# Discontinuous hydration in seeds of *Sarcomphalus joazeiro* Mart. Hauenschild (Rhamnaceae) improve seedling tolerance to water deficit

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ABSTRACT

Water is an essential abiotic factor for seed imbibition process. Seeds of several *Caatinga* species have a physiological strategy known as seed hydration memory to mitigate the effects of irregular rainfall patterns in this environment. However, the mechanisms behind are not well understood. Therefore, our study aimed to evaluate the occurrence of water memory in *Sarcomphalus joazeiro* seeds through ecophysiological, biochemical and anatomical analyzes. The seeds were subjected to different cycles (0, 1, 2, and 3) of hydration (12 hours) and dehydration (48 hours) – HD, or continuous hydration (CH) for 183 hours. The seedlings obtained of these seeds were subjected to different water suspension cycles. Our results showed that seeds subjected to HD cycles had greater germinability, higher emergence speed index, lower $T_{50}$ values, and accumulated higher proline content. Seedlings from the 0, 1, and 2 seed HD cycles showed decreased net carbon assimilation (*A*) only when subjected to severe stress after 21 days of water deficit compared to the daily irrigated plants. While in seeds exposed to 3-HD cycles after 21 days of water deficit *A* did not change compared to control. Our results evidenced that seeds subjected to 3-HD cycles conferred the plants a greater tolerance to water deficit, proving the existence of seed hydration memory in *Sarcomphalus joazeiro*.

**Key words:** *Caatinga*, gas exchange, *juazeiro*, leaf anatomy, water potential, drought

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**Availability of data and material (data transparency)**

Data is available by request to the authors.

**Code availability**

Not applicable.

**Author contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Maria Fernanda da Costa Oliveira, Emerson

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## 1. Introduction

In dry forests, such as the *Caatinga*, extreme environmental conditions, usually characterized by low rainfall patterns, high irradiance, and high temperatures, hinder the seed germination of many species (Azerêdo et al., 2016). The temporary water availability, caused by the rapid and irregular rainfall patterns associated with high evaporation from the soil surface layers, negatively affect seed germination by triggering cycles of hydration and dehydration (Nicolau et al., 2020). This environmental condition prevents the completion of the usual seed germination. Seed germination physiology is directly affected by the availability of water and its transport through the embryonic tissues. Dehydration periods during seed germination changes the seed metabolism by increasing the concentration of solutes and altering the intercellular pH. This condition in the cell trigger degenerative reactions (i.e. protein denaturation and membrane damage), increasing the occurrence of oxidative stress (Marcos-Filho, 2015).

During the dry season in the *Caatinga*, seeds with an interruption of water supply due to soil dryness can usually resume germination as soon as water is available again during the next rain (Lima et al., 2018). This is known as physiological strategy to mitigate the effects of irregular rainfall patterns which can be observed in several *Caatinga* species (Lima et al., 2018; Santos & Meiado, 2018; Melo et al., 2019; Nicolau et al., 2020). This mechanism to pause the germination metabolism during dehydration periods and continue the germination process when water is available, increases the germination and survival rates of native species in arid and semi-arid regions during short and extended drought periods. The hydration and dehydration cycles can generate an imprint or hydration memory in the seeds and contribute to their ability to counteract the physiological and biochemical changes caused by discontinuous hydration, in addition to providing uniformity and greater germination speed and formation of vigorous seedlings (Lima & Meiado, 2017).

The early stage of plant development, such as the seedling stage, is considered the most vulnerable stage to dehydration and many species exposed to this condition might have their survival compromised (Vieira et al., 2020). The primary defense mechanisms in plants under water restriction involve the stomatal control to prevent water loss, the dissipation of excess energy in the thylakoid membranes, the synthesis and accumulation of compatible osmolytes to adjust the cellular osmotic potential, and the activation of the antioxidant system to prevent oxidative stress (Vieira et al., 2021).

Usually, *Caatinga* species response to drought involve changes in the root development pattern, leaves loss, decrease in photosynthetic rates, and accumulation of compatible osmolytes (Medeiros, 2013; Prado, 2003; Sampaio, 1995). Some studies (Freitas et al., 2021; Santos Junior et al., 2021; Lima & Meiado, 2018) have shown that the occurrence of hydration memory in seeds from semi-arid environments and the propagation and continuity of this physiological strategy to the seedling provide higher drought tolerance during its initial growth stage. The mechanism behind is poorly investigated and a better understanding of how *Sarcomphalus joazeiro*, an important *Caatinga* species, deal with seed hydration and dehydration cycles can improve the propagation and growth of this species and others dry forest species, supporting the management and conservation plans to reforest degraded areas in the *Caatinga* and in other adverse environments.

This study aimed to evaluate whether seed discontinuous hydration, through different hydration and dehydration cycles, causes hydration memory in a *Caatinga* species, such as *Sarcomphalus joazeiro*, and increases seedling tolerance to water deficit using physiological, biochemical, and morphological approaches. We hypothesize that seeds subjected to longer dehydration cycles will germinate more quickly and that seedlings from these seeds will demonstrate more efficient stress tolerance mechanisms than seeds exposed to continuous hydration. Results are discussed in an ecophysiological perspective to improve propagation and growth of tree species in adverse environments.

## 2. Material and methods

### 2.1 Plant material and experimental conditions

The experiments in *Sarcomphalus joazeiro* plants were performed at the Laboratory of Physiology and Biochemistry of the "Instituto de Pesquisas Ambientais" (IPA), São Paulo, Brazil. The seeds were donated by the Caatinga Seed Network (UNIVASF, CRAD/MINISTRY OF SOCIAL INTEGRATION) and by LAFISE (Seed Physiology Laboratory of the Federal University of Sergipe, Sergipe, Brazil).

**2.2 Seed biometry and imbibition pattern**

We first analyzed the seed biometry and imbibition pattern to determine the hydration and dehydration curves. The seed moisture content used in this study was previously determined by Brazil (2009). The biometry of 200 seeds was performed using the ImageJ program and a digital caliper with 0.001 mm precision (Digimess®) to measure the length (mm) and width (mm) (Table 1). To evaluate the seed imbibition pattern, four replicates of 25 seeds each (n = 100) were used. Initially, all seeds were immersed in sulfuric acid (98%) for 120 minutes to overcome tegumentary dormancy (Diógenes et al., 2010), after the seeds were washed with tap water, weighed on an analytical balance, and placed to soak in 9-cm Petri dishes with two filter paper layers moistened with 10 ml of distilled water. The plates were kept in the laboratory at room temperature (25±5ºC). At 60-minute intervals, the seeds were removed from the Petri dishes, dried with absorbent paper, weighed to determine the fresh mass, and placed back in the Petri dishes until the imbibition cycle was completed. The imbibition rate was estimated through the variation of the seed biomass in the different time intervals evaluated.

