



**Cláudia Marisa de
Jesus**

**Salicylic acid and Drought tolerance improvement in
*Eucalyptus***

**Ácido salicílico e o aumento da tolerância à seca em
*Eucalyptus***

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Dissertação 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 coorientação da Doutora Monica Meijón Vidal, investigadora do Serviço Regional de Investigação e Desenvolvimento Agroalimentar (SERIDA), Grado, Espanha.

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à *vida*, que vale a pena ser vivida. Cair é o único caminho para nos levantarmos. Que tudo o que façamos seja em grande!

o júri

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palavras-chave

Eucalyptus globulus, ácido salicílico, seca, ecofisiologia, hormonas.

Resumo

Eucalyptus é um dos géneros florestais mais plantados em todo o mundo, contabilizando aproximadamente um total de cerca de 20 milhões de hectares. Na região do Mediterrâneo, o desempenho das plantações de eucalipto é condicionado pela baixa disponibilidade de água que limita fortemente a produtividade florestal, afetando o crescimento e desenvolvimento das plantas. O desenvolvimento de plantas tolerantes à seca pode ser conseguido através da adoção de estratégias de mitigação como a aplicação exógena de hormonas. O ácido salicílico (AS) é um regulador endógeno do crescimento de plantas, amplamente referido como um composto chave na resposta das plantas à seca. Neste estudo, investigamos se a aplicação exógena de SA melhora a tolerância à seca em *Eucalyptus globulus* e de que forma este tratamento regula a resposta da planta ao défice hídrico. AS foi aplicado por aplicação foliar de solução de salicilato de sódio nas concentrações de 0.75, 2.5 e 5 mM, três dias consecutivos antes da indução de *stress* hídrico (plantas mantidas a 15 % da capacidade de campo). Além disso, um grupo bem regado (diariamente regado a 70 % da capacidade de campo) foi analisado, com os mesmos pré-tratamentos de AS. Plantas controlo (15 e 70 %) não foram tratadas com AS. As características morfo-fisiológicas e bioquímicas foram avaliadas através dos seguintes parâmetros: potencial hídrico, peroxidação lipídica, conteúdo de pigmentos fotossintéticos, açúcares solúveis totais, fenóis, F_v/F_m , trocas gasosas e conteúdo de diferentes hormonas (ácido abscísico, ABA; ácido indol-3-acético, AIA; dihidrozeatina, DHZ; giberelina GA4; isopentenil adenina, IP, ácido jasmónico, JA; ácido salicílico, SA). Dinâmicas locais específicas do AIA e ABA nas folhas foram detetadas por imunolocalização. Os resultados mostraram que a seca afetou a fisiologia da planta. Por outro lado, o desempenho das plantas sob condições de *stress* hídrico apresentaram uma melhora significativa após a aplicação foliar de AS. A quantificação global de hormonas mostrou diferenças significativas entre os tratamentos hídricos, com o aumento das hormonas ABA e JA em plantas sob défice hídrico. Relativamente aos tratamentos com AS, as plantas sob défice hídrico mostraram diferenças nas hormonas DHZ, GA4 e IP. A distribuição local de AIA e ABA nas folhas foi claramente afetada pela indução de *stress* hídrico. A eficiência da aplicação foliar do AS depende da dose aplicada, com a concentração 5 mM a mostrar-se mais eficaz na indução da tolerância à seca. Estes efeitos positivos foram destacados no potencial hídrico, na taxa de assimilação de CO₂ e na peroxidação lipídica. Nas atuais mudanças globais, o tratamento AS poderá ser muito útil nos programas de melhoramento florestal, podendo efetivamente melhorar o efeito negativo do défice hídrico em plantas de eucalipto.

keywords

Forest; *Eucalyptus globulus*; drought; salicylic acid; physiology; hormones

abstract

Covering approximately 20 million ha, *Eucalyptus* genus is the most widely planted hardwood trees all over the world. In the Mediterranean region, the positive performance of *Eucalyptus* plantations is conditioned by low water availability that strongly limits forest productivity and alters plant growth and development. Plant drought tolerance can be managed by adopting strategies such as exogenous application of hormones. Salicylic acid (SA) is a plant endogenous regulator of growth (PGR) that has been reported as a compound playing a key role in plants responses to drought. In this study, we investigated if the exogenous application of SA improves drought tolerance on *E. globulus* and how this treatment regulates plant response to stress. SA was applied by foliar application of 0.75, 2.5 and 5 mM solution of sodium salicylate three consecutive days before water-stress (WS) induction (plants daily watered at 15% field capacity). Additionally a well-watered group (WW, daily watered at 70%) was analysed, with the same SA pre-treatments. Control plants (WW and WS) were not treated with SA. The assessed morpho-physiological and biochemical traits were: water potential, lipid peroxidation, pigments content, total soluble sugars, phenols, F_v/F_m , gas exchange and contain of different PGRs (abscisic acid, ABA; indolacetic acid, AIA; dihydrozeatin riboside, DHZ; gibberellin GA₄; isopentenyl adenine, IP; jasmonic acid, JA; salicylic acid, SA). Specific local dynamics of IAA and ABA in leaves was detected by their immunolocalization. The results showed that drought stress severely affected the plant physiology. On the other side, the performance of plants under water-stressed conditions showed a significant improvement after the foliar application of SA. Global quantification of PGRs didn't show great differences between treatments, with the exception of SA, however local distribution of IAA and ABA in leaves was clearly affected. The efficiency of exogenous SA depended on the applied dose, with 5 mM being more effective to induce the drought tolerance. These positive effects were highlighted in water potential, gas exchange (CO₂ assimilation rate) and lipid peroxidation. In the current global changes SA treatment could be very useful in breeding forest programs and can effectively ameliorate the negative effect of drought stress in *Eucalyptus* plants.

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PART I

Interest for forest and wood industry of *Eucalyptus globulus*

Covering 31% of the total land area (1), forests are between the world's most important biomes, enclosing natural, planted and regenerated forests (2). The Russian Federation, Brazil, Canada, the United States of America, China, Democratic Republic of the Congo, Australia, Indonesia, Sudan and India are the ten countries that more contribute for this value, accounting for 67% of the total forest area (3). Forests have a crucial role in the maintenance of vital ecosystems and social well-being. It is well recognised that forests protect water sources and their quality, help to maintain the fertility of the soil and terrestrial biodiversity, and are a major carbon sinks regulating global climate. In addition, forests reduce the occurrence of natural disasters, such as flooding and landslides (4). It is estimated that 1.6 billion of people depend on forests resources for their livelihoods at different levels (4). Forests industries are therefore a source of economic growth and employment, with this sector employing about 50 million people worldwide and the international trade reaching \$270 billion (4).

It is predicted that global population will to grow to 9, 4 billion in 2050 (5). With these population trends, the global demand for all wood forest products will increase , while the area of natural and semi-natural forests continues to decrease near 13 million ha annually mostly due to conversion to agriculture (6). Nearly 7% of world's forests are plantations (3). Planted forests can provide large quantities of forest related products and services to keep up with increasing world demand and, can be used to provide environmental benefits such as climate change mitigation (7). Several countries take out significant portions of raw material for industry and other uses (e.g. fuel-wood) from planted forests in order to preserve the natural forest resources (8).

The *Eucalyptus* genus belongs to the Myrtaceae family and includes about 900 species originated from Australia and nearby islands, where environmental conditions vary from moist temperate to hot arid zones (9). Because of its fast-growing feature, short rotation, high adaptability to soils and climates, the extensive variety of wood and non-wood products and high economic benefits, eucalypt is known as one of the most commonly planted tree genus in the world (10, 11). Currently, the total area of *Eucalyptus* plantations is estimated at 20 million ha over many countries, such as South America, South Africa, Asia, Australia and South-Western Europe (Fig. 1) (10). In a privileged location, Portugal has heavily invested in eucalypt plantations to meet the demand for some forest products, such as wood for pulp production. *Eucalyptus globulus* (Labill.) is the planted key species in Portugal, with approximately 811 943 ha (12). The increase of *E. globulus* plantations is mainly due to its high growth rates, superior

Drought and its impact in forest trees

Forest trees growing in our future field are likely to be exposed to a greater range and number of abiotic (e.g. drought, heat, nutrient, salinity) and biotic (e.g. bacteria, fungi, nematodes) conditions, as well as their combinations as result of an ongoing global change already predicted by Intergovernmental Panel on Climate Change (17-19). Climate change is expected to be one of the main future causes of biodiversity loss worldwide, and there is compelling evidence that climate change will result in the extinction of species from many taxa (20). Trees (and therefore forest ecosystems) are particularly sensitive to climate change as they are relatively long-lived compared to other organisms and have limited adaptive capacity to respond to rapid environmental change (17). Current and, predicted climatic conditions, such as prolonged drought, increase salinization of soil and water, low and high temperature episodes, poses a serious threat to forest productivity, affecting tree growth and survival (17). From these stress drive factors drought has been, and is becoming an acute problem most constraining plant growth, terrestrial ecosystem productivity, in many regions all over the world (21), and the increase of tree mortality due to drought and heat in Mediterranean systems is well documented (22). Productivity of forest ecosystems is severely constrained by water availability and drought may induce large-scale tree decline episodes in planted forests such as *Eucalyptus* plantations (23, 24).

Drought results in the reduction of the soil water availability (24). Drought stress can occurs whenever soil water drops below a threshold inducing restrictions to growth and transpiration (24). Thus, due to increasing frequency of this severe abiotic stress and considering the current context of global climatic changes, it is mandatory to advance in the knowledge of drought tolerance mechanism in trees. An understanding of how planted forest trees adapt to harsh environmental conditions, such as drought, is necessary in order to sustain productivity and meet future demands for commercial products (17). To respond to the demand for stress-tolerant FRM (forest reproductive material) that has never been greater (17), there is an highly current interest in improving the drought tolerance of planted forests (25).

Plants responses to drought stress occur at all levels of organization and are modulated by intensity, duration and rate of progression of the imposed stress (26). The ability to avoid drought is dependent on the tree's ability to minimize loss and maximize uptake of water (21). Some forest trees increase water uptake through deeper and more extensive root systems (27), modify a variety of leaf characteristics, including altered morphology (e.g., cuticular wax, (28)), reduce leaf area and stomatal conductance (29).

Effects during drought

Drought affects many aspects of tree physiology and metabolism, and can negatively impact tree growth, development and distribution. Responses to water deficit and tolerance mechanisms involve the prevention or alleviation of cellular damage, the re-establishment of homeostasis and growth resumption (30). Drought reduces leaf size, stems extension, and root proliferation, disturbs plant water relations and reduces water-use efficiency (Fig. 2). Active accumulation of solutes in vacuoles (i.e., osmotic adjustment) is a common physiological response to drought. As water potential declines, trees reduce transpiration by closing their stomata, but reducing water loss is done at the expense of CO₂ assimilation (31, 32). Water deficit in plants reduces water potential and turgor, elevating the solutes' concentrations in the cytosol and extracellular matrices (Fig. 2). As a result, cell enlargement decreases leading to growth inhibition. This is followed by accumulation of abscisic acid (ABA) and low-molecular-weight osmolytes, including glycine betaine, proline and other amino acids, organic acids, and sugars, that are crucial to sustain cellular functions under drought (33) (Fig. 2). Plant growth substances, such as, salicylic acid, auxins, gibberellins, cytokinins, and ABA modulate the plant responses towards drought (34). At this stage, overproduction of reactive oxygen species (ROS) and formation of radical scavenging compounds, such as, ascorbate, and glutathione further aggravate the adverse influence (35). Injury caused by ROS to biological macromolecules under drought stress is among the major deterrents to growth (33). These responses have a function in sustaining tissue metabolic activity and eventually lead to restoration of cellular homeostasis, detoxification and therefore survival under stress (36). Additionally, as the water stress becomes harsher and the acclimation mechanisms fail to ensure the proper balance, plants endure functional damage and tissue loss (21). Adaptation to stresses, such as, drought is controlled by molecular networks, and significant progress using transcriptomic, proteomic and metabolomic approaches (OMICs technology) has facilitated discoveries of abiotic stress-associated genes and proteins involved in these cascades (37). However, during this thesis I will focus on physiological and biochemical factors involved in drought stress responses.

