Genetic Modification of Lignin in Hybrid Poplar (Populus alba × Populus tremula) Does Not Substantially Alter Plant Defense or Arthropod Communities

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Subject Editor: Evan Preisser

Received 27 March 2017; Editorial decision 14 May 2017

Abstract

Lignin impedes access to cellulose during biofuel production and pulping but trees can be genetically modified to improve processing efficiency. Modification of lignin may have nontarget effects on mechanical and chemical resistance and subsequent arthropod community responses with respect to pest susceptibility and arthropod biodiversity. We quantified foliar mechanical and chemical resistance traits in lignin-modified and wild-type (WT) poplar (Populus alba × Populus tremula) grown in a plantation and censused arthropods present on these trees to determine total abundance, as well as species richness, diversity and community composition. Our results indicate that mechanical resistance was not affected by lignin modification and only one genetic construct resulted in a (modest) change in chemical resistance. Arthropod abundance and community composition were consistent across modified and WT trees, but transgenics produced using one construct exhibited higher species richness and diversity relative to the WT. Our findings indicate that modification of lignin in poplar does not negatively affect herbivore resistance traits or arthropod community response, and may even result in a source of increased genetic diversity in trees and arthropod communities.

Key words: biodiversity, genetic modification, arthropod

Lignin is a structural component of cell walls that forms a physical barrier against arthropods and pathogens and helps prevent vascular collapse when water columns are under tension (Baucher et al. 1998, Fink 1999, Boerjan et al. 2003). The structural durability of lignin also impedes access to cellulose for biofuel and pulp processing, as well as forage digestibility (Akin et al. 1991, Moore and Jung 2001, U.S. DOE 2006, Li et al. 2008). Lignin content and composition can be modified to minimize the need for chemical pretreatment, thereby improving biofuel and pulp processing efficiency and reducing costs (Chapple et al. 2007, Chandel and Singh 2011, Pu et al. 2011). Reduction of lignin content affects the amount that gets deposited in the secondary cell wall, whereas altering the monomeric composition of lignin affects the number and strength of its linkages to the polysaccharides in the cell wall. The subunits that comprise lignin in poplar are syringyl (S), guaiacyl (G), and trace amounts of p-hydroxyphenyl (H). High-S lignin is more susceptible to chemical degradation than lignin derived from wild-type (WT) plants (Campbell and Sederoff 1996, Boerjan et al. 2003, Sticklen 2008, Chandel and Singh 2011).

Modification of lignin content and composition has successfully improved processing efficiency for poplar, a woody feedstock that is widely used in the papermaking industry and has great potential as a source of biofuels (Huntley et al. 2003, Chen and Dixon 2007, Hisano et al. 2009, Sannigrahi et al. 2011). These modifications, however, have the potential for unintended consequences. Potentially negative side-effects of lignin modification include formation of novel lignin subunits and discolored tissues and collapsed xylem cells, which can affect vascular conductivity and, hence, growth (Sederoff et al. 1999, Kitin et al. 2010, Voelker et al. 2011). Arthropods are sensitive bioindicators of changes in their environment and modification of lignin has the potential to alter herbivore resistance traits and subsequent arthropod response (e.g., host selection and damage). Reduction of lignin content or an alteration in S:G ratio can contribute to reduced tissue strength and result in increased arthropod herbivory (Lowman and Box 1983, Ohmart and Edwards 1991, Steinbauer et al. 1998). Lignin modification can alter distribution of carbon to the primary chemical defenses in poplar (condensed tannins [CTs] and phenolic glycosides [PGs]), and changes to these compounds influence susceptibility to arthropod herbivory (Lindroth et al. 2001, Philippe and Bohlmann 2007).
Generalist herbivores of poplar, such as some defoliating caterpillars, also feed on a variety of other hosts and are not deterred by high levels of CTs, although their performance is reduced by PGs (Hemming and Lindroth 1995, Boeckler et al. 2011). In contrast, specialists of poplar, such as some leaf beetles, feed within a small range of chemically similar hosts and can tolerate or detoxify plant defenses (Bowers and Puttick 1988). Leaf beetles are actually attracted to and sequester PGs but do not perform well on plants high in CTs (Bingaman and Hart 1993, Donaldson and Lindroth 2004). These changes in chemical defenses can result in varying insect susceptibility and comprehensive effects on arthropod communities (Birch et al. 1999, Jørgensen and Lövei 1999, Schuler et al. 1999, Isaacs et al. 2009). Consequences of increased pest susceptibility include reduced crop yields and increased insecticide use (Painter 1941, Oerke 2006), and alteration of arthropod community dynamics may have deleterious effects on ecosystem services (Gardiner et al. 2010, Lands and Werling 2010, Duke et al. 2013).

