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TIAGO DE SOUSA LEITE

ECOPHYSIOLOGY OF CAATINGA TREE SPECIES AS A FUNCTION OF DROUGHT STRESS AND REHYDRATION

MOSSORÓ

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Tese apresentada ao Programa de Pós-Graduação em Fitotecnia da Universidade Federal Rural do Semi-Árido como requisito para obtenção do título de Doutor em Fitotecnia.

Linha de Pesquisa: Nutrição, Irrigação e Salinidade.

Orientador: Prof. Dr. Nildo da Silva Dias.

Coorientador: Prof. Dr. Rômulo Magno Oliveira de Freitas.

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BANCA EXAMINADORA

Nildo da Silva Dias, Prof. Dr. (UFERSA)
Presidente

Rômulo Magno Oliveira de Freitas, Prof. Dr. (IFRN)
Membro Examinador
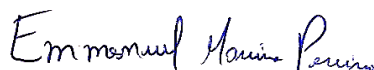
Narjara Walessa Nogueira de Freitas, Profa. Dra. (UFERSA)
Membra Examinadora

Wanderson de Sousa Mendes, Dr. (ZALF)
Membro Examinador

Emmanuel Moreira Pereira, Dr. (INSA)
Membro Examinador

Aurélio Paes Barros Júnior, Prof. Dr. (UFERSA)
Membro Examinador

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"A man is but the product of his thoughts. What he thinks, he becomes".

Mahatma Gandhi

ABSTRACT

Erythrina velutina, *Mimosa tenuiflora*, *Piptadenia stipulacea* and *Poincianella pyramidalis* are pioneer trees native to the Caatinga biome, a tropical dry forest whose composition is threatened by increasingly frequent and lasting droughts. Nevertheless, there is limited information on the recovery of these species when water availability is normalized. Therefore, their ecophysiology was studied as a function of drought stress and rehydration. Four concurrent but independent experiments were conducted under greenhouse conditions, in a split-plot design with two water regimes in the main plots (1 – control; 2 – drought followed by rehydration) and sampling times in the subplots (at varying periods of drought and rehydration). Plant water status, gas exchange parameters, biochemical attributes and subsequent growth were measured. *E. velutina* rapidly downregulated photosynthesis, reducing leaf gas exchange and improving water use efficiency to compensate for the temporary loss of xylem water transport, and recovered slowly after having a high consumption of non-structural carbohydrates. In contrast, the photosynthetic activity of *P. pyramidalis* was gradually reduced with increasing drought, but quickly recovered when rehydrated, and the leaf water potential was effectively reduced through the accumulation of proline. Although showing different mechanisms behind their drought tolerance, in both species, the full recovery of photosynthesis upon rewatering was possibly related to enhanced photoprotection by carotenoids. *M. tenuiflora* and *P. stipulacea* maintained a low leaf water potential throughout the day by accumulating compatible solutes, thus allowing a rapid and full recovery of water status when rehydrated. Even though these plants minimized water loss by closing their stomata, neither showed stomatal limitations to photosynthesis. The inhibition of this process during drought was possibly related to mesophyll limitations as well as to a reversible downregulation of photosystems, along with adjustments of their stoichiometry. Drought stress also triggered morphological adaptations at the whole plant level, leading to reduced growth, mainly of the shoots in *M. tenuiflora* and the roots in *P. stipulacea*.

Keywords: Tropical dry forest. Deciduous trees. Photosynthetic activity. Water potential. Osmotic adjustment.

RESUMO

Erythrina velutina, *Mimosa tenuiflora*, *Piptadenia stipulacea* e *Poincianella pyramidalis* são árvores pioneiras nativas do bioma Caatinga, floresta tropical seca cuja composição está ameaçada por secas cada vez mais frequentes e duradouras. No entanto, há informações limitadas sobre a recuperação dessas espécies quando da normalização da disponibilidade de água. Portanto, a ecofisiologia das mesmas foi estudada em função do estresse pela seca e da reidratação. Quatro experimentos simultâneos, mas independentes, foram conduzidos em casa de vegetação, em esquema de parcelas subdivididas com dois regimes hídricos nas parcelas (1 – controle; 2 – seca seguida de reidratação) e épocas de amostragem nas subparcelas (em períodos variados de seca e reidratação). Foram avaliados o estado hídrico das plantas, parâmetros de trocas gasosas, atributos bioquímicos e o crescimento subsequente. *E. velutina* diminuiu rapidamente a fotossíntese, reduzindo as trocas gasosas foliares e melhorando a eficiência do uso da água para compensar a perda temporária do transporte de água do xilema, e recuperou-se lentamente após um alto consumo de carboidratos não estruturais. Em contraste, a atividade fotossintética de *P. pyramidalis* foi gradualmente reduzida com o aumento da seca, mas rapidamente recuperada quando reidratada, e o potencial hídrico foliar foi efetivamente reduzido pelo acúmulo de prolina. Apesar de apresentarem diferentes mecanismos por trás de suas tolerâncias à seca, em ambas as espécies, a recuperação total da fotossíntese na reidratação está possivelmente relacionada à fotoproteção melhorada por carotenoides. *M. tenuiflora* e *P. stipulacea* mantiveram um baixo potencial hídrico foliar ao longo do dia através do acúmulo de solutos compatíveis, permitindo assim uma recuperação rápida e completa do estado hídrico quando reidratadas. Embora essas plantas minimizaram a perda de água fechando seus estômatos, nenhuma apresentou limitações estomáticas à fotossíntese. A inibição desse processo durante a seca está possivelmente relacionada a limitações do mesofilo bem como a uma regulação negativa reversível dos fotossistemas, juntamente com ajustes na estequiometria dos mesmos. O estresse pela seca também desencadeou adaptações morfológicas em toda a planta, levando à redução do crescimento, principalmente da parte aérea em *M. tenuiflora* e das raízes em *P. stipulacea*.

Palavras-chave: Floresta seca tropical. Árvores caducifólias. Atividade fotossintética. Potencial hídrico. Ajuste osmótico.

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SUMMARY

1 GENERAL INTRODUCTION

Drought-induced tree mortality has threatened the sustainability of tropical dry forests and other forest ecosystems around the world (CHOAT et al., 2018; SANTOS et al., 2014). Among the stress factors that influence forest carbon balance, drought is by far the most common, which highlights the need to understand the effect of this phenomena on tree physiology (CHOAT et al., 2018; TRUGMAN et al., 2018). Stan et al. (2021) found that tropical dry forests subjected to severe droughts may require more than two years to recover pre-stress conditions. Decreases in water availability can alter not only species composition but also their functional attributes, and these problems are often exacerbated when new droughts set in before the time required for recovery has elapsed (HUANG et al., 2018).

Although tropical dry forests can adapt to sudden changes in environmental conditions (RAYMUNDO et al., 2019; STAN et al., 2021), it is well known that droughts impair the normal growth and development of trees and, therefore, their carbon fixation (AROCA, 2012). Tropical dry forests are comprised mostly of species that show a range of drought avoidance and/or resistance mechanisms (RAYMUNDO et al., 2019). For this reason, their vegetation can usually withstand the harsh conditions of semi-arid and arid areas. Nevertheless, if these conditions were to be intensified, even such mechanisms might not be enough to avoid mass tree mortality (HUANG et al., 2018; TRUGMAN et al., 2018).

The Caatinga is an exclusively Brazilian biome, located in one of the most populous semi-arid regions in the world (BARROS et al., 2020). It covers an area of approximately 844,000 km² and it is home to about 27 million people and a remarkably rich biodiversity (CAMPOS et al., 2020; SILVA et al., 2017). Despite being the largest tropical dry forest in South America, there are projections of decrease in rainfall and increase in temperature, as well as of desertification, which will potentially affect the provision of multiple services of this ecosystem (MORO et al., 2016; SILVA et al., 2017). Climate change, including the occurrence of increasingly frequent and severe droughts, could eventually alter the composition of the Caatinga flora, favouring the prevalence of certain species to the detriment of others (CAMPOS et al., 2020; SANTOS et al., 2014).

Erythrina velutina Willd., *Mimosa tenuiflora* (Willd.) Poir., *Piptadenia stipulacea* (Benth.) Ducke and *Poincianella pyramidalis* (Tul.) L.P. Queiroz are pioneer trees native to the Caatinga biome (ALVES & FREIRE, 2019; BARROS et al., 2019; LEITE et al., 2020; SILVA et al., 2010). These species play a key role in the ecological succession of this tropical dry forest, besides being of paramount importance for the economic and social development

of local communities in the Northeast region of Brazil. Particularities aside, they share some important characteristics such as well-developed root systems and the ability to shed their leaves, which allow them to survive frequent periods with low water availability (AROCA, 2012). However, there is limited information on their response to droughts and, most of all, on their recovery dynamics following these stressful events.

Over the last few decades, the effects of water deficiency have been actively studied for Caatinga deciduous trees like *Tabebuia aurea* (CABRAL et al., 2004), *Piptadenia moniliformes* (SOUZA et al., 2010), *Handroanthus impetiginosus* (DOMBROSKI et al., 2014), *Poincianella bracteosa* (FERREIRA et al., 2015), *Anadenanthera colubrina* (BARROS et al., 2019), *Cnidoscolus quercifolius* (RAMOS & FREIRE, 2019), *Cenostigma pyramidale* (SANTOS et al., 2021), *Mimosa caesalpiniifolia* and *Myracrodruon urundeuva* (BARROS et al., 2020). However, in general, studies on the recovery of these plants from drought stress are still incipient, as pointed out by Dombroski et al. (2014).

In this context, the purpose of this study was to obtain information on the ecophysiological performance of four Caatinga tree species (*E. velutina*, *M. tenuiflora*, *P. stipulacea* and *P. pyramidalis*) in response to both drought stress and rehydration, which was possible by evaluating plant water status, gas exchange parameters, biochemical attributes and plant growth, over time, under greenhouse conditions. The results were divided into two chapters (articles). The first one compares the responses of two contrasting species in terms of drought tolerance, *E. velutina* and *P. pyramidalis*, to test the hypothesis that metabolic shifts play a key role in the regulation of plant water status for a fast recovery of photosynthesis following drought stress. The second chapter aims to elucidate some of the mechanisms used by *M. tenuiflora* and *P. stipulacea*, which are commonly accepted as drought-tolerant species, to thrive under the extremely low water availability of tropical dry forests.

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2 ECOPHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF TWO TREE SPECIES FROM A TROPICAL DRY FOREST TO DROUGHT STRESS AND RECOVERY[1]

ABSTRACT

Droughts are predicted to increase in frequency and severity, thus affecting the functioning of forest ecosystems. However, it is not clear how the recovery dynamics of *Erythrina velutina* and *Poincianella pyramidalis* work following these events. Thus, the ecophysiological and biochemical responses of these tree species to drought stress and recovery were investigated. The study was conducted under greenhouse conditions, where the effects of two water regimes (1 – control and 2 – drought stress followed by recovery) were evaluated over time. Gas exchange parameters, leaf water potentials and biochemical attributes were measured. Under drought, *E. velutina* rapidly downregulated photosynthesis, reducing leaf gas exchange and improving water use efficiency to compensate for the temporary loss of xylem water transport, and recovered slowly after having a high consumption of non-structural carbohydrates. In contrast, the photosynthetic activity of *P. pyramidalis* was gradually reduced with increasing drought, but quickly recovered when rewatered, and the leaf water potential was effectively reduced through the accumulation of proline. Although showing different mechanisms behind their drought tolerance, in both species, the full recovery of photosynthesis upon rewatering was possibly related to enhanced photoprotection by carotenoids, which can contribute to the resilience of these trees in the face of recurring droughts.

Keywords: Photosynthetic activity. Water potential. Photoprotection. Deciduous trees. Caatinga.

2.1 Introduction

Drought is one of the most important plant stressors and it is predicted to increase in frequency and severity in the future, threatening the survival of trees in tropical dry forests worldwide (EZIZ et al., 2017; GONZÁLEZ-M. et al., 2021; LIAO et al., 2018). As such, the

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Caatinga that covers almost the entire semi-arid region of Brazil commonly experiences drought years (BARROS et al., 2020; FROSI et al., 2016). Moreover, every 3–4 decades, this dry forest also undergoes severe droughts that can last up to five years (SAMPAIO, 1995). The Caatinga is mainly composed of deciduous species and is characterized by shallow soils, high potential evapotranspiration and low and erratic rainfall (SANTOS et al., 2014; SILVA et al., 2017), which combined often lead to water deficit (CHOAT et al., 2018; SAMPAIO, 1995). This condition is known to trigger tree mortality, thus affecting the structure and function of forest ecosystems (DIETRICH & KAHMEN, 2019).

