

Chlorophyll *a* fluorescence as an indicator of water stress in *Calophyllum brasiliense*

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Abstract

The objective of this study was to evaluate chlorophyll *a* fluorescence as a stress indicator in *Calophyllum brasiliense* Cambess seedlings grown with different concentrations of abscisic acid (ABA) under intermittent water deficit condition: daily irrigation without ABA (I); daily irrigation + 10 μ M ABA (I 10); daily irrigation + 100 μ M ABA (I 100); suspension of daily irrigation without ABA (SI); suspension of daily irrigation + 10 μ M ABA (SI 10) and suspension of daily irrigation + 100 μ M ABA (SI 100). The intermittent water deficit reduces water status and impairs the photochemical apparatus functioning and seedling quality. The fluorescence measurements helped identify the stress condition of water deficit in the cultivation of *C. brasiliense* and the beneficial effect of the application of 10 μ M of ABA in minimizing stress and facilitating the recovery of seedlings after re-irrigation, while maintaining the integrity and function of the photosynthetic apparatus.

Keywords: abscisic acid; Dickson quality; leaf area; photosystem II

Abbreviations: ABA: Abscisic acid; ChlF-*a*: chlorophyll *a* fluorescence; DQI: Dickson Quality Index; END: end of the experiment; F_v/F_m : potential quantum efficiency of photosystem II; F_v/F_0 : efficiency of the effective photosystem in the conversion of absorbed energy; F_0/F_m : basal quantum production of non-photochemical processes in PSII; H: height; I: irrigation; IRGA: infrared gas analyzer; P0: Photosynthesis next zero; PS II: photosystem II; RDM: root dry mass; REC: recovery; SD: stem base diameter; SI: suspension of daily irrigation; SDM: shoot dry mass; TDM: total dry mass; T0: time zero – start; WRC: water relative content

Introduction

Calophyllum brasiliense Cambess. (Clusiaceae), commonly known as ‘guanandi’, is a tree species native to Central and South America. In Brazil, it is found in several states, especially in the Amazon region and in the

Cerrado, occurring spontaneously in alluvial soils with poor drainage, periodically flooded humid places, sandy to loamy soils, and acidic soils (pH 4.5-6.0). It has ornamental, apicultural, and medicinal applications, and also has potential application in recovery of degraded areas, thus, facilitating the establishment of other species. Its wood is used in the construction of furniture, frames, and ships among other applications (Carvalho, 2003; Kalil Filho *et al.*, 2007).

Although there are some reports on the water and light requirements of this species, no information is available on its potential to tolerate water deficit when induced by abscisic acid (ABA). This information is important to extend its use economically and ecologically, especially in the recovery of degraded areas subject to seasonal periods of water deficit.

Depending on climatic conditions, plants undergo physiological, anatomical, and/or structural changes to adapt and acclimate to abiotic stresses. Responses to water deficit might include leaf area reduction, leaf abscission, stomatal closure, root growth, chlorophyll reduction, photosynthesis limitation, and seedling quality reduction among other responses (Scalon *et al.*, 2011; Asharaf and Harris, 2013; Rosa *et al.*, 2017; Nunes *et al.*, 2017; Vieira *et al.*, 2017; França *et al.*, 2017; Campelo - unpublished data).

The water deficit can change the organelle, pigment concentration and metabolites, as well as stomatal regulation (Mohammadi *et al.*, 2015). The closing of stomatal is considered a primary mechanism to regulate the water content when the plant is under dry conditions (Kowitcharoen *et al.*, 2015; Zhao *et al.*, 2015).

Studies suggest that stomatal closure under water deficit can occur under the influence of the water content of the soil and/or due to the hormonal levels of the plant (Damour *et al.*, 2010; Brodribb and McAdam, 2013). Thus, the exogenous application of some phytohormones like the ABA, you can enable the plant to adapt to hydric deficit, to mediate adaptive responses, stimulating the biosynthesis of proline and the translocation of photo assimilation (Sarafraz *et al.*, 2014).

The chlorophyll *a* fluorescence (ChlF-*a*) parameters can be used to understand the processes of tolerance and/or physiological plasticity to different water conditions (Kalaji *et al.*, 2018), since, as the only photochemical functions reflect a reduction in the efficiency of plants to capture, transfer, and convert energy (Nesterenko *et al.*, 2019) and the preservation of integrity or photo inhibitory damage in the reaction centers of PS II due to some stress factor.

