Universidade de Aveiro Departamento de Biologia
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Stress hídrico e recuperação em *Eucalyptus*: Perfis fisiológicos

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Glória Catarina Cintra da Costa Pinto, investigadora auxiliar do CESAM da Universidade de Aveiro e co-orientação da Doutora Marta Pintó i Marijuan, investigadora em pós-doutoramento do ITQB da Universidade Nova de Lisboa.

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à Isabel, ao Baltasar e à Força que nos ilumina. *Obrigada*.

o júri

presidente	Professor Doutor João António de Almeida Serôdio professor auxiliar do Departamento de Biologia da Universidade de Aveiro
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palavras-chave Floresta, *Eucalyptus globulus*, seca, recuperação, fisiologia vegetal

resumo

Em Portugal, cerca de 700,000 ha foram já plantados com clones de *Eucalyptus globulus*, selecionados pelas suas elevadas taxas de crescimento, alta produção de polpa e adaptabilidade ambiental. Contudo, a produtividade das plantações de *E. globulus* tem enfrentado sérias limitações, principalmente devido à fraca disponibilidade de água. A seca é um importante stress abiótico que afeta negativamente o crescimento e o desenvolvimento das plantas, causando um conjunto de respostas fisiológicas, bioquímicas e moleculares. Embora esteja disponível um grande número de estudos que descreve as respostas das plantas ao stress hídrico, apenas alguns trabalhos se debruçam sobre os mecanismos que permitem a recuperação. Além disso, vários estudos descrevem também como diferentes genótipos podem diferir na capacidade de lidar com a seca. Considerando que manter a produção durante o stress hídrico não é o mais relevante, mas sim a capacidade de sobreviver e recuperar rapidamente após a re-hidratação, o objetivo deste estudo foi compreender os mecanismos envolvidos na recuperação, de modo a selecionar coleções clonais adequadas a plantações sustentáveis num clima mediterrânico.

Com essa finalidade, dois clones de *E. globulus* (AL-18 e AL-126) foram submetidos a um período de três semanas em *stress* hídrico, seguido por uma semana de recuperação. Um perfil fisiológico foi obtido para cada genótipo, pela avaliação do crescimento, estado hídrico, peroxidação lipídica, respostas do aparelho fotossintético, trocas gasosas e concentração de ABA. Os principais resultados deste trabalho levam a concluir que: i) os genótipos escolhidos foram altamente tolerantes às condições testadas; ii) os clones selecionados apresentaram uma resposta similar na maioria dos parâmetros testados (exceto MDA, pigmentos, parâmetros fotossintéticos e ABA); iii) o clone AL-126 foi o mais resiliente à seca, mantendo taxas de crescimento mais elevadas em stress e após re-hidratação.

keywords Forest, *Eucalyptus globulus*, drought, recovery, plant physiology

abstract

In Portugal, about 700,000 ha have been established with *Eucalyptus globulus* clones selected for their high growth rates, high pulp yield and environmental adaptability. However, productivity in *E. globulus* plantations has encountered serious limitations, mostly because of water availability. Drought is a major abiotic stress negatively affecting plant growth and development that causes an array of physiological, biochemical and molecular responses in plants.
Apart from the great number of studies reporting on plant responses to drought stress and on the mechanisms to overcome stressful conditions, only a few reports providing evidence about the capacity of recovery and the underlying processes during recovery from drought are available. Moreover, ecophysiological studies have reported that different genotypes differ in their capacity to cope with drought. Considering that maintenance of production during drought is not the most important consideration, but rather the capacity to survive and recover rapidly after rewatering, the aim of this study was to understand the underlying mechanisms in recovery in order to select suitable clonal collections for sustainable plantations in a Mediterranean climate.
For this propose, two *E. globulus* clones (AL-18 and AL-126) were subjected to a three-week water stress period, followed by one week recovery. A physiological profile was obtained for each genotype, assessing growth, water status, lipid peroxidation, photosynthetic responses, gas exchanges and ABA concentration. The main results of this work led us to conclude that: i) the chosen genotypes were highly tolerant to the conditions tested; ii) the selected clones presented a similar response in most of the tested parameters (except for MDA, pigments, fluorescence parameters and ABA); iii) clone AL-126 was the most resilient to drought, maintaining higher growth rates under stress and after rewatering.

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Part I

Eucalyptus globulus, a key species

About one third (approximately 4 billion hectares) of the total land area in the world is covered by forests, including native or planted forests (fig. 1). Without suffering significant anthropogenic modifications, native forests have evolved and reproduced themselves naturally, while planted forests correspond to new tree plantations introduced by human action (corresponding to 180 million hectares according to the Global Forest Resources Assessment 2000) (1, 2).

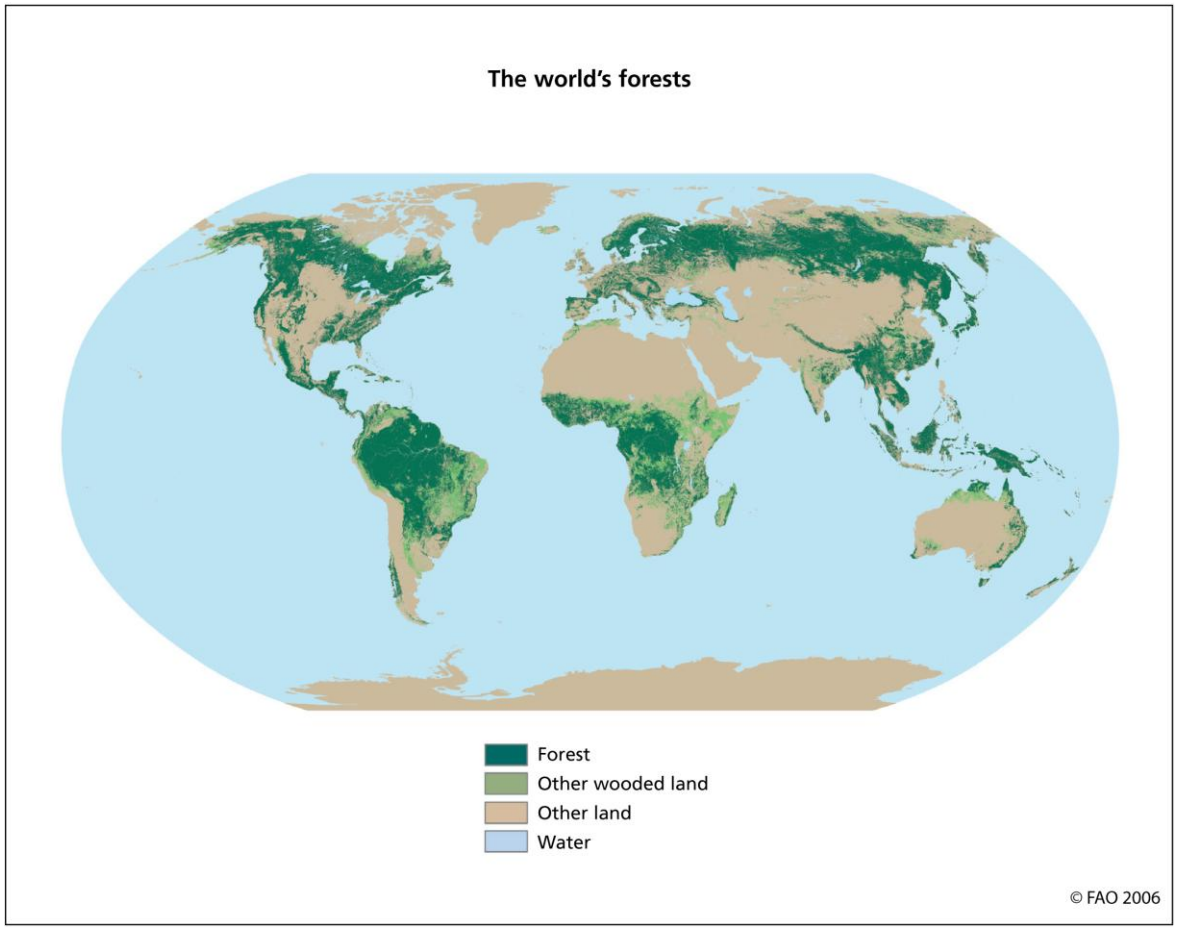

Figure 1 – Representation of forested area, other wooded land, land and water area in the world (Source: FAO Forestry Paper (3)).

Since the earliest times, forests have been major providers of humankind, supplying a complex array of vital ecological, social, and economic goods and services. According to the Food and Agriculture Organization of the United Nations, approximately one billion

people worldwide currently depend on forest resources for meeting essential fuel wood, grazing, food, fibber, medicines and other needs (2, 4). At the global economic development level, forests make an important contribution: it is estimated that 1.2 billion hectares are available for industrial wood supply, with wood and manufactured forest products adding more than $450 billion to the world market economy each year (2, 4). The International Labour Organization estimates global forest-based employment (including both industrial and nonindustrial forest, harvesting and industrialized forest products manufacture) at approximately 47 million, with special relevance for developing countries that account for almost 70% of those jobs (2), numbers that stamp the global economic significance of forests.

