle diameter) maintained at 30 °C; the isocratic mobile phase was water (pH 3.5 with acetic acid) and methanol (30:70) with a flow rate of 1 mL/min. Detection was carried out in UV at 254 nm. For the Liriodenine quantification, two calibration solution curves were performed ($y = 0.3595x - 0.0011$; $R^2 = 0.9989$ for samples with up to 10 µg of Liriodenine in the extract and $y = 0.3658x + 1.142$; $R^2 = 0.9992$ for samples larger than 10 µg) [18].

2.8. Statistical Analysis

The data were subjected to a normality test and homogeneity of variance. Subsequently, the data obtained (total alkaloids, Liriodenine, leaf carbohydrates, relative leaf water content, chlorophyll *a* fluorescence, and gas exchange) were submitted to a two-way analysis of variance (ANOVA). A two-way ANOVA was conducted to determine the effect of treatments (flooding, field capacity, and drought) with moments (stress: 30 days; recovery: 47 days) and their interaction. Means were compared using the Tukey test at the 5% significance level ($p < 0.05$).

3. Results

3.1. Effect of Water Stress on Alkaloid Metabolism

Alkaloid production in *Annona emarginata* was impacted both in stress and recovery moments (Figure 1). Drought stress stimulated almost five times more total alkaloid production (480 μg g^{-1} dry mass) than flooding and field capacity (control) (90 and 100 μg.g^{-1}, respectively).

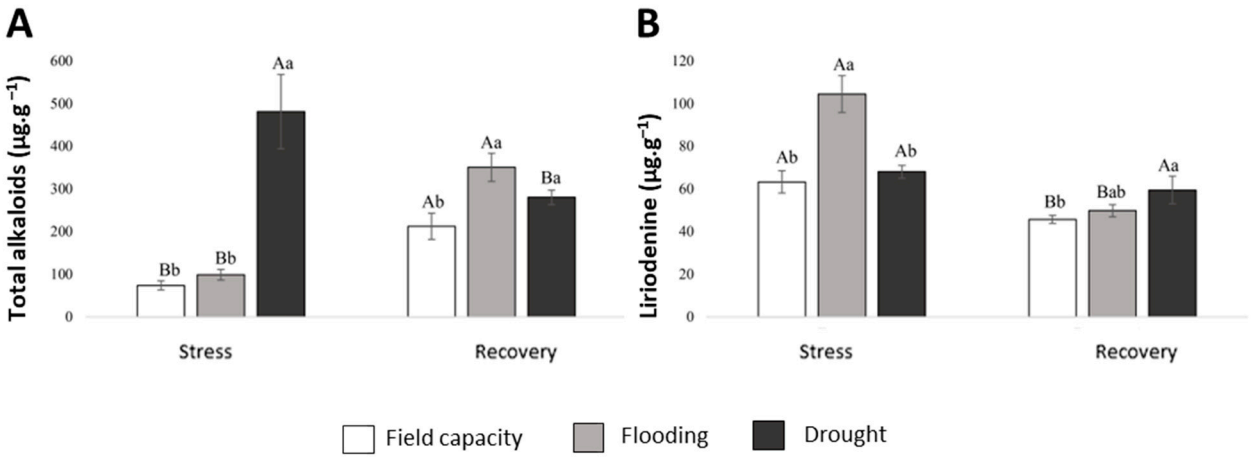

Figure 1. Concentration of (**A**) total alkaloids and (**B**) Liriodenine (means ± SD) in the roots of young *Annona emarginata* plants subjected to water stress and recovery. Means followed by capital letters indicate significant differences between assessment moments (stress × recovery), and means with lowercase letters indicate significant differences between water levels (flooding, field capacity, and drought) using the Tukey test at 5% probability (Table S1).

When plants were removed from water stress due to drought and placed at field capacity (recovery), a reduction in the concentration of total alkaloids was detected (300 μg.g^{-1}). An increase in the biosynthesis of total alkaloids was observed in plants subjected to flooding stress (350 μg.g^{-1}) without reaching the maximum production observed under drought stress conditions (480 μg.g^{-1}). Under the recovery condition, there was no difference in the amounts of total alkaloids between previously imposed drought stress and flooding; however, values were higher than those of field capacity. Thus, the results demonstrate that there is a more immediate effect at the moment of the drought imposition, with an increase in alkaloid production, while the effect of flooding occurs more slowly, being detected only during recovery.

In relation to Liriodenine, greater production was observed in flooding (100 μg.g^{-1}) than in drought stress and control (65 and 60 μg.g^{-1}). In the recovery phase, flooding and control treatments reduced the Liriodenine proportion to 50 and 45 μg.g^{-1} with no differences between them; however, in plants subjected to drought stress, the alkaloid concentration was preserved. Thus, it could be observed that flooding stress caused an increase in the Liriodenine concentration in roots, despite not having generated an increase in the total alkaloid concentration (Figure 1).

