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ORIGINAL PAPER

Ecophysiological acclimatization to cyclic water stress in *Eucalyptus*

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Abstract Drought is considered the main environmental factor limiting productivity in eucalyptus plantations in Brazil. However, recent studies have reported that exposure to water deficit conditions enables plants to respond to subsequent stresses. Thus, this study investigates the ecophysiological acclimatization of *eucalyptus* clones submitted to recurrent water deficit cycles. *Eucalyptus* seedlings were submitted to three recurrent water deficit cycles and anatomical, morphological and physiological changes were analyzed. The results were: (1) *Eucalyptus* seedlings responded to water deficits by directing carbohydrates to root and stem growth; (2) Size and number of stomata were reduced; (3) Stomatal conductance decreased which allowed the plants to reduce water losses through transpiration, increasing instantaneous water use efficiency; (4) The relationship between gas exchanges and available water contents allowed the seedlings to uptake the retained soil water at higher tensions; and, (5) Physiological recovery from subsequent water deficits became faster. As a result of these changes, the *eucalyptus* seedlings recovered from the same degree of water stress more rapidly.

Keywords Carbon partition · Drought · Gas exchange · Morpho-physiological changes · Photosynthetic apparatus

Introduction

Planted forests play a key role in supplying environmental and economic services worldwide although they represent only 7% of the global forest area (Villar et al. 2011). The genus *Eucalyptus* provides a major source for plantations in Brazil, occupying 5.63 million hectares, equivalent to 72.2% of the total area planted with trees (IBÁ 2016). *Eucalyptus* are planted throughout Brazil, exposing the numerous species to a range of abiotic stresses. Among these, drought is considered the main factor limiting productivity (Correia et al. 2014; Zhou et al. 2014).

Plant responses to drought are complex and involve diverse biochemical and physiological processes. A reduction in stomatal and/or mesophyll conductance is cited by several authors as being the main cause of reduced photosynthesis under drought stress (Grassi and Magnani 2005; Warren et al. 2011; Cano et al. 2014). The inhibition of ribulose-1,5-bisphosphate-carboxylase-oxygenase (Rubisco) activity (Tezara et al. 2002), and a reduced capacity for ribulose-1,5-bisphosphate (RuBP) regeneration (Thimmanaik et al. 2002) also play an important role in restricting photosynthesis. With the *Eucalyptus* genus, responses to low water availability include increases in photorespiration (Thumma et al. 2012), and changes in biomass allocation and cell wall reinforcement (Ladiges

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1975). However, exposure of plants to recurring stress allows them to respond and adapt to these conditions, making them more resistant to future stresses (Molinier et al. 2006; Asensi-Fabado et al. 2013).

Some researchers have reported that exposure to drought stress conditions enables plants to respond to a new stress with more rapid morpho-physiological changes contributing to a higher drought stress tolerance (Bruce et al. 2007; Ding et al. 2012; Kinoshita and Seki 2014; Iwasaki et al. 2014). Plants under water deficit or stress display morpho-physiological changes such as: (1) increasing cell wall elasticity which contributes to the maintenance of cell turgor (De Diego et al. 2013); (2) inducing leaf rolling, limiting water loss by restricting the leaf transpiration surface (Hura et al. 2012; Peguero-Pina et al. 2016); and, (3) increasing cellulose synthesis by maintaining cell wall integrity and cell turgor pressure, allowing continuous cell growth under low water potential (Ricardi et al. 2014; Le Gall et al. 2015). However, research investigating the mechanisms related to adaptation and acclimatization are still scarce, leaving this poorly understood, especially its ecophysiological aspects.

The hypothesis of this study is that the exposure of *eucalyptus* seedlings to water deficits will fine-tune their response to future drought stress. Therefore, the study investigates the ecophysiological acclimatization of *eucalyptus* clones subjected to recurrent water deficit cycles.

Materials and methods

Plant material and experimental conditions

This study was carried out under greenhouse conditions of controlled temperature and humidity at the Laboratory of Meteorology and Forest Ecophysiology, Federal University of Espírito Santo.

Seedlings of the eucalyptus clone AEC 144 (*Eucalyptus grandis* x *Eucalyptus urophylla*), the most widely used clone in plantations in Brazil, were produced in 50 cm^3 tubes with a substrate of biostabilized pine bark, vermiculite, milled charcoal and phenolic foam. This substrate is commonly used in the production of seedlings using cuttings. Within 80 days, the seedlings were planted into 50 L pots. Fertilization was carried out according to recommendations of Prezotti et al. (2007).