Beyond providing wood and other products, forests are among the most important repositories of terrestrial biological diversity and are responsible for large amounts of sequestered carbon. In addition, forests help maintaining the fertility of agricultural land, protect water sources, and reduce the risks of natural disasters such as landslides and flooding (2).

Approximately 20 million ha over many countries in several parts of the world are estimated to be covered by *Eucalyptus* plantations: South America, South Africa, Asia, Australia and South-Western Europe (5, 6). The *Eucalyptus* genus includes about 900 species native to Australia where environmental conditions vary from moist temperate to hot arid zones (5, 7). Some *Eucalyptus* species are considered the fastest-growing trees in tropical and sub-tropical areas, being the most widely used in cultivated forests, and represent an important source of hardwood for wood, paper and charcoal industries (5). *Eucalyptus globulus* (Labill.) (fig. 2) is one of the most important members of its genus and the most commonly planted in temperate regions because of its high fiber yield and rapid growth (8, 9).

It is expected that, as human populations grow and countries around the world become more affluent, the demand for wood forest products will increase as well (2). Native forests used to be important sources of eucalypt wood for pulp production, but as demands grow, well managed plantations-based production become a renewable and indispensable resource, capable of producing the required quantity of material (10). As a result, plantations have been widely established in suitable areas and have made eucalypts the most widely planted genus of angiosperm trees (10). In Portugal, about 700,000 ha

have recently been established with *E. globulus* clones selected for their high growth rates, high pulp yield and environmental adaptability (8). Apart from the world economic value, the almost completed sequencing (11) reinforces how *Eucalyptus* is set to become the second model tree genus for functional genomics (after *Populus*) (9) and illustrates the importance of investigating this species, especially in the Portuguese context.

Figure 2 – *Eucalyptus globulus*.

Water stress as a limiting factor

Abiotic stresses, especially drought (assumed to be soil and/or atmospheric water deficits), elevated temperatures and soil salinity, have led to growing agronomic, economic and ecological concerns in Europe and worldwide since a correlation between global warming and increased frequency of extreme environmental events has been found (12, 13). Among the outstanding abiotic factors, water availability is probably the most limiting, strongly affecting forest productivity and altering plantations quality and viability (14, 15)

Drought is the main cause of inter-annual variation in terrestrial carbon sequestration, causing large reductions in gross primary productivity and net ecosystem exchange of terrestrial ecosystems (16, 17). These events are especially relevant in regions subjected to natural cycles of dry and rainy seasons, or at temperate latitudes where climate change is predicted to result in reduced summer rainfalls and warmer winters (5, 16). The Mediterranean region presents a marked seasonal climate, with a dry and hot summer, when low precipitation and high evaporation lead to a decrease in moisture availability to plants (16).

Water deficit is a multidimensional stress affecting plants at various levels of their organization and involves diverse physiological, biochemical and molecular responses (18, 19). Multiple mechanisms have been developed by trees to withstand drought which cost may differ in terms of productivity (20, 21). For example, while a reduction in leaf area or stomatal closure will almost certainly mean a reduction in productivity, turgor maintenance provides the potential for maintaining metabolic processes and increasing growth (14, 20).

Plant responses to water deficit are influenced by the intensity, duration and rate of progression of the stress: these factors dictate whether mitigation processes associated with acclimation will occur or not (22). Acclimation responses under drought conditions are related to growth inhibition or leaf shedding that help to maintain plant water status and plant carbon assimilation (by restricting water expenditure by source tissues) and accumulation of compatible organic solutes that build up in response to a slowly imposed dehydration (22). These responses have a function in sustaining tissue metabolic activity and eventually lead to restoration of cellular homeostasis, detoxification and therefore survival under stress (22). On the other hand, as the water stress becomes harsher and the acclimation mechanisms fail to ensure the proper balance, plants endure functional damage and tissue loss (23).

Effects during water stress

Early drought effects are manifested as stomatal closure and leaf growth inhibition that protect plants from extensive water loss, which might result in cell dehydration, runaway xylem cavitation and ultimately, death (23). Closing stomata results from changes in turgor of guard cells relative to epidermal cells (23). As a result of osmotic adjustment, variations in turgor pressure, changes in membrane permeability or decreases in cell wall elasticity can contribute to turgor maintenance, allowing plants to take up water at low soil water potentials (14, 20, 23).

Decreased leaf water potential (ψ_L) and stomatal closure result in limited gas exchanges, reduced transpiration and photosynthesis, and limited tissues growth through cell division enlargement and differentiation (18, 24). These responses can be directly triggered by the changing water status of the tissues or induced by plant hormones (23). Abscisic acid (ABA), a phytohormone that is synthesized in both roots and leaves, is well known to mediate plant response to drought (25, 26). Early stages of water depletion are first sensed by roots that accumulate ABA able to move to the aerial parts of the plant (18, 27). As a consequence of ABA accumulation and hydraulic signals stomatal conductance is reduced (i.e., stomatal closure and limitation of gas exchange) and cellular growth is eventually restricted (18, 27). These morpho-physiological readjustments as well as other responses to drought (fig. 3) are controlled by interconnected signalling networks, which regulate profound modifications at the genome level including epigenetic and gene

expression modifications in all organs of the plant (12, 18, 28). Accordingly, molecular and genomics approaches have been employed in order to clarify the mechanisms of drought tolerance in plants, with a number of stress-responsive genes already reported (29).

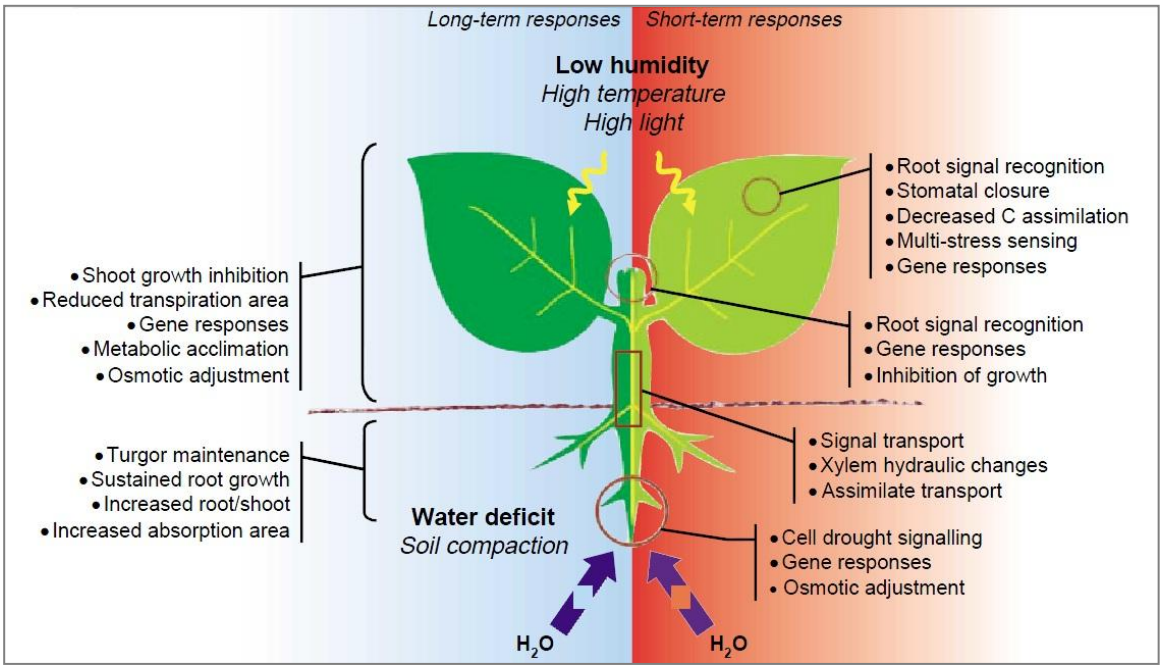

Figure 3 – Whole-plant responses (short and long-term) to drought stress (Source: Chaves *et al.* (23)).

Biomass and growth

Cell growth is considered one of the most drought sensitive physiological processes due to the reduction in turgor pressure (30) and decreased CO_2 diffusion from the atmosphere (31), for what the first and most concerning effect of drought is impaired plant growth and development, condition that may severely limit plant performance and production (32).

Growth traits have been recorded for several species under drought conditions and a reduction over stress has been consistently sustained (13, 33, 34). For example, Zhang *et al.* (35) have found that water deficit stress decreased the plant height and biomass from soybean (*Glycine max*), while Bogeat-Triboulot *et al.* (36) stated that decline of stem diameter increment was the first detected effect of soil water depletion in *Populus euphratica*.

Several reports are already available in *Eucalyptus*: three populations of *E. microtheca* presented significant decreases in their growth traits: shoot height, basal diameter, total biomass, total leaf area, root/shoot ratio, foliage area/stem cross-sectional

area ratio and specific leaf area density under water-stressed treatments (37). In *Eucalyptus globulus*, Osório *et al.* (38) reported that soil water deficits resulted in substantial reductions in total biomass and later Costa e Silva *et al.* (8) found that water stress led to a general decrease in growth which was reflected in reductions in total biomass, leaf area, number of branches and total root length.