Few studies have assessed the effects of modifying nontarget traits on herbivore resistance (Hjältén et al. 2007, Post and Parry 2011, Qin et al. 2013, Hjältén and Åxellson 2015). Many different lignin-modified poplar genetic constructs have been tested (Boerjan et al. 2003, Huntley et al. 2003, Ye et al. 2012), but the effect of transgene expression on defense traits has been evaluated in only a few. For example, altered levels of PGs have been shown to occur in some lignin-modified poplars (Coleman et al. 2008, Joshi et al. 2011). In this study, we evaluated mechanical and chemical resistance traits of lignin-modified and WT poplar and surveyed whole-community responses of arthropods present on these trees. We predicted differences in arthropod abundance, species richness, species diversity and community composition on our lignin-modified poplar, due to nontarget effects on mechanical and chemical defenses.

Materials and Methods

Plant Materials

Our experimental model was hybrid poplar clone INRA 717-1B4 (Populus alba × Populus tremula), which was modified with one construct (i.e., artificially constructed segment of DNA) that resulted in reduced lignin (i.e., reduced S and G content) and three constructs that resulted in altered S-G ratios; control trees were nontransgenic WT trees (Table 1). Lignin content and composition were genetically altered by down-regulating, via RNA-interference, or overexpressing genes encoding the enzymes that catalyze the synthesis of monolignols. The coding sequences were driven by the promoter from genes encoding the enzymes that catalyze the synthesis of monolignols. The coding sequences were driven by the promoter from the genes encoding the enzymes that catalyze the synthesis of monolignols.

Table 1. Summary of Populus alba × P. tremula lignin-modified and wild-type (WT) trees produced at Purdue University

| Constructs | No. lines | Transgene | Predicted phenotype | Trees produced |
|------------|-----------|-----------|---------------------|---------------|
| WT         | 2         | None      | Normal              | 10            |
| 1049       | 6         | C3'H      | Reduced lignin      | 10            |
| 1034       | 5         | FSH       | Low S               | 10            |
| 1036       | 5         | COMT      | Low S               | 10            |
| 1063       | 3         | FSH       | High S              | 7             |

The “constructs” column indicates designations of genetically engineered constructs (or the wild type). The “lines” column indicates the number of distinct insertions of each construct (or ancestral clones in the case of the wild type). Individual results of each line have been pooled by construct (or wild type). The “predicted phenotype” column indicates syringyl (S) and guaiacyl (G) lignin subunit content or ratio that was expected a priori from each modification.

Mechanical Resistance

In June 2012, we collected five fully expanded, undamaged leaves from each tree for analysis of mechanical resistance. Leaves were collected from the uppermost part of the plant, between leaf plastosclereid index (LPI; Larson and Isebrands 1971) 4 and 8 (for developmental synchrony). We measured leaf toughness (i.e., mechanical resistance) by puncturing each leaf at 10 veinless locations along each side of the midrib, using a leaf dynamometer (McCormick FDP 200; McCormick, Facchini, Alfonzine; Italy). Measurements from all punctures of all leaves collected from an individual tree were averaged to calculate a composite value of mechanical resistance for each tree. Constraints of the leaf dynamometer method of determining leaf toughness include: 1) inability to measure leaf-puncture displacement; and 2) leaf toughness confounded by other variables, such as flexibility (Sanson et al. 2001). For the purposes of our study, a leaf dynamometer is an appropriate surrogate for arthropod-related perforation (Feeny 1970).

Chemical Resistance

Leaves were collected from each tree for chemical analyzes at 75 and 100 days after coppicing in 2010 and 2011. Although trees were not coppiced in 2012, leaves were collected 75 days after the projected annual coppicing date. We chemically analyzed five fully expanded, undamaged leaves within a LPI of 4–8 on the central leader. Leaves were clipped at the petiole, freeze-dried and ground in a Wiley Mill (mesh size #20). A portion of ground leaves was weighed for lignin analyzes, the remaining tissue was ground more finely using a ball mill and aliquots were weighed for all other analyzes. We quantified CTs spectrophotometrically via a modified acid-butanol method (Porter et al. 1986). Standards used in CT analyzes were purified from WT P. alba × P. tremula leaves (Hagerman and Butler 1980). Qualitative and quantitative variation in PGs was assessed via an ultra high-performance liquid chromatography method with standards purified from Populus and Salix spp. WT leaves (method modified from Abreu et al. 2011). Here we report total PGs present at a concentration >0.5% dry mass. We quantified lignin levels gravimetrically via sequential extraction in a hot acid-detergent solution in an Ankrom 200 digestor and...
incubation in 72% sulfuric acid bath (Rowland and Roberts 1994). In addition to chemical resistance compounds, we also measured nitrogen, as it is a factor in host–plant preference and arthropod performance. Nitrogen levels were quantified via combustion analysis using a Flash EA1112 C/N analyzer. All chemical data are reported as percent dry mass.

