FINAL REPORT

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Project Objective: The general objective of this study is to characterize the performance and value of partially CAD-deficient wood, arising from *cad*-n1 identified in descendants of an exceptionally fast-growing pine genotype. Specific objectives are:

1. To compare lignin formation in partially CAD-deficient and "normal" trees, ranging in age from 6 years to rotation age in multiple genetic and environmental backgrounds, in both juvenile and mature wood;
2. To identify associations between CAD genotype and growth performance (growth, stem form and defect in older trees, developmental traits in younger trees);
3. To conduct laboratory studies to confirm the value of partially CAD-deficient wood for energy savings in pulp production and impact on properties of solid-wood products; and
4. To develop approaches to marker-assisted breeding for partially CAD-deficient wood.

EXECUTIVE SUMMARY

The southern US produces 58% of the nation's timber, much of it grown in intensively managed plantations of genetically improved loblolly pine. One of the fastest-growing loblolly pine selections made by the NCSU-Industry Cooperative Tree Improvement Program, whose progeny are widely planted, is also the only known natural carrier of a rare gene, cadn1. This allele codes for deficiency in an enzyme, cinnamyl alcohol dehydrogenase, which catalyzes the last step in the biosynthesis of lignin precursors. This study is to characterize this candidate gene for marker-assisted selection and deployment in the breeding program. This research will enhance the sustainability of forest production in the South, where land-use pressures will limit the total area available in the future for intensively managed plantations. Furthermore, this research will provide information to establish higher-value plantation forests with more desirable wood/fiber quality traits.

A rare mutant allele (*cad-n1*) of the *cad* gene in loblolly pine (*Pinus taeda* L.) causes a deficiency in the production of cinnamyl alcohol dehydrogenase (CAD). The effects of this allele were examined by comparing wood density and growth traits of *cad-n1* heterozygous trees with those of wild-type trees in a 10-year-old open-pollinated family trial growing under two levels of fertilization in Scotland County, North Carolina. In all, 200 trees were sampled with 100 trees for each treatment. Wood density measurements were collected from wood cores at breast height using x-ray densitometry. We found that the substitution of *cad-n1* for a wild-type allele (*Cad*) was associated with a significant effect on wood density. The *cad-n1* heterozygotes had a significantly higher wood density (+2.6%) compared to wild-type trees. The higher density was apparently due to the higher percentage of latewood in the heterozygotes. The fertilization effect was highly significant for both growth and wood density traits. While no *cad* genotype x treatment interactions was found for any of the traits studied, in the fertilized plots, the effect of the *cad-n1* allele on wood density was reduced. The study indicates that the *cad-n1* allele could be a valuable gene to the pulp and paper industry for the purpose of enhancing pulp yields through increasing wood density.

Stem growth and wood density associated with a mutant null (*cad-n1*) allele were examined in three 15-year-old loblolly pine diallel tests, established on two sites in the southern United States. In each diallel test, one or two *cad-n1* heterozygous parents were crossed with five unrelated wild-type parents, to produce five or ten full-sib families. In all, 839 trees from 20 full-sib families in four genetic backgrounds (a *cad-n1* heterozygote × 5 unrelated trees) were sampled, genotyped at the *cad* locus, and assessed for growth and wood density traits. In a combined analysis of all four genetic backgrounds, we found evidence for effects of increased wood density associated with the *cad-n1* allele at age 15 ($p=0.03$) years and height growth at ages 6 ($p=0.03$) and 15 ($p=0.005$). There were large differences in the *cad-n1* effects for the various growth and wood traits among the diallel tests. This variation may be due to either different genetic backgrounds among the parents of the different diallel tests, or for different growing environments at the field sites. Even though the *cad-n1* effect on growth and wood density was significant across genetic backgrounds, the effect was variable among full-sib families within backgrounds. We speculate that certain wild-type alleles from second parents specifically interact with *cad-n1* producing large positive effects. In addition, pleiotropic effects on growth and wood density appear to be associated with the *cad-n1* allele. While substantial

gains are possible through deployment of trees carrying *cad-n1*, these gains may be family-specific and should be verified for each cross through field testing.

