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## Effects of Increased Nitrogen Supply on the Lignification of Poplar Wood

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The short-term influence of adequate and high nitrogen fertilization on poplar lignification was investigated. The high nitrogen supply decreased lignin staining in the newly formed secondary xylem, indicating that lignin deposition was affected. Acetyl bromide determinations gave a 9–10% decrease in lignin content; however, Klason lignin content was unchanged. Thioacidolysis showed that elevated N supply affected lignin structure such that there was a reduced frequency of lignin units involved in  $\beta$ -O-4 bonds, a reduced syringyl/guaiacyl ratio, an increased frequency of *p*-hydroxyphenyl lignin units, more guaiacyl units with free phenolic groups, and more *p*-hydroxybenzoic acid ester-linked to poplar lignins. These features suggest that lignins from poplars grown under high N bear structural similarities to lignins formed during early stages of wood development. The findings also indicate that a gravitational stimulus inducing the formation of tension wood and high N availability lead to similar and additive effects on lignin content and structure.

**KEYWORDS:** Lignin; wood; hybrid poplar; *Populus trichocarpa*  $\times$  *P. deltoides*; nitrogen fertilization

### INTRODUCTION

As fast-growing species such as *Populus* species and their hybrids are being increasingly utilized in forestry and biomass production, we need to develop a better understanding of the relationships between growth conditions, production physiology, and wood properties to anticipate their impacts on end-use characteristics of wood and fiber products. The major constituents of wood are the carbon-rich polymers cellulose, hemicelluloses, and lignins. As such, wood is the final destination of a major proportion of the carbon that is fixed by trees (1).

Lignins are ubiquitous in land plants and represent the second most abundant polymers on earth after cellulose. They are essential to normal plant function because they have both structural and defense functions (2). Lignins are complex heteropolymers derived mainly from *p*-coumaryl, coniferyl, and sinapyl alcohols. When incorporated into lignins, these monolignol precursors produce *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively (3). As in most angiosperms, poplar lignins mainly consist of G and S units, together with a trace amount of H units. Lignin synthesis is initiated via the phenylpropanoid pathway, beginning with the deamination of phenylalanine by the enzyme phenylalanine ammonia-lyase (PAL). The formation of coniferyl and sinapyl alcohols involves

the hydroxylation of position 3 or of positions 3 and 5 on the aromatic ring (by C3H and F5H, respectively) followed by their methylation by *O*-methyl transferases (OMTs) (for a review see ref 4). The last steps of monolignol biosynthesis involve the formation of aldehydes by cinnamoyl-CoA reductase (CCR) followed by their reduction to alcohols by cinnamyl alcohol dehydrogenase (CAD). The oxidatively driven polymerization of the monolignols leads to a complex polymeric structure in which G and S units are interconnected by labile  $\beta$ -O-4 ether bonds and/or by resistant C–C bonds (5, 6).

It is well-known that the growth environment influences the structure and composition of xylem secondary cell walls and of wood as a whole. For example, trees adapt to external factors that cause them to lean or bend by producing reaction wood with altered fiber morphology (7), known as tension wood in poplar (8). The xylem fibers of poplar tension wood develop significantly thicker cell walls, which contain more cellulose and less lignin (8, 9). These altered properties influence the suitability of wood for pulp production.

Whereas many studies have investigated the effect of N fertilization on forest tree productivity, comparatively few studies have been carried out to assess the impact of N fertilization on wood properties (10, 11). The impact of increased nitrogen supply on lignification has been mainly documented in forage grasses in relation to their nutritional value. In most cases, the grass lignin content is increased by nitrogen fertilization (12, 13), which is attributed to the stimulation of phenylalanine biosynthesis. However, high N supply may also promote growth of new shoots that are low in lignin, thus

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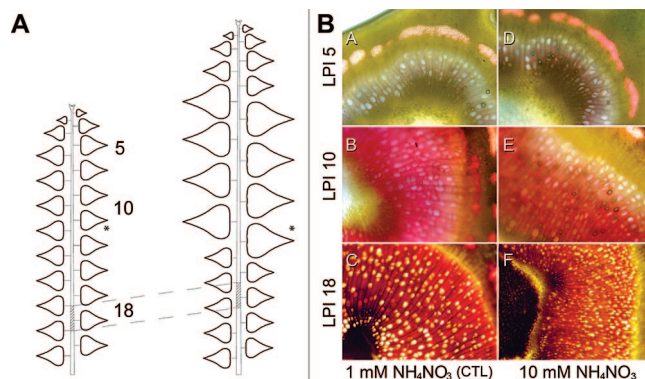
compensating for higher lignin levels in other tissues (14). The contrasting components of the response could be why some studies have reported that N fertilization has no effect on the forage lignin content (15, 16). The stage of plant development at the time of application may also modulate the plant's response to N supply. For instance, late-season N application to tall fescue maintained the forage in a physiologically younger stage with low lignin accumulation (17). In poplar, it was shown that N fertilization affected the structure of wood, for example, by decreasing its density and cell wall thickness and, in some genotypes, increasing the proportion of tension wood (10). These effects are negative for many end-use applications such as papermaking and lumber production. The impact of N supply on lignification has been less well studied. The findings to date have been inconsistent and appear to be influenced by species, developmental stage, and mode of N application. For example, in longleaf pine seedlings, N fertilization reduced the lignin content in roots only and had no effect in the aerial tissues (18). In red pine, a combined nitrogen–phosphorus–potassium (NPK) fertilization reduced the lignin content of branches (19) and gave an opposite effect in the wood of the main stem in 41-year-old Norway spruces (20).

We previously reported on the short-term responses of young poplar trees to fertilization with a high N supply. We observed that high levels of N led to the formation of wood with shorter and wider fibers, as well as altered lignin and cellulose staining, compared to trees grown with either adequate or limiting N supply (21). A very similar experimental approach has been used to characterize the effects on tree development, physiology, and gene expression (22, 23). The present work was undertaken to compare the short-term (28 days) effects of adequate and high N fertilization on the lignification of secondary xylem in poplar.

