INFLUENCE OF SYRINGYL TO GUAIACYL RATIO AND GRAVITY ON GROWTH RESPONSES AND PHYSICAL PROPERTIES IN GENETICALLY ALTERED POPLARS (*POPULUS TREMULA* X *P. ALBA*)

By

Jameel Muein Al-Haddad

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Plant Biology
Ecology, Evolutionary Biology and Behavior

2012

ABSTRACT

INFLUENCE OF SYRINGYL TO GUAIACYL RATIO AND GRAVITY ON GROWTH RESPONSES AND PHYSICAL PROPERTIES IN GENETICALLY ALTERED POPLARS (*Populus tremula* x *P. Alba*)

By

Jameel Muein Al-Haddad

Lignin is a phenolic polymer that is a fundamental part of the structure and function of plants. It provides mechanical support, acts as a hydrophobic insulation for the water conducting elements and is a strong shield against phytopathogens. Lignin is made of variable combinations of three monomers; syringyl (S), guaiacyl (G) and *p*-hydroxyphenyl (P) units. For the first time, the combined effects of modified lignin composition and gravity on the growth responses and physical properties were studied in genetically altered poplar. We hypothesized that enhancing the syringyl monomer will have an impact on the gravitropic responses and the mechanical and physiological properties of upright and inclined trees. Hybrid poplar clone 717 (*Populus tremula x P. alba*) was transformed to over express the *F5H/Cald5H* gene at different levels resulting in varying syringyl to guaiacyl ratios. Wild type poplar and the transgenic lines were either kept upright or inclined (45°) from vertical to induce gravitropic responses (tension wood formation) for three months. Over-expression of *F5H* in the tilted trees caused reduced growth rate than the upright wild type controls and a reduction in height to diameter ratio. Interestingly, the tilted stems with the highest syringyl lignin ratio responded faster and recovered back to vertical at least 8 days before the tilted wild type trees. Quantification of released growth strain showed less strain in the trees with the highest syringyl lignin percentage potentially due to a decrease in growth strain earlier than the other genetic lines at the end of the gravitropic period. When all stems were collected and mechanically tested while still fresh or oven-dried, four-point bending and compression tests showed similar elastic properties among the different genetic lines when the trees were kept upright. However, when tested until rupture, the over-expression lines had lower modulus of rupture (MOR) indicating an increase in brittleness. To test the effects of the genetic treatment and/or the gravitropic responses on the trees water conductive properties, the maximum hydraulic conductivity was measured on the stems after high pressure flushing to eliminate native emboli. Interestingly, the stems with higher syringyl lignin ratio showed improved water conductive efficiency in the upright stems. Anatomically, it was found that the lines with higher syringyl lignin abundance had larger vessel diameters and higher percent vessel lumen area. Percent total lumen area of vessels and fibers also increased whereas percent fiber area decreased. However, dry wood density was not affected by the lignin modification. Tension wood had higher fiber wall thickness but less percent total lumen area due to the formation of the gelatinous layer in the G-fibers of the tension wood. Moreover, wood samples from the different treatments and lines were analyzed for cell wall biochemical and structural properties. In the tension wood, increases in cellulose crystallinity and microfibril angle were among the expected results. Lignin quantitative analysis showed a slight decrease in total lignin with increasing syringyl fraction. The acid soluble lignin also increased significantly. Carbohydrate analyses indicated higher percent total sugars in tension wood than in opposite or normal wood. Interestingly, increasing syringyl lignin percentage resulted in slight decrease in percent total sugars. Similar results were observed for glucose and galactose percentages. Percent xylose was lower in the tension wood than in the opposite or normal woods. Before a large scale plantation of the genetically altered poplar trees can be assumed, further testing in the field will be required to verify their ability to withstand more complex stresses.

DEDICATION
My parents, my brothers and sisters and their children

ACKNOWLEDGMENTS

My utmost appreciation goes to my degree advisor Frank Telewski for his guidance and tremendous patience throughout the many years I have known him. I thank him for his continued care and concern with my success and well-being. I also thank my graduate committee members Frank Ewers, Kyung-Hwan Han and Kenneth Keegstra for their guidance and valued input. I thank the staff of the department of Plant Biology for their generous help and moral support. I thank Paul Taylor for providing the department with the annual financial support that made my conference travel and participation a possiblity. I thank Jason Kilgore for the support he provided me with academically and socially, and for his continued mentorship. Finally, I thank the staff of the Office of International Students and Scholars of Michigan State University for the help and advice.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO SYMBOLS OR ABBREVIATIONS

MOE	Modulus of elasticity
MOR	Modulus of rupture
EI	Flexural stiffness
F5H	Ferulate 5-hydroxylase
I	Second moment of cross sectional area
MFA	Microfibril angle

CHAPTER 1

INTRODUCTION

BACKGROUND

Evolution of lignin

With the evolution of terrestrial plants from aquatic ancestors around 475 million years ago (Boyce *et al*., 2004; Peter and Neale, 2004; Kendrick and Crane, 1997) (Figure 1.1) came the necessity to fortify the cell walls of the plants to withstand the different environmental stresses and to adopt an erect growth form. Studies of modern era plants showed that a complex aromatic heteropolymer (lignin) makes a vital contribution to the long-term survival of some perennials (Pedersen *et al*. 2005). Lignin provides mechanical support through cementing the secondary cell wall matrix, binding to cellulose microfibrils (Wainwright *et al*., 1982) and cross-linking other cell wall components (Martone *et al*., 2009; Ralph *et al*., 2004). In addition, it provides rigidity to vessel walls enabling them to withstand high negative pressures without collapse (Kitin *et al*., 2010). Lignin also provides a defense line against plant pathogens (Bhuiyan *et al*., 2009). The timeframe of lignin evolution is still unknown especially with the discovery of lignin in red algae which diverged from vascular plants more than one billion years ago (Martone *et al*., 2009).

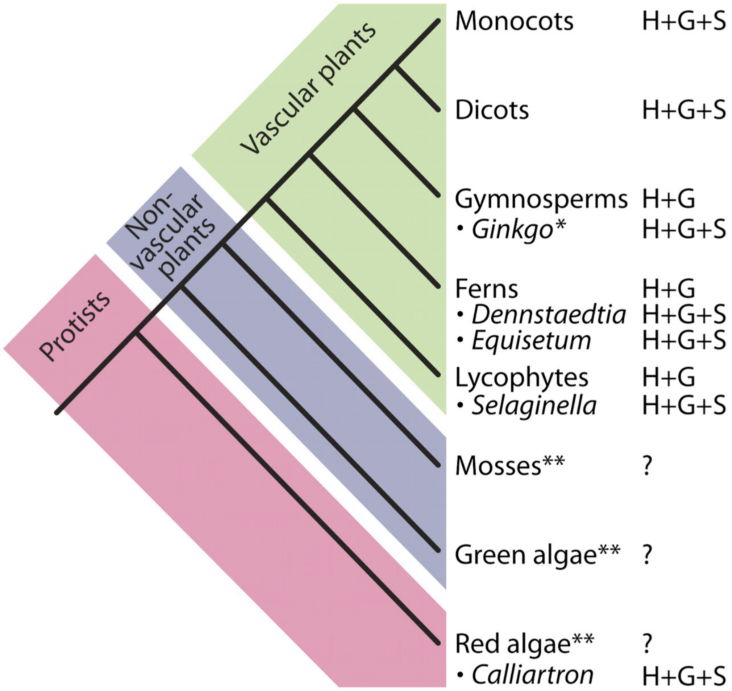

Figure 1.1 Phylogenetic tree showing the distribution of lignin monomer composition across major lineages (Vanholme *et al*. 2010).
S = syringyl lignin, G = guaiacyl lignin and H = *p*-hydroxyphenyl lignin. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Cell wall components and their roles

The first attempt to study the chemical composition of woody plants was made in 1838 by the French chemist and botanist, Anselme Payne (Schubert, 1965). He found, despite the complexity and diversity of wood species, they all contain three major chemical components. They are cellulose, the hemicelluloses and lignin.

Cell wall polysaccharides: (compiled from Liepman *et al*., 2007; Somerville, 2006; Somerville *et al*., 2004; Higuchi, 1997)

Cellulose, the most abundant organic molecule on earth, is a linear polymer that is built of 1,4-glucosidic linkage of β-D-glucose forming parallel β-1,4-glucan chains. Every 36 glucan chains are held together by hydrogen bonding to form cellulose microfibril. The length of the microfibril varies depending on species or activity of the CESA enzymes that build the glucan chains. The crystalline region of the microfibrils is the part made of those continuous chains built by the 36 CESA enzymes in the plasma membrane-embedded resette subunits. Making 50% of the cell wall, the microfibrils constitute a skeleton for other wall constituents to anchor to such as hemicelluloses and structural proteins creating a matrix in which lignin is later deposited.

Hemicelluloses. Branched heteropolymers cross-link the cellulose microfibrils in almost all plant cell wall matrices. They are divided into four classes; xyloglucans which are heavily substituted β-1,4-glucan backbone (mostly xylose and glucose), glucomannans are variably substituted β-1,4-mannan backbone (glucose and mannose), arabinoxylan made of substituted β-1,4-linked xylan backbone (mostly arabinose and xylose), and mixed linkage glucans made of unsubstituted backbone of β-1,4- and β-1,3-linkages of glucosyl residues.

Pectins. Representing one third of plant cell wall polysaccharides, pectins are major component of primary cell wall. They are a group of complex polysaccharides containing 1,4-α-D-linked galacturonic acid. Various sugars bind to the polygalacturonate including rhamnose, arabinose, fucose, galactose, and xylose.

Lignin

Lignin is a phenolic compound ranking second behind cellulose in abundance on earth (Dashtban *et al*., 2010). It acts like a cementing material in xylem tissues of trees providing support and insulation for water conduction in fibers and vessels, respectively. Lignin polymer is made of three types of monolignols; syringyl, guaiacyl and *p*-hydroxyphenyl residues (Figures 1.1). Lignin in gymnosperms is predominantly made of guaiacyl whereas *p*-hydroxyphenyl occurs at higher concentrations in the grass family but is less pronounced in hardwood (Higuchi, 1997). To understand the role played by each of the monolignols, mainly syringyl and guaiacyl, in trees mechanics and physiology numerous studies have been made (for literature refer to Table 1.1).

Types of reaction wood

Gymnosperms and angiosperms are not closely related, yet they both develop reaction wood in response to gravity and other environmental stimuli. These special tissues aid the stems and branches to move and regulate their form (Timell, 1986). Reaction wood is genetically determined and is influenced by the environment. Various factors and stimuli are known to cause plant responses and wood formation including temperature, light, water, load (ice, snow, fruit etc.), wind and gravity (Coutand, 2010; Telewski, 2006; Zimmermann and Brown, 1971). Reaction wood formed in gymnosperms is referred to as compression wood, which forms on the lower side of the leaning stems and branches. From its name, the tissue forms a compressive stress pushing the stem in the other direction. Its anatomy is characterized by round tracheids with intra-cellular spaces (Timell, 1986). The cell walls are thicker than normal and contain more lignin but less cellulose (Du and Yamamoto, 2007; Timell, 1986). In angiosperms, environmental stimuli, such as gravity, impact the tree's shape and orientation by means of formation of specialized xylem tissue called tension wood (Pilate *et al.*, 2004). In the case of inclined stems, it is formed only on the upper side of the stem while the other side is considered opposite wood. The major anatomical characteristic of tension wood is a unique fiber with an additional inner layer of gelatinous appearance (gelatinous layer), which is mostly composed of cellulose. In this layer the highly crystalline microfibrils are oriented almost parallel to the longitudinal axis of growth (Pilate *et al.*, 2004). The combined mechanical strength of lignin in opposite wood and cellulose in the gelatinous fibers of tension wood is necessary for trees to perform gravitropic responses.

Wood biomechanics

To sustain an erect growth form plants rely on their mechanical strength to counteract the gravity force. Responses to external stimuli such as snow buildup and wind require the plant stems to demonstrate capacity to flex or enhance strength in order to reduce or avoid permanent damage (Telewski and Jaffe, 1986a, 1986b; Telewski, 1995). Inside the stem, the negative pressure that exists inside the vessels or tracheids is an internal stress. This stress leads to the formation of strain in the cell walls. If the strain exceeds the elastic range the wall fails and vessel cavitation may occur leading to loss of function (Jacobsen *et al.*, 2005).

Lignin cements the cell wall structural network of cellulose, hemicellulose and structural proteins. The monomer composition of lignin was found to vary across different plant species according to the functional requirements of different cell types (Yoshinaga *et al*., 1992) and in response to environmental conditions such as drought stress (Donaldson, 2002). Parameters that influence the mechanical properties of wood have become a major concern to researchers. These parameters include lignin monomer ratio, lignin content and other cell wall structural components such as microfibril angle (MFA) (see Table 1.1 for summary of literature). Even after development and apoptosis, the strong cell wall ultrastructure is able to accommodate external stresses and adjust accordingly by means of modifying the microfibril angle (Pilate *et al*., 2004). The angle is formed by the cellulose microfibrils and the stem axis. A decrease in the angle was associated with an increase in longitudinal maturation strains in the tension wood of angiosperms (Baillères *et al.*, 1995;

Yoshida *et al.*, 2002). Nevertheless, an increase of the angle was associated with the formation of compression wood in gymnosperms (Wilson and Archer, 1977; Timell, 1986). Whether the microfibril angle and the maturation strains have a cause-effect relationship is still under investigation.

In addition to being scientifically researched, the manufacturing industry is concerned with the mechanical properties of wood, for buildings and furniture.

Water relations

The water conductive properties of vascular plants, as influenced by the lignin and other cell wall components (Boyce *et al*., 2004; Boerjan *et al*., 2003; Sederoff *et al*., 1999; Lewis and Yamamoto, 1990), are critical to the survival of the plant. These properties significantly influence the ability to withstand stresses imposed by the surrounding environment such as wind (Kern *et al*., 2005) and gravity (Pilate *et al*., 2004), as well as internal stresses such as the shrinkage of tension wood (Clair *et al*., 2011), or the negative pressure inside the xylem vessels (Voelker *et al*. 2011; Woodrum *et al*., 2003; Hacke *et al*., 2001).

Xylem vessels are responsible for conducting water from the deep roots to the highest branches and leaves (Tyree and Zimmermann, 2002). To do so, trees must maintain a continuous water column from roots to the canopy, even under extreme negative pressures (Tyree, 1997), as is the case in the tall *Eucalyptus* trees. Lignin, which makes up 20-30% of the wood, is a major contributor to these mechanical abilities of the woody plants.

Table 1.1 Summary of some literature on biomechanics and cell wall structural components

Researcher(s)	Year	Worked on	Concluded
Voelker SL, Lachenbruch B, Meinzer FC, Strauss SH	2011a	Poplar	Reductions in lignin of up to 40% gave comparable losses in wood strength and stiffness
Voelker SL, Lachenbruch B, Meinzer FC, Kitin P, Strauss SH	2011b	Poplar	*4CL*-downregulated trees had significantly lower growth efficiency
Kitin P, Voelker SL, Meinzer FC, Beeckman H, Straussss SH, Lachenbruch B	2010	Poplar	Reduced transport efficiency in low-lignin xylem was caused by blockages from tyloses and phenolic deposits within vessels rather than by xylem collapse
Wang Y, Grill J, Clair B, Minato K, Sugiyama J	2010	*Viburnum odoratissimum*	S/G ratio showed high correlation with growth strain in the lower side of branches
Coutand C, Fournier M, Moulia B	2007	Poplar (*Populus nigra* x *Populus deltoids*)	Maturation strains differ in the tension woods produced during the upward curving and decurving phases
Franke R, Hemm MR, Denault JW, Ruegger MO, Humphreys JM, Chapple C	2002	*Arabidopsis thaliana L. Heynh*.	Lignin has a role in resisting the tension generated during water transpiration
Yoshida M, Ohta H, Yamamoto H, Okuyama T	2002	Yellow poplar	Found a negative correlation between growth strain, lignin content, and the microfibril angle of cellulose in the cell wall
Franke R, McMichael CM, Meyer K, Shirley AM, Cusumano JC, Chapple C	2000	*Nicotiana tabacum*	No significant effect of lignin with high syringyl lignin on mechanical strength and water conductivity in *C4H/F5H* mutant plants

Table 1.1 (cont'd)

Baillères H, Chanson B, Fournier M, Tollier MT, Monties B	1995	*Eucalyptus*	Found a negative correlation between growth strain, lignin content, and the microfibril angle of cellulose in the cell wall
Yoshinaga A, Fujita M, Saiki H	1992	Oak	S:G ratio was found to be variable across different cell types
Boyd JD	1977	*Eucalyptus regnans, Eucalyptus sieberi,* and *Pinus radiata*	Found a strong correlation between the cellulose microfibril angle and the longitudinal shrinkage reactions due to growth strains in wood
Mosha Y,Goring DAI	1975	Hardwood	Higher syringyl in fiber lignin in contrast to higher guaiacyl in vessel lignin

Kinematics, microfibril angle and crystallinity

Efforts have also been made to correlate or to discover cause-effect relationships among the different plant cell wall components (Table 1.1). Baillères *et al*. (1995) found a negative correlation between either growth strain or lignin content and the microfibril angle of cellulose in the cell wall of *Eucalyptus*. They also found a positive correlation between growth strain and syringyl to guiacyl unit ratio. Similar results were observed during a study on yellow poplar (Yoshida *et al*., 2002). Boyd (1977) found that the cellulose microfibril angle of the cell wall S2 layer influenced the growth strains and longitudinal shrinkage reactions in wood. Coutand *et al*. (2007) studied the kinematics of inclined poplar stems as affected by the internal maturation strains of the reaction wood at different stages of stem rightening with respect to gravity. Wang et al. (2010) studied the relationship between the growth strains (GS) of the upper and lower surfaces of *Viburnum odoratissimum* var. *awabuki* branches and the microfibril angle in the cellulose and discovered a unique case of growth biomechanics where GS distribution was similar to tension and compression woods on opposite sides of the branches yet without anatomical evidence of reaction wood existence.

Modifying lignin

Due to the economic importance of poplar as a source of wood and fiber in plantation forestry, and due to the increasing interest in its wood as a potential source of renewable cellulose to serve the demand for sustainable biofuel, there have been an increasing interest in profiling the transcriptome of woody plants (Prassinos *et al.*, 2005; Ko and Han, 2004) to genetically modify the composition of wood to suit the purposes of the industry (Huntley *et al*., 2003). For better paper and pulp qualities, attempts have been made to genetically alter the lignin synthetic pathway to make lignin extraction a more feasible process (Huntley *et al*., 2003).

Due to the detrimental effect of reducing the total lignin content in poplar, lignin composition has been modified while keeping the lignin content unchanged. In one study on poplar, Huntley *et al*. (2003) found that lignin solubility is positively correlated with syringyl monomer content in the lignin matrix. As a result, bleaching chemicals can treat the matrix more effectively. Their results indicated a 60% reduction in pulping time of trees with increased S:G ratio, which potentially would increase pulp mill capacity while decreasing chemical costs. Based on the demand of the energy sector, poplar trees with down-regulated lignin have been produced to provide easier access to renewable cellulose.

As a result of 40% reduction in total lignin, Voelker *et al.* (2011) found a reduction in wood stiffness and strength. They also reported a reduction in growth and impaired xylem conductivity. An optimum balance between reduced lignin and biomass growth has been recently proposed (Novaes *et al.*, 2010).

Whether genetically modified trees can have a sustained growth and tolerate environmental and internal growth stresses is the primary interest of this investigation. *Ferulate 5-hydroxylase* (F5H) is a gene that encodes for an enzyme that acts downstream in the lignin synthesis pathway, converting 5-hydroxy-coniferylaldehyde and 5-hydroxyconiferylalcohol towards the synthesis of sinapyl alcohol, i.e. syringyl (Franke *et al*., 2000). The F5H mutants are prime candidates for use in this study, allowing us to investigate the direct influence of changed lignin monomer composition on growth responses and physical properties in a well known model system with no other apparent alterations in cell wall structure or tissue composition to bias the results.

RESEARCH OBJECTIVES

The main objective of this research was to test the ability of the genetically altered poplar trees to respond to gravity and maintain physical and growth properties that are comparable to those of the unmodified wild type trees.

The specific objectives were:

- To investigate the kinematic responses as well as to quantify the growth strains generated in the tension wood. And to evaluate the overall stem mechanical properties of the poplar lines.
- To investigate the water relations in the upright stems as well as in those inclined and formed tension wood in response to gravity. Also to investigate the leaf area changes and the anatomy of the three wood tissue types.

- To determine the changes in the wood’s carbohydrate composition and other structural features such as crystallinity and microfibril angle.

REFERENCES

REFERENCES

Andersson-Gunnerås S, Mellerowicz EJ, Love J, Segerman B, Ohmiya Y, Coutinho PM, Nilsson P, Henrissat B, Moritz T, Sundberg B (2006) Biosynthesis of cellulose-enriched tension wood in *Populus*: global analysis of transcripts and metabolites identifies biochemical and developmental regulators in secondary wall biosynthesis. Plant J **45**:144-165.

Baillères H, Chanson B, Fournier M, Tollier MT, Monties B (1995) Structure, composition chimique et retraits de maturation du bois chez les clones d'Eucalyptus. Ann Sci For **52**:157-172.

Bhuiyan NH, Selvaraj G, Wei Y, King J (2009) Role of lignification in plant defense. Plant Signal Behav **4**(2):158-159.

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol **54**:519-546.

Boyce CK, Zwieniecki MA, Cody GD, Jacobsen C, Wirick S, Knoll AH, Holbrook NM (2004) Evolution of xylem lignification and hydrogel tranport regulation. PNAS **101**(50):17555-17558.

Boyd JD (1977) Relationship between fibre morphology and shrinkage of wood. Wood Sci Technol **11**:3-22.

Clair B, Almeras T, Pilate G, Jullien D, Sugiyama J, Riekel C (2011) Maturation stress generation in poplar tension wood studied by synchrotron radiation microdiffraction. Plant Physiol **155**:562-570.

Coutand C (2010) Mechanosensing and thigmomorphogenesis, a physiological and biomechanical point of view. Plant Sci **179**:168-182.

Coutand C, Fournier M, Moulia B (2007) The Gravitropic response of poplar trunks: key roles of prestressed wood regulation and the relative kinetics of cambial growth versus wood maturation. Plant Phyiol 144:1166-1180.

Dashtban M, Schraft H, Syed TA, Qin W (2010) Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol **1**(1):36-50.

Donaldson LA (2002) Abnormal lignin distribution in wood from severely drought stressed Pinus radiata trees. J Int Assoc Wood Anatomists 23(2):161-178.

Du S, Yamamoto F (2007) An overview of the biology of reaction wood formation. J Integrative Plant Biol **49**(2):131-143.

F**ranke R, Hemm MR, Denault JW, Ruegger MO, Humphreys JM, Chapple C** (2002) Changes in secondary metabolism and deposition of an unusual lignin in the ref8 mutant of Arabidopsis. Plant J **30**(1):47-59.

Franke R, McMichael CM, Meyer K, Shirley AM, Cusumano JC, Chapple C (2000) Modified lignin in tobacco and poplar plants over-expressing the Arabidopsis gene encoding ferulate-5-hydroxylase. Plant J **22**:223-234.

Hacke UG, Sperry JS, and Pockman WT (2001) Trends in wood density and structure are linked to prevention of xylem implosion by negative pressure. Oecologia **126**:457-461.

Higuchi T (1997) Biochemistry and molecular biology of wood. Springer-Verlag, Berlin-Heidelberg-New York.

Huntley SK, Ellis D, Gilbert M, Chapple C, Mansfield SD (2003) Significant increases in pulping efficiency in C4H-F5H-transformed poplars: improved chemical savings and reduced environmental toxins. J Agric Food Chem **51**:6178-6183.

Jacobsen AL, Ewers FW, Pratt RB, Paddock III WA, Davis SD (2005) Do xylem fibers affect vessel cavitation resistance? Plant Physiol **139**:546-556.

Kendrick P, Crane PR (1997) The origin and early evolution of plants on land. Nature **389**:33–39.

Kern KA, Ewers FW, Telewski FW, Koehler L (2005) Mechanical perturbation affects conductivity, mechanical properties and aboveground biomass of hybrid poplars. Tree Physiol **25**(10):1243-1251

Kitin P, Voelker SL, Meinzer FC, Beeckman H, Strauss SH, Lachenbruch B (2010) Tyloses and phenolic deposits in xylem vessels impede water transport in low-lignin transgenic poplars: a study by cryo-fluorescence microscopy. Plant Physiol **154**:887-898.

Ko JH, Han K-H (2004) Arabidopsis whole-transcriptome profiling defines the features of coordinated regulations that occur during wood formation. Plant Mol Biol **55**:433-453.

Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Ann Rev Plant Physiol Plant Mol Biol **41**:455–496.

Liepman AH, Cavalier DM, Lerouxel O, Keegstra K (2007) Cell wall structure, biosynthesis, and assembly. In Plant Cell Separation and Adhesion, eds. Roberts, J. & Gonzalez-Carranza, Z. (Blackwell Publishing, Oxford), Annual Plant Reviews Vol. **25**, pp. 8-39.

Martone PT, Esteves JM, Lu F, Ruel K, Denny MW, Somerville C, Ralph J (2009) Discovery of lignin in seaweed reveals convergent evolution of cell-wall architecture. Curr Biol **19**:169-175.

Mosha Y, Goring DAI (1975) Distribution of syringyl and guaiacyl moieties in hardwood as indicated by ultraviolet microscopy. Wood Sci Technol **9**:45-58.

Novaes E, Kirst M, Chiang V, Windter-Sederoff H, Sederoff R (2010) Lignin and biomass: a negative correlation for wood formation and lignin content in trees. Plant Physiol **154**(2):555-561.

Pedersen JF, Vogel KP, Funnell DL (2005) Impact of reduced lignin on plant fitness. Crop Sci **45**:812-819.

Peter G, Neale D (2004) Molecular basis for the evolution of xylem lignification. Curr Opin Plant Biol **7**:737–742.

Pilate G, Chabbert B, Cathala B, Yoshinaga A, Leplé J-C, Laurans F, Lapierre C, Ruel K (2004) Lignification and tension wood. C. R. Biologies **327**:889-901.

Pittermann, J (2010) The evolution of water transport in plants: an integrated approach. Geobiology **8**:112–139.

Prassinos C, Ko J-H, Yang J, Han K-H (2005) Transcriptome profiling of vertical stem segments provides insights into the genetic regulation of secondary growth in hybrid aspen trees. Plant Cell Physiol **46**(8):1213-1225.

Ralph J, Bunzel M, Marita JM, Hatfield RD, Lu F, Kim H, Schatz PF, Grabber JH, Steinhart H (2004) Peroxidase-dependent cross-linking reactions of p-hydroxycinnamates in plant cell walls. Phytochem Rev **3**:79–96.

Schubert WJ (1965) Lignin Biochemistry. Academic Press Inc., New York.

Sederoff RR, MacKay JJ, Ralph J, Hatfield RD (1999) Unexpected variation in lignin. Curr Opin Plant Biol **2**:145-152.

Somerville C (2006) Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol **22**:53-78.

Somerville C, Bauer S, Brininstool G, Facette M, Hamann T, Milne J, Osborne E, Paredez A, Persson S, Raab T, Vorwerk S, Youngs H (2004) Toward a systems approach to understand plant cell walls. Science **306**:2206-2211.

Telewski FW (1995) Wind-induced physiological and developmental responses in trees. *In* Wind and Trees. Eds. M.P. Coutts and J. Grace. Cambridge University, Cambridge, pp. 237–263.

Telewski FW (2006) A unified hypothesis of mechanoperception in plants. Am J Bot **93**(10):1466-1476.

Telewski FW, Jaffe MJ (1986a) Thigmomorphogenesis: field and laboratory studies of *Abies fraseri* in response to wind or mechanical perturbation. Physiol Plant **66**:211–218.

