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Biochemical and morphophysiological strategies of *Myracrodruon urundeuva* plants under water deficit

L.M. SOUZA[1]*, M.R. BARBOSA[1], M.B. MORAIS[2], L. PALHARES NETO[1], C. ULISSES[1], and T.R. CAMARA[1]

Programa de Pós-Graduação em Botânica, Universidade Federal Rural de Pernambuco,
Dois Irmãos, 52171-900, Recife-PE, Brasil[1].
Programa de Pós-Graduação em Ciências Naturais, Universidade do Estado do Rio Grande do Norte,
Mossoró/RN, 59600-000, Mossoró-RN, Brasil[2]

Abstract

In view of the ecological, social, and economic importance of *Myracrodruon urundeuva* Allemão, the objective of this study was to investigate the strategies of this species under drought during its initial phase of development. Two-month-old plants were cultivated under continuous irrigation or no irrigation for 20 d. After this period, the water-stressed plants were rehydrated for 20 d. Physiological, biochemical, and anatomical variables were evaluated on 20th and 40th day. Water deficit (25 and 85 % leaf relative content) caused senescence followed by leaf abscission. Growth in height was negatively affected by water deficit (37 % reduction). A decrease in the thickness of the mesophyll was accompanied by a decrease of the total chlorophyll content. Water deficit affected saccharide metabolism and altered cellular component dynamics. Enzyme activities were higher during the rehydration period than during the water stress. There was no increase in lipid peroxidation in plants subjected to water deficit. A reduction in the stomatal opening during water stress was a strategy of reducing water loss through transpiration.

Additional key words: APX, CAT, chlorophyll, growth, leaf anatomy, relative water content, ROS, SOD, sugars.

Introduction

The ecogeographical domain of Caatinga is characterized by having sedimentary soils with poor water retention capacity, irregular rainfall, and a short warm and rainy period that is ideal for plant reproduction, and a dry period with almost no rainfall for 8 to 10 months per year (Amancio Alves *et al.* 2009, Santos *et al.* 2014, 2017). Because of its particular characteristics, Caatinga contains plant species that are adapted to water deficit by having xerophilous and deciduous leaves, a well developed root system, and closed stomata during the hottest periods of the day, which reduces transpiration (Moura *et al.* 2016, Maia *et al.* 2017). Other mechanisms used by plants under conditions of water scarcity are variations in epicuticular wax composition, stomatal density, water storage tissues, and the presence of trichomes (Barros and Soares 2013, Santos *et al.* 2014, Maia *et al.* 2017, Meira *et al.* 2017, Ribeiro *et al.* 2017)

Under conditions of water deficit, plants adjust their sugar, protein, amino acid, chlorophyll, and carotenoid content in order to protect cells. For example, sugars and amino acids may affect osmotic adjustment by acting as osmoprotectants or serve as signaling molecules to prevent oxidative stress (Nishizawa *et al.* 2008, Vieira *et al.* 2017). Plant responses to various environmental factors are related to plants ability to control oxidants content, which is highly correlated with their tolerance to environmental stresses (Munné-Bosch *et al.* 2013). An increase in the production of reactive oxygen species (ROS) can cause a partial or total oxidation of cellular components including membrane lipids and DNA, and photosynthetic damage by inducing changes in the cellular redox status (Anjum *et al.* 2011, Kar 2011).

Because of the multifunctional effects of ROS, cells need to control the accumulation of these molecules to prevent oxidative damage, so plants have developed an antioxidant defense system. Enzymatic components, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), and non-enzymatic components, such

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; LRB - leaf relative balance; MDA - malondialdehyde; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase.

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* Corresponding author; e-mail: lindomarsouza.ufrpe@gmail.com

as carotenoids, ascorbate, and glutathione ensure optimal cell function (Procházková and Wilhelmová 2007, Munné-Bosch *et al.* 2013, Labudda and Azam 2014, Anjum *et al.* 2017).

In order to sustainably use natural resources without the loss of biodiversity, it is essential to identify the variables that affect species stability in arid and semi-arid environments (Moura *et al.* 2016). Although *Myracrodruon urundeuva* Allemão is adapted to the irregularities of the climate of northeast Brazil, it is in the Red List of Endangered Flora and no studies have investigated the biochemical strategies used by this species in its initial stage of development under water-restricted conditions.

M. urundeuva is a Caatinga species that belongs to the family *Anacardiaceae* and is of a great economic value because of its bark, which has a high tannin content, which is used in traditional medicine (Viana *et al.* 2014). In addition, the trunk is widely used in constructions and leaves as forage (Pereira *et al.* 2014). *M. urundeuva* has been used in the recovery of contaminated areas (Gomes *et al.* 2013, Pereira and Morais 2014, Silva *et al.* 2015). In view of the ecological, social, and economic importance of *M. urundeuva*, the objective of the study was to identify and describe the strategies used by this species in coping with drought during its initial development phase.

Materials and methods

Plants and cultivation: The study was conducted in June and July 2016 in a greenhouse at the Federal Rural University of Pernambuco (UFRPE), Recife, Brazil (8°00'55.3"S, 34°57'04.8"W), located in Zona da Mata, Brazil, with average temperatures of 31.4 ± 2.21 °C during the day and 27.9 ± 0.7 ° C during the night, with a natural photoperiod and a mean relative humidity of 63.3 ± 6.7 % (Table 1 Suppl.). Two-month-old *M. urundeuva* plants that were obtained from the germination of diaspores at the National Semi-Arid Institute, Campina Grande, Paraíba were used. Diaspores were placed in plastic trays containing a ~4 cm layer of washed sand, which was oven sterilized at 200 °C for 120 min. At 15 days after germination, seedlings with a mean height of 2.0 cm were transplanted into 500 cm^3 polyethylene plastic bags containing washed sand. The plants received water (100 cm^3 every two days) and a nutrient solution based on Kristalon® and Calcinit® (half strenth) once a week. At the end of two months period, the plants were subjected to two water treatments for 20 d: continuous irrigation, in which pot capacity was maintained at 70 %, and water restriction. Twenty-one days after the beginning of the experiment, the water-restricted plants were irrigated. Vapor pressure deficit (Fig. 1 Suppl.) was calculated based on air temperature and relative humidity data (Tetens 1930).

