ABSTRACT

Title of Document: INFLUENCE OF NITROGEN AND SINK COMPETITION ON SHOOT GROWTH OF POPLAR.

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Directed By: Dr. Gary D. Coleman, Department of Plant Science and Landscape Architecture

Terrestrial and oceanic biomass carbon sinks help reduce anthropogenic CO_2 emissions and mitigate the long-term effect of increasing atmospheric CO_2. Woody plants have large carbon pools because of their long residence time, however N availability can negatively impact tree responses to elevated CO_2. Seasonal cycling of internal N in trees is a component that contributes to fitness especially in N limited environments. It involves resorption from senescing leaves of deciduous trees and storage as vegetative storage proteins (VSP) in perennial organs. *Populus* is a model organism for tree biology that efficiently recycles N. Bark storage proteins (BSP) are the most abundant VSP that serves as seasonal N reserves. Here I show how poplar growth is influenced by N availability and how growth is influenced by shoot competition for stored N reserves. I also provide data that indicates that auxin mediates BSP catabolism during renewed shoot growth. Understanding the

components of N accumulation, remobilization and utilization can provide insights leading to increasing N use efficiency (NUE) of perennial plants.

INFLUENCE OF NITROGEN AND SINK COMPETITION ON SHOOT GROWTH OF POPLAR

By

Chioma Egekwu.

Thesis submitted to the Faculty of the Graduate School of the
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Advisory Committee:
Professor Gary D. Coleman, PhD, Chair
Wendy A. Peer PhD
Angus S. Murphy PhD

Dedication

I dedicate this thesis to my Lord and Savior Jesus Christ who by His grace and mercy took me through this Plant Science program. I give Him all the praise and glory

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I would like to thank my committee members especially my supervisor Dr. Coleman for giving me the opportunity to work in his lab for this five years. You believed in me and trusted in my work ethics by allowing me to work independently in the lab even when it was outside of my comfort zone. I started in your lab without very limited knowledge about plant science, but I am completing my graduated program gaining a wealth of knowledge from your lab. To Drs. Murphy and Peer, I want to thank you for you support and encouragement towards my academic success.

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Table of Contents

List of Tables

Table Page

List of Figures

Figure Page

Chapter 1: Components of seasonal nitrogen accumulation and remobilization in poplar

Introduction

Perennial plants have evolved strategies for the conservation and reuse of N which provides a competitive advantage compared to annual plants in N limited environments (Rennenberg and Schmidt, 2010). Internal seasonal cycling of N is an adaptive strategy of trees that contributes to efficiency of N use and since this process occurs over the life-time of perennial plants, internal N cycling increasingly contributes to the overall N budget over time (Millard and Grelet, 2010). Because the majority of research related to internal seasonal N cycling in trees has been studied in poplar (*Populus*) this review focuses on what we know and don't know about this process in poplar.

Protein mobilization from senescing leaves and storage of bark storage protein (BSP) in bark tissues

During autumn, perennial plants including poplar, transport N from senescing leaves to perennial storage organs. In most woody plants stored N is usually sequestered in vegetative storage proteins (VSP). These VSP function as N storage reserves and are catabolized with the N remobilized to support shoot growth when nutrients are limited (Cooke and Weih, 2005). In poplar, accumulation of the Bark Storage Proteins (BSP), a class of VSP occurs in bark phloem parenchyma, xylem ray cells, and structural roots in response to short day (SD) photoperiods, low temperatures, and elevated N availability (van Cleve and Apel, 1992; Coleman et al., 1991; Langheinrich and Tischner, 1991; Sauter and Cleve, 1990; Wetzel et al., 1989).

The poplar BSP are 32 kD and 36kD proteins that are encoded by 3 genes (*BSP*A *BSP*B and *BSP*C), and the expression of these genes increase under SD and SD with low temperature (LT), in developing bud and bark (Pettengill et al., 2013). Moreover, in *in vitro* poplar plantlets grown under long day (LD) photoperiod and .44% MS media BSP was found to be synthesized in roots, stem, basal and apical leaves (Beardmore et al., 1996) further supporting the role of N availability in BSP accumulation.

Besides BSP accumulation, a number of other seasonal processes are regulated by SD photoperiods in poplar. These include the cessation of leaf expansion, internode elongation and the induction and development of apical buds. In addition, SD photoperiods are the main factor that governs poplar autumn leaf senescence (Fracheboud et al., 2009). The critical SD photoperiod required for induction of poplar leaf senescence appears to differ from that required to initiate bud development and once leaf senescence is initiated, low temperature accelerates the process (Fracheboud et al., 2009). Because both BSP accumulation and leaf senescence both occur in response to SD photoperiods, it is believed that the primary source of N used in BSP synthesis is from catabolism of leaf proteins. In addition, studies from Langheinrich and Tischner, (1991) suggested that nitrate from leaves and nitrate taken up by roots may also contribute to storage protein synthesis.

It has been proposed that environmental control of poplar BSP accumulation and seasonal nitrogen cycling is regulated by changes in metabolites and specifically by changes in amino acid contents (Wildhagen et al., 2010; Zhu and Coleman, 2001). Glutamine is the predominant amino acid that accumulates in leaves during autumn

senescence (Couturier et al., 2010; Wildhagen et al., 2010). However in poplar bark, both glutamine and arginine contents increase during autumn senescence but the patterns of accumulation of these two amino acids display important differences (Wildhagen et al., 2010). Bark glutamine levels increased in the fall, then declined during winter while bark arginine contents increased during autumn and remain elevated throughout winter (Wildhagen et al., 2010). Because glutamine is the major transport amino acid in poplar, it is possible that glutamine that accumulates in leaves during senescence is transported to bark producing the autumn associated increase in bark glutamine content.

The transport of leaf glutamine to bark would involve the export from senescing leaves and possible loading into phloem, transport to the bark, and unloading from phloem parenchyma. The poplar genome has been reported to contain 100 genes encoding amino acid transporters belonging to the amino acid/auxin permease (AAAP) and amino acid transporters belonging to the amino acid-polyamine choline families of the APC superfamily (Wu et al., 2015). Only two amino acid transporters have been characterized in poplar including a xylem associated amino acid permease, PtAAP11, and a cationic amino acid transporter PtCAT11 that is associated with leaf senescence (Couturier et al., 2010). While little is known about glutamine transport mechanism in trees, it has been suggested that Pt-CAT11 may play a role in glutamine transport during N mobilization from senescing leaves to storage tissues (Couturier et al., 2010). Additionally, genes in the arginine biosynthetic pathway are also expressed in poplar stems during autumn. Because glutamine is the major transport amino acid during leaf senescence and its transient

accumulation in bark is also associated with increased bark levels of arginine, suggests that arginine may also serve as a signaling molecular related to cellular N status.

Electron microscopy and immunogold labeling have demonstrated that during SD BSP accumulation that BSP is sequestered in single membrane protein bodies of the poplar bark parenchyma and ray cells (van Cleve and Apel, 1992; Sauter and Cleve, 1990; Wetzel et al., 1989). Maximum BSP accumulation occurs in winter when BSP abundance was about 62% of total bark soluble proteins (Coleman et al., 1991; Langheinrich and Tischner, 1991; Wetzel et al., 1989). The amount of BSP accumulated in winter may vary yearly due to environmental factors (e.g. pathogens, erratic weather conditions etc.) that can inflict severe damage to leaves and shoots, reducing the amount of mobilized N for storage protein synthesis (Sauter and Cleve, 1990).

Remobilization from storage tissues

Different molecular, cellular and physiological factors play major roles in N remobilization from storage tissues. After chilling has released bud dormancy and coincident with bud burst BSP gene expression declines in poplar bark (Wildhagen et al., 2010). Sauter and Cleve, (1990) have shown that the content of proteins bodies that accumulated in the parenchyma cells during BSP accumulation began to be diminished at the same time that smaller vesicular structures and tubular ER were observed in the storage cells. They observed that these vesicular structures and tubular ER gradually disappeared, as new leaves emerged, coinciding with BSP remobilization. Sauter and Cleve, (1990) suggested that the changes in protein bodies

were an indirect indication of proteolytic digestion of protein body contents. Evidence of proteolytic digestion of BSP during spring growth was shown by Langheinrich and Tischner, (1991) who identified potential degradation peptides with a molecular mass of 18kD, that occurred at budburst and cross-reacted with BSP antibody. Recently, multi-dimensional protein identification technology (MudPIT) identified putative vacuolar targeted cysteine and serine proteases that may play roles in BSP remobilization in poplar during spring growth (Islam et al., 2015). Additionally, transcript abundance of specific cysteine and serine proteases increased in poplar bark during LD after SD+LT treatment and coincided with BSP remobilization (data unpublished).

Sauter and van Cleve, (1992) have linked protein body hydrolysis with amino acid abundance in poplar xylem vessels during spring regrowth. Glutamine was the predominant amino acid in the xylem sap of poplar during renewed shoot growth (Sauter and van Cleve, 1992; Wildhagen et al., 2010) and likely serves as a precursor for synthesis of other amino acids, nucleic acids, polyamines and chlorophylls in plants (Suarez et al., 2002).

Utilization of stored reserve nutrients

Remobilized N from storage tissues is utilized for new growth of leaves flowers, fruits and roots (Cantón et al., 2005; Millard and Grelet, 2010; Neto et al., 2008; Tagliavini et al., 1997) linking bud expansion and shoot elongation to BSP remobilization in spring (Coleman et al., 1993; Islam et al., 2015). Although there are several studies on storage N utilization for shoot growth, the majority of these studies were not conducted in *Populus* but we can use the knowledge from these

studies to increase our understanding of N utilization and spring growth. The extent of spring growth has been shown to be correlated with the stored N reserves in apples (*Malus domestica* Borkh) (Cheng and Fuchigami, 2002). In studies of cherry (*Prunus avium*) and poplar (*Poplulus trichocarpa*), new shoot growth was shown to be two weeks dependent on previously stored N (Millard et al., 2006). In addition, the source of N that is allocated to various tissues during spring growth appears to differ. For example in pear (*Pyrus communis),* N from storage reserves was allocated to leaves during initial spring growth of leaves and as growth continued N from both stored reserves and newly assimilated N was utilized (Tagliavini et al., 1997). In contrast to vegetative growth, the majority of N allocated to flowers and fruits growth during initial shoot development was from stored N in pear (*Pyrus communis* L.) (Quartieri et al., 2002).

Future directions in seasonal N cycling research

There are several potential research avenues that can be pursued related to seasonal N cycling in poplar that could advance our understanding of N use efficiency in plants. Identifying and understanding the pathways of glutamine transport from senescing leaves to bark and how this contributes to BSP synthesis during storage protein accumulation will further contribute to our knowledge of seasonal N cycling in woody plants. To the best of our knowledge, only two amino acid transporters (PtAAP11 and PtCAT11) have been characterized in poplar, and PtCAT11 appears to be associated with the export of glutamine during leaf senescence (Couturier et al., 2010; Wu et al., 2015). Since the poplar genome contains at least100 genes encoding amino acid transporters and permeases, future

research focused on identifying and characterizing additional transporters and defining their function during seasonal N cycling would contribute to developing a system understanding of this process.

Little is known about molecular mechanism that regulates BSP gene expression, protein synthesis and accumulation. Since glutamine appears to be the predominate amino acid that is transported from senescing leaves to bark then it is possible that sensing of glutamine abundance may be critical component regulating BSP accumulation. In bacteria and most eukaryotes the P_{II} signaling protein is involved in nitrogen metabolism and functions to regulate amino acid (glutamine, arginine and histidine) biosynthesis (Chellamuthu et al., 2013). P_{II} is conserved throughout the plant and bacteria kingdom and directly regulates the activity of N-acetylglutamate kinase (NAGK), the enzyme that catalyzes the committed step in the arginine biosynthesis by relieving arginine feedback inhibition (Fallon and Weng, 2014). Studies in *C. reinhardtii* showed that binding of glutamine to the C-terminus of CrP_{II} is required for the interaction between CrP_{II} and CrNAGK. P_{II} proteins from *Physcomitrella patens* and *Oryza sativa* also exhibits glutamine-dependent activation of arginine biosynthesis suggesting that P_{II} protein may function as a glutamine sensing protein (Chellamuthu et al., 2014). If P_{II} functions as a glutamine sensor then it may be possible that regulation of poplar BSP accumulation could involve P_{II} sensing of increase bark levels of glutamine that occur during leaf senescence. P_{II} mediated binding of glutamine could activate the biosynthesis of arginine, polyamines and/or NO that then signals *bsp* expression and BSP accumulation in the xylem tissues.

Studies in poplar have identified putative proteases that are predicted to be targeted to the vacuole during regrowth and N remobilization and these proteases may function in BSP catabolism (Islam et al., 2015). Further research is needed to characterize the candidate proteases that hydrolyze BSP. Future studies need to identify the localization of these proteases, their target sites on BSP and the time-course during BSP remobilization they are activated (Müntz et al., 2001). Current studies related to BSP catabolism have demonstrated that renewed shoot growth is necessary for BSP catabolism (Coleman et al., 1993; Islam et al., 2015). It was proposed that signals emanating from expanding buds are perceived by the bark that then triggers BSP catabolism. Further research aimed towards identifying the signal molecules from expanding buds would advance the understanding of N remobilization from BSP.

Auxin is produced in developing leaves and transported basipetally to roots (Bhalerao et al., 2002; Ljung et al., 2001) and microarray analysis of genome-wide transcript changes in poplar bark during BSP catabolism and N remobilization have shown enrichment of specific auxin transporters, Auxin Response Factors (ARFs) and AUXIN RESISTANT1/LIKE AUX1 AUX/IAA genes during N remobilization (data unpublished). Identifying the regulatory components of auxin mediated N remobilization would likely provide significant advances towards understanding how N is remobilized and partitioned in relation to N source-sink relations.

Since BSP accumulates in poplar roots (Beardmore et al., 1996; Langheinrich and Tischner, 1991), future studies can focus on identifying the specific storage sites of BSP in the root and understand the mechanisms underlying BSP catabolism and N

remobilization in the poplar roots. Do the same signals from expanding shoots that mediate the catabolism of BSP in the bark also mediate the degradation of BSP and remobilization of N in the roots? Is the remobilized N from poplar roots partitioned only to root spring growth or does poplar allocate remobilized N from roots to shoot growth?

These future studies related to the regulation of seasonally stored N in poplar would advance our understanding of internal N cycling. Such advances in defining the mechanisms of N storage, remobilization and utilization in perennial plants could provide insights that can lead to increasing N use efficiency in both perennial and annual plants.

Chapter 2: Effect of N availability on poplar growth

Forest ecosystems contribution to mitigating global climate change

Increase in the burning of fossil fuels is elevating the concentration of CO_2 emitted in the atmosphere, resulting to global climate change, which can drastically affect the ecosystems and threaten human lives (Langan et al., 2011; Metzger and Hüttermann, 2008). Fortunately, research studies have shown that terrestrial and oceanic biomass are carbon sinks that can reduce anthropogenic CO_2 emissions and alleviate the possible damaging effect of increasing atmospheric CO_2 (Hansen et al., 2000; Luo et al., 2004).

The world's forest ecosystems is about 31 percent of the world's land surface and serve as a major source of food, wood, non-wood tree products; and protection from different natural disasters (Adams, 2012; Motta and Haudemand, 2000; Pimentel et al., 1997; Vinceti et al., 2013). Long term field observations and data analysis have shown forest ecosystems as significant factors in reducing anthropogenic CO_2 emissions through sequestration into biomass (Pan et al., 2011). Perennial plants such as trees have large carbon pools because of their long residence time, it takes decades to thousands of years to reach their carbon equilibrium (Luo et al., 2004). Although tree growth can reduce the increasing atmospheric CO_2, however it is also known that under elevated atmospheric CO_2, carbon is also sequestered into soil organic matter which reduces the available inorganic N in the soil for plant growth (Luo et al., 2004). Previous studies have shown that at elevated CO_2 trees produced less biomass under limited N than at N enriched environments suggesting that limited N availability negatively impacted tree response to elevated

atmospheric CO_2 over a long period of time (Norby et al., 2010; Reich et al., 2006). Since perennial plants possess high levels of N use efficiency (NUE) as a consequence of internal N cycling, fundamental knowledge related to the mechanisms and regulation of internal N cycling of perennial plants would advance our understanding of NUE in perennials and could lead to the development of strategies to improve or enhance NUE in order to improve tree biomass production in response to elevated atmospheric CO_2.

Poplar is a model organism for tree and perennial plant biology, including seasonal N cycling, in part because its genome has been sequenced and it remobilizes the majority of N from leaves to bark and sequesters this N in BSP over the winter (Jansson and Douglas, 2007; Pregitzer et al., 1990). To understand N cycling in poplar, it is important to identify the physiological characteristics influenced by N availability in order to determine what traits might be influenced by stored N remobilization.

Poplar shoot development and N availability

Studies have shown that N fertilization influences poplar growth and development. In *Populus balsamifera* ssp. *trichocarpa x deltoides* N availability changed whole-plant architecture and biomass accumulation by increasing stem height, leaf numbers, sylleptic shoots and N content of tissues (Cooke et al., 2005). In *Populus trichocarpa* (Torr and Gray) x *deltoides* (Bartr. Ex Marsh) and *Populus trichocarpa x deltoides* H11-11, N fertilization affected wood traits during secondary stem growth by increasing xylem fiber diameter and their cell wall thickness as well as wider xylem vessels with altered wood chemistry including increased cellulose and

hemicellulose content in xylem fibers (Hacke et al., 2010; Pitre et al., 2007a, 2007b; Plavcová et al., 2013). Increased N supply was also found to increase aquaporin gene expression in *Populus trichocarpa x deltoides* H11-11 and the stems of these trees were more vulnerable to cavitation (Hacke et al., 2010). Although N availability has been shown to influence a number of developmental and physiological responses in poplar, none of the previous studies have examined N availability responses in the *Populus tremula X Populus alba hybrid* clone INRA 717 IB4. Because this genotype is widely used for molecular and transgenic studies in poplar, the aim of this study was to determine how N availability influenced growth in order to determine what traits could potentially be influenced by stored N remobilization.

