Organ-coordinated response of early post-germination mahogany seedlings to drought

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Water deficit tolerance during post-germination stages is critical for seedling recruitment. In this work, we studied the effect of water deficit on morphological and biochemical responses in different organs of newly germinated mahogany (Swietenia macrophylla King) seedlings, a woody species that occurs in the Amazon rainforest. The root : shoot ratio increased under water deficit. The leaf number and water potential were not altered, although reductions in leaf area and stomatal conductance were observed. Osmotic potential became more negative in leaves of seedlings under severe stress. Water deficit increased fructose, glucose, sucrose and myo-inositol levels in leaves. Stems accumulated fructose, glucose and l-proline. Nitric oxide (NO) levels increased in the vascular cylinder of roots under severe stress while superoxide anion levels decreased due to augmented superoxide dismutase activity in this organ. Water deficit induced glutathione reductase activity in both roots and stems. Upon moderate or severe stress, catalase activity decreased in leaves and remained unaffected in the other seedling organs, allowing for an increase of hydrogen peroxide (H2O2) levels in leaves. Overall, the increase of signaling molecules in distinct organs—NO in roots, l-proline in stems and H2O2 and myo-inositol in leaves—contributed to the response of mahogany seedlings to water deficit by triggering biochemical processes that resulted in the attenuation of oxidative stress and the establishment of osmotic adjustment. Therefore, this body of evidence reveals that the development of newly germinated mahogany seedlings may occur in both natural habitats and crop fields even when water availability is greatly limited.

Keywords: drought tolerance, nitric oxide, reactive oxygen species, signaling molecules, sugars.

Introduction

Climate changes are predicted to increase global temperature and alter the precipitation pattern with the risk of more severe and prolonged water deficiency in both natural and agricultural vegetation. Drought or water deficit is one of the most important manifestations of abiotic stress that lead to reductions of plant growth and productivity. In nature, plants have developed various mechanisms to avoid or tolerate drought, represented by a series of integrated events, ranging from stress signal perception and transduction to regulation of gene expression and metabolic changes (Chaves et al. 2003).

Water deficit resistance strategies vary according to species, plant developmental stage, stress degree and duration, among others. Expansion of the root system leads to an enhancement in water uptake from soil and attenuation of water loss from cells (Shao et al. 2008). Leaf stomatal closure controls transpiration rates allowing for cell turgor maintenance (Singh and Reddy 2011). Through an osmotic adjustment mechanism, plant cells accumulate compatible solutes such as soluble carbohydrates and l-proline to improve water input in cells (Silva et al. 2010). l-Proline accumulation in plant cells experiencing water deficit has been also correlated to signaling processes that result in stress tolerance by the affected plant (Hare and Cress 1997, Carvalho et al. 2013).

The antioxidant system (enzymatic and non-enzymatic) is known to contribute to the control of reactive oxygen and nitrogen species (ROS and RNS, respectively) levels in plant cells under...
antioxidant enzymes in roots, stems and leaves of mahogany seedlings under different levels of water scarcity.

**Materials and methods**

**Plant material and water deficit imposition**

Drought-sensitive plants displayed lower catalase (CAT) and superoxide dismutase (SOD) activities in comparison to drought-resistant plants (Marok et al. 2013). The expression of genes that encode for CAT, SOD and glutathione reductase (GR) was increased in Swingle citrumelo following a severe water deficit (Carvalho et al. 2013). On the other hand, hydrogen peroxide (H₂O₂, an ROS) and nitric oxide (NO, an RNS) at relatively low amounts may act as signaling molecules of processes implicated in plant responses to a variety of stressing conditions. For instance, ex vivo experiments based on the incubation of Medicago falcata leaflets with an NO donor or H₂O₂ prior to dehydration caused an accumulation of myo-inositol in cells, which led to the resistance of leaflets to drought (Tan et al. 2013).

The overall biomass losses due to severe drought events in 1998, 2005 and 2010 evidenced the vulnerability of the Amazon rainforest to water scarcity (Coelho et al. 2012), and periods of drought are predicted to be intensified in this region as the current century progresses. The increase of drought episodes may negatively impact seedling recruitment in tropical rainforests and crop fields as well. Additionally, the increased tree mortality as a result of repeated droughts may disturb the global carbon cycle and accelerate climate change (Lewis et al. 2011). Based on these observations, we explored the responses of newly germinated mahogany (Swietenia macrophylla King) seedlings to drought. This Amazonian species is one of the most valuable and exploited tropical woody plants (Veríssimo et al. 1995), recently included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora to protect the natural population from high logging pressure (Grogan and Barreto 2005).

