CARBON DIOXIDE FLUXES AND NONSTRUCTURAL CARBOHYDRATES IN SEEDLINGS AS INFLUENCED BY HEAT, DROUGHT, AND LOW LIGHT

by

CODY MULLINS LUEDTKE

(Under the Direction of Robert O. Teskey)

ABSTRACT

Our purpose was to examine the effects of mortality-inducing heat, drought, and low light on *Pinus taeda* L. seedlings over time. We monitored net photosynthetic assimilation and stem CO_2 efflux (F_s) for 85 days, with periodic destructive harvests to measure biomass and nonstructural carbohydrates. We found a strong relationship between F_s and whole plant starch concentration among the treatments ($P<0.01$, $R^2=0.672$). Seedlings in the heat treatment survived with significantly reduced nonstructural carbohydrate concentrations and growth, but significantly elevated F_s compared with seedlings in the control treatment. Seedlings in the drought and low light treatments had significantly reduced net assimilation and F_s compared with seedlings in the control treatment, and died after 10 weeks with severely depleted levels of starch. We conclude that carbon starvation played the primary role in their mortality, with water stress contributing to mortality in the drought treatment as well.

INDEX WORDS: Carbon starvation, Hydraulic failure, Mortality, Photosynthesis, Stem CO_2 efflux, Starch, NSC, TNC, Loblolly pine

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CODY MULLINS LUEDTKE

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CODY MULLINS LUEDTKE

Major Professor:	Robert O. Teskey
Committee:	Lisa A. Donovan Mary Anne McGuire Marc W. van Iersel

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

A plant must tolerate stressful conditions to survive in a natural environment. There is substantial research investigating the response of trees to biotic and abiotic stress (Kozlowski and Pallardy 2002). However, mechanisms of tree mortality under stressful conditions remain largely unresolved (Sala et al. 2010), despite the widespread tree mortality observed in recent years (Allen et al. 2010).

Plants harness energy from the sun to convert carbon dioxide (CO_2) into carbohydrates. Nonstructural carbohydrate (NSC) products of photosynthesis may be broken down immediately to release energy or stored throughout the plant for later use. These storage compounds can serve as a substrate for respiration, establishing the links among photosynthesis, stored carbon (C), and respiration. The energy released by respiration is used for growth, maintenance processes, and ion uptake. Although many studies have focused on the effects of environmental stresses on photosynthesis and respiration of trees, relatively few studies have examined whether trees maintain C stores, store additional C, or deplete their C stores during stressful times. The purpose of this study was to evaluate how NSCs, as well as net photosynthetic assimilation (A_{net}) and dark respiration (R_d) respond in tree seedlings exposed to heat, drought, or low light treatments. The remainder of this chapter will review the properties and uses of NSCs and how they are affected by the stresses imposed by heat, drought and low light. Photosynthesis and respiration will also be discussed in this chapter.

NONSTRUCTURAL CARBON COMPOUNDS

Starch, sucrose, glucose and fructose make up more than 80% of the nonstructural C pool in temperate forest trees (Hoch et al. 2003), which excludes structural carbohydrates such as cellulose and lignin. Hoch et al. (2003) also detected raffinose and stachyose in all ten species they sampled, with the sugar alcohol pinnitol contributing a small amount to the nonstructural C pool of *Pinus sylvestris*. Lipids

were detected at concentrations of 5-12% dry mass in the branches of *P. sylvestris*, and 1-2% dry mass in stem sapwood.

Concentrations of NSCs may fluctuate seasonally. In *Pinus sylvestris*, NSCs reached a maximum of 25% dry mass in needles and 11% dry mass in branch sapwood before bud break in April, after which they gradually declined to minima of 10% and 5% dry mass, respectively (Hoch et al. 2003). The variation in NSC was primarily due to changes in starch levels because total sugars remained relatively constant, fluctuating by only 3% dry mass throughout the year. A similar trend of increasing sugar and starch concentration in cooler months followed by a decline in the summer months was observed in *Pinus radiata* leaves (Ow et al. 2010). Stem sapwood NSCs were not considerably different among seasons (Hoch et al. 2003).

Sugars are synthesized in the cytosol from triose-phosphate, the major product of photosynthesis (Lambers et al. 2009). The fate of triose-phosphate depends largely on the concentration of inorganic phosphate (P_i) in the cytosol, which is used to export triose-phosphate out of the chloroplast. High levels of P_i in the cytosol allow for more export and sucrose synthesis, whereas low levels of P_i result in triose-phosphate remaining in the chloroplast to be synthesized into starch.

Sucrose is the major sugar produced by photosynthesis and is ubiquitous in plants (Pollock et al. 1999). It may accumulate in large quantities in sink tissue, and may form the bulk of the mobile stored NSC pool (Avigad 1982). When sucrose is needed for respiration it is broken down enzymatically by invertase, producing a glucose and fructose monomer (Stitt and Steup 1985). Glucose and fructose are usually present in smaller amounts, and mainly function as metabolic intermediates or breakdown products (Pollock et al. 1999). Synthesized sugars (usually sucrose) are transported in the phloem to sinks throughout the plant where they may be used, or stored in cellular vacuoles (Stitt and Steup 1985). In addition to being the primary substrate for respiration, sugars are also used in osmotic regulation, cryoprotection, and as signaling molecules (Koch 1996; Peshev et al. 2013; Stitt and Steup 1985). Sugars are sensed by plant cells and act as long-distance messengers, causing changes in gene expression which lead to long-term changes in plant development (Koch 1996).

The term “starch” describes the compounds amylopectin and amylose, oligosaccharides of linked glucose comprising approximately 70% and 30% of the starch pool, respectively (Martin and Smith 1995). These proportions vary with plant species, variety, growth conditions, plant organ, and organ age (Martin and Smith 1995). Amylopectin is heavily branched, containing 20-40 clusters of glucose chains with about 20 glucose units each, whereas amylose is made up of highly linear chains of glucose about 1000 units long that are very infrequently branched (Martin and Smith 1995).

Starch is synthesized in plastids, including amyloplasts, chloroplasts, and chromoplasts (Martin and Smith 1995). Synthesis begins with ADP-glucose, a product of glucose-1-phosphate (supplied by either the reductive pentose phosphate pathway or synthesized from glucose-6-phosphate) and ATP (derived from photosynthesis or respiration), and progresses with the help of three enzymes: ADP-glucose pyrophosphorylase, starch synthase, and starch branching enzyme (Martin and Smith 1995). Starch synthesis in the chloroplast is strongly governed by the ratio of P_i, which is an inhibitor, to 3-phosphoglycerate (3-PGA), which is an activator of the key enzyme ADP-glucose pyrophosphorylase (AGPase) (Martin and Smith 1995).

Plants typically store starch as granules within amyloplasts, which have crystalline regions composed of amylopectin and amorphous regions composed of amylose (Jenner 1982; Martin and Smith 1995). When needed by the plant, these granules are broken down enzymatically into glucose-1-phosphate and used as a respiratory substrate (Stitt and Steup 1985). Morphological and physiological studies have described small starch granules with diurnal turnover that are present in photosynthetic cells and larger granules that are built and turned over more slowly (Jenner 1982; Stitt and Steup 1985). The turnover of NSCs often takes years in trees, and models agree that a two-pool system explains turnover most accurately; one pool with fast turnover, and one which turns over more slowly (Richardson et al. 2013).

Conceptually, stored NSCs can be broken down into three classes based on the mechanism by which they are stored: accumulation, reserves, and recyclable compounds (Chapin et al. 1990). Accumulated compounds are derived from excess C beyond the amount required for growth and

maintenance processes. Reserves are actively sequestered using C that might otherwise be used for growth or maintenance, thus competing with these processes for C. Recyclable compounds are those which normally serve a defense or growth function, but can be broken down and used for energy when needed. The actual function of stored C has some bearing on how C stores can be interpreted. For example, if control over C reserve formation in some species is an active process governed primarily by genetics, then analysis of C compounds in those species may tell us relatively little about a plant's carbon status (Sala et al. 2012).

Lipids do not usually serve a large role in tree energy storage, although some tree species build large lipid pools which may be used for storage and non-storage purposes (e.g. defensive and protective cutins and waxes) (Chapin et al. 1990). Nelson and Dickson (1981) saw an increase of triglycerides in cottonwoods from about 1 to 3% dry weight in dormancy-inducing conditions, but the function of these lipids remains unclear (Beeson and Proebsting 1988). In a study of temperate forest trees, lipids demonstrated little seasonal variation (Hoch et al. 2003). Lipids are usually discussed in reference to seeds, where they are a major source of energy and carbon skeletons during germination (Taiz and Zeiger 2010).

Sugar alcohols, including cyclitols (e.g. *myo*-inositol) and hexitols (e.g. sorbitol) usually comprise less than 10% of the nonstructural C pool (Hoch et al. 2003). These compounds often function as compatible solutes, used to maintain water potential equilibrium in plant tissues (Taiz and Zeiger 2010). They are also used as non-reducing transport compounds in the phloem (Lambers et al. 2009).

Drought can have varying effects on NSC concentrations in species depending on their drought response strategy and the severity and method of imposing drought in the experiment (Piper 2011). For example, in a study conducted with *Eucalyptus saligna*, drought-treated seedlings increased their starch concentrations almost 3-fold, although sugars showed no significant change with water status (Ayub et al. 2011). The increase in starch was attributed to the drought-induced inhibition of C export and use, coupled with the relatively smaller inhibitory effect of drought on photosynthesis, resulting in C surplus. The change in sucrose levels with drought stress is variable, with increases generally attributed to osmotic

adjustment and efforts to reduce tissue damage (Avigad 1982). On the other hand, *Pinus radiata* seedlings exposed to long-term mortality-inducing drought depleted 40% of their total NSC reserves, suggesting death caused by carbon starvation (Mitchell et al. 2013). Two *Eucalyptus* species in the same experiment did not survive as long during the drought, and had less depleted total NSCs at the time of mortality due to elevated soluble sugars. This suggests that *Eucalyptus* experienced a more intense and shorter duration drought than *Pinus radiata* because of their different physiological responses to drought stress.

Low water potentials induced by drought stress may increase hydraulic tension in the stem to the point of embolism, leading to cavitation of the xylem and/or phloem. The stem water potential at which cavitation occurs varies with species (Zimmermann 2002). Cavitation greatly hampers the plant's ability to transport water and solutes, and may lead to an inability of the plant to move carbon compounds from source to sink. The mechanisms of drought-induced mortality are disputed, but it is generally agreed that NSC starvation and/or water stress causing xylem cavitation or disruption of cellular metabolism are causal factors, often weakening the plant and allowing fatal infestation by pests or disease (Adams et al. 2013; McDowell et al. 2008). However, these mechanisms are not necessarily mutually exclusive, and the influence of each effect may vary depending on species or environment, for example (Sala et al. 2012).

Temperature can also affect plant NSC levels, with most studies reporting a negative relationship between temperature and NSC concentration. An experimental elevation in temperature was accompanied by decreased NSC and soluble sugar concentrations in 19 species in 3 functional groups, with an almost 140% decrease in sugar concentration from 7°C to 28°C in acclimated tissues, and a 192% decrease in total NSC (Campbell et al. 2007). Consistent with the previous study, high temperatures significantly reduced sugar concentration in the foliage of *Pseudotsuga menzeisii* (Hobbie et al. 2002). Starch in *Populus* leaves was broken down three times faster when leaves were moved to warmer conditions, suggesting elevated respiration rates (Huve et al. 2012). However, Tjoelker et al. (1999b) found no significant effect of growth temperature on total NSC concentration of *Pinus*.

As temperature rises, plants must meet the increased demand for energy to support associated increases in biosynthesis, transport, and protein turnover (Lambers et al. 2009) by metabolizing stored C,

which may diminish NSC stores to the point of carbon starvation, resulting in mortality (Allen et al. 2010). However, in natural environments heat-induced mortality may be a consequence of increased infestation pressure from insects and fungi whose life cycles are accelerated in warmer conditions (Allen et al. 2010).

Low light conditions show a consistent negative effect on NSC concentrations, and there is some evidence that starch synthesis is partially regulated by light intensity (Geigenberger et al. 2005). *Populus deltoides* saplings subjected to low light significantly reduced leaf and root soluble sugar concentrations, with a depletion of about 80% of total leaf NSC compounds (Wertin and Teskey 2008). Saplings in no-light treatments were affected similarly, but showed an additional 52% decrease of total root NSC concentrations. Low light may also cause mortality due to carbon starvation. When irradiance decreases below the light compensation point, plants are unable to obtain enough carbon to offset respiratory CO_2 losses. If the darkening persists, plants may rapidly deplete stored NSCs to meet their energy demands, and may use proteolysis to support metabolism after 48 hours (Brouquisse et al. 1998). Despite these examples of rapid responses to darkness, *Betula pubescens* seedlings have been observed to survive for up to 68 days in complete darkness (Hutchinson 1967).

PHOTOSYNTHESIS

Photosynthesis is the process used by plants to "fix" C, or convert it from gaseous CO_2 into a reduced form suitable for energy storage. This process is fueled with solar energy harvested by chlorophyll and accessory pigments within the mesophyll cells of leaves. The harvested energy may be transferred to the reaction centers of photosynthesis by means of the light harvesting complex of chlorophyll molecules, or lost as heat or fluorescence. The two reaction centers are photosystem I and photosystem II, each of which plays a unique role in photosynthesis. First, an electron derived from hydrolyzed water is accepted by photosystem II and from there it is transferred along an electron transport chain to photosystem I, generating a proton motive force and ATP in the process. The electron moves from photosystem I to ferredoxin, where NADH+ is reduced, forming NADPH. These processes are collectively called the "light reactions" of photosynthesis.

The "carbon reactions" of photosynthesis describe the synthesis of C compounds, and are also known as the Calvin cycle. The enzyme ribulose-1,5-bisphosphate carboxylase/oxyenase (Rubisco) uses ribulose-1,5-bisphosphate (RuBP) and CO_2 as substrates to yield phosphoglyceric acid, which is reduced to triose-phosphate using ATP and NADPH produced in the light reactions. Alternatively, Rubisco may use oxygen as a substrate for photorespiration, a process which results in a net loss of carbon. The triose-phosphate normally produced by photosynthesis may remain in the chloroplast to form starch or regenerate RuBP. Triose-phosphate may also be exported into the cytosol where it is used to make sucrose and other carbohydrates, also known as photosynthates or photoassimilates. These photosynthates are typically transported from the source to a sink where there is a demand for them.

Photosynthesis of a plant is maximal in its optimum temperature range, and decreases when temperature is elevated or reduced outside of that optimum (Lambers et al. 2009). However, a meta-analysis found that photosynthesis generally increases with increasing growth temperature (Way and Oren 2010). Photosynthetic response to temperature varies based on the habitat a plant is adapted to, and to some degree based on plant species (Medlyn et al. 2002). Wertin et al. (2010) suggested that loblolly pine photosynthesis will not be negatively affected should temperatures rise by 2-3°C within its native range. However, temperatures higher than optimum may cause disruption of photosynthesis by reducing the integrity of the photosynthetic apparatus in the chloroplast (Berry and Bjorkman 1980). Ameye et al. (2012) found significant decreases of 60-108% in photosynthesis of *Pinus taeda* during 12°C heat wave events lasting one week. Other negative effects of heat stress include membrane and protein destabilization, respiratory inhibition, reactive oxygen species production, and eventually cell death (Taiz and Zeiger 2010).

