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Calcium in Photosynthetic Restoration and Growth of *Annona emarginata* after Mechanical Damage

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Abstract: Calcium, an essential element with structural function in the cell wall and plasma membrane, in addition to being a secondary messenger, is responsible for the regulation of physiological processes in plant development and responses to biotic and abiotic stresses. This study investigated the effects of calcium variation on photosynthetic performance, growth, and enzymatic antioxidant defense system in *A. emarginata* subjected to mechanical damage. The experimental design was in 6 × 5 factorial randomized blocks. *A. emarginata* plants were submitted to the six treatments: plants grown in solution with 0 mM Ca^{2+} without mechanical damage, 0 mM Ca^{2+} with mechanical damage, 2 mM Ca^{2+} without mechanical damage, 2 mM Ca^{2+} with mechanical damage, 4 mM Ca^{2+} without mechanical damage, and 4 mM Ca^{2+} with mechanical damage, as well as five evaluation periods at 0, 15, 30, 60, and 90 days after mechanical damage. The fluorescence of chlorophyll *a*, gas exchange, total dry mass, quantitative growth, and lipid peroxidation was studied. It is concluded that the *A. emarginata* plants showed better performance in restoration after mechanical damage in the presence of Ca^{2+} and was more sensitive in the absence of the mineral. Cultivation of the species with 2 mM Ca^{2+} in complete nutrient solution was sufficient to guarantee the efficiency of the enzymatic antioxidant defense system, and photosynthetic restoration of plants subjected to mechanical damage.

Keywords: plant physiology; chlorophyll *a* fluorescence; gas exchange; calcium metabolism; antioxidant enzymes; hydrogen peroxide

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1. Introduction

Calcium (Ca^{2+}), an essential element with structural function in the cell wall and plasma membrane, is also a secondary messenger and is responsible for physiological process regulation in vegetal development and responses to biotic and abiotic stress [1]. Ca^{2+} deficiency can compromise plant growth and recovery [2,3], especially after damage and stress.

One of the pathways to stress signaling and perception is the change in calcium and reactive oxygen species (ROS) concentration in the cytoplasm, which initiates a cascade responsible for activating antioxidant enzymes that regulate ROS accumulation [4] and membrane damage, contributing to the maintenance of the photosynthetic process. This cascade coordinates intracell responses in plants and long-distance signaling [5] and can influence plant growth and development.

Calcium acts on photosystem integrity because its fast oscillation in the cytoplasm regulates the energy ratio between the quantum efficiency of photosystem II (ΦPSII) and

energy dissipation as heat observed by non-photochemical quenching (NPQ) [6]. Heat dissipation is one of the most important photoprotection pathways, and has been shown to maintain the effective quantum efficiency of *Annona emarginata* (Schltdl.) H. Rainer submitted to water deficiency [7], where the calcium presence is fundamental [6].

Plant species have different mineral demands for their metabolic functions, and low requirements can be rusticity indications [8]. *A. emarginata* grown in nutrient solution by Hoagland and Arnon [9] with half ionic strength have shown a higher CO_2 assimilation rate and equal Ca^{2+} content than plants grown in solution with 75 and 100% ionic strength, which indicates efficiency in Ca^{2+} absorption and photoassimilate synthesis [10].

Because of its rusticity, *A. emarginata* is widely used as rootstock of Atemoya (*Annona x atemoya* Mabb) [8]. Different concentrations of calcium and abiotic stress due to mechanical damage, such as in rootstock making or shoot pruning, can contribute to calcium signaling in the enzymatic antioxidant defense system and maintenance of growth after damage. We hypothesize that the slow growth and the possible Ca^{2+} reserve in *A. emarginata* ensure survival in a medium with low concentration of the nutrient, without compromising the enzymatic antioxidant system and the photosynthetic apparatus, even when the plant is subjected to mechanical damage by removal of branches.

This study investigated the calcium variation and mechanical damage caused by aerial pruning on photosynthetic performance, growth, and enzymatic antioxidant defense system in *A. emarginata*.

2. Materials and Methods

The experiment was conducted at the Department of Botany, Institute of Biosciences, UNESP, Botucatu/SP, in a greenhouse with temperature maintained at 26 °C ± 2 °C, relative humidity at 52% ± 4% and ambient light 554 ± 100 µmol m^{-2} s^{-1}, geographical coordinates 48°24′35″ W, 22°49′10″ S, 800 m altitude.

2.1. Plant Material and Experimental Conditions

A. emarginata seedlings measuring 15 cm in height were obtained from the Seedling Production Nucleus of São Bento do Sapucaí, CATI (Coordenadoria de Assistência Técnica e Integrada), São Bento do Sapucaí, SP, geographical coordinates: 45°44′11″ W, 22°41′18″ S, and 874 m altitude. After 12 months of growth in nutrient solution nº2 by Hoagland and Arnon [9] diluted to 50%, the plants were transferred to the same nutrient solution, with 100% ionic strength, containing 4 mM Ca^{2+} (control treatment) and modified to provide Ca^{2+} levels equal to 2 and 0 mM, which constituted the other treatments, where plants remained until the collection date. After 30 days of growth in the nutrient solution with the three Ca^{2+} levels, mechanical damage was applied to the plants through removal of three branches of the middle region of the aerial part.

The experimental design was 6 × 5 factorial randomized blocks with 4 repetitions, constituted by the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD, and 5 evaluation times were performed at 0 (30 min), 15, 30, 60, and 90 days after mechanical damage.

2.1.1. Chlorophyll A Fluorescence, Gas Exchange, Biomass and Growth Index

Chlorophyll ***a*** fluorescence was measured in fully expanded leaves dark-adapted for 30 min, from the region below to mechanical damage, using a pulse-amplitude modulated fluorometer (Jr PAM, Walz) with saturating irradiance of 1150 PPFD, between 9:00 and 11:00 a.m. Maximum quantum yield of PSII photochemistry (Fv/Fm), minimum Chl *a* fluorescence in the dark-adapted state (Fo), and non-photochemical quenching (NPQ = (Fm-Fm')/Fm'), were measured.

The gas exchange and chlorophyll ***a*** fluorescence in the light were measured between 9:00 and 11:00 a.m. in leaves with the same conditions using an infrared gas analyzer GFS 3000 FL, Walz. The potential quantum efficiency of open PSII center (Fv'/Fm'), effective

quantum efficiency of PSII photochemistry (ΦPSII), electron transport rate (ETR = PPFD × ΔF/Fm′ × 0.5 × 0.84), light fraction absorbed by PSII antenna that is dissipated as heat (*D*), and energy fraction not dissipated in the antenna that cannot be used for photochemistry stage (*Ex*) were measured [11]. For the gas exchange evaluation, CO_2 assimilation rate (*A*, μmol CO_2 m^{-2} s^{-1}), transpiration rate (*E*, mmol H_2O m^{-2} s^{-1}), and stomatal conductance (*Gs*, mmol H_2O m^{-2} s^{-1}) were measured. The ribulose 1,5-difosfato carboxylase oxygenase (RuBisCO) efficiency was calculated between the ratio of *A* and internal CO_2 concentration in leaves (*A*/*Ci*) [12].