Growth was evaluated by measuring stem height with a millimeter ruler, and it was a distance between the collar and the insertion point of the youngest, fully expanded leaf. Fresh shoot and root masses were determined and plant material was then placed in a forced-aeration oven at 65 °C until it reached a constant mass to determine dry mass. Leaf relative balance (LRB) was calculated according to the following formula:
LRB = (NLf -NLi) / NLi*,* where NLi corresponds to the number of leafs at initial time and NLf to the number of leafs at the end of experiment.

Relative water content (RWC) was determined according to the method proposed by Fidalgo *et al.* (2004). Ten-millimeter-diameter leaf discs were collected from four plants per treatment for the determination of fresh mass (FM) using a precision analytical balance. The disks were immersed in deionized water in Petri dishes for 6 h before being dried on a paper towel to remove excess water and a water saturated mass (WSM) was obtained. Dry mass (DM) was determined after drying the disks at 65 ºC for 48 h in a forced-aeration oven. The RWC was estimated using the following equation: RWC = (FM - DM) / (WSM - DM) × 100.

Percentage of membrane damage was estimated from electrolyte leakage according to the methodology of Azevedo *et al.* (2008). Ten 5-mm-diameter leaf disks were immersed in 20 cm^3 of distilled water in test tubes at 25 °C for 24 h, and electric conductivity was measured using a conductivity meter (*PHS3BW*, *BEL*, Piracicaba, SP, Brazil). Subsequently, the test tubes were placed in a water bath at 100 °C for 1 h, then cooled to room temperature, and a second electrical conductivity measurement was taken. The percentage of membrane damage (PD) was estimated from the readings (R) obtained from the conductivity meter using the following equation: PD = (R1 / R2) × 100.

Total chlorophyll and carotenoid content was determined by macerating 0.1 g of fresh leaves in 80 % (v/v) acetone up to the volume of 12.5 cm^3. The extract obtained was filtered through a fine-mesh nylon screen and centrifuged (*NT835*, *Novatecnica*, Piracicaba, SP, Brazil) at 2 000 *g* for 5 min. Readings for the determination of chlorophyll content were performed at 645 and 663 nm and for carotenoids at 470 nm (Lichtenthaler 1987) using a UV-VIS spectrophotometer (*220-2000UV*, *Biospectro*, Curitiba, PR, Brazil).

Total soluble sugars, saccharose, free amino acid, and phenol content were determined from 0.2 g of fresh leaves that was macerated in 80 % (m/v) ethanol. The extract was filtered into a 25 cm^3 volumetric flask and stored in a refrigerator (10 °C) until analyses. Determination of zotal soluble sugars was performed according to the method proposed by Yemm and Willis (1954) with modifications by Bezerra Neto and Barreto (2011). Determination of saccharose content was conducted as proposed by Handel (1968). All of the spectrophotometrical measurements were done at 620 nm. Determination of total free amino acid content was performed spectrophotometrically according to the method of Yemm and Cocking (1955) at 570 nm. Total phenol content was determined spectrophotometrically at 720 nm according to the methodology proposed by Magalhães *et al.* (1997).

Soluble protein content and SOD, APX, and CAT activities were determined using 0.2 g of plant material macerated in liquid nitrogen with addition of ~0.01 g of polyvinylpolypyrrolidone. This mixture was homogenized in 2 cm^3 of extraction buffer (pH 7.5) containing 100 mM potassium phosphate, 3 mM 1,4-dithiothreitol, and 1 mM ethylenediamine tetraacetic acid. Subsequently, the macerate was centrifuged at 10 000 *g* and 4 °C for 20 min and the supernatant was collected for analysis. Total soluble protein content was determined according to the method of Bradford (1976) at 595 nm. Activity of SOD was determined according to Giannopolitis and Ries (1977) at 560 nm. One unit of the SOD activity was defined as a decrease of 1 µmol min^{-1} in formation of formazan. Activity of APX was determined according to Nakano and Asada (1981) at 290 nm with a 60-s interval. Activity was calculated based on a coefficient of ascorbance of 2.8 mM$^{-1}\cdot$cm^{-1}. Activity of CAT was determined according to Berrs and Sizer (1952) at 240 nm, and calculated using a coefficient of absorbance for H_2O_2 to be 36 mM$^{-1}\cdot$cm^{-1}).

Malondialdehyde (MDA) and H_2O_2 content were determined from an extract prepared from 0.2 g of leaves macerated and homogenized in 2.0 cm^3 of trichloroacetic acid (1 %, m/v) and then centrifuged at 2 000 *g* and 4 °C for 10 min. Content of H_2O_2 was determined spectrophotometrically according to Loreto and Velikova (2001) at 390 nm. Malondialdehyde analysis was performed according to the method of Heath and Packer (1968) at 535 and 600 nm.

Leaf anatomy: Stomatal density, number of trichomes, and stomatal perimeter were determined according to the method of Franklin (1945). Fresh tissue samples (~1 cm^2) were taken from the leaf blade and fixed in the solution *FAA 70* (formaldehyde 10 %, ethanol 50 %, acetic acid 5 %, and water 35 %, volume percent) for 48 h and then stored in a 70 % (v/v) ethanol until processing. Subsequently, dissociation of the leaf epidermis was conducted by placing the samples in flasks containing glacial acetic acid and 37 % (v/v) H_2O_2 (1:1) (Franklin 1945). The flasks were placed in an oven at 60 °C for 24 h. After this process, the epidermis was washed with distilled water, stained with safranin and methylene blue, and mounted on a slide with a drop of glycerin (50 %, v/v). Subsequently, the slides were photographed under a top light optical microscope (40×) (*B2*, *Bel*®, Monza, Italy), and 20 fields were analyzed per treatment. The *ImageJ* program was used to evaluate the data (Schneider *et al.* 2012). In order to visualize the vascular tissues and bundles of the leaf blade median region, slides were prepared from plant material fixed in the *FAA 70* (formaldehyde 10 %, ethanol 50 %, acetic acid 5 %, and water 35 %, volume percent) and dehydrated in alcohol and paraffin (Johansen 1940). A rotary hand microtome (*MRP09*, *Lupetec*®, *LUPE*, São Paulo, Brazil) was used to prepare 6-µm sections, which were stained in safranin and alcian blue solution (Bukatsch 1972) and mounted in *Entellan*® (*Merck KGaA*, Darmstadt, Germany). The slides were then photographed and analyzed using *ImageJ* (Schneider *et al.* 2012).