Furthermore, from the comparison through HPLC of the standards used, it was possible to identify eleven alkaloids in roots (at stress moments), nine of which are present in all water conditions (stress as recovery), namely N-Methyl-Laurotetanine, Norglaucin, Xylopinine, Xylopine, Assimilobin, Laurotetanin, Liriodenine, Oxoglaucine, and Lanulinosin. Ten alkaloids were identified in leaves, of which seven (N-Methyl-Laurotetanine, Norglaucin, Discretin, Xylopine, Laurotetanin, Liriodenine, and Lanulinosin) were present in both stress and recovery conditions. The alkaloids Norpredicentine and Xylopinine were not detected in leaves during flooding stress or recovery. Assimilobin was not detected in leaves during both stress and recovery conditions. Oxoglaucine and Reticulin were detected only in roots (Table 1). Reticulin was only detected during the recovery from water stress.

Table 1. Presence (x) and absence (-) of alkaloids in roots and leaves of *Annona emarginata* maintained at field capacity, flooding, and drought during stress and recovery.

	Roots						Leaves					
	Stress			Recovery			Stress			Recovery		
Alkaloids	**Field Capacity**	**Flooding**	**Drought**	**Field Capacity**	**Flooding**	**Drought**	**Field Capacity**	**Flooding**	**Drought**	**Field Capacity**	**Flooding**	**Drought**
Reticulin	-	-	-	x	x	-	-	-	-	-	-	-
Norpredicentine	x	x	x	x	-	-	x	-	x	x	-	-
N-Methyl-Laurotetanine	x	x	x	x	x	x	x	x	x	x	x	x
Norglaucin	x	x	x	x	x	x	x	x	x	x	x	x
Discretin	-	x	-	x	x	-	x	x	x	x	x	x
Xylopinine	x	x	x	x	x	x	x	-	x	x	-	-
Xylopine	x	x	x	x	x	x	x	x	x	x	x	x
Assimilobin	x	x	x	x	x	x	x	-	-	x	-	-
Laurotetanin	x	x	x	x	x	x	x	x	x	x	x	x
Liriodenine	x	x	x	x	x	x	x	x	x	x	x	x
Oxoglaucine	x	x	x	x	x	x	-	-	-	-	-	-
Lanulinosin	x	x	x	x	x	x	x	x	x	x	x	x

3.2. Effect of Water Stress on Primary Metabolism

Both water stress conditions caused changes in the photosynthesis of *A. emarginata*. The greater recovery reactions of plants in water deficit (drought) indicate greater tolerance of the species to this type of stress.

The net assimilation (A_{net}) and instantaneous water use efficiency (WUE) values were reduced during drought and flooding in relation to field capacity (control), with drought also causing significantly lower values than flooding. Stomatal conductance (*gs*) and transpiration (*E*) of plants were reduced in a similar way by treatments, differing from control. However, flooding caused a reduction in the carboxylation efficiency of the Rubisco enzyme (A_{net}/Ci), differing from drought, whose plants remained similar to control (Figure 2).

