Universidade de Aveiro Departamento de Biologia
2017

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Linking *omics* and ecophysiology in *Eucalyptus*: unravelling stress tolerance in a forest species

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Glória Catarina Cintra da Costa Pinto, professora auxiliar convidada do Departamento de Biologia da Universidade de Aveiro e coorientação dos Doutores Luis Valledor González, investigador de pós-doutoramento do Programa Ramón y Cajal na Universidade de Oviedo, e Robert Douglas Hancock, investigador principal no Instituto James Hutton.

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o júri *the jury*

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um brinde à amizade! *Cheers*!
https://www.youtube.com/watch?v=W8jaOQ639ds

palavras-chave

Árvore, *Eucalyptus globulus*, fisiologia, proteómica, metabolómica, expressão genética, seca, calor, recuperação

resumo

As plantações de eucalipto estão entre as mais produtivas do mundo inteiro. Em Portugal e Espanha, são amplamente utilizadas na produção de polpa e como fonte de energia. No entanto, o clima mediterrânico da região, com secas de verão cada vez mais severas, afeta negativamente o crescimento do eucalipto e aumenta a sua mortalidade. Esta tese de doutoramento tem como objetivo desvendar a tolerância à seca da espécie *Eucalyptus globulus,* investigando e interligando informação dos processos que medeiam o défice hídrico e a reidratação, desde a regulação genética e molecular até às respostas fisiológicas e desempenho da planta, utilizando dois genótipos distintos e diferentes ensaios experimentais. Esta tese está estruturada sob a forma de estudos científicos (capítulos 2, 3, 4 e 5), precedidos por uma introdução geral (capítulo 1), e termina com as notas finais (capítulo 6). O capítulo 2 descreve um ensaio de estufa e um défice hídrico imposto lentamente, e está dividido em 3 subcapítulos. Dois genótipos (AL-18 e AL-10) foram sujeitos a um período de *stress* hídrico de 3 semanas com duas intensidades diferentes (18% e 25% da capacidade de campo), seguido de uma semana de reidratação. A recuperação foi avaliada um dia e uma semana depois da reidratação. Várias fitohormonas foram monitorizadas nas folhas, seiva xilémica e raízes, 2 h, 4 h, 24 h e 168 h depois da reidratação. A falta de água reduziu a altura, a biomassa, o potencial hídrico e as trocas gasosas. Pelo contrário, os níveis de pigmentos, parâmetros da fluorescência da clorofila e MDA aumentaram. Os níveis de ABA e de ABA-GE aumentaram, enquanto o JA diminuiu nas folhas e aumentou na seiva xilémica. Durante a recuperação, a maioria das alterações fisiológicas e bioquímicas provocadas pelo *stress* reverteram. Análises comparativas do proteoma (analisado por eletroforese em gel diferencial) e do metaboloma (analisado por cromatografia gasosa com espetrometria de massa) permitiram a separação de 2031 pontos peptídicos, dos quais 217 foram identificados, e a deteção de 121 metabolitos polares. A análise do clone resiliente AL-18, que apresentou uma rede de resposta bem distinta do clone responsivo AL-10, reforçou o papel de proteínas específicas da fotossíntese e relacionadas com a defesa como intermediários chave na tolerância à seca e revelou novos intermediários: glutamina sintetase, malato desidrogenase e isoflavona redutase. O capítulo 3 diz respeito a um ensaio em câmara climática e a uma rápida escassez de água, e está dividido em 2 subcapítulos. A expressão relativa de 12 transcritos foi analisada por PCR quantitativo em dois clones com diferentes graus de tolerância (AL-18 e AL-13) depois de 7 e 11 dias sem qualquer rega e posterior reidratação. A rápida escassez de água foi mais prejudicial para as plantas do que o défice hídrico imposto lentamente, com maior visibilidade no clone AL-13 que revelou morte de algumas plantas.

resumo (cont.)

Indicadores moleculares potencialmente ligados a uma tolerância aumentada foram identificados: rubisco ativase (RCA), ferredoxina-NADP(H) oxidorredutase (FNR), malato desidrogenase mitocondrial (mMDH) catalase peroxissomal (CAT) e isoflavona redutase (IFR). De seguida, vários marcadores bioquímicos de *stress* oxidativo e padrões de metilação do DNA foram quantificados nas folhas do clone AL-18. As alterações detetadas utilizando indicadores globais e específicos refletiram a indução de complexas modificações redox e de metilação do DNA, que ocorrem paralelamente durante a imposição e interrupção do *stress*. O capítulo 4 reporta um ensaio de campo: o conjunto de indicadores de seleção de tolerância hídrica identificado anteriormente foi testado em AL-18 e AL-13 plantados no campo. Algumas das plantas foram regadas artificialmente (IR) e outras foram deixadas nas condições ambientais de precipitação reduzida (NI) durante seis semanas e meia antes de voltar a regar. O clone AL-18 mostrou pouca variação nas condições testadas, e as alterações encontradas no clone AL-13 realçaram a indução do metabolismo fotossintético e fotorespiratório após a reidratação artificial. Estes resultados mostraram que as respostas das plantas no campo não podem ser extrapoladas a partir do estudo de um *stress* aplicado individualmente, particularmente no contexto de encontrar marcadores de seleção. O capítulo 5 descreve um ensaio em câmara climática que testou o efeito isolado e combinado de seca e calor. Alterações fisiológicas, bioquímicas e metabolómicas foram monitorizadas no clone AL-18 após 5 dias de seca consistente e/ou 4 h a 40ºC. Testar plantas em *stress* hídrico sujeitas a um choque térmico revelou uma diminuição das trocas gasosas, do potencial hídrico e do JA, nenhum efeito a nível da perda de eletrólitos, MDA, amido e pigmentos e um aumento na glutationa, em comparação com condições controlo. O *stress* combinado induziu também a produção do cinamato, uma resposta nova. Estes resultados realçam que a combinação de seca e calor fornece uma proteção significante contra os efeitos mais prejudiciais da seca isolada em eucalipto, confirmando que o *stress* combinado altera o metabolismo das plantas de uma forma nova que não pode ser extrapolada pela soma dos diferentes *stresses* aplicados individualmente. Esta tese descreve um conjunto de respostas biológicas que permitem ao eucalipto manter-se em condições de défice hídrico e revela informação útil de várias vias metabólicas a serem exploradas de modo a encontrar marcadores de tolerância ao *stress* abiótico apropriados. Apesar disso, um desafio maior permanece. Consiste na necessidade de focarmos os nossos estudos em experiências mais realistas, que mimetizem as condições de campo, pelo menos no contexto de encontrarmos marcadores de seleção ajustados a uma era de alterações climáticas.

keywords

Tree, *Eucalyptus globulus*, physiology, proteomics, metabolomics, gene expression, drought, heat, recovery

abstract

Eucalyptus plantations are among the most productive forest stands worldwide. In Portugal and Spain, they are widely used for pulp production and as an energy crop. However, the region's Mediterranean climate, with increasingly severe summer drought, negatively affects eucalypt growth and increases mortality. The aim of this doctoral thesis was to unravel drought tolerance in *Eucalyptus globulus* by investigating and interconnecting information on the processes mediating water deficit and rehydration, from gene and molecular regulation to physiological responses and plant performance, using two different genotypes and different stress trials. The thesis disclosed herein is presented in a series of research papers (chapters 2, 3, 4 and 5), preceded by a general introduction (chapter 1) and closed with concluding remarks (chapter 6). Chapter 2 describes a greenhouse trial and a slowly imposed water deficit, and is divided into three subchapters. Two genotypes (AL-18 and AL-10) were submitted to a 3-week water stress period at two different intensities (18% and 25% of field capacity), followed by 1 week of rewatering. Recovery was assessed 1 day and 1 week after rehydration. Several phytohormones were monitored in leaves, xylem sap and roots, 2 h, 4 h, 24 h, and 168 h after rewatering. Water deficit reduced height, biomass, water potential, and gas exchange. Contrarily, the levels of pigments, chlorophyll fluorescence parameters and MDA increased. ABA and ABA-GE levels increased, and JA content decreased in leaves and increased in xylem sap. During recovery, most of the physiological and biochemical responses of stressed plants were reversed. Comparative proteome (using difference gel electrophoresis) and metabolome (using gas chromatography–mass spectrometry) analyses enabled the separation and isolation of 2031 peptide spots, 217 of which were identified, and the detection of 121 polar metabolites. The analysis of the resilient clone AL-18, which presented a response network very distinct from the responsive clone AL-10, reinforced the role of specific photosynthetic and defence-related proteins as key players in mediating drought tolerance and revealed new players: glutamine synthetase, malate dehydrogenase and isoflavone reductase-like protein. Chapter 3 regards a climate chamber trial and a sudden water shortage, and is divided in two subchapters. The relative expression of 12 transcripts was analysed by quantitative PCR in two clones with different degrees of tolerance (AL-18 and AL-13) 7 and 11 days after water withholding and rehydration (2 h and 3 days after rewatering). Sudden water shortage was more detrimental to the plants than when slowly imposed, with heavier outcomes in clone AL-13, including plant death. Potential molecular indicators linked to enhanced water stress tolerance in *Eucalyptus globulus* were identified: rubisco activase (RCA), ferredoxin-NADP(H) oxireductase (FNR), mitochondrial malate dehydrogenase (mMDH), peroxisomal catalase (CAT) and isoflavone reductase (IFR).

abstract (cont.)

Afterwards, several biochemical markers of oxidative stress and DNA methylation patterns were quantified in the leaves of AL-18. The alterations detected using global and specific indicators reflected the parallel induction of redox and complex DNA methylation changes occurring during stress imposition and relief. Chapter 4 reports a field trial: the previously identified set of indicators for selection of water stress tolerance was tested in field-grown AL-18 and AL-13. Some of the plants were irrigated (IR), and others were left under environmental conditions of reduced rainfall (NI) during six and a half weeks prior to rewatering. Clone AL-18 showed few fluctuations in the conditions tested, and the alterations found in clone AL-13 highlighted an induction of photosynthetic and photorespiration metabolism after artificial rehydration. The results corroborated that responses to field conditions cannot be extrapolated from a stress applied individually in the context of developing selection markers. Chapter 5 describes a climate chamber trial that tested the isolated and combined effect of drought and heat. Physiological, biochemical and metabolomic alterations were monitored in AL-18 after a 5-day of consistent drought and/or 4 h at 40ºC. Testing drought-stressed plants subject to a heat shock revealed a decrease in gas exchange, Ψ_{pd} and JA, no alterations in electrolyte leakage, MDA, starch and pigments and increased glutathione pool in relation to control. The induction of cinnamate was a novel response triggered only by the combined stress. These results highlighted that the combination of drought and heat provides significant protection from more detrimental effects of drought-stressed eucalypts, confirming that combined stresses alter plant metabolism in a novel manner that cannot be extrapolated by the sum of the different stresses applied individually. This thesis describes a number of biological responses that enable *E. globulus* to thrive under conditions of water deficit and provides useful information of pathways to be explored in order to find suitable markers of abiotic stress tolerance in this species. Despite that, a bigger challenge remains and consists of the need to focus our studies in more realistic, field-like experiments, at least in the context of finding suitable selection markers in the climate change era.

Abbreviations

A	foliar net CO_2 assimilation rate
ABA	abscisic acid
ABA-GE	abscisic acid glucose ester
ACT	actin
ANOVA	analysis of variance
APX	ascorbate peroxidase
AsA	ascorbate
C	control
Ca^{2+}	calcium ion
C_a	ambient CO_2 concentration
CAT	catalase
CFCs	chlorofluorocarbons
Cl^-	chloride
D	drought
D*H	combined drought and heat
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DIGE	difference gel electrophoresis
df	degrees of freedom
DREB1A	dehydration response element B1A
DW	dry weight
E	transpiration rate
EL	electrolyte leakage
ET	ethylene
ETI	effector triggered immunity
FAO	food and agriculture organization of the united nations
FC	field capacity
F	steady-state fluorescence
F_0	minimum fluorescence
F_m / F'_m	maximal fluorescence
F_v / F'_v	variable fluorescence
F_v/F_m	maximum quantum yield of photosystem II photochemistry
FNR	ferredoxin-NADP(H) oxireductase
FW	fresh weight
GADPH	glyceraldehyde 3-phosphate dehydrogenase
GC-MS	gas chromatography–mass spectrometry
GCSH	mitochondrial glycine cleavage system H protein

GORK	potassium channel GORK
GR	glutathione reductase
GSH	glutathione
GSSG	oxidised glutathione
g_s	stomatal conductance
H	heat
$HO^{\cdot}$	hydroxyl radical
H2B	histone H2B
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
ICNF	instituto da conservação da natureza e das florestas
IFR	isoflavone reductase
InsP6	*myo*-inositol hexakisphosphate
IPCC	intergovernmental panel on climate change
IR	irrigated
JA	jasmonic acid / jasmonates
LECA	lightweight expanded clay aggregate
K^+	potassium ion
Max Stress	maximum stress
MDA	malondialdehyde
MDH	malate dehydrogenase
MDHAR	monodehydroascorbate reductase
midR	2 hours after rewatering
midS	7 days after water withholding
MRM	multiple reaction monitoring
MS	variance
MS/MS	tandem mass spectrometry
MS-RAPD	methylation-sensitive random-amplified polymorphic DNA
NI	non-irrigated
NPQ	non-photochemical quenching
NRQ	normalised relative quantities
$O_2^{\cdot-}$	superoxide radical
$^1O_2$	singlet oxygen
PAL	phenylalanine ammonia-lyase
PAMP	pathogen-associated molecular patterns
PAR	photosynthetically active radiation
PC	principal component
PCA	principal components analysis
PCR	polymerase chain reaction

PPFD	photosynthetic photon flux density
PRK	phosphoribulokinase
PSII	photosystem II
PTFE	polytetrafluoroethylene
PTI	PAMP-triggered immunity
PVPP	polyvinylpolypyrrolidone
qRT-PCR	quantitative real-time PCR
R	3 days after rewatering
RBOH	respiratory burst oxidase homolog
RCA	rubisco activase
RD22	responsive to desiccation protein 22
ROS	reactive oxygen species
RQ	relative quantities
RWC	relative water content
S	11 days after water withholding
SA	salicylic acid
SOD	superoxide dismutase
sPLS	sparse partial least squares regression
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TUB	tubulin
TW	turgid weight
UBQ	ubiquitin
USDA	united states department of agriculture
VPD	vapour pressure deficit
WS	water stressed
WUE	water use efficiency
WW	well-watered
1-day Rec / 1dR	1-day recovery
1-week Rec / 7dR	1-week recovery
5-mC	5-methylcytosine
Φ_{PSII}	effective quantum yield of photosystem II photochemistry
Ψ_{md}	midday water potential
Ψ_{pd}	predawn water potential

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Chapter 1

general introduction

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1. Revisiting drought impact in *Eucalyptus*: a long and yet incomplete research path

Summary

The genus *Eucalyptus* represents a keystone in the planted forests of many nations, sustaining worldwide demands for forest-based goods and services, and helping to reduce the pressure on natural forests. However, climate change represents an eminent threat, affecting forest vitality and distribution. Water limitation has been considered a major climate change-driven factor, and declines in forest productivity and tree survival are already well documented. Given the influence of the *Eucalyptus* genus in the global economy and the enormous amount of research that has been conducted focussing on drought impact in these plants, this introduction aims to review and elucidate the main outcomes of water deficit, assembling the knowledge gained over the years in relation to *Eucalyptus.* There is an extensive knowledge regarding plant response to drought, ranging from stress perception to signalling and defence; however, since earlier times researchers studying *Eucalyptus* have almost exclusively relied on the plant water status and growth. More recently, comprehensive analyses using post-genomic techniques such as transcriptomics, proteomics, and metabolomics have proved very useful to monitor and connect specific variations that increased our understanding of stress adaptation, tolerance, and complex regulatory networks. However, only a small step has been taken regarding drought tolerance in *Eucalyptus*, which constituted the basis to design the aims of this doctoral thesis, addressing them in *Eucalyptus globulus*.

World forests

"*What we are doing to the forests of the world is but a mirror reflection of what we are doing to ourselves and to one another*", Mahatma Gandhi

As stated by Keenan et al. (2015), a major indicator of the environmental condition of the planet is the area covered by trees and forests. Keeping this in mind, this review starts by showing some numbers and results from the latest Global Forest Resources Assessment of the Food and Agriculture Organization of the United Nations (FAO 2015) on the current dynamics of global forest area. Following FAO's definitions, we consider global forest area as land area that includes areas of primary forest, other naturally regenerated forest, planted forest, areas of land temporarily absent of trees but intended for forestry or conservation, and other wooded land. In 2015 the world had 3 999 million ha of forest, whilst in 1990 this area was a larger 4 128 million ha. This change reflects a decrease from 31.6 to 30.6 percent of global land area between the two considered years; or, in other words, forest has decreased from 0.8 to 0.6 ha for every person on the planet. The forest area decline *per capita* occurred mostly in the tropics, particularly in South America and Africa, but it is occurring in almost every climatic domain (not in the temperate domain) mainly driven by forest land conversion to agriculture and other land uses due to populations increase. In addition to climatic domain, trends in forest land area also vary with income category (Keenan et al. 2015): forest area is slowly increasing (about 0.05 percent per year over the 25 year period) in high income countries, stabilizing in upper middle income countries in the last few years, but continues to decline in lower middle and low income countries (to maximum loss rates of 0.6 percent per year in the worst cases). Considering country area alone, different national trends can also be found within the same continent (fig. 1). (FAO 2015)

As a primary good of forests, wood production and consumption has generally grown between 1990 and 2015. The high reliance in wood fuel remains: annual wood removals amounted to 2.8 billion m^3 in 1990 to 3.0 billion m^3 in 2011 (of which 41 and 49 percent were for wood fuel, for each year respectively), and the proportions of wood removals and total amount of wood used for fuel have increased. (FAO 2015) Multiple-use forest provides non-wood forest products, timber, range, water, recreation, and wildlife management values. Forest designated as multiple use expanded from 23 percent of total forest area to 26 percent between 1990 and 2015. In 2015 about 30 percent of the world's forest was considered as production forest, slightly increasing from the 28 percent in 1990. About 13 percent of the world's forests currently represent the focus of primary management for the conservation of biodiversity: 150 million ha of forest have been added to the

conservation category since 1990, and forests designated for the protection of soil and water represent 25 percent of the total forest area. (FAO 2015)

The State of the World's Forests report (FAO 2014) highlights the impact these numbers have on people´s lives. About 13.2 million people across the world are employed in the formal forest (captured in national statistics) sector and it is estimated that the informal (not captured in national statistics) sector employs at least another 41 million: this covers direct employment in the forest, and other activities outside the forest – transport, processing and manufacturing; in rural areas of less developed countries, wood energy represents frequently the only source of energy, being particularly significant for poor people; at least 1.3 billion people (18 percent of the world's population) have a shelter thanks to the important contribution of forest products; forests contribute greatly to food security and health by providing wood fuel to cook and sterilize water.

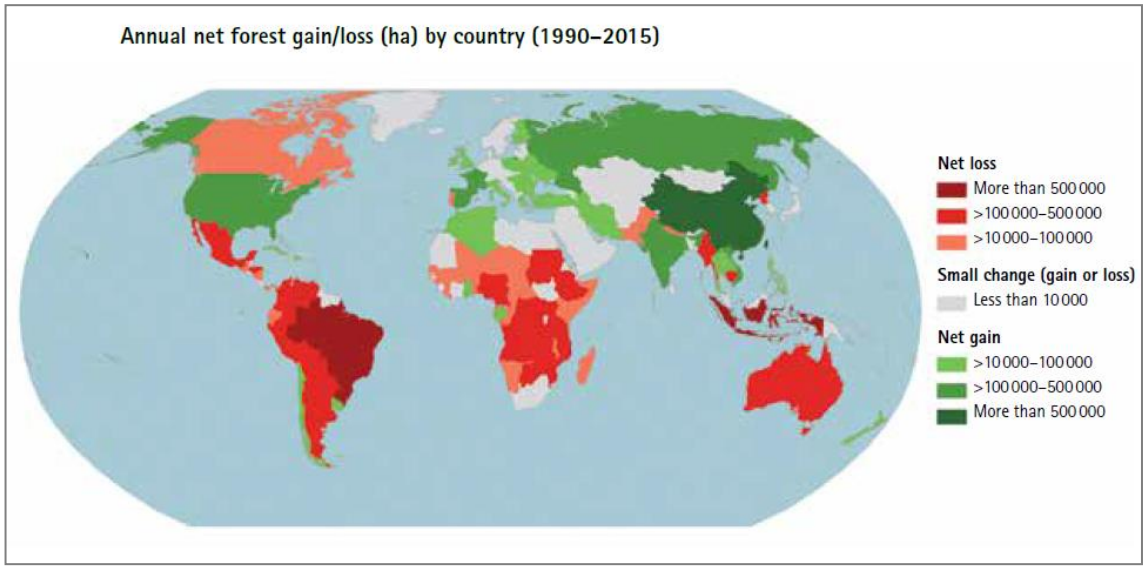

Figure 1 – Annual net forest gain/loss (ha) by country (1990-2015). Source: FAO (2015).

On the other hand, forests have many other unique properties. Their high rates of primary productivity and biological diversity separate them ecologically from other ecosystems, making them major repositories of terrestrial biodiversity and all that it represents for gene pools, pharmaceuticals, and other essential goods and services, as well as reservoirs of about 50% of the terrestrial carbon bulk (Thompson et al. 2009; Abril et al. 2011). As highlighted by Thompson et al. (2009), the special features of the forests combine the occurrence of biological structures that are able to develop in vertical and horizontal layers of plants, dead or alive, and complex processes that take place at vertical levels that range from within soil layers up to the canopy, with the capacity for self-renewal under continuous minor or major disturbances. Co-evolved plant-animal and plant-plant interactions and forest landscapes can influence on micro- and regional climates, a trait especially relevant in closed-canopy tropical forests. In addition, forests have an important role in

preserving the fertility of agricultural land and the water sources, controlling erosion and reducing the risks of natural disasters such as landslides and flooding (Abril et al. 2011).

Native *versus* planted forests

FAO (2015) describes forest area change as a process of gain (forest expansion) and loss (deforestation). As reported (FAO 2015), total forest area change is very difficult to monitor and predict: on one hand, forest gains and losses are permanently occurring; on the other hand, natural and planted forest area dynamics differ and vary dramatically across national circumstances and forest types. However, the changes of all forest resources may be combined and may provide a picture of how total forest area is affected: change in natural forest is possibly a more suitable indicator of natural habitat and biodiversity dynamics, while planted forest change explains variations in the forest product mix from natural and planted forests. For a better understanding of these ideas, some concepts must be clarified: natural forest is defined as a forest composed of native species that have naturally evolved and reproduced, without clearly visible anthropogenic modifications; planted forests are established by human action after planting and/or through deliberate seeding of native or introduced species in a process of afforestation or reforestation (FAO 2001).

Over the last 25 years, forests have globally changed: a decrease in natural forest area has been followed by increases in planted forest area. Natural forests conserve the diversity of genotypes and help to maintain the composition, structure and ecological dynamics of natural tree species. However, the continuous increase of human populations is likely to maintain high rates of conversion of forest land to agriculture despite the growing demand for forest-based products. Well-managed planted forests can therefore help to reduce the pressure on natural forests and provide important forest goods and services. Planted forest includes species mixtures and monocultures with a broad range of management intensity and purpose, and supply timber (including wood fuel), non-wood forest products and many of the environmental services provided by natural forests. (Brockerhoff et al. 2013; FAO 2015)

Countries are now more aware of their forest resources and their value, and this results in better indicators of global forest change: positive progress in forest management and lower rates of forest area loss reveal advances in current sustainable management but, at the same time, major issues remain (FAO 2015). Persistent forest conversion and unsustainable forest practices require effective incentives or enforcement of sound policies, legislation and regulation.

Natural forest and plantations around the world: the *Eucalyptus* case

One of the peculiarities of the Australian landscape is the eucalypt forest (Lima 1984). Curiously, it is said that after the First and Second World Wars soldiers that were returning by ship could smell the aroma of the eucalypt even before sighting land on the horizon (australia.gov.au). Eucalypts play a vital role to the Australian environment. Different species are used from Aboriginal use through to modern production: medicinal purposes, food, timber, and firewood for Australians, and shelter for many species of native Australian animals (australia.gov.au).

Eucalypts occur naturally across Australia (fig. 2) with a small number of species found in the north of Australia, in Papua New Guinea, Indonesia and the Philippines (Booth et al. 2015).

Figure 2 – Natural distribution of the *Eucalyptus* genus. Source: ala.org.au.

This genus includes plants with a wide range of physical forms that vary from shrubs and multistemmed mallees to trees and even the tallest flowering plants on earth and occupy a broad range of climatic and ecological niches (Potts et al. 2011; Booth et al. 2015). There are about 839 species within the genus *Eucalyptus*, and 100 species within the closely related *Corymbia* (bloodwoods, ghost and spotted gums), and this number continues to rise as new taxa are described (Coppen 2003; Booth et al. 2015).

Apart from its native value, a small number of *Eucalyptus* species has been introduced in several locations around the world and the area of eucalypt plantations has expanded from about 6 Mha in 1990 to over 20 Mha in 2011 (Harwood 2011). Harwood (2011) estimates that over 90% of world's eucalypt plantations are composed by only nine eucalypt species of the subgenus *Symphyomyrtus* (Brooker 2000) and various interspecific hybrids among these species, established by seed planting and clonal plantations. These species are *E. camaldulensis*, *E. dunnii*, *E. globulus*, *E. grandis*, *E. nitens*, *E. pellita*, *E. saligna*, *E. tereticornis*, and *E. urophylla*. Most of the world's eucalypt plantations are located in tropical, subtropical and warm temperate climates (fig. 3), which present the best climate ranges for these species to grow (Harwood 2011). Indeed, from the nine species, only *E. camaldulensis*, *E. globulus* and *E. nitens* are planted widely at latitudes further than 35º north or south from the equator because only they thrive in winter/uniform rainfall, and cool temperate climates (Harwood 2011).

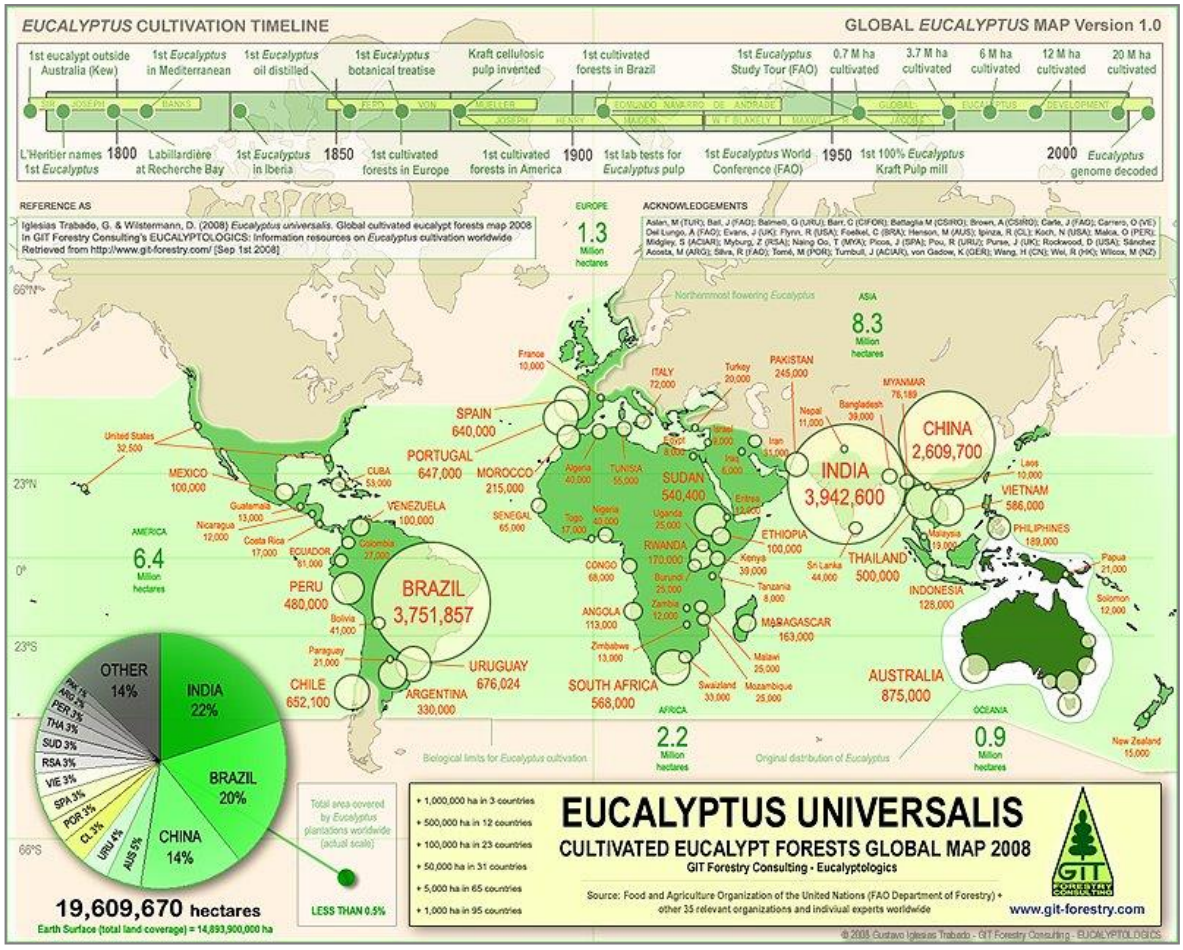

Figure 3 – Eucalyptus cultivation timeline and cultivated eucalypt forests global map 2008. Source: ncsu.edu.

Climate change

"We have changed the chemistry of our atmosphere, causing sea level to rise, ice to melt, and the climate to change. (…) The only reason we might think otherwise is to avoid committing to action" (Oreskes 2007)

Back in 1896, Professor Arrhenius was the first to calculate the contribution of carbon dioxide to the greenhouse effect and to speculate about how changing the level of this atmospheric gas would contribute to long-term variations in climate (Arrehnius 1896). Although some groups still claim that there is not good evidence that Earth's climate is being affected by human activities, the existing scientific literature show that there is a robust consensus (Oreskes 2004): the scientific community largely agrees that there is strong evidence that anthropogenic global climate change has been occurring. According to the United Nations Environment Programme, climate change has thus moved over time from being a matter of scientific discussion to becoming the major and overriding environmental concern of our time.

In 1988, the World Meteorological Organization and the United Nations Environment Programme established the Intergovernmental Panel on Climate Change (IPCC) to assess the scientific, technical, and socioeconomic information relevant to the understanding of climate change, potential impacts and appropriate response strategies (IPCC 2015). The First Assessment Report was published in 1990 and counted with the participation of several hundred working scientists from 25 countries that prepared and reviewed the scientific data (IPCC 1990). The report considered the occurrence of a natural greenhouse effect that keeps the Earth warmer than it would otherwise be, mainly due to emissions resulting from human activities that are substantially increasing the atmospheric concentrations of carbon dioxide, methane, chlorofluorocarbons (CFCs) and nitrous oxide; based on the predictions models, the outcome of these increases would be intensified with water vapour, resulting in an additional warming of the Earth's surface (IPCC 1990). This raised greenhouse effect further impacts other climate events. Hydrologic cycles are reported to be changed, which includes alterations in precipitation regimes in terms of intensity and frequency (IPCC 2015). Coupled with an increase in temperature, runoff shortages or excesses of water and changes in water availability in many regions may lead to modifications in the frequency and intensity of floods and droughts (IPCC 2015).

Almost 30 years later, these projections have been proved to be mostly right (Kerr 2007a), with some indicators showing that the risk may even have been underestimated and climate change may be responding more quickly than climate models have foreseen (Kerr 2007b). Although a scientific consensus on this fact has now been reached (at least 97% of climate scientists agree),

much of the public remains largely unaware that climate change caused by human action is happening, both in the US as well as internationally (van der Linden et al. 2015). To date, there is still a need to effectively communicate the scientific consensus to the general public so that the issue of climate change can be moved forward on the public policy agenda (van der Linden et al. 2015), as the social power distributed in the economic, political, and cultural spheres represent the barriers to action on climate change (Brulle et al. 2012).

Influence on natural distribution

Plants present species-specific physiological thresholds of temperature and precipitation, also known as `climate envelopes', which identifies climate as a primary influence on geographic distribution of species (Walther et al. 2002; McKenney et al. 2007). Considering the predicted climate change, it is therefore conceivable that plant species exhibit marked geographical displacement to track the shifting climate to the extent that dispersal and resource availability allow, especially towards the poles or higher altitudes. Fossil pollen records from the Holocene period document such responses for a variety of plant species (Malanson 1993; Williams et al. 2004).

A case study in the Swiss Alps aimed to explain the driving forces behind changes in the alpine ecosystem: increased growth rates in tree line species, compositional changes in alpine plant communities and species and increased young tree establishment in forest gaps near the tree line (Gehrig-Fasel et al. 2007). These authors suggested that land abandonment was the most dominant driver for the changes and that the relatively small effect of climate change would be attributed to anthropogenic suppression of the tree line and the short time frame of the analysed surveys, assuming that climatic effects will become more evident in the long run as forests rise towards their climatic potential.

Another study was carried out in the Iberian Peninsula, a unique hotspot of biodiversity in Europe (Benito Garzón et al. 2008). Several characteristics of this region make it particularly vulnerable to climate change: geographically isolated, constituted by a large number of endemics and species whose gene pools differ from those of their European counterparts, mountainous areas with species at the southernmost limit of their range, and large areas with Mediterranean climate subject to summer droughts (Benito Garzón et al. 2008). Modelling current and future tree distributions as a function of four IPCC scenarios, the authors anticipated serious impacts in the species distribution, including drastic reduction in the potential range of mountain conifer species, decline of temperate broad-leaved species and reductions in the areas occupied by sub-Mediterranean species, while typically Mediterranean species were predicted to be less affected for presenting a higher potential for migration.

Using the USDA Forest Service's Forest Inventory and Analysis data, Zhu et al. (2012) compared seedling and tree for 92 species in 30 longitudinal bands for 43 334 plots across the eastern United States and compared these latitudes with 20th century temperature and precipitation change and functional traits, including seed size and seed spread rate. The results highlighted the lack of evidence for climate-mediated migration by the fact that the majority of seedling extreme latitudes were less than those for adult trees, which should further increase concerns for the risks posed by climate change.

The hundreds of natural eucalypt species distributed over the Australian landscapes are known for having poor dispersal capabilities – seed dispersal distances usually similar to tree height, reduced colonization rates – about one metre per year, and limited adaptability via widespread gene-flow – mostly insect pollinated (Booth et al. 2015). The sum of these points foreshadows how the native eucalypt stands will most likely suffer with the predicted climate change.

A threat to established plantations?

Since the 18th century when the science of forestry was born in Europe because of concerns about the sustained productivity of forests in this continent that sustainability has been a concept deeply rooted in forestry (Fox 2000), which focus on two issues: maintaining soil quality and long-term site productivity, and minimising offsite impacts so that intensive management does not negatively affect adjacent ecosystems.

Planting of forests and trees are increasing at rates of 2.8 million ha/year, which reflect the widespread establishment of plantations (Chazdon 2008). Although not matching the original species composition of old-growth forests, new forests are emerging in human-impacted landscapes and if they are managed as components of a heterogeneous landscape mosaic, these can potentially promote forest restoration and regeneration, and faunal conservation, recovering many components of the original biodiversity (Chazdon 2008).

Planted forest or forest plantation refer to a man-made forest, either planted or regenerated, composed by one or very few species that are grown mainly for production of wood in blocks of varying sizes of a single age class, also being referred to as “monoculture” (Gadgil and Bain 1999). In addition to the main crop species, planted forests usually contain many other plant species, ranging from grasses to a heavy understory of woody shrubs, so the term monoculture may be somehow misleading (Gadgil and Bain 1999). However, the fact of these monocultures represent a narrow genetic base together with the often badly managed concept and implementation of industrial large-scale monocultures granted these with a bad reputation (Gadgil and Bain 1999; Paquette and Messier 2010). Conversely, tree plantations in use today are conceptually and

practically well-conceived, designed to be multi-purpose and diverse, so they can reduce pressure on natural forests, and play a key role in the fight against global warming through carbon sequestration (Paquette and Messier 2010).

Notwithstanding all the positive points of plantation forests, we have to recognise that their ultimate goal and the reason we strongly depend on them is their potential to supply the bulk of humanity's wood needs in the long run, reducing the harvest pressures on natural forests. In order to meet their goal, plantation forests must provide us with higher yield of timber than their natural counterparts on shorter rotation times (Fenning and Gershenzon 2002). This can be achieved by establishing plantations composed by selected stock bred for improved traits such as growth rate, stem straightness, branch diameter and angle, wood density, and resistance to pests and diseases (Gadgil and Bain 1999). Besides, global wood production can also be boosted by genetic engineering and biotechnology applied to the tree-improvement in many ways: resistance to biodegradable herbicides, altered lignin properties, resistance to selected pests, altered reproductive mechanisms, phytoremediation of polluted sites, production of novel chemicals or pharmaceuticals, and manipulation of wood-quality traits, photosynthetic efficiency, and tolerance to abiotic stresses (Fenning and Gershenzon 2002).

As reviewed by Gadgil and Bain (1999), some of the most healthy plantation forests are those of exotic species growing a long way from their native range (e.g., *Pinus radiata* D. Don in New Zealand) and the least healthy others are those of indigenous species (e.g., *Eucalyptus nitens* (Deane & Maiden) Maiden in Tasmania). The authors concluded that the better health of plantations results from good managing decisions, including generally good growing conditions, the proper matching of site to species grown and in the specific case of exotic species, the absence of the many pests and diseases present in their native habitat (Gadgil and Bain 1999). This underlines the need to overcome the lack of knowledge on how soil nutrition, climate and other factors (such as pest occurrence) will potentially affect productivity and survival, which will further determine the quality of decision-making processes related to plant growth, wood production and ecosystem integrity.

As reported for natural forests, it is likely that changing climate will also produce a strong impact on planted forests: rather than the direct impact of higher temperatures and elevated CO_2, forestry will certainly be impaired by the novel anthropogenic stressors, such as pollution, habitat fragmentation, land-use changes, invasive plants, outbreaks of insects and pathogens, altered fire regimes and extreme events (e.g. high winds) at local to global scales (Kirilenko and Sedjo 2007; Millar et al. 2007).

The fact that many eucalypt species have been so widely trialled within and outside Australia, often under climatic conditions that are different from those found within their natural

distributions, provides a particularly interesting opportunity to examine the likely impacts of climate change (Booth et al. 2015). In Portugal, *Eucalyptus* is believed to be planted since around 1829 albeit reliable records are only available after 1852; and, since then, over 100 *Eucalyptus* species have been introduced, mainly to supply the pulp industry (Lima 1920; de Almeida and Riekerk 1990). The *Eucalyptus* species, including the widely planted *E. globulus*, are well adapted to the ecological conditions of Portugal, although subject to several environmental challenges, mainly soil moisture (de Almeida and Riekerk 1990; Costa e Silva et al. 2004).

"*Evidentemente, não ha caixa economica que, em segurança e rendimento, se compare com a plantação d'uma arvore.*" [1] (Lima 1920)

Dead or alive? Mechanisms that dictate the tree fate after drought

From the large troupe of stressors that a tree has to cope with, water limitation has been considered a major factor influencing its distribution and productivity, and declines in forest productivity and tree survival are already well documented (Gholz et al. 1990; Chaves et al. 2002; Allen et al. 2010; Williams et al. 2013). Plant responses to drought are intricate, ranging from simple physiological changes to complex deleterious effects, and despite its implication on tree survival, water scarcity action is still not fully understood at the multiple plant level. Responses to water scarcity involve a mixture of stress escape, avoidance and tolerance strategies, and may be categorized into short-term and long-term responses: at short-term, plants seek immediate survival by minimising water loss or shifting metabolic protection against dehydration, whereas long-term responses or acclimation include new metabolic and structural adjustments that improve plant functioning under stress (Chaves et al. 2002; Chaves et al. 2003). Most of these responses are mediated by altered gene expression and occur at the leaf level, either in response to the direct stimulus generated in the leaf itself or indirectly, through signals transmitted from elsewhere in the plant (Chaves et al. 2002; Chaves et al. 2003). Ultimately, carbon assimilation and growth are negatively affected, and the integrated response at the whole plant level will decide survival and persistence or death under drought (Chaves et al. 2002; Chaves et al. 2003). Escape consists of plants successfully completing their life cycle before water deficit occurs; this prevails mainly in arid regions, where native annuals combine short life cycles with high rates of growth and gas exchange, and confers plants with a high degree of developmental plasticity (Chaves et al. 2003); however, this defence mechanism is negligible in the case of trees.

[1] *Clearly, there is no economic box that compares with the planting of a tree, both in safety and yield.*

In his commentary on "Tree responses to drought", Ryan (2011) points out our knowledge is filled with many clues and puzzle pieces of plant drought responses; however, our understanding of the whole tree coordination, including plant carbon balance and storage, and plant water relations is not enough and, given the variable nature of responses found among closely related species, we cannot predict drought response and select for drought tolerance on the basis of any simpler metric.

Stress perception: water balance and stomatal control

Plant carbon and water relations are inevitably linked by the diffusion pathway of CO_2, and water through the stomata (Schulze 1986). Tissue hydration and photosynthesis are maintained following the steady-state formulation of the soil–plant–atmosphere hydraulic continuum, which is described by the cohesion tension theory: tension generated by transpiration pulls water from the soil through the plant to the crown, diffusing it to the atmosphere (Angeles et al. 2004; McDowell et al. 2008). This means the water potential of plant tissues must be below that of the water supply in order to allow water absorption along gradients of decreasing water potential (Boyer 1968). Water potential is defined mathematically as the chemical potential of water divided by the partial molar volume (Kramer and Boyer 1995); or, as simplified by Verslues et al. (2006), a straightforward estimate of the direction of water movement in the soil/plant system that can be obtained by expressing the free energy of water and the turgor of plant cells using units of pressure. When considering a plant walled cell, the water potential is equal to the sum of the osmotic potential and the pressure potential (turgor pressure) (Verslues et al. 2006).

As mentioned before, when trees encounter insufficient soil water content, they resort to stress avoidance and tolerance mechanisms. Drought avoidance is intended to maintain tissue water potential and water content by increasing water uptake or limiting water loss such that the rates of water loss and water uptake remain balanced, whereas drought tolerance includes mechanisms to avoid cellular damage caused by water loss (Chaves et al. 2003; Verslues et al. 2006).

Although a number of abiotic stresses is characterised by altered water status, it plays a clear role regarding drought: decreased water potential hampers water uptake by the plant, which in turn switches on a range of responses that allow it to cope with the decreased water availability, either by avoiding excessive water loss, continuing water uptake at reduced water potential, or tolerating a reduced tissue water content; all together, these responses represent changes in water fluxes and relations at the cellular and the whole plant levels (Verslues et al. 2006).

The earliest and most common response to water deficit is stomatal closure, protecting plants from extensive water loss (Chaves et al. 2003). The stomatal regulation by humidity is a property of the epidermis (it was observed with isolated epidermis independently of the presence or

absence of chloroplasts in epidermal cells); and it is known that a certain stomatal aperture corresponds to steady-state conditions of turgor that represent an equilibrium between water uptake by the epidermis and the transpiration of the cuticle epidermis (Schulze 1986). Trees are aerodynamically very rough surfaces with low boundary layer resistances when compared to herbaceous vegetation, so that stomatal responses and transpiration are expected to be highly coupled to the evaporative conditions of the air and regulate trees responses (Schulze 1986). The opening and closing of stomata are regulated through changes in turgor of guard cells relative to epidermal cells, together with metabolic energy (e.g. from mesophyll photosynthesis) and changes in membrane permeability; however, the detailed mechanisms of stomatal control under drought are difficult to rationalise because stomata continuously react with the surrounding environment, such as light intensity, CO_2 concentration, or leaf water status (Chaves et al. 2003).

Water relations in guard cells function in the same way as in other cells: as a result of decreasing osmotic potential and water potential, water moves into the guard cells and pressure increases; this change in cell volume coupled with the differential thickening of guard cell walls lead to opening or closing of the stomatal pore (guard cells walls are very elastic and can reversibly increase their volume by 40 to 100% depending on the species) (Taiz and Zeiger 2002). As the reduced water content causes the concentration of existing solutes to be factored out, the turgor pressure must be assured by increasing the osmotic potential, which can be achieved by osmotic adjustment – additional solutes are actively accumulated inside the cell in response to low water potential (Verslues et al. 2006). Besides their primary role of turgor maintenance, these compatible solutes may also be involved in protecting cell membranes, stabilising proteins, and scavenging reactive oxygen species – ROS (Liu et al. 2011).

The water availability in the natural environment and the dynamic plant-environment relations have led plants to evolve two different strategies to manage the continuum of stomatal regulation of water status: isohydric and anisohydric regulation, which distinction is based on different sensitivity of their guard cells to a critical leaf water potential threshold (McDowell et al. 2008; Sade et al. 2012). Isohydric plants reduce stomatal conductance in order to limit transpiration and maintain constant midday leaf water potential when atmospheric and soil water conditions decrease. By contrast, anisohydric plants keep their stomata open even when soil water potential declines with drought, allowing midday water potential to decline as well (McDowell et al. 2008; Sade et al. 2012). This regulation is also known as risk-taking behaviour (Sade et al. 2012), and owes this nomination to the fact that anisohydric plants are able to maintain higher stomatal conductance and higher photosynthetic rates for longer periods than isohydric plants under optimal conditions and mild to moderate drought conditions, being more productive (McDowell et al. 2008; Sade et al. 2012). Determination of survival and mortality during drought may be critically

influenced by the different isohydric – anisohydric regulation of the water status (McDowell et al. 2008).

Signalling pathways: ROS, hormones and gene expression

Under stress conditions, the available energy and nutrients have to be redistributed to prioritize defence responses rather than growth processes (Prasch and Sonnewald 2015). This is drawn by a fine tuning of nutrient and energy allocation through complex signalling networks that integrate incoming developmental and environmental signals (Prasch and Sonnewald 2015) and activate the expression of appropriate responses (Bartels and Sunkar 2005). Although knowledge about stress-signal perception and transduction are of fundamental importance in stress-biology research and crucial for breeding and engineering of more tolerant trees, the precise mechanisms underlying stress perception are not yet fully understood (Harfouche et al. 2014). Despite that, the knowledge acquired from less complex plants may help us foretell some responses.

Like in other stresses, it is believed that drought is perceived at cell membrane through changes in membrane integrity and modulation of lipid synthesis, which function as primary sensing and then trigger an intracellular-signalling cascade (Kader and Lindberg 2010; Golldack et al. 2011). These cascades are regulated by protein-protein interactions and signalling molecules (such as Ca^{2+}, ROS, sugars, hormones, etc.) (Bartels and Sunkar 2005).

Calcium is an essential nutrient for plant growth and development, playing a structural role in producing plant tissues, enabling them to grow better and increasing the tissues' resistance under various stress conditions, including both biotic and abiotic stresses (Kader and Lindberg 2010). Besides, calcium works as a second messenger for a number of extracellular signals in plants, with several lines of evidence indicating it acts as a second messenger for (at least part of) ABA action in guard cells (Luan 2002). The work of Pei et al. (2000) revealed that hydrogen peroxide (H_2O_2) activates Ca^{2+}-permeable channels in the plasma membrane of *Arabidopsis* guard cells, mediating both influx of Ca^{2+} in protoplasts and increases of cytosolic Ca^{2+} in intact guard cells. This response was disrupted in the recessive ABA-insensitive mutant gca2, indicating that ABA-induced H_2O_2 production and the H_2O_2-activated Ca^{2+} channels are important mechanisms for ABA-induced stomatal closure.

In a different study, *myo*-inositol hexakisphosphate (InsP6), which levels in guard cells are known to be increased in response to ABA, inactivating the plasma membrane inward K^+ conductance in a cytosolic calcium-dependent manner, was also found to mobilise an endomembrane store of calcium in guard cells (Lemtiri-Chlieh et al. 2003). InsP6 released from caged precursor mobilises Ca^{2+} in patch-clamped guard cell protoplasts and activates vacuolar ion

channels, but has no effect on the Ca^{2+} currents in the plasma membrane activated by ABA (Lemtiri-Chlieh et al. 2003).

In addition to osmotic stress-induced signalling involving concerted changes of Ca^{2+} influx, flux changes across the plasma membrane of other key ions (H^+, K^+, Cl^-), cytoplasmic pH, and apoplastic production of ROS are also triggered after osmotic stress alters H^+-ATPase and Ca^{2+}-ATPase activities in the plasma membrane (Beffagna et al. 2005). In particular, the rise in cytosolic free Ca^{2+} concentration is considered to be an absolute requirement for evolution of the oxidative burst, as the activation of a Ca^{2+}-dependent redox system that oxidises NADPH *in vivo* occurring at the plasma membrane level was identified as the source of the oxidative burst, and the mechanism responsible for ROS production (Beffagna et al. 2005).

Reactive oxygen species (ROS) – singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO\cdot$) – result from the partial reduction of atmospheric O_2. ROS, especially singlet oxygen and the hydroxyl radical, are extremely reactive oxidising multiple cellular components, such as proteins and lipids, DNA and RNA, and will ultimately cause cell death if the cellular components are oxidised unrestrictedly (Cruz de Carvalho 2008). Part of a defence response to biotic stress is characterised by an oxidative burst that frequently triggers programmed cell death; however, the role of ROS production and control during drought stress is still not resolved (Cruz de Carvalho 2008). Multiple conditions render H_2O_2 the most likely ROS candidate to act as a secondary messenger in a stress-response signal transduction pathway: it is the most stable ROS with the ability to easily diffuse from one cellular compartment to another; it can be readily metabolised by an efficient cellular antioxidant system; it is produced at high rates under stress but rapidly reduced by an efficient antioxidant system (Cruz de Carvalho 2008). Moreover, the low toxicity of this ROS to the plant cell and its affinity to protein thiol groups suggests its possible role as a modulator of protein conformation and/or biochemical activities that enable ROS sensing (Cruz de Carvalho 2008).

Besides their major role as building units and energy providers, several sugars can also function as ROS scavengers. There is an observed correlation between sugars and oxidative stress: yet it is not a forthright positive correlation, since ROS production was shown either to be enhanced or decreased by high sugar levels, and both high and low sugar levels can evoke ROS accumulation (Keunen et al. 2013). Moreover, carbohydrates are synthesised under stress conditions, acting as osmoprotectants that stabilise cellular membranes and maintain turgor or as storage carbohydrates (Keunen et al. 2013).

Carbohydrates additionally function as tight controllers of transcriptional, posttranscriptional and posttranslational processes acting as signalling molecules *in planta* (Keunen et al. 2013). Carbohydrate-regulated genes represent a powerful mechanism for plants adjusting to

environmental changes (Koch 1996). Changes in carbohydrate allocation influence gene expression that ultimately contribute to adaptive changes in form through processes affecting import/export balance and, although slower, these gene-level responses may complement and amplify the influence of more immediate metabolic adjustments (Koch 1996). A large but specific set of genes is positively regulated by sugars: the majority of identified genes that are induced by abundant carbon resources favour genes for carbon storage, utilization, and import; carbohydrate depletion enhances expression of genes for photosynthesis, reserve mobilization, and export processes; and other important classes include defence genes, secondary product pathways, and storage proteins.