Materials and Methods

Plant growth and N treatments

The *Populus tremula X Populus alba* clone INRA 717 IB4 genotype was used for all experiments related to N availability. Experiments were conducted in the research greenhouse complex at the University of Maryland and plants were grown under LD conditions (16 h light, 8 h dark) at 20°C for 10 weeks. Supplemental lighting to extend photoperiods to a LD (minimum of 16 hrs of light) was provided by high pressure sodium lamps. Rooted *in vitro* cuttings were individually planted into 6.0L pots containing soilless media (Sunshine LC1).

Plants were randomly assigned to N treatments with 3 replicates per treatment. Plants were treated 3 times per week with 500ml of a nutrient solution (8.3mM KCl, 8.3mM $CaCl_2.2H_2O$, 1mM $MgSO_4.7H_2O$, 0.5mM KH_2PO_4, 44.8uM EDTA Fe (III) [$C_{10}H_{12}FeN_2NAO_8$], 23.1uM H_3BO_3, 4.5uM $MnCl_2.4H_2O$, 4uM $ZnCl_2$, 2.7uM

$CuCl_2.2H_2O$, 52nM $Na_2MbO_42H_2$) containing either 1.5625mM, 3.12mM, 6.25mM, 12.5mM or 25mM NH_4NO_3.

Plants were destructively harvested and shoot growth parameters were measured after 10 weeks of treatment. Leaf area was measured with an area meter (LI-3100, LiCor), stem diameter was measured with a caliper and length of new shoot growth was measured with a meter stick.

Results

N availability influences leaf area and stem diameter

N availability significantly affected poplar leaf area in *Populus tremula X Populus alba* clone INRA 717 IB4 genotype. The mean leaf area was greater in poplars fertilized with 12.5mM and 25mM NH_4N0_3 compared to 1.5625mM, 3.12mM and 6.25mM NH_4N0_3 tree (Figure 2.1, Table 2.1). Plants treated with 12.5mM and 25mM NH_4N0_3 produced leaves with a mean leaf area of 174.367 cm^2 and 199.6 cm^2 respectively, compared to 90.267 cm^2 for 1.5625 NH_4N0_3, 116.8 cm^2 for 3.12 NH_4N0_3 and 148.133 cm^2 for 6.25mM NH_4N0_3 treatments. N fertilization dependent increases in leaf area varied according to leaf development or leaf position. Leaf area increased at similar rates from LPI 1 to 4 irrespective of N availability and leaf areas were not different between N treatments for LPI 1 to 4. Differences in leaf area by leaf position between N treatments among young leaves of leaf plastichron index (LPI), LPI 1 to LPI 4; however the differences in leaf area became obvious as leaves expanded (LPI 5 and beyond) and the rate of leaf expansion was greater as N abundance increased (Figure 2.2).

Stem diameter was also influenced by increased N availability. At LPI-5 the diameter of poplar stems treated with 25mM NH_4N0_3 was larger than all treatments; although it was not significantly different from some of the N treatments (Table 2.2). Trees treated with 25mM NH_4N0_3 produced mean diameter of 6.680 cm in contrast to 4.233 cm for 1.5625mM NH_4N0_3, 6.257 cm for 3.12mM NH_4N0_3, 4.894 cm for 6.25mM NH_4N0_3 and 5.825 cm for 12.5mM NH_4N0_3 trees. At LPI-15, poplars fertilized with 12.5mM and 25mM NH_4N0_3 N produced larger mean stem diameters

than 1.5625mM, 3.12mM and 6.25mM NH_4N0_3 treatments (Table 2.3). Trees fertilized with 12.5 and 25mM NH_4N0_3 produced mean diameters with of 10.025 cm and 10.736 cm respectively compared to 6.739 cm for 1. 5625mM NH_4N0_3, 8.263 cm for 3.12mM NH_4N0_3 and 8.729 cm for 6.25mM NH_4N0_3. The mean stem diameters of trees treated with 12.5 and 25mM NH_4N0_3 were also greater at LPI-25 compared to trees that received 1.5625mM, 3.12mM and 6.25mM NH_4N0_3 (Table 2.4); their mean diameters were 12.099 cm and 13.411 respectively. In contrast 1.5625mM, 3.12mM and 6.25mM $NH4N0_3$ measured 9.821 cm, 13.005 and 12.311 respectively. The mean stem diameter at the base of trees fertilized with 12.5mM and 25mM NH_4N0_3 were also greater than those of plants treated with 1.5625mM, 3.12mM and 6.25mM NH_4N0_3 (Table 2.5). Trees of 12.5mM and 25mM N treatments produced mean diameters of 14.681 cm and 13.919 respectively; however 1.5625mM, 3.12mM and 6.25mM N treatment produced base diameters of 9.821 cm, 13.005 cm and 12.311 cm respectively.

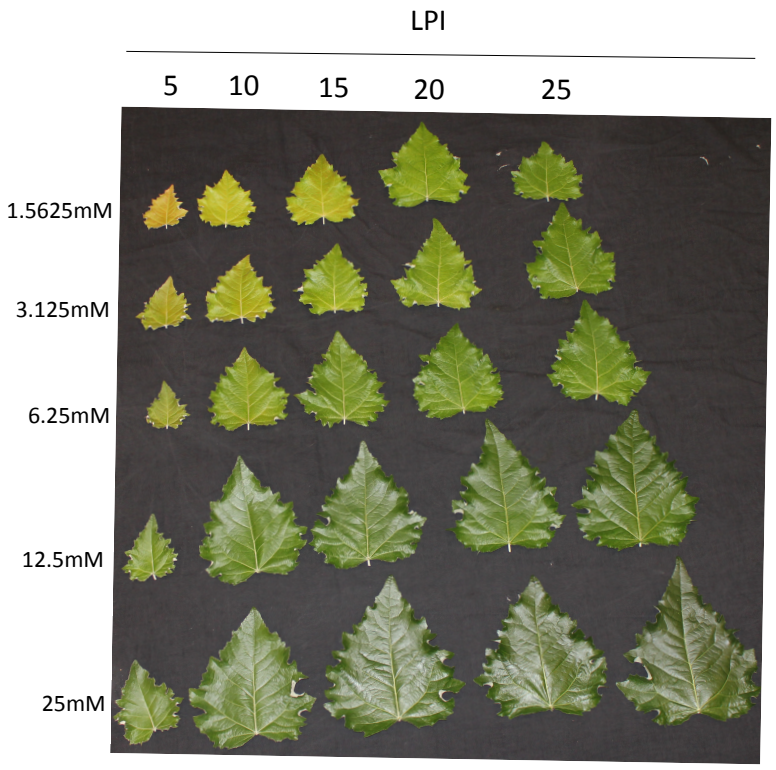

Figure 2.1: Poplar leaf samples at LPI-5, 10, 15, 20 and 25 from plants after 10 weeks of fertilization with different levels of ammonium nitrate (1.5625mM, 3.12mM, 6.25mM, 12.5mM and 25mM).

Table 2.1: Mean leaf area of poplars after 10 weeks of fertilization with 1.5625mM, 3.12mM, 6.25mM, 12.5mM or 25mM of ammonium nitrate. Means of 3 replicates. Means assigned same letters are not significantly different

Factor	Ammonium nitrate (mM)	Mean leaf area (cm^2)	*F*	*P*-value
Leaf area	1.5625	90.267 (1.852)[a]	13.54	0.0005
	3.12	116.8 (6.872)[ab]		
	6.25	148.133 (12.28)[bc]		
	12.5	174.367 (11.74)[c]		
	25	199.9 (19.13)[c]		

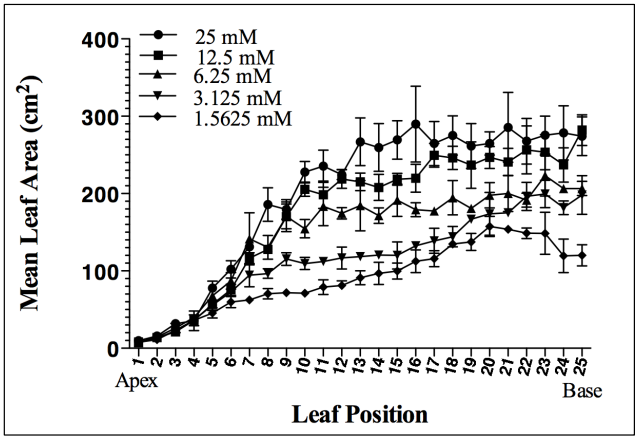

Figure 2.2: Leaf area by leaf position of poplars after 10 weeks of fertilization with 1.5625mM, 3.12mM, 6.25mM, 12.5mM or 25mM ammonium nitrate.

Table 2.2: Stem diameters at Leaf plastichron index 5 (LPI-5) of N-fertilized poplar (1.5625mM, 3.12mM, 6.25mM, 12.5mM and 25mM of ammonium nitrate). After 10 weeks of treatment Means of 3 replicates, Means assigned same letter are not significantly different.

Factor	Ammonium nitrate (mM)	Mean stem diameter (cm)	*F*	*P*-value
LPI-5	1.5625	4.233 (0.200)[a]	5.694	0.0118
	3.12	6.257 (0.771)[b]		
	6.25	4.894 (0.074)[ab]		
	12.5	5.825 (0.277)[ab]		
	25	6.680 (0.407)[b]		

Table 2.3: Stem diameters at Leaf plastichron index 15 (LPI-15) of N-fertilized poplar (1.5625mM, 3.12mM, 6.25mM, 12.5mM and 25mM of ammonium nitrate) after 10 weeks of treatment. Means of 3 replicates, Means followed by the same letter are not significantly different.

Factor	Ammonium nitrate (mM)	Mean stem diameter (cm)	*F*	*P*-value
LPI-15	1.5625	6.739 (0.449)[a]	9.297	0.0021
	3.12	8.263 (0.111)[ab]		
	6.25	8.729 (0.327)[abc]		
	12.5	10.025 (0.909)[bc]		
	25	10.736 (0.403)[c]		

Table 2.4: Stem diameters at Leaf plastichron index 25 (LPI-25) of N-fertilized poplar (1.5625mM, 3.12mM, 6.25mM, 12.5mM and 25mM of ammonium nitrate) after 10 weeks of treatment. Means of 3 replicates; Means followed by the same letter are not significantly different.

Factor	Ammonium nitrate (mM)	Mean stem diameter (cm)	*F*	*P*-value
LPI-25	1.5625	8.306 (0.404)^{a}	9.044	0.0023
	3.12	10.228 (0.094)^{ab}		
	6.25	10.854 (0.330)^{abc}		
	12.5	12.099 (1.055)^{bc}		
	25	13.411 (0.815)^{bc}		

Table 2.5: Stem diameters at the base of N-fertilized poplar (1.5625mM, 3.12mM, 6.25mM, 12.5mM and 25mM of ammonium nitrate) after 10 weeks of treatment. Means of 3 replicates, Means followed by the same letter are not significantly different.

Factor	Ammonium nitrate (mM)	Mean stem diameter (cm)	*F*	*P*-value
Base	1.5625	9.821 (0.537)^{a}	4.568	0.0234
	3.12	13.005 (0.889)^{ab}		
	6.25	12.311 (0.438)^{ab}		
	12.5	14.681 (1.358)^{b}		
	25	13.919 (0.835)^{b}		

Nitrogen availability affects sylleptic shoot growth

Trees fertilized with different levels of N displayed significant N-induced shifts in tree architecture. Poplars treated with 12.5mM and 25mM NH_4NO_3 exhibited significantly greater number of sylleptic shoots than those with 1.5625mM, 3.12mM and 6.25mM NH_4NO_3 (Figures 2.3 and 2.4). The mean number of sylleptic shoots produced in trees treated with 12.5mM and 25mM NH_4NO_3 were 17.000 and 24.667 respectively. In contrast sylleptic branches were not produced in trees fertilized with either 1.5625mM or 3.12mM NH_4NO_3. Poplars that received 6.25mM NH_4NO_3 produced syllepsis with a mean of 0.33. N levels also significantly affected growth of sylleptic shoots. The mean growth of sylleptic shoots of 12.5mM and 25mM $NH4NO_3$ were greater than 1.5625mM, 3.12mM and 6.2mM NH_4NO_3 trees. (Figure 2.5). The mean growth of sylleptic shoots of the 12.5mM and 25mM NH_4NO_3 treated plants were 8.425 cm and 11.911 cm respectively.

Figure 2.3: Sylleptic shoot growth from poplar stems after 10 weeks of fertilizations with either 1.5625mM, 3.12mM, 6.25mM, 12.5mM and 25mM of ammonium nitrate).

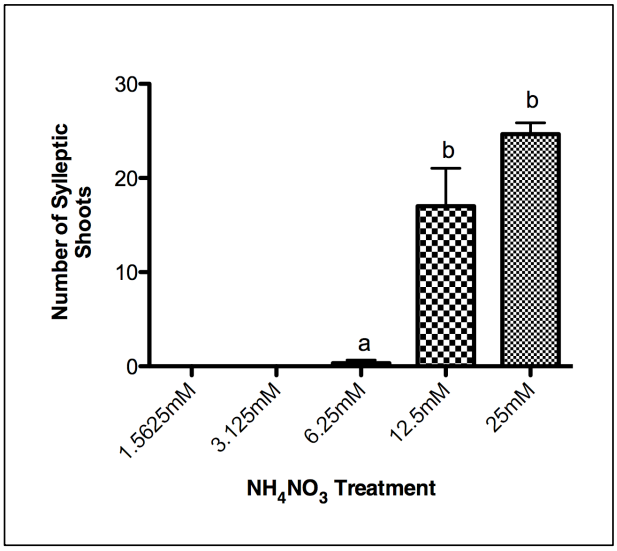

Figure 2.4: N availability influences sylleptic shoot production on poplar. Error bars display the means of five biological replicates ± standard error. Means assigned the same letter among N treatments are not significantly different (P<0.05).

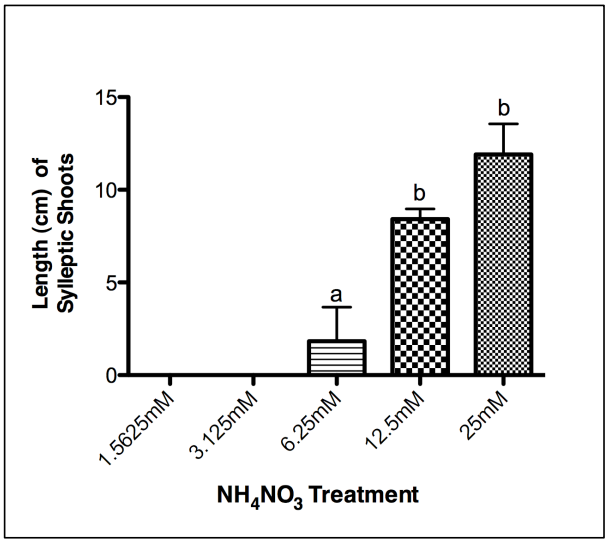

Figure 2.5: N availability influences poplar sylleptic shoot elongation. Error bars display the means of five biological replicates ± standard error. Means assigned the same letter among N treatments are not significantly different (P<0.05).

Discussion

Nitrogen induction of leaf expansion and stem growth is dependent on shoot developmental stage

Previous studies have demonstrated that differences in N abundance can change poplar phenotypes by affecting multiple growth parameters. The results of the current research confirm that the phenotype plasticity related to N availability observed in other poplar species and genotypes also occur in *Populus tremula X Populus alba* clone INRA 717 IB4. Results from this study showed N availability significantly affected poplar growth. Luxuriant N levels (12.5mM or 25mM NH_4NO_3) significantly increased leaf area of this poplar genotype. This is consistent with the results reported by Cooke et al., (2005), who investigated the developmental responses to N availability in hybrid *Populus balsamifera* ssp. *trichocarpa* x *deltoides*. Although the concentrations of N treatments and duration of experiment were different, their results showed that trees fertilized with higher N levels produced leaves with larger leaf area after 28d of N treatment. Hacke et al., (2010) have also shown that poplars fertilized with higher N concentration produced larger leaf areas than those with lower N.

The data from this study indicated a dosage effect between different levels of N treatment and their ability to increase poplar leaf area. Although increasing the N levels increased the poplar leaf area, the results of N treatment indicated that differences in leaf area related to N abundance are dependent on the developmental stage of the leaves. N availability did not influence the leaf area of the younger leaves that were nearest to the apex; however N availability influenced the leaf size of

the older leaves. Suggesting that besides the final leaf area N availability also seem to have influenced the rate of expansion of leaves. Cooke et al., (2005) reported increases in leaf area in the zone of leaf expansion of poplar treated with adequate N. Their study did not show significant differences in leaf area between N treatments at the developing leaf zone of poplar trees; however, the differences in leaf area between N treatments were measured at the zone of leaf expansion on poplar stems. These differences observed between the developing and expanding leaves in response to the differences in N treatments suggested that N is affecting the rate of leaf expansion in poplar trees. Since the leaf size of plants are affected by cell division and cell expansion it is probable that N availability influenced the factors that regulated the rate of cell division and cell expansion in the poplar trees (Gonzalez et al., 2012). The results indicated that in the presence of high N availability, poplar allocated nutrient to producing larger leaves. Larger leaf area will correspond to increased photosynthetic rate and enhanced carbon assimilation for plant metabolism and growth (Li et al., 2012).

