Morphological, growth, and photosynthetic responses of cottonwood hybrid 47-174 (*Populus trichocarpa* x *P. deltoides*) to nitrogen fertilization and leaf rust infection

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2012

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Program Authorized to Offer Degree:

School of Environmental and Forest Sciences

University of Washington

Abstract

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The growth, morphological, and photosynthetic responses of cottonwood hybrid 47-174 to nitrogen (N) stress and leaf rust stress and their interaction are summarized herein.

High nitrogen (HN) fertilization enhanced growth and photosynthesis as compared to low nitrogen (LN). HN plants developed more and larger leaves, were taller, had greater diameters, and greater dry weights as compared to LN plants. Leaves were developmentally identified by the leaf plastochron index (LPI) system. Subdividing LPIs into 4 structural-functional segments (SFS 1-4) facilitated sampling of the major stages of leaf development of this plant. Indirect measures of leaf nitrogen found higher values in HN than LN plants. However, in both plants leaf nitrogen levels were lowest in SFS 1, the top segment with newly formed leaves, and were high in the three other segments: SFS 2 - newly matured leaves, SFS 3 - fully matured leaves, and SFS 4 - mature, senescening leaves.

Both HN and LN plants were susceptible to *Melampsora* leaf rust infection. Scanning electron microscopy (SEM) of both HN and LN rust infected leaves illustrated the morphological impact of rust infection.

ACi curves (A = photosynthetic assimilation and Ci = internal CO_2 concentration) showed that healthy HN leaves had higher A rates compared to LN leaves. In analyzing interaction between N and rust infection, under favorable HN, the middle or SFS 2 segment had higher A rates whereas under LN stress the top SFS 1 had higher A rates. Infected HN leaves had higher A than infected LN leaves in all segments; however, rates were considerably lower than uninfected leaves. A general pattern was noted that HN plants had higher rates of physiological responses than LN plants regardless of whether the leaves were healthy or infected. However, it was important to divide the plants into four SFS segments as this division provided a better picture of how leaf development was influenced by and affected the plant's response to these two stresses and their interaction.

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DEDICATION

To

David R.M. Scott

For his lasting

guidance and confidence

in a heartwarming way.

ACKNOWLEDGMENTS

I have accomplished one big dream and even if done with more ways and more days, this was made possible only with the support and guidance most especially of Tom Hinckley. Tom has selflessly extended the legacy from David Scott.

I am deeply indebted to Tom Hinckley, Bob Edmonds, and Soo-Hyung Kim for their advice and feedback on the analyses and presentation of this dissertation. I am also thankful for early suggestions and pointers from Jon Johnson, Liz Van Volkenburgh, and John Leigh.

Special mention to Jon Johnson who made accessible his expertise, laboratory, and greenhouse facilities off campus at the Washington State University Experimental Station in Puyallup, WA including some amount of his Department of Energy funding. This arrangement included help from his staff: Jeff Kallestad, Marc Anderson, Yong Kim, and Shan Zhao. Thanks also to John Stark who provided extended use of facilities.

On campus work was possible with equipment and facilities provided by David Ford and technical help from his student Paul Ma. Wai Pang Chan, Doug Ewing, and Daisuke Sasatani provided technical assistance. I treasure innumerable conversations and contacts with students, faculty, and staff from other units and at the College of Forest Resources now the School of Environmental and Forest Sciences.

David Scott, Richard Walker, and Breck Byers helped me in so many ways. I appreciate encouragements from: Joan Abe, Lia Kontraros, Francine Shafer, Lew Thorson, Carla Hansen, Brice Dupoyet, Amanda Davis, Nevada Smith, and JoAnn Kocha. Overseas, I have received invaluable support from Consuelo Asis, Dolores Hernandez, and Flordeliz Uyenco.

I cannot deny that working with PhD students at the Foster School of Business has kept this PhD dream alive. Thank you to my faculty directors, other faculty, staff, and fellow students.

And at home, I value endlessly the support system and other intangibles from family and siblings and from dear Betty, Mark, and Michael and Jamie, Hunter, Jonah, and Leo Banaag.

CHAPTER 1

INTRODUCTION

1.1 Cottonwood Hybrids

The hybridization of the native western black cottonwood (*Populus trichocarpa* Torr. & Gray) with eastern cottonwood (*Populus deltoides* Bartr. ex Marsh.) has resulted in hybrids that are very productive and profitable to cultivate especially for short-rotation and biomass tree cultivation (Ceulemans, et al., 1992; Updegraff, et a., 2004). The resulting hybrids have facilitated the development of large-scale plantations (Hinckley, et al., 1998; Johnson, et al., 1999; Brown and van den Driessche, 2002; Pinon, et.al, 2006) for a wide range of products including woody biomass, pulp, paper, tissue products, fuel, lumber, plywood, and veneer. These hybrids have become very attractive to commercial interests because of their remarkable growth rates, impressive biomass production, and ease of vegetative and clonal propagation and cultivation (Heilman and Stettler, 1985; Hinckley, et al., 1989; Ceulemans, et al., 1992; Scarascia-Mugnozza, et al., 1997; Polle and Douglas, 2010). One example of a hybridization program was the Joint Poplar Research Program by Washington State University and University of Washington (WSU/UW) that developed a remarkable group of hybrids demonstrating hybrid vigor (heterosis), distinct genotypes, and remarkable phenotypes (Hinckley, et al., 1989; Johnson, et al., 1999; Bradshaw, et al., 2000; Stanton, 2001) including hybrid 47-174, the plant material used in this study.

Cottonwoods play a role in many ecological settings. For example, cottonwoods are important in maintaining the integrity of natural and restored riparian systems as they remove nutrients from ground water and decrease sediment inputs (Rood et al., 2003). Henri and

Johnson (2005) suggested that the use and subsequent harvest of hybrid cottonwoods in riparian buffer strips of agricultural systems could economically compensate farmers for the loss of agricultural land. Additional economic benefits might be realized by planting these trees between rows of agricultural crops as windbreaks. There is potential use of cottonwood for phytoremediation of pollutants in riparian zones (Dix, et al., 1997). In addition to their role in natural or restored riparian systems, cottonwoods are equally important for potentially sequestrating carbon, ameliorating environments, providing habitat (Johnson, 2000), and bioenergy production (Renenberg, et al., 2010).

Nitrogen (N) and moisture availability are not a problem in most riparian soils either due to the inherently high N availability in such systems (DeBell, 1990; Cooper and Van Haverbeke, 1990) or to the high levels of inorganic N moving in ground water towards streams (Rennenberg, et al, 2010). In contrast, one of the major requirements for growth of cottonwood hybrids in upland plantations on relatively young soils, especially in the Pacific Northwest, is the availability of N. There is now increasing attention on N nutrition of cottonwood because of the current focus on highly productive plantations in upland (and in some cases riparian soils) (Rennenberg, et al., 2010). Cottonwood tree farming in eastern Oregon and Washington may require both N and water amendments. Since N is considered a limiting factor in forest trees, there is increasing use of N fertilization to improve yields and reduce rotation age in short-rotation woody crops (Cooke, et al., 2005). On the other hand, there must be a balance between sufficient additions for optimal growth and surplus additions where N is either wasted or creates water quality problems (Chardon, et al., 2010).

In addition to the need to supply adequate resources for growth, cottonwood hybrids have to be resistant to diseases, especially leaf rust or *Melampsora* infection. Some of the hybrids

developed to date have improved disease resistance (Newcombe, et al., 1996; Fritz, et al., 1999; Stirling, et al., 2001). However, hybrid 47-174, a very fast growing hybrid, is very susceptible to leaf rust infection by *Melampsora medusae* Thuem. (Hall and Hanna, 1995). This has been observed in the field and has been well documented in greenhouse experiments (Banaag and Johnson, 2002; Kim and Johnson, 2002). Susceptibility occurs when there is a successful pathogen infection usually through the stoma as port of entry and when there is a compatible host/pathogen interaction (Aycliffe, et al., 2002); such systems are referred to as a pathosystem (Wang and van der Kamp, 1992) or host-pathogen system (Jensen and Munk, 1997), or more specifically *Populus-Melampsora* pathosystem (Tabor, et al., 2000). The unique feature of this pathosystem is the impressive degree of cellular interaction between plant and pathogen that keeps the invaded tissue alive and thus prolongs susceptibility (Heath, 1997) before resultant leaf senescence and defoliation finally occurs. Such host-parasite interactions represent an interesting struggle for survival between two organisms (Day, 1974).

Of the various diseases affecting cottonwoods and their hybrids in the Pacific Northwest, leaf rust caused by *Melampsora* may be the most widespread and damaging (Newcombe, et al., 1996) and potentially serious worldwide (Ostry, 1997), causing premature defoliation, reducing plant growth by 80% (McCracken and Dawson, 1992; Newcombe and Chastagner, 1993), volume increment by 65% (Widen and Schipper, 1981), and wood production by 60% (Ziller, 1965). Reductions in growth during the growing season also result in a subsequent lack of winter hardiness in the stems (Tabor et al., 2000). Cottonwood rusts affect all sizes and stands of cottonwood but are particularly severe in plantations and nurseries, causing heavy infection that may eventually kill the trees or make the trees predisposed to other diseases (USDA Forest Service, 1989). Repeated infections in plantations could result in the complete loss of the

plantation (Laurans and Pilate, 1999; Johnson and Kim, 2005). Therefore, it is important to study problems affecting disease occurrence and development, particularly *Melampsora* leaf rust infection. *M. medusae* infection could be a major problem for poplar growers, especially if they are using hybrid 47- 174 (or other susceptible clones), although it may be a problem for other hybrids too because a given clone may not be completely resistant to *Melampsora* (Miot, et al., 1999). Attempts are being made to select resistant clones (Laurans and Pilate, 1999); resistant clones are the best methods of control versus fungicide applications (Widin and Schiffer, 1981).

This study examines the response of cottonwood hybrid 47-174 to N fertilization (an abiotic factor) and to leaf rust infection (a biotic factor) and their individual and interactive effects on morphology, growth, and photosynthesis. These two factors were chosen because they are representative of abiotic and biotic factors that, if unfavorable, could cause considerable stress and great loss in cottonwood productivity. It is not enough to select hybrids based only on their productivity (Larocque, 1999) but also on their resistance to leaf rust infection in order to have a profitable investment in a tree farm. This study also investigates the potential negative interaction between N nutritional state of this hybrid and its susceptibility to *M. medusae* leaf rust infection. Previous preliminary study trials by this author have shown that high N fertilization rate favors leaf rust infection. It is hypothesized that there must be a period when the poplar host is most favorable to leaf rust penetration and parasitic growth before establishing a host-pathogen system wherein the rust completely dominates the host during sporulation (Figure 1.1). There is an initial physiological relation between the stoma of the host and germ tubes of the rust during infection (Siwecki and Przybyl, 1981). The N status of the host *Populus* may determine the growth of the pathogen *Melampsora* in such a way that changes in available N may change host susceptibility. This study addresses this possibility.

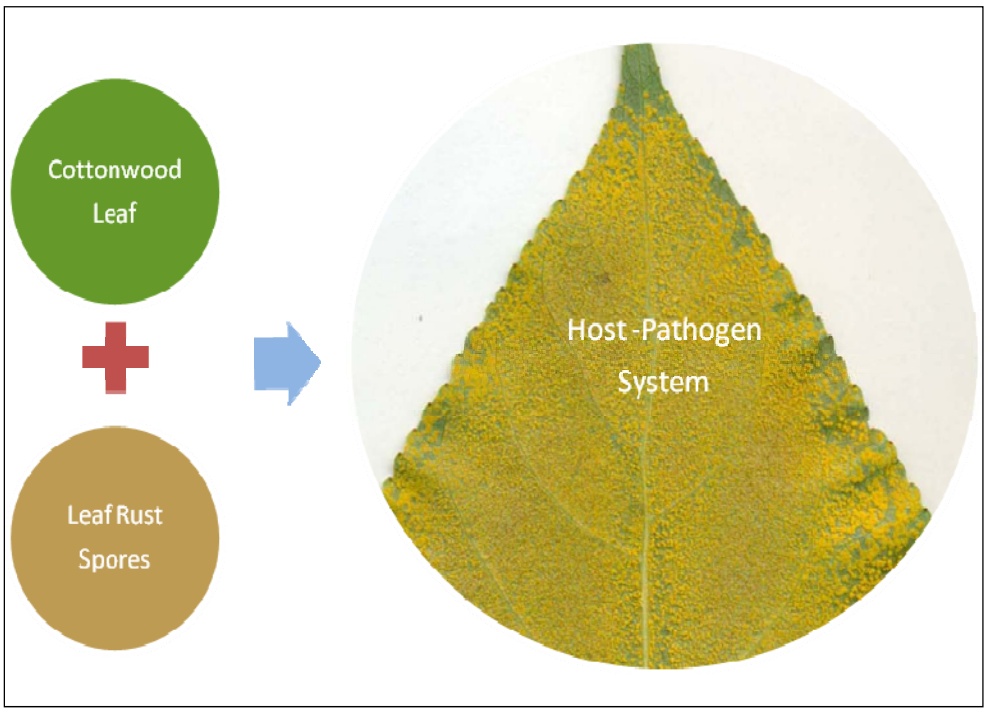


Figure 1.1 Conceptual model of my study. The biotic and abiotic status of the cottonwood leaf could favor the initial establishment of leaf rust spores leading to the prolific growth of the leaf rust forming a host-pathogen system.

1.2 Literature Review

Two broad topics are examined in the literature review: (1) Foliage morphology and development and (2) abiotic and biotic stresses.

1.2.1 Foliage Morphology and Development

The morphology of a cottonwood plant might be divided into the elongating branch and stem-leader segments or into a consideration of the architecture of the entire plant. When studying leaf function and structure, each segment of growth is often described and considered as a separate unit. Typically, the parts are the main terminal, and then the branches where there may be proleptic and sylleptic and then further into proleptics and sylleptics on proleptics (see

Ceulemans, et al., 1990). For young, experimental material, the main terminal is often the only unit of consideration. After subsequent growing seasons, more units of growth come into consideration.

For the elongating unit under consideration, foliage morphology includes the structure and features of the leaves of that unit that are emerging, are developing and maturing, are fully developed, or are senescening and abscissing. Parameters associated with describing foliage morphology and its arrangement on a plant include observable characteristics such as plant height, number and size (e.g., length and width) of individual leaves, internode length, growth unit basal diameter, and as part of this study, anatomical features including stomatal density and distribution. During harvest time additional structural features are stem dry weight, leaf dry weight, and actual leaf areas that are very helpful in analyzing data per given weight and per given leaf area.

Foliage structure and function may be expanded by consideration of branches and the accompanying new leaves that are formed. Branching is prominent in some cottonwoods that have the capacity to form sylleptic branches which are side branches formed during the current year. However, this appears not to be a factor in hybrid 47-174 because it is less branchy (Scarascia-Mugnozza, et al., 1997) or has proleptic branching or a low amount of sylleptic branching (Cline and Dong-Il, 2002).

Wait, et al. (2002) observed that cottonwoods subjected to higher N treatments showed significantly greater relative height growth rates, leaf initiation rates, and leaf area at full expansion than those grown in low N. In general, cottonwood hybrids are capable of producing large quantities of leaves very rapidly. This increases the photosynthetic leaf surface area and, in a positive feedback, increases leaf production (Van Volkenburgh and Taylor, 1996; Harvey

and van den Driessche, 1999) resulting in very impressive growth performance (Hinckley, et al., 1989). The increase in leaf area is likely accompanied by an increase in stem volume (van den Driessche, 1999). Leaf orientation, leaf area index, and leaf age are important in determining biomass productivity (Isebrands and Michael, 1986). Height is also a good predictor of total above ground biomass (Ceulemans, et al., 1987). It would be helpful then to include measurements of leaf area, number of leaves and the associated LPI number, and plant height in monitoring the effects of N on leaf morphology and plant growth.

An important measure is the specific leaf area (SLA) that is expressed as the leaf area per biomass or dry weight ($cm^2\ g^{-1}$). SLA is highly sensitive to light environment and nutrient contents (Larocque, 1999). When SLA is increased, light rays can reach carboxylation sites more easily and there is less resistance to CO_2 diffusion within the mesophyll and maintenance respiration needs are reduced (Ducrey, 1992).

Another important measure is leaf mass area (LMA) that is expressed as leaf dry weight per leaf area ($g\ cm^{-2}$ or $g\ m^{-2}$). LMA could be a measure of leaf structural toughness and could be used as an index of cell wall biomass (Onoda, et al., 2004). Typically, sun leaves with high SLA or low LMA have high rates of photosynthesis. Within the same plant, shade leaves will have higher SLAs or lower LMAs than sun leaves.

Studies of the function and structure of foliage units, because of their critical roles in gas exchange and photosynthesis, is critical to any study of plant physiology and morphology. A critical issue in these studies is how to control for difference in leaf development (i.e., consistently differentiate between developing, newly developed, mature and declining or senescening foliar units. Below I present two approaches.

1.2.1.1 Leaf Plastochron Index

In cottonwood plants, all the leaves are identified or tracked by assigning a numbering system or a Leaf Plastochron Index (**LPI**) to each leaf, starting from the top leaves to the basal leaves of a given branch. An upper (i.e, near the growing tip) leaf that is 6 cm long is commonly defined as LPI 0 or the 'index leaf' that is followed by a series of numbers from LPI 1to n depending on the total number of leaves. Smaller leaves can be given negative numbers. LPI has been used to monitor or control for differences in leaf development in studies of leaf morphology and physiology (Larson and Isebrands, 1971; Isebrands and Larson, 1973; Dickson, 1986; Dickson and Isebrands, 1991). This numbering system was derived from studies on the parent *P. deltoides* and has been applied to most cottonwood parent species and their hybrids. The assignment of an LPI number in identifying leaves is utilized in this study. This numbering system is very helpful in comparing results rather by using leaf age defined in terms of days as reported by Sharma, et al. (1980). LPI in simple terms is an index of leaf age (Marron, et al., 2008) and leaf age is determined by its branch position or LPI number (Banaag, et al., 2005). It is also used as a measure of physiological age (Noormets, et al., 2001). LPI has been used extensively in controlled environments (Marron, et al., 2008) where it serves as a foliar index during unstable growth conditions such as those imposed by drought (Marron, et al., 2003).

The LPI system approach experimentally controls the stage of leaf development as it affects structural-functional relationships in cottonwoods (Dickson and Isebrands, 1991). It has been used to efficiently describe a system of allocating and partitioning of assimilated carbon in leaf tissues (Ceulemans and Isebrands, 1996). For example, the developing leaf (LPI 6) transports carbon upward to developing leaves (LPI -2 to 5); while recently matured leaves (LPI 7-11) transport both upward and downward; fully mature leaves (LPI 12-14 or 15) transport

primarily downward (Dickson, 1986; Ceulemans and Isebrands, 1996). It is therefore important when making comparative measurements to identify the LPI number and, if possible, to include representative sample leaves from both a range of LPIs as well as from specific segments of leaf development (e.g., between LPIs 6 – 12, less than 6, and greater than 12). Physiological data on the effects of air pollutants and on nutrient mobilization and redistribution have all emphasized the importance of linking physiological measures to leaf LPI (Barker and Bryson, 2007).

1.2.1.2 Structural and Functional Segments

In this study, the LPI numbering system is not the only basis or reference point for observing and analyzing branch and leaf data. This study takes the LPI system further by subdividing each foliated segment, for example, a single branch and all the associated LPIs into four Structural-Functional Segments (SFS).

As a result, the LPIs are grouped into 4 distinct structural segments with their corresponding functional roles consisting of the following four segments: Segment 1 or SFS1 is the top segment a segment of rapid growth and expanding leaves; Segment 2 or SFS2 is the segment immediately below the top section and recently matured leaves that transport nutrients to the top; Segment 3 or SFS3 is a more mature segment below segment 2 and is made up of leaves transporting nutrients to the other parts of the branch and for storage; and Segment 4 or SFS4 is the basal segment made up of oldest leaves for downward transport and storage. All the LPIs or leaves in a given segment work in a similar fashion, thus assuming a specific functional role. Each leaf or LPI in the whole branch or segment in this sense is not entirely independent. The group of leaves in each segment would appear to be in the same development stage (Dickmann, 1971). The localization or grouping of leaves in each segment can be identified as the number of leaves constituting a particular SFS, or SFSi, with i = 1 to n. The number of

leaves in each segment depends on the maximum number or LPIs, the length of the branch or the plant, and the age of the branch or plant. It follows too that early formed LPIs will go through a succession of roles changing from SFS 1 to 4 and going through the structural-functional role associated with each segment as they become progressively older as new LPIs are formed from the top. During the development of leaves (and, therefore, their associated LPIs), a major functional change is the switch from being carbon sinks to carbon sources —this change is accompanied by the above mentioned structural and physiological changes (Dickson and Isebrands, 1991).

Parallel ways of segmenting structural and functional relationships to the SFS approach have been noted. Noormets, et al. (2001) have used age classes similar to this segmentation approach and these are young (LPI 5-8), recently mature (LPI 10-13), mature (LPI 15-21), and old (LPI 24-30). Hartmann, et al., (2000) used such an approach do document sulphur assimilation in poplar. Marron, et al. (2008) analyzed 3 leaf categories made up of expanding leaves, young mature leaves, and old leaves. Ceulemans, et al. (1995) also identified the upper, middle, and lower leaf positions in *Populus* plants with reference to stomatal studies.

This division of a branch into segments may explain more effectively various responses of a plant (Dickson and Isebrands, 1991). For example, there is apparently less leaf rust infection in SFS 1 leaves. In addition, SFS 1, made up of expanding leaves, is an active site more responsive to elevated CO_2 as compared to the other segments of the plant (Wait, et al., 1999). It could be that responses are clearer if associated with corresponding segment of a branch.

Obviously, there are differences in how many leaves there are per segment and how many of the four segments are represented as the growing season progresses. With initial leaf

emergence, there may be only two leaves and one segment (SFS1). At the end of the season, there is again only a few leaves and they may be represented by SFS4.

1.2.2 Abiotic and Biotic Stress

Diurnally, seasonally, and annually the structure and function of trees are affected by abiotic and biotic stresses. Such stresses include light, water, temperature, wind, ice and snow, nutrition, salinity, pests, pathogens, etc. Rarely do trees encounter these stresses singly – how trees then respond has been the subject of inquiry for decades (see reviews by Larcher, 2003 ; Orcutt and Nilsen, 2000). Most typically, studies focus on one stress; however, emerging research suggests that there can be cross-talk between stresses and, therefore, studies of several stresses in combination rather than a single stress have merit (Fujita, et al., 2010). In this dissertation, I examine the effects of N (an abiotic stress) and *Melampsora* leaf rust (abiotic stress) separately, and then in combination.

1.2.2.1 Nitrogen Nutrition

As a required macronutrient, N is needed in relatively large amounts by plants in the same manner as other macronutrients (P, S, K, Ca, Mg, Fe). N is the fourth most abundant element in plants (Huber and Thompson, 2007). The recognition of the essentiality of N predates modern day research since much earlier reports in the 1600s show that N fertility of the land affected the quality of crops (Barker and Bryson, 2007). In remote sensing, the variation in canopy N concentrations can be an indicator of underlying factors that influence growth rates and thus can be used to depict landscape-level spatial patterns (Ollinger and Smith, 2005).

Nitrogen in the N_2 form is abundant (~78%) in the atmosphere but is a critical limiting macronutrient because of its unavailability to plants in this form (Vance, 2001). The most

impressive system for making N accessible to plants is by having N fixers. These are bacteria or other microorganisms that convert unavailable, inorganic N to available nitrates typically in a symbiotic relationship with a plant. Plants take up N in the form of nitrate and ammonium from the soil substrate and these biologically useable forms are converted to amino acids which are the major building blocks for the synthesis of proteins. The availability of N limits forest productivity both directly by affecting the available of protein for growth and indirectly by limiting photosynthesis, which further limits growth. In addition, there must be efficient use of N already assimilated in order to sustain high productivity under conditions of both current and enriched CO_2 levels (Finzi, et al., 2007).

Proteins make up about 85% of total N in plants, mixed with about 5% in nucleic acids and 5 to 10% in other water soluble organic compounds (Barker and Bryson, 2007). In general, plants contain 2% to 5% N by dry weight (Marschner, 1986; Shuman, 2000). For example in corn, 1.46% of the dry weight of the leaf is represented by N and 92.81% is represented by carbon (C), oxygen (O), and hydrogen (H) (Epstein, 1972). The remaining 5.73% of dry matter is composed of the other macronutrients. Micronutrients are needed in relatively small amounts. Leaves, specifically the leaf blades or lamina, are chosen as a diagnostic part in assessing total N compared to including petioles or whole leaves (Barker and Bryson, 2007). In general, plants with high rates of photosynthesis and short leaf longevities tend to have more N in their leaves than plants with low rates of photosynthesis and long leaf longevity (Reich, et al., 1995).

The effect of N in plants is linked to the role of N-containing compounds in various plant physiological processes. For example, N influences photosynthesis because it is a component of compounds actively involved in photosynthesis. The N content of plants is partitioned or allocated as 30.6% in soluble protein-associated functions of CO_2 fixation, 24.6% in the

thylakoid-associated functions of light harvesting and bioenergetics, 21.4% for biosynthesis, and 23.4% as remainder (Evans and Seemann, 1989). It is notable that Rubisco (ribulose 1, 5 biphosphate carboxylase/oxygenase) or RuBPCase, a major photosynthetic enzyme, constitutes some 20 to 30% of total protein in many leaves (Evans and Seemann, 1989; Parry et al., 2002). Rubisco concentration is reported to be about 50% of soluble proteins in both sun and shade species (Hikosaka and Terashima, 1996). Rubisco may be considered as a vegetative storage protein (Millard, et al., 2007). In cottonwoods, photosynthetic rates can be related to Rubisco activity (Ceulemans, et al., 1987).

1.2.2.1.1 Nitrogen Nutrition and *Populus*

Nitrogen is an essential nutrient in the initial development of cuttings in plantations of cottonwoods in the Pacific Northwest. Heilman and Xie (1993) showed successful establishment on soils that were high in N fertility. In cottonwoods, 1.11% to 2.55% of dry matter is represented by N (Harrington, et al., 1997; Abdul Karin and Hawkins, 1999; Brown and van den Driessche, 2002). Cottonwoods developed in plantations often have the capacity for greater uptake of N and subsequently even more productivity (Forest Products, Project Fact Sheet, 2001).

Nitrogen nutrition of poplar trees has been reviewed by Rennerberg, et al. (2010). It appears that the main sources of N are mostly in the forms of nitrate or ammonium, and trace amounts in the form of organic N from the soil as free amino acids. These forms of N are made available in a variety of ways. Roots may take up biologically available N directly. Uptake of N is enhanced by the presence of ectomycorrhizal or endomycorrhizal fungi in root tips (Schultz, et al., 1983). The association between the poplar and mycorrhizal fungi is maintained in a symbiotic relationship where the autotrophic host provides photosynthates in

return for increased nutrients and water uptake provided by the heterotrophic fungus (Schultz et al, 1983; Johansson et al, 2004; Courty et al, 2010). Poplar hybrids are selective in mycorrhizal formation so that proper inoculum should be identified and introduced in preparation of planting sites (Schultz, et al, 1983). For example, *Laccaria bicolor*, an ectomycorrhizal fungus when inoculated to poplar hybrids (*Populus deltoides* x *P. trichocarpa*) adds and substitutes enzymes for the breakdown of organic matter in varying levels depending on which of the 38 F1 hybrids it is associated with (Courty et al, 2010). Poplar cuttings that were pre-treated with mycorrhiza preparations showed the same or better survival than the control; the extent of the difference varied among the 5 clones used (Galic et al, 2007). Another form of making N available is the presence of endophytic bacteria in poplars (Von Wuehlisch, 2011). There are also plant growth promoting rhizobacteria (PGPR) that enhance growth by N fixation and other mechanisms (Bhattacharyya and Jha, 2011). There are some N fixing shrubs when mixed with poplar stands may increase productivity and soil fertility not in short term but in the long run (Mao, et al., 2010).

Uptake of N is governed by ammonium transporters (AMT) and nitrate transporters (NRT) and other storage mechanisms (Lambers, et al., 1998, Rennenberg, et al., 2010). Poplars accumulate N in bark storage protein (BSP) which is involved in both seasonal and short-term N storage (Coleman, et al., 1994). An interesting feature of trees and other perennial plants is that N nutrition is sustained by a seasonal internal cycling (Poole and Douglas, 2010) and is illustrated by the seasonal assimilation, reallocation, resorption, and remobilization of N (Cooke and Weih, 2005).

1.2.2.1.2 Photosynthesis as a Physiological Indicator of Nutrition

As mentioned by Loreto, et al. (2004), the process of CO_2 acquisition by leaves and its incorporation into sugars and biochemical energy consists of CO_2 traveling from the outside (C_a) to the inside intercellular spaces (C_i), to the chloroplasts (C_c). This pathway can be described by a series of biophysical and biochemical resistances and these are the boundary layer (r_b), cuticle (r_c), stomata (r_s), mesophyll (r_m), and biochemical resistance, that is, mainly associated with Rubisco (r_b). The rate of photosynthesis is then described by an Ohm's Law analogy where the carbon dioxide gradient is divided by the series of resistances. Alternatively continuing the use of the Ohm's Law analogy, these resistances can be treated or considered as a linked series of conductances. Photosynthesis in plants is influenced by a large number of abiotic and biotic factors.

Many studies have reported a strong linear correlation between a leaf's photosynthetic capacity and N content (Field and Mooney, 1986; Wu, 1993). There is usually diminution of CO_2 uptake in conditions of N deficiency with observable chlorosis (Larcher, 1975). In addition, existing correlations between N nutrition and photosynthesis are influenced by the availability of photosynthetically active radiation (PAR). An increase in PAR can stimulate the formation of new leaves that are expected to have increasing photosynthetic capacity and N supplied to these leaves may be available from N retranslocated from senescing leaves (Wu, 1993). Photosynthetic capacity of leaves declines parallel to time averaged photon flux density (PDF) within a canopy and this relationship is most easily measured by leaf N content because most of the leaf N is associated with the photosynthetic process (Hull, 2002).

In cottonwoods the relationship between leaf N concentration and net photosynthesis varies from being strong to negligible (Ibrahim, et al., 1997). It has been reported that increased

nutrient availability increased the growth of black poplar (*Populus nigra*) but had no effect on photosynthesis (Glynn, et al., 2003). Also, it could be that an increase in N would lead to an increase in photosynthesis only when conditions are maximal for carbon fixation (Meziane and Shipley, 2001).

Biotic factors also influence photosynthesis. Leaf rust infection by *Melampsora medusae* reduced net photosynthesis of cottonwood hybrid 47-174 by as much as 23% in some preliminary experiments (Banaag and Johnson, 2002).

In cottonwoods since the harvest yield is vegetative rather than reproductive, there is added rationale to study photosynthetic leaf utilization of solar energy during the growing season (Ceulemans and Isebrands, 1996; Osmond, et al., 1998) and its conversion to biomass (Isebrands and Michael, 1986; Ceulemans, et al., 1987; Hinckley, et al., 1989). In rice, in contrast, the increase in production, yield potential, and biomass production are improved at the reproductive phase (Murchie, et al., 1999) because the harvest yield is in terms of grain or seed production. In general, N shortage has a stronger influence on biomass allocation compared to shortage of other nutrients (Lambers, et al., 1998). It would be helpful to include foliar harvests and determine the relationship between photosynthesis and the N use efficiency of individual leaves according to their stage of development (i.e., their LPIs and SFSs) in cottonwoods.

Photosynthesis is often measured using a cuvette system attached to a CO_2 gas analyzer where temperature, air flow, vapor pressure, and light exposure can be controlled. Measurements can be done instantaneously or integrated over specific time periods. In a cuvette system, as soon as the leaf is contained inside the leaf chamber, the conditions the leaf experiences change from ambient to conditions inside the cuvette, which may or may not be controlled. One way of measuring photosynthesis (designated as P or A [assimilation]) is by exposing a leaf to a series

of increasing photosynthetic light exposures and, following equilibration, determine CO_2 uptake for each light level. The resulting light response curve at some point reaches an asymptotic point wherein there is no further increase in CO_2 uptake with increasing light levels. This saturation level is defined as A_{max} or the maximum rate of net assimilation. Depending on the species it may not take a lot of light to reach the asymptote. This level of irradiance is an important ecological factor often used to define the relative shade tolerance of leaves within a canopy or the foliage of different species (Lambers, et al., 1998).

As discussed by Lambers, et al. (1998), significant photosynthetic attributes can be identified from the graph of A versus PAR including the CO_2 compensation point or the CO_2 concentration A = 0 on the graph; that is, when the production of CO_2 from respiration is fully compensated by CO_2 uptake by assimilation. CO_2 compensation point is generally higher in C_3 plants compared to the more productive and photosynthetically efficient C_4 plants.

Another way of describing the photosynthetic response of a plant is to introduce a leaf to a series of increasing concentrations of intercellular CO_2 and, just like the generation of the light response curve, for each level determine CO_2 uptake. The resulting CO_2 response curve is referred to as an A-C_i (A/C_i, A-c_i) curve or A-P_i showing that CO_2 assimilation (A) is a function of the intercellular CO_2 partial pressure (P_i). It is more commonly referred to as A-C_i curve, where C_i is referred to as the CO_2 concentration in the intercellular spaces of the leaf (Lambers, et al., 1998; Lawlor, 2002). Lawlor (2002) presented a simplified analysis of stomatal- and mesophyll-limitation factors affecting well watered to very severely stressed leaves calculated from A-C_i curves. Although there may be uncertainties in calculating C_i in stressed leaves it is not enough to invalidate assessments of its assimilation potential (A_{pot}) (Lawlor and Cornic, 2002).

1.2.2.2 Foliage Diseases

Cottonwood foliage diseases are caused by various fungi and these diseases, the causative fungi, symptoms, damage, and literature references are summarized in Table 1.1. The symptoms or visible damage reported range from initial dots or spots of various colors that eventually spread and after some time cover the entire leaf lamina. The foliage diseases are called gray spot, leaf and shoot blight, leaf rust, leaf blight and spot, powdery mildew, leaf spot, and yellow leaf blister -- all are commonly spread by airborne spores. Early infections become more serious when weather conditions, typically associated with high humidity, become favorable for further growth especially with accompanying re-infection or secondary infection. Some cause secondary infection to other plant parts in the form of cankers on twigs and main stems or cankers that blacken and curl the stem tips.

Ayres (1991) noted that rust infected plants grew more slowly than non-infected plants. Both abiotic and biotic factors affect growth. Damage to cottonwood commonly results in premature defoliation. It appears that this defoliation has less impact compared to defoliation of evergreen conifers because of the re-foliation and recovery process during the next growing season. However, there is also increased impact with year-to-year re-infection of deciduous trees especially leaf rust infection of cottonwood trees (Laurans and Pilate, 1999; Johnson and Kim, 2005). With cottonwood, pre-mature leaf drop delays the flushing time of the tree in the next growing season, which then affects wood quality (Steenackers, et al, 1996). In the parent plants, infection results in early browning and defoliation of the lower leaves that spreads eventually to the top young leaves. There is no apparent recovery during the same growing season. In fact, previous collection of spores for experimental manual inoculation was done every week until the

end of the infection cycle when no more new leaves were formed and therefore no further spread of leaf rust infection possible.

Table 1.1 List of cottonwood foliage diseases caused by various fungi with common symptoms and damage.

