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Salicylic acid application modulates physiological and hormonal changes in *Eucalyptus globulus* under water deficit

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ABSTRACT

Eucalyptus genus is the most widely planted hardwood tree, which productivity and development are limited by low water availability. Plant drought tolerance can be managed by adopting strategies, such as the exogenous application of salicylic acid. The main objective of the present study was to assess whether the exogenous foliar salicylic acid application would ameliorate the damages of water deficit on *Eucalyptus globulus* plants. Plants were watered at 70% (well water) or 15% (water deficit) of field capacity and four concentrations of salicylic acid (0, 0.75, 2.5 and 5.0 mM) were applied. Water potential, total chlorophylls and carotenoids contents, chlorophyll fluorescence parameters, leaf gas exchange, malondialdehyde, total soluble sugars, starch and total phenols contents were measured. The global hormonal content was quantified by ultra-performance liquid chromatography–mass spectrometry and specific local dynamics of indolacetic acid and abcisic acid were detected by immunolocalization in leaves. A multivariate statistical approach was used to get an overview of the plant physiological status. *E. globulus* water deficit response included growth rate decline associated with reduced in both water potential and leaf gas exchange parameters. Plant water deficit defence strategies led to an increase in total chlorophylls and carotenoids contents, lipid peroxidation, phenols and total soluble sugars. Six from the 18 hormones detected increased in water deficit plants. Exogenous salicylic acid application improved water deficit tolerance of *E. globulus* by improving water potential with a positive impact in primary metabolism (photosynthetic rate, soluble sugars) but also in secondary metabolism and defence mechanisms (higher total phenols and less lipid peroxidation) in the highest salicylic acid concentrations. Also, changes in endogenous levels of abscisic and salicylic acids, gibberellins 4 and 7, and specific cytokinins were found in water deficit plants with salicylic acid application. Our results indicated that salicylic acid application could be a potential chemical priming strategy to ameliorate water deficit effects on *E. globulus* plants.

1. Introduction

Eucalyptus genus is the most widely planted hardwood tree all over the world (Flynn, 2010) due to the large number of species, wide adaptability to soils and climates, fast-growing rates, the wide knowledge and technology for its culturing and the variety of wood and non-wood products that come from it (Brondani et al., 2012). In Portugal and Spain, *E. globulus* is the species more

planted, representing approximately 800,000 ha (ICNF, 2013) and 500,000 ha (Potts et al., 2004), respectively.

Drought stress is one of the most important environmental stresses that affects the establishment and limits forest productivity of *Eucalyptus* plantations (White et al., 2009). Preliminary studies performed by our research group already indicated the detrimental effects of drought conditions in *E. globulus* plants (decreased water potential, reduced gas exchange rates and stomatal conductance, enhanced lipid peroxidation, among others) and correlated this stress responses with changes in hormonal contents, namely abscisic acid (ABA) and jasmonic acid (JA) (Correia et al., 2014a,b). Understanding how planted forest trees tolerate low water availability, studying new insights to anticipate its impacts and

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implementing new mitigation strategies are essential tasks to undertake in *Eucalyptus* species in order to sustain productivity and meet future demands for stress-tolerant plant material. Salicylic acid (SA) is a phenolic compound that has been studied as a signal molecule mediating local and systemic defence responses against pathogens (Aimar et al., 2011). This compound was also reported as playing a role in plant responses to abiotic stress, such as drought (Singh and Usha, 2003; Bandurska and Stroinski, 2005; Hayat et al., 2008). The complex SA signalling network has an important role in plant performance by modelling key metabolic and physiological processes. SA influences photosynthesis, increasing photosynthetic rates and leaf area (Khan et al., 2003) and promoting plant growth and yield (Arfan et al., 2007). Plants pre-treated with SA also showed improved relative water content and leaf water potential, reduced electrolyte leakage and decreased level of lipid peroxidation (Hayat et al., 2008). Most of plant responses are regulated by plant hormones that never act individually (Kissoudis et al., 2014). Additive, synergistic or antagonistic interactions between hormonal pathways (Kissoudis et al., 2014) lead to changes in the biological responses for the maintenance of plant stress tolerance (Wang and Irving 2011). Growth and development regulation are essentially coordinated by cytokinins (CKs), auxins (AUXs), gibberellins (GAs), JA, brassinosteroids (BRs), ABA and SA, but the mediating biochemical mechanisms remain largely unknown (Rivas-San Vicente and Plasencia, 2011). Quantifying and monitoring the crosstalk of plant hormonal interactions can, thus, elucidate SA-induced pathways and decipher its role on plant performance. Besides, not always plant responses are successfully correlated with total hormonal content of tissues and knowledge of the precise localization of phytohormones within cell compartments would increase the understanding of its mode of action and its involvement in plant responses (Pastor et al., 1999).

The exogenous application of SA has been considered a short-term solution to improve the adverse effects of water deficit on plants (Singh and Usha, 2003), however the precise mode of SA action remains unclear (Hayat et al., 2010), in particular for trees. Research on SA application and water deficit tolerance in *Eucalyptus* species remains completely absent. The existing studies mainly focus on the responses to biotic stress drivers, namely bacteria (Ran et al., 2004) and fungi (Naidoo et al., 2013). Bearing this in mind, the main objective of this work is to investigate whether foliar application of SA can effectively ameliorate the negative effects of water deficit on *E. globulus* plants and how the plant physiology responds this treatment. Several physiological and biochemical parameters, such as water status, lipid peroxidation, pigments content, total soluble sugars and starch, phenols, F_v/F_m and ϕ_{PSII}, gas exchange and stomatal conductance, were assessed. We quantified, by ultra-performance liquid chromatography-mass spectrometry (UPLC/MS) and from the same individual sample, the global content of 18 different plant growth regulators. Moreover, changes in indole-3-acetic acid (IAA) and ABA distribution in leaf were visualized by immunolocalization to shed light about the role of these phytohormones *in situ* in plant mechanism against water deficit after SA application. In order to achieve a global view and a comprehensive picture of the explored plant parameters, PGRs levels, physiological and biochemical parameters were integrated using a multivariate analysis.

2. Material and methods

2.1. Plant material and experimental design

The experiment was conducted in a controlled climate chamber (Fitoclima 1200, Aralab, Portugal), with a temperature of 25/20 °C, a 16/8-h (day/night) photoperiod and a photosynthetic photon flux density (PPFD) of app. 600 μmol m^{-2} s^{-1}. Eighty rooted cuttings of one genotype of *E. globulus* (Al-18) with 2-months-old were obtained from the breeding program of Altri Florestal SA (Portugal). This clone was selected from an open pollination family and first test indicated very good survival results in drought prone areas (Correia et al., 2014a). The rooted cuttings were grown in 1 L plastic pots filled with 3:2 (w/w) peat:perlite and acclimatized during one-month inside the climate chamber being watered with nutritive solution (N:P:K). The pots were randomly arranged, periodically moved to the neighbouring position during the whole experiment and well-watered to 70% of field capacity, prior to application of SA. SA was administered to *E. globulus* plants by foliar spraying with 100 mL of 0.75, 2.5 and 5.0 mM SA solution (obtained from preliminary studies) for three consecutive days before the experimental set up. Sodium salicylate (Merck, Darmstadt, Germany) was used to prepare the SA solutions (adjusted to pH 7.0 with NaOH solution) dissolved in distilled water with the addition of 0.1% Tween® 20 (Sigma–Aldrich, Missouri, USA). Plants treated with 0 mM of SA solution were sprayed with distilled water containing 0.1% Tween® 20. After the last application of SA, half of the plants of each treatment were randomly assigned to each of the two water treatments as follows: (1) well-watered (WW): water supplied every evening until soil water content reached around 70% field capacity, and (2) water deficit (WD): water supplied every evening until soil water content reached around 15% field capacity. After two weeks of experiment, the followed parameters were recorded: growth, leaf gas exchange, chlorophyll *a* fluorescence and water potential. Leaves were harvested, cleaned with a moistened cloth and immediately frozen in liquid nitrogen for further biochemical analysis (estimation of lipid peroxidation, photosynthetic pigments content, total soluble sugars and total phenolic compounds and phytohormones quantification). For further immunolocalization of IAA and ABA, samples sections of leaves were fixed.