Water relations

Cell enlargement is directly dependent in the entry of water into plant tissue (13). Water absorption occurs along gradients of decreasing water potential, therefore the water potential of growing plant tissue must be below that of the water supply (13), what explains a reduction in the plant water potential under water deficit conditions.

Relative water content (RWC) is an important index of the dehydration level, very often correlated to the metabolic activity in tissues, establishing the relation between the water uptake by the roots and the water loss by transpiration (39).

Decreasing RWC in response to drought stress has been corroborated in a wide variety of plants as reported by Nayyar and Gupta (40), explaining that, once subjected to drought leaves exhibit large reductions in RWC and water potential.

In other works, drought stress was shown to decrease the leaf water potential in soybean (*Glycine max*) (35) and Norway spruce (*Picea abies*) (41). In *Eucalyptus globulus* plants, water stress also significantly decreased water potential and RWC (42).

Chlorophylls and photosynthesis

Photosynthetic pigments play an important role in plants mainly at the harvesting light complexes and producing reducing compounds (39). Chlorophylls are the major chloroplast components for photosynthesis, and relative chlorophyll content presents a positive correlation with photosynthetic rate (39).

As reviewed by Cunningham and Gantt (43), carotenoids are also essential components of the photosynthetic membranes and serve an extraordinary variety of functions in plants: react with and efficiently quench triplet chlorophyll, singlet oxygen and superoxide anion radicals, dissipate excess light energy absorbed by the antenna pigments, harvest light for photosynthesis and serve as precursors for biosynthesis of the plant growth regulator abscisic acid.

Under unfavorable conditions, plants are known to lose chlorophyll being forced to divert the absorbed light to other processes, like thermal dissipation by carotenoids to protect the photosynthetic apparatus (44). Drought stress affects photosynthetic activity in plant tissues due to an imbalance between light capture and its utilization (45). Downregulation of photosystem II (PSII) activity results in an imbalance between the generation and utilization of electrons, apparently reducing the quantum yield (44). Dissipation of excess light energy in the PSII core and antenna generates active oxygen species and leads to a drought-induced oxidative stress (44). The decrease in chlorophyll content under abiotic stress has been considered a typical symptom of oxidative stress and may be the result of pigment photo-oxidation (39). Another indicator of the prevalence of free radical reactions in tissues is the accumulation of malondialdehyde (MDA) (46). MDA is a product of membrane lipid peroxidation and shows greater accumulation under environmental stresses (47).

Photosynthetic functioning in response to various stresses, including drought, can be monitored by measuring chlorophyll fluorescence by modulated fluorometers, providing a powerful tool to assess photochemical efficiency (48). Nevertheless, the electron transport chain and its associated processes are both relatively resistant to drought stress (41). Ditmarová *et al.* (41) found significant differences in total chlorophyll content in Norway spruce (*Picea abies*) seedlings exposed to mild and severe drought stress during 47 days. Mild stress did not affect the chlorophyll content and the maximal photochemical efficiency of PSII (F_v/F_m) was stable. However, under severe stress both chlorophyll content and F_v/F_m sharply decreased (down to 36% from their initial values).

In drought-stressed eucalypts, total chlorophyll concentration increased with increasing water stress, reaching a maximum at stomatal closure and then declined only slightly with intensifying stress (49).

Gas exchanges

Leaves have small water reserves compared to the flux of water via transpiration and would be rapidly dehydrated if it was not for different mechanisms that control cellular water availability and the rate of water loss via transpiration (50). The CO_2 required for photosynthesis diffuses from the atmosphere into leaves via the stomata, water easiest way to exit and thus stomatal closure simultaneously slows the transpiration water flux (E), the

rate of CO_2 diffusion into the leaf, and in consequence the rate of photosynthesis (A – net CO_2 assimilation) (51).

Almost every response to water deficit has a metabolic cost (52) and stomatal limitation of A is accepted as one of the main limitations to plant productivity in dry-land ecosystems (53). However, it remains a point of contention whether reduced CO_2 supply, in the form of reduced intercellular CO_2 concentration (C_i) due to the stomatal closure, or impairment of mesophyll metabolism, or both, are the cause of water deficit-induced reductions in photosynthesis (54, 55). Besides, it is not easy to be certain about the detailed mechanisms of stomatal response to drought at any particular time because stomata react and adjusts continuously to a complex set of factors ranging from light intensity to CO_2 concentration in addition to leaf water status (23).

According to Ditmarová *et al.* (41) study with Norway spruce (*Picea abies*) monitoring variables under drought conditions, g_s (stomatal conductance) and net CO_2 exchange rate were the first to show decline as drought intensified, followed by ψ_L, consistent with the role of stomata in regulating plant water potential. The same happened with *Eucalyptus marginata* where partial stomatal closure was the dominant mechanism employed to cope with drought (55). However, in the same study, smaller g_s was not the sole cause of slower photosynthesis because intercellular CO_2 was unaffected. The authors expressed that this contrasts with the more common observation that water stress causes stomatal closure that in turn reduces C_i, and thus photosynthesis. They argued that these drought-induced reductions in photosynthesis which are not explained by reduced stomatal conductance are typically referred to as "non-stomatal limitation(s)" and are taken as prima facie evidence of inhibition of photosynthesis by altered metabolism (55).

Hormone signaling

Phytohormones (plant growth regulators) regulate every aspect of plant growth and development as well as the responses of plants to biotic and abiotic stresses (56). Plant hormones are compounds derived from plant biosynthetic pathways and can act either at the site of synthesis or following their transport, elsewhere in the plant (56). The five classical phytohormones are: abscisic acid (ABA), ethylene, cytokinin (CK), auxin (IAA), gibberellin (GA), jasmonate (JA), as well as brassinosteroids (BR), salicylic acid (SA),

nitric oxide (NO), and strigolactone (SL), and it is likely that additional growth regulators are yet to be discovered (56).

ABA is essential for various stress responses, including stomatal closure, stress-responsive gene expression and metabolic changes (57). There is now strong evidence that ABA plays an important role in the regulation of stomatal behaviour and gas exchanges of drought-stressed plants (26). Shinozaki *et al.* (58) have stated that ABA is produced *de novo* under water deficit conditions and plays a major role in response and tolerance to dehydration (58), while later Costa *et al.* (59) reiterated that stomatal closure is mediated by hormonal signals (ABA) travelling from dehydrating roots to shoots and that the signaling pathway triggered by ABA in guard cells is one of the better understood pathways in plants.

Granda *et al.* (60) used an hydroponic culture and an osmotic agent to study early drought mechanisms and signalling in *E. globulus* and observed a sequential increase in ABA content, first in the root xylem sap (1.5 h), followed by an increase in the stem xylem sap (3 h) and, finally, in the apical segment of the stem (12 h).

The importance of recovering

The biotic and abiotic factors of the environment that determine the wellbeing of plants are numerous and furthermore in continuous interaction in determining the fitness of a plant (61). By favouring the survival and the chance of propagation of the best adapted individuals, Darwinistic natural selection will allow annual plant species to adapt relatively fast to changing conditions (61).

For plant species growing in Mediterranean rainfed areas, summer growth is inversely correlated with survival and persistence (62). Therefore the most important strategy to consider in improved plantations is not maintenance of production during drought, but the ability to survive and recover rapidly after autumn rains (63). Not only the metabolism needs to be shifted to an alert state once an environmental factor becomes limiting, but the opposite, restoration of optimal productivity under better conditions, is just as essential (64).

Since this aspect has received relatively little research attention, it becomes essential to understand the underlying mechanisms of recovery in order to cover the urgent

need for protective steps that will allow the selection and introduction of suitable genotypes for sustainable plantations in a changing Mediterranean climate (12, 63).

Linking '*omics* and ecophysiology – unravelling stress perception

Study and research are well recognized as having a decisive impact on sustainable and productive forest management (2). Growth traits are basic components of adaptation and productivity for what understanding tree growth and development is fundamental for the management of adaptive genetic variation needed for forest survival in changing environments (2).

As already described, the impacts of water shortage on plant physiology are numerous. These impacts can be assessed at different spatial scales, ranging from the canopy to molecular processes, and approaches at finer scales are expected to improve the understanding of the processes recorded at larger scales (36).

Responses to perturbations are usually accompanied by major changes in the plant transcriptome (65, 66), proteome (5, 18, 67, 68) and metabolome (9, 69). Recent research has made efficient use of these '*omic*' approaches to identify transcriptional, proteomic and metabolic networks linked to stress perception and response – not only in the model plant *Arabidopsis* (*Arabidopsis thaliana*) but also in crop, garden and woody species (70).

Assessing specific ecophysiology profiles in conjunction with recent '*omics* approaches will allow monitoring particular variations under different environmental stresses such as water deficit. This is considered to be extremely important in breeding programs and contribute to a better understanding of phenotypic diversity and plant improvement (71, 72).