Low water availability has many negative effects on plants, limiting their growth and development (SANTIAGO et al., 2016; ZARGAR et al., 2017; ZHANG et al., 2017). Among the innumerable vital processes that it affects, drought stress has a major impact on photosynthesis, because by closing their stomata to control water loss and maintain cell turgor plants consequently reduce CO_2 uptake (DUAN et al., 2020; TAIZ et al., 2015). In addition to stomatal limitations, metabolic impairments may occur (AREND et al., 2016). Drought can lead to a decrease in photosynthetic pigments by causing their rapid breakdown. This is particularly important when damaging the antenna complexes of photosystems, thus reducing their light-harvesting efficiency and the photosynthetic capacity (ASHRAF & HARRIS, 2013). Reduced carbon assimilation under drought stress could negatively affect the long-term performance of plants in the field, especially after multiple drought and recovery cycles (PEGUERO-PINA et al., 2018).

In order to cope with drought, trees may undergo morphological, physiological and biochemical changes (BARROS et al., 2020; HU et al., 2015; TAIZ et al., 2015). These responses depend on the species and growth stage, as well as on the intensity and duration of stress (ZARGAR et al., 2017). *Erythrina velutina* Willd. (Fabaceae) and *Poincianella pyramidalis* (Tul.) L.P. Queiroz (Fabaceae) are among the most abundant species in Caatinga and other tropical dry forests in South America (BUTZ et al., 2017; LEITE et al., 2020; SILVA et al., 2010). They have been widely used for medicinal purposes, timber production and reforestation programmes (LEITE et al., 2020; OLIVEIRA et al., 2016). However, there is limited information on their response to drought stress.

Previous research has focused mainly on the morphological changes of the aforementioned species under drought. Silva et al. (2010) found that *E. velutina* seedlings grown at 25% field capacity had a higher specific leaf area, but lower shoot height, stem diameter, number of leaves, leaf area and total dry weight than those grown at 100% field capacity. More recently, Leite et al. (2020) observed that increasing drought periods led to

virtually the same morphological changes in *P. pyramidalis* seedlings, which also showed increases in the total soluble sugar and proline contents. Nevertheless, neither of these studies examined the recovery of plants upon rewatering, even though adapting to and surviving droughts depend on the rate and speed of this process (GALLÉ & FELLER, 2007). Frosi et al. (2016) assessed the physiological performance of *P. pyramidalis* under drought, evaluating plants at the highest stress level (12th day of drought) and at the fourth day after rewatering. But, unfortunately, this recovery period was not enough to restore gas exchange parameters, particularly net photosynthesis, to pre-stress levels.

Despite the social, economic and ecological importance of *E. velutina* and *P. pyramidalis*, it is not clear how their recovery dynamics work following drought periods. Understanding the response of native species to short-term drought allows us to predict how this increasingly common event will change natural ecosystems in the future (SANTOS et al., 2014; HU et al., 2015; SCHIMPL et al., 2019). Furthermore, it is also important to shed light on the underlying mechanisms of stress recovery, which can be achieved by studying this process under controlled environments (RUEHR et al., 2019). In the present work, the ecophysiological and biochemical responses of *E. velutina* and *P. pyramidalis* to drought stress and recovery were investigated under greenhouse conditions, by measuring gas exchange parameters, predawn and midday water potentials and biochemical attributes over time. The hypothesis was that metabolic shifts would play a key role in the regulation of plant water status for a fast recovery of photosynthetic activity following drought stress.

2.2 Material and methods

2.2.1 Seedling production and experimental design

The study was conducted under greenhouse conditions (5°12'16" S 37°19'29" W) with an average temperature of 29.6 °C and a relative humidity of 63.4%. *E. velutina* and *P. pyramidalis* were grown in 1.9 L polyethylene bags filled with loamy sand (Table 1), in two concurrent but independent experiments. Plants were arranged in a randomized complete block design with repeated measures over time. Each experiment had a total of 10 blocks, wherein each experimental unit was composed of eight bags. There were two water regimes as the between-subjects factor (control and drought stress followed by recovery) and evaluation dates as the within-subjects factor (8 for *E. velutina* and 9 for *P. pyramidalis*). Drought stress was induced by withholding irrigation until the net photosynthesis was close to zero (GOMES et al., 2008; LIBERATO et al., 2006). As for the evaluation dates (with 1- to 5-

day intervals), they varied according to the time necessary for photosynthetic activity to be almost completely inhibited and then restored to normal levels, at the end of the recovery phase upon rewatering, which depended on the response of each species (FREITAS et al., 2018).

Table 1 - Particle size distribution and chemical properties of the topsoil used to grow *E. velutina* and *P. pyramidalis* subjected to drought followed by rehydration.

Particle size distribution	Unity	Value
Clay	$g \ kg^{-1}$	40
Silt	$g \ kg^{-1}$	90
Fine sand	$g \ kg^{-1}$	210
Coarse sand	$g \ kg^{-1}$	660
Chemical properties	Unity	Value
pH in H_2O	-	8.28
Electrical conductivity	$dS \ m^{-1}$	0.13
Organic matter	$g \ kg^{-1}$	11.36
Nitrogen (N)	$g \ kg^{-1}$	0.35
Calcium (Ca^{2+})	$cmol_c \ kg^{-1}$	2.00
Magnesium (Mg^{2+})	$cmol_c \ kg^{-1}$	0.30
Potassium (K^+)	$mg \ kg^{-1}$	61.78
Sodium (Na^+)	$mg \ kg^{-1}$	59.38
Phosphorus (P)	$mg \ kg^{-1}$	16.13
Manganese (Mn)	$mg \ kg^{-1}$	7.06
Zinc (Zn)	$mg \ kg^{-1}$	2.27
Iron (Fe)	$mg \ kg^{-1}$	1.38
Copper (Cu)	$mg \ kg^{-1}$	0.04

Values of available nutrients

The soil was mixed with 200 mg P_2O_5 dm^{-3} and received five top dressings (in 10-day intervals, beginning at 40 days after sowing) with 100 mg N dm^{-3} and 50 mg K_2O dm^{-3}, to prevent any deficiency problems. Seeds of both species were collected from nearby trees growing in natural conditions, then mechanically scarified, by sanding their coats (FERREIRA et al., 2014; LEITE et al., 2014), and sown directly in the substrate. Three weeks later, seedlings were thinned to one per bag and irrigated twice a day before the actual

experiments, which started six months after sowing, when *E. velutina* and *P. pyramidalis* were approximately 1.1 and 0.6 m tall, respectively. For the drought stress treatment, inhibition of photosynthesis was observed when water was withheld for 10 days for *E. velutina* and 12 days for *P. pyramidalis*, whereas their recovery phases after rewatering lasted 10 days for the former and 13 days for the latter. Control plants were irrigated to field capacity throughout the whole study period.

2.2.2 Gas exchange parameters

Net photosynthesis (A), stomatal conductance (g_s), transpiration rate (E) and intercellular CO_2 concentration (C_i) were simultaneously evaluated, and based on these parameters the carboxylation efficiency (A/C_i) and the instantaneous (A/E) and intrinsic (A/g_s) water use efficiencies were calculated (FREITAS et al., 2018). Readings were taken in the middle of one new and fully expanded leaf per experimental unit per species, from 9 to 10 a.m. A LI-6400 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE, USA) was used and flow rate, CO_2 concentration and light were set at 400 µmol s^{-1}, 400 µmol mol^{-1} and 1,200 µmol m^{-2} s^{-1}, respectively, based on CO_2 and light response curves. The severity of drought stress was monitored using the net photosynthesis, which helped to determine the moment of rewatering and of the full recovery of plants (GOMES et al., 2008).

2.2.3 Leaf water potential (Ψ_w)

Plant water status was assessed based on predawn (4–6 a.m.) and midday (11 a.m.–1 p.m.) leaf water potentials, with a model 1000 pressure chamber (PMS Instrument Company, Albany, OR, USA). The third or fourth mature leaf from the top was excised from one plant per experimental unit and immediately placed in the chamber for measurement (KARIMI et al., 2015).

2.2.4 Biochemical attributes

The contents of proline, starch, soluble sugar and photosynthetic pigments (total chlorophyll and carotenoids) in newly expanded leaves were determined at the highest stress level (last day of drought), which occurred after 10 days for *E. velutina* and 12 days for *P. pyramidalis*. Leaf samples from five blocks were collected at midday, wrapped in aluminium foil and stored in a freezer (−18 °C) prior to analysis. Free proline was calculated from a standard curve, according to the method proposed by Bates et al. (1973). The contents of starch and soluble sugar were determined following phenol-sulphuric acid reaction (DUBOIS

et al., 1956). Total chlorophyll and carotenoids were estimated after acetone extraction (LICHTENTHALER, 1987), with absorbance measurements at three specific wavelengths (470, 645 and 663 nm). In addition, the relative proportion of these pigments was assessed by the chlorophyll/carotenoid ratio (GITELSON, 2020). All laboratory analyses were performed in triplicate, using five replications, and the results were expressed as unit per dry weight; except for the chlorophyll/carotenoid ratio, expressed in relative units.

2.2.5 Statistical analysis

A repeated-measures ANOVA, with water regimes as the between-subjects factor and evaluation dates as the within-subjects factor, was performed to test for drought stress followed by recovery effects. At each evaluation date, control and drought-stressed plants were compared by Student's t-test (at 5% significance level) using the Sisvar software, version 5.6 (Federal University of Lavras, Lavras, MG, Brazil). Pearson correlation analysis (at 5% significance level) was also performed between gas exchange parameters.

2.3 Results

2.3.1 Photosynthetic activity

Net photosynthesis of *E. velutina* was highly sensitive to the lack of water in the substrate, reducing by 80.6% in relation to the control after four days without irrigation. Although seedlings experienced a short drought period (10 days), it was only on the fifth day of rewatering that this parameter showed signs of recovery, which was fully accomplished five days later (Figure 1a). A similar pattern was observed for the carboxylation efficiency (Figure 1b). As for *P. pyramidalis*, an almost linear decrease was found in net photosynthesis with the increase in drought duration, but treatments did not differ significantly in the first four days and the inhibition of photosynthesis occurred only at the 12th day. There was a 13-fold increase in this parameter right after rewatering and plants were considered completely recovered within a 13-day period (Figure 1c). On the other hand, the carboxylation efficiency between treatments only differed at the highest stress level, as well as at the next two evaluation dates (Figure 1d). These results were consistent with a low intercellular CO_2 concentration, as verified for both species during drought stress and the first three days of recovery (Figure S1).
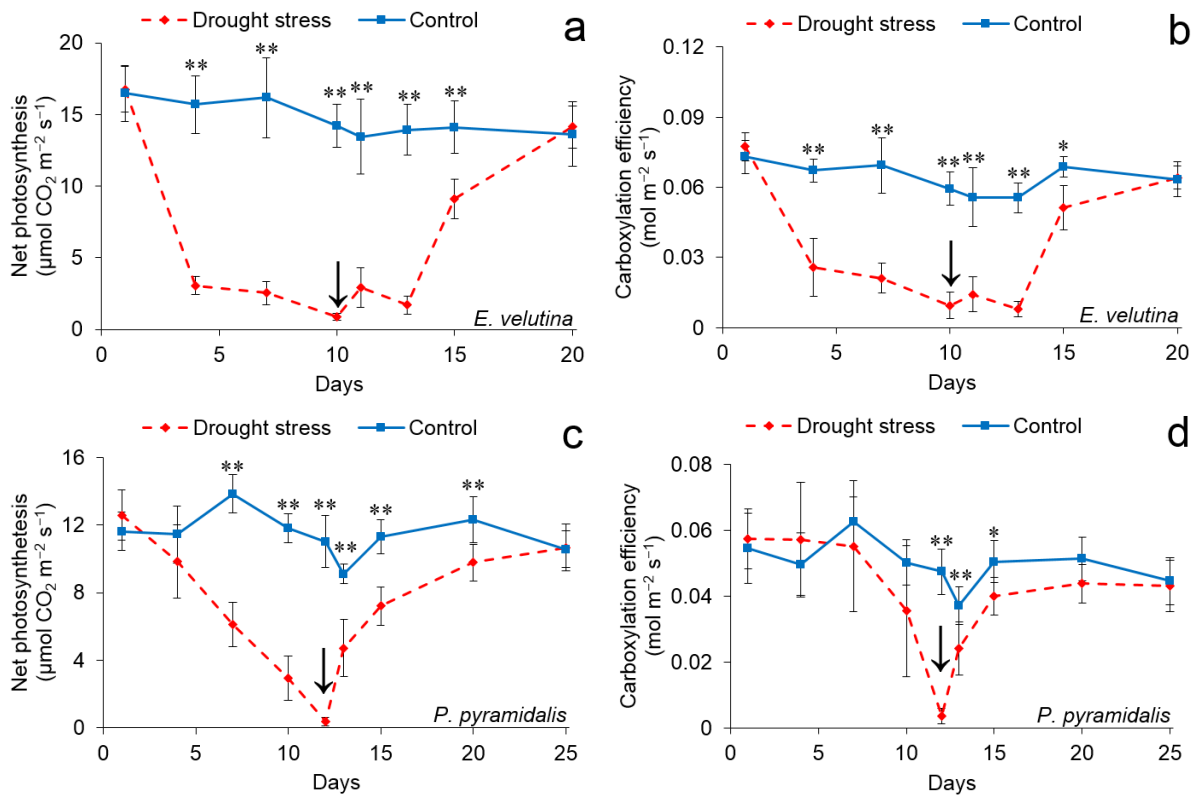
Figure 1 - Net photosynthesis (a, c) and carboxylation efficiency (b, d) of *E. velutina* and *P. pyramidalis* over time as a function of drought and rehydration. Arrows indicate the beginning of the recovery period. Values are means ± SD (*n* = 10) and asterisks denote significant differences from controls (**, *P* < 0.01; *, *P* < 0.05).

