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Ecophysiology And Carbon Allocation Of Aspen And Balsam Poplar Seedlings In Response To Drought

by

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Dedicated to my friend, partner and wife
Anayansi Cecilia Cohen Fernández.

ABSTRACT

Drought-induced forest mortality has been recorded in every forested continent. Although the effect of drought on plant growth, physiology and ecology have been extensively studied in the past, the physiological mechanism leading to plant mortality under drought conditions are far from being resolved. These mechanisms interact in very complex feedbacks between gas exchange, water relations and carbon reserves. Additionally, drought is theorized to increase plant susceptibility to other biotic stressors, such as herbivory. Questions on these issues were addressed through a series of experiments under greenhouse and outside conditions at the University of Alberta, Edmonton. Seedlings of trembling aspen (*Populus tremuloides*) and balsam poplar (*Populus balsamifera*) were artificially droughted and defoliated under controlled conditions to evaluated the effects of drought and herbivory on growth, gas exchange, water relations and carbon reserve accumulation dynamics across tissues during different time lengths.

In two separated experiments mild and severe drought treatments were imposed on aspen and balsam poplar seedlings after controlled desiccation protocols and drought targets were identified. Mild drought stress had no effect on many of the measured variables in balsam poplar seedlings and, although the impact of mild drought increased over time, results suggested that under mild drought conditions balsam poplar seedlings prioritized growth over hydraulic safety. In aspen, accumulation of carbon reserves took place under drought conditions, which is contrary to the original predictions of the current leading theory on mechanisms of plant mortality under drought conditions, the carbon starvation hypothesis (CSH).

Based on the previous results, two additional experiments were implemented to explore the effect of drought and defoliation on physiological and growth variables of both species over an extended period of time including a full growing season and a dormant period. Although both treatments affect carbon reserve dynamics, the underlying mechanisms were different. Results from these two experiments are, to our knowledge, the first experimental evidence describing some of the feedbacks between gas exchange, water relation and carbon reserve accumulation dynamics that may lead to plant mortality, and highlighted additional roles of carbohydrates, such as frost protection to roots, currently overlooked by the CSH.

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CHAPTER 1. INTRODUCTION

1.1 Background

Drought-induced changes in forest composition and forest mortality events have been reported at local and regional scale in all forested continents (Allen & Breshears 1998, Allen 2009, Allen *et al.* 2010, Michaelian 2010). The type of drought driving these changes is thought to be associated with the observed anthropogenic induced increase in global average temperature over the last five decades (Breshears *et al.* 2005, IPCC 2007, McDowell *et al.* 2008, Adams *et al.* 2009, Anderegg *et al.* 2012). Droughts are expected to increase in frequency, duration and intensity (Allen *et al.* 2010) negatively impacting forest species composition and structure (Condit *et al.* 1995, Allen *et al.* 2010), function (Dale *et al.* 2000) and services such as biodiversity, food and wood products, water, and air (Hassan *et al.* 2005, Fischlin *et al.* 2007) and may alter carbon cycling, leading to large net emissions of CO_2 to the atmosphere (Adams *et al.* 2010).

The effects of drought on forest mortality are already apparent globally and regionally (Allen *et al.* 2010, Anderegg *et al.* 2012). For example the aspen forests of the Canadian prairie provinces have shown widespread decline over the last decades (Hogg *et al.* 2008, Allen *et al.* 2010, Michaelian *et al.* 2010). We currently lack basic knowledge on many fundamental physiological processes associated with the drought-induced mortality of trees and forests (McDowell *et al.* 2008, Sala 2009, Sala *et al.* 2010, McDowell 2011, Sala *et al.* 2012). To date, the most comprehensive framework on how carbon dynamics, water relations, gas exchange and pathogen attacks may underlie drought-induced tree and forest mortality, is synthetized in the carbon starvation hypothesis (CSH, McDowell *et al.* 2008). Originally, the CSH proposed two main mechanisms driving tree mortality under drought conditions: catastrophic hydraulic failure and carbon starvation. In the sections below I will describe, *sensu lato*, these two mechanisms.

1.1.1 Catastrophic hydraulic failure

Catastrophic hydraulic failure refers to severe embolization of functional xylem tissue, where a seedling or tree irreversibly loses hydraulic conductivity. Under drought conditions embolization of the functional xylem tissue occurs as a result of a process *sensu lato*, as follows: (1) as soil dries out during drought, soil water potential (Ψ_{soil}) decreases; (2) as Ψ_{soil} becomes more negative, leaf and stem water potentials (Ψ_{leaf}, Ψ_{stem}; respectively) also become more negative; (3) to avoid reaching critically low values of Ψ_{leaf} and Ψ_{stem}, plants close their stomata; (4) if drought conditions continue, Ψ_{soil} becomes more negative and eventually evapotranspiration (E) becomes greater than water absorption rate at the roots, reaching a species-specific critical value (E_{crit}); (5) once E_{crit} is reached, water columns in xylem vessels start breaking under increased negative pressure; (6) as water columns break, air nucleation occurs at the point of brakeage and, as xylem pressure become more negative, air bubbles expand stopping water transport (i.e. xylem vessels become embolized); (7) if xylem tension becomes more negative, the number of embolized xylem vessels increases, leading to dysfunction of xylem tissue and water transport. At this point large sections of xylem tissue cavitate and (8) increased cavitation of xylem tissue decreases hydraulic conductivity at the whole stem or branch scale. Embolism is generally irreversible unless xylem tension (which is commonly expressed as negative pressure) returns to near zero or becomes positive, for long periods of time (Tyree & Zimmermann 2002). Once a branch or stem suffers irreversible embolization of the vast majority of its conductive tissue, it is said to have suffered catastrophic hydraulic failure (Tyree & Zimmermann 2002), which can leads to plant desiccation and possibly death. Catastrophic hydraulic failure is well documented in seedlings, saplings, and in branches of adult trees growing under controlled and field conditions (e.g. Rood *et al.* 1998, Awad *et al.* 2010, Anderegg 2012).

1.1.2 Carbon starvation

The original concept of carbon starvation proposed a series of physiological responses to drought that can be summarized as follows: (1) as soil dries out stomatal closure significantly reduces hydraulic failure, but it also significantly reduces carbon assimilation; (2) as carbon assimilation stops or is significantly

reduced, production of photoassimilates decline; (3) if photoassimilates demand for basic cellular process, root respiration or production of defense compounds become greater than photoassimilates production, plant reserves need to be utilized and; (4) as carbon reserves are reduced faster than it can be replenished, plant mortality occurs when carbon reserves are completely exhausted or under attack of insects or pathogens (McDowell *et al.* 2008).

In its original formulation, the CSH suggests that under drought conditions the physiological mechanism leading to plant mortality (i.e. hydraulic failure or carbon starvation) is mainly determined by the stomatal behavior of hydraulically stressed plants. Stomatal behaviour refers to a continuum of stomatal regulation of water status in plants. On the opposite end of this continuum are isohydric and anisohydric behaviours (Tardieu & Simonneau 1998). Isohydric plants close their stomata during the early onset of drought in order to maintain E below E_{crit} and avoid hydraulic failure. Isohydric behaviour increases hydraulic safety during drought conditions, but it also produces a severe reduction in CO_2 assimilation, hence, the CSH predicts that under extended periods of water stress, plant mortality in isohydric plants is more likely to occurs due to carbon starvation. Anisohydric plants on the other hand maintain their stomata open during drought conditions, which results in stem water potentials significantly more negative than in isohydric plants. This stomatal behaviour allow anisohydric plants to maintain CO_2 assimilation under drought conditions, but, as stomata remain open under increasingly more negative pressure, the CSH predicts that plant mortality in anisohydric plants is more likely to occur due to catastrophic hydraulic failure.

Originally the CSH proposed hydraulic failure and carbon starvation as mutually exclusive mechanisms that could drive plant mortality under drought conditions, this view has been recently updated (McDowell & Sevanto 2010, McDowell 2011) stating that “the binary mortality theory of McDowell *et al.*. (2008), that trees become vulnerable… via carbon starvation or hydraulic failure, is overly simplistic; more likely, the two processes are coupled.” (McDowell 2011).

1.1.3 Hydraulic failure and carbon limitation feedbacks

Currently, an increasing amount of literature supports the idea that carbon starvation and hydraulic failure are highly interconnected, rather than separate,

processes (Sala *et al.* 2010, Galvez *et al.* 2011, McDowell 2011, Anderegg 2012, Anderegg & Callaway 2012, Anderegg *et al.* 2012, Sala *et al.* 2012). In this context, a new and wider perspective exploring feedbacks between water relations, gas exchange and carbon dynamics have been presented by Sala *et al.* (2012). In their work the authors explore scenarios where carbon reserves increase instead of decrease under drought conditions and plant mortality occurs even if carbon reserves are not completely exhausted. Due to its relevance to my research, I will briefly review some of these possible feedbacks and plant responses.

1.1.4 Carbon storage as an active process

For the purpose of this section the term “active” refers to a genetically regulated process that may drive allocation of photoassimilates to storage when resources are limited, opposed to a “passive” process where carbon storage is driven solely by an imbalance between carbon supply and demand (Sala *et al.* 2012). In other words, under the “passive” storage model when photoassimilate demand to maintain metabolic processes is higher than supply from CO_2 assimilation, plant reserves are used to balance the difference, hence reserves decreases. This traditional view is been challenged by recent studies exploring the premises of the CSH working with aspen seedlings (Galvez *et al.* 2011) and adult trees (Anderegg 2012, Anderegg *et al.* 2012). These are some of the few studies that provide experimental data on carbon dynamics under drought conditions and reported significant increase in carbon reserves in tissues and severe growth reductions. These results suggest that under hydraulic stress, aspen seedlings and adult trees prioritize reserve accumulation over growth, supporting the view of reserve accumulation as an active process (*sensu* Sala 2012).

1.1.5 Starch and sugar accumulation for hydraulic repair and maintenance and transport of chemical signals

While exploring the CSH, new research paths are being explored including the important question as to why adult tree accumulate reserves. As previously mentioned, there is a growing body of literature supporting the idea that carbon dynamics and hydraulic transport are highly interconnected process. Nonetheless, there is little research been done exploring the links and

relationships between carbon dynamics and hydraulic transport. For example, although we know photoassimilates translocation between sources and sinks is done via bulk transport, water being the media of such process (Munch 1927), we do not know to what extent sugars and starch pools in xylem parenchyma cells are used to sense and repair embolized xylem vessels (Secchi *et al.* 2011). Even further, sugars may be needed for daily maintenance of hydraulic functioning even in the absence of major cavitation events or hydraulic stress (Secchi *et al.* 2011). Water in the xylem and phloem may also work as medium to transport chemical signals regulating stomata behavior and the up and down regulation of aquaporins.

1.1.6 Root ecology and drought

One of the many other subjects that have been explored in the current discussion on carbon dynamics and water relations is the role of root mortality under drought conditions. Very recently Anderegg (2012) and Landhäusser & Lieffers (2012) addressed some fundamental questions on carbon and root mortality of boreal and montane aspen forests. In their work the authors hypothesized that drought-driven decline in fine root biomass could lead to increased water stress and eventually to dieback over long periods of time. Anderegg (2012) further proposes that changes in allocation, tissue function and repair capabilities over the long-term may drive plant mortality, rather than depletion of carbohydrate reserve or hydraulic failure.

Another unexplored topic on drought-driven root mortality is the role of seasonal sugar to starch conversion on root survivorship over dormancy periods. Seasonal carbohydrate conversion is a highly regulated, synchronized and well-studied process in poplar trees (Sauter & Cleve 1994, Schrader & Sauter 2002). In this process starch accumulates in stems and roots of poplars during summer and early fall. This accumulation is followed by the conversion of almost all accumulated starch into sucrose during late fall and winter. During this period soluble sugars concentration, especially sucrose, remains high until early spring, when sucrose concentration decreases dramatically. This reduction is closely synchronized with bud expansion and leaf flush. Once leaves expand and growth initiates accumulation of starch starts again (Sauter & Cleve 1994, Schrader &

Sauter 2002). In addition to forming readily available energy pools needed to reinitiate growth in early spring, accumulation of soluble sugars during winter play a critical role in cell protection against cold stress. As part of the present work, I hypothesize that drought-induced disruption of this seasonal cycle could play a central role on plant mortality under hydraulic stress and present a possible mechanism linking seedling mortality with changes in the seasonal carbohydrate cycle.

1.1.7 Insects and pathogens as agents of plant mortality

One of the central predictions in the CSH proposes that under drought conditions severe or repeated attack of insects or pathogens may lead to increased plant mortality, even if carbon reserves are not completely exhausted or in the absence of catastrophic hydraulic failure (McDowell *et al.* 2008). This prediction is based on the documented correlation between drought and insect outbreaks (Mattson & Haack 1987, Waring & Cobb 1992, Hogg & Schewarz 1997, Hogg *et al.* 2002) and pathogens (Manion 1991).. It has been suggested that these unusually warm conditions associated with prolonged drought periods may drive an increase on insect intrinsic population growth rate, the number of generations produced per year, synchrony of key developmental phases, winter mortality, and geographic range (Ayres & Lombardero 2000, Logan & Powell 2001, Logan *et al*. 2003). Droughts conditions may also impact insect and pathogen demographics by reducing the abundance of key predators and mutualists or by changing the synchrony of emergence between them (Ayres & Lombardero, 2000.

1.2 Research rationale and outline

In my dissertation research I explored some of the many basic questions derived from the CSH (*sensu* McDowell *et al*. 2008, McDowell 2011) and the new data presented by Anderegg (2012) and Sala (2012) supporting a more integrative view of plant physiological responses at the whole plant scale to drought stress, rather than trying to test if seedling mortality is solely associated with carbon starvation or hydraulic failure. To accomplish this I designed four experiments applying simulated drought to aspen (*Populus tremuloides* Michx) and balsam

poplar (*Populus balsamifera* L) seedlings both extensively studied model species with important commercial, scenic and cultural value. I selected these species because, although species and clones in the genus *Populus* are generally considered isohydric (e.g. Ceulemans et al. 1998, Tardieu & Simonneau 1998, Silim et al. 2009), *P. tremuloides* and *P. balsamifera* have distinctly different short-term stomatal behaviours during dry down and clear differences in drought tolerance. In these experiments I performed extensive gas exchange, water relations and carbon dynamics measurements to addressed basic questions outlined as follows:

Experiment 1 (Chapter 2). Stomatal control of balsam poplar seedlings under simulated drought: functional trade-offs between hydraulic safety and root carbon dynamics. In this chapter I describe changes in physiological parameters in balsam poplar seedlings in response to 1 and 4 weeks of severe and mild simulated drought. I aimed to test the following two questions: (1) Will balsam poplar seedlings prioritize carbon production and storage over hydraulic safety and will their response fit the typical description of anisohydric species due to its short-term stomatal behaviour, and (2) over time will this prioritization scheme result into catastrophic hydraulic failure particularly under severe stress as stem water potential becomes more negative while carbon reserves continue to remain high?

Experiment 2 (Chapter 3). Root carbon reserve dynamics in aspen seedlings: does simulated drought induce reserve limitation? In this experiment I described the short-term dynamics and the interrelationships among physiological variables and root carbohydrate reserves in aspen seedlings in response to a severe 3 month drought period. I aimed to answer the following two questions: (1) How are water relations, gas exchange and root carbohydrate reserves influenced by a seasonal drought event in aspen seedlings? and (2) Is a 3-months drought, the average length of a natural drought in the prairie area of Western Canada, long enough to significantly reduce root C reserves of an aspen seedling?

Experiment 3 (Chapter 4). Low reserve accumulation during drought may lead to seedling mortality during the next growing season (or the following growth season). After the experience gained in the previous two experiments it

was possible to establish a multi-seasonal experiment collecting carbohydrate data for the two species at the whole plant scale. This work aimed to determine the response of gas exchange, water relations, growth and reserve allocation variables of two closely related tree species to simulated drought conditions. In this work I presented direct measurements of (1) concentration of nonstructural carbohydrates (NSC) at the tissue scale and NSC concentration at the whole-plant scale, (2) percentage loss of conductivity and stem water potential and, (3) changes in above and below ground mass during drought in seedlings of two *Populus* species that vary in stomatal behavior and xylem vulnerability.

Experiment 4 (Chapter 5). Combined effects of defoliation and drought on carbon reserve accumulation of seedlings in two *populus* species. Following the results obtained in our previous chapter, focused primarily on NSC dynamics under drought, in this experiment I aimed to explore the effect of defoliation, which also limits whole-plant assimilation capacity by reducing photosynthetic surface, on growth, gas exchange, water relations and NSC accumulation dynamics of trembling aspen and balsam poplar under well-irrigated and severe drought conditions. This experiment was designed to address the following questions: (1) Are growth, gas exchange, water relations and NSC accumulation dynamics of trembling aspen and balsam poplar differently affected by defoliation?, (2) Are these variables significantly different in defoliated seedlings in comparison with well-irrigated undefoliated controls? and, (3) does drought magnify the effect of defoliation on these variables?

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CHAPTER 2. STOMATAL CONTROL OF BALSAM POPLAR SEEDLINGS UNDER SIMULATED DROUGHT: FUNCTIONAL TRADE-OFFS BETWEEN HYDRAULIC SAFETY AND ROOT CARBON DYNAMICS.

2.1 Introduction

Balsam poplar (*Populus balsamifera* L.) is a fast-growing highly productive tree species that can form extensive stands along river valleys of western Canada and the United States, but is also commonly found in newly disturbed mesic and mesic to dry environments (Zasada & Phipps 1990). In Alberta, balsam poplar commonly grows in association with aspen (*Populus tremuloides* Michx.) colonizing a wide range of habitats from highly disturbed areas (Frey *et al.*. 2003) to river valleys and seepage areas on hill sites (Amlin & Rood 2003). Compared to aspen, balsam poplar is also more tolerant to cold soil temperatures (Landhäusser *et al.*. 1996; Landhäusser & Lieffers 1998) and shorter growing seasons allowing this species to grow closer to the altitudinal and latitudinal treelines in the mountains and in the subarctic. In the subarctic, balsam poplar was able to establish successfully from seed in upland tundra after a fire disturbance (Landhäusser & Wein 1993). It is unclear however, what physiological adaptations allow balsam poplar to colonize this wide range of habitats and edaphic conditions, particularly soil moisture. Balsam poplar is known as a species that can, under high vapour pressure deficits, be decoupled from the climate conditions, as stomata appear to stay open under increased evaporative demands (Bladon *et al.* 2006). This anisohydric stomatal behavior allows plants to maintain their stomata opened or partially opened during periods of stress such as drought (Tardieu & Simonneau 1998).

Anisohydry also poses a functional tradeoff at the whole-plant scale. Although keeping the stomata open may reduce the possibility of carbon limitation in the plant, it could make these plants more susceptible to catastrophic hydraulic failure during period of drought because leaf and stem water potentials become more negative more quickly as soil water potential decreases (Tyree & Sperry 1988, McDowell *et al.* 2008, McDowell 2011). Drought-induced mortality is a well-

documented cause of death for seedlings of the genus *Populus*, particularly for species of the *Tacamahaca* section (e.g. *Populus trichocarpa*, *P. angustifolia* and *P. balsamifera*) that has been related to reduced root growth in comparison with *Populus* species of the *Aigeiros* section (e.g. *Populus deltoides* and *P. fremonii*) (Mahoney & Rood 1998, Rood *et al.*. 1998). However, there could also be other physiological responses associated with water stress that may lead to seedling mortality (Hsaio 1973), such as changes in biomass allocation (Mahoney and Rood 1992; Stella and Battles 2010), xylem cavitation (Tyree *et al.* 1994), reduced stomatal conductance and photosynthesis (Horton 2001a, Amlin & Rood 2002), and seedling initial root growth rate (Mahoney & Rood 1998) (see Rood *et al*. 2003a for a review on the topic). These responses may come into play over different time scales ranging from minutes (e.g. stomatal closure; Amlin & Rood 2003) to months (e.g. altered root to shoot ratio; Stella & Battles 2010).

Even though changes in physiological parameters in response to drought have been extensively studied in members of the genus *Populus*, there is very limited experimental data on how parameters such as net assimilation, carbon dynamics, stomatal conductance and xylem cavitation interact during periods of drought stress (Sala 2010, McDowell 2011). This gap in knowledge is particularly relevant for our understanding of mortality in seedlings. In this study we describe changes in physiological parameters in the anisohydric balsam poplar seedlings in response to 1 and 4 weeks of severe and mild simulated drought. We aim to test the following two questions in these seedlings: (1) Will balsam poplar prioritize carbon production and storage over hydraulic safety fitting the proposed model for a species with anisohydric stomatal behaviour and (2) over time will this prioritization scheme result into catastrophic hydraulic failure particularly under severe stress as stem water potential becomes more negative while carbon reserves continue to remain high.

2.2 Materials and methods

2.2.1 Plant material

Sixty balsam poplar seedlings were initiated from seed collected from open pollinated seed sources near Edmonton, Alberta (53.641° N, -113.367° W).

Seedlings were established under well-watered conditions in a greenhouse at the University of Alberta, Canada in June of 2009. After 4-weeks seedlings were transplanted into individual plastic pots (4-L, 6 inch diameter), one seedling per pot, filled with Metromix media (Metro Mix 290, Terra Lite 2000; W. R. Grace of Canada, Ajax, ON, Canada). Pots had four equidistant perforations at the base to allow excess water to drain. After growing for 10 weeks under an 18-h photoperiod at 21 °C and watered daily to field capacity, 48 seedlings were randomly selected and assigned to four groups of 12 plants each. Plants were randomly reassigned in each group until no significant differences were detected ($P < 0.05$) using a one-way ANOVA for initial plant height and stem basal diameter between the four groups. These groups were then designated as the mild and severe drought treatments (referred as MLD and SEV hereafter) and two corresponding control (referred as CON hereafter) groups. Two control groups (i.e. one for MLD and one for SEV) were used because MLD and SEV groups were expected to reach its targeted stress levels at different times (in this study 3 days apart after 7 (MLD) and 10 (SEV) days, see drought treatment section below). Twenty additional seedlings were chosen and randomized the same way for destructive water potential sampling (see below).

2.2.2 Application of the drought treatment

At the beginning of the experiment, pots assigned to the MLD and SEV treatments were weighed daily using an Adam Equipment digital balance model PGW 4502e (Danbury, CT, USA). After each pot weight was recorded, plants were re-watered by adding the equivalent of half the weight that was lost from the day before. Plants in the MLD group were maintained under this water regime for 7 days until midday stem water potential (Ψ_{md}) was *c*. -1 MPa. For plants in the SEV group, the water regime was extended by 3 more days until Ψ_{md} was -1.3 MPa (see below for details). This desiccation protocol was implemented to simulate a gradual soil drying process, more similar to a natural drought event. Stem water potential measurements were performed daily on three randomly selected plants (data not shown). By allowing leaf and stem water potentials to equalize, leaf water potential can be used as a proxy for stem water potential (Begg & Tuner 1970). To accomplish this, leaves were kept inside an aluminum

foil envelope to equilibrate with the stem water potential for two hours prior to the leaf water potential measurement.

Due to the destructive nature of the measurement, midday water potential was determined using an additional set of 10 randomly selected plants for each the MLD and SEV drought conditions. Measurements were performed using a Compact Water Status Console (i.e. a portable Scholander-type pressure chamber) model 3115P40G4 (Soilmoisture Equipment Corp., CA, USA) and repeated until Ψ_{md} were -1 MPa in MLD and -1.3 MPa in SEV seedlings. These Ψ_{md} values were chosen as targets for being associated with less than 10 percent loss of hydraulic conductivity (-1 MPa; MLD group) and slightly less negative than Ψ_{md} associated with 50 percent loss of conductivity in balsam poplar seedlings growing under similar environmental conditions (-1.3 MPa; SEV group; balsam P_{50} = -1.41 MPa; Galvez and Tyree). After the Ψ_{md} target values in MLD and SEV plants were reached pots were watered daily by adding the full amount of weight lost from the day before for the rest of the experiment. Plants in CON groups were weighed daily for the first 10 days and re-watered daily to field capacity for 12 weeks.

2.2.3 Gas exchange and percentage loss of conductivity (PLC) measurements

One week after MLD plants reached their Ψ_{md} target values, CO_2 assimilation rate (A) and leaf stomatal conductance (g_s) were measured in all MLD plants and their CON group. The same variables were measured one week after the SEV plants had reached their Ψ_{md} target values. All physiological measurements were performed using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, Neb.). Measurements were performed between 0900 and 1100 hours on the youngest fully expanded leaf. Chamber's reference CO_2 concentration was set to 385 p.p.m. using a 12-g Li-Cor CO_2 cartridge as CO_2 source. Light environment in the chamber was set to 2,000 µmol m^{-2} s^{-1} after a 10-min induction period at 500 µmol m^{-2} s^{-1} using the 6400-2B red/blue LED light source of the chamber. The induction period was implemented to stabilize air humidity, flow and temperate prior exposing the measured leaf to the light-saturating photo flux density (PFD) level. Similar changes in PFD levels are common in the open

natural environments where *P. balsamifera*, a riparian pioneer trees species grows (Roden & Pearcy 1993). Measurements were taken after three minutes when A and g_s values were stable. The cuvette conditions are based on light response curves that were determined prior to measurements on three individual plants. From these curves the optimum induction time and the photon flux density to achieve maximum A was determined. A and g_s were measured on all remaining six plants in MLD, SEV and CON groups 3 weeks after the first measurements of each group were recorded (plants measured 1 week after reaching targeted stress levels were harvested to perform biomass and hydraulic measurements, see below). All measurements in the rest of this section were performed using the same number of samples and time schedule described above.

Percentage loss of hydraulic conductivity (PLC) was measured using a conductivity apparatus (Sperry *et al*. 1988) following a standardized protocol. Seedlings were cut at the stem base in the greenhouse and transported to the lab (approximately 200 m) inside black plastic bags to minimize stem dehydration. Stems cut from the pot were re-cut under water, discarding the 15-cm stem section proximal to the original cutting site in order to remove embolisms induced by cutting in air. Keeping the re-cut stem under water, five consecutive 2-cm stem segments from each stem were cut using a razor blade. Segments from each stem were mounted and measured at the same time in the conductivity apparatus. The apparatus' reservoir tank was filled with filtered (0.2 μm) 100 mM KCl solution prepared in deionized water. After the initial hydraulic conductivity (i.e., the initial value of k_h, expressed as k_i in Eq. 1) of each stem segment had been measured, the native embolism was displaced by flushing KCl solution from the reservoir under constant pressure (120 kPa) for 2 min. After being flushed, the segment's measured final hydraulic conductivity was taken as k_{max}. Preliminary tests were performed to ensure that k_{max} values did not change after repeated flushing. PLC was calculated from Eq. 1:

$$PLC = [(k_{max} - k_i) / k_{max}] \times 100 \quad (1)$$

k_s was calculated from k_i / A_w, where A_w is stem cross-sectional area.

2.2.4 Seedling and non-structural carbohydrates (NSC) measurements

At each collection, the seedling heights were recorded and plants were individually bagged in paper bags, separating leaf and root material which had been carefully washed to remove all the substrate. Stem material was not analyzed, as the stems were used to perform the PLC measurements described above. Total leaf area per plant was measured the same day using a LI-3000 leaf area meter (Li-Cor, Lincoln, Nebraska, USA). Leaf and root material was oven dried at 70 °C for 72 hours and weighed. All dried root samples were ground using a Wiley Mill to pass a 40 mesh screen and the ground root tissue was used to determine water soluble sugar and starch concentrations. Soluble sugars were extracted three times with hot 80% ethanol, followed by a reaction between the extract and phenol–sulfuric acid which allowed sugars to be measured colourimetrically (Chow & Landhäusser 2004). To measure starch concentrations, the tissue remaining after the ethanol extraction was digested with the enzymes α-amylase and amyloglucosidase followed by a colourimetrically measurable reaction with peroxidase-glucose oxidase-o-dianisidine (Chow and Landhäusser 2004). Soluble sugar and starch total root contents were calculated by multiplying the concentration values by the total root dry weight, and expressed in milligrams.