The seedlings were separated into four treatments, three subjected to water deficit cycles and one was a well-watered control:

First water deficit cycle: Only the seedlings of the 3rd water deficit group (3rd WD) were subjected to water deficit. The 1st WD, 2nd WD and control group (C_{group}) were maintained with soil moisture at field capacity. This cycle started 30 days after planting the seedlings;

Second water deficit cycle: The 2nd WD and 3rd WD groups were subjected to water deficit. The 1st WD group and the control (C_{group}) were maintained with soil moisture at field capacity. This cycle started 74 days after planting;

Third water deficit cycle: The 1st WD, 2nd WD and 3rd WD groups were subjected to water deficits. Only the plants in the C_{group} were maintained with soil moisture at field capacity. This cycle started 109 days after planting the seedlings.

The water deficit cycles started at the same time in all treatment groups. In this way, when the third water deficit cycle started, the 3rd WD, 2nd WD, and 1st WD groups were being subjected to their third, second and first water deficit, respectively. The control group was not subjected to any water deficit cycle. Physiological analyses were performed during the third water deficit cycle, and morphological analyses were carried out after the seedlings had recovered from the third water deficit.

For the purpose of this study, a water deficit cycle was defined as: “the suspension of irrigation until the photosynthetic rate of the seedlings reached zero (0 μmol CO_2 m^{-2} s^{-1})”. Irrigation (Watering) resumed immediately after zero photosynthesis was recorded.

Microclimatic characterization

An automatic meteorological station was installed for microclimatic characterization of the environment within the greenhouse, and consisted of two temperature and relative humidity sensors, model CS500 (Campbell Scientific, Inc., Logan, UT, USA). The data was stored in a data logger, model CR1000 (Campbell Scientific, Inc., Logan, UT, USA), scanning every 10 s and storing the average values every minute. Vapor pressure deficit (VPD) data was obtained by calculating the difference between the saturation values and the actual vapor pressure.

Monitoring of water stress cycles

This was performed by weighing the pots to determine field capacity (FD = 49%), wilting point (WP = 19%) and bulk density ($\rho = 0.43$ g·cm^{-3}) according to Donagema et al. (2011). Using these values, the available water capacity (AWC) was determined using the following equation:

$$AWC(mm) = 0.1x(FD - WP)x\rho$$

The AWC was transformed to AWC in liters by multiplying AWC (mm) by the pot area (m^2). The pots containing the plants were sealed with a 10-mm thick Styrofoam sheet, ensuring that only transpiration occurred with no evaporation from the pots. The ratio of the transpired fraction (weighed daily at 6:00 am) to the AWC multiplied by 100 produced the AWC (%).

Gas exchange measurements were performed every day at 8:00 am during the third water deficit cycle to determine the times when the plants should be watered, and a new cycle should begin. The time of 8:00 am was used because it is the moment of greatest photosynthetic potential under the greenhouse conditions employed, since the reduction of photosynthetic capacity throughout the day is a common phenomenon across species (Kosugi et al. 2009; Kamakura et al. 2012). The measurements were carried out on 10 plants, measuring two leaves per plant, and considering each plant as one replicate.

After watering, the seedlings remained under irrigated conditions until a photosynthetic rate of 100% of the C_{group} was achieved. To ensure the total restoration of photosynthesis after the 100% rate was reached, irrigation was maintained for a further 10 days in plants that had undergone water stress. Irrigation was then suspended, and the next water deficit cycle begun. Thus, as the cycles were synchronized at the same time, the beginning of the water deficit cycle was 10 days after the full recovery of the last group.

Gas exchange analysis

Gas exchange evaluations were carried out in two phases: (1) To monitor water stress cycles; and, (2) To identify daily patterns of photosynthesis variations in each group throughout the day on the 1st, 5th, 9th and 13th days from the beginning of the third water deficit cycle. With these analyses, net photosynthetic rate (A, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), stomatal conductance (g_s, µmol H_2O m^{-2} s^{-1}), and intercellular CO_2 concentration (Ci, µmol CO_2 mol^{-1}) were measured. The measurements were made using a portable infrared gas analyzer (IRGA, model Li COR 6400, LI-COR Inc., Lincoln, NE, USA), at a light intensity of 1500 µmol m^{-2} s^{-1} (Evans et al. 1993), and a CO_2 concentration of 400 µL L^{-1}. Evaluations were done every four hours between 8:00 am and 4:00 pm in fully expanded leaves in the upper third of the plant, using 10 replications per treatment. Each seedling was considered as one replicate, and measurements were recorded for two different leaves on each plant. The instantaneous carboxylation efficiency (EiC) was established through the relationship between the net photosynthetic rate and the intercellular CO_2 concentration (A/Ci), and the instantaneous water use efficiency (iWUE) was established through the relationship between net photosynthetic rate and transpiration rate (A/E).

