



Calcium supply alters aquaporin transcription and enhances water deficit resilience in a *Eucalyptus* hybrid

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Abstract

Key message Calcium plays a role in modulating aquaporin expression, reducing oxidative stress, preserving chlorophyll content, and maintaining membrane integrity, suggesting it may enhance eucalypt resilience to water restriction.

Abstract Drought is a significant abiotic stress that severely impacts the growth and productivity of forests worldwide. This study investigates the role of calcium (Ca^{2+}) supply in modulating aquaporin (AQP) gene expression and mitigating oxidative stress in eucalypt (*Eucalyptus urophylla* × *Eucalyptus camaldulensis*) under water restriction. Three-month-old eucalypt seedlings were subjected to water restriction, with or without Ca^{2+} supplementation. We measured mRNA expression levels of five aquaporin isoforms (*PIP1;2*, *PIP1;3*, *PIP2;1*, *PIP2;2*, and *PIP2;7*), chlorophyll content, and markers of oxidative stress [e.g., hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and the activities of catalase (CAT) and superoxide dismutase (SOD)]. Our findings reveal that initial water restriction upregulated the expression of *PIP1;2*, *PIP2;1*, *PIP2;2*, and *PIP2;7* in roots, which likely facilitated water uptake and maintained cellular hydration. However, under prolonged water limitation, plants supplemented with Ca^{2+} showed downregulation of these PIPs, possibly as a protective mechanism to reduce water loss. In contrast, plants without Ca^{2+} exhibited an upregulation of *PIP1;2*, *PIP1;3*, and *PIP2;7* under prolonged water deficit. Ca^{2+} supplementation also played a crucial role in preserving chlorophyll content and reducing oxidative damage under water limitation. Plants supplemented with Ca^{2+} had lower levels of H_2O_2 and MDA, reduced oxidative damage and membrane permeability, alongside increased CAT activity, which suggests an enhanced oxidative response. Our results provide new insights into how Ca^{2+} enhances eucalypt responses to water deprivation, enhancing our understanding of the function of Ca^{2+} in drought responses.

Keywords Abiotic stress · Gene expression · Forest · Water transport · Oxidative stress · Tree

Introduction

Eucalypt tree plantations span over 22 million hectares across 95 countries (Zhang and Wang 2021). Concerns about productivity and sustainability arise due to climate change-induced adverse weather conditions (Stape et al. 2010). Globally, drought impacts forest growth and productivity (Gazol et al. 2018; Hammond et al. 2022). As climate change accelerates, trees may struggle to adapt, resulting in increased tree mortality (Brodribb et al. 2020). Calcium (Ca^{2+}) plays a crucial role in mitigating the adverse effects of drought, enhancing plant tolerance under water stress conditions (Ranty et al. 2016; Thor 2019; Li et al. 2022). Adequate nutrition with Ca^{2+} minimizes membrane lipid peroxidation and enhances antioxidant responses, thereby protecting plants from stress-induced oxidative damage

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(Upadhyaya et al. 2011; Wu et al. 2012; Leng et al. 2020). Furthermore, Ca^{2+} supply enhances plant water status and water use efficiency (Wang et al. 2016; Kang et al. 2017).

Maintaining cellular water homeostasis in drought conditions is essential to alleviate environmental stress and ensure plant growth and development (Patel and Mishra 2021). Aquaporins (AQP) are membrane channel proteins that play a fundamental role in this process by facilitating the transport of water and specific ions across cellular membranes (Afzal et al. 2016; Kapilan et al. 2018; Patel and Mishra 2021). In plants, there are five families of AQP: PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (nodulin 26-like intrinsic proteins), SIPs (small basic intrinsic proteins), and XIPs (X-intrinsic proteins) (Maurel et al. 2015).

PIP is the largest aquaporin family in plants. Its subdivision into PIP1s and PIP2s likely occurred early in plant evolution, as both are conserved across all higher plants (Maurel et al. 2015). These two subfamilies play essential roles in regulating water transport across the plasma membrane, influencing root water uptake under both normal and stress conditions (Zargar et al. 2017; Yepes-Molina et al. 2020), and contributing to drought tolerance and recovery (Martre et al. 2002; Secchi and Zwieniecki 2014).

Although PIP aquaporins have been functionally characterized in several herbaceous species, their roles in woody plants remain less explored. In *Eucalyptus*, studies are relatively scarce, particularly under drought conditions (Amrutha et al. 2021; Feltrim et al. 2021; Seidel et al. 2024). Moreover, the effects of Ca on AQP gene expression are complex and not yet fully understood, warranting further investigation to clarify how Ca modulates aquaporin-mediated water transport in response to water deficit.

Water restriction can differentially influence AQP gene expression in roots (Afzal et al. 2016). For example, some PIPs are upregulated under water stress, facilitating water uptake and transport (Shekoofa and Sinclair 2018). Conversely, the downregulation of PIPs in roots under prolonged dry conditions may prevent the reverse flow of water from roots back into the soil (Alexandersson et al. 2005; Perrone et al. 2012; Maurel et al. 2015; Afzal et al. 2016). Ca^{2+} is known to modulate AQP functions, especially in response to environmental stress (Carvajal et al. 2000; Cabañero et al. 2006; Yang et al. 2006; Wang et al. 2016). Under water-limiting conditions, Ca^{2+} supply has been shown to enhance cell-to-cell water flow by upregulating AQP expression, thereby optimizing water use efficiency in maize (Wu et al. 2012). However, under prolonged stress, increased cytosolic Ca^{2+} concentrations have been associated with decreased AQP gene expression in pepper (*Capsicum annuum*) (Martínez-Ballesta et al. 2008) and beet (*Beta Vulgaris*) (Allewa et al. 2006). Notably, there is

limited information concerning the role of Ca^{2+} on AQPs in woody plants. In our previous work, we demonstrated that Ca availability influences the expression of Ca transporter genes in eucalypt roots under water restriction (de Barros et al. 2023). Here, we present new evidence indicating that this nutrient is involved in the regulation of AQP genes in a drought-tolerant eucalypt genotype. We investigated how Ca^{2+} supply affects the expression of *PIP1;2*, *PIP1;3*, *PIP2;1*, *PIP2;2*, and *PIP2;7* genes in eucalypt roots under water restriction. The PIP family was chosen because it is conserved throughout all higher plants and due to its crucial role in drought tolerance. We further evaluated the potential role of Ca^{2+} in mitigating oxidative stress induced by water limitation.

