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REGULAR ARTICLE

# Boron delays dehydration and stimulates root growth in *Eucalyptus urophylla* (Blake, S.T.) under osmotic stress

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**Abstract**
*Background* Water and nutritional restrictions are limiting factors for the growth of *Eucalyptus* trees in tropical climates. In the dry season, boron (B) uptake is severely affected.
*Aims* The objectives of this study were to evaluate the phloem mobility of B and whether its deficiency can increase plant sensitivity to osmotic stress. It was also tested to what extent foliar application of B could mitigate the negative effects of drought under low B supply.
*Methods* Seedlings of a drought tolerant *Eucalyptus urophylla* (*Blake*, *S. T.*) clone were grown in nutrient solution, subjected to low availability of B for 25 days, and then submitted to a progressive osmotic stress. After imposition of osmotic stress, B was applied to young or mature leaves.
*Results* B applications, mainly to mature leaf, stimulated root growth and delayed dehydration under osmotic stress and led to an increased B translocation and carbon isotopic composition. The expression of B transporters and pectin metabolism genes were also increased in water-stressed plants supplied with B by foliar application.
*Conclusions* B deficiency led to increased plant dehydration and decreased root growth under osmotic stress. The application of B to mature leaf of water-stressed plants proved effective in mitigating the negative effects of water deficit in root growth.

**Keywords** Drought tolerance · *Eucalyptus urophylla* · B mobility · Gene expression

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## Introduction

Water and nutritional restrictions are the factors that most limit growth of young *Eucalyptus* trees in Brazil. During the dry season, as boron (B) uptake is severely restricted (Mattiello et al. 2009a), B deficiency leads to shoot tips die off (drying of leaf tips) (Althoff et al. 1991), necrotic leaf tissues and pronounced lateral shoot formation. Some studies report that plants can respond differently to drought depending on the availability of B, with the

presence of this nutrient being able to mitigate the negative effects of drought, delaying dehydration in plants (Möttönen et al. 2001, 2005). Some experimental evidence suggests that B deficiency affects the functional properties of stomata, promoting permanent structural damage to guard cells resulting in non-functional stomata (Wimmer and Eichert 2013). Additionally, the uptake of some nutrients, such as K, is reduced (Schon et al. 1990), increasing the damage caused by water deficit.

The requirement of B is associated with pectin biosynthesis in the primary cell wall of plants (Hu and Brown 1994; Matoh et al. 1996). B is part of rhamnogalacturonan II (RG II) through B diester cross-links (O'Neill et al. 2004) and the majority of B in plants is linked to the RGII polymer. Under water stress there is an increase in the pectic chains RG I and RG II in the roots of drought-tolerant wheat (Leucci et al. 2008). According to these authors, these wall structures could contribute to drought tolerance as gelling and anti-desiccating agents. However, the decreased B supply to young buds could affect negatively the pectin biosynthesis in this young tissue and could be an important factor triggering bud die back.

Due to the structural complexity of RG-II, little is known about when and how borate-diester bonds are formed, as well as how B could be mobilized from this structure (Miwa and Fujiwara 2010). It is believed that pectin endocytosis could contribute to mobilization of pectin, and as consequence, B stored in its structure (Baluška et al. 2002; Amenós et al. 2009) Pectin methyl esterase (PMe) and glycosyltransferase (GT) are involved in pectin synthesis and processing, and PMes are involved in endocytosis (Röckel et al. 2008). The PMes act on the electrostatic potential, extension (Moustacas et al. 1991) and rigidity (Al-Qsous et al. 2004) of the cell wall and extension of the root system (Wen et al. 1999). The activity of these enzymes is related to the response to abiotic stress, e.g., water deficit (Pelloux et al. 2007) and low B availability (Camacho-Cristóbal et al. 2008). The GTs generally participates in reactions of sugar transfer to a large group of receptor molecules (Ross et al. 2001). The GTs of family 8 influence plant growth (Scheible et al. 2004) and their absence impairs growth and reduces adhesion between leaf and root cells, facilitating dehydration (Bouton et al. 2002). These authors mention the important role of GTs in the synthesis of pectic polysaccharides.

Several authors have emphasized the importance of the equilibrated B nutrition during water restriction (Möttönen et al. 2005; Hajiboland and Farhanghi 2011; Karim et al. 2012), but their studies did not address the mechanisms that confer increased drought tolerance to plants supplied with the nutrient and, particularly, little is known about the role of B in eucalypts during water restriction. In drought periods, B transport and uptake by the root system of eucalyptus trees become limited and the mechanisms related to the processes of B translocation and remobilization between roots and shoots remain unknown. Miwa and Fujiwara (2010) suggested that the combined action of B carriers such as NIP 5:1 (Kato et al. 2009; Tanaka and Fujiwara 2008), and of BOR1, an efflux transporter involved in carrying B to the xylem of *Arabidopsis* (Takano et al. 2002, 2005), would facilitate the acquisition and transport of B to the shoots.

Plants can be supplied with B through soil and foliar applications. In Brazil, foliar application to eucalypt plantations in the dry season have proven effective to reduce the deficiency symptoms and improve plant growth (unpublished results). Boron was usually considered a low-mobility nutrient in plants (Raven 1980), due to its structural function in pectin (O'Neill et al. 2004) and its incorporation into cell walls (Wimmer and Eichert 2013). However, nowadays several plants were identified as able to translocate B, as observed in *Prunus amygdalus*, *Malus domestica* (Brown and Hu 1996), *Sorbus aucuparia*, *Prunus padus*, *Alnus incana*, *Fraxinus excelsior*, *Betula pubescens* (Lehto et al. 2004), and even some *Eucalyptus* hybrids (Mattiello et al. 2009b). Similar results were reported by Takano et al. (2001) who observed B transport to young leaves of *Arabidopsis* plants not supplied with the nutrient. The water stress effects in B nutrition vary with the *eucalyptus* species or clone, which can be related to differences in the mobility of the nutrient (Mattiello et al. 2009b).

A major challenge when conducting experiments with concomitant water and soil micronutrient deficit is the absence of an adequate control under the stress imposed, as well as the difficulty of simulating nutrient deficiencies in small amounts similar to the ones that occurs in nature. Taken this in account, this work used the imposition of water stress in nutrient solution, where it was possible to provide the absence of B (leading to a progressive decrease in B availability in the plant), and using PEG (polyethylene glycol) to impose water restriction in a gradual and controlled manner, allowing acclimation responses, trying to mimic as much as possible conditions that would be observed in nature. Although some researchers have reported toxic effects caused by PEG (Leshem 1966) this polymer has been

successfully used to impose water stress in plants (Nepomuceno et al. 1998; Xu and Huang 2010; Sucre and Suárez 2011; O'Donnell et al. 2013) since it is a non-penetrating, non-ionic and inert polymer. The purpose of this study was to assess the phloem mobility of B and its contribution to drought tolerance in plants, and identify changes in gene expression that could contribute to a better plant performance under water and B deficiency.

## Material and methods

### Plant material and experimental design

Rooted cuttings of *Eucalyptus urophylla* Clone (i144), characterized as drought tolerant under field conditions (Plantar Company ®), were grown in a nursery and after 6 months transplanted to plastic containers with 10 L of Clark (1975) nutrient solution, pH 5.5, 5 μmol $L^{-1}$ of $^{11}B$ (99 atom %) renewed every 5 days. The experiment was conducted using 24 containers, 4 plants per container and 4 containers per treatment.

After 30 days growing in the nutrient solution with 5 μmol $L^{-1}$ of $^{11}B$ (99 atom %), the plants were separated into two groups. One group ($^1/_3$ of the plants- 32 plants - 8 containers) was transferred to 15 μmol $L^{-1}$ of $^{11}B$ (99 atom %) (**T1**+**B**) for 25 days and the other ($^2/_3$ _ 64 plants– 16 containers) was maintained in Clark solution without B (**T2** –**B**) for 25 days. Then, plants of group **T1**+**B** were treated with growth solution without B aiming to simulate the reduction of B uptake during reduction of water availability in field conditions. After that the osmotic stress was applied.

The treatments consisted of: a) 4 containers of T2's plants without water stress (water potential=0 MPa) (Control -B); b) 4 containers of T1's plants without water stress (water potential 0 MPa) (Control + B); c) 4 containers of T2's plants with drought (D-B); d) 4 containers of T1's plants with drought (D + B). Two additional treatments consisted of: e) 4 containers of T2's plants under water stress and foliar application of $^{10}B$ (99 atom %) to a mature leaf (D BML), and f) 4 containers of T2's plants under water stress and application of $^{10}B$ (99 atom %) to a young leaf (D BYL) (Fig. 1).