Relative water content (RWC)

The RWC of leaves was determined on the 1st, 5th, 9th and 13th days from the beginning of the third water deficit cycle. Approximately 15-mm circular discs were cut from leaves of eight seedlings in each treatment. The discs were collected at 5:00 am before sunrise to determine fresh weight (FW). Three leaves per plant were used for analysis and each plant was considered as one replicate. After collection, the discs were placed in the dark for 24 h in Petri dishes containing distilled water to determine the turgid weight (TW). For dry weight (DW) determination, samples were oven-dried at 60 °C until a constant weight was reached. The RWC was calculated according to the following:

$$RWC = \frac{FW - TW}{DW - TW} \times 100 \tag{1}$$

Fifteen leaf discs with diameters of 15 mm were cut per plant. Each set of 15 discs was considered as one repetition, comprising a total of eight replicates.

Dry weight, diameter and height determinations

After the recovery of the seedlings from the final water deficit cycle, dry mass (DM) was obtained after drying leaves, stems and roots at 75 °C until a constant weight was achieved. Heights were measured with a ruler and diameters with a digital caliper.

Stomata analysis

Anatomical analyses were carried out at the same time as the DM measurements. The printing method was applied which consists of pressing the leaf surface into a layer of adhesive glue. When the glue solidifies on the blade, it retains an image of the pressed surface. A total of 10 blades were prepared for each treatment using the abaxial layers of leaves from the upper third of the plants. One leaf per plant was sampled, being a new leaf grown after the final water deficit cycle. Digital 1-mm^2 images of the prints were captured using a digital camera (model AxioCam MRc5, Carl Zeiss Microscopy, Oberkochen, Germany) coupled to a light microscope (model Axio Scope. A1, Carl Zeiss Microscopy, Oberkochen, Germany), with a 50 X objective lens. Four counts and four measurements of stomatal width and length were made for each blade, totaling 40 counts per treatment. Each blade was considered one repetition.

Statistical analysis

A completely random design was used with four treatments (C_{group}, 1st WD, 2nd WD and 3rd WD) and ten replications per treatment, each represented by one seedling. Statistical analyses were carried out using the software R (R Core Team 2017). To analyze the response of each variable to the environments studied, the data were submitted to an analysis of variance (ANOVA) and, when a significant (F-test $p < 0.05$), the mean values were compared using the Tukey test ($p < 0.05$). Subsequently, in order to obtain an integrated assessment of cyclic water stress in *eucalyptus*, the data were subjected to a multivariate analysis of variance (MANOVA) and to canonical discriminant analysis (CDA) using the Candisc package (Friendly and Sigal 2014). From the CDA data, a biplot graphic was generated (Fig. 6) to evaluate the multivariate differences between the treatments and to hierarchize the contribution (weight) of each variable on physiological responses after subjection to cyclic water stress. The center of the circles on the graph represents the mean value of the first two canonical variables. When the ellipses overlap, the treatments are not statistically different. The size of the arrow represents the importance of each variable in the distinction of the treatments, and its direction indicates the positive or negative effect of the variable on the treatments. Further explanation and examples of CDA can be found in Gittins (1985). Considering that EiC results are very collinear to *A* and *Ci*, they were not included in the CDA analysis. The variable stomata, used in CDA analysis, was obtained by the multiplication of the variable sizes and widths of the stomata, and the variable shoot/root ratios were obtained from the relationship between the sum of the stem and leave dry mass and root dry mass.

Results

Microclimate conditions during the cyclic water stress

Mean air temperature (Tmean) inside the greenhouse was maintained at 30 °C ± 3 °C throughout the experimental period. Mean vapor pressure deficit (VPD) was 2 kPa ± 0.5 kPa. There were small variations in Tmean and VPD during the water deficit cycles (Table 1).

Table 1 also shows the time required for each treatment to reach zero photosynthesis. Seedlings of the 3rd WD group, when exposed to the first water deficit cycle, took 29 days to reach zero photosynthesis, whereas plants of the 1st WD group when exposed to the first cycle (i.e. in the 3rd water deficit cycle) took 6 days. It is important to note that all plants were planted on the same day and exposed to water deficit cycles in a synchronized way, so plants of the 1st WD group when exposed to the first cycle had a much larger leaf area than the plants of the 3rd WD when exposed to its respective first cycle. Thus, the time required to achieve zero photosynthesis has been reduced over time, mainly due to plant growth.

Plant dry weight, diameter and height

The water deficit cycles affected plant growth. The dry mass of the plant groups 1st WD, 2nd WD and 3rd WD were 40%, 48% and 57% lower than the controls, respectively (Fig. 1). Similar results were found for stem diameters and heights and show that seedlings that experienced a greater number of water deficit periods showed greater reductions in dry mass, diameters and heights (photos of the results are presented in Fig. S2 at the supplementary material).

The dry mass proportion (DM %) is an indicator of how the eucalyptus seedlings acclimatize to a water deficit (Fig. 2). The DM % was different in each water deficit cycle. In the C$_{group}$, leaves made up a large proportion of dry mass. The situation changed in the 1st WD group where the leaves were a smaller proportion of the dry mass, mainly due to leaf shedding during drought. However, in the 2nd WD and 3rd WD groups, there was almost no leaf abscission after the first water deficit cycle, and it was noted, that there was carbon allocated to the roots and stems as a form of acclimatization to the water deficit.