Incremental cores of 200 selected trees in a 10 years old open pollinated family trail growing under two nutrition treatments in Scotland County, North Carolina. In this study, we analyzed wood density, chemical and pulping properties Analysis of the samples with NIR allows estimation of lignin, α-cellulose and hemicellulose contents in each annual ring. Analysis of selected samples by quantitative 13C NMR showed almost no differences between *cad-n1* and wild MWL isolated from juvenile wood. However, there were some differences between between *cad-n1* and wild MWL isolated from mature wood. A micro technique is developing to allows isolation and comprehensive analysis of lignin using the amount of wood which can be obtained from each annual ring in an incremental core. Analysis of a fraction of crude MWL contained concentrated LC fragments allows detection and quantification of phenyl-glycoside bonds. Ester bonds between uronic acids and γ–position of lignin were detected in contrast to benzyl ester linkages.

Pulping result indicates heterozygote in the control plots give ~ 6% higher pulping yield than the wild type. ^{13}C NMR studies showed that the structure of the kraft lignin isolated from pulping of CAD-deficient pine is rather similar to that of the control. However, increased amounts of COOH and phenolic OH have been observed in the heterozygote lignin.

Summary of results for CAD-deficient pine and wild samples

Pulping and Bleaching

- No difference in pulping rate between the CAD and wild samples
- Mature wood was easier to pulp for both the CAD and Wild
- Mature CAD had a slightly higher yield at high kappa numbers
- Mature wood had a ~2% higher yield than juvenile wood
- No difference in bleachabilty between CAD and wild samples
- Juvenile wood was easier to bleach than mature wood

Strength properties

- Strength properties were compared for bleached and linerboard grade pulps
- No significant difference in tear-tensile relationship between CAD and Wild
- The mature wood had about 15-20% higher tear at equivalent tensile
- The fiber properties were similar for the CAD and Wild samples but the juvenile wood had a lower fiber length

These research results are detailed in the following sections, and each has its own sets of tables and figures to support these results.

THE EFFECTS OF A MUTANT GENE (*CAD-N1*) ON WOOD DENSITY AND TREE GROWTH OF LOBLOLLY PINE IN FERTILIZED AND NONFERTILIZED TREATMENTS

A rare mutant allele (*cad-n1*) of the *cad* gene in loblolly pine (*Pinus taeda* L.) causes a deficiency in the production of cinnamyl alcohol dehydrogenase (CAD). The effects of this allele were examined by comparing wood density and growth traits of *cad-n1* heterozygous trees with those of wild-type trees in a 10-year-old open-pollinated family trial growing under two levels of fertilization in Scotland County, North Carolina. In all, 200 trees were sampled with 100 trees for each treatment. Wood density measurements were collected from wood cores at breast height using x-ray densitometry. We found that the substitution of *cad-n1* for a wild-type allele (*Cad*) was associated with a significant effect on wood density. The *cad-n1* heterozygotes had a significantly higher wood density (+2.6%) compared to wild-type trees. The higher density was apparently due to the higher percentage of latewood in the heterozygotes. The fertilization effect was highly significant for both growth and wood density traits. While no *cad* genotype x treatment interactions were found for any of the traits studied, in the fertilized plots, the effect of the *cad-n1* allele on wood density was reduced. The study indicates that the *cad-n1* allele could be a valuable gene to the pulp and paper industry for the purpose of enhancing pulp yields through increasing wood density.

INTRODUCTION

The southern region of United States produces 58% of the nation's timber supply, much of it grown in intensively managed plantations of genetically improved loblolly pine (*Pinus taeda* L.) (Wear and Greis 2002). Trees grown from seeds derived from second-generation seed orchards have estimated gains from 13% to 21% in rotation-age wood volume over unimproved seed lots (Li et al. 1999). One of the most valuable loblolly pine selections made by the NCSU-Industry Cooperative Tree Improvement Program is the only known first-generation carrier of a rare muant allele (*cad-n1*) of the *cad* gene. The *cad-n1* allele confers a reduction in the amount of cinnamyl alcohol dehydrogenase (CAD, E.C. 1.1.1.195), which is significant because CAD catalyzes the final step in the biosynthesis of lignin precursors (Mackay et al. 1995, O'Malley et al. 1992).

