BÁRBARA ELIAS REIS HODECKER

COMPARISON OF DROUGHT STRESS RESPONSES OF TOLERANT AND SENSITIVE EUCALYPT GENOTYPES

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Solos e Nutrição de Plantas, para obtenção do título de Doctor Scientiae.

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Prof.: Ivo Ribeiro da Silva
(Coorientador)

Prof.: Andrew Merchant

Prof.: Leonardus Vergütz

Prof.: Wagner Luiz Araújo

Prof.: Nairam Félix de Barros
(Orientador)

Aos meus queridos pais, Carla Betânia e Geraldo Tadeu,
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Dedico

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SUMMARY

RESUMO

HODECKER, Bárbara Elias Reis, D.Sc., Universidade Federal de Viçosa, fevereiro de 2015. **Comparações das respostas relacionadas à seca em genótipos tolerantes e sensíveis de eucalipto.** Orientador: Nairam Félix de Barros. Coorientador: Ivo Ribeiro da Silva

Os plantios de eucalipto são importantes componentes dos reflorestamentos no Brasil e as respostas à deficiência hídrica têm sido fortemente estudadas pois essa restrição representa um dos maiores fatores limitantes de produção para esta cultura. Para lidar com a restrição hídrica, seleções de genótipos tolerantes à seca tem sido umas das principais estratégias adotadas nos novos plantios. Entretanto, no Brasil, a maioria das seleções genéticas tem focado principalmente nas taxa de crescimento e produtividade das fibras, sem levar em consideração o melhor entendimento das respostas morfológicas e bioquímicas em resposta ao déficit hídrico e também a influência dos nutrientes nestes processos. Assim, os principais objetivos foram comparar as respostas de clones tolerantes e sensíveis de eucalipto frente ao déficit hídrico a fim de auxiliar no entendimento destas características e futuras seleções genéticas para esta espécie. Outro objetivo visou identificar a importância da fertilização com B (boro) nos mecanismos adaptativos relacionados à tolerância à seca e o melhor entendimento das relações envolvendo a eficiência nutricional em diferentes materiais genéticos e sua influência na seleção de genótipos tolerantes, utilizando para este fim, os diagramas nutricionais. Para atingir estes objetivos, foram conduzidos quatro experimentos, sendo três deles em casa de vegetação e um em condições de campo. O objetivo do primeiro experimento foi avaliar a influência da nutrição com boro em processos relacionados à eficiência do uso da água em seis espécies de eucalipto oriundas de diferentes condições edafoclimáticas e submetidas à seca. O segundo experimento foi destinado à identificação das alterações morfológicas, fisiológicas e moleculares causadas após longo período de restrição hídrica, em quatro espécies de eucalipto em condições de campo. O objetivo do terceiro experimento foi avaliar as variáveis capazes de discriminar clones com tolerância diferencial ao estresse hídrico e fornecer marcadores para plantas jovens de eucalipto. O quarto experimento objetivou avaliar o comportamento diferencial no crescimento inicial e eficiência nutricional e da água em dez clones de eucaliptos submetidos à restrição hídrica. No primeiro capítulo, observamos elevado incremento na eficiência do uso da água em plantas sob seca e suplementadas com B, devido à combinação de alta taxa fotossintética, alta concentração de K^+

em folhas, promovendo maior fechamento estomático, menor perda de água e maior translocação de açúcares para o crescimento radicular. No experimento de campo (capítulo 2), os resultados obtidos sugerem que árvores de eucalipto crescendo sob uniforme e elevada precipitação anual mostraram se mais estressadas após longo período de déficit hídrico, comparado às árvores submetidas à períodos de estresse hídricos recorrentes. No capítulo 3, não foi possível identificar uma variável capaz de discriminar e agrupar clones de eucalipto com tolerância diferencial ao estresse hídrico, no entanto, a interação entre eficiência do uso da água, ABA, fotossíntese, transpiração e razão massa de matéria seca parte área e massa de matéria seca radicular mostraram ser importantes diferenças entre materiais genéticos. De maneira interessante, os resultados obtidos nos capítulo 4, mostram que sob estresse hídrico, clones tolerantes geralmente apresentam maior eficiência de absorção (AE), mas menor eficiência de uso (UE) de nutrientes, enquanto, clones sensíveis tiveram baixa AE, baixa UE para formação de raízes e alta AE para formação de folhas.

ABSTRACT

HODECKER, Bárbara Elias Reis, D.Sc., Universidade Federal de Viçosa, February, 2015. **Comparison of drought stress responses of tolerant and sensitive eucalypt genotypes.** Adviser: Nairam Félix de Barros. Co-adviser: Ivo Ribeiro da Silva

Eucalyptus plantations are an increasing component of tropical landscapes and water stress responses have been extensively studied since seasonal low soil water availability represents a major constraint for successful production. To cope with water restriction, the selection of drought tolerant genotypes has been the main strategy being adopted to establish new plantings. However, in Brazil, most genetic selections have focused mainly on growth rates and fiber productivity, without a clear understanding of morphological and biochemical responses of trees to water stress and the influence of nutrients on these processes. Thus, the main goals of this thesis were to compare water stress responses of tolerant and sensitive Eucalyptus genotypes in order to help to understand of Eucalyptus drought tolerance traits and assist future Eucalyptus breeding programs. Another objective was to identify the nutritional influence of B (boron) on water stress adaptive mechanisms and understand the relationship between nutritional efficiency and its influence on tolerant genotype selections using nutritional diagrams. In order to achieve these proposals, we conducted four experiments, three under controlled conditions and one under field conditions. The objective of the first experiment was to evaluate the influence of B nutrition on physiological processes related to water use efficiency in six Eucalyptus species of contrasting ecotypes under water stress. The second experiment was designed to identify the morphological, physiological and molecular changes caused by long periods of water restriction in four Eucalyptus clones under field conditions. The objective of the third experiment was to identify variables able to discriminate and group clones with differential tolerance to water stress and provide markers for young plants of eucalypt. The fourth experiment aimed to evaluate the differential behavior in the initial growth, biomass accumulation, and nutritional efficiency and water use in 10 Eucalyptus clones submitted to water stress. In the first chapter, we observed a strong increment on instantaneous WUE (water use efficiency) in D+B (drought and B supply) plants, due to the combination of higher photosynthetic rate, higher K^+ concentration in leaves promoting higher stomatal closure, lower water loss and a higher translocation of sugars and B to root growth. In our field experiment (chapter 2), our results suggest that trees growing in the area

with uniform annual high precipitation showed were stressed after a long period of drought, compared to those stands submitted to annual water-stress fluctuation period. In the chapter 3, we could not identify one single variable able to discriminate and group clones with differential tolerance to water stress, however, the interaction between WUEg (water use efficiency), ABA, A (photosynthesis), E (transpiration) and SDM/RDM (shoot dry matter/root dry matter) seemed to be the most important differences between clones under water stress. Our results from chapter 4, interestingly showed us that under water stress the drought-tolerant clone generally had high AE (absorption efficiency), but low nutrient UE (use efficiency), whereas the sensitive clone had low AE, low UE for root formation and high AE for leaf formation.

GENERAL INTRODUCTION

The genus Eucalyptus contains some 960+ species of widely contrasting ecotypes occupying regions with annual rainfall between 1800 and 300 mm in Australia (Brooker, 2000). Currently, eucalypt species have been widely planted for a range of purposes across eastern Asia, Australia, New Zealand, Europe, Africa, and North and South America (Lehto et al., 2010). More specifically, in Brazil, Eucalyptus plantations are an increasing component of tropical landscapes, with approximately 5.1 millions hectares in 2012 (Fig. 1), which represents 70 % of the total tree area plantations, and are the source of almost 5 millions direct and indirect employments (ABRAF, 2013).

Figure 1: Area and distribution of Eucalyptus plantations in Brazil. ABRAF (2013) and Pöyry Silviconsult (2013)

Due to the increasing demand of industrial forest products, the expansion of short-rotation plantations in Brazil has been directed to areas where water and nutrient restrictions are more accentuated (Fig. 2), leading to significant reductions in productivity or high tree mortality rate. Despite these negatives edaphic-climatic characteristics, the low cost of the land and the excellent soil physical and topographic properties make these areas suitable for forest implantation. However, some studies have already demonstrated the high potential of tree mortality due to changes in water availability driven by climate changes as well (Allen et al., 2010).

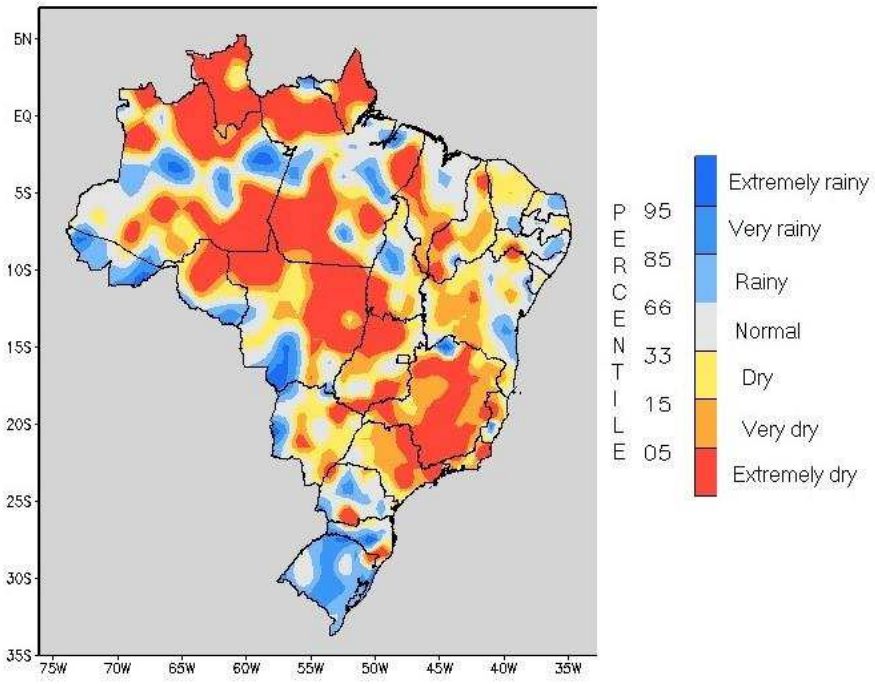

Figure 2: Percentile of precipitation observed in Brazil between January and December, 2014. INMET, 2014: http://www.inmet.gov.br/portal/index.php?r=clima/quantis2

In this way, the responses of Eucalyptus genotypes to drought stress have been intensively studied since soil drought represents a major constraint for successful production (Campion et al., 2006; Stape et al., 2008) and a range of adaptive and acclimation responses

mechanisms in response to water stress have been reported (Metcalfe et al., 1989; Pita and Pardos, 2001; Lemcoff et al., 2002; Guarnaschelli et al., 2003; Costa and Silva et al., 2004; Merchant et al., 2006; Arndt et al., 2008; Mokotedi, 2010 Warren et al., 2011).

However, it is important to emphasize that there is not just one single mechanism that will confer drought tolerance for all genotypes and it can take on several different forms, such as the efficiency of water use by plants taking account the ability to access CO_2 for photosynthesis while avoiding water loss (Condom et al., 2004), ability of the plant to access water determined by the architecture and depth of the root system (Courtois et al., 2013), as also the allocation of photosynthetic products to root growth (Costa and Silva et al., 2004) and to protect against damages, as oxidative stress (Shvaleva et al. 2005). Also, the plant response will vary greatly depending on whether it is subject to water stress for the first time or after several exposures (Vadez et al., 2013) as well as the nutrition status during the drought period.

To cope with water restriction, the selection of drought tolerant genotypes is the main strategy being adopted to establish new plantings (Bison et al., 2007). However, in Brazil, most genetic selections have focused on growth rates and fiber productivity (Navarrete-Campos et al., 2012), without a clear understanding of morphological and biochemical responses of trees to water stress and the influence of nutrients on these processes.

Highly productive genotypes often present high water and nutritional demand and may be more sensitive to drought, leading to economic losses, as observed recently in the State of Minas Gerais, Brazil (Fig. 2), where high mortality of eucalypt plantations has been reported due to a long-term water deficit. Thus, more studies focused on the Eucalyptus drought tolerance traits are required in order to assist future plant breeding programs.

In regarding of these national and abroad concerns, we conducted four experiments that took into some important morphological, nutritional, physiological and molecular mechanisms related to water deficit. In addition, we have adressed some questions related to Eucalyptus water stress responses in different drought-sensitive and tolerant genotypes and under different growth environments, under greenhouse and field conditions. Also, we pursued a better understanding of the influence of nutrition, more specifically of boron (B) fertilization, on these responses, since we recently observed the great importance of this nutrient on water use efficiency and root growth in a drought-tolerant eucalypt genotype (Hodecker et al., 2014).

The main questions and hypothesis addressed in this study are:

1. Has B the same importance to water-stress tolerance of different eucalypt species subjected to drought? (Chapter 1)

2. Has B nutrition important influence on WUE in Eucalyptus species from different ecotypes? (Chapter 1)

3. Are there different tolerance responses among eucalypt clones of high genetic similarity? (Chapter 2)

4. May eucalypt trees growing under seasonal rainfall conditions be more tolerant to the subsequent annual drought stress? (Chapter 2)

5. Are carbon and oxygen isotopic compositions important tools to separate drought sensitive and tolerant genotypes? (Chapter 2)

6. Does the high water use efficiency indicate a eucalypt clone adaptable to drought conditions? (Chapter 2)

7. Does a given physiological, biochemical and hormonal profile indicate a eucalypt clone adaptable to drought conditions? (Chapter 3)

8. Does a given nutritional efficiency profile indicate a eucalypt clone adaptable to drought conditions? (Chapter 4)

9. Is the nutritional efficiency diagram an efficient tool to separate drought tolerant and sensitive genotypes? (Chapter 4)

GENERAL OBJECTIVES

The main goals of this thesis were to compare the water stress responses of tolerant and sensitive Eucalyptus genotypes in order to help the understating of Eucalyptus drought tolerance traits and assist future Eucalyptus breeding programs. Also, the objective was to identify the nutritional influence of B on water stress adaptive mechanisms and to understand the relationship between nutritional efficiency and its influence on tolerant genotype selection.

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CHAPTER 1

BORON APPLICATION INCREASES WATER-USE EFFICIENCY OF EUCALYTPUS SPECIES OF CONTRASTING ECOTYPE

ABSTRACT

Boron (B) is a micronutrient required to maintain the normal growth and development in higher plants. Studies on B deficient-plants have provided several insights into the multifaceted function of boron in plants including the effects of insufficient B supply on important structures and processes governing plant-water status regulation. However, it remains unclear the main functions of boron in these processes and the significance of its influence over plant water use efficiency. In this context, the objective of this study was to evaluate the influence of boron nutrition on physiological processes related to water use efficiency in Eucalyptus species of contrasting ecotypes under water stress. Eucalyptus grandis, E. urophylla, E. melliodora, E. camaldulensis, E. globulus and E. cladocalyx seeds were germinated in standard commercial seed-raising mix and transplanted into 9 L pots. The experiment consisted of a factorial design 5 x 2 x 2 (5 species of Eucalyptus, absence or presence of B and absence or presence of water stress) and the evaluations were initiated 20 days after water stress imposition. The short-term B deficiency applied in our work suggests an important role of B on water use efficiency and an acute interactive response to water availability in Eucalyptus species from contrasting ecotype. We observed a strong increment on instantaneous water use efficiency in water-stressed plants supplemented with B, due to the combination of higher photosynthetic rate, higher K^+ concentration in leaves promoting faster stomatal closure, lower water loss and a higher translocation of sugars and B to root growth. Altogether, these combinations may increase the water use efficiency in B sufficient plants promoting better acclimation under drought by plants mainly during long-term water stress as observed on the field conditions. Our results reinforce the importance of B nutrition of Eucalyptus to cope with periods of water limitation.

Keywords: Boron, Eucalyptus, drought, tolerance, water use efficiency

1. INTRODUCTION

Boron (B) is micronutrient required to maintain the normal growth and development in higher plants (O'Neill et al., 2001) and it is very well known by its primary function on the formation of borate esters with apiose residues in the rhamnogalacturonan II (RG II) pectin polysaccharides (Kobayashi et al., 1999; O'Neill et al. 2004; Bolaños et al., 2004). Studies on B deficient-plants (-B) have provided several insights into the multifaceted function of boron in plants (Loomis and Durst, 1992; Goldbach, 2001; Cara et al., 2002; Bassil et al., 2004; Camacho-Cristóbal et al., 2008, Lu et al., 2014) including the effects of insufficient B supply on important structures and processes governing plant-water status regulation (Baker et al., 1956; Apostol and Zwiazek et al., 2004; Möttönen et al., 2005; Hajiboland and Bastani, 2012; Wimmer and Eichert et al., 2013; Hodecker et al., 2014). However, it remains unclear the main functions of boron in these processes and the significance of its influence over plant water use efficiency.

Regulation of water status is crucial under water-limited conditions. Describing general stress responses, plants can be categorized according to either isohydric or anisohydric responses during a short-term water stress (Tardieu and Simonneau, 1998; Maseda and Fernández, 2006). Isohydry responses are attributed to transpiration regulation by tight stomatal closure (Tardieu and Simonneau, 1998) and, in the opposite way, anisohydric plants have a loose, but not absent, stomatal control of leaf water potential, leading to these species to stave off carbon starvation (Kumagai and Porporato, 2012).

These responses can be controlled by interaction between hydraulic and chemical signals (Comstock, 2002). For B, Wimmer and Eichert (2013) proposed that it may gradually disturb stomatal aperture under short-term B deficiency or promote permanent structural damage of guard cells under severe deficiency therefore having significant influence over stomatal

conductance to water vapour. Additionally, Pollard et al. (1977) showed that activity of the K^+-stimulated ATPase in –B plants was considerably lower than that of the control +B plants. It was suggested that B acts by reducing K leakage or increasing its uptake, thus, resulting in a greater stomatal opening (Roth-Bejerano and Itai, 1981). It is noteworthy that the regulation of stomatal aperture is an important mechanism avoiding water loss in drought affected plants, which also provides an opportunity to increase water-use efficiency (Parry et al., 2005).

Plants can show distinct environmental responses due the combination of different abiotic stress, as water stress and nutritional deficiencies. Camellia sinensis plants grown under the combined effects of reduced B availability and water deficit had a reduction of the net assimilation CO_2 rate (A), increased antioxidant enzymes activity and proline content, but no changes in the stomatal conductance (Hajiboland and Bastani, 2012). Both stomatal and non-stomatal limitations were involved in the reduction of A by 53 % in –B drought stressed Brassica rapa plants (Hajiboland and Farhanghi, 2011). In Picea abies the B level neither directly affect A and/or water relations (Möttönen et al., 2001; 2005). It is evident that B may affect WUE by influencing both stomatal and non-stomatal processes leading to carbon assimilation and stomatal conductance. Hodecker et al. (2014) further suggests that B can contribute to improved water absorption, due to the greater root growth increment in +B plants, as well as improving water use efficiency and reducing dehydration in Eucalyptus urophylla during water stress. Encompassing the multifaceted processes that govern plant gas exchange, it is clear that further investigations into the influence of B on water use and photosynthesis is required to determine the mechanistic basis for these observations.

Water is the major determinant of productivity for genus Eucalyptus (Adams et al., 1996) with a range of adaptive and acclimation responses mechanisms prevalent in the genus (Metcalfe

et al., 1989; Pita and Pardos, 2001; Lemcoff et al., 2002; Guarnaschelli et al., 2003; Costa and Silva et al., 2004; Merchant et al., 2006; Arndt et al., 2008; Mokotedi, 2010 Warren et al., 2011). Eucalypts are widely planted for a range of purposes across eastern Asia, Australia, New Zealand, Europe, Africa, and North and South America (Lehto et al., 2010) where boron deficiency is commonly experienced. The genus Eucalyptus contains some 960+ species of widely contrasting ecotypes occupying regions between 1800 and 300mm of annual rainfall (Brooker, 2000). Speciation within the genus has largely been driven by climatic and edaphic conditions that influence the availability and use of water. Establishing an understanding of the molecular and physiological effects of B deficiency in Eucalyptus will not only lead to improved approaches to nutrient corrections in managed systems, but may also serve to provide a powerful tool for use in improving the resilience of Eucalyptus trees to the effects of water deficit.

The relationship between B and drought and its influence on acclimation responses in plants have been reported in the literature, however, as observed above, the results are still contradictory and it is not clear the role of B supply on isohydric responses influencing the mechanisms that regulate water use efficiency and drought-tolerance responses.

In this context, here we investigated the influence of B nutrition on physiological processes related to water use efficiency in Eucalyptus species of contrasting ecotypes under water stress.

2. MATERIAL AND METHODS

2.1 Plant growth and selection

Eucalyptus grandis (seedlot 16448), E. urophylla (seedlot 13827), E. melliodora (seedlot 17422), E. camaldulensis (seedlot 20437), E. globulus (seedlot 18673) and E. cladocalyx (seedlot 20388) seeds were obtained from the Australian Tree Seed Centre (CSIRO Forestry and Forest Products, Canberra, ACT). Seeds of Eucalyptus sp. were germinated in standard commercial seed-raising mix in November 2013 and watered every day with fresh water in a greenhouse. On February 2014, 48 uniform 3 month-old seedlings were each transplanted into 9 L pots. The pots were filled with sand and covered on the top by a wet mat to avoid loss of water. Care was taken to ensure minimal root disturbance during repotting.

The plants received every other day, during 40 days, 5 mL of a nutritive solution (Table 1). Then, the plants were separated in two groups. One group of plants received the nutrient solution with B and the other one was kept without B in a nutritive solution.

Table 1: Nutrient solution utilized during Eucalyptus seedling growth

Chemical	MW (g/mol)	Each Plant (g) / 5 mL of nutritive solution
$CaCl_2$	167	0.29
KH_2PO_4	136	0.7
K_2SO_4	174	1.05
$MgSO_4$	246	0.9
NH_4NO_3	80	3
$MnSO_4$	223	0.0165
Fe-EDTA	367	0.046
H_3BO_4	78	0.0155
$ZnSO_4$	287	0.00145
$CuSO_4$	250	0.00125
$NaMoO_4$	219	0.000165

2.2 Water deficit treatment

Forty days after splitting the plants into two groups of B nutrition regime, each group (-B and +B) was again divided, and half was kept at normal availability of water and the other half submitted to water stress. Water stress was applied by resupplying a defined fraction of the gravimetrically calculated water lost by control plants. The water stress treatment corresponded to supply of 30 % of water consumption by control (+B) plants.

The experiment consisted of a factorial design 5 x 2 x 2 (5 species of Eucalyptus, absence or presence of B and absence or presence of water deficit). Twenty days after water deficit imposition, plant tissues samples were taken from the youngest fully expanded leaves and root tips, which were initially stored at -20 °C and then at -80 °C for leaf chemical analyses. The remaining plant material was oven-dried to determine the shoot (SDM), root (RDM) and total (TDM) dry matter.

2.3 Photosynthesis and Gas-exchange related measures

Net photosynthesis (A), transpiration rate (E), stomatal conductance (g_s) and intracellular CO_2 concentration (Ci) were quantified using a fully expanded leaf of each plant at 8:00 h, 9:00 h, 10:30 h, 12:00 h, 13:00 h and 15:00 h with a LI-6400 gas exchange system (LI-COR Inc.) 20 days of water stress application. The instantaneous water use efficiency (WUE_i) and intrinsic water use efficiency (WUE_g) were calculated by the ratio between A/E, and A/g_s, respectively.

2.4 Plant height and diameter measurements

Plant height (H) and diameter (D) measurements were also performed after 20 days of treatment application. Height was measured as the distance between the stem base and the shoot tip. Stem diameter was measured 2 cm above the soil surface with an electronic caliper to 0.1 mm precision.

2.5 Phloem sap collection

Phloem sap was collected as an extract of excised phloem tissue conducted following gas exchange measurements. Phloem sap collection was made late in the photoperiod to avoid the effects on δ^{13} C of transitory starch remobilization during the night, as identified by Gessler et al. (2007). Phloem sap was obtained by excising a 1 cm length of phloem (with bark) tissue using a single-sided razor blade cutting the stem to the depth of the cambium. The tissue sample was peeled from the stem, weighed and placed into a 1 mL of deionized water and kept in the dark at room temperature for 2 hours. Phloem tissue was then removed, dried and weighed. The remaining liquid containing the phloem sap extract was then frozen at -80 ºC to be analysed latter.

2.6 Analysis of phloem sap, leaf and roots extracts for soluble carbohydrates

Leaf samples were placed in 2-mL microtubes and in the laboratory were briefly irradiated (30 s) in a standard 650-W microwave oven and then oven-dried overnight at 85 ºC. Samples were ground inside the microtubes using stainless steel ball bearings and a shaker mill (Retch, Germany). Approximately 40 mg of dried leaf material was weighed into a 2-mL screw-cap microtube. Hot water was added and incubated at 75 ºC for 60 min. The water of the extraction solution included internal standards of 0.1 % penta-erythritol. After cooling, samples were centrifuged (11,400 g) and 800 µL of the supernatant removed and placed into a clean 2-mL round-bottomed microtube.

Carbohydrates were separated and quantified by gas chromatography, triple quadrupole mass spectrometer (GC-QQQ). The deionised extracts (60 µL) were dried and re-suspended in 400 µL anhydrous pyridine to which 50 µL of trimethylchlorosilane (TMCS)/ bis-trimethylsilyl-

trifluoroacetamide mix (1:10, Sigma Aldrich, St Louis, MO) was added. Samples were incubated for 1 h at 75 ºC and analyzed by gas chromatography within 12 h.

2.7 Inorganic ion analysis

Solutes (K, Ca, Mg, P, S and B) in leaf and roots extracts were quantified by inductively coupled plasma-optical photoemission spectroscopy (ICP-OE, Varian Inc., Palo Alto, CA) as described by Merchant et al. (2010). In brief, approximately 40 mg of ground dried leaves and roots were extracted into 1 mL of hot (75 ºC) deionized water for 1h. Samples were cooled and then centrifuged at 11,400 g for 2 min. The supernatant was then removed and placed into a 1.5 mL microtube. An aliquot of 800 µL of this extract was then taken and placed into a 15 mL tube and diluted with 15 mL of deionized water ready for analysis.

2.8 Carbon isotope analysis

For the analysis of the carbon isotope composition of the soluble extract in leaves ($\delta^{13}C_{sol}$), 40 mg of ground leaf material was weighed into a 2 mL microtube to which 1 mL of hot, deionized water was added and then incubated for 1 h at 75 ºC. Samples were centrifuged at 11,400 g for 3 min and 800 µL of the supernatant transferred to a 2 mL microtube. Then 150 µL was progressively transferred into tin cups and dried at 60 ºC.

Isotope ratio mass spectrometry (IRMS) was used to determine the ^{13}C:^{12}C ratio in samples. Samples were analyzed on an Isochrom mass spectrometer (Micromass, Manchester, UK) coupled to a Carlo Erba elemental analyser (CE Instruments, Milan). Samples were dropped from an AS200 auto-sampler and combusted by Dumas-combustion in a furnace kept at 1060 ºC. Carbon isotope ratios are expressed in delta-notation, where $\delta^{13}C=R_{sample}/R_{standard}-1$, and R is the ratio of ^{13}C to ^{12}C in a sample and standard (VPDB), respectively. The precision for the standard material was between 0.06 ‰ and 0.11 ‰.

2.9 ^{13}C discrimination modeled ($\Delta_{modeled}$)

Predicted isotope values were calculated using the following equation, originally devised by Farquhar and Richards (1984):

$$\Delta_{modeled} = a_b \frac{C_a - C_s}{C_a} + a \frac{C_s - C_i}{C_a} + (b_s + a_l)\frac{C_i - C_c}{C_a} + b \frac{C_c}{C_a} - f \frac{\Gamma_*}{C_a} - e \frac{R_d}{A+R_d} \frac{C_i - \Gamma_*}{C_a} \quad (1)$$

where a_b is the fractionation caused by boundary layer (2.9 %), a is the fractionation caused by gaseous diffusion (4.4 %), and b is the effective fractionation caused by carboxylating enzymes (Rubisco and PEPC ≈ 29%), bs is the ^{13}C fractionation as CO_2 enters solution (1.1 %) and a_l is the ^{13}C fractionation resulting from diffusion of CO_2 in water (0.7 %; Oleary, 1984). f is the fractionation resulting from photorespiration (≈11 %; Lanigan et al., 2008) and Γ* is the CO_2 partial pressure at which CO_2 assimilation compensates the production of photorespiratory CO_2 calculated according to Brooks and Farquhar (1985) by:

$$_* = 44.7 + 1.88(T \quad 25) + 0.036(T \quad 25)^2 \quad (2)$$

where T is the leaf temperature (ºC). C_a was set at 400 mmol/mol^{-1} and C_i calculated using the LICOR 6400 software (LICOR, 2013).

For calculations of ^{13}C discrimination by plants, the isotopic composition of the source (i.e., the atmosphere in the growth chamber), was assumed to be –7.8 % (see Farquhar et al., 1982). Discrimination (Δ) from air was calculated using the formula:

$$= \left(\quad air \quad plant \right) \Big/ 1 + \quad plant \quad (3)$$

The water use efficiency modeled (WUE_{model}) was calculated using following equation,

$$WUE_{modeled} = \frac{C_a}{1.6}\left(\frac{b}{b\ \ a}\right) \qquad (4)$$

where a is the fractionation caused by gaseous diffusion (4.4 %), b is the effective fractionation caused by carboxylating enzymes (Rubisco and PEPC ≈ 29%).

2.10 Statistics

Effects of water deficit and B treatments were analyzed by analysis of variance using STATISTICA (2011). P values were calculated using Tukey's honestly significant difference post hoc test mean values comparing treatments within each species at 5 % probability.

3. RESULTS

3.1 Gas exchange

Differential responses to B nutrition and water deficit treatments over the photoperiod were observed on net photosynthetic rate (A), stomatal conductance (g_s) and transpiration rate (E) in Eucalyptus species of contrasting ecotype (Fig 1, 2 and 3). The highest values for A were observed at 10:00 AM in E. grandis, E. urophylla and E. melliodora. Control B deficient (C –B) plants had a decrease in A, by approximately 23 %, 20 % and 12 % in E. grandis, E. melliodora and E. globulus, respectively (Fig. 1), compared to well-watered B sufficient plants (C+B). Similarly, g_s and E were higher in both B and water sufficient-plants of all species (Fig. 2) sustaining higher g_s rate and E throughout a longer proportion of the photoperiod.

Plants under drought had different responses to B nutrition (D –B and D +B) compared to the well-watered plants (C +B). All plants reduced photosynthesis and stomatal conductance during the drought-treatment period and the sufficient B supply was extremely important, increasing A (from 40 to 70 % of increment in A) with no changes to g_s and E (Fig. 2 and 3) compared to D-B plants. These behaviors were observed across 4 of the studied species.

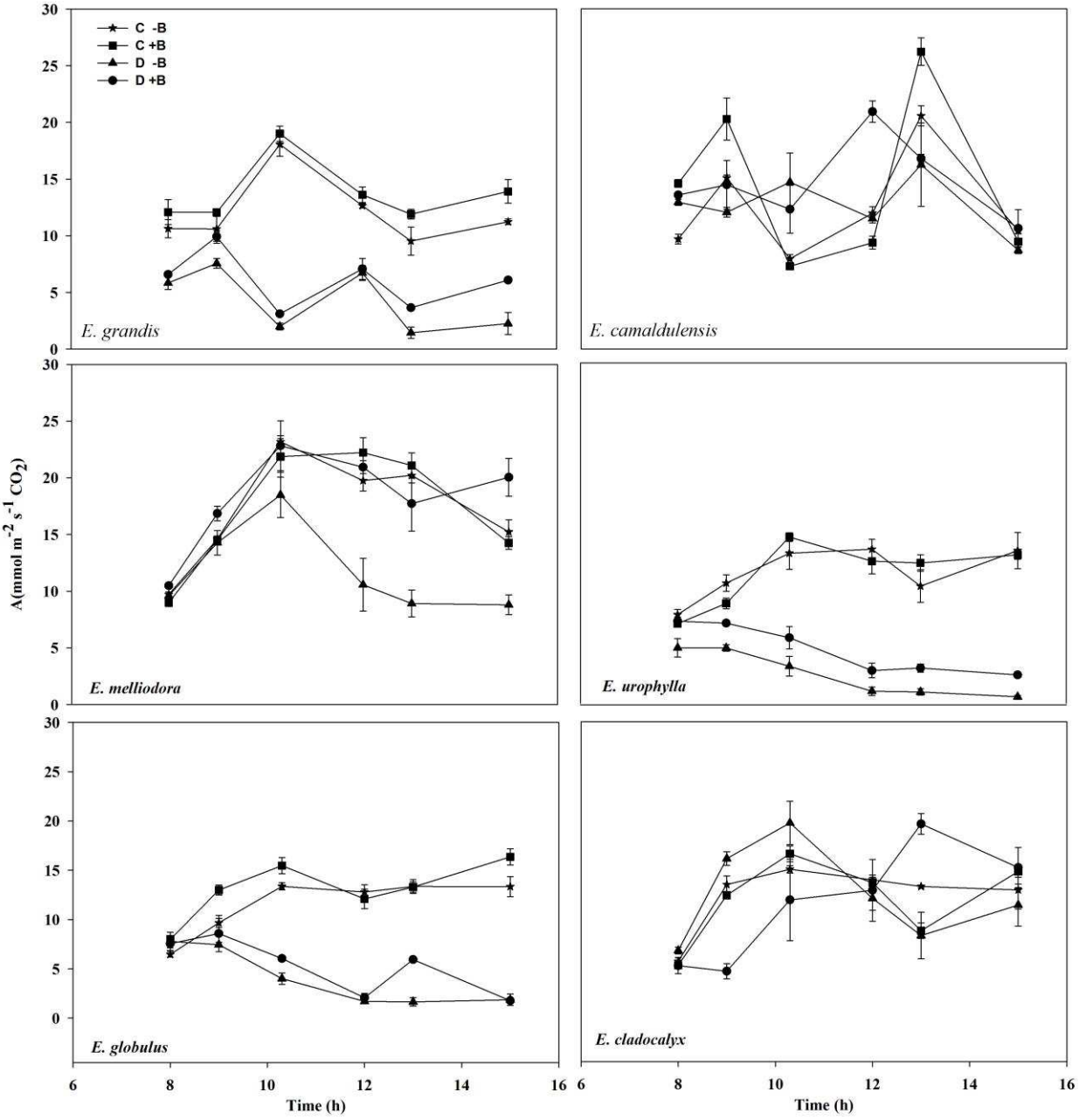

Figure 1: Changes in net photosynthetic rate (A) of six Eucalyptus species during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution.