Disease	**Caused by**	**Common Symptoms and Damage**	References
Gray spot	*Coryneum populinum*	Small, water soaked, round, ovate or irregular gray spots on leaves, also affecting petioles and tender new growth. Center of spot falls forming a shothole.	Schmutzenhofer, et al., 1996
Leaf and shoot blight	*Venturia populina and Venturia inopina sp. nov., Septotinia populiperda, Pollaccia radiosa*	Brown to blackened irregular shaped areas causing leaves to become distorted and dried forming a characteristic shepherd's crook symptom.	Berbee, 1964; Funk, 1985; Ostry, 1985; Newcombe and van Oosten, 1997; Newcombe, 2003
Leaf blight	*Linospora tetraspora*	Leaf lessions with characteristic black stromata, spreads along veins forming irregular lessions, and causes premature defoliation	Funk, 1985; Newcombe, 1998
Leaf rust	*Melampsora spp.*	Small yellow-orange pustules scattered on the lower surfaces of leaves and yellow leaf spot most visible in late summer and early fall, requiring 2 unrelated hosts to complete their life cycle.	Berbee, 1964; Funk, 1985
Leaf spot	*Mycosphaerella populicola, Mycosphaerella populurum, and other genera - Ascochyta, Epicoccum, Gleosporium, Leptosphaeria , Phyllosticta*	Circular black spots on both sides of leaves causing severe defoliation. Dead tissue creates a 'shot-hole' appearance.	Berbee, 1964; Funk, 1985
Marssonina leaf blight and leaf spot	*Marssonina brunnea, Marssonina populi, and other species M. castagnei, M. rhabdospora*	Dark brown flecks and spots, often with yellow to tan halos, borders, or margins scattered over leaf surfaces. Several spots may fuse into a large black patch. Orange spore masses develop later.	Berbee, 1964; Funk, 1985; Ostry, 1985; U Nevada Coop Ext Fact Sheet 01-27.
Powdery mildew	*Phyllactinia populi, Uncinula adunca*	Small dusty-white or gray patches or coating with small and scattered black fruit bodies that may eventually cover the entire leaf.	Berbee, 1964; Funk, 1985; Gu and Zhang, 1993
Sclerotia and ink spots	*Ciborinia whetzelii, Ciborinia spp.*	More common in aspens, discolored ring patterns showing fungus advancing through the leaf that become black bodies on brown leaves. The spots eventually fall out making 'shot hole' effect on leaves.	Berbee, 1964; Funk, 1985
Septoria leaf spot	*Septoria populi, Septoria populicola, Septoria musiva*	Distinct tan circular spot with black margins black pimples in the center.	Funk, 1985; Ostry, 1985; Sharma and Sharma, 2000
Yellow Leaf blister	*Taphrina populina, Taphrina populi-salicis, Taphrina aurea*	Golden-yellow leaf spots or raised blister like bumps on upper leaf surfaces.	Berbee, 1964; Funk, 1985; WSU Pub EB1780.

1.2.2.2.1 *Melampsora* Leaf Rust

Melampsora infections are revealed as yellow to orange pustules or uredia containing urediospores found on the abaxial or under-surface of leaves in midsummer that then progress to dark brown fungal growths in the fall (USDA Forest Service, 1989). *Melampsora* infection pathway starts from the spores lodged on the epidermis, followed by entry of the developing spore germ tubes or appressorium through the stoma to the mesophyll areas, and the eventual development of fruiting bodies emerging out to the epidermis (Littlefield and Heath, 1979; Spiers and Hopcroft, 1985; Shain and Jalfors, 1987). There may be epidermal entry of the spore germ tubes as reported by Spiers (1978) or forcible entry through the epidermal cuticle (Talbot, 1999). In *Melampsora* infected cottonwood leaves, hyphae ramify extensively throughout the internal intercellular spaces of the leaf mesophyll area (Spiers and Hopcroft, 1985, Laurans and Pilate, 1999).

It is important to determine the life stage of the infecting spores. As shown in a simplified life cycle (Figure 1.2), the winterized basidiospores that develop from teliospores in the fallen cottonwood leaves infect the primary host larch (*Larix sp*.) plants that form aeciospores and these in turn infect the alternate host *Populus*. The more common alternate host in Western Washington is Douglas-fir (*Pseudotsuga menziesii*). The rust spores used in this study are urediospores from the alternate host *Populus* plants that in turn had formed more urediospores. In this study, urediospores are from a live source of field-exposed experimental plants that were grown, maintained in the greenhouse for future infection and re-infection of other experimental plants.

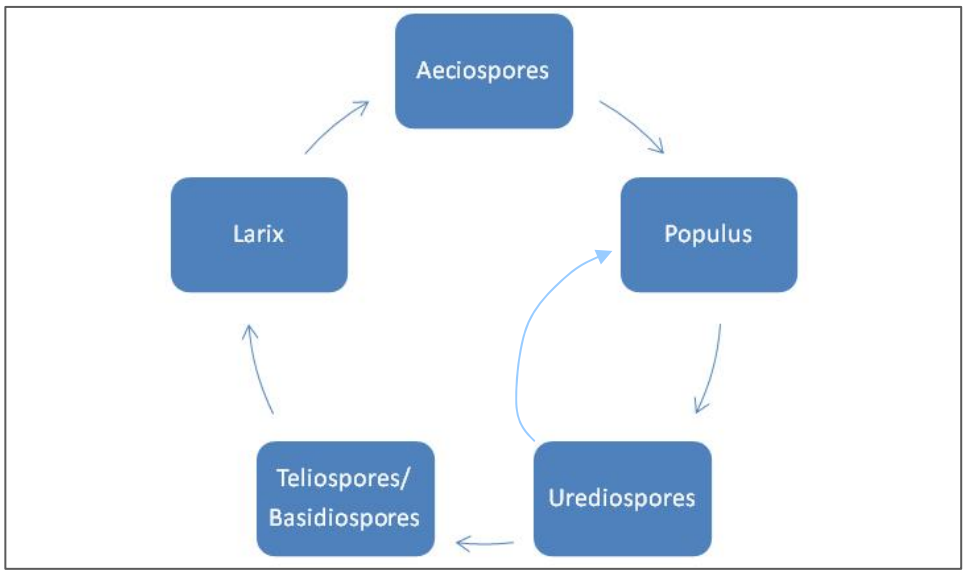


Figure 1.2 Simplified life cycle of *Melampsora* leaf rust showing *Larix* trees as the primary host and *Populus* as the alternate host. Fallen leaves form teliospores that produce basidospores that infect *Larix* that further form aeciospores and infect *Populus* that produce urediospores (Funk, 1985). Note the possibility of direct re-infection by urediospores produced on cottonwood.

The nature of the abaxial characteristics or spongy mesophyll thickness (Hinckley, et al., 1989), depending on the hybrid family, may influence sporulation or ease of spore germ tube development among the mesophyll tissues. The leaf azimuth angles (Isebrands and Michael, 1986; Hinckley, et al., 1989) may also play a role in wind-dispersed sporulation. However, this is not as critical during manual application of the spores via the brushing inoculation technique that I used in this study.

As mentioned earlier, hybrid 47-174 is susceptible to *Melampsora medusae* infection. The specific inheritance of resistance and susceptibility is known. For example, the major gene for resistance to hybrid rust *Melampsora* x *columbiana 3* has been identified as a dominant resistance gene (MXC3) and the *P. trichocarpa* parent is assumed to be heterozygous for this dominant resistance gene (i.e., MXC3/mxc3) whereas the *P. deltoides* parent is assumed to be

homozygous for the recessive allele (i.e., mxc3/mxc3) (Tabor, et al., 2000, Stirling, et al., 2001). The F_1 hybrid clone 47-174 is assumed to be homozygous recessive (i.e., mm/mm) that makes it susceptible to *Melampsora medusae* infection.

Hybrids have differences and similarities with respect to resistance vs. susceptibility to a wide range of different rust species and their hybrids or pathotypes. For example, hybrid 47-174 is susceptible to *M. medusae* but resistant to *M. occidentalis* (Kim and Johnson, 2002) while hybrid 49-177 is susceptible to *M. x columbiana* (Newcombe, et al., 2001) and *M. larici-populina* (Newcombe and Chastagner, 1993) but resistant to *M. medusae* (Banaag, et al., 2006).

1.2.2.2.2 Role that Leaf Development Plays on Leaf Rust Infection

The top segment of the branch, SFS1, has developing or expanding leaves that appear resistant to leaf rust infection. In young shoots of hybrid 47-174, LPIs 1 to 4 appear resistant to leaf rust infection; in contrast, LPI 5 to 7 leaves are fully expanded and these leaves are where leaf rusts symptoms become visible (Johnson and Kim, 2005). It could be that the resistance of younger leaves to infection is related to the morphological development of the stoma in these regions. It could be a lack of a thigmotropic response mechanism (Goodman, et al., 1986; Heath, 1997) in that stomatal ridges that initiate this response may not be formed yet in the young leaves. Or young LPI leaves may not have a detectable epidermal ridge that is a marker of an adjacent stoma initiating growth of spore appressorium. Wynn (1975) refers to this ridge as a specific physical topography that the stomata provide on the leaf surface. It may be that the younger LPI leaves of hybrid 47-174 are devoid of any physical or morphological signaling ridges during infection. In this way, the lack of physical contact or stimuli could be an explanation for apparent resistance in addition to chemical stimuli (Allen, et al., 1991). This could very well be an area that is not favorable yet to establishing a host-pathogen system in

hybrid 47-174. Alternatively, it could be that the observed higher levels of infection of older leaves are related to the time of stomatal opening because stomatal closure generally occurred earlier in young leaves than old leaves (Siwecki and Przrbyl, 1981.

1.2.2.2.3 Interaction of Nitrogen Nutrition and *Melampsora* Leaf Rust

The interaction between N nutrition and *Melampsora* leaf rust infection is not well documented. Comparison between wild and cultivated plants may show indirectly the effects of the substrate on rust infected plants, although other factors may play because rust virulence is increased with more population in a cultivated setting (Gerard, et al., 2006). As mentioned earlier there is an initial physiological relation between the stoma of the host and germ tubes of the rust during infection (Siwecki and Przybyl, 1981). The interaction may be influenced by the effect of nitrogen nutrition on the protein systems that encourage leaf rust sporulation rather than encourage resistance reactions.

The interaction is also likely dependent on the degree of susceptibility or resistance of a given cottonwood hybrid to a given *Melampsora* species. However, this has not been investigated.

1.3 Unique Aspects of the Study

This dissertation contains four unique components. These are (1) the study material, (2) the use of a modified Ingestad approach to deliver N stress (Ingestad, 1977), (3) the focus on assessing the impacts of infection stress on photosynthesis rather than just indirectly on growth, and (4) the use of a modified leaf plastochron indexing method. These are detailed below.

First, hybrid 47-174 is excellent study material because of its fast growth, ease of vegetative propagation, susceptibility to leaf rust infection, and its potential use as an agricultural

tree crop. In addition, hybrid 47-174 has been the subject of several studies. Often these studies contrast and compare results from hybrid 47-174 to other hybrids, the parents and to both F_2 offspring and backcrosses. Examples of these studies include mechanical perturbation (Pruyn, et al., 2000), pathogenic variation (Newcombe, et al., 2001), sylleptic branching (Cline and Dong-Il, 2002), and poplar leaf chemistry (Johnson and Kim, 2002).

In this dissertation, it is assumed that results observed on one-year-old material from cuttings will be representative of the response of new growth on the upper branches of older trees. It is postulated that a large *Populus* tree is basically a collection of developing one-year-old shoots (Dickson, 1986; Marron, et al., 2008). There are studies which show that this assumption may not always be valid (Hinckley, et al., 1998) although an evaluation by Wu, et al. (1998) suggests that such extrapolation may be adequate in fast growing hybrid cottonwood trees. Clonal ranking based on net photosynthesis appears to be the same for most 1 to 5 year-old poplar clones (Ceulemans, et al., 1987).

Second, a semi-hydroponic pump system, employing and modifying Ingestad's technique (Ingestad, 1977), regulates N relative addition rates (RAR) to create differences in N nutrition. This is distinct from the more commonly used fixed fertilizer application or amendment. The use of high (HN) and low (LN) N fertilization addition rates result in more realistic responses because in nature there is low probability of finding naturally occurring N deficiency symptoms, rather plants acclimate to the available nutrients (Rennenberg, et al., 2010). The semi-hydroponic pump system, which supplies the nutrients dissolved in de-ionized water, insures adequate relative water content (RWC) so that impacts on physiology and morphology are solely the results of nutrient levels (Lawlor, 2002).

Third, as demonstrated in preliminary studies (Banaag and Johnson, 2002; Johnson and Stockman, 2002), there was a lower rate of photosynthesis in infected versus un-infected hybrid 47-174. A low foliar N concentration of the infected foliage may further influence this low rate of photosynthesis. Alternatively, high N may allow leaves to compensate for infection or may accelerate or enhance infection. Uncovering the mechanism behind the response is not straightforward as foliar N levels may be affected independently of the degree of infection and the rate of photosynthesis. Knowledge about the effects of infection on structure and function as well as how N nutrition may enhance the level of infection while partially compensating for the loss of function has importance in providing a mechanistic understanding as well as improving hybrid selection and plantation management.

Such knowledge would better enable managers to realize the production promise of cottonwoods as agricultural trees (Hinckley, et al., 1989; Hinckley, et al., 1998; Bradshaw, et al., 2000; Taylor, 2002) and researchers to see that cottonwoods are model organisms for tree biotechnology, physiological and genetic studies (Haruta and Constabel, 2003; Jansson and Douglas, 2007). Additional information would be helpful too in managing poplar farming in terms of its potential as sinks for rising concentrations of atmospheric CO_2 (Finzi, et al., 2007).

Fourth and finally, this study also identifies four structural-functional segments (SFS) of a branch as an efficient way of analyzing and comparing data. Each segment is composed structurally of a group or series of leaves at a similar comparative stage of leaf development. This approach is supported by the literature (Ceulemans, et al., 1995; Hartmann, et al., 2000; Marron, et al., 2008). The sampling alternative is to treat each leaf as developmentally unique, and, thus be required to measure a great number of leaves.

CHAPTER 2

OBJECTIVES AND HYPOTHESES

The main objectives of this study are to illustrate and understand the response of cottonwood hybrid 47-174 to (1) **N stress** with regards to morphology, growth, and photosynthesis. The main objectives also include the response of cottonwood hybrid 47-174 to (2) **leaf rust stress** with regards to morphology and photosynthesis. These factors are shown in a modified Venn diagram in Figure 2.1 that also shows (3) **the probable interaction between nitrogen stress and leaf rust stress** as overlaps in the diagram.

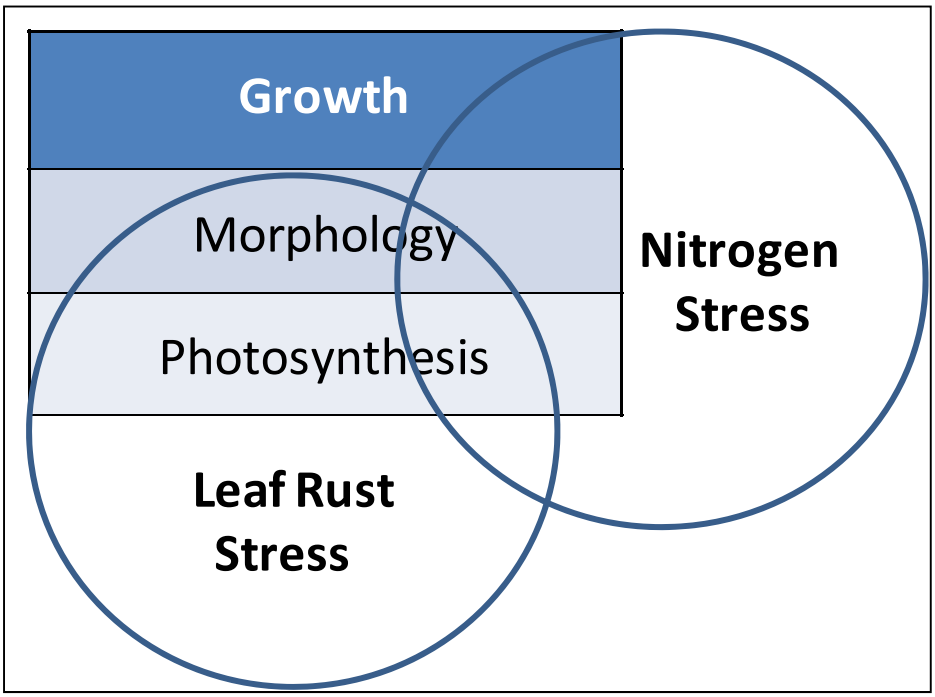


Figure 2.1 Modified Venn diagram showing the interaction of N stress, leaf rust stress, and plant processes (growth, morphology, and photosynthesis).

This modified Venn diagram (Figure 2.1) is supported by Ayers' disease triangle or pyramid with the addition of time or modification by time (Ayres, 1991). However, Ayres' (1991) representation of interactions is two-way in contrast to the diagram in Figure 2.1 that illustrates three-way interactions (overlaps). Ayers' plants-pathogens-abiotic environment corresponds to my plant (growth, morphology, photosynthesis)-pathogen (leaf rust infection)-abiotic (N fertilization) diagram.

For all studies my research approach takes into account differences in the stages of leaf development and this is defined by a leaf's LPI. Because a given plant may have more than 20 leaves, the LPI of all the leaves were identified and for sampling purposes when necessary all the leaves by LPI were divided into one of the four SFS.

2.1 Nitrogen Stress

2.1.1 Morphology

How does foliar morphology, specifically individual leaf area and stomatal density in hybrid 47-174, respond to HN and LN fertilization?

The following hypotheses are considered:

H_{01}: N fertilization has no effect on leaf area.

H_{02}: N fertilization has no effect on stomatal density.

2.1.2 Growth

How do growth processes in hybrid 47-174 respond to N nutrition? The objective was to monitor individual and total leaf area under high HN and low LN fertilization rates. In addition,

plant height, internode length, stem diameter, and stem dry weight were also evaluated. N use efficiency (NUE) was calculated and compared among LPIs and SFSs.

The following hypotheses are considered:

H_{01}: N fertilization has no effect on growth measurements.

H_{02}: N use efficiency of leaves does not vary by LPI or SFS.

2.1.3 Photosynthesis

How does photosynthesis in hybrid 47-174 respond to HN and LN fertilization rates? The objective was to verify and duplicate previously observed reduction in CO_2 uptake in LN plants compared to HN plants. Photosynthetic N use efficiency (PNUE) was calculated and compared based on LPI and SFS.

The following hypotheses are considered:

H_{01}: N fertilization has no effect on photosynthesis.

H_{02}: Photosynthetic N use efficiency of leaves does not vary with LPI or SFS.

2.2 Leaf Rust Stress

2.2.1 Morphology

How does leaf structure and features, specifically the epidermis and the mesophyll area in hybrid 47-174 respond to leaf rust infection? Gross observations were supplemented by light microscopy and scanning electron microscopy of epidermal cells, stomata, and uredia of rust infected leaves.

The following hypothesis is considered:

H_{01}: Leaf rust infection has no effect on leaf morphology.

2.2.2 Photosynthesis

How does photosynthesis in hybrid 47-174 respond to leaf rust infection? The objective was to verify and duplicate previously observed reduction in CO_2 uptake in leaf rust infected as compared to healthy leaves.

The following hypothesis is considered:

H_{01}: Leaf rust infection has no effect on photosynthesis.

2.3 Interaction between Leaf Rust and Nitrogen

2.3.1 Morphology

How does foliar morphology, specifically leaf area and stomatal density in hybrid 47-174 respond to LN and HN fertilization rates and leaf rust infection?

The following hypotheses are considered:

H_{01}: N fertilization has no effect on leaf rust infection.

H_{01}: Morphological features developed as a result of HN and LN fertilization rates have no influence on leaf rust infection.

2.3.2 Photosynthesis

How does photosynthesis in hybrid 47-174 respond to N nutrition and leaf rust infection? The objective was to verify and duplicate previously observed reduction in CO_2 uptake in infected as compared to healthy leaves from both HN and LN plants. Photosynthetic N use efficiency (PNUE) was also calculated and compared based on LPI and SFS.

The following hypotheses are considered:

H_{01}: The interaction between leaf rust infection and HN or LN fertilization rates has no effect on photosynthesis.

H_{02}: Photosynthetic N use efficiency of leaves does not vary with LPI or SFS on healthy and infected HN and LN plants.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Material

The study material is a F-1 hybrid cottonwood, 47-174, which resulted from a cross between a female *Populus trichocarpa* (ORT 80-1) and a male *Populus deltoides* (MO-243) – indicated as a T x D, or female T parent x D male parent hybridization. The provenance of the female T parent is Pierce County, WA and the provenance of the male D parent is Howard County, MO (Newcombe, et al., 2001; Johnson and Kim, 2005). This hybrid is part of the western *P. trichocarpa* accession from geographically diverse female *P. trichocarpa* sources. This hybrid is the 174th offspring from the 47th crossing (Johnson, 2000).

All study plants were rooted from cuttings taken from hybrid cottonwood 47-174 current year's new coppice growth in the clonal source plants located at Farm 1 of the Washington State University Experimental Station in Puyallup, Washington (WSU Puyallup). The source plants are close to a grove of planted larch trees that are the alternate hosts for the leaf rust. Their proximity to each other ensures yearly leaf rust infection of larch and cottonwood trees. Figure 3.1 shows the weekly growth of the coppiced parent plants from June 9 to July 14, 2009. Cuttings were taken on July 21, 2009 when the branches were mature enough to expect rooting and formation of buds (Table 3.1). Cuttings were 15 cm long sections of stems that included one node and the accompanying matured leaf that were between LPIs 8 to 12.

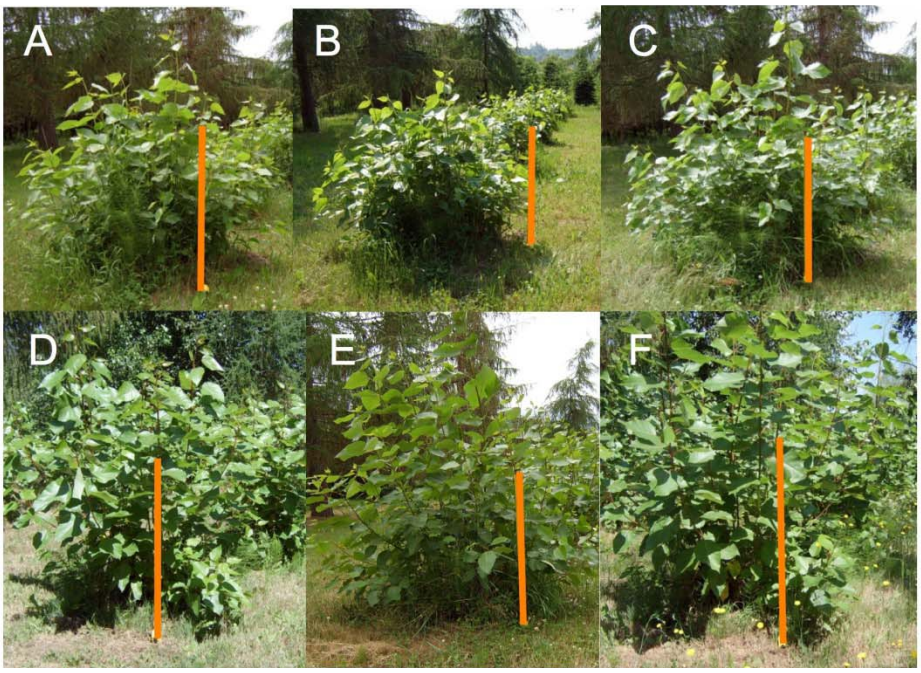


Figure 3.1 Weekly photos (A-F) of 47-174 coppiced source field cottonwood plants and larch trees in the background in Farm 1 of Washington State University Experimental Station in Puyallup, WA (WSU Puyallup). Photos were taken weekly from June 9 (A) to July 14 (F), 2009. Orange marker is 1 m.

3.2 Greenhouse Setup

Rooting of the cuttings was done under mist using small pots filled with potting soil inside the greenhouse at WSU Puyallup. Rooted plants with one primary bud were transplanted after 4 weeks on August 20, 2009 (Table 3.1) and grown in plastic pots filled with very small rocks and randomly placed in containers that were supplied weekly with complete nutrients by using a semi-hydroponic pump system (Figure 3.2). Greenhouse temperature was controlled at 20 to 22^{o}C. A calendar of subsequent steps and measurements are presented in Table 3.1.

Table 3.1 Calendar showing various key events, rooting, measurement, and harvest of cottonwood plants from July 21 to November 13, 2009. Bold calendar dates are referenced in the text.

2009													
Month/Days	**Su**	**Mo**	**Tu**	**Wed**	**Th**	**Fr**	**Sa**	**Rooting Weeks**	**Growth Weeks**	**Measurement Weeks**	**Harvest Weeks**	**Key Events (Dates)**	**Other Events (Dates)**
July			**21**	22	23	24	25	0				Cuttings planted (7/21)	
	26	27	28	29	30	31	1	1					
August	2	3	4	5	6	7	8	2					
	9	10	11	12	13	14	15	3					
	16	17	18	19	**20**	21	22	4	**0**			Rooted cuttings transplanted (8/20)	
	23	24	25	26	27	28	29		**1**				
	30	31	1	2	3	4	5		**2**				
September	6	7	8	9	10	11	12		**3**				
	13	14	15	16	17	**18**	19		**4**	**1**		Start of 7 weekly measurements (9/18-10/30)	
	20	21	22	23	24	**25**	26		**5**	**2**	**2**	Week 2 harvest (9/25)	Field plants out (9/25)
	27	28	29	30	1	**2**	3		**6**	**3**			
October	4	5	6	7	**8**	**9**	10		**7**	**4**	**4**	Week 4 harvest (10/9)	Inoculate (10/8)
	11	12	13	14	15	**16**	17		**8**	**5**			Field infected plants in (10/16)
	18	19	**20**	21	22	**23**	24		**9**	**6**	**6**	Week 6 harvest (10/23)	Inoculate (10/20)
	25	26	27	28	29	**30**	31		**10**	**7**	7	Week 7 harvest (10/30)	
November	1	2	3	4	**5**	**6**	7		**11**				CO_2 measurements (11/5) Inoculate (11/6)
	8	9	10	**11**	12	**13**			**12**		12	Final Harvest (11/13)	CO_2 measurements (11/11)

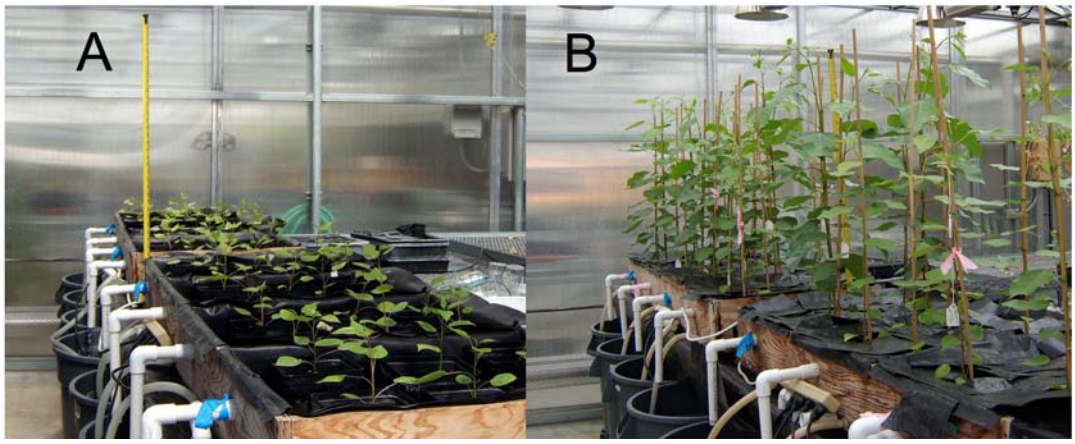


Figure 3.2 The semi-hydroponic pumping set up had tubing that circulated the nutrient solution from the dark grey plastic drum at floor level to the elevated plastic lined wooden box container partitioned into 4 units with the 6 potted plants per unit. Plastic drainage tubing (with blue tags in the photo) near the top of the containers prevented overflow by redirecting nutrient solution back to the plastic drum reservoir. Yellow marker is 1 m. A is photo at the start and B is photo at the later phase of the setup.

The rooted and transplanted plants in plastic pots were placed in 2 plastic-lined wooden containers (A and B) (Table 3.1). Each container (183 x 61 x 20 cm) had 4 separate units (46 x 61 x 20 cm) that accommodated 6 potted plants per unit (1 pot = 14 x 14 x 17 cm). In each container, 2 units were under low N (LN) and 2 units were under high N (HN). There were a total of 48 plants, 24 plants in each of the 2 containers, or 6 plants per unit in 4 HN and 4 LN units. The containers were identical. Figure 3.3 shows the various random treatments in each unit. Each of the 8 units had an independent pumping system that regularly circulated the nutrient solution every 30 minutes for 15 minutes by a timer-actuated submersible pump. The nutrient solution was replenished weekly.

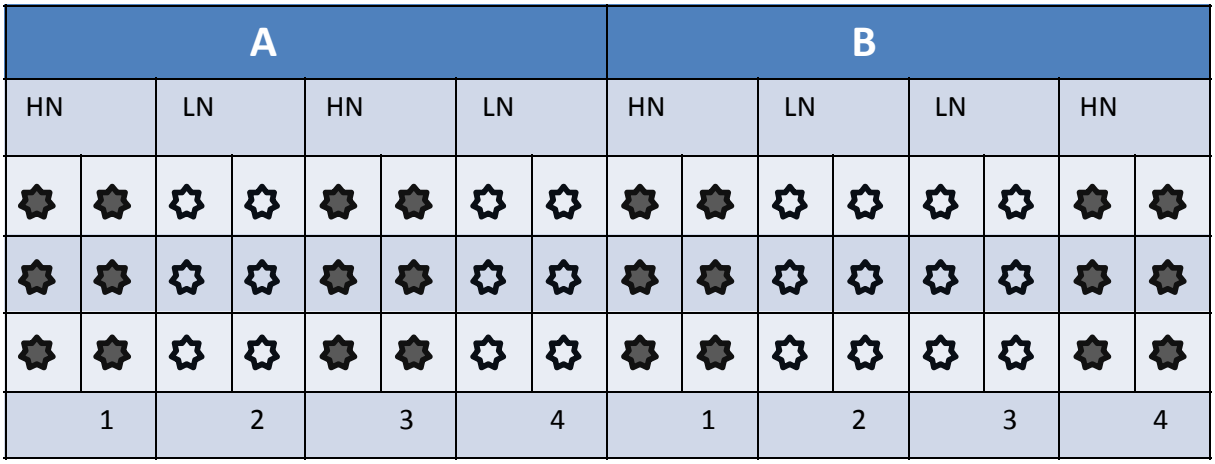


Figure 3.3 Experimental set up showing containers A and B have 4 random units each under high nitrogen (HN) or low nitrogen (LN) fertilization rates. Each of the total of 8 units contains 6 HN ( ) or 6 LN ( ) plants grown in individual pots.

3.3 Nitrogen Fertilization

Nutrients, including N, were provided by a semi-hydroponic pump system (Figure 3.2) circulating at regular intervals a complete nutrient solution from a 37.9 l plastic drum. This nutrient-solution culture set up was monitored closely, as any shut-down risked dehydrating the study material. Weekly replenishment of the nutrient solution was facilitated by the plastic drum reservoir which could be rolled, provided a continuous supply of N and other essential nutrients. The wooden potted plant containers, nutrient reservoir drums, and tubing system were covered with black plastic tarp to prevent algal growth.

Previous trials demonstrated visible differences in the growth of hybrid 47-174 cottonwood under high N (HN) versus low N (LN). HN was set at a 6% fertilization rate or relative addition rate (RAR) that exposed the plants to more N than the LN, addition rate which was set at 1.5% RAR. Under LN there were no visible symptoms of N deficiency. The N addition schedules were exponential nutrient addition patterned in principle on the Ingestad system (Ingestad, 1977; Ingestad, 1978; Ingestad and Lund, 1986) that adjusts for the rate of

plant growth and consumption. This technique assumes that the rate of external addition that is supplied according to demand or plant requirements is the decisive factor in regulating nutrition and growth rate (Ingestad, 1977; Ericsson, 1981; Ingestad and Lund, 1986; Imo and Timmer, 1992). This approach brings about internal nutrient levels that are close to "steady state" throughout the experiment (Linder and Rook, 1984) because young plants are not exposed to overabundant and unneeded amounts of nutrients and older plants do not end up with undersupply of much needed nutrients. During the exponential period of growth, nutrients were also added in exponentially increasing amounts (Ingestad and Lund, 1986). This pattern of addition was followed for both the low and high N treatments.

The nutrient composition of the N fertilization solution complete with all the essential elements is listed in Table 1, Appendix A and was administered weekly using a schedule of N RAR shown in Table 2, Appendix B (both from J. Johnson, personal communication). The recommended concentrations and balance in solution cultures for the other elements are lower than those often used in solution culture experiments (Walker, 1991). The pH values under HN ranged from 6.42 to 6.56 while under LN ranged from 7.18 to 7.44 both were measured by using a portable pH meter at the later phase of the setup. The optimum pH for hybrid poplars ranges from 5.5 to 8.0 (website of Agriculture and Agri-Food Canada).

Measurements of morphology and growth commenced on September 18, 2009 (Table 3.1) 4 weeks after transplanting to the semi-hydroponic system. Most measurements and selective destructive harvests occurred 10 weeks after transplanting on October 30, 2009. A number of plants from each treatment remained that were used for measurement of photosynthesis 12 weeks after transplanting.

3.4 Leaf Plastochron Index (LPI)

Leaves were given a LPI number to identify the developmental stage and the relative position of the leaf on the plant at a given time. Leaves were assigned a LPI number based upon a numbering system from the bud tip to the base of the plant; starting from the top, LPI 0 is assigned as the first or the "index leaf" identified in this study as the leaf that is not fully expanded with margins that are still curled adaxially inward or curled toward the upper surface. The same definition of LPI 0 was used by Dickmann (1971). LPI 0 is commonly reported as those leaves less than 6 cm long (Larson and Isebrands, 1971; Isebrands and Larson, 1973 and1977; Dickson, 1986; Newcombe, et al., 1996; and Wait, et al., 1999). In my study, LPI 0 leaf ranged from 2 to 4.5 cm in length and with widest adaxially curled margins from 0.3 to 1.7 cm in width, equivalent to leaf areas that ranged from 0.6 to 7.7 cm^2. Dickmann (1971a) identified 6 cm long leaves as LPI 3 and mentioned that because of the small size of LPI 2 or younger leaves, some measurements are only possible when they are combined with leaves of the same development stage from other plants. For example, it was necessary to harvest more leaves with the same LPI numbers from several plants in order to have sufficient plant material to determine the amount of phenolic compounds and protein from this hybrid (Banaag, et al., 2005). In some cases, for the same measurements, fewer or only one leaf was enough as the LPI number (and hence leaf size) increased. As the plant grew, new leaves were formed changing the previous LPI number and maybe become part of another SFS.

3.5 Structural-Functional Segments

As mentioned earlier, one plant with all the associated LPIs or each branch of a plant with its associated LPIs can be subdivided into 4 SFS, thereby aggregating leaves of comparable

structural and functional responses with reference to their LPIs (Dickson, 1986; Ceulemans and Isebrands, 1996). This is an efficient extension of the LPI numbering system.

Observations showed that on average, a hybrid 47-174 plant on high N (HN) fertilization would have a total of 32 LPIs and therefore dividing the plant into 4 segments would have 8 LPIs per segment. A plant on LN fertilization on the average would have a total of 20 LPIs and therefore dividing the plant into 4 segments would have 5 LPIs per segment. The use of segments is practical in that it tends to lump leaves of similar structure and function properties.