The tight spatial-temporal control of redox signalling messengers is integrated with the action of plant hormones such as abscisic acid (ABA), ethylene (ET), salicylic acid (SA), and jasmonates (JA) that generate a cascade of signal transduction networks, which ultimately determine major physiological defence events (Fujita et al. 2006; Aimar et al. 2011; Bartoli et al. 2013). The mechanisms by which hormones regulate plant development and stress tolerance comprise the hormone-dependent activation of ROS production, carried out though NADPH oxidases that are encoded in plant genomes by RESPIRATORY BURST OXIDASE HOMOLOG (RBOH) genes (Xia et al. 2015). Plant hormones occupy a prominent position in the ability of plants to adapt to changing environments, playing essential regulatory roles in plant physiology by mediating growth, development, nutrient allocation, and source/sink transitions (Aimar et al. 2011; Peleg and Blumwald 2011).

Abscisic acid is one of the most studied phytohormone in the response of plants to abiotic stress, particularly water stress, together with ABA signalling and ABA-responsive genes (Peleg and Blumwald 2011). ABA is synthesised from zeaxanthin, a C40 carotenoid (Aimar et al. 2011), one of the fastest plant responses to abiotic stress, inducing stomatal closure and triggering ABA-inducible gene expression (Peleg and Blumwald 2011) – extensive literature has identified many genes associated with ABA biosynthesis and encoding ABA receptors and downstream signals in *Arabidopsis thaliana* (reviewed by Cutler et al. (2010)).

Ethylene has a role regulating plant growth, senescence, and defence stress responses (Xia et al. 2015). ET production occurs from the metabolic precursors S-adenosylmethionine (*S*-AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC, Wang et al. (2002)) and is controlled by autocatalytic and feedback mechanisms (Xia et al. 2015). Following drought stress ET has been implicated in both stomatal opening and closure: either inhibiting ABA-induced stomatal closure, or inducing stomatal closure by promoting NADPH oxidase-mediated ROS production in stomatal guard cells (Kazan 2015).

Salicylic acid is an endogenous regulator of plant growth involved in a broad range of physiological responses (Aimar et al. 2011). There are three known routes for SA biosynthesis in

plants: from phenylalanine via cinnamic acid; from 2-hydroxylation of cinnamic acid to o-coumaric which is then decarboxylated to salicylic acid; and from chloroplastic chorismate via isochorismate (Aimar et al. 2011). SA has a role in the perception of pathogens, either through the recognition of pathogen-associated molecular patterns (PAMPs) known as PAMP-triggered immunity (PTI) or through the specific recognition of pathogen effectors (effector triggered immunity – ETI), inducing the expression of pathogen resistance genes that involve SA synthesis, the production of ROS and nitric oxide, and the activation of MAPK cascades, as well as an increase in intracellular Ca^{2+} levels (Xia et al. 2015). Application of exogenous SA improves the plant performance under water deficit (Aimar et al. 2011), also confirmed in *Eucalyptus globulus* (Jesus et al. 2015).

Jasmonic acid, its cyclic precursors and derivatives, jointly known as jasmonates (JA), comprise a family of bioactive oxylipins that regulate a variety of processes related to plant development and survival (Aimar et al. 2011). JA are produced from α-linolenic acid (α-LeA) and hexadecatrienoic acid released from plastidial galactolipids by phospholipases (Aimar et al. 2011). They are important regulators of wound stress responses in higher plants: mechanical stress is sensed by stretch-activated Ca^{2+} channels and a variety of receptors, which initiate downstream signalling through Rho-GTPases, NADPH oxidases, MAPK cascades, and JA biosynthesis (Xia et al. 2015). JA have also been implicated in promoting stomatal closure and modulating root hydraulic conductivity with limited moisture, which can contribute to water uptake from soil (Kazan 2015).

Determining how plants sense water deficit is complex: signals may be different for different developmental state, the exact locations of signal production and sensing and the processes transducing the signal into a cell or tissue response are difficult to identify, the nature of the primary mediators of cellular processes – water status, turgor, bound water, hormones, alteration in cell membranes and others – are still under discussion, a considerable overlap occurs between abiotic stress signalling pathways, and countless genes might be involved, creating a highly intricate network that reprograms plant development and dictates plant fate (Chaves et al. 2003).

Fatal events: hydraulic failure and carbon starvation

After decades of research, the physiological mechanisms by which trees succumb to drought remain a matter of discussion (Sevanto et al. 2014). Currently, there are two leading hypotheses of plant mortality mechanisms based on our understanding of plant water relations: hydraulic failure, and carbon starvation (Sevanto et al. 2014), and one hypothesis concerning biotic agent demographics (McDowell et al. 2008).

The biotic agent demographics hypothesis disregards the direct impact of drought on the tree but rather suggests that drought may drive changes in demographics of mortality agents (e.g. insects and pathogens) that may amplify or be amplified by plant physiological stress and subsequently drive forest mortality (McDowell et al. 2008).

The hydraulic failure hypothesis suggests that reduced water availability alters and threatens the liquid phase continuum integrity from soil to leaves, thereby resulting in excessive xylem water tensions, conductivity loss of the xylem and progressive cavitation (Anderegg et al. 2012; Barigah et al. 2013). This hypothesis is particularly likely when drought is so severe that plants run out of water before they deplete the carbohydrate supplies (more probable to occur in anisohydric plants, McDowell et al. (2008)). In their study, Barigah et al. (2013) suggest that xylem hydraulic failure is a causal factor of beech and poplar mortality under extreme drought conditions.

The carbon starvation hypothesis posits that stomatal closure occurring to prevent desiccation (e.g. by isohydric plants) leads to a negative carbon balance that depletes carbon storage and causes carbohydrate starvation (McDowell et al. 2008; Anderegg et al. 2012). The carbon starvation hypothesis is empirically supported by the reported link between decreased carbon availability, reduced growth and mortality (reviewed by McDowell et al. (2008)).

Despite the theoretical and conceptually very logical definitions, it is very difficult to acknowledge the actual cause of tree mortality, much discussion remains (McDowell et al. 2008; McDowell and Sevanto 2010; Sala et al. 2010), and the main agreed limitation is the lack of evidence from plants that actually die rather than plants that are stressed but survive. Anderegg et al. (2012) performed a direct and *in situ* study of the mechanisms underlying climate-induced *Populus tremuloides* forest mortality in western North America. Although focussing on a single forest type dominated by one species, which might not directly be extended to other forest die-backs, the combination of observational and experimental tests, especially direct measurements of carbohydrates and hydraulic impairment, were very convenient. They found substantial evidence of hydraulic failure of roots and branches, but suggest that further research should explore interconnections and interactions between carbon stress and hydraulic stress, since greater hydraulic vulnerability might result from carbon stress-induced declines in xylem growth (Anderegg et al. 2012). This was later supported by Sevanto et al. (2014) who hypothesised that *Pinus edulis* trees can die of co-occurring hydraulic failure and carbon starvation, and propose that loss of adequate tissue carbohydrate content required for osmoregulation may be associated with hydraulic failure, further impacting hydraulic integrity.

Drought in *Eucalyptus*

It dates back to the fall of 1913 when eucalyptus trees, especially *Eucalyptus globulus*, growing in the Arboretum of the Stanford University perished without an apparent reason (McMurphy and Peirce 1920). This fact aroused curiosity among scientists and the general public. After assuring the trees were free from fungal or bacterial infection of any sort and uncovering the more superficial part of the root system with pick and shovel, the scientists found out that the large superficial roots were seriously damaged by the plows used to clear the ground under the trees of weeds (McMurphy and Peirce 1920). This translated into a severe limitation or even impossibility of these tall and heavily foliated trees to absorb water from the upper layers of the soil, which led the scientists to conclude that the reason for the loss of the eucalyptus trees of the Arboretum was lack of water due to an impaired root system (McMurphy and Peirce 1920). Since then, many reports have sought to explain how insufficient water affects this genus in particular.

Water relations and yield indices

To the best of my knowledge, the first works reporting on drought stress responses of *Eucalyptus* date back to 1974 (Ladiges 1974a, b; Ladiges and Ashton 1974). A correlation between the mean annual rainfall, soil type of habitats and variation in naturally occurring stands of *E. viminalis* was described to occur in central Victoria, together with genetic variation in seedling growth rates related to adaptations to different soil nutrient and moisture levels (Ladiges 1974b; Ladiges and Ashton 1974). For occurring over a wide range of rainfall regimes, it was suggested that populations growing in dry habitats would be more tolerant of drought periods than populations in moist habitats (Ladiges and Ashton 1974).

The same author suggested that greater tolerance to drought would be related to physiological resistance of the protoplasm to desiccation (Ladiges 1974b), concluding later that a drought resistant *Eucalyptus viminalis* population shows a capacity to tolerate rather than avoid drought stress with a tendency to rapidly reach relatively low water potential correlating with a higher level of transpiration, thus maintaining a potential gradient between leaf and soil water without the leaf reaching critically low water contents (Ladiges 1975). Ladiges (1975) also reported thicker cell walls in the leaves in the resistant population, although we cannot tell between the environmental or genetic factors determining this trait.

Many studies have also reported on the ability of several *Eucalyptus* species to deal with drought conditions, assessing morphological, physiological and biochemical parameters, mostly relating water status and growth. In table 1, a number of these studies are briefly described.

Table 1 – Brief description of the main published studies that investigate drought responses in the *Eucalyptus* genus. Year of publication, target species, tested parameters (highlighting water relations and growth) and respective bibliography are indicated.

Year	Species	Tested parameters	Reference
1974	*Eucalyptus viminalis*	Water relations (stem water potential and leaf relative turgidity); Growth (total leaf area, total dry weight and root to shoot ratio); Mean transpiration rates and plant death.	Ladiges (1974b)
1975	*E. viminalis*	Water relations (shoot water potential, osmotic potential at full turgor and at the turgor loss point, and bulk modulus of elasticity).	Ladiges (1975)
1978	*E. robusta* *E. saligna*	Growth (plant height, total dry weight, and leaf area); Minimum stomatal resistance; Water use per unit of leaf area; Hydraulic conductivity of the intact root; Specific hydraulic conductivity of stem.	Clemens and Jones (1978)
1985	*E. blakelyi* *E. melliodora*	Water relations (predawn xylem water potential); Growth (assessment of crown size, density, epicormic growth, and dead branches).	Landsberg (1985)
1985	*E. pauciflora*	Water relations (leaf water potential, water saturation deficit, osmotic potential, and water potential at the turgor loss point); Leaf diffusive conductance; Stomatal frequency; Leaf nitrogen per unit leaf area.	Körner and Cochrane (1985)
1990	*E. globulus*	Edaphic and climatic characterisation (precipitation, runoff and soil water storage); Canopy assessment (height, crown volume and stemflow).	de Almeida and Riekerk (1990)
1992	*E. camaldulensis*	Water relations (leaf water potential, osmotic potential at full turgor, relative leaf water loss at turgor loss, and leaf water potential at turgor loss); Leaf specific weight and chlorophylls; Fluorescence measurements (maximal photosystem II (PSII) photochemical efficiency (F_v/F_m ratio), actual PSII photochemical efficiency, and photochemical efficiency of open PSII reaction centres).	Dreyer et al. (1992)
1994	*E. globulus* *E. nitens*	Water relations (predawn water potential); Growth (height and diameter, leaf area and leaf area index).	White et al. (1994)
1994	*E. camaldulensis*	Growth (leaf area and dry weight of roots, stems and leaves); Stomatal conductance.	Gibson and Bachelard (1994)
1995	*E. grandis* × *E. camaldulensis* *E. grandis* × *E.*	Water relations (xylem water potential); Transpiration; Ethylene production;	Michelozzi et al. (1995)

	robusta	Chlorophyll concentration.	
1996	*E. globulus*	Water relations (predawn and midday water potential, osmotic potential, water use rate); Growth (height and root-collar diameter); Transpiration and stomatal conductance; Plant death.	Sasse and Sands (1996)
1996	*E. globulus* ssp. *globulus* *E. nitens*	Water relations (predawn water potential, water stress integral, osmotic potential at full turgor and at turgor loss point, relative water content at turgor loss point, relative apoplastic water content, and bulk elastic modulus at full turgor)	White et al. (1996)
1997	*E. microtheca*	Water relations (leaf water potential, osmotic potential at full turgor, relative water content, bulk modulus of elasticity); Growth (seedling height, total leaf area, and stem diameter); Specific leaf density.	Tuomela (1997)
1998	*E. globulus*	Water relations (predawn and midday leaf water potential, relative water content, leaf osmotic potential, turgor potential, and osmotic potential at full turgor); Growth (shoot height, number of branches and leaf pairs, leaf area, root, stem, branch and leaf biomass, and leaf expansion rate).	Osório et al. (1998)
1998	*E. globulus* *E. nitens*	Water relations (predawn water potential); Growth (tree leaf area, sapwood area, and specific leaf area).	White et al. (1998)
2000	*E. grandis*	Water relations (predawn and midday leaf water potential) Leaf gas exchange (photosynthetic rate and transpiration rate), stomatal conductance, internal CO_2 molar fraction, and intrinsic water use efficiency.	Mielke et al. (2000)
2000	*E. tetrodonta* *E. miniata*	Water relations (predawn leaf water potential) Growth (sapling height and diameter, number of leaves, shoots and branches, branch length, and leaf area); Hydraulic conductance.	Prior and Eamus (2000)
2001	*E. globulus*	Growth (diameter at breast height – 1.30 m) and survival; Carbon isotope discrimination.	Pita et al. (2001)
2002	*E. camaldulensis*	Water relations (relative water content, osmotic potential at full turgor and at turgor loss point, maximum bulk modulus of elasticity, pressure potential at full turgor, and turgid mass to dry mass ratio); Growth (leaf area growth rate); Osmotic adjustment and stomatal conductance.	Lemcoff et al. (2002)
2003	*E. globulus*	Water relations (relative water content at turgor loss point, osmotic potential at full turgor and at turgor	Guarnaschelli et al. (2003)

		loss point, maximum bulk modulus of elasticity, apoplasmic water fraction, and turgid mass to dry mass ratio) Growth (seedling height, root collar diameter, leaf area, root mass, and total dry mass); Osmotic adjustment and stomatal conductance.	
2003	*E. microtheca*	Water relations (leaf water potential); Growth (basal diameter, shoot height, biomass, leaf area, root to shoot ratio, foliage area to stem cross-sectional area ratio, and specific leaf area density); Stomatal density and guard cell length; Carbon isotope composition; Abscisic acid concentration.	Li and Wang (2003)
2004	*E. globulus*	Water relations (predawn and midday water potential); Growth (total biomass, leaf area, number of branches, root length, dry mass of stem, branches, leaves and root, leaf area ratio, specific leaf area, and leaf to root area ratio); Transpiration and stomatal conductance; Root water flux, root hydraulic conductance, and leaf-specific hydraulic conductance.	Costa e Silva et al. (2004)
2005	*E. grandis*	Water relations (relative water content, water potential, osmotic potential at turgor loss point and at saturation, and relative water content at turgor loss point); Growth (Total dry mass increase, and root to total plant mass ratio); Stomatal conductance, transpiration and water use efficiency.	Graciano et al. (2005)
2006	*E. globulus*	Water relations (predawn and midday water potential, osmotic potential); Growth (total biomass, leaf area, root length, and leaf to root area ratio); Soluble and insoluble sugars; Leaf pigments (violaxanthin + antheraxanthin + zeaxanthin, lutein, β-carotene, and total chlorophyll); Proline and antioxidant enzymes (glutathione reductase, ascorbate peroxidase, and catalase).	Shvaleva et al. (2006)
2006	*E. globulus* ssp. *bicostata*	Water relations (predawn and midday relative water content, leaf water potential, osmotic potential at full turgor and at turgor loss point, maximum bulk modulus of elasticity, relative water content at turgor loss point, apoplasmic water fraction, and maximum turgor pressure); Growth (seedling height, root collar diameter, leaf area, specific leaf area, total biomass, and shoot to root biomass ratio); Leaf stomatal conductance.	Guarnaschelli et al. (2006)
2007	*E. marginata*	Water relations (predawn and midday leaf water	Warren et al. (2007)

		potential, osmotic pressure at full turgor and at zero turgor, relative water content at zero turgor, and turgid weight to dry weight ratio); Leaf gas exchange (photosynthetic and transpiration rates), and stomatal conductance; Carbon isotope composition; Leaf solutes.	
2007	*E. microtheca*	Growth (dry weight of leaves, stem and roots, leaf area, specific leaf area, and root to shoot ratio); Transpiration, photosynthetic rate, stomatal conductance, and water use efficiency; Chlorophyll fluorescence (F_v/F_m ratio, photochemical and non-photochemical quenching); Xylem permeability and leaf specific conductivity; Nitrogen seedling content.	Susiluoto and Berninger (2007)
2008	*E. astringens*	Water relations (predawn leaf water potential) Growth (biomass of leaves, branches, stems, and roots); Photosynthetic rate and stomatal conductance; Total osmolyte, carbohydrates, polyols (quercitol) and starch concentration.	Arndt et al. (2008)
2009	*E. globulus*	Water relations (predawn leaf water potential and water stress integral); Growth (leaf area index, annual volume increment, and growth efficiency).	White et al. (2009)
2011	*E. arenacea* *E. delegatensis* *E. nitens*, *E. regnans* *E. socialis*	Water relations (predawn leaf water potential); Growth (height); Leaf gas exchange (photosynthetic rate), chloroplast CO_2 concentration, and stomatal conductance; Epidermal conductance and transpiration; Carbon isotope discrimination; Leaf metabolites.	Warren et al. (2011b)
2011	*E. saligna*	Growth (leaf mass to leaf area ratio, fresh mass per unit area, and dry matter content); Foliar concentration of soluble sugars, starch, total non-structural carbohydrates, nitrogen and phosphorous; Leaf gas exchange (photosynthetic rate and leaf respiration in darkness and light) and stomatal conductance.	Ayub et al. (2011)
2011	*E. globulus*	Water relations (shoot and root water potential, and relative water content) and xylem sap pH; Leaf gas exchange (photosynthetic and transpiration rates), stomatal conductance, internal CO_2 concentration, intrinsic water use efficiency, and carboxylation efficiency; Hormonal content (abscisic acid and cytokinins).	Granda et al. (2011)
2011	*E. globulus*	Water relations (shoot and root water potential); Abscisic acid concentration; Total soluble proteins of the root.	Bedon et al. (2011)

2012	*E. globulus* *E. globulus* × *nitens*	Water relations (predawn xylem water potential) and plant survival; Leaf gas exchange (photosynthetic and transpiration rates), stomatal conductance, and intrinsic water use efficiency; Chlorophyll fluorescence (F_v /F_m ratio, and non-photochemical quenching).	Navarrete-Campos et al. (2012)
2014	*E. dumosa* *E. pauciflora*	Water relations (leaf predawn water potential, critical predawn water potential for the recovery of epidermal conductance to water vapour); Growth (seedling height, leaf mass per area); Leaf gas exchange (light-saturated photosynthetic rate), mesophyll conductance to CO_2, internal CO_2 concentration, intrinsic water use efficiency and stomatal conductance to water vapour; Chlorophyll fluorescence (F_v/F_m ratio); Estimation of mesophyll conductance.	Cano et al. (2014)
2014	*E. globulus*	Water relations (predawn and midday leaf water potential, relative water content, and osmotic potential); Growth (stem length and diameter, number of leaves, leaf area, aerial biomass, specific leaf area) and plant survival; Leaf gas exchange (photosynthetic and transpiration rates), stomatal conductance, internal CO_2 concentration, and intrinsic water use efficiency; Calculated hydraulic conductance; Leaf chlorophyll content; Abscisic acid concentration.	Granda et al. (2014)

Going deeper: implication of gene, genome and omics

As shown, much scientific effort has been put together over time to understand how *Eucalyptus* incorporates lack of water by adjusting its physiology, biochemistry and morphology. Yet, a lot remains to be discovered and new tools keep emerging. Since the development of the Polymerase Chain Reaction (PCR, Mullis et al. (1986)), enormous advances in both basic and applied aspects of molecular biology have been achieved, and research focussing drought in *Eucalyptus* was not an exception. For instance, the sequencing of the genome of *Eucalyptus grandis* (Myburg et al. 2014) was a major breakthrough; representing the first reference genome for the eudicot order Myrtales, it revealed the unique biology and evolutionary history of *Eucalyptus*, and provided a powerful tool to accelerate comparative biology, breeding and biotechnology.

In 2011, Villar et al. (2011), following pyrosequencing of RNA extracted from shoot apices of two *Eucalyptus* (*E. alba* and *E. urophylla* × *E. grandis*) irrigated and non-irrigated during the dry season, gathered 14 460 contigs, identifying 1 280 with differential expression between the two genotypes, 155 contigs that were differentially expressed between treatments, and 274 contigs with

significant genotype × treatment interaction. These authors reported that genes related to primary metabolism and cell organisation were over-expressed, and genes related to photosystem, transport and secondary metabolism were down regulated in the most productive genotype (Villar et al. 2011). Other studies have investigated how natural selection shaped genetic susceptibility of *E. globulus* (Dutkowski and Potts 2012) and *E. calmadulensis* (Thumma et al. 2012) to drought.

More recently, this massive gene expression analysis has been complemented with the development of omics technologies (genomics, transcriptomics, proteomics, metabolomics, …). Warren et al. (2011a) used gas chromatography coupled with mass spectrometry and capillary electrophoresis to perform a metabolomic profiling of leaf metabolites of *Eucalyptus pauciflora* and *Eucalyptus dumosa* after a long and severe water stress, and found that even species that are closely related respond differently to water stress and re-watering. Bedon et al. (2012) investigated the protein expression patterns of two *Eucalyptus* genotypes (*E. alba* and *E. urophylla* × *E. grandis*) and reported that several proteins related to molecular plasticity were identified, revealing specific adaptive mechanisms to water deficit (cell wall modification, cell detoxification and osmoregulation). On the other hand, a study that combined proteomics with plant morphology and physiology revealed that drought-tolerant *Eucalyptus globulus* showed partial leaf abscission, an outperforming root system, higher levels of stress-related hormones, accumulation of proteins related to abiotic-stress tolerance, and allocation of resources to improve survival despite the energetic cost (Valdés et al. 2013).

The reviewed examples demonstrate that taking full advantage of comprehensive analyses using post-genomic techniques such as transcriptomics, proteomics, and metabolomics has indeed favoured the scanning of specific variations under different environmental conditions and increased our understanding of stress adaptation and tolerance and the complex regulatory networks associated (Urano et al. 2010). Studies that investigate cellular processes relying solely on the identification of genes are largely defective since the functions of the genes at the biochemical level often remain unidentified (Fridman and Pichersky 2005). Besides, the metabolic control goes beyond the mRNA levels and altered levels of metabolites, proteins and post-translational protein modifications similarly influence the manifesting of the phenotypic plasticity (Weckwerth 2008). Accordingly, the application of post-genomic techniques are required for cataloguing all of the components of the cell and elucidating all the causal relationships among them — genes, proteins, and metabolites (Fridman and Pichersky 2005; Weckwerth 2008).

Technology has provided us with almost infinite information to improve our understanding of plant stress response that we got to another issue: “the phenotypic gap”. Verslues et al. (2006) points out how molecular and genetic data are increasing at a much faster pace than the identification of useful phenotypes and applications, so that we have to start considering the best

methods to integrate information and understand the phenotypes associated with stress resistance. Systems biology appears, thus, as an integrative methodology that gathers genes, metabolites, proteins, regulatory elements, fluxes and others and looks for interactions among the multiple biological components using models and/or networks (Hamanishi and Campbell 2011; Rodziewicz et al. 2014).

Thesis framework and aims

Currently, *Eucalyptus* represents the main forest occupation of the Portuguese continental area (812 thousand ha), dominated by the species *Eucalyptus globulus* (ICNF 2013). The Portuguese Forestry Sector has a very significant role in the external trade balance and economy of the country, accounting for 10% of the country's total exports, which have exceed imports by € 2 billion since 2012 (€ 2.5 billion in 2015, ICNF). Data from 2015 confirms the leading role of exports in the forest industry, revealing that pulp, paper, paperboard and their products represent 50% of the value of exports and 54% of imports; wood products, charcoal, furniture and their products account for 27% of exports and 35% of imports; cork products account for 18% of exports and 6% of imports of forest-based products, with a trade surplus of € 753.9 million (results of the Portuguese National Statistical Institute reported by ICNF).

The Portuguese National Strategy for Forests (Ordinance No. 6-B/2015) therefore seeks to maintain and increase the economic importance of the forestry industry in terms of trade balance by prioritizing minimization of the risks to forests by biotic and abiotic agents and the increase of the productivity of stands (ICNF). One of these risks is climate change driven water limitation. Located in the western edge of the Iberian Peninsula and in the transitional region between the subtropical anticyclone and the subpolar depression zones, Mainland Portugal has a mild Mediterranean climate with an Atlantic influence, and displays a very large precipitation gradient, from the northwest region that is one of the wettest spots in Europe with mean annual accumulated precipitation over 3000 mm, to the southeast regions, with less than 400 mm per year (Miranda et al. 2002; Soares et al. 2015).

Despite the efforts to decipher drought impact in *Eucalyptus*, recovery from stress, an important aspect in terms of growth and survival, is still understudied and only a few studies have been conducted (Kirschbaum 1988; Warren et al. 2011a; Cano et al. 2014; McKiernan et al. 2016). Also, several studies have revealed that different genotypes present different response profiles, water use strategies and drought tolerance (Granda et al. 2014). Assessing water deficit and restoration in different genotypes may therefore be effective in order to identify relevant pathways

and stress indicators, which will be useful in breeding programs and early selection, and contribute to maintain productivity under environmental change.

The aim of this doctoral thesis was to unravel drought tolerance in *Eucalyptus globulus* by investigating and interconnecting information on the mediating processes of water deficit and rehydration, from gene and molecular regulation to physiological responses and plant performance, using different genotypes and stress trials. This thesis aimed to answer the following questions:

- How do different genotypes adjust their physiology, biochemistry, proteome and metabolome to cope with changes in water availability?
- Are there potential molecular indicators linked to enhanced drought tolerance?
- Do stress imposition and/or relief modulate ROS, antioxidant defence and/or DNA methylation?
- Can the knowledge obtained using plants subject to controlled experiments be extended to field-grown plants?
- Can the effects of combined stress be derived from the sum of the isolated stresses?

The thesis disclosed herein is presented in a series of research papers (chapters 2, 3, 4 and 5), preceded by a general introduction (chapter 1) and closed with concluding remarks (chapter 6). Chapter 2 describes a greenhouse trial and a slowly imposed water deficit, and is divided into three subchapters: 2.1 – Water stress and recovery in the performance of two *Eucalyptus globulus* clones: physiological and biochemical profiles; 2.2 – Hormonal dynamics during recovery from drought in two *Eucalyptus globulus* genotypes: From root to leaf; and 2.3 – Integrated proteomics and metabolomics to unlock global and clonal responses of *Eucalyptus globulus* recovery from water deficit. Chapter 3 regards a climate chamber trial and a sudden water shortage, and is divided in two subchapters: 3.1 – Expression of putative stress tolerance indicators in *Eucalyptus* after water shortage and rehydration; and 3.2 – Depicting how *Eucalyptus globulus* survives drought: involvement of redox and DNA methylation events. Chapter 4 reports a field trial: Gene expression of stress tolerance indicators – can we extrapolate from the chambers to the field? Chapter 5 describes a climate chamber trial that tests the isolated and combined effect of drought and heat: Drought and heat impact on *Eucalyptus* – the combined environmental stress goes further the sum of the isolated factors.

"Não é d'isso que se trata; não se pensa em trocar pelo Eucalypto essas madeiras que formam uma aristocracia; apenas se procura auxiliar e engrandecer as plebes florestaes, associando-lhes plantas novas da sua igualha" [2] (Lima 1920)

[2] That's not what it's all about; one does not think of replacing those woods that form an aristocracy with the *Eucalyptus*; only seeks to aid and enhance the forest plebs, associating them with new plants of their own.

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Chapter 2

slowly imposed water deficit

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2.1. Water stress and recovery in the performance of two *Eucalyptus globulus* clones: physiological and biochemical profiles

Summary

Eucalyptus plantations are among the most productive forest stands in Portugal and Spain, being mostly used for pulp production and, more recently, as an energy crop. However, the region's Mediterranean climate, with characteristic severe summer drought, negatively affects eucalypt growth and increases mortality. Although the physiological response to water shortage is well characterised for this species, evidence about the plants' recovery ability remains scarce. In order to assess the physiological and biochemical response of *Eucalyptus globulus* during the recovery phase, two genotypes (AL-18 and AL-10) were submitted to a 3-week water stress period at two different intensities (18% and 25% of field capacity), followed by 1 week of rewatering. Recovery was assessed 1 day and 1 week after rehydration. Drought reduced height, biomass, water potential, NPQ and gas exchange in both genotypes. Contrarily, the levels of pigments, chlorophyll fluorescence parameters (F_v/F_m and Φ_{PSII}), MDA and ABA increased. During recovery, the physiological and biochemical profile of stressed plants showed a similar trend: they experienced reversion of altered traits (MDA, ABA, E, g_s, pigments), while other parameters did not recover (Φ_{PSII}, NPQ). Furthermore, an overcompensation of CO_2 assimilation was achieved 1 week after rehydration, which was accompanied by greater growth and re-establishment of oxidative balance. Both genotypes were tolerant to the tested conditions, although clonal differences were found. AL-10 was more productive and showed a more rapid and dynamic response to rehydration (namely in carotenoid content, Φ_{PSII} and NPQ) compared to clone AL-18.


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Water stress and recovery in the performance of two *Eucalyptus globulus* clones: physiological and biochemical profiles

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Introduction

Despite representing only 7% of the global forested area, planted forests play a key role in supplying worldwide forest based services (Villar et al. 2011). Trees from the genus *Eucalyptus* are the most widely introduced exotic species cultivated in the Mediterranean area (Navarrete-Campos et al. 2012); approximately 700,000 ha and 500 000 ha of Eucalyptus globulus have been estimated in Portugal (Bedon et al. 2011) and Spain, respectively, and more residual plantations of other eucalypt species can be found in France, Italy and Greece (Luger 2003). Although the Mediterranean climate is not optimal for this particular species, plantations are established, nowadays, mainly with improved seedlings and selected clones issued from genetic improvement programs and selected for their fibre properties and growth behaviour (Navarrete-Campos et al. 2012). However, as stated by several authors, soil and atmospheric water availability has been shown to be the major factor limiting productivity in *Eucalyptus* plantations (Shvaleva et al. 2006; Villar et al. 2011). Ecophysiological studies have reported that different genotypes differ in their capacity to cope with drought (Villar et al. 2011). This fact has led to the current practice of considering plant performance within varied environmental conditions during the genotype selection process (Navarrete-Campos et al. 2012).

Water stress causes diverse physiological, biochemical and molecular responses in plants, which depend on the rate and the intensity of the stress. This will lead first to an acclimation state and later, as the water stress intensifies, to functional damage and loss of plant parts (Chaves et al. 2003; Bedon et al. 2011). During acclimation to water stress, osmotic adjustment and increment of cell wall elasticity may occur, allowing the plant to maintain cell turgor that would otherwise be lost as a result of the water shortage (Coopman et al. 2008; Bedon et al. 2011). Leaf growth inhibition and stomatal closure take place and limit gas exchange, resulting in reduced transpiration and photosynthesis (Bedon et al. 2011) and, consequently, reduced whole plant carbon assimilation and productivity (Costa e Silva et al. 2004; Bedon et al. 2011).

Research efforts characterising the physiological effects of drought stress in *Eucalyptus* have been increasingly documented including changes in biomass allocation (Metcalfe et al. 1990), loss of turgor and osmotic adjustment (White et al. 1996), decrease of water potential (Sinclair 1980; Costa e Silva et al. 2004), stomatal closure (Sinclair 1980; Costa e Silva et al. 2004), cell wall reinforcement and water storage (Ladiges 2006), and changes in antioxidants and antioxidant enzymes, chlorophylls and carotenoids (Michelozzi et al. 1995; Shvaleva et al. 2006). Abscisic acid (ABA) is considered the principal stress hormone (Zhang et al. 2006) and has been shown to form part of a complex signalling network which mediates the physiological changes in *E. globulus* under drought stress (Granda et al. 2011).

Although there is a great number of studies reporting on the plant mechanisms to overcome drought conditions, evidence about the capacity of recovery and the underlying processes still needs research (Gallé et al. 2007). Recovery capacity emerges as a crucial topic for managing productive forests, considering both economic pressure and climate change. Climate change scenarios predict increasing frequency of drought peaks (Gallé et al. 2007; Volaire et al. 1998; Allen et al. 2010). Considering the pivotal economic importance of *E. globulus* plantations in Portugal, it is essential to understand the underlying mechanisms in drought recovery in order to select suitable clonal collections for sustainable plantations in a Mediterranean climate. Drought recovery mechanisms have been already described for other species, such as olive tree (Dichio et al. 2005), *Quercus pubescens* (Gallé et al. 2007) and *Quercus ilex* (Echevarria-Zomeno et al. 2009), however, recovery remains largely unknown in *E. globulus*, deserving further research.

It was hypothesised that different genotypes would be differently affected by water stress and require different time to recover after rewatering, namely at the photosynthetic level, which is particularly affected under moderate to severe drought conditions (Chaves et al. 2009). Considering this hypothesis, two different *E. globulus* genotypes were used to compare the effects of water shortage on morphological and physiological traits, focussing particularly on growth on biochemical and physiological processes. Water status, lipid peroxidation, pigments, chlorophyll a fluorescence, gas exchange and the ABA were assessed under greenhouse conditions, where 6-month-old rooted cuttings were subjected to a 3-week water stress with two different intensities followed by a 1-week period of rewatering. The aim of this study was to evaluate the performance of different clones to water stress and recovery in order to find sensitive turn-points that could be used for an early selection of drought tolerant genotypes. Therefore, two *E. globulus* clones were studied under water stress and during recovery with specific research questions: (1) Are there clonal differences in the responses to the different water treatments? (2) Does the time required for recovery differ for each genotype? (3) Does a given physiological profile make a clone more favourable under drought conditions?

Material and methods

Plant material and experimental design

Rooted cuttings of two genotypes of E. globulus (AL-18 and AL-10) were obtained from Altri Florestal SA (Portugal). These clones were selected according to their differential field behaviour. AL-18 is a clone selected from an open pollination family and first tests indicated very good survival results in drought prone areas. AL-10 is a clone from a control cross between two

parents from the Portuguese landrace. The first field trial results indicated low survival under severe drought conditions.

One hundred and sixty individuals of each clone, grown in plastic containers filled with 3:1 (w/w) vermiculite: peat with an initial height of 30 cm and 6 months old, were transplanted to 2 L plastic pots filled with equal weight of a 3:2 (w/w) peat: perlite mixture and transferred from a shaded house to a greenhouse. The potted cuttings were subjected to a 1-month acclimation period inside the greenhouse and were automatically watered with nutritive solution until 80% field capacity. To minimise effects of environmental heterogeneity, the pots were randomly arranged and periodically moved to the neighbouring position during the whole experiment.

During the experiment, 50 cuttings per clone were assigned to a well-watered regime (WW: water supplied every evening until soil water content reached around 80% field capacity) and the remaining cuttings (110 individuals per clone) were assigned to a water stress regime (WS 25%: water supplied every evening until soil water content reached around 25% field capacity) during 7 days. Pot weight was monitored everyday gravimetrically. After this period, half of the water-stressed cuttings (55 individuals per clone) were subjected to a harsher water stress (WS 18%: water supplied every evening until soil water content reached around 18% field capacity). The other half of the water-stressed cuttings (WS 25%) and well-watered cuttings were kept under the same water regime. This procedure lasted 14 days. At this moment, the first sampling point took place (Max Stress: maximum stress). From this moment onwards, all cuttings were equally well-watered (similarly to the WW regime described above) and recovery was monitored at two different sampling points (1-day Rec: 1-day recovery; and 1-week Rec: 1-week recovery).

At each sampling point (i.e. Max Stress, 1-day Rec, 1-week Rec), homogeneous leaves from six random individuals were immediately frozen in liquid nitrogen for further analysis (estimation of lipid peroxidation, pigment and ABA quantification). Also, homogeneous leaves from four random individuals were used for in vivo measurements of leaf gas exchange and chlorophyll fluorescence parameters (see below). Some plants were harvested for the evaluation of morphological parameters while plant water status was measured in vivo in four random individuals (see below).

The experiment was carried out at Altri Florestal R&D, located in Óbidos, from May to June 2011 under greenhouse environmental conditions (see table 1), with natural photoperiod (approximately 15 h L:9 h D) and photosynthetic active radiation, daily temperature between 16ºC (night) and 30ºC (± 3ºC) (daytime) and relative humidity between 50 and 85% (± 5%).

Table 1 – Time point values of climatic data observed in the greenhouse where water stress treatments were carried out. VPD and PAR stand for vapour pressure deficit and photosynthetic active radiation, respectively. *Measured with Li-Cor LI-6400.

	Temperature ***(^0C)***			***Humidity*** ***(%)***			***VPD*** ***(kPa)***			***PAR**** ***(µmol m^{-2} s^{-1})***
Sampling point	**0h**	**3h30**	**12h30**	**0h**	**3h30**	**12h30**	**0h**	**3h30**	**12h30**	**12h30**
Max Stress	20,4	19,2	31,6	78,5	82,9	57,1	0,51	0,38	2,00	465,0
1-day Rec	21,2	18,7	31,2	77,3	83,3	52,8	0,57	0,36	2,14	239,8
1-week Rec	20,6	19,4	35,9	74,6	74,1	41,6	0,62	0,58	3,46	440,2

Morphological and growth traits

Five plants per clone and treatment were harvested on Max Stress day and also 1-week Rec. Plant height (in cm) and number of branches were determined and the dry weight of leaves, roots and stems was recorded for biomass determination.

Plant water status

Midday shoot water potential (Ψ_{md}) was measured with a Scholander-type pressure chamber (PMS Instrument Co., Corvallis, OR, USA). Measurements were carried out in four plants per clone and treatment at 12 h 30 min (solar time) on Max Stress, 1-day Rec and 1-week Rec.

Lipid peroxidation

The extent of lipid peroxidation in leaves was estimated by measuring the amount of MDA (malondialdehyde) by the method described by Hodges et al. (1999), which takes into account the possible influence of interfering compounds in the assay for thiobarbituric acid (TBA)-reactive substances. In short, samples were extracted with 2.5 mL of TCA (trichloroacetic acid) 0.1% and hardly vortexed. After centrifugation, an aliquot of the supernatants was added to a test tube with an equal volume of either: (1) positive (+) TBA solution 0.5% (w/v) containing 20% (w/v) TCA; or (2) negative (–) TBA solution consisting in TCA 20%. Samples were heated at 95 ºC for 30 min and, after cooling and centrifuging, absorbance was read at 440, 532 and 600 nm (Thermo Fisher Scientific spectrophotometer, Genesys 10-uv S, Waltham, MA, USA). MDA equivalents (nmol mL^{-1}) were calculated as (A – B/157 000) x 10^6, where A = [(Abs 532_{+TBA}) – (Abs 600_{+TBA}) – (Abs 532_{-TBA} – Abs 600_{-TBA})], and B = [(Abs 440_{+TBA} – Abs 600_{+TBA}) × 0.0571].

Chlorophyll content and fluorescence

Total chlorophyll and carotenoid content were quantified according to Sims and Gamon (2002). Pigments were extracted with acetone/Tris (50 mM) buffer at pH 7.8 (80:20) (v/v). After

homogenization and centrifugation, supernatants were used to read absorbances at 663 nm, 537 nm, 647 nm 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 fluorimeter (Mini-PAM; Walz, Effeltrich, Germany) as described in Alves et al. (2011) 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), F_v (variable fluorescence, equivalent to $F_m - F_0$), F_v / F_m (maximum quantum yield of PSII photochemistry) and NPQ (non-photochemical quenching, equivalent to $(F_m / F'_m) - 1$).

Leaf gas-exchange measurements

Gas-exchange measurements were performed with a gas-exchange system (Li-6400 LI-Cor, Lincoln, NE, USA). Foliar net CO_2 assimilation rate (A) and transpiration rate (E), as well as stomatal conductance (g_s) were determined. Inside the chamber, the following conditions were maintained during all the measurements: C_a (ambient CO_2 concentration): 350 μL L^{-1}; air flux: 500 μmol s^{-1}; block temperature: 30ºC; relative humidity of the incoming air: 35-50%.

To find out the saturation light intensity A/PPFD (photosynthetic photon flux density; light response curves of CO_2 assimilation) rapid curves were performed with the following PPFD: 2500, 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 1000 μmol m^{-2} s^{-1}. Data were recorded when the measured parameters were stable (2 – 6 min). Homogeneous leaves from four biological replicates were analysed at each sampling point.

Abscisic acid quantification

ABA leaf content was analysed by high-performance liquid chromatography tandem mass spectrometry (HPLC MS/MS) as described by Brossa *et al.* (2011) with slight modifications. In short, approximately 100 mg of leaf were grounded in liquid nitrogen with a mortar and pestle. All following steps were performed at 4ºC. Before starting the extraction procedure, a deuterium-labelled internal standard (20 ηg ABA-d_6) was added. An aliquot of 600 μL extraction buffer [methanol-water-acetic acid (90:9:1, v:v:v)] was then added and extracts were vortexed for 10 min. Subsequently, extracts were centrifuged at 15000 × g during 15 min and supernatants were collected and stored at -80ºC until analysis. For the analysis, an aliquot of the supernatants was

filtered throughout a 0.22 μm polytetrafluoethylene (PFTE) filter (Waters, Milford, MA, USA) and 5 μL of each sample was injected into the LC system (Acquity UPLC, Waters), using a X-Bridge C18 column (3.5 μm; 100 x 2.1, Waters). The MS/MS quantification was performed on an API 3000 triple quadrupole mass spectrometer (AB Sciex, Danaher Corp, Washington, DC, USA) using multiple reaction monitoring (MRM) acquisition with the corresponding transitions for each analyte.

Statistical analysis

Principal components analysis (PCA) was carried out to explore the physiological profiles of *E. globulus* genotypes of all experimental treatments (watering regimes × sampling points), by reducing the multivariate data matrix to an interpretable bidimensional biplot that explains the highest proportion of variation in the data (ter Braak 1995). Similar approaches have been described in the literature for other morphological, reproductive and physiological data matrices (e.g. Hund et al. 2007; Rice et al. 2008; Loureiro et al. 2013). In this study, this approach allowed a global overview of the effect of watering regime during the rehydration period. Morphological and growth traits were excluded from the PCA because these parameters were not quantified on the first day of recovery (1-day Rec). Data were *a priori* centred and standardised to reduce scale effects (ter Braak 1995). PCA was conducted with CANOCO for Windows version 4.5 (Scientia Ltd., Cambridge, UK).

Two-way analysis of variance (ANOVA) was used to assess the effects of watering regime and clone, and their interaction, on height, number of stems and biomass, at Max Stress. This approach allowed comparing the effect of watering regime on the performance of the plants, while accounting for clonal differences. In order to focus on the recovery from water stress, two-way ANOVAs were employed on the morphological, biochemical and physiological traits to assess the effect of watering regime, time (sampling points along rehydration period), and their interaction. These analyses were run separately for each genotype. Whenever an interaction between watering regime and time was found, a simple main effect analysis was carried out for watering regime at each sampling point (using the error term of the two-way ANOVA; Quinn and Keough, 2002). When applicable, Tukey multiple comparison tests were employed to identify significant differences between watering regimes. Two-way ANOVAs and Tukey tests were performed with SPSS for Windows (SPSS for Windows v. 11.0, SPSS Inc., Chicago, IL, USA) and main effects were calculated with MS Excel (Microsoft, Redmond, WA, USA). Prior to analysis, data were either ln- or square root-transformed to correct non-normality and heteroscedasticity. All analyses used a significance level of 0.05.

Results

Growth and morphological traits

As expected, low watering regimes caused a significant decrease in height [$F(2, 18) = 32.2$, $p < 0.001$], number of stems [$F(2, 18) = 6.5$, $p = 0.008$] and biomass [$F(2, 18) = 43.8$, $p < 0.001$] of *E. globulus* plants at maximum stress day (figure 1). Both genotypes responded to stress in a comparable way, although clonal differences were found. Plants from clone AL-10 were consistently taller [$F(1, 18) = 20.0$, $p < 0.001$] and produced more shoots [$F(1, 18) = 30.1$, $p < 0.001$] than AL-18, at all watering regimes (non-significant clone × stress interaction). Similarly, AL-10 plants had higher biomass than AL-18 [$F(1, 18) = 6.7$, $p = 0.024$], except at the highest

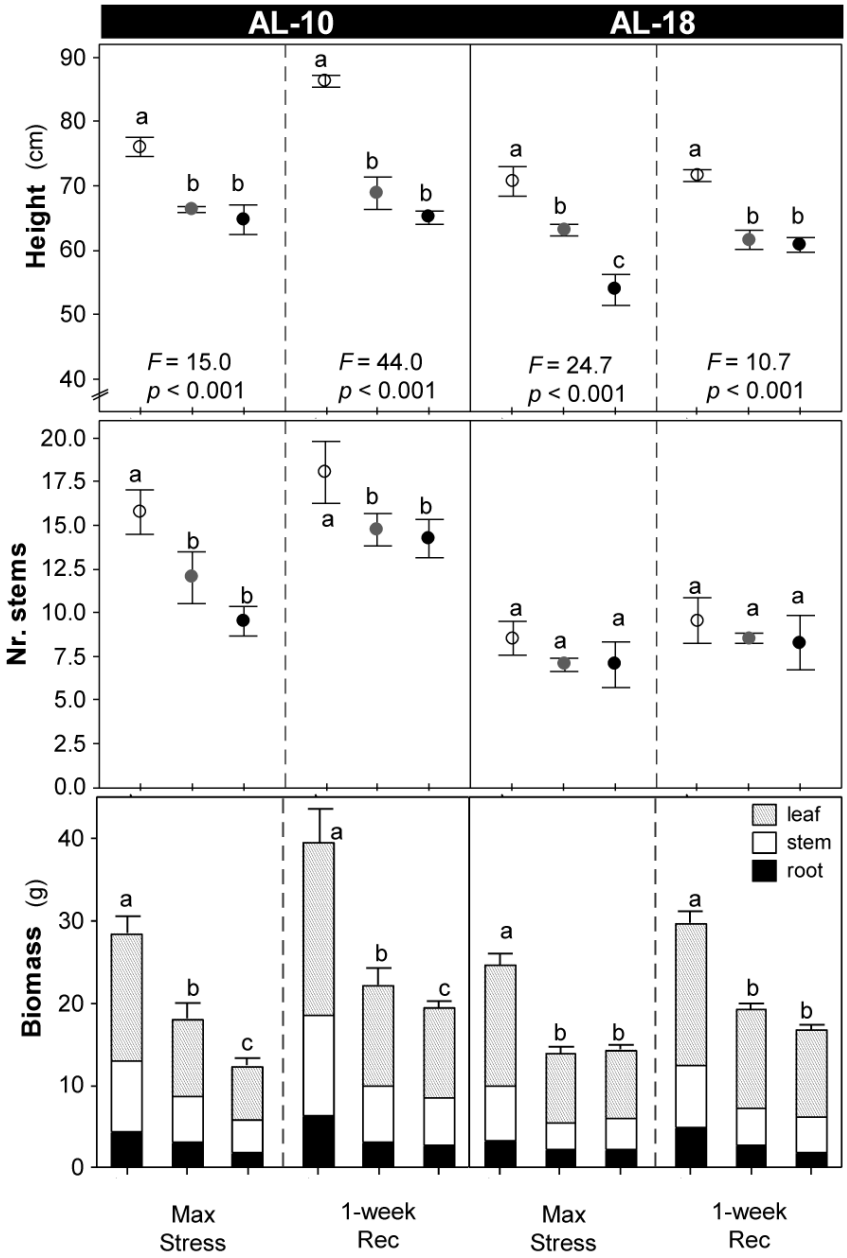

Figure 1 – Height, number of branches and biomass of well-watered (WW) and differentially water stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-10) after a three-week water stress period and one-week recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between watering regimes ($p \leq 0.05$). White circles = WW; grey circles = WS 25%; black circles = WS 18%. *F* and *p* are indicated when a simple main effect analysis was applied.

water stress level [significant clone x stress interaction, $F(2, 18) = 5.2$, $p = 0.024$].

This negative effect on growth persisted after 1 week of recovery (figure 1), with a fairly unaltered pattern (well-watered plants of both clones were overall taller and had higher biomass than stressed plants). A few differences in the response profile of genotypes were found, as clone AL-10 seemed to suffer a more drastic reduction in the number of shoots and biomass than clone AL-18 (figure 1, table 2). Clone AL-10 suffered a ca. 67% average reduction in biomass in the highest stress scenario compared to WW, which was still observed after one week (54%); clone AL-18 was more modestly affected, suffering a biomass reduction of 36% (Max Stress) and 41% (1-week Rec). As a result of this more drastic effect of water stress on the most productive genotype (higher biomass in WW conditions, AL-10), the biomasses of AL-10 and AL-18 were similar at WS 18% (see above).

Table 2 – Two-way ANOVA summary table for morphological and physiological traits of two *E. globulus* genotypes (clone AL-10 and clone AL-18). Degrees of freedom (df) and *F* value are shown for each source of variation; variance (MS) of the residual is also shown.