Four replicates of 25 seeds each were weighed on an analytical balance to obtain the initial weight and determine the dehydration curve. Subsequently, each replicate was placed in 9-cm diameter Petri dishes containing two filter paper layers moistened with 10 ml of distilled water for 26 hours, when the seeds absorbed the maximum water amount before germination, as shown by the imbibition curve. After hydration, the replicates were removed from contact with water, placed to dry in desiccators, and weighed on an analytical balance at intervals of 60 minutes until the weight of the replicates returned to the initial weight.

**2.2 Continuous hydration and hydration and dehydration (HD) cycles experiment**

To evaluate the effects of continuous hydration on the *S. joazeiro* germination, 200 seeds were subjected to 183 hours of imbibition. This treatment was carried out in Petri dishes containing 10 ml of distilled water and 25 seeds each and kept at room temperature (25± 5°C). After the beginning of seed hydration, seven collections were made at different time intervals (00 h, 13 h, 61 h, 74 h, 122 h, 135 h, and 183 h) throughout the total soaking period. During imbibition period, 30 seeds were removed at each collection, snap-frozen in liquid nitrogen and stored at -80ºC for further analysis.

To analyze the effects of hydration and dehydration (HD) cycles on the germination of *S. joazeiro*, the seeds were subjected to 0, 1, 2, and 3 cycles of HD. Each cycle corresponds to 12 hours of hydration in distilled water and 48 hours of drying (dehydration), determined through the hydration and dehydration curves (detailed in the previous session). The hydration time corresponds to half time to reach seed germination phase I (Lima et al., 2018). We used 210 seeds per treatment. The seed hydration phase was carried out in Petri dishes, which were kept in laboratory conditions at room temperature (25 ± 5°C). For the dehydration phase, the seeds were dried in Petri dishes containing two filter paper layers and kept in the desiccator for 48 hours or until they returned to their initial weight before imbibition. Seven collections were performed during the HD cycles at different time intervals (00 h, 13 h, 61 h, 74 h, 122 h, 135 h, and 183 h); 30 seeds were removed, snap-frozen in liquid nitrogen, and kept at -80ºC for subsequent biochemical analysis.

**2.3 Seed extract**

The cryopreserved seeds were lyophilized and ground in a ball mill. For the crude extract, 100 mg of dried seeds were ground with 5 mL of 0.1 M monobasic phosphate buffer solution, pH 7.0, containing 0.01 M EDTA. The crude extract was filtered through a nylon mesh and centrifuged at 4,000 g for 10 minutes. The supernatant (seed extract) was transferred to 2 mL tubes and frozen for further biochemical analysis of soluble sugars.

**2.4 Biochemical analysis of seeds**

The total soluble sugars content was determined according to Dubois et al. (1956). Seed extract (500 µL) was incubated with 5% phenol (v/v, 500 µL) and 2.5 mL of $H_2SO_4$ (concentrated) in glass tubes and vortexed. After approximately 20 minutes, the reaction mixture was read in a spectrophotometer (490 nm). The reducing sugar content was determined using the Somogyi-Nelson method (Nelson, 1944) with a 500 µL aliquot of seed extract. The results were expressed in mg/g dry mass. The seed carbohydrate profile was carried out by using the high-performance anion-exchange chromatography/pulsed amperometric detection (HPAEC-PAD) from 2 mL of seed extract. Samples were separated for purification on Dowex 50 × 8 cationic (100–200 mesh) and Dowex 1 × 8 anionic (52–100 mesh) ion exchange columns. Then, the samples were lyophilized and resuspended in 5 mL of deionized water. After sugar quantification, the concentration of each sample was adjusted to 100 µg/mL. Samples were injected into a C18 HPLC column (250 x 4.6 mm, 5 µm) with an elution gradient of sodium hydroxide (625 mM), ultrapure water (Milli Q), and sodium acetate (0.5 M). Sucrose, glucose, and fructose concentrations were determined by comparing sample peak elution times with commercial sugar standards.

The free proline content in the seeds was determined according to Bates et al. (1973). Lyophilized seed samples were ground with 3% m/v sulfosalicylic acid. The crude extract was centrifuged (3,600 g for 15 minutes at room temperature), and the supernatant (extract, 2 mL) was recovered, to which 2 mL of acid ninhydrin and 2 mL of glacial acetic acid (concentrated) were added. The reaction medium was incubated in a water bath (100°C for 1 h), and the reaction was stopped by immersion in an ice bath. Subsequently, 4 mL of toluene (concentrated) was added, followed by vigorous stirring (20 s) and the aqueous phase (superior layer) was collected for reading in a spectrophotometer (520 nm). The results were expressed in mg proline/g dry mass.

## 2.5 Obtaining seedlings and water deficit experiment

After being subjected to the five pre-germination treatments (0, 1, 2, and 3 cycles of HD and continuous hydration – CH), 100 seeds from each treatment were placed in trays with vermiculite to germinate in a BOD-type germination chamber with a 16/8 h light/dark photoperiod and day/night temperature of 25/20°C (Rocha, 2010). Germination was monitored every two days; germinability (G = %) and the emergence speed index (ESI) were evaluated using the GerminaQuant software (Marques et al., 2015). The time to obtain germination of half of the seeds placed to germinate ($T_{50}$ - days) was evaluated according to the equation: $T_{50} = ti + [(N/2 – ni) \times (tj – ti)] / (nj – ni)$, where N is the final number of seeds germinated and nj is the cumulative number of seeds germinated by adjacent counts at times tj and ti, respectively, when $ni < N/2 < nj$ (Farooq et al., 2005). Seedlings with the first pair of leaves fully expanded were transplanted into 7 L pots containing organic substrate (Natus Solos do Brasil® compost).

Only seedlings from seeds that underwent HD cycles (0, 1, 2, and 3) were used to evaluate the effects of water deficit as the seedlings from CH treatment did not have a high germination rate (12.12%). The seedlings were acclimatized for 60 days in a greenhouse and watered daily. Afterwards, the seedlings were subjected to intermittent drought through five water treatments (control − seedlings watered daily, S7 − seedlings watered at seven-day intervals, S14 − seedlings watered at 14-day intervals, S21– seedlings watered at 21-day intervals and RE – seedlings rehydrated after 21 days of water suspension and collected seven days later, on the 28$^{th}$ day of the experiment).

## 2.6 Soil moisture ($U_{soil}$) and leaf water status

The soil moisture (Usoil) was measured by Time Domain Reflectometry (TDR) using a sensor model ML2-x Delta-T Devices (Theta-Probe, Cambridge, UK). The soil moisture was measured every 7 days for the S7 treatment plants, 14 days for the S14

treatment plants, 21 days for the S21 treatment plants before watering and after rehydration at the end of 21 days of water suspension for the RE treatment. Then, plants were re-irrigated, and the soil humidity was again measured one hour after water replacement until the soil returned to values close to field capacity (approximately 20% humidity). The leaf water potential (Ψwf) was measured on fully expanded leaves of the third pair from the apex of branches in the predawn period using a Scholander-type pressure pump (model 1000, PMS InstrumentCo).

