THE EFFECTS OF INCREASED SYRINGYL TO GUAIACYL LIGNIN MONOMER RATIOS ON XYLEM HYDRAULIC PROPERTIES IN HYBRID POPLAR (*POPULUS TREMULA X P. ALBA*)

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ABSTRACT

THE EFFECTS OF INCREASED SYRINGYL TO GUAIACYL LIGNIN MONOMER RATIOS ON XYLEM HYDRAULIC PROPERTIES IN HYBRID POPLAR (*POPULUS TREMULA X P. ALBA*)

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The goal of this research was to examine how changes in the syringyl to guaiacyl (S:G) lignin monomer ratio will affect the conductive efficiency and resistance to embolism in hybrid poplar. I used two lines of hybrid poplar clone 717 (*Populus tremula* x *P. alba*) over-expressing the *Arabidopsis* gene encoding ferulate-5-hydroxylase (F5H) which resulted in an increased S:G ratio in xylem tissue. The two lines selected for use, F5H37 and F5H64, expressed 88% and 94% syringyl, respectively, compared to 66% syringyl expressed in wild type (WT) plants. To quantify the effects of increased S:G on hydraulic properties, two studies were performed. First, the hydraulic conductivity and resistance to embolism of F5H and wt lines were tested under well watered and water stress conditions. The goal of the second experiment was to examine the response of the F5H and wt lines to winter freeze-thaw stress and spring recovery. Results from this research show that increasing S:G has no significant effect on the hydraulic conductivity of xylem, but lines with increased S:G were observed to be more vulnerable to cavitation. Data for pressure at 75% embolism (P_{75}) showed that F5H64 was approximately 75% more vulnerable to cavitation and in 2009 F5H37 was twice as vulnerable to cavitation as the WT. No significant differences were found between lines in response to drought and winter freeze-thaw stress.

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CHAPTER 1

INTRODUCTION

Lignin is a complex, hydrophobic polymer that functions as a key cell wall component in vascular plants. One of the most abundant organic compounds in the world, lignin comprises approximately 1/3 of dry wood biomass. Occurring mainly in the secondary cell wall of xylem tissue, lignin contributes to the mechanical and hydraulic properties of the cell wall (Campbell and Sederoff, 1996). Lignin has also been suggested to provide protection from pathogens (Bruce and West, 1989; Hammerschmidt, 1984; Hijwegen, 1963).

Lignin can be composed of three monomers: syringyl (S), guaiacyl (G), and *p*-hydroxyphenol (H). The composition and deposition of these monomers can be highly variable across taxa (Campbell and Sederoff, 1996; Towers and Gibbs, 1953), as well as, within a species (Zobel and van Buijtenen, 1989). Lignin in Gymnosperms primarily contain guaiacyl and, to a lesser extent, *p*-hydroxyphenol. Angiosperm lignin is commonly composed of the guaiacyl and syringyl monomers (Barcelo et al., 2004; Campbell and Sederoff, 1996; Towers and Gibbs, 1953). This variation in composition of lignin monomers has also been observed between different cell types and tissues. For example, fiber cells tend to have a higher ratio of S to G (S:G) lignin, whereas vessel elements contain more guaiacyl and less syringyl (Campbell and Sederoff, 1996; Donaldson, 2001; Whetton and Sederoff, 1995; Yoshinaga et al., 1992). Environmental conditions have also been found to affect the composition of lignin (Donaldson, 2001; Donaldson, 2002).

Lignin's Role in Xylem Evolution

The evolution of the lignin biosynthetic pathway was an important step in the evolution of vascular land plants (Kenrick and Crane, 1997; Sperry, 2003). The variation in lignin deposition and content described previously has some possible implications for the overall evolution of the structure and function of xylem. The wood of Gymnosperms and primitive Angiosperms is composed of tracheids which fulfill the dual function of mechanical support and water conduction (Carlquist, 2009). Higher Angiosperms evolved distinct cell types, fiber cells and vessel elements that separately carry out the functional roles of support and water movement, respectively. Vessels, composed of multiple vessel elements, can achieve greater lumen diameter and length than tracheids. The greater diameter and length of vessels facilitates decreased resistance to the water flow and increased maximum hydraulic conductivity per wood area (Sperry, 2003; Sperry et al., 2007).

While the evolution of xylem structure and architecture has been examined extensively from a functional and ecological perspective (Bhaskar et al., 2007; Carlquist, 1975; Carlquist, 2009; Meinzer et al., 2010; Sperry, 2003; Sperry et al., 2008), there has been very little work on the role of cell wall chemistry, specifically lignin composition, in the evolution of xylem traits or how xylem evolution affected the deposition and composition of lignin (Boyce et al., 2004). Yoshinaga et al. (1992) observed that the proportion of syringyl present in the cell wall is higher in cells that function in mechanical strength compared to those that function in water conduction. This suggests that syringyl may be better suited to providing mechanical support to the cell wall and

guaiacyl functions more in conduction of water and protection from cavitation. Definitive experimental evidence for the interaction between the evolution of lignin biosynthesis and xylem structure may be difficult to obtain. Differences in anatomy and lignin composition between and within species may confound results. Yet, advances in the genetic modification of the lignin biosynthetic pathway have provided new opportunities to experimentally test the function of lignin monomers and their role in the evolution of xylem anatomy.

Xylem function and the role of lignin

In vascular plants, water is transported from the roots to the leaves through xylem tissue. The accepted method by which water is conducted through a plant is known as the cohesion-tension theory, first proposed by Dixon and Joly (1894). Transpiration at the leaf surface creates the tensional force that drives water movement through the stem (Tyree, 1997). Xylem conduits must be able to resist the negative pressures created. The mechanical strength of xylem tissue has been suggested to contribute to the hydraulic properties of xylem through regulating the vulnerability of trachiery elements to collapse (Donaldson, 2001; Jacobsen et al., 2005; Raven, 1977; Sperry, 2003). Lignin provides structural support to the conduit cell wall providing resistance to the negative pressures (Donaldson, 2001; Sperry, 2003). Fiber cells have also been shown to have a role in protecting vessel elements from implosion (Jacobsen et al., 2005). Failure of the conductive tissue to resist high negative pressures can result in collapse of the conduit walls, leading to cavitation. Formation of gas bubbles, or embolism, that cause blockage to the flow of water within conduits can result from

cavitation. Air-seeding, where gas is pulled into the conduits from neighboring gas-filled cells or intracellular spaces has also been hypothesized to cause embolism (Tyree and Zimmerman, 2002; Sperry and Tyree, 1988). Microfractures in the vessel walls, caused by high negative pressures or weakened cell walls, may also initiate nucleation of gas bubbles forming embolism (Jacobsen et al., 2005). The vulnerability of conduits to cavitation due to water stress has been traced to the permeability of intervessel pit membranes to gas diffusion (Sperry et al., 1991; Sperry and Tyree, 1988). While embolism impairs the flow of water through the conduit, embolism can be reversed (Shen et al., 2007) and may also play a developmental role in the formation of heartwood (Sperry et al., 1991).

The interplay between hydraulic efficiency and mechanical safety of the xylem has received consideration (Wagner et al., 1998; Hacke et al., 2001; Woodrum et al., 2003; Jacobsen et. al., 2005; Kern et al., 2005; Pittermann et al., 2006; Meinzer et al., 2010). It has been hypothesized that tradeoffs may occur between mechanical strength and hydraulic conductive efficiency at the tissue level (Jacobsen et. al., 2005; Wagner et al., 1998; Pittermann et al., 2006; Woodrum et al., 2003; Kern et al., 2005). While tradeoffs were observed in species of conifer (Pittermann et al., 2006), evidence for the existence of tradeoffs in angiosperm species is less conclusive (Wagner et al., 1998; Woodrum et al., 2003; Jacobsen et. al., 2005; Kern et al., 2005). The hydraulic conductivity of the stem can be influenced by frequency and diameter of conduits. According to Poiseuille's law, an increase in conduit lumen diameter will result in an increase in the hydraulic conductivity proportional to the summation of the lumen diameters to the fourth power (Ewers, 1985; Tyree and Zimmerman, 2002; Wagner et

al., 1998). While an increase in vessel diameter will result in an increase in specific conductivity, ks, this may result in decreased frequency of fiber cells, for example, in Lianas.

The Ligno-Cellulose Resource

Wood biomass represents a significant industrial resource. In 2009, global production of industrial roundwood totaled at approximately 1.424 billion cubic meters (http://faostat.fao.org/). This number actually falls short of previously projected trends in the growth of industrial wood production (Whiteman and Brown, 1999) and represents the lowest yearly production in the 2000s (http://faostat.fao.org/). Utilization of wood biomass through the harvesting of cellulose for paper products and biofuel production is impeded by the presence of lignin. Removal of lignin from wood is accomplished through the chemical (kraft) pulping process and must be performed through the use of environmentally harmful chemicals and bleaching. Global production of chemical wood pulp in 2009 was reported at 118,669,440 tons (http://faostat.fao.org/).

Advances in our understanding of the lignin biosynthetic pathway and breakthroughs in genetic manipulation of the pathway has opened up new opportunities for addressing the problems of chemical pulping (Li et al., 2008; Nehra et al., 2005; Simmons et al., 2010). A number of strategies for increasing the efficiency of industrial pulping through genetic modification of the lignin biosynthetic pathway have been attempted. These strategies include reducing the total amount of lignin content within the wood, and manipulation of lignin monomer ratios, whether through increased production of syringyl lignin or reduction in guaiacyl lignin (Baucher et al., 2003; Huntley

et al., 2003; Li et al., 2003; Li et al., 2008; Meyer et al., 1998; Nehra et al., 2005; Pilate et al., 2002; Stewart et al., 2006).

Increasing the lignin S:G ratio in poplars has been shown to positively impact the efficiency of chemical pulping (Huntley et al., 2003; Stewart et al., 2006). While lowering the total lignin content in the wood could make removal of the lignin easier, reduction to total lignin content also causes significant impacts on the physiological and morphological properties of the trees (Coleman et al., 2008; Kitin et al., 2010; Pilate et al., 2002; Voelker et al., 2011). A similar response to reduced lignin content has also been observed in non-arborescent plant species, such as *Arabidopsis* (Franke et al., 2002) and *Nicotiana* (Hepworth and Vincent, 1999). The use of genetically modified hybrid poplars has been suggested as a viable alternative for use in paper manufacturing and biofuel production (Baucher et al., 2003; Li et al., 2008; Pilate et al., 2002; Simmons et al., 2010). Modification of the lignin biosynthetic pathway to alter the ratio of lignin monomers present in the xylem tissue makes the harvesting of cellulose through pulping more efficient (Huntley et al., 2003; Pilate et al., 2002). However, it is important to examine how changes to lignin biosynthesis within the plant will affect the plant's physiological and ecological functioning.

Over-expression of the F5H gene results in an increased syringyl to guaiacyl (S:G) ratio but no changes to the overall amount of lignin in the plants (Humphreys et al., 1999; Franke et al., 2000). Over-expression does not compromise conduit function in the form of collapsed vessel elements seen in other plants with reduced overall lignin content (Franke et al., 2002; Kitin et al., 2010; Voelker et al., 2011; Zhong et al., 1998). Huntley et al., 2003, reports that there were no significant differences in the ratio of

fibers to vessels, average fiber lumen area, vessel lumen area, or cell wall thickness between wild type and hybrid poplars (*Populus tremula x P. alba*) over-expressing C4H-F5H. However, Horvath et al. (2010) found that up-regulation of CAld5H in *Populus tremuloides* Michx. resulted in decreases to stem diameter, vessel lumen diameter, and fiber length. However, vessel number and vessel lumen area fraction increased in the CAld5H mutants, though. This may suggest a response towards narrower vessel lumens to account for a change in mechanical strength of the cell wall due to the increase in the syringyl to guaiacyl ratio. How the change in vessel and fiber area affected the overall mechanical strength and hydraulic properties of the wood was not reported (Horvath et al., 2010).

Recent research has shown that reduction in total lignin content can have a drastic effect on anatomical and physiological traits, including reduced growth (Coleman et al., 2008; Voelker, 2009; Voelker et al., 2010), and increased occurrence of vessel collapse (Coleman et al., 2008; Franke et al., 2002; Kitin et al., 2010; Voelker et al., 2010; Zhong et al., 1998). Poplars with reduced lignin content were also found to have decreased hydraulic conductivity and increased vulnerability to embolism (Coleman et al., 2008; Voelker, 2009).

The genetic manipulation of the lignin biosynthetic pathway also provides an opportunity for improved experimentation on lignin's role in xylem functioning and the effects of altering lignin composition on growth and physiology. Our understanding of the role of lignin biochemistry on functional traits of the xylem is still lacking (Koehler and Telewski, 2006). There is some evidence that cell wall lignification affects ion-mediated changes in sap flow rate (Boyce et al., 2004). Previous research on lignin's

role in xylem function has had to rely on comparing variations in mechanical and hydraulic traits across different genera and species. Results of these experiments can be confounded by anatomical and developmental differences between species (Koehler and Telewski, 2006).

The overall goal of this study is to assess the role of S and G lignin monomers with respect to water transport and tree physiology, and how changes to S:G will affect the plant's response to environmental stress, specifically drought and freeze-thaw stress. This research will also assess the viability of F5H transgenic hybrid poplars for use in economic forest plantations. Previous studies have examined how increasing the S:G ratio can influence the efficiency of pulping methods and there is mixed evidence towards how genetic modification of the C4H-F5H pathway could result in changes to anatomical morphology of the transgenic wood (Huntley et al., 2003). However, attempts to experimentally analyze how changes in the S:G ratio affects mechanical and hydraulic functions within the wood have not been presented (Koehler and Telewski, 2006). To assess the viability of S:G mutants, this thesis attempts to provide more experimental evidence for lignin's role in the functioning of xylem tissue and how the ratio of lignin monomers within the cell wall contributes to this.