Increasing doses of polyethylene glycol (PEG) 6000 (0, 100, 200, 250, and 360 g/L) were added every 5 days in the containers to gradually reduce the water potential of the solution to 0, −0.15, −0.65, −1.00 and −1.5 MPa (Michel and Kaufmann 1973) and the water level was adjusted periodically every 2 days.

The experiment was conducted in a completely randomized design and the evaluations were initiated at the time of the first dose of PEG and foliar B application. For

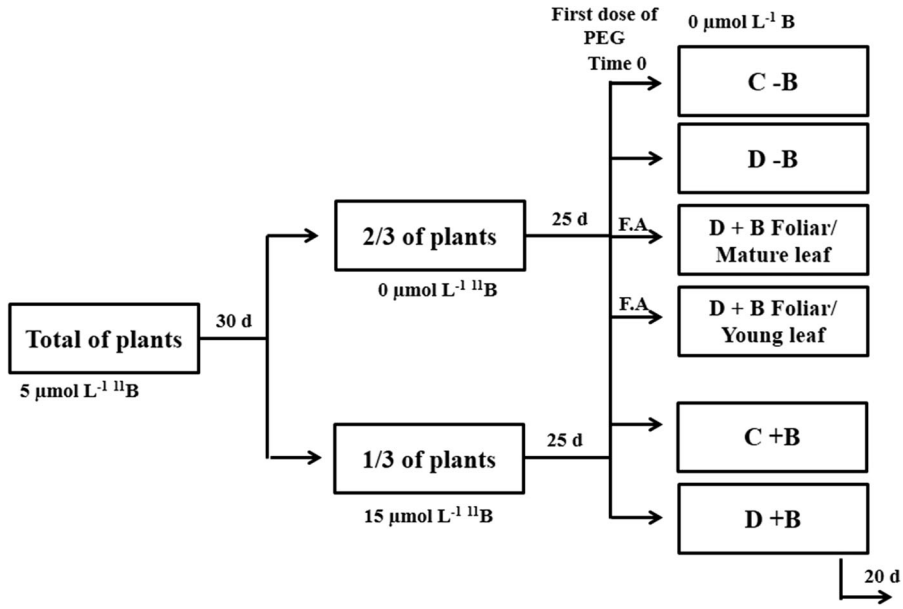


**Fig. 1** Diagram of seedling acclimation and treatment application, with different water and B availability. Plants grown under adequate water availability and acclimated in the absence (C-B) or presence (C + B) of B. Water-stressed plants acclimated to the absence (D-B) or presence (D + B) of B, and B applied to mature leaf or young leaf

foliar B treatments of young leaves, the third or fourth fully expanded leaf from the apex was used and for mature leaf treatments, the leaf from the inner side of the branch of the shoot base. The foliar application consisted of immersing the leaf in a solution (with ultra-purified water) containing $H_3BO_3$ enriched with $^{10}B$ (99 atom %) at a concentration of 1.64 g $L^{-1}$, for 1 min.

The use of the rare $^{10}B$ allows us to calculate isotopic discriminations in order to address the B translocation rate. This experimental design was used aiming to induce B deficiency as a result of decreased water availability as observed in field conditions, using osmotic stress to reduce water uptake.

The first samples were taken 1 day after application of the first dose of PEG (100 g $L^{-1}$- 0.15 MPa) for boron isotopic composition, and then plants of all groups and subgroups were harvest simultaneously 5 days after the successive PEG applications (after 5, 10, 15, and 20 days, from the −0.15, −0.65, −1.0 and −1.5 MPa solutions, in the treatments subjected to PEG, respectively). Every week the level of PEG was increased in the same containers and after each level, one plant of each container was collected, totalizing 4 plants per treatment per sampling.

Samplings were done from youngest fully expanded leaves and root tips, which were frozen in liquid $N_2$ and stored at −80 °C for RNA extraction. Additional samples were collected, dried in a convection oven at 65 °C for 72 h, and ground to determine the isotope ratio $^{11}B$:$^{10}B$ and B concentration. In the youngest fully expanded leaves, δ $^{13}C$ ($^{13}C$ composition) was also determined. The remaining plant material was oven-dried to determine the shoot, root and total dry matter. The leaf water potential was determined at the moment of each sampling, at predawn, using a Scholander pressure pump.

## Analysis of differential gene expression

### *RNA extraction*

The samples were composed of a set of four plants per treatment. The total RNA was extracted as recommended by Wang et al. (2008), in triplicate. A spectrophotometer was used for quantification and the concentration of RNA adjusted to 300 ng.μ$L^{-1}$. The physical integrity was evaluated by electrophoresis on 1 % agarose gel (Wilson and Walker 2000).

### *cDNA synthesis*

The treatment total RNA with DNAse I (RQ1 RNAse-Free DNase; Promega) was performed according to the manufacturer's recommendations. The cDNA was synthesized in triplicate for each treatment using the Super-Script II kit (Invitrogen), according to the manufacturer's instructions. A control sample (without the reverse transcription enzyme) was synthesized. The synthesis efficiency was assessed by PCR with a constitutive primer of 35S ribonucleoprotein of *Eucalyptus grandis* and histone 2B (H2B).

### *Real-time PCR expression*

The expressed sequence tags (ESTs) from orthologous genes related to the observed phenotype were obtained from NCBI GenBank (www.ncbi.gov), choosing species of the same genus or parent species (Table 1). Primers were obtained by the program Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) for a size of approximately 19–21 base pairs, Tm (melting temperature) between 54 and 60 °C, producing amplicons between 80 and 200 base pairs (Table 1).

The primers were validated on the standard curve, using a sample of the synthesized cDNA, diluted 5, 25 and 125 times in the original cDNA volume, with three replicates per dilution. The PCR reaction consisted of 0.2 μmol$^{-1}$of each primer (sense and antisense), 0.1 μL of 5 mmol $L^{-1}$ dNTPs, 2 μL 10X PCR buffer, 1.2 μL 50 μmol $L^{-1}$ $MgCl_2$, 2 μL SYBR green (100X), 0.4 μL ROX, 0.05 μL Platinum Taq DNA polymerase 5U μ$L^{-1}$(Invitrogen), 0.4 μL cDNA, completed to 20 μL with water. The PCR program consisted of 90 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 58 °C for 1 min. The dissociation curve was determined at a temperature of 60 °C, which was raised by 0.2 °C every 20 s up to 95 °C. The specificity of the amplification reactions was determined by the Tm of the amplification reaction products, and by the absence of a second dissociation curve. The primers with a logarithmic curve and $R^2$>0.98, coefficient of variation <0.1, amplification efficiency between 0.9 and 1 and Sloop of approximately −3 were considered in order to select the primer for the study of gene expression, as suggested by the manufacturer (Applied Biosystems, Foster City, USA). The relative expression was analyzed using a Step-One Plus instrument (Applied Biosystems, USA) with three technical replicates for each treatment.

**Table 1** - Primers used for the real-time PCR

| | GenBank [1] | Primer sequence | |
|---|---|---|---|
| | | Sense 5'-3' | Anti-sense 5'-3' |
| | | Primers of constitutive genes | |
| RPtnL23A | db 28432294 | AAGGACCCTGAAGAAGGACA | CCTCAATCTTCTTCATCGCA |
| H2B | AY263810 | GAGCGTGGAGACGTACAAGA | GGCGAGTTTCTCGAAGATGT |
| | | Primers of genes related to B transport | |
| BOR cl6871* | | TCAATGCCCCGGTTGACGAA | CGACACACCCTCCCACCATC |
| BOR2 | AY070067.1 | CGACCACGCGTCCGAGAATC | ACGACAGCGAGTCCACCCTC |
| BOR5 | XM_002304128 | TGGTTGGTCGCTTTGTCGCC | CCACGGGAAGAGGCTGGAG |
| | | Primers of genes related to cell wall synthesis and alteration | |
| GT8 | AY278316.1 | TTCCTGCACTGGAGAAGGTGG | TCCATGCAGGTCTCAACTGCTC |
| PME3 | DQ376133.1 | GAACCGCAAGGACCCGAACC | GGAAGCTCCCGTTTGAGGCG |

[1] Gene accession number in the database NCBI (www.ncbi.gov). * Sequence extracted from the gene bank Genolyptus-unpublished data

To normalize the relative expression, the primer for the constitutive gene RPtnL23A (ribosomal protein) was used, due to the lower standard deviation between treatments, indicating the higher amplification efficiency of this gene (Vandesompele et al. 2002). Data analysis was performed using the One Step-Plus software version 2.0 (Applied Biosystems, Foster City, USA). The Ct (threshold cycle) values of the replicates were calculated by the method of relative quantification of the copy levels of amplification (Ali-Benali et al. 2005). The plant material from the sampling of the 1st, 10th and 20th day was evaluated; for the data analysis, the plants of the group grown under 15 μmol $L^{-1}$ of $^{11}B$ without PEG were used as control in the first analysis, to compare the gene expression with other treatments and sampling times.