Previous studies have shown that poplar stem volume (plant diameter and height growth) was related to total leaf area because poplar species that produced larger total leaf area also had larger stem volume (Ridge et al., 1986). The current studies agreed with previous studies because increasing N fertilization also increased poplar stem diameter. There were no differences in stem diameter among N treatments at LPI-5, this observation was similar to leaf area measurement of the younger poplar leaves nearest to the apex (LPI 1-4). The stem diameter at LPI-5 might represent the developing zone where the poplar stem growth had not yet

transitioned to secondary cambium growth (Cooke et al., 2005; Pitre et al., 2007a) indicating that this stem position was less influenced by increased N treatments. Differences in stem diameters in response to N availability were observed at stem positions corresponding to LPI-15, -25 and at the base of the plant and these positions are likely to be located within regions where secondary stem growth had occurred (Pitre et al., 2007a). Coleman et al., (1998) examined the growth of aspen supplied with different fertilizer addition rates and observed that stem volume increased with increasing rates of fertilization, although, their measurements of stem diameter only consisted of the basal stem position and did not include measurements from other stem positions. Increased N levels may be influencing the stem diameter by affecting the size of the xylem fibers, xylem vessels, and xylem area-specific conductivities which are important in supporting the larger leaves produced on poplar stems (Hacke et al., 2010; Pitre et al., 2007a).

N availability increased sylleptic branch production on poplar

One of the important results of this study is the significant effect of N availability on the initiation and growth of sylleptic shoots. The results from this experiment showed that luxuriant levels of N (12.5mM and 25mM NH_4NO_3) produced the most sylleptic shoots in *Populus tremula X Populus alba* clone INRA 717 IB4. Cooke et al., (2005) also showed increased N availability contributed to sylleptic shoot production on poplars. Novaes et al., (2009) observed significantly increased sylleptic growth on poplars fertilized with 25mM NH_4NO_3 after four weeks of vegetative growth. The higher number of syllepsis produced by trees with luxuriant N indicated that, *Populus* increased their overall growth by producing

sylleptic branches when this nutrient is in abundance because N allocation to sylleptic shoot production can enhance tree growth (Dillen et al., 2010) by increasing crown leaf area index (Ceulemans et al., 1990). Additionally, sylleptic shoots enhance tree growth by translocating a greater proportion of photosynthate to the main tree stem and roots compared to proleptic shoots (Scarascia-Mugnozza et al., 1999). Since most of the sylleptic shoots were produced on poplars fertilized with two highest N levels, this dramatic effect of N suggested that there is a threshold level of N between 6.25mM and 12.5mM ammonium nitrate that must be reached above which results in sylleptic branch growth in *Populus tremula X Populus alba* clone INRA 717 IB4. Thus the availability of resources and in particular N is an important factor for sylleptic shoot production in poplar (Wu and Hinckley, 2001).

In this experiment N availability significantly influenced *Populus tremula X Populus alba* clone INRA 717 IB4 phenotypic plasticity. Different shoot traits of this poplar species were identified that were affected by differential N availability. Although N availability affected shoot growth traits, this effect was dependent on the shoot developmental stage and sylleptic branch elongation indicated a threshold level of N requirement.

Chapter 3: Sink competition for storage reserves

Vegetative storage proteins (VSP) in woody plants

Perennial plants have evolved adaptive traits for survival in different environmental conditions. Internal seasonal N cycling is among these traits and contributes to fitness in nutrient limited environments. Studies have shown that temperate deciduous trees conserve leaf N during leaf senescence by transporting and storing leaf N in perennial tissues such as bark and roots as vegetative storage proteins (VSP) (van Cleve and Apel, 1992; Coleman et al., 1991; Langheinrich and Tischner, 1991; Pregitzer et al., 1990; Sauter and Cleve, 1990; Wetzel et al., 1989). VSP are storage reserves synthesized when N is abundant and can subsequently be catabolized and the N can then be remobilized to support growth (Cooke and Weih, 2005). VSPs have been identified in several woody plant species including both angiosperms and gymnosperms (Gomez and Faurobert, 2002; Marie-Odile Jordan et al., 2012; Sauter and van Cleve, 1993; Wetzel and Greenwood, 1989; Wetzel et al., 1989). Studies that examined the partitioning of stored N during new shoot growth have established a possible linkage between stored N remobilization and shoot growth (Cheng and Fuchigami, 2002; Millard and Grelet, 2010; Neto et al., 2008).

Induction of bark storage proteins (BSP)

Beginning in late summer, the majority of leaf N is resorbed from senescing leaves of temperate deciduous trees and transported as glutamine (Sauter and van Cleve, 1993) to bark, wood and roots where it is eventually used in the synthesis of VSP (Bollmark et al., 1999; Clausen and Apel, 1991; van Cleve and Apel, 1992; Coleman

et al., 1991, 1993; Pregitzer et al., 1990; Sauter and van Cleve, 1993). Bark storage protein (BSP) is the most abundant VSP that accumulates in poplar bark and serves as a seasonal storage reserves (Cooke and Weih, 2005; Pettengill et al., 2013). Expression of genes encoding poplar BSP is regulated by environmental factors including photoperiod, low temperature (LT) and N availability (van Cleve and Apel, 1992; Coleman et al., 1991). Because VSP of temperate deciduous trees accumulate in the autumn and then disappear in the spring during new growth it is assumed that N is remobilized from stored VSP and utilized by the new expanding shoots (Sauter and van Cleve, 1993; Wetzel et al., 1989).

Although it is generally believed that seasonally stored N is utilized during renewed shoot growth research directed at defining the contribution of stored N to new shoot growth is lacking. The aim of this study was to further define the importance and contribution of stored N to spring shoot growth. There are two approaches that can be taken to answer this question and enhance our knowledge of the contribution of stored N to shoot growth. One approach would be to manipulate or alter the number of potential N sinks (i.e. expanding shoots) while holding N reserves constant. If a fixed amount of N reserves is partitioned to an increasing number of sinks or expanding shoots then it would be expected that as reserves becoming limiting then shoot growth would be affected. A second and complimentary approach would be to keep the number of N sinks or expanding shoots fixed but manipulate the N storage pool. In this study, the first approach was utilized in poplar to better define the role of N storage reserves to shoot growth. Understanding the function of N storage reserves to shoot growth is knowledge that is

necessary to develop strategies to manipulate or enhance N remobilization and altering plant productivity.

Materials and Methods

Plant growth

Two different poplar genotypes, *Populus trichocarpa* clone Nisqually and *Populus tremula X Populus alba* clone INRA 717 IB4 were used for this study. *Populus trichocarpa* plants were established from softwood cuttings rooted in rooting cubes in the University of Maryland research greenhouse complex mist room and *Populus tremula X Poplulus alba* were propagated *in vitro* in ½ strength LS media and rooted plants were transferred and acclimated in standard cell trays (50 cells per tray). Rooted or acclimated plants were transferred to 6.0L pots containing soilless media (Sunshine LC1). Plants were initially grown in a LD (16 h light, 8 h dark) greenhouse for six weeks and fertilized once at the beginning of LD growth with 2g of 30-day controlled release fertilizer (18-3-3, Nutricote). BSP accumulation and bud dormancy was induced by transferring plants to SD (8 h light, 16 h dark 20°C) using a 160ft^2-controlled environment chamber (EGC, Chagrin Falls, OH). After 6 weeks of SD treatment, plants were further treated with SD supplemented with low-temperatures (LT) (10°C light, 4°C dark) for 8 weeks for and additional 8 weeks to induce leaf senescence and abscission. After 8 weeks of SD combined with LT leaf senescence and abscission was complete and plants were moved to refrigerated storage (4°C) with continuous darkness for 10 weeks to provide sufficient chilling to release bud dormancy. Following the 10-week LT treatment plants were treated as described in the next section.

Sink treatments

After LT treatment to release buds from dormancy, plants of *Populus tremula* X *Populus alba* clone INRA 717 IB4 were treated by removing the apical bud and either retaining all of their lateral buds (intact) or removing all but 1 lateral bid (single lateral) which was located at the mid-point of the stem. Plants of uniform height were selected for treatment and the intact plants averaged 36.8 lateral buds and varied from a minimum of 33 lateral buds to a maximum of 39 lateral buds.

For *Populus trichocarpa* (Nisqually) plants following LT release of bud dormancy the apical bud was removed and plants were randomly assigned to one of the various treatments used to compare sink competition between lateral shoots, sylleptic shoots or between lateral and sylleptic shoots. To compare the effect of different sink numbers on resulting shoot growth from lateral buds, plants were treated such that they either consisted of 1,3, 6, 9 or 12 lateral buds by surgically removing the remaining lateral buds appropriate for each treatment. In addition, all sylleptic shoots were also surgically removed. To determine the effect of sink competition between sylleptic shoots on shoot growth the apical and lateral buds of *Populus trichocarpa* (Nisqually) plants were surgically removed and plants were treated such that they either consisted of 1, 5 or 10 sylleptic shoots. The effect of sylleptic shoot competition on the growth of lateral shoots was also examined. These treatments consisted of either 1 or 10 lateral buds left intact combined with either 1 or 10 sylleptic shoot. This resulted in 4 different treatments, 1 lateral shoot and 1 sylleptic shoot, 1 lateral shoot and 10 sylleptic shoots, 10 lateral shoots and 1 sylleptic shoot and 10 lateral shoots and 10 sylleptic shoots. The effect of lateral shoot

competition on sylleptic shoots was evaluated by treating plants such that they consisted of 1 or 10 sylleptic shoots with either 1 or 10 lateral buds. Similar to the previous treatments, this regime resulted in 4 different treatments, 1 sylleptic shoot and 1 lateral shoot, 1 sylleptic shoot and 10 lateral shoots, 10 sylleptic shoots and 1 lateral shoot and 10 sylleptic shoots and 10 lateral shoots.

Following plant manipulation to create the various treatments, plants were placed in a LD greenhouse and after 5 weeks various shoot growth attributes were measured. For experiments focused on lateral shoot growth these attributes included leaf number, leaf area, stem length, number of new sylleptic shoots and length of new sylleptic shoots. For experiments focused on sylleptic shoot growth the attributes consisted of leaf area, number of sylleptic and length of sylleptic shoots.

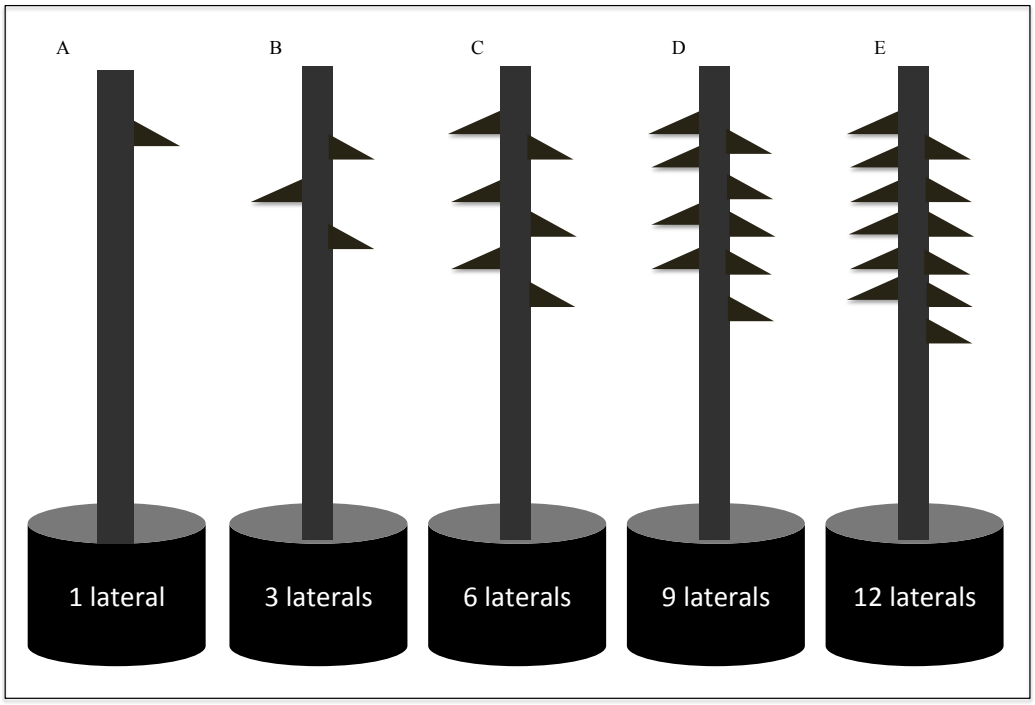

Figure 3.1 Representation of trees treated as A) 1, B) 3, C) 6, D) 9, or E) 12 lateral shoots before renewed shoot growth

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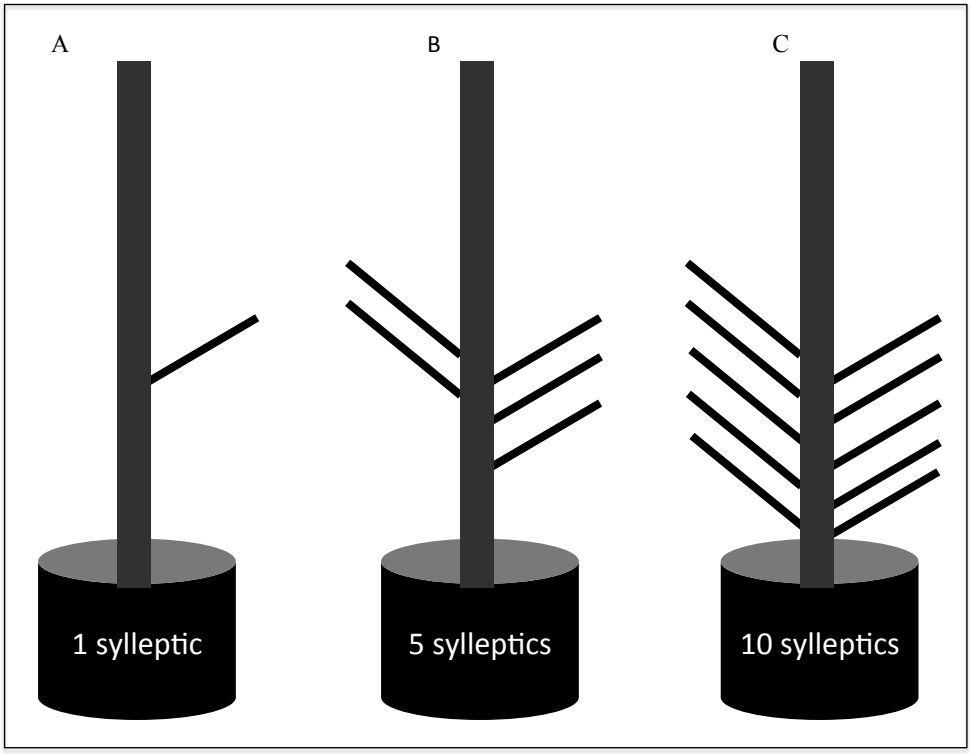

Figure 3.2. Diagrammatic representation of trees treated as A) 1, B) 5, or C) 10 lateral shoots before renewed shoot growth

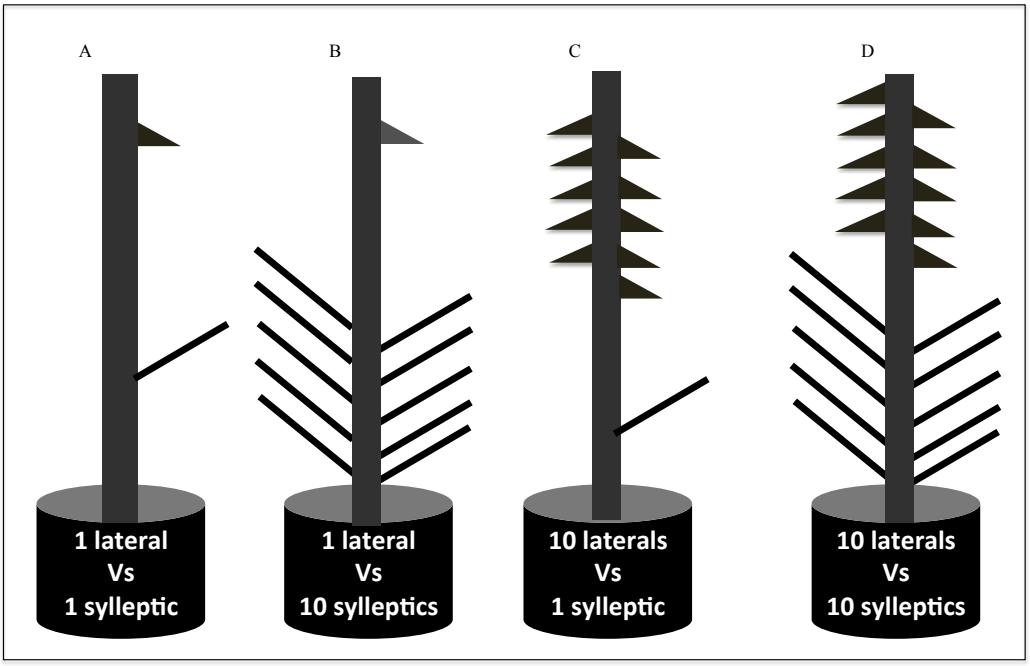

Figure 3.3. Diagrammatic representation of tree manipulation for determination of sylleptic shoot effect on lateral shoot growth and lateral shoot effect on sylleptic shoot growth before LD exposure. A) 1 lateral and 1 sylleptic shoot, B) 1 lateral and 10 sylleptic shoots, C) 10 lateral and 1 sylleptic shoot or D) 10 lateral and 10 sylleptic shoots.

