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Original Paper

Proline and antioxidant enzymes protect *Tabebuia aurea* (Bignoniaceae) from transitory water deficiency

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Abstract

Water deficiency is a major abiotic stress that limits biomass production and drives plant species distributions. We evaluate the effects of water deficiency on ecophysiological and biochemical parameters of seedlings of *Tabebuia aurea*. Plants were subjected to daily watering (control) and to stress by soil water deficiency for 29 days. Leaf area, plant biomass, gas exchange, SPAD index, maximum quantum yield (F_v/F_m), quantum yield of PSII (ΦPSII), superoxide dismutase (SOD) and L-ascorbate peroxidase (APX) activity, lipid peroxidation, and proline content were recorded. Plants responded to water deficit by reducing leaf area and accumulating proline. Stomatal conductance was reduced to limit the water loss by transpiration. However, limiting CO_2 uptake caused reduction in photosynthesis and biomass. The excess of energy unutilized by photosynthesis reduced SPAD index and ΦPSII. As a result, we observed an increase in SOD and APX activity, protecting chloroplast membranes from further damages caused by lipid peroxidation. Our results indicate that *T. aurea* have capacity to survive under water deficiency reducing stomatal aperture, but affecting the rate of CO_2 assimilation. Nevertheless, plants showed mechanisms to preventing damages to the photosynthetic apparatus. Such plasticity is an important adaptation for plants growing in dry environmental.

Key words: antioxidant system, gas exchange, tree species, water deficit, proline.

Resumo

A deficiência hídrica é um importante estresse abiótico que limita a produção de biomassa e dirige a distribuição de espécies vegetais. Nós avaliamos os efeitos da deficiência hídrica sobre parâmetros ecofisiológicos e bioquímicos de plantas jovens de *Tabebuia aurea*, uma espécie arbórea nativa da “Caatinga”, um bioma semiárido. Plantas foram submetidas à irrigação diária (controle) e ao estresse promovido por deficiência de água no solo por 29 dias. A área foliar, biomassa da planta, trocas gasosas, índice SPAD, rendimento quântico máximo (F_v/F_m) efetivo do PSII (ΦPSII), atividade da superóxido dismutase (SOD) e L-ascorbato peroxidase (APX), peroxidação lipídica e teores de prolina foram determinados. As plantas responderam ao estresse hídrico reduzindo a área foliar e acumulando prolina. A condutância estomática também foi reduzida limitando a perda de água por transpiração. Contudo, a limitação da absorção de CO2 causou reduções nas taxas fotossintéticas e na biomassa. O excesso de energia não utilizada pela fotossíntese provocou uma redução no índice SPAD e em ΦPSII. Como resultado, nós observamos um aumento nas atividades de SOD e APX, protegendo as membranas dos cloroplastos de danos causados pela peroxidação de lipídeos. Nossos resultados indicam que *T. aurea* tem capacidade de sobreviver sob deficiência hídrica reduzindo a abertura estomática, mas afetando a taxa de assimilação de CO_2. Apesar disso, as plantas mostraram mecanismos de prevenção de danos ao aparato fotossintético. Tal plasticidade é uma importante adaptação para plantas crescendo em ambientes semiáridos como a Caatinga.

Palavras-chave: sistema antioxidante, trocas gasosas, espécies arbóreas, deficiência hídrica, prolina.

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Introduction

Adaptations to water deficiency is crucial for plants growing in semi-arid environments such as the Caatinga biome of Northeastern Brazil, a seasonally dry tropical forest that has been severely degraded by human activities (Da Silva *et al.* 2017). Like many semi-arid regions, climate change is exacerbating droughts in the Caatinga (Torres *et al.* 2017), leading to increasingly harsh conditions for the native vegetation (Acosta-Salvatierra *et al.* 2017). Increased knowledge about plant responses to water deficiency is crucial for understanding the impacts of climate change and for the restoration of the Caatinga.

Trees growing in the semi-arid face a difficult trade-off between reducing water loss and growth under drought conditions. Specifically, if they close stomata to reduce transpiration, then less CO_2 will be available for photosynthesis and biomass accumulation will be reduced (Barros *et al.* 2020). The capacity to survive and grow under conditions of limited water availability is therefore critical for semi-arid vegetation and is a major driver of plant species distributions (Bykova *et al.* 2019).

In addition to stomatal closure, plants show a plethora of physiological mechanisms to tolerate periods of water deficit, resulting in alterations in gas exchange, chlorophyll index and chlorophyll *a* fluorescence (Silva-Pinheiro *et al.* 2016). Morphological adjustments are also an important strategy to adapt to water scarcity, which can last up to nine months of the year, in "Caatinga" (Da Silva *et al.* 2017). Moreover, drought episodes occur in parallel with high temperatures and light intensities further contributing to the physiological challenges of life in semi-arid ecosystems (Souza *et al.* 2010). During the dry season increasing vapor pressure deficit (VPD) increases soil water deficit, negatively affecting plant development (Queiroz *et al.* 2019). This is because both soil and atmosphere moisture are involved in stomatal responses to water deficiency (Tominaga *et al.* 2014; Barros *et al.* 2020). Plants may also reduce their leaf area in response to water deficit, probably as an additional strategy to prevent excessive loss of water (Silva-Pinheiro *et al.* 2016).

As mentioned above, high radiation levels and water deficiency occur simultaneously, and may cause damage to the thylakoid membranes, impairing the electron transfer in photosystem II (Deng *et al.* 2018). Some plants respond to these conditions by dissipating excess of absorbed light energy as heat. However, this mechanism may fail if plants are submitted to low water availability (Santos *et al.* 2013).