Leaves on HN plants were divided into the following four segments: (1) newly formed and expanding leaves (SFS 1, LPIs 1 to 8), (2) newly matured leaves (SFS 2, LPIs 9 to 16), (3) fully matured leaves (SFS 3, LPIs 17 to 24), and (4) over-matured leaves close to senescence (SFS 4, LPIs 25 to 32) (Figure 3.4). In parallel, LN plants were also divided into four segments: (1) newly formed and expanding leaves (SFS1, LPIs 1 to 5), (2) newly matured leaves **(**SFS 2, LPIs 6 to 10), (3) fully matured leaves (SFS 3, LPIs 11 to 15), and (4) over-matured leaves (SFS 4, LPIs 16 to 20). LN plants had fewer leaves than HN plants. When all of the leaves or LPIs belonging to a segment could not be measured, then randomly chosen leaves from that segment (and representative of the LPIs defined for that segment) were selected.

In terms of enabling a study of structure and function, segmentation represents a necessary and a rationale experimental shortcut (Van Pelt and Verwer, 1985). For example during a preliminary study where more leaves were needed for determination of leaf phenolic compounds and protein content of this hybrid (Banaag, et al., 2005), rather than taking samples of leaves with the same LPIs from several plants, identifying the 4 segments with the associated group of leaves provided enough plant material for each plant.

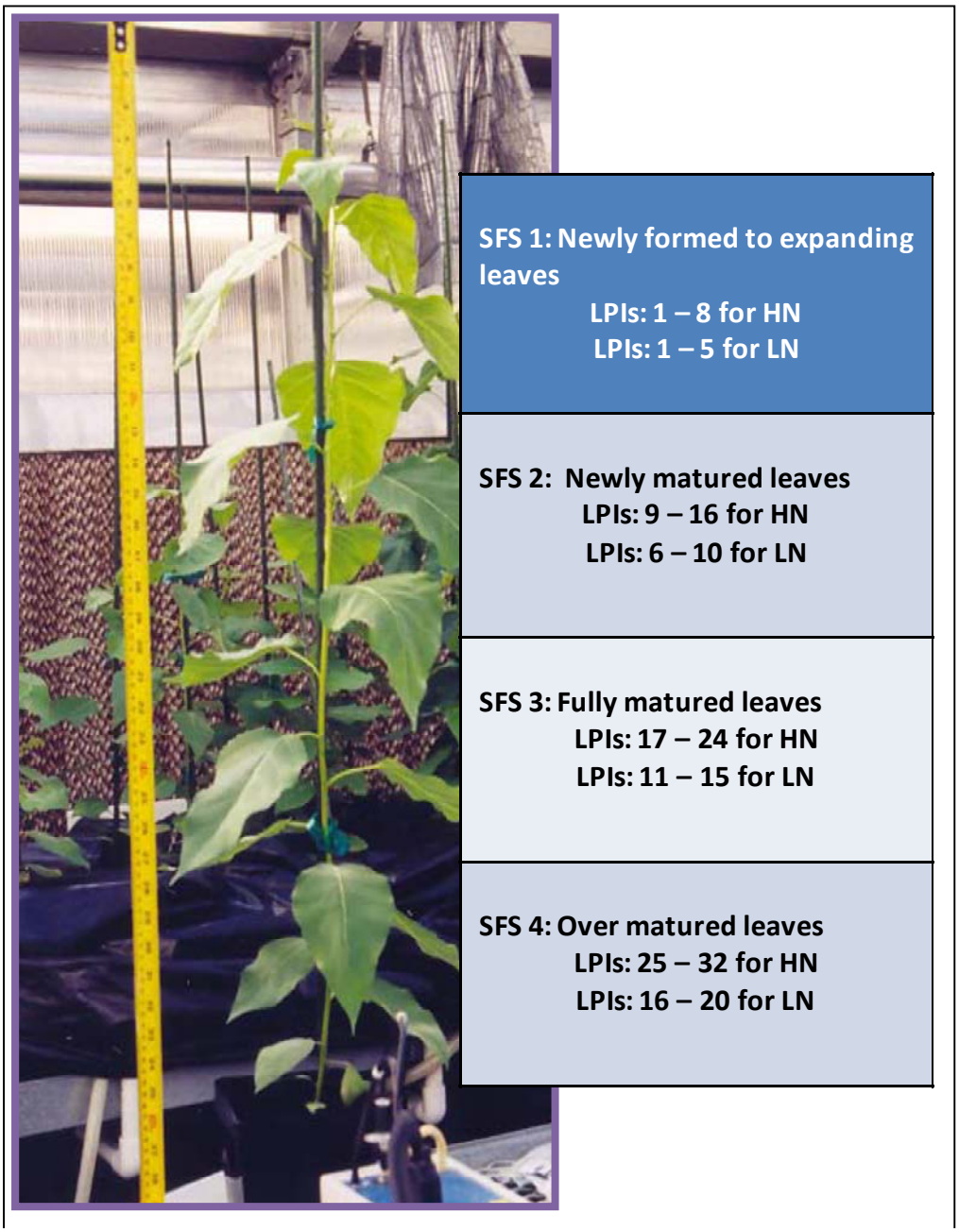


Figure 3.4 Structural and functional segments (SFS) of a branch or plant are composed of the top (SFS 1), middle (SFS 2 and SFS 3), and base (SFS 4) on high nitrogen (HN) or low nitrogen (LN) fertilization rates and their corresponding leaf plastochron index (LPI).

3.6 SPAD Measurements

A hand-held and nondestructive and noninvasive Minolta Chlorophyll Meter, model SPAD-502 was used to measure leaf chlorophyll concentration (Murchie, et al., 1999; Richardson, et al., 2002; Haile and Higley, 2003) (Soil-Plant Analysis Development Section, Minolta Corp., Osaka, Japan). N concentration of leaves was expressed as a relative value in terms of the measured SPAD values (0-100) based on reported direct correlation of SPAD chlorophyll content measurements to N concentration (Bullock, 1995; Peterson, et al., 1996; Ichie, et al., 2002; Moreau, et al., 2004; Ripullone, et al., 2003; Zhao, 2006). Weekly measurements with the SPAD-502 meter also monitored if the N levels were being maintained in the solution culture on a regular basis. Measurements were done at 3 locations of a leaf blade, clockwise from 1st on the left section, the 2nd on the tip region, to the 3rd on right section of the adaxial side of a leaf lamina with the petiole oriented at the base of an upright leaf. Leaf data were the average of measurements at these 3 locations.

The indirect measure of N as correlated to chlorophyll appears to be a good measure of N because during ashing, a necessary step in laboratory N content analysis, some amount of N disappears as N oxides (Lambers, et al., 1998). Zhao's (2006) measurements using the same SPAD-502 meter on other poplar hybrids showed a significant linear relationship between SPAD readings and N concentration; however, N and leaf development differences in leaf thickness might affect this relationship in the study material. This was not investigated. According to Moreau, et al., (2004), SPAD values accurately determined total N concentration and content (area basis) in 2 hybrids of eastern cottonwood and not influenced by clonal variety.

3.7 Leaf Area Measurements

After transplanting of the rooted cuttings, measurements began on the 9th week following initial growth and acclimation to the semi-hydroponic system (see Table 3.1). Among the plants in Container A, 8 plants or 2 plants from each of the 4 units, comprising 4 HN and 4 LN plants, were flagged for weekly monitoring of their LPI numbers and measurements of their corresponding leaf length and widest leaf width. Among the plants in Container B, 1 plant from each of the 4 units, comprising 2 HN and 2 LN plants was harvested every 2 weeks starting at the end of the 10th week. Leaves from these harvested plants were also assigned their LPI numbers. Paper tracings of the harvested leaves were done for future leaf area measurements.

Leaf area was measured by using a Leaf Area Meter (Decagon Instruments, Inc., Pullman, WA, USA) with Agimage software. The leaf area measurements of the paper cutouts from tracings of harvested leaves gave a computed correction factor that accounted for the shape of leaves from the weekly length x width measurements of intact plants. Paper tracings were done within an hour after harvest, which ensured no loss of leaf turgor and prevented cell shrinkage that would have affected the leaf area measurements. Paper tracings also prevented destructive damage or loss of leaf area when fed through the leaf area meter and allowed scheduling of leaf area measurements at a more convenient time. Cutout tracings of large leaves were cut into 2 pieces to pass through the margin restrictions of the leaf area meter. Areas of the cut pieces were combined to give the total leaf area for that particular leaf. The leaf photocopying technique (Ferris, et al., 2002) had been used in previous trials but there was too much use of toner.

3.8 Stomatal Density

Stomatal density (SD) is equal to the number of stoma per given area, expressed as the number of stoma mm^{-2}. SD counts were done by light microscopy of whole leaf impressions. Whole leaf impressions were prepared by applying a thin layer of clear nail polish on the entire abaxial side of the leaf lamina. The leaves with applied nail polish were allowed to dry inside an adequately vented laboratory hood. The dried whole leaf nail polish impressions were cut into ordered small sections, dry mounted all sections on microscope slides held in place by cover slips with clear adhesive tapes on the sides, numbered, and labeled. Measurements were taken on all of these mounted sections by making 3 random stomatal counts per section. This technique examined stomatal heterogeneity on a leaf (Weyers and Lawson, 1997) but it also provided a good measure of SD because all sections of a leaf lamina were sampled.

Whole leaf impressions were made on fully expanded leaves of LPI 11 (SFS2) of HN plants and LPI 5 (SFS1) or LPI 6 (SFS2) of LN plants. These whole leaf impressions resulted in a greater number of cut sections for HN plants because of their larger leaf areas compared to leaves from LN plants. The random stomatal counts were made from each section with a grid micrometer eyepiece using a compound Reichert microscope at 100X high power objective.

When possible, the stomatal index (SI) was also determined by counting the number of neighboring cells around the stoma. SI in this study is the % of stoma among the total neighboring epidermal cells and stoma in a given area or mm^2.

3.9 Disease Inoculation

Four plants, 2 HN and 2 LN, growing on the same semi-hydroponic setup were taken from the greenhouse on September 25, 2009, the 5^{th} week of growth after transplanting to the

location of the source plants in the field at Farm 1 and exposed to a natural source of *Melampsora medusa* leaf rust. These plants were kept in experimental 18.9 L plastic containers with the root systems continuously exposed to the same nutrient solution until infection had occurred. The nutrient solution was replenished weekly but was not circulated or refreshed because there was no pumping system. These plants were brought back to the greenhouse when infection became visible on October 10, 2009, the 8th week. These field-exposed and field-infected plants were good sources of live spores for subsequent greenhouse manual inoculations because by the time the cuttings were ready for manual inoculation the source field plants had already reached their maximum peak infection and begun to defoliate. With the infected material in the greenhouse, there was also minimal time lag from spore collection to manual inoculation.

Bright orange powdery spores were collected from the infected abaxial surface of leaves by tapping or by gently sweeping the leaf surface with a camel hair brush into a petri dish. Fresh spores from the petri dish were evenly applied in the mornings at about 0900 h on the abaxial surface of leaves with the same camel brush used in collecting the spores. This period of inoculation was optimal as humidity was high and stomata were fully open.

A transparent light plastic bag was placed over each manually infected leaf for 24 hours after inoculation to maintain high humidity conditions, a requirement for successful inoculation (Figure 3.5 A). Plants with some leaves inoculated and the whole plant bagged completely (Figure 3.5 B) did not produce successful leaf rust growth. The individual leaf inoculation using a camel brush and individual leaf bagging after inoculation allowed a uniform moderate to saturated application and efficient tracking and monitoring of inoculated leaves. Some leaves randomly selected to represent each of the 4 SFS of a plant were also inoculated. This allowed for conservative use of the available live spores. It was not feasible to infect an entire plant with

the limited supply of living spores from the infected source leaves in the greenhouse during an inoculation period. Therefore leaves representing each SFS were randomly selected which also randomized their vascular so randomized inter-connections i.e., leaf traces (Dickson and Isebrands, 1991).

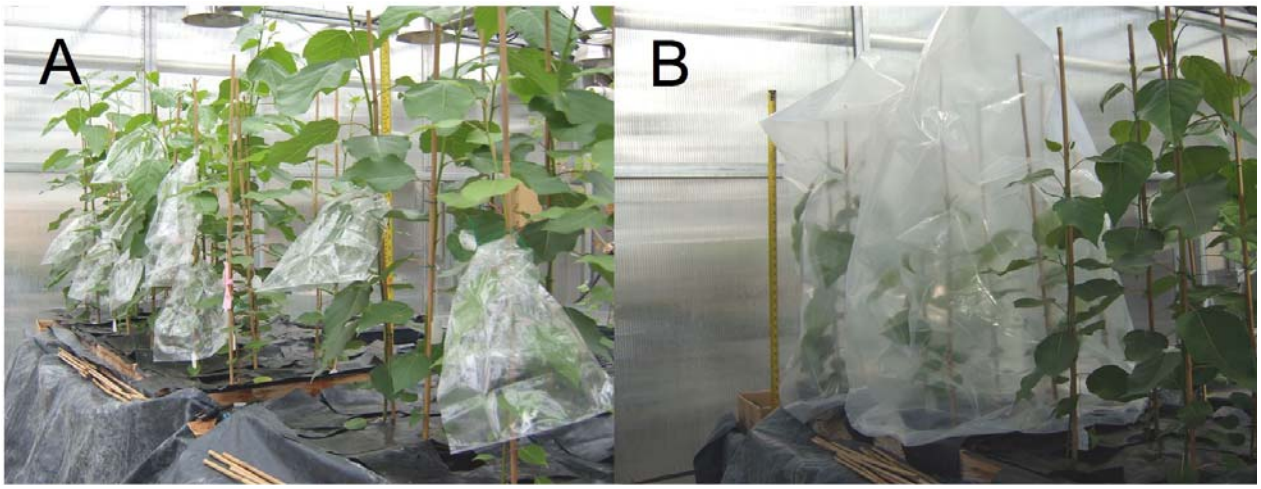


Figure 3.5 Plants with leaves bagged individually (A) or plants bagged completely (B) for 24 hours after inoculation. Note that in the photo, a black plastic tarp covers the plant pot containers, drum reservoir, and tubing of the pumping system to prevent algal growth.

The infected leaves showed visible infection one week after inoculation or 7 days post infection. Some plants were often inoculated in such a manner so has to have leaf rust infection match the schedule for photosynthesis measurements and SEM procedures. It was not necessary to re-inoculate the leaves because the brush technique insured uniform application of the spores exposing the leaves to a saturated amount of inoculums. Infection was not quantified because of the profuse and uniform leaf rust growth. In the greenhouse, there was no air circulation greatly reducing the likelihood of infecting other leaves, especially newly formed leaves. Adjacent leaves were not infected unless manually infected. It was also noticed that spores falling on the

adaxial side of the lower leaves from upper infected leaves did not result in infection. Control plants, or more appropriately control leaves, were those next neighbor leaves that were not inoculated and, thus not inflected at all in both natural and artificial ways. As noted by Dickson and Isebrands (1991), such leaves share a minimum of vascular connections.

3.10 Leaf Anatomy

Uredinial growth of leaf rust-infected leaves was observed under the scanning electron microscopy (SEM). As mentioned earlier, the brushing technique allowed a uniform moderate to saturated application that would bring about increased chances of infection. The severity of the disease can be expressed as the frequency of these uredinial pustules on a given leaf or per unit of leaf area (Wang and van der Kamp, 1991).

To accomplish the objectives, there were two options for preparing sample leaves for SEM: (1) Some infected leaves were air dried in a Hakuba KMC-11 5.5L Dry Box with 2 reusable desiccant packs (Hakuba, USA, Inc.); (2) Other infected leaves were cut into small pieces or sections that were critical point dried (CPD). CPD tissues retained surface cell contours better than air-dried tissues; however, surface wax was better preserved in air-dried tissue because no organic solvents are involved in processing (McWhorter and Paul, 1989). Samples to be critical-point dried were fixed in 4% formalin solution containing $CaCO_3$ at saturation point (Brighigna, et al., 2002), washed in distilled water, dehydrated in an ethanol series, and dried in liquid CO_2 in a critical-point dryer (Denton DCP-1 Critical Point Drying Apparatus). Sections of air-dried leaves and CPD dried samples were attached to aluminum specimen stubs by 2-sided mounting tapes that were coated in a Hummer V Sputter Coater.

Infected leaves that were air dried or fixed in a formalin solution in preparation for SEM were taken from LPIs representing developed to recently matured leaves of SFS 1 and 2 of HN

and LN plants. HN leaves were from LPIs 8 to 11 while LN leaves sampled were from LPIs 5 to 9. Infected leaves sampled were those exhibiting more than 1 week of leaf rust growth (see Table 3.1).

The SEM work was done with a JEOL JSM-840A SEM in the Biology Department, University of Washington. Some of the leaf segments were viewed at an angle during SEM analysis to see the cross-section of the mesophyll area beneath the epidermis and the external uredial leaf rust infection.

3.11 Stem Measurements

The measurement of stem variables is important because the stem constitutes the main bulk of harvested biomass and it also represents the economically viable part (Scarascia-Magnozza, et al., 1997). Three variables were measured: internode length, stem diameter, and stem dry weight.

The length of internodes and plant height were measured in 6 plants that were harvested on October 23, 2009. The LPIs associated with the internodes were noted. Such observations can be correlated with leaf length measurements, leaf area, and biomass (Pieters, et al., 1999).

Diameter of stems at 10 cm height from the soil was measured using a General dial caliper (General Tools & Instruments, NY) during the 12^{th} week after transplanting, near the end of the experiment (see Table 3.1).

The total dry weight of stems from plants harvested at the 12^{th} week was also determined after oven-drying for 48 h at 72^{o}C. This later harvest time appeared to be appropriate for indication of treatment as Cooke, et al. (2005) observed that main stem biomass values were not significantly different after 28 days in poplars exposed to varying N levels.

3.12 Growth Measurements

The effects of N fertilization rates became noticeable on August 18, 2009, 4 weeks after the rooted plants had been transplanted into the semi-hydroponic solution culture (see Table 3.1).

Growth data include measurements taken weekly for 7 weeks, harvest data taken every 2 weeks for 6 weeks, and harvest data at the end of the experiment at week 12. Growth parameters include leaf area, plant height, internode length, stem diameter at 10 cm height, and stem dry weight.

As mentioned earlier, 8 plants from Container A were measured for 7 weeks to document the increase in leaf area and plant height while at the same time monitoring leaf N content indirectly by the SPAD-502 chlorophyll meter. During the 2-week interval harvests from Container B, actual leaf areas of all the harvested leaves or LPIs were measured. Preliminary results showed that under both HN and LN levels, new leaves had matured and fully expanded in 2 to 3 weeks after which there were no more increase in leaf area.

After tracing each leaf on a piece of paper, individual leaves from harvested plants were separated and labeled with their known LPIs, put in their respective paper bags for oven drying. These harvested leaves and stems were oven-dried to determine the biomass or dry weight per given sample and harvest time.

Analyses of leaf area, leaf N, and leaf dry weight provided needed parameters for calculation of N use efficiency. N use efficiency was determined and expressed by LPI, SFS, and time of harvest.

3.13 CO_2 Uptake Measurements

Photosynthetic or CO_2 uptake (μmol CO_2 m^{-2} s^{-1}) measurements were confined to randomly chosen leaves of known LPIs from the 4 SFS. In each segment, measurements were taken on one infected leaf and a paired, uninfected or a control neighboring leaf of the same plant. Paired leaves from the same segment were an efficient way of analyzing effects of infection because of the likely similar stages of functional and morphological development resulting in a treatment. Sampling adjoining leaves was an efficient way of comparing responses of infected and uninfected leaves.

Data on CO_2 uptake were measured with a Li-Cor LI-6400 Portable Photosynthesis System (Li-Cor Ltd, Lincoln, NE, USA). This device also compares the CO_2 levels before and after passing through the cuvette. Measurements included the derivation of a light-response curve by stepping through a series of known light levels, photosynthetic active radiation (PAR), over time. In addition, an ACi curve were derived by stepping through a series of internal leaf CO_2 levels. Measurements were conducted at the School of Environmental and Forest Sciences, UW. The semi-hydroponic plants were maintained in 20-L containers under the same nutrient solution as used in the greenhouse, except that there was no continuous pumping system. The laboratory set up was convenient in maintaining and alternating charged batteries for continuous measurements with the Li-Cor LI-6400 (Figure 3.6).

Figure 3.6 Setup measuring CO_2 uptake with a Li-Cor LI-6400. A portion of the intact leaf is illuminated and flatly clamped within the cuvette in the middle of the photo.

The data include photosynthesis rate (A), stomatal conductance (gs), and calculated internal CO_2 concentration (Ci). Measurements of CO_2 uptake were associated with parallel measures of height, SPAD values, and the LPI number of leaves that had reached full leaf expansion as suggested by Wait et al. (1999).

3.14 Light Response Curves

Two plants were transported to the University of Washington campus the night before measurements, one HN and one LN plant (Table 3.1). From these plants, two leaves one from each plant were selected to develop light response curves. Measurements were made with the intercellular CO_2 set at 400 μmol CO_2 mol^{-1} (ppm). Light series were obtained with the Li-Cor LI-6400 by decreasing the absorbed photosynthetically active radiation (PAR) from 1500 to 0

μmol m^{-2} s^{-1} every 2 minutes or so until a stable reading had been obtained. The leaves selected were those that had had the highest rates of CO_2 uptake, LPI 3 from the plant on high N (HN) and LPI 4 from the plant on low N (LN) fertilization rates. The HN leaf took 5 minutes longer to stabilize between readings in the development of the light response curve compared to the LN leaf. CO_2 uptake data are expressed as μmolCO_2 m^{-2} leaf area s^{-1}.

3.15 CO_2 or ACi Response Curves

A 2nd set of plants, one HN and one LN, was transported to the UW campus for the CO_2 or ACi response curve measurements. Because measuring photosynthesis especially AC_i curves in all leaves of a given plant (Noormets, et al., 2010) takes considerable time, only one pair of leaves from the 4 SFSs of one HN and one LN plant were measured. For both HN and LN plants, these paired leaves were randomly selected from leaves of SFS 1, SFS 2, SFS 3, and SFS 4. With 4 segments per plant there were 8 leaves or LPIs per plant and therefore a total of 8 segments and 16 leaves. This paired scheme was used for HN versus LN, for HN versus HN infected, and for LN versus LN infected comparisons. Measurements were done by pairs but were taken at random per pair to account for the time lag. Each measurement was done with an absorbed photosyntheticaly active radiation set at 1500 μmol m^{-2} s^{-1}. Measurements were done at a series of intercellular CO_2 levels that started close to ambient at 400 ppm or μmol_{CO2} mol^{-1}_{air} and decreased to a level close to 50 ppm were there was no observable photosynthesis, and then increased again to a maximum level close to 700 ppm. On the average, it took 30 to 36 minutes per leaf so a total of about 10 hours to complete the measurements.

CO_2 compensation points were determined graphically from the ACi curves. The compensation point was defined as the level of intercellular CO_2 (ppm) where photosynthesis balanced respiration or net photosynthesis equaled zero.

3.16 Data Presentation and Statistical Analyses

Some leaf measurements were done on all the leaves or LPIs of a plant. Sometimes these measurements were subdivided or grouped into the 4 SFS segments for further statistical analyses. In other situations, leaf measurements were done on paired leaves representing each of the 4 SFS segments. The use of the SFS segments was the most efficient way in gathering photosynthetic assimilation data.

Data were analyzed using Microsoft Excel, statistical software Addinsoft XLSTAT 2010, Imagej, and the R program. Various tests included t-tests, Kruskal-Wallis test, regression analyses, analyses of variance (ANOVA), and analysis of covariance (ANCOVA). These resources were also helpful in preparing graphs, plots, and tables. The level of significance was set at $\alpha = 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

Within this dissertation, two stresses, one abiotic and one biotic and their interaction, were studied. Because the emphasis in this dissertation is on N, the greatest quantity of work reported will be on that.

4.1 Nitrogen Stress

4.1.1 Influence of Nitrogen on Morphology

The overarching question was: What are the responses of hybrid 47-174 to N fertilization that can be observed in leaf and stomatal characteristics? Stomata play an important role as they are the points of entry during leaf rust infection. The following hypotheses are considered:

H_{01}: N fertilization has no effect on leaf area.

H_{02}: N fertilization has no effect on stomatal density.

In response to the high N treatment (HN), there were some striking morphological differences at the whole plant scale. These plants were taller, had more and larger leaves than the low N treatment (LN) plants. The dynamics of the growth responses leading to these differences in gross morphology are detailed in the next section on the Influence of N on Growth. Differences in leaf area and stomatal density are described here.

4.1.1.1 N Fertilization and Leaf Area

The following hypothesis and its alternate were tested to see if HN compared to LN fertilization rates impacted individual plant leaf area:

H_0: N fertilization has no effect on leaf area.

H_a: N fertilization has an effect on leaf area.

As shown below, the null hypothesis (H_0) was rejected and the alternate hypothesis (H_a) was accepted.

As mentioned earlier, by the 4th week following transplanting and initiation of the experiment (see Table 3.1), visual differences between the two treatments were noted. HN plants that were harvested on the 10^{th} week of growth after transplanting had larger average individual leaf areas compared to LN plants (Table 4.1, Figure 4.1). Box plots of all the harvested leaves clearly show large differences in leaf area between HN and LN plants. There is a wide range of leaf areas because of the range of stages of leaf development from a newly formed small leaf of LPI 1 to a fully matured leaf of LPI 28 in HN plants and on a newly formed small leaf of LPI 1 to fully matured leaf of LPI 20 in LN plants. Plants with HN had a 3-fold greater leaf area than plants treated with LN – as a result HN plants had greater leaf surface area available for photosynthesis (+) and leaf rust infection (-).

Table 4.1 Individual leaf area (cm^2) of plants grown under high N (HN) and low N (LN) fertilization rates (n = 88 leaves, 4 plants, *statistically different at $\alpha = 0.05$).

Treatment	Number of observations	Mean ± SD	Maximum	Minimum
HN	53	124.5 ± 60.8*	274.9	9.6
LN	35	59.0 ± 29.0*	123.6	7.0

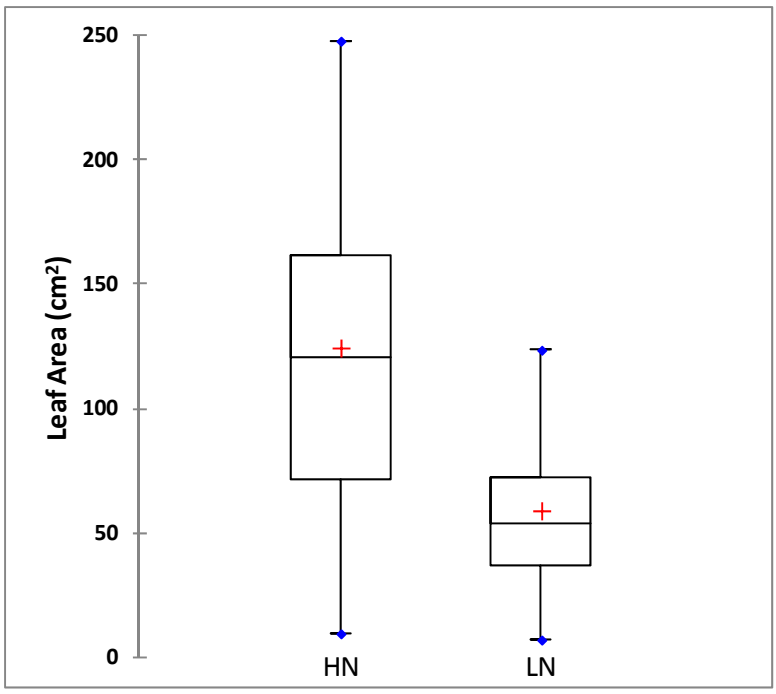


Figure 4.1 Average individual leaf area (cm^2) of plants on high N (HN) and low N (LN) fertilization rates (n = 88 leaves, 4 plants).

In order to understand the interplay between leaf development and leaf N content, it is necessary to examine the spatial changes in leaf area, leaf N, and SLA (Figure 4.2). In both plants, leaf area increased rapidly as SLA. Beyond LPI 3, leaf area was always greater in high N plants although levels of leaf N levels did not always correspond. After reaching peaks, both leaf area and leaf specific area declined. Interestingly leaf N increased slightly in both plants. There is a negative correlation between SPAD values and SLA (Figure 4.3) not easily seen in terms of LPI.

The absence of an observed correlation between N content and individual leaf is the result of leaf development. What the data demonstrate to this point is that differences in plant N content are greater than individual leaf differences in N within one plant. This condition supports SFS grouping or segmentation of leaves or LPIs based on structure and function in a

given plant. There is no correlation between N concentration (average leaf N or total N) and individual leaf area because individual leaves have unique features that may be related to their location (i.e., stage of development) and role or function on a branch. A big leaf or small leaf may have various leaf N levels depending on their development phase and location within a specific SFS.

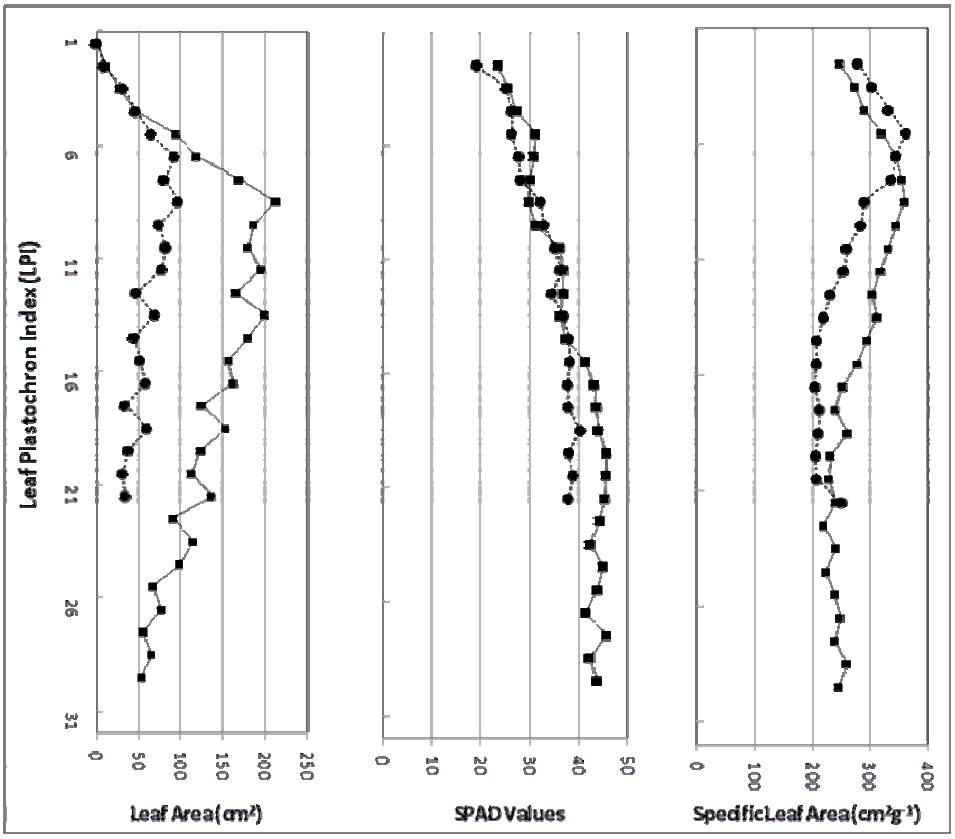


Figure 4.2 Vertical (low LPI is top of the plant) profiles of leaf area, SPAD values (leaf N), and specific leaf area (SLA) for high (solid lines and squares) and low (dash lines and solid circles) N plants. Each point is the mean of two leaves (n = 48 leaves, 2 plants).

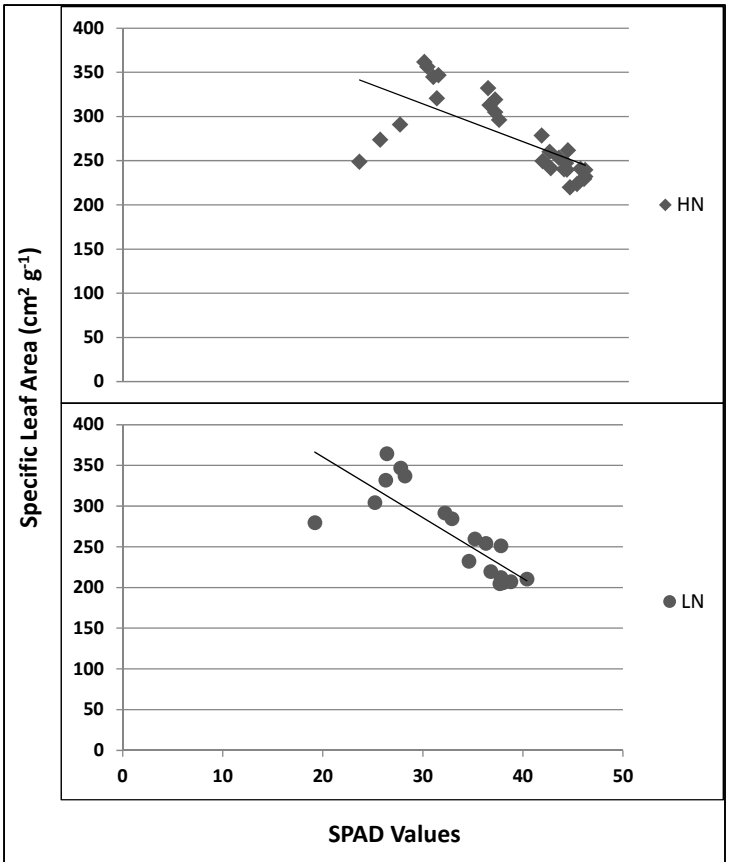


Figure 4.3 Negative correlation between SPAD values (leaf N) and specific leaf area (SLA, cm^2 g^{-1}) of leaves on high N (HN) and low N (LN) fertilization rates (n = 48 leaves, 2 plants).

As can be seen in Figure 4.2, peak leaf area (205 versus 98 cm^2) and the number of leaves (28 versus 22) were different between the HN and LN plants, respectively. As a consequence, using absolute LPI as a way of comparing leaves of similar stages of development would not work. There was better correspondence when all the leaves were grouped into 4 SFS on a given branch or plant. As illustrated in Figure 4.4, segmentation into quarters accomplished two things: it provided a better platform for comparing structural-functional relationships across treatments and, instead of one LPI leaf to another LPI leaf comparison, a group of leaves were then available for averaging, sampling and comparing.