## B concentration and determination of isotope ratio $^{11}B$:$^{10}B$

Samples of young and mature leaves and roots, collected at 5, 10, 15 and 20 days after the first PEG application were ground and the total content of B, after digestion in a muffle furnace at 550 ° C, was determined by the spectroscopic method of azomethine H at 410 nm.

Samples of young leaves and roots, collected at 1, 10 and 20 days after PEG application: (−B – no foliar application), (+B/foliar application in mature leaf) and (+B/foliar application in young leaf) were ground and 0.2 g of the material was submitted to a dry combustion at 550 °C, and the residue dissolved in 1.59 mol $L^{-1}$ of 5 mL $HNO_3$ (Merck, Darmstadt, Germany), prepared with ultrapure water. Subsequently, the solution was sieved through a 0.45 μm nylon filter and stored in plastic microcentrifuge tubes in an ultra- freezer at −80 ° C.

The isotope ratio $^{11}B$:$^{10}B$ was determined using the high resolution mass spectrometer, with inductively coupled plasma source (HR-ICP-MS, ELEMENT-Thermo Finnigan, Bremen, Germany).

Prior to reading, the equipment was calibrated. Isotopic patterns NIST SRM-951, at a $^{10}B$:$^{11}B$ ratio of 0.247, containing 99.94 μg $L^{-1}$ B, were prepared from the stock standard solution and used in the calibration phase and during the series. The standard concentration was similar to that expected in the extracts diluted from the samples. The stock solution was 1,000 mg $L^{-1}$ B and was prepared from the isotope standard ($H_3BO_3$) (NIST SRM 951) with 19.827 % $^{10}B$ and 80.173 % $^{11}B$ (accuracy of measurement 0.00001 %).

After establishing the working conditions, the isotope ratio of B ($^{11}B$:$^{10}B$) was determined in samples of eucalyptus plants, which were diluted 5 times with deionized water 18.2 MΩ $cm^{-1}$ and placed in an autosampler. Thereafter, the percentage of B derived from the leaves treated with $^{10}BH_3BO_3$ (% BDL) was calculated by the formula: **BDL**=[(**A-B**)/(**C-B**)]***100**, where: A; % of $^{10}B$ sample, B=% de $^{10}B$ control and C; % of $^{10}BH_3BO_3$ (99 atom %).

## Isotopic composition $^{13}C$ ($\delta$ $^{13}C$)

Dried samples of young leaves, corresponding to the last sampling (20 days after the first dose of PEG), were

ground and $\delta^{13}C$ was determined in relation to the international PDB (Pee Dee Belemnite) standard, using an isotope ratio mass spectrometer (ANCA-GLS,Sercom,Crewe, UK).

### Statistical analyses

The data were subjected to analysis of variance (ANOVA) and compared by the Tukey test at 5 % probability. The treatments were compared among sampling times and treatments.

## Results

### Leaf water potential and $\delta^{13}C$

The leaf water potential (Leaf-$\Psi_{lw}$) decreased significantly with increasing PEG concentration in all treatments (Fig. 2a); because the measurements were made at pre-dawn Leaf-$\Psi_{lw}$, tended to reflect the hydroponic solution potential $|\Psi_w|$. Plants under limited B availability and under water stress that received foliar B nutrition only in mature leaf had more hydrated tissues when PEG solutions of −0.65 MPa or more negative were applied. Additionally, after 20 days of water deficit, the $\delta^{13}C$ was higher in plants exposed to water stress and non-supplemented with B (−B) in the solution (Fig. 2b).

The $\delta^{13}C$ values of well-watered plants were close to −30‰ and those under stress discriminated less the $^{13}C$ ($\delta^{13}C$~−27 ‰), supporting that PEG was successful in promoting water stress in treated plants (Fig. 2b).

### B mobility and concentration

One day after the onset of water stress, the isotope ratio $^{10}B$:$^{11}B$ was approximately 0.15. The translocation of foliar-applied $^{10}B$ over time was confirmed by the transient increases in the isotope ratio $^{10}B$:$^{11}B$ of young tissues from leaf and root in eucalyptus plants (Fig. 3a and b). The application of $^{10}B$ to mature leaf resulted in greater B enrichment over time (greater ratio $^{10}B$:$^{11}B$) in both young leaves and roots of these plants, in contrast to observed for applications to younger leaf, where no significant differences were observed over time in the roots of the treated plants (Fig. 3b). The percentage of B in young leaves and roots from foliar-applied $^{10}B$ (BDL) varied according to the sampling time and plant tissue analyzed (Fig. 3c and d). When B was applied to mature leaf, BDL was

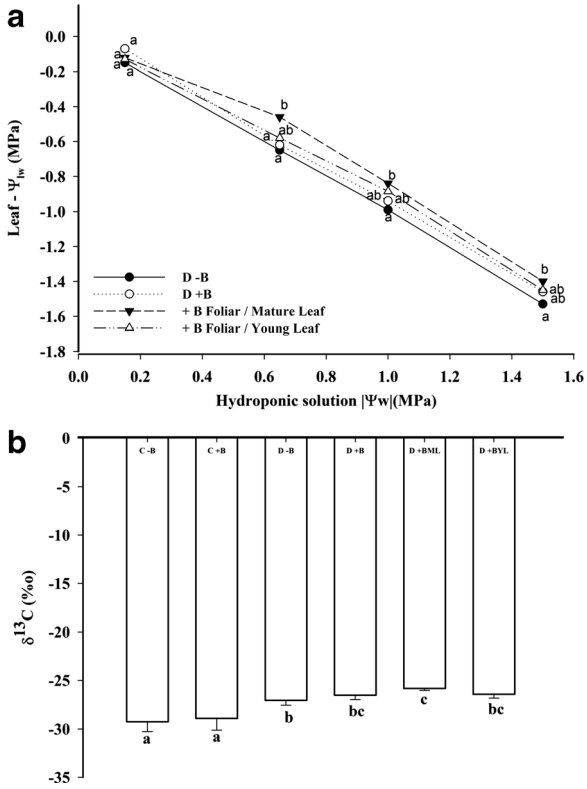


**Fig. 2** Leaf water potential (Leaf -$\Psi_{lw}$) (**a**) and $\delta^{13}C$ (**b**) in Eucalyptus according to the water and B availability. Well-watered plants (control plants) acclimated to the absence (C-B) or presence (C + B) of B. Water-stressed plants acclimated to the absence (D -B) or presence (D + B) of B, or absence in solution but with B applied to mature leaf (D BML) or young leaf (D BYL). Different lower-case letters indicate differences between treatments, according to the Tukey test at 5 % probability, ($n$=4)

approximately 26 and 6 % for young leaves and roots, respectively, 10 days after foliar application. When applied to younger leaf, the translocation to the young growing parts was lower (7 % for young leaves and no significant differences for roots). After this period, a decrease was observed in the $^{10}B$ translocation rate.

Similar to the results obtained for translocation of B under water restriction, 5 days after foliar application of B, an increase of 56 and 46 % was observed in the B content in young leaves in the treatments with application of B on mature, or younger leaves, respectively, compared to levels of B in young leaves of plants grown without B supply and under water deficit (Fig. 4a). After this period, the levels of B in young leaves decreased with the reduction of the water potential of the solution (Fig. 4a).

