Universidade de Aveiro Departamento de Biologia
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Maria Celeste Dias, Investigadora em pós-doutoramento do CESAM da Universidade de Aveiro, co-orientação da Doutora Glória Catarina Cintra da Costa Pinto, investigadora auxiliar do CESAM da Universidade de Aveiro e co-orientação da Prof. Doutora Maria da Conceição Santos, Professora associada de nomeação definitiva com agregação da Universidade de Aveiro.

aos meus pais e ao que nos Une. *Obrigada.*

o júri

Presidente	Professora Doutora Maria de Lourdes Gomes Pereira Professora Associada com Agregação da Universidade de Aveiro
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palavras-chave

Melia azedarach, plantas medicinais, défice hídrico, fisiologia, ROS, mecanismos antioxidantes

Resumo

Desde o início da civilização que muitas espécies vegetais medicinais são usadas no combate a doenças. *Melia azedarach* é uma das espécies mais utilizadas na medicina tradicional devido a várias propriedades medicinais, especialmente, propriedades antioxidantes, que contudo podem variar com fatores endógenos e ambientais. Entre os vários fatores ambientais capazes de afetar as propriedades medicinais e crescimento das plantas, o défice hídrico é provavelmente aquele com mais impacto. Está já relatado para várias espécies um aumento da atividade das enzimas desintoxicantes ou a biossíntese e/ou regeneração de metabolitos antioxidantes em condições de stress hídrico, é de grande importância avaliar os efeitos deste stress na capacidade antioxidante de *M. azedarach*. Assim, plantas de *M. azedarach* com dois meses de idade foram expostas a défice hídrico (WS) (plantas a 20% da capacidade de campo) durante 20 dias. Após este período, a *performance* das plantas foi avaliada através de medições de parâmetros fisiológicos e bioquímicos. O WS induziu o fecho dos estomas, reduziu a taxa de assimilação de CO_2 (*A*) e diminuiu a disponibilidade de CO_2 nos espaços intercelulares de mesófilo (*Ci*). O WS reduziu também a eficiência fotossintética do PSII, mas não afetou o crescimento da planta (biomassa e altura das plantas). O WS aumentou a permeabilidade da membrana e induziu uma sobre-atividade das enzimas antioxidantes, assim como um aumento da produção de metabolitos antioxidantes. Os resultados indicam que *M. azedarach* poderá ser utilizada em programas de reflorestação em zonas mais vulneráveis à seca. Além disso, a imposição do WS poderá ser uma estratégia para aumentar a capacidade antioxidante de *M. azedarach*, por exemplo, para usos medicinais, sem afetar severamente o crescimento da planta.

keywords

Melia azedarach, medicinal plants, water deficit, plant physiology, ROS, antioxidant mechanisms

abstract

Medicinal plant species are used to combat diseases from the dawn of civilization. *Melia azedarach* is one of the species most used in traditional medicine due to their several medicinal properties, especially, antioxidant properties which, however, may vary with environmental and endogenous factors. Among the various environmental factors that can affect the medicinal properties and the development of plants, water stress (WS) is probably the one that has more impact. It's been reported for several species the enhancement of the ROS-detoxifying enzymes or the biosynthesis and/or regeneration of antioxidant metabolites under WS conditions. Therefore it is of great importance to assess the effects of this stress on the antioxidant capacity of *M. azedarach*. Therefore, two months old plants of *M. azedarach* plants were exposed to WS (plants at 20% of field capacity) during 20 days. After this period, plant performance were evaluated through the measurement of physiological and biochemical parameters. WS stress induced stomatal closure, reduced the net CO_2 assimilation rate (*A*) and decreased the CO_2 availability in the intercellular spaces of mesophyll cells (*Ci*). WS also reduced the photosynthetic efficiency of PSII, but did not affect plant growth (dry weight accumulation and plant height). WS increased cell membrane permeability and induced an up regulation of the antioxidant enzymes and also an over production of antioxidant metabolites. The results indicated that *M. azedarach* could be used in re/afforestation programs for drought prone habitats. Moreover, WS imposition could be a positive strategy to increase the antioxidant capacity of *M. azedarach*, for example for medicinal uses, without affect severely plant growth.

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ABBREVIATIONS LIST

A, net CO_2 assimilation rate; AOS, active oxygen species; AOX, mitochondrial alternative oxidase; APX, ascorbate peroxidase; AsA, ascorbate; Cars, carotenoids; CAT, catalase; Chl, chlorophyll; *Ci*, intercellular CO_2; CW, cell wall; DHA, dehydroascorbate; DHAR, DHA reductase; DW, dry weight; E, transpiration rate; FD, ferredoxin; Fm, maximal fluorescence in the dark-adapted state; Fm', fluorescence during the saturating pulse in steady state; FNR, ferredoxin NADPH reductase; F_0, minimal fluorescence; F_0', minimum fluorescence after switch off the active light; F_v, maximal variable fluorescence; F_v/F_m, maximal efficiency of PSII; GLR, glutaredoxin, G-POX, glutathione peroxidase; GPX, glutathione peroxidase; GOX, glycolate oxidase; LPO, lipid peroxidation; MAPK, mitogen activated kinase; MDAR, MDA reductase; GR, glutathione reductase; *gs*, stomatal conductance; GSH, glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; OH, hydroxyl radicals; O_2-, superoxide ions; PCD, programmed cell death; PM, plasma membrane; PrsxP, peroxiredoxin, PS, photosystem; PUFA, polyunsaturated fatty acid; ROIs, reactive oxygen intermediates; ROS, reactive oxygen species; SOD, superoxide dismutase; Trx, thioredoxin; WS, water stress; WW, well watered; Φ_{PSII}, effective quantum efficiency of PSII; ψ, water potential.

INTRODUCTION

Melia azedarach, a medicinal plant

Natural products derived from plants have been used for the treatment of several diseases from ancient times and still represent the most important health care source for a large percentage of the population around the world (1,2). It is estimated that approximately 75% of people worldwide rely on traditional herbal medicine to meet their primary health care needs (2).

Treatments using herbal medicines may have some advantages over treatments using single purified chemicals as herbal medicine usually combines different therapeutic or preventive components, and so might have more activity than single products alone (1). Among the most interesting bioactivities used in herbal medicine, the antioxidant properties have been widely investigated for many plant species (3). There is an increasing interest in the measurement and use of plant antioxidants for scientific research as well as industrial (e.g. dietary, pharmaceutical and cosmetics) purposes. This is mainly due to their strong biological activity (4).

Figure 1 - *Melia azedarach* tree with flowers and fruits.

Melia azedarach Linn, usually known as melia, cinamomo or chinaberry tree (fig. 1), is native from West Asia, naturalised in several parts of the globe, such as, parts of central and southern Australia, southern Europe, southern and eastern Afria, southern USA, Mexico, Central America, the Caribbean, tropical southern America and many Pacific islands (5, 6). *M. azedarach* is present in another parts of the world, however, is not native or naturalised, but is introduced or invasive, such as in East Africa, particularly in parts of Kenya, Tanzania and Uganda (6). Taxonomically it belongs to the Filo Magnoliophyta, Class Magnoliosida, Order Sapindales, Family MELIACEAE, Genus *Melia* (7). The plant is a small-to-medium sized deciduous tree, 5 to 15 m tall and 30 to 60 cm wide in diameter. The leaves are alternate and fruits or berries are yellow, nearly round, smooth and fleshyand grows in temperate and tropical countries like India, China and Japan (8,9). *M. azedarach* is one of the most used plants in traditional medicine due to several medicinal properties (5, 10, 11). Plants of the Meliaceae family have been well documented for the ability to metabolize structurally diverse and biologically significant limonoids and triterpenoids (12). This species exhibits a wide range of biological activities of practical agricultural and pharmaceutical use (10). Extracts from different parts of *M. azedarach* are reported to exhibit antifungal (13), nematicidal (14, 15), antihelmintic (16), antilithic, diuretic (17), cytotoxic and antiproliferative (18) and antioxidant activities (18). However, the most valuable interest of this species is due of its insecticidal (e.g. 19, 20, 21, 22, 23), insect repellent properties and the several active limonoidal compounds (24). *M. azedarach* products enhance soil fertility (25, 26) and its derivatives stimulate soil microbial biomass (27, 28). The tree yields valuable timber, resistant to attack by white ants, and is often used to make furniture, plywood, toys and fuelwood. The wide range of adaptability and its usefulness makes *M. azedarach* an additional crop for afforestation programs (17). Moreover, it is also reported that *M. azedarach* can resist to heat and drought, as well as poor soil conditions (29). Thus, the multidirectional and widespread use of this species makes it one of the most important multipurpose tree species (17).

Water deficit and its effects on plant physiology

The increasing frequency of severe abiotic stresses, such as drought, salinity and high temperatures, often reported as associated with climate changes, has enhancing worldwide

concerns about the impact of these extreme environmental events on plant performance (30). Plants frequently encounter unfavourable growth conditions such as environmental stress that can disrupt cellular structures and impair key physiological functions (31). Different plant species are highly variable relatively to their optimum environments and a severe environmental condition that is harmful for one plant species, might not be stressful for another (31).

Responses to environmental stresses, including water deficit, occur at all levels of organization and are modulated by the intensity, duration and rate of progression of the imposed stress (31, 32). Among the various environmental factors that can affect the productivity and development of plants, the water deficit is probably the one with the highest impact (33). The effects of water deficit on plants have been studied for a long time and changes induced by insufficient water supply have been examined from the whole plant/plant population level to biochemical and molecular level (34). In general, most environmental stresses may have a direct impact on the photosynthetic apparatus, essentially by disrupting all major components of photosynthesis including the thylakoid electron transport, the carbon reduction cycle and the stomatal control of the CO_2 supply, together with an increased accumulation of carbohydrates, peroxidative destruction of lipids and disturbance of water balance (35). Photosynthesis, together with cell growth, is among the primary processes to be affected by water deficit. The effects of water deficit can be direct, as the decreased CO_2 availability caused by diffusion limitations through the stomata and the mesophyll or the alterations of photosynthetic metabolism, or they can arise as secondary effects, namely through the generated oxidative stress (32, 33).

Water deprivation leads to stomatal closure and leaf growth inhibition that can protect plants from extensive water loss (36). Limited gas exchanges, reduced transpiration and photosynthesis, and limited tissues growth through cell division enlargement and differentiation, are consequence of stomatal closure and decreased leaf water potential (ψ_L) (37). Closing stomata is a consequence from changes in turgor of guard cells relative to epidermal cells (36). Besides transpiration reduction, stomatal closure also limits the intercellular CO_2 concentration. In the initial phase of water deficit, the reductive effect of the stomatal closure on transpiration rate is greater than the effect on CO_2 assimilation. However, with the development of water deficit, both processes are usually strongly reduced (38, 39).

Photosynthetic pigments are important to plants mainly at the harvesting light and production of reducing compounds (35). Chlorophylls are the major chloroplast components for photosynthesis, and relative chlorophyll content has a positive relationship with photosynthetic rate. 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 (35). Carotenoids act as light-harvesting pigments, and can protect chlorophyll and membranes destruction by quenching triplet chlorophyll and removing oxygen from the excited chlorophyll–oxygen complex (40).

The activity of sucrose phosphate synthase is also reduced by water deficit and the ratio of starch/sucrose alters (41). Sucrose, glucose and fructose are important components of drought-signalling pathways (32, 33). In stressed plants by drought, the carbohydrate status depends not only on the efficiency of photosynthetic carbon reduction cycle and sucrose/starch synthesis, but it is linked to the processes of osmotic adjustment as well (42). There are classes of osmolytes accumulate in cells of plants exposed to water deficiency, such as proline (42).

Water deficit and oxidative stress

Under water deficit conditions, electrons at a high energy state can easily form reactive oxygen species (ROS, also called active oxygen species (AOS) or reactive oxygen intermediates (ROIs)). If the cell does not impairs these ROS, they causes oxidative stress damages to plant cells, once that can attack macromolecules, including lipids (e.g. membrane lipids), inactivate enzymes and damage the nucleic acids ultimately leading, in the most severe cases, to cell death (43, 44, 45, 46). ROS nomenclature includes various forms of activated oxygen, which include free radicals such as super oxide ions (O_2^-) and hydroxyl radicals (OH^-), as well as non-free-radicals species such as hydrogen peroxide (H_2O_2) (10). An increase in ROS levels can provoke a partial or severe oxidation of cellular components inducing redox status changes due to an unbalance between ROS production and scavenging (42, 44). In case of continuous stress it is necessary to increase the activity of antioxidant enzymes to protect cells from oxidative damage (47, 48). According to the literature, one of the described damages resulted from water deficit stress is the membrane injury and the

liberation of ions from the cell to the extracellular space as a consequence of an oxidative burst leading to lipid peroxidation, membrane permeabilization and cell death (49).

Plants have evolved a highly efficient defence system, as a protection against ROS, with antioxidant enzymes and non-enzymatic compounds that can neutralize free radicals and reduce the potential damages of ROS (50), but its concentration in the cell is maintained essentially by the antioxidant system (31). The different kinds of ROS that have been investigated in plants include hydrogen peroxide (H_2O_2), superoxide anion (O_2·-), hydroxyl radicals (.OH), singlet oxygen (O_2) and nitric oxide (NO) (51). H_2O_2, O_2·- and · OH can convert to one another (fig. 2). Such conversion may occur spontaneously or be catalyzed by enzymes (53).

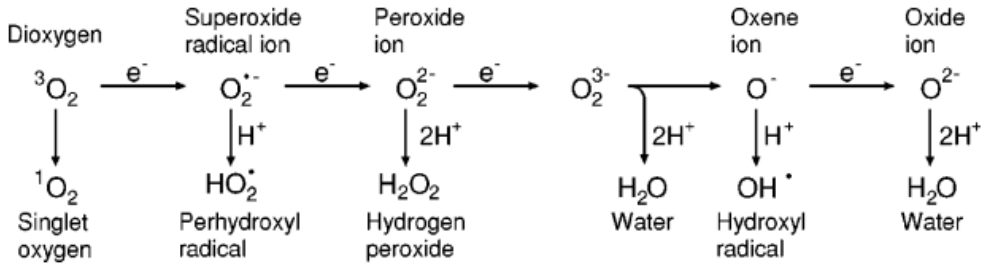

Figure 2 - Generation of different ROS by energy transfer or sequential univalent reduction of ground state triplet oxygen (Adapted: Apel and Hirt (52)).

Balance between ROS production and ROS scavenging

Water deficit is reported to increase the production of ROS and increase the oxidative load in plants (44). Several evidences have proven that H_2O_2 plays an important role in plants under severe environmental conditions, which include various biotic and abiotic stresses (54) since it participates in many resistance mechanisms, including reinforcement of the plant cell wall, phytoalexin production, and enhancement of resistance to several stresses (51).

Concerning the different parts of the plant, the major source of ROS is the chloroplast. However the production of ROS also occurs in other cell compartments such as peroxisomes, mitochondria and plasma membrane (table 1) (44).

Table 1 – Producing/ scavenging ROS in plants (Adapted: Mittler *et al*., (45)).