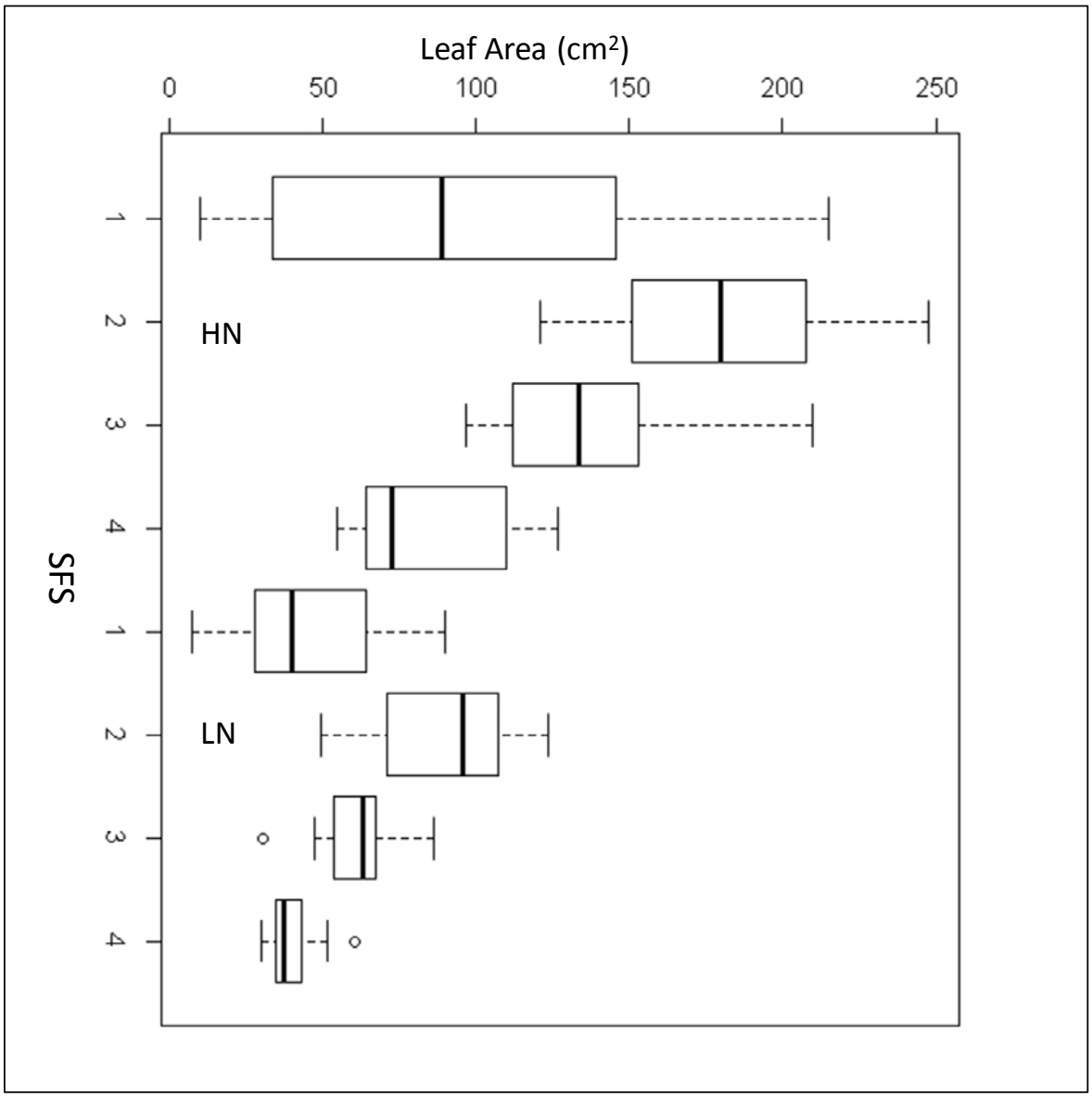


Figure 4.4 Individual leaf area (cm^2) of plants on high N (HN) and low N (LN) fertilization rates subdivided into structural functional segments (SFS) from the top (SFS1) to the base (SFS4) (n = 88 leaves, 4 plants).

4.1.1.2 N Fertilization and Stomatal Characteristics

The following hypotheses are considered when addressing the question: Is there an effect of N fertilization on stomatal density (SD)?

H_0: N fertilization has no effect on stomatal density (SD).

H_a: N fertilization has an effect on stomatal density (SD).

Results show that leaves from HN plants had higher mean SD with 218 stoma mm^{-2}, than LN plant – they had a mean SD of 179 stoma mm^{-2}. The trend, but not the magnitude of this difference (21.8% versus 14.5% increase in SD with HN) was similar to that observed in an earlier study with the same material (Banaag, et al., 2006).

A Kruskal-Wallis test rejects the null hypothesis and accepts the alternate hypothesis that SD of HN leaves is significantly greater than SD of LN leaves (Table 4.2). As noted HN plants had larger leaf areas and therefore more microscopic slide sections (135) compared to low N plants with smaller leaf areas and fewer microscopic slide sections (87). SD is the average of 3 counts from each section.

Table 4.2 Stomatal density (SD) (number of stoma mm^{-2}), mean ± SD (standard deviation), maximum, and minimum data from plants grown under high N (HN) and low N (LN) fertilization rates (n = 222 leaf sections of 4 leaves, *statistically different at $\alpha = 0.05$).

Treatment	Number of Observations	Mean ± SD	Maximum	Minimum
HN	135	218.5 ± 25.9*	280.6	170.1
LN	87	179.3 ± 29.6*	229.6	119.0

Stomatal Index (SI), a measure of the % of stoma among the total number of epidermal cells and stoma, was calculated from only those leaf nail polish impressions that showed discernible neighboring epidermal cells. HN leaves had higher mean SI of 25% compared to LN leaves with 17% SI. The data show that N fertilization has a significant effect on SI as shown by the two-tailed test summary statistics (Table 4.3).

Table 4.3 Stomatal index (SI) (%), mean ± SD (standard deviation), maximum, and minimum data from plants grown under high N (HN) and low N (LN) fertilization rates (n = 42 total leaf sections from 4 leaves, *statistically different at $\alpha = 0.05$).

Treatment	Number of Observations	Mean ± SD	Maximum	Minimum
HN	16	25.3 ± 6.4*	35.0	14.0
LN	26	16.7 ± 5.8*	29.0	10.0

Under stressful conditions there may be a decrease in stomatal density (SD) accompanied by a decrease in stomatal index (SI) as was observed in LN plants. An increase in SD and SI were noted in plants exposed to CO_2 enrichment experiments but the results were not conclusive because the responses may have been dependent on leaf position, leaf age, and genotype in poplars (Ceulemans, et al., 1995).

4.1.2 Influence of Nitrogen on Growth

Another objective of this study focuses on the growth responses of hybrid 47-174 to N fertilization.

The overarching question was: what are the growth responses of hybrid 47-174 to N nutrition? HN and LN plants were studied by analyzing the following hypotheses:

H_{01}: N fertilization has no effect on growth.

H_{02}: N use efficiency of leaves does not vary with LPI or with SFS.

The following growth parameters were measured in order to assess the impact of N fertilization rate on growth of hybrid cottonwood: plant height, leaf area, leaf dry weight, internode length, stem diameter, and stem dry weight. Measurements were initiated on September 18, 2009 after 4 weeks of acclimation (see Table 3.1).

4.1.2.1 Nitrogen Fertilization and Plant Height

High N plants harvested at the 5th week following transplanting (or week 2 from start of observations) were already taller than LN plants (Figure 4.5 A). Differences increased at the 7th week after transplanting (Figure 4.5 B) and persisted at the 9th week after transplanting (Figure 4.5 C). Height was measured because it is a good predictor of total above ground biomass (Ceulemans, et al., 1987). This visual data are for just one plant from each treatment. When all four plants from each treatment are graphed, a consistent difference is seen and the difference increased with time (Figure 4.6). The measured plants, 4 from each fertilization rate, showed differences in growth related to fertilization treatment, with an average weekly increase in height of 16.2 cm for HN as compared to 9.2 cm for LN plants. On average, the HN plants were 38% taller than LN plants. Analysis of the differences in height between the HN and LN plants were significantly different (Table 4.4) and as a result, the null hypothesis was rejected. N fertilization had a rapid, consistent, and statistically significant impact on height growth.

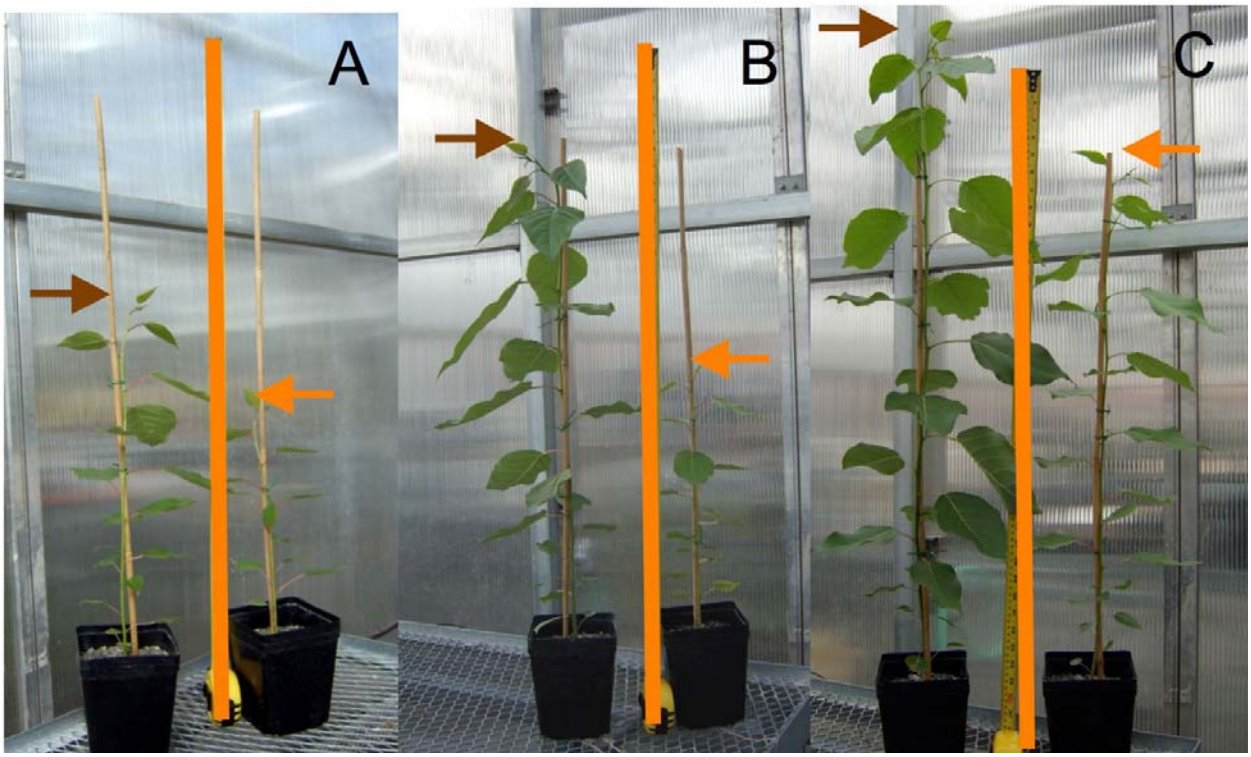


Figure 4.5 Plants harvested on the 5th week or week 2 (A), 7th week or week 4 (B), and 9th week or week 6 (C) after transplantation. HN plants are on the left-hand side and LN plants are on the right. Orange marker is 1 m. Arrows show the tip of the plants.

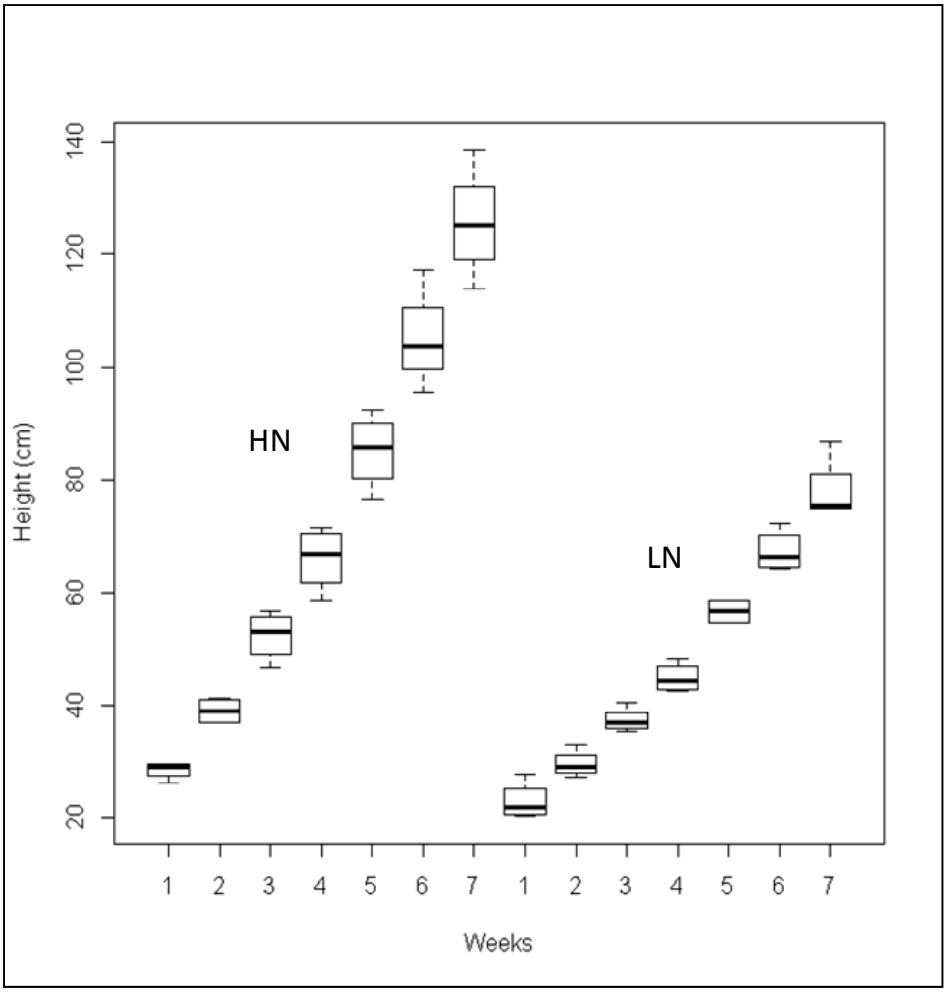


Figure 4.6 Weekly plant height (cm) for 7 weeks after acclimation in the semi-hydroponic system setup under high N (HN) and low N (LN) fertilization rates (n = 6 plants).

Table 4.4 Height (cm), mean ± SD, maximum, and minimum data of plants grown under high N (HN) and low N (LN) fertilization rates, measured on the 9th week following transplanting to the treatment system (n = 8 plants, *statistically different at $\alpha = 0.05$).

Treatment	n	Mean ± SD	Maximum	Minimum
HN	4	125.7 ± 10.2*	138.6	113.8
LN	4	78.0 ± 5.8*	86.7	74.8

4.1.2.2 Nitrogen Fertilization, Leaf Growth and Area

Leaf growth measurements required the assignment of an LPI per given time of measurement because this number changed as new leaves were formed. Therefore, it was necessary to identify a basal leaf as a reference point in determining how any new leaves had been formed after a certain period of time. Identification of the LPI number and the location of the leaf in a given SFS would document accurate changes due to time and treatment (Hull, 2002: Wait, et al., 2002).

As mentioned earlier, leaves of a given sample plant had an assigned or tagged number with leaf #1 starting from the base to track and follow the growth of individual leaves efficiently. This tagged basal leaf of course had an LPI number that is changing depending on the time of data acquisition. Results indicated that the number of leaves increased every week accompanied by a leaf area expansion of the newly formed and younger leaves during the current week of observation. There were approximately 3 to 6 new young leaves that developed every week for the LN and HN plants, respectively. As a consequence of the emergence of these new leaves, older leaves acquired increased LPI numbers by an equivalent number. In HN plants matured leaves with high LPI numbers from LPI 6 and above showed no further increase in leaf area after this initial rapid growth phase. So that in HN plants a LPI 1 leaf in one week is fully expanded and becomes a LPI 7 leaf by week 2. LN plants are somewhat delayed in maturation, in that after 1 week LPI 1 is now LPI 4, but is not completely expanded until it becomes LPI 5. This rate of leaf expansion was faster than a reported leaf expansion that required 15 days (Marron, et al., 2008).

Individual leaf areas increased as the plant grew in a span of 7 weeks from the start of observations. More new leaves were formed as the plant matured and the leaf areas are

prominent at the lower LPIs or upper segments of the plants (Figure 4.7). There were fewer leaves and reduced leaf areas during the first few weeks of growth.

The differences in leaf growth brought about by N nutritional status may be attributed to changes in the duration of cell division, changing cell number, the process of cell elongation or expansion, and changing cell size (Van Volkenburgh and Taylor, 1996; Fleming, 2002). Cottonwood hybrid 47-174 is reported to have inherited high cell number from the parent *P. deltoides* and large cell size from the parent *P. trichocarpa* (Ridge, et al., 1986). As N supply decreases, cottonwood plants show decreased leaf dry weight accompanied by fewer and smaller leaves (Heilman and Xie, 1994; Ibrahim, et al, 1997). In addition, leaf expansion rates are decreased at low N supply (Lambers, et al., 1998).

The vertical distribution of leaf areas with larger leaves on the upper SFS of the plant are also shown in Figure 4.7. This is a zone of greater light intensity. In addition, the capacity to produce more and larger leaves at full maturity increases over time. It was assumed that with time and the increase in total plant leaf area (Figure 4.8) there would be more and more carbon available to emerging upper leaves for their expansion (Scarascia et al., 1997). The general pattern of growth rate was faster in HN than LN plants and HN plants had larger total leaf areas and with more LPIs than LN plants (Figures 4.7 and 4.8).

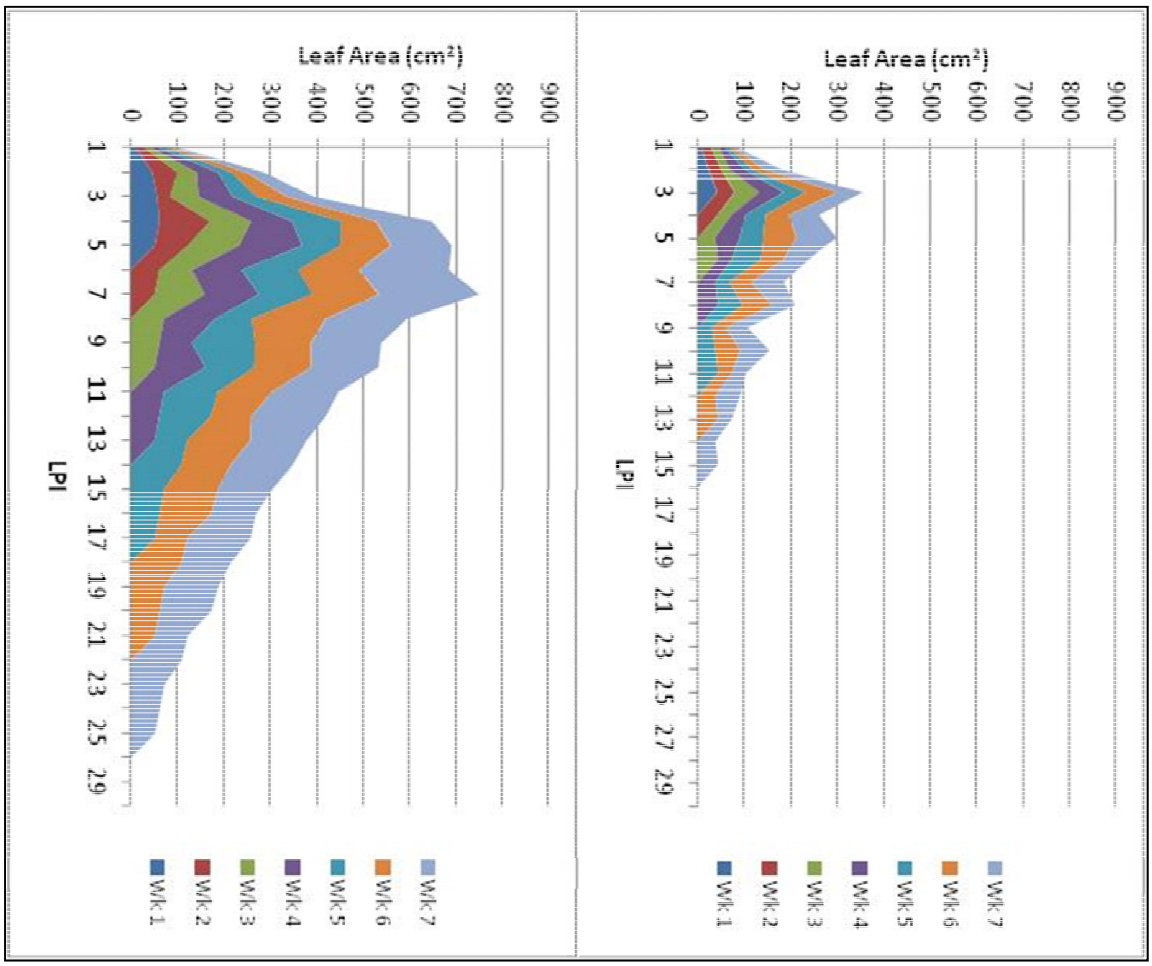


Figure 4.7 Leaf area (cm^2) of the individual leaves identified by leaf plastrochron index (LPI) of plants grown on high N (HN) and low N (LN) fertilization rates, measured weekly for 7 weeks (n = 8 plants).

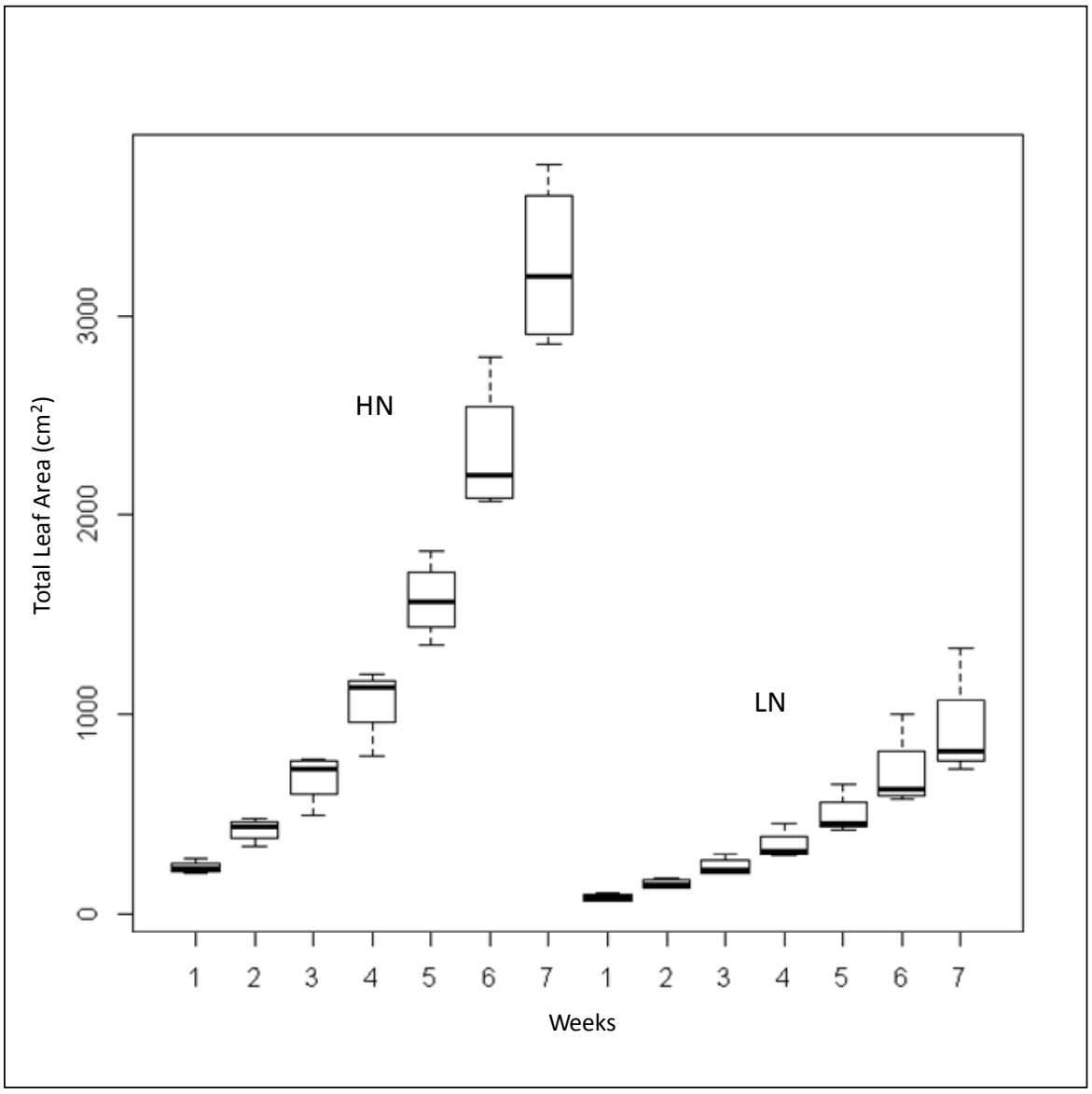


Figure 4.8 Total leaf area per plant (cm^2) on high N (HN) and low N (LN) fertilization rates during 7 weeks of growth (n = 8 plants).

Some of the variation in total leaf area shown in Table 4.5 was due to differences in average leaf N concentration (as described in the methods, the N concentration of individual leaves was determined using the SPAD-502 meter; 3 readings were taken on each leaf and then for all leaves [LPI = 0 to LPI = n], these values were average to give a plant average[1]). There was a significant relationship ($R^2 = 0.93$) between average SPAD values and total leaf area for each of the 8 plants that made up the 4 HN and 4 LN treatment plants (Figure 4.2.5). It is important to note that the four LN plants are clustered to the left and the four HN plants are clustered to the right – the greatest leaf area in the low N plant was still 1334.1 cm^2, 1511.4 cm^2 less than the smallest plant receiving HN. In contrast, average SPAD values were not greatly different although consistently less in LN treated plants.

Table 4.5 Total leaf area (cm^2) of plants grown under high N (HN) and low N (LN) fertilization rates (n = 8 plants, *statistically different at $\alpha = 0.05$).

Treatment	n	Mean ± SD	Maximum	Minimum
HN	4	3227.0 ± 454.5*	3764.4	2845.5
LN	4	933.2 ± 270.8*	1334.1	738.3

[1] No significant correlation of N concenration to individual leaf area by LPIs ($R^2 = 0.10$) or to individual leaf dry weight by LPIs ($R^2 = 0.31$) was noted.

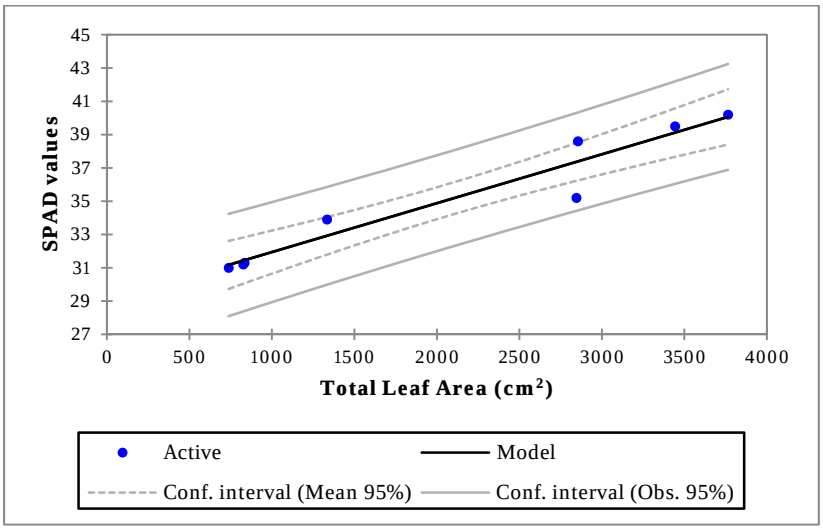


Figure 4.9 Total leaf area (cm^2) and SPAD values (leaf N) of plants on high N (right cluster) and low N (left cluster) fertilization rates (R^2 = .930, n = 8 plants).

These impressive differences in total leaf area were also noted when leaf areas of individual leaves were compared. It is important to note that HN treated plants had more leaves (n = 28) than LN plants (n= 20); therefore, differences in leaf number could account for much of the difference in total leaf area. N fertilization has a significant effect on individual as well as total leaf area (Table 4.2.2). It is important to note that although the smallest leaves were almost identical on HN and LN plants, this similarity is the result of leaf development where the newly emerging leaf will always be small – its rate of development and final size are the two variables affected by difference in N treatment.

Plants harvested every 2 weeks had greater average leaf area (cm^2) in HN plants as compared to LN plants (Figure 4.10). HN plants demonstrated a significant increase in leaf area compared to LN plants in all SFSs from week 2 to week 6. However, in both fertilization rates, SFS 2 and SFS 3 had the greatest values of average leaf areas. SFS 2 and SFS 3, which are the

middle segments of the plants, showed high values in both HN and LN fertilization rates at 6th week harvest. These segments had ideal conditions for growth because of larger leaf areas for photosynthesis with minimal shading self and interplant effects from SFS 1 and presumably re-translocation of nutrients from SFS 4. As expected there was less variation in leaf areas in SFS 4 leaves as they become over matured especially in LN plants. Leaves assigned to SFS 4 were produced early in the development of the study material and benefitted least from treatment and development effects.

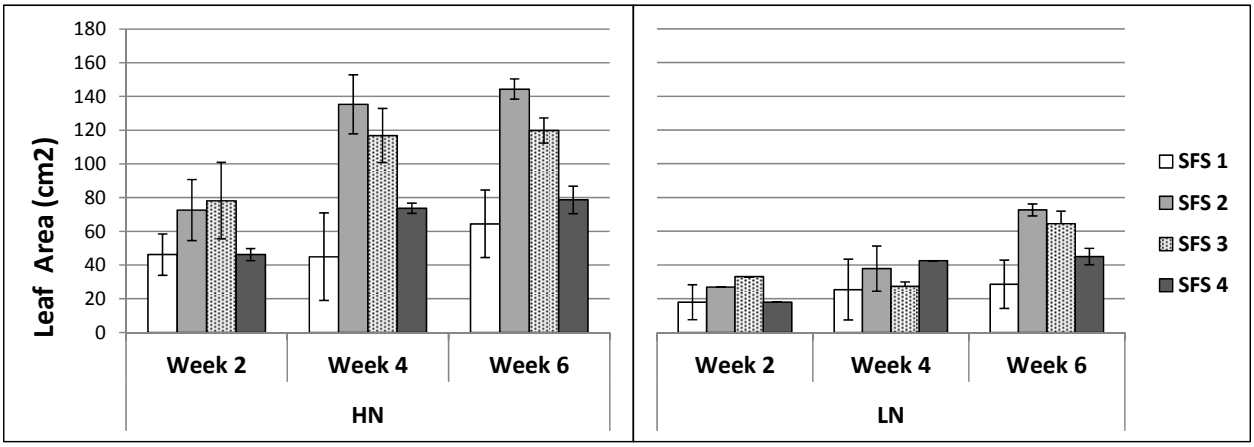


Figure 4.10 Average individual leaf area of plants harvested every 2 weeks grown on high N (HN) and low N (LN) fertilization rates, from the top segments (SFS 1) to the base segments (SFS4). Bars represent standard errors (n = 69 leaves, 6 plants).

There was a highly significant correlation between leaf dry weight and leaf area for all harvested individual leaves ($R^2 = 0.836$); in other words, large leaves have greater dry weights. These results were observed in plants harvested during the 10th week of observation (see Table 3.1); it was assumed that this relationship held for all of the leaves measured at other sampling times.

4.1.2.3 Nitrogen Fertilization and Leaf Dry Weight

The progression of dry weight accumulation from week 2 to 4 to 6 is shown in Figure 4.11. There was 3-fold increase in dry weight of all these segments over this week span. Total leaf dry weight of harvested plants show dry weights of 10.68 g in HN plants compared to 3.91 g in LN plants. SFS 2 and 3, which are the middle segments of the plants, have high total leaf dry weight values in plants from both HN and LN fertilization treatments. SFS 2 is made up of leaves that have mostly completed elongation and maturing while SFS 3 is made up of fully mature leaves.

Figure 4.11 also includes the R^2 correlation values between segments, N fertilization, and leaf dry weight as they progress from week 2, 4, to 6. The level of significance and the R^2 value increased from week 2 ($R^2 = 0.649$, $p = 0.035$) to 4 ($R^2 = 0.662$, $p = 0.001$), and finally to week 6 ($R^2 = 0.755$, $p = 0.0001$) which showed that the effects of N nutrition became more pronounced towards the later part of the experiments.

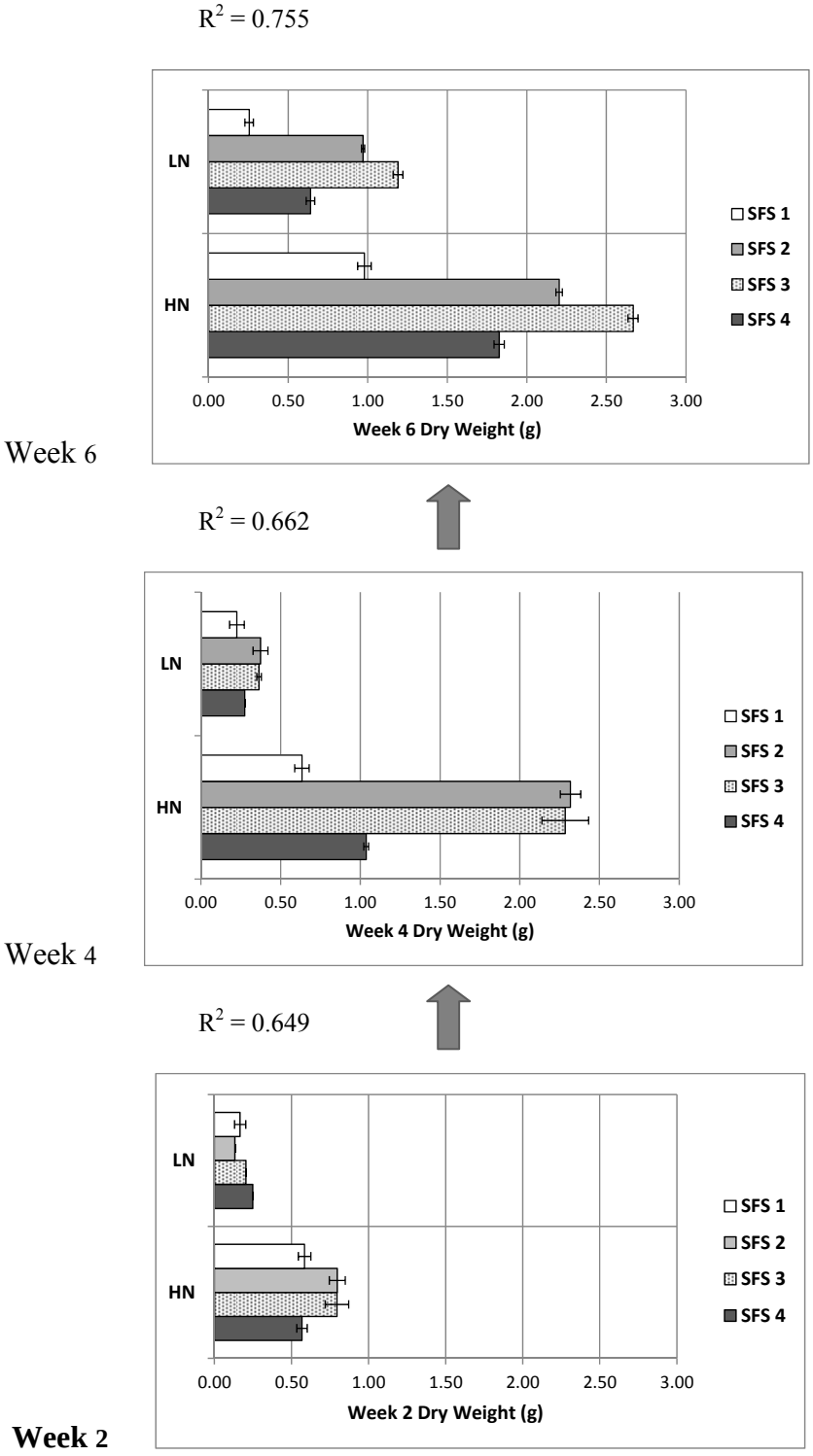


Figure 4.11 Total leaf dry weight (g) per structural functional segment (SFS) of plants harvested every 2 weeks on high N (HN) and low N (LN) fertilization rates, from the top (SFS 1) to the base (SFS 4). R^2 values are based on analysis of correlation by segments, N fertilization, and leaf dry weight. Bars represent standard errors (n = 69 leaves, 6 plants).