2.2. Growth and plant water potential

To assess growth, the height of six plants was recorded for each treatment. Midday shoot water potential (Ψ_{md}) was measured with a Scholander-type pressure chamber (PMS Instrument Co., OR) in six independent biological replicates per each treatment at 12 h 30 m (solar time) as described by Correia et al. (2014a) in order to control the plant water status.

2.3. Leaf gas-exchange measurements

Net CO$_2$ assimilation rate (A, μmol CO$_2$ m^{-2} s^{-1}), stomatal conductance (g$_s$, mol H$_2$O m^{-2} s^{-1}), transpiration rate (E, mmol H$_2$O m^{-2} s^{-1}) and intercellular CO$_2$ concentration content (C$_i$, ppm) were measured in six independent biological replicates per each treatment, using a portable infrared gas analyser (LCpro-SD, ADC BioScientific Ltd., UK) equipped with the broad leaf chamber. To find out the saturation light intensity A/PPFD (photosynthetic photon flux density; light response curves of CO$_2$ assimilation) curves were performed with the following PPFD: 2000, 1500, 1000, 750, 500, 250, 100, 50 and 0 μmol m^{-2} s^{-1}. After A/PPFD data analysis, punctual measurements at saturation light intensity were performed at 750 μmol m^{-2} s^{-1}. The following conditions were maintained inside the chamber during all the measurements: air flux: 200 mol s^{-1}; block temperature: 25 °C; and atmospheric CO$_2$ and H$_2$O concentration. Data were recorded when the measured parameters were stable (2–6 min).

2.4. Photosynthetic pigments and chlorophyll a fluorescence analysis

Total chlorophyll and carotenoid content was quantified according to Sims and Gamon (2002). Pigments were extracted

with acetone/Tris (50 mM) buffer at pH 7.8 (80:20) (v/v) in six independent biological replicates per each treatment. After homogenization and centrifugation, supernatants were used to read absorbance at 663, 537, 647 and 470 nm (Thermo Fisher Scientific Spectrophotometer, Genesys 10-uv S) and pigments' content was determined.

Steady-state modulated chlorophyll fluorescence was determined with a portable fluorometer (Mini-PAM; Walz, Effeltrich, Germany) on the same leaves as used for the gas-exchange measurements. Light adapted components of chlorophyll fluorescence were measured: steady-state fluorescence (F), maximal fluorescence (F'_m), variable fluorescence F'_v (equivalent to $F'_m - F$) and quantum yield of PSII photochemistry (ϕ_{PSII}) equivalent to $(F'_m - F)/F'_m$. Leaves were then dark adapted for at least 20 min to obtain F_0 (minimum fluorescence), F_m (maximum fluorescence), Fv (variable fluorescence, equivalent to $F_m - F_0$) and F_v/F_m (maximum quantum yield of PSII photochemistry).

2.5. Lipid peroxidation, total soluble sugars, starch and total phenolics

Lipid peroxidation on leaves was estimated using the method described by Correia et al. (2014a), which measures the amount of MDA (malondialdehyde).

Total soluble sugars (TSS) were determined by the anthrone method, as described by Irigoyen et al. (1992). Briefly, TSS were extracted from 50 mg frozen leaves using 80% (v/v) ethanol at 80 °C for 1 h. After centrifugation, the supernatant was mixed with 1.5 mL of anthrone and incubated at 100 °C during 10 min. Absorbance was read at 625 nm and TSS content was calculated against a D-glucose standard curve. The pellet resultant from the centrifugation was used to quantify starch, as described by Osaki et al. (1991). The pellet was incubated with 30% (v/v) perchloric acid at 60 °C during 1 h. The mixture was centrifuged and anthrone was added to the supernatant. After heating the mixture at 100 °C for 10 min, absorbance was read at 625 nm and starch content was determined according to a D-glucose standard curve.

Total phenolic compounds were determined according to the protocol used by Singleton and Rossi (1965). About 50 mg of frozen plant material were extracted with 80% (v/v) cold acetone and the mixture was centrifuged. Folin–Ciocalteu's phenol reagent and 7.5% (w/v) sodium carbonate were added to the supernatant and the mixture was kept at room temperature for 30 min. Absorbance was read at 765 nm and total phenolic concentration was determined according to a gallic acid standard curve. Six biological replicates of each treatment were performed.

2.6. PGRs quantification

The analysis of different plant growth regulators (PGRs) (trans-Zeatin, tZ; zeatin riboside, ZR; dihydrozeatin DHZ; dihydrozeatin riboside DHZR; isopentenyl adenine, iP; isopentenyl adenine riboside, iPR; benziladenine, BA; gibberellin GA_3, GA_4, GA_7, and, GA_9; 24-epibrassinolide, 24EB; Castasterone, BK; 28-homobrassinolide, HBI; JA; SA; ABA; IAA;) was carried out by a modified protocol based on Pan et al. (2008). Briefly, 60 mg of lyophilized tissue were ground into powder and 500 μl of 2-propanol/H_2O/concentrated HCl (2:1:0.002, v/v/v) one deuterated internal standard for each group of phytohormones (10–40 ng) were added, followed by agitation for 30 min at 4 °C. CH_2Cl_2 (1 mL) was added, followed by another 30 min of agitation at 4 °C. Two phases were formed with the plant debris between them. The lower layer was collected, concentrated in 2 mL glass vials with nitrogen flow and stored until analysis at −20 °C.

Samples were re-suspended in 200 μL of methanol (MeOH) 100% and filtered through a 0.2 μm regenerated cellulose filter (Agilent Technologies) filled with SiO_2 (15 mg). All the compounds were separated and quantified by an ultra-high performance liquid chromatography (UHPLC) in a 6460 Triple Quad LC/MS (Agilent Technologies) using the protocol described by Novák et al. (2008). A chromatographic separation was made using a reverse phase column (Zorbax SB-C18 2.1 × 50 mm column). The column was held at 40 °C and the mobile phase used in the chromatography consisted of (A) 99.9% MeOH: 0.1% COOH and (B) ammonium formate (10 mM, pH 4). A linear gradient of MeOH, from 10% to 50% in 7 min and reaching 100% in 2 min, was used to analytical elution. PGRs were quantified by dynamic multireaction monitoring of their $[M + H]^+$ and the appropriate product ions, using optimized cone voltages and collision energies for diagnosis of each PGRs analysed. Three biological replicates of each treatment were performed.

2.7. Immunolocalization

To the immunolocalization of IAA and ABA, leaves from 0 mM well-watered (WW) and all water deficit (WD) (0, 0.75, 2.5 and 5.0 mM) treatments were sampled and immediately fixed according to the method described by Meijón et al. (2010) with some modifications. The tissues were fixed for 24 h in 3% (w/v) paraformaldehyde containing 0.1% (v/v) Triton X-100 (Sigma–Aldrich Co., St. Louis, MO, USA) at 4 °C. To the mixture was also added 4% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma–Aldrich Co., St. Louis, MO, USA) to immobilize IAA and ABA by covalent binding proteins. After 24 h, samples were washed three consecutive times for 10 min in phosphate-buffered saline [PBS (137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, at pH 7.3)] to remove the fixing solution. Finally, samples were stored in PBS containing 0.1% (w/v) paraformaldehyde at 4 °C. Then, samples were introduced in a cryostat medium (Tissue-Tek, Killik; Sakura Finetek USA, Inc., Torrance, CA, USA) and were frozen at −23 °C. Finally, sections of 50 μm were cut with a sliding cryotome CM1510S (2002 Leica Microsystems, Wetzlar, Germany), collected on slides and conserved at 4 °C until the analysis. Sections were immersed for 5 min in ascending and descending 25, 50, 75 and 100% ethanol series, washed for 30 min in PBS containing 0.1% (v/v) Tween 20, and finally for 5 min in PBS. Before incubating overnight with the ABA or IAA primary antibody (polyclonal Agrisera AB, Vännäs, Sweden), samples were pre-treated with 5% (w/v) BSA in PBS for 30 min to reduce non-specific binding. After washing twice with 0.1% (v/v) Tween 20 in PBS for 10 min, sections were incubated with Alexa 488 (Molecular Probes, Göttingen, Germany) as a secondary antibody for 1 h in darkness. Samples were washed twice for 10 min with 0.1% (v/v) Tween 20 in PBS. Finally the slides were counterstained with DAPI (4′,6-diamidino-2-phenylindole; Fluka). Sections were washed in MilliQ water, and assembled on the slides with Mowiol (Sigma–Aldrich Co., St. Louis, MO, USA). In both immunochemical detection (ABA and IAA) the negative controls were obtained replacing the primary antibody by PBS. Fluorescence was visualized using a confocal microscope (Leica TCS-SP2-AOBS) connected to a workstation and the images were processed with Fiji Software (Schindelin et al., 2012).