Statistical analyses: The experimental design was completely randomized with two water regime treatments. All evaluations were performed on days 20 and 40. The data were subjected to an analysis of variance, and means compared using the Tukey test at the 5 % probability using *Assistat v. 7.7 beta* (Silva and Azevedo 2017). In addition, the software *FITOPAC 2.1* was used for principal component analysis and grouping analysis (Shepherd 2010). Principal component analysis was performed for different groups and based on their correlation matrix. Three replicates were used per treatment, in which each replicate consisted of two plants.

Results

Water deficit caused a reddish-brown coloration observed on senescent leaves (Fig. 2 Suppl.), which is typically observed in plants under conditions of water limitation. Foliar abscission was observed during water restriction and rehydration. Negative leaf relative balance that was observed in water-stressed and subsequently rehydrated plants at 40 d was related to an increase in leaf abscission during this period (Table 1). Relative water content was one of the variables that was most affected by the water regimes. Water-restricted plants had a 31.7 % lower relative RWC than those under continuous irrigation (Table 1). After the resumption of irrigation, average RWC was 79 and 74 % in plants under continuous irrigation and those that were rehydrated, respectively. Water deficit reduced stem height by 37 % (Table 1), and the rehydration resulted in a 51 % reduction in stem height (Table 1). At the end of the water restriction period, shoot and root dry masses decreased considerably (Table 1), and it was not a complete recovery after rehydration (Table 1).

Water deficit caused a 7.6 % reduction in total chlorophyll content (Table 1). After the resumption of irrigation, total chlorophyll content did not significantly differ between the treatments, with averages of 0.65 and 0.59 mg$\cdot$g^{-1}(FM) for plants under continuous irrigation and those that were stressed and rehydrated, respectively (Table 1). Carotenoid content did not significantly differ between water regimes during the entire experimental period (Table 1).

Total soluble protein content was reduced by 50.25 % in water-stressed plants. The rehydrated plants had a 56.46 % higher soluble protein content than the plants under continuous irrigation (Table 1).

At the end of the water restriction period, there was a reduction in the content of sucrose (25.8 %), total soluble sugars (42.3 %), amino acids (10.5 %), and total phenols (29.3 %) (Table 2). In rehydrated plants, only the content of amino acids did not differ between the treatments. Sucrose, soluble sugar, and phenol content remained low in rehydrated plants, with reductions of 37, 43, and 79.6 %, respectively (Table 2). Strong correlations were observed between sucrose and soluble sugars or phenols (r = 0.81 and 0.83, respectively).

Table 1. Stem height (SH), shoot dry mass (SDM), root dry mass (RDM), leaf relative balance (RLB), chlorophyll *a*+*b* content (Chl), carotenoids (CAR), total soluble proteins (SP), and relative water content (RWC) in plants of *Myracrodruon urundeuva* Allemão grown in a greenhouse under two water regimes: continuous irrigation (CI) and irrigation suspension (IS) for 20 d and then rehydration for further 20 d at 40 d. Measurements were done after 20 and 40 d. Means ± SEs, *n* = 10. Means followed by different letters are significantly different at $P < 0.05$ according to the Tukey's test. CV - coefficient of variation.

Parameters	Time [d]	Continuous irrigations	Stress + rehydration	CV [%]
Stem height [cm]	20	3.35 ± 0.14 a	2.10 ± 0.00 b	10.33
	40	4.95 ± 0.28 a	2.39 ± 0.31 b	24.57
SDM [g]	20	0.22 ± 0.02 a	0.17 ± 0.01 b	15.25
	40	0.86 ± 0.04 a	0.69 ± 0.09 a	17.72
RDM [g]	20	0.11 ± 0.01 a	0.07 ± 0.00 b	21.03
	40	0.49 ± 0.05 a	0.38 ± 0.02 a	18.66
LRB	20			
	40			
Chl [mg g^{-1}(f.m.)]	20	0.52 ± 0.00 a	0.48 ± 0.00 b	1.63
	40	0.65 ± 0.05 a	0.59 ± 0.02 a	9.81
CAR [mg g^{-1}(f.m.)]	20			
	40			
SP [mg g^{-1}(f.m.)]	20			
	40			
RWC [%]	20	95.62 ± 5.60 a	63.92 ± 8.40 b	16.72
	40	78.97 ± 2.04 a	73.77 ± 1.02 a	12.50

Table 2. The content of sucrose, soluble sugars (SS), total free amino acids (AA), total phenols, activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), content of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and membrane damage (MD) in plants of *Myracrodruon urundeuva* Allemão grown in a greenhouse under two water regimes: continuous irrigation (CI) and irrigation suspension (IS) for 20 d and then rehydration for further 20 d at 40 d. Measurements were done after 20 and 40 d. Means ± SE, *n* = 10. Means followed by different letters are significantly different at $P < 0.05$ according to the Tukey's test. CV - coefficient of variation.