**Arthropod Abundance, Species Richness, Diversity and Community Composition**

Our study site was an open plantation that allowed visitation and colonization by wild arthropod populations. Arthropod surveys were conducted twice per year (2010–2011), each before leaves were collected for chemical analysis, for a total of four surveillance periods. During each surveillance period (hereafter referred to as “date”), trees were repeatedly measured in random order for each of four consecutive days. Arthropod data from the four days were pooled by tree and date. A timed visual survey, similar to Hillstrom et al. 2014, was used for arthropod data collection. For each survey, each tree was circled and trunks, branches and foliage were visually scanned from top to bottom for 2 min. Approximately 75% of the entire canopy and trunk of each tree was inspected during each survey. We censused arthropods by counting known species using a hand-held tally (multiple tally denominator, The Denominator Company, Inc., Woodbury, CT). Unknown arthropods were counted and collected for further identification. Arthropods were collected using an aspirator and stored in 70% ethyl alcohol. The timer was stopped to record data or to collect arthropods for further identification. At the end of each survey, sedentary arthropods were manually removed from each tree to prevent repeated counts in a subsequent survey. Arthropods were broadly categorized into functional groups comprised of generalist and specialist pests of poplar and natural enemies of those pests. Arthropods were also more narrowly identified to order, family, and species or morphospecies, if species-level identification was not possible.

**Statistical Analysis**

Preliminary statistical analyzes indicated no significant differences among lines produced from a construct (i.e., all lines within a construct behaved similarly), lines were therefore pooled within each construct for all final analyzes. We tested the effect of modification on mechanical resistance using a fixed-effects model analysis of variance (ANOVA). We tested the individual and interactive effects of modification and date on chemical resistance traits using a fixed-effects model ANOVA with repeated measures. Chemical concentration data were arcsine square root-transformed (arcsin[√(% dry mass/100)]) to adjust for nonnormality before running ANOVA. We visualized similarity of chemical profiles among the various transgenic and WT trees using nonmetric, multidimensional scaling (NMDS). We statistically analyzed these similarities by calculating a Euclidean distance matrix on square root-transformed data and running ANOSIM. We identified the most abundant families within each functional group.

**Results**

**Mechanical Resistance**

Leaf toughness did not vary significantly among modified and WT trees ($F_{4,10} = 2.1, P = 0.162$). Levels of leaf toughness in the transgenic trees were within ±10% of levels in the wild type.

**Chemical Resistance**

Levels of chemical resistance traits were generally similar among modified and WT trees, with only CT levels exhibiting marginally significant differences (Table 2). Trends indicated 35% higher CT levels in “low S” trees (construct 1036) than in the wild type. Date was a significant factor influencing levels of all chemical resistance traits (Table 2) as was to be expected with seasonal changes in phenology. We tested the effect of modification on chemical levels in young *Populus* (Lindroth and Hwang 1996, Rehill et al. 2006). Post hoc tests indicated that chemistry in June 2012 was significantly different relative to chemistry at all other dates. The effect of modification on CT levels was influenced by date (significant C × D interaction; Table 2). Post hoc tests indicated that significant differences in CT levels between trees transformed with construct 1036 and the wild type occurred only in June 2012 (Fig. 1). Lastly, levels of lignin as well as nitrogen (marginally significant) also varied across transgenic and WT trees at different dates (Table 2). Post hoc tests indicated that lignin was 53% higher in 1,036 trees relative to the wild type in July 2011 (significant C × D interaction; Fig. 1). NMDS plots (not shown) indicated that chemical profiles were fairly similar among the various transgenic and WT trees. ANOSIM also indicated that chemistry was similar among the transgenic and WT trees, although chemistry differed slightly between one of the “low-S” (construct 1036) and the “high-S” trees (construct 1063) relative to the wild type ($R = 0.12,$
P = 0.031 and R = 0.25, P = 0.026, respectively). Differences identified by ANOSIM were not large enough to be clearly represented by NMDS.