The research presented in the proceeding chapters uses the hybrid poplar clone INRA 717-1B4 (*Populus tremula x p. alba*) over-expressing the *Arabidopsis* gene encoding ferulate-5-hydroxylase (F5H), driven by the cinnamate-4-hydroxylase(C4H) promoter. Chapter 2 presents results from greenhouse experiments on the response of F5H transgenic poplar to water stress. This experiment includes quantitative analysis of

the hydraulic conductivity, vulnerability to embolism, and gas-exchange properties, including transpiration, stomatal conductance, leaf water potentials, and chlorophyll fluorescence. Changes to the structural properties of the vessel elements and fibers could compromise the plant's ability to efficiently conduct water and resist increasing tension forces on the cell wall caused by transpiration. This could also lead to changes in the plant's ability to carry out other important developmental, biochemical, and physiological functions. The effects of water stress on physiological properties are well understood (Kramer, 1983; Larcher, 2003). Reduced water transport can result in decreased cell turgor pressure, stomatal closure, and inhibit photosynthetic rates (Epron et al., 1992; Hacke and Sauter, 1995; Hubbard et al., 1999; Brodribb and Field, 2008). Irrigation regimes have been found to be an important contributor towards traits involved with growth and productivity in poplar (Shock et al., 2002; Tschaplinski et al., 2006). This could have important implications towards the commercial use of transgenic poplars. Chapter 3 examines the effects of increased S:G ratios on F5H mutants' tolerance to winter freeze-thaw stress. Hydraulic conductivity and vulnerability to embolism were measured in wild type and transgenic lines during mid-winter and early spring. Possible mechanisms for recovery from freezing induced embolism in poplar are also considered. Experimental evaluation of mechanisms for freezing induced embolism in *Populus* has been addressed in a number of previous experiments (Hacke and Sauter, 1996; Sperry and Sullivan, 1992; Sperry et al., 1994). Recovery from winter induced embolism has been associated with positive xylem pressures in diffuse porous tree species (Hacke and Sauter, 1996; Sperry, 1993). Yet, *Populus* species do not demonstrate similar trends toward xylem refilling (Sperry et al., 1994) and vulnerability

to freezing induced embolism (Hacke and Sauter, 1996). Anatomical analyses of the F5H and wild type lines, including quantification of the active sapwood area of the samples, were examined. However, this data is still being analyzed and will not be presented at this time.

Summary

Genetic modification of the lignin biosynthetic pathway can provide a novel avenue for addressing the increased need for more efficient and environmentally friendly pulping processes for the wood products industry. It also provides a unique opportunity to study the role of lignin and monomer composition in xylem function. Xylem functional traits and physiological properties must be tested in order to fully evaluate the viability of modifying the lignin biosynthetic pathway in plants' for economic use. This study presents results on experiments examining the water relations of transgenic hybrid poplars over-expressing the F5H gene in response to environmental stress. These results provide a better understanding of lignin's role in the functioning and evolution of the hydraulic properties of xylem.

CHAPTER 2

HYDRAULIC CONDUCTIVITY AND VULNERABILITY TO CAVITATION IN HYBRID POPLAR (*POPULUS TREMULA X P. ALBA*) WITH INCREASED SYRINGYL TO GUAIACYL LIGNIN MONOMER RATIOS

Introduction

In woody vascular plants, water is transported from the root system to leaves through the secondary xylem tissue. Along with providing water transport, the secondary xylem also provides mechanical support to the plant. At the cellular level, this dual function of water transport and mechanical support are fulfilled by tracheids in Gymnosperms, and vessel elements and fiber cells, respectively, in higher Angiosperms. Water movement through the tracheids or vessels present within the xylem tissue is driven by transpiration at the leaf surface. Transpiration creates the tensional force that pulls the water column upward through the stem (Tyree and Ewers, 1991; Tyree, 1997). Cavitation of the water column can occur when negative pressure within the conduits becomes too high. This can result in collapse of the xylem conduit walls, and embolism, formation of gas bubbles, which cause blockage to the flow of water through the conduit. Embolism can occur when gas is pulled into the conduits from neighboring cells and intracellular spaces, known as air-seeding (Sperry and Tyree, 1988), or when nucleation of gas bubbles is initiated by microfractures in the conduit cell walls (Jacobsen et al., 2005).

Lignin is a complex, hydrophobic polymer that is deposited in the secondary cell wall of xylem cells. Lignin has been suggested to contribute to the mechanical and

hydraulic properties of the cell wall (Campbell and Sederoff, 1996).

While lignin has been shown to provide mechanical support to the cell wall (Donaldson, 2001; Sperry, 2003), lignin's role in xylem hydraulic properties is still not fully understood. Genetic modification of the lignin biosynthetic pathway provides opportunities for experiments on lignin's function. By changing lignin content and lignin monomer ratios within a single species, while maintaining other xylem anatomical properties which confound results associated with studies using different taxa can be eliminated. Some recent research has examined the effects of decreasing total lignin content on xylem function in hybrid poplar (Coleman et al., 2008; Kitin et al., 2010; Voelker et al., 2011). Results from these experiments showed that reducing the total lignin content reduces the plant's ability to efficiently transport water and resist cavitation, i.e. lower specific conductivity and increased xylem pressure at 50% embolism (Coleman et al., 2008; Voelker et al., 2011), and increases the incidence of vessel collapse (Franke et al., 2002; Kitin et al., 2010). These experiments highlight the important role that lignin plays in protecting vessel elements from the high negative pressures created within the lumen as water is transported through the vessels. However, these experiments focus on reduction in total lignin content. Yoshinaga et al. (1992) proposed that the variation in lignin monomer ratios based on cell type suggests a possible differentiation in the functional role of the lignin monomers. That is, guaiacyl is more suitable for cells that function in water movement and syringyl is more suited to cells dedicated to mechanical support.

Experiments on the mechanical properties of the increased S:G lines found a decrease in the stems modulus of rupture, MOR, but had no significant effect on the modulus of elasticity, MOE (Al-Haddad, 2012). This suggests that increasing the

syringyl content in the secondary cell wall of vessels will result in increased brittleness of the adjoining cell walls which may result in decreased ability to resist high negative pressures. This may result in microfractures in the cell wall leading to air seeding and cavitation. Jacobsen et al. (2005) found a correlation between MOR and resistance to cavitation. However, these results may result from anatomical differences within the xylem tissue between several related plant species rather than differences in the lignin content within the xylem.

In this experiment I examined the effects of increasing S:G on the hydraulic conductivity and resistance to embolism of hybrid Poplar (*Populus tremula x P. alba*) under well watered and water stressed conditions. Two genetic lines with increased S:G, along with the wild type, were used to examine their efficiency of xylem hydraulic conductivity (specific conductivity, k_S), resistance to cavitation (xylem pressure at 75% loss of hydraulic conductivity, P_{75}). To test the response to water stress, the lines were watered to field capacity and then water was withheld throughout the course of the experiment. Stem segments were sampled periodically throughout the experiment and tested for k_S and P_{75}. To observe how water stress proceeded in the living specimens, a number of physiological properties were measured throughout the experiment. These physiological processes include stomatal conductance (g_S), transpiration (E), leaf pressure potential (predawn, ψ_{pre}, and mid-day, ψ_{mid}), dark acclimated chlorophyll fluorescence (F_V/F_m), light acclimated chlorophyll fluorescence (F_V'/F_m'), and steady-state fluorescence yield (F_S). F_V/F_m, F_V'/F_m', and F_S have been shown to be useful in measuring the efficiency of Photosystem II in plants and can be useful in tracking the effects of stress on photosynthetic efficiency (Maxwell and Johnson, 2000).

It was hypothesized that an increase in the S:G would result in an increased

conductive efficiency, but would also increase the plants vulnerability to cavitation. As syringyl increases we expect to see an increase in k_S and a decrease in the resistance to embolism and P_{75} values.

It can be expected that water stress will have a greater effect on plants with decreased resistance to embolism. Thus, I hypothesize that the increased S:G lines will have a lower k_S as water stress increases. Similar effects will also be expected in physiological processes. Lines with increased k_S will show higher values for leaf physiological properties and lower values in plant lines with increased vulnerability to embolism as water stress occurs.

Methods

Transgenic lines of hybrid poplar clone INRA 717-1B4 (*Populus tremula x P. alba*) over-expressing the *Arabidopsis* gene encoding ferulate-5-hydroxylase (F5H), driven by the cinnamate-4-hydroxylase (C4H) promoter were obtained from David Ellis (formerly with CELLFOR, Richmond, BC, Canada) with permission from Clint Chapple, Purdue University (Franke et al., 2000; Material use agreement between Michigan State University and Purdue University executed 12 April 2002). Two transgenic lines expressing different percentages of syringyl were chosen for use in this experiment: F5H-37 and F5H-64, expressing 88 percent and 93 percent syringyl, respectively. The transgenic lines and wild type (WT) trees were propagated from root sprouts in early spring (March-May) and allowed to grow in a greenhouse for four months. In August, 60 trees were selected for use (20 trees each of the transgenic and WT lines). Two days before the start of the experiment all trees were watered to field capacity each day. At *t=0*, three trees of each line were selected randomly for pre-dawn and mid-day gas

exchange, water potential and fluorescence measurements. Stem samples were then harvested from the selected trees for measurements on hydraulic conductivity and vulnerability to cavitation. Remaining trees were then subjected to drought stress by withholding water. Every two days, the pre-dawn and mid-day gas exchange, water potential, and chlorophyll fluorescence were measured, as described below, on randomly selected trees (n=3 for each line). Stem samples for hydraulic conductivity measurements were collected every 12 days.

Hydraulic Conductivity and Vulnerability to Cavitation

At t=0, and every 12 days after, 9 trees (3 of each line) were randomly selected and stem samples were collected for measurement of hydraulic conductivity (k_h; kg m s^{-1} Mpa^{-1}), specific conductivity (k_S; kg m^{-1} s^{-1} MPa^{-1}), and vulnerability to cavitation. Segments, approximately 20 cm in length, were excised from the main stem underwater to avoid air infiltration into vessels and transported to the laboratory. The samples were then re-cut underwater, using a razor blade, to 14 cm long, and 1 cm of bark was removed from each end. Stem samples were then connected to a Sperry apparatus to measure the initial k_h (Sperry et al., 1988). After the initial k_h ($k_{hinitial}$) was measured, samples were then connected to a tygon tubing system and flushed with water to remove any gas emboli present in the vessel elements. To reduce the incidence of microbial growth, water used for flushing was adjusted to a pH of 2 using HCl, degassed in a glass carboy connected to a vacuum for approximately 24 hours, and passed through a 0.1-µm filter. Stems were flushed at a pressure of 14 MPa for 1 hour, and then replaced in the Sperry apparatus to obtain the maximum k_h (k_{hmax}; Sperry et al., 1988). The k_S values were determined by dividing the k_h by the sapwood cross-

sectional area (A_S; m^2) of the sample. The A_S was calculated as the wood cross-sectional area subtracted by the pith cross-sectional area. Wood cross-sectional area was calculated by taking the mean of two perpendicular diameter measurements of the wood without bark from each end of the stem sample with digital calipers. Pith cross-sectional area was calculated by averaging the measurements of the largest diameter of the pith at each end of the sample.

To determine vulnerability to cavitation, stem samples were spun in a centrifuge (RC-5B, Sorvall, Dupont Instruments) with a modified rotor built to hold the stem samples. Negative pressure, measured in MPa, was generated through spinning the rotor at specified rpms (Alder et al., 1997). The k_h was then measured at sequential negative pressures. The percent loss of conductivity (PLC) was calculated for each k_h value using the equation: $100 – \{(k_h / k_{hmax}) * 100\%\}$, and used to construct vulnerability curves. The 75 PLC (P_{75}) values were also obtained for each sample by fitting a second order polynomial model to each of the PLC values (Jacobsen et al., 2005).

Chlorophyll Fluorescence and Gas Exchange

Measurements of chlorophyll (Chl) fluorescence were obtained using a Li-6400 portable photosynthesis system with a 6400-40 Leaf Chamber Fluorometer (LI-COR Biosciences Inc.).

Every two days, 9 trees (3 each for transgenic and WT lines) were randomly selected for measurement. Measurements were taken at pre-dawn, between 4am and 6am, to ensure dark adaptation, and at mid-day, between noon and 2pm, during peak photosynthetic activity. One leaf from each tree was measured. Chl fluorescence

parameters were measured as described (Maxwell and Johnson 2000). Parameters analyzed were minimum fluorescence yield (dark adapted, F_o; light, F_o'), maximum fluorescence yield (dark adapted, F_m; light, F_m'), quantum efficiency of open photosystem II centers (dark adapted, F_v/F_m; light, F_v'/F_m'), and steady-state fluorescence yield (F_S).

Gas exchange measurements were taken directly following measurement of Chl fluorescence using the same leaves at mid-day. Transpiration, E, and stomatal conductance, g_S, were measured using a Li-1600 Steady State Porometer (LI-COR Biosciences Inc.). Before measurements, the Li-1600 porometer was activated and allowed to reach a stable relative humidity. The sensor head cuvette was attached to each leaf and held to maintain a normal orientation. The g_S was monitored until reaching equilibrium. Once the rate of conductance stabilized, measurements of E and g_S were recorded.

Leaf Pressure Potential

Leaf water potentials (predawn; mid-day) were measured using a Scholander pressure-chamber. Directly following pre-dawn and mid-day Chl fluorescence and gas exchange measurements, one leaf from each plant was harvested, by removing the base of the petiole from the stem, bagged in a Ziploc sandwich bag, and placed in a cooler containing ice packs. Leaf samples were then transported to the laboratory. The petiole of the leaf was cut using a razor blade, and then placed into the Scholander chamber.

Data Analysis

Statistical analyses were performed using SAS® software (SAS Institute Inc.). A two-way ANOVA was performed on each data set to analyze differences caused by time (day), line, and their interaction effects. A comparison of means was performed on each factor level using Tukey's HSD.

Results

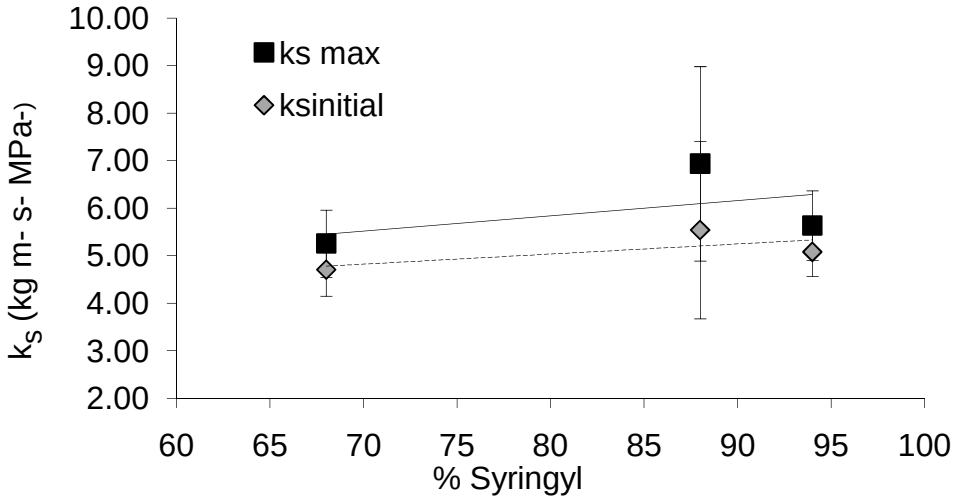
Xylem conductive efficiency

Mean specific conductivity from all four years of experiments shows a trend toward increased k_S as percent syringyl content at t = 0 (Figure 2-1a). WT and F5H64 were not significantly different from each other, but F5H37 was significantly different from the other two lines ($\alpha < 0.05$). Specific conductivity, both maximum and initial, was severely reduced by water stress (Figure 2-1b). Under the stressed treatment (t=36), F5H37 shows increased k_{smax} over the WT, and F5H64 shows a lower k_{smax} (Figure 2-1c). Statistical analysis shows no significant difference between lines in the stressed treatment, though ($\alpha = 0.3219$). Re-watered treatment plants show an increase in k_{smax} compared to stressed treatments but they do not return to k_{smax} values observed in well watered treatments (Figure 2-1b; Figure 2-1c).