Parameters	Time [d]	Continuous irrigations	Stress + rehydration	CV [%]
Sucrose [mg g^{-1}(f.m.)]	20	29.17 ± 2.57 a	21.63 ± 0.62 b	12.77
	40	23.10 ± 2.86 a	14.43 ± 0.33 b	2.63
SS[mg g^{-1}(f.m.)]	20	30.50 ± 1.10 a	17.60 ± 0.51 b	6.21
	40	21.16 ± 0.23 a	12.06 ± 2.28 b	16.87
AA [mg g^{-1}(f.m.)]	20	3.13 ± 0.05 a	2.80 ± 0.06 b	3.64
	40	3.80 ± 0.05 a	3.57 ± 0.11 a	3.50
Phenols [mg g^{-1}(f.m.)]	20	11.60 ± 0.20 a	8.20 ± 0.50 b	6.41
	40	5.90 ± 0.97 a	1.02 ± 0.40 b	35.70
SOD [U g^{-1}(f.m.)]	20	38.93 ± 0.99 a	27.40 ± 1.06 b	7.78
	40	17.30 ± 3.70 b	39.30 ± 3.02 a	20.67
APX [µmol ASA g^{-1}(f.m.)]	20	6576.20 ± 396.5 a	6158.70 ± 507.9 a	12.49
	40	3936.50 ± 553.5 a	6476.20 ± 977.4 a	26.42
CAT [mmol H_2O_2 g^{-1}(f.m.)]	20	800.00 ± 12.8 a	644.40 ± 19.2 a	10.21
	40	681.40 ± 19.6 b	740.70 ± 7.4 a	3.61
H_2O_2 [µmol g^{-1}(f.m.)]	20	24.73 ± 2.47 a	24.47 ± 0.87 a	29.37
	40	18.70 ± 13.27 a	19.70 ± 3.47 a	29.09
MDASP [nmol g^{-1}(f.m.)]	20	6.20 ± 1.30 a	5.93 ± 0.79 a	30.65
	40	4.56 ± 0.58 a	6.60 ± 0.24 a	18.84
MD [%]	20	20.90 ± 4.59 a	27.80 ± 1.47 a	28.06
	40	21.77 ± 0.02 a	24.55 ± 0.007 a	14.16

At the end of the water restriction period, there was a 29 % reduction in SOD, but APX and CAT activities did not significantly differ form controls. After rehydration, SOD activity increased by 127 % and CAT activity by 8.7 % in rehydrated plants (Table 2).

The percentage of membrane damage did not

Table 3. The means of adaxial (ADSD) and abaxial (ABSD) stomatal densities glandular trichome density on adaxial (DGTAD) and abaxial (DGTAB) surfaces, stomatal perimeter on adaxial (SPD) and abaxial (SPB) surfaces, tectorial trichome density on adaxial (TTAD) and abaxial (TTAB) surfaces, central vein perimeter (CVP), mesophyll thickness, the number of secretory ducts (SD) and secretory duct perimeter (SD) in plants of *Myracrodruon urundeuva* Allemão grown in a greenhouse under two water regimes: continuous irrigation (CI) and irrigation suspension (IS) for 20 d and then rehydration for further 20 d. Measurements were done after 20 and 40 d. Means ± SE, $n = 10$. Means followed by different letters are significantly different at $P < 0.05$ according to the Tukey's test. CV - coefficient of variation.

Parameters	Time [d]	Continuous irrigations	Stress + rehydration	CV [%]
ADSD [mm^2]	20	4.04 ± 0.23 a	4.15 ± 0.38 a	17.14
	40	3.64 ± 0.18 a	3.08 ± 0.32 a	18.23
ABSD [mm^2]	20	70.90 ± 1.81 a	75.01 ± 7.95 a	17.67
	40	4.04 ± 0.23 a	4.15 ± 0.38 a	17.14
DGTAD [mm^2]	20	1.47 ± 0.136 a	1.64 ± 0.087 a	16.38
	40	1.50 ± 0.117 a	1.12 ± 0.061 b	16.37
DGTAB [mm^2]	20	1.79 ± 0.160 a	1.74 ± 0.048 a	15.10
	40	1.80 ± 0.013 a	1.62 ± 0.019 a	21.23
SPD [µm]	20	7.20 ± 0.24 a	5.40 ± 0.15 b	7.05
	40	6.03 ± 0.103 a	6.30 ± 0.124 a	3.70
SPB [µm]	20	4.95 ± 0.026 a	4.36 ± 0.032 b	3.13
	40	7.20 ± 0.24 a	5.40 ± 0.15 b	7.05
TTAD [mm^2]	20	1.47 ± 0.098 a	1.35 ± 0.100 a	15.72
	40	1.70 ± 0.234 a	1.70 ± 0.100 a	23.53
TTAB [mm^2]	20	1.32 ± 0.000 a	1.28 ±0.00 a	33.26
	40	1.20 ± 0.007 a	1.00 ± 0.00 b	12.03
CVP [µm]	20	154.16 ± 11.9 a	163.77 ± 8.10 a	14.40
	40	91.57 ± 18.6 a	92.01 ± 16.0 a	13.57
Mesophyll thickness [µm]	20	9.24 ± 0.83 a	7.39 ± 0.49 b	9.91
	40	7.46 ± 0.46 a	5.00 ± 0.26 b	13.32
SD number	20	2.60 ± 0.40 a	3.40 ± 0.24 a	24.72
	40	2.40 ± 0.24 a	1.80 ± 0.37 a	33.67
SD perimeter [µm]	20	25.00 ± 0.34 a	23.20 ± 8.40 a	16.61
	40	16.20 ± 7.80 a	20.70 ± 7.60 a	17.26

significantly differ between the treatments and remained between 20 and 27 % throughout the experiment (Table 2). Similarly, MDA and H_2O_2 content did not significantly differ between the treatments throughout the experimental period (Table 2).

