



Full Length Article

Water Deficit Alters Morpho-Anatomical and Antioxidant Responses in Seedlings of *Campomanesia xanthocarpa* Enabling their Recovery

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Abstract

Water deficit alters plants' function include damages which may not be restored, even after irrigation is resumed. This study hypothesized that *Campomanesia xanthocarpa* presents plasticity in its morpho-anatomical and biochemical responses that enables its survival in environments subject to temporary water deficits and allow for its recovery after irrigation restoration. The effect of water deficit on the morpho-anatomy and enzymatic activity in *C. xanthocarpa* seedlings subjected to water deficit and subsequent resumption of irrigation was evaluated. The seedlings were separated into two groups: 1- control (irrigation with 70% of the water retention capacity in the substrate) and group 2- water deficit (suspension of irrigation, protection of precipitation, and resumption of irrigation at photosynthetic rate close to zero). Growth and biomass, antioxidant enzyme activity (superoxide dismutase and peroxidase), and leaf anatomy were evaluated. The seedlings of *C. xanthocarpa* under water deficit showed leaf morphoanatomical alterations as survival strategies. The seedlings also showed an active antioxidant system with increased activity of superoxide dismutase and peroxidase in both the shoots and roots, allowing the recovery of seedlings after resumption of irrigation. This behavior also justifies the occurrence of this species in environments with low water availability, proving the hypothesis that the *C. xanthocarpa* shows plasticity to adjust to temporary water deficit, favoring its use in recovery projects of degraded areas. © 2020 Friends Science Publishers

Keywords: Guabiroba; Irrigation suspension; Oxidative stress

Introduction

The life cycle of plants is subjected to adverse environmental conditions that limit the development of plants and their chances of survival. One of these adverse conditions is water deficit. As a consequence of this specific environmental condition, plants can respond with the reduction of the water potential and leaf turgescence with closure of the stomata and, consequently, a lower photosynthetic rate that reflects in the cell elongation (Nogueira *et al.* 2005) and cell growth (Jaleel *et al.* 2009). Therefore, the characteristics of the internal structure of the leaves of plants may be important to determine the level of tolerance to water stress (Batista *et al.* 2010), as well as to detect the anatomical modifications of plants subjected to treatments that involve water conditions in a phase prior to leaf wilting (Oliveira *et al.* 2014).

Plants present several modifications to adapt to the stressful conditions, such as increases in the thickness of the

epidermis, number of trichomes, deposition of the cuticle, and stomatal density in cavities and stomatal crypts (Castro *et al.* 2009), as well as a reduction in stomatal size.

In addition to leaf growth and modifications in leaf anatomy, water deficit may generate oxidative damage, caused by energy from excitation of the chlorophyll molecules not being used in photosynthesis or eliminated in the form of heat. This causes the production of reactive oxygen species (ROS), which can react with any cell molecule, causing damage to the cells and organs (Barbosa *et al.* 2014; Morales and Munné-Bosch 2016). Several studies have shown the induction of oxidative stress by water deficit and antioxidative enzyme activity, such as superoxide dismutase (SOD) and peroxidase (POD) (Wang *et al.* 2016; Rosa *et al.* 2017; Reis *et al.* 2018; Gupta *et al.* 2018).

However, it is important to emphasize that the effects of water deficit on plants are quite variable, because the effects depend on the intensity and speed of stress

imposition and the developmental stage at which it happens (Pimentel 2004). In addition, the genetic capacity of plants and their recovery potential are determinant factors for the development and survival of each species.

Campomanesia xanthocarpa O. Berg. (Myrtaceae) is a deciduous tree, popularly known as “Guabirobeira” or “Guabiroba” and according to review of Luz and Krupek (2014) it is a heliophyte and selective hygrophyte plant, with occurrence in the “capoeiras and capoeirões” on moist soils and well drained capons and gallery forests, both in flat areas and on hillsides and near water ways. It has good adaptability and may occur in dry, compact, and low-fertility soils.

The economic importance of *C. xanthocarpa* comes mainly from its use in beekeeping, human food, and medicinal products. The plants’ fruits, when ripe, are rich in vitamin C and appreciated in natural consumption and industrialization in the manufacture of liqueurs and ice creams (Carvalho 2006).

