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Role of carbohydrates metabolism in ecophysiological responses of Amazonian tree *Senna reticulata* grown under different abiotic stresses

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Orientador

A meu jacaré e minha nona dedico

“Há apenas uma coisa boa: conhecimento; e uma coisa má: ignorância. O conhecimento é indissociável da moralidade, por essa razão devemos sempre “examinar” nossas vidas. Conhece-te a ti mesmo e conhecerás o Universo de Deus”.

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SUMÁRIO

RESUMO

A vegetação ao longo do rio Amazonas é submetida a dois períodos muito bem marcados ao longo do ano: estação de cheia e estação seca. Características morfológicas e fisiológicas das espécies de várzea foram amplamente descritas, porém, uma das principais características é o metabolismo de carboidratos que tem sido muito pouco explorada. Com o objetivo de estudar a resiliência dos carboidratos não estruturais da árvore amazônica *Senna reticulata* (Leguminosae), a mesma foi cultivada sob diferentes estresses abióticos, como luz baixa combinada com elevada concentração de CO_2 atmosférico, bem como disponibilidade hídricas distintas (alagamento das raízes e seca). Os resultados encontrados reforçam o papel do amido nas folhas como principal órgão armazenador desta espécie e também a alta resiliência desta reserva, que foi mantida mesmo em condições de baixa irradiância, em alagamento e em condições de seca. Estes resultados também destacam a forte ligação entre as reservas de amido e a manutenção do crescimento. Além disso, esta espécie mostrou estratégias ecofisiológicas distintas em relação a diferentes estresses hídricos (alagamento x seca) e uma alta capacidade de se recuperar depois de um período de seca, padrão este que pode estar diretamente relacionado à manutenção do crescimento durante o estabelecimento de plantas jovens no primeiro período de fase terrestre.

Palavras-chave: estresse abiótico, carboidratos, Amazônia, alagamento, seca, elevado CO_2.

ABSTRACT

The vegetation along the Amazon River is subjected to two markedly periods: flooded and drought seasons. Morphological and physiological traits for floodplain species have been largely described; however, one of the main features is the carbohydrate metabolism, which has been poorly explored. In order to study the resilience of non-structural carbohydrates of the Amazonian tree *Senna reticulata* (Leguminosae), this species was grown under different abiotic stresses such as low light combined with elevated CO_2 and distinct water availabilities (waterlogging and drought). Results reinforce the role of starch in leaves as a main storage organ of this species and also the high resilience of this reserve under low light, waterlogging and drought conditions. These findings also highlight the tight connection between starch reserves and growth maintenance. Additionally, this species has shown very distinct eco physiological strategies to cope with different water availabilities and a high ability to recover after drought, that might be strictly related to growth maintenance during seedling establishment in the first period of terrestrial phase.

Key-words: abiotic stress, carbohydrates, Amazonia, waterlogging, drought, elevated CO_2.

INTRODUÇÃO GERAL

Plantas são organismos sésseis e não podem mudar rapidamente de habitat quando o mesmo se encontra em condição ambiental desfavorável, seja por variação climática ou pressão predatória (Sala et al., 2012). Partindo de uma terminologia física, o termo "estresse" foi adotado para descrever situações impostas que desviem de uma situação ótima (Larcher, 1994; Levitt, 1980). Larcher (1994) define o fator de estresse ou estressor como qualquer fator ambiental (biótico ou abiótico) que promova remobilização de recursos, restringindo o crescimento e/ou a reprodução de um organismo. Com frequência, a resposta a um determinado estresse não é diretamente proporcional à intensidade do estímulo e isso ocorre devido à alta variabilidade nos mecanismos de proteção que os organismos apresentam que pode ter como função atrasar ou evitar um dano (estratégia de evitação), ou ainda em uma situação onde o real estado de estresse é atingido, podendo auxiliar na tolerância aos efeitos deletérios promovidos pelo estímulo (estratégia de tolerância) (Levitt, 1980). Ambas as estratégias foram responsáveis pela ocupação das plantas nos mais diversos ambientes (Schulze et al, 2002).

Devido a esta importância somada ao desafio econômico de aumentar a produtividade de espécies cultivadas (Da Matta et al., 2010; De Carvalho et al., 2014; Rodera et al., 2014), há muitos anos os estudos sobre a resposta das plantas à diversidade de condições ambientais tem sido uma abrangente área de estudo de ecologistas e fisiologistas com o objetivo de entender estratégias de colonização e adaptação das diferentes espécies. Contudo, após a consolidação de estudos mostrando que o clima e o planeta estão passando por profundas modificações (IPCC 2007 e 2014), a natureza desses estudos foi trazida para uma nova dimensão (Menuccini, 2014).

A importância do entendimento de estudos em relação à resposta de plantas aos efeitos do tripé das Mudanças Climáticas Globais (MCGs): CO_2 atmosférico, temperatura e disponibilidade hídrica atravessaram o campo político e social envolvendo questões como biocombustíveis (Carroll & Somerville, 2009; Buckeridge et al., 2012; Grandis et al., 2014) e segurança alimentar de futuras gerações (Long et al., 2006; Tubiello & Fischer, 2007; Da Matta et al., 2009). Estudos com espécies nativas também trouxeram à tona evidências de que as MCGs estão sendo relacionados também com eventos de mortalidade de árvores e mudanças na abundância, distribuição e funcionalidade da vegetação ao longo do globo (Allen et al., 2010; Reichstein et al., 2013). Portanto, pesquisadores de diversas áreas vêm tentando entender o mecanismo de respostas de plantas nativas as MCGs com o objetivo de

modelar possíveis alterações que ocorrerão nos biomas naturais (Hirota et al., 2011) que por sua vez também acarretarão mudanças e necessidade de adaptação em todas as esferas da sociedade (Nobre et al., 2007, Malhi et al., 2008).

Recentes trabalhos relacionados à fisiologia do estresse em plantas apresentam que o entendimento de respostas de um estresse isolado não é o mesmo quando comparado ao mesmo estresse somado a outro (Knight & Knight, 2001; Mittler, 2006). Considerando que uma junção de múltiplos fatores é a situação que melhor representa condições reais de estresses para as plantas, o estudo dos mecanismos de regulação precisa ser abordado visando à integração destas respostas, como por exemplo, trabalhos onde a elevada concentração de CO_2 é acoplada a temperatura (Baker e Allen, 1993), deficiência nutricional (De Graaf et al., 2006), seca (Mikkelsen et al., 2008) e alagamento (Megonigal et al., 2005; Arenque et al., 2014).

Atualmente, o arcabouço teórico que temos em relação às respostas fisiológicas das plantas as MCGs apresenta o metabolismo de carboidratos como peça fundamental. Em plantas com metabolismo fotossintético do tipo C_3, inicialmente temos o efeito direto do aumento da razão CO_2:O_2 sobre a carboxilação da enzima Rubisco, levando a uma diminuição na função oxigenase da mesma e aumentando as taxas fotossintéticas (Saxe et al., 1998). Potencialmente este aumento observado na taxa fotossintética culminaria em maior disponibilidade de carboidratos para o crescimento, porém em diversos casos a planta se mostra incapaz de utilizar ou estocar esses açúcares (Stitt, 1991; Luo et al., 1997), promovendo um aumento do conteúdo de carboidratos não estruturais, sendo essa resposta extremamente dependente da relação fonte-dreno (capacidade de estoque e utilização) de cada espécie em determinada condição ambiental (Stitt, 1991). Uma das respostas observadas com maior frequência em estudos de plantas sob elevada concentração de CO_2 é a diminuição na condutância estomática e consequente maior eficiência do uso da água (Leakey et al., 2009; Leakey et al., 2012), ao contrário do efeito sobre a respiração que apresenta grande divergência na literatura (Wang & Curtis, 2002; Leakey et al., 2009; Watanabe et al., 2014), apesar de estudos recentes mostrarem reduções consistentes na respiração foliar (Ayub et al., 2014).

O balanço entre os três processos já mencionados: fotossíntese, respiração e transpiração, afetam diretamente os níveis de carboidratos e água nas plantas (Sage, 2002), por sua vez modulando a capacidade de investimento na aquisição de novos recursos,

reservas ou crescimento da planta. Extrapolando estas respostas para o ambiente natural e considerando que reservas (p.ex. amido e frutanos) são estritamente necessárias para tamponar períodos de estresses (Chapin et al., 1990) e que o metabolismo de carboidratos é considerado peça fundamental na sobrevivência das plantas em longo prazo (Kozlowsky & Pallardy, 2002), alterações no status de carboidratos de diferentes espécies desencadeados pelas MCGs podem influenciar diretamente nas taxas de sobrevivência de diferentes espécies.

Até o presente momento, muito tem sido descrito em relação ao efeito da elevada concentração de gás carbônico sobre a fisiologia de plantas (Leakey et al., 2012), contudo, a maioria dos trabalhos utiliza de espécies cultivadas de grande importância econômica ou plantas de regiões com clima temperado, com plantas tropicais sendo fracamente representadas (Korner, 2009; Cernusak et al., 2013).

Particularmente em relação a um importante bioma tropical como a Floresta Amazônica, esta região desempenha um papel primordial no balanço global de carbono e na ciclagem de água. Além disso, é um dos "hotspots" de alta vulnerabilidade para as MCGs (Baettig et al., 2007), tendo previsões de eventos extremos de seca (região oriental) e de alagamento (região ocidental) fortemente intensificados (Marengo et al., 2011). Contudo, apesar de tamanha relevância, ainda é uma região pouquíssimo explorada em relação as respostas das plantas ao CO_2 elevado (Arenque et al., 2014).

Dentro da Bacia Amazônica, a variação na intensa precipitação ao longo do ano acarreta flutuações sazonais nos níveis da água do Rio Amazonas e de seus maiores tributários (Junk & Piedade, 2011). Essa flutuação é responsável pela inundação periódica dos milhares de quilômetros de terras baixas marginais adjacentes a essa região que corresponde a 20-25% de toda a região da Amazônia (Junk, 1993). Este "pulso de inundação" (Junk et al., 1989) é momomodal e previsível, resultando em duas fases bem definidas ao longo do ano, um período de águas altas (fase aquática) e um período de águas baixas (fase terrestre). Comunidades vegetais que margeiam estes rios estão sujeitas a este regime periódico de alagamento e a zonação das espécies que ocorrem ao longo do gradiente de inundação são proporcionais aos níveis de tolerância aos estresses impostos pela variação na coluna d'água. Espécies ocorrentes em regiões mais altas passam menos tempo sob esse estresse de alagamento, contudo estão mais sujeitas aos períodos de seca

que ocorrem quando água começa a descer durante o início da fase terrestre (Parolin 2001b).

Senna reticulata (Willd.) H.S. Irwin & Barneby (Leguminosae) é uma espécie de hábito arbustivo-arbóreo típica de planícies alagáveis da região de várzea que se estende por todo o curso do Rio Amazonas e seus tributários (rios de água branca, rico em nutrientes) nos Estados do Amazonas e Pará (Prance, 1979). Segundo De Menezes (1978) sua ocorrência é reportada também para os estados do Amapá, Pernambuco, Bahia, Mato Grosso, Minas Gerais, Goiás, Rio de Janeiro e São Paulo, estando sempre associada a locais úmidos e bem supridos de nutrientes. Popularmente conhecida popularmente como matapasto, é considerada uma espécie pioneira e altamente eficiente na colonização de áreas abertas das regiões altas de várzea, não suportando completa submersão de todas as suas folhas. Possui altas taxas fotossintéticas e crescimento muito rápido, atingindo até 4m nos primeiros meses de sua primeira fase terrestre após o estabelecimento da plântula (Parolin, 2001b).

Essa espécie tem sido intensivamente estudada em relação a sua alta tolerância ao alagamento (Parolin 2001a, 2001b, 2002, 2005; Parolin et al., 2004), bem como sua capacidade de sobrevivência em relação ao período de seca (Parolin, 2001a, Parolin et al.,2010). Recentemente, esta espécie foi caracterizada quanto à importância de seu metabolismo de carboidratos em fase inicial de desenvolvimento da planta jovem durante a fase terrestre e início da fase aquática (Arenque et al, 2014). Neste trabalho também foi avaliado o efeito acoplado do alto CO_2 a condição de alagamento, concluindo que o elevado status de carbono (principalmente na forma de reserva de amido nas folhas) encontrado na fase terrestre contribuiu para que os efeitos deletérios do alagamento fossem atenuados, reforçando o papel do metabolismo de carboidratos na tolerância a estresses abióticos.

No presente trabalho, tivemos como proposta continuar investigando o metabolismo de carboidratos desta espécie em diferentes combinações de estresses abióticos. Primeiramente, o objetivo foi avaliar a resiliência das reservas encontradas nesta espécie acoplando a elevada concentração de gás carbônico a baixa disponibilidade de luz (Capítulo 1). Em um segundo experimento, tivemos como proposta determinar o papel destas reservas em situações de disponibilidades hídricas distintas como a seca e o alagamento (Capítulo 2). Entendemos que o estudo da plasticidade metabólica e os mecanismos de ajustes fisiológicos em resposta a diferentes estresses abióticos podem

auxiliar na compreensão de como espécies desta região evoluíram à luz deste ambiente contrastante, bem como em um maior entendimento de como espécies tropicais estão respondendo as MCGs.

CAPÍTULO 1

Ajuste temporal de carboidratos e crescimento da árvore amazônica *Senna reticulata* cultivada sob baixa disponibilidade luminosa e elevado CO_2 atmosférico

(Artigo submetido ao periódico "Tree Physiology" em maio 2014)

Colaborador

Dr. Ronan Sulpice (NUIG, Plant Systems Biology Lab, Plant and AgriBiosciences Research Centre, Botany and Plant Science, Galway, Ireland and Max-Planck-Institut für Molekulare Pflanzenphysiologie, Potsdam-Golm, Germany).

Temporal adjustments of carbohydrates and growth of Amazonian tree *Senna reticulata* cultivated under low light and elevated atmospheric CO_2

Abstract

Adjustments in carbohydrate metabolism are extremely important in helping plants to cope with daily and seasonal fluctuations of environmental conditions. These regulations lead to changes in assimilation and use of carbon (C) hardly modifying its availability for respiration, storage and growth. The aim of this study was to assess if *S. reticulata* is able to regulate its storage capacity and keep growth under low C availability and whether elevated CO_2 atmospheric can affect this response. Seedlings of the Amazonian tree *Senna reticulata* were grown in Open Top Chambers in the presence of 400 μmol CO_2 m^2 s^{-1} (control plants) and 800 μmol CO_2 m^2 s^{-1} (treated plants). These different CO_2 concentrations ($[CO_2]$) were combined or not to low light conditions (from 1500 to <100 μmols of PPFD). Data were collected at day 7 and day 14 after both CO_2 and light treatments have started. Low light decreased starch at the end of the light and the dark period in average 80%, leading to a status of C starvation, particularly under ambient CO_2. Leaf respiration and consequently leaf starch turnover were reduced probably in order to keep growth maintenance. Elevated CO_2 combined with low light decreased leaf respiration in higher extent, increased starch storage in leaves and stems and rescued 50% of biomass in comparison to ambient CO_2/LL. These results indicate that under lower C availability this species displays a great ability to re-establish starch reserves in leaves even at expenses of growth and that elevated CO_2 improve this response by strongly decreasing leaf respiration and redirecting its carbohydrates to growth.

Key words: Starch, starvation, respiration, growth, Amazon, elevated CO_2

Introduction

Floodplains correspond to approximately 25% of the whole Amazon region and this region is characterized by alternating periods of flooding cycles that is directly linked to rainfall patterns among the year (Junk, 1993). Considering that about half of the wetland area of Amazon is covered by wood vegetation (Klinge et al., 1990), the occurrence and distribution of plant species are highly dependent on length, depth, frequency, shape and predictability of inundation pulse (Junk, 1989; Junk & Piedade, 2011). According to the position among the riverbank, species can experience flooding up to 7 month in a year time and also determining the extent of drought stress during the low water period (Parolin et al., 2009). The massive water fluctuation in this environmental occur under optimal light and temperature conditions, driving plant species to develop several morphological (e.g. aerenchyma development, adventitious roots) and metabolical adaptations in order to keep or even increase growth (Parolin et al., 2004, Parolin et al., 2009).

Carbohydrate metabolism adjustments has been described as one of the most important adaptation to cope with anoxia stresses promoted by waterlogging. From a limited amount of studies, it is thought that plants use their storage system (mainly starch) in order to cope with the stressing situation imposed by the flooding pulses in the Amazon (Parolin et al., 2004). Ferreira et al. (2009) showed that the slow growing tree *H. sucuuba* is capable to survive under water for several months in Central Amazon without shedding its leaves at the expension of starch stored in roots (Ferreira et al., 2009) corroborating findings of Scarano et al. (1994) who showed that during drought period plants can display higher amount of stored carbohydrates to use in following flooding period. More recently, was demonstrated that instead starch, soluble sugars stored in roots and adventitious roots can play a key role under waterlogging and that starch storage in leaves can be crucial to increase growth during establishment of seedlings in the first terrestrial phase of *Senna reticulata* and also to cope with waterlogging when this stress was combined with elevateed CO_2 (Arenque et al., 2014).

These founds raises the importance to better understand the relationship between carbon assimilation and its partition into starch and other reserves, respiration and growth in species occurring in highly dynamic environments. The tight relationship between starch

metabolism and growth has been highlighted (Gibon et al., 2004 and 2009; Sulpice et al., 2010), and the relevance of studies in plant models other than *Arabdopsis* have been raised (Stitt & Zeeman, 2012).

Usually, CO_2 assimilation provides enough carbon to support the immediate requirements of plant growth (through sucrose synthesis and export), but yet producing a surplus of carbohydrates that lead to the accumulation of transitory starch, which is stored in chloroplasts and degraded during the night period, providing substrates for biosynthesis of sucrose that will be distributed throughout the plant, providing carbon skeletons and energy for cell and tissue growth and maintenance. It has been demonstrated that the diurnal starch accumulation/degradation mechanisms are not simply due to overflow. They are, in fact tightly regulated by the plant (Sulpice et al., 2014) and can be influenced by several factors, mainly related to growth conditions such as photoperiod length (Chaterton & Silvius, 1979; Gibon et al., 2004), photon flux density and night temperature (Hewitt et al., 1985), nutrient starvation (e.g. nitrogen or phosphorus) or excess (e.g. elevated CO_2 - Paul & Stitt, 1993).

Studying *Arabdopsis* mutants that are unable to synthesize starch, Smith & Stitt (2007) described that plants can respond in two distinct ways to changes in carbon availability: 1) An “acute” response that is experienced in conditions of sudden carbon starvation where growth stops abruptly and 2) an “Acclimatory” response that adjust the balance between supply and demand to optimize the ability to sustain growth. Stitt & Zeeman (2012) also highlighted that many signaling pathways mediated by clock and light interactions are still an emerging challenge to be explored.

In order to evaluate the plasticity of carbon metabolism of *Senna reticulata* regarding its capacity to cope with environmental fluctuation, young plants of this species were subjected to limiting light availability by shortening the period of photosynthetic active light by half. With this experiment, we expected to lead plants to near starvation and consequently be able to evaluate the resilience of physiological mechanims related to the starch storing capacity of *S. reticulata*. At the same time, the decrease in the total amount of CO_2 assimilation due to the shortened low light conditions was compensated by growing plants in open top chambers with CO_2 concentration at approximately double of the current atmospheric level (ca. 800 μmoles of $CO_2.m^{-2}.s^{-1}$ against 400 μmoles of $CO_2.m^{-2}.s^{-1}$).

General Purpose

To investigate the relationships between C availability, starch reserves and growth responses of *Senna reticulata* grown under low light combined with elevated CO_2 concentration.

Aims

1) To investigate if decreased light period promote adjustments in starch storage in leaves;
2) To investigate if carbon assimilation and growth are affected by decreased light period and whether elevated CO_2 affect this responses;
3) To assess whether elevated CO_2 reduces leaf respiration.

Material and Methods

Experiment 1. Plant material and growth in the long-term experiment (14 days)

Seeds of *Senna reticulata* were collected from trees at Universidade Federal Rural do Pará – UFRA (Belém-PA) in November 2009. The seeds were stored in an amber bottle at 5°C. In March 2011, the seeds were scarified mechanically with sandpaper and germinated in trays with vermiculite in a germination chamber at 28 °C under constant light.

Seedlings were maintained in these conditions until radicle protrusion occurred (2 days). Germinated seeds were transferred to a greenhouse and watered weekly with 100 mL per plant of nutrient solution containing 20 mM of N (modified from Epstein, 1972 according to Arenque et al., 2014).

Experimental Design

After germination the seedlings were kept one day in the greenhouse under photosynthetically active radiation (PAR) of 300 µmol photons m^{-2} s^{-1} and transferred to pots (PVC pots – 5L – one plant per pot) containing topsoil and, finally, transferred to normal day light conditions (12/12 day/night photoperiod) (Figure 1A).