Mechanism	Localization	Primary ROS
Production		
Photosynthesis ET and PSI or II	Chl	O_2^-
Respiration ET	Mit	O_2^-
Glycolate oxidase	Per	H_2O_2
Excited chlorophyll	Chl	O_2^1
NADPH oxidase	PM	O_2^-
Fatty acid β-oxidation	Per	H_2O_2
Oxalate oxidase	Apo	H_2O_2
Xanthine oxidase	Per	O_2^-
Peroxidases, Mn^{2+} and NADH	CW	H_2O_2, O_2^-
Amine oxidase	Apo	H_2O_2
Scavenging		
Superoxide dismutase	Chl, Cyt, Mit, Per, Apo	O_2^-
Ascorbate peroxidase	Chl, Cyt, Mit, Per, Apo	H_2O_2
Catalase	Per	H_2O_2
Glutathione peroxidase	Cyt	H_2O_2, ROOH
Peroxidases	CW, Cyt, Vac	H_2O_2
Thioredoxin peroxidase	Chl, Cyt, Mit	H_2O_2
Ascorbic acid	Chl, Cyt, Mit, Per, Apo	H_2O_2, O_2^-
Glutathione	Chl, Cyt, Mit, Per, Apo	H_2O_2
α – Tocopherol	Membranes	ROOH, O_2^1
Carotenoids	Chl	O_2^1

Abbreviations: Apo, apoplast; Chl, chloroplast; CW, cell wall; Cyt, cytosol; ET, electron transport; Mit, mitochondria; O_2^1, singlet oxygen; Per, peroxisome; PM, plasma membrane; PS,photosystem; Vac, vacuole.

Under normal growth conditions, the production of ROS in cells is low (240 µM s^{-1} O_2^- and a steady-state level of 0.5 µM H_2O_2 in chloroplasts), however there are many stresses that disrupt the cellular homeostasis of cells enhance the production of ROS (240–720 µM s^{-1} O_2^- and a steady-state level of 5–15 µM H_2O_2) (45). In the chloroplast, the photosynthetic electron transport system may become overactive causing a spillover of reducing power that is responsible for reduction of oxygen to different ROS (44). Another site of superoxide and H_2O_2 production are the peroxisomes. In this cellular compartment several key metabolic reactions occur, including photorespiration and are the major contributor to the cellular pool of H_2O_2 (55, 56). Superoxide and H_2O_2 production are also consequence of mitochondrial electron transport particularly under stress conditions (44). On the other hand ROS are also generated at the plasma membrane level or extracellularly in the apoplast though a transmembrane enzyme, NAD(P)H oxidase, which transfers electrons from cytoplasmic NAD(P)H to O_2 to form $O_2\cdot^-$ and subsequently H_2O_2 and $OH\cdot$ (57, 58). Besides NADPH oxidase, there are another sources of H_2O_2, such as pH-dependent peroxidase, germin-like oxalate oxidases and amine oxidase in the apoplast (59).

The chloroplasts produce $_1O^2$ at photosystem II (PSII) and $O_2\bullet^-$ at photosystem I (PSI) (50) and PSII (60) as byproducts. The mitochondria produce $O_2\bullet^-$ at complexes I and

III, also as byproducts. An estimated 1–5% of the oxygen consumption of isolated mitochondria results in ROS production (61). The peroxisomes produce $O_2^{\bullet -}$ and H_2O_2 in several key metabolic reactions (56). Finally, the NADPH oxidase in the plasma membrane produces $O_2^{\bullet -}$, which participates in several physiological processes (57).

As a protection against ROS, plants cells develop several antioxidant metabolites (e.g. ascorbate, glutathione, α-tocopherol) and, antioxidant enzymes that can neutralize free radicals and reduce the potential damage (62, 63). Antioxidants have been defined as compounds that are able to prevent and protect cells against the damaging effects of ROS preventing or slowing the oxidation of other molecules (10, 64, 65). Usually, an antioxidant can protect against metal toxicity by trapping free radicals thus terminating the chain reaction, by chelating metal ion and preventing the reaction with reactive oxygen species or by chelating metal and maintaining it in a redox state leading to its incompetency to reduce molecular oxygen (65). Antioxidants, such as ascorbic acid (AsA) and glutathione (GSH), together with ROS-scavenging enzymes have been shown to be essential for ROS detoxification during normal metabolism, and particularly during stress (66). Among the most important antioxidant enzymes there are superoxide dismutase (SOD. EC 1.15.1.1), ascorbate peroxidase (APX. EC 1.11.1.11), catalase (CAT. EC 1.11.1.6), glutathione peroxidase (G-POX. EC 1.11.1.7) and glutathione reductase (GR. EC 1.6.4.2) (10, 63). These antioxidant enzymes are not consumed during their catalytic actions, and they have high affinity and rate of reaction with ROS consequently allowing more effective protection against acute massive oxidative insults (64).

Antioxidant enzymes

Among the antioxidant enzymes superoxide dismutases (SODs) constitute the first line of defence against ROS and are present in all subcellular locations as they regulate the concentration of $O_2^{\cdot -}$ and H_2O_2 ($O_2^{\cdot -} \xrightarrow{SOD} H_2O_2 + H_2O$) (67, 68). SOD was discovered in the late 1960s and is now thought to be present in all organisms except strict anaerobes. The discovery of SOD was a crucial development leading to the widespread acknowledgement that univalent reduction of oxygen to superoxide occurred in biological systems alongside tetravalent reduction to water (69). Three isozymes of SOD, namely manganese superoxide (Mn-SOD, located in the chloroplast, cytosol and peroxisomes),

copper/zinc superoxide (Cu/Zn-SOD, located in the chloroplasts, cytosol and apoplast) and Fe-SOD (located in the chloroplasts) have been reported in various plant species (46, 70). SOD scavenges superoxide, one of the first ROS to be produced, dismutating it to oxygen and H_2O_2. Although this reaction only converts one ROS to another, and H_2O_2 also needs to be destroyed since it promptly attacks thiol proteins. The major enzymatic cellular scavengers of H_2O_2 are CAT and APX. However, they have different affinities for this ROS and seem to have different cellular roles in H_2O_2 scavenging. CAT does not need a reductant to scavenge H_2O_2 making it reducing power-free, whereas APX needs a reductant, ascorbate. On the other hand, CAT has a lower affinity for H_2O_2 (mM range) than APX (µM range) (45, 46). All this gathered has led to the hypothesis that APX, an enzyme located in every cellular ROS producing compartment, might function as a fine regulator of intracellular ROS steady-state levels, possibly for signalling purposes, whereas CAT located exclusively in the peroxisomes, might function as a bulk remover of excess ROS production under stress conditions (46).

ROS regulation in different organelles during water deficit

The various scavenging enzymes encoded by the ROS network can be found in almost every subcellular compartment (fig. 3) (71).

Chloroplast: The reaction centres of PSI and PSII in chloroplast thylakoids are a major site of ROS generation (66). The generation of superoxide ions at PSI occurs by the Mehler reaction (50). These radicals are converts into H_2O_2 by the Cu/Zn-SOD and consequently converted to water by the thylakoid-ascorbate peroxidase (tylAPX) this is also referred to as the water–water cycle (fig 3A). PrxRs associated with the thylakoid membrane together with thioredoxin (Trx) can also provide antioxidative protection enabling an alternative water–water cycle for detoxification of photochemically produced H_2O_2 in chloroplasts (72). GR is a potential enzyme of the AsA-GSH cycle and plays an essential role in defence system against ROS by sustaining the reduced status of GSH. GR catalyzes the reduction of GSH, a molecule involved in many metabolic regulatory and antioxidative processes in plants

where GR catalyses the NADPH dependent reaction of disulphide bond of GSSG and is thus important for maintaining the GSH pool (73).

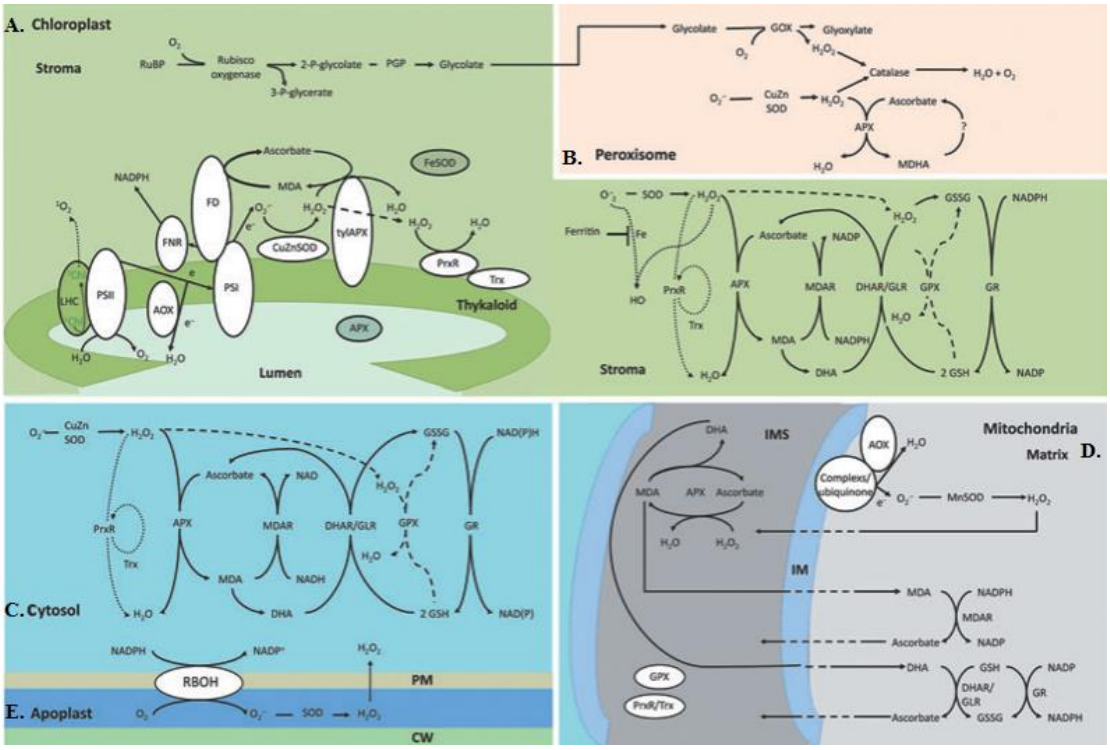

Figure 3 - Localization of ROS generation and scavenging pathways in plant cells. (A) chloroplast, (B) peroxissomes, (C) cytosol, (D) mithocondria and (E) apoplast. Abbreviations: CW, cell wall; DHA, dehydroascrobate; DHAR, DHA reductase; FD, ferredoxin; FNR, ferredoxin NADPH reductase; GLR, glutaredoxin; GPX, glutathione peroxidase;GR, glutathione reductase; GOX, glycolate oxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; LHC , light-harvesting complex; IM, inner membrane; IMS, IMspace; MDA, monodehydroascorbate; MDAR, MDA reductase; PGP, phosphoglycolate phosphatase; PM, plasma membrane; PrxR, Peroxiredoxin; PSI, photosystem I; PSII, photosystem II; RuBP, ribulose-1,5-bisphosphate; Rubisco, RuBP carboxylase oxygenase; Trx, thioredoxin; tyl, thylakoid. (Adapted: Miller *et al.*, (66)).

Peroxisomes: CATs localized mainly in peroxisomes are the major antioxidative enzymes that detoxify H_2O_2, under increased photorespiration conditions (71, 74). AsA-GSH cycle and APX can also contribute to the scavenging of H_2O_2 in peroxisomes (fig. 3B). SOD converts O_2^- into O_2 and H_2O_2 (75).

Cytosol: G-POX can be distinguished from APX in terms of differences in sequences and physiological functions (73). G-POX is another enzyme that defence against biotic stresses by consuming H_2O_2 (73). In this cellular compartment there are another antioxidant enzymes presents, such as, APX and SOD (fig. 3C).

Mitochondria: Mitochondria generates smaller amounts of ROS than chloroplasts and peroxisomes (66). Mitochondrial alternative oxidase (AOX) and Mn-SOD are key enzymes that function in controlling the signalling pathway of complex I (fig. 3D). AOX acts to maintain the reduction state of the ubiquinone (UQ) pool and lower ROS production in mitochondria, while Mn-SOD converts O_2^- into O_2 and H_2O_2 in the initial step of the ROS detoxification (61, 76, 77).

Apoplast: The main antioxidant enzyme present in the apoplast is SOD. The SODs remove O_2^- into O_2 and H_2O_2 by catalyzing its dismutation, one O_2^- being reduced to H_2O_2 and another oxidized to O_2 (fig. 3E) (73).

Non-enzymatic ROS scavenging

Among the non-enzymatic antioxidants the major cellular redox buffers are AsA and GSH (44). GSH is oxidized by ROS forming oxidized glutathione (GSSG) and ascorbate is oxidized to monodehydroascorbate (MDA) that is reduced back to AsA by either reduced ferredoxin (78), or by NAD(P)H, catalysed by MDA reductase (MDAR) (50, 79) and dehydroascorbate (DHA), which can be reduced back to GSH and ascorbate, respectively through the ascorbate-glutathione cycle (44). GSH is particularly important in plant chloroplasts because it helps to protect the photosynthetic apparatus from oxidative damage (73).

Other antioxidant compounds playing important role in ROS scavenging are α-tocopherol, flavonoids, alkaloids, carotenoids, ascorbic acid and proline (44, 73). Tocopherols are considered as a major antioxidant in biomembranes, where they play both antioxidant and non-antioxidant functions including quenching or scavenging ROS like $_1O^2$ (80). Flavonoids are among the most bioactive plant secondary metabolites. Most flavonoids outperform well-known antioxidants, such as ASH and α-tocopherol (81). This action in

ROS scavengers consists in locating and neutralizing radicals before they damage the cell thus important for plants under adverse environmental conditions (82). In all photosynthetic organisms, the carotenoids β-carotene and zeaxanthin and tocopherols serve an important photoprotective role, either by dissipating excess excitation energy as heat or by scavenging ROS and suppressing lipid peroxidation. Carotenoids, a lipid soluble antioxidant compound play a multitude of functions in plant metabolism including oxidative stress tolerance (73).

Plants accumulate compatible solutes, such as proline and glycine-betaine, in response to drought and salinity to facilitate water uptake (66). In addition to osmotic adjustments, these osmolytes were suggested to be important for protecting cells against increased levels of ROS accumulation under stress conditions (66). More than an osmolyte, proline is also considered a strong antioxidant and potential inhibitor of PCD (programmed cell death) (83).

Lipid peroxidation

The peroxidation of lipids is considered one of the most damaging processes known to occur in every living organism. Membrane damage is sometimes taken as a single parameter to determine the level of lipid destruction under several stresses (73). Lipid peroxidation (LPO) forms some products, such as MDA ketones, that react with thiobarbituric acid (TBA) to form coloured products called thiobarbituric acid reactive substances (TBARS). LPO takes place when above-threshold ROS levels are reached, thereby not only directly affecting normal cellular functioning, but also increasing the oxidative stress through production of lipid-derived radicals (84). The total process of LPO involved three distinct stages: initiation, progression and termination steps (fig. 4). In the initiation step started by the abstraction of a hydrogen atom, in an unsaturated fatty acyl chain of a polyunsaturated fatty acid (PUFA) residue, mainly by $OH^{\cdot}$ the oxygen will add to the fatty acid at the carbon-centered lipid radical to give rise to a $ROO^{\cdot}$. Once originated, $ROO^{\cdot}$ can further propagate the peroxidation chain reaction by abstracting a hydrogen atom from adjacent PUFA side chains. The resulting lipid hydroperoxide can decompose into several reactive species including: lipid alkoxyl radicals, aldehydes (malonyldialdehyde), alkanes, lipid epoxides and alcohols (fig. 4A) (73, 85). It has been found that the PUFAs (linoleic acid (18:2) and linolenic acid (18:3)) are particularly susceptible to attack to $_1O^2$

and $HO^{\cdot}$, giving rise to complex mixtures of lipid hydroperoxides (fig. 4B). Increased PUFA peroxidation decreases the fluidity of the membrane, increases leakiness and causes secondary damage to membrane proteins (86). Several aldehydes such as 4-hydroxy-2-nonenal (HNE) and MDA, as well as hydroxyl and keto fatty acids, are formed as a result of PUFA peroxidation (fig. 4C). The aldehyde breakdown products can form conjugates with DNA and protein (86).

