University of Alberta

Diverting Resources to Turn on Resistance: Influences of Biotic and Abiotic Stresses on Aspen Seedlings

by

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Dedication

I dedicate this work to my parents Mustapha Son of Abdelkader Al-Najjar and Zeyneb Daughter of Othmane El-Abidi for that which all words in all tongues fail to convey.

Abstract

The interactions between biotic and abiotic stresses and their influence on plant reserves in non-photosynthetic tissues (i.e., roots and stems) and the role of plant reserves in tree defenses are poorly understood. Aspen seedlings grown under different conditions (light, fertilizer) were grouped in three groups based on their nutrient and carbohydrate reserves. After dormancy, half of the seedlings in each group were subjected to feeding by forest tent caterpillar (*Malacosoma disstria*). We analyzed foliar and reserve chemistry and explained their effects on plant defenses and larval performance. We found that reserve TNC and nutrients can affect foliar TNC, Nitrogen, Carbon/Nitrogen ratio, defense chemistry, and the overall plant response to herbivory. Seedlings with high carbohydrate-to-nutrient reserve ratio had the greatest induction of defensive compounds and sustained the lowest insect damage. This study highlights the importance of plant defenses mediating the intricate relationship between plants and herbivores.

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Chapter I. Thesis Introduction

Trembling aspen (*Populus tremuloides Michx*) (Salicaceae) is one of the most prominent tree species in the boreal forest of Canada. It is also the main species of the parkland, the transition zone between the grassland prairies and the northern boreal forest (Brandt et al. 2003). It represents 37% of the marketed forest products in the three Prairie Provinces (Alberta, Saskatchewan and Manitoba) and aspen-based wood materials increased ten times from 1970 to 1995 in Canada (Canadian Council of Forest Ministers 1997). Aspen wood is commonly used for engineered wood products (e.g. oriented strand board) and pulp production. Combined with its economic benefits, aspen is also a keystone species that supports a number of birds, amphibians, mammals, insects and mycorhizae in the boreal forest (Stelfox 1995, Cripps 2003).

Aspen has a very large natural extension across North America where it is adapted to a wide range of environmental conditions (Little 1997). In its range, aspen has developed critical physiological and chemical defense traits that allowed it to be resistant or tolerant to herbivory (Lindroth 2001, Erwin et al. 2002). Aspen mainly relies on two families of defensive chemical compounds: phenolic glycosides and condensed tannins. Both groups of compounds are produced through the shikimic acid pathway (Lindroth 2001). Condensed tannins are mainly stored in the bark and root tissues and considered as a first line defense against pathogen infection (Lindroth and Hwang 1996). While the role of

condensed tannins in aspen defense is still debated (Wang and Constabel 2004, Barbehenn and Constabel 2011), phenolic glycosides were shown to deter herbivores (Lindroth 2001). Aspen has also developed a tolerance mechanism through increased production of shoots and leaves after herbivory (Stevens et al. 2007). A recent work suggests an eventual role of aspen carbohydrate reserves stored in non-photosynthetic tissues (i.e., roots, stem, and twigs) in compensation of lost foliage and growth after defoliation events (Landhäusser and Lieffers 2012).

Aspen has been a favorite model tree species to test plant defense theories. Bryant et al. (1983) studied aspen defenses in relation with growing conditions and resource availability, giving birth to the Carbon-Nutrient Balance (CNB) hypothesis. The CNB specified that plants rely on the most abundant resource (nitrogen or carbon) to synthesize defensive chemicals. For example, if plants have access to nitrogen, they rely heavily on nitrogen-based defensive compounds, such as alkaloids and produce less carbon-based defensive compounds, such as phenolic glycosides and tannins, or vice versa. Subsequent studies tested the CNB hypothesis and found mixed results (Koricheva et al. 1998, Koricheva 2002). A decade later, another defense hypothesis surfaced: Growth Differentiation Balance Hypothesis (GBDH, Herms and Mattson1992), which provided a larger framework for tree defenses. This hypothesis predicts a possible trade-off between growth and defense depending on the environmental conditions and resource availability. Its main premise is that under stressful conditions, plants limit their growth and allocate their resources to defense

instead; thus growth and defense compete for the same resources and this competition becomes intensive under limited resources.

More recently, there were attempts to investigate the relationship between carbon allocation dynamics and growth/defense functions in poplar in the framework of what is called "induced resource sequestration" hypothesis (Orians et al. 2011). Basically, this hypothesis states that plants subject to herbivores (mainly caterpillar) retrieve their resources from attacked tissues, such as foliage to unattacked tissues, such as stem. Babst et al. (2005) showed that induction of different species of poplar seedlings by jasmonic acid, an elicitor of herbivory response, instigated a translocation of carbon from leaves to non-photosynthetic tissues using ^{11}C tracers. A similar result using an actual herbivore (*Lymantria dispar* (L.) Lepidoptera: Lymantriidae) was further reported in poplar (Babst et al. 2008), suggesting that the mechanism of sequestration is inherent to the plant (damage induced) rather than elicited by regurgitates.

Other studies have addressed aspen defenses outside of these proposed frameworks and focused on plant genetic-defense interactions (Hamilton et al. 2001, Donaldson and Lindroth 2007). These studies reported interesting patterns between plant defenses and genetics and found differences in plant secondary chemistry among different aspen clones (each clone represents a different genotype) (Donaldson and Lindroth 2007, Stevens et al. 2007). These aforementioned studies highlight the complexity of the system and the difficulty of proposing a general framework to explain plant-insect interactions (Hamilton et al. 2001, Koricheva 2002).

Studies focusing on aspen defenses usually modify the light and fertilization regimes and measure foliar chemistry and its influence on herbivores. In many cases, fertilization improves aspen growth in common garden experiments and the treatment effect carries on up to three years after its application (van den Driessche et al. 2003). Fertilization - particularly Nitrogen (N) amendment - increases the Carbon (C) assimilation (or photosynthesis by-products, such as amino acids and proteins) making N available for different plant functions, such as defense (Liu and Dickman 1993). In contrast, other studies show that fertilization reduces aspen chemical defenses, specifically phenolic glycosides (Bryant et al. 1987) while making them more tolerant to herbivory (Stevens et al. 2007). Light is commonly used in combination with other factors, such as fertilization to manipulate plant allocation patterns. For instance, Hemming and Lindroth (2000) showed that in low nutrient conditions aspen foliage exposed to high light levels can achieve the highest synthesis of C-based compounds (starch and secondary metabolites). However, even though aspen leaves have high secondary metabolites, they are still preferred by herbivores due to their high nutrient contents, specifically N (Lévesque et al. 2002), highlighting the importance of foliar nutritional quality in insect feeding.

In addition to light and fertilization, some studies use the shoot growth inhibitor (active ingredient: Paclobutrazol) to investigate the relationship between growth and defense. For example, the application of the shoot growth inhibitor to apple trees has a delayed effect on growth (a year after application), but it immediately increased quinic acid content of woody tissues (Wang and Steffens

1987). Quinic acid is a precursor of several classes of phenolic compounds produced through the shikimic pathway (Wang and Steffens 1987), including phenolic glycosides. In the same study, the growth retardant treatment decreased cell wall cellulose while increasing other simple glycosides and overall wood carbohydrates (Wang et al. 1986a, b). Furthermore, the application of the same inhibitor on paper birch increased resistance to herbivores due to the increased amounts of condensed tannins (Chorbadjian 2009).

Most studies in aspen defenses mentioned above focused on the direct effect of growing conditions on foliar chemistry yet they did not examine the influence of nutrient and carbohydrate reserves in the non-photosynthetic tissues (roots, stems) on plant defenses. Studies in other deciduous trees have clearly demonstrated the importance of such reserves in plant defenses (Dunn et al. 1987, 1990, Canham et al. 1999, Dafoe et al. 2009). For instance, Dunn et al. (1987) found that oak trees with low root starch reserves in winter were more susceptible to attacks by the phloem feeder *Agrilus bilineatus* (Weber) (Coleoptera: Buprestidae). In another study, Canham et al. (1999) showed the negative effect of mechanical defoliation on carbohydrate reserves and seedling survival of four deciduous tree species: red maple, sugar maple, black cherries and red oak. In Eucalyptus, defoliation affected N and carbohydrate reserves in the non-photosynthetic tissues more than in the foliar reserves (Eyles et al. 2009). Combined together, these studies suggest the importance of the non-photosynthetic tissue reserves in the process of plant response to herbivory, even though the non-photosynthetic tissue is usually not the direct target of defoliators.

In the same order of suggestions, Landhäusser and Lieffers (2003) highlighted the eventual importance of reserve availability in aspen tolerance to defoliators. More recently, the effect of defoliation on carbohydrate reserves in mature aspen trees was examined and it was shown that defoliation depleted root reserves and it took aspen up to two years to recover its root carbohydrate reserves (Landhäusser and Lieffers 2012), suggesting that root reserves were translocated to other parts of plants either for growth or defense against herbivores.

Forest Tent Caterpillar (FTC, *Malacosoma disstria* Hübner) (Lepidoptera: Lasiocampidae) is a major defoliator of aspen in North America and its periodic outbreaks cause serious damage to aspen forests. While the severity of aspen decline subsequent to defoliation depends on other abiotic factors, such as drought (Hogg and Schwarz 1999; Man et al. 2008), FTC herbivory usually weakens aspen trees, making them susceptible to other insects and diseases but rarely kills trees on its own. In Canada, aspen is by far FTC's preferred host (Colasurdo et al. 2009, Wood et al. 2009). While it can also defoliate other hardwood trees, such as sugar maple (*Acer saccharum*), paper birch (*Betula papyrifera*), basswood (*Tilia Americana*), and red oak (*Quercus rubra*) in boreal forest, the fitness of FTC feeding on hosts other than aspen is significantly jeopardized (Fitzgerald 1995, Nicol et al. 1997). It has been suggested that such differences in host preference are related to the chemical composition of foliage, particularly C:N ratio and FTC disfavors diets heavily based on C (Noseworthy and Despland 2006, Colasurdo et al. 2009).

The overall objectives of this thesis are (1) to investigate whether nutrient and carbohydrate reserves in non-photosynthetic tissues of aspen seedlings influence constitutive and induced defenses of aspen, and (2) examine whether differences in plant chemistry can also affect aspen defenses against FTC. In Chapter II, open pollinated aspen seedlings were grown under different greenhouse conditions (inside and outside the greenhouse) and fertilization regimes with or without a shoot growth inhibitor application. I then analyzed the nutrients and carbohydrates stored in the seedling reserves after senescence and shortly before dormancy. In the light of the data collected I classified seedlings according to their nutrient and carbohydrate reserves into three categories. (1) Low Nutrients-Low Total Non-structural Carbohydrates (TNC); (2) High Nutrients-High TNC; and (3) High Nutrients-Medium TNC. In Chapter III, after dormancy, the same seedlings were subjected to defoliation by FTC. I analyzed foliar (primary and secondary chemistry (both constitutive and induced)) and the reserve chemistry (only primary chemistry) and explained the relationship between these two reserves and larval performance.

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Chapter II. Growing conditions affect carbohydrate and nutrient contents and concentrations of non-photosynthetic tissues in aspen seedlings

Introduction

Trembling aspen (*Populus tremuloides* Michx.) is a fast growing early successional tree species. It has a wide distribution throughout North America where it grows either in pure stands or mixed with other broadleaf and/or conifer species, such as lodgepole pine (*Pinus contorta* Douglas ex Loudon) and white spruce (*Picea glauca* (Moench) Voss), over a broad range of site and environmental conditions (Little 1997). Aspen, like other species in the genus *Populus*, is characterized by a remarkable phenotypic plasticity, a result of the interaction between its wide genotypic variation and its diverse environments (Lindroth 2001, Marron et al. 2006, Kanaga et al. 2008). This plasticity has been clearly demonstrated by its ability to change physiological and chemical attributes, and with altered growth patterns in response to various growing conditions. Relative to other boreal species, physiological adaptations of aspen and its clonal nature also contribute to its success in occupying environments that have soil moisture limitations and high disturbance frequencies such as fire: instance of this success, the clonal stands of the Great Basin thought to be regenerating for the last 8000 years (DeByle 1985).

However, the recent decline of aspen forests in North America has shown that this successful species is showing signs of weakness and a vulnerability to a

combination of various biotic and abiotic stresses. For example, warming climate, frequent drought events and heavy insect defoliation, particularly by the forest tent caterpillar *Malacosoma disstria* Hübner (Lepidoptera: Lasiocampidae) are thought to be the cause of aspen decline in the boreal and parkland forests in Canada (Hogg and Schwarz 1999, Hogg et al. 2002). Further investigations have demonstrated that changes in aspen nutrient and carbohydrate reserve status induced by biotic and abiotic stresses probably led to the observed decline, suggesting that nutrient and carbohydrate reserves might play an important role in the short-term and long-term survival of aspen forests in North America (Landhäusser and Lieffers 2003, 2012, Anderegg et al. 2012).

Aspen has widely been used as a model tree species in studies aiming to understand how genotypic and phenotypic variation influences its growth, physiology and defense (Lindroth 2001, Lindroth et al. 1993, Hemming and Lindroth 1995, 1999, Agrell et al. 2000, Donaldson et al. 2006, Couture et al. 2011). For example, changes in light intensity and soil nutrients not only influence aspen growth (Hemming and Lindroth 1999, Cole et al. 2010), but also its chemical properties (Osier and Lindroth 2001, Panzuto et al. 2001, Vigue and Lindroth 2010, Couture et al. 2011). The genotypic-environment interactions also influence aspen responses to herbivory (Hwang and Lindroth 1997, 1998).