		AL-10			AL-18		
Parameter	**Source of variation**	**df**	***F***	**Significance**	**df**	***F***	**Significance**
Height (ln)	Watering regime	2	53.1	***	2	33.2	***
	Time	1	9.10	**	1	2.92	NS
	Interaction	2	3.66	*	2	4.11	*
	Residual	18	MS = 0.002		18	MS = 0.003	
Nr stems (√)	Watering regime	2	7.89	**	2	1.04	NS
	Time	1	10.2	**	1	2.04	NS
	Interaction	2	0.80	NS	2	0.05	NS
	Residual	18	MS = 0.120		18	MS = 0.141	
Biomass (ln)	Watering regime	2	45.9	***	2	67.9	***
	Time	1	23.9	***	1	45.5	***
	Interaction	2	1.12	NS	2	1.93	NS
	Residual	12	MS = 0.018		12	MS = 0.006	
RWC (asen√)	Watering regime	2	2.40	NS	2	3.29	NS
	Time	2	10.8	***	2	20.8	***
	Interaction	4	0.87	NS	4	2.25	NS
	Residual	18	MS = 0.006		18	MS = 0.003	

Ψ_{md}	Watering regime	2	39.6	***	2	79.3	***
	Time	2	14.3	***	2	33.4	***
	Interaction	4	10.5	***	4	40.7	***
	Residual	18	MS = 0.020		18	MS = 0.008	
MDA (ln)	Watering regime	2	12.2	***	2	84.4	***
	Time	2	28.5	***	2	28.1	***
	Interaction	4	3.12	*	4	18.3	***
	Residual	24	MS = 0.018		25	MS = 0.007	
Chl (ln)	Watering regime	2	65.8	***	2	60.0	***
	Time	2	50.0	***	2	22.6	***
	Interaction	4	5.26	**	4	5.09	**
	Residual	25	MS = 0.020		25	MS = 0.027	
Carot (ln)	Watering regime	2	49.0	***	2	55.4	***
	Time	2	44.5	***	2	23.4	***
	Interaction	4	3.33	*	4	2.53	NS
	Residual	25	MS = 0.011		27	MS = 0.015	
F_v/F_m (ln)	Watering regime	2	53.4	***	2	61.2	***
	Time	2	5.50	**	2	72.4	***
	Interaction	4	5.53	***	4	9.15	***
	Residual	45	MS = 0.000031		45	MS = 0.000013	
ϕ_{PSII}	Watering regime	2	81.9	***	2	64.8	***
	Time	2	5.28	**	2	4.13	*
	Interaction	4	0.64	NS	4	3.33	*
	Residual	45	MS = 0.003		45	MS = 0.003	
NPQ (ln)	Watering regime	2	74.3	***	2	56.0	***
	Time	2	5.79	**	2	14.6	***
	Interaction	4	0.31	NS	4	4.07	**
	Residual	45	MS = 0.027		45	MS = 0.024	
A	Watering regime	2	2.19	NS	2	7.81	***
	Time	2	34.3	***	2	21.5	***
	Interaction	4	27.5	***	4	11.0	***
	Residual	66	MS = 3.40		66	MS = 2.177	

E (√)	Watering regime	2	23.4	***	2	53.8	***
	Time	2	27.2	***	2	34.9	***
	Interaction	4	19.0	***	4	9.84	***
	Residual	65	MS = 0.081		67	MS = 0.038	
g_s (√)	Watering regime	2	19.6	***	2	45.1	***
	Time	2	91.9	***	2	142	***
	Interaction	4	15.9	***	4	16.2	***
	Residual	62	MS = 0.009		65	MS = 0.007	
ABA (√)	Watering regime	2	21.4	***	2	21.2	***
	Time	2	41.8	***	2	16.2	***
	Interaction	4	3.49	*	4	3.85	*
	Residual	27	MS = 0.060		25	MS = 0.030	

*** $p \leq 0{,}001$; ** $p \leq 0{,}01$; * $p \leq 0{,}05$; NS – non significant

Multivariate approach: global overview of physiological status

PCA ordination provided an overall picture of the physiological condition of *E. globulus* plants during the experimental setup, revealing a clear separation between watering regimes (figure 2, see left-to-right gradient). Well-watered plants (WW) were grouped together (figure 2, upper right quadrant), suggesting homogeneity in the biochemistry and physiology across clones and sampling points in non-stressful conditions. However, physiological responses to watering regimes

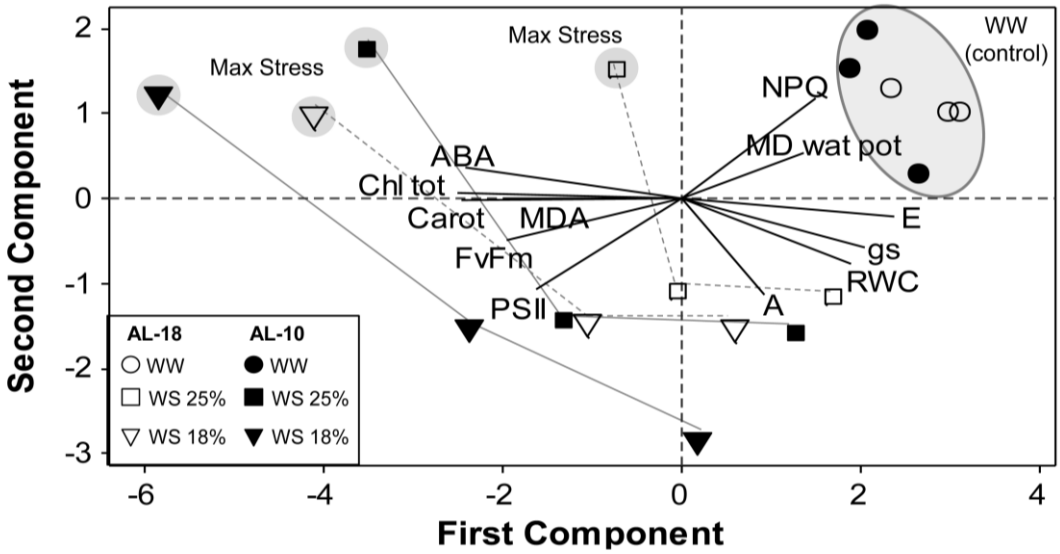

Figure 2 – PCA biplot of the physiological data of *Eucalyptus globulus* plants during the experimental setup. Circles, squares and triangles depict sample scores, while arrows show gradients resulting from biochemical and physiological profiles. Continuous (clone AL-10) and dashed (clone AL-18) lines were artificially placed in the diagram to connect sampling points (Max Stress, 1-day Rec, 1-week Rec) within the same watering regime (WW, WS 25%, WS 18%). Similarly, shaded areas were used to highlight the position specific scores (WW plants; WS plants at Max Stress).

varied between genotypes and throughout time. At Max Stress, sample scores were all located on the top left quadrant, with the plants that were subjected to the most stressful conditions being farther from the WW scores, mostly because of higher concentration of ABA, pigments and MDA and lower values for E, water potential and g_s (see arrow direction in figure 2). At Max Stress, but also during recovery, AL-10 scores were also farther from the WW plants relatively to the corresponding AL-18 score (at the same watering regime). The physiological profile of the stressed plants showed a similar trend during the recovery period: scores progressively moved from left to right (i.e. closer to the WW scores) but also downwards. The approximation to the WW scores was related to reversion of altered physiological traits (observed at Max Stress – see above). However, the downwards migration pattern of the PCA scores during recovery is related with changes that occurred in the stressed plants during the recovery period, most probably associated with vertical gradients such as A, φ_{PSII} and NPQ. These biochemical and physiological changes, which seem to take place during recovery from water stress, are analysed in more detail in the following paragraphs.

Plant water status

Although PCA analysis revealed a decline of RWC after water stress treatment, these data did not show significant differences in either watering regime or sampling point (stress duration), neither an interaction between them (table 2).

Regarding Ψ_{md}, a significant effect of watering regime and sampling point was found, as well as a significant interaction between both factors, similar in the two clones (table 2). After 3 weeks under different watering regimes, Ψ_{md} showed differential response patterns between watering regime and sampling point (figure 3). Upon dehydration, AL-18 plants showed a gradual

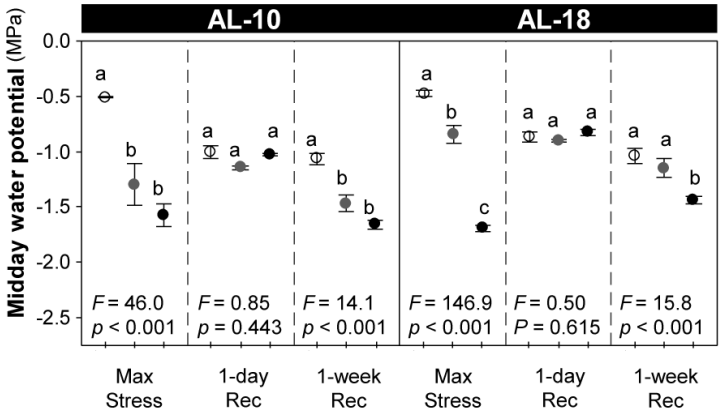

Figure 3 – Midday water potential of well-watered (WW) and differentially water-stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-10) after a 3-week water stress period and after one day and 1 week of recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between watering regimes ($p \leq 0.05$). White circles, WW; gray circles, WS 25%; black circles, WS 18%. *F* and *p* are indicated when a simple main effect analysis was applied (see section Materials and methods).

decline of Ψ_{md}, according to the stress intensity, while AL-10 plants exhibited equal values for both stress intensities (significantly different from WW condition, figure 3). In the first day of recovery, WW values were achieved in both stressed clones. After 1 week of recovery, each clone presented a similar pattern of lower water potential. These differences were only significant for clone AL-18 in WS 25%.

Lipid peroxidation

Watering regime and sampling point showed a significant effect in lipid peroxidation in both clones, as well as an interaction between the two factors (table 2). After 3 weeks, the imposed water treatments led to a significant increase in MDA concentration in the more water limiting condition (WS 18%) in both clones, while in WS 25% no significant differences were found (figure 4). After one day of full irrigation, plants under WS 25% showed a decrease of MDA in both

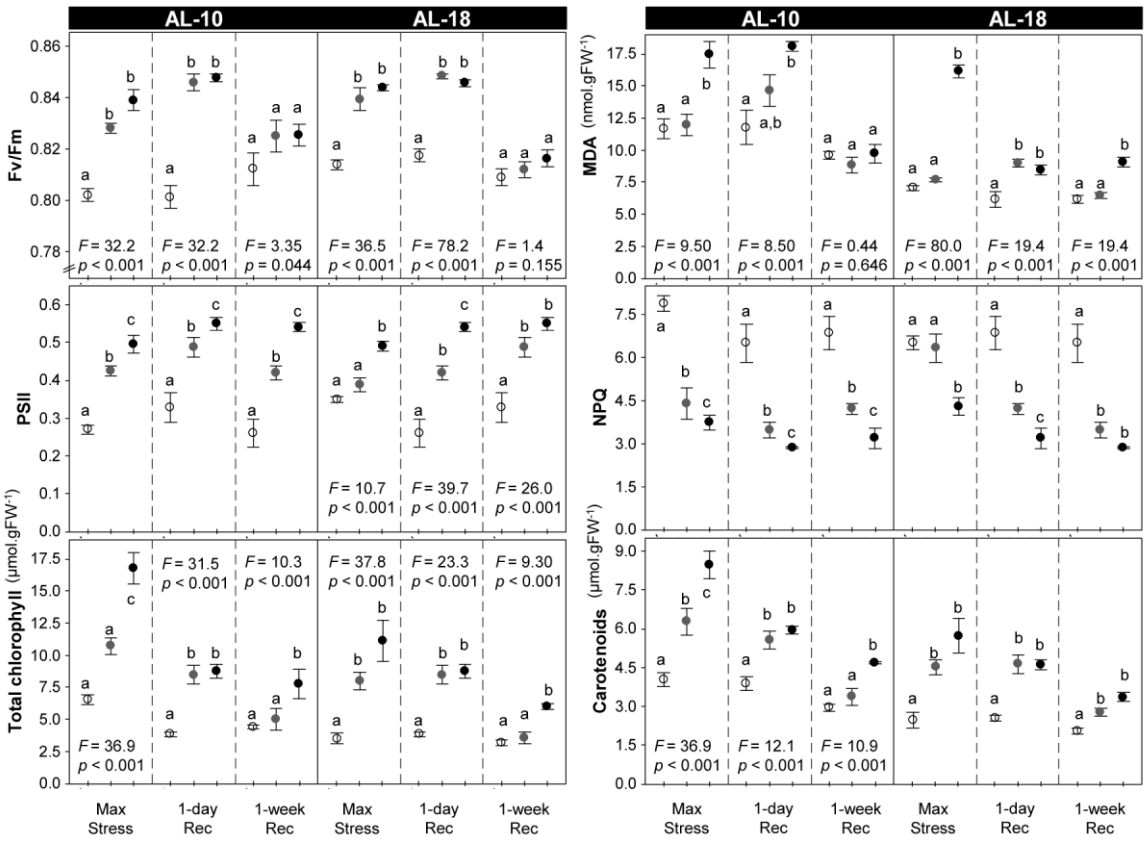

Figure 4 – F_v/F_m, Φ_{PSII} and NPQ results and MDA, total chlorophyll and carotenoid content in leaves of well-watered WW) and differentially water stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-10) after a 3-week water stress period and after one day and 1 week of recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between watering regimes ($p \leq 0.05$). White circles, WW; gray circles, WS 25%; black circles, WS 18%. *F* and *p* are indicated when a simple main effect analysis was applied (see section Materials and methods).

clones, while WS 18% kept with higher content. One week after rehydration, AL-10 drought stressed plants showed values of MDA similar to WW, while AL-18 WS 18% kept a higher MDA content than well-watered plants.

Chlorophyll content and fluorescence

In the two clones, F_v/F_m results showed a significant effect of the watering regime and sampling point and a significant interaction between the two factors. In Max Sress, the F_v/F_m ratio was significantly higher in stressed plants than in the WW group (table 2, figure 4), with no statistically significant differences between the drought stressed groups (WS 18% and WS 25%). This relation was maintained in the first day after rehydration, but after 1 week no significant differences were found between WW and water-stressed plants. Regarding Φ_{PSII}, the two clones showed significant differences. While clone AL-10 did not show differences over time, in clone AL-18 a difference was noticed in Max Stress, where only WS 18% showed a significant increase compared to WW and WS 25%, and on 1-week Rec, where both stress intensities showed similar values, higher than WW. In an identical way, clonal differences were found in relation to NPQ.

In clone AL-10 no interaction between watering regime and sampling point was observed but only a stress effect. In all sampling points, NPQ decreased with higher stress intensity. In clone AL-18WS 18%, a significant decrease compared to WW and to WS 25% was observed. One week after rewatering plants subject to both stress intensities showed similar values and lower than WW.

Regarding chlorophyll content, both clones showed a significant effect of watering regime and sampling point and a significant interaction (table 2). In Max Stress, chlorophyll content increased with stress intensity (figure 4). After stress relief, pigment concentration started to decrease and 1 week later only the plants under the WS 18% treatment showed a higher chlorophyll content. Carotenoid concentration kept the same profile in clone AL-10, while clone AL-18 carotenoid concentration had a similar concentration for both stress intensities, significantly different from WW plants.

Leaf gas exchange

With respect to A, E and g_s, significant effect of watering regime and sampling point were found, as well as a significant interaction in both clones (table 2). CO_2 assimilation revealed clonal differences. In the Max Stress day, A in the two stress intensities was similarly reduced in clone AL-10, while in clone AL-18 only WS 18% was affected (figure 5). One day after rehydration, clone AL-10 showed no significant differences in all watering regimes, while clone AL-18 exhibited higher A values in the two stress conditions compared to WW in a similar way. After 1 week, clone AL-18 kept the same profile and clone AL-10 showed increasing A according to the

previous stress intensity. Transpiration rates showed a significant difference between the clones in the Max Stress day: clone AL-10 showed significant lower values in both water-stressed groups compared to WW, while clone AL-18 plants exhibited decreased E with increasing stress intensity. After one day of rehydration the two clones maintained a similar profile of lower E in both stress intensities and after 1 week no significant differences were found compared to WW. Regarding g_s, the clones showed a similar response: in the Max Stress, a significant reduction was observed in both WS groups; in the first day after rewatering only WS 18% plants still showed a significant decrease in g_s compared to the other watering regimes and 1 week later no significant differences were found.

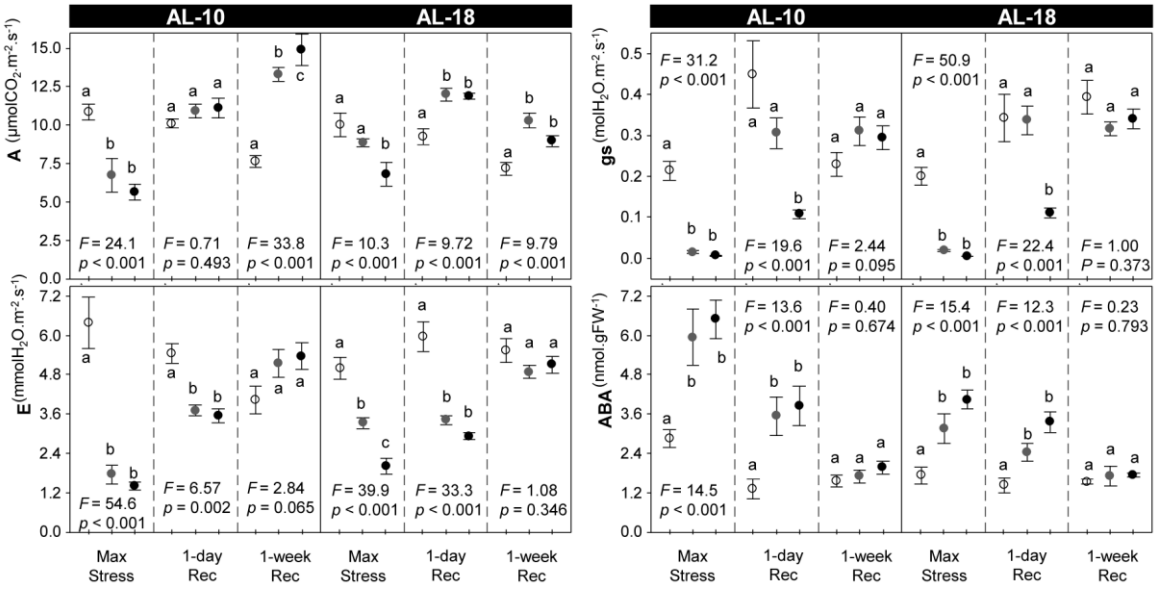

Figure 5 – Photosynthesis (A), transpiration (E), stomatal conductance (gs) and ABA content in leaves of well-watered WW) and differentially water stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-10) after a 3-week water stress period and after one day and 1 week of recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between watering regimes ($p \leq 0.05$). White circles, WW; gray circles, WS 25%; black circles, WS 18%. F and *p* are indicated when a simple main effect analysis was applied (see section Materials and Methods).

Abscisic acid

Watering regime and sampling point significantly affected leaf ABA dynamics and a significant interaction was found (table 2). Nevertheless, no clonal differences were observed. In drought stressed plants, leaf ABA concentration was significantly higher than in WW plants (figure 5). After the first day of recovery the same pattern was observed, but 1 week later no significant differences were found between watering regimes.

Discussion

The focus of this study was to identify morphological, physiological and biochemical traits that would enable to characterise water stress and recovery in two different clones of *E. globulus*.

Plant growth rates were significantly reduced by the water shortage, resulting in a reduction in total biomass, height and number of shoots in both *E. globulus* clones, which is in agreement with other reports (Li and Wang 2003; Costa e Silva et al. 2004; Granda et al. 2011). Despite that, the analysed morphological parameters showed different profiles. These differences reveal that a morphological analysis should be based on more than a few parameters in order to monitor drought stress response in a reliable way. Analysing the impact of drought using the chosen morphological parameters identified clone AL-10 as the most productive genotype.

The unsupervised multivariate data analysis approach was applied to check if the studied attributes were involved in specific responses between clones or other factors, using a similar approach as Taylor et al. (2002). PCA analysis proved to be a useful tool for finding comprehensive profiles of all data from physiological measurements, allowing a deeper interpretation of differences between clones, stress effect and recovery patterns.

Drought phase

Water deprivation had significant effects on the tissue water relations of the two clones of *E. globulus*. Midday water potential decreased under water stress conditions as previously described in other reports (Colom and Vazzana 2001; Shvaleva et al. 2006; Galmés et al. 2007a). Although the restriction in water supply was extended for three weeks, Ψ_{md} never dropped below −1.7MPa, indicating a level of water deficit less severe than that reported for other field-grown eucalypts (≈ -2.5MPa; Mielke et al. 2000). CO_2 assimilation was generally affected and E and g_s were strongly depressed, similarly to what has been reported for *E. globulus* and other species (Osório et al. 1998; Colom and Vazzana 2001; Bogeat-Triboulot et al. 2007). Stomatal closure together with leaf growth inhibition are among the earliest responses to drought, protecting plants against excessive water losses, but also restricting the diffusion of CO_2 into the photosynthetic parenchyma, usually described as a main cause of limited leaf carbon assimilation (Chaves et al. 2003; Vassileva et al. 2011).

PSII photochemistry was not negatively affected in response to drought. Moreover, an increase of F_v/F_m and Φ_{PSII} values was noticed in stressed plants. Despite this, F_v/F_m variations between WW and drought stressed plants were within the range of values of healthy plants (0.75 – 0.85; Schreiber et al., 1994). Susiluoto and Berninger (2007) also found higher F_v/F_m values in

drought stressed *Eucalyptus microtheca*. The increase of Φ_{PSII} observed in drought stressed plants could be a consequence of the higher chlorophyll content also noticed under drought stress. The increasing chlorophyll concentration could be related to the reduction in leaf mass expansion. High chlorophyll content may be explained by a putative protective role of carotenoids or other mechanisms (such as maintenance of volume in chloroplasts by osmotic adjustment; Santakumari and Berkowitz, 1991), protecting these pigments from degradation and preserving the photosynthetic capacity. These results have already been described in *E. globulus* under water stress (Michelozzi et al. 1995).

Two different pathways are underlined as a way of overcoming the stressing condition and dissipating the surplus energy: at WS 25% it seems that carotenoids and NPQ have a simultaneous action producing reinforced protecting effects; on the other hand, at WS 18% it seems that this mechanism is insufficient to overcome the stress, therefore other protective mechanisms may be triggered (e.g. antioxidant system) to counteract the negative impact of the oxidative stress (higher MDA content) observed in this treatment.

ABA showed a clear accumulation in the leaves of both clones during water stress, which is in accordance with the defined signalling role of ABA under water deficit conditions (Galmes et al. 2007a; Jiang and Hartung 2008). As reported by Jiang and Hartung (2008), higher ABA concentration is expected to match a more intensified ABA signalling, which is known to have a positive correlation with water saving.

Considering water deficit response, differences were found between the two clones in $\Psi_{md,}$ Φ_{PSII}, NPQ, pigments and gas exchange. Clone AL-10 showed no differences between WS 25% and WS 18% in Ψ_{md} and E but significant differences appeared in Φ_{PSII}, NPQ and pigments, contrarily to AL-18 that showed a more gradual and slower response. This response meets the conclusion reported by Liu et al. (2011) that argued that a better water status may benefit a higher photosynthetic efficiency. Likewise, clone AL-10 was able to keep higher growth performance, despite being more affected in CO_2 assimilation.

Recovery phase

During the rewatering period, plant water status showed a prompt recovery: Ψ_{md} of the stressed plants were restored within 1 day of rewatering. These results are in accordance with similar works in other species (Tognetti et al. 1995; Munné-Bosch and Peñuelas 2003; Vassileva et al. 2011) and showed that both clones have a good ability to recover after water becomes available. After 1 week of recovery, the two clones presented unexpected decreases in water potential, more pronounced in WS 18%. The authors hypothesised that this was the result of the elevated

photosynthetic active radiation together with a high VPD in that particular sampling day, especially pronounced in previously stressed and damaged plants.

Considering gas exchange, the lowered levels of A and E, as well as g_s, under soil water deficit had a tendency to recover quickly after rewatering. After the stress relief, A of stressed plants achieved WW values within one day of recovery, while E and gs did not fully recover to WW values until 1 week of well irrigation. The recovery of photosynthetic rates after severe water stress was shown to be different from the recovery of either g_s, mesophyll conductance or maximum rate of carboxylation of Rubisco (Galmés et al. 2007b). Our results support Galmés et al. (2007b) considering the different recovery rate between A and g_s. Fluorescence parameters were kept generally unchanged after the stress relief, except F_v/F_m that presented control values after one week of rewatering. Pigment concentration started to decrease after rewatering, probably due to tissue rehydration following shrinkage induced by water deficits.

MDA levels increased during recovery to a greater extent than during drought (WS 25%). Similar results were found by Munné-Bosch and Peñuelas (2003) in *Phillyrea angustifolia* plants. As argued, this response indicates that during the recovery phase leaves suffer oxidative stress and that increased MDA production during the first stages of recovery means that degradation processes are essential for a correct repair of photosynthetic membranes and other cellular structures.

During recovery, ABA content decrease was accompanied by the increase in stomatal conductance, reinforcing the coordinated action of ABA upon stomatal closure/opening dynamics (among other factors, such as changes in turgor of guard cells, metabolic energy and membrane permeability) (Chaves et al. 2003; Brossa et al. 2011). Clone AL-10 showed a rapid reduction in ABA concentration after rewatering, representing a faster response to water availability and greater dynamics in the ABA signalling pathway than clone AL-18.

Considering the recovering capacities, several clonal differences were observed. Clone AL-10 showed a faster and more dynamic response to rehydration regarding carotenoid content, Φ_{PSII} and NPQ compared to clone AL-18 that seems to be less reactive to water availability. Furthermore, an overcompensation of CO_2 assimilation was achieved 1 week after rehydration, which was accompanied by higher growth behaviour and re-establishment of oxidative balance. These results could be related to osmotic adjustment and antioxidative response as already highlighted in other woody species (Liu et al. 2011).

In conclusion and answering to our initial questions, the results reported here indicate that:

1. Different water treatments imposed a different response in most studied parameter – a more limiting stress often results in a more marked response that is genotype dependent.

2. Clone AL-10 showed a faster and dynamic response to water availability in growth, carotenoids, Φ_{PSII}, NPQ and A, while clone AL-18 took more time to respond.

3. Despite both clones showed to be highly tolerant to the conditions tested, biomass accumulation, together with water saving dynamics, demonstrated that clone AL-10 was able to maintain a greater performance under our experimental drought conditions and after recovery, while clone AL-18 showed to be less responsive to water availability.

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2.2. Hormonal dynamics during recovery from drought in two *Eucalyptus globulus* genotypes: from root to leaf

Summary

Drought is a limiting environmental stress that represents a growing constraint to the forestry sector. *Eucalyptus globulus* is a widely planted coppice species, which capacity to cope with water deficit has already been described. However, the capacity of this species to recover is still poorly understood. In this study, we aimed to investigate the changes in abscisic acid (ABA), ABA-glucose ester (ABA-GE) and acid jasmonic (JA) content in leaves, xylem sap and roots of two genotypes (AL-10 and AL-18) during rewatering (2 h, 4 h, 24 h, and 168 h), after a drought stress period (0 h). We wished to clarify the role of these hormones in the recovery from drought and to determine whether these hormonal relations were related to specific genotype metabolisms. Our results showed that drought caused an increase in ABA and ABA-GE levels in all analysed plant parts, while JA content decreased in leaves, increased in xylem sap and did not change in roots. Some of these responses were genotype specific. During rewatering, ABA and ABA-GE content decreased in both genotypes and all plant parts, but at different time scales, and JA levels did not greatly change. Again, the genotypes responded differently. Altogether, our results characterised the response pattern of clone AL-10 as more responsive and defended that leaf should be used in preliminary screening methods of stress tolerance. The hormonal dynamics were related to the previously documented responses of these genotypes and sustain further physiological and molecular studies of water stress in this and other tree species.


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Research article

Hormonal dynamics during recovery from drought in two *Eucalyptus globulus* genotypes: From root to leaf

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Introduction

Since their early transition from aquatic to terrestrial environments, plants have coped with periodic and unpredictable environmental stresses (Zhang et al. 2006). The exposure to these variable and often potentially damaging environmental conditions over a long evolutionary time scale has led plants to evolve complex systems of defence (Dobra et al. 2010). Also, plants have acquired mechanisms by which they can sensitively perceive incoming stresses and regulate their physiology accordingly (Zhang et al. 2006). Among environmental factors, water availability is probably the most limiting abiotic stress affecting terrestrial plants (Cornic and Massacci 2004). This is a growing concern for agriculture and forestry activities, considering the increasingly unpredictable nature of rainfall (Allen et al. 2010; Roche et al. 2009; Zhang et al. 2006), which translates into important economic losses.

Eucalyptus has been extensively grown around the world, essentially for pulp production, due to the quality of its fibre, its pulp yield, and its high growth rate (Luger 2003). Eucalypt plantations are a successful example of a fast growing coppice species in several European countries, relying almost exclusively on a single species (*Eucalyptus globulus*), used for the production of industrial biomass (Luger 2003). Many studies have reported the capacity of this important species to cope with water deficit (Bedon et al. 2011; Costa e Silva et al. 2004; Dutkowski and Potts 2012; Michelozzi et al. 1995; Navarrete-Campos et al. 2012; Pita and Pardos 2001). However, many aspects of eucalypt forestry still need research (Luger 2003). One of such aspects is the capacity to recover after a period of drought, an important and poorly understood topic (Chaves et al. 2003). We have successfully shown that recovery from water deficit involves many physiological modifications in eucalypt plants and is genotype-specific (Correia et al. 2014). However, the mechanisms or processes that mediate the plants' response to water stress and recovery still need to be clarified.

Perception of stress signals often results in a mass production of chemical compounds, including a variety of hormones, to adapt and respond to the environmental challenges (Aimar et al. 2011). Among others, two phytohormones – ABA (abscisic acid) and JA (jasmonic acid) – are known to play major roles in regulating plant defence responses against various abiotic stresses, by mediating a wide range of adaptive responses (Hirayama and Shinozaki 2010; Peleg and Blumwald 2011; Santner et al. 2009). The key phytohormone involved in the response to dehydration is ABA (Dobra et al. 2010) and its involvement in mediating drought stress has been extensively explored (Zhang et al. 2006). ABA mediates fast responses associated with regulation of the plant water status through guard cells and growth (Dobra et al. 2010), but it is also involved in slower metabolic changes, coinciding with activation of cellular dehydration tolerance pathways (Dobra et

al. 2010; Zhang et al. 2006). Moreover, ABA acts as a long-distance water stress signal (Hartung et al. 2002) as it is synthesised in dehydrated roots and transported via the xylem, thereby regulating stomatal opening/closure and leaf growth in the shoots (Zhang et al. 2006).

On the basis of some studies, other substances can also be involved in drought response (Schachtman and Goodger 2008). ABA may be conjugated with glucose, thereby forming a glucose ester (ABA-GE) in xylem sap, which has been suggested to serve as a transported form of the hormone and a stress signal (Sauter et al. 2002; Schachtman and Goodger 2008). Works reporting on the relation between drought and increased ABA-GE concentrations have already been published (López-Carbonell et al. 2009; Sauter et al. 2002). Jasmonic acid (JA) is involved in diverse plant developmental processes, such as root growth, leaf senescence or stomatal opening, and plays crucial roles in defence responses against different plant pathogens (Zhang et al. 2006). Moreover, there is increasing evidence that JA and jasmonates are also crucial signalling molecules involved in many plant responses to abiotic stress (Devoto and Turner 2003; Balbi and Devoto 2008; Wasternack 2007). De Ollas et al. (2013) reported JA as a possible precursor in the signal transduction cascade in case of drought stress, providing increased levels of ABA.

Considering the pivotal importance of *E. globulus* plantations, it is essential to understand the underlying mechanisms in drought tolerance and recovery in order to select suitable clonal collections for sustainable plantations in a Mediterranean climate. Our early work showed that two different *E. globulus* genotypes (AL-10 and AL-18) coped differently with drought and rewatering: genotype AL-10 exhibited a dynamic and responsive physiological profile, while genotype AL-18 showed a slower and less reactive metabolism (Correia et al. 2014). These same profiles were maintained with respect to ABA response in leaves. However, little is known about how other plant hormones are involved, how they are regulated in the different plant parts and whether they could be used as selective markers. Assessing water stress through analysis of cultured plant tissue or specific isolated organs may offer potential for a quick evaluation as a preliminary screening method of stress tolerance (Naik and Widholm 1993). Therefore, there is great interest to examine stress response through the analysis of different plant parts.

Bearing this in mind, the objective of this study was to investigate the changes in ABA, ABA-GE and JA content in leaves, xylem sap and roots of the two different genotypes during rewatering (2 h, 4 h, 24 h and 168 h), following a water deficit stress of three weeks (0 h). Our specific aims were 1) to clarify the role of these hormones in the response and recovery of *Eucalyptus* plants to drought, and 2) to determine whether these hormonal relations are related to specific genotype metabolisms.

Materials and methods

Plant material

Two *Eucalyptus globulus* Labill. genotypes (AL-18 and AL-10) were obtained from Altri Florestal SA (Portugal). We selected these genotypes, used in Portuguese forest plantations, because of their different physiological response profiles during recovery from drought, as already described in a previous report (Correia et al. 2014).

One hundred and fifty rooted cuttings of each genotype, grown in plastic containers filled with 3:1 (w/w) vermiculite:peat, with an initial height of 30 cm and six months old, were transplanted to 2 L plastic pots filled with equal weight of a 3:2 (w/w) peat:perlite mixture and transferred to a greenhouse, with daily records of temperature and humidity and VPD (vapour pressure deficit) determination.

Experimental design

The potted cuttings were subjected to a one-month acclimation period inside the greenhouse, being automatically watered with an NPK nutritive solution until 80% field capacity. To minimise effects of environmental heterogeneity, the pots were randomly arranged and periodically moved to the neighbouring position during the whole experiment.

During the experiment, a group of 50 plants of each genotype was assigned to a well-watered regime (WW: water supplied every evening until soil water content reached around 80% field capacity) and another group of 50 plants was assigned to a water stress regime (WS, water supplied every evening until soil water content reached around 25% field capacity) during 7 days. To gradually decrease the water content, after this period, water stressed cuttings were subjected to a harsher water stress (water supplied every evening until soil water content reached 18% field capacity). This procedure lasted 14 days before the first sampling point took place (time 0). After this period, all cuttings were rewatered until they reached the well-watered regime, and recovery was monitored for one week at four different sampling points: 2 h, 4 h, 24 h (1 day) and 168 h (1 week) recovery.

The experiment was held in Altri Florestal R&D, located in Óbidos, under greenhouse environmental conditions: natural photoperiod (± 15 h light) and photosynthetic active radiation (± 800 mmol m^{-2} s^{-1}), daily temperature between 15ºC and 29ºC (± 2ºC) and relative humidity between 50% and 85% (±5%).

Foliar net CO_2 assimilation rate

Foliar net CO_2 assimilation rate (A) was measured with a gas exchange system (Li-6400 LI-Cor, Lincoln, NE, USA) according to the protocol described by Correia et al. (2014). Six

individuals per clone and water treatment were analysed at each sampling point (i.e., 0 h, 2 h, 4 h, 24 h and 168 h of recovery).

Collection of leaves, roots and xylem sap

At each sampling point (i.e., 0 h, 2 h, 4 h, 24 h and 168 h of recovery), homogeneous leaves and roots from six random individuals of each treatment were immediately frozen in liquid nitrogen and kept at -80ºC for later ABA, ABA-GE and JA quantification. Xylem sap was collected using a Scholander-type pressure chamber (PMS Instrument Co., Corvallis, OR, USA) at midday in each of the three sampling points (0 h, 24 h and 168 h) in the recovery period and immediately frozen at -20ºC. The quantity of sap obtained by this method varied from 0.3 to 0.6 mL per shoot, higher volumes being extractable from control (WW) plants. Shoots were harvested in the third apical node, at midday (12 a.m., solar time).

Hormone quantification

Leaf, root and sap content of ABA, ABA-GE and JA were analysed by UPLC MS/MS (ultra-performance capillary electrophoresis, tandem mass spectrometry) as described by Brossa et al. (2011), with slight modifications. Standards ABA, $ABAd_6$, ABA-GE and $ABA\text{-}GEd_5$ were purchased from Plant Biotechnology Institute (National Research Council, Canada); JA and JAd_5 were obtained from OlChemIm Ltd (Olomouc, Czech Republic). In short, approximately 100 mg fresh weight of leaf/root material was ground in liquid nitrogen with a mortar and pestle. All following steps were performed at 4ºC. Before starting the extraction procedure, a deuterium-labelled internal standard (20 ng mL^{-1} of $ABAd_6$ and $ABA\text{-}GEd_5$ and 50 ng mL^{-1} of JAd_5) was added. A methanol-water-acetic acid (90:9:1, v/v/v) extraction buffer (600 µL) was then added, and extracts were vortexed for 10 min. Subsequently, extracts were centrifuged at 15000 × g during 15 min and supernatants were collected and stored at -80ºC. Sap samples were used with a water dilution 1:3 (v/v). For the analysis, an aliquot of the supernatants was filtered through a 0.22 µm polytetrafluoethylene filter (Waters, Milford, MA, USA) and 5 µL of each sample was injected into the LC (liquid chromatography) system (Acquity UPLC, Waters), using an X-Bridge C18 column (3.5 µm; 100 x 2.1 Waters). The MS/MS (tandem mass spectrometry) quantification was performed on an API 3000 triple quadrupole mass spectrometer (AB Sciex, Danaher Corp, Washington, DC, USA), using multiple reaction monitoring acquisition with the corresponding transitions for each analyte.

Statistical analysis

Principal components analysis (PCA) was carried out to explore the hormonal dynamics (ABA, ABA-GE and JA) in the different plant parts over the rehydration period (plant part × time) in both genotypes. To provide additional insights on the hormonal profiles during the experimental period and across treatments (stressed and unstressed plants), we analysed the hormonal data for each combination of hormone and plant part. First, we assessed which hormonal changes were present in stressed plants immediately after exposure to drought. To do so, two-way analyses of variance (ANOVA) were employed to hormonal data at time 0 (excluding the rehydration period), using water stress and genotype as fixed factors. Second, we focused on the hormonal changes occurring throughout recovery (i.e. during the rehydration period). These data were analysed separately for each genotype with two-way ANOVA tests, using time and water stress as fixed factors. Because the latter analyses had to be repeated twice – one for each genotype – a Bonferroni correction of significance level ($p < 0.025$) was employed (see Quinn and Keough 2002). When a significant stress × time interaction was found, the simple main effects of time and water stress had to be decomposed (see below). Otherwise, significant time effects were further analysed using a post-hoc Tukey test.

In the case of a significant stress × time interaction (see above), the effects of time at each stress level and the effects of stress at each sampling time were decomposed and compared against the error term of the two-way ANOVA or omnibus test (Quinn and Keough 2002). Because these constitute cases of a family of multiple simultaneous hypotheses (Pike 2011; Quinn and Keough 2002), we adjusted *p*-values with a graphically-sharpened procedure based on the control of false discovery rate (Benjamini and Hochberg 1995), using the spreadsheet provided by Pike (2011). When simple main effects of time were found, a post-hoc Tukey test was then performed to further discriminate differences among sampling times, again using the error term of the ANOVA test. These analyses were performed with MS Excel (Microsoft, USA). Hormone data were log-transformed – log (x + 1) – before the ANOVA tests to correct for non-normality and heteroscedasticity. All analyses used Minitab v16 (Minitab Inc., PA, USA) and a significance level of 0.05, except where otherwise noticed.

Foliar net CO_2 assimilation rate data was analysed using a Student's t-Test to discriminate significant differences between stressed and unstressed plants at each sampling time. These analyses were performed with MS Excel (Microsoft), using a significant level of 0.05. Six random individuals of each treatment were analysed.

Results

Foliar net CO_2 assimilation rate

The water stress treatment significantly decreased the photosynthetic rate in both genotypes (figure 1), showing genotype AL-10 recovering faster. During recovery, WS plants of genotype AL-10 presented levels similar to WW after 2 h of rewatering, while WS AL-18 only showed WW values after one week of rehydration (figure 1).

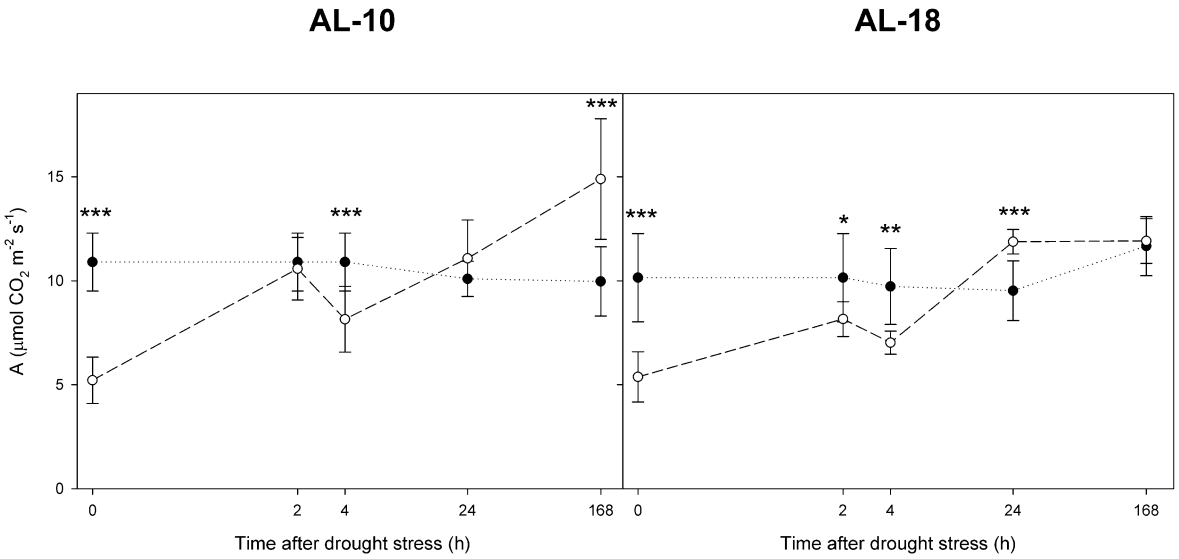

Figure 1 – Foliar net CO_2 assimilation rate (A) of water stressed (WS, dashed lines and open circles) and well-watered plants (WW, dotted line and filled circles) throughout a one-week post-stress rewatering period (i.e., recovery period). Data are shown as mean ± standard deviation; asterisks indicate significant differences between WW and WS plants (*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$).

PCA analysis

PCA ordination provides an overall picture of the hormonal variation of *E. globulus* plants following the period of drought (0 h) and during recovery, and reveals a clear separation between leaf (right side of the diagram) and the other plant parts (figure 2). This separation has to do with

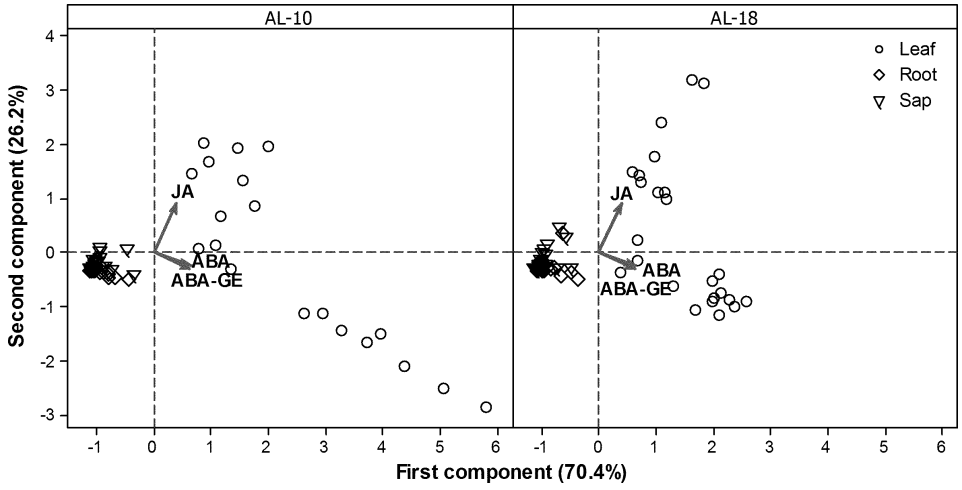

Figure 2 – PCA biplot of plant scores (from leaf, sap and root) in terms of their hormonal profile (grey arrows: ABA, ABA-GE, and JA) of two *E. globulus* genotypes (AL-10 and AL-18).

the higher hormonal content in the *Eucalyptus* leaves. Also, hormonal levels did not greatly fluctuate in root and xylem sap, showing small variations (figure 3), whereas PCA scores for leaf were more scattered (figure 3A). Leaf PCA scores for the control (WW) remained on the top left throughout the whole experiment, while stressed plants appeared on the bottom right immediately after stress and in the first hours of recovery (figure 3A). From 24 h onwards, leaf scores became closer to the control group; this reveals a clear effect of the water stress treatment, as well as a

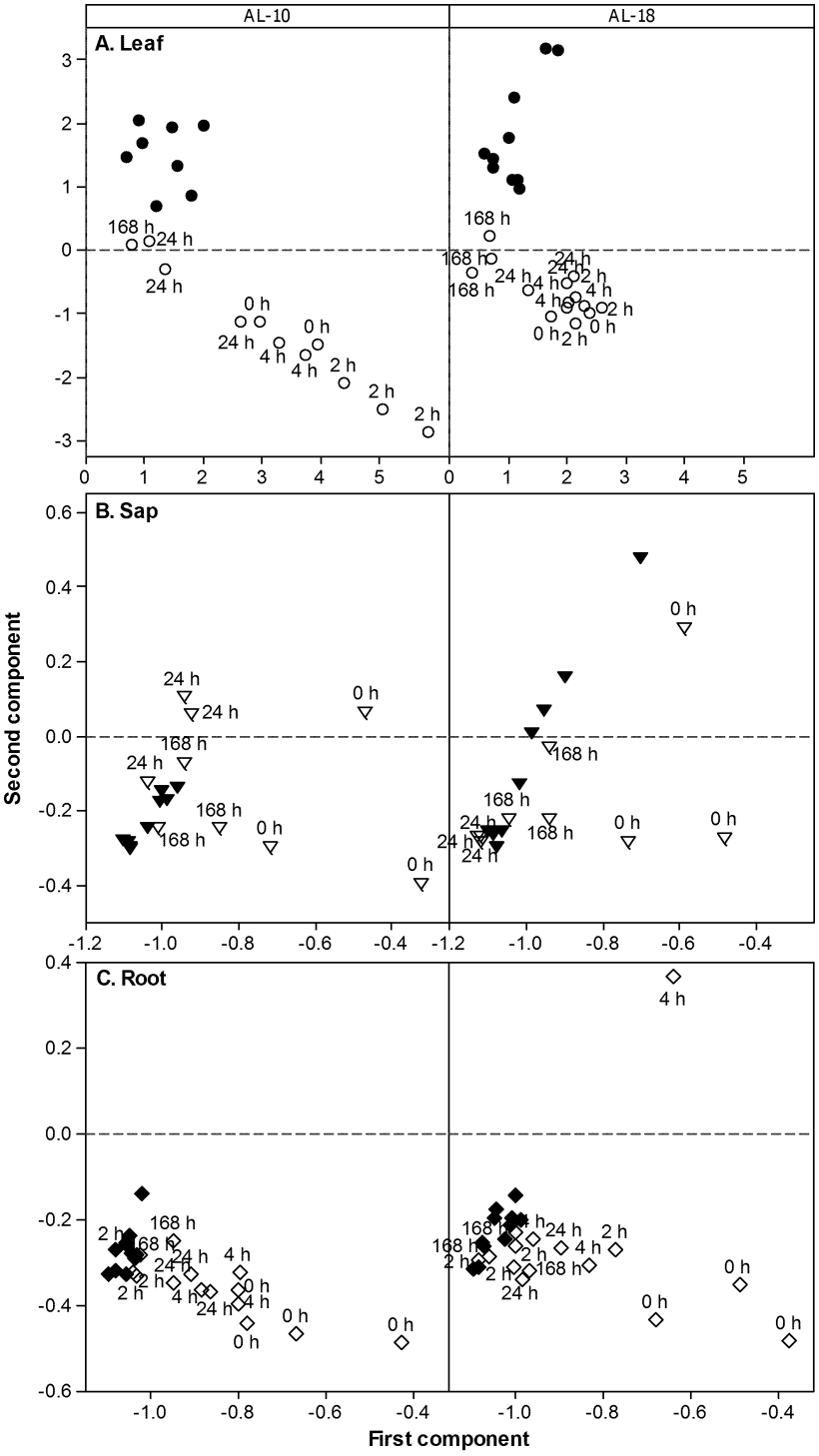

Figure 3 – Zoom in of plot of plant scores (from PCA) in terms of their hormonal profile of two *E. globulus* genotypes (AL-10 and AL-18). Each plant part is represented separately: leaf (A), sap (B) and root (C). Full symbols represent control plants (WW), while open symbols depict stressed plants (WS) before (0 h) and during the post-drought recovery period (2 h, 4 h, 24 h, and 168 h). Arrows (see figure 1) were removed for improved visualization.

distinct recovery pattern, which is much more pronounced in genotype AL-10 (figure 3A). This foliar pattern is mostly related to the increase of ABA and ABA-GE (figure 2, see gradient) during water stress and subsequent decrease during rehydration. During recovery, JA content of the leaf of stressed plants did not change over time, displaying lower scores than the controls (figure 2, see JA gradient). The PCA diagram also shows differences among genotypes, with AL-10 presenting a wider scatter – and a better definition of the gradient – compared to AL-18, especially when looking at the leaf recovery pattern. Root and xylem sap PCA scores for stressed vs. control plants grouped very closely, even when zooming in (figures 3B and C), which contrasted the clear gradient found in leaf hormones. However, there is some deviation of stressed plants at 0 h (figures 3B and C), whose scores appear slightly displaced to the right; again, this is correlated with an increase in ABA and ABA-GE content in root and xylem.

The effect of drought stress in plant hormones

Data from individual hormones corroborate the PCA ordination. When the recovery period was excluded (using only data from 0 h), a significant effect of stress was found when analysing the different plant parts in separate, except for JA in the root (table 1). Also, a significant effect of genotype was found in leaf ABA and root JA. We concluded that the two genotypes showed slight differences in the hormonal profile under stress.

Table 1 – Two-way ANOVA summary table for abscisic acid (ABA), abscisic acid glucose ester (ABA-GE) and jasmonic acid (JA) in leaf, sap and root of two *E. globulus* genotypes. Data concern the experimental period (well-watered vs. water stressed plants) before rewatering (i.e. excluding recovery, t = 0 h). Degrees of freedom (df), variance (MS), *F*-value and respective significance (*p*) are shown.

Hormone	Plant tissue	Source of variation	df	MS	*F*	*p*
ABA	Leaf	Genotype	1, 11	0.120	31.1	< **0.001**
		Stress	1, 11	0.255	66.0	< **0.001**
		Genotype × Stress	1, 11	0.00001	0.00	0.966
	Sap	Genotype	1, 8	0.0337	0.85	0.383
		Stress	1, 8	0.225	56.8	< **0.001**
		Genotype × Stress	1, 8	0.00266	0.67	0.436
	Root	Genotype	1, 12	0.000142	0.09	0.796
		Stress	1, 12	0.0704	44.9	< **0.001**
		Genotype × Stress	1, 12	0.000360	0.23	0.641
ABA-GE	Leaf	Genotype	1, 10	0.0300	4.49	0.060
		Stress	1, 10	0.262	39.1	< **0.001**

		Genotype × Stress	1, 10	0.00190	0.28	0.606
	Sap	Genotype	1, 8	0.000129	4.65	0.063
		Stress	1, 8	0.000392	14.2	**0.006**
		Genotype × Stress	1, 8	0.000037	1.32	0.284
	Root	Genotype	1, 11	0.00382	1.50	0.246
		Stress	1, 11	0.126	49.4	< **0.001**
		Genotype × Stress	1, 11	0.00254	0.68	0.426
JA	Leaf	Genotype	1, 10	0.0144	2.27	0.163
		Stress	1, 10	0.295	46.4	< **0.001**
		Genotype × Stress	1, 10	0.00010	0.02	0.903
	Sap	Genotype	1, 8	0.000130	0.02	0.898
		Stress	1, 8	0.0637	8.67	**0.019**
		Genotype × Stress	1, 8	0.00141	0.19	0.673
	Root	Genotype	1, 12	0.00773	6.69	**0.024**
		Stress	1, 12	0.000085	0.07	0.791
		Genotype × Stress	1, 12	0.000251	0.22	0.650

Stress relief in plant hormones

During rewatering, slight genotype differences in the response profile of ABA, ABA-GE and JA content were also found (figures 4, 5 and 6, respectively). The response profile of these hormones was analysed separately for each genotype (see below). With respect to ABA, WS leaves presented higher endogenous levels than WW in both genotypes, 6 nmol gFW^{-1} in genotype AL-10 and 4 nmol gFW^{-1} in genotype AL-18 (figure 4A); these levels remained higher in WS plants up to 4 h after rewatering. From 24 h onwards, ABA content in WS plants significantly decreased in both genotypes; similar values to those of WW were reached one week later (2 nmol gFW^{-1} at 168 h). In xylem sap (figure 4B), ABA content of WS samples significantly declined from 1.5 nmol mL^{-1} at 0 h to 0.25 nmol mL^{-1} at 24 h, with the latter value almost coinciding with those of WW samples for both genotypes, showing faster recovery than at the leaf level. The endogenous ABA concentrations in the WS roots of the two genotypes (about 0.4 nmol gFW^{-1}, figure 4C) were even lower than those obtained in xylem sap. After 2 h, the WS ABA concentration significantly decreased to reach practically the same concentrations obtained in WW roots. As it can be seen in figure 4, the resettlement of ABA to control levels occurred first in WS roots (2 h), followed by WS xylem sap (at 24 h), while ABA levels from WS leaves resettled later (168 h). This ABA pattern was consistent between both genotypes.