Water relations have been investigated in S. macrophylla adult plants grown in the field by determining water potential (Ψₛ), stomatal conductance in rainy and dry seasons and transitional situations as well (Silva and Lemos-Filho 2001). Additionally, the effect of drought on morphological parameters and l-proline accumulation was reported for 1-year-old S. macrophylla plants maintained in a greenhouse under drought for 20 days (Cordeiro et al. 2009). However, the response of mahogany to drought during the very early stages of development is still unknown. Considering that the initial plant development stage is critical for seedling recruitment, we investigated the extent of an integrated response to drought among organs of newly germinated mahogany seedlings. Morphological and biochemical changes in different organs would allow for the establishment of newly germinated seedlings even under severe water deficit. For this purpose, we assessed morphological adjustments, the biosynthesis and tissue localization of signaling molecules, accumulation of solutes that contribute to cellular osmotic adjustment and modulation of the activity of some oxidizing enzymes in roots, stems and leaves of mahogany seedlings under different levels of water scarcity.

**Determination of leaf area, Ψₛ, Ψᵣ and biomass**

After 45 days of water suppression, as described above, all leaves of each individual were harvested and scanned using the software SigmaScan Pro (version 5.0; SYSTAT, San Jose, CA, USA) for determining leaf area. Leaf Ψᵣ in three seedlings per water regime was measured at pre-dawn in a Scholander-type pressure chamber (PMS Instrument Company, Model 600; Albany, NY, USA). Stomatal conductance was measured at noon for five consecutive days (from the 21st to the 25th day) using a porometer (Delta-T-Devices, Model AP4, Cambridge, UK). The ratio: shoot biomass ratio of plants on different watering regimes was estimated from samples that were dried at 60 °C until reaching constant weight. The contribution of ethanol-soluble carbohydrates (ESC) to root, stem and leaf osmotic potential (Ψᵣ) was determined essentially according to Niinemets and Kull (1998). Part of Ψₛ from ESC was calculated according to the equation...
Ψρ = −C_{ESC}(DW / FW)ρRT,

where $C_{ESC}$ is the soluble carbohydrate concentration, $DW/FW$ is the tissue dry weight to fresh weight ratio, $\rho$ is water density in kg $m^{-3}$, $R$ is the universal gas constant and $T$ is the absolute temperature (293 K).

**Quantification of soluble carbohydrates and estimation of starch content**

Mahogany tissue samples were macerated in liquid nitrogen and soluble carbohydrates were extracted three times at 90 °C with 80% ethanol (1 ml per 0.2 g of plant tissue). Total and reducing carbohydrates were quantified, respectively, according to Dubois et al. (1956) and Somogyi (1945) using glucose (Sigma-Aldrich, St. Louis, MO, USA) as the standard.

Soluble carbohydrates were obtained from dried ethanolic fractions that were resuspended in ultrapure water and further purified in cationic resin Dowex 50 × 8 (100–200 mesh) followed by anionic resin Dowex 1 × 8 (52–100 mesh). After pH adjustment to 7.0, purified fractions were lyophilized and resuspended in ultrapure water. Samples were analyzed by HPAEC/PAD in an ICS3000 Dionex System (Sunnyvale, CA, USA) using a Carbo-Pac PA1 column and the soluble carbohydrates were separated by a 35-min isocratic elution with 20 mM NaOH (0.2 ml min$^{-1}$). Glucose, fructose, sucrose and myo-inositol (Sigma-Aldrich) were used as standards. The pellets obtained from extractions of mahogany tissues with 80% ethanol were dried and used to measure the starch content. Samples were subjected to enzymatic hydrolysis using a method modified by Chow and Landhäusser (2004) for woody tissues. Glucose was used as the standard.