Material and methods

Plant material and experimental design

Three-month-old seedlings of the VM01 eucalypt clone (*Eucalyptus urophylla* × *Eucalyptus camaldulensis*), propagated from mini-cuttings, were used. The plants were acclimatized in a greenhouse using polyethylene pots (3 L) containing Clark's nutrient solution (Clark 1975), modified by Locatelli et al. (1984). The composition of Clark solution consisted of: $\text{NH}_4\text{NO}_3 = 2.925 \text{ mmol L}^{-1}$; $\text{CaCl}_2 = 1.3 \text{ mmol L}^{-1}$; $\text{K}_2\text{SO}_4 = 0.3 \text{ mmol L}^{-1}$; $\text{MgSO}_4 = 0.3 \text{ mmol L}^{-1}$; $\text{KCl} = 0.53 \text{ mmol L}^{-1}$; $\text{KH}_2\text{PO}_4 = 0.138 \text{ mmol L}^{-1}$; $\text{Fe-EDTA} = 40 \text{ } \mu\text{mol L}^{-1}$; $\text{MnCl}_2 = 7 \text{ } \mu\text{mol L}^{-1}$; $\text{H}_3\text{BO}_3 = 19 \text{ } \mu\text{mol L}^{-1}$; $\text{ZnSO}_4 = 2 \text{ } \mu\text{mol L}^{-1}$; $\text{CuSO}_4 = 0.5 \text{ } \mu\text{mol L}^{-1}$; $\text{Na}_2\text{MoO}_4 = 0.086 \text{ } \mu\text{mol L}^{-1}$. In the initial phase of the acclimatization period, the nutrient solution had an ionic strength equivalent to 50% of the final solution. The ionic strength of the solution was gradually raised to 100% of the standard solution. The experiment was designed in randomized blocks, in a factorial arrangement (2×2): with Ca^{2+} (+ Ca^{2+}) or without Ca^{2+} (− Ca^{2+}) × water control (WC) or water restriction (WR), with five replicates. The experimental design and procedures were previously described by de Barros et al. (2023).

After 15 days of acclimatization, the plants were divided into two groups. The first group was maintained in a nutrient solution containing Ca^{2+} , and the second group was submitted to treatment without Ca^{2+} for 12 days. After this period, water restriction was imposed by using polyethylene glycol (PEG 6000) gradually added until the solution's water potential reached -0.65 MPa (Michel and Kaufmann 1973). Nutrient solution pH was monitored daily and maintained close to 6. All solutions were changed weekly (Pinto et al. 2011).

Plant samples were collected at two distinct time points to assess the genetic expression of AQP in the roots. The first sampling occurred five days after the nutrient solution reached the water potential of -0.15 MPa (100 g L^{-1} of PEG). The subsequent sampling was conducted at the end of the experiment, six days after the solution reached the water potential of -0.65 MPa (235 g L^{-1} of PEG), and 16 days following the initiation of water restriction imposition. Additionally, measurements of plant water potential, concentrations of photosynthetic pigments, and oxidative stress were taken at the end of the experiment (16 days after starting water restriction exposure).

Plant water status and concentration of photosynthetic pigments

Predawn leaf water potential (Ψ_w) was measured between 4:00 am and 5:00 am using a pressure chamber (Scholander, Corvallis, USA) on fully expanded leaves. Photosynthetic pigments, i.e., chlorophylls *a* and *b* and carotenoids, were measured using 100% methanol (Lichtenthaler's 1987). The absorption of the extracted pigments was then measured at specific wavelengths: 653 nm, 666 nm, and 470 nm, using a spectrometer UV/Visible Multiskan GO®.

Aquaporins gene expression

Real-time quantitative PCR (qRT-PCR) analyses were performed in roots (~5 cm length from the apex) to assess the expression level of five AQP subfamilies: *PIP1;2*, *PIP1;3*, *PIP2;1*, *PIP2;2*, and *PIP2;7*. The total RNA was extracted according to Chang et al. (1993), with a few modifications (de Barros et al. 2023), using five biological replicates. Samples were treated with DNase (Invitrogen) to remove any genomic DNA. The integrity of the RNA was checked on 1.2% (w/v) agarose gel and the concentration was verified using NanoDrop™ 2000 (Thermo Scientific™). The first cDNA strand was synthesized using the ImProm kit-II (Promega). Nucleotide sequences of target genes were obtained from NCBI (<https://www.ncbi.nlm.nih.gov/>) and Phytozome (<https://phytozome-next.jgi.doe.gov/>) platforms for *Eucalyptus grandis*. This sequence was used as a template for aquaporin primer design through the Primer Blast tool of the NCBI platform (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The gene-specific primers used for qRT-PCR are listed in Table S1. The gene that encodes proteins containing the SAND domain was used as a reference gene to normalize data in all samples. This gene was selected based on its demonstrated expression stability in *Eucalyptus* grown under water deficit, as reported by Martins et al. (2018). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Oxidative stress