Results

Growth responses to lateral shoot competition

Populus tremula X *Populus alba* plants with a single shoot produced from a single lateral bud had significantly greater mean leaf area (132.2 cm^2 for plants with single bud versus 16.87 cm^2 for intact plants) (Figure 3.4). In addition to leaf area, other growth parameters including stem length (Figure 3.5) (56.44 cm^2 for plants with single bud versus 20.41 cm^2 for intact plants), leaf fresh weight (Figure 3.7) (2.446 g for plants with single bud versus 0.2106 g for intact plants), stem fresh weight (Figure 3.8) (22.9 g for plants with single bud versus 0.8309 g for intact plants), stem dry weight (Figure 3.9) (5.174 g for plants with single bud versus 0.2558 g for intact plants) and number of sylleptic shoots produced (Figure 3.6) (4.6 for plants with single bud versus 0 for intact plants) were also significantly greater for plants with a single growing shoot compared to plants (intact) with multiple shoots (figures 3.4 and 3.5).

Similar to the results with *Populus tremula* X *Populus alba* clone INRA 717 IB4, altering shoot sink numbers in *Populus trichocarpa* Nisqually also resulted in significant changes in shoot growth after 5 weeks of renewed shoot growth. For the majority of growth traits measured, increases in the number of shoots and therefore increased sink competition, resulted in reduced leaf area, shoot length, sylleptic shoot production and growth of sylleptic shoots (Figures 3.10, 3.11 and Tables 3.1, 3.2). Maximum mean leaf area (97.24 cm^2) was observed in those plants with a single growing shoot (i.e 1 lateral bud). As the number of shoots increased the mean leaf area decreased to 58.03 cm^2, 42.99 cm^2, 34.73 cm^2 and 29.42 cm^2, for plants with 3,

6, 9, and 12 shoots, respectively (Figure 3.10). Besides leaf area, stem growth was also significantly influenced by the number of growing shoots present on the plant (Figure 3.11). Greatest stem growth (53.6 cm) was observed for the plants consisting of a single shoot. As the number of growing shoots increased, mean shoot length declined to 44.13 cm, 36.88 cm, 35.25 cm and 29.27 cm, for plants with 3, 6, 9, and 12 shoots respectively.

Similar to leaf area and stem growth, the production of sylleptic shoots also decreased as the number of growing shoots increased. The greatest number of sylleptic shoots (mean of 9.8 sylleptic shoots per plant) was observed for plants that contained a single shoot. The mean number of sylleptic shoots decreased to 7.0, 3.6, 2.4, and 0.8 for plants with 3, 6, 9 and 12 shoots respectively. Although the number of sylleptic shoots produced per treatment declined as shoot numbers increased, the growth of the sylleptic shoots was less affected by shoot numbers and significant difference were only observed between plants with a single shoot (mean shoot length 53.6 cm) and plants with 3 shoots (mean shoot length 44.13 cm). There were no significant differences in sylleptic shoot growth of plants with a single shoot versus those with 6, 9 or 12 shoots (Table 3.2).

To further determine if shoot competition influenced the growth of shoots, the leaf area of the uppermost shoot across treatments was compared. This allowed for a comparison of the effect of shoot treatments on the growth of a common shoot across treatments (Figure 3.12). This analysis revealed that the mean leaf area of the uppermost shoot was greatest in those plants with a single shoot (mean of 97.24 cm^2)

and was significantly reduced in plants with 3,6, 9 or 12 shoots (62.42 cm^2, 64.51 cm^2, 61.01 cm^2, 51.26 cm^2 respectively).

Further evidence of competition between shoots was also observed when comparing leaf area between shoot positions within each individual treatment (Figure 3.13). For plants with 3 shoots the mean leaf area was significantly greater for the first two shoots (62.42 cm^2 and 66.95 cm^2 respectively) compared to the basal shoots (44.72 cm^2). A similar pattern where leaf area was greater in the uppermost shoot compared to the remaining shoots was also observed in plants with either 6, 9 or 12 shoots (Figure 3.13).

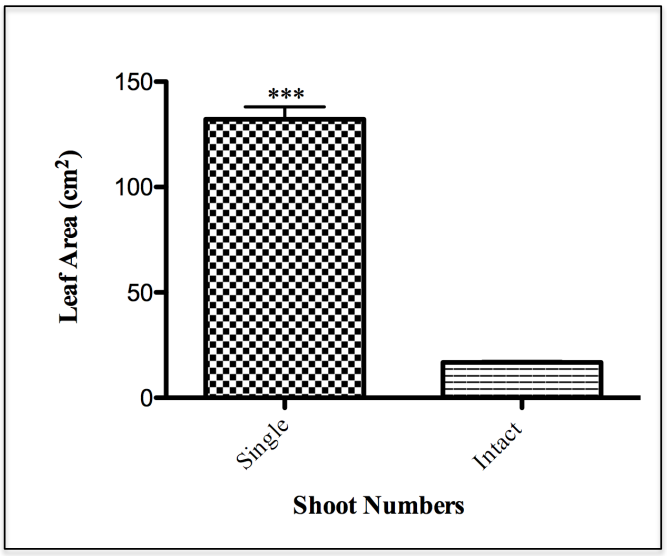

Figure 3.4 Mean leaf area (cm^2) after LD growth of *Populus tremula* X *Populus alba*, plants with a single lateral bud and plants with intact lateral buds. Values are means of five biological replicates (± standard error). Means assigned the same letter among N treatments are not significantly different (P<0.05).

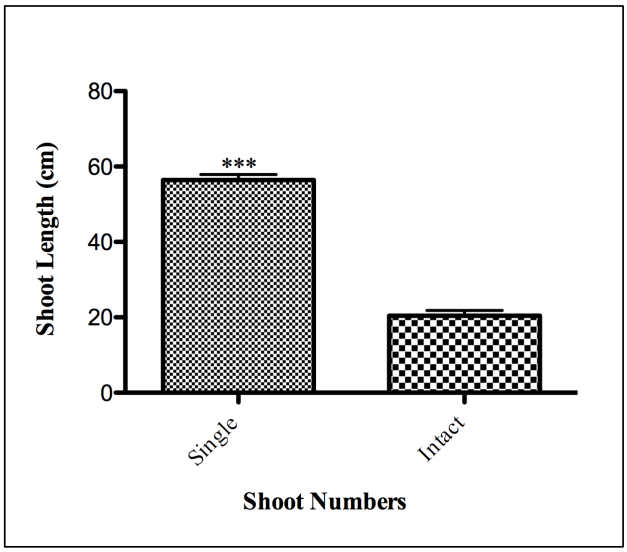

Figure 3.5 Mean shoot length (cm) after LD growth of *Populus tremula* X *Populus alba*, plants with a single lateral bud and plants with intact lateral buds. Values are means of five biological replicates (± standard error). Means assigned the same letter among N treatments are not significantly different (P<0.05).

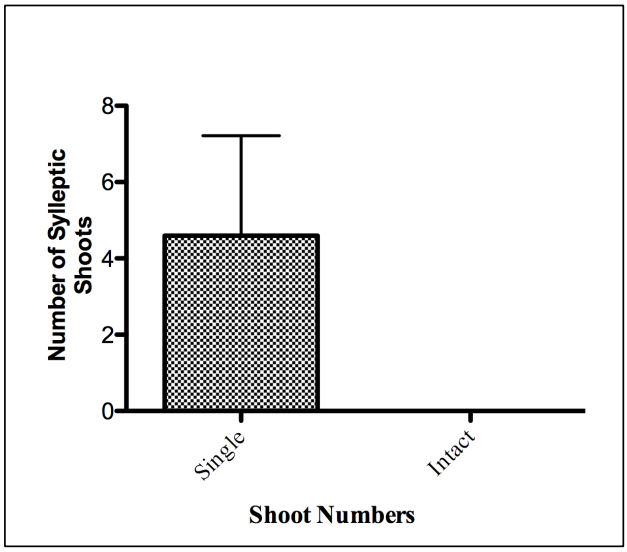

Figure 3.6 Mean number of sylleptic shoots after LD growth of *Populus tremula* X *Populus alba*, plants with a single lateral bud and plants with intact lateral buds. Values are means of five biological replicates (± standard error). Means assigned the same letter among N treatments are not significantly different (P<0.05).

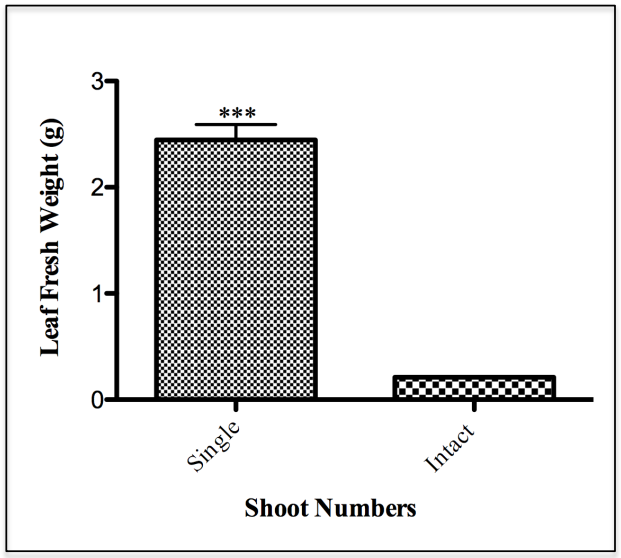

Figure 3.7 Mean leaf fresh weight (g) after LD growth of *Populus tremula* X *Populus alba*, plants with a single lateral bud and plants with intact lateral buds. Values are means of five biological replicates (± standard error). Means assigned the same letter among N treatments are not significantly different (P<0.05).

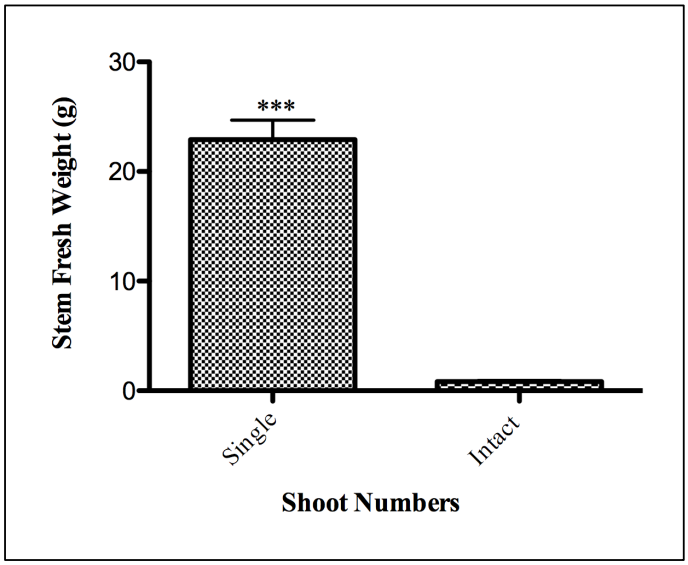

Figure 3.8 Mean stem fresh weight (g) after LD growth of *Populus tremula* X *Populus alba*, plants with a single lateral bud and plants with intact lateral buds. Values are means of five biological replicates (± standard error). Means assigned the same letter among N treatments are not significantly different (P<0.05).

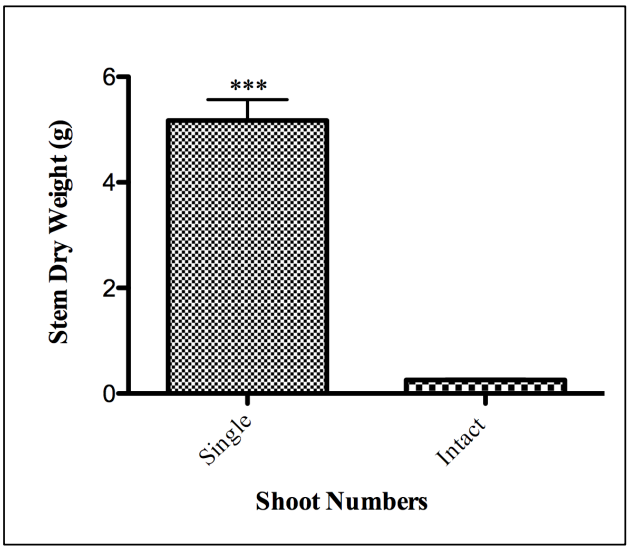

Figure 3.9 Mean stem dry weight (g) after LD growth of *Populus tremula* X *Populus alba*, plants with a single lateral bud and plants with intact lateral buds. Values are means of five biological replicates (± standard error). Means assigned the same letter among N treatments are not significantly different (P<0.05).

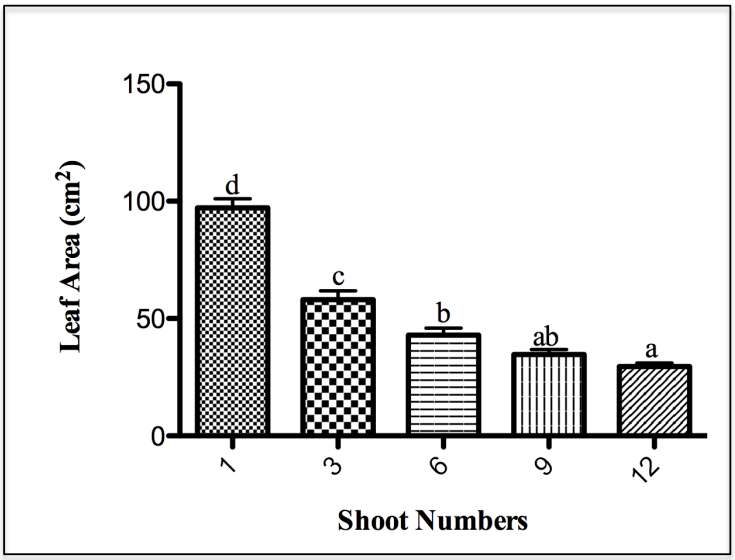

Figure 3.10 Mean leaf area (cm^2) of *Populus trichocarpa* Nisqually plants with either 1, 3, 6, 9 or 12 lateral shoots. Five biological replicates ± standard error. Means assigned the same letter among N treatments were not significantly different ($P<0.05$).

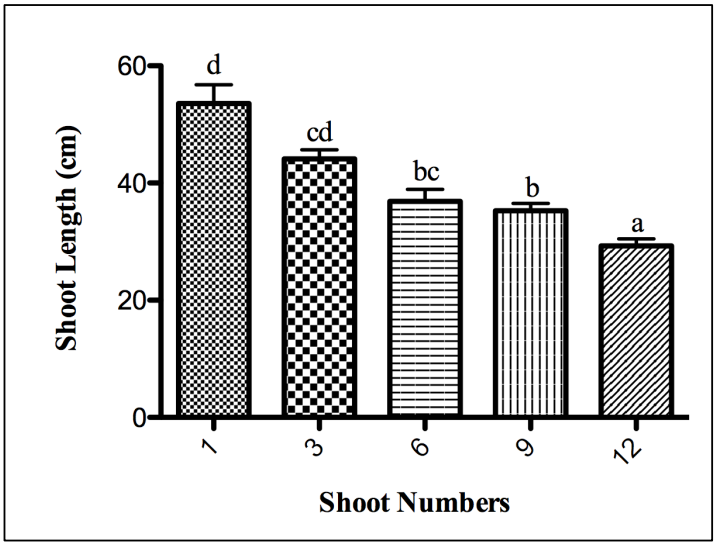

Figure 3.11 Shoot length (cm) of *Populus trichocarpa* Nisqually plants with either 1, 3, 6, 9 or 12 lateral shoots. Five biological replicates ± standard error. Means assigned the same letter among N treatments were not significantly different ($P<0$.

Table 3.1 Sylleptic shoots production in *Populus trichocarpa* Nisqually plants with either 1, 3, 6, 9 or 12 shoots. Five biological replicates ± standard error. Means assigned the same letter among N treatments were not significantly different (P<0.05).

Factor	Number of lateral shoots	Mean number of sylleptic shoots	*F*	*P*-value
Number of sylleptic shoots	1	9.8 (1.068)^{c}	12.99	<0.0001
	3	7.0 (1.549)^{bc}		
	6	3.6 (0.872)^{ab}		
	9	2.4 (0.748)^{a}		
	12	0.8 (0.490)^{a}		

Table 3.2 Sylleptic shoot growth (cm) in *Populus trichocarpa* Nisqually plants with either 1, 3, 6, 9 or 12 shoots. Five biological replicates ± standard erro. Means assigned the same letter are not significantly different (P<0.05).

Factor	Number of lateral shoots	Mean sylleptic length (cm)	*F*	*P*-value
Length of sylletpic shoots	1	8.747 (1.183)^{b}	4.051	0.0042
	3	3.501 (0.718)^{a}		
	6	4.738 (1.12)^{ab}		
	9	4.458 (1.091)^{ab}		
	12	5.775 (1.92)^{ab}		

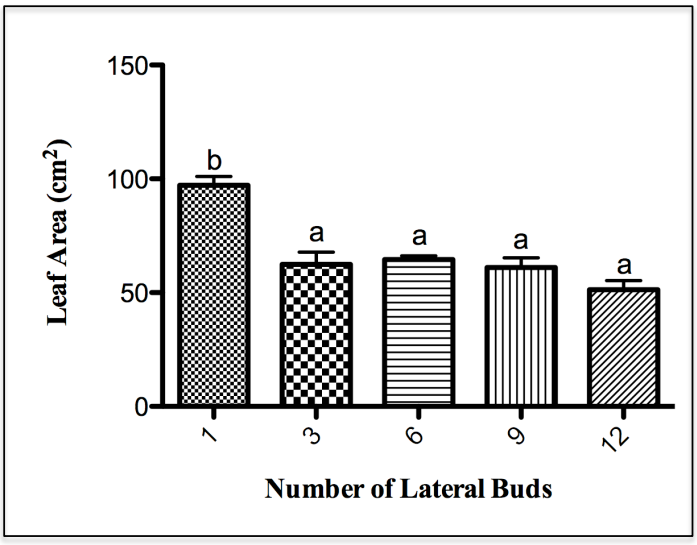

Figure 3.12 Mean leaf area ($cm^{2)}$ of the uppermost shoot of *Populus trichocarpa* Nisqually plants with either 1, 3, 6, 9 or 12 lateral shoots. Five biological replicates ± standard error. Means assigned the same letter were not significantly different ($P<0.05$).