**Determination of l-proline content**

Mahogany tissue samples were analyzed for l-proline content according to Bates et al. (1973), with modifications. Briefly, plant material was macerated in liquid nitrogen and amino acids were extracted with 2% sulfosalicylic acid (1 ml per 0.1 g of plant tissue). Homogenates were centrifuged at 10,000 $g$ for 5 min, and the supernatants were collected, following the addition of an equal volume of 80 mM ninhydrin prepared in 50% acetic acid (v/v) and 1.2 M phosphoric acid. The mixtures were incubated at 100 °C for 1 h and then cooled for the addition of two volumes of toluene. Each system was homogenized and the tolune fractions were analyzed at 520 nm. l-Proline (Sigma-Aldrich) content was determined from a standard curve.
In situ localization of superoxide anion (O\textsuperscript{2−}) and NO

Freehand cross-sections of mahogany tissues were vacuum infiltrated for 5 min with 0.1 g l\textsuperscript{−1} of nitroblue tetrazolium (NBT) solution prepared in 50 mM HEPES (pH 7.6) (Jiang et al. 2011). Sections were incubated for 2 h in the dark, washed in water, mounted in 50% glycerol and visualized through optical microscopy.

Freehand cross-sections of the main root apex were incubated in the dark with 4,5-diaminofluorescein diacetate at 10 µM. After 15 min, the sections were washed in water, mounted in Vectashield\textsuperscript{®} (Vector Laboratories, Inc., Burlingame, CA, USA) and observed with an epifluorescence Olympus BX41 microscope equipped with Olympus FITC filters (excitation at 450 nm; emission at 570 nm) and an Olympus SC30 digital camera.

Superoxide dismutase, CAT and GR assays

Mahogany tissue samples were macerated in liquid nitrogen in the presence of polyvinylpolypyrrolidone (50 mg per 1 g of plant tissue). Soluble proteins were extracted with 50 mM phosphate buffer (pH 6.8) containing 100 µM ethylenediaminetetraacetic acid (EDTA) and a protease inhibitor cocktail (Sigma-Aldrich) (1 ml of extraction buffer per 0.3 g of plant tissue). The activity of SOD was measured by incubating plant homogenates in the presence of polyvinylpolypyrrolidone (50 mg per 1 g of plant tissue). The activity of SOD was measured by incubating plant homogenates in the presence of polyvinylpolypyrrolidone (50 mg per 1 g of plant tissue). The activity of SOD was measured by incubating plant homogenates in the presence of polyvinylpolypyrrolidone (50 mg per 1 g of plant tissue). The activity of SOD was measured by incubating plant homogenates in the presence of polyvinylpolypyrrolidone (50 mg per 1 g of plant tissue).

The induced water deficit did not affect the developmental timing of newly germinated mahogany seedlings. Independently of the intensity of water stress, all seedlings presented the first mature leaves after 45 days of treatment. The number of leaves per seedling averaged 5.3 among the treatments. However, a progressive decrease in leaf area was observed upon reduction of water availability (Figure 1b). The pre-dawn leaf \( \Psi_w \) remained unchanged (−0.48 MPa) regardless of the water deficit. Part of the osmotic potential from ESC (\( \Psi_{ESC} \)) became 45% more negative in leaves and roots of seedlings under severe water stress in comparison with the corresponding control organs (Table 1). Mild water stress, however, triggered an increase in \( \Psi_{ESC} \) of stems (value less negative), while moderate or severe conditions of water deprivation did

Table 1. Effect of water deficit on osmotic potential (\( \Psi \)) from ESC and starch content in organs of mahogany seedlings. DW, dry weight; FW, fresh weight. Different letters indicate a significant difference (\( P < 0.05 \) by contrast analysis) among the watering regimes in an organ. The \( \Psi_{ESC} \) results are from experiments carried out with three seedlings per treatment and starch contents were determined from experiments carried out with four seedlings per treatment.