The seedlings of *C. xanthocarpa* cultivated under water deficit shows reduction of the leaf water potential and all the characteristics of photosynthetic metabolism, which reaches close to zero only 20 days after the suspension of irrigation. However, after resumption of irrigation, the seedlings show to recover (Bento et al. 2016). No information is available on the protective mechanisms or morphoanatomical modifications that could allow this recovery after the water deficit.

Considering the occurrence of *C. xanthocarpa* in both moist soils and environments with low water availability, we hypothesize that this species shows plasticity in its morphoanatomical and biochemical responses that enables its survival in environments subject to temporary water deficits and allow for its recovery after irrigation restoration.

This study aims to evaluate the growth, antioxidant enzyme activity, leaf anatomy, and recovery potential of *C. xanthocarpa* seedlings subjected to water deficit.

Materials and Methods

Plant and cultivation material

Fruits of *C. xanthocarpa* O. Berg. were collected in remaining Cerrado areas, located near the municipality of Dourados/MS, Brazil. After collection, the fruits were processed manually, and the seeds were washed in running water to eliminate the remnants of pulp and dried with Gemitest® paper.

The seedlings were produced from sowing in tubes 50 x 190 mm to one centimeter of depth containing Dystroferic Red Latosol, sand, and commercial substrate Bioplant® in the proportion of 1:1:1, placing one seed per tube. The seedlings were transplanted to pots with the same soil and capacity of 5 kg when reached approximately 15 cm, having been acclimated for 30 days, with water retention capacity in the substrate soil of 70% and kept in a

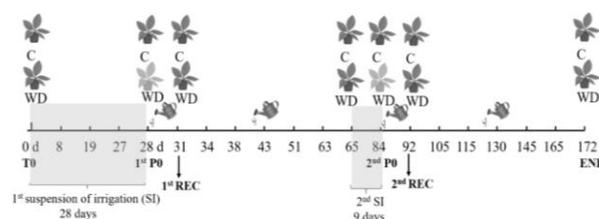


Fig. 1: Experimental scheme of the distribution of *C. xanthocarpa* plants in the two water regimes (Control-C and water deficit-WD) and in the six evaluation periods: T0 beginning of the experiment; 1st and 2nd P0 - first and second photosynthesis zero, 1st and 2nd REC - first and second recovery, and the END evaluation

greenhouse under 40% shade. During the experiment, the plants were protected from rainfall by a plastic cover.

At the beginning of the experiment (T0), the pots were distributed in two groups: Group 1 (control (C), where plants were irrigated periodically in order to maintain 70% of the water retention capacity of the substrate, and group 2 characterized by water deficit (WD), where irrigation was suspended until the photosynthetic rate reached close to zero, at which time the plants were re-hydrated with daily irrigation for one week at a rate and maintained a water retention capacity of the substrate to 70%.

The plants were evaluated in six periods: T0 (beginning of the experiment); 1st P0 (first photosynthesis zero), when the seedlings under suspension of irrigation showed photosynthetic rate close to zero (28 days); 1st REC (first recovery), when the seedlings were re-irrigated until the photosynthesis values approached the control (seven days); 2nd P0 (sec photosynthesis zero) when the seedlings were subjected to the sec cycle of suspension of irrigation and showed photosynthetic rate close to zero (nine days); 2nd REC (sec recovery), when the seedlings were re-irrigated until the photosynthesis values reached the control in the sec cycle of irrigation suspension (seven days); and the end evaluation occurred at 172 days of experiment (Fig. 1).

Plant measurements

Growth characteristics were evaluated by collecting three seedlings of each treatment and evaluated for the number of leaves (NL), shoot length (SL), and root length (RL). For the analysis of antioxidant enzyme activity, an extract was obtained from the homogenization of 1 g of the leaves and roots, removed from each treatment, fragmented in mortar in the presence of liquid nitrogen. Next, 2 ml of extraction solution was added, consisting of EDTA 0.1 mM in potassium phosphate buffer 0.1 M, pH 6.8, containing 20 mg of polyvinylpyrrolidone. Homogenized solution was centrifuged for 20 min at 4000 rpm and the supernatant collected, which was then used in the evaluations of peroxidase - POD (Macedo et al. 2005) and superoxide dismutase - SOD (Beauchamp and Fridovich 1971; Giannopolitis and Ries 1977; Longo et al. 1993).