**2.7 Gas exchange and chlorophyll *a* fluorescence**

Instantaneous measurements of net carbon assimilation rates (*A*, μmol $CO_2/m^2/s$) were assessed weekly in four plants per treatment (totaling 80 plants) using an infrared gas analyzer – IRGA (LCpro+, ACD BioScientific Ltd., Herts, UK). Measurements were performed in the middle part of the third fully expanded leaf from the apex, between 8:00 – 11:00 am. The saturating photosynthetically active radiation (PAR) used during the gas exchange measurements was 1,200 μmol photons /m2/s. The PAR was estimated in five *S. joazeiro* seedlings under optimal irrigation conditions through the light curve (Fig 1.).

Chlorophyll *a* fluorescence emission was assessed in 30-min dark-adapted leaves. Measurements were performed using a portable fluorometer (OS5p Opti-Sciences, Hudson, NH, USA). Leaves were initially exposed to a weak pulse of far-red light (1-2 μmol photons $/m^2/s$) to determine the minimum emitted fluorescence (F0) when all PSII reaction centers were in the oxidized form. Then, a saturating light pulse, with an irradiance of 3,000 μmol photons $/m^2/s^{-1}$ and duration of 1 s, was applied to temporarily promote the maximum reduction of the PSII primary electron acceptor (Qa), and the maximum fluorescence (Fm) was determined. From these measurements, the PSII maximum photochemical efficiency (Fv/Fm= (Fm - F0)/Fm) was calculated (Schreiber et al., 1994).

## 2.8 Leaf anatomy

For the anatomical analysis, fully expanded leaves were sampled from the third node of each plant of the three water treatments (control, severe stress – 21 days of drought and RE – rehydration), using two replicates for each seed HD cycle (0, 1, 2, and 3), totaling 24 leaves. The leaves from the control and RE treatments were fixed in 4% paraformaldehyde (v/v), followed by dehydration in an ethylic series (10 – 70%, v/v) and stored for the drought treatment in 100% ethanol (v/v) to avoid rehydration. Subsequently, fragments of the leaf blade, including the midrib, the margin, and the region between the margin and midrib, were obtained and subjected to dehydration in n-butyl alcohol (concentrated) and embedded in historesin (Leica Historesin Embedding Kit, Leica, Germany). Cross-sections with a thickness of 5 µm were obtained with a rotating microtome (RM 2155, Leica) and placed on histological slides. Slides were stained with periodic acid-Schiff reagent (PAS) and toluidine blue and mounted with Entellan (Merck, Germany). Sections were analyzed and photographed with a light microscope (Zeiss Axioskop 40 HBO 50, Zeiss, Germany) using AxioVision software (Version 4.8.2.0). The control and water-stressed plants were compared to diagnose structural changes; under drought conditions, especially in the most severe cases, it is expected to observe loss of cellular turgor in the tissues, reduction of chloroplasts in the mesophyll and greater lignification in vascular tissues.

## 2.9 Statistical analysis

The germination parameters were calculated by using the GerminaQuant 1.0 software (Marques et al., 2015). The different seed HD cycles were compared with an analysis of variance followed by Tukey's test. To analyze seedling development, the data were subjected to a two-factorial analysis of variance (ANOVA with two factors), represented by the seed HD cycles and the different watering treatments of the seedling experiment. Means were compared using Tukey's test ($p<0.05$). All statistical analyzes were performed using the SISVAR at a 5% significance, and the graphs were plotted using the SigmaPlot 11.0, Systat Software, Inc.

## 3.Results

### 3.1 The biometrics parameters and moisture in seeds

The seeds of *Sarcomphalus joazeiro* presented an average length of 12.76 mm and a width of 5.7 mm. The seed moisture percentage of 7.4% indicates that it is a dry seed (Table 1).

### 3.2 The germination parameters

The germination parameters were evaluated for 60 days (Table 2). Seeds that went through the three cycles of hydration and dehydration (HD) had a higher germination percentage (61.89%) than seeds that went through two HD cycles (24.12%), only one HD cycle (22.25%), or that did not go through any cycle (12.12%). Seeds that went through all three cycles had the highest emergence speed index (ESI), reaching a peak approximately 20 days after sowing, compared to seeds that went through less HD cycles or continuous hydration (CH).

The seed HD cycles decreased the $T_{50}$ (number of days necessary for germinating half of the seeds sown) proportionally according to the number of cycles where the shortest one was approximately 16 days in the 3$^{rd}$ HD cycle while the seeds under CH had the longest $T_{50}$ (45 days, Table 2).

### 3.3 Seed biochemical composition

The proline concentration of *S. joazeiro* seeds subjected to CH did not differ significantly over time (Fig. 1). While, in the seeds that went through the three HD cycles, the concentration of proline increased in the third dehydration cycle compared to previous HD cycles and the continuous hydration treatments (Fig. 1). The concentration of reducing soluble sugars decreased throughout the experiment in both seed continuous hydration and HD cycles treatments (Fig. 2A). However, it was higher in 2$^{nd}$ and 3$^{rd}$ HD cycles than in the CH seeds. The concentration of soluble sugars followed the same trend as the reducing soluble sugars. However, it was higher in the CH seeds than in the HD cycles seeds, especially after the second and third HD cycle (Fig. 2B).

The concentration of sucrose was higher in the initial periods of imbibition, corresponding to 74 hours in the seeds of continuous hydration and cycles 1 and 2 of discontinuous hydration (Fig. 3A-B). In the HD cycles seeds, sucrose concentration decreased after the second hydration cycle compared to the previous HD cycles. The levels of glucose and fructose were similar in each treatment. But they were higher in the CH and lower in the 1$^{st}$ and 2$^{nd}$ HD cycles compared to sucrose levels. In the third hydration cycle the levels of sucrose, glucose and fructose were similar, but glucose and fructose increased compared to sucrose levels in the dehydration of this cycle (Fig. 3A).

### 3.4 Seedling water status

The leaf water potential (Ψw) of control plants from the different seed HD cycles was constant over the experiment (around -0.9 MPa) (Fig. 4). The levels of Ψw in plants exposed to 14S days water deficit regime was similar to control plants regardless the seed HD cycle. Plants exposed to 7S and 21S days of water deficit regimes presented a variable leaf water potential in the different seed HD cycles. In the 3$^{rd}$ seed HD cycle, plants exposed to 21S days of water deficit regime presented lower leaf water potential (-1.2 MPa) than control while RE plants from all seed HD cycles recovered to the control levels.

**3.5 Gas exchange and chlorophyll *a* fluorescence**

Our results showed that the net carbon assimilation (*A*) of *S. joazeiro* seedlings decreased only after 21S days of water deficit regime in plants derived from seeds that underwent 0, 1 and 2 HD cycles, while in plants from 3 HD cycles this parameter was not affected by the water regimes compared to control plants (Fig 5). This result indicates a better tolerance of *S. joazeiro* seedlings (in the initial development stage) to water deficit after the seed HD cycles pre-treatment. After 21 days of water deficit, plants from all seed HD cycles were rewatered and recovered the A rates to control levels (Fig 5). The maximum quantum efficiency of PSII (Fv/Fm) did not change in all water regimes and in seedlings from all seed HD cycles (Fig. 6).