Myracrodruon urundeuva epidermal cells had sinuous walls and anomocytic stomata on both sides of the leaf, with a tendency for adaxial surface to be less conspicuous than the abaxial surface (Fig. 1*A-H*). Stomata were present on both adaxial and abaxial surfaces (amphistomatic leaves), but their higher density was on the abaxial surface, where they were throughout all the leaf blade; in contrast, on the adaxial surface, stomata were only close to the ribs. Although there were fewer stomata on the adaxial surface (Fig. 1*A*,*C*,*E*,*G*), they were larger than those on the abaxial surface (Fig. 1*B*,*D*,*F*,*H*). Water restriction did not cause a significant difference in stomatal density between adaxial and abaxial surfaces (Table 3). However, a 31.7 % decrease in RWC caused changes in stomatal perimeter with reductions of 25 and 12 % on adaxial and abaxial surfaces, respectively, and there was a strong correlation ($r = 0.83$) between RWC and stomatal perimeter. After the resumption of irrigation, a 22.9 % reduction in abaxial stomatal density of plants subjected to water deficit was observed whereas a mean stomatal perimeter on adaxial and abaxial surfaces did not differ significantly (Table 3).

Densities of glandular and tectorial trichomes did not significantly differ due to water restriction (Table 5). After rehydration, plants exhibited a 25 % reduction in glandular trichome density on adaxial surfaces and a 16.7 % reduction in tectorial trichome density on abaxial surface in comparison to control plants (Table 3).

In *M. urundeuva* veins, secretory ducts were localized near phloem (Fig. 2*A*,*B*,*D*,*G*,*H*,*J*). Cells that store phenolic compounds and calcium oxalate crystals (Fig. 2*K*) have been described in literature as common in species of the family *Anacardiaceae* (Duarte *et al.* 2009). Water restriction caused a reduction in mesophyll thickness (Table 3) and altered the conformation of palisade and spongy parenchyma cells, and bulging spongy parenchyma cells were also observed (Fig. 2*F*). In plants under water restriction, the size of the central vein was maintained (Table 3) and a greater lignification of the xylem cell walls was observed (Fig. 2*E*).

Discussion

Leaf abscission was observed in water-restricted plants, which is a remarkable characteristic of water-stressed plants, and it is one of the strategies used by *M. urundeuva* and other Caatinga species during dry periods (Barros and Soares 2013, Mendes *et al.* 2017). Leaf abscission reduces transpiration during unfavorable periods as well as allows water and nutrients to reach other parts of the plant (Padilha *et al.* 2016, Patharkar and Walker 2016). Continuing leaf abscission after irrigation resumption, as indicated by a negative leaf relative balance (Table 1), may have been related to the presence of receptors in the region of separation between the leaf and stem. The reduction in chlorophyll content during water stress (Table 1) was linked to the beginning of leaf senescence (Fig. 2 Suppl.). A leaf color change is caused by degradation of chlorophyll at the beginning of senescence, followed by protein hydrolysis (Smarth 1994, Heaton and Marangoni 1996). Water stress mobilizes nutrients to other tissues prior to leaf abscission, which help to resume growth and development after rehydration (Smarth 1994, Bilgin *et al.*

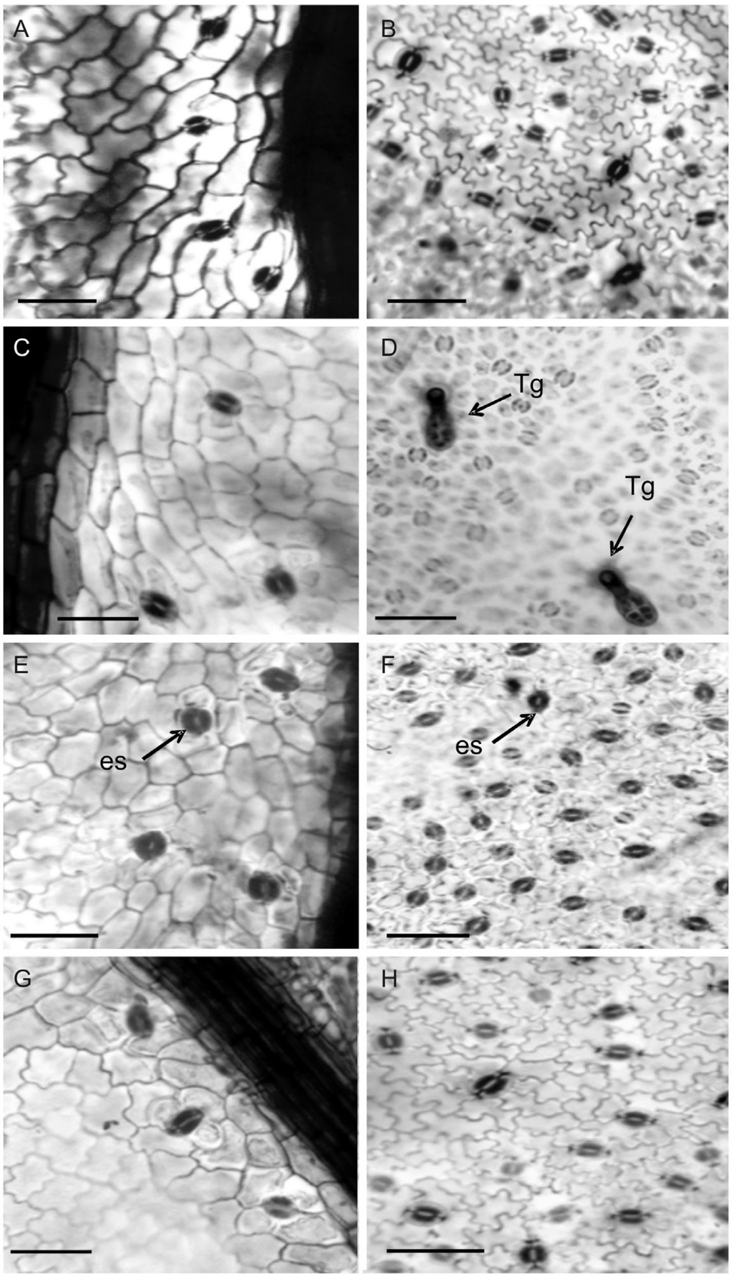

Fig. 1. Microphotographs of the adaxial surface (*A*, *C*, *E*, and *G*) and abaxial surface (*B*, *D*, *F*, and *H*) of leaves of *Myracrodruon urundeuva* grown in a greenhouse under two watering regimes: continuous irrigation for 20 d (*A* and *C*) and for 40 d (*E* and *G*) or water restriction for 20 d (*B* and *D*) followed by rehydration for further 20 d (*F* and *H*). es - stomata; Tg - glandular trichome; the *scale* is 100 µm.