## MATERIALS AND METHODS

**Plant Material.** Rooted cuttings of *Populus trichocarpa* × *Populus deltoides* clone H11-11 were generated during the summer of 2005 and maintained in standard greenhouse conditions as previously described (21). Before the onset of the experiments, the trees were fertilized weekly with a soluble 20–20–20 fertilizer corresponding to 0.2 g/L N per week. Morphological development was monitored as described (24) so that trees of similar developmental stage and size were used for each experiment.

**Nitrogen Treatments and Induction of Tension Wood.** Two different experiments were carried out. The first experiment aimed to examine the effects of elevated nitrogen fertilization upon lignification, whereas the second experiment compared the lignification in response to elevated nitrogen fertilization and during tension wood formation. In both of the experiments, the N treatments were chosen to represent adequate N fertilization (i.e., the control) and high N fertilization. The trees were grown in pots containing an artificial substrate that did not contain available nitrogen, which was provided in the form of ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ). The N control treatment (CTL) was achieved by adjusting the final concentration to 1 mM, and the elevated N treatment was adjusted 10 mM. These concentrations were determined on the basis of a previous study of whole plant physiological responses (23), which also included 0 mM N treatments. When no nitrogen was provided, there was a relatively rapid depletion of nitrogen with characteristic physiological responses and rapidly decreasing growth rates (23). Accordingly, we did not include treatment without N in our study. The trees were fertilized individually, once per day, with Hocking's complete nutrient solution (adjusting the  $\text{NH}_4\text{NO}_3$  concentration) as previously described (21–23) until runoff, over the course of 28 days. This approach allowed us to maintain a relatively constant nutrient source within the growth substrate (21). As the solution was applied daily, the amount of nitrogen corresponds to 0.2 g/L for the 1 mM  $\text{NH}_4\text{NO}_3$  control and 2 g/L for the 10 mM  $\text{NH}_4\text{NO}_3$  treatment.



**Figure 1.** (A) Representation of tree morphology and wood sampling scheme after 28 days of fertilization with 1 mM (tree on the left side) or 10 mM  $\text{NH}_4\text{NO}_3$  (on the right). The numbers (5, 10, 18) represent the leaf number (LPI) underneath each internode collected for lignin staining. (B) Stem sections were obtained from each of the internodes indicated (LPI), stained with phloroglucinol–HCl, and visualized under light microscope at 100×. Stem sections from LPI 5 and LPI 10 represent secondary xylem formed during the course of the experiment, whereas LPI 18 comprises secondary xylem formed in part before and in part during the experiment. (\* indicates stem height at the beginning of the treatment.)

Our observations indicate that the trees receiving the control treatment (CTL) grew and developed very similarly to those receiving our standard greenhouse fertilization regime (i.e., 20–20–20 weekly).

The first experiment compared the high N treatment (10 mM) and the control N treatment (1 mM) as described above and applied for a period of 28 days. At the outset of the experiment, four individual trees were randomly assigned to each treatment. The nitrogen applications were performed approximately 2 h after the beginning of the light period. After 28 days of treatment, stem segments were collected and set aside for analyses. Leaf plastochron index (LPI) was used to gauge the developmental state of the main stem (24) to compare stem sections at a similar developmental stage. For histological observations, internode segments were collected below the leaves at LPI 5, 10, and 18. For lignin analyses, the entire internodes between LPI 16 and LPI 18 were collected as outlined in **Figure 1** and debarked as previously described (21). All tissue samplings were done in the morning, 4 h after the start of the light period.

The second experiment compared the effects of N fertilization (treatments as described above) and the induction of tension wood. The young trees were grown with adequate or high  $\text{NH}_4\text{NO}_3$  fertilization (as in the first experiment) and in either a vertical or an inclined position, also over a period of 28 days and following a factorial experimental design. Tension wood formation was induced by growing the trees inclined at a 45° angle as described (25). The wood samples were collected from the same tree part as in the first experiment; however, a different wood sampling method was used in this experiment to enrich for newly formed wood. The debarked stems (10 mm in diameter on average) were scraped to produce wood shavings representing 1–2 mm of the outer part of the stem. For inclined trees, the upper side of the secondary xylem (i.e., tension wood) was collected for analyses.

**Histochemical Lignin Staining.** The presence of lignin in the stem was visualized using the Wiesner reaction (26). The phloroglucinol–HCl stain was prepared in ethanol (1:1 w/v with 13% v/v HCl). The coloration reaction was done on fresh free-hand stem cross sections taken, respectively, over LPI 5, LPI 10, and LPI 15 and visualized immediately with an Olympus BX51 light microscope (Olympus, Montreal, Canada).

**Lignin Content.** All of the lignin content determinations used air-dried wood that was ground in a Wiley mill to pass a 60 mesh. The samples were exhaustively extracted in a Soxhlet apparatus [2:1 (v/v) toluene/ethanol, ethanol, hot water] to obtain extract-free wood (EFW). Determinations of lignin content were done with two different methods. The Klason procedure was performed with 300 mg of extract-free samples (i.e., EFW), according to the standard protocol (27). The Klason

lignin content (% KL) was calculated as a percentage (w/w) of the EFW; mean values  $\pm$  standard deviation (SD) are presented. All analyses were replicated (i.e., two to four technical replicates) for each of the four independent trees to ensure a variation of <5% between analyses.

The determination of acetyl bromide (ABL) lignin was adapted from the standard protocol (28). Briefly, 5 mL of reagent, made by mixing 8.7 mL of ABL (colorless reagent, Acros) and 41.3 mL of glacial acetic acid, was added to 10 mg of EFW in a glass tube fitted with a Teflon-lined screw-cap. The reaction was then allowed to proceed for 2 h and 15 min, at 55 °C (oil bath). The reaction medium was then cooled before dilution as follows. In a dry glass tube, 0.2 mL of reaction mixture was diluted with 1.2 mL of a solution of aqueous 2 M NaOH in acetic acid (made by mixing 9 mL of 2 M aqueous NaOH and 50 mL of acetic acid), 0.3 mL of 0.5 M aqueous hydroxylamine chlorhydrate, and 3 mL of acetic acid. The lignin concentration was calculated by measuring the absorbance at 280 nm. All analyses were done in triplicate.

