Final Report

For: Exploiting Genetic Variation of Fiber Components and Morphology in Juvenile Loblolly Pine.

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Project Objective:

- *Long Term Overall Objective:* Ensure Global Competitiveness through Enhanced Forest Productivity in the U. S. South – more efficiently produces more industrial wood on limited land with targeted characteristics.
- *Short Term Objectives:* The objective of the proposed research project is to provide a molecular genetic basis for tree breeding of desirable traits in juvenile loblolly pine. This area of research represents an interface among forest science, molecular biology and material science.
  - Wood and Paper Science Group: Develop micro analytical techniques that allow rapid characterization of fiber components and morphology in a large number of samples.
  - Forest Biotechnology Group: Detect allelic variation in candidate wood property genes, develop a pilot-scale mapping microarray, and assay candidate gene single-nucleotide polymorphisms (SNPs) that segregate in the families chosen for wood property analysis.
  - Tree Improvement Group: Identify parents or families that carry one or more major genes that control wood/fiber quality and develop new breeding strategies based accordingly.

EXECUTIVE SUMMARY

In order to ensure the global competitiveness of the Pulp and Paper Industry in the Southeastern U.S., more wood with targeted characteristics have to be produced more efficiently on less land. The objective of the research project is to provide a molecular genetic basis for tree breeding of desirable traits in juvenile loblolly pine, using a multidisciplinary research approach.

We developed micro analytical methods for determine the α-cellulose and lignin content, average fiber length, and coarseness of a single ring in a 12 mm increment core. These methods allow rapid determination of these traits in micro scale.

Genetic variation and genotype by environment interaction (GxE) were studied in several juvenile wood traits of loblolly pine (*Pinus taeda* L.). Over 1000 wood samples of 12 mm increment cores were collected from 14 full-sib families generated by a 6-parent half-diallel mating design (11-year-old) in four progeny tests. Juvenile (ring 3) and transition (ring 8) for each increment core were analyzed for α-cellulose and lignin content, average fiber length, and coarseness. Transition wood had higher α-cellulose content, longer fiber and higher coarseness, but lower lignin than juvenile wood. General combining ability variance for the traits in juvenile wood explained 3 to 10% of the total variance, whereas the specific combining ability variance was negligible or zero. There were noticeable full-sib family rank changes between sites for all the traits. This was reflected in very high specific combining ability by site interaction variances, which explained from 5% (fiber length) to 37% (lignin) of the total variance. Weak individual-tree heritabilities were found for cellulose, lignin content and fiber length at the juvenile and transition wood, except for lignin at the transition wood (0.23). Coarseness had moderately high individual-tree heritabilities at both the juvenile (0.39) and transition wood (0.30). Favorable genetic correlations of volume and stem straightness were found with cellulose content, fiber length and coarseness, suggesting that selection on growth or stem straightness would results in favorable response in chemical wood traits.

We have developed a series of methods for application of functional genomics to understanding the molecular basis of traits important to tree breeding for improved chemical and physical properties of wood. Two types of technologies were used, microarray analysis of gene expression, and profiling of soluble metabolites from wood forming tissues. We were able to correlate wood property phenotypes with expression of specific genes and with the abundance of specific metabolites using a new database and appropriate statistical tools. These results implicate a series of candidate genes for cellulose content, lignin content, hemicellulose content and specific extractible metabolites. Future work should integrate such studies in mapping populations and genetic maps to make more precise associations of traits with gene locations in order to increase the predictive power of molecular markers, and to distinguish between different candidate genes associated by linkage or by function.

This study has found that loblolly pine families differed significantly for cellulose yield, fiber length, fiber coarseness, and less for lignin content. The implication for forest industry is that genetic testing and selection for these traits is possible and practical. With sufficient genetic variation, we could improve cellulose yield, fiber length, fiber coarseness, and reduce lignin content in Loblolly pine. With the continued progress in molecular research, some candidate genes may be used for selecting cellulose content, lignin content, hemicellulose content and specific extractible metabolites. This would accelerate current breeding and testing program significantly, and produce pine plantations with not only high productivity, but desirable wood properties as well.

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DEVELOPMENT OF MICRO ANALYTICAL METHODS

I. Micro analytical Method for the Characterization of Fiber Components and Morphology of Woody Plants

Summary

Micro analytical techniques were developed which allow the rapid characterization of fiber components and morphology of loblolly pine in a large number of samples. These techniques consist of extractives removal, holocellulose preparation, α-cellulose and lignin content determination, and fiber length and coarseness analyses. Greater than 95% of the non-volatile extractives from an increment core sample of loblolly pine was removed by four successive two-day acetone extractions. Fiber morphology and α-cellulose content was determined from holocellulose prepared from only 100mg of wood. Similarly a microanalytical acetyl bromide method was developed that enable the accurate determination of lignin content from less than 50 mg of wood. Through the development of these microanlytical methods, it is possible to accurately and rapidly analyze fiber morphology and chemical components in a large number of increment core samples.

Introduction

The Southeast U.S. is the largest supplier of industrial wood to the world, providing about 15% of the world's industrial roundwood (*1*) and almost 60% of the U.S. harvest (*2,3*). The region is gradually losing its global competitiveness as a result of decreasing harvest from the natural forests and the increasing competition from the Southern Hemisphere. *In order for the Southeast U.S. to meet future demands while maintaining global competitiveness, more wood with targeted characteristics has to be produced more efficiently on less land*. One viable solution to meet the future industrial wood demands is to greatly increase the productivity of the current pine plantations, leaving natural forests to be managed at low intensity, primarily for sawtimber, conservation, aesthetics and recreational ends. The productivity of the current pine plantations, which occupy less than 20% of the total forest area, must be increased two- to three-fold. This can be done through intensively managed plantation forests of genetically superior trees resulting from intensive research efforts involving biotechnology, tree breeding and silvicultural practices.

The use of intensively managed pine plantation forests as the major source of softwood pulpwood would result in a great increase in the proportion of juvenile wood utilized by the pulp mill. It is well documented that the properties of juvenile wood are quite different from those of mature wood (*4*). Compared with mature wood, juvenile wood of loblolly pine has higher lignin content, shorter average fiber length, smaller cell wall diameter, a thinner cell wall, higher compression wood content, higher microfibril angle and lower specific gravity. The thinner cell wall and smaller cell wall diameter contribute to lower refining energy requirements and better fiber bonding, thus better tensile and burst strength and better printability, making juvenile wood an ideal raw material for specific grades such as printing and writing paper, mechanical pulp, and linerboard. However, the higher lignin content, lower cellulose content and lower specific gravity, decrease the pulp yield to the extent that it significantly impacts pulp production costs.

During the past two decades there has been a gradual but steady increase in juvenile wood content in the raw material furnish of the pulp mill. The trend is expected to continue over the next decade. Given the impact of fiber components and morphology of juvenile wood on processing and end product properties, it is surprising that no systematic study has been carried out to assess the natural variations of these traits in juvenile wood, let alone taking advantage of recent advances in molecular biology and

biotechnology. The promise of biotechnology lies in the potential to make major genetic changes through genetic selection or directed modification in a short period of time. To accomplish this, analytical techniques to identify differences in fiber components and morphology need to be developed, specifically microanalytical techniques that enable rapid and accurate characterization of thousands of samples using milligrams of material. Although several spectroscopic methods, e.g. FT-Raman, near-IR, etc., exist which permit rapid structural characterization of plant materials, these techniques are not fully developed, and more importantly require time consuming wet chemistry to establish calibration sets and confirm their accuracy and precision. In this paper we describe the successive procedure developed for the easy and rapid analysis of fiber morphology and major chemical components of softwoods. The microanalytical methods developed include extractives removal, holocellulose preparation, α-cellulose and lignin content determination and fiber length and coarseness analyses.

Materials and Methods

Materials. Wood samples were from an 11-12-year old (juvenile) and a 33-year old (mature) loblolly pine. Milled wood lignin (MWL) was prepared from the 33-year old pine using the method of Björkman (5). The juvenile pine was received as 12 - mm increment cores, whereas the mature pine was a 30 cm bolt from the North Carolina State University Department of Forestry Tree breeding program.

Acetone (ACS grade), sodium chlorite (80%, tech grade), acetic acid (ACS grade), sodium hydroxide (ACS grade), acetyl bromide (99%), perchloric acid (70%, ACS grade), and sulfuric acid (ACS grade) were purchase from Aldrich Chemical Co. and used as received.

Micro Analytical Methods Developed.

Removal of extractives. The12 - mm increment cores taken in the field were immediately cut at the pith into two sections, debarked, and placed in a capped test tube (50 mL) in acetone. The samples were then transported to the lab, where after two days at room temperature, the acetone was drained and replaced. The extraction procedure was repeated at 48 h intervals for a total of six times. After each extraction, the acetone was concentrated under reduced pressure, and the amount of nonvolatile extractives was determined gravimetrically.

Preparation of wood samples. Wood meal samples of both the juvenile pine and mature pine wood was prepared using a Wiley mill, and ground to pass a various mesh screens. To minimize the modification of the wood fibers arising from the milling procedure, thin wafers from both the spring and summer wood portions of the 3-year old and 8-year old rings of juvenile pine were prepared by cutting these portions parallel to the fiber using a sharp knife or microtone. These samples represent the juvenile and juvenile-mature wood transition zone, respectively. The thin wafers were then used to make holocellulose and to determine lignin content (discussed below).

Preparation of holocellulose. Holocellulose was isolated from the extractive-free wood wafers and wood meals prepared as described above. Approximately 100 mg (OD) of wood was suspended in 2 mL of deionized water in a round-bottom flask (10 mL) with a ground glass stopper. The reaction flask was then submerged in a water bath maintained at 90 ℃. The reaction was initiated by adding 0.5 mL of sodium chlorite solution which has been prepared by dissolving 200 mg of 80% sodium chlorite into 2 mL of deionized water and 0.2 mL of acetic acid. At 30 minute intervals 0.5 mL of sodium chlorite-acetic acid solution was added to the reaction, for a total of 2 mL. At the end of 2 h (total 4 additions), the reaction was cooled in cold water bath, and filter using a sintered glass filter (coarse). The resulting holocellulose was thoroughly washed with deionized water (3x50 mL), dried in an oven at 105℃ and the yield of holocellulose determined.

To simplify the reaction protocol and enable more sample analyses to be performed, the reaction can be simplified to a single addition of the sodium chlorite-acetic acid solution. Specifically, the 100 mg (OD) of wood is suspended in 4 mL of deionized water at 90℃, and reacted with 200 mg of 80% sodium chlorite and 0.8 mL of acetic acid for 1 h. The reaction is then filtered, washed and dried in the same manner as the outlined above.

Determination of α-cellulose content. 50 mg of the oven dried holocellulose was weighed into a 10 mL beaker and left to stand at room temperature for 30 min to allow moisture equilibration, at which time 4 mL of 17.5% sodium hydroxide was added to the beaker and left reacting an additional 30 min at ambient temperature. Deionized water (4 mL) was then added and the mixture was stirred for one minute with a glass stir rod and left for another 29 min. After a total reaction time of 60 min, the fiber suspension was filter using a sintered glass filter (coarse), washed thoroughly with deionized water (3x30 mL), and soaked in 1 . 0 M acetic acid solution for 5 minutes. The neutralized α-cellulose was then thoroughly washed with deionized water (3x30 mL) and the yield was determined after oven drying at 105℃.

Determination of fiber length and coarseness. Fiber length and coarseness was determined on the isolated holocellulose using a FQA (Fiber Quality Analyzer, Op Test Equipment, Inc., Hawkesbury, ON) and Kajaani FS-200 (Valmet Automation, Inc., Norcross, GA). For FQA analysis, exactly 10-16 mg of the oven-dried holocellulose (it is critical to record the exact weight) was suspended in 20 mL of deionized water and defibrated for 5 min. The liberated fibers were then quantitatively transferred into a 400 mL beaker using 50-100 mL of deionized water. The fiber suspension was diluted with an appropriate volume of water so that a total of 200 mL of deionized water had been added. The suspension was vigorously stirred and 10 mL of the suspension was extracted using a glass pipet and subjected to fiber length and coarseness analysis. For the Kajaani fiber analysis, 3-4mg of fibers is required (versus the 0.5-0.8 mg for FQA), therefore 60-80 mg of wood is required.

Determination of lignin content. The lignin content of the various wood samples was determined using a modified acetyl bromide method. Accordingly, 10 mL of freshly prepared 25% (w/w) acetyl bromide/acetic acid solution was added to a 20 mg (OD) wood wafer or wood meal sample in a 25 mL Erlenmeyer flask with a ground glass stopper. This was immediately followed by the addition of 4 mL of 70% (w/w) perchloric acid solution. The flask was stoppered and sealed with Teflon tape, quickly shaken, and placed it into a water bath maintained at 70℃. The reaction was left for 30 min with intermittent shaking at 10 min intervals. At the end of 30 min, the reaction was stopped by cooling the flask in cold water. The reaction mixture was then quantitatively transferred using acetic acid to a volumetric flask (250 mL) containing 10 mL of 4 . 0 M sodium hydroxide and 50 mL of acetic acid. The volume was made up to 250 mL with acetic acid and the UV absorbance at 280 nm of this acetic acid solution was determined. NOTE: It is extremely important that the UV analysis be recorded within 10 min after the beginning of the dilution.

The determination of lignin content was conducted using a calibration line made by subjecting known amounts of holocellulose and MWL (both from mature pine) to the acetyl bromide method described above. As a result, the gram extinction coefficient of lignin treated by acetyl bromide is not needed. The calibration line is shown in Figure 1. The calibration line shows the correlation between the total lignin content in the mixtures and the UV absorbance at 280 nm obtained by subjecting the mixtures to this microacetyl bromide method. The total lignin content in the mixtures is calculated from the MWL content using the values obtained by the Klason lignin determination (*6*): 5.1% for holocellulose prepared from mature pine (0.9% for Klason lignin (KL), 4.2% for acid soluble lignin (ASL)) and 91.0% for MWL prepared from mature pine (KL 89.9%, ASL 1.1%).

Microanalytical Procedure. Using the microanalytical techniques developed above, a successive procedure for the analysis of fiber components and morphology of softwoods has been established (Figure

2). Using this method, fiber morphology and chemical components of increment cores are easily and rapidly analyzed.

Results and Discussion

Removal of extractives. To analyze the main chemical components in our wood samples, the extractives had to be removed. Furthermore, as our goal was to survey an enormous number of wood samples, a simple extraction procedure needed to be developed. The conventional extraction procedure, using a Soxhlet apparatus, is extremely tedious and likely not necessary for juvenile wood. In addition, considering the hazards associated with the traditional extraction solvents used (ethanol and benzene), a process modification was warranted. For these reasons, we tried to develop an effective and convenient method for the removal of extractives from increment core wood samples.

The results obtained from repeated acetone extraction are shown in Table 1. A total of 1270 mg of nonvolatile extractives was obtained from a single increment core sample. The oven dried weight of the increment core sample after the extractions was 26.4 g, indicating that the yield of the nonvolatile

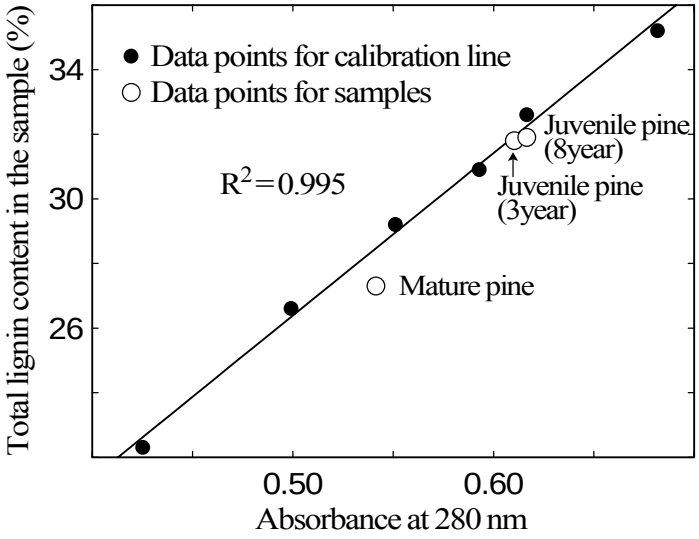

Figure 1. Calibration line used for the determination of lignin content in juvenile pine and data points for actual samples.

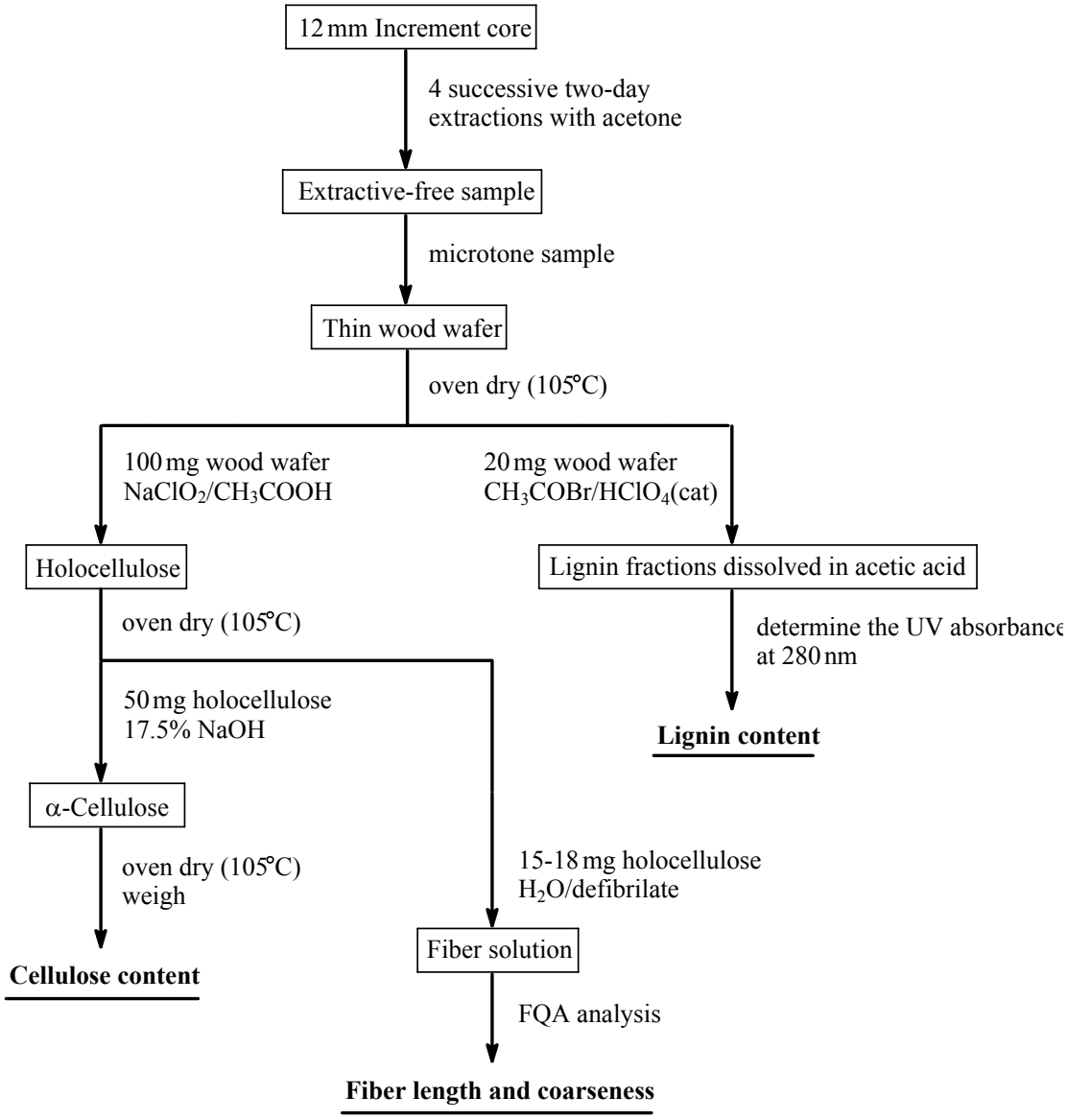

Figure 2. Flow chart of micro analytical method developed.

extractives based on extractive-containing wood was 4.7%. A significant observation was made that four successive two-day soakings with fresh acetone can remove over 95% of the non-volatile extractives in the increment core sample, and has been adopted as the new method. Moreover, this procedure can be initiated in the field as the increment core samples are being collected, greatly reducing the time required.

Table 1. Amount of non-volatile extractives obtained by successive two-day soakings with acetone.

No. of successive two-day extraction	Amount of non-volatile extractives (mg)
1	802
2	334
3	52
4	21
5	31
6	30
Total	1270

Table 2 illustrates the results obtained for both an acetone and an ethanol/benzene (1:2) extraction of two increment wood cores. Included, are the results from a conventional ethanol/benzene (1:2) Soxhlet extraction of a corresponding wood meal. No difference was observed in the amount of extractives removed using either the acetone extraction procedure or the conventional ethanol/benzene (1:2) Soxhlet procedure.

Table 2. Comparison of acetone extraction of 12 mm increment wood cores with conventional ethanol/benzene (1:2) extraction of wood meal.

	12 mm Increment wood core		**Wood Meal**
	Acetone Extraction (4x2days)	Ethanol/Benzene (96 hrs)	Ethanol/Benzene (24 hrs)
Sample 1	298 mg	305 mg	---
Sample 2	458 mg	---	390 mg

Preparation of holocellulose. To determine α-cellulose content, fiber length and fiber coarseness, holocellulose has to be prepared. Traditionally, the preparation of holocellulose requires 5 g of wood meal (*7*). As obtaining 5 g of wood from any ring in a 12 mm increment core is not possible, a micro technique had to be established.

We first ran the conventional holocellulose procedure using 100 mg (OD) of wood sample and the appropriate chemical additions; 3.2 mL of water; 37.5 mg of 80% sodium chlorite and; 10 µL of acetic acid. Analogous to the original method, the sodium chlorite and acetic acid were added 4 times at 60 minute intervals and the temperature was maintained at 70-80°C. The yield of holocellulose obtained was

82.2 ± 1.2%. These results indicate that the holocellulose contains a considerable amount of lignin (5-10% based on mature L. Pine), which could possibly affect the following α-cellulose content, fiber length and fiber coarseness analyses.

In the preparation of holocellulose yields of approximately 70-74% are expected (*7*). Therefore, we adjusted the reaction conditions of time, temperature and chemical addition to obtain holocellulose yields of 70-74%. Using the protocol outlined above, satisfactory holocellulose yields of 73.5 ± 0.3% and 71.0 ± 0.6% can be obtained using the multiple and single chemical application respectively. The holocellulose yield of the single addition microanalytical method is slightly lower than that of the multiple addition method and is likely not an accurate determination of holocellulose content. However it has the advantage for α-cellulose, fiber length, and fiber coarseness determinations, in that a large number of samples can be easily analyzed.

Determination of α-cellulose content. In the traditional method for the preparation of α-cellulose, holocellulose is kneaded in the early stage of the treatment with 17.5% sodium hydroxide solution. However, the omission of such a laborious step would greatly increase the ability to perform high-throughput analyses. Therefore, the effect of kneading on the yield of α-cellulose was examined, and the results are shown in Table 3. As can be seen, kneading has almost no effect on the yield of α-cellulose. Thus, this process can be eliminated from the protocol.

The α-cellulose content of the thin wood wafers of juvenile pine and the wood meal of the mature pine were 42.4 ± 0.4% and 46.2 ± 0.4%, respectively. These values are in good agreement with reported values for Loblolly pine (*4*).

Table 3. Effect of kneading in the early stage of the preparation of α-cellulose on its yield. Four samples of juvenile pine were used.

Sample no.	Yield of α-cellulose based on holocellulose	
	with kneading	without kneading
1	63.2	62.9
2	63.5	64.4
3	64.7	63.9
4	64.3	63.4
average	63.9 ± 0.7	63.7 ± 0.6

Determination of fiber length and coarseness. Fiber length and coarseness are very important factors impacting pulp production cost and quality. Results of FQA and Kajaani FS-200 analysis of the holocellulose isolated from the various wood preparations outlined above are shown in Figure 3 and Table 4.

An important factor in the determination of fiber length and coarseness is the particle size of the sample being measured, which is dependent on the techniques used for its isolation. Therefore, we conducted experiments to find the minimum particle size in which fiber length is not adversely affected. The holocellulose samples prepared from the mature pine wood meal were fractionated by particle size into 6 fractions: not wiley-milled; wiley-milled -10+20 mesh (pass 10- and retained by 20-mesh screen); -20+35 mesh; -35+60 mesh; -60 mesh; and unfractionated wiley-milled. Holocellulose samples prepared from the thin wafers of the juvenile pine from the 3 and 8-year old rings were also used. In addition, the not wiley-milled mature and juvenile pine wafers were divided into spring and summerwood before preparing holocellulose. Figure 3 shows the average fiber length and coarseness values obtained from FQA and Kajaani FS-200 analyses of the various wood preparations.

Table 4. Weight weighted average of fiber length and fiber coarseness of mature and juvenile pines observed by FQA and Kajaani FS-200. Every cell shows the average of three time analyses and the standard deviation.

Sample	Fiber length (mm)		Fiber Coarseness (mg/m)	
	FQA	Kajaani	FQA	Kajaani
Mature pine A	2.995 ± 0.082	2.75 ± 0.06	0.374 ± 0.013	0.654 ± 0.075
Mature pine B	2.882 ± 0.086	2.74 ± 0.10	0.455 ± 0.023	0.542 ± 0.001
Mature pine C	3.005 ± 0.191	2.88 ± 0.07	0.350 ± 0.015	0.488 ± 0.028
Juvenile pine 3-year spring	0.980 ± 0.033	1.03 ± 0.02	0.176 ± 0.002	0.235 ± 0.007
Juvenile pine 3-year summer	1.148 ± 0.043	1.21 ± 0.02	0.194 ± 0.001	0.256 ± 0.006
Juvenile pine 8-year spring	1.028 ± 0.010	1.08 ± 0.02	0.160 ± 0.009	0.218 ± 0.009
Juvenile pine 8-year summer	1.705 ± 0.044	1.78 ± 0.01	0.238 ± 0.006	0.319 ± 0.002

The results indicate that particle size of the sample significantly affects their fiber lengths. The larger the particle size becomes, the longer the fiber length. The fiber length of the fraction -10+20 mesh, the largest particle size examined, is even shorter than that of the not wiley-milled sample. FQA tends to give a little shorter fiber length and a clearly lower coarseness than Kajaani FS-200 for the exactly identical sample.

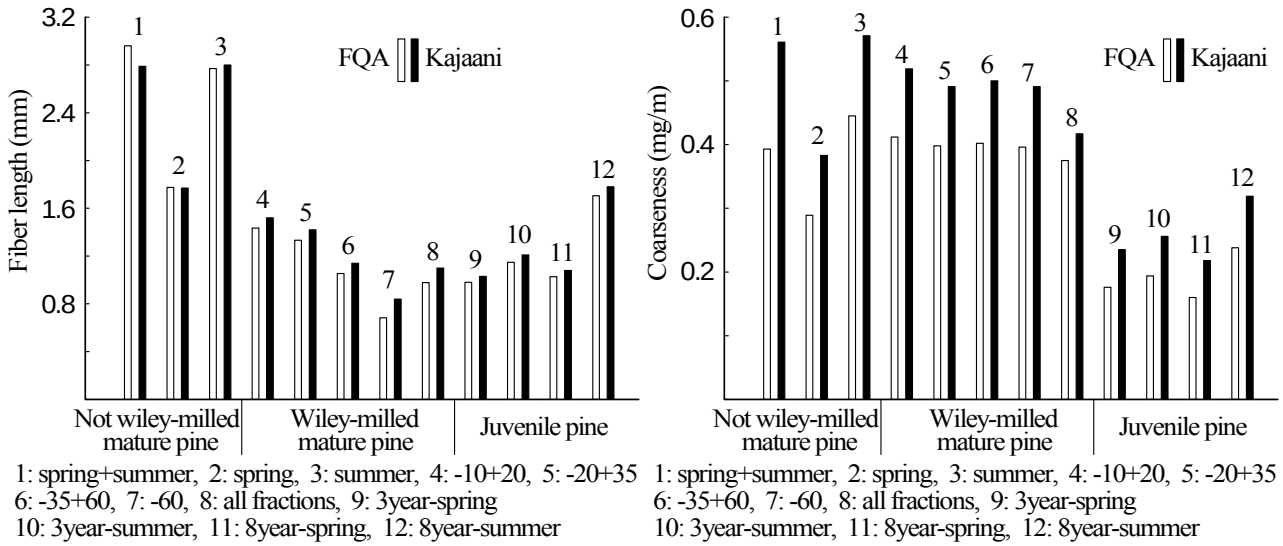

Fig. 3. Weight weighted average of fiber length and fiber coarseness of mature and juvenile pines observed by FQA and Kajaani FS-200.

Table 4 shows the values obtained for three mature pine wood meal samples (not Wiley milled) and juvenile pine thin wafer samples for spring and summer wood from years 3 and 8. There is little difference in the fiber length and coarseness between the spring and summer wood in the juvenile pine of the 3-year old ring. However in the mature pine and the juvenile pine of the 8-year old ring (the transition from juvenile to mature) the fiber length and coarseness values of the summer wood are larger than those observed for the spring wood. Finally, the mature pine wood has a much higher fiber length and coarseness than the juvenile pine wood.

Although similar results are obtainable from both FQA and Kajaani FS-200 analysis, FQA is being adopted as the instrument of choice because of the lower amount of fiber required per analysis (0.5-0.8 mg for FQA versus 3-4 mg for Kajaani FS-200).