When examining k_S separately for each year, results are inconsistent. Figure 2-2 shows how mean k_{smax} changed over time for 2009 and 2010 experiments. In 2009, both F5H overexpression lines had a lower mean k_{smax} then the WT for t=1 and t=8. ANOVA results show that for 2009 day, line and the interaction effect were significant for both $k_{sinitial}$ and k_{smax} (Table 2-1; Table 2-2). For 2010 data, there is no difference in k_{smax} between lines but by t=14 F5H64 has a higher k_{smax} then the WT and F5H37 lines. A Tukey's HSD shows that at t=14 F5H64 is significantly different from F5H37 but

not different from the WT (α< 0.05). The interaction effect between day and line was significant for 2010 but line effect was not (Table 2-1; Table 2-2).

Figure 2-1. Mean specific conductivity (k_S) as a function of percent syringyl content. a) k_{smax} (*solid line*) and $k_{sinitial}$ (*dashed line*) for t=0. b) k_{smax} against percent syringyl for watered (*solid line*), stressed (*dashed line*), and re-watered (*dashed-dot line*) treatments. c) k_{smax} against percent syringyl for stressed (*dashed line*) and re-watered (*dashed-dot line*) treatments. WT and F5H64 n=12; F5H37 n=6.
a)



b)

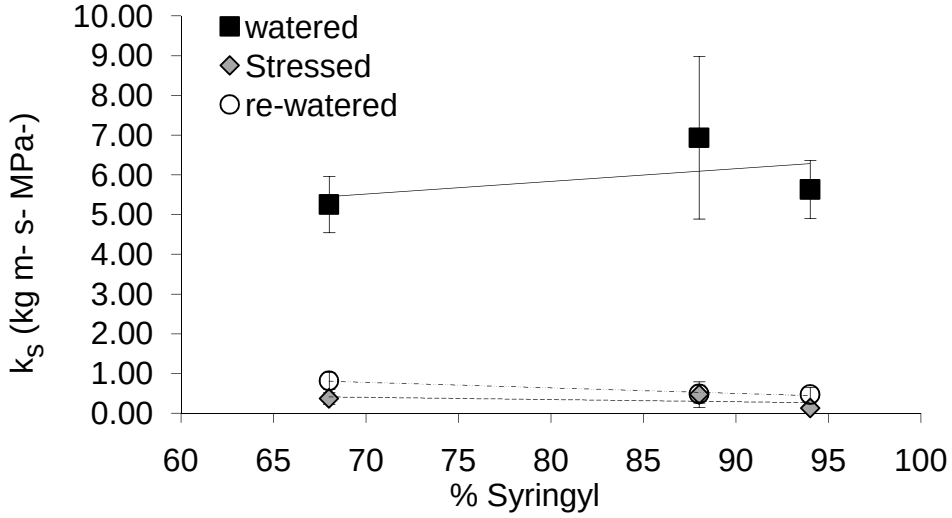


Figure 2-1 continued.

c)

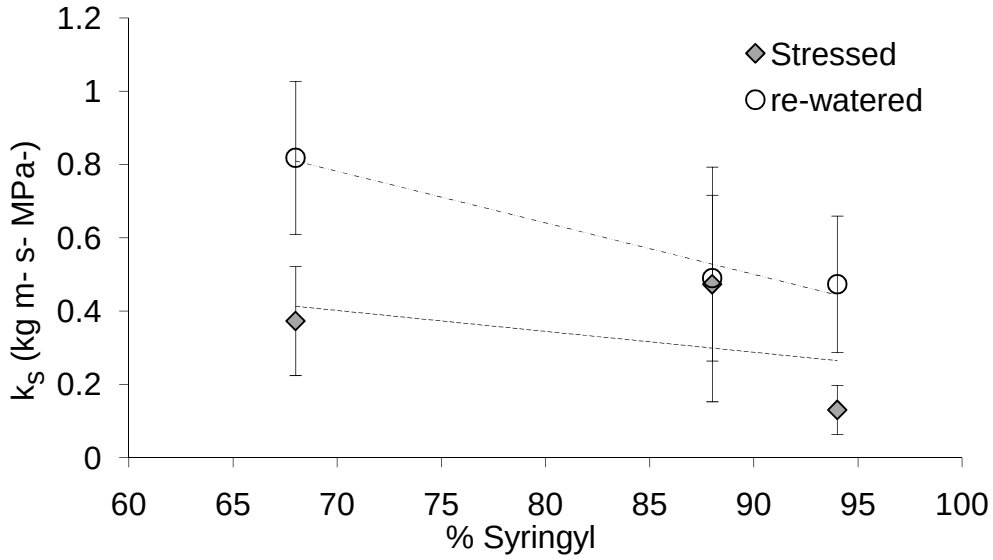


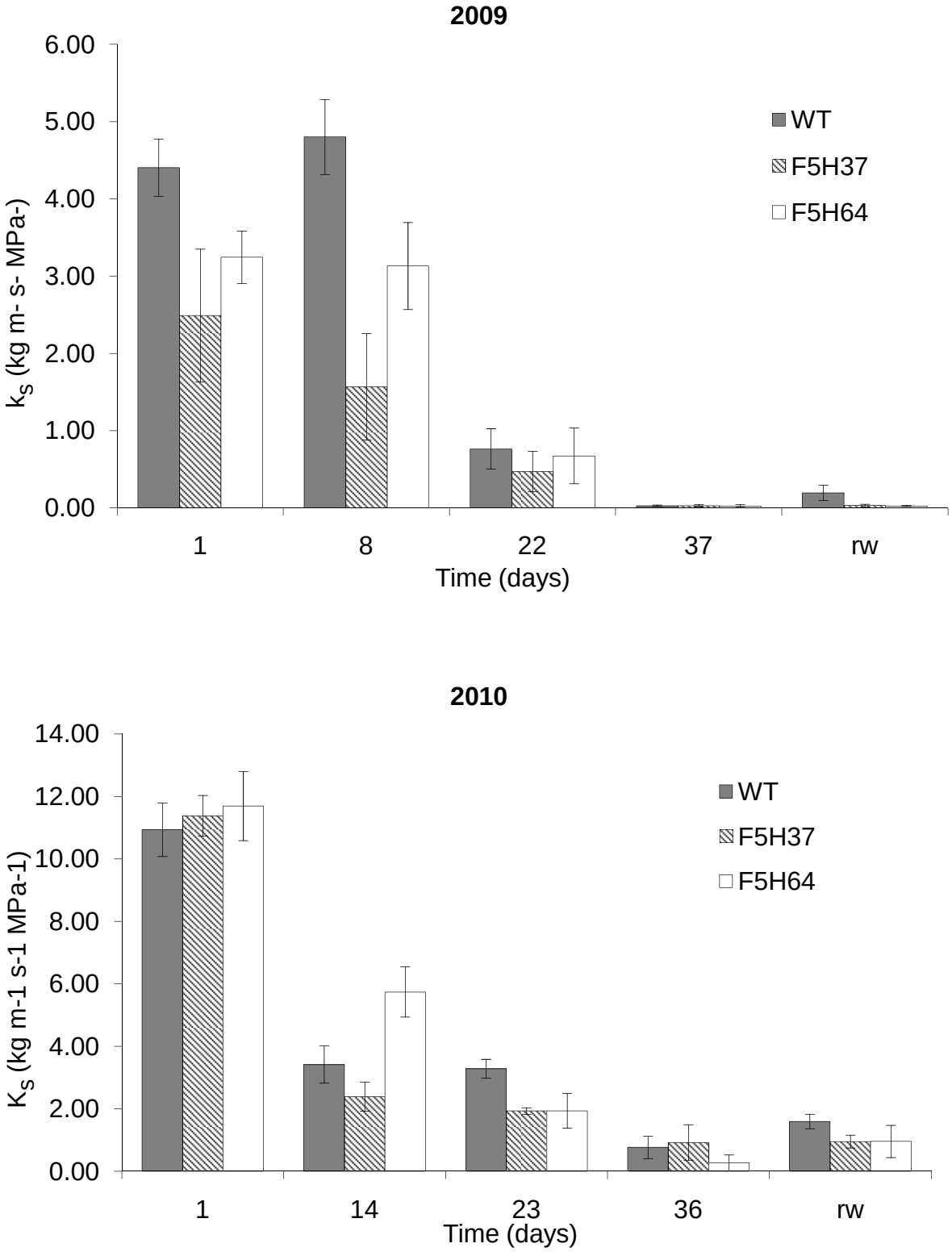
Table 2-1. ANOVA results for yearly initial specific conductivity ($k_{sinitial}$) for WT, F5H37, and F5H64 lines exposed to increasing water stress over time (day). Significant (α<0.05) terms are in bold.

Response	Year	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
$k_{sinitial}$	2007	0.5432	<.0001	**Day**	**3**	**94.6248**	**14.45**	**<.0001**
				Line	1	5.7678	2.64	0.1119
				Line*Day	4	3.4491	0.53	0.6666
				Residuals	40	87.3359		
	2008	0.8492	<.0001	**Day**	**3**	**112.8121**	**81.58**	**<.0001**
				Line	1	0.0057	0.01	0.9118
				Line*Day	**3**	**4.3346**	**3.13**	**0.0344**
				Residuals	46	21.2048		
	2009	0.8697	<.0001	**Day**	**3**	**45.7281**	**39.61**	**<.0001**
				Line	**2**	**8.6529**	**11.24**	**0.0004**
				Line*Day	**6**	**7.2485**	**3.14**	**0.0204**
				Residuals	24	13.7036		
	2010	0.9574	<.0001	**Day**	**3**	**387.3637**	**173.68**	**<.0001**
				Line	2	0.4907	0.33	0.7221
				Line*Day	**6**	**12.8769**	**2.89**	**0.0291**
				Residuals	24	17.8431		

Table 2-2. ANOVA results for yearly initial specific conductivity (k_{smax}) for WT, F5H37, and F5H64 lines exposed to increasing water stress over time (day). Significant (α<0.05) terms are in bold.

Response	Year	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
k_{smax}	2007	0.4760	0.0003	**Day**	**3**	**74.1750**	**11.60**	**<.0001**
				Line	1	0.2819	0.13	0.7180
				Line*Day	3	2.9619	0.46	0.7094
				Residuals	40			
	2008	0.8463	<.0001	**Day**	**3**	**124.0421**	**80.12**	**<.0001**
				Line	1	0.0058	0.01	0.9163
				Line*Day	**3**	**4.9796**	**3.22**	**0.0313**
				Residuals	46	23.7403		
	2009	0.8807	<.0001	**Day**	**3**	**79.7400**	**46.55**	**<.0001**
				Line	**2**	**11.1174**	**9.74**	**0.0008**
				Line*Day	**6**	**10.2737**	**3.00**	**0.0249**
				Residuals	24	13.7036		
	2010	0.9581	<.0001	**Day**	**3**	**598.3527**	**176.33**	**<.0001**
				Line	2	3.4418	1.52	0.2387
				Line*Day	**6**	**19.4217**	**2.86**	**0.0301**
				Residuals	24	27.1466		

Figure 2-2. Maximum specific conductivity (k_{smax}) for WT, F5H37, and F5H64 lines over time for 2009 (*top*) and 2010 (*bottom*). Rw signifies re-watered treatments. Mean ± SE. n=3.



Resistance to cavitation

Vulnerability curves for the watered treatments from 2009 and 2010 show some variation between the two years (Figure 2-5). Both F5H37 and F5H64 are more vulnerable to cavitation than WT in 2009. In 2010 there is no difference in vulnerability between WT and F5H37, but F5H64 shows a slight increase in vulnerability. No clear trend was observed between P_{75} values and percent syringyl content for watered, stressed and re-watered treatments (Figure 2-3). ANOVA results for 2009 showed significance for day and line effects, but the day effect was the only significant variable for 2010 ($\alpha < 0.05$; Table 2-3). When comparing P_{75} values for watered treatment from 2009 and 2010, WT and F5H64 lines were similar, but F5H37 showed higher vulnerability to cavitation in 2009 then 2010 (Figure 2-4; Figure 2-6).

Table 2-3. ANOVA results for yearly xylem pressure at 75% loss of conductivity (P_{75}) for WT, F5H37 and F5H64 lines exposed to increasing water stress over time (day). Significant ($\alpha<0.05$) terms are in bold.

Response	Year	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
P_{75}	2009	0.5277	0.0330	**Day**	**3**	**1.4639**	**3.35**	**0.0357**
				Line	**2**	**1.4944**	**5.13**	**0.0140**
				Line*Day	6	0.9464	1.08	0.4003
				Residuals	24	3.4953		
	2010	0.6782	0.0009	**Day**	**3**	**1.8853**	**10.63**	**0.0001**
				Line	2	0.3685	3.12	0.0627
				Line*Day	6	0.7365	2.08	0.0941
				Residuals	24	1.4191		

Figure 2-3. Pressure at 75 percent loss of conductivity (P_{75}) as a function of percent syringyl content from watered (*solid line*), stressed (*dashed line*), and re-watered (*dashed-dot line*) for 2009 (*top*), 2010 (*bottom*). Mean ± SE. n=3.