The master thesis: main purposes

In a rapidly changing climate the ability and rate of recovery after whatever stressing situation are important aspects in terms of growth and survival. Considering the pivotal importance of *Eucalyptus globulus* plantations in Portugal and the current need to understand the underlying mechanisms in drought perception and recovery, it becomes essential to explore the impacts of water shortage and restoration on plant physiology and on the aforementioned finer scales (transcriptome, proteome and metabolome).

Hypothesising that different genotypes would present different response profiles, two different *E. globulus* clones widely established in field plantations were used to compare effects of water shortage on morphological and physiological traits, focusing particularly on growth, water relations, photosynthetic responses and the ABA role. Rooted cuttings at the nursery phase were subjected to a 3-week water stress with two different intensities, in greenhouse conditions, followed by a 1-week period of rewatering.

The aim of this study was to evaluate the performance of different genotypes in order to ensure the first step of characterizing the water stress recovery capacities in *E. globulus*.

It is expected that this first task would be complemented with molecular, proteomic and metabolomic approaches in order to investigate and interconnect information on the mediating processes of drought tolerance, from gene regulation to physiological responses and plant performance, to better understand climate changes and forest trees responses. In fact, this strategy has already been properly applied: assessing epigenetic variations in *Quercus suber* plants exposed to heat stress and recovered allowed illustrating the biological meaning of the studied mechanisms in heat tolerance. This work has been recently accepted for publication in PLOS ONE journal (PONE-D-12-26577 – Correia B *et al.* "Is the interplay between epigenetic markers related to the acclimation of cork oak plants to high temperatures?").

The master thesis disclosed herein is presented as a research paper and it is prepared according to the authors' instructions from the Journal of Plant Physiology.


Clone AL-18
WW
WS 25%
WS 18%


Clone AL-126
WW
WS 25%
WS 18%

Part II

Responses during water stress and recovery in two *Eucalyptus* clones: Physiological profiles

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Summary

In Portugal, about 700,000 ha have been established with *Eucalyptus globulus* clones selected for their high growth rates, high pulp yield and environmental adaptability. However, productivity in *E. globulus* plantations has encountered serious limitations, mostly because of water availability. Drought is a major abiotic stress negatively affecting plant growth and development that causes an array of physiological, biochemical and molecular responses in plants.

A number of studies reporting on plant responses to drought stress is currently available, but only a few reports provided evidence about the plants' capacity of recovering and the underlying processes. Moreover, ecophysiological studies have reported that different genotypes differ in their capacity to cope with drought. Considering that the capacity to survive and recover rapidly after drought should be the most important consideration in plant productivity, the aim of this study was to characterize the water perception and gather physiological features about the recovery capacities in different genotypes, contributing with new data that support a future early selection of suitable clonal collections for sustainable plantations in a Mediterranean climate.

For this propose, two *E. globulus* clones (AL-18 and AL-126) were subjected to a three-week water stress period, followed by one week recovery. Each genotype was analysed and a physiological profile was obtained for each one. Growth, water status, lipid peroxidation, photosynthetic responses, gas exchange and ABA concentration were assessed during maximum stress day and also after one day and one week of recovery. The main results of this work led us to conclude that: i) the chosen genotypes were highly tolerant to the conditions tested; ii) the selected clones presented a similar response in most of the tested parameters (except for MDA, pigments, fluorescence parameters and ABA); iii) clone AL-126 was able to maintain higher growth rates under stress and after rewatering.

Key words

Forest; drought; genotypes; rewatering; ecophysiology

Abbreviations

1-day Rec, 1-day recovery; 1-week Rec, 1-week recovery; A, foliar photosynthetic rate; ABA, Abscisic acid; C_a, ambient CO_2 concentration; E, foliar transpiration rate; F, steady-state fluorescence; F_0, minimum fluorescence; F_m, maximum fluorescence; F'_m, maximal fluorescence; F_v, dark adapted variable fluorescence; F'_v, variable fluorescence; F_v / F_m, maximum quantum yield of PSII photochemistry; g_s, stomatal conductance; Max Stress: maximum stress; MDA, malondialdehyde; NPQ, non-photochemical quenching; PPFD, photosynthetic photon flux density; RWC, relative water content; VPD, vapour pressure deficit; WS, water stressed; WW, well-watered; ϕ_{PSII}, quantum yield of PSII photochemistry; Ψ_{pd}, predawn water potential; Ψ_{md}, midday water potential

Introduction

Despite only representing 7% in the global forested area, planted forests constitute a key role in supplying worldwide forest based services (1). *Eucalyptus* genera are the most widely introduced and cultivated in the Mediterranean area (2), with approximately 700.000 ha of *Eucalyptus globulus* (Labill.) planted in Portugal (3). Although the Portuguese climate is not optimal for this particular species, it has been chosen over other eucalypt species because of its high fibre quality and fast growth (4, 5). Portuguese plantations have been established with clonal collections of select genotypes. Founding generations of plants were chosen mainly for their fibre properties and growth behaviour (2). However, as stated by several authors, soil and atmospheric water availability has been shown to be the major factor limiting productivity in *Eucalyptus* plantations (1, 6). Moreover, ecophysiological studies have reported that different genotypes differ in their capacity to cope with drought (1). This fact has led to the current practice of considering plant performance maintenance within varied environmental conditions during the genotype selection process (2).

Reduced water availability (drought) is a major abiotic stress negatively affecting plant growth and development (3, 7). Water stress causes diverse physiological, biochemical and molecular responses in plants, which first achieve an acclimation state and later, as the water stress intensifies, endure functional damage and loss of plant parts (3, 8). During acclimation to water stress, osmotic adjustment and changes in cell wall elasticity occur, allowing the plant to maintain cell turgor that would otherwise be lost as a result of the water shortage (3, 9). Stomatal closure takes place and limits gas exchanges, resulting in reduced transpiration and carbon assimilation (3) and, consequently, reduced leaf growth inhibition and significant reductions in productivity (3, 4).

Many studies have been conducted to test the effect of drought stress in *Eucalyptus* and the main obtained responses include changes in biomass allocation (10), loss of turgor and osmotic adjustment (11), decrease of water potential (4, 12), stomatal closure (4, 12), cell wall reinforcement and water storage (13), and changes in antioxidants, antioxidant enzymes, chlorophylls and carotenoids (6, 14).

Phytohormones are essential for the ability of plants to respond and adapt to abiotic stresses (15). Abscisic acid (ABA) is considered the principal stress hormone (16) and has

been shown to form part of a complex signalling network which mediates the physiological changes in *Eucalypus globulus* under drought stress (7).

Apart from the great number of studies reporting on the plant mechanisms to overcome drought conditions, only a few reports providing evidence about the capacity of recovery and the underlying processes are available (17). However, in a rapidly changing climate the ability and rate of recovery are important aspects in terms of growth and survival, so that maintenance of production during drought is not the most important consideration, but rather the capacity to survive and recover rapidly after rewatering (17, 18). Since this aspect of drought perception has received little research attention and considering the pivotal importance of *Eucalyptus globulus* plantations in Portugal, it is essential to understand the underlying mechanisms in recovery in order to select suitable clonal collections for sustainable plantations in a Mediterranean climate.

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 that is particularly affected by drought conditions (reviewed in (19)). Considering this hypothesis, two different *E. globulus* clones widely established in field plantations were used to compare effects of water shortage on morphological and physiological traits, focusing particularly on growth, water relations, photosynthetic responses and the ABA role. Rooted cuttings at the nursery phase were subjected to a 3-week water stress with two different intensities, in greenhouse conditions, followed by a 1-week period of rewatering. The aim of this study was to evaluate the performance of different genotypes to water stress and during recovery at the nursery phase in order to characterize the water perception and gather physiological features about the recovery mechanisms that would be useful for an early selection of genotypes to be introduced in locations affected by reduced water availability.

Therefore, two *E. globulus* clones were studied under water stress and during recovery, testing in particular: 1. whether a clone shows different responses between different water treatments; 2. whether the time required for recovering a studied parameter differs for each genotype; 3.whether the level of a given parameter differs between the genotypes under the same watering regime; 4. whether a given physiological profile makes a clone more favourable in drought conditions.

Material and methods

Plant material and experimental design

Rooted cuttings of two *E. globulus* clones largely used in forest plantations in Portugal (AL-18 and AL-126) were obtained from Altri Florestal SA (Portugal). One hundred and sixty replicate cuttings of each clone, grown in plastic containers filled with 3:2 (w/w) peat:perlite, with an initial height of 30 cm and six months old, were transplanted to 2L plastic pots filled with equal weight of a 3:2 (w/w) peat:perlite mixture and transferred from a shaded house to a greenhouse, with daily records of temperature, humidity and VPD (vapour pressure deficit). The potted cuttings were subjected to a one-month acclimatization period inside the greenhouse being watered with nutritive solution. To minimize effects of environmental heterogeneity, the pots were randomly arranged and periodically moved to the neighbouring position during the whole experiment.