**Structural Lignin Investigations.** Structural analyses of lignins were made using thioacidolysis as previously described (29). Determination of the lignin-derived monomers was made by gas chromatography–mass spectrometry (GC-MS, Saturn 2000, Varian) of their silylated derivatives.

The analysis of low molecular weight phenolics was performed after mild alkaline hydrolysis, as described in ref 29 with slight modifications. Twenty-five (25) milligrams of EFW was treated with 2 M aqueous NaOH under nitrogen at 37 °C, with magnetic stirring. Ethylvanillin (0.1 mg) was added to the mixture as an internal standard. After acidification and solvent extraction (29), the released phenolics were identified by high-performance liquid chromatography–mass spectrometry (HPLC-MS) and quantified by HPLC-PDA (UV–visible photodiode array).

The frequency of free-end phenolic groups in lignins was determined after thioacidolysis of methylated samples. A modification of the original permethylation protocol was used (30). In a glass tube sealed with a Teflon-lined screw-cap, 20 mg of dried EFW was put in contact with 5 mL of methanol and 0.5 mL of the reagent trimethylsilyldiazomethane (2 M hexane solution, Aldrich) under magnetic stirring (in the dark, at room temperature). Three additions of the reagent were made during the 24 h reaction time. The insoluble permethylated sample was then washed (MeOH) and dried before analysis by thioacidolysis. The percentage of lignin-derived monomers methylated at C4 (i.e., originating from free phenolic and methylatable units) or trimethylsilylated at C4 (i.e., originating from etherified internal units) was determined as previously described (30).

**HPSEC Analysis of Thioacidolysis Reaction Mixture.** The distribution of lignin-derived monomers and oligomers was investigated using high-performance size exclusion chromatography (HPSEC) of the thioacidolysis reaction mixture. For HPSEC, thioacidolysates of EFW were dried under N<sub>2</sub> and resuspended in tetrahydrofuran (THF). HPSEC analysis of the thioacidolysates was performed by injecting 200  $\mu$ L on a PL-Gel column (Polymer Laboratories, 5  $\mu$ m, 600  $\times$  7.5 mm) with THF as the eluent (stabilized THF, J. T. Baker; 1 mL min<sup>-1</sup>) and 280 nm UV detection (31).

**Data Analysis.** Most data were obtained from analyses of two to four technical replicates of four independent trees ( $n$  = four biological replicates) per treatment. Statistical comparisons between the control (CTL) and the high N treatment were performed with Student's  $t$  test using SPSS v. 12.0 for Windows (SPSS Inc., Chicago, IL).

## RESULTS

Several independent greenhouse experiments were conducted with the *P. trichocarpa*  $\times$  *P. deltoides* clone H11-11, using standardized conditions previously described (21). The trees were fertilized daily with Hocking's complete nutrient solution supplemented with NH<sub>4</sub>NO<sub>3</sub> adjusted to a final concentration of either 1 mM [referred to as control (CTL)] or 10 mM (high N treatment) for a period of 28 days (see Materials and Methods). As previously reported (22, 23), the treatments rapidly gave contrasting growth rates that were highly repeatable across

five independent fertilization experiments (23). The analyses reported here were carried out on samples from two such experiments. In each experiment, we analyzed separately four randomly selected trees per N treatment. To focus on the effects of the treatment upon secondary xylem growth, lignin analyses were not carried out on the whole stem but were restricted to its basal part as wood was collected from the internodes between leaves LPI 16 and LPI 18. With the samples from one of the experiments, whole debarked internodes were analyzed, whereas, in the second experiment, the newly formed wood (also from LPI 16 to LPI 18 internodes) was scraped from the stem to minimize any dilution of the wood formed after the onset of the N fertilization experiment by the pre-existing wood. In our previous paper (21), we pointed to specific similarities between wood produced under a high N fertilization treatment and tension wood. Therefore, in the second experiment presented here, poplar trees were grown either in the vertical position or inclined at an angle of about 45° to induce the formation of tension wood, so as to investigate the cumulative effects, if any, of high N supply and gravitational stimulus.

**Lignin Deposition and Content.** The deposition of lignin in secondary xylem was monitored with the Wiesner reaction, which uses phloroglucinol–HCl as a stain, giving a red color indicative of coniferaldehyde end-groups in lignins. Three internode segments were collected along the stem (LPI 5, LPI 10, and LPI 18) and stained (Figure 1A–D). Not unexpectedly, the red lignin staining was more intense in the lower segments (Figure 1B–F). In all of the experiments, we observed differences between the LPI 18 cross sections from the control compared to the high N samples. In the control, these sections gave a rather uniform staining. In contrast, a heterogeneous staining was observed in the high N samples in which more lightly stained areas were visible next to the bark. The stem sections from LPI 5 and LPI 10 contained secondary xylem formed only during the experiment (Figure 1), whereas the sections from LPI 18 comprised secondary xylem produced in part before the experiment and in part during the experiment. These observations suggested that the high N supply altered lignin deposition in the newly formed secondary xylem.

Two methods of lignin determination, namely, the gravimetric Klason procedure and the spectrophotometric acetyl bromide (ABL) method, were applied to the extract-free wood (EFW) from the whole basal internodes (LPI 16–LPI 18) of the first experiment. The CTL and the 10 mM N samples had similar Klason lignin contents (20.80% for the CTL and 21.29% for the 10 mM treatment) based on the analysis of four trees (i.e., biological replicates) per treatment and three to four analytical replicates per sample (Table 1). As the proportion of acid-soluble lignins may be affected by the frequency of S units (32), we also determined the so-called acid-soluble lignin. The acid-soluble fractions also gave similar results between the CTL and the high N treatment (1.39 and 1.52%, respectively). On the other hand, the ABL determination gave a small but significant decrease in lignin content from 22.05% in the 1 mM CTL samples to 20.63% in the 10 mM N-fertilized ones. This ABL result seems to be more consistent with the histochemical observations performed on stem segments (LPI 18) close to those subjected to lignin analyses.