2.8. Statistical analysis

The results of physiological and biochemical parameters are presented as mean ± standard deviation (SD) of six independent biological replicates. All statistics procedures were performed using SigmaPlot (SigmaPlot for Windows v. 11.0, Systat Software Inc.). For these parameters two-way analysis of variance (ANOVA) followed by post-hoc multiple comparisons using Holm-Sidak test was employed to estimate the significance of the results ($p \leq 0.05$). For PGRs quantification, three biological replicates were analysed

and a Student's *t*-test was carried out to assess significant differences between WW and WD in non-treated plants. To find out significant differences in the WD plants treated with different SA concentrations, a one-way ANOVA was used, followed by a post-hoc Tukey test. In every case, different lowercase letters indicate significant differences between SA concentrations in WW and WD conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

To reduce the dimensionality and complexity of PGRs data, Heatmap–Clustering was performed normalizing data for each PGR. Principal components analysis (PCA) was carried out to integrate physiological and biochemical data with PGRs profile of *E. globulus* plants by reducing the multivariate data matrix to an interpretable bidimensional biplot that explains the highest proportion of variation of the data (ter Braak and Verdonschot, 1995). SA treatments under WW condition were excluded from PCA because these treatments were not evaluated to global contain of PGRs. Data were a *priori* centered and normalized to reduce scale effects (ter Braak and Verdonschot, 1995). Heatmap and PCA were conducted using heatmap2 and mixOmics packages with the R programming language running under the open-source computer software RStudio: Integrated development environment for R (RStudio Boston, MA. Available on http://www.rstudio.org/).

3. Results

3.1. Growth and plant water status

Exposure of plants to water deficit led to a general decrease in the height, excepting the plants sprayed with 5.0 mM SA that presented similar height in both water regimes (Table 1). Water potential is useful variable to evaluate the physiological water status of plants. In comparison with WW plants, the WD plants showed a significant decrease in the ψ_{md} values. However, the plants sprayed with SA were significantly less affected than non-treated ones (Fig. 1). Moreover, plants treated with 5.0 mM SA presented the highest water status under water deficit condition (Fig. 1).

3.2. Chlorophyll and carotenoid quantification and fluorescence

Under WW condition, plants with and without SA application presented similar values (Table 2). Under water deficit conditions, the total chlorophyll concentration was significantly higher than in well-watered plants, with the exception of the plants sprayed with 5.0 mM SA (Table 2). Moreover, in WD condition, the total chlorophylls tended to decrease with the increasing of SA concentration (Table 2). In the WW condition, after an increase in the carotenoid content at 0.75 mM SA, these compounds diminished as the concentration of SA increased, reaching similar values to the non-treated plants at 2.5 and 5.0 mM (Table 2). In the WD plants, carotenoid concentration was significantly lower in the SA-treated plants compared to non SA-treated (Table 2). Regarding
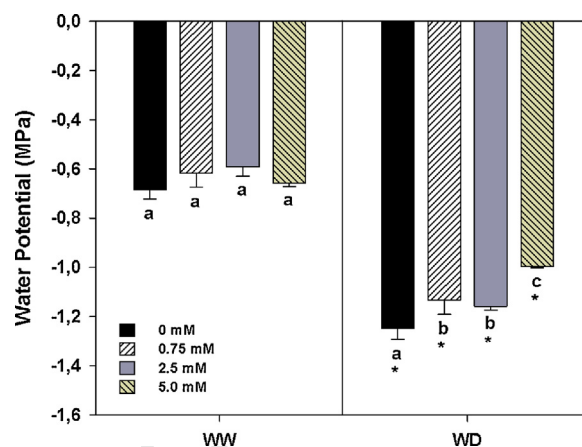

Fig. 1. Midday water potential (Ψ_{md}) in well-watered (WW) and water-stressed (WS) plants of *E. globulus* after a two-week water deficit period. Data are presented as mean ± SD ($n = 6$). Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

the F_v/F_m (maximum yield of PSII photochemistry) and ϕ_{PSII} (quantum yield of PSII photochemistry) ratios, there were no statistically significant differences between water regimes, neither in plants with or without SA treatment (Table 2).

3.3. Leaf gas-exchange measurements

Water deficit reduced net CO_2 assimilation rate (A) (Fig. 2A), stomatal conductance (g_s) (Fig. 2B), transpiration rate (E) (Fig. 2C) and intercellular CO_2 concentration (Ci) (Fig. 2D) of leaves. Under WW condition, SA treatments led to an increase of A and E (Fig. 2A and C), while Ci showed lower values compared to non-treated SA plants (Fig. 2D). Regarding WD condition, A and E increased as the concentration of SA increased, presenting values significantly higher than non-treated plants (Fig. 2A and C).

3.4. Lipid peroxidation, starch, TSS and total phenolics content

Lipid peroxidation was measured in terms of MDA concentration. The plants subjected to the water deficit exhibited a significant increase in MDA levels when compared to the WW ones (Fig. 3A). In both cases, the MDA levels decreased as the SA concentration increased (Fig. 3A).

Relatively to starch quantification, no significant differences were observed independently of water treatment and SA application (Fig. 3B). Significant differences in the phenols content were observed in non-treated plants between WW and WD (Fig. 3C). In WW plants, the SA application significantly increased the total phenolics (Fig. 3C). In WD plants, significant differences were observed in 2.5 and 5.0 mM SA, which showed higher levels of phenols compared to 0 and 0.75 mM SA treatments (Fig. 3C).

Relative to TSS content, significant increases were found in plants subjected to WD, except for 5.0 mM SA treatment, which maintained similar values in both water regimes (Fig. 3D).

The accumulation of TSS was significantly higher in plants with SA application both in WW and WD (Fig. 3D). In WW condition, the TSS levels increased as the SA concentration increased. However, in plants subjected to low water availability, the levels of TSS decreased as the SA concentration increased (Fig. 3D).

3.5. PGRs quantification, IAA and ABA immunolocalization

For PGRs quantification, we used a highly sensitive and high-throughput method for the simultaneous analysis of 18 molecular

Table 1

Height in well-watered (WW) and water deficit (WD) plants of *E. globulus* after a two-week water deficit period. Data are presented as mean ± SD ($n = 6$). Different lowercase letters indicate significant differences between SA concentrations in WW and WD conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

[SA] (mM)	Height (cm)	
	WW	WD
0	38.27 ± 2.22a	34.50 ± 0.87a*
0.75	41.03 ± 0.66a	35.60 ± 2.17a*
2.5	38.2 ± 2.08a	34.63 ± 1.97a*
5.0	33.45 ± 1.53b	33.22 ± 1.46a