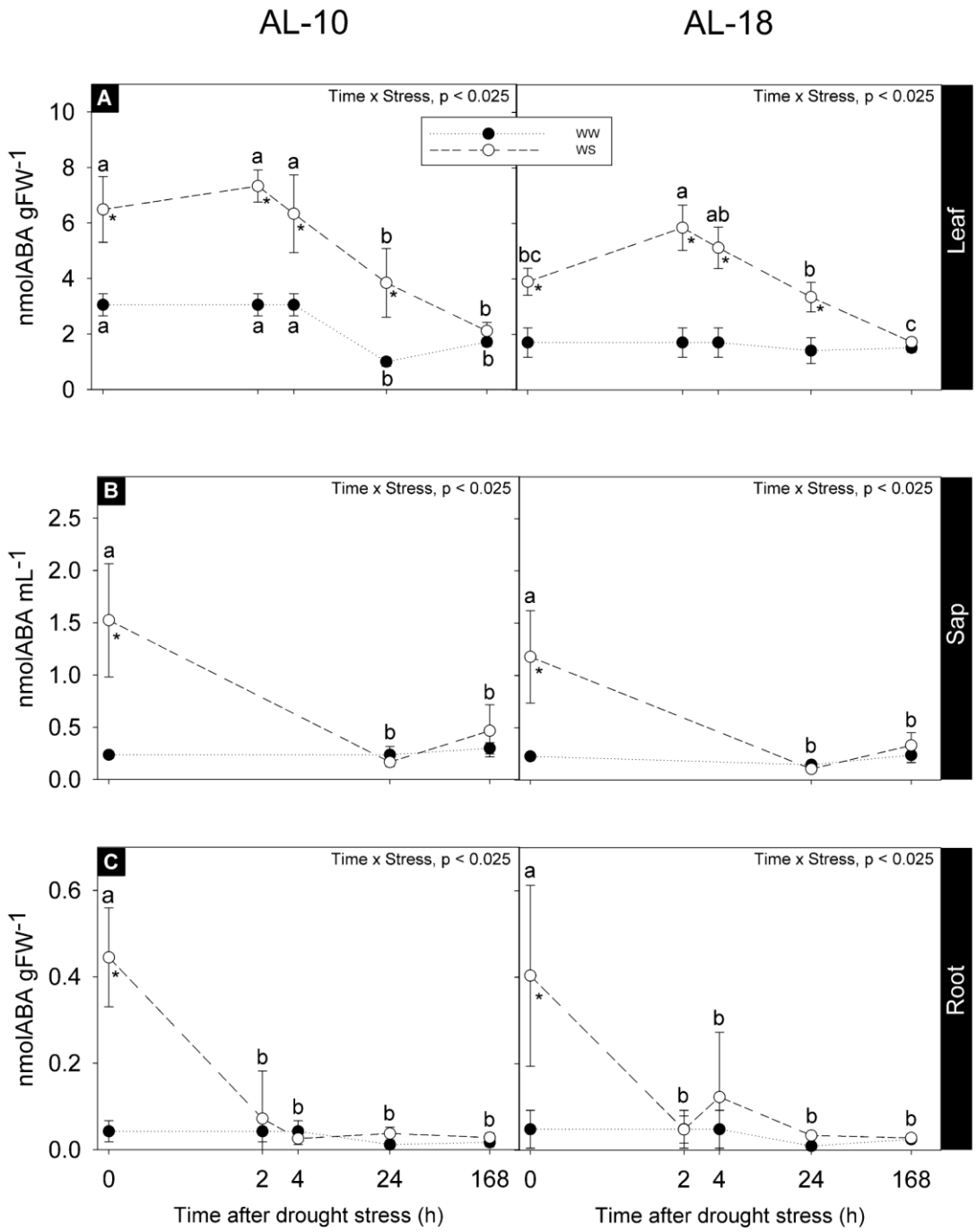

Figure 4 – Abscisic acid (ABA) content in the leaf, sap and root of water stressed (WS, dashed lines and open circles) and well-watered plants (WW, dotted line and filled circles) throughout a one-week post-stress rewatering period (i.e., recovery period). Data are shown as mean ± standard error; when significant, terms of the two-way ANOVA (time × stress) are shown. Different letters represent significant temporal differences in hormone levels for each water stress treatment; asterisks represent significantly different hormone levels in stressed plants relatively to the control treatment (well-watered plants), at each sampling time. Time (x) is shown in logarithmic [log (x + 1)] scale.

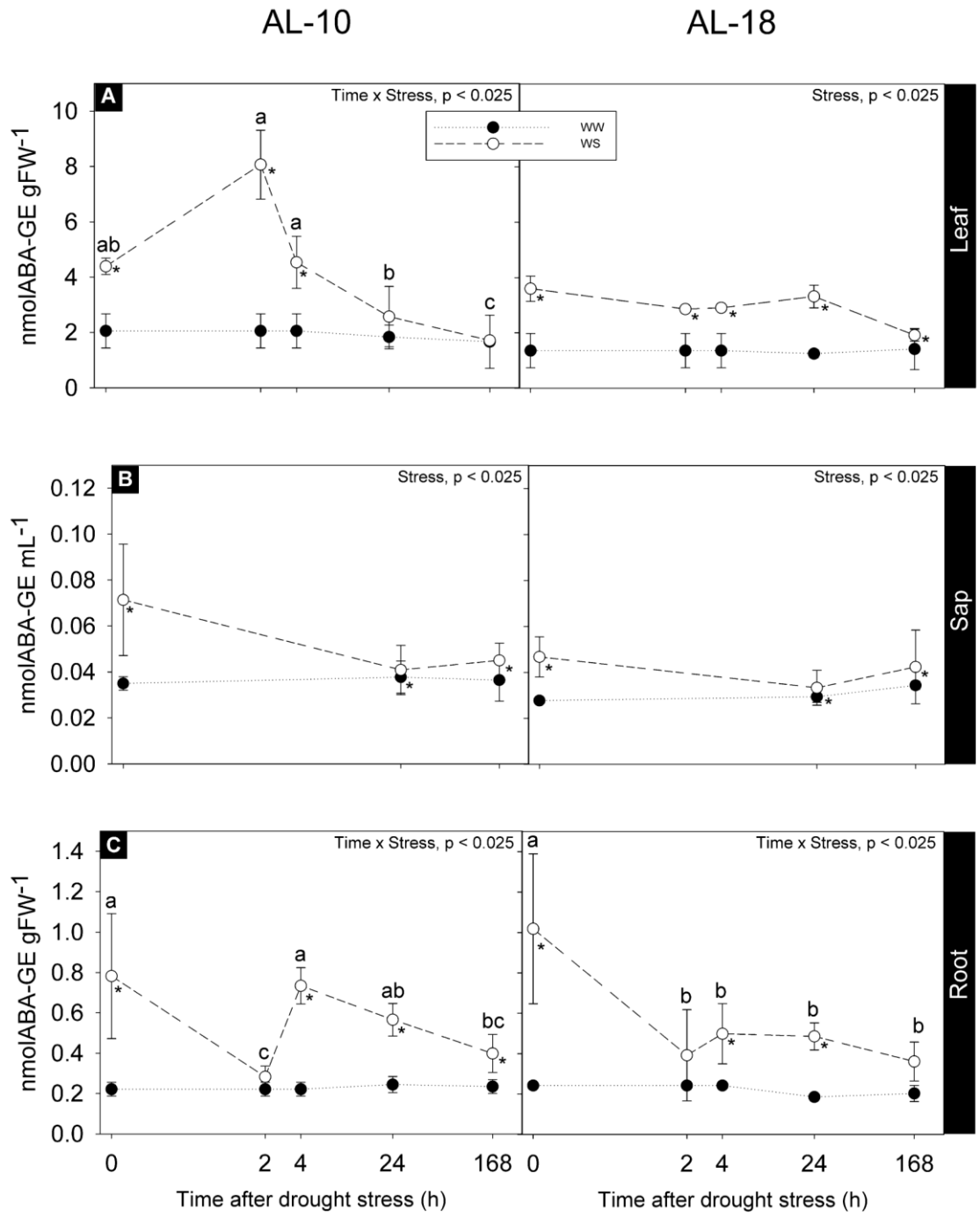

Figure 5 – Abscisic acid glucose ester (ABA-GE) content in the leaf, sap and root of water stressed (WS, dashed lines and open circles) and well-watered plants (WW, dotted line and filled circles) throughout a one-week post-stress rewatering period (i.e., recovery period). Data are shown as mean ± standard error; when significant, terms of the two-way ANOVA (time × stress) are shown. Different letters represent significant temporal differences in hormone levels for each water stress treatment; asterisks represent significantly different hormone levels in stressed plants relatively to the control treatment (well-watered plants), at each sampling time. Time (x) is shown in logarithmic [log (x + 1)] scale.

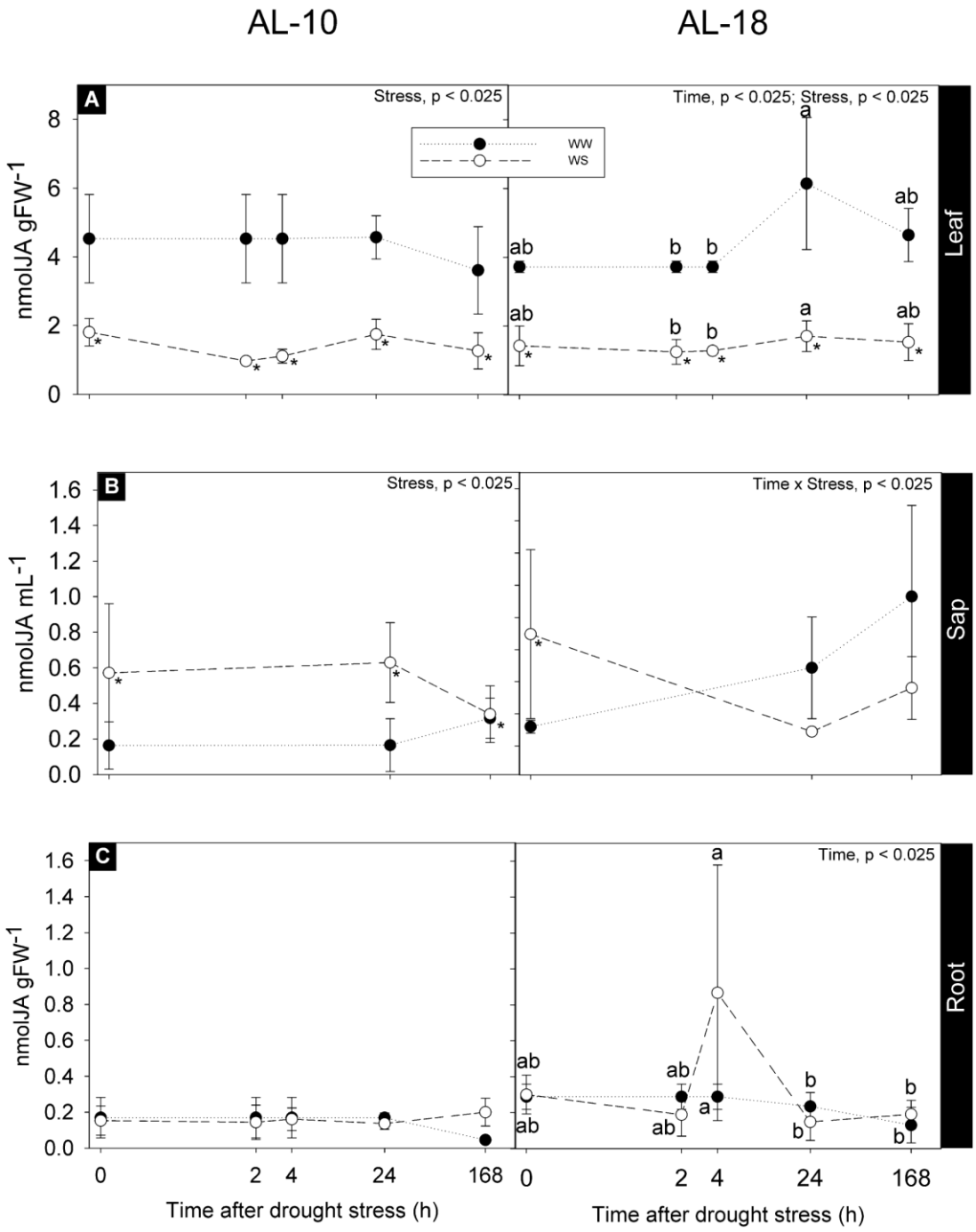

Figure 6 – Jasmonic acid (JA) content in the leaf, sap and root of water stressed (WS, dashed lines and open circles) and well-watered plants (WW, dotted line and filled circles) throughout a one-week post-stress rewatering period (i.e., recovery period). Data are shown as mean ± standard error; when significant, terms of the two-way ANOVA (time × stress) are shown. Different letters represent significant temporal differences in hormone levels for each water stress treatment; asterisks represent significantly different hormone levels in stressed plants relatively to the control treatment (well-watered plants), at each sampling time. Time (x) is shown in logarithmic [log (x + 1)] scale.

The changes in ABA-GE concentrations differ in terms of their response profile in leaves (figure 5A) and roots (figure 5C). Water stressed leaves of the AL-10 genotype showed a high ABA-GE content at 2 h (8 nmol gFW^{-1}, figure 5A), which significantly decreased over time to values similar to those of WW plants after 24 h of recovery (2 nmol gFW^{-1}). In contrast, WS leaves from AL-18 did not show any significant response, with a constant level of ABA-GE (4 nmol gFW^{-1}) during the early rehydration period. Although still statistically different, the concentration of WS leaves was only comparable to that of WW plants after 168 h (figure 5A), demonstrating a slower response of this genotype to rehydration. Regarding the endogenous ABA-GE concentrations of the xylem sap, slight but consistent differences over time were found between WS and WW samples, with the former showing higher ABA-GE content in both genotypes (figure 5B). The ABA-GE of WS roots also exhibited a similar profile in both genotypes (figure 5C). The high content observed after the stress period (about 1 nmol gFW^{-1}) decreased immediately after 2 h of rewatering, but increased again at 4 h; from this point onwards, the ABA-GE content decreased to 0.4 nmol gFW^{-1} (at 168 h), which is close to the concentrations observed in WW roots (figure 5C).

The endogenous JA concentrations were fairly consistent between genotypes, whereas differences were found among plant parts, as shown in figures 6A (leaf), 6B (xylem sap) and 6C (root). Inversely to ABA and ABA-GE, JA content of the WS leaves was significantly lower than that of WW plants (figure 6A). Indeed, these differences were consistent throughout the experimental period to both genotypes (2 nmol gFW^{-1} in WS and 4 nmol gFW^{-1} in WW leaves). In both genotypes, the JA content in the xylem sap (figure 6B) exhibited a higher concentration in WS samples compared to WW at 0 h. During the rehydration period, differences between WS and WW plants tended to disappear, although this general pattern is sometimes obscured by the large variation in the data. JA content of the roots persisted low (0.2 nmol gFW^{-1}) before and during the rehydration period (figure 6C).

Discussion

Several authors (Chaves et al. 2009; Furlan et al. 2012; Kirschbaum 1988) have stressed the need for comprehensive studies on plant responses to rehydration, considering that plant recovery from drought is a basic trait that accounts for plant survival, reflecting the balance between damaged structures reconstruction and adequate metabolism restoration. The capacity of *E. globulus* to recover after a drought period was already reported by Correia et al. (2014) and it is highlighted here by the results of foliar net CO_2 assimilation rate, which reflect the effect of water deficit in the photosynthetic performance and the prompt recovery after rewatering, clearly dependent on the genotype (figure 1). The regulation of plant responses to biotic and abiotic stress

by the interaction of phytohormones has been well studied (Brossa et al. 2011; De Ollas et al. 2013; Fujita et al. 2006; Moons et al. 1997). These studies demonstrate the interplay between ABA and JA in several species (e.g., *Citrus*, rice, *Arabidopsis*), with different signalling interaction pathways being reported. Moreover, several authors (Bari and Jones 2009; Jackson 1997) have raised an important issue on how different hormone-mediated responses are regulated in specific tissues. Thus, we have focused our research on the hormonal interactions that occur among leaves, xylem sap and roots of the *E. globulus* genotypes AL-10 and AL-18 taking place after a mid-term drought stress and during the rehydration period. On the basis of a previous study (Correia et al. 2014), which enabled us to characterise these two genotypes' profiles, we now expect to relate hormonal dynamics to specific clonal metabolism.

Our preliminary PCA analysis shows that the hormonal variations (especially ABA and ABA-GE) are more pronounced depending on the plant part rather than water stress treatment or genotype. Although several studies have already reported the hormonal dynamics taking place from root to shoot (e.g. Hartung et al. 2002; Jackson 1997; Sauter et al. 2001), this dependence constitutes a novel observation and underlines the need to take the plant part into account when analysing the hormonal response during stress.

Following this result, leaves from genotypes AL-10 and AL-18 showed a similar pattern of ABA dynamics under WS conditions. WS led to foliar ABA increases by two-fold if compared to that of WW leaves; also, these levels were slightly higher in genotype AL-10 than in genotype AL-18. As suggested (Correia et al. 2014), this could be related to specific water saving dynamics of AL-10 genotype. Additionally, WS induced a two-fold ABA-GE increase in AL-10 genotype leaves, which was not observed in AL-18 leaves. On the other side, the JA content in WS leaves was lower than that of WW plants in both genotypes. Regarding the differences between the genotypes, clone AL-10 exhibited more ABA/ABA-GE and less JA content compared to clone AL-18 after the dehydration period. This result is opposite to the obtained in other works, in which JA has been shown to increase after a drought period in spear tips of *Asparagus officinalis* (Gapper et al. 2002), *Carica papaya* seedlings (Mahouachi et al. 2007) and *Pinus pinaster* plants (Pedranzani et al. 2007). Regarding the function of JA in plant defence against stress, it has been suggested that JA may interact with ABA synthesis under water stress conditions (Adie et al. 2007; Bandurska et al. 2003; De Ollas et al. 2013). Moreover, the role of JA in the regulation of ABA biosynthesis has been addressed (Brossa et al. 2011; Kazan and Manners 2008) and several authors suggested that ABA regulates JA biosynthesis in ABA-treated *Arabidopsis* plants (Adie et al. 2007; Kazan and Manners 2008).

The crosstalk between ABA, ABA-GE and JA is in agreement with the work of Anderson et al. (2004), which explored the antagonistic interplay between ABA and the JA-ethylene signalling

pathways in the dehydration response in *Arabidopsis*. Also, the absence of significant interactions between clone and water stress in each hormone-plant part may reveal a well-conserved drought response mechanism.

During rewatering, levels of ABA decreased in each plant part of both genotypes thus confirming its role as a dehydration signal which moves from the root to the leaf (Hartung et al. 2002; Wilkinson and Davies 2002). Accordingly, after the stress relief, the decrease in ABA content takes place first in root and xylem sap and finally in leaves. On the other hand, ABA-GE exhibits a more dynamic response between the genotypes. The ABA-GE content of genotype AL-10 decreased first in leaves than roots, while in genotype AL-18 this decrease was consistent between these two tissues. In xylem sap, no remarkable variations were observed. These results support the role of ABA-GE as long distance transport from root to leaves (Wilkinson and Davies 2002) in genotype AL-18 and add the role of ABA-GE as an ABA metabolite and an additional hormonal stress signal (Hansen and Dörffling 1999) in genotype AL-10. It is important to note that ABA-GE levels do not always vary in parallel to the change in ABA levels, since conjugation can be regulated in particular tissues and conditions (Nambara and Marion-Poll 2005). Because ABA-GE is a precursor of ABA and its levels also increased in WS plants from genotype AL-18, we suggest that the observed increase in ABA content could be related, at least in part, to its breakdown from endogenous ABA-GE in this genotype. Sauter et al. (2002) already pointed that β-D-glucosidase releases ABA from ABA-GE in some plant species.

Regarding JA, no clear effects during drought relief were observed either in genotype AL-10 or in genotype AL-18; thus, water stress reduced foliar JA content, but the stress relief did not alleviate this decrease. Given the distinct pattern observed relatively to ABA and ABA-GE, we suggest that ABA is not likely to be responsible for the JA reduction in the dehydration response as previously reported (Anderson et al. 2004), therefore other mechanisms should be involved. Hereupon, other phytohormones and their interactions should be assessed in further research.

In conclusion, our work addresses some questions about ABA, ABA-GE and JA variations on leaves, xylem sap and roots of AL-10 and AL-18 *E. globulus* genotypes. Our results showed that genotypes AL-10 and AL-18 responded differently to water stress, characterising the response pattern to drought of clone AL-10 as a more responsive one. More studies are needed to clarify the way plants regulate the balance between ABA synthesized *de novo* and ABA released from ABA-GE. Besides, we argue that leaf should be the plant part to be used as a preliminary screening method of stress tolerance, not only because it offers a better perspective of the variations, but also because it is easier to obtain (less damaging to the plant), when compared to root or xylem sap. The hormonal dynamics were related to the previously documented responses of these two *E. globulus* genotypes and sustain further physiological and molecular studies of water stress in this and other

tree species. Despite our findings, an exhaustive research on hormone signalling is required for a better comprehension of different metabolisms.

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2.3. Integrated proteomics and metabolomics to unlock global and clonal responses of *Eucalyptus globulus* recovery from water deficit

Summary

Water availability is well known for impacting productivity of *Eucalyptus* but comprehensive knowledge on cellular pathways involved in recovery and tolerance is scarce. In this context, we aimed to unveil putative mechanisms that account for drought recovery of *Eucalyptus globulus*, and to identify specific strategies that make a clone more adapted to water deficit. We resorted to comparative proteome (using difference gel electrophoresis) and metabolome [using gas chromatography–mass spectrometry (GC-MS)] analyses in two *E. globulus* clones that exhibit physiological differences in their capacity to tolerate water shortage and restoration; also, interpretable networks were constructed coupled with previously assessed physiological matrices in order to interrogate the large datasets generated and develop a clear and integrative analysis. Our study enabled the separation and isolation of 2031 peptide spots, 217 of which were identified. GC-MS yielded the detection of 121 polar metabolites. Water shortage negatively affected photosynthesis, gene regulation, cell growth and secondary metabolites; enhanced photo protection, osmoprotection, and other defence-related pathways; and caused a shift from chloroplastic to mitochondrial energy generation. Recovery was characterized by upregulation of all previously described pathways. The analysis of the resilient clone AL-18, which presented a network very distinct from the responsive clone AL-10, reinforced the role of specific photosynthetic and defence-related proteins as key players in mediating drought tolerance and revealed new players: glutamine synthetase, malate dehydrogenase and isoflavone reductase-like protein. This study provides a set of novel proteins and pathways involved in drought stress that represent potential drought tolerance markers for early selection of *Eucalyptus*.

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ORIGINAL ARTICLE

Integrated proteomics and metabolomics to unlock global and clonal responses of *Eucalyptus globulus* recovery from water deficit

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Introduction

Eucalypts represent one of the most significant pulpwood planted genera in the world (Freeman et al. 2013). *Eucalyptus globulus* Labill. is successfully cultivated in the Mediterranean region, but experiencing environmental stress is unavoidable for this plant species particularly in light of a rapidly changing environment where drought is one of the major abiotic factors limiting plant growth (Galmés et al. 2007). Water deficit can disrupt cell structures and impair biological functions leading to inhibition of photosynthesis, metabolic dysfunction, and damage to membranes and proteins (Krasensky and Jonak 2012).

Due to its global significance, the impact of drought stress on *Eucalyptus* has received much scientific interest and most of the morphological and physiological responses that occur during water deficit are already documented (Costa e Silva et al. 2004; Shvaleva et al. 2006; Coopman et al. 2008; Granda et al. 2011; Correia et al. 2014a, b). Earlier studies from our group indicate that *E. globulus* responds to drought mainly by reducing stomatal conductance, gas exchange, height, biomass and water potential; whilst pigments (chlorophylls and carotenoids), malondialdehyde (MDA) and some hormones, such as abscisic acid (ABA) increase (Correia et al. 2014a, b). However, studies that explore the capacity for drought recovery of this species are less abundant (Correia et al. 2014a, b; McKiernan et al. 2015) and little is known about the cellular pathways regulating recovery from stressful conditions in *Eucalyptus*.

As previously stressed by other authors, proteomic and metabolomic variations are far less researched in forest species than in herbaceous plant species (Warren et al. 2011a; Valdés et al. 2013). Valdés et al. (2013) analysed two *E. globulus* provenances with contrasting drought tolerance and found differences in morphology and accumulation of endogenous contents of ABA and proteins involved in abiotic stress processes in the tolerant provenance. Warren et al. (2011a) used gas chromatography–mass spectrometry (GC–MS) to examine the response of leaf metabolites to a 2 month water stress in two species of *Eucalyptus* (*E. pauciflora* and *E. dumosa*) reporting that metabolites were differentially affected during both water stress and rewatering with species-related variations. McKiernan et al. (2014) investigated the influence of water availability on a range of foliar secondary metabolites in juvenile *E. globulus* and *E. viminalis*. These authors concluded that significant species variation occurred but with a minimal treatment effect. These studies highlight the need to incorporate multidisciplinary approaches that investigate plants' ability to recover and unveil the cellular pathways involved. In order to reduce this gap in our knowledge of the cellular processes taking place in *E. globulus* after drought imposition and rewatering, we performed comparative proteome and metabolome analyses using two clones that exhibit differences in their capacity to tolerate water shortage and restoration. In addition,

considering the large amount of experimental data generated and the need for a clear, integrated and meaningful view of the investigated biological events, we resorted to the construction of interpretable networks coupled with previously assessed physiological matrices. This approach has led to novel insights regarding the processes involved in drought tolerance and recovery in *E. globulus*. Our main goals were: (i) to unveil putative mechanisms that account for the extraordinary ability of *E. globulus* to recover from water deficit, and (ii) to identify specific strategies that make a clone more adapted to water deficit conditions.

Materials and methods

Plant material and experimental design

This study was undertaken in two clones of *Eucalyptus globulus* Labill. (AL-18 and AL-10) obtained from Altri Florestal SA (Portugal). These clones are planted in Portuguese areas and were chosen because of their differential physiological and biochemical responses to water shortage and restoration (Correia et al. 2014b; Correia et al. 2014a). Clone AL-18 was identified as a resilient clone, less reactive to water availability, and AL-10 is considered as more sensitive and responsive in responding to drought stress and rehydration.

Six month old rooted cuttings of each clone grown in plastic containers filled with 3:1 (w/w) vermiculite:peat with an initial height of 30 cm were transplanted to 2 L plastic pots filled with 350 g of a 3:2 (w/w) peat:perlite mixture and transferred from a shaded house to a greenhouse for acclimation for one-month where plants were automatically watered with nutritive solution to 80% field capacity (FC). To minimise effects of environmental heterogeneity, the pots were randomly arranged and periodically moved to the neighbouring position during the whole experiment.

After the acclimation period, AL-18 and AL-10 cuttings were divided into two groups: one group was assigned to a well-watered regime (WW: water supplied daily until soil water content reached 80% FC) and a second group was assigned to a water stress regime (WS: water supplied daily until soil water content reached 25% FC) for 7 days. Pot weight was monitored everyday gravimetrically. After this period, the stress in the WS group was intensified (water supplied daily until soil water content reached 18% FC). This lasted for 14 days prior to the initial sampling (stress point, WW and WS plants sampled). After this period, the WS cuttings were re-watered to the well-watered regime and recovery was monitored one day (1dR) and seven days (7dR) after rehydration. For each treatment group (i.e. WW, WS, 1dR, 7dR), homogeneous leaves from six random individuals were immediately used for physiological analyses or alternatively frozen in liquid nitrogen for further biochemical, proteomic and metabolomic analysis.

Physiological analysis

Previously published physiological data (Correia et al. 2014b, chapter 2.1) were introduced into our data analysis in order to obtain an integrated and meaningful interpretation of the proteome and metabolome datasets. Data are summarised in supplementary table S1 and full experimental details are published elsewhere (Correia et al. 2014b). The physiological status of the plants was assessed by monitoring several parameters, which included: height, number of stems and biomass; water potential and relative water content (RWC), content of MDA, pigments (total chlorophylls and carotenoids) and abscisic acid (ABA); effective (Φ_{PSII}) and maximum (F_v/F_m) quantum yield of photosystem II and non-photochemical quenching (NPQ); gas exchange (photosynthetic (A) and transpiration (E) rates), and stomatal conductance (g_s).

Proteomics analysis

Protein separation and analysis

The proteomic analysis described by Vítámvás et al. (2012) was undertaken with slight modifications. Foliar tissue (200 mg) was ground in 2 mL microtubes with a bead grinder 3 times (30 s with 1 min in liquid nitrogen between cycles). Powder was then resuspended in 2 mL ice-cold 10% trichloroacetic acid in acetone and incubated for 1 h at – 20°C. After centrifugation at 10000 ×g for 5 min (4°C), the resulting pellet was washed twice with cold acetone and then dried in a speed vac. The dry pellet was resuspended in 0.6 mL SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl pH 8.0, 5% 2-mercaptoethanol) and 0.6 mL phenol (Tris-buffered pH 8.0) and vortexed for 30 min at 1200 rpm. Samples were then centrifuged at 10000 ×g for 5 min and 300 µL of the upper phenol phase was transferred to a fresh microtube. Five volumes of ice-cold methanol containing 0.1 M ammonium acetate were added to the phenol phase and stored for 30 min at −20°C. Precipitated proteins were recovered by centrifugation at 10000 ×g for 5 min and the pellet was washed twice with ice-cold methanol-0.1 M ammonium acetate and then twice with ice-cold 80% acetone. The pellet was finally dried in a speedvac and dissolved in labelling buffer (30 mM Tris, 7 M urea, 2 M thiourea, 2% w/v CHAPS). The pH of the lysate was adjusted to 8.5 by the careful addition of 50 mM sodium hydroxide and protein concentration was quantified by the Bradford method (Bradford 1976) using BSA as standard.

Protein labelling for DIGE analysis and protein migration

Proteins, 30 µg for each sample, were labelled with the CyDyes minimal labelling method (GE Healthcare) following the procedure reported by Printz et al. (2013) and separated in two dimensions as described in the same study. Cy2 was used for the internal standard, composed of a

balanced amount of each of the samples, whereas Cy3 and Cy5 were used to label the different samples, applying a dye swap to avoid biasing of the results due to differential labelling. The labelling was carried out according to the manufacturer's instructions. Labelled samples were pooled into groups of three such that each pool contained an equal ratio of proteins marked with Cy2, Cy3, and Cy5, and lysis buffer was added to reach a final volume of 120 µL. Finally, 0.72 µL of Destreak Reagent (GE Healthcare) and 2% (v/v) Biolyte (pH 3−10) (Bio-Rad) were added. For each clone and treatment, 1 additional charged gel with a total protein load of 300 µg was run and dedicated to picking. The samples used for running the charged gels consisted of 270 µg of unlabelled protein and 30 µg of proteins labelled with one of the CyDyes. The inclusion of labelled protein in the charged gels allowed the matching of the charged gels with the gel images used for the differential expression analysis. Lysis buffer was added to a final volume of 450 µL with further addition of 2.7 µL of Destreak Reagent and 2% (v/v) Biolytes.

Gel scanning and analysis

Gels were scanned using a Typhoon FLA 9500 (GE Healthcare) at 100 µm resolution following the manufacturers' instructions. Gels containing four biological replicates were analysed using the software Decyder 2D v7.0 (GE Healthcare). A first selection was based on a one-way ANOVA (analysis of variance) to compare all the samples. Then, spots of different samples were compared by calculating the ratio between the average intensity in the different conditions. Only absolute fold changes superior to 1.5 (Student's t test, p-value ≤ 0.05) were considered as significantly different between the conditions.

Spot picking, digestion and MS analysis

Picking, digestion and MALDI spotting were carried out using the Ettan Spot Handling Workstation (GE Healthcare) according to the protocol described by Printz et al. (2013). After extraction, the peptides were dried and spotted on MALDI-TOF target plates and a MALDI peptide mass spectrum was obtained using the Applied Biosystems 5800 TOF/TOF (Applied Biosystems). The 10 most abundant peaks, excluding known contaminants, were selected and fragmented. MS and MS/MS spectra were submitted for database-dependent identification using the NCBInr database (downloaded on the 30th of October 2014 with the taxonomy viridiplantae (http://www.ncbi.nlm. nih.gov, 2447481 sequences) using an in-house MASCOT server (Matrix Science, www.matrixscience.com, London, U.K.). A secondary search was carried out against Eucalyptus genome version 1 (downloaded from Phytozome, 277890 sequences). The parameters used for these searches were mass tolerance MS 100 ppm, mass tolerance MS/MS 0.50 Da, fixed modifications cysteine carbamidomethylation and variable modifications methionine oxidation,

double oxidation of tryptophan and tryptophan to kynurenine. Proteins were considered as identified when at least two peptides passed the MASCOT-calculated 0.05 threshold score (= 40). However, when a high-scoring peptide (score > 2 times the threshold score) was matched and a protein p-value of $< 1 \times 10^{-5}$ was obtained, the protein was also accepted. The MASCOT-based results were manually validated afterwards. In order to decrease the potential of co-migrating non-identified proteins and the impact this could have on the interpretation of the gel images, the list of matched peptides was compared to the MS spectrum. When high-intensity peaks were not matched to the identified protein, the corresponding MS/MS spectra were researched and/or the sequence of the peptide was de novo determined. Spots with peaks in the MS spectrum matched to a second protein were excluded from biological interpretation. A matched peptide that contained one of the oxidized forms of tryptophan was also checked by analysing the presence of peaks corresponding to the same peptide with other oxidation products of this residue (Trp; Trp +4 Da = kynurenin, Trp +16 Da = oxidized Trp; Trp +32 Da = N-formylkynurenin). This enabled the delineation of several signal and transit peptides and the discovery of different molecular forms of the same protein in some of the spots. Finally, the MS/MS spectra presenting a score around the MASCOT threshold ($p < 0.05$) were validated by matching high intensity peaks and presenting well-known sequence-dependent characteristics. These easy-to-recognise spectral features included the presence of a peak corresponding to the C-terminal arginine and the presence of the neutral loss of 64 Da from peptides containing oxidized methionine. The impact specific residues (most notably proline and aspartic acid) had on the intensity of fragment peaks was respected. When high quality spectra did not result in a significant identification, manual de novo sequence analysis was applied and/or extra peaks were fragmented to confirm near-to-threshold identifications.

Metabolomics analysis

Metabolite separation and analysis

Metabolites were analysed according to an adaption of the procedure previously reported by Weckwerth et al. (2004). Using a mortar and a pestle, polar metabolites were extracted from frozen leaves (50 mg fresh weight) in 1 mL of cold extraction buffer [methanol:chloroform:10% acetic acid (2.5:1:0.5)]. After vortexing, samples were centrifuged at 15000 × g for 10 min at 4ºC. The supernatants were transferred to new tubes containing 800 µL of water: chloroform (1:1) and vortexed. The mixtures were then centrifuged at 15000 × g for 10 min at 4ºC and the upper layer (polar phase) containing water-soluble metabolites were transferred to new tubes and completely dried by speed vac. Samples were derivatised before injection as described in Furuhashi et al. (2012). Twenty microliters of methoximine hydrochloride (40 mg mL^{-1} in pyridine) was added and the samples incubated for 90 min at 30°C while shaking. This was followed by addition of 80 µL

of MSTFA and further 30 min incubation at 37°C while shaking. The mixture was finally centrifuged at 21000 g for 2 min, and the supernatant was transferred into a glass micro-vial.

GC-MS procedure and analysis

GC-MS measurements were carried out following the protocol previously developed (Valledor et al. 2013) on a triple quad (TSQ Quantum GC, Thermo) instrument. In brief, 1 µL per sample was injected and GC separation was performed on a HP-5MS capillary column (30 m × 0.25 mm × 0.25 mm; Agilent Technologies). Oven temperature increased from 80ºC to 200ºC at 3ºC min^{-1} then from 200ºC to 250ºC at 10ºC min^{-1} and was finally held at 250ºC for 3 min. Temperature was held at 300ºC for 4 min as post run condition. The mass spectrometer was operated in electron-impact (EI) mode at 70 eV in a scan range of m/z 40-600. Metabolites were identified based on their mass spectral characteristics and GC retention times, by comparison with retention times of reference compounds from an in-house reference library. Peak areas corresponding to each metabolite were normalised to the total peak area in the sample.

Statistical and multivariate analysis

All statistical procedures described were performed using the software R v3.1.2 (R Core Team 2014) core functions plus the package mixOmics following the recommendations of Valledor and Jorrin (2011).

Two-way ANOVA were applied to each variable of proteomic and metabolomic datasets (supplementary material, tables S2-4), considering clone and water treatment as factors. Proteomic and metabolomic datasets were analysed following log transformation to meet the requirement for normality and homocedasticity. Differences were set for a significance level of 0.05. Due to the low number of comparisons, false discovery rate was not controlled.

A complete dataset comprising proteomic, metabolomic and previously reported physiological data (Correia et al. 2014b) from WW, WS, and 7dR was subjected to principal component analysis (PCA) and correlation network analysis. These analyses were conducted employing the R package mixOmics v.4.0.2 (González et al. 2011). In order to identify clonal specific differences, PCA and network analyses were separately conducted for the clones AL-18 and AL-10.

sPLS was used to integrate physiological and molecular data with the aim to highlight the interactions between the different levels of organization within the system. Specifically, proteomics data was used as predictor for metabolomics and physiological data. Generated networks were visualized and filtered (only nodes with correlations equal or higher than 0.8 were maintained) in

Cytoscape v.2.8.3, using the Force Directed layout, which is based on the "force-directed" paradigm.

Results

Proteome and metabolome profiling

The proteome profile of the two *E. globulus* clones after drought and during rewatering was established using proteins extracted from leaves of 32 individuals (4 biological replicates of WW, WS, 1dR and 7dR from clones AL-18 and AL-10). DIGE enabled the separation and isolation of 2031 spots (Supplementary Table S2), 217 of which could be identified (Supplementary Tables S3, S4). Considering the identified proteins, the abundance of 124 was significantly altered because of the watering dynamics and 68 revealed significant differences between the genotypes. The abundance of 32 proteins was significantly affected by interaction between watering dynamics and tree clone.

The foliar metabolic profiles of the two genotypes subjected to drought and rewatering were compiled using GC–MS. The analysis of 24 samples (4 biological replicates of WW, WS and 7dR from clones AL-18 and AL-10) yielded the detection of 121 polar metabolites (Supplementary Table S5). Of these, exact metabolite identities could be assigned to 62, 44 were characterised according to their molecular composition and 15 remained as unknown compounds. From the identified or characterised metabolites, 35 showed significant changes due to the watering dynamics and 15 metabolites were differently abundant between genotypes. Only two metabolites exhibited a clone × watering interaction in their abundance.

Drought-induced changes in primary metabolism reflect decreased photosynthesis that is rapidly restored after rewatering, and enhanced photo and osmoprotection

At the photosynthetic electron transfer chain level, photosystem II (PSII) reaction centre PSB28, plastocyanin 1 and cytochrome b559 increased in WS, a trend that was maintained during rehydration (table 1). Ferredoxin–thioredoxin reductase also increased during WS and recovery. Taken together, these results indicate the induction of cyclic electron transfer through PSII, relieving overreduction of the electron transport chain and thereby suppressing the generation of $^{3}P680^{*}$ and $^{1}O_2$. The latter occurs under conditions of excess excitation energy caused by a reduced availability of CO_2, lower CO_2 assimilation rates and a lower demand for reducing power (Asada 2006).

Several proteins involved in the Calvin cycle were significantly altered in abundance in response to drought stress (table 1). The RuBisCO (ribulose-1,5-bisphosphate

carboxylase/oxygenase) large subunit, plastidic aldolase, transketolase, and phosphoribulokinase were all less abundant under WS conditions, however the RuBisCO small subunit increased in abundance at this sampling point. This reduction in Calvin cycle enzyme abundance was concomitant with the parallel accumulation of ribulose-1,5-bisphosphate (ribulose-1,5-BP). Similarly, fructose, galactose, xylose, arabinose, myoinositol, ribose, pentose, hexose, and pentitol accumulated during stress (table 1). The decrease in abundance of photosynthesis-related proteins is a common stress response (Wade et al. 2002; Kottapalli et al. 2009; Sergeant et al. 2011). Beyond that, it is suggested that an overall reduction of photosynthesis-related proteins during water stress may occur in tolerant genotypes that correlated with a rapid decrease in transpiration and photosynthetic rates (Kottapalli et al. 2009). These results in *E. globulus* are in line with results of other *Eucalyptus* described by Warren et al. (2011b).

Table 1 – Impact of drought and rewatering on proteins associated with photosynthetic electron transport and carbon fixation, and primary carbohydrates. Abundance data is presented on a scale relative to the lowest value among sampling points and *p*-values for significance differences dependent on clone, watering regime or the interaction between clone and watering regime (C × W) as estimated by two-way ANOVA are indicated.

	Relative abundance (WW-WS-1dR-7dR)		**Two-way ANOVA**		
Protein	*AL-18*	*AL-10*	*Clone*	*Water*	*C x W*
Photosystem II reaction centre PSB28			0.169	**0.043**	0.834
Plastocyanin 1			0.482	**0.000**	0.587
Cytochrome b559			0.603	**0.004**	0.743
RuBisCO large subunit			0.379	**0.036**	**0.037**
RuBisCO small subunit			**0.000**	**0.000**	**0.020**
Plastidic aldolase			0.909	**0.021**	0.696
Transketolase			**0.003**	**0.000**	**0.004**
Phosphoribulokinase			0.625	**0.000**	0.608
RuBisCO activase			0.224	**0.043**	0.169
Ferredoxin-thioredoxin reductase			0.111	**0.001**	**0.015**

	Relative abundance (WW-WS-7dR)		**Two-way ANOVA**		
Metabolite	*AL-18*	*AL-10*	*Clone*	*Water*	*C x W*
Ribulose-1,5-BP			0.882	**0.018**	0.514
Fructose			0.916	**0.000**	**0.035**
Galactose			0.607	**0.000**	0.129
Xylose			0.967	**0.001**	0.601
Arabinose			0.483	**0.017**	0.369
Myo-inositol			**0.009**	**0.032**	0.507
Ribose			0.822	**0.004**	0.111
Pentose			0.880	**0.009**	0.561
Hexose			0.327	**0.000**	0.410
Pentitol			0.822	**0.002**	0.862

The RuBisCO large subunit and phosphoribulokinase increased, and RuBisCO activase showed a maximum peak following 1 day of rewatering (1dR, table 1). Most of the carbohydrates that increased during water deficit diminished after rewatering, whereas pentitol and other unknown sugars maintained a high content during rehydration (table 1). The increased abundance in some spots containing RuBisCO small subunit may indicate either degradation of RuBisCO during exposure to abiotic stress, as reported in other studies (Feller et al. 2008; Sergeant et al. 2011) or it can be related to the different cellular generation between large and small RuBisCO subunits. The maintenance of pentitol and other unspecified sugars after water deficit relief may suggest a strategy to maintain higher osmotic potential to permit stomatal opening and gas exchange under future water stress (Warren et al. 2011a).

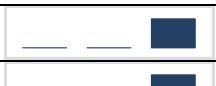
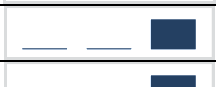
Gene regulation and cell growth were affected by water availability

The impact of drought stress on cell growth was confirmed with a reduction in the relative abundance of actin, which was recovered after prolonged rehydration (7dR, table 2). Proteome remodelling, and its related metabolism and growth pattern reprogramming, is the consequence of a complex regulatory process involving signal transduction, gene regulation, and protein biosynthesis and degradation. Regarding signal transduction mechanisms, different proteins were responsive to water availability. Calmodulin, plastid fibrillin 2, and 20 kDa chaperonin increased with water stress (table 2), representing potential indicators of enhanced cellular oxidation as a result of the environmental stress. Furthermore, transcriptional regulation was also affected: water deficit caused a decline in mRNA binding protein but rewatering led to an increment, also shown by a

maximum peak of RNA-binding family protein isoform 2 at 1dR (table 2). We were unable to detect changes at the protein translation level as we could not identify ribosomes or eukaryotic initiation factors. On the other hand, we did observe an increase in a 20 kDa chaperonin following water deficit and recovery, indicative of an increased translation level as we could not identify ribosomes or eukaryotic initiation factors. On the other hand, we did observe an increase in a 20 kDa chaperonin following water deficit and recovery indicative of an increased requirement for protein refolding. Similarly, an increase in the abundance of a polyubiquitin 4-like protein after prolonged recovery (7dR) indicated a potential requirement for protein degradation and turnover during recovery (table 2).

Table 2 – Impact of drought and rewatering on proteins associated with growth, gene regulation and plastid metabolism, and related metabolites. Abundance data is presented on a scale relative to the lowest value among sampling points and *p*-values for significance differences dependent on clone, watering regime or the interaction between clone and watering regime (C × W) as estimated by two-way ANOVA are indicated.

	Relative abundance (WW-WS-1dR-7dR)		**Two-way ANOVA**		
Protein	*AL-18*	*AL-10*	*Clone*	*Water*	*C x W*
Actin			0.184	**0.001**	0.383
Calmodulin			**0.023**	**0.011**	**0.009**
Plastid fibrillin 2			0.187	**0.003**	0.415
20 kDa chaperonin, chloroplastic			0.404	**0.000**	0.977
mRNA-binding protein			0.711	**0.015**	0.211
RNA-binding family protein isoform 2			0.655	**0.000**	0.272
Polyubiquitin 4-like protein			0.074	**0.001**	0.184
Soluble inorganic pyrophosphatase			0.612	**0.000**	0.579
Malate dehydrogenase, glyoxysomal-like			**0.030**	**0.000**	0.102

ATP-dependent Clp protease ATP-binding subunit ClpC			0.157	**0.007**	0.810
ATP synthase CF1 alpha subunit			0.355	**0.012**	0.262
ATP synthase D chain, mitochondrial			0.982	**0.000**	0.686
Glutamine synthetase precursor			**0.016**	**0.006**	0.131
	Relative abundance (WW-WS-7dR)		**Two-way ANOVA**		
Metabolite	*AL-18*	*AL-10*	*Clone*	*Water*	*C x W*
Glycine			0.948	**0.030**	0.601
Serine			0.180	0.132	0.275
Valine			0.303	**0.005**	0.289
Proline			0.884	**0.033**	0.068
Alanine			0.191	0.267	0.361
Glyceric acid			0.271	**0.027**	0.310

Water stressed E. globulus exhibit a shift from chloroplastic to mitochondrial energy generation

Our dataset suggested a reduction in cellular metabolism, particularly at the chloroplast level. We observed a decrease in the relative abundances of inorganic pyrophosphatase, malate dehydrogenase, ATP-dependent Clp protease ATP binding subunit ClpC and ATP synthase CF1 following water stress (table 2). The reduction in the chloroplastic ATP synthase CF1 was accompanied by a rise in the mitochondrial ATP synthase D (table 2), which may indicate an alteration in the primary site of ATP production. After rewatering, most of these proteins increased to WW or kept their high abundance (table 2).

Multiple stress and defence related pathways are involved in alleviating drought stress effects

Several proteins implicated in stress and defence showed an enhanced accumulation during drought and rewatering (table 3): 2-cys-peroxiredoxin, peroxiredoxin, thioredoxin superfamily protein, oxidoreductase, zinc-binding dehydrogenase family protein isoform 1, putative quinone reductase, phi class glutathione S-transferase protein and a putative translationally-controlled tumor protein. Thioredoxin and thioredoxin–peroxiredoxin systems are best characterised in plant

chloroplasts, where they have been demonstrated to function in the antioxidant water–water cycle as an alternative in place of APX (Asada 2006). Moreover, the maintenance of these proteins during recovery may also be involved in RuBisCO activase modulation in response to rehydration, as was observed for light regulation of RuBisCO (Zhang and Portis 1999). Epoxide hydrolase 2-like, probable protein Pop3 and heat shock protein 70 (HSP 70) declined in WS and increased after rehydration (table 3).

Table 3 – Impact of drought and rewatering on proteins associated with stress and defence, and secondary metabolism, and related metabolites. Abundance data is presented on a scale relative to the lowest value among sampling points and *p*-values for significance differences dependent on clone, watering regime or the interaction between clone and watering regime (C x W) as estimated by two-way ANOVA are indicated.

	Relative abundance (WW-WS-1dR-7dR)		**Two-way ANOVA**		
Protein	*AL-18*	*AL-10*	*Clone*	*Water*	*C x W*
2-cys-peroxiredoxin, partial			**0.031**	**0.001**	0.162
Peroxiredoxin			**0.014**	**0.011**	0.119
Thioredoxin superfamily protein			**0.006**	**0.023**	0.704
Oxidoreductase, zinc-binding dehydrogenase family protein			0.439	**0.003**	0.782
Putative quinone reductase, partial			0.210	**0.050**	0.410
Phi class glutathione S-transferase protein			0.957	**0.031**	0.421
Putative translationally-controlled tumor protein			**0.041**	**0.044**	0.479
Epoxide hydrolase 2-like			**0.007**	**0.000**	0.199
Heat shock protein 70			**0.048**	**0.001**	0.329
Isoflavone reductase homolog Bet v 6.0101			**0.013**	**0.000**	0.242
Linalool synthase			0.377	**0.025**	0.042

	Relative abundance (WW-WS-7dR)		**Two-way ANOVA**		
Metabolite	*AL-18*	*AL-10*	*Clone*	*Water*	*C x W*
Shikimic acid			0.344	**0.001**	0.699
3-trans-caffeoylquinic acid			0.479	**0.007**	0.733
Quercetin			0.089	**0.000**	0.490
Piceatannol			0.090	**0.020**	0.047

Secondary metabolites were less abundant under water stress

Secondary metabolism was negatively affected by the imposed water deficit. Key enzymes required for isoflavonoid (isoflavone reductase) and terpenoid (linalool synthase) biosynthesis were reduced in abundance during water stress (table 3). Similarly, the abundance of phenolic compounds and their precursors, including shikimic acid, a key precursor for the biosynthesis of aromatic amino acids that form the entry precursor to phenylpropanoid biosynthesis, the hydroxycinnamate 3-transcaffeoylquinic acid, the flavonoid quercetin and the stilbenoid piceatannol were diminished after water stress (table 3). The majority of the metabolites remained at low concentration following rewatering, although levels of piceatannol increased after the reapplication of water.

Integrated proteomic, metabolomic and physiological analysis reveal topological differences in correlation networks between the responsive and resilient clone

Drought stress induced clone specific proteome and metabolome profiles in parallel with the previously reported physiological behaviour (Correia et al. 2014b). Clone AL-18 exhibited a greater abundance in many proteins and metabolites, particularly those associated with the Calvin cycle and elements of the photosynthetic electron transfer chain, irrespective of water stress or abundance. A number of stress and defence-related proteins/metabolites also highlighted clonal differences.

To integrate the different omics datasets and physiological responses, we applied multivariate analysis integrating previously described physiological measurements (Correia et al. 2014b). This analysis provided us a comprehensive overview of plant stress responses, identifying clone-dependent interaction networks. Initial comparison based on principal component analysis (PCA) exhibited a clear separation between well-watered and water stressed samples in both *E. globulus* clones (figure 1). Further examination of PCA plots indicated that variability assigned to principal component (PC 1) was primarily related to biological variability between individuals. On the other hand, PC2 describes variability due to watering regime where well-watered plants were clearly separated from water-stressed and recovered plants.