Arthropod Abundance, Species Richness, Diversity and Community Composition

The total count of arthropods surveyed on all trees from all dates combined was 7,127 individuals. Arthropods belonging to the Formicidae family were the most abundant group present on all of the trees (Supp Table 1 [online only]).

Total arthropod abundance per unit time did not vary significantly among modified and WT trees, although trends indicated 25% higher abundance on the “reduced lignin” trees (construct 1049), relative to the wild type (Table 3, Fig. 2). Abundance of individual functional groups and orders also did not vary significantly among the various transgenic and WT trees. As with chemistry, date was also a significant factor in total arthropod abundance and abundance of individual functional groups and orders (Table 3). Post hoc tests indicated that total arthropod abundance was higher in June 2011 than all other dates (Buhl 2013). Post hoc tests also indicated differences in abundance of individual functional groups and orders among dates. Total arthropod abundance and abundance of individual functional groups and orders did not vary significantly among transgenic or WT trees at any date (no significant C × D interaction; Table 3).

Species richness varied significantly among modified and WT trees (F₄,₄₂ = 11.42, P < 0.001). Diversity, as calculated by Simpson’s index of diversity, also varied significantly due to modification (F₄,₄₂ = 2.75, P = 0.040). Both species richness and diversity were higher on trees engineered with construct 1049, relative to the wild type (Table 4). Results from NMDS (Fig. 3) as well as ANOSIM (Buhl 2013) indicated similar species richness across all

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### Table 2. Summary of analyses of variance (ANOVA) with repeated measures examining the individual and interactive (C × D) effects of genetic construct and sample date on foliar chemical resistance traits and nitrogen

| Trait          | dfₙ,d | F     | P    | dfₙ,d | F     | P    |
|----------------|-------|-------|------|-------|-------|------|
| Condensed tannin| 4.12  | 2.9   | 0.072| 4.5   | 3.3   | 0.115|
| Date           | 4,181 | 412.6 | <0.001| 4,190 | 60.1  | <0.001|
| C × D          | 16,181| 3.0   | <0.001| 16,190| 1.5   | 0.109|

Significant values are in bold.

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![Fig. 1. Levels of foliar chemical resistance traits and nitrogen for each genetic construct or wild-type at each sampling date. All trees were coppiced and allowed to reflush before surveys in 2010 and 2011, but not in 2012. Coppicing “resets” tree age and chemistry responds accordingly (Stevens et al. 2012) effectively making our trees phenologically ~1-year-old in 2010 and 2011 but 2-years-old in 2012. Lines represent mean chemistry levels of each transgenic or wild-type (WT) poplar tree (n = 7–10 replicate trees) at each date. Dashed lines represent modified constructs and solid lines represent wild types. Asterisks indicate dates at which levels of resistance traits varied among constructs (significant construct × date interactions).](https://example.com/fig1.png)


**Table 3.** Summary of ANOVAs with repeated measures examining the individual and interactive (C × D) effects of genetic construct and sample date on total arthropod abundance and abundance of individual arthropod orders and functional groups (each per unit time).

| Construct | Total arthropods | Generalists | Specialists | Natural enemies |
|-----------|------------------|-------------|-------------|-----------------|
|           | df_{n,d} F P     | df_{n,d} F P | df_{n,d} F P | df_{n,d} F P    |
| Date      | 4.6 0.4 0.780    | 4.13 0.9 0.479 | 4.10 0.2 0.932 | 4.12 0.5 0.724  |
| C × D     | 3,143 14.2 <0.001 | 3,157 4.7 0.004 | 3,150 93.3 <0.001 | 3,153 10.8 <0.001 |

Significant values are in bold.

Community composition of functional groups and families was similar among modified and WT trees (Figs. 4 and 5). Trends indicated 34% more generalists and 55% fewer natural enemies on a “low-S” trees (construct 1034), relative to the wild type, although abundance of each group did not differ significantly between 1,034 trees and the wild type (Table 3, post hoc results not shown). The majority of generalists belonged to the families Cicadellidae, Psychidae, Tortricidae, and Buprestidae. Specialists made up the largest proportion among functional groups and consisted solely of cottonwood leaf beetles (Chrysomelidae). The majority of natural enemy species was parasitic Hymenoptera followed by species belonging to the families Salticidae, Cantharidae, and Coccinellidae.