Stomatal conductance and transpiration rate were positively correlated ($r^2 \geq 0.98$, Table S1), showing virtually the same behaviour over time (Figure 2) and indicating that water was lost largely through stomata. In addition, there was a positive correlation between the former parameter and intercellular CO_2 concentration ($r^2 \geq 0.79$), suggesting that stomatal aperture determined CO_2 availability. Already in the fourth day of drought, compared with the control, *E. velutina* leaves had their stomatal conductance (Figure 2a) and transpiration rate (Figure 2b) nearly ceased (with reductions of 94.5 and 90.7%, respectively). This condition persisted for three days after rewatering, with a noticeable recovery from the fifth day onwards. Conversely, in *P. pyramidalis*, only at the 10th and 12th day of drought these parameters decreased by over 86.8% (Figures 2c and 2d).
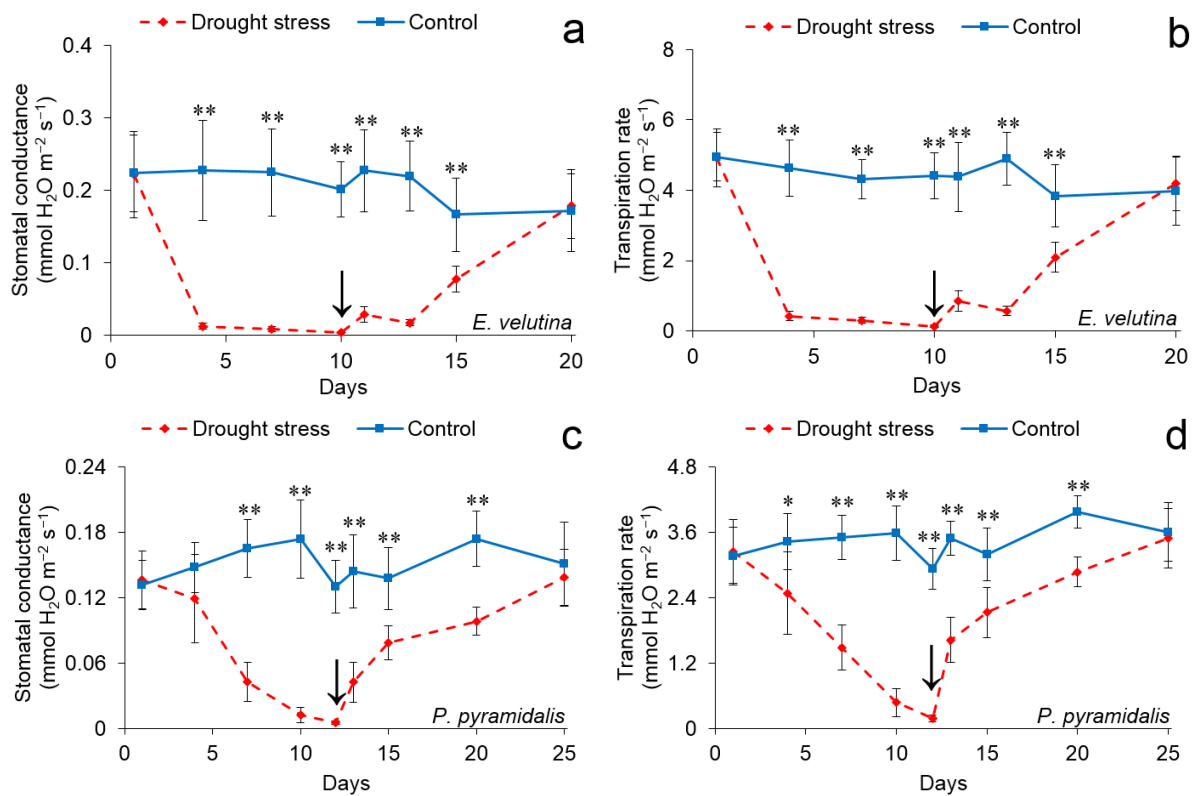
Figure 2 - Stomatal conductance (a, c) and transpiration rate (b, d) of *E. velutina* and *P. pyramidalis* over time as a function of drought and rehydration. Arrows indicate the beginning of the recovery period. Values are means ± SD (*n* = 10) and asterisks denote significant differences from controls (**, *P* < 0.01; *, *P* < 0.05).

The leaf level water use efficiency was negatively correlated with net photosynthesis (Table S1) and regardless of the way used to calculate it, whether depending on both stomatal conductance and vapour pressure deficit (instantaneous) or only on the former (intrinsic), *E. velutina* had increased values under drought (Figures 3a and 3b). In fact, its intrinsic water use efficiency was higher in stressed than in well-watered plants even five days after rewatering (Figure 3b). *P. pyramidalis* did not show a stable response pattern for these parameters, but at the 10th day of drought there were marked increases of 89.6% in instantaneous (Figure 3c) and 168.6% in intrinsic water use efficiencies (Figure 3d) in relation to control plants. Interestingly, despite few changes in water use efficiency, this species was able to maintain stomata open (to some extent) in the early days of drought stress and recovery. In order to elucidate this fact, plant water status was also assessed using leaf Ψ_w.
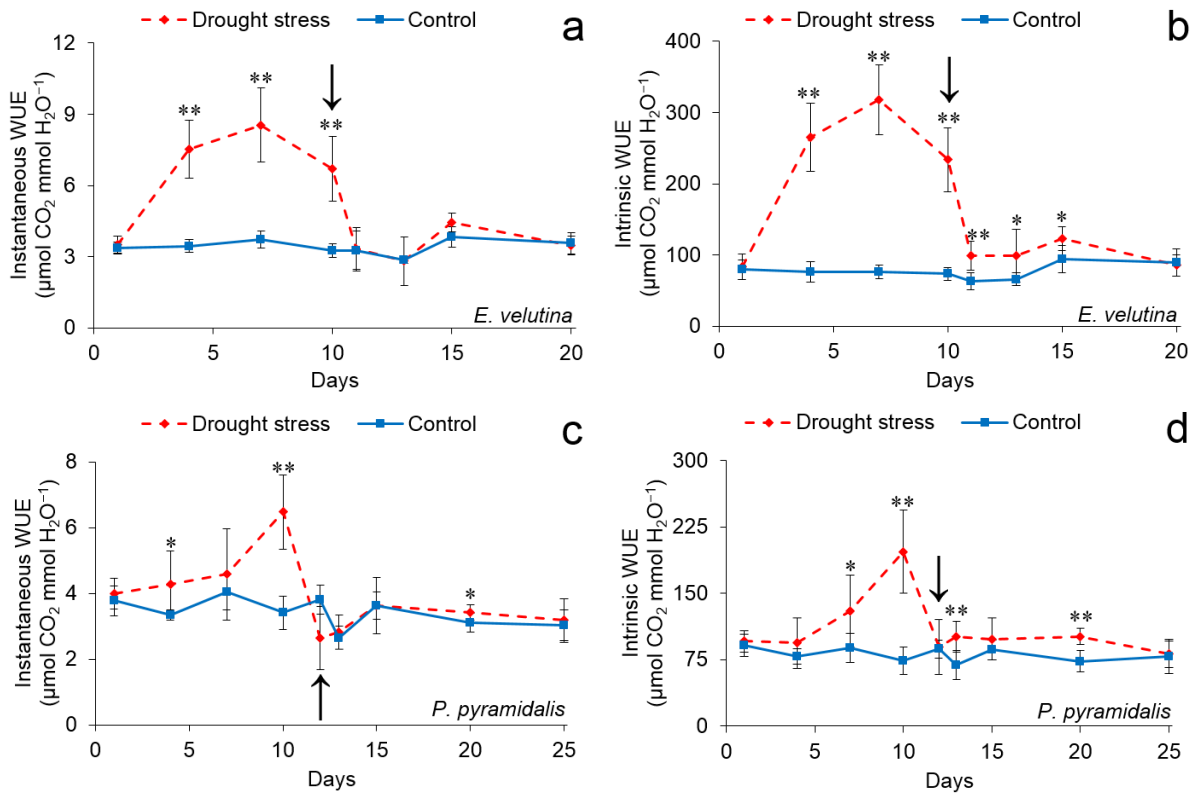
Figure 3 - Instantaneous (a, c) and intrinsic (b, d) WUE – water use efficiencies of *E. velutina* and *P. pyramidalis* over time as a function of drought and rehydration. Arrows indicate the beginning of the recovery period. Values are means ± SD ($n = 10$) and asterisks denote significant differences from controls (**, $P < 0.01$; *, $P < 0.05$).

2.3.2 Plant water status

Two different responses were observed regarding plant water status. For *E. velutina*, there was a subtle change at predawn, where stressed plants reduced their leaf Ψ_w from −0.33 to a minimum of −0.79 MPa after ten days of drought (Figure 4a). Curiously, apart from the first and last evaluation dates, the midday leaf Ψ_w (Figure 4b) of control plants was actually lower than that of those subjected to drought (albeit with no statistical difference at the last day of this condition and at the first day after rewatering). *P. pyramidalis*, on the contrary, revealed a high capability of absorbing water from the substrate by presenting naturally low Ψ_w. The first significant differences between control and treated plants appeared only after seven days without irrigation and the lowest values were recorded at the highest stress level, when drought led to 5.9- and 2.7-fold decreases in predawn (Figure 4c) and midday (Figure 4d) leaf Ψ_w, respectively. Moreover, the water status of this species was normalized immediately after rewatering, as observed at the sixth evaluation date.
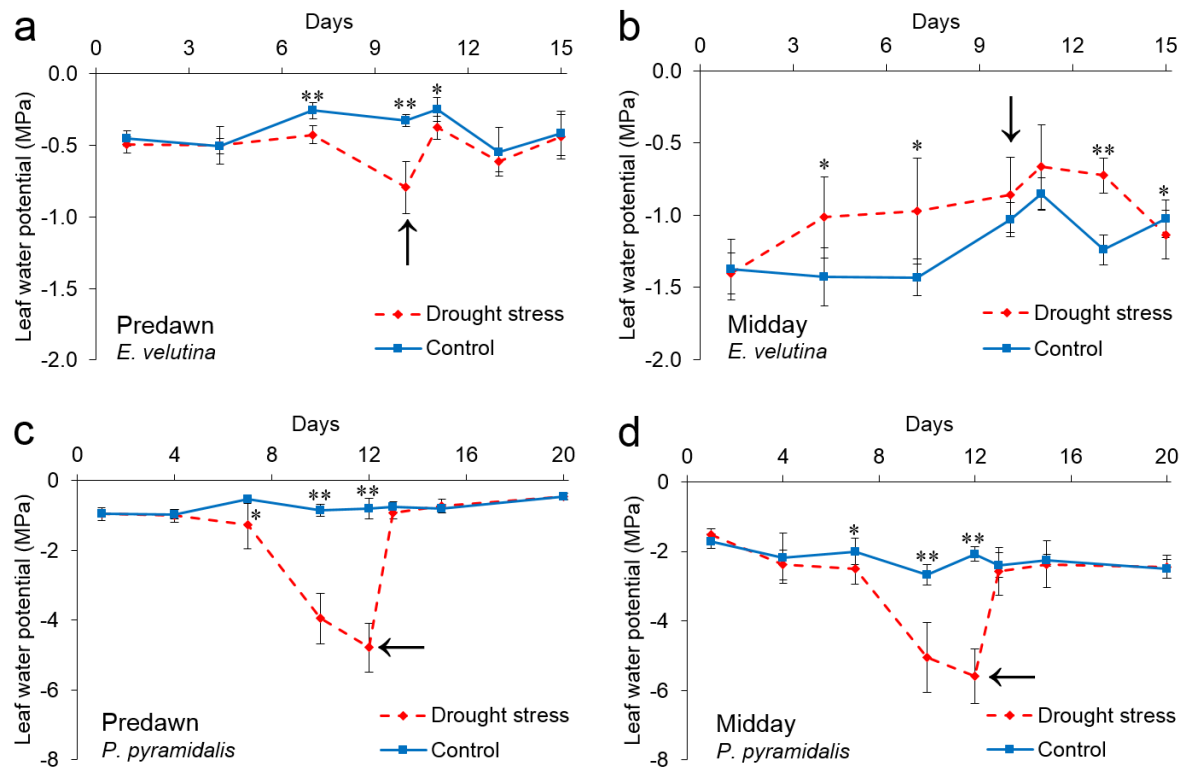
Figure 4 - Predawn (a, c) and midday (b, d) leaf water potentials of *E. velutina* and *P. pyramidalis* over time as a function of drought and rehydration. Arrows indicate the beginning of the recovery period. Values are means ± SD (*n* = 10) and asterisks denote significant differences from controls (**, *P* < 0.01; *, *P* < 0.05).