Determination of lignin content. A microanalytical method for the determination of lignin content was developed based on the modified acetyl bromide method by Iiyama and Wallis (*8*). Again, a method modification was made to make it possible to analyze a large number of samples more easily. Although the modified acetyl bromide method for the determination of lignin content is known to be precise, our modification dictated that we reconfirm its accuracy. In the microanalytical technique developed, the analysis of lignin content required a calibration line which was obtained by subjecting various mixtures of known amounts of holocellulose and MWL (both from the mature pine) to our microanalytical acetyl bromide method. Therefore, the gram extinction coefficient of lignin treated by acetyl bromide was not used.

Figure1 shows the calibration line and data points of the juvenile and mature pines examined. The total lignin content in the three samples was determined by the Klason method: 31.8% for the 3-year old ring of juvenile pine (KL 30.6%, ASL 1.2%); 31.9% for the 8-year old ring of juvenile pine (KL 30.8%, ASL 1.1%); and 27.3% for mature pine (KL 27.0%, ASL 0.3%). Although the calibration line is made by analyzing the mixtures of holocellulose and MWL prepared from mature pine, the data points representing the three samples (mature wood; juvenile wood year 3, and juvenile wood year 8) correlate

well with the calibration line. Thus, the microanalytical acetyl bromide method developed here is suitable to make rapid analysis of lignin content in softwoods in a large number of samples.

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II. Rapid Prediction of Solid Wood Lignin Content Using Transmittance Near Infrared Spectroscopy

Summary

A rapid transmittance near infrared (NIR) spectroscopic method has been developed to characterize the chemical composition of solid wood. The NIR monochromator equipped with a InTact Single Tablet module was used in this study. Wood samples of varying lignin content were prepared by blending milled wood lignin and holocellulose. Using simple, multivariate, and partial least square statistical analysis, the lignin content of wood sample can be compared and quantified. Wood wafers taken from the increment cores were also used directly to develop the regression model. The developed NIR method can be used on small (less than 100 mg) solid samples. Strong correlations were obtained between the predicted NIR results and those obtained from traditional chemical methods. In addition to the experimental protocol and method development, NIR results from wood samples with different particle sizes and varying lignin content were discussed.

Introduction

Increasing global population is placing substantial pressures on the forest industry. The demand for forest products is increasing, however environmental concerns and loss of available land for forest plantations is making the ability to meet that demand more challenging. In order for the world forest industry to meet future demand, and for the U.S. forest industry to maintain global competitiveness, more wood with targeted characteristics will have to be produced more efficiently on less land. In addition, the majority of the softwood pulpwood supply in this country will come from intensively managed fast-growing plantation forests. This will result in a substantial increase in the proportion of juvenile wood entering the pulp mill, significantly impacting both production cost and product quality. Genetic improvement must focus not only on the quantity, but also on the quality of the raw material, and must develop raw materials uniquely suited for a particular process and product.

Variation in most wood properties within a species is under a relatively high degree of genetic control (*1*). Wood properties, which are related to product qualities include density, tracheid diameter and cell wall thickness, tracheid length, and chemical composition. Paper properties such as burst, tear strength and tensile strength are closely related to fiber morphology (*2*), while cost of processing in the mill and resultant profitability may be significantly affected by chemical properties and/or pulp yield (*3*). Despite the fact that wood quality is both very important and under a high degree of genetic control, there has been only limited emphasis on wood quality traits in most operational breeding programs due to high costs of sampling and assessment. Today, world and market conditions are making wood properties more important, and technological developments make the outlook for rapid, precise, and cost-effective assessment of wood properties quite positive.

Near-infrared spectroscopy (NIR) is a rapid, non-destructive technique. One general advantage of NIR spectroscopy over IR spectroscopy is that the NIR region is associated with higher energy transition. These have smaller absorptions so that longer pathlengths are possible. The penetration depth of IR radiation is about 10-100 micrometers, whereas for NIR radiation it is several millimeters (*4*). In contrast to the fundamental bands of IR spectra, the NIR spectra are mainly composed of overtones and combination bands. By incorporating the fibre optical technique and chemomertics, NIR has been used extensively both quantitatively and qualitatively in the food science and pharmaceutical industries (*5,6*). Within the forestry field, it has been used for applications such as foliar analysis for nitrogen and lignin (*7*), determining extractive content in wood of *Larix sp.* (*8*), determining chemical composition of *Eucalyptus globulus* and *E. nitens* (*9*), analyzing wood chemistry of sweetgum (*Liquidambar styraciflua*)

and loblolly pine (*10*), predicting the pulp properties (*11,12*), and wood identification (*13*). All these works were done by the reflectance mode near-infrared spectroscopy.

Generally, the reflectance measurements penetrate only 1-4 mm of the front surface of ground samples, this small penetration of energy into a sample brings greater variation when measuring nonhomogeneous samples, such as wood, than transmittance techniques, which the entire pathlength of sample is integrated into the spectral measurement. In transmittance, higher frequency energy is most commonly used due to its greater depth of penetration into samples (*14*). And less quantity of sample is required for transmittance mode NIR.

In this paper, we describe a new approach by using transmittance mode near-infrared spectroscopy, which is built on previous demonstrations of the potential of NIR (reflectance) for characterizing fiber or wood properties. The main focus of this paper is to develop a quick and micro-analytical wood lignin assessment by using transmittance mode NIR method, which can be further adapted by the tree-breeding program.

Materials and Methods

Materials. The wood meal samples used were the mature section (sapwood) of a 33-year old loblolly pine (*Pinus taeda*). The wood chips were ground by a Wiley Mill (standard model #3) into wood meals. The wood meals were further screened into fractions of different particle size using a stack of various mesh screens. Milled wood lignin (MWL) was prepared from the same batch of wood meals according to a previously published method (*15*).

Wood increment core samples were collected from 12-year-old loblolly pine received from the Tree Breeding Program, Department of Forestry, North Carolina State University. The extractives of wood cores were removed by acetone extraction as described in the previously research (*16*). The extractives-free wood cores were soaked in D.I. water for overnight and cut into thin wood wafers from the 3rd-year-ring springwood by the microtone. The wet-thickness was 200μm. The wood wafers were dried with P_2O_5 under vacuum overnight before NIR measurement. The NIR spectra of all dried wafers were taken before the lignin content determination.

Holocellulose preparation. Wood meal, 5 g oven-dried weight, was weighed into a 500 ml Erlenmeyer flask. 200 ml of 90°C D.I. water was then added, followed by 10 ml of acetic acid and 2.5 g of 80% (w/w) $NaClO_2$. The flask was kept at 90°C for 30 min. An additional 10 ml of acetic acid and 2.5 g of 80% (w/w) $NaClO_2$ were added in to flask at the end of 30 min. The cycle was repeated over the course of 2 hr. At the end of the 2 hr period, the flask was stoppered and the reaction was cooled with cold water to stop the reaction. The reaction mixture was then filtered using a course crucible and dried at 105°C until the crucible weight was constant, and the holocellulose yield calculated.

Lignin content determination. The lignin content was determined by Klason lignin method. Accordingly, 1 g (oven-dried) of wood meal was placed in a 100 ml beaker to which 15 ml of 72% H_2SO_4 was added. The mixture was left at room temperature for 2 hr with occasional stiring. The solution was then transferred to a 1 L Erlenmeyer flask, diluted with 560 ml of DI water to a H_2SO_4 concentration of 3%, and refluxed for 4 hr. The solution was then filter and the acid insoluble lignin determined gravimetrically. The filtrate was diluted to 1 L with DI water, and the acid soluble lignin was calculated from the UV absorbance reading at 205 nm. The extinction coefficient used was 110 AU*L/g*cm(*17*). The reduced amount of all experimental conditions were applied to wood wafer samples. The starting weight was 0.1 g (oven-dried). The pooled standard error for Klason lignin method (Lab error) of 7 duplicate samples is 0.30.

NIR sample preparation. Wood meal samples for NIR analysis were prepared using a pellet method. The wood meals or holocellulose sample (100 mg OD weight) were put in a stainless capsule and vibrated

in an amalgamator (Zenith, model Z-1A) for 1 min to produce a uniform particle size. The powder sample, 75 mg OD weight, was then pressed into a pellet, with a pellet diameter of 1.3 cm.

For holocellulose and MWL blended samples, the amalgamated samples were separately weight and mechanically mixed in different ratios using a mortar and pestle prior to being pressed into pellets for NIR analysis. The holocellulose/MWL ratio was varied between 100/0 to 60/40 in the samples.

For wood wafer samples, the dried wafers were placed on the sample holder and scanned directly. The wafer diameter is the same as the increment core diameter, 1.2 cm.

Near infrared spectroscopy. A Foss NIRSystems Near infrared spectrometer equipped with an InTact Single Tablet Module (NR-1650) and a monochromator (NR-6500-V/H) was used to analyze the various samples. The spectra obtained were absorbance spectra. 32 scans were applied to all samples with the spectrum interval of 600-1900 nm and the data collecting interval to be 2.0 nm

Calibration development and statistics. All regressions and predictions were performed by using VISION software (Version 2.51) from Foss NIRSystems. All statistics were also obtained from this software. All spectra were converted to the 2nd derivative mode with a segment of 10 nm and a gap of 0 nm prior to any calibration development. Multiple Correlation Coefficient (R^2) and the Standard Error of Calibration (SEC) were used to evaluate how well a calibration of simple or multiple linear regression fits the data. SEC is the standard deviation for the residuals due to the difference between the actual lab values and the fitted values of samples within the calibration set (*18,19*).

For the Partial Lest Squares (PLS) regression, the regression models were developed with four cross validation segments and a maximum of 16 factors. The best number of PLS factors for the model is determined by the PRESS (Prediction Residual Error Sum of Squares) value, which is the sum of all squared differences between lab and predicted values (*8*). A number of PLS factors that yields the lowest PRESS value is chosen to establish a model (*8,12*). Addition to R^2 and SEC, Standard Error of Cross Validation (SECV) is also used here to evaluate the PLS calibration performance. SECV can be an indication of how well an equation will predict samples which were not used to generate the calibration equation when there are not sufficient external validation samples (*20*). In cross validation, samples in the original calibration set are grouped into 4 subsets. One subset is withheld to simulate the external validation set while a calibration equation is created using the remaining samples. The resulting equation is used to predict the samples in the withheld subset. The standard error between the predicted values and the lab values is calculated. The first subset is returned to the calibration set and the process is repeated for each group. The standard errors are then combined to produce the SECV (*21,22*).

Results and Discussion

Chemical components in the NIR spectra. In order to apply the simple regression method, which is based on the Lambert-Beer law, to build the prediction calibration line, it is of great importance to distinguish each of the component peaks in the NIR spectra. To do this, the mature pine wood meal, holocellulose, mill wood lignin (MWL), and cellulose powder were pressed into pellets and analyzed by the transmittance NIR spectroscopy. The corresponding 2nd derivative NIR spectra are shown in **Figure 1**.

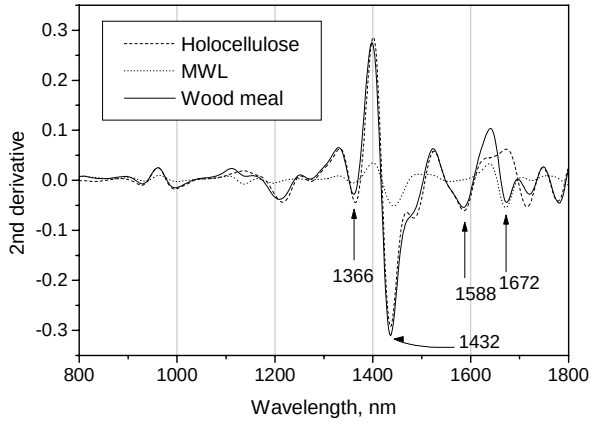

Figure 1. The 2nd derivative NIR spectra of wood chemical components.

The 2nd derivative spectra are the inverse of the original absorbance spectra, in which the peaks are project downward and better resolved than the original spectra. Comparing these three spectra with the pure cellulose spectrum (spectra not show), the peak at 1672 nm can be assigned to lignin, and the peaks at 1366, 1432, and 1588 nm to carbohydrates. These peak assignments are similar to the previous research done by diffuse reflectance NIR (*23*). Although the peak at 1432 nm has been assigned to both cellulosic OH and OH contributed by absorbed water (*23,24*), we still consider this peak as the carbohydrates peak due to our spectra were obtained by oven-dried samples.

Particle size effects. To assess the effect of particle size on the observed NIR spectra, we fractionated the wood meal obtained from the Wiley Mill. The results are shown in **Figure 2**. Contrary to the results reported in the literature wherein variations in particle size between 30 to 60 mesh did not have a significant effect on the NIR spectra using diffuse reflectance (*18*), we found that as the particle size changed from –10~+20 mesh to –60 mesh, the characteristics peak intensities increased.

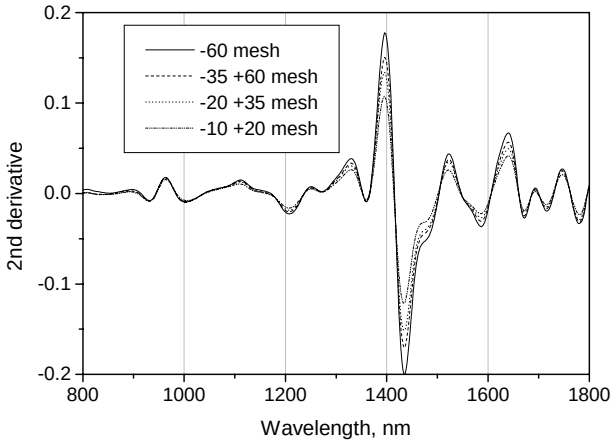

Figure 2. The 2nd derivative NIR spectra of the different mesh wood meals.

This means that using different particle size wood meals may affect the regression results, particularly if one single peak is to be utilized to develop a calibration line. The peak intensity of the finer particle size wood meal is stronger than that of the larger particle size wood meal. Erroneous results regarding lignin concentration or holocellulose content could result if individual peak heights are utilized to determine component concentrations. This can be eliminated by utilizing the ratio of the component peaks, rather than the individual peak data. For the same wood meal, which has the same lignin content, the peak ratio of the lignin peak intensity to the carbohydrate peak intensities is the same and is independent of particle size. This result is shown in **Figure 3**.

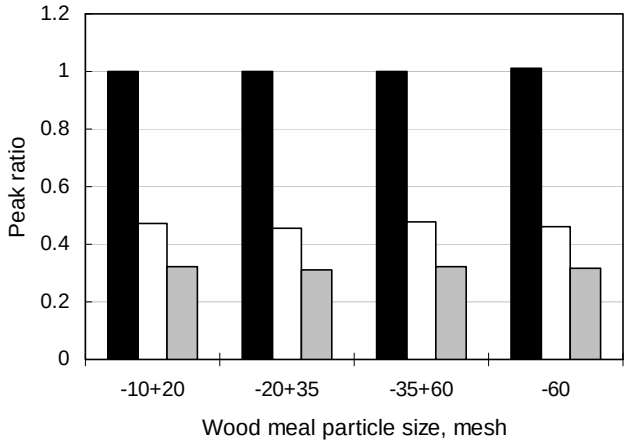

Figure 3. The relative peak ratio of different particle size wood meals. Black bar, 1672/1366; White bar, 1672/1432; Grey bar, 1672/(1366+1432).

Simple and multiple regression method. Different lignin content samples were made by blending holocellulose and MWL in different ratios. The 2nd derivative NIR spectra are shown in **Figure 4**. The lignin peak (1672 nm) intensity increases as the percentage of lignin increases. There are also linear relationships between some of the selected peak ratios for lignin and carbohydrates peaks (**Figure 5**).

They both show very good linearity, for example, the R^2 is 0.9964, and the SEC (Standard Error of Calibration) is 0.80, for 1672/1366 peak ratio to lignin %. These relationships can be used to predict the lignin content of the mature pine wood meals, which are the same wood sources as holocellulose and MWL used to build the models. The results are shown in **Figure 6**. The total lignin contents of the two wood samples determined by the Klason lignin methods are 27.2% and 27.7% respectively. The predicted values from the 1672/1366 regression line are around 30%, and from 1672/1432 regression line are around 29%.

To increase the prediction accuracy, multiple linear regression analysis was applied. This model combines the two simple regression terms used in the previous section. An $R^2 = 0.9986$ with SEC=0.56 was obtained. The prediction results are also shown in **Figure 6**. The predicted values are much closer to the laboratory values than the values obtained from the simple regression.

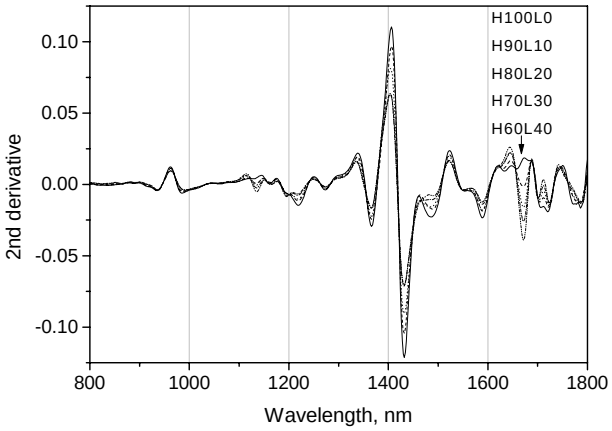

Figure 4. The 2nd derivative NIR spectra of different lignin content blending samples.

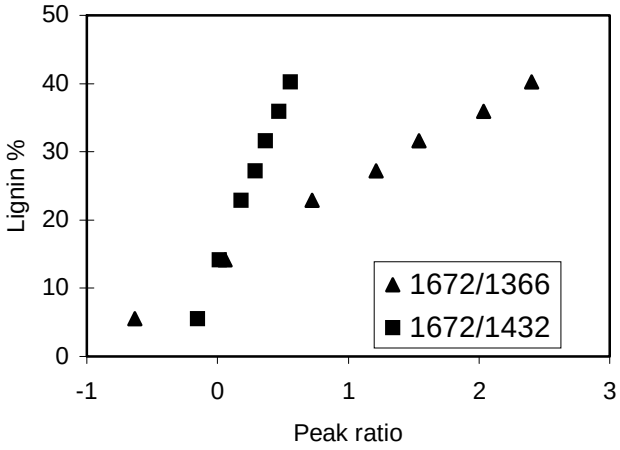

Figure 5. The linear relationships of some selected peak ratio. Triangle, y=11.3238x+13.4941 (R^2=0.9964, SEC=0.80); Square, y=48.738x+13.435 (R^2=0.9986, SEC=0.50).

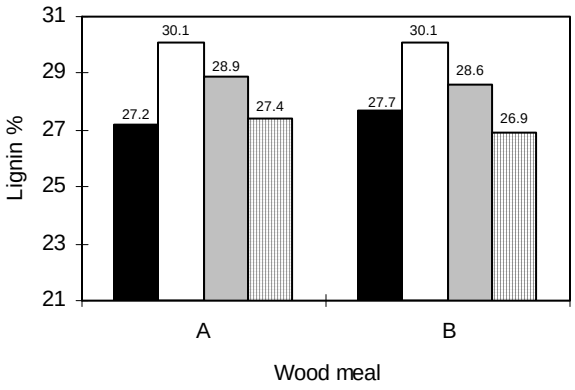

Figure 6. NIR predicted lignin content by simple and multiple regression method. Black bar, Lab data; White bar, 1672/1366; Grey bar, 1672/1432; Grid bar, 1672/1366+1672/1432.

Partial least square analysis. From our previous analysis, the prediction results depends on the peaks chosen and the regression methods used. For a complex matrix material like wood, the NIR spectra are composed of many overtones and combination bands. If we can utilize the entire spectra in the regression, in which each individual piece of spectral information can be taken into consideration, we can obtain a more powerful regression line. Hence, the partial least square (PLS) method was used to obtain a calibration line. Additional 30 standard samples were made, where the lignin content varied from 6.8% to 41.8%. The regression result can be explained by 5 PLS factors with the R^2=0.9948 and SEC=0.74. The SECV (Standard Error of Cross Validation) of this model is 1.05. The correlation between the standard lab values and the NIR fitted values of the calibration set is shown in **Figure 7**, the slope of this correlation is very close to 1. The lignin content prediction of the previous two wood samples by this regression method is shown in **Figure 8**. The lignin content predictions of both wood meals are very close to the lab values.

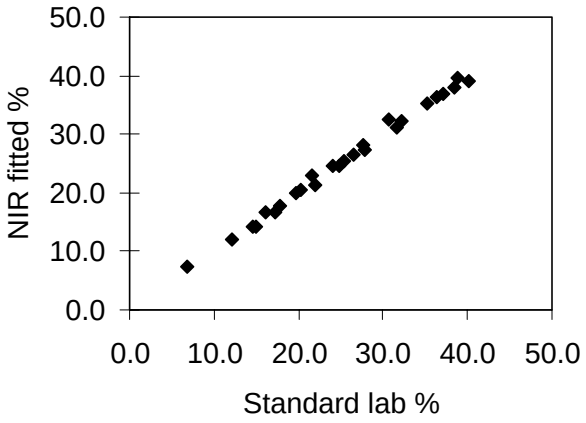

Figure 7. The correlation between the standard lab values and the fitted values of the calibration set from holocellulose and MWL blending samples. (y=0.9948x+0.1314, R^2=0.9948, SEC=0.74).

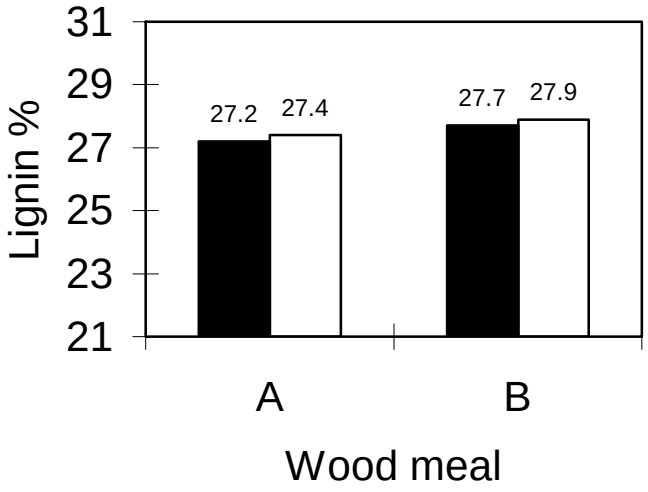

Figure 8. NIR predicted lignin content by PLS method. Black bar: Lab data; White bar: NIR prediction.

For the lignin content prediction of other wood samples, which are not the same sources for holocellulose and MWL, no matter the simple regression or the PLS model, the prediction values are not as good as the same source ones, and the prediction of juvenile wood samples were even worse. These means the calibration model is too specific, and only good for the prediction of the same sources of woods, which were used to make the holocellulose and MWL. The detail chemical compositions of other woods might be different from the woods used to build the models, and this might cause the prediction error. Beside the bad prediction of other wood samples, the preparation of blending samples is a tedious process. Those samples need to be grinded, mixed and pressed into pellets before any NIR scanning. So a new regression model based on wood wafers from the increment cores were further developed.

Increment core model. The increment core model was built by 32 different wood cores. 15 pieces of thin wood wafers were obtained from the 3rd ring springwood of each wood core. All the dried wafers were scanned directly by transmittance NIR, and each set of 15 spectra was averaged to represent the 3rd ring springwood NIR spectrum. The total lignin content of each set of 15 wafers was determined by Klason method. The total lignin contents of the 3rd ring springwood of these 32 wood cores range from 28.6% to 32.9%. Partial least square method was applied to this model, and the correlation between the lab lignin values and the NIR fitted lignin values of the increment core model is shown in **Figure 9**. The regression result can be explained by 6 PLS factors with R^2=0.8121 and SEC=0.47. The residual values (the difference between the predicted value and lab value) of all the calibration samples are within one-percentage differences. The SECV for this model is 0.73. The prediction results of some external validation samples are shown in **Figure 10.** The prediction values are very close to the lab values. This indicates that a good regression model could be obtained by using the transmittance mode NIR with wood wafers taken from increment cores. The grinding, screening, and other tedious preparation procedures of the wood meal regression models can be eliminated and replaced by taking the wood wafers and subsequently direct transmittance NIR measurement. This new procedure could reduce analysis time and chemical costs, and provide a quick lignin assessment for the tree-breeding project.

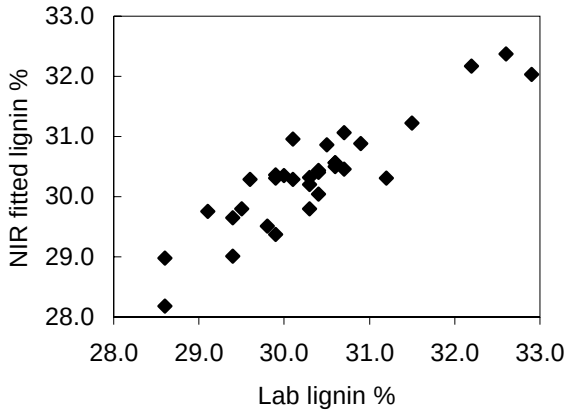

Figure 9. The correlation between the lab lignin values and the NIR fitted lignin values of the increment core model. (y=0.8121x+5.701, R^2=0.8121, SEC=0.47).

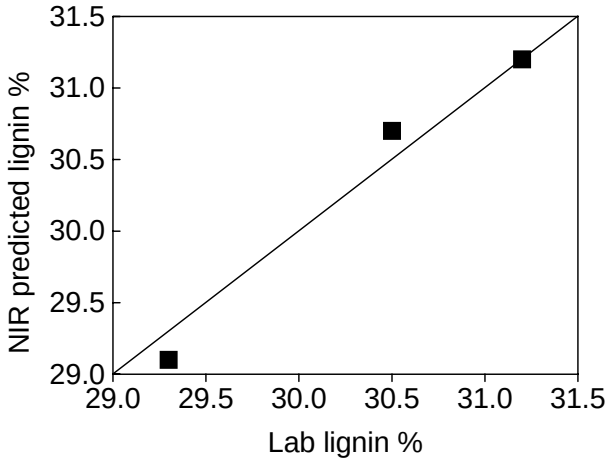

Figure 10. The prediction results of the external validation wood wafer samples by using the wood wafer calibration model.

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III. Rapid Screening of Wood Chemical Component Variations Using Transmittance Near Infrared Spectroscopy

Summary

A rapid method using transmittance near infrared (NIR) spectroscopy has been developed to predict the chemical composition variations of solid wood. Using stacked wood wafers obtained from increment cores, the minimum sample preparation, quantity (less than 100 mg), and NIR acquisition time were determined. Strong correlations were obtained between laboratory wet chemistry values and NIR fitted values. The calibration sets were used to predict the α-cellulose content and lignin content of the validation sets. It was demonstrated that transmittance NIR spectroscopy combined with the stacked wafer method could be used to rapidly screen the variations of wood chemical components when compared to single-wafer method reported before. In addition to the experimental protocol and method development, the improvements of the calibration error are also discussed.

Introduction

In order to ensure the global competitiveness of the Pulp and Paper Industry in the Southeastern U.S. more wood with targeted characteristics have to be produced more efficiently on less land. One viable solution to meet the future industrial wood demands is to greatly increase the productivity of the current pine plantations, leaving natural forests to be managed at low intensity, primarily for saw timber, conservation, aesthetics and recreational ends. To enable the efficient utilization of the fast growing plantation wood, tree breeders need to accurately and rapidly screen the large breeding population for a variety of phenotypic traits.

Wood properties related to product quality, include density, tracheid diameter and length, cell wall thickness and chemical composition. Paper properties such as burst, tear strength and tensile strength are closely related to fiber morphology (*1*), while processing costs and resultant profitability are more significantly affected by the chemical compositions (*2*), in particular α-cellulose and lignin content. Traditional wet chemistry methods for α-cellulose and lignin content are quite costly and very time-consuming (*3*).

Recently, we reported a rapid transmittance Near Infrared (NIR) spectroscopic method for the determination of lignin content in solid wood (*4*). Using simple, multiple regression, and partial least square statistical analysis the lignin content of wood wafers, taken from 12mm increment cores and synthetic wood, prepared by blending milled wood lignin and holocellulose were compared and quantified. Strong correlations were obtained between the predicted NIR results and those obtained from traditional chemical methods. Unfortunately, only the wood wafer increment core model satisfactorily predicted lignin values for samples not included in the model development. The development of a *universal calibration line* for the prediction of lignin content using *synthetic wood* was not feasible.

Recently, Sykes et al.(*5*) applied this method to predict fiber length, coarseness, α-cellulose and lignin content of loblolly pine. However, due to the large laboratory standard error of the lignin measurements, lignin content could not be adequately predicted using this model.

A limitation to this method is the fact that it utilizes averaging 15 single-wafer NIR spectra from each ring of an increment wood core. Although the scanning speed of modern NIR instruments is rapid, when considering screening the enormous amount of samples from a tree-breeding project, minimal sample preparation is crucial. In this paper the new method of stacking several wafers from the same year ring together and collecting a single NIR spectrum is proposed and compared to the averaged single-wafer model. We report the better correlation between lab and NIR measurements by this stacked wafer method for □-cellulose and lignin content and discuss potential sources of error.

Materials and Mehtods

Materials. Wood increment core samples were collected from thirteen 9-year-old loblolly pine (*Pinus taeda*) received from the Tree Breeding Program, Department of Forestry, North Carolina State University, and from thirty-seven 4-year-old aspen (*Populus trichocarpa*) received from Oak Ridge National Laboratory, Tennessee. The increment wood core extractives were removed by acetone extraction as described previously (*6*). The extractives-free increment wood cores were then soaked in deionized water overnight and microtoned into wood wafers (13mm in diameter and 200μm in thickness) (*4*). The wood wafers were dried with P_2O_5 under vacuum overnight before NIR measurements and subsequent chemical analysis.