Telewski FW, Jaffe MJ (1986*b*) Thigmomorphogenesis: anatomical, morphological, and mechanical analysis of genetically different sibs of *Pinus taeda* in response to mechanical perturbation. Physiol Plant **66**:227-233.

Timell TE (1986) Compression wood in gymnosperms. Vol. I, Springer-Verlag, Berlin.

Tyree MT (1997) The cohesion-tension theory of sap ascent: current controversies. J Exp Bot **48**(315):1753-1765.

Tyree MT, Zimmermann MH (2002) Xylem structure and the ascent of sap, 2nd ed., Springer-Verlag, Berlin.

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W (2010) Lignin biosynthesis and structure. Plant Physiol **153**:895-905.

Voelker SL, Lachenbruch, B, Meinzer FC, Kitin P, Strauss SH (2011b) Transgenic poplars with reduced lignin show impaired xylem conductivity, growth efficiency and survival. Plant Cell Environ **34**:655-668.

Voelker SL, Lachenbruch B, Meinzer FC, Strauss SH (2011a) Reduced wood stiffness and strength, and altered stem form, in young antisense 4CL transgenic poplars with reduced lignin contents. New Phytol **189**(4):1096-1109.

Wainwright SA, Biggs WD, Currey JD, Gosline JM (1982) Mechanical design in organisms. Princeton University Press, Princeton, New Jersey.

Wang Y, Grill J, Clair B, Minato K, Sugiyama J (2010) Wood properties and chemical composition of the eccentric growth branch of *Viburnum odoratissimum* var. *awabuki.* Trees **24**:541-549.

Wilson BF, Archer RR (1977) Reaction wood: induction and mechanical action. Ann Rev Plant Physiol **28**:23-43.

Woodrum CL, Ewers FW, Telewski FW (2003) Hydraulic, Biomechanical, and Anatomical Interactions of Xylem from Five Species of Acer (*Aceraceae*). Am J Bot **90**(5): 693-699.

Yoshida M, Ohta H, Yamamoto H, Okuyama T (2002) Tensile growth stress and lignin distribution in the cell walls of yellow poplar, Liriodendron tulipifera Linn. Trees Struc Funct **16**:457-464.

Yoshinaga A, Fujita M, Saiki H (1992) Relationships between cell evolution and lignin structural varieties in oak xylem evaluated by microscopic sectrophotometry with separated cell-walls. Mokuzai Gakkaishi **38**:629-637

Zimmermann MH, Brown CL (1971) Trees: Structure and Function. Springer-Verlag, Berlin.

CHAPTER 2

EFFECT OF SYRINGYL LIGNIN RATIO ON GROWTH RESPONSES AND MECHANICAL PROPERTIES OF INCLINED GENETICALLY-ALTERED POPLARS (*POPULUS TREMULA* X *P. ALBA*)

ABSTRACT

Lignin is an important phenolic polymer of secondary cell walls that is known to influence the mechanical properties of secondary xylem (wood). The major monomers of angiosperm lignin are syringyl and guaiacyl. This study evaluates the effect of modifying lignin composition on the response of trees to gravity following inclination. Changes in kinematics and mechanical properties were investigated in one-year old hybrid poplar clone 717 (*Populus tremula* x *P. alba*) that was transformed with *C4Hprom::F5H* resulting in an increased syringyl lignin ratio. Wild type poplars and the transgenic lines were inclined 45° from vertical for three months to induce a negative gravitropic response (stem curvature and tension wood formation). Inclination, and the resulting gravitropic response stimulated tension wood formation in stems and caused decreases in tree allometry and stem growth. Interestingly, increasing the syringyl lignin content of stems resulted in a faster adjustment to gravity, by up to eight days, compared to the unmodified wild-type controls. The stems of the upright and inclined trees had similar elastic properties. However, the stems of the inclined trees were stiffer when the tension wood side was under tension during the bending test. Evaluating the gravitropic response of the transgenic poplar lines to inclination improved our understanding of the mechanical and physiological role of lignin monomer composition in normal and tension wood.

INTRODUCTION

The mechanical strength of wood is partially attributed to its lignin content. In angiosperms, lignin is primarily composed of two monomers, syringyl (S) and guaiacyl (G) units. The monomer composition of lignin was found to vary across different plant species, according to the functional requirements of different cell types (Yoshinaga *et al*., 1992), and in response to environmental conditions such as drought stress (Donaldson, 2002). Huntley *et al*. (2003) found that lignin solubility is positively correlated with syringyl monomer content in the lignin, making the wood more amenable to bleaching chemicals. They also reported a 60% reduction in pulping time for wood with increased S:G ratio, leading to increase pulp mill capacity while decreasing chemical and energy costs.

Poplar trees have the advantage of being the most common model for perennial plants serving experimental research (Stettler et al., 1996; Herschbach and Kopriva, 2002) in addition to their economic importance as a source of wood and fiber in plantation forestry. This same perennial model is also being used to study the different aspects of xylem composition and function and the trees reaction to mechanical stimuli imposed by the environment (Kern et al., 2005; and reviewed in Telewski, 2006). Environmental stimuli, such as gravity, impact the shape and form of trees by means of longitudinal growth stresses which can reorient the stem to a new upright position aided by the formation of specialized xylem tissue called tension wood (Pilate et al., 2004). The major anatomical characteristic of tension wood in poplar is the unique fibers with an additional cell wall layer of gelatinous appearance (gelatinous layer) that is predominantly made of highly crystalline cellulose microfibrils (Pilate *et al*., 2004). The combined mechanical strength of lignin in the opposite wood and cellulose microfibrils in the gelatinous fibers of tension wood enables the trees to adjust to mechanical or environmental stimuli such as gravity (Pilate et al., 2004).

The mechanical properties of tension wood have not been extensively studied despite their distinctive physical properties. These properties are evident in the differential shrinkage characteristics of tension wood, and the difficulty to achieve a smooth surface in wood samples that contain it (Illston et al., 1979; Hoadley, 2000). In a study comparing tension wood to normal wood in young poplar, Coutand et al. (2004) reported higher Young's modulus and higher level of longitudinal residual strain as proof of the unique mechanical properties of this specialized tissue.

Similarly, little is known about the effect of modified lignin content and composition on the mechanical properties of woody plants such as poplar. The ability of the trees to sustain mechanical support of their own crown weight was reported to been affected by the lignin content (Gindl and Teischinger, 2002). Due to the relatively longer time to form a useable volume of wood, it was necessary to utilize young transgenic trees to investigate the effect of modified lignin content on trees wood properties. To test the mechanical properties of young genetically modified trees, newly developed testing techniques were tailored to the age and size of these new stock plants (Kasal et al., 2007). Subsequently, Horvath et al. (2010) studied the mechanical properties of young genetically modified aspen trees with modified lignin content or structure. They found a significant reduction in the mechanical properties due to reduced lignin content, and a slight reduction due to increased S:G ratio. A more recent study showed comparable losses in wood strength and stiffness and lower growth efficiency due to reduced lignin content in young poplar (Voelker *et al*., 2011a and b). Other similar studies have focused on the effects of either lignin modification or the formation of tension wood in trees. However, no study has investigated the interaction between lignin modification and tension wood formation. Therefore, this chapter describes, for the first time, a study that combined the effects of both tension wood formation and lignin structure modification on the mechanical properties of stems of a perennial model plant. The study was carried out by investigating the gravitropic response to displacement with respect to gravity leading to tension wood formation in the transgenic hybrid poplar (*Populus tremula* x *P. alba*) trees with modified lignin. Investigating the impact of lignin composition in transgenic lines of the same species will minimize possible bias introduced by other anatomical parameters when different plant species or different growth forms of the same species are compared. It was hypothesized that genetically modified lignin monomer ratio will have an impact on hybrid poplar growth responses in the form of altered allometry and kinematics. In addition, significant changes in the mechanical properties of the wood are also expected. It was predicted that elevation of syringyl lignin ratio and inclination of the trees, leading to the formation of tension wood in the stems, would cause increases in the modulus of elasticity (MOE) and modulus of rupture (MOR) in poplar stems.

MATERIAL AND METHODS

Experimental Plan

This study was conducted on wild type hybrid poplar clone 717 (*Populus tremula* x *P. alba*) and transgenic lines altered to over-express *C4Hprom::F5H* at different levels (Huntley *et al*., 2003). The transgenic lines were obtained from David Ellis and Clint Chapple laboratories and have been vegetatively-propagated over multiple generations using root sprouts. Young trees (100-120 cm) were grown under greenhouse conditions on Michigan State University campus. Pots (5-liter) with soil-less medium (Baccto, Michigan Peat Company, Houston, TX) were used with no additional fertilization or supplemental lighting. The trees were grown for three months under two different regimes: (1) undisturbed, and (2) displaced with regard to gravity. In each experiment, six trees of each line were used with mole percent syringyl ranging from 67% on average (unmodified hybrid poplar clone 717) to 94% on average (genetically modified line with lignin almost entirely made of syringyl) at the beginning of the experiment. Allometric and kinematic responses to gravity, longitudinal growth strain, and mechanical properties of the stems were analyzed. The experiment was conducted twice in two separate growth seasons to reproduce the results. The basic experimental design included the concurrent testing of all hypotheses in every experimental setting. The kinematics of response to gravity and the release of longitudinal strain were measured in separate experiments.

Morphology and Allometry

Stem height and basal stem diameter were measured on all trees at the beginning and end of each experiment to track allometric changes. The thinnest and thickest diameters were measured using a digital vernier caliper at the soil level of each stem. The average of the two extreme diameters was used in the analysis. In addition, the most recent fully expanded leaf was labeled on each tree to mark the beginning of secondary growth as well as to allow for quantification of the new growth.

Kinematics of Response to Gravity

Digital images were captured individually for all tilted trees from a fixed angle and location during the experimental period (Sep 08 2010-Nov 03 2010). An method used to study the kinematics of curvature fields in tilted 2-year-old pine trees (*Pinus pinaster* Ait.) (Sierra-de-Grado et al., 2009) was adapted to investigate the trees kinematic responses in this study. . Using image analysis software ImageJ (ver. 1.43u, Wayne Rasband, National Institutes of Health, USA) five angles were determined after virtually dividing the stems into three major segments; the first segment was represented by the basal part of the stem with only radial growth, the second segment was represented by the middle part of the stem, and the last segment was represented by the upper stem part with least secondary growth (Figure 2.1). The deviation of each of these stem segments from horizontal was measured (absolute angles A01, A12 and A23, respectively). And the relative angles formed by each two adjacent stem segments, the lower two stem segments (A012) and upper two segments (A123), were also measured.

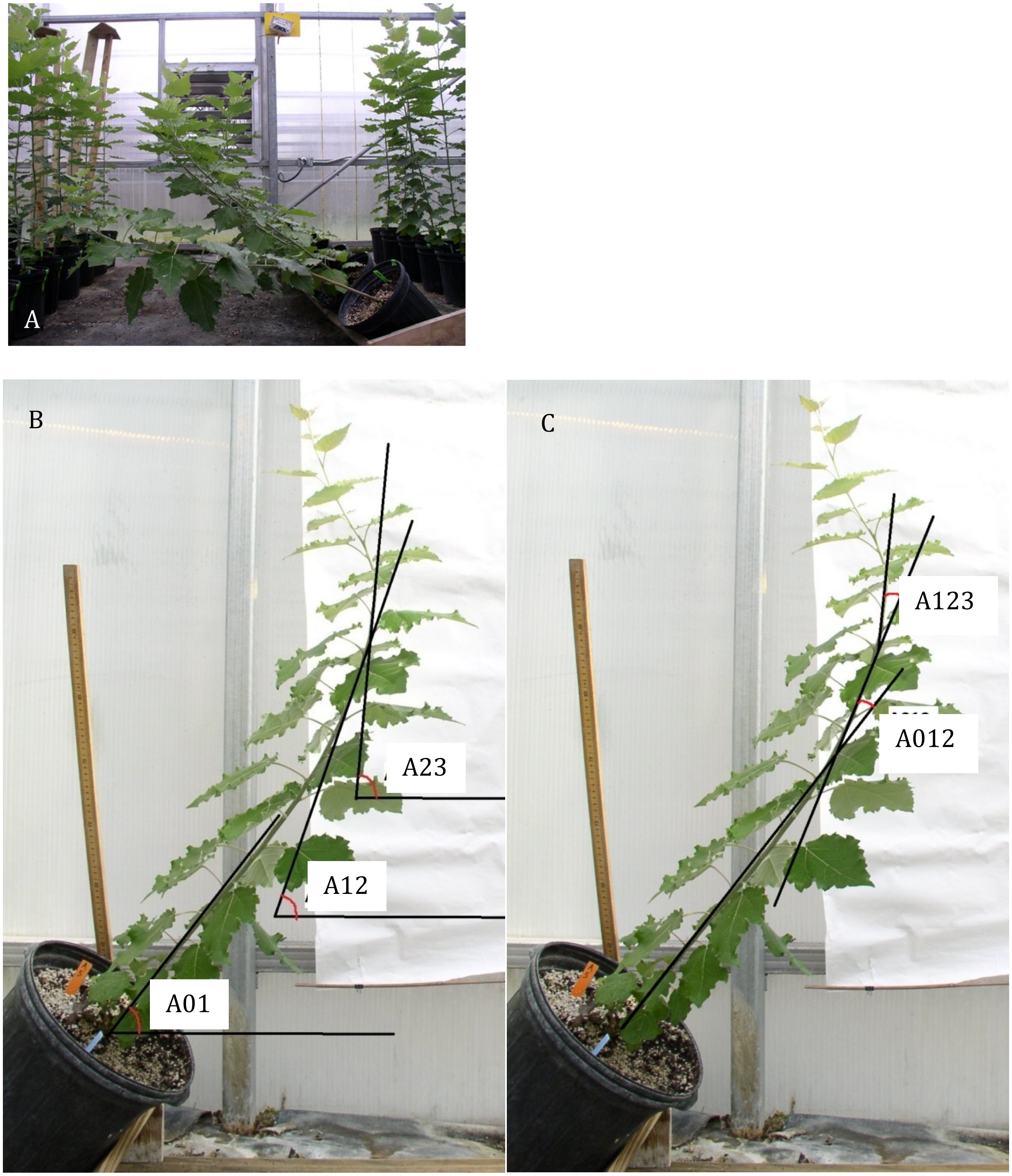

Figure 2.1. A. Wild-type and transgenic poplar trees inclined 45° from vertical. Trees of different S:G ratio respond to gravity at varying degrees. Digital images were taken at different stages and were used to draw three absolute angles (B) and two relative angles (C) using three stem segments; lower, middle and upper.

Measuring growth Strains

A strain gauge method (Yoshida and Okuyama, 2002) was used to measure growth strain in poplar trees in which strain gauges were glued to fresh, bark-free stem surfaces under which tension wood was expected to form (upper surface of the inclined poplar stems). The gauges (EP-08–250BG-120, Vishay Precision Group, Malvern, Pennsylvania, USA) were bonded to the wood surfaces longitudinally along the grain (Figure 2.2) using a contact adhesive compatible with wet surfaces (M-Bond 200, bonding was catalyzed by Catalyst-C 200, Vishay Precision Group, Malvern, Pennsylvania, USA). A three-wire quarter-bridge circuit was used to allow for automatic compensation for the effects of temperature changes in the lead wires on the bridge balance, and for increased measurement sensitivity. To reduce tension induced by wires, terminal strips (CEG-75C) were glued to the xylem surfaces and connected to the strain gauges using thin copper wires (134-AWP) as was recommended by the manufacturer (Vishay Precision Group, Malvern, Pennsylvania, USA). To allow recovery of wood shrinkage, cuts were made above, below and underneath each gauge to a depth equivalent to about half the radius of the respective stems. Continuous readings were taken every second during the cutting process using a strain indicator and recorder (P3 Strain Indicator and Recorder, Vishay Precision Group, Malvern, Pennsylvania, USA) until a stabilized reading was achieved. The terminal readings were considered for subsequent analyses and comparisons.

Figure 2.2. (Left) A strain gauge was bonded to the wood surface longitudinally along the grain of inclined hybrid poplar trees that recovered the vertical position. (Right) Released strain was measured every second during the cutting process using a P3 Strain Indicator and Recorder.

Mechanical properties

Intact green and oven-dried debarked stems were used for mechanical testing. The data allowed for the calculation of the modulus of elasticity (MOE), modulus of rupture (MOR) and flexural stiffness (*EI*). Also known as Young's Modulus, MOE is equal to the flexural stiffness divided by the second moment of xylem cross sectional area (Niklas, 1992). The *EI* and MOR were calculated, and MOE was derived from *EI* and the second moment of cross sectional area (*I*). Whereas MOE was calculated within the elastic range of the stems, stems were loaded until failure in order to determine MOR (Wagner *et al*., 1998). Six-tree replication was used in 2006, and 22-tree replication in 2008.

Four-point bending. Bending generates a combination of tensional forces at the lower side of the sample and compressive at the upper side. In the case of four-point bending, no biasing shear forces were generated. Before debarking the stems, they were cut to be more than ten times longer than their diameter, as was required for the bending tests (Niklas, 1992, Pruyn et al., 2000) and in compliance with the testing standard ASTM D143

(1994). Using an Instron® Universal Machine (Model 4202, Instron Corporation, Canton, MA, USA) (Figure 2.3) equipped with a 10 kN load cell and a crosshead speed of 20 mm/min, 14-cm segments of green stems were loaded within the elastic range. The stems from the inclined trees were tested with the tension wood side facing away from the crosshead, and again while facing the crosshead. The same stems were then dried to a constant weight, equilibrated to room temperature and moisture level, and then loaded again until rupture allowing for the calculation of MOR. To calculate the second moment of area, an idealized oval shape was assumed for the cross sections. This depended on the respective diameters of the axes, which, in the inclined stems, increased parallel to gravity rather than perpendicular.

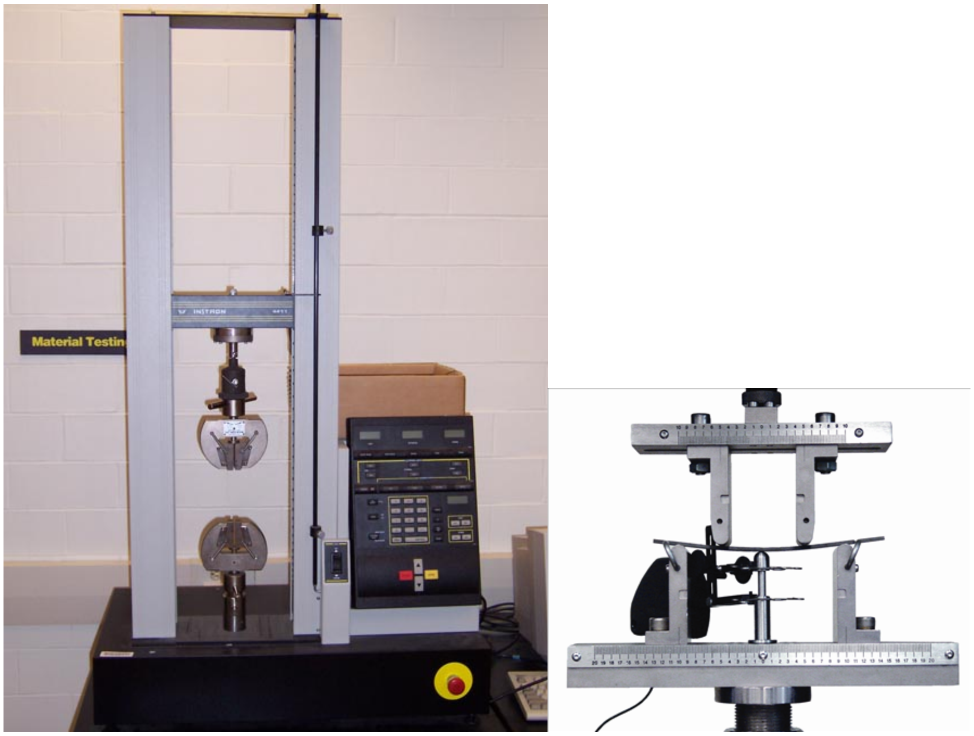

Figure 2.3. An Instron® Universal Machine was used for mechanical testing (left) and can be fitted with a crosshead specific for four-point bending tests (right).

Compression testing. The compressive modulus was measured through testing the stems under compressive force. Cylindrical stem segments from upright trees, twice as long as their diameter, were exposed to compressive force parallel to the grain using the same Instron® Universal Machine equipped with 0.5 kN load cell and a crosshead speed of 3 mm/min.

Replication and Data Analysis

The design included a wild-type and three *F5H* over-expression lines for each of the two treatments (upright vs. inclined), and with a replication of six trees, the total was 48 trees for the experiment. The kinematics experiment included wild-type (~75 mol.% syringyl) and two genetic lines (~83 and ~94 mol.% syringyl). To test for stems' mechanical properties in 2008, 22-upright tree replication for each poplar line was used. Given the balanced design, a 2-way analysis of variance (ANOVA) was used. Data were analyzed using the software R (R Development Core Team, 2007). Assumptions of normality and homogeneity of variances were tested. The kinematic responses data were analyzed using repeated measures ANOVA.

RESULTS

The allometric data from the poplar stems were collected in 2006 after the stems were allowed to respond to gravity and restore the vertical position. Increasing syringyl lignin percentage resulted in a decrease in stem height to diameter ratio in upright and inclined trees (*P*=0.016, correlation coefficient= -0.29) (Figure 2.4A). The slopes of the two regression lines were statistically similar. Therefore, the difference in the intercept represented the difference between the upright and inclined trees. Based on the estimated intercepts, the inclined trees showed a decrease in their height-to-diameter ratio by as much as 16.69 ± 5.12 (± *SE*), relative to the upright trees (*P*=0.002). During the experiment, both the upright and the inclined trees grew at a statistically similar rate (Figure 2.4B). The two regression lines had similar slopes and intercepts. However, a slower growth rate was noticed with increasing syringyl lignin percentage in all of the tested trees (*P*=0.008, correlation coefficient= -0.17).

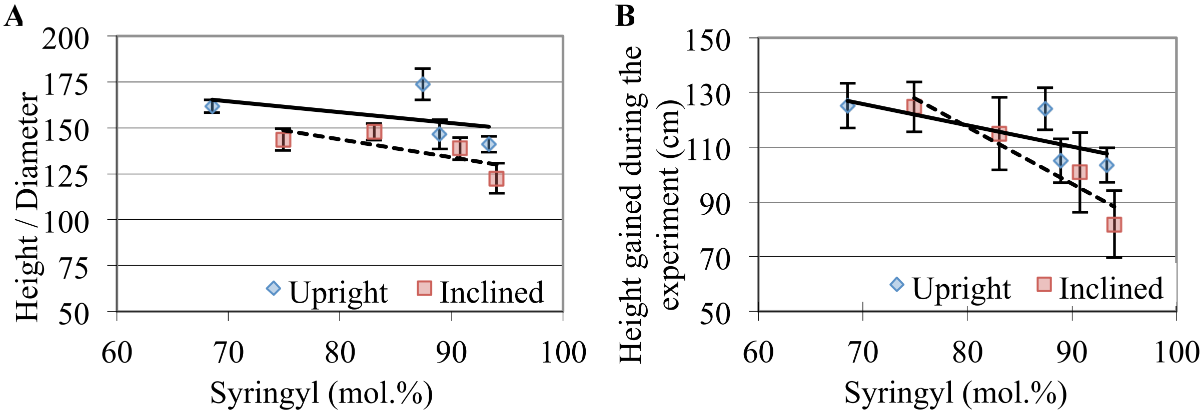

Figure 2.4. Poplar tree allometry results of an experiment, and biochemical analyses, that were conducted in 2006. The syringyl mole percentages that were used with the inclined poplar trees were based on the analysis of the tension wood side of each stem. **A**: The calculated ratio was based on an average of the maximum and minimum diameters at the stem base. **B**: The gained height was measured from the first fully expanded leaf that was marked at the beginning of the experiment. Error bars represent means ± *SE* with *n* = 6.

The kinematics of response to gravity in the wild-type and the three transgenic lines are presented in Figures 2.5-2.9. Figure 2.5 shows the changes in angle A01 that represents the deviation of the basal part of the stem from the horizontal line (check Figure 2.1B) tracked throughout the experimental period. The trees spent the first four days of inclination in the latent phase (sagging behavior) before they started the gravitropic phase. During the gravitropic response, the trees with the highest mole percent syringyl (94 mol.%) maintained a bigger angle (*P*<0.001) than the trees with the lower syringyl lignin ratio (83 mol.%), or the unmodified wild-type trees (75 mol.%).

Based on repeated measure ANOVA, the wild-type trees showed different linear (*P*=0.004) and quadratic (*P*=0.001) trends over time when compared to the two genetic lines with higher syringyl lignin percentage.

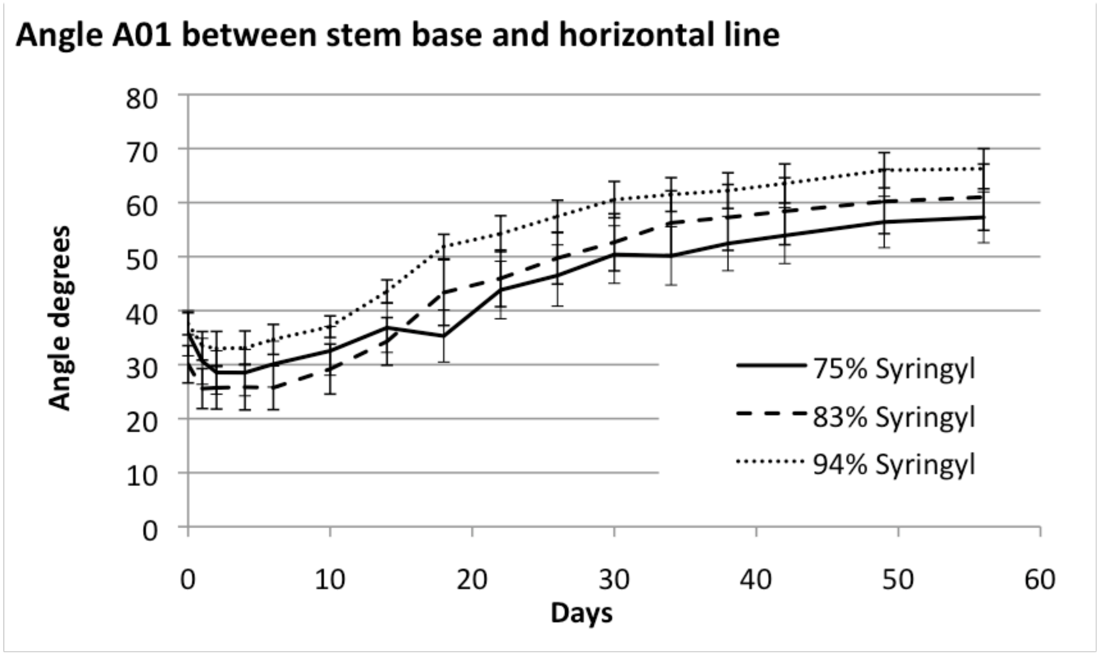

Figure 2.5. A time course of the response of the trees' basal stem segments to inclination as represented by angle A01 between the basal part of the stems and the horizontal line. Poplar trees were genetically modified to increase their production of syringyl lignin from 75 mol.% to 83 mol.% and 94 mol.%. Each data point is an average of six trees. Error bars represent the standard error.

Represented by the absolute angle A12 (Figure 2.6) between the middle stem segment and an arbitrary horizontal line, it took two days for the stems to exit the latent phase (saggy stems) and start the gravitropic responses. Interestingly, the trees with highest mole percent syringyl (94 mol.%) adjusted the middle part of the stem, to gravity, significantly faster (P<0.001) than the stems of the trees with unmodified syringyl (75 mol.%). The fast responding trees adjusted their stems back to a vertical position, with respect to gravity, eight days or less than the trees with the unmodified lignin. All trees reached, and maintained, a maximum angle of 100° from horizontal line, indicating overshooting the perfectly vertical position without further re-adjusting. Similar pattern was observed for the upper segment of the stems A23 (Figure 2.7).