During the experiment, fifty 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. 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 stress cuttings (WS 25%) and well-watered cuttings were kept in the same watering regime. This procedure lasted 14 days and the first sampling point took place (Max Stress: maximum stress). After this period, all cuttings were rewatered until reach well-watered regime and recovering was monitored for one week at two different sampling points (1-day Rec: 1-day recovery and 1-week Rec: 1-week recovery).

At each sampling point (i.e., maximum stress day, one-day recovery and one-week recovery), homogeneous leaves from four biological replicates were immediately frozen in liquid nitrogen for further analysis (estimation of lipid peroxidation, pigment and abscisic acid quantification).

The experiment was carried out from May to June 2011 under greenhouse environmental conditions (see table 1): natural photoperiod and photosynthetic active

radiation, daily temperature between 16ºC and 30ºC (± 3ºC) and relative humidity between 50% and 85% (± 5%).

Table 1 – Climatic data recorded in the greenhouse

	Temperature (^{0}C)			*Humidity (%)*			*VPD (kPa)*			*PAR (µmol m^{-2} s^{-1})*
Sampling day	**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	28,3	74,6	74,1	55,9	0,62	0,58	1,70	440,2

Growth and morphological traits

Five plants per clone and treatment were harvested on the maximum stress day and also after one-week recovery. Plant height and number of stems were determined and dry weight of leaves, roots and stems was recorded for biomass determination.

Plant water status

Predawn (Ψ_{pd}) and midday (Ψ_{md}) shoot water potential were measured with a Scholander-type pressure chamber (PMS Instrument Co., Corvallis, OR). Measurements were carried out in three plants per clone and treatment at 03h30 and 12h30 (solar time) on the maximum stress day, one-day recovery and one-week recovery.

For the same sampling points, four leaf discs (diameter = 11 mm) per individual (six individuals per watering regime) were collected at midday and used to determine relative water content (RWC), using the following equation: RWC = (FW-DW) / (TW-DW) × 100, where FW is the fresh weight, TW is the turgid weight after rehydrating the leaf discs for 24 h at 4ºC in darkness, and DW is the dry weight after oven-drying the leaf discs at 70ºC until constant weight.

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*. (20), 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 95ºC for 30 min and, after cooling and centrifuging, absorbance was read at 440, 532 and 600 nm. MDA equivalents (nmol mL^{-1}) were calculated as (A – B/157 000) × 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

Chlorophyll/carotenoid content was quantified according to Sims and Gamon (21). 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.* (22) 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 (ϕPSII) equivalent to F'_v / 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). 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 (light response curves of CO_2 assimilation) curves were performed with the following PPFD (photosynthetic photon flux density): 2500, 2000, 1500, 1000, 750, 500, 250, 100, 50 and 0 μmol m^{-2} s^{-1}. After A/PPFD data, 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.

Abcisic acid quantification

Leaf content of ABA was analysed by HPLC MS/MS as described by Brossa *et al.* (23) with slight modifications. In short, approximately 100 mg were ground in liquid nitrogen with a mortar and pestle. All following steps were performed at 4ºC. Before starting the extraction procedure, deuterium-labeled internal standard (20 ηg ABA-d_6) was added. 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, Milford, MA, USA), using a X-Bridge C18 column (3.5 µm; 100 x 2.1 Waters, Milford, MA, USA). The MS/MS quantification was performed on an API 3000 triple quadrupole mass spectrometer (AB Sciex, Danaher Corp, Washington, DC) using multiple reaction monitoring (MRM) acquisition with the corresponding transitions for each analyte.

Statistical analysis

The results presented are the mean with standard errors of three to six independent replicates. Data are presented as mean ± SE (standard error). All statistical procedures were performed using SPSS for Windows (SPSS for Windows v. 11.0, SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by post hoc multiple comparison using Tukey test (employed when appropriate) was performed to estimate the significance of the results. Different lowercase letters indicate significant differences between water treatments, different capital letters indicate significant differences between sampling points and asterisks indicate significant differences between clones ($p < 0.05$).

Results

Growth and morphological traits

Water stress led to a general decrease in growth that was reflected in reductions in height, number of branches (Table 2) and total biomass (fig. 1). Clone AL-126 presented higher growth values than clone AL-18 (height, number of branches and biomass) in control and both water stressed conditions either at maximum stress or recovery. When comparing the effect of the water recovery after the stress, more important differences between sampling points were detected in the WS 18%; highest increase in height in the AL-18 and highest increase in number of branches in AL-126.

Table 2 – Height and number of branches in well-watered (WW) and differentially water stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-126) after a three-week water stress period and one-week recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between water treatments, different capital letters indicate significant differences between sampling points and asterisks indicate significant differences between clones ($p < 0.05$).

		Height (cm)		*Number of branches*	
		Max Stress	**1-week Rec**	**Max Stress**	**1-week Rec**
AL-18	**WW**	70,6 ± 2,19 aA	71,50 ± 0,96 aA*	8,50 ± 0,96 aA*	9,50 ± 1,32 aA*
	WS 25%	63,0 ± 0,94 aA*	61,50 ± 1,55 bA*	7,00 ± 0,41 aA*	8,50 ± 0,29 aB*
	WS 18%	53,7 ± 2,39 bA*	60,75 ± 1,25 bB*	7,00 ± 1,29 aA	8,25 ± 1,60 aA*
AL-126	**WW**	76,0 ± 1,47 aA	86,25 ± 0,95 aB*	15,75 ± 1,25 aA*	18,00 ± 1,78 aA*
	WS 25%	66,18 ± 0,44 bA*	68,75 ± 2,46 bA*	12,00 ± 1,47 abA*	14,75 ± 0,95 aA*
	WS 18%	64,63 ± 2,27 bA*	65,00 ± 1,00 bA*	9,50 ± 0,87 bA	14,25 ± 1,11 aB*

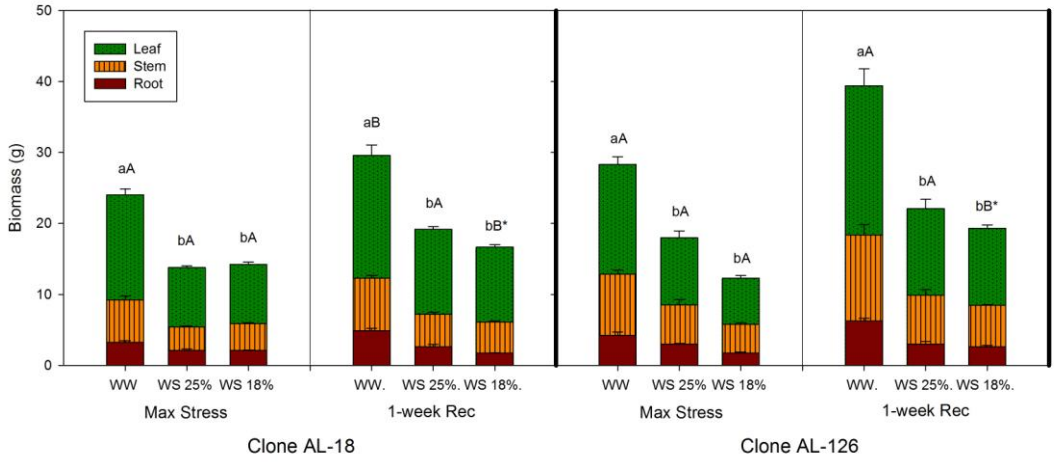

Figure 1 – Total biomass in well-watered (WW) and differentially water stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-126) after a three-week water stress period and one-week recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between water treatments, different capital letters indicate significant differences between sampling points and asterisks indicate significant differences between clones ($p < 0.05$).

Plant water status

In figure 2, RWC of the plants during the experiment is reported. In controls, RWC was constant during the experimental period, and differences between clones were not significant. In water stressed plants at WS 18%, RWC values declined (73,16 ± 3,29 in AL-18 and 78,15 ± 2,32 in AL-126), but no significant differences between clones were found. In the first day of recovery, RWC of water stressed plants reached control values and these levels were kept until the end of one week recovering. Again no significant differences were found between genotypes.

After three weeks under different watering regimes, Ψ_{pd} of control and water stressed plants remained stable within the range of -2.5 to -4.5 MPa for both genotypes either in maximum stress day or after rewatering (fig. 3). On the other hand, Ψ_{md} presented differential response patterns between the clones and the same sampling points (fig.3). Upon dehydration, AL-18 plants showed gradually decreased values according to the stress intensity, while AL-126 plants exhibited equal values for both stress intensities (different from control condition). In the first day of recovery, control values were achieved in both clones. After one week of recovery, each clone presented a similar pattern of decreased water potential values according to the previous stress intensity, but only significant for clone AL-126.