To evaluate the impacts of water restriction and Ca^{2+} limitation on plants, we performed the following oxidative stress analysis: hydrogen peroxide (H_2O_2), malondialdehyde, electrolyte leakage (EL), superoxide dismutase (SOD) and catalase (CAT). The H_2O_2 content was analyzed following the procedure outlined by Velikova et al. (2000). The reaction mixture consisted of 1500 μL of 0.1% trichloroacetic acid (TCA), 2.5 mM K_2HPO_4 at pH 7, and 0.5 M potassium iodide (KI). The absorbance was recorded at 390 nm using a UV/Visible Multiskan GO® spectrometer. The H_2O_2 content was determined on the authentic H_2O_2 standard curve. Lipid peroxidation was assessed by measuring the accumulation of MDA following the method described by Heath and Packer (1968), with modifications. Lyophilized leaves (35 mg) were homogenized with 1.5 mL of 0.1% TCA. The resulting extract was then centrifuged at 12,000 g for 15 min at 4 °C. Subsequently, 500 μL of the supernatant was collected and mixed with 1.5 mL of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA. The mixture was incubated at 95 °C for 30 min. After centrifugation (10,000 g at 4 °C for 10 min), the absorbance of the supernatant was read at two wavelengths (535 and 600 nm) using a UV/Visible Multiskan GO® spectrometer. The MDA content was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

The EL was evaluated following the method described by Sairam and Srivastava (2002). Ten leaf discs with a diameter of 1.1 cm were collected and washed with deionized water. Subsequently, they were immersed in 20 mL of deionized water at room temperature. After 24 h, the initial electrical conductivity (IC) was measured using a conductivity meter (Orion, Model 115). The samples were then subjected to a temperature of 90 °C for 2 h in a drying oven. The final conductivity (FC) was measured after cooling. The EL was calculated using the following equation: $\text{EL} = (\text{IC}/\text{FC}) \times 100$.

To assess SOD and CAT activity, 35 mg of lyophilized leaf samples were homogenized with 0.1 M potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 180 mM PMSF, and 1% (w/v) PVPP (Ribeiro et al. 2012). The homogenate was filtered and centrifuged at 12,000 g for 15 min at 4 °C, and the supernatant was used to assess SOD and CAT activities. SOD activity was determined by measuring the enzyme's ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), as described by Giannopolitis and Ries (1977). To determine CAT activity, 50 mM potassium phosphate buffer at pH 7.0 and 12.5 mM H_2O_2 were added to the enzymatic extract (Ribeiro et al. 2012). CAT activity was estimated by the change in absorbance during the first minute of the reaction at 30 °C using the following molar extinction coefficient: 240 nm, $\epsilon = 36 \text{ mmol L}^{-1} \text{ cm}^{-1}$.

Statistical analysis

Data were analyzed using a variance analysis (ANOVA), and means were compared using Tukey's test at the 5% significance level. The Shapiro–Wilk and Bartlett's tests were applied to verify ANOVA assumptions of residual normality and homogeneity, respectively. All statistical analyses were performed using R software version 4.0.0 (R Core Team 2020).

Results

Leaf water potential (Ψ_w) was reduced in all treatments regardless of Ca^{2+} supply (Fig. 1A), consistent with the patterns described by de Barros et al. 2023. Ca^{2+} supply increased the expression of *PIP1;2*, *PIP2;1*, *PIP2;2*, and *PIP2;7* genes under water restriction (-0.15 MPa).

Conversely, the expression of the *PIP1;2* and *PIP2;7* genes increased in plants grown without Ca^{2+} under water restriction (i.e., WR $-\text{Ca}^{2+}$ treatment) compared to WC plants (Fig. 1B, F). Plants grown without Ca and under water restriction (i.e., WR $-\text{Ca}^{2+}$ treatment) showed an increase in the expression of the *PIP1;2* and *PIP2;7* genes, compared to WC plants (Fig. 1B, F). On the other hand, WR did not affect the expression of the *PIP1;3*, *PIP2;1*, and *PIP2;2*, compared to the control treatment (Fig. 1C–E), and the expression of *PIP2;7* was lower in $+\text{Ca}^{2+}$ plants compared to $-\text{Ca}^{2+}$ plants, regardless of the water condition (Fig. 1F).

After 16 days of water restriction (-0.65 MPa), plants grown with Ca^{2+} and water restriction (i.e., WR $+\text{Ca}^{2+}$ treatment) showed decreased expression of most aquaporin PIP subfamilies genes in the roots compared to the control treatment (i.e., WC $+\text{Ca}^{2+}$ treatment) (Fig. 2). On the other hand, a similar expression of most PIP genes was found when comparing WC $-\text{Ca}^{2+}$ and WR $-\text{Ca}^{2+}$ plants. *PIP1;2* and