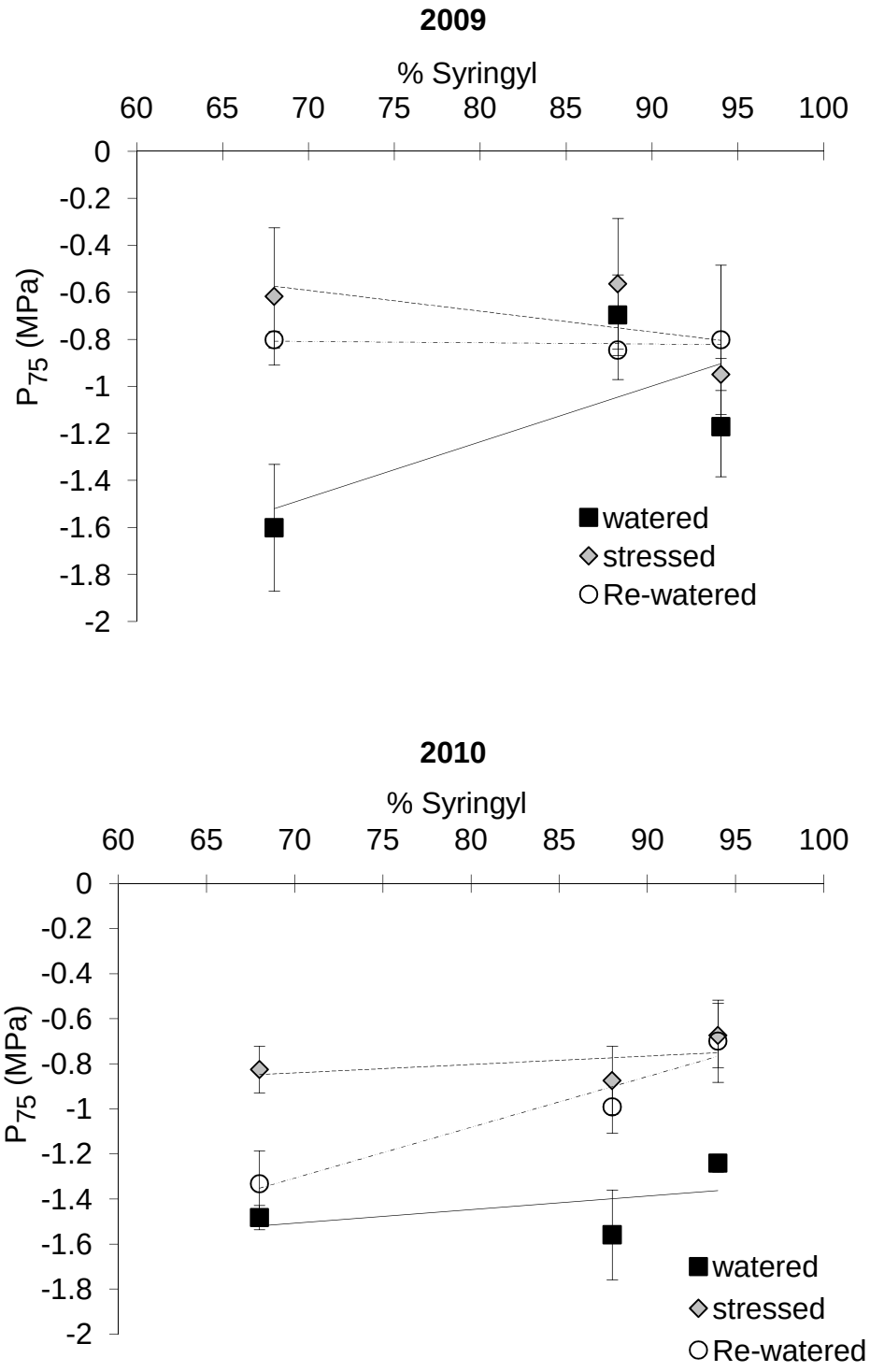


Figure 2-4. Comparison of 2009 (*solid line*) and 2010 (dashed line) P_{75} as function of % syringyl. Mean ± SE. n=3.

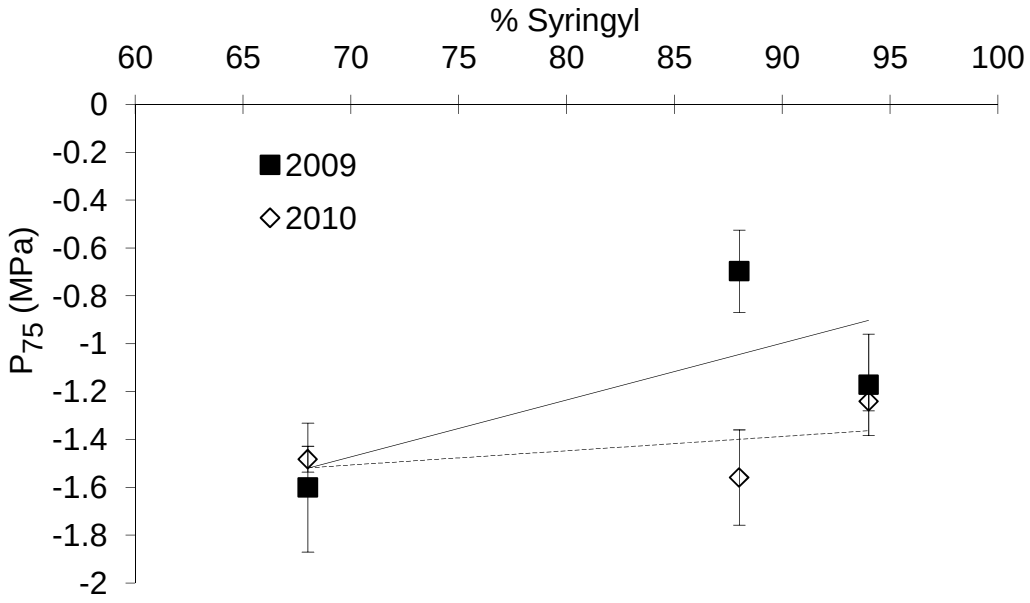
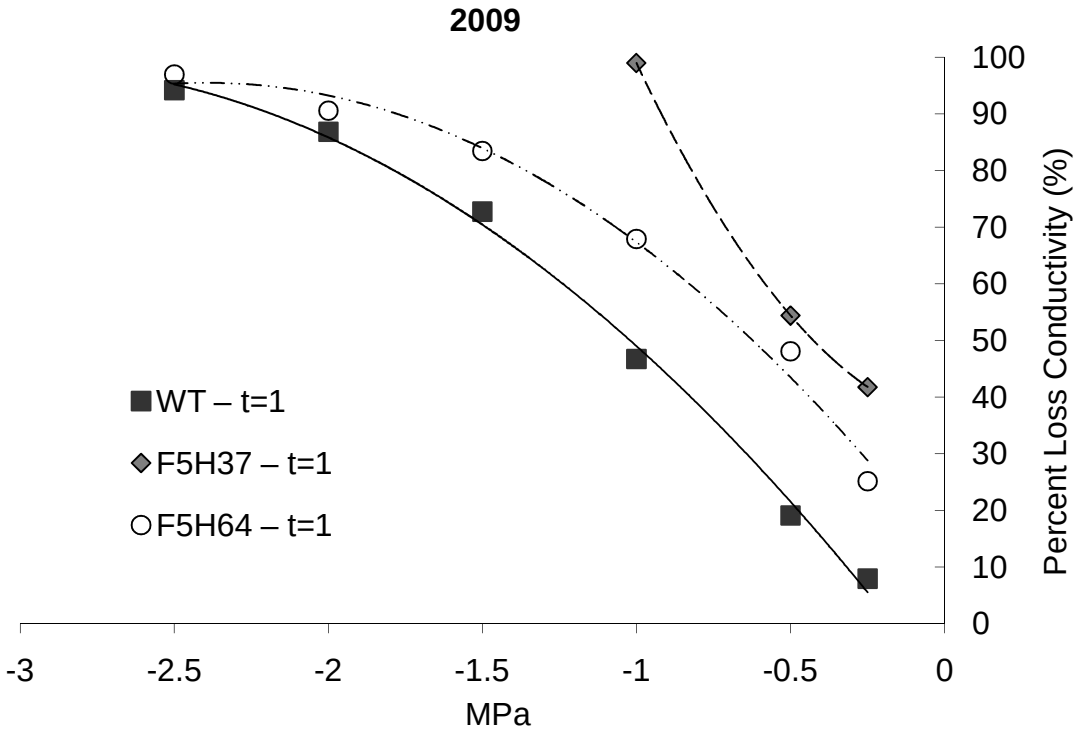


Figure 2-5. Vulnerability curves for WT (*solid line*), F5H37 (*dashed-dot line*), and F5H64 (*dashed line*) lines for 2009 (*top*) and 2010 (*bottom*) well watered treatments (t=1). n=3.



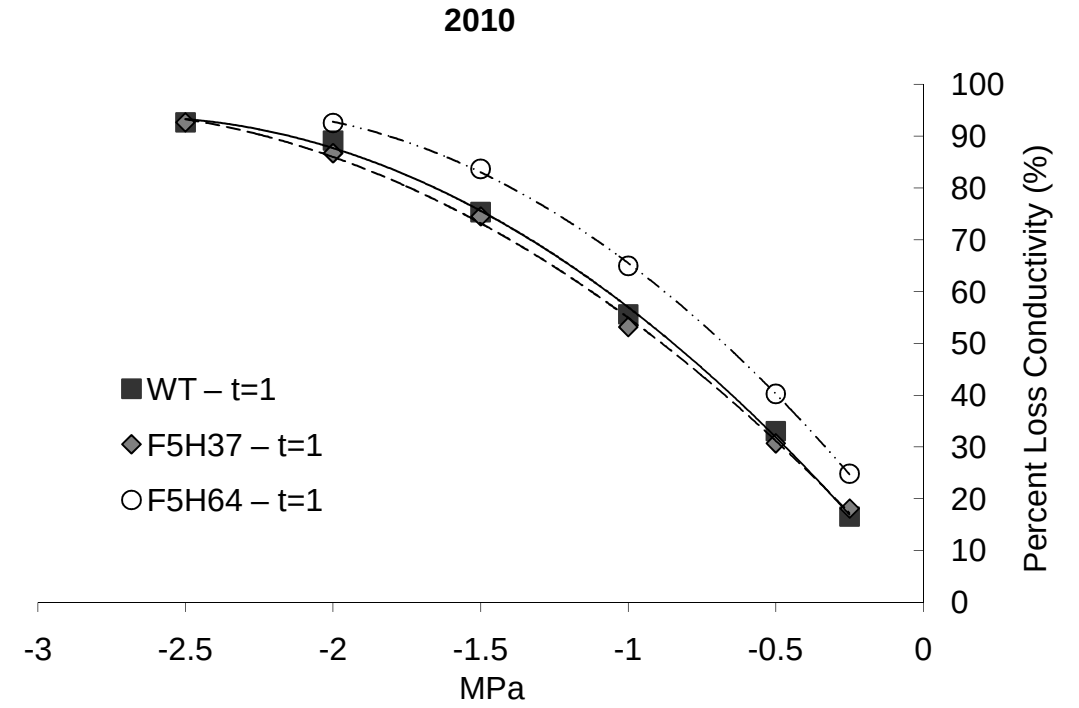
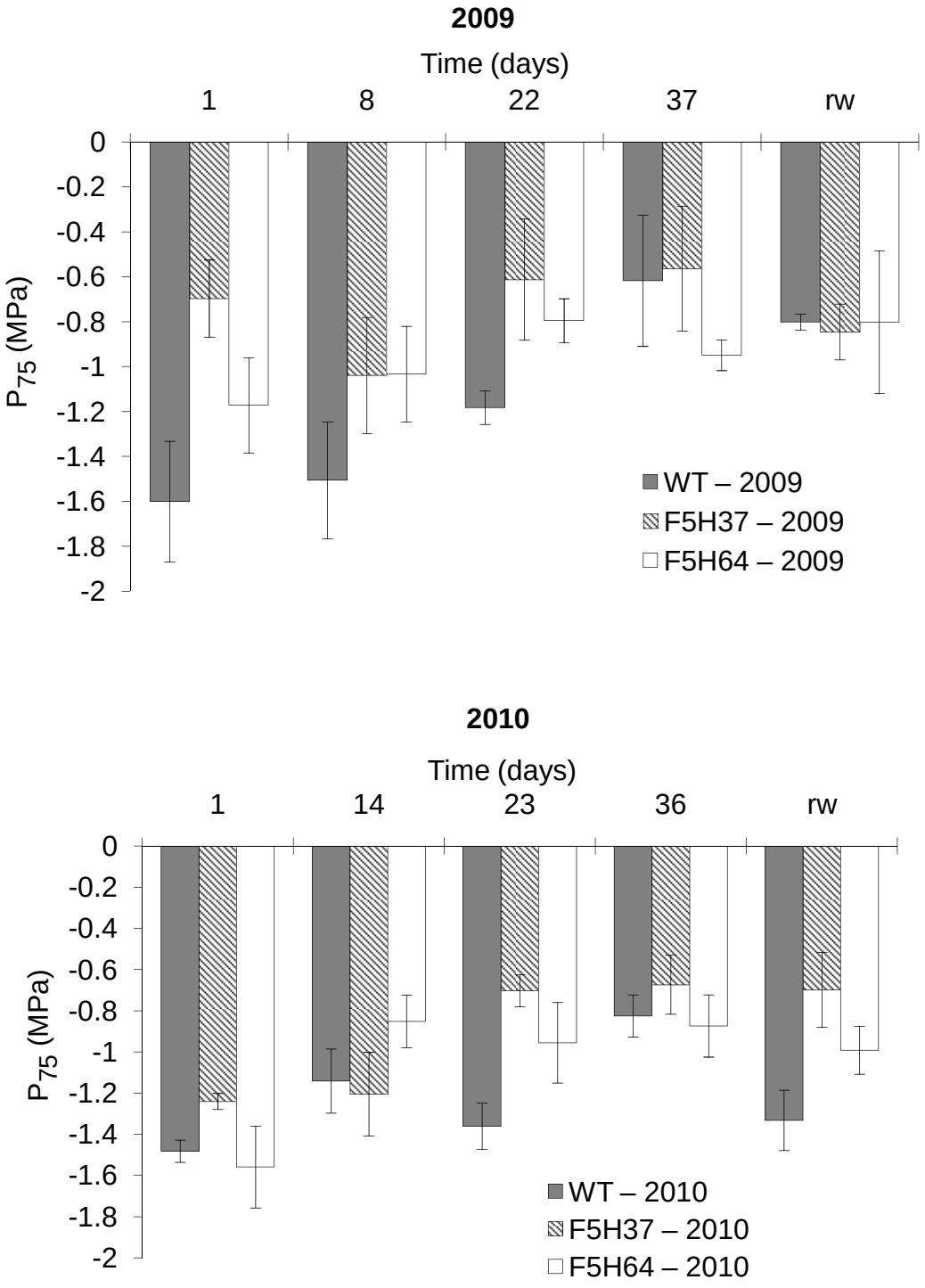


Figure 2-6. Xylem pressure at 75 percent loss of conductivity (P_{75}) for 2009 (*top*) and 2010 (*bottom*) for wild type (WT), F5H37, and F5H64 lines over time. Rw signifies re-watered treatment. Mean ± SE. n=3.