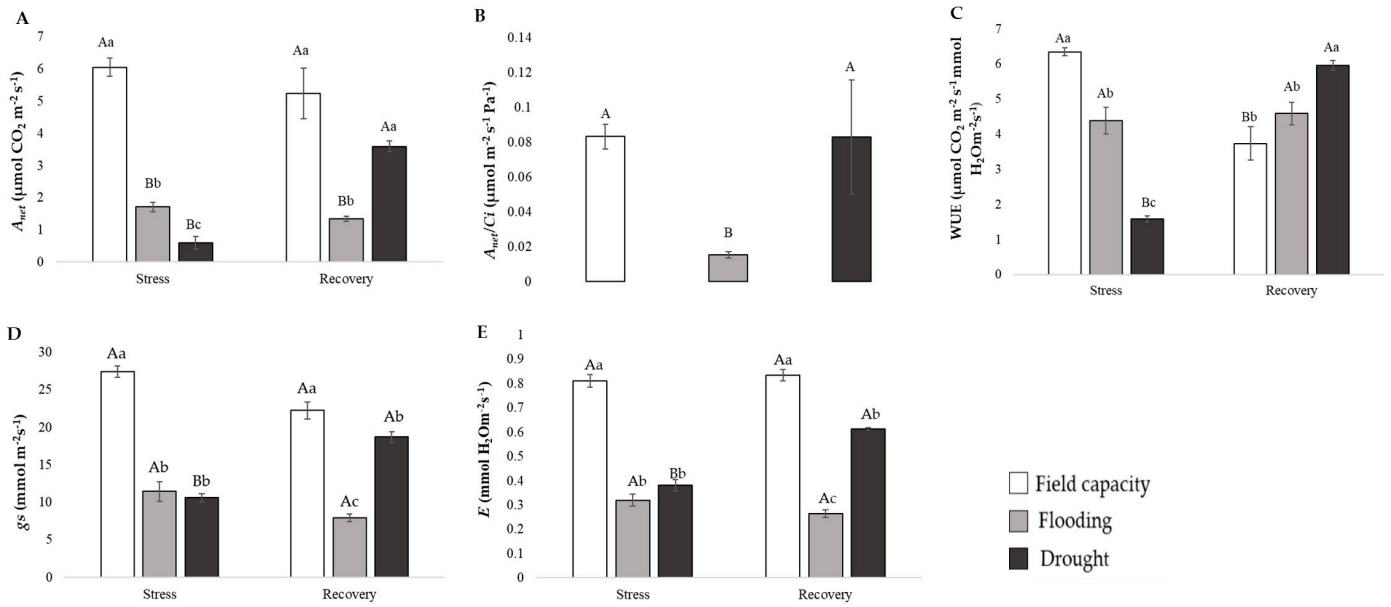

Figure 2. (**A**) Carbon assimilation rate (A_{net}), (**B**) Rubisco carboxylation efficiency (A_{net}/Ci), (**C**) instantaneous water use efficiency (WUE), (**D**) stomatal conductance (*gs*), and (**E**) transpiration (*E*) (means ± SD) in *Annona emarginata* plants maintained at field capacity, flooding, and drought during stress and subsequent recovery. Means followed by capital letters indicate significant differences between assessment moments (stress × recovery), and means with lowercase letters indicate significant differences between water levels (flooding, field capacity, and drought) using the Tukey test at 5% probability (Table S2).

At the same time, at the stress moment, the impact of treatments on chlorophyll *a* fluorescence was observed, with a reduction in maximum quantum yield (Fv/Fm) and the energy that cannot be dissipated by photosystem II (Ex), especially caused by flooding.

Furthermore, both drought and flooding caused similar reductions in potential quantum efficiency (FV′/FM′), electron transport rate (ETR), effective quantum yield (*F*PSII), and non-photochemical quenching (NPQ), and increased energy dissipated in the form of heat (D) (Figure 3).