Table 1: Water potential (Ψ_w) due to the evaluation periods between irrigated seedlings of *C. xanthocarpa* and subjected to water stress conditions

	T0 ¹	1 st P0	1 st REC	2 nd P0	2 nd REC	END
Control	-0.1 Aa*	-0.1Aa	-1.3 Ba	-0.1 Aa	-1.9 Ca	-1.9 Ca
Water deficit	-0.1 Aa	-2.6 Db	-2.7 Da	-2.3 Cb	-2.3 Ca	-1.9 Ba

Uppercase letters buy the different evaluation time in the same water condition and lowercase letters bought the different hydric conditions at the same evaluation time. ⁽¹⁾T0 beginning of the experiment; 1st and 2nd P0 - first and second photosynthesis zero, 1st and 2nd REC - first and second recovery, and the END evaluation

Free-hand para dermal and transversal cuts were made in the median region of completely expanded leaves of each treatment (n=8). Cross-sectional cuts were performed free-hand with a steel blade in fresh material being stained with astra blue and safranin, according to Bukatsch's (1972) proposal modified and assembled between lamina and laminula in glycerinated gelatin. From this cross-sectional sections, the thickness of the cuticle adaxial (CAD; μm) and abaxial (CAB; μm), epidermis adaxial (EAD; μm) and abaxial (EAB; μm), palisade (PP; μm), and lacunous (PL; μm) were determined.

The paradic impressions were prepared in the morning, between 8:00 and 11:00 h, with Super Bonder[®] glue of samples from the median region of the limbus on the adaxial and abaxial surfaces, being determined: polar diameter (PD μm) and equatorial diameter of the stomata (ED μm), ostiolar aperture (OA μm), stomatal functionality (considered the polar diameter/equatorial diameter ratio of stomata), and stomatal index (SI%) calculated using the formula proposed by Salisbury (1928). The laminary obtained was photographed with the aid of a Moticam 2000 digital camera coupled to the optical microscope by means of the MoticImage 2000 program and adjusted scales in the appropriate optical conditions.

Experimental design and statistical analysis

The experimental design was completely randomized in a factorial scheme with 6 evaluation periods (T0, 1stP0, 1st REC, 2nd P0, 2nd REC, and END) and 2 water regimens (control and water deficit) with four replications, where each repetition corresponded to a seedling.

The data were subjected to analysis of variance. Significant effects on the means of the water regimens were compared by the T test with a 5% probability and on the averages of the evaluation periods were compared by the Scott-Knott test at 5% probability.

Results

Effects on Water potential (Ψ_w)

The interaction was observed for water potential between evaluation time and water regimens (Table 1). In the first and sec cycle of photosynthesis close to zero (1st and 2nd P0), significant reductions were observed for Ψ_w . At the end of the experiment, the stressed seedlings did not differ from the control.

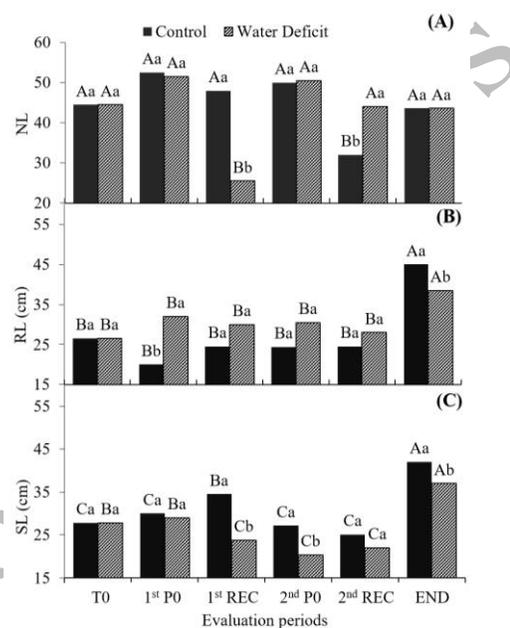


Fig. 2: Number of leaves - NL (A) root length - RL (B) and shoot length - SL (C) between irrigated seedlings of *C. xanthocarpa* and water deficit conditions. Lowercase letters compare the different water regimens in the same period of evaluation by the T test ($P < 0.05$). Uppercase letters compare the control treatment in the different evaluation periods. Uppercase letters in italics compare the stress treatment in the different evaluation periods, both by the Scott-Knott test ($P < 0.05$)

Effects on Growth characteristics

The seedlings of *C. xanthocarpa* under water deficit conditions showed reduction in the number of leaves (NL) in the first cycle of suspension/resumption of irrigation when compared to control (Fig. 2A). However, at the end of the experiment, the NL of seedlings under water deficit did not differ from control.