2.3.3 Biochemical responses

At the last day exposed to drought (highest stress level), there was no significant difference between the proline contents of control and treated *E. velutina* seedlings. *P. pyramidalis* under drought accumulated 443.9% more of this amino acid when compared to control plants (Figure 5a). The dynamics of reserve substances showed very similar responses, where withholding irrigation of *E. velutina* resulted in decreases of 87.3% in starch content (Figure 5b) and 62.3% in soluble sugar content (Figure 5c). However, there were no significant effects on *P. pyramidalis* despite an apparent increase in these variables for stressed plants. Regarding photosynthetic pigments, treatments did not influence their contents, which had the following means (mg g^{-1}): *E. velutina* – total chlorophyll of 0.60 ± 0.11 and carotenoids of 0.17 ± 0.04; *P. pyramidalis* – total chlorophyll of 1.64 ± 0.48 and carotenoids of 0.60 ± 0.15. Nevertheless, drought stress altered the relative proportion of these pigments, causing the chlorophyll/carotenoid ratio of *E. velutina* and *P. pyramidalis* to decrease by 10.2 and 19.3%, respectively (Figure 5d).
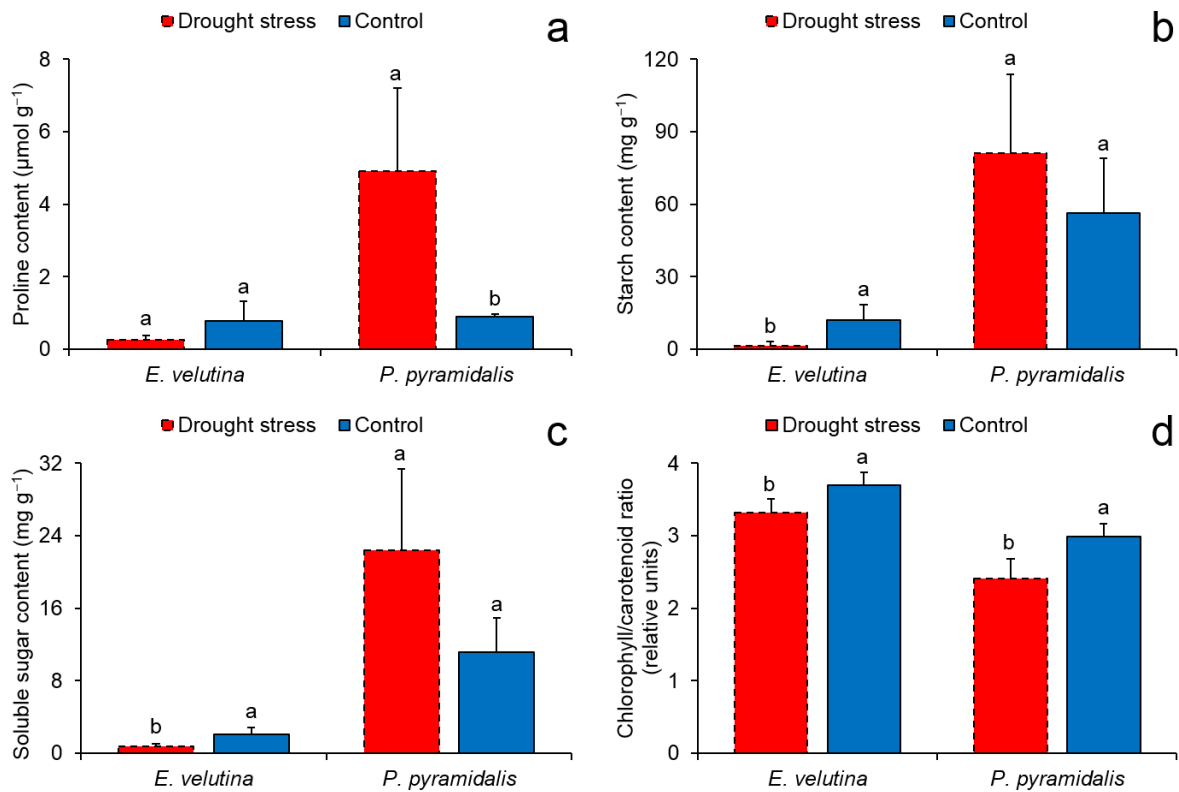
Figure 5 - Proline (a), starch (b) and soluble sugar (c) contents and chlorophyll/carotenoid ratio (d) in leaves of *E. velutina* and *P. pyramidalis* after 10 and 12 days of drought, respectively. Values are means ± SD ($n = 5$) and, for each species, different letters indicate significant differences from controls ($P < 0.05$).

2.4 Discussion

Photosynthetic responses are of great importance for understanding plant tolerance to drought, as the dehydration of leaf tissues causes stomatal closure and downregulates photosynthesis (GALLÉ & FELLER, 2007; ZHANG et al., 2017). Apart from the instantaneous and intrinsic water use efficiencies, all gas exchange parameters studied here were also negatively affected by drought, but the extent of its effect differed between species, whether during the stress period or at the recovery phase. The net photosynthesis and the carboxylation efficiency of *E. velutina* were considerably reduced without irrigation and had a slow recovery upon rewatering. In *P. pyramidalis*, these two parameters were little sensitive and quickly recovered. In both plants, the reductions were accompanied by a low intercellular CO_2 concentration, indicating that a decline in CO_2 availability into the sub-stomatal chamber might have reduced its fixation by Rubisco (HUANG et al., 2018).

Reduced net photosynthesis under drought stress could result from a decrease in stomatal conductance (CORNIC & MASSACCI, 1996; HAIDER et al., 2018). The low intercellular CO_2 concentrations observed even during the first days after rewatering were in fact a consequence of drought-induced stomatal closure, which was responsible for regulating and maintaining transpiration at reduced levels, particularly in *E. velutina*. Notwithstanding the large decreases in transpiration rate after withholding irrigation, there were only small reductions in intercellular CO_2 concentration, presumably because stomatal closure affects more transpiration than CO_2 diffusion into the leaf (ASHRAF & HARRIS, 2013). Carbon and water relations are closely coupled, but while stomatal limitations determine carbon assimilation under mild drought stress, non-stomatal factors (e.g. metabolic impairments) may play a role as the water restriction increases (DRAKE et al., 2017; GALLÉ & FELLER, 2007). Given that stomatal conductance and intercellular CO_2 concentration were positively correlated, it can be assumed that there were indeed stomatal limitations to photosynthesis, as a decrease in CO_2 availability in the chloroplasts of drought-stressed plants could contribute to their reduced photosynthetic activity (HUANG et al., 2018). However, this does not exclude the possibility that metabolic impairments were also involved.

Although reductions in net photosynthesis and stomatal conductance under drought stress are rather classical phenomena, their underlying mechanisms are not fully understood (NIINEMETS, 2016). Stomatal closure is one of the first responses to drought and it is mediated by an increase in abscisic acid concentration (MCKIERNAN et al., 2016; XU et al., 2010). Thus, a residual effect of this phytohormone could prevent a rapid recovery of photosynthesis upon rewatering (DIETRICH & KAHMEN, 2019), as observed for *E. velutina*. However, drought stress is known to affect the function of photosystems I and II, thus hindering the electron transfer process and the production of ATP and NADPH, which are essential for carbon fixation. In addition, it can ultimately compromise the whole structure of the photosynthetic apparatus by damaging thylakoid membranes (ASHRAF & HARRIS, 2013). It is also noteworthy that plants may be exposed to more than one stressor at the same time. For example, because it limits photosynthesis, drought is frequently followed by photoinhibition (NIINEMETS, 2016).

Increases in the instantaneous and intrinsic water use efficiencies showed that transpiration and stomatal aperture, respectively, were reduced more than photosynthesis, mainly during the stress period. As water became a limiting factor, plants improved their photosynthetic performance to use less of this resource (LIU et al., 2010). In fact, *E. velutina* and *P. pyramidalis* performed much better than other Caatinga tree species under similar

conditions, considering that the instantaneous water use efficiency of *Leucaena leucocephala*, *Mimosa caesalpiniifolia* and *Prosopis juliflora* actually decreased in relation to control seedlings after only seven days without irrigation (BARROS et al., 2020). The results described here corroborate previous reports on the increase in water use efficiency because of drought-induced stomatal closure (DRAKE et al., 2017; SURESH et al., 2012; XU et al., 2010), as well as on the negative correlation between net photosynthesis and water use efficiency under drought (HAIDER et al., 2018). However, the observed changes were more pronounced and persistent in *E. velutina*. Improving water use efficiency did not appear to be crucial for *P. pyramidalis*, because this plant was capable of overcoming the decrease in soil water availability.

Predawn and midday assessments of plant water status revealed that *P. pyramidalis* could effectively reduce leaf Ψ_w in response to drought to maintain cell turgor, especially during the day, which explains why its photosynthetic activity was not much affected throughout the experiment. This plant was not only able to substantially decrease its Ψ_w under drought stress but also to normalize it immediately upon rewatering. This is an indicator that xylem water transport was not compromised, seeing that this limitation develops early in sensitive plants (RUEHR et al., 2019). For *E. velutina*, there was an evident loss of hydraulic conductivity at midday, given that the leaf Ψ_w of stressed seedlings was actually higher than that of control plants, as previously reported for this species after a month of drought (SILVA et al., 2010). Thus, in this case, there seems to be a relatively narrow margin between the Ψ_w promoting stomatal closure and that causing leaf xylem embolism.

By closing their stomata, plants reduce transpiration and tend to relieve xylem tension (CHOAT et al., 2018; HÁJÍČKOVÁ et al., 2017). Hence, stomatal closure can effectively restrict water loss and prevent cavitation during mild droughts. On the other hand, a more severe water deficit may cause embolism, which in turn limits the recovery of leaf gas exchange (PEGUERO-PINA et al., 2018). Accordingly, a short but severe drought treatment led to a loss of hydraulic conductivity in *Castanopsis chinensis, Schima superba* and *Syzygium rehderianum* (DUAN et al., 2019). Despite having this problem, once rewatered, *E. velutina* was also able to fully recover its photosynthetic activity, which indicates that xylem water transport was not permanently compromised. Therefore, the slow recovery of stomatal conductance observed in these seedlings could have facilitated vessel refilling upon the occurrence of xylem cavitation (SCHIMPL et al., 2019). A possible explanation for the differences discussed so far between the species studied here could lie in their biochemical responses.

Progressive drought leads to increased electrolyte leakage, an indicator of oxidative damage to cell membranes after the overproduction of reactive oxygen species (LIAO et al., 2018). *E. velutina* did not accumulate proline at the highest stress level. This might have implicated on the limited ability of this plant to reduce leaf Ψ_w and to attenuate oxidative stress. Besides effectively reducing the osmotic potential of the cell, proline also acts as a molecular chaperone, stabilizing proteins, and as a scavenger of reactive oxygen species, maintaining the balance between oxidants and antioxidants as well as cell integrity (HAIDER et al., 2018). As a result, several studies have shown increases in leaf proline content of tree species under drought stress (KHALEGHI et al., 2019; LIU et al., 2011; SPIEß et al., 2012), including *P. pyramidalis* (FROSI et al., 2016).

Ten days of drought led to decreases in the starch and soluble sugar contents of *E. velutina*. Under severe drought conditions, the carbon balance may become negative and induce the consumption of non-structural carbohydrates, thus providing the energy and carbon needed for growth and development (SOUDEN et al., 2020). Drought stress up-regulated several genes with protective function in *Quercus robur*, including those involved in α- and β-amylase activities, thus responsible for starch breakdown (SPIEß et al., 2012). Furthermore, Schimpl et al. (2019) observed a decrease in starch content along with an increase in soluble sugar content, indicating that the former was broken down to the latter to support lower Ψ_w values in *Bertholletia excelsa*. Here, the drought-induced reduction in starch content was much greater than that in soluble sugar content, which suggests that such breakdown did occur. Nevertheless, it was not enough to support a decrease in leaf Ψ_w like observed in *P. pyramidalis*, especially when considering that there were large differences in the starch and soluble sugar contents of the two species. The threshold between accumulation and consumption of reserve substances depends on the severity of the drought (LIU et al., 2011; SOUDEN et al., 2020), consequently it can be deduced that for *E. velutina* it is low.

By using the stress-recovery framework proposed by Ruehr et al. (2019), it was possible to distinguish two different realities in the present study. *E. velutina* proved to be more sensitive to drought by showing a moderate stress level. Although completely restoring its gas exchange parameters after 10 days of rewatering, a rather delayed recovery and a likely occurrence of leaf xylem embolism during the hottest hours of the day were observed. Significant losses in xylem hydraulic conductivity increase the time needed to normalize stomatal conductance. This condition requires the activation of repair mechanisms, leading to the consumption of non-structural carbohydrates. Since at the highest stress level there was basically no carbon assimilation, the observed decreases in starch and soluble sugar contents

might indicate that they were used to allow for repair. On the other hand, *P. pyramidalis* experienced only a mild stress and had a fast recovery of photosynthetic activity, with no apparent loss of hydraulic conductivity. Therefore, until the 12th day of drought, this plant did not need to use reserve substances, as evidenced by the biochemical results.