Table 1 Characteristics of the greenhouse microclimate with controlled temperature and relative humidity over 97 days. T mean—mean temperature, VPD—vapor pressure deficit. (Treatments: WD cycle 1; WD cycle 2; WD cycle 3; 3rd WD—submitted to three water deficit cycles; 2nd WD—submitted to two water deficit cycles; 1st WD—submitted to one water deficit cycle)

Treatments		Time (days)	T mean (°C)	VPD (kPa)	Thermal amplitude (°C)
WD cycle 1	3rd WD	29	30.7	1.9	4.1
WD cycle 2	2nd WD	16	31.1	2.0	4.8
	3rd WD	20	31.1	2.0	4.8
WD cycle 3	1st WD	6	31.6	2.2	5.5
	2nd WD	11	31.5	2.1	5.7
	3rd WD	12	31.5	2.1	5.7

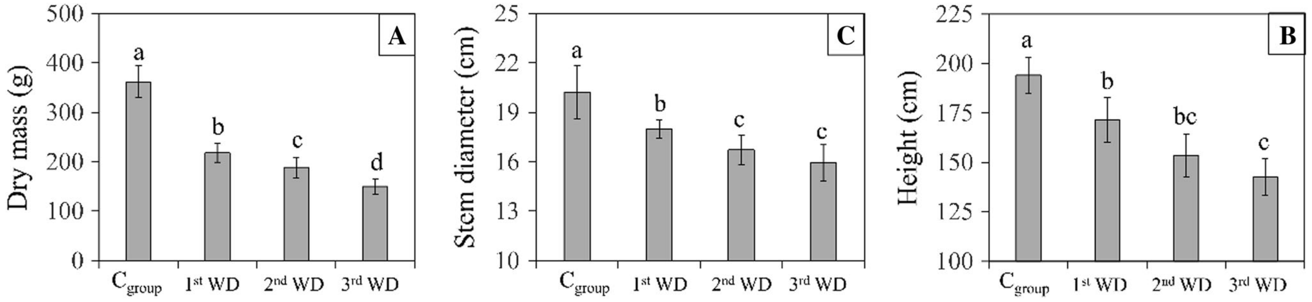


Fig. 1 Dry mass (**a**), stem diameter (**b**) and height (**c**) of eucalyptus plants submitted to three water deficit cycles. Data are mean ± SD. Letters represent statistically significant differences (Tukey, at $p < 0.05$). $n = 10$

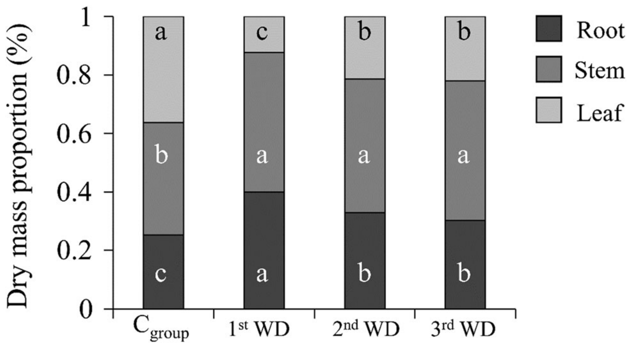


Fig. 2 Dry mass proportion (DM %) of eucalyptus plants submitted to three water deficit cycles Different letters in the same organ represent statistically significant differences (Tukey, at $p < 0.05$; $n = 10$)

Stomata

Stomatal number, length and width were other morphological changes observed in eucalyptus under water deficits (Fig. 3). The number of stomata was reduced in the 2nd WD group. In the 3rd WD group, there was also a reduction in length and width.

Gas exchange during the period of water deficit and rewatering

Measurements of leaf gas exchange are fundamental to understanding eucalyptus adaptation to water stress. In this study, plants in the 1st WD group showed a rapid fall in *A*, and by the 5th day, their assimilation rate was completely impaired (Fig. 4). This result is a consequence of the high rates of *E* since the 1st day, which caused a rapid reduction of available water and RWC (Fig. S1). On other hand, plants in the 2nd WD and 3rd WD groups showed a reduction of g_s from the 1st day of water deficit and this, together with the lowest proportion of leaves, allowed the plants to reduce their *E* rates, and as a consequence, maintain high RWC and *A* rates longer.

On the 9th day, the *A* in the 3rd WD group was higher throughout the day than in the 2nd WD group. With the 8:00 am measurements, this can be explained by the g_s, which was higher at 8:00 am in the 3rd WD plants, resulting in a greater Ci value which represents a greater amount of substrate (CO_2) for carrying out photosynthesis. However, from 12:00 am, the g_s was the same in both treatments. Thus the highest *A* rates in the 3rd WD plants were due to their higher RWC (see supplementary data).