<table>
<thead>
<tr>
<th>Watering regime</th>
<th>Organ</th>
<th>DW/FW</th>
<th>ESC (kg kg\textsuperscript{−1} DW)</th>
<th>Part of ( \Psi ) from ESC (MPa)</th>
<th>Starch (%DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13% FC</td>
<td>Root</td>
<td>0.51 ± 0.08 A</td>
<td>0.04 ± 0.01 A</td>
<td>−0.28 ± 0.07 B</td>
<td>0.11 ± 0.05 A</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>0.23 ± 0.00 B</td>
<td>0.07 ± 0.03 A</td>
<td>−0.22 ± 0.07 B</td>
<td>0.08 ± 0.02 B</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>0.06 ± 0.01 B</td>
<td>0.33 ± 0.08 A</td>
<td>−0.29 ± 0.07 B</td>
<td>0.08 ± 0.01 A</td>
</tr>
<tr>
<td>26% FC</td>
<td>Root</td>
<td>0.36 ± 0.09 B</td>
<td>0.03 ± 0.01 A</td>
<td>−0.14 ± 0.03 A</td>
<td>0.03 ± 0.01 B</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>0.19 ± 0.03 C</td>
<td>0.09 ± 0.02 A</td>
<td>−0.23 ± 0.01 B</td>
<td>0.10 ± 0.04 B</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>0.28 ± 0.00 A</td>
<td>0.04 ± 0.01 C</td>
<td>−0.15 ± 0.04 A</td>
<td>0.05 ± 0.01 B</td>
</tr>
<tr>
<td>53% FC</td>
<td>Root</td>
<td>0.46 ± 0.05 A</td>
<td>0.02 ± 0.00 B</td>
<td>−0.10 ± 0.02 A</td>
<td>0.02 ± 0.01 B</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>0.21 ± 0.00 C</td>
<td>0.02 ± 0.00 B</td>
<td>−0.05 ± 0.01 A</td>
<td>0.27 ± 0.17 A</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>0.06 ± 0.00 B</td>
<td>0.17 ± 0.01 B</td>
<td>−0.14 ± 0.01 A</td>
<td>0.04 ± 0.01 B</td>
</tr>
<tr>
<td>93% FC</td>
<td>Root</td>
<td>0.20 ± 0.00 B</td>
<td>0.06 ± 0.01 A</td>
<td>−0.15 ± 0.04 A</td>
<td>0.05 ± 0.02 B</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>0.26 ± 0.00 A</td>
<td>0.05 ± 0.01 A</td>
<td>−0.18 ± 0.04 B</td>
<td>0.18 ± 0.03 B</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>0.29 ± 0.00 A</td>
<td>0.05 ± 0.01 C</td>
<td>−0.20 ± 0.03 A</td>
<td>0.05 ± 0.01 B</td>
</tr>
</tbody>
</table>
not affect ($P > 0.05$) this trait. An increase by 87% in the root : shoot ratio was verified in seedlings grown under severe water deficit (13% FC; Figure 1c), while mild (53% FC) or moderate (26% FC) water deficit did not significantly affect this parameter in 45-day-old mahogany plants. Both moderate and severe water deficit conditions diminished stomatal conductance in leaves when compared with control seedlings (93% FC treatment; Figure 1d).

**Soluble carbohydrate and starch levels in mahogany tissues upon water deficit**

The total soluble carbohydrate levels remained unchanged in roots and stems of seedlings grown under moderate or severe stress when compared with control seedlings but significantly decreased ($P < 0.05$) in such organs of seedlings subjected to mild water deficit (Figure 2). On the other hand, an increment in the levels of this class of metabolite was detected in...

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**Figure 2.** Accumulation of soluble carbohydrates in different organs of mahogany seedlings under water deficit. Newly germinated mahogany seedlings were subjected to watering regimes of 93 (control), 53, 26 or 13% of FC for 45 days, after which samples were harvested. Bars represent means + standard deviations of experiments carried out with three seedlings per treatment. Data presented in the panels on the left were subjected to variance analysis (one-way ANOVA) followed by mean separation using the Scott–Knott test. Different letters in the panels on the left indicate a significant difference among the treatments. Data presented in the panels on the right were subjected to variance analysis (two-way ANOVA) or Kruskal–Wallis followed by mean separation using the Scott–Knott test. Different lowercase letters in the panels on the right indicate a significant difference between carbohydrates extracted from tissues of the same watering regime while distinct uppercase letters show a significant difference with the watering regime treatments. Fru, fructose; Glu, glucose; Myo, myo-inositol; Suc, sucrose.
leaves of seedlings challenged with mild or severe water stress (Figure 2).

The levels of reducing carbohydrates decreased in plantlet roots upon different intensities of water deficit when compared with control seedlings (Figure 2). In contrast, stems of seedlings under moderate stress presented roughly twice as much reducing carbohydrates as control ones (Figure 2). The amount of such carbohydrates in leaves increased three- and sixfold when seedlings were grown under mild or severe water deficit, respectively (Figure 2).