However, the majority of these studies focused only on the response of foliage chemistry to herbivory, while the role of nutrients and carbohydrate reserves stored in non-photosynthetic tissues, such as stems and roots, has been widely overlooked even though nutrients and carbohydrates in structural non-

photosynthetic tissues of plants can be active participants of various plant functions. For example, carbon and nutrient reserves in non-photosynthetic tissues can be altered by defoliators (Dunn et al. 1987, 1990, Kosola et al. 2001, Landhäusser and Lieffers 2003, 2012), as well as by changes in growing conditions such as drought (Galvez et al. 2011) and light (Snedden et al. 2010). The role of these resources in aspen defenses has received little attention even though a clear understanding of how these reserves contribute to plant defense is essential to unravel the complex interactions among aspen, its changing environment and herbivores.

My objective in this chapter was to manipulate the growing conditions of aspen seedlings, to generate seedlings with different nutrient and carbohydrate reserves in their non-photosynthetic tissues. These seedlings will then be used to test whether plants with different reserves will have different defense responses to herbivory by forest tent caterpillar (Chapter III).

Materials and Methods

Treatment applications and seedling generation

To grow aspen seedlings with different carbohydrate and nutrient reserves in their non-photosynthetic tissues, seeds from open pollinated seed sources were collected near Edmonton (AB, Canada) (53˚31’01.32”N, 113˚29’56.55”W,

elevation 655 m) and were sown May 29, 2009 into eight 5-15 styroblocks (Beaverplastic, Edmonton, AB) with 66 cavities (5 cm diameter and 15 cm depth and a soil volume of 220 ml). The planting substrate used was five parts peat, one part perlite and ½ part clay particles. The greenhouse conditions were 18h day length (supplemented when necessary with artificial lighting), 24°C and 60% relative humidity during the course of the experiments. Germination of seeds occurred within two days and germinants were misted with water during the first two weeks. On June 14, a single fertilization took place using 10-52-10 (N-P-K) with chelated micronutrients (Plant Prod Co. Brampton, ON, Canada) at a concentration of 1 g L^{-1}. A fertilizer with high phosphorus concentration was used to facilitate early establishment of seedlings. Between June 28 and July 12 all seedlings were fertilized twice (15-30-15 N-P-K fertilizer with chelated micronutrients (1 g L^{-1})).

On July 15, half the blocks (four) were moved outside of the greenhouse while four blocks stayed inside the greenhouse. Apart from the lower light levels (30% - 50% lower than outside), the greenhouse conditions also likely influenced other environmental factors such as temperature, wind, and vapour pressure deficit. Two of the inside and two of the outside blocks were assigned to either low (0.2 g L^{-1}) or high (2 g L^{-1}) fertilization (15-30-15 N-P-K) regime. Blocks were fertilized at their respective concentrations once a week until August 10. One week after the fertilizer treatments started, seedlings in one block of each treatment combination were treated with a shoot growth inhibitor (active ingredient: paclobutrazol; Bonzi®, Plant Growth Regulator, Syngenta Crop

Protection Canada, Inc. Guelph, Ontario). Seedlings were treated once with 20 mg L^{-1} paclobutrazol by adding 5 ml Bonzi per liter of water in which the root systems were submerged.

On August 16, the seedlings inside the greenhouse were also moved to the outside to go through the natural hardening and dormancy process. By mid-November, when seedlings were fully dormant, seedlings were packed in plastic bags, put in wax-coated cardboard boxes and stored at -4˚C until April 12, 2010. These seedlings were used for the herbivory experiment described in the Chapter III. From the packed seedlings ten seedlings from each treatment combination (total 80 seedlings) were randomly picked to evaluate dormant seedlings characteristics as described below.

Measurement of seedling characteristics

The ten seedlings taken from each treatment combination were used to assess shoot height, root collar diameter (RCD), root volume, total dry weight of root and shoot, root to shoot ratio (RSR). Total non-structural carbohydrates and nutrients were analysed in the root and shoot tissues combined. The roots were thoroughly washed to remove the soil. The shoot height was determined by measuring the length of a shoot from the root collar to the tip of the terminal bud. The root volume was measured using the water displacement method (Harrington et al. 1994). After these measurements, the roots and shoots were separated and oven dried at 70˚C until constant weight was reached (72 hrs. on average). The

dry weight was recorded for each of shoot and root per seedling and RSR was calculated.

To determine the content (gr/seedling) and concentration (mg/gr tissue) of total non-structural carbohydrate (TNC) (sum of water soluble sugars and starch) and total nutrients (the sum of Phosphorous (P), Manganese (Mn), Magnesium (Mg), Iron (Fe), Zinc (Zn), Aluminium (Al), Nitrogen (N), Potassium (K), Sulphur (S), Copper (Cu), Lead (Pb) and Calcium (Ca)) of the non-photosynthetic tissues (shoots and roots), dried root and shoot tissues were combined and ground together in a Wiley Mill (Thomas Scientific Wiley Laboratory Mill, Swedesboro, NJ) to pass 40 mesh (0.4 mm). TNC was determined following the method described by Chow and Landhäusser (2004). Briefly, water soluble sugars were extracted three times from ground samples using hot 85% ethanol and then analysed colorimetrically using phenol–sulphuric acid at 490 nm. Following sugar extraction, the starch in the remaining residue was digested with α-amylase (ICN 190151, from *Bacillus lichenformis*) and amyloglucosidase (Sigma A3514, from *Aspergillus niger*) and glucose equivalents were determined colorimetrically with peroxidase-glucose oxidase-o-dianisidine (Sigma Glucose Diagnostic Kit 510A). The same ground tissues were also analysed for total N using the Kjeldahl method (Bremner and Mulvaney 1978) and for 11 other nutrients in an Elemental Combustion Analyzer (ECS 4010 Elemental Combustion System CHNS-O, Costech Analytical Technologies Inc., Valencia, CA.).

Data Analysis

For the factorial design I used three-way ANOVA and LSD mean test in order to determine the treatment effects on the seedlings characteristics measured. Treatment factors, shoot growth inhibitor (yes and no), fertilization (low and high), and location (inside and outside greenhouse), were used to examine the relationship between seedlings characteristics and their chemistry. Each treatment combination was replicated 10 times and statistical analyses were completed using R VEGAN and MASS packages (Oksanen et al., 2011). To determine the treatment effects on TNC, total N and other nutrients in the non-photosynthetic tissues, I used graphical vector analysis (GVA) (Timmer and Stone 1978). Since this technique requires a control treatment for all treatments, the seedlings with the conventional nursery growing conditions (under high fertilization and high light) were used as the control. GVA took content and concentration of TNC and nutrients into account to explain interactions between total dry weight of shoots and roots.

In order to group seedlings in terms of their similarity in TNC, total N and nutrient contents and concentrations across the eight treatments I used a linear discriminant analysis (LDA). In the current study, LDA incorporated different seedling characteristics, including growth (total dry weight, vertical and radial growth) and chemistry (micro and macronutrients and TNC).

Results

Impact of growing conditions on seedlings characteristics

All measured aspen seedling growth characteristics are summarized in Table 2.1. Statistical analyses are summarized in Tables A1 in the Appendix section.

Seedling shoot height was significantly influenced by all three treatment factors (shoot growth inhibitor, location (inside or outside the greenhouse), and fertilization) or their binary or tertiary interactions, except by the binary combination between shoot growth inhibitor and fertilization (Appendices Table A1). Seedlings treated with a high fertilization and grown inside the greenhouse (Treatments:1 , 3) had the highest shoot growth, regardless of the shoot growth inhibitor application. While the seedlings grown outside and that had been treated with a combination of low fertilization and/or an addition of the shoot growth inhibitor (Treatments: 2, 6, 8) were the shortest. As expected the shoot growth inhibitor application reduced shoot height of seedlings in both inside and outside the greenhouse, even under a high fertilization regime (Treatments: 5, 7).

All three treatment factors, their binary and tertiary interactions had significant impact on the root volume, except the binary combination between location and shoot growth inhibitor and between location and fertilization (Appendix Table A1). Root volume was greatest in fertilized seedlings regardless of the presence or absence of the other two factors (Treatments: 1, 5, 6), with the

exception of Treatment 3, where seedlings had been grown inside the greenhouse and were treated with high fertilization but not treated with the shoot growth inhibitor. On the other hand, root volume was the lowest in low fertilizer treatment regardless of the presence or absence of the shoot growth inhibitor or any location effect (Treatments: 2, 4, 6, 8).

Root collar diameter (RCD) was influenced by all three treatment factors, their binary or tertiary interactions, except the binary interactions between shoot growth inhibitor and fertilization or between the location and fertilization (Appendix Table A1). Overall, high fertilization had a positive effect on radial growth (i.e. RCD), however, seedlings kept outside the greenhouse and treated with high fertilization without the shoot growth inhibitor application had the largest RCD, while the seedlings in low fertilization without the shoot growth inhibitor application had the lowest RCD regardless of whether they were grown inside or outside the greenhouse (Treatments: 2, 4, 6, 8).

Only fertilization, its interaction with shoot growth inhibitor or tertiary interactions among three treatments significantly affected total dry weight (TDW) of aspen seedlings (Appendix Table A1). TDW was exclusively driven by fertilization and the seedlings that received the high fertilization regardless of the presence or absence of the other two factors had the largest TDW (Treatments: 1, 3, 5, 7) while seedlings treated with the low fertilization had the lowest TDW (Treatments: 2, 4, 6, 8).

With the exception of interactions between shoot growth inhibitor and the location or tertiary interactions, all individual treatments and two of the binary interactions (growth inhibitor x fertilization and the location × fertilization) significantly affected the root-to-shoot ratio (RSR) (Appendix Table A1). Seedlings grown outside and treated with the shoot growth inhibitor had the highest RSR, and fertilization had no impact on RSR (Treatments: 5, 6). Aspen seedlings with the lowest RSR were grown inside the greenhouse and treated with a high fertilizer regardless of whether they had received the shoot growth inhibitor or not (Treatments: 1, 3). All measured aspen chemical characteristics are summarized in Tables 2.2. and 2.3. Statistical analyses are shown in Tables A2 – A3 in the appendices section.

TNC content varied with all three individual treatments, binary interaction between the location and fertilization and tertiary interactions among three treatments (Appendix Table A2). In general, the aspen seedlings treated with high fertilization contained more carbohydrate content in their non-photosynthetic tissues than seedlings treated with low fertilization. In contrast to TNC content, TNC concentration was influenced by the shoot growth inhibitor or fertilization treatments or binary interactions among three treatments. The seedlings grown inside the greenhouse and treated with high fertilization with or without the shoot growth inhibitor had the lowest TNC concentration.

Total nutrient concentration was only influenced by shoot growth inhibitor. Total nutrient content was significantly influenced by the shoot growth inhibitor and fertilization, their binary interaction and tertiary interactions among

three treatments. In general, total nutrients and total N concentrations were higher in aspen seedlings under high fertilization regime. In contrast, the aspen seedlings treated with low fertilization had the lowest total nutrients and total N concentrations. There was no apparent effect of the location or shoot growth inhibitor on nutrient concentration. N, P and K contents of non-photosynthetic tissues were similar to the total nutrients or total N concentrations and aspen seedlings with high fertilization regimes contained higher N, P and K.

Differentiation of seedlings stock types

The linear discriminant analysis (LDA) revealed three distinct groups of aspen seedlings in terms of nutrient and carbohydrate contents or concentrations in their non-photosynthetic tissues (Fig. 2.1): (1) Low Nutrients-Low TNC, (2) High Nutrients-High TNC, and (3) High Nutrients-Medium TNC.

I further conducted the graphical vector analysis (GVA) to illustrate the combined effect of the location, fertilization and shoot growth inhibitor treatments on both content and concentration of TNC and total nutrients in non-photosynthetic tissues and total dry weight of roots and shoots (Figs. 2.2, 2.3). GVA analysis showed that aspen seedlings with low fertilization clustered together by having comparable total dry weight and similar content and concentrations of total nutrients (Fig. 2.2). In contrast, seedlings with high fertilization showed a different pattern. Although total dry weight was similar among high fertilized seedlings, they were different in terms of content and

concentrations of total nutrients: seedlings grown inside the greenhouse had the lowest content and concentrations of total nutrients, followed by seedlings treated with the high fertilization and grown outside the greenhouse. Further, seedlings grown inside the greenhouse and under high fertilization with the application of shoot growth inhibitor had the highest TDW, yet had relatively lower content and concentrations of total nutrients.

GVA patterns for the TNC content-concentration interactions and total nutrient content and concentration interactions were similar to each other (Fig. 2.3). There was only one exception, seedlings grown inside the greenhouse that had been treated with shoot growth inhibitor and high fertilization had similar TDW as the seedlings grown outside the greenhouse that had been treated with high fertilization and the shoot growth inhibitor, but the former had lower TNC content and concentrations than the latter. Under the outside conditions and high fertilization regime, the shoot growth inhibitor treatment increased TNC contents and concentrations compared to the control, while inside the greenhouse conditions, both concentration and content of TNC decreased, yet the decrease was less severe in seedlings treated with the shoot growth inhibitor. Under low nutrient regime TNC content decreased tremendously while the concentration was overall maintained by the outside conditions and the shoot growth inhibitor treatment. TNC concentration decreased in the absence of these two treatments.