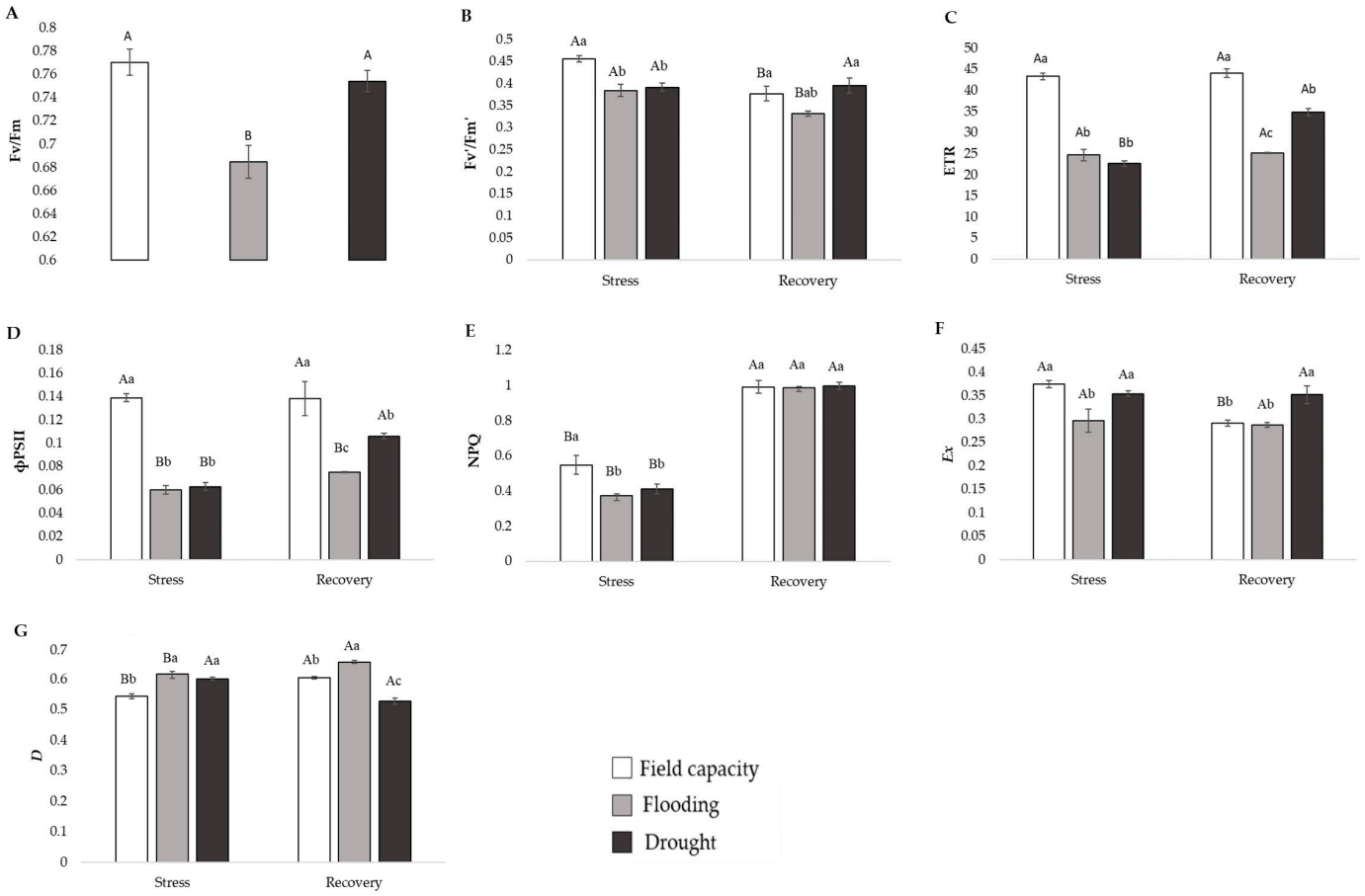

Figure 3. (**A**) Maximum quantum yield (Fv/Fm), (**B**) potential quantum efficiency (Fv′/Fm′), (**C**) electron transport rate (ETR), (**D**) effective quantum yield (ΦPSII), (**E**) non-photochemical quenching (NPQ), (**F**) energy of photosystem II that cannot be dissipated (Ex), and (**G**) energy dissipated in the form of heat (**D**) (means ± SD) of *Annona emarginata* plants maintained at field capacity, flooding, and drought during stress and subsequent recovery. Means followed by capital letters indicate significant differences between assessment moments (stress × recovery), and means with lowercase letters indicate significant differences between water levels (flooding, field capacity, and drought) using the Tukey test at 5% probability (Table S3).

The photosynthesis of plants placed in recovery reinforces the understanding of what occurred at the stress moment. Gas exchange (A_{net}, *gs*, E, WUE), chlorophyll *a* fluorescence [electron transport rate (ETR), effective quantum yield (*F*PSII), and non-photochemical quenching (NPQ)] values were higher in recovering plants (rehydration) than at the stress moment, indicating the ability of plants to recover and restore photosynthetic efficiency (Figures 2 and 3). The carboxylation efficiency (A_{net}/Ci) of plants in drought remained similar to control regardless of the assessment moment, indicating that despite stomatal closure and lower CO_2 assimilation caused by drought, no damage to the photosynthetic apparatus that would prevent CO_2 incorporation by the Rubisco enzyme was observed.

Plants submitted to flooding presented greater recovery difficulties since A_{net}, *gs*, E, and WUE values were lower than those of plants maintained in drought and field capacity, which was also observed in relation to A_{net}/Ci, regardless of whether under stress or during recovery. Similarly, *F*PSII and ETR values were maintained below values

from plants submitted to drought conditions and field capacity, and Ex data were below the values of plants under drought conditions, while D values increased under flooding conditions (Figure 3), which indicates damages related to flooding stress.

Variations caused by water conditions in photosynthetic metabolism were reflected in the synthesis of leaf sugars. Under drought stress conditions, lower total sugars, reducing sugars, sucrose, glucose, and fructose concentrations, and higher trehalose concentrations were observed, both in relation to flooding and control. Arabinose and starch in drought presented higher concentrations compared to plants maintained at field capacity (control group) without, however, differing from plants under flooding stress (Figure 4).