Lignin is conventionally defined as a complex hydrophobic network of phenylpropanoid units derived from the oxidative polymerization of one or more of three types of hydroxycinnamyl alcohol precursors (Sederoff et al. 1994). As such lignin plays an important role in plant biology, especially for mechanical support, water transport, and pathogen resistance (Vance et al. 1980). In trees, high levels of lignin are contained in wood and account for 15% to 36% of the dry weight of wood (Whetten and Sederoff 1995). In the pulp and paper industry, lignin must be removed by chemical treatments that are costly to the mill and the environment (Dean and Eriksson 1994).

Progeny from the *cad-n1* founder are widely planted throughout the southern United States in operational and experimental plantations. Breeding work using this selected genotype produces three types of trees with respect to the *cad* locus. With self-pollination, partially CAD-deficient heterozygotes (*Cad/cad-n1*), wild-type homozygotes (*Cad/Cad*), and totally CAD-deficient homozygotes (*cad-n1/cad-n1*) are produced (MacKay et al. 1997, Ralph et al. 1997). Only heterozygotes and wild-type homozygotes are produced with cross-pollination. Wu et al. (1999) reported that heterozygous *cad-n1* trees produced 14% more debarked wood volume at age 4

years, compared to wild-type trees. In addition, Dimmel et al. (2001) reported that homozygous *cad-n1* trees had poor growth and low pulp yields (due at least in part to their inbred nature), compared to other genotypes, although they produced wood that was more easily delignified.

Previous studies of *cad-n1* heterozygotes yielded inconsistent results on pulping and bleaching. In one study, kraft cooks of 4- and 6-year-old heterozygous trees resulted in kappa numbers (i.e., lignin contents) that were significantly lower than wild-type trees. Additionally, significantly less energy was required (15% to 25% lower H-factor) to pulp to a given kappa number than for wild-type trees, and the pulp of the heterozygotes was brighter and stronger (Dimmel et al. 2001). Conversely, Dimmel et al. (2002) found no apparent differences in ease of delignification or pulp yield between heterozygous and wild-type trees that were 14-years-old.

The effects of the *cad-n1* allele in loblolly pine are caused by a frame shift mutation in the coding region of the *cad* gene (Gill et al. 2003) that causes a reduction of *cad* mRNA and CAD enzyme expression (Stasolla et al. 2003). Heterozygous and homozygous *cad-n1* trees have only 50% and 1% of the normal (i.e., homozygous wild-type) levels of CAD expression (MacKay et al. 1997). There are also differences in the chemical composition of lignin between *cad-n1* heterozygous and wild-type trees, resulting in a large difference in the extractability of lignin and potential benefits to the pulp and paper industry (MacKay et al. 1999, Lapierre et al. 2000).

To date, no studies have focused on the effect of the *cad-n1* allele on physical properties of wood, although one study measured wood density of a few *cad-n1* heterozygous trees (Dimmel et al. 2002). Wood density (i.e., specific gravity) is an important trait in loblolly pine because it is highly correlated with wood strength, wood stiffness, and pulp yield (Haygreen and Bowyer 1996, Faust et al. 1999). For example, a change of 0.02 in wood specific gravity is equivalent to a change of 23 kg of dry weight per m^3 (Zobel and Jett 1995). In this paper, we report on the effects of this mutant allele by comparing wood density and growth traits of *cad-n1* heterozygotes with those of wild-type trees. These measurements were made in a 10-year-old open-pollinated family trial growing under two levels of fertilization. The specific objectives of the study are: 1) to analyze the quantitative effect of the mutant *cad-n1* allele on growth and wood density; and 2) to examine the magnitude of *cad* genotype by nutrient interactions on these traits.