To specifically analyze the young secondary xylem formed after the onset of the N fertilization experiment, the younger secondary xylem was scraped from the debarked poplar stems segments (the internodes between LPI 16 and LPI 18) from the second experiment using the same N treatment as above. For this second experiment, lignin content was determined only by



**Table 1.** Lignin Content of Extract-free Wood (EFW) from Poplars Grown with Different Levels of N Fertilization<sup>a</sup>

| treatment (ammonium nitrate) | tree | acid-soluble lignin (% EFW) | Klason lignin (% EFW) | acetyl bromide lignin (% EFW) |
|------------------------------|------|-----------------------------|-----------------------|-------------------------------|
| 1 mM (CTL)                   | a    | 1.25 (0.34)                 | 21.10 (1.09)          | 22.43 (0.66)                  |
|                              | b    | 1.56 (0.16)                 | 20.23 (0.35)          | 22.83 (0.40)                  |
|                              | c    | 1.35 (0.17)                 | 20.91 (0.14)          | 21.69 (0.16)                  |
|                              | d    | 1.40 (0.08)                 | 20.95 (0.28)          | 21.26 (0.31)                  |
| av $\pm$ SD                  |      | 1.39 $\pm$ 0.20             | 20.80 $\pm$ 0.39      | 22.05 $\pm$ 0.71              |
| 10 mM                        | a    | 1.46 (0.10)                 | 21.29 (0.22)          | 20.70 (0.45)                  |
|                              | b    | 1.69 (0.39)                 | 21.50 (0.29)          | 19.62 (0.75)                  |
|                              | c    | 1.37 (0.11)                 | 21.44 (0.54)          | 20.74 (0.81)                  |
|                              | d    | 1.58 (0.04)                 | 20.91 (0.11)          | 21.47 (0.70)                  |
| av $\pm$ SD                  |      | 1.52 $\pm$ 0.14             | 21.29 $\pm$ 0.26      | 20.63 $\pm$ 0.76*             |

<sup>a</sup> EFW was obtained from whole stem wood samples (basal segments, LPI 16–18) as described under Materials and Methods (first experiment). Mean values  $\pm$  SD are presented for four independent trees (a–d) per treatment. The standard deviations between replicate analyses run on each tree ( $n$  = three or four technical replicates for Klason and acetyl bromide lignin;  $n$  = two technical replicates for acid-soluble lignin) are given in parentheses. Statistical significance between CTL and the 10 mM N treatment is based on Student's  $t$  test (\*,  $p$  < 0.05).

**Table 2.** Lignin Content of Extract-free Wood from Poplars Submitted to N Fertilization Treatments and Tension Wood Induction<sup>a</sup>

| treatment nitrogen and position (upright or tension) | acetyl bromide lignin |
|--|-----------------------|
| 1 mM normal wood                                     | 20.5 $\pm$ 2.0        |
| 10 mM normal wood                                    | 18.6 $\pm$ 0.1        |
| 1 mM tension wood                                    | 12.52 $\pm$ 0.3       |
| 10 mM tension wood                                   | 8.27 $\pm$ 0.1        |

<sup>a</sup> EFW was obtained from wood shavings of the outer part of the stem (basal segments LPI 16–18) as described under Materials and Methods (second experiment). Normal wood is from trees grown in an upright position; tension wood is from the upper side of the stem of trees grown at a 45°. Mean values  $\pm$  SD are presented for two trees per treatment, and analyses were run on each tree ( $n$  = three or four analytical replicates).

the ABL method (two trees per N level) as the Klason method requires more starting tissue. In agreement with the finding from the previous experiment (**Table 1**), the high N supply seemed to reduce the ABL lignin content of the vertically grown trees, as shown in **Table 2** (18.6% ABL lignin in the high N treatment and 20.5% in the CTL). In this analysis, using wood shavings, the difference between the treatment and the control was slightly larger. This experiment also included tension wood induction to compare the simultaneous influence of the gravitational stimulus and the high N supply on lignification. Not unexpectedly, the shavings from the tension wood from both the CTL and high N fertilization treatment had a strongly decreased lignin content relative to the samples grown in the vertical position (**Table 2**). Such a decreased lignin content of tension wood is well documented (for a review see ref 9). Again, the lignin content of the CTL (12.9% EFW) was significantly larger than that of the high N treatment (8.9% EFW). Overall, the ABL determinations carried out on the newly formed wood consistently suggested that a high N supply reduced the lignin level not only in normal wood (upright trees) but also in tension wood (inclined trees).

**Structural Alterations in Lignins.** We investigated the structure of the lignin polymers with thioacidolysis, a technique that cleaves the  $\beta$ -O-4 bonds, the major linkage present in poplar lignins. Thioacidolysis was carried out on four trees per treatment and in duplicate for each tree. When calculated on the basis of the Klason lignin content, the thioacidolysis yield was significantly lower for the samples grown under high N