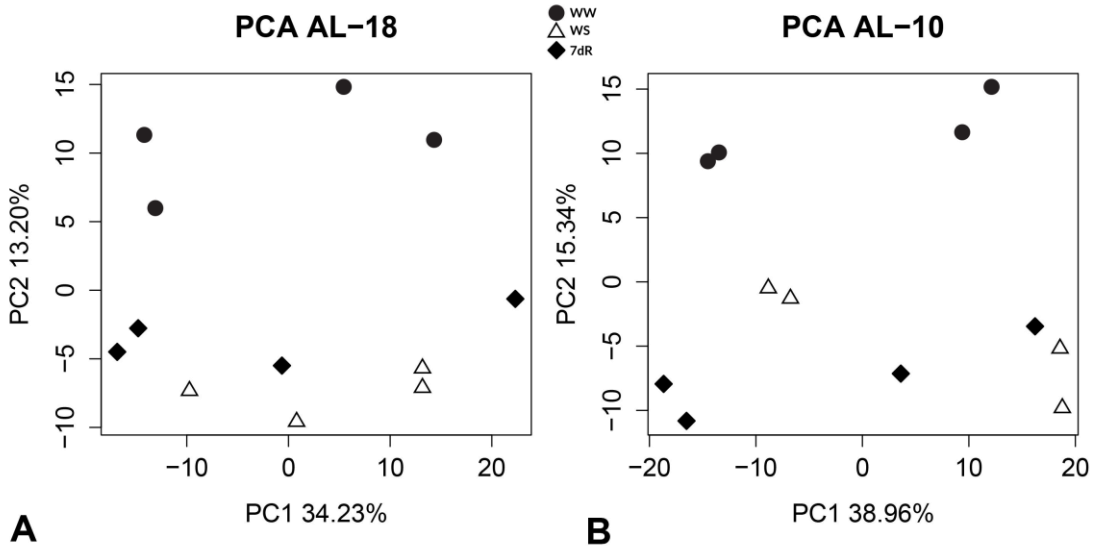


Figure 1 – Principal Component Analysis of the AL-18 (A) and AL-10 (B) datasets. First two components are plotted in the main graph. The proportion of variance explained by each component is indicated on axes labels.

The construction of correlation networks based on sparse partial least squares (sPLS) allowed the determination of the specific components behind the observed phenotypical changes considering proteomic, metabolomic and physiological levels. These networks (figures 2 and 3) showed clone-specific topologies and provided a comprehensive visualization of the differential responses to stress between the analysed clones. Clone AL-18 (figure 2) showed an inverse correlation of height, biomass, and water potential to the studied pigments, MDA, pentitol and sugars. This connection was mediated by specific aldolases, glutamine synthetase, malate

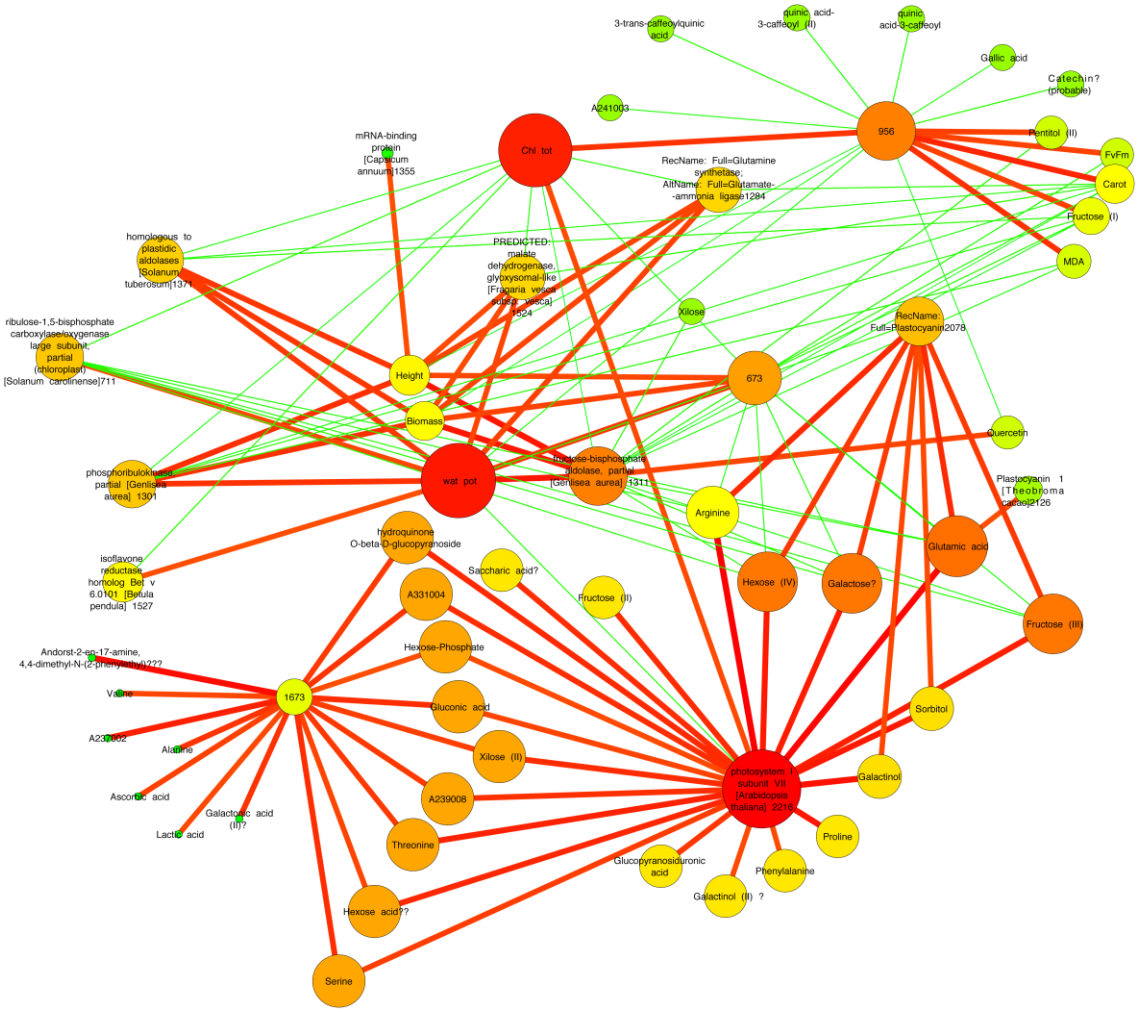


Figure 2 – sPLS-based interaction network of water deficit and rehydration in clones AL-18. Network is presented using the Force Directed layout, which is based on the ‘‘force-directed’’ paradigm. Colour and size of nodes illustrate radiality on a small to large and green to red scale (radiality of a node is calculated by computing the shortest path between the node and all other nodes in the graph); colour and size of edges reflect weight with red and green edges illustrating positive and negative correlations, respectively.

dehydrogenase and phosphoribulokinase. Isoflavone reductase was highly correlated with water potential, having a negative impact on chlorophyll contents. RuBisCO large subunit was positively correlated to water potential and negatively correlated to sugars and glutamic acid. Glutamic acid, arginine, galactose, hexose and fructose were positively correlated with plastocyanin and photosystem I subunit VII.

The interaction network of clone AL-10 (figure 3) showed a structure different from AL-18, with glyceric acid, operating quantum yield of PSII (UPSII), and water potential amongst the nodes showing higher centralities. Among correlations, RuBisCO small subunit, plastocyanin 1, phi (II) class glutathione S-transferase and other unidentified proteins positively correlated with glycolic acid. Many of the aforementioned unidentified proteins and plastocyanin I also correlated with glyceric acid. Small ubiquitin-related modifier 2 isoform 1 and peptidyl-prolyl cis-trans isomerase are positively correlated with Φ_{PSII} and negatively correlated with water potential and NPQ.

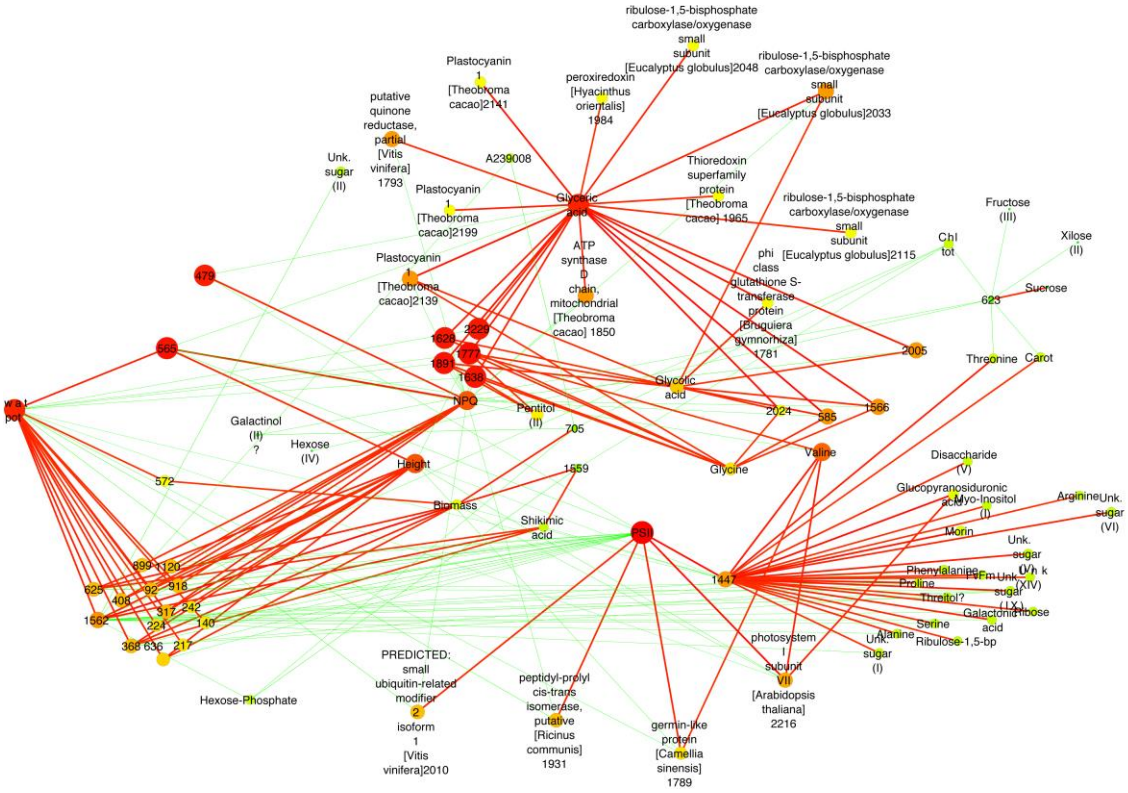


Figure 3 – sPLS-based interaction network of water deficit and rehydration in clones AL-18. Network is presented using the Force Directed layout, which is based on the ‘‘force-directed’’ paradigm. Colour and size of nodes illustrate radiality on a small to large and green to red scale (radiality of a node is calculated by computing the shortest path between the node and all other nodes in the graph); colour and size of edges reflect weight with red and green edges illustrating positive and negative correlations, respectively.

The potential biological implications of the detected relationships are explored in the discussion section.

Discussion

In this work, we combined an analysis of the proteome and metabolome with previously obtained physiological parameters in two *E. globulus* clones under drought and rehydration. The physiological characterisation of the response of the studied clones to drought and recovery demonstrated different degrees of tolerance (Correia et al. 2014b). We identified clone AL-18 as resilient and less reactive to water availability, and AL-10 as more sensitive and responsive in responding to water shortage and restoration. These differences were mainly related to growth, carotenoids, Φ_{PSII}, NPQ and photosynthetic rate, which rapidly varied in response to water availability in clone AL-10, while clone AL-18 took more time to respond (Correia et al. 2014b).

Through a comprehensive analysis of the proteomic and metabolomic datasets, we can now identify mechanisms behind these altered physiological responses, which confirmed previous knowledge but, most importantly, revealed novel responsive mechanisms.

First, we have confirmed several reported results that include decreased photosynthesis, disrupted cell growth, and alterations in gene regulation, in parallel with enhanced photo and osmoprotection, and the activation of other stress and defence related pathways (Wade et al. 2002; Chaves et al. 2003; Kottapalli et al. 2009; Brossa et al. 2015; Kattam et al. 2016). However, our results also indicate novel responses that have not been reported previously. These include a shift of energy metabolism away from chloroplasts and towards mitochondria as evidenced by changes in the abundance of subunits of chloroplastic or mitochondrial ATP synthases. Similarly, the observation that secondary metabolism and particularly phenylpropanoid metabolism was downregulated by drought stress is not commonly reported. Phenylpropanoids are implicated in defence against biotic stresses (Cheynier et al. 2013) and hence this result has significance in terms of the impact of abiotic stress on biotic interactions. For example, it has previously been reported that water stress can negatively influence defence against aphid infestation (Foyer et al. 2016).

Second, we verified that rehydration is mainly characterised by a reversion of the altered parameters, well-illustrated by our PCA analysis and once again confirming our physiological profiles: scores from recovered (4R) plants tend to migrate to WW scores, with AL-10 scores keeping farther from the WW plants relative to AL-18.

Finally, we were able to unveil interesting *E. globulus* responses described before (Correia et al. 2014b) such as overcompensation of CO_2 assimilation rate after rehydration, and the different

response profiles displayed by the clones, more easily understood after integration with omics datasets and network analysis.

Considering CO_2 assimilation rate, we hypothesise that it can be explained by a tightly controlled mechanism involving specific proteins of the Calvin cycle and photosynthetic electron transfer chain, which together with several defence-related proteins act to prevent oxidative damage to the photosynthetic machinery, reducing photosynthesis as the water deficit occurs, and later to reinforce photosynthetic rates. Our hypothesis is based on our results that show a specific relation among RuBisCO, RuBisCO activase, phosphoribulokinase and proteins of the ferredoxin–thioredoxin systems.

A decrease in photosynthetic enzymes in conjunction with an increase in thiol-mediated defence systems prevents oxidative damage during stress. The positive induction of both photosynthetic and redox defence systems during recovery is probably responsible for the re-activation of photosynthesis at several levels: photosynthesis is already known to be regulated through phosphoribulokinase/ferredoxin/thioredoxin system (Wolosiuk and Buchanan 1978) or to respond to light modulation by RuBisCO activase/thioredoxin (Zhang and Portis 1999), processes possibly occurring after rehydration and leading to an overcompensation of CO_2 assimilation. Interestingly, although both clones exhibited a reduced glycine to serine ratio, indicative of reduced photorespiration, following drought stress and recovery, this value was higher for the resilient clone AL-18, suggesting higher photorespiration under conditions of abiotic stress, possibly as protective sink against excess oxidative stress.

These global responses are differently triggered in each clone and lead to differences in tolerance to drought stress as reflected in the interaction networks. These plots illustrated the different physiological and biochemical rearrangements induced by drought in the two studied clones, confirming previous results and providing additional information concerning the underlying molecular causes of observed physiological differences.

Focussing our attention in the network relative to clone AL-18, we found the involvement of key proteins, namely specific aldolases, glutamine synthetase, phosphoribulokinase, isoflavone reductase, malate dehydrogenase and RuBisCO large subunit, mediating a close inverse correlation among height, biomass and water potential, on one hand, and pigments, MDA, pentitol and sugars on the other hand. These results strengthen our conclusions that specific photosynthetic and defence-related proteins are key players in mediating drought tolerance in *E. globulus* and reveal new players. Recently, Kaminski et al. (2015) suggested that cytosolic glutamine synthetase genes are important for limiting nitrogen loss due to photorespiration in potato under well-watered conditions possibly by improving photosynthetic and water use efficiency. The involvement of glutamine synthetase was also reported by Bernard and Habash (2009), which found that cytosolic

glutamine synthetase overexpression enhances water stress resistance by promoting photorespiratory activity, and providing a protective sink for electrons from photosynthetic reaction centres. Confirming this, malate dehydrogenase is also described as significant regulator of respiratory rate in plants (Tomaz et al. 2010) and appears with a role in adaptation to drought stress (Pastore et al. 2007). Finally, the down-regulation of isoflavone reductase-like protein was also described as a novel drought-responsive mechanism (Wade et al. 2002).

Interestingly, regarding the network relative to clone AL-10, we found a completely different scenario: not only none of these ‘‘tolerance players’’ is present, not reflecting any pathways described above, as the correlations found are weaker and poorly supported by physiological markers. The lack of activation of stress-responsive pathways in AL-10 may explain the increased sensitivity to drought compared to AL-18. Moreover, the observed correlations among glyceric acid and several plastocyanins and antioxidant-related proteins, coupled with the involvement of several key amino acids (valine, proline and alanine) appears to be more related to increased salinity tolerance rather than drought (Sanchez et al. 2008).

Conclusion

We reported here a forward-looking approach that enabled us to go further than a classic descriptive analysis of omics results and look for an integrative and meaningful view of the biological events that take place not only during water deficit but also after rewatering. This approach represents a powerful tool for monitoring changes in response to environmental disturbances (Kottapalli et al. 2009; Liu et al. 2013; Scalabrin et al. 2015), and in our hands allowed not only to survey of global biological alterations that characterise *E. globulus* response to drought and rewatering (i), but also to successfully identify specific strategies that make a clone more adapted to water deficit conditions (ii) (figure 4). This work increases our understanding of drought tolerance in *E. globulus* and it may lead to applications in breeding for enhanced drought tolerance in this and other species. Specifically, this study provides a set of new relevant proteins and pathways involved in drought stress that can be the subject of further research in order to check their relevance as tolerance markers for early selection.

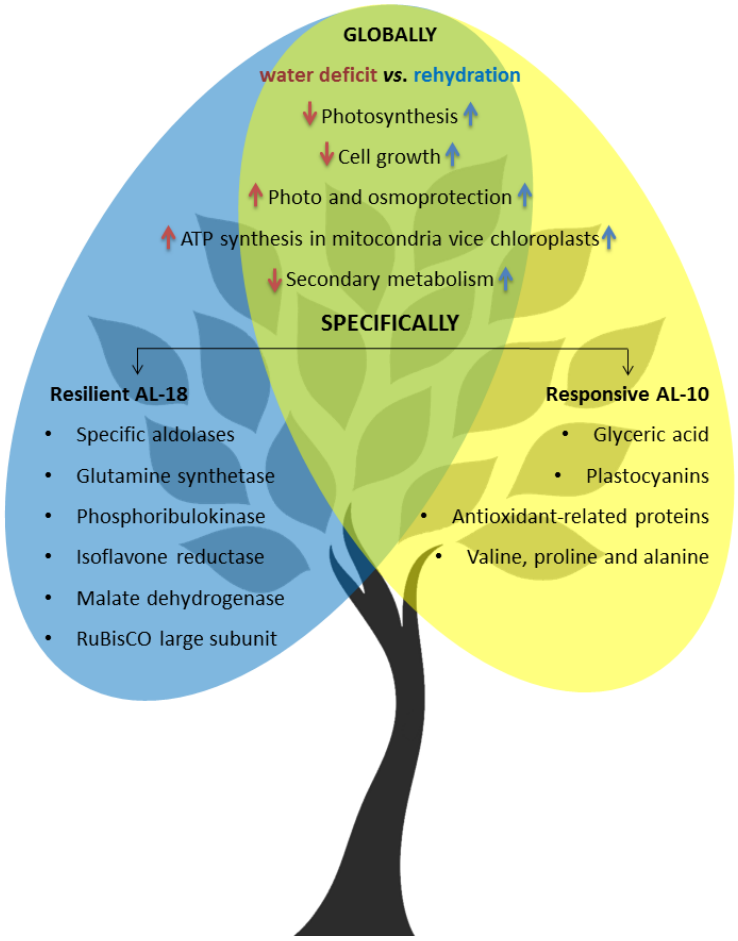


Figure 4 – Schematic illustration of the main conclusions of the work. Global pathways altered by water deficit and rehydration responses in E. globulus are indicated by red arrow and blue arrows, respectively. The direction of the arrows indicates whether pathways are up- or down-regulated. Also indicated are elements of the specific responses of clones AL-18 and AL-10 achieved following network analysis and highlighting key players that mediate each response profile.

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Supplementary Data

Table S1: Physiological response to water deficit and rehydration of clones AL-18 and AL-10, as it was described by Correia et al. 2014b.

Table S2: Summary of the proteomic dataset. Abundance values represent the average spot abundance of the biological replicates for each sampling time and studied clone. *p*-values of a Two-Way ANOVA analysis considering clone and treatment as fixed factors are also indicated.

Table S3: Simplified list of identified proteins that are differentially accumulated during the experiment including its abundance values and statistical significance. Detailed identification data including scores, identified peptides, and coverages is available on Table S4.

Table S4: Protein identification matrix including theoretical and experimental Mr/pI, identified peptides, difference in masses, and protein score.

Table S5: Summary of the metabolomic dataset. Abundance values represent the average spot abundance of the biological replicates for each sampling time and studied clone. *p*-values of a Two-Way ANOVA analysis considering clone and treatment as fixed factors are also indicated.

Table S6: Principal Component Analysis. Importance of each component (a) and variables showing higher loadings to PC1 and PC2 (b) in each clone.

Chapter 3

sudden water shortage

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3.1. Expression of putative stress tolerance indicators in *Eucalyptus* after water shortage and rehydration

Summary

Eucalyptus globulus is a key forest species for which the capacity to cope with water deficit is largely investigated. Previous knowledge suggested the involvement of specific pathways/proteins that could be identified as potential molecular indicators linked to enhanced tolerance in this species. To validate that, the relative expression of 12 transcripts was analysed by quantitative PCR in two clones with different degrees of tolerance (AL-18 and AL-13) subject to sudden water shortage (7 and 11 days after water withholding) and rehydration (2h and 3 days after rewatering): rubisco activase (RCA), phosphoribulokinase (PRK), and ferredoxin-NADP(H) oxireductase (FNR), mitochondrial (mMDH) and peroxisomal (pMDH) malate dehydrogenase, mitochondrial glycine cleavage system H protein (GCSH), peroxisomal catalase (CAT), mitochondrial superoxide dismutase (SOD), isoflavone reductase (IFR), responsive to desiccation protein 22 (RD22), dehydration response element B1A (DREB1A), and the potassium channel GORK (GORK). Water shortage was very detrimental to *Eucalyptus*, resulting in decreased water potential and gas exchange, and high oxidative damage, with heavier outcomes in clone AL-13 that also presented plant loss. Both clones recovered by reversing previous alterations. Regarding the transcript alterations, early stress over expression of DREB1A and GORK of AL-13 coincided with silencing of DR22, RCA, PRK, FNR, mMDH, pMDH and CAT; most of these stabilised at later stress, prior to sharply re-decrease during early recovery and then showing a strong induction at late recovery. In clone AL-18, the activation of RD22, DREB1A and GORK at early stress concurred with silencing of RCA and GCSH, and induction of FNR, mMDH and CAT at late stress; RCA increased at early recovery followed by PRK later, but recovery was mainly characterised by a general re-settlement of gene expression. IFR was down-regulated in AL-18 and highly over-expressed in AL-13. This experiment identified RCA, FNR, mMDH, CAT and IFR as potential molecular indicators linked to enhanced water stress tolerance in *Eucalyptus globulus*, but the specific involvement of these transcripts must be validated through functional studies.

Introduction

Altered water status is a plant response to several abiotic stresses with the most obvious implication in relation to drought (Verslues et al. 2006). When water becomes insufficient, plants have to decrease their water potential to allow extraction of water from the soil and immediately close their stomata to limit water loss through transpiration. This results in decreased gas exchange and CO_2 assimilation, therefore directly impacting plant growth and productivity.

In a World under heating, heat waves and extremes of heavy precipitation are becoming more frequent in high latitudes, in parallel with decreases of precipitation and more droughts in lower latitudes (Kerr 2007). Given the heavy impact water deficit has on plants' metabolism and productivity, mainly considering the European territory (Ciais et al. 2005), it is of utmost importance to find novel ways of working around this issue. One of the strategies may be the introduction of selected plants with improved tolerance to specific locations and this is dependent on finding suitable selection criteria – which is based in the proper interpretation of the obtained knowledge on key proteins and metabolites involved in plant growth, development and stress response that ultimately should identify potential molecular indicators linked to enhanced tolerance (Rodziewicz et al. 2014).

The *Eucalyptus* genus was imported from its native area and is widely distributed and planted over 20 M hectares across the globe (White et al. 2014); from this, *Eucalyptus globulus* Labill. is one of the species which capacity to deal with water deficit has been largely investigated (David et al. 1997; Costa e Silva et al. 2004; Bedon et al. 2011; Correia et al. 2016). After the large body of research obtained, it is now time to seek for suitable molecular indicators that would enable the proper characterisation of more tolerant genotypes.

On the basis of previous works, we found that a more resilient *E. globulus* profile would relate to specific photosynthesis-, photorespiration-, hormonal- and antioxidative- related mechanisms (Correia et al. 2014a; Correia et al. 2014b; Correia et al. 2016). Thus, we decided to look for specific variations in key transcripts involved in these pathways. Rubisco activase, phosphoribulokinase, and ferredoxin-NADP(H) oxireductase were chosen to test a possible modulation that justifies a protection of the photosynthetic machinery during stress imposition and enables an enhanced recovery of photosynthesis after stress relief. A possible role of photorespiration and other antioxidative and defence mechanisms led us to test mitochondrial and peroxisomal malate dehydrogenase, mitochondrial glycine cleavage system H protein, peroxisomal catalase and mitochondrial superoxide dismutase and isoflavone reductase. Finally, considering the proven role of abscisic acid (ABA) in drought stress signalling (Zhang et al. 2006; Correia et al.

2014a), we tested three ABA-responsive genes: responsive to desiccation protein 22, dehydration response element B1A and the potassium channel GORK.

With the aim of checking if any of the previous transcripts would be modulated by water deficit and/or rehydration and be identified as potential molecular indicators linked to enhanced water stress tolerance in *Eucalyptus globulus*, we performed qRT-PCR in two clones with different degrees of tolerance (AL-18 and AL-13) subject to sudden water shortage and subsequent rehydration. Also, mortality rate determination and several physiological measurements (water potential, lipid peroxidation and gas exchange) were carried out in order to check the stress progression and intensity.

Materials and methods

Plant material and experimental design

Rooted cuttings of *Eucalyptus globulus* (two different clones: AL-18 and AL-13) were obtained from the breeding program of Altri Florestal SA (Portugal). AL-18 has been tested before, it is identified as a resilient clone, less reactive to water availability; although tested here for the first time, clone AL-13, which is related to the previously used AL-10, is expected to be more sensitive responding to drought stress and rehydration. Five month old plants were transferred from an outdoor shaded house and plastic containers filled with 3:1 (w/w) vermiculite:peat, and transplanted to 1 L plastic pots filled with an equal weight of a 3:2 (w/w) peat:perlite mixture. The experiment was conducted in a climate chamber (Fitoclima 1200, Aralab, Portugal) under the following conditions: 25/20 ºC (day/night), 16/8 h (day/night) photoperiod, 50% relative humidity and 600 µmol m^{-2} s^{-1} photosynthetic photon flux density. The potted cuttings were acclimatised over three weeks inside the climate chamber during which time they were watered to 70% field capacity with nutritive solution (N:P:K, 5:8:10). In order to minimise the effects of environmental heterogeneity, the pots were randomly and periodically moved to the neighbouring position during the whole experiment.

During the experiment, environmental conditions inside the climate chamber were maintained as in the acclimation period and only watering was altered: control plants were kept under well-watered conditions (WW: water supplied every evening until soil water content reached 70% field capacity; 46 plants); and a second group was randomly assigned to sudden water shortage (WS: watering was discontinued; 46 plants). Control (WW) and water stressed (WS) plants from each clone (AL-18 and AL-13) were sampled 7 (midS) and 11 days (S) after water withholding. After the last sampling point, WS plants were watered to WW levels and recovery was monitored by sampling plants 2 h (midR) and 3 days (R) after rewatering.

At each sampling point (i.e., midS, S, midR and R), homogeneous leaves from ten random individuals of each watering group were used for *in vivo* measurements of leaf gas exchange parameters (see below) and five random individuals of each watering group were measured to determine the plant water status (see below). Also, fully expanded leaves from five independent individuals of control (WW) and stressed (WS) plants were collected, immediately frozen in liquid nitrogen and stored at -80ºC for subsequent analyses (estimation of lipid peroxidation, and RNA extraction).

Mortality rate

The percentage of plant mortality was recorded per clone at each sampling point (i.e., midS, S, midR and R).

Water potential

Shoot water potential (Ψ_{md}) was measured using a Scholander-type pressure chamber (PMS Instrument Co., Corvallis, OR, USA). Measurements were carried out in five plants per clone (AL-18 and AL-10) and watering group (WW and WS) around midday (solar time) at each sampling point (i.e., midS, S, midR and R).

Lipid peroxidation

Lipid peroxidation was estimated by measuring the amount of MDA (malondialdehyde) in leaves following the protocol described by Hodges et al. (1999) from 40 mg of frozen leaves per mL of extraction buffer.

Gas exchange and stomatal conductance

Net CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and stomatal conductance (g_s, mol H_2O m m^{-2} s^{-1}) were determined using a portable infrared gas analyser (LCpro-SD, ADC BioScientific Ltd., UK) equipped with the broad leaf chamber. To determine the saturation light intensity, light response curves of CO_2 assimilation were performed with the following PPFD (photosynthetic photon flux density): 2000, 1500, 1000, 750, 500, 250, 100, 50 and 0 µmol m^{-2} s^{-1}. Punctual measurements were performed at saturation light intensity (1000 µmol m^{-2} s^{-1}, defined after analysis of light response curves). Ambient temperature, CO_2 and H_2O concentration, and air flow 200 µmol s^{-1} were maintained inside the chamber during all the measurements. Data were recorded when the measured parameters were stable (2 – 6 min).

RNA extraction and cDNA synthesis

Total RNA was extracted from 50 mg of frozen leaves using the Spectrum™ Plant Total RNA Kit (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. To remove any contaminating traces of genomic DNA, RNA samples were treated with rDNase (Macherey-Nagel, Düren, Germany) and further purified using the NucleoSpin® RNA Clean-up (Macherey-Nagel) protocol. RNA integrity was checked by electrophoresis on an agarose gel and yield and purity were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For each sample, single-stranded cDNA was synthesised from 1.5 µg of total RNA with the Maxima Reverse Transcriptase (Thermo Fisher Scientific) and random hexamer primer (Thermo Fisher Scientific).

Quantitative Real-Time PCR (qRT-PCR)

Target genes were defined by their putative role as stress markers under drought stress and considering former studies (Correia et al. 2014b; Correia et al. 2016). They were first chosen from the *Arabidopsis* Information Resource – www.arabidopsis.org (Berardini et al. 2015) and then localised within the *Eucalyptus grandis* genome, the closest species with a sequenced genome, using the BLAST tool from Phytozome ver. 11.0 (Goodstein et al. 2012). Gene-specific primers were designed using the Primer3Plus' interface in Geneious Pro 4.8.2 (Biomatters Ltd, Auckland, New Zealand; Kearse et al. (2012)) and are listed in table 1. Quantitative PCR was performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Reaction mixtures in a final volume of 20 µL contained 1× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fischer Scientific), 0.48 µM of gene-specific primer and 1.5 µL of a 10-fold dilution of the previously synthesised single strand cDNA. The two-step cycling protocol was used for all reactions: a first cycle of 95ºC for 10 min and then 45 cycles of 95ºC for 15 and 60ºC for 60 s. Fluorescence detection took place at the end of each one of these 45 cycles. To confirm the presence of a single amplicon a dissociation step was also carried out. Prior to qRT-PCR cDNA samples were pooled in groups of three biological replicates, and analysed by triplicate.

Five potential housekeeping genes were tested in order to select the best ones to normalise data: actin (ACT), tubulin (TUB), histone H2B (H2B), ubiquitin (UBQ), and glyceraldehyde 3-phosphate dehydrogenase (GADPH) – primers sequences in table 1. Their constitutive expression was validated with geNorm v3.5 (Vandesompele et al. 2002), separately for each clone. H2B and GADPH were chosen as reference genes and their relative mean expression was used for data normalisation of clone AL-18, and ACT and GADPH were used in clone AL-13. Normalised Relative Quantities (NRQ) and Standard Errors of RQ were determined according to Hellemans et al. (2007).

Table 1 – List of target and housekeeping genes and primers sequences used in the qRT-PCR.

Protein	**Target gene**	**Forward primer sequence (5'-3')**	**Reverse primer sequence (5'-3')**
RuBisCO activase	RCA	CTGGGCACCAACT CGCGAAGAC	AGCACGCAGGGC ACCAAAGAAG
Chloroplastic phosphoribulokinase	PRK	GAGGCAACCCGGA CTCGAACAC	GGCCCTTGGGTCA AGTGCAGTC
Ferredoxin-NADP(H) oxireductase	FNR	ACCACGGAGGCTC CAGCTAAGG	CGTGTTGAGGAG GCACCTTCCAATG
Mitochondrial malate dehydrogenase	mMDH	AGTGCTGCGGATC GTGGTGAAATG	AGTGCTGCGGATC GTGGTGAAATG
Peroxisomal malate dehydrogenase	pMDH	CCACCGCAGCCGA AGTTTTCAAG	CCTGCATGACCCC CAACAACTGG
Mitochondrial glycine cleavage system H	GCSH	CGATCATGCGCAA GACCATTTGGG	GGTGGCCTTGACG CTCTCAACC
Peroxisomal catalase	CAT	CAGGGGAGCGAGC GCAAAAGG	TAAGGGTTTCAGG GCTGCCACG
Mitochondrial superoxide dismutase	SOD	GGATGGGTGTGGC TTGGTGTGG	GAACCAAACTTG GTCCCTTGGTCAC
Isoflavone reductase	IFR	ACCGACATCCACC GCCTTCTTG	CAGCTCTAGTCCT GGGCTGCAC
Responsive to desiccation 22	RD22	TCACGGTGATTCTG GCGACTTCC	TGGATTTTGGCAT TTGCGTGTTGGG
Dehydration response element B1A	DREB1A	CCGGGAGCCCAAC AAGAAGACC	AGTCGGCGAAATT GAGGCACGC
Potassium channel GORK	GORK	TCTTTGTGCAGCCC GTGGCAAG	ACATGAAGTGGG GTTCGGTGGTC
Actin	ACT	AGAGCATCCCGTG CTCCTCACC	GAGCACGGCTTG AATGGCAACG
Tubulin	TUB	CCAGCTCGAGCGA GTGAACGTG	GTGCGCAGGCTGT CCATAGTCC
Histone H2B	H2B	GCGGGTGAAGAAG AGCGTGGAG	AGGCCTCCTGGGC GAGTTTCTC
Ubiquitin	UBQ	GGGATTTCGCCGG ATGAGATTGTTC	AAATGTGGGCTCG TGGGGCAAG
Glyceraldehyde 3-phosphate dehydrogenase	GADPH	CGCCTTCCCTCCAG CTTCAACG	ACAGGCTGAATG CTCCTGACAGAGG

Statistical analysis

All physiological measurements are presented as mean ± standard error (SE) from 5 or 10 plants per treatment. At each sampling point, significant differences between WS and respective WW are indicated (* in the WS column) at $p \leq 0.05$, according to a Student's t test, which was performed using SigmaPlot (SigmaPlot for Windows v. 11.0, Systat Software Inc., San Jose, CA, USA).

Results

The imposed water deficit had different outcomes in both clones starting with distinct mortality rates. While all plants from the clone AL-18 dealt with water shortage (figure 1b), 15% of the plants from clone AL-13 were lost in the 11th day after water withholding (figure 1d). Recovery was also not fully achieved, with AL-13 plants continuing to die: 22% of the plants were not recovered after rewatering. No deaths were recorded in clone AL-18 until the end of the experiment.

Figure 1 – *Eucalyptus globulus* plants from clone AL-18 (a, b) and clone AL-13 (c, d) under well-watered conditions (WW, a, c) and 11 days after water withholding (WS, b, d).

Considering water potential, water withholding caused a depression similar in both clones after 7 days, which evolved to even lower levels after 11 days, more pronounced in clone AL-13 than in AL-18 (figure 2a, b). Both clones reacted to rewatering by increasing their water potential 2 h after rehydration, although maintaining lower levels than WW plants until 3 days after rewatering (R, figure 2a, b).

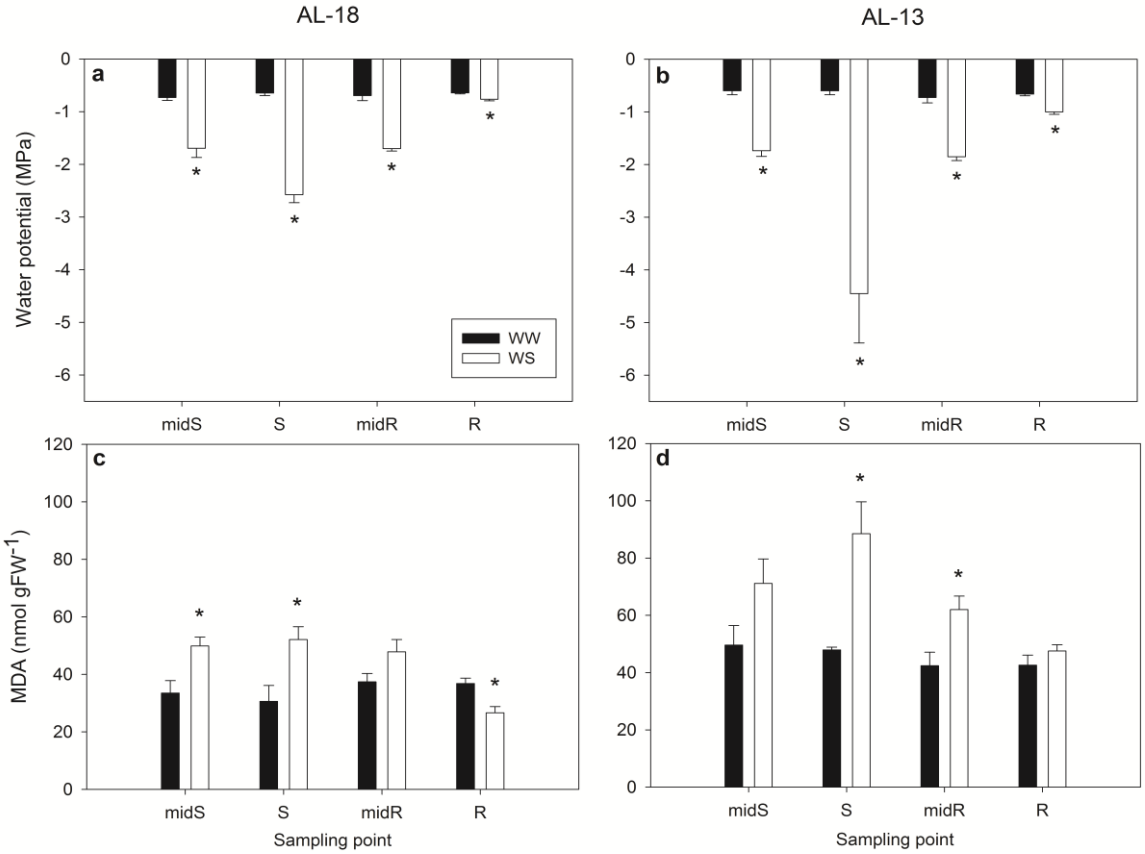

Figure 2 – Shoot water potential (a, b) and leaf MDA content (c, d) of two *Eucalyptus globulus* clones (AL-18, left side, and AL-13, right side) exposed to water withholding (midS – 7 days and S – 11 days) and rewatering (midR – 2 h and R – 3 days). Abbreviations: WW, well-watered plants; WS, water stressed plants. Significant differences between WS and respective WW are indicated: *, $p \leq 0.05$.

Water withholding caused an increase in lipid peroxidation that decreased promptly after rewatering (figure 2c, d). Once again, the effect during stress was more pronounced in clone AL-13 (figure 2d), but after 3 days both clones had recovered. Clone AL-18 showed MDA content even lower than respective WW (R, figure 2c).

Water stress resulted in a decrease of the photosynthetic (figure 3a, b) and transpiration (figure 3c, d) rates, together with a sharp reduction in stomatal conductance of both clones (figure 3e, f), although more distinct in clone AL-13. This clone showed higher photosynthetic and transpiration rates in WW conditions, which decreased to a greater extent after water withholding when compared to clone AL-18 (figure 3). Despite showing increasing gas exchange after rewatering, none of the clones recovered to WW levels after 3 days of rehydration (figure 3).

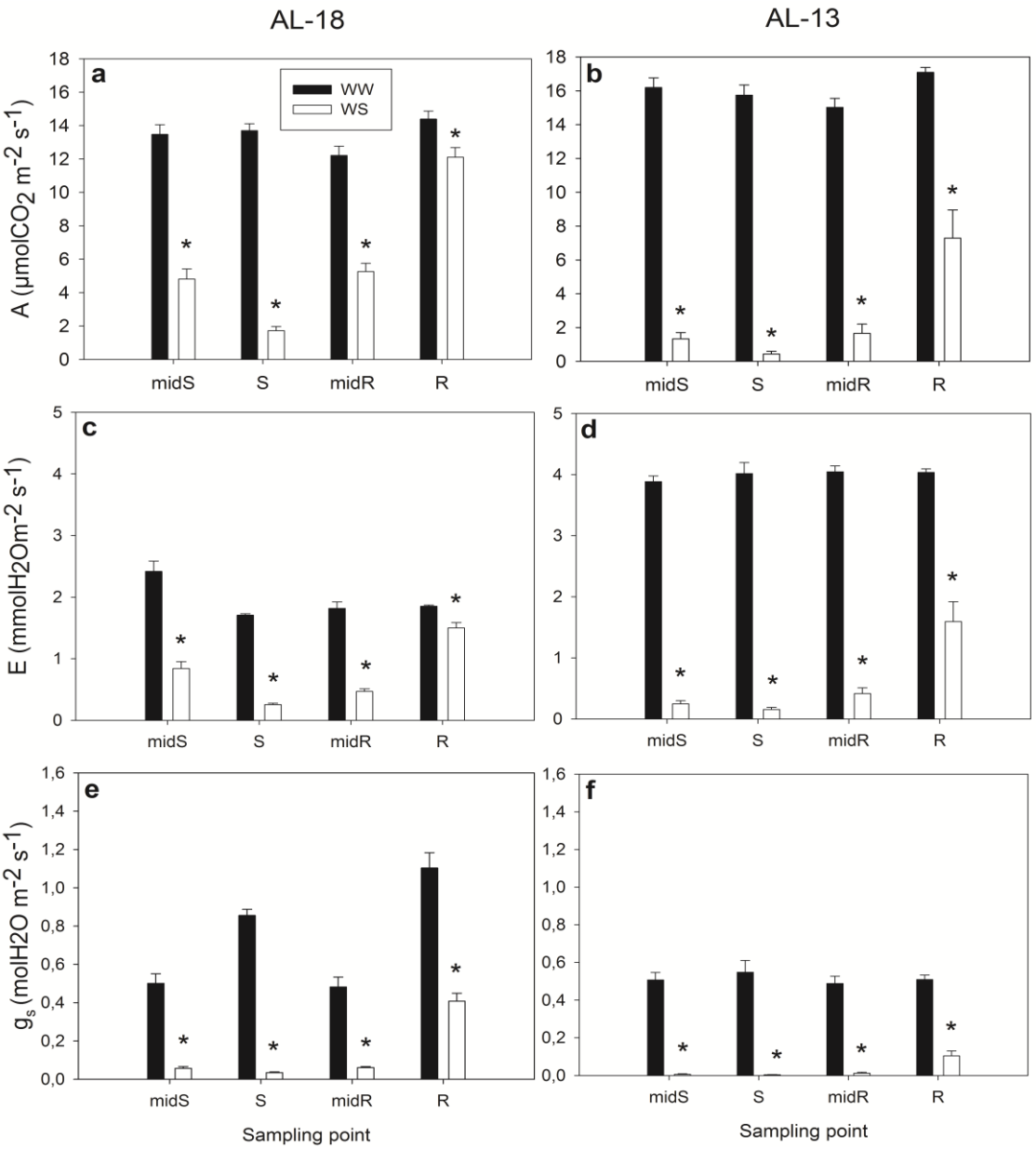

Figure 3 – Net photosynthetic rate (A), transpiration rate (E), and stomatal conductance (g_s) of two *Eucalyptus globulus* clones (AL-18, left side, and AL-13, right side) exposed to water withholding (midS – 7 days and S – 11 days) and rewatering (midR – 2 h and R – 3 days). Abbreviations: WW, well-watered plants; WS, water stressed plants. Significant differences between WS and respective WW are indicated: *, $p \leq 0.05$.

The relative abundances of the transcripts related to the photosynthetic machinery are shown in figure 4. The expression of RuBisCO activase (RCA) was heavily depressed in both clones 7 days after water withholding, maintaining lower expression at the 11th day in clone AL-18 (figure 4a), but increasing to WW levels in clone AL-13 (figure 4b). This AL-13 pattern was similar in phosphoribulokinase (PRK, figure 4d) and ferredoxin-NADP(H) oxireductase (FNR, figure 4f). However, in clone AL-18, these two transcripts were less reactive to water shortage,

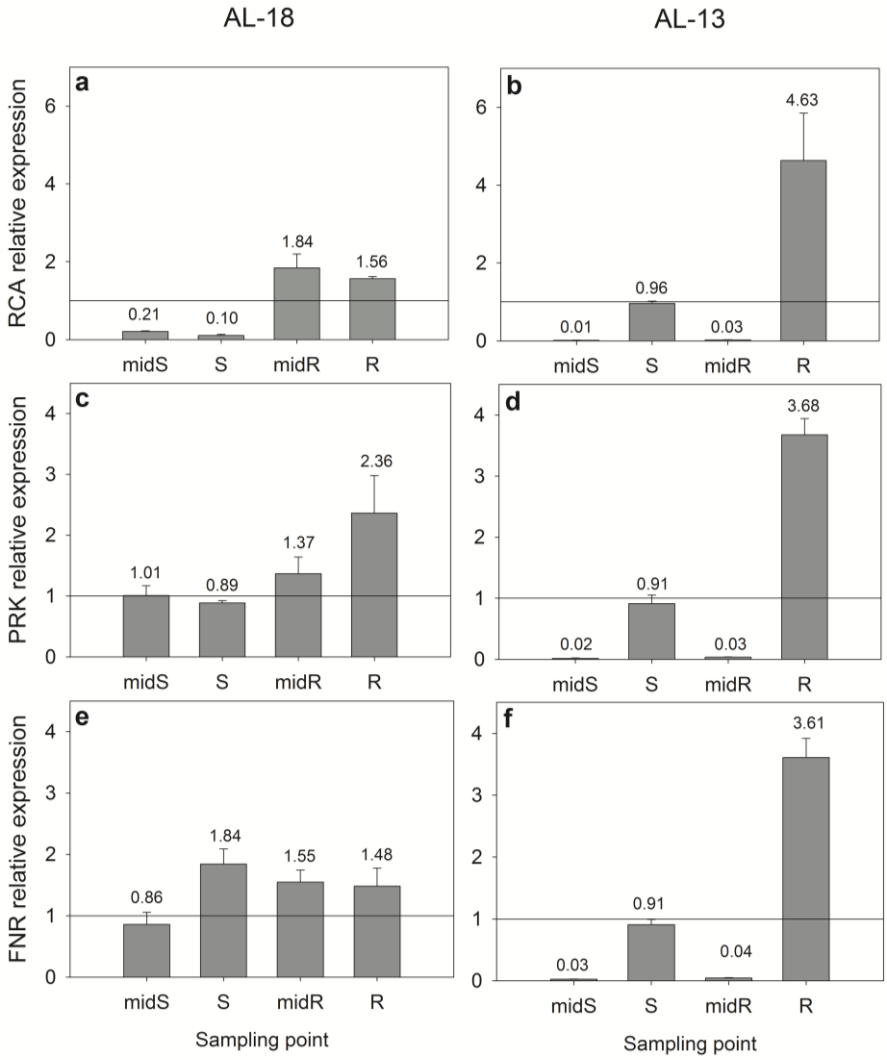

Figure 4 – Relative mRNA abundances [RCA: RuBisCO activase (a, b); PRK: chloroplastic phosphoribulokinase (c, d); and FNR: ferredoxin-NADP(H) oxireductase (e, f)] in leaves of two *E. globulus* clones (AL-18, left side, and AL-13, right side) exposed to water withholding (midS – 7 days and S – 11 days) and rewatering (midR – 2 h and R – 3 days). Transcript relative expression is represented as fold change of water stressed (WS) samples to respective control (well-watered – WW) at each sampling point.

showing a steady expression (figure 4 c, e). After rewatering, RCA expression increased in clone AL-18 (figure 4a), PRK and FNR kept a stable expression (figure 4 c, e), with PRK showing a slight tendency to increase 3 days after rewatering (figure 4 c). On the other side, these three transcripts (RCA, PRK and FNR) showed the same profile in clone AL-13: strongly repressed 2 hours after rehydration, and overexpressed 3 days later (figure 4b, d, f).

Considering the transcripts related to photorespiration, the relative mRNA abundances of the five studied genes are shown in figure 5. In clone AL-18, mitochondrial malate dehydrogenase (mMDH) showed an overexpression (2.5-fold) 11 days after water withholding that decreased to WW levels during recovery (figure 5a), and peroxisomal malate dehydrogenase (pMDH) kept a steady expression during drought imposition and relief (figure 5c). On the other hand, these transcripts (mMDH and pMDH) exhibited a similar expression profile in clone AL-13 as seen in photosynthesis related-transcripts: heavily depressed 7 days after water withholding, increasing at the 11th day, then strongly downregulated 2 hours after rehydration, and over expressed 3 days later (figure 5 b, d).

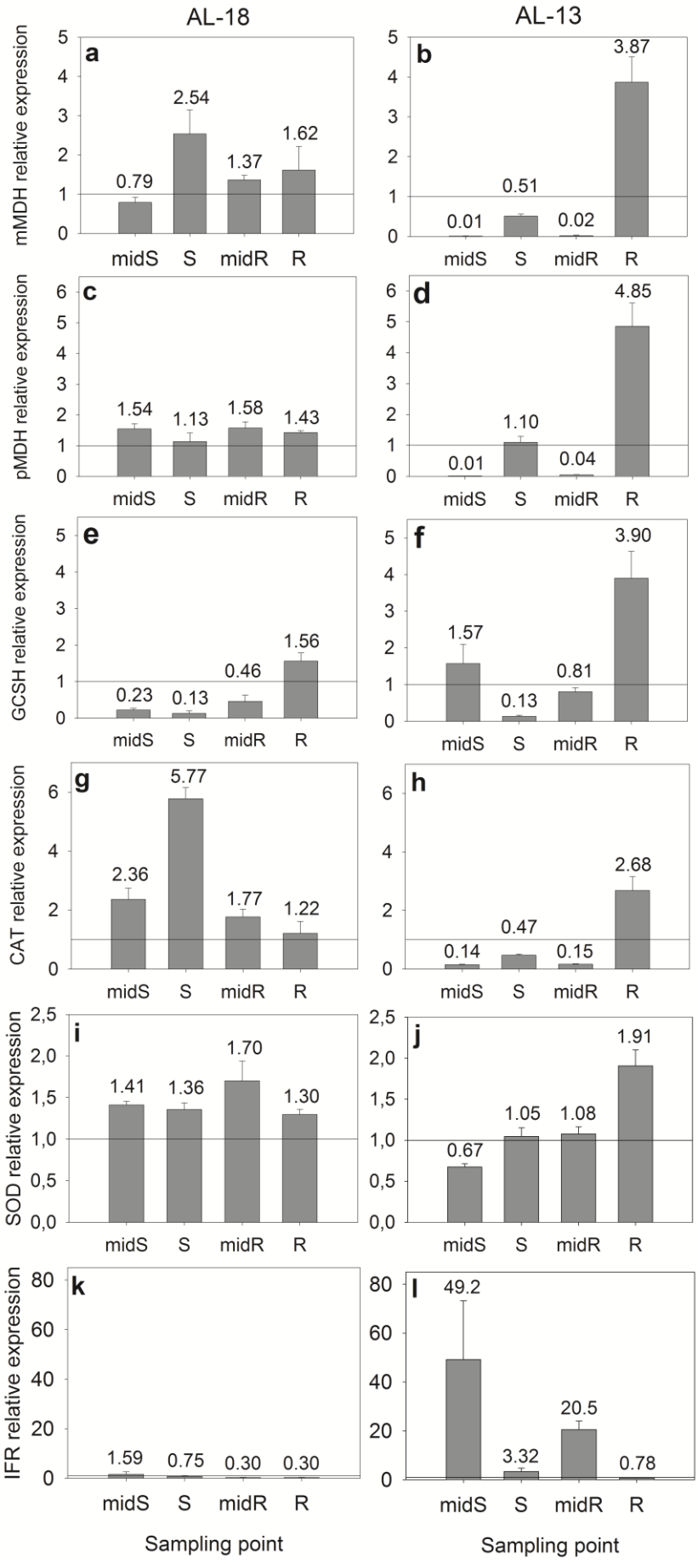

Figure 5 – Relative mRNA abundances [mMDH: mitochondrial malate dehydrogenase (a, b); pMDH: peroxisomal malate dehydrogenase (c, d); GCSH: mitochondrial glycine cleavage system H (e, f); CAT: peroxisomal catalase (g, h); SOD: mitochondrial superoxide dismutase (i, j); and IFR: isoflavone reductase (k, l)] in leaves of two *E. globulus* clones (AL-18, left side, and AL-13, right side) exposed to water withholding (midS – 7 days and S – 11 days) and rewatering (midR – 2 h and R – 3 days). Transcript relative expression is represented as fold change of water stressed (WS) samples to respective control (well-watered – WW) at each sampling point.