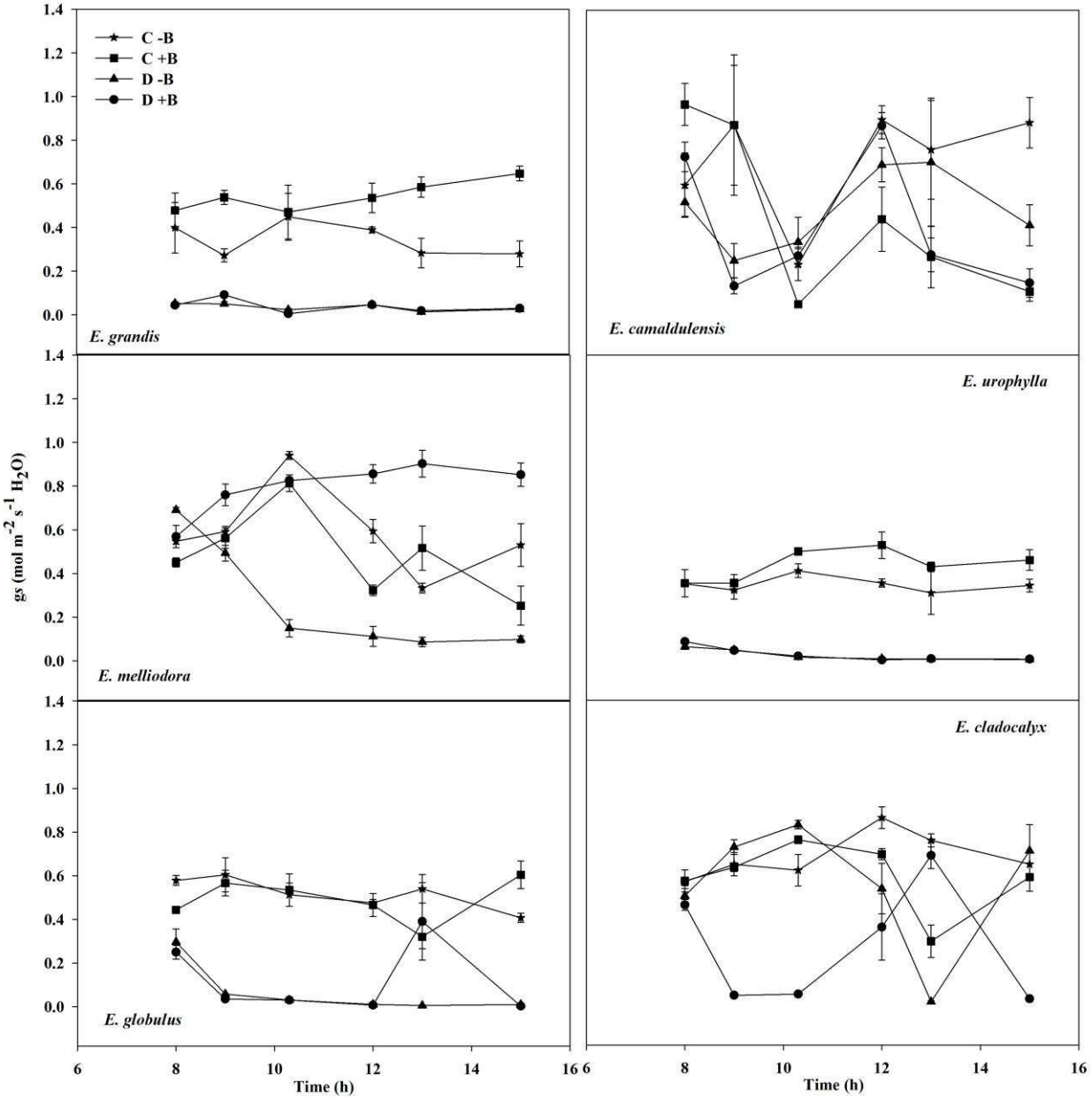

Figure 2: Changes stomatal conductance (g_s) of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution.

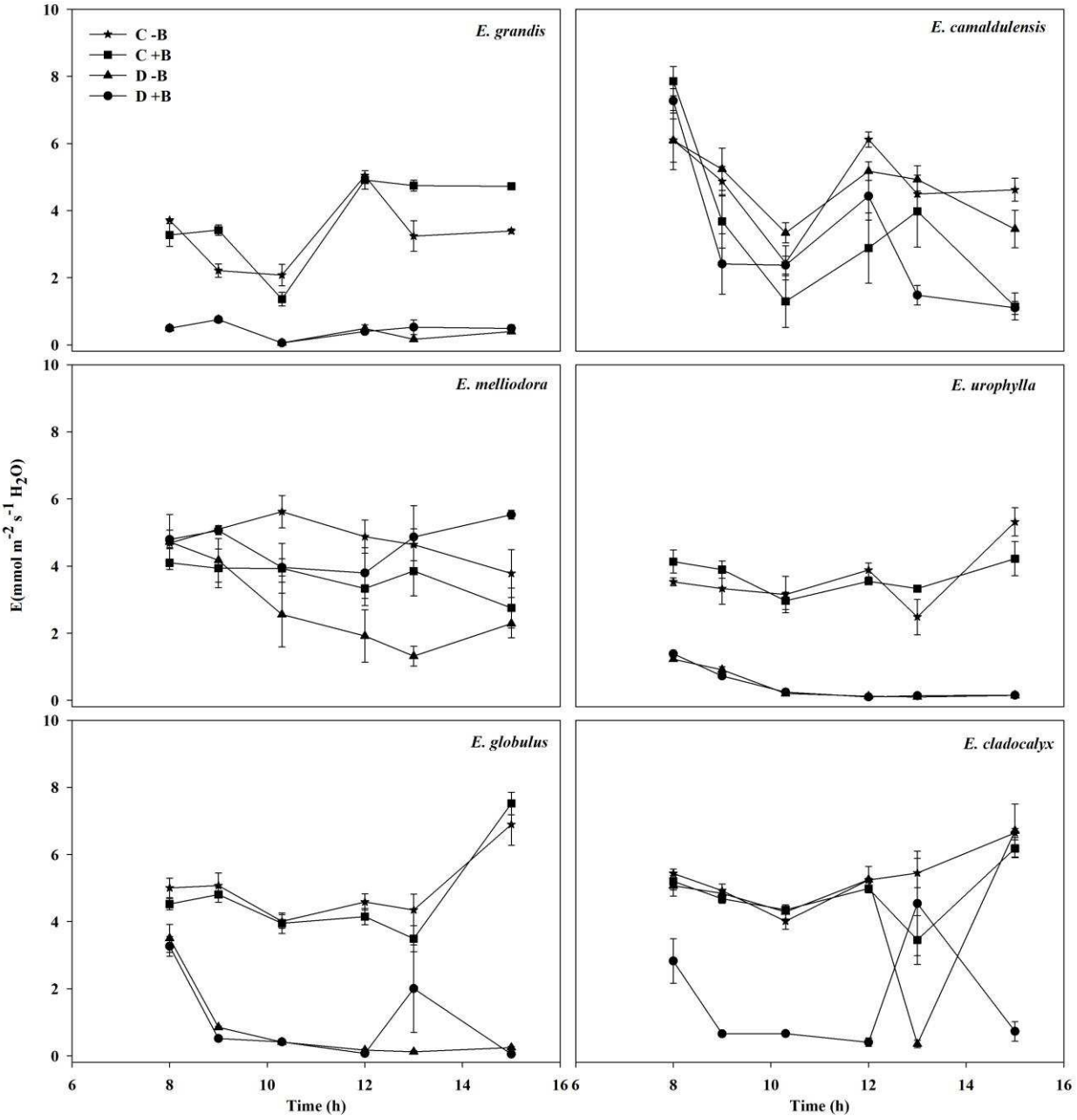

Figure 3: Changes in transpiration rate (E), of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution.

3.2 Plant Growth

Plant height (H), diameter (D), shoot dry matter (SDM), root dry matter (RDM) and total dry matter (TDM) were greatly influenced by water deficit and, or, low B supply, across all species, except for E. melliodora, for which no differences were observed for these growth parameters (Table 1).

SDM in E. grandis and E. camaldulensis was reduced by approximately 60 % and 35 %, in D-B plants and D+B plants, respectively, compared to C+B. B deficiency decreased SDM in E. camaldulensis and E. globulus, up to 50 and 70 %, respectively, under higher water availability (Table 1). After 4 weeks of water restriction, E. grandis root dry matter was 1.6 times higher in D+B plants than D-B plants and no significant difference was observed comparing D+B and C+B plants. Similarly, 50 % of RDM reduction was observed in E. camaldulensis under low B supply and normal water availability (Table 1).

TDM was reduced by approximately 50 % for each species, except for E. melliodora, when subjected to water deficit. However no differences were observed on B deficient and sufficient plants of E. camaldulensis, E. melliodora, E. urophylla and E. cladocalyx. For E. grandis was observed a reduction by 30 % of the TDM in D –B plants compared to D+B plants. Similar response to low B supply was observed in well-watered E. globulus plants.

Table 1: Height (H), diameter (D), shoot dry matter (SDM), root dry matter (RDM) and total dry matter (TDM) of six Eucalyptus species seedlings during 20 days of: control (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. ± represents standard error of the mean (n=4)

	Treatment	E. grandis			E. camaldulensis			E. melliodora			E. urophylla			E. globulus			E. cladocalyx		
H (cm)	C -B	86.67	± 2.32	a	81.33	± 3.17	c	45.25	± 5.68	a	91.00	± 0.71	a	49.75	± 3.79	b	31.75	± 2.02	ab
	C +B	86.67	± 6.56	a	118.33	± 4.87	a	57.75	± 3.97	a	89.67	± 1.31	a	66.00	± 6.87	a	36.00	± 2.16	a
	D -B	48.00	± 1.47	b	94.00	± 7.75	bc	47.33	± 3.06	a	64.75	± 2.46	b	50.25	± 4.50	b	25.50	± 0.87	b
	D +B	56.25	± 1.44	b	105.50	± 5.14	ab	53.75	± 3.84	a	68.00	± 1.63	b	49.25	± 2.95	b	21.00	± 1.47	b
D (mm)	C -B	8.19	± 0.09	a	7.46	± 0.44	b	3.97	± 0.11	a	8.27	± 1.03	b	6.09	± 0.69	a	4.20	± 0.43	ab
	C +B	8.27	± 0.31	a	9.52	± 0.52	a	4.28	± 0.27	a	11.82	± 0.29	a	7.72	± 0.66	a	5.27	± 0.51	a
	D -B	5.55	± 0.24	b	7.03	± 0.26	b	3.89	± 0.57	a	7.68	± 0.19	b	6.12	± 0.16	a	2.90	± 0.80	b
	D +B	5.74	± 0.29	b	7.55	± 0.26	b	4.27	± 0.42	a	8.63	± 0.32	b	6.44	± 0.36	a	3.91	± 0.29	ab
SDM (g/plant)	C -B	33.62	± 0.94	a	9.59	± 2.54	bc	4.02	± 0.66	a	29.12	± 1.69	a	6.17	± 0.70	b c	7.28	± 0.65	ab
	C +B	33.04	± 1.37	a	18.29	± 3.31	a	4.92	± 0.45	a	32.39	± 1.24	a	20.20	± 2.92	a	8.56	± 1.46	a
	D -B	13.72	± 1.99	c	6.70	± 0.22	c	5.10	± 0.78	a	17.67	± 0.87	b	8.46	± 0.41	b	2.58	± 0.27	b
	D +B	19.65	± 0.57	b	12.40	± 1.09	b	5.45	± 0.53	a	15.60	± 0.86	b	9.57	± 0.29	b	3.06	± 0.54	b
RDM (g/plant)	C -B	6.82	± 0.13	b	3.39	± 0.36	b	1.79	± 0.23	a	11.82	± 2.74	ab	2.76	± 0.86	a	2.75	± 0.63	a
	C +B	10.85	± 0.95	a	6.84	± 0.67	a	1.84	± 0.21	a	10.00	± 0.75	a	4.19	± 0.98	a	2.89	± 0.71	a
	D -B	7.31	± 0.55	b	3.35	± 0.62	b	2.95	± 0.85	a	7.90	± 0.18	b	4.13	± 0.50	a	1.09	± 0.43	a
	D +B	12.16	± 1.58	a	5.75	± 0.49	b	1.54	± 0.17	a	8.11	± 0.17	b	5.47	± 0.93	a	1.56	± 0.38	a
TDM (g/plant)	C -B	40.44	± 1.06	a	12.97	± 2.88	a	5.81	± 0.88	a	40.94	± 1.01	a	8.92	± 2.54	b	10.03	± 1.01	a
	C +B	43.90	± 1.61	a	25.12	± 3.98	a	6.76	± 0.48	a	42.39	± 3.27	a	24.39	± 1.98	a	10.73	± 2.06	a
	D -B	21.02	± 2.26	c	10.05	± 0.84	b	8.05	± 1.58	a	25.57	± 0.85	b	12.59	± 0.76	b	3.67	± 0.69	b
	D +B	31.82	± 1.41	b	18.15	± 1.39	ab	6.98	± 0.38	a	23.72	± 1.16	b	15.04	± 0.81	b	4.62	± 0.92	b

3.3 Tissue soluble B concentration

Tissue soluble B concentration, as expected, was also reduced at absence B supply, more in young leaves (up to 96 %) than in the roots (up to 85 %) (Fig. 4 and 5). Eucalyptus grandis, E. urophylla and E. globulus had the highest B concentration in leaves of D+B plants and E. grandis, E. urophylla, E. melliodora and E. cladocalyx in the roots of D +B plants.

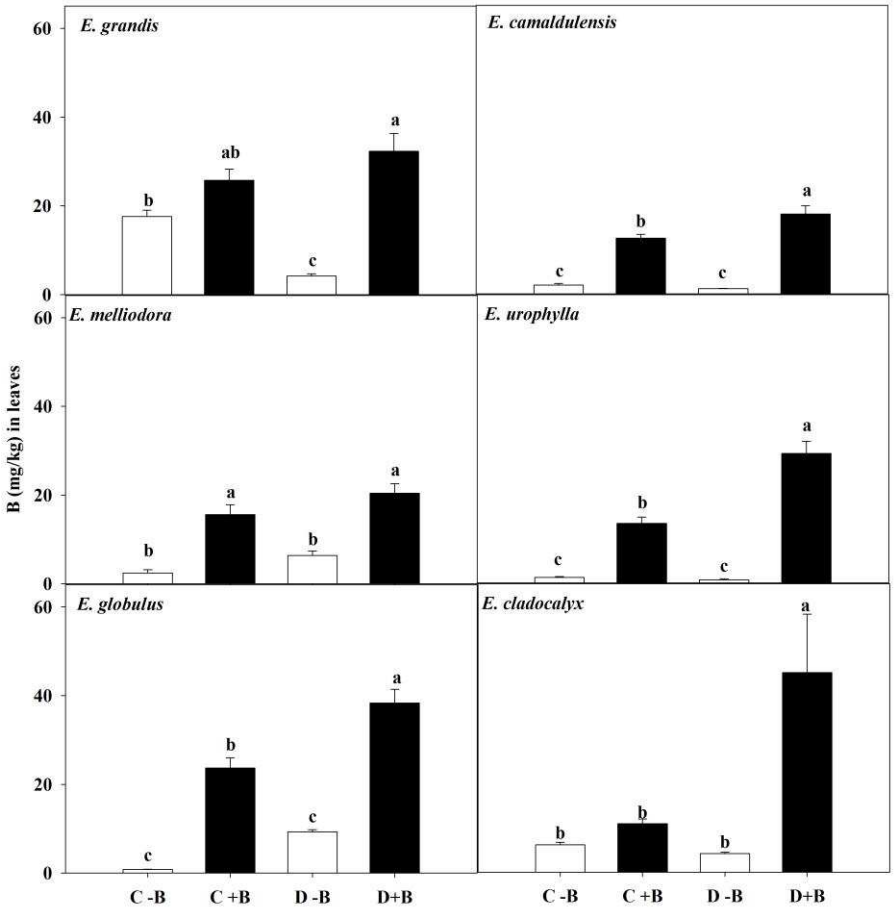

Figure 4: Soluble boron concentration in leaves (B) of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

Higher concentration of B in young roots, in B sufficient plants subjected to water stress (D +B), was observed for all species (Fig. 5).

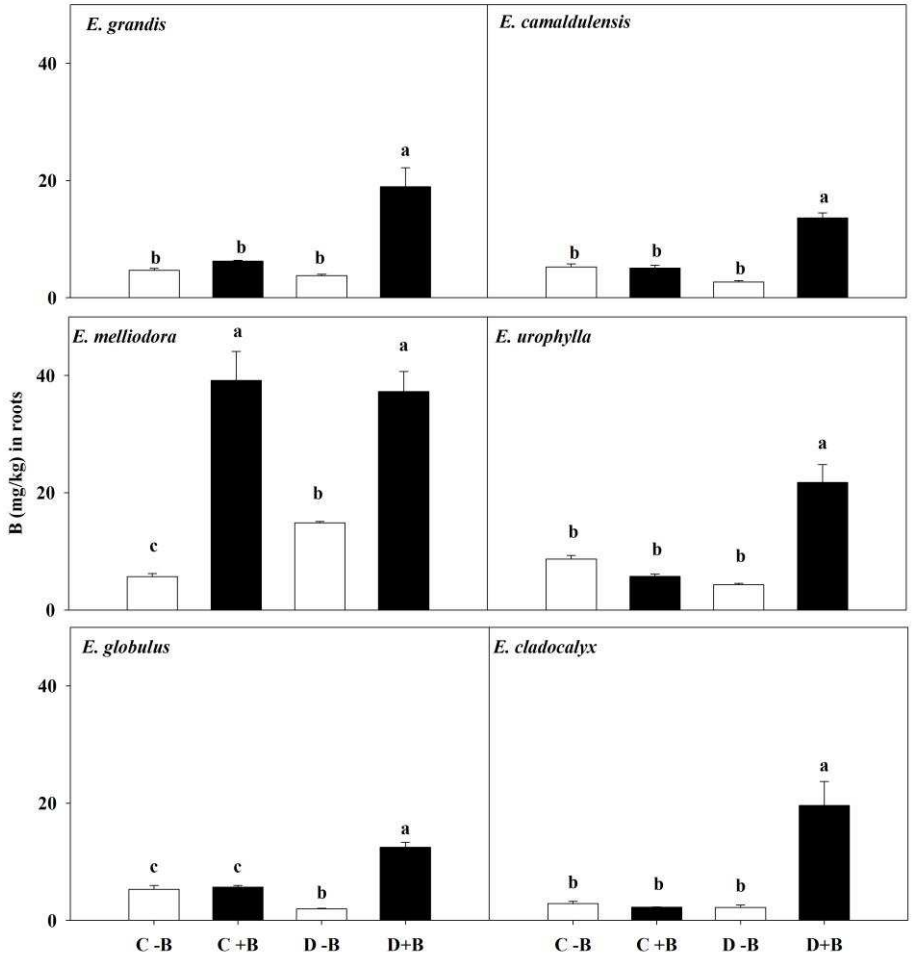

Figure 5: Soluble boron concentration in roots (B) in six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

3.4 Leaf and root nutrient content

The absence of B in the nutrient solution significantly reduced leaf Ca, P and S concentration in all Eucalyptus species, except for E. urophylla (Table 2). Whilst, D+B plants had the largest increment of leaf K concentration in E. globulus, E. grandis, E. camaldulensis seedlings, 20, 26.5, 34 %, respectively, as compared to the D –B plants, and 45, 27 and 30 % higher, respectively, than C+B plants (Table 2).

Overall, major root solutes did not differ between treatments and species.

Table 2: Major leaves nutrients of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. ± represents standard error (n=4)

Nutrients	Treatment	*E. grandis*	*E. camaldulensis*	*E. melliodora*	*E. urophylla*	*E. globulus*	*E. cladocalyx*
K (g/kg)	C -B	4.1 ± 0.2 c	7.111 ± 0.83 b	9.75 ± 0.8 b	5.45 ± 0.2 bc	5.38 ± 0.3 c	5.22 ± 0.3 b
	C +B	5.96 ± 0.4 b	7.828 ± 0.35 b	9.55 ± 0.3 b	4.96 ± 0.2 c	5.24 ± 0.1 c	5.31 ± 0.7 b
	D -B	6.01 ± 0.6 b	7.965 ± 0.24 b	12 ± 0.2 a	6.53 ± 0.3 ab	7.5 ± 0.3 b	9.67 ± 0.2 a
	D +B	8.17 ± 0.5 a	11.19 ± 0.28 a	10.9 ± 0.3 ab	6.94 ± 0.2 a	9.45 ± 0.2 a	11.3 ± 0.6 a
Ca (g/kg)	C -B	0.27 ± 0.02 b	0.211 ± 0.01 ab	0.72 ± 0.09 a	0.49 ± 0.07 ab	0.35 ± 0.05 a	0.32 ± 0.01 b
	C +B	0.53 ± 0.09 a	0.307 ± 0.01 a	0.58 ± 0.06 ab	0.66 ± 0.06 a	0.38 ± 0.05 a	0.21 ± 0.03 b
	D -B	0.32 ± 0.04 b	0.224 ± 0.04 ac	0.44 ± 0.04 ab	0.3 ± 0.05 b	0.29 ± 0.02 a	0.28 ± 0.04 b
	D +B	0.44 ± 0.03 ab	0.177 ± 0.01 b	0.37 ± 0.02 b	0.64 ± 0.04 a	0.38 ± 0.04 a	0.48 ± 0.03 a
Mg (g/Kg)	C -B	0.5 ± 0.04 b	0.551 ± 0.06 ab	1.01 ± 0.1 a	1.19 ± 0.17 a	0.99 ± 0 a	0.52 ± 0 a
	C +B	1.02 ± 0.10 a	0.593 ± 0.07 ab	0.97 ± 0 a	0.89 ± 0.08 a	1.04 ± 0.1 a	0.4 ± 0 a
	D -B	0.8 ± 0.02 ab	0.392 ± 0.03 b	0.97 ± 0.1 a	0.67 ± 0.09 a	0.91 ± 0.1 a	0.46 ± 0 a
	D +B	0.99 ± 0.08 a	0.855 ± 0.04 a	0.84 ± 0.1 a	1.11 ± 0.08 a	1.18 ± 0.1 a	0.57 ± 0 a
P (g/kg)	C -B	0.4 ± 0.03 c	0.589 ± 0.05 b	1.08 ± 0 bc	0.71 ± 0.08 a	0.53 ± 0 b	0.66 ± 0 b
	C +B	0.66 ± 0.03 b	0.777 ± 0.04 ab	0.86 ± 0 c	0.55 ± 0.01 a	0.47 ± 0.1 b	1.43 ± 0.3 ab
	D -B	0.43 ± 0.04 c	0.947 ± 0.13 a	1.39 ± 0 a	0.55 ± 0.02 a	0.8 ± 0 a	1.84 ± 0.2 a
	D +B	1.41 ± 0.05 a	0.953 ± 0.03 a	1.22 ± 0.1 ab	0.63 ± 0.01 a	0.9 ± 0.1 a	1.81 ± 0.1 a
S (g/kg)	C -B	0.21 ± 0.02 b	0.371 ± 0.03 b	0.36 ± 0 b	0.31 ± 0.03 b	0.44 ± 0.1 b	0.45 ± 0 c
	C +B	0.48 ± 0.08 ab	0.704 ± 0.03 a	0.39 ± 0.1 ab	0.54 ± 0.08 b	0.44 ± 0 b	0.53 ± 0.1 bc
	D -B	0.51 ± 0.04 ab	0.24 ± 0.01 b	0.41 ± 0 ab	0.43 ± 0.03 b	0.24 ± 0 b	0.82 ± 0.1 ab
	D +B	0.8 ± 0.14 a	0.722 ± 0.07 a	0.56 ± 0 a	1.01 ± 0.07 a	1.13 ± 0.1 a	0.95 ± 0.1 a

Table 3: Major roots nutrients of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. ± represents standard error (n=4)

Nutrients	Treatment	*E. grandis*	*E. cam*	*E. melliodora*	*E. urophylla*	*E. globulus*	*E. cladocalyx*
K (g/kg)	C -B	16 ± 0.4 a	11 ± 1 b	17 ± 1.3 a	9.5 ± 0.6 b	9.1 ± 0.1 b	16 ± 1.3 a
	C +B	15 ± 0.9 a	7.5 ± 0.3 b	22 ± 0.9 a	17 ± 1 a	13 ± 1.1 a	13 ± 1.9 a
	D -B	9.7 ± 0.2 b	20 ± 0.2 a	21 ± 2.5 a	5.1 ± 0.4 b	9 ± 0.9 b	24 ± 3.9 a
	D +B	10 ± 0.9 b	22 ± 3.8 a	24 ± 3.7 a	15 ± 1.7 a	10 ± 0.8 ab	25 ± 3.7 a
Ca (g/Kg)	C -B	0.32 ± 0.02 b	0.37 ± 0.02 a	0.50 ± 0.06 b	0.45 ± 0.03 a	0.54 ± 0.06 a	0.75 ± 0.05 a
	C +B	0.42 ± 0.05 b	0.29 ± 0.03 a	0.53 ± 0.06 ab	0.68 ± 0.06 a	0.47 ± 0.03 ab	0.63 ± 0.07 a
	D -B	0.63 ± 0.05 a	0.49 ± 0.05 a	0.59 ± 0.10 ab	1.58 ± 0.01 a	0.31 ± 0.02 b	0.69 ± 0.03 a
	D +B	0.45 ± 0.01 ab	0.45 ± 0.06 a	0.93 ± 0.12 a	0.88 ± 0.11 a	0.57 ± 0.06 a	0.70 ± 0.04 a
Mg (g/kg)	C -B	1.24 ± 0.16 b	1.07 ± 0.19 a	0.85 ± 0.04 b	1.06 ± 0.10 a	1.06 ± 0.07 a	1.30 ± 0.11 a
	C +B	2.10 ± 0.39 b	0.72 ± 0.08 a	1.01 ± 0.07 b	1.50 ± 0.08 a	1.47 ± 0.28 a	1.55 ± 0.04 a
	D -B	1.42 ± 0.09 a	0.66 ± 0.06 a	1.19 ± 0.14 b	0.99 ± 0.05 a	0.89 ± 0.08 a	1.43 ± 0.11 a
	D +B	1.92 ± 0.14 ab	0.80 ± 0.16 a	1.82 ± 0.13 a	1.91 ± 0.28 a	1.28 ± 0.17 a	1.38 ± 0.13 a
P (kg/g)	C -B	0.74 ± 0.04 a	1.51 ± 0.14 a	1.44 ± 0.11 a	1.07 ± 0.26 a	0.51 ± 0.03 b	2.24 ± 0.21 a
	C +B	0.71 ± 0.05 a	0.84 ± 0.06 a	0.80 ± 0.05 b	0.72 ± 0.14 a	0.85 ± 0.10 ab	2.47 ± 0.09 a
	D -B	0.76 ± 0.14 a	1.14 ± 0.19 a	1.18 ± 0.04 ab	0.62 ± 0.23 a	0.57 ± 0.10 b	1.39 ± 0.12 b
	D +B	0.78 ± 0.18 a	1.47 ± 0.08 a	1.59 ± 0.12 a	1.06 ± 0.05 a	0.98 ± 0.05 a	1.87 ± 0.07 ab
S (g/kg)	C -B	1.90 ± 0.28 a	2.32 ± 0.20 a	2.50 ± 0.32 b	2.20 ± 0.26 a	1.38 ± 0.10 b	1.05 ± 0.26 b
	C +B	1.88 ± 0.55 a	1.92 ± 0.14 a	2.09 ± 0.20 b	3.32 ± 0.12 a	1.80 ± 0.31 ab	1.20 ± 0.12 b
	D -B	2.33 ± 0.04 a	3.04 ± 0.15 a	3.73 ± 0.57 ab	1.80 ± 0.11 a	2.41 ± 0.20 ab	2.85 ± 0.31 ab
	D +B	2.76 ± 0.28 a	2.30 ± 0.20 a	4.61 ± 0.32 a	3.12 ± 0.26 a	2.69 ± 0.10 a	2.85 ± 0.26 a

3.5 Sugars concentration

Eucalyptus grandis, E. globulus and E. urophylla leaves under normal water supply and –B treatment showed higher contents of glucose and fructose than the leaves of + B plants (Fig. 6 and 7). The D-B treatments in E. grandis and E. cladocalyx plants had the opposite effect as to the glucose concentration. While the first species had a decrease on this sugar content, the second one had an increment of approximately 45 %. B fertilization and water deficit had no effect on the myo-Inositol concentration in leaves, except for E. globulus (Fig. 8).

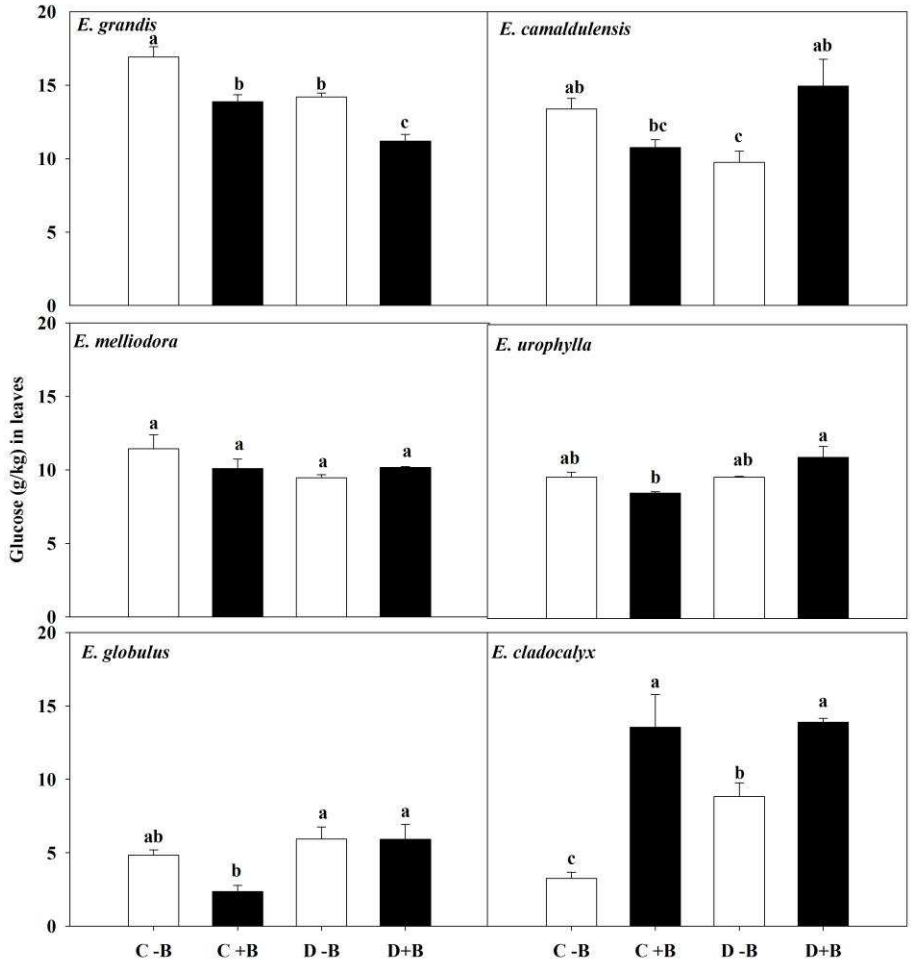

Figure 6: Glucose concentration in leaves of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

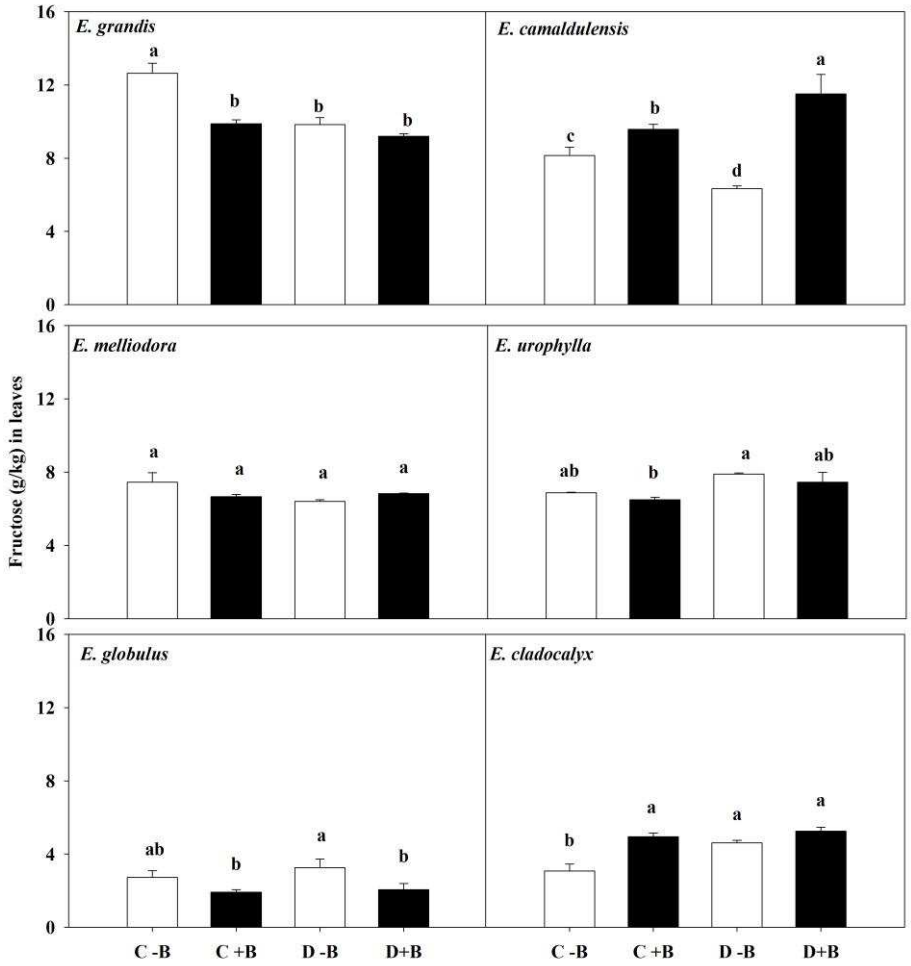

Figure 7: Fructose concentration in leaves of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

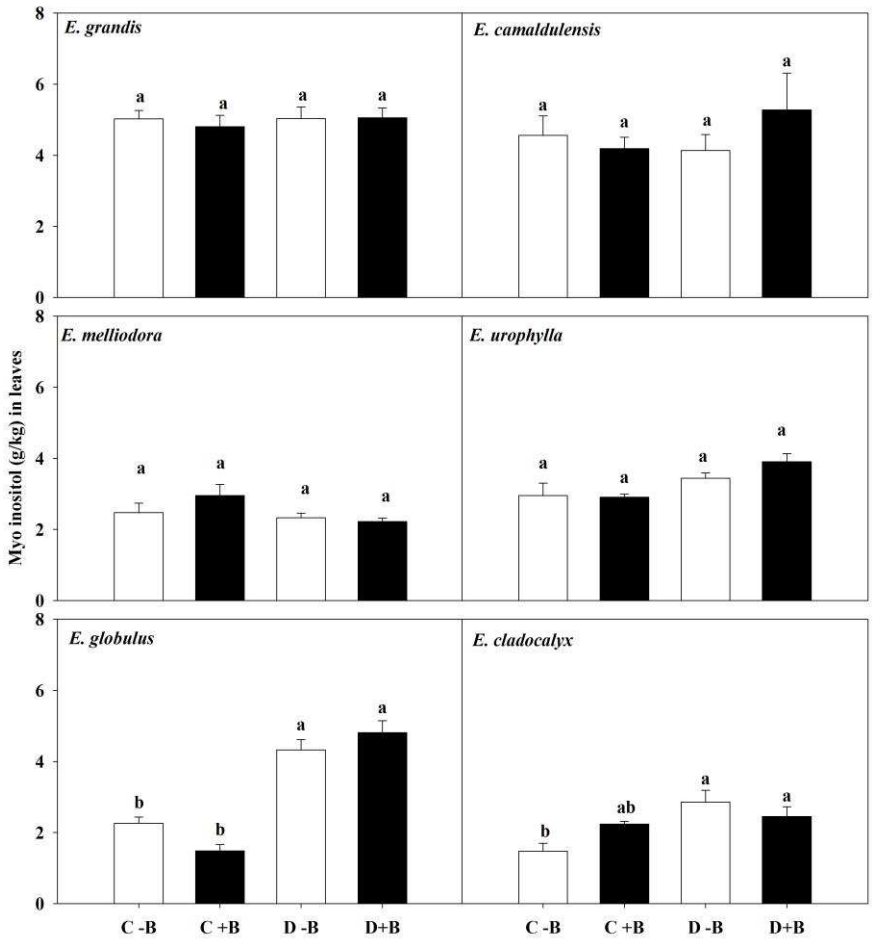

Figure 8: Myo-Inositol concentration in leaves of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

Contrasting to the responses observed in leaves, B application increased the root glucose concentration in E. grandis, E. camaldulensis, E. urophylla and E. globulus (Fig. 9). Similar effects were observed to fructose and myo-Inositol concentrations in roots (Fig. 10 and 11).