Considering the natural habitat of this species, we hypothesized that their seedlings are sensitive to water deficit, which can be minimized by the application of ABA, and that ChlF-*a* can indicate stress condition, which reflects on the quality of the seedling. Therefore, the aim of the present study was to evaluate ChlF-*a* as a stress indicator in *C. brasiliense* seedlings grown with different concentrations of ABA and under intermittent water deficit condition.

Materials and Methods

Plant and cultivation material

The experiment was carried out in a protected environment where the seedlings of *C. brasiliense* Cambess were maintained under 30% shade and protected from rainfall using plastic cover. Seven-month old seedlings (after emergence), of mean height 14.32 cm and with 9.33 leaves, were grown in 7L pots. All the pots were irrigated at 70% water retention capacity (WRC) of the substrate until the characterization of the seedlings at time zero, with two seedlings per pot.

The treatments included suspension of daily irrigation without the addition of ABA (SI 0 ABA), suspension of daily irrigation + 10 μ M ABA (SI 10 ABA), suspension of daily irrigation + ABA 100 μ M (SI 100 ABA), daily irrigation without ABA (I 0 ABA), daily irrigation + 10 μ M ABA (I 10 ABA), and daily irrigation + 100 μ M ABA (I 100 ABA). Each treatment consisted of 22 pots, in addition to the six separate seedlings used for evaluation at time zero.

For the irrigation treatments, the seedlings were divided into two groups. The first group was irrigated daily throughout the experimental period and the soil was maintained at 70% WRC; this group was considered control based on the concentration of ABA. The second group was subjected to water deficit until the photosynthetic rate approached zero, considered the first zero photosynthesis (1st P0).

From the 1st P0 period, all the pots were irrigated daily, and the plants were maintained at 70% WRC until recovery (REC), which was considered the stage when the seedlings under water deficit presented photosynthesis rates similar to those of irrigated seedlings (data not shown). After REC, another cycle of irrigation suspension was performed, and the seedlings were evaluated until the photosynthetic rate approached zero again, considered the second photosynthesis zero (2nd P0). To determine the 1st P0 and 2nd P0, photosynthesis was monitored every two days using portable LCIPRO-SD (IRGA - Infra Red Gas Analyzer) (ADC BioScientific Ltd model), considering favorable climatic conditions. Subsequently, the pots were irrigated again until REC, according to the previously established standard and final evaluation that occurred 165 days after the start of the experiment (END).

On day 17, based on the photosynthetic rate of approximately $2 \mu\text{molm}^{-2}\text{s}^{-1}$, according to pre-tests, ABA was applied at the predetermined concentrations. The results were evaluated at five periods: T0, time zero (beginning of experiment); 1st P0, first photosynthesis zero (day 23); 2nd P0, second photosynthesis zero (day 82); REC, recovery (day 120); and END, final evaluation (day 165). The following parameters were evaluated:

Evaluations

Chlorophyll *a* fluorescence: Using a portable fluorometer (OS-30p; Opti-Sciences Chlorophyll Fluorometer, Hudson, NY, USA), we determined the potential quantum efficiency of photosystem II (PS II) (F_v/F_m), efficiency of the effective photosystem in the conversion of absorbed energy (F_v/F_0), and basal quantum production of non-photochemical processes in PSII (F_0/F_m). Fluorescence was determined between 8:00 and 11:00 AM was the second pair of fully expanded leaves. To determine their fluorescence, the leaves were subjected to a 30 min period of dark adaptation using adaptive clips, to ensure that all the reaction centers in this leaf region were open, that is, complete oxidation of the photosynthetic system of electron transport.

Relative water content (RWC): The relative water content in the leaves was determined using four leaves of each treatment, using the formula: $\text{RWC} = 100 \times (\text{fresh mass} - \text{dry mass} / \text{saturated mass} - \text{dry mass})$. The leaves were collected between 7:00 and 10:00 AM and cut into discs of known area. After weighing the fresh mass, they were placed in Petri dishes with distilled water for 24 h for saturation. After weighing the saturated discs, they were dried for determine the dry mass.

Chlorophyll index and leaf area: The chlorophyll index was determined using achlorophyll meter (SPAD 502; MINOLTA) (8:00 and 11:00 AM), and leaf area was determined using a leaf area integrator (Li 3100, Area Meter).