Growth and leaf gas exchange

Plant growth is one of the most drought-sensitive physiological processes due to the reduction of turgor pressure (38) and decreased CO₂ diffusion from the atmosphere (39), 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 (38). Growth traits have been recorded for several species under drought conditions and a reduction over stress has been consistently sustained (40, 41). Several reports about *Eucalyptus* growth responses under drought are already available: three populations of *E. microtheca* presented significant decreases

in 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 (42). Costa e Silva et al. (16) found that low water availability led to a general decrease in growth which was reflected in reductions in total biomass, leaf area, number of branches and total root length.

Photosynthesis, together with cell growth, is one of the primary processes to be affected by drought (43). The effects can be directly, parallel to the decrease of CO₂ availability, caused by diffusion limitations through the stomata and the mesophyll (44) or the alterations of photosynthetic metabolism (45). Photosynthetic responses to drought stress are highly complex because stomata react and adjust continuously to a complex set of factors ranging from light intensity to CO₂ concentration in addition to leaf water status (21). Many authors report a decrease in gas exchange parameters in *Eucalyptus*: Guarnaschelli et al. (46) showed that *E. globulus* seedlings exposed to water deficit exhibited a rapid decline in stomatal conductance (g_s). Imposition of drought stress in five species of *Eucalyptus* led to declines on stomatal conductance (g_s), photosynthesis (A) and transpiration rates in all the species (47).

Water relations and osmolyte accumulation

Cell enlargement is directly dependent in the entry of water into plant tissue (48). 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 (48), what explains an increase in the plant water potential under water deficit conditions. Increasing water potential in response to drought has been confirmed in wide reports (40, 49, 50). Water deprivation showed to affect two clones of *E. globulus* plants, presenting lower water potential (15). Also, Guarnaschelli et al. (46) showed that *E. globulus* plants under water deficit exhibited a significantly decrease in water potential.

Osmotic adjustment in terms of accumulating compatible solutes has been considered an important physiological adaptation for plant to resist drought (51). This facilitates extracting water from dry soils and maintaining cell turgor, gas exchange and growth in very dry environments (21, 52). Besides their roles in osmotic adjustment, compatible solutes may protect membranes from damages induced by stress conditions and stabilize the structures and activities of proteins and enzymes (52). Soluble sugars are one of the most important compatible solutes in plants. As described by many authors, drought-stressed plants accumulate the total concentration of soluble sugars in the leaves as an important physiological adaptation to minimize the detrimental effects of water deficits (53-55).

Photosynthetic pigments

Photosynthetic pigments are important to plants mainly for harvesting light complexes and production of reducing powers (56). Chlorophylls are the major chloroplast components for photosynthesis, and the relative chlorophyll content presents a positive correlation with photosynthetic rate (57). The decrease in chlorophyll content under drought stress has been considered a typical symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation (57). Carotenoids are also essential components of the photosynthetic membranes and serve an extraordinary variety of function 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 ABA (58).

Under unfavourable 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 (59). However, Michelozzi *et al.* (60) revealed that drought-stressed eucalyptus presented increases in total chlorophyll concentration with increasing water deficit, reaching a maximum at stomatal closure and then declined only slightly with intensifying stress (60). Increasing total chlorophyll and carotenoids concentrations in drought condition were also reported by Correia *et al.*(15).

Oxidative stress

Drought stress induces oxidative stress in plants by generation of reactive oxygen species (ROS) that can directly attack biological structures and induce lipid peroxidation (61). The overproduction of ROS induced by drought leads to the accumulation of malondialdehyde (MDA) which is considered as a suitable marker for membrane lipid peroxidation (57). A decrease in membrane stability reflects the extent of lipid peroxidation caused by ROS (57). Correia *et al.* (15) showed that *E. globulus* exposed to different levels of water deficit exhibited an increase in the content of MDA. Other authors support this observations, thereby that Xu and Zhou (62) in a study testing the effects of water stress and high temperature in *Leymus chinensis* showed that lipid peroxidation levels increases in severe and extreme water deficit. Farooq *et al.* (63) also has pointed out the accumulation of MDA as indicator of lipid peroxidation in plants under drought stress.

Plants typically produce a diverse group of antioxidants as a protective mechanism against oxidative compounds which are produced in response to numerous stresses (drought, heat, light, salinity, etc) and known to have a damaging effect on membranes, organelles and macromolecules (61). A significant part of antioxidants produced by plants in response to stress is secondary metabolites, including a vast array of simples and complex phenolic compounds

(flavonoids, anthocyanin, carotenoids, and phenolic acids) derived primarily via the phenylpropanoid pathway. Phenolic compounds possess ideal structural chemistry for radical-scavenging activities and have been reported to be effective in a lot of works (64, 65). Ayaz and Turgut (66) demonstrated that water stress induced the increase of phenolic acids in *Ctenanthe setosa* (Rosc.) Eichler.

Hormones signalling

Plants respond to exogenous stimuli, including drought, by modulation of their behaviour, which is partially mediated by plant growth regulators (PGRs). PGRs are compounds derived from plant biosynthetic pathways and can either at the site of synthesis or following their transport, elsewhere in the plant (34). Understanding of the role of different hormones could improve the choice of a suitable strategy for enhancement of plant stress tolerance (67). PGRs like ABA, jasmonic acid (JA), auxins, salicylic acid (SA), cytokinins (CKs) and gibberellins (GAs) amend the plant response towards drought stress (33).

ABA and JA are known to play major roles in regulating plant defence responses against abiotic stress, by mediating a wide range of adaptive responses (34). ABA has been shown to form part of a complex signaling network, which mediates the physiological changes in *E. globulus* under drought stress (15, 68). There is increasing evidence that jasmonic acid (JA) and jasmonates are also crucial signalling molecules involved in many plant responses to abiotic stress (69-71). CKs and auxins are plant hormones predominantly associated with stimulation of cell division and control of plant growth and development and play important role in stress responses (72, 73). Indole-3-acetic acid (IAA) is one of the predominant naturally occurring forms of auxins in plants (74). An increased level of IAA has been reported in leaves of cucumber and in hypocotyls of squash in response to water stress (75, 76). SA is a phenolic compound with key roles in a broad range of physiologic and metabolic responses in plants (77). Under drought, higher endogenous SA content was observed in leaves of *Phillyrea angustifolia* L. plants (78) and in roots of barley plants (79). GAs are a class of plant hormones that exert profound and diverse effects on plant growth and development (80). In response to water stress, reduced accumulation of GAs has been observed in some dicots but not in others (81, 82).

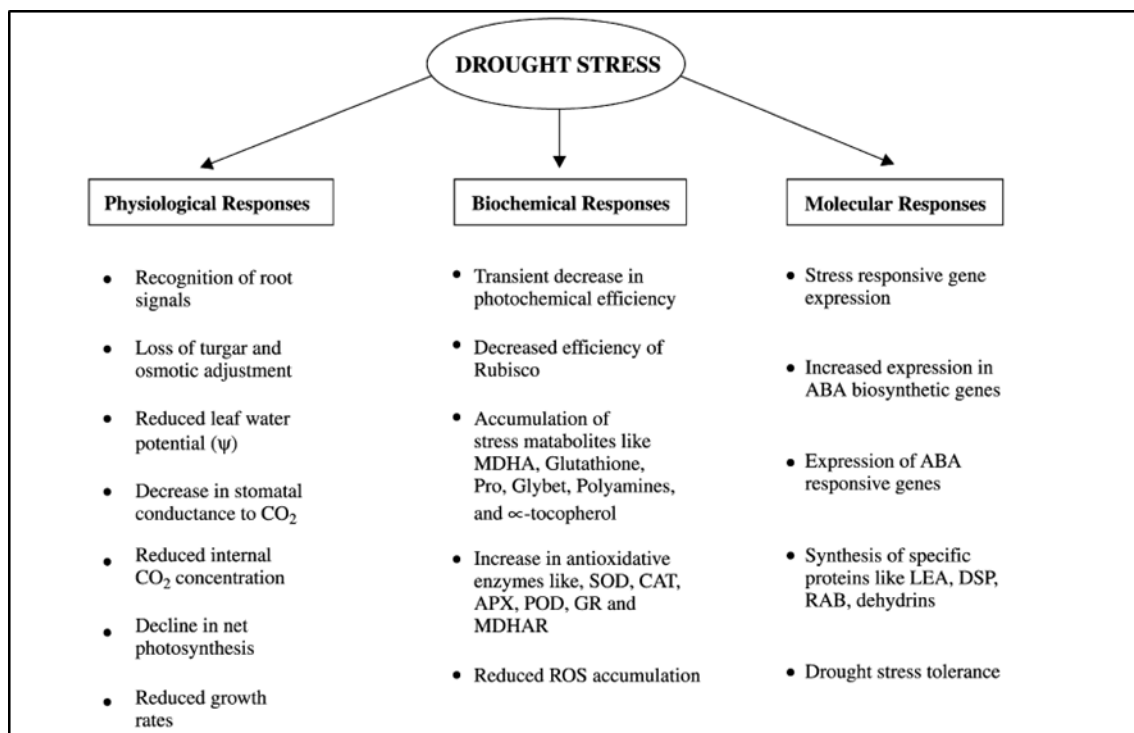


Figure 2 - Physiological and molecular basis of drought stress tolerance (adapted from Reddy *et al.* (59)).

Exogenous application of salicylates as a strategy to improve drought tolerance

Great efforts have been made with the purpose of developing mechanisms that help plants to counteract the adverse effects of drought (83-85). One of those mechanisms is the exogenous application of PGRs to the plants. Many studies have shown that the exogenous application of SA is an 'effective therapeutic agent' for plants, just like in mammals (79, 86-88). Plants are one of the world's richest sources of natural medicines (89). One of the main

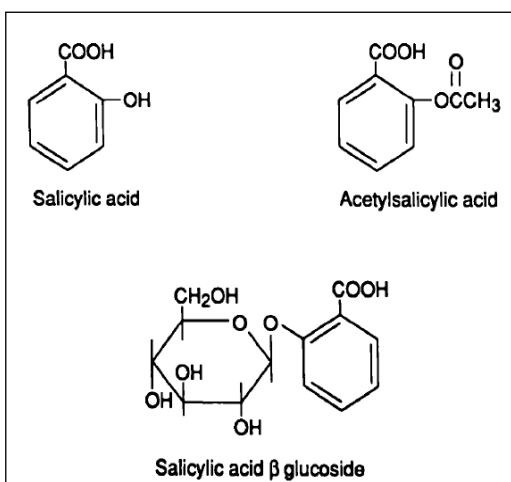


Figure 3 – Chemical structure of salicylic acid and several of its derivatives (adapted from Malamy and Klessig (89)).

historical discoveries in the pharmaceutical field related to the use of extracts from plants is the aspirin, a synthetic derivative of SA (Fig. 3) (90).

A wide-ranging of prokaryotic and eukaryotic organisms including plants produce SA (91). Chemically, SA or *ortho*-hydroxybenzoic acid belongs to a wide variety of phenolic compounds synthesized by plants that possess an aromatic ring with a hydroxyl group or its functional derivate (88) (Fig. 3). Free SA is a crystalline powder, moderately soluble in water but highly soluble in polar organic solvents (92).

The physical properties of SA suggest that it could be actively transported, metabolized or conjugated in the plants. Moreover, the exogenously applied SA seems to be carried away from the sites of its initial application to different other tissues of the plants to generate response (93).

SA is a natural derivative of chorismic acid, an intermediate in shikimic acid pathway, operative for the synthesis of phenolic compounds in plants. However, SA can be synthesized through two possible and distinct routes: the phenylpropanoid and the isochorismate pathways (Fig. 4) (90, 94). In the phenylpropanoid pathway that occurs in the cytoplasm, SA is synthesized from cinnamic acid by two pathways. One involves the decarboxylation of side chain of cinnamic acid to generate benzoic acid that undergoes hydroxylation, at C-2 position to SA. Alternatively through *o*-coumaric acid occurs the hydroxylation of cinnamic acid to *o*-coumaric acid followed by its decarboxylation to SA (92, 95). Thus, the difference between the two routes is the hydroxylation of the aromatic ring before or after the chain-shortening reactions. The isochorismate pathway is pathogen-induced and occurs in the chloroplast. In this route, the chorismic acid is converted to SA in a two-step process involving isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) (90, 94).