Figure 2 also shows quantitative data for soluble carbohydrates in different mahogany tissues upon water deficit. Fructose, glucose and sucrose levels in roots and stems were similar within a water deficit treatment, except for control seedlings whose amounts of fructose and glucose in roots were lower than those of sucrose (Figure 2). Water deficit caused a decrease in the levels of such carbohydrates in roots and an increase of fructose and glucose in stems of seedlings under moderate or severe water deficit. The water deficit increased the amounts of the investigated carbohydrates in leaves (Figure 2). Sucrose and glucose levels were, on average, sixfold higher in leaves of seedlings under mild or severe stress in relation to control. In comparison to control seedlings, fructose levels increased fivefold, on average, in leaves independent of the stress intensity. The myo-inositol levels were roughly the same in roots, stems and leaves of control seedlings (1.9 mg g\(^{-1}\) DW) (Figure 2). The amount of myo-inositol decreased by ~50% in roots under moderate and severe stress and remained unchanged in stem tissues. On the other hand, the levels of this alcohol carbohydrate increased by about eightfold in leaves of seedlings under mild or severe stress (Figure 2).

The severe stress caused an increase of starch by 60% in leaf and 220% in root tissues (Table 1); mild or moderate stress did not affect the starch levels in any of these tissues. Increase of starch by 50% was verified in stems under mild stress with no changes in the level of this non-structural carbohydrate in stems under the other watering regimes in relation to control (Table 1).

### L-Proline levels in mahogany tissues upon water deficit

Alteration in l-proline levels as a result of water deficit occurred only in stem tissues (Figure 3). Under no water stress, mahogany stems presented 178.3 nmol l-proline g\(^{-1}\) DW, which increased up to 1286.1 nmol g\(^{-1}\) DW in seedlings under severe water deficit. Water deficit did not affect (\(P > 0.05\)) l-proline levels in roots and leaves, which averaged 292 and 254 nmol, respectively.

#### Effect of water deficit on \(O_2^-\) and NO production

The production of \(O_2^-\) was assessed using NBT that, in the presence of the referred ROS, is converted to the corresponding formazan, a blue-colored compound here referred to as dark, sharp staining. The presence of \(O_2^-\) was detected in all root tissues, except in the vascular cylinder (Figure 4). The intensity of the dark, sharp staining and the number of stained cells considerably decreased in roots under severe water deficit when compared with those of seedlings under mild, moderate or no water deficit at all (Figure 4).

This indicates that the lowest water availability negatively affected the production of \(O_2^-\) in root tissues. Production of \(O_2^-\) was detected in secondary phloem (Sp), radial system in secondary xylem (Rssx) and primary xylem (Px) of stems and did not appear to vary following different intensities of water deficit (Figure 5). Epidermal (Ep) and phloem fiber (Pf) cells from control leaves and few Pf cells of seedling leaves under mild water stress were positive for the production of \(O_2^-\) (Figure 5e–h).

Since mahogany roots are the first organ to experience water deficit, the production and localization of NO, a signaling molecule, was investigated in root tips of seedlings under different water deficit intensities. Nitric oxide was mainly detected in vascular cylinder cells of control roots (Figure 4). The levels of this RNS increased with the increment of water deficit as determined by the notorious augmentation of the intensity of green-yellowish fluorescence (here described as bright patches) in the vascular cylinder. Some Ep cells of roots under different levels of water deficit also produced NO (Figure 4).

#### Effect of water deficit on the activity of SOD, CAT and GR

The severe water deficit condition stimulated by twofold the activity of SOD in roots (82.8 ± 8.6 U min\(^{-1}\) mg\(^{-1}\) protein;
However, no difference ($P > 0.05$) in SOD activity was found in stems and leaves of seedlings, irrespective of the intensity of the imposed stress (Figure 6). Catalase activity decreased by 47% in leaf cells of seedlings grown under severe stress in comparison to control ones (Figure 6). The activity of this enzyme was not affected ($P > 0.05$) in stem or root cells following water deficit. All the water deprivation conditions tested triggered a more than twofold increase in GR activity of stem cells (Figure 6). A 2.5-fold increase in GR activity was observed in roots of seedlings under mild or moderate stress.

Figure 4. In situ localization of $O_2^-$ and NO in transverse sections of mahogany roots as a function of water deficit. Newly germinated mahogany seedlings were subjected to watering regimes of 93 (control), 53, 26 or 13% of FC for 45 days after which samples were harvested. The presence of $O_2^-$ (panels on the left; bars = 100 µm) is characterized by the dark, sharp staining within cells. The presence of NO in cells (panels on the right; bars = 200 µm) is characterized by the bright patches.