Near infrared spectroscopy. A Foss NIRSystems Near infrared spectrometer equipped with an InTact Single Tablet Module (NR-1650) and a monochromator (NR-6500-V/H) was used to analyze the wood wafers. Absorbance spectra totaling 32 scans were collected at 2.0 nm intervals over the range of 600-1900 nm.

NIR sample preparation and measurement. The dried wood wafers were analyzed using a modified sample holder as reported previously (*4*). The NIR and wet chemistry measurement were based on ring-by-ring bases, especially in Pine. Due to the small amount of material available per ring of the increment cores for chemical analysis, the wood wafers were separated into springwood rings 2, 4, 6 and 8 for pine α-cellulose measurements, and springwood rings 3, 5, and 7 for pine lignin measurements, and for aspen springwood ring 3 for aspen lignin determination. The same pile of wood wafers obtained from the same year ring of the wood core was considered one individual sample. The total sample population numbers are 55 for pine α-cellulose measurement, 59 for pine lignin measurement, and 62 for aspen lignin measurement. Depending on the availability of wood wafers in a typical experiment, 10 pine wafers (corresponding to about 80mg of wood) or 14 aspen wafers (corresponding to about 100mg of wood) were stacked together and placed on the NIR sample holder, and directly scanned. The NIR spectra of all dried wafers were taken before any wet chemistry determination.

For the averaged single-wafer model, 10 or 14 wood wafers were scanned individually by NIR spectrometer, and the average of these 10 or 14 spectra was used to represent the sample spectrum. Whereas in the stacked-wafer model, 10 or 14 wafers were stacked together, only one NIR spectrum was taken and used to represent the sample spectrum. During the regression model developments, both the averaged single-wafer spectrum and the stacked-wafer spectrum from the same pile of wood wafers are corresponding to the same reference data obtained from the wet chemistry analyses.

Holocellulose preparation. The isolation of holocellulose was carried out according to the protocol of Yokoyama et al. (*6*) utilizing a total of 10 wood wafers per analysis. Specifically, 100mg of wood wafers (OD) were suspended in 4mL of deionized water at 90°C, and reacted with 200mg of 80% sodium chlorite and 0.8ml of acetic acid for 1 hour. The reaction mixture was then filtered using a course crucible, washed, and dried at 105°C until the crucible weight was constant, and the holocellulose yield was calculated. The chemicals used were reduced proportionally according to the exact weight of 10 wafers.

α-cellulose preparation. □-cellulose was prepared as per the protocol of Yokoyama et al. (*6*) wherein 50mg of the holocellulose, obtained from previous section, was reacted with 4ml of 17.5% sodium hydroxide for 30 min, then diluted with 4ml of deionized water and the reaction mixture was left for 30 min. After a total reaction time of 1 hour, the fiber suspension was filtered with a coarse crucible, washed thoroughly with deionized water, and soaked in 1.0M acetic acid for 5 min. This neutralized α-cellulose was then washed with deionized water. The yield was calculated after drying in 105°C until the crucible weight was constant.

Lignin content determination. The lignin content was determined by Klason lignin method. Traditionally, 1 g (oven-dried) of wood meal was reacted with 15 ml of 72% H_2SO_4. The mixture was left at room temperature for 2 hr with occasional stirring. The solution was then diluted with 560 ml of DI water to a 3% H_2SO_4, and refluxed for 4 hr. The solution was then filter and the acid insoluble lignin determined gravimetrically. The filtrate was diluted to 1 L with DI water, and the acid soluble lignin was calculated from the UV absorbance reading at 205 nm. The extinction coefficient used was 110 AU*L/g*cm (*7*). The acid insoluble and acid soluble lignin were combined to get the total lignin content.

In order to facilitate the small wafer weights and to speed up the large numbers of measurements, the 1/10 reduced amount of all experimental conditions were applied to wood wafer samples, and the 4-hour reflux was changed to 121°C, 2 atm in a commercial available pressure cooker for 1 hour. Our preliminary results showed no statistically significance difference in the total lignin content between the 4-hour reflux and 1-hour cook, and the total reaction time could be reduced by half.

Calibration development and statistics. The calibration models are developed using the Vision software (Version 2.51, Foss NIRSystems). The Vision software starts with the sample selection by finding the outlier samples. Outlier samples are identified using a Mahalanobis distance algorithm that measures how far a sample is from the center of a cluster of spectra. A sample is considered to be an outlier when its probability level exceeds the 0.95 threshold value (*8*). The remaining samples were further split into 75% for the calibration set and 25% for the prediction set using an algorithm that measures a Euclidean distance between samples. The Vision software calculates the Euclidean distance around a sample, and identifies which samples are closer than the threshold value. Such redundant samples are moved into the prediction set (*8*). A statistical summary of the calibration and prediction sets is given in **Table 1**.

Table 1. Summary statistics for α-celluloses and total lignin contents for the calibration and prediction sets.

Chemical compositions (%*)	Calibration set					Prediction set				
	n	Min	Max	Avg	Std[$]	n	Min	Max	Avg	Std
Pine α-cellulose										
Stacked-wafer model	38	35.6	47.3	42.4	2.4	12	38.2	46.4	42.0	2.5
Singel-wafer model	38	35.6	47.3	42.4	2.4	12	38.2	43.8	41.0	2.0
Pine Total lignin	39	28.0	32.0	30.0	0.9	14	28.5	32.0	29.8	0.9
Aspen Total lignin	39	20.9	28.6	25.3	2.3	14	21.4	27.1	24.6	1.9

*: Based on extractive-free, OD wood weight.
[$]: Standard deviation.

The original spectra were converted to the 2nd derivative spectra with a segment of 10 nm and a gap of 0 nm prior to any calibration development. The calibrations were developed using a Partial Lest Squares (PLS) regression with four cross validation segments and a maximum of 16 factors. The best number of PLS factors for the model was determined by the PRESS (Prediction Residual Error Sum of Squares) value, which is the sum of all squared differences between the lab and predicted values (*9*). The PLS factors that yield the lowest PRESS values are then chosen to establish a model (*9,10*).

The coefficient of determination (R^2), the standard error of calibration (SEC), and the standard error of cross validation (SECV) were used to evaluate the calibration performance. SEC is the standard deviation for the residuals due to the difference between the actual lab values and the fitted values of samples within the calibration set (*11,12*). SECV can be an indication of how well an equation will predict samples which were not used to generate the calibration equation in cross validation (*8,13,14*).

The standard error of prediction (SEP) was used to evaluate how well the calibration predicts the interested constituent value for a set of unknown samples that are different from the calibration set (*15*). The predictive ability of calibration was evaluated by the ratio of performance to deviation (RPD), which is calculated from the ratio of standard deviation of the reference data of prediction data set to the SEP (*16*). The RPD should be as high as possible. A value of greater than 10 is excellent and equivalent as reference method, and values of 5-10 are adequate for quality control. An RPD value of 2.5 or greater is satisfactory for screening for breeding programs (*11,16*), but research has showed that with an RPD of approximately 1.5, NIR spectroscopy can be used as an initial screening tool (*15,17,18*).

Results and Discussion

Stacked-wafer model versus averaged single-wafer model. Our previous model (*4*) was established by averaging 15 single-wafer NIR spectra from a single growth ring of an increment wood core. As we all know that wood is not a homogeneous material, the large number of samples analyzed per ring were used to fully represent the entire year of growth, while providing enough material for the following wet chemistry measurements. In an attempt to minimize data collection time, instead of scanning the wafers one by one, 10 wafers from the same year ring were stacked together and one NIR spectrum was taken. The 2nd derivative NIR spectra of an averaged 10-single wafer and 10-stacked wafers are compared in Figure 1. The intensity of the 2nd derivative NIR spectrum from the 10-stacked wafers is far more intense than that from the averaged 10-single wood wafer. In addition to the improved signal-to-noise, which will help reduce the calibration error (*19*), the intense NIR absorption bands of the stacked spectra will enhance regression development.

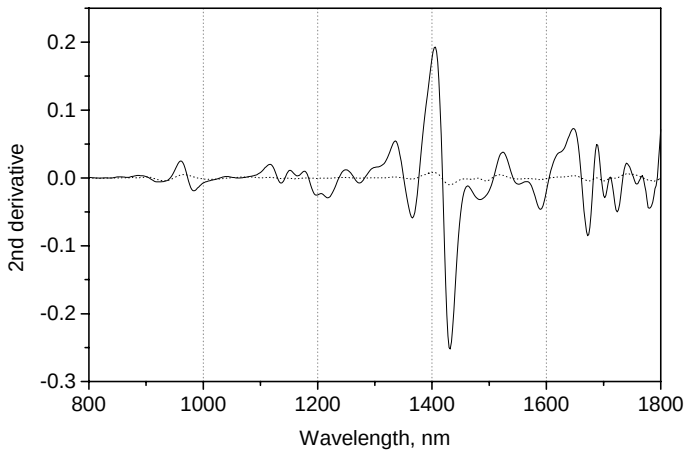

Figure 1. **The 2nd derivative NIR spectra of (___) 10-stacked wafers, and (.....) averaged 10-single wafer**

Pine wood wafers of α-cellulose data set were further used to develop two calibration models, the stacked-wafer model and the averaged single-wafer model. Figure 2 shows the calibration results of the α-cellulose content of loblolly pine for the stacked-wafer model (Figure 2a) and the averaged single-wafer model (Figure 2b) respectively. A stronger correlation is obtained for the stacked-wafer model (R^2=0.82) than the averaged single-wafer model (R^2=0.80). The SEC is 1.05 for stacked-wafer model and 1.17 for single-wafer model. This means that the regression models a better fit in the stacked-wafer model than the averaged single-wafer model. The SECV, which is a better measurement of calibration error (*18*), shows the same trends between both models, i.e. smaller calibration error in the stacked-wafer model (SECV = 1.21) than in the single-wafer model (SECV = 1.87). The considerably larger SECV than SEC in the averaged single-wafer model could possibly be due to over fitting (*18*). This might arise from the interference noises being modeled during the calibration development of the averaged single-wafer model and reduces the SEC. The results from these two models show that using stacked wafers a reduction in the calibration error may be achieved, likely the result of the higher signal to noise ratio of the spectra, as showed in Figure 1.

Prediction of α-cellulose content of loblolly pine. The α-cellulose content calibrations were tested on the prediction sets from another set of loblolly pine wood wafer samples (12 spectra). The relationship between the lab measured α-cellulose content and the NIR predicted α-cellulose content is quite good using the stacked-wafer model (R^2=0.75). As shown in Figure 3a, the SEP is 1.42, which is slightly higher than the SECV (1.21). The RPD is 1.77 indicating that the stacked-wafer model could be used as a screening tool for estimating the α-cellulose content of increment core samples. Figure 3b shows that the correlation between the measured α-cellulose values and the NIR predicted values for the averaged single-wafer model (R^2=0.65) is not as good as for the stacked-wafer model. Furthermore, the SEP is closer to the SEC than SECV, and the RPD (1.54) is lower than that of the stacked-wafer model. Thus, the predictive ability of the single-wafer model is weaker than the stacked-wafer model, however they both fulfill the initial screening criterion (RPD = ~1.5).

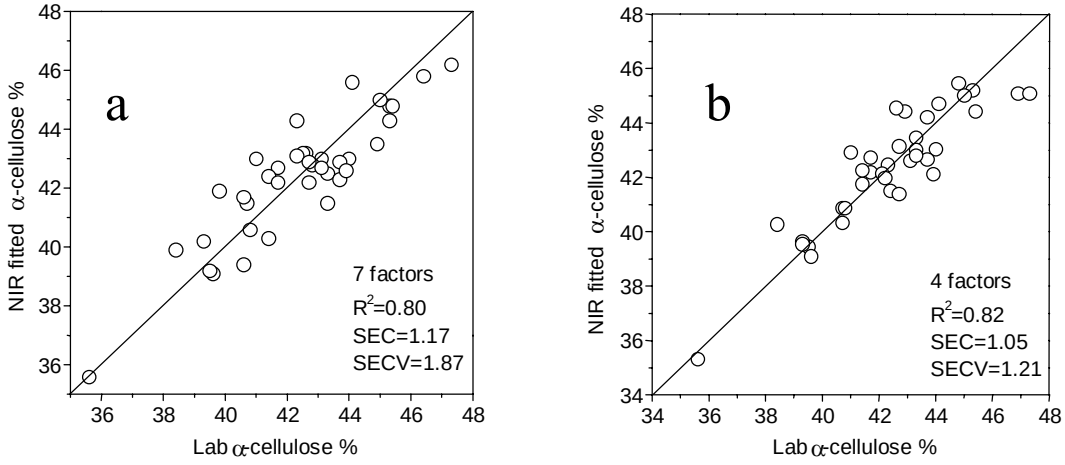

Figure 2. The correlation between the lab α-cellulose content and the NIR fitted α-cellulose content of the (**a**) stacked-wafer model, and (**b**) averaged single-wafer model

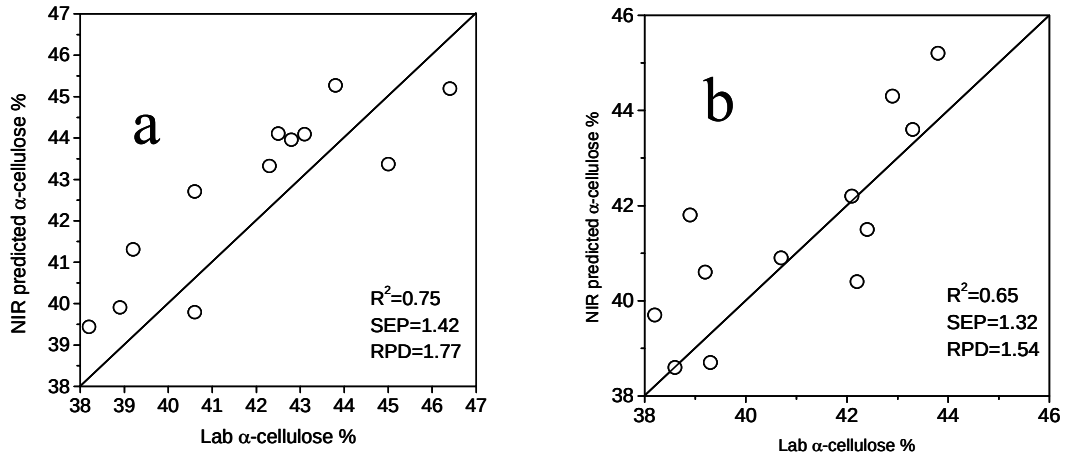

Figure 3. The correlation between lab measured α-cellulose content and the NIR predicted α-cellulose content using (**a**) stacked-wafer model, and (**b**) averaged single-wafer model.

PLS calibrations based on lignin content. The stacked wafer method was also applied to develop two lignin content calibration models based on loblolly pine and aspen, the two common wood species for pulp and paper industries. The calibration results are shown in Figure 4. The correlation of the pine lab lignin values and the NIR fitted lignin values is quite good (R^2=0.73, Figure 4a). The SEC and SECV are 0.47 and 0.58, respectively. The considerable lower SEC and SECV values here compared to pine α-cellulose models (Figure 2a) might be explained by the lower laboratory error for the reference methods, as 0.55 for lignin measurement and 1.05 for α-cellulose determination). Interestingly, the correlation obtained for the aspen data set was very strong (R^2=0.95, Figure 4b), with the SEC and SECV being 0.56 and 0.66, respectively. The better calibration performance (R^2=0.95) of the aspen data set compared to pine data set (R^2=0.73) might be due to the evenly distributed constituent range. The more uniformly distributed and broader range of lignin concentration of the aspen, 21-29% lignin versus the pine, 28-32% lignin could increase the calibration performance (*11*,*19*). The pine samples used in this research are juvenile wood cores, and the natural lignin content variations of juvenile loblolly pine wood normally range from 28~32%(*20*). That means the natural variation of aspen lignin is larger than that of pine lignin. Further efforts need to be done to collect more extreme lignin contents of pine wood cores, and hence the calibration performance might be improved.

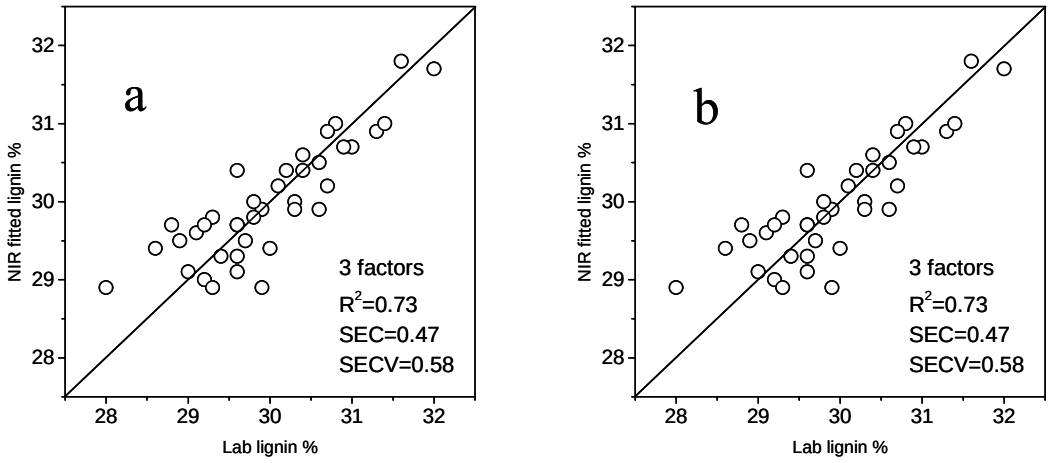

Figure 4. The correlation between the lab measured total lignin content and the NIR fitted lignin content for (**a**) loblolly pine, and (**b**) aspen.

Prediction of wood lignin content. Both lignin content calibrations were tested on prediction sets utilizing additional loblolly pine and aspen samples (14 spectra). The results are shown in Figure 5. The correlation (R^2=0.52, Figure 5a) for pine lignin prediction was considerably lower than that from the calibration set (R^2=0.73). But the RPD was 1.49, thus, the calibration model can still be used for initial screening. The relationship between the lab lignin value and the NIR predicted lignin value of the aspen data set is very stronger (R^2=0.89, Figure 5b), and the RPD is 2.58. This high RPD value indicates this calibration model can be used successfully for screening the lignin variation in aspen.

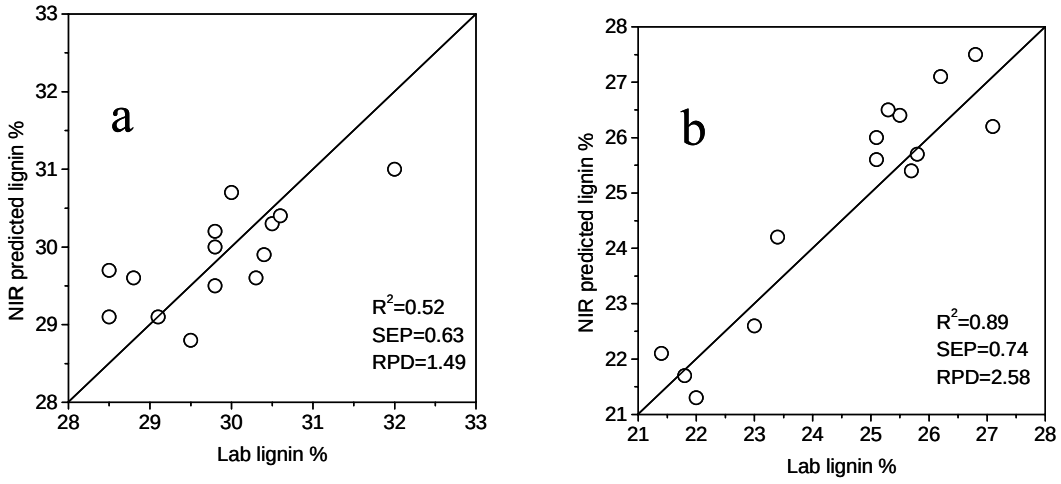

Figure 5. The correlation between lab measured total lignin content and the NIR predicted lignin content for (**a**) loblolly pine, and (**b**) aspen.

Improving the sources of error. The work presented in this study demonstrates that it is feasible to develop a good calibration model using stacked wood wafers. The calibration error can be reduced by some technical improvements, such as increasing the signal intensities, increase the accuracy of the reference method, or broadening the variability of the calibration set, but generally the greatest source of error in any calibration is from the error associated with the reference laboratory data (*11*,*19*).

Generally, the wet chemistry methods involved in the determination of α-cellulose or lignin content rely on first breaking down the wood into fine wood meals. This provides a more uniform material and increases the accessibility of the chemicals during the respective reactions. However, to facilitate rapid screening, minimal processing of the wood is required. Further, to reduce introducing unnecessary variation between the NIR and laboratory measurements, which would cause a negative influence on the calibration model (*18*), our method involves performing the wet chemistry analysis directly on the stacked wafers used in the NIR analyses. Therefore, complete dispersion and mixing of chemicals throughout the sample is crucial, particularly for klason lignin measurements (*21*) where care needs to be taken to thoroughly knead and stir the sample mixtures.

In α-cellulose determination the system is more susceptible to error due to the two-step reaction procedure utilized. First, holocellulose must be isolated from the wood wafers by acetic acid and sodium chloride. As with the lignin analysis care must be taken to ensure sufficient mixing and introduction of reagent chemicals. The resulting holocellulose is then further reacted with $NaOH_{(aq)}$ to produce α-cellulose. These processes involve very frequent weighting, kneading, and stirring. In each step, care must be taken to minimize the possible sources of error.

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IV. Prediction of Wood Properties of Loblolly Pine using Transmittance Near Infrared Spectroscopy

Abstract

Near infrared (NIR) spectroscopy is a rapid non-destructive technique that has been used to characterize chemical and physical properties of a wide range of materials. In this study, transmittance NIR spectra from thin wood wafers cut from increment cores were used to develop calibration models for the estimation of α-cellulose content, fiber length, coarseness, and lignin content measured in the laboratory. Eleven-year-old trees from two sites were sampled using 12 mm increment cores. Earlywood and latewood of ring-3 and ring-8 from these samples were analyzed in the laboratory using micro analytical methods for cellulose content, fiber length, coarseness, and lignin. Calibrations of NIR and laboratory measurements based on one site were generally reliable, with coefficients of determination (R^2) ranging from 0.54 to 0.88 for fiber length and α-cellulose content, respectively. Predicting ring-8 properties using ring-3 calibration equations showed potential for α-cellulose content and coarseness with R^2 values of approximately 0.60, indicating the potential for early selection. Predicting the wood properties using the calibration equations from one site to predict another showed moderate success for α-cellulose content ($R^2 = 0.64$) and coarseness ($R^2 = 0.63$), but predictions for fiber length were relatively poor ($R^2 = 0.43$). Lignin content prediction using transmittance NIR was not as reliable in this study, which was partially due to low variation in lignin content in these wood samples and to large lignin measurement errors in the laboratory.

Introduction

Improvement for growth, stem straightness, disease resistance, and specific gravity have been the primary focus of tree breeding due to their economic importance and ease of measurement (Zobel and Talbert 1984). Large genetic gains have been documented for loblolly pine (*Pinus taeda* L.) for growth, stem form, and rust infection from two cycles of breeding (Li et al. 1999), but relatively little work has been done on the improvement of wood properties in this species, except specific gravity. Chemical and morphological wood properties, including α-cellulose content, fiber length, and fiber coarseness can have major impacts on pulp and paper products (Kerekes and Schell 1995; Kube and Raymond 2002; Zobel and Talbert 1984). The α-cellulose content is highly correlated with pulp yield (Kube and Raymond 2002). Increasing the cellulose content in wood would reduce pulping costs and increase the efficiency of the pulp and paper mills (Zobel and Talbert 1984). Fiber length also plays an important role in the pulp and paper industry. Long fibers provide paper with greater tensile and tear strength for products such as cardboard and paper bags (Myers 2001). Short fibers are preferred for products such as fine printing paper where surface smoothness and ink bleeding is important. Coarseness is the total mass of a sample of fibers divided by the total length of all fibers. Coarser fibers tend to have large lumens and, therefore, it is difficult to separate the effects of lumen size and wall thickness (Kerekes and Schell 1995). Lower coarseness results in easier fiber collapse causing better bonding of fibers and the formation of dense paper with a smooth surface. Wood with higher coarseness values yield pulp and paper products with higher bulk, which is beneficial to products requiring higher absorbance and/or higher bending stiffness.

One of the limitations in genetic improvement of wood is the difficulty in measuring these traits on many trees and progeny trials. To obtain an accurate assessment of a breeding population, analyses of many trees are required in progeny tests. Traditional laboratory methods for the measurement of chemical properties of wood are expensive and time-consuming. Recently, a microanalytical technique has been developed to screen the chemical properties of progeny test trees using a small sample of wood (Yokoyama et al. 2002). Using this method, genetic variation for a number of properties was identified in

a large, loblolly pine progeny test (Sykes et al. 2003). Although this analysis is more rapid than previous chemical analyses, it is still costly and time consuming, particularly for a large progeny test. Therefore, a more rapid and inexpensive screening technique is needed to incorporate wood properties into breeding programs.

Near infrared (NIR) spectroscopy is a rapid, non-destructive technique that has been used to characterize diverse substances, such as pharmaceuticals and wood. NIR spectroscopy has successfully been used to characterize wood and fiber properties (Kelly et al. 2004, Michell 1995; Raymond et al. 2001a; Schimleck and Evans 2003; Wright et al. 1990). Most NIR studies used reflectance NIR spectroscopy to predict wood properties of wood meal or solid wood (Jones et al. 2005; Raymond et al. 2001b), but reflectance measurements suffer from several limitations, the most serious being the small penetration depth (1-4 mm) into the sample. Thus, when using reflectance measurements the uniformity of particle size becomes very important (Schimleck et al. 2001; Schimleck and Evans 2002; Schimleck et al. 2003). The milled wood must be screened to remove the larger particles to ensure the uniformity of the sample. For non-homogeneous samples such as wood, this limited penetration can result in variable results and a strong dependence on sample size and preparation technique. Furthermore, because a large amount of wood meal is typically used to represent the sample of interest, it is not possible for independent measurements of individual rings for earlywood and latewood.

In contrast, transmittance techniques can fully penetrate the sample (depending on thickness), are less sensitive to particle size effects, and permit analysis of smaller wood samples. Yeh et al. (2004) recently reported the use of transmittance NIR spectroscopy to study lignin content in earlywood and latewood from 12-mm increment wood cores. In this paper, we examined the use of transmittance NIR spectroscopy to measure α-cellulose content, average fiber length, coarseness, and lignin content of juvenile wood and transition wood in loblolly pine. Using 12-mm increment cores of 11-year-old loblolly pine trees, earlywood and latewood of juvenile and transition rings were evaluated by transmittance NIR spectroscopy, and their relationships with laboratory data were tested to determine the usefulness of this technique for predicting wood properties in breeding programs.

Materials and Methods

Wood Sample Collection

Wood samples of loblolly pine trees from field-grown progeny tests were collected and processed as described **previously** (Sykes et al. 2003). Briefly, fourteen full-sib families generated by a six-parent half-diallel mating design were tested in the Piedmont of South Carolina. A randomized, complete-block design with six replications was used in the field. Each full-sib family was laid out in six-tree row plots in each replication. Wood core samples from 11-year-old trees were collected from two sites in South Carolina. Increment cores (12 mm) were taken from each tree at breast height (approximately 1.30 m) using generator-powered drills. Wood cores having visible limbs, curves, resin pockets, compression wood, or rust infections were avoided. The samples were placed into plastic storage bags, labeled, and placed in coolers to retain moisture during the material collection and transportation. In this study, 40 trees from two full-sib families were sampled on site A, and another 30 trees from three full-sib families were sampled on site B. The two sites were within 15 miles of each other in South Carolina, USA. Site A was located in a river bottom with faster growth and greater uniformity than site B, which was established in the upland Piedmont.

Measurements of Wood Properties

The bark and cambium were removed from the wood cores and the cores were split at the pith into two halves. Thin wafers (200 μm) were cut with a microtome from ring-3 earlywood (3E), ring-3 latewood (3L), ring-8 earlywood (8E), and ring-8 latewood (8L) from each wood core. These rings were

chosen to represent the juvenile (ring-3) and transition (ring-8) sections of the tree (Sykes et al. 2003). With 70 increment cores and 4 ring segments per core, there were approximately 280 samples. Each ring sample consisted of wood wafers that weighed at least 300 mg. Sixteen wood wafers were randomly taken from each ring and wood type, measured using NIR, and then saved for cellulose determination. The wafers were pressed between glass microscope slides under small weights to ensure flatness during the drying process. Wafers with noticeable thickness variation were discarded. Prior to NIR analysis, each sample was placed in a vacuum desiccator with P_2O_5 for two days to remove moisture.

Wood wafers were analyzed using a NIR spectrometer (Foss Model 6500). A single wafer was scanned to measure the amount of NIR light (600-1900 nm) that is transmitted through the sample. To ensure uniformity, fiber orientation was kept constant for all samples, and samples were analyzed in a dry condition. The 16 spectra for each ring and wood type were averaged to create one spectrum, which was used for the calibration equations with α-cellulose content, average fiber length, coarseness, and lignin content estimated from the laboratory (Sykes et al. 2003).

These wood wafers were used for laboratory chemical analysis. Using a microanalytical technique developed by Yokoyama et al. (2002), several wood properties including lignin content, α-cellulose content, average fiber length, and coarseness were determined for each ring (ring-3 and ring-8) and wood types earlywood and latewood. Descriptive statistics for the wood properties were calculated and reported in Table 1. Laboratory standard errors were calculated by repeating measurements of α-cellulose content, average fiber length, and coarseness three times and lignin content two times for approximately 50 samples.

Table 1. Descriptive statistics of wood properties obtained from wet-chemistry data for in site A, site B, and both sites combined.