Mitochondrial glycine cleavage system H (GCSH) gene was under expressed during the water shortage and first 2 hours of rewatering in clone AL-18, showing an increased expression after 3 days of recovery (figure 5e). In clone AL-13, GCSH expression was only decreased at the 11th day after water withholding, increasing after rewatering to ≈ 4-fold at the 3rd day (figure 5f).

In relation to peroxisomal catalase (CAT) and mitochondrial superoxide dismutase (SOD), different expression profiles were found in each clone. CAT expression increased in clone AL-18 following water shortage up to ≈ 6-fold, decreasing to control levels after rewatering (figure 5g), whereas SOD presented only a slight upregulation at all sampling points (figure 5i). In clone AL-13, CAT was down expressed after water withholding, showing an increase 3 days after rewatering (figure 5h), and SOD presented only a slight upregulation at the same sampling point (figure 5j).

Isoflavone reductase (IFR) gene expression was downregulated in clone AL-13 after the 7th day of water withholding to even a lower extent during rewatering (figure 5k). On the other hand, IFR transcript showed an over-accumulation in clone AL-13 following water shortage, ≈ 50-fold at maximum expression at the earliest stress point and ≈ 3-fold after 11 days without water (figure 5l). Following the first 2 hours of rewatering, IFR showed a new overexpression in clone AL-13 (20-fold), decreasing to control levels 3 days later (figure 5l).

The expression of the three studied ABA-responsive genes is indicated in figure 6.

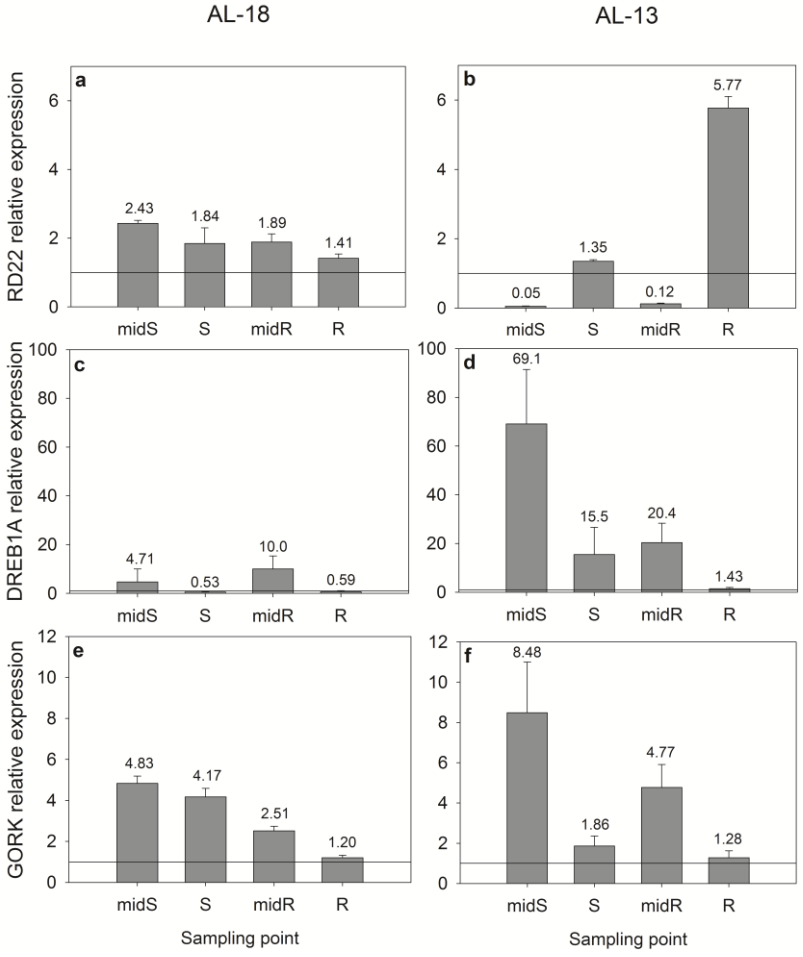

Figure 6 – Relative mRNA abundances [RD22: responsive to desiccation 22 (a, b); DREB1A: dehydration response element B1A (c, d); and GORK: potassium channel GORK (e, f)] in leaves of two *E. globulus* clones (AL-18, left side, and AL-13, right side) exposed to water withholding (midS – 7 days and S – 11 days) and rewatering (midR – 2 h and R – 3 days). Transcript relative expression is represented as fold change of water stressed (WS) samples to respective control (well-watered – WW) at each sampling point.

Responsive to desiccation 22 (RD22) showed an upregulation during drought imposition and early recovery in clone AL-18 (figure 6a), contrary to clone AL-13, which revealed a downregulation of RD22 during early water shortage and rehydration, and an over-accumulation 3 days after rewatering (figure 6b).

Dehydration response element B1A (DREB1A) was overexpressed at early water stress and early recovery and downregulated at late stress and recovery in clone AL-18 (figure 6c). In clone AL-13, DREB1A transcript showed an over-accumulation after water withholding and early recovery (more pronounced at early stress), falling to control expression levels 3 days after rewatering (figure 6d). Finally, the relative expression of potassium channel GORK (GORK) was enhanced following water shortage, decreasing after rewatering down to control levels after 3 days of recovery in clone AL-18 (figure 6e). In clone AL-13, this transcript was also reactive to water shortage, presenting an over induction at early stress and early recovery (figure 6f).

Discussion

In opposition to a slowly imposed water deficit (chapter 2), inducing water stress by water withholding was more detrimental to *Eucalyptus*. Clone AL-13, although quickly responding to water deficit by limiting water loss through decreasing stomatal conductance and gas exchange at the earlier stage of stress, was not able to avoid a major depression in water potential. This inevitably translated in greater oxidative damage as indicated by increased MDA content and plant death. On the other hand, clone AL-18 managed to maintain higher gas exchange and water potential than AL-13, limiting oxidation impairment and avoiding plant death. Other works have investigated drought resistance mechanisms using drought-tolerant and drought-sensitive clones/species of *Eucalyptus* (Costa e Silva et al. 2004; Cano et al. 2014). Costa e Silva et al. (2004) observed that a drought-tolerant clone possessed higher stomatal and hydraulic conductance than a drought sensitive clone; and Cano et al. (2014) discussed how the hydraulic system of a tolerant species is more resistant to cavitation, which together with higher mesophyll conductance is able to increase intrinsic water use efficiency and maintain lower oxidative stress under prolonged water stress compared to a drought sensitive species.

Plants from both clones reacted promptly to rehydration, reversing water potential, lipid peroxidation and gas exchange after 2 hours. Lipid peroxidation fully recovered at this point in clone AL-18 but recovery of AL-13 was also remarkable, mainly regarding water potential. Clones displayed a considerably slower recovery of the gas exchange capacity, especially AL-13. Given the full recovery of water potential and lipid peroxidation, the known resistance of the photosynthetic apparatus to desiccation (Cornic and Massacci 2004), and results obtained in other

experiments (Correia et al. 2014b; chapter 2), this is a revealing result and confirms the severity of the imposed drought stress (Warren et al. 2011).

In order to validate a potential role of key proteins/pathways that were previously defined using an integrated approach of proteomics, metabolomics and physiology (Correia et al. 2016), chapter 2.3) as suitable molecular indicators of drought tolerance, we investigated the relative abundance of specific transcripts. Most of the studied transcripts were specifically modulated by water shortage and restoration.

Earlier findings have already confirmed a consistent accumulation of ABA in water stressed *Eucalyptus* (Correia et al. 2014a; chapter 2.2) and the results here validate the drought-induction of gene expression governed by ABA-dependent regulatory systems (Shinozaki and Yamaguchi-Shinozaki 2007). DREB1A encodes a transcription factor with a known role in increased tolerance to drought (Zhao et al. 2007; Bhatnagar-Mathur et al. 2014), GORK encodes an outwardly rectifying channel in guard cell membrane that controls stomatal movements and allows the plant to reduce water loss via transpiration (Hosy et al. 2003), and RD22 is a known responsive to dehydration gene mediated by ABA (Yamaguchi-Shinozaki and Shinozaki 1993). As expected, the three ABA-responsive genes increased with water deficit, resettling during recovery. The more responsive profile of clone AL-13 compared to AL-18 aligns with previous results (Correia et al. 2014a; chapter 2.2), but the repression of RD22 at early stress in clone AL-13 appears as an unexpected and intriguing result.

The higher drought tolerance of AL-18 compared to AL-13 may be partly supported by the different expression profiles of photosynthesis and photorespiration related genes. On one hand, the early stress over expression of ABA-responsive genes DREB1A and GORK of AL-13 coincided with DR22, photosynthesis related RCA, PRK, FNR, and photorespiration related mMDH, pMDH and CAT were repressed; most of these stabilised at later stress. This indicates a global downregulation of photosynthesis and photorespiration that takes place before water deficit becomes overpowering. On the other hand, the activation of all ABA-responsive genes RD22, DREB1A and GORK at early stress in clone AL-18 concurred with silencing of RCA and GCSH and induction of FNR, mMDH and CAT at late stress. This result illustrates a rather different regulation mechanism. The down-regulation of RCA during the stress extension together with the steady expression of PRK and induction of FNR is in accordance with previous works (Correia et al. 2016; chapter 2.3), confirming a tightly controlled regulation of the photosynthetic machinery, protected by an active antioxidant defence (Scheibe et al. 2005; Zhao et al. 2011). Besides, the activation of mMDH and CAT during late stress validates the essential role of mitochondria and photorespiration for redox balancing during photosynthesis under stress conditions, both as an

alternative sink for excess oxidative load (Noctor et al. 2002), and reinforcing its contribution to acclimation (Watkinson et al. 2003; Scheibe et al. 2005).

During early recovery, most of the early drought silenced genes of clone AL-13 sharply re-decreased, showing a strong induction at late recovery. Again, AL-13 reacted to water availability with a transcriptional shutdown of the genes encoding photosynthesis and photorespiration key enzymes. Although appearing less implicated during drought imposition, the enhancement of these transcripts in the later post-drought recovery may be determinant in the superior capacity of these plants to recover (Xu et al. 2013). Considering clone AL-18, recovery is mainly characterised by a general re-settlement of gene expression, although RCA increased its expression at early recovery followed by PRK later, probably to support re-activation of the photosynthetic machinery (Hayano-Kanashiro et al. 2009). The up-regulation of these genes and the re-establishment of GORK are in accordance with the increase in the rate of photosynthesis and stomatal conductance.

These results may also indicate that the proteins of clone AL-13 get turned over during the drought stress and hence transcriptional machinery needs to be strongly induced to replace them following rewatering. Arguably AL-18 protects the proteins during the stress and thus recovery does not require a strong induction of the genes.

Finally, IFR was selected for representing a potential indicator of drought tolerance mechanism: the down-regulation of isoflavone reductase-like protein was previously suggested as a novel drought-responsive mechanism (Wade et al. 2002; Correia et al. 2016; chapter 2.3), declining markedly in abundance as the glutathione level rises (Wade et al. 2002). Our results come upon the initial assumption as the tolerant clone AL-18 presents a down-regulated IFR along the stress in opposition to a highly over-expressed gene in the responsive clone AL-13.

This experiment has enabled us to identify potential molecular indicators linked to enhanced water stress tolerance in *Eucalyptus globulus*: genotypes with down-regulation of RCA and IFR, and over expression of FNR, and mitochondrial MDH and CAT after water shortage are more likely to express increased tolerance. Despite the relevance of these conclusions, we are unable to know if these gene expression changes modulate or are modulated by water stress, so the specific involvement of each one of these transcripts must be must be confirmed in field stressed plants and validated through functional studies.

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3.2. Depicting how *Eucalyptus globulus* survives drought: involvement of redox and DNA methylation events

Summary

Eucalyptus globulus Labill. is widely cultivated and used by industry but its productivity is currently restricted by drought events, so research focussing on supporting programs to breed adapted germplasm is needed. In the present work we monitored severity of acute drought stress (7 and 11 days after water withholding) and relief (2 h and 3 days after rewatering) by quantifying several biochemical markers of oxidative stress and DNA methylation patterns in leaves. Water withholding imposed a mild oxidative stress as estimated by redox shifts in the major antioxidant pools and increased lipid peroxidation. At the DNA level, global 5-methylcytosine distribution increased over the dehydration period especially in vascular tissue as estimated by immunolocalisation. Using methylation-sensitive RAPD analysis, which discriminates methylation changes occurring in specific DNA sequences, we found a high number of specific demethylation events also taking place. Immunolocalisation indicated a rapid reduction in global DNA methylation 2 h after rehydration; however, a large number of de novo methylation events were still detected by methylation-sensitive RAPD. These events were associated with decreased lipid peroxidation and high cellular GSH pools relative to unstressed plants. Our results indicate the parallel induction of redox and complex DNA methylation changes occurring during stress imposition and relief.

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Depicting how *Eucalyptus globulus* survives drought: involvement of redox and DNA methylation events

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Introduction

Eucalyptus globulus Labill. is a widely cultivated species with multiple advantageous characteristics, such as rapid biomass production and stress tolerance. It is grown for its wide range of industrial applications and has also been identified as an energy crop, providing biomass that may reduce dependence on fossil fuels (Luger 2003). However, productivity of *E. globulus* within its current cultivation area is mainly limited by drought, which is also the primary environmental constraint preventing expansion of its cultivation range. Further, drought stress is becoming more frequent and of greater intensity in some of the major productive regions (Booth 2013). Deciphering the mechanisms behind the drought adaptive response is, therefore, an urgent task in order to provide new tools that will support breeding programs to maintain productivity under environmental change (Villar et al. 2011).

A plethora of physiological, biochemical and molecular studies have elucidated the main responses of *E. globulus* to water deficit conditions. Water stress is known to affect plant metabolism by causing loss of cell turgor and osmotic adjustment (Pita and Pardos 2001), decreased water potential (White et al. 1996), stomatal closure and limited gas exchange (Correia et al. 2014), reduced carbon assimilation and changes in biomass allocation (Osório et al. 1998). In parallel, water deficit induces alterations in gene expression (Gamboa et al. 2013), as well as changes in several phytohormones (Granda et al. 2011), metabolites (Shvaleva et al. 2006) and proteins (Valdés et al. 2013).

Drought stress also leads to perturbations in the operation of the photosynthetic and respiratory electron transport chains, and increases rates of photorespiration resulting in increased production of reactive oxygen species (ROS) and changes in the redox balance of the key cellular redox buffers ascorbate and glutathione (Sofo et al. 2005). ROS and redox signalling pathways interact with a range of other signalling components including calcium signals, phosphorylation and dephosphorylation cascades and hormonal signalling pathways to influence gene expression (Foyer and Noctor 2009). Redox modulation of chromatin remodelling in animal cells has also been demonstrated and is known to be necessary for active remodelling of the transcriptome and proteome required for adaptation to stressful environments (Rahman et al. 2004). However, the precise mechanisms by which redox signals are transduced to changes in gene expression in plants continue to be elucidated, and the number of redox regulated transcription factors identified continues to grow (Dietz 2014).

Plants exhibit dynamic and reversible epigenetic modifications to integrate intrinsic and environmental cues (Chinnusamy and Zhu 2009; Mirouze and Paszkowski 2011; Gutzat and Scheid 2012). These epigenetic responses have been proposed to mediate phenotypic plasticity and

adaptive capacity, and are most intensively studied with respect to the stress defence mechanisms of primed plants, which show that epigenetic modifications produce persistent alterations in patterns of gene expression (Pastor et al. 2013). These covalent modifications of DNA and histones occur without changing original nucleotide sequence via several mechanisms of which DNA methylation is the most studied (Boyko and Kovalchuk 2008). DNA methylation is a well characterised epigenetic regulatory strategy that maintains genomic plasticity under environmental stimuli by controlling gene expression in a relatively rapid way, affecting the local structure of chromatin (Guangyuan et al. 2007; Tan 2010). Generally, increased methylation relates to gene silencing and demethylation correlates with active transcription (Guangyuan et al. 2007).

Several studies have already shown that environmental perturbations induce epigenetic and genetic changes that trigger methylation. Water stress (Gourcilleau et al. 2010; Han and Wagner 2013; Rico et al. 2014), heavy metals (Aina et al. 2004), aluminium (Choi and Sano 2007), salt stress (Kovarik et al. 1997; Guangyuan et al. 2007) and heat stress (Correia et al. 2013) were demonstrated to cause changes in levels of DNA methylation throughout the genome at specific loci. However, the mechanisms driving changes in DNA methylation in response to environmental stresses remain largely unknown in forest species (Gourcilleau et al. 2010; Bräutigam et al. 2013) and whether stable epigenetic alterations are transgenerationally inheritable is still a matter of discussion (Mirouze and Paszkowski 2011). Considering that most of these environmental drivers result in significant changes in ROS production and signalling, we chose to examine whether ROS, antioxidant defence and DNA methylation changes concur during stress imposition and/or relief. It has already been speculated that ROS act as possible triggers of specific demethylation activated upon stress in plants (Choi and Sano 2007). As long-lived organisms with complex life cycles and large genomes, epigenetic mechanisms of gene regulation are likely to be prominent in forest trees (Bräutigam et al. 2013). Given the economic significance and stressful environmental context in which *E. globulus* is cultivated, an understanding of the involvement of oxidative signalling and epigenetic regulation under environmental stress is of key importance, particularly with respect to future breeding programs.

Previously, in a different experiment that aimed to evaluate the performance of different clones to water stress, we demonstrated that a moderate water deficit had a negative impact of both leaf transpiration and CO_2 assimilation, but did not affect the health of the photosynthetic apparatus (Correia et al. 2014). Also, we hypothesised that *E. globulus* would trigger antioxidant or other defence mechanisms in order to overcome the negative impact of a more restrictive water deficit. In the present work, we aimed to depict the role of redox and DNA methylation mechanisms in a resilient *E. globulus* clone after a more intense and acute water deficit and rewatering addressing two specific goals: to decipher the impact of water deficit and rehydration on the redox status of *E.*

globulus, and to look for specific or global DNA methylation changes taking place due to the water deficit and/or rehydration.

Materials and methods

Plant material and experimental design

Eucalyptus globulus Labill. rooted cuttings from the genotype AL-18 were obtained from the breeding program of Altri Florestal SA (Portugal). Five-month-plants, grown in plastic containers filled with 3:1 (w/w) vermiculite:peat, were transplanted to 1 L plastic pots filled with an equal weight of a 3:2 (w/w) peat:perlite mixture, and transferred from an outdoor shaded house to a climate chamber (Fitoclima 1200, Aralab, Portugal). The experiment was conducted under the following conditions: 25/20ºC (day/night), 16/8 h (day/night) photoperiod, 50% relative humidity and 600 $\mu molm^{-2}\ s^{-1}$ photosynthetic photon flux density. The potted cuttings were acclimatised over three weeks inside the climate chamber during which time they were watered to 70% field capacity (FC) with nutritive solution (N:P:K, 5:8:10). In order to minimise the effects of environmental heterogeneity, the pots were randomly and periodically moved to the neighbouring position during the whole experiment.

During the experiment, control plants were kept under well-watered conditions (WW, water supplied every evening until soil water content reached 70% FC). A second group was randomly assigned to acute water deficit conditions (WS, in which watering was discontinued). Control and water stressed plants were sampled 7 (midS) and 11 days (S) after water withholding. After the last sampling point, WS plants were watered to WW levels and recovery was monitored by sampling plants 2 h (midR) and 3 days (R) after rewatering. At each sampling point (i.e. midS, S, midR and R), fully expanded leaves from independent individuals of control (WW) and stressed (WS) plants were collected, immediately frozen in liquid nitrogen and stored at -80ºC for subsequent analyses. Leaf sections were also fixed in paraformaldehyde for immunohistochemical detection of DNA methylation. Fresh leaf samples were used for determination of relative water content.

Plant water status and photosynthetic performance

Relative water content (RWC) was determined using four leaf discs (11mm diameter) per plant (four plants per experimental group). Fresh weight (FW) was recorded and leaf discs were transferred to tubes containing de-ionised water and kept overnight at 4ºC in the dark before recording turgid weight (TW). Finally, leaf discs were dried at 80ºC until constant weight and dry weight (DW) recorded. RWC was calculated using the equation: RWC (%) = (FW – DW)/(TW –

DW) × 100. Midday shoot water potential (Ψ_{md}) was also measured in four plants per treatment with a Scholander-type pressure chamber (PMS Instrument Co., Corvallis, OR, USA).

Steady-state modulated chlorophyll fluorescence was determined with a portable fluorimeter (Mini-PAM; Walz, Effeltrich, Germany). Leaves from 10 plants per treatment were dark-adapted for 30 min to obtain F_0 (minimum fluorescence) and F_m (maximum fluorescence), and then F_v (variable fluorescence, equivalent to $F_m - F_0$) and F_v/F_m (maximum quantum yield of photosystem II (PSII) photochemistry) were determined.

All measurements were carried out in control and stressed plants collected at midday in each sampling point (i.e. midS, S, midR and R).

Hydrogen peroxide quantification

The extraction of hydrogen peroxide (H_2O_2) was carried out according to the procedure described by Kerchev et al. (2012). Extracted H_2O_2 was immediately quantified by fluorimetry using the commercially available Amplex Red Hydrogen Peroxide/Peroxidase Assay kit according to the manufacturer's instructions (Invitrogen Ltd, Paisley, UK). Five biological replicates per sampling point (i.e. midS, S, midR and R) and watering group (i.e. control (WW) and stressed (WS)) were analysed.

Nonprotein redox couples ascorbate and glutathione

Ascorbate (AsA) and dehydroascorbate (DHA) concentrations, as well as oxidised (GSSG) and total (GSH) glutathione were determined according to the microplate method described by Queval and Noctor (2007) in five biological replicates per sampling point (i.e. midS, S, midR and R) and watering group (i.e. control (WW) and stressed (WS)).

Enzymes of the plant ascorbate-glutathione cycle

Specific activities of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) were determined according to the microplate method described by Murshed et al. (2008) with a slight modification. The ascorbate-glutathione recycling enzymes were extracted together from 250 mg of leaf material into 1 mL of the described extraction buffer that additionally contained 5% (w/v) polyvinylpolypyrrolidone (PVPP). Five biological replicates per sampling point (i.e. midS, S, midR and R) and watering group (i.e. control (WW) and stressed (WS)) were analysed.

Lipid peroxidation

The level of lipid peroxidation was estimated by quantifying malondialdehyde (MDA). MDA was determined as thiobarbituric acid (TBA)-reactive substances according to the method described by Hodges et al. (1999). Five biological replicates per sampling point (i.e. midS, S, midR and R) and watering group (i.e. control (WW) and stressed (WS)) were analysed.

Methylation-sensitive random-amplified polymorphic DNA (MS-RAPD)

Total DNA was extracted from 50 mg of frozen leaves according to the combined protocol described by Valledor et al. (2014). Five biological replicates per sampling point (i.e. midS, S, midR and R) and watering group (i.e. control (WW) and stressed (WS)) were analysed. DNA yield and purity were assessed by spectrophotometry and electrophoresis on agarose gels by direct comparison with phage λ DNA. To study the methylation changes in specific DNA sequences, we resorted to the procedure previously reported by our group (Correia et al. 2013). Briefly, DNA was digested in parallel with the two methylation-sensitive isoschizomers HpaII and MspI and, after digestion, a standard RAPD procedure was used to amplify the restriction fragments, using two 'arbitrary' primer sets (OPC and OPH, Eurofins Operon, Luxembourg). The products of RAPD assay were resolved on agarose gels and the interpretation of MS-RAPD bands followed the representation reported by Valledor et al. (2010), appearance/disappearance of bands was used to identify three categories: *de novo* methylation, demethylation, and *de novo* methylation-demethylation events.

Differentially expressed MS-RAPD bands were excised and purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Pittsburgh, PA, USA) according to the manufacturer's instructions. The purified product was then cloned into plasmid pJet 1.2 (Thermo Fisher Scientific, Waltham, MA, USA) following the recommended protocol. The cloned PCR products were sequenced and chromatograms were analysed using Geneious Pro 4.8.2 (Biomatters Ltd, Auckland, New Zealand). Resulting sequences (available in supplementary table S7) were identified using the BLAST tool from Phytozome ver. 10.2 (Goodstein et al. 2012) and the genome of *Eucalyptus grandis* W.Hill ex Maiden, the most related species with available genome. These sequences were mapped into the recently available methylome of *E. grandis* (Schmitz laboratory, unpubl. data) to define whether they were located in genomic regions potentially regulated by DNA methylation (browsing of this data is permitted provided that no genome-wide analyses are to be performed, see http://epigenome.genetics.uga.edu/aj/Schmitzlab_Plant_Methylome_DB/Methylome_E_grandis.html, accessed 30 October 2015).

Immunohistochemical detection

Methylated DNA was immunolocalised according to the procedure described by Valledor et al. (2010). Half cross sections of leaf were fixed and sectioned at 50 mm thickness using a cryomicrotome Leica CH 1510–1 (Leica Microsystems, Wetzlar, Germany). The samples were mounted on slides coated with APTES (3-aminopropyltriethoxysilane; Sigma, St Louis, MO, USA). The leaf sections were permeabilised, blocked with bovine serum albumin and incubated with mouse antibody anti-5-methylcytidine (anti-5-mdC, Eurogentec, Liege, Belgium) diluted 1:50 in 1% blocking solution. Unbound antibodies were washed with 0.1% Tween 20 in PBS. Alexa Fluor 488-labelled anti-mouse polyclonal antibody (Invitrogen, Carlsbad, CA, USA) diluted 1:25 was used as secondary antibody for the 5-methylcytidine detection. The slides were counterstained with DAPI (6-diamidino-2-phenylindole; Sigma). Fluorescence was visualised using a confocal microscope (Leica TCS-SP2-AOBS; Leica Microsystems). Five biological samples were analysed per treatment and sampling point and maximal projection from stack of six slides per sample was acquired using Fiji Software (Schindelin et al. 2012).

Statistical analysis

When applicable, results are presented as mean ± SD. Data were analysed using SigmaPlot for Windows ver. 11.0 (Systat Software Inc., San Jose, CA, USA) by performing Student's t-tests at each sampling point to compare WW(control) and WS (stressed) groups. Significant differences between WS (stressed plants) and WW (control plants) group are indicated: *, $p \leq 0.05$. Some data were previously transformed in order to meet the requirements of normality and homogeneity of variances. MS-RAPD results were analysed by performing a χ^2 test using R Environment 3.1 (R Development Core Team, Vienna, Austria) using core packages.

Results

Watering dynamics affect the redox status of E. globulus

Drought was characterised by a significant reduction in RWC and water potential after 7 days of water withholding (figure 1A, B, midS) and that continued to decrease until the 11th day (Fig. 1A, B, S). This reduction did not affect F_v/F_m (figure 1C, midS and S), nor was it accompanied by an induction of leaf H_2O_2 accumulation, since no significant differences were found between control and stressed plants after watering was discontinued (figure 2, midS and S). Water stress resulted in an increase in the total AsA pool that was accompanied by a shift to a more oxidised status (figure 3; table 1). Similarly, the total GSH pool was increased during water stress (figure 3; table 1). Changes in AsA and GSH pool sizes coincided with a large induction in DHAR activity (figure 4), whereas APX, GR and MDHAR did not show any significant alteration during

the stress phase. Coincident with the shift towards greater oxidation in the AsA and GSH pools, MDA content significantly increased during the stress phase (figure 5).

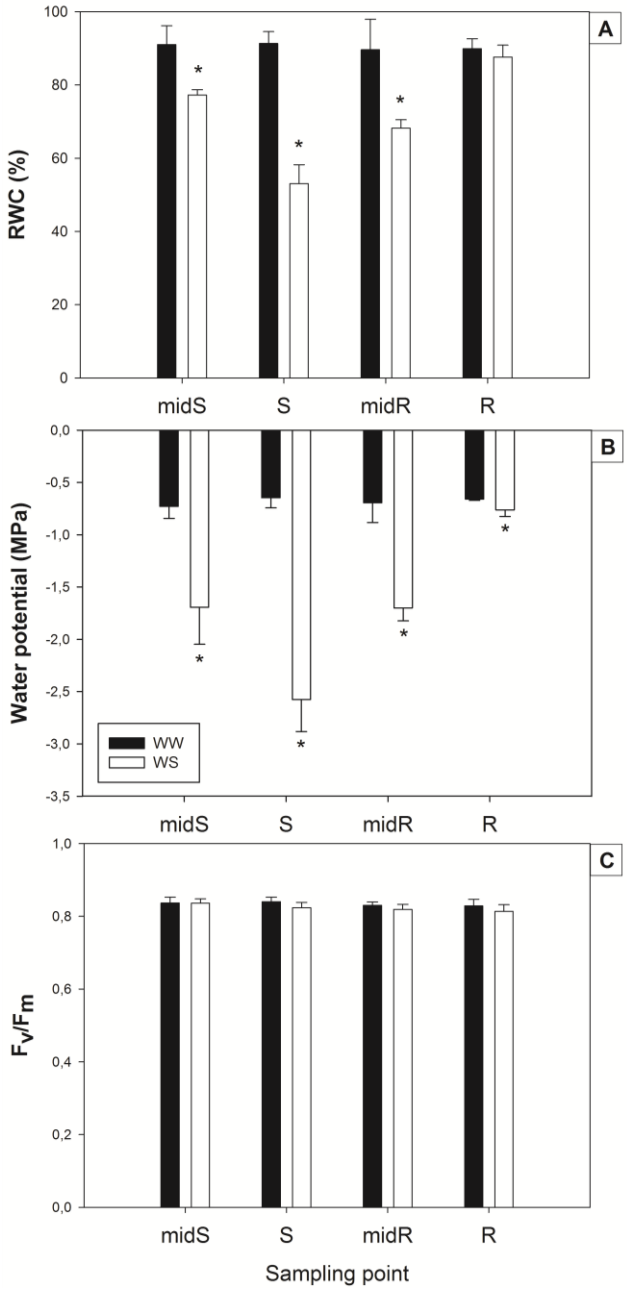

Figure 1 – Relative water content (RWC, A), water potential (B), and maximum quantum yield of PSII (F_v/F_m, C) of *Eucalyptus globulus* exposed to water withholding (midS – 7 days and S – 11 days) and rewatering (midR – 2 h and R – 3 days). Abbreviations: WW, well-watered plants; WS, water stressed plants. Significant differences between WS and WW plants are indicated: *, $p \leq 0.05$.

Following rewatering, plants showed a prompt response with RWC of stressed plants increasing within 2 h and fully recovering within 3 days (figure 1A). Water potential also decreased quickly within 2 h of rewatering and almost fully recovered to WW values in the last sampling point (figure 2B). Once again, F_v/F_m and H_2O_2 levels did not show any significant differences between treatments (figures 1C, 2). 2 h after rewatering the leaf ascorbate content and redox status of the stressed group was indistinguishable from that of leaves that had not received the water stress (figure 3; table 1). The glutathione pool remained elevated due to an increase in GSH for the remainder of the experiment with the result that recovering plants had a more reduced glutathione pool (figure 3; table 1). This was associated with a reduction in APX activity during early recovery while the activity of other enzymes of the ascorbate-glutathione cycle was unchanged from control plants during the entire recovery period (figure 4). During the recovery phase, MDA rapidly decreased to that observed in control plants (midR, figure 5) and following three days of rewatering were lower than those measured in unstressed plants (R, figure 5).

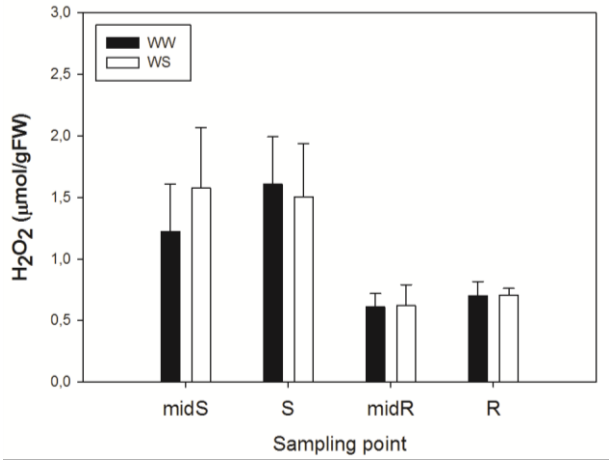

Figure 2 – Hydrogen peroxide (H_2O_2) content in the leaves of *Eucalyptus globulus* exposed to water withholding (midS – 7 days and S – 11 days) and rewatering (midR – 2 h and R – 3 days). Abbreviations: WW, well-watered plants; WS, water stressed plants. Significant differences between WS and WW plants are indicated: *, $p \leq 0.05$.

DNA methylation changes due to the watering dynamics

Estimation of changes in DNA methylation was carried out using two different methods. MS-RAPD was used to discriminate methylation changes occurring in specific DNA sequences (5'-CCGG-3'), and immunolocalisation was applied to survey the global distribution of 5-methylcytosine (5-mC) in the tissue. Both techniques were conducted in leaf tissue and each one exhibited a specific response. MS-RAPD revealed that drought progression was characterised by a large number of demethylation events, mostly during the transition from control to the early stressed conditions (table 2, WW-midS). Later during water stress, demethylation was still the dominant event, but the total number of demethylation events was significantly lower (table 2, midS-S). In contrast, the recovery period was dominated by *de novo* methylation events that occurred at a higher rate during the early recovery period (table 2).

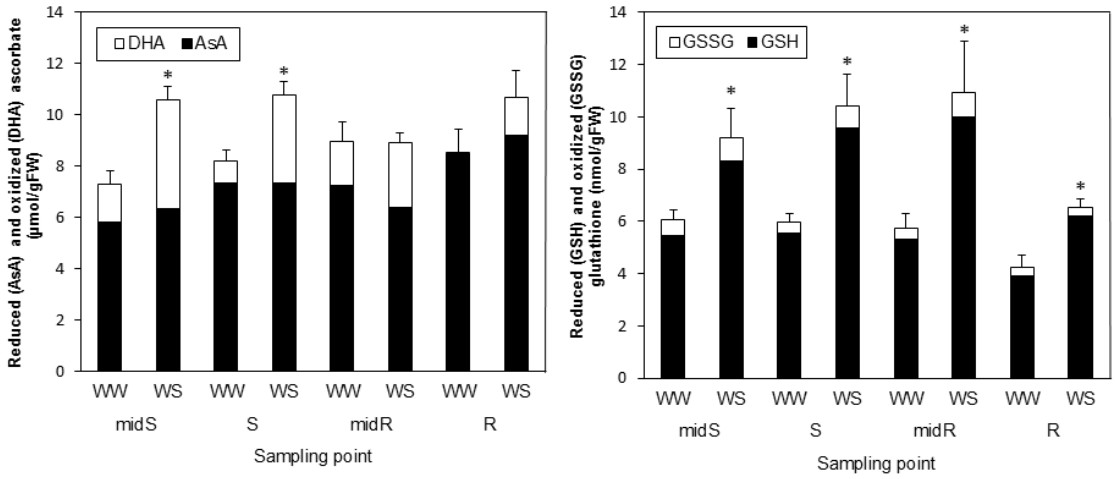

Figure 3 – Total ascorbate (incorporating reduced (AsA) and oxidised (DHA) forms), and total glutathione (incorporating reduced (GSH) and oxidised (GSSG) forms) quantified in the leaves of *Eucalyptus globulus* exposed to water withholding (midS – 7 days and S – 11 days) and rewatering (midR – 2h and R – 3 days). Abbreviations: WW, well-watered plants; WS, water stressed plants. Data are presented as mean values ± SE. Significant differences in the total ascorbate or glutathione pools between WS and WW plants are indicated: *, $p \leq 0.05$.

Table 1 – Oxidation status (%) of the ascorbate and glutathione pools quantified in the leaves of *E. globulus* exposed to water withholding (midS – 7 days and S – 11 days) and rewatering (midR – 2 h and R – 3 days). WW – well watered plants; WS – water stressed plants. An asterisk (*) in WS result indicates a significant difference with respect to the equivalent WW sample ($p \leq 0.05$).

		midS	S	midR	R
Oxidation ascorbate pool (%)	**WW**	20.5	10.3	19.1	15.2
	WS	40.1*	31.9*	28.2	13.8
Oxidation glutathione pool (%)	**WW**	9.7	7.4	7.4	7.5
	WS	9.6	8.2	4.9	5.0*

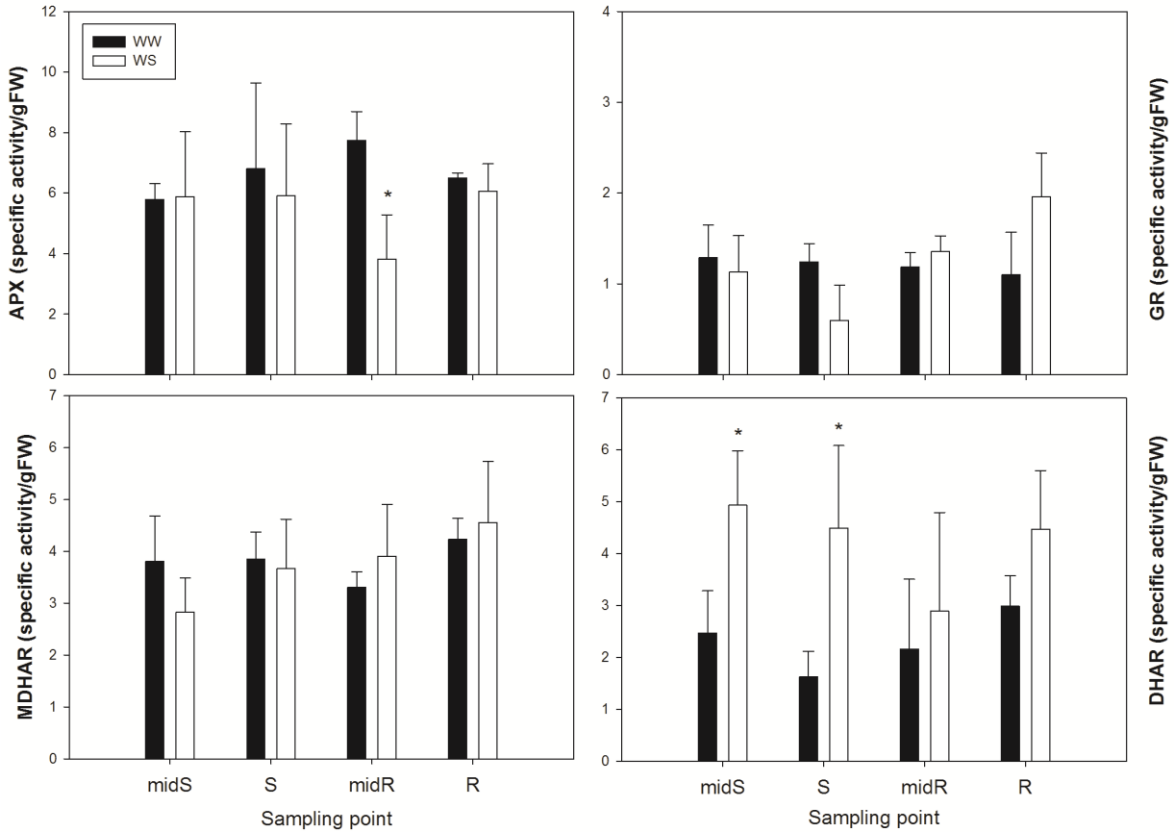

Figure 4 – Specific activity of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), and dehydroascorbate reductase (DHAR) quantified in the leaves of *Eucalyptus globulus* exposed to water withholding (midS – 7 days and S – 11 days) and rewatering (midR – 2 h and R – 3 days). Abbreviations: WW, well-watered plants; WS, water stressed plants. Significant differences between WS and WW plants are indicated: *, $p \leq 0.05$.

Table 2 – MS-RAPD profiles of *E. globulus* leaves exposed to water withholding and rewatering. Interpretation of MS-RAPD bands followed the representation of MSAP (methylation sensitive amplified polymorphisms) detected by HpaII/MspI endonuclease digestion according to Valledor et al. (2010). Appearance-disappearance of bands was used to study the variation of methylation between treatment steps (WW-midS; midS-S; S-midR; midR-R. WW: well-watered conditions; midS: 7 days after water withholding; S: 11 days after water withholding; midR: 2 h after rewatering; R: 3 days after rewatering). Fragment analysis enabled the classification into three categories: *de novo* methylation, demethylation and *de novo* methylation-demethylation (Met-Dem) events. A λ^2 test was used to analyse the data and the result is indicated below.

	MS-RAPD				
	de novo methylation	Demethylation	Met-Dem	Stable	Total
WW-midS	5 (2.9%)	19 (11.2%)	1 (0.6%)	145 (85.3%)	170
midS-S	0	6 (3.5%)	0	164 (96.5%)	170
S-midR	10 (5.9%)	1 (0.6%)	0	159 (93.5%)	170
midR-R	3 (1.8%)	8 (4.7%)	0	159 (93.5%)	170

χ^2	**WW-midS**	**midS-S**	**S-midR**	**midR-R**
WW-midS	×			
midS-S	0,003	×		
S-midR	2,771E-5	8,383E-5	×	
midR-R	0,012	0,011	0,027	×

The DNA methylation changes reported at this point were found to be highly different from the ones related to the stress progression (χ^2 test, table 2). Finally, analysing the recovery progression (midR-R) showed a new increase in demethylation compared with *de novo* methylation events, but with a reduced significance (χ^2 test, table 2). From the differentially expressed MS-RAPD bands, eight were purified and sequenced (table 3). Out of these sequences three could be localised next to annotated genes (table 3). Genes potentially affected by drought-responsive DNA methylation were an F-Box protein, Eucgr.E01419.1 (unannotated gene), and asmbl_9220 (unannotated gene). The other five bands were either close to unannotated gene models or in intergenic regions (figure 6). Furthermore, the overlap between identified bands and *E. grandis* methylome showed an interconnection with regions of high rates of DNA methylation (figure 6). Immunohistochemistry complemented the results obtained using MS-RAPD and additionally provided significant information regarding the tissue distribution of DNA methylation. Under control conditions, 5-mC was equitably distributed over the tissues of the sample (WW, figure 7), while drought progression coincided with an increase in intensity of methylated cytosine, concentrated near vascular vessels (midS and S, figure 7).

Conversely, after rewatering, a prompt reduction in 5-mC signal occurred (midR, figure 7), reaching a similar pattern to the one obtained in control leaves but showing the upper layer of the leaf more strongly marked (midR and R, figure 7).

Table 3 – Identification of differential MS-RAPD bands between well-watered (WW), water stressed (midS and S) and rewatered (midR and R) plants. Bands were sequenced and identified against *Eucalyptus grandis* genome (v. 1.1) available at Phytozome.) Associated modifications – Meth.: *de novo* methylation event; Demeth.: demethylation event.

Band ID	**Associated modification**	**Location and e-value (*E. grandis* genome v 1.1)**	**Annotation**
MSB_01	Meth. WW/ S and demeth. S/midR	scaffold_4:17557132..17557493 (3.2e-174)	Overlaps: Eucgr.D00967.1, F-Box associated domain 2kb upstream of Eucgr.D00966 cyclic nucleotide gated channel,
MSB_02	Demeth. S/midR	scaffold_7:20725390..20730370 (0.0)	
MSB_03	Demeth. WW/midS	scaffold_5:16097232..16097898 (0.0)	Eucgr.E01419.1, non annotated 2kb upstream of Eucgr.E01417.1
MSB_04	Demeth.WW/midS and meth. midR/R	scaffold_4:36337623..36338317 (0.0)	Downstream of Eucgr.D02214.1 (non annotated)
MSB_05	Meth.WW/midS	scaffold_2:16254399..16256434 (0.0)	Intergenic region
MSB_06	Meth. WW/midS	scaffold_7:42648684..42649012 (7.5e-86)	1kb upstream of asmbl_9240
MSB_07	Demeth. WW/midS and meth. midR/R	scaffold_9:13145759..13145962 (5.7e-2)	Intergenic region
MSB_08	Demeth. WW/midS	scaffold_9:30580299..30582334 (0)	Intergenic region

Discussion

The impact of water deficit and the ability to respond after rehydration are important issues when considering *E. globulus* productivity. Relating cellular water status to metabolism becomes possible by using a robust, easily measured indicator of water status, RWC (Lawlor and Cornic 2002).


a
b
c
d
e
f
g
h
Tracks
E_grandis Baseline
Gene Models (E_grandis)
mc_E_grandis
CG
CHG
CHH

Figure 6 – The specific loci corresponding to sequenced MS-RAPDbands (MSB_01 (*a*) . . . 08 (*h*); table 3) were compared with available *Eucalyptus grandis* methylome (Schmitz laboratory, unpubl. data) to validate its presence in 5-mC rich regions. *E. grandis* methylome was visualised using GBrowse available at http://epigenome.genetics.uga.edu/aj/Schmitzlab_Plant_Methylome_DB/Methylome_E_grandis.html, accessed 30 October 2015. Thick red horizontal bars (below) represent the sequences of the analysed genes, localised in the *E. grandis* sequenced genome (upper bar) and *E. grandis* sequenced methylome (middle bar with thin vertical lines that indicate methylated cytosines). In all cases, gene sequences overlap with 5-mC rich regions.

In our work, the pronounced decrease in RWC and water potential confirmed the progression and intensity of the acute water deficit. Upon water loss, growth rates are directly affected by water potential owing to the role of turgor in cell enlargement (Boyer 1968). The decrease in cellular water availability also reduces the potential for metabolic processes as it causes crowding of cytoplasmic components, increasing the potential for molecular interactions that can cause protein denaturation and other harmful events (Hoekstra et al. 2001).

Drought typically results in lower stomatal conductance and reduced CO_2 assimilation leading to over-reduction of the photosynthetic electron transport chain and the induction of photo-oxidative stress within the chloroplasts (Pintó-Marijuan and Munné-Bosch 2014). The chlorophyll fluorescence measurements corroborate a lack of induction of photo oxidative damage within PSII in *E. globulus* leaves, already described in a previous experiment, but showing this persists even under a more severe water deficit. Consistently, we were unable to detect any increase in leaf H_2O_2 although we were able to detect enhanced levels of lipid peroxidation following water stress (figure 5) suggesting an enhanced oxidative load. Similarly, we observed an induction of antioxidant systems that is consistent with an increased cellular oxidative load. Water stress resulted in an increase in the leaf ascorbate content primarily resulting from an increase in DHA and a shift towards a more oxidised status of the ascorbate pool (figure 3). This is consistent with an increased requirement for ascorbate to act as a cofactor for the reduction of ROS leading to enhanced oxidation of the ascorbate pool and subsequent increase in ascorbate synthesis to match rising demand. The total GSH pool size was also enhanced but the pool shifted towards greater reduction under water deficit (figure 3). We observed little change in the activity of the majority of enzymes of the ascorbate-glutathione cycle however DHAR activity was strongly induced by water deficit (figure 4). These data suggest induction of DHAR activity to maintain the redox status of the ascorbate pool associated with increased synthesis of the cofactor GSH. The rate limiting step of GSH biosynthesis is catalysed by the plastid localised glutamate-cysteine ligase which exhibits greater activity when oxidised (Galant et al. 2011); our observation that leaf glutathione content is increased in water stressed plants therefore supports the hypothesis that under these conditions, the

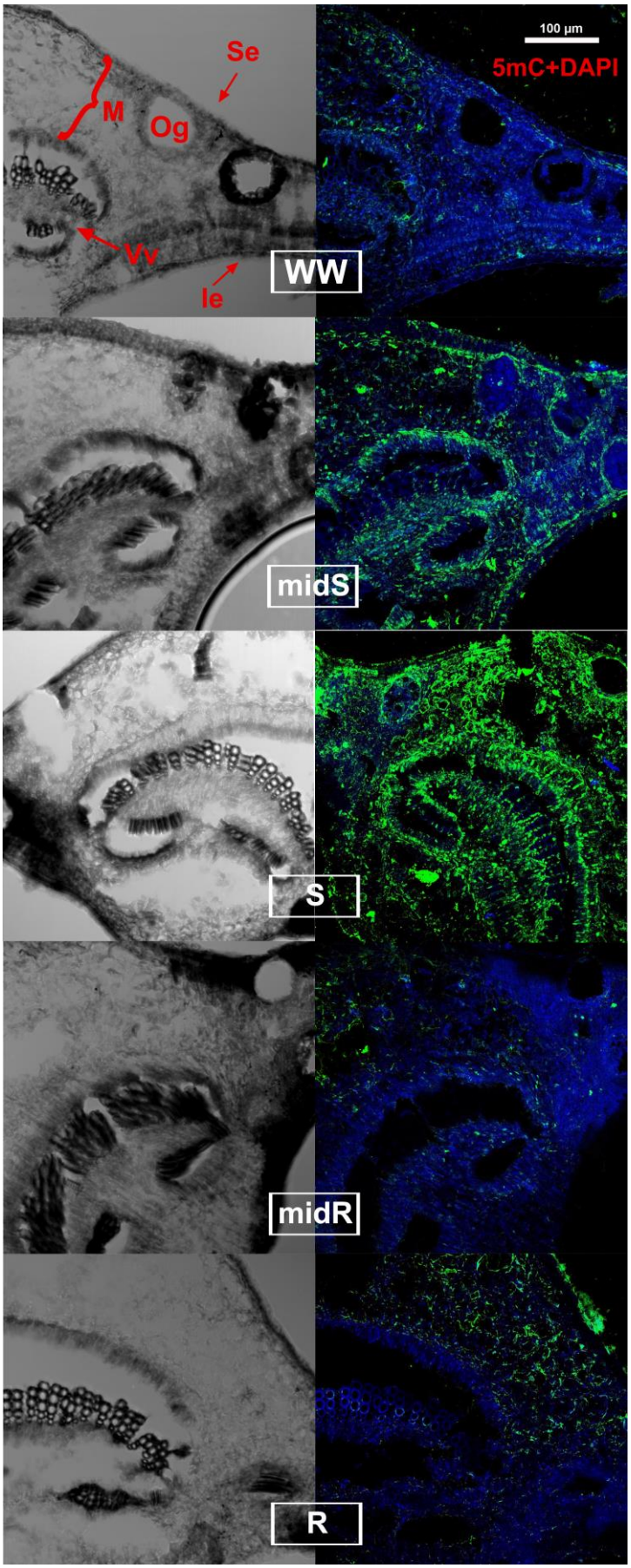

Figure 7 – Immunodetection of 5-mC (5-methylcytosine) in sections of *Eucalyptus globulus* leaf in transversal axis using a confocal microscope (20 ×). On the left-hand side: differential interference contrast (DIC) of leaf section at (WW) well-watered conditions; (midS) 7 days after water withholding; (S) 11 days after water withholding; (midR) 2 h after rewatering; and (R) 3 days after rewatering. On the right-hand side: 5-mC labelling (green signals) and DAPI (blue signals) images were merged in transversal leaf sections. Abbreviations: Se, superior epidermis; Ie, inferior epidermis; Vv, vascular vessel; M, mesophyll; Og, oil gland.

plastid environment may become more oxidising. The data also imply that the pool of reduced nucleotides was sufficient to maintain glutathione reduction status, consistent with our observation that photosynthetic electron transport was maintained under severe water deficit. Taken together, our results suggest that water stress in *E. globulus* results in enhanced superoxide production within PSI leading to an induction of the water–water cycle to reduce ROS. We propose that this induction is sufficient to maintain electron flow through PSII thereby preventing over-reduction, maintaining apparent PSII quantum efficiency and preventing photo-oxidative damage. These data indicate that selection of genotypes with a high capacity for water-water cycle induction may represent a strategy for enhancing drought tolerance in *E. globulus* clones. Furthermore, the data suggest that water stressed leaves were only under a mild oxidative stress that was almost entirely compensated by induction of leaf antioxidant systems.