Figure 6)
Discussion

The applied drought treatments in newly germinated mahogany seedlings promoted a reduction of leaf area and an increase in the root:shoot ratio. The reduction in leaf area in mahogany seedlings under 53% FC, accompanied by no alteration in stomatal conductance (and likely photosynthesis as well), is consistent with the fact that root signals control shoot growth and development (Davies and Zhang 1991, Munns et al. 2000, Davies 2007). Reduction in leaf area was also described by Cordeiro et al. (2009) in studies performed with 1-year-old
mahogany plants grown under drought (soil $\Psi_w$ of $-3.5$ MPa) for 20 days. Besides stomatal closure, reduction in leaf growth also comprises a mechanism to diminish transpiration rate. An increase by over 80% in the root:shoot ratio in seedlings under severe water deficit indicates a higher accumulation of biomass in root systems since leaf area and stem length (data not shown) decreased upon seedling stress. The carbon allocation to root growth, also confirmed by the accumulation of starch in this organ, allows for the exploration of a larger soil area by the plant under water deficit conditions in an attempt to increase water uptake. Indeed, it was found that the number of secondary/lateral roots considerably increased in seedlings under moderate or severe water deficit when compared with seedlings under the other watering regimes. The stomatal conductance decreased upon moderate to severe water deficit. These values found in 45-day-old mahogany leaves were, in general, lower than those reported by Silva and Lemos-Filho (2001) and Cordeiro et al. (2009) in experiments carried out with the same plant species. Such differences may be attributed to the distinct developmental stages of the mahogany plants used: adults in field experiments (Silva and Lemos-Filho 2001) or 1-year-old plants in greenhouse experiments (Cordeiro et al. 2009). The levels of water deficit imposed on mahogany seedlings did not affect the pre-dawn $\Psi_w$ of leaves, the value of which indicated good water status in cells. Overall, these results suggest that mahogany is able to perform osmotic adjustment to maintain cell turgor to cope with water deficit conditions during the first 45 days of development.

To test this hypothesis we quantified soluble carbohydrates and determined the osmotic potential ($\Psi_{ESC}$) and the levels of l-proline in cells of different mahogany organs. Evidence of occurrence of osmotic adjustment was found in leaf cells, whose soluble carbohydrate levels dramatically increased upon water deficit. The levels of total carbohydrates increased in leaves of seedlings upon mild or severe water stress, but not in stems or roots at any of the tested water deficit intensities. A pronounced development of mahogany secondary/lateral roots under moderate water deficit, as observed for other plants (Shao et al. 2008), likely improved water uptake by seedlings, excluding the need for osmotic adjustment in tissues of this organ. The levels of reducing carbohydrates, which on average comprised 30% of total carbohydrates in the evaluated organs of control seedlings, increased only in stems of seedlings under moderate water deficit and in leaves of seedlings under mild or severe water deficit. However, only leaves really exhibited a significant increase in total soluble carbohydrates. The contribution of soluble carbohydrates to the $\Psi_s$ of cells from the studied organs was determined, and the results revealed a more negative value in leaves (but not in stems) under severe stress in comparison with the respective control. This indicates that the increase of soluble carbohydrates by almost sevenfold in leaves was essential for cells performing osmotic adjustment when exposed to