The 150 pots were then randomly distributed into four Open Top Chambers (OTCs). Each chamber was 1.5 meters in diameter by 3 meters in height, fitted with an air circulation system (Fig. 1B) according to De Souza et al. (2008). Two OTCs were coupled to carbon dioxide (CO_2) feeding in order to maintain the atmosphere inside the chamber around 800 µmol.$mol^{-1}CO_2$. In the other two chambers, only atmospheric air was injected naturally containing ca. 400 µmol.$mol^{-1}CO_2$. The gas concentration inside the chambers was monitored three times per week with a portable CO_2 measurer (Testo® model 435). Half of the plants (ca. 75 pots) were grown in current CO_2 and the other half under atmosphere with an increased CO_2 concentration (~800 µmol.mol^{-1}).

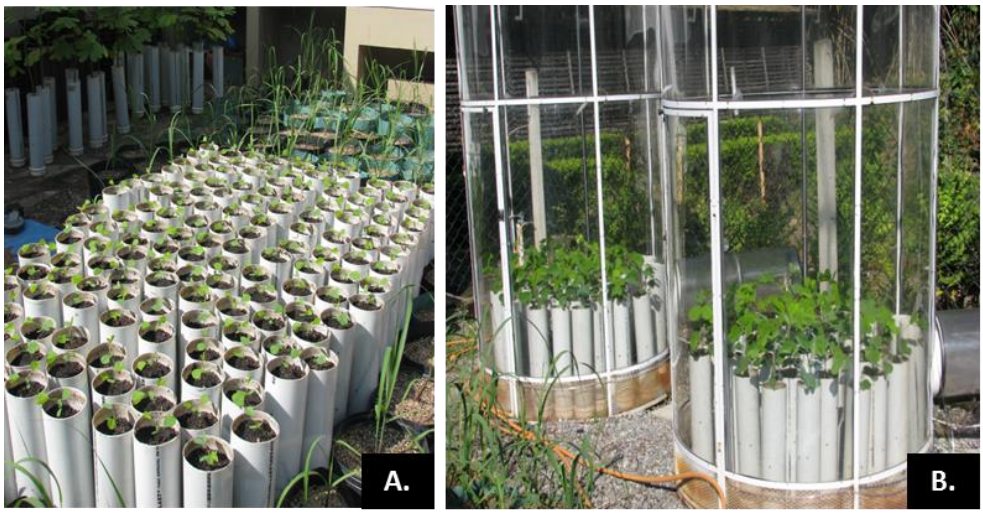

Figure 1. A. *Senna reticulata* growing in pots and **B.** inside Open Top Chambers at LAFIECO after 60 days. Two chambers were kept with ~400 μmol.mol^{-1} and the other two with enriched atmosphere of ~800 μmol.mol^{-1}.

After 12 days at ambient and elevated CO_2, two of the OTCs (one ambient – 400 μmol.mol $^{-1}$ CO_2 and one elevated – 800 μmol.mol^{-1}.CO_2) were covered with black plastic tarps in order to obtain a treatment under lower light (LL) conditions (Figure 2). This treatment aimed at interfering in transitory starch accumulation in leaves (C availability) by decreasing the length of time plants received photosynthetically active light intensity. The tarps cover were installed every day at 12 am and removed from the OTC at 7 pm during 15 days. This interference provided differences in light intensity from 1500 μmol of PPFD on a sunny day (control chambers) to 100 μmol of PPFD (lower light availability - LL), with a total of six hours under full light, six hours under low light and 12 hours of nighttime. The non-covered control chambers were maintained in normal photoperiod (12 hours of light and 12 night hours). This combination allowed four treatments: 1) ambient CO_2 and normal day light conditions – **amb CO_2;** 2) ambient CO_2 coupled with low light treatment – **amb CO_2/LL;** 3) elevated CO_2 and normal day light conditions – **elev CO_2**; and 4) elevated CO_2 coupled with low light treatment **– elev CO_2/LL.**

Figure 2. Low Light treatment (LL) using covering with plastic tarps of OTCs under ambient and elevated CO_2.

Harvestings

Both treatments ("elevated CO_2" and "low light-LL") lasted 14 days. During this period, harvests on day **0** (31 April, 2011 – "LL" started), on day **7** (6 May) and on day **14** (13 May) were performed.

At each harvest day (0, 7 and 14) measurements of gas exchange were performed (**1** - CO_2 amb, **2** - CO_2 amb/LL, **3** - CO_2 elev and **4** - CO_2 elev/LL) at 4 am and 10 am, with an experimental **n** of 12 individuals for each treatment. On the same day, at 6 pm and the following day at 6 am, we sampled six plants for metabolic analyses.

Plants were removed from pots by inversion on a screen; the soil was carefully removed from the roots, allowing the minimum loss of plant material. The roots were washed over trays containing tap water and subsequently dried with a paper towel to remove surplus water. With pruning shears, the material was separated into: youngest leaf fully expanded a pool of remaining leaves, stems and roots. Different organs were weighed to determine fresh weight. They were wrapped in plastic bags and immediately immersed in liquid nitrogen. The material was freeze-dried and weighed to determine the dry mass. After this procedure the material was pulverized in a ball mill and stored at room temperature for subsequent biochemical analysis.

Environmental data

The climatic data were obtained from the Laboratory of Plant Physiological Ecology (Lafieco), Department of Botany (IB-USP), through a system of sensors coupled to the RICS Software (RICS ®-Integrated Remote Control System), which monitored temperature and humidity inside of OTCs every 10 minutes. These data were averaged for 24 hours periods.

Gas exchange

Gas exchange measurements were taken using a portable photosynthesis system (model LI 6400 XTR, Li-Cor) composed of an open system with an infrared gas analyzer (IRGA) that infers the differential concentration of CO_2 and H_2O in an air flow passing through the chamber where the leaf unit is being analyzed.

Measurements were performed on the youngest fully expanded leaf (12 plants for each treatment). The leaf temperature was set at 25°C, airflow was 300 µmol during the night measurements (4 am) and 500 µmol during the day (10 am), and the photosynthetically active radiation (PAR) followed the day light pattern (days 0 and 7 – 800 µmol and day 14 - 500 µmol). The CO_2 concentration used for the gas exchange analysis was the same as the respective growth conditions of the plants in the OTCs (400 or 800 $\mu mol.CO_2\, m^2 s^{-1}$).

Non-structural carbohydrates

The non-structural carbohydrates (soluble sugars and starch) were analyzed along plant development. Soluble sugars were extracted from 10 mg of powdered samples in 80% ethanol at 80 °C for 20 min and this procedure was repeated four times. The supernatants were dried under vacuum and the dried pellets were re-suspended in 1 mL of deionized water. The leaf pigments of the samples were extracted by addition of 0.5 mL of 99% chloroform. Soluble sugars (glucose, fructose and sucrose) were quantified in the water phase by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC/PAD) using a Dionex-DX500 system (Dionex Corporation, Sunnyvale-CA,

USA) equipped with a CarboPac PA1 column. Chromatography was performed using 100 mM NaOH as eluent, with a flow rate of 1 mL min^{-1}.

Starch contents were measured by the enzymatic method described by Amaral et al. (2007). After removal of soluble sugars, samples were treated with 0.5 mL of thermostable α-amylase (120 U mL^{-1}) from *Bacillus licheniformis* enzyme (cod. E-ANAAM, MEGAZYME) diluted in MOPS buffer 10 mM pH 6.5 during 30 minutes at 75˚C. The incubation was repeated. Samples were cooled to 50°C and treated with 0.5 mL amyloglucosidase (30 U mL^{-1}) from *Aspergillus niger* enzyme (cod. E-AMGPU, MEGAZYME) diluted in sodium acetate buffer 100 mM pH 4.5 for 30 minutes. This procedure was repeated and 100 µL 0.8 M perchloric acid was added to stop the reactions. After hydrolysis, the starch content was measured based on the amount of free glucose using the glucose oxidase reaction kit (CENTERLAB, Brazil). The samples were incubated with this solution for 15 minutes at 30°C and glucose content was measured at 490 nm. Commercial glucose (SIGMA) was used as standard.

Chlorophyll, amino acids, nitrate, malate and glucose-6-phophate

Ethanolic extraction was performed following three steps: 1) 20 mg of fresh weight (FW), with the addition of 250 µl 80% ethanol/10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) pH 5.9, then shaken and heated for 30 min at 120°C (heating blocks in robot system) and centrifuged 10 min (3,500 rpm). 2) Pellet addition of 150 µl 80% ethanol/10mM MES pH 5.9 and step 1 repeated. 3) Pellet addition of 250 µl 50% ethanol/10mM MES pH 5.9 and step 1 repeated. Following this, all supernatants were combined in a polystyrene 96-deep well plate for the determination of:

Chlorophylls a and b

For the assay using a Sarstedt microplate®, 50 µL of ethanolic extract and 120 µL 98% ethanol were dispensed. Then, after mixing, optic distance measurements were taken at 645 and 665. Chlorophyll content was calculated using the formula adapted for the Sinergy HT reader®: Chl a (µg/well) = 5.48*A665 – 2.16*A645, Chl b (µg/well) = 9.67*A645 – 3.04*A665.

Amino acids

For the amino acid assay, 2 µL of ethanolic extract, 15 µL of borate buffer, 90 µL fluorescamine and 100 µL water were dispensed in a black microplate (Bantan-Polak et al., 2001). Then, after mixing and incubating at room temperature for five minutes, fluorescence was taken with 405 nm of excitation and 485 nm of emission. Glutamate was used as standard (0, 100, 200, 400 and 800 µM in 70% ethanol).

Malate

Malate content was measured following the protocol described by Cross et al. (2006). Using a Sarstedt microplate®, we dispensed 50 µL of Tricine buffer, 10 µL of MTT (tetrazole), 10µL NAD+, 5 µL PES (electron coupling reagent), 5µL Triton X-100 and 10 µL diluted extracted. Then, protected from light the material was mixed and absorbance read at 570 nm on the open plate reader. After 20 minutes 1 µL of MDH (malate dehydrogenase, 1000 U mL^{-1}, in Tricine/KOH 200 mM pH 9/$MgCl_2$ 10 mM [-80°C]) was added and, when OD stabilised, absorbance was measured again. Malate was used as standard (0, 125, 250 and 500 µM in 70% ethanol).

Nitrate

For the nitrate assay using a Sarstedt microplate®, we dispensed 10 µL of phosphate buffer, 0.5 µL of NADPH, 1 µL nitrate reductase, 83.5 µL of water and 5µL of diluted extracted (1:20) (Cross et al., 2006). Then, protected from light the material was mixed and incubated at 25°C for 20 minutes. After that, 15 µL of phenazine methosulphate (PMS) was added and incubated again at 25°C for 20 minutes. Finally, 50 µL of sulphanilamide and 50 µL of N-(1-naphtyl) ethylendiamine dihydrochloride (NNEDA) were added and incubated at 25°C for 10 minutes. Samples were read at 540 nm.

Glucose-6-phosphate (G6P)

G6P assay (Gibon et al., 2002) was split into two reactions: 1) Using a Sarstedt microplate®, 5 µL of ethanolic extract, 18 µL Assay buffer, 2 µL G6PDH (glucose-6-phosphate-dehydrogenase), 2 µL $NADP^+$ 2.5 mM and 23 µL of water were dispensed. Then, after mixing, the plate was incubated at 25°C for 20 minutes. After that, 20 µL NaOH was added and in order to destruct the remaining NADP+ from first reaction, the plate was

incubated at 95°C for five minutes and then 20 µL of HCl buffer was added to stop the reaction. 2) For the second reaction, in the same plate 18.5 µL of water, 10 µL of Tricine buffer pH 9, 10 µL MTT, 4 µL EDTA, 2 µL G6P and 0.5 µL G6PDH were added. After mixing and protecting the plate from light, absorbance was read at 570 nm. Glucose-6-phosphate was used as standard (0, 2, 4 and 10 µM).

Proteins

Extraction and quantification of proteins were performed according to Hendriks et al. (2003). After ethanolic extraction, pellet was re-suspended with 400 µL 0.1 M NaOH, stirred vigorously and plant material was heated at 120°C for 30 min (heating block in robot system). Subsequently, samples were left at room temperature to cool, stirred again and centrifuged at 106 *g* for 10 min. For the assay (Sarstedt microplate®), 3 µL of supernatant and 180 µL diluted Bradford solution (1:5) were dispensed. Then, after briefly shaking and waiting for five min, optic distance measurements were taken at 595 nm. Bovine serum albuminum was used as Standard (0, 250, 500 and 1000 µg.mL in 0.1 M NaOH).

Data Analysis

Differences between treatments were tested using a generalized linear model (GLM). The model considered two distinct factors: CO_2 concentration (amb/elev), light (light/LL) and also their interaction considering a P value <0.05. Test of Tukey was used a posteriori. Results were considered significant for $P \leq 0.05$.

The change in non-structural carbohydrates during nighttime was named “turnover” and defined by Equation 1, where the difference between the total amount of carbohydrate (e.g. glucose, fructose, sucrose or starch) produced at 6 pm and the total carbohydrate found at 6 am of the following day indicate positive turnover (amount of carbohydrate released during the night) or negative turnover (synthesis or remobilize) in each organ.

$$(\text{average of leaf carbohydrate}_{6pm} - \text{average of leaf carbohydrate}_{6am}) \qquad \text{(Equation 1)}$$

The variables: leaf starch, assimilation and respiration were correlated with nitrogen compounds (leaf soluble protein, nitrate, chlorophyll and amino acids were measured at 6

pm) and total biomass. All correlations were performed considering data from day 14. Pearson's correlation was considered significant when $P \leq 0.05$.

All data analyses were performed using Minitab® 14 application (Statistical Software© 1972–2004 Minitab Inc. USA) and graphed with GraphPad Prism 5 (GraphPad Software© 1984-2012 GraphPad Inc. USA).

In order to better understanding the hypothesis of "First chapter" I have included this second experiment (Experiment 2) as part of my methodology, results and discussion but I would like to clarify that they were extracted from my master degree (Arenque, 2010) and from Grandis (2010).

Experiment 2. Daily course experiment (24 hours cycle)

In 2009, an experiment was performed with plant growth conditions exactly as described for long-term experiment (2010), except for the fact that plants were grown in bigger pots (10 L pots of 9 cm diameter and 1,10 m height) and left for 60 days under current ambient atmospheric CO_2 concentration (380 µmol mol^{-1}), and treated plants were kept in two other OTCs under elevated CO_2 concentration (760 µmol mol^{-1}) (Arenque et al., 2014).

At day 60, plants were sampled (n=5) every four hours in a diurnal cycle (6 am, 10 am, 12 pm, 2 pm, 6 pm, 10 pm and 2 am). Assimilation measurements and non-structural carbohydrates statistical analyses were performed exactly as described for long-term experiment.

Results

Diurnal time course

Assimilation was slightly higher under elevated CO_2 treatment at 12 pm and night respiration was slightly lower at 2 am in this same treatment (Fig. 3A). As a result of CO_2 elevation, leaf starch was found to be higher (2 fold on average) at all times during the day and night in elevated CO_2 in comparison with ambient CO_2, except at noon (Fig. 3B). Whereas in ambient CO_2 sucrose contents in leaves and stem are unrelated, in elevated CO_2 the lower concentrations of sucrose in leaves coincided with higher concentrations of this sugar in stem (Fig. 3C). This is probably related to the fact that in elevated CO_2 more sucrose is exported from leaves to stem due to the higher carbohydrate status.

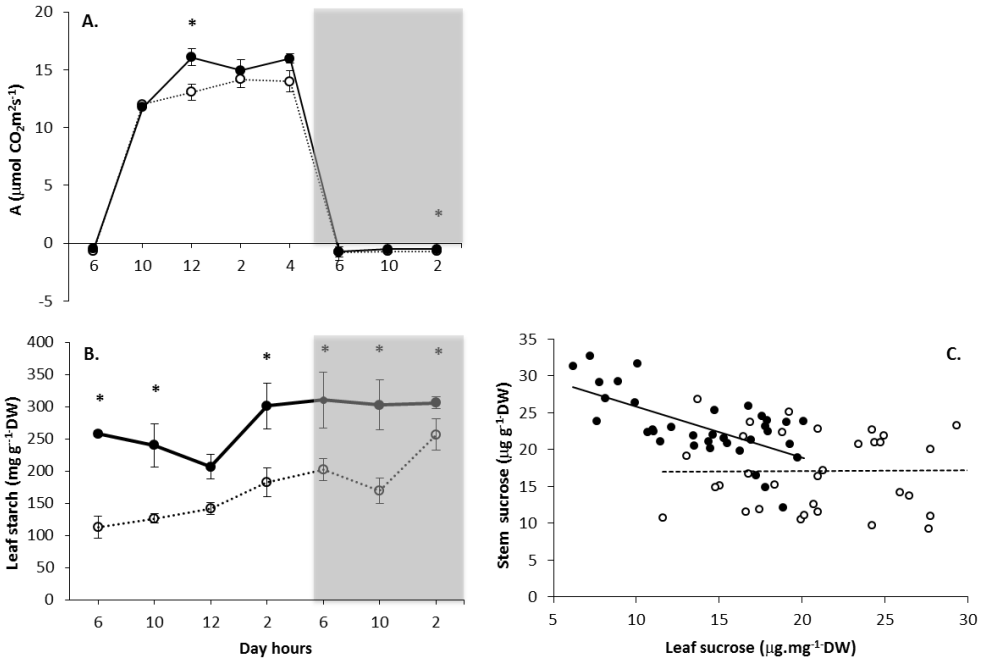

Figure 3. A. Carbon assimilation (µmol CO_2 m^2s^{-1}) and **B.** Starch contents in leaves ($mg.gDW^{-1}$). Points are means and bars standard error (n= 5). **C.** Pearson's correlation between the contents of sucrose in leaves and stem taking into account all data recorded for each treatment: ambient CO_2 (empty circles - R=0.654, *P* = 0.000) and elevated CO_2 (filled circles - R=0.012, *P*=0.946) (n=30).

Environmental data

Temperature and relative humidity were followed inside the OTC during 45 days of experiment (Fig. 4). Relative humidity (RH%) varied from 58 to 80%. For the whole period, temperature average was of 22.7°C, with a maximum of 28.7°C during the first three weeks and a minimum average of 15.3°C in later stages. At the harvest stages (days 0, 7 and 14), RH was on average 68% and temperature 22.2°C. We observed unexpressive variation between chambers for environmental parameters, regardless of the treatment (amb CO_2, amb CO_2/LL, elev CO_2 and elev CO_2/LL).

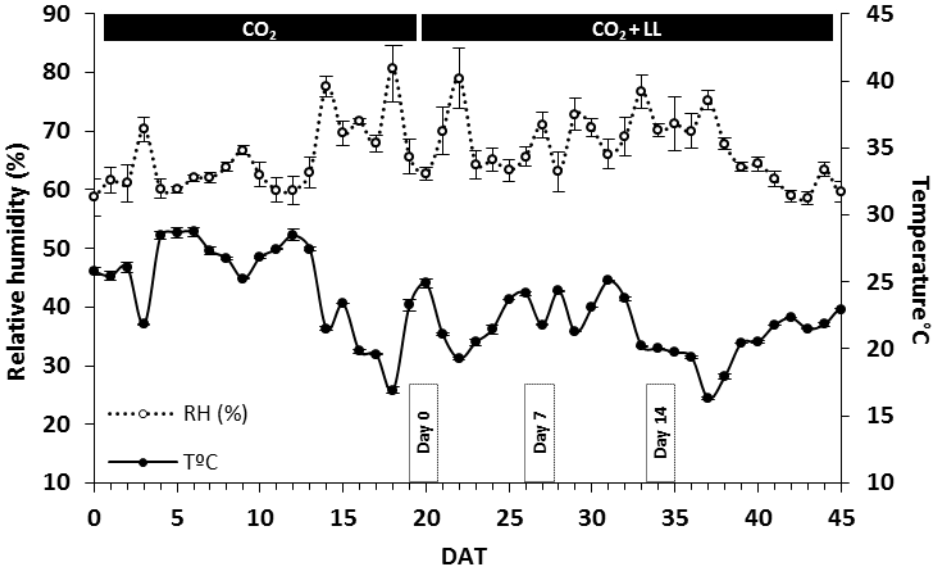

Figure 4. Daily report of air temperature (continuous line – T °C) and relative humidity (dashed line - RH%) inside OTCs. The experiment was performed from April 10th (started with CO_2 treatment) to May, 23rd , 2011. Days highlighted (inside the boxes) indicate the three harvest points (n=4).

Gas exchange

Rates of CO_2 assimilation measured under elevated CO_2 were consistently different from ambient CO_2 among all the experiment (Fig. 5A). Assimilation was higher at day 0 (17%), at day 7 (13%) and at day 14 (55%) under elevated CO_2. Interestingly, this parameter was significantly higher in LL treatments in comparison with unshaded treatments at day 7

(17% in comparison with amb CO_2 and 7% in comparison with elev CO_2), but this effect was not observed at day 14.