Dickson Quality Index (DQI): was calculated using the following equation $\text{DQI} = \text{TDM} / [(\text{H}/\text{SD}) + (\text{SDM}/\text{RDM})]$, where TDM = total dry mass (g); SDM= shoot dry mass (g); RDM=root dry mass (g), H = height (cm) and SD = stem base diameter (mm) (Dickson 1960).

Experimental design and statistical analysis

The data were evaluated in a completely randomized design with subdivided plots, where the plots represented the form of irrigation (daily irrigation - I and irrigation suspension - SI); each subplot included the three concentrations of ABA (0, 10, and 100 μM ABA); and the sub-sub-plot included the five evaluation periods (T0, 1stP0, 2ndP0, REC, and END). The results were subjected to the analysis of variance (ANOVA), and when significant effect was observed according to the F test, the means of the plots were subjected to the *t*-test of Bonferroni ($p \leq 0.05$) and the averages of subplots and sub-subplots were subjected to Tukey test ($p \leq 0.05$), using the SISVAR statistical program (Ferreira, 2014).

Results

The F_v/F_m was lower in the seedlings under water deficit at the 1st P0 period in relation to that of the irrigated seedlings (Figure 1A). The non-irrigated seedlings treated with 10 μ M ABA presented a higher F_v/F_m (0.797) than that of the seedlings subjected to other treatments, including SI 0 ABA (0.768) and SI 100 μ M ABA (0.738), and was close to that of the control I 10 μ M ABA treatment (0.801) (Figure 1B). The seedlings subjected to the SI 100 μ M ABA treatments presented the lowest F_v/F_m values, especially at the 1st P0 period, with an average of < 0.75 .

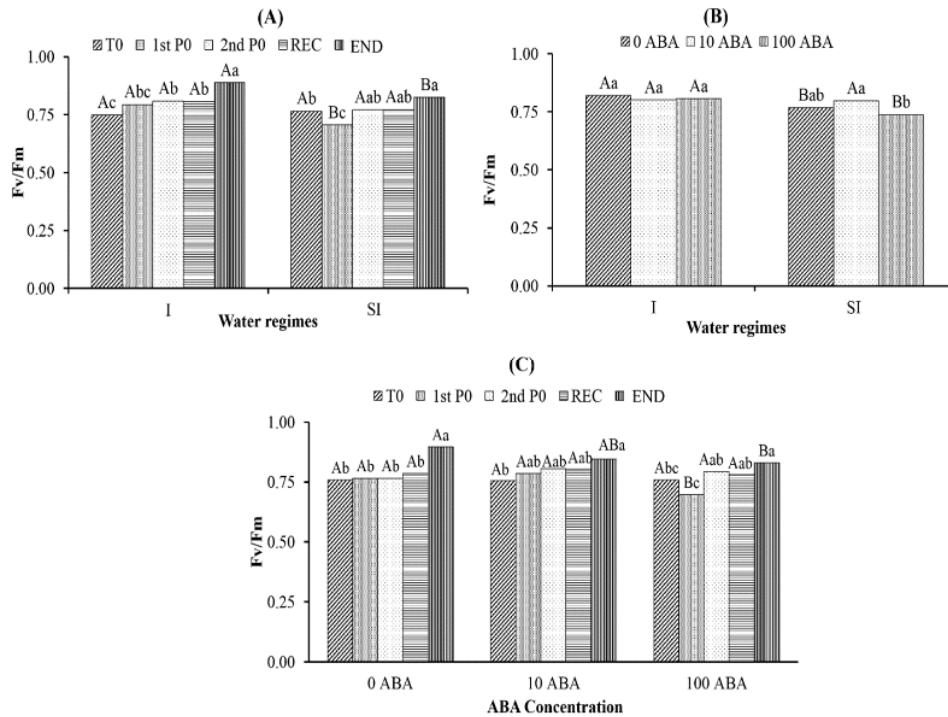


Figure 1. Quantum efficiency of photosynthetic II (F_v/F_m) (A, B, and C) seedlings of *Calophyllum brasiliense* subjected to different water regimes (irrigated-I and suspension of irrigation-SI), ABA concentrations (0, 10 and 100 μ M) and evaluation periods (time zero-T0, first and second photosynthesis near zero - 1stP0 and 2ndP0, recovery-REC and final evaluation END). Lowercase letters compare different periods of evaluation (A) and doses of ABA (B) in the same water regime (A, B) and different periods of evaluation in the same ABA concentration (C). Capital letters compare the same evaluation periods (A) and concentration of ABA (B) in different water regimes (A, B) and the same evaluation periods in different concentration of ABA (C)