Both species-specific increases and decreases were observed in phloem sugars and sugar alcohol concentrations (Fig. 12, 13 and 14) although no discernable pattern emerged among the species.

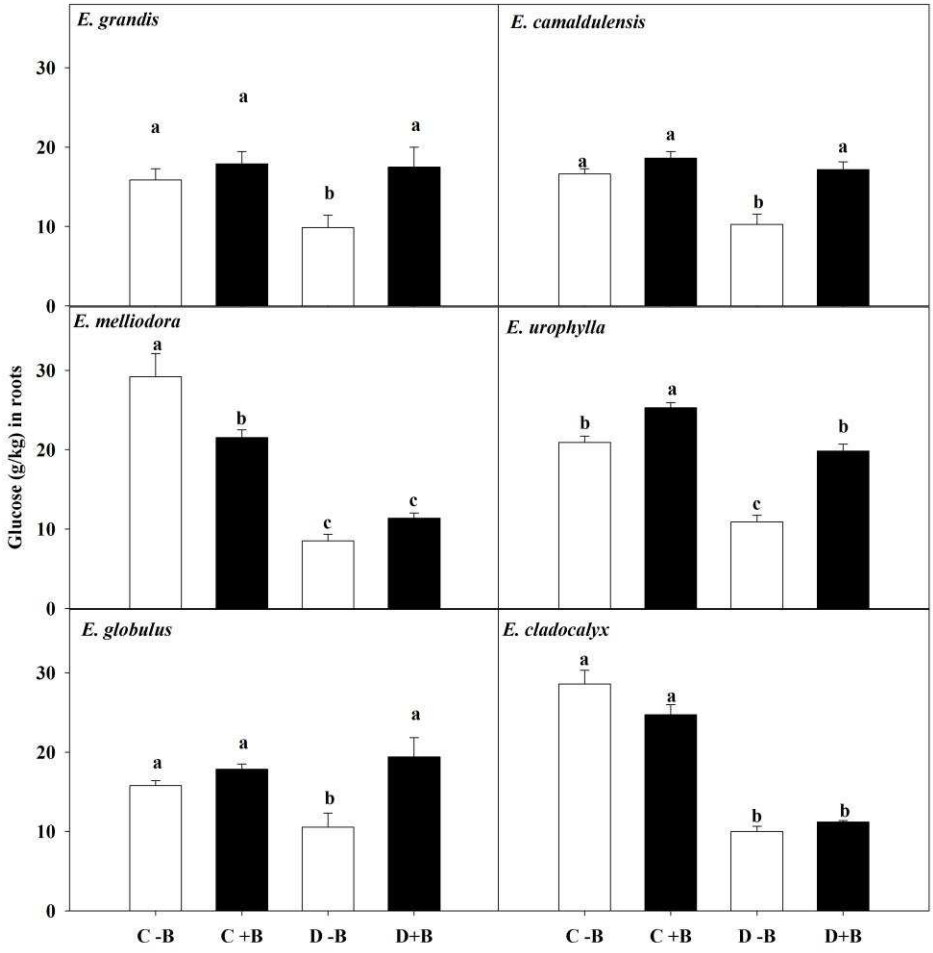

Figure 9: Glucose concentration in roots of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

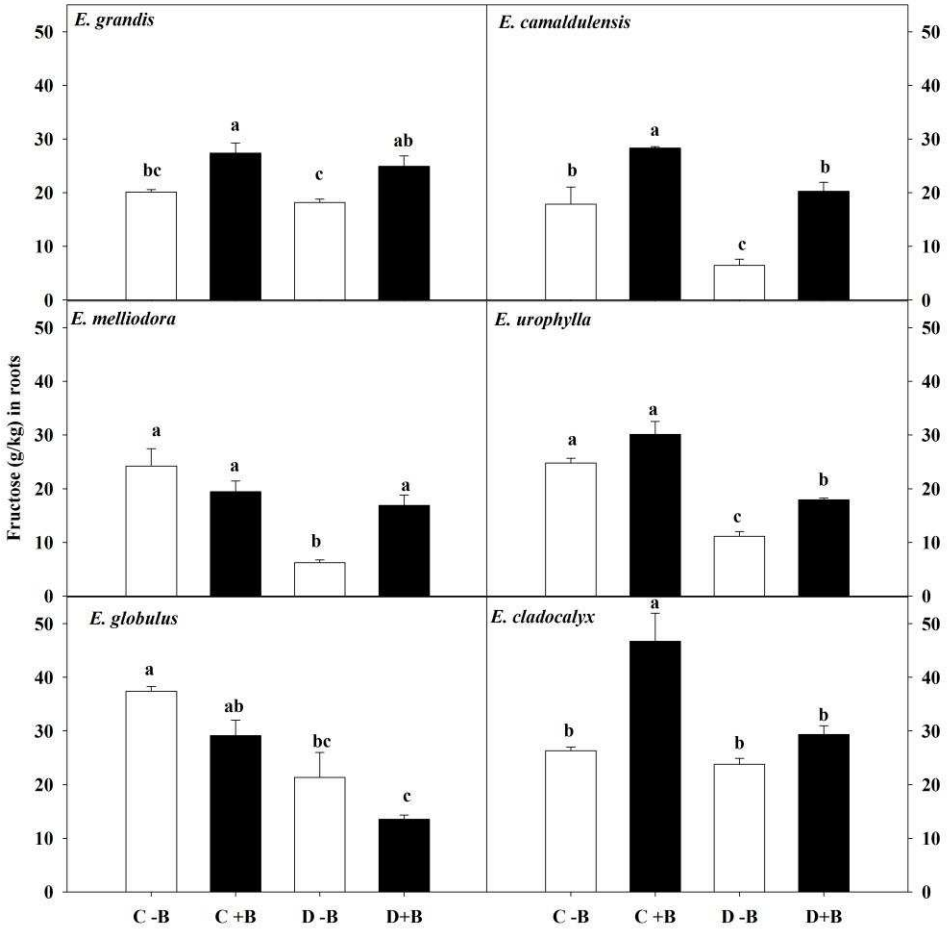

Figure 10: Fructose concentration in roots of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

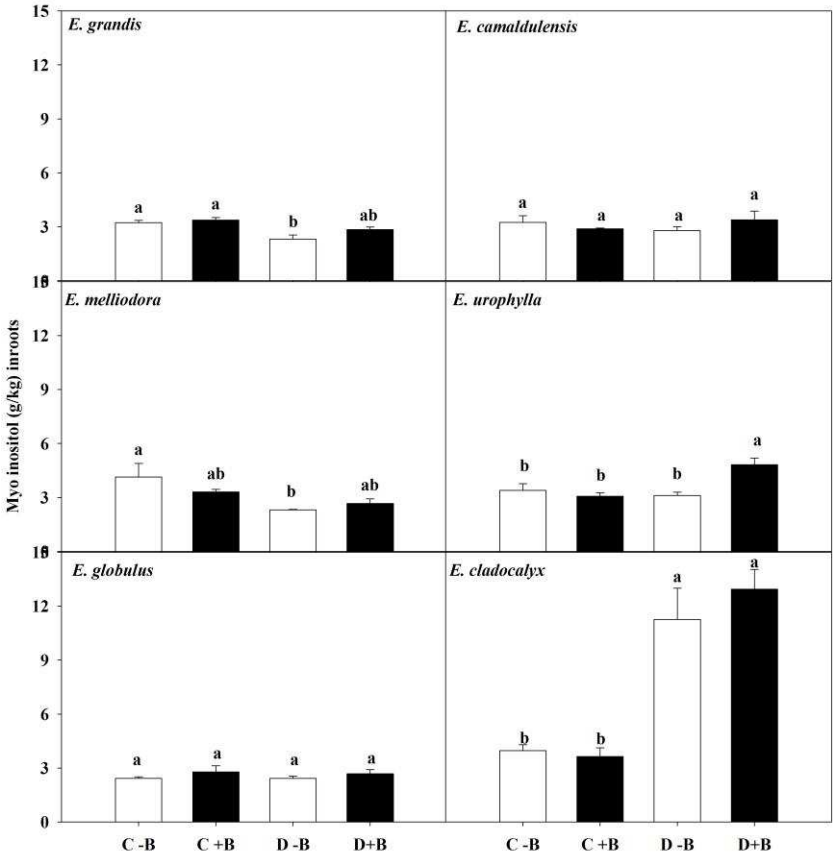

Figure 11: Myo-Inositol concentration in roots of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

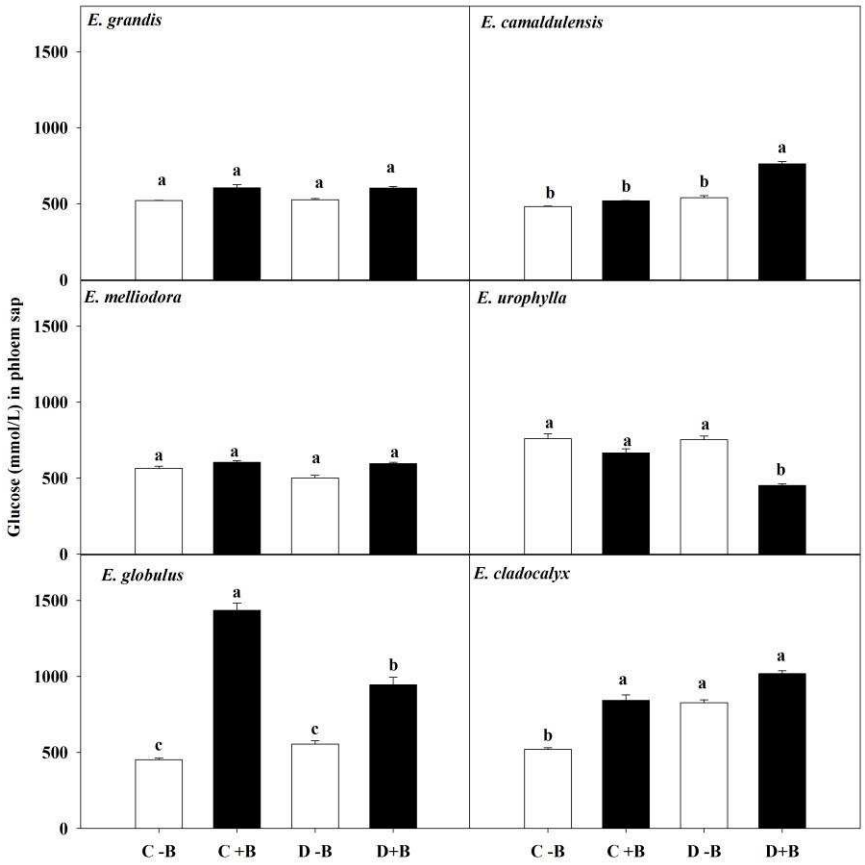

Figure 12: Glucose concentration in phloem sap of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

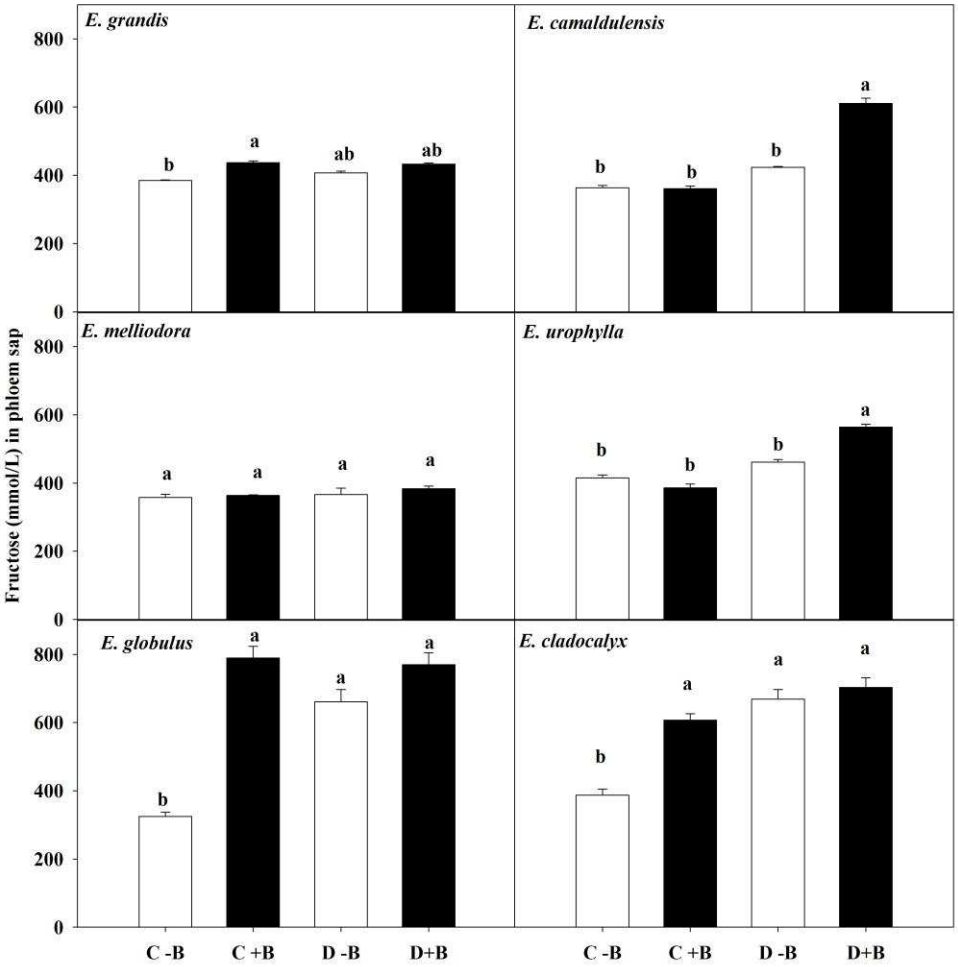

Figure 13: Fructose concentration in phloem sap of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

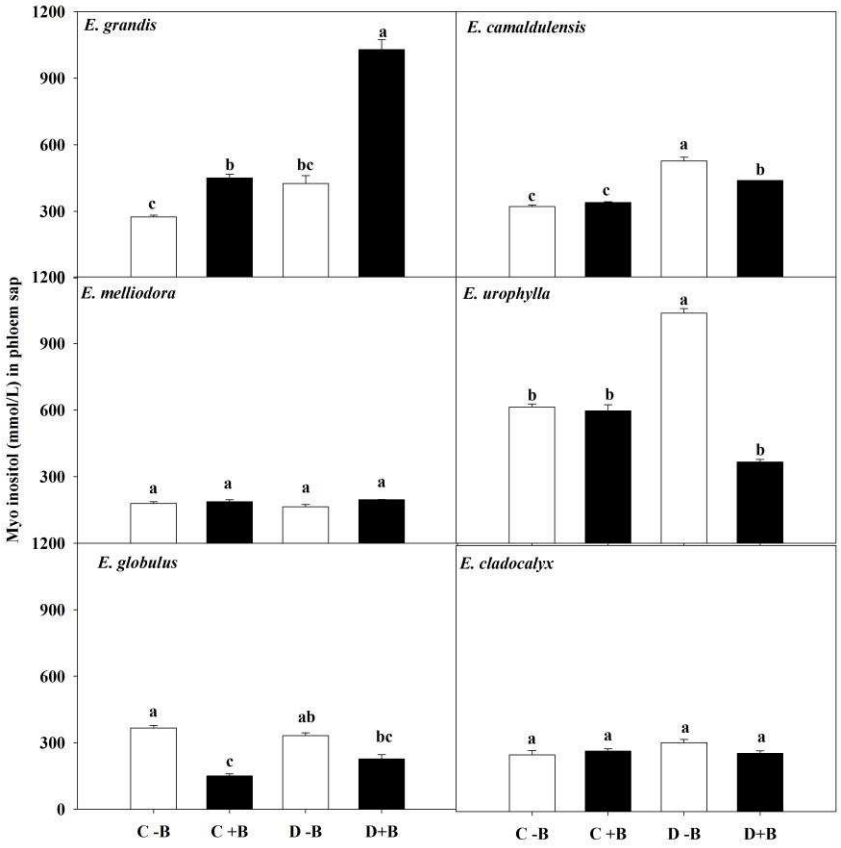

Figure 14: Myo-Inositol concentration in phloem sap of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

3.6 δ ^{13}C and instantaneous water use efficiency (WUE_i)

As expected, water restriction increased the enrichment of $^{13}CO_2$ in the leaves major carbon pool (Fig. 15). The δ ^{13}C values of well-watered plants were close to −30 ‰ and those under stress discriminated less against the abundance of ^{13}C ($\delta^{13}C$ ranged between ~ -25 and -27‰). After 20 days of water deficit, in E. camaldulensis and E. globulus, the $\delta^{13}C$ was higher in plants exposed to water stress and supplemented with B.

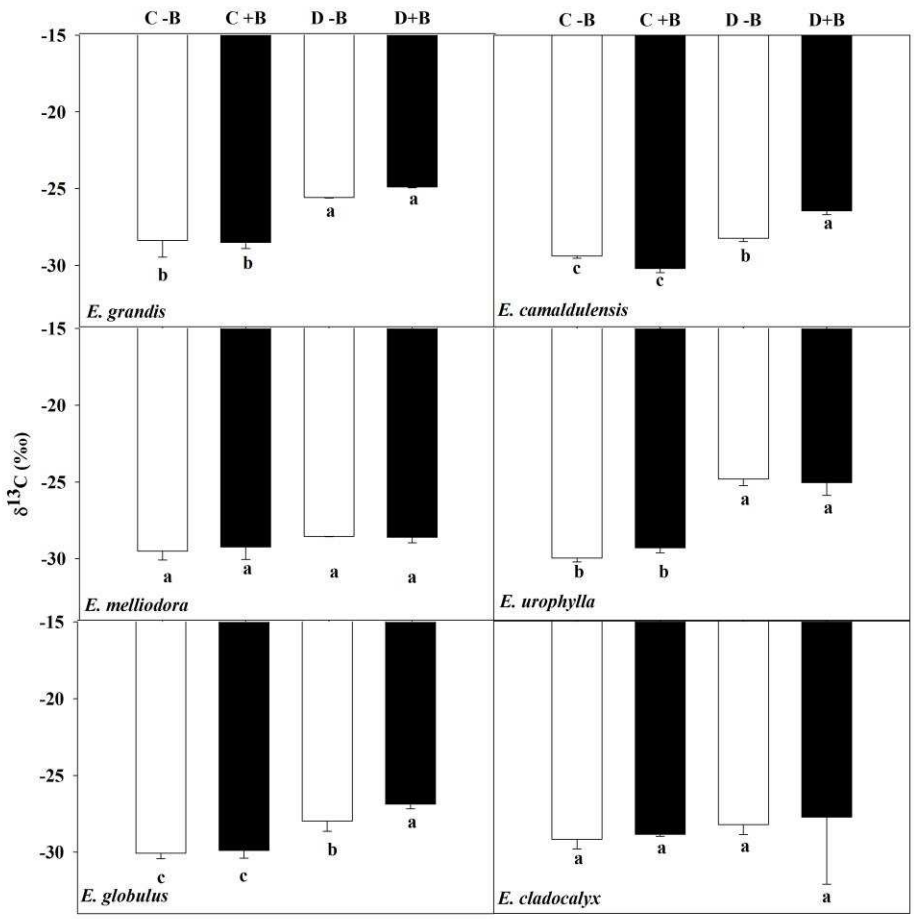

Figure 15: Carbon isotopic composition ($\delta^{13}C$) of six Eucalyptus species seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Lower case letters indicate differences among treatments within the same species, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

The instantaneous water use efficiency (WUE_i) had a great improvement across Eucalyptus species, except in E. melliodora, in plants under water deficit but supplemented with B, throughout most of the photoperiod (Fig.16). The WUE in E. camaldulensis and E. uropylla was approximately 2 times higher in D+B plants than D –B plants. Similarly, in E. grandis and E. globulus the WUE increment was 5 times more in D+B plants as compared to the D-B plants (Fig. 16).

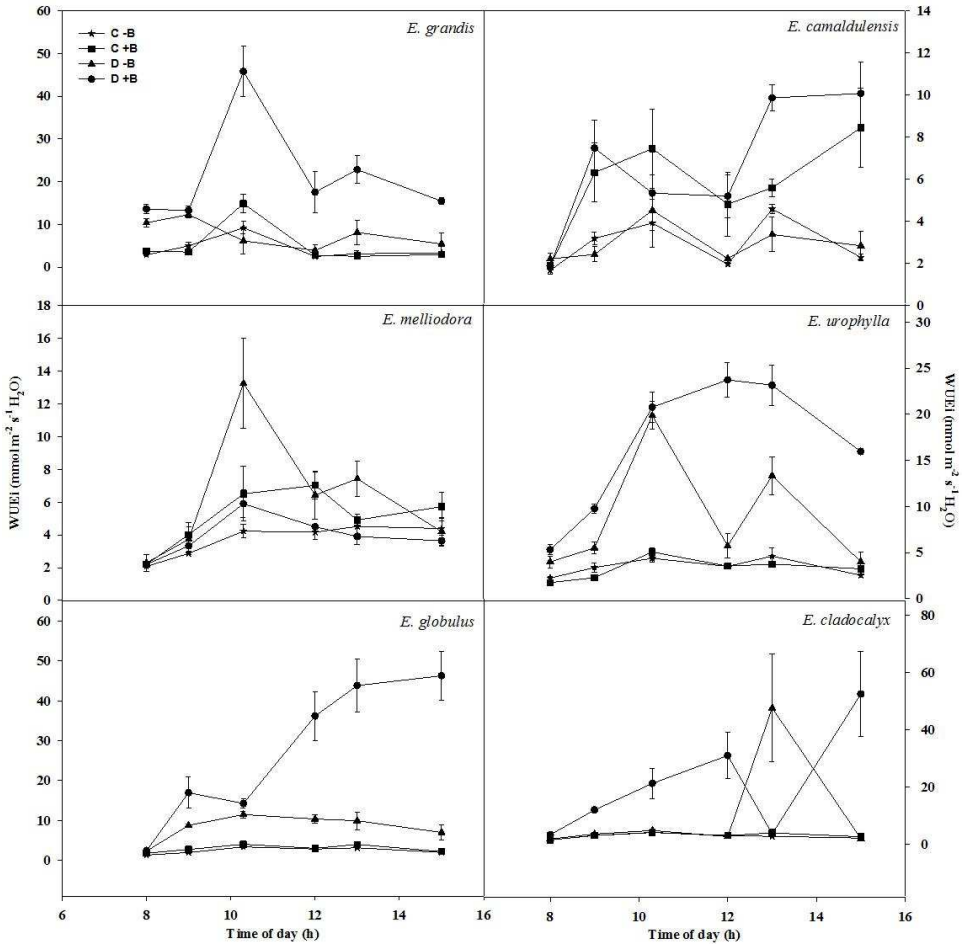

Figure 16: Instantaneous Water Use Efficiency (WUEi) in six Eucalyptus seedlings during 20 days of: normal water supply (C); water restriction (D); at absence (-B) or presence (+B) of boron in the nutrient solution. Bars represent standard error (n=4).

3.7 $\Delta\ ^{13}C$ versus modeled values and WUE_g versus modeled values

$\Delta\ ^{13}C$ from the leaves major carbon pool correlated well and positively with modeled values across control and water-stressed plants under differential B nutrition (Table 4) indicating the validity of the isotopic technique. The highest correlation was observed for E. urophylla and E. grandis ($r= 0.92$ and 0.89, $p< 0.01$, respectively).

Significant correlations were found between WUE_g and intrinsic WUE modeled considering all treatments (Table 5). The correlation between intrinsic WUE and WUE modeled

was higher (up to r=0.98, p<0.01) than that between Δ ^{13}C and Δ ^{13}C modeled (up to r=0.92 p<0.01) (Tables 4 and 5).

Table 4: Correlation between Δ modeled and intrinsic Δ measured in six Eucalyptus species seedlings during 20 days of: normal water supply; water restriction; in the absence (-B) or presence (+B) of boron in the nutrient solution.

E. grandis	$\Delta_{modeled} = -29.62 + 2.484^{*}\Delta$	r = 0.89
E. camaldulensis	$\Delta_{modeled} = 18.38 + 0.094^{ns}\Delta$	r = 0.045
E.melliodora	$\Delta_{modeled} = -19.77 + 1.914^{*}\Delta$	r = 0.43
E. urophylla	$\Delta_{modeled} = -11.58 + 1.538^{*}\Delta$	r = 0.92
E. globulus	$\Delta_{modeled} = 27.99 - 0.3428^{*}\Delta$	r = 0.53
E. cladocalyx	$\Delta_{modeled} = 27.77 - 0.258^{ns}\Delta$	r = 0.31
6 species	$\Delta_{modeled} = -4.47 + 1.203*\Delta$	r=0.54

* = Significant at 0.1% according to t test ns= no significant

Table 5: Correlation between intrinsic WUE and intrinsic WUE modeled in six Eucalyptus species seedlings during 20 days of: normal water supply; water restriction; in the absence (-B) or presence (+B) of boron in the nutrient solution.

E. grandis	$WUE_{modeled} = 23.22 + 0.84^{*}WUE$	r = 0.90
E. camaldulensis	$WUE_{modeled} = 24.74+ 0.86^{*}WUE$	r = 0.91
E.melliodora	$WUE_{modeled} = 8.50 + 1.17^{*}$ WUE	r = 0.98
E. urophylla	$WUE_{modeled} = 88.70 – 0.043^{ns}WUE$	r=0.03
E. globulus	$WUE_{modeled} = 18.122 - 0.68^{*}$ WUE	r = 0.82
E. cladocalyx	$WUE_{modeled} = 20.67 - 0.69\ ^{*}WUE$	r = 0.78
6 species	$WUE_{modeled} = 27.966 – 0.71\ ^{*}WUE$	r = 0.75

* = Significant at 0.1% according to t test ns= no significant

4. DISCUSSION

The responses of Eucalyptus plants to short-term B deficiency applied here indicates an important role of B on water use efficiency and an acute interactive response to water availability in Eucalyptus species from contrasting ecotype. For the species tested here, B had the same importance to water-stress tolerance in almost all species subjected to drought suggesting the fundamental role(s) that B plays in plant processes and the significance of B nutrition for the genus Eucalyptus.

4.1 B increases A and reduces g_s in drought stressed plants

A decrease in A, g_s and E have been observed in many B-deficient plants (Han et al. 2008, 2009; Mukhopadhyaya et al., 2013; Lu et al., 2014). Similarly, B deficiency in Eucalyptus species generally induced notable inhibition of CO_2 assimilation, reduced g_s and led to accumulation of nonstructural carbohydrates in leaves and decreased their concentration in roots, supporting previous results observed in other species (Zhao and Oosterhuis; 2002; Han et al. 2008, 2009; Lu et al., 2014). The accumulation of sugars in leaves is known to be a result of a decreased sink demand most likely because of the decreased growth observed in B deficient plants (Han et al. 2008, 2009). Furthermore, B deficiency reduces the concentration of chloroplastic pigments (Sharma and Ramchandra, 1990; Tewari et al., 2010) and aggravates oxidative stress in some species through intensified generation of reactive oxygen species (ROS) (Tewari et al., 2010; Mukhopadhyay et al., 2013). All these responses may have promoted either a feedback repression over photosynthetic rate or damage to the photosynthetic apparatus in Eucalyptus species subjected to B deficiency.

Interestingly, even though the g_s reduction was more prominent in C-B compared to C+B plants, when Eucalyptus plants were subjected to the combination of drought stress and B

treatment, D +B leaves presented higher A than D –B leaves (up to 70 % higher), however no differences were observed on g_s of most species. Although there is still no strong evidence of B influencing stomatal opening in the literature, it has been suggested that B deficiency may be associated with the reduction of K^+ uptake into guard cells and loss of membrane integrity stimulating passive leakage of K^+ from guard cells (Pollard, 1977; Roth-Bejerano and Itai, 1981; Hajiboland and Farhangi, 2010). In our work we found that B supply under water deficit condition greatly increased K^+ concentration in leaves of Eucalyptus plants, suggesting an important role of B in stomatal aperture and isohydric responses, since K regulates the stomatal functioning under drought-stress conditions (Kant and Kafkafi, 2002) (scheme on Fig. 17). It is noteworthy that stomatal closure is an important mechanism to avoid water loss by water stressed plants, hence, increasing plant water-use efficiency (Parry et al., 2005), which is an important strategy during drought. These results highlight the importance of adequate K fertilization, specifically in low fertility tropical soils, in order to achieve the benefits of B supply under water deficit conditions.

In addition, B deficiency has been shown to decrease the amount of aquaporins (Goldbach et al., 2001), potentially reducing water flow and creating a further disadvantage for plants under drought conditions. Eucalyptus B deficient plants were more severely damaged by drought than B supplied plants with high reduction of A and growth rate. Similar observations were described for Picea abies, Helianthus annuus, Brassica rapa and E. urophylla (Möttönen et al. 2001, 2005; Hassan and El-Sayed, 2011; Hajiboland and. Farhanghi, 2011; Hodecker et al., 2014).