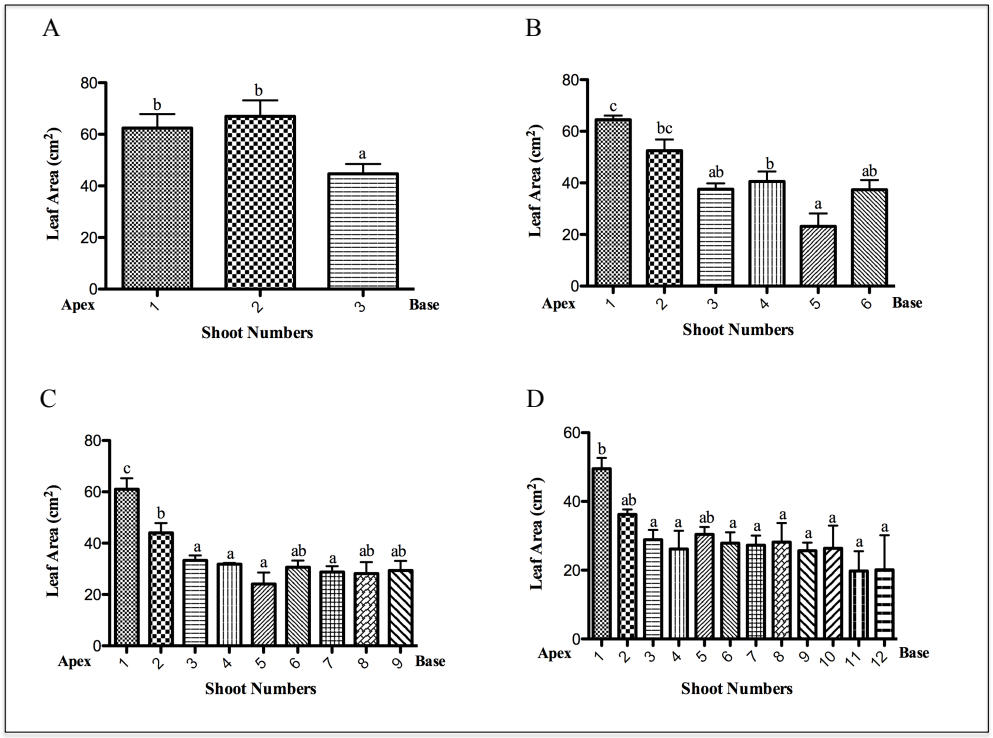

Figure 3.13 A to D, Mean leaf area of *Populus trichocarpa* Nisqually plants with either 3, 6, 9 or 12 lateral shoots; A) Mean leaf area of lateral bud 3 treatment, B) Mean leaf area of lateral bud 6 treatment, C) Mean leaf area of lateral bud 9 treatment, and D) Mean leaf area of lateral bud 12 treatment. Five biological replicates ± standard error. Means assigned the same letter were not significantly different ($P<0.05$).

Growth responses to sylleptic shoot competition

Populus trichocarpa clone Nisqually readily produces sylleptic shoots during growth and was used to determine if these shoots compete with each other by comparing the growth of sylleptic shoots following bud dormancy of plants with either 1, 5 or 10 sylleptic shoots (Figures 3.2 and 3.14, 3.15 and 3.16). Similar to the effects of lateral bud/shoot competition an increase in the number of sylleptic shoots significantly decreased mean leaf area from 35.17 cm^2 for plants with a single sylleptic to18.55 cm^2 and 14.68 cm^2 for plants with 5 or 10 sylleptics respectively (Figure 3.14). Increased sylleptic shoot number also tended to reduce the number of secondary sylleptic shoots produced (2.6, 0.2 and 0.6 for 1, 5 or 10 sylleptic shoots) (Figure 3.15) but had no effect on shoot growth (sylleptic shoot growth) (3.14 cm, 0.3 cm, 1.97 cm for 1, 5, or 10 sylleptic shoots respectively) (Figure 3.16).

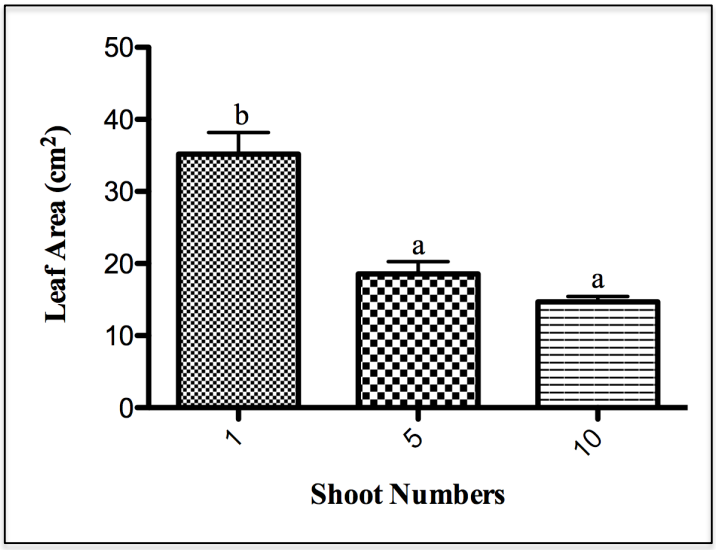

Figure 3.14 Mean leaf area (cm^2) after LD growth of *Populus trichocarpa* Nisqually plants with either 1, 5 or 10 sylleptic shoots. Five biological replicates ± standard error. Means assigned the same letter were not significantly different ($P<0.05$).

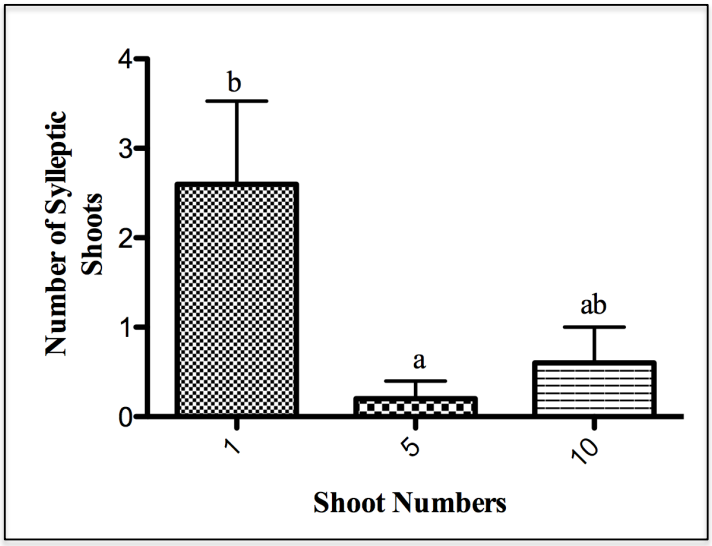

Figure 3.15 Mean number of sylleptic shoots after LD growth of *Populus trichocarpa* Nisqually plants with either 1, 5 or 10 sylleptic shoots. Five biological replicates ± standard error. Means assigned the same letter were not significantly different ($P<0.05$).

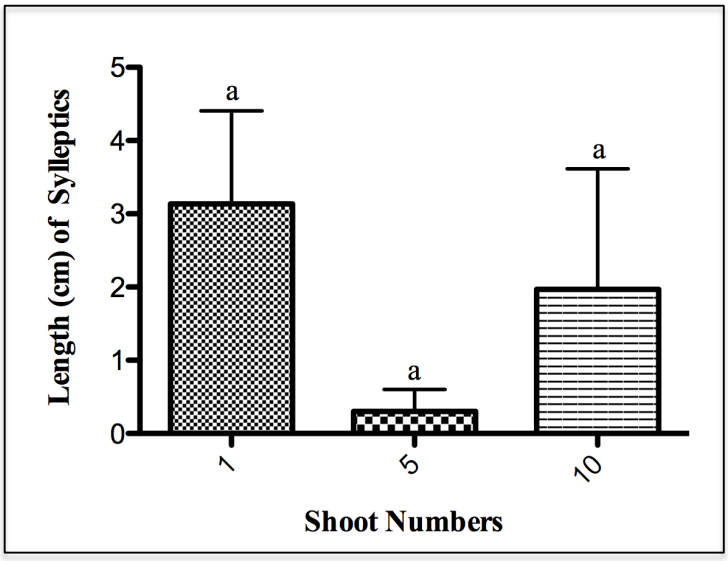

Figure 3.16 Mean length of sylleptic shoots after LD growth of *Populus trichocarpa* Nisqually plants with either 1, 5 or 10 sylleptic shoots. Five biological replicates ± standard error. Means assigned the same letter were not significantly different ($P<0.05$).

Effect of sylleptic shoot number on the growth of shoots of laterals

To determine if shoots arising from lateral buds and sylleptic shoots compete during growth following dormancy plants of *Populus trichocarpa* clone Nisqually were manipulated to consist of plants with either 1 or 10 shoots from lateral buds combined with either 1 or 10 shoots from sylleptic shoots (described in materials and methods) (Figures 3.3). These plants were then used to determine if the growth of shoots from lateral buds was influenced by the presence of sylleptic shoots (Figures 3.17 and 18). As seen previously, increasing the number of lateral shoots significantly reduced the mean leaf area from 76.83 cm^2 for plants with 1 lateral shoot and 1 sylleptic shoot to 31.47 cm^2 for plants with 10 lateral shoots and 1 sylleptic shoot and from 67.97 cm^2 for plants with 1 lateral shoot and 10 sylleptic shoots to 27.27 cm^2 for plants with 10 lateral shoots and 10 sylleptics (Figure 3.17). However mean leaf area of shoots from lateral buds was not significantly different between plants with 1 lateral bud and 1 sylleptic shoot compared to plants with 1 lateral shoot and 10 sylleptic shoots (76.83 cm^2 versus 67.97 cm^2). Likewise, leaf area of shoots from lateral buds were also not significantly different between plants with 10 lateral shoots and 1 sylleptic shoot compared to 10 lateral shoots and 10 sylleptic shoots (31.47 cm^2 versus 27.27 cm2). A similar lack of difference was also observed in stem growth of shoots from lateral buds (Figure 3.18). Consistent with previous results, increasing the number of shoots from 1 lateral bud to 10 lateral buds decrease mean shoot length from 57.12 cm to 31.07 cm in plants with 1 sylleptic shoot and from 54.96 cm to 27.34 cm of plants with 10 sylleptic shoots. Although increased lateral shoots reduced shoot growth, increase sylleptic shoot number had little effect on

mean shoot length of plants with 1 lateral shoot and either 1 sylleptic or 10 sylleptic shoots (57.12 cm versus 54.96 cm) or plants with 10 lateral shoots and either 1sylleptic or 10 sylleptic shoots (31.07 cm versus 27.34 cm). Further support of the lack of sylleptic shoot effects on the growth of shoots from lateral buds can be observed by examining the partitioning of variance by 2-way ANOVA (Tables 3.3 and 3.4). For both leaf area and stem length the effect of lateral bud number was significant ($p<0.0001$) while the number of sylleptic shoots or the interaction between sylleptic and lateral shoots was not significant.

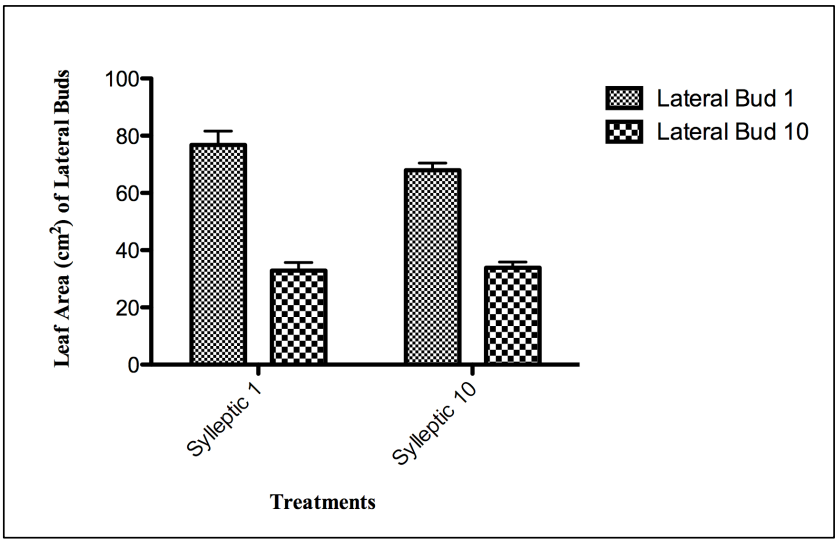

Figure 3.17 Comparison of lateral shoots mean leaf area (cm^2) in the presence of either 1 or 10 sylleptic shoots. Five biological replicates ± standard error ($P<0.05$).

Table 3.3 Two-way Analysis of Variance of the effect of sylleptic shoots on leaf area of *Populus trichocarpa* Nisqually plants with wither 1or 10 lateral buds.

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Interaction	1	121.4	121.4	2.400	0.1408
Lateral buds	1	7612	7612	150.5	<0.0001
Sylleptics	1	77.44	77.44	1.531	0.2338
Residual	16	809.1	50.57		

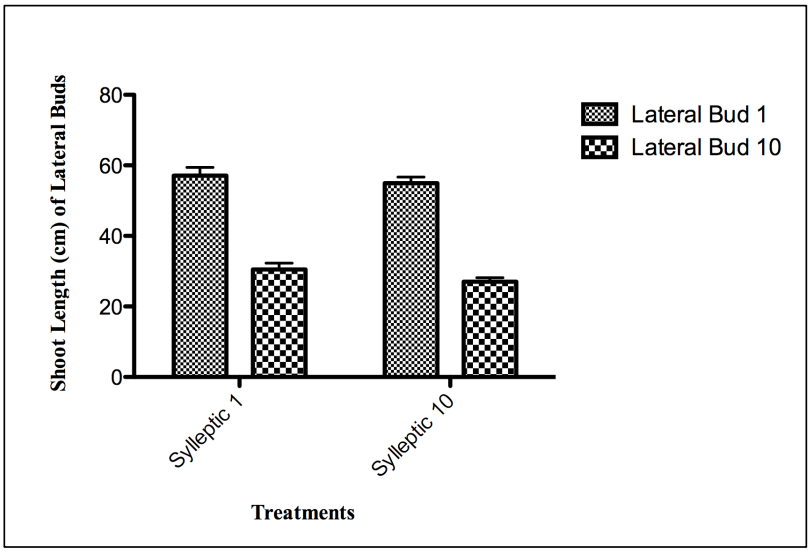

Figure 3.18 Comparison of lateral shoots mean shoot length (cm) in the presence of either 1 or 10 sylleptic shoots. Five biological replicates ± standard error ($P<0.05$).

Table 3.4 Two-Way Analysis of Variance of the effect of sylleptic shoots on shoot length of *Populus trichocarpa* with either 1 or 10 lateral buds.

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Interaction	1	2.010	2.010	0.1234	0.7300
Lateral buds	1	3719	3719	228.3	<0.0001
Sylleptics	1	39.03	39.03	2.396	0.1412
Residual	16	260.6	16.29		

Effect of lateral shoot number on growth of sylleptic shoots

To determine if sylleptic shoot growth was influenced by lateral shoot growth a reciprocal experiment was conducted where shoot growth was compared between plants with either 1 or 10 sylletic shoots and either 1 or 10 shoots from lateral buds (Figure 3.3). In contrast to the absence of an effect of sylleptic shoot number on the growth of shoots from lateral buds, the growth of sylleptic shoots was significantly reduced by an increase in lateral shoot number (Figures 3.19 and 20). The mean sylleptic shoot leaf area of plants with 1 sylleptic shoot decreased from 29.2 cm^2 for plants with 1 lateral shoot to 11.71 cm^2 for plants with 10 lateral shoots (Figure 3.19). Although mean sylleptic shoot leaf area also declined for plants with 10 sylleptic shoots and 1 lateral buds (14.26 cm^2) compared to plants with 10 lateral shoots (10.87 cm^2), the decrease was not as great as that observed for plants with 1 sylleptic shoot. Similar to leaf area, the number of shoots from lateral buds also significantly influenced the stem length of sylleptic shoots (Figure 3.20). Mean shoot length of plants consisting of 1 sylleptic shoot decreased from 25.41 cm in plants with 1 lateral shoot to 7.969 cm in plants with 10 lateral shoots. A similar pattern of reduced shoot growth was also observed for plants with 10 sylleptic shoots where stem length declined from 15.5 cm for plants with 1 lateral shoot to 7.696 cm for plants with 10 lateral shoots. Further conformation of the effect of shoots from lateral buds on the growth of sylleptic shoots comes from examining the partitioning of variance by 2-way ANOVA (Tables 3.5 and 3.6). For sylleptic shoot leaf area the number of sylleptic shoots ($p<0.0001$) and lateral shoots ($p<0.0007$) had a significant effect and the interaction between these two variables was also significant ($p<0.0017$). A

similar pattern was observed for sylleptic stem length with both lateral shoot number ($p< 0.0166$) and sylleptic shoot number ($p<0.0001$) and their interaction ($p<0.0223$) having significant effect.