There is evidence that loblolly pine exhibits thermal acclimation of respiration, but conflicting reports on thermal acclimation of photosynthesis (Nedlo et al. 2009; Ow et al. 2010; Teskey and Will 1999). A study of loblolly pine grown at different temperatures found that maximum net photosynthetic assimilation (A_{net}) was greatest when measured at the growth temperature and lower when measured at other temperatures, indicating some degree of thermal acclimation (Teskey and Will 1999). However, in

that study acclimation was not complete because photosynthesis was greatest for plants grown and measured at 25°C, and lower for the 30°C and 35°C treatments.

Water deficit also reduces photosynthetic rate, with more severe droughts having a greater effect (Galmes et al. 2007; Limousin et al. 2013). When plants experience drought stress they decrease stomatal conductance, limiting the influx of CO_2 through stomata. The resulting decrease in intracellular CO_2 limits the amount of C that is available to be fixed through photosynthesis, thus lowering photosynthetic rate. Drought can also cause increased hydraulic resistance, reduced cell and leaf expansion, leaf abscission, altered carbon partitioning, cytorrhysis (collapse of plant cell wall and consequent cell shrinkage due to water loss), cavitation, membrane and protein destabilization, reactive oxygen species production, ion cytotoxicity, and cell death (Taiz and Zeiger 2010).

Low light imposes a stress on plants by limiting photosynthetic rate, and thus limiting C gain. Below the level of light saturation of photosynthesis, a reduction in light intensity reduces A_{net} in plants. For example, a 65 percent shade treatment of loblolly pine reduced A_{net} by about half (Zhang et al. 1997). A 51% reduction in maximum photosynthesis was observed in *Abies amabilis* trees after 100 days of treatment with an 80% light reduction from ambient (Brooks et al. 1994). When light levels are below the light compensation point, plants have a negative carbon balance. The light compensation point for trees ranged from 2-12 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) in a study of 13 tropical trees, and a study of four temperate tree species found light compensation points from 30-120 μmol m^{-2} s^{-1} PAR (Groninger et al. 1996; Kitajima 1994).

Light level has a significant influence on various aspects of plant size, allocation, morphology, and architecture (P<0.001) (Poorter 1999). When individual branches of an herbaceous plant were darkened, those branches died or had significantly modified growth and development (Kemball et al. 1992). However, darkening an entire *Arabadopsis* plant did not immediately induce senescence of leaves, suggesting that whole plant light status may affect leaves differently than individual leaf light status (Weaver and Amasino 2001). As long as enough light is available for the plant to survive, leaves acclimate to the light conditions in which they are grown, with shade leaves developing lower light

compensation points and lower light-saturated photosynthetic rates compared with sun leaves (Kitajima 1994). Shade leaves are also typically thinner, and have lower rates of photosynthesis per unit area.

RESPIRATION

Respiration produces ATP and biosynthetic precursors, and can facilitate nitrogen assimilation, photosynthesis, stress acclimation, programmed cell death, fruit ripening, and thermogenesis (Plaxton and Podesta 2006). During respiration, energy is released from storage molecules through complex oxidation and temporarily stored in the form of adenosine triphosphate (ATP). This chemical energy is used for plant growth, maintenance, and ion uptake (Lambers 1985).

Respiratory processes can be grouped into glycolysis, the oxidative pentose phosphate pathway, the tricarboxylic acid (TCA) cycle (also called the citric acid or Krebs cycle), and electron-transport pathways. Respiratory substrates can include carbohydrates, lipids, organic acids, and proteins, but sucrose or starch usually serve as the substrate. Other whole-plant biochemical processes and the quantity of substrate available strongly influence the efficiency of respiration. When sufficient oxygen is not available, respiration will proceed through the anaerobic fermentation pathway, producing lactate or ethanol and oxidizing NADH into NAD^+.

Glycolysis describes the oxidation of a carbohydrate or sugar alcohol into malate or pyruvate and a small amount ATP and NADH. This process is controlled enzymatically with adenylates, which allow it to accelerate when metabolic demand for ATP increases (Plaxton and Podesta 2006). The oxidative pentose phosphate pathway, which accounts for 10-25% of glucose breakdown, appears to be most important for producing intermediate metabolic products and NADPH, and does not seem to be controlled by energy demand (Lambers et al. 2009; Taiz and Zeiger 2010).

In the mitochondria, malate or pyruvate are oxidized in the TCA cycle to generate the reducing compounds NADH and $FADH_2$. Carbon dioxide is released in this second phase of respiration. Substantial control is exerted on this point in the process by the major enzyme pyruvate decarboxylase. Next, the electron-transport chain accepts two electrons from NADH (or NAD(P)H produced elsewhere), and establishes an electrochemical gradient through a series of electron transfers. This process includes

the transfer of an electron by cytochrome c, hence it is called the cytochrome pathway (Lambers et al. 2009). The proton gradient generated here is used to synthesize ATP, and the entire process is called oxidative phosphorylation.

The non-phosphorylating cyanide-resistant pathway may be entered into at the ubiquinone transfer point in the electron-transport chain. This cyanide-resistant alternative oxidase path produces only one third the amount of ATP that would be generated by the cytochrome path. It is activated in strongly reducing conditions such as low light, low temperature, drought, osmotic stress, wounding, or pathogen attacks (Plaxton and Podesta 2006). This cyanide-resistant pathway may prevent some damage inflicted by reactive oxygen intermediates, which are produced in response to stress. The alternative pathway may also be involved in oxidizing excess sugars (Lambers 1985).

Respiration is governed by a plant's respiratory capacity, energy demand, substrate availability, and oxygen supply, as well as environmental conditions (Covey-Crump et al. 2002). Control over respiration can be described as fine or coarse (Plaxton and Podesta 2006). Coarse control comprises longer-term differences in the enzyme concentration in cells, controlled by genetic expression or protein turnover rates. Fine control involves the regulation of enzyme activity by adjusting the rate of metabolite flux through respiratory pathways. Maintenance respiration, which represents the component of respiration associated with protein and membrane repair, and the maintenance of ion gradients, is sensitive to changes in temperature, water status, pollutants, and CO_2 concentration (Ryan 1991). Maintenance respiration rate is also highly correlated with the amount of protein present in the leaf, representative of energy demand. Reich et al. (2006) observed an isometric increase in total plant respiration with total plant N. Growth respiration, representing the cost of new tissue synthesis, is sensitive to environmental factors affecting growth, such as water stress, pollution, plant nutrient status, and CO_2 concentration (Ryan 1991). Different tissues exhibit different respiration rates. For example, measured CO_2 efflux in mature *Pinus radiata* was highest in growing foliage and mature foliage (1.7-3.0 and 1.5-2.8 μmol kg^{-1} s^{-1}), followed by fine and coarse roots (0.8-1.6 and 0.2-1.5 μmol kg^{-1} s^{-1}), and lowest in stem and branch sapwood (0.05-0.18 and 0.06-0.11 μmol kg^{-1} s^{-1}) (Vose and Ryan 2002).

Environmental factors can have a significant effect on the respiration rate of plant tissues. Dark respiration of leaves decreases as plants experience greater drought stress, reflecting a general decrease in metabolic processes and demand, specifically with regard to the decrease in C assimilation and growth (Lambers et al. 2009). This effect seems to vary greatly with species. In a study conducted by Galmes et al. (2007), Mediterranean herbs reduced dark respiration in response to drought more so than shrubs, which the authors attributed to a shorter leaf lifespan and greater need to optimize carbon balance. A study with *Populus nigra* found a 40% decrease in dark respiration with a reduction of transpirable soil water from 100% to 5% (Centritto et al. 2011). In contrast, other studies have found no effect of drought treatments on dark respiration (Limousin et al. 2013; Mitchell et al. 2013), although daily carbon balance was negatively affected by decreasing predawn water potentials. A meta-analysis of temperature-corrected respiration rates of woody species found a negative relationship with mean annual precipitation (Wright et al. 2006). Plants also have long-term responses to drought. To acclimate to drought events, plants may adjust the osmotic potential of their cells to maintain turgor, and over the long term they may adjust the elasticity of cell walls in new growth to lower the point at which turgor is lost.

Temperature has a positive correlation with respiration of woody plant tissues measured at ambient temperatures (Way and Oren 2010; Wright et al. 2006). Respiration generally exhibits a positive relationship to increases in temperature due to kinetic increases in enzymatic activity leading to increased energy demand (Lambers et al. 2009). The relationship between temperature and respiration is often represented using Q_{10} values, indicating the change in respiration rate with a 10°C increase in temperature. For example, the Q_{10} of loblolly pine grown at 30°C was 2.7, although Q_{10} varies with both growth and measurement temperature (Teskey and Will 1999; Tjoelker et al. 2001).

Respiration often thermally acclimates in trees grown in both controlled environments and field conditions (Atkin and Tjoelker 2003; Ow et al. 2010). The thermal acclimation response of respiration is similar in many species and functional groups (Campbell et al. 2007), although there is considerable variability among species (Loveys et al. 2003). The degree of thermal acclimation also varies depending on the plant's metabolic status and growing conditions (Atkin et al. 2005). Teskey and Will (1999) found

substantial thermal acclimation of respiration in *Pinus taeda* seedlings grown at three different measurement temperatures, with dark respiration at the growth temperature differing little among treatments. In an air- and soil-warming experiment with *Picea mariana*, a 5°C increase in temperature had no effect on rates of photosynthesis, foliar respiration, or stem CO_2 efflux, indicative of thermal acclimation (Bronson and Gower 2010). However, a few experiments have not generated support for thermal acclimation. For example, Dillaway and Kruger (2011) saw dark leaf respiration rate rise along a 12°C growth temperature gradient in a study of temperate and boreal trees.

Leaf respiration on both an area and mass basis can decrease with large decreases in irradiance (Feng et al. 2004). For example, *Populus deltoides* saplings significantly reduced their dark respiration rates in both low-light (150 μmol m^{-2} s^{-1} PAR) and dark conditions over a period of 7 days by about 40% and 70%, respectively (Wertin and Teskey 2008). Branches of *Abies amabilis* trees in an outdoor stand had a 53% reduction in leaf dark respiration after 100 days of an 80% light reduction (Brooks et al. 1994). A consistent reduction of respiration was also seen in *Fagus sylvatica* seedlings growing in reduced-light conditions (Rodriguez-Calcerrada et al. 2010).

Temperature-corrected leaf respiration rates of woody species had a positive relationship with irradiance in a meta-analysis by Wright et al. (2006). In a study of 11 juvenile tree species, the effect of reduced canopy openness was more pronounced in angiosperms than conifers, which exhibited a respiration response on only a mass basis (Lusk and Reich 2000). A study of the evergreen shrub *Buxus sempervirens* found that leaf dark respiration, photorespiration, and photosynthesis were all significantly lower in understory individuals at low light levels (Letts et al. 2012), suggesting an overall slowing of plant metabolism in response to low irradiance. In low light conditions, plants may acclimate by producing new leaves that are thinner, larger, and/or have fewer chloroplasts per unit area.

STEM CARBON DIOXIDE EFFLUX

Stem CO_2 efflux (F_s), while easily measured, is not a direct representation of stem respiration. The stem has barriers to CO_2 diffusion, causing a large amount of locally respired CO_2 to dissolve in the xylem sap (Teskey and McGuire 2002). The dissolved CO_2 in stems may originate from root or stem

tissues and possibly the rhizosphere (Aubrey and Teskey 2009; Bloemen et al. 2013), and may be fixed by woody tissue photosynthesis, transported with the xylem sap toward the foliage, or diffuse out of the stem into the atmosphere (Teskey et al. 2008). When temperature is corrected for, F_s exhibits a positive relationship with stem CO_2 concentration (Saveyn et al. 2008). Stem respiration can be more accurately calculated using a mass balance approach, although it is not yet possible to make these measurements in small seedlings (Teskey and McGuire 2002).

Stem CO_2 efflux has varying responses to temperature which are seemingly dependent on experimental design, climate, functional group, and/or species. A study using field-grown *Pinus ponderosa* did not find differences in F_s between desert and montane trees, with F_s per unit sapwood remaining constant among treatments and temperatures (Carey et al. 1997). However, a study of ten field-grown tropical tree individuals of different species found significant positive and negative relationships between temperature and F_S, as well as some individuals with F_s completely decoupled from temperature changes (Zach et al. 2010). Another study using nursery-grown *Populus deltoides* trees found a positive relationship between temperature and F_s over several days, but a weak relationship between stem respiration and temperature (Saveyn et al. 2008). Lastly, four conifer species growing in contrasting climates had similar positive responses of F_s to temperature, with an estimated Q_{10} of 1.73 (R^2=0.78) (Ryan et al. 1995).

The response of F_s to drought is also variable. Measurements of F_s in tropical trees showed variable responses to seasonal precipitation changes, but many trees had lower F_s in the wet season (Zach et al. 2010). Another study of trees in a mature tropical forest saw no effect of a drought treatment on F_s, although the lowest rates of F_s were measured during the peak of the dry season (Nepstad et al. 2002). In contrast with the forest studies above, an experiment with 3-year-old *Quercus robur* seedlings generated a clear reduction in F_s when stem water potential was reduced by withholding water (Saveyn et al. 2007).

There are few studies examining the response of F_s to large reductions in irradiance. However, stem CO_2 efflux decreased by 78% in a low light treatment and 65% in a dark treatment of *Populus deltoides* (Wertin and Teskey 2008).

CARBON DIOXIDE FLUXES AND NONSTRUCTURAL CARBOHYDRATE RELATIONSHIPS

There have been varying results regarding the relationship of photosynthesis to NSCs. Ow et al. (2010) found a significant negative relationship between NSC concentration and photosynthesis in *Pinus radiata*, and Ribeiro et al. (2012) found a similar negative relationship in citrus (R^2=0.55 in winter, R^2=0.81 in summer). However, soluble sugar concentration had no relationship to photosynthesis in a study of grasses, forbs, and evergreen shrubs and trees (Campbell et al. 2007). Photosynthesis and respiration were both decreased in *Eucalyptus globulus* during drought, but accumulation of starch suggests that these plants were sink-limited (Pinkard et al. 2011). Based on their observations, Wertin et al. (2010) suggested that plants reduce their dark respiration rate as a survival mechanism to conserve C stores when A_{net} is reduced.

There is evidence that mass-based shoot respiration is strongly positively affected by total NSC concentration (Tjoelker et al. 1999b). Also, soluble sugar concentration was significantly positively related to dark respiration rate in leaves (Lee et al. 2005), although both responses were strongly linked to changes in temperature, making causation difficult to pin down. There was a positive relationship between respiratory Q_{10} and sugar and starch concentrations in *Pinus radiata*, and rate of respiration at ambient temperature was positively correlated to soluble sugar concentration (Ow et al. 2010). Sugar concentration in leaves is positively related to respiration rate during the dormant season, suggesting a similar positive relationship between leaf sugar concentration and maintenance respiration rate (Ogren 2000). In contrast, shade leaves of two *Quercus* species exhibited significantly lower basal rates of dark respiration compared with those grown at full irradiance, although the two treatments had comparable levels of soluble sugars (30-50 mg g^{-1}) (Zaragoza-Castells et al. 2008).