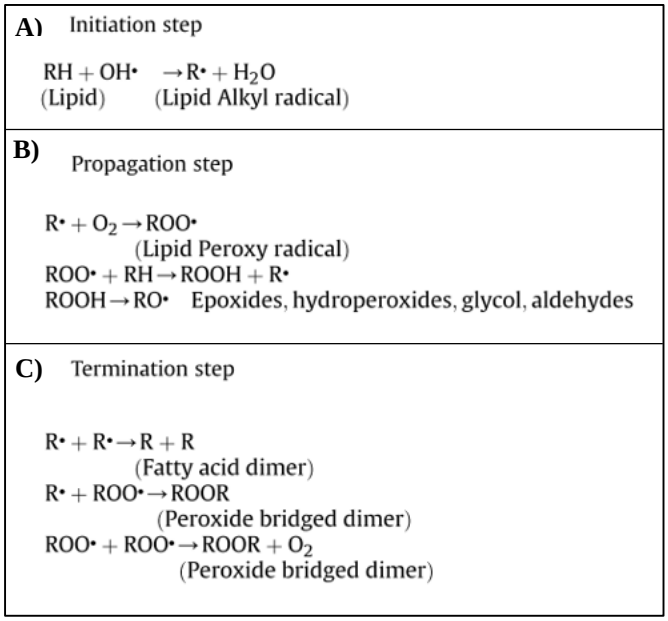

Figure 4 - Different stages of LPO. (A) initiation step, (B) propagation step and (C) termination step (Adapted: Gill and Tuteja (73)).

Oxidative signalling

Typically, more damages are observed under stress conditions when the ROS levels are increased. At the same time the oxidized products can be important secondary signalling molecules, and in such cases damages and signalling are two sides of the same story (fig. 5) (86).

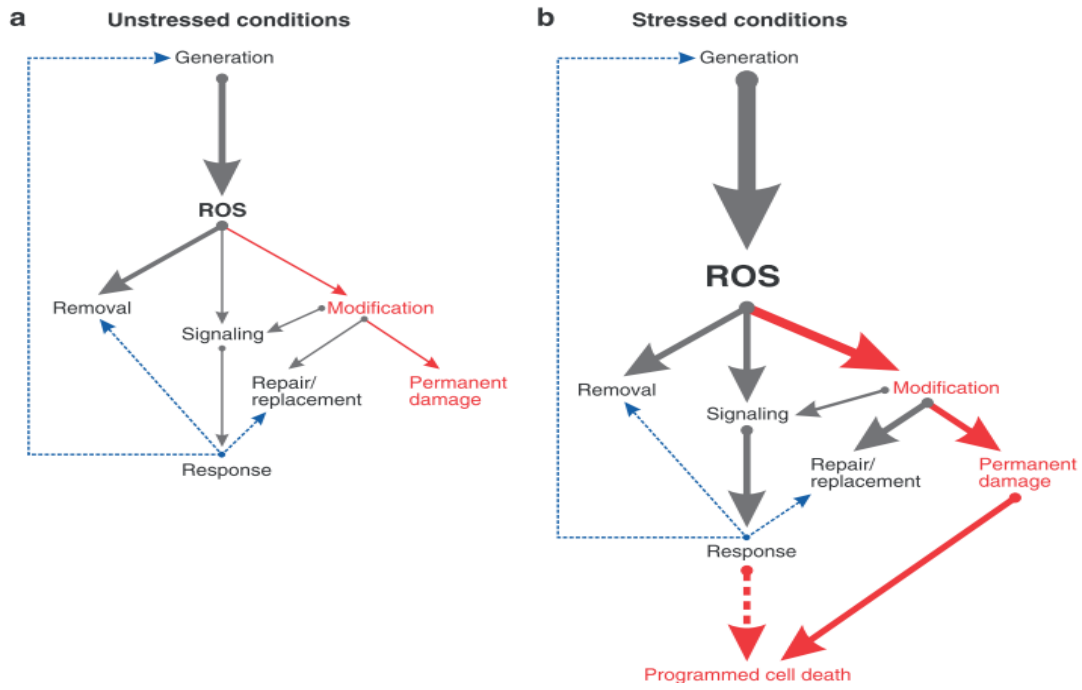

Figure 5 - The relationship between ROS production, removal, modification, signaling, and damage in plant cells under (A) unstressed and (B) stressed conditions. The arrow from "Modification" to "Signalling" indicates that some of the modified molecules are secondary signal molecules (Removed: Møller *et al.*, (86)).

At low and moderate concentrations, ROS have been implicated as second messengers in intracellular signalling cascades that mediate several plant responses in plant cells, including stomata closure (87, 88), programmed cell death (45), gravitropism, and acquisition of tolerance to both biotic and abiotic stresses (89). ROS can be sensed directly also by key signalling proteins such as a tyrosine phosphatase through oxidation of conserved cysteine residues (90). ROS can also modulate the activities of many components in signalling, such as protein phosphatases, protein kinases and transcription factors (91) and communicate with other signal molecules and the pathway forming part of the signalling network that controls response downstream of ROS (89).

Relative stability of H_2O_2 compared with other ROS and its ability to cross the membrane makes it suitable for signalling. It can induce the intracellular ROS scavenging system by activating the antioxidant enzymes and also by modulating the expression of genes of these enzymes (87). For the purpose of signalling, either in case of stress responses or growth and development, the place and amount of ROS production should be under tight control (44). Biochemical evidence indicated that a plant mitogen activated kinase (MAPK) cascade is responsible for relaying the H_2O_2 signal (91) once that H_2O_2 can modulate gene expression via activation of transcription factors (44). However, plants possess an unusually high number of MAPKs, and the kinase network can be a convergence as well as a divergence point for different stress factors (92). Oxidative stress-activated MAP triple-kinase 1 (OMTK1) is a more specific MAPK kinase that can be activated only by H_2O_2 and not by abiotic stresses or hormones in alfalfa (93). OMTK1 can specifically activate the downstream MAP kinase MMK3, which results in cell death. MMK3 can be activated also by ethylene and elicitors, thus serving as a convergence point of the cell death network (fig. 6) (93).

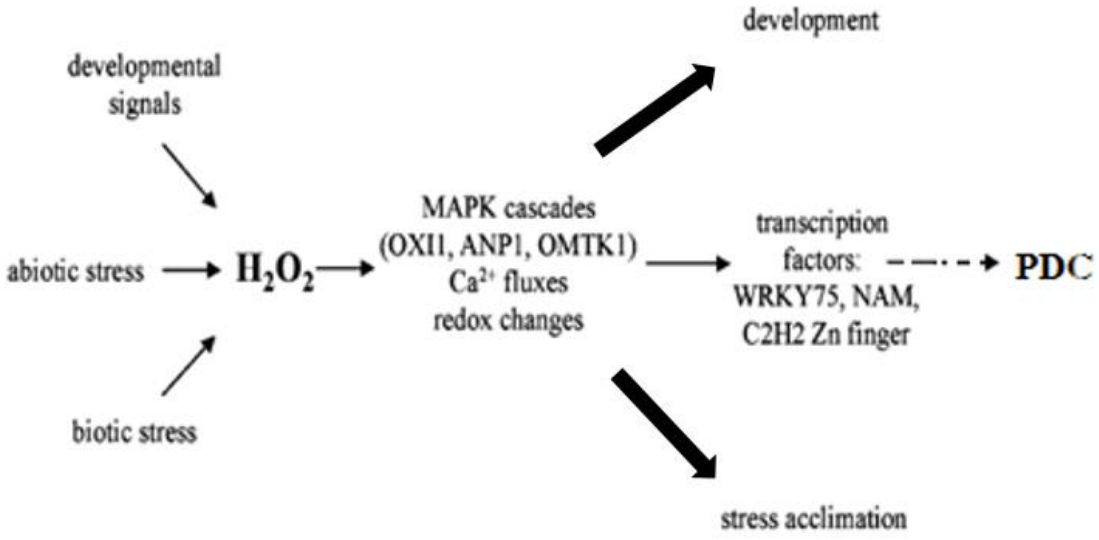

Figure 6 - Biological processes leading to and regulated by H_2O_2. Abbreviations: MAPK, mitogen-activated protein kinase; OXI1, oxidative signal inducible kinase; ANP1, Arabidopsis mitogen-activated protein kinase kinase kinase; OMTK1, oxidative stress-activated MAP triple-kinase 1; WRKY75, putative WRKY transcription factor 75; NAM, no apical meristemtranscription factor; C2H2 Zn finger, zinc finger (C2H2 type) family protein (ZAT11); PDC, programmed cell death (Adapted: Gechev and Hille (91)).

THESIS MAIN PURPOSES

Medicinal plants have been worldwide explored due to their high amounts of antioxidants that scavenge free radicals with their concomitant oxidation and formation of a more stable free radical. In particular, the medicinal plant *M. azedarach* exhibited a great antioxidant activity. This species is largely used in etnobotany practices. Thus, since the enhancement of the antioxidant capacity was reported in several species under abiotic stress conditions it is important to assess the effects of drought stress on the antioxidant capacity of *M. azedarach.* The putative enhancement of the antioxidant capacity in this kind of species under stress conditions could be of a great value, not only for re/afforestation of drought prone areas and but also for medicinal and human feeding purposes. In the present study, the effects of drought stress imposition on physiological and antioxidant capacity characteristics, including the water potential, plant growth, photosynthesis, the content on pigments, H_2O_2, proline, the activities of several antioxidant enzymes, non-enzymatic antioxidant contents, cell membrane permeability and the lipid membrane peroxidation in *M. azedarach* were investigated to test the hypothesis that drought stress enhances the antioxidant capacity of *M. azedarach*.

MATERIALS AND METHODS

Plant material and culture conditions

Seeds of *Melia azedarach* L. from a mature tree in the centre of Portugal (Aveiro), were germinated in a mixture of turf and vermiculite (2:1). Cultures were maintained in a growth chamber with a temperature of 20 ± 2°C, a 16/8-h (day/night) photoperiod and a photosynthetic photon flux density (PPFD) of app. 400 ± 20 µmol m^{-2} s^{-1}. Prior to water deficit treatment, all plants were well-watered. Two months old *M. azedarach* plants of similar size were randomly assigned to each of the two treatments as follows: (1) well-watered (WW): plants were watered daily at 80 % of field capacity, and (2) water stress (WS): plants were watered at 20% of field capacity. After 20 days plant performance was evaluated through assessment of physiological parameters and oxidative stress parameters.

Plant water status and plant growth

After 20 days of treatment, plant water potential (Ψ) was measured with a Scholander-type pressure chamber (PMS Instrument Co., OR) on abscised stems just above the soil surface, according to Scholander *et al.*, (94).

Plants height was determinate at the end of the experiment. Additionally, leaves and roots dry weight (DW) was recorded for total biomass determination.

Pigment contents

Pigments were extracted in a cold acetone/Tris 50 mM pH 7.8 buffer solution and centrifuged according to Sims and Gamon (95). Absorbances at 470, 537, 647 and 663 nm were determined with a Thermo Fisher Scientific (Waltham, USA) spectrophotometer (Genesys 10-uv S). For the determination of anthocyanins, leaf disks were extracted with a methanol/HCl 1% water buffer according to Sims and Gamon (95). Absorbances at 529 and 650 nm were determined with a Thermo Fisher Scientific (Waltham, USA) spectrophotometer (Genesys 10-uv S). The contents of chlorophyll (Chl) *a*, Chl *b*,

carotenoids (Cars) and anthocyanins were calculated using the formulae described by Sims and Gamon (95).

Gas exchange and Chlorophyll a fluorescence

In situ leaf gas exchange measurements (net CO_2 assimilation rate: *A*, transpiration rate: *E*, and the intercellular CO_2 concentration: *Ci*) were performed using a portable infrared gas analyser (*LCpro+*, *ADC*, Hoddesdon, UK), operating in open mode under growth chamber conditions. The stomatal conductance (g_s) were automatically calculated according to Von Caemmerer and Farquhar (96). Measurements were always performed in the middle of the photoperiod at growth temperature (24 ± 2 ºC) and atmospheric CO_2 concentration in the youngest fully developed leaf.

Chl *a* fluorescence measurements were determined *in situ* in full expanded leaves of WW and WS plants, with a portable fluorimeter Mini-PAM (Walz, Effeltrich, Germany). Minimal fluorescence (F_0) was measured in 30 min dark-adapted leaves by applying a weak modulated light and maximal fluorescence (F_m) was measured after a 1 s saturating pulse of white light (>1500 μmol m^{-2} s^{-1}) in the same leaves. In light adapted leaves, steady state fluorescence (F´), maximal fluorescence (F_m´) after 1 s saturating pulse (>1500 mol m^{-2} s^{-1}) and minimal fluorescence (F_0´) measured when actinic light was turned off, were determinate. Definitions of fluorescence parameters (F_v/F_m - maximal efficiency of PSII and Φ_{PSII} - effective quantum efficiency of PSII) were used as described by Van Kooten and Snel (97).

Soluble sugars, starch and proline

Soluble sugars were extracted with 80 % (v/v) of ethanol at 80 ºC for 20 min as described by Correia *et al*., (98). Glucose, fructose and sucrose were quantified using a spectrometric enzyme-coupled assay described by Jones *et al*., (99) and starch was quantified in accordance with Stitt *et al*., (100) in a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S).

Free proline was extracted and determined as described by Khedr *et al.,* (101). Leaf samples were extracted with 3% sulphosalicylic acid. Extracts were maintained for 1h at 100ºC with the addition of glacial acetic acid and acid ninhydrin. Cold toluene was added afterwards and shaken. Absorbance was read at 520 nm. The amount of proline was determined from a standard curve.

Cell membrane permeability and lipid peroxidation

Electrolyte leakage was used to assess cell membrane permeability as described by Lutts (102). Leaf segments were detached, washed with deionized water, placed in closed vials containing 20 mL of de-ionized water and incubated over night at 25ºC, on a rotary shaker. Electrical conductivity of the bathing solution (L_t) was determined after 24h. Samples were then autoclaved at 120ºC for 20 min and a last conductivity reading (L_0) was obtained upon equilibration at 25ºC. The electrolyte leakage was defined as L_t/ L_0 and expressed as percentage.

Lipid peroxidation on leaves was obtained by measuring malondialdehyde (MDA) production (103). Approximately 0.5 g of leaves were homogenized with 5 mL of 0.1% TCA (w/v) and centrifuged according to Dias *et al.,* (62). After centrifugation, 1 mL of supernatant was mixed with 4 mL 20% TCA (w/v) in 0.5% TBA (w/v) and incubated for 30 min at 95ºC. The extract was then cooled immediately on ice to stop the reaction and centrifuged. MDA concentration was estimated by subtracting the nonspecific absorption at 600 nm from the absorption at 532 nm using an absorbance coefficient of extinction (ε), 155 mM^{-1} cm^{-1}.