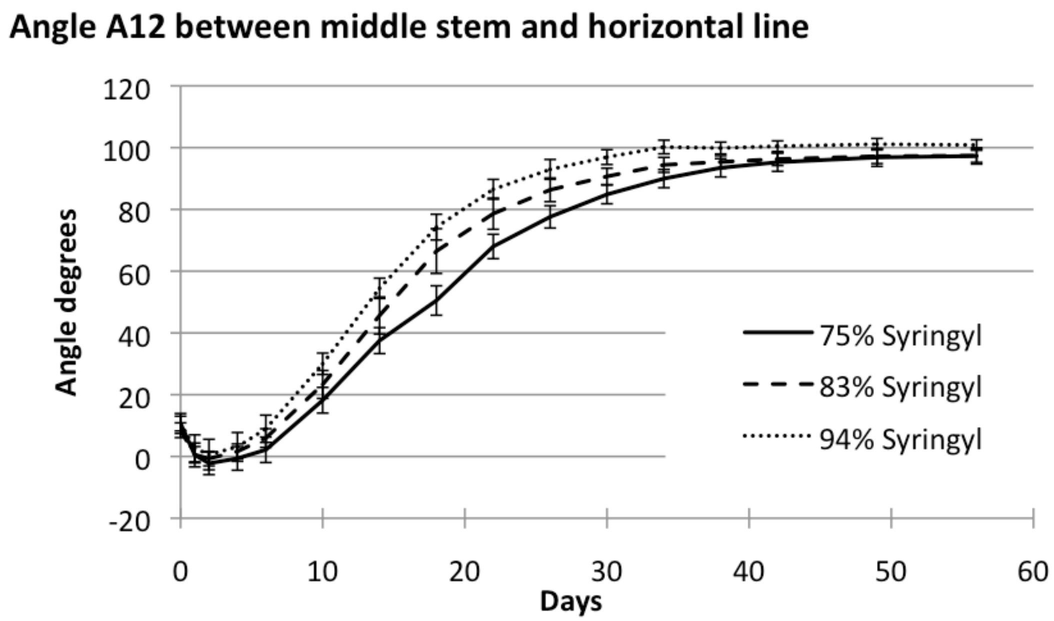

Figure 2.6. A time course of the response of the trees middle stem segments to inclination as represented by angle A12 between the middle part of the stems and the horizontal line. Poplar trees were genetically modified to increase their production of syringyl lignin from 75 mol.% to 83 mol.% and 94 mol.%. Each data point is an average of six trees. Error bars represent the standard error.

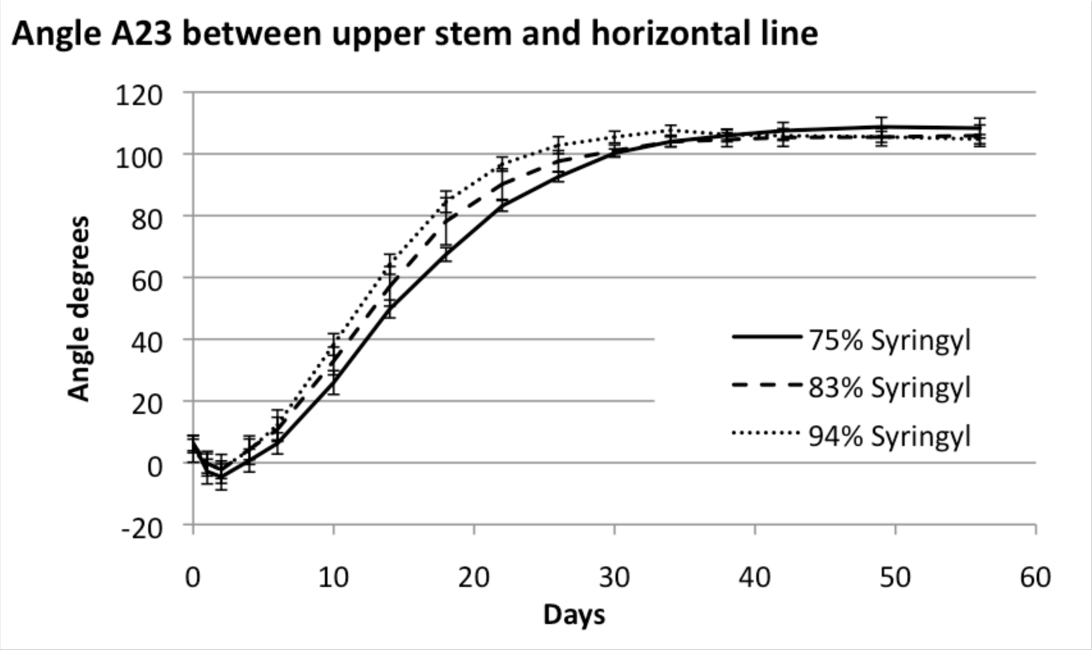

Figure 2.7. A time course of the response of the trees upper stem segments to gravity and inclination in the three hybrid poplar lines as represented by angle A23 between the upper part of the stems and the horizontal line. Poplar trees were genetically modified to increase their production of syringyl lignin from 75 mol.% to 83 mol.% and 94 mol.%. Each data point is an average of six trees. Error bars represent the standard error.

The relative angle between the lower and middle stem segments (A012, check Figure 2.1C) was different among the poplar lines that show different syringyl lignin ratios. Increasing the syringyl monomer ratio in lignin polymer seemed to increase the rate at which the trees adjusted their stems with respect to gravity (Figure 2.8). The initial increase in angle degrees in the negative direction coincided with the trees going through the latent phase (saggy stems) in addition to self-loading. Six days after inclination the trees started a noticeable gravitropic response. The trees with the highest syringyl monomer ratio (94 mol.%) showed a significantly faster response and reached the maximum angle 26 days after inclination. The wild-type control trees achieved their maximum relative angle 8 days later. The other relative angle, between the middle and upper stem segments (A123, check Figure 2.1C), was similar for the three poplar lines during the first 14 days followed by a differential response during the autotropic phase. Figure 2.9 shows poplar trees with the highest syringyl lignin percentage demonstrating earlier onset of straightening (autotropic phase) in the upper stem parts. None of the poplar lines achieved a perfectly vertical position at the conclusion of the recovery.

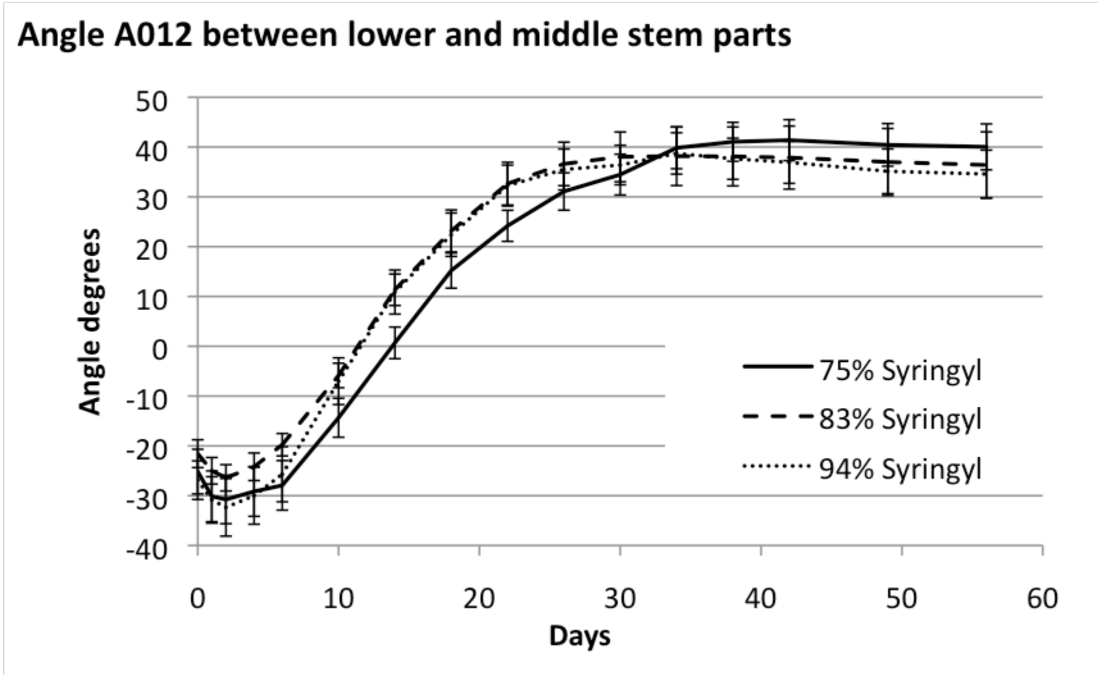

Figure 2.8. A time course of the response of the trees stem segments to inclination as represented by angle A012 between the lower and middle parts of the stems. Poplar trees were genetically modified to increase their production of syringyl lignin from 75 mol.% to 83 mol.% and 94 mol.%. Each data point is an average of six trees. Error bars represent the standard error.

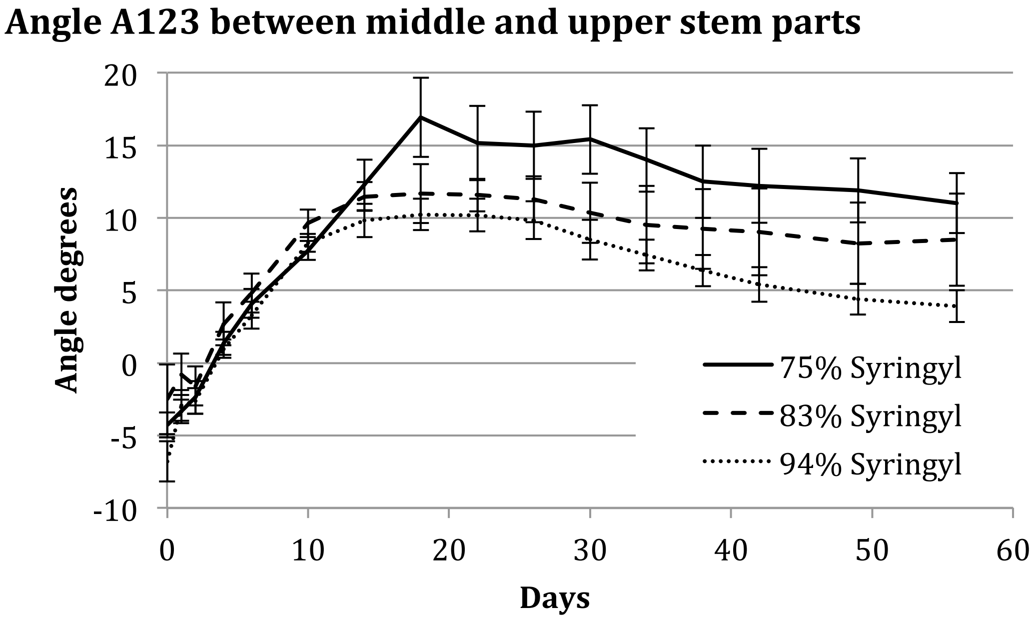

Figure 2.9. A time course of the response of the trees stem segments to inclination as represented by relative angle A123 between the middle and upper parts of the stems. Poplar trees were genetically modified to increase their production of syringyl lignin from 75 mol.% to 83 mol.% and 94 mol.%. Each data point is an average of six trees. Error bars represent the standard error.

The quantitative results of the released tensile force in the tension wood side of the leaning poplar stems are presented in Figure 2.10. The wild-type trees, with ~74 mol.% syringyl lignin in the tension wood side, released the most strain. After the two cuts were made above and below the stain gauge to calculate the difference in released strain, the wild-type trees had a released strain of as much as 277.3±35.6 $\mu\varepsilon$ (mean±*SE*, *n*=6), whereas the two transgenic lines with increased syringyl lignin (83 mol.% and 94 mol.%) had a released strain of as much as 229.5±29.6 $\mu\varepsilon$ and 214.2±13.6 $\mu\varepsilon$, respectively. However, the variation between the wild-type and the transgenic lines was statistically non-significant.

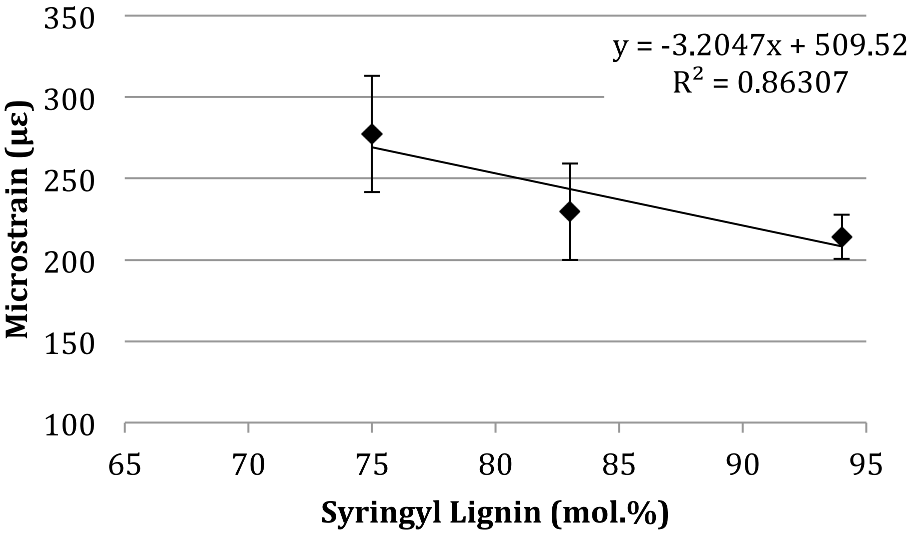

Figure 2.10. Quantification of the released strain in the tension wood side of leaning hybrid poplar stems. The trees have been genetically modified to increase their production of syringyl lignin from 75 mol.% to 83 mol.% and 94 mol.%. Data points are means±*SE*, *n*=6 stems per treatment line.

In an experiment conducted in 2006, the poplar stems of the upright and the inclined trees were harvested and mechanically tested (Figure 2.11). The calculated modulus of elasticity (MOE) was 8189±504 $N.mm^{-2}$ (mean±*SE*) for the freshly harvested stems of the upright wild-type trees (68.5 mol.% syringyl lignin). However, the inclined stems with tension wood facing upward (under compression) towards the crosshead had a calculated MOE of 7639±901 $N.mm^{-2}$. And when the same stems were mechanically loaded while their tension wood was facing downward (under tension) away from the crosshead their calculated MOE was 9139±526 $N.mm^{-2}$, significantly higher than the upright control stems or the inclined stems with tension wood side under compression ($p<0.001$, correlation coefficient=0.30). Except for the transgenic poplar line with the highest syringyl lignin percentage (93.4-94.1 mol.%), which averaged a higher MOE

($P<0.01$), the other two transgenic lines had statistically similar MOE to the wild-type stems.

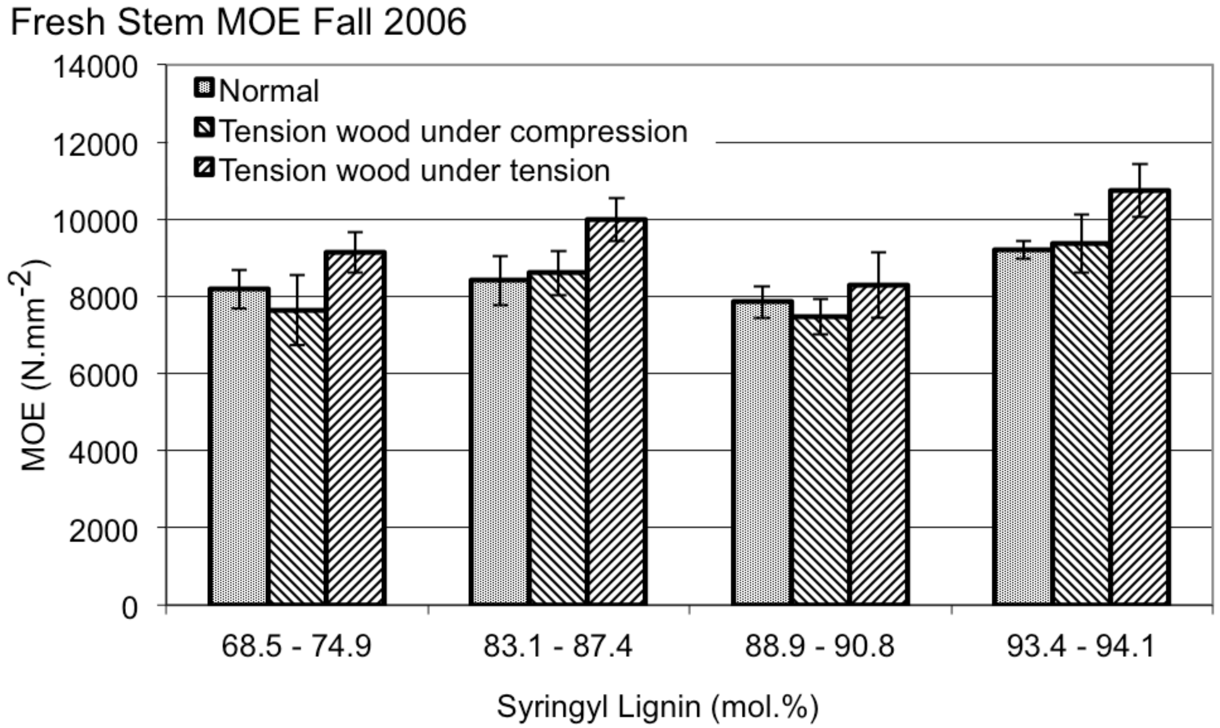

Figure 2.11. Modulus of elasticity (MOE) based on four-point bending of fresh upright and inclined wild type and transgenic hybrid poplar stems based on data collected in 2006, 14-cm long stems were tested immediately after harvest. Three data sets represent the upright stems with normal wood, inclined stems with their tension wood side facing down away from the crosshead, and inclined stems with their tension wood side facing up towards the crosshead. Error bars represent means±*SE*, $n=6$ stems per treatment line.

The calculated elastic modulus (MOE) of the dried stems was determined to be 13681±910 N.mm^{-2} (mean±*SE*) for the upright wild-type stems, and 14884±531 N.mm^{-2} for the inclined stems (Figure 2.12A). The flexural stiffness *EI*, which is influenced by the second moment of the cross sectional area *I*, was 2421627±442523 N.mm^{2} for the upright wild-type stems, and 3027796±376899 N.mm^{2} for the inclined stems (Figure 2.12B). For both MOE and *EI*, the upright stems were statistically similar to the inclined stems in both the wild-type and the transgenic lines with higher syringyl lignin percentages. The upright and the inclined trees were also similar in dry stem strength properties as was represented by the calculated modulus of rupture (MOR) in Figure 2.13. The MOR for the wild-type trees with stems remaining upright (68.5 mol.% syringyl lignin) was 405.4±17.2 N.mm^{-2} (mean±*SE*) compared to 428.6±19.2 N.mm^{-2} for those with inclined stems (74.9 mol.% syringyl lignin). Under the experimental conditions of the 2006 trial, increasing the percentage of syringyl lignin had no effect on the MOR for the transgenic trees.

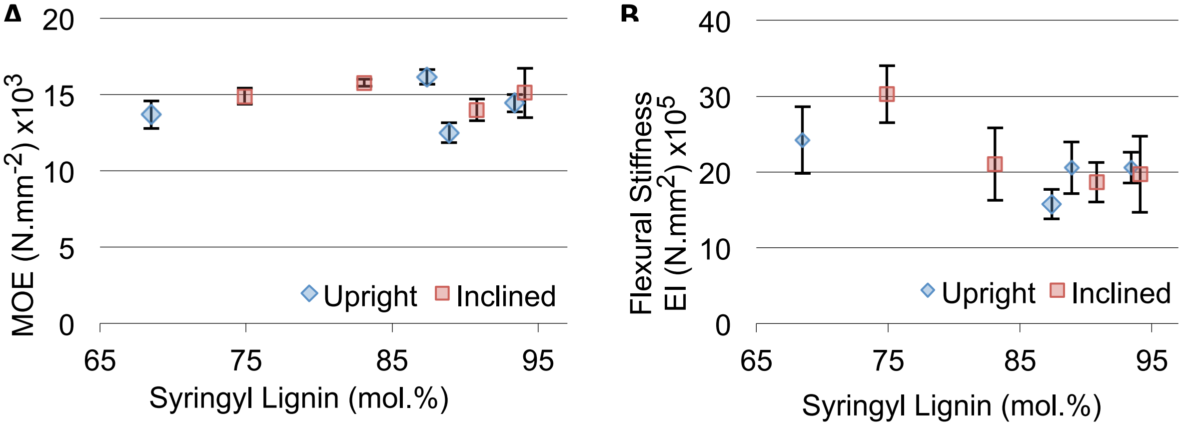

Figure 2.12. **A.** Modulus of elasticity (MOE) and **B.** flexural stiffness (*EI*) of dry upright and inclined wild type and transgenic hybrid poplar stems based on data collected in 2006, 14-cm long stems were allowed to dry to constant weight. Error bars represent means±*SE*, *n*=6 stems per treatment line.

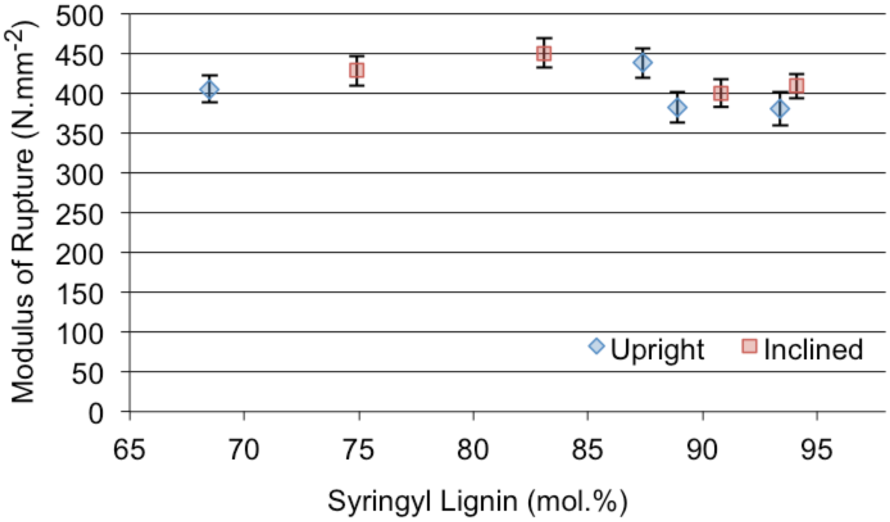

Figure 2.13. Modulus of Rupture (MOR) of upright and inclined wild type and transgenic dry hybrid poplar stems based on data collected in 2006, 14-cm long stems were allowed to dry to constant weight. Error bars represent means±*SE*, *n*=6 stems per treatment line.

The mechanical tests were repeated in 2008. For higher replication, twenty-two trees were used to represent the wild-type clone and similar number was used for each of the three transgenic lines. Similar to the earlier-presented 2006 results, the MOE for the fresh stems of wild-type trees (68.5 mol.% syringyl lignin) was 8460.5±373.2 N.mm^{-2} (mean±*SE*) (Figure 2.14a). The fresh stems of the transgenic polar lines with higher syringyl lignin (87.4-93.4 mol.% syringyl lignin) had MOE ranging from 8123.7±415.3 N.mm^{-2} to 9070.0±396.9 N.mm^{-2} while remaining statistically similar to the fresh wild-type stems. Flexural stiffness (*EI*) of the upright stems was 577871.2±46288.8 N.mm^{2} for the fresh wild-type stems, and it ranged from 585985.9±46715.5 N.mm^{2} to as high as 665723.5±64388.2 N.mm^{2} for the fresh transgenic stems (Figure 2.14b) while remaining statistically similar to the wild-type controls.

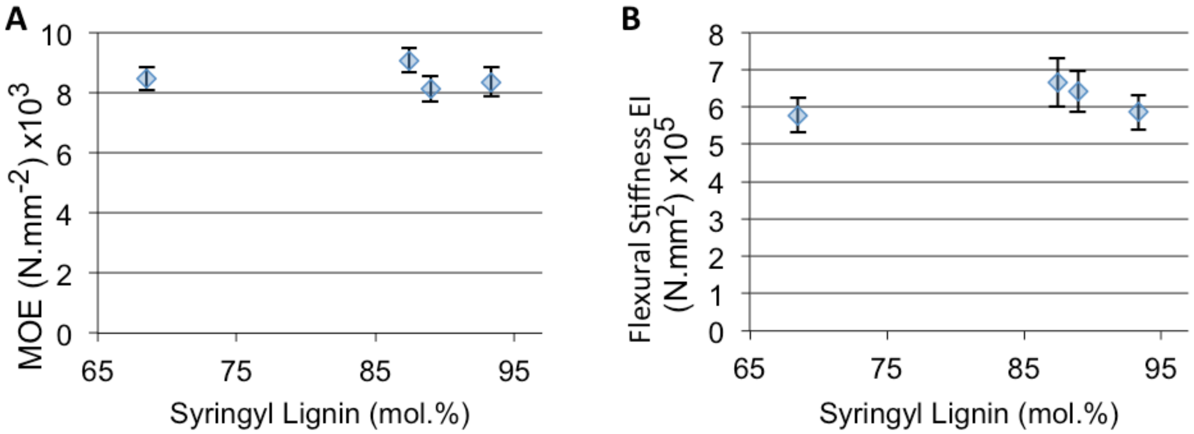

Figure 2.14. **A**. Fresh stem modulus of elasticity (MOE) and **B**. flexural stiffness (*EI*) of upright wild-type and transgenic hybrid poplar stems based on data collected in 2008, 14-cm long stems were tested immediately after harvesting. Error bars represent means±*SE*, *n*=22 stems per line.

The same stems were then oven-dried and the mechanical testing was repeated. The modulus of elasticity (MOE) and the flexural stiffness (*EI*) were higher for the dry stems compared to the freshly harvested green stems. The MOE for the wild-type upright stems (68.5 mol.% syringyl lignin) was 10136.5±382.3 N.mm^{-2} (mean±*SE*), and for the transgenic lines with higher syringyl lignin (87.4-93.4 mol.% syringyl lignin) it ranged from 9960.7±406.5 N.mm^{-2} to 10455.8±479.9 N.mm^{-2} while remaining statistically similar to the wild-type trees (Figure 2.15a). Similarly, *EI* for the wild-type stems was 706133.1±57543.3 N.mm^2, and for the transgenic lines it ranged from 750407.3±66992.1 N.mm^2 to 793275.2±65803.0 N.mm^2 while remaining to be statistically similar to the wild-type trees (Figure 2.15b).

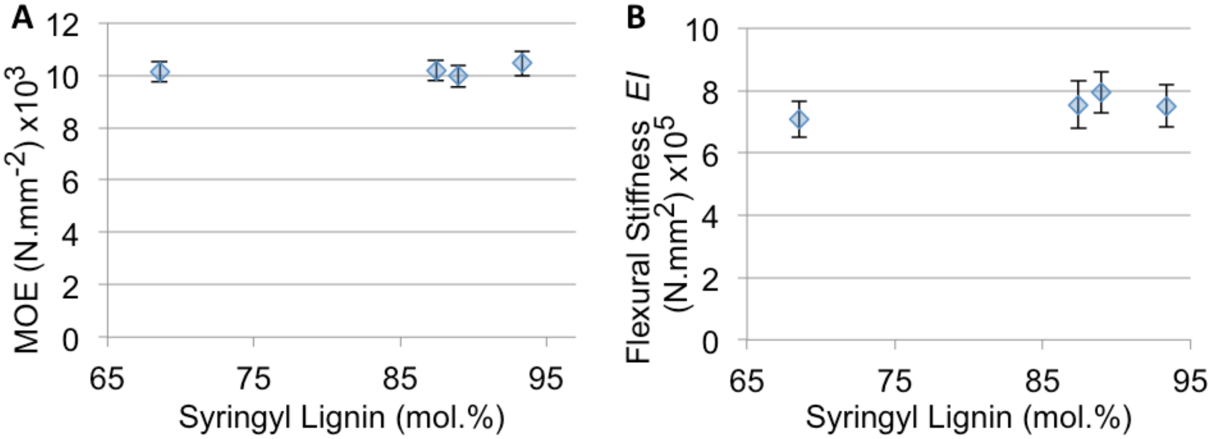

Figure 2.15. **A**. Dry stem modulus of elasticity (MOE) and **B**. flexural stiffness (*EI*) of upright and inclined wild type and transgenic hybrid poplar stems based on data collected in 2008, 14-cm long stems were allowed to dry to constant weight. Error bars represent means±*SE*, *n*=22 stems per line.