Stomatal conductance was lower under elevated CO_2 at day 0 (-42%) and day 7 (-62%) in comparison with ambient CO_2. LL treatment promoted a significant increase at day 7 (amb CO_2 46% – and elev CO_2 - 91%) and at day 14 (amb CO_2 - 37% and elev CO_2 - 49%) in comparison with unshaded treatments (Fig. 5B).

Estimative of leaf respiration during the night (LL CO_2 efflux – Fig. 5C) demonstrates lower rates at ambient (-44%) and elevated CO_2 (-20%) combined with LL in comparison with unshaded treatments at day 7 (Fig. 5C). This decrease was more pronounced in elevated CO_2/LL at day 14 (-70%). At day 14, a strong reduction also occurred under elevated CO_2 (0.55 µmol CO_2 m^2s^1) in comparison with ambient CO_2 (1.02 µmol CO_2 m^2s^{-1}).

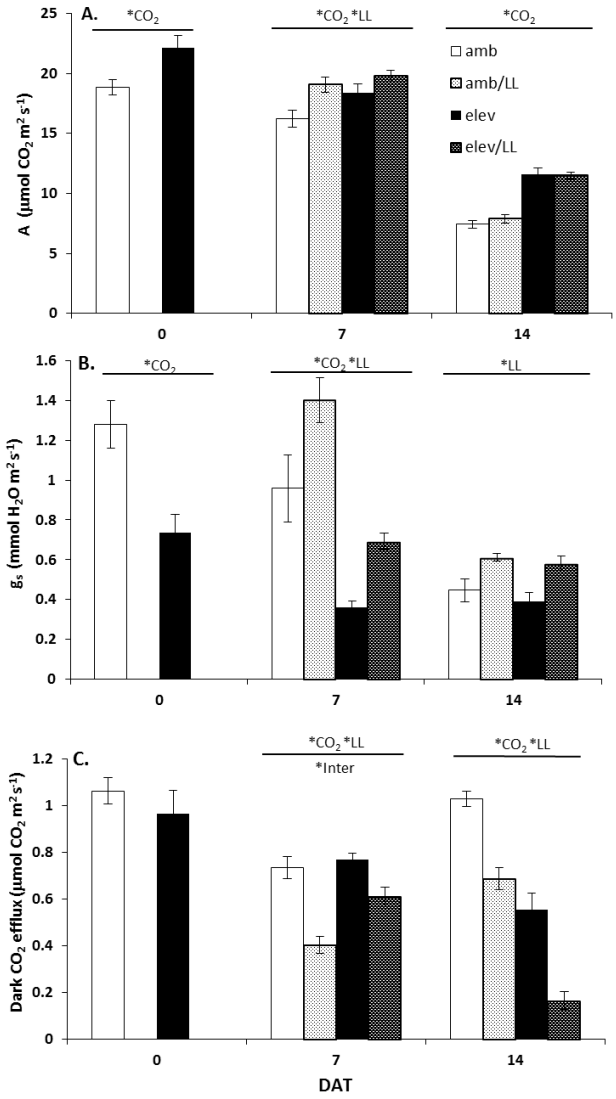

Figure 5. **A.** Photosynthetic assimilation and **B.** Stomatal conductance measured between 10 am and 12 pm for all treatments and **C.** Respiration measured as CO_2 efflux from leaves at 4 am for all treatments among the days after treatment (DAT). Lines above the graph indicate ANOVA Two-Way results: "*CO_2" – significance for CO_2 factor; "*LL" – significance for Light factor; and "*Inter- significance for interaction between CO_2 and Light factors. Bars are means with standard error (n=12).

Non-structural carbohydrates

Elevation of CO_2 induced a strong increase of starch content in leaves sampled at 6 am at day 0 (72%), 7 (96%) and 14 (28%) (Fig. 6). Under LL conditions starch level was abruptly decreased in comparison with unshaded treatments at day 7: amb/LL -98% and elev/LL -95%, and at day 14: amb/LL -65% and elev/LL -32%.

Measurements at 6 pm displayed exactly the same pattern as 6 am leaves. Starch turnover under elevated CO_2 was similar to ambient CO_2 at day 0 and 14 and slightly lower at day 7 (Fig. 6). LL treatments showed a strong effect on starch turnover, with lower turnover in comparison with unshaded treatments especially at day 14 (in average - 60% for both CO_2 treatments).

Sucrose content decreased at 6 am and 6 pm in LL treatments (amb CO_2/LL -60% and elev CO_2/LL -54%) at day 7, and these reductions were less pronounced at day 14. Sucrose turnover was different among treatments only at day 14, with amb/LL displaying the highest value (ca. 5μg) whereas elev/LL did not show any turnover, as well the other treatments (Fig. 6).

Monossacharides glucose and fructose were increased under LL treatments in both times (6 am and pm) and at day 14 this response was intensified under elev CO_2 /LL. Glucose turnover varied from being positive at day 0, negative at day 7 and at day 14 was positive for elevated treatments and negative for ambient treatment. Fructose turnover also showed a wide range of variation among time and between treatments for example at day 7 where turnover was positive under unshaded treatments and negative under LL (Fig.6).

In stems sampled at 6 am and 6 pm, all non-structural carbohydrates (TNC) content decreased in low light treatments at day 7 (Fig. 7). Related to CO_2 effect, only sucrose (30.6 μg.mg DW^{-1}) and starch (15 μg.mg $^{-1}$DW) showed higher content at elevated CO_2, rather than ambient CO_2 (sucrose – 19.2 μg.mg^{-1}DW, starch – 4 μg.mg^{-1}DW). Glucose and fructose displayed a very similar pattern of turnover in stems, with lower rates under elev CO_2 at day 0 and higher rates at day 14 in this same treatment. Sucrose did not present positive turnover under elevated CO_2 in any of the harvesting points. This pattern was very similar to starch turnover in this organ that showed positive turnover only in

amb CO_2 at day 0 in contrast to increasing amount under elevated CO_2. Negative turnover (synthesis or remobilization) for starch was rather higher under LL treatment in comparison to unshaded treatments at day 14 (Fig. 7).

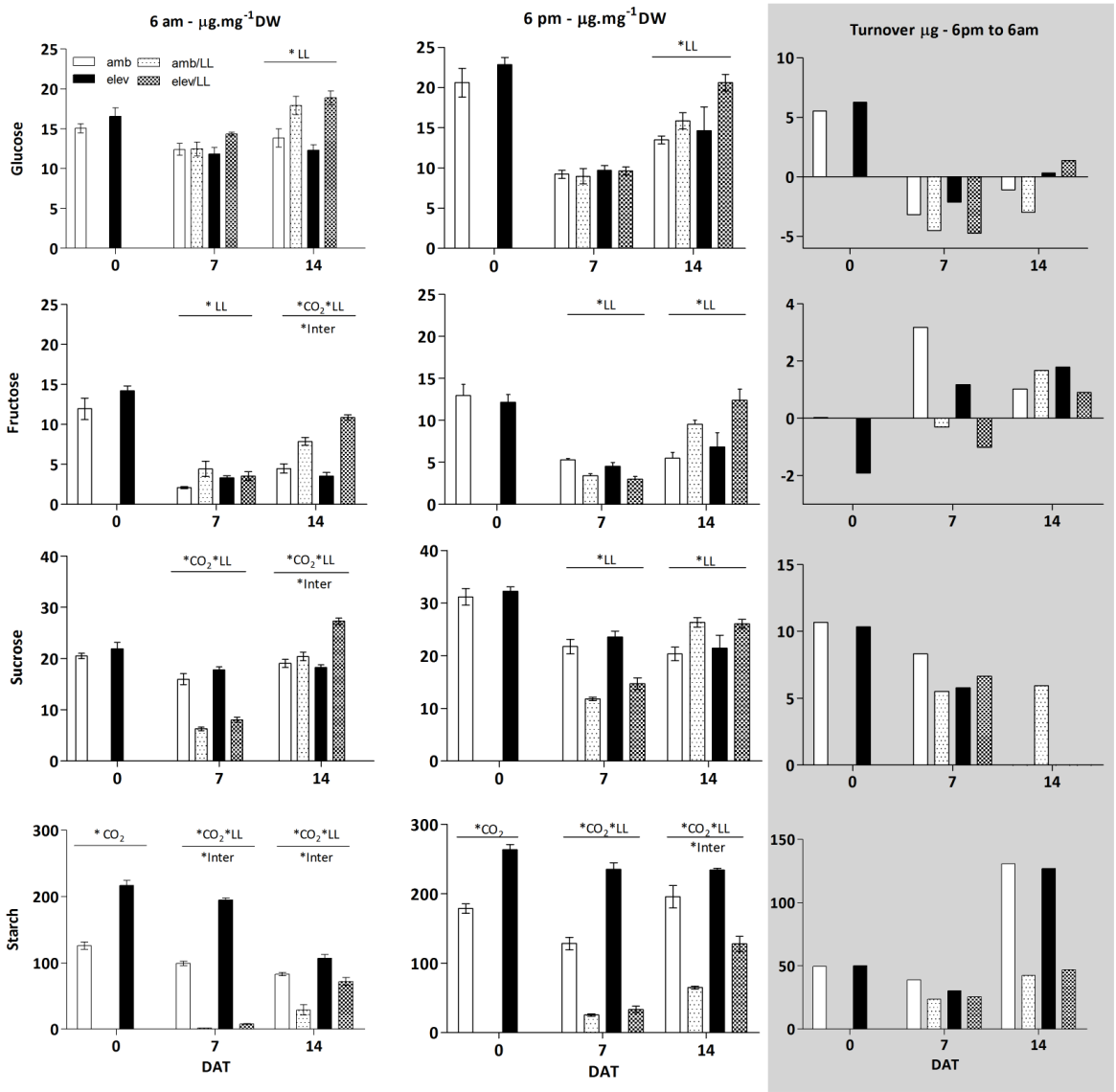

Figure 6. Leaf non-structural carbohydrates (glucose, fructose, sucrose and starch – μg.mg^{-1}DW) measured at 6 am (left), 6 pm (middle) and turnover (right) among days after treatment (DAT). Lines above the graph indicate ANOVA Two-Way result: "*CO_2" – significance for CO_2 factor; "*LL" – significance for Light factor; and "*Inter" – significance for interaction between two factors. Bars are means with standard error (n=6).

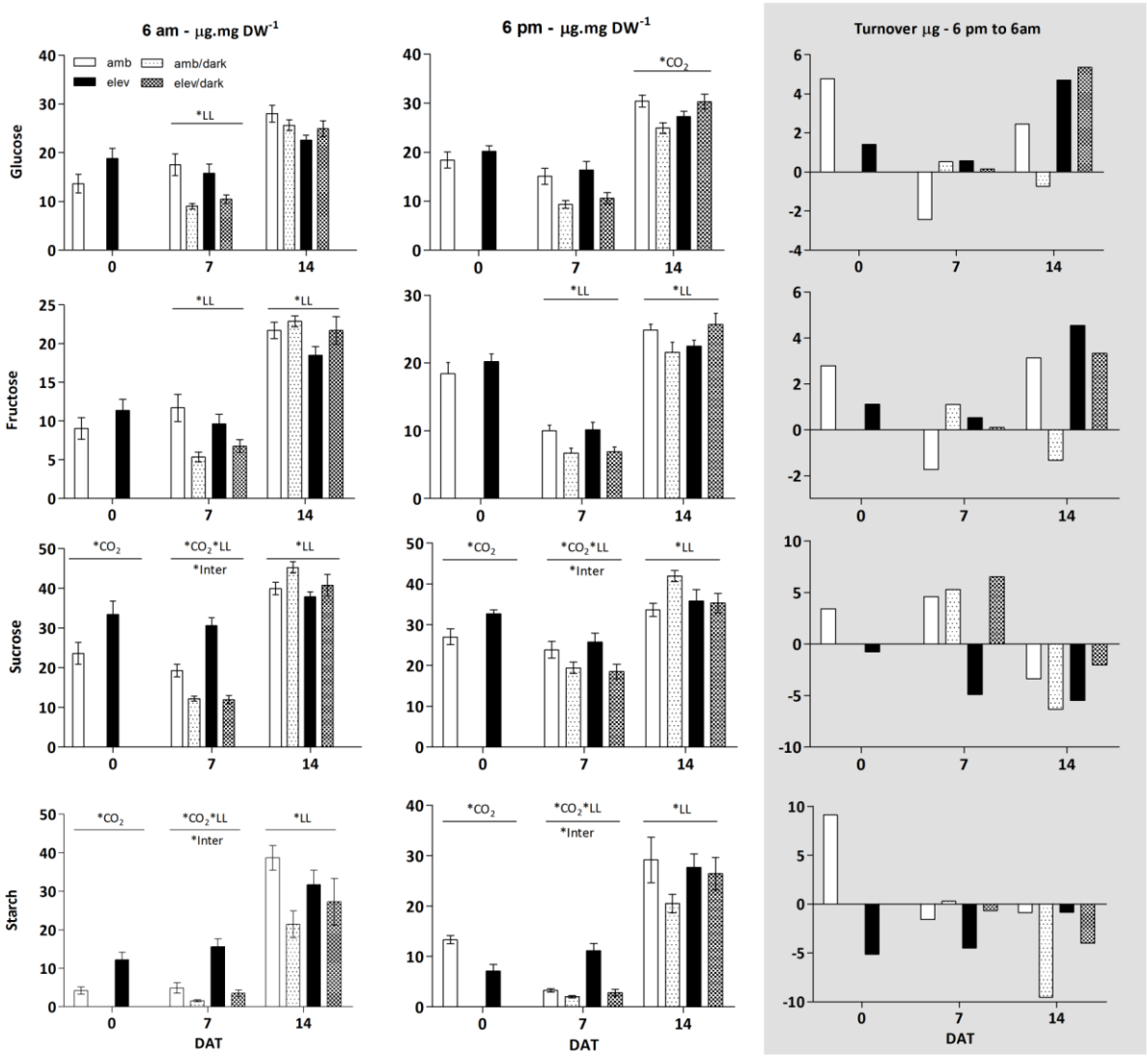

Figure 7. Stem non-structural carbohydrates (glucose, fructose, sucrose and starch µg.mg^{-1}DW) measured at 6 am (left), 6 pm (middle) and turnover (right) among days after treatment (DAT). Lines above the graph indicate ANOVA Two-Way results: "*CO_2" – significance for CO_2 factor; "*LL" – significance for Light factor; and "*Inter" – significance for interaction between CO_2 and Light factors. Bars are means with standard error (n=12).

Similar to stems, roots TNC decreased in both times (6 am and 6 pm) at day 7. Elevated CO_2 increased starch content in LL and unshaded treatments, in comparison with ambient CO_2 conditions (Fig. 8). Glucose was degraded/mobilized in all treatments at days 7 and 14, except for ambient CO_2 at day 7, which was increasing levels of this sugar at night. A similar pattern occurred with fructose levels. At day 0, sucrose and starch increased levels during nighttime and considering starch, this difference was almost three times higher than

in ambient CO_2 treatment. Elevated CO_2 displayed positive starch turnover at day 14 while all other treatments where increasing starch level during the night in roots (Fig. 8).

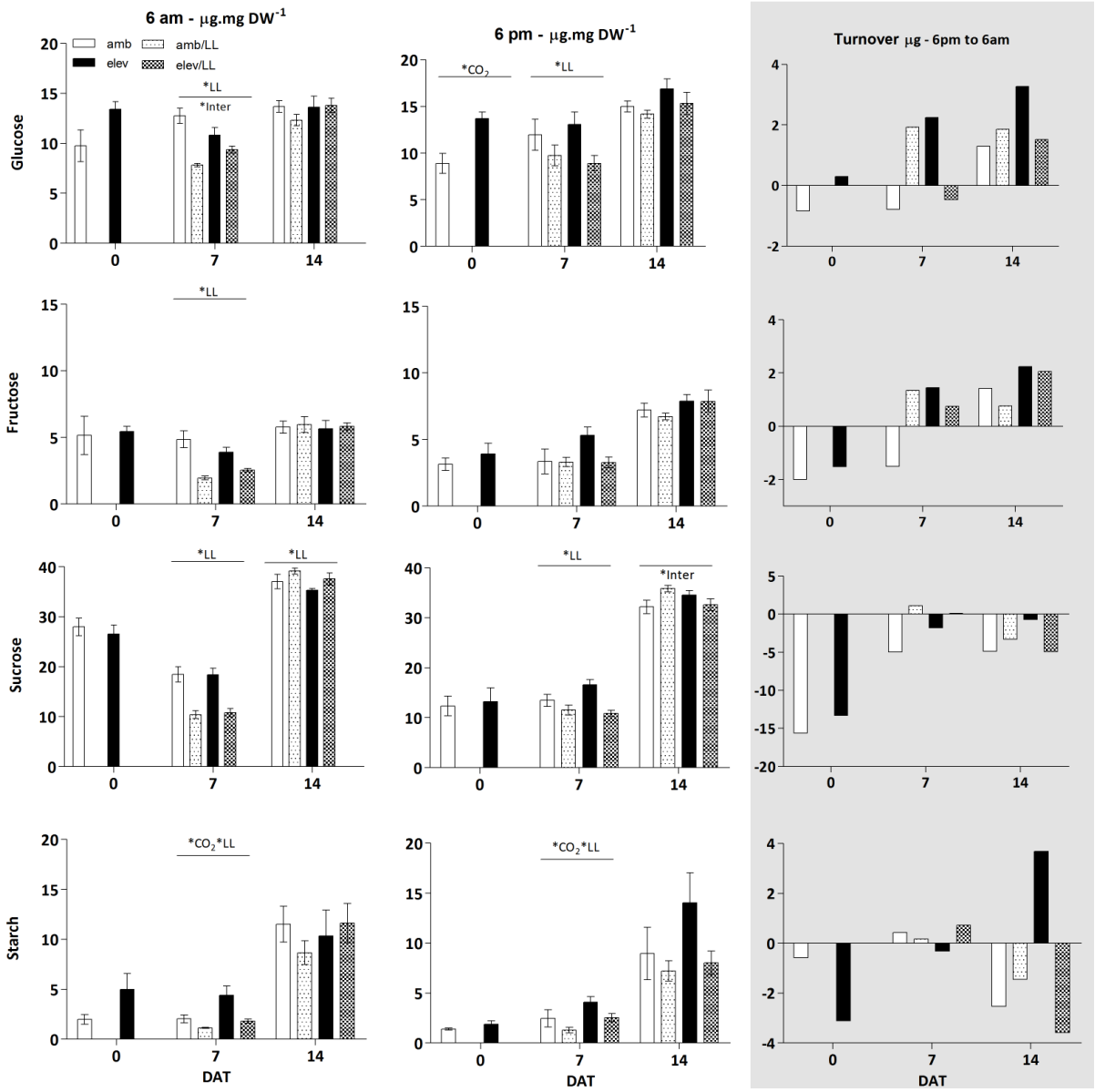

Figure 8. Root non-structural carbohydrates (glucose, fructose, sucrose and starch – µg.mg^{-1}DW) measured at 6 am (left), 6 pm (middle) and turnover (right) among days after treatment (DAT). Lines above the graph indicate ANOVA Two-Way result: "*CO_2" – significance for CO_2 factor; "*LL" – significance for Light factor; and "*Inter" – significance for interaction between two factors. Bars are means with standard error (n=12).

Summarizing, under elevated CO_2, starch doubles in all organs at 6 am and 6 pm, despite of the fact that it is much higher (ca. 10 fold) in leaves than in stem and root. Lower light decreased the accumulation of starch in leaves and this phenomenon was apparently independent of the response of the leaves to elevated CO_2. The LL treatment dramatically decreased starch accumulation in leaves, leading starch concentration to near zero in leaves (Fig. 6) It is noticeable that glucose and fructose vary independently from the other two transporting (sucrose) and storage (starch) compounds of the plant.

Chlorophyll, nitrate, malate, and glucose-6-phophate

Despite of CO_2 concentration, all seven measured metabolites were affected by LL treatment in relation to unshaded treatment (Table 1). In leaves, there was an increase in chlorophyll *b* and proteins at 6 am, and an increase in amino acids and glucose-6-phosphate (G6P) at 6 pm. Chlorophyll *a*, malate and nitrate were increased in both day times (6 am and 6 pm). In roots, decreases in malate and protein occurred at 6 pm whereas increases in G6P at 6 am and nitrate at 6 pm were observed.

Except for nitrate in the roots, all effects of elevated CO_2 in both organs occurred at 6 pm in comparison with ambient CO_2. In leaves, chlorophyll *a* decreased under elevated CO_2 in comparison with ambient and elevated CO_2/LL decreased in relation to ambient CO_2/LL. Chlorophyll *b* showed exactly the opposite pattern, increasing under elevated CO_2 in comparison with ambient CO_2 treatments. Protein content was reduced and G6P increased under elevated CO_2 treatments. Nitrate increased under elevated CO_2 in unshaded conditions, whereas under LL treatment elevated CO_2/LL decreased this compound in comparison with ambient CO_2/LL. In roots, there was an extreme reduction in amino acids and nitrate under elevated CO_2 treatments (Table1).