SUMMARY

In summary, the stresses imposed by drought, heat, and low light can all affect the content and concentration of NSC in plant tissues, in large part by affecting rates of photosynthesis and respiration. Nonstructural carbohydrate concentrations are generally reduced by heat or low light. The response of NSCs to drought is variable, with some studies reporting elevation and others depletion of NSCs

depending on the experimental design and study species. Photosynthesis is generally negatively related to NSC concentration, while respiration and NSCs have a consistently positive relationship. Stem CO_2 efflux seems to have a positive relationship with NSC concentration as well.

The purpose of this study was to evaluate how photosynthesis, respiration, and NSCs of one-year-old loblolly pine (*Pinus taeda* L.) seedlings responded to severe stresses imposed by withholding water, reducing light levels to near zero, or increasing daytime temperature to 40°C for nearly three months. Nonstructural carbohydrate content was measured periodically over 85 days, along with rates of photosynthesis, stem CO_2 efflux, and leaf water potential.

CHAPTER 2

CARBON DIOXIDE FLUXES AND NONSTRUCTURAL CARBOHYDRATES IN SEEDLINGS AS INFLUENCED BY HEAT, DROUGHT, AND LOW LIGHT[1]

[1]Luedtke, C. L., M.A. McGuire, A.G. Roberts, M.W. Ingwers and R.O. Teskey. 2013. To be submitted to *Tree Physiology.*

ABSTRACT

Widespread forest mortality has occurred in recent years. However, mechanisms of tree mortality are not fully resolved, despite the substantial body of research investigating the response of trees to abiotic and biotic stresses. The purpose of this study was to examine the effects of mortality-inducing heat, drought, and low light on *Pinus taeda* L. seedlings over time. We monitored net photosynthetic assimilation and stem CO_2 efflux for 85 days, with periodic destructive harvests to measure biomass accumulation and nonstructural carbohydrate compounds in leaves, stems and roots. We found a strong statistical relationship between stem CO_2 efflux and whole plant starch concentration among the treatments ($P<0.01$, $R^2=0.672$). Seedlings in the heat treatment survived for the entire experiment, but had significantly reduced starch concentrations and growth compared with seedlings in the control treatment. Overall, mean stem CO_2 efflux of seedlings in the heat treatment was elevated over that of seedlings in the control treatment, but net assimilation was comparable. Seedlings in the drought and low light treatments had significantly reduced net assimilation and stem CO_2 efflux compared with seedlings in the control treatment, and died after 10 weeks with severely depleted levels of starch. We conclude that carbon starvation played the primary role in their mortality. We suspect that hydraulic failure played a secondary role in the mortality of seedlings in the drought treatment because they had significantly more sugars remaining in their tissues at the time of death compared with seedlings in the low light treatment.

Key words: carbon starvation, hydraulic failure, mortality, photosynthesis, stem CO_2 efflux, starch, NSC, TNC, loblolly pine

INTRODUCTION

Exposure to environmental stresses often triggers physiological changes in trees, including altered metabolic processes, reduced growth, or even mortality (Kozlowski and Pallardy 2002). There is substantial research investigating the response of trees to biotic and abiotic stress (Kozlowski and Pallardy 2002). However, mechanisms of tree mortality under stressful conditions remain largely unresolved (Sala et al. 2010), despite the widespread tree mortality observed in recent years (Allen et al. 2010). This study will specifically examine the effects of heat, drought, and low light on loblolly pine

(*Pinus taeda* L.) seedlings, with the intent of gaining insights into mortality mechanisms and the relationships between stress, metabolic processes, and nonstructural carbohydrate (NSC) concentrations.

Heat alters photosynthetic net assimilation (A_{net}), and supra-optimal heat reduces A_{net} by causing disruption of the photosynthetic apparatus in the chloroplast (Berry and Bjorkman 1980). In loblolly pine seedlings, there appears to be some degree of thermal acclimation of A_{net}, observed as an adjustment of maximum A_{net} to match growth temperature (Teskey and Will 1999). In response to a change in temperature, dark respiration demonstrates a large degree of thermal acclimation in many species and functional groups (Campbell et al. 2007; Ow et al. 2010). Coniferous tree species typically exhibit greater thermal acclimation than broadleaf tree species (Tjoelker et al. 1999a), and studies have found thermal acclimation of dark respiration in loblolly pine (Nedlo et al. 2009; Teskey and Will 1999). Stem CO_2 efflux (F_s) is a useful proxy to measure combined respiration of the stem and roots, although a small amount of dissolved CO_2 in the rhizosphere may also be transported into stems and contribute to F_s (Aubrey and Teskey 2009; Bloemen et al. 2013; Teskey et al. 2008). A portion of root-respired CO_2 also diffuses out of the roots and into the soil and atmosphere, depending on sap flow and soil CO_2 concentration (Teskey et al. 2008). The relationship of F_s to temperature has proved difficult to quantify, showing inconsistent responses diurnally, seasonally, and among species and individuals (Ryan et al. 1995; Zach et al. 2010).

Net photosynthetic assimilation is reduced when plants experience drought, with more severe droughts having a greater effect (Galmes et al. 2007; Limousin et al. 2013). Respiration measured on a darkened leaf also typically decreases when plants are stressed by a lack of water (Centritto et al. 2011; Galmes et al. 2007; Pinkard et al. 2011). While most studies have reported large reductions in dark respiration under water stress, others have found no effect of drought on dark respiration (Limousin et al. 2013; Mitchell et al. 2013), although daily carbon balance is significantly reduced by decreasing predawn water potentials.

A reduction in light intensity below the light saturation point reduces A_{net}. Low light conditions greatly reduce dark respiration as well, with up to a 70% reduction of respiration after 3-5 days in

complete darkness (Brooks et al. 1994; Wertin and Teskey 2008). Stem CO_2 efflux of *Populus deltoides* in low light conditions was reduced by up to 78% (Wertin and Teskey 2008). Growth in low light conditions also causes morphological changes, including modified growth and development and, if light levels are extremely low, leaf and branch senescence (Kemball et al. 1992; Ryser and Eek 2000; Weaver and Amasino 2001).

Plants in stressful conditions are sometimes unable to fix sufficient C to meet the energetic demands of tissue maintenance. These plants may use stored NSCs to meet these demands. Plants show a variable response of NSC concentrations to stressful conditions, including depleting or building their NSC reserves, which is likely linked to changes in metabolic activity (Laureano et al. 2008; Lee et al. 2005; Tjoelker et al. 1999b).

Drought may have different effects on plant NSC stores depending on the severity and duration of the water deficit and the physiological response of the plant. During a less severe drought, trees may increase their NSC stores as a result of initial C surplus caused by reduced demand by growth processes (Ayub et al. 2011). Longer, mortality-inducing drought can deplete up to 85% of stored NSCs in trees by the time of death (Mitchell et al. 2013). Heat has varied effects on plant NSC stores, with studies reporting increased sugars and total NSC stores (up to 192%) (Campbell et al. 2007), decreased sugar concentrations (Hobbie et al. 2002), or no significant effect of growth temperature on total NSC concentration (Tjoelker et al. 1999b). In an experiment with *Populus deltoides*, NSC compounds in leaves were 80% depleted after 7 days in a low light treatment, and those in a dark treatment were affected similarly but also showed a 52% decrease in total root NSC concentration (Wertin and Teskey 2008).

Exposure to extreme and/or long-term stresses results in tree mortality. Low intensity heat may increase enzymatic activity, leading to increased demand for energy to support biosynthesis, transport, and protein turnover (Amthor 2000). Both high temperatures and low light conditions may diminish NSC stores to the point of carbon starvation (Allen et al. 2010). Drought-induced mortality may occur as a result of carbon starvation, hydraulic failure, or some combination of the two (McDowell et al. 2008). Which of these mechanisms is more physiologically relevant is still being disputed, and seems to vary

depending on species and drought treatment (Sala et al. 2012). The physiological effects of abiotic stress often weaken the tree, making it susceptible to potentially lethal biotic attack by insects and fungal pathogens (Allen et al. 2010).

The purpose of this study was to evaluate the metabolic and NSC response of tree seedlings to heat, drought, and low light stress treatments, with the aim of gaining insights into mortality mechanisms. We hypothesized that seedlings in the stress treatments would exhibit reduced rates of A_{net} and F_s. We also hypothesized that seedlings in the stress treatments would deplete all mobilizable NSC stores by the time of death, with carbon starvation rather than hydraulic failure acting as the primary mechanism of mortality. We expected seedlings in the low light treatment to die of carbon starvation. To determine the mechanism of mortality in drought-stressed seedlings, we compared their nonstructural carbohydrate concentrations to those of seedlings in the low light treatment.

METHODS

In February 2012, we obtained bare-root one-year-old half-sib loblolly pine (*Pinus taeda* L.) seedlings of mixed genotypes originating in the Georgia piedmont (Georgia Forestry Commission, Byromville, GA) which were stored at 6°C until planting. On May 3, 300 seedlings were inoculated with the symbiotic ectomycorrhizal fungus *Pisolithus tinctorius* (PHC® Root Dip, Orchard Valley Supply, Inc., Harrisburg, NC) and planted in 8 L pots in a calcinated illite and silica clay substrate (Turface® MVP®, Profile Products LLC, Buffalo Grove, IL). After thorough watering, the seedlings were placed in greenhouses maintained at ambient temperature for establishment and initial growth. Each pot was fitted with a 2.0 L hr^{-1} drip emitter and watered for 15 minutes at 08:00, 11:00, 14:00, and 17:00.

Each pot was fertilized with 229 mg Peters® Excel 15-5-15 Cal Mag in solution every 8 days, with the first treatment on May 14 and the last on June 21. On May 29, each pot was treated with 0.75 g Sprint 138 Iron Chelate Micronutrient (6% Fe), and on July 3 each pot was treated with 45 g of slow release (12-14 month) Osmocote® 15-9-12 fertilizer. Seedlings were moved into three walk-in growth chambers (model GC36, Environmental Growth Chambers, Chagrin Falls, OH) on May 22. Environmental conditions in the chambers were 25°C during the light period and 20°C during the dark

period, 65% relative humidity and 400 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) with a light period of 14 hours.

TREATMENTS

On September 19, after 139 days of pre-treatment conditions, we selected 96 seedlings and randomly assigned them to each of four treatment groups: control, heat, drought, and low light. Seedlings in the control and drought treatments were moved into one chamber, and seedlings in the low light and heat treatments were moved into two additional separate chambers. Every two weeks, the seedlings were rotated among four growth chambers. Seedlings in the control, heat, and low light treatments were irrigated at a rate of 500 mL H_2O day^{-1}, administered over a 15-minute period one hour after the dark period was initiated. Water was completely withheld from seedlings in the drought treatment. All chambers were maintained at 65% relative humidity. Vapor pressure deficit was typically 2.6 kPa during the light period in the heat treatment, and 1.5 kPa during the dark period. Vapor pressure deficit of the control, drought, and low light treatments was typically 1.5 kPa during the light period and -0.8 during the dark period. Heat, drought, and control treatments received 400 µmol m^{-2} s^{-1} PAR for 14 hours per day from fluorescent and incandescent lamps. The irradiance in the low light chamber was 5 µmol m^{-2} s^{-1} PAR for 14 hours per day. This irradiance level was below the light compensation point, as determined by a light curve derived from net assimilation measured incrementally from 0-2000 µmol m^{-2} s^{-1} PAR before the study began. Drought, control, and low light treatments were maintained at 30°C during the light period and 20°C during the dark period. The heat treatment was maintained at 40°C during the light period and 30°C during the dark period. Carbon dioxide concentrations were monitored in each chamber with non-dispersive infrared sensors (model GMP222, Vaisala, Helsinki, Finland) and recorded using a datalogger (model CR23X, Campbell Scientific, Logan, UT). Mean CO_2 concentration averaged 471 ppm among the chambers. Carbon dioxide was not manipulated during the experiment, but extra seedlings were placed in the low light and heat chambers to match CO_2 concentrations to that of the control/drought chamber. Six pots in each treatment were fitted with Decagon EC-5 soil moisture sensors calibrated specifically for the soil media and read with the datalogger. Mean volumetric water content stabilized at

22, 23, 11, and 27% in weeks 6-14 in the control, heat, drought, and low light treatments, respectively. Needles were removed from the lower 15 cm of stems to facilitate measurements of F_s. On November 7, aphids in the genus *Cinara* were found on seedlings in the low light treatment, and all plants in all treatments were sprayed with bifenthrin 7.9% at a rate of 20.5 mL L^{-1} (Menace 7.9% Flowable, Nufarm Americas Inc., Chicago, IL) to exterminate the aphids.

MEASUREMENTS

Every seven days, needle respiration (R_n), stem CO_2 efflux, and needle water potential were measured on the same three individuals in each treatment during the dark period (one hour before the light period), and net assimilation at 400 µmol m^{-2} s^{-1} PAR (A_{400}) and needle water potential were measured after eight hours of irradiation. A portable photosynthesis system (model LI-6400, LI-COR Biosciences, Lincoln, NE) was used to quantify gas exchange. Net assimilation and respiration were measured on fully-expanded needles using a leaf chamber equipped with an LED light source (model 6400-02B, LI-COR Biosciences). Cuvette temperature was set to match the ambient temperature of the growth chamber for each treatment. Flow rate in the cuvette was adjusted based on rate of gas exchange, and varied between 350-500 µmol s^{-1}. To measure rates of photosynthesis in all treatments under a common environment, cuvette irradiance was set to 400 µmol m^{-2} s^{-1} PAR, equivalent to the PAR incident on the foliage in the control chamber. Needle area was calculated according to Fites and Teskey (1988) using measurements of needle radius taken immediately after A_{400} was measured. Needle area was used to calculate A_{400} and R_n on an area basis. After examination of the data, measurements of R_n were determined to be unreliable and were not included in this analysis. Stem CO_2 efflux was measured on the lower 10-15 cm of the stem using a conifer chamber (model 6400-05, LI-COR Biosciences). Stem diameter was measured at the top and bottom of the chamber immediately after F_s measurements were taken. These measurements were used to calculate stem area using the formula for the lateral surface area of a frustum. Stem CO_2 efflux was expressed on an area basis. Because our seedlings were grown in a soilless media, heterotrophic soil respiration was probably negligible. Thus, autotrophic root respiration or respiration of plant symbionts and associates was probably the only source of any stem CO_2 efflux

derived from dissolved CO_2 in the rhizosphere. Although some root-respired CO_2 inevitably fluxed from the roots into the soilless substrate and was lost to the atmosphere, we loosely interpret F_s as a proxy for combined stem and root respiration. A pressure chamber (model 600, PMS Instrument Company, Albany, OR) was used to measure needle xylem pressure potential, interpreted as needle water potential. On a 14 day interval, the twelve seedlings from the corresponding week's gas exchange measurements were destructively harvested, and their tissues separated into young needles (current year), old needles (first year), stem, coarse roots (≥1mm diameter), and fine roots (<1mm diameter) for NSC quantification. Our experiment took 14 weeks to complete. Week one measurements represent pre-treatment initial data, and measurements in succeeding weeks were taken on seedlings that were continually exposed to their respective stress treatments. Gas exchange data were discarded for days 13, 20, and 55 due to an equipment malfunction.