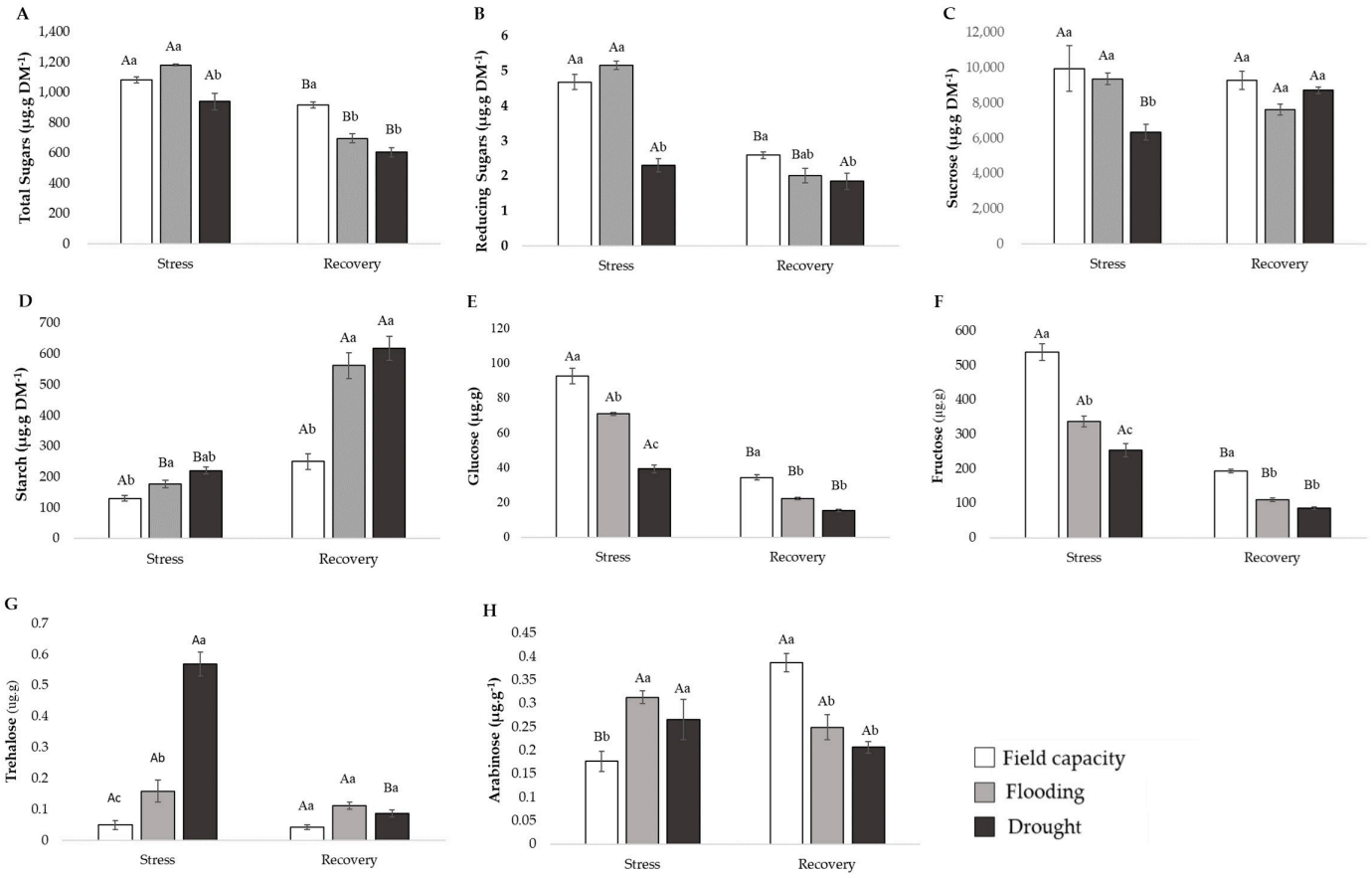

Figure 4. (**A**) Total sugars (µg.g DM^{-1}), (**B**) reducing sugars (µg.g DM^{-1}), (**C**) sucrose (µg.g DM^{-1}), (**D**) starch (µg.g DM^{-1}), (**E**) glucose (µg.g^{-1}), (**F**) fructose (µg.g^{-1}), (**G**) trehalose (µg.g^{-1}), and (**H**) arabinose (µg.g^{-1}) (means ± SD) of *Annona emarginata* plants maintained at field capacity, flooding, and drought during stress and subsequent recovery. Means followed by capital letters indicate significant differences between assessment moments (stress × recovery), and means with lowercase letters indicate significant differences between water levels (flooding, field capacity, and drought) using the Tukey test at 5% probability (Table S4).

4. Discussion

This work highlights the existence of a differential response of the primary and specialized metabolism of *Annona emarginata* to variations in water regimes.

It was observed that *A. emarginata* maintains a pool of "constitutive alkaloids" regardless of water conditions and the consequences of photosynthesis. This set is associated with at least seven alkaloids common in leaves and roots. No solid evidence of the production of alkaloids stimulated by any type of stress was detected. Plants under drought produced the greatest amount of total alkaloids, and plants under flooding produced the particular alkaloid Liriodenine in amounts that were reduced during recovery but remained at least

equal to control. These variations were associated with the adjustment of the photosynthetic metabolism.

A. emarginata has been considered drought-tolerant, which is one of the reasons why it is an excellent rootstock for atemoya production [38]. Furthermore, field observations suggest that the species can grow in flooded areas, which led to the hypothesis that the species is also flood-tolerant. However, this work demonstrates that water variations modulated the photosynthetic metabolism of *A. emarginata* in a different way, with greater difficulty in maintaining primary productivity after being subjected to flooding, which indicates less tolerance for this condition.

Water stress (drought and flooding) led to a reduction in stomatal conductance (*gs*) and transpiration of *A. emarginata*, which was expected since stomatal closure increases resistance to the diffusion of water vapor into the atmosphere [51]. However, these mechanisms that lead to stomatal closure vary with the type of stress. In drought, stomatal closure is related to the action of abscisic acid (ABA) on guard cells as a protective mechanism to prevent water loss [52]. Under flooding, the induction of stomatal closure may occur through an increase in ABA and ethylene synthesis induced by the decrease in available O_2 levels due to soil hypoxia, in addition to leading to the accumulation of reactive oxygen species [53,54].

In addition to reducing transpiration, the decrease in *gs* can also interfere with the carbon assimilation rate (A_{net}) due to the increased resistance to CO_2 entry due to stomatal closure, as observed in *A. emarginata* plants subjected to water restriction [11]. However, the reduction of A_{net} may also be related to internal CO_2 accumulation (which reduces the potential for diffusion into the leaf) due to damage to the photosynthetic apparatus and the lower carboxylation efficiency of the Rubisco enzyme, which affects the generation of energy used to reduce CO_2 [55].