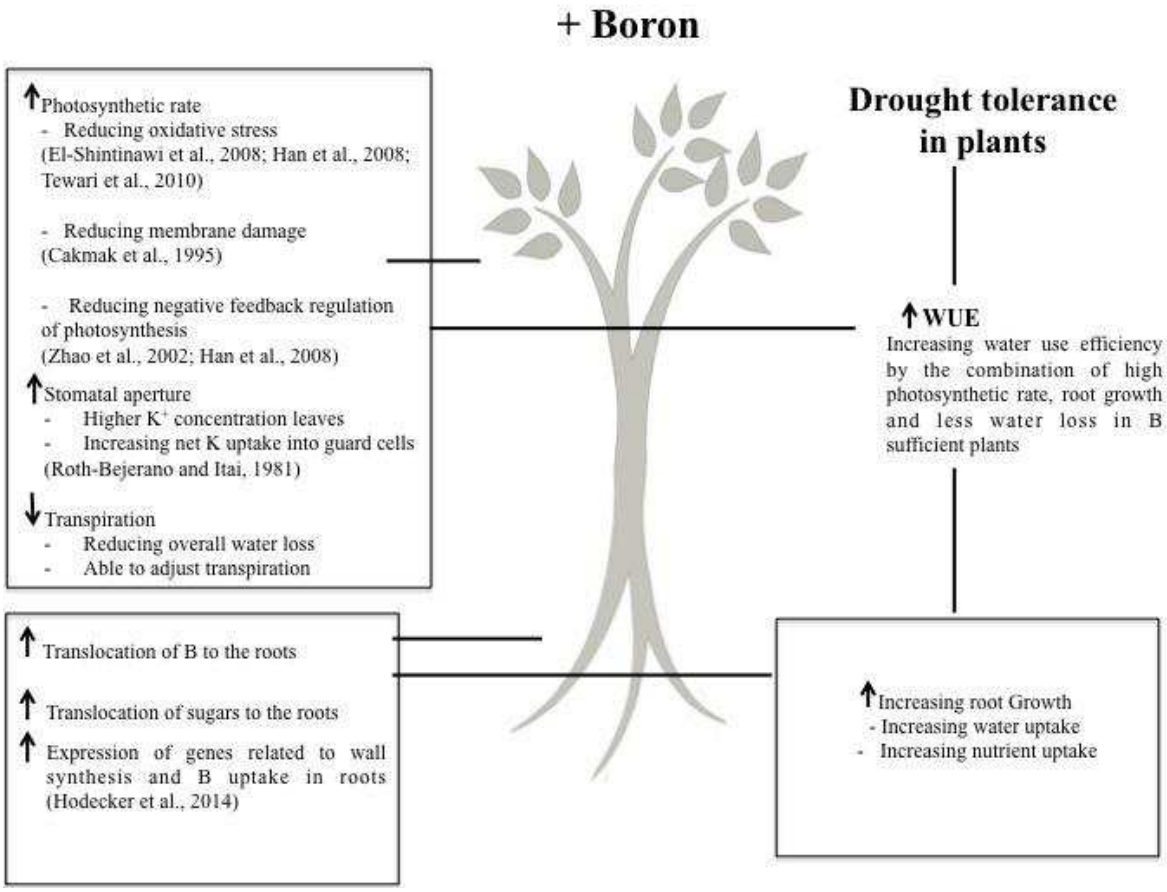

Figure 17: Proposal scheme on the influence of B nutrition on water-use efficiency in Eucalyptus plants subjected to drought. B sufficient plants have higher photosynthetic rate possibly reducing oxidative stress and membrane damage under drought stress. The higher concentration of K^+ in leaves may promote the increment of K uptake into guard cells leading a better stomatal control and reducing overall water loss. In addition, the translocation of sugars and B to roots, combined with a higher expression of genes related to cell wall biosynthesis and boron uptake by roots may promote a better root growth leading to an increment of water and nutrient absorption area. Altogether, these physiological and morphological responses will lead to an increase in water use efficiency and improved drought tolerance by plants.

4.2 Sugar and B translocation to roots is enhanced by B

The starvation of B and drought stress affected photosynthesis and carbohydrate metabolism of Eucalyptus plants leading to reduced growth rate. The increase of shoot dry matter in E. grandis and E. camaldulensis seedlings was significantly reduced by water

restriction and absence of B (60 and 35 %, respectively). However, the first species greatly increased root dry matter, by 1.6 times higher in D +B plants than D-B plants, supporting our previous results for E. urophylla under drought and foliar B application (Hodecker et al., 2014). Similarly, Picea abies (Möttönen et al., 2001, 2005; Räisänen et al., 2007) and Brassica rapa (Hajiboland and Farhanghi 2011) also showed a greater root growth in +B plants compared –B ones.

The indicative of higher translocation of sugars and B to roots, observed in almost all B sufficient plants, may be an important mechanism to induce new root growth under long water limitations periods (scheme on Fig. 17). Hodecker et al. (2014) suggested that in Eucalyptus, B would contribute to a better water absorption (larger root system), as well as to improve WUE and to reduce dehydration during water stress, which were also observed by some Eucalyptus species in our work.

4.3 Isotopic signals and intrinsic water use efficiency in Eucalyptus plants are related in plants under water and B treatments

Alterations in the abundance of ^{13}C is driven by discrimination in stomatal conductance to CO_2 (diffusional), biochemical processes such as photosynthetic assimilation or combination of both (see for example: Farqhuar et al., 1989) and has been used as a proxy for intrinsic water use efficiency at leaf level (Farquhar et al., 1982; Lloyd and Farquhar 1994; Martin et al., 1999; Moghaddam et al., 2013). Although affected by similar processes, the carbon discrimination and intrinsic or instantaneous water-use efficiency may vary independently; most likely because the isotopic signal depends on mesophyll conductance in addition to gs, whilst instantaneous water use efficiency depends on evaporative demand in addition to gs (see review Seibt et al., 2008).

As expected, we observed an enrichment of $^{13}CO_2$ in leaf tissues as water limitation increased, as shown by the extracted metabolites. Interestingly, E. camaldulensis and E. globulus showed significant higher enrichment of ^{13}C in D+B plants than D-B plants, with increment ranging from 20-30 % of K^+ concentration, which could be due changes in stomatal conductance rather than increment of photosynthetic rate.

Using the Δ ^{13}C predicted, we evaluated the correlation between intrinsic WUE and modeled WUE, using either water or B treatments, and we found high correlation between these parameters, which indicate higher influence of water, but also of the B supply, on intrinsic WUE. Despite being common to find poor relationships between ^{13}C from some major soluble carbon pools and ^{13}C predicted by C_i/C_a (Merchant et al., 2011), we found high correlation, except for E. camaldulensis, between both parameters. These results indicate that to +B Eucalyptus plants this technique offered a valid prediction on leaf gas exchange and represented good reflection when compared with carbon sources.

4.4 B promotes increased instantaneous water use efficiency by plants under drought

The relationships between B and water status have been intensively studied (Baker et al., 1956; Möttönen et al., 2001; Apostol and Zwiazek et al., 2004; Möttönen et al., 2005; Hajiboland and Bastani, 2012; Wimmer and Eichert, 2013; Hodecker et al., 2014), however, most of these responses resulted from long-term B deficiency (varying from 10 up to 29 weeks of -B).

The long-term B deficiency may promote a reduced number and deformed shapes of stomata (Rosolem and Leite, 2007), structural damages to the conducting tissues, impair root water uptake and others mal-formations (see review Wimmer and Eichert, 2013) which will interfere on a clear picture of the primary roles of B on water relations in plants.

The short-term B deficiency (approximately near 8 weeks) imposed in our work suggests an important role of B on water use efficiency. All genotypes expressed significant influence of B nutrition when plants were subjected to drought stress, although these responses were more pronounced on E. grandis, E. camaldulensis, E. globulus and E. urophylla.

We observed a strong increment on instantaneous WUE in D+B plants, except for E. melliodora, due to the combination of higher photosynthetic rate, higher K^+ concentration in leaves promoting higher stomatal closure, lower water loss and a higher translocation of sugars and B to root growth (scheme on Fig. 17). Altogether these combinations may increase the water use efficiency in B sufficient plants promoting better acclimation under drought by plants mainly during long-term water stress as observed on the field conditions. Our results reinforce the importance of B nutrition of Eucalyptus to cope with periods of water limitation.

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CHAPTER 2
WATER AVAILABILITY FLUCTUATION DEFINES THE TOLERANCE OF EUCALYPTUS CLONES TO DROUGHT

ABSTRACT

Eucalyptus plantations are an increasing component of tropical landscapes in Brazil and drought stress responses have been extensively studied since soil drought represents a major constraint for successful production. The main objectives of this study were to identify the morphological, physiological and molecular changes caused by large periods of water restriction in four Eucalyptus clones under field conditions. The study sites are located in north-eastern Brazil in commercial Eucalyptus stands (between 5 and 6 year old) located in two regions with an average rainfall of 800 mm and 1500 mm per year. In 2013, the year of assessment, the precipitation decreased to approximately 500 mm and 1150 mm, respectively. At each rainfall regime, the following clones were evaluated: 1404 (Eucalyptus urophylla) and 1407 (E. urophylla x E. camaldulensis), both considered drought-tolerant, and 1296 and 6500 (E. grandis x E. urophylla), considered sensitive to drought. Our results suggest that those trees growing in the area with more uniform annual high precipitation were more stressed after a long period of drought, compared to those stands submitted to annual water-stress fluctuation period. The studied genetic materials showed distinct responses to drought, which allowed their separation in two groups, 1404 and 1407 (group 1, clones of E. urophylla and E. urophylla x E. camaldulensis, respectively) and 6500 and 1296 (group 2, E. grandis x E. urophylla). The group 1 had similar responses growing at the drier site (500 mm) as, lower SLA and MDA, higher leaf $\delta^{18}O$ enrichment, no differences in $\delta^{13}C$, decreased in starch concentration, compared to the other two genetic materials. The group 2 had higher values of SLA, $\delta^{13}C$, glucose, and MDA concentration and lower leaf cyclitols content in that site. The two groups growing under 1150 mm of precipitation had different responses to water stress.

Keywords: Eucalyptus, drought, field condition, tolerance

1. INTRODUCTION

Eucalyptus plantations are an increasing component of tropical landscapes in Brazil, covering approximately 6.6 millions hectares in 2012, which represents 70 % of the total tree area plantations, and the forest sector is the source of almost 5 million direct and indirect employments (ABRAF, 2013). A study across a geographic gradient in Brazil found that the productivity of eucalypt increased with increasing rainfall, and the water supply affects the biomass allocation to roots, stems and leaves (Stape et al., 2004). Despite these aspects, due to the increasing demand of industrial forest products, the expansion of planted forests in Brazil has been directed to areas where water restrictions are more accentuated, as the north eastern region, leading to significant reductions in productivity or high tree mortality rate. Additionally, some studies have already demonstrated the high potential of tree mortality due to changes in water availability driven by climate changes (Allen et al., 2010). To cope with water restriction the selection of drought tolerant genotypes is the main strategy being adopted to establish new plantings (Bison et al., 2007). However, in Brazil, most genetic selections have been only focused on growth rates and fiber productivity (Navarrete-Campos et al., 2012), without taking into account a clear understanding of morphological and biochemical responses of trees to water stress.

The main morphological and physiological alterations observed in drought-tolerant Eucalyptus submitted to water deficiency are: leaf area reduction (Metcalfe et al., 1989; Guarnaschelli et al., 2003; Mokotedi, 2010), photoassimilates allocation to root growth (Costa and Silva et al., 2004), regulation of stomatal closure and transpiration (Pita and Pardos, 2001; Tausz et al., 2008; Mokotedi, 2010), activation of antioxidants mechanisms (Shvaleva et al. 2005), changes in chlorophyll and carotenoids content (Michelozzi et al. 1995; Shvaleva et al.,

2005), and osmotic and elastic adjustment (Lemcoff et al., 2002; Merchant et al., 2006; Arndt et al., 2008; Callister et al., 2008; Warren et al., 2011). These alterations are important to minimize water loss or optimize its acquisition and use during dry periods.

In addition, in order to overcome oxidative stress during drought periods, plants have developed defense mechanisms to scavenge ROS (Smirnoff, 1993) resulting in lower malondialdehyde (MDA, a product of lipid peroxidation) content, an indicator of higher resistance to drought (Shinozaki et al., 2003; Dhanda et al., 2004). The combination of the expression analysis of oxidative stress-related genes (as the CuZnSOD, APX) and lipid peroxidation may indicate the cell stability and integrity in plants under drought.

Furthermore, others important molecular signaling changes can be induced by water stress and are important to understand drought tolerance mechanisms. The RD26 (responsive to dehydration) transcription factor gene, a member of the NAC-domain family, was identified by Fujita et al. (2004) and it was one of the most strongly expressed genes in transcriptome sequencing of E. camaldulensis subjected to water stress (Thumma et al., 2012). This gene apparently has an important role mediating cross talk between ABA signaling during drought (Shinozaki et al., 2007). Another important transcription factor gene is the MYB2, which has also an important role as transcriptional activators in ABA-inducible genes expression during drought stress (Abe et al., 1997, Abe et al., 2003, Finkelstein et al., 2002) and its overexpression was related with osmotic stress tolerance in transgenic plants (Shinozaki et al., 2007).

Responses of Eucalyptus plants to drought not only vary with stress severity, duration (Bedon et al., 2011) and genetic material (Li and Wang, 2003; Shvaleva et al., 2005; Merchant et al., 2007a; Merchant et al., 2007b; Warren et al., 2011; Navarrete-Campos et al., 2013; Correia et al, 2014), but also may change with developmental stage (Buchanan et al., 2000). Most of

these previous studies were carried out under controlled conditions in greenhouse and there are some limitations to up scaling the findings to the field situation, where the pace of water stress development is different from that under controlled conditions. Furthermore, it is still not clear whether the responses observed in drought-tolerant species during seedling stage are applicable to the stresses responses under field conditions, where plants are older and exposed to prolonged periods of water shortage. It's well known that plants submitted to a slowly increasing water stress may have enough time to adapt to the drought (Spieß et al., 2012).

Therefore, the main objectives of this study were to identify the morphological, physiological and molecular changes caused by large periods of water restriction in four Eucalyptus clones under field conditions. This study, carried out in the field, aimed to answer the following questions: (1) May eucalypt trees growing under seasonal rainfall conditions be more tolerant to the subsequent drought stress events? (2) Are there different tolerance responses among eucalypt clones of high genetic similarity? (3) Does a given physiological profile indicate a eucalypt clone adaptable to drought conditions?

2. MATERIAL AND METHODS

2.1. Study area

The study sites are located in north eastern Brazil (areas owned by Bahia Specialty Cellulose Group) in commercial Eucalyptus stands (between 5 and 6 year old) located in two sites with an average rainfall of 800 mm (dry site) and 1500 mm (rainy site) per year. In 2013, the year of assessment, the precipitation decreased to approximately 500 mm and 1150 mm, respectively, in the dry and rainy sites (Fig. 1). Maximum and minimum annual temperatures of 30.0 and 20.9 ºC characterized this tropical area, and the mean annual temperature at the both areas was 25.5 ºC.

At each rainfall regime site, the following clones were evaluated: 1404 (Eucalyptus urophylla) and 1407 (E. urophylla x E. camaldulensis), both considered drought-tolerant, and 1296 and 6500 (E. grandis x E. urophylla), considered sensitive to drought (Table 1). In each site, a rectangular inventory plot of 400 m^2 was established and we selected 4 trees (replicates) with height and diameter corresponding to the average of the population. The assessments were performed at the end of the dry season (February, 2013), after 10 months without substantial rain. Samples of youngest fully expanded leaves from the upper part of the canopy were collected and immediately frozen in liquid N_2 and stored at -80 °C for further analysis. Leaf samples from the same position were placed in plastic bags and stored in an ice chest for subsequent leaf area measurement and subsamples were dried in the oven to determine the nutrient content.

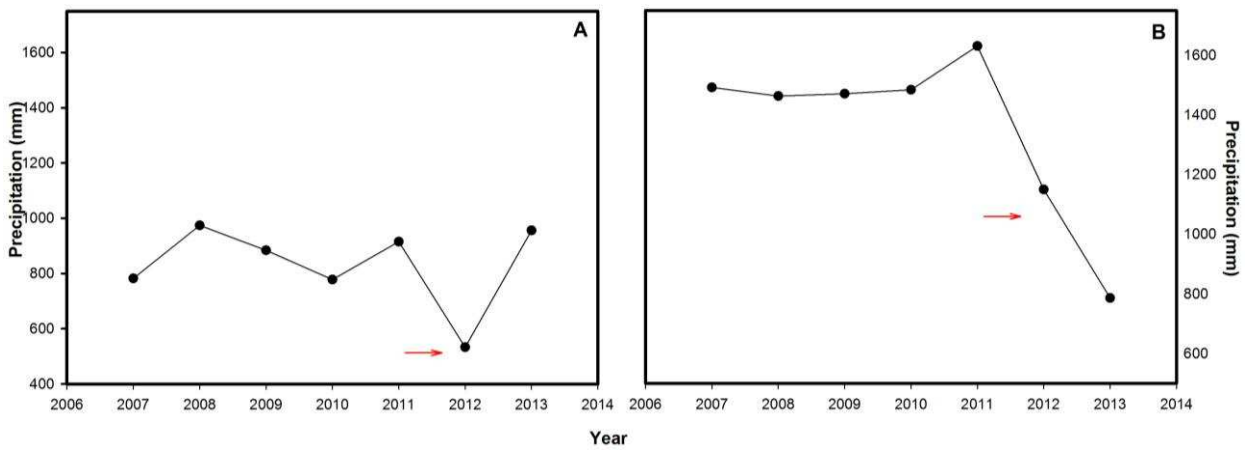

Figure 1: Annual average precipitation at the two studied sites (arrows indicate sampling time).

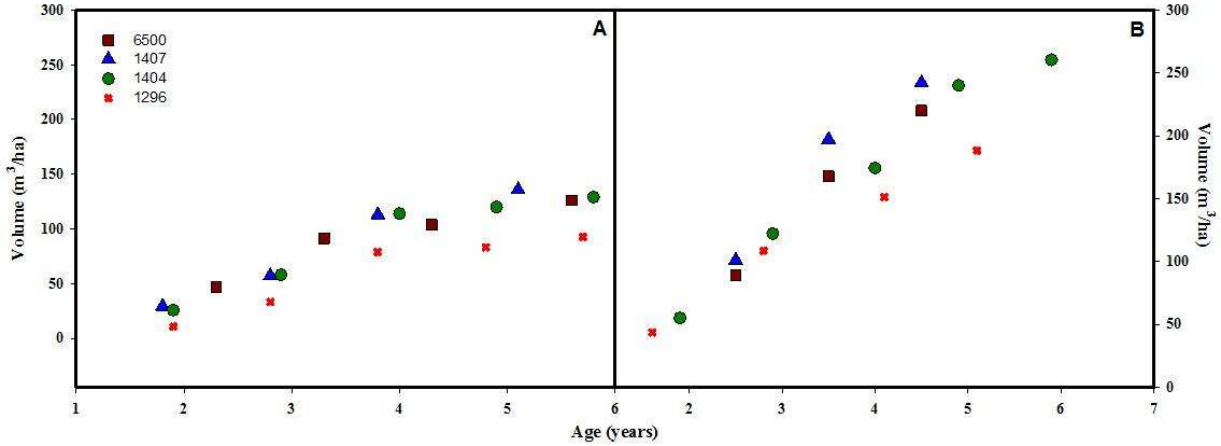

Figure 2: Wood volume (without bark) according to stand age and rainfall of the planting sites (A = 500 mm; B = 1150 mm).

Table 1: Volume mean annual increment (MAI) and age of the plantations of Eucalyptus clones according to the rainfall of the sites

Clone	Precipitation (mm/year)	MAI (m³/ha/year)	Stand age (year)
1296	500 (dry site)	16.28	5.7
6500	500 (dry site)	22.67	5.6
1404	500 (dry site)	22.04	5.8
1407	500 (dry site)	24.67	6.1
1296	1150 (rainy site)	36.9	5.1
6500	1150 (rainy site)	48.88	5.9
1404	1150 (rainy site)	44.13	4.5
1407	1150 (rainy site)	53.73	4.5

2.2. Analysis

2.2.1. Concentration of nutrients

Leaves samples were digested by a nitro-perchloric acid mixture. The Ca and Mg concentrations in the digests were determined by atomic absorption spectrophotometry, P by colorimetry and K by emission flame photometry. The B content, after digestion in a muffle furnace at 550 °C, was determined by the spectroscopic method of azomethine H^+ at 410 nm (Wolf, 1974).

2.2.2. Specific leaf area

The sampled leaves were scanned, digitalized, weighed and leaf area measured using the Image Pro-Plus version 4.1, for Windows® (Media Cybernetics, Silver Spring, MD, USA).

2.2.3. $\delta^{13}C$ and $\delta^{18}O$ determination

Dried leaf samples were macerated and the isotope ratio ^{13}C:^{12}C (expressed as $\delta^{13}C$), in a per mill basis was determined in relation to the international standard PDB (Pee Dee Belemnite) using isotope ratio mass spectrometry (ANCA-GLS, Sercon, Crewe, UK) and the ^{18}O:^{16}O isotope ratio (expressed as $\delta^{18}O$) determined using a Delta V Plus IRMS (Thermo Scientific).

2.2.4. Glucose, sucrose, raffinose and myo-Inositol contents

Frozen leaf samples were briefly irradiated (30 s) in a standard 650 W microwave oven, oven-dried overnight at 70 °C and stored at -86 °C. The non-structural carbohydrates were extracted and quantified according to Merchant et al. (2006) using a GC-QQQ. 60 µl of deionized methanol-chloroform-water extracts were dried and resuspended in 400 µl anhydrous pyridine to which 50 µl of trimethylchlorosilane (TMCS)/bis-trimethylsilyl-trifluoroacetamide mix (1:10 v/v, Sigma Aldrich, Sydney, Australia) was added. Samples were incubated for 1 h at 75 ºC and analyzed by GC within 12 h. GC analysis was performed using a Shimadzu 17A series

gas chromatograph using a DB1 (30 m) column. Split injection was made at 300 ºC with an initial oven temperature program of 60 ºC for 2 min ramping to 300 ºC at 10 ºC min^{-1} and maintained for 10 min. Column flow rate was maintained at 1.5 ml min^{-1}. Peak integration was carried out using Class VP analysis software (Shimadzu Corporation Limited, Columbia, MD, USA).

2.2.5 Clorophyll a, b and carotenoids contents

The leaf chorophyll (Chl) and carotenoids (Car) were extracted by dimethylsulfoxide (DMSO) in the dark, for 4 hours at 65 ºC. The absorbance was then determined at 480, 649.1 and 665.1 nm and concentrations of Chl a, Chl b and Car were calculated using the method proposed by Wellburn (1994).

2.2.6 Malondialdehyde (MDA) content

The concentration of malondialdehyde (MDA) accumulated in leaves was determined by extraction by trichloroacetic acid (TCA) 0.1% (Hodges et al. 1999). The homogenized sample was centrifuged, and 0.5 mL from the supernatant was added the solution of TCA 0.1 % with or without (blank) thiobarbituric acid (TBA). The samples were mixed, incubated in water bath (90 ° C, 30 min), cooled, and re-centrifuged. Absorbance was measured at 440 nm, 532 nm and 600 nm. MDA equivalents were calculated as described by Hodges (1999).

2.2.7 Gene expression analysis by real-time qPCR

Approximately 0.5 g of leaf samples was ground in liquid N_2. Then 1.2 ml of extraction buffer was added (Plant RNA Reagent - Invitrogen), and carried out according to the manufacturer recommendations. Total RNA was quantified in a Fluorometer (Qubit® Invitrogen) and its integrity was assessed by electrophoresis in 2% agarose gel (Wilson and Walker, 2000). The total RNA was treated with DNase I (Invitrogen) in order to eliminate contaminating

genomic DNA, following the manufacturer's protocol. The cDNA synthesis was performed with Superscrip II (Invitrogen), according to the manufacturer's instructions.

Sequences from the target genes were obtained at NCBI (ncbi.gov) and Phytozome (phytozome.net) websites, and primers were designed at the website Primer Blast (ncbi.nlm.nih.gov/tools/primer-blast), as described in Table 2.

Table 2: Primers used in real-time qPCR analysis

Gene	Function	GenBank[1]	Primer Sequence	
			Sense 5'-3	Anti sense 5'-3
RD26	RD26 NAC domain containing protein 2; transcription factor	AT4G27410.3	CAACACACCT CCGCAATATG	TCCCAACCT TCCAACTTCAC
EgBCL	Eucalyptus globulus ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene	HM849985.1	AAGCAAGGG CTGTGGTAGC	CAGCAACAGGT TCGATGTGG
EgCuZnSOD	Eucalyptus grandis x Eucalyptus urophylla Cu/Zn superoxide dismutase (Cu/ZnSOD1)	JX138573.1	TTGTTGGA AGGGCTGTGGTT	GTCCACCCTT GCCAAGATCA
EcAPX	Eucalyptus camaldulensis ascorbate peroxidase mRNA	DQ839645.1	CCCGGAA GAGAGGACAAACC	GTCACAGCC CTTGGTAGCAT
Myb2	New transcriptional activator from Eucalyptus xylem, regulates secondary cell wall formation and lignin biosynthesis	GOICOECHEA et al., 2005	GCGGATGGA GATTCTGTACA	AACGCCCTTC CCTACTAAGA

[1]Gene accession number in the database NCBI (www.ncbi.gov)

The validation of primers was performed on the standard curve using cDNA synthesized from sample diluted to the original volume of the cDNA, 5, 25 and 125 times, with three replicates for each dilution. The specificity of the amplification reactions was determined by the melting curve of the reaction products of amplification. Primers were considered valid for linear equation with R^2> 98%, coefficient of variation <10% and, amplification efficiency between 90

and 110% of slope about -3.2, as suggested by the manufacturer (Applied Biossystems, Foster City, U.S.).

Polymerase chain reactions were carried out in an optical-96 well plate on Step One Plus Platform (Applied Biosystems, U.S.), using 5 μL of Fast Sybr® Green (Life Technologies) to monitor dsDNA synthesis. To the reaction mixture it was added 50 ng of cDNA, 0.4 μL of each 2.5 μM primers and the volume was then completed to 10 μL with ultrapure water. Reaction mixtures were incubated for 20 s at 95 ºC, followed by 40 amplification cycles of 3 s at 95 ºC and, 30 s at 60 ºC. Polymerase chain reaction (PCR) efficiencies and optimal cycle threshold (ct) values were estimated using the online real-time PCR Miner tool (Zhao and Fernald 2005). For all reference genes studied, four independent biological samples of each experimental condition were evaluated in technical duplicates.

2.3 Statistical Analyses

Data were subjected to analysis of variance and means compared by the Tukey test, at 5% probability, using the software STATISTICA 8.0.

3 RESULTS

3.1 Specific leaf area (SLA), pigments, gene expression, MDA and total phenols

In the dry region, the SLA was significantly lower for 1404 plants, whereas there were no significant differences between the other clones. At 1150 mm, the highest SLA was found for clone 1296 ($p < 0.05$) (Fig.3). Clone 1407, at 1150 mm, had a lower SLA compared to the trees at 500 mm.

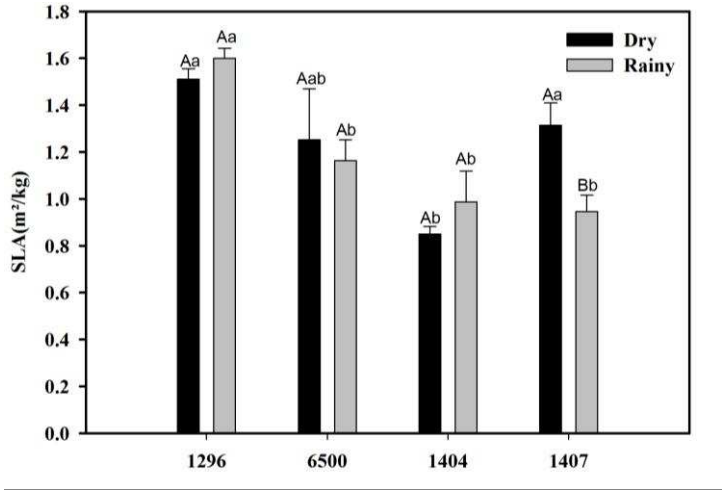

Figure 3: Specific leaf area (SLA) of Eucalyptus clones in regions with different rainfall, after 10 months without rain. Capital letters mean significant differences within the same clone in different precipitation and lower case letters indicate differences among clones within the same precipitation, according to Tukey's test at 5 % probability. Bars represent standard error (n=4).

Clone 1407 had the highest concentration of Chl a and carotenoids at the rainy site (Table 2). However, at lower precipitation, there were no significant differences in these parameters between clones. No differences between plants in same or different rainfall conditions were found to Chl b concentration.

The expression of the EgBCL gene was highest to 1404 plants collected in the region with higher rainfall (Table 3). At the dry site, clone 6500 had the lowest RuBP relative gene expression.

Table 3: Chlorophyll a (Chl a) and b (Chl b), Carotenoids, Malondialdehyde (MDA), and total phenols gene expression of Eucalyptus clones in regions with different rainfall after 10 months without rain. Capital letters mean significant differences within the same clone in different precipitation and lower case letters indicate differences among clones within the same precipitation, according to Tukey's test at 5% probability

Clone	Sites	Chl *a* (µg/g FW[1])	Chl *b* (µg/g FW)	Carotenoids (µg/g) FW	MDA (nmol/g) FW	Total Phenols (mg/g) FW
1296	Dry	183.1 ±12.94Aa	213.2 ±27.72Aa	1438.2 ± 47.64Aa	29.6± 2.65Ba	31.4 ± 1.28Aa
6500	Dry	231.2 ±12.60Aa	228.1 ±23.46Aa	2452.2 ± 437.86Aa	27.9 ± 3.08Aa	27.0 ± 1.34Aa
1404	Dry	217.4 ±25.05Aa	124.2 ±23.06Aa	1504.4 ±321.92 Aa	14.1 ±1.01 Bb	31.4 ± 1.02Aa
1407	Dry	221.3 ±17.28Ba	221.7± 13.76Aa	1591.3± 147.55Ba	23.6 ± 0.39Bab	27.4 ± 0.66Aa
1296	Rainy	226.7 ±15.49Ab	254.9 ±25.81Aa	1635.8± 154.87Ab	38.2 ± 1.14Aa	31.4 ± 0.23Aa
6500	Rainy	230.54 ±9.05Ab	181.8 ±9.30Aa	1674.4 ± 69.35Ab	34.2 ± 3.61Aa	29.28 ± 0.15Aa
1404	Rainy	216.5 ±9.03Ab	202.4 ±38.95Aa	1549.6 ± 68.53Ab	31.9 ± 3.03Aa	28.08 ± 1.88Aa
1407	Rainy	306.08 ±32.73Aa	307.7 ±32.41Aa	3466 ± 903.50Aa	36.2 ± 1.28Aa	29.9 ± 0.36Aa

Mean ± standard error. Uppercase letters mean significant differences within the same clone in regions with different precipitation and lower case letters indicate differences among clones within the same precipitation, according to Tukey's test at 5% probability. Abbreviations: FM= fresh weight. Standard error (n=4).

Table 4: Myb2, RD26, EcAPX, CuZnSOD and EgBCL gene expression of Eucalyptus clones in regions with different rainfall after 10 months without rain. Capital letters mean significant differences within the same clone in different precipitation and lower case letters indicate differences among clones within the same precipitation, according to Tukey's test at 5% probability

Clone	Sites	Myb2 (u.a)	RD26	EcAPX (u.a)	CuZnSOD (u.a)	Eg BCL
1296	Dry	1.01 ±0.14 Aa	0.74 ± 0.09 Aa	0.29±0.13Aa	0.25±0.03 Ba	60.49±8.0Aa
6500	Dry	1.03 ± 0.11 Aa	1.254± 0.22 Aa	0.19±0.06Aa	0.06±0.02Bb	53.93±3.3Aa
1404	Dry	1.25 ± 0.08 Aa	1.13 ± 0.16 Aa	0.14±0.02Aa	0.06±0.009Bb	81.03±7.8Ba
1407	Dry	1.08 ± 0.34 Aa	0.99 ± 0.18 Aa	0.11±0.02Aa	0.10±0.05Bb	70.14±9.0Aa
1296	Rainy	1.00 ±0.18 Aa	1.09 ± 0.17 Aa	0.14±0.02Aa	6.29±3.6Aa	82.21±15.7Ab
6500	Rainy	1.01±0.06 Aa	1.07±0.30 Aa	0.15±0.05Aa	0.63±0.12Ab	86.11±20.4Ab
1404	Rainy	1.12 ±0.10 Aa	0.93± 0.012 Aa	0.18±0.02Aa	2.03±0.49Aab	169.81±20.0Aa
1407	Rainy	1.06±1.38E-01 Aa	0.88± 0.12 Aa	0.12±0.008Aa	0.88±0.21Ab	73.01±11.8Ab

Mean ± standard error. Uppercase letters mean significant differences within the same clone in regions with different precipitation and lower case letters indicate differences among clones within the same precipitation, according to Tukey's test at 5% probability. Abbreviations: FM= fresh weight. Standard error (n=4).

After 10 months of drought in both sites, the superoxide dismutase gene expression (CuZnSOD) was significantly increased in all genotypes at the rainy site (Table 3). Clone 1296

had the highest CuZnSOD gene expression, increased 25 fold at as compared to the other genetic materials in the same growth condition.

The MDA content was reduced by 34 and 55 % in clones 1404 and 1407, respectively, in the drier region as compared with the rainy one (Table 2). These genetic materials had the lowest concentration of MDA, at the dry site as compared to 6500 and 1296 clones, and no differences between genetic materials were found in trees growing in the rainy site. There were no significant differences in total phenolics concentrations between genetic materials and different precipitations conditions.

There were no statistical differences between clones and, or, different precipitations, for ascorbate peroxidase (EcAPX) and the transcription factors (RD26 and Myb2) gene expression (Table 3).

3.2. Leaf sugars, cyclitol and starch concentration

It was observed distinct responses among the genetic materials on leaf sugars and cyclitol contents for the two contrasting sites (Figs. 4A and 4B). Glucose concentration was increased by 31 and 34 % for clones 1296 and 6500, respectively, at the drier site, but the opposite was found for sucrose. Clone 1404 had the lowest glucose concentration in plants growing in the drier site and the highest at raining site as compared to other genetic materials.

All genetic materials had higher concentrations of raffinose and myo-Inositol at the higher precipitation site, except for clone 1407 (Figs. 4A and 4B). An increase of 38 % was observed in the leaf raffinose concentration for clone 1296 grown at the rainy site (Fig. 4A). The highest concentration of myo-Inositol was observed for 1296 and 6500 clones growing in the rainy sites (Fig. 4B)

In general, no difference was found in leaf sugars and cyclitol content, for clone 1407 at both sites; the lowest values for sucrose, myo-Inositol and raffinose were found for clone 6500 growing in the dry site (Fig. 4).