ENZYMATIC ANALYSIS

Samples were prepared for total NSC analysis as described in Hoch et al. (2002). Samples were heated in a microwave oven to halt enzymatic activity and dried in a forced air oven at 75°C until mass was consistent (usually about 3 days). Each sample was weighed, and the smaller of either 4 g or the entire sample was ground to a fine powder using a ball mill (model 8000-D Mixer/Mill, Spex Sample Prep, Metuchen, NJ) and stored in an airtight scintillation vial.

Samples were analyzed for NSC content using a method modified from Zhao et al. (2010). Approximately 15 mg of woody or 17 mg of needle dry powder was placed in a 1.5 mL microcentrifuge tube and soluble sugars were extracted three times with 0.5 mL of 80% EtOH for 15 min in an 80°C water bath. The final extract volume was brought to 1.5 mL and centrifuged for 15 min at 13,500 × *g*. The supernatant was incubated at 30°C with a sequence of enzymes in 96-well plates to quantify glucose, fructose, and sucrose. Samples were analyzed for glucose content using glucose assay reagent (Sigma-Aldrich G3293), a combination of hexokinase and glucose-6-phosphate dehydrogenase, along with the coenzymes ATP and NAD. Phosphoglucose isomerase (Sigma-Aldrich P5381) was added to the enzyme mixture to quantify fructose. Sucrose was quantified with the addition of invertase (Sigma-Aldrich I9274)

to the sample wells. The insoluble starch remaining in the pellet after removal of the supernatant was solubilized and then reduced to glucose using the enzymes α-amylase (Sigma-Aldrich A3403) and amyloglucosidase (Sigma-Aldrich 10113). Starch samples were analyzed for glucose content as described above. The absorbance of each sample was read photometrically at 340 nm using a 96-well plate reader (Flexstation3 Benchtop Multi-mode Multiplate Reader, Molecular Devices, Sunnyvale, CA). This wavelength corresponds to the peak absorbance of NADH, which is produced during the conversion of glucose-6-phosphate to 6-phosphogluconate in the last reaction step (Supplemental Figure 1). NSC components were quantified according to standard curves derived from D-(+)-glucose standards that were run with each plate. Samples were run in triplicate, with one sample blank for each sugar sample and three for each starch sample. We chose to run starch blanks in triplicate to compensate for high absorbance variation in the starch sample supernatant.

STATISTICAL ANALYSIS

Differences among treatments were determined by ANOVA using Fisher's least significant difference test and an α=0.05 significance level. Linear and nonlinear regression were used to model the relationships between NSC data and gas exchange data. To meet the assumptions of normality, nine F_s measurements from the heat, drought, and low light treatments were excluded from the analysis based on their high Studentized residuals. Normality was confirmed using the Shapiro-Wilk test. Due to the impossibility of obtaining a negative NSC concentration for any of the components, any negative NSC concentrations were changed to zero for statistical analyses.

We performed a regression analysis to establish if weekly relative growth rate (calculated as: $(\ln M_2 - \ln M_1)/(t_2 - t_1)$, where M_1 and M_2 are plant dry mass at times t_1 and t_2) was related to whole plant starch concentration throughout the experiment. We also performed a regression analysis of weekly change in whole plant starch concentration (calculated as: $[\text{starch}]_{x+1} - [\text{starch}]_x$, where x is the week number) and the weekly change in F_s (calculated as: $F_{s_{x+1}} - F_{s_x}$, where x is the week number).

Statistical analyses were performed using SAS software Version 9.3 of the SAS® System for Windows (copyright © 2010 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service

names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC). Figures were generated using SigmaPlot (Systat Software, San Jose, CA, USA).

RESULTS

GROWTH

Seedlings in the control treatment nearly tripled their biomass over the course of the experiment, growing to an average of 77.54 g (Figure 2.1). Young needles made up 51% of total biomass at the last harvest in week 14, old needles made up 1%, stems made up 24%, coarse roots made up 14%, and fine roots made up 10%. Predictably, old needle mass did not change significantly in any of the treatments over the course of the experiment, but all other tissues significantly increased their biomass over the course of the experiment.

Seedlings in the heat treatment also gained biomass over the course of the experiment (Figure 2.1). For the first 8 weeks they not differ significantly from seedlings in the control treatment, but in subsequent weeks their biomass was significantly less than seedlings in the control treatment. By week 14 they were, on average, 35% smaller than seedlings in the control treatment. Young needles gained significantly less biomass than those of seedlings in the control treatment. Coarse roots were similar in mass to those of seedlings in the control treatment. Fine roots and stem masses of seedlings in the heat treatment were not significantly different from seedlings in the control treatment by the end of the experiment.

All of the seedlings in the drought and low light treatments died by week 10. Their needle water potentials were either lower than -3.5 MPa or unmeasurable by week 10 due to needle desiccation and senescence. The stems and roots of seedlings in the drought treatment were dry and brittle, and the bark of seedlings in the low light treatment readily peeled off, indicating necrosis and possible decomposition. The roots of seedlings in the low light treatment were similarly soft and easily broken. Our indicators of mortality were similar to those of Hartmann et al. (2013).

Mean biomass of seedlings in the drought treatment did not increase or decrease during the experiment (Figure 2.1). At the time of their death in week 10, seedlings were 67% smaller than seedlings

in the control treatment. When seedlings in the drought treatment died, their young needles, stems, and roots had significantly less biomass than those of seedlings in the control treatment.

Mean biomass of seedlings in the low light treatment also did not increase or decrease during the experiment, with the exception of the seedlings harvested in week 2, which were smaller than pre-treatment seedlings (Figure 2.1). By week 10, when all seedlings in the low light treatment had died, they were 72% smaller than seedlings in the control treatment on average. Young needles, stems, and roots of seedlings in the low light treatment had significantly lower biomass than those of seedlings in the control treatment by week 10. From week 4 to week 10, seedlings in the low light treatment were of comparable mass to those in the drought treatment, and there were no significant differences between the two treatments in the mass of individual tissues.

NET ASSIMILATION

Mean A_{400} measured over the treatment period was 3.09, 3.04, -1.44, and 0.54 μmol CO_2 m^{-2} s^{-1} for seedlings in the control, heat, drought, and low light treatments, respectively. Mean A_{400} for seedlings in the control and heat treatments did not differ, while seedlings in the low light treatment had significantly lower A_{400} than control and heat treatments, and seedlings in the drought treatment had significantly lower A_{400} than all other treatments. Net assimilation was measured at 400 μmol m^{-2} s^{-1} PAR in all treatments to compare photosynthetic capacities among treatments at a common light level, but seedlings in the low light treatment (5 μmol m^{-2} s^{-1} PAR) were grown below the light compensation point. Except for the brief measurements made at 400 μmol m^{-2} s^{-1} PAR, net assimilation of seedlings in the low light treatment was negative, averaging -0.07 μmol CO_2 m^{-2} s^{-1} (measured at 5 μmol m^{-2} s^{-1} PAR in weeks 7-10). Seedlings in the control and heat treatments maintained comparable A_{400} throughout the experiment (Figure 2.2). Seedlings in the drought and low light treatments had significantly lower A_{400} than seedlings in the heat and control treatments at each weekly measurement (Figure 2.2). Seedlings in the drought treatment had significantly lower A_{400} than seedlings in the low light treatment at most weekly measurements. Mean A_{400} of seedlings in both the drought and low light treatments had dropped to near 0 μmol CO_2 m^{-2} s^{-1} by week 10 when the seedlings were pronounced dead.

STEM CO_2 EFFLUX

Mean stem CO_2 efflux (F_s) over the treatment period was 1.10, 1.33, 0.03 and 0.23 μmol CO_2 m^{-2} s^{-1} for seedlings in the control, heat, drought, and low light treatments, respectively. Mean F_s averaged over the entire experiment was significantly different among all treatments. Despite having a significantly higher mean F_s overall, F_s of seedlings in the heat treatment was not significantly different from F_s of seedlings in the control treatment during weekly measurements, with one exception in week 7 (Figure 2.2). Seedlings in the drought treatment had significantly lower F_s than seedlings in the control and heat treatments throughout the experiment, as did seedlings in the low light treatment, except in week 5, when F_s of seedlings in the low light treatment was comparable to F_s of seedlings in the control treatment (Figure 2.2). Stem CO_2 efflux of seedlings in the drought treatment had dropped to near 0 μmol CO_2 m^{-2} s^{-1} by week 2, which paralleled their negative A_{400} values, indicating a rapid physiological response to the drought treatment.

Predawn needle water potential of seedlings in the drought treatment dropped more gradually than their rates of gas exchange, from the pre-treatment mean of -0.6 MPa to -1.9 MPa in week 2, probably because stomatal conductance was rapidly reduced from 49 to 8 to mmol H_2O m^{-2} s^{-1}. Predawn needle water potential of seedlings in the drought treatment continued to decline gradually to less than -3.5 MPa by week 7, and F_s remained near 0 μmol CO_2 m^{-2} s^{-1}. There was a positive relationship between F_s and predawn needle water potential of seedlings in the drought treatment (Figure 2.3). Needle water potential remained high in all other treatments, and had no relationship to F_s (data not shown).

TREATMENT EFFECTS ON WHOLE-PLANT NONSTRUCTURAL CARBOHYDRATES

Pre-treatment mean whole-plant total NSC was 4.43% (dry mass basis) (Figure 2.4). The concentration of pre-treatment total sugars was 2.64%, representing 60% of initial total NSC. Starch initially comprised the largest portion of total NSCs at 1.79% (40% of total NSC), followed by sucrose at 1.53% (35% of total NSC), glucose at 0.59% (13% of total NSC), and fructose with the smallest proportion at 0.52% (12% of total NSC). The whole plant starch and sugar pools are presented in Supplemental Figures 2 and 3. Whole plant starch concentration had a significant positive relationship to

whole plant mass, indicating that larger seedlings typically had higher starch concentrations ($P<0.01$, $R^2=0.553$) (Figure 2.5).

By the last harvest on week 14, whole-plant concentrations of glucose, fructose, sucrose, total sugars, starch and total NSCs of seedlings in the control treatment were 1.27, 0.86, 1.46, 3.59, 5.88, and 9.48%, respectively (Figure 2.4). Seedlings in the control treatment had significantly increased concentrations of glucose, fructose, total sugars, starch, and total NSC by week 14 (Figures 2.4). Seedlings in the control treatment had significantly and consistently elevated starch concentrations after three weeks. Change in NSC component concentrations on a whole plant basis is presented in Supplemental Figure 4.

Whole-plant total sugar concentrations in the control treatment increased from pre-treatment levels (Figure 2.4). Sucrose concentrations remained relatively constant throughout the experiment, with the exception of a significant decrease in week 10 compared with pre-treatment levels. Fructose concentration in seedlings in the control treatment did not change significantly until the last harvest, when it was elevated above the pre-treatment level. In contrast to the other sugars, glucose concentration gradually increased in seedlings in the control treatment, and was significantly higher than the pre-treatment level from week six until the end of the experiment. At the end of the experiment, starch made up 62% of total NSC in seedlings in the control treatment, followed by sucrose at 15%, glucose at 13% and fructose at 9%.

At the last harvest date, whole-plant concentrations of glucose, fructose, sucrose, total sugars, starch and total NSCs in seedlings in the heat treatment were 1.32, 0.88, 1.92, 4.12, 3.27, and 7.39%, respectively (Figure 2.4). Glucose, fructose, total sugars, starch, and total NSC were all significantly elevated in week 14 compared with pre-treatment data. By the end of the experiment, seedlings in the heat treatment had significantly lower concentrations of starch and total NSC compared with seedlings in the control treatment. Starch concentration was typically lower in seedlings in the heat treatment compared with seedlings in the control treatment, but that trend did not become significant until week 12.

Concentrations of glucose, fructose, sucrose, and total sugars in seedlings in the heat treatment were not significantly different from concentrations in the control treatment at the last harvest date on week 14, although they were all significantly higher in seedlings in the heat treatment on weeks 10 and 12 (Figure 2.4). At the end of the experiment, starch made up 44% of total NSC in seedlings in the heat treatment, followed by sucrose at 26%, glucose at 18% and fructose at 12%.

At the time of their mortality in week 10, concentrations of glucose, fructose, sucrose, total sugars, starch and total NSC in seedlings in the drought treatment were 0.65, 0.25, 1.19, 2.08, 0.57, and 2.56%, respectively (Figure 2.4). Concentrations of fructose, starch, and total NSC were significantly lower than pre-treatment concentrations. In week 10, total NSC and starch concentrations were significantly lower than the control treatment. The starch concentration of seedlings in the drought treatment was significantly lower than concentrations in the control treatment by week 2, but the difference in total NSC concentrations did not develop until week 4. Starch concentrations in the drought treatment continued to decline until week 10.

In week 10, glucose and fructose concentrations of seedlings in the drought treatment were significantly lower than seedlings in the control treatment, but sucrose was comparable between the two treatments (Figure 2.4). In week 2, seedlings in the drought treatment had significantly elevated total sugars compared with control concentrations. Individual sugar concentrations were not significantly higher than the control treatment in week 2, but they were significantly higher than pre-treatment concentrations. Seedlings in the drought treatment had similar levels of glucose, fructose and sucrose compared with seedlings in the control treatment until week 8, when the glucose concentration of seedlings in the drought treatment was significantly lower by comparison. In week 10, the concentration of fructose also became significantly lower than the control treatment. When seedlings were pronounced dead in week 10, sucrose made up 45% of total NSC in seedlings in the drought treatment, followed by glucose at 25%, starch at 22%, and fructose at 9%.

When seedlings in the low light treatment were pronounced dead in week 10, concentrations of glucose, fructose, sucrose, total sugars, starch and total NSCs were 0.13, 0.05, 0.17, 0.34, 0.46, and 0.80%

dry mass, respectively (Figure 2.4). Concentrations of all NSC components were significantly lower than those of seedlings in the control treatment beginning in week 2, and remained significantly lower until their death in week 10. At that time, starch made up 57% of total NSC in seedlings in the low light treatment, followed by sucrose at 21%, glucose at 16% and fructose at 6%.

Concentrations of starch, fructose, and total NSCs were similar between seedlings in the drought and low light treatment by week 10, but seedlings in the drought treatment had significantly higher glucose, sucrose, and total sugars (Figure 2.4). In general, NSC components were more rapidly depleted in seedlings in the low light treatment compared with seedlings in the drought treatment, but at the time of death starch concentrations were very low in both treatments: 0.57% in the drought treatment and 0.46% in the low light treatment.