Discussion

The results of this study suggest that nutrient availability appears to be the most important driver of the measured seedling characteristics measured. Seedlings grown under high resources conditions increased their radial and vertical growth, root volume, and total dry weight, however, nutrient content and concentrations in their non-photosynthetic tissues were low. Further, a possible antagonistic relationship between the location where seedlings were grown and the use of a shoot growth inhibitor existed in nutrient accumulation in the non-photosynthetic tissues of aspen seedlings. Aspen seedlings treated with the shoot growth inhibitor inside the greenhouse contained higher nutrient and carbohydrate contents and concentrations compared to those grown inside the greenhouse, but without a shoot growth inhibitor application. Surprisingly this interaction between shoot growth inhibitor and location did not exist in outside-grown seedlings, suggesting that the shoot growth inhibitor mimics outside conditions for seedlings when grown inside. Similar antagonistic relationships have been reported between light availability and shoot growth inhibitor in other plant systems (Pardos et al. 2005, Kurepin et al. 2007) and details are discussed in the paragraphs below.

Plant growth

Among the three treatment factors, the fertilization had overall the most influence on aspen seedling characteristics in the current study. Particularly seedlings that

received high fertilization had higher radial and vertical stem growth, root volume, and total dry weight. Earlier studies similarly reported that the use of NPK fertilizer enhanced aspen seedling growth (DesRochers et al. 2003, Pinno et al. 2012). The location (inside vs. outside) had also an impact on aspen seedling characteristics, including shoot height, root volume, root collar diameter and root:shoot ratio. Among these, inside greenhouse conditions reduced root volume, root collar diameter and root:shoot ratio, while outside conditions only reduced shoot height. How much of this response is driven directly by light or other conditions is not clear, as other environmental factors such as light quality, variations in day and night temperature and vapour pressure deficits are greater outside of the greenhouse.

In the current study, although the shoot growth inhibitor directly influenced both physical and chemical characteristics of seedlings, its interaction with fertilization resulted in mixed results as both factors had almost opposite effects on seedlings. For example, when seedlings received the high fertilization without the shoot growth inhibitor grew taller with larger root collar diameter. In contrast, when the same seedlings received both fertilization and the shoot growth inhibitor, high fertilization had no impact on the seedling characteristics. Further, the shoot growth inhibitor also reduced shoot height and radial growth particularly in fertilized seedlings inside the greenhouse. Low fertilized seedlings set bud earlier. In addition, total dry weight was overall higher in seedlings treated with the shoot growth inhibitor and subjected to a high fertilization treatment. This increase in total mass was likely the result of increases in root

mass and the overall carbohydrate content. Similar positive effects of shoot growth inhibitor on root growth were found in another aspen species (*Populus tremula* L.) (Žiauka and Kuusienė 2010).

Seedlings reserves (nutrients and carbohydrates) in non-photosynthetic tissues

Nutrients and carbohydrate content and concentrations in dormant tissues of aspen seedlings were also mostly responsive to the level of fertilization. The graphical vector analysis suggested that aspen seedlings grown under low fertilization (regardless of the presence/absence of the other two treatments) represent a distinct group in terms of their carbohydrate and nutrient contents and concentrations. Furthermore, the aspen seedlings grown under high fertilization were more responsive to the other treatments, such as shoot growth inhibitor or the greenhouse conditions (Figures 2.2, 2.3). This is likely because seedlings with low nutrient set bud earlier and therefore the other treatments were not effective. Interestingly, regardless of fertilization treatment, the shoot growth inhibitor had a positive effect on nutrient accumulation in aspen seedlings, particularly for seedlings grown outside the greenhouse. These observations are similar to studies that reported an increase in total nutrient accumulations in the terminal shoots of fructifying peach trees (Blanco et al. 2002) and in potassium accumulation in olive tree leaves (Navarro et al. 1989) and wheat tillers (Hajihashemi et al. 2009) after the shoot growth inhibitor application.

In the terms of carbohydrate reserves, the shoot growth inhibitor had a particularly positive effect on TNC accumulation in non-photosynthetic tissues when seedlings had high nutrients availability and were grown outside. This suggests that outside conditions also induced terminal bud set allowing seedlings to accumulate nutrients and TNC in their tissues. Under greenhouse conditions where the shoot growth inhibitor played the role of shoot growth termination and bud set, TNC in tissues did not accumulate even though total dry weight of the seedlings grown inside the greenhouse was higher. Similarly, Vu and Yelenosky (1992) found that the application of paclobutrazol, the same active ingredient of the shoot growth inhibitor used in the current study, increased the accumulation of TNC in orange tree (*Citrus sinensis* (L.) Osbeck cv. Valencia) roots, while decreased foliar TNC reserves. Further, the application of paclobutrazol enhanced carbohydrate accumulation in the woody tissue of Macadamia trees (*Macademia integrifolia* Maiden and Betche) (Stephenson et al. 1989). Further, a recent study in aspen reported an accumulation of TNC in root tissues following the application of the paclobutrazol or through the artificial shortening day length (Landhäusser et al. 2012). This earlier study further suggested that root TNC seems to be a reliable predictor of seedling establishment and early growth after planting. In terms of tree defenses, root TNCs were a better predictor of tree resistance to wood borers (Dunn et al. 1987, 1990).

While it is commonly known that carbohydrate reserves are highly affected by seasonality and phenology of plants, our findings also suggest that seedling reserves are significantly affected by the growing conditions. A recent

investigation with *Populus* species has suggested a possible molecular mechanism for the observed differences in carbohydrate reserves in non-photosynthetic tissues (Raj et al. 2011). This work showed that clonal poplars grown in different environments had different transcriptomes, including the TNC transcriptome, and tsuggested that this unexpected difference in metabolism between clones was mediated by resource availability, such as light and fertilization. While the time frame of this later study stretched over several growing seasons, the present study shows that the differentiation in reserve status starts from the very first growing season. The cumulative effect of time could be a venue for further investigation.

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Chapter II tables

Table 2. 1. Matrix of the different treatments obtained through the combination of different levels of fertilization and shoot growth inhibitor application inside and outside the greenhouse conditions and their corresponding aspen seedling classes.

Results are presented as (Mean ± Standard Error). LSD test was conducted. Means with the same letter within a column are not considered significantly different at p=0.05 (n=10).

Treatment No	Seedling Group	Treatments			Seedling Characteristics				
		Location	Fertilizer	Shoot growth inhibitor	Shoot height (cm)	Root Vol (cm^3)	RCD [a] (mm)	TDW [b] (g)	Root:Shoot Ratio
1	High Nut-High TNC	Inside	High	Yes	56.1±1.9 a	12.1±0.9 a	5.0±0.2 b	6.3±0.3 a	1.5±0.1 de
2	Low Nut-Low TNC	Inside	Low	Yes	25.3±1.5 cd	3.0±0.3 c	2.9±0.1 d	1.6±0.1 b	2.4±0.1 c
3	High Nut-High TNC	Inside	High	No	58.5±2.3 a	6.9±1.0 bc	5.6±0.2 ab	4.9±0.5 a	0.7±0.2e
4	Low Nut-Low TNC	Inside	Low	No	31.2±1.8 c	4.1±0.3 c	3.9±0.1 c	2.2±0.1 b	1.7±0.1 d
5	High Nut-Med TNC	Outside	High	Yes	29.5±1.5 c	13.4±1.6 a	5.3±0.2 ab	5.3±0.5 a	3.8±0.3 a
6	Low Nut-Low TNC	Outside	Low	Yes	20.0±0.8 d	4.8±0.5 c	3.7±0.1 c	2±0.1 b	3.5±0.2 ab
7	High Nut - Med TNC	Outside	High	No	45.9±3.2 b	9.9±0.9 ab	5.8±0.2 a	5.6±0.5 a	1.5±0.2 d
8	Low Nut-Low TNC	Outside	Low	No	23.5±0.7 cd	3.8±0.3 c	3.6±0.2 cd	1.9±0.1 b	2.8±0.2 bc
F-value					65.35	22.38	44.63	33.49	41.89
P-value					<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a] RCD: Root Collar Diameter. [b] TDW: Total Dry Weight. [c] TNC: Total Non-structural Carbohydrates.

Table 2. 2. Concentration (mg/gr tissue) and content (g/seedling) of total non-structural carbohydrate (TNC), total nutrients (micro and macronutrients including Nitrogen) of aspen seedlings treated with the combination of different levels of fertilization and shoot growth inhibitor application inside and outside the greenhouse conditions.

Results are presented as (Mean ± Standard Error). LSD test was conducted. Means with the same letter within a column are not considered significantly different at p=0.05.

Treatment No	Seedling Group	Seedling Characteristics			
		TNC [a] (mg/gr tissue)	TNC [b] (g/seedling)	Total Nutrients (mg/gr tissue)	Total Nutrients (g/seedling)
1	High Nut-High TNC	286 ± 1.1 cd	1.83 ± 0.1 ab	205.2 ± 0.8 bc	129.5 ± 6.6 a
2	Low Nut-Low TNC	353 ± 1.0 ab	0.57 ± 0.1 d	210.2 ± 0.3 b	33.5 ± 2.3 c
3	High Nut-High TNC	248 ± 0.8 d	1.26 ± 0.2 bc	180.6 ± 0.4 c	88.0 ± 8.5 b
4	Low Nut-Low TNC	305 ± 1.3 bc	0.66 ± 0.1 cd	200 ± 0.3 bc	43.3 ± 2.4 c
5	High Nut-Med TNC	393 ± 1.8 a	2.1 ± 0.2 a	242.8 ± 1.2 a	124.7 ± 7.7 a
6	Low Nut-Low TNC	335 ± 1.3 bc	0.66 ± 0.1 cd	207.1 ± 0.4 bc	41.6 ± 2.6 c
7	High Nut-Med TNC	348 ± 1.3 ab	1.94 ± 0.2 a	199.4 ± 0.7 bc	108.9 ± 7.9 ab
8	Low Nut-Low TNC	352 ± 0.7 ab	0.67 ± 0.1 cd	179.3 ± 0.4 c	34.0 ± 2.0 c
F-value		15.437	23.45	10.69	51.92
P-value		<0.001	<0.001	<0.001	<0.001

[a] TNC: Total Non-structural Carbohydrates (sum of starch and soluble sugars)
[b] Only roots and shoots were included in this estimation.

Table 2. 3. Nitrogen, Phosphorus and Potassium content of non-photosynthetic tissues of aspen seedlings treated with the combination of different levels of fertilization and shoot growth inhibitor application inside and outside the greenhouse conditions.

Results are presented as (Mean ± Standard Error). LSD test was conducted. Means with the same letter within a column are not considered significantly different at p=0.05.

Treatment No	Seedling Group	Seedling Characteristics					
		Nitrogen (mg/g tissue)	Nitrogen [a] (mg/seedling)	Phosphorus (mg/g tissue)	Phosphorus (mg/seedling)	Potassium (mg/g tissue)	Potassium (mg/seedling)
1	High Nut-High TNC	7.12±0.3 bc	44.93±2.4 a	1.98±0.1a	8.6±0.5 ab	4.96±0.2 bcd	31.33±1.6 a
2	Low Nut-Low TNC	6.64±0.26 bc	10.48±0.6 c	1.71±0.1ab	2.57±0.2 c	5.37±0.1 ab	8.63±0.7 c
3	High Nut-High TNC	6.64±0.3 bc	31.93±2.9 b	1.7±0.1 b	6.72±0.7 b	4.26±0.1 d	20.69±2.0 b
4	Low Nut-Low TNC	6.29±0.2 bc	13.57±0.7 c	1.7±0.1 b	3.62±0.2 c	4.97±0.2 bc	10.75±0.6 c
5	High Nut-Med TNC	10.12±0.8 a	50.8±2.5 a	1.67±0.1 b	10.19±0.6 a	5.77±0.2 a	30±2.4 a
6	Low Nut-Low TNC	6.71±0.2 bc	13.5±0.9 c	1.61±0.1 bc	3.41±0.3 c	5.41±0.2 ab	10.86±0.8 c
7	High Nut - Med TNC	7.89±0.5 b	42.81±3.3a	1.38±0.1 c	9.29±0.7 a	4.75±0.1 bcd	26.22±2.2 ab
8	Low Nut-Low TNC	6.03±0.2 c	11.38±0.7 c	1.36±0.1 c	3.24±0.2 c	4.38±0.1 cd	8.34±0.6 c
F-value		11.65	66.32	10.62	42.07	11.67	40.74
P-value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a] Only roots and shoots were included in this estimation.

Figure 2. 1. Linear Discriminant Analysis (LDA) based on the growth and chemical characteristics of aspen seedlings prior to dormancy. Aspen seedlings were treated with the combination of different levels of fertilization and shoot growth inhibitor grown either inside or outside the greenhouse.

✳ indicates centroids of different classes of seedlings, **SDW:** Shoot Dry Weight, **RDW:** Root Dry Weight, **SH:** Shoot Height, **TNK:** Total Nitrogen content, **TNC:** Total Non-structural Carbohydrates contents (sum of soluble sugars and starch), **RCD:** Root Collar Diameter.