Under water deficit, root length (RL) showed an increment during the 1st P0 period (Fig. 2B); however, at the end of the experiment, this increment was lower than the control. The shoot length (SL) was reduced during 1st REC, 2nd P0, 2nd REC, and at the end of the 90 days of irrigation resumption (Fig. 2C).

Effects on antioxidant enzyme activity

The enzymatic activity of superoxide dismutase (SOD) and

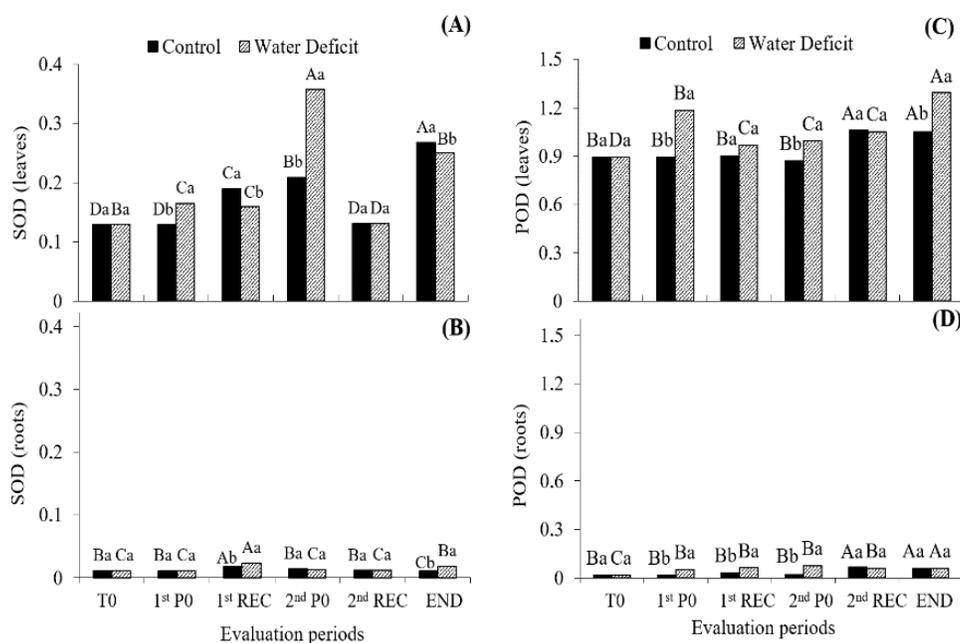


Fig. 3: Antioxidant activity of superoxide dismutase (SOD - U of SOD) enzymes in leaves (A) and roots (B); and Peroxidase (POD - $\mu\text{mol g FM}^{-1}$) in leaves (C) and roots (D) between irrigated seedlings of *C. xanthocarpa* and water deficit conditions

peroxidase (POD) was influenced by the interaction between the control and stress treatments (Fig. 3). Water deficit showed an increase in the enzymatic activity of SOD in the leaves during periods of 1st P0 and 2nd P0, respectively. A significant increase in the activity of this enzyme was also observed in control seedlings in the first cycle of suspension/resumption of irrigation and at the end of the experiment (Fig. 3A). However, the SOD activity in the root was higher in the 1st REC periods and at the end of the experiment (Fig. 3B).

In relation to POD activity in the leaves, an increase was observed during the 1st P0 and 2nd P0 periods and at the end of the experiment in the seedlings under water deficit (1.18 mM, 0.99 mM, and 1.29 mM, respectively) (Fig. 3C). In the roots, this increase was seen in the 1st P0, 1st REC, and 2nd P0 periods (Fig. 3D).