Figure 6. Effect of water deficit on the activity of SOD, CAT and GR in different organs of mahogany seedlings. Newly germinated mahogany seedlings were subjected to watering regimes of 93 (control), 53, 26 or 13% of FC for 45 days after which samples were harvested. Bars represent means + standard deviations of experiments carried out with four seedlings per treatment. Asterisks (*) indicate $P < 0.05$ in relation to the respective control samples according to variance analysis (one-way ANOVA) or Kruskal–Wallis followed by mean separation using the Scott–Knott test. GSH, glutathione.
critical water deficit for 45 days. Interestingly, the \( \psi_s \) of root cells under severe water deficit was much more negative than that of control roots, which would suggest that soluble carbohydrates played a major role in the osmotic adjustment in such cells even though quantitative data revealed that the levels of soluble carbohydrates in roots were not affected by water deficit. These contrasting results may be explained by the differences in the DW/FW ratio, the parameter used for calculating \( \psi_s \) from the van't Hoff equation (Ninemets and Kull 1998); the DW/FW ratio for roots under severe stress was 0.51, while the value obtained for those under no treatment (control) was 0.29 (Table 1). This set of results indicates that osmotic adjustment based on carbohydrate accumulation has occurred in leaf cells of mahogany seedlings in response to water deficit. The starch content in leaves somehow increased in response to severe water deficit. Although starch degradation is a common response involved in sugar accumulation triggered by water deficit (Lee et al. 2008), a recent report shows that leaves of the woody plant Lycium barbarum accumulate starch and ultimately sucrose upon increasing water stress (Zhao et al. 2013). The survival and growth of all seedlings 45 days post severe water deficit indicates that the net photosynthesis in seedling leaves was greater than zero. Thus, part of the glucose originating from photosynthesis (and still from starch cleavage) would be directed to sucrose biogenesis as fructose levels in this organ decreased, while the remaining free glucose could contribute to the augmentation of cell osmolarity. Starch content increased by 220% in roots of seedlings under severe water deficit, but this event was not accompanied by an increment in the levels of soluble carbohydrates. Galvez et al. (2011) also detected an increase of starch in roots of the woody plant Populus tremuloides under drought. The same authors also disclosed that accumulation of starch in roots of P. tremuloides and Populus balsamifera may contribute to seedling survival when under drought during the dormant season (Galvez et al. 2013). Interestingly, water deficit triggered the accumulation of the sugar alcohol myo-inositol (3.5-fold on average) only in leaf cells of seedlings under mild or severe stress. Accumulation of myo-inositol was also observed in leaves of 5- to 7-week-old Actinidia deliciosa under salt stress (Klages et al. 1999). The role of myo-inositol as a structural basis for a series of second messengers in plants is known in which its metabolism was recently shown to influence drought tolerance, carbohydrate metabolism and biomass accumulation in tomato plants (Khodakovskaya et al. 2010). Stem tissues of mahogany under moderate or severe water deficit presented, respectively, 50 and 34% more fructose and glucose than stems of control seedlings. Differently from the findings in leaves, sucrose levels in stems were not affected by water deficit. As expected, the levels of soluble carbohydrates in leaves were much higher than those of roots or stems since leaves, at this developmental stage, are notably sources of photoassimilates while roots and stems act as sinks.

The water deficit caused a considerable increment in \( \Gamma \)-proline levels in stems, but not in roots or leaves. As observed for Anacardium occidentale (Silveira et al. 2003), \( \Gamma \)-proline levels in mahogany roots were not affected by water deficit. Our results contrast those reported by Cordeiro et al. (2009), in which 1-year-old mahogany plants accumulated \( \Gamma \)-proline in leaves, but not in stems. Such contrasting results observed for the same plant species may be explained by the great difference in the developmental stage. The accumulation of \( \Gamma \)-proline in plant leaves under drought is well documented. A study showed that the amount of \( \Gamma \)-proline accumulated in stems of in-vitro propagated 30-day-old Solanum tuberosum plants in response to salinity stress was higher than that accumulated in leaves, in relation to the respective controls (Potluri and Prasad 1993). The observed accumulation of \( \Gamma \)-proline in stems of S. tuberosum was found to contribute to the osmotic adjustment in cells (Potluri and Prasad 1993). On the other hand, Hare and Cress (1997) found that increases in the \( \Gamma \)-proline pool in cell cytoplasm following some stress lead to accumulation of 1-\( \delta \)-pyrroline-5-carboxylate and subsequent transfer of redox potential between plant tissues. This phenomenon was proposed to comprise another metabolic signaling pathway in higher plants. Indeed, recent studies showed that the increased production of \( \Gamma \)-proline in transgenic Swingle citrulmo under severe water deficit triggered the accumulation of transcripts that encode for enzymes of the antioxidant system (in particular GR) in comparison to non-transformed plants (Carvalho et al. 2013). Our findings demonstrate that, in addition to \( \Gamma \)-proline accumulation, mahogany stems exhibited increased GR activity upon water deficit. Then, in addition to osmotic adjustment, the increased \( \Gamma \)-proline levels in mahogany stems contribute to the signaling of gene expression with activation of biochemical routes that will help cells to cope with moderate-to-severe water scarcity.