The recovery of physiological processes after rewatering is another important difference among the treatments. 3 days after rewatering, the 1st WD group (9th day) had not

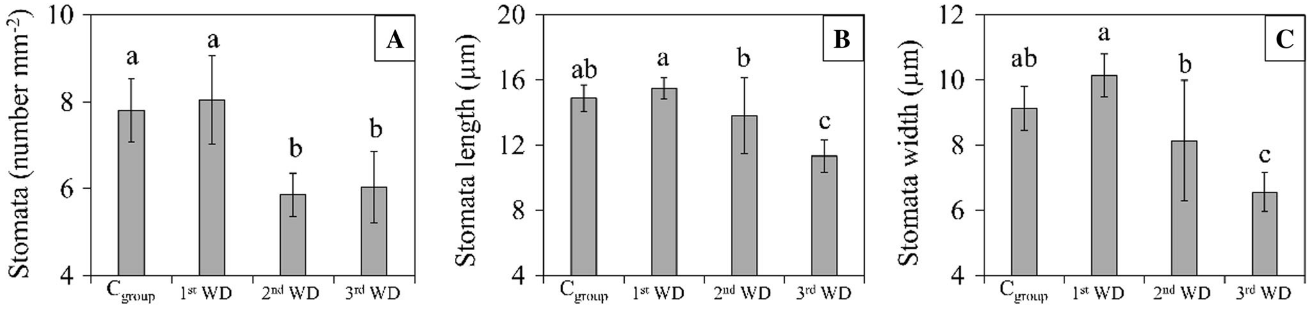


Fig. 3 Stomata number, length and width in eucalyptus plants submitted to three water deficit cycles. Data are mean ± SD. Different letters represent statistically significant differences (Tukey, at $p < 0.05$; $n = 10$)