Table 2

Total chlorophylls and carotenoids content, F_v/F_m and ϕ_{PSII} ratios in well-watered (WW) and water deficit (WD) plants of *E. globulus* after a two-week water deficit period. Data are presented as mean $\pm$ SD ($n = 6$). Different lowercase letters indicate significant differences between SA concentrations in WW and WD conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

| [SA] (mM)	Total Chlorophylls$_{(a+b)}$ ($\mu mol\ g^{-1}$ FW)		Carotenoids ($\mu mol\ g^{-1}$ FW)		F_v/F_m		ϕ_{PSII}	
	WW	WD	WW	WD	WW	WD	WW	WD
0	2.33 ± 0.12a	3.09 ± 0.21a*	0.74 ± 0.03ac	1.03 ± 0.06a*	0.84 ± 0.01a	0.84 ± 0.01a	0.76 ± 0.03a	0.78 ± 0.01a
0.75	2.55 ± 0.18a	2.87 ± 0.11ab*	0.83 ± 0.06b	0.94 ± 0.03b*	0.84 ± 0.02a	0.83 ± 0.01a	0.76 ± 0.02a	0.78 ± 0.01a
2.5	2.39 ± 0.27a	2.81 ± 0.24ab*	0.77 ± 0.06ab	0.89 ± 0.03b*	0.84 ± 0.01a	0.84 ± 0.01a	0.79 ± 0.02a	0.79 ± 0.01a
5.0	2.65 ± 0.24a	2.63 ± 0.18b	0.69 ± 0.05c	0.69 ± 0.05c	0.84 ± 0.01a	0.84 ± 0.01a	0.78 ± 0.01a	0.78 ± 0.01a
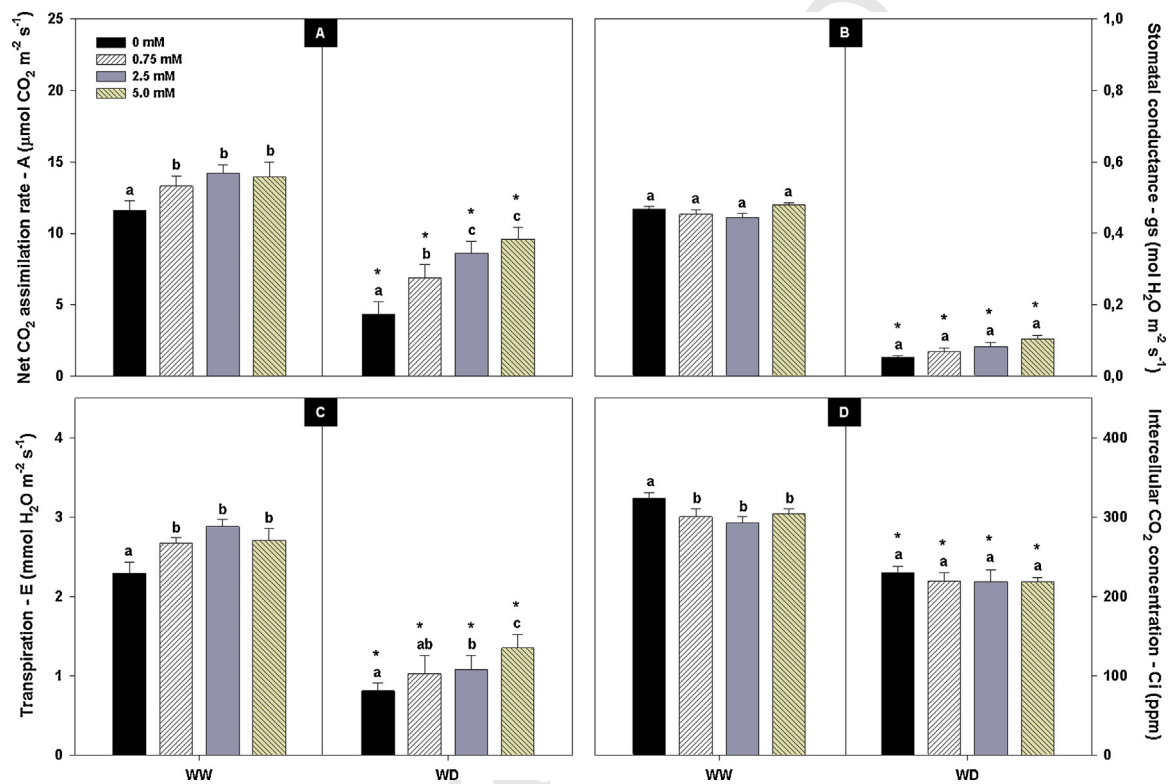
Fig. 2. (A) Net CO_2 assimilation rate—A; (B) stomatal conductance—g_s; (C) Transpiration rate—E; (D) intercellular CO2 concentration—Ci, in well watered (WW) and water deficit (WD) plants of *E. globulus* after a two-week water deficit period. Data are presented as mean $\pm$ SD ($n = 6$). Different lowercase letters indicate significant differences between SA concentrations in WW and WD conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

species of CKs, IAA, GAs, JA, BRs, ABA and SA. Plants subjected to WD regime presented higher levels of ABA, IAA, JA, iP, GA_4 and GA_7 (Table 3). Additionally, SA treatments influenced ABA, SA, DHZ, iP, GA_4 and GA_7 amounts. Endogenous ABA content increased linearly with the dose of exogenous SA application, although the differences were only significant between 0.75 and 5.0 mM SA concentrations. Also, SA content increased with the dose of exogenous SA application reaching significant differences at 5.0 mM (Table 3). DHZ (active cytokinin) presented significant higher concentration at 2.5 mM SA, although the differences were only significant in relation to 0.75 mM SA (Table 3). And finally, iP, GA_4 and GA_7 significantly decreased in most SA treatments (Table 3) in relation to WD control (0 mM). Since the hormonal system represents an intricate network involving functional crosstalk between signalling and metabolism, a comprehensive and integrative analysis of plant hormone changes is required. The global content of the 18 plant hormone-related compounds was represented by heatmap clustering (Fig. 4). Through this representation a clear different hormone profile between WW and WD treatments was shown. Moreover, within WD in SA treatments, it

was also observed a differential distribution of the quantified phytohormones species, indicating this specific plant hormone activity not only in relation to water stress but also in relation to SA-doses applied. In the heatmap representation and in relation to phytohormone profile, we observed that 0.75 and 2.5 mM SA treatment were the treatments closer to WW plants, while 5.0 mM SA and WD control plants (0 mM SA) showed greater distance to WW plant in terms of global phytohormone content. Additionally, through immunolocalization studies it was shown that plants response to water deficit induced variation both in concentration and distribution of ABA and IAA. Thereby, under water deficit, ABA signal was unequally distributed and mainly accumulated in vascular tissues, oil glands and epidermis (Fig. 5B). On the other hand, in the WD plants pre-treated with 0.75 mM SA, there was a slight redistribution of ABA into the mesophyll, while with 2.5 and 5.0 mM SA a strong ABA accumulation was observed both around vascular tissues and mesophyll (Fig. 5D and E). Different IAA distribution was also observed under water deficit and SA treatments. In WW plants, IAA signal was mainly located near vascular tissues (Fig. 6A). However, under stress, IAA was equally
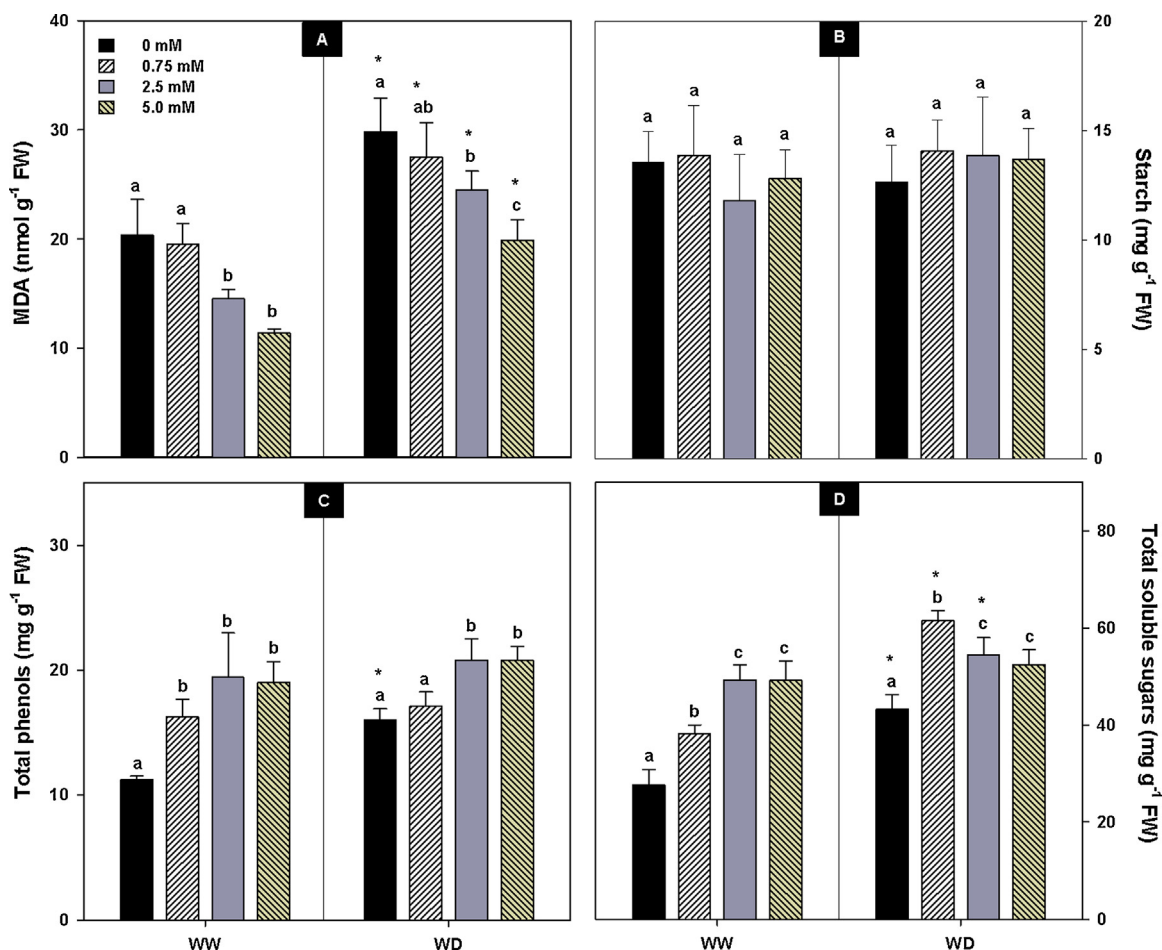
Fig. 3. (A) MDA content; (B) starch content; (C) total phenolics content and (D) total soluble sugars—TSS in well-watered (WW) and water-stressed (WD) plants of *E. globulus* after a two-week water deficit period. Data are presented as mean ± SD (*n* = 6). Different lowercase letters indicate significant differences between SA concentrations in WW and WD conditions and asterisks indicate significant differences between water treatments (*p* ≤ 0.05).