It is well documented that SA as a PGR (95) exert their influence on physiological and biochemical processes including ion uptake and photosynthesis (96, 97), membrane permeability, enzyme activities, flowering, heat production, growth and development of plants (93). However, its effect on some of these processes may be indirect because SA can modify the synthesis and/or signalling of other plant hormones including JA, ethylene (ET) and auxin (90). SA is also considered as a signalling molecule in plant immune response (90), mediating the local and systemic plant defence responses to pathogens (94, 98). Studies in plant species have shown that pathogen infection leads to SA accumulation not only in infected leaves but also in uninfected leaves that develop systemic acquired resistance (SAR) (99). In recent years, exogenous application of SA has also received much attention due to its role in plant responses to abiotic stresses such as heat (100), oxidative stress (101) salt and osmotic stress (102) and drought (87).

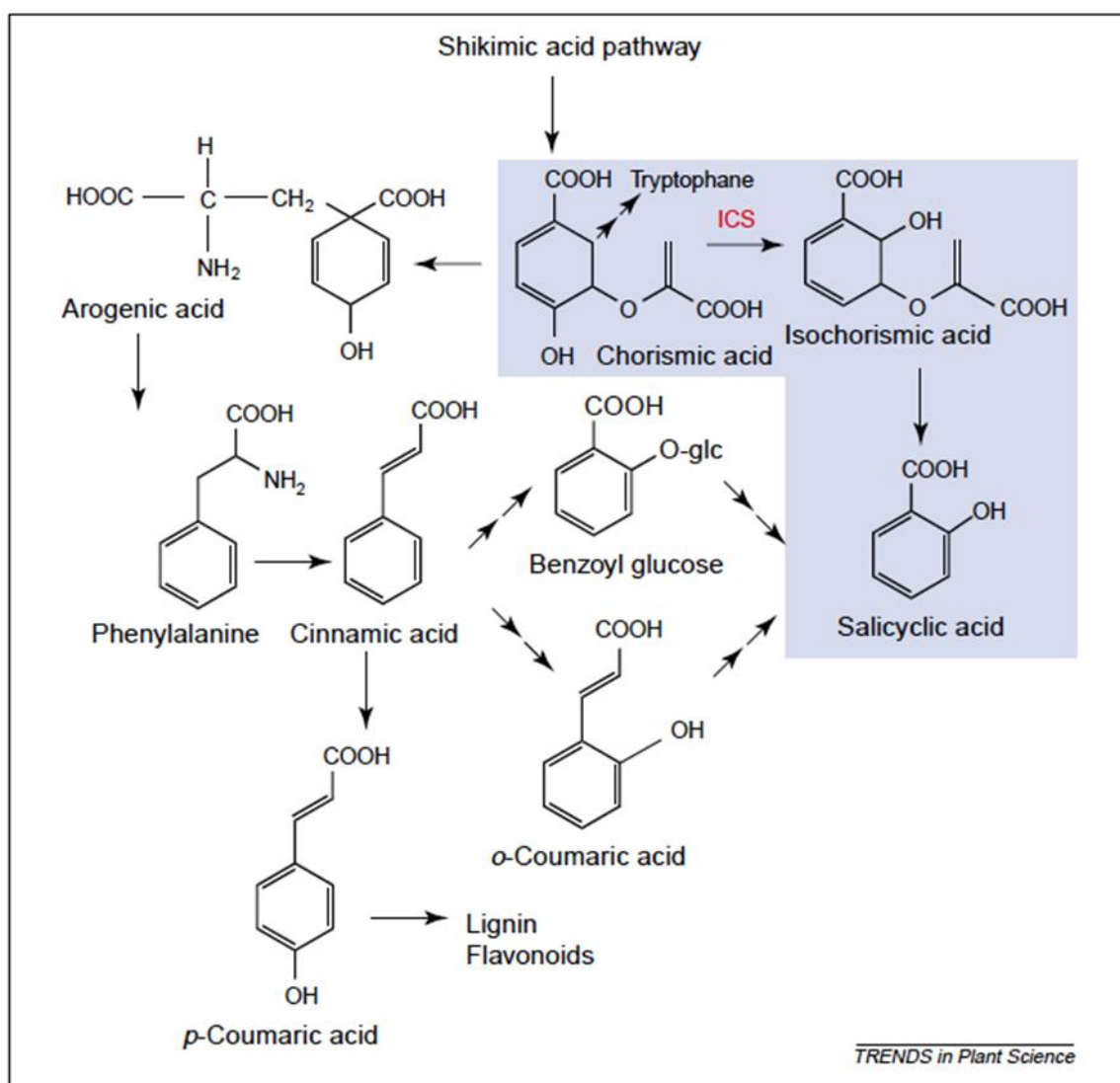


Figure 4 – The biosynthetic pathways for salicylic acid biosynthesis. The part highlighted in blue was originally described in bacteria and has now be shown to take place in the chloroplast of plants. Abbreviation: ICS, isochorismate synthase (adapted from Métraux 2002).

The results reported by Singh and Usha (87) revealed that wheat seedlings treated with SA and subjected to water stress exhibited, in general, a higher moisture content, carboxylase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), superoxide dismutase (SOD) activity and total chlorophyll content compared to the untreated seedlings. Exogenous application of salicylic acid also alleviated the damaging effects of water deficit on cell membranes of barley plants and concomitantly increased the ABA content in leaves, which might have contributed to the enhanced tolerance of plants to drought (79). Hayat *et al.* (88) evaluated the influence of salicylic acid application in the *Lycopersicon esculentum* L. (tomato) plants exposed to water stress. The results of their experiments revealed that SA enhanced significantly the photosynthetic parameters, membrane stability index, leaf water potential, chlorophyll and relative water contents and the level of antioxidant system (catalase, peroxidase,

SOD and proline) under water stress and well-watered conditions. Rao *et al.* (103) also conducted a similar study to perceive the protective role of different SA treatments (100, 150 and 200 ppm) in maize plants under water deficit. The authors concluded that relative water content, leaf membrane stability index, chlorophyll and potassium content were significantly higher in plants treated with 100 ppm compared with other treatments of salicylic acid and control plants.

Besides providing tolerance against drought stress, the exogenous application of SA also increased the plants' sensitivity to drought. Németh *et al.* (104) investigated if SA induced drought tolerance in maize and wheat plants under the same conditions that it induced chilling tolerance. The authors verified that the same SA pre-treatment, which increased the chilling tolerance, caused a dramatic increase in the electrolyte leakage and a decrease in certain photosynthetic parameters in maize and wheat. In another study, an experiment was conducted to assess whether exogenously applied SA through the rooting medium could mitigate the adverse effects of water stress in wheat plants. Although exogenous application of SA have had growth promoting effects under well-watered conditions, it did not mitigate the adverse effects of drought stress on growth (105).

In spite of the diverse physiological roles of SA in plants, as well as its potentially alleviating effect on the devastating damages generated by drought stress, the exact mode of action of SA still requires a lot of work to be carried out (77). One of the main reasons for this is the common inconsistency of the effects of salicylic acid on plant resistance to abiotic stress. The efficiency of the SA action also depends on the developmental stage of the plant, the method of application and the concentration of SA (106). As already discussed, the same pre-treatment with exogenous SA results in opposite responses in different plant species (107). Even the same SA concentration that promotes resistance to one kind of stress may decrease the resistance to another stress (104).

Thesis main purposes

Considering the crucial importance of *Eucalyptus globulus* plantations in Portugal, it becomes essential to develop mitigation strategies that improve the performance of the *Eucalyptus* planted forests. Currently, there has been a major effort to unravel the role played by PGRs in alleviating the negative impact of drought stress on plants' physiology and yield (33). SA is an endogenous regulator of growth involved in a wide range of physiological and metabolic response in plants (77). Although exogenous application of SA to plants has been considered a short-term solution to ameliorate the adverse effects of drought stresses on plants (87), the exact mode of action of SA still remains unclear (77). In addition, research that combines SA application, drought stress and *Eucalyptus* species is scarce. Bearing this in mind,

the main objective of this work is to investigate whether SA can effectively ameliorate the negative effects of drought stress on *E. globulus* plants. Here, I present a research work that assessed water status, lipid peroxidation, pigments content, total soluble sugars, phenols, F_v/F_m , gas exchange on *Eucalyptus globulus* plants pre-treated with different SA levels and exposed to drought stress. Moreover, I hypothesized that hormonal dynamic variations explain drought stress tolerance. For this reason, I proceeded to the simultaneous quantification of multiple classes of phytohormones by liquid chromatography-mass spectrometry system (HPLC/MS). For better understanding of the role of IAA and ABA during stress, changes in their distribution in tissues were visualized by immunolocalization.

PART II

Salicylic acid and Drought tolerance improvement in *Eucalyptus globulus*

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Abstract

Covering approximately 20 million ha, *Eucalyptus* genus is the most widely planted hardwood trees all over the world. In the Mediterranean region, the positive performance of *Eucalyptus* plantations is conditioned by low water availability that strongly limits forest productivity and alters plant growth and development. Plant drought tolerance can be managed by adopting strategies such as exogenous application of hormones. Salicylic acid (SA) is a plant endogenous regulator of growth (PGR) that has been reported as a compound playing a key role in plants responses to drought. In this study, we investigated if the exogenous application of SA improves drought tolerance on *E. globulus* and how this treatment regulates plant response to stress. SA was applied by foliar application of 0.75, 2.5 and 5 mM solution of sodium salicylate three consecutive days before water-stress (WS) induction (plants daily watered at 15% field capacity). Additionally a well-watered group (WW, daily watered at 70%) was analysed, with the same SA pre-treatments. Control plants (WW and WS) were not treated with SA. The assessed morpho-physiological and biochemical traits were: water potential, lipid peroxidation, pigments content, total soluble sugars, phenols, F_v/F_m , gas exchange and contain of different PGRs (abscisic acid, ABA; indolacetic acid, IAA; dihydrozeatin riboside, DHZ; gibberellin GA₄; isopentenyl adenine, IP; jasmonic acid, JA; salicylic acid, SA). Specific local dynamics of IAA and ABA in leaves was detected by their immunolocalization. The results showed that drought stress severely affected the plant physiology. On the other side, the performance of plants under water-stressed conditions showed a significant improvement after the foliar application of SA. Global quantification of PGRs didn't show great differences between treatments, with the exception of SA, however local distribution of IAA and ABA in leaves was clearly affected. The efficiency of exogenous SA depended on the applied dose, with 5 mM

being more effective to induce the drought tolerance. These positive effects were highlighted in water potential, gas exchange (CO₂ assimilation rate) and lipid peroxidation. In the current global changes SA treatment could be very useful in breeding forest programs and can effectively ameliorate the negative effect of drought stress in *Eucalyptus* plants.

Keywords: Forest, *Eucalyptus globulus*, drought, salicylic acid, physiology, plant hormones, immunolocalization

Abbreviations

A, foliar photosynthetic rate; **ABA**, Absciscic acid; **Ci**, intercellular CO₂ concentration; **DHZ**, dihydrozeatin riboside; **E**, foliar transpiration rate; **F₀**, minimum fluorescence; **F_m**, maximum fluorescence; **Fv/Fm**, maximum quantum yield of PSII photochemistry; **g_s**, stomatal conductance; **HLPC/MS**, liquid chromatography-mass spectrometry system; **GA4**, gibberellin; **IP**, isopentenyl adenine; **JA**, jasmonic acid; **SA**, salicylic acid; **IAA**, indole-3-acetic acid; **MDA**, malondialdehyde; **PPFD**, photosynthetic photon flux density; **WS**, water stressed; **WW**, well-watered; **Ψ_{md}**, midday water potential.