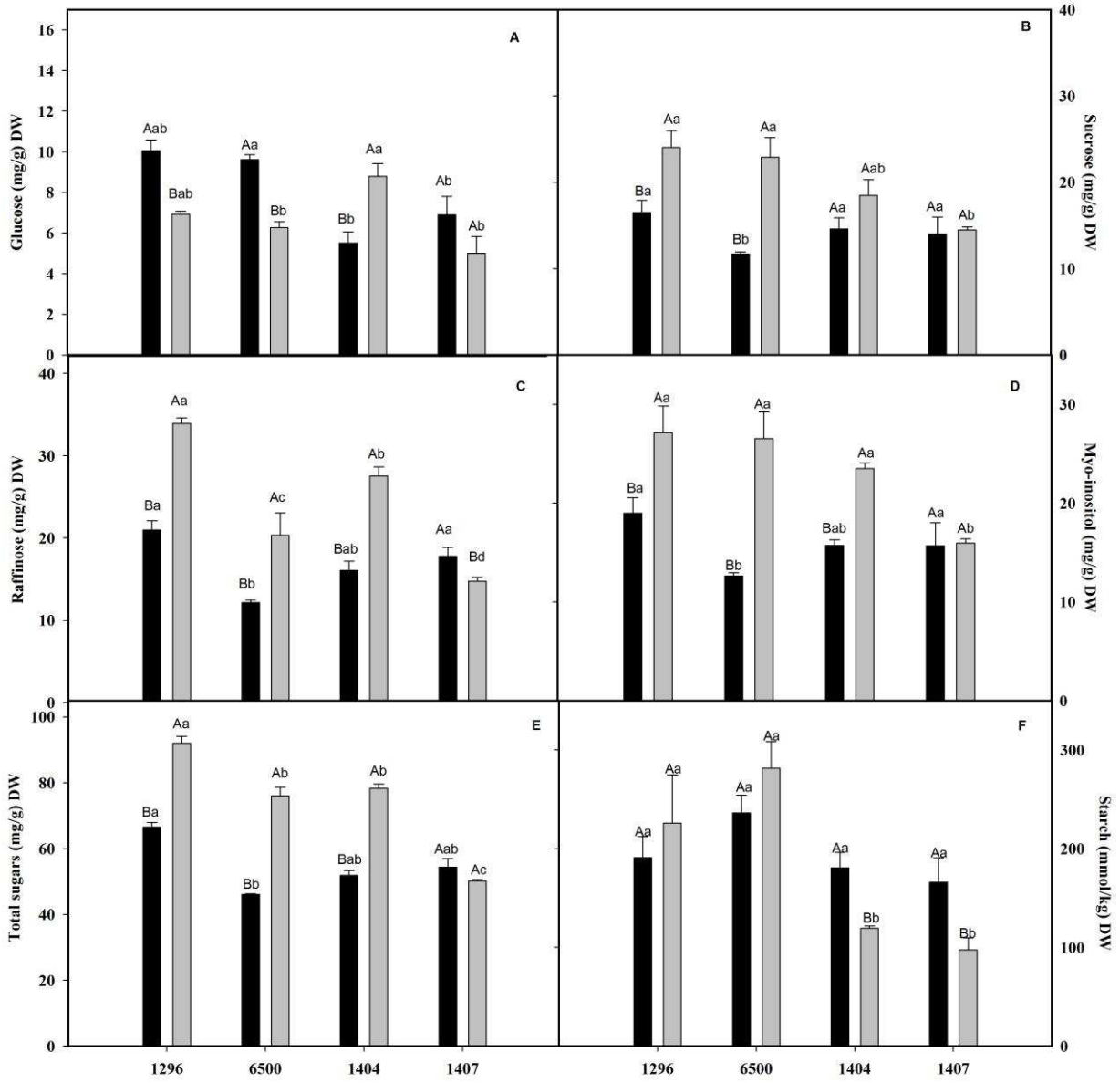

Figure 4: Glucose (4A), sucrose (4B), raffinose (4C), myo-Inositol (4D), total sugars (4E) and starch (4F) concentration of Eucalyptus clones in regions with different rainfall, after 10 months without rain. Capital letters mean significant differences within the same clone in different precipitation and lower case letters indicate differences among clones within the same precipitation, according to Tukey's test at 5% probability. Bars indicate error (n=4).

All genetic materials had higher concentrations of total sugars at the higher precipitation site, except for clone 1407 (Fig. 4E).

Leaf starch concentration was higher under the condition with lower precipitation only for 1404 and 1407 (Fig.4F) by 33 % and 41 %, respectively, growing at the dry site.

3.3. Leaf $\delta^{13}C$ and $\delta^{18}O$

Leaf $\delta^{13}C$ and $\delta^{18}O$ were different between plants from the two studied sites and showed a clear genotype differentiation, which allowed their separation in two groups: group 1 - 1404 and 1407 clones; group 2 - 6500 and 1296. Significant differences in leaf $\delta^{13}C$ were found among populations under different rain regimes, where decreases in isotopic carbon composition were observed in plants collected in the regions with highest rainfall, except for clones 1404 and 1407. There were no significant differences among genetic materials evaluated at the same precipitation regime (Fig.5A).

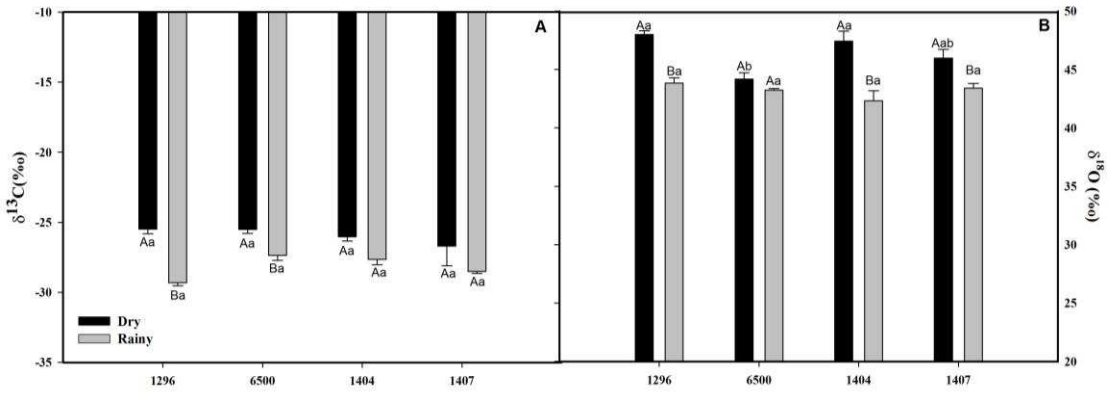

Figure 5: Isotope ratio $\delta^{13}C$ and $\delta^{18}O$ of Eucalyptus clones leaves collected in regions with different rainfall, after 10 months without rain. Capital letters mean significant differences within the same clone in different precipitation and lower case letters indicate differences among clones within the same precipitation, according to Tukey's test at 5% probability. Bars represent standard error (n=4).

Decrease in $\delta^{18}O$ enrichment under higher rainfall condition was observed in all genotypes, except for clone 6500. The lower enrichment in leaf $\delta^{18}O$ was found for 6500 at the drier region (Fig 5B). There were no $\delta^{18}O$ differences between genetic materials in the rainy site (Fig 5B). Analysis of data from all clones from both dry and rainy sites indicated that $\delta^{18}O$ was positively correlated to $\delta^{13}C$ (Fig. 6).

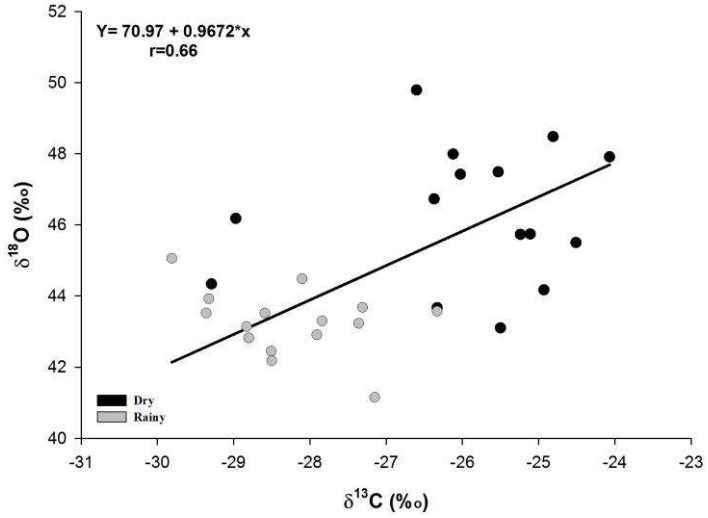

Figure 6: Relationship between $\delta^{13}C$ and $\delta^{18}O$ for plants grown in different precipitation regimes, after 10 months without rain. * Significant at 10 % of probability.

3.4. Nutrient content

There were no significant differences in K, Ca, Mg and B concentrations, among genetic materials grown at the drier site (Table 5). For clones 1404 and 1296, decreasing in water availability promoted an increased of B concentration in leaves.

Table 5: Nutrient concentration in leaves collected at the middle part of Eucalyptus plants growing in dry and rainy regions, after 10 months without rain

Clones	Site	Nutrient Concentration			
		K (g/kg)	Ca (g/kg)	Mg (g/kg)	B (mg/kg)
1296	Dry	8.22 ± 0.79Aa	6.38 ± 0.69Aa	3.64 ± 0.06Aa	98.64 ± 5.12Aa
6500	Dry	7.28 ± 0.46Aa	6.93 ± 0.48Aa	3.55 ± 0.18Aa	88.31 ± 9.03Aa
1404	Dry	8.18 ± 0.30Aa	6.02 ± 0.76Aa	3.87 ± 0.09Aa	112.15 ± 3.44Aa
1407	Dry	9.01 ± 0.99Aa	7.01 ± 0.88Aa	3.84 ± 0.11Aa	86.41 ± 8.95Aa
1296	Rainy	9.01 ± 1.00Aa	6.57 ± 0.44Aa	3.35 ± 0.28Aab	75.70 ± 6.36Ba
6500	Rainy	9.46 ± 0.39Aa	7.11 ± 0.20Ab	3.05 ± 0.19Aab	93.27 ± 9.80Aa
1404	Rainy	10.17 ± 0.87Aa	7.48 ± 0.30Aab	3.66 ± 0.16Aa	82.67 ± 9.57Ba
1407	Rainy	9.87 ± 1.53Aa	6.47 ± 0.49Ab	2.82 ± 0.07Bb	87.86 ± 7.22Aa

Mean ± standard error. Uppercase letters mean significant differences within the same clone in regions with different precipitation and lower case letters indicate differences among clones within the same precipitation, according to Tukey's test at 5% probability.

4. DISCUSSION

The results show clear physiological and morphological differences among clones growing at different precipitation regimes. It is important to emphasize that the stand age in 2013 ranged from 4.5 to 5.9 years old and during the growth period trees had been submitted to 800 (dry) or 1500 mm (rainy) annual mean rainfall. During 10 months previous to data collection (February of 2013) trees experienced water restriction, as the precipitation showed a decrease of 300 and 350 mm, respectively, at the drier and rainier sites. However, trees growing in the area with lower precipitation in regular years, had been submitted to higher water availability fluctuation which started soon after the first year of planting, contrastingly the trees growing at the rainy site were under more constant and high water supply along the rotation, except during the year before the sampling period (Fig. 1), when high reduction of water availability was observed.

4.1 Plants submitted to water deficit are morphologically and physiologically adapted to subsequent drought

Plants can be grouped according to different successful strategies to withstand drought stress, such as plasticity, avoidance, acclimation or resistance mechanisms. A new concept named “stress imprint” is used to refer to plant genetic or biochemical modifications resulting from previous stress exposure reflecting responses to future stresses (Bruce et al., 2007). Those previous modifications may provide the benefit of increased protection during future stress periods (Hulten et al., 2006). Our results suggest that those eucalypts trees growing in the site with usually higher precipitation (during the first 5 years of growth) were more stressed after a long period of drought (10 months without rain) as compared to those plants submitted more regularly to annual water-deficit situations.

The studied genetic materials showed distinct responses to drought, which allowed their separation in two groups, group 1, clones 1404 and 1407 (E. urophylla and E. urophylla x E. camaldulensis, respectively) and group 2, clones 6500 and 1296 (E. grandis x E. urophylla). In general, the group 1 clones had similar responses growing at the drier site as lower SLA and MDA, higher $\delta^{18}O$ enrichment, no differences in leaf $\delta^{13}C$, decreased in starch concentration as compared to the other two genetic materials. The group 2 had higher values of SLA, leaf $\delta^{13}C$, glucose, and MDA concentration and lower leaf cyclitols content in the drier site. The two groups growing in the more humid site had different responses to water stress.

Decreased leaf area (Mokotedi, 2010), higher antioxidants activity (Shvaleva et al., 2005) leading to lower lipid peroxidation and osmotic adjustment processes (measured by sugars accumulation) (Lemcoff et al., 2002; Merchant et al., 2006; Arndt et al., 2008; Callister et al., 2008; Warren et al., 2011), observed in this work, seems to be some of the important morphological and physiological adaptations in order to mitigate the water stress damage in drought-tolerant genotypes.

The lower specific leaf area observed for clones 1404 and 1407 is an important morphological parameter found in plants adapted to xeric environments (Abrams 1994; Teklehaimanot, 1998; Lamont et al., 2002, Miller et al., 2002) and have been described in some cultivars produced to better cope with drought condition (Cornish et al., 1991; Mencuccini and Comstock, 1999). In the rainy site, a reduction of SLA in clone 1407 indicates that the reduced water availability during 10 months, occurred in 2012/2013 contributed to diminish carbon assimilation by leaves, inhibiting cell expansion and subsequently resulting in slower growth rates (Metcalfe et al., 1990; Gonçalves and Passos, 2000; Pita and Pardos, 2000).

Eucalyptus clones with large leaves must be classified as high-risk clones for plant in water restriction prone areas (Pita et al., 2001). Thus, a lower SLA is an important drought-tolerance characteristic, resulting in a lower transpiration area and a reduced potential for water loss. No differences in SLA, between areas, were found for clones 1296 or 6500, and the highest SLA observed for clone 1296 could classify it as a high-risk clone to drought-prone areas.

During water deficit, the reduction of intercellular CO_2 concentration due to stomatal closure, promotes an imbalance between capture and utilization of light energy in the photosynthetic process, promoting excessive electron accumulation in chloroplasts membranes and leading to the formation of reactive oxygen species (ROS) (Fu and Huang, 2001; Ozkur et al., 2009; Ben Ahmed et al., 2009). In order to overcome oxidative stress, plants have developed defense mechanisms to scavenge ROS (Smirnoff, 1993) resulting in lower malondialdehyde (MDA, a product of lipid peroxidation) content, an indicator of higher resistance to drought (Shinozaki et al., 2003; Dhanda et al., 2004). The combination of the expression analysis of oxidative stress-related genes (as the CuZnSOD) and lipid peroxidation may indicate the cell stability and integrity in plants under drought.

Our results showed a significantly increase in CuZnSOD gene expression in all genotypes at rainy site, and clone 1296 had the highest expression value to this gene at both studied areas. Conversely, the MDA content was reduced by 34 % and 55 % in clones 1404 and 1407, respectively, in the drier site. It can be hypothesized that plants growing in the rainy site were not as much acclimated to water deficit as plants growing at drier site. Therefore, the negative effects of low water availability were more severe for plants growing in the primer than in the later site, as indicated by higher CuZnSOD gene expression and higher lipid peroxidation at the rainy site. Besides, the genetic materials 1404 and 1407 had the lowest concentration of MDA, at the drier

site as compared to clones 6500 and 1296. Altogether, these results suggest that the clones 1407 and 1404, known for year to be more tolerant to drought under field conditions, were coping better with further reduced water availability, showing higher resistance to drought and higher cell stability and integrity as compared to the other genotypes. Since a long-term reduction in productivity in drought-affected plants resulted from decreased leaf area and intercepted solar radiation (Osório et al., 1998), the lowest SLA found for clones 1404 and 1407 could have promoted lower radiation intercepted by canopy and, consequently, lower light energy in the photosynthetic apparatus, leading to a lower formation of reactive oxygen species (ROS) and resulting in lower lipid peroxidation, as supported by theirs low MDA content.

The drought-tolerant plants have different physiological adaptations, avoiding water loss, and, or, facilitating its uptake. As an example of these processes, a reduction in the leaf osmotic potential may occur as a result of the accumulation of compatible organic compounds, such as sugars, alcohols sugar, prolines, and betaines (Serraj and Sinclair, 2002). Higher concentrations of these solutes can contribute to reduce cell osmotic potential, allowing the movement of water molecules into the cells (Tyree and Jarvis, 1982; Bray, 1993). These solutes can also protect the cell membranes and protein complexes allowing normal activity of the metabolic machinery (Chaves et al., 2003).

Our data suggest that large and sudden reduction of water availability in 2012 at the rainy site and the long period without rain (10 months) promoted an increase in sucrose, myo-Inositol and raffinose concentration in leaves for clones 1407, 1296 and 6500. Clone 1404 had the highest glucose concentration at this site, compared to the other genetic materials. However it presented the the lowest starch concentration, indicating changes in C partitioning, as observed for other tolerant genotypes under drought (Dichio et al., 2006; Maraghni et al. 2013). It's

noteworthy that part of the observed glucose increment might be resulted from starch degradation (Chaves, 1991). However, it may be possible that only starch degradation contribution would not be enough to increase the glucose concentration after a long term water stress.

The lowest sucrose concentration was found for clone 6500 in the dry site. Increase on sucrose concentration under stress may be an important strategy for resuming growth because it can be metabolically recycled after periods of stress (Merchant et al., 2006).

All genetic materials had higher concentrations of raffinose and myo-Inositol in the rainy site, except for clone 1407, which could be important to the leaf osmotic adjustment processes.

A high expression of the RD26 gene was found by Thumma et al. (2012) after 2 months of water stress in Eucalyptus camaldulensis seedlings. However, at the present work we have found no differences in this gene expression. The absence of response could be due the tree development stages and the long term of drought leading to secondary responses after some months without raining.

4.2 Clone sensitive to drought does not change the stomatal aperture during water stress.

The carbon isotopic composition ($\delta\ ^{13}C$) can be used as proxy for the WUE (Farquhar et al., 1989) given the high correlation between WUE and $\delta\ ^{13}C$ that has been found in C3 species (Zhang et al. 1997, Martin et al., 1999 Chen et al. 2007; Moghaddam et al., 2013). Higher less negative $\delta\ ^{13}C$ indicates higher WUE and a higher WUE is extremely relevant to Eucalyptus forests, specially for those planting in more drought-prone areas.

In our study, clones 1296 and 6500 had their leaf $\delta\ ^{13}C$ values decreased with increasing water availability, which was expected, since in water limiting condition generally the C_i/C_a ratio and, or, the photosynthetic rating reduced, reflecting a decreased carbon discrimination in plants

(Farquhar et al., 1984). Similar results were found for different Eucalyptus species along a rainfall gradient in Australia (Miller et al., 2001; Schulze et al., 2006).

Simultaneous measurements of $\delta\ ^{13}CO_2$ and $\delta\ ^{18}O_2$ composition in plant tissue could provide important information about the physiological and environmental factors that regulate water-use efficiency (Flanagan and Farquhar, 2013), and seem to be a useful tool in the selection of drought tolerant genotypes (Cabrera-Bosquet et al., 2011; Yousfi et al., 2012). The $^{18}O/^{16}O$ composition of plant tissues may help separate the photosynthesis rate or stomatal conductance effects on C discrimination, as it is not thought to be strongly influenced by photosynthetic rate (Barbour and Farqhuar, 2000; Barbour, 2007; Farquhar et al., 2007). In agreement with several experimental system conditions (Switsur et al., 1996; Barbour and Farquhar, 2000), in the current study we observed higher $\delta^{18}O$ enrichment in leaf tissues from plants under lower water availability, indicating that if the $\delta^{18}O$ of source water is the same in both areas, a greater stomatal closure occurred on trees at the drier site. As theory explained by Barbour and Farquhar (2000), plants growing at drier sites are more enriched in ^{18}O than plants growing in more rainy conditions.

The positive correlation observed in our study between $\delta^{18}O$ and $\delta^{13}C$ in plant tissues was expected and has been also observed by other authors under field conditions (Sternberg et al., 1989; Saurer et al., 1997; Flanagan and Farquhar, 2013) or controlled conditions (Barbour and Farquhar, 2000). These results indicate that most of the $\delta^{13}C$ variation in Eucalyptus leaves was driven by changes in stomatal conductance (Barbour et al., 2002; Barbour, 2007).

Striking responses were found analyzing simultaneously $\delta^{13}C$ and $\delta^{18}O$ for clones 1404, 1407 and 6500. There was no difference in $\delta\ ^{13}C$ for 1404 and 1407, considered drought-tolerant genetic materials, growing at the two sites. However, both genetic materials had higher

enrichment of $\delta^{18}O$ at the driest area. As $\delta\ ^{13}C$ can be driven by changes in g_s or/and A (Farquhar et al., 1984), and a decreased gs was found in our study (higher $\delta^{18}O$ enrichment), possibly due to a reduction in the photosynthetic rate occurred to keep unaltered $\delta\ ^{13}C$ in those clones. An efficient stomatal closure is an important mechanism to avoid plant water loss, although it reduces C fixation and plant productivity, such growth loss may be preferable as compared to high tree mortality rate of the plantations subjected to intense and long duration drought.

Clone 1296 presented both stomatal conductance and photosynthetic rate as indicated by the high enrichment of $\delta\ ^{13}C$ and $\delta^{18}O$ at 500 mm. Interestingly, clone 6500, considered a drought-sensitive genotype, showed no difference in $\delta^{18}O$ enrichment of leaf tissues in both precipitation regimes, indicating lower ability to stomatal control under different water supplies. Therefore, this clone would not be considered a potential genetic material for drought-prone areas; however, it can be recommended for planting in areas without water restriction because of its high productivity rate under high rainfall sites.

5. CONCLUSIONS

Eucalyptus genotypes showed different abilities to acclimate to water stress under field conditions. Our results suggest that trees growing in areas with more and higher uniform annual precipitation were more stressed after a long period of drought in comparison to those stands more used to drier regimes and more frequent period. Previous exposure to water deficit may provide the benefit of increased defense protection during future water deficit stress. The studied genetic materials showed distinct responses to drought, which allowed their separation in two groups, clones 1404 and 1407, considered tolerant, and clones 6500 and 1296, more sensitive to drought stress. The combination of $\delta^{13}C$ and $\delta^{18}O$ extracted from leaves seems to be important tool to separate sensitive and tolerant genotypes to drought.

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CHAPTER 3

PRE-SELECTION OF EUCALYPTUS CLONES TO DROUGHT: PHYSIOLOGICAL APPROACH

ABSTRACT

Water stress is one of the abiotic stresses that most affect Eucalyptus growth and productivity in Brazil. The selection of tolerant genotypes has been the main way of ensuring the survival of the clones in water-deficient environments and is crucial for the high productivity. The work aimed to investigate physiological approach to pre-select tolerant eucalypt plants for drought by the evaluation in the gas exchange, chlorophyll a fluorescence, pigment content, ABA concentration and growth of seven Eucalyptus clones subjected to water stress. The clones were grown in Clark's nutrient solution with polyethylene glycol to simulate the water stress. The Eucalyptus clones responded differently to water stress with differentiated photosynthetic limitations in drought-stressed Eucalyptus clones. The photosynthetic rates, stomatal conductance, transpiration and internal CO_2 concentration were reduced in all genotypes under stress condition. Clone i144 had a smaller reduction in the evaluated characteristics besides showed increase in the root system weight when subjected to drought. It was followed in order by the clones 3367 and i224, potentially tolerant. The clones i042 and i182 were highly drought-susceptible, with greatly photosynthetic and growth reduction, even though showed a high increase in the intrinsic water efficiency and a thermal dissipation as a mechanism to reduce the damage on the photochemical components. The interaction between WUEg, ABA, A, E and SDM/RDM seemed to be the most important differences between clones under water stress.

Keywords: abiotic stress, clonal variability, clones, cluster analysis, Eucalyptus.

1. INTRODUCTION

Eucalypt short rotation plantations have expanded in Brazil since it was introduced in 1903. Nowadays, they represent 71 % (5,102,030 ha) of the total commercial forest area (ABRAF, 2013). The rapid growth of the forestry sector in Brazil and the high demand for forest products has led to expansion of new enterprises to areas with limited water supply, resulting in reduced growth and survival rates of trees, especially of the most drought-sensitive clones. Water stress is one of the limiting factors of production and survival of Eucalyptus (Campion et al., 2006; Stape et al., 2008). Thus, the selection of more tolerant genotypes has been the main way of ensuring the survival of clones in these water restrictive environments, since errors in the genotype choice or the forest management can lead to widespread death of stands during drought periods (White et al., 2009).

To cope with water stress, plants can trigger processes that prevent dehydration, maintaining higher water potential, or that allow tissues to tolerate a lower water potential (Chaves et al., 2003). In the first case, the plants use strategies that minimize water loss through the regulation of transpiration via stomatal control (Pita and Pardos, 2001; Macfarlane et al., 2004). Given that the stomata is the first barrier for the entrance of CO_2 for photosynthesis, reductions in the stomatal conductance may lead to reductions in the photosynthesis rate. This means that photosynthesis is the physiological process primarily affected by water stress (Roupsard et al., 1996; Warren et al., 2004; Warren et al., 2007). The reduction in the photosynthetic rate in plants subjected to mild or moderate and short-term water stress is usually due to stomatal closure (Chaves, 1991; Roupsard et al., 1996; Pita and Pardos, 2001; Flexas et al., 2002; Flexas et al., 2004; Macfarlane et al., 2004; Erismann et al., 2008; Chaves et al., 2009) mediated by the hormone ABA (Liu et al., 2005; Schachtman and Goodger 2008). In response to

stresses such as drought, this substance regulates adaptive responses to the restrictive conditions (Chaerle et al., 2005; Wasilewska et al., 2008).

Under severe and long-term drought, the reduction in the photosynthetic rate may also be the result of biochemical limitations in the photosynthetic metabolism (Lawlor, 2002; Lawlor and Cornic, 2002), such as limitations in phosphorylation (Lawor and Tezara et al., 2009), ribulose-1,5-bisphosphate (RuBP) regeneration (Medrano et al., 2002) and Rubisco carboxylation (Zhou et al., 2007). Both processes favor a reduction of C assimilation by plants and lead to super excitation and accumulation of reducing power in the leaves (Epron et al., 1992). Therefore, protection mechanisms against excess reducing power are important strategies for drought-stressed plants. These mechanisms compete with the photochemical phase by the absorbed energy, leading to a decrease in the quantum yield of photosystem II (Genty et al., 1989), increase in thermal dissipation (Demming-Adams and Adams, 1996) and protection of the photosynthetic apparatus against oxidative damage and photoinhibition (Barber and Andersson, 1992; Niyogi et al., 1999; Alves et al., 2002; Adir et al., 2003).

Other important tolerance mechanisms are known, such as increased activity of antioxidant enzymes (Lima et al., 2002; Mittler, 2002), adjustment of the osmotic potential (Hare et al., 1999; Anderson et al., 2001; Sakamoto et al., 2002; Merchant et al., 2006; Arndt et al., 2008; Warren et al., 2011), alteration in the production of polysaccharides present in root cell walls (Leucci et al., 2008), among others, and have been suggested as means of adaptation or tolerance of plants to drought.

In addition, the simultaneous measurement of gas exchange and chlorophyll a fluorescence is becoming an important tool in understanding the relationship between the use efficiency of light, CO_2 assimilation and photoinhibition (Maxwell and Johnson, 2000), aside

from being an important indicator of water stress in plants (Epron et al., 1992; Flexas et al., 1999).

Understanding the physiological, biochemical and hormonal responses resulting from drought effects on different Eucalyptus genotypes, and their discrimination in genotypes with differential tolerance to water stress may be fundamental for the selection of drought-tolerant clones. Thus, the purpose of this study was to evaluate variables able to discriminate and group clones with differential tolerance to water stress and provide markers for screening young eucalypt plants.

2. MATERIAL AND METHODS

2.1 Cultivation and harvesting conditions

Seedlings of seven Eucalyptus sp clones (1528, 3367, gg157, i042, i144, i182, i224), were grown in a greenhouse in plastic pots (8 L) and acclimated in Clark nutrient solution (Clark, 1975). The clones i042 and i144 are featured in the field as drought-sensitive and tolerant, respectively, and were used as reference for comparison with other genotypes in this study. After 30 days of acclimation, the plants were separated into two groups. Half of the plants remained in solution with water potential near 0 MPa and the other half was subjected to water stress, by the gradual addition of polyethylene glycol 6000 (PEG 6000).

The PEG 6000 doses were added every five days to gradually reduce the water potential of the solution (to -0.16, -0.32, -0.65, and -1.0 MPa) (Michel and Kaufmann, 1973). The experiment was arranged in a randomized block, in a 10 x 2 factorial design scheme, with four replications.

Five days after application of a PEG 6000 dose to reduce the water potential of the solution to -1 MPa, the plant height (cm) and stem diameter (SD) were measured. Subsequently, the plants were harvested and separated into leaves, stem and roots. The materials were wrapped in paper bags, oven-dried and weighed to determine the shoot dry matter (SDM), shoot and root dry matter ratio (SDM/RDM) and total dry matter (TDM).

2.2 Determination of gas exchange

The gas exchange was measured in fully expanded leaves. We determined the net photosynthetic assimilation rate (A), stomatal conductance (g_s), transpiration rate (E), and the ratio between internal and external CO_2 concentration (C_i/C_a) with a portable photosynthesis

system LI-6400 (LI-COR Bioscience Inc., Lincoln, NE, USA). The instantaneous water use efficiency (WUEi; A/E) was calculated as the ratio between A and E and the intrinsic water use efficiency (WUEg; A/g_s) was calculated as the ratio between A and $/g_s$. The measurements were performed in a greenhouse between 8:00 and 12:30 am, under constant photosynthetically active radiation (PAR) (1400 μmol photons m^{-2} s^{-1}), atmospheric CO_2 concentration (C_a) (~372μmol mol^{-1}), at environmental temperature (22 - 28 °C) and humidity (48-66 %).

2.3 Chlorophyll a fluorescence analysis

Variables of chlorophyll a fluorescence were measured in the same leaf area as the gas exchange, with the IRGA (LI-6400xt). The leaves were dark-adapted so that the reaction centers were completely open (all primary electron acceptors oxidized) with minimum heat loss. The variables initial fluorescence (F_0) and maximal fluorescence (F_m) of fluorescence induction were measured. From these data, the potential quantum yield of photosystem II (PSII), $F_v/F_m = (F_m-F_0)/F_m$ was calculated (Genty et al., 1989). Variables of the slow fluorescence induction phase were obtained gradually by applying an actinic light and actinic saturating light pulse to determine fluorescence variables in a sample that was light-adapted prior to the saturation pulse (F) and maximum fluorescence in a light-adapted sample (F_m'). From these data, the minimum fluorescence of the illuminated plant tissue was calculated by the expression: $F_0' = F_0/[((F_m-F_0/F_m)+(F_0/F_m'))]$ (Oxborough and Baker, 1997), to determine the coefficient photochemical quenching by the model lake, which provides an estimate of the open PSII reaction centers by $q_L = (F_m'-F)/(F_m'-F_0').(F_0'/F)$ (Kramer et al., 2004). The effective quantum yield of photochemical energy conversion in PSII, $Y_{II} = (Fm'-F)/Fm'$, was calculated as proposed by Hendrickson et al. (2004). The apparent electron transport rate was also estimated using Y_{II}, by the formula: ETR

$=Y_{II}$.PAR x abs x 0.5 (Bilger et al., 1995), where PAR is the photon flux (µmol m^{-2} s^{-1}) incident on the leaf; the value 0.5 corresponds to the energy fraction of excitation distributed to the PSII (Laisk and Loreto, 1996); and, abs, leaf absorbance corresponding to the fraction of incident light absorbed by the leaves (Ehleringer, 1981).

2.4 Quantification of Abscisic Acid (ABA)

ABA was extracted from ground frozen leaf material (100 mg) using methanol (80 %, 1 mL) as extraction solvent, according to the protocol of Durgbanshi et al. (2009). Deuterated ABA (ABAd4) standard was added during the extraction process to correct the data. An aliquot of 200 µL of the sample extract had the methanol removed in a speedvac at 30 °C. Water was added to give a total volume of 500 µL and the pH was corrected to 3.0 (100 µl of 10% (v/v) acetic acid). Diethyl-ether (500 µl) was added to allow phases separation. The organic phase was collected in a tube and the extract was washed again in diethyl-ether, repeating the process. The extract was dried using a heat block in fume hood. The samples were dissolved in LC-MS buffer (same solvent as start of HPLC gradient). The leaf extracts containing ABA were analyzed in a Triple Quadrupole LC-MS (6430, Agilent Technologies) with the following settings: column (Agilent Eclipse Plus, RRHD, 1.8 um, 2.1x50 mm with guard column), solvents (A: Acetonitrile + 0.1% formic acid, B: Water + 0.1% formic acid), flow (0.3 mL/min) for 7 min (gradient time/B%: 0/81, 3/50, 4/10, 4.25/10, 4.5/81). The detection and quantification of ABA in the samples were made by multiple reaction monitoring (MRM) by means of selecting the transition density of the molecule of interest (ABA 263 → 153; ABAd4 267 → 156 (Dwell 200, Fragmentor 60, CE 6, Accelerator voltage 7, Negative). The data were processed using the software MassHunter.