In view of the likely occurrence of oxidative stress in drought treated plants, it is important to highlight that this problem is counteracted by a defence system that includes both enzymatic and non-enzymatic antioxidants (KHALEGHI et al., 2019). As part of the second group, carotenoids are pigments of great importance for photoprotection against oxidative damage. In particular, the xanthophyll cycle plays a key role in the dissipation of excess excitation energy through non-photochemical quenching (SOFO, 2011). Since photosynthesis is limited under drought, plants might experience an accumulation of reducing power when exposed to light. In order to avoid potential damages to photosystems, this excess energy must be safely dissipated, which can be achieved through the action of protective pigments (TAIZ et al., 2015). Zhang et al. (2017) reported the activation of photoprotective carotenoids in response to increasing drought stress in *Q. ilex*. Here, at the highest stress level, both tree species changed their chlorophyll/carotenoid ratio towards a higher proportion of the second pigment, thus protecting their photosystems against photoinhibition during the stress period and allowing photosynthesis to be fully recovered upon rewatering.

2.5 Conclusions

E. velutina and *P. pyramidalis* show different mechanisms behind their drought tolerance. *E. velutina* rapidly downregulates photosynthesis, reducing leaf gas exchange and improving water use efficiency to compensate for the temporary loss of xylem water transport. Nevertheless, this species recovers slowly and is prone to starvation during prolonged drought periods due to the consumption of non-structural carbohydrates. In contrast, the photosynthetic activity of *P. pyramidalis* is gradually reduced with increasing drought, but quickly recovered when water is no longer a limiting factor. This outcome does not depend on high water use efficiency, as leaf Ψ_w can be effectively reduced through the accumulation of proline. In both species, the full recovery of photosynthesis upon rewatering is possibly related to enhanced photoprotection by carotenoids. Altogether, such mechanisms could ensure the resilience of these trees in the face of recurrent droughts. Further research on this topic is needed to improve predictions of drought-induced changes in the Caatinga flora.

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3 THE INTERPLAY BETWEEN LEAF WATER POTENTIAL AND OSMOTIC ADJUSTMENT ON PHOTOSYNTHETIC AND GROWTH PARAMETERS OF TROPICAL DRY FOREST TREES[2]

ABSTRACT

Mimosa tenuiflora and *Piptadenia stipulacea* are commonly accepted as drought-tolerant species but little is known about their response to drought followed by rehydration. Therefore, the interplay between leaf water potential and osmotic adjustment on photosynthetic and growth parameters of these species was examined. A greenhouse study was conducted in a split-plot design with two water conditions in the main plots (control; drought followed by rehydration), and eight sampling times in the subplots (1, 4 and 7 days of drought, and 1, 3, 6, 12 and 17 days of rehydration). Plant water status and biochemical changes were assessed as well as leaf gas exchange and subsequent growth. Under drought stress, both species maintained a low leaf water potential throughout the day by accumulating compatible solutes, thus allowing a rapid and full recovery of water status when rehydrated. Although these plants minimized water loss by closing their stomata, neither showed stomatal limitations to photosynthesis. The inhibition of this process during drought was possibly related to mesophyll limitations as well as to a reversible downregulation of photosystems, along with adjustments of their stoichiometry. Water deficits also triggered morphological adaptations at the whole plant level, leading to reduced growth, mainly of the shoots in *M. tenuiflora* and the roots in *P. stipulacea*.

Keywords: Drought stress. Rehydration. Gas exchange. Plant biomass.

3.1 Introduction

Drought is one of the main factors causing tree mortality and forest decline, thus altering the carbon balance of terrestrial ecosystems (CHOAT et al., 2018; JIAO et al., 2021; SERRA-MALUQUER et al., 2018). Therefore, further information on the ability of trees to survive low water availability is of paramount importance to predict changes in carbon cycling (PRITZKOW et al., 2020; SANTIAGO et al., 2016). Drought stress triggers

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physiological, biochemical and molecular alterations in plants (ASHRAF & HARRIS, 2013). Unfavourable water status induces turgor loss and consequently reduces cell growth (FOX et al., 2018). Furthermore, it usually promotes stomatal closure as well as non-stomatal limitations to photosynthesis, inhibiting carbon assimilation (ANTEZANA-VERA & MARENCO, 2021; DUAN et al., 2020; GOMES et al., 2008). Under field conditions, drought may also lead to several other abiotic stresses such as light, temperature and nutrient stress.

Similar to what is observed in other tropical dry forests, drought events are frequent in the Brazilian Caatinga (SAMPAIO, 1995). The annual rainfall ranges from 250 to 750 mm and is distributed over three to four months, followed by a dry season that lasts the rest of the year (BARROS et al., 2020; SILVA et al., 2010). In addition to low water availability, Caatinga plants face high irradiance and temperatures, making water deficits a common phenomenon (DOMBROSKI et al., 2014; SILVA et al., 2010), especially because the potential evapotranspiration exceeds 1,500 mm $year^{-1}$ (SAMPAIO, 1995). Nevertheless, water deficits will intensify in the following years, with impacts on flora characteristics (FROSI et al., 2016). In fact, Campos et al. (2020) reported an increase in tree mortality from 2009 to 2019, along with a decrease in biomass production of a Caatinga fragment.

Mimosa tenuiflora (Willd.) Poir. (Mimosaceae) and *Piptadenia stipulacea* (Benth.) Ducke (Mimosaceae) are woody species widely distributed in tropical dry forests from Mexico to Brazil. These pioneer trees play a key role in the ecological succession of the Caatinga dry forest vegetation (ALVES & FREIRE, 2019; BARROS et al., 2019). However, it is unclear how they cope with the extreme conditions of this semi-arid habitat, particularly low and erratic rainfall (SAMPAIO, 1995). To thrive in such environment, species depend on the ability to withstand droughts and on the capacity to recover (GALLÉ et al., 2007; XU et al., 2010). This is especially important when considering the occurrence of short but frequent drought events. Yet there is far more information available on drought stress than on stress recovery, although the latter may determine survival (CHOAT et al., 2018; SANTIAGO et al., 2016).

Despite being regarded as drought-tolerant, little is known about the responses of *M. tenuiflora* and *P. stipulacea* to drought followed by rehydration. Lima and Meiado (2018) assessed the effect of hydration and dehydration cycles on the germination of *M. tenuiflora* and concluded that seedlings benefited from a discontinuity in the imbibition process, showing increased shoot heights, stem diameters and total dry weight. Alves and Freire (2019) evaluated the physiological response of one-year-old seedlings to water deficits and rewatering and found that drought-induced changes in relative water content and gas

exchange parameters were normalized within three days of rehydration. As for *P. stipulacea*, apart from germination tests, there are few studies on biomass production and allocation as affected by drought (BARROS et al., 2019; CAMPOS et al., 2020), with no information on biochemical and physiological responses of this species nor on its recovery from drought stress.

Drought-triggered tree mortality can result in vegetation shifts with unknown environmental consequences (SERRA-MALUQUER et al., 2018; THAMMANU et al., 2021). There is a wide variation in the response of plants to this stress as well as in their recovery dynamics on rewatering (NIINEMETS, 2016; TAIZ et al., 2015; YORDANOV et al., 2000). Understanding these intrinsic characteristics may be useful in the implementation of management practices to ensure the resilience of tropical dry forests under climate change conditions (SERRA-MALUQUER et al., 2018; STAN et al., 2021; XU et al., 2010). To elucidate some of the mechanisms behind the drought tolerance of *M. tenuiflora* and *P. stipulacea*, the interplay between leaf water potential and osmotic adjustment on photosynthetic and growth parameters of these plants was studied by evaluating their responses to drought followed by rehydration.

3.2 Material and methods

3.2.1 Plant material and experimental conditions

A greenhouse study was conducted in Mossoró, Brazil (5°12'16" S 37°19'29" W) where *M. tenuiflora* and *P. stipulacea* were grown in polyethylene bags (1.9 L). A Thermo Recorder TR-72U (T&D Corporation, Matsumoto, Nagano, Japan) monitored air temperature and relative humidity, which had average values of 28.8 °C and 62.4%, respectively. Two independent experiments, one for each species, were performed in a split-plot design with two water conditions in the main plots (1 – control and 2 – drought followed by rehydration) and eight sampling dates in the subplots (1, 4 and 7 days of drought, and 1, 3, 6, 12 and 17 days of rehydration). Treatments were laid out in a randomized complete block design, replicated 10 times, with eight bags per experimental unit.

M. tenuiflora and *P. stipulacea* seeds were immersed in hot water at 100 °C for 4 minutes to break dormancy (BENEDITO et al., 2017, 2019), sown in a substrate of topsoil (see Table 1 in subsection 2.2.1) mixed with 200 mg P_2O_5 dm^{-3} and subjected to five top dressings (at 40, 50, 60, 70 and 80 days after sowing), totalling 500 mg N dm^{-3} and 250 mg K_2O dm^{-3}. Thinning was carried out 21 days after sowing to one seedling per bag. The plants

were watered to field capacity in the early mornings and late afternoons for six months. Half were then subjected to the treatment shown in Figure 6. While control plants were kept well-watered, drought was imposed by suspending irrigation for seven days, causing net photosynthesis of both species to be almost completely suppressed (FREITAS et al., 2018; SURESH et al., 2012). Drought-stressed plants were then rehydrated until their photosynthetic activity returned to control levels (GOMES et al., 2008), which took 17 days for *M. tenuiflora* and 12 days for *P. stipulacea*.
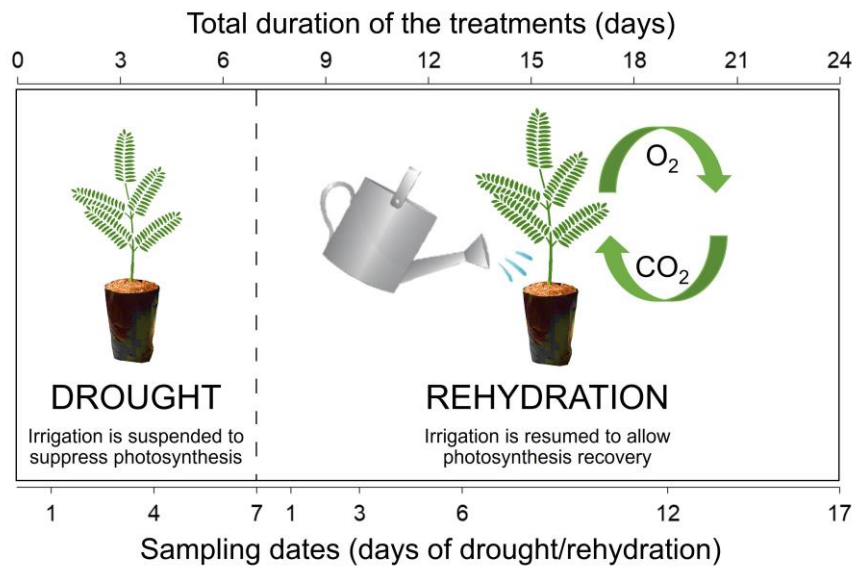
Figure 6 - Schematic of the drought followed by rehydration. The dash line indicates the transition between the two periods.

3.2.2 Water potential (Ψ_w) measurements

To assess plant water status, fully expanded leaves from the middle-third (SCHIMPL et al., 2019) of one plant per treatment were used for measurements with a Scholander pressure chamber (PMS Instrument Company, Albany, OR, USA). Predawn and midday Ψ_w (leaf water potential) were recorded immediately after excision (KARIMI et al., 2015), from 4 to 6 a.m. and from 11 a.m. to 1 p.m.

3.2.3 Biochemical assay

On the seventh day of drought, the highest stress level, newly expanded leaves of plants from five replications were collected and stored at −18 °C for subsequent analysis of proline, soluble sugars and amino acids and photosynthetic pigments (chlorophyll *a* and *b*). To ensure the accuracy of the results, data were collected in triplicate and expressed on a dry

weight basis. Proline accumulation was determined after reaction with acid-ninhydrin solution (BATES et al., 1973). Soluble sugars and amino acids were estimated using the phenol-sulphuric acid method (DUBOIS et al., 1956) and the ninhydrin method (YEMM et al., 1955), respectively. Chlorophyll (Chl) *a* and *b*, and their ratio, were calculated from absorbance (A) values at 645 and 663 nm following acetone extraction (LICHTENTHALER, 1987), where: Chl $a = 12.25 \times A_{663} - 2.79 \times A_{645}$ and Chl $b = 21.5 \times A_{645} - 5.1 \times A_{663}$.

3.2.4 Leaf gas exchange

Gas exchange parameters were measured between 9 and 10 a.m. in one newly expanded leaf of each experimental unit using a LI-6400 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE, USA). After setting light to 1,200 μmol m^{-2} s^{-1}, CO_2 concentration to 400 μmol mol^{-1} and flow rate to 400 μmol s^{-1}, the following parameters were simultaneously recorded: stomatal conductance, transpiration rate, net photosynthesis, intercellular CO_2 concentration and carboxylation efficiency. Net photosynthesis was used to establish the length of the drought and rehydration periods, as proposed by Freitas et al. (2018), and it was also plotted against stomatal conductance and intercellular CO_2 concentration data to assess their relationships.