4.1.2.4 Nitrogen Fertilization and SPAD Values

During the 10th week after transplanting, there was a significant difference in leaf N content as a result of treatment differences in the quantity of N supplied (Table 4.6) with LN plants consistently having lower N (SPAD values) than HN plants. Interestingly when examining the within plant variation, differences in SPAD values are more defined when illustrated by SFS levels (Figure 4.12). SFS 1 was the segment of low SPAD values (< 30) which also corresponds to the segment with young to developing leaves compared to higher SPAD values (> 30) in segments SFS 2 to 4.

Table 4.6 SPAD values (N concentration) of plants grown under high N (HN) and low N (LN) fertilization rates measured on the 15th week following transplanting to the treatment system (n = 8 plants, *statistically different at $\alpha = 0.05$).

Treatment	Number of Observations	Mean ± SD	Maximum	Minimum
HN	28	38.9 ± 6.3*	46.2	23.3
LN	20	33.1 ± 5.2*	40.4	21.2

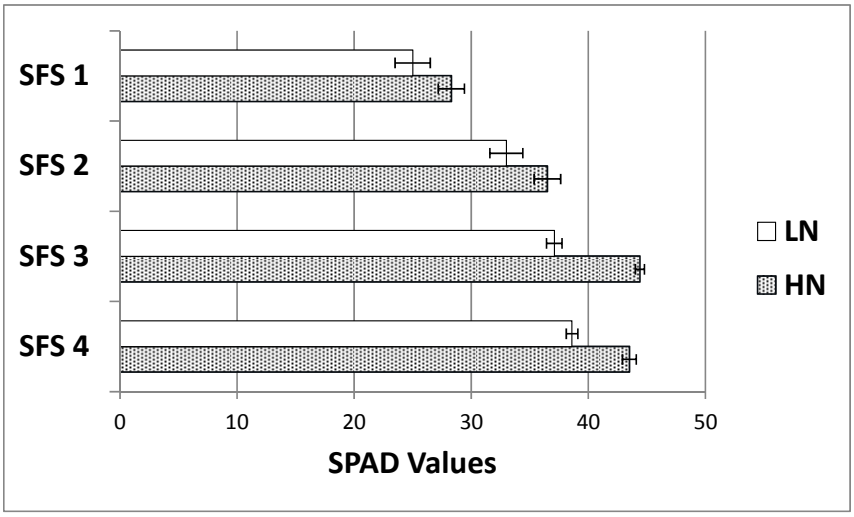


Figure 4.12 SPAD values (leaf N) of leaves on high N (HN) and low N (LN) fertilization rates subdivided into structural functional segments (SFS) from the top (SFS1) to the base (SFS4). Bars represent standard errors (n= 48 leaves, 2 plants).

These observations are in contrast to those of Mooney and Winner (1991) who found that N concentrations were always highest in the youngest leaves. In addition, they also found that N levels decreased in older foliage, particularly foliage undergoing senescence. In my study, N content did not decrease with leaf age perhaps as a result of a lack of pronounced shading of the lower leaves (Hikosaka and Terashima,1996). As mentioned above, LN plants showed lower N or SPAD values than HN plants in all 4 SFS. As mentioned earlier, leaves from SFS 1 had SPAD values less than 30 while leaves from SFS 2-4 had SPAD values greater than 30. These plants were not canopy light restricted because of the presence of a single vertical stem without branches in the greenhouse setting. With further development, a combination of self-shading and leaf aging would result in pronounced decreases in leaf N in SFS 4 (Field, 1983).

4.1.2.5 Nitrogen Fertilization and Internode Length

The maximum internode length (i.e., distance between two successive LPIs) measured was 6.5 cm on HN plants which is longer than the maximum internode length reported by Pieters et al. (1999) in *Populus euramericana* (~3.5 cm). Consistently, the longest internode lengths were noted in the middle segments (SFS 2 and SFS 3) of HN and LN plants (Figure 4.13).

LN plants had fewer and smaller leaves and fewer internodes than HN plants – since the number or sum of the internodes determines the total height, LN plants were shorter than HN plants (Figure 4.13). N fertilization not only affects leaf area and number, but also internode length and number, an example of an allometric relationship (Dickson and Isebrands, 1991). In some hybrids or parental plants, short plants can occur when there are short internodes even with the same number of leaves (Busov, et al., 2003).

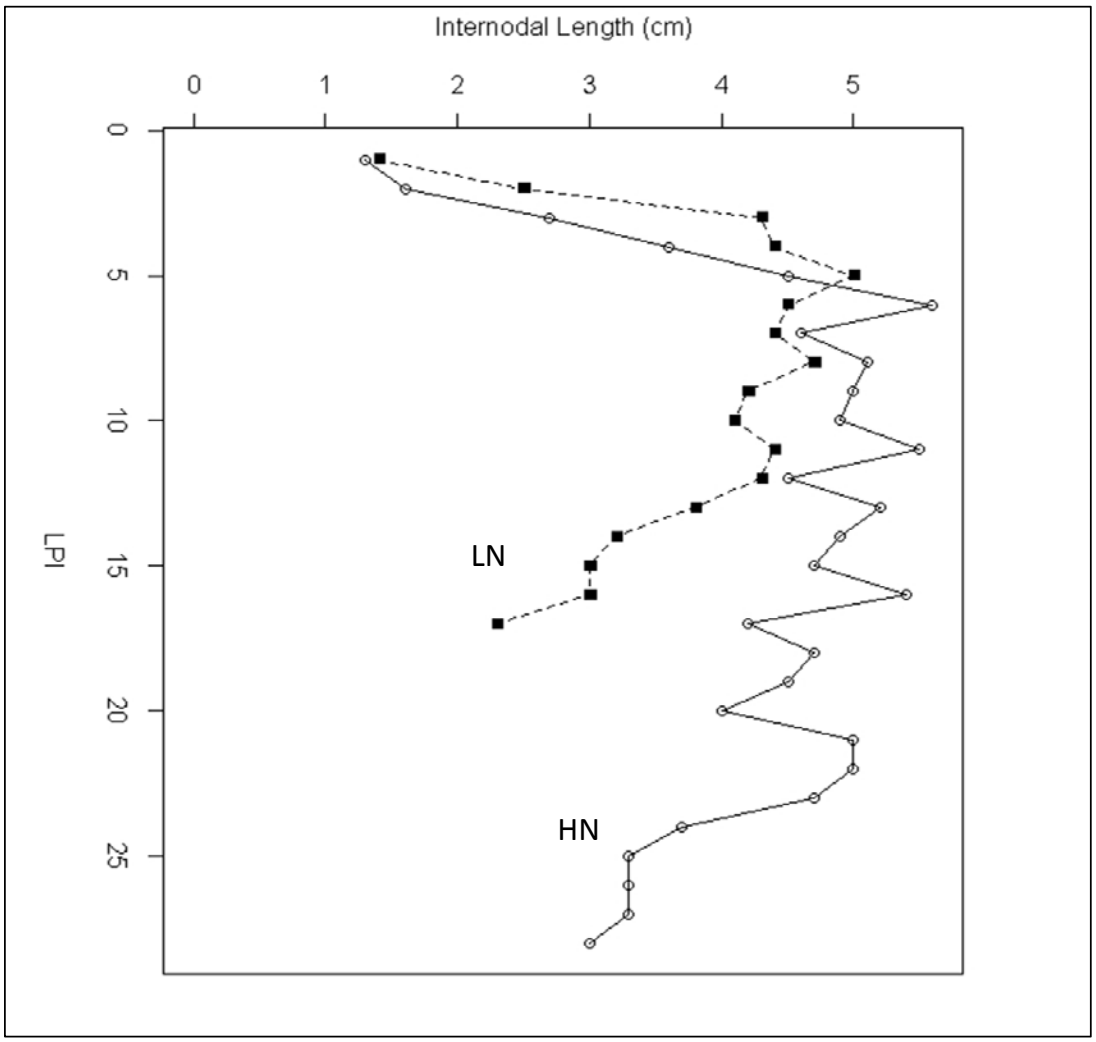


Figure 4.13 Internodal length (cm) of plants per given leaf plastochron index (LPI) under high N (HN) and low N (LN) fertilization rates (n = 6 plants).

4.1.2.6 Nitrogen Fertilization and Stem Diameter

Stem diameters were measured at around 10 cm height from the base of the plant, a position that corresponded to near the basal most leaf. Mean stem diameter increased from 6.3 mm in LN plants to 9.4 mm in HN plants (Figure 4.14, Table 4.7). A two-tailed test, concluded that N fertilization has an effect on stem diameter ($p = 0.05$). Interestingly, the largest LN plant has a smaller diameter than the smallest HN plant (6.6 vs 7.15 mm). In addition, plants with

large diameters were taller and greater leaf areas than plants with smaller diameters. Clones that retained green leaves late in the fall were observed to have measurable photosynthetic production that contributed significantly to late season stem diameter increment (Nelson, et al., 1982).

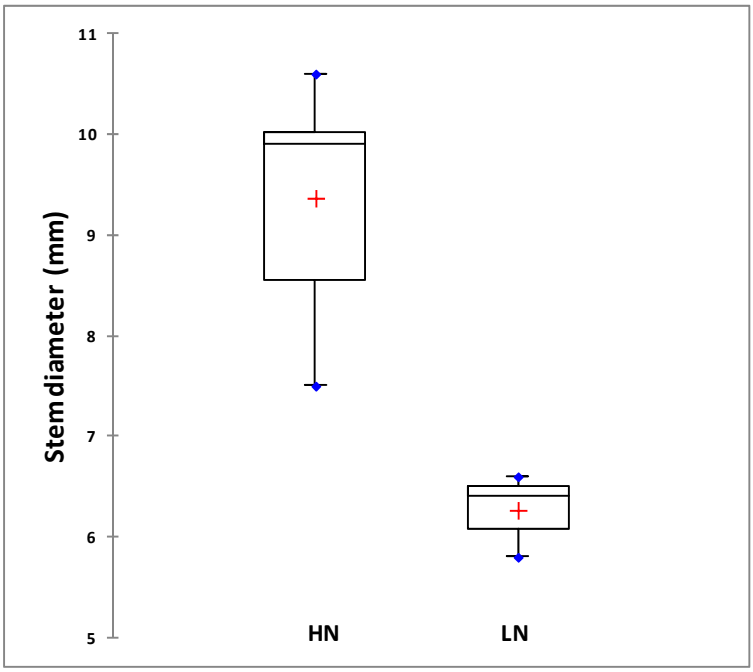


Figure 4.14 Stem diameter (mm) of plants on high N (HN) and low N (LN) fertilization rates (n = 24 plants).

Table 4.7 Stem diameter (mm), mean ± SD, maximum, and minimum data at 10 cm stem height of plants grown under high N (HN) and low N (LN) fertilization rates measured on the 12th week following transplanting to the treatment system (n = 24 plants, *statistically different at α = 0.05).

Treatment	n	Mean ± SD	Maximum	Minimum
HN	12	9.4 ± 1.0*	10.6	7.5
LN	12	6.3 ± 0.3*	6.6	5.8

4.1.2.7 Nitrogen Fertilization and Stem Dry Weight

As already noted plants receiving a greater amount of N were taller, had greater diameters, had more leaves, had larger individual leaves, and had greater total leaf area than

those receiving less N. Stem dry weights were greater in HN vs. LN plants (mean 7.1 vs. 2.6 g, respectively, Table 4.8). A two-tailed t-test, concludes that N fertilization has an effect on stem dry weight ($p = 0.05$).

The increased stem dry weight observed for plants on HN fertilization could be brought about by increased photosynthesis per unit leaf area and per leaf (see Section 4.3) in HN plants. A number of authors have observed a significant relationship between leaf area and plant biomass (Ridge, et al, 1986; Ceulemans, et al., 1992; Barigah, et al., 1994). Stem biomass values also increased significantly in *Populus balsamifera ssp. trichocarpa x deltoides* after 28 days on short-term intermediate and luxuriant N levels (Cooke and Weih, 2005). Stem growth was lowest priority in the above ground biomass distribution sequence, starting from the active growth centers in leaves followed by the increased growth relayed to branches as sinks and eventually to the stems (Scarascia-Mugnozza, et al., 1997). The increase in stem dry weight may be accompanied by thickening of the xylem fiber cell walls and wood that contains more holocellulose (Pitre, et al., 2007).

Table 4.8 Total stem dry weight (g), mean ± SD, maximum, and minimum data of plants grown under high N (HN) and low N (LN) fertilization rates measured on the 12th week following transplanting to the treatment system (n = 6 plants, *statistically different at $\alpha = 0.05$).

Treatment	n	Mean ± SD	Maximum	Minimum
HN	3	7.1 ± 2.4*	9.8	5.0
LN	3	2.6 ± 0.6*	3.2	2.1

4.1.2.8 Nitrogen Use Efficiency

N use efficiency (NUE) can be expressed as the amount of leaf area or leaf dry weight produced per unit of N available in the same photosynthesizing leaf or group of leaves per given harvest time. It can be expressed as:

$$NUE = \frac{LPILAi}{LPINi} \text{ or } \frac{LPIBi}{LPINi} \text{ or } \frac{\sum SFSBi}{\sum SFSNi} \qquad (1)$$

This formula is adapted from the series of equations presented by Finzi, et al. (2007) and where LPILA = leaf area and LPIB = leaf biomass or dry weight of a given LPI number i and LPIN = leaf N concentration of a given LPI number i. LPI is the leaf plastochron index. NUE can also be expressed as combined (Σ) biomass or dry weight of leaves in a given segment (SFSB) per combined (Σ) N concentration (SFSN) of a given segment i. In each segment, leaf area and leaf biomass can also be expressed as the average leaf area or average dry weight of the segment. N uptake (Finzi, et al., 2007) is replaced by N absorbed in response to nutrient treatment (Ingestad, 1979; Birk and Vitousek, 1986) expressed as the relative amount of N (SPAD = 0 to100) in a given LPI or SFS at the time of harvest. NUE is therefore expressed as a relative value based on leaf area or leaf dry weight that is in g $SPAD^{-1}$.

NUE is defined as the amount of organic biomass produced divided by the amount of N accumulated or the N content of the biomass (Birk and Vitousek 1986; Berendse and Aerts, 1987). In contrast, others (e.g., Chapin, 1980; Shaver and Melillo, 1984) define NUE as the inverse; NUE is the N concentration in the biomass (g $mg^{-1}g^{-1}$). For my work, I used the former definition and I focused on leaves so that I could express NUE relative to either leaf dry weight or area. It is important to remember that N concentration as measured by the SPAD method is relative, so that NUE is a relative value.

NUE gives an index of the amount of N needed to bring about a particular morphological feature. The following hypotheses are considered:

H_0: NUE does not vary with LPI or SFS in leaves of HN or LN plants.

H_a: NUE varies with LPI or SFS in leaves of HN or LN plants.

The general result rejects H_o and accepts H_a. NUE of leaves varies with LPIs and with SFS. NUE in this study was expressed as (1) the size of leaf area developed per SPAD value and (2) the amount of dry matter produced per SPAD value.

In terms of leaf area, SFS 1 and SFS 2 composed of developing and recently matured leaves exhibited high NUE for both HN and LN plants (Figure 4.15). These segments are composed of LPIs 1 to 16 in HN plants and LPIs 1 to 11 in LN plants.

When NUE is based on leaf dry weight, SFS 2 composed of newly maturing to matured leaves had high NUE values for both HN plants and LN plants (Figure 4.16). SFS 3 also has higher NUE values as compared to SFS 1 for HN plants. However, NUE based on leaf dry weight does not seem to be as long term compared to evergreens that have leaf longevity favoring long term N storage (Warren and Adams, 2004). Highest levels of NUE in SFS 2 and SFS 3 likely resulted from the combined effects of development (newly matured) and physiological function (i.e., with optimal physical positioning for photosynthesis). As mentioned earlier, leaves in SFS 2 and SFS 3 are translocating to newly developing leaves in SFS 1.

Also, the high NUE in the top to middle segments could be based upon increased N uptake efficiency, N assimilation efficiency, N utilization efficiency, or N remobilization efficiency associated with leaves in these segments (Chardon, et al., 2010). The same pattern of higher NUE in younger sections of the plant is apparent for both HN and LN plants. However, HN plants had higher NUE than LN plants.

The analysis of NUE based on leaf area (Figure 4.15) resulted in the division of plant into the top half with high NUE and base half with lower NUE. In contrast, NUE based on dry

weight (Figure 4.16) resulted in a different pattern with the top and base segments having low NUE and the two middle segments having highest NUEs..

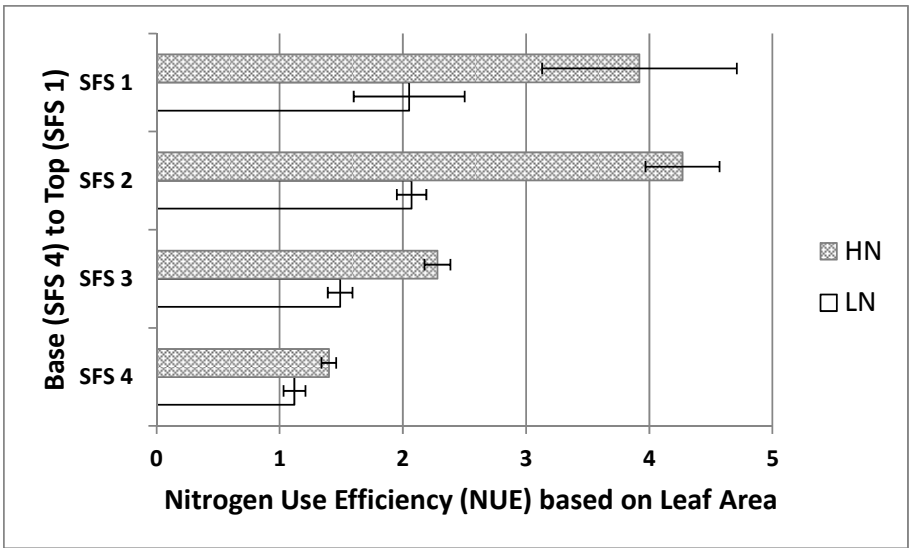


Figure 4.15 Nitrogen use efficiency (NUE) based in leaf area of plants grown on high N (HN) and low N (LN) fertilization rates, from the base segment (SFS 4) to the top segment (SFS 1). Bars represent standard errors (n = 8 plants).

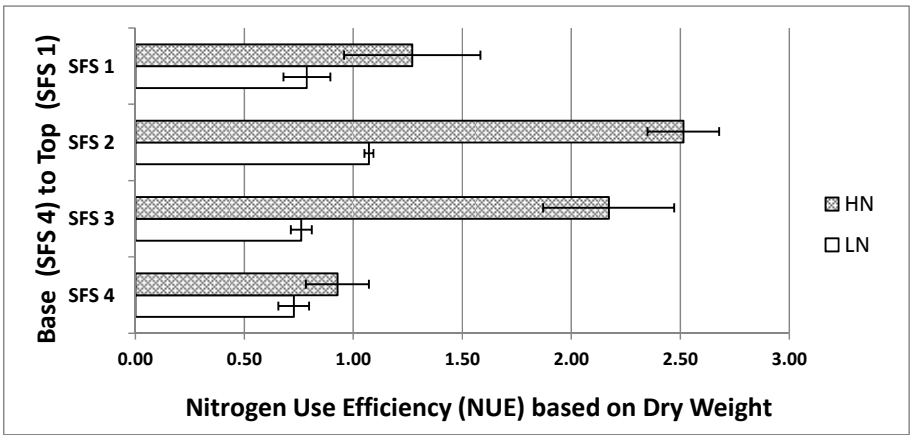


Figure 4.16 N use efficiency (NUE) based on dry weight of plants grown on high N (HN) and low N (LN) fertilization rates, from the base segment (SFS 4) to the top segment (SFS 1). Bars represent standard errors (n = 4 plants).

4.1.3 Influence of Nitrogen on Photosynthesis

The third objective of this study focuses on the photosynthetic responses of hybrid 47-174 to N fertilization. The overarching question was: what are the photosynthetic responses of hybrid 47-174 to N nutrition? The following hypotheses are considered:

H_{01}: N fertilization has no effect on photosynthesis.

H_{02}: Photosynthetic N use efficiency (PNUE) of leaves does not vary with LPI or SFS.

4.1.3.1 Nitrogen Fertilization and CO_2 Uptake

The HN and LN plants were compared based on photosynthetic values mesured at the very end of growth and morphological observations (i.e., weeks 11 and 12, see Table 3.1). As mentioned above, the following hypotheses are under consideration:

H_0: N fertilization has no effect on photosynthesis.

H_a: N fertilization has an effect on photosynthesis.

N fertilization had a positive effect on photosynthesis (Figure 4.17). Analysis of N (i.e., SPAD values) versus CO_2 uptake resulted in a R^2 value of 0.55. Plants with high N content tended to have higher CO_2 uptake values compared to plants with low N content. These results suggest that the null hypothesis (H_0) is rejected and the alternate hypothesis (H_a) is accepted.

These results are not unexpected because about half or most of the total leaf N is used for photosynthesic enzymes or associated with photosynthesic processes (Hull, 2002; Onoda, et al., 2004). It is expected that high N fertilization rates would increase available N that would favor production of more N containing photosynthetically related compounds that would increase CO_2

uptake. The most abundant and important of these photosynthetic compounds or enzymes is ribulose-1, 5-biphosphate carboxylase/oxygenase or Rubisco.

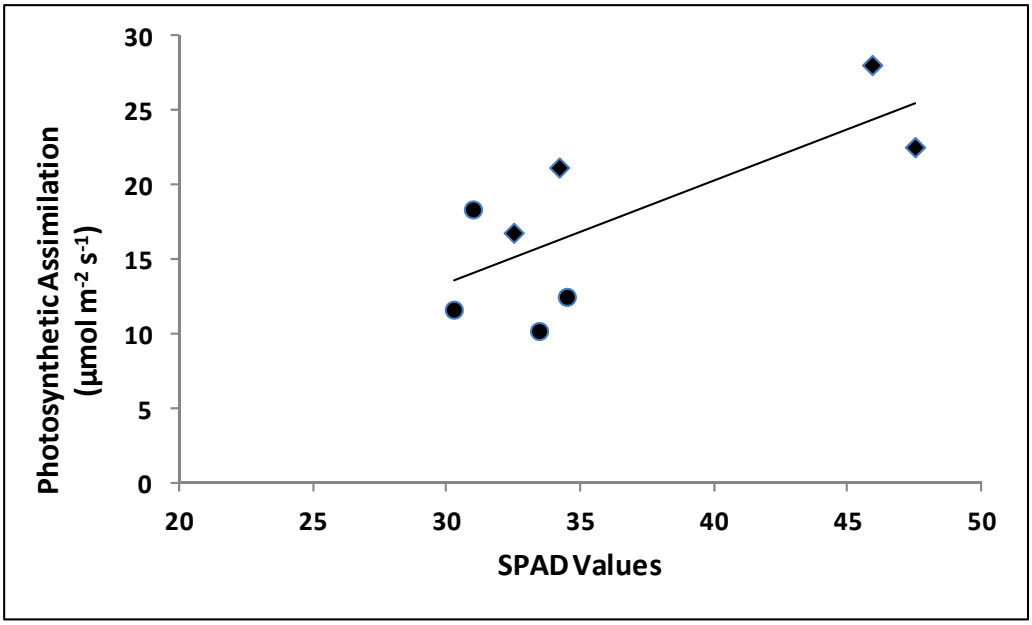


Figure 4.17 Photosynthetic assimilation (µmol $m^{-2}s^{-1}$) (CO_2 uptake) and SPAD values (leaf N) of plants on high N (◆) and low N (●) fertilization rates (R^2 = .55, n = 8 leaves, 2 plants).

In an effort to understand the interaction between N concentration in a leaf and photosynthesis as leaves mature or as leaves are in different developmental segments, Figure 4.18 illustrates the relationship between photosynthesis or assimilation and N level in each of the four segments for high and low N leaves. If one ignores the lines and focuses on the points, one could imagine a significant, positive relationship between N and A, as noted for data in Figure 4.17; however, when one adds the lines, the relationship is not so clear. The solid line is for the high N plant and the dashed line for the low N. Arrows indicate the developmental sequence (from segment 1 to 4). Segments from the two treatments differ considerably. Segment 2 (SFS 2) had the highest rate of photosynthesis for HN plants. In contrast, SFS 1 had the highest rate of photosynthesis for LN plants. For LN plants, N increased from SFS 1 to 2 to 3, but

photosynthesis declined. For the HN plants both N and photosynthesis increased from SFS 1 to 2. There must be other factors that lead to reductions in CO_2 uptake given adequate N levels in LN plants. Maybe the N levels observed in the lower leaves are in stored N not made up of photosynthetically and actively related CO_2 uptake compounds. Also, the reduced CO_2 uptake in LN segments with high N levels may occur because LN plants, as noted by Tsai et al. (1986) in maize may cannibalize photosynthetic ribulose biphosphate carboxylase (rubisco), phosphoenolpyruvic acid (PEP), and structural proteins (hydroxyproline-rich glycoproteins as sources of N, which greatly decreases photosynthesis (Huber and Thompson, 2007). Also, plants appear to synthesize more Rubisco than is needed for C assimilation (Stitt and Schulze, 1994).

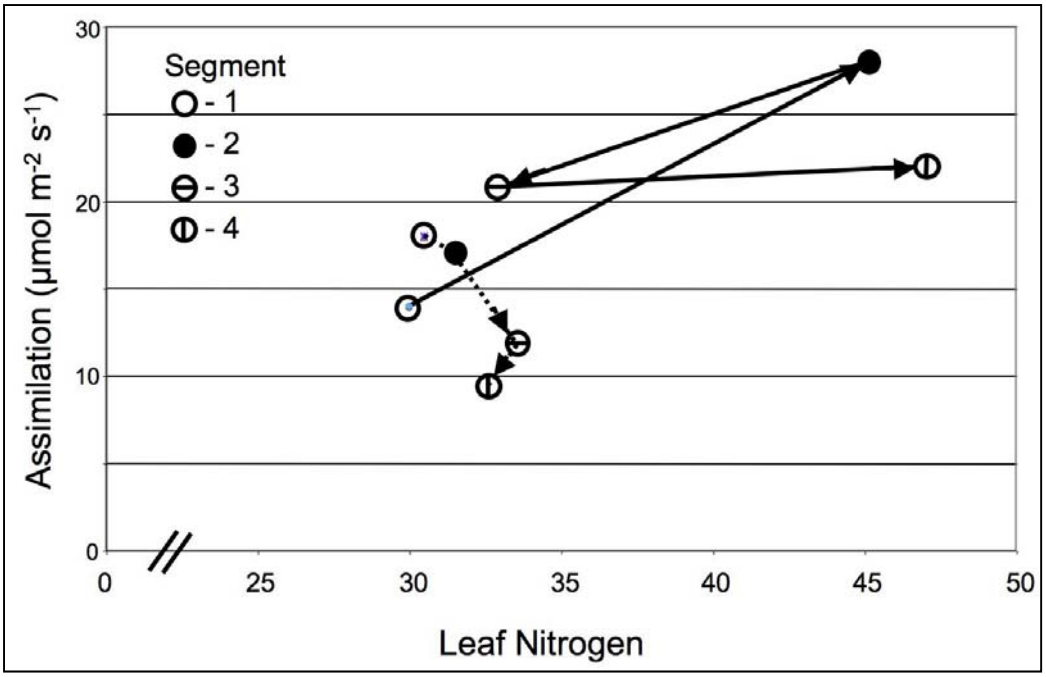


Figure 4.18 SPAD values (leaf N) and CO_2 uptake (Assimilation) in high N (HN) and low N (LN) plants from the top (Segment 1) to the base (Segment 4). Dashed line is for LN plants, solid for HN (n = 8 leaves, 2 plants).

Fortunately, there are two complete sets of photosynthetic measurements from hybrid 47-174 available for comparison; preliminary data from 2003 was measured using the PP Systems TPS-1 Portable Photosynthesis System and data from 2009 using the Li-Cor 6400 system (Figure 4.19). In 2003, data were from only segments 1 and 2 whereas in 2009, data were from all four segments. As a result, photosynthetic rates in the HN plants were greater in 2003 than 2009. In addition, rates for the LN plants in 2003 were lower than for 2009. However, differences between years for a given N level were not significant, whereas there were significant differences between high and low N plants for both years. These data strongly suggest that the null hypothesis must be rejected as N affected photosynthetic rates.

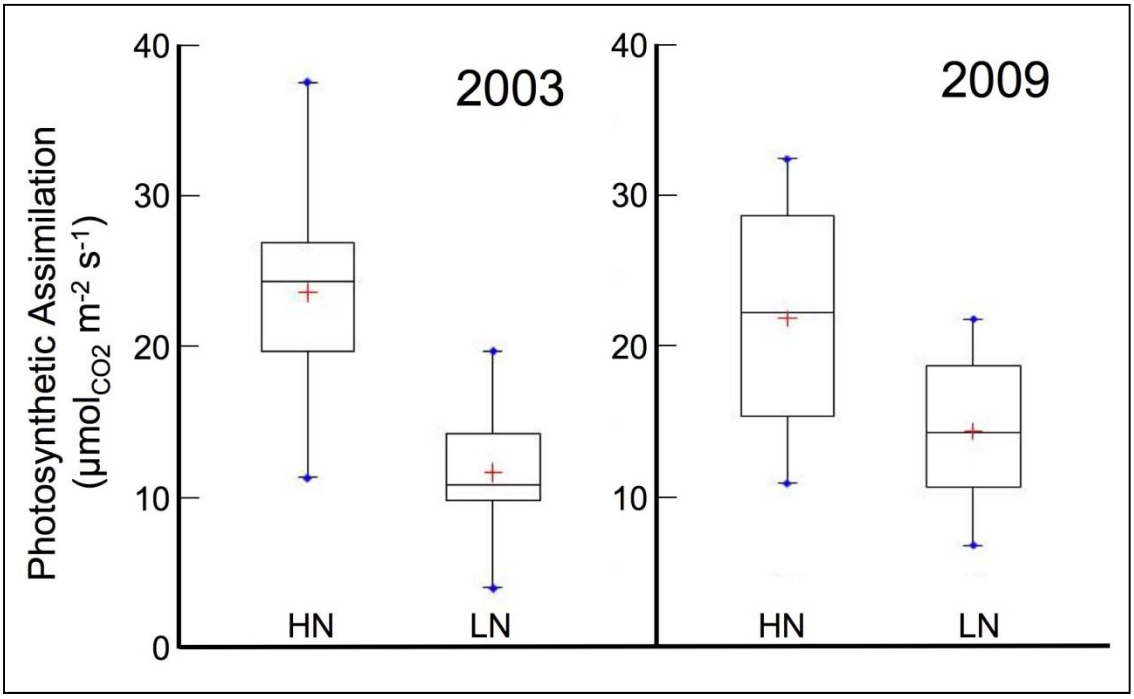


Figure 4.19 Photosynthetic assimilation (CO_2 uptake) of leaves on high N (HN) and low N (LN) plants measured in 2003 and 2009 (2003, n = 17 leaves, 4 plants; 2009 = 8 leaves, 2 plants).

4.1.3.2 Light Response Curves

A comparison of CO_2 uptake from LPI 4 leaves over a wide range of absorbed PAR levels of a LN vs. a HN plant shows a slight difference at PAR values greater than 800 μmol m^{-2} s^{-1} (Figure 4.20). The slightly higher assimilation in the LN plant could be attributed to a more rapid leaf development and a greater stomatal conductance in LN (data not shown).

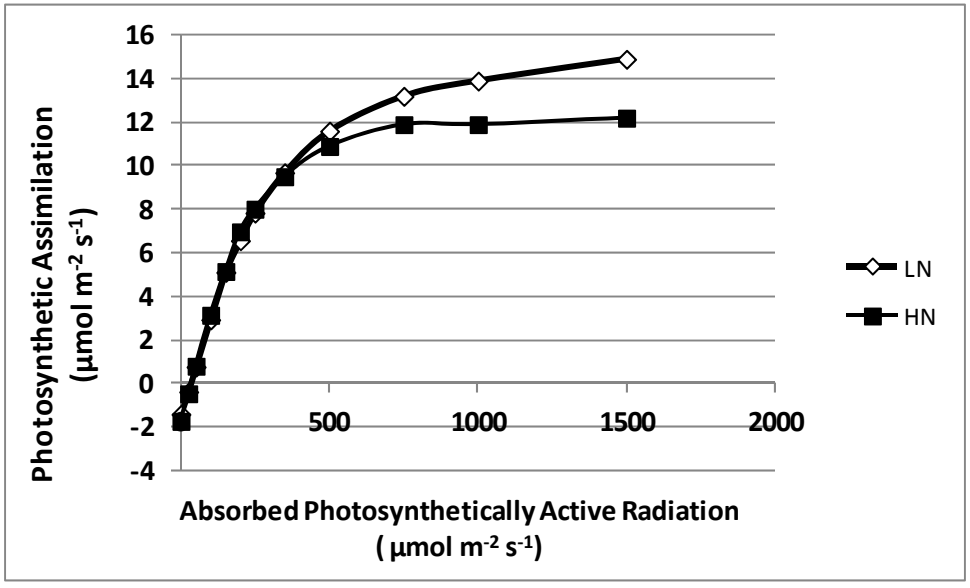


Figure 4.20 Light response curve of a LPI 4 leaf of a plant on low N (LN) and another plant on high N (HN) fertilization rates.

4.1.3.3 CO_2 or ACi Response Curves

The influence of ambient CO_2 on CO_2 uptake is seen when a photosynthesizing leaf is exposed to a series of increasing internal CO_2 levels resulting in a CO_2 or ACi response curve.

A comparison of ACi response curves was prepared from LPI 4 leaf of a HN vs. a LN plant. HN leaves showed a higher rate of CO_2 uptake or photosynthetic assimilation when exposed to increasing levels of intercellular CO_2 (μmol mol^{-1}) as compared to LN leaves (Figure 4.21). There was no significant difference in photosynthesis between HN and LN leaves at

lower intercellular CO_2 level. However, a large difference developed for levels of intercellular CO_2 greater than 300 μmol mol^{-1}. There was no significant difference in the stomatal conductance (gs) of the HN and LN leaves (Figure 4.22). An increase in assimilation in the HN leaf compared to the LN leaf was small, but consistent between 200 and 350 μmol mol^{-1} CO_2. Above 350 μmol mol^{-1} CO_2, differences became greater.

Lambers, et al. (1998) suggested that Rubisco is not saturated during the initial linear increase of photosynthesis with increasing CO_2 however, it becomes limiting at the saturating region or asymptotic region of photosynthesis versus CO_2. Increasing leaf N increases Rubisco levels, hence the higher rates of photosynthesis in the HN versus LN plants.

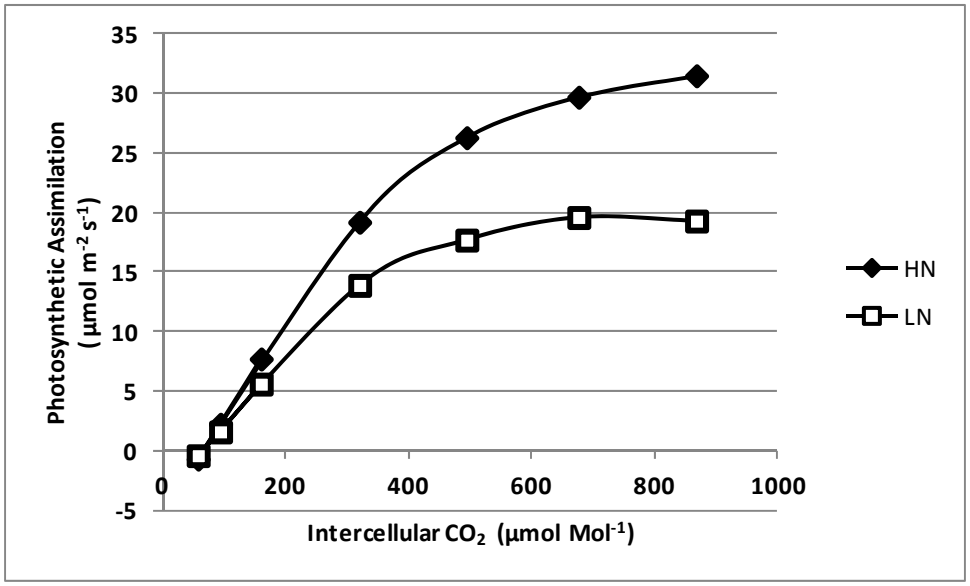


Figure 4.21 CO_2 or ACi response curve of a LPI 4 leaf of a plant on high N (HN) and another plant on low N (LN) fertilization rates.