2.5 Cloroplastidic Pigments

The pigment contents (chorophyll a, chorophyll b and carotenoids) were determined using dimethyl sulfoxide (DMSO) as extractor as described by Wellburn (1994). Two leaf discs were extracted in 5 mL of DMSO saturated with calcium carbonate ($CaCO_3$) under dark condition. After four hours in a water bath at 65 ºC, the absorbance was measured on the wavelength: 480, 649.1 e 665.1 nm in spectrophotometer. Pure DMSO was used as blank. Absorbance values were used to estimate chorophyll a, chorophyll b and total carotenoids.

Chorophyll a (Ca): $12.47 \times A_{665,1} - 3.62 \times A_{649,1}$

Chorophyll b (Cb): $25.06 \times A_{649,1} - 6.5 \times A_{665,1}$

Total carotenoids: $(1000 \times A_{480} - 1.29 \times Ca - 53.78 \times Cb)/220$

The pigments concentration was expressed by leaf area.

2.6 Statistical Analysis

The ANOVA (F test, $p < 0.05$) analyses were performed using STATISTICA Software (7.0). Hierarchical clustering and heat maps were performed on mean-centered data scaled to unit variance using MEV (MultiExperiment Viewer) software v.4.9.0. with Pearson's correlations and complete linkage.

3. RESULTS

3.1 Gas exchange, pigments, ABA and WUE

The net CO_2 assimilation rate (A) of drought-stressed Eucalyptus plants was reduced in all genotypes (Figure 1A). The largest reductions in A (74.8 % and 81.19 %) were observed for clones gg157 and i042, respectively, reinforcing the sensitivity of clone i042 to water stress, as reported by Nunes (2010) for field conditions. In drought-stressed clones 3367 and i144, the negative effect on A was still large but less pronounced (reduction of 40.36 % and 30.82, respectively) than in the control plants (Figure 1A). Similarly as in the case of the reduction of the photosynthetic rate, water stress caused a reduction in stomatal conductance (gs), ratio between internal and external CO_2 concentration (C_i/C_a) and transpiration rate (E) (Figure 1B, 1C and 1D) ($p < 0.05$).

The largest reductions in gs (93.28 %) and E (87.94 %) were observed in clone i042. Clones 3367, i144 and i224, on the other hand, had the highest E (2.14, 2.81 and 2.02 mmol m^2s^{-1} H_2O, respectively) under water stress (Figures 1B and 1D).

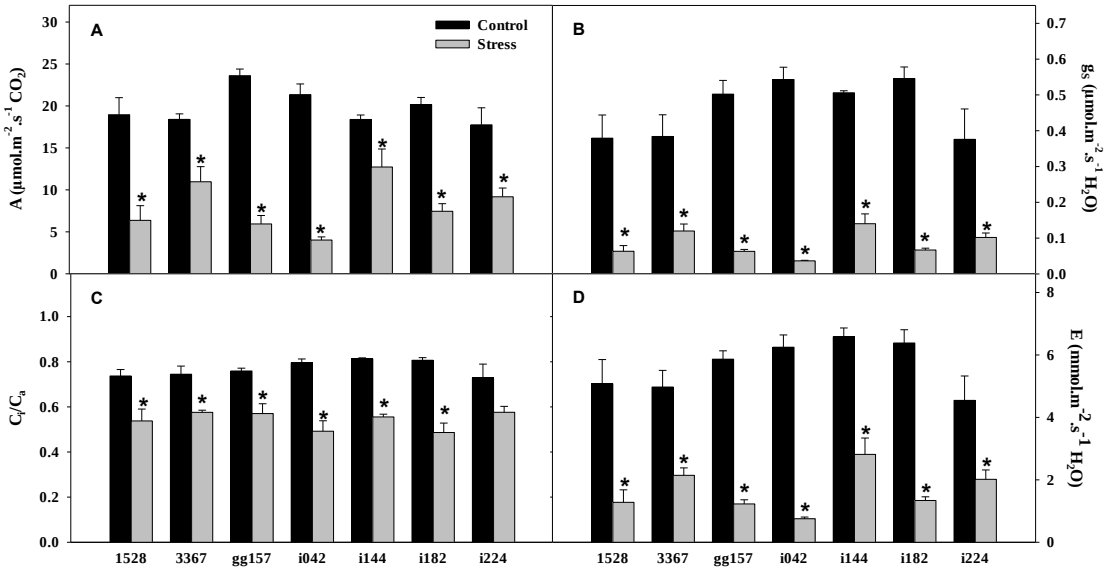

Figure 1: Net CO_2 assimilation rate (A; A); stomatal conductance (gs; B); ratio between internal and external CO_2 concentration (C_i/C_a; C); transpiration rate (E; D) in Eucalyptus clones under sufficient water supply (control) or subjected to water stress (stress). Bars indicate mean±SE (n=4). Asterisks (*) means significant difference between water stress and control plants of the same clone, $p< 0.05$ (F test, Statistic 7.0).

The chlorophyll a concentration increased in the clones i042 (26.1 %), i144 (34.6 %) and i182 (37.2 %) subjected to drought (Fig. 2A). The greatest increase in the chlorophyll b concentration was observed in clone i144 (41.8 %) followed by clone 3367 (41.1 %) whereas the lowest increase was observed in clone i182 (34.6 %) (Fig. 2B). No differences in the chlorophyll b concentrations were found for clones 1528 and gg157. The clone i144 had the highest reduction on the total carotenoids (-10.1 %) and the other clones showed no difference on this characteristic (Fig. 2C).

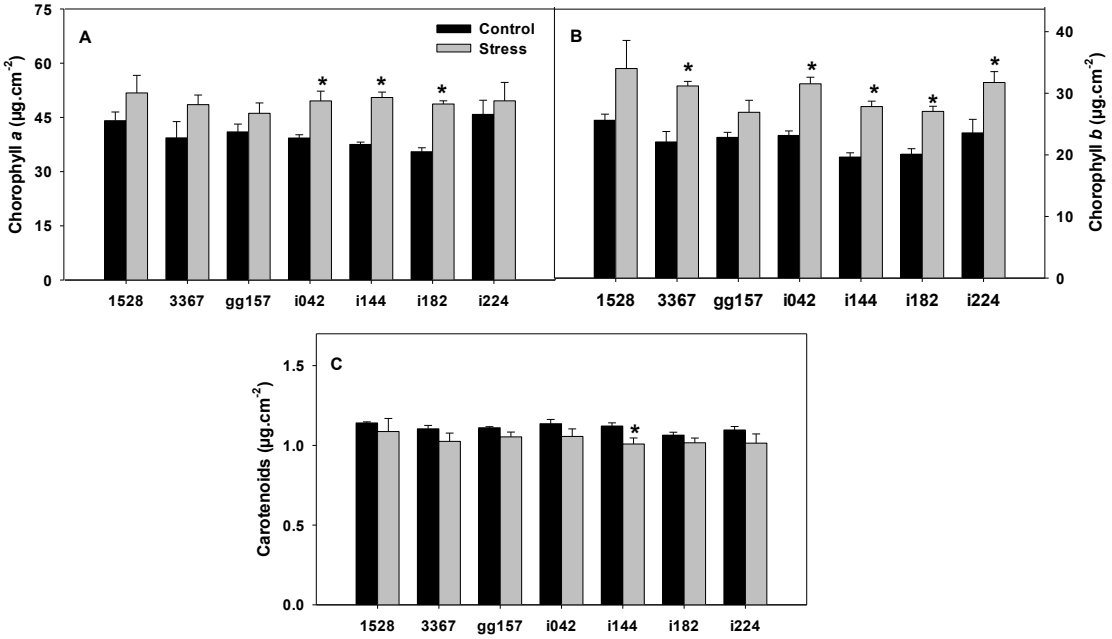

Figure 2: Chlorophyll a (A), chlorophyll b (B) and carotenoids (C) content in Eucalyptus clones under sufficient water supply (control) or subjected to drought (stress). Bars indicate mean±SE (n=4). Asterisks (*) means significant difference between water stress and control plants of the same clone, $p< 0.05$ (F test, Statistic 7.0).

Water deficit increased the ABA levels in leaves, except in clones 1528, gg157, 3367 and i182. The largest increase was observed in clones i224, i144 and i042 (153.2, 75.6 and 74.2 %, respectively) (Fig. 3A). We find no correlation between ABA and g_s (Fig. 3B).

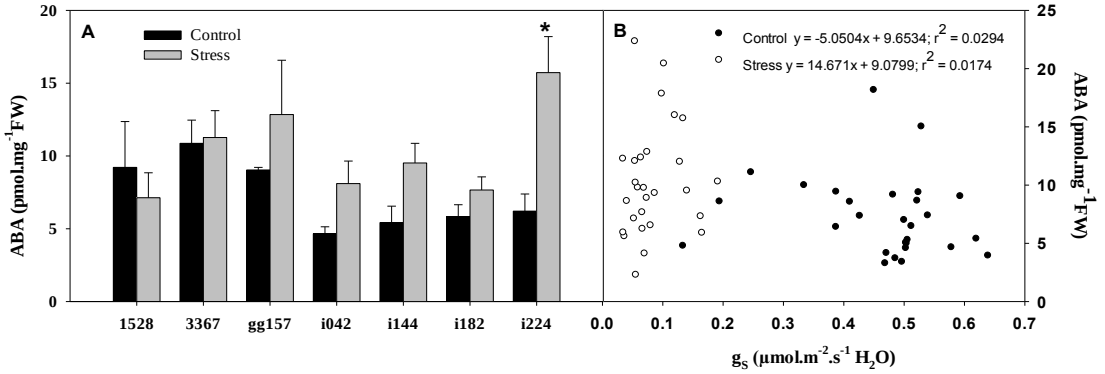

Figure 3: Abscisic acid (ABA) content (A) and ABA versus gs (B) in Eucalyptus clones under sufficient water supply (control) or subjected to drought (stress). Bars indicate mean±SE (n=4). Asterisks (*) means significant difference, between water stress and control plants of the same clone, $p< 0.05$ (F test, Statistic 7.0).

The instantaneous water use efficiency (WUEi) as well as of intrinsic water (WUEg) increased in drought-stressed plants (Figs. 4A and 4B) indicating that the decreases in transpiration and conductance rates were proportionally higher than that of the photosynthesis. The greatest increases in WUEg were measured in clones i042 (2.8 fold) and i182 (3-fold) and the lowest (1.6-fold) in clone i224 (Fig. 4B). Increases in WUEi were greatest for clone i182 (42 %) and i144 (39 %) and the smallest for clones gg157 and i224 (18.31 % and 11.38 %, respectively) (Fig 4A).

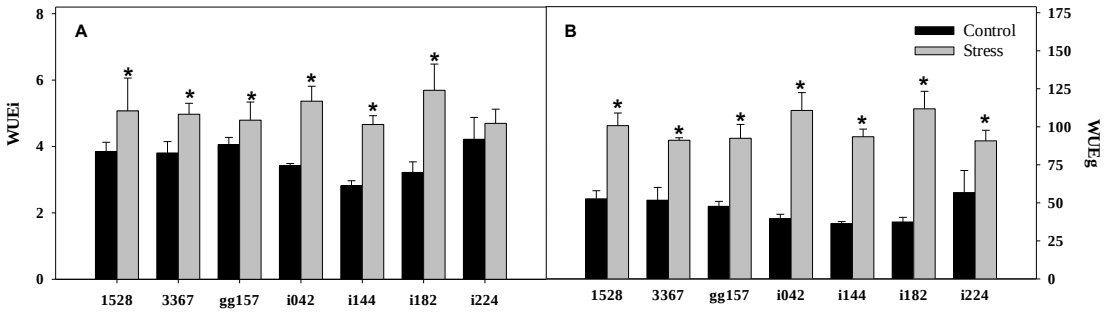

Figure 4: Instantaneous water use efficiency (WUEi) (A) and intrinsic water (WUEg) (B) in Eucalyptus clones under sufficient water supply (control) or subjected to drought (stress). Bars indicate mean±SE (n=4). Asterisks (*) means significant difference between water stress and control plants of the same clone, $p< 0.05$ (F test, Statistic 7.0).

3.2 Chlorophyll a fluorescence

In general, the initial chlorophyll a fluorescence (F_0) was not affected in drought-stressed plants (Figure 5A). Reductions of 7.91 % and 7.00 % were observed in the clones i182 and 3367, respectively. No differences were observed in the potential quantum efficiency of PSII (F_v/F_m) of plants under adequate or restricted water supply ($p < 0.05$) (Figure 5B).

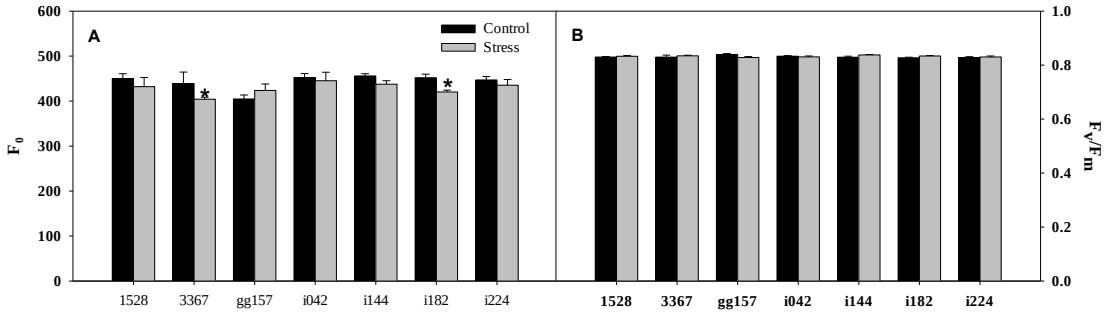

Figure 5: Initial chlorophyll a fluorescence (F_0; A); potential quantum yield of PSII (F_v/F_m; B) in Eucalyptus clones under sufficient water supply (control) or drought (stress). Bars indicate mean±SE (n=4). Asterisks (*) means significant difference between water stress and control plants of the same clone, $p < 0.05$ (F test, Statistic 7.0). $p < 0.05$ (F test, Statistic 7.0).

The responses in the electron transport rate (ETR) were reduced dramatically by the drought onset, with the largest reduction (96.42 %) for clone i042, and the lowest (48.94 %) for clone i144 (Figure 6A). The estimate of open centers (qL) increased significantly in clone i144 (82.79 %) and decreased in clones 3367, i042 and i224 (14.54 %, 42.82 %, 8.28 %, respectively) (Figure 6B). No changes were observed in the qL of the other clones ($p < 0.05$).

The coefficient of non-photochemical quenching (NPQ) increased by 59.62 %, 67.38 %, 48.75 %, 56.25 % in the clones 1528, gg157, i042 and i182, respectively (Figure 6C). In the clones 3367, i144 and i224, no changes in NPQ were detected.

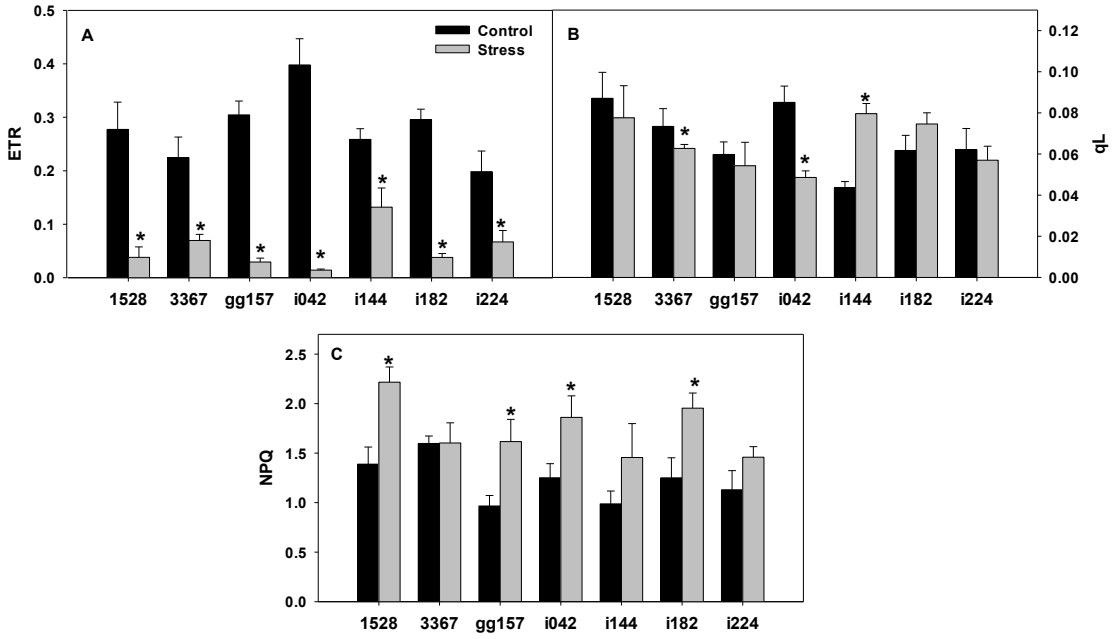

Figure 6: Electron transport rate (ETR; A); coefficient photochemical quenching (qL; B); coefficient of non-photochemical quenching (NPQ; C) in Eucalyptus clones under sufficient water supply (control) or drought (stress). Bars indicate mean±SE (n=4). Asterisks (*) means significant difference, $p < 0.05$ (F test, Statistic 7.0).

The A/Ci and ETR/A ratios were reduced in almost all clones under water stress (Figure 7A and 7B). The greatest reductions were observed in clone i042 (69.02 % and 80.05 %, respectively).

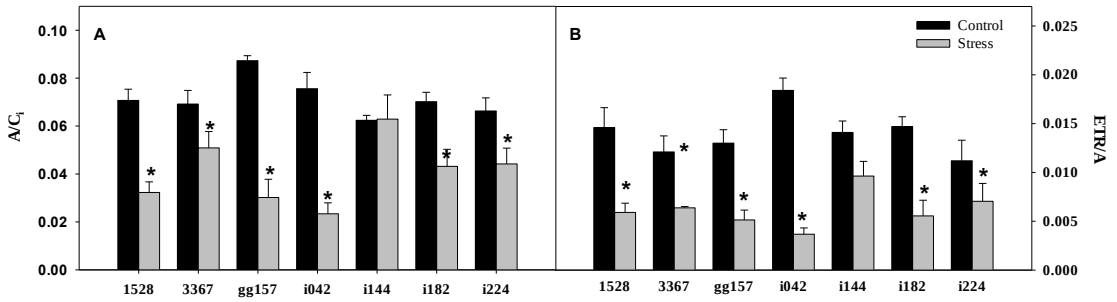

Figure 7: A/Ci (A) and ETR/A ratios (B) in Eucalyptus clones under sufficient water supply (control) or drought (stress). Bars indicate mean±SE (n=4). Asterisks (*) means significant difference between water stress and control plants of the same clone, $p < 0.05$ (F test, Statistic 7.0).

3.3 Growth and SDM/RDM ratio

After a short period of water deficit, height (H, data not shown), shoot dry matter (SDM) and total dry matter (TDM) were most affected in clone i042, with reductions of 26.2 %, 52.7 % and 47.9 %, respectively (Figure 8A and 8C). The reduction in SDM (31.49 %) was the lower in clone i224 (Figure 8A).

The lowest ratios of shoot and root dry matter (SDM/RDM) were found for clones 1528 and gg157 under drought (Figure 8B). This SDM/RDM decreased most in clone gg157 (54.12 %) and least in clone 3367 (13.17%), compared to plants under adequate water supply (Figure 8B).

No significant changes in the TDM of plants of clones 1528, gg157, i144 and i224 were detected under drought (Figure 8C).

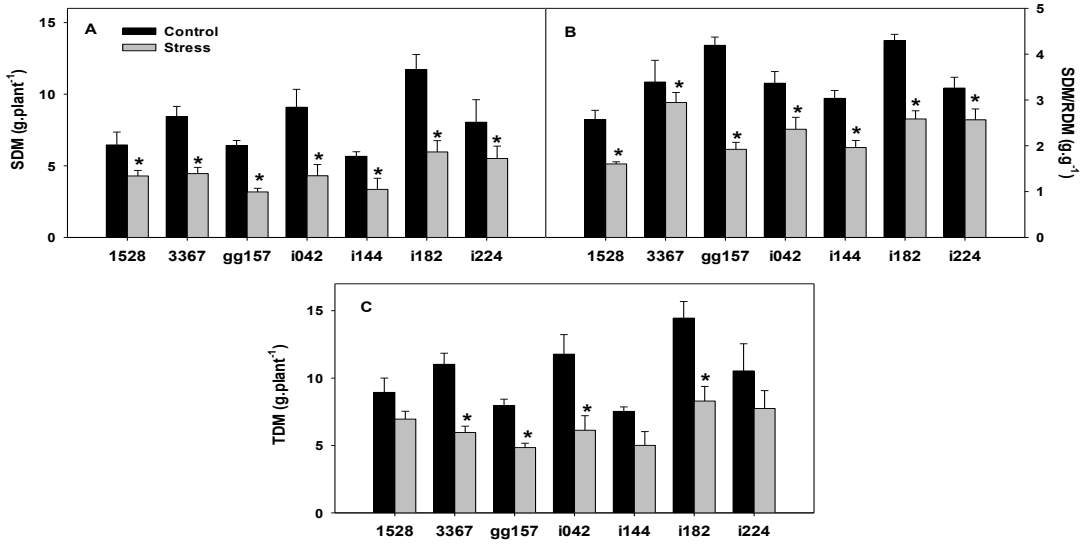

Figure 8: Shoot dry matter (SDM; A), shoot and root dry matter ratio (SDM/RDM; B) and total dry matter (TDM; C) in Eucalyptus clones under sufficient water supply (control) or drought (stress). Bars indicate mean±SE (n=4). Asterisks (*) means significant difference between water stress and control plants of the same clone, $p < 0.05$ (F test, Statistic 7.0).

3.4 Clustering by principal component analysis

By the multivariate analysis of 15 physiological and 3 morphological variables, the studied clones could be discriminated according to their similarity. The data obtained from principal component analysis (PCA) were supported and extended by cluster analysis (Figure 9). Responses were grouped into 2 general main clusters: the first cluster was composed of ABA content, pigment contents and fluorescence parameters and the second was comprised of gas-exchange-related responses and growth variables.

The principal component analysis separated the clones into the following order: i042 = gg157 < i182 = 1528 < 3367 < i224 < i144 (Fig. 9). Generally, the SDM/RDM, gs, E, A, ETR,

A/Ci, Ci/Ca, SDM and TDM presented a decreased in the water deficit treatment, while NPQ, A/gs, Chl a and b, Carotenoids and ABA had an increased under such condition.

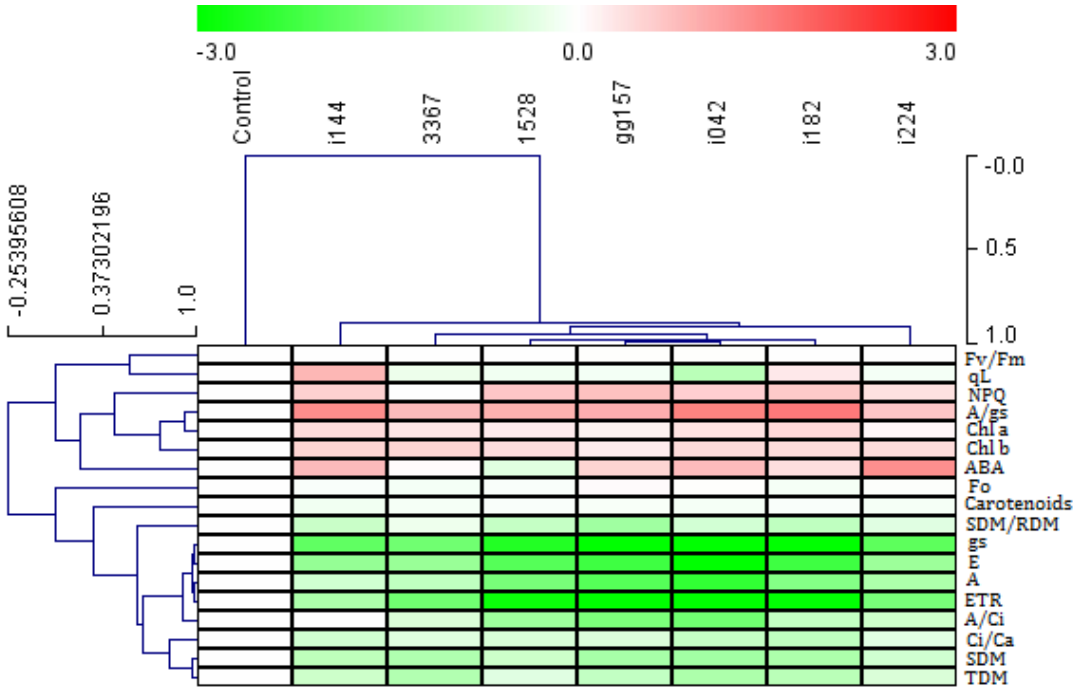

Figure 9: Hierarchical clustering analysis of physiological and growth variables in Eucalyptus clones under sufficient water supply (control) or drought (stress). The clustering analysis was performed by Pearson's correlation, mean centered, and scaled to unit data. Columns correspond to Eucalyptus clones, and rows correspond to physiological and growth variables. The colors indicate response values below (in green), equal to (white) or above (red) the samples' average normalized by the respective control. Experimental replicates were used to obtain dendrograms (n=4).

4. DISCUSSION

4.1 Relation of water deficit and Gas exchange, ABA and water use efficiency

Water deficiency is one of the most limiting abiotic factors to Eucalyptus productivity (Campion et al., 2006; Stape et al., 2008). In this genus, the physiological and morphological disturbances caused by low water availability reduce the leaf area (Mokotedi et al., 2010), photosynthetic rate (Warren et al., 2011), metabolite production (Warren et al., 2012), growth, and productivity (Macfarlane et al., 2004), among other interferences. In this study, gas exchange as well as biomass accumulation were affected differently in drought–stressed Eucalyptus genotypes.

Photosynthesis is one of the primary processes initially affected by water restriction (Chaves, 1991). The imposition of drought to clones resulted in reductions in A, Ci, E and gs, which were more pronounced in clones i042 (sensitive), gg157 and i182. Similar results were observed in five drought-stressed Eucalyptus species (Warren et al., 2010).

The reduction in stomatal conductance is the first and fastest response to reduced water supply (Chaves et al., 2003) and can be mediated by higher ABA concentrations (Liu et al., 2005; Schachtman and Goodger 2008), leading to increased water use efficiency (Parry et al., 2005) and avoiding unnecessary water loss. Clones i042, i144 and i224 contained higher ABA levels in the leaves and greatly reduced stomatal conductance than the others clones, however no significant correlation were obtained between ABA and gs. For the genotypes 1528, gg157, 3367 and i182 however, there were no differences in ABA leaf concentrations, but also greatly reduced stomatal conductance under drought. These results indicating that stomatal closure in these genotypes was not mediated by ABA or that after its effect on stomata, this hormone was

rapidly metabolized during the first stages of drought without effective deposition and accumulation in the leaves (Jiang and Hartung, 2008).

Higher ABA concentrations are usually associated with stronger tolerance signaling pathways for this hormone (Jiang and Hartung, 2008) and have a positive effect on water saving and increase the agricultural quality (Dodd et al., 2006). Clone i144 had an increase in ABA concentration of approximately 40 %, possibly leading to an increase of 39 % in WUEi. Increases in water use efficiency are considered an important elasticity strategy observed in drought-tolerant genotypes (Li et al., 2009).

4.2 Differential photosynthetic limitations in drought-stressed Eucalyptus clones

In this study, we found clonal different photosynthetic limitations caused by water stress. In the long term and under moderate stress, the gs reduction can induce a decrease in the CO_2 concentration at the carboxylation sites of RUBISCO (Flexas et al., 2004; 2007), with consequent stomatal limitation that reduces the photosynthetic rates. However, in this study, the CO_2 concentrations (Ci) and A/C were not proportionately reduced for most clones (1528, gg157, i042, i182), indicating, in this situation, the occurrence of changes or damage to the photosynthetic metabolism (Lawlor and Cornic, 2002), resulting in non stomatal limitation of A. These biochemical limitations are generally associated with reduced RUBISCO activity (Parry et al., 2002), limitation of phosphorylation (Tezara et al., 1999) and of RuBP regeneration (Gimenez et al., 1992).

The RuBP regeneration capacity is limited by drought, which may be due to a reduction in the electron transport rate and, consequently, in NADPH (Flexas et al., 2004). These authors found that the decrease in the photochemical efficiency was evidenced by a high reduction in the

electron transport rate (ETR), accompanied by a reduction in photochemical quenching (qL), phenomena especially observed in the drought-sensitive clone i042 under water stress.

Decreases in A and ETR are generally proportional, indicating a strong link between the photosynthetic processes (Foyer et al., 1990). On the other hand, a decrease in A and maintenance of ETR, leading to an increased ETR/A ratio, indicate that water stress caused no inhibition of mechanisms of the photochemical reactions (Lal et al., 199; Flexas et al., 1998; Flexas et al., 1999; Singh and Reddy, 2011). Rather, it was associated with increases in photorespiration (Wingler et al., 2011) to avoid excessive excitation energy and protect the photosynthetic apparatus from photoinhibition (Bai et al., 2008; Guan and Gu, 2009). However, for almost all genotypes, the ETR/A and A/C ratios decreased, especially for clones i042 and i182. These responses suggest that the reduction in the electron transport rate was as high as the drop in the photosynthetic rate during drought. Although the possible biological limitations were not measured in this study, the drastic changes in the above mentioned processes indicate predominance of non-stomatal limitations in these genotypes.

Conversely, in the drought-tolerant genotype i144 the reductions in ETR and increase in qL were less drastic, apart the unchanged A/C and ETR/A ratios, indicating absence of metabolic limitations, while the small reduction in A may have occurred due to stomatal limitations. Clones 3367 and i224 were similar to i144 in these characteristics. These results suggest that these genotypes have a greater potential of post-stress recovery, for not requiring the recovery of the photosynthetic apparatus, which can be damaged in drought-sensitive clones during water stress periods.

The higher photosynthetic rate during the early drought stages also increases plant survival and dry matter accumulation (Parry et al., 2005) and in genotypes with higher A under water stress, photoinhibition of photosynthesis is reduced (Singh and Reddy, 2011).

Another energy dissipation mechanism resulting from reducing power accumulation (NADPH) and ATP produced in the photochemical phase is its release in the form of thermal energy, measured by NPQ. Thermal dissipation is directly related to the xanthophyll cycle in the antenna complex of photosystem II, and is an important protection mechanism of photosynthesis, reducing the formation of singlet oxygen and other reactive oxygen species (ROS) (Niyogi, 1999). At high levels, ROS can hamper the cell integrity, for being able to promote oxidation and depolymerization of nucleic acids, peroxidation of lipids in the plasma membrane, and protein denaturation (Arora et al., 2002). The dissipation of absorbed energy through thermal dissipation (NPQ) increased most in clones 1528 and i182. In these genotypes, the thermal dissipation may have been important to avoid photoinhibition.

4.3 Genotype growth and classification

In general, the clones used in this study are grown in the Northern, Midwestern and Southeastern of Brazil and few were studied for drought susceptibility or tolerance. Similar to the field observations (Nunes, 2010), the clones i042 (sensitive) and i144 (tolerant) remained in opposite groups in the principal component analysis. The other genotypes were found in intermediate positions between the two above clones.

Clone i144, considered drought-tolerant in the field, performed well under drought in this study. This good performance may have been the result of minor changes in A, gs, C_i/C_a, and minor damage to the photosynthetic apparatus and lower SDM/RDM ratio. The lower SDM/RDM ratio indicates expansion of the root system at the expense of shoot growth and

allows a better recovery of Eucalyptus after water stress (Mokotedi et al., 2010). To increase water uptake, many plants increase root growth, laterally or in depth (Le et al., 2011) and in Eucalyptus species, the difference in drought tolerance was attributed to differences in root growth in depth (Agency et al., 2007; Mokotedi et al., 2010) and the hydraulic conductivity of the root system (Costa and Silva et al., 2004). Thus, the above characteristics may have led to a greater increase in WUEi and WUEg and continued growth despite water stress. The performance of the clones 3367 and i224 was similar to that of the tolerant clone (i144) in most characteristics evaluated.

The largest reductions in growth and photosynthetic rate observed for clone i042 reinforce its drought-susceptibility. It is noteworthy that although clones i042 and i182 were in closed groups by multivariate analysis, the last genotype is moderately drought-tolerant in the field, but this potential was not expressed under the greenhouse conditions in this study. By the observed distribution of the dendrograms and univariate analysis of studied traits, the other genotypes i224, 3367, 1528 were classified as moderately drought-tolerant.

Physiological (e.g., photosynthesis, gas exchange, fluorescence of chlorophyll a), biochemical (e.g., pigments contents) and hormonal (e.g., ABA content) variables are important attributes to be considered in the evaluation of water stress tolerance. Even though, from those measurements, we were not able to select a single variable to be a marker of drought-tolerance in early Eucalyptus stage. The interaction between WUEg, ABA, A, E and SDM/RDM seemed to be the most important differences among clones under water stress.

In conclusion, the method utilized in the present work and the physiological, biochemical, morphological and hormonal characteristics identified in different Eucalyptus genotypes, should be considered as a preliminary study in that it readily identifies a reduced pool

of genetic materials for the much-needed further analyses of heritability and genotypic variation that must be addressed.