		Wood Property			
Dataset	Parameter	ACY (%)	COA (mg/mm)	FLW (mm)	LIG (%)
Among wood samples					
Combined n=240	Minimum	35.6	0.223	0.491	24.1
	Maximum	55.2	0.699	3.04	32.63
	Mean	44.7	0.369	1.598	29.24
	Standard deviation	4.3	0.101	0.531	1.44
Site A n=128	Minimum	35.6	0.223	0.85	24.1
	Maximum	54.3	0.547	3.04	32.63
	Mean	43.3	0.337	1.751	29.09
	Standard deviation	4.5	0.071	0.494	1.45
Site B n=112	Minimum	36.6	0.242	0.491	26.5
	Maximum	55.2	0.699	2.66	32.37
	Mean	46.3	0.406	1.423	29.41
	Standard deviation	3.6	0.116	0.521	1.42
Among repeated measures of the same wood samples					
Laboratory Standard Error	SE	1.805	0.011	0.034	0.638

Note: ACY is α-cellulose content, COA is coarseness, FLW is average fiber length, and LIG is lignin content. Laboratory standard errors were calculated by repeating measurements of α-cellulose content, average fiber length, and coarseness three times and lignin content two times for approximately 50 samples. Sites were significantly different for ACY, COA, and FLW, but not for LIG at the p = 0.05 significance level.

Calibration of NIR Data

The NIR spectrometer produced spectra from 600-1900 nm in 2 nm increments, which were converted to the second derivative to remove baseline offset and sloping effects that are common in NIR spectra (Thosar et al. 2001). The second derivative data showed noise at both ends of each spectrum (Figure 1) and, therefore, calibration equations were based on a reduced spectrum. Michell and Schimleck (1996) report one of the major bands for cellulose prediction occurred at 1477 nm, whereas a major band for lignin prediction occurred at 1668 nm. Following this report, calibrations for coarseness, fiber length, and α-cellulose content were based on wavelengths from 800-1600 nm (Figure 1), and lignin calibrations were based on wavelengths from 800-1750 nm.

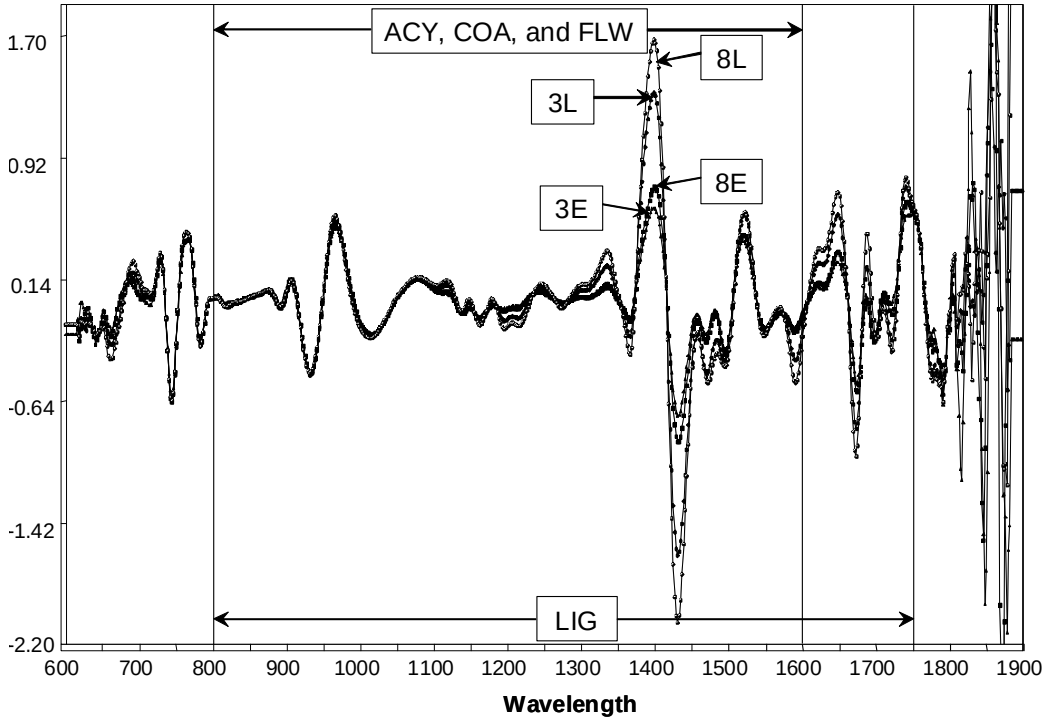

Figure 1. Examples of second derivative NIR spectra for ring-3 earlywood (3E), ring-3 latewood (3L), ring-8 earlywood (8E) and ring-8 latewood (8L) for one increment core. Cut-off points used for α-cellulose content (ACY), coarseness (COA), and weighted fiber length (FLW) were wavelengths 800-1600nm, whereas cut-off points for lignin content were wavelengths 800-1750nm.

The coefficient of determination (R^2) was estimated between the laboratory chemistry data and the NIR predicted values using partial least squares (PLS) regression models. The number of factors to be used in each calibration model was chosen by determining when the mean square error of cross validation reached a minimum and started to plateau (Miller 1989). In addition to R^2, the standard error of calibration (SEC) was used to evaluate how precise the regression line fit the data:

$$[1] \quad SEC = \sqrt{\frac{\sum_{i=1}^{NC} (\hat{y}_i - y_i)^2}{(NC - k - 1)}}$$

where $\hat{y}_i$ is the value of the constituent for validation sample *i* estimated by the calibration model; y_i is the known value of the constituent of sample *i*; NC is the number of samples used in the development of the calibration equation; and *k* is the number of factors used to develop the model (Raymond and Schimleck 2002, Workman 1992). SEC is the standard deviation for the residuals due to the difference between wet-chemistry data from the laboratory and the NIR predicted values within the calibration set (Bailleres et al. 2002; Mark and Workman 1991). The standard error of cross validation (SECV) was also determined to measure how well the calculated equations predicted the samples that were not used to create the calibration set (NIRSystems 2001). The SECV is given by:

$$[2] \quad SECV_j = \frac{\sum_{i=1}^{n} (\hat{x}_{ij} - x_i)^2}{n}$$

Where *j* is the number of factors used to develop the calibrations equation; x_i is the known value of the constituent for sample *i*; $\hat{x}_{i,j}$ is the value of the constituent for sample *i* predicted by the model (excluding sample *i*) developed with *j* factors, and *n* is the number of samples (Schimleck et al. 1999). Cross validation involves dividing the calibration set randomly into four equal-sized segments. One segment is excluded and the other three segments are used to develop a PLS model. This model is then checked to determine how well it fits the samples in the excluded set. This process is repeated four times to validate all the samples and to provide an estimate of the SECV (Schimleck et al. 1999). A prediction function was used to predict a set of unknown data using the previously established calibration equations to produce the standard error of prediction (SEP) as well as the R^2 for the prediction set. The SEP is given by:

$$[3] \quad SEP = \sqrt{\frac{\sum_{i-1}^{NP} (\hat{y}_i - y_i)^2}{(NP - 1)}}$$

where $\hat{y}_i$ is the value of the constituent for prediction sample *i* predicted by the calibration model; y_i is the known value of the constituent of sample *i*; NP is the number of samples used in the prediction set (Workman 1992).

Calibration models for each dataset (combined data, individual site data, individual ring data, and earlywood/latewood data) were developed using 75% of the samples, whereas the remaining 25% of samples were used for validation of the models. Each ring sample (3E, 3L, 8E, and 8L) was analyzed individually to test whether earlywood or latewood produced different results. The calibration equations from site A with higher R^2 were used to predict properties in site B Ring-3 equations were used to predict ring-8 data for examining how well the equations can be extrapolated on relatively different datasets. Similarly, calibration equations based on site A were used to predict data collected from site B.

To minimize the thickness variation of wood wafers and variation within the ring, several mathematical options were tested. The PLS analysis was completed by using the multiplicative scatter correction, whereas the second derivative was used to standardize the data and eliminate some of the variation in NIR intensity due to thickness differences. Using multiplicative scatter correction, the light scattering or change in path length for each sample is estimated relative to that of an ideal sample effectively reducing the spectral noise (Næs et al. 1990). Although several other regression options were examined, these did not result in improved calibration equations and, thus, are not reported.

Results

NIR Calibrations

The fitted calibration models of α-cellulose content, coarseness, and fiber length with NIR spectra showed linear relationships between NIR data and wet-chemistry (Table 2). For the combined site analysis, regression models based on the whole data set with samples 3E, 3L, 8E, and 8L resulted in higher R^2 values than subsets broken out by earlywood/latewood or ring-3/ring-8 (Table 2). The range of R^2 values for α-cellulose content was between 0.56 to 0.63 for the two wood types and the two rings, whereas the coefficient increased to 0.75 for the whole core data set (Figure 2). Results from NIR spectroscopy explained lower percentages of variation in coarseness (R^2 = 0.64) and fiber length (R^2 = 0.43) laboratory measurements.

The calibration models for lignin content had low R^2 values, i.e., low association (R^2 = 0.16) of laboratory data for site B with the NIR predictions (Figure 3), and low R^2 value (R^2 = 0.37) with the complete data. Due to the poor association of lignin content with NIR spectra on site B, lignin content was not included in further analysis.

NIR Calibrations by Sites

Regression models based on site A data showed stronger R^2 values than those based on site B (Table 2). Relationships between the NIR data and laboratory α-cellulose contents were the strongest for all the individual datasets and ranged from R^2 = 0.68 to R^2 = 0.88. Relationships between NIR data and laboratory measurements for coarseness and fiber length were moderately strong for site A. The latewood and ring-8 datasets yielded the highest coefficients of determination, with slightly higher standard errors than the earlywood and ring-3 datasets on site A.

Table 2. Linear regression models fitted to predict wet-chemistry measured wood properties from NIR spectra for A) site A, B) site B, and C) both sites combined for latewood (3L and 8L), earlywood (3E and 8E), ring-3 (3E and 3L), ring-8 (8E and 8L), and for combined data (3E, 3L, 8E, and 8L). The number of factors for these calibration equations ranged from 3 to 6.

A) Site A

		ACY (%)			COA (mg/mm)			FLW (mm)		
Dataset	n	R^2	SEC	SECV	R^2	SEC	SECV	R^2	SEC	SECV
Latewood	70	0.88	1.882	2.322	0.61	0.053	0.037	0.71	0.231	0.345
Earlywood	65	0.73	1.545	1.305	0.54	0.022	0.026	0.67	0.255	0.275
Ring-3	70	0.68	1.617	1.715	0.74	0.029	0.038	0.60	0.203	0.222
Ring-8	65	0.83	1.811	2.074	0.74	0.042	0.046	0.68	0.283	0.285
Combined	135	0.86	1.931	2.212	0.73	0.044	0.045	0.72	0.309	0.354

B) Site B

		ACY (%)			COA (mg/mm)			FLW (mm)		
Dataset	n	R^2	SEC	SECV	R^2	SEC	SECV	R^2	SEC	SECV
Latewood	55	0.63	2.037	3.099	0.24	0.084	0.079	0.42	0.363	0.278
Earlywood	60	0.70	1.435	1.692	0.47	0.054	0.064	0.48	0.282	0.203
Ring-3	55	0.51	2.189	1.853	0.41	0.076	0.098	0.54	0.235	0.292
Ring-8	60	0.46	2.606	3.100	0.35	0.098	0.055	0.55	0.448	0.475
Combined	115	0.73	1.992	2.135	0.63	0.067	0.079	0.55	0.297	0.372

C) Combined Sites

		ACY (%)			COA (mg/mm)			FLW (mm)		
Dataset	n	R^2	SEC	SECV	R^2	SEC	SECV	R^2	SEC	SECV
Latewood	120	0.63	2.718	2.882	0.36	0.076	0.068	0.38	0.390	0.453
Earlywood	120	0.60	2.341	1.890	0.39	0.041	0.058	0.27	0.367	0.350
Ring-3	140	0.57	2.162	2.232	0.44	0.056	0.053	0.18	0.266	0.303
Ring-8	110	0.56	2.510	3.400	0.52	0.070	0.089	0.27	0.505	0.560
Combined	250	0.75	2.417	2.134	0.64	0.059	0.066	0.43	0.387	0.392

Note: R^2 is coefficient of determination, SEC is standard error of calibration, SECV is standard error for cross validation, ACY is α-cellulose content, COA is coarseness, and FLW is average fiber length. N values are approximate due to missing values.

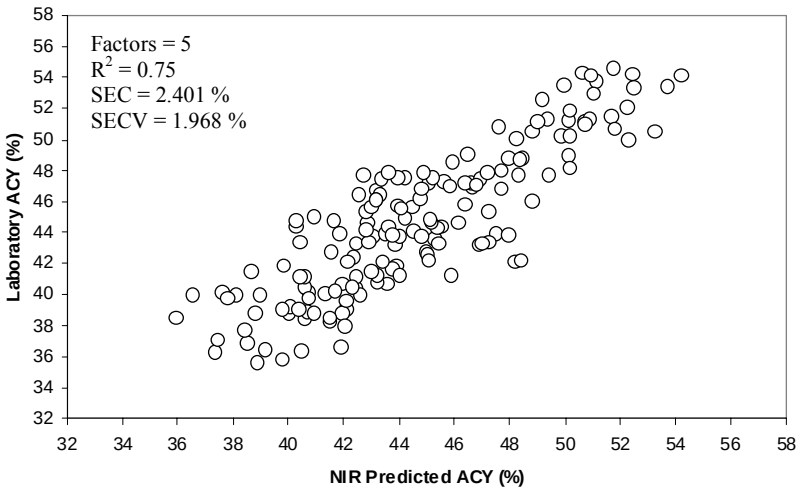

Figure 2. Calibration data for combined sites to predict α-cellulose content (ACY). Standard error of calibration (SEC), standard error of cross validation (SECV) and R^2 of each model are given with the scatter plot (n ≈ 180).

Regression models based on site B data showed smaller R^2 values for all traits. The best calibration equation for α-cellulose content had a R^2 value of 0.70 for the earlywood data on site B, compared to $R^2 = 0.73$ for site A (Table 2). In general, all standard errors for site B were higher than those from site A. Standard errors (SEC and SECV) for coarseness on site B were approximately twice those reported from site A. Similarly, standard errors for fiber length on site B were generally 1.5 times higher than those from site A. When all ring measurements are combined into one data set (3E, 3L, 8E, and 8L), calibrations resulted in stronger relationships for both sites, with R^2 ranging from 0.72 to 0.86 on site A and 0.55 to 0.73 on site B (Table 2).

NIR Predictions between Sites

Whole core calibration equations based on site A resulted in higher R^2 values and were used to predict the wood properties of the trees on site B. Using the α-cellulose content and coarseness calibrations of Site A to predict the NIR spectra obtained from the site B data, yielded $R^2 = 0.64$ and $R^2 = 0.63$, respectively (Figure 4) compared to $R^2 = 0.73$ and $R^2 = 0.63$ for the Site B equations. The SEP for α-cellulose content was approximately 1.5 times greater than the SEC found on site A and the SEP of coarseness was twice the SEC (Table 2). Fiber length on site B was not well predicted from site A, with a low $R^2 = 0.43$ and SEP that was nearly twice the SEC from site A.

Ring-3 calibration equations were used to predict the values for ring-8. Alpha cellulose content predictions resulted in a positive relationship with $R^2 = 0.60$ and a SEP of 3.53% (Figure 5). Ring-3 prediction equations for coarseness of ring-8 resulted in a higher R^2 (0.68) than using the ring-8 data independently ($R^2 = .52$) with a SEP of 0.08 (Table 2). A noticeable separation of earlywood and latewood was apparent on the coarseness distribution (Figure 5). Latewood coarseness values were higher than those of earlywood, and separated into clusters on the graph. Fiber length predictions were not made due to the lower R^2 values.

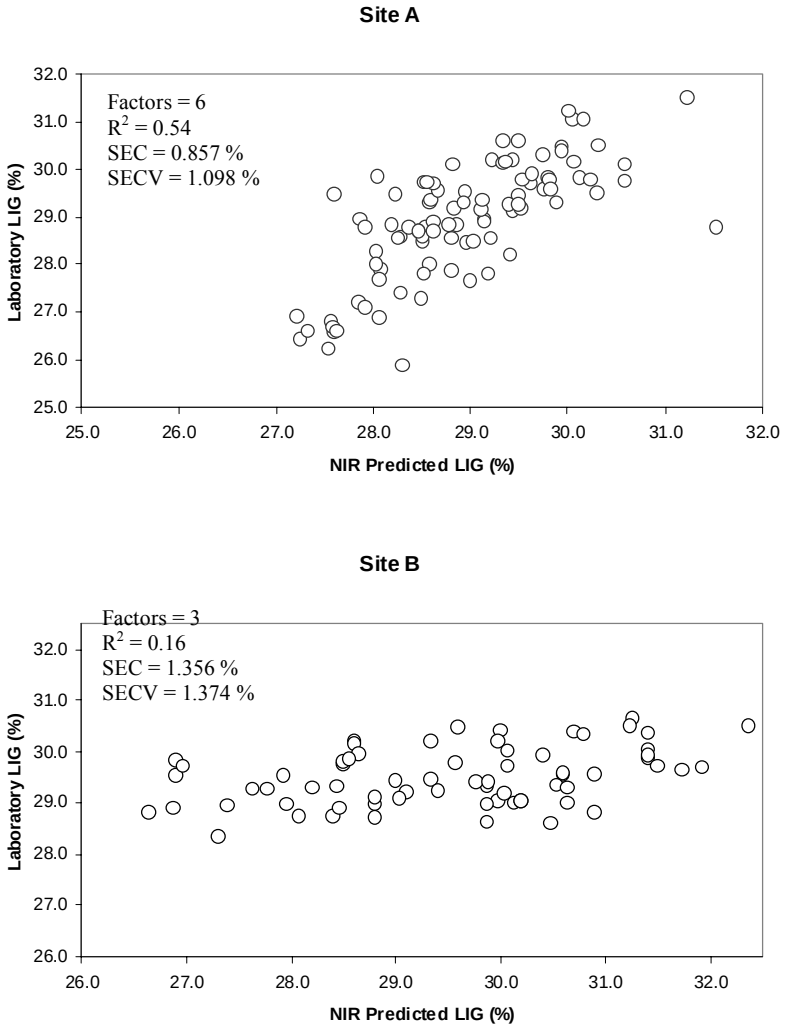

Figure 3. Calibration data for individual sites to predict lignin content (LIG) using NIR spectra. Standard error of calibration (SEC), standard error of cross validation (SECV) and R^2 of each model are given with the scatter plots ($n \approx 120$ and $n \approx 110$ respectively)

Discussion

NIR transmittance provided relatively reliable calibrations for predicting α-cellulose content in juvenile loblolly pine (Figure 2, Figure 5). The standard errors of calibration and prediction ranged from 1.43% to 3.55%, depending on the dataset that was used (Table 2, Figure 5). The standard errors for α-cellulose content were higher, with slightly lower R^2 values than those reported by Raymond and Schimleck (2002) using *Eucalyptus globulus* and Schimleck et al. (2004) using *Eucalyptus nitens*. Different laboratory methods for determining cellulose values may lead to the higher errors in this study. Another difference was that the wood samples were maintained separately (3E, 3L, 8E, and 8L) for NIR analysis, whereas other studies used ground wood meal for cellulose determination.

Reliable cellulose determination is important to the pulp and paper industry because cellulose content is highly correlated with pulp yield (duPlooy 1980; Kube and Raymond 2002; Schimleck and Michell 1998; Wallis et al. 1996a; Wallis et al. 1996b; Wright et al. 1990). If tree breeders can use NIR

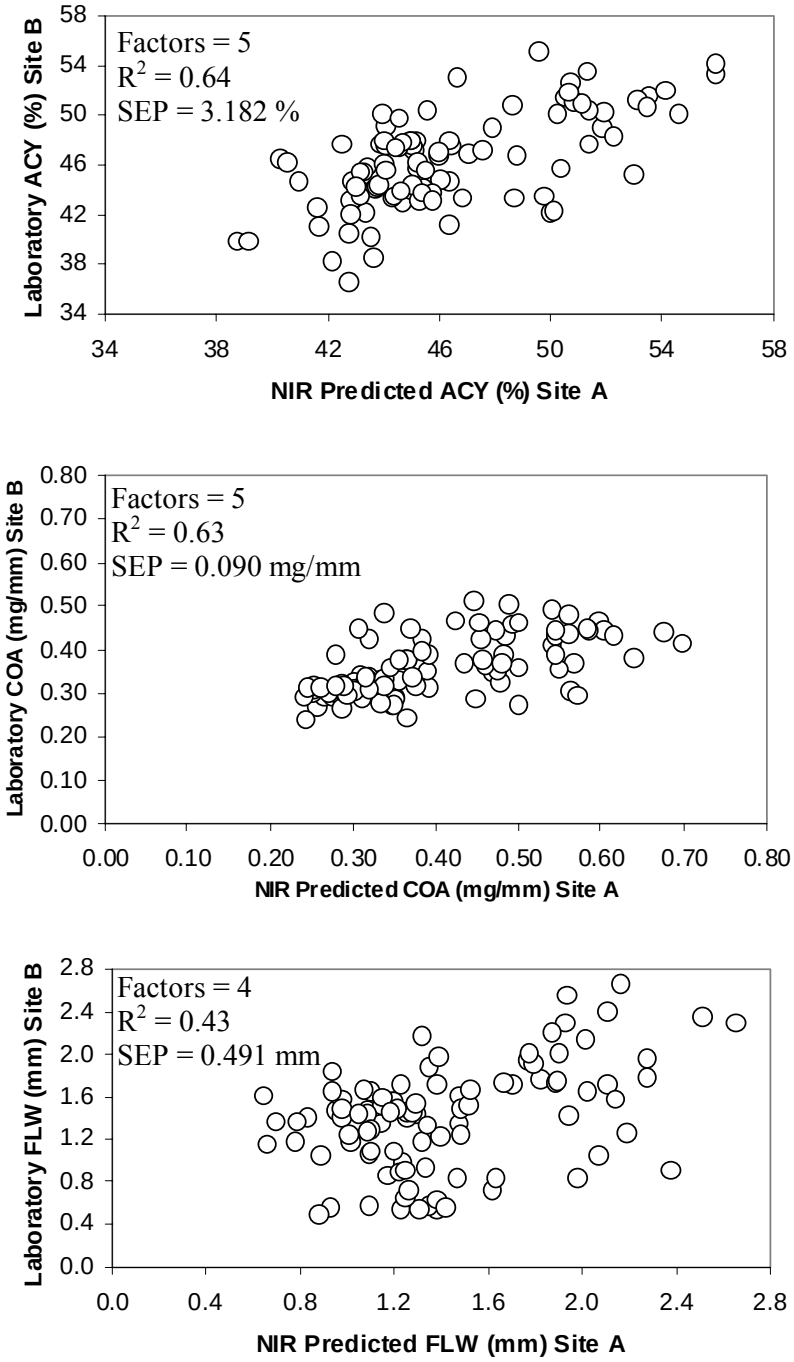

Figure 4. Comparison of wood properties predicted using NIR calibration models from site A and laboratory measurements for samples from site B for α-cellulose content (ACY), coarseness (COA) and average fiber length (FLW). Standard error of prediction (SEP) and R^2 of each model were given with the scatter plots. (n = 99).

spectroscopy to rapidly and cost effectively screen progeny tests to select trees with high α-cellulose content, selected trees can be bred to improve pulp yields in plantations. Because a relatively high degree of genetic control was found in a population of juvenile loblolly pine (heritability ranged 0.38-0.55 for juvenile and transition wood; Sykes et al. 2003), α-cellulose content could be improved in a breeding program by using NIR for rapid screening and selection.

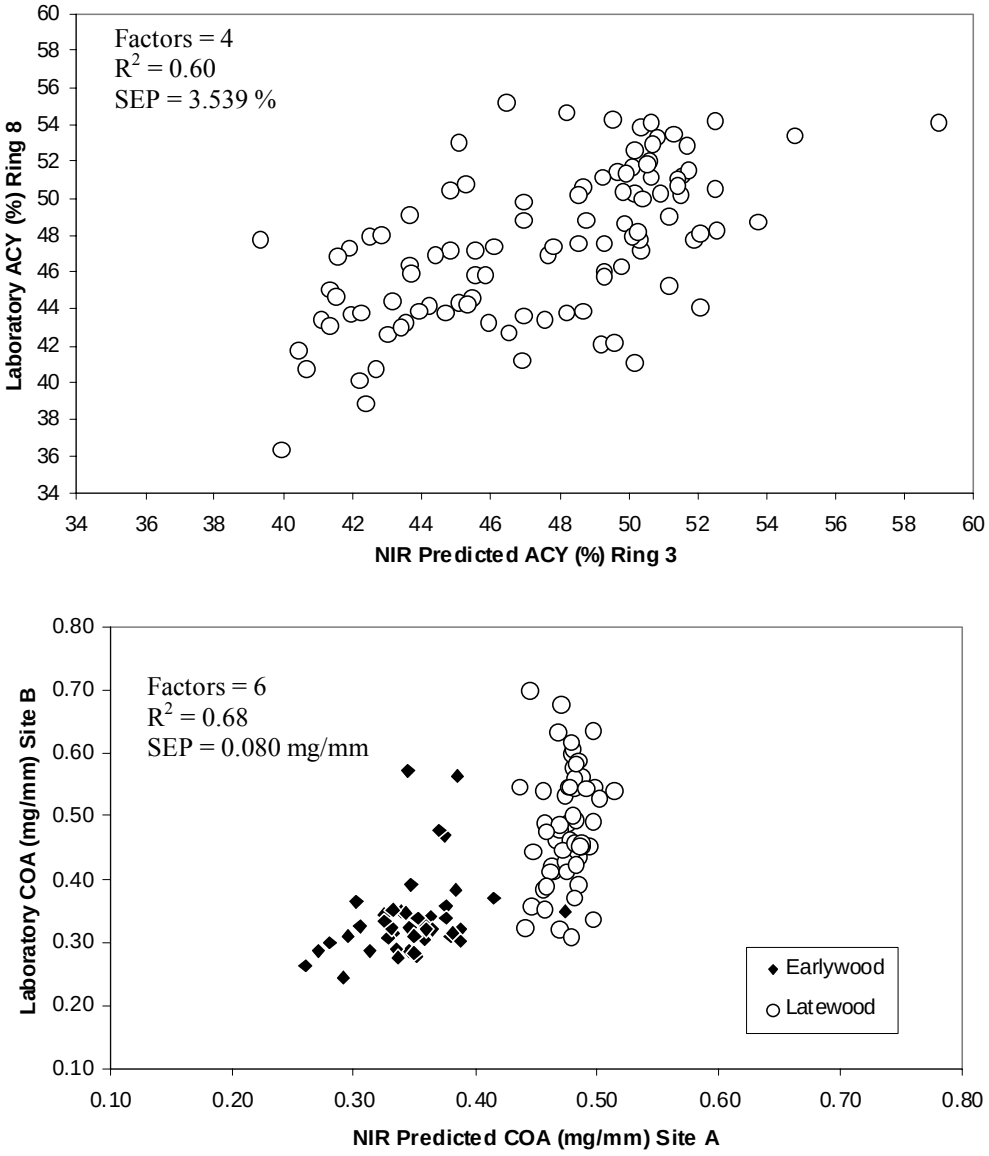

Figure 5. Comparison of wood properties predicted using NIR calibration models from juvenile wood (ring-3) and laboratory measurements for samples from transition wood (ring-8) for α-cellulose content (ACY) and coarseness (COA). Standard error of prediction (SEP) and R^2 of each model were given with the scatter plots. (n = 103).

Fiber length calibrations varied in R^2 values, from 0.40 on site B to 0.70 on site A (Table 2). The results suggested that environmental factors such as site differences may affect fiber length more than the other two traits. This was evident when data from both sites were used to create calibration equations. Correlations based on the whole core data set were lower than those from individual sites and the corresponding SEC was higher (Table 2).

NIR spectroscopy is mainly used to measure overtone and combination bands of the fundamental stretching vibrations of O-H, N-H, and C-H functional groups which contain chemical and physical information about a sample (Barton 1989). However, in this study two physical properties of wood, coarseness and average fiber length, were predicted moderately well using transmittance NIR. This may be due to their genetic correlations with α-cellulose content, $r_g = 0.37$ and $r_g = 0.40$ for fiber length and coarseness respectively (Sykes et al. 2003). Phenotypic correlations between α-cellulose content and fiber length ($r_p = 0.52$) and coarseness ($r_p = 0.33$) were similar. The predictability of coarseness and fiber length may be due to pleiotropic effects, in which the same genes affect multiple traits simultaneously. Although calibration equations for fiber length and coarseness had lower R^2 values than α-cellulose content equations, there were moderate associations that may be useful for using NIR spectra in predicting coarseness and fiber length.

The poor lignin calibration in this study may be due to the high laboratory standard error (Table 1). The site difference in the calibration equations may be due to environmental effects of these two sites and the high laboratory standard error. Yeh et al. (2004) used transmittance NIR to predict the lignin content of 12 mm wood cores ($R^2 = 0.81$) for ring-3 earlywood using the Klason lignin method. The Klason lignin method had less than half the standard error (SE = 0.30 %) (Yeh et al. 2004) compared to the acetyl bromide method in our study (SE = 0.64 %, Table 1). Kelly et al. (2004) found slightly lower associations, with a correlation of 0.81 ($R^2 = 0.66$) for predicting lignin content using reflectance NIR on ground wood of one ring in loblolly pine. Similar prediction results were obtained in this study ($R^2 = 0.83$) when the dataset was restricted to site A and ring-3 earlywood (data not shown). Thus, it seemed that lignin content prediction using NIR transmittance could be promising, but accuracy in prediction may be depend on the methods of lignin determination and the quality of the laboratory data.