Plants may also respond to water deficiency by accumulating compatible solutes and by activating their antioxidant system. These mechanisms contribute to maintaining plant cell turgor and protecting the integrity of the membranes (Nounjan *et al.* 2018). The antioxidant system also protects against the toxic effects of reactive oxygen species (ROS) as O_2^- and H_2O_2 (Martins *et al.* 2018) and includes enzymes such as superoxide dismutase (SOD) and L-ascorbate peroxidase (APX) functionally scavenging ROS (Sharma *et al.* 2012).

Studies of plant physiological responses to short (transitory) episodes of water deficiency are important to understand how drought-adapted plants deal with carbon assimilation and biomass distribution among shoots and roots during conditions of low soil water availability. This information is crucial for Caatinga conservation, since effective restoration of this highly degraded biome will depend upon detailed knowledge of the physiological tolerances and ecology of native plant species. Tree species are particularly important in this respect because they act as 'nurse plants' for many other species, facilitating the growth and reproduction of other native species (Moura *et al.* 2013).

Tree seedlings are usually used to recover degraded areas subject to water deficiency. The ability to tolerate water scarcity in the early stages of development is the key characteristic for choosing plant species for the recovery of degraded lands (Vieira *et al.* 2017). One of the most promising tree species for restoration projects is the drought tolerant *Tabebuia aurea* (Silva Manso) Benth. & Hook.f. *ex* S.Moore (Bignoniaceae), which is widely distributed in tropical and subtropical regions of the Americas (Lorenzi 1992). Previous studies have indicated that leaf water potential of *T. aurea* rapidly recovers after a short-term stress provoked by water deficiency. Specifically, Silva-Pinheiro *et al.* (2016) subjected plants to water deficit by suppressing irrigation until photosynthesis reached zero, and then plants were re-watered until carbon assimilation rates reached those values of irrigated plants. They recorded that plants supported an average of about -4.0 MPa of leaf water potential, indicating considerable capacity to adapt to water scarcity conditions.

Here, we investigate the physiological response of *T. aurea* to short-term water deficiency (29 days) under greenhouse conditions. Specifically, we focus on the effects of water deficiency on gas exchange, dissipation of energy, proline accumulation and the antioxidant system. We aimed to assess the responses of plants after a controlled period of water deficit, not submitting individuals to total drought. Our results have direct for the effective use of *T. aurea* for the restoration of degraded areas of the semi-arid Caatinga biome.

Material and Methods

Experimental site and plant material

We collected seeds of *Tabebuia aurea* from a seasonally dry tropical forest region known as "Caatinga" (09°21'49"S, 37º14'54"W). Seeds were initially germinated in soil in plastic trays. We selected 18 apparently healthy seedlings and transferred them into 25 L plastic pots (one plant per pot) containing 20 kg of soil. The soil was collected from an area located at the Agricultural Science Center (Federal University of Alagoas). The soil was characterized as sand = 674 g Kg^{-1}, (26% thin sand and 74% thick sand), clay = 296 g Kg^{-1} and silte = 30 g Kg^{-1}, as medium texture soil. The main chemical elements were P = 207 mg dm^{-3}, K = 3.6 mg dm^{-3}, Ca^{2+} = 41 $cmol_c$ dm^{-3}, Mg^{2+} = 13 $cmol_c$ dm^{-3}, MO = 25 dag Kg^{-1}.

The experiment was performed under greenhouse conditions at the Federal University of Alagoas, Brazil (09°28'02"S, 35°49'43"W). Microclimatic data was recorded by an automatic weather station (WS - GP1, Delta-T Devices, Cambridge, England) installed inside the greenhouse (data shown in Fig. 1a-b). The mean temperature during the experiment was 30.29 ºC, the mean relative humidity and radiation was 68.92% and 569 µmol m^2 s^{-1} respectively, and the mean VPD was 1.61 KPa.

Six month-old plants were subjected to two treatments, with nine replicates each: (1) control: plants daily irrigated to field capacity; (2) stress: plants subjected to controlled water deficit maintained at soil water potential (Ψw_{soil}) below -1.5 MPa (Fig. 2) for 29 days. The analysis started when the water potential of the soil reached values equal to or below 1.5 MPa. The maintenance of the water potential was performed with the replacement of the water lost daily. For this purpose, pots were weighed daily with the use of a semi-analytical balance in order to replace the water lost by evapotranspiration. The Ψw_{soil} was measured daily using a WP4C - Dew Point Potential Meter (Decagon Devices, Pullman, USA) with soil samples collected through a PVC tube inserted in each pot at a depth of 10 cm, based on previous studies of Silva-Pinheiro *et al.* (2016).

Dry mass, number of leaves, leaf area, leaf water status, gas exchange, relative chlorophyll content, and photochemical efficiency of photosystem II (PSII) were measured 29 days after the imposition and maintenance of treatments. We also collected leaf material to quantify the levels of soluble proteins, proline, the activity of the antioxidant enzymes SOD and APX and malondialdehyde-equivalents (MDA).

Dry mass accumulation and leaf area

Dry mass was measured with a semi-analytical balance AD3300 (Marte Científica, Santa Rita do Sapucaí, Brazil) after sectioning and drying stems, leaves and roots for three days at 65 ºC in a forced air oven . Leaf area was determined using a Leaf Area Meter LI-3100C (LI-COR, Richmond, New Zealand).