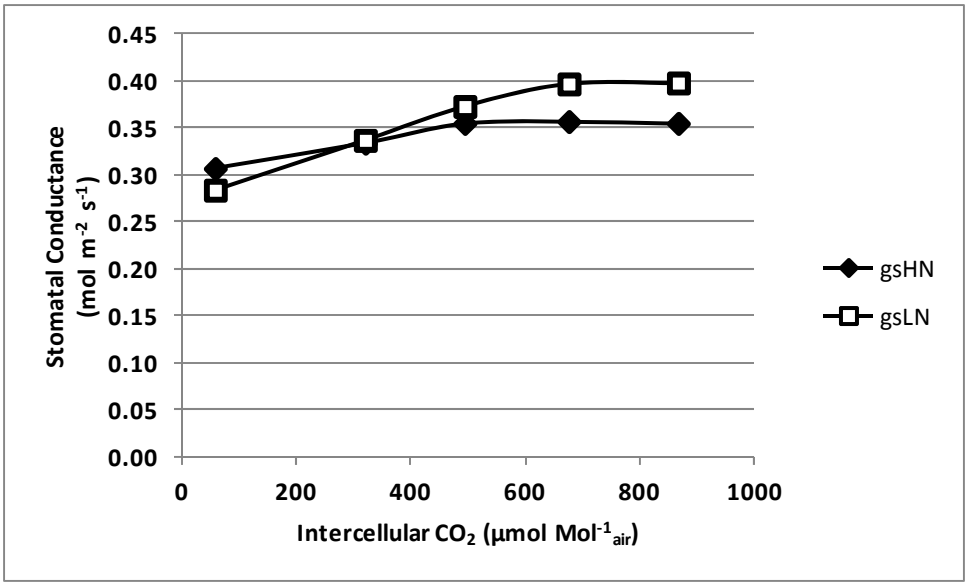


Figure 4.22 Stomatal conductance (gs) during ACi curve measurements of a LPI 4 leaf of a plant on high N (HN) and low N (LN) fertilization rates.

Similar to the results shown from the single LPI 4 leaf, results comparing leaves from the four SFSs of the HN and LN plants illustrated clear differences (Figure 4.23). Interestingly, the leaf from top SFS 1 of the LN plant had higher rates of photosynthesis at all levels of CO_2 in comparison to SFS 1 of the HN plant. N data shown in Figure 4.18 suggested higher levels from leaves from SFS 1 in LN versus HN plants. The expected higher CO_2 uptake in HN leaves was noted after the leaves had further matured in SFS 2, 3, and 4. Leaves, especially those from the base SFS 4, demonstrated large differences in photosynthesis between HN and LN treated plants. A general pattern of high CO_2 uptake in HN plants is seen when all the SFS data are combined in Figure 4.24 and Figure 4.25. These data suggest that N effects should become more pronounce with increases in atmospheric CO_2 (Figure 4.24) and that N levels resulted in sustaining ability to fix carbon in older, more mature leaves (Figure 4.25).

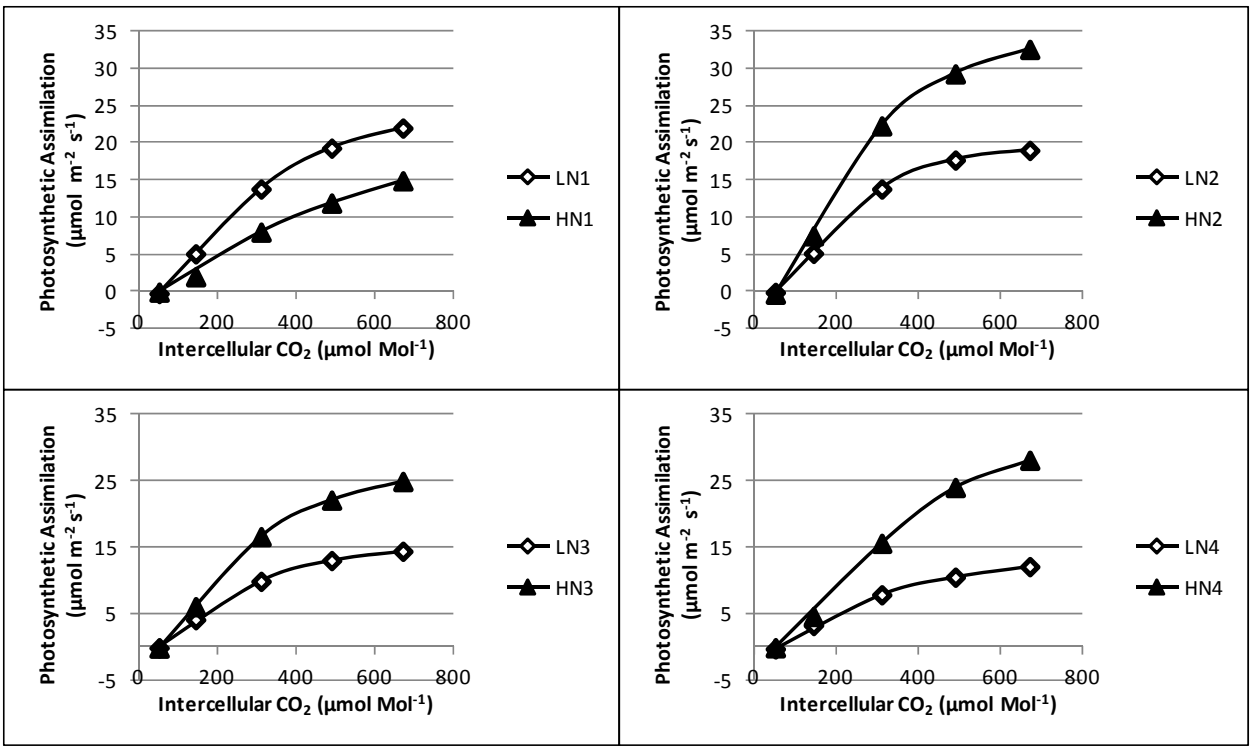


Figure 4.23 CO_2 or ACi response curve of leaves representing SFS (1-4) of plants on high N (HN1-4) and low N (LN1-4) fertilization rates at 1500 µmol m^{-2} s^{-1} PAR (n = 2 plants).

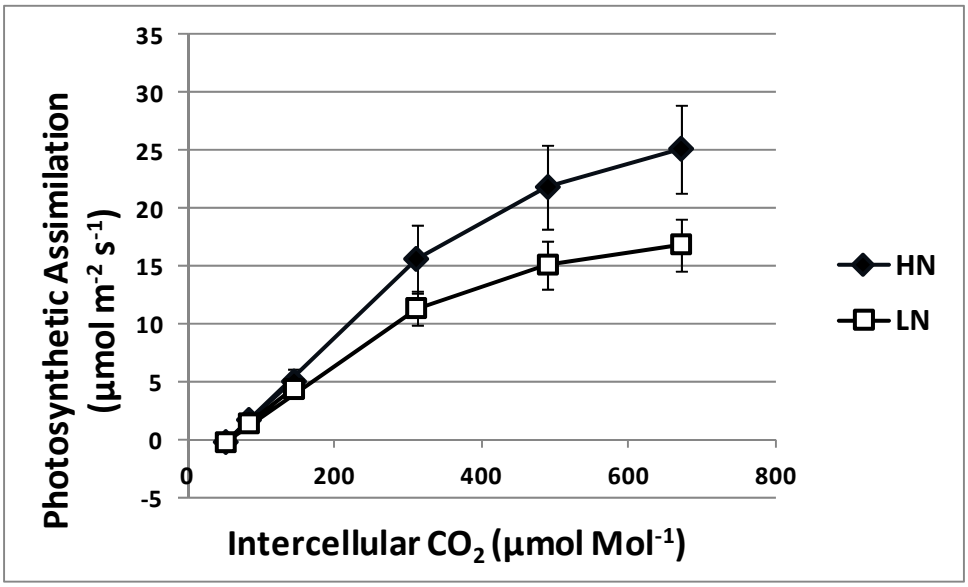


Figure 4.24 CO_2 or ACi response curve of plants on high N (HN) and low N (LN) fertilization rates at 1500 µmol m^{-2} s^{-1} PAR. Bars represent standard errors (n = 8 leaves, 2 plants).

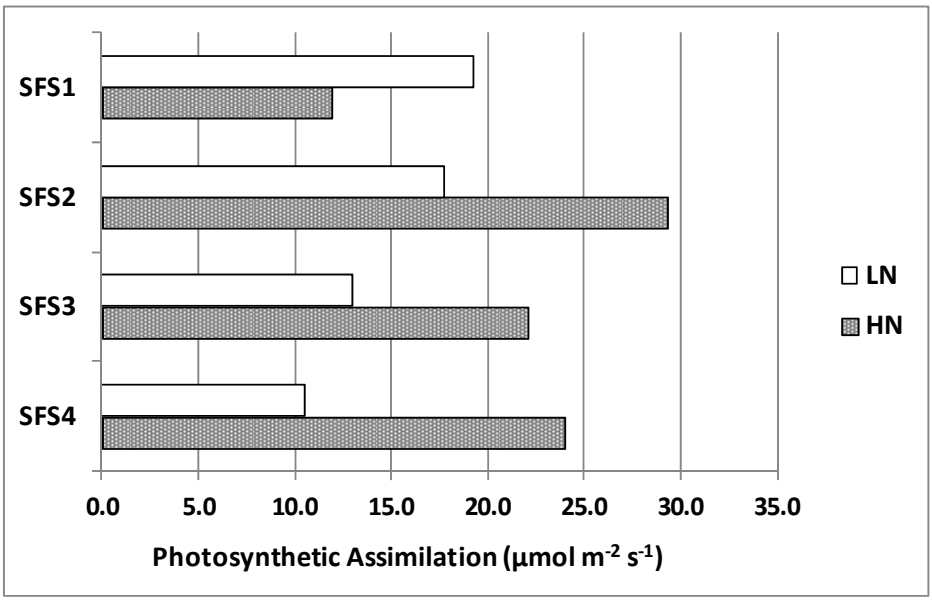


Figure 4.25 Photosynthetic assimilation **(**µmol m^{-2} s^{-1}) of plants on high N (HN) and low N (LN) fertilization rates at 1500 µmol m^{-2} s^{-1}PAR and 490 intercellular CO_2 (µmol Mol^{-1}) (n = 8 leaves, 2 plants).

4.1.3.4 Photosynthetic N Use Efficiency

Photosynthetic N use efficiency (PNUE) is defined as the amount of CO_2 fixation per given N content of a given leaf, that can also be determined per leaf LPIi or SFSi. It is expressed as:

$$\text{PNUE} = \frac{\text{Pi}}{\text{Ni}} \quad \text{or} \quad \frac{\text{Ai}}{\text{Ni}} \quad \text{or} \quad \frac{\sum \text{SFSi}}{\sum \text{NSi}} \qquad (2)$$

where P or A = photosynthetic capacity or measured CO_2 fixation and N = leaf N concentration of a given LPI number i. This is adapted from a series of equations presented by Finzi,, et al. (2007). PNUE can be determined per segment of the branch in terms of the summation of all the photosynthetic capacity of the LPIs of a given segment SFSi. As mentioned in the NUE section, N uptake (Finzi, et al., 2007) is replaced by N absorbed in response to nutrient treatment

(Ingestad, 1979; Birk and Vitousek, 1986) expressed as the relative amount of N (SPAD = 0 to100) in a given LPI or SFS at the time of photosynthetic measurement. N concentration is a relative value (SPAD = 0 to 100). PNUE is therefore expressed also as a relative value that is (μmol CO_2 m^{-2} s^{-1}) $SPAD^{-1}$. A notable feature of PNUE compared to NUE is that PNUE is an instantaneous measure while NUE is a measure of carbon gain and loss (Lambers, et al.,1998) over a given time period.

HN and LN plants were compared based on their values of PNUE expressed as leaf CO_2 uptake per leaf N content. This was determined at the later part of the experiment during the 11^{th} and 12^{th} week after transplanting (Table 3.1). The following hypotheses are considered:

H_0: PNUE does not vary with SFS in leaves of HN or LN plants.

H_a: PNUE varies with SFS in leaves of HN or LN plants.

The results showed that the null hypothesis (H_0) is rejected and the alternate hypothesis (H_a) is accepted. PNUE of leaves varied with N fertilization in cottonwood hybrid 47-174 (R^2 =0.822, p 0.388). The middle segments, SFS 2 and SFS 3 had higher PNUE compared to the top (SFS 1) and basal (SFS 4) segments of the HN plants. However in LN plants the top segments (SFS 1 and SFS 2) had high PNUE compared to the lower segments (SFS 3 and SFS 4) (Figure 4.26).

Except for SFS 1, as discussed earlier, PNUE was greater in HN vs LN plants. PNUE was positively correlated with area-based leaf N in *Populus x euroamericana* (Ripullone, et al., 2003).

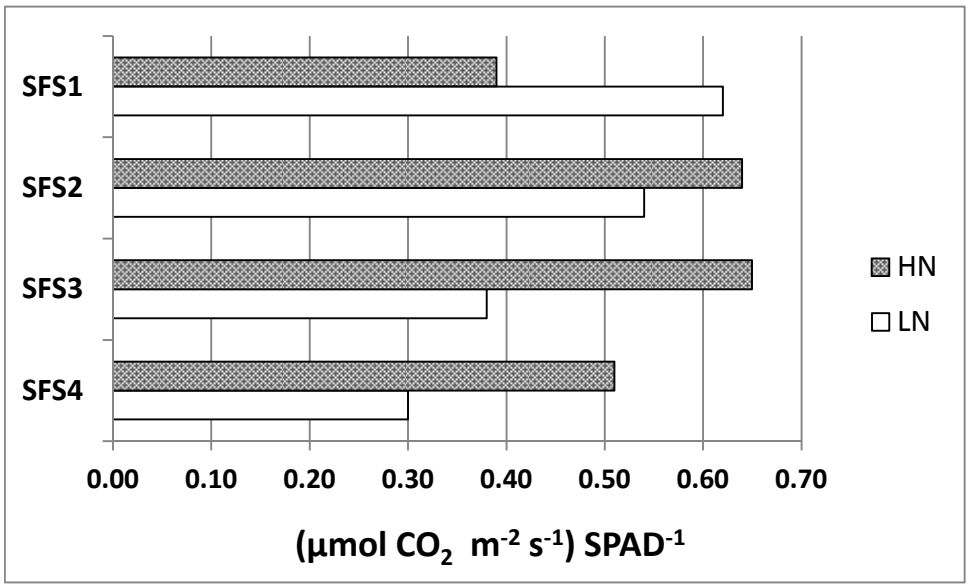


Figure 4.26 Photosynthetic N use efficiency (PNUE) of high N (HN) and low N (LN) plants expressed as [(µmol CO_2 m^{-2} s^{-1}) $SPAD^{-1}$] of leaves located in the 4 segments of a branch from the base (SFS4) to the top (SFS1) (n = 8 leaves).

There is a significant correlation between specific leaf area (SLA) and PNUE (R^2 = .76 at p. 060) (data not shown) because as mentioned earlier, increased SLA favors more light rays reaching the carboxylation sites (Ducrey, 1992). As shown above, PNUE is high in the middle SFS 2 and SFS 3 of HN leaves while PNUE is high in SFS1 and lower toward the base in SFS 4 of LN leaves. There is increased PNUE in SFS 1 of LN leaves with high SLA which could be related to higher Rubisco contents in slow-growing species (Poorter and Evans, 1998, Onoda, et al., 2004) like the LN plants. Also, other protein complexes other than Rubisco may have been partitioned differently in tissues with lower N that influenced their proportion toward increased efficiency (Poorter and Evans, 1998). In evergreen leaves, there is a tendency to decreased

PNUE because of conservative use of water and nutrients and tolerance of water and nutrient stress (Warren and Adams, 2004).

PNUE can be correlated also to leaf mass area (LMA). As mentioned earlier, LMA is a measure of leaf structural toughness and can be used as an index of cell wall biomass (Onoda, et al., 2004). There is close correlation between PNUE and LMA of HN and LN plants in SFS 2, SFS 3, and SFS 4 (data not shown). SFS 1 is an exception because of its greater PNUE in LN leaves compared to PNUE of HN leaves. However the difference in higher photosynthesis in LN SFS1 was not significant. Also, LMA of HN leaves showed higher values in all segments compared to LMA of LN leaves.

4.2 Leaf Rust Stress

In nature, the more prominent and noticeable diseases are those occurring on plant foliage. In this study I focus on *Melampsora* leaf rust infection that, as mentioned earlier, is the most common, serious, and widely distributed disease affecting cottonwood in nurseries, plantations, and forests worldwide (Berbee, 1964; Newcombe, et al., 1996; Ostry, 1997; Sharma and Sharma 2000).

4.2.1 Influence of Leaf Rust on Morphology

Leaf rust infected leaves, especially HN leaves, were dried and died after 2 weeks of leaf rust infection or 14 days post infection (dpi). It was not possible to take prolonged photosynthetic measurements on leaves that were on 14 dpi. As a consequence, data were obtainable for both HN and LN leaves at 6 dpi when the infection was not so advanced. The curling of the infected leaves and eventual drying process was predictable after manual infection

by the brushing technique because the whole leaf became completely infected that showed prolific and profuse leaf rust growth.

4.2.1.1 Leaf Rust Sporulation

After viewing infected leaves via the naked eye, microscopically, and scanning electron microscopy (SEM) the following pattern of infection emerged. Spores are reported to enter through a single stoma and after development, hyphal growth occurred in the mesophyll followed by sporulation or formation of the uredium that emerged from the inside through the epidermis (Littlefield and Heath, 1979). This process of emergence is clearly shown in Figure 4.27 (and Figures 1 and 2, Appendix C). The SEM image of the abaxial side shows that the uredium and urediospores emerged from the lower surface of the leaf by breaking through or tearing up the epidermis and the associated stomata.

The initial pushing or breaking of the epidermis has been reported to be aided by elongated, often capitate paraphyses formed in the uredium or uredinium (Laurans and Pilate, 1999). As shown in an enlarged detail of a uredium or pustule (Figure 4.27 B, Figures 3 and 4, Appendix C), there are stoma on the torn up or broken portion of the epidermis. Obviously this tear or structural opening of the epidermis would create a stressful situation for the leaf in terms of impairment of maintaining regulatory processes especially those controlled and dependent on stomatal integrity, like water balance. The observed tearing of the epidermal layer is in strong contrast to the cellular interactions and responses to injury presented by Ayres (1991).

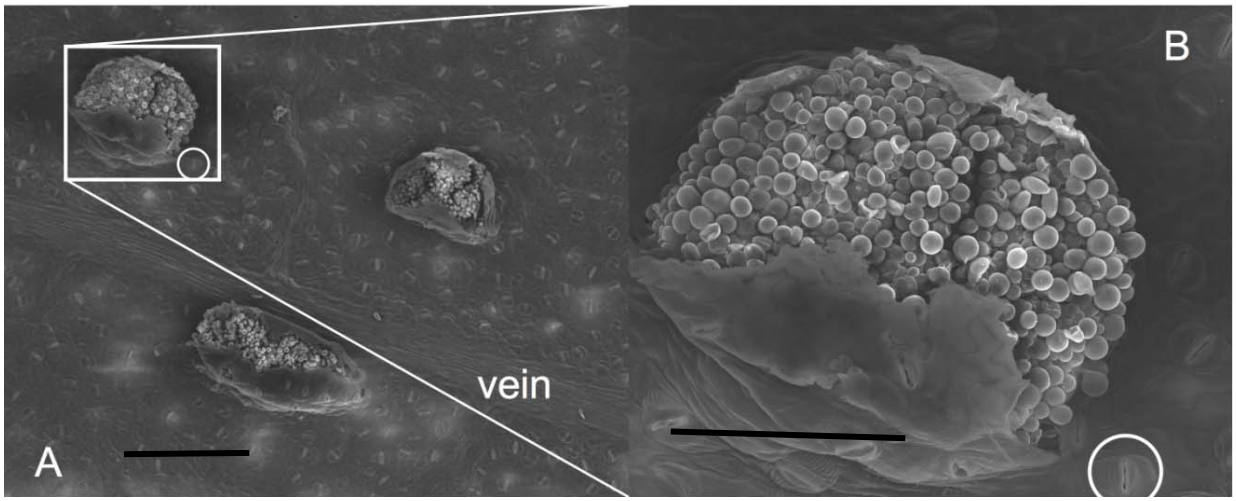


Figure 4.27 A. Scanning electron microscopy (SEM) of the abaxial side of a leaf showing leaf rust pustules or uredia with urediospores emerging among numerous stoma. A leaf vein is seen across on the side of one of the pustules (bar = 300 μm). B. Enlargement of a leaf rust pustule or uredium from panel A showing associated numerous urediospores, emerging or erupting from a torn leaf epidermis with visible stoma and neighboring epidermal cells. Circle indicates the same stoma on both panels (bar = 150 μm).

SEM images of air-dried leaf sections from HN and LN plants similarly showed comparable levels of leaf rust infection (Figure 4.28). Spores are seen scattered on the leaf surface of these air-dried leaves because the surface was not cleared by a fixing solution and the subsequent washing and dehydration with ethanol. Figure 4.28 also shows uredia that are starting to emerge or erupt from the epidermis.

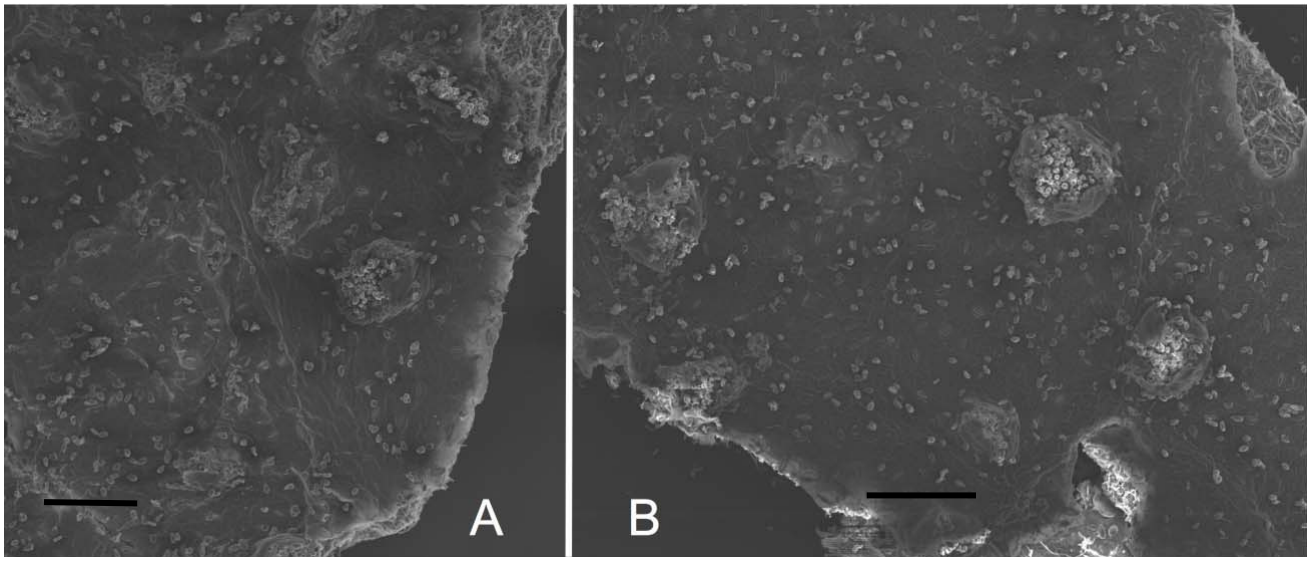


Figure 4.28 Scanning electron microscopy (SEM) of air-dried leaf rust infected leaf sections from (A) high N (HN) and (B) low N (LN) fertilization rates. Spores are scattered on the surface because air-drying did not use clearing effects of washing the fixing solution and subsequent dehydration with ethanol. Some uredia are newly emerging or erupting from the epidermis (bar = 300 μm).

Analysis of more SEMs from infected leaves shows some mesophyll areas with rust hyphae close to the pustules or uredia. Sections of HN and LN leaves in Figure 4.29 show a cleaner razor blade cut in LN versus HN. This difference suggests that infected LN leaves are sturdier and more rigid than HN leaves. Spores observed in the mesophyll were probably spread during razor blade cutting of the sections.

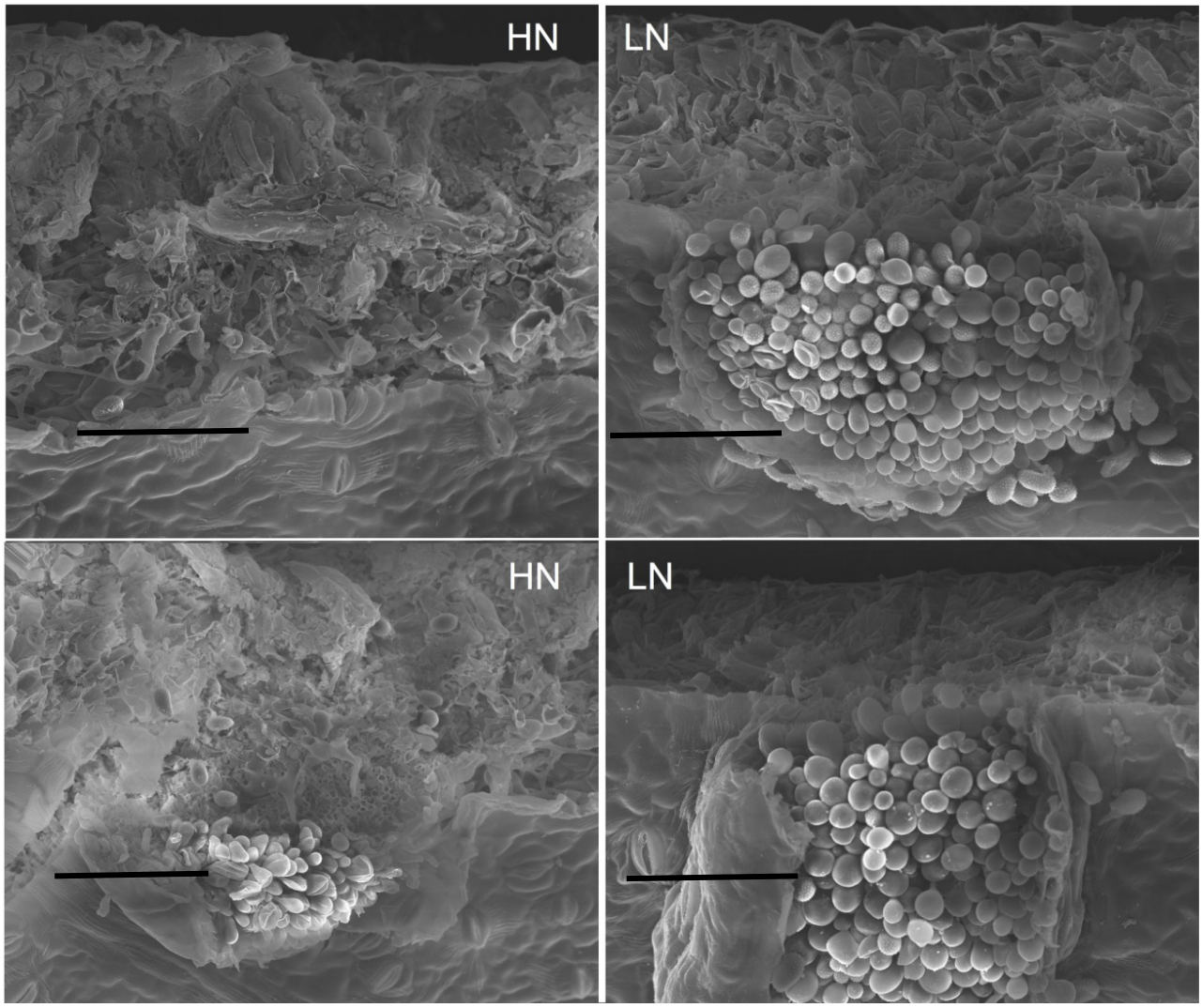
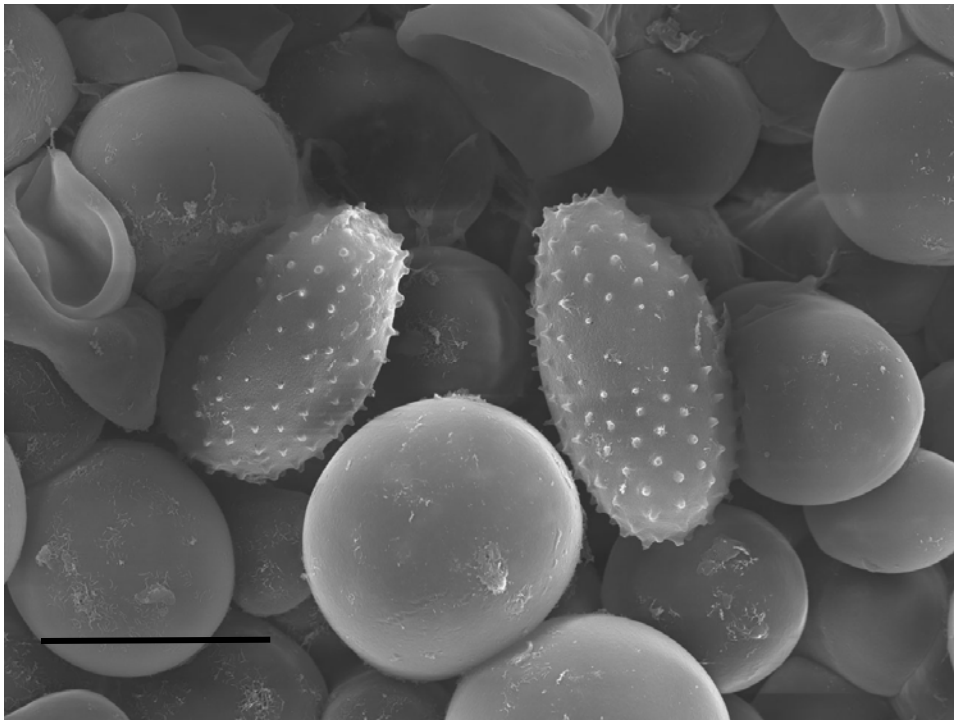


Figure 4.29 Scanning electron microscopy (SEM) of leaf sections (A, B) from high N (HN) and low N (LN) plants. For the HN SEMs: Palisade cells are visible in the top image and a uredium with spores is on the foreground of the lower image. For the LN SEMs: Uredia with spores are in the foreground. Palisades cells are visible in the top SEM and in the lower SEM, there is a piece of epidermis on the left side of the uredium (bar = 150 μm)**.**

Although LN leaves were as susceptible as HN leaves, LN leaves may be better pathogen anchors or hosts because they were sturdier with more persistent tissues that might ensure longer life span of the pathogen and, therefore, rust occupation. The infected HN leaves, which were less sturdy and larger, began drying after one week of infection.

Profuse leaf rust development appeared to emerge from the inside of the mesophyll to the outer epidermis on the abaxial side. As mentioned earlier, the emerging uredia tear up a large section of a leaf. In addition, the urediospores were bundled together like a bunch of grapes and were a mixture of young smooth and older rough or echinulated spores with projections (Figure 4.30). As the smooth urediniospores mature, the cell walls thicken, and ornamentation or echinulation develop (Littlefield and Heath, 1979; Laurans and Pilate, 1999). Figure 4.30A shows two mature echinulated spores with the characteristic equatorial smooth spot (ESS) of *Melampsora medusa* Thumen. (Newcombe, et al., 2000).

A



B

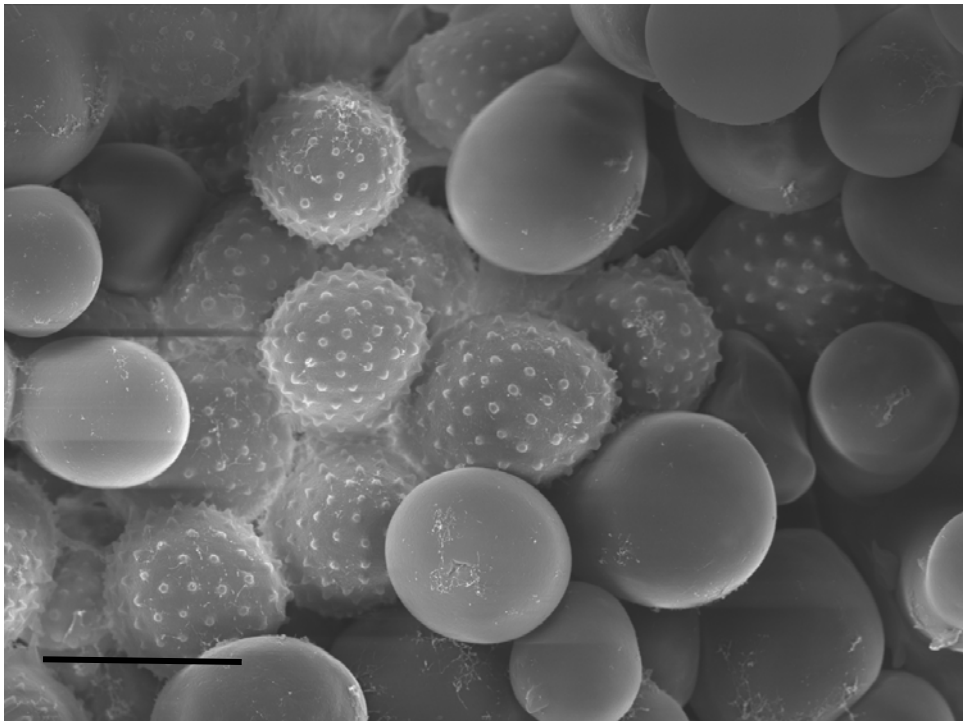


Figure 4.30 Scanning electron microscopy (SEM) of leaf rust spores (A, B) that are a mixture of young smooth and older echinulated spores (bar = 10 μm)**.**

4.2.1.2 Interaction between Leaf Rust and Leaf Anatomy

The anatomical features of a leaf starting from the epidermal cells and the associated stomata to the internal mesophyll layers that are the sites of gas exchange are also key regions in the growth and development of leaf rust infection. The stomata in hybrid 47-147, located on the abaxial side of leaves, play an important role because they are the main ports of entry of leaf rust spores on the leaf surface. At the same time, stomata are able to change their aperture and control water loss and gas exchange (Evans and von Caemmerer, 1996). CO_2 once inside the mesophyll areas diffuses to the intercellular air spaces and sites of carboxylation. Open stoma would enhance CO_2 diffusion and the rate of photosynthesis.

However, in cottonwood plants, one might expect better growth of the *Melampsora* spore germ tubes through the open stoma. Once inside the leaf the gas exchange mechanisms inside the mesophyll region would be also affected, if not severely impacted by the profuse growth of leaf rust hyphae that develops from the germ tubes inside the mesophyll region. Finally, the same abaxial side becomes the ports of exit of the fruiting bodies, visible as the orange pustules of leaf rust infected leaves.