(**Table 3**). This result suggests that the corresponding lignins are slightly enriched in resistant interunit bonds. To further support this result, we subjected the thioacidolysis reaction mixture to HPSEC to extend our evaluation to the lignin-derived dimers and oligomers. The HPSEC traces were normalized relative to the thioacidolysis monomer peaks (eluted at 16.2 min, **Figure 2**). These traces showed that high N fertilization increased the relative abundance of the dimers and oligomers, which were eluted as larger and earlier peaks, compared to the monomer reference peak. This finding confirms that high N supply increases the frequency of resistant interunit bonds in poplar lignins. The relative frequencies of the thioacidolysis S and G monomers varied little between the nitrogen treatments (**Table 3**). The S/G molar ratio tended to be lower with the elevated N treatment (1.69) relative to the control 1 mM N treatment (1.83), but the difference was not significant due to the biological variability between the trees. In contrast, the CTL and high N samples could be discriminated by the frequency of the H lignin-derived monomers, which are recovered in low amount from poplar lignins. The high N treatment led to a weak but nevertheless significant increase in the frequency of H thioacidolysis monomers (18%, relative increase) compared to the control (**Table 3**). In the experiment combining fertilization and tension wood induction (**Table 4**), we observed essentially the same trends in response to the high N supply compared to the control. The thioacidolysis yield, when calculated on the basis of the lignin content, was slightly reduced, and so was the S/G ratio, whereas the frequency of H thioacidolysis monomers was increased in the upright-grown trees. In addition to the impact of N supply on lignin structure, this experiment showed that tension wood formation was associated not only with a substantially lower lignin content but also with changes in lignin structure, mainly a lower frequency of S lignin units. As a consequence, the formation of tension wood and high N treatment had cumulative effects on the S/G molar ratio, and the lowest S/G ratio (1.28, **Table 4**) was obtained in the tension wood of high N trees.

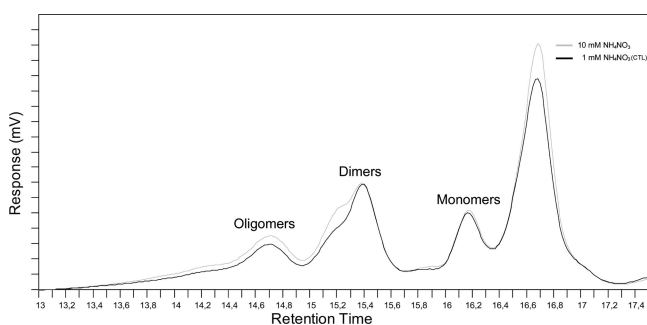
We examined the proportion of H, G, and S  $\beta$ -O-4-linked lignin units that occurred as free phenolic terminal units or etherified internal units (**Table 5**) based on the recovery of thioacidolysis monomers from exhaustively permethylated samples (four trees per treatment, basal section LPI 16–18). As expected, the  $\beta$ -O-4-linked H units were essentially terminal units with free phenolic groups (i.e., 80–90% of H monomers were methylated at C4), S units were prominently internal units (only 2% could be methylated at C4), and G units displayed an intermediate behavior (17–20% of  $\beta$ -O-4-linked G units could be methylated at C4). The high N treatment led to a decreased proportion of G monomers methylated at C4, relative to the total amount of thioacidolysis G monomers (17.6  $\pm$  0.3% for the 10 mM versus 19.3  $\pm$  0.5% for the CTL). Although the differences are relatively small in magnitude, they are statistically significant. This result establishes that the distribution of  $\beta$ -O-4-linked G units as free phenolic units located at the periphery of the polymer or as internal units with etherified phenolic groups is affected by the treatment. In contrast, there was a slightly increased proportion of H units with free phenolic groups (5% relative increase), which suggests that the minor amount of H units that were added as an effect of N fertilization are essentially terminal units.

Poplar lignins are specifically acylated by *p*-hydroxybenzoic acid (33, 34). The amount of *p*-hydroxybenzoic acid released by subjecting the extract-free wood sample (basal internodes from trees in **Tables 1** and **3**, analyses run in duplicate) to a

**Table 3.** Lignin-Derived H, G, and S Monomers Released by Thioacidolysis of Extract-free Wood (EFW) from Poplars Grown with Different Levels of N Fertilization<sup>a</sup>

| treatment<br>(ammonium nitrate) | tree        | G + S + H<br>( $\mu\text{mol g}^{-1}$ of EFW) | G + S + H<br>( $\mu\text{mol g}^{-1}$ of KL) | S/G             | % H              | % G            | % S            |
|---------------------------------|-------------|---|--|-----------------|------------------|----------------|----------------|
| 1 mM (CTL)                      | a           | 539 (13)                                      | 2555 (61)                                    | 1.72 (0.01)     | 0.26 (0.01)      | 36.7 (0.1)     | 63.0 (0.1)     |
|                                 | b           | 534 (33)                                      | 2642 (164)                                   | 1.93 (0.03)     | 0.27 (0.01)      | 34.1 (0.3)     | 65.6 (0.4)     |
|                                 | c           | 564 (6)                                       | 2698 (28)                                    | 1.90 (0.03)     | 0.28 (0.01)      | 34.4 (0.3)     | 65.3 (0.3)     |
|                                 | d           | 546 (3)                                       | 2607 (14)                                    | 1.78 (0.04)     | 0.30 (0.00)      | 35.9 (0.5)     | 63.8 (0.5)     |
|                                 | av $\pm$ SD | 548 $\pm$ 22                                  | 2634 $\pm$ 104                               | 1.83 $\pm$ 0.09 | 0.28 $\pm$ 0.02  | 35.2 $\pm$ 1.2 | 64.5 $\pm$ 1.2 |
| 10 mM                           | a           | 510 (8)                                       | 2395 (36)                                    | 1.61 (0.00)     | 0.33 (0.01)      | 38.2 (0.4)     | 61.5 (0.4)     |
|                                 | b           | 500 (0)                                       | 2326 (0)                                     | 1.84 (0.00)     | 0.32 (0.00)      | 35.1 (0.0)     | 64.6 (0.0)     |
|                                 | c           | 525 (15)                                      | 2448 (70)                                    | 1.53 (0.08)     | 0.36 (0.02)      | 39.4 (1.2)     | 60.3 (1.3)     |
|                                 | d           | 469 (23)                                      | 2240 (109)                                   | 1.77 (0.00)     | 0.38 (0.00)      | 35.9 (0.0)     | 63.7 (0.0)     |
|                                 | av $\pm$ SD | 501 $\pm$ 29                                  | 2356 $\pm$ 118*                              | 1.69 $\pm$ 0.14 | 0.35 $\pm$ 0.03* | 37.1 $\pm$ 2.0 | 62.5 $\pm$ 2.0 |

<sup>a</sup> EFW was obtained from wood samples (basal segments LPI 16–18) as described under Materials and Methods. Yields are calculated as micromoles per gram of EFW of Klason lignin (KL) or of acetyl bromide lignin (ABL). Mean values  $\pm$  SD are presented for four trees per treatment (trees a–d are the same as in Table 1). The standard errors between duplicate analyses run on each sample are given in parentheses. Statistical significance between CTL and the 10 mM N treatment is based on Student's *t* test (\*,  $p < 0.05$ ).