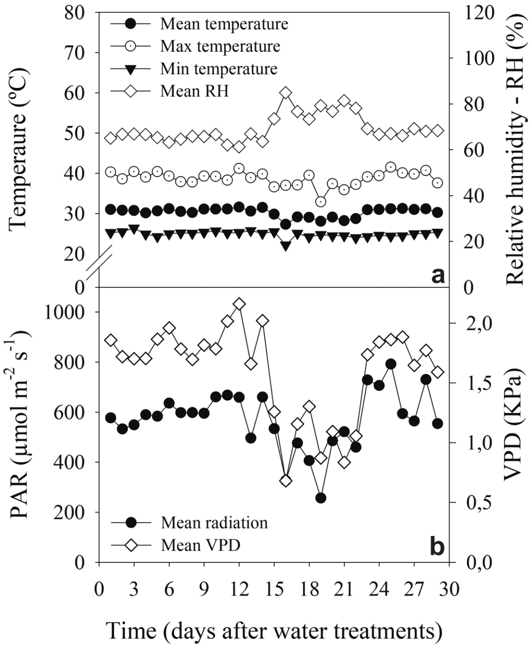

Figure 1 – a-b. Greenhouse microclimatic data during the experiment – a. temperature (ºC) and relative humidity (%); b. radiation (photosynthetically active radiation - PAR) (µmol m^{-2} s^{-1}) and vapour pressure deficit - VPD (KPa). Each point (day) represents a mean of 24 measurements (hour-hour).

Gas exchange measurements

Leaf gas exchange was evaluated between 08:00 and 10:00 a.m. using an infrared CO_2 gas analyzer model Li-6400XT (LI-COR, Lincoln, USA). Photosynthetic rate (A), transpiration rate (E) and stomatal conductance (gs) were determined for healthy, fully expanded leaves from the middle of the plant using the following settings: 1000 μmol m^{-2} s^{-1} photosynthetic photon flux density (determined through a light curve), we used a buffer volume to dampen fluctuations in concentration of atmospheric CO_2, block temperature 27 ºC. Water use efficiency (instantaneous - iWUE and intrinsic - IWUE) was calculated as A/E (iWUE) and A/g_s (IWUE).

Photochemical efficiency of photosystem II (PSII), relative chlorophyll content (SPAD index) and leaf water status

Quantum yield of PSII (ΦPSII) was evaluated at midday with a fluorometer PAM-2500 (Walz Heinz GmbH, Effeltrich, Germany). The maximum quantum yield of PSII (F_v/F_m) was also determined at midday using a saturation pulse of actinic light (8000 μmol m^{-2} s^{-1}) during one second to promote PSII reaction center closing, following Maxwell & Johnson (2000). The readings of F_v / F_m began 20 min after leaf adaptation to darkness, using special clips. We determined relative chlorophyll content (SPAD index) using a portable chlorophyll meter SPAD-502 (Minolta, Plainfield, USA). Leaf water potential (Ψw_{leaf}) was used to verify plant water status and was measured using a pressure chamber (SoilMoisture Equipment, Santa Bárbara, USA) (Scholander *et al.* 1965) at predawn and midday. All measurements were made on the same leaves that were used to evaluate gas exchange.

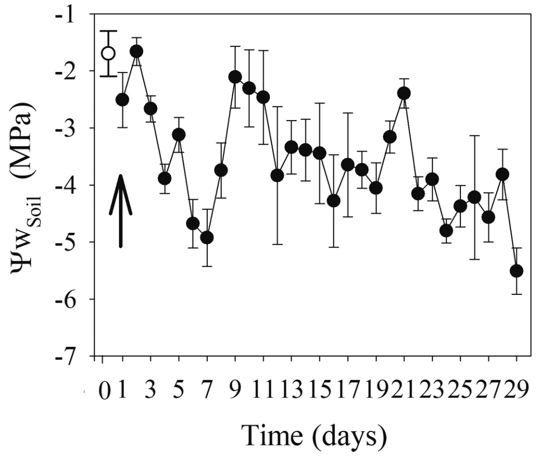

Figure 2 – Soil water potential (Ψw_{soil}) of *Tabebuia aurea* soil under water deficit during the experiment. The arrow indicates the day when plants reached Ψw_{soil} below -1.5 MPa (first day of the experiment). * means significance at $P < 0.05$ between treatments using the t test. n = 9.

Biochemical analyses

We collected leaf samples and immediately stored them at -80 °C until analysis. Proline was extracted from lyophilized leaves and determined according to Bates *et al.* (1973). Acid ninhydrin solution was prepared by heating 1.25 g of ninhydrin in 20 mL of 6 M phosphoric acid and 30 mL of glacial acetic acid with continuous stirring until mixture was completely homogenized. We mixed 0.1 g of triturated leaf with 118 mM sulfosalicylic acid. Homogenized samples were centrifuged at 3000 g for 10 min at a temperature of 25 ºC, and the supernatant was used for subsequent analysis. Absorbance was read at wavelength of $\lambda = 520$ nm by using toluene blank and standards of known concentration of proline.

SOD activity was measured following the method described by Giannopolitis & Ries (1977). 300 mg of fresh weight leaves were homogenized in a mortar by using liquid N_2 with polyvinylpyrrolidone (PVP) and extracted with pH 7.8 phosphate buffer. The assay was carried out using the following reaction mixture: 50 mM potassium phosphate buffer (pH 7.8), 100 nM ethylenediaminetetracetic acid (EDTA), 75 mM nitro blue tetrazolium (NBT), 13 mM methionine and 2 mM riboflavin and 50 μL of plant extract. Subsequently, the homogenate was centrifuged at 14.000 g for 15 min at 4 °C. After this step, the reaction mixture was exposed to white light for 10 minutes and absorbance was measured at 560 nm. SOD activity of the extract was expressed as SOD mg^{-1} protein.