3.2.5 Growth traits

Once considered recovered from drought (after rewatering *M. tenuiflora* for 17 days and *P. stipulacea* for 12 days), all plants were irrigated to field capacity for a further two months. This allowed for the evaluation of the effects of drought stress on subsequent biomass production and allocation within the plant. Leaves, stems and roots of two seedlings per treatment were harvested and placed in a forced-air drying oven at 65 $\pm$ 2 °C for three days. Based on their dry weights, the total dry weight and the shoot/root ratio were calculated, where: shoot = leaf + stem.

3.2.6 Statistical analysis

Data were analysed by Student's *t*-test at the 5% level, comparing control and drought stress conditions at each sampling date using the Sisvar software, version 5.6 (Federal University of Lavras, Lavras, MG, Brazil). Regression and Pearson correlation analyses (at 5% significance level) were also performed when assessing the relationships between photosynthetic parameters.

3.3 Results

3.3.1 Predawn and midday Ψ_w

Leaf measurements revealed that these pioneer species had a high ability to adjust leaf water potential (Ψ_w) to cope with decreasing soil water availability. In general, the recorded values were naturally lower at midday than at predawn (as observed for control plants), but suspending irrigation resulted in much greater reductions over time. After seven days of drought, the Ψ_w of *M. tenuiflora* ranged from −5.0 MPa at predawn (Figure 7a) to −6.3 MPa at midday (Figure 7b). Intriguingly, the former decreased even further to −6.0 MPa on the first day following rehydration, suggesting a lingering response to drought. It was only on the sixth sampling date that leaf Ψ_w returned to control levels. At maximum stress, compared with well-watered plants, there were 5.9 and 2.8-fold decreases in predawn (Figure 7c) and midday (Figure 7d) Ψ_w of *P. stipulacea*, respectively. Nevertheless, this species rehydrated relatively rapidly, and water status was normalized three days after rewatering.
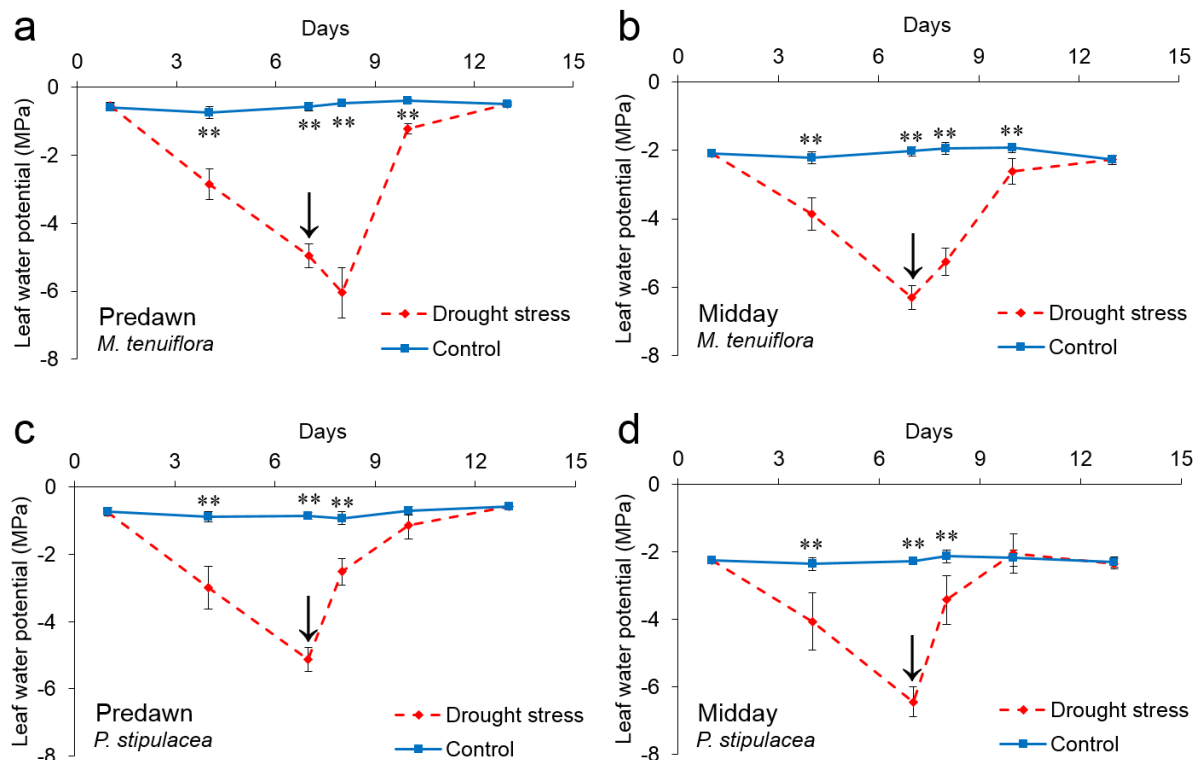
Figure 7 - Predawn (a, c) and midday (b, d) leaf water potentials of *M. tenuiflora* and *P. stipulacea* over time as a function of drought and rehydration. Arrows indicate the beginning of the recovery period. Values are means ± SD (*n* = 10) and asterisks denote significant differences from controls (*P* < 0.01).

3.3.2 Biochemical changes

Suspending irrigation for seven days resulted in significant accumulation of compatible solutes by augmenting the levels of proline, soluble sugars and soluble amino acids in the seedlings. The highest differences between control and treated plants were in proline content, with 30.7 and 32.6-fold increases for *M. tenuiflora* and *P. stipulacea*, respectively (Figure 8a). Unlike free proline, the accumulation of soluble sugars (Figure 8b) and amino acids (Figure 8c) was proportionally higher in the former species. After the drought treatment, chlorophyll *a* of *M. tenuiflora* went from 1.12 ± 0.20 to 2.15 ± 0.87 mg g^{-1} ($P < 0.05$) and there was also a significant increase of chlorophyll *a/b* ratio (Figure 8d). The other photosynthetic pigments analysed did not differ statistically and their contents were as follows (mg g^{-1}): *M. tenuiflora* – chlorophyll *b* of 0.62 ± 0.13; *P. stipulacea* – chlorophyll *a* of 2.33 ± 0.66 and chlorophyll *b* of 0.39 ± 0.07.
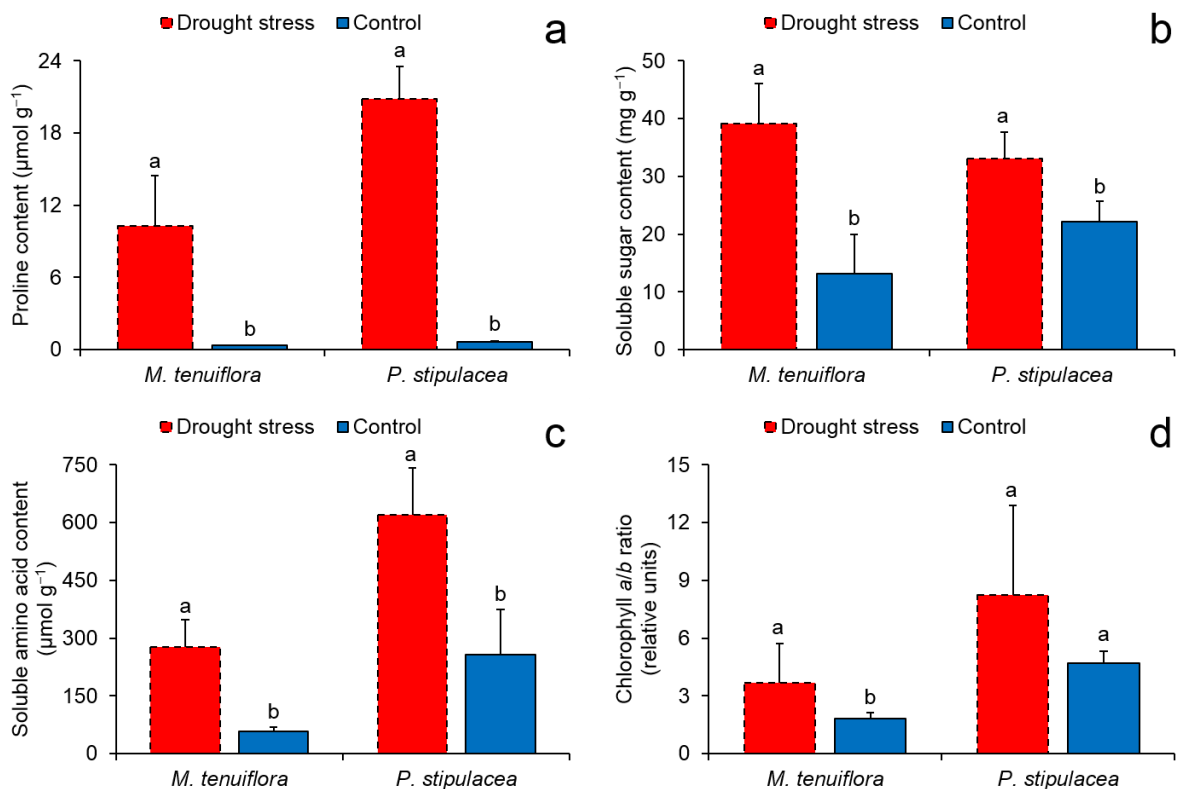
Figure 8 - Proline (a), soluble sugar (b) and soluble amino acid (c) contents and chlorophyll *a/b* ratio (d) in leaves of *M. tenuiflora* and *P. stipulacea* after seven days of drought. Values are means $\pm$ SD ($n = 5$) and, for each species, different letters indicate significant differences from controls ($P < 0.05$).

3.3.3 Photosynthetic responses

The response patterns of stomatal conductance and net photosynthesis to drought were similar, suggesting that photosynthetic activity was driven by changes in stomatal aperture and perhaps limited by a low CO_2 uptake. After only four days without irrigation, the stomatal conductance of *M. tenuiflora* seedlings decreased by 90.1% relative to the controls (Figure 9a), which also led to a 79.3% decrease in net photosynthesis (Figure 9b). This last parameter was almost completely suppressed within seven days of drought, requiring a 17-day period to fully recover. The performance of *P. stipulacea* under stress was analogous to that of *M. tenuiflora*, except that its photosynthetic activity was completely restored by the 12th day of rehydration (Figures 9c and 9d). For both species, stomatal closure resulted in a decrease in transpiration rate (Figure S2).
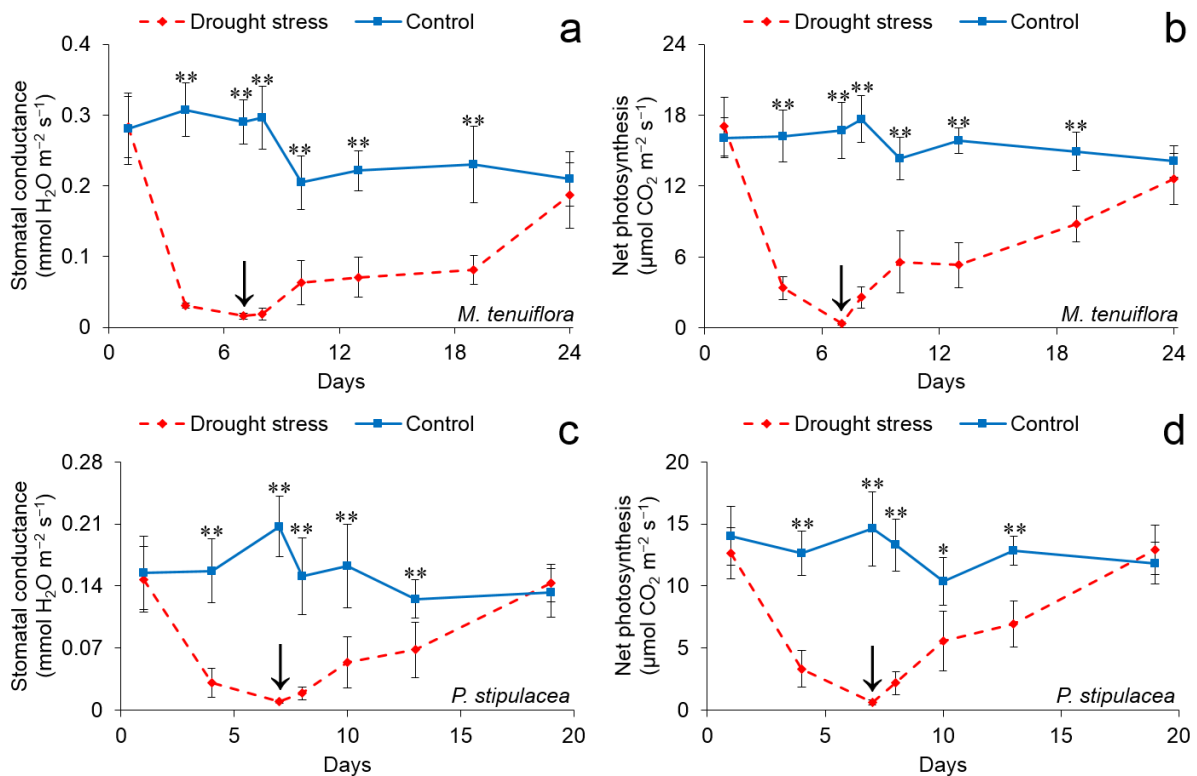
Figure 9 - Stomatal conductance (a, c) and net photosynthesis (b, d) of *M. tenuiflora* and *P. stipulacea* over time as a function of drought and rehydration. Arrows indicate the beginning of the recovery period. Values are means ± SD ($n = 10$) and asterisks denote significant differences from controls (**, $P < 0.01$; *, $P < 0.05$).