**Figure 2.** High-performance size exclusion chromatography (HPSEC) elution profile of lignin oligomers, dimers, and monomers released by thioacidolysis. Representative samples are shown for each level of nitrogen fertilization; the last peak (16.7 min of elution) corresponds to reagent-derived products, and the authenticity of each compound was determined by injecting appropriate standards.

mild alkaline hydrolysis was determined by HPLC coupled to UV–visible photodiode array (PDA) and mass spectrometry detection. The *p*-hydroxybenzoic acid was more abundant in the sample from the high N supply (Figure 3). On the basis of the HPLC-PDA determination, high N fertilization led to a 30% increase ( $0.22 \pm 0.01\%$ /EFW) when compared to the CTL ( $0.16 \pm 0.01\%$ /EFW). Similarly and albeit occurring as a trace component, the amount of ferulic acid (peak at 10.5 min) was approximately doubled in response to the 10 mM treatment ( $0.01 \pm 0.002\%$ /EFW). In contrast, vanillin and syringaldehyde, which are degradation products released from lignins upon mild alkaline hydrolysis, were recovered in similar amounts from the extract-free woods of the two treatments.

## DISCUSSION

In plants, the allocation and partition of carbon resources are related to growth conditions (35). The impact of N fertilization on carbon allocation in woody species has been relatively well investigated; however, the impact on carbon partitioning into major wood compounds such as lignin, cellulose, and hemicelluloses is poorly documented. In a previous study, we showed that the formation of the secondary xylem cell wall may vary significantly in response to N fertilization. Significant variations were observed in fiber width and length, as well as histology, suggesting that cell wall deposition was affected by N availability (21). In a separate study, transcript accumulation varied for a number of genes including genes coding enzymes involved

in the formation of secondary cell walls (22). The work presented in this paper was undertaken to examine and compare the structure of poplar lignins following fertilization with adequate and high levels of ammonium nitrate.

**Decreased Lignin Deposition and Content in Response to High Nitrogen Fertilization.** In this study, the first indication that nitrogen fertilization could affect lignin deposition came from the less intense Wiesner lignin staining in the newly formed wood of trees receiving the high ammonium nitrate fertilization (Figure 1). The intensity of the Wiesner reaction is affected not only by lignin content but also by the structure of the lignin polymer as the key chromophoric groups involved in the reaction with phloroglucinol–HCl are the *p*-OH cinnamaldehyde end-groups (36–38). However, when expressed on the basis of the lignin content or the extract-free wood, thioacidolysis did not reveal any increase in coniferaldehyde or sinapaldehyde end-groups in the 10 mM N fertilized trees (data not shown). Accordingly, the lighter Wiesner staining in the newly formed xylem more likely came from a lower lignin level.

As there is no single method that provides an accurate measure of the total lignin in a given sample (39), we applied two different methods to determine the lignin content of the extract-free LPI 18–LPI 20 internodes, which includes both the pre-existing wood and the wood neosynthesized after the onset of the N supply experiment. We used the gravimetric Klason method, which relies on the treatment of the extract-free cell walls by concentrated sulfuric acid and the recovery of the so-called Klason lignin as an insoluble residue (corrected for ash components if any). As some lignin fragments may have been solubilized in the acid medium, the acid-soluble lignin fraction was evaluated from the absorbance of the sulfuric supernatant at 205 nm, to avoid any interference from furfurals derived from polysaccharides (see Materials and Methods). The acetyl bromide method is based on the complete solubilization of lignins in 25% acetyl bromide in glacial acetic acid and the determination of their absorbance in the UV, which is then converted into lignin using either an arbitrary specific absorption coefficient and/or an appropriate standard lignin preparation. These two methods gave slightly different results. The sulfuric acid method (including the acid-insoluble Klason lignin and the so-called acid-soluble lignin) did not show any differences between the treatments. In contrast, the spectrometric ABL method suggested a reduction of lignin content after high N fertilization. Similarly, slight discrepancies between Klason

**Table 4.** Lignin-Derived Monomers Released by Thioacidolysis Content of Extract-free Wood (EFW) from Poplars Submitted to Nitrogen Fertilization Treatments and Tension Wood Induction<sup>a</sup>