The levels of B in mature leaves (Fig. 4b) after the imposition of water deficit in the foliar application

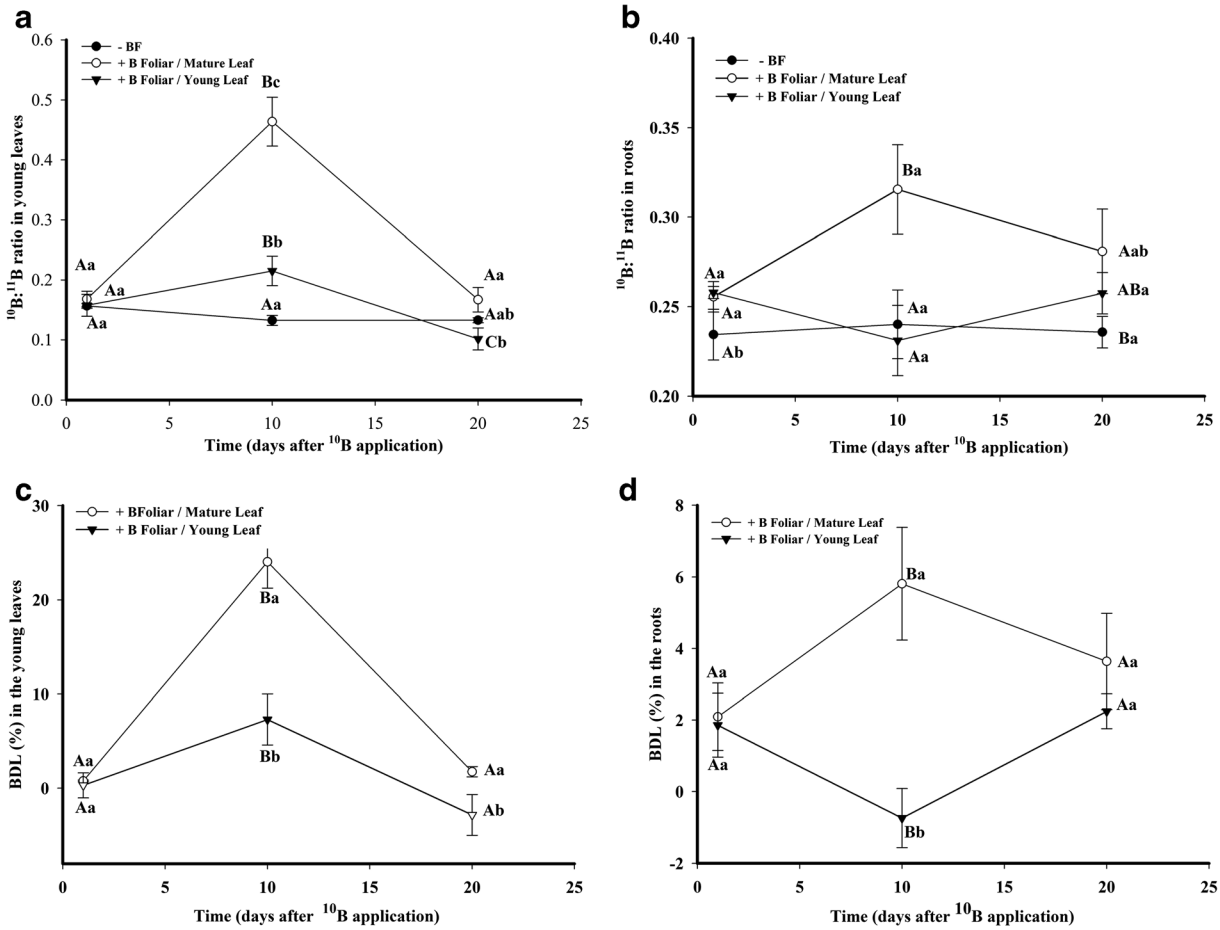


**Fig. 3** Isotope ratio $^{10}$B:$^{11}$B in young leaves (**a**), roots (**b**) and percentage of B derived from the source leaf (BDL) in young leaves (**c**) and roots (**d**) in treatments under water restriction and foliar application of B, measured 1, 10 and 20 days after imposition of water stress. Capital letters indicate differences between the sampling times and lowercase letters differences between treatments, by the Tukey test at 5 % probability, ($n$=4)

treatments were similar to those obtained in plants acclimated with B and without water stress.

## Shoot, root and total dry matter

The increase in seedling dry matter was significantly reduced by water restriction and, or, absence of B in solution for 25 days (Fig. 5a). Omission of B alone caused reduction, in average, by 50 % of total dry matter (TDM), whereas under -1 MPa or −1.5 MPa promoted a 48 % reduction in TDM at 20 days (Fig. 5a). These effects were more strongly observed in shoots where B deficiency resulted in reduction by 50 % in shoot dry matter (SDM) (Fig. 5b) at 20 days (−1.5 MPa). Application of B to mature or young leaves resulted in lower decrease in SDM, but only under −0.15 MPa or −0.65 MPa (Fig. 5b).

The application of B to mature or young leaves during severe water stress resulted in a complete recovery of root growth, but only when applied to mature leaves (Fig. 5c). This effect was not observed in shoots. After 20 days of water deficit, the increase in RDM after B application to mature leaf was approximately twice that of plants under combined water deficit and absence of B (Fig. 5c).

## Gene expression

Virtually nothing is known about Eucalyptus B transporters, and expression data could be helpful to distinguish the functions between the different genes. The B effect on gene expression of different B transporters in

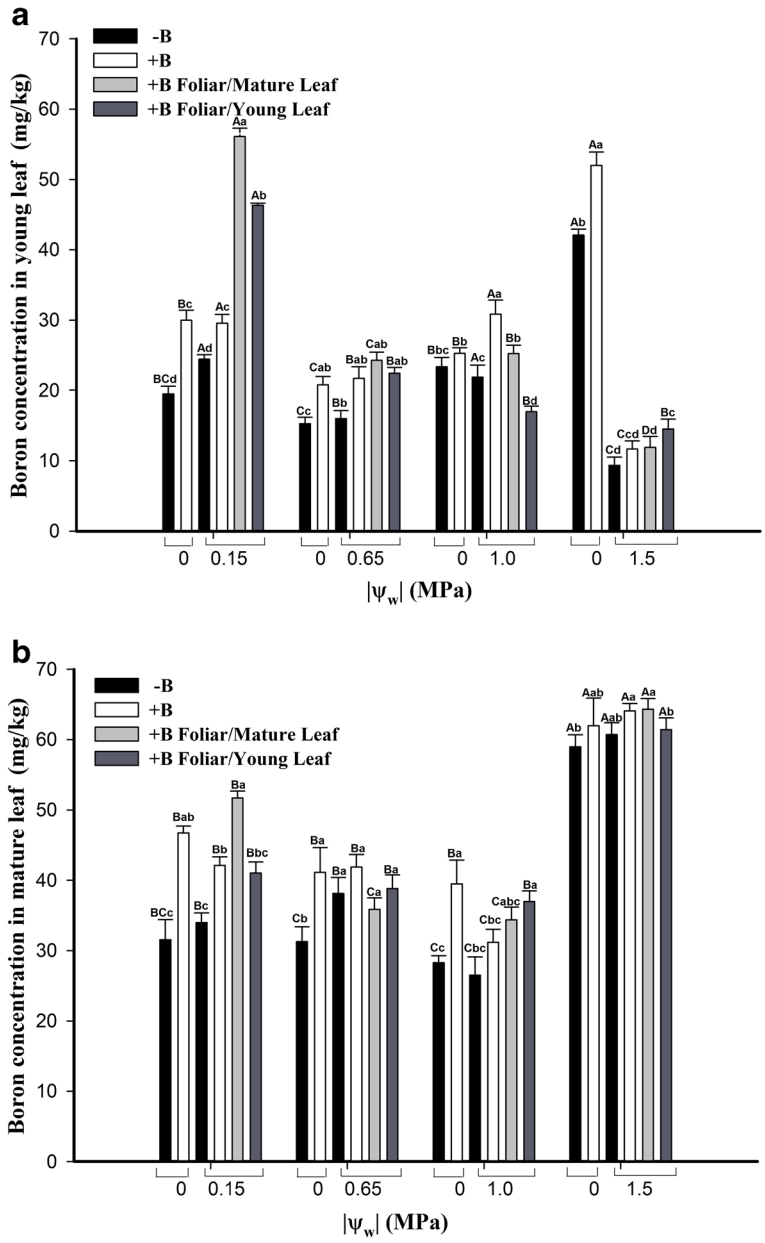


**Fig. 4** Boron concentration in young (A) and mature leaves (B) of *Eucalyptus* in nutrient solutions at different water potentials ($|\Psi_w|$) acclimated in treatments with (+B) or without B (− B) and B application to mature or young leaves. Bars=standard deviation. Capital letters indicate differences between the sampling times and lowercase letters differences between treatments, by the Tukey test at 5 % probability, ($n$=4)

leaves and roots revealed differences between the genes and allowed separation of the three genes in two groups (Fig. 6a-f). In Group I, the genes, including BOR5 and BOR cl6871, were induced at higher B levels, both in leaves and roots (Fig. 6a–d), whereas BOR2 (group II) was induced by B deficiency (Fig. 6e and f). Foliar application of B only in mature leaves induces its mRNA accumulation of BOR5 and BOR cl6871 mainly in leaves (Fig. 6a and c). Despite that, it is interesting to observe that the highest values of gene expression for both genes in both plant organs occur when foliar application of B occurred in the plants under water stress and low B level.