The experimental design was analyzed as a 4 × 2 factorial design with four drought treatments (MLD, SEV and corresponding CON treatments) and two collection times (Week 1 and Week 4 after the drought treatment had been established). All growth data were normally distributed and variances were equal. Two-way ANOVA were performed for height, leaf area, leaf number, PLC, stem water potential and root dry weight response variables, using statistical software package SigmaStat 4 (Systat Software Inc, Chicago, IL). Differences between means were considered significant at an α=0.05. When significant differences between the means were detected, all-pairwise multiple comparisons using the Holm-Sidak procedure was performed. CO_2 assimilation rate, stomatal conductance, sugar and starch content and concentration datasets failed tests for normality or independence of variance. These response variables were fitted with a linear mixed-effects model using the functions *lme* and *varIdent* from the *nlme* R package (Pinherio *et al*. 2010) to allow different variance structure for

Time and Treatment. Once the datasets were fitted, they were analyzed using ANOVA procedures with statistical package R (R-CRAN). Differences between means were considered significant at an α=0.05. To help visualize tendencies of change in time, the difference between the mean value of all the experimental variables and their corresponding controls was calculated and plotted as scatter from a zero-value line.

2.3 Results

2.3.1 Effects of drought on seedling growth

Both the MLD and SEV seedlings slowed their height growth compared to CON seedlings; however, seedlings in both treatments continued to grow in height and after four weeks the MLD seedlings almost caught up to CON seedlings while SEV seedlings added much less in height over the same time period (Figure 2.1a).

After Week 1, average leaf area in the MLD seedlings was not different from its corresponding CON seedlings; however after four weeks, the leaf area was lower in the MLD seedlings compared to CON seedlings (Figure 2.1b). Only one week into the treatment, SEV seedlings had lower leaf area than CON seedlings; however, no leaf loss was observed, and during the following three weeks no additional leaf area was added (Figure 2.1b). Similarly to leaf area, the average number of leaves in the MLD seedlings after the first week was not different from the CON seedlings; however, in the following weeks new leaves were added in the MLD seedlings but the addition of new leaves lagged behind the controls (Figure 2.1c). In SEV seedlings, leaf number was lower compared to the CON seedlings in the first week and seedlings did not add any new leaves to the shoot in the following three weeks (Figure 2.1c).

After one week, root dry weight in MLD and SEV seedlings was not different from the CON seedlings. Over the following three weeks root dry weight decreased to 65% of the CON in MLD seedlings (2.99 g) and to 43.5% of the control in SEV seedlings (3.49 g) (Figure 2.1d).

2.3.2 Gas exchange and water relations response to drought

At Week 1, leaf stomatal conductance (g_s) of MLD seedlings was only reduced by 18% from 0.426 mol H_2O m^{-2} s^{-1} in the controls to 0.349 mol H_2O m^{-2} s^{-1}, while in SEV seedlings g_s was reduced by 70% (Figure 2.2a). At Week 4, g_s in the MLD seedlings had decreased by 79% while in the SEV seedlings it had decreased by 97 % compared to the g_s measured in its corresponding CON seedlings (Figure 2.2a). At Week 1, CO_2 assimilation rate (A) in MLD seedlings was not different from CON seedlings (P=0.91), while in SEV seedlings A was reduced by 65% (Figure 2.2b). After and additional three weeks of drought A was reduced by 68% in the MLD seedlings and by 95% in the SEV seedlings (Figure 2.2b).

At Week 1 midday stem water potential (Ψ_{md}) in the drought treated seedlings was much lower than in CON seedlings, which coincided with the anticipated targeted values used for this study (i.e. MLD = -1 MPa, SEV = -1.3 MPa, Controls < -0.5 MPa; see also Material and Methods section for details) (Figure 2.3a). After an additional 3 weeks, Ψ_{md} in the MLD seedlings remained unchanged while it was further reduced to -1.98 MPa in the SEV seedlings (Figure 2.3a). Despite the significant differences in Ψ_{md} at Week 1, percentage loss of conductivity (PLC) in MLD and SEV seedlings was not different from CON seedlings (P>0.34) (Figure 2.3b). Even after the following three weeks, PLC values for MLD seedlings were not different from CON seedlings but increased from 10 % in the controls to 54.2% in SEV seedlings (Figure 2.3b; P<0.001).

2.3.3 Content and concentration of soluble sugars and starch in roots

At Week 1 soluble sugar content of MLD and SEV seedlings was no different from CON seedlings (54.2 mg). Over the following three weeks sugar content was about half that of CON seedlings in both the MLD seedlings (245 mg) and SEV seedlings (377 mg) (Figure 2.4a). At Week 1 starch content in MLD and SEV seedlings was not different from CON seedlings, but over the following three weeks starch content in CON seedlings increased much faster than in the treated seedlings where the MLD seedlings reached only 26.4% of the CON seedlings (306 mg; P=0.006) while the SEV reached only 18.4% of the CON seedlings (383 mg; P<0.001) (Figure 2.4b).

At Week 1 sugar concentration of MLD seedlings were not different from CON seedlings (both $P>0.27$). Over the following three weeks sugar concentration in MLD seedlings were with 8.84% lower than the CON seedlings (7.04%) ($P=0.234$) while it increased to 11.5% in the SEV seedlings compared to CON seedlings with 9.69% ($P=0.167$) (Figure 2.4c). Overall, at Week 1 starch concentrations in MLD, SEV and their CON seedlings were very low (<1%), but over the following three weeks starch concentration increased significantly in droughted and CON seedlings. However, in the MLD and SEV seedlings, starch concentrations were only about half of the CON seedlings ($P<0.05$) (Figure 2.4d).

2.4 Discussion

Drought stress (SEV and MLD) did not result in a decrease of leaf area through leaf abscission or a cessation of height growth in balsam poplar seedlings, although drought significantly reduced stem water potential and increased the risk of hydraulic failure. This was particular evident in the MLD treatment, where seedlings continued to grow in height and added new leaves even after four weeks of drought exposure when A and g_s were already reduced by 75%. These results indicate that maintaining growth and leaf area under hydraulic stress appears to be an important short-term adaptation for balsam poplar seedlings.

Interestingly at Week 1, A and g_s in MLD seedlings appeared to be decoupled from the stem water potential as A and g_s were little affected by the drought treatment while stem water potential decreased by 100%. These results are consistent with findings of potted balsam poplar hybrids (Larchevêque *et al*. 2011) and mature balsam poplar (Bladon *et al*. 2006) where under water stress, stomatal behaviour was found to be decoupled from climatic (VPD) or soil moisture conditions, as stomata stayed open even under increased evaporative demands. This may provide a functional advantage over species with a more coupled stomatal control where stomata are closed under increased evaporative demand (e.g. *Populus tremuloides*; Galvez *et al*. 2011). Interestingly, this stomatal behavior in balsam poplar (although overall clearly isohydric) could be characterized as somewhat anisohydric, as it prioritized the carbohydrate

production for continued growth over hydraulic safety, particularly under the mild stress conditions.

After the first week of drought there were no significant differences in root growth between droughted and control seedlings detectable regardless of drought severity. However, after four weeks the root mass in the control seedlings had doubled, while root mass in droughted seedlings remained similar to Week 1. This may imply that the root system did not respond to the drought treatments and that root growth had stopped throughout the four week drought treatment. This might indicate that even a mild drought in balsam poplar does not result in greater allocation to the root system. This supports observation made on poplar seedlings of the *Tacamahaca* section (i.e. *Populus trichocarpa*, *P. angustifolia* and *P. balsamifera*) which had low initial root growth rates (Mahoney and Rood 1998). In addition, since root reserves had increased in both drought treatments by about 5% in week 4, a potential root loss could have been masked in droughted seedling. The lack of allocation of carbon to the root system under drought conditions, as well as balsam poplar's anisohydric behavior (see above) is consistent with recent findings of Larchevêque *et al*. (2011) for balsam poplar and two hybrids. In their work, the authors reported that, even though *P. balsamifera* had higher CO_2 assimilation rate than *P. balsamifera x P. trichocarpa* and *P. balsamifera x P. maximowiczii* hybrids when growing at a gravimetric water content near zero, the photosynthates produced were not used to grow roots. However, the authors did not speculate on the ultimate fate of the photoassimilates produced during the drought period.

Nonetheless after four weeks of hydraulic stress in the SEV seedlings, this prioritization scheme of balsam poplar seedlings resulted in catastrophic hydraulic failure for the xylem. Catastrophic hydraulic failure in the SEV seedlings can be assumed, since the percentage loss of hydraulic conductivity (PLC) was greater than 50% and stem water potential had reached -1.98 MPa (Figure 2.3a,b), a value significantly above the species' specific P_{50} value ($P_{50} \approx$ -1.4 MPa) (Tyree *et al*. 1994, Hacke & Sauter 1996). Percentage loss of hydraulic conductivity (PLC) and midday stem water potential (Ψ_{md}) maintain a sigmoidal relationship, known as a vulnerability curve, where PLC increases as Ψ_{md} decreases (Tyree & Sperry *et al*. 1988, Tyree & Zimmerman 2002). Vulnerability

curves for balsam poplar (Tyree *et al*. 1994, Arango *et a.*. 2011) show that the vulnerability curve is very steep at near the Ψ_{md} values corresponding to P_{50} hence the species with anisohydric behaviour are very prone to runaway embolism. We speculate that runaway embolism may outweigh any possible advantage associated with maintaining stomatal conductance and CO_2 assimilation at this level of hydraulic stress. These results support the idea that *P. balsamifera*, as other members of the section *Tacamahaca*, are not very hydraulically adapted (i.e. the species is prone to catastrophic hydraulic failure at relatively high water potentials) to resist severe drought stress (Tyree *et al*. 1994, Sparks & Black 1999).

One week into the experiment, root sugar and starch content and concentration were similar in droughted (MIL or SEV) and CON seedlings. This suggests that regardless of drought intensity a one-week drought period was not enough to significantly impact balsam poplar's root carbon reserves and their dynamics. Interestingly, four weeks into the drought treatments, MLD seedlings had significantly lower reserves (sugars and starches) than the controls, whereas in the SEV treatment concentration of soluble sugars continued to be similar to the controls and only the starch reserves were much lower. This apparent switch in reserve dynamics in SEV seedlings may suggest the potential onset of osmotic adjustment, a well-documented response to drought in *Populus* species (Gebre *et al.* 1998; Tschaplinski *et al.* 1998) while in MLD seedlings it suggests that balsam poplar seedlings continued to prioritized the use of sugar to maintain stem and leave growth over root growth or reserve accumulation. Although root starch content and concentration in MLD and SEV seedlings increased from Week 1 to 4, values were one order of magnitude lower than in controls which is likely related to the significantly reduced net assimilation rate and provides additional support to the idea that under stress conditions reserve accumulation (i.e. starch) is a low priority for balsam poplar seedlings.

The results of our study also provide support to the idea that carbon dynamics and water relations in plants are not only closely coupled processes with continuous and dynamic feedbacks (McDowell 2011) but also are driven by the ecology of the species investigated. These results are in stark contrast to a closely related species, trembling aspen (*Populus tremuloides* Michx.). In this

species, seedlings growing under severe drought conditions ceased height growth, immediately reduced stomatal conductance, and significantly reduced leaf area, while increasing root reserves by 4 times in comparison with well irrigated growing seedlings (Galvez *et al*. 2011). We hypothesize that the difference of stomatal behavior between balsam poplar and trembling aspen in response to drought may be explained by their contrasting preferred habitats. Both species can grow in similar sites when site conditions are moderate in moisture, nutrients and soil temperatures (Peterson & Peterson 1996, Landhäusser *et al*. 2002, 2003). However aspen also occupies the dryer extremes of the spectrum, while balsam poplar occupies the moister (flood plains) and cooler extremes (Rood *et al*. 2003a, Rood *et al.* 2007). As a result of the drier growing conditions, adaptations of aspen to frequent disturbances such as fires are necessary. As aspen readily regenerates from its root system, root reserves play a significant role in its regeneration after disturbance and root starch accumulation is an important strategy for survivorship (Schier & Campbell 1978, Schier & Smith 1979, Burns & Honkala 1990). As a result aspen might show more of an isohydric stomatal behaviour and has more negative P_{50} value (-2.25 MPa) than balsam poplar (-1.4 MPa); Cai & Tyree 2010, Galvez & Tyree unpublished). On the other hand, balsam poplar is a common species of riparian and lowland seepage areas which experience disturbance regimes such as flooding, sedimentation, and slides which requires establishment from seed (Mahoney & Rood 1998, Rood *et al*. 1998, Amlin & Rood 2003, Rood *et al.* 2003a). However, despite the contrasting responses between these two species, our results suggest that in both species carbon optimization (i.e. starch accumulation in aspen and aboveground growth in balsam poplar) may be prioritized over hydraulic safety under drought conditions which concurs with the hypothesis presented by Cowan & Farquhar (1977) and Raven (2002) proposing that the optimization of C versus water loss plays an important role in plant evolution.

2.5. References

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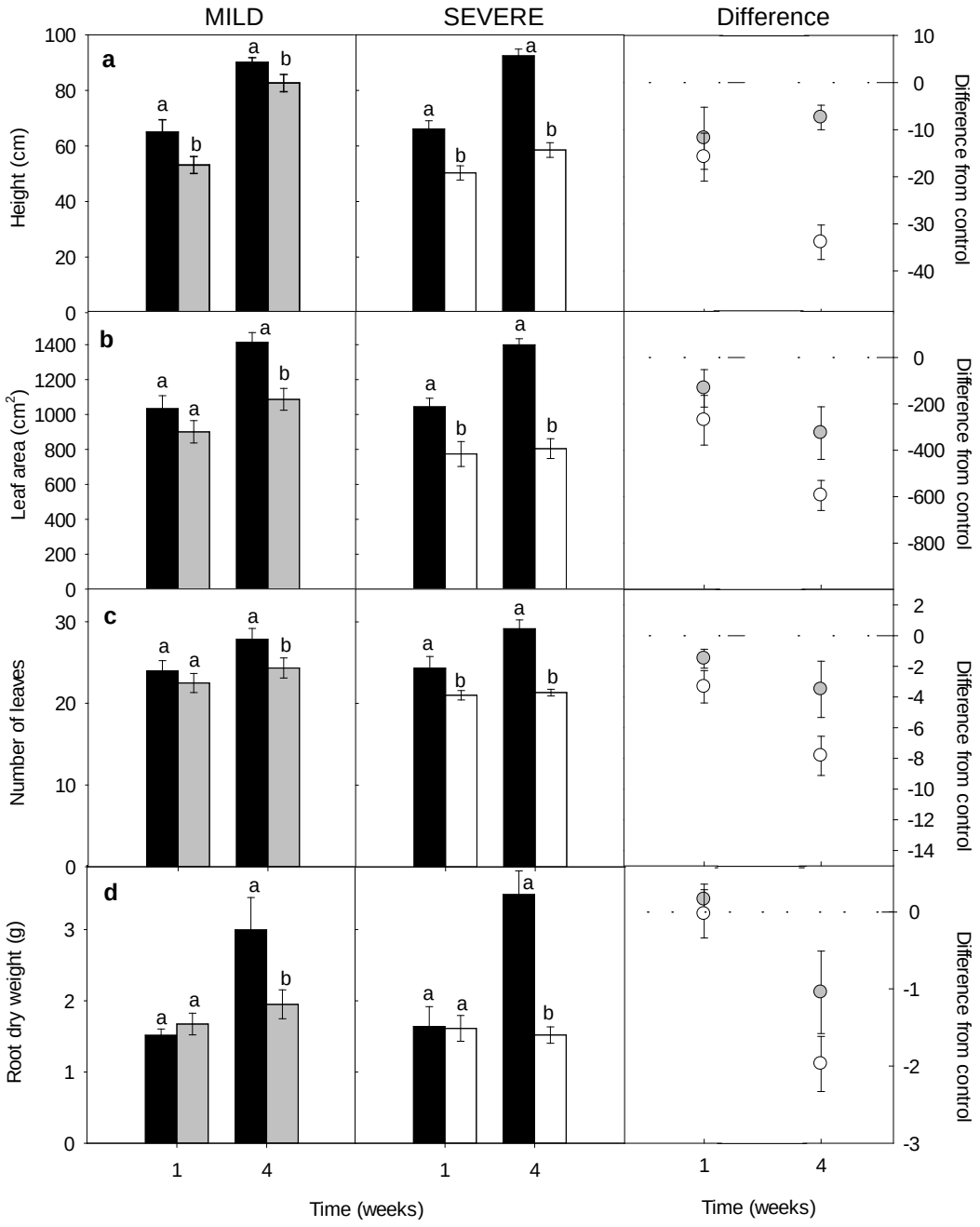

Figure 2.1 Mean (± SE) of plant height (a), leaf area (b), total leaf number (c) and root dry weight (d) of control balsam poplar seedlings (black bars) in response to mild (gray bars) and severe (open bars) drought conditions and their difference from their respective well-irrigated controls (third column), (n=6). Means followed by a different letter within each bar pair were significantly different ($P < 0.05$).

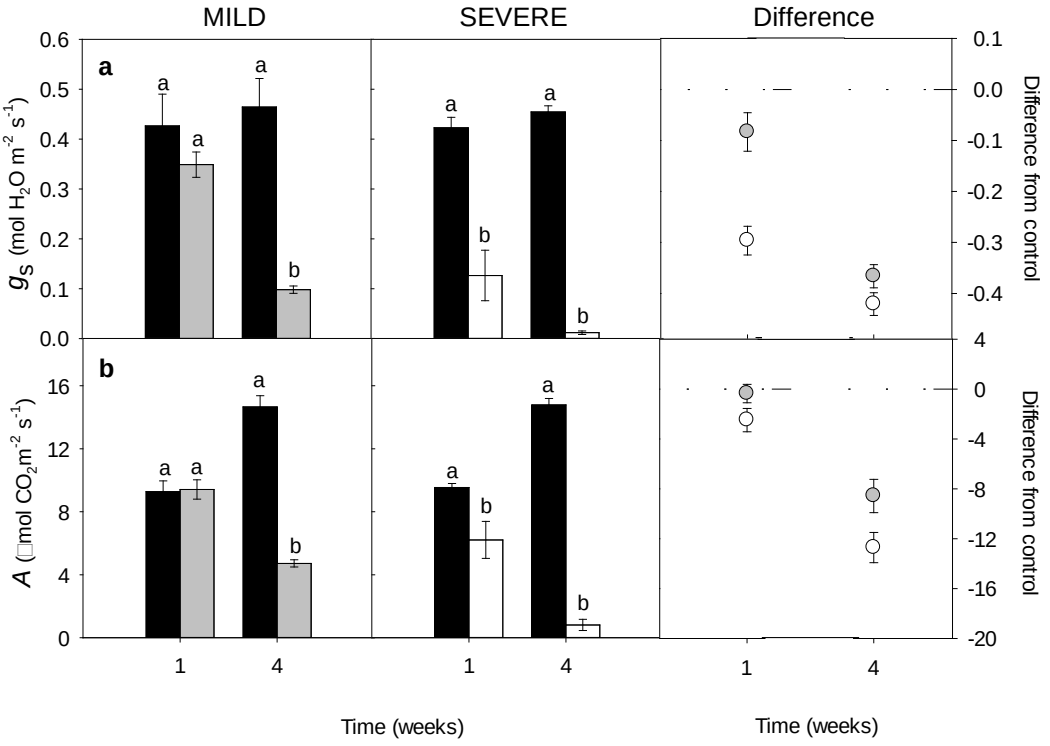

Figure 2.2 Mean (± SE) of leaf stomatal conductance (a) and assimilation rate (b) of control balsam poplar seedlings (black bars) in response to mild (gray bars) and severe (open bars) drought conditions and their difference from their respective well-irrigated controls (third column), (n=6). Means followed by a different letter within each bar pair were significantly different ($P < 0.05$).

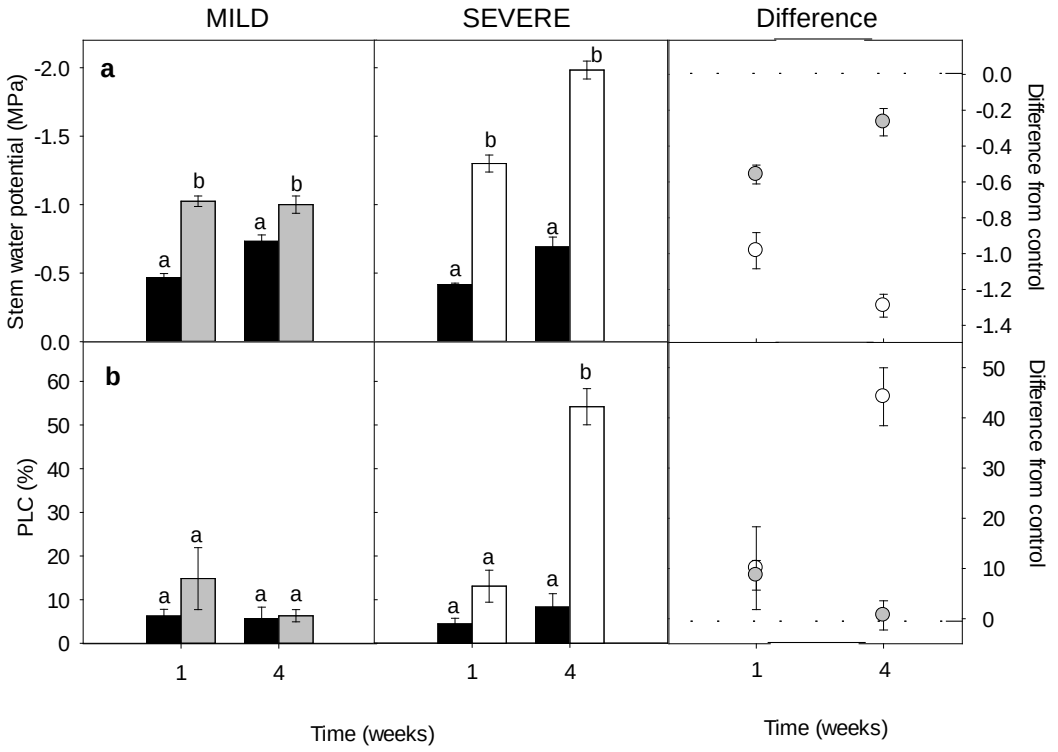

Figure 2.3 Mean (± SE) of stem water potential (a) and percentage loss of conductivity (PLC; b) of control balsam poplar seedlings (black bars) in response to mild (gray bars) and severe (open bars) drought conditions and their difference from their respective well-irrigated controls (third column), (n=6). Means followed by a different letter within each bar pair were significantly different ($P < 0.05$).

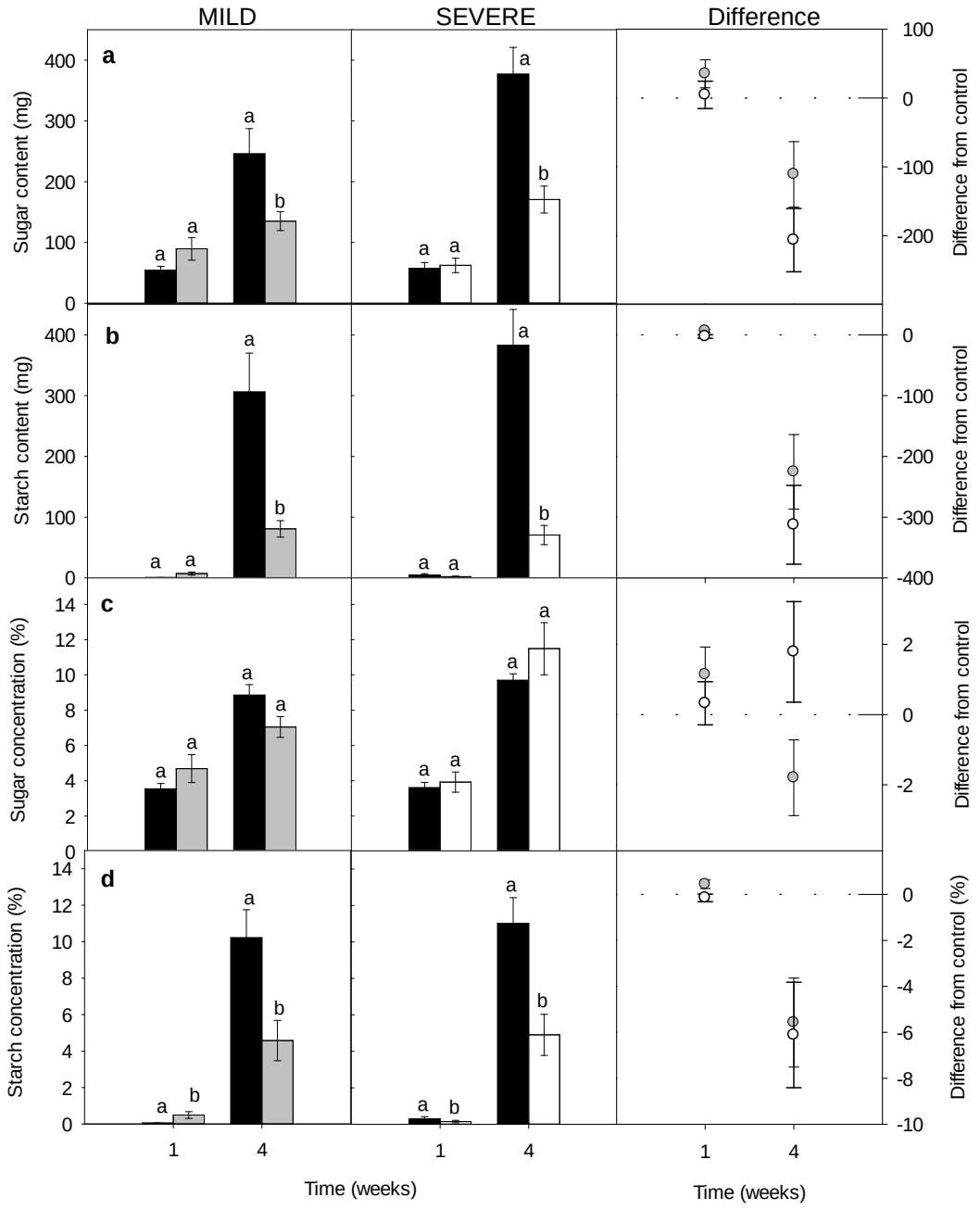

Figure 2.4 Mean (± SE) of sugar and starch root content (a, b) and concentration (c, d) of control balsam poplar seedlings (black bars) in response to mild (gray bars) and severe (open bars) drought conditions and their difference from their respective well-irrigated controls (third column), (n=6). Means followed by a different letter within each bar pair were significantly different ($P < 0.05$).

CHAPTER 3. ROOT CARBON RESERVE DYNAMICS IN ASPEN SEEDLINGS: DOES SIMULATED DROUGHT INDUCE RESERVE LIMITATION?[1]

3.1 Introduction

Changes in the intensity, length and frequency of drought events have recently been associated with forest and tree mortality at the global level (Allen *et al.* 2010). In boreal forests, these changes have been characterized as rapid non-linear events occurring at a faster rate than previously predicted (Soja *et al.* 2007) and have been associated with drought-induced mortality of trembling aspen (*Populus tremuloides* Michx.) observed across a million hectares in Saskatchewan and Alberta (Hogg *et al*. 2008). Currently the processes leading to this mortality are widely discussed and are thought to be related to mechanisms of water transport and/or carbon (C) limitation (McDowell *et al*. 2008).