Table 3
PGRs quantification in 0 mM WW and 0, 0.75, 2.5 and 5.0 mM WD plants of *E. globulus* after a two-week water deficit period. Data are presented as mean ± SD (*n* = 3). Different lowercase letters indicate significant differences between SA concentrations in WD condition and asterisks indicate significant differences between water treatments (WW and WD) to 0 mM (*p* ≤ 0.05).

	0 WW	0 WS	0.75 WS	2.5 WS	5.0 WS
SA (ng g^{-1} DW)	790.9 ± 554.6	904.0 ± 323.6a	806.78 ± 355.7a	2079.1 ± 1184.2a	13304.5 ± 8912.4b
GA$_3$ (ng g^{-1} DW)	195.5 ± 101.1	73.7 ± 53.6a	214.2 ± 56.6a	149.6 ± 33.4a	345.3 ± 309.3a
IAA (ng g^{-1} DW)	5.6 ± 0.1	18.5 ± 3.6a*	15.8 ± 10.0a	13.9 ± 2.3a	28.5 ± 20.0a
ABA (ng g^{-1} DW)	232.0 ± 84.6	853.6 ± 79.8a*	999.3 ± 58.2a	1045.6 ± 13.9ab	1205.5 ± 59.0b
JA (ng g^{-1} DW)	3.5 ± 0.3	10.1 ± 1.39a*	7.9 ± 1.0a	7.9 ± 2.0a	8.5 ± 1.4a
DHZ (ng g^{-1} DW)	1.0 ± 0.2	5.1 ± 3.0ab	1.5 ± 0.6 a	6.9 ± 1.3b	3.6 ± 1.5ab
RDHZ (ng g^{-1} DW)	0.8 ± 0.6	1.6 ± 0.5a	0.6 ± 0.2a	1.4 ± 0.5a	1.6 ± 0.3a
tZ (ng g^{-1} DW)	1.89 ± 0.6	3.0 ± 1.9a	1.5 ± 0.4a	3.8 ± 1.7a	3.9 ± 1.1a
GA$_7$ (ng g^{-1} DW)	21.2 ± 15.4	90.5 ± 21.1a*	20.3 ± 8.2b	18.9 ± 2.8b	39.9 ± 35.8ab
BA (ng g^{-1} DW)	7.2 ± 2.8	15.4 ± 4.9a	8.5 ± 0.6a	11.6 ± 2.8a	10.1 ± 3.5a
GA$_4$ (ng g^{-1} DW)	1356.9 ± 434.3	3182.7 ± 994.8a*	1574.6 ± 301.5b	1627.3 ± 186.5b	2242.0 ± 397.2ab
iP (ng g^{-1} DW)	2.9 ± 0.9	10.3 ± 0.9a*	5.7 ± 1.2b	5.4 ± 0.7b	5.1 ± 1.5b
iPA (ng g^{-1} DW)	32.3 ± 17.3	53.6 ± 20.7a	30.4 ± 19.3a	26.9 ± 1.9a	21.3 ± 18.4a
24EB (ng g^{-1} DW)	1192.5 ± 415.8	877.1 ± 347.5a	307.8 ± 35.1a	668.7 ± 394.6a	676.4 ± 274.3a
HBI (ng g^{-1} DW)	24.7 ± 9.1	24.9 ± 15.2a	9.6 ± 2.5a	12.2 ± 9.2a	16.7 ± 13.4a
RZ (ng g^{-1} DW)	23.9 ± 6.9	20.6 ± 10.0a	28.5 ± 10.7a	25.4 ± 7.7a	26.4 ± 14.7a
GA$_9$ (ng g^{-1} DW)	89.5 ± 21.5	91.4 ± 39.9a	82.4 ± 46.1a	61.4 ± 23.3a	76.1 ± 37.1a
BK (ng g^{-1} DW)	2285.5 ± 695.9	2029.6 ± 370.4a	1641.1 ± 1262.9a	1471.6 ± 240.2a	1902.2 ± 171.3a

accumulated around all the leaf-tissues (Fig. 6B). Differences on IAA accumulation between non-treated and SA treated plants were less clear (Fig. 6B–E). However, at the higher SA concentration a special accumulation can be noticed (Fig. 6E), which was in accordance with IAA global quantification by LC–MS methodology.

3.6. Multivariate approach: global overview of physiological status

PCA ordination provided an overall picture of the physiological/biochemical condition of *E. globulus* plants during the experimental setup, revealing a clear separation between water regime and
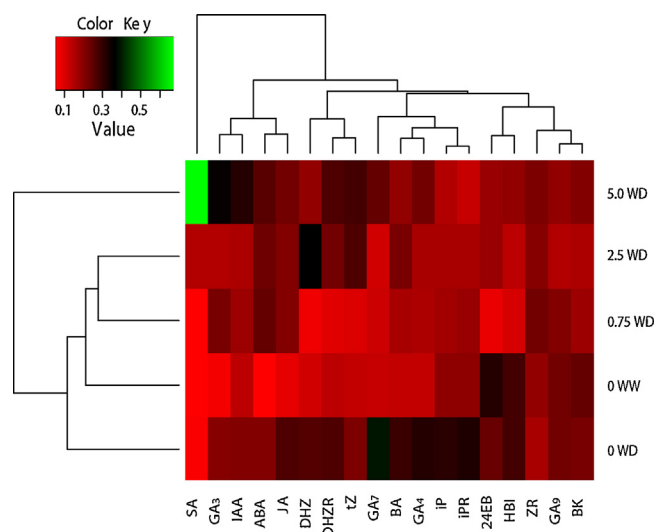
Fig. 4. Heatmap of PGRs content in 0 mM WW and 0, 0.75, 2.5 and 5.0 mM WD plants of *E. globulus* after a two-week water deficit period. The relative accumulation patterns are shown in the heat map based on the average value (*n* = 3) for each plant hormone. Red and green colours indicate lower and higher concentrations, respectively. The colour scale is shown at the top.