Leaf anatomy

The leaf anatomy of *C. xanthocarpa* seedlings was influenced by irrigation conditions (Fig. 4). The seedlings under water deficit showed significant reductions in the thickness of the cuticle of the adaxial face from the 1st P0 period and remained same until the 2nd REC period. At the end of the experiment, the previously stressed seedlings showed an increase in the thickness of the cuticle of the adaxial face (Fig. 4A). For the cuticle of the abaxial face, the seedlings under water deficit showed an increase in thickness in 2nd REC period (Fig. 4B).

In relation to the epidermal faces, the adaxial and abaxial faces showed an increase in the thicknesses due to the water deficit. For the epidermis of the adaxial face, this

increase was observed in the 2nd P0 and 2nd REC periods and at the end of the experiment (Fig. 4C) and for the epidermis of the abaxial face, in the periods of 1st P0 and at the end of the experiment (Fig. 4D).

As for the brackets, the seedlings under water deficit showed reductions in the thickness of the palisade parenchyma in the 1st P0 period and at the end of the experiment (Fig. 4E). However, in the 2nd P0 period, an increase in thickness with a value of 19.3 μm was observed. For the lacunous parenchyma, reductions in the thickness were observed during the 1st REC and 2nd REC periods and at the end of the experiment in seedlings subjected to water deficit when compared with the control seedlings (Fig. 4F).

Effects on stomatal characteristics

The ostioles aperture and the polar diameter of the stomata were influenced by irrigation conditions (Fig. 5). For the ostioles aperture, reductions observed were found in the periods of higher water stress: 1st P0 and 2nd P0 (Fig. 5A). In relation to the polar diameter, a reduction was seen in the 1st P0; however, in the 2nd P0 period, an inversion in the behavior was observed, with an increase in the polar diameter of the stomata in the stressed seedlings seen compared to control (Fig. 5B).

The equatorial diameter was influenced by the factors alone, and they were smaller in seedlings subjected to water deficit, reduced from the first cycle of suspension/resumption of irrigation and remaining with an average value of 2.96 μm until the end of the evaluation period (Fig. 5C, D).

The stomatal index in the seedlings under water deficit

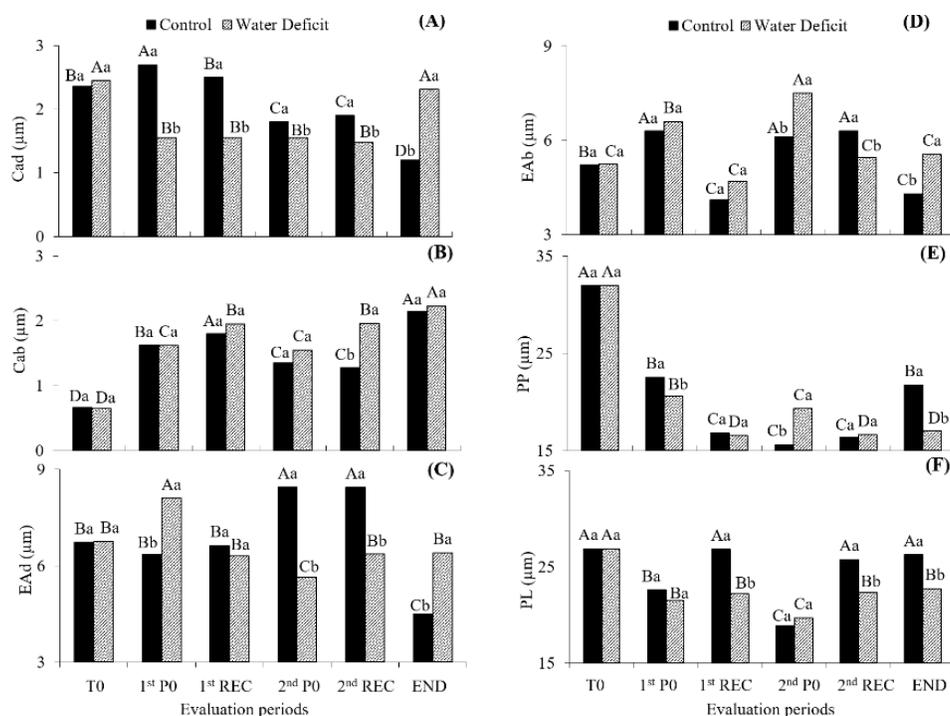


Fig. 4: Cuticle adaxial – Cad (A) and abaxial - Cab (B), epidermis adaxial – Ead (C) and abaxial – Eab (D), palisade parenchyma – PP (E) and lacunous – PL (F) of leaf structures/tissues between irrigated seedlings of *C. xanthocarpa* and water deficit conditions