The activity of APX was determined following Nakano & Asada (1981) with some modifications. The extract was obtained from 100 mg of fresh leaf material homogenized in 2 mL of the extraction mixture, composed by deionized water, 50 mM monobasic potassium phosphate (pH 7.5) buffer, 20 mM ascorbate, 0.1% Triton-x 100, 0.2 mM EDTA, and 300 mg of PVP, with liquid N_2. Subsequently, the homogenate was centrifuged at 14.000 g for 15 min at 4 °C and the supernatant was used for determination of APX activity. Then, 75 µL of homogenate was added to a reaction mixture containing deionized water, 50 mM monobasic potassium phosphate (pH 7.5) buffer, 0.5 mM ascorbate and 0.05 mM hydrogen peroxide. Absorbances were measured for one minute at 290 nm and the extinction coefficient used for ascorbate was 2.8 $mM^{-1}cm^{-1}$ at 290 nm. Results were expressed as APX min^{-1} mg^{-1} protein. Total protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (Sigma Chemical Company) as a standard.

Lipid peroxidation in leaf tissues was measured according to method described by Cakmak & Horst (1991). Grounded leaves (100 mg of fresh leaf) were homogenized using a pestle and mortar with liquid N_2 and 4 mL of trichloroacetic acid (TCA) 0.1% w/v. Subsequently, the homogenate was centrifuged at 15.000 g for 15 minutes at 4 °C. Malondialdehyde-equivalents (MDA) quantification was as follows: 1.5 mL of the thiobarbituric acid solution (0.5% TBA made in 20% TCA) was added to homogenate that was heated for 5 minutes at 95 °C. The concentration of MDA was calculated from the absorbance of 530 nm by using the extinction coefficient of 155 Mm^{-1} cm^{-1} and was expressed as nmol g^{-1} FW.

Statistical analysis

Data from different treatments were compared using *T* test ($P < 0.05$). Was used 9 (nine) replicates in the analysis of gas exchange, biomass, leaf area, number of leaves, Ψw_{leaf}, SPAD Index, F_v / F_m and ΦPSII. Was used 8 (eight) replicates for proline content analysis and 5 (five) replicates in MDA, APX and CAT analysis. We used Pearson's correlation analysis to investigate possible correlations between Ψw_{leaf}, proline content, Ψw_{soil}, SPAD Index, ΦPSII, F_v / F_m, SOD, APX, MDA, *A*, g_s and *E* in each treatment, using software R (R Development Core Team 2020) 3.4.3. Photochemical, biochemical and gas exchange parameters were subjected to a principal component analysis (PCA) using Past3 software. Pearson's correlation and PCA analysis were made with 5 (five) replicates from each variable. We used SigmaPlot (free trial) to plot graphics.

Results

Plant morphology and Gas exchange

On the 29th day after the suspension of irrigation, significant effects of water regimes on *T. aurea* biomass were detected. The leaf dry mass of plants under water deficit was 38% lower than the control ($P = 0.004$) and stem ($P = 0.0002$) and root ($P = 0.003$) dry mass was 29% lower than the control (Fig. 3a). No significant differences were observed for the root: shoot ratio between treatments ($P = 0.3$) (Fig. 3b). Leaf area and number of leaves were 31% lower in plants under water deficit than in the control ($P = 0.03$) (Fig. 3c,d). The rate of stomatal conductance (g_s) was reduced by 91% in plants under water deficit ($P = 0.000005$), affecting transpiration by 90% reduction ($P = 0.000004$) and photosynthesis by 85% reduction ($P = 0.000001$) (Tab. 1). We observed a positive correlation between g_s and *A* ($r = 0.90$ *) (Tab. 2). The iWUE had an increase of 52% ($P = 0.002$) and IWUE increased 76% ($P = 0.002$) in plants under water deficit (Tab. 1).

Photochemical efficiency of photosystem II & relative chlorophyll content

On the 29th day, the SPAD index was 8% lower in plants under water deficit ($P = 0.03$), though F_v / F_m did not differ significantly between this treatment and the control ($P = 0.41$). ΦPSII was impaired by water deficit, with reductions of around 48% ($P = 0.001$) (Fig. 4). Furthermore, we found positive correlation between F_v / F_m and the SPAD index ($r = 0.90$ *) (Tab. 2).

Leaf water potential & proline content

Ψw_{leaf} values decreased significantly after the reduction in soil water potential. Predawn Ψw_{leaf} levels were around -0.7 MPa in control plants and -1.7 MPa in plants under water deficit (Fig. 5a). At midday, Ψw_{leaf} declined to -2.9 MPa in plants under water deficit and to -0.9 MPa in control plants. Leaf proline content did not differ between treatments at the predawn. By midday the leaf of the plants under water deficit showed significant increase of proline of 69% when compared to the control, both lyophilized before analysis (Fig. 5b). We observed an inverse correlation between proline and Ψw_{leaf} ($r = -0.89$ *) and APX ($r = -0,88$ *) in plants under water deficit (Tab. 2).

Concentration of antioxidant enzymes

The indicative values of the concentration of SOD did not differ between control and water deficit treatments at predawn, but had increased significantly (61%) at midday in plants under water deficit. In contrast, the results obtained from APX was higher at predawn (28% higher) than at midday (24% higher) in plants under water deficit. MDA content in leaves was not affected by water deficiency (Fig. 6). However, MDA showed an inverse correlation with A (r = -0.93 *) (Tab. 2).

Plants under the two treatments were clearly separated on the PCA analysis. The first two main components of the PCA explained 92.4% of the total variation. Gas exchange (A, g_s and E) and Ψw_{leaf} made the higher contributions to PC1, which explained 75.6% of the variation and managed to separate the groups of control and water deficit plants. PC2, that explained 16.8% of the variation, accounted primarily for antioxidant enzymes (SOD and APX), MDA, proline content, and F_v / F_m, indicating a gradient of separation between plants, independent from watering regime (Fig. 7).