Leaf area was measured using a leaf area integrator Area Meter 3100C LICOR. Growth indices were calculated through the relationship between dry mass and leaf area, and the net assimilation rate (NAR), which reflects liquid photosynthesis, and the relative growth rate (RGR), which reflects growth in relation to pre-existing plant material [13], were determined.

Net Assimilation Rate (NAR)

$$\mathrm{NAR}\ (\mathrm{g/dm^2\ .\ day}) = \frac{(\mathrm{b} + 2\mathrm{ct}).\ \mathrm{a.e}^{(\mathrm{bt}+\mathrm{ct}^2)}}{\mathrm{a}_1.\mathrm{e}^{(\mathrm{b_t}+\mathrm{c}\ \mathrm{t}^2)}} \quad (1)$$

Relative Growth Rate (RGR)

$$\mathrm{RGR}\ (\mathrm{g/g\ .\ day}) = \mathrm{d}\ \ln \frac{\mathrm{a.e}^{(\mathrm{bt}+\mathrm{ct}^2)}}{\mathrm{dt}} \quad (2)$$

2.1.2. Antioxidant Enzyme Activity, Hydrogen Peroxide Quantification and Lipid Peroxidation

Leaves were collected between 9:00 and 11:00 a.m. and frozen in liquid nitrogen until evaluation. Antioxidant enzymes and protein extraction were performed according to Kar and Mishra [14]. The obtained extract was separated in microtubes and stored at −20 °C for later determination. The enzyme activities were determined as follows: superoxide dismutase (SOD, EC 1.15.1.1) [15], total peroxidase (POD, EC 1.11.1.7) [16], and catalase (CAT, EC 1.11.1.6) [17]. Total protein [18] hydrogen peroxide (H_2O_2) was determined using trichloroacetic acid (TCA) and reading the absorbance in a spectrophotometer at 390 nm [19] and lipid peroxidation were quantified using thiobarbituric acid and TCA and expressed by the formation of malonaldehyde (MDA) [20].

2.1.3. Calcium Concentration in Plant Tissue

The foliar and stem Ca^{2+} concentrations were determined in growth plants at 0 and 2 mM Ca^{2+}, with or without mechanical damage, 90 days after damage. Ca^{2+} determination was realized through flame atomic absorption instruments, model Varian 55B-Agilent [21].

2.1.4. Statistical Analysis

Levene's test was used to test homogeneity of variances between treatments using the software Sigmaplot 12.5. The results of variables were subjected to analysis of variance. The averages of treatments 0 mM Ca^{2+} intact, 0 mM Ca^{2+} w/MD, 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact and 4 mM Ca^{2+} w/MD, in 0 (30 min), 15, 30, 60, and 90 days after mechanical damage were compared using the Tukey test at the level of 5% probability [22].

Software used to create the heat map was MetaboAnalyst (v4.0, https://www.metaboanalyst.ca/ (accessed on 23 May 2022). Variables were standardized and the Euclidean distance between treatments was considered.

3. Results

3.1. Fluorescence Analysis of Chlorophyll A, Gas Exchange and Biomass

Plants 0 mM Ca^{2+} intact showed lower Fv/Fm (Figure 1A) and higher Fo (Figure 1B) at time 0 (30 min), in relation to the other evaluation times.

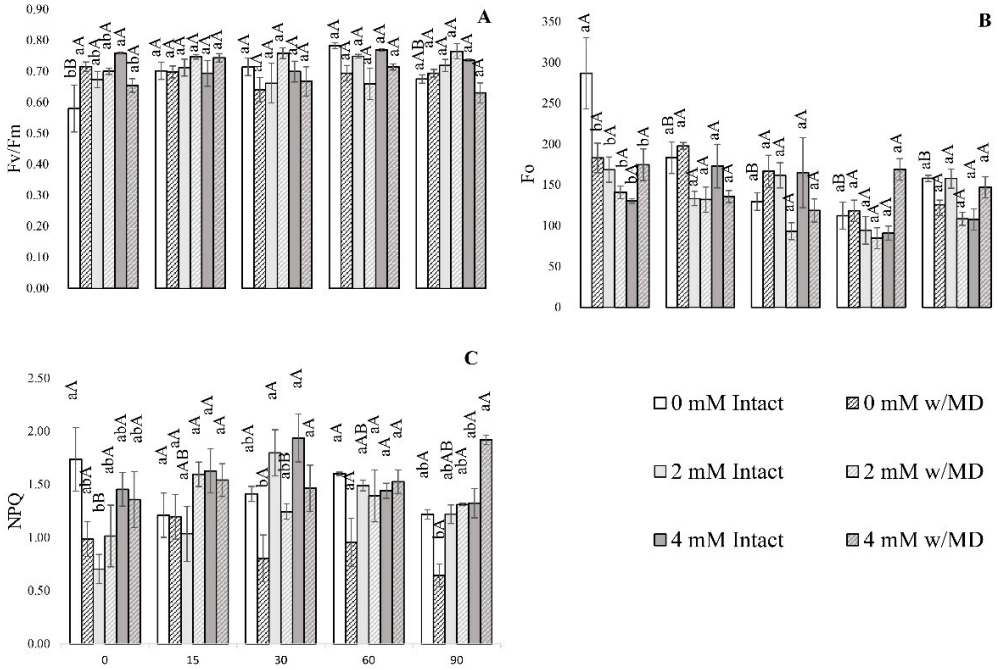

Figure 1. (**A**) Maximum quantum yield of PSII photochemistry (Fv/Fm) $p < 0.028$; (**B**) Minimum Chl a fluorescence yield in the dark-adapted state (Fo) $p < 0.033$; (**C**) Energy dissipation as heat observed by non-photochemical quenching (NPQ) $p < 0.036$ in Annona emarginata plants submitted to the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD, at evaluation times: 0 (30 min) 15, 30, 60, and 90 days. Values correspond to means ± SE ($n = 4$). Lowercase letters indicate differences between treatments within each evaluation time and uppercase indicate differences between each treatment over evaluation time.

At time 0, plants 0 mM Ca intact showed lower Fv/Fm compared to plants 0 mM Ca^{2+} w/MD and plants 4 mM Ca^{2+} intact. Plants 0 mM Ca^{2+} intact showed higher Fo compared to the others.

Lower NPQ was observed in plants 2 mM Ca^{2+} intact compared to plants 0 mM Ca^{2+} intact at time 0 (Figure 1C). At 30 days, lower NPQ was presented by plants cultivated with 0 mM Ca^{2+} w/MD, compared to plants 2 mM Ca^{2+} intact and plants 4 mM Ca^{2+} regardless of the damage. At 90 days, plants 4 mM Ca^{2+} w/MD showed higher NPQ compared to plants 0 mM Ca^{2+} w/MD.