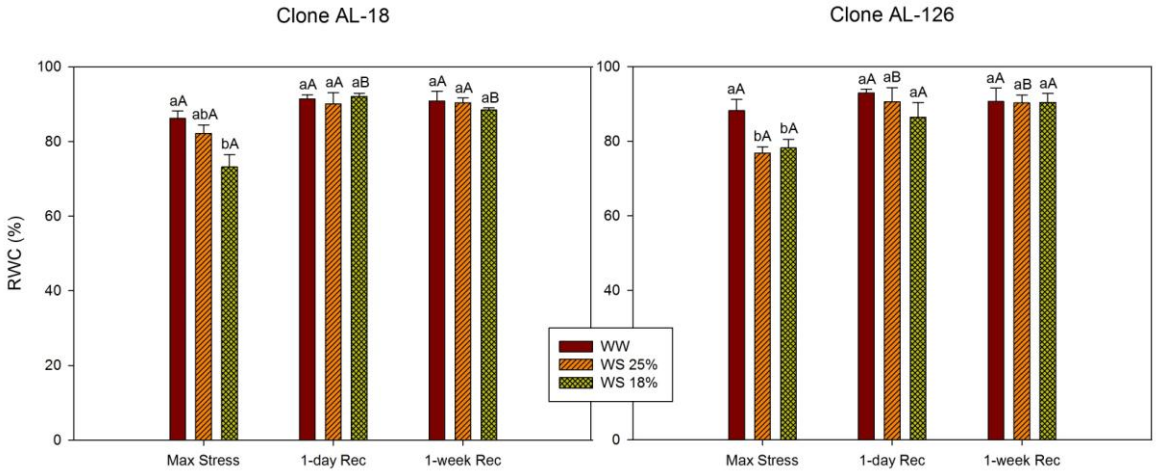

Figure 2 – Relative water content (RWC) in well-watered (WW) and differentially water stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-126) after a three-week water stress period and after one day and one week of recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between water treatments, different capital letters indicate significant differences between sampling points and asterisks indicate significant differences between clones ($p < 0.05$).

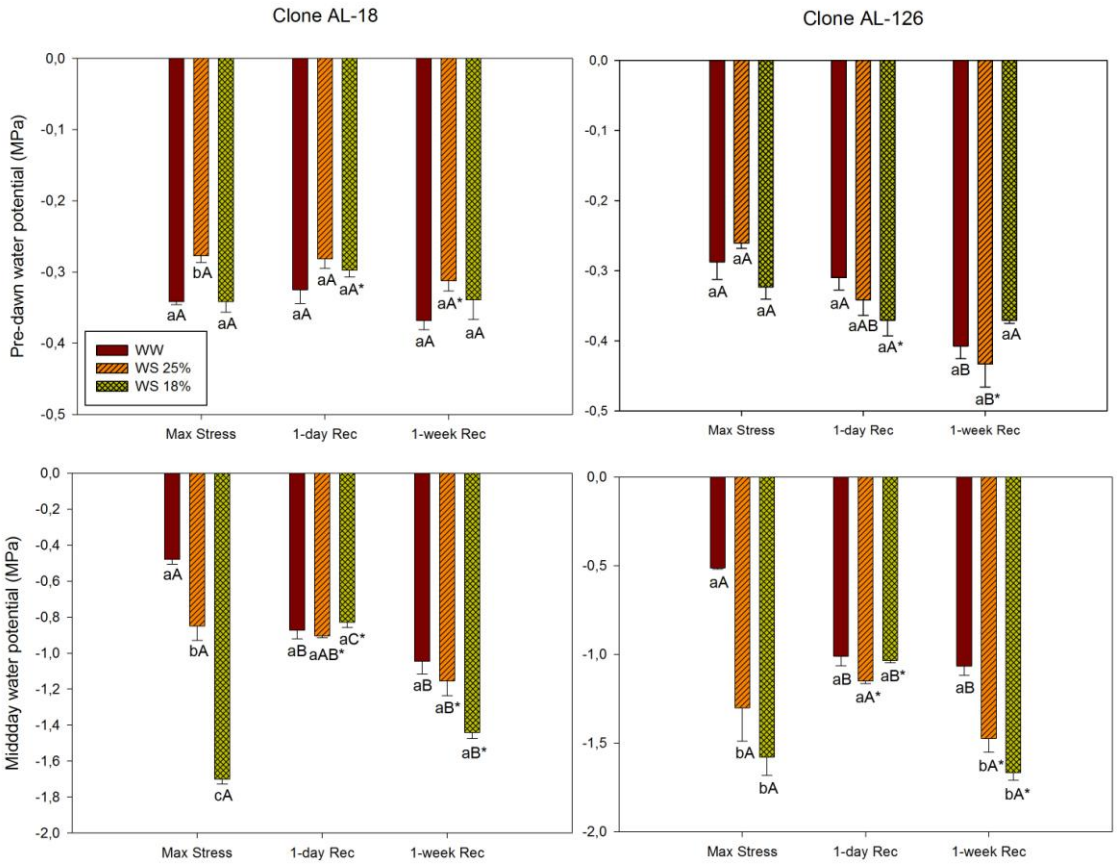

Figure 3 – Predawn (top) and midday (bottom) water potential in well-watered (WW) and differentially water stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-126) after a three-week water stress period and after one day and one week of recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between water treatments, different capital letters indicate significant differences between sampling points and asterisks indicate significant differences between clones ($p < 0.05$).

Lipid peroxidation

In what concerns to control condition, clone AL-126 presented about one third higher levels of MDA during the whole experiment. After the three-week experiment, the imposed water treatments showed 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. After one day of full irrigation, only AL-18 plants under WS 18% significantly decreased the MDA content, while plants under WS 25% showed an increase in both clones. One week after rehydration, AL-126 droughted plants presented control values while AL-18 WS 18% kept higher MDA content.

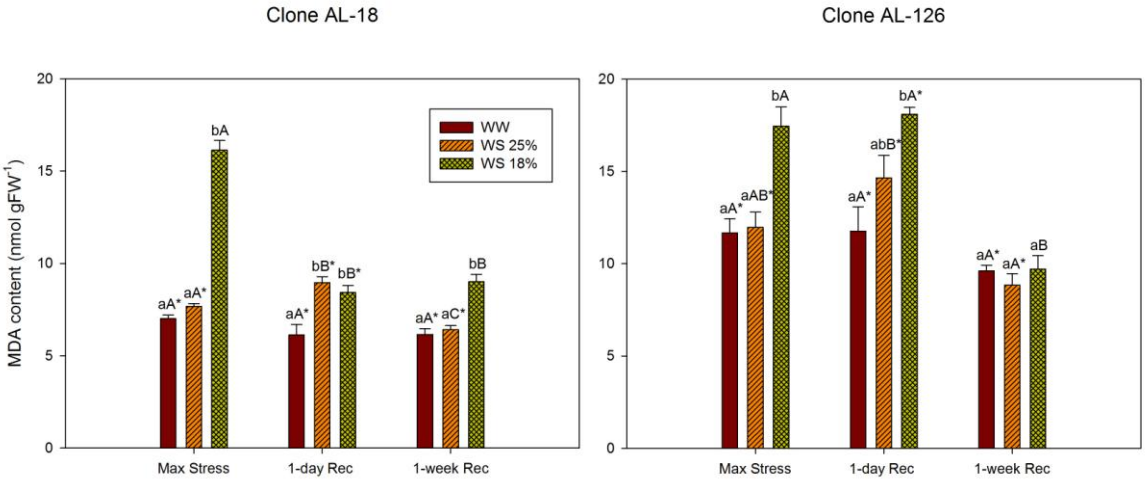

Figure 4 – MDA content in leaves of well-watered (WW) and differentially water stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-126) after a three-week water stress period and after one day and one week of recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between water treatments, different capital letters indicate significant differences between sampling points and asterisks indicate significant differences between clones ($p < 0.05$).

Chlorophyll content and fluorescence

In the maximum stress day, the F_v/F_m ratio was higher in stressed plants than in the control group. Stressed conditions from both clones differed significantly from the control group but there was no statistically significant difference between the drought stress groups in the clone AL-18, while clone AL-126 exhibited higher F_v/F_m values at the more intense stress condition. In the same way and in what concerns to ϕ_{PSII}, stressed plants presented higher values than well watered plants. However, this increment was only true for the more intense stress condition in clone AL-18, while AL-126 plants exhibited higher values with increasing stress intensity. On the other hand, non photochemical quenching was lower in stressed plants for both clones, but clone AL-18 only displayed this decrease in the more intense stress deficit, while clone AL-126 showed this decrement in both stress conditions. During the recovering sampling points, the previous parameters maintained a similar profile, without achieving control values after one week recovery (see table 3).

In both clones chlorophyll and carotenoid concentrations expressed per area of leaf were significantly higher in water stressed plants than in well watered plants (see table 3). After the stress relief pigment concentration started to decrease but only WS 25% from both clones achieved control values after one week recovery. Clone AL-126 kept higher pigment concentration during the whole experiment compared to clone AL-18.

Table 3 – F_v/F_m, ϕ_{PSII}, NPQ, total chlorophyll and carotenoids content in well-watered (WW) and differentially water stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-126) after a three-week water stress period and after one day and one week of recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between water treatments, different capital letters indicate significant differences between sampling points and asterisks indicate significant differences between clones ($p < 0.05$).