In this experiment, results suggest that the reduction in A_{net} in plants under flooding was due to damage to the photosynthetic apparatus since a significant reduction in the carboxylation efficiency of the Rubisco enzyme was observed (A_{net}/Ci). These results differ from those observed under drought conditions, as even with lower A_{net} and *gs*, plants maintained carboxylation efficiency, demonstrating greater tolerance to this condition, as demonstrated with *A. crassiflora* [56], which also maintained carboxylation efficiency under conditions of reduced CO_2 entry into leaves during drought stress.

The literature presents evidence that a water deficit leads to an increase in specialized metabolites, including alkaloids [28,57–59]. In general, water restriction events lead to stomatal closure, reduced CO_2 entry, and, as a consequence, a decrease in the NADPH + H^+ consumption for CO_2 fixation in the Calvin Cycle, which results in excess and accumulation of this reducing equivalent [28]. Thus, the increase in NADPH + H^+ alters reactions that involve its consumption, and to avoid damage caused by its excess, the reducing equivalent is involved in the synthesis of highly reduced carbon compounds, such as alkaloids [28]. This fact may explain the increase in the concentration of total alkaloids when there is less carbon assimilation under dry conditions, differing from plants under flooding. In plants under flooding, although there was an intermediate A_{net} between drought and control, a reduction in the carboxylation efficiency of the Rubisco enzyme was observed, which could also lead to NADPH + H^+ accumulation and greater production of alkaloids; however, the investment in specialized metabolism was lower, with a particular increase in Liriodenine. The alkaloid biosynthesis depends on the availability of carbon and nitrogen and the energy provided by the primary metabolism, demonstrating a high degree of connectivity between primary and specialized metabolisms [60].

The reduction in A_{net}/Ci caused by flooding, indicating damage to the photosynthetic apparatus, is proven by changes in chlorophyll *a* fluorescence patterns, changing the light energy dissipation paths, as proposed by Kalaji et al. [61]. Even without detecting damage to the photosynthetic apparatus under drought stress conditions, it was possible to verify variations in chlorophyll *a* fluorescence patterns, which may explain the differential production of alkaloids between types of stress.

Therefore, it is known that the light energy absorbed by chlorophylls can be used in three ways: (i) boosting photosynthesis through photochemical reactions; (ii) dissipating excess energy in the form of heat; and (iii) re-emitting it in the form of fluorescence [62]. These processes occur concurrently, so any increase in one will result in a decrease in the others [39].

A decline in the maximum quantum yield (Fv/Fm) of plants kept under flooding in relation to those under drought and control was observed. This reduction indicates that there was a decline in the photochemical efficiency of PSII and disturbance or damage to the photosynthetic apparatus of *A. emarginata*, since the FV/FM ratio is an estimate of the maximum quantum efficiency of the photochemical activity of PSII when the centers of reaction are open and has even been used to evaluate other types of stress, such as salt stress [63]. The same behavior was observed for *Annona crassiflora* plants under flooding [56], indicating that this variable measures the intrinsic functioning of PSII, reflecting the high sensitivity of this photosystem to environmental stresses [39]. *A. emarginata* plants in the absence of stress showed an FV/FM ratio around 0.78, similar to several species (0.83) [64].

Unlike the maximum quantum yield (FV/FM), a reduction in the potential quantum efficiency (Fv′/Fm′) of plants under flooding and drought was observed, indicating that there was a lower proportion of light absorbed under both conditions. Therefore, considering that this measurement estimates the proportion of absorbed light effectively used in the photochemical phase of PSII [64], there was less light for the photochemical phase in both water stress conditions, which resulted in a reduction in the photochemical efficiency of PSII (ΦPSII).

In this context, in stressed *A. emarginata* plants (flooding and drought), there was a reduction in the energy fraction absorbed by chlorophyll associated with PSII that was used in the photochemical activity, indicating a reduction in the amount of transported electrons and a reduction in photosynthesis. Thus, a lower electron transport rate (ETR) is observed, directly reflecting a lower effective quantum yield (ΦPSII), as proposed by Tian et al. [65]. It is noteworthy that, although in both stress conditions there was a reduction in PSII efficiency, only plants submitted to drought demonstrated recovery, achieving responses similar to control, indicating that flooding was more harmful to *A. emarginata* plants.

The reduction in light energy destined for photosynthesis provides evidence that it follows other paths [39,62,64], which is confirmed by the increase in energy dissipation in the form of heat (D) for both stress conditions. Non-photochemical extinction (NPQ) values were also expected to be high, considering that NPQ is responsible for converting a large amount of absorbed light energy into heat [66]. However, what was observed is that under stress, NPQ values are low, which seems to be characteristic of the species, as also observed by Mantoan et al. [11]. Furthermore, subtle changes in ETR values can modulate NPQ performance [64].