Mechanical loading of the dry stems was extended until stem rupture, hence allowing for the calculation of the modulus of rupture (MOR). The MOR for the upright wild-type stems was 329.59±7.55 N.mm^{-2} (mean±*SE*) (Figure 2.16), and for the transgenic trees with increased percentage of syringyl lignin in the stems (>68.5 mol.%) the MOR ranged from 258.18±9.81 N.mm^{-2} to 290.19±10.84 N.mm^{-2}. Based on the regression analysis, increasing the percentage of syringyl lignin in poplar stems led to a significant decrease in MOR ((p<0.001, correlation coefficient=0.19).

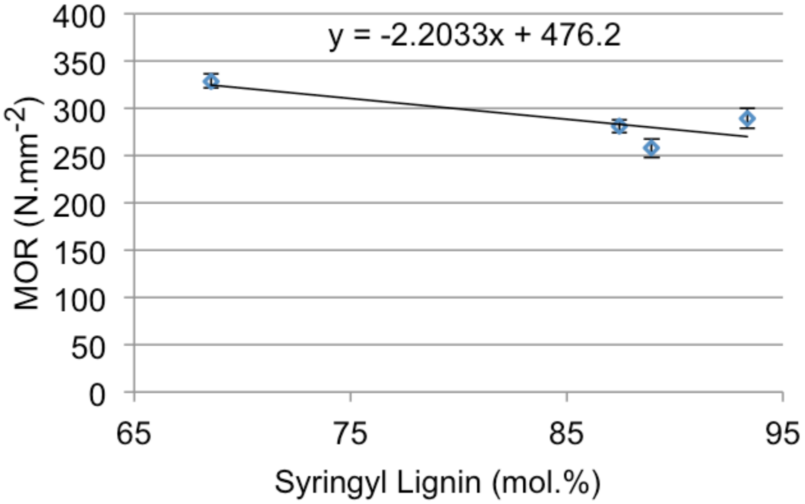

Figure 2.16. Modulus of Rupture (MOR) of upright wild type and transgenic dry hybrid poplar stems based on data collected in 2008, 14-cm long stems were allowed to dry to constant weight. Error bars represent means±*SE*, *n*=22 stems per line.

The compressive modulus *E* for the dry stems was calculated after conducting compressive loading parallel to the grain (parallel to stem axis) and was found to be 754.7±35.2 $N.mm^{-2}$ (means±*SE*) for the wild-type stems compared to 758.1±33.6 $N.mm^{-2}$ (means±*SE*) for the transgenic line *F5H64* with the highest syringyl lignin percentage (93.4mol.%), (Figure 2.17). The compressive modulus for the two stem groups was almost identical; therefore the two means were statistically similar.

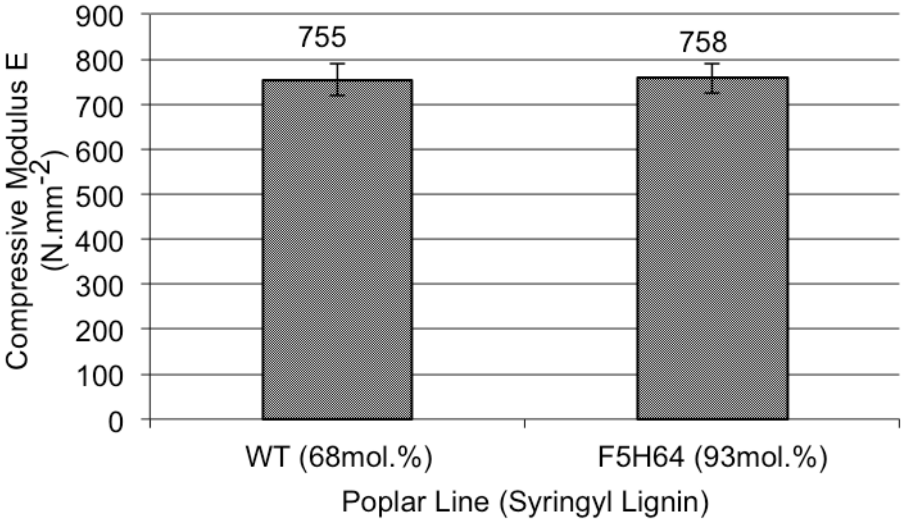

Figure 2.17. Young's modulus based on compressive loading parallel to the grain (also known as compressive modulus) of hybrid poplar stems dried to constant weight. Error bars represent means±*SE*, *n*=22 stems per line.

DISCUSSION

Wild type hybrid poplar trees (*Populus tremula* x *P. alba*) and three genetically modified lines, over-expressing *F5H* gene to different levels, were used in our study. The gene modification caused a higher incorporation of synapyl alcohol into the lignin polymer thus increasing the syringyl to guaiacyl ratio (S:G) in the stems. The greenhouse-grown poplars (i.e. the wild-type and the transgenic lines) were kept upright or inclined from vertical for three months to induce tension wood formation and stem reorientation to the vertical position. After an experimental trial in 2006, the stems that had been inclined had a lower height to diameter ratio (Figure 2.4A), the result of additional radial growth due to tension wood formation Figure 2.4B). The stems of the inclined trees had the same growth rate compared to the upright trees during the experiment. The inclined trees with the highest syringyl lignin percentage gained less height throughout the experiment compared to the inclined wild-type control trees. This suggests that the synthesis of more syringyl lignin monomers, and less guaiacyl monomers, can potentially be costly to the tree growth. The connection between the disruption of lignin biosynthesis and plant dwarfing has been previously reviewed (Bonawitz and Chapple, 2010). Major efforts have been made to discover correlations between the different plant structural components (e.g. lignin properties and cellulose microfibril angle) and growth responses. These responses are induced by growth stresses due to environmental changes. Some of the major findings were, in chronological order: Boyd (1977) found a strong correlation between cellulose microfibril angle in the S_2 layer and the longitudinal shrinkage reactions due to growth strains in leaning stems of *Eucalyptus regnans*, *Eucalyptus sieberi* and *Pinus radiate.* Baillères et al. (1995) found a negative correlation between growth strain, lignin content, and the microfibril angle of cellulose in the S_2 layer of the fiber cell walls of hybrid *Eucalyptus*. They also found a positive correlation between growth strain and the S:G ratio in lignin. Similar results were obtained in a study that used yellow poplar (Yoshida *et al*., 2002). That, in addition to recent findings by Wang et al. (2010), had suggested an important role for lignin structure in plants adaptation to environmental changes.

The modified syringyl lignin percentage in the xylem had an effect on the kinematic responses of the leaning poplar trees. Tracking the angular changes along the stems revealed interesting results. During the gravitropic response, the trees with the highest syringyl lignin percentage (94 mol.%) demonstrated an early lead indicating a faster formation of tensile force on the upper side of their leaning stems. Those faster responding trees adjusted their stems to a vertical position with respect to gravity at least 8 days earlier than the trees with unmodified syringyl percentage. All of the trees reached and maintained a maximum angle of 100° from horizontal line indicating overshooting the vertical without further re-adjusting. As was demonstrated by the angle A123 in Figure 2.9, the trees that had the highest syringyl lignin percentage started the decurving events sooner, and were able to close the angle at a faster rate.

Tracking these angles across the leaning stems proved to be useful. According to Coutand et al. (2007), it is important to study the gravitropic response of trees along the entire stem length rather than just one part (e.g. stem tip) due to the differing proportions of tissue types (i.e. mostly radial secondary growth in the lower part of the stem versus predominantly elongating primary growth in the upper part of the stem), and due to stem tip correction (decurving) occurring before over-shooting the vertical, which would provide minimum information on the decurving process in tissues with secondary growth. Autotropic stem decurving below the stem tip was associated with shifts in the location of compression wood in the leaning stems of *Pinus* (Wilson and Archer, 1973; Sierra-de-Grado et al., 2009), or the location of tension wood in the stems of *Populus nigra* x *Populus deltoides*, 'I4551' (Coutand et al., 2007), indicative of simultaneous curving and decurving events. The kinematic results presented here suggest a change in the structural and functional properties of the poplar stems as a result of artificially modifying the lignin composition. The resulting change can potentially be at either side of the leaning stems (the opposite wood at the lower side of the leaning stems or the tension wood on the upper side) or it could be at both sides.

Tension wood is usually characterized by fibers with a gelatinous layer rich in highly crystalline cellulose instead of a lignified S3 layer in the secondary wall. The cellulose microfibril angle, in tension wood fibers, change to being almost parallel with the stem axis during shrinkage events, indicating the presence of longitudinal growth stress. This force helps stems reorient in response to gravity. And due to the resistance of the older wood tissue, the trapped growth strain can be quantified and correlated with the microfibril angle (Boyd, 1977; Clair *et al*., 2011). The results from measuring the microfibril angles are presented in a separate chapter.

The differential response of the three poplar lines with varying syringyl to guaiacyl ratio in lignin to gravity may be attributed to differential tensile forces inside the stems. Measurements of the released strain inside these stems showed lack of significant differences among the poplar lines. When the data were fitted to a regression model, a significant interaction existed between percent syringyl lignin and the larger diameter of the stem ($P=0.04$) where the stain gauge was attached. The change in released strain in the tension wood side due to syringyl lignin modification was negatively affected by the stem thickness, perpendicular to the tension wood surface. The best model explaining the released strain is presented here.

Fitted model:

$$(\mathit{Released\ strain})^2 \sim \mathit{Diameter} + \mathit{Syringyl} + \mathit{Diameter{:}Syringyl} \quad (r^2 = 0.54,\ P<0.01)$$

Where: ‘Released strain’ is the difference between the released strain from the cuts above and below the strain gage, ‘Diameter’ is the stem diameter that is perpendicular to the tension wood surface, and ‘Syringyl’ is the mole percent syringyl lignin.

These strain gauge readings were recorded at the end of the experiment after the poplar trees had adjusted to gravity and approached the vertical position. The pulling forces, which were attributed to tension wood, were reported to decrease gradually when the leaning stems approached the upright position (Coutand *et al*., 2007). Therefore, the stems that respond to gravity faster reach lower level of internal forces (relaxation) sooner than the slower responding stems. This might explain the lower growth strain recorded in the faster responding poplar trees, with the highest syringyl lignin percentage, at the end of the experiment.

The mechanical analyses of stems using four-point bending revealed interesting changes in some of the properties. Wild-type and genetically modified stems from the upright and the inclined trees were tested when still fresh and after they were air-dried. There were no differences between the stems of the upright trees and those of the inclined trees. However, the fresh stems of the inclined trees scored different results when the orientation of the tension wood side was accounted for. When tested with the tension wood side facing upward and under bending compression (closer to the crosshead) the results indicated stiffer stem properties compared to testing them with the tension wood side facing down and under tension (away from the crosshead). In a study that used artificially tilted 1-year-old poplar trees, Coutand *et al*. (2004) attributed higher Young's modulus (or MOE) to the microfibril angle in the G-layer of tension wood. The change in MFA may account for the change in the elastic properties of the inclined stems that were tested here. Testing irregularly shaped stems of small diameter has been a constant challenge and often led to the discard of a significant portion of the samples and data (Kasal *et al*., 2007). Due to the lack of proper tools, the inability to isolate and independently test the tension wood tissue, all subsequent mechanical tests were done on upright stems. Therefore, the mechanical tests were repeated in 2008 using 22 upright stems of each of the transgenic poplar lines and the wild-type clone. Young's modulus (MOE) and the flexural stiffness (*EI*) remained similar despite the elevated syringyl lignin percentage in the fresh stems (Figure 2.14), and after drying to constant weight (Figure 2.15). Interestingly, when the 14-cm long dry hybrid poplar stems, that were used during the four-point bending tests, were loaded until failure the modulus of rupture decreased suggesting an increase in brittleness due to the higher ratio of syringyl units in the lignin polymer (Figure 2.16). When tested using compressive loading, the compressive modulus of the wild-type stems and the transgenic line with the highest percentage of syringyl lignin (*F5H64*) was statistically similar (Figure 2.17). Despite the structural difference between the lignin polymers, with 67 mol.% syringyl units (wild-type) and 93 mol.% syringyl units, the genetically modified poplar trees maintained similar flexural properties compared to the unmodified controls. The change in the stem strength properties (i.e. MOR) suggests a potential change in the anatomical properties and/or cell wall biochemistry and structural components (each is discussed in a separate chapter).

CONCLUSION

Poplar stems, after inclination with respect to gravity, adjusted their stems back to vertical position by means of tension wood formation on the upper side of the tilted stems. Subsequently, they became shorter with thicker stems where tension wood was formed. Modifying lignin composition so as to contain higher percentage of syringyl lignin caused the stems to grow less during the experiment than the unmodified controls. When the tilted trees began to respond to the gravitropic stimulus they went through three stages: latent phase, gravitropic phase and autotropic phase. Increasing the syringyl lignin percentage in the stems led to shorter latent phase and faster overall kinematic response. The stems with the highest syringyl lignin ratio corrected their stems and completed the curving and decurving responses eight days earlier than the unmodified controls. Contrary to the expectation, the released growth strain on the tension wood surface was lower in the stems with the highest syringyl lignin percentage, when measured at the end of the experiment, potentially due to faster recovery to the vertical position. The mechanical analyses of stems of the upright and inclined trees showed no change in the elastic properties in response to the lignin modification, suggesting a limited role for lignin composition in determining stem elastic properties, or the trees ability to cope by means of other structural and/or compositional changes. Nevertheless, a slight increase in brittleness was observed due to enhancing the syringyl lignin percentage in the xylem of the dried poplar stems. Investigating the interaction between tension wood formation and modified lignin monomer composition in hybrid poplar stems unveils novel information for the scientific community, and conforms with the increasing interest in utilizing wood as an industrial feedstock.

ACKNOWLEDGEMENTS

We thank David Ellis (National Center for Genetic Resources Preservation) and Clint Chapple (Purdue University) for providing us with the transgenic poplar lines used in this study, Bradley Marks and Steve Marquie (Michigan State University) for providing access to their Instron® Universal Machine. This research was supported by the National Research Initiative of the USDA CSREES grant number 2005-35103-15269

REFERENCES

REFERENCES

Archer RR, Wilson BF (1973) Mechanics of the compression wood response. II. On the location, action and distribution of compression wood formation. Plant Physiol **51**:777–782.

ASTM D143 (1994) Standard methods of testing small clear specimens of timber. ASTM International, West Conshohocken, PA, USA.

Baillères H, Chanson B, Fournier M, Tollier MT, Monties B (1995) Structure, composition chimique et retraits de maturation du bois chez les clones d'Eucalyptus. Ann Sci For **52**:157-172.

Bonawitz ND and Chapple C (2010) The genetics of lignin biosynthesis: connecting genotype to phenotype. Annu Rev Genet **44**:337-363.

Boyd JD (1977) Relationship between fibre morphology and shrinkage of wood. Wood Sci Technol **11**:3-22.

Clair B, Almeras T, Pilate G, Jullien D, Sugiyama J, Riekel C (2011) Maturation stress generation in poplar tension wood studied by synchrotron radiation microdiffraction. Plant Physiol **155**:562-570.

Coutand C, Fournier M, Moulia B (2007) The Gravitropic response of poplar trunks: key roles of prestressed wood regulation and the relative kinetics of cambial growth versus wood maturation. Plant Phyiol **144**:1166-1180.

Coutand C, Jeronimidis G, Chanson B, Loup C (2004) Comparison of mechanical properties of tension and opposite wood in Populus. Wood Sci Technol **38**:11-24.

Donaldson LA (2002) Abnormal lignin distribution in wood from severely drought stressed Pinus radiata trees. J Int Assoc Wood Anatomists **23**(2):161-178.

Fang C-H, Clair B, Gril J, Liu S-Q (2008) Growth stresses are highly controlled by the amount of G-layer in poplar tension wood. IAWA **29**(3):237-246

Herschbach C and Kopriva S (2002) Transgenic trees as tools in tree and plant physiology. Trees **16**:250-261

Hoadley RB (2000) Understanding wood: a craftsman's guide to wood technology, 2nd ed. Taunton Press, Newtown, Connecticut, USA.

Huntley S, Ellis D, Gilbert M, Chapple C, Mansfield SD (2003) Significant increases in pulping efficiency in C4H-F5H transformed poplars: Improved chemical savings and reduced environmental toxins. J Agric Food Chem **51**:6178-6183.

Illston JM, Dinwoodie JM (1979) Concrete, timber, and metals: the nature and behaviour of structural materials. Van Nostrand Reinhold, New York, USA.

Kasal B, Peszlen I, Peralta P, Li L (2007) Preliminary tests to evaluate the mechanical properties of young trees with small diameter. Holzforschung **61**:390-393.

Kern KA, Ewers FW, Telewski FW, Koehler L (2005) Mechanical perturbation affects conductivity, mechanical properties and aboveground biomass of hybrid poplars. Tree Physiol **25**(10): 1243-1251.

Niklas KJ (1992) Plant biomechanics: an engineering approach to plant form and function. University of Chicago Press, Chicago, Illinois.

Pilate G, Chabbert B, Cathala B, Yoshinaga A, Leplé J-C, Laurans F, Lapierre C, Ruel K (2004) Lignification and tension wood. C. R. Biologies **327**:889-901.

Pruyn ML, Ewers BJ, Telewski FW (2000) Thigmomorphogenesis: changes in the morphology and mechanical properties of two Populus hybrids in response to mechanical perturbation. Tree Physiol **20**:535-540

R Development Core Team (2007) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Sierra-de-Grado R, Pando V, Martínez-Zurimendi P, Peñalvo A, Báscones E, Moulia B (2009) Early selection of stem straightness in *Pinus pinaster ait* based on the straightening process. 6th Plant Biomechanics Conference, Cayenne, French Guiana.

Stettler RF, Bradshaw HD Jr., Heilman PE, Hinckley TM (1996) Biology of Populus and its implications for management and conservation. NRC Research Press, Ottawa, Ontario, Canada.

Telewski FW (2006) A unified hypothesis of mechanoperception in plants. Am J Bot **93**(10):1466-1476.

Voelker SL, Lachenbruch B, Meinzer FC, Strauss SH (2011a) Reduced wood stiffness and strength, and altered stem form, in young antisense 4CL transgenic poplars with reduced lignin contents. New Phytologist **189**(4):1096-1109.

Voelker SL, Lachenbruch, B, Meinzer FC, Kitin P, Strauss SH (2011b) Transgenic poplars with reduced lignin show impaired xylem conductivity, growth efficiency and survival. Plant Cell Environ **34**:655-668.

Wagner KR, Ewers FW, Davis SD (1998) Tradeoffs between hydraulic efficiency and mechanical strength in the stems of four co-occurring species of chaparral shrubs. Oecologia **117**:53-62.

Wang Y, Grill J, Clair B, Minato K, Sugiyama J (2010) Wood properties and chemical composition of the eccentric growth branch of *Viburnum odoratissimum* var. *awabuki.* Trees **24**:541-549.

Yoshida M, Ohta H, Yamamoto H, Okuyama T (2002) Tensile growth stress and lignin distribution in the cell walls of yellow poplar, Liriodendron tulipifera Linn. Trees Struc Funct **16**:457-464.

Yoshida M, Okuyama T (2002) Techniques for measuring growth stress on the xylem surface using strain and dial gauges. Holzforschung **56**:461-467.

Yoshinaga A, Fujita M, Saiki H (1992) Relationships between cell evolution and lignin structural varieties in oak xylem evaluated by microscopic sectrophotometry with separated cell-walls. Mokuzai Gakkaishi **38**:629-637.

CHAPTER 3

INFLUENCE OF SYRINGYL TO GUAIACYL RATIO ON ANATOMY AND WATER RELATIONS IN GENETICALLY ALTERED POPLARS (*POPULUS TREMULA* X *P. ALBA*)

ABSTRACT

Lignin is an important phenolic polymer of secondary cell walls that is known to influence the physiological properties of secondary xylem (wood). Plants have been shown to alter their lignin composition to meet internal functional requirements as well as due to external environmental stimuli. Angiosperm lignin is majorly composed of two monomers, syringyl and guaiacyl units. The role these monomers play to help the plants maintain their vital hydraulic functions were investigated. It was hypothesized that increasing the syringyl content in the xylem will improve water conductivity and resistance to implosion without affecting the xylem's anatomical properties. Hybrid poplar clone 717 (*Populus tremula* x *P. alba*) transformed to over express the *C4H::F5H* gene at different levels resulting in varying syringyl to guaiacyl ratios was used in this study. Wild type poplar trees and the transgenic lines were kept upright or inclined (45°) from vertical for three months to induce tension wood formation. Specific water conductivity was improved in the upright poplar trees with higher syringyl lignin ratio but not in the inclined trees. On the other hand, changing the lignin monomer ratio did not affect the upright and the inclined stems vulnerability to cavitation under negative pressure. Increases in the average leaf area, vessel diameter and percent vessel lumen area in response to increased syringyl lignin ratio in normal wood were detected, and coincided with the changes in water conductive properties. The anatomical properties of tension wood were not affected by

the lignin modification, nor did xylem density. Evaluating the response of poplar trees to inclination and altered lignin composition improves our understanding of the role of lignin monomers in affecting the xylem anatomy and physiological properties in normal and tension wood.

INTRODUCTION

The mechanical and water conductive properties of vascular plants are critical to the survival of the plant and are influenced by the lignin and other cell wall components (Boyce *et al*., 2004; Boerjan et al., 2003; Sederoff et al., 1999; Lewis and Yamamoto, 1990). These properties significantly influence the ability of a plant to withstand external and internal such as wind (Kern et al., 2005) or gravity (Pilate et al., 2004), the shrinkage of tension wood (Clair et al., 2011), and the negative pressure inside the conducting xylem vessels (Voelker et al. 2011; Woodrum et al., 2003; Hacke et al., 2001; Amrhein et al., 1983).

Gravity is an external stimulus to which terrestrial plants can sense and respond. Woody plants respond through formation of reaction wood; termed compression wood in gymnosperms, or tension wood in angiosperms (Timell, 1986; Boyd, 1977). Poplar (*Populus*) trees form tension wood on the upper side of their leaning stems to correct displacement with respect to gravity by means of pulling forces created inside the tension wood (Pilate et al., 2004). Tension wood is characterized as containing fibers with a thickened secondary cell wall layer, reduced lignin content and increased crystallization of near vertically (0°) oriented cellulose microfibrils. This modified secondary cell wall is termed the G-layer (Pilate et al., 2004). Tension wood also has fewer and smaller diameter vessel elements (Jourez et al., 2001). Internally, xylem vessels are responsible for conducting water from the deep roots to the high branches and leaves. To do so, trees must maintain a continuous water column throughout the conducting system, under extreme negative pressures (Tyree, 1997). Lignin, which makes up 20-30% of the wood chemical components, is a major contributor to these physical abilities of woody plants.

The evolution of syringyl, guaiacyl and *p*-hydroxyphenyl lignin coincides with a division in function for each specific monomer in different plant species, growth forms or tissues and cells. For example, vessel cell walls are rich in guaiacyl and fibers are rich in syringyl (Mosha and Goring, 1975). Thus, the contribution of these monolignols to plant structure and function has been a great interest to plant researchers starting as early as the mid nineteenth century (Schubert, 1965) and continues until this day. With equal interest, the impact of lignin with higher syringyl content on the water relations and anatomy of *Populus* has been investigated, the results of which are presented and discussed in this chapter.

The *F5H* mutants are prime candidates for use in this study, allowing us to investigate the direct influence of modified lignin monomer composition on water conductivity and xylem vulnerability to vessel implosion in a well-known model system with no other apparent alterations to bias the results. It has been reported that lignin impacts vulnerability of the vessels to implosion (Franke et al., 2002). To investigate if lignin composition impacts similar traits, it was hypothesized that *Populus,* with increased syringyl lignin ratio, will show enhanced water conductivity but more susceptibility to implosion in normal wood. In addition, it was hypothesized that lignin modification or the over-expression of *ferulate*-5 *hydroxylase* (*F5H*) will not alter the anatomy of tension wood or normal wood in *Populus*. Due to the limited abundance of vessel cells in tension wood, it was hypothesized that modifying lignin composition in the tissue will have no effect on its water conductive properties.

MATERIALS AND METHODS

Plant Material

This study was conducted on wild type hybrid poplar clone 717 (*Populus tremula* x *P. alba*) as well as on transgenic lines that were altered to over-express the *F5H* gene at different levels resulting in higher syringyl to guaiacyl ratio ranging from 68.5 mol.% in wild type to as high as 93.4 mol.% in the over-expression lines. *F5H* over-expression lines were obtained from David Ellis and Clint Chapple laboratories and have been vegetatively-propagated over multiple generations using root sprouts. Young trees were grown under greenhouse conditions on the Michigan State University campus. Pots (18-liter) with soil-less medium (Baccto, Michigan Peat Company, Houston, Texas, USA) were used with no additional fertilization or supplemental lighting. The modified lignin composition was proven to remain stable over several years of growth. Less than a year old trees with 50-60 cm long stems were inclined 45° from vertical to induce the gravitropic responses including tension wood formation, and they were grown for three months along with control trees kept in the upright position. Fourteen-centimeter long stem segments were taken at eight centimeters distance from the growth medium surface to the lower end of the sample.

Water transport and Vulnerability

Measurement of hydraulic conductivity and evaluation of the vulnerability to embolism were conducted to determine if the altered syringyl lignin ratio impacts the conductive properties of vertical and displaced *Populus* stems compared to an unmodified wild type control. Six stems of each genetic line and treatment were harvested and re-cut under water to obtain 14 cm long stem segments. The stem segments were connected to a tubing system and water flushed in the apical direction with high-pressure (14.5 kPa) de-ionized water (adjusted to pH 2.0 using HCl and passed through a 0.2 μm filter) for one hour to remove native gas emboli in the vessels. Water specific conductivity k_S (in Kg $MPa^{-1}s^{-1}m^{-1}$) was calculated by gravimetric measurement of k_h divided by xylem cross sectional area (Tyree and Ewers, 1991). Vulnerability curves were created to calculate the negative pressure achieving 50% loss of hydraulic conductivity (P_{50}). To achieve that, centrifugal force was used to create negative pressure in the xylem to measure vulnerability to implosion and xylem cavitation as described by Alder *et al*. (1997). The 14-cm long stems were spun in a centrifuge (RC-5B Refrigerated, Sorvall, Kendro Laboratory Products, Newtown, Connecticut, USA) fitted with a modified rotor that was designed for stems with small diameter. A known negative pressure (pressure potential, Ψ_{xp}) was created on the water columns inside the xylem vessels using pre-determined rotation speeds. Water conductivity was measured after each increase in the rotation speeds until at least a 90% loss of conductivity (PLC) was achieved in each stem due to the generated emboli. The pressure level achieving 50% PLC was graphed against the mole percent syringyl content of the stem samples. Correction due to cavitation fatigue was carried out when necessary; the water conductivity measured after the centrifuge-generated pressure of -0.5MPa was considered the maximum conductivity k_{max}.

Xylem anatomical measures

A sliding microtome was used to create traverse sections of the stems. The sections were cut at 30 μm thickness, wet mounted and observed under a compound microscope fitted with a digital camera. The randomly captured digital images were used to measure a sample size of 200 vessels and 100 fibers from each stem using Scion Image (Scion Corporation).