Discussion

The results of this experiment with *Tabebuia aurea* demonstrated that plants had tolerance to soil water deficiency. The induced water deficit significantly affected growth, gas exchange and biochemical parameters. As anticipated, the plants adaptively responded with a drought avoidance strategy by reducing leaf area and closing stomata. Although the chlorophyll index and effective quantum efficiency of photosystem II reduced under water restriction, values of F_v / F_m indicated that the photochemical apparatus continued to be active. Possibly, the activity of SOD and APX prevented damage to the chloroplast membranes. Such drought tolerance mechanisms are among the strategies adopted by semi-arid plants to tolerate long periods of water shortage, as also the individual characteristics related to their own physiological plasticity of each species (Souza *et al.* 2010).

This is a strategy to prevent excessive loss of water, thereby increasing the ability of young plants to survive water scarcity (Nascimento *et al.* 2011; Silva-Pinheiro *et al.* 2016). Silva-Pinheiro *et al.* (2016) also observed that water deficiency reduced the leaf area of *Bauhinia monandra* Kurz and *T. aurea*. Several plant species native to the Caatinga lose their leaves just before flowering, which typically coincides with a period of drought,

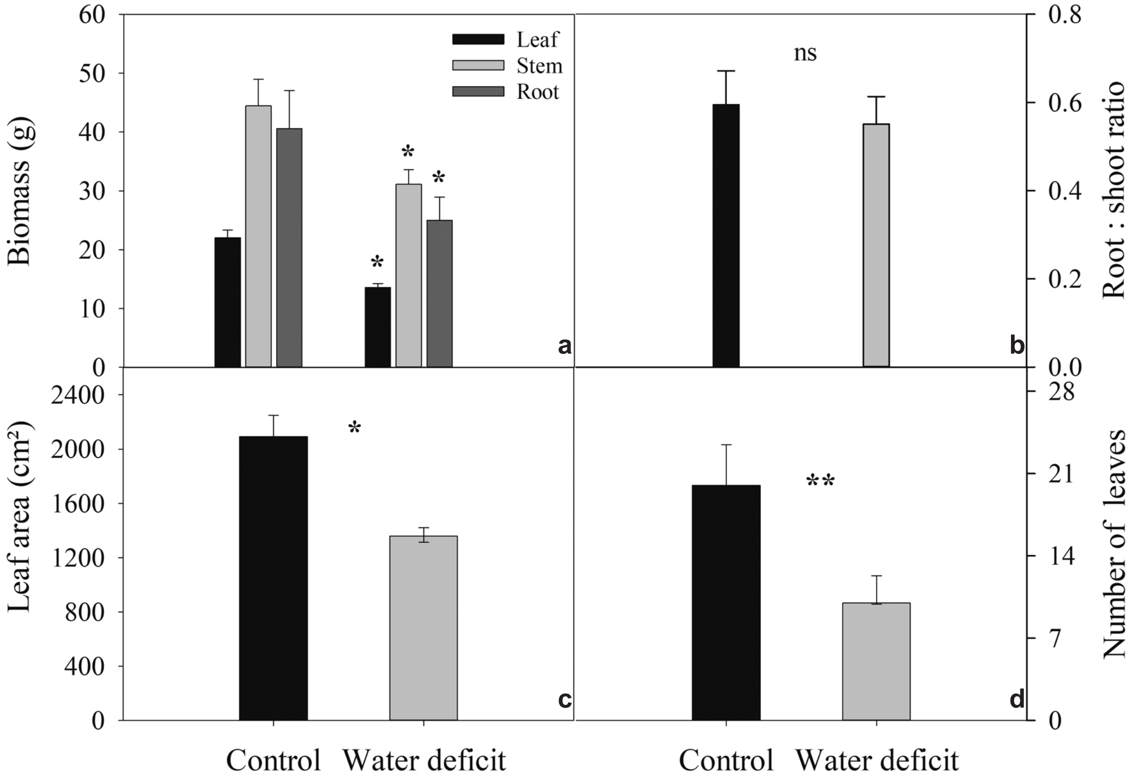

Figure 3 – a-d. *Tabebuia aurea* seedlings under two water regimes: control and water deficit – a. biomass; b. root : shoot ratio; c. leaf area; d. number of leaves. * means significance at $P < 0.05$ between treatments by T test; ** mean significance at $P < 0.01$, ns = not significant. n = 9.

Table 1 – Stomatal conductance (g_s), transpiration (E), photosynthesis (A), instantaneous water-use efficiency (iWUE) and intrinsic water-use efficiency (IWUE) of *Tabebuia aurea* seedlings under two water regimes: control and water deficit.

Parameter	**Control ± SD**	**Water deficit ± SD**
g_s (mol H_2O m^{-2} s^{-1})	0.07 ± 0.006	0.006 ± 0.002*
E (mmol H_2O m^{-2} s^{-1})	3.78 ± 0.3	0.37 ± 0.1*
A (µmol CO_2 m^{-2} s^{-1})	8.53 ± 0.67	1.27 ± 0.34*
iWUE (µmol CO_2 mmol H_2O^{-1})	2.25 ± 0.1	3.42 ± 0.89*
IWUE (µmol CO_2 mol H_2O^{-1})	116.7 ± 6.07	206.3 ± 24.8*

* means significance at $P < 0.01$ by Student's T test, respectively. SD = Standard deviation; n = 9.

producing new leaves at the beginning of the rainy season (Mendes *et al.* 2013). We observed that reductions in leaf area occurred concomitantly with reductions in root biomass, suggesting that *T. aurea* did not invest in root growth as an adaptive strategy to access more water.

Under water deficiency, *T. aurea* reduced stomatal conductance, which consequently affected transpiration rate and photosynthesis. However, lower rates of transpiration and stomatal conductance can play a beneficial role by increasing leaf iWUE (that is most responsive for environmental changes such as VPD) and IWUE (that is regulated by plants physiology state or genotype) respectively (Mujawamariya *et al.* 2018). Both parameters are commonly analyzed

Table 2 – Pearson's correlation coefficients between physiological and biochemical variables of *Tabebuia aurea* submitted to two water regime (control and water deficit): Ψw_{leaf}, proline (Pro), Ψw_{soil}, SPAD Index (SPAD), F_v / F_m, SOD, APX, MDA, A, g_s and E. Up-right diagonal refers to the control treatment and down-left diagonal refers to the water deficit treatment. n = 5.