**3.6 Leaf anatomy**

The leaves of *S. joazeiro* are flat, dorsiventral and hypostomatic, with a prominent midrib on the abaxial surface, with a convex contour (Fig. 7-1A, C-E). The epidermis is unistratified and has cells with thickened external periclinal walls (Fig. 7-1A, C-E); these cells are generally periclinally elongated and are similar in size throughout their length (Fig. 7-1C-E), except for the midrib. In the midrib, the epidermal cells on the abaxial surface are smaller and the rounded shape predominate, and it was not detected stomata in this region (Fig. 7-1A). Moreover, we can notice in the midrib a large vascular bundle is evident, in addition to mesophyll cells presenting cortical cells with a rounded shape (Fig. 7-1A); and few cortical cells on the adaxial face (Fig. 7-1A-B) interrupting the continuation of the chlorophyll parenchyma.

The vascular bundle of the midrib is collateral, presenting an arched shape and surrounded by fibers (Fig. 7-1B). In the remainder of the lamina, the mesophyll is differentiated into palisade parenchyma, which is unistratified and spongy with three to four cell layers (Fig. 7-1C-E). The vascular bundles in the remainder of the lamina are also collateral, but they are smaller than that of the midrib and have a rounded shape. Some of these larger caliber bundles are also surrounded by fibers (Fig. 7-1D). The leaf margin has a rounded shape (Fig. 7-1D). In the control conditions (seed HD cycles 0 to 3), as there was no water restrictions, the cells were turgid, and the cells of the palisade and spongy parenchyma in the mesophyll had a large number of chloroplasts (Fig. 7-1C-E), causing a darker coloration in these tissues (Fig. 7-1C-E).

However, under drought conditions, we observed that in seedlings from seeds that went through 0, 1, and 2 HD cycles an initial turgor loss in the epidermal cells of the abaxial surface and/or reduction in the number of starch grains in the chloroplasts (Fig. 7-1C-D). This is probably related with a lighter coloration of the palisade and spongy parenchyma (Fig. 7-2C-E). In these first cycles, the mesophyll cells could also be more widely spaced, even the palisade ones (Fig. 7-2D), forming conspicuous intercellular spaces.

All these effects were intensified in seedlings originating from seeds that went through three HD cycles, where the epidermal cells of the abaxial face and mesophyll showed greater turgor loss (Fig. 7-2E). In all drought treatments, the fibers that surround the midrib vascular bundle were darker (Fig. 7-2A-B) than those of the control (Fig. 7A-B) and rehydration (Fig. 7-2B) treatments.

## 3. Discussion

Biometric is a morphological parameter that allows for the identification of environmental influences on seed germinative characteristics as well as variation among plant species (Santos Júnior et al., 2023). Larger seeds tend to produce healthier seedlings, which increases their survival rate during the initial development period (Silveira et al., 2022). The biometric data found falls within the range described in the characterization performed by Araujo et al. (2015).

The percentage of moisture found in the seeds (7.4%) indicates that it is an orthodox species (Table 1), a physiological characteristic found in various species of the *Caatinga*. This behavior facilitates easier storage, as high moisture can damage the embryo during this period. Santos Júnior et al. found similar moisture values (7.07%) in seedlings of *Piptadenia moniliformis*, a tree species native to the Brazilian dry tropical forest.

*Sarcomphalus joazeiro* seeds have tegumentary dormancy (Ursulino et al., 2019). Some studies (Araujo et al., 2015; Diógenes et al., 2010) suggested mechanical scarification to standardize and accelerate seed germination; however, without the pre-germination treatment with hydration and dehydration cycles (HD), the germination rate in continuous hydration (CH) seeds was only 12.12%. Other studies also observed greater efficiency in germination parameters when subjecting seeds of species that inhabit semi-arid environments to different hydration and dehydration cycles (Rito et al., 2009; Lima & Meiado, 2017; Lima et al., 2018). The HD cycles reduce the period necessary for germination in those species, as observed in the lower $T_{50}$ values in this study and for four *Caatinga* tree species (*Anadenanthera colubrina*, *Enterolobium contortisiliquum*, *Pityrocarpa moniliformis,* and *Pterogyne nitens*) subjected to different temperatures and HD cycles (Nascimento et al., 2021). This result suggests a positive metabolic change in response to HD cycles during seed germination.

Proline is an amino acid with osmoregulatory function frequently evaluated in stress studies since it maintains turgor in different plant tissues subjected to low water availability in the soil (Sena et al., 2021). In our research, the seed HD cycles induced greater proline accumulation in the seeds when subjected to drought events. Besides its role in osmotic adjustment, proline also performs a pivotal role in stabilizing membranes during stress conditions, preventing cellular electrolyte linkage, controlling reactive oxygen species (ROS) levels, and regulating general protein synthesis (Hayat et al., 2012; Kishor et al., 2015). Thus, the increase of proline concentration in seeds from the 3$^{rd}$ dehydration cycle suggests a better protection against the damage caused by dehydration during germination.

The reduction of total soluble sugars concentration in the seeds, especially sucrose, in the 2$^{nd}$ and 3$^{rd}$ dehydration cycles indicates that they were metabolized during

the germination, and/or degraded into glucose and fructose. According to Gill et al. (2002), reduced germination under water stress conditions may be attributed to the effect that seeds seemingly develop an osmotically enforced "dormancy" under water stress conditions, which may be an adaptive strategy for seeds to prevent germination under stressful environments thus ensuring a proper establishment of the seedling. However, our results show that the effect of successive drying cycles can accelerate reserve consumption, increase germination rate and emergency speed index. Furthermore, the increase in glucose, fructose and proline concentrations in the 3$^{rd}$ dehydration cycle may be a protective mechanism of cellular structures against drought. During the germination, the reactivation of metabolism occurs during the phase II of water imbibition. In this phase, the mobilization of sugars from starch degradation increase providing energy to respiration and embryo growth. According to Buckeridge et al. (2000), soluble sugars promote the formation of a glassy state which act as solutes capable of reducing chemical reactions harmful to cellular structures during dehydration.

The metabolic changes faced during germination may cause an imprint in the seedlings, preparing them to perform better in further adverse environments, as drought periods. Seedlings from seeds subjected to successive cycles of dehydration showed greater tolerance to water deficit, showing that drought memory seems to be present in *Sarcomphalus joazeiro*. The leaf water potential exhibited little variation in plants submitted to 1-3 HD cycles when compared to 0 HD cycle. It is possible that the osmotic regulation resulting from the degradation of seed reserves was translocated to the seedlings, maintaining a higher leaf Ψw. Sustaining a higher water status allowed seedlings from 1-3 HD cycles seeds to maintain higher levels of *A*, when compared to seedlings from 0 HD cycle seeds. Our results also demonstrated that even during the

initial growth of *S. joazeiro*, periods of moderate stress (7 to 14 days) were not enough to disturb the photosynthetic performance.