Introduction

Eucalyptus genus is the most widely planted hardwood trees all over the world (1) due to their large number of species, wide adaptability to soils and climates, fast-growing rates, the wide knowledge and technology for their culturing and the variety of wood and non-wood products that come from them (2). The total area of *Eucalyptus* plantations is estimated to cover approximately 20 million ha (3). In the temperate and Mediterranean climates, which present dry hot summers with high sunlight irradiance and low water availability (4), fast-growing forestry species have developed a relevant role in productivity and afforestation plans (5). *Eucalyptus globulus* (Labill.) is considered to produce high quality pulp and is a suitable species for temperate areas (6). In Portugal and Spain, *E. globulus* has been planted in approximately 800 000 ha (7) and 500 000 ha (8), respectively.

Since drought stress is one of the most important environmental stresses that affect the establishment and limits forest productivity of *Eucalyptus* plantations (9), new insights and deep studies drive to understand the forest drought tolerance are essential. Drought tolerance is a complex process that involves a large number of morpho-physiological, biochemical and molecular responses (10). Almost every response to water deficit has a metabolic cost (11). The effects of drought are manifested by leaf growth inhibition and stomatal closure, which limit gas exchange, resulting in reduced transpiration and photosynthesis (10) and, consequently, reduced whole plant carbon assimilation and productivity (10, 12). Ecophysiological studies in

Eucalyptus species have increasingly showed the effects of drought stress. These include: changes in biomass allocation (13), accumulation of soluble sugars (14), decrease of water potential (15), stomatal closure (12), cell wall reinforcement and water storage (16), changes in antioxidants and antioxidant enzymes, chlorophylls and carotenoids (17, 18), increase of lipid peroxidation and membrane deterioration (15).

Perception of stress signals often results in hormonal dynamics variations, to adapt and respond to environment changes (19). Hormones such as abscisic acid (ABA), jasmonic acid (JA), cytokinins (CKs), auxins, salicylic acid (SA) and gibberellins (GAs) can modulate the plant responses towards drought. ABA and JA are known to play major roles in regulating plant defence responses against abiotic stress, by mediating a wide range of adaptive responses (20). ABA has been shown to form part of a complex signaling network, which mediates the physiological changes in *E. globulus* under drought stress (15, 21). There is increasing evidence that jasmonic acid (JA) and jasmonates are also crucial signalling molecules involved in many plant responses to abiotic stress (22-24). Cytokinins (CKs) and auxins are plant hormones predominantly associated with stimulation of cell division and control of plant growth and development and play important role in stress responses (25, 26). Indole-3-acetic acid (IAA) is one of the predominant naturally occurring forms of auxins in plants (27). An increased level of IAA has been reported in leaves of cucumber and in hypocotyls of squash in response to water stress (28, 29). SA is a phenolic compound with key roles in a broad range of physiologic and metabolic responses in plants (30). Under drought, higher endogenous SA content was observed in leaves of *Phillyrea angustifolia* L. plants (31) and in roots of barley plants (32). Gibberellins are a class of plant hormones that exert profound and diverse effects on plant growth and development (33). In response to water stress, reduced accumulation of GAs has been observed in some dicots but not in others (34, 35).

Among mitigation strategies to counteract drought effects, the role played by plant growth regulators (PGRs) in alleviating the negative impact of drought stress on plants yield has been investigated (36). In the last years, salicylic acid (SA) has been studied as a signal molecule mediating local and systemic defence responses against pathogens (19). This compound was also reported as playing a role in plants responses to abiotic stress, such as drought (32, 37, 38). SA also has been shown to take part in several physiological processes including nutrients uptake and photosynthesis, membrane permeability, enzyme activities, flowering, heat production, growth and development of plants (39-41). These studies suggested that SA enhance multiple types of stress tolerance in plants; it was reported that SA cause interactive effects on several functional molecules or other signalling molecules, participating in more complex responses (42). The efficiency of exogenous SA action depends on the developmental stage of the plant, the method of application and the concentration of SA (43).

Although exogenous application of SA has been considered a short-term solution to improve the adverse effects of drought stresses on plants (37), the precise mode of SA action remains unclear (30). Research that combines SA application and drought stress in *Eucalyptus* species is scarce. Bearing this in mind, the main objective of this work is to investigate whether foliar application of SA can effectively ameliorate the negative effects of drought stress on *E. globulus* plants. Several physiological and biochemical parameters, such as water status, lipid peroxidation, pigments content, total soluble sugars, phenols, F_v/F_m , gas exchange and stomata conductance, were assessed. Moreover, we hypothesized that hormonal dynamic variations explain drought stress tolerance. For this reason, we proceeded to the simultaneous quantification of multiple classes of phytohormones by liquid chromatography-mass spectrometry system (HPLC/MS). For better understanding of the role of IAA and ABA during stress, changes in their distribution in tissues were visualized by immunolocalization in plants exposed to water deficit.

Material and Methods

Plant material and experimental design

The experiment using 1L plastic pots filled with 3:2 (w/w) peat:perlite and under different water treatments was conducted in the controlled climate chamber (Fitoclima 1200, Aralab, Portugal), with a temperature of 20 °C, a 16/8-h (day/night) photoperiod and a photosynthetic photon flux density (PPFD) of app. 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Eighty rooted cuttings of *E. globulus* (clone AL-18) with 2-months-old were obtained from the breeding program of Altri Florestal SA (Portugal). The potted cuttings were acclimatized during one-month period inside the climate chamber being watered with nutritive solution (N:P:K). The pots were randomly arranged and periodically moved to the neighbouring position during the whole experiment and well-watered to 70% of field capacity, prior to foliar application of SA. SA was administered to *E. globulus* plants by foliar spraying with 100 mL of 0.75, 2.5 and 5 mM SA solution (obtained from previous studies) for three consecutive days before experimental set up. Sodium salicylate (Merck, Darmstadt, Germany) was used to prepare the SA solutions (adjusted to pH 7.0 with NaOH solution) dissolved in distilled water with the addition of 0.1 % Tween® 20 (Sigma-Aldrich, Missouri, USA). Control plants for SA treatment (0 mM) were sprayed with distilled water containing 0.1 % Tween® 20. After the last application of SA, half of the plants of each treatment were randomly assigned to each of the two water treatments as follows: (1) well-watered (WW): water supplied every evening until soil water content reached around 70% field capacity, and (2) water stress (WS): water supplied every evening until soil water content reached around 15 % field capacity. After two weeks of stress exposure, the followed

parameters were recorded: growth, leaf gas exchange, chlorophyll fluorescence and water potential. Leaves were harvested, cleaned with moistened cloth and immediately frozen in liquid nitrogen for further biochemical analysis (estimation of lipid peroxidation, photosynthetic pigments content, total soluble sugars and total phenolic compounds and phytohormones quantification). Further immunolocalization of IAA and ABA samples sections of leaves were fixed.

Morphological aspects, growth and plant water potential

The morphological aspect of plants was evaluated in the end of experiment by visual observations in order to assess plant quality. To assess growth the height of six plants were recorded for each treatment. Midday shoot water potential (Ψ_{md}) was measured with a Scholander-type pressure chamber (PMS Instrument Co., OR) in six plants per treatment at 12h30m (solar time) as described by Correia *et al.* (15).

Leaf gas-exchange measurements

Stomatal conductance (g_s , mmol H₂O m⁻² s⁻¹), transpiration rate (E , mmol H₂O m⁻² s⁻¹), intercellular CO₂ concentration content (C_i , ppm) and photosynthesis (A , μ mol CO₂ m⁻² s⁻¹) were measured in six plants using a portable infrared gas analyser (LCpro-SD, ADC BioScientific Ltd., UK) equipped with the universal photosynthesis chamber. The following conditions were maintained inside the chamber during all the measurements: C_a (ambient CO₂ concentration): 350 μ l L⁻¹; air flux: 500 μ mol s⁻¹; block temperature: 25 °C; 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 CO₂ assimilation) curves were performed with the following PPFD: 2500, 2000, 1500, 1000, 750, 500, 250, 100, 50 and 0 μ mol m⁻² s⁻¹. After A/PPFD data analysis, punctual measurements at saturation light intensity were performed at 750 μ mol m⁻² s⁻¹. Data were recorded when the measured parameters were stable (2–6 min).

Chlorophyll fluorescence and photosynthetic pigments analysis

Chlorophyll fluorescence was measured on the same leaves as used for the gas-exchange measurements, using a portable fluorometer (Mini-PAM; Walz, Effeltrich, Germany). The maximum quantum yield of PSII photochemistry (F_v/F_m) was estimated as described by Alves *et al.* (44),

$$F_v/F_m = (F_m - F_0) / F_m \quad (1)$$

where F_0 and F_m are the minimum and the maximum chlorophyll fluorescence yields in the dark-adapted state, respectively.

Total chlorophyll and carotenoid content was quantified according to Sims and Gamon (45). 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, 537, 647 and 470 nm (Thermo Fisher Scientific Spectrophotometer, Genesys 10-uv S) and pigments' content was determined.

Lipid peroxidation

Lipid peroxidation on leaves was estimated by measuring the amount of MDA (malondialdehyde) by the method described by Hodges *et al.* (46), which takes into account the possible influence of interfering compounds in the assay for thiobarbituric acid (TBA)-reactive substances. Briefly, samples were extracted with 2.5 mL of TCA (trichloroacetic acid) 0.1 % and 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 °C 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). MDA content was estimated by the formulae presented by Hodges *et al.* (46)..

Total soluble sugars quantification

Total soluble sugars (TSS) were determined by using the anthrone method. TSS were quantified as described by Irigoyen *et al.* (47). Briefly, total soluble sugars extraction from frozen leaves was performed using 80 % (v/v) ethanol at 80 °C for 1h. After centrifugation, the supernatant was mixed with 1.5 ml of anthrone and incubated at 100 °C during 10 min. Absorbance was read at 625 nm and TSS content was calculated against a D-glucose standard curve.

Total phenolics quantification

Total phenolic compounds were determined according to the protocol used by Singleton and Rossi (48). About 50 mg of frozen plant material were extracted with 80 % (v/v) cold acetone and the mixture was centrifuged. Folin-Ciocalteu's phenol reagent and 7.5 % (w/v) sodium carbonate were added to the supernatant and the mixture was kept at room temperature for 30 min. Absorbance was read at 765 nm and total phenolic concentration was determined according to a gallic acid standard curve.

Hormones quantification

The analysis of different PGRs (abscisic acid, ABA; indolacetic acid, IAA; dihydrozeatin riboside, DHZ; gibberellin GA₄; isopentenyl adenine, IP; jasmonic acid, JA; salicylic acid, SA) was carried out by a modified protocol based on Pan *et al.* (49), briefly: 60 mg of lyophilized tissue were ground into powder and 500 µl of 2-propanol/H₂O/concentrated HCl (2:1:0.002, v/v/v) with internal standards (10-40 ng) were added, followed by agitation for 30 min at 4 °C. CH₂Cl₂ (1 mL) was added followed by another 30 min of agitation at 4 °C. Two phases were formed with the plant debris between them. The lower layer was collected, concentrated in 2 ml glass vials with nitrogen flow and stored until analysis at -20 °C.

Samples were re-suspended in 200 µL of methanol (MeOH) 100% and filtered through a 0.2 µm regenerated cellulose filter (Agilent Technologies) filled with SiO₂ (15 mg). All the compounds were separated and quantified by an ultra-high performance liquid chromatography (UHPLC) in a 6460 Triple Quad LC/MS (Agilent Technologies) using the protocol described by Novak *et al.* (50) for cytokinins and performed for the plant growth regulators analyzed. A chromatographic separation was made using a reverse phase column (Zorbax SB-C18 2.1 x 50 mm column). The column was held at 40 °C and the mobile phase used in the chromatography consisted of (A) 99.9 % MeOH: 0.1 % COOH and (B) ammonium formate (10 mM, pH 4). A linear gradient of MeOH from 10 % to 50 % and then reaching 100 % in 7 and 2 minutes, respectively, was used to analytical elution. PGRs were quantified by dynamic multireaction monitoring (MRM) of their [M+H]⁺ and the appropriate product ions, using optimized cone voltages and collision energies for diagnosis of each PGRs analyzed.