Genetic control plays an important role adjusting the genotype to the adapted phenotype by regulating genes to express only in specific cells under specific situations (Correia et al. 2013). One of the most important gene expression control mechanisms is DNA methylation. This is a dynamic epigenetic modification that is key in controlling chromatin structure and function and regulating cell development (Solís et al. 2012; Valledor et al. 2015) as well as having an active role in stress response (Pascual et al. 2014). Considering our results, specific DNA methylation changes occurred due to water deficit. To provide a more complete understanding of DNA methylation dynamics, we utilised two different and complementary strategies: the immunodetection of 5 mC identifies and localises all methylated cytosines present; whereas MS-RAPD can only discriminate methylation of cytosines present in specific CCGG sites, which are present in a small part of the genome of the plants, and frequently located in gene promoter regions (Correia et al. 2013). MS-RAPD results revealed that the initial period of drought was highly dynamic and dominated by demethylation events suggesting that under initial stress conditions the *E. globulus* cell reorganises its DNA structure and compaction by demethylation to promptly activate defence mechanisms. Demethylation events continued to predominate throughout the stress induction although with lower frequency as the duration of stress increased. The demonstration that MS-RAPD coincided with regions with high DNA methylation validated the approach we followed.

Although immunohistochemical methods applied to study cytosine methylation status give accurate measures of the degree of DNA methylation, one of the notable aspects of these approaches is that they provide information on tissue specific patterns. Several reports using immunolocalisation coupled with confocal microscopy in order to localise 5-mC and its involvement in plant development are already available (e.g. Meijón et al. 2009; Testillano et al. 2013). Our study indicated that drought-induced 5-mC changes occur mainly in vascular tissues. The DNA methylation signal increased near the vascular bundle sheath cells and this signal became

stronger as the water deficit intensified. Bundle sheath cells are associated with vascular tissues and have a well-recognised role in both plant drought response and ABA metabolism, interconnecting vascular parenchyma cells with activation of ABA-dependent signal cascade (Endo et al. 2008). This aspect was recently corroborated by Jesus et al. (2015) that reported specific local dynamics of ABA detected by immunolocalisation in leaves of *E. globulus* under water deficit. Moreover, we note that these events could be related to the way secondary stress drivers, such as oxidative stress, are perceived and how signals are transmitted from vascular tissues (probably the first line of defence) to the whole plant level. Interestingly, ROS involvement was also reported in bundle sheath cells in leaves of *Arabidopsis* exposed to excess light (Karpinski et al. 1999; Fryer et al. 2003).

Altogether, the DNA methylation results are in accordance with those described in the literature (Gourcilleau et al. 2010; Raj et al. 2011; González et al. 2013; Wang et al. 2014). They reveal that although stress induces a hypermethylation in the genome of stressed plants, which reveals a global inactivation of normal growth responses, many loci are found hypomethylated (Boyko et al. 2010; Correia et al. 2013), indicating an active regulation of specific genes induced in response to stress.

The *E. globulus* ability to recover from drought stress was remarkable. Although RWC of recovered plants usually reach WW levels within several days of rewatering (Davidson and Reid 1989; Pérez-Pérez et al. 2007), plants exhibiting an increase in RWC and water potential after 2 h of rewatering showed a prompt reaction to water availability. We have previously demonstrated that *E. globulus* rapidly recovers the capacity for photosynthetic carbon assimilation following alleviation of moderate drought stress (Correia et al. 2014). These data further support our hypothesis that the induction of antioxidant mechanisms during drought prevents photo oxidative damage within the electron transport chain. Here, we demonstrated the capacity of the plant to also rapidly rebalance the antioxidant systems, such that the ascorbate pool of stressed plants resembled that of unstressed plants within 2 h of rewatering (figure 3) and the activities of ascorbate-glutathione cycle enzymes similarly recovering within a short period of time (figure 4). Following rewatering, lipid peroxides were rapidly reduced and within 2 h levels were similar to those in unstressed plants suggesting a highly active system for their removal. α-Tocopherol plays an essential role in the reduction of lipid peroxides and plants accumulate this compound and its oxidation products under high light stress (Piller et al. 2014). The primary oxidation product is the a-tocopheroxyl radical which is rapidly reduced in vitro by ascorbate in a spontaneous reaction that produces monodehydroascorbate (Scarpa et al. 1984) that is subsequently regenerated via the ascorbate-glutathione cycle (Munné-Bosch 2005). It is therefore conceivable that the accumulation

of soluble antioxidants not only protects the photosynthetic machinery under conditions of drought but also contributes to the removal of lipid peroxides upon recovery.

During early recovery, the MS-RAPD pattern was reversed with de novo methylation predominating indicative of a rapid adjustment to the new environmental condition. The immediate decrease in global DNA methylation after stress relief (2 h) is also noteworthy and stresses the highly dynamic and reversible nature of this epigenetic mechanism that enables a quick adaptation to constantly changing environmental conditions.

The data presented here suggest and reinforce that *E. globulus* has a strong adaptive capacity to water deficit. This is characterised by a rapid boosting of antioxidant defences thereby preventing the induction of oxidative stress and protecting the capacity of electron transport through PSII. Furthermore, it is also clear that DNA methylation exhibits a highly rapid and dynamic response to water deficit-induced mild oxidative stress, mirroring those alterations observed in the antioxidant systems. It seems likely that the resulting capacity for global metabolic and physiological reprogramming in response to stress enables this species to thrive in harsh and changeable environments.

The relation between oxidative stress, DNA methylation and altered cellular function is well studied in human cells (Franco et al. 2008); however, research into the relationship between these events in plant cells is still poorly investigated with only a few reports available (Causevic et al. 2006; Filek et al. 2008; Cyr and Domann 2011). Indeed, although extensive research has demonstrated crosstalk between cellular redox homeostasis and plant growth regulators, much remains to be discovered with regard to the regulation of genetic and epigenetic factors by individual redox mediators with specific roles (Considine and Foyer 2014).

We describe the parallel induction of redox and DNA methylation changes and show that changes in DNA methylation can occur even under only mildly oxidising conditions. Furthermore, the link between stress and epigenetic regulation of an uncharacterised F-box protein, associated with protein ubiquitination and signal transduction, provides a potential target for continuing investigations of the relationship between stress signalling, epigenetics, and adaptive processes. However – and despite the parallelism of this relationship – the study was not designed to determine causality and it is possible that both redox and DNA methylation alterations were independently but similarly affected by water stress. To identify the redox and other signals that control DNA methylation and to define any causal link between cellular redox status, reactive oxygen signalling and changes in DNA methylation, and to identify the underlying molecular mechanisms of signal transduction should therefore be key targets in further research.

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Supplementary Data

Table S7: Sequencing data of differentially expressed MS-RAPD bands.

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Chapter 4

field trial

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4. Gene expression of stress tolerance indicators – can we extrapolate from the chambers to the field?

Summary

Given the gap that exists between the controlled conditions used in the laboratory and the heterogeneous environment that plants encounter in the field, we decided to test if the results of the previously set selection indicators of water stress tolerance, defined in a controlled climate chamber experiment, could be extrapolated when tested in field-grown *Eucalyptus globulus*. Therefore, plants from previously tested clones (AL-18 and AL-13) were planted in the field and allowed to grow independently. When summer dry weather began, some of the plants were irrigated (IR), and others were left under environmental conditions of reduced rainfall (NI) during six and a half weeks prior to rewatering. No significant changes were detected regarding height and water potential between IR and NI, but both clones showed lower photosynthetic and transpiration rates and decreased stomatal conductance in NI, recovering partly after rewatering. Regarding the analysed transcripts, clone AL-18 showed few fluctuations in the conditions tested, and the alterations found in clone AL-13 highlighted the impact of early rewatering rather than the long-term water deficit, which indicated an induction of photosynthetic and photorespiration metabolism after artificial irrigation. The results obtained here using field-grown *E. globulus* draw a very distinct picture when compared with a controlled climate chamber experiment, and ultimately corroborate that the knowledge acquired from imposing the stress individually to test stress-tolerant plants cannot be extrapolated to field-grown plants in the context of developing selection markers.

Introduction

Widely established throughout the Mediterranean area, maintenance and sustainability of *Eucalyptus* plantations present concerns because of the region's climate, with characteristic severe summer drought that negatively affects eucalypt growth (Villar et al. 2011; Navarrete-Campos et al. 2012). Current plantations are composed by clonal collections that have resulted from selection of genetic material based on empirical growth rates and fibre productivity from field trials (Navarrete-Campos et al. 2012). However, most of these genetic selections have disregarded plant physiological responses to potential changes in environmental conditions, such as water availability, so we now need to maximize survival and improve growth rates via enhancing environmental stress resistance (Villar et al. 2011; Navarrete-Campos et al. 2012).

Following this idea and based on early research (Correia et al. 2014a; Correia et al. 2014b; Correia et al. 2016b; chapter 2), our latter study aimed to find specific variations in key transcripts involved in putative pathways that could be identified as potential molecular indicators linked to enhanced water stress tolerance in two *Eucalyptus globulus* Labill. clones following a controlled experiment in a climate chamber (chapter 3.1). Our results validated the modulation of specific photosynthesis- (RCA: chloroplastic RuBisCO activase; PRK: chloroplastic phosphoribulokinase; and FNR: ferredoxin-NADP(H) oxireductase), photorespiration and antioxidative- (mMaDH: mitochondrial malate dehydrogenase; pMaDH: peroxisomal malate dehydrogenase; GCSH: mitochondrial glycine cleavage system H; CAT: peroxisomal catalase; SOD: mitochondrial superoxide dismutase; and IFR: isoflavone reductase), and hormonal- (RD22: responsive to desiccation 22; DREB1A: dehydration response element B1A; and GORK: potassium channel GORK) related genes in drought stress, with a major involvement during stress progression in the most tolerant clone and a central role in the recovery in the least tolerant one. The experiment identified RCA, FNR, mMDH, CAT and IFR as potential molecular indicators linked to enhanced water stress tolerance in *Eucalyptus globulus.*

Despite the relevance of these conclusions, several authors defend that testing stress-tolerant plants by imposing the stress individually, although valuable, might be unsuitable in the context of developing selection markers that enable plants to thrive and maintain a high yield in field conditions (Atkinson and Urwin 2012). In the field, conditions are unlike the controlled conditions used in the laboratory and plants are most likely to be subjected to a combination of different abiotic stresses: for example, when considering drought plants frequently also encounter heat stress (Mittler 2006; Mittler and Blumwald 2010), which is the case for the typical Mediterranean summer droughts. As the majority of abiotic stress studies are performed under laboratory controlled conditions without reflecting the actual field conditions, the knowledge

gained by these studies cannot be extrapolated to develop plants with enhanced tolerance to field conditions and a considerable knowledge gap exist (Mittler 2006).

Keeping this in mind, this study aims to test the hypothesis that the results of the previously set selection indicators of water stress tolerance, defined in a controlled climate chamber experiment, can be extrapolated when tested in field-grown *Eucalyptus globulus*. Therefore, plants from the same tested clones with different degrees of tolerance (AL-18 and AL-13), with the same age and production method as used before, were planted in the field during winter and allowed to grow independently in a typically Mediterranean plantation site. When summer dry weather began, some of the plants were given supplementary and artificial irrigation, and others were left under environmental conditions of reduced rainfall. Irrigated (IR) and non-irrigated (NI) plants were analysed at the end of the summer and after rehydration (2 h and 3 days after artificial irrigation), and the previously defined potential molecular indicators linked to enhanced water stress tolerance in *Eucalyptus globulus* were analysed by quantitative PCR. Stress intensity and progression was also verified by measuring growth, water potential and gas exchange.

Materials and methods

Plant material and experimental design

Five month old rooted cuttings of *Eucalyptus globulus* (two clones: AL-18 and AL-13) were obtained from the breeding program of Altri Florestal SA (Portugal). The plants were transferred from an outdoor shaded house and plastic containers filled with 3:1 (w/w) vermiculite:peat, and transplanted to the experimental field (40.075122ºN, 8.811844ºW – central Coastal region of Portugal) in early November 2015. As the field presented a substantial slope, we made sure to plant all plants subject to natural conditions at higher elevation and the ones to be irrigated in a lower area. Plants were distributed homogenously so that all plants had the same orientation to the sun, with a minimum distance among them of 1.5 m. The plots and their surroundings were manually maintained clear of vegetation that could have interfered with the plant growth. Until the experiment started, all plants were grown under rain-fed conditions or were given supplementary irrigation using a 5 L bucket every two days at evening (after early July, reduced rainfall) in order to maintain well-watered conditions and avoid transplantation stresses. In the meantime, they received starter fertilization and insecticide treatment.

The experiment was conducted from late July 2016 until early September 2016 under natural environmental conditions (Supplementary Figure S1). During the experiment, watering was partially controlled: plants were kept under well-watered conditions (IR: artificial irrigation each couple days at evening); or subject to environmental conditions of reduced rainfall (NI:

supplementary irrigation was restricted, occasional light rain showers). Irrigated (IR) and non-irrigated (NI) plants from each clone (AL-18 and AL-13) were sampled six and a half weeks after supplementary irrigation was restricted (S). After this sampling point, NI plants were watered to IR levels and recovery was monitored by sampling plants 2 h (midR) and 3 days (R) after rewatering. The first sampling day (S and midR) was representative of a hot summer day, whereas the third day (R) was cooler.

At each sampling point (i.e., S, midR and R), homogeneous leaves from five individuals of each watering group were used for *in vivo* measurements of leaf gas exchange parameters (see below) and were measured to determine plant water potential (see below) at midday. At the same time, fully expanded leaves from the same individuals of control (IR) and non-irrigated (NI) plants were collected, immediately frozen in liquid nitrogen and stored at -80ºC for subsequent analyses (RNA extraction and gene expression analysis).

Plant height

Height of IR and NI plants was separately recorded for each clone at the beginning and end (sampling point S) of the six and a half week period of controlled supplementary irrigation. Growth is expressed as the difference between final and initial height.

Water potential

Shoot water potential (Ψ_{md}) was measured using a Scholander-type pressure chamber (PMS Instrument Co., Corvallis, OR, USA). Measurements were carried out in five plants per clone (AL-18 and AL-10) and watering group (IR and NI) around midday (solar time) at each sampling point (i.e., S, midR and R).

Lipid peroxidation

Lipid peroxidation was estimated by measuring the amount of MDA (malondialdehyde) in leaves following the protocol described by Hodges et al. (1999) from 40 mg of frozen leaves per mL of extraction buffer.

Gas exchange and stomatal conductance

Net CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and stomatal conductance (g_s, mol H_2O m m^{-2} s^{-1}) were determined using a portable infrared gas analyser (LCpro-SD, ADC BioScientific Ltd., UK) equipped with the broad leaf chamber. To determine the saturation light intensity, light response curves of CO_2 assimilation were performed with the following PPFD (photosynthetic photon flux density): 2000, 1500, 1000, 750, 500, 250,

100, 50 and 0 µmol m^{-2} s^{-1}. Punctual measurements were performed at saturation light intensity (1000 µmol m^{-2} s^{-1}, defined after analysis of light response curves). Ambient temperature, CO_2 and H_2O concentration, and air flow 200 µmol s^{-1} were maintained inside the chamber during all the measurements. Data were recorded when the measured parameters were stable (2 – 6 min) and were carried out in five plants per clone (AL-18 and AL-10) and watering group (IR and NI) around midday (solar time) at each sampling point (i.e., S, midR and R).

RNA extraction and cDNA synthesis

Total RNA was extracted from 50 mg of frozen leaves using the Spectrum™ Plant Total RNA Kit (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. To remove any contaminating traces of genomic DNA, RNA samples were treated with rDNase (Macherey-Nagel, Düren, Germany) and further purified using the NucleoSpin® RNA Clean-up (Macherey-Nagel) protocol. RNA integrity was checked by electrophoresis on an agarose gel and yield and purity were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For each sample, single-stranded cDNA was synthesised from 1.0 µg of total RNA with the Maxima Reverse Transcriptase (Thermo Fisher Scientific) and random hexamer primer (Thermo Fisher Scientific).

Quantitative Real-Time PCR (qRT-PCR)

Target genes were defined by their putative role as stress markers under drought stress and considering former studies (Correia et al. 2014b; Correia et al. 2016b); chapter 3.1). Gene-specific primers are listed in table 1. Quantitative PCR was performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Reaction mixtures in a final volume of 20 µL contained 1× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fischer Scientific), 0.48 µM of gene-specific primer and 1.5 µL of a 10-fold dilution of the previously synthesised single strand cDNA. The two-step cycling protocol was used for all reactions: a first cycle of 95ºC for 10 min and then 45 cycles of 95ºC for 15 and 60ºC for 60 s. Fluorescence detection took place at the end of each one of these 45 cycles. To confirm the presence of a single amplicon a dissociation step was also carried out. Prior to qRT-PCR cDNA samples were pooled in groups of three biological replicates, and analysed by triplicate.

Five potential housekeeping genes were tested in order to select the best ones to normalise data: actin (ACT), tubulin (TUB), histone H2B (H2B), ubiquitin (UBQ), and glyceraldehyde 3-phosphate dehydrogenase (GADPH) – primers sequences in table 1. Their constitutive expression was validated with geNorm v3.5 (Vandesompele et al. 2002), separately for each clone. H2B and ACT were chosen as reference genes and their relative mean expression was used for data

normalisation of clone AL-18, and ACT and GADPH were used in clone AL-13. Normalised Relative Quantities (NRQ) and Standard Errors of RQ were determined according to Hellemans et al. (2007).

Table 1 – List of target and housekeeping genes and primers sequences used in the qRT-PCR.

Protein	**Target gene**	**Forward primer sequence (5'-3')**	**Reverse primer sequence (5'-3')**
RuBisCO activase	RCA	CTGGGCACCAACT CGCGAAGAC	AGCACGCAGGGC ACCAAAGAAG
Chloroplastic phosphoribulokinase	PRK	GAGGCAACCCGGA CTCGAACAC	GGCCCTTGGGTCA AGTGCAGTC
Ferredoxin-NADP(H) oxireductase	FNR	ACCACGGAGGCTC CAGCTAAGG	CGTGTTGAGGAG GCACCTTCCAATG
Mitochondrial malate dehydrogenase	mMDH	AGTGCTGCGGATC GTGGTGAAATG	AGTGCTGCGGATC GTGGTGAAATG
Peroxisomal malate dehydrogenase	pMDH	CCACCGCAGCCGA AGTTTTCAAG	CCTGCATGACCCC CAACAACTGG
Mitochondrial glycine cleavage system H	GCSH	CGATCATGCGCAA GACCATTTGGG	GGTGGCCTTGACG CTCTCAACC
Peroxisomal catalase	CAT	CAGGGGAGCGAGC GCAAAAGG	TAAGGGTTTCAGG GCTGCCACG
Mitochondrial superoxide dismutase	SOD	GGATGGGTGTGGC TTGGTGTGG	GAACCAAACTTG GTCCCTTGGTCAC
Isoflavone reductase	IFR	ACCGACATCCACC GCCTTCTTG	CAGCTCTAGTCCT GGGCTGCAC
Responsive to desiccation 22	RD22	TCACGGTGATTCTG GCGACTTCC	TGGATTTTGGCAT TTGCGTGTTGGG
Dehydration response element B1A	DREB1A	CCGGGAGCCCAAC AAGAAGACC	AGTCGGCGAAATT GAGGCACGC
Potassium channel GORK	GORK	TCTTTGTGCAGCCC GTGGCAAG	ACATGAAGTGGG GTTCGGTGGTC
Actin	ACT	AGAGCATCCCGTG CTCCTCACC	GAGCACGGCTTG AATGGCAACG
Tubulin	TUB	CCAGCTCGAGCGA GTGAACGTG	GTGCGCAGGCTGT CCATAGTCC
Histone H2B	H2B	GCGGGTGAAGAAG AGCGTGGAG	AGGCCTCCTGGGC GAGTTTCTC

Ubiquitin	UBQ	GGGATTTCGCCGG ATGAGATTGTTC	AAATGTGGGCTCG TGGGGCAAG
Glyceraldehyde 3-phosphate dehydrogenase	GADPH	CGCCTTCCCTCCAG CTTCAACG	ACAGGCTGAATG CTCCTGACAGAGG

Statistical analysis

All physiological measurements are presented as mean ± standard error (SE) of five plants per treatment. At each sampling point, significant differences between NI and respective IR are indicated (* in the NI column) at $p \leq 0.05$, according to a Student's t test, which was performed using SigmaPlot (SigmaPlot for Windows v. 11.0, Systat Software Inc., San Jose, CA, USA).

Results and discussion

Growth of IR and NI plants (AL-18 IR: 34.6 ± 2.9 cm, NI: 27.8 ± 2.4 cm; AL-13 IR: 45.8 ± 2.0 cm; NI: 33.6* ± 2.9 cm) revealed clone AL-13 growing more in IR conditions, but also more affected in NI than clone AL-18. This result largely aligns with the work of Reis et al. (2006) who compared several clones of other *Eucalyptus* spp., and found differences in height growth among clones, despite no significant difference in growth between irrigation treatments. Regarding water potential and MDA concentration, no significant changes were detected when comparing irrigated (IR) and non-irrigated (NI) field-grown *E. globulus* after six and a half weeks subject to a typically Mediterranean summer dry weather (S), as shown in figure 1.

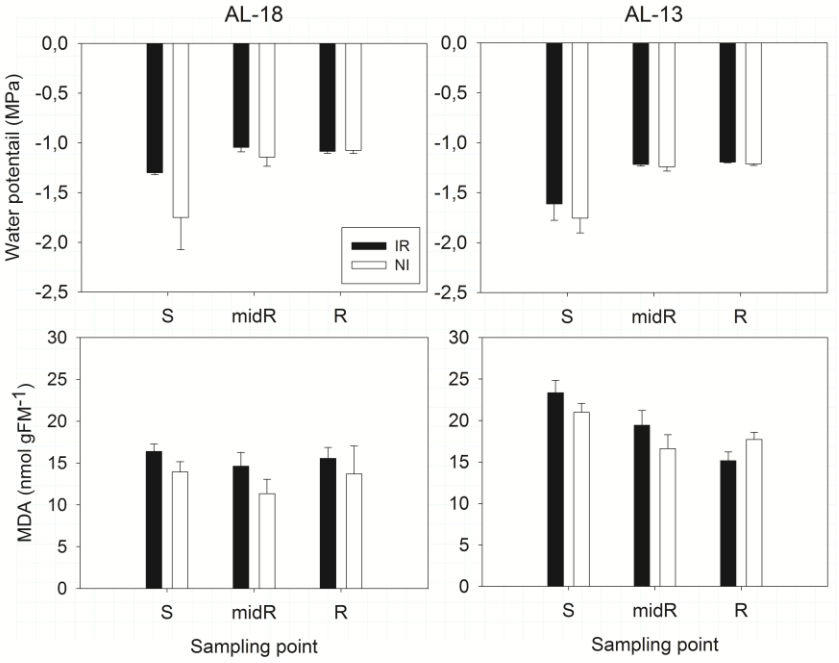

Figure 1 – Shoot water potential and leaf MDA content of two field-grown *Eucalyptus globulus* clones (AL-18, left side, and AL-13, right side). Plants were artificially irrigated (IR, black column) or subject to environmental conditions of reduced rainfall (NI, white column) during six and a half weeks (S). Water potential after recovery (midR – 2 h and R – 3 days) is also shown. If present, significant differences between NI and respective IR are indicated: * $p \leq 0.05$.

The non-significant tendency of decreased water potential found at S may be explained by IR values lower than would be expected. The reason for this result might be the fact that the first sampling day was a very clear and hot summer day: even under artificial irrigation, plants were suffering from the environmental conditions, which cancelled major differences we could find between IR and NI plants. This explanation concurs with leaf MDA content, which presents slightly higher levels in IR plants. The values of IR and NI plants at midR, which took place 2 h after S, reinforce this, indicating that irrigating at the middle of a hot summer day alleviated both IR and NI plants of the two clones considering water potential, and reduced MDA levels in clone AL-13. Despite that, IR plants of clone AL-18 were less affected than AL-13 plants (comparing IR at S with the other sampling points, midR and R) and both clones presented minimum values of midday water potential less negative than those reported in the literature for other eucalypt species (-2.8 MPa in *Eucalyptus grandis* Hill ex. Maiden, Mielke et al. (2000)). This indicates the plants were only suffering a moderate water stress, which *E. globulus* is reported to tolerate in the field (White et al. 1996).

Gas exchange results, on the other hand, highlight significant differences between IR and NI plants, with both clones showing lower photosynthetic (A) and transpiration (E) rates in NI (figure 2). This response was also accompanied by decreased stomatal conductance (figure 2). Following rewatering, plants recovered partly, with greater evidence regarding E of clone AL-18. Our minimum (NI) values of A and g_s were close to the range described for a different commercial eucalypt species (*E. grandis*) growing under field conditions (Mielke et al. 2000), but the maximum (IR) values were higher. Once again, IR values at S reveal some stress at this point: lower A and higher E in S than in midR and R, mainly in clone AL-13 (figure 2). This reinforces the prevailing role of a very clear and hot summer day: Mielke et al. (2000) discuss how A and g_s are highly controlled by the microclimatic variables photosynthetic active radiation and vapour pressure deficit, even in a winter moderate water deficit. Another explanation for reduced photosynthesis in conditions of summer high light inhibition could be leaf respiration inhibition, which was found to occur at greater degree in droughted than well-watered *Eucalyptus saligna* (Crous et al. 2012). These authors explored how variation in light inhibition of respiration affected predictions of the carbon balance of individual leaves, finding that even a moderate level of light inhibition of leaf respiration can result in substantial decreases in ratios of respiration to light-saturated photosynthesis (Crous et al. 2012). However, our current knowledge (Correia et al. 2016a; chapter 3) lead us to disregard such conclusion, once we found *E. globulus* with a high capacity for water-water cycle induction that most probably supplies additional ATP for the photorespiratory pathway. Anekonda et al. (1999) has already shown that selective environmental

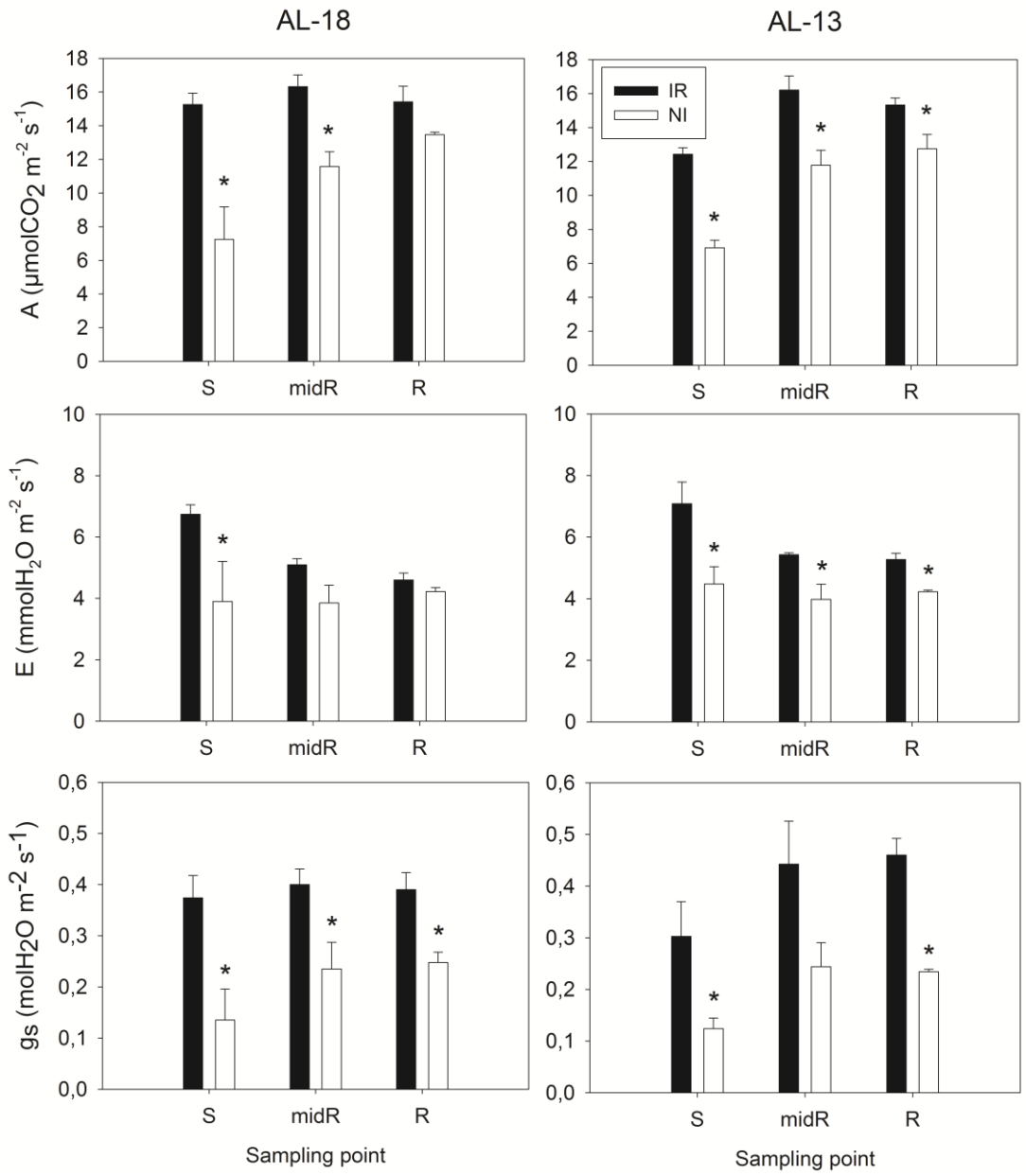

Figure 2 – Net photosynthetic rate (A), transpiration rate (E), and stomatal conductance (g_s) of two field-grown *Eucalyptus globulus* clones (AL-18, left side, and AL-13, right side). Plants were artificially irrigated (IR, black column) or subject to environmental conditions of reduced rainfall (NI, white column) during six and a half weeks (S) and rewatered (by artificial irrigation, midR – 2 h and R – 3 days). If present, significant differences between NI and respective IR are indicated: *, $p \leq 0.05$.

forces, most likely climate and specifically temperature, have shaped distinct respiratory metabolic adaptation in different eucalyptus subgenera and species.

The relative abundances of the transcripts related to the photosynthetic machinery are shown in figure 3. RuBisCO activase (RCA) was the only overexpressed transcript in clone AL-18

after six and a half weeks subject to a typically Mediterranean summer dry weather (S) (figure 3a). In clone AL-13, RCA was overexpressed at S and 2 hours after rewatering (midR, figure 3b), and phosphoribulokinase (PRK, figure 3d) and ferredoxin-NADP(H) oxireductase (FNR, figure 3f) presented an induction at midR. Considering the transcripts related to photorespiration, the relative mRNA abundances of the five studied genes are shown in figure 4. In clone AL-18, these transcripts did not experience any remarkable alterations.

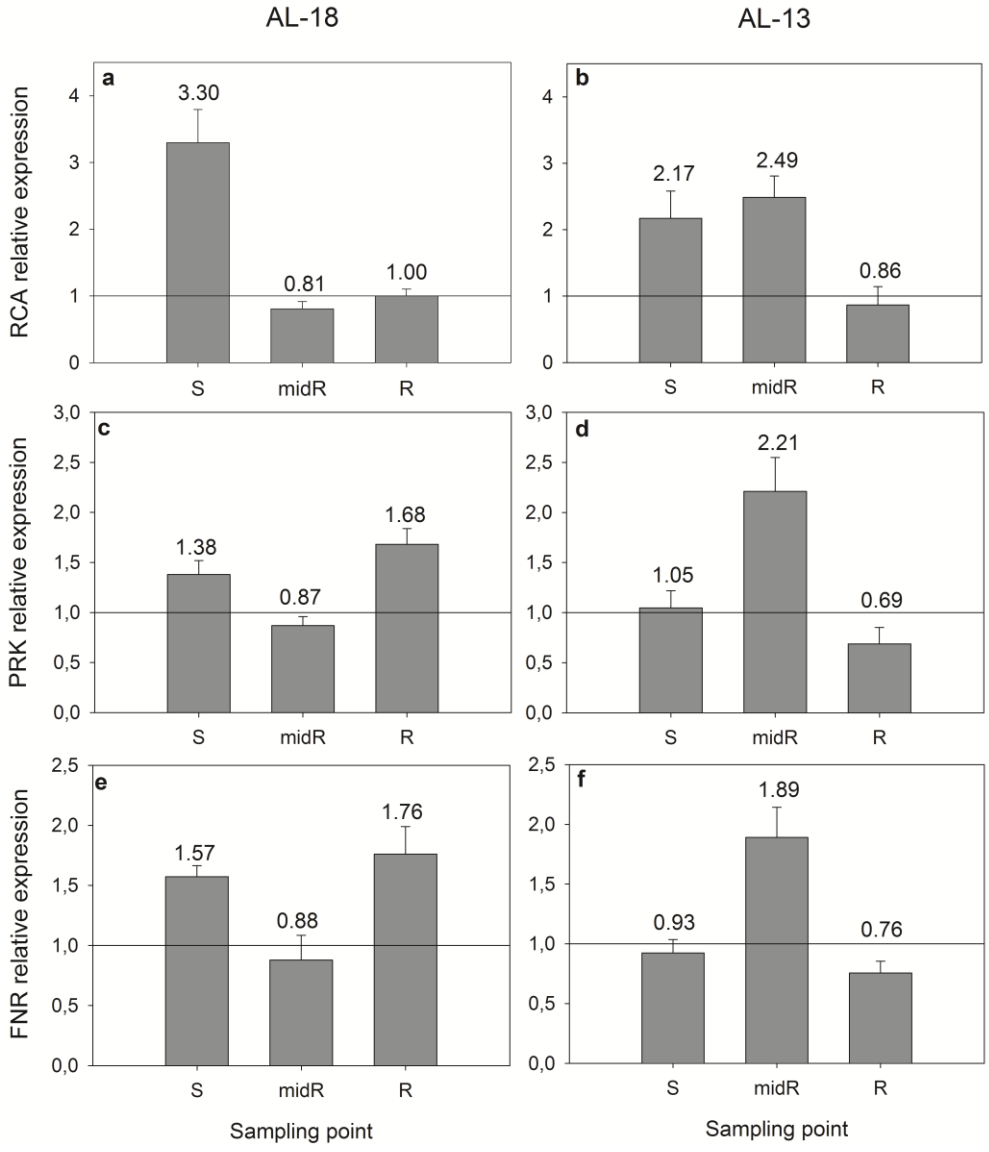

Figure 3 – Relative mRNA abundances [RCA: RuBisCO activase (a, b); PRK: chloroplastic phosphoribulokinase (c, d); and FNR: ferredoxin-NADP(H) oxireductase (e, f)] of two field-grown *Eucalyptus globulus* clones (AL-18, left side, and AL-13, right side). Plants were artificially irrigated (IR) or subject to environmental conditions of reduced rainfall (NI) during six and a half weeks (S) and rewatered (by artificial irrigation, midR – 2 h and R – 3 days). Transcript relative expression is represented as fold change of NI samples to respective control – IR at each sampling point.

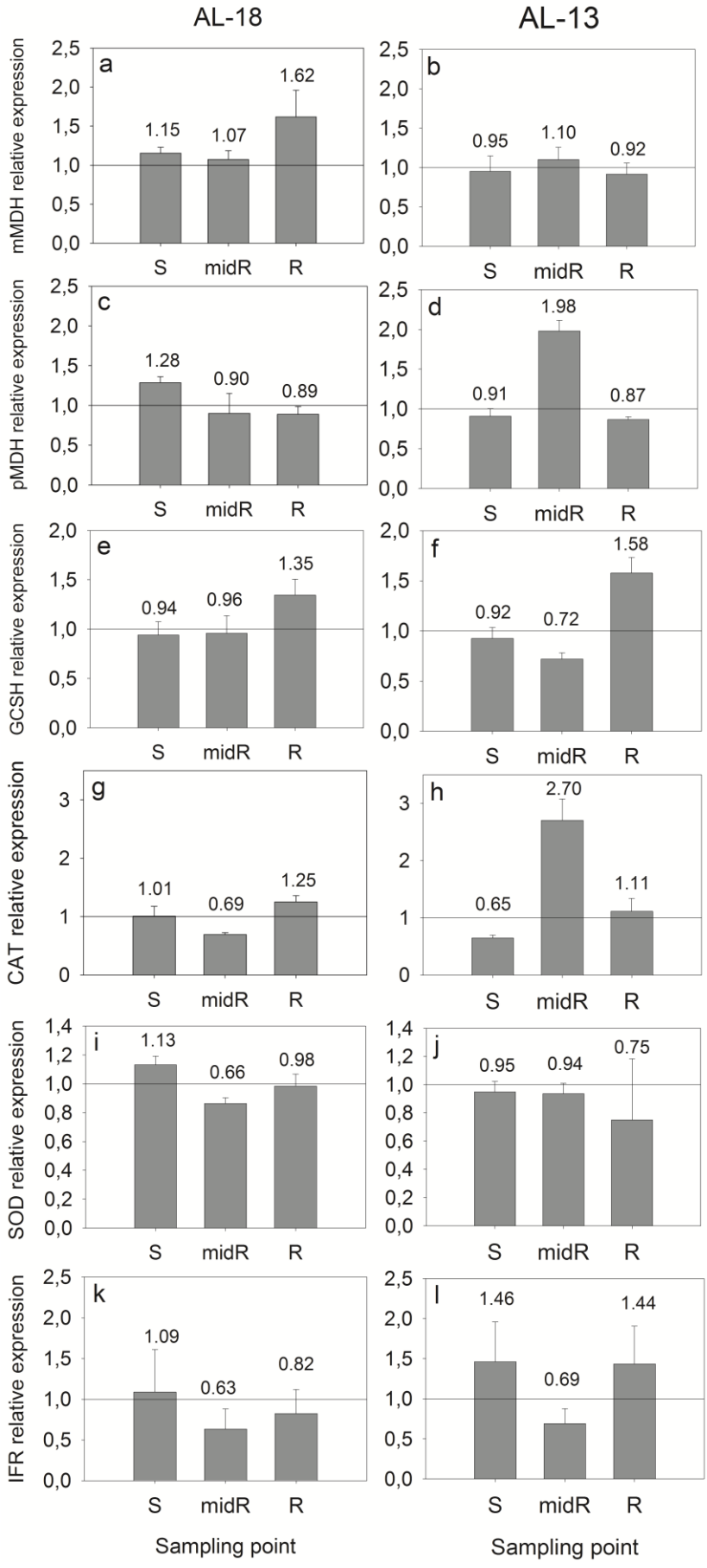

Figure 4 – Relative mRNA abundances [mMDH: mitochondrial malate dehydrogenase (a, b); pMDH: peroxisomal malate dehydrogenase (c, d); GCSH: mitochondrial glycine cleavage system H (e, f); CAT: peroxisomal catalase (g, h); SOD: mitochondrial superoxide dismutase (i, j); and IFR: isoflavone reductase (k, l)] of two field-grown *Eucalyptus globulus* clones (AL-18, left side, and AL-13, right side). Plants were artificially irrigated (IR) or subject to environmental conditions of reduced rainfall (NI) during six and a half weeks (S) and rewatered (by artificial irrigation, midR – 2 h and R – 3 days). Transcript relative expression is represented as fold change of NI samples to respective control – IR at each sampling point.

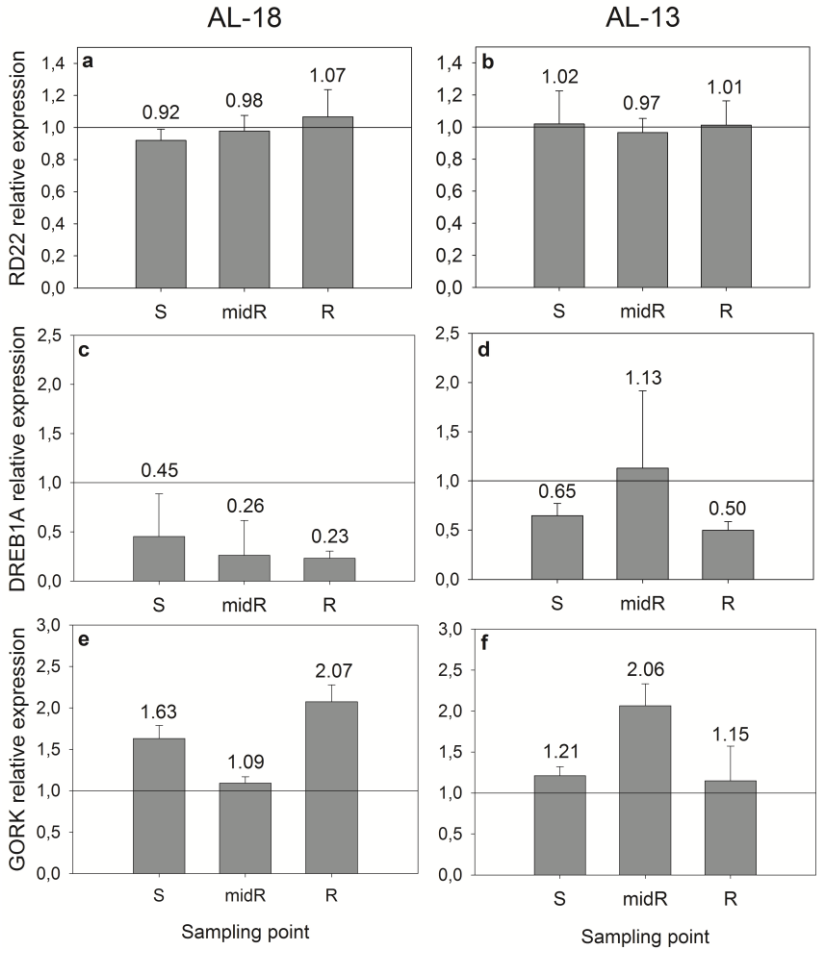

Figure 5 – Relative mRNA abundances [RD22: responsive to desiccation 22 (a, b); DREB1A: dehydration response element B1A (c, d); and GORK: potassium channel GORK (e, f)] of two field-grown *Eucalyptus globulus* clones (AL-18, left side, and AL-13, right side). Plants were artificially irrigated (IR) or subject to environmental conditions of reduced rainfall (NI) during six and a half weeks (S) and rewatered (by artificial irrigation, midR – 2 h and R – 3 days). Transcript relative expression is represented as fold change of NI samples to respective control – IR at each sampling point.

Clone AL-13 revealed an induction of peroxisomal malate dehydrogenase (pMDH, figure 4d) and catalase (CAT, figure 4h) at midR and mitochondrial glycine cleavage system H (GCSH, figure 4f) after 3 days of rewatering (R). Clone AL-13 did not show great fluctuations in photosynthesis related transcripts, but as reviewed by Chaves et al. (2009), the number of stress responsive genes or proteins is very dependent on the stress intensity. Besides, genes or proteins associated with photosynthetic pathways are generally less altered by the stress (Chaves et al. 2009). In clone AL-13, these results indicate that artificial irrigation after a long-term typical Mediterranean summer drought positively regulated photosynthetic (RCA, PRK and FNR) and photorespiratory (pMDH, CAT and GCSH) metabolism. *Quercus pubescens* was also identified for having a high capacity to protect and preserve its photosynthetic apparatus during the rewatering phase, observed through overnight reversibility of midday photo-inhibition (Gallé et al. 2007). Besides, we know that preventing photo-inactivation of photosynthetic electron transport relies on the interplay of photoassimilatory and photorespiratory electron transport (Heber et al. 1996), and that enzymes from leaf peroxisomes are fundamental to the light-dependent process of photorespiration (Donaldson et al. 2001).

The expression of the three studied ABA-responsive genes is indicated in figure 5. Responsive to desiccation 22 (RD22) kept unchanged at all analysed sampling points in both clones (figure 5a, b). Dehydration response element B1A (DREB1A) was downregulated, with a marked response in clone AL-18 (figure 5c). The relative expression of potassium channel GORK (GORK) was enhanced at S and R in clone AL-18 (figure 5e), but only at midR in clone AL-13 (figure 5f). These changes were not as evident as we would expect from the previous experiment (chapter 3.1), but genes involved in stress sensing or signalling were probably missed because this experiment regards long-term and acclimation responses to drought (Bogeat-Triboulot et al. 2007).

Taking the results together, three major points stand out: (i) transcripts of clone AL-18 are less reactive to the conditions tested, which is in line with previous studies (Correia et al. 2014b; Correia et al. 2016b; chapter 2); (ii) the alterations found in clone AL-13 highlight the impact of early rewatering rather than the long-term effect of six and a half weeks subject to a typically Mediterranean summer dry weather, and indicate an induction of photosynthetic and photorespiration metabolism that take place after artificial irrigation; (iii) the results obtained here using field-grown *Eucalyptus globulus* draw a very distinct picture when compared with a controlled climate chamber experiment. This study ultimately corroborates that the knowledge acquired from imposing the stress individually to test stress-tolerant plants cannot be extrapolated to field-grown plants in the context of developing selection markers. Further studies should therefore address plant tolerance to naturally occurring environmental conditions by developing more realistic experiments that mimic the field environment.

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Supplementary Data

Supplementary Figure S1 – Daily minimum (white circle) and maximum (black circles) air temperature (ºC) and respective humidity (white and black squares indicate relative humidity (%) measured at the time of the minimum and maximum temperature, respectively) (A); daily precipitation (mm) and maximum radiation (KJ/m2) (B) measured at a meteorological station placed near the experimental field from July 23 to September 9, 2016. Data were obtained from IPMA, I.P. – the Portuguese Institute for Sea and Atmosphere. A vertical line indicates a sampling day (Sept 6 and 9).

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Chapter 5

combined drought and heat

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5. Drought and heat impact on *Eucalyptus* – the combined environmental stress goes further the sum of the isolated factors

Summary

Aiming to mimic a more realistic field condition and to determine convergent and divergent responses of individual stresses in relation to their combination, we explored physiological, biochemical and metabolomic alterations after drought and heat stress imposition (alone and combined), and recovery using a drought-tolerant clone (AL-18). Regarding drought alone, the main responses included reduced pre-dawn water potential (Ψ_{pd}), gas exchange, jasmonic acid (JA), fructose-6-phosphate, glucose-6-phosphate, α-glycerophosphate, and shikimate, and increases in malondaldehyde (MDA), glutathione pool, abscisic acid (ABA), amino acids, starch, and non-structural carbohydrates. On the other hand, heat alone triggered an increase in relative water content (RWC), Ψ_{pd}, mannitol, sorbitol, inositol and several amino acids that were accompanied by a reduction in the photosynthetic rate and pigments, water use efficiency (WUE), starch, fructose-6-phosphate, glucose-6-phosphate, α-glycerophosphate, salicylic acid (SA), and JA. Activation of the shikimic acid pathway prevailed under this condition, but was downregulated under drought conditions. Drought-stressed plants subject to a heat shock revealed a decrease in gas exchange (sharp), Ψ_{pd} and JA, no alterations in electrolyte leakage, MDA, starch and pigments and increased glutathione pool in relation to control. Comparing this with drought stress alone, subjecting drought stressed plants to an additional heat stress alleviated Ψ_{pd} and MDA, maintaining an increased glutathione pool and reducing starch content and non-structural carbohydrates. A novel response triggered by the combined stress was the induction of cinnamate. Regarding recovery, most of the parameters affected by each stress condition reversed after re-establishment of control growing condition. These results highlight that the combination of drought and heat provides significant protection from more detrimental effects of drought-stressed eucalypts, confirming that combined stresses alter plant metabolism in a novel manner that cannot be extrapolated by the sum of the different stresses applied individually.

Introduction

Forest trees as all sessile plants have evolved many mechanisms that enable them to thrive in variable environmental conditions, ranging from circadian regulation (Dodd et al. 2005) up to recovery from overpowering stress (Brodribb and Cochard 2009). Despite that, the long life-span of trees does not allow for rapid adaptation to environmental changes, rendering forests particularly susceptible to climate change (Lindner et al. 2010). Therefore, climate-driven forest vulnerability and tree die-off have become emerging concerns for forest sustainability worldwide (Anderegg et al. 2012; Allen et al. 2015).

Decades of research have significantly improved our understanding of how abiotic stresses that plants encounter in the field, such as drought and heat stress, affect plant development and growth (Rennenberg et al. 2006). However, predominant abiotic stress factors have been mostly tested in an individual way and under controlled laboratory conditions (Mittler and Blumwald 2010). In contrast, relatively little attention has been given to the combined effects of abiotic stresses: in the field, water deficit, for example, does not occur alone but associated with high temperature or high light (Chaves et al. 2002).

There is a growing body of evidence that the impacts of combined stresses on plant functioning traits are unique, not necessarily representative of the sum of the individual parts, and can act synergistically, additively or antagonistically (Bansal et al. 2013; Pandey et al. 2015). The high degree of complexity results from the fact that when two stresses co-occur, plant adaptation to the stress combination is governed by the interaction of the two stresses, controlled by different signalling pathways that may interact, inhibit one another or be prioritized differentially by the plant (Zandalinas et al. 2017).

A particular abiotic stress induces a plant response tailored to that specific environmental condition and, when encounters different combined stresses, a plant might actually require conflicting adjustments (Mittler and Blumwald 2010). Under combined drought and heat stress, for example, plants have to act and balance stomatal responses between preventing water loss and cooling their leaves by transpiration, meaning that a proper defence response depends simultaneously on decreasing and increasing stomatal conductance (Mittler and Blumwald 2010).

The previous example leaves no doubt on the challenging task of researching abiotic stress combination. Several studies have already researched this subject mainly focussing on the drought and heat combination (Valladares and Pearcy 1997; Dobra et al. 2010; Silva et al. 2010; Arend et al. 2013; Duan et al. 2014). The results indicate a plethora of plant responses ranging from stomatal and non-stomatal limitations to photosynthesis (Arend et al. 2013), photo-inhibition (Valladares and Pearcy 1997), changes in key stress signalling components, such as reactive oxygen species

(Silva et al. 2010) and plant hormones (Dobra et al. 2010), up to rapid mortality through loss of stem hydraulic conductivity (Duan et al. 2014). Furthermore, the conclusions are very species/experiment dependent: elevated temperature is benefiting, but detrimental when drought occurs simultaneously (Arend et al. 2013); elevated temperature triggers rapid mortality through hydraulic failure, which is induced by drought (Duan et al. 2014); drought greatly disturbs photosystem II activity and oxidative metabolism, which are strongly stimulated by heat stress (Silva et al. 2010). Given the known impact of abiotic stress on the plant metabolome (Warren et al. 2011; Hochberg et al. 2013), we would also expect extensive research on this topic, but the available knowledge is short (Obata et al. 2015).