Activities of antioxidant enzymes

For the determination of antioxidant enzyme activities, leaves (0.5 g) were homogenized in 5 mL of extraction buffer in a pre-chilled mortar and pestle by liquid nitrogen. The extraction buffer contained 0.1 M potassium phosphate buffer (pH 7.5), 0.5 mM Na_2EDTA, 1% PVP (m/v), PMSF 1 mM, 0.2% Triton X-100 (v/v) and 2 mM DTT. Homogenates were centrifuged in a refrigerated centrifuge at 8000 g for 15 min filtered through four layers of cheesecloth and centrifuged at 15000 g for 30 min at 4ºC. The

supernatant obtained was used for enzyme assays (SOD, CAT, APX, GR and G-POX). SOD (EC 1.15.1.1) was determined according to the method of Agarwal *et al.,* (104). The reaction mixture contained 13.3 mM methionine, 63 µM NBT, 0.1 mM EDTA, 50 mM potassium phosphate buffer (pH 7.8), 50 mM Na_2CO_3, and an aliquot of extract. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT in comparison with control. CAT (EC 1.11.1.6) activity was assayed at 25ºC as described by Beers and Sizer (105). Assay mixture contained 0.1 M potassium phosphate buffer (pH 7.0) and enzyme extract. To start the reaction, 20 mM H_2O_2 ($\varepsilon = 39.4$ mM^{-1} cm^{-1}) was added and the decrease of absorbance at 240 nm was recorded. APX (EC 1.11.1.11) activity was determined at 25ºC by recording the decrease in absorbance at 290 nm due to ascorbic acid ($\varepsilon = 2.8$ mM^{-1} cm^{-1}) oxidation to dehydro-ascorbate by H_2O_2, according to the method of Nakano and Asada (106). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, and an aliquot of enzyme extract, and 0.5 mM H_2O_2 was added to start the reaction. The expression of one unit of APX activity was mmol of ascorbic acid oxidized per minute, calculated using its extinction coefficient (ε) 2.8 mM^{-1} cm^{-1}. GR (EC 1.6.4.2) activity was determined at 30ºC according to Sgherri *et al.,* (107). The reaction mixture contained 0.2 M potassium phosphate (pH 7.5), 0.2 mM Na_2 EDTA, 1.5 mM $MgCl_2$, 0.25 mM of oxidized glutathione (GSSG), and enzyme extract. The reaction was started with 25 µM NADPH, and the decrease in absorbance at 340 nm was monitored. The GR activity was calculated using the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}). For G-POX (EC 1.11.1.7) determination, the reaction mixture contained 10 mM phosphate buffer (pH 6.1), 12 mM hydrogen peroxide, 96 mM guaiacol and 50 µL enzyme extract. Absorbance was recorded at 470nm according to Castillo *et al.,* (108) and the specific activity was calculated using the 26.6 mM^{-1} cm^{-1} molar extinction coefficient.

Content of AsA-GSH cycle non-enzymatic antioxidants

Total AsA (TA, AsA + DHA), reduced AsA and DHA (dehydroascorbate) concentrations were determined as Jin *et al*., (109) with some modifications. Briefly, frozen samples were extracted with TCA 5% and centrifuged. TA was determined in a mixture of 0.2 mL of supernatant, 0.25 mL of 150 mM phosphate buffer (pH 7.4) (containing 5 mM

EDTA), and 0.1 mL of dithiothreitol (DTT) 10 mM. After incubation for 10 min at room temperature, 0.05 mL of 0.5% (w/v) N-ethylmaleimide was added. The AsA was assayed in a similar manner except that instead of DTT, 100 µL of ultra pure water were added. Color was developed in both reaction mixtures after the addition of 0.2 mL of 10% (w/v) TCA 10%, 0.2 mL of 44% (v/v) phosphoric acid, 0.2 mL of α,α'-dipyridyl in 70% (v/v) ethanol, and 0.1 mL of 3% (w/v) $FeCl_3$. After incubation at 40◦C for 40 min, the absorbance was read at 532 nm using AsA as a standard. Standard curves for total AsA and AsA were established. DHA was estimated from the difference between TA and AsA.

Total glutathione (reduced glutathione (GSH) plus GSSG) and GSSG were measured according to Ma *et al.*, (110) with some modifications. Frozen samples were homogenized with 5-sulphosalicylic acid and then centrifuged. Total glutathione was measured in a reaction mixture (1 mL) consisting of 100 µL supernatant, 200 mM phosphate buffer solution (pH 7.2) containing 5 mM EDTA and 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.2 mM NADPH and 1U GR. The reaction mixture was then incubated at 27ºC for 30 min and quantified at 412 nm. GSSG was analysed in a similar manner, except that the supernatant was previously incubated with 0.17 M 2-vinylpyridine and 3.6% triethanolamine at 27◦C for 1h. Standard curves for GSH and GSSG were established. For each sample, GSH was estimated from the difference between total glutathione and GSSG.

Quantification of H_2O_2

The concentration of H_2O_2 was measured according to Zhou *et al.*, (111). Briefly, half gram of a leaf tissue was ground in 5 mL precooled 5% TCA (w/v) and activated charcoal. After centrifugation, extracts were adjusted to pH 8.4 with 17 M ammonia, and H_2O_2 was spectrophotometrically quantified following its reaction with 4-amino-antipyrine and phenol to form a stable red product in the presence of 150 U mg^{-1} peroxidase. Blanks containing 8 µg CAT (EC 1.11.1.6) were run for each sample as well as for the calibration with H_2O_2 standards, which were added to the extraction medium in parallel to the samples.

Data analysis

The experiment was repeated twice (two independent assays): for each experiment, 20 plants were used, 10 plants were maintained under WW conditions and the other 10 plants were exposed to WS. Data were analysed by t-test at a significant level set to 0.05 using Sigma Stat for Windows version 3.1.

RESULTS

Plant water status and plant growth

Twenty days after the beginning of the WS treatment plants became wilted (visual observation). No visual symptoms of chlorosis or necrosis were observed. Moreover, morphologically both WW and WS plants looked similar. Plant water status was evaluated by the measurement of the Ψ and the results corroborated with visual observation: the Ψ decreased 27% in plants under WS conditions compared to plants under WW conditions (p<0.05) (table 1).

Concerning plant growth, no significant differences were observed in plant height and total plant DW accumulation between plants under WW and WS conditions (table 1).

Table 1 – Ψ (MPa), height (cm), plant total DW (g), maximal efficiency of PSII (F_v/F_m), effective quantum yield of PSII (ΦPSII), sugar content (µmol g^{-1}FW) and pigment contents (mmol g^{-1}FW) in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (n= 6). Different letters indicate significant differences between tratments (p<0.05).

Parameter	WW	WS
Ψ	-0.76 ± 0.10[a]	-1.04 ± 0.08[b]
Height	14.0 ± 2.53[a]	13.8 ± 1.96[a]
Plant total DW	3.11 ± 0.99[a]	2.42 ± 0.62[a]
F_v/F_m	0.79 ± 0.02[a]	0.74 ± 0.10[a]
Φ_{PSII}	0.45 ± 0.02[a]	0,29 ± 0.03[b]
Glucose	25.8 ± 5.22[a]	66.9 ± 4.35[b]
Fructose	31.1 ± 2.92[a]	41.5 ± 8.82[a]
Sucrose	28.8± 2.01[a]	15.2 ± 3.60[b]
Starch	234.7 ± 12.8[a]	237.6 ± 10.9[a]
Chl *a*	271.1 ± 44.07[a]	250.2 ± 13.18[a]
Chl *b*	101.5 ± 18.25[a]	98.8 ± 1.98[a]
Carotenoids	187.4 ± 21.96[a]	183.5 ± 4.85[a]
Anthocyanins	0.02 ± 0.008[a]	0.03 ± 0.005[a]

Photosynthetic parameters, pigment, sugar and proline content

No significant differences were observed in the F_v/F_m values between WW and WS plants (table 1). The Φ_{PSII} decreased significantly 35% in WS plants relatively to plants under WW conditions (table 1). Plants under WS conditions showed a significantly lower *A*, g_s, *E* and C_i values than plants under WW conditions (fig. 1A, B, C and D). WW plants showed a *A* approximately 4 times higher than WS plants.

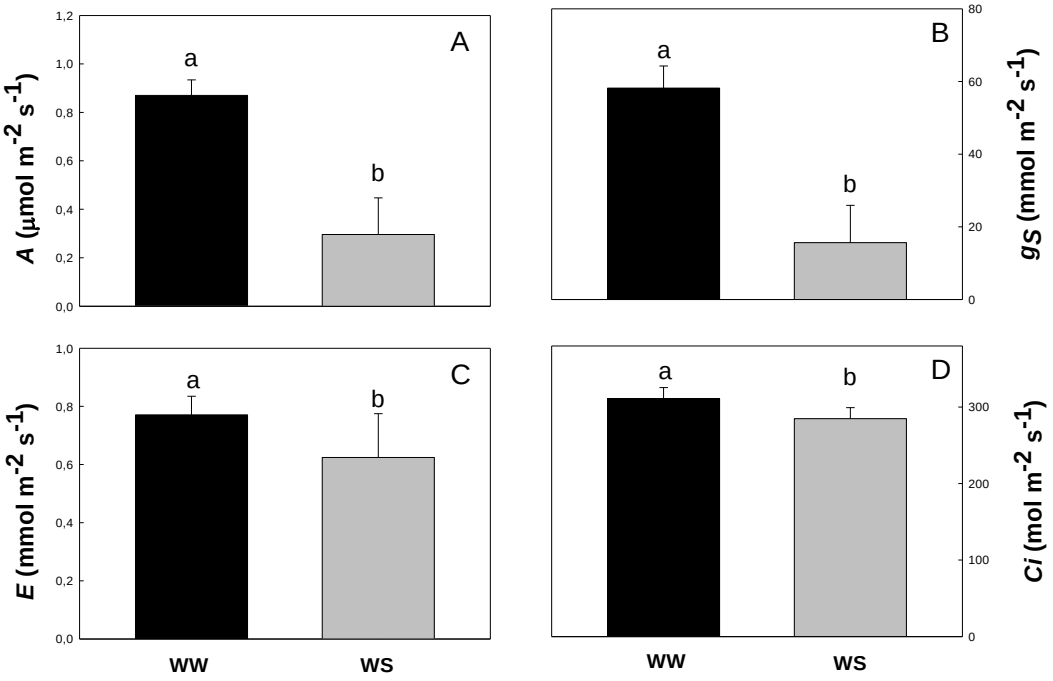

Figure 1 – Net CO_2 assimilation rate (*A*) (A), stomatal conductance (g_s) (B) transpiration rate (*E*) (C) and intercellular CO_2 concentration (*Ci*) (D) in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (n=12). Different letters indicate significant differences between tratments (p<0.05).

Plants exposed to WS conditions showed a similar Chl *a*, Chl *b*, and carotenoids content to plants under WW conditions (p>0.05) (table 1). Concerning the anthocyanin contents, no significant differences were observed between WW and WS plants (table 1).

Relatively to sugar content, significantly differences were found between treatments for glucose and sucrose content (table 1). Glucose content significantly increased (2.6 times, relatively to WW plants) in WS plants, while sucrose content significantly decreased (1.9 times, relatively to WW plants) in WS plants. In contrast, no significantly differences in the fructose and starch content were observed between treatments (table 1).

A strong decreased (83%) in proline content were observed in plants under WS conditions compared to WW plants (p<0.05) (fig. 2).

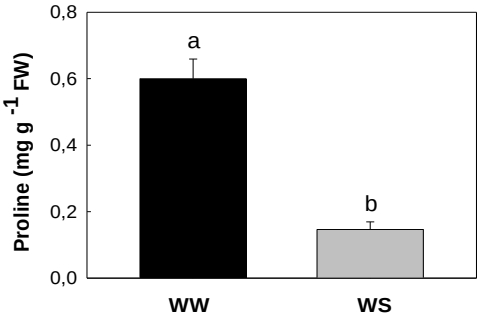

Figure 2 - Proline content in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (n= 6). Different letters indicate significant differences between tratments (p<0.05).

Cell membrane stability, lipid peroxidation and H_2O_2 content

Lipid peroxidation was measured in terms of MDA content and cell membrane permeability was determined through measurement of electrolyte leakage. No significant differences were observed in the MDA content between WW and WS plants (fig. 3A). Contrarily, plants under WS conditions showed significantly higher cell membrane permeability than plants under WW conditions (fig. 3B).

The levels of H_2O_2 in plants under WW and WS were similar (p>0.05) (fig. 3C).

Antioxidant enzyme activities and non-enzymatic antioxidant contents of AsA–GSH cycle

The antioxidant enzyme system was assessed by the measurement of the activities of five enzymes: SOD, CAT, APX, Gr and G-POX. A significant increase in the activity of CAT, SOD, APX and Gr (37%, 55%, 89% and 91%, respectively) was observed in plants under WS conditions compared to those under WW conditions (fig. 3D, E, F and G). No significant differences were observed in the activity of G-POX between WW and WS plants (fig. 3H).

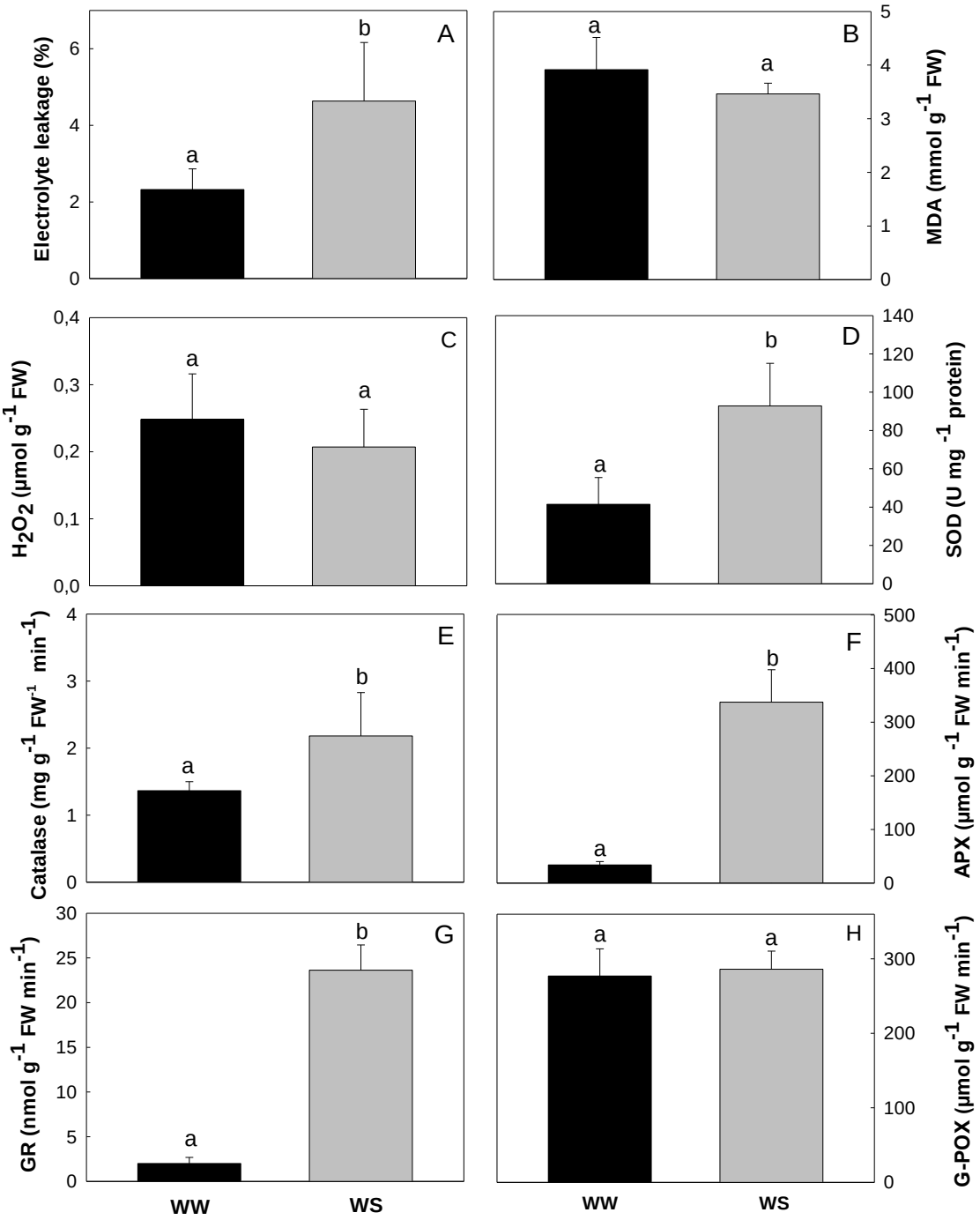

Figure 3 – Electrolyte leakage (A), MDA (B), H_2O_2 (C), SOD (D), CAT (E), APX (F), GR (G) and G-POX (H) activities in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (*n*= 6). Different letters indicate significant differences between tratments (p<0.05).