To avoid damage caused by excess energy, there was greater dissipation in the form of heat (D) mainly in flooding plants, similar to results obtained in *A. crassiflora* [56], a condition that continued even with recovery from stress. It is also noteworthy that the lower photosystem II energy values that cannot be dissipated (Ex) during flooding may indicate a reduction in the flow of electrons that would be destined for the production of specialized metabolites, such as alkaloids [67], which only occurred in this experiment in plants with water deficits both in stress and recovery, which showed high *Ex* values. *Ex* is energy that is in the photosystem, driving the transport of electrons and the oxidation–reduction mechanism of reaction centers, and its excess is accumulated when there is no oxidizing agent, as there is no possibility of dissipation in the form of heat. In this way, this energy can be directed to alternative drains such as the synthesis of specialized metabolites, for example, alkaloids in *A. emarginata*, which did not occur under flooding since the *Ex* value was reduced.

In summary, in both water stress conditions, there was less light available for the photochemical phase (FV′/FM′), which resulted in lower electron transport rate (ETR)

and effective quantum yield values and, therefore, less photosynthesis. The energy from the unused photochemical phase accumulated (*Ex*) can be diverted to the synthesis of total alkaloids in drought plants and only to Liriodenine in flooding plants, where Ex is more reduced. During recovery, drought plants began to divide their energy flow between photosynthesis (effective quantum yield values increased) and the production of alkaloids, which reduced the production of these metabolites. In contrast, flooding plants maintained the lowest photosynthesis values and high D values, and although they maintained low *Ex* in relation to drought, there is evidence that energy has been targeted to the production of specific alkaloids, such as Liriodenine.

Increased alkaloid production in plants subjected to drought has been reported in several species, such as *Catharanthus roseus* [68], *Senecio jacobaea* [69], *Nicotiana tabacum* [70], and *Phellodendron amurense*, in which an increase in the concentration of benzylisoquinoline alkaloids was observed [71], the same group of alkaloids commonly found in species of the genus *Annona* [26].

In this experiment, regardless of drought or flooding stress, the presence of the alkaloids N-Methyl-Laurotetanine, Norglaucin, Laurotetanin, Lanulinosin, Liriodenine, and Xylopine was detected in the roots and leaves of all treatments, both in stress and recovery. These alkaloids may have played an antioxidant role, and, in the specific case of isoquinoline alkaloids, there is the potential to inhibit lipid peroxidation through the elimination of singlet oxygen [72]. Ovile Mimi et al. [6] worked with two *A. emarginata* morphotypes, "terra-fria" and "mirim", and also detected the presence of these alkaloids in the root material.

Particularly in the genus *Annona*, it has been described that drought increases Liriodenine production [36], an oxoaporphinic alkaloid recorded in more than 250 species [18], which has potential antibacterial [17], antiprotozoal [21,22], cytotoxic [22,23], and antifungal activities against phytopathogens [24]. In *A. emarginata*, the increase in Liriodenine production only occurred in plants submitted to drought stress and then returned to field capacity, while flooding stimulated Liriodenine production already at the time of stress establishment. Flooding caused an increase in Liriodenine concentration (around 110 $\mu\ g^{-1}$) in roots, around twice as much as in plants maintained in drought, which is the first report of the presence and quantification of Liriodenine in species of the genus *Annona* under flooding.

The increase in alkaloid production under drought conditions may indicate plants' strategies to perform osmotic adjustment and ensure tissue hydration. This osmotic adjustment occurs due to the accumulation of osmolytes, such as amino acids and sugars, in roots [59], in addition to total alkaloids production, which increased in roots. With the increase in alkaloids in roots, reductions in root water potential could occur, which could favor water absorption even under water stress or maintenance of cellular water status. Thus, in this experiment, under reduced water conditions, an increase in alkaloid production was observed, perhaps with the aim of mitigating the effect of drought.

In relation to the presence of osmolytes, plants subjected to drought showed a higher trehalose concentration (found only in some plants). This reducing disaccharide acts on the stabilization of enzymes, proteins, and lipid membranes against denaturation in situations of water restriction [73,74]. According to Paul et al. [74], trehalose is also related to starch accumulation, as the trehalose-6-phosphate enzyme (T6P) can reflect conditions of high supply of assimilates, and, in this case, it activates the AGPase enzyme through a redox activation mechanism, boosting starch formation.

Thus, drought increased the starch content in *A. emarginata* leaves, as reported by Matos Filho and Carvalho [75], however, not differing from flooding plants, corroborating Honório et al. [56]. This carbohydrate is part of the carbon "pool" within the plant [75], and it is worth mentioning that, in photosynthetic cells, starch can be synthesized and temporarily stored in chloroplasts, which is a "transitory" starch; therefore, it can be synthesized and degraded in other sugars within a 24-hour window, influencing carbon allocation throughout the plant and thus mitigating the negative effect of stress [76–78].