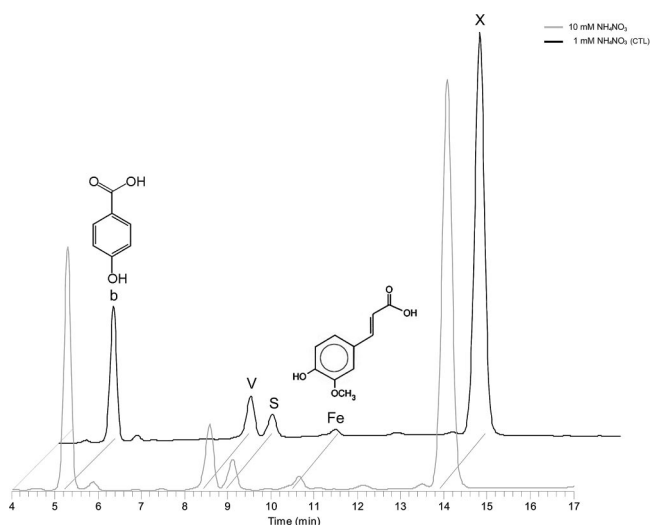
| treatment nitrogen and position (upright or tension) | G + S + H ( $\mu\text{mol g}^{-1}$ of EFW) | G + S + H ( $\mu\text{mol g}^{-1}$ of ABL) | % H             | % G              | % S              | S/G             |
|--|--|--|-----------------|------------------|------------------|-----------------|
| 1 mM normal wood                                     | 435 $\pm$ 10                               | 2136 $\pm$ 165                             | 0.13 $\pm$ 0.00 | 34.88 $\pm$ 2.62 | 64.99 $\pm$ 2.62 | 1.88 $\pm$ 0.22 |
| 10 mM normal wood                                    | 376 $\pm$ 8                                | 2021 $\pm$ 43                              | 0.18 $\pm$ 0.02 | 38.51 $\pm$ 1.11 | 61.31 $\pm$ 1.13 | 1.59 $\pm$ 0.08 |
| 1 mM tension wood                                    | 274 $\pm$ 6                                | 2184 $\pm$ 5                               | 0.23 $\pm$ 0.01 | 39.15 $\pm$ 0.47 | 60.62 $\pm$ 0.48 | 1.55 $\pm$ 0.03 |
| 10 mM tension wood                                   | 148 $\pm$ 5                                | 1864 $\pm$ 45                              | 0.25 $\pm$ 0.02 | 43.8 $\pm$ 1.50  | 57.53 $\pm$ 1.56 | 1.28 $\pm$ 0.03 |

<sup>a</sup> EFW was obtained from wood shavings from the outer part of the stems, as described under Materials and Methods (second experiment). Mean values  $\pm$  SD were based on individual analyses of two trees per treatment.

**Table 5.** Impact of Adequate or High N Supply on the Percentage of Free Phenolic Groups in B-O-4-Linked H, G, and S Lignin Units, Determined by Thioacidolysis of CH<sub>2</sub>N<sub>2</sub> Methylated Samples<sup>a</sup>

| treatment (ammonium nitrate) | tree | % free phenolic groups in $\beta$ -O-4-linked H, G, or S units |                    |                  |
|------------------------------|------|--|--------------------|------------------|
|                              |      | H  | G                  | S                |
| 1 mM (CTL)                   | a    | 80.78  | 19.48              | 2.00             |
|                              | b    | 79.28  | 18.60              | 1.73             |
|                              | c    | 80.95  | 19.62              | 1.78             |
|                              | d    | 80.87  | 19.61              | 1.91             |
| av $\pm$ SD                  |      | 80.47 $\pm$ 0.80   | 19.33 $\pm$ 0.49   | 1.86 $\pm$ 0.12  |
| 10 mM                        | a    | 87.21  | 17.76              | 2.07             |
|                              | b    | 83.31  | 17.26              | 2.01             |
|                              | c    | 84.43  | 17.85              | 2.08             |
|                              | d    | 84.83  | 17.57              | 2.07             |
| av $\pm$ SD                  |      | 84.82 $\pm$ 0.26**   | 17.61 $\pm$ 0.26** | 2.06 $\pm$ 0.04* |

<sup>a</sup> The extract-free wood (basal segment LPI 16–18) was obtained from wood samples as described under Materials and Methods (first experiment). Mean values  $\pm$  SD are presented for four trees per treatment (trees a–d are the same as in Tables 1 and 2). Statistical significance between CTL and the 10 mM N treatment is based on Student's *t* test (\*, *p* < 0.05; \*\*, *p* < 0.01).

**Figure 3.** Phenolic compounds released by mild alkaline hydrolysis. Photodiode array spectra were obtained following high-performance liquid chromatography. Representative samples are shown for each level of nitrogen fertilization. (b, *p*-hydroxybenzoic acid; V, vanillin; S, syringaldehyde; Fe, ferulic acid; X, internal Standard.)

lignin and ABL lignin levels have been repeatedly reported in past studies, particularly in the case of grass samples (39).

This discrepancy may come from an overestimate of the Klason method and/or from an underestimate of the ABL method. For instance, if the 10 mM sample contained more proteins that are not fully solubilized in the solvent extraction step, the proteins may coprecipitate with the Klason lignin

fraction (40, 41), which could lead to an overestimate of the Klason lignin. The occurrence of noticeable amounts of proteins associated with gravimetric lignins has been often addressed, more particularly in the case of the so-called acid-detergent fiber methods and in the case of foliar tissues (42, 43). The putative contamination of the high N sample-derived Klason lignins by proteins is supported by the unusual foam observed when the corresponding diluted sulfuric reaction medium is refluxed during the Klason lignin protocol. It is also supported by studies demonstrating that in poplar stems, vegetative storage protein synthesis is increased under a high N supply (22, 23, 44–47). With regard to the ABL method, the lignin content was determined after the complete solubilization of lignins in the reagent and the measurement of the absorbance at 280 nm. As suggested in a comprehensive study (48), this absorbance was then converted to lignin equivalent by using the specific absorption coefficient determined by subjecting an acidic dioxan poplar lignin fraction extracted from poplar wood to the ABL protocol and measuring the resulting absorption coefficient ( $20.0 \pm 0.2 \text{ L g}^{-1} \text{ cm}^{-1}$ ). The coefficient that we obtained was similar to the one most often used in the literature. However, it has not been clearly established whether this extinction coefficient is relevant whatever the lignin origin and developmental stage (41). As the ABL method is consistent with the reduction in Wiesner lignin staining, the most likely explanation is that of an overestimate of the Klason lignin level in the high N samples.