In the END evaluation, this ratio tended to increase in the seedlings of all ABA treatments, with 0.785, 0.845, and 0.781, and reached a maximum of 0.896, 0.845 and 0.830, respectively. Furthermore, the F_v/F_m values of seedlings without ABA application varied significantly only at the END evaluation period (Figure 1C). Stressed seedlings recovered to the values close to those of the control plants when treated with 10 μ M ABA. The F_v/F_0 was higher at T0 (4.47) than at the 1st and 2ndP0 (3.76 and 4.02, respectively) (Figure 2A). The F_v/F_0 value of seedlings subjected to the SI 0 ABA treatment was lower (3.75) than that of the seedlings subjected to the I 0 ABA control (4.65) (Figure 2B). Stressed seedlings recovered only after re-irrigation at the end of evaluation and when treated with ABA.

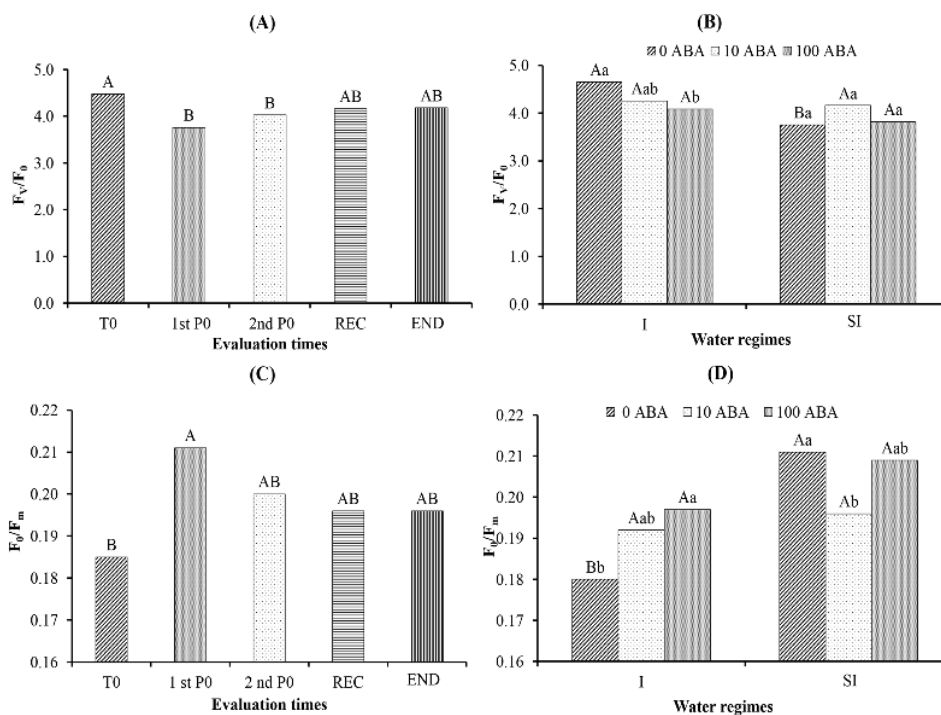


Figure 2. Efficiency of the effective photosystem in the conversion of absorbed energy (F_v/F_0) (A, B) and basal quantum production of non-photochemical processes in PSII (F_0/F_m) (C, D) seedlings of *Calophyllum brasiliense* subjected to different water regimes (irrigated-I and suspension of irrigation-SI), ABA concentrations (0, 10 and 100 μM) and evaluation times (time zero-T0, first and second photosynthesis near zero - 1st P0 and 2nd P0, recovery-REC and final evaluation END). Capital letters comparing the same dose of ABA in different water regimes (B, D). Lowercase letters compared different doses of the same ABA water regime (B, D).

The F_0/F_m was higher at the 1stP0 period, but not significantly different from that at the 2ndP0, REC, and END periods (Figure 2C). The seedlings subjected to the SI 0 ABA treatment presented the F_0/F_m value (0.16) higher than that of the seedlings subjected to the SI10 ABA treatments (0.195), which reached values close to that of the seedlings subjected to the I 0 ABA control treatment (0.192 higher) (Figure 2D). We also observed that the F_0/F_m values of the seedlings subjected to the SI 0 ABA treatment was 0.031-times higher than those of the seedlings subjected to the I 0 ABA treatment. Stressed plants recovered to values close to that of control seedling after re-irrigation when treated with ABA.