			F_v/F_m	ϕ_{PSII}	NPQ	Chl $_{a+b}$ (μmol cm^{-2})	Carot (μmol cm^{-2})
Max Stress	AL-18	*WW*	0,717 ± 0,009 aA*	0,349 ± 0,008 aA*	6,500 ± 0,244 aA*	3,48 ± 0,86 aA*	2,44 ± 0,60 aA*
		WS 25%	0,781 ± 0,014 bA	0,388 ± 0,019 aA	6,331 ± 0,503 aA*	7,99 ± 1,40 bA*	4,51 ± 0,58 bA*
		WS 18%	0,806 ± 0,004 bA	0,490 ± 0,013 bA	4,295 ± 0,307 bA	11,11 ± 3,23 bA*	5,72 ± 1,34 bA*
	AL-126	*WW*	0,687 ± 0,010 aA*	0,269 ± 0,011 aA*	7,877 ± 0,265 aA*	6,50 ± 0,72 aA*	4,02 ± 0,56 aA*
		WS 25%	0,753 ± 0,012 bA	0,425 ± 0,012 bA	4,389 ± 0,550 bA*	10,72 ± 1,29 bA*	6,27 ± 1,02 bA*
		WS 18%	0,805 ± 0,006 cA	0,495 ± 0,023 cA	3,720 ± 0,268 bA	16,79 ± 2,49 cA*	8,46 ± 1,08 cA*
1-day Recovery	AL-18	*WW*	0,665 ± 0,008 aB	0,261 ± 0,037 aA	6,859 ± 0,590 aA	3,81 ± 0,42 aA*	2,53 ± 0,23 aA*
		WS 25%	0,764 ± 0,005 bA	0,419 ± 0,018 bAB*	4,204 ± 0,183 bB*	8,45 ± 1,49 bA*	4,63 ± 0,74 bA
		WS 18%	0,810 ± 0,013 cA	0,541 ± 0,011 cAB	3,173 ± 0,367 bB	8,73 ± 1,01 bAB*	4,61 ± 0,38 bAB*
	AL-126	*WW*	0,654 ± 0,014 aA	0,328 ± 0,039 aA	6,504 ± 0,663 aA	5,95 ± 0,80 aA*	3,89 ± 0,54 aA*
		WS 25%	0,758 ± 0,010 bA	0,488 ± 0,025 bA*	3,461 ± 0,265 bA*	11,46 ± 0,69 bAB*	5,56 ± 0,71 bA
		WS 18%	0,787 ± 0,009 bA	0,550 ± 0,017 bA	2,847 ± 0,030 bA	11,76 ± 0,21 bB*	5,94 ± 0,16 bB*
1-week Recovery	AL-18	*WW*	0,654 ± 0,014 aB	0,328 ± 0,039 aA	6,504 ± 0,663 aA	3,17 ± 0,45 aA*	2,03 ± 0,25 aA*
		WS 25%	0,758 ± 0,010 bA	0,488 ± 0,025 bB*	3,461 ± 0,265 bB*	3,52 ± 0,77 aB	2,74 ± 0,30 bB
		WS 18%	0,787 ± 0,009 bA	0,550 ± 0,017 bB	2,847 ± 0,030 bB	5,97 ± 0,42 bB	3,34 ± 0,35 bB*
	AL-126	*WW*	0,665 ± 0,008 aA	0,261 ± 0,037 aA	6,859 ±0,590 aA	4,36 ± 0,37 aB*	2,95 ± 0,27 aA*
		WS 25%	0,764 ± 0,005 bA	0,419 ± 0,018 bA*	4,204 ± 0,183 bA*	4,97 ± 1,44 abB	3,36 ± 0,64 aB
		WS 18%	0,810 ± 0,013 cA	0,541 ± 0,011 cA	3,173 ± 0,367 bA	7,73 ± 1,15 bB	4,68 ± 0,05 bB

Leaf gas-exchange measurements

In the maximum stress day, as shown in figure 5, A, E and g_s presented significant lower values in the stressed group compared to control conditions, except in clone AL-18 WS 25%. After the stress relief, CO_2 assimilation of stressed plants achieved control values within one day recovering, while transpiration and g_s did not fully recovered to control values until one week of well irrigation. The two clones showed a similar response profile in the CO_2 assimilation and stomatal conductance. Clone AL-126 presented lower transpiration ratios (E) at the maximum water stress, but showed a rapid and significant response after one day recovering and still increasing rates until last day of sampling.

Abcisic acid quantification

In drought stressed plants, ABA concentration was significantly higher than in control plants (fig. 5). AL-126 plants presented higher content of ABA than clone AL-18. After the first day of recovery, ABA concentration was significantly decreased only in clone AL-126, showing a faster response to water availability. Control values were reached for both clones after one week.

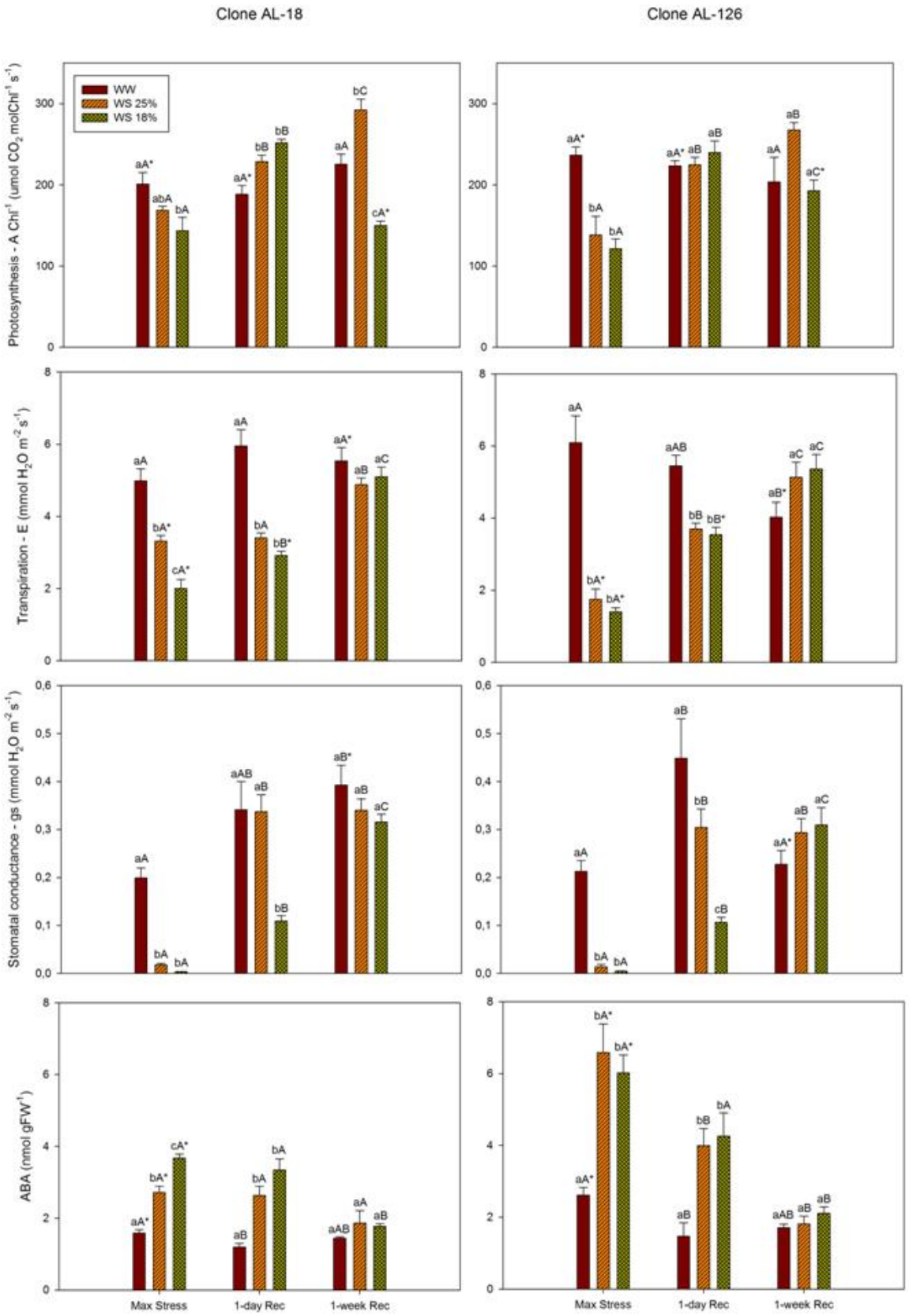

Figure 5 – Photosynthesis, transpiration, stomatal conductance and ABA content in leaves of well-watered (WW) and differentially water stressed (WS 25% and WS 18%) plants of two different *Eucalyptus globulus* clones (AL-18 and AL-126) after a three-week water stress period and after one day and one week of recovery. Data are presented as mean ± SE. Different lowercase letters indicate significant differences between water treatments, different capital letters indicate significant differences between sampling points and asterisks indicate significant differences between clones ($p < 0.05$).