Even though by the third sampling date stomatal conductance was reduced to near zero, there were increases in intercellular CO_2 concentration, indicating that CO_2 was not a

limiting factor for photosynthesis. Moreover, the fact that at that time net photosynthesis was suppressed, despite a great availability of CO_2, resulted in extremely low carboxylation efficiency values. At the highest stress level, intercellular CO_2 concentration of *M. tenuiflora* was 76.0% higher than that of control seedlings (Figure 10a), and its carboxylation efficiency was normalized by the 17th day of rehydration (Figure 10b). *P. stipulacea* showed essentially the same responses but the increase in intercellular CO_2 concentration was of 105.7% with a significant difference shortly afterwards (Figure 10c), and it took only 12 days to recover its carboxylation efficiency (Figure 10d).
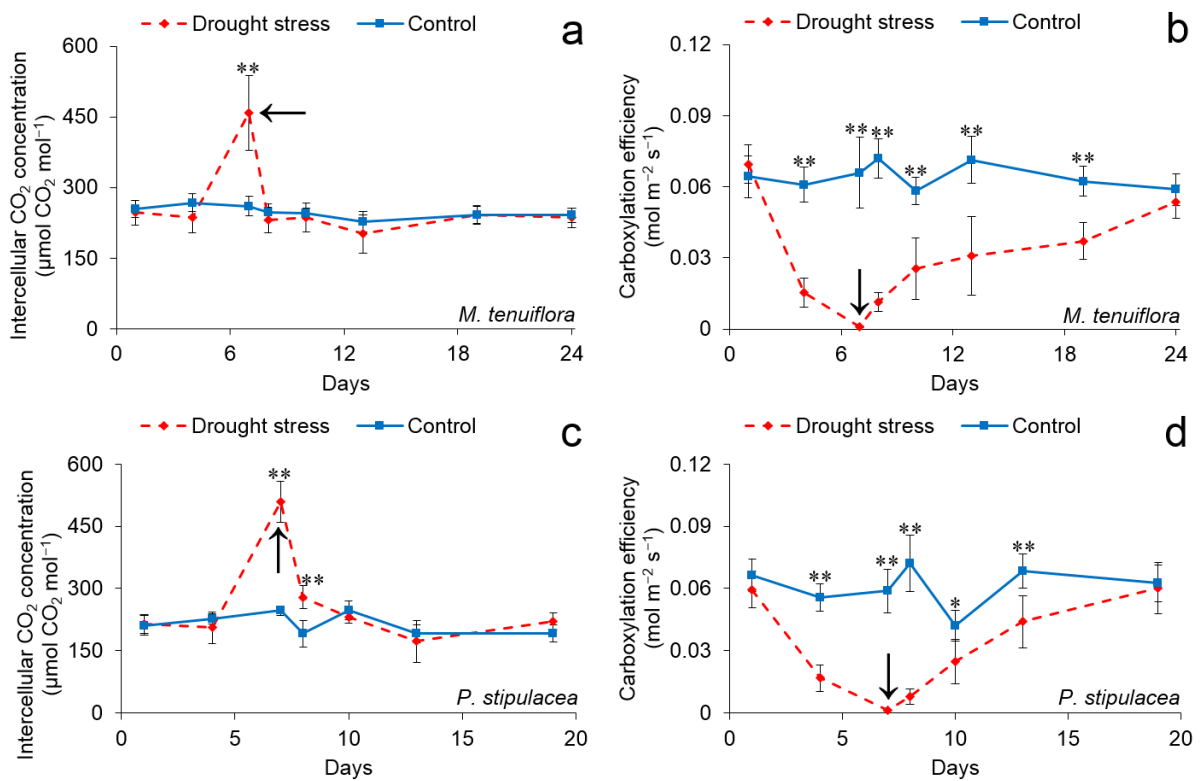
Figure 10 - Intercellular CO_2 concentration (a, c) and carboxylation efficiency (b, d) of *M. tenuiflora* and *P. stipulacea* over time as a function of drought and rehydration. Arrows indicate the beginning of the recovery period. Values are means $\pm$ SD ($n = 10$) and asterisks denote significant differences from controls (**, $P < 0.01$; *, $P < 0.05$).

As indicated previously, drought-induced stomatal closure did not have a negative effect on intercellular CO_2 concentration. Nevertheless, by plotting net photosynthesis against stomatal conductance and intercellular CO_2 concentration data, it was confirmed that photosynthetic activity was indeed coupled with stomatal aperture but not limited by a low CO_2 influx into the sub-stomatal chamber. For both *M. tenuiflora* and *P. stipulacea*, there was

a high correlation between the first two parameters, while the third was weakly and negatively correlated with the first one (Table S2). Regression analysis showed a quadratic relationship between net photosynthesis and stomatal conductance, where the photosynthetic activity increased with stomatal opening (Figures 11a and 11c). On the other hand, most of the net photosynthesis occurred between 150 and 300 µmol CO_2 mol^{-1}, with basically no carbon assimilation above this optimal range (Figures 11b and 11d).
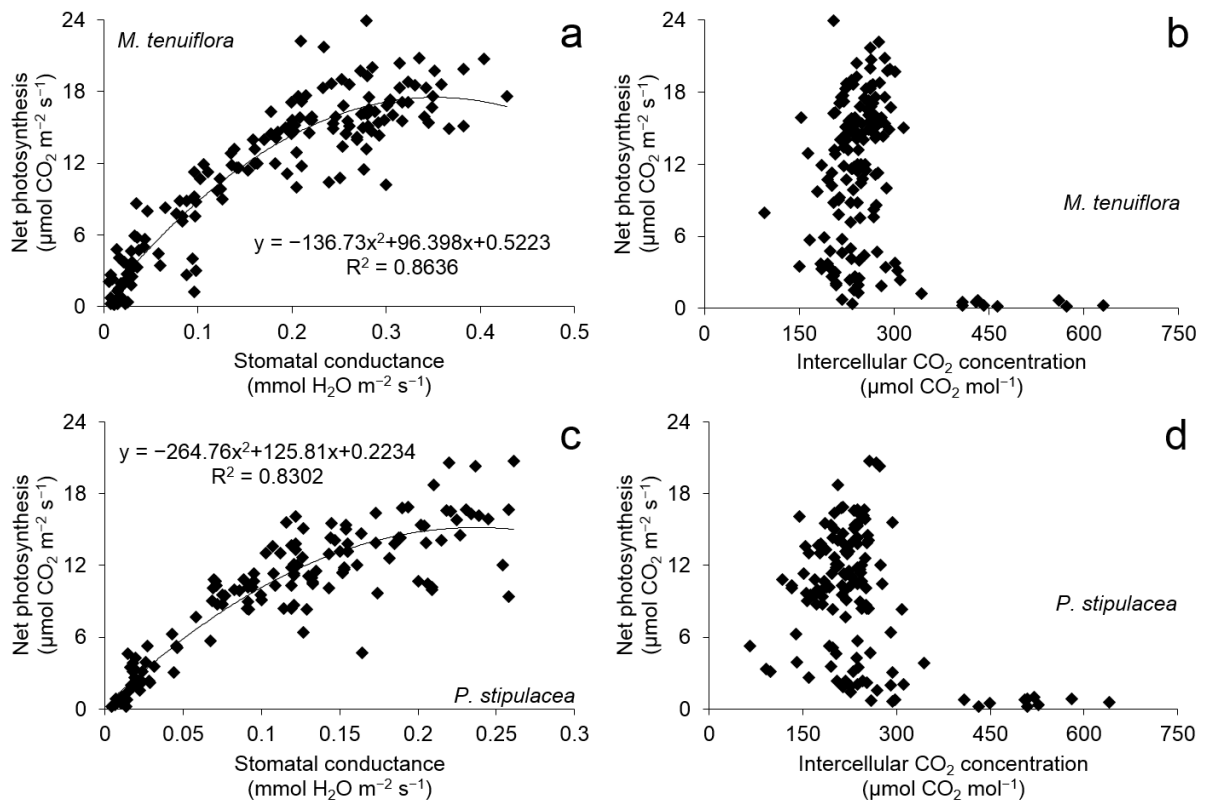
Figure 11 - Relationships between net photosynthesis and stomatal conductance (a, c) and net photosynthesis and intercellular CO_2 concentration (b, d) of *M. tenuiflora* and *P. stipulacea* under drought and rehydration.

3.3.4 Biomass production and allocation

With regards to subsequent biomass production as compared to controls, treated plants invested considerably less in leaf and stem tissues and, in the case of *P. stipulacea*, also in root tissues. Consequently, the total dry weight of the two species was negatively affected by approximately 30%. Furthermore, exposure to a single drought period was enough to alter biomass allocation within the plant. Although the reductions in shoot and root dry weight (the latter not significant) of *M. tenuiflora* were relatively equivalent, they were quite different for *P. stipulacea*, where the shoot dry weight of previously stressed plants was 28.9% smaller

than that of well-watered seedlings, whereas the root dry weight was affected by 45.0%. Therefore, there was an increase in the shoot/root ratio, apparently favouring shoot growth at the expense of root growth (Table 2).

Table 2 - Biomass production and allocation in *M. tenuiflora* and *P. stipulacea* two months after a drought period.

Species	Water condition	LDW (g plant^{-1})	SDW (g plant^{-1})	RDW (g plant^{-1})	TDW (g plant^{-1})	S/R (relative units)
M. tenuiflora	Drought stress	8.57 ± 2.00 b	17.59 ± 3.29 b	6.67 ± 2.29 a	32.84 ± 5.81 b	4.38 ± 1.69 a
	Control	10.69 ± 1.47 a	26.15 ± 5.70 a	8.61 ± 2.13 a	45.46 ± 7.39 a	4.39 ± 0.77 a
	LSD	1.12	5.38	2.80	7.57	1.57
	CV (%)	11.49	24.32	36.27	19.10	35.37
P. stipulacea	Drought stress	5.12 ± 2.03 b	25.23 ± 5.79 b	6.63 ± 1.89 b	36.99 ± 8.46 b	4.69 ± 0.78 a
	Control	7.41 ± 1.60 a	35.28 ± 6.44 a	12.05 ± 3.33 a	54.74 ± 9.88 a	3.66 ± 0.65 b
	LSD	1.27	5.27	1.91	7.03	0.67
	CV (%)	19.99	17.23	20.17	15.16	15.89

LDW – leaf, SDW – stem, RDW – root and TDW – total dry weight and S/R – shoot/root ratio. Values are means ± SD ($n = 10$) and different letters in the columns indicate significant differences from controls ($P < 0.01$).

3.4 Discussion

One of the first responses of plants to drought stress is a decrease in leaf water potential (Ψ_w) (HAIDER et al., 2018), which allows for a rapid recovery of cell turgor and photosynthetic activity upon rewatering (NIINEMETS, 2016; RUEHR et al., 2019). *M. tenuiflora* and *P. stipulacea* coped with the increasing water deficit by considerably reducing their Ψ_w throughout the day. In fact, their predawn and midday Ψ_w after seven days of drought were much lower than those recorded for six Caatinga trees in the dry season (DOMBROSKI et al., 2011), as well as for other tropical species under drought stress, such as *Hevea brasiliensis*, −1.85 MPa at predawn (FALQUETO et al., 2017), *Bertholletia excelsa*, −4.7 MPa at midday (SCHIMPL et al., 2019), and *Erythrina velutina*, −0.31 MPa at predawn and −0.89 MPa at midday (SILVA et al., 2010). However, despite showing similar responses, the rehydration of *P. stipulacea* was completed three days earlier than in *M. tenuiflora*.

The recovery of Ψ_w precedes, and is usually faster than, that of gas exchange parameters, occurring within hours to a few days (DUAN et al., 2020; RUEHR et al., 2019). For this reason, water relations of drought-stressed trees can benefit even from short rainfall events (DIETRICH & KAHMEN, 2019). This is particularly important in the Caatinga dry

forest, where plants experience low and irregular rainfall (DOMBROSKI et al., 2011; SAMPAIO, 1995). The sooner the plant water status is normalized, the faster the full recovery of photosynthetic activity will be (YORDANOV et al., 2000), as demonstrated in this study for *P. stipulacea*. To withstand droughts, plants have evolved a series of adaptive strategies. An important mechanism is the accumulation of compatible solutes like proline, soluble sugars and amino acids, which helps to maintain cellular homeostasis and promotes osmotic adjustment (LIAO et al., 2018). Therefore, the equal ability of *M. tenuiflora* and *P. stipulacea* to decrease their Ψ_w can be explained by the biochemical changes observed in these plants at the highest stress level.