Plants grown with 2 mM Ca^{2+} w/MD showed higher Fv'/Fm', ΦPSII and Ex and lower *D* compared to plants 4 mM Ca^{2+} w/MD at time 0 (Figure 2). Plants with 2 mM Ca^{2+} w/MD showed lower Fv'/Fm' and *Ex* and higher *D* at 15 and 30 days compared to time 0.

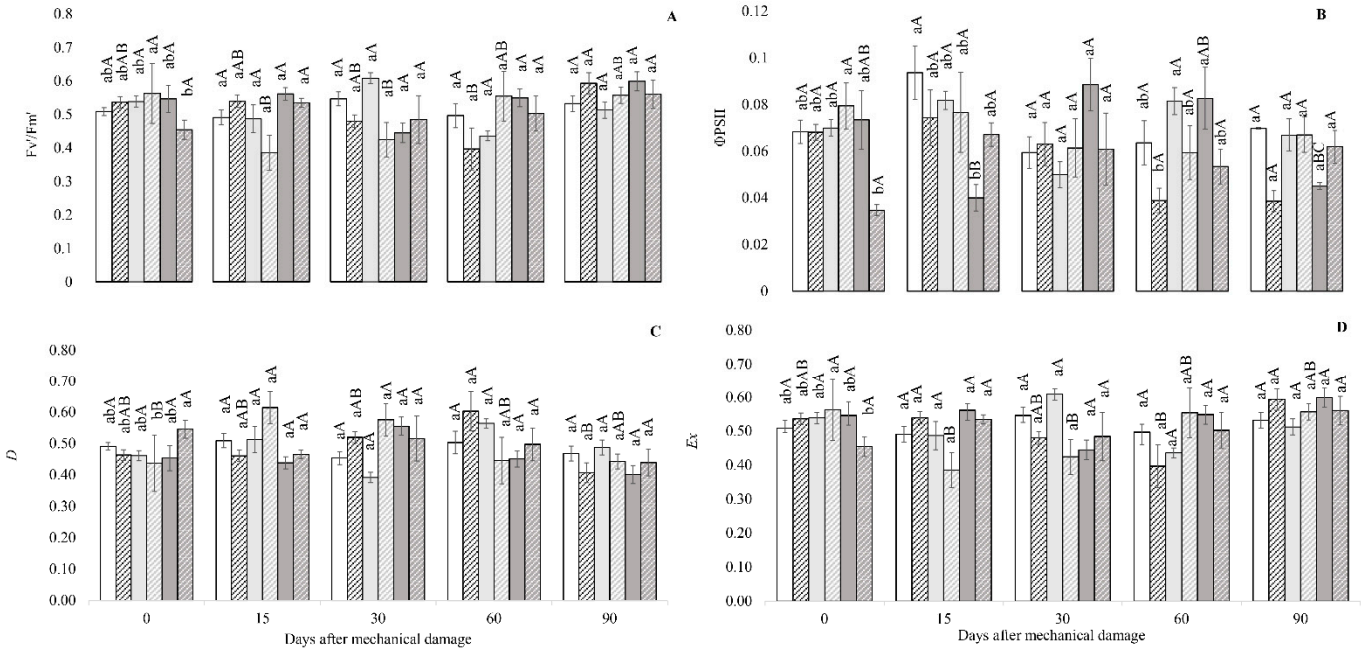

Figure 2. (**A**) Potential quantum efficiency of open PSII center (Fv′/Fm′) $p < 0.012$; (**B**) Effective quantum efficiency of PSII photochemistry (ΦPSII) $p < 0.003$; (**C**) Light fraction absorbed by PSII antenna that is dissipated as heat (**D**) $p < 0.012$; (**D**) Energy fraction not dissipated in the antenna that cannot be used for photochemistry stage (Ex) $p < 0.012$, in *Annona emarginata* plants submitted to the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD, at evaluation times: 0 (30 min) 15, 30, 60, and 90 days. Values corresponding to means ± SE ($n = 4$). Lowercase letters indicate differences between treatments within each evaluation time and uppercase indicate differences between each treatment over evaluation time.

Plants 2 and 4 mM Ca^{2+} intact showed higher A compared to plants 4 mM Ca^{2+} w/MD at time 0 (Figure 3). At 90 days, plants 0 mM Ca^{2+} w/MD presented the lower *A* values. Plants with 2 mM Ca^{2+} w/MD had lower *A* at 15 and 30 days compared to 90 days, and plants with 4 mM Ca^{2+} w/MD had higher *A* at 90 days compared to 0 and 15 days.

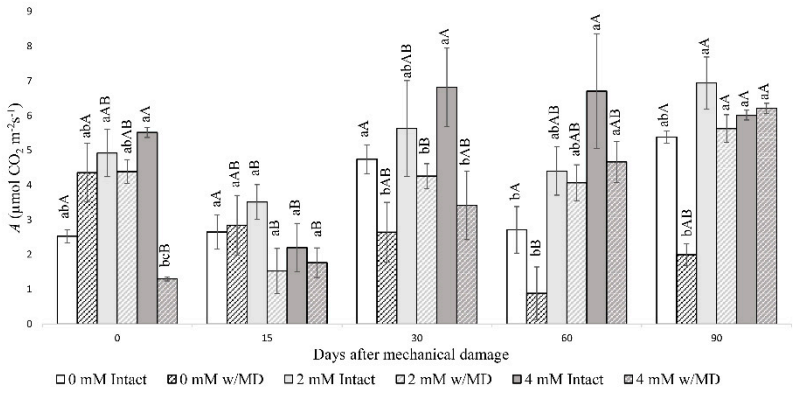

Figure 3. CO_2 assimilation rate (*A*, μmol CO_2 $m^{-2}s^{-1}$) $p < 0.001$ in *Annona emarginata* plants submitted to the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact; 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD, at evaluation times: 0 (30 min) 15, 30, 60, and 90 days. Values correspond to means ± SE ($n = 4$). Lowercase letters indicate differences between treatments within each evaluation time and uppercase indicate differences between each treatment over evaluation time. Lowercase letters indicate the differences between the treatment and capital letters between evaluation times.

Plants grown with 0 and 4 mM Ca^{2+} w/MD showed lower *E*, *Gs* and *A/Ci* compared to plants with 4 mM Ca^{2+} intact (Figure 4). Plants with 2 mM Ca^{2+} intact and w/MD did not differ in *E*, *Gs* and *A/Ci*, results also revealed with plants 0 mM Ca^{2+}.