Wood density

Wood density was determined using the method described by ASTM Standard Method D2395 with modification. Wood samples (14-cm long) were oven dried at 60°C until constant weight then left to equilibrate to room moisture level. The samples were weighed before and after they were dip-coated with liquefied paraffin. The added paraffin volume was accounting for by diving the weight change for each sample by the density of paraffin. Using the water displacement method, the determined volume of the wood samples was used to calculate wood density.

Mäule staining

For differential staining of cell types and tissues with modified lignin a Mäule staining method was used as was described by Chapple et al. (1992). Transverse sections were fixed in 4% glutaraldehyde for 60 minutes and rinsed with water. Then, they were treated with 10% HCl for 5 minutes and rinsed with water before they were mounted in concentrated NH4OH. Digital images were captured using a light microscope that was fitted with a digital camera.

Leaf area

Before the beginning of the 45°inclination treatment of the trees, a permanent marker was used to label the first fully expanded leaf of each tree (leaf plastochron index, LPI) to facilitate tracking of the new growth, and to mark the beginning of the secondary growth (Erickson and Michelini, 1957). Three months later, the leaves above the mark were harvested and their adaxial surface areas were measured using a leaf area meter LI-3100 (LI-COR Biosciences, Inc., Lincoln, Nebraska, USA). Total and average leaf areas above the mark were determined for the upright and inclined trees.

Data analysis

The collected data were statistically fitted with linear models then tested with Analysis of Variance (ANOVA) for balanced experimental design. Assumptions of normality and homogeneity of variances were verified. Data transformation was conducted when necessary. Statistical analyses were done using the software R (R Development Core Team, 2007).

RESULTS

In an experiment conducted in 2006, the specific conductivity of hybrid poplar stems did not change with increasing mole percent syringyl in the inclined trees (Figure 3.1). However, in upright stems, increasing syringyl lignin percentage from 66 mol.% to 94 mol.% resulted in near linear increase in specific conductivity ($P<0.01$).

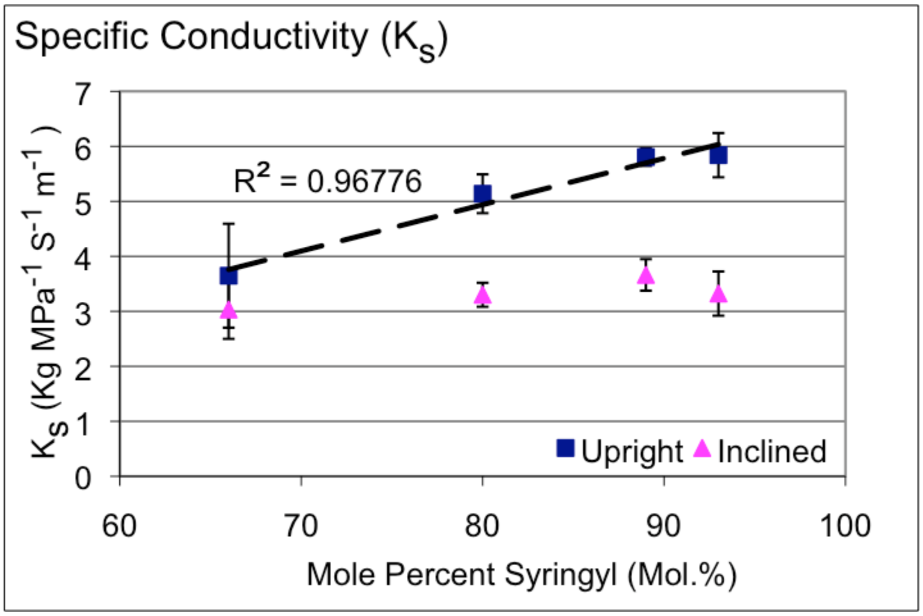

Figure 3.1. Specific conductivity (K_S) was measured on freshly harvested 14-cm long upright and inclined hybrid poplar stems. Means ± *SE* are shown, $P < 0.01$, $n = 5$ stems per treatment line.

After the same stems, used to determine the specific conductivity, were flushed to remove native emboli, they were tested for vulnerability to embolism by exposing them to increasing negative pressures while repeatedly measuring their water conductivity (Figures 3.2 to 3.4). The negative pressure achieving 50% loss of water conductivity (vulnerability to embolism) was used when comparing the hybrid poplar lines that were grown in two conditions, upright or inclined (Figure 3.2). Under both conditions, increasing syringyl lignin percentage had no effect on stem vulnerability to embolism. Both, upright trees with normal wood and inclined trees with opposite and tension wood, performed similarly in the vulnerability test.

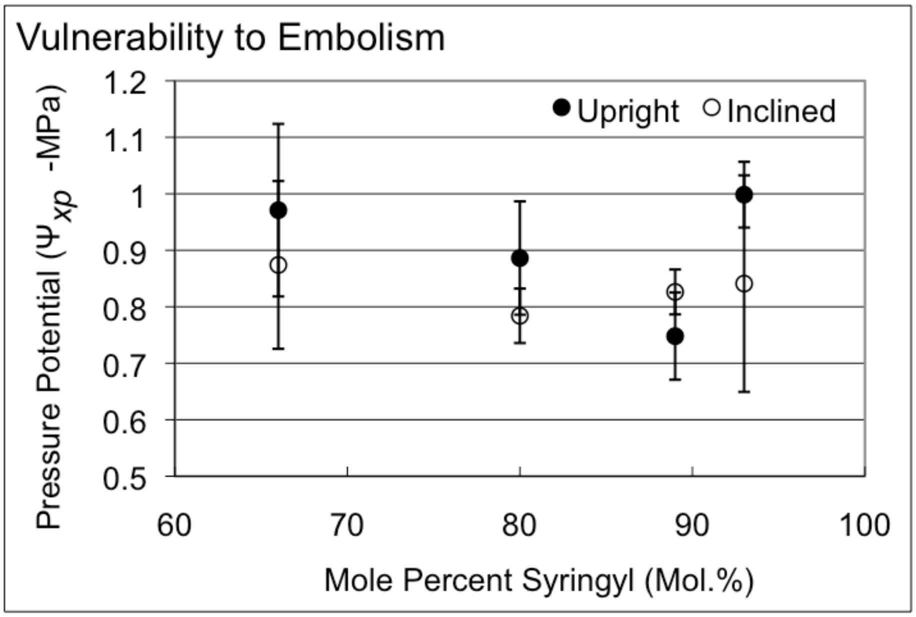

Figure 3.2**.** Poplar stems' vulnerability to embolism as represented by the negative pressure achieving 50% loss of their water conductivity. Means ± *SE* are shown, *n* = 6 stems per treatment line.

Figures 3.3 and 3.4 present the response of the stems of the different *Populus* lines to the increasing negative pressure potential (Ψ_{xp}), that they have been exposed to, and its impact on their hydraulic conductivity. These stems were harvested from upright trees (Figure 3.3) or from inclined trees with tension wood (Figure 3.4). The curves were used to calculate the pressure potential that achieved 50% loss of hydraulic conductivity. The graphs show no clear effect for increasing mole percent syringyl on the trees percent loss of conductivity under the current experimental conditions and gradual change in pressure potential.

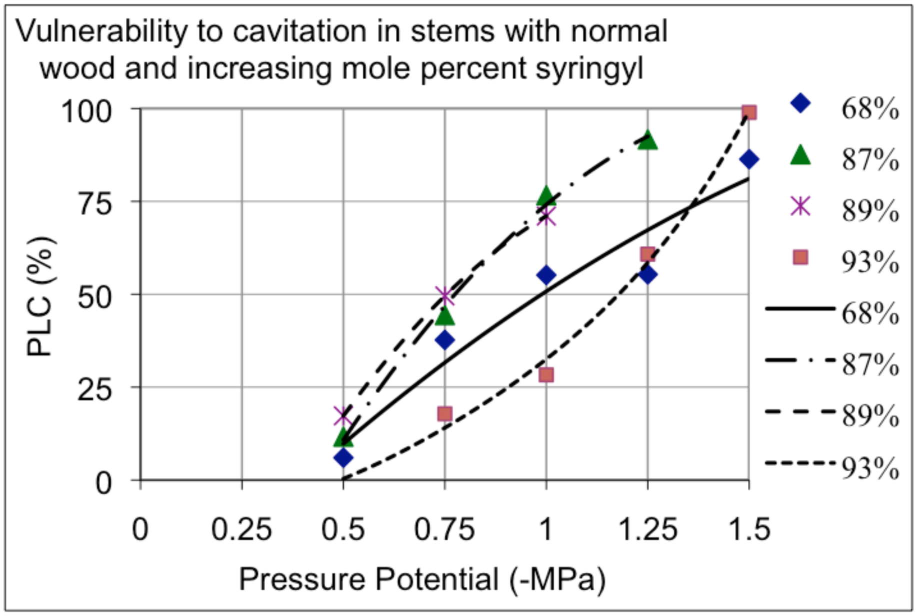

Figure **3.3**. Percent loss of hydraulic conductivity (PLC) as a function of xylem pressure potential of upright stems with normal wood and increasing mole percent syringyl. Each data point represents the average of six stems. The graph was used to calculate the pressure potential that achieves 50% loss of conductivity.

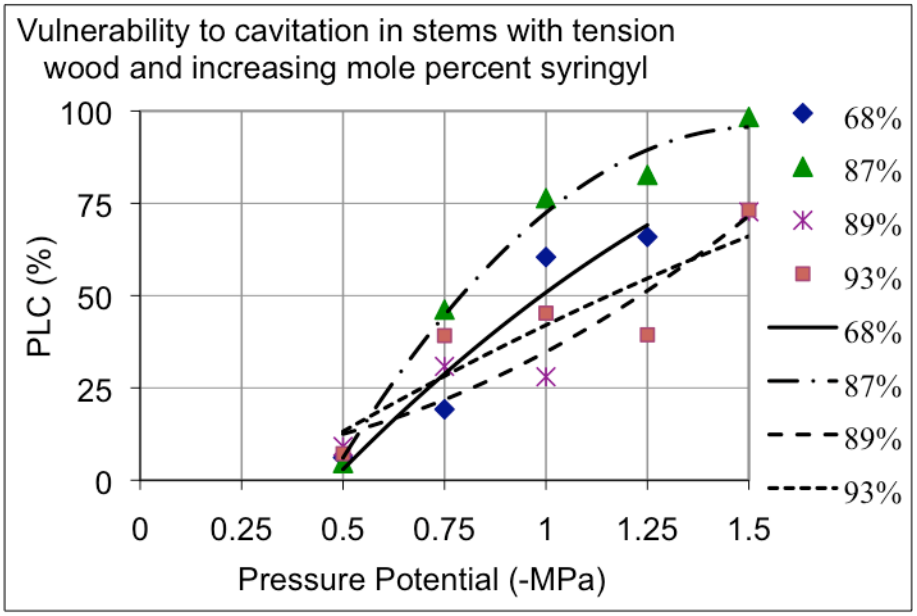

Figure **3.4**. Percent loss of hydraulic conductivity (PLC) as a function of xylem pressure potential of inclined stems with tension wood and increasing mole percent syringyl. Each data point represents the average of six stems. The graph was used to calculate the xylem pressure at which there was 50% loss of conductivity. Mole percent syringyl in this graph was measured before stem inclination treatment, for values after inclination period refer to Figure 4.1.

The stems were harvested at the end of the experiment, and following their response to gravity and recovery back to the vertical position. The leaves that were formed during the experiment were also collected, counted, and their adaxial surface areas were measured (Figures 3.5 and 3.6). When mole percent syringyl was enhanced from 68% to 94%, the average leaf area of the upright poplar trees increased by up to 41% (P=0.027) (Figure 3.5). No statistically significant increase in average leaf area was observed in the inclined trees due to high data variance within the replicates. When the areas of all leaves, that developed during the experiment, were added up for each tree, no change was observed due to syringyl lignin enhancement, or due to inclination and tension wood formation (Figure 3.6A). In a separate experiment, the leaf count for each stem did not change with changing percent syringyl in upright or inclined stems (Figure 3.6B). On average, there were more leaves on the stems of upright trees than on those of the inclined trees (P<0.01).

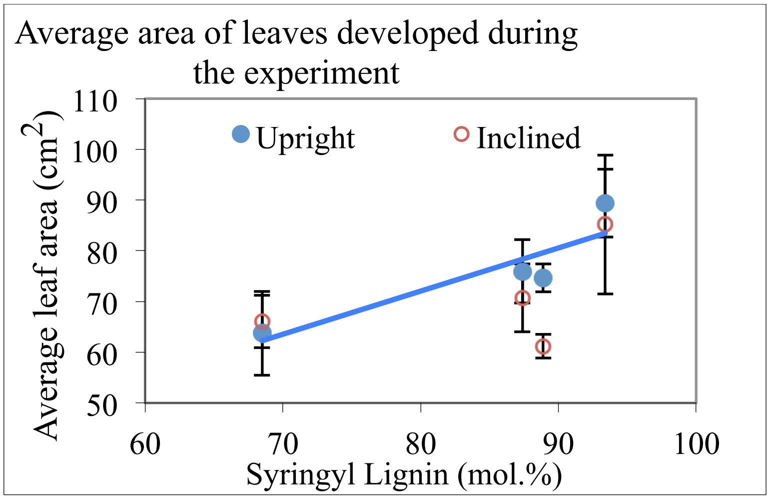

Figure **3.5**. Average leaf area of upright and inclined poplar trees three months after the inclination treatment. Leaves were collected from portions of the stems that developed during the experiment. Means ± *SE* are shown with *n* = 3 poplar trees per treatment line. The experiment was conducted in 2005 inside a greenhouse.

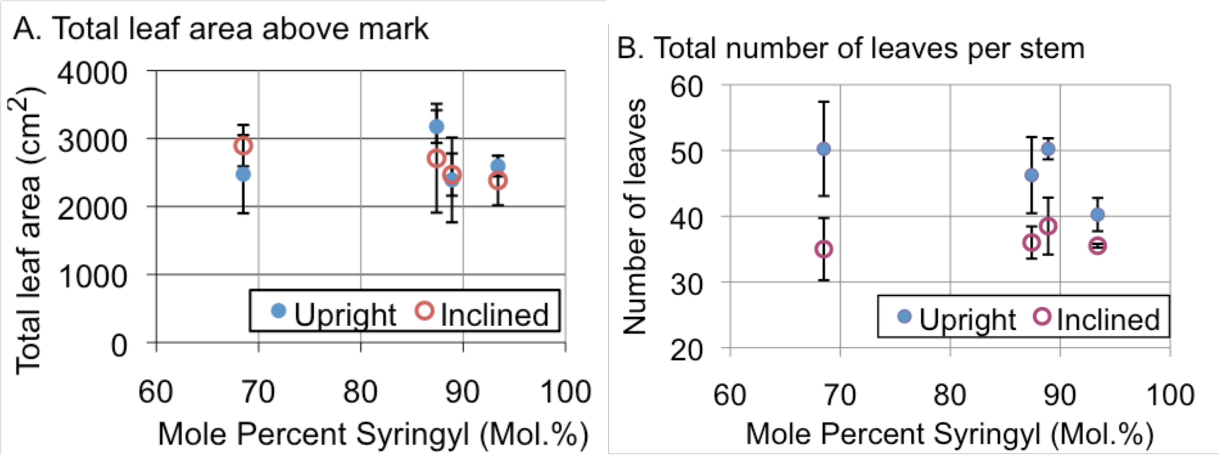

Figure **3.6**. **A**: Total adaxial surface area of leaves, of upright or inclined poplar trees that developed during the experiment. The first fully expanded leaf (leaf plastochron index) was marked at the beginning of an experiment, which was conducted in 2005 and lasted for three months. **B**: All leaves per stem were counted in a separate experiment in 2006. Means ± *SE* are shown with *n* = 3 or 4 trees per treatment line.

Visual observations and anatomical measurements were collected to determine if the increase in hydraulic conductivity could be attributed to changes in xylem anatomy

(Figures 3.7 through 3.11). Initially, to visually compare transverse sections of the different *Populus* lines, the Mäule staining method was used to highlight xylem tissue areas that are rich in syringyl lignin (Figure 3.7). It was expected that the increase in syringyl content would lead to darker red staining as was previously illustrated (Chapple *et al*., 1992). The transverse sections of wild type stems with an average of 68.5 mol.% syringyl (Figure 3.7 C) stained less than those of the over-expression lines with higher syringyl lignin (Figure 3.7 D and E). In separate transverse sections, methylene blue staining was used to compare the inclined stems with tension wood (Figure 3.7 B) to upright stems with normal wood (Figure 3.7 A). The tension wood was identified and subsequently labeled.

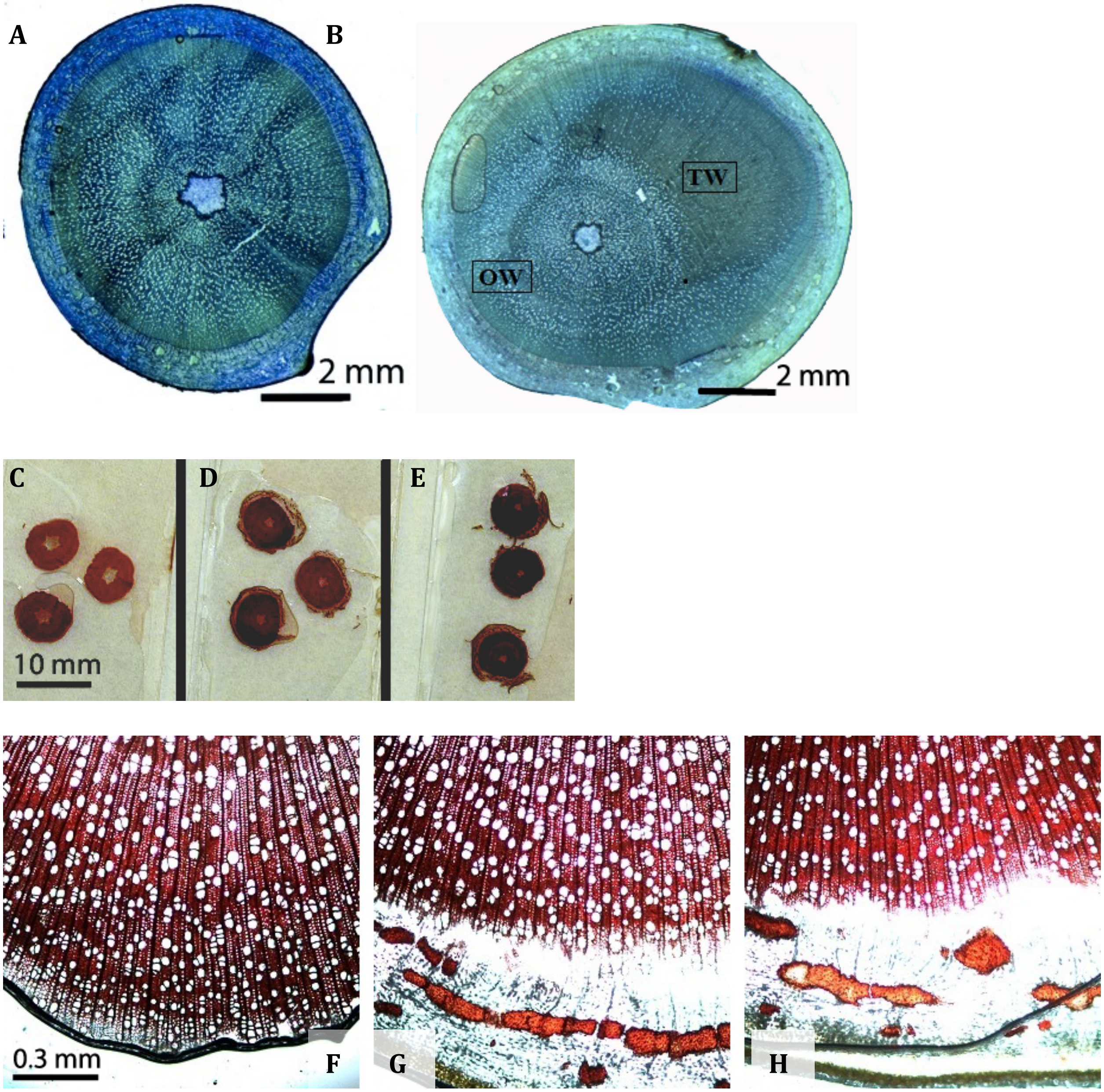

Figure **3.7**. Transverse sections of poplar stems three months after inclination. Genetically unmodified trees with 68.5 mol. % syringyl in normal wood (**A**, **C** and **F**), tension wood (**B**) and *F5H* over-expression lines with 88.9 mol.% (**D** and **G**) or 93.4 mol.% (**E** and **H**) syringyl. Mäule staining was used for all sections except in **A** and **B** where methylene blue staining was used. The bark from the sections of wild type stems (**C** and **F**) was lost during handling.

A visual comparative analysis was performed on tissues (normal, opposite and tension woods) from wild type (left column in Figure 3.8) and *F5H* over-expression line

F5H64 (right column). Visualized with a light microscope, it was unclear whether there were overall differences in tissue types among the *Populus* lines due to syringyl enhancement. Subsequently, detail morphometric analyses were conducted on the digital images of the tissues, the results of which are presented in Figures 3.9 through 3.11.

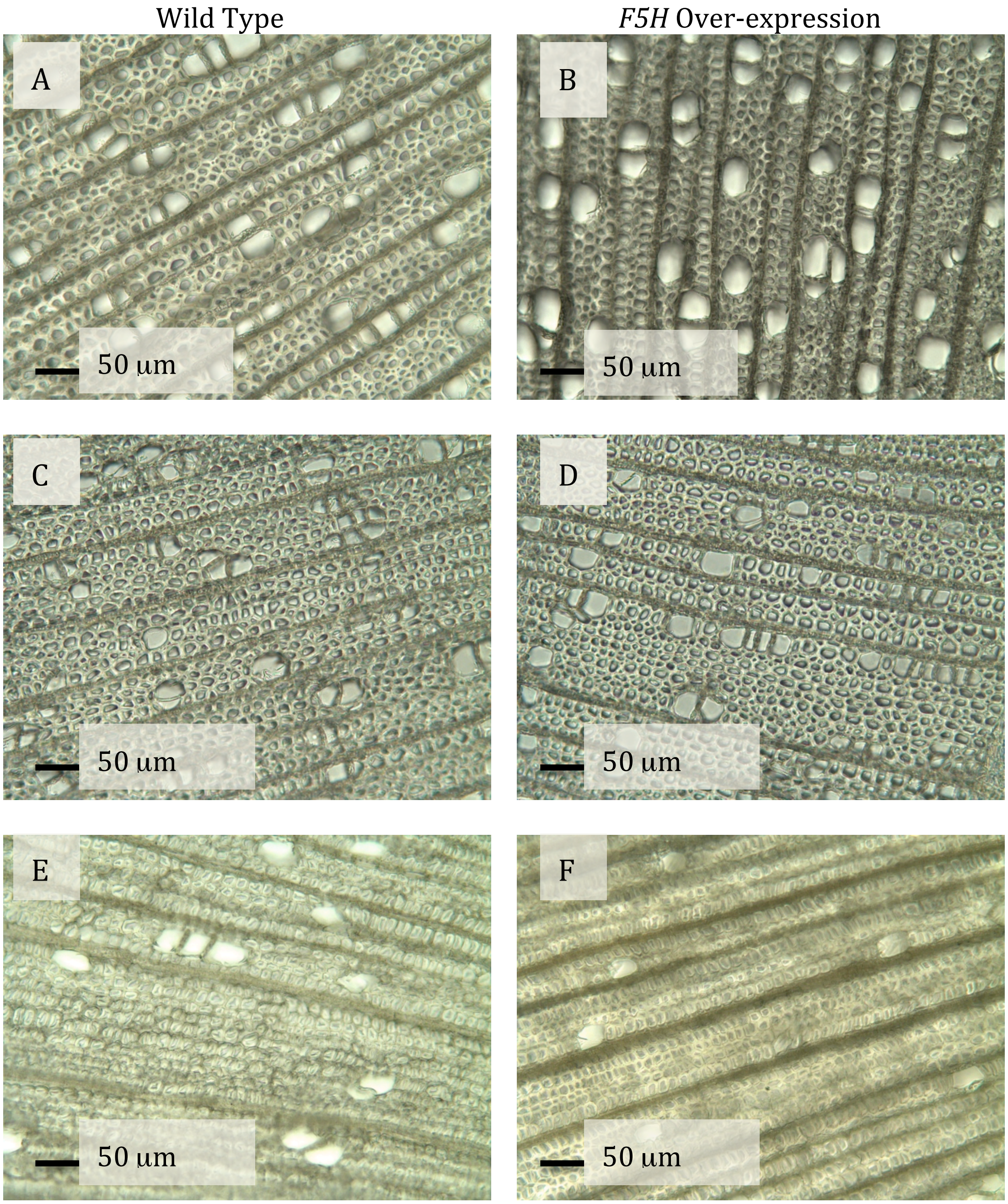

Figure **3.8.** Transverse sections of poplar stems three months after inclination. Genetically unmodified trees (wild type) with ~68 mol. % syringyl and an *F5H* over-expression line *F5H64* with ~93 mol. % syringyl. **A** and **B** show normal wood of upright control stems, **C** and **D** show opposite wood of inclined stems and **E** and **F** show tension wood of inclined stems. Images of tension wood (E and F) were less sharper due to the uneven section surface of the gelatinous fibers.

When the cross sectional areas of the transverse sections were measured and compared, they were found to be statistically similar in the upright and inclined trees, and across the poplar lines with different mole percent syringyl. However, the inclined trees had, on average, 21% larger transverse sections (*P*=0.014) than the upright trees. The area of tension wood relative to the whole transverse section, in the inclined or upright trees, was the same across all poplar lines (Figure 3.9). However, and as was expected, percent area tension wood was higher in the transverse sections of the inclined trees, averaging 18.2%, than those of the upright trees with average 3.9% (*P*<0.001).

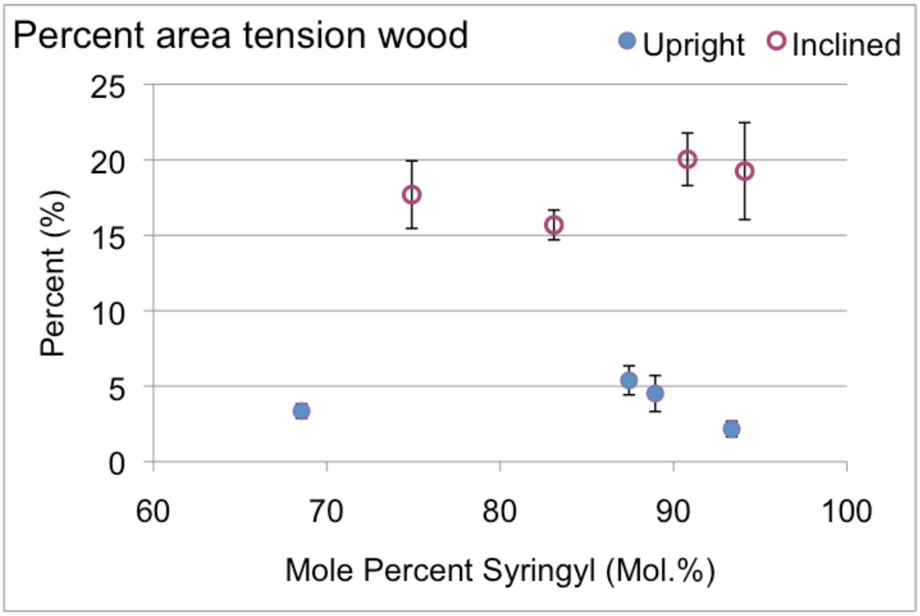

Figure **3.9**. Percent area tension wood in transverse stem sections of upright and inclined poplar trees with different levels of mole percent syringyl. Means ± *SE* are shown with *n* = 6 poplar trees per treatment line.