Osmotic adjustment may take up to three weeks to achieve (SPIEß et al., 2012). Nevertheless, after only seven days of drought, both species showed increased proline, soluble sugar and soluble amino acid contents. Proline had by far the highest increase, presumably because of its importance both as an osmolyte and as an antioxidant (HU et al., 2015; KHALEGHI et al., 2019). Plants under drought stress must contend not only with the loss of cell turgor, but also with the overproduction of reactive oxygen species, which increases the risk of irreversible damages to major macromolecules such as lipids, proteins and carbohydrates by oxidative stress (GALLÉ & FELLER, 2007). With regards to soluble sugars, short periods of drought induce the accumulation of readily metabolizable carbohydrates (SPIEß et al., 2012). Thus, the increased contents observed here may indicate an important adaptation to prepare for recovery. During drought, soluble sugars are important for osmoregulation and as signalling molecules to induce stress responses. But once no longer needed for these purposes, they can be essential to supply carbon and energy for repair and regrowth (KHALEGHI et al., 2019). This could also explain the accumulation of soluble amino acids, which would support subsequent protein synthesis (TAIZ et al., 2015).

Decreases in photosynthetic pigments are commonly reported in drought-stressed plants and attributed to a slow biosynthesis or a fast degradation of chlorophylls (FOX et al., 2018; HAIDER et al., 2018; LIAO et al., 2018). Like the findings of this study, there seems to be no major impact on the chlorophyll content of certain tree species (DUAN et al., 2020; FREITAS et al., 2018; FROSI et al., 2016), which suggests a lesser impact on their photosynthetic apparatus (GALLÉ et al., 2007). The increase in chlorophyll *a* and, consequently, in the chlorophyll *a/b* ratio of *M. tenuiflora* under stress could also indicate an adjustment of photosystem (PS) stoichiometry towards a higher PSI/PSII ratio, reducing light harvesting and avoiding photoinhibition (LIU et al., 2011). In *P. stipulacea*, because there was no significant change in chlorophyll levels, and net photosynthesis recovered much

earlier, it is presumed that these functional units may have been downregulated but preserved during drought.

Altogether, the biochemical changes in *M. tenuiflora* and *P. stipulacea* contributed to the rapid and complete recovery of their photosynthetic activities after rehydration. Such recovery is an indicator of drought tolerance and demonstrates a high physiological plasticity (SCHIMPL et al., 2019), emphasizing the ability of these plants to withstand drought events. To prevent extreme water loss in leaf tissues during the stress period, both species reduced transpiration by closing their stomata, which in some circumstances affects CO_2 uptake (HAIDER et al., 2018; LIAO et al., 2018; YORDANOV et al., 2000). Peguero-Pina et al. (2018) observed that the reduction in stomatal conductance of *Quercus ilex* consisted in a limitation to carbon assimilation. Although it was initially considered the possibility of drought-induced stomatal limitations to photosynthesis, it was later confirmed that this was not the case in this study, indicating exclusively the occurrence of mesophyll or biochemical limitations.

At the highest stress level, the reduction in net photosynthesis, despite increased CO_2 availability, could be attributed to a low gas use efficiency of chloroplasts (ASHRAF & HARRIS, 2013). Increased resistance in the mesophyll layer could reduce CO_2 diffusion to carboxylation sites (ELFERJANI et al., 2021). In addition, plants under increasing water deficits, if still exposed to light, are likely to experience photoinhibition (ZARGAR et al., 2017). Drought can lead to the degradation of the D1 protein, a key subunit of PSII, thus causing the inactivation of its reaction centre (ASHRAF & HARRIS, 2013). In this case, the photosynthetic activity of drought-sensitive plants might not be fully recovered (RUEHR et al., 2019). Conversely, Gallé and Feller (2007) reported enhanced photoprotection in *Fagus sylvatica* after a reversible downregulation of PSII accompanied by an increase in the dissipation of excess excitation energy. This helped to maintain a functional photosynthetic apparatus, allowing the complete recovery upon rewatering, as observed here for both species.

While the magnitude of the effects of stomatal and non-stomatal limitations on photosynthetic capacity cannot be easily distinguished (ASHRAF & HARRIS, 2013), *M. tenuiflora* and *P. stipulacea* were found to be mainly affected by the latter, because at no time was photosynthesis limited by low CO_2 availability. Besides, there were no positive correlations between stomatal conductance and intercellular CO_2 concentration (Table S2). At the highest stress level, the fact that photosynthesis was almost completely suppressed explains, in part, the increases in CO_2 availability, given that this substrate was no longer being used. However, since stomatal conductance was restricted, this increase was probably

due to respiration rather than a high influx of CO_2. In any case, the extremely low carboxylation efficiency observed at that point could be the result of declines in mesophyll conductance and/or in the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (ELFERJANI et al., 2021; ZHOU et al., 2014).

Based on several studies, Elferjani et al. (2021) estimated that stomatal, mesophyll and biochemical limitations accounted for 49, 39 and 12%, respectively, of the reductions in photosynthesis of *Populus* spp. Nevertheless, the impacts of short-term drought stress on these proportions vary greatly among tree species (ZHOU et al., 2014). Apparently, drought stress affects stomatal conductance and net photosynthesis at the same rate, leading to a high correlation between these parameters. However, the extent of stomatal closure was insufficient to limit CO_2 influx into the sub-stomatal chamber, unlike that observed for another Caatinga species (DOMBROSKI et al., 2014). Therefore, neither of the species studied here presented stomatal limitations to photosynthesis, which is in accordance with Alves and Freire (2019) for *M. tenuiflora*. Unfortunately, there seems to be no study on the effect of drought on photosynthesis of *P. stipulacea*, which highlights the need for further research.

In addition to altering photosynthetic activity, drought stress affected growth patterns. Changes in biomass production and allocation can play an important role in adaptation to future drought events (GALLÉ & FELLER, 2007; NIINEMETS, 2016). Under the conditions described here, previously stressed plants invested less in shoot (*M. tenuiflora* and *P. stipulacea*) and root (*P. stipulacea*) growth. Thus, even a relatively short drought period of seven days can induce morphological adaptations in these species. Despite reporting a strong compensation growth upon rewatering, Spieß et al. (2012) observed that long-term drought had a negative effect on the subsequent growth of *Q. robur*, affecting mainly the shoots. Curiously, root growth in *P. stipulacea* is more reduced than shoot growth, as suggested by Barros et al. (2019). A meta-analysis revealed that, under drought stress, roots are not as affected as leaves and stems, and their biomass generally increases to facilitate water uptake (EZIZ et al., 2017). However, this is more common for herbaceous than woody species, and may not even occur under extreme water deficit (EZIZ et al., 2017; XU et al., 2010). Moreover, there are reports on the increase of shoot/root ratios during drought (BUENO et al., 2021; SANTIAGO et al., 2001). In contrast, Barros et al. (2020) found no differences in the shoot/root ratio of four woody species after rehydration, as shown here for *M. tenuiflora*.

3.5 Conclusions

The drought tolerance of *M. tenuiflora* and *P. stipulacea* is associated with their ability to respond quickly and effectively to this stress. These species maintain a low leaf water potential throughout the day by accumulating compatible solutes, thus allowing a rapid and full recovery of water status upon rewatering. Nonetheless, because the rehydration of *P. stipulacea* occurs at a faster rate, its photosynthetic activity recovers earlier. Although both species minimize water loss by closing their stomata, neither showed stomatal limitations to photosynthesis. The inhibition of this process during drought is probably related to mesophyll limitations as well as to a reversible downregulation of photosystems, along with, in the case of *M. tenuiflora*, adjustments of their stoichiometry. Moreover, water deficit triggers morphological adaptations in these species, leading to reduced subsequent growth, mainly of shoots in *M. tenuiflora* and roots in *P. stipulacea*. Future studies may help to elucidate the gene expression and antioxidant enzyme activity underlying this drought tolerance.

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APPENDIX – SUPPLEMENTARY MATERIAL

Table S1 - Results of simple correlation analysis between gas exchange parameters of *Erythrina velutina* and *Poincianella pyramidalis* as a function of drought and rehydration.

Plant species	Parameter[a]	C_i	E	A/E	A/g_s
E. velutina	A	—	—	−0.62*	−0.73**
	g_s	0.79**	0.99**	—	—
P. pyramidalis	A	—	—	−0.16[ns]	−0.56*
	g_s	0.90**	0.98**	—	—

[a] A – Net photosynthesis; g_s – Stomatal conductance; C_i – Intercellular CO_2 concentration; E – Transpiration rate; A/E – Instantaneous water use efficiency; A/g_s – Intrinsic water use efficiency. **, $P < 0.01$; *, $P < 0.05$; [ns], $P \geq 0.05$.

Table S2 - Results of simple correlation analysis between gas exchange parameters of *Mimosa tenuiflora* and *Piptadenia stipulacea* as a function of drought and rehydration.

Plant species	Parameter[a]	g_s	C_i
M. tenuiflora	A	0.97**	−0.33*
	g_s	—	−0.21*
P. stipulacea	A	0.96**	−0.58*
	g_s	—	−0.46**

[a] A – Net photosynthesis; g_s – Stomatal conductance; C_i – Intercellular CO_2 concentration. **, $P < 0.01$; *, $P < 0.05$.
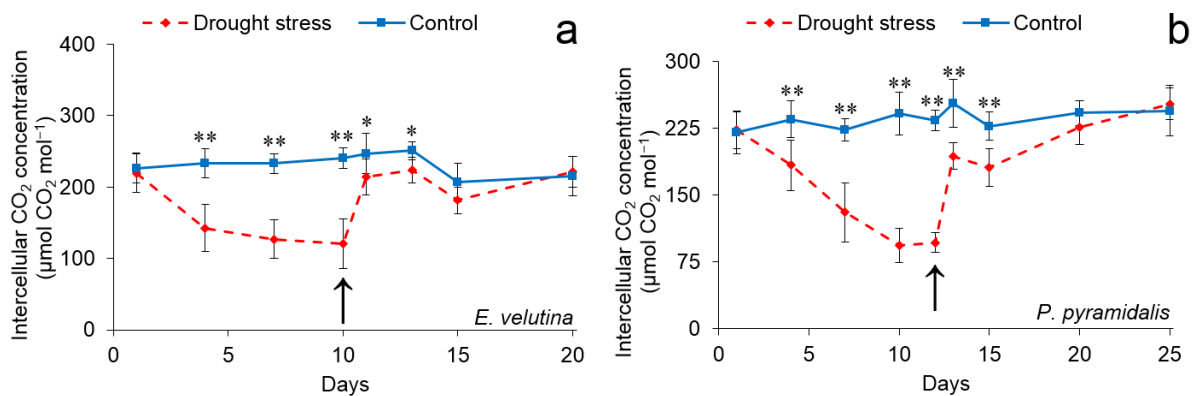
Figure S1 - Intercellular CO_2 concentration of *Erythrina velutina* (a) and *Poincianella pyramidalis* (b) over time as a function of drought and rehydration. Arrows indicate the beginning of the recovery period. Values are means ± SD (n = 10) and asterisks denote significant differences from controls (**, $P < 0.01$; *, $P < 0.05$).
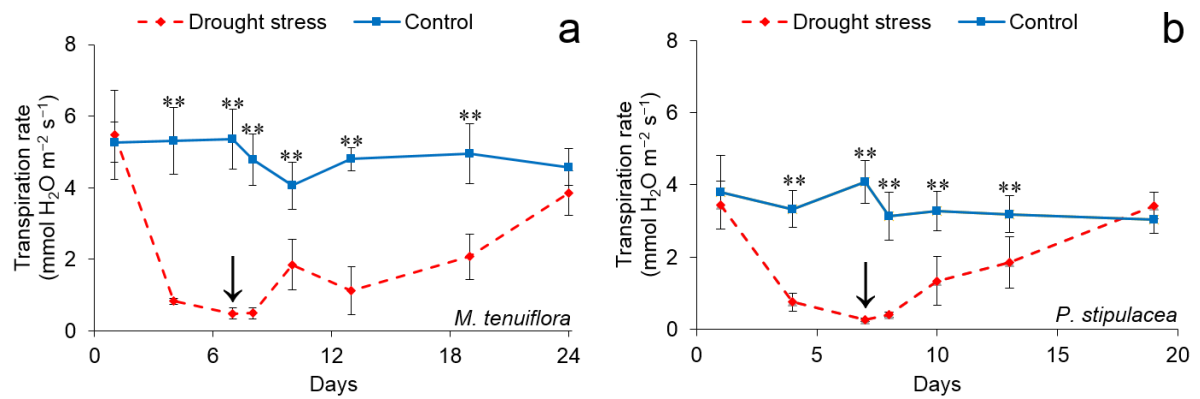
Figure S2 - Transpiration rate of *Mimosa tenuiflora* (a) and *Piptadenia stipulacea* (b) over time as a function of drought and rehydration. Arrows indicate the beginning of the recovery period. Values are means $\pm$ SD ($n = 10$) and asterisks denote significant differences from controls ($P < 0.01$).