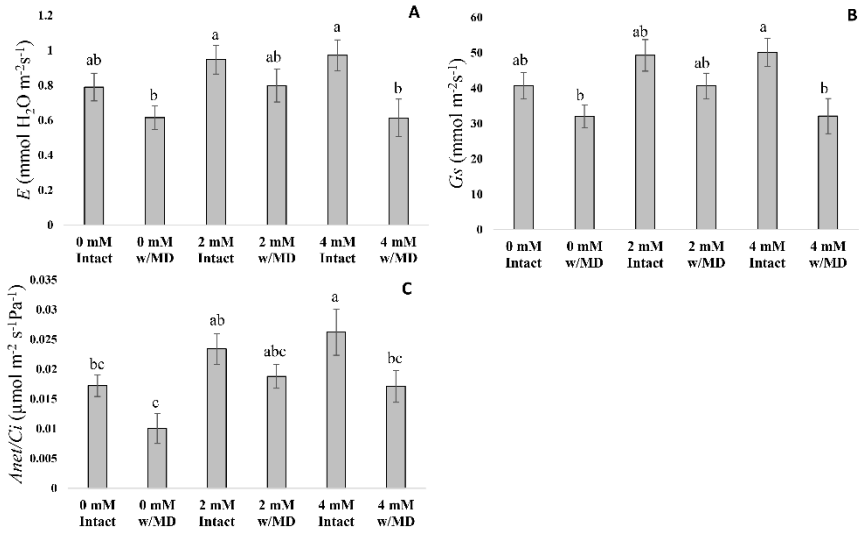

Figure 4. (**A**) Transpiration rate (*E*, mmol H_2O $m^{-2}s^{-1}$) Mechanical damage $p < 0.001$; (**B**) Stomatal conductance (*Gs*, mmol $m^{-2}s^{-1}$), MD $p < 0.001$; (**C**) RuBisCO's carboxylation efficiency (*A/Ci*, µmol $m^{-2}s^{-1}Pa^{-1}$), Ca x evaluation time $p < 0.027$, in *Annona emarginata* plants submitted to the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD, at evaluation times 0 (30 min) 15, 30, 60, and 90 days. Values correspond to means ± SE (n = 20). Lowercase letters indicate differences between treatments within each evaluation time and uppercase indicate differences between each treatment over evaluation time. Lowercase letters indicate the differences between the treatment and capital letters between evaluation times.

Plants grown with 0 mM Ca^{2+}, regardless of mechanical damage, showed constant total dry mass throughout the evaluation period (Figure 5). However, at 90 days, plants with 0 mM Ca^{2+} showed signs of deficiency in this element, such as small and necrotic leaves and apical stem and root death (Figure 6B). The difference between the total dry mass was observed at 60 days and plants with 4 mM Ca^{2+} intact had higher total dry mass than the others, with the exception of those cultivated with 2 mM Ca^{2+} intact.

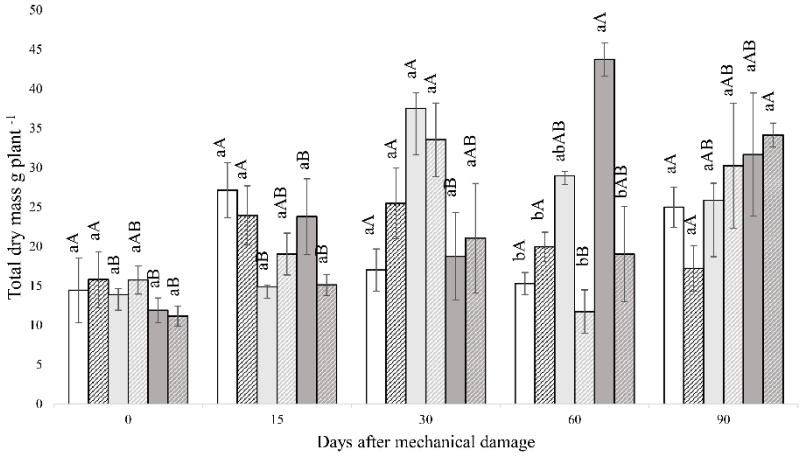

Figure 5. Total dry mass (g) $p < 0.001$ in *Annona emarginata* plants submitted to the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD, at evaluation times: 0 (30 min) 15, 30, 60, and 90 days. Values correspond to means ± SE ($n = 4$). Lowercase letters indicate differences between treatments within each evaluation time and uppercase indicate differences between each treatment over evaluation time.

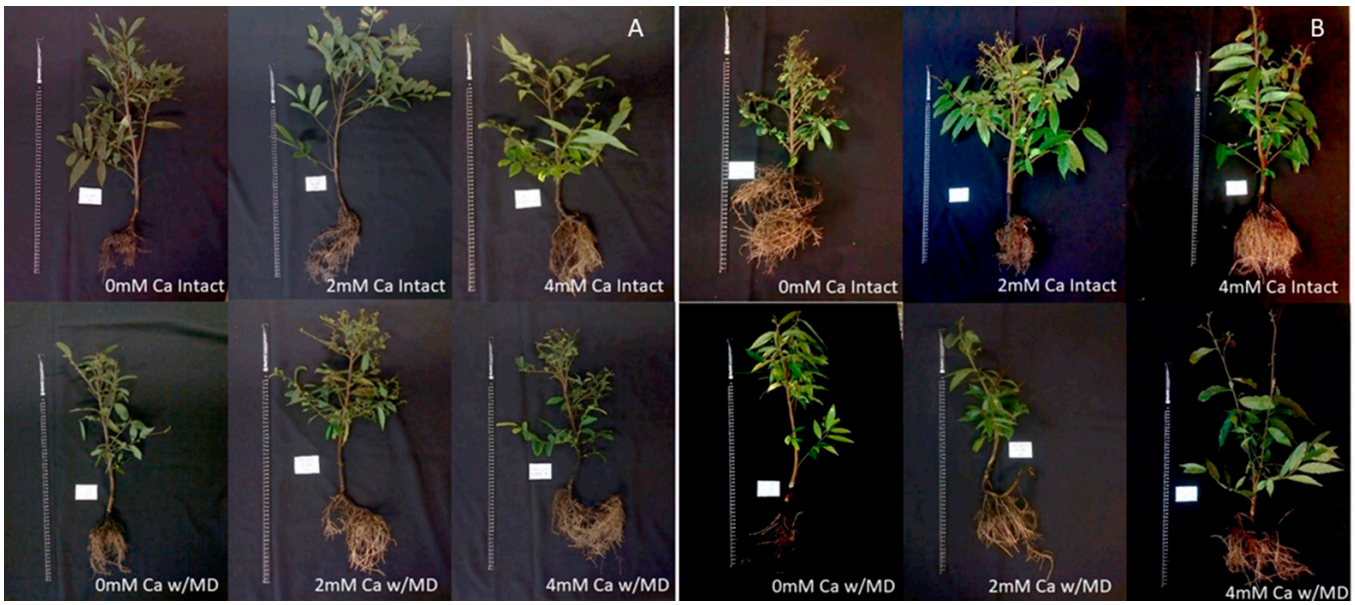

Figure 6. Annona emarginata plants submitted to the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD, at evaluation times: (**A**) 0 (30 min) and (**B**) 90 days.