Among the forest plantations, *Eucalyptus* species play an increasingly essential role to guarantee the world's demand for wood products, and assessing the impact of drought and heat on such economically important plants is highly pertinent since both factors are considered the main drivers controlling vulnerability of *Eucalyptus* plantations (Booth 2013). Our previous research which compared results from a controlled climate chamber experiment (chapter 3) with field-grown *Eucalyptus globulus* Labill. (chapter 4) corroborated that the knowledge acquired from imposing the stress individually to test stress-tolerant plants cannot be extrapolated to field-grown plants. This ultimately urged us to evaluate the impact of combined drought and heat stress in *Eucalyptus globulus* plants, mimicking a more realistic field condition. Since assessing recovery may also be very informative and provide better insights of the severity of the combined stress than observations done at the stress imposition (Mitchell et al. 2013), we have also included a post stress period.

This study hence arises from the need to elucidate the major responses that take place in *Eucalyptus globulus* under combined drought and heat stress. Aiming to determine convergent and divergent responses of the individual stress in relation to their combination, we explored physiological and biochemical alterations after stress imposition (alone and combined) and recovery using a drought-tolerant clone. An additional key goal was to add an extra dimension by identifying major metabolomic alterations.

Materials and methods

Plant material and experimental design

Rooted cuttings of *E. globulus* (clone AL-18) were obtained from the breeding program of Altri Florestal SA (Portugal) and transplanted to 1 L plastic pots filled with equal weight of a 3:2 (w/w) peat:perlite mixture. The potted cuttings were then divided and placed in two climate chambers (Fitoclima 1200, Aralab, Portugal) for a one-month acclimation period, under the

following conditions: 25/20ºC (day/night), 16/8 h (day/night) photoperiod, 50% relative humidity and 600 µmol m^{-2} s^{-1} photosynthetic photon flux density. During the acclimation period, plants were watered up to 70% field capacity (FC) and fertilized every week with a NPK (5:8:10) nutritive solution. Pot weight was monitored every day and the percentages of FC were maintained by adding the amount of water lost. During the experiment, environmental conditions inside the climate chambers were maintained as in the acclimation period and only watering was altered. Half of the cuttings in each climate chamber was assigned to a control well-watered regime (C: water supplied every day until soil water content reached 70% FC) and the other half was assigned to a drought regime (D: water supplied every day until soil content reached 18% FC). This lasted for 5 days. Air temperature inside the second climate chamber was then gradually increased, and plants from both groups (C and D) were subject to 40ºC (heat stress) during 4 h (H and D*H, respectively). At this moment, the first sampling point took place: C and D plants were sampled in the first climate chamber, H and D*H plants were sampled in the second climate chamber. In order to perform a realistic experiment, corresponding with the dawn, heat exposure treatment began with an increasing temperature gradient from 20 to 40°C for 3 h, which was then maintained for 4 h. Lightweight expanded clay aggregate (LECA©), together with a refrigeration system, was used around the pots in order to mimic a fresher field soil temperature under heat stress. After that, environmental conditions inside the climate chambers were restored and all cuttings were well-watered (70% FC). The recovery of all groups was then evaluated at the second sampling point after 4 days under environmental and watering control conditions. In order to minimise the effects of environmental heterogeneity, the pots were periodically moved to the neighbouring position during the whole experiment.

At each sampling point (first sampling point: stress; second sampling point: recovery), five plants per group (i.e., C, D, H and D*H) were used to evaluate plant water potential, homogenous leaves from six individuals were used for *in vivo* measurements of leaf gas exchange parameters and subsequently used to determine plant relative water content and electrolyte leakage. Also, homogeneous leaves from six individuals were immediately frozen in liquid nitrogen and kept at -80ºC for further analysis (lipid peroxidation, redox couples ascorbate and glutathione, quantification of starch and pigments, hormonal alterations and metabolomics).

Water relations

Predawn water potential (Ψ_{pd}) was measured using a Scholander-type pressure chamber (PMS Instrument Co., Corvallis, OR, USA). Four leaf discs (diameter = 11 mm) per individual were also collected to determine relative water content (RWC), by using the equation: RWC = (FW - DW) / (TW - DW) × 100, in which FW is the fresh weight, TW is the turgid weight after

rehydration of the leaf discs for 24 h at 4ºC in the dark, and DW is the dry weight after oven-drying the leaf discs at 70ºC until they reach a constant weight.

Gas exchange and stomatal conductance

Leaf gas exchange measurements were performed on fully expanded leaves using an infrared gas analyser, LCpro-SD (ADC BioScientific Ltd., UK), equipped with the broad leaf chamber. Measurements were performed maintaining the following conditions inside the chamber: ambient temperature, CO_2 and H_2O concentration, air flow 200 µmol s^{-1} and saturation light intensity 400 µmol m^{-2} s^{-1}. Data were recorded when the measured parameters were stable (2 – 6 min). Net CO_2 assimilation rate (A), transpiration rate (E), stomatal conductance (g_s) and internal CO_2 concentration (C_i) were determined. Water use efficiency (WUE) was calculated based on leaf gas exchange, using the formulae WUE = A/E.

Starch quantification

Starch concentration was determined by using the anthrone method. Total soluble sugars were extracted from 50 mg of frozen leaves in 80% (v/v) ethanol for 1 h at 80ºC. After centrifugation, the pellet was used to quantify starch, as described by Osaki et al. (1991). The pellet was resuspended with 30% (v/v) perchloric acid and incubated at 60ºC during 1 h. The mixture was then centrifuged and anthrone was added to the supernatant. After heating the mixture at 100ºC for 10 min, absorbance was read at 625 nm (Thermo Fisher Scientific Spectophotometer, Genesys 10-uv S) and starch concentration was determined according to a D-glucose standard curve.

Pigments quantification

Concentration of chlorophyll a, b, and carotenoids was determined according to Sims and Gamon (2002). Pigments were extracted using cold acetone:50 mM Tris buffer pH 7.8 (80:20) (v/v). Following centrifugation, supernatants absorbance was read at 470, 537, 647 and 663 nm (Thermo Fisher Scientific Spectophotometer, Genesys 10-uv S). Chlorophyll a, b, and carotenoids were then quantified by using the formulae presented by the author.

Electrolyte leakage and lipid peroxidation

To determine electrolyte leakage (EL), four leaf discs (diameter = 11 mm) were collected. Conductivity was measured (CONSORT C830, Consort bvba, Turnhout, Belgium) and EL was determined using the equation: $EL = (C_i - W_c) / (C_f - W_c) \times 100$, in which W_c represents water conductivity, C_i is the initial conductivity of water plus the leaf discs, and C_f is the final conductivity of water plus the leaf discs after 5 min at 121ºC and 24 h at 4 ºC.

The extent of lipid peroxidation on leaves was estimated by measuring the amount of malondialdehyde (MDA), following an adaptation of the procedure described by Hodges et al. (1999). About 100 mg of leaves were ground in 2.5 mL of cold 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged. A 250 µL aliquot of the supernatant was added to 1 mL of 20% (w/v) TCA containing 0.5% (w/v) TBA (positive control), and another 250 µL was added to 1 mL of 20% (w/v) TCA was added (negative control). Both positive and negative controls per sample were heated at 95ºC for 30 min. After stopping the reaction on ice and centrifuging, absorbance was read at 440, 532 and 600 nm (Thermo Fisher Scientific Spectophotometer, Genesys 10-uv S, Waltham, MA, USA), and MDA content was determined by the formulae presented by the author.

Nonprotein redox couples ascorbate and glutathione

Ascorbate (reduced, AsA) and dehydroascorbate (DHA) concentrations, as well as oxidised (GSSG) and reduced (GSH) glutathione were determined according to the microplate method described by Queval and Noctor (2007).

Hormones quantification

Abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) were extracted and analysed following the procedure described by Durgbanshi et al. (2005), with slight modifications. Frozen-dried tissue (50 mg) was added with 100 ng of abscisic acid-d_6 ($ABAd_6$), 100 ng of salicylic acid-d_6 (SAd_6) and 100 ng of dihydrojasmonic acid and homogenized with 5 mL of distilled water. After cold centrifugation, supernatants were recovered and pH adjusted to 3 with 30 % acetic acid. The acidified water extract was partitioned twice against 3 mL of diethyl ether. The organic upper layer was recovered and vacuum evaporated in a centrifuge concentrator (SpeedVac, Jouan, Saint Herblain, France). The dry residue was then resuspended in a 10% methanol solution by gentle sonication. The resulting solution was passed through 0.22 µm regenerated cellulose membrane syringe filters (Albet S.A., Barcelona, Spain) and directly injected into a UPLC system (Acquity SDS, Waters Corp., Milford, MA, USA). Analytes were separated by reversed-phase (Nucleodur C18, 1.8 µm 50 × 2.0 mm, Macherey- Nagel, Barcelona, Spain) using a linear gradient of ultrapure water (A) and methanol (B) (both supplemented with 0.01% acetic acid) at a flow rate of 300 µL min^{-1}. The gradient used was: (0-2 min) 90:10 (A:B), (2-6 min) 10:90 (A:B) and (6-7 min) 90:10 (A:B). Hormones were quantified with a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) connected online to the output of the column through an orthogonal Z-spray electrospray ion source. The analytes were quantified after external calibration against the standards.

Metabolomics analysis

Metabolites were extracted, derivatised and analysed by gas chromatography-mass spectrometry (GC-MS), as previously described by Foito et al. (2013). *Eucalyptus* frozen leaves were lyophilised and 100 mg of dried material were weighed into glass tubes. Lyophilized material was homogenised and extracted sequentially in methanol, water, chloroform for 30 min at 37ºC each. Internal standards (aqueous ribitol and methanolic *n*-nonadecanoic acid) were added during the initial methanol extraction step. Finally, an additional aliquot of water was added and the polar and non-polar phases were separated, evaporated to dryness and derivatized independently. Metabolite profiles of the polar and non-polar fractions were acquired following separation of compounds on a DB5-MSTM column (15 m × 0.25 mm × 0.25 μm; J&W, Folsom, CA, USA) using a Thermo-Finnigan DSQ II GC-MS system (Thermo Finnigan, UK). The samples were analysed as a single batch, in a randomized order, while quality control samples as well as blanks were incorporated at the beginning and the end of the sequence. Peak areas were calculated in relation to respective internal standard and normalised to respective extracted weight.

Statistical analysis

Data are presented as mean ± SE (standard error) of three to six independent biological replicates. All statistical procedures were performed using SigmaPlot (SigmaPlot for Windows v. 11.0, Systat Software Inc., San Jose, CA, USA), except metabolites that were analysed using GenStat v12 (VSN International Ltd, Hemel Hempstead, UK). One-way analysis of variance (ANOVA) followed by the Fisher's LSD post-hoc all pairwise multiple comparison tests were employed separately for each sampling point (i.e. stress and recovery) to estimate the significance of the results. Different lower cases indicate significant differences between treatments (C, D, H, D*H) at $p \leq 0.05$.

Results

The effect of drought (D) and heat (H) stress applied alone and combined (D*H) in *E. globulus* plants was analysed at multiple scales, assessing physiological, biochemical, hormonal and metabolomic alterations after stress imposition and recovery. The plant water status was evaluated by Ψ_{pd} and RWC. Drought and combined stress induced a significant reduction in Ψ_{pd}, less significant in the combined stress, whereas heat showed a tendency of higher Ψ_{pd} when compared to control conditions (figure 1A). After recovery, although increased, drought and combined stress were still lower than the control (figure 1A). In relation to RWC, it was only slightly decreased after the drought treatment (not significant), significantly increased in heat, and

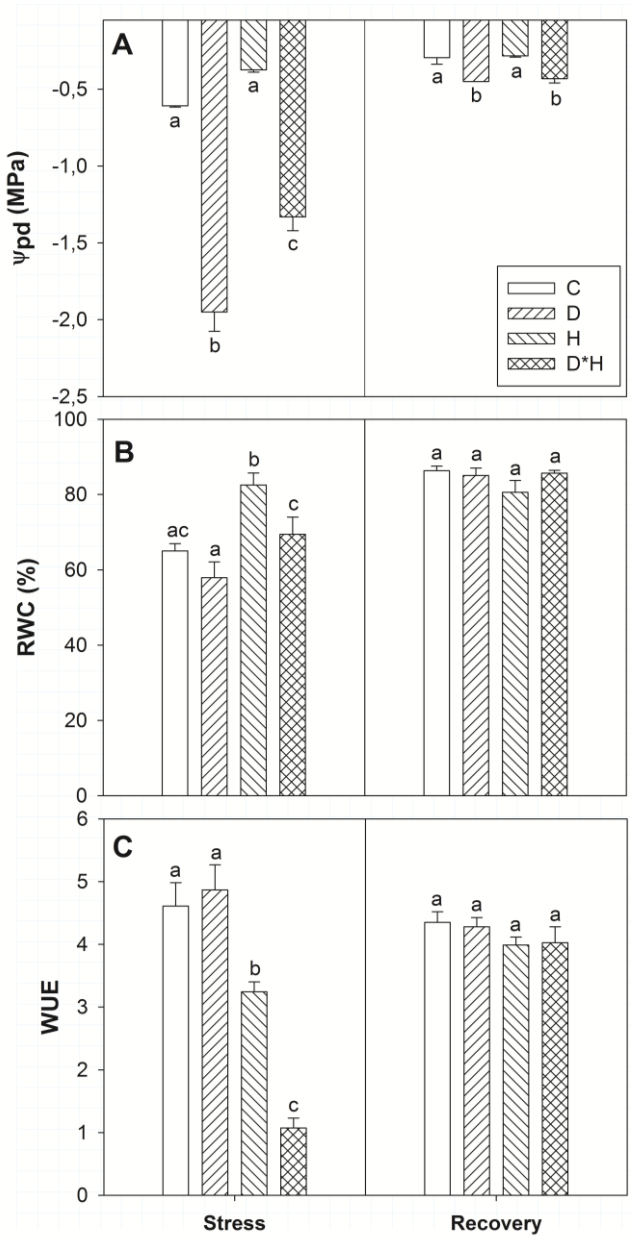


Figure 1 – Predawn shoot water potential (Ψ_{pd}, A); relative water content (RWC, B) and water use efficiency (WUE, C) of *Eucalyptus globulus* exposed to control conditions (C), drought (D), heat (H) and combined stress (D*H) and respective recovery. Different lowercase letters indicate significant differences among the four treated groups (C, D, H and D*H), after stress and after recovery, separately ($p \leq 0.05$).

was unaffected in the combined stress (figure 1B). On the other hand, WUE revealed a reduction in heat and combined stresses, very sharp in the combined one (figure 1C). Both RWC and WUE show no differences after recovery (figure 1B, C).

Gas exchange varied in response to the imposed stresses (figure 2). Net photosynthetic rate (A, figure 2A) revealed an overall reduction, heavier in the combined stress (D*H), followed by drought, and to a minor extent, heat stress. Transpiration rate (E) and stomatal conductance (g_s) were similarly affected: only drought and combined stresses showed a decrease (figure 2B, C). On the other hand, internal CO_2 concentration (C_i) significantly increased in drought, and decreased in heat, keeping unchanged in the combined stress (figure 2D). Although reversing, most of these profiles persisted after recovery (figure 2A, B and C). In particular, g_s, E and C_i increased after recovery from heat (figure 2B, C and D).

Leaf starch content is shown in figure 3: increased in drought, decreased in heat, and slightly decreased in the combined stress. After recovery no further changes were found (figure 3).

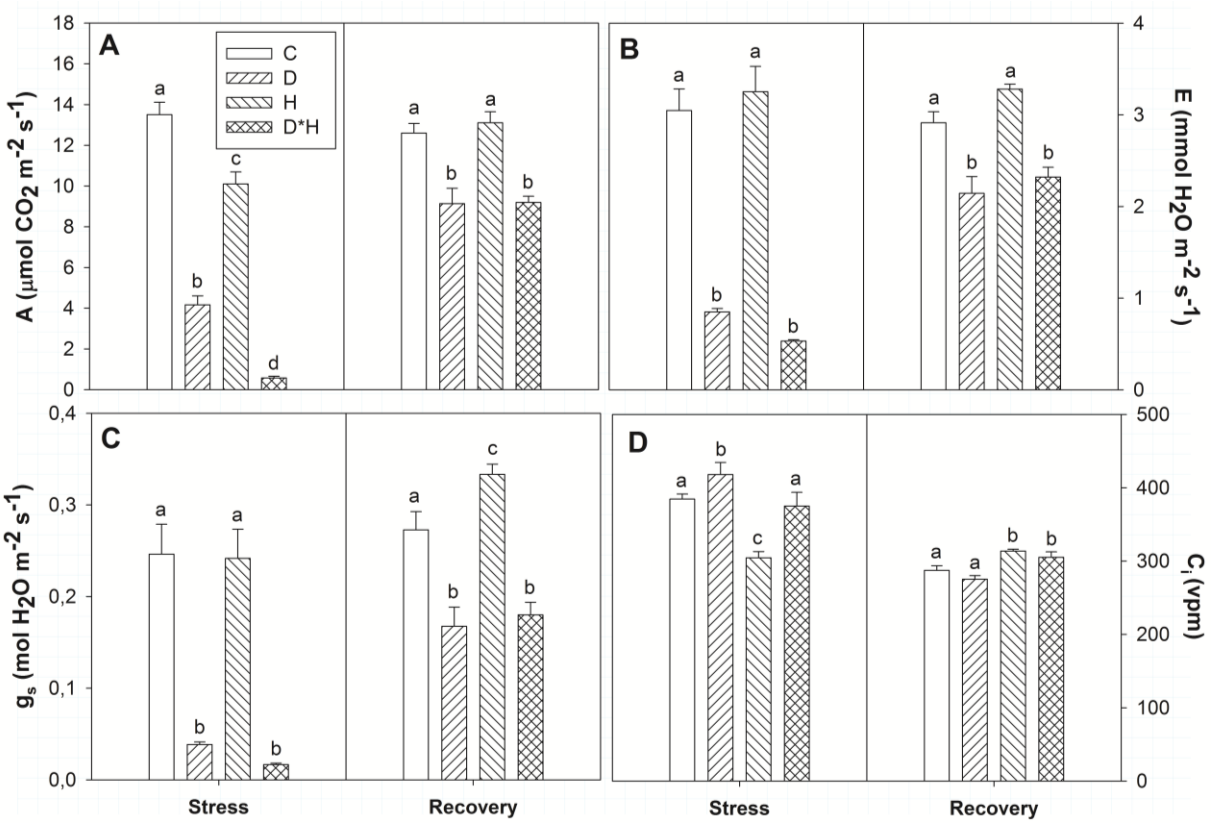


Figure 2 – Net photosynthetic rate (A, A); transpiration rate (E, B); stomatal conductance (g_s); and internal CO_2 concentration (C_i, D) of *Eucalyptus globulus* exposed to control conditions (C), drought (D), heat (H) and combined stress (D*H) and respective recovery. Different lowercase letters indicate significant differences among the four treated groups (C, D, H and D*H), after stress and after recovery, separately ($p \leq 0.05$).

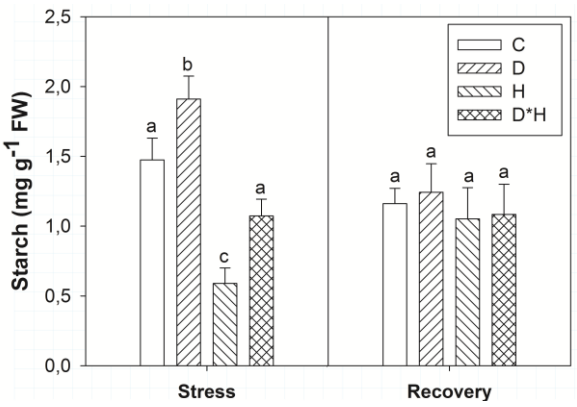


Figure 3 – Starch of *Eucalyptus globulus* exposed to control conditions (C), drought (D), heat (H) and combined stress (D*H) and respective recovery. Different lowercase letters indicate significant differences among the four treated groups (C, D, H and D*H), after stress and after recovery, separately ($p \leq 0.05$).

Chlorophyll a, b and carotenoids were differentially modulated by the imposed stresses (figure 4). Chlorophyll a decreased in drought, and heat but not when the combined stress was imposed (figure 4A), chlorophyll b was only reduced after the heat treatment, and no changes occurred in the combined stress (figure 4B). After recovery, chlorophyll a revealed an increase in drought stressed plants (figure 4A, B). Regarding carotenoids, their abundance profile matches chlorophyll b: a major reduction only in the heat stress and an increase in drought stressed plants after recovery (figure 4C).

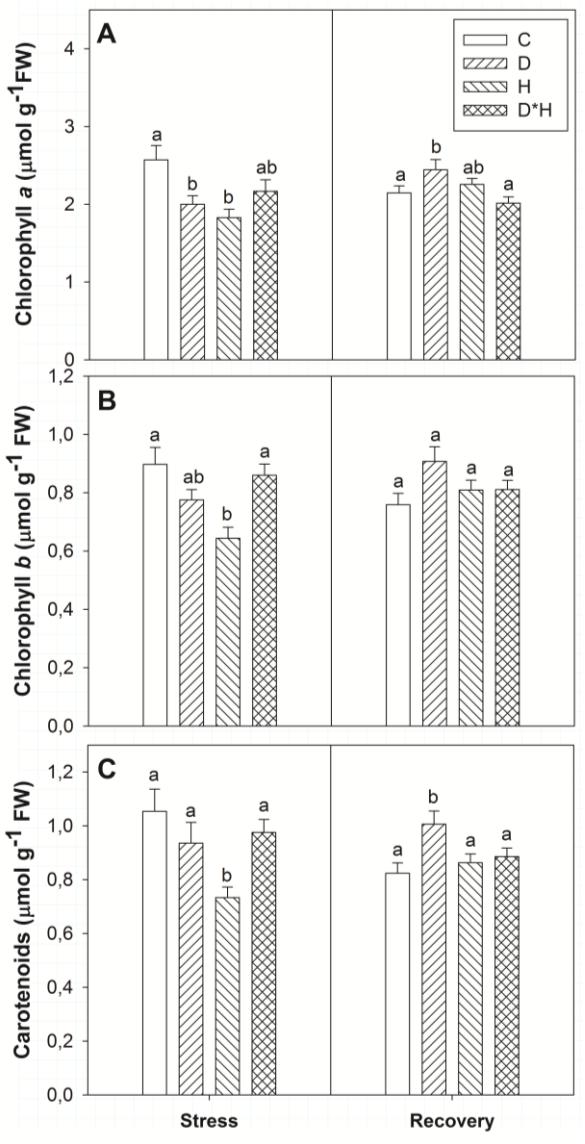


Figure 4 – Chlorophyll a (A); chlorophyll b (B); and carotenoids (C) of *Eucalyptus globulus* exposed to control conditions (C), drought (D), heat (H) and combined stress (D*H) and respective recovery. Different lowercase letters indicate significant differences among the four treated groups (C, D, H and D*H), after stress and after recovery, separately ($p \leq 0.05$).

Membrane integrity was assessed by leaf electrolyte leakage (EL) and MDA accumulation. From these, only EL revealed a significant increase in drought stress (figure 5A), although MDA also showed a slightly higher tendency at the same point (figure 5B). After recovery, no differences were found considering both EL and MDA (figure 5A, B).

The total ascorbate pool was not affected by the imposed stresses, on the contrary the total glutathione pool was

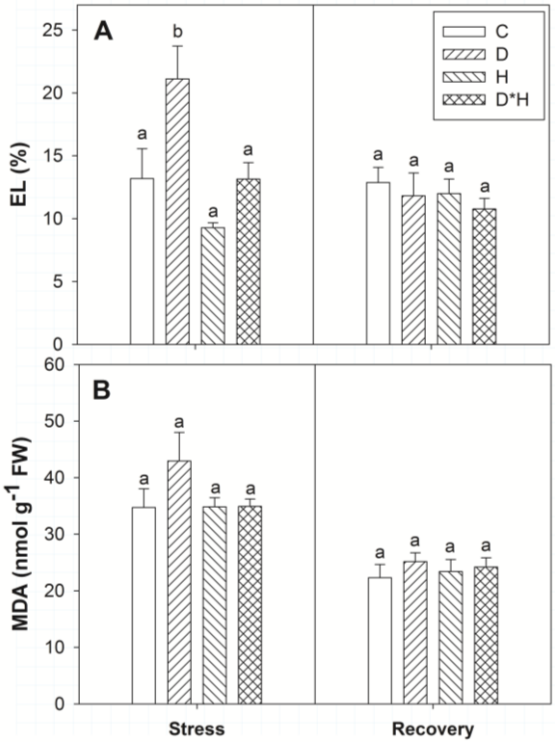


Figure 5 – Electrolyte leakage (EL, A); and malondialdehyde content (MDA, B) of *Eucalyptus globulus* exposed to control conditions (C), drought (D), heat (H) and combined stress (D*H) and respective recovery. Different lowercase letters indicate significant differences among the four treated groups (C, D, H and D*H), after stress and after recovery, separately ($p \leq 0.05$).

increased in the drought and combined stresses (figure 6). This induction was not accompanied by an increase in the oxidised pool (figure 6). After recovery, the DHA pool revealed an induction in the combined stress, without major alterations in the total ascorbate pool, and glutathione pool reversed to control levels (figure 6). The imposed stresses significantly affected the leaf hormonal dynamics and major differences were found regarding abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA), as shown in figure 7.

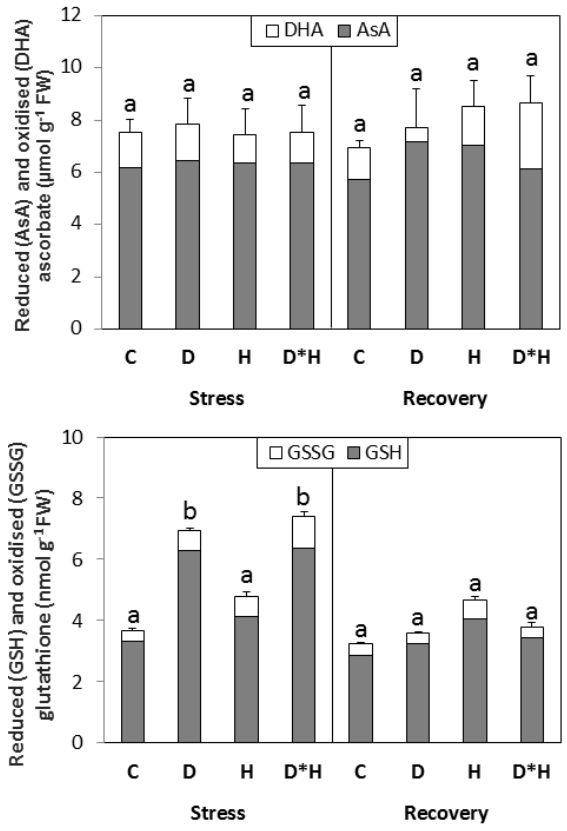


Figure 6 – Total ascorbate (incorporating reduced (AsA) and oxidised (DHA) forms), and total glutathione (incorporating reduced (GSH) and oxidised (GSSG) forms) quantified in the leaves of *Eucalyptus globulus* exposed to control conditions (C), drought (D), heat (H) and combined stress (D*H) and respective recovery. Different lowercase letters indicate significant differences in the total ascorbate or glutathione pools among the four treated groups (C, D, H and D*H), after stress and after recovery, separately ($p \leq 0.05$).

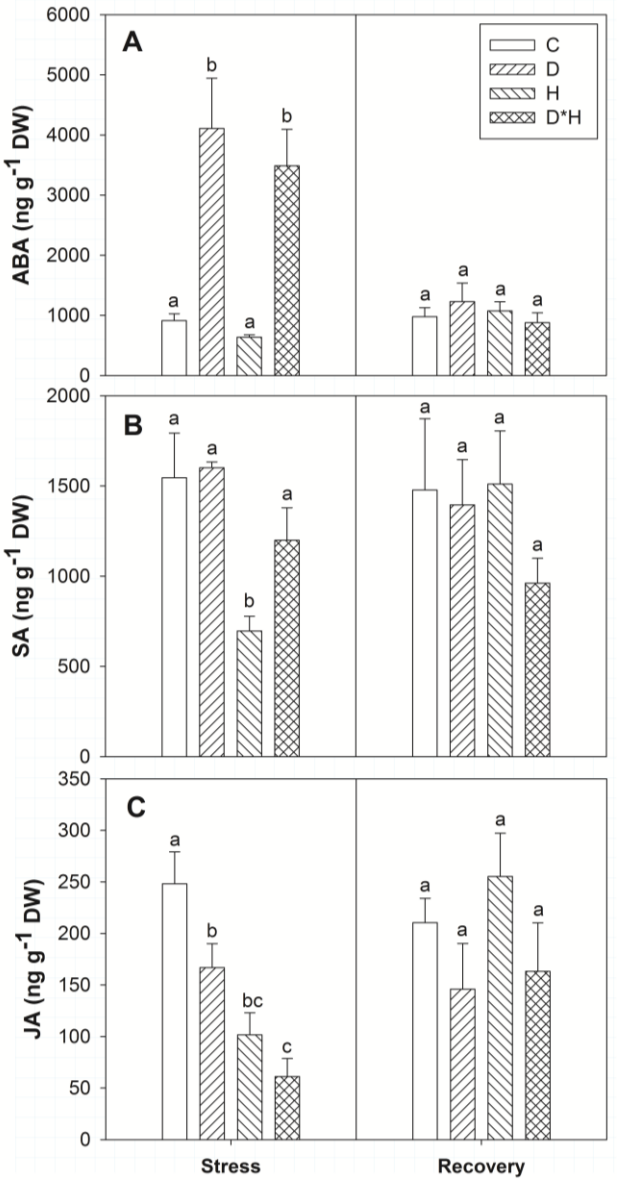


Figure 7 – Abscisic acid (ABA, A); salicylic acid (SA, B); and jasmonic acid (JA, C) of *Eucalyptus globulus* exposed to control conditions (C), drought (D), heat (H) and combined stress (D*H) and respective recovery. Different lowercase letters indicate significant differences among the four treated groups (C, D, H and D*H), after stress and after recovery, separately ($p \leq 0.05$).

On one hand, ABA significantly accumulated in drought and combined stresses (Figure 7A). On the other hand, SA levels decreased exclusively after heat (Figure 7B) and JA content decreased under all stress conditions in a descending order: drought, heat and combined stress (Figure 7C). No hormonal differences were detected after recovery from stress.

The foliar metabolomic profile of *E. globulus* subjected to drought, heat, combined stress and recovery were compiled using GC–MS. This analysis yielded the detection of 106 metabolites (Supplementary Table S8), distinguishing between 64 polar and 42 non-polar metabolites. Only a small part of the detected metabolites could not be identified after data processing (7 polar and 5 non-polar metabolites). From the identified metabolites, 48 showed significant changes due to the applied stresses and/or recovery, including 12 carbohydrates (table 1), 5 organic acids and 17 amino acids (table 2), 2 phenolic acids, 6 fatty acids/alcohols, 1 phytosterol and 5 unknown metabolites (table 3).

Regarding carbohydrates (table 1), mannose, galactose and two separate peaks assigned to glucose increased exclusively after drought. Mannitol, sorbitol and inositol contents increased under all stress conditions, and maltose increased in drought and combined stress. On the contrary, abundances of fructose-6-phosphate and glucose-6-phosphate were negatively affected by drought, heat and combined stresses. Dihydroxydihydrofuranone showed the same profile as the previous ones, but with lower magnitude. After recovery, both fructose-6-phosphate and glucose-6-phosphate of all stressed plants reversed to control levels, but drought stressed plants still kept significantly lower content (table 1). All other carbohydrates reversed the alterations caused by stress after recovery, except inositol that maintained higher levels in the previously drought and combined stressed plants (table 1).

Five organic acids – succinate, malate, citrate, quinate, and glyceric acid – were modulated by stress (table 2). Succinate, quinate, and glyceric acid abundances were reduced under both drought and combined stresses. From these, glyceric acid was also decreased under heat. Citrate was elevated in response to drought, heat, and combined stress (table 2). Malate abundance was enhanced by drought and reduced after heat, staying unchanged in the combined stress. Following recovery, none of the organic acids showed significant changes relative to control pants (table 2).

Amino acids constitute the largest group of compounds showing significant differences under stress, mainly the combined one (table 2). Aspartate, glutamate, leucine, isoleucine and proline abundances were significantly increased in drought and heat, and with a greater extent in the combined stress. Threonine, lysine, histidine, tryptophan, methionine and GABA were only positively regulated under drought and combined stresses, and valine showed an over accumulation only in the combined stress. After recovery, only oxoproline (generated from glutamine during the derivatisation procedure), tryptophan and methionine revealed significant alterations (table 2).

Oxoproline decreased in previously droughted and combined stressed plants, tryptophan could only be detected in previously drought stressed plants, and methionine decreased in the combined stress, although showing a slight decrease in previously heat stressed plants. Drought, heat and combined stresses positively induced urea levels, which were restored after recovery (table 2).

Table 1 – Metabolomic analysis, relative abundance of carbohydrates. Abundance data is presented on a scale relative to the lowest value among treatments. Different lowercase letters indicate significant differences between the control (C), drought (D), heat (H) and combined stress (D*H) plant groups, under stress and after recovery; absent relative abundance indicates that no differences were found ($p \leq 0.05$).

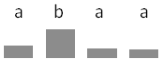
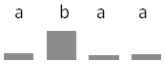
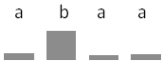
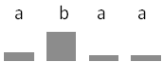
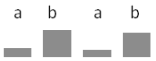
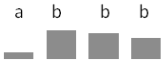
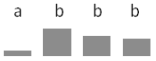
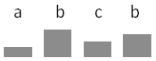
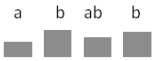
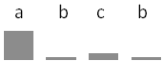
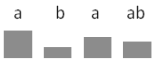
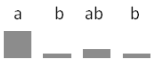
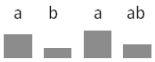
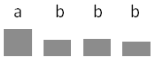
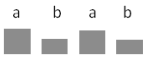
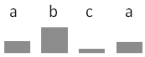
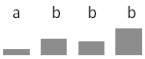
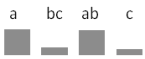
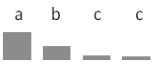
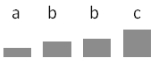
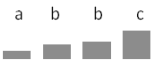
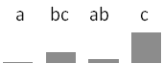
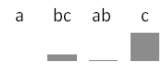
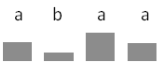
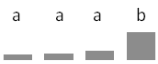
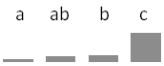
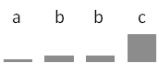
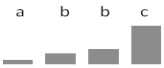
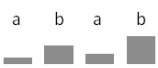
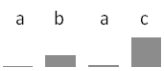
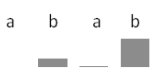
	Relative abundance (C – D – H – D*H)	
Carbohydrates	*Stress*	*Recovery*
Mannose		----------
Galactose		----------
Glucose 1		----------
Glucose 2		----------
Maltose		----------
Mannitol		----------
Sorbitol		----------
Inositol		
Fructose-6-phosphate		
Glucose-6-phosphate		
Dihydroxydihydrofuranone		----------
α-Glycerophosphate		----------

Table 2 – Metabolomic analysis, relative abundance of organic acids and amino acids. Abundance data is presented on a scale relative to the lowest value among treatments. Different lowercase letters indicate significant differences between the control (C), drought (D), heat (H) and combined stress (D*H) plant

groups, under stress and after recovery; absent relative abundance indicates that no differences were found ($p \leq 0.05$).

	Relative abundance (C – D – H – D*H)	
Organic acids	*Stress*	*Recovery*
Succinate		----------
Malate		----------
Citrate		----------
Quinate		----------
Glyceric acid		----------
Amino acids		
Aspartate		----------
Glutamate		----------
Asparagine 1		----------
Asparagine 2		----------
Oxoproline	----------	
Valine		----------
Leucine		----------
Isoleucine		----------
Proline		----------
Threonine		----------
Lysine		----------
Histidine		----------

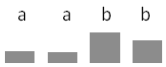
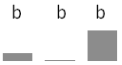
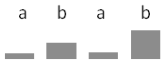
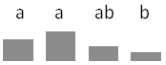
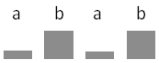
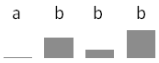
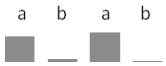
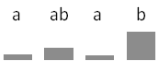
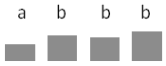
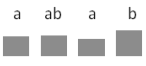
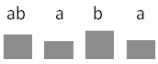
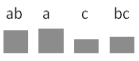
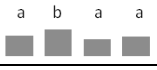
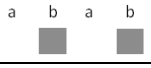
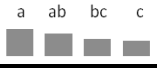
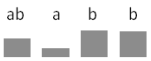
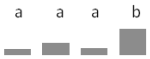
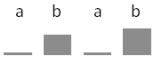
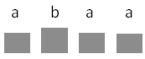
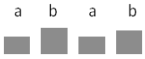
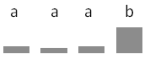
Phenylalanine		----------
Tryptophan		
Methionine		
GABA		----------
Urea		----------

Table 3 – Metabolomic analysis, relative abundance of phenolic acids, fatty acids/alcohols, phytosterols, and unknown metabolites. Abundance data is presented on a scale relative to the lowest value among treatments. Different lowercase letters indicate significant differences between the control (C), drought (D), heat (H) and combined stress (D*H) plant groups, under stress and after recovery; absent relative abundance indicates that no differences were found ($p \leq 0.05$).

	Relative abundance (C – D – H – D*H)	
Phenolic acids	*Stress*	*Recovery*
Shikimate		----------
Cinnamate		----------
Fatty acids, fatty alcohols		
C14:0		
C18:2		----------
C21:0	----------	
C23:0	----------	
C29:0	----------	
C26 alcohol	----------	
Phytosterols		

Avenasterol	----------	
Unknowns		
pU1585		----------
pU1598		----------
pU2020		----------
pU2367		----------
npU1680	----------	

Regarding phenolic acids (table 3), shikimate decreased under drought and combined stress. Conversely, cinnamate abundance enlarged under combined stress, although a slight increase was also observed in drought. Neither of these phenolic acids showed significant differences after recovery. Alterations in fatty acids and fatty alcohols were mainly detected in recovery (table 3), with the exception of C14:0, which increased under every stress, and C18:2, which slightly reduced under drought and combined stresses. After recovery, C14:0 abundance of previously stressed plants largely reversed to control levels and only plants previously subject to combined stress still presented a significantly higher content. Recovery from drought induced C23:0 and C29:0 accumulation; recovery from heat reduced C21:0 and C26 alcohol; and recovery from combined stress resulted in enhanced C29:0 and reduced C26:0 alcohol. Avenasterol was the only identified phytosterol showing significant changes (table 3); however, the difference observed is due to a lower level of avenasterol on drought in relation to heat and combined stresses, but it represents only a slight decrease when comparing with control.

From the unidentified metabolites (table 3), pU2020 increased under drought and pU1585 raised under combined stress, pU1598 and pU2367 increased under both conditions. Moreover, npU1680 revealed an increase after recovery from combined stress.

Discussion

Considerable research advances have been accomplished focussing on plant responses to single stress factors under controlled environments (Mittler and Blumwald 2010). However, plants growing in the field encounter a number of different co-occurring abiotic stresses that most

probably cannot be extrapolated by the sum of the different stresses applied individually, altering plant metabolism in a novel manner (Rizhsky et al. 2002; Zandalinas et al. 2016; chapter 4). Keeping this in mind, we aimed to determine convergent and divergent responses of the individual stresses in relation to their combination, evaluating the impact of drought and heat stress (alone and combined) and respective recovery using a drought-tolerant *Eucalyptus globulus* clone.

Regarding drought stress alone, the main responses included reduced Ψ_{pd}, gas exchange, JA, fructose-6-phosphate, glucose-6-phosphate, α-glycerophosphate, and shikimate, and increases in MDA, glutathione pool, ABA, amino acids, starch and non-structural carbohydrates. Most of these results are in agreement with other reports that analysed the isolated effect of drought on *Eucalyptus globulus* (Warren et al. 2011; Correia et al. 2014b; Correia et al. 2016a; Correia et al. 2016b; chapter 2; and chapter 3), and indicate that water deficit negatively affects plant water relations and photosynthesis, causing a moderate oxidative stress, and inducing enhanced osmoprotection and other defence-related pathways.

On the other hand, heat stress alone triggered an increase in RWC, Ψ_{pd}, mannitol, sorbitol, inositol and several amino acids that were accompanied by a reduction in the photosynthetic rate and pigments, WUE, starch, fructose-6-phosphate, glucose-6-phosphate, α-glycerophosphate, SA, and JA. The reduction in the photosynthetic rate and pigments in parallel with unaffected transpiration rate and stomatal conductance confirms the particular sensitivity of photosynthesis to heat stress (Sharkey 2005), even in a short heat shock (4 h at 40ºC). It also indicates that the main limitations are non-stomatal and mostly related to heat-induced alterations in enzyme activity (Larkindale et al. 2005). A decrease in photosynthetic pigments, fructose-6-phosphate, glucose-6-phosphate, and starch was also documented in potato leaves growing at a moderate 30ºC (Hancock et al. 2014). No major oxidative impairment was detected and this can be explained by the shifts in the polyols mannitol, sorbitol, inositol and several amino acids, such as proline, possibly indicating that these compatible solutes were effective hydroxyl radical scavengers (Smirnoff and Cumbes 1989; Wang et al. 2003). In addition to their role as radical scavengers, the accumulation of the polyols under heat stress is most likely responsible for the observed increase in RWC and Ψ_{pd}, reinforcing their primary role as osmoprotectants (Bokszczanin et al. 2013).

The heat induced reduction in SA and JA is an unexpected result since both hormones are reported to play an important role as signal molecules in abiotic stress tolerance (Horváth et al. 2007; Xu et al. 2016) and SA as been reported to protect plants from heat stress (Wang et al. 2014). However, the downregulation of JA has already been described in *Eucalyptus globulus* under water deficit (Correia et al. 2014a; chapter 2.2). Our results further confirm the downregulation of JA under drought stress, highlighting a similar response triggered by heat stress regarding not only JA but also SA. The way these abiotic stresses influence these two phytohormones in *E. globulus* is yet

to be discovered. However, SA and JA, together with ethylene, are known to play major roles in regulating plant defence: SA is usually associated with the activation of defence against biotrophic and hemi-biotrophic pathogens, and the establishment of systemic acquired resistance (SAR), and JA and ethylene are generally involved in defence against necrotrophic pathogens and herbivorous insects (reviewed by Bari and Jones (2009)). Hence, this result has significance in terms of the impact of abiotic stress on biotic interactions, revealing that these abiotic stresses can negatively influence defence against other biotic threats.

A divergent response between isolated drought and heat stress is related to changes in the TCA cycle intermediates. In heat, citrate increase went along with reduced malate, whereas drought-induced increases of citrate and malate were accompanied by reduced succinate. Together with the different amino acids that accumulate in each stress this result highlights two different metabolic regulations. In heat, the TCA cycle flux appears to be changed to two weakly connected branches, with malate functioning as a mitochondrial respiratory substrate to produce citrate, which is then converted to glutamate and proline. Similar cases of the non-cyclic flux mode of TCA cycle has been reviewed elsewhere (Sweetlove et al. 2010). However, the prevailing pathway activated under this condition appears to be the shikimic acid pathway, revealed by the over accumulation of shikimate and phenylalanine. Conversely, the shikimic acid pathway is downregulated under drought conditions. In this stress scenario, an induction in the first steps of the TCA cycle likely supplies higher demands for citrate that is metabolized to amino acids of the glutamate family; and succinate is converted to malate, which in turn is redirected to produce amino acids of the oxaloacetate/aspartate family.

Still on this subject, comparing the isolated stresses with the combined one reveals novel responses. In the combined conditions of drought and heat stress, the highest accumulation of citrate was accompanied by reduced succinate without major alterations in malate. The higher content of α-glycerophosphate together with the major accumulation in amino acids of the glutamate family, the oxaloacetate/aspartate family and leucine/valine indicates that glycolysis is enhanced in this combined condition, sustaining the higher demand for amino acids. The absence of significant changes in the fatty acids/alcohols and phytosterols detected at this new stress state does not support the premise of a regulation by changes of membrane lipids as we could assume (Falcone et al. 2004).

A novel response triggered by the combined effect of drought and heat was the induction of cinnamate. Cinnamate originates all phenylpropanoids through the action of phenylalanine ammonia-lyase (PAL) on phenylalanine (Dixon and Paiva 1995). We are uncertain of which phenylpropanoids are generated under this condition since a number of different phenylpropanoids

can be involved (Dixon and Paiva 1995), but given that SA was unaffected by the combined condition, we can rule out this metabolite.

Drought-stressed plants subject to a heat shock revealed a decrease in gas exchange (sharp), WUE, Ψ_{pd} and JA, no alterations in EL, MDA, starch and pigments and increased glutathione pool in relation to control. Comparing with drought stress alone, this reveals that subjecting drought stressed plants to an additional heat stress alleviated Ψ_{pd} and MDA, maintaining an increased glutathione pool and reducing starch content and non-structural carbohydrates. Interestingly, and in contrast to the expected negative effect of the stress combination on plant growth reported for other species (Silva et al. 2010), these results highlight that the combination of drought and heat provides significant protection from more detrimental effects of drought-stressed eucalypts. A similar conclusion has been described for tomato plants under the combined effect of salinity and heat (Rivero et al. 2014).

Regarding recovery, most of the parameters affected by each stress condition reversed after re-establishment of the control growing condition. This is a common reported response (Correia et al. 2014a; Correia et al. 2014b; Correia et al. 2016b; chapter 2). Gas exchange and some carbohydrates reversed at a slower pace after drought and combined stress, which reveals the sensitivity of the photosynthetic apparatus (Chaves et al. 2009) and points out the most restrictive effect of these two stress conditions. On the other side, the different modulation of several fatty acids/alcohols and phytosterols after recovery from drought and combined stresses uncovers a putative regulation that allows restoration after stress through changes of membrane lipids (Falcone et al. 2004).

At present, information on the combined effect of heat and drought stress in *Eucalyptus* is rather limited although much needed from the application point of view (e.g. finding suitable markers for selecting the most tolerant genotypes to field establishment, chapter 4). In this work, we have reported different physiological, biochemical and metabolic adjustments that enable *Eucalyptus globulus* to thrive under conditions of drought and heat applied alone or in combination. Although a few mechanisms were convergent to all stress conditions, the response magnitude was very dependent on the specific stress, and most of the metabolic pathways showed to be unique to each specific stress. Rather than presenting an additive outcome, the combination of heat stress ameliorated part of the negative effect of drought. The information collected here confirms that the biological processes switched on by an environmental factor is very specific to that exact condition and is likely to differ from those activated by a slightly different environmental condition (Mittler 2006; Atkinson and Urwin 2012). The need for studies that focus on the actual field stress conditions is thus evident and imperative for selecting plants with enhanced tolerance to naturally occurring environmental conditions.

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Supplementary Data

Table S8: Metabolomic dataset. Area ratio per leaf dry weight of all detected metabolites with respective identification.

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Chapter 6

concluding remarks

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Main findings

This doctoral thesis successfully describes several mechanisms and processes mediating drought tolerance in *Eucalyptus globulus*. Comparing two different genotypes using different stress trials and state-of-the-art procedures allowed scanning water deficit and rehydration at different levels and unravelling global and specific responses that enable *E. globulus* to thrive under changing environmental conditions.

After describing most of the already known responses of this genus under drought conditions, the next step was to characterise how genotypes known for occurring in drought prone areas with differential field behaviour adjust their physiology, biochemistry, proteome and metabolome to cope with changes in water availability (first slowly reduced and then restored in a greenhouse trial). This was accomplished in chapter 2 and revealed global and specific responses. Some of the global responses reported were already known to be involved in drought tolerance, while others were novel: a shift of energy metabolism away from chloroplasts and towards mitochondria and the downregulation of secondary metabolism, particularly the phenylpropanoid pathway. Both clones were highly tolerant to the conditions tested, but each clone was identified with a specific response profile. Clone AL-10 was more responsive to water availability compared to clone AL-18. The response profile of AL-10 implicated correlations among glyceric acid and several plastocyanins and antioxidant-related proteins, coupled with the involvement of several key amino acids (valine, proline and alanine). The response profile of AL-18 involved key photosynthetic and defence-related proteins, increased photorespiration, and revealed new tolerance players: glutamine synthetase, malate dehydrogenase and isoflavone reductase-like protein.

Later, the involvement of the previously defined pathways/proteins was tested to check if potential molecular indicators linked to enhanced tolerance could be found: RCA, FNR, mMDH, CAT and IFR were successfully identified by analysing AL-18 and AL-13 subject to sudden water shortage and subsequent rehydration. An additional task aimed to depict the role of redox and DNA methylation mechanisms in the resilient AL-18. The alterations detected using global and specific indicators reflected the parallel induction of redox and complex DNA methylation changes occurring during stress imposition and relief, providing an interesting avenue for continuing investigations of the relationship between plant stress signalling, epigenetics, and adaptive processes. The controlled climate chamber stress trial was more detrimental to the plants when compared to that slowly imposed in a greenhouse. However, when replicating this experiment in the same field-grown plants, the plants were not as affected as in the prior experiment. Furthermore, none of the molecular indicators identified in the climate chamber trial exhibited the

same expression magnitude in the field trial. However, several conclusions were drawn from the field trial: transcripts of AL-18 are less reactive to the conditions tested, which is in accordance with the previous tests; the alterations found in AL-13 highlighted the impact of early rewatering rather than the long-term water deficit and indicated an induction of photosynthetic and photorespiration metabolism after artificial irrigation; the knowledge acquired from imposing the stress individually to test stress-tolerant plants cannot be extrapolated to field-grown plants in the context of developing selection markers.

In keeping with the previous premises, the isolated and combined effect of drought and heat was tested in the drought-tolerant clone AL-18 in a climate chamber trial designed with the aim of mimicking a more realistic field condition and determining convergent and divergent responses of individual stresses in relation to their combination. This experiment highlighted that the combination of drought and heat provided significant protection from more detrimental effects of drought-stressed eucalypts and induced novel responses. Ultimately, confirming that combined stress alters plant metabolism in a novel manner helped to understand that one cannot extrapolate the effects of combined stress by the sum of the different stresses applied individually. It also supported the previous observations of field-grown eucalypts being less affected by restricted irrigation than when water was restricted in a controlled climate chamber experiment.

Final considerations

Assessing water deficit and restoration in different genotypes and stress trials proved to be appropriate for detecting relevant pathways involved in stress tolerance and identifying stress indicators. This thesis thereby describes a number of biological responses that enable *E. globulus* to thrive under conditions of water deficit. However, one must consider that controlled climate chamber conditions are rather different than the scenario a plant encounters when growing in the field. On one hand, controlled experiments are useful in providing and increasing a more fundamental knowledge on the specificity of an isolated stress factor. On the other hand, considering the key goal of obtaining knowledge that allows finding suitable markers of abiotic stress tolerance in this species, useful in breeding programs and early selection, and contribute to maintain productivity under environmental change, a bigger challenge remains. It consists of the need to focus our studies in more realistic, field-like experiments, at least in the context of finding suitable stress markers in the climate change era.

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