In addition, seeds subjected to three HD cycles with periods of up to 21 days of water deficit did not reduce $CO_2$ assimilation, which may indicate the possible acquisition of physiological memory in the plants after the dehydration events in the seeds. It is possible that stomatal closure has partially occurred, allowing carbon assimilation to have been maintained at minimal levels, without severe damage to the PSII. This fact is confirmed by the absence of photochemical damage according to the results obtained for Fv/Fm. Nascimento et al. (2019) evaluating seedlings of *Hevea brasiliensis* under water deficit, showed a decrease in net carbon assimilation, with photosynthesis values very close to zero. Santos *et al*. (2014) assessed the photosynthetic parameters of *S. joazeiro* under field conditions in a semi-arid region and verified a decrease in net carbon assimilation rates throughout the day, with negative values after 02:00 pm associating this response with a reduction a stomatal limitation. Likewise, Trovão et al. (2007) evaluated the photosynthetic parameters of 10 species from the *Caatinga*, including *S. joazeiro*. They did not find a reduction in the PSII quantum efficiency in this species, similar to our results; all values for Fv/Fm were within those proposed by Maxwell & Johnson (2000).

The maintenance of water status and carbon assimilation may also be related to the morphological/anatomical attributes of *S. joazeiro* seedlings. Although there was a loss of turgor in the palisade and spongy parenchyma as the drying cycles intensified, the leaves of *S. joazeiro* preserved water in the tissues, preventing cell collapse. In addition, the decrease in the amount and size of starch grains corroborates with the hypothesis that the degradation of leaf reserves results in a higher concentration of soluble sugars. The leaves of *S. joazeiro* are hypostomatic, meaning that stomata are restricted to the abaxial face. This leaf trait represents a protection to water loss under dry environments with high irradiance as faced by this species.

According to Vieira et al. (2022), the detrimental effects of excessive light on the photosynthetic apparatus are mitigated by the curling of leaves inward, which presents the palisade tissue on the inner side of the leaf. This alteration in the leaf orientation proves highly effective in protecting photosynthetic tissues from high light stress.

A similar result was found by Cabral et al. (2004) evaluating the leaf anatomy of *Tabebuia aurea*, a species that tolerates high luminosity and water deficit. The location of the stomata on the abaxial face contributes to a better development in periods of water stress, considering that it promotes an economy in the amount of water present in the plant tissues (Lemos et al., 2020). Regarding to the vascular bundles, the darker color of the fibers in the midrib of the leaves under drought indicates greater lignin deposition (Vieira et al., 2017), which provides resistance to the leaves, preventing senescence, even with the loss of turgor in the epidermis and mesophyll.

The morphophysiological and metabolic changes observed in this study suggest a high adaptive capacity of *S. joazeiro* to periods of water limitation, which may confer greater drought tolerance during seed discontinuous hydration cycles. Our findings can provide support for species propagation studies focusing on management and conservation of plants. Furthermore, our data indicate that drought memory in seeds certainly result in higher germination rates under favorable environmental conditions, as well as in the production of more vigorous seedlings with attributes that enable greater resistance to environmental stresses.

These attributes may provide better survival rates and success in the reintroduction of *S. joazeiro* for the reforestation of degraded areas in the *Caatinga*. Based on our results and the existing information on *Caatinga* species, we recommend the implementation of seed discontinuous hydration as a strategy for the reintroduction of nurse-woody species to degraded areas in dry forests like *Caatinga*. This involves on-site seed planting immediately upon maturity, utilizing direct sowing in open spaces, and direct planting of seedlings.

## 5. Conclusions

The seed hydration memory in *S. joazeiro* promoted seedlings more tolerant to drought. The photosynthetic indicators in this species strongly decreased after 21 days of water deficit. The recovery of these indicators and the restoration of all photosynthetic characteristics of the reirrigated plants occurred within seven days.

Irrigation intervals of up to 14 days associated with cycles of discontinuous hydration in the seeds do not compromise the production and survival of *S. joazeiro* seedlings. Both conditions can favor specific parameters such as germination rate, contributing to a greater and more vigorous seedling production and help restoration programs in *Caatinga* degraded areas.

## Conflicts of interest/Competing interests

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

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**Tables**

**Table 1**. Length, width and moisture content of seeds of *Sarcomphalus joazeiro* Mart.

**Table 2**. Germinability (%), emergency speed index (ESI), and the number of days for the germination of 50% of the seeds ($T_{50}$) of *Sarcomphalus joazeiro* Mart. subjected to different pre-germination treatments (0, 1, 2, and 3 hydration and dehydration cycles − HD and continuous hydration − over a total period of 183 hours)

**Figures**

**Fig. 1** Proline concentration (mg $g^{-1}$ DM) in *Sarcomphalus joazeiro* Mart. seeds subjected to hydration and dehydration cycles (HD) and continuous hydration. Seven collections were made at different time intervals (00 h, 13 h, 61 h, 74 h, 122 h, 135 h, and 183 h) throughout the total soaking period in seeds under HD cycles and the control (continuous hydration). Different lowercase letters compare treatments within the same evaluation period, and uppercase letters compare treatments over collections according to Tukey's test ($P<0.05$)

**Fig. 2** A- Reducing sugars concentration (mg g-1 DM) and B- soluble sugars concentration (mg g-1 DM) in *Sarcomphalus joazeiro* Mart. seeds submitted to hydration and dehydration cycles (HD) and continuous hydration. Seven collections were made at different time intervals (00h, 13h, 61h, 74h, 122h, 135h, and 183h) throughout the total soaking period in seeds under HD cycles and the control (continuous hydration). Different lowercase letters compare treatments within the same evaluation period, and uppercase letters compare treatments over collections according to Tukey's test ($P<0.05$)

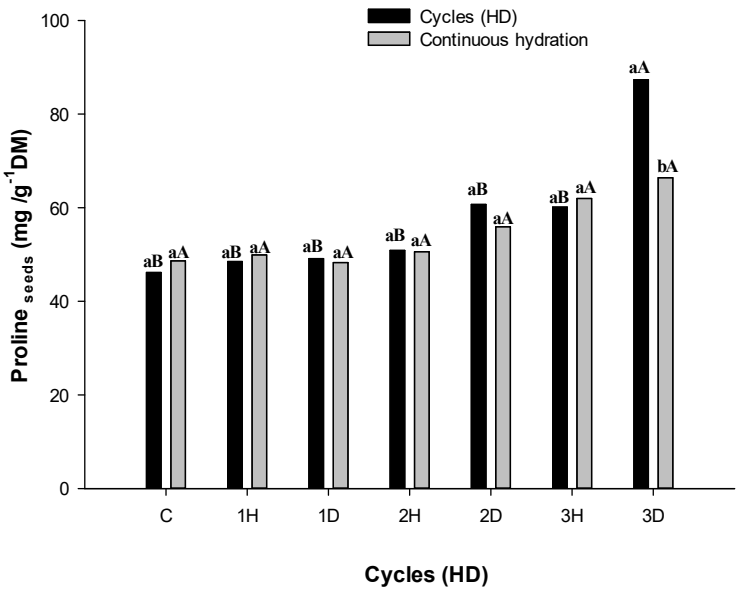
**Fig. 3** Glucose, fructose, and sucrose concentration (mg g-1 DM) in *S. joazeiro* seeds subjected to cycles of hydration and dehydration (A) and continuous hydration (B) and continuous hydration. Seven collections were made at different time intervals (00h, 13h, 61h, 74h, 122h, 135h, and 183h) throughout the total soaking period in seeds under HD cycles and the control (continuous hydration). Uppercase letters show differences between evaluation periods and lowercase letters between sugars analyzed in each collection. Equal letters do not differ by Tukey's test at a 5% probability