Plants grown with 2 mM Ca^{2+} intact and 0 mM Ca^{2+} w/MD showed a rapid decline in NAR and RGR (Figure 7). Plants with 4 mM Ca^{2+} intact showed slow growth, observed by the stabilization of RGR after 30 days. In these plants, NAR showed a decrease, but later, at 90 days. Plants with 2 and 4 mM Ca^{2+} w/MD and 0 Ca^{2+}intact plants showed constant RGR. Plants with 0 mM Ca^{2+} intact and plants with 4 mM Ca^{2+} w/MD showed an increase in NAR over time.

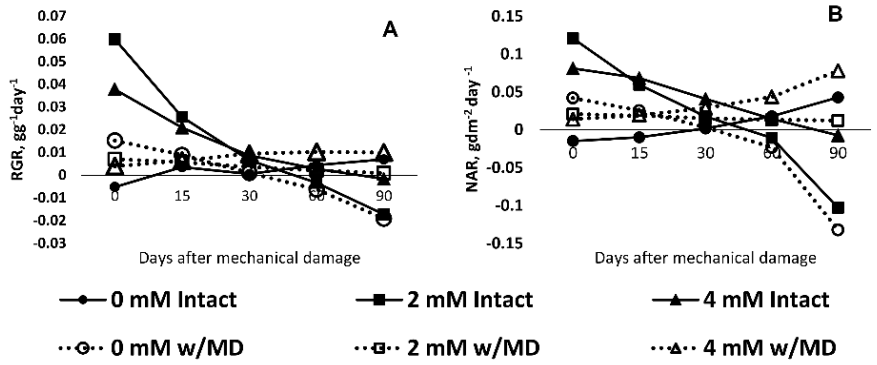

Figure 7. (**A**) Relative growth rate (RGR); (**B**) Net assimilatory rate (NAR) in *Annona emarginata* plants submitted to the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD, at evaluation times: 0 (30 min) 15, 30, 60, and 90 days. Values correspond to means ± SE (n = 4). Relative growth rate (RGR); 0 mM Ca^{2+} intact: $y = -0.0004x^2 + 0.0048x - 0.0082$; $R^2 = 0.7558$; 0 mM Ca^{2+} w/MD: $y = -0.0009x^2 - 0.0031x + 0.0189$; $R^2 = 0.9961$; 2 mM Ca^{2+} intact: $y = 0.0036x^2 - 0.0396x + 0.0941$; $R^2 = 0.9896$; 2 mM Ca^{2+} w/MD: $y = 6 \times 10^{-5}x^2 - 0.002x + 0.0091$; $R^2 = 0.9956$; 4 mM Ca^{2+} intact: $y = 0.0022x^2 - 0.0232x + 0.0585$; $R^2 = 0.9982$; 4 mM Ca^{2+} w/MD: $y = -0.0006x^2 + 0.0049x - 0.0007$; $R^2 = 0.9848$; B. Net assimilatory rate (NAR); 0 mM Ca^{2+} intact: $y = 0.0032x^2 - 0.005x - 0.0129$; $R^2 = 0.9995$; 0 mM Ca^{2+} w/MD: $y = -0.0135x^2 + 0.0413x + 0.0076$; $R^2 = 0.9635$; 2 mM Ca^{2+} Intact: $y = -0.0035x^2 - 0.0309x + 0.1477$; $R^2 = 0.9726$; 2 mM Ca^{2+} w/MD: $y = 0.0003x^2 - 0.004x + 0.0248$; $R^2 = 0.989$; 4 mM Ca^{2+} intact: $y = -0.0012x^2 - 0.0159x + 0.1003$; $R^2 = 0.9924$; 4 mM Ca^{2+} w/MD: $y = 0.0046x^2 - 0.0121x + 0.023$; $R^2 = 0.99$.

3.2. Hydrogen Peroxide Concentration (H_2O_2)

Plants grown with 0 and 2 mM Ca^{2+} and mechanical damage showed high concentrations of H_2O_2 compared to the intact. Plants grown with 4 mM Ca^{2+} showed the same concentration of H_2O_2, regardless of mechanical damage (Figure 8).

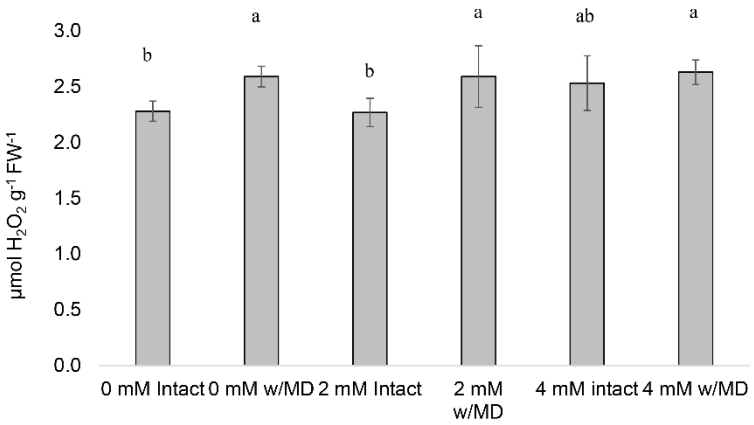

Figure 8. Hydrogen peroxide (H_2O_2) in *Annona emarginata* plants submitted to the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD. Values correspond to means ± SE (n = 20). Lowercase letters indicate differences between treatments.

3.3. Antioxidant Enzyme Activity

Plants grown with 0 mM Ca^{2+} intact and 4 mM Ca^{2+} w/MD showed lower SOD activity compared to 2 mM Ca^{2+} plants regardless of damage at time 0 (Figure 9A). At 90 days, plants with 0 mM Ca^{2+} intact showed the highest SOD and POD activity of the evaluated period (Figure 9A,B).