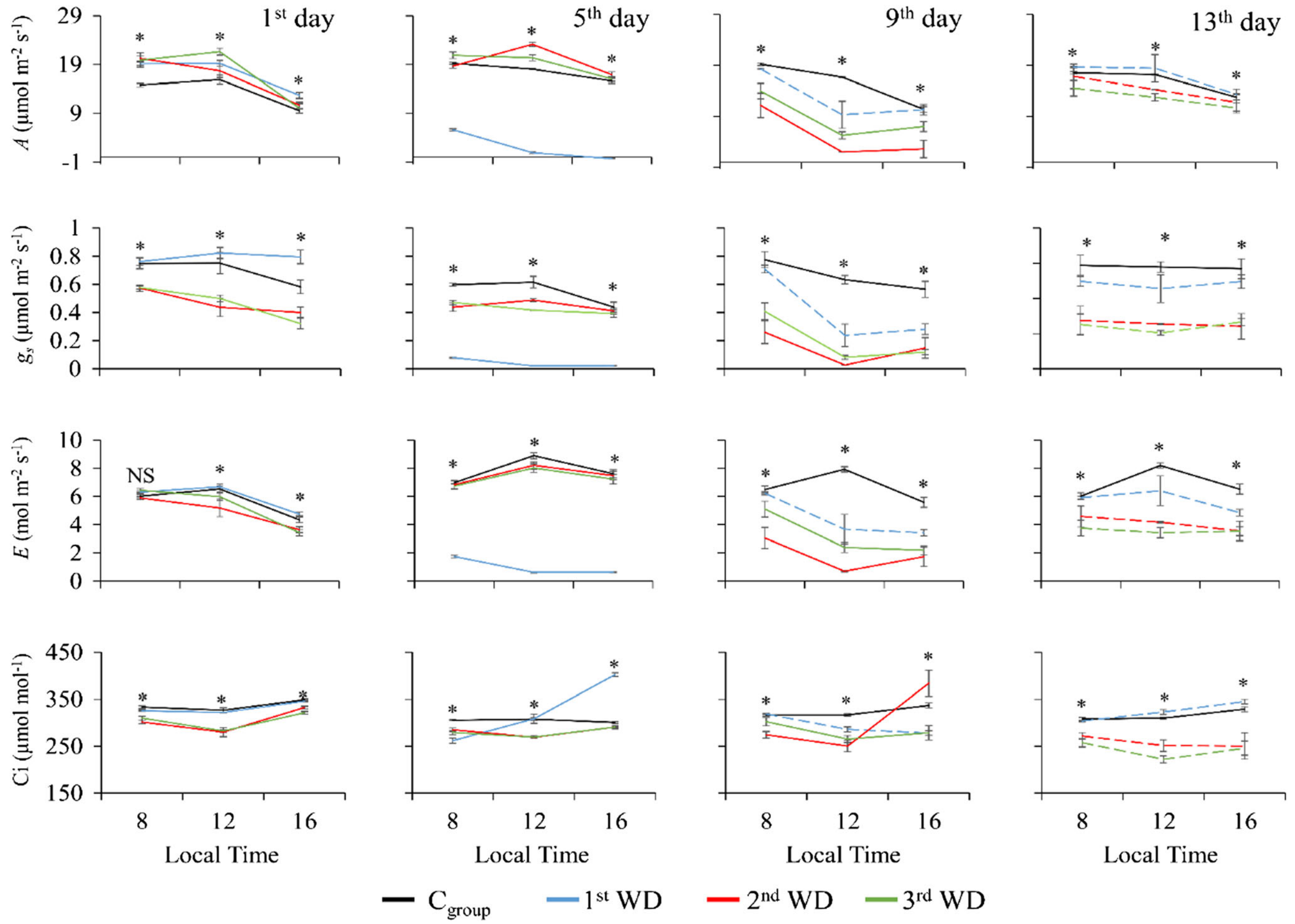


Fig. 4 Diurnal variation of net photosynthesis (*A*), stomatal conductance (g_s), transpiration rates (*E*) and intercellular CO_2 concentration (Ci) for the eucalyptus clone AEC 144, under three water deficit cycles, on the 1st, 5th, 9th, and 13th days of experimentation. Data are mean ± Standard Error. NS: no significance, *: significant (ANOVA, $p < 0.05$). n = 10. Whole lines represent plants under water deficit and dashed lines indicate plants in recovery period

fully recovered their photosynthetic potential. On the other hand, one and 2 days after rewatering (13th day), the 3rd WD and 2nd WD groups, respectively, had *A* and RWC values similar to their respective well-irrigated controls.

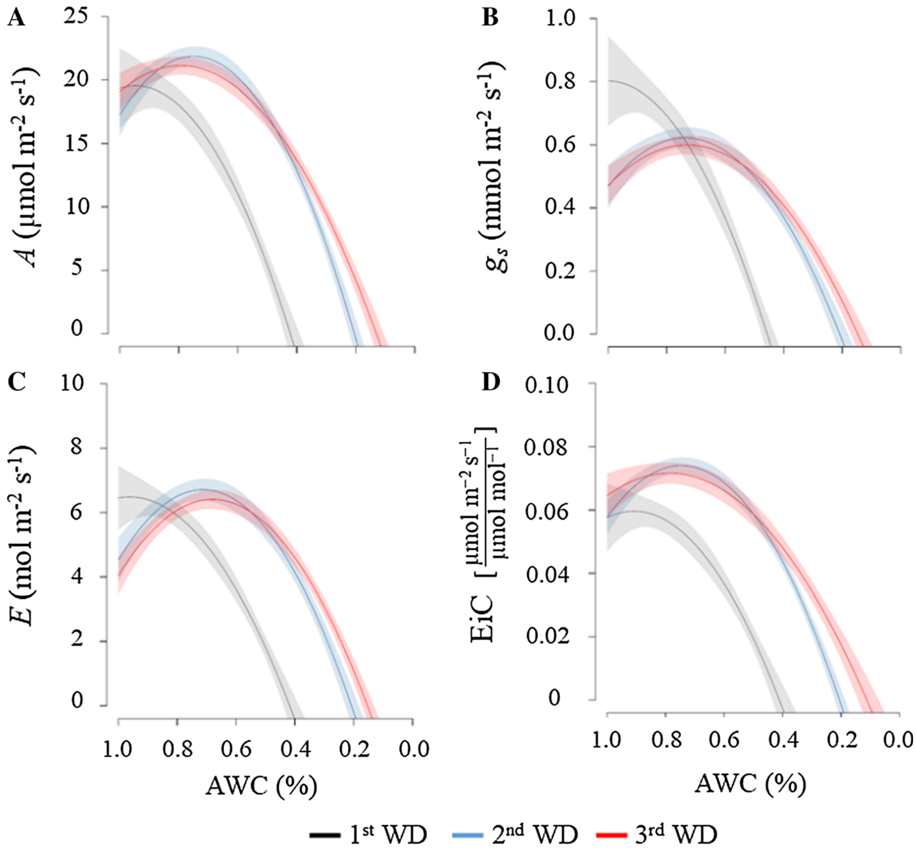
Available water content and its impact on gas exchange

Monitoring of soil moisture verified that seedlings in the 2nd WD and 3rd WD groups could utilize soil moisture even under low water availability. To make this physiological acclimatization more explicit, the relationship between *A*, g_s, *E* and EiC and the available water content (AWC) was determined (Fig. 5). The 1st WD group showed null values of *A*, *gs*, *E* and EiC (0 μmol m^{-2} s^{-1}) when the AWC was 40%. On other hand, plants in the 2nd WD and 3rd WD groups reached the same values when the AWC was 20% and 15%, respectively. Figure 6 clearly shows that acclimatized plants better control *gs* and *E*, which makes the carboxylation process more efficient, so that the plant becomes able to assimilate the same amount of CO_2 while losing less water.