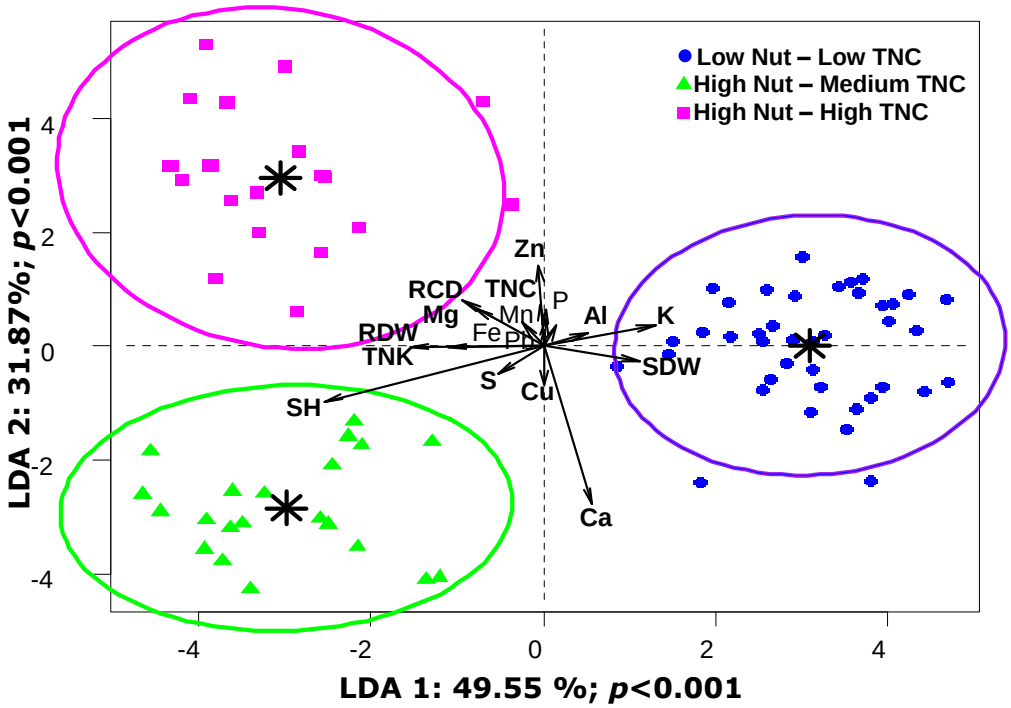


Figure 2. 2. Graphical vector analysis of total nutrients reserves (micro and macro-nutrients) in the non-photosynthetic tissue of aspen seedlings. Aspen seedlings were treated with the combination of different levels of fertilization and shoot growth inhibitor applications and grown in either inside or outside the greenhouse.

(H: High, L: Low, F: Fertilization, L: Location, GR: Shoot growth inhibitor, ▲: Seedlings with High Nutrient contents and average TNC contents in non-photosynthetic tissues. ■: Seedlings with High Nutrient and High TNC contents in non-photosynthetic tissues. ●: Seedlings with Low Nutrient contents and Low TNC contents in non-photosynthetic tissues. ⊕: Control)

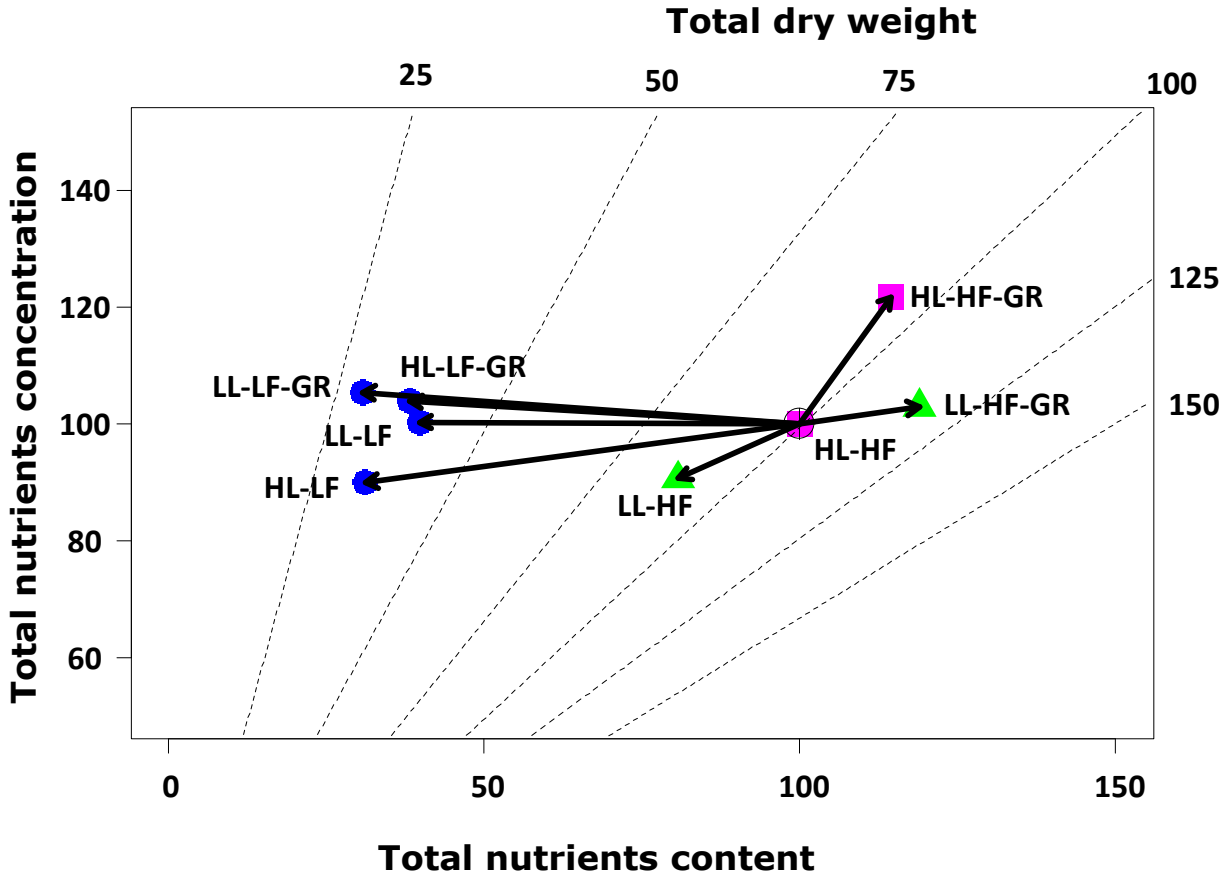
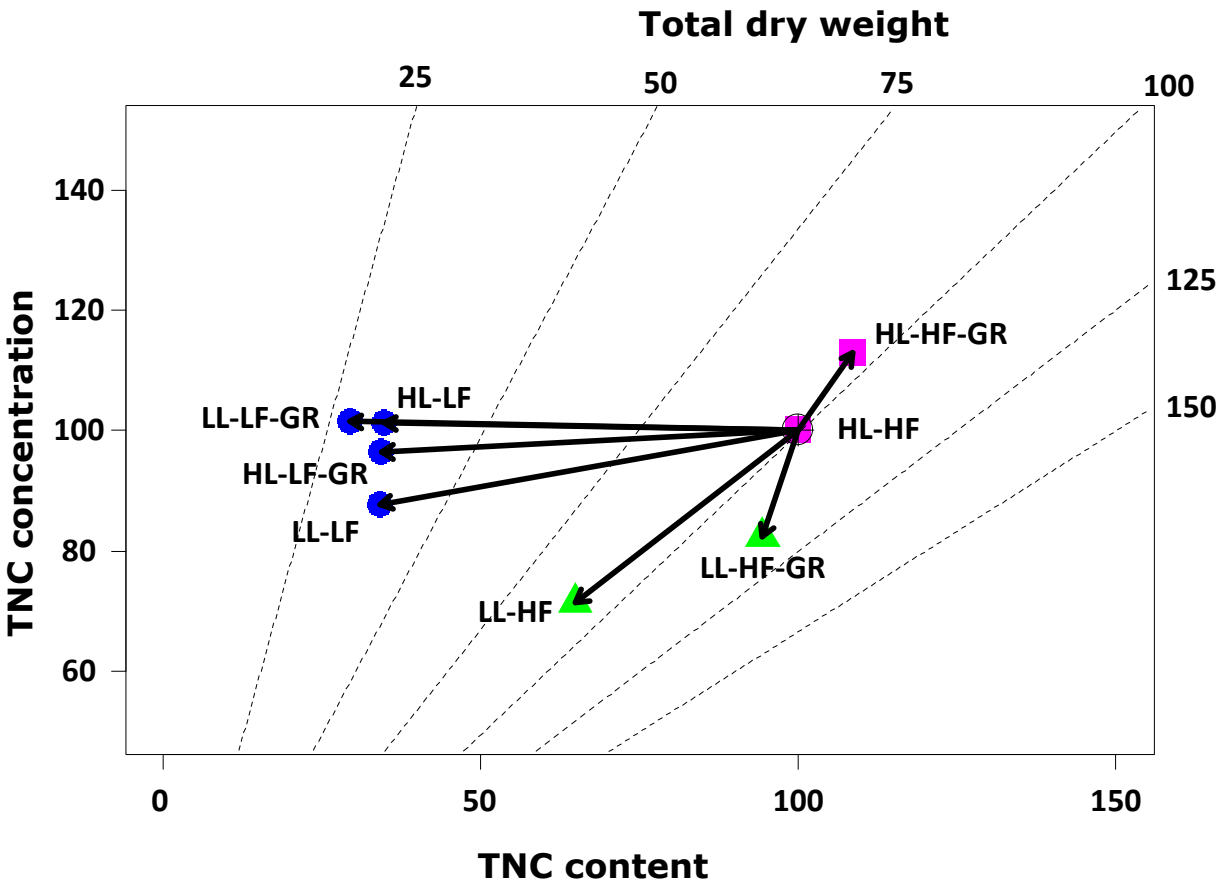


Figure 2. 3. Graphical vector analysis of total non-structural carbohydrates (TNC) contents in non-photosynthetic tissues of aspen seedlings. Aspen seedlings were treated with the combination of different levels of fertilization and shoot growth inhibitor applications and grown in either inside or outside the greenhouse.

H: High, L: Low, F: Fertilization, L: Location, GR: shoot growth inhibitor. ▲: Seedlings with High Nutrient contents and average TNC contents in non-photosynthetic tissues. ■: Seedlings with High Nutrient and High TNC contents in non-photosynthetic tissues. ●: Seedlings with Low Nutrient contents and Low TNC contents in non-photosynthetic tissues. ⊕: Control)



Chapter III. The role of reserves of non-photosynthetic tissues in plant defense. Impact on our current understanding of plant defense theories

Introduction

Trees deploy a combination of constitutive (pre-existing) and induced (post-attack), structural and biochemical mechanisms against herbivores and pathogens (Franceschi et al. 2005, Ralph 2009, Eyles et al. 2010). Constitutive defenses are always present in a tree to discourage attackers, whereas induced responses are triggered by tissue damage and aim to limit further damage from attacking organisms (Bonello et al. 2006, Eyles et al. 2010, Reid and Purcell 2011). However, the development of defense mechanisms against these forces is metabolically costly for plants and requires resources in form of carbohydrates or nutrients (Frost et al. 2008).

In deciduous plants, carbohydrates, such as starch and lipids, and nutrients, such as amino acids and macro and micro nutrients, can be reserved in non-photosynthetic tissues. These reserve tissues can play important roles in the growth or regrowth of new foliage (Sprugel 2002, Oyarzabal and Oesterheld 2009, Landhäusser and Lieffers 2012), defense (Dunn et al. 1987, 1990), and symbiosis (Kozlowski 1992). These reserves can also serve as a safeguard for plants during times of stress induced by biotic such as insect herbivory; or abiotic factors such as drought or cold (Kaplan and Guy 2004, McDowell et al. 2008, McDowell 2011, Anderegg et al. 2012, Sala et al. In-press). This is particularly

relevant for perennial species that are exposed to multiple stresses throughout their lifetime.

Currently two major theories along with others are proposed that generate seemingly divergent predictions between growth and defense in plants: the Carbon-Nutrient Balance (CNB) (Bryant et al. 1983, Bazzaz et al. 1987) and the Growth Differentiation Balance (GDB) (Herms and Mattson 1992) hypotheses. The CNB hypothesis predicts that the carbon/nitrogen (C/N) ratio in plants determines which type of secondary metabolites will be synthesized. For instance, if plants growing in carbon-limited environment, such as shading, but can access nitrogen, rely on nitrogen-based toxins, whereas plants growing in nitrogen-poor soils can utilize carbon-based defenses. Under this hypothesis plants can switch their defense types (carbon vs. nitrogen-based) in response to the available resources. The GDB hypothesis proposes that chemical defenses are only produced by plants when carbon is available (after growth), and therefore growth and defense compete for the same resources in response to resource availability. For example, carbon supply in plants growing in a photosynthesis-limiting environment, as a result of low water, light, or nutrient availability, could limit both growth and defense. In principle, this hypothesis predicts that rapidly growing plants contain lower levels of secondary metabolites and vice versa.

There are numerous examples supporting both hypotheses. However, recent reviews have revealed numerous exceptions to each hypothesis, and emphasize the need to better define the specific plant biochemical pathways and insect behavioral traits under which each is likely to operate and develop synthetic

models (e.g., Larsson 1989, Gershenzon 1994, Herms and Raffa 1995, Koricheva et al. 1998, Stamp 2003, Boege and Marquis 2005). One shortcoming of these hypotheses is that they discount the importance and function of non-photosynthetic tissues as a reservoir that could be available for plant defenses and growth during times of reduced photosynthesis. I submit that this omission could compromise our understanding of plant defenses against herbivores. Although relatively few studies have examined the role of reserves stored in the non-photosynthetic tissues and their potential role in plant defenses (Dunn et al. 1987, 1990, De Souza Cândido et al. 2011, Sampedro et al. 2011, Landhäusser and Lieffers 2012), evidence is emerging that the strength of constitutive and induced defenses can be partly driven by the available resources in these tissues. Collectively, these studies suggest that severe carbon stress resulting from limitations (e.g. due to drought) in photosynthetic function or destruction of photosynthetic tissue (e.g. defoliation) may constrain growth and defense, thus highlighting the importance of plant reserves in non-photosynthetic tissues in plant defense and growth (Simms 1992, Redman et al. 2001). For example, when leaves are damaged or removed by herbivores, especially during repeated and severe defoliations, the basic functions of plant and the renewal of the foliage are supported by resources stored in the non-photosynthetic tissues (Babst et al. 2005, 2008, Donaldson et al. 2006, Thiébeau et al. 2011). In fact, if these reserves are depleted or low before new photosynthetic tissue is fully functional, plant growth and survivorship could be compromised even under resource-rich environments (Man et al. 2008, Zhao et al. 2008, Landhäusser et al. 2012). However, neither

the role of long-distance transport of reserves from and to different non-assimilating parts in woody plants nor the impact of reserve proximity to carbon sinks are clearly understood, particularly for large plants such as mature trees (Landhäusser and Lieffers 2003, 2012, Sala et al. In-press).