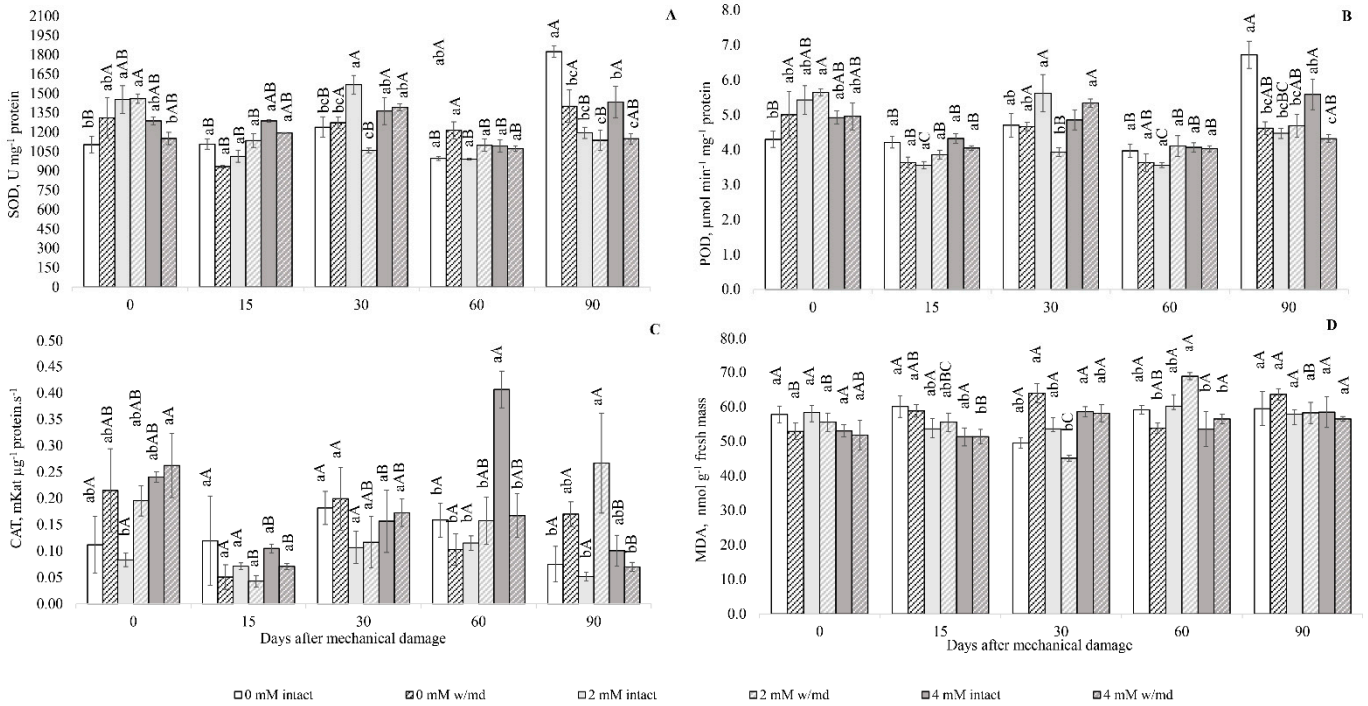

Figure 9. Antioxidant enzyme activity (**A**) Superoxide dismutase (SOD) $p < 0.001$; (**B**) Catalase (CAT) $p < 0.001$; (**C**) Peroxidase (POD) $p < 0.001$; (**D**) Lipid peroxidation expressed by the formation of malonaldehyde (MDA) $p < 0.001$ in *Annona emarginata* plants submitted to the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD, at times: 0 (30 min) 15, 30, 60, and 90 days. Values correspond to means ± SE ($n = 4$). Lowercase letters indicate differences between treatments within each evaluation time and uppercase indicate differences between each treatment over evaluation time.

The CAT activity was constant in plants grown with 0 mM Ca^{2+}, regardless of damage (Figure 9C). In damaged 2 mM Ca^{2+} plants, CAT activity was higher at 90 days than at 15 days. Plants with 4 mM Ca^{2+} intact showed higher CAT activity at 60 days compared to 15, 30, and 90 days. Plants with 4 mM Ca^{2+} w/MD showed higher CAT activity at time 0 compared to 15 and 90 days.

At 90 days, plants grown with 2 mM Ca^{2+} w/MD showed higher CAT activity compared to plants with 0 and 2 mM Ca^{2+} intact and plants 4 mM Ca^{2+} w/MD.

3.4. Lipid Peroxidation Quantification

Regardless of the calcium supplied, plants without mechanical damage showed no change in MDA concentration over the period (Figure 9D). Plants 0 mM Ca^{2+} w/MD showed higher MDA at 30 and 90 days compared to time 0. Plants 2 mM Ca^{2+} w/MD showed higher MDA at 60 days. Plants 4 mM Ca^{2+} w/MD showed lower MDA at 15 days, compared to 30, 60 and 90 days.

At 60 days, among plants that received mechanical damage, plants 2 mM Ca^{2+} showed higher MDA. At 90 days, MDA did not differ in plants.

3.5. Calcium Concentration in Plant Tissue

A. emarginata plants had an equal leaf calcium concentration when grown in the presence of 2 mM Ca^{2+} or absence of the ion. Lower leaf calcium concentrations were found in plants subjected to mechanical damage compared to intact plants. A higher Ca^{2+} concentration in the stems was observed in plants grown in the presence of 2 mM Ca^{2+}, and with no variation verified through mechanical damage (Table 1).

Table 1. Calcium concentration in the leaves and stems of *Annona emarginata* cultivated with 0 and 2 mM Ca^{2+}, with or without mechanical damage at 90 days after mechanical damage.

		Ca^{2+} Leaves, g kg^{-1}	
2 mM Ca^{2+}	11.438 ± 1.1805 ^{ns}	Intact	13.37 ± 1.2609 a [1]
0 Ca^{2+}	11.91 ± 1.2525	W/MD	9.978 ± 0.4938 b
		Ca^{2+} Stems, g kg^{-1}	
2 mM Ca^{2+}	4.993 ± 0.1866 b	Intact	5.743 ± 0.4553 ^{ns}
0 Ca^{2+}	6.182 ± 0.3756 a	W/MD	5.432 ± 0.3168

[1] Averages followed by different letters differ from each other by the Tukey 5% significance test. ^{ns} not significant. Values correspond to means ± SE (n = 4).

3.6. Heat Map

The heat map of the variables shows two large groups at time 0 (Figure 10A). One group with plants 0 mM Ca^{2+} intact and 4 mM Ca^{2+} w/MD, and another group, divided into two sub-groups, which showed greater similarity in responses to each other. The first group (0 mM Ca^{2+} intact and 4 mM Ca^{2+} w/MD) showed similarity in chlorophyll *a* fluorescence and gas exchange responses, with a negative influence on these variables. However, NPQ and *D* were positively influenced. The variables that distinguished them most in their responses were yield, ETR, H_2O_2, CAT, and MDA. The first subgroup of the second group matched plants with 0 mM Ca^{2+} w/MD and 4 mM Ca^{2+} intact, and the second subgroup matched plants 2 mM Ca^{2+} regardless of damage. Plants in the second group showed similar responses to chlorophyll *a* fluorescence variables and gas exchange. Plants 2 mM Ca^{2+} w/MD showed a positive influence for Fv'/Fm' and *Ex* and a negative influence for *D*. SOD and POD activities showed less similarity between the two subgroups (positive influence with 2 mM Ca^{2+}) and MDA (negative influence on plants with 0 mM Ca^{2+} w/MD and 4 mM Ca^{2+} intact). At time 0, therefore, among plants with mechanical damage, those cultivated with 4 mM Ca^{2+} had a negative effect on chlorophyll *a* fluorescence and gas exchange, with a positive influence on H_2O_2 and CAT and a negative effect on MDA. Among intact and damaged 0 mM Ca^{2+} plants, damage stimulated gas exchange and chlorophyll *a* fluorescence, with the exception of *A/Ci*, NPQ, Fv'/Fm' and *Ex*.