2016). The reduction in chlorophyll content during water restriction (Table 1) could be considered as an adaptive mechanism for low-energy absorption because the excess energy stored in chlorophyll molecules in their triplet state can be transferred to oxygen molecules and lead to the formation of singlet oxygen ($^1O_2$), which is a ROS that can cause membrane-lipid peroxidation (Vieira *et al.* 2017).

The resumption of irrigation promoted the recovery of total chlorophyll content in plants (Table 1). Protein degradation in senescent leaves is an important hydrolytic process that plays a crucial role in nutrient recycling, particularly nitrogen. Many genes that are associated with senescence and encode proteases are synthesized *de novo* or are induced during senescence (Smarth 1994, Deo *et al.* 2006). The reduction in protein content may be related to an imbalance between their synthesis and degradation, which may result in a decrease in the content of enzymes, such as Rubisco, resulting in a reduction in photosynthetic rate and a lower sugar production (Table 2) (Thimann *et al.* 1977, Smarth 1994, Tezara *et al.* 1999). Chlorophyll content reduction may lead to a decline in sugar content, *e.g.*, glucose, which in turn may lead to a reduction in synthesis of amino acids, which are precursors for protein synthesis (Oaks and Hirel 1985, Heaton and Marangoni 1996).

The imbalance between the production and the use of sugars in plants under moderate drought plays an important role in the signaling and modulation of sugar content in different plant organs (Pinheiro and Chaves 2011). Reduction in soluble sugar content (Table 2) may

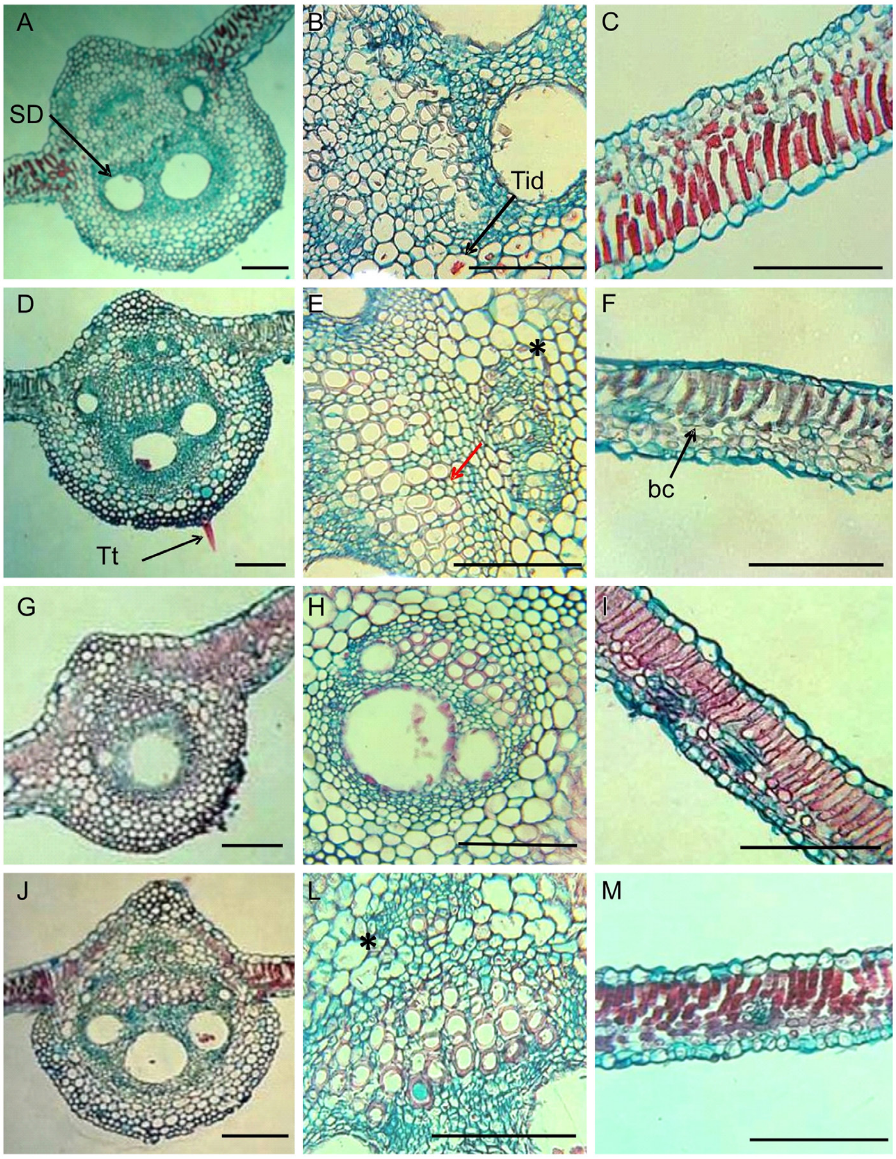

Fig. 2. Microphotographs of the cross sections of *Myracrodruon urundeuva* leaves grown in a greenhouse under two water regimes: continuous irrigation for 20 d (*A* - *C*) or 40 d (*G* - *I*) and water restriction for 20 d (*D* - F) followed by rehydration for further 20 d (*J* - *M*). SD - secretory ducts, Tid - taniferous idioblasts, Tt - tectorial trichome, * - crystals, bc - braciforms cells; the *red arrow* - lignification of conducting vessels; the *scale* is 100 µm.

affect several metabolic pathways, including the pentose-phosphate pathway, reflecting changes in amino acid and phenolic compounds profiles (Martin *et al.* 1988, Bolouri-Moghaddam *et al.* 2010); therefore, a limited sugar supply could play an important role in regulating metabolic pathways and in the production of compounds of primary and secondary metabolism, which modulates plant responses to different environmental stressors.