Treatment Effects on Nonstructural Carbohydrates in Tissues

Before the treatments were applied, total NSC concentration in coarse roots was significantly higher than the concentrations in fine roots, young needles, stems, and old needles (Table 2.1). Pre-treatment concentration of starch was significantly higher in roots compared with stems and foliage. Total sugar concentration was significantly lower in fine roots compared with all other tissues before treatments were applied. Sucrose, fructose, and glucose concentrations in tissues varied, but were relatively low in fine roots and high in coarse roots. Some tissues contributed more than other tissues to the calculation of whole plant NSC concentration due to their greater mass. Averaged across all harvests and treatments, young needles, old needles, stems, coarse roots, and fine roots contributed about 50%, 4%, 19%, 15%, and 13% of total NSC (Figure 2.4).

Seedlings in the control treatment had significantly elevated total NSC concentrations in all tissues in week 14 compared with pre-treatment (Table 2.1). Starch concentration significantly increased in needles, stems, and coarse roots, with a greater than 420% increase in foliage. Total sugar concentration showed significant increases in the stem and roots of seedlings in the control treatment by week 14. Sucrose increased significantly in coarse roots, but remained similar to pre-treatment in all other

tissues. The stem and roots had significantly elevated fructose concentration compared with pre-treatment, and glucose concentrations increased significantly in all tissues except old needles.

Young needles of seedlings in the heat treatment had a significantly lower total NSC concentration compared with seedlings in the control treatment, although other tissues had similar concentrations (Table 2.1). Compared with seedlings in the control treatment, starch concentrations of seedlings in the heat treatment were significantly lower in young needles and stems, but were similar in other tissues. Total sugar concentrations in all tissues were not significantly different between seedlings in the heat and control treatments in week 14 (Table 2.1). Sucrose concentration in stems and coarse roots of seedlings in the heat treatment was significantly higher compared with the control treatment, but similar in other tissues. Fructose was significantly higher in young needles of the heat treatment, but other tissues had similar concentrations compared with control. Stems and fine roots of seedlings in the heat treatment had significantly lower glucose concentrations than seedlings in the control treatment, but other tissues were similar.

By the time they were pronounced dead in week 10, seedlings in the drought treatment had significantly lower total NSC concentrations in young needles and stems compared with seedlings in the control treatment, but other tissues had similar total NSC concentrations (Table 2.2). Starch concentrations of seedlings in the drought treatment in week 10 were significantly lower compared with control in all tissues except stems, which were similar. Total sugar concentrations in stems in the drought treatment were significantly lower compared with seedlings in the control treatment in week 10, whereas fine roots had significantly higher total sugars, and other tissues were similar (Table 2.2). Sucrose was also significantly higher in the fine roots of seedlings in the drought treatment. In contrast, fructose was depleted in young needles and stems compared with the control treatment, but similar in other tissues. Glucose was significantly lower in the drought treatment compared with the control treatment in stems, but other tissues had similar concentrations. During week two, seedlings in the drought treatment had elevated levels of sugars in many tissues compared with control and pre-treatment concentrations (data

not shown). Total sugars concentrations were significantly higher than control in stems (4.51%), coarse roots (6.05%), and fine roots (2.51%), and were 24% higher in young needles (3.62%).

In week 10 in the low light treatment, total NSC concentrations in most tissues were significantly lower compared with the control treatment, except in old needles which had similar concentrations (Table 2.2). By week 4 total NSCs had been rapidly depleted in all tissues of seedlings in the low light treatment compared with control, a pattern that was significant in most tissues and on most harvest dates until week 10. More details of changes in NSC concentrations in specific tissues over time are presented in additional analyses in Supplemental Figure 5. Starch was significantly lower in all tissues compared with the control treatment in week 10. Total sugar concentrations in young needles, stems, and fine roots of seedlings in the low light treatment were significantly lower in week 10 compared with seedlings in the control treatment, but old needles and coarse roots had similar concentrations (Table 2.2). Sucrose was only significantly lower in stems of seedlings in the low light treatment, and concentrations in all other tissues were similar to seedlings in the control treatment. Stems and young needles of seedlings in the low light treatment had significantly lower concentrations of fructose compared with control in week 10, while other tissues were similar. Glucose concentrations were significantly lower than control in all tissues except old needles.

By comparison, seedlings in the low light treatment had significantly lower total NSC concentrations in stems and fine roots than seedlings in the drought treatment, but similar concentrations in other tissues at the time of death in week 10 (Table 2.2). Much of the difference in total NSC between the two treatments arose from the significantly lower concentrations of total sugars in all tissues of seedlings in the low light treatment except old needles. However, starch concentrations were depleted to a similar extent in all tissues in the low light treatment compared with the drought treatment. Sucrose concentrations of seedlings in the low light treatment were significantly lower in all tissues except old needles when compared with seedlings in the drought treatment. Fructose was significantly lower in seedlings in the low light treatment compared with the control treatment in fine roots only, but glucose was lower in young needles and roots of seedlings in the low light treatment.

GAS EXCHANGE AND NONSTRUCTURAL CARBOHYDRATE RELATIONSHIPS

Net assimilation had a significant positive relationship with glucose, fructose, starch, total sugars, and total NSC (Table 2.3). The relationships of A_{400} to starch and total NSC were stronger than relationships with the individual sugars. Stem CO_2 efflux had significant positive relationships with all NSC components (Table 2.3). The non-linear relationship between F_s and whole plant starch was the strongest, and showed a steep decline in F_s below about 2% starch (Figure 2.6). The relationship between F_s and stem starch concentration was slightly less strong (R^2=0.605, P<0.01) than with whole plant starch concentration. Stem CO_2 efflux of seedlings in the drought treatment showed a much more rapid decrease than starch concentrations, a pattern that was not present in any other treatments. In comparison, both F_s and starch concentration in the low light treatment declined rapidly.

We found a weak positive linear relationship between relative growth rate and whole plant starch concentration, indicating that faster growing plants had slightly higher levels of starch in their tissues (P=0.03, R^2=0.136). We did not detect a significant relationship between the weekly change in whole plant starch concentration and the weekly change in stem CO_2 efflux (P=0.17, R^2=0.043).

DISCUSSION

Our findings indicate that drought and low light stress had similar effects on C depletion, suggesting that C starvation played an important role in seedling mortality in both situations. When exposed to these conditions, loblolly pine seedlings exhibited rapid reductions in A_{400}, F_s, and NSC concentrations. We also observed significant positive relationships of A_{400} to glucose, fructose, starch, total sugars, and total NSC. The relationships between F_s and glucose, fructose, sucrose, starch, total sugars, and total NSC were also positive and significant.

MECHANISMS OF MORTALITY

A comparison of the effects of drought and low light on the seedlings suggests that the drought-treated seedlings died primarily of C starvation. Considering the near-zero carbon balance of seedlings (rates of A_{400} near the zero assimilation point) in the low light treatment coupled with their almost complete depletion of NSCs, and adequate supply of water and nutrients, it follows that mortality of

seedlings in the low light treatment was driven mostly by C depletion leading to C starvation. This mechanism is thought to be a causal factor in widespread forest mortality (Breshears et al. 2009). Seedlings in the low light and drought treatments depleted statistically similar amounts of starch by the time they died in week 10, suggesting that seedlings in the drought treatment also suffered mortality-inducing C depletion. Starch levels decreased more slowly in seedlings in the drought treatment compared with seedlings in the low light treatment, perhaps due to the rapid decline in F_s in the drought treatment, which may reflect a reduced metabolic demand for C. In contrast to our results, a 10 week study of both an isohydric and anisohydric conifer species native to the southwestern US found no significant depletion of starch in a drought treatment (Anderegg and Anderegg 2013). This difference in drought response between loblolly pine and the southwestern US species may be due to different adaptations to specific habitats.

Loblolly pine is an isohydric species that prioritizes the maintenance of high midday needle water potentials when soil water potentials are low. High water potential is preserved by reducing stomatal conductance, a response that occurs quickly in soils with high porosity such as the calcinated illite and silica clay substrate used in this experiment (Hacke et al. 2000; Van Iersel 2013). Consequently, isohydric species tend to be more susceptible to C starvation than anisohydric species when subjected to drought (McDowell et al. 2008; Mitchell et al. 2013). However, it is likely that even isohydric species experience some degree of both C starvation and hydraulic failure because carbon is needed to maintain hydraulic systems, and hydraulic systems are needed to transport mobilized C to sinks (McDowell et al. 2013).

Starch concentration of seedlings in the drought treatment dropped to near-minima by week 4, while needle water potentials reached minima closer to week 7, but seedlings were not considered dead until week 10. The timing of these events suggests a multi-faceted mortality mechanism that includes both C starvation and hydraulic failure. Loss of 50% of xylem conductivity has been observed at -3.3 MPa in branches of loblolly pine (Domec et al. 2010), and more negative water potentials were measured in this study, suggesting that our seedlings suffered a greater than 50% loss of conductivity during the experiment. It is unknown if loblolly pine could recover from a stress of this magnitude upon rewatering,

but evidence suggests that conifers have a lower capacity for refilling emboli compared with angiosperms (Meinzer and McCulloh 2013).

The initial elevation and maintained presence of sugars in seedlings in the drought treatment may be attributed the seedlings' attempts to maintain turgor through osmotic adjustment. Sugar elevation may also reflect the inability of seedlings to utilize these sugars due to loss off cell hydration, which may limit cellular metabolism, or transport limitation because of damage to the xylem and phloem (Allen et al. 2010; Meier et al. 1992; Sala and Hoch 2009). There is also evidence suggesting that sugars are used in the refilling mechanism of xylem emboli (Salleo et al. 2009).

GROWTH

The impact of the stress treatments on seedlings was demonstrated by our growth data. Seedlings in the control treatment were on average 55% larger than seedlings in the heat treatment, 292% larger than seedlings in the drought treatment, and 389% larger than seedlings in the low light treatment by the end of the study. These differences suggest that seedlings in drought and low light treatments were severely stressed, and seedlings in the heat treatment experienced only mild stress. Our observation of a weak positive relationship between relative growth rate and whole plant starch concentration is contrary to the findings of Piper et al. (2009), who found no association between relative growth rate on a height basis and total NSC concentration in leaves (P=0.7) or roots and stems (P=0.9) of two *Nothofagus* species. We may have detected a relationship where Piper et al. (2009) did not because we measured both starch concentration and plant size on a mass basis.

GAS EXCHANGE

Seedlings in the drought and low light treatments reduced their metabolic activity, as demonstrated by significantly reduced A_{400} and F_s. Our results are consistent with the findings of several other studies. Rapid and large reductions in A_{net} and F_s have also been observed in *Populus deltoides* in response to a low light treatment (Wertin and Teskey 2008). Another study found a smaller reduction of A_{net} in loblolly pine in response to less severe drought (Wertin et al. 2010). In a drought study using oak, F_s was reduced in parallel with stem water potential (Saveyn et al. 2007). The rapid reduction of F_s of seedlings in the

low light treatment may indicate a greater stress response compared with the seedlings in the drought treatment. However, the reduction in F_s of seedlings in the drought treatment may reflect a reduction in xylem hydration and sap flow, limiting the contribution to F_s of CO_2 transported in sap from the roots.

Seedlings in the heat treatment had elevated grand mean F_s compared with seedlings in the control treatment. Meanwhile, A_{400} of seedlings in the heat and control treatments was similar. A heat-induced increase in the rate of CO_2 diffusion from the stems of seedlings in the heat treatment may have contributed to elevated F_s. However, elevated F_s may also indicate increased respiration throughout the plant, in which case the additional C loss by respiration in seedlings in the heat treatment was the likely cause of their reduced growth compared with seedlings in the control treatment. In contrast, a study of heat-treated *Picea mariana* seedlings found significantly elevated rates of A_{net} and daytime needle respiration ($P<0.01$) compared with seedlings in the control treatment, but no difference in night needle respiration (Way and Sage 2008). Our data may indicate that the heat treatment we applied (40°C in the light period/ 30°C in the dark period) was not a severe stress, and may have been within the optimum range for photosynthesis (Wertin et al. 2010). Our data may also indicate that thermal acclimation of A_{400} took place in loblolly pine, in contrast to the findings of Nedlo et al. (2009).

NONSTRUCTURAL CARBOHYDRATES

Total NSC, starch, and glucose concentrations all increased significantly in seedlings in the control treatment by the end of the experiment. In contrast, seedlings in the heat treatment had a smaller increase in total NSC and starch. The smaller increase in NSC indicates that these seedlings had greater energetic demands compared with seedlings in the control treatment. Glucose, fructose, and sucrose concentrations of seedlings in the heat treatment were intermittently elevated above those in the control treatment, possibly indicating elevated metabolic activity. In contrast, *Picea mariana* seedlings in a low temperature treatment had significantly higher levels of glucose and fructose compared with seedlings in a heat treatment ($P<0.05$) (Way and Sage 2008). This difference in results may be the result of differing severity of treatments applied, and/or species-specific responses to temperature.

Total NSC and starch were significantly depleted in seedlings in the drought and low light treatments by the end of the experiment. The decreases of NSCs in the drought and low light treatments indicate that stored NSCs were consumed to meet respiratory demands. Seedlings in the low light treatment also consumed glucose, fructose, and sucrose, but seedlings in the drought treatment did not. Wertin and Teskey (2008) saw a similar significant depletion of soluble sugars and total NSC in leaves and roots of seedlings in low light and no light treatments (with the exception of total NSC in roots of seedlings in the low light treatment). A study by Hartmann et al. (2013) found a similar depletion of NSCs in saplings which were C-starved by removing atmospheric CO_2, compared with less-depleted NSCs in drought-stressed saplings. Drought-stressed *Pinus edulis* trees also had significantly reduced total NSCs and soluble sugars over a long-term experiment, but increased starch concentrations (Adams et al. 2013). Changes in NSC concentration in response to drought may be species specific, as demonstrated by a drought study of two *Nothofagus* species in which the more drought-susceptible species depleted starch and the more drought-tolerant species increased starch (Piper 2011).

Whole plant starch concentration had a significant positive relationship with whole plant mass, suggesting ontogenetic changes in NSC. A similar positive relationship of tree size to branch NSC concentration was seen in multiple age classes of *Pseudotsuga menzesii* trees, with the strongest correlation seen in the autumn (R^2=0.83, P<0.0001) (Woodruff and Meinzer 2011). In contrast, root total NSC concentration was negatively correlated to diameter in two tropical *Macaranga* tree species (R^2=0.46 and 0.19, P<0.05) (Kenzo et al. 2013).

Gas Exchange and Nonstructural Carbohydrates

Carbon gained through photosynthesis is often stored as NSCs, and stressed plants will use this stored C to maintain physiological processes. To determine how strongly metabolic processes are linked to C balance, we investigated the relationships between CO_2 fluxes and NSC components and found that A_{400} had a positive relationship with glucose, fructose, and total sugars, with a stronger positive relationship to starch and total NSC (Table 2.3). Adams et al. (2013) found a similar positive relationship between sugar

concentration and A_{net} in *Pinus edulis* (P<0.05). In contrast, little or no relationship was found between total NSC concentration and A_{net} in two *Nothofagus* species (Piper 2011).