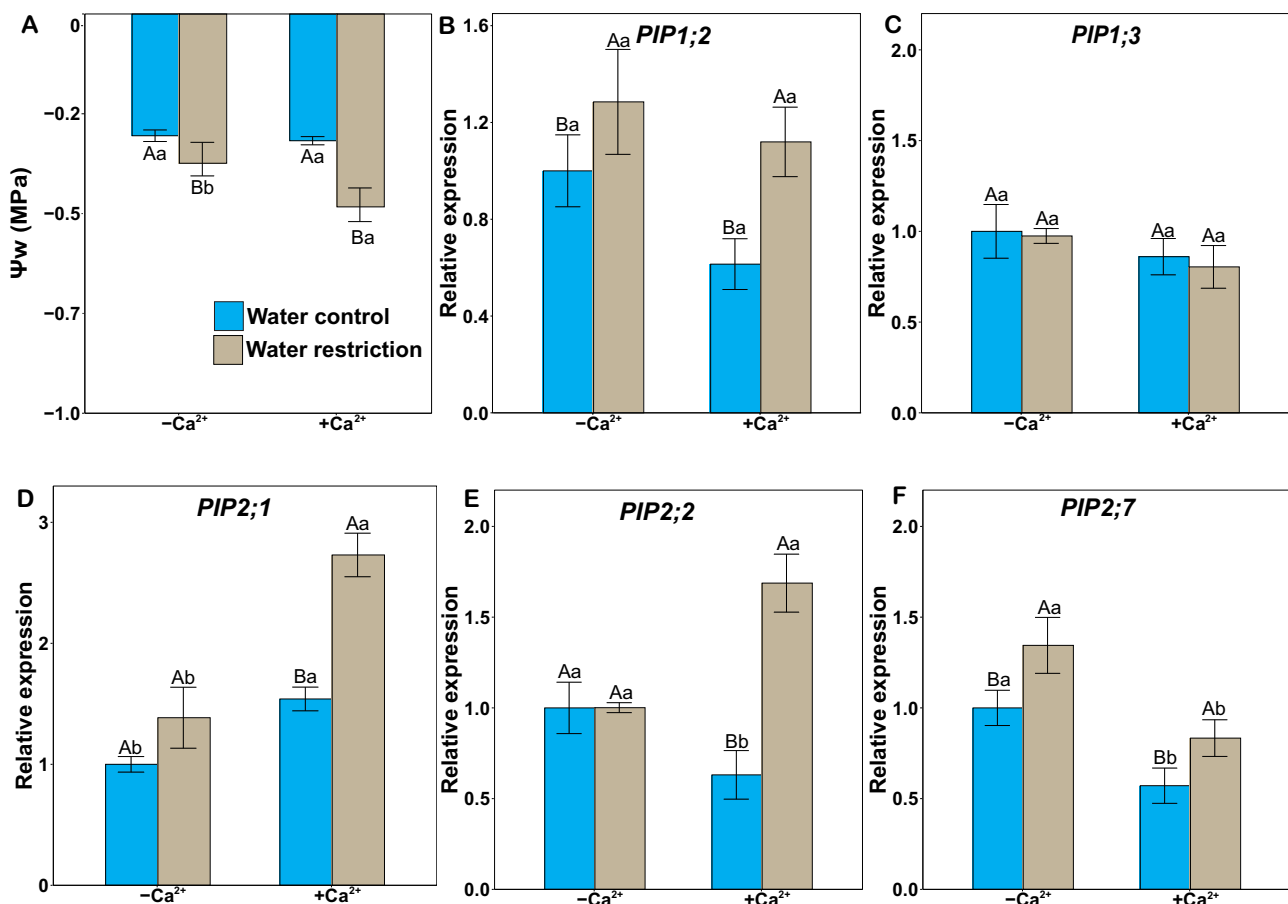
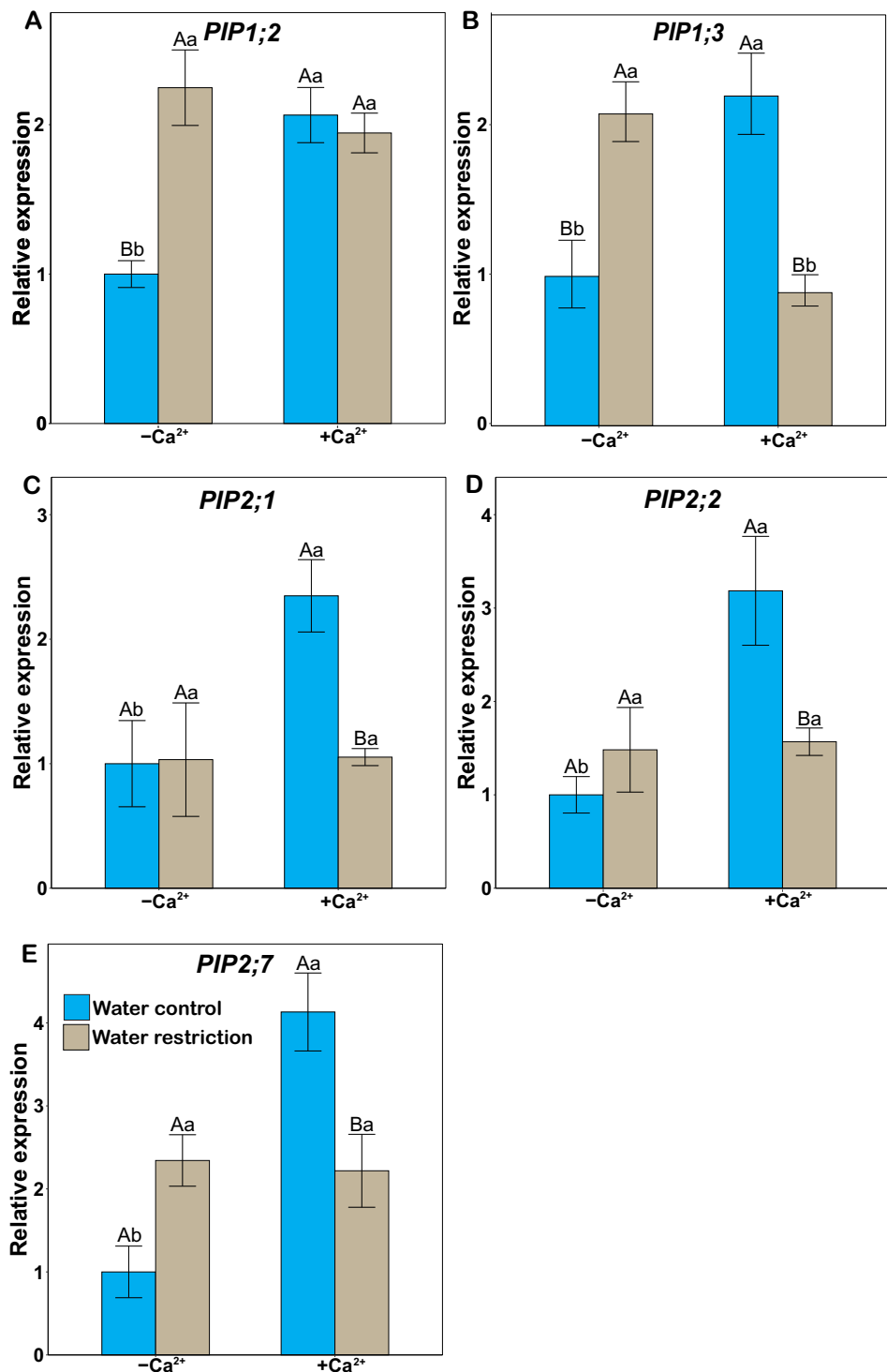