SA application (Fig. 7). Non treated well watered plants (0 WW) were grouped together (Fig. 7, left side) suggesting homogeneity in the physiology and biochemistry of plants in non-stressful conditions. Sample scores of WD plants were all located on the right side, with the plants that were not pre-treated with SA (0 WD) being located in the upper right part (Fig. 7). The horizontal left-to-right movement observed in WD is mostly explained by lower Ci, gs, E, water potential, and height and higher JA, IAA, MDA, and total chlorophyll compared to WW (Fig. 7). SA treated WD scores progressively moved from top-to-bottom (Fig. 7). The downwards migration pattern of PCA scores under increased SA treatment is most probably associated with vertical gradients, such as increased ABA, SA, TSS, and total phenols and decreased IP, GA₄ and carotenoids (Fig. 7).

4. Discussion

4.1. Water deficit effect in E. globulus

Impaired plant growth and development are the first and most concerning effects of low water availability in plants. In fact, water stressed plants of *E. globulus* showed a marked reduction in height (Table 1), which is in agreement with other reports (Silva et al., 2004; Granda et al., 2011; Correia et al., 2014a). Cell growth is mainly affected by a reduction in turgor pressure and decreased CO_2 diffusion from atmosphere (Pinheiro and Chaves 2011). Following this assumption, WD plants showed decreased water potential values (Fig. 1), as already documented in other works (Guarnaschelli et al., 2006; Correia et al., 2014a), as well as Ci, CO_2 assimilation, g_s and E (Fig. 2). Stomatal limitation under water deficit conditions is accepted as one of the main limitations of plant photosynthesis and further productivity (Silim et al., 2009) and it has been well documented (Bogeat-Triboulot et al., 2007; Ditmarová et al., 2009). Maximum and effective PSII photochemistry performance, assessed as F_v/F_m and ϕ_{PSII}, respectively, was not negatively affected in response to water deficit (Table 2) however, according to Correia et al. (2014a), total chlorophylls and carotenoids increased with WD (Table 2). As argued, the increasing chlorophyll content could be related to a reduction in leaf mass expansion and to a protective role of carotenoids or other

mechanisms, which protected these pigments from degradation and preserve the photosynthetic capacity. The peroxidation of lipids is the most obvious symptom of oxidative stress in plants. Drought induced oxidative stress leads to the generation of reactive oxygen species (ROS). The prevalence of free radicals reaction in membranes is indicated by the accumulation of MDA (Fig. 3A), which is expected to show greater accumulation under environmental stresses (Cakmak and Horst, 1991; Xu and Zhou, 2006). Another response to oxidative stress is the accumulation of polyphenols (Fig. 3C). These compounds possess ideal structural chemistry for free radical scavenging activities (Rice-Evans et al., 1997) and are reported as accumulating in several abiotic stresses (Alexieva et al., 2001; Rivero et al., 2009). Total soluble sugars content (Fig. 3D) rose in response to water deficit. This is a common response in water deficit stress considering that accumulation of compatible solutes is an important physiological adaptation and enables osmotic adjustment including in eucalypts plants (Parida et al., 2007; Mohammadkhani and Heidari, 2008). In fact, soluble sugars do not only function as metabolic resources and structural constituents of cells, they also act as signals and interact with stress pathways into a complex network to modulate metabolic plant responses (Rosa et al., 2009). Plant hormones are essential molecules able to modify plant physiology and biochemistry in rapid response to changes in their environment, a critical requirement for their survival as sessile organisms. Morpho-physiological responses as growth reduction or stomatal closure (among others) under water deficit are outcomes regulated by a complex network of hormone signalling pathways. The major and the best-known player, ABA, acts in concert with jasmonates, ethylene, AUXs, and CKs (Nemhauser et al., 2006; Huang et al., 2008). From the studied hormones, ABA, IAA, JA, iP, GA₄, and GA₇ increased under water deficit conditions (Table 3). ABA is considered a key hormone in the response to drought stress (Zhang et al., 2006) and in *E. globulus* has been shown to form part of a complex signalling network which mediates the physiological changes induced by drought stress (Granda et al., 2011). Correia et al. (2014a,b) also found higher ABA accumulation in the leaves of *E. globulus* during drought stress, being in accordance with the defined signalling role of ABA under water deficit conditions (Galmés et al., 2007; Jiang and Hartung, 2008). Regarding JA and its metabolically active derivatives (jasmonates), there is increasing evidence that they are also crucial signalling molecules involved in many plant responses to biotic and abiotic stresses (Brossa et al., 2011). In our work, ABA and JA hormones seem to have a synergistic interaction in response to water deficit stress, which is according to other studies (Fujita et al., 2006; Harb et al., 2010; Brossa et al., 2011). JA may interact with ABA synthesis under water deficit conditions (Bandurska et al., 2003) and this interaction could regulate stomatal closure (Acharya and Assmann, 2009). Our results also showed an increase on IAA content in plants subjected to water deficit, which explained that under moderate osmotic stress IAA promotes water uptake into the protoplasts (Pustovoitova et al., 2003). IAA and ABA are probably involved in turn in the process of drought adaptation and perform phase-specific functions (Pustovoitova et al., 2003). In fact, IAA is considered for some authors as the most representative 'water deficit signal' (De Diego et al., 2012). De Diego et al. (2013) also confirmed the efficiency of immunolocalization techniques as a tool to understand the translocation of IAA and ABA in plants subjected to different water stress situations. In our study, the immunolocalization of ABA and IAA concurred with the global quantification results showing an accumulation under water deficit conditions of these hormones (Figs. 5 and 6). Additionally, the immunolocalization data indicated that eucalypts response to water deficit also induced a variation in ABA and IAA distribution. Thus, we observed that under water deficit ABA is mainly translated into the tissues
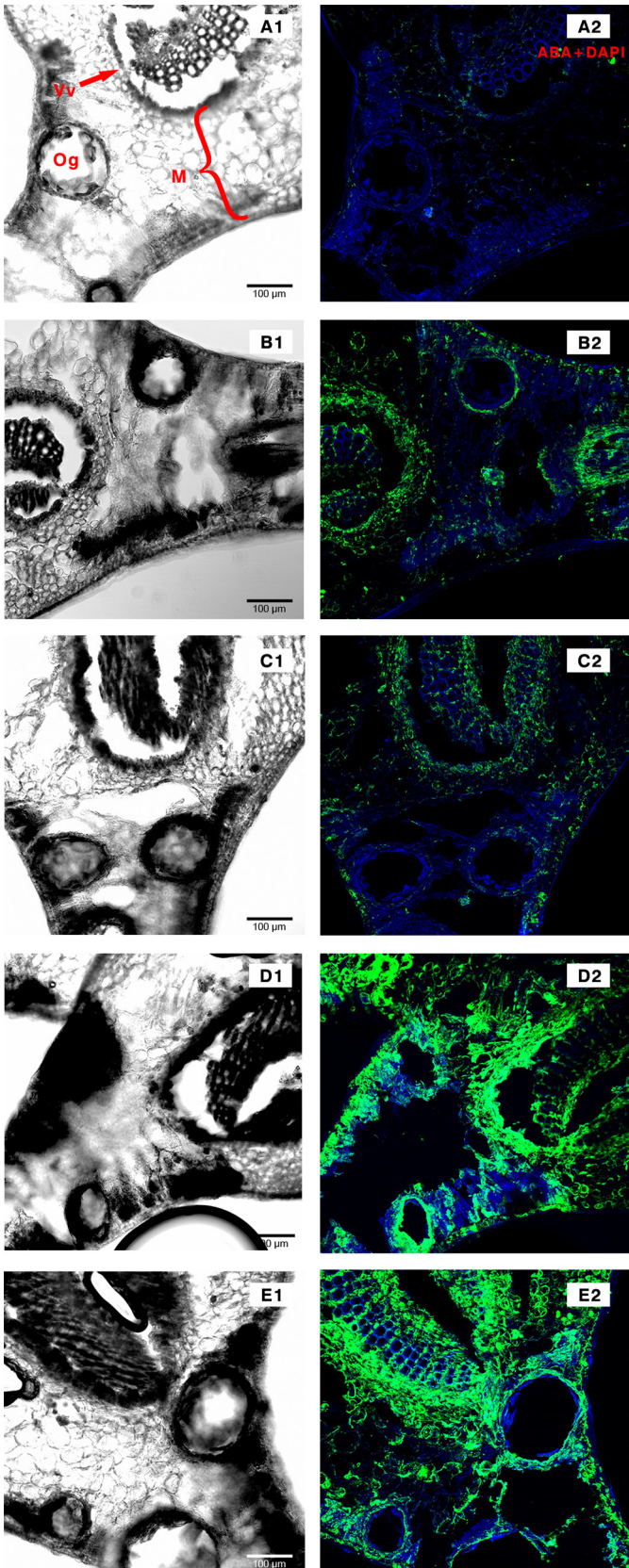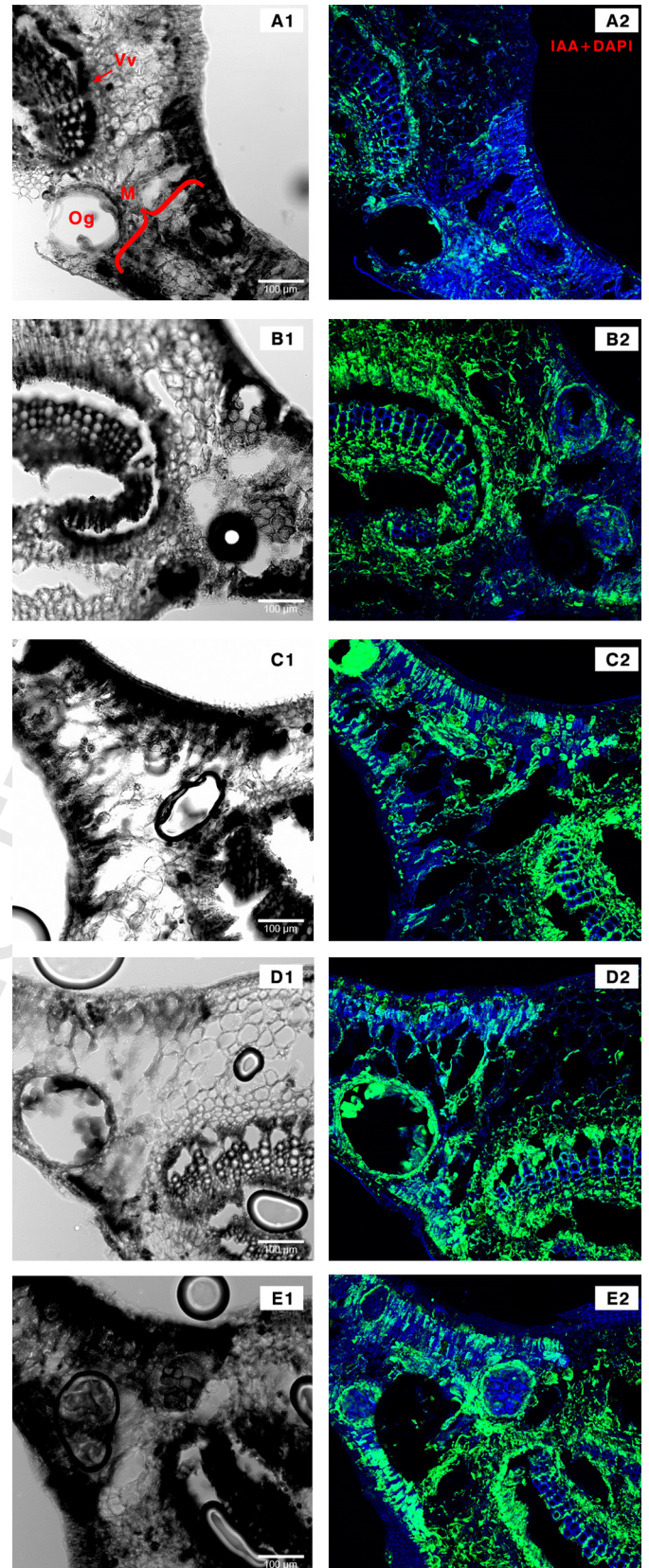
Fig. 5. Immunodetection of ABA in section of *E. globulus* leaves using confocal microscope. Differential interference contrast (DIC) (A1, B1, C1, D1 and E1) and immunolocalization of ABA (A2, B2, C2, D2 and E2). ABA labelling: DAPI (blue signals) and ABA (Green signals) merged in transversal leaf section. (A1 and 2) −0 mM WW; (B1 and 2) −0 mM WD; (C1 and 2) −0.75 mM WD; (D1 and 2) −2.5 mM WD; (E1 and 2) −5.0 mM WD. Abbreviations: Vv = Vascular vessels; M = Mesophyll; Og = Oil gland. Three biological replicates of each treatment were performed.