MATERIALS AND METHODS

1. Plant materials

The field test is located in Scotland County, North Carolina, adjacent to the USDA Forest Service / N.C. State University Southeast Tree Research and Education Site (SETRES). The test was established in November and December of 1993 with container-grown seedlings from 10 open-pollinated families of loblolly pine. The soil is very infertile and somewhat excessively drained. The fertilized and unfertilized (i.e., control) plots, each consisting of 100 trees, were replicated over 10 randomized complete blocks. The trees were planted at a 1.5 x 2.1 meter spacing, with a 12-meter buffer around each treatment plot to minimize the influence of adjacent fertilizer treatments. Fertilizer was applied annually to maintain an optimum supply of all nutrients (including micros) to stimulate rapid growth in fertilized plots, as determined by foliar analysis. Through the first 10 growing seasons the major nutrient additions (kg/ha) have been

817 N, 83 P, 87 K, 9 Ca, 52 Mg, and 179 S as well as micronutrient additions (kg/ha) of 1.7B, 2.0 Cu, 5.0 Fe, 5.0 Mn, and 2.0 Zn.

2. Growth measurements

Height was measured annually through age 10 years (except for years 7 and 9), and DBH (diameter at breast-height) was measured annually starting in year 3. Thirty-three trees (20 and 13 from control and fertilized treatments, respectively) were randomly selected for destructive sampling at age 10 to generate an equation to estimate total inside-bark volume (V, m^3) from total height (H, m) and DBH (D, mm). Once felled, each tree was bucked at heights of 0, 1.4, and 2.4 meters and every 1.2 meters thereafter. Inside and outside bark diameters were measured for each stem section. Inside bark diameters were used in Smalian's log volume equation to estimate the inside-bark volume of each bolt (Avery and Burkhart 1994). The equation is V = L * (B + b)/2, where V= total section volume, L=length of bolt, B=basal area of bolt's large end, and b=basal area of bolt's small end. The stem section volumes were summed to produce a whole tree, inside-bark volume. The whole tree volume was then used to develop a prediction equation, $V = B_0 + B_1 (DBH^2 * H)$, as suggested by Spurr (1952). The explanatory variables fertilizer (X_1), $X_1 * D^2H$, and $(D^2H)^2$ were added to the equation based on their significance ($p \leq 0.05$), to produce the following equation:

[1] $V = 4.258133*10^{-9}(DBH^2H)-0.0204(X_1)+1.93733*10^{-9}(X_1*DBH^2H)-7.9859*10^{-17}(DBH^2H)^2$

where $X_1 = 1$ for the fertilized treatment, and $X_1 = 0$ for the control .

The variation explained by this model ($R^2 = 0.98$) was highly significant ($p < 0.0001$).

3. Wood density measurements

The seed parent used in this study is a selected second-generation descendant of the *cad-n1* founder that is known to be a *cad-n1* heterozygote. In August 2003, a total of 200 healthy trees were randomly selected from 5 blocks (20 trees from each treatment per block). A 12-mm core was sampled from each tree at breast height for wood quality analyses, and phloem/cambium tissue was collected for DNA isolation and analysis.

Cores were sectioned longitudinally to produce a strip approximately 2-mm thick. The samples were conditioned to a uniform moisture content of 8% before they were scanned. Wood density was measured using X-ray densitometry. Each strip was scanned from pith to the bark on a QMS Tree Ring Analyzer® (Model Qtrs-01x, Quintek Measurement Systems, Inc.). The last growth ring was excluded due to missing latewood on cores collected in mid-summer. For each ring scanned, the following intra-ring wood density characteristics were determined: average ring density, earlywood density, latewood density, latewood percentage, and cambial age. Weighted average wood density traits were calculated by weighting ring mean density with total ring basal area, which approximates the average density of a disk sample wood taken at breast height.

4. Genotyping

Inner-bark (phloem/cambium) tissue was collected from each of the sample trees for genotyping. DNA was isolated from each tissue sample using DNAeasyKits® (Qiagen, Inc.). Each DNA sample was then PCR-genotyped for the *cad* locus using forward primer CADF8 and reverse primer CADR2 or CADF4 (Gill et al. 2003). Amplified PCR products were resolved and detected on an ABI 3100 Genetic Analyzer, as recommended by the manufacturer (Applied

Biosytems, Inc.). Peak sizes were determined with GeneScan® and inspected and scored with Genotyper® software (Applied Biosystems, Inc.). Homozygous wild-type trees produce a single peak, whereas heterozygous trees produce two peaks, the same peak as in the homozygote plus a peak that is two base pairs (bp) longer. These results are consistent with the *cad* sequence data that indicates that the *cad-n1* allele contains a 2 bp insertion compared to the wild-type allele (Gill et al. 2003). Putative *cad-n1* homozygous trees that were identified by the presence of only the longer peak were re-tested with a different reverse primer (usually CADF4) to verify their *cad* genotype. In all cases, the second test resulted in two peaks rejecting the homozygous *cad-n1* interpretation in favor of heterozygous *cad-n1*.