Analyzing the effect of water deficit alone in the gene expression of these transporters, we could see that both genes of group I showed a modest induction of gene expression in leaves (15 to 70 % increase), this induction being more notorious in roots (20–300 %), depending on the level of stress. BOR 2 was also induced by drought, both in leaves as in roots.

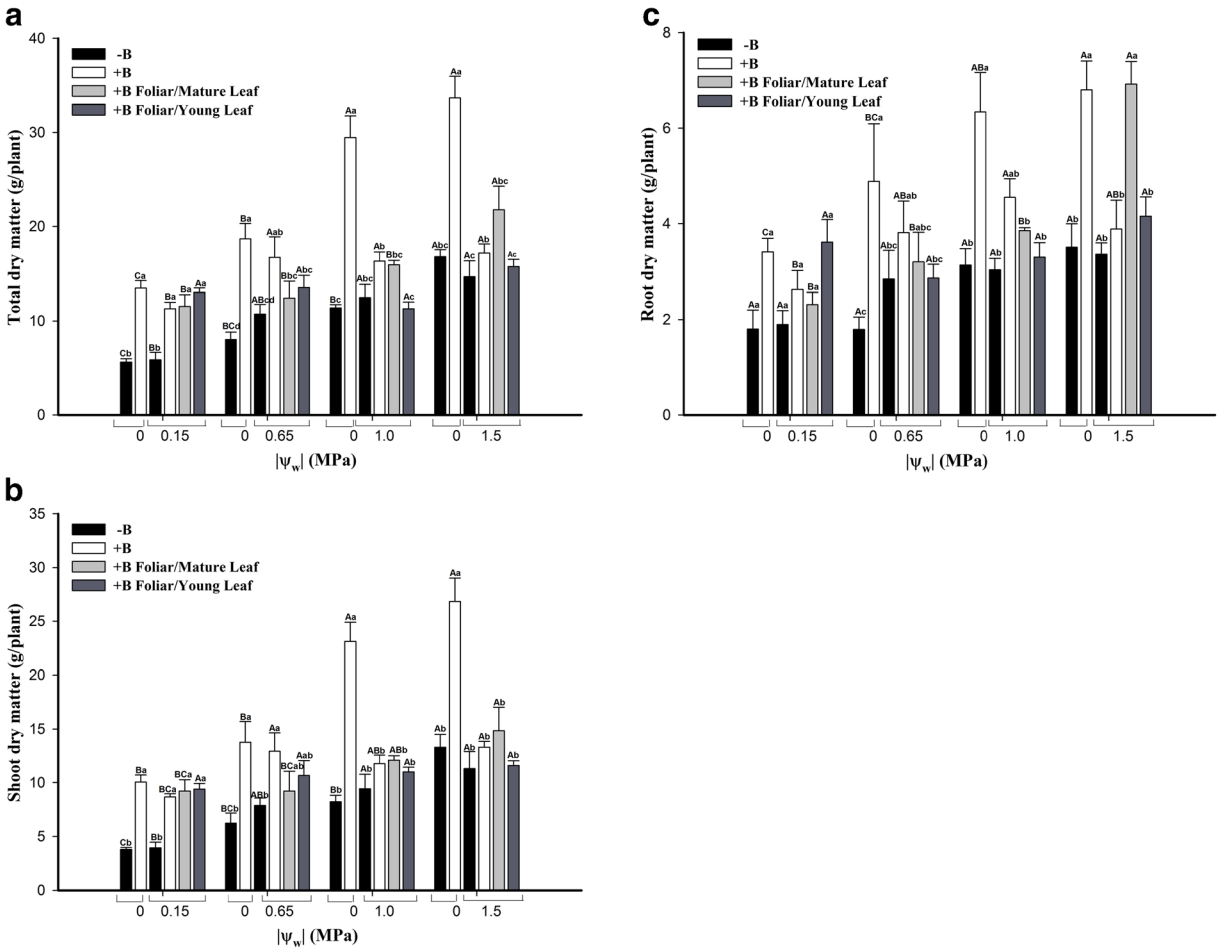


**Fig. 5** Total dry matter (TDM), (**a**), shoot dry matter (SDM), (**b**), and root dry matter (RDM), (**c**) of *Eucalyptus* in nutrient solutions at different water potentials ($|\Psi_w|$) acclimated in treatments with (+B) or without B (− B) and B application to mature or young leaves. Bars=standard deviation. Capital letters indicate differences between the sampling times and lowercase letters differences between treatments, by the Tukey test at 5 % probability, ($n$=4)

B is mainly found linked to pectin in plant cell walls, but little is known whether pectin metabolism or degradation could contribute to boron mobility in plants, or if reduction in pectin in apical shoot due to reduction in B availability could result in shoot die back in Eucalyptus. B deficiency reduced expression of pectin metabolism genes in both, leaves and roots, by around 50 % (Fig. 7a–d) with or without water deficit. As for the B transporters of group I, foliar application in mature leaf induced higher levels of accumulation of both genes than the application in young leaf.

Water stress affected differently the expression of two pectin metabolism genes (Fig. 7a–d). Whereas severe water stress induced the expression of GT8 in both organs, this effect in PMe3 was visible only in roots (Fig.7b). Under −0.65 MPa only increased GT8 expression in roots (Fig. 7d). At -0.15 MPa, the opposite behavior in leaves and roots was observed (Fig. 7c and d).

## Discussion

### Foliar application of B delays dehydration and promotes root growth

In the field, B uptake by the root system of water-stressed plants becomes limited due to the reduced transpiration flux (Mattiello et al. 2009a). Therefore, to mimic the circumstances in nature (reduced B uptake during drought), we decided to remove B from the nutrient solution after the onset of drought. Under this condition, it was observed that B application to mature