Immunolocalization

To immunolocalization of IAA and ABA, leaves from 0 mM well-watered and all water-stressed (0mM, 0.75mM, 2.5mM and 5mM) treatments, were sampled and immediately fixed according to the method described by Meijón *et al.* (51) with some modifications. The tissues were fixed for 24h in 3% (w/v) paraformaldehyde containing 0.1% (v/v) Triton X-100 (Sigma-Aldrich Co., St Louis, MO, USA) at 4 °C. To the mixture was also added 4% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich Co., St Louis, MO, USA) to immobilize IAA and ABA by covalent binding proteins. After 24h, samples were washed three consecutive times for 10 min each in phosphate-buffered saline [PBS(137mM NaCl, 2.7mM KCl, 7.9mM Na₂HPO₄ and 1.5mM KH₂PO₄, at pH 7.3)] to remove the fixing solution. Finally, samples were stored in PBS containing 0.1% (w/v) paraformaldehyde at 4 °C. Then, samples were introduced in a cryostat medium (Tissue-Tek, Killik; Sakura Finetek USA, Inc., Torrance, CA, USA) and were frozen at -23 °C. Finally, sections of 50 µm were cut with a sliding cryotome CM1510S (2002 Leica Microsystems, Wetzlar, Germany), collected on slides and

conserved at 4 °C until the analysis. Sections were immersed for 5 min in ascending and descending 25, 50, 75 and 100% ethanol series, washed for 30 min in PBS containing 0.1% (v/v) Tween 20, and finally for 5 min in PBS. Before incubating overnight with the ABA or IAA primary antibody (polyclonal Agrisera AB, Vännäs, Sweden), samples were pre-treated with 5% (w/v) BSA in PBS for 30 min to reduce non-specific binding. After washing twice with 0.1% (v/v) Tween 20 in PBS for 10 min, sections were incubated with Alexa 488 (Molecular Probes, Göttingen, Germany) as a secondary antibody for 1 h in darkness. Samples were washed twice for 10 min with 0.1% (v/v) Tween 20 in PBS. Finally the slides were counterstained with DAPI (4', 6-diamidino-2-phenylindole; Fluka). Sections were washed in MilliQ water, and assembled on the slides with Mowiol (Sigma-Aldrich Co., St Louis, MO, USA). In both immunochemical detection (ABA and AIA) the negative controls were obtained replacing the primary antibody by PBS. Fluorescence was visualized using a confocal microscope (Leica TCS-SP2-AOBS) connected to a workstation and the images were processed with Fiji Software (52).

Statistical analysis

The results presented are the mean with standard deviation of six to ten independent replicates. Data are presented as mean \pm SD (standard deviation). All statistics procedures were performed using SigmaPlot (SigmaPlot for Windows v. 11.0, Systat Software Inc.). Two-way analysis of variance (ANOVA) followed by post-hoc multiple comparisons using Holm-Sidak test was employed to estimate the significance of the results, except for hormones quantification ($p \leq 0.05$). In the latter case, a Student's t-Test was carried out to assess significant differences between WW and WS in non-treated plants. To find out significant differences in the WS plants treated with different SA concentrations, a one-way ANOVA was used, followed by post-hoc Tukey test. Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

Principal components analysis (PCA) was carried out to explore the morpho-physiological profile of *E. globulus* plants of WW and WS control plants and SA treated WS plants, by reducing the multivariate data matrix to an interpretable bidimensional biplot that explains the highest proportion of variation of the data (53). SA treatments under WW condition were excluded from PCA because these parameters were not quantified to phytohormones and confocal. Data were *a priori* centered and standardized to reduce scale effects (53). PCA was conducted with the R programming language running under the open-source computer software RStudio: Integrated development environment for R (RStudio Boston, MA. Available from <http://www.rstudio.org/>

Results

Morphological aspects, growth and plant water status

At morphological level, all SA pre-treated plants showed local signals of damage in leaves that were more evidence in higher concentration (fig. 1).

Exposure of plants to water deficit for two-weeks led to a general decrease in growth that was reflected in reductions in height, except for the plants sprayed with 5 mM SA treatment (fig.2, table 1). *E. globulus* plants sprayed with 5 mM presented similar height in both water regimes (fig. 2, table 1). Independently of SA treatment, Ψ_{md} of well-watered plants remained identical while the plants under WS showed a decrease of the Ψ_{md} values (fig. 3). However, under WS, plants without SA application (0 mM) were significantly more affected than the plants sprayed with SA (fig. 3, table 1). Moreover, plants with 5 mM SA treatment presented less negative water potential values under drought (fig. 3).



Figure 1 – Morphological aspect of *Eucalyptus globulus* after SA foliar application: a) 0 mM; b) 0.75 mM; c) 2.5 mM; d) 5.0 mM.

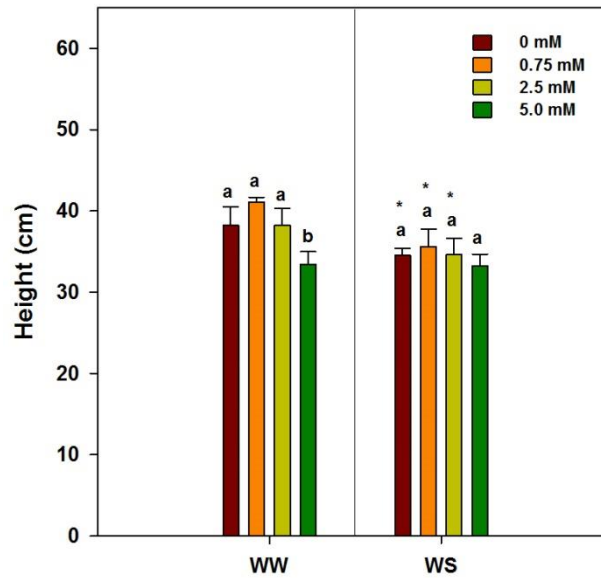


Figure 2 - Height in well watered (WW) and water stressed (WS) plants of *E. globulus* after two-week water stress period. Data are presented as mean \pm SD. Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

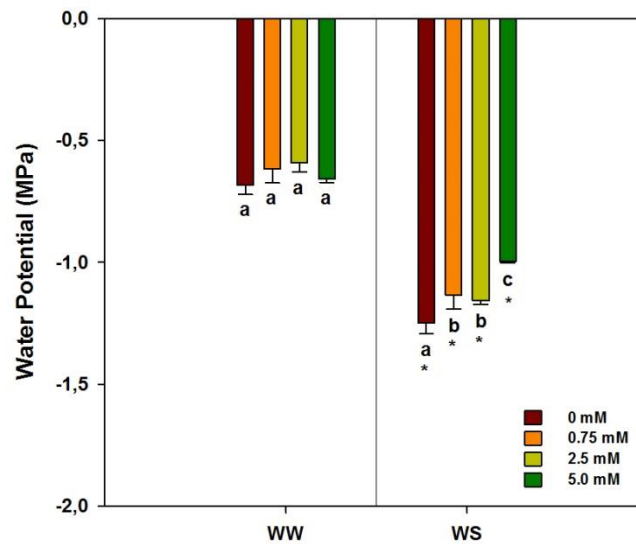


Figure 3 - Midday water potential (Ψ_{md}) in well watered (WW) and water stressed (WS) plants of *E. globulus* after two-week water stress period. Data are presented as mean \pm SD. Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

Photosynthetic pigments and chlorophyll fluorescence

The total chlorophyll and carotenoid content concentrations were significantly higher in water stressed plants than in well-watered plants, except for plants sprayed with 5 mM SA (fig.

4). Under WW condition, total chlorophyll content was greater in the 5mM SA treatment than in the plants control and others SA treatments (fig. 4, table 1). In WS condition, plants treated with 0.75 mM showed more responsive, presenting higher total chlorophyll values than other treatments (fig. 4, table 1). Carotenoid concentration in WW condition diminished as the concentration of SA increased, reaching control values at 2.5 and 5 mM treatments (fig. 4, table 1). In the water-stressed plants, carotenoid concentration was significantly lesser in varying SA treatments compared to control plants. In this case, plants without application of SA, which presented higher concentration of this pigment, and 5 mM were the concentrations less responsive to drought (fig. 4, table 1). In the F_v/F_m ratio there were no statistically significant differences between water treatments neither in plants with or without SA treatment (fig. 5, table 1).

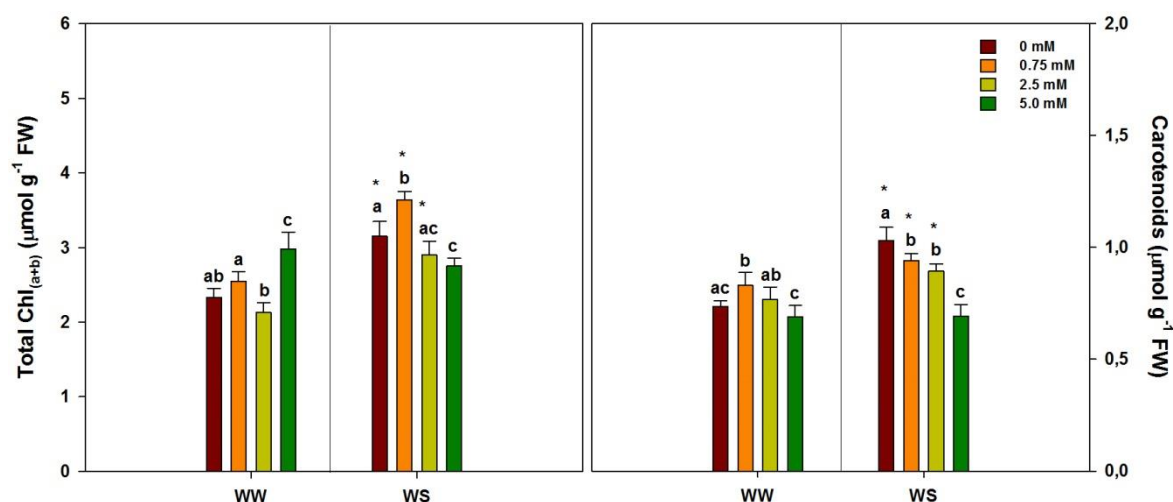


Figure 4 – Total chlorophyll and carotenoids content in well watered (WW) and water stressed (WS) plants of *E. globulus* after two-week water stress period. Data are presented as mean \pm SD. Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

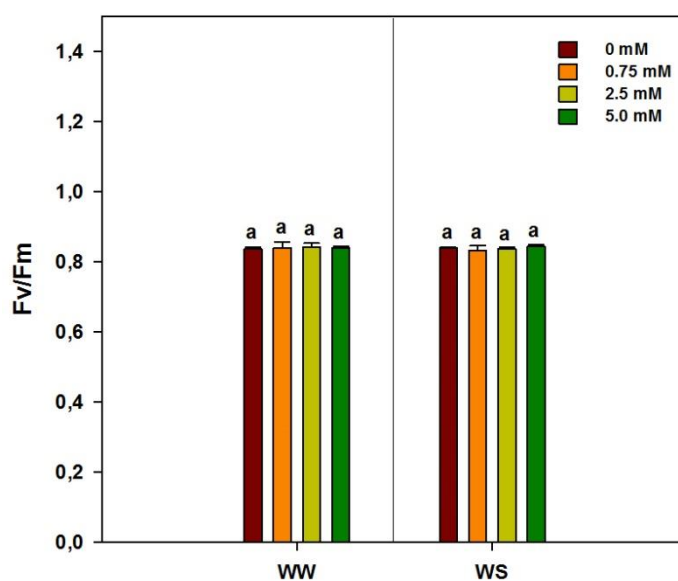


Figure 5 – Fv/Fm ratio in well-watered (WW) and water stressed (WS) plants of *E. globulus* after two-week water stress period. Data are presented as mean \pm SD. Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

Leaf gas-exchange measurements

Water deficit reduced photosynthesis (A), stomatal conductance (g_s), transpiration rate (E) and intercellular CO₂ concentration (Ci) of leaves (fig. 6, table 1). SA treatments caused significant differences in A, E and Ci in leaves under WW condition. SA treatments led to

increase of A and E, while to Ci showed lower values compared to control plants (0 mM) (fig. 6, table 1). Regarding WS condition, E and A increased as the concentration of SA increased, presenting values significantly higher than control plants (fig. 6, table 1).