On the cellular level, a slight increase in vessel diameter was observed in response to increased mole percent syringyl in normal wood (*P*=0.046, when a linear regression model was fitted separately to normal wood vessel diameter data) (Figure 3.10 A). Wild type trees had vessel mean diameter of 32.96 ± 1.09 μm (± *SE*), while the *F5H* over-expression lines had vessel mean diameters reaching up to 36.95 ± 1.36 μm. In opposite and tension wood, vessel mean diameters of the over-expression lines (≥ 83 mol.% syringyl) were statistically similar to the genetically unmodified controls. Percent vessel lumen area also increased with the increase in mole percent syringyl in normal wood (P<0.001) (Figure 3.10 B), but not in opposite or tension wood. No significant effect was found for increasing mole percent syringyl on fiber wall mean thickness (Figure 3.10 C) or percent fiber wall area (Figure 3.10 D) within each of the three wood tissues. However, tension wood had higher averages (statistically referred to as main effects) compared to normal or opposite wood (P<0.001) for the two parameters. The fiber wall average thickness in tension wood was 3.64 ± 0.08 μm (± *SE*), while in normal wood and opposite wood it was 2.03 ± 0.07 μm and 1.88 ± 0.05 μm, respectively. Tension wood had the highest percent fiber wall area of 72.35 ± 0.80 % (± *SE*) compared to normal wood or opposite wood with 41.46 ± 1.23 % and 48.53 ± 2.16 %, respectively.

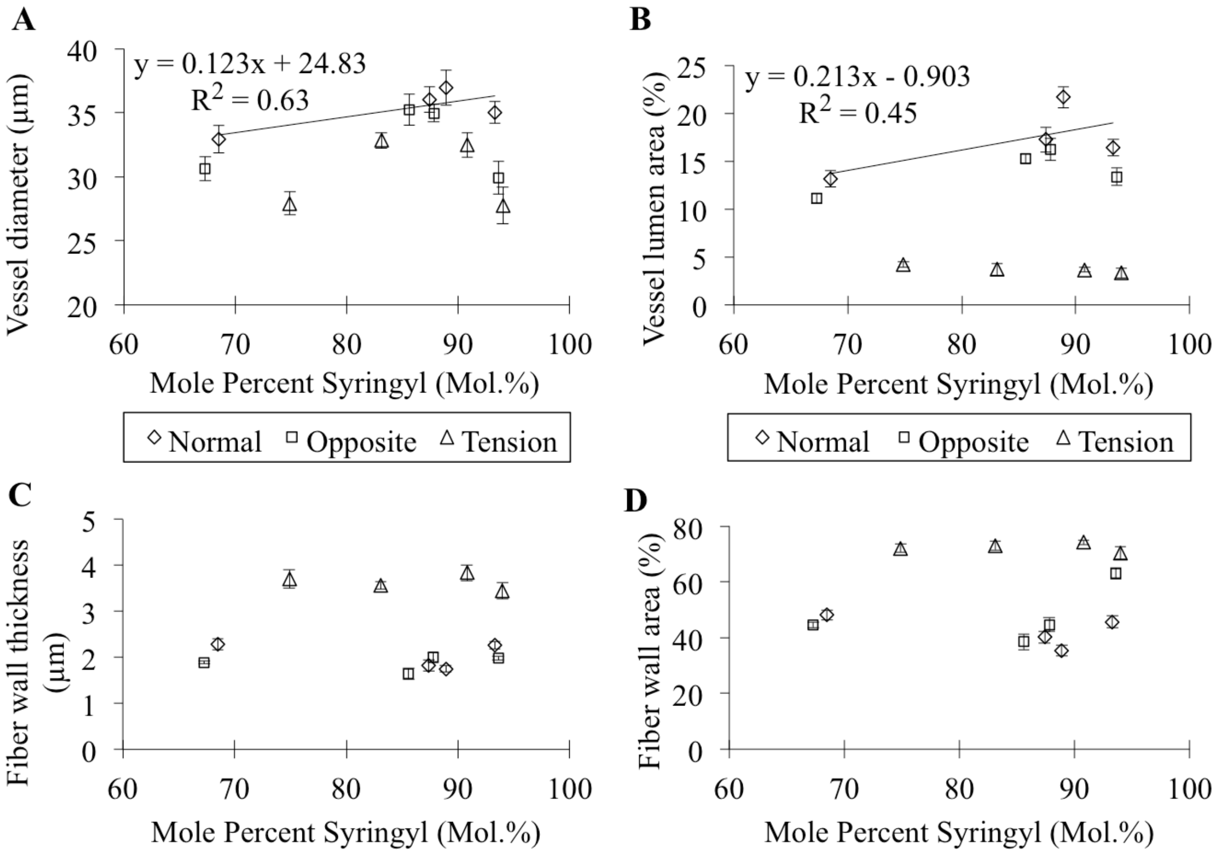

Figure 3.10. Vessel and fiber properties in normal wood collected from upright poplar trees, and opposite and tension wood from inclined trees. Wild type clone (<80 mol.%) and *F5H* over-expression lines (>80 mol.%) with higher levels of syringyl were used. Means ± *SE* are shown with *n* = 6 poplar trees per treatment line. Trendlines in A and B represent significant trends in normal wood only.

The percent fiber area decreased with increasing mole percent syringyl in normal wood ($P<0.001$) (Figure 3.11 A). In tension wood, percent fiber area was similar at all levels of syringyl. But the average fiber area (main effect) in tension wood was 88.24 ± 0.42 % (± *SE*) which is higher compared to 77.45 ± 0.86 % in normal wood or 79.45 ± 0.49 % in opposite wood. The percent parenchyma area was not affected by changes in mole percent syringyl in any of the wood tissues (Figure 3.11 B), while this value was slightly higher in tension wood ($P<0.001$). The latter wood type had percent parenchyma area of 8.03 ± 0.30 % compared to 5.94 ± 0.19 % in normal wood and 6.05 ± 0.19 % in opposite wood. Similarly, increasing syringyl lignin percentage in any of the three wood tissues had no effect on percent total lumen area in the transverse sections (Figure 3.11 C). Tension wood had significantly lower percent total lumen area (P<0.001) with an average of 19.61 ± 0.76 % (± *SE*) compared to 52.60 ± 1.28 % in normal wood and 45.42 ± 2.20 % in opposite wood. Vessel implosion resistance $(t/b)^2$ was also estimated, but showed high variability across the different *Populus* lines (Figure 3.11 D). Nevertheless, when a linear regression model was fitted separately to the $(t/b)^2$ data of normal wood, a slight, but significant (P=0.0101) decrease in vessel implosion resistance was observed with increasing percentage of syringyl lignin in the xylem.

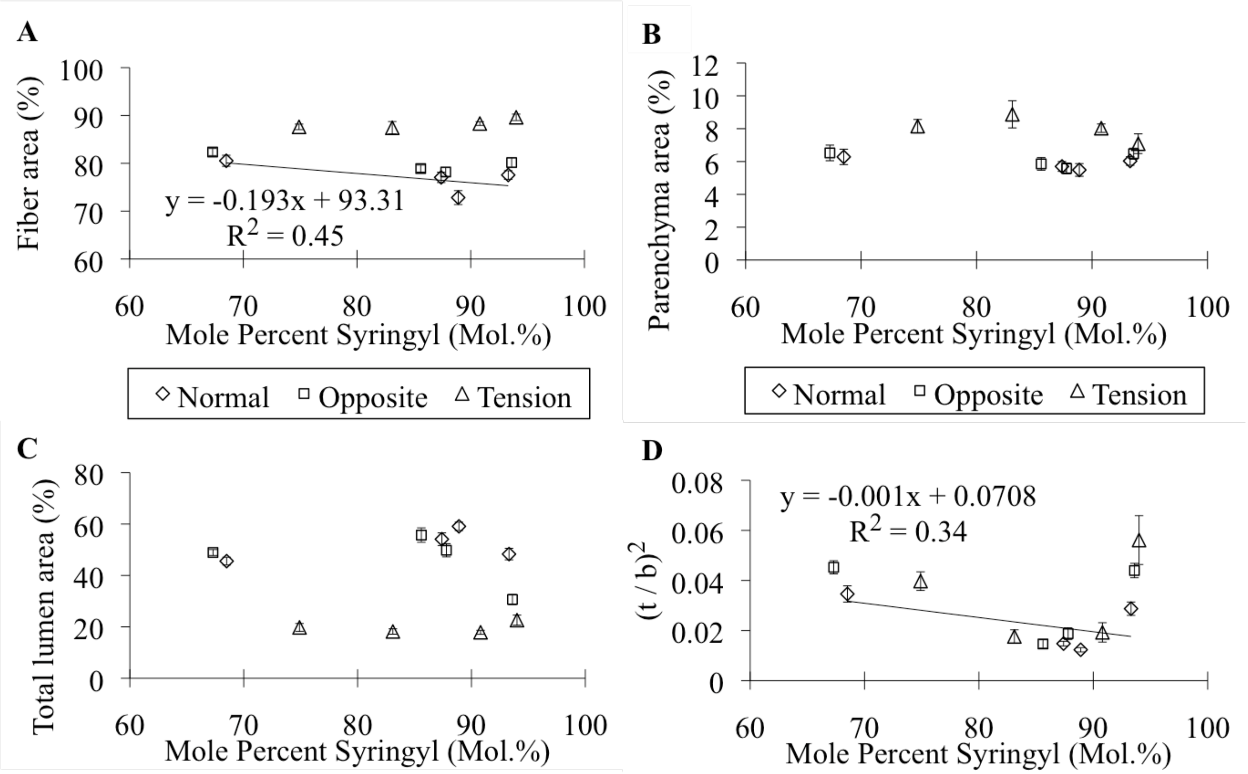

Figure 3.11. Anatomical properties of normal wood in upright poplar trees versus opposite and tension wood in inclined trees. Wild type and *F5H* over-expression lines with higher levels of syringyl were used. The vessel implosion resistance $(t/b)^2$ was determined for vessel pairs with t as the thickness of adjoining vessel walls and b the

lumen diameter of the vessel. Means ± *SE* are shown with *n* = 6 poplar trees per treatment line. Trendlines in A and D represent significant trend in normal wood only.

The stems of wild type trees (68 mol.% syringyl lignin) and those of the over-expression line with highest syringyl lignin percentage (93 mol.%) were harvested from upright trees with normal wood. The stems were used to measure xylem density, which was then plotted against their modulus of rupture (MOR). As illustrated in Figure 3.12, the upright stems with higher mole percent syringyl had lower MOR but similar xylem density compared to the wild type stems (refer to Figure 2.17 for full MOR results).

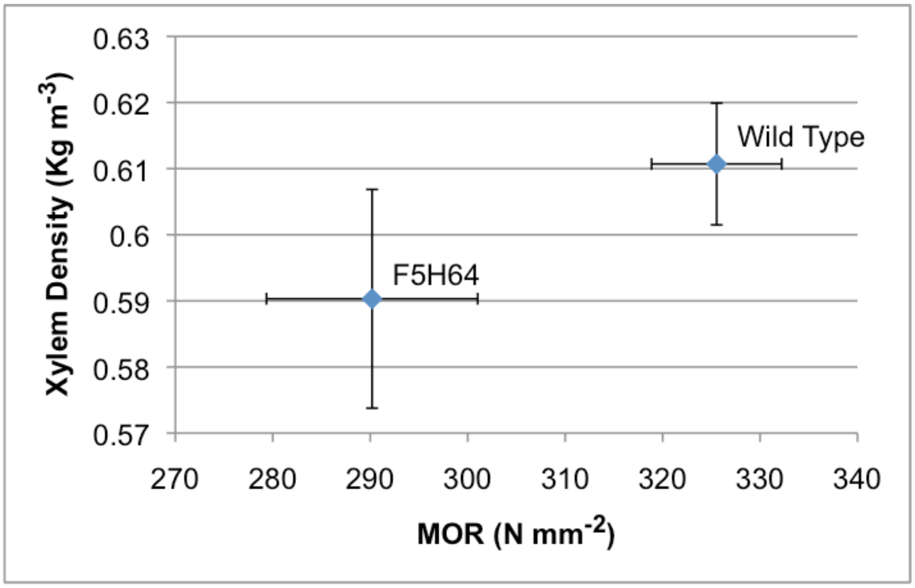

Figure 3.12. Xylem density and modulus of rupture (MOR) of normal wood of both, wild type poplar trees (68 mol.% syringyl lignin) and an *F5H* over-expression line *F5H64* (93 mol.% syringyl lignin). Oven-dried debarked stems were used. Means ± *SE* are shown with *n* = 16 poplar stems for xylem density and *n* = 22 poplar stems for MOR. For the method of determining MOR refer to chapter 2.

DISCUSSION

Over-expressing the *F5H* gene leads to higher syringyl to guaiacyl unit ratio in the lignin of poplar stems (Franke et al., 2000). To investigate the effect of the lignin modification on the hydraulic properties in upright trees as well as in stems that formed tension wood due to inclination treatment. The results in an experimental trial conducted in 2006 showed a clear increase in the specific conductivity in the upright stems with increasing mole percent syringyl in lignin (Figure 3.1). This finding supported our hypothesis; however, the increase in syringyl ratio did not affect the water conductivity in the inclined trees. The improved conductivity in the upright stems could be attributed to the wider vessels and/or increased vessel frequency. Koehler and Telewski (2006) analyzed UV-microspectrophotometric scans of the same poplar over-expression lines that were used in our investigation and found more syringyl-rich lignin in fiber walls of *Populus* xylem while guaiacyl-rich lignin was more abundant in the vessel walls. The higher hydrophilic properties of guaiacyl are less likely to account for the level of increase in water conductivity in our upright stems with higher syringyl to guaiacyl ratio. Attempts to reproduce the results in 2008 failed due to lack of uniform plants and instrumental inaccuracy.

Elevating the syringyl ratio in lignin had no effect on vulnerability to embolism in the *Populus* stems. Tension wood formation also did not affect vessel vulnerability to embolism. These findings are consistent with Gartner el al. (2003) who reported tension wood formation in *Quercus ilex* seedlings had no impact on vulnerability to embolism or specific conductivity. Based on the presented results, treatments or stimuli that affect stem conductivity may not affect its vulnerability to embolism, and vice versa. Jacobsen et al. (2005) found no tradeoff between cavitation resistance and specific conductivity in chaparral shrubs. Cavitation in the vessels is more related to the mechanical properties of the vessel walls (Hacke et al. 2001). In chapter 2, elevated syringyl ratio had no effect on the elastic properties of the cell wall and the stem overall. However, the modified ratio caused a slight increase in the oven-dried stem brittleness (Figure 2.17).

During the three months of the experiment, the upright poplar trees showed an increase in average leaf area in response to elevated syringyl to guaiacyl ratio (Figure 3.5), an effect that was not observed in the inclined trees. The total leaf area remained the same during the same time period (Figure 3.6), indicating that trees with higher syringyl lignin ratio had fewer leaves than the wild type trees. The average leaf area, and the number of leaves, might have influenced the trees water conductive properties. Recently, Voelker et al. (2011) associated the increases in the leaf area-to-sapwood area ratio, and the wood specific conductivity with less biomechanical demand on the xylem in stalked poplar trees. Kitin et al. (2010) reported impeded water transport in low-lignin poplar trees due to tyloses and phenolic deposits. These, and other similar studies, suggest that the biomechanical properties of the trees, also affected by lignin, are significantly correlated with water transport in the xylem.

Measurements of percent area tension wood showed no differences among the *Populus* lines that were kept upright, or among those that were inclined with respect to gravity (Figure 3.9). This suggests that modifying the ratio of syringyl lignin may not affect the level of tension wood being developed in these stems.

Anatomical changes can potentially lead to significant structural and functional alterations in the trees. Thus, the cell wall and lumen of fibers and vessels were measured in normal, opposite and tension wood of the wild type and the three over-expression lines. A slight increase in vessel diameter and percent vessel lumen area may be correlated with the increase in syringyl lignin percentage in normal wood of the upright stems (3.10 A and

B). Tension wood had an overall lower vessel diameter and percent vessel lumen area compared to normal or opposite wood. Fiber wall thickness and percent fiber wall area were similar across all poplar lines, but were higher in tension wood, as was expected, due to the addition of the relatively thick gelatinous layer in the G-fibers. Similar results were reported by Jourez et al. (2001) who compared the anatomy of tension wood to that of opposite or normal wood in young poplar *Populus euramericana* cv 'Ghoy'. However, their study did not take into consideration the aspects of lignin content or composition, or their potential interaction. The percent fiber area decreased with increasing syringyl in normal wood. However, normal wood and opposite wood had significantly lower percentages than tension wood (Figure 3.11). A study conducted by Horvath et al. (2010) reported similar increase in both vessel and fiber lumen area fractions as a result of increased S:G ratio in aspen trees. In addition, they reported an increase in vessel frequency and lower vessel lumen diameter in the trees with higher syringyl lignin ratio compared to the unmodified controls. The percent parenchyma area reported in transverse sections was similar across the poplar lines, whereas it was higher in tension wood. In addition, tension wood had significantly lower percentage of overall lumen area, which may be attributed to the thick gelatinous layer of the G-fibers. The vessel implosion resistance $(t/b)^2$ in normal wood slightly decreased (Figure 3.11). But due to high variance, the $(t/b)^2$ results of opposite wood or tension wood were considered inconclusive. The fore-mentioned anatomical changes refuted our hypothesis of no change in the wood anatomy as a result of the lignin modification.

The wild type trees and those of the highest mole percent syringyl (93 mol.%) were tested for change in xylem density due to changes in anatomical properties. The results indicated no significant change in xylem density due to the lignin modification (Figure 3.12). This finding, and the change in anatomy, falls short of explaining the slight increased in dry stem brittleness when the modulus of rupture (MOR) was estimated in the previous chapter.

CONCLUSION

The water conductive efficiency tests indicated a possible role for syringyl in facilitating water conductivity in the vessels. One hypothesis suggested that a less electric polarity in the vessel walls, associated with a syringyl-rich lignin, to be behind the enhanced water conductivity. Yet, that was not evident with the inclined stems that formed tension wood. The anatomical analyses showed an increase in vessel diameter and percent area vessel lumen in normal wood but not in tension wood. These results can partially explain the increased water conductivity of the stems. There was no clear evidence for an effect on trees vulnerability to embolism due to syringyl enhancement or tension wood formation. It can be hypothesized that higher syringyl lignin ratio in vessel walls may provide additional mechanical support against vessel wall fatigue, which is one of the causes of cavitation. To test this hypothesis, mechanical testing of individual vessel walls will be required. However, this is beyond the capacity of this study. As a result of the higher syringyl lignin ratio, the change in normal wood anatomy may have led to the slight decrease in the stems modulus of rupture (MOR, previous chapter), incurring increased stem brittleness. However, the lignin modification did not affect xylem density in the stem samples that were tested.

Whether these genetically altered poplar trees are suitable for plantation forestry is a question that cannot be answered until the trees are tested outdoors in a natural environment, rather than under the modified conditions of the greenhouse. Based on the findings of this study, it would appear that under field conditions, trees with a higher syringyl lignin can potentially perform similarly, or better, than the unmodified wild type trees.

ACKNOWLEDGEMENTS

We thank David Ellis (National Center for Genetic Resources Preservation) and Clint Chapple (Purdue University) for providing us with the transgenic poplar lines used in this study, Amy Wilson and Emily Flynn (Michigan State University) for their help with the anatomical measurements. This research was supported by the National Research Initiative of the USDA CSREES grant number 2005-35103-15269

REFERENCES

REFERENCES

Alder NN, Pockman WT, Sperry JS, Nuismer S (1997) Use of centrifugal force in the study of xylem cavitation. J Exp Bot **48**, 665-674.

Amrhein N, Frank G, Lemm G, Luhmann HB (1983) Inhibition of lignin formation by L-alpha-aminooxy-beta-phenylpropionic acid, an inhibitor of phenylalanine ammonia-lyase. Eur J Cell Biol **29**(2):139-144.

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Ann Rev Plant Biol **54**:519-546.

Boyce CK, Zwieniecki MA, Cody GD, Jacobsen C, Wirick S, Knoll AH, Holbrook NM (2004) Evolution of xylem lignification and hydrogel transport regulation. PNAS **101**(50):17555-17558.

Boyd JD (1977) Basic cause of differentiation of tension wood and compression wood. Aust Forest Res **7**:121-143.

Capple CS, Vogt T, Ellis BE, Somerville CR (1992) An *Arabidopsis* mutant defective in the general phenylpropanoid pathway. Plant Cell **4**:1413-1424.

Clair B, Almeras T, Pilate G, Jullien D, Sugiyama J, Riekel C (2011) Maturation stress generation in poplar tension wood studied by synchrotron radiation microdiffraction. Plant Physiol **155**:562-570.

Erickson RO, Michelini FJ (1957) The plastochron index. Am J Bot **44**:297–305.

Franke R, Hemm MR, Denault JW, Ruegger MO, Humphreys JM, Chapple C (2002) Changes in secondary metabolism and deposition of an unusual lignin in the ref8 mutant of *Arabidopsis*. Plant J **30**(1), 47-59.

Franke R, McMichael CM, Meyer K, Shirley AM, Cusumano JC, Chapple C (2000) Modified lignin in tobacco and poplar plants over-expressing the Arabidopsis gene encoding ferulate 5-hydroxylase. Plant J **22**:223-234.

Gartner BL, Roy J, Huc R (2003) Effects of tension wood on specific conductivity and vulnerability to embolism of *Quercus ilex* seedlings grown at two atmospheric CO2 concentrations. Tree Physiol **23**(6):387-395.

Hacke UG, Sperry JS, and Pockman WT (2001) Trends in wood density and structure are linked to prevention of xylem implosion by negative pressure. Oecologia **126**:457-461.

Horvath B, Peszlen I, Peralta P, Kasal B, Li L (2010) Effect of lignin genetic modification on wood anatomy of aspen trees. IAWA **31**(1):29-38.

Jacobsen AL, Ewers FW, Pratt RB, Paddock III WA, Davis SD (2005) Do xylem fibers affect vessel cavitation resistance? Plant Physiol **139**:546-556

Jourez B, Riboux A, Leclercq A (2001) Anatomical characteristics of tension wood and opposite wood in young inclined stems of poplar *Populus euramericana* cv 'Ghoy'. IAWA **22**(2):133-157.

Kern KA, Ewers FW, Telewski FW, Koehler L (2005) Mechanical perturbation affects conductivity, mechanical properties and aboveground biomass of hybrid poplars. Tree Physiol **25**(10): 1243-1251.

Kitin P, Voelker SL, Meinzer FC, Beeckman H, Strauss SH, Lachenbruch B (2010) Tyloses and phenolic deposits in xylem vessels impede water transport in low-lignin transgenic poplars: a study by cryo-fluorescence microscopy. Plant Physiol **154**:887-898.

Koehler L, Telewski FW (2006) Biomechanics and transgenic wood. Am J Bot **93**(10):1433-1438.

Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Ann. Rev. Plant Physiol. Plant Mol Biol **41**:455–96.

Mosha Y, Goring DAI (1975) Distribution of syringyl and guaiacyl moieties in hardwood as indicated by ultraviolet microscopy. Wood Sci Technol **9**:45-58.

Pilate G, Déjardin A, Laurans F, Leplé JC (2004) Tension wood as a model for functional genomics of wood formation. New Phytol **164**:63-72.

R Development Core Team (2007) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Sederoff RR, MacKay JJ, Ralph J, Hatfield RD (1999) Unexpected variation in lignin. Curr Opin Plant Biol **2**:145-152.

Schubert WJ (1965) Lignin Biochemistry. Academic Press Inc., New York.

Timell TE (1986) Compression wood in gymnosperms. Vol. I, Springer-Verlag, Berlin.

Tyree MT (1997) The cohesion-tension theory of sap ascent: current controversies. J Exp Bot **48**(315):1753-1765.

Tyree MT, Ewers FW (1991) The hydraulic architecture of trees and other woody plants. New Phytol **119**:345-360.

Voelker SL, Lachenbruch, B, Meinzer FC, Kitin P, Strauss SH (2011) Transgenic poplars with reduced lignin show impaired xylem conductivity, growth efficiency and survival. Plant Cell Environ **34**:655-668.

Woodrum CL, Ewers FW, Telewski FW (2003): Hydraulic, Biomechanical, and Anatomical Interactions of Xylem from Five Species of *Acer* (*Aceraceae*). Am J Bot **90**(5): 693-699.

CHAPTER 4

CHEMICAL RESPONSES TO MODIFIED LIGNIN COMPOSITION IN TENSION WOOD OF HYBRID POPLAR (*POPULUS TREMULA* X *P. ALBA*)

ABSTRACT

The effect of altering the syringyl lignin monomer ratio was investigated in three wood tissues (normal, opposite and tension wood) of one-year old hybrid poplar clone 717 (*Populus tremula* x *P. alba*) transformed with the *C4Hprom::F5H* gene. Wild-type and three transgenic poplar lines were grown either upright or inclined 45° from vertical for three months to induce tension wood formation. Normal wood in the stems of upright trees, and the tension and opposite wood in the stems of inclined trees, were analyzed separately for carbohydrates, lignin, cellulose crystallinity, and microfibril angle. In wild-type poplar stems, tension wood had, on average, 6.4% higher mole percent syringyl lignin than normal wood, and 7.6% higher than opposite wood. These differences diminished in the transgenic trees with increased ectopic syringyl monomer composition. The lignin modification resulted in a slight decrease in the total lignin in normal wood. In tension wood, the addition of a gelatinous layer in the fiber walls resulted in a consistently lower percent of total lignin in the tissue. Acid soluble lignin was observed to increased by up to 2.3 fold in the transgenic lines. Compared to normal and opposite wood, cell wall crystallinity in tension wood was higher and the microfibril angle was smaller, as expected, with no evidence of an effect for modifying syringyl monomer abundance. Tension wood in all lines expressed consistently higher total sugar and glucose percentages compared to

normal wood within the respective lines. However, both sugar and glucose percentages were lower in the tension wood of transgenic lines when compared to the tension wood of wildtype trees. Evaluating the response of trees with altered lignin monomer composition to gravity will improve our understanding of the changes in cell wall chemistry and ultrastructural properties of normal, opposite and tension wood in plants.

INTRODUCTION

Gravity induces the formation of a special type of wood called tension wood in the branches and displaced stems of porous wood in angiosperms. In inclined trunks, it is formed only on the upper side of the stem, whereas wood formed on the lower side is termed opposite wood. Generally, tension wood has more cellulose, and less lignin and hemicelluloses than normal wood (Timell 1969). Tension wood is also characterized anatomically by wood with more fibers and fewer vessels of smaller diameter. In most woody angiosperms, tension wood fibers have secondary cell walls with up to two S-layers and an additional layer of gelatinous appearance (G-layer). In the G-layer, highly crystalline microfibrils are oriented almost parallel to the longitudinal axis of fiber, or what is referred to as “approaching zero degrees” (Pilate et al. 2004). The microfibrils are of high tensile strength and the angle they form correlates with shrinkage events in the tension wood at maturation (Boyd 1977). The major components of the G-layer in the tension wood of woody dicots are, by dry weight of the acid insoluble residue (AIR), ~70% cellulose, ~15% xyloglucan, ~2% mannan, ≤8% pectin, and traces of lignin. In normal wood, the secondary cell walls have three S-layers, S_1, S_2 and S_3. In these layers, cellulose makes 50% of the dry weight of the AIR. Other major components include 20-30% xylan, ~5% glucomannan and 10-20% lignin. Composition summaries of wood tissue and cell wall layers can be found in Nishikubo et al. (2007) and Mellerowicz and Gorshkova (2012).