The dynamics of stem water potential, stomatal conductance, and photosynthesis are dramatically affected during drought and are proposed to lead to C depletion and C limitation in plants (Mc Dowell *et al.* 2008, Sala *et al.* 2010). This becomes especially important for isohydric species which close their stomata early to maintain leaf evapotranspiration (*E*) below a critical value (Tardieu & Simonneau 1998) to avoid catastrophic xylem failure (Tyree & Zimmermann 2002). This physiological response should be closely linked to carbon reserves (McDowell *et al*. 2008) particularly in the root system which is a large sink for non-structural carbohydrates, as it is entirely dependent on the autotrophic parts of the plant and might require up to 50% of the produced photosynthates (Lambers *et al.* 2008).

Drought modulates C dynamics through a complex cascade of events: (i) as soil dries and stomata close, photosynthesis is reduced, (ii) if a plant's C demand is larger than the C supply (via photosynthesis) overall C-balance becomes

[1] A version of this chapter was published on June 2011. Galvez D.A., Landhäusser S.M., Tyree M.T. 2011. Root carbon reserve dynamics in aspen seedlings: does simulated drought induce reserve limitation? Tree Physiology 31:250-257.

negative, (iii) leading to a consumption of stored C reserves (C depletion) in order to maintain metabolic processes, some of which are critical under water stress (e.g. osmotic regulation), (iv) if drought conditions continue, C reserves become limiting to physiological processes (C limitation) (e.g. Sala *et al*. 2010).

Simultaneous changes in multiple physiological domains (i.e. water relations, gas exchange and root carbohydrate reserves) in response to drought are scarcely documented in the literature, normally including measurements of only one or two of these domains (e.g. Guehl *et al*. 1993, Carpenter *et al*. 2008) and are mostly presented comparing initial and final values (e.g. Runion *et al*. 1999), missing the dynamic and interdependent changes of these proxies over time. As a result, specific information on how drought events affect the dynamics of carbohydrate reserve accumulation and consumption in time and linking them to other physiological variables in tree species is limited (see McDowell & Sevanto 2010; Sala *et al*. 2010 and references within).

Aspen and other members of the genus *Populus* have become key model tree species that are comprehensively used for research in plant molecular biology, ecology and physiology as they are economically important, easy to propagate from seeds or cuttings, and grow quickly. Specific mechanisms for drought tolerance have also been studied in *Populus* species, however, mainly focusing on gas exchange and water relations (Bassman & Zwier 1991, Silim *et al*. 2009), genetics (Street *et al.* 2006, Bonhomme *et al.* 2009) and growth (Strong & Hansen 1991, Huang *et al*. 2008) and not on the interaction between photosynthesis and water relation and carbon accumulation under drought conditions.

Here we describe the short-term dynamics and the interrelationships among physiological variables and root carbohydrate reserves in aspen seedlings, an isohydric species, in response to a severe 3 month drought period. We aim to answer the following two questions: (1) How are water relations, gas exchange and root carbohydrate reserves influenced by a seasonal drought event in aspen seedlings? and (2) Is a 3-months drought, the average length of a natural drought in the prairie area of Western Canada, long enough to significantly reduce root C reserves of an aspen seedling?

3.2 Materials and Methods

3.2.1 Plant material

Ninety *Populus tremuloides* seedlings were established from seed under well-watered conditions in a greenhouse at the University of Alberta, Canada in April of 2009. After 4-weeks seedlings were transplanted into individual 4-l plastic pots, one seedling per pot, filled with Metromix media (Metro Mix 290, Terra Lite 2000; W. R. Grace of Canada, Ajax, ON, Canada). Pots had four equidistant perforations at the base to allow excess water to drain. After growing for 10 weeks under an 18-h photoperiod at 21 °C and watered daily to field capacity, 72 seedlings were randomly selected and assigned to two groups of 36 plants each. A one-way ANOVA was performed to check whether there were significant differences in initial plant height or stem basal diameter between the groups. Plants were randomly reassigned in each group until no significant differences were detected (P = 0.203). These groups were then designated as droughted (DRY) and control (CON) groups.

3.2.2 Application of the drought treatment

At the beginning of the experiment (referred hereafter as Week 0), pots assigned to the DRY treatment were weighed daily using an Adam Equipment digital balance model PGW 4502e (Danbury, CT, USA). After each pot weight was recorded, plants were re-watered by adding the equivalent of half the weight lost from the day before. This water regime was maintained for 10 days until midday stem water potential (Ψ_{md}) was *c*. -2.25 MPa (see below for details). This desiccation protocol was implemented to simulate a gradual soil drying process, more similar to a natural drought event. Midday stem water potential was determined daily in ten randomly selected plants from each treatment (data not shown). Measurements were performed on one leaf per plant. Leaves were kept inside an aluminum foil envelope for two hours prior to the measurement to allow leaf and stem water potentials to equalize so that leaf water potential can be used as a proxy for stem water potential. Measurements were performed using a Compact Water Status Console (i.e. a portable Scholander-type pressure chamber) model 3115P40G4 (Soilmoisture Equipment Corp., CA, USA) and

repeated until midday stem water potential (Ψ_{md}) in the DRY group was -2.15 ± 0.0428 (MPa; mean ± SE, n=10). This Ψ_{md} value was chosen as a target for being slightly less negative than Ψ_{md} associated with 50 percent loss of conductivity in aspen seedlings growing under similar environmental conditions (P_{50} = -2.25 MPa; Cai & Tyree 2010). After the Ψ_{md} target value in DRY plants was reached, DRY pots were watered daily by adding the full amount of weight lost from the day before for the rest of the experiment. Plants in the CON group were weighed daily for the first 10 days and re-watered daily to field capacity for 12 weeks.

3.2.3 Gas exchange and percentage loss of conductivity (PLC) measurements

At Week 0, CO_2 assimilation rate (A) and leaf stomatal conductance (g_s) were measured in ten randomly selected plants of each group using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, Neb.). Measurements were performed between 0900 and 1100 hours on the youngest fully expanded leaf. Chamber's reference CO_2 concentration was set to 385 p.p.m. using a 12-g Li-Cor CO_2 cartridge as CO_2 source. The light environment in the chamber was set to 2,000 µmol m^{-2} s^{-1} after a 10-min induction period at 500 µmol m^{-2} s^{-1} using the 6400-2B red/blue LED light source of the LI-6400's chamber. The induction period was implemented to stabilize air humidity, flow and temperate prior exposing the measured leaf to the light-saturating photo flux density (PFD) level. Similar changes in PDF levels are common in natural environments where *P. tremuloides*, a fast growing pioneer trees species, germinate and growth. Measurements were logged after three minutes at 2,000 µmol m^{-2} s^{-1} when A and g_s values were stable. The cuvette conditions were based on three light response curves that were determined on individual plants in which the optimum induction time and the photon flux density to archive maximum A were determined. Measurements were taken on six randomly selected plants in each treatment after 4, 6, 8, 10 and 12 weeks (no repeated measures). All measurements in the rest of this section were performed using the same number of samples and time schedule described above.

Percentage loss of hydraulic conductivity (PLC) was measured using a conductivity apparatus (Sperry *et al.* 1988) following a standardized protocol. Seedlings were cut at the stem base in the greenhouse and transported to the lab (approximately 200 m) inside black plastic bags to minimize stem dehydration. Stems cut from the pot were re-cut under water, discarding the 15-cm stem section proximal to the original cutting site in order to remove embolisms induced by cutting in air. Keeping the re-cut stem under water, five consecutive 2-cm stem segments from each stem were cut using a razor blade. Segments from each stem were mounted and measured at the same time in the conductivity apparatus. The apparatus' reservoir tank was filled with filtered (0.2 µm) 100 mM KCl solution prepared in deionized water. After the initial hydraulic conductivity (i.e., the initial value of k_h, expressed as k_i in Eq. 1) of each stem segment had been measured, the native embolism was displaced by flushing KCl solution from the reservoir under constant pressure (120 kPa) for 2 min. After being flushed, the segment's measured final hydraulic conductivity was taken as k_{max}. Preliminary tests were performed to ensure that k_{max} values did not change after repeated flushing. PLC was calculated from Eq. 1;

$$PLC = [(k_{max} - k_i) / k_{max}] \times 100 \quad (1)$$

k_s was calculated from k_i / A_w, where A_w is stem cross-sectional area.

3.2.4 Seedling and non-structural carbohydrates (NSC) measurements

At each collection, the seedling heights were recorded and plants were individually bagged in paper bags, separating leaf and washed root material (stems were used to perform the PLC measurements described above). Total leaf area per plant was measured the same day using a LI-3000 leaf area meter (Li-Cor, Lincoln, Nebraska, USA). Leaf and root material were oven dried at 70 °C for 72 hours and weighed. All dried root samples were ground in a Wiley Mill to pass a 40 mesh screen and water soluble sugar and starch concentrations were determined for the root tissues. Soluble sugars were extracted three times with hot 80% ethanol, followed by a reaction between the extract and phenol–sulfuric acid which allowed sugars to be measured colourimetrically (Chow & Landhäusser 2004). To measure starch concentrations, the tissue remaining after the ethanol extraction was digested with the enzymes α-amylase and

amyloglucosidase followed by a colourimetrically measurable reaction with peroxidase-glucose oxidase-o-dianisidine (Chow & Landhäusser 2004). Since leaf area of seedlings changed over the 12 week experimental period, soluble sugar and starch root content was scaled by leaf area to account for these changes in leaf area. Therefore we present root sugar and starch as content per seedling leaf area (mg cm^{-2}) and referred hereafter as specific sugar (SSUC) and specific starch content (SSTC), respectively. To illustrate the conversion dynamics between water soluble sugars and starch during the experimental period, the ratio of sugar to starch concentrations (SSTR) was calculated.

The experimental design was analyzed as a 2 × 6 factorial design with two drought treatments (DRY and CON) and five collection times (4, 6, 7, 10 and 12 weeks). All response variables of seedlings growing in DRY and CON treatments were contrasted at Week 0 (starting point) using a t-test to identify potential differences between them before the drought treatment was applied. T-tests were performed with statistical software package SigmaStat 4 (Systat Software Inc, Chicago, IL). Values of all response variables at weeks 4, 6, 8, 10 and 12 were fitted using a linear mixed-effects model using the functions *lme* and *varIdent* from the *nlme* R package (Pinherio *et al*. 2010). The *lme* function allowed the use of time as random variable while *varIdent* allowed for a different variance structure for each level of the random variable (time). Once the data was fitted, it was analyzed using ANOVA procedures with statistical package R (R-CRAN). Differences between means were considered significant at an α=0.05.

3.3 Results

3.3.1 Effects of drought on seedling growth

At the beginning of the experiment (Week 0), height and total leaf area of seedling in DRY and CON groups were not significantly different (Figure 3.1a,b; *P*>0.05). Height of CON seedlings gradually increased over the entire duration of the experiment from 43.1 ± 2.0 cm at Week 0 to 98.3 ± 1.9 cm in Week 12 (mean ± SE; *n*=6), while height in DRY seedlings increased by 14% over the first four

weeks but then remained the same until the end of the experiment (Figure 3.1a; P>0.05).

Total leaf area of CON seedlings increased threefold during the length of the experiment from 994 ± 103 cm^2 at Week 0 to 3215 ± 128 cm^2 in Week 12 (mean ± SE; n=6) (Figure 3.1b). In contrast, total leaf area in DRY seedlings decreased over time. At Week 4, average leaf area was 32.8% less than at Week 0. During Week 2, all DRY seedlings had shed approximately the bottom third of their leaves, and they gradually shed more leaves during the rest of the experiment. Total leaf area in DRY seedlings decreased 52.7% over the whole experiment, from 850 ± 132 cm^2 at Week 0 to 403 ± 80 cm^2 at Week 12 (mean ± SE; n=6) (Figure 3.1b).

3.3.2 Gas exchange and water relations response to drought

Leaf stomatal conductance (g_s) of DRY seedlings decreased 86.6% during the first six weeks from 0.15 ± 0.008 mol H_2O m^{-2} s^{-1} to 0.02 ± 0.001 mol H_2O m^{-2} s^{-1} (mean ± SE; n=6) remaining without significant change during the rest of the experiment. (Figure 3.2a; P>0.05 n=6). The relatively abrupt stomatal closure in DRY seedlings during the first weeks of the experiment concatenated with a more gradual reduction in CO_2 assimilation rate (A) which did not plateau until Week 12, showing a 82% reduction by the end of the experiment (Figure 3.2b). A and g_s values in CON seedlings increased 29.7% and 90.1% respectively during the course of the experiment, relative to values recorded at Week 0 (Figure 3.2a,b).

Values of midday stem water potential (Ψ_{md}), a good integrator of soil and plant water stress, were not significantly different between CON and DRY seedlings at Week 0 (P>0.05 n=6). Seedlings under the DRY treatment showed a significant and abrupt reduction in midday stem water potential (Ψ_{md}) closely following the reduction in leaf stomatal conductance reported above, from -0.54 ± 0.05 MPa in Week 0 to -2.15 ± 0.04 MPa by Week 4 followed by a more gradual reduction to -2.43 ± 0.06 MPa (mean ± SE; n=6) at the end of the experiment. After four weeks in the DRY treated aspen seedlings lost 72.4% of stem conductivity. This value of percentage loss of conductivity (PLC) remained relatively constant during the rest of the experiment (Figure 3.3b). There were no significant changes in stem water

potential and PLC values in CON seedlings over the whole experiment (Figure 3.3a,b; $P>0.05$ $n=6$).

3.3.3 Concentration and content of soluble sugars and starch in roots

At week 0 soluble sugar and starch concentration of CON and DRY seedlings were not different ($P>0.05$), with soluble sugar concentrations being much higher than starch concentrations. During the experimental period sugar and starch concentrations in CON seedlings increased from 3.17 and 0.29 percent at week 0 to 5.57 and 9.64 percent respectively at week 12. In the roots of CON seedlings sugar concentration only increased between week 4 and 6 and remained relatively constant until week 12 (Figure 3.4a). In contrast starch concentrations increased slowly between week 4 and 8 and more rapidly between week 10 and 12 (Figure 3.4b). In DRY seedlings root sugar concentration increased from 4.13 percent at week 0 to a maximum of 10.17 percent at week 6 followed by a reduction at week 8 with a slight recovery to week 12 (Figure 3.4a). Starch concentration increased rapidly from 0.14 percent at week 0 to 17.72 percent at week 6 and then appeared to fluctuate somewhat between week 8 and 12 (Figure 3.4b). Overall, total nonstructural concentrations (sum of soluble sugars and starch) in root tissues were 73.69 percent higher in DRY than in CON seedlings.

At week 0, neither specific sugar (SSUC) nor specific starch content (SSTC) in roots of aspen seedlings were significantly different between CON and DRY seedlings (Figure 3.5a,b). Specific sugar and starch content of CON seedlings increased only slightly from 0.004 mg g^{-1} cm^{-2} to 0.099 mg cm^{-2} and from 0.037 mg cm^{-2} to 0.061 mg cm^{-2}, respectively (Figure 3.5a,b). Whereas SSUC and SSTC of DRY seedlings increased by two orders of magnitude from 0.005 mg cm^{-2} to 0.815 mg cm^{-2} and from 0.061 mg cm^{-2} to 0.416 mg cm^{-2}, respectively (Figure 5a,b).

At Week 0, sugar to starch ratio (SSTR) in roots of aspen seedlings was not significantly different between the CON and DRY treatments (Figure 3.5c) and in both treatments SSTR had declined steeply by week 4. After week 4, SSTR remained relatively constant for the rest of the experimental period but SSTR in CON seedlings was on average 2.7 times higher in DRY seedlings.

3.4 Discussion

Contrary to what might be expected from an isohydric species, reduced carbon assimilation as a result of severe drought stress did not result in a significant reduction of root carbon reserves in aspen seedlings. The results from our experiment quantified the complex interrelationship between water relations, gas exchange, and root carbon dynamics, showing that roots of aspen seedlings growing under severe water stress increased soluble sugar and starch reserves in the root system. This suggest a different use of the limited photoassimilates produced under drought conditions which were likely used for additional height growth and leaf area in the non-stressed seedlings. DRY seedlings had proportionally more carbon stored in the form of starch than the CON seedlings. Accumulation of starch during the initial stages of the drought period can be seen as a critical process because once a certain threshold of water deficit is reached, starch can be utilized to maintain a necessary concentration of soluble sugars needed for osmoregulation and osmoprotection (Chaves 1991). The higher SSTR in DRY seedlings could indicate more efficient C production but also could suggest a prioritization of starch accumulation for osmoregulatory processes over growth under drought conditions, as has suggested for other plants (Chaves *et al.* 2003).

Overall, this prioritization scheme may also constitute a strategy for aspen to prioritize root system survival, which can actively resprout (sucker) from its root system after a disturbance or stress such as fire, defoliation, or drought killed the aboveground portions (Bailey & Whitham 2002, Wan *et al*. 2006, Worrall *et al*. 2010).This idea concurs with a hypothesis presented by Cowan and Farquhar (1997) and Raven (2002) proposing that the optimization of C uptake *versus* water loss plays an important role in plant evolution. Additional support for evolutionary pressure favoring C optimization *versus* water loss can be found in the strong relationship between maximum stomatal conductance and leaf nitrogen concentration, and hence photosynthetic capacity, as reported by Schulze *et al.* (1994) over a range of vegetation types. The increase in sugar and starch reserves we observed in the roots of DRY seedlings indicates that a >50% loss of hydraulic conductance in the xylem caused by water stress was not sufficient to limit C translocation; although reduced conductance has been

reported as a possible mechanism regulating reserves and root mortality (Sung & Krieg 1979, Marshall 1986).

The duration of our stress period (3-months) is also ecologically significant for boreal forests because it spans the normal frost-free growth period in Alberta. Our results showed that even under this severe water stress (i.e. significantly reduced leaf stomatal conductance and CO_2 assimilation, stem water potential near the species' specific P_{50} value, and terminated growth) reserves in the roots of young aspen seedlings increased over the three month drought period.

This study highlights some of the intricacies of C dynamics in plants under stress: while some symptoms of C limitation in sink tissues were observed in DRY seedlings (e.g. terminated height growth and reduction in leaf area, and stomatal conductance), there was no indication of C depletion in the root system. The increase in soluble sugar concentrations in DRY seedlings may also suggest the potential onset of osmotic adjustment, a well-documented response to drought in *Populus* species (Gebre *et al*. 1998, Tschaplinski *et al.* 1998) in order to maintain higher (less negative) values of leaf water potentials. However, we caution against over-extending the significance of our results based on the first-year growth of seedlings. Both adult trees and seedlings facing several consecutive seasons of drought are likely to behave very differently. We recognize that there may be additional ecological and environmental factors modulating the conditions that potentially lead to C limitation and C depletion.

Aspen seedlings in the DRY treatment started to show indications of water stress one week after the drought treatment was initiated (e.g. reduction of leaf blade angle relative to seedling stem, reduction of leaf blade apparent turgor). Two weeks later all seedlings in this treatment shed approximately the basal third of their leaves. Shedding of basal leaves during periods of drought stress is a well-documented mechanism in poplars that simultaneously reduces transpiring surface area while making the limited water supply available to growing leaves and meristems in the seedling's distal section (Chen *et al*. 1997, Rood *et al.* 2000, Giovannelli *et al*. 2007). Leaf shedding may also play several additional roles besides reducing transpirational surface such as remobilization of nutrients during stress (Munne-Bosch & Alegre 2004), prevention of runaway embolisms by maintaining a favorable water balance at the whole-plant level, and leaf

temperature control (see Chaves *et al*. 2003 for a review on the subject). By Week 4, drought stressed seedlings had terminated height growth (i.e. terminal bud set) and with that the growth of new leaf area which is a well-documented response of aspen to drought (Hogg & Hurdle 1995). Reduced leaf area in combination with stomatal closure also triggers a series of physiological responses such as changes in ion uptake and transport, osmotic adjustment, nitrogen metabolism, starch reallocation from leaves (Iljin 1957, Hsiao 1973) and reduction of leaf water potential (Farquhar & Sharkey 1982). As leaf water potential decreased, stem water potential also decreased, reaching values near 50 percent loss of conductivity (P_{50}). Traditionally, conductivity values near P_{50} have been utilized as a proxy for severe whole-plant stress (Hacke *et al*. 2006) because compromising hydraulic conductivity may limit carbon gain, growth and productivity (Tyree 2003). DRY seedlings reached an average PLC value of 80 percent (Figure 3.3b) after Week 8; however this level of PLC did not decrease the reserves in roots and is unlikely to affect short-term survival of aspen seedlings. Lu *et al.* (2010) has shown that aspen seedlings of the same age could be droughted to ≥90% PLC and survive after re-watering; hence we tentatively reject the possible notion that hydraulic failure could confound our results.

Canadian boreal forests have suffered record periods of drought and corresponding tree die-offs during the past decade and this trend is expected to continue dramatically impacting forest composition in Western Canada (Hogg *et al*. 2008, Mbogga *et al*. 2009, Allen *et al.* 2010). More information is needed about the dynamics of carbon accumulation and depletion before, during, and after multiple annual-cycles of drought and dormancy especially in the case of aspen, a clonal species with remarkable capacity for regenerate via root sprouts. Also we need to gain a better understanding of the role of physiological (e.g. isohydric and anisohydric stomata behaviors), anatomical (e.g. pit membrane size and distribution) and environmental factors (e.g. length and intensity of seasonally repeated drought periods) modulating the overall impact of drought on carbon reserves.

3.5 References

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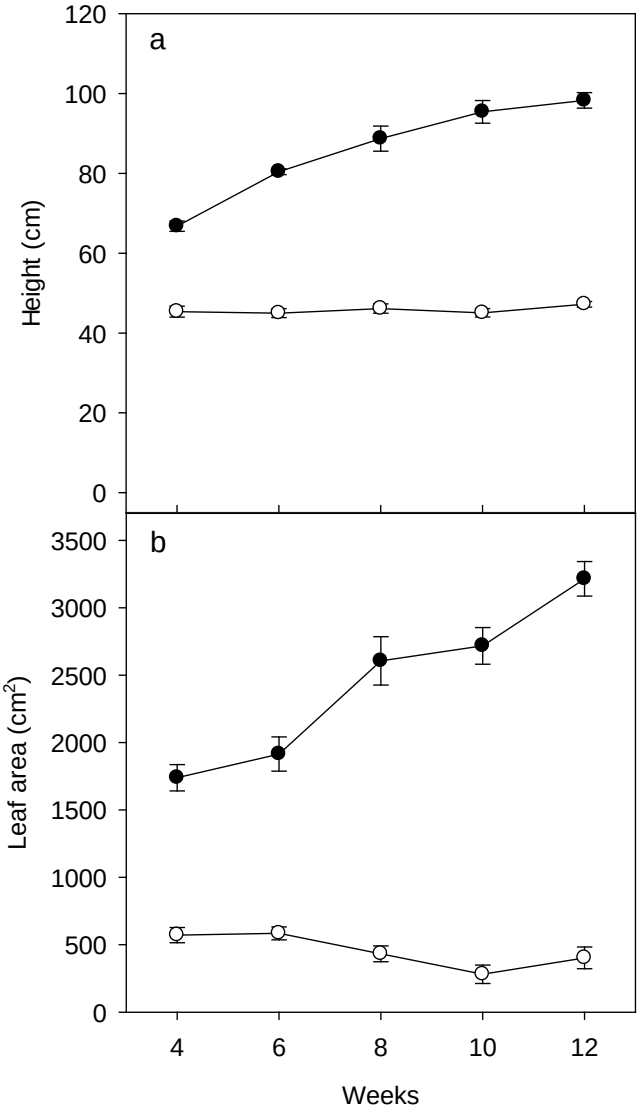

Figure 3.1 Mean (± SE) of plant height (a) and total leaf area (b) of aspen (*Populus tremuloides*) seedlings growing under drought (open circles) and well irrigated (solid circles) conditions for 12 weeks. Data are means ± SE; n=6.

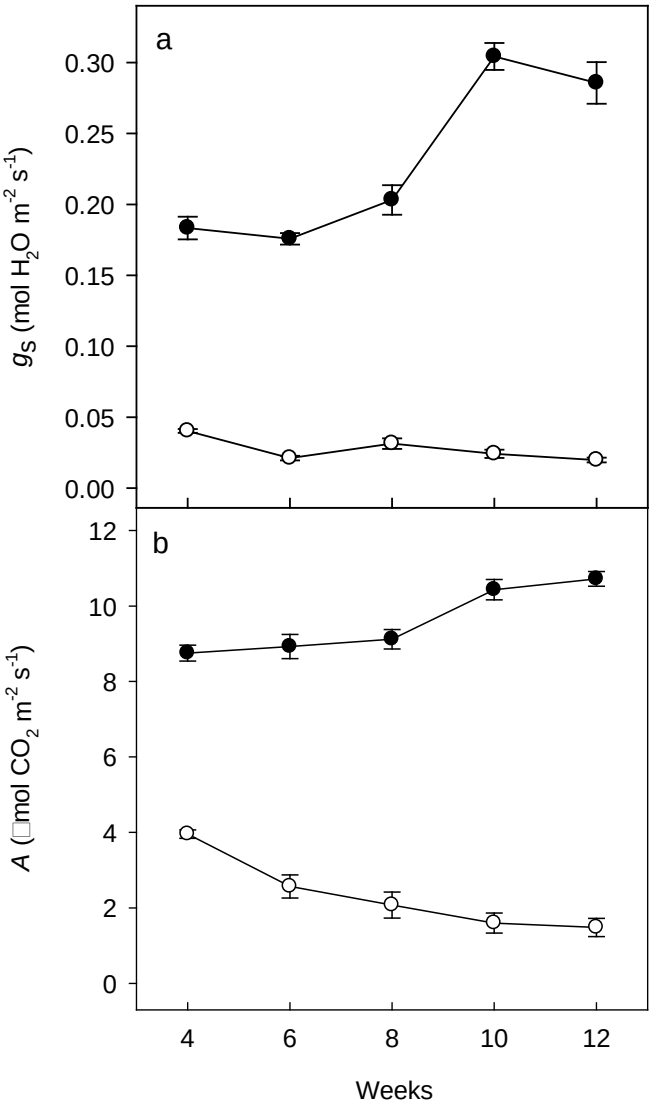

Figure 3.2 Mean (± SE) of leaf stomatal conductance (a) and assimilation rate (b) of aspen seedlings growing under drought (open circles) and well irrigated (solid circles) conditions for 12 weeks. Data are means ± SE; n=6.