Physiological Properties

F_v/F_m did not decline until late in the experimental water stress treatment. F_v/F_m stayed high in all lines until approximately 25 days (Figure 2-7). After this point leaves began to turn yellow and F_v/F_m values declined. ANOVA results show significant day effect for all four years. Line effects were significant for 2009 but not for any other year. F_v'/F_m' and F_s showed a much more linear decline over time (Figure 2-7; Figure 2-8). Day effects were significant for all four years but only 2010 showed significance from line effects (Table 2-5).

Figure 2-7. a) 2010 dark acclimated chlorophyll fluorescence (F_v/F_m). b) light acclimated chlorophyll fluorescence (F_v'/F_m') versus time. Mean ± SE. n=3.

a)

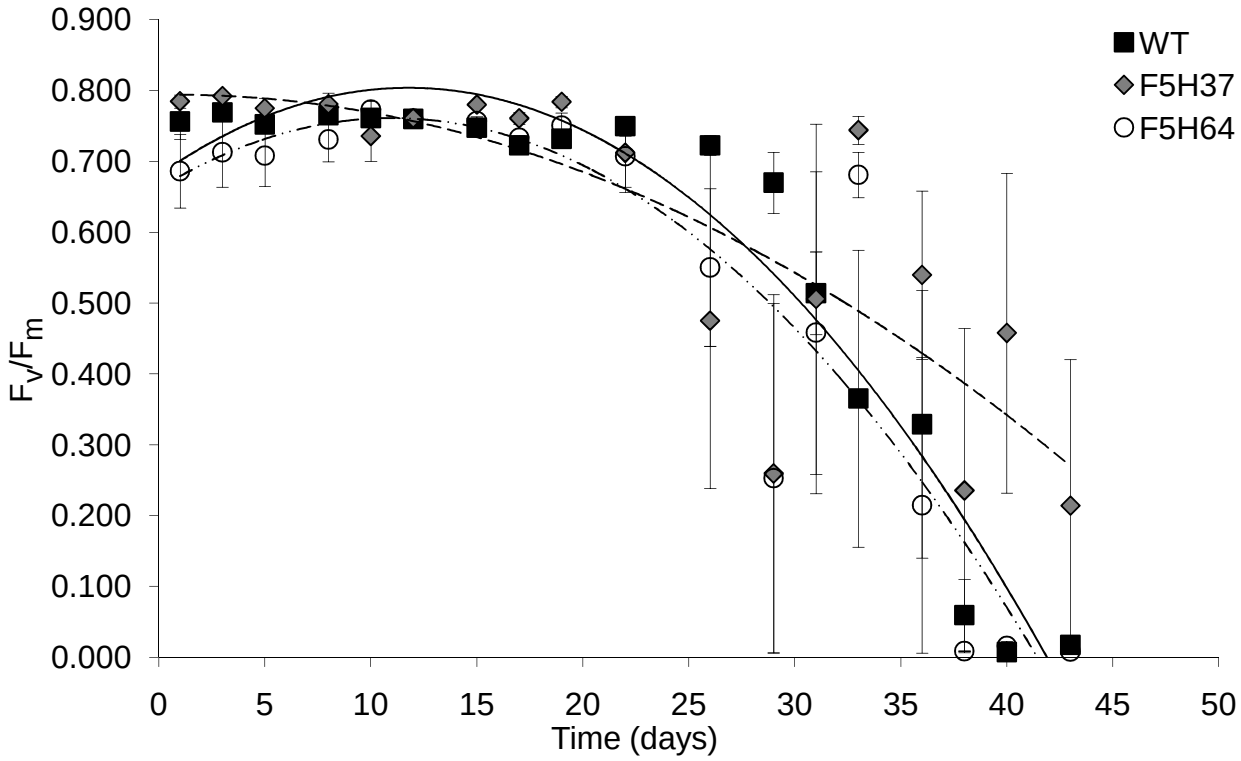


Figure 2-7 continued.

b)

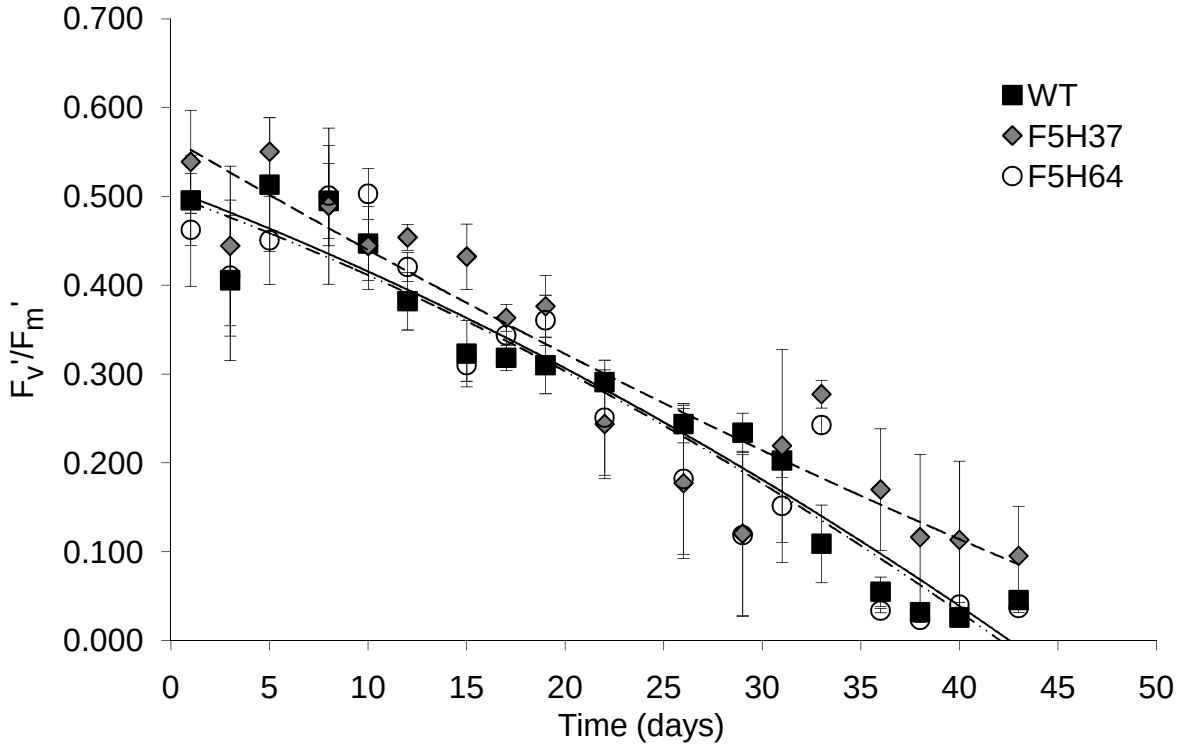


Figure 2-8. 2010 steady-state fluorescence (F_S) versus time. Mean ± SE. n=3.

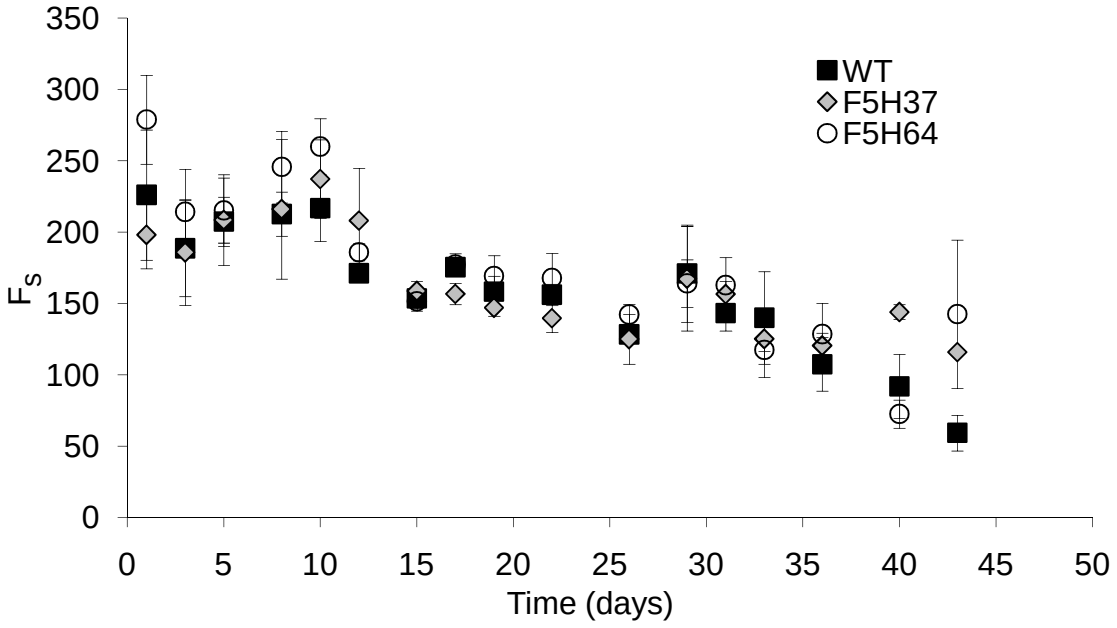


Table 2-4. ANOVA results for yearly dark acclimated chlorophyll fluorescence (F_v/F_m) and light acclimated chlorophyll fluorescence (F_v'/F_m') for WT, F5H37 and F5H64 lines exposed to increasing water stress over time (day). Significant ($\alpha<0.05$) terms are in bold.

Response	Year	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
F_v/F_m	2007	0.1988	0.2084	Day	7	0.1480	1.46	0.1943
				Line	1	0.0179	1.23	0.2701
				Line*Day	7	0.1219	1.20	0.3119
				Residuals	80	1.1597		
	2008	0.7074	<.0001	**Day**	**11**	**9.5599**	**24.78**	**<.0001**
				Line	1	2.06E-003	0.06	0.8091
				Line*Day	11	0.6136	1.59	0.1099
				Residuals	120	4.2086		
	2009	0.7660	<.0001	**Day**	**7**	**5.8358**	**18.32**	**<.0001**
				Line	**2**	**0.5993**	**6.59**	**0.0030**
				Line*Day	14	0.7153	1.12	0.3635
				Residuals	48	2.1841		
	2010	0.7448	<.0001	**Day**	**17**	**9.3137**	**16.01**	**<.0001**
				Line	2	0.2105	3.08	0.0502
				Line*Day	34	1.2576	1.08	0.3708
				Residuals	108	3.6949		
F_v'/F_m'	2007	0.4525	<.0001	**Day**	**8**	**0.3765**	**6.88**	**<.0001**
				Line	1	0.0136	1.99	0.1622
				Line*Day	**8**	**0.1185**	**2.17**	**0.0374**
				Residuals	90	0.6153		
	2008	0.7077	<.0001	**Day**	**10**	**4.2571**	**26.72**	**<.0001**
				Line	1	6.97E-003	0.44	0.5096
				Line*Day	10	0.1235	0.77	0.6527
				Residuals	114	1.8163		
	2009	0.8801	<.0001	**Day**	**5**	**1.7628**	**48.98**	**<.0001**
				Line	2	0.0265	1.84	0.1735
				Line*Day	10	0.0423	0.59	0.8128
				Residuals	35			
	2010	0.8271	<.0001	**Day**	**17**	**4.0531**	**28.69**	**<.0001**
				Line	**2**	**0.0615**	**3.70**	**0.0279**
				Line*Day	34	0.1777	0.63	0.9386
				Residuals	108	0.8974		

Table 2-5. ANOVA results for yearly steady-state chlorophyll fluorescence (F_S) for WT, F5H37 and F5H64 lines exposed to increasing water stress over time (day). Significant (α<0.05) terms are in bold.

Response	Year	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
F_s	2007	0.9471	<.0001	**Day**	**8**	**2.18E+006**	**199.97**	**<.0001**
				Line	1	9.86E+001	0.07	0.7886
				Line*Day	8	1.48E+004	1.36	0.2271
				Residuals	90	1.23E+005		
	2008	0.4890	<.0001	**Day**	**9**	**2.94E+005**	**9.70**	**<.0001**
				Line	**1**	**1.45E+004**	**4.31**	**0.0403**
				Line*Day	9	3.07E+004	1.01	0.4334
				Residuals	104	3.50E+005		
	2009	0.9167	<.0001	**Day**	**6**	**2.59E+005**	**63.30**	**<.0001**
				Line	2	2.55E+003	1.87	0.1683
				Line*Day	12	9.62E+003	1.18	0.3340
				Residuals	37	2.52E+004		
	2010	0.6650	<.0001	**Day**	**16**	**2.66E+005**	**10.90**	**<.0001**
				Line	2	7.56E+003	2.47	0.0893
				Line*Day	32	3.54E+004	0.72	0.8500
				Residuals	102	1.56E+005		

Stomatal conductance and transpiration showed no difference between lines (Table 2-6). Water stress significantly affected the rates of g_S and E over time. Figure 2-9 shows g_S over the first 22 days of 2010 experiment. By day 10 stomatal closure resulted in a significant decline in g_S and E.