In addition to stomatal opening, stomatal density (SD) may also influence the extent of infection. The mesophyll areas subsequently play a major role too being the region of profuse hyphal growth of the leaf rust before the fruiting period or sporulation (Spiers and Hopcroft, 1985). Therefore, N nutrition and its influence on leaf area and leaf size may also affect both stomatal density and spongy mesophyll volume. These morphological changes may in turn affect the intensity and rate of rust infection.

4.2.1.3 Stomatal Density and Leaf Rust Infection

Both HN and LN plants showed remarkable leaf rust infection in both naturally field infected plants and in manually brushed infected plants. The results were positive regardless of stomatal density (SD). Although leaves from HN plants had significantly higher mean SD (218 stoma mm^{-2}) than LN plants (179 stoma mm^{-2}) both plants after manual brushing infection had profuse leaf rust growth (Figure 4.31). Plants under high N fertilization may be prone to increased infection because of increased SD exposing more ports for spore entry (Harvey and van den Driessche, 1999).

Either as a result of my inoculation technique or the fact that my LN treatment as not sufficiently stressed, I was unable to see an effect of SD on infection. In contrast to my observations, Suzuki (1973) found that when plants were grown on N deficient solution culture or in solution culture completely lacking N, low N levels appeared to inhibit infection.

SEM observations of appresorium development in infected leaves may be the best way to compare successful rust infection and stomatal density. Is the region of high SD similar to the region of successful infection? This study may be done by monitoring the process of successful infection in natural field infected plants. The region of early uredial growth maybe the region of high SD or the patchiness of uredial growth at the start of field infection may be explained by the patchiness of stomatal distribution.

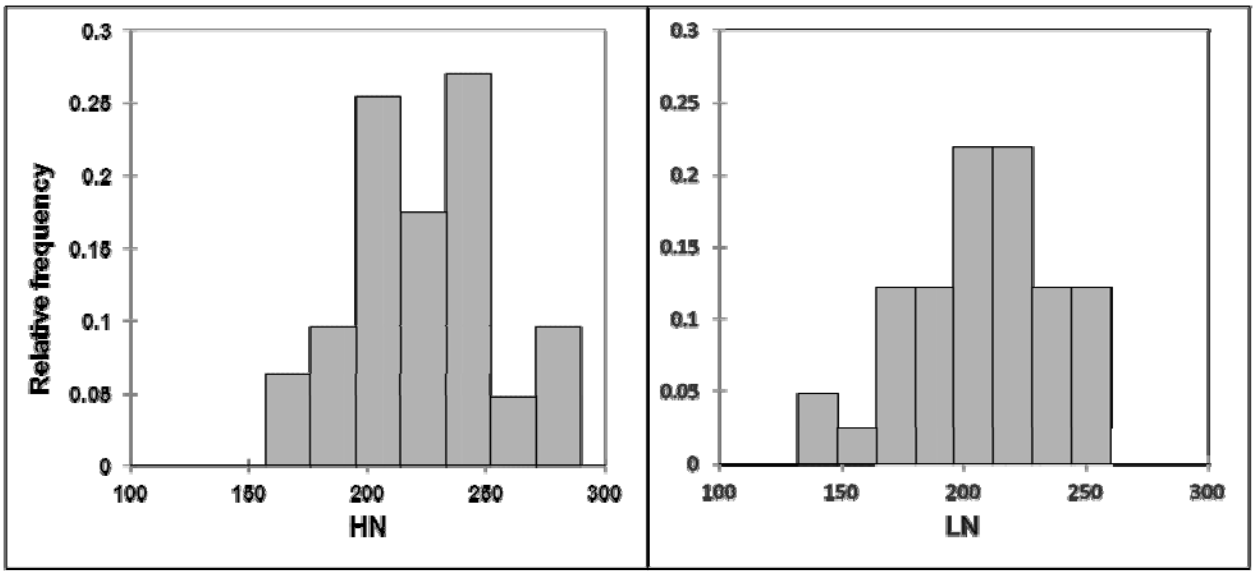


Figure 4.31 Histograms showing the relative frequency (x 100%) of stomatal density (SD) (stoma mm^{-2}) of leaves from plants on high N (HN) and low N (LN) fertilization rates (n = 222 leaf sections of 4 leaves).

4.2.2 Influence of Leaf Rust on Photosynthesis

During the 9th week following transplanting on October 20, 2009 (Table 3.1) the greenhouse plants were successfully infected and the effects of leaf rust infection on photosynthetic rates were measured. The following hypotheses are considered:

H_0: Leaf rust infection has no effect on photosynthesis.

H_1: Leaf rust infection has an effect on photosynthesis.

Leaf rust infection had a significant effect on photosynthesis. The results were obtained from direct measurements that have not yet been reported for poplar during pathogen infection (Major, et al., 2010).

Leaf rust infection likely influenced the rate of photosynthesis in a number of ways. For example, there may be increased pathway resistance (i.e, stomatal and mesophyll) brought about by an obvious hyphal growth in the mesophyll areas. Hyphal growth could also reduce light

exposure, depriving photosynthesis of adequate photons. Figure 4.32 shows that healthy uninfected leaves have higher photosynthetic assimilation rates than infected leaves.

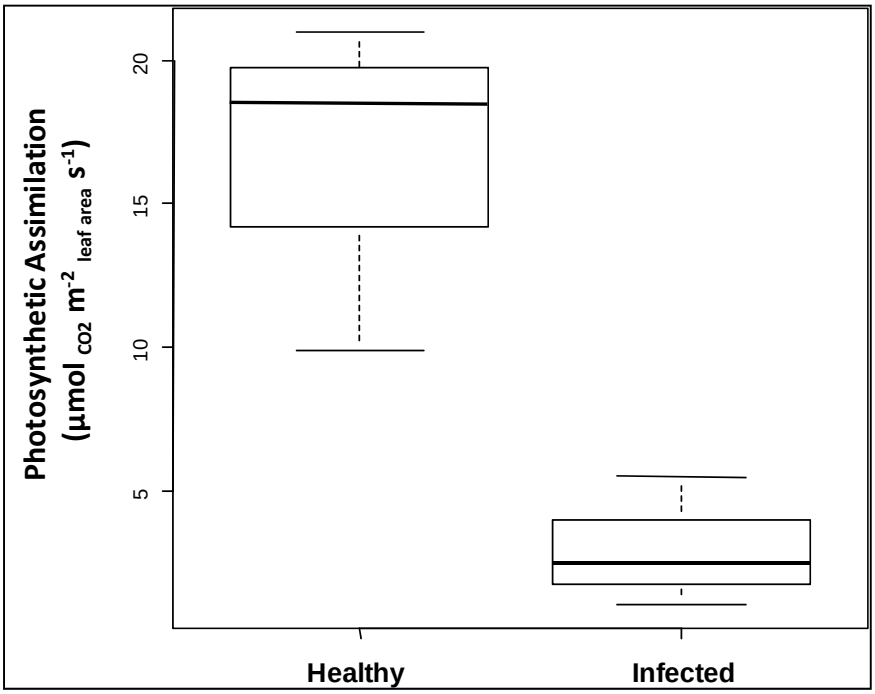


Figure 4.32 Photosynthetic assimilation or CO_2 uptake of healthy versus infected leaves (n = 16 leaves).

In a previous trial, leaf rust infection decreased CO_2 fixation by 23% (Banaag and Johnson, 2002). This time, leaf rust infection decreased CO_2 fixation by 75%. The main reason was the extent of infection. With the spray inoculation (previous trial), the amount of infection was not as prolific as when the brushing technique was used (this study). As previously described, the brush technique both saturated and more evenly exposed the leaf to rust spores and thereby insuring maximum infection. By comparison the decrease in CO_2 fixation brought about by low N fertilization was 35%.

4.2.2.1 Light Response Curves

A comparison of CO_2 uptake over a wide range of absorbed PAR levels for healthy LN (LPI 4) and infected LN (LNi) (LPI 7) leaves shows a significant difference (Figure 4.33, Table 4.9). LPI 4 showed fairly high rate of CO_2 uptake. This could be attributed to a greater stomatal conductance (gs) in healthy leaves compared to a somewhat inactive or unchanging stomatal conductance of infected leaves (Figure 4.34) plus the possibility of hyphal impedance to light absorption and impaired stomatal control in ruptured epidermal cells of infected plants.

It was not possible to measure the light response curve of an infected HN leaf at 14 days post infection like the LN plants because the infected leaves were dried too fast and could not be exposed to prolonged photosynthetic measurements.

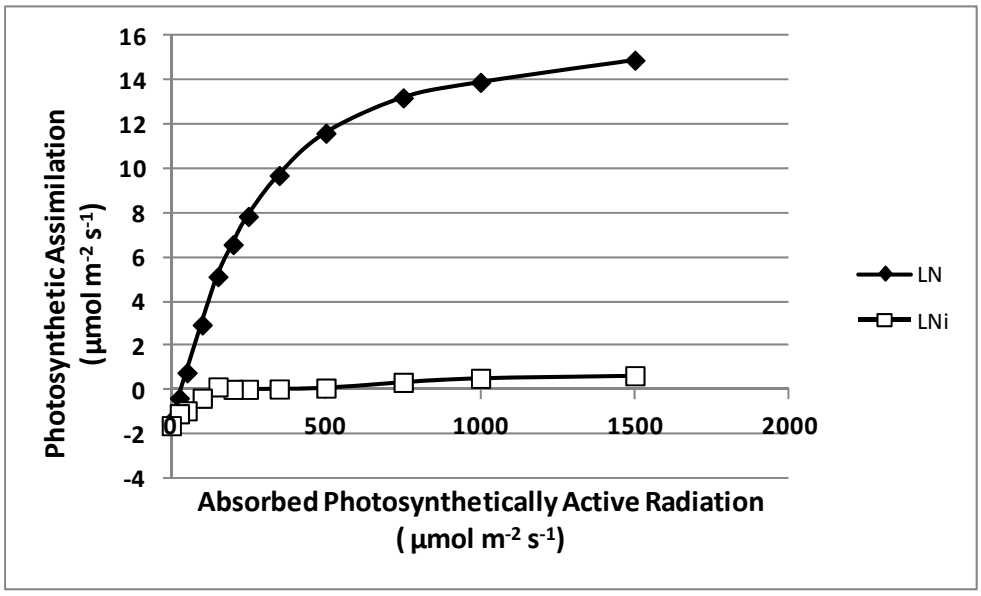


Figure 4.33 Light response curve of a healthy LPI 4 leaf (LN) versus an infected LPI 7 leaf (LNi) from the same plant on low N (LN) fertilization rate.

Table 4.9 Photosynthetic assimilation or CO_2 uptake (µmol CO_2 m^{-2} s^{-1}), mean ± SD, maximum, and minimum data based on light response curve of healthy low N (LN) leaf LPI 4 and infected low N (LNi) leaf LPI 7 from the same plant n (n = 24 measurements, *statistically different at α = 0.05).

Treatment	Number of Observations	Mean ± SD	Maximum	Minimum
HN	12	7.1 ± 5.7*	14.9	-1.4
LN	12	-0.18 ± 0.7*	0.6	-1.6

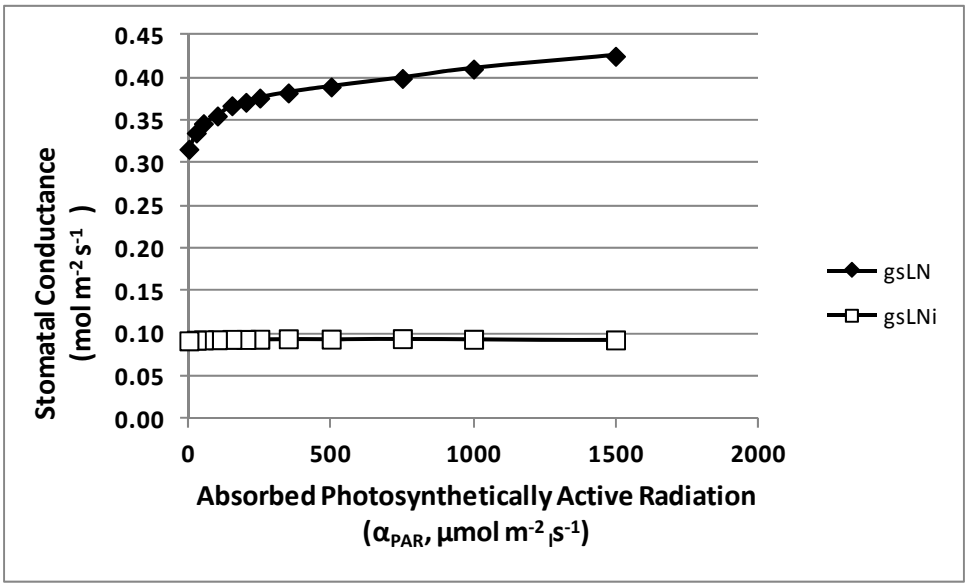


Figure 4.34 Stomatal conductance (gs) during light response curve measurements of a healthy LPI 4 leaf (LN) and an infected LPI 7 leaf (LNi) from the same plant on low N (LN) fertilization rate.

4.2.2.2 CO_2 or ACi Response Curves

The ACi response curves showed a higher rate of CO_2 uptake or photosynthetic assimilation in healthy versus infected leaves 6 days post infection when exposed to increasing intercellular levels of CO_2 from 50 to 800 intercellular CO_2 (Figure 4.35). There is significant difference between healthy and infected leaves, with the risk to reject the null hypothesis is less

than 0.11 % at $\alpha = 0.05$. For a few of the infected leaves, only a few data points had dried quickly and could not be exposed to prolonged photosynthetic measurements. Measurements were done at 6 dpi when the leaves were not as far advanced in infection therefore, the infected leaves could withstand prolonged photosynthetic measurements.

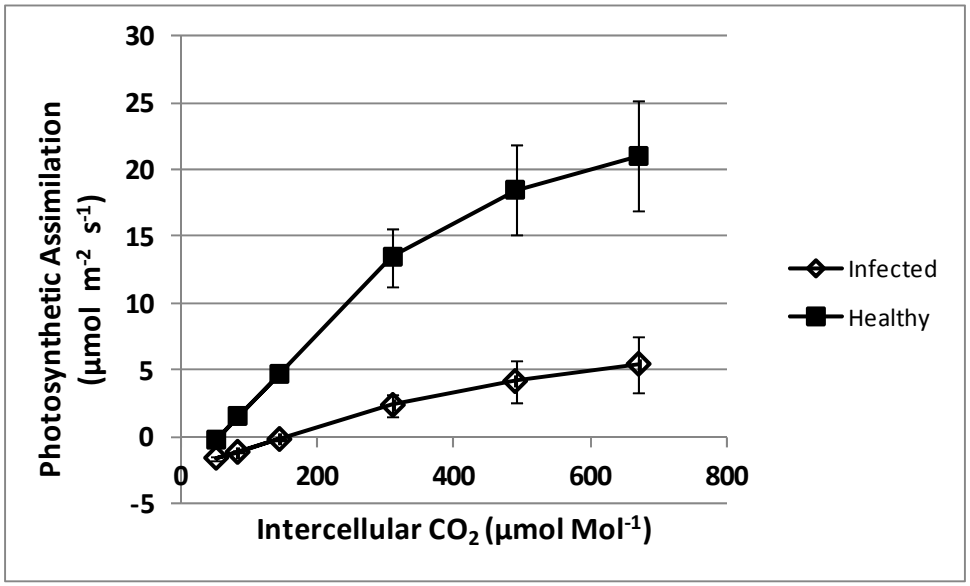


Figure 4.35 CO_2 or ACi response curve of healthy vs. infected leaves.
Bars represent standard errors (n = 16 leaves).

4.3 Interaction between Leaf Rust and Nitrogen

4.3.1 Healthy Versus Infected Leaves

As mentioned earlier, each plant brought to the laboratory for measurements consisted of healthy and diseased leaves from each of the 4 SFS and representing HN and LN plants. A leaf located in each of the SFS had been inoculated and was paired with a healthy leaf immediately below it. All leaves had been inoculated at the same time. This procedure was vastly better for four reasons: (1) it dealt with time constraints in making measurements, (2) it insured that there were enough spores to inoculate leaves representing the four segments or representative of the whole plant, (3) it provided a more robust comparison as only four leaves were infected versus

the whole plant, and (4) the proximity of the paired leaves to each other offered clearer comparison of healthy versus diseased leaves. Figure 4.36 is a photograph of one plant showing the location of 3 of the 4 infected leaves in the region of SFS 2, SFS 3, and SFS 4.

The overarching questions were: What are the responses of hybrid 47-174 on HN and LN fertilization to *Melampsora* leaf rust infection. Do rust infected leaves influence the photosynthetic process. The following hypotheses are considered:

H_{01}: N fertilization and leaf rust infection has no effect in SFS.

H_{02}: PNUE does not vary with N nutrition and leaf rust infection in SFS.

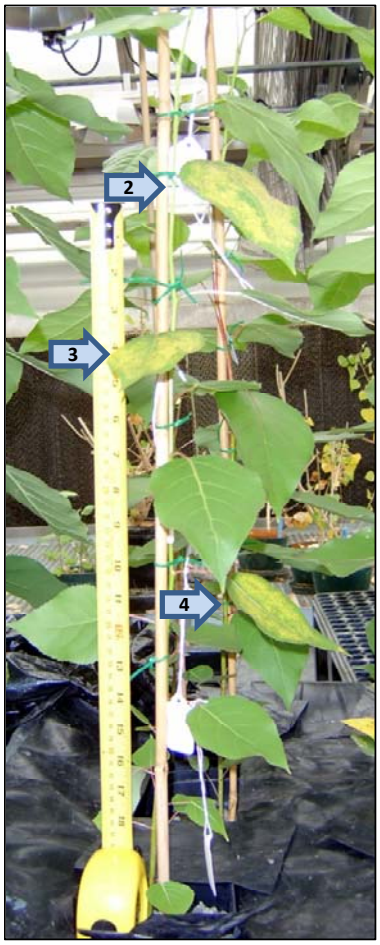


Figure 4.36 Photo of a plant showing 3 rust infected yellowish leaves (with numbered arrows) representing SFS 2 (2) from the top, SFS 3 (3) in the middle, and SFS 4 (4) at the base. On top of SFS 2 is an infected leaf representing SFS 1 not shown in the photo.

4.3.2 Influence of Leaf Rust and Nitrogen on Rust Development

In terms of the N fertilization, the following hypotheses were considered when answering the question: Did fertilization have an effect on leaf rust infection?

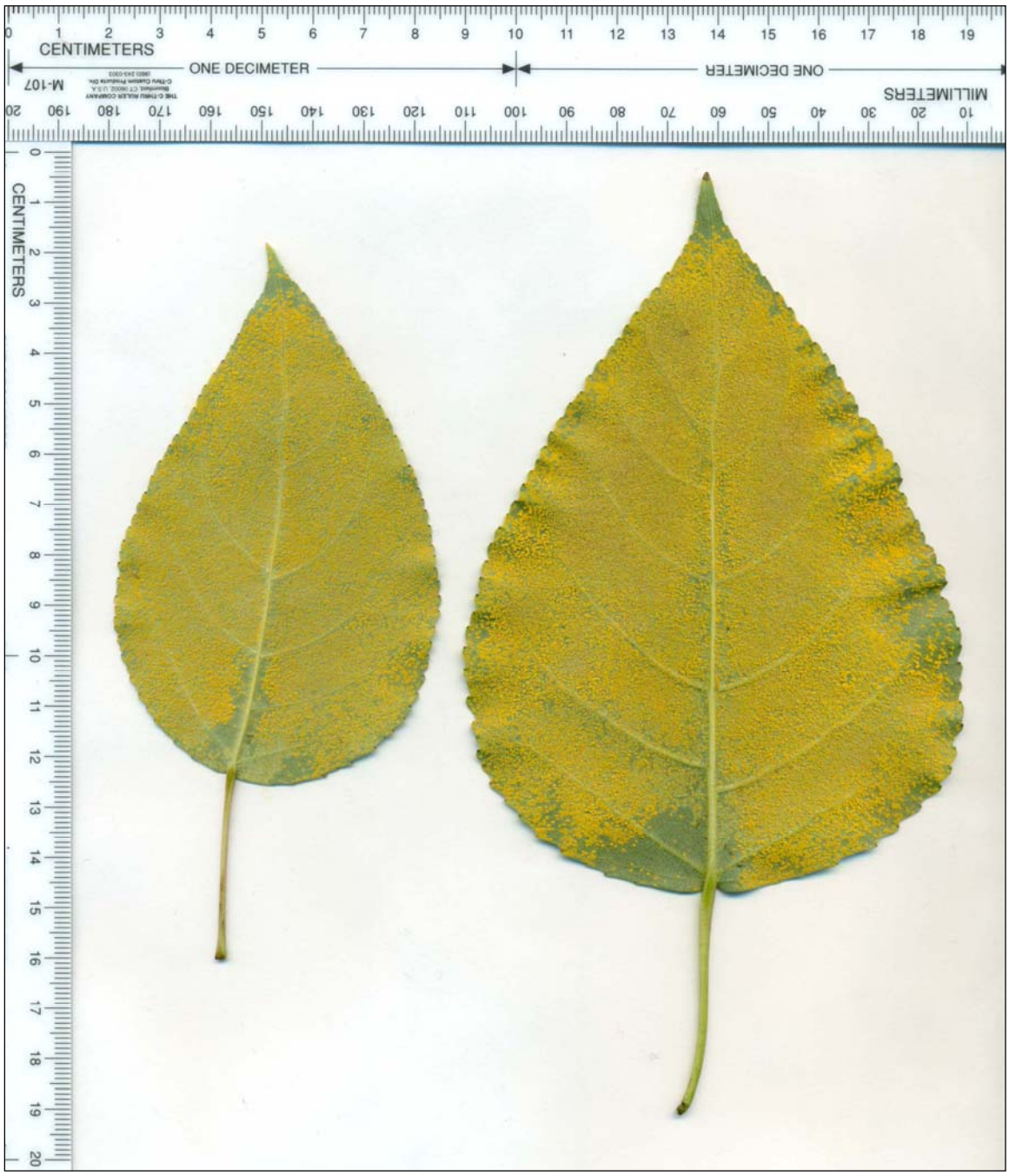
H_0: N fertilization has no effect on leaf rust infection.

H_a: N fertilization has an effect on leaf rust infection.

Rigorous testing of the null hypothesis and its alternate turned out to be quite difficult. There was a surprisingly good growth of leaf rust on both LN and HN greenhouse potted semi-hydroponic plants that were manually inoculated and on the same group of plants that were brought out and field inoculated in the area of the source plants' natural location at Farm 1. The same potted plants were brought back to the greenhouse for spore seeding or for maintaining fresh supply of spores for subsequent inoculations. The same good leaf rust growth, which had been observed in the field, was continuously observed in the greenhouse during the subsequent inoculation of LN plants and HN plants. On the positive side, the use of fresh spores resulted in consistent inoculation success. Storing spores and then using them to subsequently inoculate plants has been shown to be difficult because, unless stored under special conditions, spores are extremely short-lived (Gottlieb, 1950).

As shown in fresh-leaves removed from SFS 3 of plants from each treatment (Figure 4.37), leaf rust infection occurred in both HN and LN grown leaves after manual inoculation using a brushing technique. The photograph of the abaxial side of both LN and HN illustrated profuse leaf rust growth, which means that the null hypothesis that N fertilization has no effect on leaf rust infection was not rejected. As mentioned earlier, these leaf rust growths were not

quantified. Success in inoculation had been brought about by this brushing technique that approximated the dry lodging of wind-blown spores on field-grown plants. However, an important condition or requirement for successful inoculation following manual brushing was the bagging of each leaf in clear and soft plastic overnight to maintain a certain amount of moisture and humidity (see Figure 3.5). Inoculated leaves that were not bagged with clear plastic overnight did not develop successful leaf rust growth. The plastic bag accumulates and traps moisture that promotes spore germination because water, either in its liquid or vapor phase, is a prime factor in spore germination (Gottlieb, 1950). Temperatures between 18^{o} and 23^{o}C and relative humidity close to 100% are the most conducive micro-meteorological conditions for leaf rust spore germination and infection (Spiers, 1978). Inhibition of germination by increasing light intensity, most pronounced at 25^{o}C, is reversed when urediospores are incubated in the dark (Singh and Heather, 1982).



LN HN

Figure 4.37 Photos of the abaxial side of hybrid 47-174 leaves grown on low N (LN) and high N (HN) fertilization rates showing profuse leaf rust growth. Rulers have cm and mm markings. Note that the HN leaf is 67% larger than the LN leaf from the same middle SFS 3 of the plants. Also note the yellow-orange spots indicating that uredia are erupting from the surface.

Huber and Thompson (2007) stated that cereal rust fungus, mildew fungi, and other foliar pathogens show increased infection as the rate of N availability increases as in HN plants. However, the LN plants may favor infection too because the amount of N is not yet at a deficiency level. In fact, the LN fertilization rate was set at a level to not show N deficiency symptoms.

It was also noted that there was growth of spores through the adaxial or upper surface of the leaf (Figure 4.38). Because of the asymmetric distribution of stomata, spores may not enter from the adaxial side but some tend to emerge through this side. This was noticeable at a later stage of maturation of leaf rust growth -- at this time, there were numerous uredial growths ready to emerge.

Because of the very careful and consistent way I inoculated leaves in the greenhouse, temporal patterns or differences in rates of inoculation were not possible to observe. However, field observations permitted additional observations when the entire leaf area did not become infected all at once. Instead, the leaf rust infection followed a developmental pattern when some areas of the leaf get infected initially followed by the advance of rust growth to the entire leaf. Also, once the first uredia emerged, then they could initiate more growth of the leaf rust by a recycling back to the internal mesophyll especially on the abaxial side without maybe even leaving the leaf. The natural pattern of patchy leaf rust development may not coincide with the patchiness too of stomatal patterning that was hypothesized earlier (Banaag, et al., 2006) as shown in the SEM analysis.

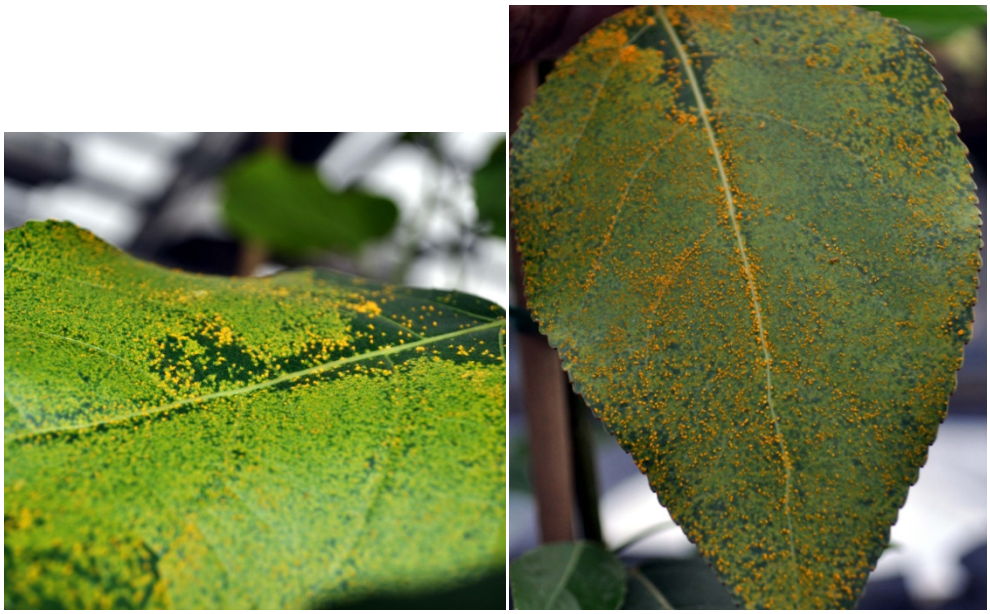


Figure 4.38 Adaxial side of infected leaves showing yellowish-orange powdery outgrowth of leaf rusts.

4.3.3 Influence of Leaf Rust and Nitrogen on Photosynthesis

Data shown using box plots (Figure 4.39) demonstrated that uninfected HN leaves have higher rate of CO_2 uptake than infected leaves (HNi) and uninfected LN leaves have higher CO_2 uptake rates than infected leaves (LNi). At the same time, HN leaves have higher CO_2 uptake than LN leaves as shown earlier. The box plots, however, show a range of values and this range could be the result of leaf development as it affects differences in leaf structure and thus function. With infection there are also the epidermal breaks that could impact gas exchange. The extent of rupture of the epidermis could be one explanation why Robert, et al. (2004) reported that photosynthesis was severely inhibited within the rust infected areas than in the rest of the uninfected wheat leaf. In this study, greater uredial growth (estimated via LM, SEM) appeared to be associated with lower CO_2 uptake measurements. Severity of infection is usually

expressed as frequency of uredinial pustules (Wang and Van der Kamp, 1992). Differences in CO_2 uptake could also be influenced by the rates of rust respiration of infected leaves. Infected leaves show a much lower rate of photosynthesis for both HN and LN plants.

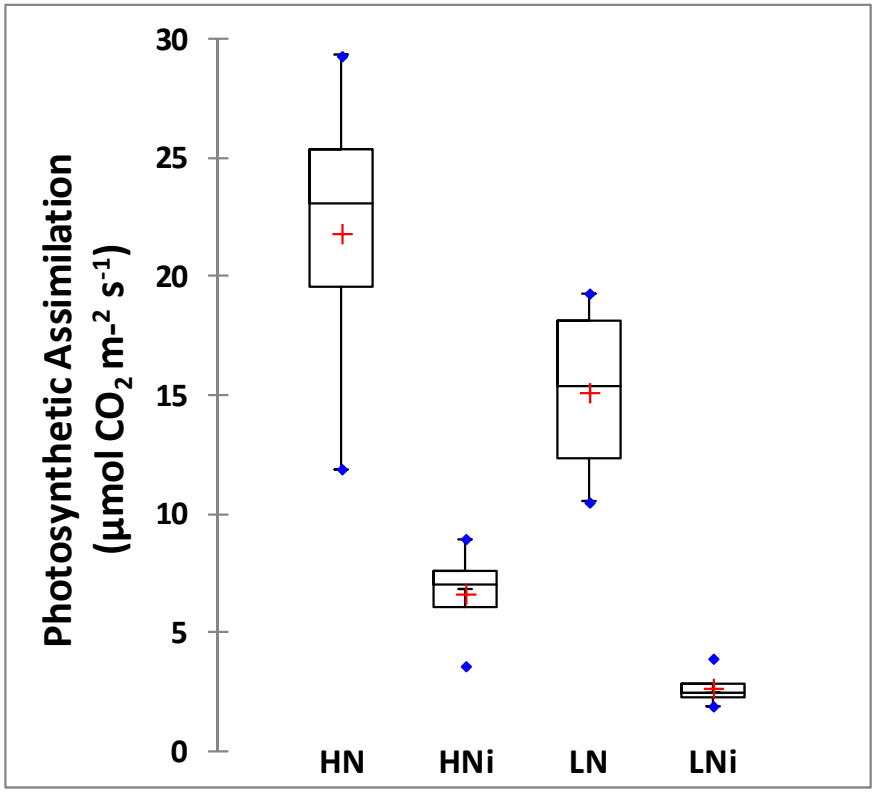


Figure 4.39 Photosynthetic assimilation or CO_2 uptake of healthy and infected (i) leaves on high N (HN) and low N (LN) fertilization rates (n = 16 leaves).

Infected leaves show a much lower rate of photosynthesis for both HN and LN plants. Leaf rust infection likely influenced the rate of photosynthesis in a number of ways. For example, there may be increased stomatal resistance brought about by an obvious hyphal growth in the mesophyll areas. The same hyphal growth could reduce light exposure, depriving photosynthesis of adequate photons. Finally, internal leaf water loss could have directly affected photosynthesis both through changes in stomatal conductance as well as changes in metabolic pathways.

4.3.3.1 CO_2 or ACi Response Curves

The ACi response curves of healthy and infected leaves showed a higher rate of CO_2 uptake or photosynthetic assimilation when exposed to increasing levels from 50 to 800 intercellular CO_2 (μmol_{CO2} $Mol^{-1}{}_{air}$) (Figure 4.40). However, it is very noticeable that infected leaves showed the lowest rate of CO_2 uptake and in general HN had higher values than LN for both healthy and infected leaves. There was a significant difference between healthy and infected leaves, with the risk to reject the null hypothesis is less than 0.11 % at $\alpha = 0.05$. There was also significant difference in the stomatal conductance between healthy and infected leaves. A few infected leaves of HN plants had dried quickly and could not be exposed to prolonged photosynthetic measurements. HNi leaves, that were not as far advanced in infection could withstand prolonged photosynthetic measurements.

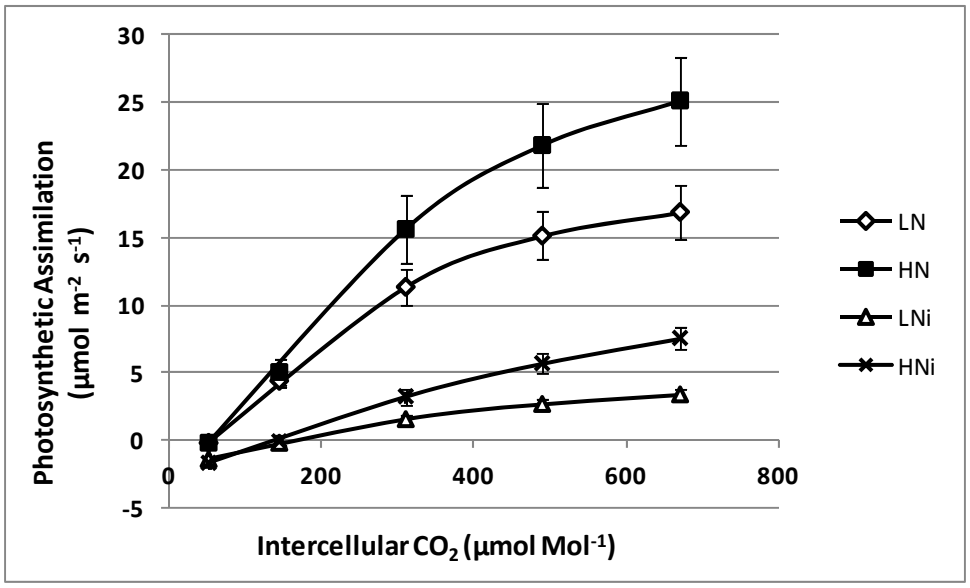


Figure 4.40 CO_2 or ACi response curve of healthy vs. infected (i) leaves of plants on high N (HN, HNi) and low N (LN, LNi) fertilization rates. Bars represent standard errors (n = 16 leaves).

4.3.3.2 PNUE, Leaf Rust Infection and Nitrogen Nutrition

The following hypothesis and alternative hypothesis are considered with reference to PNUE, N nutrition and leaf rust infection:

H_0: PNUE does not vary with leaf rust infection (infected vs. not infected) and N in SFS.

H_a: PNUE does vary with leaf rust infection (infected vs. not infected) and N in SFS.

It is possible to compare PNUE of healthy and infected leaves only because of the time constraints in analyzing NUE measurements (long and must be integrated) versus PNUE.

The results showed that the null hypothesis (H_0) is rejected and the alternate hypothesis (H_a) is accepted. PNUE of leaves varied with leaf infection in leaves of similar LPI or SFS in cottonwood hybrid 47-174.

The middle segments, Segments 2 and 3 had higher photosynthetic N use efficiencies (PNUE) compared to the top (Segment 1) and basal (Segment 4) segments of the high N (HN) plants. However in LN plants the top segments (SFS 1 and SFS 2) have high PNUE compared to the lower segments (SFS 3 and SFS 4) (Figure 4.41). Infected HN leaves has higher PNUE in SFS 1 and lower in the other segments, while infected LN leaves had lowest PNUE values in SFS 3. The R^2 regression value of variable PNUE = 0.84, p = 0.001 (ANCOVA) based on N fertilization, segments, and leaf rust infection. The association of high N fertilization rates with high PNUE was noted in SFS 2, 3, and 4, but not in SFS 1.