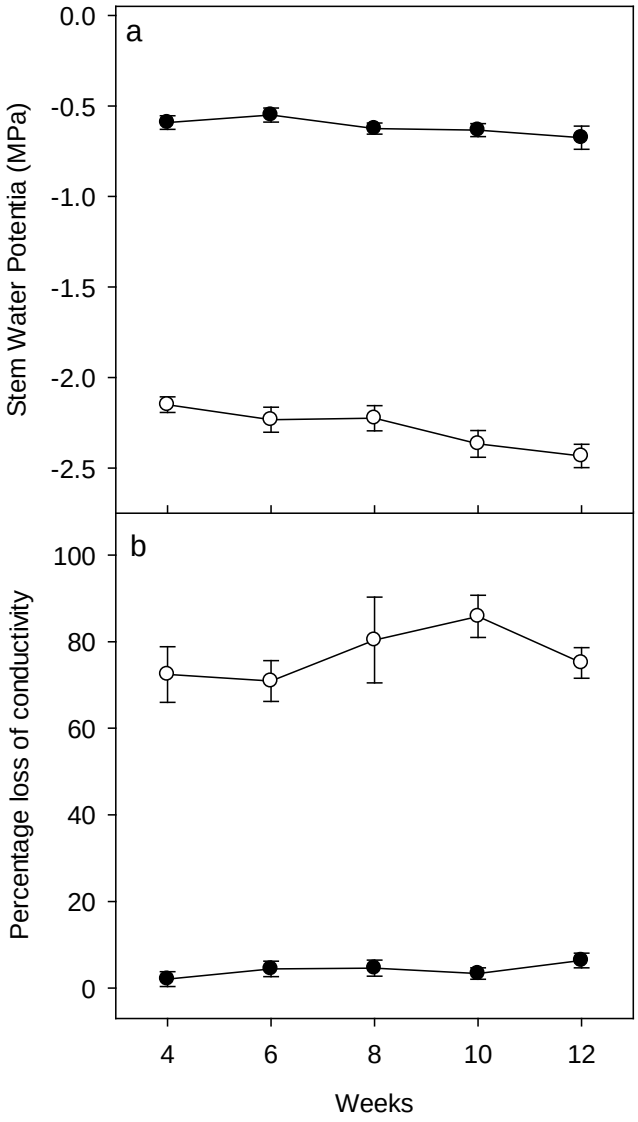

Figure 3.3 Mean (± SE) of stem water potential (a) and percentage loss of conductivity (b) in aspen seedlings growing under drought (open circles) and well irrigated (solid circles) conditions for 12 weeks. Data are means ± SE; n=6.

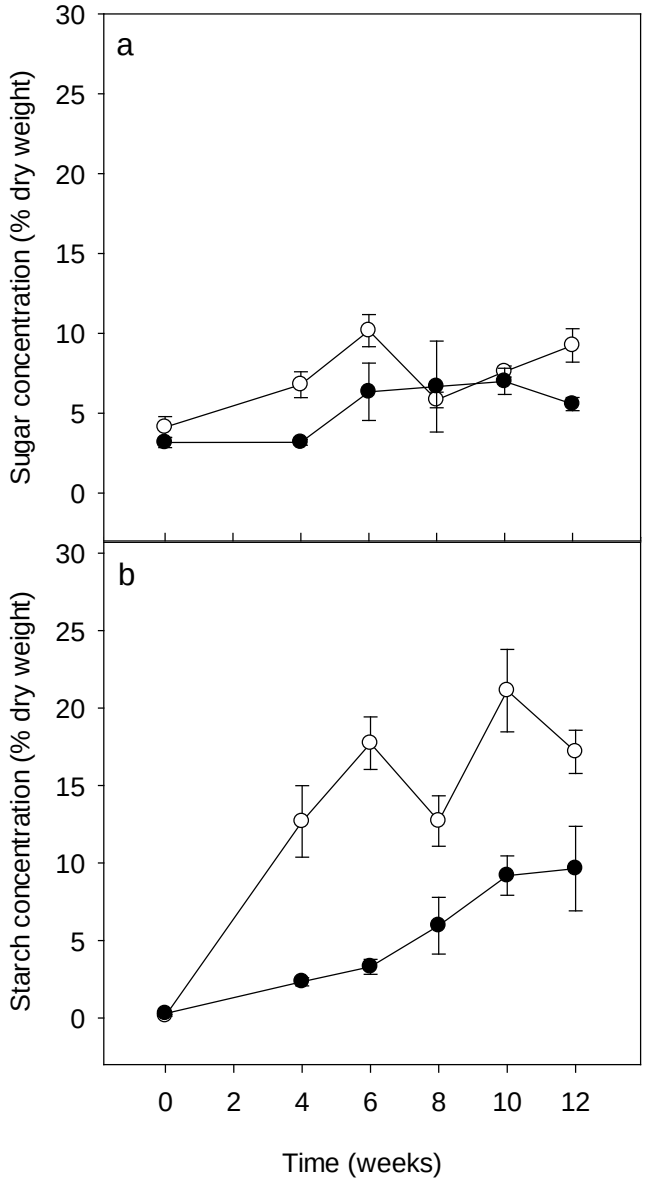

Figure 3.4. Mean (± SE) of sugar (a) and starch (b) concentration (% dry weight) in roots of aspen seedlings growing under drought (open circles) and well irrigated (solid circles) conditions for 12 weeks. Data are means ± SE; n=6.

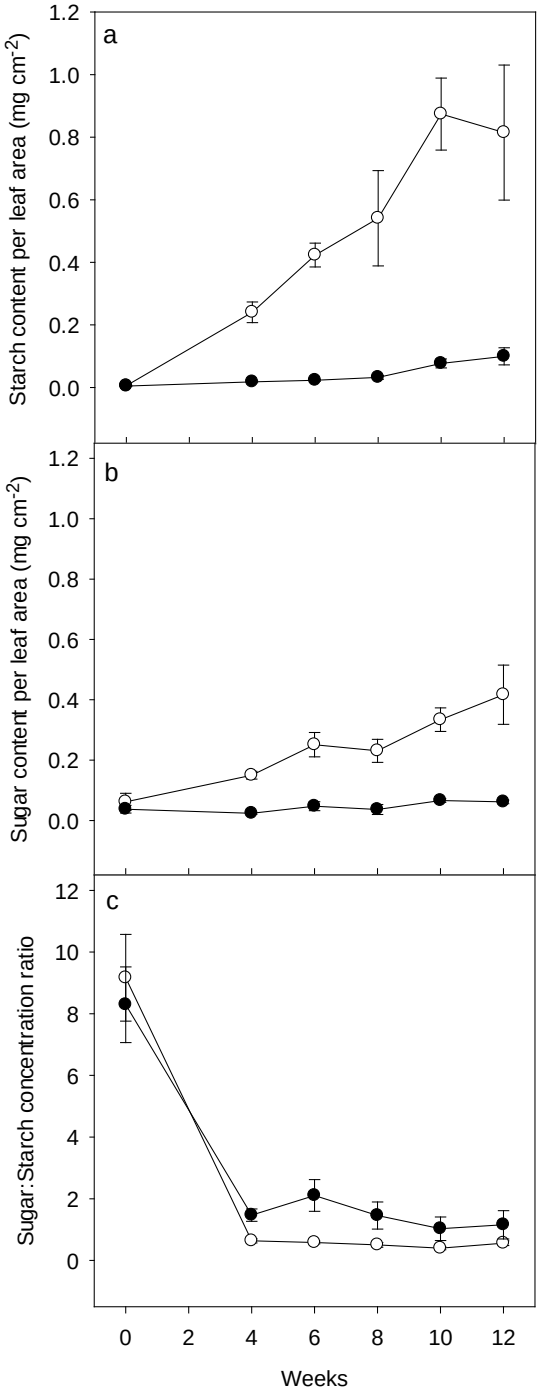

Figure 3.5 Mean (± SE) of starch (a) and sugar (b) content per unit leaf area and soluble sugar to starch ratio (c) in roots of aspen seedlings growing under drought (open circles) and well irrigated (solic circles) conditions for 12 weeks. Data are means ± SE; n=6

CHAPTER 4. LOW RESERVE ACCUMULATION DURING DROUGHT MAY LEAD TO SEEDLING MORTALITY DURING FOLLOWING GROWTH SEASON

4.1 Introduction

Non-structural carbon (NSC) reserves play a fundamental role in plant germination, growth, reproduction, defense and survivorship under stress. Although most of these roles have been studied for more than a century (e.g. Brown & Escombe 1898, Halsted 1902), the interaction between NSC reserves and water transport, especially under hydraulic stress, has not gained attention until recently. The ecological relevance of this interaction was initially highlighted by the carbon starvation hypothesis (McDowell *et al.*, 2008), which proposed two broad mechanisms, carbon starvation and catastrophic hydraulic failure, as the drivers of tree mortality under severe drought stress. Furthermore, Anderegg *et al.* (2012) suggested that a complex interaction between these two mechanisms is responsible for the widespread climate-induced die-off in aspen forests across Colorado, USA.

Although these proposed mechanisms aim to understand ecological events at the stand and ecosystem level, the underlying interrelationships between carbon dynamics and water transport at the organ and whole-plant level are far from understood (McDowell *et al.* 2008, Sala *et al.* 2010, Ryan 2011). Furthermore, the notion that under drought stress carbon allocation and water transport are likely closely-coupled processes is just starting to be recognized (Galvez *et al.* 2011, McDowell 2011, McDowell *et al.* 2011, Landhäusser & Lieffers 2012, Sala *et al.* 2012). In a recent review Sala *et al.* (2012) suggested that carbon reserves in trees are accumulated above a threshold, which is needed to maintain metabolic processes and hydraulic integrity, particularly under episodes of severe drought. The authors further speculate that maintaining hydraulic integrity is prioritized in order to avoid hydraulic failure, which would make tissue carbon reserves irretrievable (i.e. if xylem suffers catastrophic levels of embolism) after water transport ceases and the potential remobilization of reserves from sources to sinks would not be possible.

The interaction between carbon dynamics and water transport under drought conditions is likely more complex than what is currently understood. With a few exceptions (e.g. Galvez *et al.* 2011, Anderegg 2012), the interaction between carbon and water have been traditionally assessed using functional proxies to the whole-plant carbohydrate and hydraulic status (e.g. mass accumulation, CO_2 assimilation and stomatal conductance) instead of direct by measurements of them (i.e. content and concentration of NSC and percentage loss of conductivity (PLC)).

Xylem vulnerability to hydraulic failure and stomatal behavior (e.g. the timing and cues of stomatal opening and closure) are important drivers of plant responses to drought stress and can vary with species (Nardini *et al.* 2001, Sperry & Pockman 1993). Trembling aspen (*Populus tremuloides* Michx) and balsam poplar (*Populus balsamifera* L) are both fast growing boreal forest trees species that can coexist in sites that have similar mesic edaphic and climatic conditions. However, both species have different tolerances to drought stress, with aspen being the more tolerant species than balsam poplar. During water limitation, leaf stomatal conductance (g_s) in aspen decreased parallel with soil water content, maintaining a relatively constant stem water potential (i.e. isohydric behavior (Galvez *et al.* 2011)), while in balsam poplar g_s remained relatively unchanged when soil water content decreased until it dropped below a threshold, resulting in an abrupt change in stem water potentials (i.e. anisohydric behaviour; Larchevêque *et al.* 2011). Both species also appear to have different vulnerability to hydraulic failure, with balsam poplar being more subject to hydraulic failure than aspen, under relatively mild drought conditions (Tyree *et al.* 1994).

Our work aimed to determine the response of gas exchange, water relations, growth and reserve allocation variables of two closely related tree species to simulated drought conditions. In this work we present direct measurements of (1) concentration of non-structural carbohydrates (NSC) at the organ scale and NSC concentration at the whole-plant scale, (2) percentage loss of conductivity and stem water potential, (3) changes in above and below ground mass and, (4) seedling survivorship after a growing and dormant period in seedlings of two *Populus* species that vary in stomatal behavior and xylem vulnerability.

4.2 Materials and Methods

4.2.1 Plant material

One hundred and twenty trembling aspen (*Populus tremuloides* Michx; hereafter Pt) and balsam poplar (*Populus balsamifera* L; hereafter Pb) seedlings were grown from open pollinated seed sources collected near Edmonton, Alberta (53.6° N, -113.3° W). Seedlings were established under well-watered conditions in a greenhouse at the University of Alberta, Canada in April of 2011. Greenhouse conditions were a 18-h photoperiod at 21/18 °C with a humidity of approximately 60%. After 4 weeks seedlings were transplanted into individual plastic pots (4-L, 6 inch diameter with four equidistant perforations at the base to allow excess water to drain), filled with Metro Mix media (Metro Mix 290, Terra Lite 2000; W. R. Grace of Canada, Ajax, ON, Canada), and one seedling per pot.

After transplanting, plants were watered daily and fertilized with 200 ml of 10-52-10 NPK solution (1g L^{-1} pot^{-1}) every two weeks for four weeks. After 4 weeks, plant height and stem basal diameter of all seedlings was recorded and a distribution curve for these variables was constructed for each species. Once the average of each variable per species was calculated, 84 Pt and Bp seedlings each with height and basal diameter closest to their respective average were kept and the rest of the seedlings discarded. The 84 remaining seedlings per species were randomly selected and assigned to seven groups of 12 plants each. Plants were randomly reassigned in each group until no significant differences (tested with one-way ANOVA) in initial plant height and stem basal diameter among the six groups were detected. Six plants within each group were randomly selected and assigned to a well-watered control treatment (hereafter referred as CON seedlings) and the remaining six plants were assigned to a drought treatment (hereafter referred as DRY seedlings). On June 1, all plants were moved outside the greenhouse into cold frames. Transparent lids were attached to the frames allowing them to be closed in the event of rain. There were only few rain events and therefore light availability was not significantly reduced throughout the experiment. Lids did not close completely and allowed for sufficient air circulation preventing heating within the cold frame.

4.2.2 Application of the drought treatment

Following the same methodology developed during past experiments (Galvez *et al.* 2011) the DRY seedlings were slowly desiccated in a controlled process. Starting on June 26, 2011, DRY seedlings were weighed daily using an Adam Equipment digital balance model PGW 4502e (Danbury, CT, USA). After each weight was recorded, DRY seedlings were re-watered by adding the equivalent of half the weight that was lost from the day before. This process was repeated for 10 days. This desiccation protocol was implemented to simulate a gradual soil drying process. Leaf water potential measurements were performed at day 8, 9 and 10 on three randomly selected plants of each species using one leaf per plant (data not shown). By allowing leaf and stem water potentials to equalize, leaf water potential can be used as a proxy for stem water potential (Begg & Tuner 1970). To accomplish this, leaves selected to be measured were kept inside an aluminum foil envelope for two hours prior to the leaf water potential measurement to allow the stem water potential to equilibrate with the leaf water potential. Water potential measurements were performed using a Compact Water Status Console (i.e. a portable Scholander-type pressure chamber) model 3115P40G4 (Soilmoisture Equipment Corp., CA, USA). By the end of the desiccation period, midday stem water potential Ψ_{md} in DRY Pt was approx. -2.2 MPa and -1.2 MPa in DRY Pb. These Ψ_{md} values are slightly less negative than Ψ_{md} associated with 50 percent loss of conductivity (P_{50}) in these species growing under similar environmental conditions (P_{50} -2.2 to -2.3 MPa in Pt and -1.3 to -1.4 MPa in Pb) (Hacke & Sauter 1995; Lu *et al.* 2010). After the initial 10-day period DRY seedlings were watered with the average full amount of daily water loss determined at the 10^{th} day of the 10-day drying period. Control seedlings were kept well watered throughout the growing season. Watering was discontinued after October 15, 2011 as soils were frozen and seedlings had shed their leaves. A layer of straw (20 cm) was placed on top and around the remaining pots to preserve soil moisture and provide some thermal insulation (see below).

4.2.3 Gas exchange and percentage loss of conductivity (PLC) measurements

On July 11, 2011 CO_2 assimilation rate (A) and leaf stomatal conductance (g_s) were measured in 6 randomly selected DRY and seedlings of each Pt and Bp. All gas exchange measurements were performed using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, Neb.). All measurements were performed between 0900 and 1100 hours on the youngest fully expanded leaf. Chamber's reference CO_2 concentration was set to 385 p.p.m. using a 12-g Li-Cor CO_2 cartridge as CO_2 source. Light environment in the chamber was set to 1,800 µmol m^{-2} s^{-1} after a 10-min induction period at 500 µmol m^{-2} s^{-1} using the 6400-2B red/blue LED light source of the LI-6400's chamber. The induction period was implemented to stabilize air humidity, flow and temperate prior exposing the measured leaf to the light-saturating photo flux density (PFD) level which the seedlings experienced in the open conditions. Measurements were taken after three minutes when A and g_s values were stable. The cuvette conditions were based on light response curves that were determined prior to measurements on three individual plants (data not shown). From these curves the optimum induction time and the photon flux density to achieve maximum A was determined. These same measurements were repeated on July 24, August 8, 22 and September 5 (i.e. 2, 4, 6 and 8 weeks after the first set of measurement was taken). All measurements in the rest of this section were performed using the same number of samples and time schedule described above.

Percentage loss of hydraulic conductivity (PLC) was measured using a conductivity apparatus (Sperry *et al.*, 1988) following the standardized, and now traditional, protocol for this widely used equipment. Seedlings were cut at the stem base in the cold-frames and transported to the laboratory (approximately 200 m), wrapped in damp towel paper inside black plastic bags to minimize stem dehydration. Stems cut from the pot were re-cut under water, separating the 15-cm stem section proximal to the original cutting site in order to remove embolisms induced by exposing open xylem vessels to atmospheric pressure when cut from the stem base. Keeping the re-cut stem under water, five consecutive 2-cm stem segments from each stem were cut using a razor blade. Segments from each stem were mounted and measured at the same time in the conductivity apparatus. The apparatus' reservoir tank was filled with filtered (0.2 µm) 100 mM KCl solution prepared in deionized water. After the initial hydraulic

conductivity (i.e., the initial value of k_h, expressed as k_i in Eq. 1) of each stem segment had been measured, the native embolism was displaced by flushing KCl solution from the reservoir under constant pressure (120 kPa) for 2 min. After being flushed, the segment's measured final hydraulic conductivity was taken as k_{max}. Preliminary tests were performed to ensure that k_{max} values did not change after repeated flushing. PLC was calculated from Eq. 1;

$$PLC = [(k_{max} - k_i) / k_{max}] \times 100 \quad (1)$$

k_s was calculated from k_i / A_w, where A_w is stem cross-sectional area.

4.2.4 Seedling and non-structural carbohydrates (NSC) measurements

At each sampling period the height of the selected seedlings of Pt and Pb were recorded and plants were separated into roots, stems and leaves. Roots were carefully washed to remove all substrate. Once cleaned, roots were patted dry with paper towels and left exposed to air circulation for 5 minutes. After this time, root systems were attached to a small metal clamp by the root collar and carefully submerged inside a water reservoir placed on top of a digital balance. Root volume was determined by water displacement recording the weight reading before and after root immersion, verifying that lateral roots were not pressing against the bottom or sides of the container. After root volume was determined, each root was pat-dried again, individually bagged, labeled, stored cooled and sent to the lab for immediate processing.

Leaves from each plant were detached from stems, bagged, labeled and sent to the lab for processing. Stem material was processed as indicated in the previous section to perform PLC measurements. After PLC measurements were conducted, all segments of each stem were bagged, labeled and send to the lab for processing. Total leaf area per plant was measured the same day using a LI-3000 leaf area meter (Li-Cor, Lincoln, Nebraska, USA), after measurements leaf, stem and root material was oven dried at 70 °C for 72 hours and weighed. Dry weight of roots was used to calculate specific root weight by dividing the dry weight of each individual root system by its volume. All dried samples were individually ground using a Wiley Mill to pass a 40 mesh (0.4 mm) screen and the ground tissues were used to estimate water soluble sugar and starch

concentrations. Soluble sugars were extracted three times with hot 80% ethanol, followed by a reaction between the extract and phenol–sulfuric acid which allowed sugars to be measured colourimetrically (Chow & Landhäusser 2004). To measure starch concentrations, the tissue remaining after the ethanol extraction was digested with the enzymes α-amylase and amyloglucosidase followed by a colourimetrically measurable reaction with peroxidase-glucose oxidase-o-dianisidine (Chow & Landhäusser 2004). Soluble sugar and starch concentration of roots, stems and leaves is presented by organ and as total concentration of non-structural carbohydrates at the whole plant scale.

4.2.5 Seedling dormancy and leaf reflush

To explore the effect of drought after a growth and dormancy season on seedlings survivorship and NSC dynamics, all remaining seedlings were left inside their cold frames until January 15, 2012. On that date, all seedlings were relocated inside a greenhouse with environmental conditions similar to the previously used during seed germination. After soils were thawed (2 days) another set of 6 dry and 6 control seedlings for each species was collected and all response variables other than leaf measurements were taken as previously described. The last remaining CON and DRY seedlings were well-watered daily using the same amount of water used during the earlier part of the experiment until leaves flushed. On February 27, once all control seedlings had flushed and expanded their leaves, all response variables were measured.

The experimental design was analyzed as a 2 × 2 × 7 factorial design with two species, two drought treatments (droughted and control seedlings) and seven collection times. All growth data were normally distributed and variances were equal. Three-way ANOVA were performed for height, leaf area, leaf number, PLC, stem water potential and root dried weight response variables, using statistical software package SigmaStat 4 (Systat Software Inc, Chicago, IL). Differences between means were considered significant at an α=0.05. When significant differences between the means were detected, all-pairwise multiple comparisons using the Holm-Sidak procedure was performed. CO_2 assimilation rate, stomatal conductance, sugar and starch concentration datasets failed tests for normality or independence of variance. These response variables were fitted

with a linear mixed-effects model using the functions *lme* and *varIdent* from the *nlme* R package (Pinherio *et al.* 2010) to allow different variance structure for Time and Treatment. Once the datasets were fitted, they were analyzed using ANOVA procedures with statistical package R (R-CRAN). Differences between drought treatments, species and collection times stated in the following Result and Discussion section were statistically significant at an α=0.05, unless mentioned otherwise. ANOVA tables are presented in Appendix 1.

4.3 Results

4.3.1 Gas exchange during controlled desiccation

CO_2 assimilation (*A*) and leaf stomatal conductance (g_s) in well irrigated Pt and Pb seedlings remained fairly constant during the controlled desiccation protocol. In Pt seedlings CO_2 assimilation of DRY seedlings started to deviate from well irrigated seedlings four days after the desiccation started and continued decreasing until July 5 when the seedlings reached the desired drought target (Figure 4.1a,b). Stomatal conductance in DRY Pt seedlings started deviating from controls after only two days and continued decreasing until July 5. Seedlings of Pb showed a distinctly different stomatal behavior than Pt seedlings. In Pb seedlings *A* and g_s remained similar to well irrigated controls until eight days after the desiccation protocol started, after which both variables showed a decline. In both species *gs* decreased more than *A* relative to the well irrigated controls (Figure 4.1). At the end of the desiccation process, *gs* and *A* in DRY seedlings of both species was lower than in the correspondent well-irrigated controls. At that time, there was no significant difference between *gs* in DRY Pt and DRY Pb seedlings but *A* in DRY Pb seedlings was higher than in DRY Pt (Figure 4.1a,b).

4.3.2 Effects of drought on seedling growth

Regardless of species and drought treatment, height growth of all seedlings stopped about four weeks into the experiment, likely the result of the exposure to the natural photoperiod and temperature conditions that signal the end of active

height growth period in August (Figure 4.2a). However, during the first four weeks, height of CON Pt seedlings increased from 65.2 cm to 89.3 cm. Height growth in CON Pb seedlings was slower than in Pt, increasing from 32.2 cm to 47.6 cm over the same period of time. Average height of DRY Pt and Pb seedlings was lower than in the corresponding well-irrigated controls throughout the whole experiment. Height growth of both species was reduced during the initial desiccation process and at the start of the experiment DRY Pt seedlings increased from 59 cm to 69.5 cm and from 27 cm to 35.9 cm in DRY Pb seedlings, respectively, and remained then constant for the rest of the experiment. Similarly to seedling height, average number of leaves of control seedlings stopped increasing around August 3 and remained relatively constant for the rest of the experiment (Figure 4.2b). During the first four weeks of the experiment, average number of leaves in CON Pt and Pb seedlings increased from 24 to 28 and from 18 to 21, respectively and remained relatively constant until September 5. During the following weeks of fall, seedlings of both species shed their leaves. Seedlings were leafless in January 15 when they were moved inside the greenhouse. After reflush (February 27, 2012) the average number of leaves in CON Pt and Pb seedlings was similar to the average number of leaves the seedlings had in before leaves were shed in September, suggesting no significant damage to the bud meristems over the winter period. The DRY Pt seedlings steadily decreased their number of leaves during the first six week of the experiment from 25 to 10, but increasing again to 16 leaves by September 5. The average number of leaves in DRY Pb on the other hand seedlings steadily decreased during the whole growth season from 17 to 9. After the dormant period, no new leaves redeveloped in DRY Pt and Pb seedlings (Figure 4.2b). Average leaf area of CON Pt seedlings increased from 938.3 cm^2 to 987.8 cm^2 and from 454.8 cm^2 to 475.6 cm^2 in CON Pb seedlings during the first 4 weeks of the experiment and remained stable for the rest of the growing season. After leaf flush in 2012, average leaf area of control seedlings was 518.9 cm^2 for Pt and 446.7 cm^2 for Pb seedlings. Average leaf area in DRY seedlings steadily decreased from 923.1 cm^2 to 66 cm^2 in Pt seedlings and from 480.4 cm^2 to 97 cm^2 in Pb seedlings (Figure 4.2c).

Average root volume of CON Pt seedlings increased from 27.4 cm^3 to 37.3 cm^3 during the first four weeks and remained constant until September 5. Average

root volume of CON Pb seedlings increased during the first six weeks of the experiment from 20.9 cm^3 to 32.3 cm^3 and remaining constant until September 5 (Figure 4.2d). On January 5, 2012 average root volume of CON Pt seedlings had decreased to 23.2 cm^3 and to 15.7 cm^3 in CON Pb seedlings. Visual inspection of roots during cleaning suggested a reduction in number of fine roots (< 1 mm in diameter) of both species during the dormant period, but no diameter-based quantification was performed. Between January 5 and February 27, 2012 average root volume increased from 23.2 to 29.7 cm^3 and from 15.7 to 27.5 cm^3 in CON Pt and Pb seedlings, respectively (Figure 4.2d). Average root volume in DRY seedlings of both species increased only during the first two weeks from 15.5 cm^3 to 20.2 cm^3 in Pt seedlings and from 7.59 cm^3 to 16.2 cm^3 in Pb seedlings. By September 5, average root volume of DRY seedlings decreased in both species to 8.49 cm^3 and to 3.44 cm^3 for Pt and Pb seedlings, respectively. Average root volume of DRY seedlings decreased even more after the dormant period to 4.75 cm^3 in Pt seedlings and to 2.1 cm^3 in Pb seedlings. Average root volume of DRY seedlings remained stable for the rest of the experiment (Figure 4.2d). As in the control seedlings, visual inspection of roots during cleaning suggested a reduction in number of fine roots in both species during the dormant period.

4.3.3 Gas exchange and water relations response to drought

Average CO_2 assimilation rate (A) in control seedlings of both species steadily decreased seasonally during the eight weeks of the experiment, from 12.1 µmol CO_2 m^{-2} s^{-1} to 6.24 µmol CO_2 m^{-2} s^{-1} in Pt seedlings and from 13.9 µmol CO_2 m^{-2} s^{-1} to 7.46 µmol CO_2 m^{-2} s^{-1} in Pb seedlings (Figure 4.3a). In 2012 after the control seedlings flushed in the greenhouse, A was 7.06 µmol CO_2 m^{-2} s^{-1} in Pt seedlings and 8.74 µmol CO_2 m^{-2} s^{-1} in Pb seedlings. After the 10 day adjustment period, A in was reduced to 3.91 µmol CO_2 m^{-2} s^{-1} and 1.09 µmol CO_2 m^{-2} s^{-1} in DRY Pt and Pb seedlings, respectively. Both DRY species also showed a seasonal decline in A to 1.29 µmol CO_2 m^{-2} s^{-1} in Pt and to -0.775 µmol CO_2 m^{-2} s^{-1} in Pb seedlings (Figure 4.3a). As no new leaves were produced in 2012 after the dormant period, no additional measurements of A, g_s or Ψ_{md} could be taken in DRY seedlings of both species. Average leaf stomatal conductance (g_s) in

control seedlings also seasonally decreased during the first eight weeks of the experiment, but this reduction was significantly steeper in Pb seedlings than in Pt seedlings. During this time g_s decreased from 0.272 mol H_2O m^{-2} s^{-1} to 0.189 mol H_2O m^{-2} s^{-1} in Pt seedlings and from 0.525 mol H_2O m^{-2} s^{-1} to 0.171 mol H_2O m^{-2} s^{-1} in Pb seedlings. In DRY seedlings of both species, average g_s also showed a seasonal decline, but remained below 0.1 mol H_2O m^{-2} s^{-1} throughout the whole experiment (Figure 4.3b).