At time 90, plants 0 mM Ca^{2+} w/MD formed an isolated group, in which chlorophyll *a* fluorescence (except Fv/Fm, Fv'/Fm' and *Ex*), gas exchange, and total dry mass had a negative influence (Figure 10B). The other treatments formed a large group, subdivided into two. The first subgroup joined plants 2 mM Ca^{2+} w/MD and plants 4 mM Ca^{2+} intact, which resembled each other mainly by positive influence on H_2O_2 and negative influence of Fo and MDA. The second subgroup joined plants 0 and 2 mM Ca^{2+} intacts and plants 4 mM Ca^{2+} w/MD, which showed similarity in the responses of *E*, *Gs* and Fo, with positive and negative influence for CAT and H_2O_2. However, plants 0 mM Ca^{2+} intact showed a positive influence on SOD and POD in relation to other plants in the sub-group. Therefore at 90 days, plants 0 mM Ca^{2+} w/MD had a negative effect on chlorophyll *a* fluorescence and gas exchange and a positive effect on MDA.

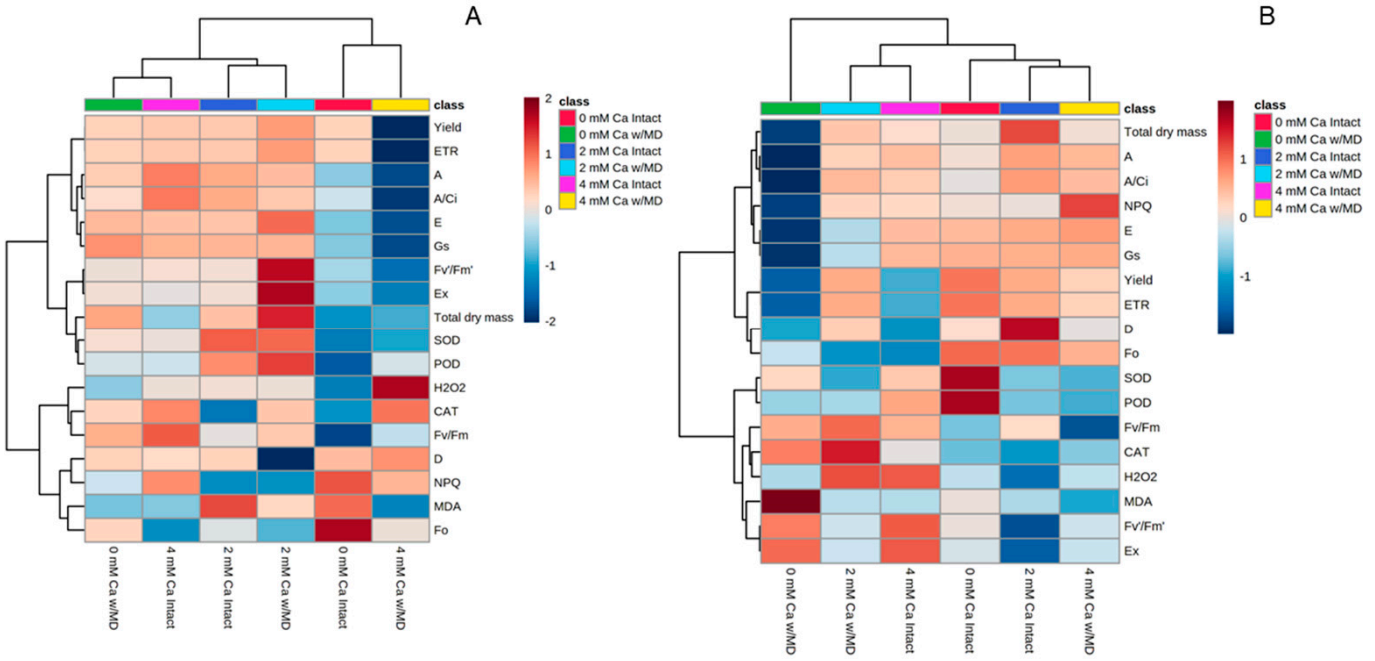

Figure 10. Heat map. Hierarchical cluster analysis presented as a heatmap on evaluations of maximum quantum yield for PSII photochemistry (Fv/Fm), energy dissipation as heat observed by non-photochemical quenching (NPQ), minimum Chl a fluorescence yield in the dark-adapted state (Fo), potential quantum efficiency of open PSII center (Fv'/Fm'), electron transport rate (ETR), effective quantum efficiency of PSII photochemistry (yield), light fraction absorbed by PSII antenna that is dissipated as heat (D), energy fraction not dissipated in the antenna that cannot be used for photochemistry stage (Ex), CO_2 assimilation rate (A), transpiration rate (E), stomatal conductance (Gs), RuBisCO's carboxylation efficiency (A/Ci), total dry mass, foliar concentration of hydrogen peroxide (H_2O_2), antioxidant enzyme activity of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), and lipid peroxidation expressed by the formation of malonaldehyde (MDA) in *Annona emarginata* plants submitted to the treatments: 0 mM Ca^{2+} without mechanical damage (intact), 0 mM Ca^{2+} with mechanical damage (w/MD), 2 mM Ca^{2+} intact, 2 mM Ca^{2+} w/MD, 4 mM Ca^{2+} intact, and 4 mM Ca^{2+} w/MD, at evaluation times: (**A**). 0 (30 min) and (**B**). 90 days.

4. Discussion

Manifestation of deficiency signs in *A. emarginata* was late, a result consistent with the low nutritional demand of the species [8]. Over time, plants grown in the absence of Ca^{2+} showed death of apical buds, a condition aggravated in the last evaluation. NPQ and Fo were high, while ΦPSII did not vary. However, plants 0 mM Ca^{2+} intact revealed *A* and *A/Ci* similar to plants 2 mM Ca^{2+}, which may be an indication that these plants have the capacity to store enough calcium to maintain baseline functions [23].

In plants 0 mM Ca^{2+} intact, the concentration of H_2O_2 was low, contributing to the efficiency of the enzymes SOD, CAT, and POD in controlling membrane damage. The development of these plants was compromised, since the relative growth rate showed constant behavior and a net assimilatory rate increasing, indicating that the photoassimilates were mainly directed to leaf production. Although plants grown without Ca^{2+} were kept in this condition for 120 days, the ion amount stored in the leaf did not differ from that of plants 2 mM Ca^{2+}. In the absence of calcium, there was no limitation in the accumulation of total dry mass at the end of the evaluation period, and the growth was constant, as observed by the growth analysis. Same calcium concentration in the stems was observed in intact plants and in those with mechanical damage, perhaps due to its low mobility. These verified results agree with those found in the literature that describe that a low Ca^{2+} concentration can interfere with development [24]. The low concentration of H_2O_2 contributed to the

efficiency of antioxidant enzymes and was not enough to signal and stimulate growth, considering that these ROS are reported in the literature as signaling agents in plants [25].