Different patterns of activity of antioxidant enzymes were observed among the studied organs in response to water deficit. Various studies have shown that the activity of a certain antioxidant enzyme increased in some plant species and decreased or was unaffected in other ones when subjected to water deficit (reviewed by Cruz de Carvalho 2008). The duration and intensity of stress and the plant age and its degree of tolerance to water stress are believed to differentially affect the activity of antioxidant enzymes. In the present study, severe water deficit more than doubled SOD activity in mahogany roots, while the activity of this enzyme in stem and leaf cells remained constant. Stimulation of SOD leads to an increase of \( O_2^- \) dismutation to \( H_2O_2 \). This finding is in agreement with that of \( O_2^- \) detection in root tissues, in which the intensity of dark, sharp staining characteristic of \( O_2^- \) presence was considerably reduced in roots under severe water deficit in relation to control roots. Also, the basal SOD activity in stems and leaves corroborates the observation that \( O_2^- \) levels...
were apparently unaffected in such organs regardless of the water deficit intensity. Only leaf cells exhibited an alteration in CAT activity, which decreased in seedlings under moderate or severe stress.

Lignification was found to increase in leaf cells under water deficit to prevent xylem cavitation and excessive water loss by cells (Tyree and Sperry 1988). The substrate for CAT, H$_2$O$_2$, plays a role not only in cell wall lignification (Olson and Varner 1993), but also as a signaling molecule when produced at relatively low concentrations in plants under stressing conditions (Hung et al. 2005). The decrease of CAT activity in leaves upon moderate or severe stress implicates some increase in the H$_2$O$_2$ pool in cells since the SOD activity in this organ was not affected by water deficit. However, the increment in the H$_2$O$_2$ pool was not probably high enough to induce oxidative stress; otherwise seedlings would have failed to grow. Then, H$_2$O$_2$ accumulated in leaves could drive lignin biosynthesis and also induce signaling pathways related to myo-inositol biosynthesis as proposed by Tan et al. (2013). Indeed, myo-inositol content dramatically increased in leaves upon the water regime studied, as it occurred in M. falcata upon treatment with H$_2$O$_2$.

Glutathione reductase activity was stimulated in mahogany roots and stems and unchanged in leaf cells. Glutathione reductase activity leads to the formation of reduced glutathione, which is indispensable for the prevention of lipid and protein oxidation (Szalai et al. 2009) and the regeneration of ascorbate, another antioxidant molecule (Mittler 2002).

Control mahogany seedlings exhibited NO production in the vascular cylinder of roots. Production of NO increased in such root tissue in seedlings under mild or severe water stress, as determined by the increase of bright patches. The presence of NO was confirmed in the epidermis, cortex and endoderm tissues of root cells under severe water deficit. The signaling role of NO in the synthesis of secondary walls of xylem (Gabaldón et al. 2005) and the importance of xylem in the recovery of plants after exposure to extreme water deficit (Brodribb et al. 2010) are documented. Nitric oxide is also recognized as an important signaling molecule in plant response to biotic and abiotic stresses (Modolo et al. 2005, Siddiqui et al. 2010). Then, NO produced in different tissues of mahogany roots under moderate or severe water deficit contributes to local (increase of root surface; Correa-Aragunde et al. 2006) and systemic signaling pathways in stems and leaves. As for the systemic signaling process, translocation of NO from roots to leaves through the xylem may induce the expression of the myo-inositol phosphate synthase (MIPS) gene in shoots with subsequent accumulation of myo-inositol to increase drought tolerance. This is supported by the recent report of Tan et al. (2013) in which accumulation of myo-inositol in excised M. falcata leaflets under dehydration was shown to be stimulated by exogenous NO via induction of MIPS expression to confer drought tolerance.

Overall, severe water deficit conditions triggered reductions in mahogany seedling leaf area, remarkable control of transpiration and carbon allocation toward root systems with accumulation of reserve. The water scarcity orchestrated the differential accumulation of signaling molecules among the studied organs. Thus, the increase of NO in roots, l-proline in stems, and H$_2$O$_2$ and myo-inositol in leaves of mahogany under water scarcity led to signaling events in the whole seedling that ultimately allowed for attenuation of oxidative stress and accumulation of high amounts of soluble carbohydrates in leaf cells for osmotic adjustment purposes. l-Proline accumulation exclusively in stems could also account for osmotic adjustment in this organ under severe stress. The similar pattern found for organs of seedlings under control conditions and under moderate stress for most of the evaluated parameters may be a result of mahogany acclimation to water deprivation as moderate stress induced the formation of various secondary/lateral roots. In summary, our results clearly demonstrate that these organ-coordinated responses of mahogany seedlings to water deficit during the very early stages of development guarantee the success of plant establishment when water availability is greatly limited in natural habitats or crop fields.

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**Conflict of interest**

None declared.

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