Average PLC in control seedlings of both species remained below 10% for the whole first growing season; however, PLC increased to 35.8% in Pt seedlings and 31.2 % in Pb seedling after the dormant period at the end of the experiment (Figure 4.3c). In DRY seedlings, average PLC remained similar to PLC in control seedlings only for the first two weeks and by the end of the experiment the stem xylem was more than 80% embolized in both species (Figure 4.3c). Average stem water potential (Ψ_{md}) in control seedlings of both species remained above -1 MPa for the whole experiment. Average Ψ_{md} decreased from -2.16 MPa to -2.66 MPa in Pt DRY seedlings and from -1.2 MPa to -1.5 MPa in DRY Pb seedlings (Figure 4.3d).

4.3.4 Concentration of soluble sugars and starch at tissue level

Leaves

Average soluble sugar concentration in leaves ($SugConc_{leaf}$) in control seedlings increased from 12.2 % to 21.6 % in Pt seedlings and from 13.1 % to 20.8 % in Pb seedlings during the first eight weeks of the experiment. During reflush after the dormant period, $SugConc_{leaf}$ was 15.6 % in CON Pt seedlings and 14.4 % in CON Pb seedlings (Figure 4.4a). In DRY seedlings $SugConc_{leaf}$ also increased during the first eight weeks of the experiment, but only from 13.7 % to 17.4 % in Pt seedlings and from 10.5 % to15.5 % in Pb seedlings. Average leaf starch concentration ($StaConc_{leaf}$) in CON Pt seedlings increased from less than 1 % (i.e. at the detection limit) to 3.79 % during the first four weeks of the experiment only to decrease below 1 % just before the dormant period. In Pb, $StaConc_{leaf}$ remained at about 2% just before the dormant season. After reflush, $StaConc_{leaf}$ was 1.4 % in CON Pt seedlings and less than 1 % in CON Pb seedlings.

$StaConc_{leaf}$ of Pt and Pb DRY seedlings remained below 1% for the entire experiment (Figure 4.4b).

Stems

At the beginning of the experiment, stem soluble sugar concentration ($SugConc_{stem}$) in CON Pt seedlings was 8.86 % and remained relatively unchanged during the growing season. In CON Pb seedlings $SugConc_{stem}$ increased from 9.38 % to 12.9 % during the same period of time. In the dormant period $SugConc_{stem}$ was higher in both species with 12.6 % in CON Pt and 15.1 % in Pb seedlings. Shortly after leaf flush, $SugConc_{stem}$ was somewhat lower compared to the first growing season with 8.64 % in Pt and 10.4 % in the Pb seedlings. During the first six weeks of the experiment $SugConc_{stem}$ in DRY seedlings increased from 6.53 % to 10.5 % in Pt seedlings and from 6.19 % to14 % in Pb seedlings. Contrary to the control seedlings, $SugConc_{stem}$ in DRY seedlings was lower during the dormant period and then stayed the same in Pt or was slightly higher in Pb at the end of the experiment (Figure 4.4c). During the first eight weeks of the experiment stem starch concentration ($StaConc_{stem}$) in CON Pt and Pb seedlings increased from less than 1 % to 4.54 % in Pt and to 4.58 % in Pb. $StaConc_{stem}$ in control seedlings decreased to 1 % in both species during the dormant period and was close to 0 % during leaf flush. $StaConc_{stem}$ of DRY Pt and Pb seedlings increased slightly towards the end of the growing season, but was close to 0% during the dormant season and at the end of the experiment (Figure 4.4d).

Roots

At the beginning of the experiment average root sugar concentration ($SugConc_{root}$) in control seedlings was 8.21 % in Pt seedlings and 9.25 % in Pb seedlings; and remained relatively unchanged during the first eight weeks of the experiment. During the dormant period, $SugConc_{root}$ in control seedlings had increased two-fold to 20.6 % in Pt and to 25.4 % in Pb seedlings. After leaf flush $SugConc_{root}$ were similar to levels measured in the first growing season (8.6 % in CON Pt and 12.4 % in CON Pb) (Figure 4.4e). This steep increase in $SugConc_{root}$ during the dormant period coincided with an equally steep decrease in root starch concentration ($StaConc_{root}$) at the end of the growing season in both

control species (Figure 4.4f). $SugConc_{root}$ in DRY Pt seedlings was 7 % at the beginning of the experiment and slightly increased to 10 % over the first four weeks. The following four weeks $SugConc_{root}$ in DRY Pt seedlings steadily decreased for the rest of the growing season and continued to decrease to 2.8 % during the dormant season and the last measurement. In DRY Pb seedlings $SugConc_{root}$ increased from 5.1 % to 9.3 % during the first four weeks and then remained stable until after the dormant period when it was 6.1 %. DRY seedlings did not have the large fluctuation in $SugConc_{root}$ and $StaConc_{root}$ before and after the dormant period, compared to the control seedlings (Figure 4.4e,f). Average root starch concentration ($StaConc_{root}$) steadily increased in control seedlings of both species during the first eight weeks of the experiment. In CON Pt seedlings $StaConc_{root}$ increased from 6.41 % to 19.1 % and from 5.25 % to 19.7 % in Pb. During the dormant period $StaConc_{root}$ decreased to 4.82 % in CON Pt and to 5.18 % in CON Pb. In DRY seedlings of both species, $StaConc_{root}$ increased during the first four weeks and then slowly decreased until the dormant period and remained at 2% at the end of the experiment (Figure 4.4f).

4.3.5 Total non-structural carbohydrate concentration at the whole plant level

Average total non-structural carbohydrate concentration at the whole plant level ($TotConc_{plant}$) increased throughout the growing season in control seedlings and decreased only after the dormant period during the reflush of leaves (Figure 4.5). In DRY Pt seedlings, $TotConc_{plant}$ increased only during the first four weeks reaching concentrations similar to the Pt control, but after that $TotConc_{plant}$ decreased for the rest the experimental period. In DRY Pb seedlings, $TotConc_{plant}$ also increased during the first four weeks, but remained constant during the remainder of the season only to decrease during the dormant period (Figure 4.5).

4.4 Discussion

After the onset of drought stress in Pt and Pb seedlings, our results suggest that a cascade of responses are taking place in the droughted seedlings where: i) soil desiccation limited A and g_s, which, ii) limited stem growth and with that the

production of new leaves and fine roots, iii) as seedling stopped growing, photoassimilates were initially re-directed to accumulate in stem and root tissues, and iv) while hydraulic conductivity continued to be compromised and the season progressed toward dormancy, reserves did not continue to accumulate significantly and were only half that of the non-droughted control during the dormant season. However, decreasing photosynthesis and increasing reserves in woody tissues appear also to be a seasonal process in well-watered seedlings growing under outside conditions. Assimilation also declined in these seedlings throughout the growing season, while NSC in stem and root tissues accumulated once height growth had terminated after the first four weeks of growth (Figure 4.4).

In the four weeks after shoot growth had ceased, root growth increased in the control seedlings. The cues for these seasonal changes in *Populus* are likely shortened day length and cooler night temperatures, which are known to induce tissue hardening and dormancy (Ibáñez *et al*. 2010). Similar late seasonal root growth has been observed in mature aspen stands (Landhäusser & Lieffers 2003). At the end of the growing season and prior to the dormant season, NSC reserves in the droughted seedlings did not reach or surpass the NSC levels of the control seedlings. This pattern was in contrast to that described in an earlier study that was conducted under greenhouse conditions (Galvez *et al.* 2011). In that study the well-watered Pt seedlings grew continuously and the newly acquired carbon was likely used to maintain height growth, while the droughted seedlings stopped growing and accumulated NSC reserves in their tissues; however, this study did not incorporate natural growing season conditions and the seedlings were not exposed to the seasonal change in climate and conditions.

Droughted seedlings in our current study show clear signs of carbon starvation, particularly in the root system; however, its effect was only revealed after the dormant period. After re-watering following the dormant period, it became clear that the roots in the droughted seedlings were dead. This could not have been detected during the dormant season measurement, as roots were preserved by the frozen soil conditions. However, root systems turned black after they had thawed out, and no live roots segments, new root tips, or roots suckers were

detected on these root systems. Although root reserves were not completely depleted, the low concentration of NSC and the heavily embolized stems likely compromised the re-initiation of leaves on the shoot and/or the development of root suckers, and new root growth after the dormant period. The impact of low NSC concentrations can manifest itself in poor frost protection (i.e. low concentration of soluble sugars compared to the control) and/or low NSC reserves, which did not meet the basic cell tissue needs, such as increased respiration at the start of the following growing season (Regier *et al.* 2010).

Although at the beginning of the experiment stem water potentials (Ψ_{md}) in DRY seedlings of both species were not different from their corresponding controls, four weeks later Ψ_{md} were near the species' specific tension associated with 50 percent loss of conductivity. We can safely assume that by September 5 (i.e. our last sampling period of 2011) droughted seedling had suffered catastrophic hydraulic failure, since the percentage loss of hydraulic conductivity (PLC) was above 90% in Pt and above 80% in Pb (Figure 4.3c). At that time we observed significant stem necrosis, which should be expected (Lu *et al.* 2010). In light of the heavily embolized stems, the likelihood of flushing from the shoot in both species was very small, perhaps unless re-watering happened in September, which was not attempted. As a result the failure of Pt to regrow or sucker in our study appears to be in contrast to an experiment by Lu *et al*. (2010) who desiccated first year Pt seedlings in a short-term drought until all leaves were shed and stems were necrotic (PLC averaged 90%). When re-watered, these plants were able to re-sprout from axial buds or from their roots. The shoot symptoms matched the conditions of our droughted seedlings in September. However in their study, seedling carbon reserves and the dormant season performance were not measured (Lu *et al*. 2010). This clearly suggests that high PLC alone was not enough to kill the seedlings during a short-term drought experiments, and other factors such as NSC concentration are important drivers of mortality. Both *Populus* species have the ability to regenerate from adventitious root sprouts, a still functioning root system with sufficient NSC reserves should have been able to produce new shoots (Snedden *et al.* 2010, Landhäusser *et al.* 2006). Clearly this was not the case. To our knowledge, this is the first study to present experimental data supporting the idea that both carbon

limitation and hydraulic failure will lead to seedling mortality under severe drought conditions following a winter dormancy period.

Interestingly the changes in NSC (i.e. soluble sugars + starch) concentration at the whole plant scale ($TNSC_{plant}$) as a result of drought was predominately driven by NSC concentration in the root tissues (Figure 4.4,4.5); hence, any stress affecting NSC accumulation in the roots of the seedlings has likely a direct effect on the whole plant performance. Our results also showed a clear seasonal effect on root starch accumulation in seedlings, likely driven by the outdoor growing conditions. The findings in this current study are in clear contrast with our previous work, where well-watered Pt seedlings showed no reduction in neither growth nor gas exchange variables when growing under constant 18-hr photoperiod in a greenhouse (Galvez *et al.* 2011). Although our earlier work gave us unique information and insight into root NSC dynamics of droughted and non-droughted Pt seedlings under optimal and controlled light and temperature growing conditions, it appears not to represent what happens under natural photoperiod and temperature fluctuations, which is especially important for species growing in environments with distinct seasons as is common in the boreal and temperate zones.

Average leaf soluble sugar concentration ($SugConc_{leaf}$) in DRY seedlings increased during the first four weeks of the experiment even though *A* in DRY seedlings remained at least 50% lower than in control seedlings. This initial increase in $SugConc_{leaf}$ may suggest the onset of osmotic adjustment, a well-documented response to drought in *Populus* species (Gebre *et al.* 1994, 1998) and other tree species (Tschaplinski *et al.* 1998). Although $SugConc_{leaf}$ in DRY seedlings remained above 15% for the rest of the experiment, it is possible that leaf soluble sugars and starch remained unavailable for any other plant organ once loss of conductivity (PLC) was higher than 80%. We hypothesized that the increase in SugConcstem of DRY seedlings measured during the growth period was an osmotic response that might up regulate xylem pressure potential which is a suggested mechanism for repairing xylem embolism (Secchi *et al*. 2011). Maintaining NSC in the stems is also important because as shoots need access to reserves for the new leaf flush after the dormant season (Landhäusser 2011). The fact that by the end of the experiment only control seedlings had produced

new leaves and increased root volume, highlights the relevance of starch accumulation during the growing season and starch-to-sugar conversion during the cold hardening period (Levitt 1980, Sauter 1988) (Figure 4.4). Starch is a compound with no other biological function in plants besides storage and it is needed to buffer periods of stress (Kozlowski & Pallardy 2002). Starch-to-sugar conversion is a temperature-dependent adaptive mechanism well studied in *Populus* (Sauter 1988, Sauter and van Cleve 1991). This conversion plays an important role in maintenance of cell membranes at low temperatures and increases freezing tolerance (Levitt 1980). In our study, after control seedlings thawed, $SugConc_{root}$ in control seedlings declined as apical and lateral meristems became active and new leaves expanded, a process previously observed in other *Populus* and *Salix* species (e.g. Sauter 1988, Von Fircks & Sennerby-Forsse 1998).

Although Pt and Pb can overlap on sites with similar mesic edaphic and climatic conditions (Peterson & Peterson 1996, Landhäusser *et al*. 2002, Landhäusser & Lieffers 2003) they thrive in distinctive habitats (Burns & Honkala 1990). Pt forms extensive stands in mesic to dry mesic upland sites, while Pb occupies the moister (flood plains or seepage areas) and cooler extremes (Rood *et al.* 2003a, Rood *et al.* 2007). This difference in habitat is also reflected by the different stomatal adaptation to drought. Stomatal behavior in response to drought was distinctively different between Pt and Pb seedlings during the desiccation process (Figure 4.1). In DRY Pt seedlings, g_s declined earlier and faster than in DRY Pb seedlings. The decline of g_s in DRY Pt seedlings suggest an isohydric behavior (i.e. leaf stomata conductance decreased as soil desiccation progressed) (Tardieu & Simonneau 1998) in comparison with a more anisohydric response in DRY Pb seedlings, which maintained similar g_s to well-irrigated controls for a period of eight days before g_s started to decrease. This anisohydric stomatal behavior is consistent with findings by Larchevêque *et al.* (2011) working with Pb seedlings under drought conditions.

To our knowledge, our work is the first study to present experimental data illustrating a complex feedback between stomatal behaviour, gas exchange, water relations and carbon reserve accumulation dynamics. These responses and feedbacks are clearly modulated by seasonality, making the role of drought

stress as a driver of plant mortality dependent of the interaction between phenology and physiology, and very likely the ontogeny of plants.

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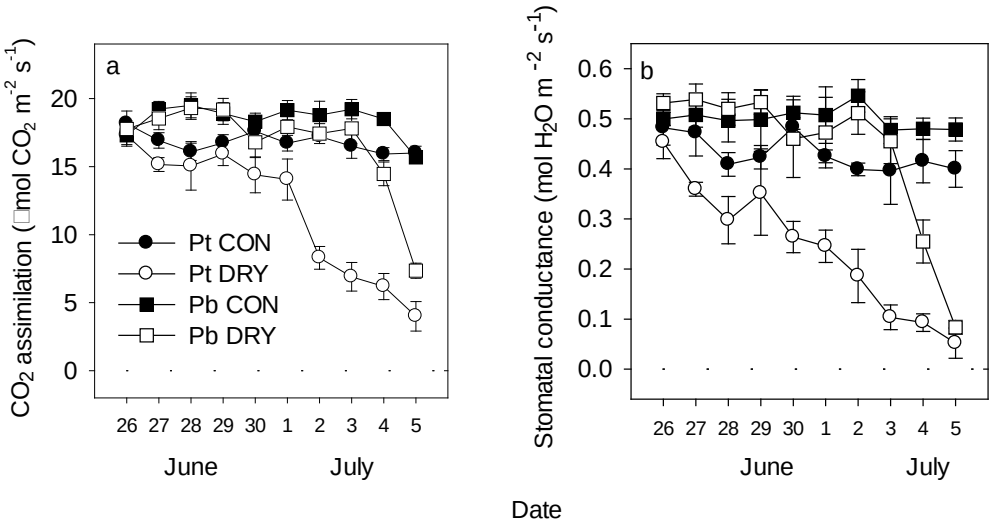

Figure 4.1 Mean (± SE) of CO_2 assimilation (a) and leaf stomatal conductance (b) of *Populus tremuloides* (Pt) and *Populus balsamifera* (Pb) seedlings under drought (open symbols) and well-irrigated (solid symbols) conditions, during the desiccation process, (n=6). Statistical analysis is presented in Appendix 1.

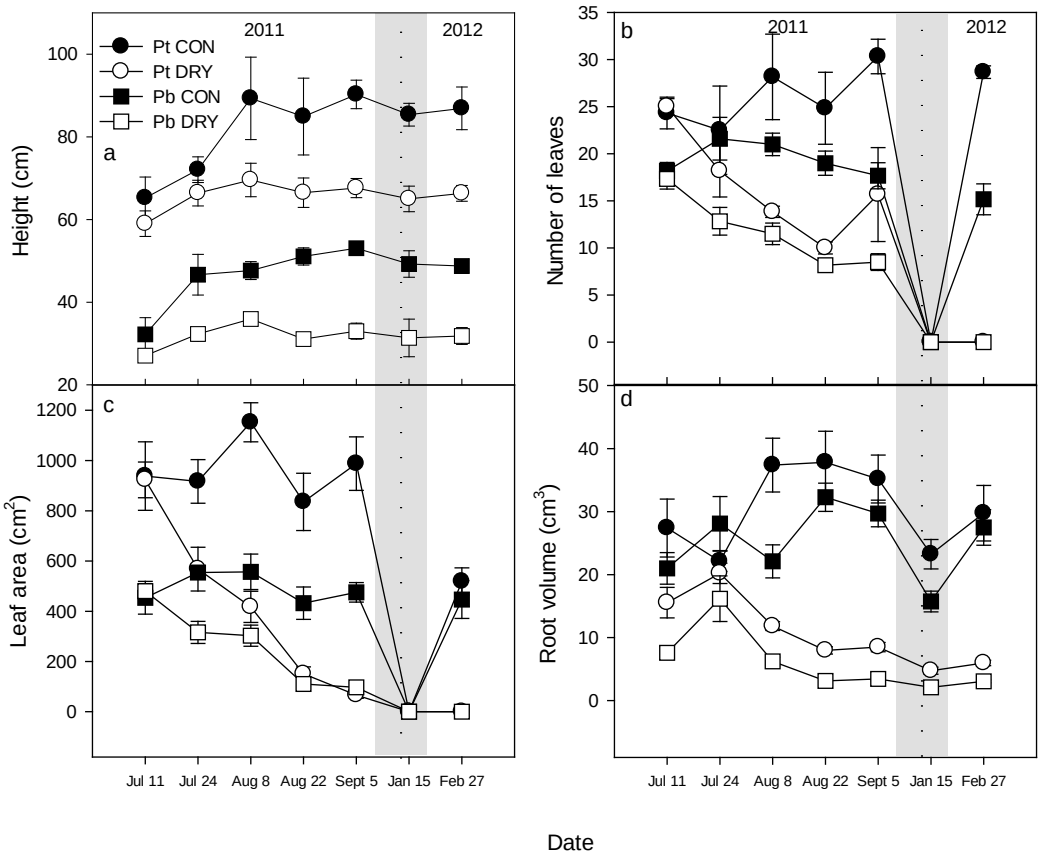

Figure 4.2 Mean (± SE) of plant height (a), number of leaves (b), leaf area (c) and root volume (d) of *Populus tremuloides* (Pt) and *Populus balsamifera* (Pb) seedlings under drought (open symbols) and well-irrigated (solid symbols) conditions, (n=6). Gray area indicates dormant period (not at scale). Dotted line indicates approximate date when seedlings were moved inside the greenhouse. Statistical analysis is presented in Appendix 1.

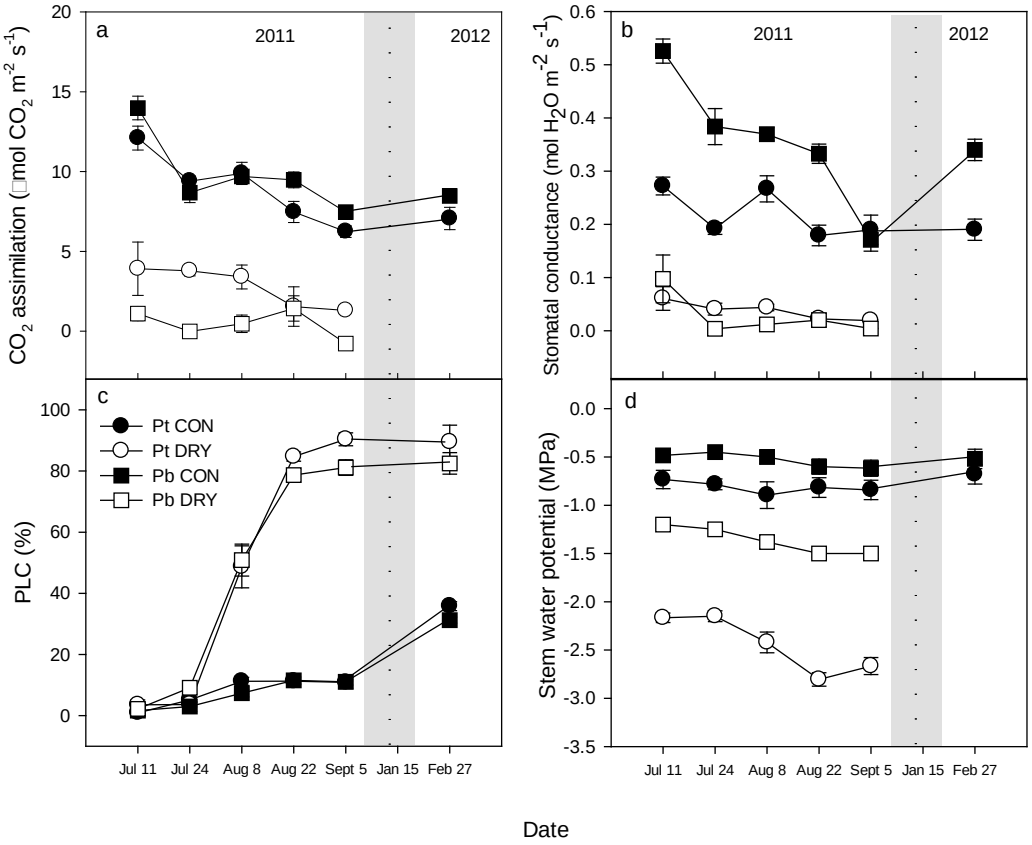

Figure 4.3 Mean (± SE) of CO_2 assimilation (a), leaf stomatal conductance (b), percentage loss of conductivity (PLC, c) and stem water potential (d) of *Populus tremuloides* (Pt) and *Populus balsamifera* (Pb) seedlings under drought (open symbols) and well-irrigated (solid symbols) conditions, (n=6). Gray area indicates dormant period (not at scale). Dotted line indicates approximate date when seedlings were moved inside the greenhouse. Statistical analysis is presented in Appendix 1.

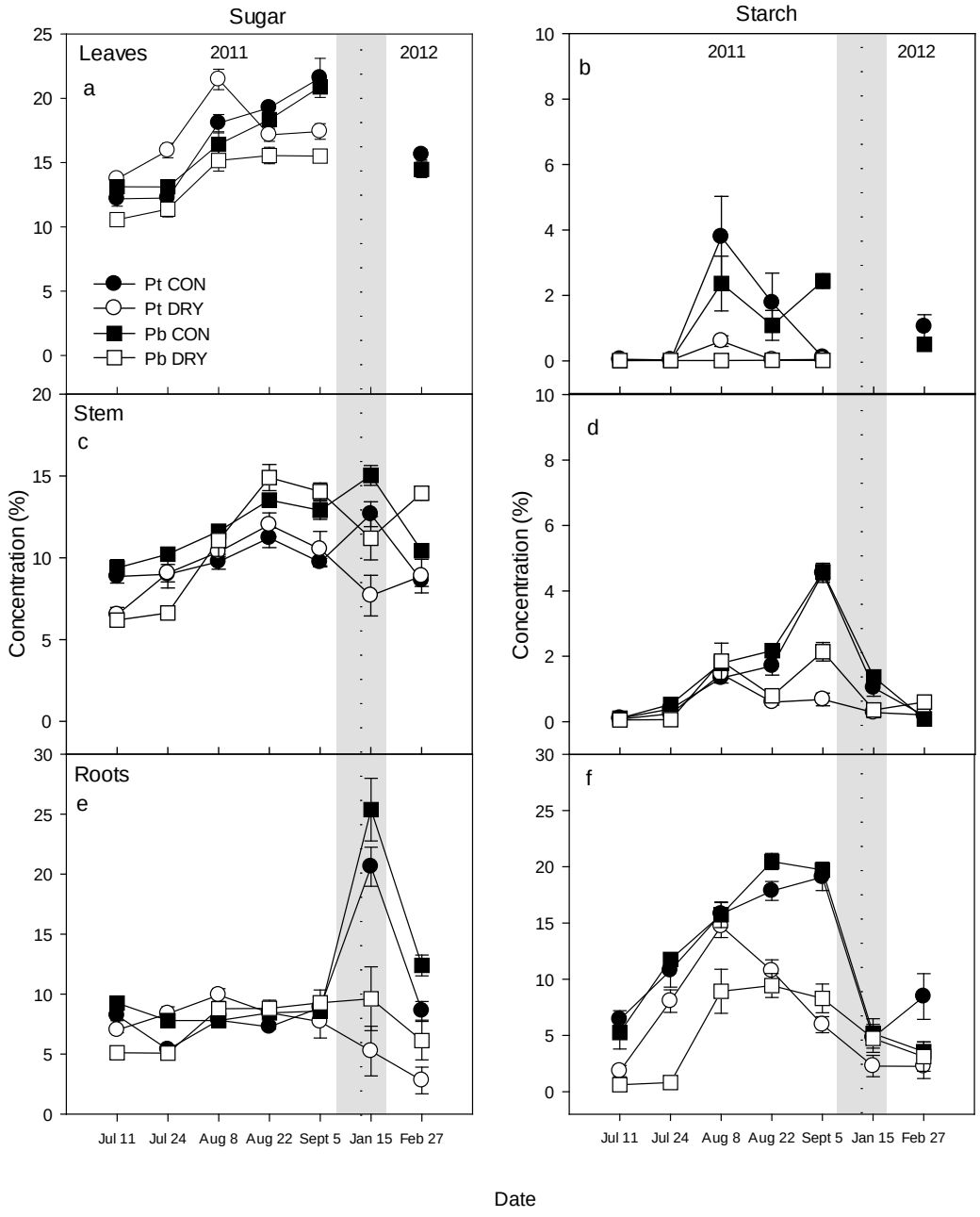

Figure 4.4 Mean (± SE) of sugar (left column) and starch (right column) concentration in leaves (a,b), stems (c,d) and roots (e,f) of *Populus tremuloides* (Pt) and *Populus balsamifera* (Pb) seedlings under drought (open symbols) and well-irrigated (solid symbols) conditions, (n=6). Gray area indicates dormant period (not at scale). Dotted line indicates approximate date when seedlings were moved inside the greenhouse. Statistical analysis is presented in Appendix 1.