**Discussion**

Genetic modification of lignin content and composition had no detectable effect on mechanical resistance and only a moderate effect on chemical resistance traits. Modification of lignin did not significantly influence arthropod abundance or community composition, but did affect species richness and diversity. Richness and diversity were highest on the “reduced-lignin” trees (construct 1049) relative to all other trees. In short, moderate effects of lignin modification on defense translated to mostly insubstantial effects on arthropod communities.

In contrast to our predictions, we found trends toward higher mechanical resistance in our “reduced-lignin” trees (construct 1049) relative to all other modified and WT trees. This finding reinforces the notion that reducing S and G content may not result in total lignin reduction due to a compensatory increase in H lignin. Because H lignin is naturally present in such trace amounts in Populus, we did not expect a large effect on H content in response to reduction of S and G content. Modification of lignin content or composition also did not substantially alter levels of chemical resistance. The only transgenic trees exhibiting altered chemical resistance relative to the wild type were the “low-S” trees (construct 1034). We conclude that these modifications did not have substantial effects on mechanical or chemical defenses in poplar but we caution that this may not be the case for other lignin modifications and each must be evaluated separately.

Modification had no effect on mechanical resistance and only a moderate effect on chemical resistance, and as a consequence, whole arthropod community response did not substantially vary among
our modified and WT trees. Species richness and diversity were the only arthropod response factors that varied among our trees. We documented higher species richness and diversity on our “reduced-lignin” trees (construct 1049) relative to all other modified and WT trees. As mentioned previously, 1,049 trees were modified for reduced lignin but levels of foliar lignin did not differ between 1,049 and WT trees, possibly due to increases in H lignin. Neither reduced mechanical nor chemical resistance could be implicated as mechanisms underlying higher arthropod species richness and diversity on trees produced using this construct over all other engineered trees. Higher species richness and diversity may have resulted from factors influencing herbivore attraction rather than resistance. Determinants of host–plant preference include moisture content, olfactory stimuli (i.e., volatiles), and visual stimuli (e.g., apparency, coloration, architecture; Williams 1954, Patt and Sétamou 2007). We did not, however, observe noticeable physical differences in appearance among our experimental trees.

Arthropod community composition was statistically similar among modified and WT trees, although some noticeable trends emerged in “low-S” trees (construct 1034). Composition of functional groups on trees engineered with this construct shifted toward more generalist pests and fewer natural enemies, relative to composition on all other transgenic and WT trees. Abundance, but not richness and diversity, of generalists and natural enemies differed between trees produced using construct 1034 and all other transgenic and WT trees. Although not significant, low abundance of natural enemies on 1,034 trees may have resulted in more generalist pests. As with trees produced using construct 1034, those produced with construct 1036 were also modified for reduced S lignin but the latter did not demonstrate a similar trend in functional group composition. This observation speaks to the variable outcomes possible for trees with similar modifications. Genotype is known to determine chemical profiles in plants, which, in turn, influence arthropod community composition (Fritz and Simms 1992, Wimp et al. 2007, Robinson et al. 2012). Variation in chemistry across genotypes (in this case, genetically modified trees), however, is not always sufficient to influence arthropods (Brown 1956, Prittinen et al. 2003, Donaldson and...
Lindroth 2004). Levels of some chemical resistance traits varied among our modified and WT trees by as much as 53%, but these differences were insufficient to translate to effects on arthropods.

Genetic modification has proved to be beneficial for the enhancement of target traits in biofuel and forestry crops (James 2008, Mannon and Morse 2012). The ultimate goal of lignin modification is to improve the efficiency of lignin extraction, but such work should also attempt to avoid disruption of plant–arthropod dynamics. Genetic enhancement of crops is a valuable tool but may come at a cost if pest susceptibility or arthropod ecosystem services are negatively affected. The arthropod communities identified in this study represent what might be expected in one cycle of a short-rotation coppice plantation of lignin-modified poplar (Figs. 4 and 5). Results from this research suggest that genetic modification of lignin in *Populus* does not substantially alter nontarget traits in leaves that confer resistance against arthropod pests or negatively influence arthropod communities.

Supplementary Data
Supplementary data are available at *Journal of Insect Science* online.

Acknowledgments
This work was supported by University of Wisconsin Hatch grant WIS01336 to R.L.L. We thank Burk Thompson and all other staff at the Purdue Department of Forestry and Natural Resources’ Lugar Forestry Farm for use of equipment and facilities. We also thank members of the Lindroth Lab for assistance with experimental design (M. Hillstrom); chemical analyzes (K. Rubert-Nason and M. Crossley); and statistical analysis (J. Couture, L. Holeksi, and M. Jamieson).

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