The NIR spectra from site A may be used to predict properties at site B for α-cellulose content and coarseness with a moderate R^2 of 0.63. Although, these R^2 values were lower than the individual site predictions, they may be sufficient for screening a large number of progeny test trees for ranking and selection purposes. On the other hand, the model for fiber length from one site may not be reliable for predicting fiber length in another site, as shown with a low R^2 (0.43) and a high SEP (0.48 mm).

Predictions between ring-3 and ring-8 were moderate for α-cellulose content ($R^2 = 0.60$) and for coarseness ($R^2 = 0.68$). Ring-3 calibration equations explained 60% of the variation in ring-8 α-cellulose content equations. Similarly, 68% of the variation in ring-8 coarseness was explained by ring-3 equations. The moderate R^2 values for α-cellulose content and coarseness indicated that the NIR spectra may be used for early selection in wood properties. However, before these NIR prediction models can be used in a breeding program, more wood samples need to be sampled to validate the predictions. More sites from different environments need to be sampled to estimate the environmental effects and genotype by environment interaction.

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GENETIC VARIATION OF JUVENILE WOOD PROPERTIES IN A LOBLOLLY PINE PROGENY TEST

Summary

Genetic variation in several juvenile wood traits of loblolly pine (*Pinus taeda* L.) was investigated utilizing 14 full-sib families generated by a 6-parent half-diallel mating design. Wood samples of 12 mm increment cores were collected from 11-year-old trees from one progeny test. Earlywood and latewood of ring three (juvenile wood) and ring eight (transition wood) for each increment core were analyzed for alpha cellulose content (ACY), average fiber length (FLW), coarseness (COA) and lignin content (LIG). Transition wood of ring eight had significantly higher ACY, FLW and COA but lower LIG than juvenile wood at ring three. Latewood of both rings had higher ACY, FLW and COA than earlywood. Loblolly pine families differed significantly for ACY, FLW and COA but not for LIG. In general, additive genetic effects explained greater percentages of family variation than dominance genetic effects in these traits. For all traits, genetic variation increased from juvenile to transition wood (ring three to ring 8). While weak individual and family heritabilities were found for ACY, FLW and COA for the juvenile wood, heritability estimates for the transition wood were moderate. Positive genetic correlations of wood density with ACY, FLW and COA were found; indicating genetic improvement of wood density might help to improve these important wood and fiber traits. Results from this study showed that genetic improvement for these important traits for pulp and paper production could be possible by selection based on the transition wood assessments.

Introduction

The demand for timber production and pulp in the Southeast of the United States has increased greatly, as harvesting has decreased on public lands in other areas of the United States (*1*). As urban sprawl increases and rural land is converted into urban areas, the land available for wood production has decreased. In order to meet the demand for wood in the United States, the remaining timberland must be managed efficiently to increase the productivity through intensive silvicultural practices and genetic improvement (*2*).

Intensive silvicultural practices and genetic improvement of trees have increased forest plantation productivity significantly in the southeast U.S. for loblolly pine (*Pinus taeda L.*) (*3*). Improved growth has allowed rotation ages to be reduced to about 20 to 25 years for intensively managed loblolly pine plantations, compared with 40 to 50 years in natural stands. Consequently, the percent of juvenile wood from plantations has increased (*4*). Compared to mature wood, juvenile wood has low wood density, shorter tracheid length and higher lignin content (*5, 6*). The pulp and paper industry has experienced reduced yields and higher pulping costs due to the increased use of juvenile wood (*7*).

Most studies on wood properties have concentrated on the genetic control of wood density (*8, 9,* and *10*). Zobel and Sprague (*11*) compiled a list of narrow-sense heritabilities for loblolly pine specific gravity that ranged from 0.20 to 1.00. Genetic control concerning transition age from juvenile to mature wood for loblolly pine has been studied. Loo et al. (*12*) reported that loblolly pine families produce juvenile wood through age six, with a transition period of ages 6 to 14, and mature wood production beyond age 14. Szymanski and Tauer (*7*) reported similar results (*12*) for transition age, and Hodge and Purnell (*9*) reported the average transition age for slash pine as 9.4 rings from the pith.

Genetic variation in juvenile wood properties in loblolly pine is very important in improving the quality and uniformity of solid and chemical wood products. Specific gravity, cellulose content, lignin content, average fiber length, and coarseness are several key wood properties that affect the quality and quantity of wood produced either in plantations or in natural stands. Favorable juvenile wood properties

may be obtained through a breeding program, if there is significant genetic variation among families and populations. Large genetic variations in holo-cellulose content, alpha-cellulose content, average fiber length, and lignin content have been reported in juvenile wood for loblolly pine and other species (*13, 14, 15, 16, 17,* and *18*). This genetic variation allows the breeder to select trees with desirable and uniform juvenile wood properties, that will increase yield, improve product properties and lower pulping costs (*19*).

Studies of chemical wood properties have been completed on other species such as the study by Yu et al. (*16*), where the physiochemical wood properties of hybrid aspen (*P. tremula x P. tremuloides*) clones were measured. Those authors tested two sites and found the family variance for alkali-soluble lignin accounted for 0.0 % and 48.6% of the total variance on sites. Jett et al. (*20*) reported weak inheritance of cellulose yield using a limited number of samples with most of the variance inherited in a non-additive manner. Zobel et al. (*21*) found similar results for inheritance of cellulose yield. Loo et al. (*12*) studied the inheritance of tracheid length of 15-year-old loblolly pine and found weak individual tree and moderate family heritabilities.

This study was to quantify genetic variation of important juvenile wood quality traits of loblolly pine. Specific objectives were to determine among and within family genetic variation in alpha-cellulose content, average fiber length, coarseness, and lignin content, study genetic variation of earlywood and latewood and estimate genetic parameters, and examine correlations among these chemical and morphological wood traits. This information would benefit the tree improvement program and the pulp and paper industry by allowing selection and planting of trees with desirable traits. Genetically improved trees with uniform and desired juvenile wood properties would also allow mills to process wood more efficiently and economically (*19*).

Materials and Methods

Material and data collection

Fourteen full-sib families generated by a six parent half-diallel mating design were tested in the Piedmont of South Carolina. A Randomized complete block design with six replications was used in the field. Each full-sib family was laid out in six-tree row-plots for each replication. Wood core samples from one site in Florence Co. (South Carolina) were collected from 11-year-old trees. Wood samples of 12 mm increment cores were taken from each tree at breast height (about 1.30 m) using generator-powered drills. Wood cores having visible limbs, curves, resin pockets, compression wood, or rust infections were avoided. The samples were placed into sealed plastic storage bags and stored on ice in coolers to retain moisture during the material collection.

The bark and cambium were removed from the wood cores, and the cores were split at the pith into two halves. Chemical analysis was done using microanalytical techniques developed by Yokoyama et al. (*22*), which allow the rapid characterization of fiber components and morphology of loblolly pine in a large number of samples. Briefly, the techniques involved are extractive removal, holocellulose preparation, alpha-cellulose and lignin content determination, and average fiber length and coarseness analyses. Nonvolatile extractives greater than 95% were removed from the increment core by four successive two-day acetone extractions (*22*). Extractives were removed from all cores using the successive acetone method. The increment cores were then soaked in water overnight before ring samples were taken.

Within-core samples were taken from ring three and ring eight to study chemical properties of juvenile wood (ring 3) and transition wood (ring 8), respectively. Thin wafers from ring three earlywood, ring three latewood, ring eight earlywood, and ring eight latewood were taken using a microtome. At

least 300-500 mg of each sample were taken from the earlywood and latewood of each. Each sample was oven-dried 12 hours.

Statistical analyses

Earlywood and latewood means of wood traits within each ring were compared using T-tests. Analysis of variance was conducted to compare full-sib families using the GLM procedure of SAS (*23*). A standard general linear mixed model for diallel analysis (*25*) was used to analyze the data, which included fixed effect of replication, and random effects of general combining ability (GCA), specific combing ability (SCA), their interactions with replication, plot, and experimental error. Variance components of random effects, which measure the magnitude of variation, were estimated using the SAS PROC MIXED procedure developed for diallel analysis (*25*).

The coefficients of variation for GCA and SCA were estimated by dividing the square roots of the estimates with their means. Family least squares means with 95% confidence intervals were estimated. Earlywood and latewood of ring three (juvenile wood) and ring eight (transition wood) were compared for each wood trait. Using variance components from the mixed model, individual (h^2_i), half-sib family (h^2_{hs}), full-sib family (h^2_{fs}), and within full-sib family (h^2_{wfs}) heritabilities were estimated.

$$h_i^2 = \frac{4\sigma_{GCA}^2}{2\sigma_{GCA}^2 + \sigma_{SCA}^2 + \sigma_{plot}^2 + \sigma_E^2} \quad \text{[Eq. 2]}$$

$$h_{hs}^2 = \frac{\sigma_{GCA}^2}{\left(p\sigma_{GCA}^2 + \sigma_{SCA}^2 + \frac{\sigma_{plot}^2}{b} + \frac{\sigma_E^2}{bn}\right)\left(\frac{1}{p-1}\right)} \quad \text{[Eq. 3]}$$

$$h_{fs}^2 = \frac{2\sigma_{GCA}^2}{2\sigma_{GCA}^2 + \sigma_{SCA}^2 + \frac{\sigma_{plot}^2}{b} + \frac{\sigma_E^2}{bn}} \quad \text{[Eq. 4]}$$

$$h_{wfs}^2 = \frac{2\sigma_{GCA}^2}{\left(\frac{b-1}{b}\right)\sigma_{plot}^2 + \left(\frac{bn-1}{bn}\right)\sigma_E^2} \quad \text{[Eq. 5]}$$

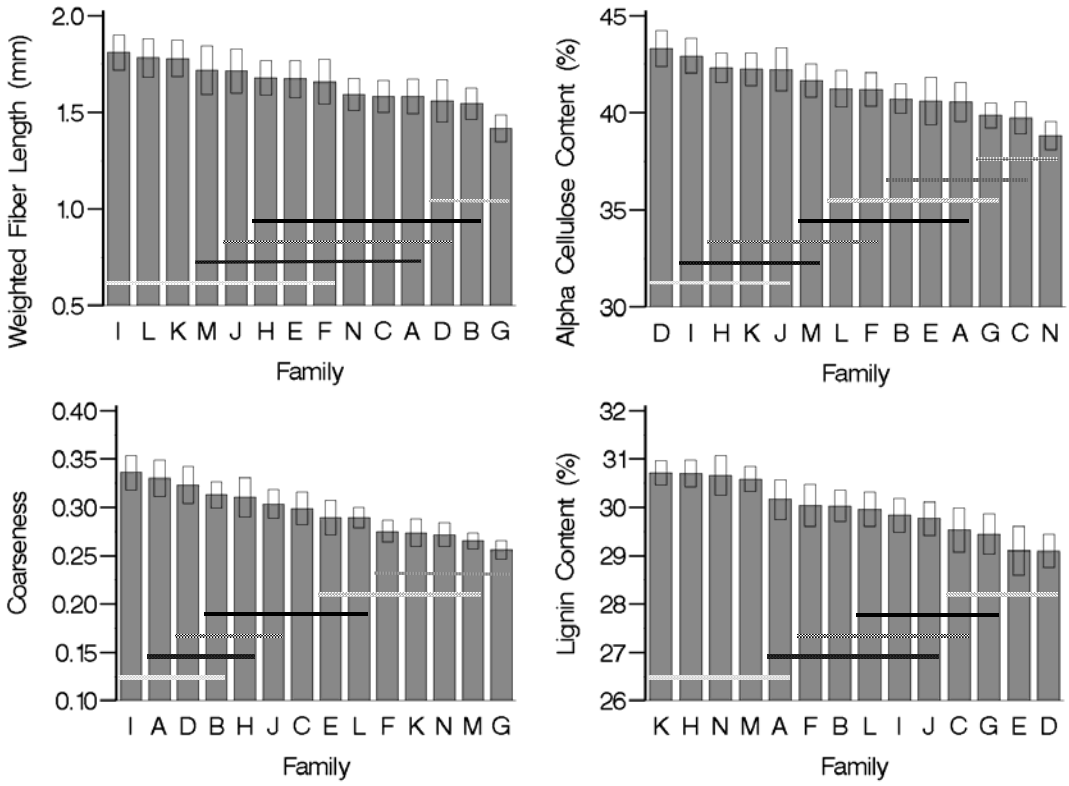
where σ^2_{GCA} is genetic variance, σ^2_{SCA} is dominance variance, σ^2_{plot} is SCA x replication variance, σ^2_e is error variance, p is # of parents in the diallel, b is # of blocks, and n is # of trees per family per replication. Product-moment correlations and genetic correlations were estimated among these traits and with wood density (26) that was determined by the standard volumetric method. Standard errors of heritabilities and genetic correlations were calculated using the Delta Method (27) and ASREML software (28).

Results

Differences of earlywood and latewood, rings and families

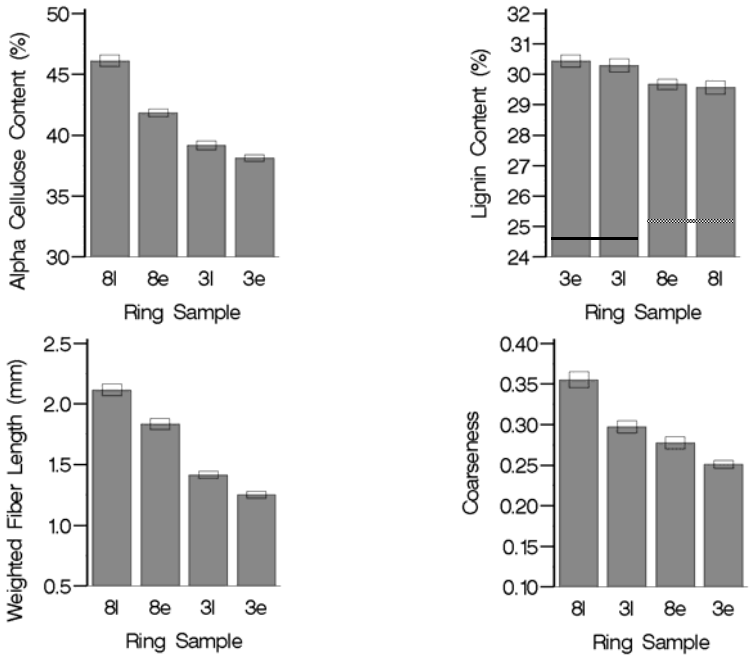
Latewood had significant greater ACY, FLW and COA than earlywood in ring three (juvenile wood) (Figure 1). The differences between latewood and earlywood for ACY, FLW, and COA were more pronounced in ring eight (transition wood) compared to ring three. Latewood of ring eight had greater ACY, longer average fiber length and greater coarseness than earlywood. FLW for latewood of ring eight was 13.4% longer than earlywood of the same ring. Earlywood and latewood of ring eight were not different for LIG.

Families differed significantly for all the chemical and morphological wood properties at the probability of <0.001 level (data not presented). The interactions of family by earlywood or latewood within a ring were not significant. Family means for FLW ranged from 1.42 (family I) to 1.81 mm (family G) (Figure 2). There was considerable variation among families for COA. Family I had the greatest COA, whereas family G had the lowest values. Families I and G were consistent in ranking for FLW and COA. Family means for alpha cellulose content ranged from 38.8 to 43.3 %, with family D having the highest value and family N the lowest. Very little variation was found among families for lignin. Family E had the lowest lignin content (29.1 %), while family K had the highest lignin content (30.7 %) (Figure 2).



Note: Families within the same line were not significantly different at 5% significance level.

Figure 1. Least squares means and 95% confidence intervals of average fiber length, alpha cellulose content, coarseness, and lignin content of juvenile wood (ring 3) for earlywood and latewood (3E and 3L) and transition wood (ring 8, 8E and 8L)



Note: Families within the same line were not significantly different at 5% significance level.

Figure 2. Rankings of 14 full-sib family means (A - N) and 95% confidence intervals for average fiber length, alpha cellulose content, coarseness, and lignin content

Phenotypic and genetic parameters

Coefficients of genetic variation (CV) for fiber length, cellulose content, coarseness and lignin content were generally large for these traits (Figure 3). In general, additive genetic effects (GCA variance) explained greater variation for fiber length, coarseness and cellulose content than dominance genetic effects (SCA variance). The CV of SCA for fiber length was essentially zero for earlywood and latewood of juvenile and transition wood. The CV of GCA was clearly greater than that of SCA for FLW, ACY, and COA for latewood or ring three and ring eight. There was no genetic variance for lignin in juvenile wood, but the CV of SCA for transition wood was higher that of GCA for both earlywood and latewood. In general, GCA variance increased from earlywood to latewood of the same ring and from ring three to ring eight. However, the CV of SCA was inconsistent from one ring to another.

Moderately high family heritabilities were estimated for ACY, FLW and COA (Table 1). Weak individual and within full-sib family heritabilities were observed for early wood of ring three. Heritabilities could not be estimated for LIG for earlywood and latewood of ring three due to lack of GCA variance. Family heritabilities were higher then individual heritabilities for all traits. Latewood heritabilities for ring three were higher than earlywood heritabilities. In contrast to ring three, heritabilities for earlywood and latewood of ring eight were similar but higher for all traits. Among all the traits studied, COA and ACY had the highest heritabilities, whereas lignin was more controlled by non-genetic effects in this study.

Table1. Heritabilities and standard errors for fiber length (FLW), α-cellulose yield (ACY), coarseness (COA) and lignin content (LIG) traits for earlywood and latewood of ring 3 and ring 8 (3E, 3L, 8E and 8L)

		Ring # and Wood Type			
Heritability[1]	Trait[2]	3E	3L	8E	8L
h^2_i	FLW	0.05 ± 0.08	0.09 ± 0.10	0.23 ± 0.22	0.25 ± 0.20
	ACY	0.07 ± 0.14	0.14 ± 0.17	0.32 ± 0.23	0.36 ± 0.31
	COA	0.08 ± 0.11	0.31 ± 0.22	0.23 ± 0.19	0.22 ± 0.18
	LIG	-[3]	-	0.04 ± 0.11	0.16 ± 0.21
h^2_{HS}	FLW	0.04 ± 0.31	0.52 ± 0.27	0.61 ± 0.20	0.67 ± 0.13
	ACY	0.37 ± 0.44	0.53 ± 0.29	0.72 ± 0.09	0.66 ± 0.15
	COA	0.44 ± 0.34	0.71 ± 0.10	0.66 ± 0.14	0.66 ± 0.14
	LIG	-	-	0.26 ± 0.56	0.54 ± 0.31
h^2_{FS}	FLW	0.26 ± 0.29	0.35 ± 0.31	0.47 ± 0.30	0.58 ± 0.24
	ACY	0.21 ± 0.36	0.37 ± 0.34	0.68 ± 0.20	0.57 ± 0.27
	COA	0.27 ± 0.32	0.66 ± 0.21	0.55 ± 0.25	0.55 ± 0.26
	LIG	-	-	0.13 ± 0.36	0.38 ± 0.38
h^2_{WFS}	FLW	0.03 ± 0.04	0.05 ± 0.06	0.14 ± 0.14	0.15 ± 0.13
	ACY	0.04 ± 0.08	0.08 ± 0.10	0.19 ± 0.14	0.23 ± 0.21
	COA	0.05 ± 0.07	0.18 ± 0.14	0.13 ± 0.12	0.13 ± 0.11
	LIG	-	-	0.02 ± 0.06	0.10 ± 0.13

[1] h^2_i individual, h^2_{HS} half-sib , h^2_{FS} full-sib and h^2_{WFS} within full-sib heritability

The chemical and morphological wood properties had higher heritabilities for transition wood (ring 8) than for juvenile wood (ring 3) (Table 2). For example, the family heritability for ACY was 0.38 for juvenile wood, whereas it was 0.55 for transition wood. Heritabilities for ACY and COA were similar and higher than those of FLW, both for ring three and ring eight. Heritabilities for lignin were essentially zero for ring three, but moderate for ring eight. When data from both rings were combined, lower heritabilities were observed for all traits compared to the individual ring data.

Table 2. Heritabilities and standard errors for fiber length (FLW), alpha cellulose yield (ACY), coarseness (COA) and lignin content (LIG) traits for earlywood and latewood of ring 3 and ring 8 (3E, 3L, 8E and 8L) for juvenile wood (ring 3), transition wood (ring 8), and for whole core.

		Traits			
	Heritability[1]	FLW	ACY	COA	LIG
Ring 3 (Juvenile)	h^2_i	0.09 ± 0.10	0.13 ± 0.18	0.32 ± 0.25	-[2]
	h^2_{HS}	0.54 ± 0.21	0.48 ± 0.33	0.69 ± 0.12	-
	h^2_{FS}	0.38 ± 0.26	0.32 ± 0.35	0.61 ± 0.23	-
	h^2_{WFS}	0.05 ± 0.06	0.08 ± 0.11	0.20 ± 0.16	-
Ring 8 (Transition)	h^2_i	0.28 ± 0.23	0.43 ± 0.32	0.37 ± 0.29	0.13 ± 0.19
	h^2_{HS}	0.65 ± 0.15	0.70 ± 0.11	0.68 ± 0.13	0.48 ± 0.35
	h^2_{FS}	0.55 ± 0.26	0.64 ± 0.23	0.60 ± 0.25	0.31 ± 0.37
	h^2_{WFS}	0.17 ± 0.15	0.28 ± 0.23	0.24 ± 0.20	0.08 ± 0.12
Whole Core	h^2_i	0.08 ± 0.06	0.12 ± 0.10	0.16 ± 0.12	0.00 ± 0.08
	h^2_{HS}	0.52 ± 0.18	0.58 ± 0.16	0.61 ± 0.15	0.01 ± 0.78
	h^2_{FS}	0.35 ± 0.21	0.43 ± 0.22	0.48 ± 0.22	0.00 ± 0.32
	h^2_{WFS}	0.04 ± 0.04	0.07 ± 0.06	0.09± 0.07	0.00 ± 0.04

[1] h^2_i individual, h^2_{HS} half-sib , h^2_{FS} full-sib and h^2_{WFS} within full-sib heritability

[2] Heritabilities could not be estimated due to zero GCA variance

Average fiber length had a high genetic correlation with wood density (r_g=0.95) (Table 3). Positive and moderate genetic correlations were observed between wood density, cellulose content, lignin content and coarseness. ACY had a high negative genetic correlation with lignin content. Lignin content was also negatively correlated with fiber length. Genetic correlation between FLW and COA was

positive and high (r_g = 0.99). Genetic correlations could not be estimated for ring three due to lack of genetic variation.

Wood density of the wood core was phenotypically correlated with cellulose content but not with other traits. Phenotypic correlations between FLW, COA and ACY were significant. None of the traits had significant phenotypic correlation with lignin content.

Table 3. Genetic correlations (above diagonal) and phenotypic correlations (below diagonal) among fiber length (FLW), alpha cellulose yield (ACY), coarseness (COA), lignin content (LIG) and wood density for transition wood (ring 8).

	FLW	ACY	COA	LIG	Density
FLW		0.37 ± 0.48	0.99 ± 0.01	-0.39 ± 0.66	0.95 ± 0.25
ACY	0.52 ***		0.40 ± 0.45	-0.99 ± 0.01	0.56 ± 0.51
COA	0.13 *	0.33 ***		0.57 ± 0.51	0.32 ± 0.55
LIG	-0.03	-0.10	-0.11		-[1]
Density	0.10	0.13 *	0.12	-0.04	

*, ***: Coefficients are significant at 0.05 and 0.001 level respectively. Number of observations used ranged from 183 to 242

[1] Genetic correlation was not estimable due to zero GCA variance for lignin

Discussion

The chemical and morphological wood properties evaluated in this study are very important for pulp and paper production. Alpha cellulose content relates to the amount of pulp that is obtained from wood. The higher the alpha cellulose content in a tree, the more pulp the tree will produce (*29*). Increasing the amount of cellulose content of wood will reduce pulping costs and increase the efficiency of the pulp and paper mill. Average fiber length also plays an important role in the pulp and paper industry. Long fibers as well as short fibers are favored depending on the product being produced. Long fibers give paper greater tensile and tear strength, for products such as cardboard and paper bags (*30*). Short fibers are favored for products such as fine printing paper, where surface smoothness is of importance. Coarseness is related to thickness of fiber wall. The thicker the fibers are, the higher the coarseness values. Lower coarseness values result in better fiber collapse, tighter fiber bonds, and therefore formation of dense paper with a smooth surface. Trees with higher coarseness values yield pulp and paper products with higher bulk, which is beneficial to products requiring higher absorbance and/or higher bending stiffness. One of the most important properties in wood is lignin content. In order to separate the wood fibers lignin must be removed (*31*). This requires chemical breakdown of lignin, which

is a very expensive process. Reducing lignin content in wood could save processing costs for the pulp and paper industry.

Results from this study showed that latewood had greater cellulose content, longer average fiber length and higher coarseness compared to earlywood of the same rings. Juvenile wood at ring three appeared to be less desirable for these chemical wood traits compared to ring eight, which is considered as a transition wood rather than mature wood for loblolly pine (*7*). There was an increasing trend of cellulose content, fiber length and coarseness from juvenile wood to transition wood. Genetic selection based on latewood of the growth ring could be an effective way to manipulate chemical and morphological wood properties.

Considerable genetic variation in the chemical and morphological wood properties was found between earlywood and latewood, except for lignin. A considerable portion of the genetic variance for cellulose content, fiber length and coarseness was explained by additive genetic effects (CV of GCA, Figure 3). Additive genetic variation (GCA) in cellulose content was higher than that found by Jett et al. (*20*). Lignin had little to no GCA variance for both juvenile and transition wood (Figure 3). In order to make genetic improvement on cellulose content and average fiber length, controlled crosses or vegetative propagation techniques should be used to capture non-additive genetic variance (*6*). Dominance genetic effects (SCA) explained about 50% of total genetic variance for these two traits in transition wood. Lowering lignin content through breeding will involve considerable effort and cost. In this study with limited sample size, selecting the family with the lowest lignin content would cause less than a two percent reduction in lignin when compared to the family with the highest lignin content (Figure 2).

Cellulose content had a high genetic correlation with wood density, suggesting that increasing wood density would increase cellulose content (Table 3). Similarly, coarseness and fiber length had positive genetic relationships with density. Because wood density can be measured relatively easy and less expensive, it may be possible to use it to improve other traits indirectly. However, wood density was positively correlated with lignin in this study, and selecting for higher wood density might also increase lignin content. Latewood fibers are thicker and longer than earlywood fibers and result in higher alpha cellulose content and coarseness values (*32*). In this study, a high and positive genetic correlation (0.99) was observed between fiber length and coarseness, indicating that long fiber is associated with thicker fiber wall.

Family heritabilities were moderate to high for all chemical and morphological traits, except for lignin content of juvenile wood, which could not be estimated due to lack of additive genetic variation. Genetic parameters estimated from this study may be biased due to a small population size (6parents and 14 full-sib families) because of the genetic drift. These estimates may be biased upward because one test was measured and the genotype by environmental interaction may be confounded in the genetic variance. Nevertheless, these moderate estimates suggested that genetic improvement for cellulose content and fiber length could be realized through genetic improvement. Heritabilities for fiber length from this study were similar to those found by Loo et al. (*12*) for loblolly pine. Loo et al. (*12*) reported 0.31 and 0.37 for individual heritabilities, and 0.45 and 0.51 for family heritabilities for transition wood at two sites. Increasing cellulose content will result in the production of more paper per cubic meter of wood.

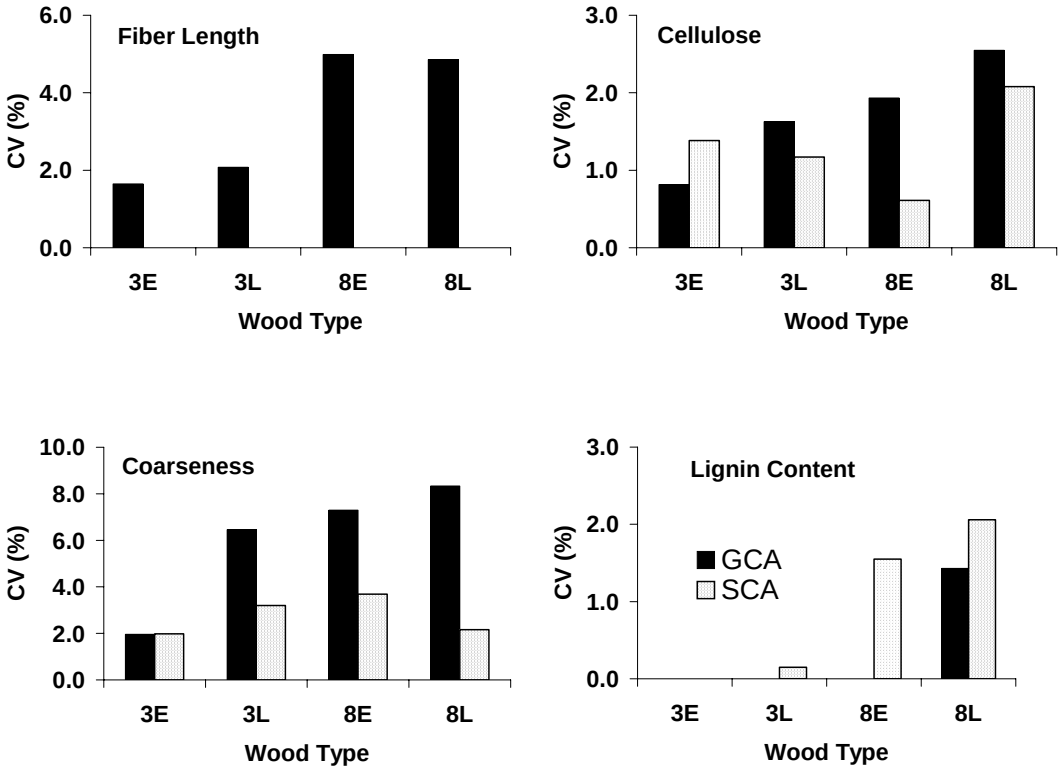


Figure 3. Coefficients of variation (CV) for general combining ability (GCA) and specific combining ability (SCA) estimates for fiber length (FLW), alpha cellulose yield (ACY), coarseness (COA) and lignin content (LIG) at early and latewood of rings three and eight (3E, 3L, 8E and 8L)

Transition wood heritabilities may be more meaningful than those of juvenile wood, as they are closer to the age (age six) where most selections are made for the North Carolina State University-Industry Tree Improvement Coop *(3)*. As transition wood is formed, the wood properties become more uniform, which may contribute to more accurate estimates of genetic parameters. Juvenile wood (ring 3) seems to be more affected by environmental conditions such as moisture content and resin. For example, expression of genetic differences for lignin content was observed in transition wood, but not in juvenile wood. At ring eight, genetic differences for lignin appeared to be more important in this study.