Table 1. Metabolites measured at DAT 14 (6 am and 6 pm) in leaves and roots. Chlorophyll *a*, *b* (mg.g^{-1}FW), malate (µmol.g^{-1}FW), glucose–6-phosphate (nmol.g^{-1}FW), protein (mg.g^{-1}FW), amino acids (µmol.g^{-1}FW) and nitrate (µmol.g^{-1}FW). ANOVA Two-Way analysis for "CO_2", "LL" and interaction between CO_2 and Light factors are described with P value and adjusted R^2.

			Treatments				Factor			
		Time	amb	amb/LL	elev	elev/LL	CO_2	Light	CO_2*Light	R^2_{adj} (%)
Chl a	**Leaves**	**6 am**	1.6 ± 0.09	2.99 ± 0.01	1.4 ± 0.06	2.7 ± 0.30	0.173	***0.000***	0.859	84.65
		6 pm	1.6 ± 0.09	3.02 ± 0.10	1.3 ± 0.09	2.6 ± 0.20	0.043	***0.000***	0.639	90.12
Chl b	**Leaves**	**6 am**	0.1 ± 0.00	0.00 ± 0.01	0.08 ± 0.02	0.1 ± 0.02	0.143	***0.043***	***0.013***	58.63
		6 pm	0.1 ± 0.01	0.11 ± 0.04	0.18 ± 0.01	0.2 ± 0.03	***0.046***	0.836	0.587	21.23
Malate	**Leaves**	**6 am**	100.6 ± 4.42	141.7 ± 2.54	92.6 ± 2.40	135.0 ± 3.78	0.064	***0.000***	0.853	93.30
		6 pm	71.2 ± 2.28	150.8 ± 7.97	107 ± 3.43	126.2 ± 6.6	0.318	***0.000***	***0.001***	90.60
	Roots	**6 am**	141.0 ± 11.2	119 ± 4.0	109 ± 5.5	127 ± 13	0.240	0.856	***0.058***	24.42
		6 pm	127.0 ± 1.79	126 ± 11.2	140 ± 4.7	90 ± 11	0.195	***0.015***	***0.020***	60.46
Glucose6P	**Leaves**	**6 am**	1347 ± 238	1188 ± 81.7	1092 ± 22	1643 ± 96	0.481	0.184	***0.031***	37.25
		6 pm	1919 ± 216	2319 ± 109	2325 ± 73	3114 ± 95	***0.001***	***0.002***	0.162	80.08
	Roots	**6 am**	1354 ± 114	1513 ± 120	1119 ± 62	1428 ± 2.37	0.109	***0.030***	0.420	41.92
		6 pm	1102 ± 98.5	1419 ± 7.05	1444 ± 34	843 ± 66.2	0.096	0.051	***0.000***	84.69
Protein	**Leaves**	**6 am**	15.7 ± 2.66	23.9 ± 3.35	14.0 ± 1.91	23.9 ± 0.40	0.737	***0.005***	0.736	52.06
		6 pm	26.5 ± 1.29	31.1 ± 3.83	20.3 ± 0.79	24.3 ± 2.19	***0.023***	0.101	0.895	43.07
	Roots	**6 am**	7.7 ± 0.50	6.6 ± 0.08	6.7 ± 0.12	6.4 ± 0.72	0.220	0.150	0.350	17.22
		6 pm	7.0 ± 0.31	6.1 ± 0.37	7.2 ± 0.51	6.1 ± 0.26	0.932	***0.026***	0.734	29.34
Amino acids	**Leaves**	**6 am**	108.7 ± 4.99	91.7 ± 5.89	108.9 ± 9.78	107.0 ± 5.14	0.284	0.197	0.294	12.44
		6 pm	67.8 ± 4.07	79.5 ± 5.37	55.4 ± 3.26	104.7 ± 4.59	0.182	***0.000***	***0.003***	85.66
	Roots	**6 am**	65.3 ± 17.7	68.1 ± 8.37	67.3 ± 5.79	57.2 ± 7.04	0.695	0.745	0.566	0.00
		6 pm	64.0 ± 20.4	85.7 ± 11.6	34.8 ± 1.21	27.2 ± 3.78	***0.006***	0.568	0.253	53.17
NO_3^-	**Leaves**	**6 am**	10.2 ± 0.78	59.0 ± 2.05	22.0 ± 5.81	33.6 ± 1.36	0.065	***0.000***	***0.000***	91.99
		6 pm	8.8 ± 3.32	74.2 ± 1.47	30.2 ± 4.97	26.3 ± 6.87	***0.020***	***0.000***	***0.000***	90.62
	Roots	**6 am**	83.7 ± 3.49	158.3 ± 19.2	96.6 ± 5.15	72.7 ± 11.7	***0.014***	0.062	***0.003***	72.70
		6 pm	66.9 ± 10.1	104.3 ± 7.6	69.4 ± 5.08	88.5 ± 13.5	0.506	***0.019***	0.364	39.18

Biomass

Plant dry weight increased under elevated CO_2 in all organs, mainly at days 0 and 14. The average increase in leaves was of 30%, in stems of 24% and in roots of 58% (Fig. 9). At day 7, LL treatment decreased the biomass of leaves, stem, roots and total biomass, despite of CO_2 concentration. However, at day 14, biomass was also decreased in all organs, but displaying less pronounced effect of elevated CO_2 treatments in comparison to ambient CO_2 plants (e.g total biomass going from 7.4 to 4.7 g under elevated CO_2 and from 5.8 to 3.4 g under ambient CO_2).

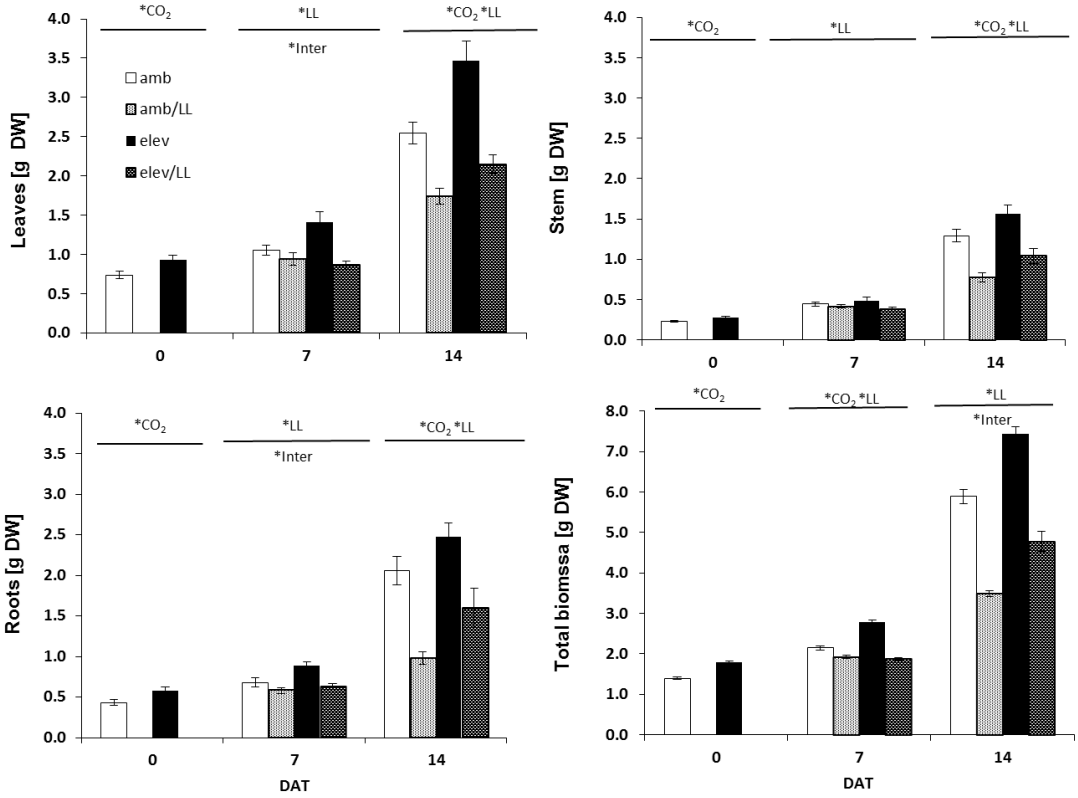

Figure 9. Dry weight in different organs (leaves, stems, roots) and total biomass among days after treatment (DAT). The lines above each graph indicate statistical differences obtained from ANOVA Two-Way analyses: "*CO_2" – significance for CO_2 factor; "*LL" – significance for Light factor; and "*Inter" – significance for interaction between CO_2 and Light factors. Bars are means with standard error (n=12).

Correlations

Considering all treatments, negative correlations were observed between starch in leaves and three nitrogen containing compounds: leaf protein (*P*=0.059), nitrate (*P*=0.000) and chlorophyll a (*P*=0.000) (Table 2). Assimilation was negatively correlated with leaf protein (*P*=0.030). Total biomass was positively correlated with leaf starch (*P*=0.001).

Table 2. Pearson correlation considering all treatments between nitrogen compounds measured at 6 pm: leaf protein (mg.g^{-1}FW), amino acids (μg.g^{-1}FW), nitrate (μg.g^{-1}FW) and chlorophyll *a* (mg.g^{-1}FW) and total biomass (g); and measurements of: Leaf starch (mg.g^{-1}DW), Dark night respiration (μmol CO_2 m^2s^{-1}) and Assimilation (μmol CO_2 m^2s^{-1}). P-value in parenthesis (n=6) and bold letters indicate significance *P*<0.05.

	Leaf Starch		Dark night resp		Assim	
Leaf protein	-0.559	*(0.059)*	-0.202	*(0.529)*	**-0.625**	***(0.030)***
NO_3^-	**-0.862**	***(0.000)***	0.089	*(0.784)*	-0.315	*(0.319)*
Amino acids	0.108	*(0.739)*	-0.318	*(0.314)*	-0.454	*(0.139)*
Chllorophyll	**-0.850**	***(0.000)***	0.303	*(0.338)*	-0.256	*(0.421)*
Total Biomass	0.835	***(0.001)***	-0.108	*(0.738)*	0.385	*(0.216)*

Discussion

This study show that reduced light intensity decreased starch accumulation in leaves at the end of the light and the dark period, in average by 80%. Interestingly, leaf starch turnover was unaffected by shaded treatment at day 7 but only at day 14, which could explain why biomass accumulation was unaffected in these plants at day 7 and impaired only at day 14. Elevated CO_2 combined with low light strongly decreased leaf respiration, increased starch storage in leaves and stems and rescue 50% of biomass in comparison to ambient CO_2/LL. This is probably related to the higher photosynthesis levels and as a consequence to the higher leaf sucrose exportation rates observed during the day in elevated CO_2 plants. These results indicate that under lower C availability this species re-establish starch reserves in leaves at the expense of growth and that elevated CO_2 does not influence this response but strongly decreased leaf respiration and directed its carbohydrates to growth. Figure 9 summarizes the findings of this work.

S. reticulata growing under C avaiability

Starch levels in leaves of Senna reticulata during the first hour of the day never remained lower than 10% of dry weight, which represents 80% of maximum starch amount found at 6 pm (Fig. 6). The persistence of high starch levels during nighttime indicates that the competition between the two main sink strengths - growth and reserve - for this species is rather high, and could be explained by the environmental pressure in which this plant occurs. Under these extreme conditions the use and allocation of carbohydrates is tightly connected to the need of carbon reserves in order to tolerate and adjust to water stresses (Crawford, 1992; Ferreira et al., 2009; Arenque et al., 2014; see Chapter 2).

Although higher photosynthetic assimilation rates has been observed (day 7), low light treatments decreased TNC carbohydrates levels in all organs, at 6 am and 6 pm (Figs. 5A, 6, 7 and 8), this response being even more pronounced for starch accumulation, especially in leaves. These results do not corroborate findings in literature that have shown increase in the rates of starch synthesis under shorter photoperiods (Matt et al., 2001; Gibon et al., 2004) and under low light environments (Lichtenthaler et al., 1981). Moreover,

since we fixed harvesting times at 6 pm (without considering the end of the day for each treatment – e.g. 12 pm for day 7 of harvest), it was not possible to assess whether shaded plants synthesized the same amount of starch than unshaded plants at the end of the light period. However, this is unlikely the case as the carbohydrate adjustment in this species seems not going towards an increase of starch synthesis during the day time but more towards a decrease in its night usage by reducing leaf respiration, thus leading to lower starch turnover as described for plants grown under shorter photoperiods (Gibon et al., 2009).

Decreases in nitrogen compounds usually decrease respiration rates in a near-linear way (van der Werf et al., 1992). Therefore, the amount of starch that is required to keep respiration during the night is lower, probably being strictly related with decreases in leaf starch turnover found under low light treatments (Fig.6). However, we have found an increase of all nitrogen compounds under shaded conditions in leaves of *S. reticulata* (Table 1). Considering that *S. reticulata* is highly adapted to high light intensities, it is possible that chlorophyll content (as well chloroplast number and density of thylakoids; Lichtenthaler et al., 1981) have increased at low light conditions is associated to an increase light capture capacity that is required to sustain high relative growth rates inherent to this species (Parolin, 2001). Therefore, it is unlikely that the observed down regulation of leaf dark respiration would be correlated with protein adjustments in this experiment (Table 2).

Sulpice et al. (2014) did not find any change in protein contents under shorter photoperiods whereas Gibon et al. (2009) showed a decrease in protein contents. The later authors discuss the possibility that although C availability affects protein synthesis, it is possible that the light-signaling/C-signaling interaction also plays a significant role in protein reduction (Nozue & Maloof, 2006). Thus, since in our experiment photoperiod was not affected, only the photosynthetic active light availability, and that high protein amount did not promote higher respiration rates at day 14 either (Table 1 and Fig. 5C), it is reasonable to assume that other mechanisms may be involved in the regulation of protein synthesis and also respiration rates in this species.

Approximately half of whole-plant respiratory CO_2 release takes place in leaves (Ayub et al., 2014) so variations in leaf respiration are extremely important for the functioning of photosynthetic tissues (discussed above) but also to the overall carbon economy of individual leaves and whole plants (Atkin et al., 2013). Despite the fact that

very low amount of starch was observed in leaves on day 7 at 6 pm (Fig.6), decreased leaf respiration (Fig. 5C) observed at the same time was sufficiently great to avoid complete depletion of its main C reserve.

Degradation of nighttime starch reserves was probably used for growth as the total biomass was only slightly affected by low light treatment on day 7 (Figs. 6 and 9). However, on day 14 a large reduction in biomass was observed under low light treatments and at the same time an increase in starch reserves in leaves and stems (Figs. 6 and 7). These results indicate that in response to decreased C availability this species display an "acclimatory" response (Stitt & Smith, 2007) firstly decreasing leaf respiration and spending its reserve for growth maintenance and secondly increasing leaf starch reserves at expenses of growth, corroborating findings of Wiley et al. (2013) whom conclude that the prioritization of reserves can be considered as an important adaption to increasing carbon stress. These results highlight the capacity of *S. reticulata* to cope with starvation periods, what likely implicates a high ability of this species to maintain a balance between reserve and growth investments probably strictly related to the disturbed environment of this species occurrence (Fig. 10A).

Elevated CO_2 combined with low light

Plant response to elevated CO_2 can help to explain the balance between non-structural reserves and growth, most commonly named sink strength relationship that determines whether a plant is able to invest in structural growth or not. Furthermore, CO_2 combined with some environmental stresses such as low nutrients, low light, drought (Leakey et al., 2006) and flooding (Arenque et al., 2014) are likely to enhance the CO_2 positive effects. Thus, elevated CO_2 promotes some direct and indirect physiological responses that allow plants to face better some adverse situations, or, inclusively, to be more responsive to elevated CO_2 (Würth et al., 1998). In this study, elevated CO_2 showed a "rescue" capacity from low light stressing effects and this response was strictly linked to leaf respiration and carbohydrates adjustments.

The starch content of Senna reticulata increased whereas a tendency of decrease in soluble sugars in all organs was found (Figs. 6, 7 and 8, Arenque et al., 2014). Azcon Bieto & Osmond (1983) and Hrubec et al. (1985) showed that increased glucose and fructose levels

in leaves of plants grown under elevated CO_2, were well correlated with higher respiration rates. In our study, the export of soluble sugars from leaves to stems seemed to promoted under elevated CO_2 in comparison with ambient CO_2 plants, because a negative correlation between soluble sugars in the leaves and stems along a diurnal time course was only apparent under high $C0_2$ treatment and not ambient $C0_2$ (Fig. 3C; Grandis, 2010; Arenque, 2010). These findings corroborate observations made in *Ricinus communis* during nighttime (Grimmer & Kommer, 1999; Long et al., 2004). Thus, the carbohydrate adjustment in leaves of plants grown under elevated CO_2, e.g. the ability to decrease leaf soluble sugars amount by increasing starch reserve and exporting the soluble sugars from leaves, might help to explain the decreased leaf respiration observed during nighttime in both Experiment 1 and Experiment 2 (Figs. 6 and 5C).

Decreased soluble protein content in leaves and amino acids in leaves and roots of plants grown under elevated CO_2 (Table 1) corroborates literature that shows consistent decrease in protein content (increase in nitrogen-use-efficiency – NUE) and a following increase in C:N ratio in experiments under elevated CO_2 (Saxe et al., 1998; Ineson et al., 1998; Leakey et al., 2009). Lower nitrogen contents are also able to decrease the construction costs (the amount of C required to produce a unit of biomass) (Gibon et al., 2009), since protein synthesis is responsible for 20–70% of respiration costs in plant tissues (Penning de Vries, 1975; Hachiya et al., 2007).

Negative correlations between starch leaf and nitrogen compounds such as leaf protein, nitrate and chlorophyll, were observed at day 14 (Table 2), indicating that, regardless the CO_2 concentration (amb or elev) and light conditions (LL or unshaded) higher amounts of starch in leaves were found to be associated with low levels of nitrogen containing compounds. Sulpice et al. (2009) hypothesized that starch turnover and C allocation would play a central role in the network that coordinates metabolism with growth in 94 Arabidopsis thaliana accessions. They found that starch and total protein (to a lesser extent) integrate the metabolic status of the whole plant. Additionally, they noted that the regulatory network of starch and protein changes contributes to the regulation of biomass. In our study, we found that starch levels were highly correlated to biomass (Table 2).

Under elevated CO_2, increases in growth and TNC would alter the availability of respiratory substrates and/or the ATP consumption (Lambers et al., 2005; Watanabe et al.,

2014) but in this study, *S. reticulata* was shown to have decreased amounts of soluble sugars under elevated CO_2 in leaves concomitantly with increasing starch synthesis and sucrose export to stems (Fig. 3C). Additionally, ca. 20% lower protein content was found in leaves at 6 pm in comparison to ambient CO_2 plants (Table 1). Thus, changes in the levels of nitrogen compounds, driven by carbohydrates adjustments occurred under elevated CO_2, might have played a role in respiration and growth rates.

These results suggest that, linked to a reduction in leaf respiration, plants grown under elevated CO_2/LL partition preferentially leaf starch and also recently fixed photoassimilates (sucrose during the day) to growth, even in a low C availability in comparison to unshaded plants. Thus, it is likely that the modification of the C status of the plant, enhanced under elevated CO_2 due to higher photosynthesis, lead to a modification of ratio of the allocation of the resources towards growth and not storage. These findings corroborate Ayub et al. (2014), a recent study exploring the relationship between leaf respiration of soybean plants and growth in different CO_2 concentrations (290, 400 and 700 μmols). Their results showed that a greater proportion of the carbon fixed by leaf photosynthesis was released by leaf respiration in plants grown under low CO_2 than under current/future CO_2 (Fig. 10B).

In this regard, it is possible that management of carbon balance by reducing leaf respiration and redirection of carbohydrates to growth could be considered a key mechanism that contribute to “rescue/buffer” responses when plants are subjected to environmental stresses combined with elevated CO_2.