Fig. 6. Immunodetection of IAA in section of *E. globulus* leaves using confocal microscope. Differential interference contrast (DIC) (A1, B1, C1, D1 and E1) and immunolocalization of IAA (A2, B2, C2, D2 and E2). IAA labelling: DAPI (blue signals) and IAA (Green signals) merged in transversal leaf section. (A1 and 2) −0 mM WW; (B1 and 2) −0 mM WD; (C1 and 2) −0.75 mM WD; (D1 and 2) −2.5 mM WD; (E1 and 2) −5.0 mM WD. Abbreviations: Vv = Vascular vessels; M = Mesophyll; Og = Oil gland. Three biological replicates of each treatment were performed.
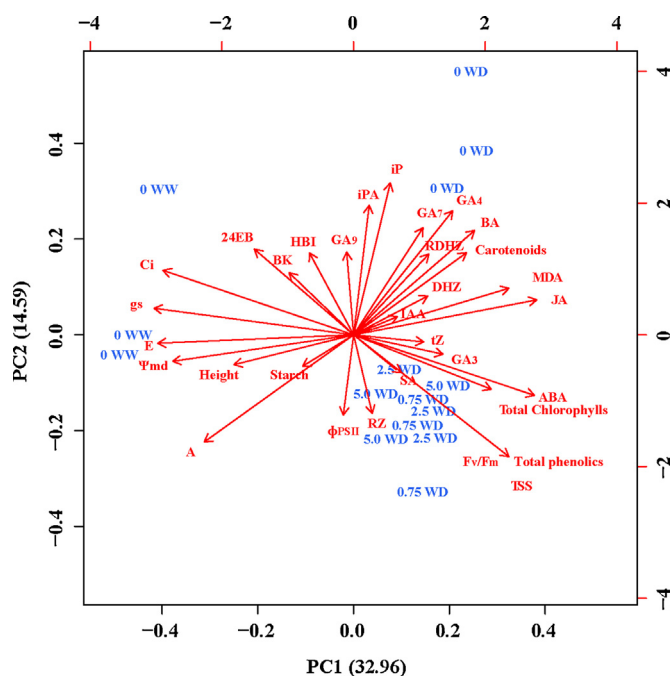
Fig. 7. PCA biplot of the physiological data of *Eucalyptus globulus* plants in theexperimental setup. Squares areas were used to highlight the position specific stress (0 WW plants; 0 WD plants and 0.75, 2.5 and 5.0 mM WD). Loading plots for the first axis (explained variation is 32.96%) and second axis (explained variation is 14.59%).

that are more in contact with the external environment (vascular tissues, oil glands and epidermis) while IAA is indifferently translocated into all the tissues of the leaf. The rapid mobilization of IAA could be related to the fact that IAA changes are frequently due to crosstalk with other hormones (Chandler, 2009) especially with CKs, other essential hormone to plant response to stress. It is well established that CKs can regulate several processes of plant growth and development, including the response of plant to abiotic stress (Pospíšilová et al., 2000). It has been described that CKs play an important role in the development of the plant photosynthetic apparatus by directly affecting the chloroplast and reducing chlorophyll degradation (Rivero et al., 2010). In fact, according Dwivedi et al. (2014) the increase of the total chlorophyll content observed in our data could also be directly related to the higher level of iP hormone observed in WD plants. Moreover, CKs may partially ameliorate negative effects of water stress by stimulating osmotic adjustment (Merewitz et al., 2012), such as increasing carbohydrate content (Sarafraz-Ardakani et al., 2014), and directly or indirectly scavenging ROS (Stoparić and Maksimović, 2008). Last but not least, a classical growth hormones, the GAs (GA4 is one of the predominant bioactive forms) are crucial targets for stress-induced growth modulation and there is increasing evidence for their involvement in growth regulation under particular abiotic stress (Colebrook et al., 2014). There are relatively little published studies on the influence of water deficit on GA metabolism and crosstalk with other hormones. In our study GA4 and GA7 act in synergy with ABA unlike most of the reports (Colebrook et al., 2014). As far as we know, this is the first report where such complete quantification of hormones, as well as immunolocalization of ABA and IAA was performed for *E. globulus* exposed to water deficit.