fluctuated throughout the experimental period, as seen through reductions in the index during the 1st P0, 2nd P0, and 2nd REC periods, and through increases in the 1st REC period and at the end of the experiment (Fig. 5E).

It was only possible to observe stomatal functionality (PD/ED) differences promoted by the water deficit from the 2nd P0 period, (Fig. 5F) in which the seedlings under deficit showed a value for this parameter superior to that observed for the control seedlings, which remained until the end of the experiment.

Discussion

In this study, the seedlings of *C. xanthocarpa* under water deficit showed a reduction in water potential (Ψ_w 2.4 MPa) when the photosynthetic rate was reduced to values close to zero. After the resumption of irrigation, there was recovery of the Ψ_w of the seedlings previously maintained under water restriction, reaching values close to of the control plants, as observed for other tree species (Bento *et al.* 2016; Rosa *et al.* 2017; Reis *et al.* 2018).

The water deficit negatively affected the growth of seedlings of *C. xanthocarpa*, as evidenced by the reduction in the new leaves even after the first cycle of suspension/resumption of irrigation, likely reflecting the reduction of cell division and expansion caused by the low availability of water (Martins *et al.* 2010).

However, in the 2nd REC cycle, the seedlings maintained under water deficit produced a higher new

leaves. This phenomenon is known as “hardening”, which allows the plant a greater osmotic adjustment when it has already undergone a first cycle of stress due to lack of water. The solutes accumulated in the first water deficit are not readily assimilated and allow a higher osmotic accumulation and adjustment in the sec cycle of water deficit (Kramer and Boyer 1995), allowing the continuity of the development processes.

As a response to the water deficit (1st P0), the seedlings showed higher root production as a strategy to adapt to stress, since the aerial part of the plant showed reduced growth. This may be related to the recognition and signaling of stress, through the production of abscisic acid and reactive oxygen species (ROS) (Gupta *et al.* 2018), both of which induce root growth and stimulate the emergence of lateral roots while suppressing growth of the aerial parts.

However, after the 1st P0 period, the stressed seedlings ceased the growth of roots and reduced the aerial portion of the plants, a state that remained until the end of the experiment. This behavior can justify the reduction of leaf water potential in this period and signals the sensitivity of *C. xanthocarpa* to water deficit. We emphasize that the effects of water deficit on seedling growth are due to alterations in metabolism, with observed reductions of stomatal conductance, transpiration rate, and photosynthesis (Bento *et al.* 2016).

The decrease in water availability affects the growth of plants by controlling the opening of the stomata (Ashraf 2010), which blocks the influx of CO₂ into the leaves, thus,