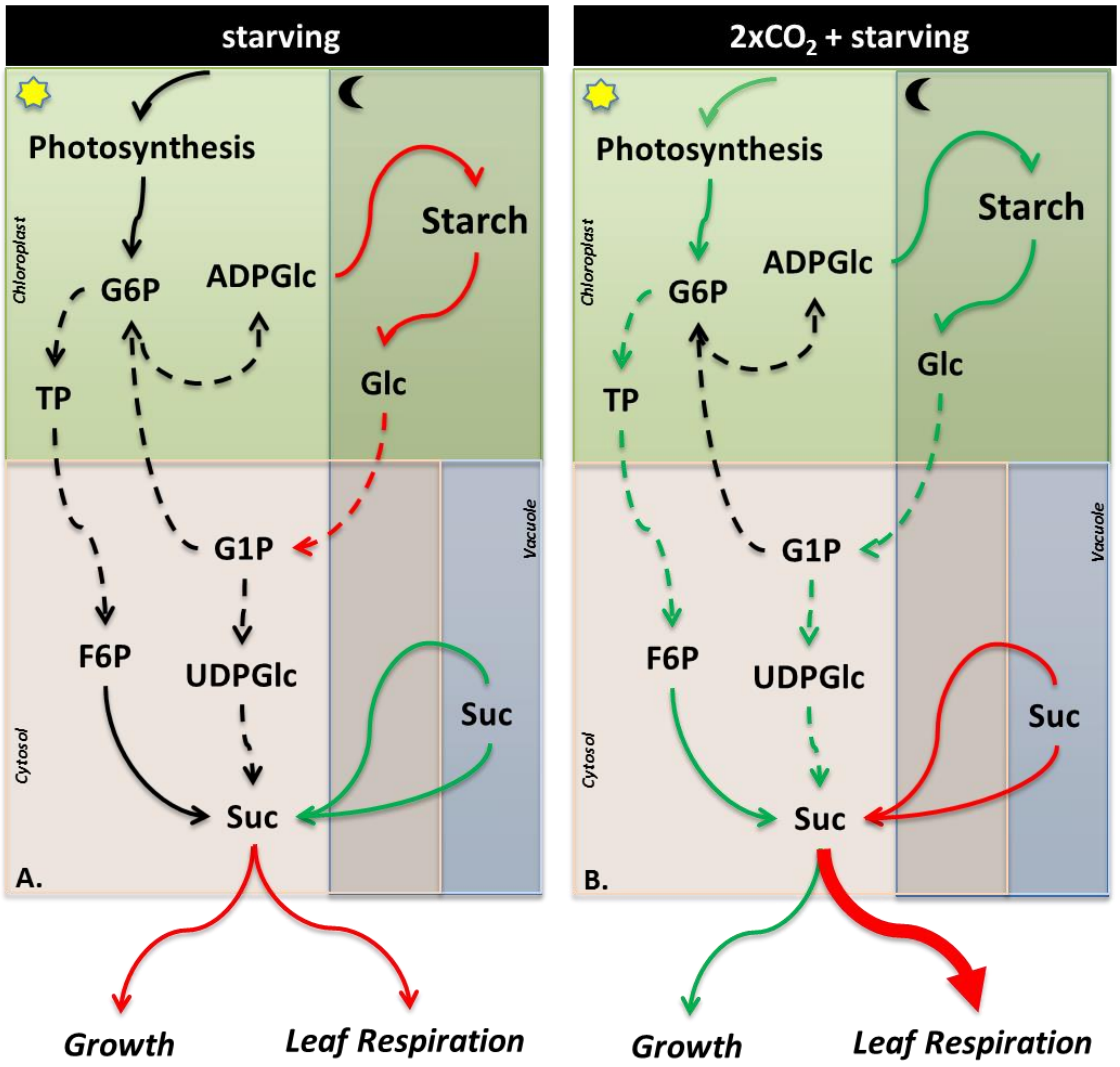

Figure 10. Main responses of *Senna reticulata* leaves grown under low light and elevated CO_2 for 14 days. Continuous lines indicate measured variables and dashed lines indicate non-measured variables. Red color highlights reduction whereas green color indicates increases **A.** Low light (starving) in comparison to unshaded plants: Low light promoted reduced C availability by reducing starch amount and starch turnover, culminating in lower growth. Leaf respiration was decreased **B.** Low light (starving) combined with elevated CO_2 in comparison to shaded plants: Elevated CO_2 decreased leaf respiration in a higher extent, reduced sucrose turnover and increased photosynthesis, glucose-6-phopshate (G6P), starch reserves, starch turnover and growth. Triose-phosphate (TP), fructose-6-phosphate (F6P), ADP-glucose (ADPGlc), glucose-1-phosphate (G1P), UDP-glucose (UDPGlc) and Sucrose (Suc).

Conclusions

Reduced light availability led *S. reticulata* to starvation, in a first moment decreasing respiration, reducing the levels of starch to very low amounts and keeping growth. After 14 days, plants under low light were able to increase starch levels in leaves at expenses of growth, reinforcing the relevance of starch storage in this species. When elevated CO_2 was combined with low light, plants have apparently remodelled metabolism by strongly decreasing respiration but increasing photosynthesis in comparison to amb CO_2/LL. These findings demonstrate that under lower C availability this species displays a great ability to re-establish starch reserves in leaves even at expenses of growth, higlighting the fact that this may be a result of the evolution in an environmental tension zone in Amazon floodplains. Interestingly, elevated CO_2 allowed the plants to decrease leaf respiration and redirect the gained carbohydrates to growth.

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CAPÍTULO 2

Papel dos carboidratos não estruturais na tolerância a seca e ao alagamento da árvore amazônica *Senna reticulata*

(Artigo a ser submetido ao periódico “New Phytologist”)

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Role of non-structural carbohydrates in drought and flood tolerance of an Amazonian tree, *Senna reticulata*

Abstract

Morphological and physiological traits characteristic of floodplain species have been described in the literature, however, one of the main features is the carbohydrate metabolism, which has been poorly explored. Constantly, the balance between providing sugars for fermentation process under flooding period and increase carbohydrate production to avoid water loss in drought is faced. We studied *Senna reticulata* (Leguminosae) seedlings grown in 10 L pots, well-watered for 30 days, and submitted to drought and waterlogging treatments for the following 60 days. After this period the treatments were inverted – waterlogging to drought, and drought to waterlogging - for a period of 20 days. Harvests were performed every 10 days (Day 0 to 60) and photosynthesis, leaf respiration, leaf water potential, non-structural carbohydrates (glucose, fructose, sucrose and starch) and biomass components were measured during the day (10 am) and night (5 am). Results showed that soluble sugars tended to be higher in leaves under drought (day 10) and waterlogging treatments (among all days) when compared to control. After 60 days of treatment, assimilation rates in flooded plants were equal or higher than in control plants, while drought-stressed plants displayed a decrease (approx. 30%) in assimilation. Concomitantly, this same treatment showed a decrease in leaf water potential (ca. 50%) compared to control and flooded plants. Biomass was significantly different with flooded and droughted plants presenting lower (approx. 35%) biomass than control plants at day 60. *S. reticulata* is able to cope with both waterlogging and drought stress using different physiological mechanisms, both tightly connected to sugar metabolism. Starch stored in leaves and sucrose levels in roots are important to maintain metabolism under waterlogging, whereas high levels of soluble sugars also seems to play an important role under drought, especially in earlier stages. In our study, drought was considered as important as waterlogging in relation to growth limitation at seedling stage but the tolerance mechanisms regarding carbohydrate metabolism, hydraulic properties and gas exchange were quite different under distinct water stresses for *S. reticulata.* After

rewatering, droughted plants displayed a very fast ability to recover that must be crucial for establishment of *S. reticulata* seedlings during the dry season of Central Amazonian floodplains.

Key-words: floodplain, starch, storage, trees, várzea.

Introduction

Amazonian floodplains are subjected to an annual monomodal flooding pulse that is determined by seasonal variation in the rain pattern providing two well-marked seasons: Wet (December to April) and Dry (June to October) (Ribeiro & Adis, 1989). Forest communities that occur in the river basin are subject to these two seasons (Prance, 1979), and those on the floodplains experience prolonged flooding. Since in this environment plants are waterlogged from 50 to 270 days every year (Junk et al., 1989), the predictability of the flooding period is recognized as a key driver for a range of adaptive responses that allow species to cope with different water availability during the year. Distinct to temperate wetlands, the aquatic phase occurs during a period where temperature and light availability are optimal for plant growth and development, requiring adaptations at different levels of physiological, phenological and structural traits (Parolin et al., 2004; Worbes, 1989, 1997; Parolin et al., 2002; Schöngart et al., 2002).

The flood pulse causes changes in the availability of oxygen levels, promoting hypoxic and often anoxic soil conditions (Amstrong et al., 1991, Jackson & Armstrong, 1999), interfering with root respiration and inducing anaerobic processes (Sioli, 1954; Crawford, 1983; Kozlowski, 1984; Ernst, 1990; Larcher, 1994). Increased anaerobic respiration results in a decrease of carbohydrate reserves. However, many plant species can accumulate sugars in their tissues during the dry season displaying an important metabolic adaptation to anoxia conditions during the flooding season (Albrecht et al., 2004; Piedade et al., 2009). Several authors have reported that the tolerance of plants to anoxia is proportional to the availability of sugars in their tissues (Su et al., 1998; Crawford, 1992; Schlüter & Crawford, 2001). In seedlings of the Amazonian tree *Himatanthus sucuuba* subjected to flooding combined with dark, starch levels decreased concomitantly with the increase of soluble sugars, indicating a rapid mobilization of sugars related to use under increased anaerobic respiration (Ferreira et al., 2009). However, in seedlings of the weed *Cyperus rotundus,* the main reserve found in roots related to flooding tolerance were soluble sugars (mainly sucrose) instead starch (Peña- Fronteras et al., 2009).

The timing of seasonal carbohydrate reserve accumulation and depletion may be expected to differ between species from habitats with different flooding regimes. In fact,

Scarano et al. (1994) quantified the content of glucose and starch in young roots of 18 tree species typical of lowland region. The study was conducted during the peak of the dry season and high level of sugars was observed only in part of the species. Regarding the fact that C reserves are indeed important for survival in flooded trees, this finding suggests that accumulation of carbohydrates may also occur during the rainy season for later use in the dry season, depending on the strategy of each species, as different levels of adaptations result in tree zonation along the gradient flooding and sedimentation (Wittmann et al., 2002).

Species that occur at higher sites of riverbank are subjected to shorter times of flooding period and longer times of dry periods in a year time, compared with plants living at lower sites. During the dry season, river water levels drop concomitantly with the lower level of precipitation (between September and November – Parolin, 2001b). Drought has been considered one of the most important forces that drive species distribution in other tropical floodplains (Lopez & Kursar, 2007) and tropical forests (Engelbrecht et al., 2007, Curran et al., 2013). In particular, the seedling stage is considered the critical phase of the plant life cycle that affects survival rates (Kelly & Purvis, 1993; Poorter & Markesteijn, 2008). Moreover, seedlings are more sensitive to drought in comparison to adult plants, likely related to their shallow roots that can limit water uptake (Tyree et al., 2003). Drought resistance of seedlings is therefore thought to be very relevant for rainforest community composition (Engelbrecht et al., 2007), especially in Amazonian floodplains where germination and seedling establishment mostly after floodwaters recede, at the start of the dry season. Information about how dry periods affect Amazonian species is thus highly relevant but still very incomplete (Parolin, 2001b; Oliveira et al., 2005; Parolin et al., 2010)

Importantly, in many cases flooding can be thought of as a “physiological drought” for many species. One of the main question addressed in flooding experiments with Amazonian species is whether phenological and some physiological effects are observed in response to flooding predictability or is a function of water stress caused by restricted water uptake under flooding period (Parolin et al., 2005). Studies have reported that even in well-adapted plants, adjustments in stomatal conductance, carbon assimilation, leaf water potential and hydraulic properties (e.g. increases in hydraulic safety) during flooding periods play an important role in avoiding water losses and keeping growth maintenance (Zotz et al., 1997; Parolin et al., 2005).

The dry and wet seasons represent strongly contrasting conditions for Amazonian floodplain species, and interactions between drought and flooding stresses may exist due to impacts of the preceding period on the physiological status of the plant, or to common signaling pathways. Signaling mechanisms of water deficit constitute a complex network, interconnected at many levels and the known "cross resistance" to different stresses (Knight & Knight, 2001; Chaves et al., 2003) may play an important role in drought and flood tolerance of Amazonian floodplain species. Interestingly, it has been proposed that floodplains have been refuges for upland species during previous times of more frequent and prolonged drought (Baraloto et al., 2007) so that it is reasonable to expect to find traits that enhance survival under drought conditions, and particularly so if those traits also help cope with flooding events.

One of the main species already explored about physiological responses under waterlogging and drought conditions is *Senna reticulata* (Leguminosae). In a broader approach, including other six species, *S. reticulata* was found to be one of the most flooding tolerant but also displayed low survival rates when subjected to prolonged drought period (Parolin, 2001b). In this study, we aimed at understanding the role of the carbohydrate metabolism in the tolerance of *Senna reticulata* to drought and flooding conditions, addressing three main questions: 1) Do carbohydrates adjustments play a role in drought tolerance in this species? 2) Can flooding be regarded a "physiological drought" in terms of physiological and metabolic adjustments? 3) Is *Senna reticulata* able to recover from changes promoted by previous water regime?

General Purpose

To investigate the role of non-structural carbohydrates in the tolerance of *Senna reticulata* seedlings of drought and waterlogging.

Aims

1) To investigate whether drought promotes an increase in the TNC content in different tissues of this species, contributing to osmoregulation.
2) To assess if waterlogging resembles a physiological drought condition for *S. reticulata* in terms of the photosynthetic process and carbohydrates adjustments.
3) To evaluate whether starch stored in leaves and stems of this species can be used as a substrate for metabolic processes during waterlogging.
4) To investigate whether drought promotes a greater reduction in growth in comparison to waterlogging.
5) To assess whether physiological and metabolic changes promoted by an adverse condition (drought or waterlogging) affect tolerance under a subsequent stress.
6) To investigate changes in hydraulic structure in roots when subjected to waterlogging and drought conditions.

Material and Methods

Plant material

Seeds of *Senna reticulata* were collected from trees along the Rio Solimões (Manaus-AM) in June 2011. The material was placed in amber bottles and stored at 5°C for 6 months. Then, seeds were scarified mechanically with sandpaper in order to provide greater consistency for soaking and germination times. The seeds were placed in trays with vermiculite in a germination chamber with constant temperature (28°C) and 12 h photoperiod.

Seedlings were maintained in the germination chamber for 3 days (radicle protrusion occurred after the second day inside the chambers), and transferred to the greenhouse. Were added 100 mL of a nutrient aqueous solution (Epstein, 1972, modified accordingly to Arenque et al., 2014), once a week.

Environmental data

The climatic data were obtained from the Laboratory of Ecological Physiology of Plants (Lafieco), Department of Botany (IB-USP) through a system of sensors (temperature and humidity) coupled to the RICS Software (RICS®-Integrated Remote Control System), which monitored, every 10 minutes, temperature and humidity parameters in a weather station placed at 300 meters away from the greenhouse. Relative water content of the soil (RWC_{soil}) was determined weighing soil samples from different depths: top, middle and bottom of the pot and calculated using the following equation: ((weight of fresh soil − weight of dry soil)/weight of fresh soil) × 100.

Experimental Design

After one day of light acclimation (with photosynthetically active radiation (PAR) of 300 µmol photons m^{-2} s^{-1}), seedlings were transferred to PVC pots (10 L) containing organic soil (a mixture of peat, wood *Pinus* cheaps and vermiculite) and kept under full sunlight (up

to 2000 µmol m^{-2} s^{-1} PAR) in a greenhouse with retractable roof (Fig. 1). Plants were kept for 30 days in equal conditions of light and were watered once a day to field capacity. After this period, drought and waterlogging treatments were started. The dry treatment was established by withholding water for 60 days. The waterlogging treatment was achieved by applying epoxy (Durepoxi®) to pot drainage holes to avoid water drainage (waterlogging consisted of a watermark at the level of the root-shoot transition – Fig. 2B). In two additional treatments, aimed at verifying the effect of possible carbohydrate depletion on plant physiology under water stress, drought and waterlogging treatments were reversed after 30 days of growth. Plants subjected to drought conditions were flooded and plants subjected to waterlogging were allowed to drain and dry down. A well-watered control (soil at field capacity) was maintained throughout the experiment.

The retractable roof of the greenhouse was opened during daytime assuring high light intensity (up to 2000 µmol m^{-2} s^{-1} PAR) and closed during the night or rainy days, avoiding interference of rain water on the drought treatment (Fig. 1A and B).

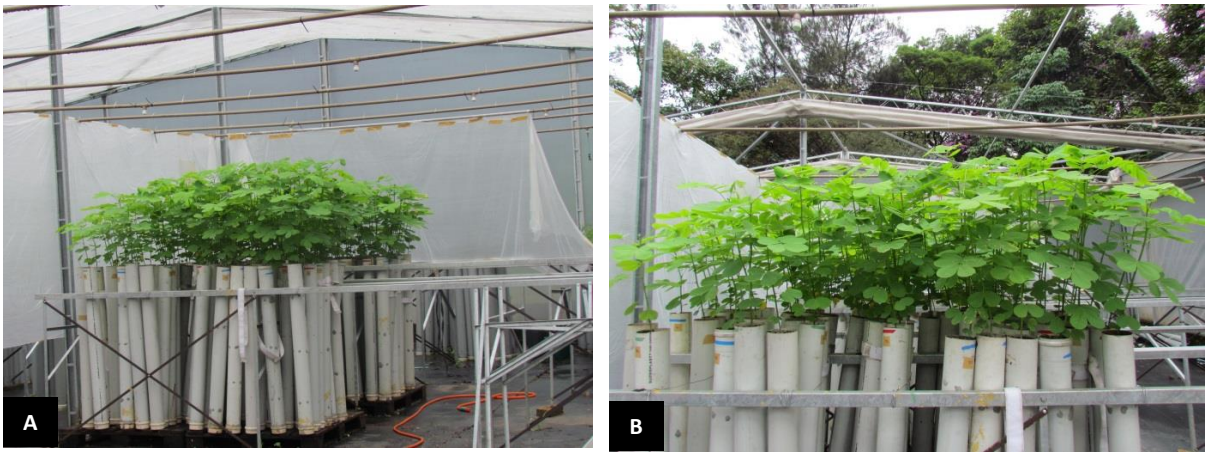

Figure 1. A. *Senna reticulata* seedlings growing in a greenhouse with a retractable roof **B.** Opened roof.

Figure 2. Plant growth. **A.** 30 days after germination **B.** Waterlogging treatment.

Harvests

Harvests were performed at seven different time points. The first harvest was 30 days after planting in pots (day 0). The following four harvests were made during drought and waterlogging treatments (days 10, 20, 30, 40) with three treatments: well watered (**ww**), drought (**dro**) and waterlogging (**wtg**). After treatments inversion, there were five treatments: ww, dro, wtg, drought followed by waterlogging (**dro+wtg**) and waterlogging followed by drought (**wtg+dro**) on days 50 and 60 for non-destructive analysis (gas exchange, leaf area); and on day 60 for destructive analysis (carbohydrates, leaf water potential and biomass). At each time point analysis were performed using six plants for each treatment (n=6). For destructive sampling, plants were removed from the pots by inversion on a metal screen and soil was carefully removed from the roots avoiding plant material losses. Shoots were separated into fully expanded leaf, other leaves and stem. Roots were washed in trays containing tap water and subsequently dried with a paper towel, and separated into roots and adventitious root, when present. After determining fresh weight (FW) of the different organs, these were put inside plastic bags and immediately immersed in liquid nitrogen. This material was freeze-dried and weighed to determine the dry weight (DW). Dried plant material was pulverized in a ball mill and stored at room temperature for further biochemical analyses (non-structural carbohydrates).

Gas exchange and fluorescence measurements

Leaf gas exchange measurements: net photosynthesis (A); dark respiration (R_d); transpiration (E); stomatal conductance (g_s) and fluorescence parameters were performed at 4 am and 10 am on the day of the harvest. Gas exchange was measured using the LI-6400 XTR portable photosynthesis system (Li–Cor, Lincoln, Nebraska) consisting of an open system containing an infrared gas analyzer (IRGA). Measurements were performed on the youngest fully expanded leaf (n=6). Leaf temperature was kept at 28°C during daytime measurements and 25° C at nighttime measurements. Airflow was set at 400 μmol s^{-1} at 10 am and 300 μmol s^{-1} at 4 am. Light intensity was set at 800 μmol m^{-2} s^{-1} PAR) for daytime measurements. CO_2 concentration was set at 400 μmol mol^{-1}. Whole-plant transpiration was also measured, by weighing pots (Transp –– g H_20 $plant^{-1}$ day^{-1}) two days prior to the gas exchange measurements at days 20, 40 and 60, as follows: (pot weight (g) – pot weight after 24 hours (g)).

Concomitantly to measuring gas exchange, fluorescence of chlorophyll *a* measurements were performed using in the LICOR chamber using the fluorescence unit. Maximum fluorescence in the dark (F0 and Fm) was measured at 4 am and maximum fluorescence in the light (F0' and Fm') was measured at 10 am. These measurements were used for subsequent calculation of: Fv/Fm ratio, Fv'/Fm' ratio, maximum quantum yield of PSII (ΦPSII), electron transport rate (ETR – μmol m^{-2} s^{-1} - (Earl & Tollenaar, 1998) and non-photochemical quenching (NPQ), according to (Van Kooten & Snel, 1990; Bolhar-Nordenkampf & Oquist, 1993).

Leaf water potential and plant hydraulic conductance

Leaf water potential was determined using the youngest fully expanded leaf (same leaf as for A, Rd and carbohydrates measurements) at 4 am (predawn) and at midday (between 10 am and 12 pm) using a pressure chamber (PMS, Albany, OR, USA). Total plant hydraulic conductance (K_T) was estimated using the following equation: $K_T = T / (\Psi_{leaf} - \Psi_{soil})$, where T is total plant transpiration (mmol s^{-1}, estimated by multiplying measured whole-plant leaf areas and Licor-measured leaf transpiration rates), Ψ_{leaf} is leaf water potential and

Ψ_{soil} is soil water potential (estimated from pre-dawn leaf water potential) (Sands & Theodorou, 1978).