Fig. 1 Predawn leaf water potential (Ψ_w) (A), relative expression of *PIP1;1* (B), *PIP1;2* (C), *PIP2;1* (D), *PIP2;2* (E) and *PIP2;7* (F) in roots of eucalypt clone VM01 grown in nutrient solution with ($+\text{Ca}^{2+}$) or without ($-\text{Ca}^{2+}$) calcium, under conditions of water restriction or control. Measurements were taken five days after the onset of water restriction (nutrient solution $\Psi_w = -0.15$ MPa). Different letters indi-

cate significant differences among treatments ($p < 0.05$, Tukey's test). Uppercase letters compare water regimes (control vs. restriction) within each Ca^{2+} condition, while lowercase letters compare Ca^{2+} treatments within each water regime. Bars represent the mean $\pm$ standard error

Fig. 2 Relative expression of *PIP1;1* (A), *PIP1;2* (B), *PIP2;1* (C), *PIP2;2* (D) and *PIP2;7* (E) in roots of eucalypt clone VM01 grown in nutrient solution with (+Ca²⁺) or without (−Ca²⁺) calcium, under conditions of water restriction or control. Measurements were taken 16 days after the onset of water restriction (nutrient solution $\Psi_w = -0.65$ MPa). Different letters indicate significant differences among treatments ($p < 0.05$, Tukey's test). Uppercase letters compare water regimes (control vs. restriction) within each Ca²⁺ condition, while lowercase letters compare Ca²⁺ treatments within each water regime. Bars represent the mean $\pm$ standard error



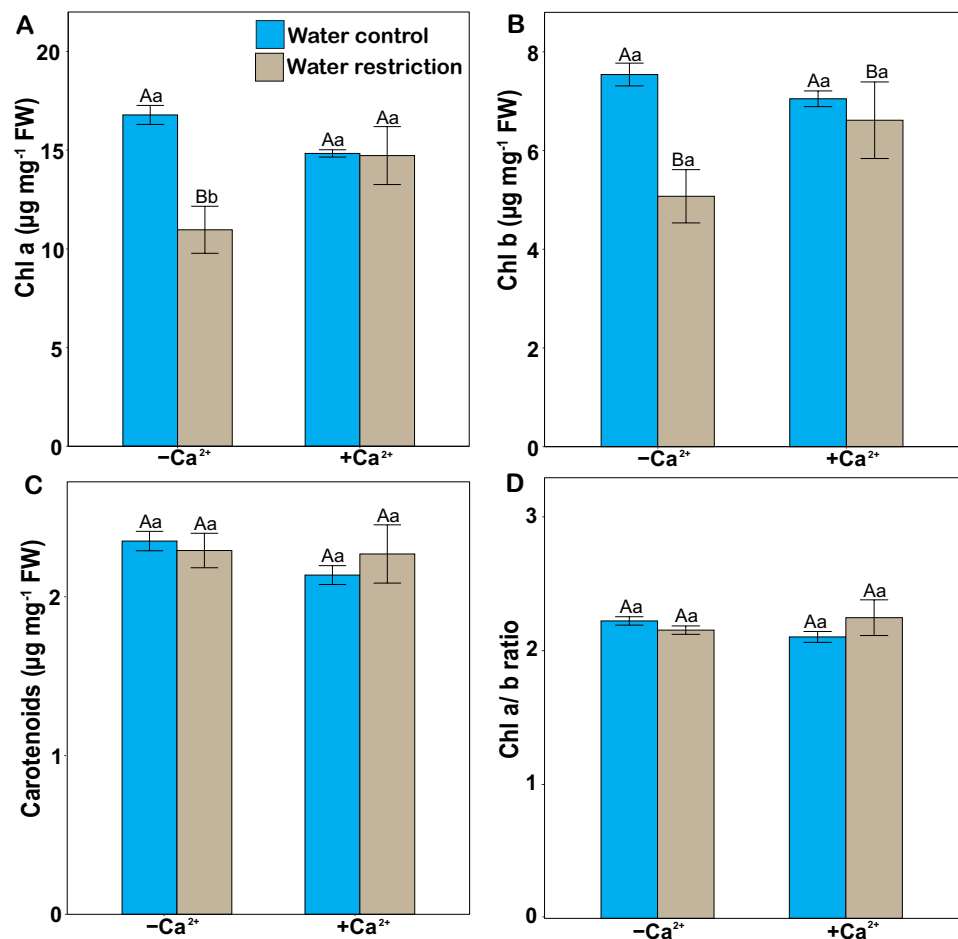
PIP1;3 genes were upregulated with the imposition of water restriction expression compared to the respective control (i.e., WC +Ca²⁺ treatment) (Fig. 2B, C).

The chlorophyll *a* (Chl *a*) content was reduced by 35% under water restriction in $-Ca^{2+}$ plants (Fig. 3A). Chlorophyll *b* (Chl *b*) content decreased in all plants subjected to water restriction. In $-Ca^{2+}$ plants, this reduction was 32.8%,

whereas in $+Ca^{2+}$ plants, the decrease was 6.16% compared to the WC within the same Ca²⁺ supply (Fig. 3B). The carotenoid was not affected by treatments (Fig. 3C).