Under flooding, *A. emarginata* plants accumulate total sugars, reducing sugars and sucrose similar to results found by Henrique et al. [79], and, according to these authors, in this type of stress, there is a reduction in the translocation rate of carbohydrates from leaves to roots, in addition to a reduction in plant growth and metabolic activities; thus, the demand for carbohydrates decreases, leading to the accumulation of these photosynthates in leaves. On the other hand, the sucrose concentration was lower in plants submitted to drought, and this situation can be explained, according to Kuai et al. [80], due to the fact that this sugar can be used in two ways: (i) directly via glycolysis; or (ii) translocated within the plant through the phloem to draining tissues, so that when recovery was imposed on plants, the sucrose concentration did not differ between treatments.

With regard to arabinose, the high concentration of this monosaccharide in plants subjected to stress, whether due to excess or lack of water, corroborates the results found by Moore et al. [81]. These authors argue that the accumulation of this sugar induces the flexibilization of the cell walls of photosynthetic cells during water stress, facilitating subsequent plant recovery, and this may be one of the reasons why the concentration of this sugar was lower when plants were submitted to recovery.

In summary, it could be concluded that stress (drought and flooding) caused changes in variables such as gas exchange and chlorophyll *a* fluorescence more markedly in flooded *Annona emarginata* plants "terra fria" morphotype. What draws attention is that plants maintained in drought did not reduce carboxylation efficiency even with a reduction in stomatal conductance and A_{net}, indicating that there was no damage to the photosynthetic apparatus, which was confirmed by the maintenance of maximum quantum yield (FV/FM) at levels similar to plants kept at field capacity. It is noteworthy that except for FV/FM and *Ex*, all other fluorescence variables were reduced or increased by stress treatments, but only plants subjected to drought showed recovery of variables such as effective quantum efficiency, ETR, and gas exchange, which generally represent greater photosynthesis.

During stress establishment, it was also evident that the energy not used in photochemistry was dissipated in the form of heat (increase in D) by plants in both treatments. Furthermore, a reduction in non-photochemical extinction in the antenna complexes was observed (reduction in NPQ), releasing thermal energy in both treatments. However, the maintenance of high *Ex* values only in drought plants suggests that this energy was used to increase the synthesis of total alkaloids, and during flooding, it was directed to the synthesis of Liriodenine. However, in the recovery, *Ex* values remained high in drought plants, D was reduced, and the quantum yield was increased to intermediate values (higher than that of flooding plants and lower than control), indicating that part of the energy was used to maintain photosynthesis and part was used for the synthesis of specialized metabolites, such as alkaloids.

Therefore, the results suggest that the use of the *Annona emarginata* "terra fria" morphotype should be better evaluated as rootstock for regions with high water regimes while confirming its suitability for soils with greater water restrictions. Furthermore, the variation in alkaloid production can be managed depending on the water regime, which can be interesting in the search for molecular biodiversity, which deserves to be further investigated.

5. Conclusions

The primary metabolism of *Annona emarginata* is differentially affected by drought and flooding conditions, which results in the modulation of the alkaloid metabolism. Flooding in general causes greater damage to gas exchange as well as to chlorophyll *a* fluorescence, which results in greater plant recovery difficulties. However, flooding promotes an increase in the concentration of Liriodenine but not of total alkaloids. On the other hand, water stress due to drought causes less damage to the photosynthetic apparatus and greater plant recovery capacity. Under this condition, there is an increase in the biosynthesis of total alkaloids. Some alkaloids are constant in *Annona emarginata* plants regardless of stress (N-Methyl-Laurotetanine, Norglaucin, Xylopine, Laurotetanin, Liriodenine, and Lanulinosin). However, other alkaloids (Reticulin, Norpredicentine, Discretin, Xylopinine, Assimilobin,

and Oxoglaucine) are observed differently depending on water levels during stress and recovery periods. Although it is a species native to the Atlantic Forest, a biome with high water availability, *Annona emarginata* adapts better to drought than flooding periods.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10030220/s1. Table S1: *p* and f values (two-way ANOVA) are indicated. Analyzed the interaction between water levels (field capacity, flooding, drought) × moments (stress and recovery) on the concentration of total alkaloids and Liriodenine; Table S2: *p* and f values (two-way ANOVA) are indicated. Analyzed the interaction between water levels (field capacity, flooding, drought) × moments (stress and recovery) on the carbon assimilation rate (A_{net}), Rubisco carboxylation efficiency (A_{net}/Ci), instantaneous water use efficiency (WUE), stomatal conductance (*gs*), and transpiration (E); Table S3: *p* and f values (two-way ANOVA) are indicated. Analyzed the interaction between water levels (field capacity, flooding, drought) × moments (stress and recovery) on the maximum quantum yield (Fv/Fm), potential quantum efficiency (Fv′/Fm′), electron transport rate (ETR), effective quantum yield (ΦPSII), non-photochemical quenching (NPQ), photosystem II energy that cannot be dissipated (Ex), and energy dissipated in the form of heat (D); Table S4: *p* and f values (two-way ANOVA) are indicated. Analyzed the interaction between water levels (field capacity, flooding, drought) × moments (stress and recovery) on the total sugars, reducing sugars, sucrose, starch, glucose, fructose, trehalose, and arabinose.

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