The RWC of the leaves decreased in the seedlings under water deficit with the lowest values in the seedlings subjected to the SI without ABA and SI 100 μM ABA treatments; however, the seedlings subjected to the 10 μM ABA treatment presented values similar to those of the control (Figure 3A). There was a significant reduction in RWC at both 1st and 2nd P0 periods in the seedlings grown under water deficit with elevation after re-irrigation, although it did not reach the values close to those of control seedlings (Figure 3B).

The leaf area of seedlings subjected to the 10 μM ABA treatment and those subjected to irrigation treatments increased and was maintained relatively high throughout the experiment (Figures 3C, D). The highest leaf area was observed in the END evaluation, and the irrigated seedlings (I) presented 105.35 cm^2 more area than that of the stressed seedlings (SI). Stressed seedlings did not recover the leaf area after re-irrigation, presenting significantly lower values than that of the control plants.

The non-irrigated seedlings without ABA application presented relatively low chlorophyll index, which was low even at the 1st P0, 2nd P0, and REC periods, although the index increased, it did not reach the values to

that of the control seedlings. The application of ABA favored the maintenance of relatively high chlorophyll index when cultivated under water stress (Figures 4A, B, C).

The lowest chlorophyll index was observed at the 1st P0 evaluation period in all the treatments with or without ABA application, especially in the stressed seedlings, which was 44.51% lower than that in the irrigated seedlings (Figures 4B, C).

Dickson Quality Index (DQI) increased during the experiment under both irrigation conditions. In the irrigated treatments, the values were higher than the stressed seedlings with significant differences at 2nd P0, REC, and END evaluation periods. However, stressed seedlings even after rehydration did not attain values close to those of the control seedlings (Figure 4D), suggesting that the period might not have been enough for the seedlings to recover.