A significant increase in AsA/DHA, TA, AsA and DHA (46%, 49%, 59% and 34%, respectively) was observed in plants under WS conditions compared to those under WW

conditions (Fig. 4A, B, C and D). However, no significant differences were observed in the level of GSSG, GSH, total glutathione and GSH/GSSG between WW and WS plants (table 2).

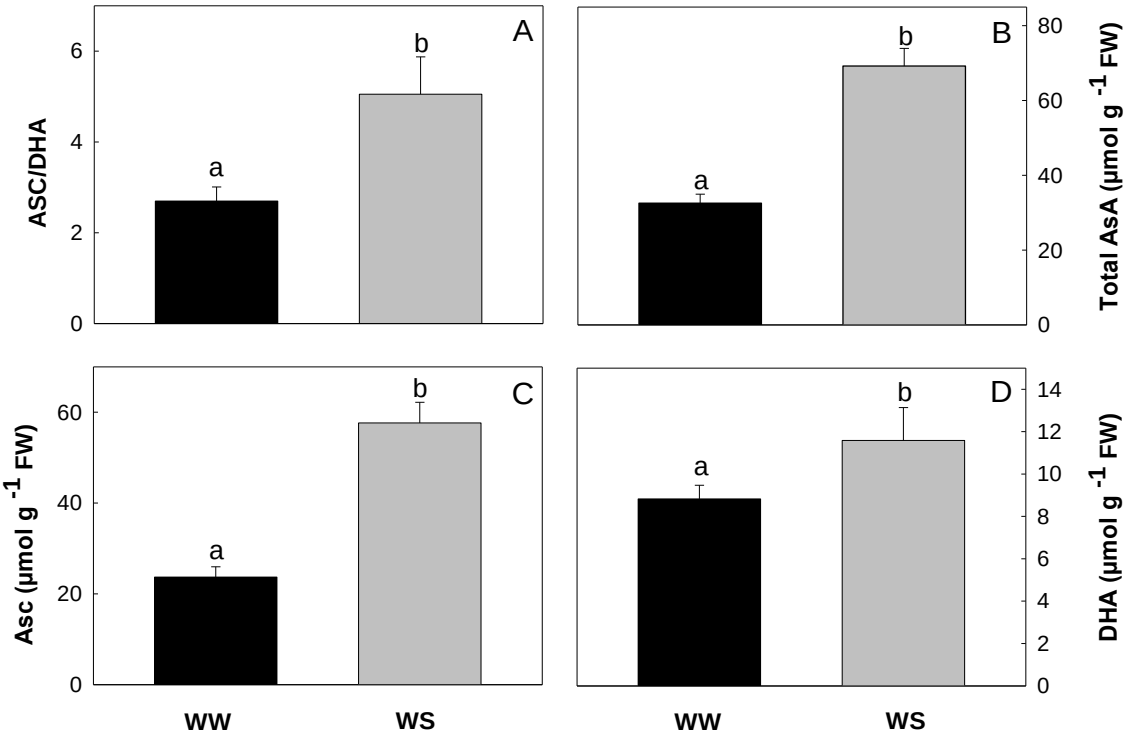

Figure 4 - Ratio of AsA to DHA (ASC/DHA) (A), total content of ascorbate (TA) (B), content of ascorbate (AsA) (C), and content of DHA (DHA) (D) in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (*n*= 8). Different letters indicate significant differences between tratments (p<0.05).

Table 2 – Concentration of GSSG (µg mL^{-1}), GSH (µg mL^{-1}), total glutathione (µg mL^{-1}) and the ratio of GSH to GSSG in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (*n*= 4). Different letters indicate significant differences between tratments (p<0.05).

Parameter	WW	WS
GSH	34.3 ± 5.6[a]	19.5 ± 1.4[a]
GSSG	20.9 ± 3.8[a]	14.6 ± 2.8[a]
Total glutathione	45.6 ± 8.4[a]	34.2 ± 1.6[a]
GSH/GSSG	0.86 ± 0.16[a]	0.76 ± 1.19[a]

DISCUSSION

Due to global climate change, scientific climate prediction appointed that drought may become more common in the future (112). Over the last years the impact of these climate changes (in particular, drought and temperature increase) on plant performance have been studied (113, 114), but the effects of WS events on the antioxidant capacity of medicinal plants have been poorly explored. To the best of our knowledge this is the first report that evaluated the effects of WS on the physiological status and antioxidant capacity of the medicinal plant *M. azedarach*.

After the WS imposed, plants presented a wilted aspect (visual observation). Plant water status was evaluated by the measurements of the Ψ and corroborated with visual observation: WS imposition resulted in reduced plant Ψ. This result in further stomatal closure (*E* and g_s decreased) and *A* impairment. Stomatal closure is among the earliest responses to WS, protecting plants from extreme water loss, which can results in cell dehydration and eventually in plant dead (36). WS induced stomatal closure in *M. azedarach* to prevent water loss, but also limited the CO_2 availability in the intercellular spaces of the mesophyll cells, as evidenced by the low C_i. Although WS affected negatively the *A*, these effects were already not so remarkable in the plant growth (total DW and plant height).

Sugars are the final products of photosynthesis. WS led to the decrease of succrose, which could be in part related to the general tendency of increased glucose and fructose. Sucrose decrease could result from an increased carbon demand of sink organs probably due to a reduction in carbohydrate concentration availability or due to a decrease in sucrose phosphate synthase enzyme activity (115). However, WS did not affect the levels of the reserve sugar, starch.

The F_v/F_m characterizes the maximal efficiency of excitation energy capture by "open'' PSII reaction centres and this parameter is usually used as a sensitive indicator of plant photosynthetic performance (116). WS did not affected this parameter and the F_v/F_m values obtained are typical of healthy plants (117). Analyses of the Φ_{PSII} revealed that the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry is reduced by WS. This reduction may be mainly related to a decrease of the proportion of the PSII reaction centres in the "open" state (q_P), since Chl *a* levels were not affected. Correia *et al.* (118) also observed that WS impositions in sunflowers did not affect

the F_v/F_m but reduced the Φ_{PSII}. Moreover, the Φ_{PSII} was found to be reduced in several plant species under WS conditions (119, 120, 121).

Photosynthetic pigments are important to plants mainly for harvesting light and production of reducing powers, nonetheless carotenoids have additional roles and partially help the plants to withstand adversaries of drought (122). Carotenoids act as light-harvesting pigments, and can protect chlorophyll and membranes destruction by quenching triplet chlorophyll and removing oxygen from the excited chlorophyll–oxygen complex (40). WS imposition in *M. azedarach* did not affect the level of carotenoids and chlorophylls. Besides carotenoids, also anthocyanins are important pigments in plant photoprotection. Several studies have presented evidences that anthocyanins protect the photosynthetic apparatus from photoinhibition by absorbing green light and thereby reducing excess excitation energy (123, 124). Moreover, anthocyanins seem to have a high ability to quench oxidants and to mitigate the formation of ROS (124, 125, 126). According to our results, anthocyanins seem not to have an important role in *M. azedarach* plant protection under stress conditions.

One of the most common stress responses in many plants species exposed to different abiotic stresses is an enhanced production of different types of compatible organic solutes such as proline, glycine-betaine and choline (121). Proline is a compatible solute that can have a major role in osmotic adjustment but also have a number of other protective roles such as membrane structures protection ROS scavenging (121, 127). In *M. azedarach*, proline does not seem to have an important role as an osmoprotectant or even as a ROS scavenging. Contrarily to our results, proline accumulation has been shown in different abiotic stressed species, including water deficit (127, 128). It is possible that in *M. azedarach* other osmolytes, e.g. glycine-betaine, may have a more important role in osmoprotection during stress.

Plant redox status depends on the delicate equilibrium between ROS production and scavenging at the proper site and time (129). Under stress conditions, this equilibrium is highly dependent on the antioxidant machinery activity. In general, our results revealed that WS induced oxidative stress in *M. azedarach* plants: despite the similar levels of lipid peroxidation (MDA) observed in WS and WW plants, cell membrane permeability analysis clearly revealed that WS stress induced membrane damages. Plants can protect themselves against oxidative damage by antioxidant system including antioxidant enzymes and non-enzymatic compounds (73). In the present work SOD activities increased in WS *M.*

azedarach plants, but the maintenance of the similar levels of H_2O_2 in both WS and WW plants could be attributed to the strong increased of APX, CAT and Gr activities under WS conditions. In particular, APX and Gr appear to be in the front line to detoxify cells against H_2O_2. Contrarily, G-POX, one of the major enzymes that scavenges H_2O_2 in chloroplasts (73), seems not to play an important role in ROS detoxification in *M. azedarach*. AsA and GSH are essential plant metabolites that regulate major cellular functions and play a pivotal role in antioxidant defence (130). AsA is present in most cellular compartments (131), serves as an electron donor and reacts with ROS. GSH exerts its antioxidant function by reaction with superoxide radicals, peroxy radicals and singlet oxygen followed by the formation of oxidized glutathione and other disulfides (131). Our results indicated that WS increase the AsA/DHA, TA, AsA and DHA in *M. azedarach*. However, WS did not affected the GSSG, GSH, total glutathione and the ratio of GSH to GSSG. Candan and Tarhan (132) and Zhu *et al.* (133) also found increased levels of AsA in medicinal plants exposed to WS, but the levels of GSH were not changed (133).

Despite the prompt response of the antioxidant enzyme system (e.g. CAT, APX and Gr activities) and the strong increased of antioxidant pools (e.g. AsA), WS still induced oxidative damages and decreased the photosynthetic performance of *M. azadarach* plants.

FINAL CONSIDERATIONS

The present work demonstrated that WS stress conditions reduced some photosynthetic parameters, and eventually may reduce plants' photosynthesis. The *A* reduction may be related to stomatal closure and to the decrease of CO_2 availability in the intercellular spaces of mesophyll cells (*Ci*). WS also reduced the photosynthetic efficiency of PSII. Despite these reductions, WS did not statistically affect plant growth (DW accumulation and plant height). Thus, *M. azedarach* showed potential characteristics to be a candidate species for re/afforestation programs in drought prone habitats.

WS also induces some oxidative stress in *M. azedarach* plants. However, and as hypothesized, the antioxidant capacity of *M. azedarach* increased strongly under WS conditions. WS induced an up regulation of the antioxidant enzymes, CAT, APX and Gr and also an over production of antioxidant metabolites (e.g. AsA). Therefore, the imposition of controlled WS periods could be a positive strategy to increase the antioxidant capacity of *M. azedarach*, for example for medicinal uses, without severe effects on plant growth.

Future scientific investigation related to the of *M. azedarach* antioxidant capacity *in situ* under stress conditions are of great importance to test putative changes in medicinal properties. Molecular and epigenetic studies could also be carried on in order to understand underlying mechanisms involved in the *M. azedarach* antioxidant capacity.

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ANNEX

Part of this work was presented in conferences and/or under preparation for submission to a scientific journal.

Annex 1 Poster 3º Workshop BioPlant. 4 e 5 de dezembro 2012 (page 23 of abstract book).
Annex 2 Poster V Jornadas de Genética e Biotecnologia. 20, 21 e 22 de fevereiro 2013 (page 65 of abstract book).
Annex 3 Poster V Jornadas de Genética e Biotecnologia. 20, 21 e 22 de fevereiro 2013 (page 66 of abstract book).
Annex 4 Oral presentation 4º Workshop BioPlant. 18 e 19 de julho 2013 (page 9 of abstract book).
Annex 5 paper under preparations for submission to a scientific journal (to be submitted).

Annex 1

Poster #16

Effects of water stress on the physiological performance of *Melia azedarach*

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Melia azedarach is one of the most used plants in traditional medicine. A number of biological activities have already been described for crude extracts. Among the various environmental factors that can affect plant performance, the water stress (WS) is probably the one that has more impact. WS affects photosynthesis, biomass production reducing plant growth and productivity. Timor is a country with an unstable climate due to different latitudes that causes rainfall and drought cycles. A consequence of this climate is deforestation that leads to soil erosion and floods. This work aims to analyse the physiological performance of one species found in Timor, Melia azedarach, under WS conditions for future use of this species in reforestation programs in this island. Plants of *M. azedarach* were exposed to WS (plants at 20% of field capacity) during 20 days. After this period plant performance were evaluated: the relative water content, water potential and the effective quantum yield of PSII were significantly reduced by WS; pigment content and biomass were not affected by WS. Moreover, WS did not affect cell cycle of *M. azedarach* leaves. These preliminary results indicated that WS induce a decrease of photosynthetic efficiency and affect the water relations of M. azedarach. However, others physiological parameters should be performed in order to have an in deep/complete knowledge of the performance of *M. azedarach* under WS conditions.

Annex 2

P18

Efeito do défice hídrico na *performance* fisiológica de *Melia azedarach*

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Keywords: Défice hídrico, *performance* fisiológica, *Melia azedarach*.

Melia azedarach é uma das espécies mais utilizadas na medicina tradicional. Várias atividade biológicas têm sido descritas para os extratos brutos desta espécie. Entre os vários fatores ambientais que podem afetar a *performance* das plantas, o défice hídrico é provavelmente o de maior impacto. O défice hídrico afeta a fotossintese, a produção de biomassa, reduz o crescimento da planta e a sua produtividade. Timor é um país com um clima instável devido às diferenças de latitude que causam ciclos de seca e chuva. Uma consequência deste tipo de clima, é a desflorestação que leva à erosão dos solos e cheias.

Este trabalho tem como objetivo analisar a *performance* fisiológica de uma das espécies encontradas em Timor, *M. azedarach*, em condições de défice hídrico para futuro uso desta espécie em programas de reflorestação nesta ilha. As plantas de *M. azedarach* foram expostas a défice hídrico (solo a 20% da capacidade de campo), durante vinte dias. Após este período, observou-se uma redução do teor relativo em água e do potencial hídrico nas plantas sujeitas a défice hídrico. O rendimento efetivo do PSII (fotossistema II) também foi negativamente afetado, mas, a biomassa e o conteúdo em pigmentos não foram afetados pelo défice hídrico. Além disso, o défice hídrico não afetou o ciclo celular das folhas de *Melia azedarach*. Estes resultados preliminares indicam que o défice hídrico induz a redução da eficiência fotossintética e afeta as relações de água de *Melia azedarach*. Contudo, outros parâmetros fisiológicos devem ser realizados de forma a existir um profundo e completo conhecimento da *performance* de *Melia azedarach* sob condições de défice hídrico.