Leaf area and biomass

The total leaf area of each plant was calculated from the sum of the areas of all the leaflets. Leaflet area was calculated as the product of the length, the maximum width and the empirical factor of 0.76, which was established by equation. Leaf size was calculated dividing the total leaf area by the number of leaves of each plant.

For biomass determination all organs were weighed to determine fresh weight (FW), packaged in plastic bags and immediately immersed in liquid nitrogen. Thus, material was freeze dried and weighed to determine the dry weight (DW).

Non-structural carbohydrates

All leaves, stems and roots were sampled between 10 am and 12 noon. Soluble sugars were extracted from 10 mg of powdered samples in 80% ethanol at 80 °C for 20 min and this procedure was repeated 4 times. The extracts were dried under vacuum and the dried pellets were re-suspended in 1 mL of deionized water. Remaining leaf pigments were removed by addition of 0.5 mL of 99% chloroform. Soluble sugars (glucose, fructose and sucrose) were quantified in the water phase by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC/PAD) using a Dionex-DX500 system (Dionex Corporation, Sunnyvale-CA, USA) equipped with a CarboPac PA1 column. Chromatography was performed using 100mM NaOH as eluent, with a flow rate of 1 mL min^{-1}.

Starch analysis was performed with an enzymatic method described by Amaral et al. (2007). After removal of soluble sugars, samples were treated with 0.5 mL of thermostable α-amylase (120 U) from *Bacillus licheniformis* (cod. E-ANAAM, MEGAZYME) diluted in 10 mM MOPS buffer at pH 6.5 during 30 minutes at 75˚C. The incubation was repeated. Samples were cooled to 50°C and treated with 0.5 mL amyloglucosidase (30 U) from *Aspergillus niger* (cod. E-AMGPU, MEGAZYME) diluted in 100 mM sodium acetate buffer at pH 4.5 for 30 minutes. This procedure was repeated and 100 μL of 0.8 M perchloric acid was

added to stop the reactions. After hydrolysis, the starch content was measured based on the amount of free glucose using the glucose oxidase reaction kit (CENTERLAB, Brazil). The samples were incubated with this solution for 15 minutes at 30°C and glucose content was measured at 490 nm. Commercial glucose (SIGMA) was used as a standard.

Root hydraulic conductance

At day 60, pieces of tap roots were immersed in formalin, acetic acid and ethanol solution (FAA 70° – Johansen, 1940) and stored at 5°C. The fixed material was dehydrated in ascending ethanol series (35%, 50%, 70%, 95%, 100% (v/v)) for 1 hour, and then, infiltrated in historesin (Leica Historesin Kit - Jung®).

Cross sections of 5 μm were produced using a semi-automatic microtome (Leica RM 2145) and subsequently subjected to histological staining with toluidine blue (0.025%), pH 4.0 (Vidal, 1977). Cross sections were mounted using Entellan® for observation under a light microscope (OLYMPUS BX51). Conduit density (CD) and specific hydraulic conductivity (K_s) of whole root cross section were calculated following Hagen-Poiseuille equation (Nonweiler, 1975) using Roxas 1.5.0.3 version running on Image-ProPlus® (version 6.3, 2008) platform (Arx & Dietz, 2005).

Data analysis

Differences between treatments were tested using ANOVA test. Results with p values <0.05 were considered significant. Tukey tests for differences between means were used a posteriori.

All statistical analyses were performed using JMP (SAS Inc. USA) and Minitab (Minitab Inc. USA) and graphed with GraphPad Prism 5 (GraphPad Software Inc., EUA)

Results

Environmental data

Outside conditions of temperature and relative humidity at the greenhouse were followed during all the experiment (Fig. 3A). Mean day relative humidity varied from 48 to 95%. Temperature showed considerable variation during the experiment, with an overall average of 23.7°C, a maximum of 32.6°C (accompanied by lowest relative humidity) at day 40 and minimum of 16.4°C at day 55. Soil moisture was 50% on average in well watered pots, and decreased to 25% in droughted pots at day 60. Droughted pots followed by waterlogging (dro+wtg) changed soil moisture from 35% to 60% in 20 days. Soil moisture of waterlogged pots that were drained and not watered anymore (wtg+dro) decreased from 60% to 38% in the same period (Fig. 3B).

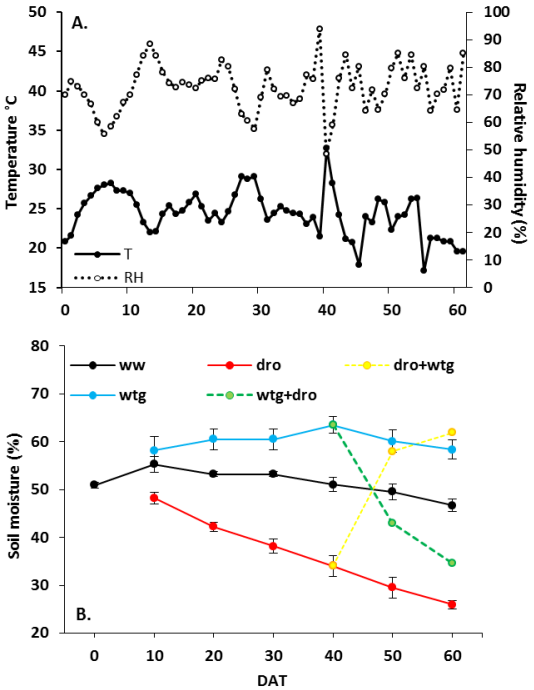

Figure 3. A. Air temperature (filled line – T °C) and relative humidity (dashed line – RH %) at a meteorological station near the greenhouse **B.** Soil moisture (%) during the experimental period (days after treatments - DAT) for each treatment: well watered (ww, black); waterlogging (wtg, blue); drought (dro, red); waterlogging followed by drought (wtg+dro, green) and drought followed by waterlogging (dro+wtg, yellow).

Gas exchange

Rates of CO_2 assimilation were decreased under drought conditions at days 40 (-38%), 50 (-80%) and 60 (-65%) in comparison to well watered plants. When drought was applied after waterlogging (dro+wtg), significant reductions were observed at days 50 (-42%) and 60 (-20%) in comparison to ww plants. In contrast, waterlogging did not reduce carbon assimilation rates and waterlogging after drought caused a quick recovery, doubling the CO_2 assimilation (Fig. 4A).

Nighttime leaf respiration (R_d) was decreased in droughted plants at all times, except on day 30 in comparison to well watered plants, whereas waterlogged plants only had lower rates than the well-watered plants on day 10. Droughted plants increased respiration rates in the first 10 days after waterlogging establishment in the dro+wtg treatment (day 50) but quickly returned to lower values on day 60. Waterlogging followed by drought decreased respiration after 20 days without watering (Fig. 4B). It is worth mentioning the exceptionally low rate observed on day 40 that seem to coincide with a sudden drop in temperature (Fig 3A).

Stomatal conductance was decreased in droughted plants from day 20 (-49% on average). Waterlogged plants only had lower g_s than well-watered plants at the beginning and end of the experiment (-48% and -50%). Both wtg+dro and dro+wtg treatments showed a fast response of g_s upon changing the water regime (Fig. 4C), maintaining the pattern observed before treatments inversion.

Transpiration rates displayed similar patterns as stomatal conductance, both at the leaf level (E – Fig. 4D) and the whole plant level (Transp – Fig. 4E).

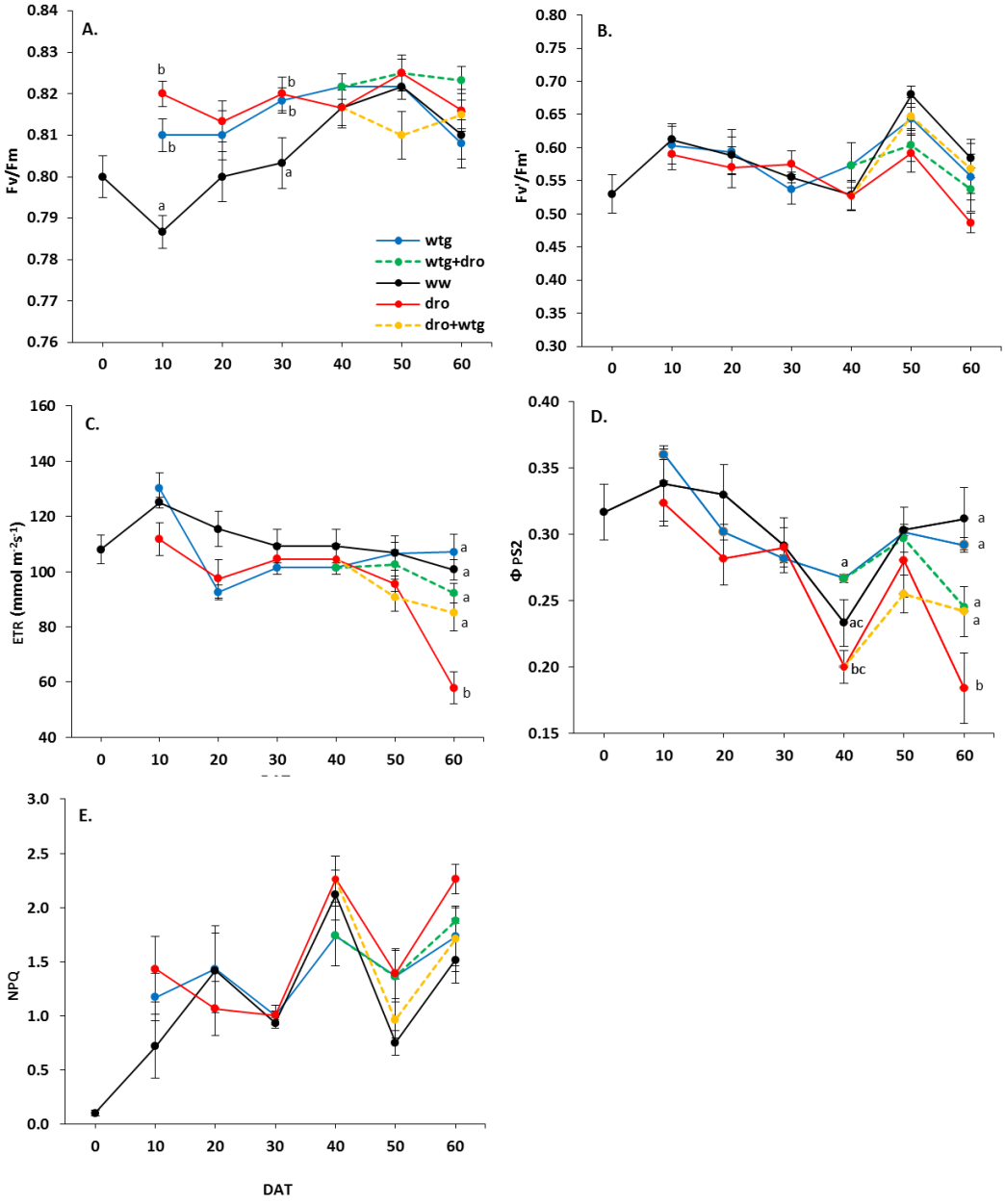

Figure 4. Gas exchange measurements. **A.** Carbon assimilation - A (µmol CO_2 m^{-2} s^{-1}) **B.** Dark respiration - R_d (µmol CO_2 m^{-2} s^{-1}) **C.** Stomatal conductance – g_s (mol H_2O m^{-2} s^{-1}) **D.** Transpiration (mmol H_2O m^{-2} s^{-1}) and **E.** Transpiration (g H_20 $plant^{-1}$ day^{-1}) measured in leaves of *Senna reticulata* grown under different water regimes: well watered (ww, black); waterlogging (blue); drought (red); waterlogging followed by drought (green) and drought followed by waterlogging (yellow). Days after treatment (DAT). Means are shown with standard errors (n=6). Different letters indicate significance of $P<0.05$ in Tukey analysis applied on each time point.

Whole plant hydraulic conductance and leaf water potential

Hydraulic conductance of the whole plant (K_T) was in average 80% lower in droughted plants than well watered plants at day 50 and 60 (Fig. 5A). Waterlogged plants displayed differences in K_T only at day 60. Differences in water potential were generally small and inconsistent, except on the first day (tendency of reduction in both water stresses) and the last day when droughted plants had significantly lower values than others and in waterlogged plants that displayed an increment of ca. 20% in comparison to drought and well watered plants on day 40 (Fig. 5B).

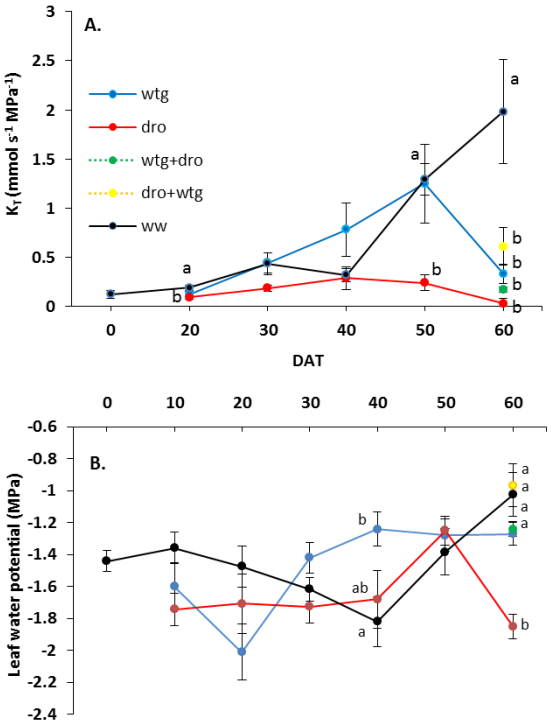

Figure 5. **A.** Whole plant hydraulic conductance (K_T) and **B.** Water potential (MPa) measured in leaves of *Senna reticulata* grown under different water regimes: well watered (ww, black); waterlogging (wtg, blue); drought (dro, red); waterlogging followed by drought (wtg+dro, green) and drought followed by waterlogging (dro+wtg, yellow). Days after treatment (DAT). Means are shown with standard errors (n=6). Different letters indicate significance of $P<0.05$ in Tukey analysis applied on each time point.

Chlorophyll fluorescence

Under drought and waterlogged treatments, the ratio Fv/Fm was slightly higher than well watered plants, suggesting no serious damage to photosystems as they are all around 0.8 (Fig. 6A). Fv'/Fm' did not show any difference (Fig. 6B). Quantum yield of photosystem II (ΦPSII) was decreased under drought in comparison with flooded plants at day 40 and day 60. Dro+wtg treatment was able to recover partially ΦPSII at day 60 (Fig. 6D).

Electron transport rate (ETR) was lower in drought treatment only at day 60 showing the same pattern of recovering after waterlogging was applied (Fig. 6C). Non-photochemical quenching (NPQ) did not display significance in any time of measurement, showing a tendency of increase in drought treatment after day 40 (Fig. 6D).

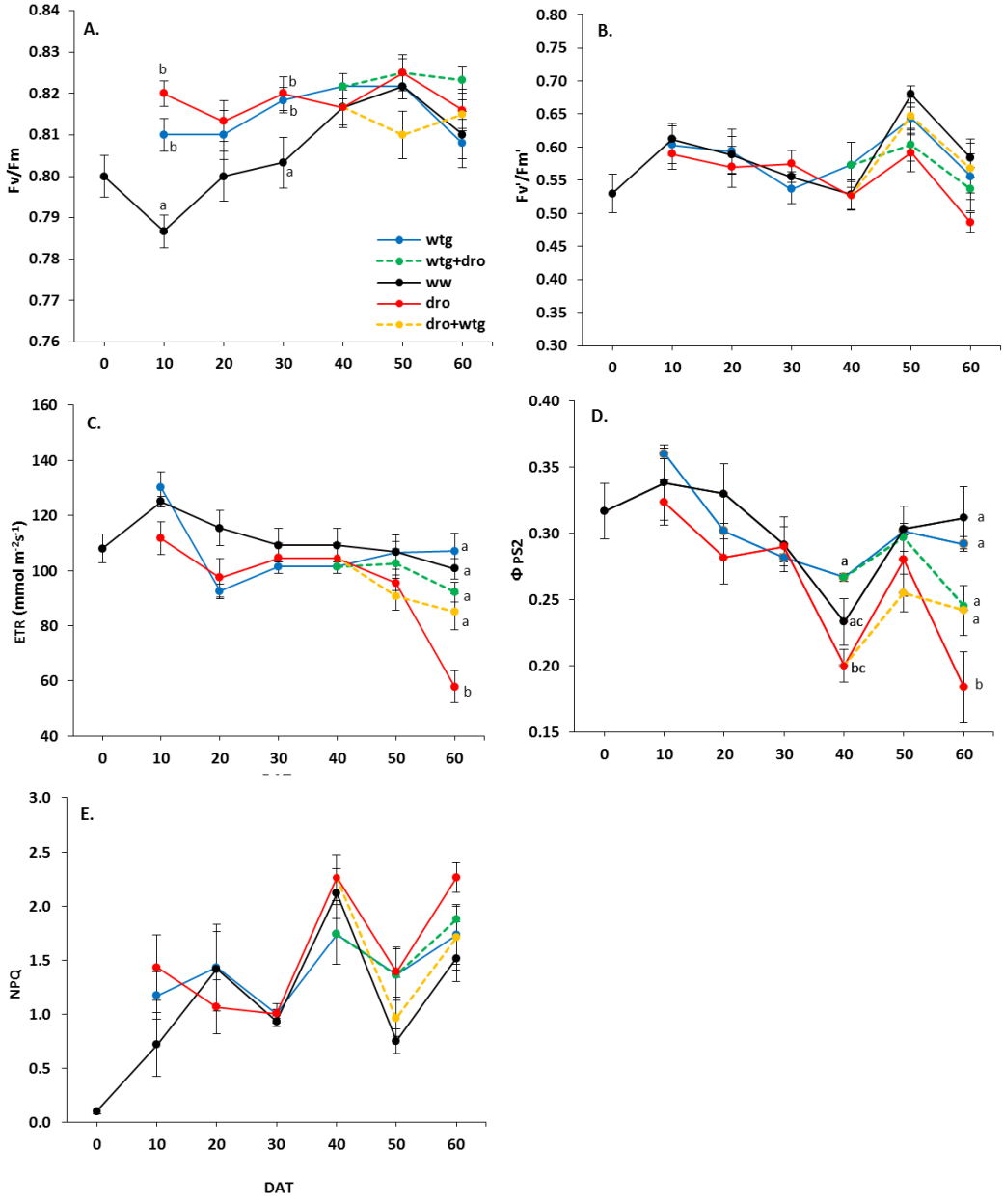

Figure 6. Fluorescence measurements **A.** Fv/Fm ratio **B.** Fv'/Fm' ratio **C.** Quantum yield of photosystem II – ΦPSII **D.** Electron transport rate - ETR (µmol $m^{-2}s^{-1}$) **E.** Non photochemical quenching (NPQ), all measured in leaves of *Senna reticulata* grown under different water regimes: well watered (ww, black); waterlogging (wtg, blue); drought (wtg, red); waterlogging followed by drought (wtg+dro, green) and drought followed by waterlogging (dro+wtg, yellow). Days after treatment (DAT). Means are shown with standard errors (n=6). Different letters indicate significance of $P<0.05$ in Tukey analysis applied on each time point.

Leaf area

Waterlogged and droughted plants had tendency of higher values of average leaf size on day 50 (Fig. 7B) because both displayed lower leaf number (Fig. 7C), higher rate of shed leaves (Fig. 7D) that resulted in lower total leaf number (Fig. 7E) at the same time. Plants in all treatments displayed ca. 40% lower leaf area (Fig. 7A) and lower values of average leaf size in comparison to well watered plants on day 60 (Fig. 7B).