The combined measure of a whole wood core may not provide meaningful information on genetic variation on these traits. By averaging over rings, the genetic variation of different components of rings, earlywood and latewood cannot be determined. This information is important for selection if one component has higher genetic variation, i.e., wood trait in latewood at ring eight. Considerations should be taken into account before using chemical and morphological wood properties in a tree-breeding program. Costs for sampling each tree from a progeny test and processing wood samples for chemical analysis in the lab can be prohibitively expensive. New methods for measuring chemical traits that are cheaper and less time consuming need to be developed. One such method is using a near infrared reflectance (NIR) machine to scan the sample to predict the wood properties. The technology has been used on other species, such as eucalyptus, but not for loblolly pine.

Operational use of trees with genetically improved wood properties also poses a problem. Many forest industry companies are selling their land bases for various reasons. The largest gains can be obtained by using the best families with the desired wood properties, if family information in plantations are well documented. While this may be simple on industry land, that is not where all the wood is harvested. Private landowners currently provide much of the wood that is used in pulp and paper mills. Genetic differences among families for chemical wood traits provide an opportunity for family forestry. Planting in family blocks will allow the industry to grow individual families for the specific chemical properties needed, but company land bases will need to be maintained.

In conclusion, considerable genetic variation was detected for all chemical and morphological traits in this loblolly pine progeny test. Heritabilities were moderate to high for all traits, indicating potential for genetic improvement. High positive genetic correlations between cellulose content, fiber length and wood density suggested simultaneous improvement of chemical wood properties and wood density may be possible. Further studies with large population size are on the way to confirm the genetic variation and develop breeding strategies for improving these wood quality traits.

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SELECTIVE LIGNIN CHARATERIZATION

Summary

Milled wood and milled wood lignin (MWL) samples were subjected to DFRC and thioacidolysis. Despite the fact that both methods selectively cleave aryl ether bonds, substantial differences in results were obtained. Lignin thioacidolysis gave total molar yields of degradation monomer products in the range 3.5 - 7 mol% higher than DFRC. GPC analysis showed that the thioacidolysis treated lignin was degraded to a lower average molecular weight than that by DFRC. Contrary to results reported for lignin model compounds, these results indicate that the DFRC method does not completely or efficiently degrade the lignin polymer. In fact, the DFRC degraded lignin retained much of the characteristics of the original MWL. Elemental analysis revealed the presence of bromine in the DFRC treated lignin. These results indicate inefficiency in the chemistry of the method, probably due to steric constraints of the polymeric nature of lignin. Thioacidolysis was used to analyze wood obtained from families extreme in α-cellulose content. Results obtained revealed no significant diference diference in monomer units released.

Introduction

Lignin, arguably the second most abundant natural polymeric material on earth is extremely complicated, and its structure has not yet been completely elucidated. Lignin *in situ* has no structural regularity. Unlike most natural polymers, which consist of a single intermonomeric linkage, lignin is a network polymer made-up of many carbon-to-carbon and ether linkages. The tight physical binding and chemical linkages between lignin and cell wall polysaccharides also practically prevent its isolation in unaltered form. This makes it very difficult to use degradative or non-degradative methods for structural determination. As a result, our understanding of the structure of lignins, either *in situ* or in pulps, is formed as a sum of the information obtained from different fields of lignin research: studies concerning the elucidation of the mechanisms of lignin biosynthesis (*1*); and analytical data obtained in studies with isolated lignin specimens (*2*,*3*).

Most of the conclusion pertaining to the bonding pattern of native and residual lignins have been derived from degradative methods such as hydrogenolysis (*4*), acidolysis (*3*), and thioacidolysis (*5*-*7*). Through careful analyses of the low molecular weight compounds generated a detailed picture of the original lignin can emerge. Unfortunately, these methods, which utilize gas chromatography (GC) to detect and quantify the degradation products, only analyze the monomeric or dimeric degradation products. They are confined to phenylpropanoid units that are linked via aryl ether linkages. Therefore, only noncondensed lignin structures are quantified. Nonetheless, a large amount of structural information can still be obtained.

Of the various degradative methods, thioacidolysis is proving to be one of the most widely utilized and effective diagnostic methods in the study of lignin structure. It is routinely used to estimate the amount and composition of uncondensed aryl ether structures. Thioacidolysis is an acid-catalyzed solvolysis reaction in dioxane-ethanethiol catalyzed by boron trifluoride etherate, which leads to the depolymerization of lignins. Specifically, thioacidolysis proceeds by either cleavage of α-ethers or substitution of α-hydroxyl groups by the thioethyl group, followed by β-aryl ether cleavage and ultimately the formation of trithioethyl monomeric products. However, thioacidolysis uses malodorous ethanethiol and is therefore not desirable for many researchers to use.

In 1997, Lu and Ralph (*8*-*10*) introduced a new selective α,β-aryl ether cleavage protocol, derivatization followed by reductive cleavage (DFRC). The DFRC method uses mild conditions and no malodorous chemicals. DFRC consists of three simple steps: bromination of the benzylic position and concomitant acetylation of free hydroxyl groups by acetyl bromide; reductive cleavage of the β-aryl ether bonds via zinc metal coordination; and acetylation of newly generated phenolic hydroxyl groups for quantification by gas chromatography. However, as with thioacidolysis the amount of structural

information obtainable from DFRC is limited to noncondensed structural moieties. Recently, Tohmura and Argyropoulos (*11*) and Ikeda *et al.* (*12*) have modified the DFRC protocol to further enhance the amount of structural information obtainable.

The DFRC method has been reported as being quantitative in the yields of low molecular weight products, with >92% yield of targeted products in model compound studies (*9*). By contrast, thioacidolysis monomer yields are reportedly much lower (*5,6*). However, both methods have recently been questioned as to the completeness of aryl ether cleavage and the quantitative recovery of the desired monomers (*12*). Therefore, a detailed investigation into the completeness of aryl ether cleavage and monomer yield from DFRC and thioacidolysis is warranted. In this study, vibratory-milled wood and milled wood lignin prepared from loblolly pine was used as samples of native lignin and an isolated lignin, respectively. Both lignins were subjected to DFRC and thioacidolysis, and monomer yields were quantified by GC analysis and gel permeation chromatography (GPC). Finally, analysis of families extreme in α-cellulose content were analyzed and the diference in lignin structure discussed.

Materials and Methods

Materials. *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald), acetyl bromide, glacial acetic acid, zinc dust (50 mesh), ethanethiol, boron trifluoride etherate, sodium hydrogen carbonate, bis(timethylsilyl)trifluoroacetamide (BSTFA), deuteriochloroform, propionic anhydride and tetracosane were all purchased from Aldrich (Milwaukee, WI) and used as received. Diethyl ether, methanol, ethanol, potassium hydroxide, methylene chloride, acetic anhydride and pyridine were purchased from Fisher Scientific and used as received. 1,4-Dioxane was purchased from Fisher Scientific (Suwanee, GA) and distilled over $NaBH_4$ and blanketed with argon before use.

Milled wood lignin and vibratory-milled wood were produced from Loblolly pine (*Pinus taeda* L.) sapwood. The sapwood was ground to pass a 20 mesh screen in a Wiley mill and Soxhlet extracted with ethanol/benzene (1:2, v/v) for 24 h, followed by ethanol for an additional 24 h. The milled wood (100g) was ground for 1 week in a 1-gallon porcelain jar under a nitrogen atmosphere using glass balls. This was followed by 48 h of vibratory milling under a nitrogen atmosphere in the presence of toluene in a vibratory ball mill (Siebtechnik GMBH, Mulheim, Germany) with stainless steel balls (vibratory milled wood). MWL was then isolated from the vibratory milled wood according to the method of Björkman (*13*).

DFRC Procedure. The DFRC and modified DFRC procedures were identical to those reported in the literature (*9,12*).

Thioacidolysis Procedure. The thioacidolysis and modified thioacidolysis procedures were performed as described elsewhere (*5,14*).

Gas Chromatography analysis. Monomeric products produced from the DFRC and thioacidolysis treatments were quantitatively determined by gas chromatography (GC) (Hewlett Packard 6890). The analytical column was a 30m x 0.32 mm i.d. HP-1, (Hewlett Packard). The carrier gas was helium with a flow rate of 2.0 mL/min. The GC conditions were as follows: injection temperature 220 ^{o}C with a split ratio of 10:1, FID detector temperature was 310 ^{o}C, the column temperature was 100 ^{o}C, which was held for 1 min, raised at 3^{o}C/min to 240^{o}C, held for 1 min, raised at 30 ^{o}C/min to 300 ^{o}C, and held for 5min. The amount of individual monomers were determined using response factors derived from pure compounds relative to tetracosane as the internal standard (*12,15*).

Sample Preparation. Prior to GPC, NMR and elemental analysis, reaction solvents were removed and the samples were exhaustively dried. Specifically, the solvent was evaporated at 40 ^{o}C and 20 mTorr reduced pressure until the presence of any solvent (e.g. acetic anhydride or pyridine in the case of DFRC) could no longer be detected. The samples were then placed in a vacuum dessicator and dried at 40 ^{o}C for

72 h. The samples were then ground to a fine powder and stored in a drying pistol over P_2O_5 and refluxing chloroform until analyses were performed.

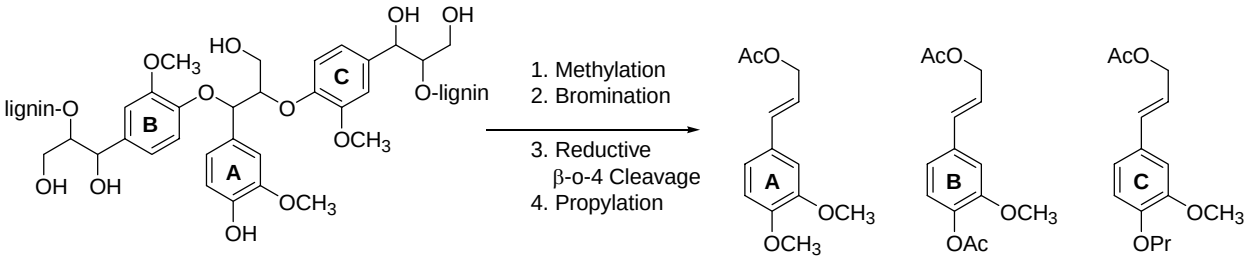
Gel Permeation Chromatography. GPC analyses were performed on a Waters HPLC system at ambient conditions using two μ-Styragel columns (HR-4 and 5E) connected in series. THF was the eluent (0.5 mL/min), and fractions were monitored using refractive index (Waters Refractometer Model No. 410) and UV absorbance at 280 nm (Waters UV Spectrometer Model No. 484). All lignins were analyses after excess reagent was removed by evaporation. The derivatized lignins and the DFRC and thioacidolysis product mixtures were dissolved in THF at a concentration of 1 mg/mL and 120 μL of this solution were then injected into the HPLC. Molecular weight determinations were made using polystyrene as a calibration standard.

Bromine Analysis. Bromine analysis was performed by Complete Analysis Laboratories, Inc. Parsippany, NJ.

Results and Discussion

Comparison of Monomer Yields from Thioacidolysis and DFRC Treatment of Milled Wood and MWL. Recently, we demonstrated that the DFRC method could be modified to provide additional structural information through the determination of three different structural monomeric products originating in lignin (*12*). In this protocol, the free phenolic hydroxyl groups in lignin are methylated by diazomethane followed by the same acetyl bromide treatment and zinc reduction steps as in the original DFRC procedure. In the final step, propionic anhydride, instead of acetic anhydride, is used to esterify the newly generated phenolic hydroxyl groups. Thus, the modified DFRC method allows the quantitative determination of three different monomeric units in lignin; the uncondensed phenolic β–*O*–4 (Unit A), the uncondensed α–*O*–4 (Unit B) and the uncondensed etherified β–*O*–4 (Unit C) structures (**Figure 1a**). While DFRC is a flexible method due to its three distinctly separate steps, thioacidolysis does not have this utility. Thioacidolysis follows a pathway similar to kraft pulping in which the thiol group displaces the α-hydroxy or α-ether group and the β-aryl ether to form an episulfide-type intermediate (**Figure 1b**). Because these reactions all proceed during the single thioacidolysis step, such derivatizations as in the modified DFRC method cannot be performed. Therefore, thioacidolysis can only differentiate between phenolic hydroxyl groups (Unit A) and aryl ethers, which will be referred to as Units B+C. The mechanism by which thioacidolysis and DFRC degrade various lignin structures has been thoroughly investigated using specific lignin model compounds (*7,16*). In both thioacidolysis and DFRC the predominant β-aryl ether structures in lignins are selectively degraded.

(a)



(b)

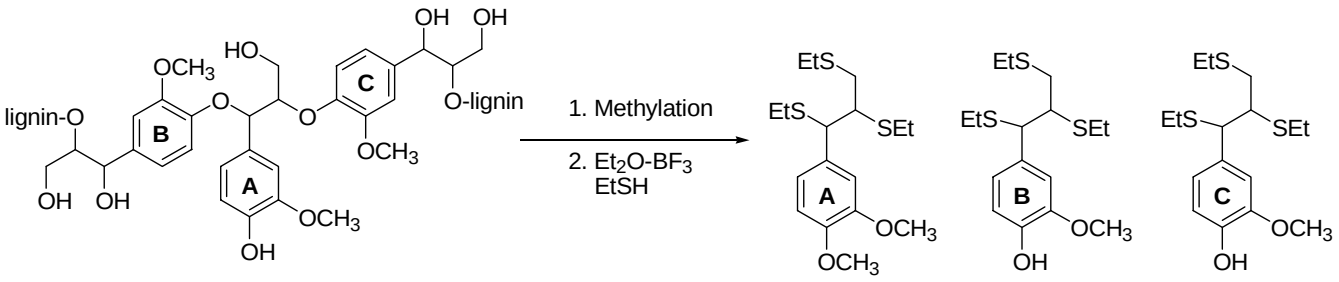


Figure 1. Modified (a) DFRC method; (b) thioacidolysis (Unit A: from phenolic β-O-4 units; Unit B: from α-O-4 units; Unit C: from etherified β-O-4 units) (12).

Each method releases monomeric products from an uncondensed portion of the lignin (i.e., a monomer linked by a β-*O*-4 linkage through both the β- and the 4-positions) or a β-*O*-4 linked monomer end group. Therefore, it is expected that the yield of monomeric degradation products detected would be comparable. However, **Table 1** shows that the modified thioacidolysis gives much higher monomer yields than the modified DFRC; 21.8 mol% vs 14.8 mol%, respectively for the vibratory milled wood; and 18.1 mol% vs 14.5 mol% respectively for the MWL. These results are in good agreement with literature values (*8-10,17,18*) and clearly indicate that the DFRC method is inefficient at cleaving β-aryl ether linkages.

Table 1. Total Yields and Unit Composition (mol %) Data for modified DFRC and Thioacidolysis analysis of Vibratory-Milled Wood and MWL.

	Modified DFRC				Modified Thioacidolysis		
	Unit Composition				Unit Composition		
	Unit A %	Unit B %	Unit C %	Total Yield mol %	Unit A %	Units B+C %	Total Yield mol %
Milled Wood	26.7	7.8	65.5	14.8	21.8	78.2	21.8
MWL	41.3	7.7	51.0	14.5	33.4	66.6	18.1

According to the reaction mechanism proposed for DFRC degradation of β-*O*-4 linkages, a *cis* orientation of the bromine and β-*O*-4 ether oxygen is required. The inability to obtain such geometry would preclude the reductive cleavage of the β-*O*-4 ether linkage and the α-brominated structure would persist. In fact Iiyama and Wallis (*19*) reported a 9% bromine content in the acetyl bromide dissolution of wood pulp (*Pinus radiata*). Likewise, Ralph et al (*20*) showed that cinnamyl model compounds on DFRC treatment gave rise to brominated aryl propanes. Bromine analysis of the DFRC degraded lignin revealed a 3 mol% bromine content. Another possible explanation for the ineffective cleavage of β-*O*-4 linkages by the DFRC method may be due to hydrolysis of the α-bromide on etherified phenylpropanoid units prior to reductive cleavage (*21*). Prolonged exposure of the lignin to AcBr, or the presence of water can facilitate the elimination of the α-bromo group, and the replacement with an α-OAc group.

Comparison of the yields of phenolic hydroxyl groups (Unit A) obtained from the modified DFRC and thioacidolysis reveals that the Unit A composition from the DFRC method is higher than from thioacidolysis for both the vibratory milled wood and the MWL. The percent increase for both samples is essentially the same, i.e., 18% for the milled wood and 19% for the MWL. This percent increase may be

explained by the fact that there are β-*O*-4 linkages remaining in the DFRC degradation product. These linkages are likely of the etherified β-*O*-4 type (Unit C), rather than end groups (Unit A). Therefore, complete cleavage would result in a higher relative Unit C composition and a lower relative Unit A composition.

Effect of Thioacidolysis and DFRC on the Molecular Weight Distribution of Lignin. Figure 2 shows the GPC chromatograms of the MWL before and after thioacidolysis and DFRC treatment. Comparison of the chromatographs of the two treatments reveals substantial differences. Thioacidolysis has much more completely degraded the lignin than DFRC. As can be seen in **Figure 2**, thioacidolysis degraded the lignin polymer to a significant extent and it no longer has any resemblance to the initial MWL molecular weight profile. The GPC chromatogram shows the presence of three sharp peaks, which calibrated against polystyrene standards are below an average relative molecular mass of 700 Da. The first peak is centered near 650 Da and is probably comprised of dimeric material. The second is centered near 350 Da and probably represents the uncondensed monomer released from aryl ether cleavage. The final peak is centered near 250 Da and represents other monomeric material. This observation implies that thioacidolysis caused significant depolymerization of the lignin and that a significant number, if not all of the β-*O*-4 inter-unit linkages have been cleaved. Again, as with the DFRC protocol, incomplete cleavage could occur in lignin thioacidolysis if the method is not carefully optimized (*22*).

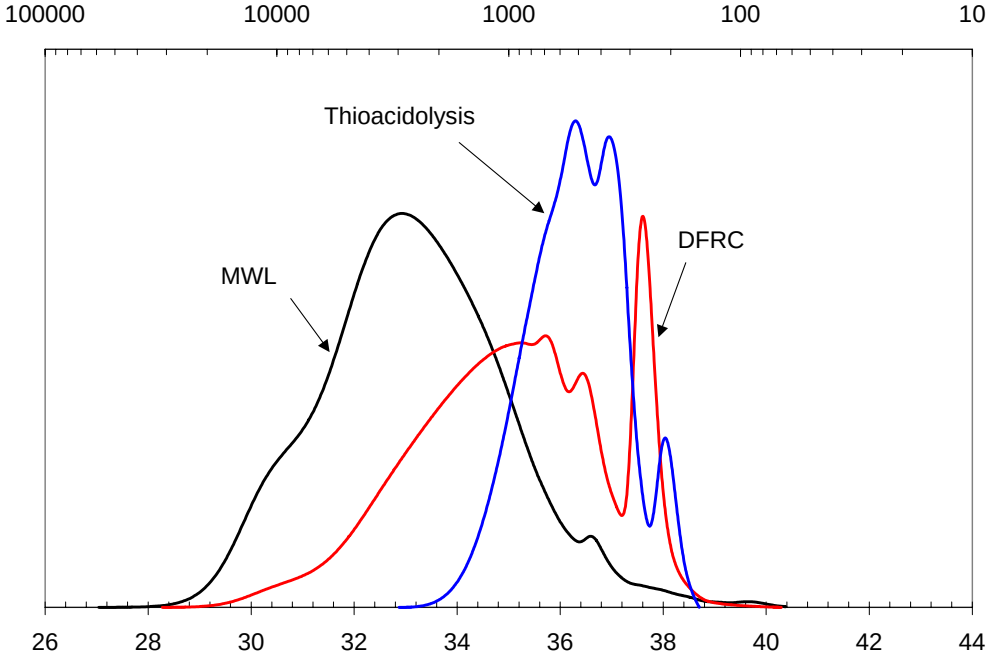


Figure 2. GPC chromatographs of MWL before and after thioacidolysis and DFRC treatment.

By comparison, the DFRC treated MWL exhibits a GPC trace that maintains much of the characteristics of the original MWL curve. Like the thioacidolysis treated MWL, the DFRC treated MWL has two peaks that are representative of low relative molecular weight moieties, monomers and dimers. However, a large amount of high relative molecular weight highly polydispersed material similar to the initial MWL is still present. Probably due to incomplete β-aryl ether cleavage, these findings are consistent with and support the lower monomer yields detected by GC analyses from DFRC degradation relative to thioacidolysis. In addition, the lower relative incidence of monomers from etherified β-*O*-4

linkages (Unit C) verify that the decrease in yield is attributable to internally located moieties that remain uncleaved, and result in the observed higher degree of polymerization. It should be noted that the relative intensities of the monomeric peaks by no means imply concentration. The UV absorbance will be dependent on the compound structure, with compounds having double bonds having stronger absorbance than those without or the higher relative molecular weight fractions.

Thioacidolysis of milled wood and MWL produced 7 mol% and 3.5mol%, respectively, higher monomer yields than DFRC. GPC analysis revealed the thioacidolysis treated lignins were degraded to a lower average molecular weight than by DFRC. In fact, the DFRC treated MWL retained much of the characteristics of the original lignin. In addition, the DFRC treated MWL had a ~3 mol% bromine content. Contrary to results reported for lignin model compounds, these findings indicate that the DFRC method does not completely or efficiently degrade the lignin polymer. The presence of elemental bromine within the lignin, combined with the existence of β-*O*-4 inter-unit linkages and the high average relative molecular weight, suggests the DFRC treatment of the lignin may be affected by the rigid 3-dimensional structure of the lignin macromolecule; restricted rotational mobility of certain C9 units precludes the formation of the necessary geometry to enable reductive cleavage of all of the β-*O*-4 inter-unit linkages. As a result

Thioacidolysis of families with extreme a-cellulose content. As shown in the previous section chemical analysis of the various wood samples showed that families differed significantly for all the chemical and morphological wood properties at the probability level of <0.001. There was considerable variation among families for fiber coarseness as well as fiber length, and a large variation was observed between family means for alpha cellulose content, which ranged from ~38.8 to ~43.3 %, with families D and I having the highest value and family N the lowest, whereas very little variation was found among families for lignin, varying between 29.1% for family E and 30.7% for family K. It was quite surprising that lignin content did not vary between families despite the large variation in alpha cellulose content. However, as important as low lignin content is, lignin structure is of equal importance. That is, if the lignin structure consists of highly condensed inter-unit linkages, which are not responsive to conventional processing technologies, these trees will not be useful. Therefore, chemical analysis of extremes in alpha cellulose content, i.e. families N (low alpha cellulose) and I (high alpha cellulose) were analysed using thioacidolysis. **Table 2** shows the results obtained from thioacidolysis of families N and I. Thioacidolysis shows that the amount of monomeric units released are essentially the same (within experimental error) between the two families ~ 1100 umol/g. This implies that both families likely have a similar amount of condensed linkages. As a pre methylation step was utilized, differences in uncondensed phenolic units (A) can be seen; slightly lower phenolic unit (unit A) content can be seen for family N as compared to family I.

Table 1. Thioacidolysis results for the high and low alpha cellulose content families (6-10 samples per family were tested and run in triplicate)

sample	Average		
	Thioacidolysis		
	Unit A ,%	Unit B+C%	total yield, umol/g
N	23.6 ± 0.95	76.4 ± 0.95	1177.8 ± 45.4
I	26.0 ± 0.68	74.0 ± 0.68	1071.1 ± 84.0

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CORRELATION OF GENE EXPRESSION IN DIFFERENTIATING XYLEM WITH SPECIFIC CHEMICAL PROPERTIES OF WOOD IN LOBLOLLY PINE (*PINUS TAEDA* L.)

Summary

Methods have been developed for the correlation of transcript level for specific genes with the chemical properties of wood on a population level in loblolly pine. To do this we have carried out microarray analysis for 2107 specific cDNAs and carried out chemical analysis for alpha-cellulose, hemicellulose and lignin on a set of 52 trees from the NCSU loblolly pine breeding program. We have used correlation analysis to identify specific genes whose transcript levels are positively or negatively correlated with specific wood properties or specific metabolites. Many significant correlations are detected. The correlations may be due to metabolic relationships, genetic linkage, or due to developmental or adaptive variation in the specific population and environment. The 10 most significant gene-trait associations for each trait, both positive and negatively correlated, are presented in Tables III-X. Microarrays provide a powerful tool for the investigation of molecular phenotypes in a breeding population, and these results are an important first step to demonstrate feasibility, identify genes of interest, and guide further experiments. Methods were developed for genetic mapping of sites regulating transcript level during wood formation and for the correlation of metabolites with gene expression during wood formation. These results provide a molecular basis for complex quantitative traits where the action of many genes contributes to the phenotype.

INTRODUCTION

The physical and chemical properties of wood must be the consequence of the organization and composition of the cell walls of the tissue formed by terminal differentiation during xylogenesis. In turn, the properties of the cell walls must result from the expression and products of specific genes that provide the organization and materials for the formation of the wood cell walls (1). The purpose of this work is to identify specific genes that determine the variation in wood properties so that these genes may be used to improve selection during breeding or for directed genetic manipulation to modify wood properties.

Our experiments have been carried out on loblolly pine because it is the most important commercial species of southern pine, and is the most intensively studied gymnosperm. Loblolly pine serves as a model system for gymnosperms and woody plants in spite of the technical disadvantages of size, generation time and genome size (2). Although much information on gene expressions during xylogenesis will come from model plant systems such as *Arabidopsis* and *Zinnia* (3-8), the information needed to select or modify wood properties must also be based on the unique biology of gymnosperms and breeding populations loblolly pine itself.

In the past decade, new genomic technology has made it possible to isolate and identify tens of thousands of genes expressed during wood formation (9-11) and to demonstrate a high level of homology between these genes and those inferred from the genome sequence of *Arabidopsis* (12,13). The identification of large numbers of expressed genes has led to the development of massively parallel gene expression analysis where thousands of genes may be analyzed simultaneously by molecular hybridization on glass slides (gene chips) to determine the level of transcript associated with specific genes (14). The availability of cDNAs for genes expressed during wood formation has led to the development of microarrays for loblolly pine that provide information on variation in gene expressions during xylogenesis, due to variation in genotype, developmental stage, or environmental influence.

A major advance in the application of this technology has been the use of quantitative genetic methods to examine variation in transcript level (15-20). In this way, it is possible to treat the transcript level of each gene as a quantitative trait, and to dissect the components of variation due to genotype or environment. It is also possible to use transcript level variation to map regulatory sites, discover

coordinate control, and to estimate the components of epistasis (15, 18-23). Correlate the variation in the expression of specific transcripts with phenotype have identified candidate genes that have a major role in the determination of wood properties in a hybrid population of *Eucalyptus* (15).

Therefore, we have begun experiments to test this approach in breeding populations of loblolly pine, to identify genes whose transcript level variation is associated by correlation analysis with variation in wood properties in loblolly pine. We expect this to be a technical challenge because of the levels of genetic variation within populations of loblolly pine is substantially less than that of a *Eucalyptus* hybrid. The limits of resolution of microarray analysis, and the limited availability of appropriate experimental material contribute to the challenge.

In this report we demonstrate the first application of quantitative analysis of transcript level in breeding populations of loblolly pine and the correlation of specific transcript levels with the content of cellulose and lignin. We are able to define transcript level variation associated with levels of cellulose, hemicellulose lignin and related metabolites. The purpose of these experiments has been to demonstrate feasibility, to direct our attention to new genes of interest that determine or regulate wood properties, or to identify genes that may serve as markers that could predict wood properties.

MATERIALS AND METHODS

Plant material.

Four loblolly pine families were selected from a six parent half-diallel located at Rock Hill, South Carolina (site 2). Two families were chosen for their high cellulose contents and two families had low cellulose contents (Table 1). A total of 52 individuals were analyzed from the four families. The families and individuals also were analyzed for their lignin content (Table 1). The trees were 10 years old when sampled for wood forming tissue. Wood chemistry was measured for different years using the appropriate locations in the seasonal rings.

Chemical analysis of wood properties

Extractives are removed from increment cores with acetone and specific rings and regions are assayed. Lignin content was estimated by a micro acetyl bromide method (24). The holocellulose is fiberized using a micro blender, washed and dried, and the content is determined gravimetrically. Oven dried holocellulose is equilibrated with moisture and hemicelluloses are extracted by 17.5% NaOH. The product is filtered, washed and dried (24) and the α-cellulose content is determined gravimetrically.