Annex 3

P19

Efeito do défice hídrico na capacidade antioxidante de *Melia azedarach*

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Keywords: Défice hídrico, capacidade antioxidante, *Melia azedarach*.

As plantas medicinais são parte da sociedade Humana no combate às doenças desde o início da civilização. *Melia azedarach* é uma das espécies mais utilizadas na medicina tradicional. Para os seus extratos brutos, provenientes das folhas, têm sido descritas várias atividades biológicas, tais como propriedades antitumorais, atividades antivirais e propriedades antioxidantes. Entre os vários fatores ambientais que podem afetar a *performance* das plantas, o déficehidrico é provavelmente o de maior impacto.

O défice hídrico impede o desenvolvimento e o crescimento das plantas. Para sobreviver a tais condições ambientais severas, as plantas desenvolvem estratégias que envolvem programação molecular, celular e metabólica. Os efeitos do défice hídrico podem ser diretos, tal como a diminuição da disponibilidade CO_2 e alterações do metabolismo fotossintético ou podem surgir efeitos secundários, como stresse oxidativo. O stresse oxidativo caracteriza-se por um desequilíbrio entre a produção de espécies reativas de oxigénio (ROS, do inglês *reactive oxygen species*)e a sua eliminação por parte dos componentes antioxidantes. Um aumento dos níveis de ROS pode levar à oxidação severa ou parcial dos componentes celulares (ex. DNA, RNA, proteínas, lípidos).

O objetivo deste trabalho foi estudar a capacidadeantioxidante de *M. azedarach* em condições de défice hídrico. As plantas de *M. azedarach* foram expostas a défice hídrico (solo a 20% da capacidade de campo) e a sua capacidade antioxidante foi avaliada através da análise dos seguinte parâmetros: a) atividade das enzimasantioxidantes e b) metabolitos. Os resultados obtidos sugerem que existe um aumento significativo da capacidade antioxidante das plantas sujeitas a défice hídrico apoiado pelo aumento da atividade das enzimas antioxidantes SOD e APX.

Annex 4

O4 Antioxidant capacity of *Melia azedarach* under water deficit conditions

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This work aims to evaluate the effects of water stress (WS) on the antioxidant capacity and on the photosynthetic apparatus of the medicinal plant *Melia azedarach* L. Two month old plants of *M. azedarach* were exposed to WS (plants at 20% of field capacity) during 20 days. After this period, plant performance was evaluated through the measurement of the water potential, plant growth, chlorophyll *a* fluorescence, gas exchange, pigment content, H_2O_2, proline, the activities of antioxidant enzymes and antioxidant metabolites, cell membrane permeability and lipid peroxidation. IRGA analysis demonstrated that WS induced stomatal closure, reduced the net CO_2 assimilation rate (*A*) and decreased the CO_2 availability in the intercellular spaces of mesophyll cells (*Ci*). However, WS did not affect the photosynthetic efficiency of PSII or plant growth (dry weight accumulation and plant height). WS increased cell membrane permeability and induced an up-regulation of the antioxidant enzymes, CAT, APX and Gr and also an over production of antioxidant metabolites. Despite these effects on photosynthetic apparatus and increased antioxidant capacity, plant growth was not affected, supporting that this species may be used in re/afforestation programs for drought prone habitats. Moreover, it would be interesting to analyse if the stress-induced increase of the antioxidant capacity may be used as a positive strategy to increase antioxidant properties of this species.

Annex 5 (to be submitted)

Antioxidant capacity of *Melia azedarach* under water deficit conditions

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Summary

This work aims to evaluate the effects of water stress (WS) on the antioxidant capacity and on the photosynthetic apparatus of the medicinal plant, *Melia azedarach*. Therefore, two months old plants of *M. azedarach* were exposed to WS (plants at 20% of field capacity) during 20 days. After this period, plant performance were evaluated through the measurement of the water potential, plant growth, chlorophyll *a* fluorescence, gas exchange, pigment content, H_2O_2, the activities of antioxidant enzymes and antioxidant metabolites, cell membrane permeability and the lipid peroxidation. WS stress induced stomatal closure, reduced the net CO_2 assimilation rate (*A)* and decreased the CO_2 availability in the intercellular spaces of mesophyll cells (*Ci*). WS also reduced the photosynthetic efficiency of PSII, but did not affect plant growth (DW accumulation and plant height). WS increased cell membrane permeability and induced an up-regulation of the antioxidant enzymes, CAT, APX and Gr and also an over production of antioxidant metabolites. The results indicated that despite the prompt response of *M. azedarach* to WS by increasing their antioxidant activity, these increase was not sufficient for remedying the damage of the oxidative stress caused by the WS and the photosynthetic apparatus was negatively affected. However, since plant growth was not affected WS imposition to increase the antioxidant capacity of *M. azedarach* could be a positive strategy, for example, for medicinal uses. The physiological

characteristics displayed under WS conditions support the use of *M. azedarach* in afforestation programs for drought prone habitats.

Key words

Melia azedarach, water deficit, oxidative stress, photosynthesis, ROS, antioxidant system.

Abbreviations

A, net CO_2 assimilation rate; APX, ascorbate peroxidase; AsA, ascorbate; Cars, carotenoids; CAT, catalase; Chl, chlorophyll; *Ci*, intercellular CO_2; DHA, dehydroascorbate; DW, dry weight; E, transpiration rate; Fm, maximal fluorescence in the dark-adapted state; Fm', fluorescence during the saturating pulse in steady state; F_0, minimal fluorescence; F_0', minimum fluorescence after switch off the active light; F_v, maximal variable fluorescence; F_v/F_m, maximal efficiency of PSII; G-POX, glutathione peroxidase; GR, glutathione reductase; *gs*, stomatal conductance; GSH, glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; PS, photosystem; ROS, reactive oxygen species; SOD, superoxide dismutase; WS, water stress; WW, well watered; Φ_{PSII}, effective quantum efficiency of PSII; ψ, water potential.

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Introduction

Plants have been exploited worldwide as an important source of medicine for the treatment of several diseases for thousands of years. Even today, plants still play a pivotal role in the health care of modern cultures. Approximately 80% of people still rely on traditional medicine (e.g. use plant extracts) for their primary healthcare needs (Demiray *et al.,* 2009). *Melia azedarach* Linn., usually known as melia, cinamomo or chinaberry tree, is native to West Asia and is one of the most used plants in traditional medicine due to several medicinal properties (Ahmed *et al.,* 2008; Khan *et al.,* 2011; NCBI 2003; Orhan *et al.,* 2012). This species exhibit a wide range of biological activities of practical agricultural and pharmaceutical use. Extracts from different parts of *M. azedarach* are reported to exhibit antifungal (Carpinella *et al.,* 2005), nematicidal (Hasabo and Noweer, 2005; Ntalli *et al.,* 2010a), antihelmintic (Maciel *et al.,* 2006), antilithic, diuretic (Husain *et al.,* 2009), cytotoxic, antiproliferative, and antioxidant activities (Ntalli *et al.,* 2010b). Also the carboxylic acids and aldehydes from *M. azedarach* fruits, and in particular furfural, 5-hydroxymethylfurfural and furfurol have proven to be strong nematicides (Ntalli *et al.,* 2010c). However, the most valuable interest of this species is due of its insecticidal (e.g. Akhtar and Isman 2007; Charleston *et al.,* 2005; Hammed and McAuslane 2006; Isman 2006; Rachokarn *et al.,* 2008), insect repellent properties and the several active limonoidal compounds (Huang *et al.,* 1996). *M. azedarach* products enhance soil fertility (Noble and Rndall 1996; Toselli *et al.,* 2010) and its derivatives stimulate soil microbial biomass (Marino *et al.,* 2008; Spyrou and Menkissoglu-Spirouli 2009). The tree yields valuable timber, resistant to attack by white ants, and is often used to make furniture, plywood, toys and fuelwood. The wide range of adaptability and its usefulness makes *M. azedarach* an additional crop for afforestation programmes. Thus, the multidirectional and widespread use

of this species makes it one of the most important multipurpose tree species (Husain *et al.*, 2009).

Water deficit is one of the main environmental factors that adversely affect the growth and development of plants. Water deficit invariably leads to the accumulation of reactive oxygen species (ROS), which causes oxidative stress damages to plants. ROS can directly attack the membrane lipids, inactivate enzymes and damage the nucleic acids leading, in some cases, to cell death (Mittler 2002). As a protection against ROS, plants have evolved a highly efficient defence system, with antioxidant enzymes and non-enzymatic compounds that can neutralize free radicals and reduce the potential damages of ROS (Asada 2006). Superoxide dismutase is an antioxidant enzyme that converts the $O_2^{\bullet-}$ radical into H_2O_2 and water. The accumulation of H_2O_2 is prevented in the cell by its reduction to water through the actions of CAT, G-POX or APX (Apel and Hirt 2004). Beside the enzymatic antioxidant system, plants also possess non-enzymatic antioxidants, such as, AsA, GSH, carotenoids, anthocyanins and proline. Among numerous physiological roles attributed to these compounds, their most prominent and best established functions are the removal of ROS (Liu *et al.*, 2011). AsA scavenges most dangerous forms of ROS e.g. $^{\cdot}OH$, O^{2-} and H_2O_2 and dismutates H_2O_2 through the action of APX (Silva *et al.*, 2013). GSH participates in the regeneration of AsA via dehydroascorbate reductase and it also reacts with singlet oxygen and OH radicals to protect protein thiol groups thus representing the major cytoplasmic thiol disulfide redox buffer in the plants (Gill and Tuteja 2010).

Several medicinal plants have been explored due to their high quenching activity of ROS (Gonçalves *et al.*, 2013). This high antioxidant capacity is mainly attributed to the high amounts of antioxidants, in particular phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes (Cai *et al.*, 2004; Maisuthisakul *et al.*, 2007; Nahak and Sahu

2010). These compounds are described as antioxidant molecules that scavenge free radicals with their concomitant oxidation and formation of a more stable free radical. Also *M. azedarach* contains a high amount of phenolic compounds and exhibited a great antioxidant activity (Nahak and Sahu 2010). Thus, since the enhancement of the ROS-detoxifying enzymes or the biosynthesis and/or regeneration of antioxidant metabolites was reported in several species under abiotic stress conditions (Fini *et al.,* 2012; Selote *et al.,* 1965; Uzilday *et al.,* 2012) it is important to assess the effects of drought stress on the antioxidant capacity of *M. azedarach.* The putative enhancement of the antioxidant capacity in this kind of species under drought stress conditions could be of a great value both for afforestation of drought prone areas and for medicinal and human feeding purposes. Moreover, the increasing need of medicinal plants for the primary health care strongly justified an in deep knowledgement of the effects of WS on the antioxidant capacity but also at the physiological level (plant productivity). Thus, in the present study, the effects of drought stress imposition on physiological and antioxidant capacity characteristics, including the water potential, plant growth, photosynthesis, the content on pigments, H_2O_2, the activities of antioxidant enzymes, and the lipid membrane peroxidation in *M. azedarach* were investigated to test the hypothesis that drought stress enhances the antioxidant capacity of *M. azedarach*.

Materials and Methods

Plant material and culture conditions

Seeds of *Melia azedarach* were germinated in a mixture of turf and vermiculite (2:1). Cultures were maintained in a growth chamber with a temperature of 20 ± 2°C, a 16/8-h (day/night) photoperiod and a photosynthetic photon flux density (PPFD) of app. 400 ± 20 µmol m^{-2} s^{-1}. Prior to water deficit treatment, all plants were well-watered. Two months old

M. azedarach plants of similar size were randomly assigned to each of the two treatments as follows: (1) well-watered (WW): plants were watered daily at 80 % of field capacity, and (2) water stress (WS): plants were watered at 20% of field capacity. After 20 days plant performance was evaluated through assessment of physiological parameters and oxidative stress parameters.

Plant water status and plant growth

After 20 days of treatment, plant water potential (Ψ) was measured with a Scholander-type pressure chamber (PMS Instrument Co., OR) on abscised stems just above the soil surface, according to Scholander *et al.*, 1965.

Plants height was determinate at the end of the experiment. Additionally, leaves and roots dry weight (DW) was recorded for total biomass determination.

Pigment contents

Pigments were extracted in a cold acetone/Tris 50 mM pH 7.8 buffer solution and centrifuged according to Sims and Gamon 2002. Absorbances at 470, 537, 647 and 663 nm were determined with a Thermo Fisher Scientific (Waltham, USA) spectrophotometer (Genesys 10-uv S). For the determination of anthocyanins, leaf disks were extracted with an acetone/Tris buffer according to Sims and Gamon 2002. Absorbances at 529 and 650 nm were determined with a Thermo Fisher Scientific (Waltham, USA) spectrophotometer (Genesys 10-uv S). The contents of chlorophyll (Chl) *a*, Chl *b*, carotenoids (Cars) and anthocyanins were calculated using the formulae described by Sims and Gamon 2002.

Gas exchange and Chlorophyll a fluorescence

In situ leaf gas exchange measurements (net CO_2 assimilation rate: *A*, transpiration rate: *E*, and the intercellular CO_2 concentration: *Ci*) were performed using a portable infrared gas analyser (*LCpro+*, *ADC*, Hoddesdon, UK), operating in open mode under growth chamber conditions. The stomatal conductance (g_s) were automatically calculated according to Von Caemmerer and Farquhar 1981. Measurements were always performed in the middle of the photoperiod at growth temperature (24 ± 2 ºC) and atmospheric CO_2 concentration in the youngest fully developed leaf.

Chl *a* fluorescence measurements were determined *in situ* in full expanded leaves of WW and WS plants, with a portable fluorimeter Mini-PAM (Walz, Effeltrich, Germany). Minimal fluorescence (F_0) was measured in 30 min dark-adapted leaves by applying a weak modulated light and maximal fluorescence (F_m) was measured after a 1 s saturating pulse of white light (>1500 μmol m^{-2} s^{-1}) in the same leaves. In light adapted leaves, steady state fluorescence (F´), maximal fluorescence (F_m´) after 1 s saturating pulse (>1500 mol m^{-2} s^{-1}) and minimal fluorescence (F_0´) measured when actinic light was turned off, were determinate. Definitions of fluorescence parameters (F_v/F_m - maximal efficiency of PSII and Φ_{PSII} - effective quantum efficiency of PSII) were used as described by Van Kooten and Snel 1990.

Soluble sugars and starch

Soluble sugars were extracted with 80 % (v/v) of ethanol at 80 ºC for 20 min as described by Correia *et al*., 2005. Glucose, fructose and sucrose were quantified using a spectrometric enzyme-coupled assay described by Jones *et al*., 1977 and starch was quantified in

accordance with Stitt *et al*., 1978 in a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S).

Cell membrane permeability and lipid peroxidation

Electrolyte leakage was used to assess cell membrane permeability as described by Lutts 1996. Leaf segments were detached, washed with deionized water, placed in closed vials containing 20 mL of de-ionized water and incubated over night at 25ºC, on a rotary shaker. Electrical conductivity of the bathing solution (L_t) was determined after 24h. Samples were then autoclaved at 120ºC for 20 min and a last conductivity reading (L_0) was obtained upon equilibration at 25ºC. The electrolyte leakage was defined as L_t/ L_0 and expressed as percentage.