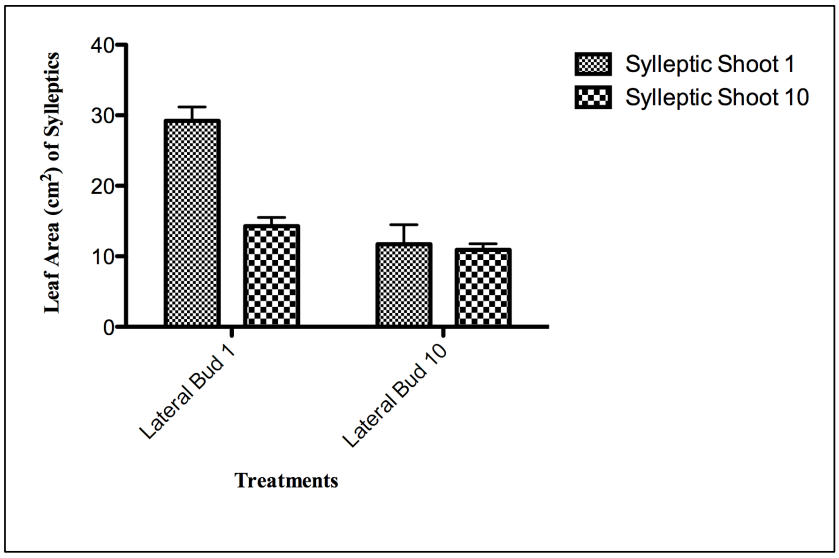

Figure 3.19 Comparison of sylleptic shoots mean leaf area (cm^2) in the presence of either 1 or 10 lateral shoots. Five biological replicates ± standard error ($P<0.05$).

Table 3.5 Two –Way Analysis of Variance of the effect of lateral buds on the leaf area of *Populus trichocarpa* Nisqually plants with either1 or 10 sylleptic shoots.

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Interaction	1	248.8	248.8	14.12	0.0017
Lateral buds	1	311.4	311.4	17.68	0.0007
Sylleptics	1	545.5	545.5	30.96	<0.0001
Residual	16	281.9	17.62		

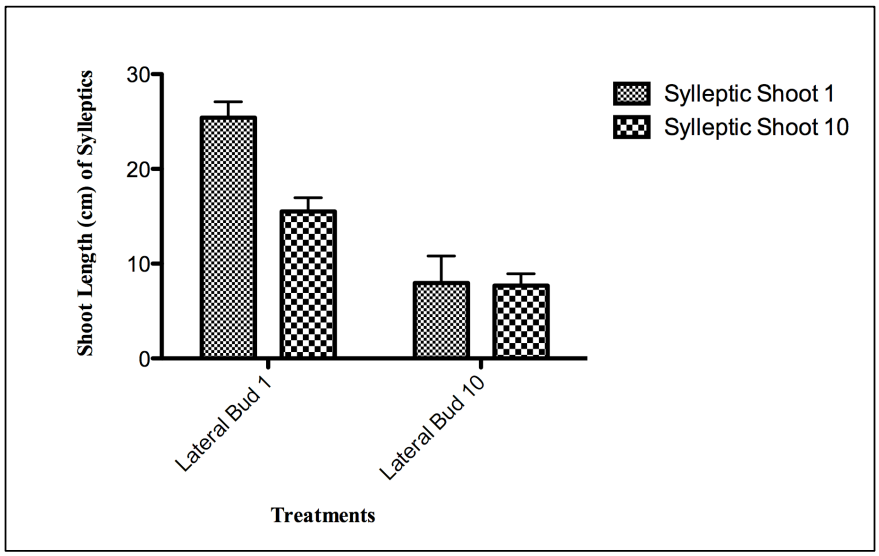

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Figure 3.20 Comparison of sylleptic shoots shoot length (cm) in the presence of either 1 or 10 lateral shoots. Five biological replicates ± standard error ($P<0.05$)

Table 3.6 Two-Way Analysis of Variance of the effect of lateral buds on shoot length of Populus trichocarpa with either 1 or 10 sylleptic shoots.

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Interaction	1	116.1	116.1	6.402	0.0223
Lateral buds	1	129.6	129.6	7.148	0.0166
Sylleptics	1	797.1	797.1	43.95	<0.0001
Residual	16	290.2	18.13		

Discussion

Increasing the number of sink tissues reduced poplar shoot growth

Remobilization of stored N has been shown to contribute to shoot growth in the absence of a current N supply during spring growth. In a study in apple (*Malus domestica* Borkh) trees Cheng and Fuchigami, (2002) investigated the effect of current N supply in the spring on utilization of N reserved from previous-year N fertilization during spring growth and found total leaf area of trees in spring increased with increasing N supplied the previous year. They estimated that the current N supply for spring growth slightly but significantly increased total leaf area to about 10%. Their data indicated stored N sets the potential for initial spring growth by contributing significantly to N economy during new shoot growth (Cheng and Fuchigami, 2002). Millard and Proe, (1993) also showed the importance of N storage to spring growth by examining whether N remobilization in the spring was preconditioned by the size of the N store or whether it was subject upon the spring N supply in *Picea sitchensis*. They observed that the amount of N remobilized to new growth was dependent on the amount of N provided in the previous year, but the amount of remobilized N was not affected by the current-year's N supply. Additional studies have also shown that N remobilization from storage reserve occurred before N uptake from soil in *Betula pendula* (Millard et al., 1998). Studies in *Populus* have focused on characterizing N resorption from leaves and storage in perennial organs (Cooke and Weih, 2005) and other studies have shown that seasonal stored N is utilized first before N uptake from soil during spring growth by measuring the timing of ^{15}N enrichment in the xylem sap (Millard et al., 2006). However, no actual

measurements have been done on how seasonal stored N is utilized for poplar spring growth with respect to the growth of lateral and sylleptic shoots/branches.

Results from the current study agreed with previous studies on the importance and contribution of stored N to spring shoot growth. Data from 717 lateral shoot experiments, Nisqually lateral shoot and sylleptic shoot experiments showed seasonally stored reserve contributed to spring growth, in addition results showed competition from multiple sinks significantly reduced poplar shoot growth. Because no additional nutrients were provided to the plants during renewed growth, it is likely that the effects of shoot numbers on growth is a consequence of competition between shoots for stored nutrients. It is possible that increasing shoot numbers on trees limited the available nutrient for each individual shoots, which reduced shoot growth traits resulting in reduced leaf area and shorter stem length. This reduced shoot growth coincided with the phenotype seen in poplars when supplied with limited levels of N (Cooke et al., 2005; Pitre et al., 2007a). Jordan et al., (2011) who evaluated the effect of fall N supply on N uptake, storage and cycling during spring growth of peach (*Prunus persica* Batsch, cv. GF305) trees and found that N reserves were used to support the initial growth flush and differences in the N storage pool contributed to differences in biomass accumulation of shoots and fruits. Nutrient storage also appears to be important in coniferous species since pruning of needle branches of *Pinus cembra* L. showed enhanced growth of current-year needles, although nonstructural carbohydrate concentration was reduced during the growing season (Li et al., 2002).

Analysis of growth traits from this study revealed shoot competition across lateral shoot treatments during renewed shoot growth following dormancy influenced growth of poplars by significantly reducing the mean leaf area of the apical shoots as the number of shoots per plant increased, suggesting that those multiple shoots were in competition for stored reserves. Although increasing sink numbers reduced the uppermost shoot leaf area relative to poplars with a single shoot, data showed competition within lateral shoots treatments increased the growth of the uppermost shoots compared to the lower lateral shoots. Competition resulted to the uppermost shoots becoming stronger sinks for stored nutrient, which could be due to a higher rate of metabolism in the uppermost shoots. Competition between shoots has been observed to result in establishment of shoot dominance and vigorous growth of some shoots, but slower development in other shoots of *Salix* (Remphrey and Pearn, 2006). The ability of the uppermost shoots to become stronger sink tissues resulted in apical control, which is when lateral branches in the upper regions of the stem reduce shoot growth of the lower lateral branches by limiting hormones, water or available assimilate needed for growth (Wilson, 2000). Previous studies in poplar have shown evidence that the uppermost shoots limit the available nutrient allocated to the lower branches which reduces the growth of the lower shoot branches (Cline et al., 2008).

Increasing sink competition reduced sylleptic shoot production on poplar

Sink competition studies from 717 and Nisqually experiments showed sylleptic shoot initiation was affected by nutrient competition. Previous studies have showed that in the midst of high N availability poplar trees allocated nutrient to sylleptic shoot production, however when poplar trees were fertilized with low N

sylleptic shoot production was reduced (Cooke et al., 2005). The current study on sink competition for stored nutrient showed that limiting seasonal stored nutrient availability by increasing the number of sink tissues influenced sylleptic shoot production in a manner that was influenced by N availability. When nutrient is in abundance as in the case for the trees with a single shoot, poplars were able to allocate nutrient to producing sylleptic shoots, however when nutrient was limiting as in the case of poplars with many shoots, there were fewer sylleptic shoots produced. These results indicated that N is the seasonally stored nutrient that limited poplar shoot growth. Another factor that may have regulated the outgrowth of sylleptic shoots on poplar during spring growth is the phytohormone auxin (Cline and Dong-Il, 2002; Cline and Harrington, 2007; Wilson, 2000). Research studies in poplar have shown that basepital transport of auxin from growing shoots repress the growth of lateral buds (Cline and Dong-Il, 2002). Simulation models by Prusinkiewicz et al., (2009) suggested 'successively activated lateral shoots export auxin at a high rate and thus inhibit activation of buds below them.' This proposed mechanism of auxin inhibition of lateral bud outgrowth indicates that the high rate of auxin transport in poplar trees with multiple shoots inhibited the growth of lateral buds produced during the renewed shoot growth thereby reducing the number of sylleptic shoot growth on poplar trees.

Lateral shoots exhibit greater sink competition compared to sylleptic shoots

The effect of shoot competition between different shoots types (lateral and sylleptic) may indicate that competition between these shoot types is somehow different. Measurement of shoot related traits of lateral branches of plants that

consisted of 1 or 10 lateral shoots with plants possessing 1 or 10 sylleptic shoots showed the mean leaf area and shoot length of lateral shoots was not significantly influenced by the presence of either 1 or 10 sylleptic branches. This suggested sylleptic shoots exerted competition on other sylleptic shoots but do not compete with lateral shoots for stored reserve during spring growth. In contrast, the interaction between sylleptic and lateral shoots displayed by 2-way ANOVA analysis of sylleptic shoot growth, suggested that lateral shoots not only competed with lateral shoots, but lateral shoots also competed with sylleptic shoots for stored nutrient during spring growth. The mean leaf area and mean shoot length were significantly reduced when increased number of lateral shoots competed with sylleptic shoots. This indicated that lateral shoots are likely to out-compete sylleptic shoots for stored reserves, including N, during spring growth.

These differences in growth between lateral and sylleptic shoots indicated the different shoots have different N sink strengths. Lateral shoots appeared to be stronger sinks, or can outcompete sylleptic shoots for storage reserves during spring growth. This may also suggest that poplar may preferentially allocate a greater proportion of storage reserves for lateral shoot elongation during spring growth. In poplar the total branch biomass of lateral (proleptic) shoots is greater than sylleptic shoot growth during most growing seasons (Ceulemans et al., 1990). This differential allocation of nutrient reserves in poplar could be influenced by the branch position of lateral shoots relative to sylleptic branches (Sprugel, 2002). In the current study of lateral and sylleptic shoots competition for stored reserve, the sylleptic branches were located near base of the tree while the lateral branches were nearer to the apex. This

high position of the lateral shoots may have contributed to the higher proportion of resources allocated to these branches for growth. In *Cleyera japonica*, the growth of shoots differed based on their vertical position locations where the tree upper crown produced longer shoots compared to the lower crown (Suzuki, 2002). In a crown architectural study in *Salix* that followed the fate of buds and shoots development. It was observed that self pruning of branches drastically reduced the number of shoots located closest to the base of the tree (Remphrey and Pearn, 2006). In addition, the differences in growth between sylleptic and lateral shoots could also be that remobilization of stored nutrient is a localized event where the main stem stored reserve is utilized for lateral shoot growth while the nutrient stored in the short sylleptic stems is utilized for poplar sylleptic shoot growth.

Seasonally stored nutrient reserves contributed significantly to spring shoot growth of poplar and shoot competition from multiple sinks resulted to differences in shoot growth. The growth parameters measured during renewed shoot growth were influenced by limiting storage reserves in a manner that were influenced by N availability suggesting that N is the limiting nutrient in the storage reserves. These studies also indicated that lateral shoots and sylleptic shoots have differential sink strengths for stored N during spring growth. These results have further enhanced our knowledge of how stored N reserve contributes to spring growth in poplar plants and how seasonally internal N recycling contributes to perennial nitrogen use efficiency (NUE).

Chapter 4: Auxin induction of BSP catabolism

Auxin hormone

The phytohormone auxin is a key regulator of plant growth and development (Smet et al., 2011). Auxin is produced predominantly in young tissues (Ljung et al., 2001) and transported to various tissues and cells where it regulates many aspects of plant growth and development (Gallavotti, 2013). Auxins are weak acids that require the assistance of transport proteins to cross the plasma membrane in their charged forms (Blakeslee et al., 2005; Zažímalová et al., 2010). Long distance transport of auxin involves mass flow through the phloem and while short distance transport (cell-to-cell) is mediated by transporters. The asymmetrical distribution of these transporters along the cell membrane is an important component of polar transport (Zažímalová et al., 2010). There are three main classes of auxin transporters that include the PIN FORMED (PIN) protein family and ATP Binding Cassette subfamily B (ABCB) which function primarily as auxin efflux carriers. The third class of auxin transport proteins are the AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) permeases that function as auxin influx carriers (Blakeslee et al., 2005; Peer et al., 2011; Petrášek and Friml, 2009; Zažímalová et al., 2010).

Auxin regulates a wide range of plant developmental processes including embryogenesis, organogenesis, apical dominance, root development and tropism (Cheng et al., 2007; Gallavotti, 2013; Kimura and Kagawa, 2006; Möller and Weijers, 2009; Peer et al., 2011; Tanaka et al., 2006). Although the majority of studies related auxin responses have been conducted in *Arabidopsis*, in poplar polar

auxin transport has been implicated in wood formation and cambium growth cessation and dormancy (Baba et al., 2011; Nilsson et al., 2008). Recently, microarray data indicated that the RNA abundance of a number of auxin signaling components including auxin transporters, auxin responsive factors (ARFs) and AUX/IAA genes increase in poplar bark during the resumption of growth and N remobilization following dormancy. The differential gene expression for many of these auxin signaling components coincided with BSP catabolism and shoot growth (Lin, unpublished) and suggest that auxin may have a role in woody plant physiology related to BSP degradation and N remobilization.

Role of auxin on renewed shoot growth of woody plants

BSP catabolism in poplar requires bud expansion and shoot growth following dormancy (Coleman et al.,1993). Plants lacking buds failed to show declines in BSP content while plants with intact buds resulted in BSP catabolism, which coincided with bud expansion and shoot growth. This observation indicated that an internal signal for BSP degradation is emanating from expanding buds and this signal is then perceived by bark tissues to induce BSP catabolism. This observations combined with the results from transcriptome analysis suggests that auxin synthesis and transport from expanding buds and shoots to bark may play a regulatory role in bark N remobilization during shoot regrowth. The aim of this study was to determine the role of auxin in mediating BSP remobilization following dormancy by inhibiting auxin transport and by treatment with exogenous auxin in *Populus trichocarpa*.

Materials and Methods

Plant growth

Populus trichocarpa (clone Nisqually) were established from softwood cuttings at University of Maryland research greenhouse. Rooted plants were transferred to 6.0L pots containing soilless media (Sunshine LC1). Plants were initially grown in a LD (16 h light, 8 h dark) greenhouse for six weeks and fertilized once at the beginning of LD growth with 2g of 30-day controlled release fertilizer (18-3-3, Nutricote). BSP acclimation and bud dormancy were induced by transferring plants to SD (8 h light, 16 h dark and 20°C) using a 160ft^2-controlled environment chamber (EGC, Chagrin Falls, OH). After 6 weeks of SD treatment, plants were further treated with SD supplemented with low-temperature (LT) (10°C light, 4°C dark) for 8 weeks for induction of leaf senescence and abscission. After 8 weeks of SD combined with LT leaf senesce and abscission was complete and plants were moved to refrigerated storage (4°C) with continuous darkness for 10 weeks to provide sufficient chilling to release bud dormancy.

Poplar shoot growth treatment

To determine the importance of the presence of elongating shoots in BSP catabolism, a similar experiment by Coleman et al., (1993) was conducted to compare BSP decline in bark of poplars with shoots and in bark of poplars without shoots. Experiments were conducted after plants were released from bud dormancy using low temperature treatment. The first uppermost 5 lateral buds and the apical bud were left intact while the remaining lateral buds were surgically removed from the stem of plants. In plants without expanding shoots, all lateral and apical buds were surgically

removed and plants were moved to LD photoperiod conditions for growth of shoots. Bark tissues were subdivided into 2 sections: 1) the region around the tree apex 'upper bark' and 2) the region close to the basal portion of the tree 'lower bark'. Bark samples were collected at the beginning of the LD treatment (day 0) and after 5, 10 15 and 21 days of LD treatment. At each sampling date four biological replicates were collected. Bark was peeled from stems and immediately frozen in liquid N_2 and stored in -80°C freezer until used for protein extraction.

Blocking auxin transport from developing shoots with NPA treatment

To determine a possible role for auxin in BSP catabolism and N remobilization two approaches were used. If auxin synthesized in expanding buds and shoots that is then transported to bark plays a role in regulating BSP catabolism then it is possible that blocking or inhibiting auxin transport with the auxin transport inhibitor, 1-*N*-naphthylpthalamic acid (NPA) could inhibit BSP catabolism and N remobilization. To test this hypothesis, NPA treatments were applied to plants following low temperature treatment to overcome bud dormancy and prior to LD treatment. First the uppermost 5 lateral buds and the apical bud were left intact while the remaining lateral buds were surgically removed from the stem of plants and then a subset of the plants were treated with either 1) a ring of 50 mM NPA in lanolin paste (Spicer et al., 2013); 2) applied 20 cm below the proximal lateral bud, 2) a ring of lanolin paste with 0 mM NPA applied 20 cm below proximal lateral bud. Plants were randomly assigned each treatment. NPA (Chem Services, West Chester PA) was first dissolved in DMSO and then mixed with lanolin paste to give a final concentration of 50mM. The lanolin paste control (without NPA) contained the same volume of

DMSO. The area of application was immediately covered with aluminum foil and plants were transferred to LD greenhouse conditions. Lanolin paste treatments were reapplied at weekly intervals. Bark tissue was collected at the beginning of the LD treatment (day 0) and after 5, 10 15 and 21 days of LD treatment. At each sampling date four biological replicates were collected. For the NPA and lanolin control treatments, bark tissues were subdivided into 3 sections that included the region containing lateral and apical buds (termed shoot zone), the 20 cm region below the shoot zone but above the site of NPA application, and bark from below the site of treatment. Bark was peeled from stems and immediately frozen in liquid N_2 and stored in -80°C freezer until used for protein extraction.