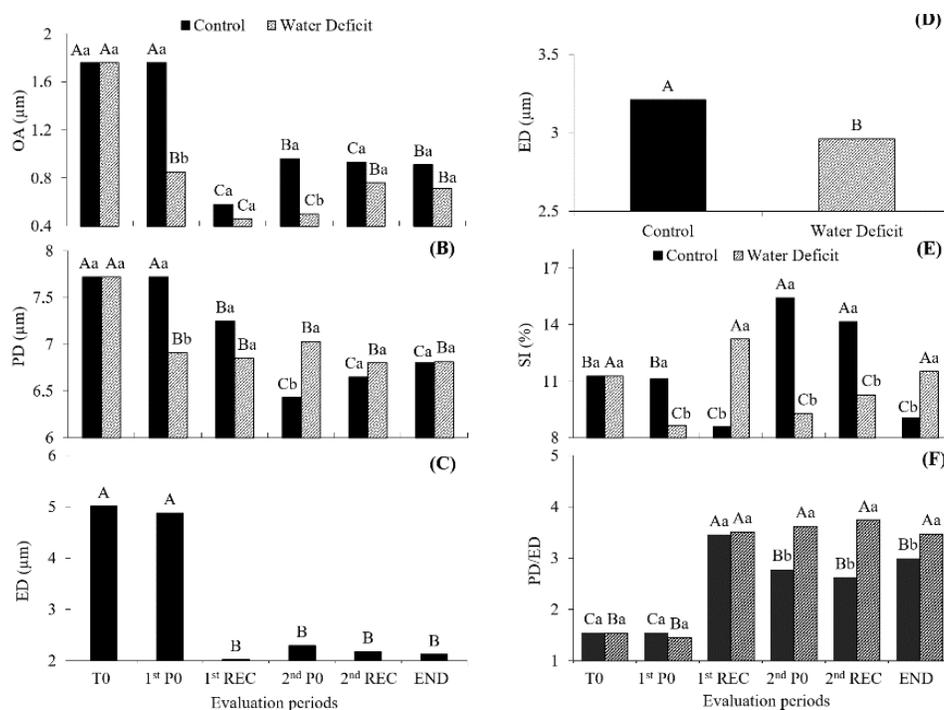


Fig. 5: Ostiolar aperture - OA (A), polar diameter - PD (B) and equatorial diameter of the stomata - ED (C, D), and stomatal index – SI (E) stomatal functionality – PD/ED (F) (polar diameter/equatorial diameter ratio of stomata) of leaf structures/tissues between irrigated seedlings of *C. xanthocarpa* and water deficit conditions

affecting photosynthetic activity and biomass production.

In the face of environmental stresses, plants which exhibit tolerance tend to minimize damage to their metabolism through the regulation of enzymatic activity, showing an increase in the activity of these enzymes that inactivate and/or transform ROS immediately after stress. Oxidative stress can act as an important regulator of vegetative growth, reproduction, defense, and survival of the plant (Morales and Munné-Bosch 2016).

In present study, the highest SOD activity was recorded in the periods of higher water deficit (1st P0 and 2nd P0). This behavior proves that an increase in enzymatic activity is a protective response to oxidative stress that *C. xanthocarpa* presents in the face of increased levels of ROS. This response is produced because of water deficit, since SOD efficiently performs the removal of hydrogen peroxide (H₂O₂), which generates the toxic superoxide radical (O₂⁻) (Silva et al. 2012; Hura et al. 2015).

In addition to SOD, high POD activity was observed in the leaves of seedlings under water deficit for the same periods of high water deficit. This high POD activity is associated with an increase in the intracellular level of H₂O₂ owing to removal of the superoxide radical (Pereira et al. 2012; Taiz et al. 2017). The POD has high affinity for H₂O₂ when at low concentrations (Gechev et al. 2006; Jaleel et al. 2009; Locato et al. 2010).

It should be emphasized that the increase in the activity of these antioxidant enzymes was also found in the

young plants of *Jatropha curcas* (Arcoverde et al. 2011; Silva et al. 2012), *Copaifera langsdorffii* Desf. (Rosa et al. 2017) e *Calophyllum brasiliense* Cambess. (Reis et al. 2018), in which such behavior was related to the occurrence of a favorable adjustment of the activity of antioxidant enzymes or new synthesis of proteins (Jin et al. 2009) as a form of defense and tolerance of plants against stress.

Hydrogen peroxide is a species of ROS that may play two important roles in plants. In low concentrations, H₂O₂ acts as a molecular sign involving signaling acclimatization, triggering tolerance to various biotic and abiotic stresses. At high concentrations, it causes the programmed death of the cell (Scandalios 2005; Bhattacharjee 2012; Taiz et al. 2017). Thus, the log of the SOD and POD activity in the seedlings of *C. xanthocarpa*, outside the periods of water restriction, is likely related to the presence of O₂⁻ and H₂O₂ formed according to the stressful environmental conditions recorded in the previous periods, at levels harmful to the plants.