Lipid peroxidation on leaves was obtained by measuring malondialdehyde (MDA) production (Dhindsa and Matowe 1981). Approximately 0.5 g of leaves were homogenized with 5 mL of 0.1% TCA (w/v) and centrifuged according to Dias *et al*., 2011. After centrifugation, 1 mL of supernatant was mixed with 4 mL 20% TCA (w/v) in 0.5% TBA (w/v) and incubated for 30 min at 95ºC. The extract was then cooled immediately on ice to stop the reaction and centrifuged. MDA concentration was estimated by subtracting the nonspecific absorption at 600 nm from the absorption at 532 nm using an absorbance coefficient of extinction (ε), 155 mM^{-1} cm^{-1}.

Activities of *antioxidant enzymes*

For the determination of antioxidant enzyme activities, leaves (0.5 g) were homogenized in 5 mL of extraction buffer in a pre-chilled mortar and pestle by liquid nitrogen. The extraction buffer contained 0.1 M potassium phosphate buffer (pH 7.5), 0.5 mM Na_2EDTA, 1% PVP (m/v), PMSF 1 mM, 0.2% Triton X-100 (v/v) and 2 mM DTT. Homogenates were

centrifuged in a refrigerated centrifuge at 8000 g for 15 min filtered through four layers of cheesecloth and centrifuged at 15000 g for 30 min at 4ºC. The supernatant obtained was used for enzyme assays (SOD, CAT, APX, GR and G-POX). SOD (EC 1.15.1.1) was determined according to the method of Agarwal *et al.,* 2005. The reaction mixture contained 13.3 mM methionine, 63 µM NBT, 0.1 mM EDTA, 50 mM potassium phosphate buffer (pH 7.8), 50 mM Na_2CO_3, and an aliquot of extract. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT in comparison with control. CAT (EC 1.11.1.6) activity was assayed at 25ºC as described by Beers and Sizer 1952. Assay mixture contained 0.1 M potassium phosphate buffer (pH 7.0) and enzyme extract. To start the reaction, 20 mM H_2O_2 ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$) was added and the decrease of absorbance at 240 nm was recorded. APX (EC 1.11.1.11) activity was determined at 25ºC by recording the decrease in absorbance at 290 nm due to ascorbic acid ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$) oxidation to dehydro-ascorbate by H_2O_2 , according to the method of Nakano and Asada 1981. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, and an aliquot of enzyme extract, and 0.5 mM H_2O_2 was added to start the reaction. The expression of one unit of APX activity was mmol of ascorbic acid oxidized per minute, calculated using its extinction coefficient (ε) 2.8 $mM^{-1}\ cm^{-1}$. GR (EC 1.6.4.2) activity was determined at 30ºC according to Sgherri *et al.,* 1994. The reaction mixture contained 0.2 M potassium phosphate (pH 7.5), 0.2 mM Na_2 EDTA, 1.5 mM $MgCl_2$, 0.25 mM of oxidized glutathione (GSSG), and enzyme extract. The reaction was started with 25 µM NADPH, and the decrease in absorbance at 340 nm was monitored. The GR activity was calculated using the extinction coefficient of NADPH (6.22 $mM^{-1}\ cm^{-1}$). For G-POX (EC 1.11.1.7) determination, the reaction mixture contained 10 mM phosphate buffer (pH 6.1), 12 mM hydrogen peroxide, 96 mM guaiacol and 50 µL enzyme extract. Absorbance was

recorded at 470nm according to Castillo *et al.*, 1984 and the specific activity was calculated using the 26.6 mM^{-1} cm^{-1} molar extinction coefficient.

Content of AsA-GSH cycle non-enzymatic antioxidants

Total AsA (TA, AsA + DHA), reduced AsA and DHA (dehydroascorbate) concentrations were determined as Jin *et al.*, 2008 with some modifications. Briefly, frozen samples were extracted with TCA 5% and centrifuged. TA was determined in a mixture of 0.2 mL of supernatant, 0.25 mL of 150 mM phosphate buffer (pH 7.4) (containing 5 mM EDTA), and 0.1 mL of dithiothreitol (DTT) 10 mM. After incubation for 10 min at room temperature, 0.05 mL of 0.5% (w/v) N-ethylmaleimide was added. The AsA was assayed in a similar manner except that instead of DTT, 100 µL of ultra pure water were added. Color was developed in both reaction mixtures after the addition of 0.2 mL of 10% (w/v) TCA 10%, 0.2 mL of 44% (v/v) phosphoric acid, 0.2 mL of α,α'-dipyridyl in 70% (v/v) ethanol, and 0.1 mL of 3% (w/v) $FeCl_3$. After incubation at 40◦C for 40 min, the absorbance was read at 532 nm using AsA as a standard. Standard curves for total AsA and AsA were established. DHA was estimated from the difference between TA and AsA.

Total glutathione (reduced glutathione (GSH) plus GSSG) and GSSG were measured according to Ma *et al.*, 2008 with some modifications. Frozen samples were homogenized with 5-sulphosalicylic acid and then centrifuged. Total glutathione was measured in a reaction mixture (1 mL) consisting of 100 µL supernatant, 200 mM phosphate buffer solution (pH 7.2) containing 5 mM EDTA and 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.2 mM NADPH and 1U GR. The reaction mixture was then incubated at 27ºC for 30 min and quantified at 412 nm. GSSG was analyzed in a similar manner, except that the supernatant was previously incubated with 0.17 M 2-vinylpyridine and 3.6% triethanolamine

at 27◦C for 1h. Standard curves for GSH and GSSG were established. For each sample, GSH was estimated from the difference between total glutathione and GSSG.

Quantification of H_2O_2

The concentration of H_2O_2 was measured according to Zhou *et al.*, 2006. Briefly, half gram of a leaf tissue was ground in 5 mL precooled 5% TCA (w/v) and activated charcoal. After centrifugation, extracts were adjusted to pH 8.4 with 17 M ammonia, and H_2O_2 was spectrophotometrically quantified following its reaction with 4-amino-antipyrine and phenol to form a stable red product in the presence of 150 U mg $^{-1}$ peroxidase. Blanks containing 8 µg CAT (EC. 1.11.1.6) were run for each sample as well as for the calibration with H_2O_2 standards, which were added to the extraction medium in parallel to the samples.

Data analysis

Data were analysed by t-test at a significant level set to 0.05 using Sigma Stat for Windows version 3.1.

Results

Plant water status and plant growth

Twenty days after the beginning of the WS treatment plants became wilted (visual observation). Plant water status was evaluated by the measurement of the Ψ and the results corroborated with visual observation: the Ψ decreased 27% in plants under WS conditions compared to plants under WW conditions (p<0.05) (table 1).

Concerning plant growth, no significant differences were observed in plant height and total plant DW accumulation between plants under WW and WS conditions (table 1).

Photosynthetic parameters, pigment and sugar content

No significant differences were observed in the F_v/F_m values between WW and WS plants (table 1). The Φ_{PSII} decreased significantly 35% in WS plants relatively to plants under WW conditions (table 1). Plants under WS conditions showed a significantly lower *A*, g_s, *E* and C_i than plants under WW conditions (fig. 1A, B, C and D). WW plants showed a *A* approximately 4 times higher than WS plants.

Plants exposed to WS conditions showed a similar Chl *a*, Chl *b*, and carotenoids content to plants under WW conditions (p>0.05) (table 1). Concerning the anthocyanin contents, no significant differences were observed between WW and WS plants (table 1).

Relatively to sugar content, significantly differences were found between treatments for glucose and sucrose content (table 1). Glucose content significantly increased (2.6 times, relatively to WW plants) in WS plants, while sucrose content significantly decreased (1.9 times, relatively to WW plants) in WS plants. In contrast, no significantly differences in the fructose and starch content were observed between treatments (table 1).

Cell membrane stability, lipid peroxidation and H_2O_2 content

Lipid peroxidation was measured in terms of MDA content and cell membrane permeability was determined through measurement of electrolyte leakage. No significant differences were observed in the MDA content between WW and WS plants (fig. 2A). Contrarily, plants under WS conditions showed significantly higher cell membrane permeability than plants under WW conditions (fig. 2B).

The levels of H_2O_2 in plants under WW and WS were similar (p>0.05) (fig. 2C).

Antioxidant enzyme activities and non-enzymatic antioxidant contents of AsA–GSH cycle

The antioxidant enzyme system was assessed by the measurement of the activities of five enzymes: SOD, CAT, APX, Gr and G-POX. A significant increase in the activity of CAT, SOD, APX and Gr (37%, 55%, 89% and 91%, respectively) was observed in plants under WS conditions compared to those under WW conditions (fig. 2D, E, F and G). No significant differences were observed in the activity of G-POX between WW and WS plants (fig. 2H). A significant increase in AsA/DHA, TA, AsA and DHA (46%, 49%, 59% and 34%, respectively) was observed in plants under WS conditions compared to those under WW conditions (fig. 3A, B, C and D). However, no significant differences were observed in the level of GSSG, GSH, total glutathione and GSH/GSSG between WW and WS plants (table 2).

Discussion

Due to global climate change, scientific climate prediction appointed that drought may become more common in the future (IPCC 2007). Over the last years the impact of climate changes (in particular, drought and temperature increase) on plant performance have been studied (e.g. Correia *et al.*, 2013; Dias and Brüggemann 2007), but the effect of WS events on the antioxidant capacity of medicinal plants have been poorly explored (Jaleel *et al*., 2007a, b, c; Zhu *et al*., 2009). To the best of our knowledge this is the first report that evaluated the effects of WS on the physiological status and antioxidant capacity of the medicinal plant *M. azedarach*.

As expected, WS imposition resulted in reduced plant Ψ, which further induce stomatal closure (*E* and g_s decreased) and *A* impairment. Stomatal closure is among the earliest responses to WS, protecting plants from extreme water loss, which can results in cell dehydration and eventually in plant dead (Chaves *et al.*, 2003). WS induced stomatal closure

in *M. azedarach* to prevent water loss, but also limited the CO_2 availability in the intercellular spaces of the mesophyll cells, as evidenced by the low C_i. Although WS affected negatively the *A*, these effects were already not remarkable in plant growth as indicated by the similar total DW and plant height observed in WS plants compared to those under WW conditions. Photosynthesis, together with cell growth, is among the primary processes to be affected by drought (Chaves 1991). WS depress gas-exchange characteristics to a varying extent thereby affecting overall photosynthetic capacity of most plants (Ashraf and Harris 2013). For example, Dias and Brüggemann 2010, Lawlor and Cornic 2002 and Silva *et al*., 2010a, b, observed a strong decrease of the *A*, *E* and g_s in several species as the leaf water potential and/or relative water content decreased. Moreover, Cechin *et al*., 2006 and Sapeta *et al*., 2013 reported that WS affected negatively gas-exchange characteristics and even substantial decreased plant growth.

In addition to the changes observed in leaf gas exchange, *M. azedarach* photochemistry was also negatively affected by WS. The reduction of the *A* was accompanied by a decrease of the Φ_{PSII}. However, the F_v/F_m did not changed, indicating that non-reversible photoinhibition damage to PSII reactions centres did not occur. Φ_{PSII} reduction may be mainly related to a decrease of the proportion of the PSII reaction centres in the "open" state or to decreased efficiency of excitation energy capture by open PSII reaction centres (Correia *et al*., 2006; Osório *et al*., 2006), since Chl *a* levels were not affected under WS conditions. Correia *et al*., 2006 also observed that WS impositions in sunflowers did not affect the F_v/F_m but reduced the Φ_{PSII}. These authors concluded that drought-induced depression on Φ_{PSII} was a result of decreased efficiency of excitation energy capture by open PSII reaction centres. Cechin *et al*., 2006 also did not found changes in the F_v/F_m in WS sunflower plants, but in spite of the strong reduction of *A*, WS did not affect Φ_{PSII}.

Under WS conditions leaf Chl contents often decline due to Chl degradation (Jaleel *et al*., 2009; Martínez-Ferri *et al*., 2004; Pompelli *et al*., 2010). However, and similar to the findings of Sapeta *et al*., 2013, in *Jatropha curcas,* in the present experimental conditions we found no reduction in Chl content. Carotenoids act as light-harvesting pigments and can protect chlorophyll and membranes destruction by quenching triplet chlorophyll and removing oxygen from the excited chlorophyll–oxygen complex (Young 1991). Anthocyanins are also important pigments and have a major role in plant photoprotection. Several studies have presented evidences that anthocyanins protect the photosynthetic apparatus from photoinhibition by absorbing green light and thereby reducing excess excitation energy (Pietrini *et al.,* 2002; Zhang *et al.,* 2010). Moreover, anthocyanins seem to have a high ability to quench oxidants and to mitigate the formation of ROS (Hatier and Gould 2008; Sperdouli and Moustakas 2012; Zhang *et al.,* 2010). The absence of photoinhibition symptoms in *M. azedarach* plants under WS conditions, despite the high decrease of the *A*, and the similar content of carotenoids and anthocyanins, suggests that other photoprotection mechanisms are displayed under WS conditions.

One of the most common stress responses in plants is overproduction of different types of compatibles solutes (e.g. proline, glycine-betaine and sugars) (Ashraf and Foolad 2007). Osmolyte accumulation in plant results in a reduction in cell osmotic potential and thus improves water absorption and cell turgor pressure, which might help sustain physiological processes, such as photosynthesis and growth under WS conditions (Ashraf and Foolad 2007). In response to the WS, there was a small accumulation of soluble sugars (glucose) in leaves of *M. azedarach*. However, WS lead to the decrease of sucrose, which could be in part related to the general tendency of increased glucose and fructose. Sucrose decrease could also results from an increased carbon demand of sink organs probably due to a

reduction in carbohydrate concentration availability or due to a decreased in sucrose phosphate synthase enzyme activity (Rosa *et al.,* 2009).

Oxidative stress is regarded as a major damaging factor in plants exposed to biotic stresses, in particular under drought stress. Plant redox status depends on the delicate equilibrium between ROS production and scavenging at the proper site and time (Sharma and Dubey 2007). Under stress conditions, this equilibrium is highly dependent on the antioxidant machinery activity. According to our results, WS induced oxidative stress, as observed by the up-regulation of the antioxidant enzymes and increased cell membrane permeability in *M. azedarach* plants. Plants can protect themselves against oxidative damage by antioxidant system including antioxidant enzymes and non-enzymatic compounds (Gill and Tuteja 2010). SOD activities increased in *M. azedarach* plants under WS conditions, but the similar levels of H_2O_2 in WS, compared to WW plants, may be attributed to the efficient removal of H_2O_2 by increased activity of APX, CAT and Gr. In particular, APX and Gr appear to be in the front line to detoxify cells against ROS. Contrarily, G-POX, one of the major enzymes that scavenge H_2O_2 in chloroplasts (Gill and Tuteja 2010), seems not to play an important role in ROS detoxification in *M. azedarach* since the activity of this enzyme were not changed under WS conditions. A general increased of the levels of antioxidant enzymes under WS conditions are reported by several authors in various species, including medicinal plants (Arbona *et al*., 2008, Candan and Tarhan 2012). Jaleel *et al*., 2007a reported that the cultivation of the medicinal plants, *Catharanthus roseus,* under WS conditions increased the antioxidant defense system and the level of active principles. Also, Zhu *et al*., 2009 observed that *Bupleurum chinense* exhibit effective antioxidative protection mechanism to withstand WS and conclude that drought-induced saikosaponins accumulation may be involve in mitigating the oxidative damage due to its high anti-lipid peroxidation capacity.