The hemicelluloses help maintain the structure of the primary and secondary cell walls. Several models have been proposed on how this is achieved, including cross-linking the cellulose microfibrils (Atalla et al. 1993, Cosgrove 2000), covalently linking to pectins (Keegstra et al. 1973), and/or interacting with lignin (Sugino et al. 2004). The amount of hemicellulose varies among species, cell types and cell wall layers. Their backbones share a similar structure that is made up of glucose, mannose, or xylose. In dicots, the backbones may be linked to branches consisting of associated sugars to make xyloglucans, xylans, mannans, and glucomannans. Xyloglucans are the most abundant hemicelluloses in the primary cell walls of most plants, and play an important role in the G-layer of secondary cell walls (Nishikubo et al. 2007). Their glucan backbones are substituted with xylose sidechains that often terminate with arabinose and galactose residues (reviewed in Fry 1989). Xylans are the dominating non-cellulosic polysaccharides in the secondary walls of woody angiosperms (Ebringerová and Heinze 2000, McCartney et al. 2006, Bowling and Vaughn 2008, Kim and Daniel 2012). They make up 25-35% of the dry biomass of woody dicots, and are comprised of mostly of xylose and a lower level of arabinose. Xylans are also implicated in strengthening the secondary walls against xylem collapse and failed water transport (Bernal et al. 2007, Persson et al. 2007). Mannans are composed of a mannose backbone whereas glucomannans also contain glucose. These two hemicellulose moieties are usually less abundant in gymnosperms and angiosperms. In poplar secondary cell walls, glucomannan was mostly found in the S-layers while mannan was found to a lesser extent in the G-layer (Kim and Daniel 2012, Mellerowicz and Gorshkova 2012). Hemicelluloses are of economic significance as they influence biomass saccharification and thus increase the level of biofuel produced. To improve production efficiency in plant tissues, several researchers have attempted to modify the composition of hemicelluloses (reviewed in Scheller and Ulvskov 2010).

Lignins are important phenolic polymers that are abundant in the middle lamellae and secondary cell walls of terrestrial plants (Boerjan et al. 2003). Generally, they have been perceived to provide rigidity and a hydrophobic surface to conductive cells in the xylem - (Hacke et al. 2001), contributing to the mechanical and physiological importance in woody tissues. The mechanical strengths of lignin in the opposite wood and cellulose in the gelatinous fibers of tension wood are necessary for angiosperm trees to establish the gravitropic response (Fratzl et al. 2008). Lignins are derived from the random polymerization of three monolignols; coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol, and are commonly referred to as guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units once incorporated into the lignin polymer. Across the plant kingdom, where lignin is synthesized, the composition and monomer ratios vary substantially (reviewed in Vanholme et al. 2010). These ratios have been found to change in response to external stimuli such as wind (Koehler and Telewski 2006), and gravity (Baillères et al. 1995). To understand the contribution of the monomers to tree form and function, several studies have looked at the differing ratios of monomers in different tissues or plant species (Yoshinaga et al. 1992, Chabbert et al. 1997, Hoffmann et al. 2003). However, the combined effects of other variables such as anatomy and the chemical composition of these tissues and species complicates the interpretation of the role of lignin monomer composition on structure and function. Investigating the affect of lignin composition in transgenic lines of the same species minimizes the possible bias of other structural and anatomical parameters introduced when different plant species or different growth forms of the same species are compared.

Ferulate 5-hydroxylase (F5H) is a key enzyme in regulating lignification and, specifically, in controlling lignin monomer composition in woody plants (Franke et al. 2000). Using coniferaldehyde and coniferyl alcohol as substrates, F5H diverts phenylpropanoid flux towards the synthesis of syringyl lignin monomers, and away from guaiacyl synthesis (Chen et al. 2000). Over-expression of *F5H*, therefore, drives the lignin biosynthetic pathway and alters the inherent syringyl to guaiacyl ratio (S:G) without changing the overall lignin content (Huntley et al. 2003, Stewart et al. 2009). The objective of this research was to investigate the effect of altering the syringyl lignin percentage in poplar on cell wall components in normal wood, as well as in opposite and tension wood. In this study, transgenic hybrid poplar was used as a model for a woody perennial angiosperm containing different levels of syringyl lignin.

MATERIALS AND METHODS

Plant material and sampling

This study was conducted using wild-type hybrid poplar clone 717 (*Populus tremula* x *P. alba*) and three lines of transgenic trees (*F5H37*, *F5H64* and *F5H82*) previously described by Franke et al. (2000) over-expressing the Arabidopsis *F5H* gene. The syringyl monomer abundance in the normal wood ranged from 68.5 mol.% in the wild-type to a high of 93.4 mol.% in *F5H64* mutant. Young trees of verified lignin composition were grown under greenhouse conditions in the summer at Michigan State University. Pots (18-liter) with a soil-less medium (Baccto, Michigan Peat Company, Houston, TX) were used with no additional fertilization or supplemental lighting. Trees with 100-120 cm long stems were inclined 45° from vertical to induce tension wood formation, and were grown for three months along with paired upright controls. For chemical analyses, stem samples were taken 15 cm above the root collar. They were debarked and air-dried at 60°C to achieve a constant weight. Stem segments, taken from the inclined trees, were cut longitudinally to separate tension wood from opposite wood for independent chemical analyses.

Crystallinity and microfibril angle

Microfibril angle and cell wall crystallinity were determined by X-ray diffraction according to Coleman et al. (2009) using a Bruker D8 Discover X-ray diffraction unit equipped with an area array detector (GADDS). Briefly, diffractograms were obtained from samples of radial face wood sections precision cut (1.69 mm) from the growing stem isolated 15 cm above the root collar. Wide-angle diffraction was used in transmission mode, and the measurements were performed with CuKα1 radiation ($\lambda = 1.54$ Å), the X-ray source was fit with a 0.5 mm collimator and the scattered photon collected by a GADDS detector. Both the X-ray source and the detector were set to theta = 0° for microfibril angle determination, whereas the 2 theta (source) was set to 17° for wood crystallinity determination. The average T-value of the two 002 diffraction arc peaks was used for microfibril angle calculations, as per the method of Ukrainetz et al. 2007, whereas crystallinity was determined by mathematically fitting the data using the method of Vonk (1973). Two radii were taken from each tree and these values were averaged for each tree.

Lignin and carbohydrate analysis

Extractive-free ground wood was prepared by grinding representative samples of wood in a Wiley mill to pass through a 40 mesh sieve, and Soxhlet extracted overnight with acetone at 70°C. The extract free wood was then dried over P_2O_5 and retained for complete chemical analysis. A modified Klason method was employed to determine total carbohydrate and lignin ratios (Huntley et al. 2003). The acid-insoluble residual (AIR) was quantified gravimetrically after acid hydrolysis, whereas the acid-soluble lignin was quantified using absorption spectroscopy at 205 nm (TAPPI Useful Method UM-250, 1991). Carbohydrate concentration was determined by high-performance liquid chromatography (Dionex BioLC, Dionex, CA) equipped with an ion exchange PA1 column, a pulsed amperometric detector with a gold electrode, and a AS50 auto-injector. Prior to injection, samples were filtered through 0.45 mm filters (Millipore, Bedford, MA). A 20 mL volume was loaded on the column equilibrated with 250 mM NaOH and eluted with de-ionized water at a flow rate of 1.0 mL min^{-1}, followed by a post column addition of 200 mM NaOH at a flow rate of 0.5 mL min^{-1}. Each sample was determined in triplicate.

Syringyl to guaiacyl to *p*-hydroxyphenyl ratios were determined by thioacidolysis (Robinson et al. 2009) using 10 mg samples of extractive-free wood. Gas chromatography analyses were performed on a HP 5890 Series II fit with a 15 m x 0.25 mm DB-5 column (J&W Scientific) and FID detector. The GC method used a 2.0 μL injection volume, an initial injector temperature of 250°C, and a detector temperature of 270°C. The initial oven temperature was set to 130°C (held for 3 min.) and thereafter ramped at a rate of 3°C min^{-1} to 260°C and held for 5 min.

Data analysis

Three individual trees for each genetic line and treatment were sampled for statistical analyses. Data were analyzed using the software R (R Development Core Team 2007). Wood type and line effects were evaluated by a two-way analysis of variance (ANOVA). Assumptions of normality and homogeneity of variances were tested.

RESULTS

Effects of genetic and environmental modifications on total lignin and lignin components

Inclined hybrid poplar trees of the wild-type origin and the three F5H over-expression lines formed tension wood on the upper side of the leaning stems. When compared to normal wood, the tension wood of wild-type (non-transformed controls) poplar stems had a 6.4 mol.% increase (P=0.04) in syringyl monomer percentage at the expense of guaiacyl monomer (Figure 4.1, A). Transgenic line *F5H64* had a statistically similar percentage of ~93 mol.% in all three wood tissues. In wild-type hybrid poplar, percent total lignin in tension wood was significantly lower (P=0.011) than in both normal or opposite wood (Figure 4.1, B). Among normal wood, percent total lignin was 2.6% higher in wild-type trees than in transgenic trees (P=0.005, correlation coefficient = -0.56). Both wood type and syringyl monomer abundance affected the percent acid soluble and insoluble lignins (Figure 4.1, C and D). On a wood sample weight basis, in addition to lower total lignin, tension wood consistently had a lower percentage of soluble and insoluble lignins compared to normal or opposite wood (both P<0.001). In the normal wood of trees expressing an increasing ratio of syringyl monomer, lignin acid solubility increased by 2.3 fold (P<0.001, correlation coefficient = 0.90), whereas acid insoluble lignin (Klason) decreased by as much as 5.5% (P<0.001, correlation coefficient = -0.88).

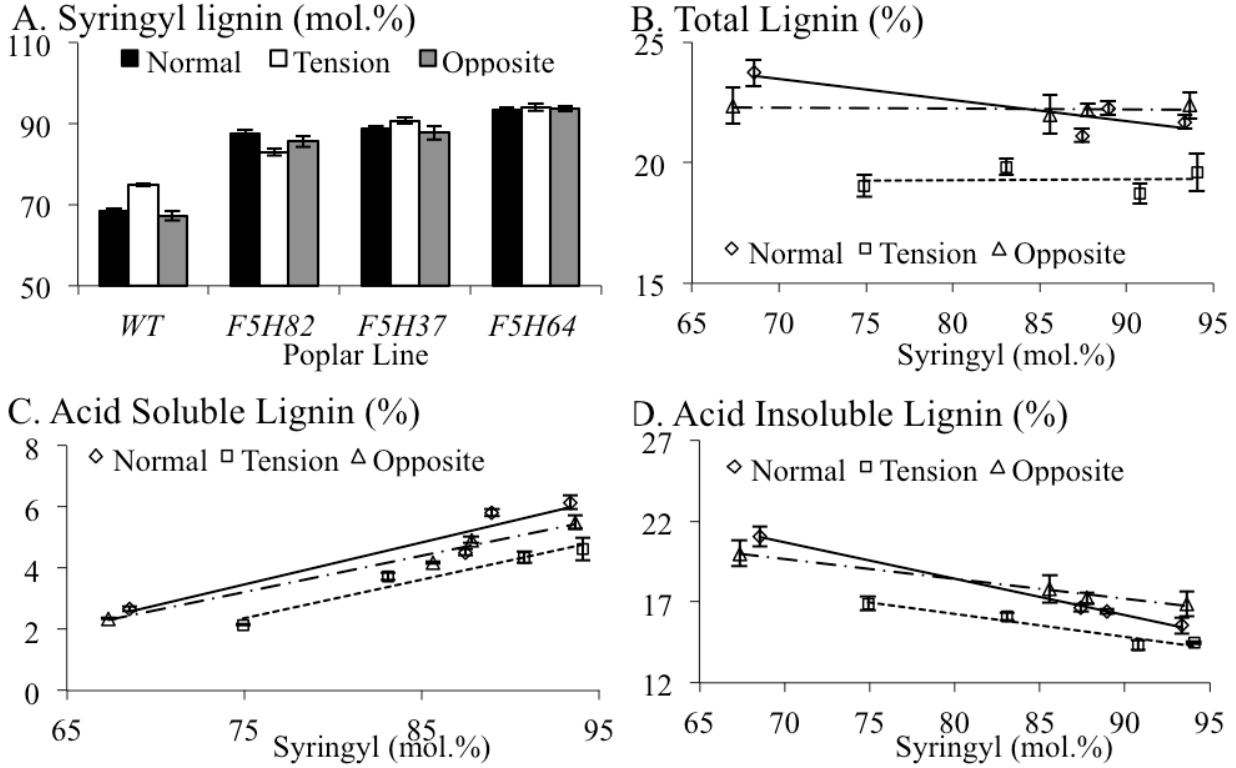

Figure 4.1. Lignin ratio and composition of hybrid poplar clone 717 (*Populus tremula* x *P. alba*) wild-type and transgenic lines *F5H37*, *F5H64*, and *F5H82*. Trees were kept upright or inclined with respect to gravity to form tension wood. Wood samples of normal, opposite and tension wood were dried and analyzed separately. (A) Poplar lines showing different levels of mole percent syringyl. (B) Effect of modified mole percent syringyl on percent total lignin. (C) Percent lignin acid solubility and (D) acid insolubility, as affected by syringyl enhancement. Error bars represent means±*SE* (*n*=3 trees).

Effect on cellulose microfibril angle (MFA) and cellulose crystallinity

Both wild-type and transgenic poplar trees which were displaced with respect to the gravational vector, produced a typical gravitropic response. This included the producing of tension wood on one side of the stem resulting in the reorientation of the displaced stem to the vertical position. (Figure 4.2). Within any wood tissue, increased syringyl monomer abundance did not affect the cellulose MFA (Figure 4.3, A), while tension wood had a significantly lower MFA ($P<0.001$). Cell wall crystallinity, which does not appear to be affected by the change in lignin composition, was significantly higher in tension wood ($P<0.001$) compared to opposite or normal wood (Figure 4.3, B).

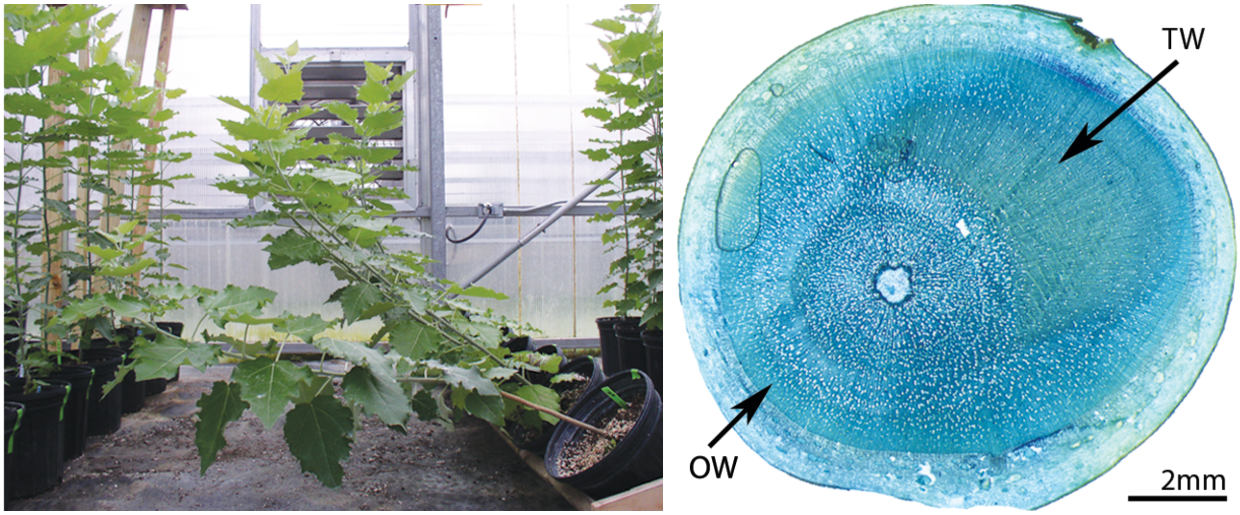

Figure 4.2. Poplar trees, of varying mole percent syringyl, responding to gravity (left). Transverse section of wild-type poplar stem after three months of inclination treatment (right). Tension wood (TW) and opposite wood (OW) were labeled, and methylene blue staining was used.

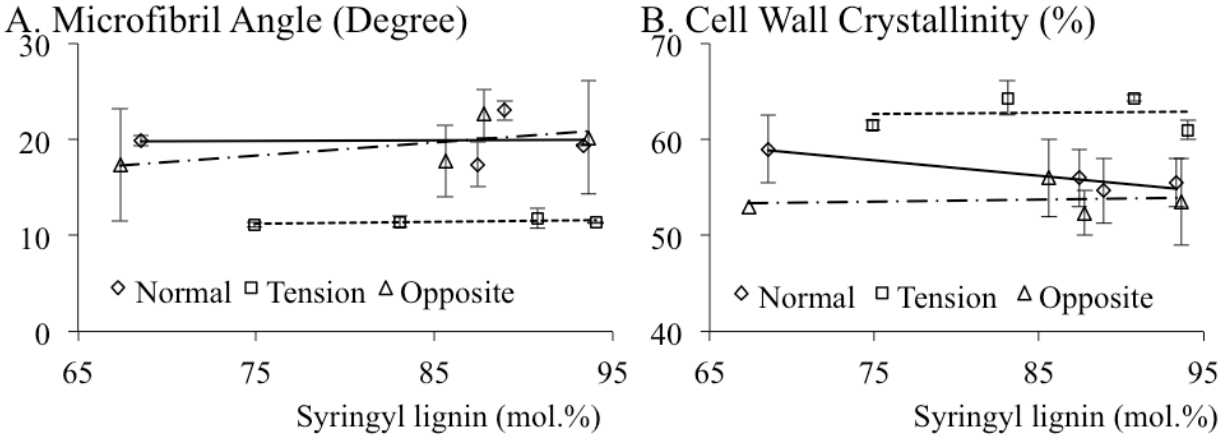

Figure 4.3. Effect of varying mole percent syringyl on (A) microfibril angle and (B) cell wall crystallinity in hybrid poplar clone 717 (*Populus tremula* x *P. alba*) expressing *C4Hprom::F5H* at different levels. Trees were kept upright or inclined with respect to gravity to form tension wood. Wood samples of normal, opposite and tension wood were dried and analyzed separately. Error bars represent means±*SE* (*n*=3 trees).

Effect on wood carbohydrate content

In wild-type and transgenic hybrid poplar lines, normal wood had a significantly lower percentage of total sugars (*P*<0.001) than tension or opposite wood (Figure 4.4, A). Interestingly, there was a slight, yet significant, interaction between percent syringyl monomer in the lignin fraction and percent total sugar (to dry mass) in tension wood (*P*=0.038, correlation coefficient = -0.63); a 19.1 mol.% increase in syringyl monomer abundance in tension wood was accompanied by a 3.6% decrease in total sugar relative abundance. A similar trend was observed when the lignin modification was accompanied by a 4.1% decrease in glucose relative abundance (Figure 4.4, B). On average, percent galactose was higher in tension wood than in normal wood (*P*=0.038) or opposite wood (*P*=0.004)(Figure 4.4, C). However, percent galactose in tension wood decreased with increasing abundance of syringyl lignin and equalled its level in normal wood. The overall average percent xylose in tension wood was 2.2% lower than in normal wood (*P*<0.001). Altering the syringyl monomer percentage did not significantly affect xylose relative abundance in the three wood tissues (Figure 4.4, D), whereas a significant interaction was shown between percent xylose and percent total lignin in any of the wood tissues (*p*≤0.006) (Figure 4.5). The average rhamnose percentage was higher in normal wood (*P*<0.001) than in either tension or opposite wood. However, in spite of the differing monomer ratios in their lignin, the relative abundance of rhamnose within each wood type remained constant (Figure 4.4, E). Manipulating the syringyl monomer ratio in poplar trees did not affect the relative abundance of mannose (Figure 4.4, F) or arabinose (data not shown) in the three wood types.

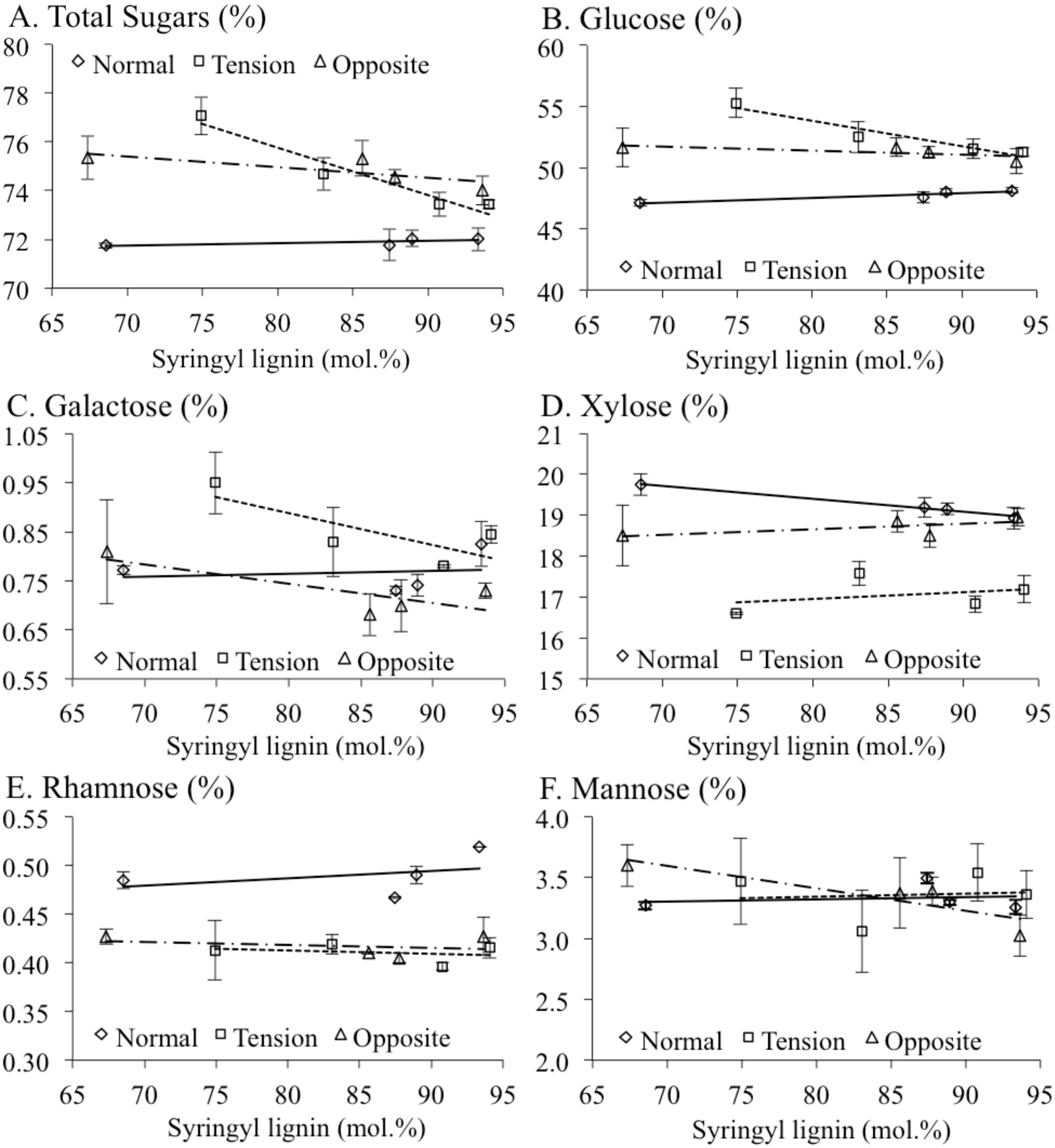

Figure 4.4. Effect of varying mole percent syringyl on the percentages, to dry mass, of (A) total sugars, (B) total glucose, (C) galactose, (D) xylose, (E) rhamnose, and (F) mannose in hybrid poplar clone 717 (*Populus tremula* x *P. alba*) expressing *C4Hprom::F5H* at different levels. Trees were kept upright or inclined with respect to gravity to form tension wood. Wood samples of normal, opposite and tension wood were dried and analyzed separately. Error bars represent means±*SE* (*n*=3 trees).

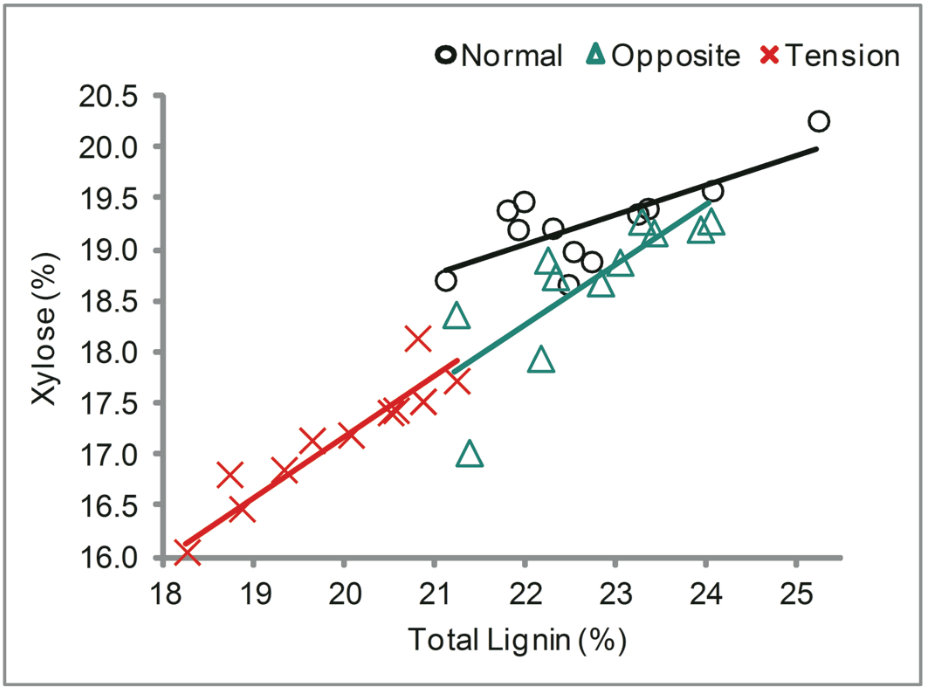

Figure 4.5. Regression plot showing significant correlation between percent total lignin (soluble and insoluble) and percent xylose in three wood types: normal (Norm), opposite (Oppos) and tension (Tens) in hybrid poplar clone 717 (*Populus tremula* x *P. alba*) expressing *C4Hprom::F5H* at different levels. The trees were inclined with respect to gravity to induce tension wood formation whereas upright trees were used as controls. In addition to normal wood in upright trees, opposite and tension wood from each inclined tree were separated, dried and analyzed separately.

DISCUSSION

Environmental stimuli, such as gravity, alter the shape and form of angiosperm trees often resulting in stem reorientation to a new upright position driven by the tensile force of specialized xylem tissue called tension wood (Pilate et al. 2004). Different approaches have been adopted to induce tension wood formation. Andersson-Gunnerås et al. (2006) and Foston el al. (2011) induced the formation of tension wood through bending the stems (higher mechanical stress) compared to displacing the trees with respect to gravity (lower mechanical stress), a method employed in this study. Inclination of unsupported angiosperm stems is usually followed by tension wood formation on the upper side, but only in the lower part of the stem, leading to stem re-orientation to the vertical position. Bending the stems leads to the formation of a wider spread of homogenous tension wood on the side under tension. The effect of stem bending is analogous to that of snow build up on the stem or branches, and the effect of tree inclination is similar to that of sudden changes to land surface topography following landslides. A major anatomical characteristic of tension wood in poplars is a fiber with an additional non-lignified cell wall layer of gelatinous appearance (G-layer) that is mostly composed of highly crystalline cellulose microfibrils. Previous analyses using *Populus tremula* x *alba* reported 24% lignin in tension wood compared to 27% in opposite or normal wood (Foston et al. 2011). Despite the fact that tension wood presents a problem to the lumber industry, its low lignin and high cellulose characteristics are of interest as a feedstock for alternative energy production (Ragauskas et al. 2006).