In this study I tested whether the interaction between plants and their insect defoliator is mediated by the resources stored in the non-photosynthetic. I used trembling aspen (*Populus tremuloides* Michx.) – Forest tent caterpillar (*Malacosoma disstria* Hübner) (Lepidoptera: Lasiocampidae) system to investigate how nutrients and carbohydrate reserves stored in non-photosynthetic tissues function during herbivory. I selected aspen, because it is one of the most prominent tree species in the boreal forest of North America. Furthermore, this species has been investigated to explore the complex interactions between growing conditions and defensse in the view of plant defense-growth-resource interactions, linking to the CNB and GDB hypotheses. In these studies the trade-offs between defense and growth under different CO_2, fertilizer, or light levels were tested (Bryant et al. 1987, Osier and Lindroth 2001, Lévesque et al. 2002, Donaldson et al. 2006, Donaldson and Lindroth 2007). However, they focused only on the quality of the foliage material and did not account for reserves in the non-photosynthetic tissues of aspen.

Forest tent caterpillar (FTC) is one of the most important defoliators of aspen forests in the North America. It is native to North America and frequently outbreak and causes serious damage to aspen in the boreal forest (Colasurdo et al. 2009, Wood et al. 2009). Although low or medium defoliations rarely kill aspen

(Hogg et al. 1999) they weaken the trees making them susceptible to other insects, diseases, or abiotic stresses such as drought (Man et al. 2008).

The objective of this study was to investigate whether nutrients and carbohydrates stored in the non-photosynthetic tissues of aspen seedlings prior to leaf flush could alter the constitutive and induced foliar defenses, and to examine whether differences in aspen defenses could affect the larval development of FTC.

Materials and Methods

Aspen seedlings prepared in the Chapter II were utilized in the following feeding experiment. Seedlings characteristics were reported in Tables 2.1-2.3 in the Chapter II. For the details how seedlings were prepared, please see Material and Method section of the Chapter II.

Feeding experiment

On April 12 2010, 128 dormant seedlings generated in 2009 were thawed and potted in 500mL pots, containing soil mix with peat:clay:perlite (1:0.2:0.2) and transferred to a growth chamber. The growth chamber conditions were set at 22˚C, 60% RH and a 16:8 light:dark period. Seedlings were arranged in a completely randomized block design where every block contained two representative seedlings from each of the eight seedling types (seedling types were described in Tables 2.1-2.3). One seedling of each of the seedlings types in each block was randomly assigned to the herbivory treatment while the other served as an untreated control.

FTC egg masses were collected from an outbreak population in Prince George, BC, Canada by Dr. Staffan Lindgren (University of Northern British Colombia). Egg masses were stored at 4˚C and shortly before use, the egg masses were disinfected in 6% sodium hypochlorite (Grisdale 1985). When buds started to flush, egg masses of FTC were tied to the seedlings to mimic the natural synchrony between hatching and bud break. Once the egg mass was placed, mesh bags were put over seedlings (including the untreated control) to prevent the caterpillars from escaping (Fig. 3.1). Larval emergence occurred from May 3 to May 13, 2010 with a 100% hatching success. The maximum instar attained on each seedling was recorded at the end of the experiment (mid July 2010). The average maximum instar reached was used as an indicator of larval development. At the end of the experiment, foliage of the aspen was used to determine their phenolic glycoside, TNC and N concentrations.

I did not conduct any chemical analysis in non-photosynthetic tissues of aspen seedlings after the feeding experiment and rather used the seedling characteristics prior to the dormancy as described in the Chapter II (Tables 2.1-2.3).

Extraction and quantification of phenolic glycosides of aspen foliage

Once the feeding experiment was terminated, foliage of herbivory and control seedlings were collected and placed into paper bags and stored at -40˚C until further processing. Foliage was freeze dried for 72 hrs and ground in liquid

nitrogen. The resulting powder was stored at -40˚C until chemical extraction. For the chemical extraction, about 25 mg of leaf tissue powder was added to 1.5 ml methanol and placed in an ultrasonic bath at 4˚C for 30 min, and then centrifuged at 13000 rpm for 15 min. The supernatant was transferred to 1.5 ml glass vials and stored at -20˚C.

The analysis of phenolic glycosides was performed on Alliance 2690 HPLC separation module (Waters, Milford, MA, USA) equipped with an auto sampler and a Waters 996 Photodiode Array detector using Thermo ODS Hypersil, 250 mm length, 4.6 mm inner diameter and 5µM particle size. The autosampler was set at 4˚C and the column temperature was 28-30˚C. The binary mobile phase was water/acetic acid (98:2, v/v) (Phase A) and methanol/acetic acid (98:2, v/v) (Phase B). At a flow rate of 1ml/min, the elution program was as follows (percentages refer to the proportion of Phase B): 0 – 35% (0 – 20 min), 35 – 65% (20 – 35 min), 65 – 80% (35 – 36 min), 80 – 100% (36 – 37 min), 100% (37 – 38 min), 100 – 0% (38 – 39 min), 0% (39 – 40 min). The injection volume was 15µl and the scanning range was 200 – 400 nm with a monitoring and a processing of the quantification data performed at 274nm.

Figure 3. 1. Experimental setting with the mesh bags applied on all the seedlings.

The quantification was made possible through the use of pure standards: tremulacin, salicin and salicortin were provided by APIN chemicals Ltd. (Oxfordshire, UK) while tremuloidin was provided by Dr. Richard Lindroth (University of Wisconsin-Madison, USA). Standards were pooled in a concentrated stock solution that was diluted to generate a standard curve with five points. The standard pools were run three times and an average mean was recorded and a linear regression line fitted with an $R^2 = 0.989$. Both the extraction and the quantification of phenolics were developed and optimized in Dr. Bonello's Lab at the Ohio State University.

Analyses of total non-structural carbohydrate (TNC) and nitrogen (N) of aspen foliage

Foliage was dried for three days at 70˚C then ground in mini Willey Mill (Thomas Scientific Willey Laboratory Mill, Swedesboro, NJ) to pass a 40 mesh (20 mm). The resulting powder was extracted for soluble sugars three times using hot 85% ethanol and then analysed colorimetrically using phenol–sulphuric acid determined at 490 nm (Chow and Landhäusser 2004). Following sugar extraction, the starch in the remaining residue was digested with α-amylase (ICN 190151, from *Bacillus lichenformis*) and amyloglucosidase (Sigma A3514, from *Aspergillus niger*) and glucose equivalents were determined colorimetrically with peroxidase-glucose oxidase-o-dianisidine (Sigma Glucose Diagnostic Kit 510A). Combined the soluble sugars and starch formed the TNC concentrations. Foliar N was quantified by Elemental Combustion Analyzer (ECS 4010 Elemental Combustion System CHNS-O, Costech Analytical Technologies Inc., Valencia, CA.).

Derived variables

In the Chapter 2 and in this chapter, I measured nutrient and TNC concentrations and contents of non-photosynthetic tissues (stem and roots pooled), and only the concentrations of foliar N and foliar TNC. From these and the dry weight measurements I derived the following variables: (a) nutrient content and concentration of non-photosynthetic tissues (micro and macronutrients stored in non-photosynthetic tissues), (b) foliar N (N concentration in foliage), (c) TNC of non-photosynthetic tissues (TNC stored in non-photosynthetic tissues), (d) foliar

TNC (TNC stored in foliage), (e) TNC/N ratio of non-photosynthetic tissues (TNC concentration of non-photosynthetic tissue / N concentration of non-photosynthetic tissue), and (f) foliar TNC/N ratio (foliar TNC concentration / foliar N concentration).

Statistical Analysis

The statistical design for the seedling production was a three factorial design with location (inside and outside), fertilizer (high and low) and shoot-growth inhibitor treatment (presence and absence) treatments. The herbivory experiment was designed as a randomized block design where every block had two representatives from each of the seedling type.

After foliage was flushed I investigated the effect of both TNC and nutrient concentration of non-photosynthetic tissues and the carbohydrate and N concentrations of newly flushed foliage on the constitutive chemicals of foliage using RDA (rdaTest package in R (Legendre and Durand, 2010). RDA is a non-symmetric method that operates by extracting the components of the explanatory matrix in way that makes them as closely correlated with the response matrix and then it does the same with the components extracted from the response matrix in order to correlate them with the explanatory matrix. In general, RDA requires two matrices: First, the explanatory variables matrix which included Calcium (Ca), Potassium (K), Magnesium (Mg), Phosphorus (P), Manganese (Mn), and TNC/N ratio of non-photosynthetic tissues in this study. Second, the response

variables matrix included four phenolic glycoside compounds, tremulacin, tremuloidin, salicin, and salicortin, and TNC/N ratio in foliage.

Pearson coefficient of correlation was used to assess the effect of phenolic glycosides on larval performance. To explore the roles of nutrients and TNC in non-photosynthetic tissues, foliar nitrogen and TNC, and induced total phenolic glycosides on the larval response, multiple linear regression techniques were applied (Microsoft Excel 2011).

Results

As I described in the Chapter II, aspen seedlings were grouped into three distinct groups depending on the TNC and nutrient contents of the non-photosynthetic tissues of the dormant aspen seedlings (Fig. 2.1, Tables 2.1-2.3): (1) Low Nutrient (Nut) – Low TNC, (2) High Nut – Medium TNC, and (3) High Nut – High TNC. Group 1 (Low Nut-Low TNC (seedling types: 2, 4, 6, 8)) was characterized by low nutrients, particularly Aluminium (Al), Ca, K, P, low TNC, and small seedling size. Groups 2 (High Nut-Medium TNC (seedling types: 5, 7)) and 3 (High Nut-High TNC (seedling types: 1, 3)) were characterized by their high nutrient contents, particularly by Nitrogen (N) and Mg, Mn, Iron (Fe), Lead (Pb), Sulfur (S), Copper (Cu), and Zink (Zn). However, these two groups were distinguished from one another by their TNC contents, where the seedlings in the Group 3 had much higher TNC than the seedlings in the Group 2.

Influence of TNC and nutrient contents of non-photosynthetic tissues on the constitutive foliar chemistry

Overall, aspen seedlings in the Groups 2 and 3 (High Nut – Medium TNC and High Nut – High TNC respectively) had higher foliar TNC/N ratio than seedlings in the Group 1 (Low Nut – Low TNC) (Fig. 2.1, Table 3.1). I did not observe any interaction between nutrient content of non-photosynthetic tissues and foliar phenolic glycosides as indicated by the distribution of the centroids of the different seedling groups.

In a more detailed analysis, the RDA indicated significant interactions between TNC and nutrient content of non-photosynthetic tissues and the constitutive phenolic glycosides (p=0.04 for vertical and p=0.004 for horizontal separations) (Fig. 3.2, Table 3.1). There were several significant interactions between nutrients and TNC/N ratio of non-photosynthetic tissues as well as between foliar TNC and foliar phenolic glycosides. First, I observed an inverse relationship between nutrient content (P, Ca, K, Mg, and Mn) and TNC/N ratio in the non-photosynthetic tissues where seedlings with higher nutrients had lower TNC/N ratio (Groups 2 and 3) and vice versa (Fig. 3.2). Second, seedlings with higher nutrients or lower TNC/N ratio in non-photosynthetic tissues produced foliage with higher TNC/N ratio and lower constitutive phenolic glycosides (Fig. 3.2). In contrast, seedlings with lower nutrients or higher TNC/N ratio in non-photosynthetic tissues (Group 1) produced more salicortin in their foliage, but there was no relationship for the remaining phenolic compounds (Fig. 3.2). Third, foliage with higher TNC/N ratio had more salicin and tremuloidin but less

tremulacin and salicortin contents compared to the foliage with low TNC/N ratio. When foliar TNC/N ratio was low, tremulacin was high. Fourth, RDA showed a significant positive relationship between Mn content in non-photosynthetic tissues and the amount of tremulacin produced in the foliage.

Influence of TNC and nutrient content of non-photosynthetic tissues on the induced foliar chemistry

Overall, at the induced level, aspen seedlings in Groups 2 and 3 had less phenolic glycosides, than seedlings in Group 1 (Fig. 3.3, Table 3.1).

In a more detailed analysis to understand the role of TNC and nutrient content of non-photosynthetic tissues on the foliar phenolic glycosides, I kept the same explanatory matrix as described in the previous section (*Influence of TNC and nutrient contents of non-photosynthetic tissues on the constitutive foliar chemistry*) and replaced the foliar phenolic glycosides associated with constitutive defense with the induced in the response matrix. The RDA indicated that seedlings with high TNC/N ratio in the non-photosynthetic tissues had higher phenolic glycosides and lower TNC/N ratio in the foliage. Further, foliage with high TNC/N ratio (Groups 2, 3) had less induced phenolic glycosides than foliage with lower TNC/N ratio (Group 1).