Cyclic water stress impacts on eucalyptus seedlings: a multivariate approach

The multivariate approach allowed an integrated assessment of cyclic water stress on the eucalyptus seedlings (Fig. 6). CDA showed that the first two canonical variables (CV) were responsible in explaining 98.2% of the data variance induced by cyclic water stress. This resulted in a reduction of *A*, *Ci*, g_s and *E*, as well as the size of the stomata and in the shoot/root ratio. Furthermore, it can be seen that the iWUE increased from the 2nd WD plants. The variables *A*, *Ci*, g_s and *E* were more important to distinguish the treatment in the CV1, while the relation shoot/-root and iWUE, respectively, were the most relevant variables in the CV2.

Fig. 5 The relationship between photosynthetic rate (*A*), transpiration rate (*E*), stomatal conductance (g_s) and instantaneous carboxylation efficiency (EiC) with available water content (AWC) for the eucalyptus clone AEC 144 under three water deficit cycles. The polygons correspond to an area of the confidence interval 95%. Statistical analyzes are presented in Table S1 of the supplementary data



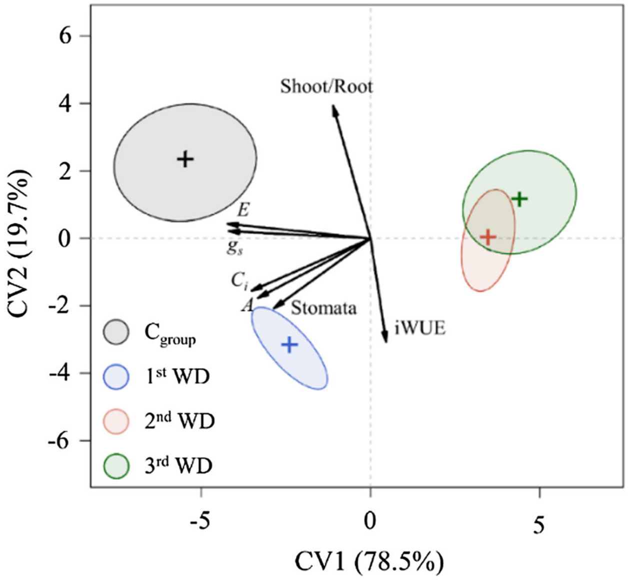


Fig. 6 Biplot of the loadings of the original variables in the first two canonical variables. The percentage of total variance explained by each canonical variable is indicated in parentheses

All of the adaptations by the eucalyptus seedlings contributed to their ability to withstand water deficits. Because of these adaptations, the duration of the third water deficit cycle varied among the treatments. The 1st WD group reached zero photosynthesis 6 days after the beginning of the third water deficit, while the 2nd WD and 3rd WD groups stopped photosynthesizing 11 and 12 days, respectively, after the beginning of the third water deficit, showing their acquired resistance to periods of drought.

Discussion

Eucalyptus seedlings became increasingly adapted to water deficits in each drought period. This was due to progressive morphological and physiological acclimatization that occurred in the plants. They responded to drought by apportioning biomass differentially to different organs, but did not redistribute existing biomass (Reynolds and Thornley 1982). Our results also show that the seedlings responded to water deficits by directing carbohydrates to root and stem growth, which is in agreement with results of Maseda and Fernández (2015). This change in allocation, promoted by water stress, is a known acclimation response for eucalyptus (Costa et al. 2004).

When water becomes limiting, leaf expansion is reduced before photosynthesis is affected. This reduces carbon and energy consumption and a higher proportion of plant assimilates can be allocated to root and stem growth (Taiz and Zeiger 2013). With the best developed roots and stems, the seedlings tended to optimize water absorption and then to maximize the growth rate under these conditions (Bloom et al. 1985). However, a reduction in leaf dry mass can also have a negative impact on carbon gain in eucalyptus (Fig. 1), damaging the growth volume rate which can lead to an increase in production costs.

The adaptation to water deficits not only involved changes in the dry mass proportions contributed by different organs but also in anatomical and morphological changes. In general, the transpiration rate may be considered to be directly proportional to stomatal size and density, and so to better control transpiration rates, the seedlings reduced the size and number of their stomata (Fig. 3), consistent with previous studies by Xu and Zhou (2008) and Mansouri and Radhouane (2015). Plants with less than half the normal number of stomata conserve moisture and are highly tolerant to drought (Hepworth et al. 2015). Another important aspect is stomatal conductance since this plays a key role in gas exchange between vegetation and the atmospheric interface (Xu et al. 2010).