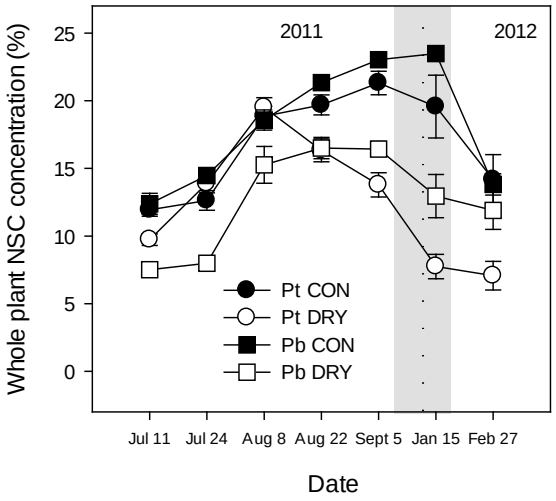

Figure 4.5 Mean (± SE) of whole plant non-structural carbohydrates concentration of *Populus tremuloides* (Pt) and *Populus balsamifera* (Pb) seedlings under drought (open symbols) and well-irrigated (solid symbols) conditions, (n=6). Gray area indicates dormant period (not at scale). Dotted line indicates approximate date when seedlings were moved inside the greenhouse. Statistical analysis is presented in Appendix 1.

CHAPTER 5. COMBINED EFFECTS OF DEFOLIATION AND DROUGHT ON CARBON RESERVE ACCUMULATION OF SEEDLINGS IN TWO *POPULUS* SPECIES

5.1 Introduction

Exposure to herbivory is ubiquitous during the lifespan of a plant. The overall impact of herbivory on plant growth, reproduction, and survivorship can be modulated by mechanical (e.g. frequency, intensity and type of tissue damage), ecological (e.g. competition, resource availability) and climatic (e.g. temperature and drought) factors at the time of injury (e.g. see reviews by Schowalter *et al.* 1986, Wise and Abrahamson 2007, Schmitz 2008, Fornoni 2011). There is increasing evidence that large insect outbreaks often follow or coincide with severe drought events (Mattson and Haack 1987, Hogg *et al.* 2002, Fettig *et al.* 2007). The coincidence of insect outbreak and drought events and their negative effects on plants is hypothesized to be additive, which might result in increased plant mortality (Koricheva *et al.* 1998, Allen *et al.* 2010). However, the mechanisms by which affected trees die are not fully understood and might be related to reduced plant resistance to stress, increased susceptibility to pathogens, as well as interfere with whole-plant hydraulics and non-structural carbohydrate (NSC) reserve dynamics (McDowell 2011, Landhäusser & Lieffers 2012).

Trembling aspen and balsam poplar are commonly defoliated by forest tent caterpillar (FTC) (*Malacosoma disstria* Hubner). Major FTC outbreaks in the Canadian prairie provinces (i.e. Alberta, Saskatchewan and Manitoba) are strongly decadal (Duncan & Hodson 1958, Sutton & Tardif 2007) and have a severe negative economic (Sutton & Tardif 2007) and ecological impact (Hogg and Schwarz 1999, Hogg *et al.* 2002) on forest stands. The occurrence of insect outbreaks have been studied in trembling aspen (*Populus tremuloides* Michx) (Hogg & Schwarz 1999, Hogg *et al.* 2002) and in balsam poplar (*Populus balsamifera* L) (Sutton & Tardif 2007) and were also found to be correlated with unusually warm and dry years prior or during the outbreak in the boreal forest region. Although the overall negative effect of insect outbreaks on aspen forest is

hypothesized to increase during severe drought, most manipulative experiments have focused on defoliation (Hart *et al.* 2000) or on drought alone and few studies have explore NSC dynamics under these conditions (Anderegg & Callaway 2012, Landhäusser & Lieffers 2012). Therefore the precise mechanisms driving NSC dynamics under the combined drought and herbivory conditions are not well understood and experimental work on the physiological feedbacks between gas exchange, water relations and NSC dynamics are lacking (McDowell *et al.* 2008, McDowell 2011).

Leaf area loss is also a common response of plants to drought (Braatne *et al.* 1992). During severe drought, both trembling aspen and balsam poplar abscised leaves (see previous chapters); hence, both herbivory and drought can lead to leaf area loss and may be seen as analogs to conditions limiting carbon assimilation (Landhäusser & Lieffers 2012). However, the environmental and physiological conditions leading to the leaf area loss are very different. Under drought, stem hydraulic conductivity is severely limited through xylem cavitation (Tyree & Zimmermann 2002) while defoliation might not negatively impact water relations at the whole-plant scale and might lead to compensatory effects (Hart *et al*. 2000). However, when occurring together at the early stages of drought, herbivory under drought conditions may have a palliative effect on drought stress and xylem cavitation, because leaf area reduction at the onset of drought might also reduce the water demand for evapotranspiration.

Xylem vulnerability to hydraulic failure and stomatal behavior can vary by species (Sperry & Pockman 1993, Nardini *et al.* 2001). Trembling aspen and balsam poplar have different tolerances to drought stress, with aspen being the more tolerant species than balsam poplar (Rood *et al*. 2003a). In the short-term during soil drying, leaf stomatal conductance (g_s) in aspen decreased parallel with soil water content, maintaining a relatively constant stem water potential (i.e. isohydric behavior (Galvez *et al.*. 2011)), while in balsam poplar g_s remained initially unchanged, until it dropped abruptly following the low g_s of aspen (Larchevêque *et al.* 2011, Galvez *et al.* unpublished).

Based on the results obtained in chapter 4, which focused on NSC dynamics in trembling aspen and balsam poplar under only drought conditions, we aimed to

explore the effect of defoliation alone and the combined effect of defoliation and severe drought on plant responses. As both factors limit whole-plant assimilation capacity, their impact on growth, gas exchange, water relations and NSC dynamics was investigated and compared between both species.

5.2 Materials and Methods

5.2.1 Plant material

Trembling aspen (*Populus tremuloides* Michx; hereafter Pt) and balsam poplar (*Populus balsamifera* L; hereafter Pb) seedlings were grown from seed collected from open pollinated seed sources near Edmonton, Alberta (53.65° N; 113.38° W). Seedlings of both species were established under well-watered conditions in a greenhouse at the University of Alberta, Canada in April of 2011. Greenhouse conditions were a 18-h photoperiod at 21/18 °C with a humidity of approximately 60%. After 4 weeks one seedling each was transplanted into individual plastic pots (4-L, 6 inch diameter with four equidistant perforations at the base to allow excess water to drain), Pots were filled with Metromix media (Metro Mix 290, Terra Lite 2000; W. R. Grace of Canada, Ajax, ON, Canada). After transplanting, plants were kept well watered and were fertilized twice over the next four weeks with 200 ml pot^{-1} of a 10-52-10 N-P-K (1g L^{-1}) solution. After 4 weeks plant height and stem basal diameter of all seedlings was recorded and a size distribution curve for these variables was constructed for each species. Once the average of each variable per species was calculated, 126 seedlings of each Pt and Bp were selected that had their height and basal diameter closest to the average. Of the 126 seedlings, seedlings were randomly selected and assigned to seven replicate sets of 18 plants each. Plants were randomly reassigned among sets until no significant difference (tested with one-way ANOVA) in initial plant height and stem basal diameter among the seven sets was detected. Within each set, six plants were then randomly selected and assigned to either a well-watered control treatment (hereafter referred as CON seedlings), a defoliation treatment (hereafter referred as DEF seedlings) or a defoliation and drought treatment combination (hereafter referred as DEF+DRY seedlings). On June 1, 2011 all plants were moved into cold frames to outside conditions. Transparent lids were

attached to these frames allowing them to be covered during rain events. As there were only few rain events in the summer months of 2011, lids were rarely closed. Air temperatures were not affected by the cover as lids did not close completely and allowed for sufficient air circulation.

5.2.2 Application of the drought and defoliation treatments

To apply the drought in the DEF+DRY treatment, seedlings were slowly desiccated in a controlled process from June 26 to July 6 of 2011, as described in the previous chapter. Control (CON) and defoliated only (DEF) seedlings were maintained at well-watered conditions throughout the growing season. Once DEF+DRY seedlings reached their targeted level of stress (i.e. average midday stem water potential (Ψ_{md}) in DEF+DRY Pt -2.2 MPa and -1.2 MPa in DEF+DRY Pb; see Chapter 4 for details), the defoliation treatment was apply to the DEF and DEF+DRY treatments in both species. Defoliation was applied *after* seedlings reached the target levels of drought stress for two reasons: (i) this order of events simulated defoliation under drought conditions (*i.e*. a biotic agent of plant mortality proposed by McDowell *et al*. 2008) and (ii) soil desiccation in potted seedlings was mainly driven by leaf evapotranspiration, hence, defoliating seedling before applying the drought treatment would significantly slow down the desiccation process. DEF and DEF+DRY seedlings were defoliated by removing all leaves below the first fully expanded mature leaf, which lead to an approximately 64% reduction of leaf area in Pb and 73% of leaf area in Pt seedlings compared to undefoliated seedlings. Leaf removal was done manually detaching each leaf from the stem by the pulvinus, avoiding tearing of the epidermal and cambial tissues. This precaution was taken in order to minimize xylem “air seeding”, which could potentially increase stem embolization over time (Sperry & Tyree 1988, Galvez & Tyree 2009). In addition, the terminal tissues were maintained to allow seedlings to continue growing. Defoliation treatments were maintained during the experimental period, by removing additional leaves approximately one time per week to maintain a relatively constant leaf area. Watering of all seedlings was discontinued after October 15, 2011, as all seedlings had shed their leaves and soils were continuously frozen. A layer of

straw (20 cm) was placed on top and around the remaining pots to preserve soil moisture and provide some thermal insulation.

5.2.3 Gas exchange and percentage loss of conductivity (PLC) measurements

On July 11, 2011 (i.e. 5 days after the defoliation treatment was applied to DEF and DEF+DRY seedlings) CO_2 assimilation rate (*A*) and leaf stomatal conductance (g_s) were measured in 6 randomly selected seedlings in the three treatments and for both species. All gas exchange measurements were performed using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, Neb.). All measurements were taken between 0900 and 1100 hours on the first fully expanded leaf. The reference CO_2 concentration in the leaf chamber was set to 385 p.p.m. using a 12-g Li-Cor CO_2 cartridge as CO_2 source. Light environment in the chamber was set to 1,800 µmol m^{-2} s^{-1} after a 10-min induction period at 500 µmol m^{-2} s^{-1} using the 6400-2B red/blue LED light source of the LI-6400's chamber. The induction period was implemented to stabilize air humidity, flow and temperate prior exposing the measured leaf to the light-saturating photo flux density (PFD) level which the seedlings experienced in the open conditions. Measurements were taken after three minutes when *A* and g_s values were stable. The cuvette conditions were based on light response curves that were determined prior to measurements on three individual plants (data not shown). From these curves the optimum induction time and the photon flux density to achieve maximum *A* was determined. These same measurements with the same number of replicates were repeated on July 24, August 8, 22 and September 5 (i.e. 2, 4, 6 and 8 weeks after the first measurement were taken).

Percentage loss of hydraulic conductivity (PLC) was measured using a conductivity apparatus (Sperry *et al.*. 1988) following the standardized, and now traditional, protocol for this widely used equipment. Seedlings were cut at the stem base in the greenhouse and transported to the laboratory (approximately 200 m), wrapped in damp towel paper inside black plastic bags to minimize stem dehydration. Stems were re-cut under water, discarding a 15-cm stem section proximal to the original cutting site in order to remove embolisms induced by exposing open xylem vessels to atmospheric pressure when cut from the stem

base. Keeping the re-cut stem under water, five consecutive 2-cm stem segments from each stem were cut using a razor blade. Segments from each stem were mounted and measured at the same time in the conductivity apparatus. The apparatus' reservoir tank was filled with filtered (0.2 μm) 100 mM KCl solution prepared in deionized water. After the initial hydraulic conductivity (i.e., the initial value of k_h, expressed as k_i in Eq. 1) of each stem segment had been measured, the native embolism was displaced by flushing KCl solution from the reservoir under constant pressure (120 kPa) for 2 min. After being flushed, the segment's measured final hydraulic conductivity was taken as k_{max}. Preliminary tests were performed to ensure that k_{max} values did not change after repeated flushing. PLC was calculated from Eq. 1;

$$PLC = [(k_{max} - k_i) / k_{max}] \times 100 \quad (1)$$

k_s was calculated from k_i / A_w, where A_w is stem cross-sectional area.

5.2.4 Seedling and non-structural carbohydrates (NSC) measurements

At each sampling period the height of the selected Pt and Pb seedlings were recorded and plants were separated into roots, stems and leaves. Roots were carefully washed to remove all the substrate. Once cleaned, roots were patted dry with paper towels and left exposed to air circulation for 5 minutes. After this time, root systems were attached to a small metal clamp by the root collar and carefully submerged inside a water reservoir placed on top of one of the digital balances previously described. Root volume was determined by water displacement, recording the weight reading before and after root immersion, verifying that lateral roots were not pressing against the bottom or sides of the container. After root volume was determined, each root was pat-dried again, individually bagged, labeled, stored cooled and sent to the lab for immediate processing.

Leaves from each plant were detached from stems, bagged, labeled and sent to the lab for processing. Stem material was processed as indicated in the previous section to perform PLC measurements. After PLC measurements were conducted, all segments of each stem were bagged, labeled and send to the lab for further processing. Total leaf area per plant was measured the same day

using a LI-3000 leaf area meter (Li-Cor, Lincoln, Nebraska, USA). Leaf, stem and root material was then oven dried at 70 °C for 72 hours and weighed. All dried samples were individually ground using a Wiley Mill to pass a 40 mesh (0.4mm) screen and the ground root tissue was used to estimate water soluble sugar and starch tissue concentrations. Soluble sugars were extracted three times with hot 80% ethanol, followed by a reaction between the extract and phenol–sulfuric acid which allowed sugars to be measured colourimetrically (Chow & Landhäusser 2004). To measure starch concentrations, the tissue remaining after the ethanol extraction was digested with the enzymes α-amylase and amyloglucosidase followed by a colourimetrically measurable reaction with peroxidase-glucose oxidase-o-dianisidine (Chow & Landhäusser 2004). Soluble sugar and starch concentration of roots, stems and leaves is presented by tissue and as total concentration of non-structural carbohydrates at the whole plant scale.

5.2.5 Seedling dormancy and leaf reflush

To further explore the treatment effects after the first growing season, seedlings were measured at the end of the dormant season and early in the second growing season after leaf flush. All remaining seedlings that were left in their cold frames were relocated on January 15, 2012 inside a greenhouse with environmental conditions similar to those described above for seed germination and initial establishment. After soils had thawed (2 days) a set of 6 seedlings each for the three treatments and both species was collected and all response variables other than the leaf measurements were taken as previously described. The remaining seedlings in the three treatments were all well-watered using the same amount of water used during the earlier part of the experiment until seedlings finished flushing. On February 27, 2012 once all CON seedlings had flushed and fully expanded their leaves, all response variables were measured on the remaining seedlings.

The experimental design was analyzed as a 2 × 3 × 7 factorial design with two species, three defoliation treatments (CON, DEF and DEF+DRY seedlings) and seven collection times. All growth data were normally distributed and variances were equal. Three-way ANOVA were performed for height, leaf area, leaf number, PLC, stem water potential and root dry weight response variables, using

statistical software package SigmaStat 4 (Systat Software Inc, Chicago, IL). Differences between means were considered significant at an α=0.05. When significant differences between the means were detected, all-pairwise multiple comparisons using the Holm-Sidak procedure was performed. CO_2 assimilation rate, stomatal conductance, sugar and starch concentration datasets failed tests for normality or independence of variance. These response variables were fitted with a linear mixed-effects model using the functions *lme* and *varIdent* from the *nlme* R package (Pinherio *et al*. 2010) to allow different variance structure for collection time and defoliation. Once the datasets were fitted, they were analyzed using ANOVA procedures with statistical package R (R-CRAN). Differences between means were considered significant at an α=0.05. ANOVA tables are presented in Appendix 2.

5.3 Results

5.3.1 Effect of drought and defoliation on seedling growth

Defoliation (DEF) and the combination of defoliation and drought (DEF+DRY) reduced height growth in Pt and Pb seedlings in comparison with the well-irrigated CON seedlings throughout the whole experiment (Figure 5.1a,b). At the beginning of the experiment DEF+DRY seedlings of Pt and Pb seedlings were slightly smaller than their corresponding CON and DEF seedlings, but this difference was not significant. Regardless of species, the average height in DEF seedlings was slightly higher than in DEF+DRY seedlings, but they were not different from each other (Figure 5.1a,b). In all three treatments both species stopped height growth four weeks into the experiment (Aug 8), signaling the end of active height growth under the natural photoperiod and temperature conditions (Figure 5.1a,b). During the first four weeks, height in CON Pt seedlings increased from 65.2 cm to 89.3 cm, while height growth in CON Pb seedlings was somewhat slower than in Pt, increasing from 32.2 cm to 47.6 cm over the same period of time. During the first four weeks average height of Pt seedlings increased by approximately 4 cm from 65.1 cm to 69.3 cm in DEF and from 57.7 cm to 61.3 cm in DEF+DRY seedlings. Average height of Pb seedlings increased by 3 cm from 32.8 cm to 35.4 cm in DEF and by 8 cm from 26.2 cm to 34.2 cm in

DEF+DRY seedlings over the same period of time. After the first four weeks average height in all seedlings and treatments remained relatively constant (Figure 5.1a,b).

Average number of leaves in CON Pt seedlings increased from 24 to 28 and from 18 to 21 leaves in CON Pb seedlings during the first four weeks of the experiments and remained relatively constant until early fall (September 5). In fall, seedlings of both species shed their leaves. After re-flush in February 27, 2012 the average number of leaves in CON Pt and Pb seedlings was similar to the average number of leaves the seedlings had in September 5 before leaves were shed, suggesting no significant damage to the bud meristems over the winter period. Average number of leaves in Pt seedlings increased from 7 to 12 in DEF seedlings and from 7 to 11 in DEF+DRY during the first four weeks of the experiment. In Pb seedlings average number of leaves increased from 6 to 9 in DEF seedlings and from 6 to 7 in DEF+DRY during the same period of time. Average number of retained leaves in DEF and DEF+DRY seedlings of both species remained relatively constant for the rest of the experiment. After reflush, the average number of leaves in DEF seedlings of both species was similar to the average number of leaves of CON seedlings measured in late summer. Both species in the DEF+DRY treatment produced no new leaves after the dormant period (Figure 5.1c,d).

Average leaf area of CON Pt seedlings increased from 938 cm^2 to 988 cm^2 and from 455 cm^2 to 476 cm^2 in CON Pb seedlings during the first 4 weeks of the experiment and remained relatively constant during the rest of the growth season. Average leaf area of Pt seedlings was 142 cm^2 in DEF seedlings and 121 cm^2 in DEF+DRY seedlings. In Pb seedlings average leaf area was 123 cm^2 in DEF seedlings and 105 cm^2 in DEF+DRY seedlings. After flush in 2012, average leaf area of CON seedlings was 519 cm^2 for Pt and 447 cm^2 for Pb seedlings. No leaf area redeveloped in DEF+DRY Pt and Pb seedlings after the dormant period, while leaf area in DEF seedlings was in 400 cm^2 in Pb and 310 cm^2 in Pt seedlings (Figure 5.1e,f). Average leaf area of the retained leaves in the DEF and DEF+DRY seedlings of both species was manually maintained fairly constant during the first growing season (Figure 5.1e,f). Nevertheless, the total leaf area removed during the growing season through manual defoliation (i.e.

from July 11 to September 5) was larger than the total area of undefoliated seedling. By the end of the growing season 963 cm^2 and 1290 cm^2 were manually removed from DEF+DRY and DEF Pt seedlings, while 722 cm^2 and 703 cm^2 were removed from DEF+DRY and DEF Pb seedlings.

Average root volume in CON Pt seedlings increased from 27.4 cm^3 to 37.3 cm^3 during the first four weeks and then remained constant until September 5. Average root volume in CON Pb seedlings increased during the first six weeks of the experiment from 20.9 cm^3 to 32.3 cm^3 and remaining similar until September 5 (Figure 5.1g,h). After the dormant season (January 5, 2012) average root volume in CON Pt seedlings had decreased to 23.2 cm^3 and to 15.7 cm^3 in CON Pb seedlings. By February 27, average root volume had increased to 29.7 cm^3 and to 27.5 cm^3 in CON Pt and Pb seedlings, respectively. At the beginning of the experiment, average root volume of Pt seedlings was with 23.2 cm^3 in DEF seedlings was similar to CON seedlings, but much lower (10.2 cm^3) in the DEF+DRY seedlings. Root volumes in Pt remained relatively constant during the first four weeks in both treatments, but decreased significantly to 15.9 cm^3 in DEF and to 7.05 cm^3 in DEF+DRY seedlings by the end of the growing season and to 7.76 cm^3 in DEF seedlings and 4.48 cm^3 in DEF+DRY seedlings by the end of the dormant period (Figure 5.1g). After re-flushing, root volume of Pt recovered in the DEF seedlings to 14.4 cm^3, but not in the DEF+DRY seedlings. At the beginning of the experiment average root volume of Pb seedlings in the DEF and DEF+DRY treatments was much lower than in the CON seedlings (Figure 5.1h). In the first two weeks both treatments showed a slight increase in root volume; however, in the DEF+DRY seedlings root volume declined to 2.03 cm^3, while in the DEF seedlings root volume increased slowly over the remaining growing season to 11.9 cm^3 (Figure 5.1h). After the dormant season and after re-watering, the root volume in DEF+DRY Pb seedlings did not recover, while root volume in DEF seedlings recovered from roots lost over the dormant season to levels similar to the late summer (Figure 5.1h).

5.3.2 Gas exchange and water relations response to drought and defoliation

Even though both species, regardless of treatment had a seasonal decrease of the average CO_2 assimilation rate (A) and leaf stomatal conductance (g_s) per unit leaf area, the DEF and DEF+DRY treatments affected these physiological variables differently. Defoliated seedlings of both species had higher A and g_s than in their corresponding CON seedlings for most of the experiment, while in the DEF+DRY seedlings these variables were reduced by more than 50% (Figure 5.2a-d). After re-flush and re-watering in 2012, average A and g_s in CON and DEF seedlings of both species were similar (Figure 5.2a,b). As no new leaves were produced in the DEF+DRY seedlings after the dormant period, leaf physiological variables could not be determined.

Although g_s of DEF and DEF+DRY seedlings was relatively similar between the two species, g_s of CON Pb seedlings remained consistently higher than of CON Pt seedlings during most of the experiment. During the growing season, average g_s in Pt seedlings decreased from 0.423 mol H_2O m^{-2} s^{-1} to 0.197 mol H_2O m^{-2} s^{-1} in DEF seedlings, from 0.272 mol H_2O m^{-2} s^{-1} to 0.189 mol H_2O m^{-2} s^{-1} in CON seedlings and from 0.127 mol H_2O m^{-2} s^{-1} to 0.007 mol H_2O m^{-2} s^{-1} in DEF+DRY seedlings. During the same period of time average g_s in Pb seedlings decreased from 0.537 mol H_2O m^{-2} s^{-1} to 0.257 mol H_2O m^{-2} s^{-1} in DEF seedlings, from 0.525 mol H_2O m^{-2} s^{-1} to 0.171 mol H_2O m^{-2} s^{-1} in CON seedlings and from 0.081 mol H_2O m^{-2} s^{-1} to 0.003 mol H_2O m^{-2} s^{-1} in DEF+DRY seedlings. After flush in 2012, average g_s in CON and DEF Pt seedlings was similar to the g_s seedlings had in September 5, but average g_s in CON and DEF Pb seedlings was higher after leaf flush than at September 5 (Figure 5.2c,d).

Average PLC in CON and DEF seedlings of both species remained below 10% for the whole growing season. However, after the dormant season, PLC in Pt seedlings increased to 35.8 % in CON seedlings and to 29.5 % in DEF seedlings. PLC also increased in Pb seedlings after the dormant season to 31.2 % in CON seedlings and to 27.7 % in DEF seedlings. In DEF+DRY Pt seedlings PLC rapidly increased after the second week reaching 80.6 % of by the end of the growing season. In DEF+DRY Pb seedlings PLC increased more gradually reaching 67.9 % during the same period of time. DEF+DRY seedlings of both species were completely embolized after the dormant season (Figure 5.2e,f). Average stem water potential in CON and DEF seedlings of both species

remained above -1 MPa for the whole experiment. During the growing season average stem water potential decreased from -1.98 MPa to -2.62 MPa in DEF+DRY Pt seedlings and from -1.01 MPa to -1.66 MPa in DEF+DRY Pb seedlings (Figure 5.2g,h).

5.3.3 Concentration of soluble sugars at tissue level

Leaves

Average leaf soluble sugar concentration ($SugConc_{leaf}$) of Pt increased in all three treatments during the first four weeks of the experiment. After four weeks, $SugConc_{leaf}$ continued to increase in the CON seedlings, while it slightly decreased in DEF seedlings and remained relatively unchanged in DEF+DRY seedlings. Overall, during the growing season $SugConc_{leaf}$ in Pt seedlings increased from 12.2 to 21.6 % in CON seedlings, from 11.8 to 14.3 % in DEF seedlings and from 13.5 to 17.2 % in DEF+DRY seedlings. During the same period of time $SugConc_{leaf}$ in Pb seedlings increased from 13.1 to 20.8 % in CON seedlings, from 13.7 to 17.9 % in DEF seedlings and from 12.8 to 15.2 % in DEF+DRY seedlings. After flush in 2012, $SugConc_{leaf}$ in Pt and Pb seedlings were similar to levels found at the start of the first growing season (Figure 5.3a,b).

Stems

At the start of the experiment $SugConc_{stem}$ in Pt and Pb seedlings was higher in CON seedlings than in DEF and DEF+DRY seedlings, but there was no difference among treatments by week six. Average stem soluble sugar concentration ($SugConc_{stem}$) increased during the first six weeks of the experiment in all seedlings regardless of treatment or species. After the initiation of drought and defoliation, seedlings in the DEF+DRY treatment had the lowest $SugConc_{stem}$ in Pt and Pb seedlings, but those concentrations increased more quickly over the first six weeks than the $SugConc_{stem}$ in the DEF and CON seedlings. In the DEF treatment $SugConc_{stem}$ were also lower than the CON, but also increased faster over the next six weeks than the CON seedlings. Across all three treatments $SugConc_{stem}$ remained relatively unchanged in Pt seedlings for the rest of the experiment. $SugConc_{stem}$ remained similar in CON and DEF Pb

seedlings during the dormant period, but after the dormant season $SugConc_{stem}$ decreased to concentrations similar to measurements in August. In DEF+DRY Pb seedlings, $SugConc_{stem}$ started decreasing after week six and slightly increased after the dormant season (Figure 5.3b,c).