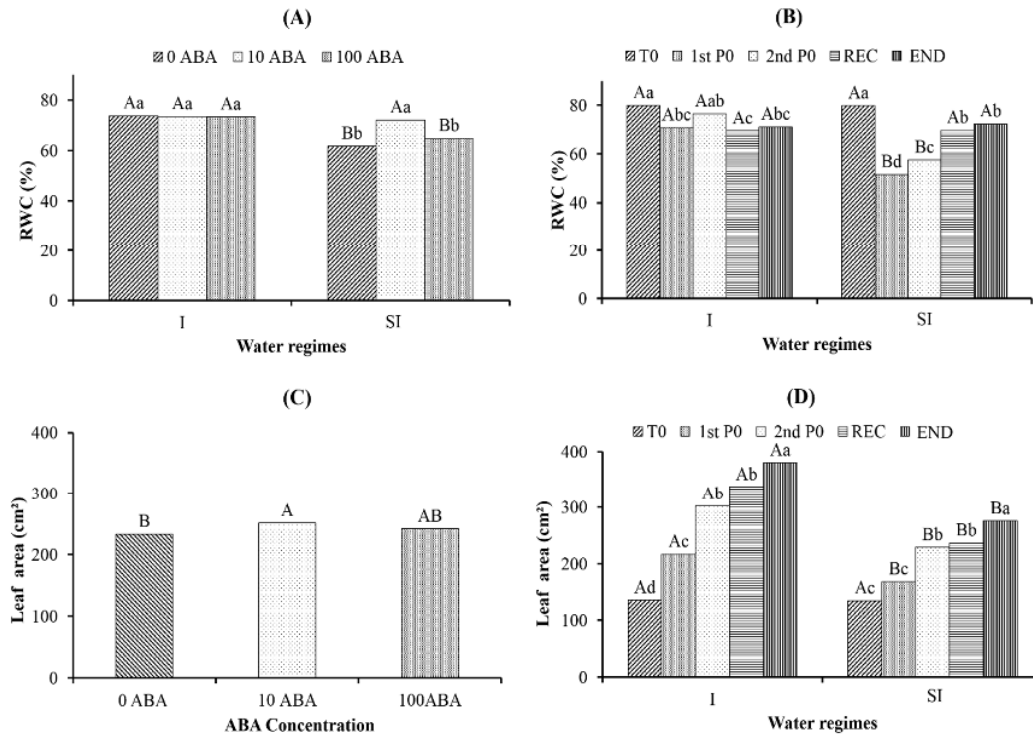


Figure 3. Relative water content (RWC) (A-B) and leaf area (C-D) of *Calophyllum brasiliense* subjected to different water regimes (irrigated-I and suspension of irrigation-SI), ABA concentrations (0, 10 and 100 μ M) and evaluation times (time zero-T₀, first and second photosynthesis near zero - 1st P0 and 2nd P0, recovery-REC and final evaluation END). Capital letters compare different water regimes in the same concentration of ABA (A) and evaluation period (B-D). Lowercase letters compare different concentrations of ABA (A) and evaluation periods (B-D) in the same water regimes.

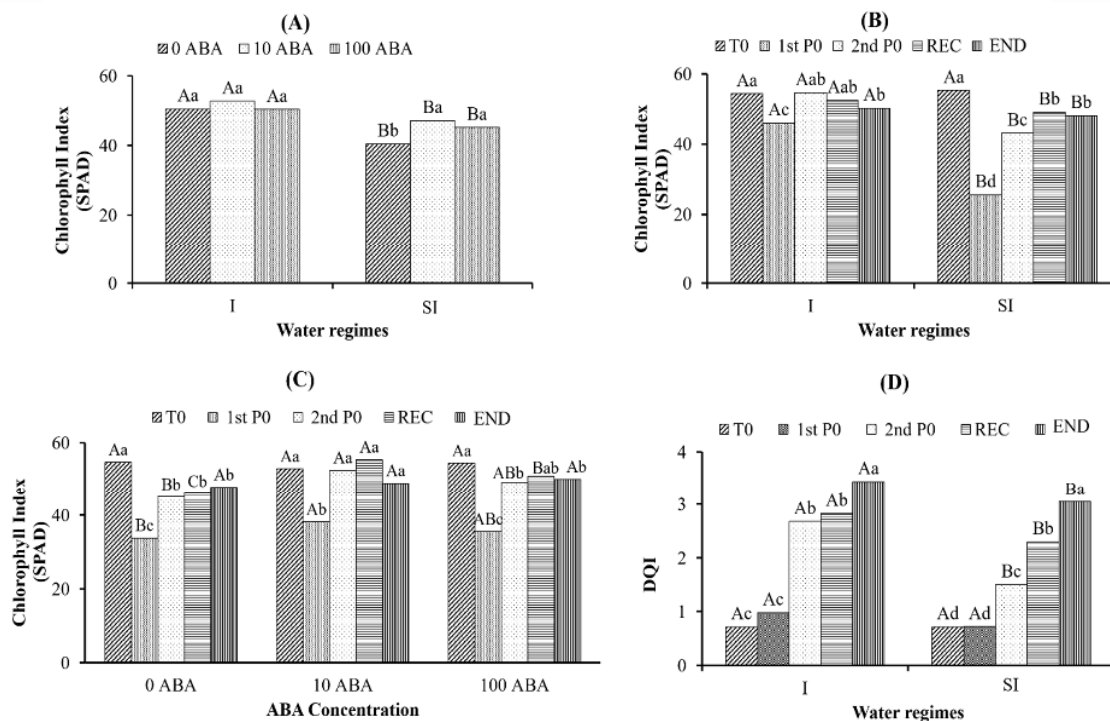


Figure 4. Chlorophyll index (SPAD)(A-B-C) and Dickson quality index (DQI) (D) seedlings of *C. brasiliense* subjected to different water regimes (irrigated-I and suspension of irrigation-SI), ABA concentrations (0, 10 and 100 μM) and evaluation periods (time zero-T0, first and second photosynthesis near zero - 1st P0 and 2nd P0, recovery-REC and final evaluation END). Capital letters compare different water regimes (A) and evaluation periods (B, D) in the same concentration of ABA and the same evaluation periods and the same evaluation times in different concentration of ABA (C). Lowercase letters compare different concentrations of ABA (A) and evaluation periods (B, D) in the same water regimes and the same concentration of ABA in different evaluation periods (C)

Discussion

The intermittent water deficit reduces the water status of *C. brasiliense*, which impairs the functioning of the photochemical apparatus and the quality of the seedlings. After the reirrigation of the seedlings and until the end of the evaluations, most of the characteristics evaluated reached values close to that of the control seedlings, showing that the damage was not irreversible. However, for the quality of the seedlings and the leaf area, the period evaluated may not have been sufficient for the metabolic adjustment, which may have occurred after the second reirrigation, to reflect the growth and consequently the quality of the seedlings.