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CHAPTER 4

NUTRITIONAL EFFICIENCY IN EUCALYPTUS CLONES UNDER OSMOTIC STRESS

ABSTRACT

Plant nutrition is affected by the water supply level. Under water stress, the transport of nutrients in the soil is hampered and nutrient uptake by plants is reduced, which can affect plant growth. The magnitude of this process is dependent on plant morphology and genetic characteristics. The objective of this study was to evaluate the differences in growth, biomass accumulation, and nutritional efficiency of Eucalyptus clones under water stress. Seedlings of 10 Eucalyptus clones (1528, 1641, 3367, gg157, i042, i144, i182, i224, PL040, and vc865), were grown in a greenhouse in Clark's nutrient solution. Two Eucalyptus genotypes, one classified as drought-sensitive (i042) and the other as drought-tolerant (i144) in the field, were used as reference for comparison with other eight genotypes. After an adjustment period, the plants were separated into two groups. The water potential of half of the seedlings was maintained close to 0 MPa and the other half was subjected to water stress by the addition of polyethylene glycol 6000 (PEG 6000). Thus, the experiment consisted of factorial 10 x 2 laid out in randomized block design, with four replications. Each experimental unit consisted of three plants per pot. Five days after the addition of a PEG 6000 dose corresponding to -1 MPa, the plant material was harvested. Water stress reduced the dry matter (DM) of young leaves, roots and whole plant drastically as well as the nutritional efficiency of most clones. Clone PL040 was the least efficient in nutrient uptake and use and the most sensitive to water stress. Interestingly, under water stress the drought-tolerant clone generally had high AE (absorption efficiency), but low nutrient UE (use efficiency), whereas the sensitive clone had low AE, low UE for root formation and high AE for leaf formation. Considering the growth and water and nutrient use efficiencies, clones vc865, i182, i144, gg157 can be grouped as drought-tolerant, gg157, 1528 and i224 as moderately drought-tolerant and the others, i042, 1641, 3367 and PL040, particularly the latter, as drought-sensitive.

Keywords: nutrient use efficiency, water use efficiency, drought tolerance, Eucalyptus

1. INTRODUCTION

Brazil has one of the highest forest productivity in the world (Poke et al., 2005), mainly of the Eucalyptus plantations (Júnior and Ahrens, 2010). Eucalyptus plantations have expanded into areas with low annual rainfall, which has directly affected the efficiency of uptake and use of growth resources such as nutrients, CO_2 and water (Stape et al., 2004).

Reductions in water supply can greatly alter the nutrient uptake (Brouder and Volenec, 2008), since water is the driving vehicle, interfering with nutrient uptake and use by the plant (Ferreira et al., 2008). Also, when water supply is reduced, the microbial activity in the soil is limited (Borken and Matzner 2009), reducing the availability of some nutrients as, for example, N. Additionally, prolonged drought periods can change and inhibit cell growth, resulting in smaller root surface and reduced nutrient uptake. Nutritional deficiency can impede the normal functioning of the plant metabolism and increase damage caused by drought. Morphological and physiological changes such as stomatal closure, osmotic adjustment, amount of water uptake channels, changes in root plasma membrane and root growth can be compromised in drought-stressed plants, since these characteristics are usually affected by the deficiency of certain nutrients (Roberts, 1998; Möttönen et al., 2001; Henzler et al., 2004; Möttönen et al., 2005; Teixeira et al., 2008; Hodecker et al., 2014).

The relationship of nutrient supply, plant physiology and growth has been registered in several works. Calcium is known to participate actively in signaling pathways of several environmental events and is believed to be involved in the regulation of various processes in response to abiotic stresses (Trewavas et al., 1998; Taiz and Zeiger, 2009), as, for example, the transduction of the process that regulates stomatal opening, mediated by the hormone ABA (Song et al., 2008). The nutrient plays, also, a fundamental role in the resumption of cambium

growth of the trees after water or heat stress (Fromm, 2010). In two eucalyptus species, Ca was important in reducing the negative effects caused by water deficit (Silva et al., 2009) and increased growth in common bean during water stress (Ballester-Fernandez et al., 1997).

Phosphorus has been considered the main limiting nutrient for Eucalyptus tree growth in Brazil (Barros and Novais, 1990). It is an essential constituent of ATP synthesis, for which its deficiency can cause reduction in CO_2 uptake and assimilation (Yong-Fu et al., 2006). Potassium controls stomatal closure and its deficiency can decrease photosynthetic rates (Mengel and Kirkby, 1982; Hawkesford et al., 2012), negatively affecting the production and accumulation of assimilates. On the other hand, the role of B in increasing drought tolerance was observed in different species (Möttönen et al. 2001, 2005; Hassan and El-Sayed, 2011; Hajiboland and Farhanghi, 2011; Hodecker et al., 2014), and our studies confirmed the great influence of B on the increase of water use efficiency in Eucalyptus urophylla (Hodecker et al., 2014). Further details on increasing the water use efficiency by nutrient management are laid out in the review of Waraich et al. (2011).

Given the above and since a reduced water supply can hamper nutrient uptake (Brouder and Volenec, 2008), knowledge on nutrient efficiency (nutrient uptake, transport and use) and water use efficiency in different Eucalyptus genotypes could represent an additional and important strategy of forest management in regions where water stress is common.

For many years, Brazilian forest companies used only the mean annual increment in stem volume as well as fiber yield as breeding criteria (Navarrete-Campos et al., 2012), without taking into consideration the demand of the growth factors, namely water and nutrients. Several studies addressed the main mechanisms of drought tolerance by Eucalyptus species (Pita and Pardos, 2001; Li and Wang, 2003; Costa and Silva et al., 2004; Macfarlane et al., 2004; Campion et al.,

2006; Merchant et al., 2006; Chen et al., 2007; Tausz et al., 2008; Granda et al., 2011; Warren et al., 2011; 2012). However, there is no primary mechanism of drought tolerance that could underlie the selection of tolerant genotypes, which hampers breeding, due to the complexity of drought-tolerance-related traits, low genetic variance in the components that define the response to stress conditions and the lack of efficient selection techniques (Blum 1998; Blum et al., 1999). In addition, this process is time-consuming, due to the lengthy cultivation cycle.

In this paper, we present a methodology to compare eucalypt genotypes, in the seedling stage, in terms of drought tolerance. A grouping strategy is presented, based on clone efficiency of nutrient uptake and use under sufficient or insufficient water supply.

Therefore, the objective of this study was to evaluate the differential behavior in the initial growth, biomass accumulation, and nutritional and water use efficiency of 10 Eucalyptus clones subjected to water stress.

2. MATERIAL AND METHODS

Seedlings of 10 Eucalyptus clones (1528, 1641, 3367, gg157, i042, i144, i182, i224, PL040, and vc865), were grown in a greenhouse in plastic pots (8 L) containing Clark nutrient solution (Clark, 1975). Clones i042 and i144 were confirmed, respectively, as drought-sensitive and tolerant in the field (Nunes, 2010; Barros Filho, 2014), and used as a reference for comparison with other genotypes in this study. After 30 days in nutrient solution, the plants were separated into two groups. Half of the plants remained in solution with water potential near 0 MPa and the other half was subjected to water stress, by the addition of polyethylene glycol 6000 (PEG 6000).

The PEG 6000 doses were added every five days to gradually reduce the water potential of the solution (to -0.16, -0.32, -0.65 and -1.0 MPa) (Michel and Kaufmann, 1973). Thus, the experiment was arranged in factorial 10 x 2 design and laid out in randomized blocks, , with four replications, in experimental units with three plants per pot.

Five days after application of the PEG 6000 dose to reduce the water potential of the solution to -1 MPa, root length was measured (cm). Subsequently, the plants were separated into old leaves, new leaves, stems, and roots, packed in paper bags, oven-dried and weighed to obtain the dry matter of the young leaves (YLDM), root dry matter (RDM) and total dry matter (TDM).

To quantify the nutrient content in leaves and roots, the dry plant material was ground in a Wiley mill, calcined in a muffle and the minerals extracted with HCl (0.1 mol mol.L^{-1}). The nutrients P, Ca and Mg, were analyzed by plasma emission spectrometry; K by a flame emission photometer and B with a spectrophotometer, by the method of Azometina-H. (Wolf et al., 1974).

Nutrient absorption efficiency (AE) (Swiader et al., 1994), translocation efficiency (TE) (Li et al., 1991) and use efficiency for leaf (UEL) and root production (UER) (Siddiqui and Glass, 1981), was calculated using the equations 1, 2, 3 and 4, respectively.

$$AE = \frac{Nutrient\ Content\ (shoot\ and\ root)\ (mg)}{Root\ Dry\ Matter\ (g)} \quad (1)$$

$$TE = \frac{Shoot\ Nutrient\ Content\ (mg)}{Total\ Nutrient\ Content\ (g)} \quad (2)$$

$$UEL = \frac{(Leaf\ Dry\ Matter(g))^2}{Leaf\ Nutrient\ Content\ (mg)} \quad (3)$$

$$UER = \frac{(Root\ Dry\ Matter\ (g))^2}{Root\ Nutrient\ Content\ (mg)} \quad (4)$$

The isotopic composition of $^{13}CO_2$ in young leaves was analyzed for abundance of ^{13}C ($\delta^{13}C$ ‰), with a mass spectrometer (IRMS), and the breakdown of ^{13}C calculated as proposed by Farquhar and Richards (1984), by the following formula:

$$\Delta = \frac{\delta^{13}C_{atm} - \delta^{13}C_{plant}}{1 + \delta^{13}C_{plant}}$$

The gas exchange in fully expanded leaves was measured and the intrinsic efficiency of water use (WUEi) was calculated as the ratio between net photosynthetic assimilation rate and transpiration rate, obtained by a portable photosynthesis system LI-6400 (LI-COR Bioscience Inc., Lincoln, NE, USA). Measurements were performed between 8:00 and 12:30 AM, in a greenhouse, under constant photosynthetically active radiation (PAR) (1400 μmol photons m^{-2} s^{-1}), atmospheric CO_2 concentration (C_a) (~372 μmol mol^{-1}), at environment temperature (22 - 28 °C) and humidity (48-66 %).

Diagrams were used to group the clones in terms of nutritional efficiency under sufficient water supply or drought. We used the mean absorption efficiency and use of K, Ca, Mg, P, and B to separate the diagram into quadrants, generating nutritional efficiency groups. In addition, a diagram was used representing the percentage in relation to the control of the young leaf and root dry matter of drought-stressed plants. The percentage compared to the control (CC) was calculated as follows:

$$\%\,CC = \frac{(Water\ Stress - Sufficient\ Water\ Supply) * 100}{Sufficient\ Water\ Supply}$$

Data were subjected to analysis of variance, the treatments tested at 5% probability by the F test, and the correlation between WUEi and $\Delta^{13}C$ and multivariate weights calculated using software Statistica 7.0.

3. RESULTS

3.1 Growth, $^{13}CO_2$ and efficiency of water and nutrient use

The Eucalyptus clones differed in terms of growth, and water and nutrient use efficiency when exposed to water deficit. The dry matter of young leaves (YLDM) was severely affected by water deficit, resulting in a decrease of 26.1% for clone i182, and up to 42.0 % and 50.4 % for the clones 1641 and 3367, respectively (Fig. 1A). Nonetheless, the YLDM of the other genotypes was however not reduced during the water stress period (Fig.1A).

Root growth, i.e., dry matter (RDM) and root length, were very sensitive to water stress (Fig. 1B and 1C). The RDM of the clones 1641, 3367, i042, i224 and PL40 was significantly reduced (up to 51.0 % for clone PL40). On the other hand, the RDM of clone vc865 increased 19.6 % (Fig. 1B) under water deficit condition and maintained the root length (Fig. 1C).

In general, the total dry matter (TDM) under water stress was most reduced in clone PL040, i.e., approximately 64 % (Fig. 1D). The TDM was least reduced in clone 3367, and remained unchanged in clones 1528, i144, i224, gg157 and vc865 when compared to plants under sufficient water supply.

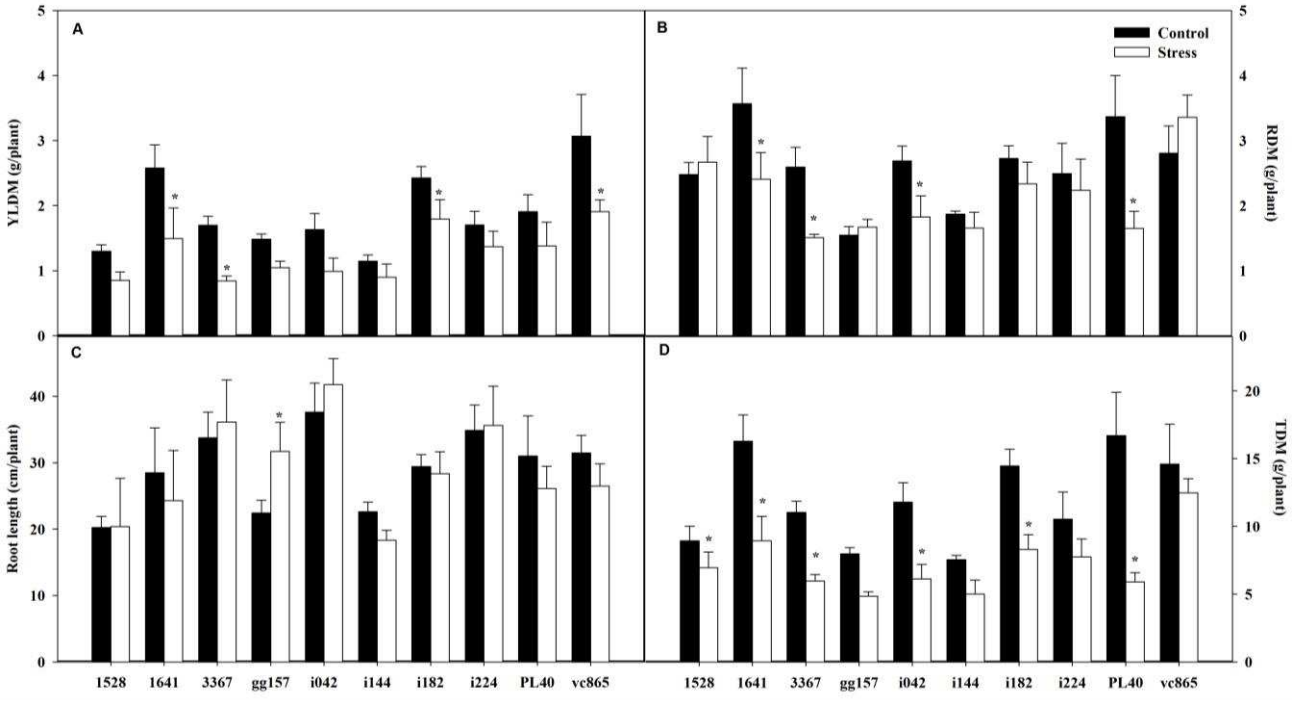

Figure 1: Young leaf dry matter (YLDM), (A); Root length (B); Root dry matter (RDM), (C); and plant total dry matter (TDM), (D) of 10 Eucalyptus clones under sufficient water supply (Control) or water stress. Bars indicate standard error (n = 4). * Significant at 5% by the F test.

The distribution of clones in terms of variation in YLDM and RDM compared to the control (Fig. 2) enabled the construction of a diagram and separation of the clones into four groups of differential growth under drought. Clones i144, i182, i224 and gg157 were clustered in group I, which contains the clones with least significant reduction in YLDM and increase in RDM, which are potentially most drought-tolerant (i144, reference of drought tolerance, based on growth traits). Group II composed by the genotypes vc865 and 1528, characterized by smaller reductions in RDM and YLDM. Group III contained clone PL40, due to the negative effect on YLDM when grown under water stress. The reductions in the traits studied were most drastic for clones i042, 3367 and 1641, of group IV, characterizing them as most sensitive to water stress (i042, as reference of drought sensitivity).

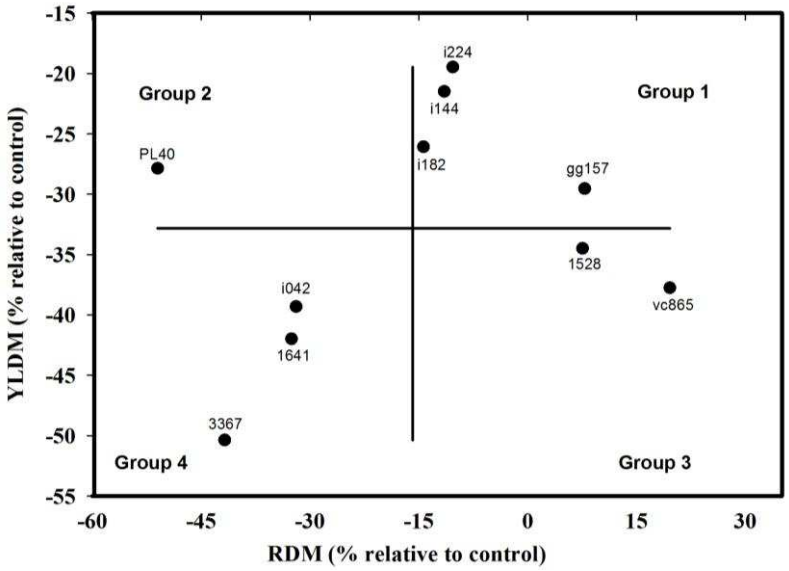

Figure 2: Distribution diagram of Eucalyptus clones according to the reduction percentage in young leaf dry matter (YLDM) and root dry matter (RDM) due to water stress as compared to control (seedlings under no water stress).

The intrinsic water use efficiency (WUEi) was only significantly increased under water stress in the genotypes 3367, i042, i144, i182, PL040, and vc865 (Fig. 3A). Clones vc865 and i144 had the greatest increases in WUEi (39.9 % and 40 % respectively), while the lowest increase was observed for clone 3367 (22 %).

Under appropriate water supply, minor isotope $^{13}CO_2$ discrimination was observed in clone 1641, with no difference from the water-stressed plants (Fig. 3). Clones i144, i224 and PL040 did not show difference in the isotopic $^{13}CO_2$ discrimination regardless water supply condition. In all other drought-stressed genotypes there was a reduction on $^{13}CO_2$ discrimination, with largest values (13.7 and 13.5 %, respectively) in clones i042 and i182 (Fig. 3B). A high correlation was observed between WUEi and $\Delta^{13}CO_2$ ($r = 0.84$) (Fig. 4).

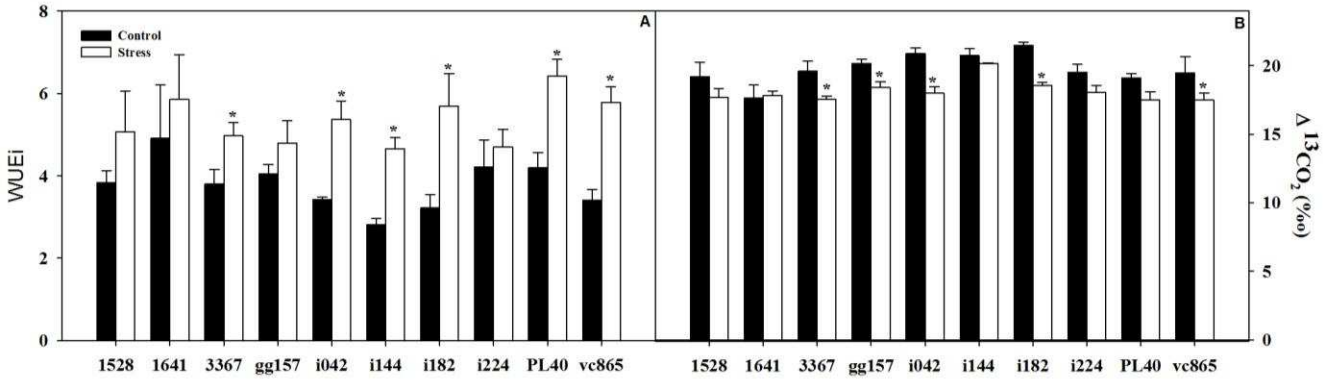

Figure 3: Intrinsic water use efficiency (A) and isotope $^{13}CO_2$ discrimination (Δ^{13} CO_2) (B) in 10 Eucalyptus clones under sufficient water supply (control) or subjected to water stress. Bars indicate standard error (n = 4). * Significant at 5% by the F test.

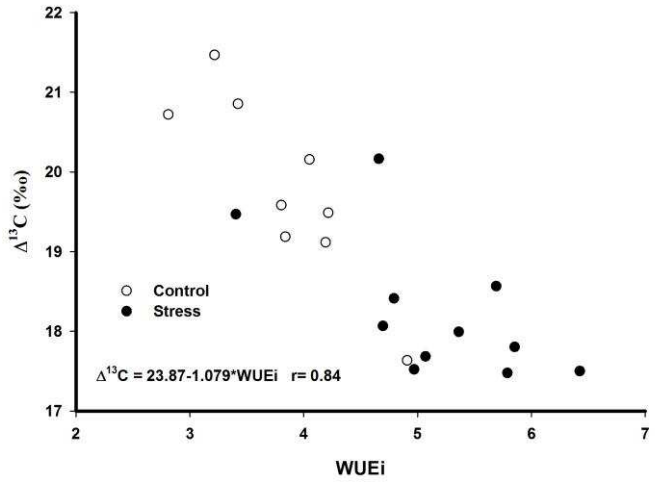

Figure 4: Relation between intrinsic water use efficiency (WUEi) and isotope $^{13}CO_2$ discrimination ($\Delta^{13}CO_2$) in 10 Eucalyptus clones under sufficient water supply (control) or under water stress.

In general, under adequate water supply, the absorption efficiency (AE) of the analyzed nutrients varied among genotypes, with highest values for gg157, i182, i144 and vc865 (Table 1). In the plants exposed to drought, nutrient AE decreased, which was most pronounced for

clone gg157 in relation to K (61.4 %). Clone 3367 showed the lowest reductions in AE for K (21.8 %), Mg (20.5 %) and P (15.1 %) and an increase in AE for Ca (26.4 %) in drought-stressed, compared to control plants (Table 1). Clone gg157 had the greatest reductions in AE for all evaluated nutrients, followed by clone vc865.

The nutrient translocation efficiency was less significantly changed in plants exposed to water stress than the absorption efficiency of the clones (Table 1). The lowest translocation efficiency of K (TEK) to shoots was observed for clone i144 (11.4 %).

Among clones grown under good water supply, TE was similar for Ca, P and B (Table 1). Under drought, however, TE was reduced for most nutrients, most markedly for Ca, in clones 1528 (26.7 %), gg157 (28.9 %) and i042 (25.6 %) (Table 1).

The nutrient UE, in general, for leaf formation in Eucalyptus plants exposed to water stress, was highest for clones i224 and vc865, and the lowest for PL040 (Table 2).

For root formation, nutrient UE was highest for clones vc865 and gg157, while no significant differences were observed among clones in UE of Ca and Mg for root formation in drought-stressed plants (Table 2).

Table 1: Absorption efficiency (AE) and translocation efficiency (TE) of nutrients in 10 Eucalyptus clones under sufficient water supply (Contr.) or under water stress. * significant at 5%, by the F test.

Clone	AE (Cont.)	AE (Stress)		TE (Cont.)	TE (Stress)		AE (Cont.)	AE (Stress)		TE (Cont.)	TE (Stress)	
	mg g^{-1}						mg g^{-1}					
	Potassium						**Boron**					
1528	45.11	23.84	*	0.83	0.84		0.17	0.15		0.75	0.63	*
1641	59.09	45.42		0.83	0.81		0.25	0.22		0.80	0.73	
3367	65.35	51.13		0.81	0.85		0.23	0.20		0.79	0.74	
gg157	79.80	30.83	*	0.87	0.84		0.30	0.16	*	0.77	0.67	*
i042	68.60	41.58	*	0.83	0.78	*	0.19	0.16		0.78	0.52	*
i144	59.60	33.36		0.81	0.72		0.22	0.14		0.78	0.70	
i182	81.35	47.01	*	0.86	0.81	*	0.24	0.20		0.81	0.69	*
i224	60.96	43.69	*	0.82	0.82		0.21	0.17		0.81	0.70	*
PL40	67.93	48.45	*	0.85	0.87		0.25	0.17	*	0.80	0.69	*
vc865	75.84	48.98	*	0.84	0.81		0.25	0.15	*	0.85	0.79	
	Calcium						**Magnesium**					
1528	8.01	7.43		0.85	0.62	*	3.81	2.40	*	0.84	0.77	*
1641	6.84	6.95		0.88	0.76		4.74	3.69	*	0.88	0.85	
3367	5.12	6.48		0.91	0.74	*	3.74	2.98		0.87	0.85	
gg157	10.95	7.07	*	0.89	0.63	*	5.21	2.16	*	0.89	0.81	*
i042	6.99	6.43		0.88	0.66	*	4.29	2.77	*	0.89	0.84	*
i144	9.76	8.63		0.86	0.70		5.28	3.50		0.87	0.76	
i182	7.98	6.70		0.94	0.72	*	6.20	3.31	*	0.91	0.84	*
i224	7.71	7.85		0.84	0.67	*	4.67	3.15	*	0.82	0.82	
PL40	7.49	7.02		0.92	0.71	*	4.74	2.67	*	0.90	0.86	
vc865	9.28	6.64	*	0.89	0.75	*	5.85	3.08	*	0.86	0.82	
	Phosphorus											
1528	16.69	10.85	*	0.78	0.75							
1641	22.32	17.91		0.80	0.76							
3367	21.72	18.45		0.78	0.79							
gg157	29.70	13.06	*	0.83	0.75	*						
i042	18.18	13.02	*	0.79	0.72	*						
i144	21.18	14.69		0.82	0.71							
i182	27.44	18.67	*	0.83	0.76	*						
i224	23.00	15.89	*	0.79	0.80							
PL40	23.46	16.10	*	0.79	0.77							
vc865	24.40	15.49	*	0.79	0.73	*						

Table 2: Efficiency of nutrient use for leaf (UEL) and root production (UER) in 10 Eucalyptus clones under sufficient water supply (Contr.) or under water stress. * significant at 5%, by the F test.

Clone	UEL (Cont.)	UEL (Stress)		UER (Cont.)	UER (Stress)		EUL (Cont.)	UEL (Stress)		UER (Cont.)	UER (Stress)	
	$g^2\ mg^{-1}$						$g^2\ mg^{-1}$					
	Potassium						**Boron**					
1528	0.27	0.21		0.34	0.79	*	65.71	32.41	*	64.18	48.16	
1641	0.46	0.25		0.37	0.28		86.88	47.43	*	73.13	42.33	
3367	0.30	0.18		0.22	0.20		68.15	42.24		61.75	31.99	*
gg157	0.21	0.12		0.15	0.37	*	47.27	25.33		22.91	34.04	
i042	0.29	0.17		0.24	0.24		98.04	51.95		76.86	26.54	
i144	0.21	0.14		0.17	0.18		43.86	29.74		41.50	41.73	
i182	0.39	0.21	*	0.24	0.26		125.24	55.09	*	62.31	39.81	
i224	0.28	0.22		0.23	0.31		70.65	52.42		71.46	48.34	
PL40	0.51	0.15		0.36	0.27		115.78	42.28	*	66.90	32.88	*
vc865	0.43	0.32	*	0.23	0.38		105.07	82.03		76.66	107.92	*
	Calcium						**Magnesium**					
1528	1.79	1.11		2.18	0.98		2.63	1.85		4.27	5.12	
1641	4.49	1.98		4.59	1.46		5.12	2.41	*	6.73	4.21	
3367	3.16	1.83		6.17	0.91	*	4.13	2.59		5.79	3.53	
gg157	1.58	0.86		1.29	0.66		2.51	1.37		2.78	4.33	
i042	2.96	1.69		3.49	0.87		3.76	2.02		5.72	4.79	
i144	1.45	0.73		1.43	0.64		1.92	1.09		2.73	1.98	
i182	4.10	2.24	*	5.55	1.34	*	4.58	2.70		4.93	4.69	
i224	2.64	1.88		2.29	0.90		3.33	2.65		3.15	5.17	
PL40	4.93	1.56	*	6.05	0.81	*	6.26	2.15	*	8.17	4.59	*
vc865	3.98	2.85		2.73	2.05		5.39	4.02		3.60	6.79	
	Phosphorus											
1528	0.90	0.63		0.70	1.00							
1641	1.31	0.68	*	0.84	0.59							
3367	0.96	0.64		0.59	0.39							
gg157	0.67	0.41		0.31	0.53							
i042	1.16	0.68		0.73	0.53							
i144	0.59	0.38		0.48	0.38							
i182	1.16	0.64		0.58	0.52							
i224	0.81	0.73		0.54	0.86							
PL40	1.77	0.59	*	0.72	0.45							
vc865	1.59	1.17		0.56	0.82							

3.2 Nutrient efficiency groups

The analysis of the mean nutrient AE and UE for leaf and root production, according to the water supply condition, allowed the grouping of Eucalyptus clones (Figure 5). We used the AE and UE diagrams for K, Ca, Mg, P, and B. Group 1 is composed by clones with high nutrient AE and UE; group 2 by clones with low AE and high UE; group 3 by clones with high AE and low UE and group 4 by clones with low AE and UE.

Clones PL040, vc865 and i182, under adequate water supply, were characterized as nutritionally efficient for K (group 1) for leaf formation. Even under water stress, clones vc865 and i182 remained in group 1, maintaining a high AE and UE for K for leaf formation (Fig. 5A and 5C). Water stress reduced UE of the clones PL040, 1528 and gg157 for leaf and root production, unaffected AE for K for PL040 and increased AE for the other clones (group 3; Fig. 5C and 5D).

On the other hand, clone 1528 was inserted in the group with low AE and UE for leaf formation, when under appropriate water supply (group 4; Fig. 5A) and showed a significant increase in UE for leaf production when subjected to water stress, being characterized by low AE, but high UE for K (group 2; Fig 5C.).

Clone i144, our reference for drought tolerance, was characterized, regardless water condition, as inefficient in AE and UE of K for leaf and root production (group 4) (Fig. 5).

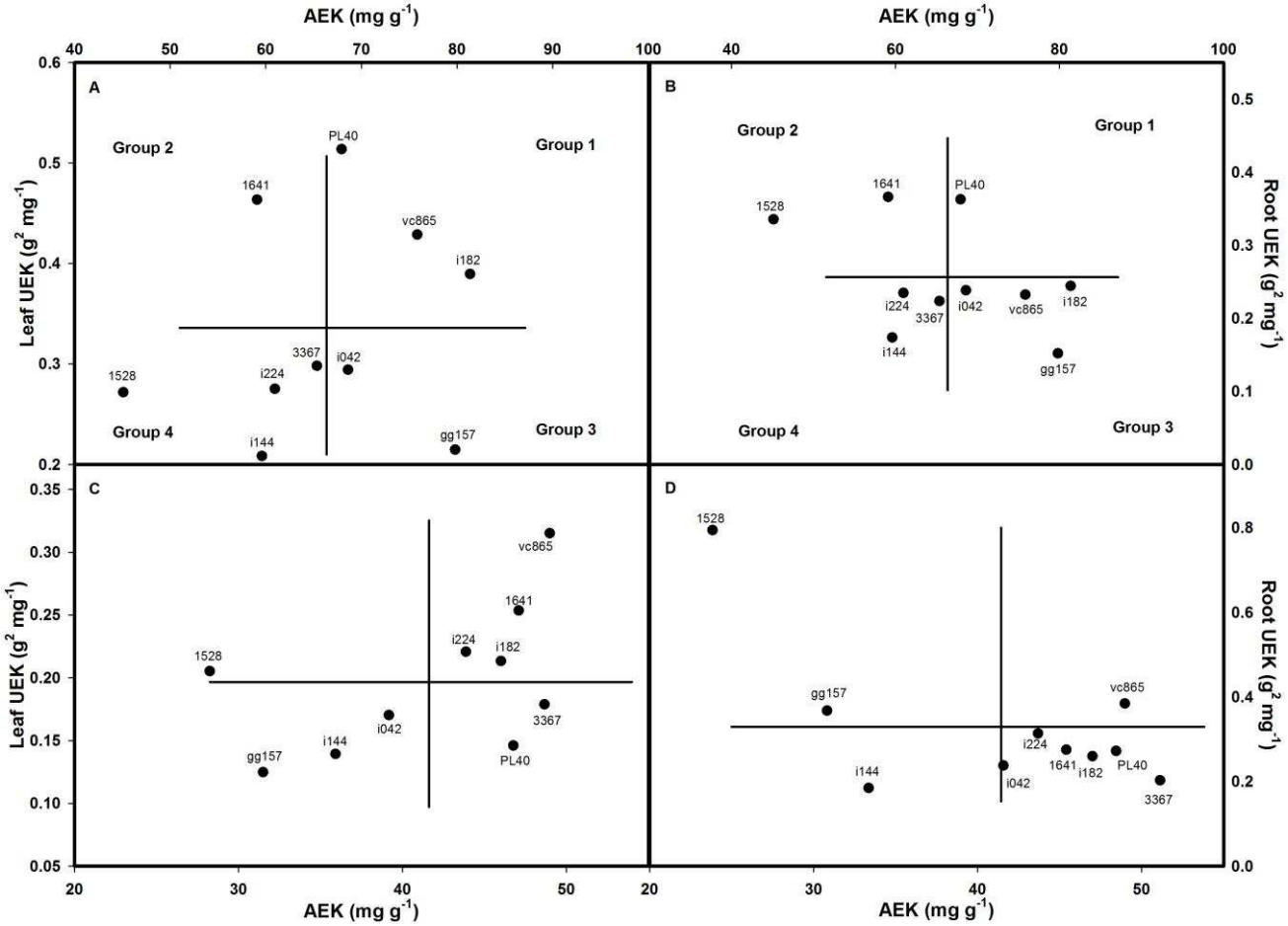

Figure 5: Distribution diagram of Eucalyptus clones in the absorption efficiency (AEK) and K use in leaves (leaf UEK), (A), and roots (root UEK), (B) under sufficient water supply and in function of the absorption efficiency (AEK) and K use in leaves (leaf UEK), (C), and roots (root UEK), (D) in seedlings subjected to water stress.