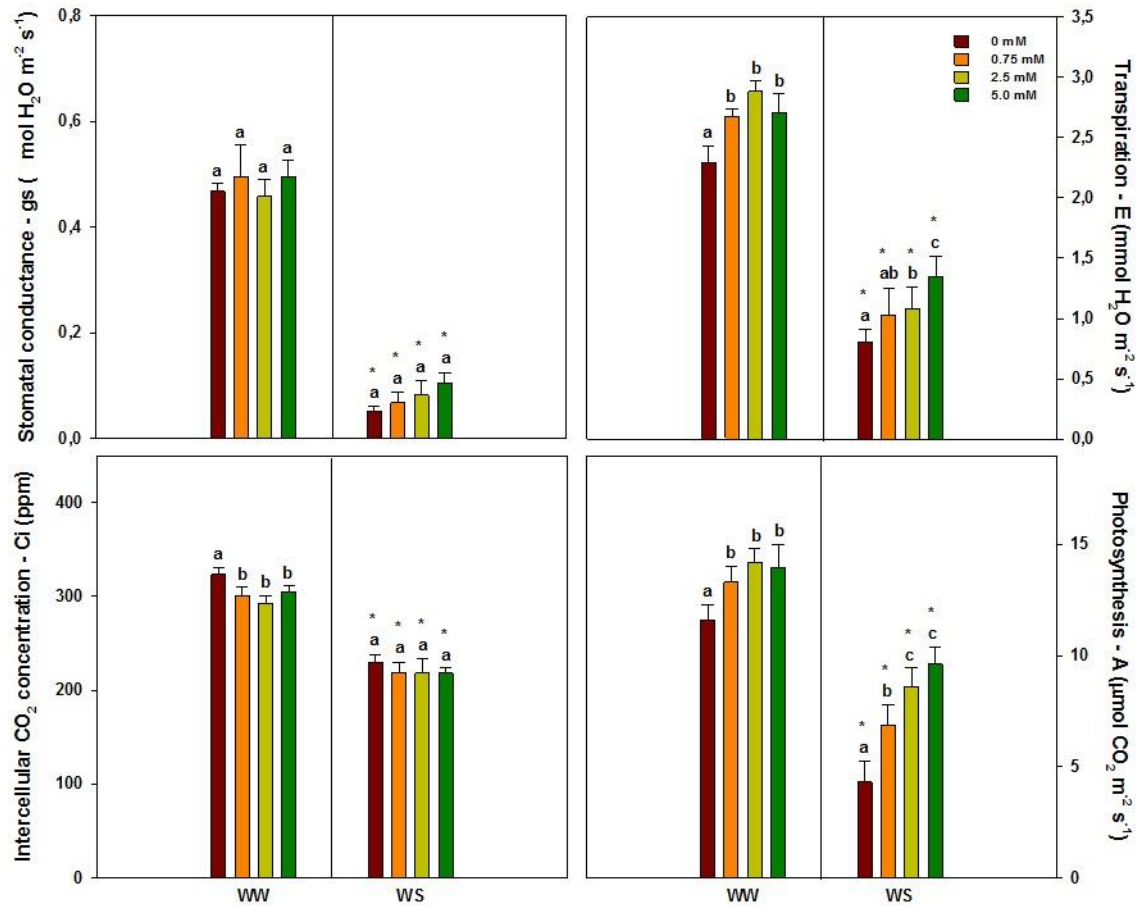


Figure 6 – Photosynthesis (A), transpiration (E), stomatal conductance (g_s) and intercellular CO₂ concentration (Ci) in well watered (WW) and water stressed (WS) plants of *E. globulus* after two-week water stress period. Data are presented as mean \pm SD. Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

Lipid peroxidation

Lipid peroxidation was measured in terms of MDA content. After the two-week experiment, the imposed water stress treatment showed a significant increase in MDA concentration compared to well-watered condition (fig. 7, table 1).

In what concerns to WW and WS condition, plants sprayed with 2.5 and 5 mM presented levels of MDA significantly lesser than control plants and the 0.75 mM (fig. 7, table 1).

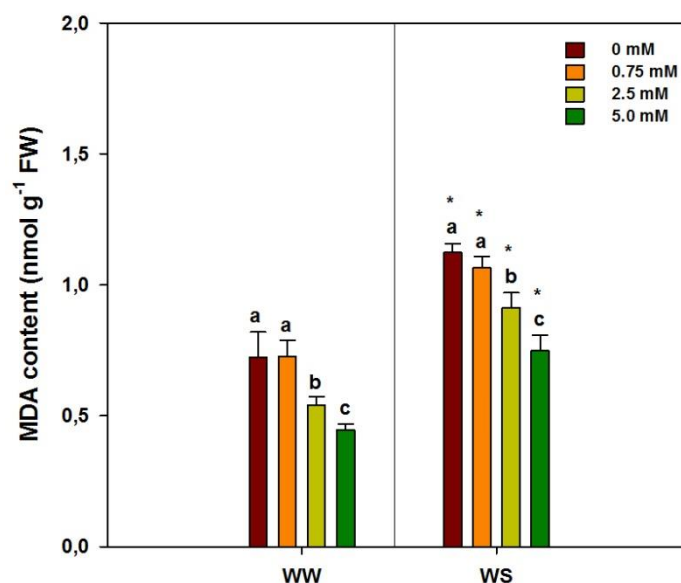


Figure 7 – MDA content in well watered (WW) and water stressed (WS) plants of *E. globulus* after two-week water stress period. Data are presented as mean \pm SD. Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

Total soluble sugars

Relatively to total soluble sugars content, significantly differences were found between water treatments. In general, plants in WS conditions increased the levels of total soluble sugars (TSS) compared to the plants under WW condition, except for the plants treated with 5mM SA which remained similar values in both water regimes (fig. 8, table 1).

The accumulation of TSS was significantly higher in plants with SA application in both water treatments (fig. 8, table 1). In WW condition the accumulation increased as the SA concentration increased. However in plants subjected to drought, the levels of TSS decreased as the SA concentration increased (fig. 8, table 1).

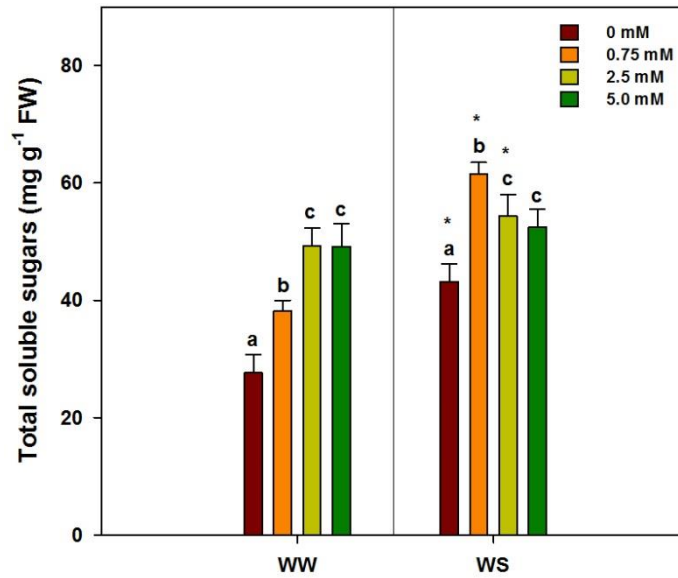


Figure 8 – Total soluble sugars content in well watered (WW) and water stressed (WS) plants of *E. globulus* after two-week water stress period. Data are presented as mean \pm SD. Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

Total Phenols

Significant differences were observed only in phenols content between WW and WS in control plants (0 mM) (fig. 9, table 1). The levels of phenols in WW conditions were similar in all SA concentrations, presenting values significantly higher compared to control plants (fig. 9, table 1). In WS plants, significant differences were observed in 2.5 and 5 mM SA treatments, which showed higher levels of phenols compared to control plants and plants with 0.75mM SA (fig. 9, table 1).

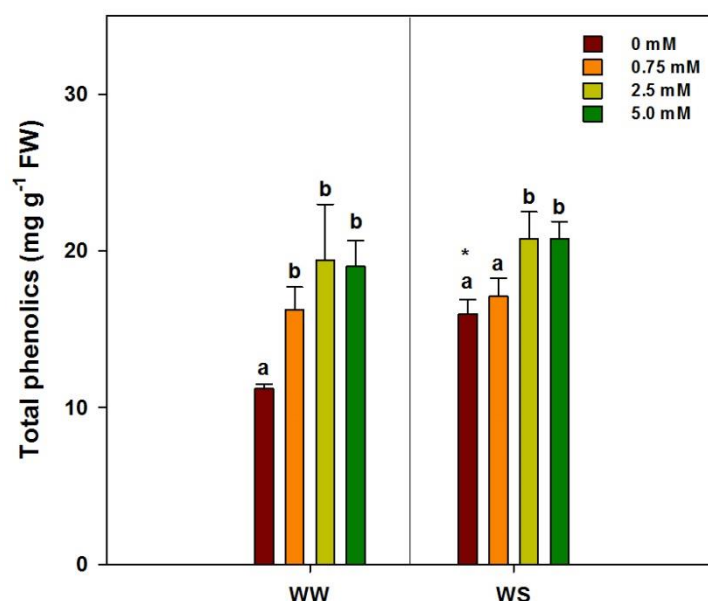


Figure 9 – Total phenolics content in well watered (WW) and water stressed (WS) plants of *E. globulus* after two-week water stress period. Data are presented as mean \pm SD. Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments ($p \leq 0.05$).

Table 1 - Two-way ANOVA summary table for morphological and physiological characteristics of the *Eucalyptus globulus* plants subjected to two water treatments (WW and WS) with foliar application of sodium salicylate. Degrees of freedom (df) and F value are show for each source of variation; variance (MS) of residual is also showed. Values in bold represent differences in the factors analysed ($p \leq 0.01$).

Parameter	Source of variation	df	F	Significance
Height	SA	3	14,483	<0,001
	Water treatment	1	26,504	<0,001
	Interaction	3	3,125	0,045
	Residual	24	MS = 2,842	
Ψ	SA	3	12,962	<0,001
	Water treatment	1	979,101	<0,001
	Interaction	3	11,275	<0,001
	Residual	16	MS = 0,00151	
Total Chl	SA	3	0,832	0,484
	Water treatment	1	32,573	<0,001
	Interaction	3	4,440	0,009
	Residual	39	MS = 0,134	
Carotenoids	SA	3	33,942	<0,001
	Water treatment	1	66,006	<0,001
	Interaction	3	14,170	<0,001
	Residual	26	MS = 0,00215	

Table 1 – continued

	SA	3	0,800	0,503
F_v/F_m	Water treatment	1	0,275	0,604
	Interaction	3	0,809	0,499
	Residual	31	MS = 0,0000857	
	SA	3	37,450	<0,001
A	Water treatment	1	431,654	<0,001
	Interaction	3	4,781	0,009
	Residual	26	MS = 0,683	
	SA	3	2,624	0,070
g_s	Water treatment	1	1455,243	<0,001
	Interaction	3	1,276	0,302
	Residual	28	MS = 0,000990	
	SA	3	19,395	<0,001
E	Water treatment	1	1000,297	<0,001
	Interaction	3	4,126	0,015
	Residual	28	MS = 0,0218	
	Salicylic	3	8,116	<0,001
Ci	Water treatment	1	698,180	<0,001
	Interaction	3	1,607	0,211
	Residual	27	MS = 87,380	
	SA	3	63,285	<0,001
MDA	Water treatment	1	343,687	<0,001
	Interaction	3	1,413	0,261
	Residual	26	MS = 0,00298	
	SA	3	52,959	<0,001
TSS	Water treatment	1	115,884	<0,001
	Interaction	3	18,234	<0,001
	Residual	22	MS = 8,859	
	SA	3	17,077	<0,001
Total phenolics	Water treatment	1	8,181	0,010
	Interaction	3	0,888	0,466
	Residual	18	MS = 2,995	

Hormone global quantification, IAA and ABA immunolocalization

With respect to plant hormones global quantification, water stress treatment only affected ABA and JA, which content increased under drought conditions (Table 2). Additionally SA treatments influenced over SA, DHZ, GA4 and IP amounts. Thus, endogenous SA content increased linearly with the dose of exogenous SA application, although the differences were only significant at 5.0 mM (Table 2). DHZ presented significant higher concentration at 2.5 mM (Table 2). And finally, GA4 and IP significantly decreased in all SA treatments (Table 2) in relation to WS control (0 mM). In relation to ABA immunolocalization analysis different ABA levels and spatial distribution in leaves in response to water stress and SA treatment was detected (fig. 10). Under WW conditions, ABA was equitably distributed over the tissues of the samples. An increasing in intensity of ABA was observed after drought imposition. The SA application did not significantly change ABA levels but a clear redistribution was observed:

ABA was more concentration near vascular vessels (fig. 10). IAA global quantification did not show any alterations with drought imposition neither SA treatment (Table 2), however by immunolocalization differential distribution was observed between WW and WS plants showing special accumulation of IAA around of vascular vessels and oil gland in 5 mM SA treatment (fig. 11).