5. Statistical analysis

The data were analyzed according to the following linear model:

[1] $Y_{ijlk} = \mu + b_i + t_j + g_l + bt_{ij} + gt_{jl} + gbt_{ijl} + e_{ijlk}$

where Y_{ijlk} is the observed value for the k^{th} tree of the l^{th} *cad* genotype in the i^{th} block in the j^{th} treatment; μ is the overall mean; b_i is the effect of i^{th} block; t_j is the effect of the j^{th} fertilizer treatment; g_l is the effect of the l^{th} *cad* genotype; bt_{ij} is the effect of the interaction between the i^{th} block and the j^{th} treatment; gt_{jl} is the effect of the interaction between the l^{th} *cad* genotype and the j^{th} treatment; gbt_{ijl} is the effect of the interaction between the l^{th} *cad* genotype and i^{th} block and the j^{th} treatment; and e_{ijkl} is the tree-to-tree effect within plot. All terms except for e_{ijkl} were considered as fixed. The genotypic effects (i.e., the difference between heterozygous mutant and wild-type trees) were estimated by analysis of variance (ANOVA) using PROC GLM (SAS Institute 1996). The SAS procedures PROC CORR and PROC REG were used to assess the linear relationships between the studied traits in both heterozygous and wild-type trees.

RESULTS

Open-pollinated progeny from the *cad* heterozygous seed parent resulted in two *cad* genotypes: wild-type (107 trees) and heterozygous mutant (93 trees). Chi-square analysis indicated no significant difference from the expected 1:1 allele segregation ratio (χ^2=0.9800, P = 0.3222).

The analyses of variance for growth and wood traits are given in Table 1. Volume growth responses to fertilization were large and highly significant (Table 1 and 2). Height and DBH were 45.9% and 38.2% greater, respectively, in fertilized plots, and volume growth differences were even more dramatic: 168% greater in the fertilized plots. The effect of *cad* genotype was not significant for any growth traits at age 10 years (Tables 1 and 2, and Figure 1). In contrast, the fertilizer effect was significant for all wood traits, where weighted wood density, weighted latewood density, weighted earlywood density and weighted latewood percentage were 9.7 %, 4.2 %, 7.6 % and 17.1 % lower, respectively, in fertilized plots compared to the controls (Table 1).

The effect of the *cad* genotype was significant for all wood traits, except for weighted latewood density (Table 1, Figure 2). For the first 9 years, the weighted wood density for heterozygous trees was 2.6% higher than that of wild-type trees. The heterozygotes also had 6.3%, 1.5%, and 0.2% greater weighted latewood percentage, earlywood density and latewood density,

respectively, compared to the wild-type trees. *cad* genotype x fertilizer treatment interactions were not significant for any growth or wood density trait, and the weighted wood density for the first 9 years was 2.6% and 1.3% greater for heterozygous trees than for wild-type trees on control and fertilized treatments combined, respectively. When analyzed by fertilizer treatment, these differences were significant only for the control plots (P= 0.002), and not in the fertilized plots (P=0.501).

Wood density, latewood density and latewood percentage for each ring in the control and fertilized trees increased from pith to bark (Figure 3). Earlywood density plotted against age showed little variation and remained more or less constant with age. ANOVA results in Table 3 indicate significant main effects of fertilizer and *cad* genotype for ring wood density traits for several ages. The ring density in the last three years (cambium age 7 to 9) and latewood percentage at cambium age 9 were significantly different between the heterozygous and wild-type trees. Fertilizer had continuously affected ring density and earlywood density, and the effect was significant for all wood density traits at age 9. *cad* genotype x fertilizer treatment interactions were not significant for any wood traits at any cambium age, except for latewood percentage at cambium age 5 (P = 0.045).