WR increased lipid peroxidation in all treatments (Fig. 4). In $-Ca^{2+}$ plants, there was a 25.3% increase in the MDA content, whereas in the presence of Ca²⁺, this increase was approximately 11% compared to their respective controls.

Fig. 3 Chlorophyll *a* (Chl *a*) (A), Chlorophyll *b* (Chl *b*) (B), Carotenoids (C) and Chlorophyll *a/b* ratio (D) in VM01 grown in nutrient solution with (+Ca²⁺) or without (−Ca²⁺) calcium, under conditions of water restriction or control. Measurements were taken 16 days after the onset of water restriction (nutrient solution $\Psi_w = -0.65$ MPa). Different letters indicate significant differences among treatments ($p < 0.05$, Tukey's test). Uppercase letters compare water regimes (control vs. restriction) within each Ca²⁺ condition, while lowercase letters compare Ca²⁺ treatments within each water regime. Bars represent the mean $\pm$ standard error



All +Ca²⁺ plants had lower MDA content (Fig. 4A). Electrolyte leakage (EL) increased in WR −Ca²⁺ plants when compared WC +Ca²⁺ (Fig. 4B). Calcium supply also reduced H₂O₂ production in WR plant leaves (Fig. 4C).

WR did not affect catalase activity (CAT) in −Ca²⁺ treatments (Fig. 4E). On the other hand, Ca²⁺ supply increased CAT by 20% in WR plants. Superoxide dismutase (SOD) activity was not affected by treatments (Fig. 4D).

Discussion

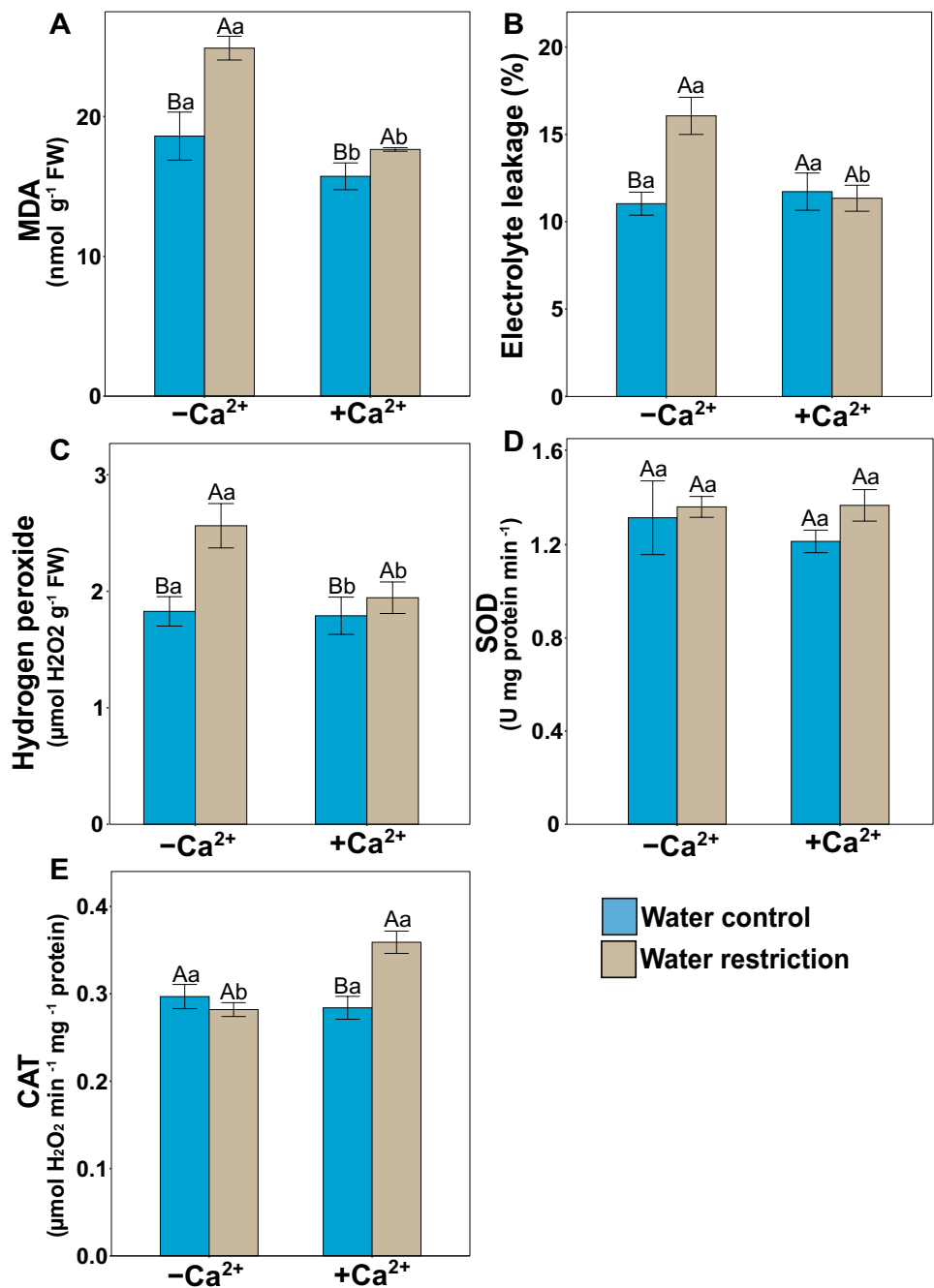
Maintaining optimal water levels amid dynamic environmental variations is a critical survival strategy for plants (Mahdich et al. 2008). Our findings suggest that Ca²⁺ supply modulates, directly and/or indirectly, the expression of PIPs in roots, which are essential facilitators of water transport across cells (Maurel et al. 2015; Yaneff et al. 2015). We observed that Ca²⁺ supply enhances leaf hydration, reduces drought symptoms (Figure S1), and mitigates oxidative stress under water-restricted conditions.