As observed in the heat map, at 90 days, plants 0 mM Ca^{2+} w/MD showed high Fv/Fm and Fv'/Fm' and low NPQ and Fo, conditions that, according to the literature, can lead to membrane damage [26,27]. These ROS, generated by low energy dissipation in the form of heat and fluorescence, with a lower ETR and ΦPSII, can cause damage to photosystems, according to studies in which ROS were generated by interruption of electron flow in photosystems [28,29]. This condition, in the present study, may justify low *A*, *Gs*, and *E* in plants 0 mM Ca^{2+} with mechanical damage, since, with the reduction in the ETR, there is a decrease in the synthesis of NADPH + H^+, which is required for CO_2 reduction [30].

While some studies demonstrate that the presence of ROS induces a cascade of signals, with the involvement of ABA and kinases, which contributes to stomatal closure [31] and limits H_2O vapor output and CO_2 input, other studies reveal that ROS act in the signaling of physiological processes, which can accelerate plant growth and development [32]. In this study, the accumulation of ROS may have acted both in signaling stomatal closure and in growth [33,34], since, even with less stomatal conductance, plants cultivated without calcium and with mechanical damage revealed a marked decrease in the curves of the net assimilation rate and relative growth rate, indicative conditions of self-shading and, therefore, rapid decline. This can explain the lower Ca^{2+} concentration in the leaves of plants grown without calcium and subjected to mechanical damage, indicating that this element may have been used to overcome stress from mechanical damage and not to increase the total dry mass.

Plants 2 mM Ca^{2+} w/MD showed ΦPSII and *A* similar to those presented by plants 4 mM Ca^{2+} intact, and with high Fv/Fm. This indicates high efficiency in energy use and less need for energy dissipation as a protection mechanism [28,35], which indicates that plants 2 mM Ca^{2+}, even with mechanical damage, showed highly efficient photochemistry.

In these plants, 2 mM Ca^{2+} w/MD, a low variation of the total dry mass was observed, in addition to constant NAR and RGR, indicating that their photoassimilates were not reversed for growth and development. The high concentration of H_2O_2 may have signaled the directing of photosynthetic resources to overcome stress from mechanical damage, as previously observed [33]. In addition, antioxidant enzymes were effective in controlling the concentration of H_2O_2, preventing membrane damage, observed at 90 days.

Plants 2 mM Ca^{2+} intact showed, in general, high ΦPSII, which estimates the amount of energy available for carbon reduction. This condition may have favored high *A*, indicating that 2 mM Ca^{2+} was sufficient for plants without damage to maintain their quantum yield without photoinhibition [36], since these plants presented adequate use of the luminous resource to produce reducing agents and carbon assimilation, without limiting stomatal conductance. In these plants, the amount of H_2O_2 was low, and the activity of the enzymes SOD, CAT and POD efficiently neutralized ROS, avoiding membrane damage [37]. The rapid decrease in curves of NAR and RGR also indicate good performance of these plants, which agrees with other studies carried out with the same species [9]. Thus, 2 mM Ca^{2+} is sufficient to guarantee intact plant growth. *A. emarginata* recovers from mechanical damage in the presence of 2 mM Ca^{2+} but stoppage growth.

Plants 4 mM Ca^{2+} intact showed a high ΦPSII. This allows the production of reducing agents, which together with the high *Gs* and *A*, contributed to high *A/Ci*, indicative of appropriate functioning of the photosynthetic apparatus as observed in other studies [36,38]. In these plants, the high concentration of H_2O_2, which may be due to photochemical activity, did not cause an increase in membrane damage. The activity of antioxidant enzymes controls reactive oxygen species, especially catalase, which is the main enzyme involved in the detoxification of H_2O_2 [34,37], as observed at 60 days.

These plants with 4 mM Ca^{2+} intact showed a high accumulation of total dry mass, although the slight decreases in the NAR and RGR revealed late self-shading and slow growth.

Plants 4 mM Ca^{2+} w/MD showed high NPQ, attributed to a photoprotection mechanism, to avoid damage to photosystems, and is in accordance with the literature [28,35]. Such protection may have contributed to the *A* that increased over time and with antioxidant enzymes, efficient in combatting the high concentration of H_2O_2, avoiding damage to membranes [37].

In addition, these reactive species may have participated in mechanical damage stress signaling [39], directing photoassimilates to overcome and restore the plant. This can be confirmed by the practically constant relative growth rate and the increasing net assimilation rate, indicative of leaf investment.

According to the literature, mechanical damage and photosynthesis can generate ROS [27], that were efficiently neutralized by the antioxidant system which depend on calcium signaling. This system's action can be confirmed by the evaluation of lipid peroxidation. In the present study, the absence of significant variation in the concentration of malondialdehyde, at 90 days, suggests that *A. emarginata*, without supplying Ca^{2+} and with damage, may have stored the ion in the form of calcium oxalate crystals when necessary [23] and as already verified for other *Annona* species [40], or it may have been stored in chloroplast, since this element is essential for the functioning of the photosynthetic apparatus [28]. This is in line with what was observed in the present study, in which the plants, regardless of the Ca^{2+} concentration, did not alter the concentration of the leaf element and in the condition of mechanical damage presented less accumulation of ions in the leaf, a condition that may have contributed to signaling and defense against the stress generated by mechanical damage, although other functions exerted by calcium, such as growth, may be impaired. It is known that the species *A. emarginata* presents strategies for overcoming abiotic stress, such as high energy dissipation in the form of heat, observed by the NPQ and maintaining effective quantum efficiency, conditions already observed in a study with water deficiency [8]. The high NPQ can also be indicative of calcium storage in the tissue, and this ion is involved in the energy balance between heat dissipation and the production of reducing agents [7].

The greater resistance of *A. emarginata* plants to mechanical damage may be the result of their slower growth, demonstrated by growth rate curves, with growth arrest when calcium was not supplied. In plants subjected to mechanical damage and with calcium, photoassimilates were used for restoration. Intact plants without calcium stopped growth, and their slower metabolism contributed to the restoration of the photosynthetic rate. In these plants, the stored calcium may have been sufficient to maintain the antioxidant system. Mechanical damage in the absence of calcium may have stimulated the use of the element previously stored in plant tissue.

We suggest that mechanical damage and calcium are related to activation of the defense system in *A. emarginata*. In absence of the element, the acceleration of growth did not influence the dry mass production, which was low due to the investment of resources to overcome this damage. In that case, calcium may have come from storage as calcium oxalate crystals, or from calcium stored in the chloroplast. The presence of calcium in plants with mechanical damage contributed to a signaling cascade, allowing photosynthetic restoration with growth arrest.

5. Conclusions

It was concluded that *Annona emarginata* showed better performance in restoration after mechanical damage in the presence of Ca^{2+} and was more sensitive in the absence of the mineral.

The cultivation of the species with 2 mM of Ca^{2+} in the complete nutrient solution was sufficient to guarantee the efficiency of the enzymatic antioxidant defense system, and photosynthetic reestablishment of plants subjected to mechanical damage.

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