**Nitrogen Availability Modulates Lignin Structure and Xylem Differentiation.** In addition to the slight decrease in lignin deposition, we found that the lignins formed in response to high N fertilization shared several similarities with lignins formed at early stages of cell wall development. These findings led us to hypothesize that high N supply results in a greater proportion of cells in earlier stages of differentiation. This increased proportion of developing secondary xylem cells could be a consequence of an increased rate of xylem cell production and/or a prolonged period of development. Partly differentiated xylem cells would indeed have accumulated less lignin relative to fully mature xylem cells, because lignification in developing cells is generally restricted to the middle lamella/primary wall (40). The structural differences between lignin of control and high N treated trees that are characteristic of lignins formed at the early stages of cell wall development are outlined below.

Lignins from high N samples are slightly enriched in resistant bonds (referred to as the condensed bonds) as revealed not only by the lower thioacidolysis yield, when calculated on the basis of the Klason or ABL lignin content (Tables 1–4), but also by the HPSEC trace of the thioacidolysis reaction mixture (Figure 2). Evidence showing that lignins formed at the early stage of cell wall development have a higher degree of condensation comes from the pioneering microautoradiographic studies of Terashima's group in hardwoods, including poplar samples, as well as in softwoods (49, 50). This specific trait of lignins deposited in the middle lamella and primary wall areas and at



the onset of the secondary xylem lignification was confirmed in more recent studies (40, 51).

Another specific structural trait of hardwood lignins formed in the early stage of cell wall formation is the occurrence of higher amounts of *p*-hydroxyphenyl H units (52). H units are minor components of hardwood lignins and usually receive little attention. They may nevertheless play an important role as these units might contribute to form a highly condensed lignin network within the pectin-rich middle lamella region. A diagnostic fingerprint of the lignin produced under high N is the significantly increased amount of H monomers (by 18%) incorporated in the polymer, a characteristic that is thus reminiscent of lignins deposited at the early steps of cell wall development.

The lignins from high-N trees tended to have lower S/G ratios relative to the controls. That G units are deposited at the onset of the lignification has been established by many past studies (for a review see ref 53). Accordingly, the trend observed in this study toward lower S/G ratios is also consistent with the hypothesis that high N fertilization promotes a rapid cambial growth together with a delayed lignification in this cambial area.

A more specific trait of poplar lignins is the occurrence of *p*-hydroxybenzoic acid ester-linked to lignin units (33, 34, 54). The incorporation of these esters into poplar lignin has been examined by tracer methods. It was possible to establish that their incorporation was greater at the early lignification stages (55). Accordingly, the higher proportion of *p*-hydroxybenzoic esters of lignins of high N samples is similar to lignins formed near the cambium, at the early developmental stage of the secondary xylem.

Another structural feature that discriminates middle lamella lignins, deposited at the onset of lignification, and secondary wall lignins, which are deposited later, was shown in the case of softwood samples by ultraviolet microscopy (56) and analytical pyrolysis (57). Both methods consistently suggested that G lignins from middle lamella regions have a substantially lower content (2-fold lower) of free phenolic groups than G lignins from secondary walls. Relative to the control samples, poplar lignins from the high N treatment also displayed a lower frequency of G units with free and thereby methylable phenolic groups. If the results from softwood samples can be extrapolated to hardwoods, a delayed lignification in response to high N fertilization could account for a relative enrichment in early developmental stage-type lignins, typified by a lower content in free phenolic groups.

Both the fertilization treatment and the induction of tension wood affected lignin deposition (Tables 1 and 2). It has been repeatedly shown that tension wood contains less lignin (10–20% decrease) than normal wood produced on vertically growing stems (9, 58). Nitrogen depletion of poplar cuttings lowered the proportion of fibers possessing a thick gelatinous (G-) layer (59). By contrast and in agreement with the present results, an increased growth rate resulted in an increased number of gelatinous fibers, rich in cellulose and with a lower lignin content. In our experiment, the combination of high N fertilization and tension wood induction treatments led to a strong decrease in lignin content (Table 2). Our analysis was performed on secondary xylem shavings enriched in wood formed during the experiment; therefore, it was likely more representative of the lignin formed in response to the treatments. A recent survey of the genes differentially expressed in tension wood showed that many genes are affected (60). Several of the genes down-regulated in tension wood encoded enzymes involved in lignin biosynthesis and more specifically in the formation of S precursors (ferulate-5-hydroxylase and caffeic acid-*O*-methyltransferase). Similarly, a previous paper showed that the levels

of *CCoAOMT* mRNA transcripts are reduced in response to nitrogen fertilization (22). When combined, the high N and the tension wood induction gave a further decrease of the lignin content and S/G ratio, thus suggesting additive effects of these two factors (Table 4).

As a preliminary assessment of the impact N fertilization on wood utilization, we also carried out assays evaluating the susceptibility of poplar wood to cellulolysis. Interestingly, our preliminary results indicated that the digestibility was increased in trees supplemented with the high N treatment. A previous study has also shown that the suitability of poplar in biofermentation for the production of bioethanol was significantly influenced by relatively small variations in lignin content and structure (61).

In conclusion, our results highlight the plasticity of wood formation and in lignin deposition in response to N fertilization as well as gravitational stimuli in young poplar trees. We observed that trees supplied with high levels of nitrogen produce secondary xylem fibers with more juvenile lignin characteristics, consistent with their accelerated rate of growth. When combined with a gravitational stimulus (inducing the formation of tension wood), the effects of nitrogen fertilization on lignin structure are even greater, indicating the two environmental factors have additive effects. Further investigation may be aimed at defining pathways that mediate the responses to these external factors and the mechanisms through which they modulate wood formation and structure.

## ABBREVIATIONS USED

N, nitrogen;  $\text{NH}_4\text{NO}_3$ , ammonium nitrate; NPK, nitrogen–phosphorus–potassium; H, *p*-hydroxyphenyl; G, guaiacyl; S, syringyl; LPI, leaf plastochron index; PAL, phenylalanine ammonia-lyase; OMT, *O*-methyl transferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; CTL, control; EFW, extract-free wood; KL, Klason lignin; ABL, acetyl bromide lignin; GC-MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; PDA, photodiode array; HPSEC, high-performance size exclusion chromatography; UV, ultraviolet.

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