4.2. Foliar application of SA and water deficit tolerance

At the morphological level, all SA pre-treated plants showed necrotic leaf lesions in leaves due to direct contact to SA, which was not transported into the tissue (Bandurska and Stroinski, 2005), being more evident in the highest concentration.. In general, the foliar application of the phenolic compound: SA ameliorated the negative damages of water deficit. SA application alleviated water potential in WD plants to a great extent, without affecting WW plants (Fig. 1). This response was enhanced according to the higher concentrations of SA.). In parallel, the studied compatible solutes, TSS, also increased in response to SA application (Fig. 3D). The strong correlation between accumulation of compatible solutes and water relation components indicates the involvement of those solutes to balance plant water status under drought (Farooq et al., 2009). Total phenols levels similarly increased in response to SA treatment (Fig. 3C). This increase may also be part of the explanation to the lower levels of MDA in SA-treated WW and WD plants (Fig. 3A). Our results support that the decreased membrane damage may be related with the induction of antioxidant responses triggered by SA, including soluble sugars and phenols accumulation, which in turn protect plants against ROS and membrane injury or may affect synthesis of other substances, having a protective effect on plants under stress (Bandurska and Stroinski 2005). Fayez and Bazaid (2014) showed that SA application alleviate lipid peroxidation in barley plants in contrast to the untreated plants under drought stress condition. Carotenoids in WW and WD plants decreased when treated with SA (Table 2) reflecting stress relief as they play a critical role in preventing photo-oxidative damage. Although the total chlorophylls in WW plants was similar between non-treated and SA treated plants, in WD plants a pronounced response occurred due to the SA application. As argued before, water deficit induced the increase of these pigments in *E. globulus*. Thus, we defend that SA application could alleviate the water deficit effects on chlorophylls content. As SA induced water deficit relief, a reduction in leaf mass expansion and the protective role of carotenoids or other mechanisms to protect the chlorophylls from degradation and preserve the photosynthetic capacity was not necessary. Bearing this in mind, the application of SA caused an increase in A and E (Fig. 2A and C), slightly induced gs (Fig. 2B) and did not change Ci (Fig. 2D) on *E. globulus*. The observed increase between A and gs in this study partly support Galmés et al. (2007), suggesting that SA-induced photosynthesis may depend on stomatal control. Furthermore, and as explained above, SA is probably inducing several non-stomatal responses, preventing major metabolic impairment and carboxylase inactivation of RuBisCO. It is possible that SA-induced an increase in RuBisCO activity, which could be responsible for the increase in the photosynthetic rate, as it has been previously described by Singh and Usha (2003) for wheat plants. Understanding the complicity of plant hormone cross-linking networks is important to unravel how plants adapt to environmental changes. In this work, we could observe that SA application increased SA, ABA and DHZ and decreased GA4, GA7 and iP levels. Other works also showed that through PGR exogenous application not only plant physiology is altered but also the general hormone status of the plant (Meijón et al., 2011a). As expected, SA concentration also drastically increased with SA application (Table 3) demonstrating the effective exogenous absorption of SA by the eucalypts plants. However, the treatments of plants with SA also caused a clear increase of ABA content and redistribution in *E. globulus* leaves tissues in relation to SA-doses applied. So, with the increase of the SA concentration, ABA is expanded along all the leaf tissues. Bandurska and Stroinski (2005) defended that the increased ABA levels after SA application and before drought stress imposition may be responsible for the alleviation of membrane injury in barley plants when subjected to drought stress. Shakirova et al. (2003) also argued that the SA-induced increase in ABA might contribute to a pre-adaptation of wheat plants to stress. According with the authors, ABA serves as an intermediate in the sign of the

protective action of SA. SA-treatment induced a higher accumulation of ABA, which in turn induces a wide spectrum of anti-stress programs in the plants. On the other hand, the negative correlation between iP and DHZ under SA treatment could imply a displacement of the CK biosynthesis route in relation to the decrease of growth and cell division (Table 1). iP is the initial precursor of CKs biosynthesis and by means of side-chain trans-hydroxylation reactions is converted in DHZ, very active CK. The large increase in free bases of the Z-type species is related to the activity of CKs (Meijón et al., 2011a,b). Experiments have shown that the free form of Z-type species, but not its derivative riboside or ribotide, binds directly to the receptor CYTOKININ RESPONSE 1 (CRE1) (Yamada et al., 2001), indicating that the free base is the only active form of this CK. Furthermore, the decrease on iP could also be related to the lower level of total chlorophyll content verified on WD plants treated by SA, as previously explained. The reduction in GA_4 and GA_7 (Table 3) was expected as a correlation between SA treatment and inhibition gibberellin synthesis. Xie et al. (2007) showed that SA suppressed GA-induced expression in barley.

The clear distribution revealed by PCA-analysis (Fig. 7) came upon the previously described results providing an overview of the all data obtained in this work. First, the horizontal gradient (PC1) revealed a well-defined separation between WW and WD. Component 1 is mainly supported by the decrease in height, gas exchange and water potential, the most sensitive physiological evidence of water deficit, as previously explained. Besides, the water deficit was also marked by increased lipid peroxidation, chlorophylls, JA and IAA, which are also known responses after water deficit imposition. On the other hand, the effect of SA application is highlighted by the vertical gradient (PC2). Through the loadings of component 2 it is revealed that SA increase could be linked to an increase in total phenols and TSS, which in turn seem to be related to a biased MDA decrease and CO_2 assimilation increase. Additionally, by component 2 heft, it is again confirmed that SA response to water stress in eucalypts plants is also connected to ABA stress response.

5. Conclusions

Our results show that WD generally affected *E. globulus* performance but the pre-treatment with exogenous SA increased the tolerance of these plants to low availability water. SA application ameliorated the damaging effects of water deficit on *E. globulus*, probably by inducing different anti-stress programs that were expressed by improving some physiological and biochemical attributes. The complicity of plant hormone cross-linking networks makes extremely difficult to study individual role of each phytohormone in specific physiological processes. However, through this work, we could demonstrate that by combining uni- and multivariate statistical approaches, despite of the complex PGRs response to drought, it is possible to get an overview of PGRs dynamics in water deficit response of eucalypts plants. We proved that the efficiency of exogenous SA action depend on the SA concentrations getting the best result in the plants treated with higher SA concentrations. Various strategies can be considered to maximize *Eucalyptus* productivity and water use efficiency under environmental stress such as drought. A fundamental one is to develop drought-tolerant plants through genetic means (by genetic engineering or conventional breeding). However such processes are very time consuming. An alternative and technical simpler approach with short-term results is to induce drought tolerance through exogenous application of compounds such as SA. In practice this investment can be adopted in nurseries production management and initial field establishment to improve plant quality, maintaining healthy trees that is important for ensuring future sustainability of the forestry industry. Here, we are able to conclude that foliar application of SA could be used as a potential chemical priming strategy in *E. globulus*.

Uncited references

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