In plants, water deficit affects water potential, nutritional status, leaf gas exchange, the efficiency of capture, transfer and conversion of energy by photosystems, mechanisms and physiological processes related to growth (Roháček, 2002; Zananndrea *et al.*, 2006; Campelo *et al.*, 2015; Rosa *et al.*, 2017; Nunes *et al.*, 2017; França *et al.*, 2017).

Studies have provided reference values related to chlorophyll *a* fluorescence, which has been used to predict stress condition. The reference values for the F_v/F_m range between 0.750 and 0.850 (Baker and Rosenqvst, 2004). However, these values are subject to a range of variation that depends, on the species, its physiological mechanisms and growth (Li *et al.*, 2004; Zananndrea *et al.*, 2006). For the F_v/F_0 , the values that reflect the maintenance of good state of functionality of the PS II reaction centers are between 4 and 6; values

below this range indicate stress. This characteristic can be used as an indicator of the maximum efficiency of the photochemical process in PSII and/or potential photosynthetic activity. Similarly, the reference for the F_0/F_m is between 0.14 and 0.20, suggesting that the increase in this ratio is indicative of stress (Roháček, 2002).

Thus, we can use these fluorescence characteristics as reliable parameters to evaluate the cultivation condition of *C. brasiliense* seedlings, which indicated the stress condition in the present study due to water deficit. This was reinforced by the changes in other characteristics evaluated, such as leaf area, chlorophyll index, and seedling quality.

Under water deficit condition, *C. brasiliense* seedlings presented a reduction in the F_v/F_m , which is similar to the findings of other studies with different species under water stress, such as *Hancornia speciosa* (Scalon *et al.*, 2015), *Swietenia macrophylla* King, *C. brasiliense* and *Handroanthus albus* (Cham.) Martos (Campelo *et al.*, 2015), *Hymenaea coubaril* L. (Freitas *et al.*, 2018).

The higher F_v/F_m reduction observed at the 1stP0 period reinforces the stress condition of the non-irrigated *C. brasiliense* seedlings. However, after re-irrigation, the F_v/F_m ratio was restored, and the ABA concentration in the END evaluation period was higher than 0.75, indicating that there were no permanent damages to the photosynthetic apparatus.

The F_v/F_0 ratio was also lower at the 1stP0 evaluation period. However, at the REC and END evaluation periods, the seedlings presented values close to those of the control, indicating the efficiency of *C. brasiliense* seedlings in tolerating stress. The F_0/F_m ratio increased significantly in the 1stP0 period, and after re-irrigation, the tendency of reduction was observed. However, the values were maintained above the reference values and those of the control, except in the seedling treated with 10 μ M ABA.

Calophyllum brasiliense, *S. macrophylla* and *H. albus* showed higher sensitivity to water deficit than that of other species. This was reflected by higher reductions in gas exchange and the photochemical efficiency of PS II (Campelo *et al.*, 2015). Freitas *et al.* (2018) working on *H. coubaril* with different concentrations of ABA, verified water stress attenuation, thus, a reduction in the functions of PS II.

The seedlings of *C. brasiliense* under water deficit condition presented reduced leaf area in relation to the plants irrigated from the 1stP0; although the leaf area increased at the end of the experiment, the seedlings under stress did not reach values close to those of the control plants (I).

Abscisic acid increased leaf area and did not cause leaf abscission in *C. brasiliense* seedlings. The ABA alters the growth and development of plants and regulates the adaptive responses under conditions of low water availability, such as opening and closure of the stomata, leaf abscission, and root growth (Zhu, 2002; Tardieu *et al.*, 2010; Vieira *et al.*, 2017). The leaf area of *C. brasiliense* seedlings treated with 10 μ M ABA was higher than that of the seedlings not treated with ABA.

Furthermore, Tardieu *et al.* (2010) suggest that the positive effect of ABA on leaf expansion is attributed to the increase in the hydraulic conductivity of the root system at the same time as it leads to the stomatal closure and consequently to the maintenance of leaf turgescence.