Sample collection and RNA preparation

Differentiating xylem was collected in May 2003, by cutting away a 4 inch by 8 inch window of bark and phloem. Rapid scrapping of differentiating xylem was carried out within 30 seconds and the tissue frozen in liquid nitrogen, followed by storage at minus 80^{o} C. We have extracted RNA from all of the 52 genotypes. Xylem tissue from each tree (about 300 mg) was ground to powder in liquid nitrogen using a mortar and pestle. RNA was extracted using a combination CTAB extraction buffer is heated to 65^{o}C (25) 30 ul of beta-mercaptoethanol is added, the solution is vortexed briefly, and held at 65^{o}C for 5 min. An equal volume of chloroform/isoamyl alcohol (24:1) is added, vortexed, and centrifuged to separate the phases. The extraction is repeated twice and the final supernate was placed in a QIAshredder column. The remaining steps are carried out according to the manufacturers instructions. The final product is resuspended in 30ul of RNase free water, and a sample is run on 1% agarose to determine size. Concentration and purity was monitored by UV absorbance at 260/280 nm.

Labeling, hybridization and image scanning

RNA is converted into cDNA and amplified as double stranded DNA using a SuperSMART™ PCR cDNA Synthesis Kit (Clonetech, Palo Alto CA). This procedure has been optimized for small amounts (500 to 900 ng) of starting total RNA. The resulting double stranded cDNA was labeled using

fluorescently labeled nucleotides and hybridized. 20µl of cDNA solution was diluted in 48 µl of water, denatured at 95°C for 5 min, quenched on ice, then 20µl of either dUTPCy3 or dUTPCy5 solutions (30 µM), followed by 10µl hexanucleotide mix ((Roche Molecular Biochemicals, Indianapolis IN) and 10 units of Klenow fragment (Amersham, Piscataway, NJ). The mixture uses the recommended buffer for Klenow fragment, and is incubated at 37°C overnight. Paired Cy3 and Cy5 samples are combined and purified using the PCR purification kit from Qiagen. Labeled target (80µl) in 50% deionized formamide was applied to slides and placed in Corning microarray slide chambers and incubated overnight at 42°C. The first post hybridization wash is 1XSSC, 0.2%SDS at 42°C for 4 min, followed by 0.1XSSC, 0.2%SDS (4 min), and finally 0.1XSSC (3X 4 min). Slides are dried and scanned using a ScanArray 4000. Gridding was carried out n raw intensities using Quantarray (GSI Lumonics Moorpark, CA).

cDNA selection and annotation

A *Pinus taeda* unigene set of cDNAs containing 2,109 unique ESTs were selected containing genes associated with wood formation. ESTs were selected from contigs, and rpresent relatively abundant transcripts in differentiating xylem. A number of contigs were selected that gave no hits in gene databases, because they might be novel or unique genes in pine or in wood formation. Within each contig we select an EST that would have the highest potential of being gene specific, chosing short ESTs that had their 5' end closest to the 3' end of the transcript, based on the University of Minnesota web site (http://web.ahc.umn.edu/biodata/nsfpine). Genes on the current array represent 17 different functional categories, based on homology to inferred genes of *Arabidopsis thaliana* (11,26).

Array construction

For probe preparation and printing, glycerol stocks were diluted and clone inserts were PCR amplified in 50 µl reactions in 96-well reaction plates using M13 forward and reverse universal primers. Each 50 µl reaction contained 39.1 µl ddH_2O, 5 µl 10x PCR buffer containing 15 mM $MgCl_2$ ((Roche Molecular Biochemicals), 1 µl dNTPs (10mM each), 1 µl each M13 forward and reverse primers (10 µM), 0.4 µl TAQ polymerase (5U/µl) (Roche Molecular Biochemicals), and 2.5 µl of 10X diluted glycerol stocks. Amplifications were done in a MJ Research thermocycler (Waltham, MA). Denaturation was performed at 95 °C for 5 minutes, primer annealing at 57 °C for 1min and chain elongation at 72 °C for 4 min for 35 cycles. Final chain elongation took place at 72 °C for 10 min. PCR products were viewed on 1.0 % agarose gels, stained with ethidium bromide, under UV light. Amplifed cDNA was purified using 96-well multiscreen filter plates (Millipore Corp. Bedford, MA) and re-dissolved in 40 µl H_2O. Dimethyl sulfoxide was added to 50% for spotting and PCR products were printed on CMT™-GAPS-II aminosilane-coated glass microscope slides (Corning, Corning, NY, USA) using a LUCIDEA printer (Amersham, NJ). After printing, slides were dried and spotted cDNA was UV-crosslinked (250 mJoules) using a Stratalinker® (Stratagene, LA Jolla, CA, USA). Slides were then baked at 75 °C for 2 hours and stored at room temperature.

Experimental design and analysis

A loop design (27) was used to compare all 52 individuals in these families in pair-wise comparisons on microarrays, pairing the highest and lowest individuals on the same arrays to maximize the power of the comparisons. The loop design allows all pair-wise comparisons to be made and to compare gene expression for other traits in future comparisons. A replicate set of RNAs were purified, hybridized and analyzed from 6 individuals within the original 52 as a pilot experiment with paired dye swap tests within a small loop representing technical replicates. Data shown here represents results from the combined analysis. Analysis of variance (ANOVA) methods for microarray provides more robust statistical assessment of differences in gene expression (28,29). These approaches permit much more flexibility in the experimental design, and generate statistical measurements (p-values), which describe

the significance of observed differences in gene expression. The procedure described by Wolfinger et al. (29) and Jin et al. (30) is based on two sequential ANOVA models. The first step of the analysis is a global normalization that corrects for effects of slide, dye, and slide-dye interactions. The residual from this model is applied in a second analysis (gene model) to carry out statistical inference for each gene.

Normalization Model

$$y_{dsa} = \mu + D_d + A_a + (DA)_{da} + \varepsilon_{dsa}$$

Where: y_{dsa} is the $\log_2$ of the intensity measurement for dye d at spot s on array a. μ represents the overall mean value. D is the main effect for dye. A is the main effect for array. DA is the interaction effect of arrays and dyes, and ε is stochastic error. Because the array and dye are sufficient to identify a tree we do not include a treatment (tree) effect in the model.

The SAS PROC MIXED statements used for normalization are:

```
class array dye;
model log2i=dye;
random array dye*array;
```

Gene Model

$$r_{gats} = \mu_g + \beta_g X_t + A_{ga} + S(A)_{gas} + \varepsilon_{gtas}$$

All effects in this model are indexed by g, the gene identifier, indicating that we are now working at the gene level. X represents the value of the trait being modeled (e.g. lignin). We include the $S(A)_{gas}$ effect to account for variation between spots on one array which have the same gene (technical replicates within the array). β is the slope of the correlation of the variation in gene expression and the trait of interest.

The SAS PROC MIXED statements used for the gene model are:

```
class array spot;
model log2i=value;
random array spot(array);
estimate "Slope" value 1;
```

Where array is an identifier for each array, spot is a spot number within each array, and value is the value for the trait in question.

Metabolite profiling:

The profiling method on xylem tissue was conducted according to Morris et al 2004 (37). Metabolite data was normalized based on the experimental design, utilizing site, clone, and site by clone. During the normalization process, site was treated as a fixed effect to remove environmental effects. The new normalized values were applied in the principal component analysis (PCA). Based on the PCA results, there were distinct clusters created for samples from the top and bottoms of the tree. However, there was no distinct clustering on the basis of site and clone. Statistical analysis used principal component analysis in SAS JMP 4.04 and analysis of variance using SAS 8.02.

Correlation of gene effects and wood chemistry phenotypes

The level of expression for each gene on the array was estimated for each tree and then a correlation (slope) was calculated for each gene with the chemical determination of wood for alpha cellulose, hemicellulose and lignin content. We used trait value as a continuous variable in the mixed

model. We ran the mixed model to obtain estimates of transcript level for each gene. We selected significant genes based on the application of q value approach of Storey and Tibshirani (31) for a false discovery rate of 0.001. We obtain a correlation of genes and traits from the mixed model, and a p value for the data under the null hypothesis that give us confidence that the correlations are real (Tables III-VIII).

RESULTS

These results provide the first information on correlation of wood property phenotypes with gene transcript level for individuals from breeding populations of loblolly pine. Correlation of gene expression of trees in populations has been a significant technical and conceptual challenge. It was not previously known whether the variation within populations was sufficient to detect effects and it was also not known if the precision of the method would be adequate to detect it. The precision of microarrays is now at a level where population effects can be examined. To do this requires applying the concepts of quantitative trait analysis (32) where continuous variables can be analyzed for genetic and environmental effects. We find the technical and theoretical application of quantitative microarray analysis provides a rich source of information about the populations and gene expression associated with wood phenotypes.

The families used in this analysis were selected because they show high variation between families for average cellulose content. We have also determined the lignin content and estimated hemicellulose content as well. We have done this because there is a substantial variation within families for all three traits, although the differences between families for lignin content are not significant, for example.

Significant effects of transcript level variation

Large numbers of genes show significant changes of transcript level assayed by microarrays (Table II). A major problem in understanding wood formation and the genetic basis of wood properties, is that many abundant genes expressed during wood formation are of unknown function. The genes showing significant effects on transcript level may be the result of several factors. We expect that some of the responses are due to primary effects in the developmental program of wood formation, or on related events in control of the metabolic pathways involved in precursor biosynthesis. Correlation may come from pleiotropic effects, based on biochemical or developmental effects that are downstream from the primary events. Correlated responses may also come from genetic linkage, where alleles that have increased transcript levels are linked and only one of them has a functional relationship to the phenotype

Table I. Mean values for cellulose (□lpha-cellulose) and lignin.

Family	Number of Individuals	Mean Alpha-Cellulose Yield (%)± SE	Mean Lignin Content (%) ± SE
D (1590x1612)	10	44.4 ± 0.46 high	28.9 ± 0.17
I (1612x1649)	19	42.3 ± 0.45 high	29.8 ± 0.17
N (1634x1652)	14	38.8 ± 0.37 low	30.8 ± 0.21
G (1626x1634)	9	39.8 ± 0.32 low	29.3 ± 0.21

Table II. Number of significant gene effects.
Number of significant genes up regulated or down regulated and correlated with alpha-cellulose, hemicelluloses, and lignin content. Numbers represent combined data from two experiments. Significance was based on a q value of 0.001 (31). P-value of 0 means the p value was less than 0.000000001.

	Genes correlated With trait	Up regulated	Down regulated	% genes of unknown function
Alpha-cellulose	265	104	161	37
Hemicelluloses	86	25	61	33
Lignin	101	75	26	33

. Examination of Tables III-VIII show effects on transcript levels of many genes. Correlations of gene transcript level and phenotype are often as much as 0.3, which suggests a strong relationship. These relationships may 'explain " substantial proportions of the phenotypic variance. The large number of genes of small effect also suggests interactions or epistasis, but analysis for such effects has not yet been conducted. Significant effects are found for many genes associated with cell wall biosynthesis, but many effects are found for genes encoding enzymes in metabolism and energy relationships. Variaton in transcript level of transcription factors are also apparent. Alternative members of gene families are differentially expressed as one may be up regulated for cellulose, while another member of the family may be up regulated for lignin. Cellulose synthase clones show this effect.

Association of gene expression with abundance of specific metabolites:

We have used the data from microarrays and metabolite profiles to look for correlated effects of gene specific transcripts and abundant metabolites. Data is obtained from differentiating xylem, cambial scrapings collected in 2003 and analyzed as described previously for metabolites and for transcript levels for 2109 genes expressed during wood formation. The results in Table III show correlations for 12 abundant metabolites and 728 specific gene effects.

Table III. Genes identified by correlation with specific metabolites. Normalized levels of gene expression determined on an individual tree basis are correlated with the levels of specific metabolites using a q test criteria. Metabolite significant genes ($p < 0.05$).

Metabolite	Up regulated	Down regulated
Coniferin	70	58
B-D-pyranose	11	12
Citrate	11	12
D-fructose	56	68
D-glucose	63	68
Inositol	29	41
Malate	16	20
Ononitol	56	23
Pinitol	21	26
Quinate	12	4
Shikimate	19	10
Sucrose	14	8

As an example, the significant genes identified are for sucrose level are shown below a t a *p value* of less than or equal to 0.05 after correction for multiple tests. The first column is the slope of the correlated effect and the second column is the individual p value. Of interest are two putative genes, one implicated in sucrose metabolism (sucrose synthase) and a lignin biosynthetic gene (cinnamyl alcohol dehydrogenase).

Mapping using microarrays:

Methods have been developed for mapping using microarrays. The methods use the quantitative variation present between alleles that are heterozygous in parents and are assayed quantitatively. Relatively small differences can be used if sufficient progeny are available in a mapping population. A pre-exisiting genetic map is required. The method is analogous to QTL mapping and uses the same software as phenotypic QTLs. In our most commonly used tree species, the level of variation in expression between alleles allows for about 10% of genes to be mapped with this method in any particular intra- species cross. In interspecies comparisons, the variation is about double, and larger numbers of loci can be mapped. These QTL localizations are useful to map the coding sequences of specific genes, but also can map regulators of transcript level. To distinguish these alternatives, two approaches are followed. Master regulators control more than one gene, often in a coordinately regulated pathway. For example, many genes in the lignin biosynthetic pathway may be regulated by a master QTL. In such cases, the location of the structural gene is different from that of the site of the regulator.

Methods for the mapping using microarrays, may now be designed using short oligo arrays, where oligos contain alternative sequences for different SNP alleles. The principle is the same as oligo arrays made commercially, and depends on specific association of cDNA with short oligos. The technology has advanced so that SNP based mRNA mapping is a demonstrated technology. It has he advantage of being able to map the site of the coding sequence and to locate variation in regulators at other sites in the genome. Testing and application of this method for mapping populations of trees will depend on future experiments.

DISCUSSION

These results provide the basis for investigations of the genetic basis of wood property phenotypes and the identification of genes that determine specific wood properties in specific individuals and breeding populations. The results show effect on a diverse set of genes with many functional associations. It comes as no surprise that the genetic variation underlying complex traits such as wood properties may be complex. Analysis of microarray data from the perspective of quantitative traits provides useful technology for analysis and useful models for understanding complex molecular processes. Growth rate is also complex trait and may affect the average density of wood. The quantitative variation in wood properties may be related to competition for carbon flow into lignin, cellulose and hemicelluloses, and the competition between cell division and differentiation. Lignin, cellulose and hemicelluloses are the major carbon sinks in the formation of the wood cell wall (1, 33) Lignin biosynthesis requires the formation of monolignol precursors from aromatic amino acids synthesized in the plastids. Cellulose is derived from sucrose and hexoses are the precursors for hemicelluloses. Sucrose, derived from the phloem, is transported into the cytosol of wood forming cells while glucose and fructose are derived from sucrose through invertase action (34). Sucrose also stimulates cell division through activation of cyclins (35,36). Although growth may be controlled at least in part by sucrose, there are many ways the composition of the walls might be regulated. Our further work will attempt to correlate specific metabolites associated with cell wall biosynthesis with transcript level and wood properties.

CONCLUSIONS

1. Transcript level variation between individuals and families of loblolly pine may be estimated with high statistical precision.
2. Transcript level of specific genes may be associated with wood properties, particularly, cellulose content, hemicellulose content and lignin.
3. Hundreds of genes may contribute to the variation of these traits, however, major effects may be correlated with a small number of genes.

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TABLES

Table IV: The ten most significant examples of differential gene expression positively correlated with alpha cellulose content. The identifier for the cDNA clone is the clone ID. The slope shows the correlation of the trait and the transcript level of the gene, and the p-value is the estimate of the probability that the correlation is due to chance. The p value is the probability that this result could be obtained if there were no correlation.

Clone ID	Annotation	Slope	p-value
NXCI002C10	putative dehydrin	0.079	6E-009
NXSI007B11	cellulose synthase catalytic subunit	0.064	5E-009
NXSI103A01	predicted GPI-anchored protein	0.061	0
ST19E08	nonspecific lipid-transfer protein precursor - like	0.06	7.18E-007
NXSI089H07	putative nonspecific lipid-transfer protein	0.059	6.23E-007
NXCI093F03	anther development protein	0.049	7.1515E-005
NXSI130F05	caffeoyl-CoA O-methyltransferase - like protein	0.048	3.8442E-005
NXCI076A10	H+-transporting ATP synthase chain 9 - like	0.048	8E-009
NXCI054A10	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase	0.046	0
ST21H08	L-ascorbate peroxidase	0.046	1.36E-007

Table V: The ten most significant examples of differential gene expression negatively correlated with alpha cellulose content.

Gene id	Annotation	Slope	p-value
NXCI096G01	putative oxido reductase (at1g65560)	-0.134	0
NXNV158A10	coatomer-like protein	-0.124	0
NXSI036A10	putative coatomer epsilon subunit	-0.113	0
ST01E09	putative actin-depolymerizing factor	-0.109	0
NXLV105E07	cellulose synthase catalytic subunit	-0.102	1E-009
NXNV055G05	putative coatomer complex subunit	-0.087	1.4E-008
NXSI066B08	14-3-3 protein epsilon	-0.083	1.823E-006
NXSI096H09	60S ribosomal protein L39	-0.081	6E-009
NXSI115C08	importin alpha	-0.079	8.435E-005
NXSI125H09	beta tubulin	-0.075	6.6733E-005

Table VI: The ten most significant examples of differential gene expression positively correlated with hemicellulose content.

Gene id	Annotation	Slope	p-value
ST01E09	putative actin-depolymerizing factor	0.116	2.4918E-005
NXSI115C08	importin alpha	0.109	1.1487E-005
ST06H05	lectin - like protein	0.082	2.313E-006
ST23G07	putative auxin-induced protein	0.082	3.6475E-005
NXSI100C03	heat shock protein 21	0.076	1.11E-007
ST04F03	PHD-finger protein	0.064	3.45E-006
ST31B03	putative auxin-induced protein	0.06	8.926E-006
ST06C03	putative NADPH oxidoreductase	0.041	3.026E-006
ST02B03	cinnamyl alcohol dehydrogenase -like protein	0.037	8.81E-007
NXSI080E08	putative auxin-induced protein	0.036	1.17E-007

Table VII: The ten most significant examples of differential gene expression negatively correlated with hemicellulose content..

Gene id	Annotation	Slope	p-value
NXCI093F03	anther development protein	-0.129	7.2E-008
ST19E08	nonspecific lipid-transfer protein precursor - like	-0.123	5.4E-008
NXSI089H07	putative nonspecific lipid-transfer protein	-0.122	8.3E-008
ST15D07	pollen-specific protein - like	-0.106	5.933E-006
NXSI130F05	caffeoyl-CoA O-methyltransferase - like protein	-0.103	8.363E-006
NXSI103A01	predicted GPI-anchored protein	-0.102	0
NXSI007B11	cellulose synthase catalytic subunit	-0.101	3.172E-006
NXSI053G04	alpha galactosyltransferase-like protein	-0.086	2.236E-006
NXNV068B08	40S ribosomal protein S30 homolog (emb|CAB79697.1)	-0.073	1.889E-005
NXNV086B04	pectinesterase like protein	-0.069	8.7E-008

Table VIII: The ten most significant examples of differential gene expression positively correlated with lignin content.

Gene id	Annotation	Slope	p-value
NXLV105E07	cellulose synthase catalytic subunit	0.317	5.8E-008
ST01E09	putative actin-depolymerizing factor	0.3	0
NXNV055G05	putative coatomer complex subunit	0.277	1.96E-007
NXSI125H09	beta tubulin	0.276	1.91E-007
NXNV133D04	ERD3 protein (ERD3)	0.267	9.78E-006
NXSI066B08	14-3-3 protein epsilon	0.26	2.1611E-005
NXCI132E09	putative MYB family transcription factor	0.259	1.6E-007
NXNV149G02	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	0.256	1.87E-007
NXNV066E09	NAD(P)H oxidoreductase	0.256	2.884E-006
NXSI036A10	putative coatomer epsilon subunit	0.243	1.4416E-005

Table IX: The ten most significant examples of differential gene expression negatively correlated with lignin content.

Gene id	Annotation	Slope	p-value
NXSI101E11	histone H2A - like protein	-0.181	0
NXCI048F02	putative LEA protein	-0.142	2.751E-006
NXPV055E04	beta-glucosidase -like protein	-0.142	0
ST21H08	L-ascorbate peroxidase	-0.133	1.2323E-005
NXSI116A11	adenylate translocator	-0.13	6.404E-006
NXSI041H12	putative ribosomal protein S4	-0.116	1.7618E-005
NXCI054A10	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase	-0.114	4.436E-006
NXNV015H07	putative calmodulin	-0.111	1.1927E-005
NXSI002G12	L-ascorbate peroxidase	-0.101	7.027E-006
NXCI056E09	superoxidase dismutase	-0.088	1E-009

Table X: The ten most significant examples of differential gene expression positively correlated with sucrose content.

Clone ID	Annotation	Slope	individual p value
ST13F05	no hit – unknown sequence	0.448	2.8E-008
ST12G02	unknown protein	0.337	3.27E-007
NXCI155E06	transketolase - like protein	0.282	4.7E-007
NXSI103A01	predicted GPI-anchored protein	0.28	5.63E-007
NXSI117C08	no hit unknown sequence	0.262	1.837E-005
NXCI106C10	putative sucrose synthetase	0.254	1.045E-006
ST14B10	heat shock protein 18	0.241	1.493E-006
NXSI112B07	delta tonoplast integ. prot.	0.234	6.82E-007
NXCI118F05	SAR DNA-binding protein - like	0.206	1.1246E-005
ST02B03	cinnamyl alcohol dehydrog. -like	0.131	6.256E-006

Table XI: The ten most significant examples of differential gene expression negatively correlated with sucrose content.

Gene ID	Annotation	Slope	p-value
NXSI109B06	60S ribosomal protein L37A like	-0.117	6.4616E-005
NXSI037F05	Ca-dependent memb-bind. annexin	-0.128	2.1135E-005
ST02E09	cytoplasmic aconitate hydratase	-0.138	4.9E-008
NXSI104E11	ornithine aminotransferase	-0.146	9.682E-006
NXRV132G06	putative glucosyltransferase	-0.146	6.8526E-005
NXLV022H09	cytoplasmic aconitate hydratase	-0.154	6.1226E-005
NXRV128C10	PRL1 protein	-0.166	1.5449E-005
NXSI113E06	xyloglucan endo-1	-0.223	7.2188E-005
NXNV096C09	glutamine-dep. asparagine synth.	-0.241	1.3754E-005
NXCI018F10	putative NADPH oxidoreductase	-0.267	2.2061E-005

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GENETIC VARIATION AND GENOTYPE BY ENVIRONMENT INTERACTION OF JUVENILE WOOD PROPERTIES IN *PINUS TAEDA* L.

Summary

Genetic variation and genotype by environment interaction (GxE) were studied in several juvenile wood traits of loblolly pine (*Pinus taeda* L.). Wood samples of 12 mm increment cores were collected from 11-year-old trees in four progeny tests. Juvenile (ring 3) and transition (ring 8) for each increment core were analyzed for α-cellulose and lignin content, average fiber length, and coarseness. Transition wood had higher α-cellulose content (46.1%), longer fiber (1.98 mm), and higher coarseness (0.34). but lower lignin (29.7%) than juvenile wood (cellulose 40.9%, fiber length 1.4 mm, coarseness 0.28 and lignin 30.3%). General combining ability variance for the traits in juvenile wood explained 3 to 10% of the total variance, whereas the specific combining ability variance was negligible or zero. There were noticeable full-sib family rank changes between sites for all the traits. This was reflected in very high specific combining ability by site interaction variances, which explained from 5% (fiber length) to 37% (lignin) of the total variance. Weak individual-tree heritabilities were found for cellulose, lignin content and fiber length at the juvenile and transition wood, except for lignin at the transition wood (0.23). Coarseness had moderately high individual-tree heritabilities both at the juvenile (0.39) and transition wood (0.30). Favorable genetic correlations of volume and stem straightness were found with cellulose content, fiber length and coarseness, suggesting that selection on growth or stem straightness would results in favorable response in chemical wood traits.

Introduction

Genetic improvement and intensive silviculture of loblolly pine (*Pinus taeda L.*) have increased forest plantation productivity significantly in the southern United States [1]. With improved growth, rotation ages have been reduced to about 20 to 25 years compared with 40 to 50 years in natural stands. Consequently, the percent of juvenile wood from plantations has increased [2]. Juvenile wood typically has less desirable wood properties than mature wood, e.g., low wood density, shorter tracheid length and higher lignin content. These wood properties are associated with low pulp yield and high pulping costs [3, 4, 5]. However, if there is large genetic variation in these juvenile wood properties in loblolly pine, it is possible to improve the juvenile wood for solid and chemical wood products through a breeding program.

Sykes *et al.* [3] reported large genetic variation in some chemical wood properties in loblolly pine. Considerable genetic variation in α-cellulose content, average fiber length, and lignin content have been reported in juvenile wood for loblolly pine and several other tree species [4-8]. However, there is little information on how genotypes interact with different environment for those chemical wood properties. It is essential to determine the magnitude of genotype by environment interaction (GxE) for a tree breeding program to be effective. The level of GxE would help tree breeders and practical foresters to decide how to effectively design breeding programs and how genotypes with desirable wood traits can be planted over different environments. This information would benefit tree improvement programs of loblolly pine and the pulp and paper industry by allowing selection and planting suitable trees on suitable sites. If GxE is deemed to be negligible, then selected genotypes could be used for plantations to produce uniform wood under different environmental conditions. This would increase yield, improve product properties and lower pulping costs [9].

This study was to determine genetic variation of key juvenile chemical wood properties in loblolly pine on four test sites and examines GxE interaction. Specific objectives were (1) to compare genetic variation in α-cellulose content (ACY), fiber length (FLW), coarseness (COA), and lignin content (LIG)

on four field test sites, (2) examine GxE interaction of chemical wood traits, and (3) study relationships between growth, stem quality, wood density with chemical wood traits.

MATERIALS AND METHODS

Materials and data collection

Fourteen full-sib families generated by a 6-parent half-diallel mating design were tested on four sites in the Piedmont of South Carolina. A randomized complete block design with six replications was used in the field. Each full-sib family was laid out in 6-tree row-plots in each replication. Wood core samples were collected when the trees were 11-year-old. Increment cores (12 mm) were taken from each tree at breast height (about 1.30 m above ground) using generator-powered drills. Wood cores having visible limbs, curves, resin pockets, compression wood, or rust infections were avoided. The samples were placed into sealed plastic storage bags and stored in coolers to retain moisture during the material collection.

The bark and cambium were removed from the wood cores, and the cores were split at the pith into two halves. Chemical analysis was done using microanalytical techniques developed by Yokoyama *et al*. [10], which allow the rapid characterization of fiber components and morphology of loblolly pine in a large number of samples. Briefly, the techniques involved are extractive removal, holo-cellulose preparation, α-cellulose and lignin content determination, and average fiber length and coarseness analyses. Nonvolatile extractives greater than 95% were removed from increment cores by four successive two-day acetone extractions [10]. Extractives were removed from all cores using the successive acetone method. The increment cores were then soaked in water overnight before ring samples were taken.

Within-core samples were taken from ring 3 and ring 8 to study chemical properties of juvenile wood (ring 3) and transition wood (ring 8), respectively. Thin wafers from ring 3 earlywood, ring 3 latewood, ring 8 earlywood, and ring 8 latewood were taken using a microtome. At least 300 mg of each sample were taken from the earlywood and latewood of each ring. Each sample was oven-dried for 12 hours [3]. Subsequent chemical analyses were carried on wafers.

Statistical analyses

Juvenile wood and transition wood were compared for micro wood traits using the paired t-tests. A general linear mixed model was fitted to data to estimate variance components for combined sites.

$$[1] \qquad \mathbf{Y} = \mathbf{X\beta} + \mathbf{Z\gamma} + \boldsymbol{\varepsilon}$$

where, $\mathbf{Y}$ is the vector of individual observations, $\boldsymbol{\beta}$ is the vector of fixed-effects parameters (overall mean, site and blocks within site), $\boldsymbol{\gamma}$ is the vector of random-effects parameters including general combining ability (GCA) for female and male, specific combining ability (SCA), GCA by site interaction, SCA by site interaction, plot-to-plot error. The $\boldsymbol{\varepsilon}$ is an unknown random error vector, $\mathbf{X}$ and $\mathbf{Z}$ are known design matrices for fixed and random effects, respectively. The $\boldsymbol{\varepsilon}$ and $\boldsymbol{\gamma}$ are assumed to have normal distributions with 0 mean and variances. The variance of the $\mathbf{Y}$ vector is $\mathbf{V}=V(\mathbf{Y})=\mathbf{ZGZ}^T+\mathbf{R}$ (Littell 1996). It is also assumed that $\mathbf{G}$ is the diagonal variance-covariance matrix of random effects and $\mathbf{R}$ is the variance-covariance matrix of random errors ($\mathbf{R}=\sigma^2\mathbf{I}_n$). The diallel genetic analyses were carried out using a SAS Proc Mixed approach (Xiang and Li 2001). Using variance components from the mixed model, individual-tree (h^2_i), half-sib family (h^2_{hs}), and full-sib family (h^2_{fs}) heritabilities were estimated for micro wood traits as follows:

$$[2] \qquad h^2_i = 4\,\sigma^2_g / (2\,\sigma^2_g + \sigma^2_s + 2\sigma^2_{gl} + \sigma^2_{sl} + \sigma^2_{plot} + \sigma^2_e)$$

[3] $h^2_{hs} = \sigma^2_g / [(p\sigma^2_g + \sigma^2_s + p\sigma^2_{gl} / t + \sigma^2_{sl} / t + \sigma^2_{plot} / tb + \sigma^2_e / tbn) 1/(p-1)]$

[4] $h^2_{fs} = 2\sigma^2_g / (2\sigma^2_g + \sigma^2_s + 2\sigma^2_{gl} / t + \sigma^2_{sl} / t + \sigma^2_{plot} / tb + \sigma^2_e / tbn)$

where, σ^2_g is the GCA variance, σ^2_s is the SCA variance, σ^2_{gl} and σ^2_{sl} are the GCA by site and SCA by site interactions, σ^2_{Plot} is the plot variance, σ^2_e is the residual variance, t is the number of sites (t=4), b is the number of blocks within sites (b=6), p is the number of parents (p=6) and n is the harmonic mean number of trees within plot (n=2.83). Standard errors of heritabilities were estimated by the Delta Method (Lynch and Walsh 1998).