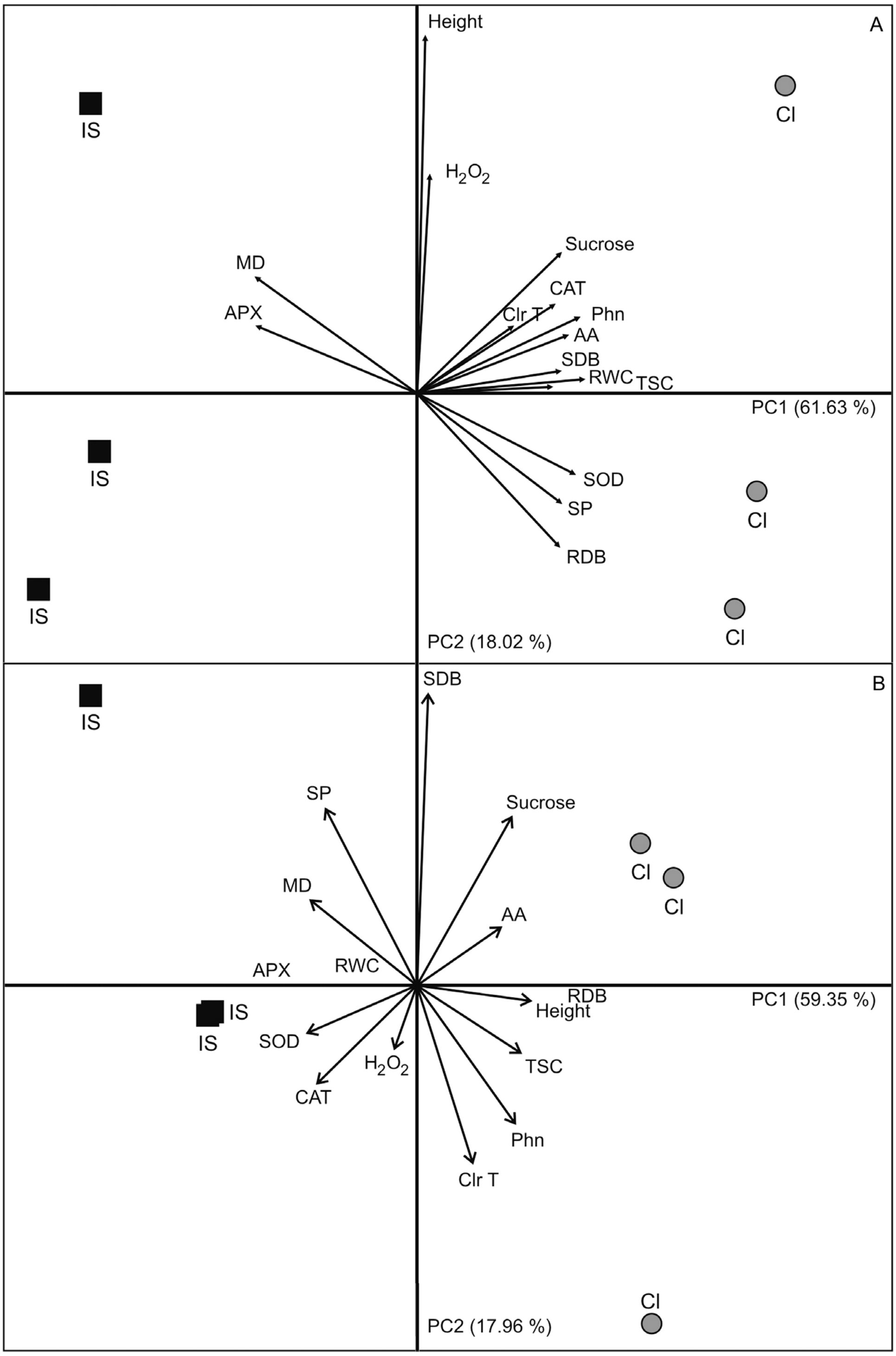

Fig. 3. The projection in the first two main components of the biochemical variables of *Myracrodruon urundeuva* plants grown in a greenhouse under two water regimes: continuous irrigation (CI) and irrigation suspension for 20 d following by rehydration for 20 d (IS). Measurements were done after 20 d (*A*) and after 40 d (*B*). AA - amino acids, APX - ascorbate peroxidase, CAT - catalase, Chl - chlorophyll (Chl), MDA - malondialdehyde, MD - membrane damage, RWC - relative water content, RDB - root dry mass, SDB - shoot dry mass, SP - soluble proteins, SOD - superoxide dismutase, TSC - total soluble sugars, and Phn - total phenols.

Phenolic compounds exhibit antioxidant activity that may be related to plant tolerance with respect to the prevention of lipid peroxidation under stressful conditions because flavonoids can chelate metals, act as radical sequestrants, and interact with membrane surfaces to prevent oxidants accessing the lipid bilayer (Verstraeten *et al.* 2003). However, our results do not indicate that the stress imposed on *M. urundeuva* plants caused an increase in lipid peroxidation, as evaluated by the MDA content and percentage of membrane damage.

Under adverse environmental conditions, plants activate enzymatic and non-enzymatic antioxidant defense systems to avoid a severe damage caused by oxidizing molecules to their structures. Enzymatic antioxidants mainly consist of SOD, APX, CAT, and peroxidase whereas non-enzymatic antioxidants consist of glutathione, ascorbic acid, and carotenoids (Vieira *et al.* 2017). Although water restriction led to increased antioxidant enzyme activities, reductions in enzyme activities may occur during senescence as observed in *M. urundeuva* control plants at the end of the experimental period (Table 2). These adjustments do not happen as a cause but rather as a consequence of this process (Jimenéz *et al.* 1998, Procházková and Wilhelmová 2007). A low SOD activity in plants subjected to water restriction, and the maintenance of APX and CAT activities, irrespective of treatment (Table 4), suggest that, after 20 d of water stress, there was no increase in superoxide (O^-_2), which is a substrate for SOD, production. This was confirmed by no increase in H_2O_2 content (Table 2). There was also no increase in membrane lipid peroxidation as indicated by the maintenance of the MDA content and the percentage of membrane damage (Table 2). An increase in SOD and CAT activities in rehydrated plants (Table 2) suggests that this antioxidative response was related to a greater production of O^-_2 and H_2O_2 (Oliveira *et al.* 2014, Choudhury *et al.* 2017). During rehydration, SOD activity may be influenced by the activities of NADPH oxidases, present in the membrane, which play an important role in ROS production, particularly O^-_2, and may cause an oxidative stimulus during rehydration (Silveira *et al.* 2017).