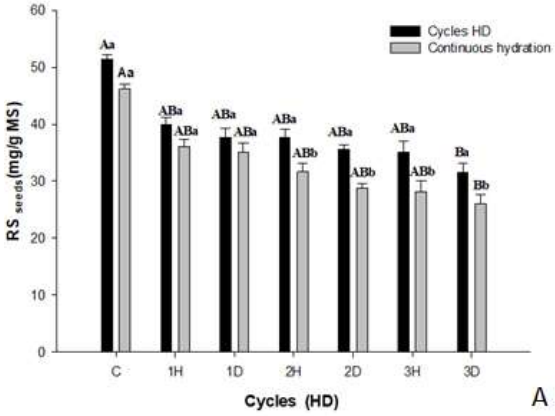
**Fig.4** Leaf water potential (Ψw MPa) in *Sarcomphalus joazeiro* Mart. from seeds that underwent HD hydration and dehydration cycles (0-A, 1-B, 2-C, and 3-C HD cycles) subjected to different water treatments (Control - plants watered daily, moderate stress - plants watered between intervals of 7 days and 14 days, severe stress – plants watered between intervals of 21 days and RE – plants subjected to rehydration after 21 days of water suspension). Equal lowercase letters between HD cycles and uppercase letters between water treatments did not differ from each other by Tukey's test at a 5% probability

**Fig. 5** Net CO2 assimilation (*A*) in *Sarcomphalus joazeiro* Mart. from seeds that underwent HD hydration and dehydration cycles (0-A, 1-B, 2-C, and 3-C HD cycles) subjected to a dry cycle (A) through different water treatments (Control - plants watered daily, Moderate stress – plants watered between intervals of 7 days and 14 days, Severe stress – plants watered between intervals of 21 days and RE– plants subjected to rehydration after 21 days of water suspension). Equal lowercase letters between HD cycles and uppercase letters between water treatments did not differ from each other by Tukey's test at a 5% probability

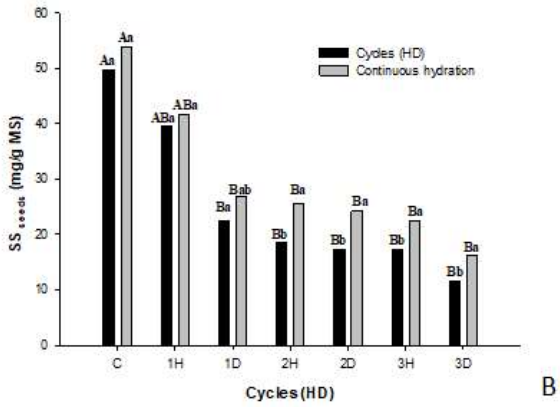
**Fig.6** Maximum quantum efficiency of the PSII (Fv/Fm) in *Sarcomphalus joazeiro* Mart. from seeds that underwent HD hydration and dehydration cycles (0-A, 1-B, 2-C, and 3-C HD cycles) subjected to a dry cycle (A) through different water treatments (Control - plants watered daily, Moderate stress – plants watered between intervals of 7 days and 14 days, Severe stress – plants watered between intervals of 21 days and RE– plants subjected to rehydration after 21 days of suspension of watering). Equal lowercase letters between HD cycles and uppercase letters between water treatments did not differ from each other by Tukey's test at a 5% probability

**Fig. 7** 1- Leaf anatomy of *S. joazeiro* in seedlings from seeds subjected to different cycles of hydration and dehydration (HD) under normal irrigation conditions (control). HD treatments: cycle 1 (A-B, D), cycle 0 (C), cycle 3 (E). A. General aspect of the midrib. B. Detail of the midrib showing the vascular bundle and cortical cells. C and E. General aspects of the region between the midrib and the margin. D. General aspect of the margin. (Arrows indicate crystals; Arrowheads indicate starch grains in cortical cells; Square indicates cortical cells; C, cortex; Es, estomata; Fi, fibers; M, mesophyll; Ph, phloem; PP, palisade parenchyma; SP, spongy parenchyma; VB, vascular bundle; X, xylem). Scale bars: A (100 µm); B-E (50 µm). 2- under drought conditions. HD treatments: cycle 2 (A-B, D), cycle 0 (C), cycle 3 (E). A. General aspect of the midrib. B. Detail of the midrib showing the vascular bundle; note the darker coloring of the fibers. C-E. General aspects of the region between the midrib and the margin: in C there is a loss of turgor in the epidermal cells of the abaxial surface; in E there is a loss of turgor in the epidermal cells of the abaxial surface and the mesophyll; in D and E it is noted that the mesophyll cells are spaced apart; in the three images it is shown that the mesophyll has a lighter color, indicating a decrease in the amount of starch grains in the chloroplasts. (Arrows indicate crystals; Arrowheads indicate starch grains in cortical cells; C, cortex; Fi, fibers; M, mesophyll; Ph, phloem; PP, palisade parenchyma; SP, spongy parenchyma; VB, vascular bundle; X, xylem). Scale bars: A (100 µm); B-E (50 µm)