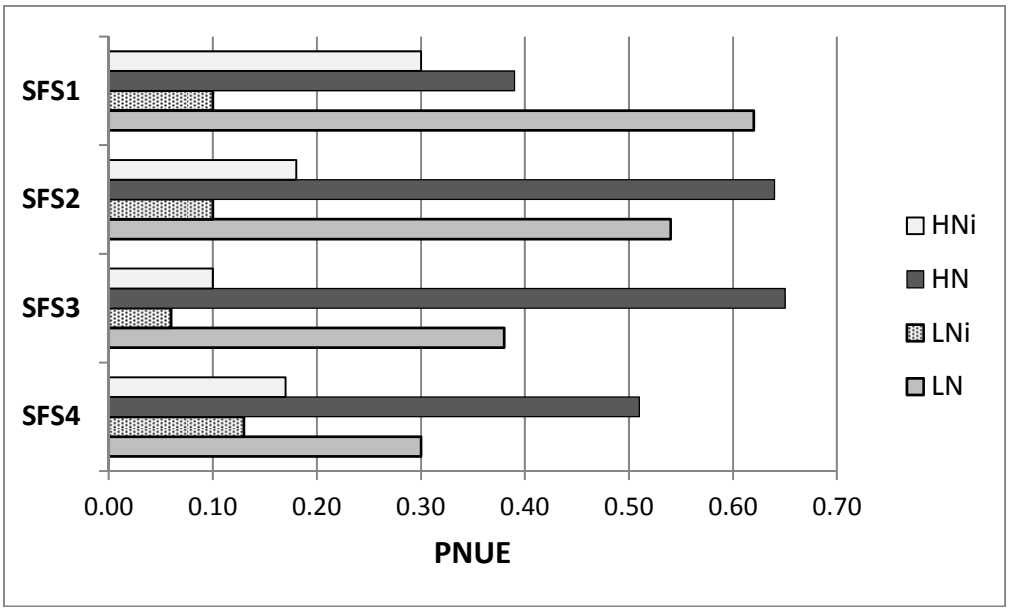


Figure 4.41 Photosynthetic N use efficiency (PNUE) of healthy high N (HN) and low N (LN) and infected (HNi and LNi) plants expressed as [(μmol CO_2 m^{-2} s^{-1}) $SPAD^{-1}$] of leaves in the 4 segments of a branch from the base (SFS 4) to the top (SFS 1) (n = 16 leaves).

4.3.3.3 CO_2 Compensation Point

The CO_2 compensation point (Γ) was determined from the A-C_i curves of the different LPIs. CO_2 compensation point is the point where CO_2 uptake = 0 or photosynthesis equals respiration, but not necessarily photorespiration (Lawlor and Cornic, 2002).

Figure 4.42 is a box plot of Γ values for healthy and infected HN and LN leaves. There is wider range of values and higher Γ values in LN plants; these same plants had lower maximum CO_2 uptake values and therefore have less efficient photosynthesis. There is no significant difference between HN and LN plants but there is significant difference between healthy and infected leaves (Table 4.10). The average Γ for healthy leaves is 50 μmol_{CO2} mol^{-1}_{air} (or ppm) while the average Γ for infected leaves is 123 μmol_{CO2} mol^{-1}_{air}.

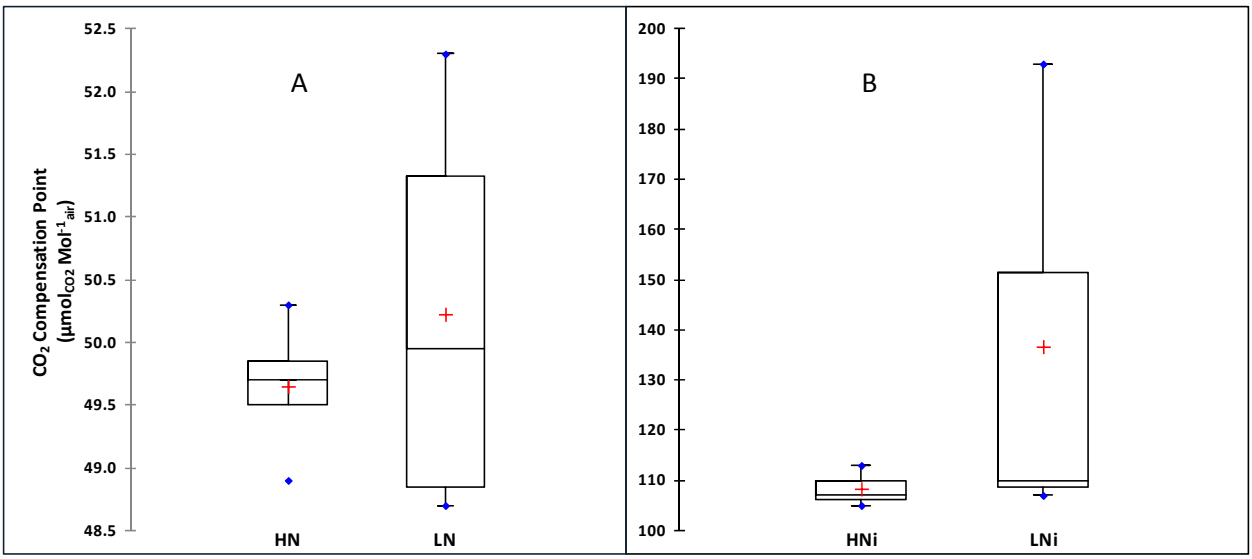


Figure 4.42 CO_2 compensation point values of healthy (A) and infected (B) LPIs of plants on high N (HN) and low N (LN) fertilization rates (n = 16 leaves, 2 plants).

Table 4.10 CO_2 compensation points ($\mu mol\ mol^{-1}_{air}$), mean ±SD, maximum, and minimum data of healthy leaves and infected leaves (n = 16 leaves, 2 plants, *statistically different at $\alpha = 0.05$).

Treatment	Number of Observations	Mean ± SD	Maximum	Minimum
Healthy	8	49.9 ± 1.2*	52.3	48.7
Infected	6	122.5 ± 36.7*	193.0	105.0

CHAPTER 5

SYNTHESIS AND CONCLUSION

The influence of two stresses and their interaction on the growth, morphology and photosynthesis of hybrid 47-174, a *Populus trichocarpa* x *P. deltoides* hybrid, was examined in this dissertation. Rooted cuttings were exposed between weeks 1 and 12 to N stress and were periodically exposed to leaf rust inoculum. From week 4 to 12, a combination of repetitive and period measurements was taken. In summary, 12 week-old plants exposed to higher levels of N had bigger leaves (124.5 vs. 59.0 cm^2), more leaves (32 vs 20), and, therefore, greater leaf area (3227.0 vs. 933.2 cm^2) than LN plants. In addition, they were taller (125.7 vs. 78.0 cm), had greater leaf and stem dry weights (10.68 vs. 3.91 g and 7.1 vs. 2.6 g , respectively), and diameters (9.4 vs. 6.3 mm). In addition, HN plants had greater rates of photosynthesis (21.8 vs. 14.5 μmol m^{-2} s^{-1}). Leaf infection by leaf rust suppressed photosynthesis and greatly shortened the life of a leaf. Almost all of the study's null hypotheses, that is, each stress and their combination would have no impact on the variables measured, were found to be unsupported by the data. These specific findings are summarized in the next three sections of this chapter. Sections on study shortcomings and future studies follow this.

5.1 Nitrogen Stress and Leaf Development Patterns

Figure 5.1 illustrates the influence of N stress on morphology, growth and photosynthesis of the four structural-functional segments (SFS) of hybrid cottonwood clone 47-174. As the results from this figure illustrate, it is important to identify the location (and, therefore, the developmental status) of the leaves from a given plant because the deciduous leaves of *Populus*

develop, mature, and undergo senescence rather quickly. Newly formed and rapidly expanding leaves of SFS 1 underwent further, but at greatly reduced rates as they transitioned to SFS 2. In contrast, SFS 3 were fully mature and typically demonstrated maximum leaf areas and photosynthesis. Leaves from SFS 4 were often demonstrating signs of senescence.

As shown in Figure 5.1, all the structural-function segments (SFS) from 1 to 4 have greater leaf area and greater leaf dry weight for HN compared to LN plants. On the other hand, Segments 2 to 4 have greater CO_2 uptake or assimilation (A) for HN as compared to LN plants; in contrast, SFS 1 for LN plants had greater A. SFS 2 to 4 for HN plants and SFS 1 for LN plants appeared to be the regions of the highest rates of photosynthesis. Others have demonstrated that foliar stresses (e.g., ozone) accelerated the rate of leaf physiological, but not necessarily morphological maturation (Reich, 1984). In general, HN plants have increased levels of morphological, growth, and photosynthesis measures compared to LN plants.

Photosynthetic assimilation (A) or CO_2 uptake was 35% greater in HN plants compared to LN plants. CO_2 uptake measurements of HN plants were 39%, 41%`, and 56% greater in SFSs 2, 3, and 4, respectively, as compared to their counterparts in LN plants. Previous measurements had A 58% greater in HN plants compared to LN plants (Banaag, 2006).

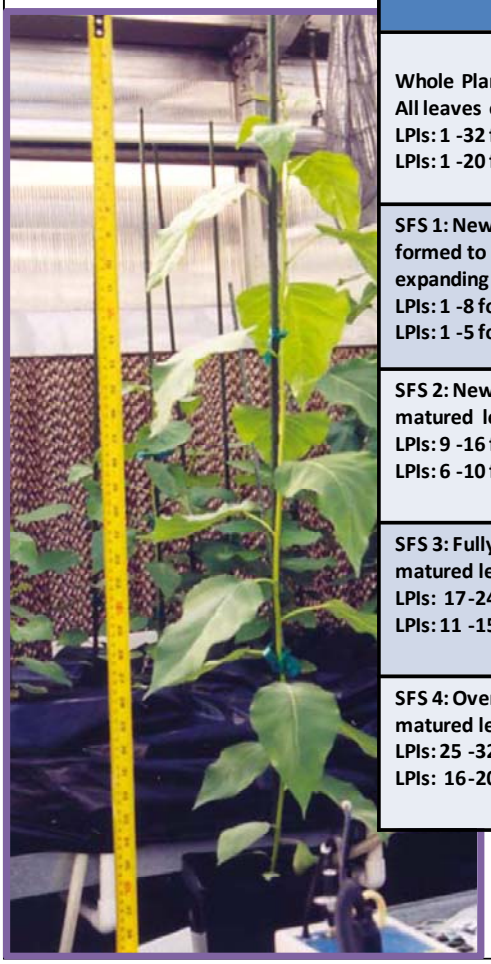
5.2 Nitrogen Stress and Growth Parameters

The following conclusions can be drawn from the influence of N stress on growth of hybrid cottonwood. N fertilization affects a number of growth parameters (Table 5.1).

1. There is rapid and consistent impact of N fertilization on plant growth as measured by an increased number of internodes (i.e., leaves) and distances between internodes. This resulted in more than a 61% increase in height in HN vs. LN plants (125.7 vs. 78.0 cm).

2. Total plant leaf area increases exponentially in both N treatments; however, the rate was much greater in the HN vs. LN plants (Figure 4.8: reaching means of 3227 and 933 cm 2, respectively, after seven weeks of observation). Total plant leaf area was significantly correlated with plant average N levels (from SPAD measurements) (Figure 4.9).
3. There is progression of leaf dry weight accumulation that showed higher values in HN plants compared to LN plants and, for both HN and LN plants, the greatest gains were in the middle segments of plants.
4. LN plants had fewer and smaller leaves and shorter internodes than HN plants.
5. There is increased stem dry weight (Table 4.8) and stem diameter (Table 4.7) on HN plants compared to LN plants.
6. N use efficiency (NUE) based on leaf dry weight and leaf area demonstrated strong differences based upon which SFS was considered. On a leaf area basis and for both HN and LN plants, the greatest NUE was noted in SFSs 1 and 2 (Figure 4.15) whereas on a leaf dry weight basis, NUE was greatest in SFS 2 and decreased in both directions from this segment (Figure 4.16).
7. NUE was greater for all SFSs in HN versus LN plants.

Most of the growth measurements focused on vegetative growth with an emphasis on above-ground plant characteristics. Data were not collected on root growth as plants were not grown separately in the containers. Because carbon allocation is known to change with N nutrition, having measures of root biomass and biomass increment would have provided a more comprehensive picture of the effects of fertilization on growth and allocation. Future work with root systems should use completely separated plants.



LPI/SFS		Nitrogen Stress (NS)		Leaf Rust Stress (RS)	Interaction* (NS x RS)
		HN	LN		
Whole Plant All leaves or LPIs LPIs: 1 -32 for HN LPIs: 1 -20 for LN	M	>Leaf Area > SD, >SPAD	< Leaf Area < SD, < SPAD	< Infection < SPAD	> Infection < SPAD
	G	> Plant Ht > Stem Diam	< Plant Ht < Stem Diam		
	A	> A, > PNUE	< A , < PNUE	< A	< A
SFS 1: Newly formed to expanding leaves LPIs: 1 -8 for HN LPIs: 1 -5 for LN	M	> Leaf Area > NUE, < SPAD	< Leaf Area		
	G	> Plant Ht	< Plant Ht		
	A	< A, < PNUE	> A, > PNUE	< A, < PNUE	< A, < PNUE
SFS 2: Newly matured leaves LPIs: 9 -16 for HN LPIs: 6 -10 for LN	M	> Leaf Area > NUE, > SPAD	< Leaf Area	> Infection	> Infection
	G	> InternodeL	< InternodeL		
	A	> A, > PNUE	< A	< A, <PNUE	< A
SFS 3: Fully matured leaves LPIs: 17-24 for HN LPIs: 11 -15 for LN	M	> Leaf Area > SPAD	< Leaf Area < NUE	> Infection	> Infection
	G	> Stem Dry Wt	< Stem Dry Wt		
	A	> A, >PNUE	< A	< A, <PNUE	< A
SFS 4: Over matured leaves LPIs: 25 -32 for HN LPIs: 16-20 for LN	M	> Leaf Area > SPAD	< Leaf Area < NUE		
	G	> Stem Diam	< Stem Diam		
	A	> A, > PNUE	< A	< A, <PNUE	< A

Figure 5.1This summary figure contains 7 parts. (1) At the far left is a photograph of a growing hybrid cottonwood plant. Immediately to its right is the (2) first column with five boxes that either consider (a) the whole plant with all its leaves or (b) the plant divided into four different structural and functional segments (SFS 1-4). Within each of these five boxes, the specific leaf plastochron indexes associated are indicated. (3) Next to the right is a column with the letters M, G, and A repeated (M – morphology, G – growth, A – assimilation). Continuing to the right are columns labeled N stress (4) – high N [HN] and (5) – low N [LN], (6) a leaf rust stress column and (7) an interaction column. Each response may show greater (>) or lesser (<) values. All responses are compared horizontally where the HN column is treated as the control. A – assimilation or CO_2 uptake, NUE – N use efficiency, PNUE – photosynthetic N use efficiency, SD – stomatal density, and SPAD – the device that infers N concentration via its measurement of chlorophyll content. *< - Impact due to interaction between the two stresses (LN and leaf rust) was greater than that observed for either stress singly; that is, the value was lower.

Table 5.1 Percentage values based on various measurements of plants on high N (HN) compared to plants on low N (LN) fertilization rates and healthy (HN, LN) compared to leaf rust infected leaves (HNi, LNi).

Variable	Percentage Difference	Not rust infected	Not vs. rust infected
	Morphology		
Leaf Area	53%	HN > LN	
Stomatal density	18%	HN > LN	
Stomatal index	30%	HN > LN	
	Growth		
Leaf dry weight	49%	HN > LN	
Height	38%	HN > LN	
Internode length	26%	HN > LN	
Stem diameter	33%	HN > LN	
Stem dry weight	63%	HN > LN	
	Physiology		
Leaf nitrogen	17%	HN > LN	
CO_2 uptake (A)	35%	HN > LN	
CO_2 uptake (A)	75%		HN > HNi
CO_2 uptake (A)	82%		LN > LNi

5.3 Leaf Rust Stress and the Host-Pathogen System

The study also generated important new as well as supportive data regarding the influence of leaf rust stress on morphology and photosynthesis of cottonwood hybrid 47-174 (Table 5.1). In general, HN plants had larger leaf area and greater stomatal density and stomatal indices than LN plants. Photosynthetic assimilation (A) or CO_2 uptake was greater in healthy HN plants by 75% and in healthy LN plants by 82% than infected plants, respectively.

Establishment of an experimentally successful host-pathogen infection-system completely dominated by leaf rust occurred over a short time span of 2 weeks or 14 days post inoculation (Figure 5.2). This uniformly successful infection-complex was observed in both HN and LN plants within some morphological characteristics unique to plants from each N level. The most dramatic effect of infection, as demonstrated by scanning electron microscopy (SEM), was the process of sporulation where the uredia with young to mature urediospores tear through the epidermis and erupt on the surface. In an extensively infected leaf, this disruption caused so much damage (i.e., direct exposure of the inside of the leaf) that by the beginning of the third week following inoculation the leaf had dried up, curled and died. Defoliation occurred soon thereafter. This was most pronounced for HN plants.

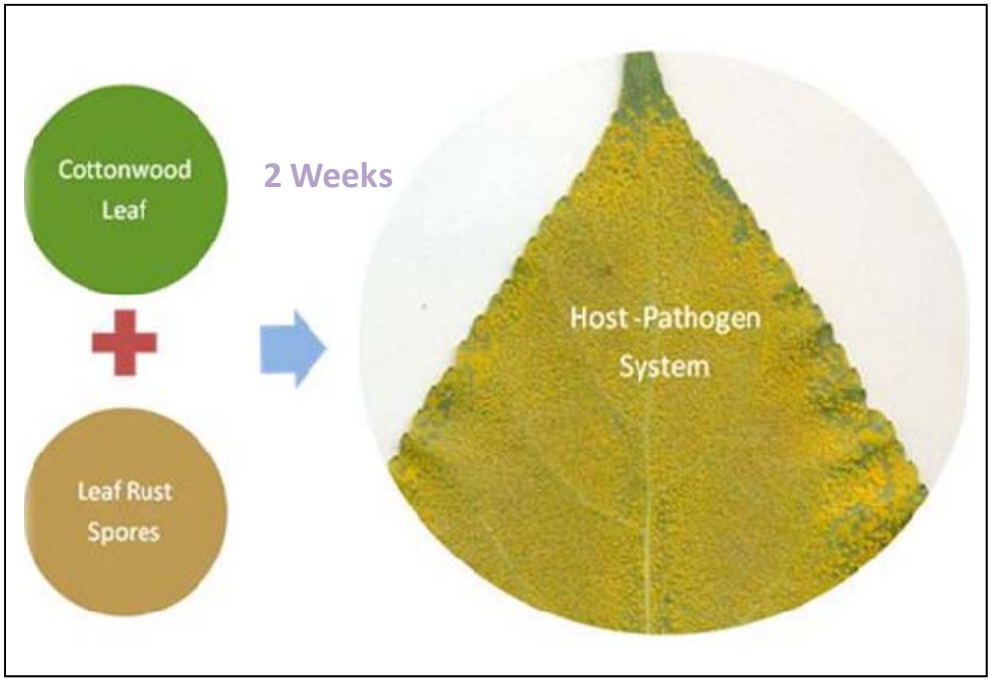


Figure 5.2 The hybrid cottonwood leaf host is sensitive and easily favored the initial establishment of leaf rust spores leading to the eventual prolific growth of the leaf rust thus forming a host-pathogen infection-complex in 2 weeks.

The deleterious effect of leaf rust stress was observed, but not uniformly, in all 4 structural-functional segments of the plant. Over-matured leaves (i.e., from SFS 4 or those about to senesce) appeared to be better able to tolerate leaf rust stress. In contrast, less developmentally mature foliage was both more sensitive to infection and, as a result the speed of infection and leaf death were faster leading to insufficient time to withdraw nutrients (resorption) before abscission (Wright and Cannon, 2001). Such developmental differences in ability to tolerate abiotic and biotic stresses have been described by Marron, et al., (2008). The pre-mature defoliation eliminated leaves that have not reached their full potential for photosynthesis (Shaw and Kile, 1991).

Nitrogen stress affected the durability of leaves to leaf rust infection. LN plants developed smaller and thicker leaves that were sturdier and more durable when leaves were infected. In contrast, HN plants developed larger and thinner leaves but were less able to survive when infected. In fact, HN leaves that were extensively infected tended to dry and wither on or before the 15th day post inoculation compared to LN infected leaves that remained viable for sometimes as long as 22 days post inoculation. In both cases, there was a remarkable tearing of the epidermis and associated epidermal cells and neighboring stoma complex when the leaf rust uredium erupted from the mesophyll. LN leaves were able to persist longer than high N leaves perhaps as a result of a combination of differences including leaf morphology and the speed of leaf rust and uredia development. Finally, leaf rust infection severely impacted net carbon exchange (A) in both HN and LN supplied plants (Table 5.1).

5.4 Experimental Concerns

In spite of the general clarity of the results noted, three experimental concerns remained. The first deals with the size of the cutting used. The cutting came from a 5 cm-long section of

one of several main coppiced stems. It was sufficiently long to have at its top a bud initial and then a sub-tending section also long enough to hold the stem in place when planted in the rooting soil medium. It was definitely smaller than the 20 cm-long cuttings used by Marron, et al. (2008). It was also felt that such a short cutting increased the likelihood of greater uniformity in initial growth and decreased the presence of stored N (e.g., Cooke and Weih, 2005). The second concern is partially related to the first in that one assumes that the source of the cutting, its size and the time that it is taken assure uniformity in response. Replicationed and repeated experiments that are initiated during different times of the growing season or different years may also not assure initially uniform material for study. For each of these concerns, a combination of a series of preliminary studies and care were developed so that these concerns were greatly lessened during the final series of experiments.

5.5 Procedural Contribution

There were four procedural developments of sufficient significance to be documented here. The first involved the successful and consistent rearing of material for the various study experiments. Five cm cuttings were taken from selected uniform sections of coppice stems grown in the field. These cuttings were then rooted in a misting bench, selected again for uniformity, transplanted and grown in individual pots. They were then randomly assigned to treatments. The second involved the use of a modified Ingestad system for supplying nutrients in a highly repeatable and controlled setting to the study material (Ingestad, 1977; Ingestad, 1979; Ingestad and Lund, 1986). Experimental cuttings were exposed to the two different N supply regimes immediately following transplanting. The third important procedural result involved demonstrating the effectiveness of using representative foliage from four structural-functional segments (SFS) along a growing shoot in contrast to sampling every leaf according to its leaf

plastochron index (LPI). In order to use a SFS system, preliminary studies were necessary where morphological and physiological measurements were taken from every leaf using the LPI scheme —once these patterns were understood, then segments and their transitions could be identified and justified. This comparison was done for high and low N plants. In both cases, the utility of using SFS was demonstrated. The fourth contribution featured an inoculation system that resulted in rapid, relatively uniform and extensive rust infection that made good study material for direct measurements of photosynthesis during leaf rust infection, a direction for future study suggested by Major et al (2010). Unfortunately, this successful system precluded understanding how N nutrition might affect leaf rust development under field conditions.

5.6 Future Research

A number of investigators have described many of the physiological and morphological attributes likely resulting in hybrid vigor as noted in many of the cottonwood hybrids developed in the Pacific Northwest including hybrid 47-174 (e.g., Stettler, et al., 1996); however, to date, there have not been corresponding biochemical studies to address hybrid vigor. For example, is there an equivalent concentrating mechanism in hybrid cottonwoods that makes CO_2 easily accessible to the photosynthesizing unit (i.e. specifically to Rubisco) as noted by Millard et al. (2007) in some woody shrubs and small trees with C_4 photosynthesis? Results in this study did not demonstrate enhanced photosynthesis in HN vs. LN plants based upon light curves generated at ambient CO_2 levels. In contrast, A-Ci curves from HN plants did show greatly elevated rates of CO_2 fixation vs. those from LN plants. The entire photosynthetic system in hybrid plants deserves more study whether from the stand point of reduced mesophyll resistance to carbon dioxide movement to sites of utilization or changes in source – sink dynamics leading to changes

in feedbacks on Calvin cycle enzyme dynamics (Mayrhofer, et al., 2004). These are suggestions to supplement the photosynthetic expression of genes presented by Major et al. (2010).

It would also be interesting to observe leaf rust growth patterns in field plants. Future research could include responses that may arise if N were added in excess amounts in the form of luxury consumption (Heilman and Xie, 1993, Tripler, et al., 2002). During previous trials (Banaag 2006), field grown one-year-old material would not support a sufficient number of spores to collect for subsequent infection of laboratory plants. Older field grown material was much more susceptible. Perhaps the period of leaf susceptibility for infection was not in synchrony with the period of greatest activity of the leaf rust spores (Marschner, 1986). In some years, high rust incidence was observed in the source plants as was reported in other locations by Widin and Schiffer (1981) and Wang and van der Kamp (1992). Clearly a better understanding of host-pathogen dynamics including the role of the over-wintering host is needed. And perhaps the effect of N fertilization on leaf rust infection may be more observable in resistant clones, where reduced N levels may prevent the availability of nitrogen-related protein systems that aid in resistance reactions and therefore may exhibit an increase in leaf rust infection because of less than 100% resistance to leaf rust infection.

There are two important take home messages for those involved in the commercial use of hybrid cottonwoods. N nutrition and absence of rust infection are both clearly critical for optimum productivity. The fact that there is an interaction between N nutrition stress and leaf rust stress suggests both additional laboratory and field studies.

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APPENDICES

Appendix A Nutrient Composition

Table 1 Composition of Solution A and Solution B that were kept in separate containers and kept refrigerated (Johnson, personal communication).

Salt	Moles	Mo. Wt.	g2L
Ingestad Solution A			
NH_4NO_3	1.25	80.05	100.0625
KNO_3	0.76	101.10	76.8360
KH_2PO_4	0.23	136.09	31.3007
K_2HPO_4	0.25	174.18	43.5450
K_2SO_4	0.28	174.26	88.7928
Ingestad Solution B			
$Ca(NO_3)_2$*4-hydrate	1.12	164.10	183.7920
$Mg(NO_3)_2$*hydrate	0.82	148.32	121.6224
$Fe(NO_3)_2$	0.0125	241.87	3.0234
$Mn(NO_3)_2$	0.0073	178.95	1.3063
H_3BO_3	0.0185	61.84	1.14440
$Zn(NO_3)_2$	0.00092	189.38	0.1742
$Cu(Cl_2)$	0.00047	134.45	0.0632
$Na_2(MO)O_4$	0.000073	205.92	0.0150

Appendix B Nitrogen Fertilization Addition Rates (6% and 1.5%)

Table 2 Amount of Ingested Solution A and Ingested Solution B added per week (Johnson, personal communication).

Week	6% Addition Rate		1.5% Addition Rate	
	ml Sol. A*	ml Sol. B*	ml Sol. A*	ml Sol. B*
1	1.20	1.20	1.20	1.20
2	1.80	1.80	1.35	1.35
3	2.70	2.70	1.45	1.45
4	4.10	4.10	1.65	1.65
5	6.10	6.10	1.80	1.80
6	9.20	9.20	2.00	2.00
7	13.85	13.85	2.25	2.25
8	20.85	20.85	2.50	2.50
9	31.35	31.35	2.75	2.75
10	57.15	57.15	3.25	3.25
11	132.50	132.20	4.20	4.20
12	241.00	241.00	4.65	4.65
13	362.35	362.35	5.15	5.15

*If plants are removed from a container, the volume of nutrients is prorated: e.g., if at week 10, 2 plants are removed, add only 4/6 x 57.15.

Appendix C More Scanning Electron Microscopy

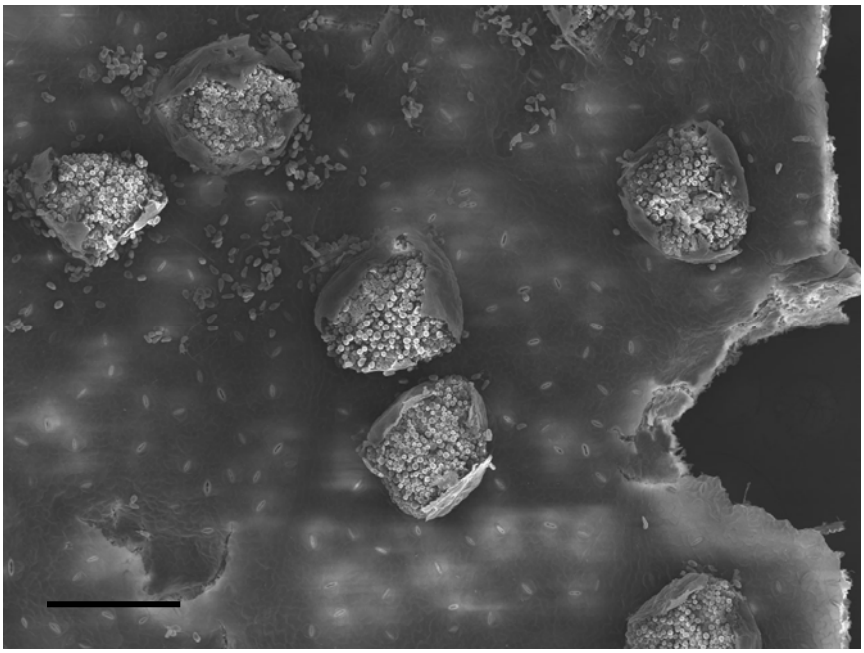

Figure 1 Uredia breaking out of the epidermis. Stomata are also seen as part of the epidermis (bar = 300 μm).

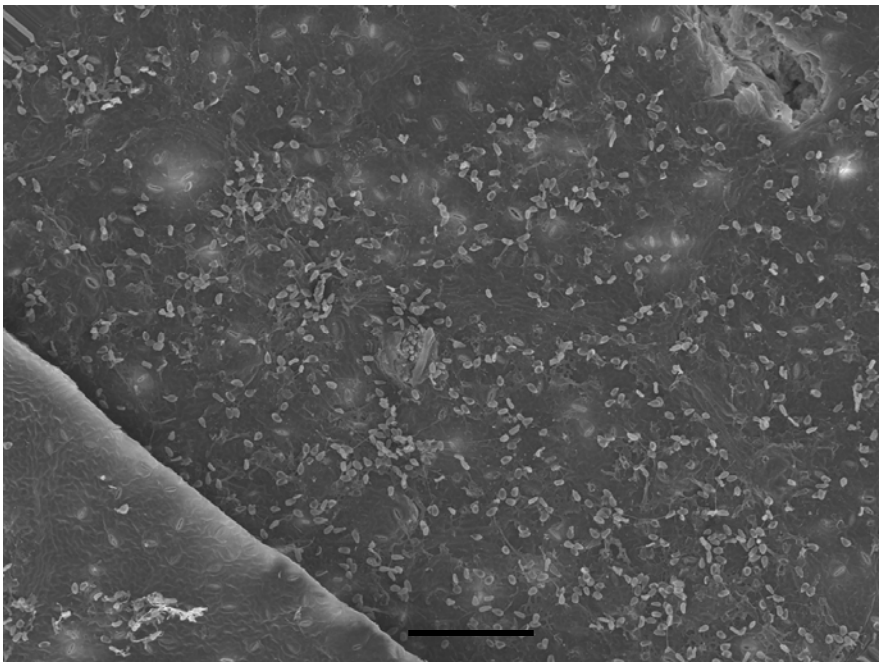

Figure 2 Spores scattered on the surface of a leaf that was air-dried in a dessicator. One uredium in the middle is starting to break through the epidermis (bar = 300 μm).

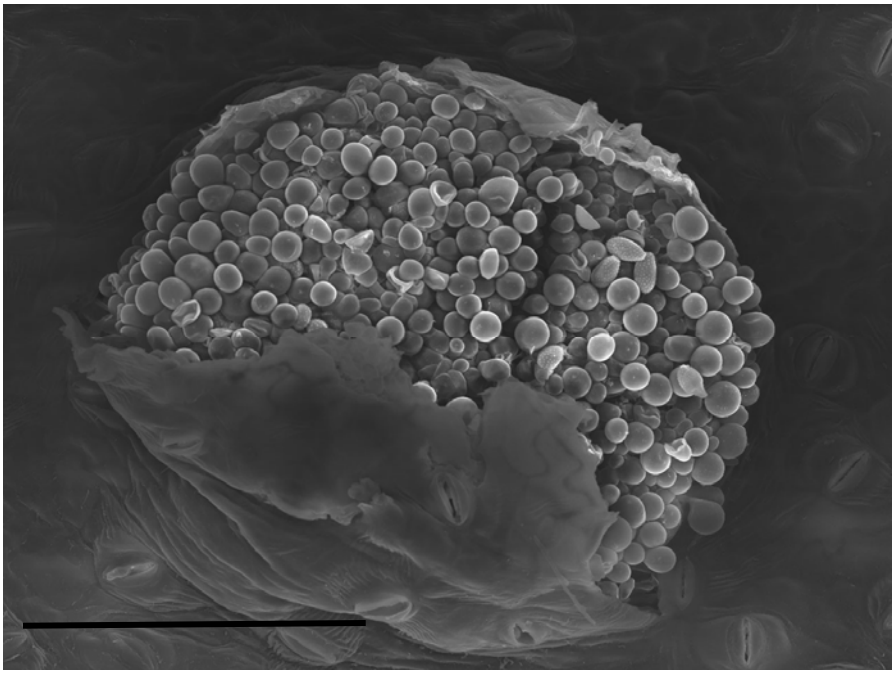

Figure 3 A uredium showing the epidermis and stomata still draped over it after breaking through (bar = 150 μm)

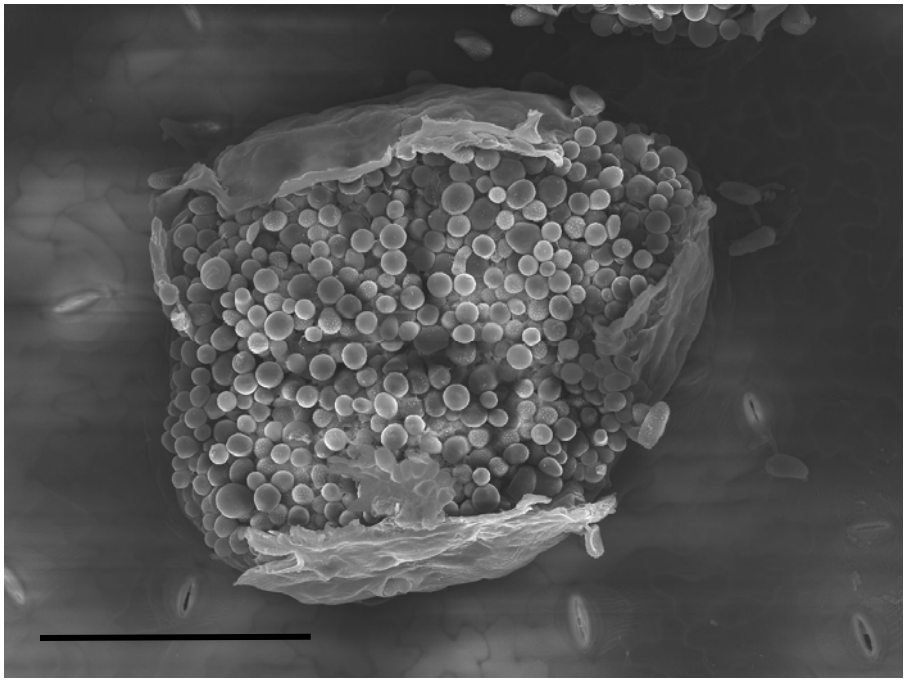

Figure 4 Another uredium showing the numerous urediospores developing as it emerged through the epidermis (bar = 150 μm)

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