The maintenance of carotenoids under water deficit (Table 1) indicates that they are important for plants to tolerate stress because under adverse environmental conditions, changes in the content of these pigments are one of the first responses of plants against oxidative damage caused by ROS (Heaton and Marangoni 1996, Park and Jung 2016). Under water-stress, an increase in content of the main carotenoids (zeaxanthin, violaxanthin, neoxanthin, and lutein), suggests that they are important under water-restricted conditions (Mibei *et al.* 2016). Changes in the content of these antioxidants may or may not reflect changes in the total carotenoid content, so comprehensive studies of variations in carotenoid profiles in *M. urundeuva* plants under water deficit are necessary to understand how variations in the content of these pigments can prevent oxidative stress caused by environmental stressors.

Loss of water by transpiration depends, among other factors, upon stomatal size and density on leaf surface, as well as structures that protect the stomata (Ferri 1955). Although some studies have investigated stomatal density in *M. urundeuva* under conditions of differential water availability, Figueirôa *et al.* (2004) stated that future studies of this species should also consider stomatal size because they reported that stomata tend to shrink under water restriction.

Although there was no reduction in stomatal density (Table 3), a decrease in the stomatal perimeter (Table 3) was an important event in the regulation of gas exchange because leaves with small stomata are more efficient in the use of water (Melo *et al.* 2007). In addition to the component necessary for cell growth (Santos and Carlesso 1998), water contributed to the maintenance of cell turgidity, which may interfere with cell size and morphology (Table 3). A reduction in stomatal perimeter that is not accompanied with an increase in stomatal density may lead to a reduction in gas exchange capacity (Alves *et al.* 2001). Therefore, changes in stomatal perimeter, a reduction in mesophyll thickness, and a low total chlorophyll content might contribute to a reduction in the production of sugars in *M. urundeuva* leaves (Table 3). A study by Drake *et al.* (2013) proposed that, for some species, smaller stomata have faster stomatal movement as they allow faster changes of solutes and control of stomatal conductance in relation to larger guard cells.

Trichomes on leaf surface protect plants by reducing water loss and reflecting excess radiation (Reis *et al.* 2014). Reduction in trichome density in rehydrated plants (Table 5) can be explained by the re-establishment of water availability because the density of trichomes can be affected by temperature, radiation, and water and nutrient availability (Xiao *et al.* 2017). The sinuosity observed in the epidermal cells on the abaxial surfaces of *M. urundeuva* leaves may have been related to a greater adaptation to surface tension because of their greater flexibility (Medri and Lleras 1980).

Anatomical variations in plants under water deficit occur as a form of protection and adaptation to stresses (Grisi *et al.* 2008). An apparent increase in xylem cell wall lignification in plants under water deficit (Fig. 2*E*) may prevent pot cavitation, allowing water to be trapped in plants under water deficit (Scatena and Scremin-Dias 2006, Marcondes and Garcia 2009).

The presence of calcium oxalate crystals (Fig. 2*E*,*K*) in plants is mainly related to herbivory, but they are also involved in osmoregulation, calcium tissue regulation, ionic detoxification, and the provision of CO_2 for photosynthesis. The latter usually occurs under water-limited conditions, when there is a greater control of stomatal opening that limits the entry of CO_2 from the atmosphere (Rotondi *et al.* 2003, Tooulakou *et al.* 2016).

The preservation of the size of the central vein probably contributed to the fact that secretory ducts did not undergo any morphological changes (Table 3). The presence of internal secretory ducts is common in species of family *Anacardiaceae*, and they are involved in the secretion of lipophilic material (Castro and Machado 2006, Duarte *et al.* 2009). Depending on the chemical nature of the material secreted, these substances can promote plants to adapt to certain environmental conditions (Aguiar-Dias and Cardoso-Gustavson 2011). The reduction of only 31.7 % in relative water content of water-restricted plants (Table 1) enabled biochemical, physiological, and anatomical adjustments to survive, and a rapid recovery when irrigation resumed.

Principal component analysis (Fig. 3) helped us to understand the differences and similarities among the responses of plants under different water regimes. The evidence for stress is in the axis IS with 20 days of water restriction and in the axis CI suggesting metabolic responses differentially modulated. The distance between the treatment points out the response divergence between them, and especially the negative correlation with the groups IS and CI (Fig. 3*A*). In opposite, the set of responses was enough to ensure a better performance in the rehydration phase of water restriction. It is graphically noted that there was a higher correlation with RWC and although the evidences for oxidative stress are in the same axis, samples were more related to enzymatic metabolism (Fig. 3*B*). The different water regimes analyzed present alternative pathways and molecular mechanisms which, in turn, may produce different phenotypic responses.

In conclusion, *M. urundeuva* is tolerant to water restriction during its initial phase of development and has a capacity to recover after a long-term water restriction. The water restriction affected metabolic processes and reduced content of soluble sugars, chlorophyll, amino acids, soluble proteins, and total phenols (Fig. 3 Suppl.) during *M. urundeuva* initial phase of development, taking plants to a stage of latency until the environmental conditions were more favorable. Although antioxidant enzymes exhibited reduced activities during water stress, carotenoid content was maintained highlighting the efficiency and importance of non-enzymatic antioxidants for protection against oxidative stress.

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