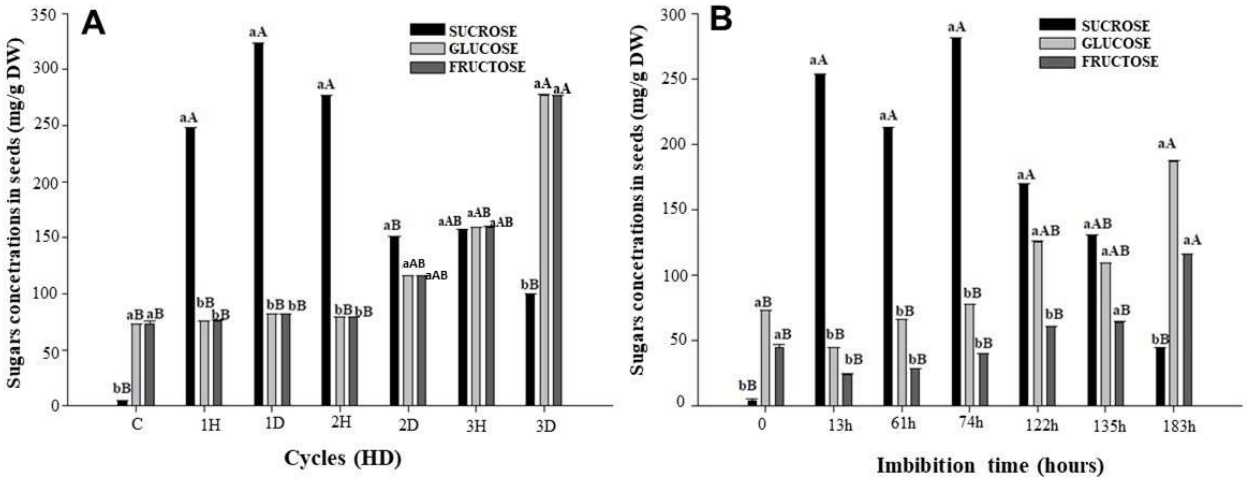

Cycles (HD)
Continuous hydration
Proline seeds (mg /g-1DM)
100
80
60
40
20
0
aB aA
aB aA
aB aA
aB aA
aB aA
aB aA
aA bA
C
1H
1D
2H
2D
3H
3D
Cycles (HD)

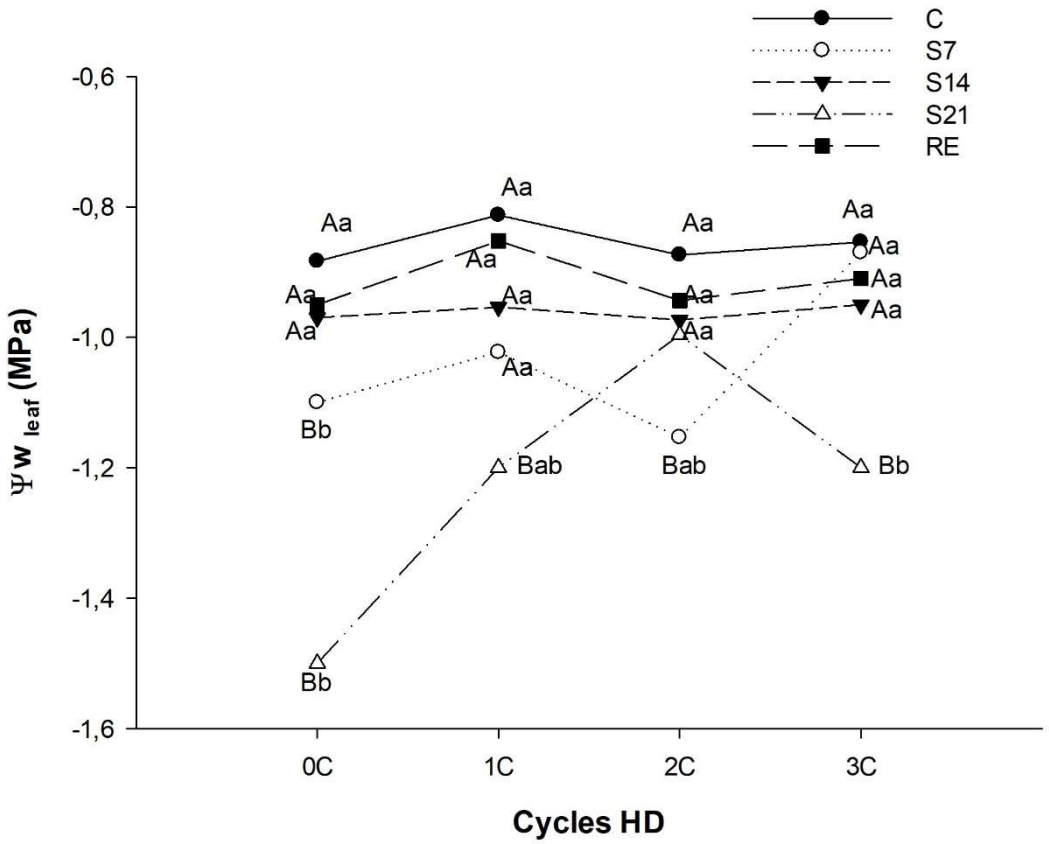

Cycles HD
Continuous hydration
RS seeds(mg/g MS)
60
50
40
30
20
10
0
Aa Aa
ABa ABa
ABa ABa
ABa ABb
ABa ABb
ABa ABb
Ba Bb
C
1H
1D
2H
2D
3H
3D
Cycles (HD)