Effect on lignin

Sarkanen and Hergert (1971) reported an increase in syringylpropane units in tension wood compared to normal wood in birch (*Betula* sp.) and madrona (*Arbutus* sp.). Foston et al. (2011) reported an increase in syringyl to guaiacyl ratio in tension wood compared to normal wood in hybrid poplar (*Populus tremula* x *alba*), a change that is equivalent to 12.5% based on NMR data. Other studies observed a similar trend (Bland and Scurfield 1964, Baillères et al. 1995, Yoshida et al. 2002, Aguayo et al. 2010). The presented results in hybrid poplar concur with these reports, and show a typical response to the gravitropic stimulus. Wild-type poplar had an average of 6.4% increase in syringyl monomer ratio in tension wood when compared to normal wood, and an average of 7.6% increase compared to the paired opposite wood section. A similar increase in the monomer ratio was also reported for the same type of hybrid poplar trees as a result of mechanically perturbing them to simulate wind sway (Koehler and Telewski, 2006). Gravity-induced increase in syringyl lignin abundance in tension wood diminished in the transgenically modified trees in the presented study. Exogenous *C4Hprom::F5H* over-expression in these trees led to a near-saturation of lignin with the syringyl monomer.

A negative correlation existed between percent syringyl monomer and percent total lignin in normal wood in poplar stems. In a study using 13 different poplar clones (*P. deltoides*, *P. deltoides* x *P. maximowiczii* F1 hybrids, *P. deltoides* x *P. nigra* F1 hybrids and undisclosed *Populus* species), it was found that a strong negative correlation existed between S:G ratio and lignin content (Bose et al. 2009). In the presented study, percent total lignin (soluble and insoluble) was significantly higher in normal and opposite wood, compared to the paired tension wood samples. Tension wood incorporates a mostly cellulosic layer that adds to the total mass of the cell wall. A study employing eucalyptus showed similar results where tension wood formation had impaired lignification compared to normal wood (Aguayo et al. 2010). In spite of the slight decrease in percent total lignin in normal wood of poplar over-expression lines in the current study, acid soluble lignin percentage increased by up to 2.3 fold in the same tissue. The increase in lignin acid solubility was an expected outcome and is in agreement with previous studies as syringyl moieties are more acid labile than guaiacyl (Stewart et al. 2006). As a result of the positive correlation between lignin acid solubility and syringyl monomer abundance, increasing the proportion of syringyl lignin resulted in more than 60% reduction in pulping time and/or a concurrent reduction in the processing chemicals (Huntley et al. 2003).

Effect on cellulose microfibril angle (MFA) and cellulose crystallinity

Cellulose microfibril angle and crystallinity were found to correlate with total lignin, growth stresses, and shrinkage events in the stems (Boyd 1977, Baillères et al. 1995, Yoshida et al. 2002). In the hybrid poplar trees that were evaluated in this study, the MFA decreased (closer to the parallel with the fiber axis) in tension wood and the cellulose crystallinity increased. These two ultrastructural changes can be correlated with the formation of the cellulose-rich gelatinous layer. These findings are consistent with previously reported characteristics of tension wood (Boyd 1977, Pilate et al. 2004). Altering the syringyl monomer percentage in the transgenic lines did not influence secondary cell wall MFA or crystallinity in the different wood tissues. The results reported here suggest that lignin monomer composition, in angiosperm wood, does not affect these ultrastructural traits.

Effect on wood monosaccharides

In *Populus alba* L*,* xylose is a component of xyloglucan, a hemicellulose that exists in the G-layer of tension wood fibers. Xyloglucan makes up 10-15% of the layer dry mass, making it the most abundant non-cellulose carbohydrate of the layer (Nishikubo et al. 2007, Mellerowicz et al. 2008, Kaku et al. 2009, Mellerowicz and Gorshkova 2012). The data presented here show significantly lower xylose percentage in tension wood (16.6% of dry mass) compared to opposite (18.5%) or normal wood (19.8%). These results are similar to previous reports (Fuji et al. 1982, Foston et al. 2011). However, other studies showed higher xylose in developing tension wood of poplar (Andersson-Gunnerås et al. 2006), and in 8-year old eucalyptus (Aguayo et al. 2010). The latter study also found an increase in the content of hemicelluloses in tension wood contrary to an earlier report by Timell (1969). In some instances the same plant yields different results when analyzed at different developmental stages. Mature, light-grown pea (*Pisum sativum* L. cv. Alaska) stems yielded less xyloglucan than elongating, dark-grown stems indicating either less xyloglucan later in the tissue or less accessibility to extraction enzymes due to altered structure (Pauly et al. 2001). Similarly, tension wood samples may return differing results when analyzed at varying developmental stages. Andersson-Gunnerås et al. (2006) reported higher xylose in developing tension wood of field-grown hybrid aspen (*Populus tremula* L.) 11 days after bending, whereas samples of fully developed tension wood of hybrid poplar (*Populus tremula* L. x *P. alba* L.) bent for 60 days showed significantly lower xylose than those of normal wood (Foston et al. 2011). This would indicate that standardization for tissue age may be required for comparative studies on cell wall composition. Notable differences between tension wood and normal wood exist when the different cell wall layers are analyzed separately. In most woody angiosperms, the G-layer of tension wood fiber, which replaces the S3 layer in normal wood fiber, has distinct cellulose ultrastructure, non-cellulose carbohydrates, and lignin content (reviewed in Mellerowicz and Gorshkova 2012). These features give tension wood its distinct chemical and structural properties.

A decrease in mannose and 1,4-β-mannan in tension wood compared to normal wood of aspen was previously reported (Andersson-Gunnerås et al. 2006, Hedenström et al. 2009), whereas no significant change in percent mannose was observed between the two tissues in 8-year old eucalyptus (*Eucalyptus globulus* Labill.) sapwood (Aguayo et al.

2010). The mannose quantification results in the present study were inconclusive due to high variance among the replicates. Galactose is a major component of several polymers in plant cell walls. In the G-layer, galactose and arabinose are components of both arabinogalactan II and xyloglucan sidechains. The latter were reported to cross-link the G-layer to the adjacent S_2 Layer (Nishikubo et al. 2007). Similar to the results presented here for hybrid poplar, galactose was found to be higher in tension wood than in normal wood of beech species, and therefore has been proposed to be an indicator of the presence of tension wood (Ruel and Barnoud 1978, Azuma et al. 1983). However, a significant decrease in percent galactose was observed in tension wood of 8-year old eucalyptus sapwood (Aguayo et al. 2010) indicating contrasting results from different species or tissues. The increase in syringyl lignin abundance in poplar in this study was correlated with a slight decrease in galactose abundance in tension wood. This decrease may be a result of reduced galactose binding to xyloglucan and/or arabinogalactan II in the G-layer due to lower galactosyltransferases activity, or due to reduced epimerization of glucose into galactose. Glucose abundance was also found to slightly decrease with increasing syringyl monomer abundance in the same samples of tension wood. Similar to galactose, different studies reported contrasting results for rhamnose; one fraction of alkaline cell-wall extracts was found to contain higher level of rhamnose in tension wood than normal wood of Japanese beech, (*Fagus crenata* Blume) (Azuma et al. 1983), whereas lower levels were found in tension wood in eucalyptus sapwood (Aguayo et al. 2010). Rhamnose and galacturonic acid make up the backbone of rhamnogalacturonan I (RG I) pectins. In poplar (*Populus alba* L), rhamnose was reported to exist in isolated G-layer of tension wood (Nishikubo et al. 2007). In American beech (*Fagus grandifolia* Ehrh.), rhamnose was part of galactan in the G-fiber of tension wood suggesting the presence of RG I in these fibers (Kuo and Timell 1969). However, according to Mellerowics and Gorshkova (2012), a biochemical proof of the presence of RG I in tension wood is still lacking.

The estimated glucose percentages in this study take into account the abundance present in both cellulose and hemicelluloses. In tension wood, a slight decrease in extractable glucose would be expected due to the previously reported decrease in hemicelluloses. This decrease is usually offset by the increase in cellulose deposition in the G-layer; which explains the higher percent glucose of tension wood compared to normal wood. However, the slight, yet statistically significant, decrease in tension wood glucose and overall sugar percentages in response to syringyl monomer enhancement raises further questions as to whether the ferulate 5-hydroxylase (F5H) enzyme, or any of its products, is involved in the signaling process for tension wood formation, or that the elevated expression of F5H protein interferes with the conversion of sucrose into glucose. During tension wood formation, carbon may be utilized towards building the different components of the gelatinous layer (Schubert 1965). This allocation of carbon may explain the reduction in total sugar and glucose percentages observed in the *F5H* over-expressing poplar lines used in this study. Given that these percentages are relative abundances, a quantitative change in any structural component results in a cascade shift in the calculated percentages of the remaining components.

CONCLUSION

This study confirms the results of previous studies on tension wood and provides a new insight into the role that altering monolignol ratio in lignin has on the cell wall chemistry of hybrid poplar in normal, tension and opposite wood. Both wild-type and transgenic trees responded to gravity by producing tension wood on the upper side of the stem and corrective growth returning displaced stems to the vertical position. Confirming earlier studies are the observations that tension wood in the wild-type poplar is characterized by being higher in percent cellulose, cellulose crystallinity, and percent syringyl monomer, and being lower in microfibril angle and percent total lignin compared to normal wood. Increasing the syringyl lignin by over-expressing *C4Hprom::F5H* in hybrid poplar in the transgenic lines also increased the acid solubility of lignin. However, increasing the syringyl lignin did not alter cellulose crystallinity or microfibril angle in tension wood, opposite wood or normal wood compared to the wood in the wild-type line where lignin was unmodified. Similar to the wild-type line, normal wood of the transgenic lines also had lower percent total sugar than tension or opposite wood. In contrast to the wild-type line, transgenic lines exhibited no additional change in the monolignol percentage when comparing the three different types of wood (tension, opposite and normal). In normal wood, total lignin percentage in wild-type trees was higher than in the transgenic lines.

Results reported here focus new attention on the carbohydrate content of the different wood tissues of the tested poplar lines. Tension wood in the transgenic lines exhibited reduced relative abundance of total sugars and glucose when compared to tension wood in the wild-type trees. Percent galactose was higher in tension wood than in normal wood or opposite wood regardless of syringyl monomer percentage. Although higher in normal wood of each respective line, percent galactose decreased in tension wood samples as syringyl monomer percentage increased. Syringyl abundance did not correlate with the percentages of xylose, rhamnose, mannose or arabinose in the three wood types. The percentages of both xylose and rhamnose were lower in tension wood than in normal and opposite wood.

With a few exceptions, the data on the cell wall composition of tension wood vs. normal and opposite wood reported here support earlier studies. Some of the conflicting results reported in earlier studies and reviewed in this study could be explained by the different experimental designs used to induce tension wood formation and in the timing of tissue harvest resulting in the analysis of tissues of different developmental age. In general, increasing the syringyl monomer abundance in lignin does not significantly affect cellulose crystallinity or microfibril angle when compared to the wild-type hybrid line. However, increasing percent syringyl lignin does inhibit further enrichment of the syringyl monomer in tension wood. This could be the result of saturating the sinapyl alcohol biosynthetic pathway. What is unclear is why increasing syringyl lignin reduced the relative abundance of total sugars and glucose or why galactose percentage decreased in tension wood with increasing syringyl lignin. These trends in carbohydrate chemistry might provide some insight into the role of galactose as a possible link between different monolignols within the complex lignin molecule and the cellulose matrix of the secondary cell wall.

ACKNOWLEDGMENTS

We thank Clint Chapple from Purdue University and David Ellis from the USDA Plant Genetic Resources Preservation Program National Center for Genetic Resources Preservation Fort Collins, CO for providing the transgenic poplar lines. We also thank Peter H. Carrington for his assistance in reviewing this manuscript.

Funding

This research was supported by the National Research Initiative of the USDA CSREES grant number 2005-35103-15269

REFERENCES

REFERENCES

Aguayo MG, Quintupill L, Castillo R, Baeza J, Freer J, Mendonça RT (2010) Determination of differences in anatomical and chemical characteristics of tension and opposite wood of 8-year old *Eucalyptus globulus*. Maderas. Ciencia y tecnología **12**(3):241-251.

Andersson-Gunnerås S, Mellerowicz EJ, Love J, Segerman B, Ohmiya Y, Coutinho PM, Nilsson P, Henrissat B, Moritz T, Sundberg B (2006) Biosynthesis of cellulose-enriched tension wood in *Populus*: global analysis of transcripts and metabolites identifies biochemical and developmental regulators in secondary wall biosynthesis. Plant J **45**:144-165.

Atalla RH, Hackney JM, Uhlin I, Thompson NS (1993) Hemicelluloses as structure regulators in the aggregation of native cellulose. Int J Biol Macromol **15**:109-112.

Azuma J, Fujii M, Koshijima T (1983) Studies on hemicelluloses in tension wood II. Structural studies on xylans from tension, opposite and side woods of Japanese beech (*Fagus crenata* Blume). Wood Res **69**:12-21.

Baillères H, Chanson B, Fournier M, Tollier MT, Monties B (1995) Structure, composition chimique et retraits de maturation du bois chez les clones d'*Eucalyptus*. Ann Forest Sci **52**:157-172.

Bernal AJ, Jensen JK, Harholt J, Sørensen SO, Moller I, Blaukopf C, Johansen B, de Lotto R, Pauly M, Scheller HV, Willats WG (2007) Disruption of *ATCSLD5* results in reduced growth, reduced xylan and homogalacturonan synthase activity and altered xylan occurrence in *Arabidopsis*. Plant J **52**:791-802.

Bland DE, Scurfield G (1964) Chemistry of reaction wood: IV. Distribution and nature of the lignin in seedlings of hardwood. Holzforschung **18**:161-166.

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol **54**:519–546.

Bose SK, Francis RC, Govender M, Bush T, Spark A (2009) Lignin content versus syringyl to guaiacyl ratio amongst poplars. Bioresource Technol **100**:1628-1633.

Bowling AJ, Vaughn KC (2008) Immunocytochemical characterization of tension wood: Gelatinous fibers contain more than just cellulose. Am J Bot **95**(6):655-663.

Boyd JD (1977) Relationship between fibre morphology and shrinkage of wood. Wood Sci Technol **11**:3-22.

Chabbert B, Monties B, Rowe NP, Speck T (1997) Variability of lignin composition and lignification pattern in the lianescent and self-supporting growth phase of the liana *Condylocarpon guianense*. In: Jeronimidis G, Vincent JFV (eds) Plant biomechanics: Conference proceedings, Vol. 1. Centre for Biomimetics, University of Reading, Reading, UK, pp 73–78.

Chen C, Meyermans H, Burggraeve B, de Rycke RM, Inoue K, de Vleesschauwer V, Steenackers M, van Montagu MC, Engler GJ, Boerjan WA (2000) Cell-specific and conditional expression of caffeoyl-coenzyme A-3-O-methyltransferase in poplar. Plant Physiol **123**:853-867.

Coleman HD, Yan J, Mansfield SD (2009) Sucrose synthase affects carbon partitioning to increase cellulose production and altered cell wall ultrastructure. Proc Natl Acad Sci USA **106**(31):13118-13123.

Cosgrove DJ (2000) Expansive growth of plant cell walls. Plant Physiol Bioch **38**:109-124.

Ebringerová A, Heinze T (2000) Xylan and xylan derivatives- biopolymers with valuable properties. I. Naturally occurring xylans structures, isolation procedures and properties. Macromol Rapid Commun **21**:542–556.

Foston M, Hubbell CA, Samuel R, Jung S, Fan H, Ding S-Y, Zeng Y, Jawdy S, Davis M, Sykes R, Gjersing E, Tuskan GA, Kalluri U, Ragauskas AJ (2011) Chemical, ultrastructural and supramolecular analysis of tension wood in *Populus tremula* x *alba* as a model substrate for reduced recalcitrance. Energy Environ Sci **4**:4962-4971.

Franke R, McMichael CM, Meyer K, Shirley AM, Cusumano JC, Chapple C (2000) Modified lignin in tobacco and poplar plants over-expressing the *Arabidopsis* gene encoding ferulate 5-hydroxylase. Plant J **22**:223-234.

Fratzl P, Elbaum R, Burgert I (2008) Cellulose fibrils direct plant organ movements. Faraday Discuss **139**:275-282.

Fry SC (1989) The structure and functions of xyloglucan. J Exp Bot **40**(210):1-11.

Fujii M, Azuma J, Tanaka F, Kato A, Koshijima T (1982) Studies on hemicelluloses in tension wood: I. Chemical composition of tension, opposite and side woods of Japanese beech (*Fagus crenata* Blume). Wood Res **68**:8-21.

Hacke UG, Sperry JS, Pockman WT, Davis SD, McCulloh KA (2001) Trends in wood density and structure are linked to prevention of xylem implosion by negative pressure. Oecologia **126**:457-461.

Hedenström M, Wiklund-Lindström S, Öman T, Lu F, Gerber L, Schatz P, Sundberg B, Ralph J (2009) Identification of lignin and polysaccharide modifications in *Populus* wood by chemometric analysis of 2D NMR spectra from dissolved cell walls. Mol Plant **2**:933-942.

Hoffmann B, Chabbert B, Monties B, Speck T (2003) Mechanical, chemical and x-ray analysis of wood in the two tropical lianas *Bauhinia guianensis* and *Condylocarpon guianense*: variations during ontogeny. Planta **217**: 32–40.

Huntley S, Ellis D, Gilbert M, Chapple C, Mansfield SD (2003) Significant increases in pulping efficiency in C4H-F5H transformed poplars: Improved chemical savings and reduced environmental toxins. J Agr Food Chem **51**:6178-6183.

Kaku T, Serada S, Baba K, Tanaka F, Hayashi T (2009) Proteomic analysis of the G-layer in poplar tension wood. J Wood Sci **55**:250-257.

Keegstra K, Talmadge KW, Bauer WD, Albersheim P (1973) Structure of plant-cell walls. III. Model of wall of suspension-cultured sycamore cells based on interconnections of macromolecular components. Plant Physiol **51**:188-196.

Kim JS, Daniel G (2012) Distribution of glucomannans and xylans in poplar xylem and their changes under tension stress. Planta **236**:35-50.

Koehler L, Telewski FW (2006) Biomechanics and transgenic wood. Am J Bot **93**(10):1433-1438.

Kuo CM, Timell TE (1969) Isolation and characterization of a galactan from tension wood of American beech (*Fagus grandifolia* Ehrl.). Svensk Papperstid **72**:703-716.

McCartney L, Blake AW, Flint J, Bolam DN, Boraston AB, Gilbert HJ, Knox JP (2006) Differential recognition of plant cell walls by microbial xylan-specific carbohydrate-binding modules. P Natl Acad Sci USA **103**:4765–4770.

Mellerowicz EJ, Gorshkova TA (2012) Tensional stress generation in gelatinous fibers: A review and possible mechanism based on cell-wall structure and composition. J Exp Bot **63**(2):551-565.

Mellerowicz EJ, Immerzeel P, Hayashi T (2008) Xyloglucan: The molecular muscle of trees. Ann Bot **102**:659-665.

Mosha Y, Goring DAI (1975) Distribution of syringyl and guaiacyl moieties in hardwood as indicated by ultraviolet microscopy. Wood Sci Technol **9**:45-58.

Nishikubo N, Awano T, Banasiak A, Bourquin V, Ibatullin F, Funada R, Brumer H, Teeri TT, Hayashi T, Sundberg B, Mellerowics EJ (2007) Xyloglucan endotransglycosylase (XET) functions in gelatinous layers of tension wood fibers in poplar – A glimpse into the mechanism of the balancing act of trees. Plant Cell Physiol **48**:843-855.

Pauly M, Qin Q, Greene H, Albersheim P, Darvill A, York WS (2001) Changes in the structure of xyloglucan during cell elongation. Planta **212**:842-850.

Persson S, Caffall KH, Freshour G, Hilley MT, Bauer S, Poindexter P, Hahn MG, Mohnen D, Somerville C (2007) The *Arabidopsis irregular xylem8* mutant is deficient in glucuronoxylan and homogalacturonan, which are essential for secondary cell wall integrity. Plant Cell **19**:237-255.

Pilate G, Chabbert B, Cathala B, Yoshinaga A, Leplé J-C, Laurans F, Lapierre C, Ruel K (2004) Lignification and tension wood. C R Biol **327**:889-901.

R Development Core Team (2007) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, URL http://www.R-project.org.

Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick Jr WJ, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T (2006) The path forward for biofuel and biomaterials. Science **311**:484–489.

Robinson AR, Mansfield SD (2009) Rapid analysis of poplar lignin monomer composition by a streamlined thioacidolysis procedure and near-infrared reflectance-based prediction modeling. Plant J **58**:706-714.

Ruel K, Barnoud F (1978) Recherches sur la quantification du bois de tension chez le hêtre: Signification statistique de la teneur en galactose. Holzforschung **32**:149-156.

Sarkanen KV, Hergert HL (1971) Classification and distribution. In: Sarkanen KV, Ludwig CH (eds) Lignin: Occurrence, formation, structure and reactions. Wiley-Interscience, New York, pp 43-95.

Scheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol **61**:263–289.

Schubert WJ (ed) (1965) Lignin biochemistry. Academic Press Inc., New York, pp 53-75.

Stewart JJ, Akiyama T, Chapple C, Ralph J, Mansfield SD (2009) The effects on lignin structure of overexpression of ferulate 5-hydroxylase in hybrid poplar. Plant Physiol **150**:621-635.

Stewart JJ, Kadla JF, Mansfield SD (2006) The influence of lignin chemistry and ultrastructure on the pulping efficiency of clonal aspen (*Populus tremuloides* Michx.). Holzforschung **60**:111-122.

Sugino H, Shigematsu M, Tanahashi M, Shinoda Y (1994) Affinities between lignin precursors and hemicelluloses. Proceedings of the 39th Lignin Symposium, Fukuoka, Japan, pp 145-146.

TAPPI Useful Method UM250 (1991) Acid-soluble lignin in wood and pulp. In: Useful Methods. TAPPI Press, Atlanta, USA.

Timell TE (1969) The chemical composition of tension wood. Svensk Papperstidning **72**:173-181.

Ukrainetz NK, Ritland K, Mansfield SD (2007) Identification of quantitative trait loci for wood quality and growth across eight full-sib coastal Douglas-fir families. Tree Genet Genomes **4**:159-170.

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W (2010) Lignin biosynthesis and structure. Plant Physiol **153**:895–905.

Vanholme R, Morreel K, Ralph J, Boerjan W (2008) Lignin Engineering. Curr Opin Plant Biol **11**:278-285.

Vonk CC (1973) Investigation of nonideal two-phase polymer structures by small *x*-ray scattering. J Appl Crystallogr **6**:81-86.

Yoshida M, Ohta H, Yamamoto H, Okuyama T (2002) Tensile growth stress and lignin distribution in the cell walls of yellow poplar, *Liriodendron tulipifera* Linn. Trees-Struct Funct **16**:457-464.

Yoshinaga A, Fujita M, Saiki H (1992) Relationships between cell evolution and lignin structural varieties in oak xylem evaluated by microscopic spectrophotometry with separated cell-wall. Mokuzai Gakkaishi **38**:629-637.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The growing awareness of the limited availability of energy sources like oil and coal has brought simultaneously a significant energy price hikes and increased interest in finding alternative sources. In the industry, the production costs also increased but so did the need to reduce the chemical wastes that are partially blamed for changing the global climate. The paper and pulp industries started looking for ways to increase the efficiency of the operations and to reduce the chemicals used along the process. Lignin extraction is costly in terms of energy and use of hazardous chemicals. A research team had modified the lignin composition in poplar trees allowing more than 60% increase in lignin extraction efficiency while using less disposable chemicals. The trees were genetically modified to over-express *ferulate*-5-*hydrovylase* (*F5H*) gene which encodes an enzyme that diverts the conversion of lignin intermediates into producing more syringyl than guaiacyl. In angiosperms, lignin in the secondary cell walls is majorly made of these two monomers. The ratio of which was found to change across different plant species and in response to environmental stimuli such as gravity. The influence of changing the syringyl to guaiacyl ratio and gravity on poplar trees growth responses and physical properties was investigated in this study.

Increasing syringyl to guaiacyl ratio in hybrid poplar trees (*Populus tremula* x *P. alba*) was found to result in a lower plant height to stem diameter ratio. The trees with the higher syringyl content gained less height than the control trees throughout the experiment. However, when the syringyl-enriched trees were tilted they spent eight days less than the wild type controls to adjust to gravity and recover back to vertical. Whether that was due to higher tensile force in the tension wood this research was not able to confirm. Growth strain measurements showed less strain released from the tension wood of the faster responding trees. That could be due to the measurements being done at the end of the gravitropic phase when the tensile force is at its minimum. Four-point bending and compression tests showed no differences in the elastic properties due to the lignin modification when the stems were tested while fresh or after drying. Accordingly, two assumptions were made; one assumption was that the elastic properties of the stems are more influenced by the cellulose microfibrils than by the lignin structure. The other assumption was that the trees demonstrated another example of their ability to adapt or compensate to changes in the lignin structure. When the dry stems were loaded to rupture a decrease in the modulus of rupture was found. This increase in brittleness raised further questions about the possible changes in the tissue anatomy and the chemical and structural properties of the cell walls.

Dry Wood density estimation using the water displacement method showed a decrease in the density in the poplar line with the highest syringyl content when compared to the wild type. Wood density is considered a good predictor of the woods mechanical properties. The brittle nature of the trees with increased syringyl content may be explained by the decrease in the density. Transverse sections were created and stained before they were visualized under a light microscope. Percent area tension wood was similar among all tested poplar lines. Digital images were captured for further measurements to be done on the cellular level. Major findings were a decrease in percent fiber wall in the upright trees with higher syringyl. Moreover, it was found that those trees have higher percent vessel diameter and percent vessel lumen, which raises a question whether these anatomical changes, as a result of the syringyl enrichment, will cause changes in the water conductive properties in poplar. Among the expected results were lower total lumen in the tension wood and an increase in percent fiber wall, which can be explained by the fact that fibers in the tension wood have an additional cell wall layer of gelatinous nature hence the name G-fibers.

To investigate the influence of syringyl over-expression and gravity on the water conductive properties of the poplar trees maximum hydraulic conductivity (k_{max}) was measured in 14-cm long stem segments allowing for calculation of water specific conductivity (k_s). It was found that the upright trees with the higher syringyl conducted water more efficiently that the wild type. This answered the question whether the larger vessel lumen, in poplar lines with higher syringyl, increases water conductivity. To determine if the vessel walls had been affected by the lignin modification vulnerability curves were constructed but failed to lead to a conclusion due to the high variance of the data.

Further analyses of the wood were carried out through analyzing the lignin and carbohydrate composition of the cell wall. Increasing syringyl content was found to lead to a slight drop in total lignin. It cannot be rules out that this slight change in total lignin might have some effect on the results of the mechanical analyses. Increasing syringyl content did increase the percent acid soluble lignin but had no impact on cell wall crystallinity or microfibril angle. An interesting biochemical result was a slight decrease in percent sugars with increasing syringyl in the tension wood. Similar result was observed for percent glucose and galactose. Percent xylose came out lower in the tension wood than in the normal wood which was contrary to what was reported by a different group. No effect of syringyl enrichment was found on percent xylose in any of the tissue types.

An attempt had been made during the study to create new genetic lines with RNAi-suppressed *F5H* gene. If successful a broader spectrum of syringyl to guaiacyl would allow for higher resolution data. In addition, there have been very few studies on the influence of guaiacyl enrichment on the plants characteristics and behavior. Even though the transformation attempt had been successful, no significant change occurred on the syringyl to guaiacyl ratio in the modified poplar stems. A change in the design of the transformation construct might have been necessary. Another possible explanation was that the introduced siRNA was successful at hybridizing with the RNA of only one out of two copies of the *F5H* gene in poplar.

Overall, no major altercations due to syringyl enrichment were detected in the tested poplar trees under the current greenhouse setting. Before a large scale plantation of the genetically altered poplar trees can be assumed, further testing in the field will be required to verify their ability to withstand more complex stresses.