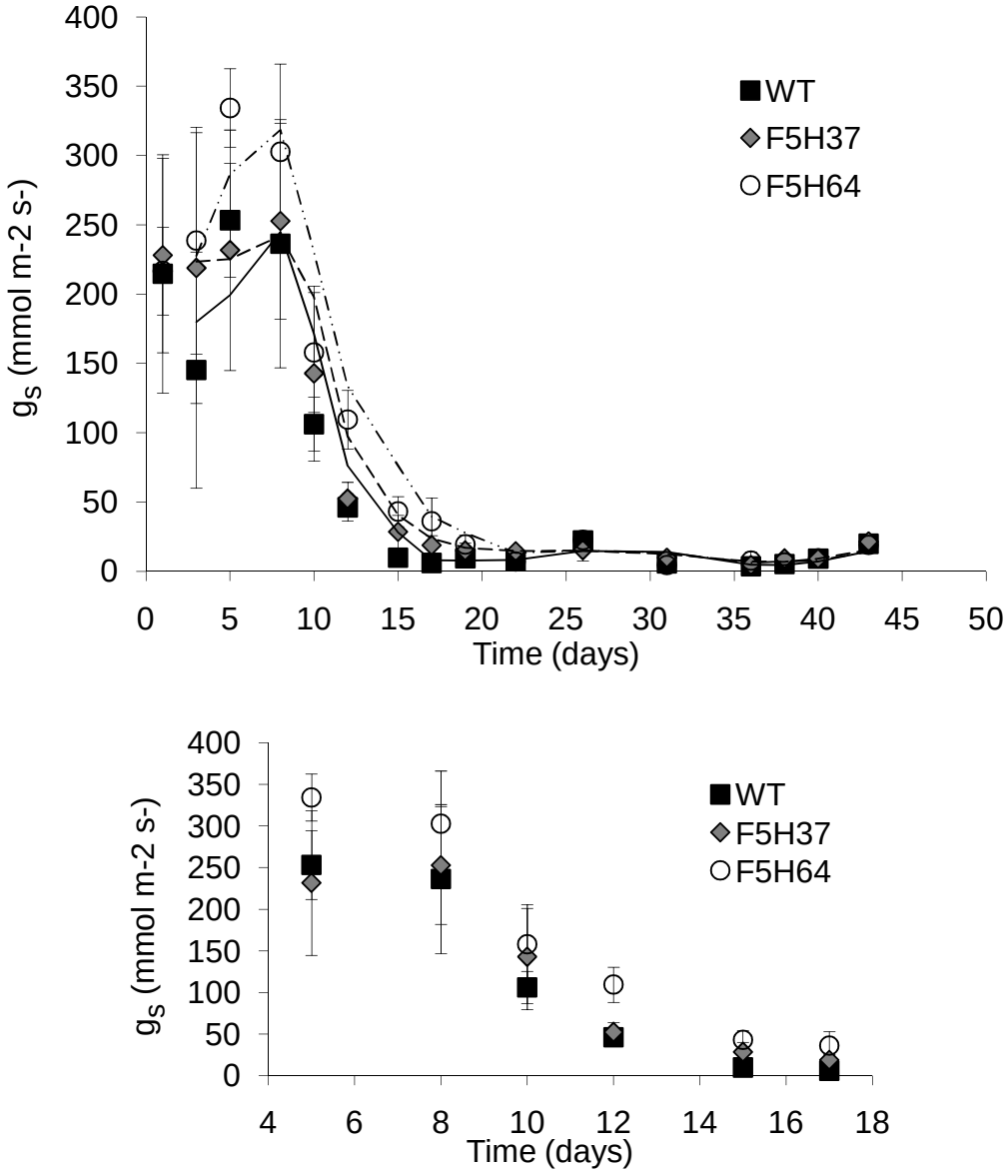


Figure 2-9. 2010 stomatal conductance (g_S) for WT (*solid line*), F5H37 (*dashed-dot line*), and F5H64 (*dashed line*) lines over 22 days (top) and g_S for days 5 to 17 (*bottom*). Mean ± SE. n=3.

Table 2-6. ANOVA results for yearly stomatal conductance (g_S) and transpiration (E) for WT, F5H37 and F5H64 lines exposed to increasing water stress over time (day). Significant (α<0.05) terms are in bold.

Response	Year	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
g_s	2007	0.6943	<.0001	**Day**	**7**	**4.59E+006**	**21.69**	**<.0001**
				Line	**1**	**1.87E+005**	**6.20**	**0.0150**
				Line*Day	7	2.76E+005	1.30	0.2603
				Residuals	74			
	2009	0.7472	0.0004	**Day**	**2**	**2.58E+005**	**22.22**	**<.0001**
				Line	2	2.28E+004	1.96	0.1694
				Line*Day	4	2.81E+004	1.21	0.3411
				Residuals	18	1.05E+005		
	2010	0.7684	<.0001	**Day**	**15**	**1.37E+006**	**20.43**	**<.0001**
				Line	2	1.80E+004	2.01	0.1398
				Line*Day	30	3.66E+004	0.27	0.9999
				Residuals	96	4.30E+005		
E	2007	0.6919	<.0001	**Day**	**7**	**231.2952**	**21.82**	**<.0001**
				Line	**1**	**7.9924**	**5.28**	**0.0244**
				Line*Day	7	11.5671	1.09	0.3776
				Residuals	74			
	2009	0.3036	0.4813	Day	2	3.5688	1.50	0.2499
				Line	2	1.9789	0.83	0.4515
				Line*Day	4	3.7944	0.80	0.5426
				Residuals	18	21.4242		
	2010	0.7613	<.0001	**Day**	**15**	**157.4923**	**19.51**	**<.0001**
				Line	2	2.3332	2.17	0.1200
				Line*Day	30	4.9323	0.31	0.9998
				Residuals	96	51.6531		

Predawn and midday ψ increased over time as water stress increased. For 2009, F5H37 ψ_{pre} was significantly higher from F5H64 but not from the WT. No difference in lines was detected in ψ_{mid} for 2009 (Figure 2-10a). In 2010, both ψ_{pre} and ψ_{mid} for F5H37 were significantly lower than WT and F5H64 lines (Figure 2-10b). ANOVA results also show a significant interaction effect between lines and day effects for 2010 ψ_{mid} (Table 2-7).

Figure 2-10. 2010 mean leaf pressure potential versus time. a) Predawn pressure potential. b) midday pressure potential. n=3.

a)

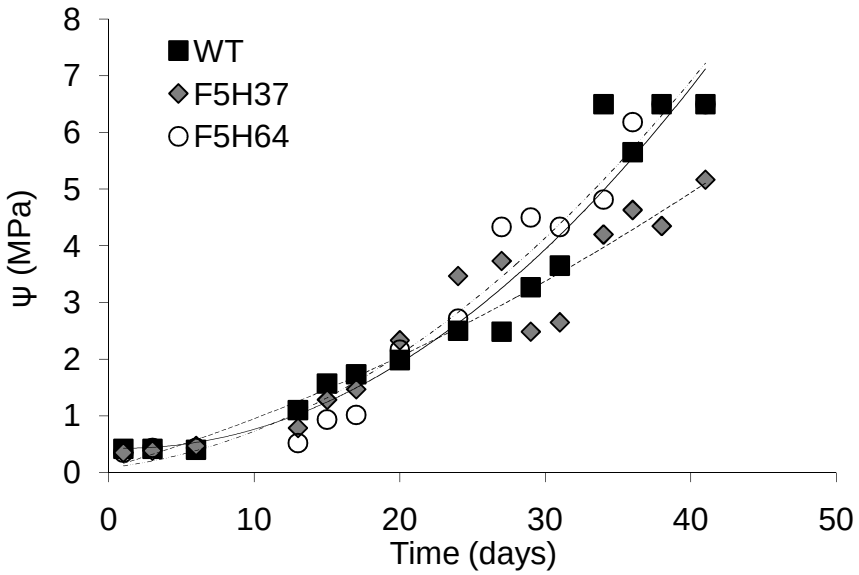


Figure 2-10 continued.

b)

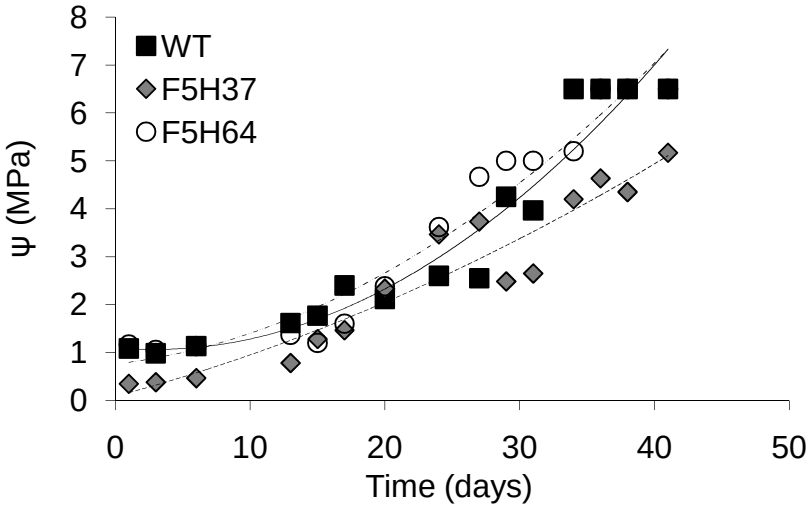


Table 2-7. ANOVA results for yearly predawn (ψ_{pre}) and midday (ψ_{mid}) pressure potentials for WT, F5H37 and F5H64 lines exposed to increasing water stress over time (day). Significant ($\alpha<0.05$) terms are in bold.

Response	Year	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
Ψ_{pre}	2007	0.3300	0.0112	**Day**	**4**	**1.21E+003**	**5.61**	**0.0008**
				Line	1	2.98E+001	0.55	0.4603
				Day*Line	4	8.72E+001	0.41	0.8036
				Residuals	50	2.69E+003		
	2008	0.7264	<.0001	**Day**	**7**	**3.84E+004**	**29.34**	**<.0001**
				Line	1	6.67E-001	0.00	0.9526
				Day*Line	7	1.31E+003	1.00	0.4396
				Residuals	80	1.50E+004		
	2009	0.7441	<.0001	**Day**	**5**	**1.77E+004**	**16.95**	**<.0001**
				Line	**2**	**2.00E+003**	**4.79**	**0.0143**
				Day*Line	10	2.17E+003	1.04	0.4347
				Residuals	36	7.53E+003		
	2010	0.8574	<.0001	**Day**	**14**	**5.37E+004**	**35.69**	**<.0001**
				Line	**2**	**7.51E+002**	**3.49**	**0.0346**
				Day*Line	28	3.70E+003	1.23	0.2309
				Residuals	90	9.68E+003		
Ψ_{mid}	2007	0.4252	<.0001	**Day**	**7**	**2.19E+003**	**7.65**	**<.0001**
				Line	1	4.59E+001	1.12	0.2921
				Day*Line	7	1.84E+002	0.64	0.7196
				Residuals	80	3.27E+003		
	2008	0.6723	<.0001	**Day**	**7**	**2.72E+004**	**23.19**	**<.0001**
				Line	1	4.67E+001	0.28	0.5990
				Day*Line	7	1.26E+003	1.08	0.3857
				Residuals	84	1.41E+004		
	2009	0.7795	<.0001	**Day**	**4**	**1.53E+004**	**23.15**	**<.0001**
				Line	2	1.03E+003	3.11	0.0592
				Day*Line	8	1.19E+003	0.90	0.5255
				Residuals	30	4.95E+003		
	2010	0.8327	<.0001	**Day**	**14**	**4.16E+004**	**27.23**	**<.0001**
				Line	**2**	**1.50E+003**	**6.89**	**0.0016**
				Day*Line	**28**	**5.79E+003**	**1.90**	**0.0125**
				Residuals	90	9.82E+003		

Discussion

Analysis of the k_{smax} under well watered conditions averaged over the four experimental years shows an increase in k_{smax} with increased percent syringyl (Figure 2-1). However, no significant difference was found between the WT and F5H64 lines, but F5H37 was significantly higher. When data from each year was examined separately results for k_S were contradictory. For 2009, F5H37 was observed to have lower k_{smax} than the WT. Both F5H lines were also found to be more vulnerable to cavitation in 2009 (Figure 2-5). This is in contrast to 2010 where no significant difference was observed between the WT and F5H lines for both k_S and P_{75} values.

The maximum specific conductivity declined as water stress increased over time in all lines. Theoretically, gas bubbles present in vessels should be completely removed after pressurized flushing by de-gassed solution (Sperry, 1988). This inability for specific conductivity values in stressed plants to return to the levels observed in well watered plants suggests that vessels are becoming permanently non-functional. This could be caused by tylosis or gum formation in gas-filled vessels, effectively closing them off from neighboring functional vessels (Tyree and Zimmerman, 2002). Formation of tyloses has been previously observed in hybrid *Populus* with reduced lignin content (Kitin et al., 2010).

A recent study on the methods of measuring hydraulic conductivity has questioned the efficacy of using high positive pressure to flush out gas embolism to obtain stable measurements of maximum conductivity (Espino and Schenk, 2011). If the solution used for flushing is not thoroughly degassed or if stem samples were not flushed for long enough gas bubbles may still be present within the xylem reducing conductivity. For these experiments, the solution used in flushing experiments were

degassed overnight until no visible gas bubbles were observed in the solution. To ensure complete removal of emboli from samples used in these experiments, multiple flushing events and measurements were performed on samples until the k_h value stopped increasing and reached its maximum. While these precautions were taken to avoid problems it is still possible some emboli may have remained after flushing (Espino and Schenk, 2011).

WT and F5H lines were found to be more vulnerable to cavitation in stressed plants compared to well watered plants. P_{75} values were observed to become less negative in all lines as when exposed to water stress (Figure 2-3; Figure 2-6). Cavitation events may weaken vessels making them subsequently more vulnerable to cavitation (Hacke et al., 2001) and microfractures in the cell wall (Jacobsen et al., 2005). Cavitation fatigue in *Populus* species due to stress has been previously observed (Hacke et al., 2001). Repair of cavitation fatigued vessels may be accomplished during the natural refilling of vessels in some plants, such as *Helianthus* (Stiller and Sperry, 2002). However, with the exception of the WT line from 2010, P_{75} values from re-watered treatments showed no significant difference from stressed treatments (Figure 2-3). This suggests that cavitation fatigue may be a permanent condition and native processes in intact plants are unable to reverse cavitation fatigue.

The reduction in gs and E corresponded with the decline in k_S values around 7 to 14 days after the beginning of the water stress regime. Regulation of stomata in response to water stress has been observed in a number of studies (Cochard et al., 2007; Meinzer and Grantz, 1990). This relationship between stomatal closure and xylem conductivity may be a method of maintaining leaf water status (Cochard et al., 2007). This could explain the delayed response in F_V/F_m data and the slower decline

observed in F_v'/F_m', F_s, ψ_{pre}, and ψ_{mid}.

Results from these experiments show that increased S:G does not significantly affect hydraulic efficiency and physiological properties in both healthy plants and those experiencing water stress. However, increasing syringyl content does appear to have an effect on vulnerability to embolism. This is likely caused by changes in the mechanical properties of the vessel cell wall causing reduced ability to withstand negative pressures induced by movement of the water column.

CHAPTER 3

HYDRAULIC RESPONSE TO FREEZE-THAW STRESS IN GENETICALLY MODIFIED HYBRID POPLAR WITH INCREASED SYRINGYL:GUAIACYL LIGNIN MONOMER RATIOS

Introduction

The effects of below freezing temperatures on the xylem function are important to consider when examining the growth and ecology of temperate woody plants. Stress caused by freezing and thawing of xylem sap can result in increased incidence of xylem embolism (Sperry, 1993). Xylem embolism can be quite extensive throughout winter months with up to 80 percent loss of conductivity observed in some deciduous trees (Sperry, 1993).

Embolism induced by freezing stress occurs when dissolved gas within the sap, which is insoluble in ice, freezes out, forming bubbles. When the sap thaws, the tensional forces in the xylem may cause the gas bubbles to expand inducing cavitation (Sperry and Sullivan, 1992; Tyree and Sperry, 1989).

Xylem structure plays a significant role in the response and vulnerability to freeze-thaw stress. Increased vulnerability to freezing induced embolism has been correlated with increased conduit diameter (Ewers, 1985; Sperry and Sullivan, 1992). Conifers, diffuse-porous, and ring-porous species respond to freeze-thaw stress differently (Sperry and Sullivan, 1992; Sperry, 1993; Sperry et al., 1994; Hacke and Sauter, 1996). Diffuse-porous species have been found to refill embolised vessels after winter freezing. However, ring-porous species are unable to refill their embolised

vessels and must develop a new ring of vessels in order to begin conducting water again in the spring (Sperry and Sullivan, 1992; Sperry, 1993; Sperry et al., 1994).