Our data did not show a significant relationship between sucrose and A_{400}. Biochemically, the levels of sucrose in the cytosol and rate of photosynthetic activities in the cytoplast are not directly linked (Duffus and Duffus 1984). However, sucrose levels do seem to be under strict control in trees, remaining relatively high with little diurnal or seasonal variation, perhaps due to the important roles sucrose plays in plant biochemical and physiological processes (Magel et al. 2000). Seedlings in our low light treatment had low concentrations of both sucrose and starch. They may not have had enough mobilizable starch to maintain desirable sucrose levels. With the exception of seedlings in the low light treatment, which had significantly reduced sucrose by week 2, there was little variation in sucrose concentrations among treatments until week 10. This lack of variation may have contributed to the poor relationship with A_{400}.

Stem CO_2 efflux had a positive relationship with all NSC components. The strongest relationship detected in this study was between F_s and whole plant starch concentration (Figure 2.6). Maier et al. (2010) saw similarly strong correlations between mean daily F_s and both soluble sugars (R^2=0.57, P<0.0001) and starch (R^2=0.61, P<0.0001) in the stems of young loblolly pine trees.

Also of note is the rapid decrease of F_s in seedlings in the drought treatment, followed by more gradual declines in starch concentration and water potential. We suspect that this combination of responses might indicate a rapid metabolic acclimation to drought stress, resulting in reduced stem and/or root respiration. As a result, starch concentrations were maintained longer than those of seedlings in the low light treatment, which responded with a smaller reduction in F_s.

SUMMARY

Our hypothesis that seedlings in the stress treatments would reduce rates of A_{net}, and F_s was supported for the drought and low light treatments, but seedlings in the heat treatment showed a weak elevation of F_s and no change in A_{400}. Growth data indicated that seedlings in the heat treatment were not as severely stressed as seedlings in the drought or low light treatments, so metabolic response to stress appears to depend on the severity of the stress experienced by the plant. We found a strong positive relationship

between starch concentration and both F_s and A_{400} in pooled data from all treatments. Our hypothesis that seedlings in the stress treatments would deplete all mobilizable NSC stores by the time of death was supported in the low light treatment. The depletion of comparable levels of starch in the seedlings in the drought and low light treatments supports C starvation as the primary cause of mortality, but seedlings in the drought treatment had significantly higher concentrations of mobile sugars present in tissues at the time of death. This discrepancy indicates that seedlings in the drought treatment may have been unable to mobilize the sugars remaining in their tissues due to dehydration, or that they maintained higher sugar concentrations as part of an osmotic regulation mechanism. The data suggest that C depletion leading to C starvation was the primary mechanism of mortality in seedlings in both the low light and the drought treatments. Low light prevented carbon gain and caused depletion of stored NSC and mortality. Water stress in the drought treatment resulted in a remarkably similar response, preventing carbon gain and causing a similar depletion of stored NSC and mortality within the same time period. This study enhances our understanding of the causes of drought-induced mortality, the thermal tolerance of loblolly pine, and the relationship of CO_2 fluxes to NSC.

CHAPTER 3

CONCLUSION

In our study, we evaluated metabolic and nonstructural carbohydrate responses of loblolly pine (*Pinus taeda* L.) seedlings to heat, drought, and low light stresses. We measured photosynthesis and dark stem CO_2 efflux once a week over a period of 85 days, with destructive harvests every two weeks to quantify nonstructural carbohydrate concentrations of young needles, old needles, stems, coarse roots, and fine roots. The drought and low light treatments caused stress that was severe enough to induce mortality by week 10 and the heat treatment induced stress but not mortality. We conclude that seedlings in the low light treatment died of C starvation. Based on similar depletion of starch, we conclude that seedlings in the drought treatment also died primarily of C starvation, but remaining sugars suggest that hydraulic failure or other effects of inadequate water may have also played a role.

We also found a strong positive relationship between starch concentration and both F_s and A_{400} in pooled data from all treatments, indicating that the links between C balance and photosynthesis and respiration were significant. We hypothesized that seedlings in the stress treatments would reduce their rates of A_{400} and F_s. This hypothesis was supported for the drought and low light treatments, but not for the heat treatment. We also hypothesized that seedlings in the stress treatments would deplete all mobilizable NSC stores by the time of death, and that hypothesis was supported in low light treatment, but not for the drought or heat treatments.

This study would have benefitted from the use of a planting substrate with a smaller pore size. A planting substrate with smaller pores would have allowed a more gradual application of the drought treatment, resulting in higher resolution measurements of the decline of CO_2 fluxes, nonstructural carbohydrates, and needle water potentials. It may also have allowed for finer control of soil water potential. This study would also have been improved by regulating CO_2 concentration in the growth chambers with a datalogger-controlled system. Optimally, the heat treatment would have been maintained

at a higher humidity level, thus matching vapor pressure deficit in the heat treatment to vapor pressure deficit in the other treatments.

A study that implements a drought treatment eliciting stomatal closure, a low light treatment below the light compensation point, and a treatment with CO_2 concentrations below the CO_2 compensation point, in conjunction with quantification of NSC concentration would further elucidate the mechanisms of drought-induced mortality. In this type of experiment, multiple causes of C starvation could be compared. Different types of drought treatments (e.g. rapid vs. gradual, mild vs. severe, steady vs. cyclic) could also be evaluated, helping to draw parallels to naturally occurring drought. Of course, similar studies using other species and functional groups would also allow researchers to gain insights into C starvation and the relationships between metabolism and NSC across the plant kingdom.

TABLES

Table 2.1: Mean concentrations (percent dry mass) of nonstructural carbohydrate (NSC) components of loblolly pine seedlings before treatment and after 14 weeks in control and heat treatments. Abbreviations are: YN=young needles, ON=old needles, S=stems, CR=coarse roots, FR=fine roots. Significant differences among treatments at week 14 for each NSC × tissue combination are indicated by capital letters, and are read across rows (α=0.05). Significant differences among tissues for each NSC × treatment combination are indicated by lowercase letters, and are read down columns (α=0.05). Significant differences between a specific post-treatment NSC × treatment × tissue mean and its corresponding pre-treatment mean are indicated with asterisks.

		Pre-treatment		Control			Heat		
Glucose	**YN**	0.63	b	0.99	-	b *	1.23	-	b *
	ON	1.1	a	1.53	-	ab	1.49	-	ab
	S	0.37	c	1.39	A	ab *	1.06	B	b *
	CR	0.84	ab	2.15	-	a *	2.12	-	a *
	FR	0.48	c	1.29	A	b *	0.89	B	b *
Fructose	**YN**	0.46	bc	0.51	B	-	0.88	A	b *
	ON	0.07	c	0.54	-	-	0.37	-	c
	S	0.64	ab	1.49	-	- *	0.87	-	b
	CR	0.82	a	1.29	-	- *	1.29	-	a *
	FR	0.38	c	0.50	-	- *	0.33	-	c
Sucrose	**YN**	1.66	a	1.19	-	c	1.35	-	cd
	ON	1.22	bc	1.93	-	ab	1.89	-	bc
	S	1.70	a	1.64	B	bc	2.39	A	b *
	CR	1.38	ab	2.40	B	a *	3.42	A	a *
	FR	0.90	c	1.24	-	bc	1.13	-	d
Total Sugars	**YN**	2.75	a	2.69	-	b	3.46	-	bc *
	ON	2.78	a	4.00	-	ab	3.75	-	b
	S	2.72	a	4.53	-	ab *	4.33	-	b *
	CR	3.05	a	5.84	-	a *	6.83	-	a *
	FR	1.76	b	3.03	-	b *	2.35	-	c
Starch	**YN**	1.61	b	8.40	A	a *	4.02	B	ab *
	ON	1.28	b	6.83	-	ab *	5.13	-	a *
	S	1.60	b	1.96	A	c *	1.12	B	b *
	CR	2.37	a	4.50	-	abc *	3.33	-	ab
	FR	2.64	a	3.95	-	bc	3.78	-	ab
Total NSC	**YN**	4.36	b	11.09	A	a *	7.48	B	ab *
	ON	4.05	b	10.83	-	a *	8.89	-	ab *
	S	4.32	b	6.49	-	b *	5.45	-	b *
	CR	5.42	a	10.34	-	ab *	10.16	-	a *
	FR	4.40	b	6.97	-	ab *	6.13	-	ab *

Table 2.2: Mean concentrations (percent dry mass) of nonstructural carbohydrate (NSC) components of loblolly pine seedlings before treatment and after 10 weeks in control, heat, drought, and low light treatments. Abbreviations are: YN=young needles, ON=old needles, S=stems, CR=coarse roots, FR=fine roots. Significant differences among treatments at week 10 for each NSC × tissue combination are indicated by capital letters, and are read across rows (α=0.05). Significant differences among tissues for each NSC × treatment combination are indicated by lowercase letters, and are read down columns (α=0.05). Significant differences between a specific post-treatment NSC × treatment × tissue mean and its corresponding pre-treatment mean are indicated with asterisks.

		Pre-treatment		Control			Heat			Drought			Low Light		
Glucose	**YN**	0.63	b	0.93	AB	ab	1.42	A	*	0.67	B		0.11	C	b *
	ON	1.10	a	0.51		b	1.36			0.94			0.66		a
	S	0.37	c	1.16	A	a *	0.96	A	*	0.25	B		0.03	B	b
	CR	0.84	ab	0.94	A	ab	1.58	A	*	1.00	A		0.15	B	b *
	FR	0.48	c	0.79	B	ab *	1.72	A	*	0.95	B	*	0.15	C	b *
Fructose	**YN**	0.46	bc	0.47	A	b	0.69	A	ab	0.07	B	*	0.05	B	*
	ON	0.07	c	0.24		b	0.36		b	0.06		*	0.02		*
	S	0.64	ab	0.95	A	a	0.99	A	ab *	0.26	B	*	0.04	B	*
	CR	0.82	a	0.41	B	b	1.24	A	a	0.59	AB		0.05	B	*
	FR	0.38	c	0.29	BC	b	1.04	A	a *	0.61	B		0.05	C	*
Sucrose	**YN**	1.66	a	0.92	AB	b *	1.60	A		1.00	A	*	0.20	B	ab *
	ON	1.22	bc	0.64		b	1.57			1.10			0.75		a
	S	1.70	a	1.51	B	a	2.15	A		1.00	B	*	0.03	C	b *
	CR	1.38	ab	1.07	AB	ab	1.90	A		1.56	A		0.14	B	ab *
	FR	0.90	c	0.81	B	b	1.89	A	*	1.88	A	*	0.12	B	b *
Total Sugars	**YN**	2.75	a	2.31	B	b	3.71	A	*	1.74	B	*	0.37	C	ab *
	ON	2.78	a	1.39		b	3.30			2.10			1.44		a
	S	2.72	a	3.63	A	a	4.10	A	*	1.51	B	*	0.11	C	b *
	CR	3.05	a	2.42	AB	ab	4.72	A		3.16	A		0.34	B	b *
	FR	1.76	b	1.90	C	b	4.66	A	*	3.44	B	*	0.32	D	b *
Starch	**YN**	1.61	b	4.28	A	*	3.64	A		0.36	B		0.37	B	
	ON	1.28	b	2.68	A	*	2.86	A	*	0.40	B		0.69	B	
	S	1.60	b	1.33	A		1.15	AB		0.82	AB	*	0.47	B	*
	CR	2.37	a	4.09	A		3.19	A		0.40	B		0.46	B	
	FR	2.64	a	4.34	A	*	4.19	A	*	0.84	B	*	0.65	B	*
Total NSC	**YN**	4.36	b	6.60	A		7.35	A	*	2.10	B	*	0.73	B	b *
	ON	4.05	b	4.07	AB		6.16	A		2.50	B		2.13	B	a
	S	4.32	b	4.96	A		5.26	A		2.34	B	*	0.58	C	b *
	CR	5.42	a	6.51	AB		7.90	A		3.57	BC		0.80	C	b *
	FR	4.40	b	6.28	B		8.85	A	*	4.28	B		0.97	C	ab *

Table 2.3: Regressions between nonstructural carbohydrate (NSC) components and gas flux measurements of loblolly pine seedlings (A_{400}=net photosynthesis at 400 µmol m^{-2} s^{-1} PAR, F_s=stem CO_2 efflux measured in the dark). Significant (P<0.05) relationships are bolded. The type of best-fit regression is also indicated (E=exponential rise to maximum, L=linear, S=sigmoidal). Regression equations are presented in Supplemental Table 1.

	A_{400}		F_s	
NSC Component	R^2	*Fit*	R^2	*Fit*
Glucose	**0.130**	L	**0.395**	S
Fructose	**0.049**	L	**0.366**	S
Sucrose	0.038	L	**0.290**	E
Starch	**0.440**	E	**0.672**	E
Total Sugars	**0.085**	L	**0.332**	L
Total NSC	**0.388**	S	**0.600**	S

FIGURES

Figure 2.1: Mean dry mass (g) of tissues by treatment over time in loblolly pine seedlings in control (C), heat (H), drought (D), and low light (L) treatments. Significant (α=0.05) differences between treatments in a specific week are represented by different letters, and significant (α=0.05) differences from week one (pre-treatment) data are indicated with an asterisk ($*$).

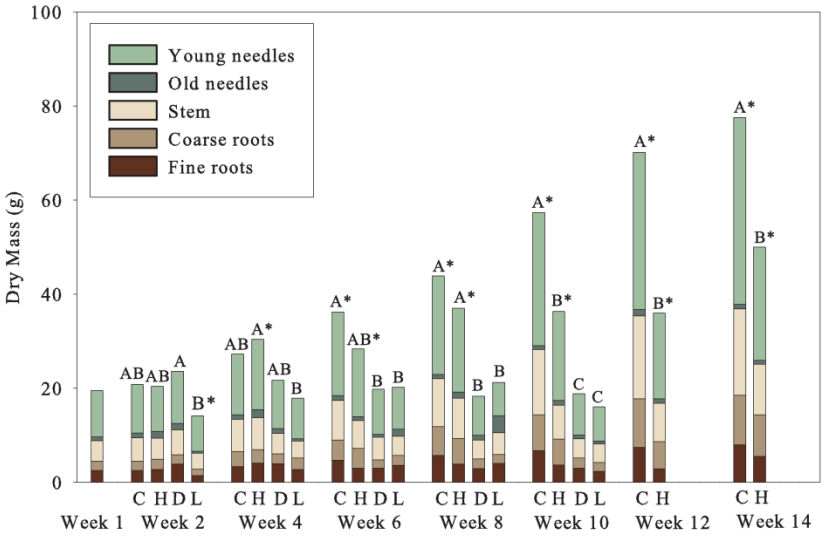

Young needles
Old needles
Stem
Coarse roots
Fine roots
Dry Mass (g)
100
80
60
40
20
0
C H D L
Week 1
Week 2
Week 4
Week 6
Week 8
Week 10
Week 12
Week 14

Figure 2.2: Mean net photosynthetic assimilation measured at 400 μmol m^{-2} s^{-1} PAR (A_{400}) and dark stem CO_2 efflux (F_s) of loblolly pine seedlings in control, heat, drought, and low light treatments over time. Error bars indicate one standard error and different letters indicate treatments with significantly different (α=0.05) means on a given measurement date.