Auxin manipulation treatment using exogenous IAA

An alternative approach to test the possible role of auxin during N remobilization involved the exogenous application of IAA at the apex of completely debudded poplars. If auxin plays a role in BSP catabolism then application of exogenous IAA to the shoot apex of poplars lacking buds could induce BSP catabolism and remobilization of N. In this experiment, plants were debudded and randomly assigned to two treatments prior to LD treatment that included application of 30 uM IAA in lanolin to the shoot apex or treatment with lanolin alone. Following lanolin application (with or without IAA), the apex of plants was covered with aluminum foil. Plants were retreated at weekly intervals. At the beginning of the experiment (day 0) and after 5, 10, 15 and 21 days of LD treatment bark from four biological replicates was collected, immediately frozen in liquid N and stored at -

80°C. Bark was collected from two different stem locations that included 20 cm immediately below the site of treatment and 20-40 cm below the site of application.

Auxin manipulation treatment with exogenous NAA

Another alternative approach to test the possible role of auxin in N remobilization following dormancy involved application of NAA to the stems of poplars containing lateral buds. In this experiment, stems of plants consisting of the 5 uppermost lateral and the apical bud were first ringed with 50 mM NPA at 20 cm below the proximal lateral bud. Following NPA 1 of 5 NAA treatment (0uM, 1uM, 5uM, 10uM, 15uM in lanolin paste) were used to ring the stems 5 cm below the site of NPA treatment. After applying the appropriate NPA and NAA treatment the treatment site was covered with aluminum foil. Plants were retreated at weekly intervals. At the beginning of the experiment (day 0) and after 5, 10 and 15 days of LD treatment bark from four biological replicates was collected, immediately frozen in liquid N and stored at -80°C. Bark was collected from two different stem locations that included (1) 20 cm immediately below the last lateral bud and (2) bark from 20-40 cm below the site of NAA application.

Protein analysis

Bark samples were ground using a Freezer/Mill 6970EFM (Spex SamplePrep Metuchen, NJ). Ground bark samples (0.2-0.4g fresh weight) from three biological replicates were used for protein extraction. Protein was extracted from bark as previously described (Coleman et al., 1991; Wetzel et al., 1989), but with minor modifications. Frozen bark was mixed with 10 volumes of extraction buffer (50mM sodium borate, 50mM ascorbic acid, [pH 9.0] 1% β-mercaptoethanol and 1mM

PMSF added just before homogenizing), vortexed for 10 seconds and then homogenized for 30 seconds and maximum speed using a tissue homogenizer (PowerGen 125, Fisher Scientific). The homogenized mixture was then centrifuged for 30 minutes at 15,000 x g and the supernatant was transferred to a clean 50ml centrifuge tube and five volumes of 0.1M ammonium acetate in -20°C methanol was added to each protein sample. Proteins were then precipitated overnight at -20°C. Precipitated proteins were collected by centrifuging for 26 minutes at 15000 x g, washed twice with 0.1M ammonium acetate in -20°C methanol and twice with -20°C acetone. The protein pellet was resuspended in Laemmli lysis buffer (Laemmli, 1970), held in boiling water bath for 5 minutes and cooled at room temperature. Proteins were stored at -20°C for long-term storage and at 4°C shortly before used for analysis.

Protein quantitation

Protein quantification was determined determined using the Bicinchoninic acid (BCA) assay with minor modifications (Brown et al., 1989; Smith et al., 1985). Duplicate protein samples were prepared by mixing 5ul of total protein with water to a volume of 1ml solution. Triplicates of protein standards were prepared from Pierce BCA protein assay kit product. Samples and standards were precipitated from Laemmli buffer with 100ul of deoxycholate (DOC) (0.15%, w/v) and 100ul trichloroacetic acid (TCA) (72%, w/v) followed by centrifugation at 5000 x g for 15 minutes. Protein pellets were then resuspended in 50ul solution containing 5% SDS and 0.1N NaOH. To the resuspended protein solution was added 225ul of BCA working reagent (WR) from Pierce Thermo scientific (BCA Reagent A product No.

23227 and BCA Reagent B; WR ratio = 1:8). Samples were then vortexed and incubated at 37°C for 30 minutes. The absorbance of each sample was measured using μ Quant spectrophotometer (Bio-Tek Instruments, Inc). Linear regression using Prism software was used to calculate protein concentrations.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE using a Bio-Rad Mini-protean cell and 12 resolving and 4% stacking gels. 5 ug of total protein (5ug) was electrophoresed at 180V for 1 h. Gels were stained with commassie and destained in water. Gel images were visualized using a VersaDoc imaging system (Bio-Rad).

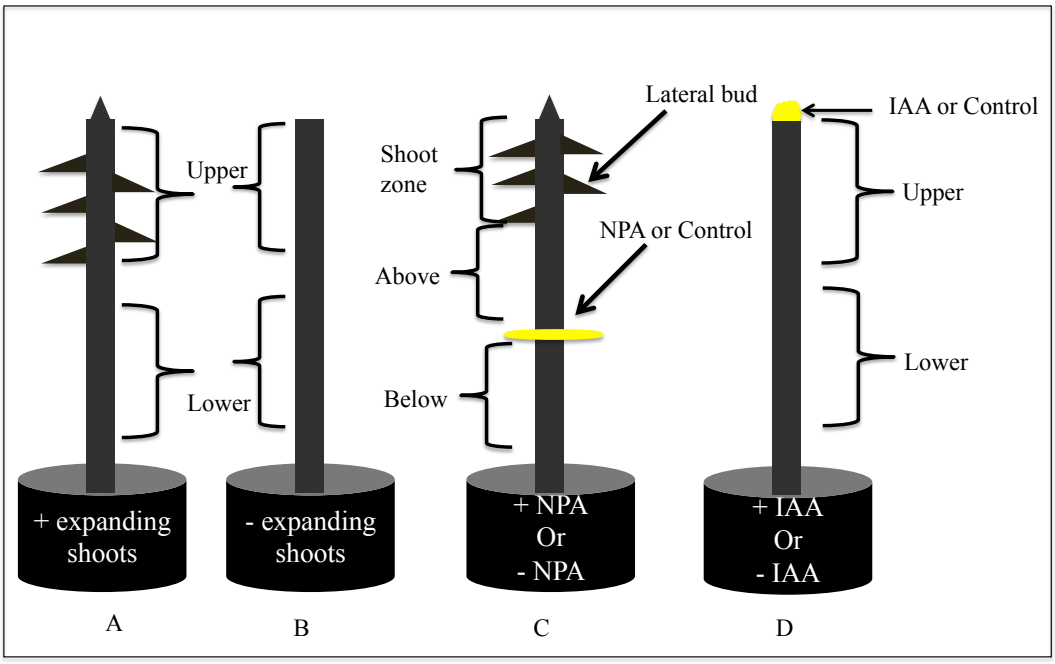

Figure 4.1 Diagrammatic representation of A) poplars with expanding shoots B) Poplars without expanding shoots C) poplars ringed NPA and controls ringed with DMSO D) poplars treated with IAA and control treated wit ethanol before renewed shoot growth.

Results

Differential steady-state BSP disappearance between poplars with and without growing shoots

Difference in BSP decline was observed between poplars with shoots and those that lacked shoots within 15 days of LD treatment (Figure 4.2). After 21 days of LD treatment BSP was nearly undetectable in trees where bud break and shoot growth had occurred while high levels of BSP were still detected in plants lacking growing shoots.

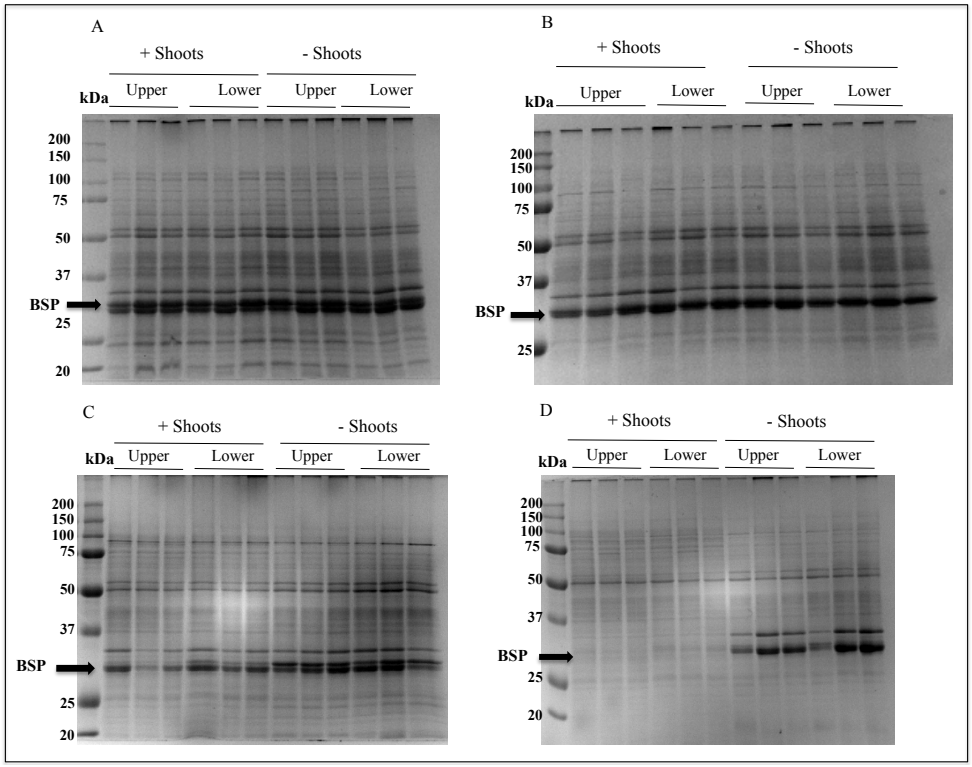

Figure 4.2 A to D, Effect of shoot growth on BSP abundance in poplar bark at two different stem positions during LD treatment following dormancy release. Plants with shoots or without shoots were treated with LD for either A) 5 days, B) 10 days, C) 15 days or D) 21 days.

NPA treatment inhibited BSP degradation

Differences in BSP content between different bark locations of NPA treated plants were detected after 10 days of LD treatment (Figure 4.1). In plants treated with 50mM NPA BSP was more abundant in bark below the site of treatment compared to bark located in the shoot zone or above the site of NPA treatment (Figure 4.1E). Continued treatment in LD for 15 and 21 days resulted in greater differences in BSP abundance between the bark below the site of NPA treatment and bark above the site of treatment as well as bark located in the shoot zone (Figures 4.1 G and I). After 21 days of LD treatment little BSP was detected in bark corresponding to the shoot zone bark and in above the site of NPA treatment. Compared to bark located above the site of NPA treatment, BSP abundance remained constant and declined little in bark located below the site of NPA treatment over the 21 day LD treatment. In contrast to NPA treated stems, no differences were apparent for the different stem locations during the 21 days of LD treatment for plants treated with lanolin that lacked NPA (Figure 4.1D) and after 21 days the majority of BSP was catabolized (Figure 4.1B, D, F, H). Bud break of these plants occurred after 5 days of LD and no differences in the timing of bud break were observed for the different treatments.

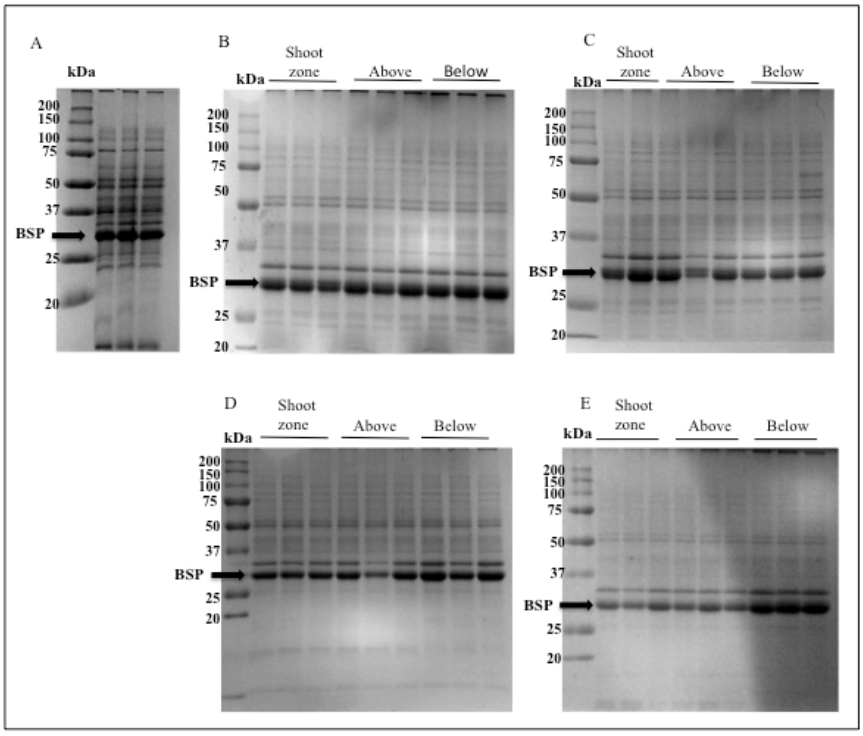

Figure 4.3 A to E, Effect of NPA treatment on BSP abundance in poplar bark at different stem positions during LD treatment following dormancy release. A) Following LT treatment just before LD treatment, B) control plants (0mM NPA) after 5 days of LD treatment, C) plants treated with 50 mM NPA after 5 days of LD treatment, D) control plants (0 mM NPA) after 10 days of LD treatment and E) plants treated with 50 Mm NPA after 10 days of LD treatment.

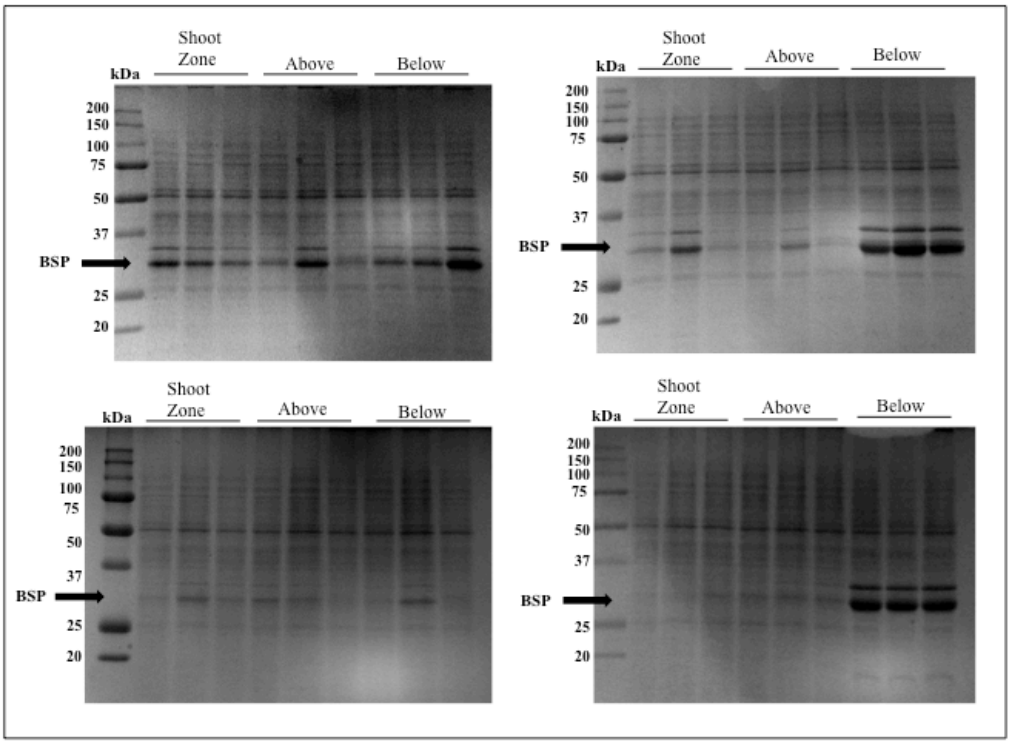

Figure 4.2 Continued F to I, Effect of NPA treatment on BSP abundance in poplar bark at different stem positions during LD treatment following dormancy release, F) control plants (0 mM NPA) after 15 days of LD treatment, G) plants treated with 50 mM NPA after 15 days of treatment, H) control plants (0 mM NPA) after 21 days of LD treatment and I) plants treated with 50 mM NPA after 21 days of LD treatment

Effect of exogenous IAA application on BSP remobilization

Protein analysis of IAA treated plants did not show any noticeable decline in BSP abundance between treatment control plants and those treated with 1AA at any of the sampling time points (Figure 4.3).