Discussion

The focus of this study was to investigate water perception in *E. globulus* plants and gather physiological features that enable to characterize water stress and recovery at the nursery phase. In this sense, two different genotypes were subjected to drought under two different intensities of water deficit and subsequently rewatered in a greenhouse experiment, and several physiological measurements were carried out.

Drought phase

In general, *E. globulus* plants were affected by the imposed water treatments. However, water deprivation had not significant effects on all of the tissue water relations measured. Midday water potential and RWC was decreased under water stressed conditions as expected and previously described (6, 24, 25) but predawn water potential did not significantly illustrate the water deficit as it would be expected (6, 9, 24). The authors assumed that this fact may be due to the high humidity conditions in the greenhouse during the night, hypothesising that high atmospheric water availability induces diffusion of gaseous air water into leaves and, together with the evening watering, allows leaf water potentials to return each night to a higher level ensuring a 'daily half-recovery'.

As expected (4, 26, 27), 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. The general decline in growth was accompanied by significant reductions in stomatal conductance, transpiration and CO_2 assimilation which is in accordance with other studies (24, 27, 28). 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 carbon assimilation (8, 29).

Lipid peroxidation indicates the prevalence of free radical reactions in tissues and MDA content is often used as an indicator of the extent of lipid peroxidation resulting from oxidative stress (30). After three weeks under water stress (18%), MDA increased for both clones (clone AL-126 presenting a lower percentage of increasing in relation to control than clone AL-18), suggesting that prolonged intense water deficit caused membrane lipid peroxidation. The increases observed in leaf MDA contents of drought plants after a

prolonged period (3 weeks) were in agreement with results of other studies (31, 32). Fu and Huang (31) examined the involvement of lipid peroxidation in two cool-season grasses and found out that MDA content increased in both grasses under full drying soil but not under surface drying. The absence of MDA accumulation under WS 25% could indicate that both clones are capable of adapting to the imposed water treatment which could be explained by an effective antioxidative system under the environmental conditions or other protecting mechanisms.

Photochemical efficiency monitored by measuring chlorophyll fluorescence enabled to assess photosynthetic functioning in response to drought and the analysed parameters (F_v/F_m, ϕ_{PSII} and NPQ) showed that stressed plants had more efficient photochemical reactions. Our results are in contrast to the typically accepted that down regulation of photosynthesis under environmental constraints concurs with lower F_v/F_m and ϕ_{PSII} ratios and higher NPQ (33-35). Cornic and Massacci (36) explained how the reduction of CO_2 into the photosynthetic parenchyma due to stomatal closure causes a decrease in photochemical yield of open PSII centres and, consequently, an increase of thermal dissipation of the excitons trapped in PSII units. However, our results are supported by Susiluoto and Berninger (37) where drought stress in *E. microtheca* was accompanied by an increase in F_v/F_m and lower NPQ. Higher chlorophyll content under drought stress is also an unusual response (8) but has already been described in other works (14) and, in addition to carotenoids accumulation, seems to be a defence mechanism in *E. globulus*. It is suggested that the xanthophyll cycle has a major role in energy dissipation (38), effectively protecting photosynthesis and, along with other mechanisms such as chlorophyll accumulation or maintenance of volume in chloroplasts by osmotic adjustment (39) may preserve photosynthetic capacity and prevent injury to chloroplasts from toxic concentrations of ions, as described for other woody species (40).

Relative to ABA, there was an evident accumulation in the leaves of both clones (clone AL-126 presenting a higher percentage of increasing in relation to control than clone AL-18) during water stress which is in accordance to the defined role of ABA under water deficit conditions (29, 41). As explained by Wilkinson and Davies (42), under root perturbations the xylem vessels transfer their contents (including their ABA) to the leaf apoplast. The transpiration stream carries ABA inside the leaf around and/or through the mesophyll cells until reaching the stomatal guard cells in the epidermis, and then induces

an internal signal transduction cascade usually involving increases in both externally and internally sourced cytoplasmic calcium, which eventually reduces guard cell osmotic potential to cause stomatal closure. Higher ABA concentration (clone AL-126) is expected to match a more intensified ABA signalling which is known to have a positive correlation with water saving and quality improvement (41).

Recovery phase

During the rewatering period, plant water status showed a prompt recovery: RWC (fig. 2) and water potential (fig. 3) of the stressed plants were restored within one day of rewatering. These results are in accordance with similar works (28, 43, 44). After one week of recovery, each clone presented a similar tendency of decreased water potential according to the previous stress intensity. The authors believe that this was the result of the elevated photosynthetic active radiation together with a high VPD in that particular sampling day.

Considering gas exchanges, the lowered levels of all the three parameters (A, E and g_s) under soil water deficit had a tendency to recover. The same was observed in other studies (45, 46). Miyashita *et al*. (45) analysed photosynthesis, transpiration, and stomatal conductance in kidney bean after rewatering and observed a recovery pattern and Galmés *et al.* (46) subjected ten Mediterranean species to water stress and rewatering and showed that all studied species recovered from severe drought. The authors defended that the different recovery rates might reflect different adaptations to water-stress periods under Mediterranean conditions. After the stress relief, CO_2 assimilation of stressed plants achieved control values within one day recovering, while transpiration and g_s do not fully recovered to control values until one week of well irrigation. According to Galmés *et al.* (46), the different extents of recovering photosynthetic rates after severe water stress are accompanied by different extents in recovery of stomatal conductance, mesophyll conductance or maximum rate of carboxylation of Rubisco. Considering the different recovery rate between A and g_s, our results partly support Galmés *et al.* (46) remarks and exclude stomatal conductance as being the strongest limitation on photosynthesis recovery, remaining the question about mesophyll conductance or maximum rate of carboxylation of Rubisco. However, as stated by Chaves *et al.* (8), stomatal response to drought and the detailed mechanisms are not easy to rationalise because in addition to leaf water status

stomata keep responding to a complex set of factors (ranging from light intensity to CO_2 concentration). ABA content followed the same pattern as 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) (8, 23). Clone AL-126 showed a rapid reduction after rewatering, representing a faster response to water availability and greater dynamics in the ABA signalling.

MDA levels increased during recovery to a greater extent than during drought (WS 25%). This result is sustained by Munné-Bosch and Peñuelas (44) that found the same response in *Phillyrea angustifolia* plants. As they 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.

Fluorescence parameters kept generally unchanged after the stress relief and pigment concentration, even decreasing, did not achieve control values after one week of well watering. As previously explained, the system consisting of pigment accumulation (and xanthophyll cycle) may be of extreme importance in *E. globulus* to overcome drought and the maintenance of a high pigment level may represent an adaptive response of a species accustomed to periods of low water availability in the Mediterranean climate (46).

In conclusion, the results reported in this paper about two different *E. globulus* clones that were studied under water stress and during recovery indicate that:

1. Different water treatments imposed a different response in almost every tested parameter – a more limiting stress often results in a more marked response.
2. The time required for recovering the tested parameters did not differ between the genotypes.
3. The level of MDA, pigments and ABA content and fluorescence parameters differed between the genotypes under the same watering regime.
4. Despite both clones showed to be highly tolerant to the conditions tested, biomass accumulation demonstrated that clone AL-126 is able to maintain a greater performance under drought and recovery. It is expected that the different levels of MDA, pigments, ABA and fluorescence parameters between the genotypes may have a role in this response.

However, other aspects than the ones evaluated in this study should be considered to understand how this clones have a markedly different growth behaviour if presenting a similar response profile. Molecular and epigenetic studies as well as proteomic and metabolomic studies should be helpful clarifying how clone AL-126 is able to maintain a greater performance under drought and after recovery if both clones had a similar response profile.

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Part III

Final considerations

This study arises from the current need of investigating water stress perception and most importantly understanding the underlying mechanisms in plant recovery from constraint conditions that become more and more frequent in the changing Mediterranean area. This concern was addressed to *Eucalyptus globulus*, a worldwide established forest tree, with especial relevance for Portugal, where the great economic value and the prospective headline as second model tree genus for functional genomics meet.

The aim of the work was, thereby, to investigate water shortage perception in young *E. globulus* plants and gather physiological features that enable to characterize water stress and recovery at the nursery phase and ensure the first step of understanding the water stress recovery capacities in *E. globulus*. For that, two different genotypes were subjected to drought under two different intensities of water deficit in a greenhouse experiment and several physiological measurements were carried out.

The physiological profile obtained for each genotype, assessing growth, water status, lipid peroxidation, photosynthetic responses, gas exchanges and ABA concentration led us to conclude that the chosen genotypes were highly tolerant to the conditions tested. The selected clones presented a similar response in most of the tested parameters, exception made for MDA, pigments, fluorescence parameters and ABA. These differences, together with the higher growth rates, identify clone AL-126 as the most resilient to the imposed conditions.

Other aspects than the ones evaluated in this study should be considered in a next step to explain how clone AL-126 is able to maintain a greater performance under drought and after recovery if both clones had a similar response profile. Molecular and epigenetic studies should be carried on, as well as proteomic and metabolomic analyses in order to constitute a complete monitoring and assure the fulfilment of the proposed goals.

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