The response of *Populus* to freeze-thaw stress has been examined previously (Sperry and Sullivan, 1992; Sperry et al., 1994; Hacke and Sauter, 1996). Sperry and Sullivan (1992) observed an increase in percent of embolised vessels throughout the winter and reaching 90 percent embolism by late March in *Populus tremuloides*. Results examining the recovery from winter embolism have been inconsistent in *Populus*. Evidence for both embolism reversal and a lack of recovery in Populus has been observed in experiments from successive years (Sperry et al., 1994; Hacke and Sauter, 1996). The method of embolism reversal in *Populus* is still not fully understood. Positive xylem pressure was not observed in *Populus balsamifera* (Hacke and Sauter, 1996).

While xylem anatomy has been studied previously, the role of cell wall chemistry, specifically lignin content, in the response to freeze-thaw stress has not been characterized. Lignin provides mechanical support to the cell wall and aids in resisting negative pressures associated with water movement. Since tension increases within the xylem during leaf expansion in the spring, changes to the mechanical strength of conduits through modified lignin content may also affect recovery from winter embolism in the spring.

The goal of this experiment was to quantify the effects of increased syringyl monomer ratio on the response to freeze-thaw stress in hybrid poplar. The hydraulic conductivity and vulnerability to cavitation were measured in WT and F5H over-expression lines of hybrid poplar clone 717 (*Populus tremula x P. alba*) in winter and early spring. I hypothesized that increasing the syringyl:guaiacyl (S:G) ratio will result in

reduced hydraulic conductivity in the spring compared to winter treatments and an increased vulnerability to cavitation due to freeze-thaw embolism in both winter and spring treatments. As the frozen sap begins to thaw, tensional forces increase within the vessels. I predict that the increased brittleness of the cell wall caused by the increase in S:G will be more vulnerable to microfracturing and cavitation as tension forces increase during thawing events. Bubble manometers were also attached to excised branches to test for the presence of positive xylem pressure as a source of embolism reversal.

Methods

The trees used in this experiment consisted of wild type (WT) and the F5H transgenic lines previously described in Chapter 2 (F5H64 and F5H37). Trees were grown in pots and moved to an open air courtyard located in the greenhouses at Michigan State University in 2007. Experiments were performed over two years, during the winter and spring of 2009 and 2010. In 2009, three trees each of the WT, F5H64, and F5H37 lines were selected from the courtyard populations for experimentation. To examine the response of young trees that have not experienced freezing stress, 18 one-year old, single stemmed trees, six of each line, were taken from the greenhouse and transplanted into the courtyard in the November, 2009. These trees were added to the 2010 experiment, along with the three trees of each line previously growing in the courtyard.

Hydraulic conductivity and Vulnerability to Embolism

The trees moved to the courtyard in 2007, all had multiple lateral branches

present. For both experiments, two branches were selected each year from each tree and a stem samples were collected from one branch in February and from the other branch in May. Since the 18 transplanted one-year old trees were single stemmed with no branching, samples were collected from only 9 trees, 3 of each line, in February and May. Branches were cut from the courtyard plants 0.5 to 1m below the previous year's terminal bud scale scars. The branches were then quickly moved to a plastic container filled with water. One segment, 20 to 25 cm long, was then cut from each branch from the one year old growth underwater to avoid inclusion of embolised vessels. Segments were stored in the underwater in the plastic container, and transported to the laboratory. Once in the Laboratory, the samples were cut into 14 cm long separate segments underwater using razor blade. The excess stem sections were cut down to 4 to 6 cm in length, perfused with 0.1% crystal violet stain and stored in a freezer for later use in anatomical measurements. The 14 cm samples were used for measurement of hydraulic conductivity (k_h; kg m-1 s-1 MPa-1) as described by Sperry et al. (1988). The specific conductivity (k_S) values were determined by dividing the k_h by the sapwood cross-sectional area (A_S; m^2) of the sample. The A_S was calculated as the wood cross-sectional area subtracted by the pith cross-sectional area. Wood cross-sectional area was calculated by taking the mean of two perpendicular diameter measurements of the wood without bark from each end of the stem sample with digital calipers. Pith cross-sectional area was calculated by averaging the measurements of the largest diameter of the pith at each end of the sample. Vulnerability to cavitation and P_{75} values were calculated as described in Chapter 2.

Positive Xylem Pressure

Manometers were constructed using 0.5 ml glass pipettes sealed at the terminal end by melting the tip over a Bunsen burner. The pipettes were connected to 2mm 3-way polypropylene stopcocks (Bel-Art products) with Nalgene tubing. The manometers were partially filled with water using vacuum infiltration before attachment to each plant. Manometers were attached to the branch that was cut for hydraulic conductivity samples in February, 2010 one hour after the cut was made using a 3cm section of Nalgene tubing. Adjustable metal clamps were attached to the tubing where it connected the pipette and the plant to ensure a tight seal. After attachment to the stem, the 3-way stopcock was opened to vent any pressure created during attachment and then closed to allow only a 2-way flow between the pipette and the plant stem. To measure the xylem pressure, the length of the bubble within the pipette (L_X) was recorded while the stopcock was closed. The stopcock was then opened, and the length of the bubble at atmospheric pressure (L_{atm}) was measured. Xylem pressure (P_X) was then calculated as: $P_X = 100*[(L_{atm}/L_X)-1]$ (Ewers et al., 1997; Fisher et al., 1997). Xylem pressure was measured daily, between 11am and 3pm, beginning in mid-February when the winter k_h stem samples were collected until May when the spring k_h samples were collected. Temperature data was recorded using a HOBO U10 temperature data logger (Onset Computer Corporation).

Data Analysis

The response of $k_{sinitial}$, k_{smax}, and P_{75} were analyzed using a two-way ANOVA with line, treatment, and their interaction effects as factors using SAS® software (SAS Institute Inc.). Tukey's HSD was performed to compare means at each factor level. For positive xylem pressure, P_X, a two-way ANOVA was performed using time (day) and line

as factors, and Tukey's HSD was performed on each factor in SAS.

Results

The response of specific conductivity to winter and spring treatments for F5H and wt lines was inconsistent between both years of experiments. No significant difference was found for initial k_S for line, treatment, or interaction effects during either year. In 2009 the average maximum k_S increased for all lines from winter to spring treatments. Mean values for k_{smax} for wt, F5H37 and F5H64 lines were 3.7619, 3.7701, and 2.8553, respectively, in winter and 4.0759, 4.5234, and 4.1694, respectively, in the spring (Figure 3-1). However, the 2010 data showed a declining trend in mean k_{smax} from winter to spring treatments. Mean values of k_{smax} for all lines ranged from 2.7 – 3.27 in the winter and 1.51 – 2.0 in the spring (Figure 3-1). ANOVA results for $k_{sinitial}$ and k_{smax} showed no significant difference between line or treatment effects for 2009, but treatment effects were significant ($\alpha < 0.05$) for 2010 data (Table 3-1; Table 3-2). Figure 3-1 shows mean k_{smax} for winter and spring treatments plotted against percent syringyl content.

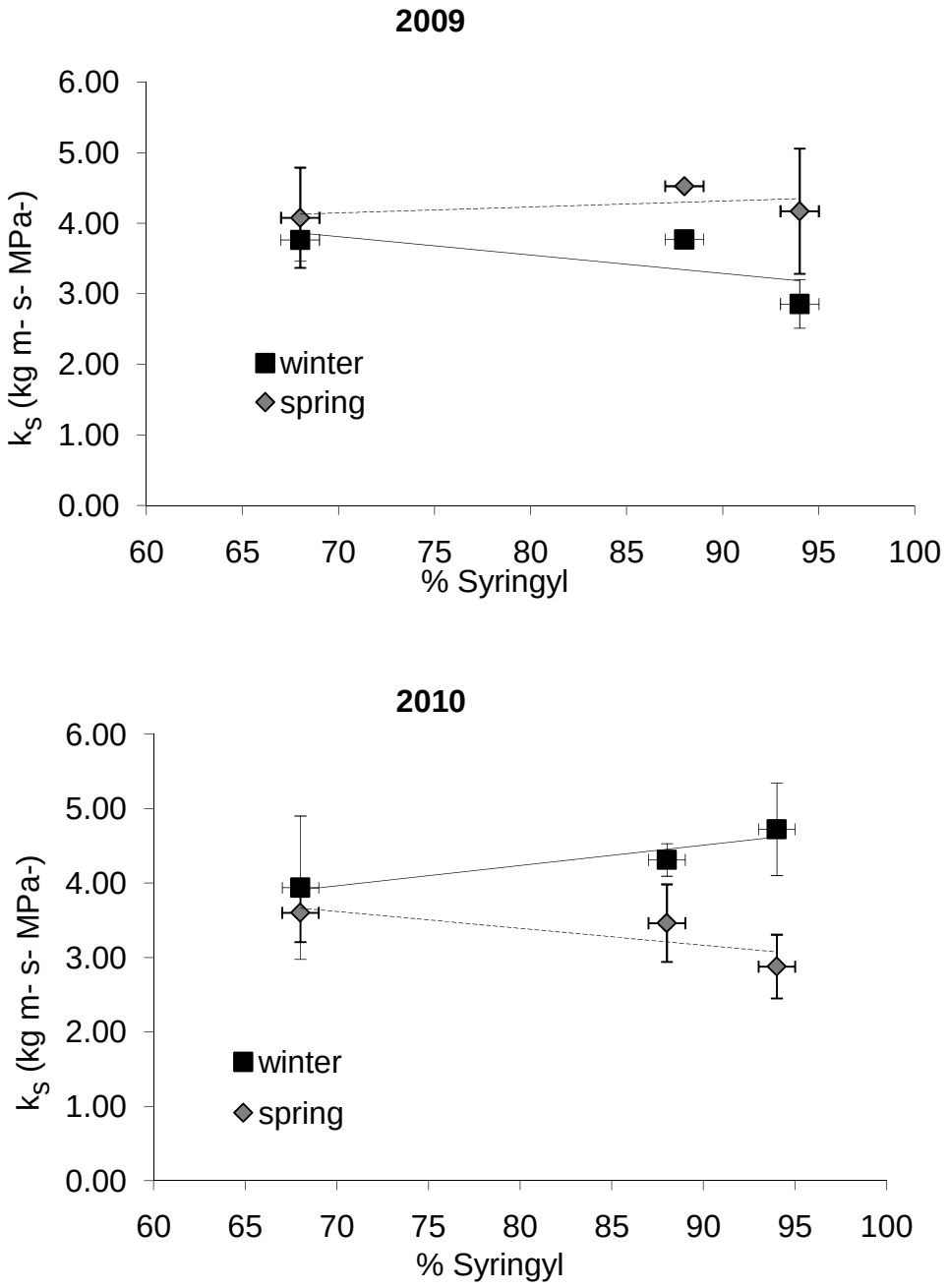
Table 3-1. ANOVA results for yearly initial specific conductivity ($k_{sinitial}$) for WT, F5H37, and F5H64 lines for winter and spring treatments (Trt). 2010 data includes analysis on effects of transplanted versus non-transplanted plants (tran). Significant (α<0.05) terms are in bold.

Response	Year	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
$k_{sinitial}$	2009	0.4522	0.3452	Line	2	1.5949	1.21	0.3473
				Trt	1	1.7148	2.60	0.1453
				Line*Trt	2	0.1268	0.10	0.9093
				Residuals	8	5.2707		
	2010	0.8245	<.0001	Line	2	0.1668	0.13	0.8819
				Trt	1	1.0824	1.64	0.2125
				tran	**1**	**70.3011**	**106.53**	**<.0001**
				Line*Trt	2	0.6835	0.52	0.6023
				Line*tran	2	0.9021	0.68	0.5144
				Trt*tran	1	0.4975	0.75	0.3939
				Line*Trt*tran	2	0.7478	0.57	0.5749
				Residuals	24	15.8379		

Table 3-2. ANOVA results for yearly maximum specific conductivity (k_{smax}) for WT, F5H37, and F5H64 lines for winter and spring treatments (Trt). 2010 data includes analysis on effects of transplanted versus non-transplanted plants (tran).Significant (α<0.05) terms are in bold.

Response	Year	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
k_{smax}	2009	0.2984	0.6511	Line	2	0.8145	0.36	0.7077
				Trt	1	1.7014	1.51	0.2542
				Line*Trt	2	0.7518	0.33	0.7260
				Residuals	8	9.0215		
	2010	0.8288	<.0001	Line	2	0.5686	0.38	0.6895
				Trt	**1**	**14.2018**	**18.86**	**0.0002**
				tran	**1**	**70.1044**	**93.11**	**<.0001**
				Line*Trt	2	1.3386	0.89	0.4242
				Line*tran	4	0.2096	0.14	0.8708
				Trt*tran	2	0.5439	0.72	0.4038
				Line*Trt*tran	4	0.5243	0.35	0.7095
				Residuals	24	18.0706		

Figure 3-1. Linear regression of maximum specific conductivity (k_{smax}) against syringyl content (%) for winter (*solid line*) and spring (*dashed line*) treatments from 2009 (*top*) and 2010 (*bottom*). For 2009, WT and F5H64 n=3; F5H37 n=1. For 2010, WT, F5H37, and F5H64 n=3.