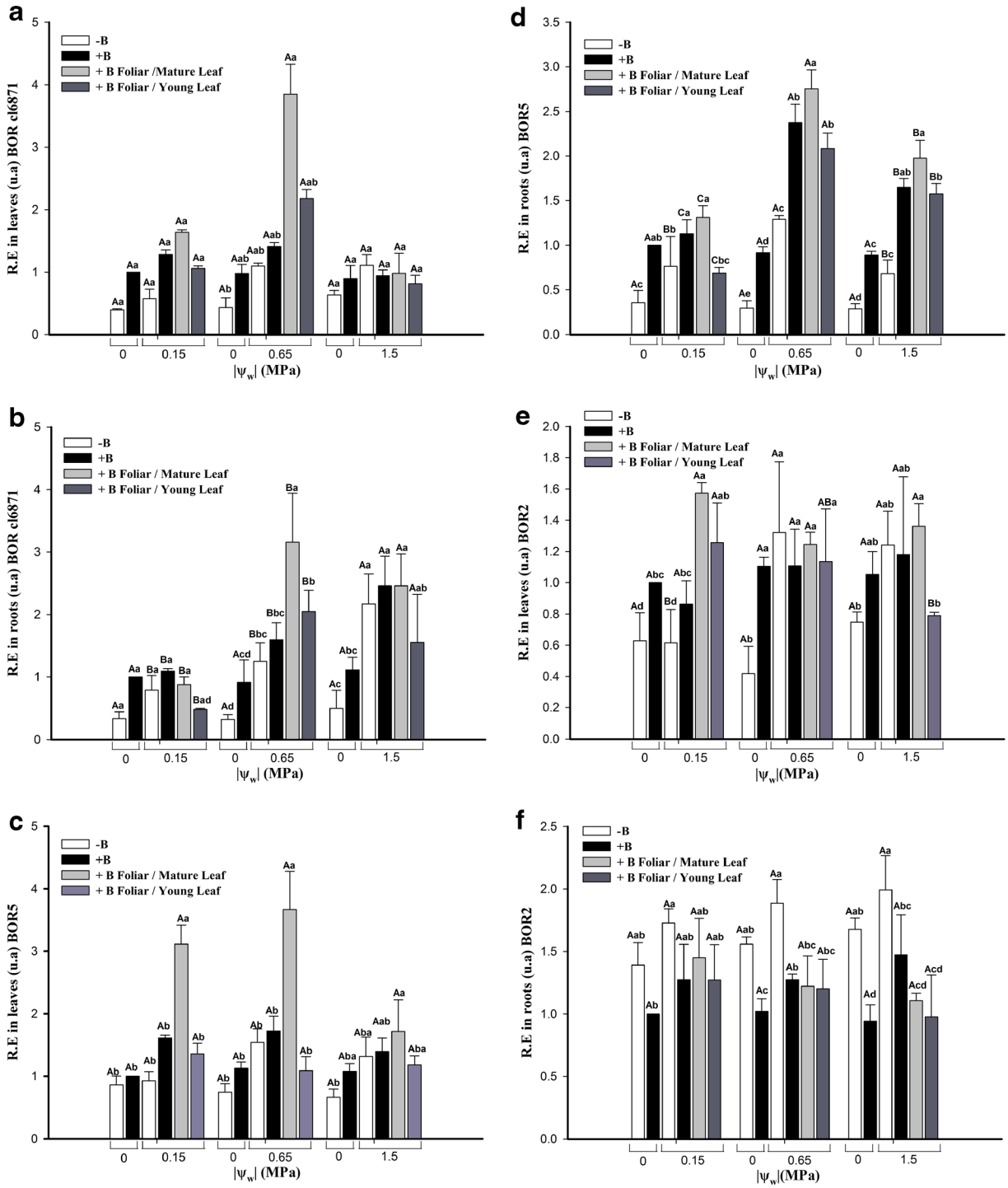


**Fig. 6** Relative expression of genes (RE) transporters B (BOR cl6871, BOR5 and BOR2) in leaves (A, C and E, respectively) and roots (B, D, F, respectively) of *Eucalyptus* in nutrient solutions at different water potentials ($|\Psi_w|$) acclimated in treatments with (+B) or without B (−B) and B application to mature or young leaves. Bars=standard deviation. Capital letters indicate differences between the sampling times and lowercase letters differences between treatments, by the Tukey test at 5 % probability, ($n$=3)

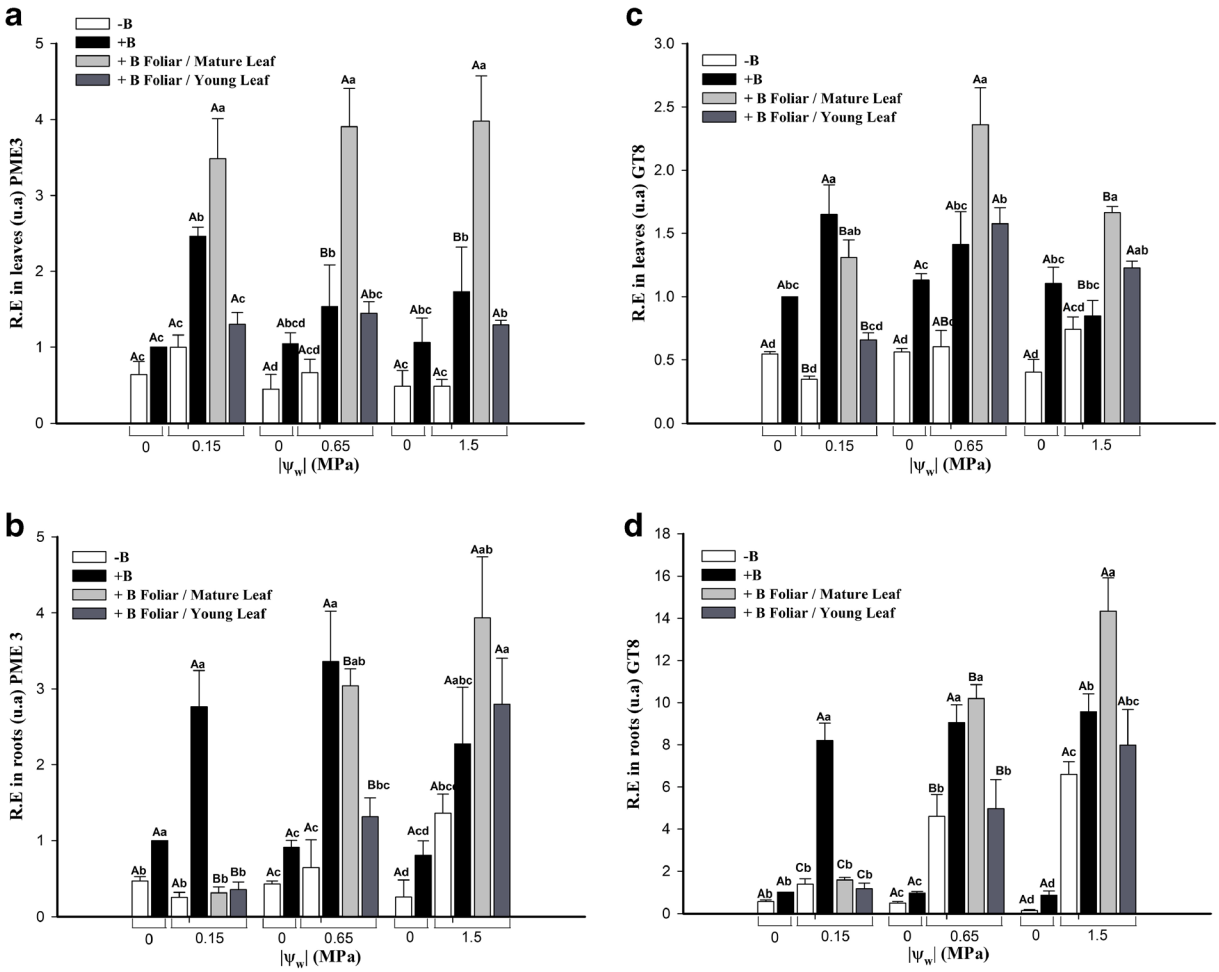


**Fig. 7** Relative expression of genes (RE) related to the formation of cell wall formation; pectin methylesterase (PMe3) and glycosyltransferase isoform 8 (GT8) in leaves (A and C respectively) and roots (B and D respectively) of *Eucalyptus* seedlings in nutrient solutions at different water potentials ($\Psi_{wf}$) acclimated in treatments with (+B) or without B (−B) and B application to mature or young leaves. Bars=standard deviation. Capital letters indicate differences between the sampling times and lowercase letters differences between treatments, by the Tukey test at 5 % probability, ($n$=3)

leaves was the most effective in improving water use efficiency and delaying plant dehydration, as shown in long term treatments by the highest $\delta\ ^{13}C$ (Farquhar et al. 1982) and in the short term by the less negative Leaf-$\Psi_{wf}$. On the other hand, the same treatment in young leaves was not as effective. The $\delta\ ^{13}C$ can be used as a proxy for the water use efficiency in plants (Farquhar et al. 1989) given a high correlation between WUE and $\delta\ ^{13}C$ has been found in C3 species (Zhang et al. 1997, Chen et al. 2007), where higher $\delta\ ^{13}C$ indicates higher WUE.

Applying B to the mature leaf also intensified root growth during water deficit, contributing to lower plant dehydration during water stress periods, as similarly observed for *Picea abies* (Möttönen et al. 2001, 2005) which also showed restricted root growth under B deficiency (Räisänen et al. 2007). In *Brassica rapa*, root growth was reduced by 74 % in water-stressed plants under low B availability, while in plants supplied with the nutrient, root growth reduction was lower (56 %) (Hajiboland and Farhanghi 2011).

B deficiency can affect water relations of plants, altering water uptake by roots, transport through the shoot, as well as facilitating the wilting in plants under water stress (Wimmer and Eichert 2013). In this study, a higher WUE (as inferred from higher $\delta\ ^{13}C$) and lower $\Psi_{wf}$ were observed in response to foliar B application in plants under water stress, suggesting that in *Eucalyptus*,

B would contribute to a better water absorption (larger root system), as well as an improved WUE and reduction of dehydration during water stress.

## B foliar application promotes nutrient translocation and changes in expression of boron transporters

Although B is considered a nutrient with low mobility in various plants (Raven 1980), supported by its higher concentration and binding in the cell wall, reflecting its major role in structural functions (O'Neill et al. 2004), the retranslocation of this nutrient in some species is significant (Brown and Hu 1996; Lehto et al. 2004). Our previous study showed that B mobility depends on the plant genotype and is induced by its deficiency (Mattiello et al. 2009b), being higher when B is chelated with mannitol in foliar application (São José et al. 2009). The difference in its retranslocation may be associated with a differential expression of B transporters in leaves.

Boron application to leaves increased the expression of B transporters (BOR5 and BORcl6871) in leaves, suggesting that these are candidate genes involved in B retranslocation. Furthermore, the application to mature leaf resulted in larger increments of $^{10}B$ in other leaves and roots, demonstrating higher mobility of the nutrient when applied to mature leaf. Aside from the action of B carriers facilitating root uptake, Sun et al. (2012) suggested that carriers also have the function of a more generalized B retranslocation in different cell types and multiple tissues, including vascular and parenchyma tissues.