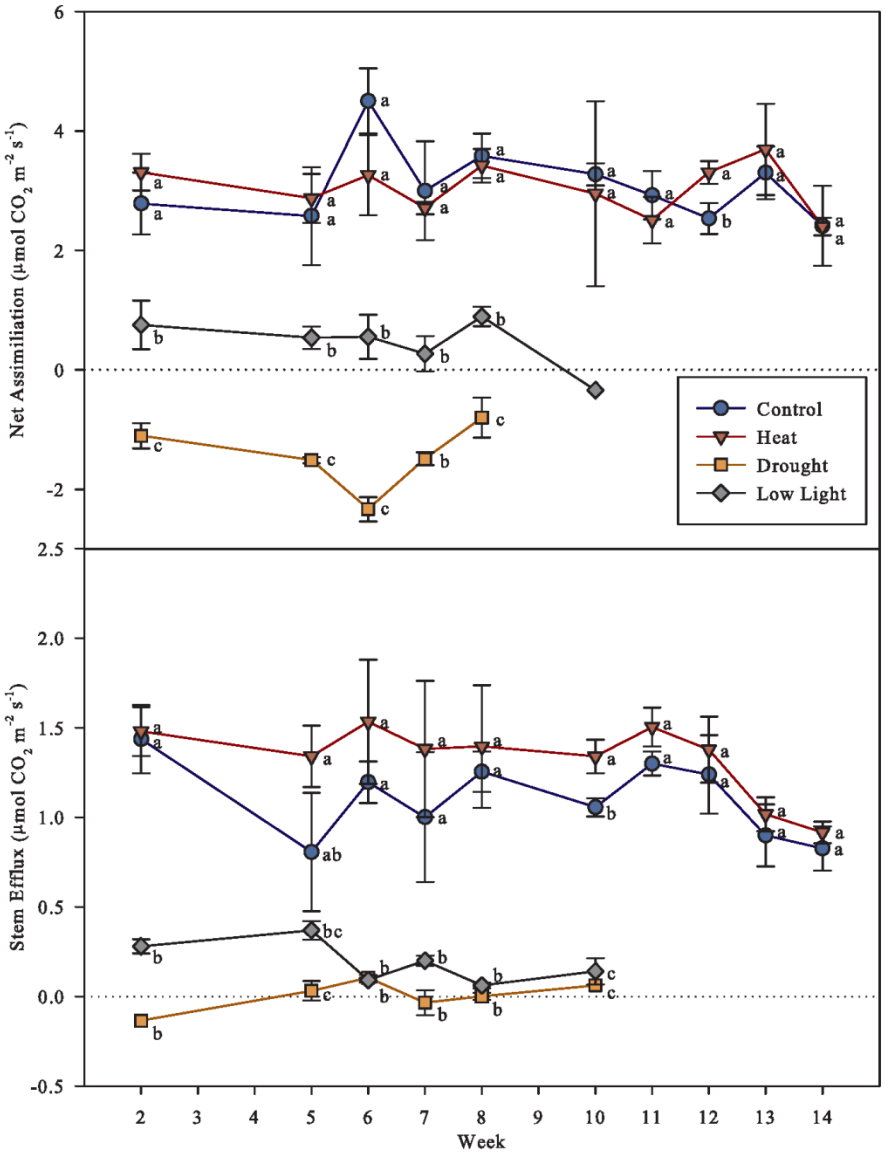

Net Assimiliation (µmol CO2 m-2 s-1)
Stem Efflux (µmol CO2 m-2 s-1)
Week
Control
Heat
Drought
Low Light

Figure 2.3: Regression quantifying the relationship between dark (pre-dawn) needle water potential (Ψ_{needle}, MPa) and stem CO_2 efflux (μmol CO_2 m^{-2} s^{-1}) of drought-treated loblolly pine seedlings. The line indicates the linear relationship: $y = 0.932 + 0.361x$.

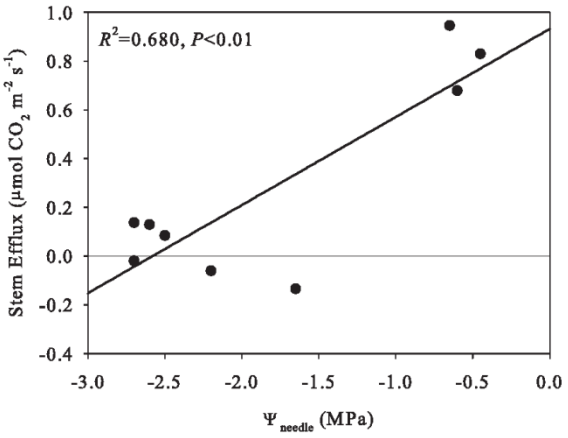

R^2=0.680, P<0.01
Stem Efflux (μmol CO_2 m^{-2} s^{-1})
Ψ_{needle} (MPa)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0

Figure 2.4: Mean whole-plant concentration (percent dry mass) of glucose, fructose, sucrose, total sugars, and starch by treatment over time of loblolly pine seedlings in control (C), heat (H), drought (D), and low light (L) treatments. Mass-weighted individual tissue concentrations (represented by different colors in each bar) indicate the percent of whole-plant NSC component concentration contributed by each tissue. Significant (α=0.05) differences in whole-plant concentration among treatments in a specific week are indicated with different letters, and significant (α=0.05) differences from week one (pre-treatment) means are indicated with an asterisk ($*$).

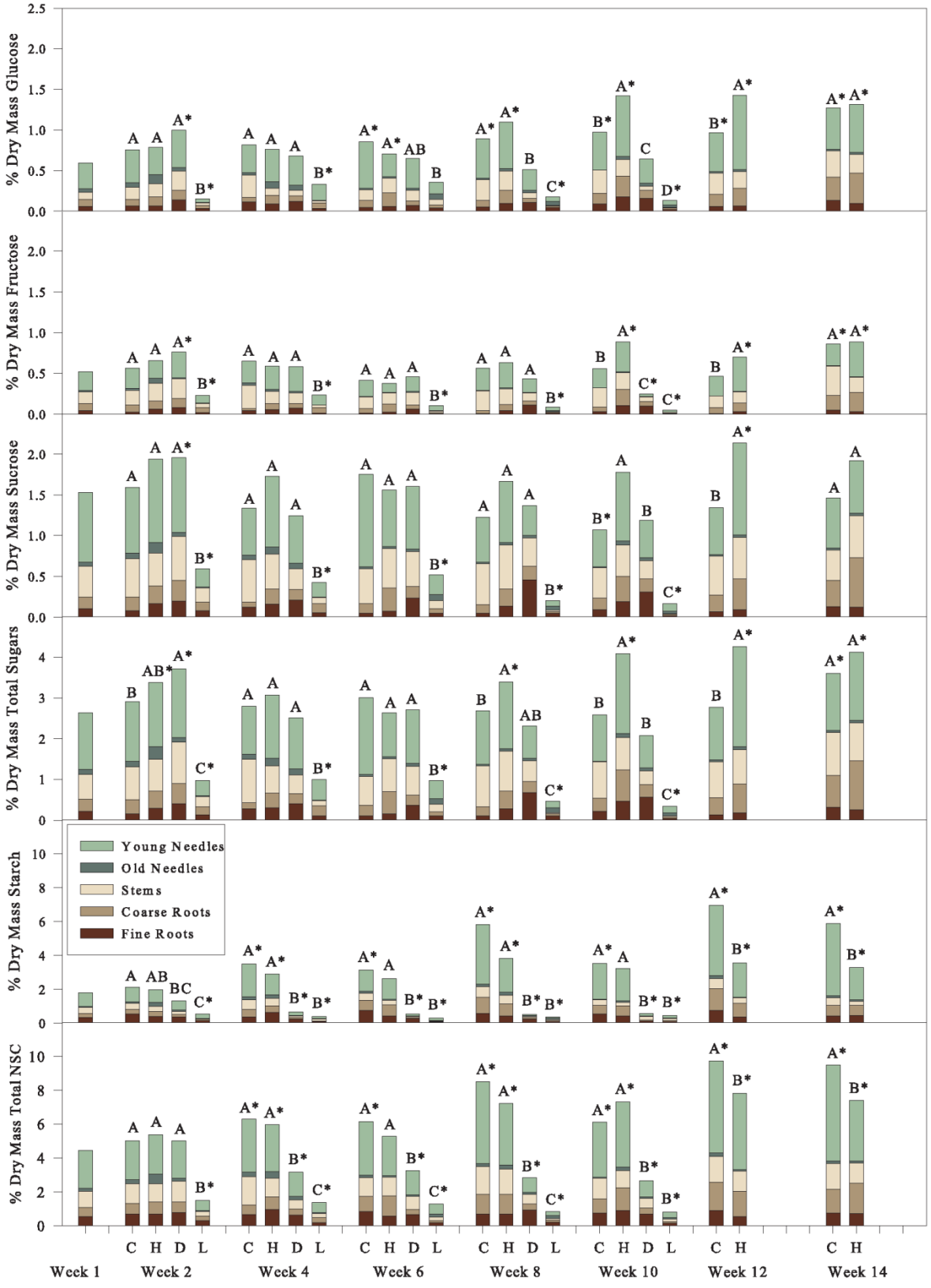

% Dry Mass Glucose
% Dry Mass Fructose
% Dry Mass Sucrose
% Dry Mass Total Sugars
% Dry Mass Starch
% Dry Mass Total NSC
Young Needles
Old Needles
Stems
Coarse Roots
Fine Roots
C H D L
Week 1
Week 2
Week 4
Week 6
Week 8
Week 10
Week 12
Week 14

Figure 2.5: Relationship of whole-plant starch concentration (percent dry mass) to whole plant mass (g) of loblolly pine seedlings in control, heat, drought, and low light treatments measured every two weeks for 14 weeks. The line indicates the linear relationship: $y = 14.805 + 6.556x$.

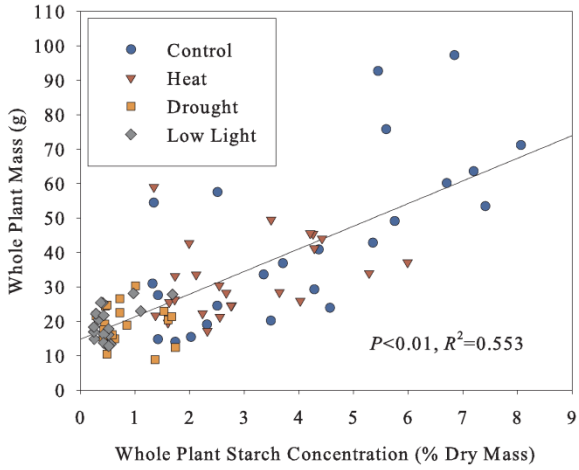
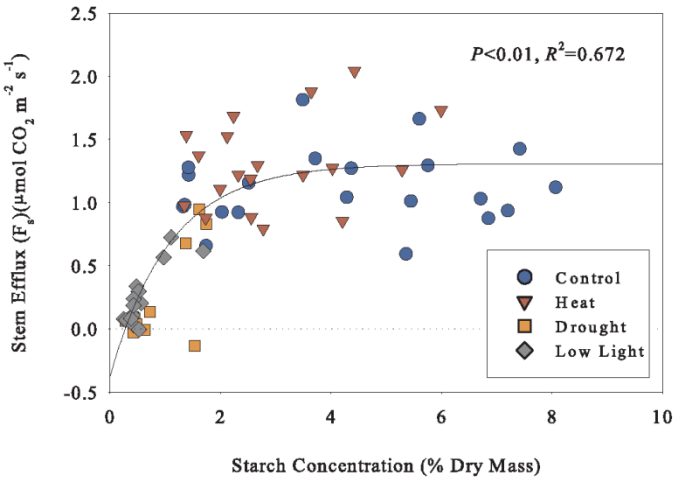

Control
Heat
Drought
Low Light
Whole Plant Mass (g)
Whole Plant Starch Concentration (% Dry Mass)
P<0.01, R2=0.553
0
10
20
30
40
50
60
70
80
90
100
110
0
1
2
3
4
5
6
7
8
9

Figure 2.6: Regression of whole-plant starch concentration (percent dry mass) and stem CO_2 efflux (μmol CO_2 m^{-2} s^{-1}). The line represents the best-fit equation for an exponential rise to maximum curve and its formula is given in Supplemental Table 1.


P<0.01, R2=0.672
Control
Heat
Drought
Low Light
Stem Efflux (Fs)(μmol CO2 m-2 s-1)
Starch Concentration (% Dry Mass)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0
2
4
6
8
10