Product-moment correlations were estimated among the micro wood, stem straightness and growth traits. Approximate genetic correlations among traits were calculated using individual-tree breeding values of traits. The magnitude of genotype by environment interactions was estimated by analyzing a pair of sites under the general linear mixed model given in equation [1]. Type B correlations for additive effects (r_{B_gca}) and dominance effects (r_{B_sca}) were estimated. A correlation coefficient close to 1.0 indicates no interaction, whereas small coefficients indicate significant rank changes among the genotypes from one location to another.

[5] $r_{B\_gca} = \sigma^2_g / (\sigma^2_g + \sigma^2_{ge})$

[6] $r_{B\_sca} = \sigma^2_s / (\sigma^2_s + \sigma^2_{se})$

Results

Comparison of wood types

Cellulose content (%), fiber length (mm) and coarseness increased from the earlywood to latewood within a ring (Figure 1). In contrast, lignin content (%) did not change. Transition wood (ring 8) had significantly (Pr>0.0001) greater percentage of cellulose, longer fiber and greater coarseness and less lignin than juvenile wood (ring 3) (Table 1, Figure 2). Transition wood had about 5.2% more cellulose than juvenile wood. The difference between two wood types for lignin was less than 1% and significant. Transition wood had about 0.58 mm longer fibers than juvenile wood. We observed two distinct variation patterns for the juvenile and transition wood (Figure 2). Variation in cellulose, coarseness and fiber length was greater in the transition wood than in juvenile wood. In contrast, variation in lignin content was similar for two wood types. Tough, lignin was less variable in transition than juvenile wood.

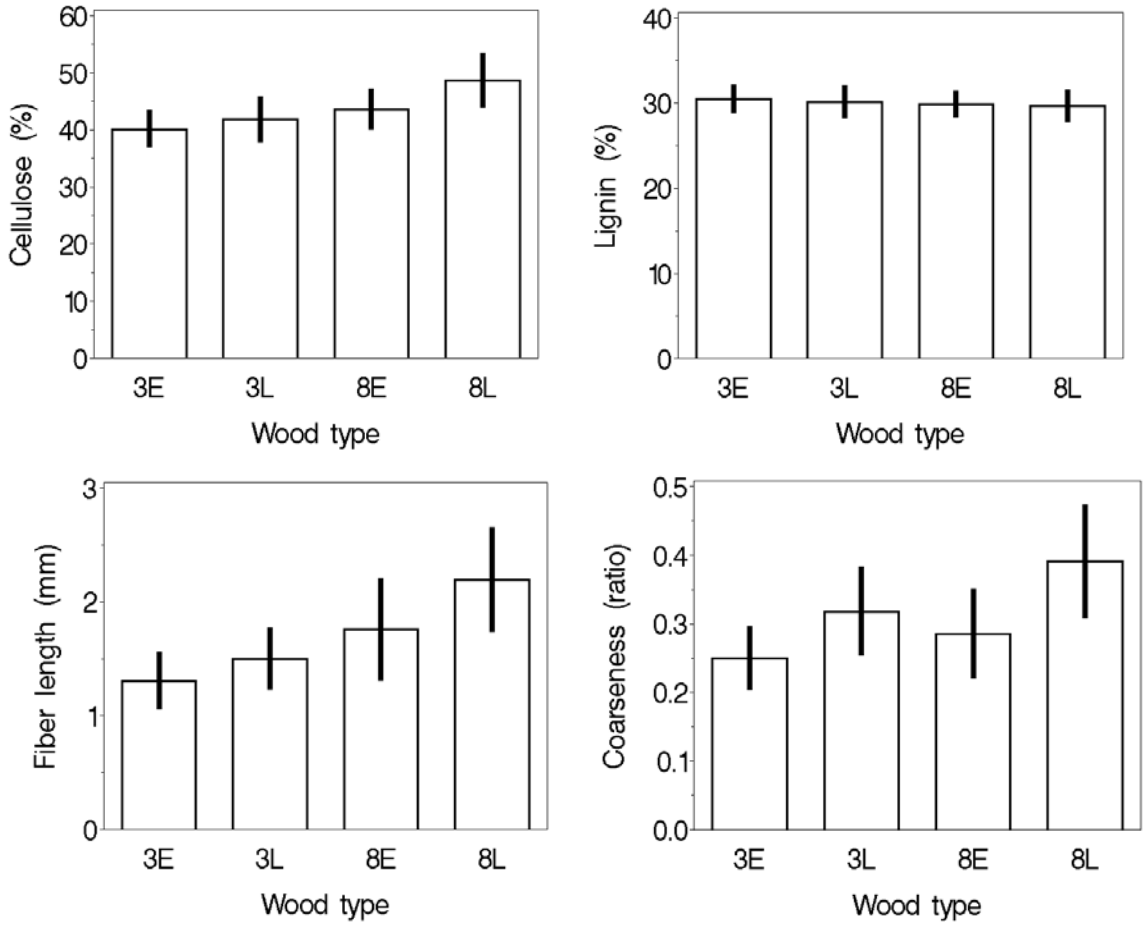

Figure 1. Variation in α-cellulose (%), lignin (%), fiber length (mm) and coarseness for the earlywood and latewood of two rings. The thick horizontal bars in the middle of the boxes are the median, lower and upper edge of the boxes are quartiles

Genetic parameters

Percentages of variance components over the total variance and heritability estimates for each trait were presented in Table 2. Additive genetic effects (σ^2_g) explained 3% (lignin) to 10% (coarseness) of the total phenotypic variance for the juvenile wood. For the transition wood, variation explained by the additive genetic effects was smaller for the cellulose and coarseness, but was higher for lignin and fiber length compared to the juvenile wood. Non-additive genetic effects were zero for all the traits at the juvenile wood. For the transition wood, however, non-additive genetic effects for lignin and coarseness were as high as additive genetic effects.

Heritabilities for juvenile and transition wood were presented in Table 2. Coarseness was the most heritable trait among the four studied chemical wood traits. Weak individual–tree heritabilities were found for all the traits for the juvenile wood, except for coarseness. Cellulose had smaller individual-tree and family heritabilities for the transition wood than the juvenile wood. In contrast, heritabilities of lignin were higher in the transition wood than juvenile wood. Fiber length and coarseness had similar heritability for both wood types.

Table 1. Comparisons of juvenile (ring 3) and transition (ring 8) wood for α-cellulose (ACY), lignin (LIG), fiber length (FLW) and coarseness (COA) of loblolly pine across four sites.

Variable	**Ring 3 (**mean ±SE)	**Ring 8 (**mean ±SE)	**DF**	**t Value**	**Pr > |t|**
ACY (%)	40.9 ± 0.09	46.1 ± 0.12	3347	-35.7	<.0001
LIG (%)	30.3 ± 0.04	29.7 ± 0.04	3702	9.4	<.0001
FLW (mm)	1.40 ± 0.006	1.98 ± 0.012	2715	-42.5	<.0001
COA (ratio)	0.28 ± 0.0015	0.34 ± 0.0022	3091	-20.6	<.0001

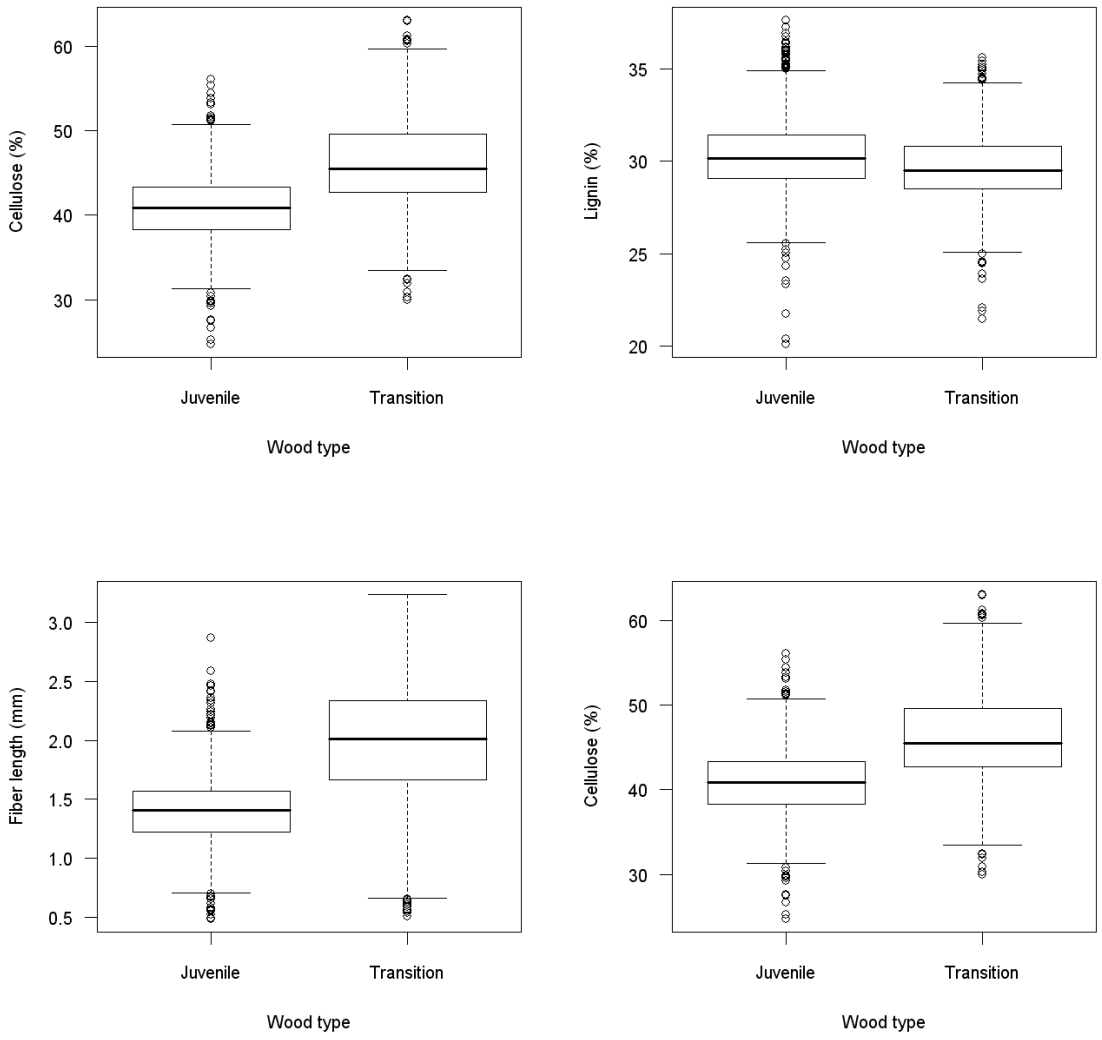

Figure 2. Variation patterns in cellulose, lignin, fiber length and coarseness for juvenile wood and trasition wood

Table 2. Percentages of variance components over the total variance, individual-tree and full-sib family means heritabilities (± standard error) for chemical wood traits of loblolly pine.

a) Juvenile wood (ring 3)

Estimate	Cellulose	%	Lignin	%	Fiber length	%	Coarseness	%
σ^2_g	0.29	4	0.08	3	0.0016	3	0.00021	10
σ^2_s	0	0	0	0	0	0	0	0
σ^2_{ge}	0	0	0.08	3	0.0006	1	0	0
σ^2_{se}	1.80	23	1.01	37	0.0026	5	0.00068	31
σ^2_{plot}	1.30	17	0.12	4	0.0079	15	0.00025	11
σ^2_e	4.41	57	1.44	53	0.0398	76	0.00103	47
h^2_i	0.15 ± 0.14		0.12 ± 0.17		0.12 ± 0.10		0.39 ± 0.28	
h^2_{fs}	0.50 ± 0.25		0.35 ± 0.33		0.63 ± 0.22		0.68 ± 0.19	
h^2_{hs}	0.63 ± 0.40		0.47 ± 0.43		0.66 ± 0.40		0.72 ± 0.42	

b) Transition wood (ring 8)

Estimate	Cellulose	%	Lignin	%	Fiber length	%	Coarseness	%
σ^2_g	0.25	2	0.14	6	0.0059	4	0.00026	7
σ^2_s	0.27	2	0	0	0.0009	1	0	0
σ^2_{ge}	0	0	0.13	5	0.0007	0	0.00020	6
σ^2_{se}	3.26	28	0.81	32	0.0159	10	0.00071	20
σ^2_{plot}	1.47	13	0.06	2	0.0236	15	0.00032	9
σ^2_e	6.49	55	1.37	54	0.1096	70	0.00201	57
h^2_i	0.09 ± 0.13		0.23 ± 0.24		0.15 ± 0.13		0.30 ± 0.26	
h^2_{fs}	0.29 ± 0.37		0.49 ± 0.29		0.60 ± 0.28		0.62 ± 0.24	
h^2_{hs}	0.46 ± 0.46		0.56 ± 0.41		0.67 ± 0.41		0.62 ± 0.39	

Genotype by environment interaction

General combining ability by site interaction variance (σ^2_{ge}) was zero for the cellulose and coarseness for the juvenile wood (Table 2). For the lignin, the σ^2_{ge} variance explained 3% of the total phenotypic variance while it was negligible for fiber length (1%). At transition wood, the σ^2_{ge} was considerable (5%) for lignin and coarseness (6%) but was zero for cellulose and fiber length. Specific combining ability by site interaction variance (σ^2_{se}) was very high for all traits both for the juvenile and transition wood. The σ^2_{ge} variance explained 5% (fiber length) to 37% (lignin) of the total phenotypic variance at juvenile wood. The range for the transition wood was from 10% (fiber length) to 32% (lignin).

Type B genetic correlations as a measure of genotype by environment interactions for the juvenile and transition wood and for the combined rings were presented in Table 3. The higher the correlation coefficient is the less significant genotype by environment interaction. High non-additive genetic effects (full-sib family) by site interactions were reflected in weak non-additive type B genetic correlations (r_{B_sca}) both for the juvenile and transition wood. Most type B correlations were not estimable because of zero specific combining ability genetic variances or because of zero general and specific combining ability by site interaction variances. Full-sib families changed the rank from one site to another considerably for all the traits as shown by near zero non-additive type B genetic correlations. On the other hand parent trees were relatively stable. The estimated additive type B genetic correlations (r_{B_gca}) were in the range of 0.51 (lignin) to 0.95 (fiber length).

Table 3. Type B additive (r_{B_gca}) and non additive (r_{B_sca}) genetic correlations for cellulose, lignin, fiber length, and coarseness for the juvenile wood (ring 3), transition wood (ring 8) and for the combined.

Wood Type	Correlation	Cellulose	Lignin	Fiber length	Coarseness
Juvenile	r_{B_gca}	-[1]	0.51	0.72	-[1]
	r_{B_sca}	-[1]	-[1]	-[1]	-[1]
Transition	r_{B_gca}	-[1]	0.52	0.89	0.56
	r_{B_sca}	0.08	-[1]	0.06	-[1]
Combined	r_{B_gca}	-[1]	0.53	0.95	0.77
	r_{B_sca}	0.01	-[1]	0.05	-[1]

-[1] Correlation was not estimable due to zero genetic variance or zero genotype by site interaction variance.

Product-moment phenotypic and additive genetic correlations

Phenotypic relationships between pairs of chemical wood traits were presented in Figure 1. As cellulose, fiber length and coarseness increase, lignin percentage decreases. The relationships of lignin with other three traits were in the range of -0.20 to -0.23. Cellulose had positive correlations with fiber length (0.57) and coarseness (0.47). The relation between fiber length and coarseness was also positive. All the product-moment correlations were significantly different from zero (Pr<.0001). Chemical wood traits were not genetically correlated with growth, stem straightness and rust infection. However, additive genetic correlations between chemical wood traits were moderately high and significantly different from

zero both for juvenile wood and transition wood (Table 4). The signs of the genetic correlations were parallel with the product-moment correlations, i.e., the relationships of lignin with other three traits were all negative. On the other hand, cellulose, fiber length and coarseness had positive genetic correlations. Genetic correlations among chemical wood traits for the juvenile and transition wood were similar in magnitude and in sign.

Table 4. Genetic correlations based on individual-tree breeding values for juvenile wood (above diagonal) and transition wood (below diagonal) among fiber length (FLW), α-cellulose content (ACY), coarseness (COA), lignin content (LIG), height at age 6 (HT6), volume at age 6 (VOL6), fusiform rust infection at age 6 (RUST6), and straightness at age 6 (STRT6) for combined sites

	ACY	COA	FLW	LIG	HT6	VOL6	RUST6	STRT6
ACY		0.69***	0.50***	-0.73***	0.01	-0.01	-0.01	0.00
COA	0.67***		0.37***	-0.53***	0.02	-0.03	-0.01	0.00
FLW	0.50***	0.34***		-0.74***	0.01	0.00	-0.01	0.00
LIG	-0.73***	-0.52***	-0.73***		-0.02	-0.02	0.01	-0.01
HT6	-0.01	0.00	-0.01	0.01		0.78***	0.00	-0.09***
VOL6	-0.03	0.01	-0.02	0.01	0.78***		0.06*	-0.02
RUST6	0.00	-0.01	0.00	0.00	-0.01	-0.07**		0.14***
STRT6	-0.02	-0.01	-0.02	0.00	-0.09***	-0.03	0.14***	

*, **, ***: Correlations are significant at 0.05, 0.01, and 0.001 probability level respectively. Number of observations used ranged from 515 to 550

[1] Genetic correlation was not estimable due to zero general combining ability variance (σ^2_g).

Discussion

Earlywood within a growth ring of loblolly pine had more desirable micro wood properties than latewood. Latewood appeared to be more desirable because cellulose content was higher and lignin content was lower than earlywood. In addition, longer fibers and higher coarseness make latewood desirable for solid wood products compared to earlywood of the same rings. The results based on combined four sites from this study were parallel to the results by Sykes *et al.* [3] that were based on limited sample size from one site. Genetic selection based on percentage of latewood in a growth ring could be an effective way to manipulate chemical and morphological wood properties. However, the difference between two wood types within ring 8 (transition wood) was more pronounced for cellulose content and fiber length compared the difference within ring 3.

Juvenile wood (ring 3) appeared to be less desirable for chemical wood traits compared to transition wood (ring 8). Ring 8 from the pith of trees at breast height is considered as a transition wood rather than mature wood for loblolly pine [15]. There was an apparent increasing trend of cellulose content, fiber length and coarseness from juvenile wood to transition wood. In contrast, there was slight decrease of lignin content from juvenile wood to transition wood.

Considerable variation in the chemical wood properties was found between juvenile and transition wood, except for lignin content (Figure 2). The results suggested that non-additive genetic effects have negligible effects on micro wood traits but these traits are mainly controlled by the additive genetic effects. Genetic variation in cellulose content was higher than that found by Jett *et al*. [16], with both additive and dominance components. Selection against lignin content or selection for longer fiber and higher cellulose content through breeding could yield modest genetic gains due to weak heritabilities. Although, individual-tree heritabilities were generally weak, selection based on half-sib or full-sib family means may help to improve chemical wood traits.

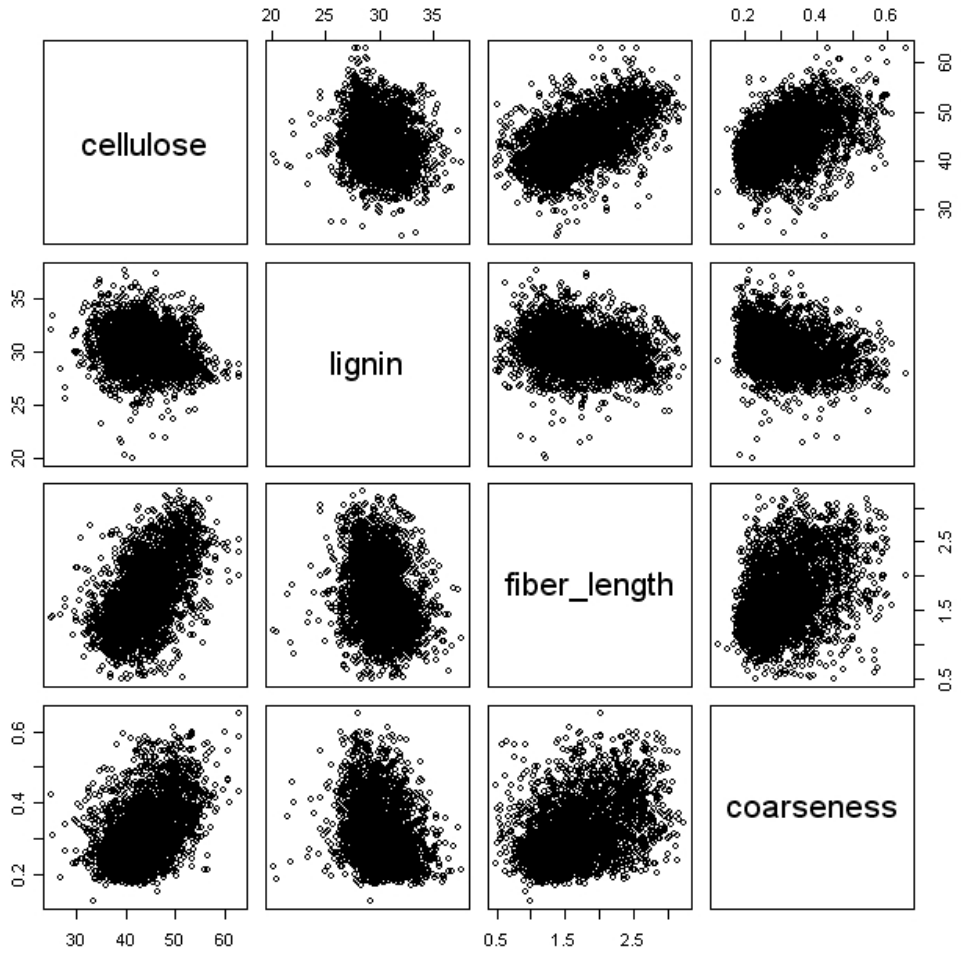

Figure 3. Relationships between pairs of micro wood traits. Cellulose and lignin were not correlated. However, cellulose had positive and significant relationships with fiber length and coarseness.

The results suggested that genetic improvement for cellulose content and coarseness may be realized based on transition wood because phenotypic variation was higher for transition wood than juvenile wood. Transition wood heritabilities may be more meaningful than those of juvenile wood, as they are closer to the age (age 6) where most selections are made for the North Carolina State University-Industry Cooperative Tree Improvement Program (1). The heritabilities based on combined sites were unbiased lower than those from Sykes *et al*. [3], because genotype by site interaction was taken into account in estimations. Heritabilities for fiber length were lower than those found by Loo *et al*. [4] for loblolly pine (Table 1). Loo *et al*. reported 0.31 and 0.37 individual, and 0.45 and 0.51 family heritabilities for transition wood fiber length at four sites.

Lignin had moderately high and negative (favorable) genetic correlations with cellulose and fiber length. The favorable correlations indicated that selection for cellulose content or fiber length could decrease lignin content in a breeding and selection program. Increasing cellulose content may result in the production of more paper per cubic meter of wood, less lignin and more efficient of pulping and bleaching. However, before considering one or two wood traits for breeding and selection, more efforts are needed. Tree improvement programs must decide which traits will be of most importance in the future before incorporating them into their breeding programs.

Standard errors of all the heritabilities were high due to limited number of parents in the experiment and possible random genetic drift in the sampling. The results reported in this study should be considered cautiously and may be repeated with greater sampling size of parents. Isik et al. [16] reported considerable variation in heritability estimates from different diallel groups of the same breeding populations. To have more reliable genetic parameters for the breeding populations, the study should be repeated with a larger sample number of parents. Measurement of chemical wood traits is costly and time consuming. Unless laboratory measurement techniques are improved, it is unlikely to increase sample size for more reliable estimation of genetic parameters.

There was essentially no site by general combining ability interactions for juvenile wood and negligible interaction for cellulose and fiber length for transition wood. The results suggested that parent trees do not interact noticeably with different sites for the micro wood traits. In contrast, very high specific combining ability by site interaction variances were observed for all micro wood traits (Table 2). Full-sib families changed the rank for all traits considerably from one site to another (data were not presented). However, these results are based on the limited samples. Until further evidences are obtained for unexpectedly high levels of interactions, the results should be viewed cautiously.

Genotype by environment interactions may play a larger role as the trees mature, and need to be investigated further (*17, 18*). Our results suggested that loblolly pine full-sib families might not be stable across different site conditions for micro wood properties. If site by specific combining ability interactions continue to be important, as shown in this study, breeding for full-sib family deployment strategy may be considered for the improvement of micro wood traits.

In conclusion, chemical wood traits were under additive genetic control. Non-additive genetic effects appeared to be negligible. Individual-tree heritabilities were generally weak, but family heritabilities were moderate. Specific combining ability by site interaction variance was very high for all the traits, explaining about 30% of the total phenotypic variance. Micro wood traits had weak relationships with height growth, volume and stem straightness. Genetic correlations of lignin with cellulose and fiber length were moderately high and negative; suggesting that selection for cellulose content or for fiber length may lead to decrease in lignin content in the breeding population. The results from this study were based on a small number of parents (6 parents). Further research is needed to fully understand the genetic basis of micro wood traits and their potential to include in tree improvement programs.

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Isik, F. and Bailian Li. 2004. Rapid Assessments of Wood Density in Progeny Trials. Abstract, p. 337, In Li, Bailian and Steven McKeand (eds.) Forest Genetics and Tree Breeding in the Age of Genomics: Progress and Future, IUFRO Joint Conference of Division 2, Conference Proceedings. 2004. 490 p.

Cumbie, P. 2002. Genetic Variation of wood density traits in rooted cuttings and seedlings of Loblolly Pine. M.S. Thesis, Department of Forestry, N. C. State University December, 2002. p. 109.

Milestone Status Table

Our application for a no-cost extension of the project for one year beyond the original termination date of Feb. 11, 2004 has been approved. The extension will allow us to complete the project as planned. Completion dates for some tasks in the Milestone Table have been modified accordingly.

ID Number	Task / Milestone Description	Planned Completion	Actual Completion	Comments
1	Method Development			
1.1	Resistograph	3/31/01	12/31/00	Completed
1.2	Extractive Removal	6/30/03		Completed
1.3	Holocellulose & α-cellulose	3/31/01	3/31/01	Completed
1.4	Fiber Analysis	3/31/01	3/31/01	Completed
1.5	Lignin Analysis, acetyl bromide method	3/31/01	3/31/01	Completed
1.6	Lignin Analysis, FTIR method	6/30/01	9/30/01	Completed
1.7	Modified DFRC	12/31/01	12/31/01	Completed
1.8	Molecular markers	12/31/02		completed
2	Sample Collection			
2.1	For Tree Improvement Group	2/11/03		Completed
2.2	For Wood and Pulping Chemistry Group	2/11/03		Completed
2.2.1	Site 1	6/30/02	3/31/02	Completed
2.2.2	Site 2	12/31/01	12/31/01	Completed
2.2.3	Site 3	12/31/02	3/31/02	Completed
2.2.4	Site 4	6/30/03	3/31/02	Completed
2.3	For Forest Biotechnology Group	2/11/05	12/31/04	Completed
3	Sample Analysis and Sequencing			
3.1	Wood density	2/11/04	9/30/03	Completed
3.2	Fiber Morphology and Chemistry	2/11/05		On-going
3.2.1	Site 1	12/31/04	12/31/04	Completed
3.2.2	Site 2	6/30/02	12/31/02	Completed
3.2.3	Site 3	6/30/03	7/31/03	Completed
3.2.4	Site 4	12/31/04	1/31/05	Completed
3.3	Relationship of Gene Expression in Differentiating Xylem and Specific Wood Properties	2/11/05	1/31/05	
3.3.1	Verify Effects of Candidate Genes Using RT PCR	2/11/05	12/31/04	Completed
3.3.2	Correlate Gene Expression with Wood Properties	2/11/05	12/31/04	Completed
3.3.3	Examine the Effects of Alternative Sites on Gene Expression	2/11/05	2/11/05	Completed
3.3.4	Analyze Gene Expression Effects for within Family and between Family Variations	2/11/05	12/31/04	Completed Completed
3.4	Selective Lignin Characterization	2/11/05	2/11/05	Completed
4	Field Trial and Strategic Development	2/11/05	2/11/05	Completed
5	Final Report	6/30/05	6/30/05	Completed

Budget Data (as of date): The approved spending should not change from quarter to quarter. The actual spending should reflect the money actually spent on the project in the corresponding periods. A table such as the following could be used. **This is the final budget data.**

			Approved Spending Plan			Actual Spent to Date		
Phase / Budget Period			DOE Amount	Cost Share	Total	DOE Amount	Cost Share	Total
	From	To						
Year 1	2/12/01	2/11/02	195,142	175,848	370,990	143,845.75	171,818.00	315,663.75
Year 2	2/12/02	2/11/03	195,142	25,848	220,990	88,296.25	25532.24	113,828.49
Year 3	2/12/03	2/11/04	195,142	25,848	220,990	147,540.18	30598.26	178138.44
Year 4	2/12/04	2/11/05	0	0	0	205,375.71	19,679.13	225,054.84
Totals			585,426.00	227,544.00	812,970.00	585,057.89	247,627.63	832,685.52