WS is frequently related to an increase in membrane permeability, affecting membrane integrity and cell compartmentation (Campos *et al*., 2003, Dias *et al*., 2011). Cell membrane permeability and MDA have been widely used to evaluate membrane damage following stress. In particular, MDA is one of the final products of stress induced lipid peroxidation of poliunsaturated fatty acids (Leshem 1987), and has been considered a marker for stress sensitivity. Increased lipid peroxidation and cell membrane permeability were reported in the present work for *M. azedarach* and also in other species (Candan and Tarhan 2012; Dias *et al*., 2011; Zhu *et al*., 2009) under WS conditions. Increased cell membrane permeability under WS conditions may result from the appearance of membrane domains presenting different configurations due to stress-induced changes in lipid phases (Leshem 1992), and not from damage of membrane, particularly as regards lipids (Harwood 1997) since our data demonstrated that MDA content was not affected by WS.

AsA and GSH are key antioxidant compounds that play an essential role in plant protection against oxidative damage and are also involved in plant cell redox status maintenance (Gill and Tuteja 2010). The balance between GSH and GSSH is a central component in maintaining cellular redox state. GSH is necessary to maintain the normal reduced state of cells so as to counteract the inhibitory effects of ROS induced oxidative stress (Gill and Tuteja 2010). Additionally, GSH plays an important role in the antioxidative defense system by regenerating another potential antioxidant, AsA, via AsA/GSH cycle. The regeneration of AsA is extremely important because fully oxidized dehydroascorbate acid has a short half-life and would be lost unless it is reduced back. According to our data, WS induced an increase in the levels of AsA and AsA/DHA, but did not affect the levels of GSH. Moreover, the GSH/GSSG, that gives information about the redox status, was also similar in plants under WW and WS conditions. It is possible that these similar levels of GSH and

GSH/GSSG may be attributed to a putative increased rate of GSH degradation (Noctor and Foyer 1998) since under this condition Gr activity increased sharply and higher levels of AsA and AsA/DHA are maintained under WS conditions in *M. azedarach*. Similar to our results, a decline in GSH levels due to an increased rate of GSH degradation and/or to its decreased rate of synthesis, were reported in rice under WS conditions (Sharma and Dubey 2005). Candan and Tarhan 2012 and Zhu *et al*., 2009 also found increased levels of AsA in medicinal plants exposed to WS, but the levels of GSH were not changed (Zhu *et al*., 2009).

Conclusion

The present work demonstrated that 20 days under WS conditions affects the physiological performance and induce oxidative stress in *M. azedarach* plants. Although WS affects negatively the photosynthetic apparatus, plant growth (DW accumulation and plant height) still not being affected and as hypothesized, the antioxidant capacity of *M. azedarach* increased strongly. Our results demonstrated that the impositions of controlled WS periods (e.g. 20 days) could be a positive strategy to increase the antioxidant capacity of *M. azedarach* for medicinal uses, without affect severely plant growth. Moreover, *M. azedarach* showed elevated potential characteristics to be a candidate plant for re/afforestation programs in drought prone habitats.

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Tables and Figures

Table 1 – Ψ (MPa), height (cm), plant total DW (g), maximal efficiency of PSII (F_v/F_m), effective quantum yield of PSII (ΦPSII), sugar content (μmolg^{-1}FW) and pigment contents (mmol g^{-1}FW) in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (*n*= 6). Different letters indicate significant differences between tratments ($p<0.05$).

Parameter	WW	WS
Ψ	-0.76 ± 0.10[a]	-1.04 ± 0.08[b]
Height	14.0 ± 2.53[a]	13.8 ± 1.96[a]
Plant total DW	3.11 ± 0.99[a]	2.42 ± 0.62[a]
F_v/F_m	0.79 ± 0.02[a]	0.74 ± 0.10[a]
Φ_{PSII}	0.45 ± 0.02[a]	0,29 ± 0.03[b]
Glucose	25.8 ± 5.22[a]	66.9 ± 4.35[b]
Fructose	31.1 ± 2.92[a]	41.5 ± 8.82[a]
Sucrose	28.8± 2.01[a]	15.2 ± 3.60[b]
Starch	234.7 ± 12.8[a]	237.6 ± 10.9[a]
Chl *a*	271.1 ± 44.07[a]	250.2 ± 13.18[a]
Chl *b*	101.5 ± 18.25[a]	98.8 ± 1.98[a]
Carotenoids	187.4 ± 21.96[a]	183.5 ± 4.85[a]

Anthocyanins	$0.02 \pm 8.67 \times 10^{-3}$ [a]	$0.03 \pm 5.36 \times 10^{-3}$ [a]

Table 2 – Concentration of GSSG (μg mL^{-1}), GSH (μg mL^{-1}), total glutathione (μg mL^{-1}) and the ratio of GSH to GSSG in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (*n*= 4). Different letters indicate significant differences between tratments (p<0.05).

Parameter	WW	WS
GSH	34.3 ± 5.6[a]	19.5 ± 1.4[a]
GSSG	20.9 ± 3.8[a]	14.6 ± 2.8[a]
Total glutathione	45.6 ± 8.4[a]	34.2 ± 1.6[a]
GSH/GSSG	0.86 ± 0.16[a]	0.76 ± 1.19[a]

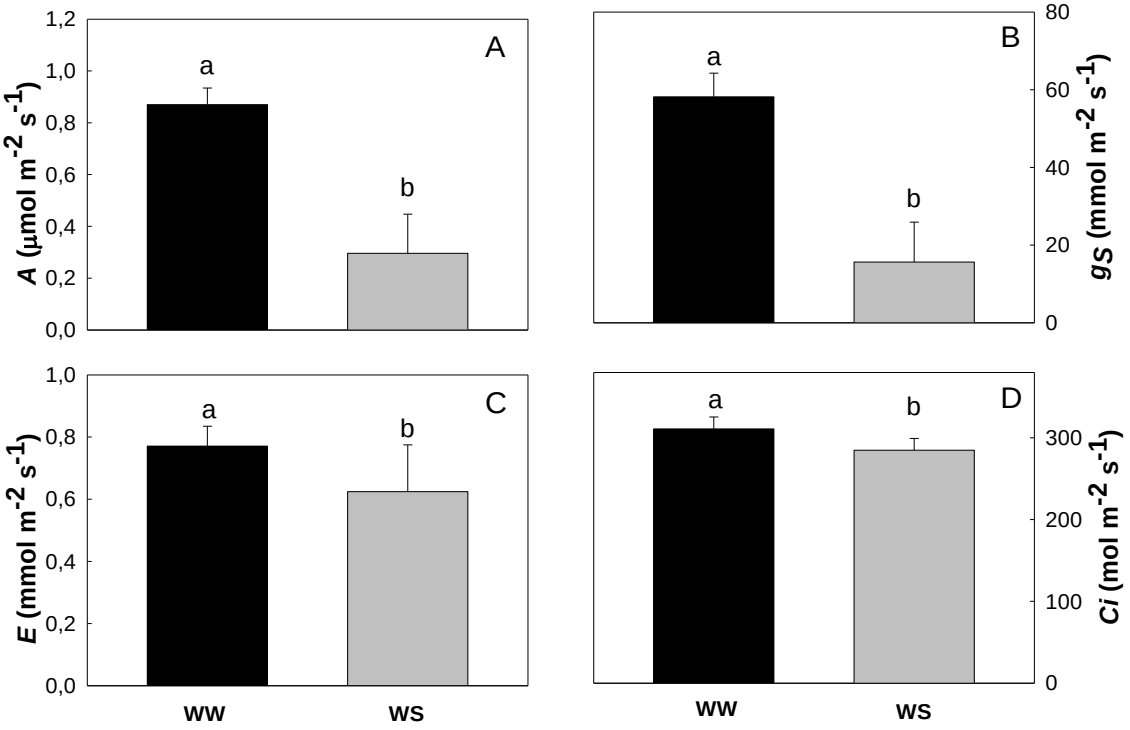

Figure 1 – Net CO_2 assimilation rate (*A*) (A), stomatal conductance (g_s) (B) transpiration rate (*E*) (C) and intercellular CO_2 concentration (*Ci*) (D) in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (*n*=12). Different letters indicate significant differences between tratments (p<0.05).

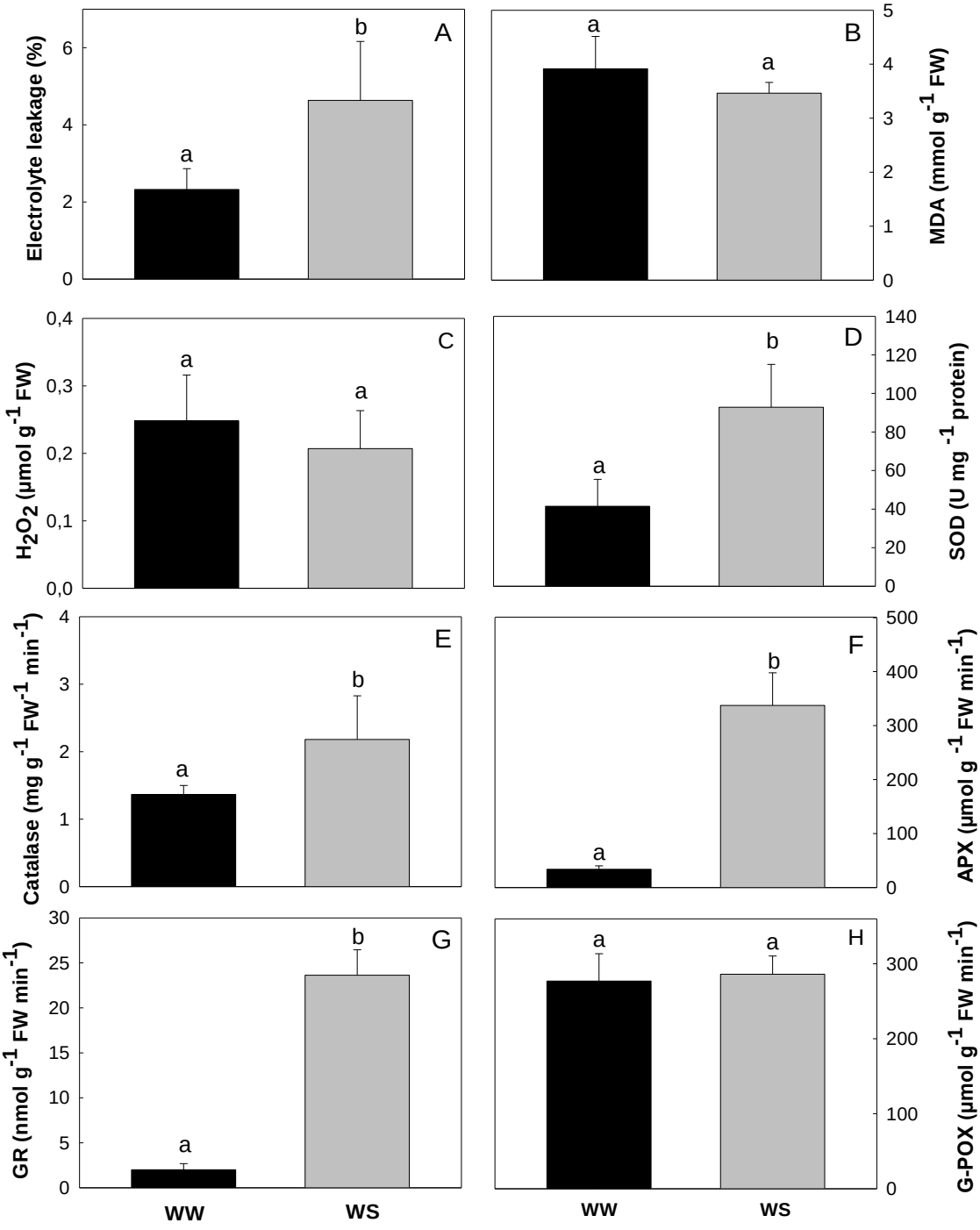

Figure 2 – Electrolyte leakage (A), MDA (B), H_2O_2 (C), SOD (D), CAT (E), APX (F), GR (G) and G-POX (H) activities in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (*n*= 6). Different letters indicate significant differences between tratments (p<0.05).

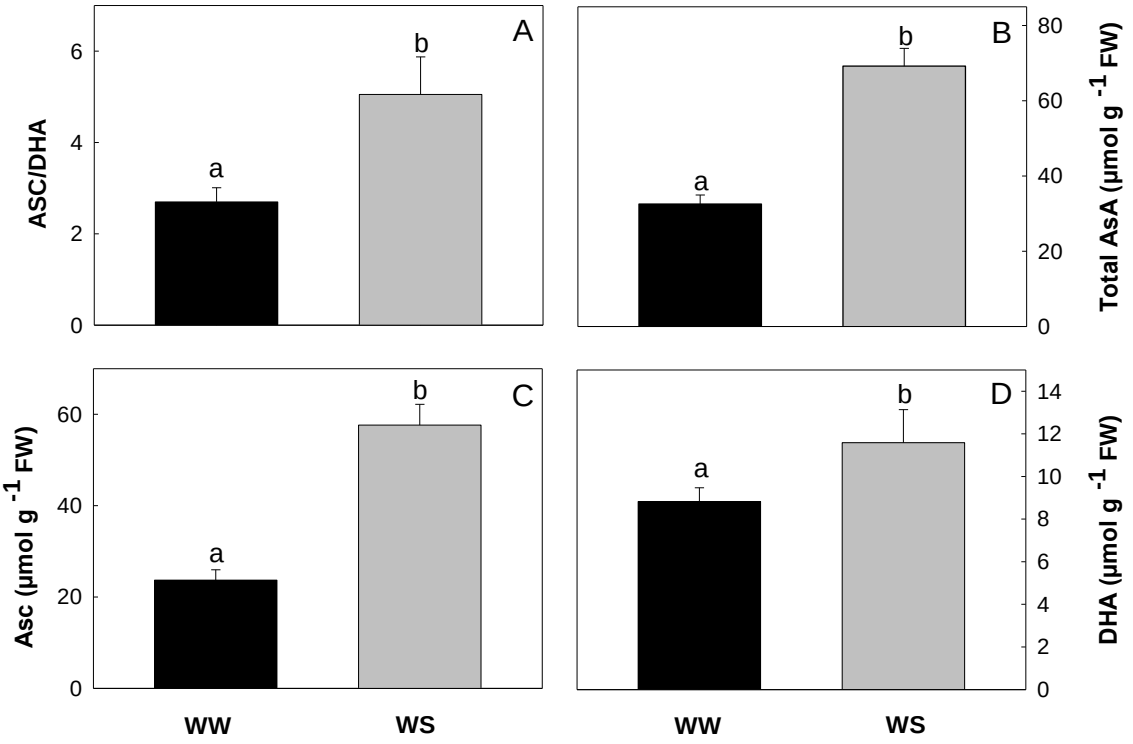

Figure 3 - Ratio of AsA to DHA (ASC/DHA) (A), total content of ascorbate (TA) (B), content of ascorbate (AsA) (C), and content of DHA (DHA) (D) in WW and WS *M. azedarach* plants. Data are presented as mean ± SD (n= 8). Different letters indicate significant differences between tratments (p<0.05).