The diversification in the expression patterns of different AQP subfamilies in roots may enable plants to respond

flexibly to environmental changes (Zargar et al. 2017). Aquaporin expression in roots is regulated under water deficit conditions and is closely linked to the intensity and duration of stress (Alexandersson et al. 2005; Afzal et al. 2016; Zargar et al. 2017).

Under low water tension (−0.15 MPa), the expression of aquaporin-encoding genes (*PIP1;2*, *PIP2;1*, *PIP2;2*, and *PIP2;7*) was upregulated in the roots of +Ca²⁺ plants (Fig. 5). The positive regulation of PIPs in roots is a key strategy to increase membrane permeability and water transport to cells, thereby maintaining tissue hydration and supporting proper physiological processes (Zargar et al. 2017; Paudel et al. 2019). The overexpression of PIP aquaporins has been shown to enhance plant performance under water deficit. For instance, *Arabidopsis thaliana* expressing *Vicia faba* VfPIP1 developed longer primary roots and more lateral roots, traits that may contribute to improved drought tolerance (Cui et al. 2008). Similarly, overexpression of OsPIP1, a member of the PIP1 subfamily from rice, in *Arabidopsis thaliana* increased root hydraulic conductivity and improved drought tolerance (Guo et al. 2006). Additionally, studies have reported positive regulation of PIP genes under drought conditions, such as in *Phaseolus vulgaris* L. roots

Fig. 4 Malondialdehyde content (MDA) (**A**), electrolyte leakage (EL) (**B**), hydrogen peroxide (H_2O_2) (**C**), superoxide dismutase (SOD) activity (**D**), and catalase (CAT) activity (**E**) in VM01 grown in nutrient solution with ($+\text{Ca}^{2+}$) or without ($-\text{Ca}^{2+}$) calcium, under conditions of water restriction or control. Measurements were taken 16 days after the onset of water restriction (nutrient solution $\Psi_w = -0.65$ MPa). Different letters indicate significant differences among treatments ($p < 0.05$, Tukey's test). Uppercase letters compare water regimes (control vs. restriction) within each Ca^{2+} condition, while lowercase letters compare Ca^{2+} treatments within each water regime. Bars represent the mean $\pm$ standard error

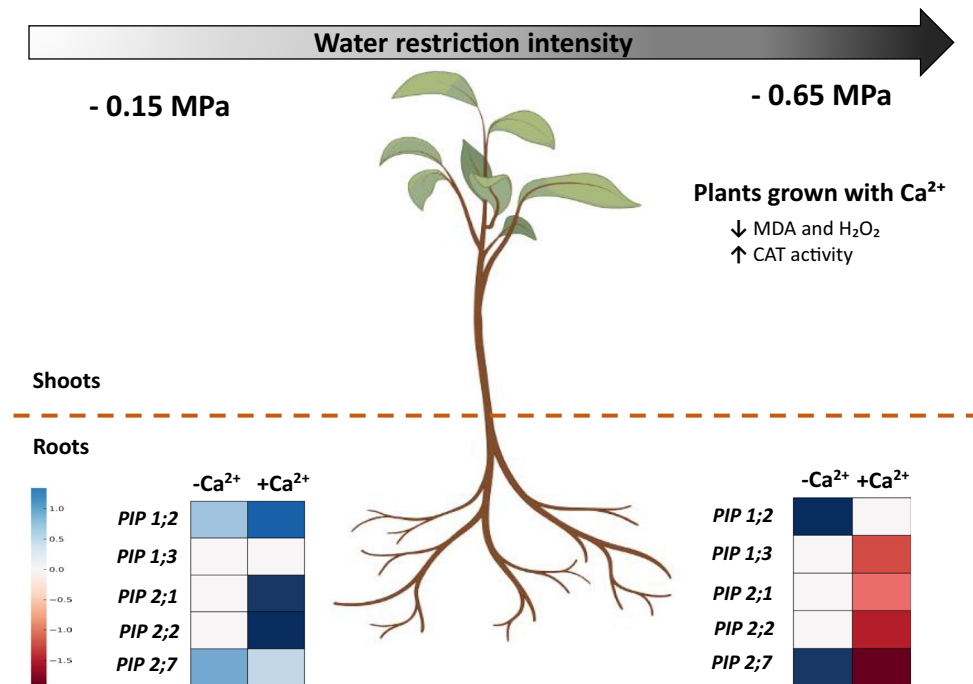


subjected to water deficit (Aroca et al. 2007), and in *Zea mays* L. roots evaluated 2 and 8 h after the onset of water restriction induced by PEG addition to the nutrient solution (Hachez et al. 2012).