The activity of SOD and POD enzymes was inferior in the roots of the plants to the leaves. This is because the enzymatic activity may vary in the different organs of the plants dependent on the diversified sites of activity of each enzyme (Gechev et al. 2006; Jaleel et al. 2009; Maia et al. 2012). Furthermore, the production of H₂O₂ in peroxisomes and leaf chloroplasts during environmental stresses may be 30–100 times faster than in mitochondria (Foyer and Noctor 2003; Bhattacharjee 2012). The greater activity of enzymes in the leaves than in the roots before the water deficit

indicates that the signaling for defense against this stress in *C. xanthocarpa* is exercised first in the leaves and then translated into other parts of the plant.

The observation of the antioxidant activity of SOD and POD in the roots of the stressed seedlings in the 1st REC period is related to the reduction of the relative humidity of the air recorded during this period (data not presented). It is known that some enzymes are constitutively expressed while others are induced by environmental stresses, as shown in studies of low stress activities which result in less severe symptoms and high stress activities that result in more severe symptoms (Barbosa *et al.* 2014). Thus, the environmental conditions recorded for the 1st REC period induced a continuity of protection from oxidative damage provided by the enzymes on the seedlings, in the face of the production of new toxic molecules.

The water deficit promoted morpho-anatomical alterations, reflected in the reduction of ostiolar aperture in the polar and equatorial diameters. Such modifications (density, index, aperture, size, and diameters) are related to the regulation of gas exchanges under stressful situations (Castro *et al.* 2005, 2009; Souza *et al.* 2010). During such situations, leaves that have smaller stomata and are under water deficit conditions have greater capacity for efficiency in water use due to smaller stomatal pore size, thus, conditioning a lower loss of water due to transpiration (Boeger and Wisniewski 2003). In case of *C. xanthocarpa*, the reduction in the size of the stomata was another strategy adopted by the plant to avoid water loss in the face of stress. This may indicate that the decrease observed for ostiolum opening had a greater effect on the diffusion of water than of CO₂, thereby maintaining the influx of CO₂ and lower loss of water by transpiration (Taiz *et al.* 2017).

The alterations evidenced in stomata size, index, and functionality, along with other modifications in the leaf structures, indicate that *C. xanthocarpa* can be subjected to severe water restriction situations. However, some of these alterations occur on a structural and irreversible level and therefore require time (Casson and Gray 2008), as evidenced from the 1st REC by the increase of the stomatal index.

In present study, the increased functionality of the stomata observed for the stressed plants demonstrate that the stomata acquired a more elliptical morphology in relation to the plants maintained under irrigation, which favored the low water loss (Melo *et al.* 2014).

This emphasize that these alterations may serve as an indicator of plant tolerance response to their hydric state and can prove the adaptive value of the plant in investing in the protection of chlorophyllous tissues and reduce transpiration. It is interesting to note that the assessed values of some anatomical characteristics of the stressed plants were not restored to the values of the control until the end of the experiment. These results indicate that the initial priority of the species is the maintenance of turgescence, even if reduced, as a way of maintaining metabolism, and that, perhaps with a longer period of evaluation, the plants can

increase metabolism or completely recover the evaluated parameters. Similar behavior was observed in other species (Arcoverde *et al.* 2011; Melo *et al.* 2014; Bento *et al.* 2016; Reis *et al.* 2018).

C. xanthocarpa showed morpho-anatomical and metabolic changes which favored minimizing water loss. This behavior can justify the occurrence of this species in environments with low water availability, proving our hypothesis that the species has plasticity to adjust to temporary water deficit.

Conclusion

The *C. xanthocarpa* is a native species sensitive to water deficit but presents strategies to adapt to a temporary water restrictive environment. The seedlings of *C. xanthocarpa* showed leaf morphoanatomical alterations as survival strategies to temporary water deficit. The species showed an active antioxidant system with increased activity of the enzymes superoxide dismutase and peroxidase in both the shoots and roots, allowing the recovery of seedlings after resumption of irrigation. This information is important because it can assist viverists or farmers, minimizing the mortality rates of seedlings and consequently the costs of production. It will also favor the success of management practices in projects of the recovery of degraded areas.

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Author Contributions

Larissa FB Araújo, Ferenanda S Junglos and Mário S Junglos collected data, Larissa FB Araújo and Daiane M Dresch conducted statistical analysis, Fernanda S Junglos e Rosilda Mara Mussury conducted leaf anatomy, Silvana de Paula Quintão Scalon supervised the experimental work and all authors wrote the article.

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