As B acquisition by the root system during periods of water stress is limited, the energy-dependent B carriers may act to facilitate nutrient uptake through roots (Stangoulis et al. 2001; Takano et al. 2002; Tanaka and Fujiwara 2008). Several studies addressed the function of the transporters located in the plasma membrane of the root vascular cylinder, which are involved in the transport of B to the xylem (Takano et al. 2002; Takano et al. 2005; Miwa & Fujiwara 2010). These transporters facilitate B acquisition and transport to shoots, especially at low B availability in the soil solution (Reid 2014).

The genes AtBOR1 (*Arabidopsis thaliana*) and OsBOR1 (*Oryza sativa*), also related to the ion transport, are required for uninterrupted growth at low B availability (Takano et al. 2002; Nakagawa et al. 2007). The results reported here for BOR2 in roots are similar to those obtained by these authors, who reported increased expression of the gene in the absence of B. The expression of two other carriers, BOR5 and BORcl6871, was increased with increasing B levels in leaves as well as roots, as reported for AtBOR2 (Nakagawa et al. 2007). It is noteworthy that the mechanisms and action of B transporters are still unknown and further studies on this issue are urgently needed (Park et al. 2008; Reid 2014).

There are several functions for B, including the development of the cell wall by participating actively in RG II, by the formation of B-diester bonds responsible for the integrity of the pectin chain (O'Neill et al. 2004). The B requirement is associated with the amount of pectin in the primary cell wall of plants (Hu and Brown 1994; Matoh et al. 1996).

The results of this study suggest that the presence of B and water stress induced pectin synthesis in drought-tolerant Eucalyptus clones, based in the specific increased expression of PMe3 and GT8 in leaves and roots, two enzymes associated with pectin biosynthesis. The increase in the synthesis of pectin chains could increases drought tolerance, since under water stress they have been proposed as acting as gelling and anti-desiccating agents (Leucci et al. 2008). Additionally, the increased synthesis of pectic chains can prevent drying of leaf tips in eucalyptus, by avoiding malformation of the cell wall in meristematic regions (Althoff et al. 1991).

We also found that the absence of B reduced the transcription rate of gene PMe3 by around 20 % and 50 % in leaves and roots, respectively, in well-watered plants (Fig. 6). These results are similar to those obtained for the roots of *Arabidopsis thaliana* where the expression of PMe2 was reduced by 50 % in plants with restricted B supply (Camacho-Cristóbal et al. 2008). Both foliar B application and water stress increased the transcriptional rate of this gene suggesting that its increased expression plays an important role in root development (Wen et al. 1999) and the results presented here suggests an important role of PMe to avoid die-back as consequence of drought.

The greater expression of GT8 in leaves and roots under water stress periods and after foliar application of B plays an important role in water-stressed *Eucalyptus*. Its absence can result in a reduced adhesion between leaf and root cells, increasing their dehydration, as suggested by the knockout of this gene (Bouton et al. 2002).

Water deficit and B application to mature leaves increased the expression of these two genes (PMe3 and GT8). Apparently, there was a synergistic action

of these two factors on the expression of these genes and their greater expression in leaves and roots could be associated with modification of cell wall, as needed for an improved root growth, or helping to decrease leaf and apical tip dehydration in *Eucalyptus*. Several proteomic and transcriptomic studies have found evidence for the importance of cell wall modification in drought tolerance in plants (Vicent et al. 2005; Yoshimura et al. 2008; Ranjan et al. 2012). For example, expansin, a cell-wall loosening protein, was detected as a drought induced protein, and its over expression resulted in changes in leaf anatomy and higher tolerance to water stress (Dai et al. 2012; Lü et al. 2013).

## Conclusion

Altogether, our results suggest that foliar application of B in mature leaves was the most effective in increasing the water stress tolerance in *Eucalyptus urophylla* resulting in a bigger root system, higher WUE, increased cell wall gene expression (GT8 and PMe3) and higher translocation of B to growing tissue under water stress. Thus, foliar application of B during prolonged periods of water stress may be an important silvicultural practice in order to increase water stress tolerance in *Eucalyptus urophylla* plantations.

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## References

Ali-Benali MA, Alary R, Joudrier P, Gautier MF (2005) Comparative expression of five Lea Genes during wheat seed development and in response to abiotic stresses by real-time quantitative RT-PCR. Biochim Biophy Acta 1730:56–65. doi:10.1016/j.bbaexp.2005.05.011

Al-Qsous S, Carpentier E, Klein-Eude D et al (2004) Identification and isolation of a pectin methylesterase isoform that could be involved in flax cell wall stiffening. Planta 219:369–378. doi:10.1007/s00425-004-1246-1

Althoff P, Oliveira AC, Morais EJ, Fonseca S (1991) Eucalypt dieback in cerrado areas in North-Northwest of Minas Gerais. In: Schonau, A.P.G. (Ed), Symposium on Intensive Forestry: The role of eucalypts, pp 598–609

Amenós M, Corrales I, Poschenrieder C et al (2009) Different effects of aluminum on the actin cytoskeleton and brefeldin A-sensitive vesicle recycling in root apex cells of two maize varieties differing in root elongation rate and aluminum tolerance. Plant Cell Physiol 50:528–540. doi:10.1093/pcp/pcp013

Baluška F, Hlavacka A, Šamaj J (2002) F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A-induced compartments. Plant Physiol 130(1):422–431

Bouton S, Leboeuf E, Mouille G et al (2002) QUASIMODO1 encodes a putative membrane-bound glycosyltransferase required for normal pectin synthesis and cell adhesion in Arabidopsis. Plant Cell 14:2577–2590. doi:10.1105/tpc.004259.bone

Brown P (1996) Phloem mobility of boron is species dependent: evidence for phloem mobility in sorbitol-rich species. Ann Bot 77:497–506. doi:10.1006/anbo.1996.0060

Camacho-Cristóbal JJ, Herrera-Rodríguez MB, Beato VM et al (2008) The expression of several cell wall-related genes in Arabidopsis roots is down-regulated under boron deficiency. Environ Exp Bot 63:351–358. doi:10.1016/j.envexpbot.2007.12.004

Chen S, Bai Y, Lin G, et al (2007) Isotopic carbon composition and related characters of dominant species along an environmental gradient in Inner Mongolia, China. J Arid Environ 71:12–28. doi:10.1016/j.jaridenv.2007.02.006

Dai F, Zhang C, Jiang X et al (2012) RhNAC2 and RhEXPA4 are involved in the regulation of dehydration tolerance during the expansion of rose petals. Plant Physiol 160:2064–2082. doi:10.1104/pp. 112.207720

Farquhar G, O'Leary M, Berry J (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Aust J Plant Physiol 9:121. doi:10.1071/PP9820121

Farquhar G, Ehleringer J, Hubick K (1989) Carbon isotope discrimination and photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 40:503–537. doi:10.1146/annurev.pp. 40.060189.002443

Hajiboland R, Farhanghi F (2011) Effect of low boron supply in turnip plants under drought stress. Biol Plant 55:775–778. doi:10.1007/s10535-011-0186-4

Hu H, Brown PH (1994) Localization of boron in cell walls of squash and tobacco and its association with pectin (evidence for a structural role of boron in the cell wall). Plant Physiol 105:681–689

Karim MR, Zhang Y-Q, Zhao R-R et al (2012) Alleviation of drought stress in winter wheat by late foliar application of zinc, boron, and manganese. J Plant Nut Soil Sc 175:142–151. doi:10.1002/jpln.201100141

Kato Y, Miwa K, Takano J et al (2009) Highly boron deficiency-tolerant plants generated by enhanced expression of NIP5;1, a boric acid channel. Plant Cell Physiol 50:58–66. doi:10.1093/pcp/pcn168

Lehto T, Räisänen M, Lavola A et al (2004) Boron mobility in deciduous forest trees in relation to their polyols. New Phytol 163:333–339. doi:10.1111/j.1469-8137.2004.01105.x

Leshem B (1966) Toxic effects of carbowaxes (polyethylene glycols) on Pinus halepensis mill. Seedlings 2:3–5

Leucci MR, Lenucci MS, Piro G, Dalessandro G (2008) Water stress and cell wall polysaccharides in the apical root zone of wheat cultivars varying in drought tolerance. J Plant Physiol 165:1168–1180. doi:10.1016/j.jplph.2007.09.006