	Ψw_{leaf}	**Pro**	Ψw_{soil}	**SPAD**	F_v / F_m	**SOD**	**APX**	**MDA**	A	g_s	E
										Control treatment	
Ψw_{leaf}	---	0.10	0.78	-0.53	-0.55	-0.07	0.72	-0.71	-0.71	0.05	0.11
Pro	-0.89*	---	0.02	-0.88*	-0.59	-0.62	-0.48	-0.29	-0.59	-0.41	-0.39
Ψw_{soil}	0.41	-0.12	---	-0.31	-0.44	-0.22	0.59	-0.28	0.04	0.59	0.61
SPAD	0.31	-0.23	0.68	---	0.66	0.43	0.43	0.67	0.67	0.36	0.30
F_v / F_m	0.64	-0.58	0.55	0.90*	---	0.85	0.18	0.15	0.81	0.36	0.38
SOD	-0.44	0.35	0.38	0.21	-0.16	---	0.59	-0.32	0.56	0.23	0.30
APX	0.67	-0.88*	-0.35	-0.08	0.33	-0.59	---	-0.60	0.22	0.42	0.50
MDA	-0.45	0.15	0.00	0.37	0.09	0.76	-0.21	---	0.37	0.22	0.11
A	0.37	-0.20	-0.30	-0.63	-0.30	-0.78	0.38	-0.93*	---	0.82	0.81
g_s	0.23	-0.19	-0.42	-0.85	-0.56	-0.51	0.38	-0.72	0.90*	---	0.99**
E	0.24	-0.20	-0.43	-0.84	-0.55	-0.52	0.39	-0.72	0.91*	0.99*	---
	Water deficit treatment										

* means significance at $P < 0.05$ and ** means significance at $P < 0.01$.

together with carbon isotope discrimination to select more productive or tolerant plant genotypes (Centritto *et al.* 2009; Broeckx *et al.* 2014; Medrano *et al.* 2015; Wang *et al.* 2020). Increasing water-use efficiency during periods of water deficit as seen on this study is an important characteristic that can increase plant tolerance to drought (Wang *et al.* 2020). It is important to highlight that the water-use efficiency measured at leaf level such as by gas exchange has its limitations due be always measured over short periods and so, can reflect only short-term variations in environmental conditions and the current physiological state of the leaves (Landsberg 1999).

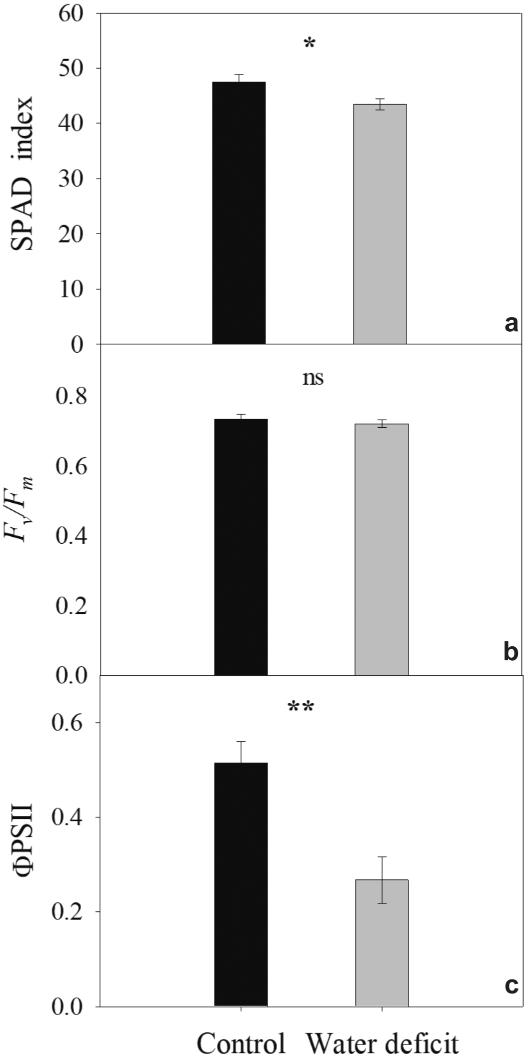

Figure 4 – a-c. *Tabebuia aurea* seedlings under two water regimes: control and plants under water deficit – a. SPAD index; b. F_v / F_m; c. ΦPSII. * means significance at $P < 0.05$ between treatments by T test and ** means significance at $P < 0.01$, ns = not significant. n = 9.

Stomatal opening is proportional to the amount of CO_2 demanded by photosynthesis and the amount of water loss by transpiration. Stomatal closing is thus a mechanism to reduce transpiration and decrease gas exchange, with negative consequences for carbon fixation and biomass accumulation (Barros *et al.* 2020). Stomatal effects seem to be the main limiting factor for photosynthesis, since other parameters were not severely affected. It is noteworthy that the plants were able to remain photosynthetically active even under dry conditions throughout the experiment. Nevertheless, the decrease in stomatal conductance under water deficit suggests that the g_s response is a key mechanism of drought tolerance. The positive correlation observed in these variables shows that stomatal closure was linked to reductions in C_i and photosynthetic rates in stressed plants.