Table 2 – Hormones quantification in 0 mM WW and WS plants (0, 0.75, 2.5 and 5.0 mM) of *E. globulus* after two-week stress period. Data are presented as mean \pm SD. Different lowercase letters indicate significant differences between SA concentrations in WW and WS conditions and asterisks indicate significant differences between water treatments without SA application ($p \leq 0.05$).

	0 mM WW	0 mM WS	0.75 mM WS	2.5 mM WS	5.0 mM WS
ABA (ng g ⁻¹ DW)	5.5E+06 \pm 1.9E+06	2.1E+07 \pm 2.0E+06 *a	2.5E+07 \pm 1.5E+06 a	2.4E+07 \pm 4.3E+06 a	2.5E+07 \pm 7.0E+06 a
IAA (ng g ⁻¹ DW)	3.3E+05 \pm 2.0E+05	3.5E+05 \pm 8.7E+04 a	3.3E+05 \pm 1.5E+05 a	3.5E+05 \pm 5.9E+04 a	4.1E+05 \pm 1.5E+05 a
JA (ng g ⁻¹ DW)	8.8E+04 \pm 3.8E+03	2.5E+05 \pm 3.5E+04 *a	2.0E+05 \pm 2.6E+04 a	2.0E+05 \pm 5.1E+04 a	2.1E+05 \pm 3.6E+04 a
SA (ng g ⁻¹ DW)	2.3E+07 \pm 1.3E+07	2.5E+07 \pm 4.8E+06 a	2.1E+07 \pm 7.8E+06 a	5.2E+07 \pm 3.0E+07a	3.3E+08 \pm 1.6E+08 b
DHZ (ng g ⁻¹ DW)	5.6E+04 \pm 5.4E+04	6.0E+04 \pm 1.9E+04 a	3.3E+04 \pm 9.2E+03a	2.2E+05 \pm 3.2E+04 b	4.0E+04 \pm 1.2E+04 a
IP (ng g ⁻¹ DW)	1.8E+05 \pm 1.4E+05	2.6E+05 \pm 2.4E+04 a	1.5E+05 \pm 1.9E+04 b	1.4E+05 \pm 1.8E+04 b	1.4E+05 \pm 1.8E+04 b
GA4 (ng g ⁻¹ DW)	3.4E+07 \pm 1.1E+07	8.0E+07 \pm 2.5E+07 a	4.0E+07 \pm 7.5E+06 b	4.1E+07 \pm 4.7E+06 b	5.6E+07 \pm 9.9E+06 b

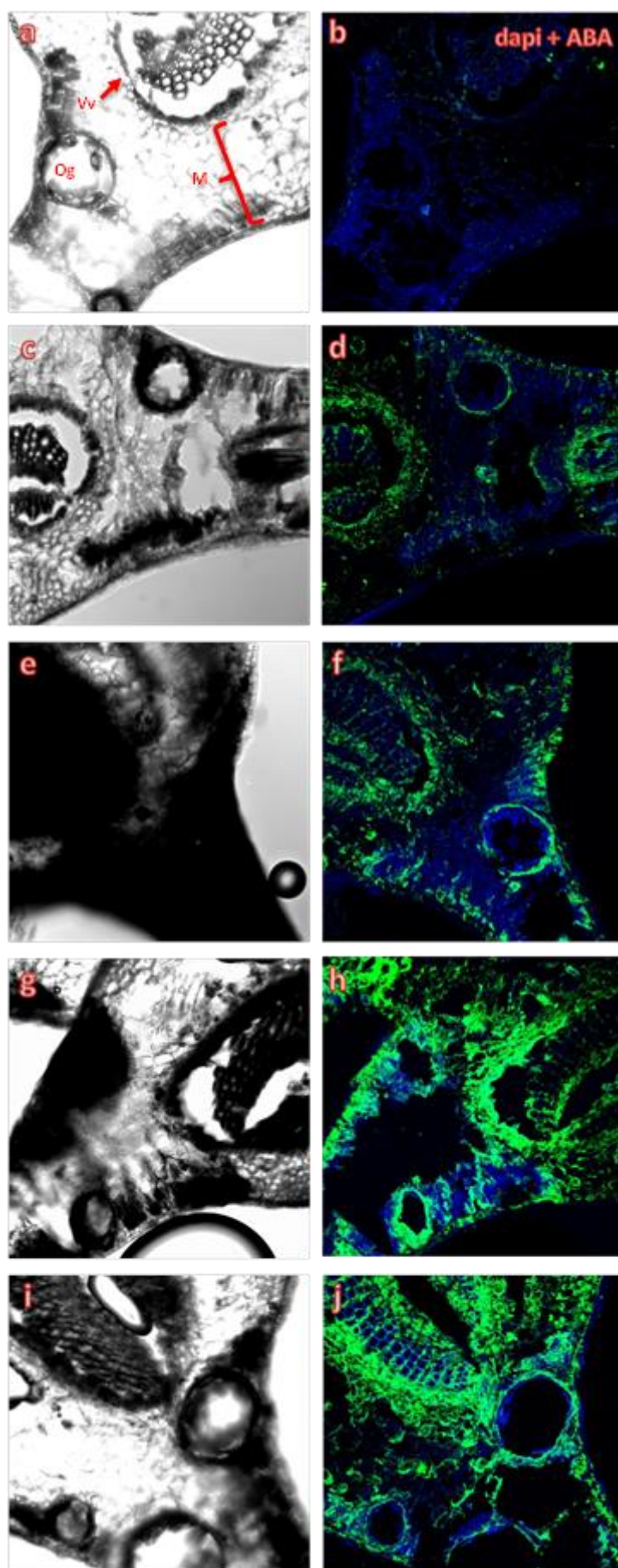


Figure 10 – Immunodetection of ABA in section of *E. globulus* leaves using confocal microscope. Differential interference contrast (DIC) (a, c, e, g and i) and immunolocalization of ABA (b, d, f, h and j). ABA labelling: DAPI (blue signals) and ABA (Green signals) merged in transversal leaf section. (a,b) – 0 mM WW; (c,d) – 0 mM WS; (e,f) – 0.75 mM WS; (g,h) – 2.5 mM WS; (i,j) – 5.0 mM WS. Abbreviations: Vv= Vascular vessels; M =Mesophyll; Og = Oil gland

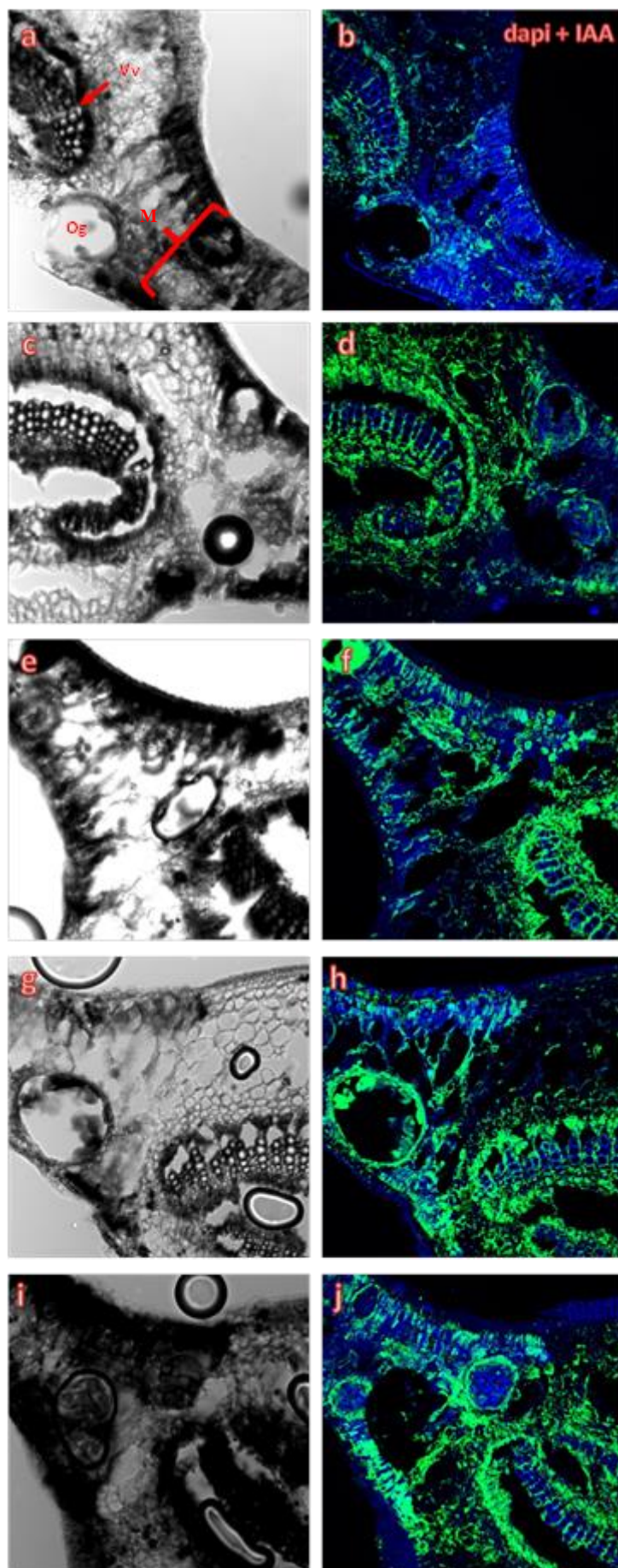


Figure 11 – Immunodetection of IAA in section of *E. globulus* leaves using confocal microscope.

Differential interference contrast (DIC) (a, c, e, g and i) and immunolocalization of IAA (b, d, f, h and j). IAA labelling: DAPI (blue signals) and IAA (Green signals) merged in transversal leaf section. (a,b) – 0 mM WW; (c,d) – 0 mM WS; (e,f) – 0.75 mM WS; (g,h) – 2.5 mM WS; (i,j) – 5.0 mM WS. Abbreviations: Vv= Vascular vessels; M =Mesophyll; Og = Oil gland

Multivariate approach: global overview of physiological status

PCA ordination provided an overall picture of the physiological/biochemical condition of *E. globulus* plants during the experimental setup, revealing a clear separation between water treatment and SA application (fig. 12). Non treated well watered plants (WW) were grouped together (fig. 12, left side) suggesting homogeneity in the physiology and biochemistry of plants in non-stressful conditions. Sample scores of WS plants were all located on the right side, with the plants that were not pre-treated with SA being located in the upper right part (fig. 12). The horizontal left-to-right movement observed in WS is mostly explained by lower C_i , g_s , E , water potential and height and higher JA, ABA, MDA and total chlorophyll compared to WW (fig. 12). SA treated WS scores progressively moved from top-to-bottom (fig. 12). The downwards migration pattern of PCA scores under increased SA treatment is most probably associated with vertical gradients, such as increased DHZ, SA, TSS and total phenolic compounds and decreased IP, GA4 and carotenoids (fig. 12).