The reduction of g_s or stomatal conductance, which causes a reduction in Ci (intercellular CO_2 concentration), is suggested to be the main limiting factor of photosynthesis under water deficits (Bota et al. 2004; Cano et al. 2014). In this study, after undergoing water deficit cycles, the plants had a lower g_s, and initially, this did not affect *A* (net photosynthesis). Thus, with a lower g_s, the plants reduce their losses of water to the atmosphere, which guarantees their survival for longer periods under a water deficit. With the intensification of the water deficit, there was a decrease in *A*, *E* and RWC and an increase in Ci, (which occurred at different times for each treatment group), which suggests that the photosynthetic machinery was impaired by a water deficit. Some studies have suggested that the deactivation of Rubisco under water stress (Flexas et al. 2006; Cano et al. 2014), and the reduced capacity for ribulose-1,5-bisphosphate (RuBP) regeneration (Thimmanaik et al. 2002) are the main explanation to high Ci values.

Focusing on the relationship between gas exchange and available water content, it may be inferred that exposure to water deficits enables the seedlings to uptake retained water at higher tensions. This capacity may be a function of a change in root water potential (ψ_w). The adjustment of ψ_w, either through the absorption of ions from the soil or the translocation of solutes from other organs to the roots, may be responsible for this change in water uptake ability (Steudle 2000a, b; Merchant et al. 2010), which is also documented for eucalyptus (Christina et al. 2015). However, changes in root morphology, structure and anatomy (Kadam et al. 2015; Maseda and Fernández 2015), changes in the transport pattern or mechanism of water flow (Costa et al. 2004), and changes in the activity of water channels (Steudle 2000b) may also be responsible for changed water uptake capacity of the roots.

The cyclic water deficit also influenced the rewatering period. The *A* (net photosynthesis) and RWC (relative water content) values showed a prompt recovery in all treatments and as the plants were subjected to subsequent water deficits, the recovery period became faster. These results corroborate those obtained by Correia et al. (2014), and show that the ability to recover after a water deficit period increases after passing through previous periods of water deficit. The recovery of *A* was shown to be different to both g_s and *E* (transpiration rates), in agreement with the results of Galmés et al. (2007). Plants take a longer time to recover g_s and *E*; however, they can quickly recover rates of *A*. Galmés et al. (2007) hypothesized that, after a severe water deficit, plants can make better use of intercellular CO_2 (Ci), making the carboxylation process more efficient and thus allowing the plant to maintain high rates of *A*.

Several studies have demonstrated that moderate stomatal closure is an extraordinary adaptation to various environmental stresses in plants (Keenan et al. 2013; Li et al. 2014; Adams et al. 2016). When plants encounter drought stress, stomatal conductance usually decreases earlier than photosynthesis, leading to an increase of instantaneous water use efficiency (iWUE) in plants (Condon et al. 2002; Chaves and Oliveira 2004). Our studies show that, after cyclic water stress, the iWUE in eucalyptus seedlings became higher, even if the plant was not under water deficit (Fig. S1), thus contributing to the plant having water available longer.

The canonical discriminant analysis allows an overview of the importance of each morpho-physiological variable in the treatments differentiation (Fig. 6). After they pass through the first water deficit cycle, the main changes occur in the relationship between shoot and root. Therefore, the first changes are related to the reduction of the transpiration area, which are also found for several others species (Baldocchi and Xu 2007; Peguero-Pina et al. 2014). A positive aspect of the transpiration area reduction is the decrease in aerodynamic resistance of leaves. This leads to a better coupling between leaf temperatures and air temperatures (Peguero-Pina et al. 2016), enhancing the control of transpiration by stomata (Jarvis and McNaughton 1986). From the second water deficit cycle, the plants stomatal conductance controls water loss and increases iWUE. This stomatal response may be mediated by non-hydraulic signals (such as abscisic acid) (Davies and Zhang 1991), leading to the maintenance of a relatively stable leaf water potential (Amdt et al. 2001). Thus, even if the plant is not under water stress, it has better control of the transpiration process, allowing water consumption to decrease, which ensures that the plant has water available longer.

According to White et al. (2009), management practices help to mitigate the consequences of water deficits in planted forests. The results in this study might be a first indication that a good way to mitigate the consequences of water stress in eucalyptus would be to subject the seedlings, prior to planting, to moderate water deficits in order to develop mechanisms to survive subsequent drought stress. However, we believe that more studies on the subject are needed to extrapolate these findings to field conditions where different soil and climatic conditions exist. Besides, our studies indicate that there is a loss of biomass accumulation during water deficit cycles which can cause plants to take longer to reach the ideal harvest volume or to be ready for planting in the case of nurseries, causing an increase in production costs. On the other hand, thinking only of gain tolerance to drought, these results are directly applicable to nurseries responsible for the production of eucalyptus seedlings and that use controlled atmospheric conditions and similar substrates as in this work.

The results of this study support the hypothesis that subjecting eucalyptus seedlings to water deficit changes their ecophysiological aspects and makes them more resistant to subsequent water deficits. These changes allow the plants to survive longer periods of water deficit. Acclimatization by the exposure of eucalyptus to water deficit, during initial growth, can contribute to successful plantation establishment, reduce seedling mortality and production and maintenance costs and thus making forest plantations sustainable and profitable.

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