Lu P, Kang M, Jiang X, Rhexpa R (2013) RhEXPA4, a rose expansin gene, modulates leaf growth and confers drought and salt tolerance to Arabidopsis. Planta 237:1547–1559. doi:10.1007/s00425-013-1867-3

Matoh T, Kawaguchi S, Kobayashi M (1996) Ubiquity of a borate-rhamnogalacturonan II complex in the cell walls of higher plants. Plant Cell Physiol 37:636–640. doi:10.1093/oxfordjournals.pcp.a028992

Mattiello EM, Ruiz HA, Silva I et al (2009a) Transporte de boro no solo e sua absorção. Rev Bras Ci Solo 33:1281–1290. doi:10.1590/S0100-06832009000500021

Mattiello EM, Ruiz HA, Silva IR et al (2009b) Phloem mobility of Boron in two eucalypt clones. Rev Bras Ci Solo 33:1695–1704. doi:10.1590/S0100-06832009000600018

Michel BE, Kaufmann MR (1973) The osmotic potential of polyethylene glycol 6000 1. Plant Physiol 51:914–916. doi:10.1104/pp.51.5.914

Miwa K, Fujiwara T (2010) Boron transport in plants: co-ordinated regulation of transporters. Ann Bot 105:1103–1108. doi:10.1093/aob/mcq044

Möttönen M, Aphalo PJ, Lehto T (2001) Role of boron in drought resistance in Norway spruce (Picea abies) seedlings. Tree Physiol 21:673–681

Möttönen M, Lehto T, Rita H, Aphalo PJ (2005) Recovery of Norway spruce (Picea abies) seedlings from repeated drought as affected by boron nutrition. Trees 19:213–223. doi:10.1007/s00468-004-0384-1

Moustacas AM, Nari J, Borel M et al (1991) Pectin methylesterase, metal ions and plant cell-wall extension. The role of metal ions in plant cell-wall extension. Biochem J 279:351–354

Nakagawa Y, Hanaoka H, Kobayashi M et al (2007) Cell-type specificity of the expression of Os BOR1, a rice efflux boron transporter gene, is regulated in response to boron availability for efficient boron uptake and xylem loading. The Plant Cell 19:2624–2635. doi:10.1105/tpc.106.049015

Nepomuceno A, Oosterhuis D, Stewart J (1998) Physiological responses of cotton leaves and roots to water deficit induced by polyethylene glycol. Environ Exp Bot 40(1):29–41. doi:10.1016/S0098-8472(98)00018-5

O'Donnell NH, Møller BL, Neale AD, Hamill JD, Blomstedt CK, Gleadow RM (2013) Effects of PEG-induced osmotic stress on growth and dhurrin levels of forage sorghum. Plant Physiol and Biochem : PPB/Soc Fr de Physiol Végétale 73:83–92. doi:10.1016/j.plaphy.2013.09.001

O'Neill MA, Ishii T, Albersheim P, Darvill AG (2004) Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. Ann Rev Plant Biol 55:109–139. doi:10.1146/annurev.arplant.55.031903.141750

Park J, Kim Y, Martinoia E, Lee Y (2008) Long-distance transporters of inorganic nutrients in plants. J Plant Biol 51:240–247. doi:10.1007/BF03036122

Pelloux J, Rustérucci C, Mellerowicz EJ (2007) New insights into pectin methylesterase structure and function. Trends Plant Sci 12:267–277. doi:10.1016/j.tplants.2007.04.001

Räisänen M, Repo T, Lehto T (2007) Cold acclimation was partially impaired in boron deficient Norway spruce seedlings. Plant Soil 292:271–282. doi:10.1007/s11104-007-9223-7

Ranjan A, Pandey N, Lakhwani D et al (2012) Comparative transcriptomic analysis of roots of contrasting Gossypium herbaceum genotypes revealing adaptation to drought. BMC Genomics 13:680. doi:10.1186/1471-2164-13-680

Raven JA (1980) Short- and long-distance transport of boric acid in plants. New Phytol 84:231–249. doi:10.1111/j.1469-8137.1980.tb04424.x

Reid R (2014) Understanding the boron transport network in plants. Plant Soil. doi:10.1007/s11104-014-2149-y

Röckel N, Wolf S, Kost B et al (2008) Elaborate spatial patterning of cell-wall PME and PMEI at the pollen tube tip involves PMEI endocytosis, and reflects the distribution of esterified and de-esterified pectins. Plant J 53:133–143. doi:10.1111/j.1365-313X.2007.03325.x

Ross J, Li Y, Lim E, Bowles DJ (2001) Higher plant glycosyltransferases. Genome Biol 2:1–6. doi:10.1186/gb-2001-2-2-reviews3004

São José J, Silva I, Barros N et al. (2009) Boron mobility in eucalyptus clones. Rev Bras Ci Solo 1733–1744. doi: 10.1590/S0100-06832009000600022

Scheible W-R, Pauly M (2004) Glycosyltransferases and cell wall biosynthesis: novel players and insights. CurrOpin Plant Biol 7:285–295. doi:10.1016/j.pbi.2004.03.006

Schon MK, Novacky A, Blevins DG (1990) Boron induces hyperpolarization of sunflower root cell membranes and increases membrane permeability to K+. Plant Physiol 93:566–571

Stangoulis JC, Reid RJ, Brown PH, Graham RD (2001) Kinetic analysis of boron transport in Chara. Planta 213:142–146. doi:10.1007/s004250000484

Sucre B, Suárez N (2011) Effect of salinity and PEG-induced water stress on water status, gas exchange, solute accumulation, and leaf growth in Ipomoea pes-caprae. Environ Exp Bot 70(2–3):192–203. doi:10.1016/j.envexpbot.2010.09.004

Sun J, Shi L, Zhang C, Xu F (2012) Cloning and characterization of boron transporters in Brassica napus. Mol Biol Rep 39:1963–1973. doi:10.1007/s11033-011-0930-z

Takano J, Yamagami M, Noguchi K et al (2001) Preferential translocation of boron to young leaves in *Arabidopsis thaliana* regulated by the BOR1 gene. Soil Sci Plant Nutr 47:345–357. doi:10.1080/00380768.2001.10408398

Takano J, Noguchi K, Yasumori M et al (2002) Arabidopsis boron transporter for xylem loading. Nature 420:337–340. doi:10.1038/nature01139

Takano J, Miwa K, Yuan L et al (2005) Endocytosis and degradation of BOR1, a boron transporter of Arabidopsis thaliana, regulated by boron availability. Proc Natl Acad Sci U S A 102:12276–12281. doi:10.1073/pnas.0502060102

Tanaka M, Fujiwara T (2008) Physiological roles and transport mechanisms of boron: perspectives from plants. Pflügers Archiv : Eur J Physiol 456:671–677. doi:10.1007/s00424-007-0370-8

Vandesompele J, De Preter K, Pattyn F et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Gen Biol 3:1–12. doi:10.1186/gb-2002-3-7-research0034

Vincent D, Lapierre C, Pollet B et al (2005) Water deficits affect caffeate O-methyltransferase, lignification, and related enzymes in maize leaves. Plant Physiol 137:949–960. doi:10.1104/pp. 104.050815

Wang X, Tian W, Li Y (2008) Development of an efficient protocol of RNA isolation from recalcitrant tree tissues. Mol Biotechnol 38:57–64. doi:10.1007/s12033-007-0073-6

Wen F, Zhu Y, Hawes MC (1999) Effect of pectin methylesterase gene expression on pea root development. Plant Cell 11: 1129–1140. doi:10.1105/tpc.11.6.1129

Wilson K, Walker K (2000) Principles and techniques of practical biochemistry, 5ath edn. Cambridge University Press, Cambridge

Wimmer MA, Eichert T (2013) Review: mechanisms for boron deficiency-mediated changes in plant water relations. Plant Sci 203–204:25–32. doi:10.1016/j.plantsci.2012.12.012

Xu C, Huang B (2010) Differential proteomic responses to water stress induced by PEG in two creeping bentgrass cultivars differing in stress tolerance. J Plant Physiol 167(17):1477–1485. doi:10.1016/j.jplph.2010.05.006

Yoshimura K, Masuda A, Kuwano M et al (2008) Programmed proteome response for drought avoidance/tolerance in the root of a C(3) xerophyte (wild watermelon) under water deficits. Plant Cell Physiol 49:226–241. doi:10.1093/pcp/pcm180

Zhang J, Feng Z, Cregg B, Schumann C (1997) Carbon isotopic composition, gas exchange, and growth of three populations of ponderosa pine differing in drought tolerance. Tree Physiol 17(7):461–466. doi:10.1093/treephys/17.7.461