There was no clear relationship between DBH and weighted wood density for either wild-type or heterozygous trees in fertilized and control treatments, but there were significant correlations between weighted wood density and latewood percentage (Figure 4). Latewood percentage of each genotype was positively related to its weighted wood density in the regression analysis, with R^2 values from 0.75 to 0.85 at fertilized and control plots (Table 4). The only regression models that differed significantly were the two regressions for wild-type trees in fertilized and control plots. The slope for the wild-type trees (b=2.93) in fertilized plots was significantly different (P = 0.007) from that in the control plots (b=3.74).

DISCUSSION

We found that the *cad-n1* allele is associated with a significant increase in wood density in 10-year-old trees (Table 1). This increase results from a higher earlywood density and a greater proportion of latewood. Previous studies associated the *cad-n1* allele with a severe reduction of *cad* mRNA levels and CAD enzyme activity (e.g., Stasolla et al. 2003). In other studies, there were also major differences in the chemical composition of lignin between the mutant and wild-type trees (Mackay et al. 1999, Lapierre et al. 2000), but no definitive studies have investigated the effect of *cad-n1* on wood density. The segregation ratio for *cad-n1* mutant and wild-type alleles was consistent with the expected ratio of 1:1 (Wu et al. 1999). This suggests that *cad-n1* is selectively neutral for survival and adaptability through age 10 in our research planting, and that our sampling strategy was random. To date, *cad-n1* has not been identified in any tree outside of the pedigree of the original founder parent.

Fertilization effect

It was not surprising to observe faster growth in the fertilized plots (Table 2), as previous analyses had shown similar results (McKeand et al. 2000). Silvicultural treatments, including fertilization, can affect wood density by modifying growth conditions. While increases in tree volume, resulting from intensive silvicultural inputs, have been associated with changes in wood properties, the effects of fertilization on wood properties for southern pines are difficult to generalize. Early reports found that aerial fertilization with N, P and K temporarily reduced

wood density in loblolly pine (Beckwith and Reines 1985), whereas P and NP treatments that were applied to planted slash pine increased wood density compared to non-treated trees (Rockwood et al. 1985; Megraw 1985; Zobel and van Buijtenen 1989).

The weighted average wood density through 9 years on fertilized plots was 9.7 % less than that on control plots. Larson et al. (2001) reported that fertilization at the time of planting increased height and crown development, which usually results in a temporary decrease in wood density and latewood proportion. In this case, the large difference in wood density between fertilized and control treatment can be attributed to the continued prescribed annual applications of fertilizer throughout the 10-year growing period.

In this study, fertilization significantly decreased all wood density traits, irrespective of *cad* genotype (Figure 3, Table 3). The increase in radial growth under fertilization was due primarily to an increase in earlywood width. As an effect of greater earlywood width, the proportion of latewood decreased in both heterozygous and wild-type trees. There was a significant negative correlation between earlywood width and late wood percentage ($r = -0.73$, $P < 0.001$). Previous studies indicated that such a decrease was partly caused by a shift in the relative width of earlywood and the proportion of latewood (Blair and Olson 1984, Zobel and van Buijtenen 1989). Zobel and van Buijtenen (1989) found the most consistent change in wood properties attributed to fertilization appears to be a short-term adjustment in the earlywood/latewood ratio. In this study, the high correlation between wood density and latewood percentage agree with this finding (Figure 4).

cad-nl *allele effect*

While Wu et al. (1999) found that *cad-n1* heterozygous trees appeared to grow faster than wild-type trees, we did not find a significant association between *cad* genotype and growth. These conflicting results likely arise from differences in the uniformity of the field trials or sampling methods. In the present study, we sampled 200 trees randomly from the two treatments in contrast to the systematic sample of 158 at one site in the earlier report. For valid estimates, further studies require a sufficient number of samples to be tested in sufficient number of trials, replicated in time.