Conversely, we found that prolonged water restriction led to a downregulation of most *PIP* genes in plants supplied with Ca^{2+} , which may help prevent water loss by reducing membrane permeability (Maurel et al. 2015; Afzal et al. 2016). This strategy of reducing membrane permeability in root cells is essential to prevent water loss from roots to the soil under prolonged drought conditions. In agreement with our

findings, Amrutha et al. (2021) observed negative regulation of *PIPs* in the roots and leaves of *Eucalyptus camaldulensis* following eight days of drought. Similarly, Lopez-Zaplana et al. (2022) reported that water deficit reduced the expression of various *PIP* subfamilies in the roots of *Cucumis melo* L. In contrast, plants grown without Ca^{2+} under water restriction showed an upregulation of *PIP1;2*, *PIP1;3*, and *PIP2;7*, deviating from the patterns observed with Ca^{2+} supplementation (Fig. 5). This suggests a complex regulatory response involving Ca^{2+} supply in modulating AQP expression, potentially mediated by cytosolic Ca^{2+} levels affecting

Fig. 5 Graphical summary of the regulation of aquaporin genes from the PIP subfamily in eucalypt roots and the effect of calcium (Ca^{2+}) on oxidative stress mitigation in the shoot. This schematic illustration represents findings obtained in VM01 eucalypt clone grown under different intensities of water restriction in the presence and absence of Ca^{2+} . Box colors depict the expression response compared to well-hydrated control conditions: white indicates no change, red indicates a decrease, and blue indicates an increase in gene expression. Results are based on five biological replicates ($n=5$ per treatment)



AQP pore closure. For example, in *Capsicum annum* L. under salt stress, Martínez-Ballesta et al. (2008) observed that an increase in cytosolic Ca^{2+} due to stress perception could lead to AQP pore closure. Conversely, they noted that salt stress reduced Ca^{2+} concentration in plants, while Ca^{2+} supplementation resulted in positive AQP regulation. Similarly, applying Ca^{2+} to plasma membrane vesicles in *Beta vulgaris* L. roots reduced water permeability by up to 95% (Alleva et al. 2006). These authors reported that the cytoplasmic face of AQPs is sensitive to Ca^{2+} , a behavior also observed in *Arabidopsis thaliana* (Gerbeau et al. 2002), suggesting an inhibitory effect of Ca^{2+} on specific aquaporins. Moreover, divalent cations, particularly Ca^{2+} , inhibited the activity of PIP subfamilies in *A. thaliana* (Verdoucq et al. 2008).

Chlorophyll content is a crucial physiological parameter for photosynthesis in plants under water deficit (Ma et al. 2012). Our results showed that Chl *a* content decreased in plants grown under water limitation without Ca^{2+} . We also observed a significantly more pronounced reduction in Chl *b* content in plants without Ca^{2+} supplementation. Previous studies have reported the protective effect of Ca^{2+} on chlorophyll structure under various stresses, including salinity, heat, and temperature fluctuations (Jiang and Huang 2001; Rahman et al. 2016; Li et al. 2017; Min et al. 2021). Furthermore, in our previous study (de Barros et al. 2023), we demonstrated that Ca^{2+} supply alleviated the decline in photosynthetic rate and plant growth under water-limited conditions.

The degradation of chlorophyll in plants under water deficit can result from increased accumulation of reactive

oxygen species (ROS) (Pintó-Marijuan and Munné-Bosch 2014). Excessive ROS production can lead to lipid peroxidation, alterations in plasma membrane integrity, and chloroplast damage, ultimately inhibiting photochemical reactions (Cruz de Carvalho 2008). Key indicators of oxidative damage levels in plant cells caused by stress include malondialdehyde (MDA) and electrolyte leakage (EL) (Garg and Manchanda 2009; Zhang et al. 2014).

Our study demonstrates that Ca^{2+} supplementation reduced membrane permeability and H_2O_2 content in eucalypt leaves under water restriction. Moreover, there was lower oxidative damage and lipid peroxidation of membranes, reflected in reduced MDA content. These results indicate that Ca^{2+} provides structural and functional protection of biological membranes in eucalypt plants experiencing water limitation. Reduced MDA levels have also been reported in drought-tolerant eucalyptus clones compared to sensitive ones, underscoring oxidative stress mitigation as a key trait associated with drought resilience (Hodecker et al. 2018; Pita-Barbosa et al. 2023).

Several studies have highlighted the role of Ca^{2+} in maintaining the structural and functional integrity of cell membranes under adverse conditions, consequently enhancing plant drought tolerance (Hawkesford et al. 2012; Xu et al. 2017; Gazol et al. 2018; Xue et al. 2018; Leng et al. 2020). The increase in antioxidant enzyme activity in response to stress is crucial for reducing MDA accumulation and maintaining the balance between ROS production and scavenging. Ca^{2+} supplementation has also been shown to activate the antioxidant enzymatic system under stress conditions in various plant species (Rezayian et al. 2018; Khushboo et al.

2018; Leng et al. 2020). This fact aside, data for eucalypt remains limited.

Our results show that eucalypt plants grown with Ca^{2+} under water restriction exhibited increased CAT activity. CAT is essential for decomposing and removing excess H_2O_2 in plants, serving as the primary enzyme in mitigating the deleterious effects of ROS (Laxa et al. 2019). The significant increase in CAT activity in $+\text{Ca}^{2+}$ plants under water limitation highlights the essential role of Ca^{2+} in bolstering antioxidative defense mechanisms in eucalypt plants during drought. By activating the antioxidant enzymatic system, Ca^{2+} helps mitigate oxidative stress by efficiently neutralizing ROS, protecting cellular components from oxidative damage, and maintaining cellular homeostasis. Our findings align with previous research that underscores the importance of Ca^{2+} in enhancing antioxidant enzymatic activity and stress tolerance in plants (Rezayian et al. 2018; Khushboo et al. 2018; Leng et al. 2020).

Conclusions

Our study demonstrates the indispensable role of Ca^{2+} in fortifying water restriction resilience in eucalypt plants through a multifaceted mechanism that spans from molecular regulation to physiological protection. Ca^{2+} supply not only modulates AQP gene expression to optimize water transport in response to water limitation but also enhances antioxidant defenses, notably through CAT activity, which mitigates the harmful effects of ROS. This regulation supports the structural integrity of chlorophyll and reduces membrane lipid peroxidation, preserving vital photosynthetic capacity and membrane stability under stress conditions. Together, these mechanisms highlight how Ca^{2+} fosters a dynamic response, promoting immediate adaptation and sustained protection against prolonged water limitation.

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Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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