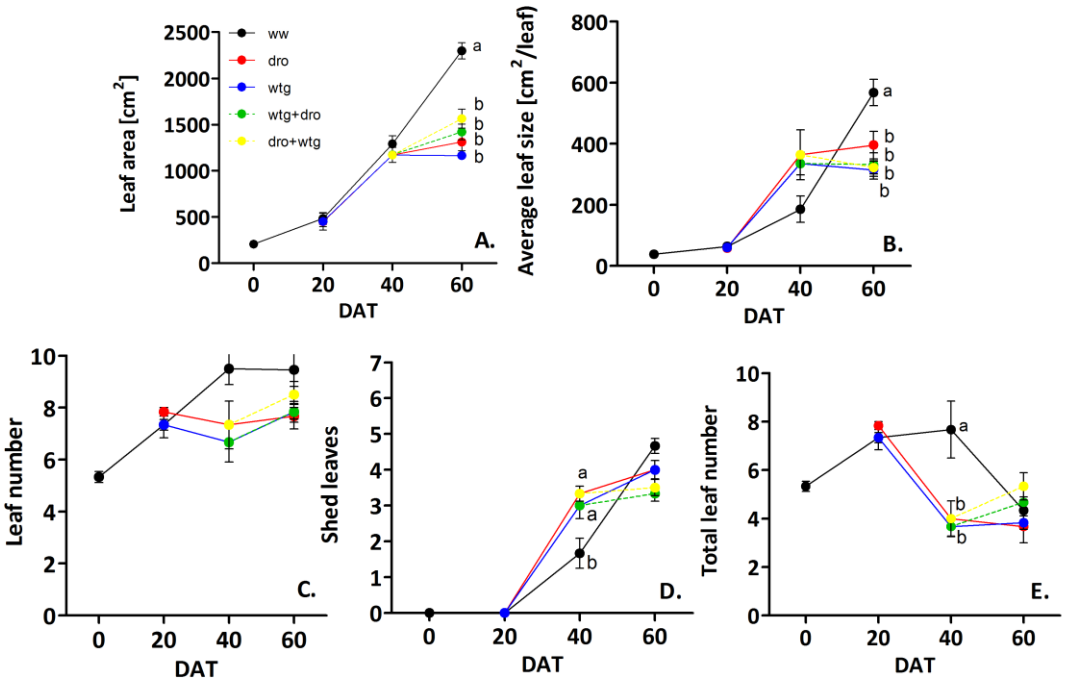

Figure 7. A. Leaf area (cm^2) **B.** Average leaf size **C.** Leaf number **D.** Shed leaves and **E.** Total leaf number of *Senna reticulata* cultivated under different water regimes: well watered (ww, black); waterlogging (wtg, blue); drought (dro, red); waterlogging followed by drought (wtg+dro, green) and drought followed by waterlogging (dro+wtg, yellow). Days after treatment (DAT). Means are shown with standard errors (n=6). Different letters indicate significance of $P<0.05$ in Tukey analysis applied on each time point.

Non-structural carbohydrates

The levels of non-structural carbohydrates found in leaves sampled during the day and night were quite similar, except for sucrose that was two-fold lower during the

nighttime (Fig. 8). Droughted plants displayed an increase in glucose and fructose during the day and glucose, fructose and sucrose during the night at day 10 in comparison to well watered plants. Waterlogged plants showed a tendency to increase in soluble sugars in almost all harvests but only significantly so at day 40: glucose and fructose in both day and nighttime. Droughted plants had lower daytime starch concentrations from day 40 and lower nighttime concentration at 20, 50 and 60 days. Waterlogged plants had starch levels similar to well-watered plants except on day 40 when they were unexpectedly low. Starch levels changed upon treatment inversion as expected: increasing in dro+wtg and decreasing in wtg+dro.

In stems, glucose was increased ca. 30% in average at days 10 to 30 in drought and waterlogging treatments in comparison to well watered treatments. Fructose also increased in stems at day 10 under waterlogging and sucrose in stems was increased on days 10-30. In waterlogged plants, starch was higher at day 20 and 40 and displayed lower levels at day 60 in comparison to well watered plants (Fig. 9).

Similar to leaves, roots of droughted plants displayed an increase in glucose and fructose on day 10 and in fructose at days 40 and 50 as well. Waterlogged plants showed an increase in glucose on days 40 and 50 and a strike increase in sucrose levels up to day 50 (average of 200%) (Fig. 9).

Adventitious roots displayed very similar starch level to the roots (less than 0.7% of dry weight) but resembled stems in the higher levels of glucose and fructose (ca. 2% of dry weight).

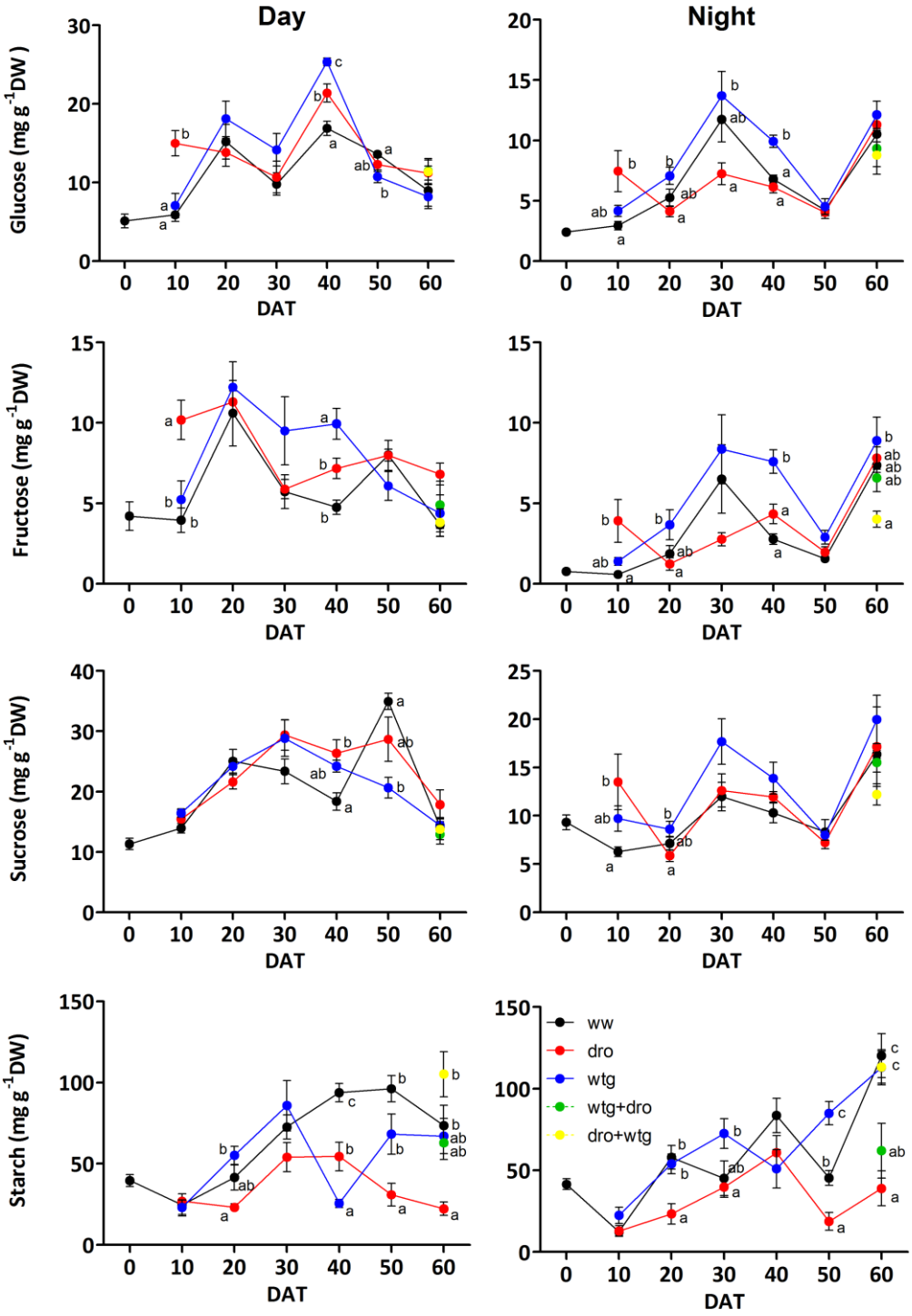

Figure 8. Non-structural carbohydrates (Glucose, fructose, sucrose and starch) measured during day time (10 am) and nightime (4 am) in leaves of *Senna reticulata* cultivated under different water regimes: well watered (ww, black); waterlogging (wtg, blue); drought (dro, red); waterlogging followed by drought (wtg+dro, green) and drought followed by waterlogging (dro+wtg, yellow). Days after treatment (DAT). Means are shown with standard errors (n=6). Different letters indicate significance of $P<0.05$ in Tukey analysis applied on each time point.

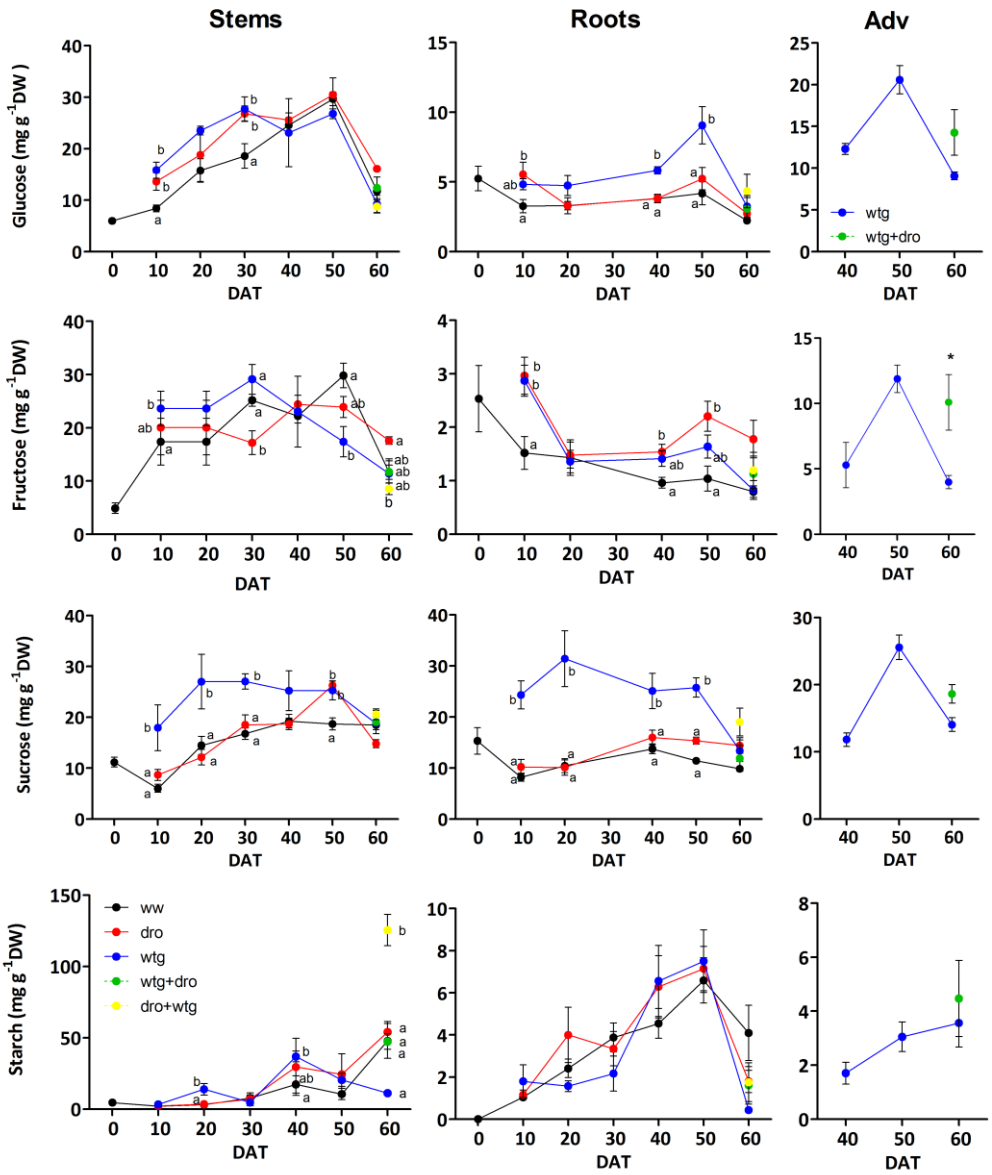

Figure 9. Non-structural carbohydrates (Glucose, fructose, sucrose and starch) measured at 10 am in stems, roots and adventitious roots of *Senna reticulata* cultivated under different water regimes: well watered (ww, black); waterlogging (wtg, blue); drought (dro, red); waterlogging followed by drought (wtg+dro, green) and drought followed by waterlogging (dro+wtg, yellow). Days after treatment (DAT). Means are shown with standard errors (n=6). Different letters indicate significance of $P<0.05$ in Tukey analysis applied on each time point.

Biomass and allocation

After 40 days, drought presented lower leaf biomass on days 40, 50 and 60 compared to well-watered plants. Inverted treatments did not affect leaf biomass (Fig. 10A).

Compared to well-watered plants, stems of flooded and droughted plants had less biomass but this was only significant for droughted plants. Changes in this organ under waterlogging were unexpected. Waterlogging applied after drought increased stem biomass at day 60 (Fig. 10B).

Root biomass was decreased compared to well-watered plants at days 40 and 60 in both drought and flooded treatments (Fig. 10C), being the main organ that contributes to total biomass (Fig. 10D). Adventitious roots were produced under flooded treatment since day 40. However, from 50 to 60 days a sharp increase was observed probably related with lower investment in roots during same period.

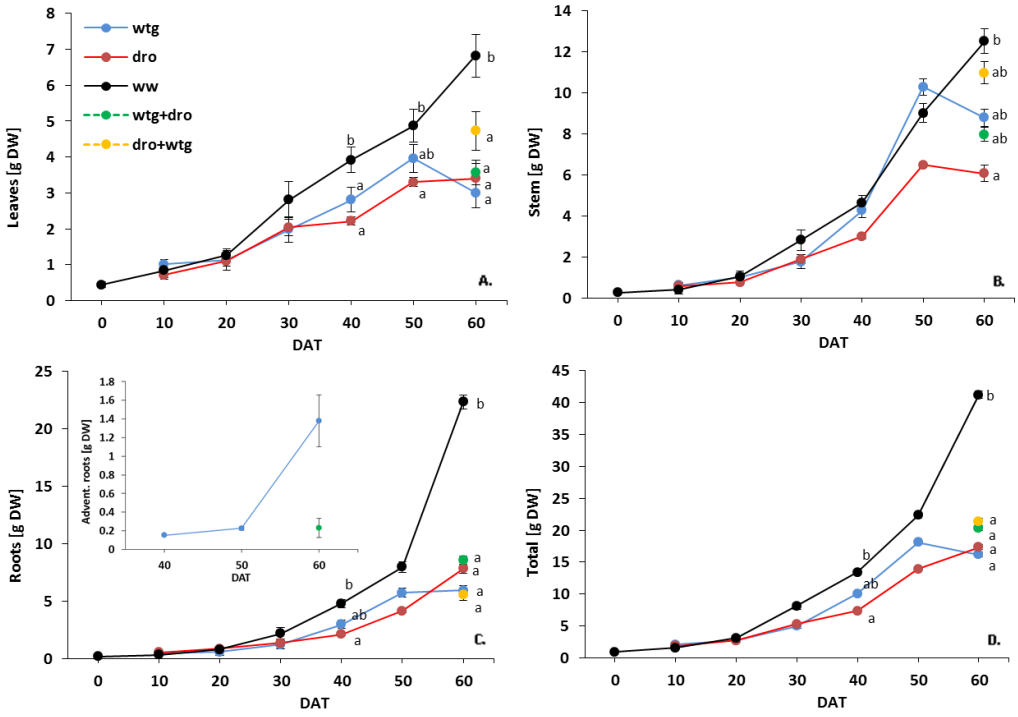

Figure 10. Leaves, stem, roots and total biomass of *Senna reticulata* cultivated under different water regimes: well watered (ww, black); waterlogging (wtg, blue); drought (dro, red); waterlogging followed by drought (wtg+dro, green) and drought followed by waterlogging (dro+wtg, yellow). Days after treatment (DAT). Means are shown with standard errors (n=6). Different letters indicate significance of $P<0.05$ in Tukey analysis applied on each time point.

In well-watered plants, stem and root biomass fractions slowly increased over time, at the cost of the leaf fraction (Fig. 11A). All treatments followed these trends, but waterlogging caused a stronger increase in the root fraction while the stem fraction was more stable (Fig. 11B,C). Early in the drought treatment, the roots fraction was significantly higher compared to the well-watered and waterlogged treatments (Fig. 11C). Inversion of treatments had effects on stems and root fractions that were opposite to those expected on the basis of the flooded and droughted treatments.

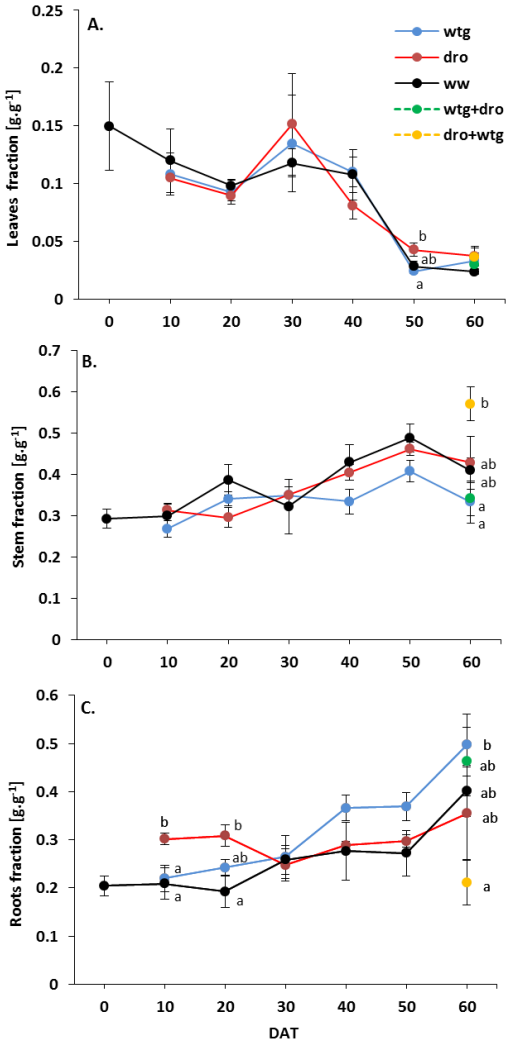

Figure 11. Fraction of leaves, stem and roots (g g^{-1}) and total biomass of *Senna reticulata* cultivated under different water regimes: well watered (ww, black); waterlogging (wtg, blue); drought (dro, red); waterlogging followed by drought (wtg+dro, green) and drought followed by waterlogging (dro+wtg, yellow). Days after treatment (DAT). Means are shown with standard errors (n=6). Different letters indicate significance of $P<0.05$ in Tukey analysis applied on each time point.

Root hydraulic

Conduit density was not statistically different among treatments (Fig. 12B) whereas specific hydraulic conductivity (K_s) was ca. 40% lower under drought and 80% lower under flooded in comparison to well watered plants (Fig. 12C). Upper panel illustrate maintenance of vessel diameters in droughted plants and decrease in waterlogged plants in comparison to well watered plants.

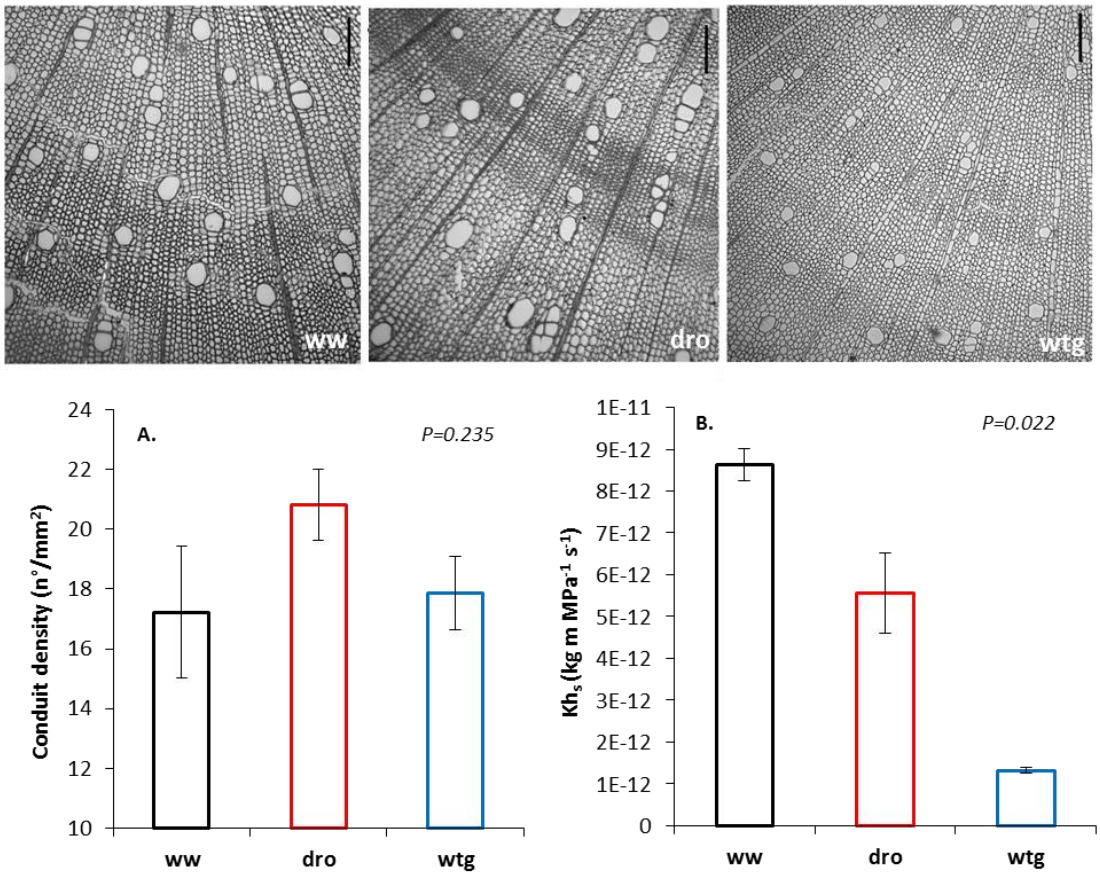

Figure 12. A. Conduit density (n°/mm^2) and **B**. Estimated specific hydraulic conductivity - K_s (kg m MPa^{-1}s^{-1}). On the upper panel pictures (scale bar 2μm) of roots cross sections under well watered (ww), droughted (dro) and waterlogged (wtg) treatments sampled at day 60.