Stomatal closure is a strategy to minimize the negative effects of water deficiency and is an important characteristic of drought tolerant species (Lawlor & Tezara 2009; Berry *et al.* 2010), as observed in *Carapa guianensis* Aubl. (Carvalho *et al.* 2009). Various studies (*e.g.*, Pinheiro *et al.* 2005; Deuner *et al.* 2008; Nascimento *et al.* 2008; Broeckx *et al.* 2014) suggest that stomatal closure is the primordial strategy to minimize water loss during the day period. Galmés *et al.* (2007) working with eight Mediterranean species submitted to water deficiency also verified decreases in stomatal conductance. Certainly, decreases in stomatal conductance are important to minimize water loss, but with the consequence that the rubisco catalytic site may be deprived of CO_2 leading to a decrease in carbon assimilation. Thus, acclimation of *T. aurea* during drought stress is primarily dependent on stomatal control, since CO_2 availability is crucial to ensure photosynthesis. Under regular watering, open stomata permit high levels of gas exchange enabling vegetative growth, as observed in our study. Generally, g_s of species from semiarid environments have more sensitive drought responses before a change in leaf water content is detectable (Souza *et al.* 2010).

The multivariate analysis (PCA) corroborates the results found by Pearson's correlation, that shows a correlation between the gas exchange variables and supports the role of the control of the stomatal opening and its reflexes on the A and E as an important drought tolerance strategy adopted by the plants of *T. aurea* under water deficit in this experiment. In a review of 20 water stress indicator parameters, Füzy *et al.* (2019) concluded that gas exchange is one of the parameters set that are the most useful indicators of stress, preferably when the plant biomass is constant between treatments (not observed in this experiment), which reinforces the data obtained from the PCA.

High levels of radiation can cause reductions in gas exchange due to photoinhibition, when excess excitation energy absorbed by plant leaves exceeds the photosystem II repair cycle (Nixon *et al.* 2010). However, we observed a similar F_v / F_m ratio in plants submitted to water deficiency and well-watered plants. Although *T. aurea* did not exhibit significant differences of F_v / F_m between treatments, there was a reduction in ΦPSII in plants under water deficit, which may have contributed to the decrease in photosynthesis. Reductions in ΦPSII probably impair efficiency of photosystem II, reducing the proportion of energy absorbed by plants (Maxwell & Johnson 2000).

The increase in SOD and APX activity observed after 29 days of water deficiency probably reflects an adaptation to avoid chronic photooxidative damage. SOD is the first barrier against the toxic effect of ROS (Mittler 2002). Oxidative stress induced by an excess of energy in photosystems may limit photosynthesis and lead to an over-excitation of the reaction centers of photosystem II leading to a production ROS (Carvalho 2008; Santos *et al.* 2013). An increase in SOD and APX activity was verified in plants stressed by water deficit. These enzymes are involved in antioxidant defense system, scavenging ROS and reducing damage to chloroplasts membranes and photosystem (Rena & Maestri 2000; Grisi *et al.* 2008; Deng *et al.* 2018). When considered with the similarity in MDA content in both treatments, these results indicate that antioxidant system of *T. aurea* is able to regulate oxygen metabolism avoiding chronic damages to the chloroplast membrane system and protecting proteins complexes that catalyzes the light reactions (Queiroz *et al.* 2002; Oliveira *et al.* 2014).

Besides the ROS detoxification system, plants can develop other strategies to tolerate water deficit, such as reduction of chlorophyll synthesis or of chloroplast pigment content (Silva-Pinheiro *et al.* 2016). The observed reduction in SPAD index in drought stressed plants suggests that chlorophyll content was affected by the water deficit. A decrease in chlorophyll content under drought stress has been considered as a typical symptom of oxidative stress and may result from photooxidation of the photosynthetic pigments (Anjum *et al.* 2011; Abdoli & Saeidi 2013).

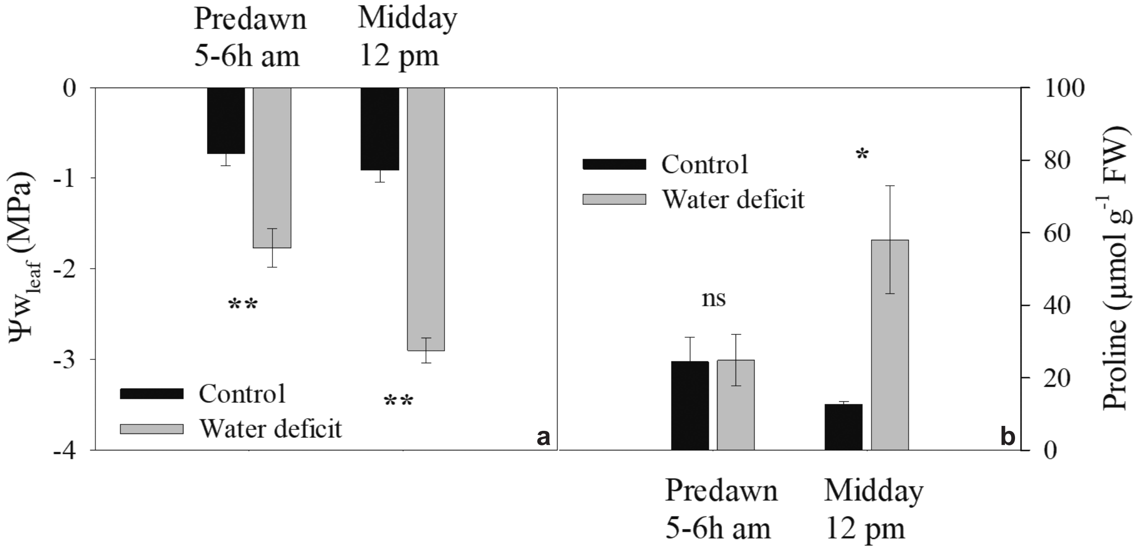

Figure 5 – a-b. *Tabebuia aurea* seedlings under two water regimes: control and plants under water deficit, at predawn (5–6 a.m. before sunrise) and at midday (12 p.m.) – a. leaf water potential (Ψw_{leaf}); b. leaf proline content. * means significance at $P < 0.05$ between treatments by T test and ** means significance at $P < 0.001$, ns = not significant. n = 9 for Ψw_{leaf}, n = 8 for proline content.