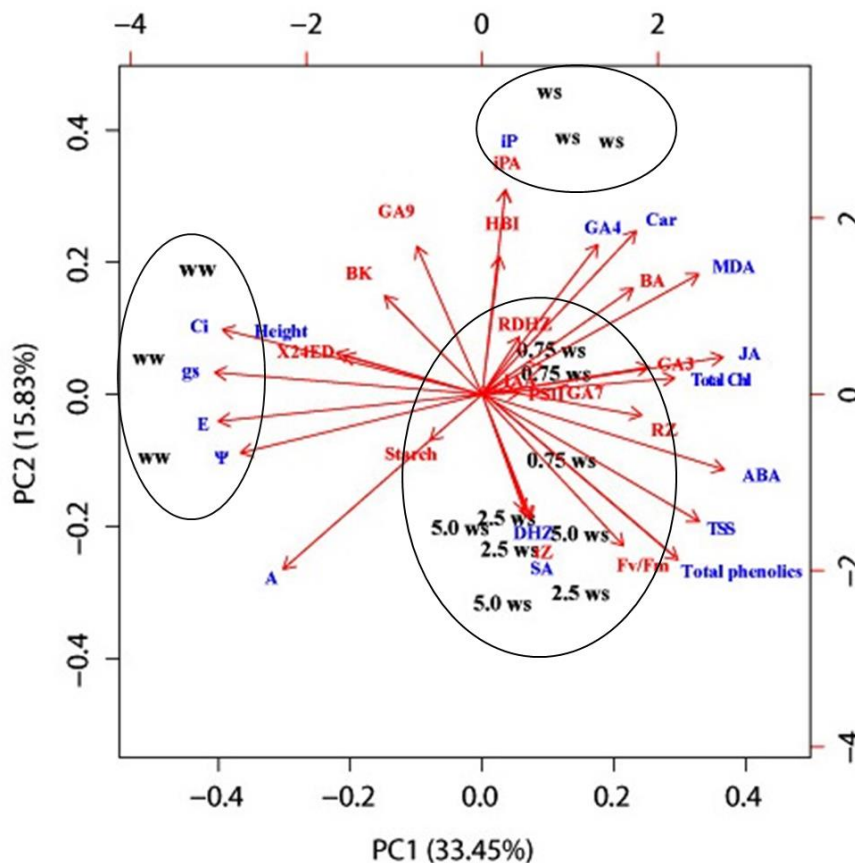


Figure 12 – PCA biplot of the physiological data of *Eucalyptus globulus* plants in the experimental setup. Circles areas were used to highlight the position specific stress (WW plants; WS plants and 0.75, 2.5 and 5mM WS). Parameters in blue represent the parameters that showed differences when using post-hoc analysis ($p \leq 0.05$). Loading plots for the first axis (explained variation is 33.45%) and second axis (explained variation is 15.83%).

Discussion

Drought stress effect in *Eucalyptus globulus*

Impaired plant growth and development are the first and most concerning effects of drought. In fact, water stressed plants showed a marked reduction in height of *E. globulus* plants (fig. 2), which is agreement with other reports (12, 21). Cell growth is mainly affected by a reduction in turgor pressure and decreased CO₂ diffusion from atmosphere (54). Following this assumption, WS plants showed decreased water potential values (fig. 3), as already documented in other works (15, 55), as well as Ci, CO₂ assimilation, g_s and E (fig. 6). Stomatal limitation of A under water deficit conditions is accepted as one of the main limitations of plant productivity (56) and it has been reported in many studies (57, 58).

PSII photochemistry, assessed as Fv/Fm, was not negatively affected in response to drought (fig. 5). Moreover, total chlorophyll and carotenoids increased with WS (fig. 4). These results are in accordance with Correia *et al.* (15). In this study, WW and WS plants exhibited Fv/Fm values within the range of healthy plants however WS plants showed an increase in the pigments concentration. As argued, the increasing chlorophyll content could be related to a reduction in leaf mass expansion and to a protective role of carotenoids or other mechanisms, which protected these pigments from degradation and preserve the photosynthetic capacity.

Drought induced oxidative stress leads to the generation of ROS. The prevalence of free radicals reaction in membranes is indicated by the accumulation of MDA, which is expected to show greater accumulation under environmental stresses (59, 60). Our results support these studies: MDA levels increased in WS plants (fig. 7). Another response to oxidative stress is the accumulation of polyphenols. These compounds possess ideal structural chemistry for free radical scavenging activities (61) and are reported as accumulating in several abiotic stresses (62, 63). In our study, total phenols also increased probably as defence mechanism against drought induced oxidative stress (fig. 9).

Soluble sugars content raised in response to water deficit (fig. 8). This is a common response in drought stress considering that accumulation of compatible solutes is an important physiological adaptation and enables osmotic adjustment (64, 65).

From the studied phytohormones, ABA and JA increased under water deficit conditions (Table 1). ABA is a phytohormone that is extensively involved in responses to abiotic stresses such as drought, low temperature, and osmotic stress (66). In addition, there is increasing evidence that jasmonic acid (JA) and its metabolically active derivatives (jasmonates) are also crucial signalling molecules involved in many plant responses to biotic and abiotic stresses (67). In our work, these two phytohormones seem to have a synergistic interaction in response to

drought stress. Other authors (66-68) already defended this conclusion. The other quantified phytohormones did not alter in response to water deficit, for what we considered these should not have a clear active role in response to this abiotic stress. However, the immunolocalization of IAA showed redistribution under drought, with accumulation of IAA near both oil glands and vascular vessels (fig. 11). On the other hand, ABA immunolocalization concurred with ABA quantification and presented an accumulation under drought conditions (fig. 10).

Foliar application of SA and drought stress tolerance

At morphological level, all SA pre-treated plants showed local signals of damage in leaves due to direct contact to SA which was not transported into the tissue (32). The application of some chemicals to the plants is known to enhance the mechanisms that plants resort to counteract the adverse effects of drought. In general, the foliar application of the phenolic compound SA ameliorated the overpowering damages of water deficit (fig. 3). SA application alleviated water potential in WS plants to a great extent, without affecting WW plants (fig. 3). This response was enhanced according to the higher concentrations of SA. The beneficial effect of SA in plant water relations was reported in previous studies (36, 69).

In parallel, the studied compatible solutes, total soluble sugars, also increased in response to SA application (fig. 8). This response is in agreement with Farooq *et al.* (36). As explained by these authors, the strong correlation between accumulation of compatible solutes and water relation components indicates the involvement of those solutes to balance plant water status under drought.

Total phenols also increased in response to SA treatment (fig. 9). Considering that SA is a phenolic compound, this is an expected response and it has already been documented (70). This increase may also be part of the explanation to the lower levels of MDA in SA-treated WW and WS plants (fig. 10). Our results support that the decreased membrane damage may relate to the induction of antioxidant responses triggered by SA, including soluble sugars and phenols accumulation, which in turn protect plants against ROS and membrane injury or may affect synthesis of other substances, having a protective effect on plants under stress (32).

SA application induced a decrease in the pigment content (fig. 4). Both chlorophylls and carotenoids in WW and WS plants decreased when treated with SA (fig. 4). This result is contradictory to some earlier studies carried out in wheat (71) and tomato (38). Considering that drought stress induced these compounds in *E. globulus*, we defend that SA application reduced them to WS levels.

Exogenously applied SA caused an increase in A and E of plants treated with SA, slightly induced g_s and did not affected C_i (fig. 6). These responses were more pronounced in WS plants. SA-induced alterations in A can be associated with stomatal and non-stomatal

limitations (fig. 6). The observed increase between A and g_s in this study partly support Galmés *et al.* (72), suggesting that SA-induced photosynthesis may depend on stomatal control. Furthermore and as explained above, SA is probably inducing several non-stomatal influence, preventing major metabolic impairment and carboxylase inactivation of RuBisCO.

With respect to plant hormones, SA application increased SA and DHZ and decreased GA₄ and IP. As expected, endogenous SA concentration increased with SA application (Table 1). The negative correlation between IP and DHZ under SA treatment could imply a displacement of the cytokinins biosynthesis route in relation to the decrease of growth and cell division (Table 1). As explained in a different work with *Pinus radiata* (73), in this metabolic pathway, IP as an initial compound, by means of side-chain *trans*-hydroxylation reactions is converted in DHZ. The reduction in GA₄ (Table 1) was expected as a correlation between SA treatment and inhibition gibberellin synthesis has been already reported (74). Xie *et al.* (74) showed that SA suppressed GA-induced expression in barley. Due the involvement of GA₄ in cell elongation (75) the decrease of GA₄ detected in stressed plants in this work could be also related to the reduction of height observed in the SA-treatments.

The clear distribution revealed by PCA-analysis came upon the previously described results (fig. 12). First, the horizontal gradient revealed a well-defined separation between WW and WS. This response is mainly due to decreases in height, gas exchange and water potential, the most sensitive physiological evidence of water deficit, as previously explained. Besides, the water deficit was also marked by increased lipid peroxidation, chlorophylls, JA and ABA, which are also known responses after drought imposition. The effect of SA application is highlighted by the vertical gradient. In this case, SA increase may explain an increase in phenolics and TSS, which in turn seem related to a biased MDA decrease and CO₂ assimilation increase.

Conclusions

Our results show that WS generally affected *E. globulus* performance but the pre-treatment with exogenous SA increased the tolerance of these plants to drought. SA application ameliorated the damaging effects of drought on *E. globulus*, probably by inducing different anti-stress programs that were expressed by improving some physiological and biochemical attributes. This could be due to the activation of the antioxidant system, which in turn protect the plants against ROS and membrane damage, or to the synthesis of other substances with a protective role under stress. We also proved that the efficiency of exogenous SA action depended on the SA concentrations getting the best result in the plants treated with 5 mM SA. Therefore, we are able to conclude that foliar application of 5 mM SA could be used as a

potential chemical priming strategy to enhance *E. globulus* drought tolerance in the breeding forest programs.

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PART III

Final considerations

This study arises from the current need to investigate new approaches to relieve the detrimental effects of water deficit on *Eucalyptus globulus*, a worldwide established forest tree, with especial relevance for the Portuguese forest industry. Due to global change, it is increasingly accepted that future climate conditions in the Mediterranean countries will experience an increase in aridity, exacerbating negative effects in forest productivity. Therefore, it is essential to develop mitigation strategies, effective not only at the economic level, but also in terms of *E. globulus* quality and environmental sustainability. Within this topic, the scientific community has undertaken a major effort to investigate the effect of PGRs in the improvement of drought tolerance.

The aim of this work was, thereby, to investigate whether SA can effectively ameliorate the negative effects of drought on *E. globulus* plants and gather morpho-physiological and biochemical features that enable to characterize their mechanism at the nursery phase. For that, *E. globulus* plants were pre-treated with different SA concentrations and subjected to drought in a climate chamber experiment. Several morpho-physiological and biochemical measurements were carried out.

After measuring growth, water status, lipid peroxidation, photosynthetic responses, gas exchange, osmolyte accumulation, total phenolic compounds content and hormones quantification and dynamics, I can concluded that SA application ameliorated the damaging effects of drought on *E. globulus*. This response probably results from the induction of different anti-stress programs that were expressed by improving some physiological and biochemical attributes. I also proved that the best results were achieved on plants pre-treated with 5 mM SA. The selected SA concentration presented higher photosynthetic rates and water potential, and a decrease in lipid peroxidation. Therefore, the foliar application of 5 mM SA could be used as a potential chemical priming strategy to enhance *E. globulus* drought tolerance in the breeding forest programs.

Other aspects than the ones evaluated in this study should be considered in a next step to explain how plants treated with SA are able to maintain a greater performance under drought and to explore SA effects during recovery from stress. Besides, it is necessary to conduct more experiments using a large SA concentration range between 2.5 and 5.0 mM, such that we found a perfect balance among morphological and physiological features. Also, it is important to check if the SA application maintains a positive action on the plants in a period of drought longer than two weeks and monitoring a field experiment to see the further performance of *E. globulus* treated with SA.

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