In the present study, the plants under water deficit condition exhibited reduced leaf area at the 2ndP0 period. Although the leaf area of seedlings under stress increased, which can be attributed to the natural growth of the plant, it did not attain the control values. The reduction in the leaf area of woody plants as a response to low soil water availability has been proven in several species. Similar results were observed for *Guazuma ulmifolia* (Scalon *et al.*, 2011) and *Vatairea macrocarpa* (Benth.) Ducke (Vieira *et al.*, 2017).

The chlorophyll index varied according to the treatments, however, stressed seedlings treated with 10 μ M ABA maintained higher SPAD index in relation to that of the seedlings subjected to other treatments under stress, observing also hormonal action between the evaluation periods. The 1stP0 was the time that presented the lowest chlorophyll index in all the treatments with or without ABA application. At the dose of 10 μ M ABA, the values were higher than that of the treatment without ABA at the 2ndP0 and REC periods. At the other periods of evaluation, there was an increase in the SPAD index; however, they did not attain the control values.

Generally, plants under water stress present reduced photosynthetic pigment content due to oxidative damages, thus affecting photosynthesis (Asharaf and Harris, 2013) and consequently the production of dry mass and leaf area expansion, which reflected the reduction of DQI, to *C. brasiliense* seedlings.

Seedlings of *Hymenaea coubaril* treated with 10 μ M ABA both in the photosynthesis close to zero and in the recovery period, presented SPAD index close to that of the control seedlings. However, chlorophyll index values showed reduced for plants treated with 100 μ M ABA, independent of water availability and the DQI did not differ significantly among the different treatments throughout the experimental period (Freitas *et al.*, 2018).

Under water deficit condition, *Schinus terebinthifolius* showed reduced chlorophyll index (SPAD) when the seedlings reached almost zero photosynthesis (1st P0) and did not recover after irrigation (Nunes *et al.*, 2017). Furthermore, *Copaifera langsdorffii* seedlings showed the lowest SPAD index at 25% and 100% FC (Rosa *et al.*, 2017). Other species such as *Khaya ivorensis*, *Calophyllum brasiliense*, *Astronium fraxinifolium*, *Handroanthus albus*, and *Simarouba amara* also presented a reduction in chlorophyll content due to water deficit (Campelo - unpublished data).

Dickson quality index of *C. brasiliense* seedlings under water deficit condition at T0 and 1st P0 periods did not change significantly from that of the seedlings subjected to irrigation treatments. At the other evaluation periods, the seedlings under stress presented lower DQI, which might be due to lower biomass production for growth and target metabolism for defense mechanisms, such as increased enzyme activity (data not shown). Dickson Quality Index indicates robustness of seedlings; the higher the value, the better the quality (Moraes *et al.*, 2012; Gordin *et al.*, 2016).

Schinus terebinthifolius at different irrigation depths (8, 10, 12, and 14 mm) also showed lower DQI when they received the lowest amount of water (Moraes *et al.*, 2012). Similar results were observed in *Hancornia speciosa* seedlings when grown at 25%, 50%, 75% and 100% substrate water retention capacity, they presented lower DQI at 25% and 50% RWC (Gordin *et al.*, 2016). As stressed seedlings, even after rehydration, did not attain quality indexes similar to those of control seedlings, we believe that the period might not have been sufficient for them to recover.

In natural habitat, *C. brasiliense* is found in phytophysiology that present hyper seasonality, that is, dry and rainy seasons throughout the year, causing oscillations in the soil water status (Souza *et al.*, 2018), causing the species to grow plants subjected to the water deficit condition at certain periods. Under reduced soil water availability (dry season - 1st P0 and 2nd P0), *C. brasiliense* seedlings reduced the efficiency of activities in PSII, indicating stress conditions for maintenance of metabolic processes, as well as physiological plasticity by reversing photochemical damage, at the end of evaluation period.

The application of ABA in adequate amounts is a practice that substantially contributed to the mitigation of photochemical damage, maintaining the integrity of photosynthetic apparatus until the rainy season, characterized by re-irrigation, ensuring the development of seedlings and restoration of ecosystem services. Thus, study of plant photochemical responses to environmental variants contributes understanding of processes resulting from PSII under adverse conditions, aiming at the *in situ* and *ex situ* conservation.

Conclusions

The fluorescence measurements helped identify the stress condition of water deficit in the cultivation of *C. brasiliense* and the beneficial effect of the application of 10 μ M ABA in minimizing stress and in facilitating the recovery of seedlings after re-irrigation, while, maintaining the integrity and function of the photosynthetic apparatus.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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