Influence of TNC and nutrient content of non-photosynthetic tissues and foliar chemistry on FTC herbivory

Overall the highest instars of FTC was reached when larvae fed on seedlings in Groups 2 and 3 which contained high nutrients and medium or high TNC content in the non-photosynthetic tissues (Fig. 3.4, Table 3.1). I found a strong inverse relationship between the constitutive foliar phenolic glycosides and the overall larval performance, as shown by the Pearson coefficient of correlation and the corresponding significant *P*-value (Fig. 3.4, r= - 0.81, p=0.01). Whereas the correlation between the induced foliar phenolic glycosides and the larval performance was less important (Fig. 3.5, r= - 0.63, p=0.09).

Multiple regressions indicated that the overall model was significant and suggested that 87% of the variation in the average instar stages observed across different seedling groups was due to the variation in foliar TNC, N, and phenolic glycosides (F= 8.58, p=0.03, Table 3.2). The model also indicated that N concentration of aspen foliage had a positive impact (t=3.71, p=0.02) while phenolic glycosides had a negative impact on the larval performance (t=-3.54, p=0.02).

Since there was a negative association between foliar phenolic concentration (both at constitutive and induced levels) and nutrients in the non-photosynthetic tissues and a positive association between induced foliar phenolic glycosides and TNC/N ratio in non-photosynthetic tissues, I further investigated the relationship between larval performance and the nutrient contents in the non-photosynthetic tissues. I added a component of plant growth (change in the plant

height from the initial seedling height to the final seedling height) in order to highlight a possible trade-off between defense and plant growth. In a multiple regression, I included nutrients and TNC content of the non-photosynthetic tissues and plant growth as predictor variables and the larval performance as a response variable. Larval performance was positively related to the nutrient content ($t=5.43$, $p=0.005$) while it was inversely related to TNC ($t=-2.97$, $p=0.04$) and plant growth ($t=-4.77$, $p=0.009$) ($R^2=94\%$, $F=20.52$, $p=0.007$, Table 3.3).

Since the inverse relationships between phenolic glycosides and larval performance and between larval performance and TNC contents in non-photosynthetic tissues of the seedlings, I further analyzed the relationship between foliar phenolic glycosides and foliar N and foliar TNC concentrations using multiple regressions by replacing larval performance by phenolic glycosides as response variable. There was no relationship between the induced level of phenolic glycosides and foliar N ($t=0.45$, $p=0.64$) or foliar TNC ($t=-1.51$, $p=0.19$) ($R^2=35\%$, $p=0.34$, $F=1.36$, Table 3.4).

Using different explanatory variables, overall 88% of the phenolic glycosides variation was explained by nutrients and TNC in non-photosynthetic tissues and plant growth ($R^2=88\%$, $F=10.07$, $p=0.025$, Table 7). However, there was no relationship of the phenolic glycosides with nutrients in the non-photosynthetic tissues ($t=-0.96$, $p=0.39$), with TNC in the non-photosynthetic tissues ($t=0.35$, $p=0.74$), or with plant growth ($t=-0.13$, $p=0.91$) (Table 3.5).

Discussion

Neither the CNB nor the GDB hypotheses currently recognize the influence of reserves and the allocation of these reserves from the non-photosynthetic tissues (stems and roots) to the foliar tissues and their impact on the plant response to herbivory. The results of the current study provide several new lines of evidence that TNC and nutrients stored in the dormant stems and roots of aspen seedlings can affect the TNC, N and defense chemistry of foliage, and the overall plant responses to herbivory.

First, the TNC/N content ratio was four times higher in aspen seedlings in the Group 1 (Low Nut-Low TNC) than the seedlings in the Groups 2 and 3, despite the high TNC and nutrient contents in stems and roots of the latter groups. However, the high TNC/N ratio in dormant stems and roots did not translate into increased levels of constitutive foliar phenolic glycoside compounds identified. I found only one positive correlation between the TNC/N ratio with salicortin. On the other hand, tremuloidin and salicin content were only high when the foliar TNC/N ratio was high, while tremulacin and salicortin were only high when foliage TNC/N ratio was low, suggesting a complex relationship between foliar TNC/N ratio and the individual phenolic glycoside compounds. In contrast, induced phenolic glycosides were correlated only with the TNC/N ratio in non-photosynthetic tissues, not with the foliar TNC/N ratio. In an earlier study, Karowe and Grubb (2011) suggested a strong relationship between the foliar CN ratio and the foliar soluble phenolics at both the constitutive and induced levels in *Brassica rapa* although the relationship between the TNC/N ratio of the non-

photosynthetic tissue and foliar phenolics was not reported. The result in the present study suggest that TNC/N ratio of stems and roots vs. the foliage play different roles in the synthesis of secondary compounds at the constitutive and induced levels, and that TNC and nutrient contents of both tissue types should be further investigated to strengthen the current understanding of the role of carbohydrate and nutrient reserves in different tissues in plant-insect interactions.

The positive relationship between Mn contained in the non-photosynthetic tissues and tremulacin in foliage is interesting and suggests that stored Mn might be a co-factor for tremulacin synthesis and possibly for other defense compounds in aspen. The role of Mn in phenolic accumulation has been shown in wheat, where seedlings fertilized with Mn synthesized more phenolics and showed improved resistance to pathogens (Rengel et al. 1993). However, since I did not determine the foliar Mn content and its possible interaction with tremulacin in the current study, this result should be viewed with caution.

Second, there were significant interactions between TNC content in stems and roots of aspen seedlings and the foliar TNC/N ratio, and phenolic glycosides at the constitutive and induced levels. At the constitutive level, aspen seedlings with high stem and root TNC had also high TNC concentrations in their foliage, suggesting a possible allocation pathway of TNC between non-photosynthetic tissues and foliage within aspen seedlings. Likewise, Landhäusser (2011) has recently demonstrated that TNC reserves in twigs are sufficient to support the new flush of foliage in aspen seedlings and that twigs become quickly carbon autonomous. In another study it is suggested that higher carbon reserves in the

roots of aspen allow for new fine root growth and therefore more efficient transport of water and nutrients to the leaves resulting in higher photosynthetic rates and reserves (Landhäusser and Lieffers 2012). In the current study, when aspen seedlings with high foliar TNC were challenged with herbivory, the level of TNC in defoliated seedlings was reduced and became similar for all seedling types regardless of their initial TNC content in their non-photosynthetic tissues. I suspect that the high TNC and high nutrient concentrations in seedlings of Groups 2 and 3, were possibly used for growth rather than defense, as these seedlings had the lowest induced phenolic glycosides. This suggests that aspen with abundant resources (high nutrients and high TNC in non-photosynthetic tissues) would favor tolerance over resistance against defoliators (Donaldson and Lindroth 2007). This preferential resource allocation to tolerance likely supported the additional growth in aspen to recover from damage, as I found higher root volume and radial growth of aspen seedlings in Groups 2 and 3 after defoliation relative to the aspen seedlings in the Group 1. In contrast, seedlings with the low nutrient and low TNC reserves showed resistance by investing in induced chemical defenses (Stevens et al. 2007, 2008). Similar compensatory growth has been reported in other systems (Hjältén et al. 1993, Lovelock et al. 1999, Bast and Reader 2003, Rogers and Siemann 2003, Baker et al. 2005, Zou et al. 2005, King et al. 2008).

Alternatively, the depletion of foliar TNC after defoliation in the aspen seedlings with high TNC and high nutrients in the non-photosynthetic tissues could also portray a "strategic retreat" of resources (Orians et al. 2011).

According to this ricent view, plants reallocate and store their resources from the damaged photosynthetic tissues to non-photosynthetic tissues to avoid total loss of these resources to herbivory. After jasmonic acid, a plant hormone known to mimic herbivory damage, was applied to poplar, Babst et al. (2005) tracked down starch stored in leaves using ^{11}C isotope and found that the starch was moved from the foliage to the stems and roots. I currently do not know whether this mechanism also applies to aspen and about the fate of these retracted resources, but they could have been used either to increase tree resistance against FTC or support post-herbivory plant tolerance.

Third, there was an inverse relationship between N content of non-photosynthetic tissues and foliar N concentration, where the latter was higher in seedlings with low N content in stem and root tissues. However, after herbivory, foliage N content was similar among the three seedling groups. This suggests two possible mechanisms of N depletion in the foliage: N either decreased due to the direct consumption of foliage by the FTC (i.e., more N was consumed along with the defoliated foliage) or N was used for the production of phenolic glycosides increase tree resistance. The current study provided evidence for both possibilities. First, I found that aspen seedlings with N rich foliage was defoliated more than those with N poor foliage, suggesting that foliage quality could act as feeding simulants and be as important as plant secondary chemicals for herbivory (Lindroth et al. 1993, Hwang and Lindroth 1997, Roth el al. 1998, Noseworthy and Despland 2006). Likewise, earlier studies found that FTC larvae can thrive on foliage with high amounts of carbohydrates and nutrients or low TNC/N ratio

even though the same foliage also contained high amounts of secondary metabolites (Lindroth and Bloomer 1991, Fitzgerald 1995, Couture et al. 2011). Furthermore, aspen seedlings that had high TNC/N ratio in their non-photosynthetic tissues were able to produce foliage with a high amount of phenolic glycosides, which reduced the larval growth in the current study. Whereas the seedlings with low TNC/N ratio in non-photosynthetic tissue – even though they had high amounts of carbohydrates and nutrients – sustained more damage from defoliation, contained less phenolic glycosides and supported longer larval feeding. Interestingly phenolic glycosides were linked to TNC and nutrients in non-photosynthetic tissues, which might indicate that translocation of reserves from non-photosynthetic tissues is necessary to synthesize the phenolic glycosides in the foliage.

In the case of our other prediction about allocation N to support plant resistance, aspen seedlings with high foliar N produced more phenolic glycosides in response to defoliation than seedlings with low foliar N. Even though phenolic glycosides are carbon-based defenses, this type of defense sometimes requires N-based enzymes to achieve their synthesis (Gershenzon 1994). These results contradict the CNB hypothesis, where carbon-based compounds, such as phenolic glycosides should be favored by carbohydrate accumulation, but penalized by N accumulation (Bryant et al. 1983). The positive relationship between the nutrient content of stems and roots in seedlings and the foliar TNC/N ratio at the induced level also suggests a possible N mobilization from the non-photosynthetic tissues to the foliage for the production of phenolic glycosides.

Finally, larval performance was positively influenced by the nutrient content of non-photosynthetic tissues, but negatively affected by TNC content of stem and roots. When I used plant TNC reserves and plant growth as predictors in the multiple regression analysis, 88% of the variation in phenolic glycosides was explained by these two predictors and the taller seedlings with high nutrients and high TNC contents in non-photosynthetic tissue produced less phenolic glycosides. In the current study, aspen seedlings contained two major, tremulacin and salicortin, and two minor, salicin and tremuloidin, phenolic glycoside compounds. Among these tremulacin and salicortin are the main defensive chemicals and can have a negative impact on the development and survival of defoliators (Hemming and Lindroth 2000, Lindroth 2001). In contrast, the seedlings with low nutrients and TNC content in non-photosynthetic tissues produced more phenolic glycosides. These results support the predictions in the CNB hypothesis.

Genetics and environment may also have influenced the levels of defense in aspen seedlings in the current study. There was a negative correlation between the constitutive level of phenolic glycosides and larval performance, whereas the same relationship was not statistically significant at the induced level. This could be attributed to the large genetic variation in the levels of induction between aspen seedlings as they were generated from open pollinated seed sources. I suspect that sexually regenerated aspen has greater induced response than aspen regenerated asexually through suckering, because sexual reproduction can enhance aspen genotypic character acquisition and genetic crossover. Perhaps this

could explain why I experienced such variation in induced defenses in aspen seedlings that was lacking in other studies used aspen clones in their experiments (Stevens and Lindroth 2005, Donaldson and Lindroth 2007). Thus, I suspect that aspen generated via seeds may have more plasticity in terms of chemical defenses and resource allocation between the non-photosynthetic tissues and foliage than what can be observed in aspen generated through clonal reproduction. However, I need to emphasize that the combination of genetics and the environment may also influence the induction response in aspen, because only aspen seedlings in the low nutrients and low TNC reserves were induced and maintained this response.

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Table 3. 1. Primary and secondary chemistry of damaged (induced) and undamaged (constitutive) leaves. The same seedling categories were obtained from the LDA analysis (Fig. 1).

All values are presented as mean content ± standard error.

Seedling categories	Nitrogen		Total Non-structural Carbohydrates		Phenolic Glycosides	
	Constitutive	Induced	Constitutive	Induced	Constitutive	Induced
High Nut – Medium TNC	1.31±0.07	1.52±0.1	30.27±1.1	20.65±1.3	199.26±10.3	215.95±15.1
Low Nut – Low TNC	1.86±0.09	1.68±0.1	21.83±0.6	20.13±0.5	218.07±5.4	299.78±8.8
High Nut – High TNC	1.25±0.1	1.49±0.2	30.9±0.9	23.66±1.0	206.19±8.4	220.13±15.9

	Salicin		Salicortin		Tremulacin		Tremuloidin	
	Constitutive	Induced	Constitutive	Induced	Constitutive	Induced	Constitutive	Induced
High Nut – Medium TNC	5.52±0.7	6.2±0.5	95.85±5.8	100.06±7.4	68.81±4	81.16±8.12	29.08±3.7	28.53±1.9
Low Nut – Low TNC	9.58±0.6	11.23±0.8	105.07±4.3	133.02±4.7	68.59±2.2	114.81±4.7	34.82±2.6	40.72±3.2
High Nut – High TNC	4.29±0.4	6.35±0.5	106.02±4.9	106.52±7.4	69.87±3.0	77.78±8.4	26.02±2.3	29.48±2.0

Table 3. 2. Results of multiple regression analysis of the induced foliage chemistry of aspen seedlings and the observations of the forest tent caterpillar performance.