Roots

At the beginning of the experiment average root sugar concentration ($SugConc_{root}$) in Pt and Pb seedlings was higher in CON seedlings than in DEF and DEF+DRY seedlings. $SugConc_{root}$ in Pt seedlings remained relatively constant during the growing season in all treatments. $SugConc_{root}$ in Pt seedlings sharply increased during the dormant period from 8.87 % to 20.6 % in CON seedlings and from 7.73 % to 16.8 % in DEF seedlings. Nevertheless in DEF+DRY Pt seedlings $SugConc_{root}$ only increased from 7.48 % to 11.1 % during the same period of time. After the dormant season $SugConc_{root}$ in CON, DEF and DEF+DRY Pt seedlings was similar to the concentration they had at the beginning of the experiment but $SugConc_{root}$ in CON Pt seedlings was almost twice than in DEF+DRY Pt seedlings (Figure 5.3e). $SugConc_{root}$ in CON Pb seedlings remained relatively unchanged (9.25 %) for the whole growing season. While in DEF Pb seedlings $SugConc_{root}$ increased from 5.75 % to 10.5 % during the same period of time. $SugConc_{root}$ sharply increased during the dormant period in CON and DEF Pb seedlings, while Pb DEF+DRY seedlings did not show this increase in $SugConc_{root}$. As in Pt seedlings $SugConc_{root}$ in CON Pb seedlings was almost twice than in DEF+DRY Pt seedlings (Figure 5.3f).

5.3.4 Concentration of starch at tissue level

Leaves

During the first four week of the experiment average leaf starch concentration ($StaConc_{leaf}$) of CON Pt seedlings increased from less than 1 % (i.e. at the detection limit) to 3.79 %, decreasing to less than 1 % by the end of the growing season. $StaConc_{leaf}$ of CON Pb seedlings increased from less than 1% to 2.36 % during the first four weeks and remained relatively constant for the rest of the growing season. $StaConc_{leaf}$ of DEF and DEF+DRY Pt and Pb seedlings remained near detection limit for the whole experiment. $StaConc_{leaf}$ of CON and

DEF Pt and Pb seedlings remained below 1 % after the dormant period. (Figure 5.4a,b).

Stems

Average stem starch concentration ($StaConc_{stem}$) increased during the first four weeks of the experiment in Pt and Pb seedlings regardless of treatment and remained relatively unchanged during the following two weeks. However in the following weeks later in the growing season $StaConc_{stem}$ of CON and DEF Pt and Pb seedlings more than doubled and then decreased over the dormant and the begin of the 2nd growing period to concentrations similar to the beginning of the experiment (Figure 5.4c,d). During that same period $StaConc_{stem}$ of DEF+DRY seedlings of both species did not show the accumulation of starch in the stems later in the growing season (Figure 5.4c,d).

Roots

After the initiation of the drought and defoliation treatments CON seedlings of both species root had higher root starch concentration ($StaConc_{root}$) than the CONDEF and the DEF+DRY seedlings. However, four weeks into the study the roots of DEF and DEF+DRY Pt seedlings had similar concentrations of starch than the CON. This was not the case in Pb, where the roots of the DEF+DRY seedlings had only about 30% of the starch concentration than the CON and DEF seedlings (Figure 5.4e,f). While $StaConc_{root}$ in CON and DEF Pt and Pb seedlings continued to increase in the following weeks, it quadrupled in both species relative to the start of the study, $StaConc_{root}$ sharply declined in the DEF+DRY of both species and remained below the 2% for the rest of the study. Over the dormant period $StaConc_{root}$ in CON and DEF seedlings of both species decreased below 5 %. After reflushing, $StaConc_{root}$ in Pt seedlings increased again to 8.45 % in CON seedlings and to 7.72 % in DEF seedlings. In Pb seedlings $StaConc_{root}$ in CON and DEF seedlings continued to remain below 5 % after the dormant period (Figure 5.4e,f).

5.3.5 Total non-structural carbohydrate concentration at the whole plant level

Average total non-structural carbohydrate concentration at the whole plant level ($TotConc_{plant}$) of Pt and Pb seedlings was higher in CON seedlings than in DEF seedlings and $TotConc_{plant}$ of DEF seedlings was higher than the DEF+DRY seedlings (Figure 5a,b). $TotConc_{plant}$ of Pt seedlings steadily increased until the last measurement of 2011 from 11.9 % to 21.3 % CON seedlings and from 9.7 % to 19.1 % in DEF seedlings. $TotConc_{plant}$ of Pb seedlings increased from 12.3 % to 23 % CON seedlings and from 9.79 % to 21.1 % in DEF seedlings over the growing season. In both species $TotConc_{plant}$ of CON and DEF seedlings remained relatively unchanged during the dormant period and decreased more than 30% during the first three weeks of flush. In contrast, in both species $TotConc_{plant}$ of DEF+DRY seedlings of increased only during the first four weeks and remained relatively stable at 15% in Pt and 12% in Pb. After the dormant season $TotConc_{plant}$ in DEF+DRY Pt seedlings dropped significantly to below 10% while $TotConc_{plant}$ of DEF+DRY Pb remained relatively unchanged but also below 10% (Figure 5.5a,b).

5.4 Discussion

Defoliation alone can be considered a carbon-limiting event, since the reduction in leaf area and with that the overall assimilation capability of the seedling resulted in lower carbon reserves and potentially in a loss of roots. However, we cannot discard the possibility that loss in root volume of DEF+DRY Pb seedlings (Figure 5.5h) was partially an artifact resulting from problems associated with retrieval and cleaning of root systems. Collecting root systems of droughted seedlings is specially challenging because under drought, small roots become more brittle and prompt to be inadvertently detached from primary roots. Although defoliation negatively impacted reserve accumulation, some of these potential losses appear to be offset by carbon gains through enhanced physiological responses of the remaining leaf tissue and its ability to provide enough reserves to the tissues to allow for the survival and growth in the following season. Clearly these responses to defoliation were modulated by the water status of the seedling. In the absence of drought, defoliation improved water relations and gas exchange in the remaining leaf and xylem tissues, but

greatly reduced above and belowground growth and reduced whole plant NSC accumulation. Under drought conditions not only the above and belowground growth was reduced, but also photosynthesis, stomatal conductance, and NSC accumulation were greatly reduced at the organ and whole plant scale. To our knowledge, our work is the first study that comprehensively shows that the carbon reserve allocation at the tissue and whole plant scale differs between defoliation and drought and that these effects are very much tissue dependent and have very different effects on the physiology and growth of Pt and Pb seedlings.

In our study, the severe and lethal drought treatment appears to be the main stressor driving most of the detrimental effects of overall seedling performance (see also chapter 4). Since the drought treatment appears to be lethal by itself, quantifying any additional detrimental effect of defoliation under drought conditions seems to be difficult. However, the difference in NSC concentration at the whole plant scale between control and defoliated seedling (Figure 5.5) also support the notion that likely under less severe chronic drought conditions, repeated or severe defoliation events will likely play a significant role in plant mortality and reduction in carbon reserves at the whole plant and root level (McDowell *et al.* 2008, McDowell 2011, Landhäusser & Lieffers 2012). Defoliation resulted in reduced carbon reserves and potentially in loss of root mass; however, our defoliation treatment alone did not result in critically low reserves and did not compromise the ability of the root system to absorb water and nutrients. Nonetheless depending on carbohydrate reserve availability prior defoliation, plant size and defoliation intensity, repeated defoliation in the same growing season, or over multiple seasons, would inevitably become detrimental to the overall plant performance. Repeated defoliation can result in significant reduction of growth, above-ground (Tschaplinski & Blake 1994, Agrawal *et al.* 2002,) and below-ground (Kosola *et al.* 2001) and NSC concentration (Anderegg & Callaway 2012, Landhäusser & Lieffers 2012).

Interestingly, while defoliation has a clear impact on the root system by reducing the root volume of seedlings by up to 50% over the experimental period, the water availability to the remaining leaf tissue appears not to be impacted. This may suggest that the reduced root volume more than compensated the initial

loss of leaf area (i.e. 64% in Pb and 73% in Pt) and therefore still provided an ample amount of water and nutrients, and actually increasing carbon assimilation and stomatal conductance rates in the remaining leaf area. This increased photosynthetic capacity has been observed in earlier studies and is known as compensatory photosynthesis. It has been reported in aspen (Hart *et al.* 2000, Baret & DesRochers 2011) and several other woody species (e.g. Ovaska *et al.* 1992, Lovelock *et al.* 1999, Ozaki 2004) after defoliation events. Additionally, defoliation seemed to have also promoted sink activity at the apical meristems, which we infer from the large amount of leaf area manually removed through the growing season (see section 5.3.1). It is possible that this increased sink activity in apical meristems was a contributing factor limiting resources to promote root growth in DEF seedlings. Trembling aspen appears to have a somewhat stronger compensatory response than balsam poplar (e.g. leaf stomatal conductance and stem water potential in Pt DEF seedlings remained higher for longer time than in Pb DEF seedlings (Figure 5.2 c,d); however, overall the differences in response to the defoliation treatments were small between the two species. Defoliation in the early season can promote the production of new leaves (Braatne *et al*. 1992), but in our study we continuously adjusted leaf area negating the ability of seedlings to add new functional leaf area (i.e. defoliated seedlings did produce new leaf area but, by manually removing leaves as soon as they became fully expanded, these leaves never became fully functional sources of photoassimilates). Similar to the control seedlings, defoliated seedlings started reallocating resources from growth to reserves once shoot height growth had ceased; however, carbon reserves did not recover to the same levels as in the control seedlings, which is likely due to the much reduced leaf area and the inability of the remaining leaf area to fully compensate for this loss.

The compensatory response described above assumes that water is readily available and that the functional transport tissues are maintained to translocate assimilates and water between above and belowground tissues. The drought treatment compromised or stopped these functions and exacerbated the negative effect on carbon reserve accumulation due to reduced gas exchange variables. Defoliation alone had no negative effect on PLC and stem water potential. This was likely the result of improved water relations associated with the changes in

leaf area to root ratio and enhanced gas exchange (see above; Figure 5.2a-h) maintaining soluble sugars and starch concentrations in the stems. Soluble sugars in stems have been proposed to play an important role on osmotic regulation of stem water potential and to help repairing xylem embolism under drought conditions (Secchi *et al.* 2011).

Although the effect of defoliation on sugar and starch accumulation was not uniform across tissues and species, it resulted in an overall reduction of NSC reserves at the whole plant scale in both species (Figure 5.5a,b). These results support the idea proposed by Landhäusser & Lieffers (2012) that over time, repeated defoliation events will have an overall negative effect on plant performance even in the absence of drought conditions, and that carbon reserves, in particular play a vital role in the maintenance of the root systems in perennial plants. Further, this current study supports the idea that a negative feedback loop exists between drought and defoliation, which limit carbon assimilation and the carbon reserve status and root maintenance and survival of perennial plants.

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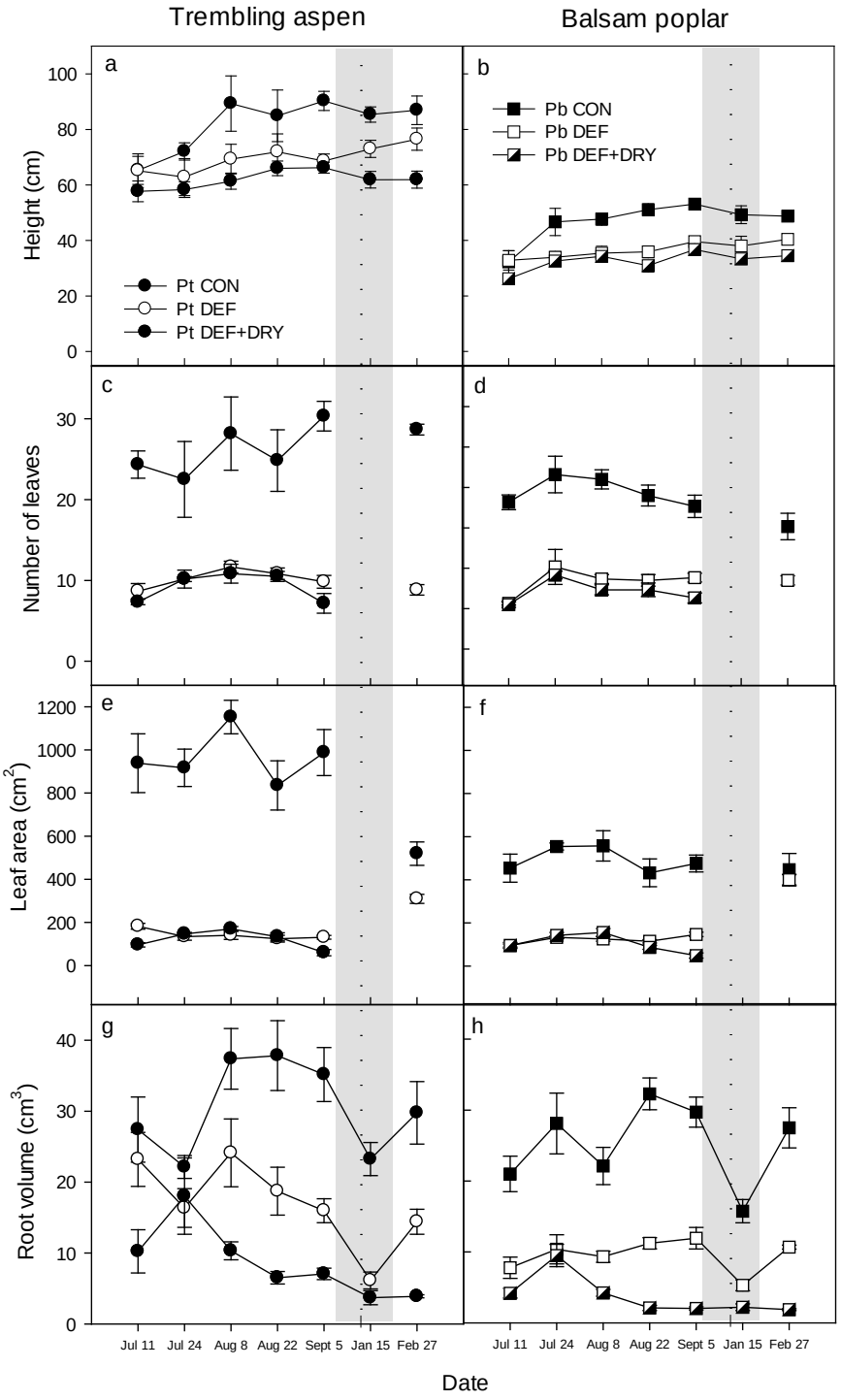

Figure 5.1 Mean (± SE) of plant height (a,b), number of leaves (c,d), leaf area (e,f) and root volume (g,h) of *Populus tremuloides* (Pt, left column) and *Populus balsamifera* seedlings (Pb, right column) in response to well-irrigated (CON), defoliation (DEF) and defoliation plus drought (DEF+DRY) conditions, (n=6). Statistical analysis is presented in Appendix 2.

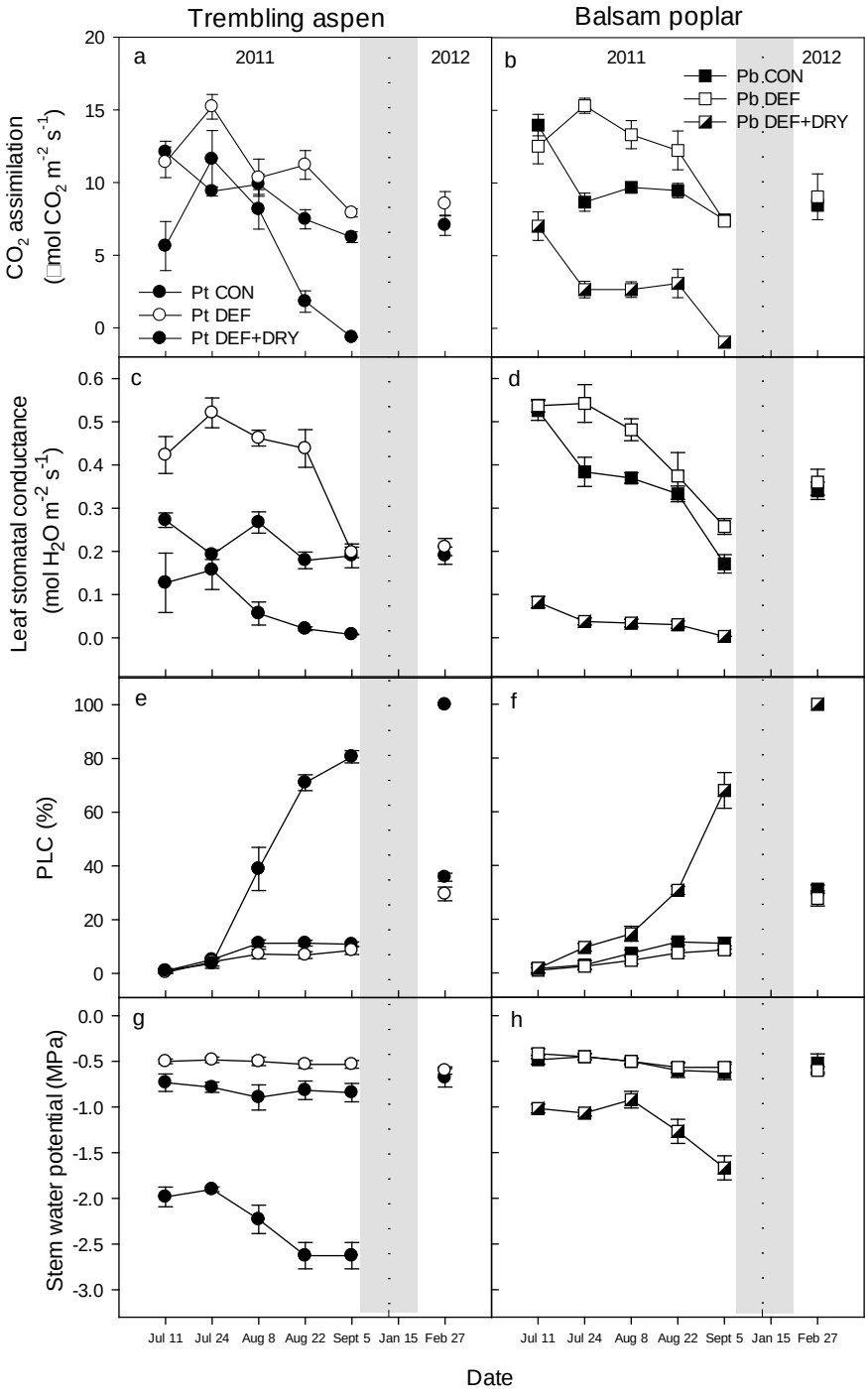

Figure 5.2 Mean (± SE) of CO_2 assimilation (a,b), leaf stomatal conductance (c,d), percentage loss of conductivity (PLC) (e,f) and stem water potential (g,h) of *Populus tremuloides* (Pt, left column) and *Populus balsamifera* seedlings (Pb, right column) in response to well-irrigated (CON), defoliation (DEF) and defoliation plus drought (DEF+DRY) conditions, (n=6). Statistical analysis is presented in Appendix 2.

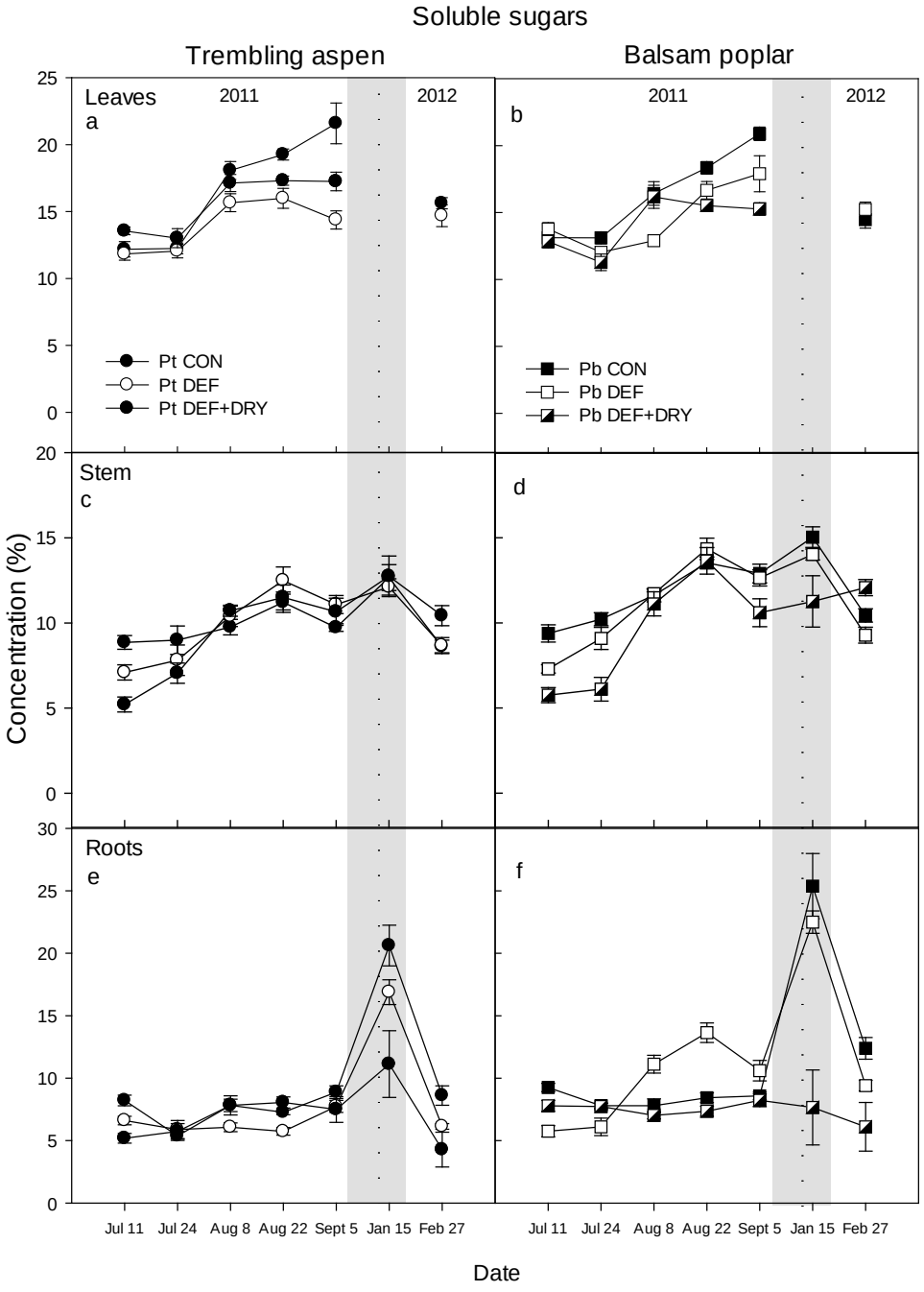

Figure 5.3 Mean (± SE) of soluble sugar concentration in leaves (a,b), stems (c,d) and roots of *Populus tremuloides* (Pt, left column) and *Populus balsamifera* seedlings (Pb, right column) in response to well-irrigated (CON), defoliation (DEF) and defoliation plus drought (DEF+DRY) conditions, (n=6). Statistical analysis is presented in Appendix 2.

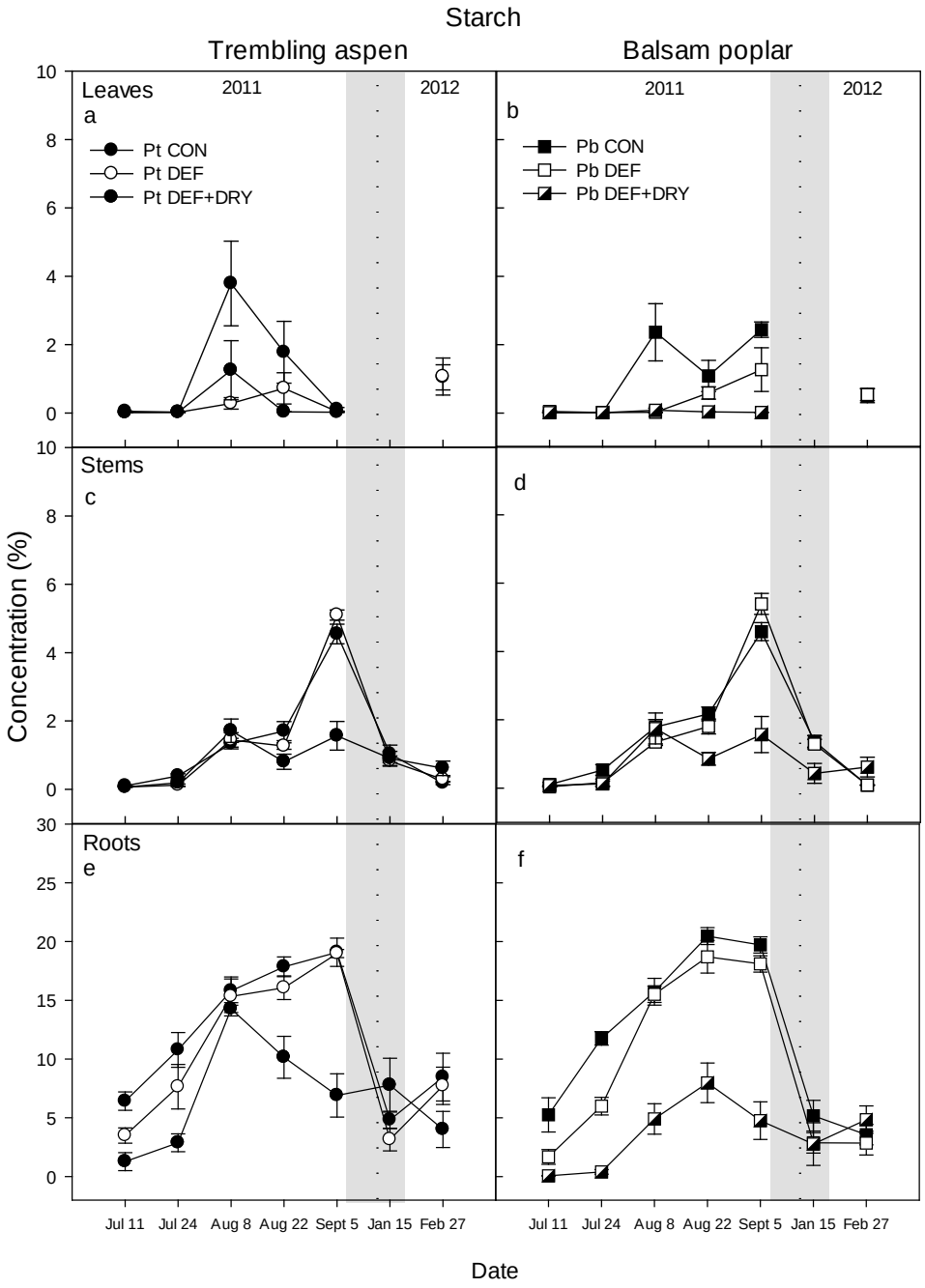

Figure 5.4 Mean (± SE) of starch concentration in leaves (a,b), stems (c,d) and roots of *Populus tremuloides* (Pt, left column) and *Populus balsamifera* seedlings (Pb, right column) in response to well-irrigated (CON), defoliation (DEF) and defoliation plus drought (DEF+DRY) conditions, (n=6). Statistical analysis is presented in Appendix 2.

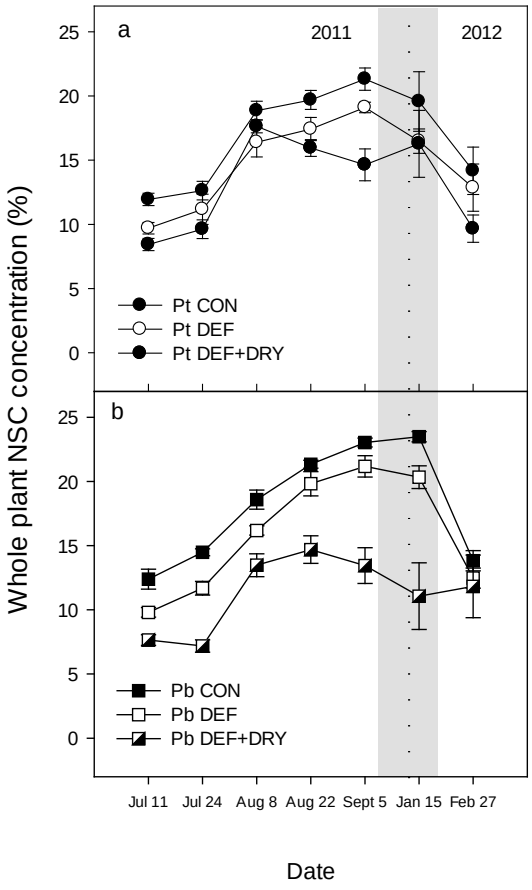

Figure 5.5 Mean (± SE) of whole plant non-structural carbohydrates concentration of *Populus tremuloides* (a) and *Populus balsamifera* (b) in response to well-irrigated (CON), defoliation (DEF) and defoliation plus drought (DEF+DRY) conditions, (n=6). Statistical analysis is presented in Appendix 2.