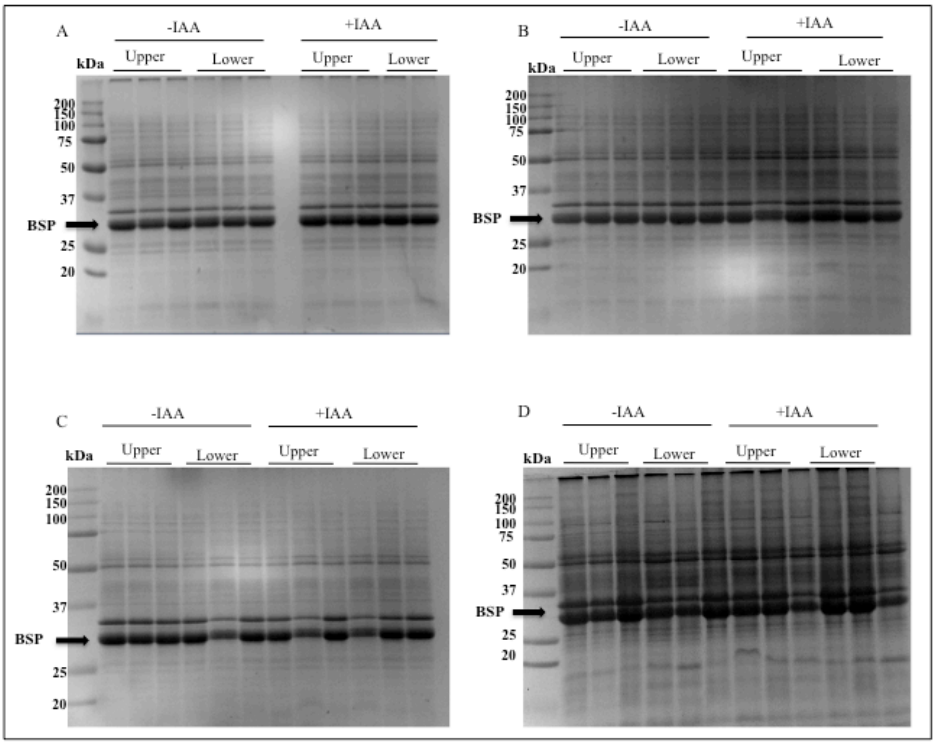

Figure 4.4 A to D, Effect of IAA treatment on BSP abundance in poplar bark at two different stem positions of plants during LD treatment following dormancy release. Plants without buds were treated with 20 uM IAA and LD for either A) 5 days, B) 10 days, C) 15 days or D) 21 days.

Effect of NAA application on BSP remobilization

Protein analysis of NAA treated plants revealed that there were no significant decline in BSP abundance associated with NAA treatment (Figure 4.5). The results are similar to what was observed with IAA treated plants (Figure 4.4).

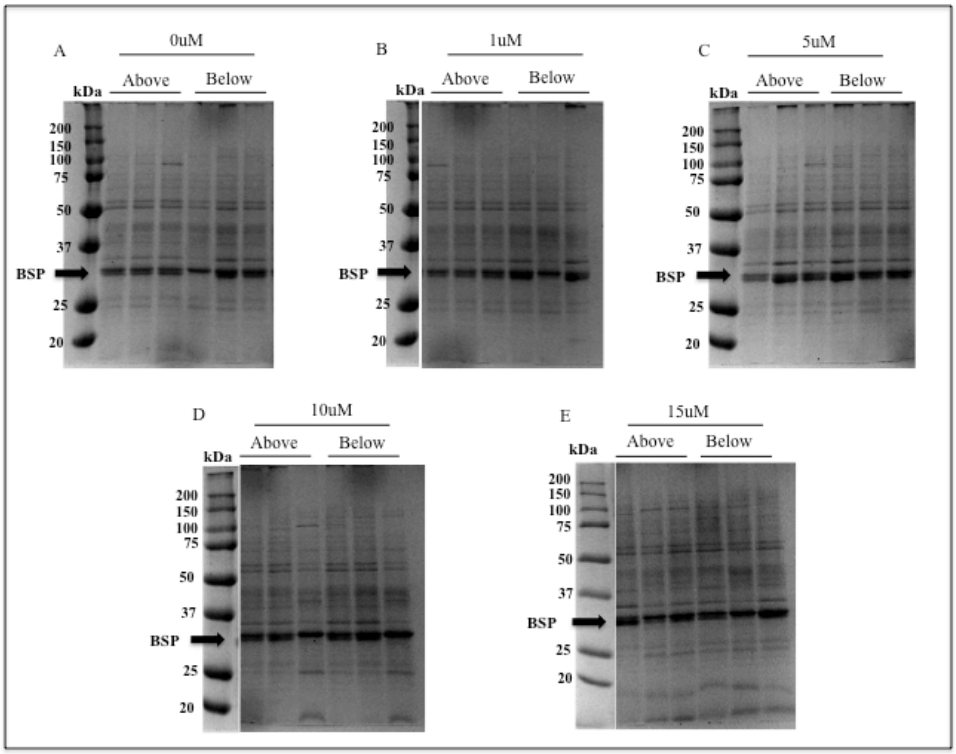

Figure 4.5 A to E, Effect of NAA treatment on BSP abundance in poplar bark at two different stem positions of plants during LD treatment following dormancy release. Plants with the apical and uppermost 5 buds were treated with 50 mM NPA 20 cm below the lowest lateral bud and then treated with either 1, 5, 10 or 15 uM NAA in lanolin below the site of NPA treatment. A) 0 uM NAA, B) 1 uM NAA, C) 5 uM NAA, D) 10 uM NAA and E) 15 uM NAA treatments after 5 days of LD treatment.

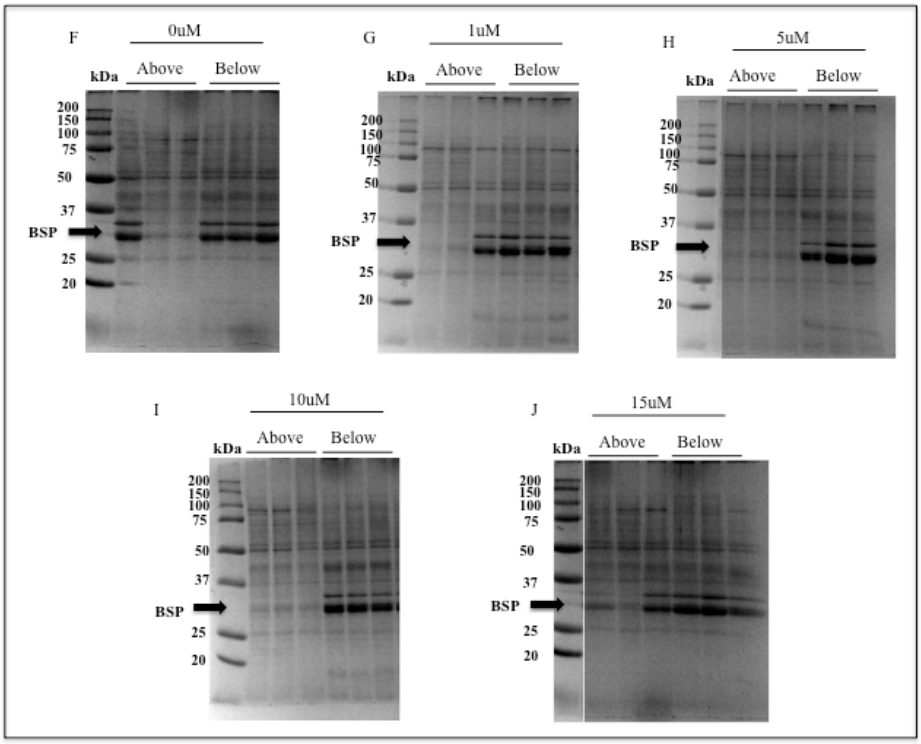

Figure 4.5 continued F to J, Effect of NAA treatment on BSP abundance in poplar bark at two different stem positions of plant during LD treatment following dormancy release. Plants with the apical and uppermost 5 buds were treated with 50 mM NPA 20 cm below the lowest lateral bud and then treated with either 0, 1, 5, 10 or 15 uM NAA in lanolin below the site of NPA treatment. F) 0 uM NAA, G) 1 uM NAA, H) 5 uM NAA, I) 10 uM NAA and J) 15uM treatment after 10 days of LD treatment.

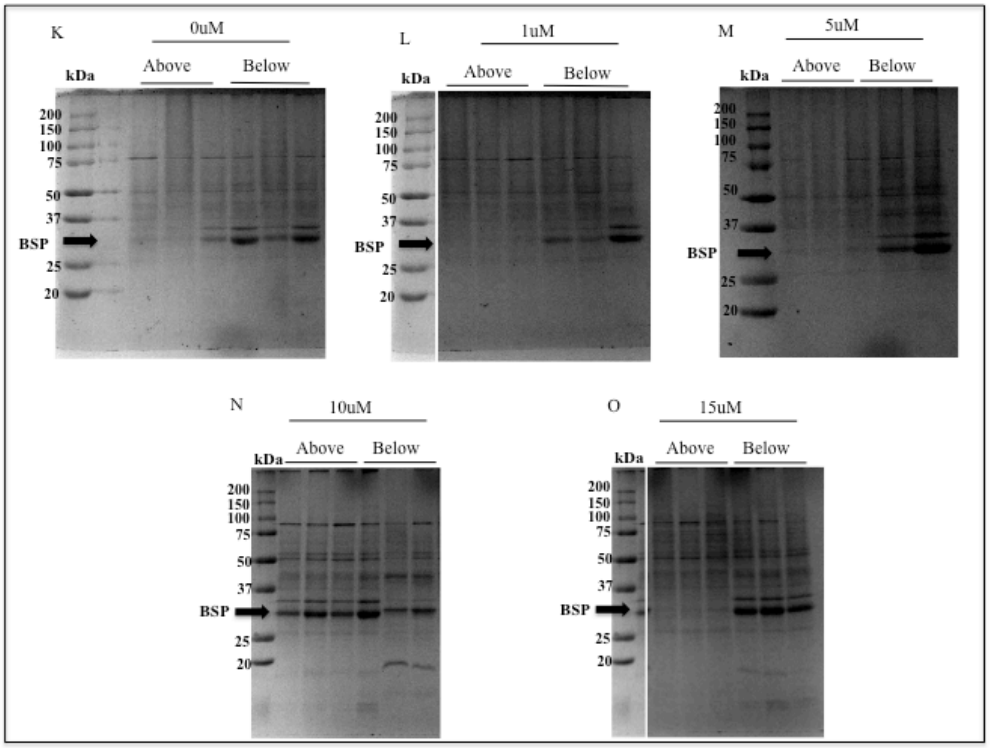

Figure 4.5 continued K to O Effect of NAA treatment on BSP abundance in poplar bark at two different stem positions of plants during LD treatment following dormancy release. Plants with the apical and uppermost 5 buds were treated with 50 mM NPA 20 cm below the lowest lateral bud and then treated with either 0, 1, 5, 10, or 15 uM IAA in lanolin below the site of NPA treatment. K) 0 uM NAA, L) 1 uM NAA M,) 5 uM NAA, N) 10 uM NAA and O) 15 uM NAA treatments after 14 days of LD treatments.

Discussion

Differential steady-state disappearance resulted in poplar bark treated with NPA

Poplar bark BSP levels with either expanding buds and shoots or those lacking buds was similar to what was observed by (Coleman et al., 1993). The decline in BSP content in trees with intact buds after 21 days of LD exposure suggested that expanding buds play a major role in BSP catabolism during spring shoot growth.

After 5 days of LD treatment there was no obvious bud or shoot growth which corresponded to a lack of substantial changes in bark BSP which is consistent with the previous studies focused on the proteomic changes that occur during bark N remobilization (Islam et al., 2015). This proteomic analysis showed that BSPA, BSPB and BSPC spectra counts were spectra counts were greater after 1 week of LD treatment compared to 3 weeks. This would indicate that there was little protease activity directed at BSP catabolism during the initial exposure to LD conditions following dormancy release (Islam et al., 2015). Islam et al., (2015) also observed that the abundance and diversity of putative proteases increased in poplar bark after 3 weeks of LD growth. The increased abundance of certain cysteine and serine proteases was also correlated with increased transcript abundance of their respective mRNAs. It is likely that the decrease in BSP abundance in bark above the site of NPA treatment is associated with increased abundance or activity of some of the proteases identified by Islam et al.,(2015).

As bud expansion and new shoot growth commenced the BSP levels began to decline in bark samples located above the site of NPA treatment but not in bark below the site of treatment. The auxin content of bark after 1 week of LD treatment was

below detectable levels regardless of stem location or NPA treatment but increased in bark above the site of NPA treatment with continued LD treatment (Gen Li personal communication and manuscript in preparation). This increase in auxin levels observed in bark above the site of NPA treatment coincides with when bud expansion and shoot growth commences, suggesting auxin is produced in those tissues and transported via the phloem to bark (Bhalerao et al., 2002; Blakeslee et al., 2005; Ljung et al., 2001). This transported auxin appears to be required for BSP degradation and N remobilization which may involve the regulation of protease gene expression or activity.

The result from IAA and NAA experiment were inconclusive. However, it cannot be concluded that exogenous auxin application to debudded plants does not induce BSP degradation. It could be speculated that the auxin application method used was unsuccessful in delivering exogenous auxin to bark tissues or perhaps, auxin was readily oxidized in the plant allowing for sufficient auxin to be transported to bark tissues to induce BSP degradation.

Several evidence from transcriptomic analysis of poplar bark genes, protein analysis comparing BSP content of poplars with and without growing shoots, poplars treated with auxin transporter inhibitor NPA, and auxin concentration analysis of bark samples have indicated that auxin produced in developing shoots and transported to the bark mediated BSP degradation in poplar during spring growth. Understanding the mechanisms and regulations of seasonal N storage, remobilization and utilization in perennial plants could provide insights that can lead to increasing N use efficiency in both perennial and annual plants.

Appendix

One-Way ANOVA Tables For Nitrogen Feeding Experiment

Table A.1 ANOVA Leaf Area

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Leaf area	4	22923	5731	13.54	0.0005
Residual	10	4231	423.1		
Total	14	27153			

Table A.2 ANOVA of Stem Diameter

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Stem diameter	4	27.65	6.913	11	0.0011
Residual	10	6.284	0.6284		
Total	14	33.94			

Table A.3 ANOVA of Number of Sylleptic Shoots

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Number of sylleptics	4	1634	408.6	38.07	<0.0001
Residual	10	107.3	10.73		
Total	14	1742			

Table A.4 ANOVA of Growth of Sylleptic Shoots

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Sylleptic shoot length	4	353.7	88.44	23.17	<0.0001
Residual	10	38.16	3.816		
Total	14	39.19			

Appendix B One-Way ANOVA - Tables For Sink Competition Experiment Between Lateral Buds Using *Populus trichocarpa* clone Nisqually

Table B.1 ANOVA of in Leaf area

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Lateral buds	4	28705	7176	42.40	<0.0001
Residual	140	23697	169.3		
Total	144	52402			

Table B.2 ANOVA of in Stem length

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Lateral buds	4	4928	1232	16.02	<0.0001
Residual	137	10535	76.90		
Total	141	15463			

Table B.3 ANOVA of Number of New Sylleptics Shoots

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Lateral buds	4	265.0	66.26	12.99	<0.0001
Residual	20	102.0	5.100		
Total	24	367.0			

Table B.4 ANOVA of Length of New Sylleptic Shoots

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Sylleptic shoot length	4	643.7	160.9	23.17	0.0042
Residual	112	4449	39.72		
Total	116	5093			

Table B.5 ANOVA of Leaf Area of the First Lateral Shoot Position Between Treatments

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Competition between lateral buds	4	6124	1531	18.87	<0.0001
Residual	20	1623	81.14		
Total	24	7746			

Table B.6 ANOVA of Leaf Area within Lateral Bud Treatment 3

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Lateral bud 12	2	1380	689.9	5.032	0.0259
Residual	12	1645	137.1		
Total	14	3025			

Table B.7 ANOVA of Leaf Area within Lateral Bud Treatment 6

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Lateral bud 6	5	5005	1001	15.65	<0.0001
Residual	22	1407	63.97		
Total	27	6412			

Table B.8 ANOVA of Leaf Area within Lateral Bud Treatment 9

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Lateral bud 9	8	4987	623.4	12.23	<0.0001
Residual	32	1631	50.98		
Total	40	6619			

Table B.9 ANOVA of Leaf Area within Lateral Bud Treatment 12

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Lateral bud 12	11	3104	282.2	3.188	0.0029
Residual	45	3983	88.51		
Total	56	7087			

Appendix C One-Way ANOVA Tables For Sink Competition Experiment Between Sylleptic Shoots Using *Populus trichocarpa* clone Nisqually

Table C.1 ANOVA of Leaf Area

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Sylleptic shoot treat.	2	1185	592.5	28.00	<0.0001
Residual	12	253.9	21.16		
Total	14	1439			

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Sylleptic shoot treat.	2	1185	592.5	28.00	<0.0001
Residual	12	253.9	21.16		
Total	14	1439			

Table C.2 ANOVA of Number of New Sylleptics

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Sylleptic shoot length	2	20.34	10.17	1.386	0.2874
Residual	12	88.03	7.336		
Total	14	108.4			

Table C.3 ANOVA of Length of New Syllepsis

Source of Variation	Df	Sum of Squares	Mean Square	F	P value
Sylleptic shoot length	2	16.53	8.267	4.679	0.0315
Residual	12	21.20	1.767		
Total	14	37.73			

Appendix D T-test For Sink Competition Experiment Between Lateral Buds Using *Populus tremula X Populus alba* hybrid clone INRA 717 IB4

T-test for Leaf Area

Number of Lateral buds	N	Mean	Std. Error Mean
Single	5	132.2	5.835
Intact	5	16.87	0.4204

T-test for Shoot Length

Number of Lateral buds	N	Mean	Std. Error Mean
Single	5	56.44	1.479
Intact	5	20.41	1.436

T-test for Leaf Fresh Weight

Number of Lateral buds	N	Mean	Std. Error Mean
Single	5	2.446	0.1459
Intact	5	0.2106	0.006393

T-test Out-put Analysis of Differences in Stem Fresh Weight

Number of Lateral buds	N	Mean	Std. Error Mean
Single	5	22.90	1.776
Intact	5	0.8309	0.1081

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