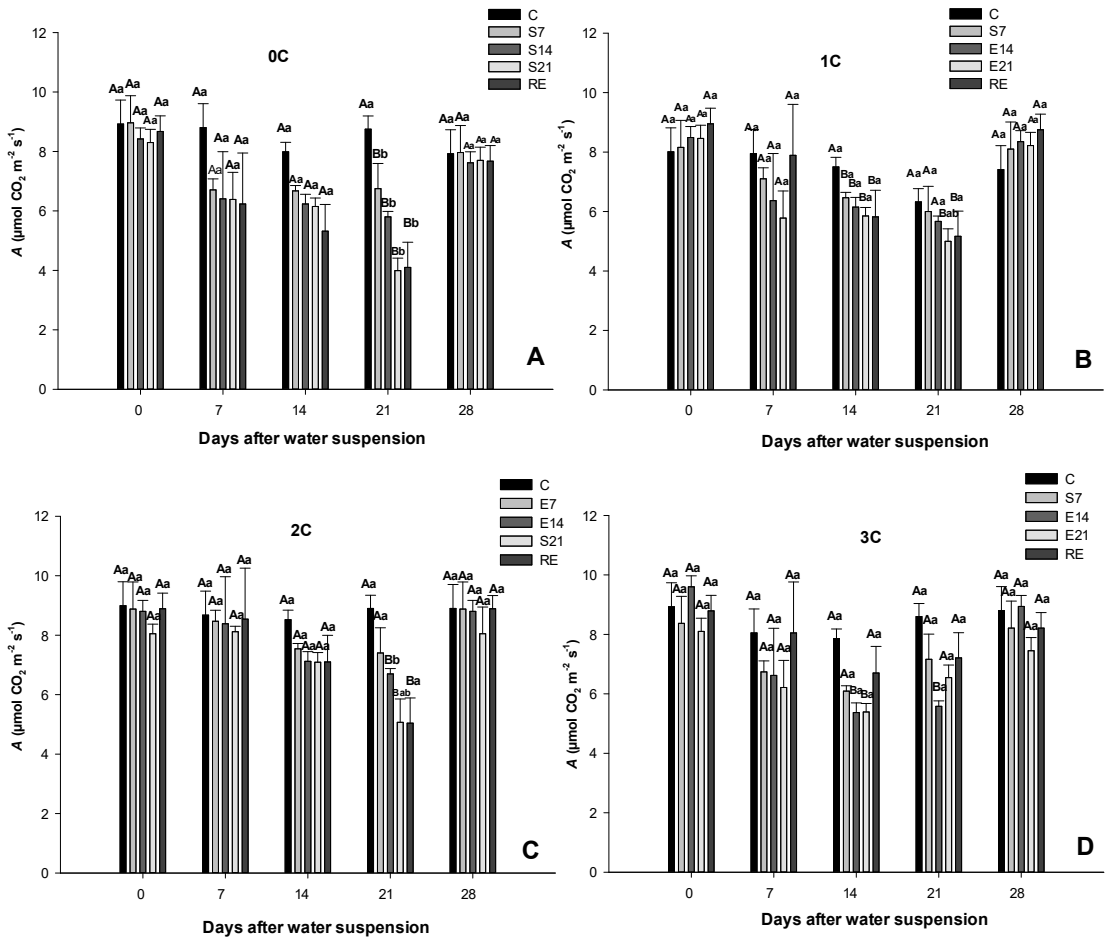
A

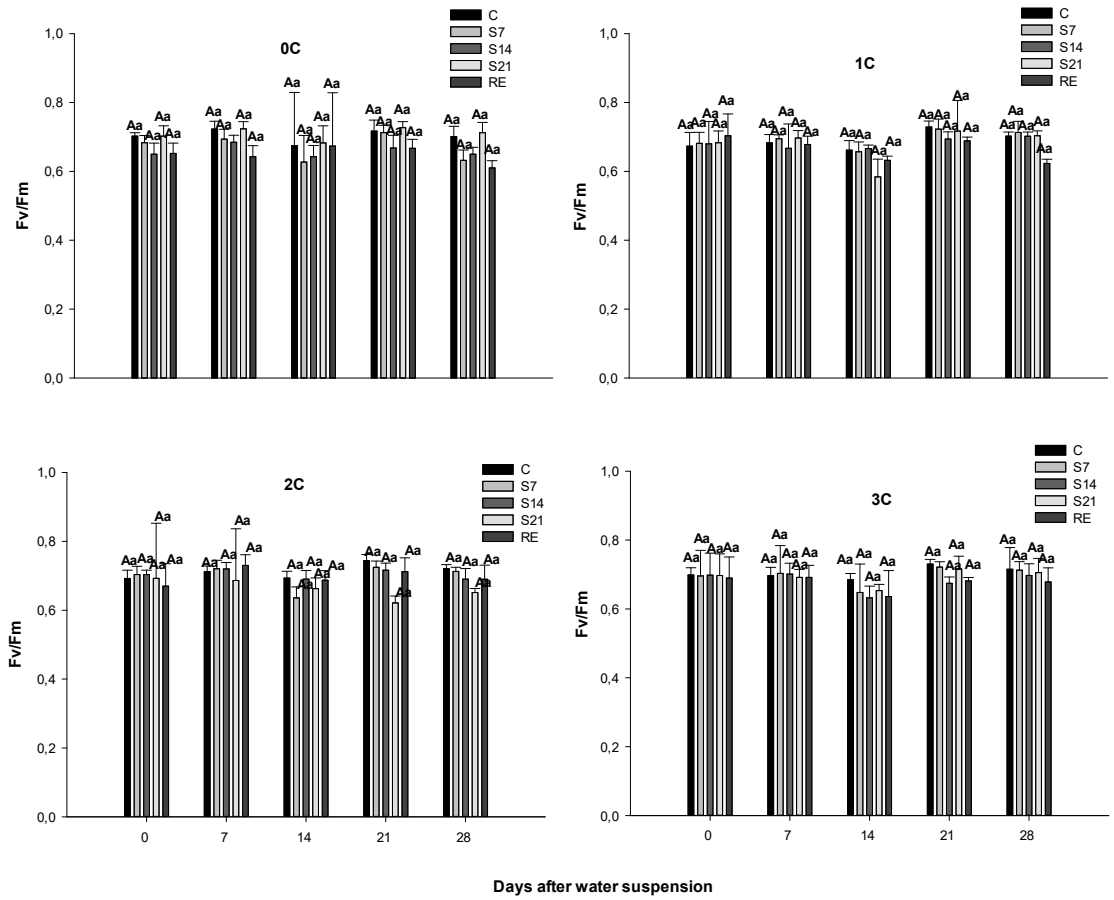

Cycles (HD)
Continuous hydration
SS seeds (mg/g MS)
60
50
40
30
20
10
0
Aa Aa
ABa ABa
Ba Bab
Bb Ba
Bb Ba
Bb Ba
Bb Ba
C
1H
1D
2H
2D
3H
3D
Cycles (HD)

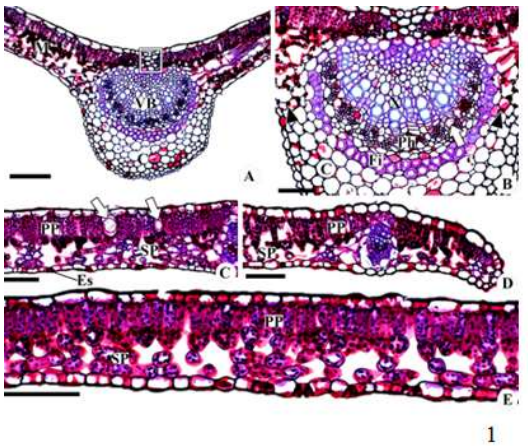
B


A
SUCROSE
GLUCOSE
FRUCTOSE
Sugars concetrations in seeds (mg/g DW)
Cycles (HD)
C
1H
1D
2H
2D
3H
3D
B
Imbibition time (hours)
0
13h
61h
74h
122h
135h
183h

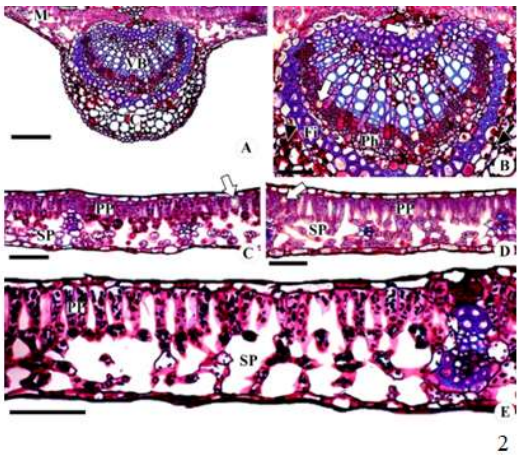

C
S7
S14
S21
RE
Ψw leaf (MPa)
Cycles HD
0C
1C
2C
3C


0C
1C
2C
3C
A
B
C
D
C
S7
S14
E7
E14
E21
S21
RE
A (μmol CO2 m-2 s-1)
Days after water suspension
0
7
14
21
28


0C
1C
2C
3C
C
S7
S14
S21
RE
Fv/Fm
1,0
0,8
0,6
0,4
0,2
0,0
Aa
0
7
14
21
28
Days after water suspension


VB
PP
SP
Es
A
B
C
D
E

1


VB
PP
SP
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2