CHAPTER 6. SYNTHESIS AND FUTURE RESEARCH

6.1 Dissertation Overview

This dissertation investigated the intricate feedbacks between gas exchange, water relations and the dynamics of carbon reserve accumulation of trembling aspen (*Populus tremuloides*) and balsam poplar (*P. balsamifera*) seedlings under drought conditions. Overall, the dissertation explored two main topics of relevance for plant ecology and physiology under current and future drought events, both likely occurring as result of anthropogenic climate change. The first topic was to get a better understanding of how gas exchange and water relations modulate carbon reserve accumulation under drought, while the second issue aimed to understand how the dynamics of carbon reserve accumulation under such conditions change with time.

Current and future drought conditions are expected to play a central role altering species and community composition in forests globally. Trembling aspen and balsam poplar are both tree species that, although closely genetically related, thrive in different edaphic and climatic conditions and have different stomatal behaviour. Based on these differences we hypothesized that variables in our three domain of interest will follow different response trajectories to drought conditions, resulting in different patterns of reserve accumulation over time and, ultimately, in different seedling survivorship. These responses were explored in four different experiments, each one building up and expanding from the previous one, implemented under simulated drought conditions in greenhouse and outside conditions.

Our results will contribute to increase our current understanding of the intricate feedback between variables of these three domains and their role in seedling survivorship under drought conditions. Some of the methodologies, criterion and results resulting from these experiments can be used to explore additional questions in seedlings of other species and as a general reference while working with adult trees or under natural conditions. In the following section I summarize

and discuss some of the general implications of each of our four experiments, as presented in the preceding chapters.

6.2 Research synthesis and implications

6.2.1 A brief preamble

Although gas exchange, water relations and the dynamics of carbon reserve accumulation are commonly studied separately from each other, they can be also seen as parts of a single process starting at a common anatomical structure, the stomata, and ultimately driven by natural selection. The notion that these three physiological domains can be seen as parts of a single process is central to our work and justifies the rationale behind the wide range of variables measured in our experiments. To our knowledge, this tri-domain approach was the first experimental attempt to test some of the central assumptions of the carbon starvation hypothesis (CSH) which is the current theoretical framework on how current and future drought conditions will modify plant species and community composition.

6.2.2 Experiment 1. Stomatal control, water relations and root carbon dynamics of balsam poplar seedlings under simulated drought

In this first experiment we explored two main questions based on some the CSH premises for anisohydric species. These premises were: 1) balsam poplar seedlings will maintain production of photoassimilates to the expense of hydraulic safety under mild drought and 2) they will hydraulically fail under severe drought. Is important to notice that, although balsam poplar is not an anisohydric species *sensu stricto*, it has a significantly less sensitive stomatal control than trembling aspen.

Overall the results in this experiment showed a partial agreement with the CSH premises. Seedlings under mild drought maintained higher CO_2 assimilation and shoot growth than seedlings under severe drought, but this higher CO_2 assimilation did not resulted neither in higher root starch concentration in comparison with seedlings under drought conditions nor in clear signs of

increased hydraulic risk. Seedlings under severe drought conditions did hydraulically fail but had a higher root sugar concentration and similar root starch concentration than seedlings under mild drought. This first experiment also worked as a *proof of concept* for many of the techniques and ideas that were used in subsequent experiments, among them: 1) it is possible to desiccate soil in a controlled and gradual manner, 2) it is possible to stop the desiccation process at predefined targeted levels of water stress and to maintain such targeted levels relatively constant for weeks, 3) water stress targets (e.g. mild or severe) can be defined using stem water potentials and vulnerability curves and 4) these targeted levels of water stress were associated with distinctively different plant responses in our three domains of interest.

In addition to these results, this first experiment provided us with experimental evidence supporting the idea of very complex feedbacks between gas exchange, water relations and carbon accumulation dynamics. These results suggested that the original binary mortality theory of McDowell *et al.* (2008) proposing that trees become vulnerable to pests and extreme climate via carbon starvation or hydraulic failure, was probably overly simplistic; hence, some of the CSH premises need to be revisited.

6.2.3 Experiment 2. Root carbon reserve dynamics in aspen seedlings: does simulated drought induce reserve limitation?

Based on the results of our previous experiment, in our second investigation we used trembling aspen seedlings, a species with isohydric stomatal behaviour. In this experiment we explored the response of gas exchange, water relations and root carbon dynamics to a 3-months drought, the average length of a natural drought in the prairie region of Western Canada. We were particularly interested in determine if a 3-months drought event will significantly reduce root carbon reserves, as predicted by some of the CSH premises for isohydric species.

The main results in this second experiment were somehow unexpected: contrary to what we could expect from an isohydric species, reduced carbon assimilation as a result of severe drought stress did not significantly reduced root carbon reserves in these aspen seedlings. These results suggested that under severe

drought conditions aspen seedlings shifted allocation patterns from growth to reserve accumulation. These findings may support the idea that under drought stress, growth is sink limited (i.e. low water availability results in turgor-limited cell expansion) hence, drought reduces growth more than photosynthesis (Körner 2003). Nonetheless, very recently Wiley and Helliker (2012) have suggested that accumulation of reserves at the cost of reducing growth could be an adaptive response to avoid carbon starvation (sensu McDowell 2011). The authors further suggested that because photosynthesis is reduced with increasing water deficit, low levels of reserves may lead to carbon starvation and death during severe drought. Additionally, reduced CO2 assimilation could also reduce carbon reserve allocation to defense, potentially diminishing the ability of trees to defend or recover from insect attacks in the long term. In our experiment severe drought conditions significantly reduced gas exchange parameters stopping aboveground ground growth. Once shoot growth and leaf production and expansion stopped, photoassimilates were allocated to reserves in roots (stems were not measured in this study), resulting in the fourfold increase of root starch concentration measured in droughted seedlings.

One of the central premises of the CSH states that under severe drought conditions, once gas exchange is significantly reduced, plants will start consuming carbon reserves to maintain respiratory and osmoregulatory cost; hence, we were intrigued by the increase in root carbon measured in our seedlings. We speculated that these results could be one of the key mechanisms behind aspen's remarkable capacity for regrowth from root suckers after severe stress events compromising aboveground growth (e.g. fire or severe browsing).

Nonetheless, these results only posted further and more complex questions. It became clear that in order to answer more ecologically relevant questions we needed to explore the carbon accumulation patterns at the whole plant scale, outside the greenhouse and using more than one species.

6.2.4 Experiment 3. Low reserve accumulation during drought may lead to seedling mortality during following growth season

For our third experiment we grew trembling aspen and balsam poplar seedlings, , at severe drought under more natural conditions outside. After growing for three months, we allowed the seedlings to enter into hardening and dormancy phases from September 2011 to January 2012, and brought them back inside a greenhouse, where we re-watered them in order to determine their ability to continue growing. With this experiment we were interested in exploring: 1) concentration of nonstructural carbohydrates (NSC) at the tissue and whole-plant scale, 2) percentage loss of conductivity and stem water potential and, 3) changes in above and below ground mass under severe drought conditions.

This experiment generated many interesting and new results. To our knowledge this is the very first experiment quantifying the full cycle of drought stress, from non-drought conditions to seedling death in two species. The experiment provided a very detailed description of gas exchange depression during the onset of drought conditions, followed by the effect of drought on variables of three different physiological domains during one growing season and one dormancy period and, after seedlings were re-watered, confirmed seedling mortality. Although the response of carbon accumulation to drought was somehow unexpected (i.e. increase of reserves accumulation, instead of decrease due to carbon starvation), it was the same than in our previous experiment in both species, but the magnitude of response was clearly different. This result posted obvious questions: why did droughted seedlings accumulated fewer reserves? Did one of these experiments have an experimental artifact?

We interpreted these physiological results in the context of the species' phenology. We speculated that seedlings in our previous experiment accumulated more reserves, due to much favorable growing conditions in the greenhouse (i.e. 18 hrs of light and constant temperature). Growing conditions in the greenhouse were in clear contrast with the outside conditions the seedlings had in this experiment (i.e. natural day length became shorter and temperature cooler from July to September). These less optimal growing conditions gradually reduced total daily production of photoassimilates and hence, reserve accumulation.

We consider that the detailed quantification and description of how growing conditions, stomatal behaviour, gas exchange, water relation and carbon accumulation interacted together, ultimately determining seedling survivorship over the dormant period, is a main contribution of this experiment. We speculate that a cascade of feedbacks between these factors determinate seedling survivorship: 1) differences in stomatal behaviour determined how severely gas exchange variables were reduced during the onset of drought (i.e. balsam poplar's more anisohydric behavior resulted in a more severe depression of CO_2 assimilation and leaf stomatal conductance), 2) as total daily production of photoassimilates decreased through summer and fall, reserve accumulation decreased too, 3) as drought progressed, hydraulic conductivity was severely compromised in droughted seedlings, 4) during fall a starch-to-sugar conversion takes place in roots, 5) reduced root starch accumulation in droughted seedlings resulted in reduced root sugar accumulation during the starch to sugar conversion, 6) low root sugar accumulation in droughted seedlings provided limited frost protection of roots and limited resources to maintain root respiration during winter, 7) when droughted seedlings were moved inside the greenhouse and re-watered, a significant amount of root tissue might have been frost damaged compromising reactivation of root growth, 8) translocation from and to any surviving root tissue was compromised by the highly embolized xylem tissue and 9) as resource translocation from and to roots was significantly reduced and xylem tissue was highly embolized activation and growth of aboveground and belowground meristems was also compromised, ultimately resulting in seedling mortality.

6.2.5 Experiment 4. Combining defoliation and drought: Impact on the carbon reserve dynamics of two *Populus* species

In our last experiment we explored the effect of defoliation and drought, both limiting factors of growth and considered as drivers of plant mortality by the CSH, on variables of our three domains of interest. As in our previous experiment we used seedlings of trembling aspen and balsam poplar. We applied drought treatment similar to the one in our previous experiment but added a defoliation treatment. In this experiment we were interested in determine if defoliaiton and

defoliation under drought conditions would differentially impact our variables of interest and if defoliation would magnify the effect of drought on those variables.

Our results suggested defoliation had no negative effect on gas exchange and water relations variables but reduced reserve accumulation and root volume, although at a lesser degree than the combined defoliation and drought treatment did. The combined effect of drought and defoliation negatively impacted all variables, but based on our previous results, we hypothesized drought is by far the stronger driver of the detrimental effects. These results are in concordance with one of the central prediction of the CSH, which suggest that once plant vigor and overall function of physiological and growth variables are negatively affected by drought, pathogen attacks can significantly increase plant mortality. We hypothesized the mechanism leading to plant mortality under drought and defoliation followed a similar cascade of events as the one described in the previous section. Nonetheless, under the combined effect of these two factors reserve accumulation was compromised even further because, while drought reduced gas exchange, defoliation reduced the leaf area where this limited gas exchange occurred, hence total daily production of photoassimilates was reduced even more.

6.3 Research Limitations and Future Research Directions

By definition, extrapolating results from experiments using potted seedlings to adult trees growing under natural environments is difficult, if not risky. Pot-based experiments impose logistical challenges associated with the possibility of root restriction, pest management, continuous tracking of weather conditions (if growing under outside conditions) and general maintenance of hundreds of seedlings. However, this is one of the few feasible methods, within our manpower and budget, to impose and maintain a controlled desiccation treatment as the one used in these experiments. Another obvious limitation of our work is that our seedlings grew for only one growing season, making impossible to estimate the potential effect of previous growing conditions, poor or favorable, on reserve accumulation dynamics. Nonetheless, this experimental setting allowed us to have a very homogenous initial seedling stock, controlled drought

conditions, quantification of reserves at the whole plant scale with high certitude on collection of root biomass and to avoid quantifying reserves accumulated in years prior to the application of drought and to avoid the possible transfer of carbon or water between neighbour trees.

An additional challenge of growing plants using a soilless media with added peat and perlite, as I did in my experiments, is the unavoidable growth of some roots into such materials, which could potentially compromise the retrieval and cleaning of root systems. This potential problem becomes especially relevant while collecting root systems of droughted seedlings because under drought, small roots become more brittle and prompt to be inadvertently detached from primary roots. The use of soilless media with added organic matter may also make the identification and retrieval of detached dead roots more difficult due to the similar appearance of these roots and the added organic materials.

Another important limitation of my experiment was that it was not possible to know when seedlings died; were they already dead at some point during the first growing season (although leaf and root tissues appeared to be alive), during winter, or after re-watering in the spring? A simple solution to this problem could have been to re-water additional sub-sets of droughted seedlings at each collection period in order to identify when re-growth was no longer possible (i.e. seedling mortality). Unfortunately this was beyond a manageable scope and size of the study.

I have identified two potential shortcomings within the implementation of the experimental treatments. First, although at the beginning of the experiment stem water potentials (Ψ) in droughted seedlings remained close to their corresponding targeted values, by the end of the experiment Ψ had become more negative than Ψ values associated with the species-specific P_{50}. This procedural weakness limited my ability to identify in a more conclusive way the individual role of catastrophic hydraulic failure as a driver of seedling mortality. Second, although the randomization process implemented at the beginning of the experiment helped homogenizing seedling height within experimental groups, it was impossible to ensure root volume was also homogenized. This second limitation may help explaining some of the differences detected in the root

volumes measured in Pb DEF seedlings at the beginning of the experiment (Figure 5.1h).

Ideally, with a very large budget, a multi seasonal experiment could be established under natural conditions using saplings growing in large containers placed on, or buried in, forest plots. Such experiment could run for multiple years exposing trees to fully natural climatic conditions. Using dendrological techniques, year-by-year reserves accumulation in roots and boles could be assessed by analyzing tree rings individually and exploring correlations with weather patterns. Such analysis will provide accumulation patterns over time and, if combined with hydraulic transport measurements, it could provide information of how many reserves become permanently sequestered (i.e. irretrievable for the plant regardless current physiological needs) in non-functional xylem. In this type of large scale experiment multiple species can be used including highly drought tolerant species and slow growing conifer. Such questions are completely open roads for future research and results are highly needed.

Finally, another very wide road for future research is to include molecular and biochemical analysis on collected samples. We still have a very poor understanding on how phytohormones, starch-associated proteosynthesis and proteolysis, and aquaporins change over time in different tissues under an experimental setting as the one used in our work.

6.4 References

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APPENDIX 1

Table A1.1 ANOVA table for variable Height Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	3770.258	942.564	6.936	<0.001
Species	1	34850.208	34850.208	256.460	<0.001
Treatment	1	6223.680	6223.680	45.800	<0.001
Time x Species	4	226.441	56.610	0.417	0.796
Time x Treatment	4	1010.024	252.506	1.858	0.124
Species x Treatment	1	0.616	0.616	0.00454	0.946

Table A1.2 ANOVA table for variable Number of leaves. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	261.467	65.367	2.558	0.043
Species	1	1484.033	1484.033	58.068	<0.001
Treatment	1	2502.533	2502.533	97.921	<0.001
Time x Species	4	67.967	16.992	0.665	0.618
Time x Treatment	4	862.800	215.700	8.440	<0.001
Species x Treatment	1	97.200	97.200	3.803	0.054

Table A1.3 ANOVA table for variable Leaf area. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	1742724.7	435681.182	6.783	<0.001
Species	1	3829066.2	3829066.236	59.611	<0.001
Treatment	1	4811122.436	4811122.436	74.899	<0.001
Time x Species	4	202936.416	50734.104	0.790	0.534
Time x Treatment	4	1581449.108	395362.277	6.155	<0.001
Species x Treatment	1	1051627.847	1051627.847	16.372	<0.001

Table A1.4 ANOVA table for variable Root volume. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	234.199	58.550	37.517	<0.001
Species	1	36.931	36.931	23.665	<0.001
Treatment	1	338.142	338.142	216.672	<0.001
Time x Species	4	4.863	1.216	0.779	0.541
Time x Treatment	4	185.043	46.261	29.643	<0.001
Species x Treatment	1	0.565	0.565	0.362	0.549

Table A1.5 ANOVA table for variable CO_2 assimilation. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	218.915	54.729	19.014	<0.001
Species	1	16.097	16.097	5.592	0.020
Treatment	1	1828.866	1828.866	635.397	<0.001
Time x Species	4	36.385	9.096	3.160	0.017
Time x Treatment	4	56.902	14.225	4.942	0.001
Species x Treatment	1	78.230	78.230	27.179	<0.001

Table A1.6 ANOVA table for variable Leaf stomatal conductance. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	0.264	0.0660	28.857	<0.001
Species	1	0.120	0.120	52.652	<0.001
Treatment	1	1.965	1.965	859.116	<0.001
Time x Species	4	0.0854	0.0214	9.336	<0.001
Time x Treatment	4	0.0808	0.0202	8.832	<0.001
Species x Treatment	1	0.161	0.161	70.383	<0.001

Table A1.7 ANOVA table for variable Percentage loss of conductivity (PLC). Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	45771.290	11442.823	318.771	<0.001
Species	1	54.783	54.783	1.526	0.220
Water status	1	42958.238	42958.238	1196.720	<0.001
Time x Species	4	139.109	34.777	0.969	0.428
Time x Water status	4	30175.025	7543.756	210.152	<0.001
Species x Water status	1	3.968	3.968	0.111	0.740

Table A1.8 ANOVA table for variable Stem water potential. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	1.862	0.466	14.174	<0.001
Species	1	14.407	14.407	438.619	<0.001
Treatment	1	46.875	46.875	1427.056	<0.001
Time x Species	4	0.137	0.0343	1.043	0.389
Time x Treatment	4	0.816	0.204	6.210	<0.001
Species x Treatment	1	5.059	5.059	154.028	<0.001

Table A1.9 ANOVA table for variable Leaf sugar concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	838.171	209.543	83.507	<0.001
Species	1	107.380	107.380	42.793	<0.001
Treatment	1	37.976	37.976	15.134	<0.001
Time x Species	4	34.159	8.540	3.403	0.012
Time x Treatment	4	149.470	37.368	14.892	<0.001
Species x Treatment	1	78.748	78.748	31.383	<0.001

Table A1.10 ANOVA table for variable Leaf starch concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	45.136	11.284	11.239	<0.001
Species	1	0.0691	0.0691	0.0688	0.794
Treatment	1	35.576	35.576	35.433	<0.001
Time x Species	4	14.789	3.697	3.682	0.008
Time x Treatment	4	31.531	7.883	7.851	<0.001
Species x Treatment	1	0.203	0.203	0.202	0.654

Table A1.11 ANOVA table for variable Stem sugar concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	438.684	109.671	54.789	<0.001
Species	1	54.071	54.071	27.013	<0.001
Treatment	1	7.170	7.170	3.582	0.061
Time x Species	4	65.622	16.406	8.196	<0.001
Time x Treatment	4	69.995	17.499	8.742	<0.001
Species x Treatment	1	6.780	6.780	3.387	0.069

Table A1.12 ANOVA table for variable Stem starch concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	129.460	32.365	129.349	<0.001
Species	1	2.594	2.594	10.367	0.002
Treatment	1	25.954	25.954	103.725	<0.001
Time x Species	4	2.494	0.623	2.492	0.048
Time x Treatment	4	43.643	10.911	43.605	<0.001
Species x Treatment	1	0.166	0.166	0.663	0.418

Table A1.13 ANOVA table for variable Root sugar concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	69.085	17.271	8.313	<0.001
Species	1	0.00210	0.00210	0.00101	0.975
Treatment	1	0.251	0.251	0.121	0.729
Time x Species	4	10.361	2.590	1.247	0.296
Time x Treatment	4	61.009	15.252	7.341	<0.001
Species x Treatment	1	22.764	22.764	10.956	0.001

Table A1.14 ANOVA table for variable Root starch concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	2155.694	538.924	81.294	<0.001
Species	1	25.643	25.643	3.868	0.052
Treatment	1	1662.508	1662.508	250.782	<0.001
Time x Species	4	118.710	29.677	4.477	0.002
Time x Treatment	4	308.881	77.220	11.648	<0.001
Species x Treatment	1	69.508	69.508	10.485	0.002

Table A1.15 ANOVA table for variable Total NSC concentration at the whole plant scale. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	1483.851	370.963	118.070	<0.001
Species	1	5.252	5.252	1.672	0.199
Treatment	1	417.225	417.225	132.794	<0.001
Time x Species	4	88.562	22.140	7.047	<0.001
Time x Treatment	4	109.733	27.433	8.731	<0.001
Species x Treatment	1	66.835	66.835	21.272	<0.001

APPENDIX 2

Table A2.1 ANOVA table for variable Height Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	3688.337	922.084	7.280	<0.001
Species	1	46115.207	46115.207	364.091	<0.001
Defoliation	2	8421.690	4210.845	33.246	<0.001
Time x Species	4	378.005	94.501	0.746	0.562
Time x Defoliation	8	1431.564	178.945	1.413	0.195
Species x Defoliation	2	148.079	74.039	0.585	0.559

Table A2.2 ANOVA table for variable Number of leaves. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	253.756	63.439	4.900	<0.001
Species	1	789.606	789.606	60.984	<0.001
Defoliation	2	8965.811	4482.906	346.230	<0.001
Time x Species	4	85.644	21.411	1.654	0.164
Time x Defoliation	8	48.744	6.093	0.471	0.875
Species x Defoliation	2	486.544	243.272	18.789	<0.001

Table A2.3 ANOVA table for variable Leaf area. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	182878.529	45719.632	1.133	0.343
Species	1	1729161.028	1729161.028	42.850	<0.001
Defoliation	2	16387450.812	8193725.406	203.047	<0.001
Time x Species	4	28496.167	7124.042	0.177	0.950
Time x Defoliation	8	144048.162	18006.020	0.446	0.891
Species x Defoliation	2	2872379.730	1436189.865	35.590	<0.001

Table A2.4 ANOVA table for variable Root volume. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	259.472	64.868	52.485	<0.001
Species	1	36.592	36.592	29.606	<0.001
Defoliation	2	499.851	249.925	202.215	<0.001
Time x Species	4	9.324	2.331	1.886	0.116
Time x Defoliation	8	200.217	25.027	20.249	<0.001
Species x Defoliation	2	1.806	0.903	0.731	0.483

Table A2.5 ANOVA table for variable CO_2 assimilation. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	0.822	0.206	42.649	<0.001
Species	1	0.0847	0.0847	17.582	<0.001
Defoliation	2	4.154	2.077	431.063	<0.001
Time x Species	4	0.0489	0.0122	2.536	0.042
Time x Defoliation	8	0.251	0.0314	6.518	<0.001
Species x Defoliation	2	0.229	0.114	23.740	<0.001

Table A2.6 ANOVA table for variable Leaf stomatal conductance. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	870.701	217.675	45.494	<0.001
Species	1	1.724	1.724	0.360	0.549
Defoliation	2	1815.034	907.517	189.669	<0.001
Time x Species	4	137.192	34.298	7.168	<0.001
Time x Defoliation	8	234.738	29.342	6.132	<0.001
Species x Defoliation	2	110.497	55.248	11.547	<0.001

Table A2.7 ANOVA table for variable Percentage loss of conductivity (PLC). Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	21960.493	5490.123	154.612	<0.001
Species	1	1232.345	1232.345	34.705	<0.001
Defoliation	2	26535.591	13267.796	373.646	<0.001
Time x Species	4	1424.208	356.052	10.027	<0.001
Time x Defoliation	8	23814.784	2976.848	83.834	<0.001
Species x Defoliation	2	1767.622	883.811	24.890	<0.001

Table A2.8 ANOVA table for variable Stem water potential. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	0.959	0.240	5.527	<0.001
Species	1	12.456	12.456	287.282	<0.001
Defoliation	2	44.386	22.193	511.866	<0.001
Time x Species	4	0.377	0.0943	2.174	0.075
Time x Defoliation	8	0.422	0.0527	1.216	0.293
Species x Defoliation	2	13.548	6.774	156.243	<0.001

Table A2.9 ANOVA table for variable Leaf sugar concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	927.814	231.953	89.486	<0.001
Species	1	5.375	5.375	2.074	0.152
Defoliation	2	154.170	77.085	29.739	<0.001
Time x Species	4	33.575	8.394	3.238	0.014
Time x Defoliation	8	160.217	20.027	7.726	<0.001
Species x Defoliation	2	33.448	16.724	6.452	0.002

Table A2.10 ANOVA table for variable Leaf starch concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	41.381	10.345	10.897	<0.001
Species	1	0.00496	0.00496	0.00522	0.943
Defoliation	2	36.105	18.053	19.015	<0.001
Time x Species	4	21.492	5.373	5.660	<0.001
Time x Defoliation	8	43.577	5.447	5.738	<0.001
Species x Defoliation	2	1.304	0.652	0.687	0.505

Table A2.11 ANOVA table for variable Stem sugar concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	748.630	187.158	91.888	<0.001
Species	1	60.634	60.634	29.769	<0.001
Defoliation	2	66.604	33.302	16.350	<0.001
Time x Species	4	17.886	4.471	2.195	0.072
Time x Defoliation	8	86.577	10.822	5.313	<0.001
Species x Defoliation	2	14.955	7.478	3.671	0.028

Table A2.12 ANOVA table for variable Stem starch concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	317.480	79.370	266.352	<0.001
Species	1	0.701	0.701	2.352	0.127
Defoliation	2	27.546	13.773	46.219	<0.001
Time x Species	4	0.685	0.171	0.575	0.681
Time x Defoliation	8	73.362	9.170	30.774	<0.001
Species x Defoliation	2	0.357	0.178	0.598	0.551

Table A2.13 ANOVA table for variable Root sugar concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	84.756	21.189	9.760	<0.001
Species	1	3.909	3.909	1.801	0.182
Defoliation	2	85.324	42.662	19.651	<0.001
Time x Species	4	3.671	0.918	0.423	0.792
Time x Defoliation	8	74.069	9.259	4.265	<0.001
Species x Defoliation	2	50.942	25.471	11.733	<0.001

Table A2.14 ANOVA table for variable Root starch concentration. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	4305.439	1076.360	143.760	<0.001
Species	1	50.572	50.572	6.754	0.010
Defoliation	2	2607.909	1303.955	174.158	<0.001
Time x Species	4	77.610	19.402	2.591	0.039
Time x Defoliation	8	484.936	60.617	8.096	<0.001
Species x Defoliation	2	137.115	68.557	9.157	<0.001

Table A2.15 ANOVA table for variable Total NSC concentration at the whole plant scale. Differences between means were considered significant at an α=0.05

Source of Variation	DF	SS	MS	F	P
Time	4	2442.552	610.638	176.695	<0.001
Species	1	0.0233	0.0233	0.00675	0.935
Defoliation	2	803.874	401.937	116.305	<0.001
Time x Species	4	36.373	9.093	2.631	0.037
Time x Defoliation	8	122.741	15.343	4.440	<0.001
Species x Defoliation	2	89.830	44.915	12.997	<0.001