	Coefficients	*Std. Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	3.06	4.37	0.70	0.52	-9.05	15.18
Foliar TNC	-0.01	0.15	-0.05	0.96	-0.43	0.42
Foliar Nitrogen	2.91	0.79	3.71	0.02	0.73	5.09
Phenolic Glycosides	-0.02	0.006	-3.54	0.02	-0.04	-0.005

Table 3. 3. Results of multiple regression analysis of the induced foliage chemistry of aspen seedlings and the observations of the forest tent caterpillar performance.

TNC: Total non-structured carbohydrates. Height was calculated as a difference between the final and the initial height of seedlings.

	Coefficients	*Std. Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.95	0.34	2.78	0.05	-0.0002	1.90
Total Nutrients	0.17	0.03	5.43	0.005	0.081	0.25
TNC	-3.42	1.15	-2.97	0.041	-6.610	-0.22
Height	-1.99	0.42	-4.77	0.009	-3.153	-0.83

Table 3. 4. Results of multiple regression analysis of the primary foliage chemistry (nitrogen (N) and total non-structural carbohydrates (TNC) of aspen as predictor variables and the phenolic glycosides as response variable.

	Coefficients	*Std. Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	511.20	223.76	2.28	0.07	-64.01	1086.40
Foliar TNC	-14.05	9.26	-1.51	0.190	-37.87	9.77
Foliar N	28.05	56.13	0.45	0.64	-116.24	172.35

Table 3. 5. Results of multiple regression analysis of the primary foliage chemistry (nitrogen (N), total non-structural carbohydrates (TNC) and height of aspens as predictor variables and the phenolic glycosides as response variable.

Height was calculated as a difference between the final and the initial height of seedlings.

	Coefficients	*Std. Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	337.81	26.56	12.72	<0.001	264.07	411.56
Total Nutrients	-1.59	1.65	-0.96	0.39	-6.16	2.98
TNC	35.40	100.65	0.35	0.74	-244.05	314.85
Height	-0.31	2.50	-0.13	0.91	-7.25	6.63

Figure 3. 2. Regularized Discriminant Analysis of the constitutive level of primary and secondary chemistry of aspen foliage in relationship to the nutrient and carbohydrates status of seedlings prior to dormancy.

Each point represent the centroid of a treatment (n=8 replicates). Continuous vectors: response matrix, dashed lines: explanatory matrix.

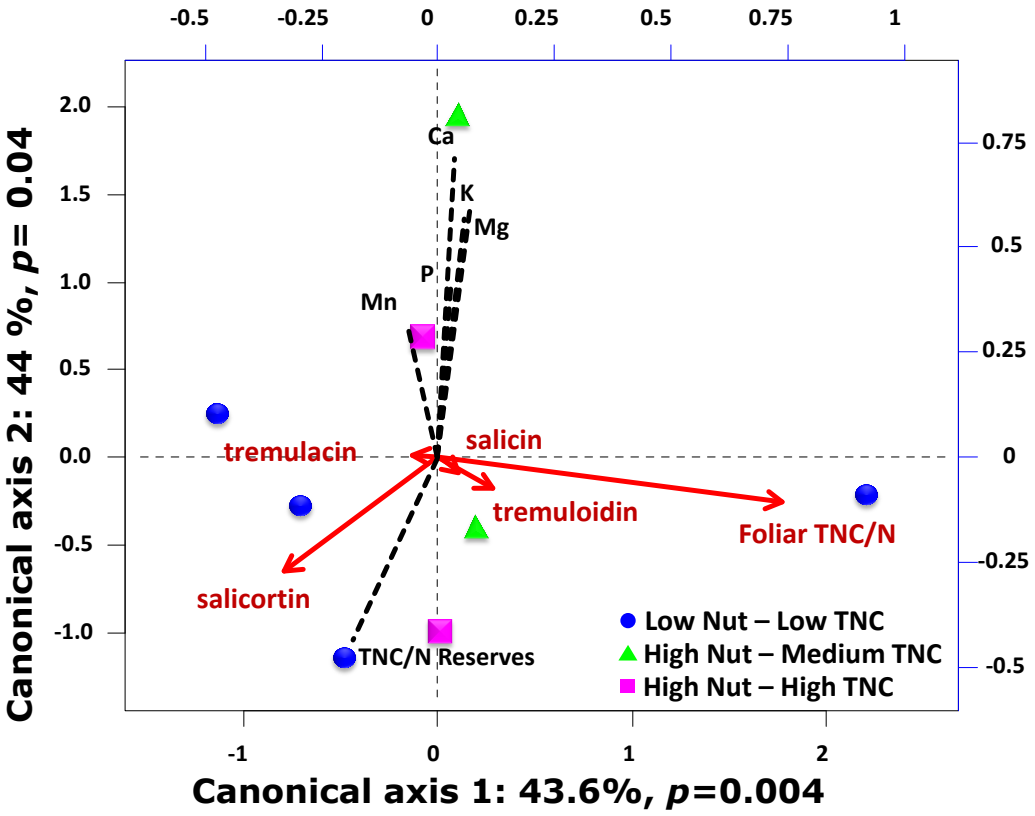


Figure 3. 3. Regularized Discriminant Analysis of the induced level of primary and secondary chemistry of aspen foliage in relationship to the nutrients and carbohydrates status of seedlings prior to dormancy.

Each point represent the centroid of a treatment (n=8 replicates). Continuous vectors: response matrix, dashed lines: explanatory matrix.

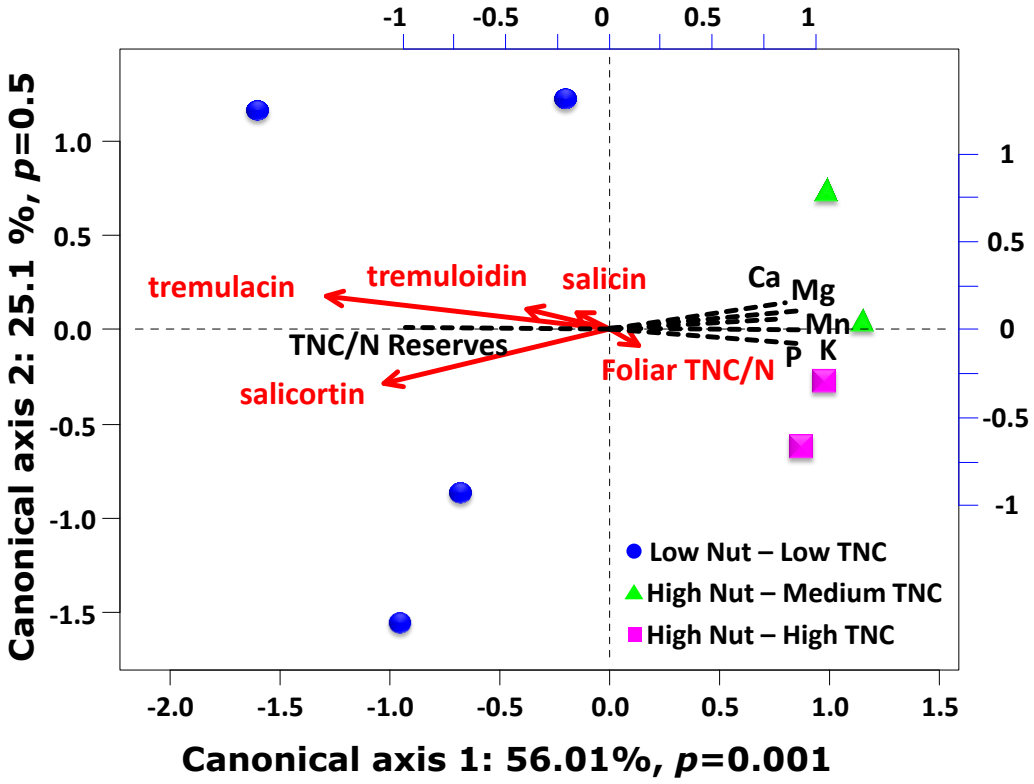


Figure 3. 4. Relationship between the constitutive level of foliar phenolic glycosides and the average instar achieved by forest tent caterpillars.

Each point represent the mean of a treatment (n=8 replicates).

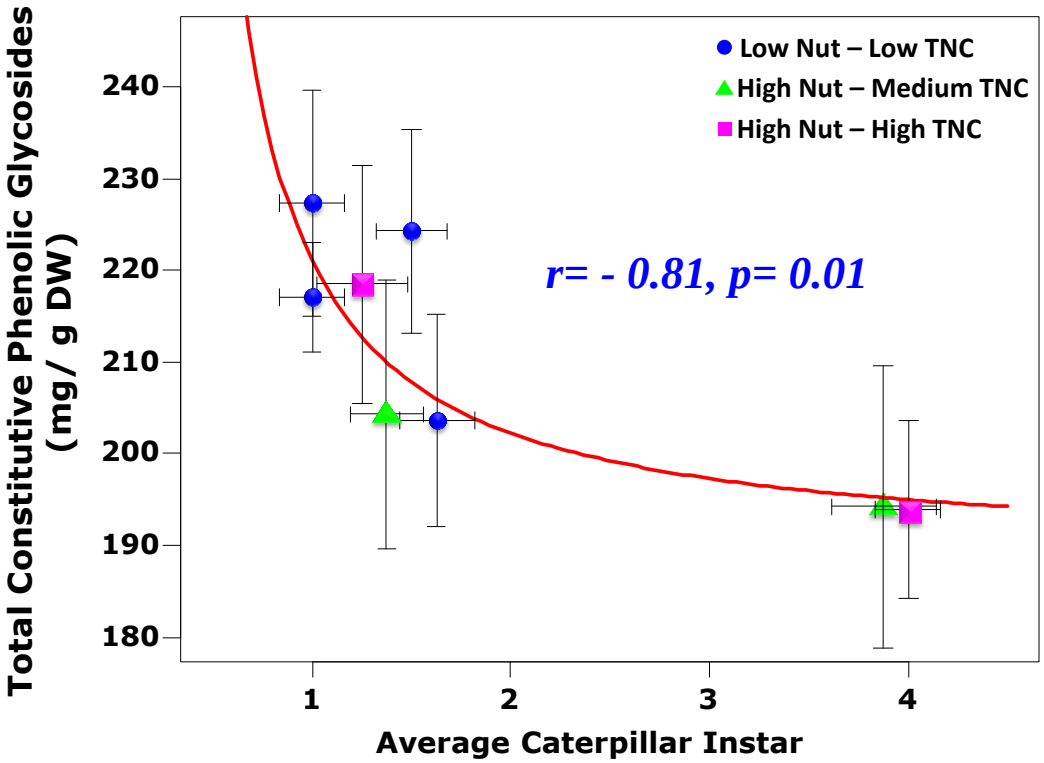
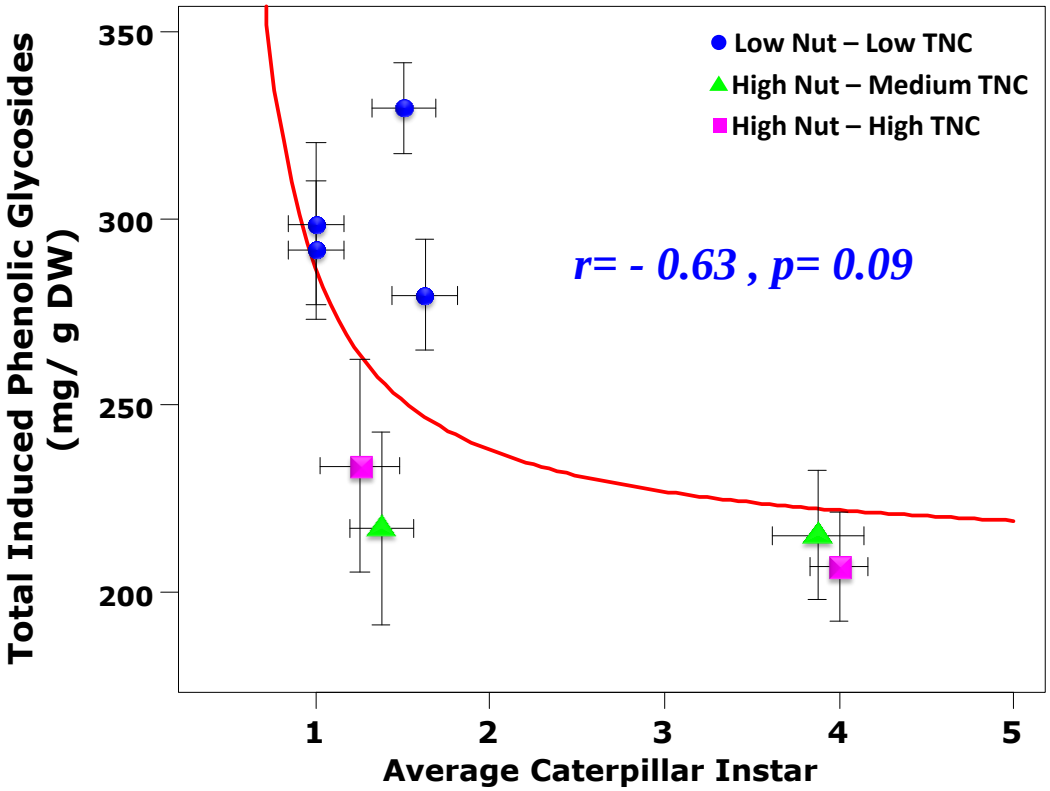


Figure 3. 5. Relationship between the induced level of foliar phenolic glycosides and the average instar achieved by the caterpillars.