Clone PL040, despite belonging to group 2, efficient in Ca and B use, for both leaf and root production under sufficient water supply, decreased AE and UE drastically under drought, compared to the other genotypes (Fig. 6 and 7). AE for B was reduced under water stress (Fig. 6D) for clones i182, 3367, 1641, i144, gg157, and PL40 for root formation

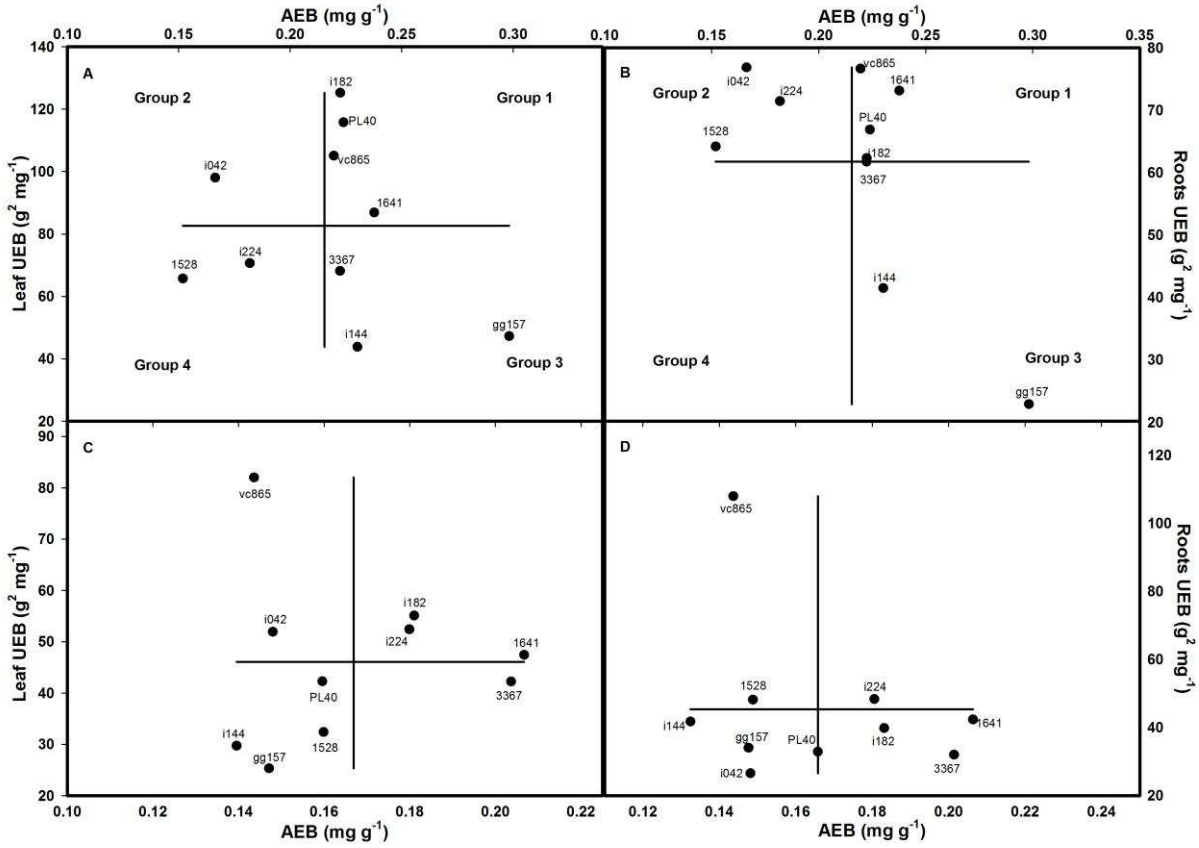

Figure 6: Distribution diagram of Eucalyptus clones in the absorption efficiency (AEB) and B use in leaves (leaf UEB), (A), and roots (root UEB), (B) under sufficient water supply and in function of the absorption efficiency (AEB) and B use in leaves (leaf UEB), (C), and roots (root UEB), (D) in seedlings subjected to water stress.

The clones 1641 and 3367, for leaf production and clones 1641 and i182, for root production, independent of the water supply level, were grouped and characterized as low AE and high UE for Ca (group 2; Fig. 7). Clones i042 and vc865 showed an increase in UE for Ca when subjected to water stress, for leaf (group 2; Fig. 7C) and root formation (group 2; Fig. 7D), respectively. Under the same adverse conditions, i224 and 1528 clones showed an increase in Ca AE for the formation of both organs (Fig. 7).

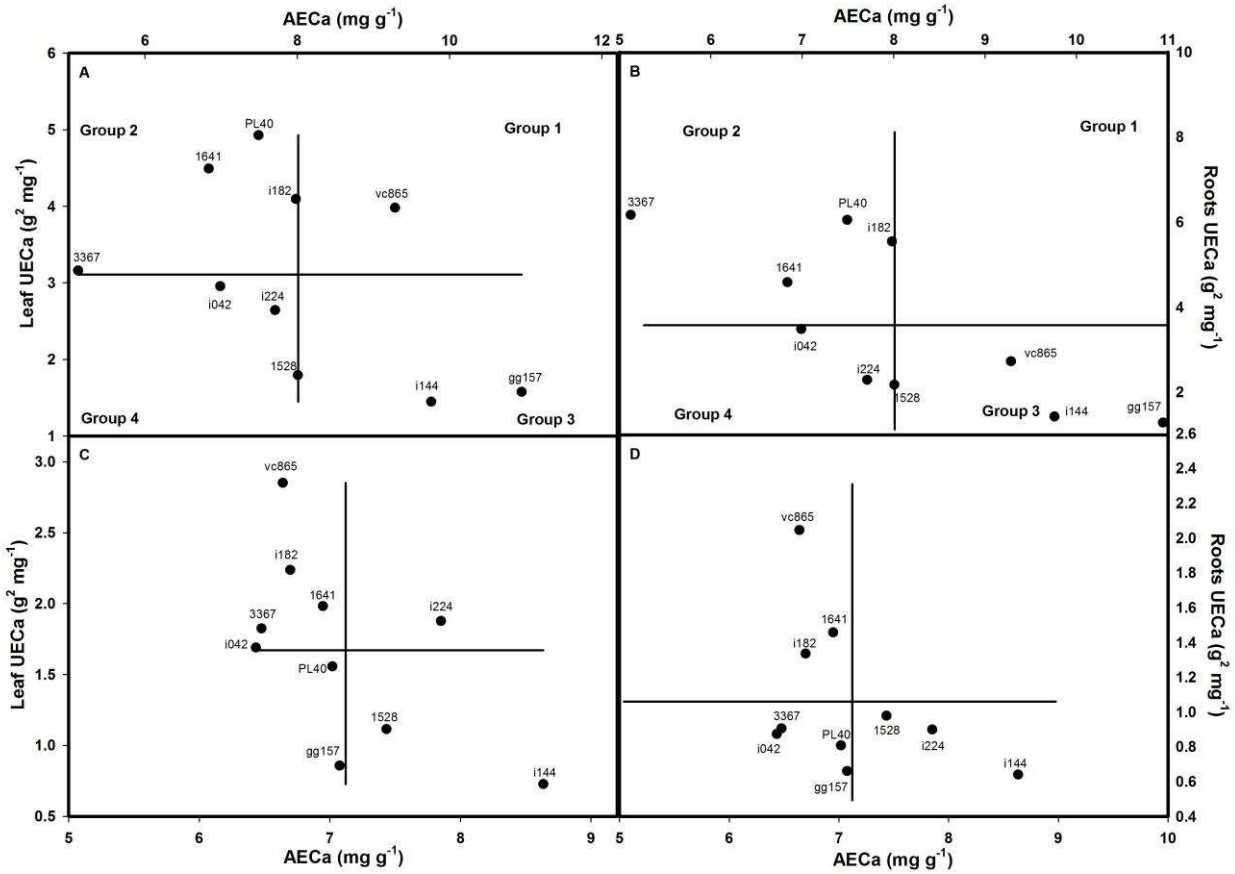

Figure 7: Distribution diagram of Eucalyptus clones in the absorption efficiency (AECa) and Ca use in leaves (leaf UECa), (A), and roots (root UECa), (B) under sufficient water supply and in function of the absorption efficiency (AECa) and Ca use in leaves (leaf UECa), (C), and roots (root UECa), (D) in seedlings subjected to water stress.

Clones i224, i182 and vc865 presented the highest Mg UE for leaf and root production (Fig. 8). On the other hand, clones i042 (reference for high drought sensitivity), 1528, PL040 and 3367 were considered the least efficient in Mg uptake and use for leaf formation during water stress (Fig. 8).

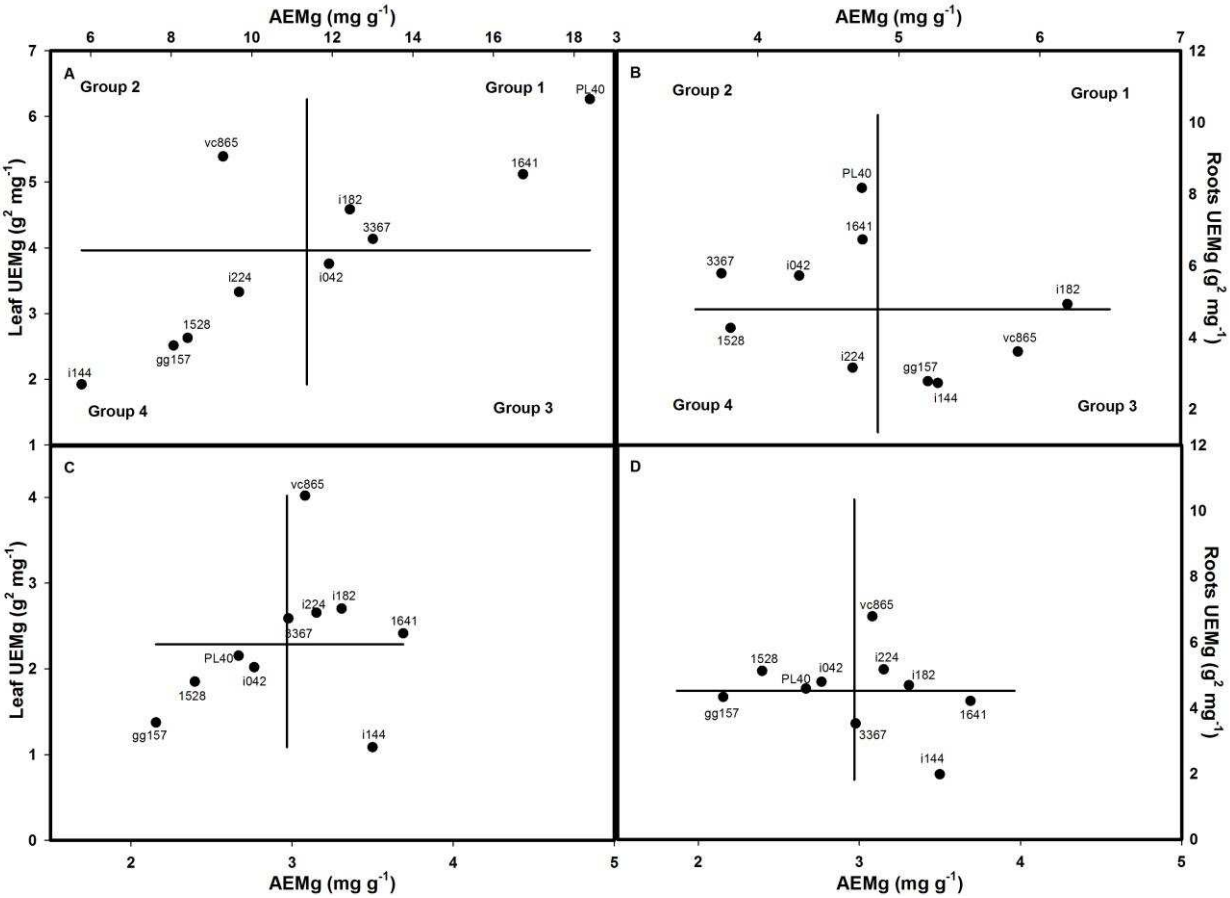

Figure 8: Distribution diagram of Eucalyptus clones in the absorption efficiency (AEMg) and Mg use in leaves (leaf UEMg), (A), and roots (root UEMg), (B) under sufficient water supply and in function of the absorption efficiency (AEMg) and Mg use in leaves (leaf UEMg), (C), and roots (root UEMg), (D) in seedlings subjected to water stress.

PL040 clone was most efficient in P use for formation of leaves and roots under sufficient water supply, whereas under water stress, clones i224 and vc865 were more efficient (Fig. 9).

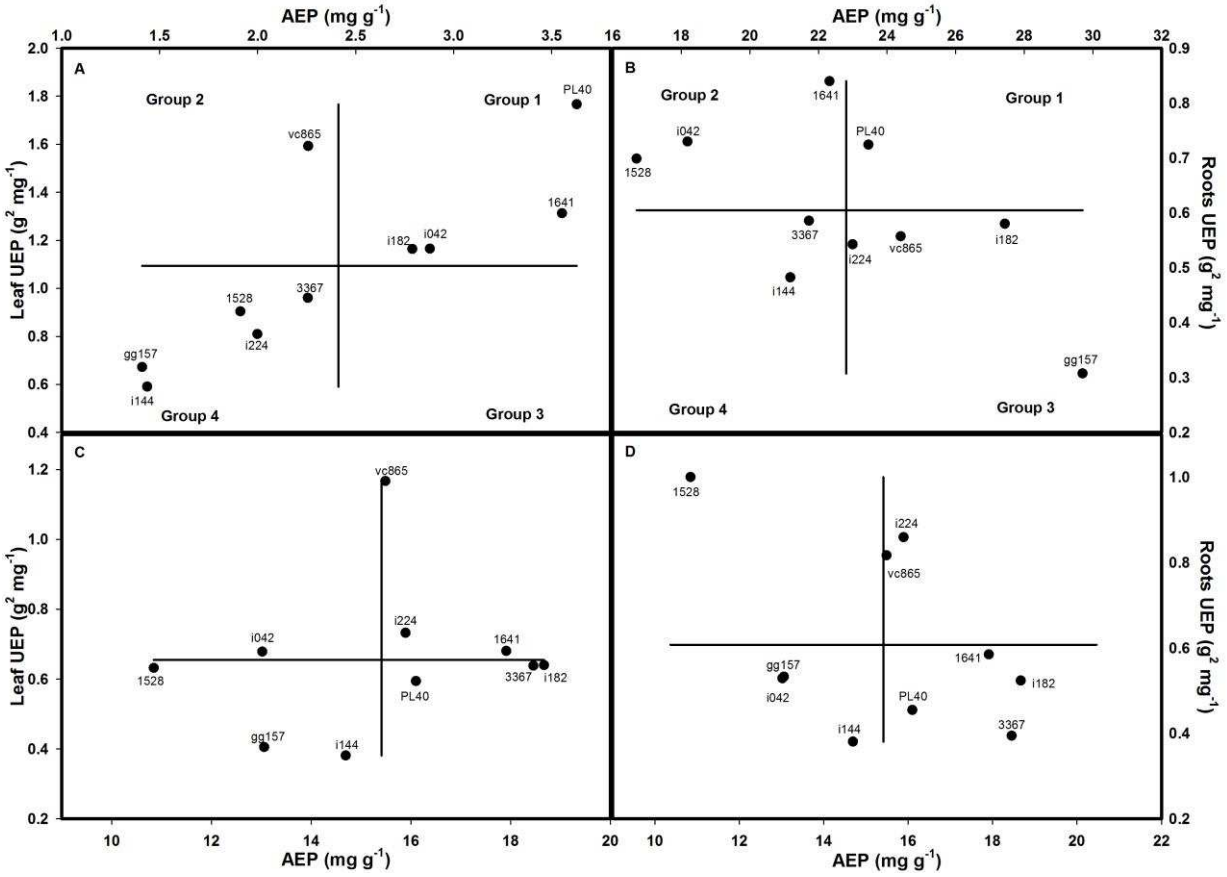

Figure 9: Distribution diagram of Eucalyptus clones for absorption efficiency (AEP), and P use in leaves (leaf UEP), (A), and roots (root UEP), (B) under sufficient water supply and in function of the absorption efficiency (AEP) and P use in leaves (leaf UEP), (C), and roots (root UEP), (D) in seedlings subjected to water stress.

4. DISCUSSION

4.1 Plant growth

Based on the distribution diagram of genotypes according to the percentage reduction in YLDM and RDM of water-stressed plants, clones were separated into four groups with different drought tolerance. The reference of drought tolerance (i144), as expected, was assigned to Group 1, which contained clones with less significant reduction in YLDM and RDM, potentially more drought-tolerant. The genotypes i182, i224 and gg157 were also clustered in this group, with great similarity in growth variables to the tolerant clone. However, the drought-sensitive clone (i042) was allocated in Group 4, which also contained genotypes 3367 and 1641, which were most affected in growth when subjected to water stress. During drought periods, the water content in plant tissues is reduced, promoting cell contraction and loss of turgor (Cosgove., 1997; Jallel et al, 2009), leading to reductions in leaf expansion (Metcalfe et al, 1990; Gonçalves and Steps 2000; Pita and Pardos, 2000). Thus, one of the signals of water stress is a reduction in leaf area, of young expanding leaves and plant biomass as a whole. The results in this study were similar to those mentioned in the literature above, as the dry matter of young leaves (YLDM) was severely affected by water stress, resulting in a reduction of up to 50.4 %.

In general, the amount of root dry matter (RDM) was more sensitive to water stress than root length, with greater multivariate weight than the other variables. In clone vc865, despite maintaining root length, RDM increased by 19.6 % in. The significant increase in RDM (vc865) or root length (gg157, 41.2 %) of drought-stressed plants indicated that, under these circumstances, some genotypes direct photosynthates for root growth (Wu and Cosgrove, 2000; Sharp, 2002; Silva et al, 2004). Carbon allocation to the root system becomes important for plant development, since the maintenance thereof enables greater

exploitation of the soil profile and allowing increases in nutrient and water uptake (Forrester et al., 2010).

4.2 Efficiency of water and nutrient use

The continued growth observed in some of the drought-stressed genotypes may have been the result of increased efficiency of use of resources such as water and nutrients. The efficiency of water use (WUE) is considered an effective adaptive mechanism of evolution, by which the plant becomes more elastic to support possible adverse water conditions, and is therefore an important trait in the selection of water-stress-tolerant species (Li et al., 2009). On the other hand, the isotopic $^{13}CO_2$ discrimination in plant dry matter is the result of transpiration efficiency, expressed as the ratio of the photosynthetic rate by the amount of water transpired throughout the growing period, i.e., the period of dry matter assimilation (Araus et al., 2003). In studies testing short drought periods, isotopic $^{13}CO_2$ discrimination may not adequately represent the plant response to the stress. A strong correlation between UE and $^{13}CO_2$ leaf discrimination was found in some C3 species (Zhang et al., 1993, Chen et al., 2007) and also in this study ($r = 0.84$, $p < 0.05$). However, some studies pointed out that the relationship between $^{13}CO_2$ discrimination and drought tolerance is not sufficiently confirmed, since other traits may indicate increased drought susceptibility or tolerance of a genotype, such as the ability to avoid water loss (Zhang et al., 1997).

In our study, isotopic $^{13}CO_2$ discrimination was lowest in clone 1641, under appropriate water supply as well as in drought-stressed plants. Plants of clone i144 with adequate or insufficient water supply did not differ in isotopic $^{13}CO_2$ discrimination, indicating maintenance of stomatal conductance during water stress periods, since growth was not reduced under stress conditions. This genotype also had the largest increase in WUEi (40 %) under drought, which may be due to the higher photosynthetic rate and reduced water loss, leading to the maintenance of plant growth even under stress.

In this study, the AE and UE of the analyzed nutrients generally varied between genotypes and according to the water supply. Higher AE and UE resulted in higher biomass yield, allowing maintenance of productivity (Machado, 2000), mainly on nutritionally unfavorable soils. Thus, genotypes with greater capacity of nutrient absorption and/or use are more desirable (Godoy and Rosado, 2011), since, in addition to a better exploitation of the fertilized nutrients, smaller amounts of nutrients are required for growth (Luca, 1997).

In the short term, the reduction in AE observed in drought-stressed plants causes a decrease in the plant transpiration rate, with reduction in the nutrient transport from the soil by diffusion and mass flow (Blouder and Volenec, 2008), while a long-term effect is reduced root growth, resulting in less nutrient absorption and consequent translocation in the plant. An efficient nutrient translocation to the shoot is however of utmost importance to ensure the nutrient supply of the photosynthetically active sites, while maintaining an adequate plant metabolism (Abichequer and Bohnen, 1998; Pinto et al., 2011), and mainly avoiding unnecessary water loss and maintenance of the plant water status, especially in drought periods.

4.3 Nutritional Diagrams

The analysis of the mean nutrient AE and UE for leaf and root formation and according to the water supply condition allowed the grouping of Eucalyptus clones. In situations of lack of resources, e.g., low nutrient availability, desirable clones would have high nutrient absorption and use capacity, increasing the chances of survival under unfavorable growth conditions (Godoy and Rosado, 2011). However, some specific observations about the UE of some nutrients under drought can be made.

Comparing all genotypes and analyzing the UE and AE for K, clones vc865 and i182, under sufficient water supply, were characterized as efficient for leaf formation. On the other hand, clone 1528, inserted in the group with low AE and UE for leaf production under

appropriate water conditions (group 4), showed a significant increase in UE for leaf formation under water stress, characterized by low AE, but high K UE (group 2). In plantations in low-rainfall areas, fertilization increased K nutritional status of the plants and can minimize the drought damage. This is possible because adequately K-supplied plants have more efficient anti-oxidative mechanisms (Cakmak, 2005; Wang et al., 2013), better stomatal regulation (Cochrane & Cochrane, 2009) and osmotic pressure in the vacuoles, promoting water maintenance in the tissues, even under adverse conditions (Hawkesford et al, 2012), and therefore higher efficiency in water uptake from the soil (Waraich et al., 2011).

The tolerant clone (i144) was characterized, both under sufficient water supply and drought, as ineffective in K use for leaf and root formation (group 4), although WUEi was high. A question raised in this study was about how far the higher UE for K for leaf formation indicates better drought tolerance. Clones with higher K UE can develop in soils with low K availability without affecting biomass production (Pinto et al., 2011), but to support drought periods, higher K concentrations in the leaves are required (Hawkesford et al., 2012; Wang et al, 2013). In this way, the nutritional economy reflected in higher leaf production per unit of nutrient, may not be advantageous in these adverse situations, but rather a higher K content, resulting in better stomatal control during water restriction. Potassium affects the stomatal control directly, since its deficiency causes reduction in the osmotic pressure of the guard cells, thus reducing their swelling, leading to partial stomatal closure (Graham and Ulrich, 1972; Cochrane & Cochrane, 2009; Hawkesford et al., 2012).

Potassium and phosphorus are nutrients with most frequent incidence of deficiency in Eucalyptus stands (Silveira et al., 2004), so that the nutritional demand of the plantations must be met by fertilization. The maintenance of leaf water potential, increased stomatal conductance (Brück et al., 2000), photosynthesis (Ackerson, 1985), and root growth (Waraich, 2011) are positively influenced by P in plants under drought stress. In this sense,

some studies reported considerable growth increase in drought-stressed plants fertilized with P (Studer, 1993; Garg et al., 2004). In this study, the clones with most efficient P use for leaf and root formation during water stress were i224 and vc865.

During the dry season, with stomatal closure, the photosynthetic capacity of plants is restricted. Nutrients such as magnesium may be important in such restriction periods, since it participates in the structure of chlorophyll and its deficiency leads to carbohydrate accumulation in leaves, resulting in inhibition of the carbon metabolism and restricting CO_2 fixation (Asada, 2006). In addition, the nutrient promotes root growth and increase in specific area by facilitating water and nutrient acquisition by roots (Waraich et al., 2011). Clones i042, 1528, PL040 and 3367 were considered least efficient in Mg uptake and use for leaf formation and clones i224, i182 and vc865 most efficient during water stress. The low nutrient use efficiency of these genotypes may have decreased their photosynthetic capacity, thus resulting in reductions in TDM, as observed in this study.

Similarly to the other nutrients, the importance of calcium and boron for the tolerance of plants under drought has been shown in several studies (Trewavas et al., 1998; Möttönen et al. 2001, 2005; Taiz and Zeiger, 2009; Hajiboland and Farhanghi, 2011; Hassan et al., 2011; Barros Filho, 2014; Hodecker et al., 2014). Clone PL040, although belonging to group 2, efficient in Ca and B use, both for leaf and root formation under sufficient water supply, showed strong decreases in AE and UE when subjected to drought, compared to the other genotypes. These nutrients are extremely important for root formation (Demidchik et al., 2002, Räisänen et al., 2007), particularly under water stress (Möttönen et al., 2001, 2005; Hodecker et al., 2014).

Additionally, boron has been reported to minimize damage caused by water stress by promoting sugar transport in the plant (Waraich et al., 2011, Wimmer and Eichert, 2013) and cell extension of growing tissues (O'Neill et al., 2004). Results of our group have shown the

importance of this nutrient in enhancing the efficiency of water use and root growth (Hodecker et al., 2014). In clones i182, 3367, 1641, gg157, and PL40, boron UE for root production was reduced under water stress. Thus, the lower UE of this nutrient in these clones may have been due to the lower B absorption and, consequently, concentration, especially in the root system, resulting in a drastic reduction of root growth, as observed in this study.

Clones i042 and vc865 showed an increase in Ca UE when subjected to water stress, for leaf (group 2) and root formation (group 2), respectively. Under the same adverse conditions, clones i224 and 1528 showed an increase in Ca AE for the formation of both organs. Due to its low mobility in the plant, Ca translocation is extremely dependent on the transpiration rate (Wilson et al., 1980), which is strongly reduced under water stress. In addition, this nutrient promotes increased growth under water stress, especially of roots (Ballester-Fernandez et al., 1997; Matos et al., 2012). Unlike proposed for K, the greater Ca UE for root production may be an important strategy of drought tolerance, since the expansion of the root surface allows greater water and nutrient uptake. However, we emphasize that a high growth rate of organs with low transpiration rates, increases the risk that the content of these nutrients required for cell wall stabilization and membrane integrity would drop below the critical level (Hawkesford et al., 2012).

Interestingly, the drought-tolerant clone under water stress generally had high AE, however low nutrient UE, while the drought-sensitive clone under stress had low AE, low UE for root formation and high AE for leaf formation. The higher AE and lower UE observed in the tolerant clone under drought may be an adaptive strategy of nutrient accumulation in unfavorable soil-climate situation and subsequently increased translocation and use of these under favorable growth conditions.

In general, clones with high nutrient AE and UE under sufficient water supply could not maintain the high AE and UE when subjected to drought and can therefore be planted in

regions with low nutrient availability, but high water supply. It should be emphasized that a specific clone will not have the highest AE and UE for all nutrients and that in general, clones i224, vc865 and i182 were considered the most efficient in nutrient absorption and use for leaf and root formation during water stress. However, clone PL040 was observed to be the most inefficient in nutrient absorption and use under low water supply, resulting in lowest growth under limiting water conditions, based on the high reduction in TDM (64 %) in drought-stressed plants.

Analyzing all variables and using the comparative diagrams, clones with greatest similarity to the reference genotypes were identified. Based on the growth variables, isotope discrimination of $^{13}CO_2$, nutritional efficiency and the diagrams, clones vc865, i182, i144 and gg157 could generally be grouped as drought-tolerant, with least growth reduction and highest nutrient efficiency under drought; clones 1528 and i224 as moderately tolerant and the other genotypes, 1641, 3367, PL040 and i042, especially the last two, as drought-sensitive.

It is worth mentioning that the strategy used in this study is interesting when clones with contrasting drought tolerance are used, as reference for comparison with other genotypes and this study should be considered as an initial step in the study of nutrition and growth characteristics of these genotypes, with a view to address the most outstanding genotypes in further studies. However for forest companies, which have no prior knowledge about the susceptibility of their genotypes to water and nutrient restrictions, the rapid identification and strategic distribution of the clones most susceptible to drought and nutrient limitation in their areas is extremely important. In this sense, the methodology of genotype separation used in this experiment allows the grouping of genotypes and their separation based on two previously known genotypes with contrasting drought tolerance levels.

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FINAL CONSIDERATIONS

The responses of Eucalyptus genotypes to drought stress have been investigated since soil drought represents a major constraint for successful production. In some regions of Brazil, the frequent low water availability has caused high mortality rate of eucalypt plantations leading to high economic impacts to the forest companies. The selection of drought tolerant genotypes is the main strategy being adopted to cope with water restriction and to establish new plantings, but more studies needs to be addressed on the Eucalyptus drought tolerance traits in order to assist future plant breeding programs.

In our work we have focused on a better understanding of water stress responses on two lines of drought tolerance. The first research line was related to the influence of plant nutrition on drought-tolerance mechanisms, including the importance of boron fertilization (Chapter 1) and the influence of nutrient uptake and use efficiency to separate tolerant and sensitive-drought genotypes (Chapter 4). Even though there are a great number of studies focused on water stress responses, few of them have given enough attention to plant nutrition and its important influence to mitigate the water stress damage in plants.

Regarding of these abroad concerns, we observed (Chapter 1) an important role of B on water use efficiency and an acute interactive response to water availability in Eucalyptus species from contrasting ecotype. In previous works, our group observed that B is phloem-mobile in Eucalyptus species and B-fertilization may be carried out during low water availability periods by foliar applications of B, since in drought periods, B transport and uptake by the root system of Eucalyptus trees become limited. In this thesis, we have got a clear picture why B is extremely important to Eucalyptus plants to overcome water stress periods. We observed a strong increment on instantaneous water use efficiency in +B water stressed-plants, due to the combination of higher photosynthetic rate, higher K^+ concentration in leaves promoting higher stomatal closure, lower water loss and a higher translocation of

sugars and B to root growth. Altogether, these combinations may increase the water use efficiency in B sufficient plants promoting better acclimation under drought mainly during long-term water stress, as observed in field conditions. In this way, our results reinforce the importance of B fertilization of Eucalyptus to cope with periods of water limitation and we strongly suggest its fertilization during these stressful periods.

In this same line, we present (Chapter 4) a comparative methodology for grouping genotypes as regard to their similarities or contrast, using selected genetic materials previously characterized as drought tolerant. Clones were grouped based on their efficiency of nutrient uptake and use under sufficient or insufficient water supply. In this experiment, interestingly, the drought-tolerant clone under water stress generally had high uptake efficiency, however low nutrient use efficiency, while the drought-sensitive clone under stress had low uptake efficiency, low nutrient use efficiency for root formation and high use efficiency for leaf formation. We concluded that the higher uptake efficiency and lower use efficiency observed in the tolerant clone under drought may be an adaptive strategy of nutrient accumulation in unfavorable soil-climate situation and subsequently increased translocation and use of of nutrient under favorable growth conditions. We also observed that lower K use efficiency for leaf formation may indicate higher drought tolerance, because higher K concentrations in the leaves are required to plant support drought periods. In this way, the nutritional economy, reflected in higher leaf production per unit of nutrient, may not be advantageous in these adverse situations, but rather a higher K content, resulting in better stomatal control during water restriction. We could observe that clones with high nutrient uptake and use efficiency under sufficient water supply, will not maintain the high efficiency when subjected to drought and can, therefore, be planted in regions with low nutrient availability, but high water availability.

The second research line was related to physiological responses to drought of different Eucalyptus genotypes under field and greenhouse conditions (Chapters 2 and 3). Eucalyptus genotypes had different acclimations to water stress in field conditions (Chapter 2). Our results suggest that trees growing in an area with uniform annual high precipitation are more stressed (high MDA and oxidative stress genes, low growth rate, etc.) after a long period of drought, compared to those stands submitted to annual water-stress fluctuation period and previous modifications may provide the benefit of increased defense protection during the future stress. The studied genetic materials showed distinct responses to drought, which allowed their separation in two groups of tolerance (sensitive and tolerant). Interestingly, the sensitive-drought genotype had no differences in $\delta^{18}O$ enrichment of leaf tissues in both precipitation regimes, while the tolerant genotypes had an increment in this oxygen isotopic composition, indicating lower ability to control the stomatal closure in different water supplies. An efficient stomatal closure is an important mechanism to avoid plant water loss, although it reduces the productivity, which may be preferable as compared to high mortality rate of the plantations caused by drought. The sensitive genotype would not be considered a potential genetic material for drought-prone areas; however, it can be recommended for planting in areas without water restriction because of it anysohydric characteristic leading a high productivity rate in high rainfall sites. We observed that the combination of $\delta^{13}C$ and $\delta^{18}O$ extracted from leaves seems to be important tool to separate sensitive and tolerant genotypes to drought and may be used in future studies and genotypes selections.

Further in this research line, the high number of clones and physiological variables investigated in Eucalyptus genotypes submitted to drought (Chapter 3) allowed us to use the multivariate statistics and to obtain a hierarchical clustering and heat maps comparing all studied genotypes. Responses were grouped into 2 general main clusters: the first cluster was composed of ABA, pigment contents and fluorescence parameters and the second comprised

gas-exchange-related responses and growth variables. In this study, we found that the genotypes had different photosynthetic limitations caused by water stress and interestingly for the drought-tolerant genotypes were observed absence of metabolic limitations (the opposite result was observed to sensitive-drought genotypes), while the small reduction in A occurred due to stomatal limitations. This response indicates that these genotypes have a greater potential of post-stress recovery, for not requiring the recovery of the photosynthetic apparatus, which can be damaged in drought-sensitive clones during water stress periods.

We analysed a large number of physiological (e.g., photosynthesis, gas exchange, fluorescence of chlorophyll a), biochemical (e.g., pigments contents) and hormonal (e.g., ABA content) variables, however we were not able to select a single variable to be a marker of drought-tolerance in early Eucalyptus stage, but the interaction between water use efficiency, ABA, A, E and SDM/RDM seemed to be the most important indicators of clones tolerance to water stress. More studies need to be focused in small number of genotypes, long period of water stress, however less stress severity to comprove their tolerance or sensitive to drought and to obtain a higher number of responses during the water stress.

The tools utilized in our studies, such as nutritional diagrams, $\delta^{13}C$, $\delta^{18}O$, hierarchical clustering, heat maps and the results obtained in our experiments may be useful to guide future water stress studies in Eucalyptus. In addition, our plant nutrition results reinforce the important of correct fertilization to better acclimation of plants during water stress periods.

The high genotypic variability in the Eucalyptus germplasm existing within forest companies enable the selection of new eucalypt genotypes and our results may help to guide the future selections of drought tolerant genotypes.