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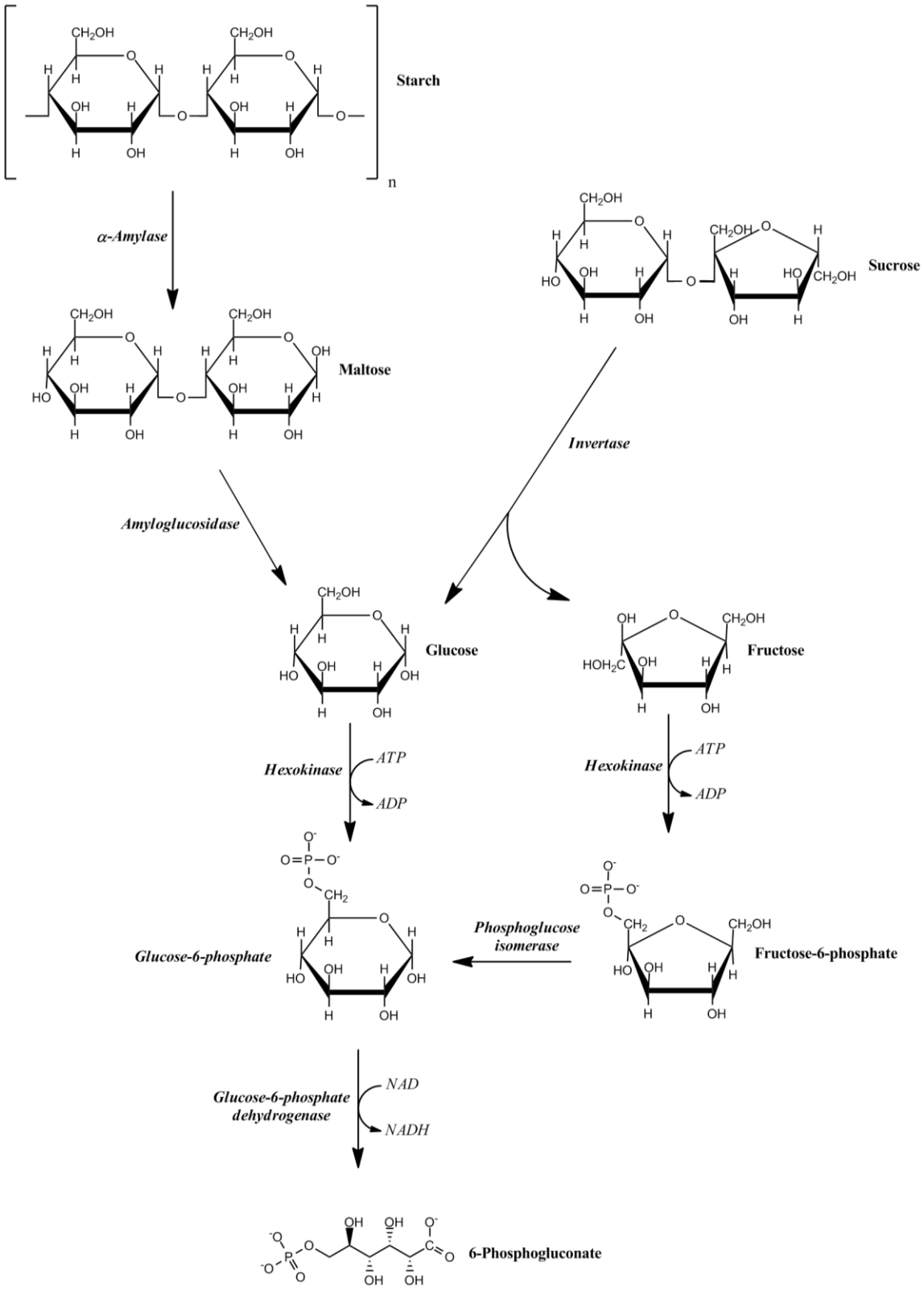
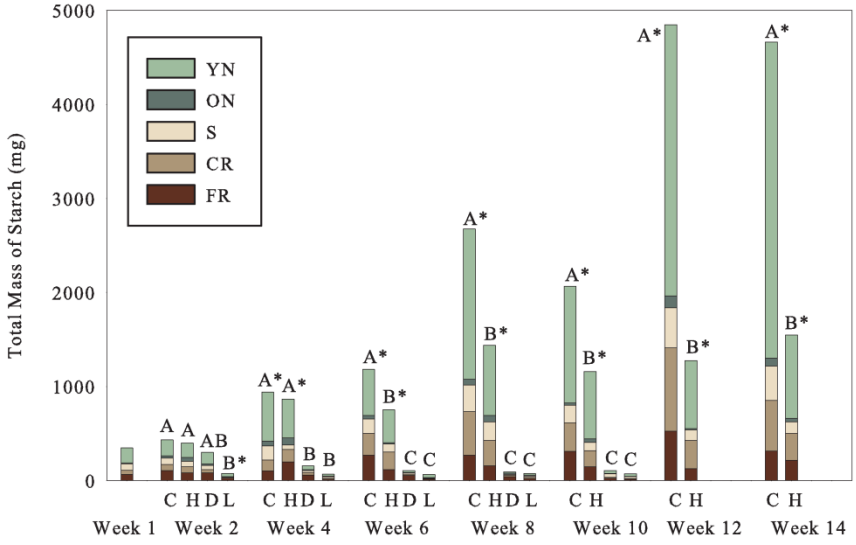
APPENDIX A: SUPPLEMENTAL FIGURES

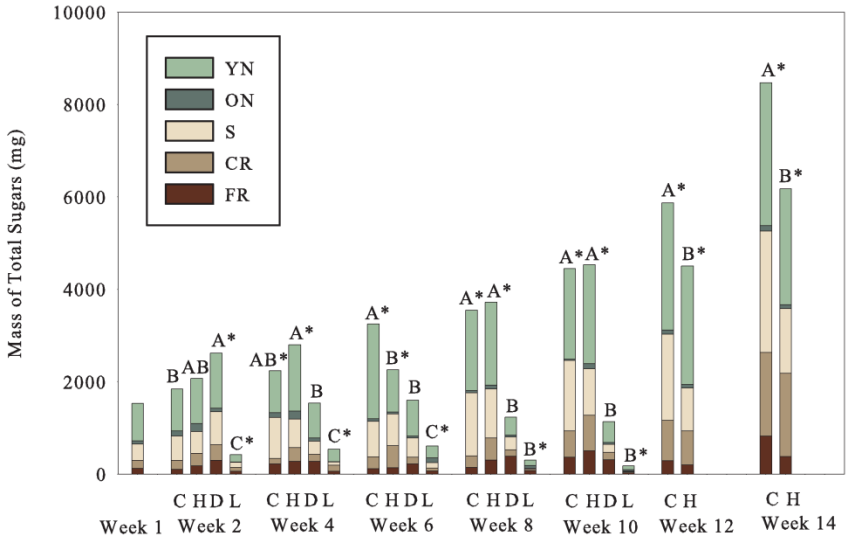
Supplemental Figure 1: Pictorial representation of the chemical pathway involved in the enzymatic quantification of nonstructural carbohydrate components. Carbohydrates are in **bold**, enzymes are in ***bold italics***, and reactants are in *italics*.


Starch
n
α-Amylase
Maltose
Sucrose
Invertase
Amyloglucosidase
Glucose
Fructose
Hexokinase
ATP
ADP
Hexokinase
ATP
ADP
Glucose-6-phosphate
Phosphoglucose isomerase
Fructose-6-phosphate
Glucose-6-phosphate dehydrogenase
NAD
NADH
6-Phosphogluconate

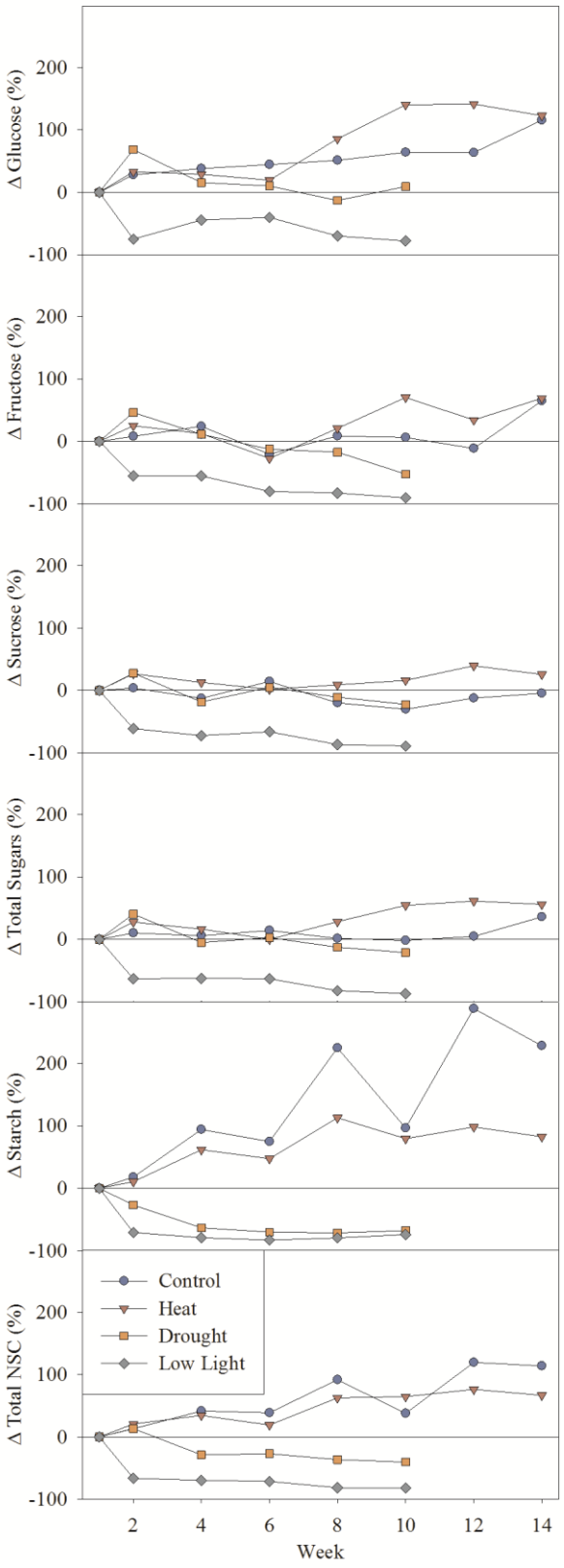
Supplemental Figure 2: Mean total mass of starch (mg) in loblolly pine seedlings in control (C), heat (H), drought (D), and low light (L) treatments over time. The mass of starch in each tissue is also indicated (YN=young needles, ON=old needles, S=stems, CR=coarse roots, FR=fine roots).



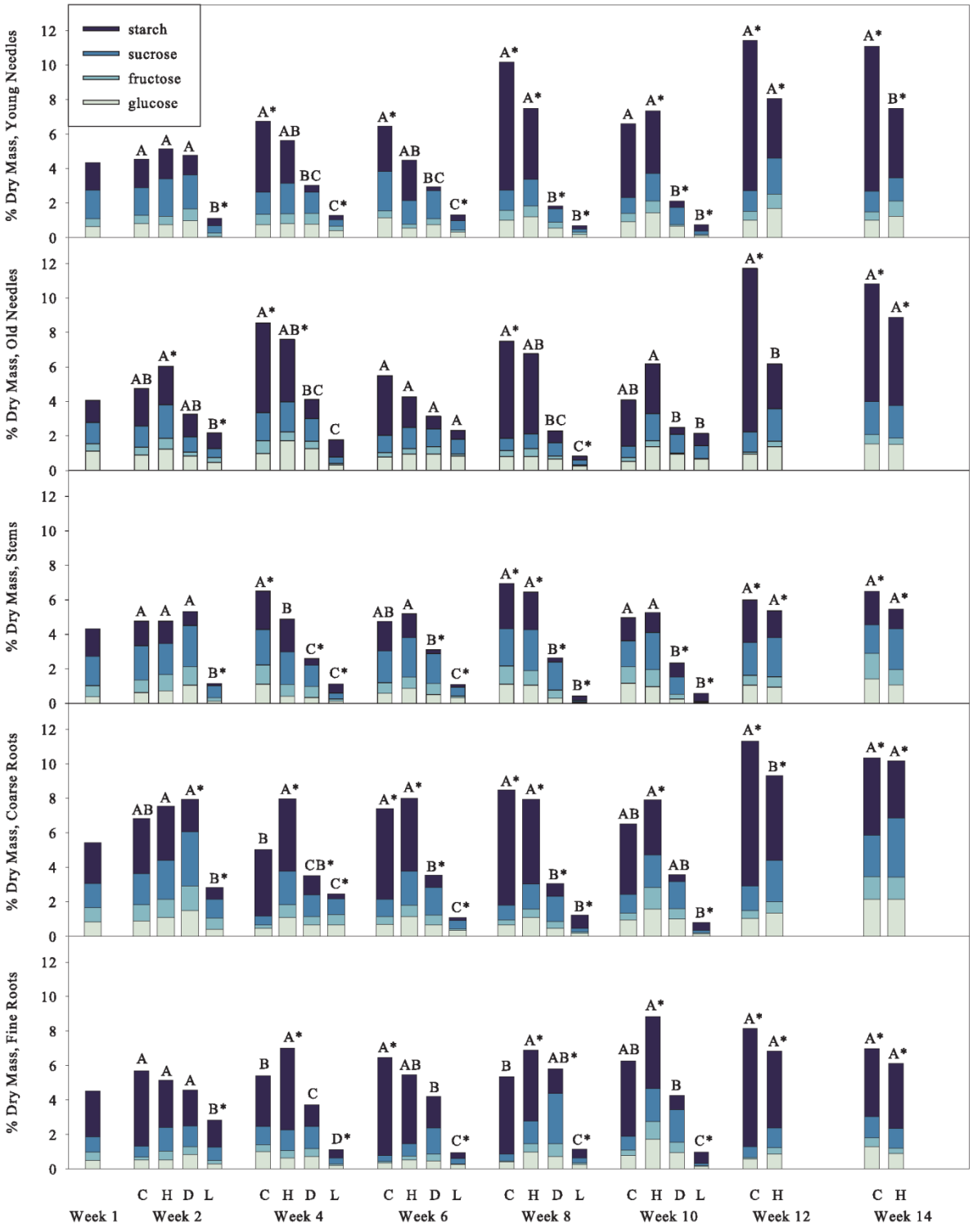
Supplemental Figure 3: Mean total mass of total sugars (mg) in loblolly pine seedlings in control (C), heat (H), drought (D), and low light (L) treatments over time. The mass of total sugars in each tissue is also indicated (YN=young needles, ON=old needles, S=stems, CR=coarse roots, FR=fine roots).



Supplemental Figure 4: Mean change in each nonstructural carbohydrate component concentration over time by treatment in loblolly pine seedlings. The change was calculated by dividing the mean of each treatment at each measurement date by the week one (pre-treatment) means and subtracting one, representing the percent change since the start of the experiment.


Δ Glucose (%)
Δ Fructose (%)
Δ Sucrose (%)
Δ Total Sugars (%)
Δ Starch (%)
Δ Total NSC (%)
200
100
0
-100
Control
Heat
Drought
Low Light
2
4
6
8
10
12
14
Week

Supplemental Figure 5: Mean concentration (percent dry mass) of total nonstructural carbohydrates in each tissue type of loblolly pine seedlings in control (C), heat (H), drought (D), and low light (L) treatments over time. Concentrations of glucose, fructose, sucrose, and starch are represented by different colors in each bar. Significant (α=0.05) differences among mean total NSC concentration of treatments in a specific week and nonstructural carbohydrate component are indicated with different letters, and significant (α=0.05) differences from week one (pre-treatment) means are indicated with an asterisk ($*$).


starch
sucrose
fructose
glucose
% Dry Mass, Young Needles
% Dry Mass, Old Needles
% Dry Mass, Stems
% Dry Mass, Coarse Roots
% Dry Mass, Fine Roots
C H D L
Week 1
Week 2
Week 4
Week 6
Week 8
Week 10
Week 12
Week 14

APPENDIX B: SUPPLEMENTAL TABLES

Supplemental Table 1: Equations for regressions between the nonstructural carbohydrate components and gas flux measurements of loblolly pine seedlings depicted in Table 2.3. The type of best-fit regression is also indicated (E=exponential rise to maximum, L=linear, S=sigmoidal)

	A_{400}		F_s	
NSC Component	*Equation*	*Fit*	*Equation*	*Fit*
Glucose	$y = 0.509 + 1.762x$	L	$y = 0.159 + \frac{0.948}{\left(1 + e^{\frac{-(x-0.488)}{0.080}}\right)}$	S
Fructose	$y = 0.925 + 1.855x$	L	$y = 0.113 + \frac{0.927}{\left(1 + e^{\frac{-(x-0.299)}{0.028}}\right)}$	S
Sucrose	$y = 0.797 + 0.771x$	L	$y = -0.231 + 1.468\ (1 - e^{-1.135x})$	E
Starch	$y = -1.703 + 4.859\ (0.390^x)$	E	$y = -0.553 + 1.839\ (1 - 0.345^x)$	E
Total Sugars	$y = 0.4314 + 0.5354x$	L	$y = 0.1002 + 0.2812x$	L
Total NSC	$y = 2.872\ (1 - e^{-2.406x})^{12230.037}$	S	$y = 0.115 + \frac{1.058}{\left(1 + e^{\frac{-(x-3.696)}{0.311}}\right)}$	S