Discussion

This study has demonstrated contrasting physiological and biochemical adjustments in *S. reticulata* when subjected to different types of water stresses. Drought reduced all gas exchange parameters and leaf starch storage was reduced, whereas waterlogging increased soluble sugars concentration in all organs (especially sucrose in roots) and decreased root hydraulic conductivity. In addition, the ability to recover from drought stress was very impressive whereas drought applied after waterlogging did not promote changes quickly. Despite the contrasting physiological responses, plants in both treatments reached the same biomass at the end of the experiment, thus different strategies are discussed.

Drought and carbohydrates adjustments

In contrast to expectations, the concentrations of soluble sugars were not increased in the drought treatment, with the exception of increases that were observed mainly in glucose and fructose (sucrose in a lesser extent) in all organs, but only at the very beginning of drought treatment – day 10 (Figs. 8 and 9). These transient high sugar concentrations appear to have decreased water potential of leaves on day 10 (Fig. 5). Osmolyte accumulation (OA) in plant cells decrease osmotic potential and help to maintain water absorption and cell turgor pressure, which can contribute to sustaining physiological processes such as stomatal opening, photosynthesis and growth (Morgan, 1984; Blum, 1996, Chaves et al., 2003). In our experiment, it is likely that plants may not have experienced severe drought stress as gas exchanges decreased gradually and there was no strong trend in water potential. The quick increase in soluble sugars observed in response to mild drought may play an important role in maintenance of water status even if growth is not yet threatened as supposed by Serraj & Sinclair (2002).

Previous studies have shown that *Senna reticulata* is highly adapted to waterlogging (Parolin, 2001a) but apparently susceptible to complete submersion and drought (Parolin, 2001b). In the present study, *S. reticulata* was capable of maintaining growth at least until day 30 (Fig. 9), and maintaining concentrations of soluble sugar similar to those in well watered plants in all organs until day 60. Apparently, despite lower assimilation rates

(especially after day 30 – Fig. 4) the soluble sugars concentrations were well regulated at the expense of starch and this is very likely to be related to growth maintenance.

Dark leaf respiration was reduced at day 10 (Fig. 4) which may have contributed to the higher levels of soluble sugars found in leaves. Drought-stressed plants also had lower levels of leaf starch from day 20 to 60, when starch drastically declined (-73%) in comparison to ww plants (Fig. 8). This result indicates that photoassimilate partitioning under drought conditions might be occurring in favor of soluble sugars rather than leaf starch in order to maintain soluble sugars status, mainly glucose and fructose in all organs, which presumably contribute to sustaining turgor pressure. The latter has been described as a critical component in the maintenance of cell wall integrity and as a central integrator allowing plant cells to monitor and coordinate metabolic processes in response to environmental stimuli (Chaves, 1991; Hamann et al., 2009; Wormitt et al., 2012)

Waterlogging tolerance and carbohydrates

Senna reticulata had high levels of soluble sugars in roots, which increased overtime in flooded roots. It did not have high starch concentrations in roots, unlike other floodplain species (Ferreira et al., 2009), but instead had starch in leaves. These patterns confirm trends in Arenque et al. (2014) and also with the previous ideas that the tolerance of plants to waterlogging is directly proportional to sugar levels in the roots Schlüter & Crawford (2001).

Sucrose is the main carbohydrate found in roots of *S. reticulata*, and is shown to be increased under waterlogging conditions (Fig. 9). Peña-Fronteras et al. (2009) studied non-structural carbohydrates in tubers of *Cyperus rotundus* from two distinct populations (upland – more sensitive and lowland – more tolerant to waterlogging) and found that higher tolerance of lowland plants is likely due to higher amount of soluble sugars and higher amylase activity found in their bigger tubers in comparison to smaller tubers that displayed starch as the main reserve. These observations are consistent with the idea that plants that store larger quantities of soluble sugars, rather than starch, have readily available substrate to cope with anaerobic fermentation under waterlogging, increasing their tolerance Peña-Fronteras et al. (2009). Furthermore, sucrose breakdown has been recently described as one of the key traits that enhance anoxia tolerance in roots of two

Eucalyptus species (Kogawara et al., 2014). In addition, soluble sugars can play a role also under post-hypoxia/anoxia stresses, decreasing reactive oxygen species (ROS - especially hydrogen peroxide and superoxide which are a major cause of post-anoxic injury), usually promoted by re-oxygenation of plant tissues (Blokhina et al., 2003; Coueé et al., 2006). Thus, *S. reticulata*'s ability to store larger quantities of soluble sugars in stem and roots during waterlogging must be linked with better efficiency in use of its reserve for anaerobic respiration and with ROS protection, partly helping to explain why this species is considered one of the highest flooded tolerant in Amazon floodplains (Parolin, 2001a).

While the advantage of high soluble sugar levels in roots is clear, the mechanisms that maintain these high levels are less clear. Our results suggest that high sugar levels are likely to be due to starch degradation (starch levels in leaves are low after day 40, Fig. 8); differential photoassimilates partitioning between starch and sucrose or by decreased leaf respiration rates (Fig. 4). Thus, the increase in soluble sugar concentrations may be the result of lower respiration rates in other organs (rather than leaves), or breakdown and remobilization from other compounds not measured in this experiment. It is also possible that sugars accumulated due to sink limitation of growth, i.e. due to a greater decrease in growth rate than in net assimilation, in comparison to well watered plants, which seems likely in the second half of the experiment (Fig. 10).

Even with accumulation of starch in leaves rather than roots, this reserve seemed to play an important role under waterlogging conditions, especially at day 40 when starch was strongly reduced in leaves (Fig. 8). Concomitantly with this reduction a shift in biomass allocation from stem to roots was observed (Fig. 11), indicating that starch breakdown could occur in order to provide substrate for this process. In addition, at this time adventitious roots started to grow, possibly using mobilized leaf starch. The abrupt increase in starch concentration in leaves and stems when droughted plants were subjected to waterlogging reinforce the relevance of it under this condition (Fig. 8 and 9).

The levels of sucrose in adventitious roots were particularly high (Fig. 9) and some of the regular roots are green (personal observation), suggesting the possibility that some photosynthetic activity in both types of roots must be explored as active chlorophylls were found as an important source of O_2 and potentially carbohydrates in *Tecticornia pergranulata* (Chenopodiaceae) under flooded and submerged habitats (Rich et al., 2008)

Interactions between waterlogging and drought responses

It has been discussed that signaling pathways in response to abiotic stresses constitute a complex network, interconnected at many levels (Knight & Knight, 2001; Valluro & Ende, 2011). For example the induced response by cold, drought and salt stresses are all mediated by ABA signaling (Knight & Knight 2001; Schulze et al., 2002) helping to explain why plants can share similar responses under differential stresses or one stimuli being able to trigger several responsive genes expression (Cushman & Bohnert, 2000). Flooding that may cause a physiological drought, decreasing water potential and promoting leaf shedding in order to decrease transpiration rates (Parolin et al., 2005). However, in our experiment we found a very similar trend in both water regimes in relation to phenology. Both treatments decreased transpiration area firstly decreasing total leaf number (Fig. 7E) and later reducing leaf area (Fig. 7A). These responses were probably triggered by lower g_s and lower water potential found in leaves under both water regimes at the very beginning of the experiment and suggest that this species share similar mechanisms in both water stresses in order to reduce energy consumption and leaf transpiration (De Simone et al., 2003, Chapter 1).

Despite of this similarity, we observed a very distinct pattern of other physiological responses in both water conditions. Stomatal conductance (g_s) was reduced over time under drought treatment, whereas flooded plants did not show changes consistently (Fig. 4). Along with the maintenance of g_s in the waterlogging treatment, assimilation, dark respiration in leaves, transpiration, a ΦPSII and leaf area (Figs. 4, 6 and 7) were also maintained. Thus, the pattern in flooded plants was opposite to those in droughted plants, which had higher root hydraulic conductivity and lower stomatal conductance.

As expected, in our experiment, roots of droughted plants displayed higher allocation to roots in comparison to other treatments (Fig. 11C). It is known that plants can actively adjust root growth to maximize uptake of water under drought events (Thornley, 1972; Cannell & Dewar, 1994). Changes in root morphology (e.g. increase in specific root length and surface area) of several Amazonian species were also reported to enhance water uptake under seasonal and long term drought events Metcalfe et al. (2008).

Under drought, *S. reticulata* also displayed slightly lower Ks in comparison to well watered plants (Fig. 10C), whereas plants in the waterlogging treatment displayed

significant lower values in comparison to drought and well watered plants (Fig. 12C). Since vessel diameter is positively correlated with the volume of water transported and inversely correlated with the safety (lower risk of cavitation) of the conductive system (Carlquist, 1980; Vaselatti et al., 2001; Kim et al., 2014) it seems that this species did not show plasticity to change vessel diameter probably associated with lower hydraulic security in comparison to waterlogged plants. It is also important to highlight that values of whole-plant hydraulic conductance (K_T) were similar to those of droughted plants (Fig. 5A). The poor correlation between root specific conductivity and whole plant conductance suggest that roots contribute a relatively small part to whole plant resistance to water flow.

All these results indicate that waterlogging promoted very distinct physiological responses in *Senna reticulata* seedlings in comparison to drought. Although both water stresses triggered decreases in leaf area and biomass, apparently it is not likely that flooded plants were experiencing a "physiological drought" since g_s and E were maintained up to day 50. However, it is important to highlight that this ability to maintain g_s and E can be related to enhancement in hydraulic conductivity promoted by changes in hydraulic architecture in roots of flooded plants, avoiding and/or delaying a possible water stress. On the other hand, after rewatering was applied in droughted plants, *S. reticulata* displayed a strong ability to recover and reestablished carbon assimilation, leaf respiration, transpiration, leaf water potential, ΦPSII and starch concentration in leaves and stems (Figs. 4,5,6,8, 9 and 13). These results indicate that the ability to recover from drought stresses may be extremely important to maintain physiological status of seedlings during establishment in the first terrestrial phase at Central Amazonian floodplains.

We conclude that drought and waterlogging (both increased gradually over time) can be considered equally relevant for growth maintenance in seedlings of *S. reticulata,* as both treatments reached similar decreases in biomass accumulation (Fig. 10). However, the tolerance/avoidance mechanisms to cope with flood and drought events are quite different in this species (Fig. 13).

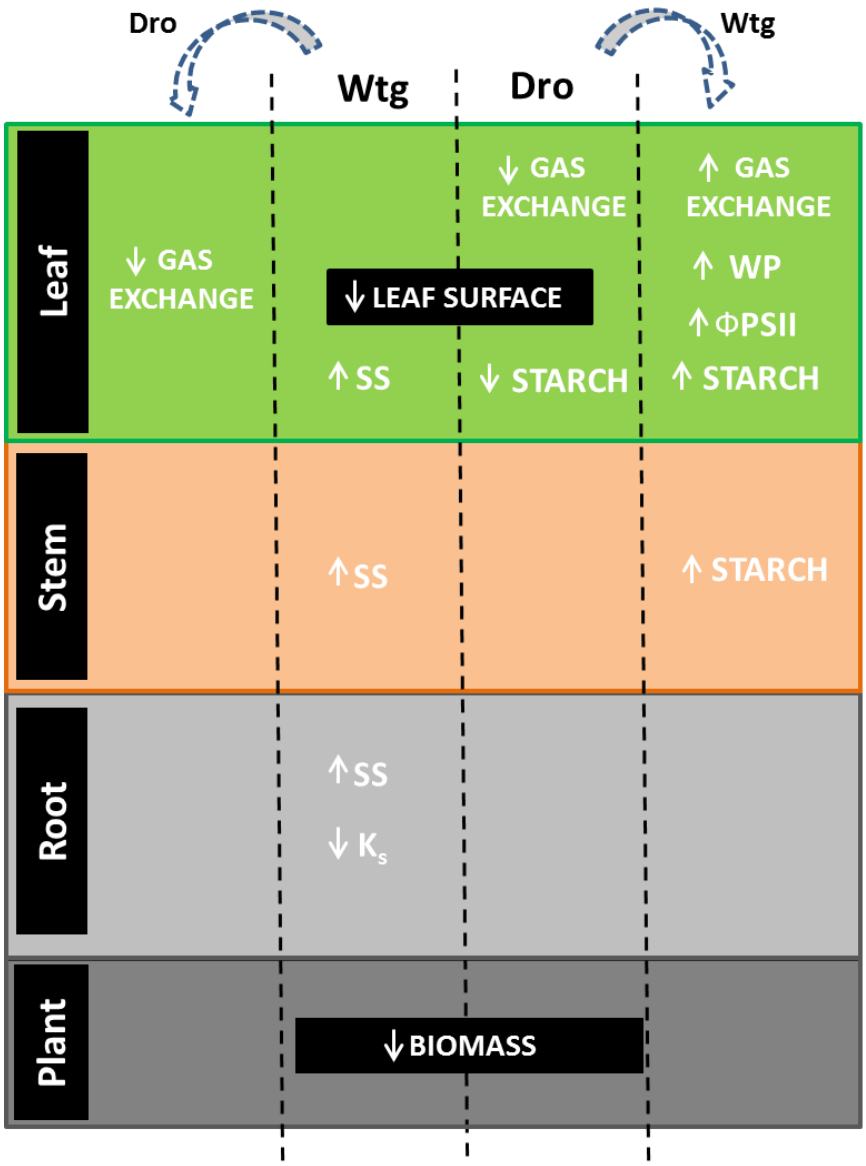

Figure 13. Schematic response of *Senna reticulata* when subjected to drought and waterlogging for 60 days and inverted treatments: waterlogging after drought and drought after waterlogging for 20 days. Changes are represented in relation to well watered plants at distinct levels: Leaf, Stem, Roots and Total Plant. Droughted and waterlogged plants decreased leaf surface by shedding old leaves and reducing leaf area and both treatments decreased biomass at the same rate at day 60. Droughted plants down regulated gas exchange and leaf starch reserves whereas flooded plants increased soluble sugars (SS) concentration in all organs and decreased root hydraulic conductivity (Ks). Waterlogging applied after drought enhanced gas exchange, ΦPSII, leaf water potential (WP) and starch storage, whereas drought applied after waterlogging decreased gas exchange.

Conclusion

In our study, drought was considered as important as waterlogging in relation to growth limitation at seedling stage of *S. reticulata* but the tolerance mechanisms regarding carbohydrate metabolism, hydraulic properties and gas exchange were quite different under distinct water stresses. When re-watered, droughted plants displayed a very fast ability to recover that must be crucial for establishment of *S. reticulata* seedlings during the dry season of Central Amazonian floodplains.

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CONSIDERAÇÕES FINAIS

A regulação encontrada no metabolismo de carboidratos de *Senna reticulata* em relação a uma situação de baixa disponibilidade de carbono corrobora a recente literatura que demonstra o armazenamento de carbono não somente como um produto do desequilíbrio entre fornecimento (fonte) e demanda (dreno) de carbono, mas também resultante de um específico controle genético (Sala et al., 2012; Ronan et al., 2014). Trabalhos prévios postulavam que durante o dia todo o carbono recém-fixado seria dirigido para processos metabólicos de crescimento e respiração e não para armazenamento. Porém, avanços nessa área de estudo vêm demonstrando que órgãos especializados em armazenamento podem, de fato, ser considerados drenos competidores em relação ao crescimento mesmo durante o período de luz (Webb & Kilpatrick, 1993; Smith & Stitt, 2007).

Ao contrário da maior parte das espécies de planícies alagáveis amazônicas já estudadas, que apresentam a raiz como órgão armazenador (Scarano et al., 1994; Ferreira et al., 2009), a espécie abordada neste estudo *S. reticulata*, tem a folha como principal local de reserva (Arenque et al., 2014). No presente trabalho a diminuição no período de exposição à luz (tratamento de baixa luz) reduziu o aporte de carbono total para a planta, culminando em acentuada diminuição nos níveis de amido foliar em um primeiro momento e uma visível recuperação dos níveis dessa reserva na coleta seguinte. Esse padrão de recuperação sugere uma alta resiliência (persistência – Walker et al., 1999) em seu sistema de armazenamento, o que pode estar diretamente associado à necessidade de lidar com as flutuações ambientais inerentes ao seu local de ocorrência, ainda mais se considerarmos que um alto investimento em reservas não era esperado para esta planta já que árvores pioneiras e de rápido crescimento, normalmente particionam a maior parte de seu carbono diretamente para crescimento (Poorter et al., 1997).

Quando a elevada concentração de CO_2 foi acoplada a baixa disponibilidade de carbono (promovida pela baixa luz), foi possível observar as principais alterações que o CO_2 promoveu nas taxas fotossintéticas, respiração foliar e crescimento. A proeminente redução da respiração foliar parece ter sido peça fundamental nessa regulação, direcionando carboidratos para aumento de reserva e de crescimento, reforçando a partição equilibrada entre esses dois processos. Esses resultados adicionam elementos no entendimento de como ocorreu o efeito de tamponamento observado por Arenque et al. (2014) quando a mesma espécie foi submetida à elevada concentração de CO_2 acoplada ao alagamento,

reforçando a ideia de que a plasticidade presente no sistema de armazenamento da planta (partição entre crescimento e armazenamento equilibrado), bem como a possibilidade de uso destas reservas (disponibilidade) são fatores relevantes a manutenção do crescimento (Bustan et al., 2011; Sala et al., 2012).

A relevância e resiliência das reservas foliares dessa espécie puderam ser observadas também ao longo do segundo experimento (capítulo 2), onde o armazenamento de carboidratos nas folhas continuou sendo preservado mesmo em condições ambientais adversas promovidas pela variação na disponibilidade de água. Em condição de alagamento, o conteúdo de amido nas folhas não sofreu variação alguma em relação ao tratamento controle, exceto aos 40 dias que provavelmente se relaciona com o início da produção de raízes adventícias neste tratamento. Durante a seca, reduções nestas reservas foram significativas somente após os 40 dias, concomitantemente com a redução de biomassa. Estes dados reforçam o importante papel que a reserva de amido exerce sobre a manutenção do crescimento. Mesmo que pouco elucidados, trabalhos mostram que os mecanismos que regem essa relação são provavelmente mediados por reguladores do metabolismo do amido ou ainda por sinais produzidos que agiriam integrando metabolismo e crescimento da planta (Sulpice et al., 2009).

Em resumo, os dados provenientes desta tese auxiliam no entendimento da relação fonte-dreno de espécies tropicais que ocorrem naturalmente em ambientes altamente perturbados como é o caso das planícies alagáveis da Amazônia Central. Além disso, cientes de que a extrapolação dos resultados para o ambiente natural é complexa, ainda sim este estudo contribui para a compreensão de como plantas tropicais responderão às MCGs. Os dados gerados adicionam elementos na discussão sobre o papel da disponibilidade de carboidratos na mortalidade de plantas no sentido de corroborar as ideias de Sala (2009) e Sala et al. (2010), indicando que o motivo da morte de indivíduos em condições de seca extrema estão muito mais relacionados com incapacidade de uso das reservas por limitações hidráulicas do que por total depleção de carboidratos em uma condição de estresse severo. Esse argumento parece ter ainda mais fundamento para espécies onde a plasticidade de resposta foi moldada em um ambiente com flutuações altamente previsíveis como, por exemplo, a várzea amazônica. Contudo, uma questão a ser abordada em futuros trabalhos é quão resiliente seria essa plasticidade em locais com variações ambientais menos previsíveis (p.ex. terra firme amazônica). Este e outros estudos poderiam auxiliar,

inclusive, em modelagens climáticas que vêm buscando incluir dados de vegetação em seus algoritmos, não interessados somente na flutuação das variáveis físicas (vento, irradiância, temperatura, precipitação), mas também adicionando a influência da do comportamento fisiológico da comunidade vegetal sobre o clima, e incluindo nisso as respostas das mesmas às MCGs. Adicionalmente, tem sido considerada a importância da inclusão de dados sobre alocação de carbono entre diferentes compartimentos em modelos que descrevem produtividade primária das comunidades vegetais (Litton et al., 2007; Davi et al., 2009), indicando que a produtividade primária não está relacionada somente com o balanço entre fotossíntese e respiração, mas também com um balanço ativo entre fotossíntese, respiração, crescimento e reserva respondem por boa parte da capacidade de um bioma continuar crescendo.

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