Each point represent the mean of a treatment (n=8 replicates).



Chapter IV. Discussion

Open pollinated aspen seedlings grown under different greenhouse conditions and fertilization regimes with or without the shoot growth inhibitor application contained different carbohydrate and nutrient reserves in their non-photosynthetic tissues (roots and stems). Fertilization application had the biggest influence on plant shoot and root growth although other factors, such as the shoot growth inhibitor influenced the aspen seedlings depending on the fertilization levels applied. Under low fertilization, fertilization overpowered the effect of other treatments, while under high fertilization regimes the effects of the inhibitor application on carbohydrate and nutrient reserves were apparent. I grouped aspen seedlings in three distinct groups depending on their nutrient and carbohydrate contents or concentrations in non-photosynthetic tissues: (1) Low Nutrients-Low TNC, (2) High Nutrients-High TNC, and (3) High Nutrients-Medium TNC.

The reserve status of (i.e. carbohydrates and nutrients) influenced the foliar TNC, N and phenolic glycosides, and the overall performance of the forest tent caterpillar. Aspen seedlings with low nutrients-low TNC reserves (or high TNC/N ratio) contained higher amounts of phenolic glycosides, were less palatable to FTC since they were not able to maintain feeding. In fact in this seedling group, larvae were not able to pass the second instar. In contrast, the foliage of seedlings with low TNC/N ratio reserves (groups 2 and 3), even though it had relatively high carbohydrate and nutrient reserves, had relatively lower phenolic glycosides. This foliage supported longer larval feeding (up to fourth instar). I further found that the performance of the caterpillars was explained by both primary (carbohydrates and nitrogen) and secondary (phenolic glycosides) chemistry of aspen seedlings, suggesting that foliar quality measured in its nutrient concentration is equally important as its defensive chemistry. In addition, the concentration of phenolic glycosides in foliage was related to the reserves (carbohydrates and total nutrients, including nitrogen) stored in the non-photosynthetic tissues, not the foliar reserves (carbohydrates and nitrogen).

Overall these results suggest multifunctional roles of plant nutrient and carbohydrate reserves in plant defenses as well as mediating plant-herbivory interactions.

I also evaluated the CNB hypothesis in terms of resource availability and defense (Bryant et al. 1983). The CNB hypothesis is built around the assumption that the production of secondary metabolites of aspen depends on its foliar chemistry. One of its premises presumes an increase of foliar TNC/N ratio usually resulting in increased carbon-based secondary metabolites. CNB also assumes that carbohydrate/nutrient reserves in the non-photosynthetic tissue have no role in active plant defenses. In this thesis, the aspen seedlings with high reserve TNC/N ratio produced more phenolic glycosides; this is consistent with the general principle of CNB. In contrast to the CNB hypothesis, reserve TNC/N ratio in the present study predicted the phenolic glycoside in the foliage, while foliar TNC/N ratio did not. The strong positive relationship between reserves TNC/N ratio in non-photosynthetic tissues and phenolic glycosides and the non-existent or negative relationship between foliar and reserve TNC/N ratios in non-photosynthetic tissues further emphasizes the critical role of reserve chemistry in foliar defenses. Parallel to the results of my study, a number of studies reported results inconsistent with the CNB hypothesis (Fajer et al. 1992, Koricheva et al. 1998, Hamilton et al. 2001, Koricheva 2002). We explained how GDB also failed to predict our results in certain respects in the Chapter III. However the present study was not designed to test these specific defense hypotheses. GDB is the most comprehensive defense theory established in the literature (Stamp 2003), however it is very difficult to test (Stamp 2004): one of the obstacles of testing it, is that it uses resource availability and relative growth rates (RGR) while those parameters are not well defined and often overlap with other parameters (Stamp 2004). Our study suggests that the use of TNC/N ratio of the reserve tissue could provide a more reliable measure for resource availability that would make it easier to test the GDB.

Phenolic glycosides play major roles in the deterrence of herbivores (Palo 1984, Scriber et al. 1989, Boeckler et al. 2011). Parallel to the earlier studies, phenolic glycosides were linked to reduced herbivory damage in the current project and aspen seedlings with the low foliar TNC/N ratio had the highest phenolic glycoside contents. While previous studies have shown that phenolic glycoside is a clonal trait (i.e., plant genotype influences phenolic production) (Lindroth and Hwang 1996, Hwang and Lindroth 1997), my results further suggest that phenolic glycoside production is influenced by the relative concentrations of nitrogen and carbohydrate reserves, as well as other micro- and macro-elements in non-photosynthetic tissues. Further, carbohydrate reserve status, particularly of the roots, is also known to play an important role in the clonal growth strategy, which is key to its success in re-colonizing disturbed sites (Barnes 1966, Schier and Zasada 1973, Landhäusser and Lieffers 2002).

The current study also suggests an interesting contrast between aspen seedlings generated from open-pollinated seeds and from vegetative (clonal) reproduction. Aspen seedlings in the current project demonstrated strong induced defense responses while other studies found no such response in aspen clones under similar growing conditions (Stevens and Lindroth 2005, Donaldson and Lindroth 2008). Thus I suspect that aspen adaptation to herbivory is enhanced by genetic crossover and genotypic character acquisition through sexual reproduction. This outcome sheds light on factors contributing to the aspen decline in North America. In nature, aspen sexual reproduction is less frequent than vegetative reproduction (DeByle 1985, Frey et al. 2003, Landhäusser et al. 2010). Even if instances of vegetative mutations exist they are rare compared to the genetic variation induced by sexual reproduction and seed establishment (Bazzaz et al. 1987). I speculate that this clonal propagation strategy, also called an ancient legacy (e.g. the clones of Great Basin originated from seeds 8000 years ago), may drive the aspen decline because clonally propagated aspens often lack the necessary phenotypic and genotypic plasticity to adapt stresses such as herbivory and diseases.

Future Implications

The results of the present study as well as others (Landhäusser and Lieffers 2003, 2012) made it necessary to rethink the function of the non-photosynthetic tissues in plant reserve allocation and potential defenses as well as plant tolerance against herbivores. Non-photosynthetic tissues should not simply be viewed as inert transport tissues or storage media though should be regarded as an active contributing tissue shaping aspen interactions with its biotic and abiotic environment. Thus, the outcome of this project has important implications to improve seedling quality for areas such as agroforestry, intensive plantations and forest reclamation.

First, initial seedling establishment and seedling mortality are one of the biggest challenges in reforestation and afforestation (Macdonald et al. (in press)). The outcome of this project and similar projects conducted in Dr. Landhäusser lab (Landhäusser et al. 2012a) suggest that seedlings with high carbohydrate and nutrient reserves might have a better survival rate than seedlings without such reserves. Therefore, plantation managers should target such seedlings to improve seedling quality and out planting performance particularly on stressful sites (Landhäusser et al. 2012b). Specifically the current project reported that TNC/N ratio in seedling reserves can be used to target specific seedling characteristics. For example, high reserve TNC/N ratio would produce well defended leaves but would yield humble growth rates while low TNC/N ratio will speed up growth and enhance canopy cover, yet the same seedlings may not be as defended. However, further studies are needed to identify whether and how long the effect of the stored reserves would last.

Second, the current project suggested superiority of seedlings generated from open pollinated seeds to the seedlings generated from clonal propagation. The current project found that open pollinated seedlings can demonstrate strong induce chemical defense responses compared to clones even though the treatments were similar (Lindroth 2001). Seedlings with such phenotypic

plasticity are a precious material for an improved survival in unpredictable environments under the global climate change. I suspect that such seedlings can be incorporated in agroforestry for better survival and development of more robust forest stands in the future.

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Appendices. ANOVA Tables

Table A. 1. ANOVA table of physical responses of aspen seedlings treated with different fertilization regimes and shoot growth inhibitor (SGI) and grown inside or outside greenhouse.

P values are significant at $p<0.05$. For details of statistical analysis, please see data analysis subtitle in Methods and Materials section.

(A) Shoot Height	Df	Sum Sq	Mean Sq	F value	P-values
SGI	1	0.917	0.917	35.017	<0.001
Location	1	2.530	2.530	96.641	<0.001
Fertilizer	1	7.745	7.745	295.854	<0.001
SGI:Location	1	0.138	0.138	5.274	0.025
SGI:Fertilizer	1	0.015	0.015	0.559	0.457
Location:Fertilizer	1	0.222	0.222	8.498	0.005
SGI:Location:Fertilizer	1	0.229	0.229	8.752	0.004
Residuals	72	1.885	0.026		

(B) Root Volume	Df	Sum Sq	Mean Sq	F value	P-values
SGI	1	0.87	0.87	6.63	0.010
Location	1	1.12	1.12	8.53	<0.001
Fertilizer	1	18.35	18.35	140.16	<0.001
SGI:Location	1	0.02	0.02	0.14	0.710
SGI:Fertilizer	1	1.53	1.53	11.65	<0.001
Location:Fertilizer	1	0.03	0.03	0.22	0.640
SGI:Location:Fertilizer	1	1.19	1.19	9.09	<0.001
Residuals	72	9.43	0.13		

(C) Root Collar Diameter	Df	Sum Sq	Mean Sq	F-value	P-value
SGI	1	4.97	4.97	18.73	<0.001
Location	1	1.19	1.19	4.47	0.040
Fertilizer	1	71.56	71.56	269.61	<0.001
SGI:Location	1	1.36	1.36	5.13	0.030
SGI:Fertilizer	1	0.08	0.08	0.31	0.580
Location:Fertilizer	1	0.00	0.00	0.00	0.970
SGI:Location:Fertilizer	1	1.41	1.41	5.31	0.020
Residuals	72	19.11	0.27		

(D) Total Dry Weight	Df	Sum Sq	Mean Sq	F value	P-value
SGI	1	0.00	0.00	0.01	0.920
Location	1	0.00	0.00	0.02	0.890
Fertilizer	1	21.88	21.88	351.21	<0.001
SGI:Location	1	0.00	0.00	0.02	0.880
SGI:Fertilizer	1	0.31	0.31	4.97	0.030
Location:Fertilizer	1	0.04	0.04	0.67	0.410
SGI:Location:Fertilizer	1	0.62	0.62	10.01	<0.001
Residuals	72	4.49	0.06		

(E) Root Shoot Ratio	Df	Sum Sq	Mean Sq	F value	P-values
SGI	1	6.61	6.61	106.05	<0.001
Location	1	8.28	8.28	132.93	<0.001
Fertilizer	1	4.61	4.61	74.02	<0.001
SGI:Location	1	0.00	0.00	0.00	0.980
SGI:Fertilizer	1	1.49	1.49	23.90	<0.001
Location:Fertilizer	1	0.82	0.82	13.19	<0.001
SGI:Location:Fertilizer	1	0.16	0.16	2.64	0.110
Residuals	72	4.49	0.06		

Table A. 2. ANOVA table of total non-structural carbohydrates (TNCs) concentration and content of aspen tissues of seedlings grown at different growing and nutrient conditions and treated with a shoot growth inhibitor (SGI).

P values are significant at $p<0.05$. For details of statistical analysis, please see data analysis subtitle in Methods and Materials section.

(A) Concentration	Df	Sum Sq	Mean Sq	F value	P-value
SGI	1	0.02	0.02	11.16	<0.001
Location	1	0.07	0.07	48.34	<0.001
Fertilizer	1	0.01	0.01	4.18	0.040
SGI:Location	1	0.00	0.00	2.83	0.100
SGI:Fertilizer	1	0.00	0.00	2.27	0.140
Location:Fertilizer	1	0.04	0.04	27.21	<0.001
SGI:Location:Fertilizer	1	0.01	0.01	4.71	0.030
Residuals	72	0.10	0.00		

(B) Content	Df	Sum Sq	Mean Sq	F value	P-value
SGI	1	0.13	0.13	1.51	0.220
Location	1	0.73	0.73	8.31	0.010
Fertilizer	1	19.14	19.14	217.09	<0.001
SGI:Location	1	0.04	0.04	0.50	0.480
SGI:Fertilizer	1	0.54	0.54	6.08	0.020
Location:Fertilizer	1	0.17	0.17	1.88	0.170
SGI:Location:Fertilizer	1	0.34	0.34	3.85	0.050
Residuals	72	6.35	0.09		

Table A. 3. ANOVA table of total nutrients concentration and content of aspen tissues of seedlings grown at different growing and nutrient conditions and treated with a shoot growth inhibitor (SGI).

P values are significant at $p<0.05$. For details of statistical analysis, please see data analysis subtitle in Methods and Materials section.

(A) Concentration	Df	Sum Sq	Mean Sq	F-value	P-value
SGI	1	3.17	3.17	8.22	0.010
Growing location	1	0.18	0.18	0.45	0.500
Fertilizer	1	0.01	0.01	0.03	0.860
SGI:Location	1	0.03	0.03	0.07	0.790
SGI:Fertilizer	1	0.42	0.42	1.10	0.300
Location:Fertilizer	1	1.10	1.10	2.85	0.100
SGI:Location:Fertilizer	1	1.50	1.50	3.88	0.050
Residuals	72	27.77	0.39		

(B) Content	Df	Sum Sq	Mean Sq	F-value	P-value
SGI	1	8.90	8.90	9.57	<0.001
Location	1	0.86	0.86	0.93	0.340
Fertilizer	1	384.67	384.67	413.88	<0.001
SGI:Location	1	0.00	0.00	0.00	0.950
SGI:Fertilizer	1	11.52	11.52	12.40	<0.001
Location:Fertilizer	1	1.21	1.21	1.30	0.260
SGI:Location:Fertilizer	1	9.64	9.64	10.38	<0.001
Residuals	72	66.92	0.93		