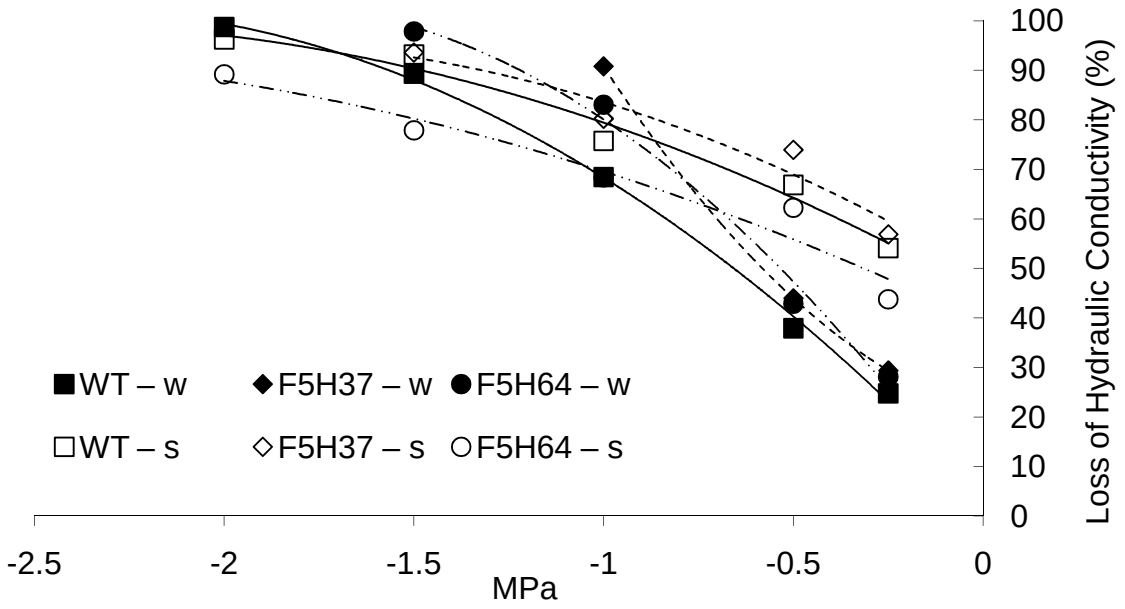
Vulnerability to cavitation also differed for each year of experiments. The vulnerability curve for 2009 shows spring treatments more vulnerable to cavitation at lower pressures compared to the winter treatment (Figure 3-2a). In 2010, however, winter treatments were more vulnerable to cavitation than the spring treatment (Figure 3-2b). P_{75} values for the winter treatment were similar for both years ranging from -1.15 to -0.86 MPa (Figure 3-3). P_{75} values for spring treatment in 2009 show a trend of decreasing P_{75} values as syringyl content increases with mean P_{75} for WT and F5H37 lines reaching 75 percent embolism at higher pressures than the winter treatments (Figure 3-3a). In 2010 mean P_{75} values for all lines were significantly lower than winter treatments and no clear trend can be observed between lines (Figure 3-3b). ANOVA results show no significant effects for 2009, but the treatment effect was significantly different in 2010 (Table 3-3).

Table 3-3. ANOVA results for yearly xylem pressure at 75% loss of conductivity (P_{75}) for WT, F5H37, and F5H64 lines for winter and spring treatments (Trt).Significant ($\alpha<0.05$) terms are in bold.

Response	Year	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
P_{75}	2009	0.2296	0.7848	Line	2	0.1277	0.23	0.8019
				Trt	1	0.0918	0.33	0.5837
				Line*Trt	2	0.4198	0.75	0.5047
				Residuals	8	2.2519		
	2010	0.5051	0.0005	Line	2	0.0109	0.06	0.9415
				Trt	**1**	**2.7474**	**30.37**	**<.0001**
				Line*Trt	2	0.0115	0.06	0.9386
				Residuals	30			

Figure 3-2. Percent loss of conductivity versus xylem pressure (P_X) for winter (*solid*) and spring (*empty*) treatments from a) 2009 and b) 2010. For 2009, WT and F5H64 n=3; F5H37 n=1. For 2010, WT, F5H37, and F5H64 n=3.

a)



b)

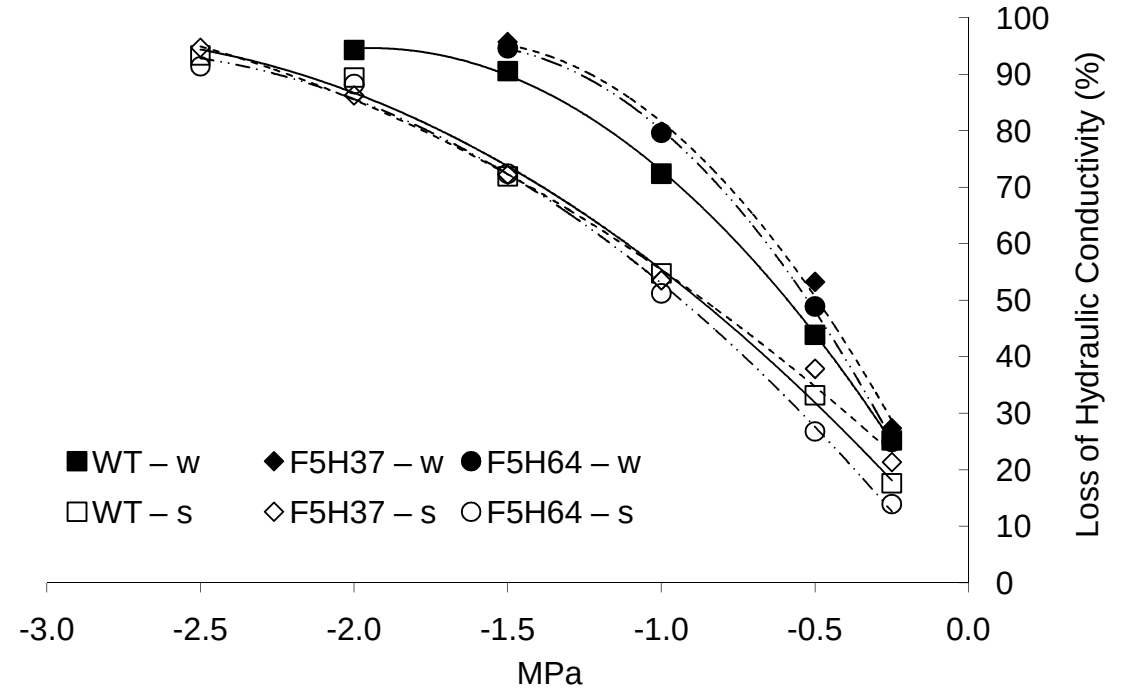
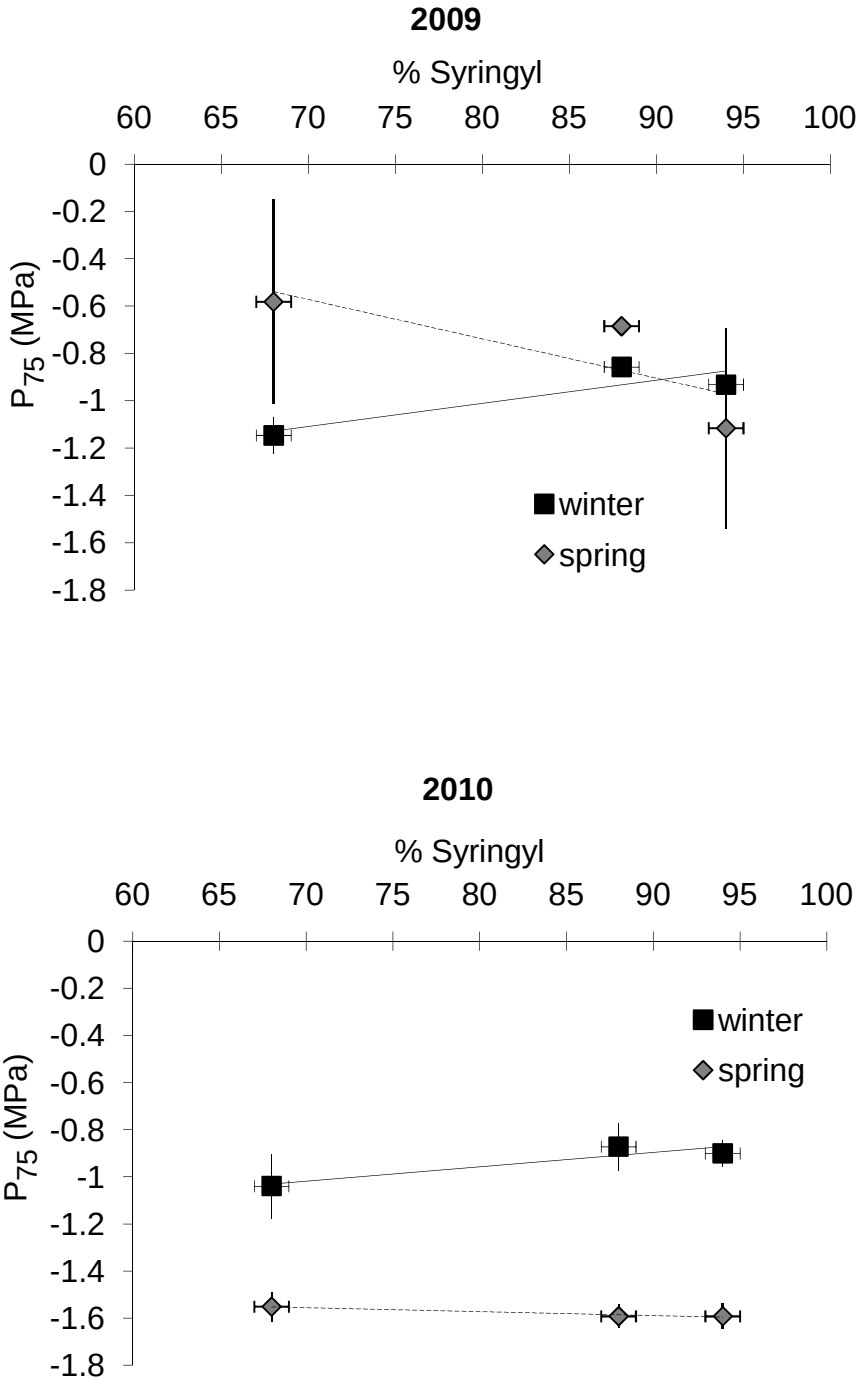


Figure 3-3. Regression of xylem pressure at 75% loss of conductivity (P_{75}) against syringyl content (%) for winter (*solid line*) and spring (*dashed line*) treatments from 2009 (*top*) and 2010 (*bottom*). For 2009, WT and F5H64 n=3; F5H37 n=1. For 2010, WT, F5H37, and F5H64 n=3.



Positive xylem pressure was not detected in any of the lines throughout the 2010 experiment. ANOVA results showed no significant difference in xylem pressure over time or between lines (Table 3-4).

Table 3-4. ANOVA results for positive pressure manometer measurements of xylem pressure (P_X) for WT, F5H37, and F5H64 lines. Significant ($\alpha<0.05$) terms are in bold.

Response	Adj R^2	F (*p*-value)	Term	Df	Type III SS	F-value	*P*-value
P_x	0.2598	0.3216	day	50	25.5861	1.15	0.2341
			Line	2	0.9208	1.03	0.3566
			Line*day	100	45.2663	1.02	0.4462
			Residuals	459	204.4966		

Discussion

Based on these results we can conclude that increasing syringyl content has no significant effect on the plant's response to freeze-thaw stress and spring recovery. All lines showed similar responses in both k_S and P_{75} values. The lack of significant difference between k_S values between winter and spring treatments in 2009 suggests that embolism reversal did not occur. In 2010, spring k_S values were significantly lower than the winter treatment. Samples in the spring were collected as leaves were beginning to expand. The increase in tension created by transpiration returning as leaves become active could be causing the decreased k_S values observed in 2010. The inconsistent response of xylem embolism in poplar from one year to the next has been observed previously (Sperry et al., 1994; Hacke and Sauter, 1996).

Lack of positive xylem pressures in *Populus* is in agreement with observations by Hacke and Sauter (1996). Refilling of embolised vessels in Populus could be influenced by the high occurrence of vessels in contact with each other. This increases the probability of embolised vessels located beside several water-filled vessels which could aid in dissolution of gas bubbles (Hacke and Sauter, 1996).

In summary, increased S:G does not affect the response of hybrid poplars to freeze-thaw stress. Both the response to winter freeze-thaw induced embolism and spring recovery are not affected by lignin monomer content and are likely influenced by xylem anatomical structure as reported by previous studies (Ewers, 1985; Sperry and Sullivan, 1992).

CHAPTER 4

GENERAL CONCLUSIONS

The overall objective of this research was twofold: to experimentally evaluate the function of lignin monomers with respect to water transport in hybrid poplars and how genetic modification of lignin monomer ratios, specifically increasing S:G, affects water transport and physiological response to drought and freezing stress. Results from these experiments can also be used to assess the viability of using transgenic hybrid poplars with increased S:G in economic forest plantations.

It was hypothesized that increased S:G would result in an increased hydraulic efficiency, i.e. increased k_S. While $k_{sinitial}$ and k_{smax} was observed to have a positive trend with increased S:G when averaged across all four experimental years, this trend was not statistically significant. It may be valuable to incorporate RNAi transgenic lines that have a reduced S:G ratio in future experiments. This positive trend observed, while not significant here, may be only part of the picture, and data from transgenic lines with lower S:G may help illuminate the broader role that S and G lignin play in the hydraulic functions of xylem. No significant difference in hydraulic conductivity was calculated between lines for well watered treatments, thus the hypothesis that increased S:G will increase k_S can be rejected. P_{75} values show a positive relationship with increasing S:G. This is consistent with the hypothesis that increasing S:G would increase vulnerability to cavitation. F5H37 showed significantly less negative P_{75} than WT and F5H64 suggesting reduced resistance to embolism. It is unclear why F5H37 is more vulnerable to cavitation than F5H64 at this time. Further research that incorporates a

larger number of transgenic lines with a gradient of multiple S:G values, as well as, RNAi lines with decreased S:G is needed to better understand how lignin monomers fully affect the function of vessels with respect to vulnerability to cavitation. The increased vulnerability to cavitation is likely caused by increased brittleness and susceptibility to microfracture in the cell wall as a result of changes to the mechanical properties of vessel element cell walls and neighboring fiber cells (Jacobsen et al., 2005).

Water transport and physiological properties were all significantly reduced by water stress. However, the response of F5H lines to water stress was not significantly different from the WT for both k_S and P_{75} values. Plants that underwent the re-watered treatment were unable to restore k_{smax} values to those observed in well watered treatments.

Physiological properties were not significantly different between lines under both well watered and stressed treatments. From a whole plant perspective, this shows that any changes to xylem functioning caused by increased S:G were not significant enough to effect leaf gas exchange and photosynthesis.

Response to winter freeze-thaw stress was not affected by S:G. Results for k_S and P_{75} for winter and spring treatments were inconsistent between study years. This lack of significant difference between F5H and WT lines and inconsistency between years suggests that other factors may be involved in the response to freeze-thaw stress and spring recovery. Xylem structure, specifically vessel lumen diameter, most likely has a stronger affect on determining the plant's response to freeze-thaw stress as reported in previous literature (Ewers, 1985; Sperry and Sullivan, 1992).

Overall, these results show that modification of S:G in hybrid poplars will reduce

xylem resistance to embolism but has no significant effect on leaf physiology, efficiency of water transport, and response to drought and winter freezing stress at the tissue level. Further research is needed, however, to fully evaluate the effects of changes to S:G on xylem functioning at the cellular level. Taken all together, these results show that increasing S:G does not significantly alter xylem functioning and physiological properties. Thus, the use of transgenic hybrid poplars with increased S:G will not have reduced biomass production compared to the WT and should be considered as a viable alternative for use in economic tree plantations.

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