Although, the positive correlation between F_v / F_m and SPAD index implies that water deficiency reduces chloroplast pigment content and may damage PSII activity, there is evidence that water stressed plants can prevent photoinhibitory damage by reducing chlorophyll content (Kyparissis *et al.* 1995). Similar results have been reported in other studies with tree species under water deficiency (Alves 2007; Huseynova *et al.* 2016).

Proline content increased in the leaves of plants under water deficit, and we can suggest that that *T. aurea* has an osmotic adjustment mechanism based on proline accumulation; there was a positive correlation between proline and Ψw_{leaf}. These changes are consistent with other studies that show that increased proline can benefit plant metabolism, like maintaining cell turgor by osmoregulation and protecting membranes and macromolecules from damage caused by desiccation (Hayat *et al.* 2012; Hassan *et al*. 2021). Our results do not support the strategy of maintaining the turgor since there was a reduction in plant growth and closure of stomata, but it indicates that proline may have acted in protecting membranes and as a reserve of carbon and nitrogen for subsequent synthesis of organic compounds after rehydration (Aranjuelo *et al.* 2011; Hemaprabha *et al.* 2012).

Our results suggest that the analyzed physiological and biochemical variables were adjusted to attenuate the effects of water deficiency. Stomatal conductance was reduced, but it probably was essential for plants to remain photosynthetically active during the 29 days of water deficit. Despite a reduction in biomass accumulation due decreases in photosynthesis and leaf area, antioxidant activity avoided significant damage to the thylakoid membranes.

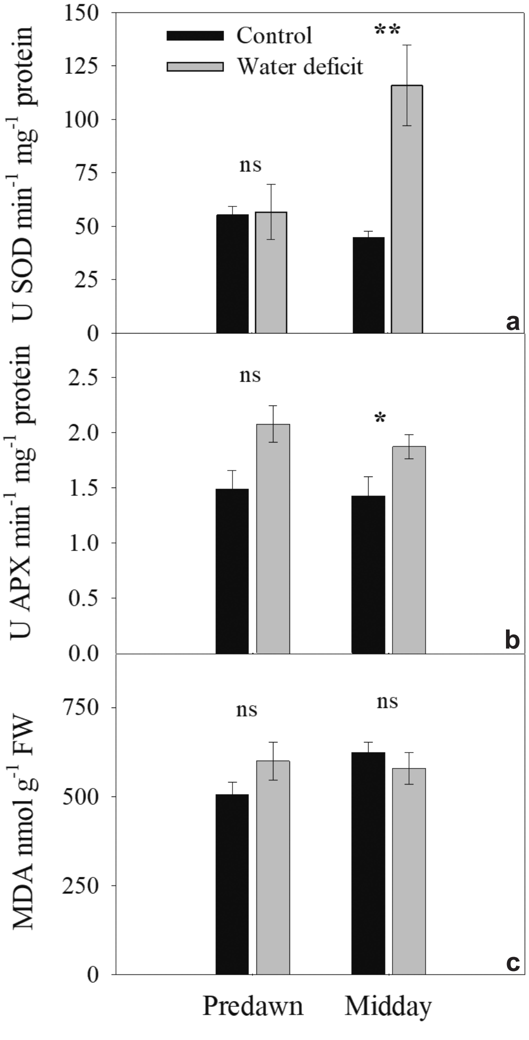

Figure 6 – a-c. Leaves of *Tabebuia aurea* seedlings under two water regimes: control and plants under water deficit, at predawn (5-6 a.m. before sunrise) and midday – a. SOD activity; b. APX activity; c. malondialdehyde-equivalents content (MDA). * means significance at $P < 0.05$ between treatments by T test; ** mean significance at $P < 0.01$, ns = not significant. n = 5.

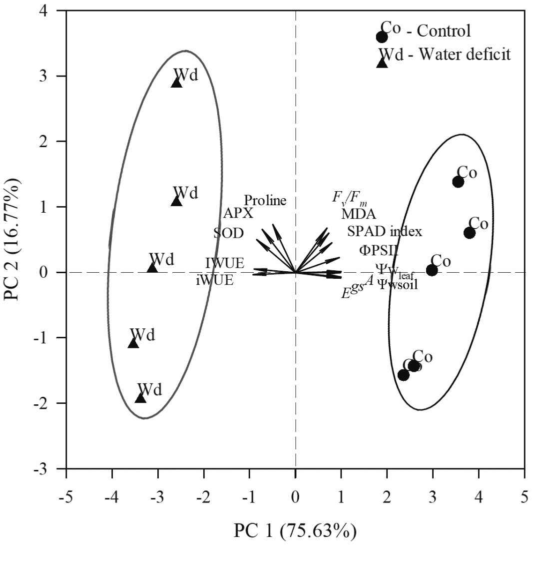

Figure 7 – Principal component analysis (PC1 and PC2) applied to the variables of gas exchange (A, g_s, E, iWUE, IWUE), photochemical (ΦPSII, F_v / F_m, SPAD index), Ψw_{leaf}, Ψw_{soil}, proline content in leaves, antioxidant enzimes (APX, SOD) and MDA of *Tabebuia aurea* seedlings under two water regimes: control and plants under water deficit.

Tabebuia aurea responded to experimental water deficiency via stomatal regulation, resulting in a decrease in biomass accumulation. Nevertheless, plants were able to maintain low rates of photosynthesis supported by the protective effects of an antioxidant mechanism and synthesis of proline. These physiological adaptations make this species a good candidate for restoration initiatives in degraded areas of the semi-arid Caatinga biome of northeast Brazil.

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