Significant differences for wood density traits were observed between trees with alternative *cad* genotypes, except for latewood density (Figure 2). The weighted wood density for heterozygotes was 2.6 % higher than wild-type trees. Dimmel et al. (2002) found average specific gravities for disks of 14-year old loblolly pine were 0.435 and 0.427 (difference of 0.008 or 1.8%) for heterozygous and wild-type trees, respectively, but the difference was not statistically significant. Zobel and Jett (1995) indicated that a change of 0.02 in specific gravity results in a change of 23 kg per m^3 of dry processed kraft pulp. In the present study, the *cad-n1* allele had a significant effect on increasing the proportion of latewood, suggesting the potential for a significant impact on pulp yield. Previous studies indicated that latewood produces 2 to 7% more pulp than earlywood (Gladstone et al. 1970), but lignin, holocellulose and alphacelluose in latewood are about the same as in earlywood (Gladstone et al. 1970, Sykes et al. 2003).

Weighted wood density through year 9 was greater for heterozygotes than for wild-type trees in both the control and fertilized treatments, but these differences were significant only in the control plots. We suspect that the lack of significance between wild-type and heterozygous trees in the fertilized plots for wood density could be strongly influenced by nutrient availability. The

large increase in the earlywood/latewood ratio in the fertilizer treatments may obscure the influence of the *cad-n1* allele on decreasing the ratio. The *cad-n1* allele decreases the earlywood/latewood ratio, but fertilization increased the ratio to a much greater degree. Despite this apparent conflict, there was no significant *cad* genotype x treatment interaction for any wood density or growth traits.

Wood density can be thought of as a direct combination of earlywood density, latewood density, and latewood percentage. The general trend in wood density of loblolly pine is an increase from pith outwards reaching a maximum value around ring 14. Loo et al. (1985) reported that loblolly pine families produce juvenile wood for the first 6 years, transition wood between 6 and 14 years and mature wood beyond age 14. In this study, the mean ring density, as well as the earlywood and latewood density for both heterozygous and wild-type trees, followed a continuously increasing trend in both the control and fertilized plots.

The effect of *cad-n1* on the wood density traits appeared to be consistent with tree growth (Figure 3). This was expected due to the lack of a *cad* genotype by fertilizer treatment interaction effect. Heterozygotes showed consistently higher ring wood density than wild-type trees throughout most of the years through cambium age 9 (Figure 3, Table3). For both heterozygous and wild-type trees, latewood density increased rapidly from the pith. Megraw (1985) indicates that latewood density increases rapidly with ring number from the pith until values are almost up to their characteristically high level. Earlywood density plotted against age showed little variation and remained more or less constant with age. This relatively constant pattern of earlywood density over time has been reported previously (e.g., Megraw 1985, Hodge and Purnell 1993, Bucur et al. 1994).

Our results indicate that the higher wood density for heterozygous trees is most likely attributed to the higher percentage of latewood. The weighted latewood percentage for heterozygotes was 6.3% higher than for wild-type trees, while average earlywood and latewood ring densities were only 1.5%, and 0.2% higher, respectively. Significant correlations were found between weighted wood density and latewood percentage for heterozygous trees in both fertilized and control treatments (Figure 4). Regression model for wild-type trees in fertilized plots is significantly different from wild-type trees in control plots ($P < 0.001$). The result indicates that the fertilizer treatment may have caused a greater difference in wild-type than in heterozygous trees (Table 4).

For both heterozygous and wild-type-trees, our results indicated no meaningful relationship between growth (DBH) and wood density in both control and fertilize treatment ($r=0.04$ to $r=0.00$). As has been generally found with loblolly pine (Zobel and Jett 1995), little or no relationship exists between growth rate and wood density at the genetic level. If trees heterozygous for *cad-n1* are grown and have higher wood density, as predicted based on these data, we also would expect no concomitant reduction in growth.

CONCLUSIONS

In this study, we found that the substitution of *cad-n1* for a wild-type allele is associated with a significant increase in weighted wood density, and especially the proportion of latewood in loblolly pine. The *cad-n1* allele displayed a greater influence on earlywood density and latewood percentage throughout tree development, which resulted in high total wood density for heterozygotes, as compared to wild-type trees. The mechanisms for this are unknown. Wu et al.

(1999) speculated that trees with *cad-n1* may invest fewer resources in the production of monolignols, which is an energy consuming process, providing additional resources for tree growth and wood production. If so, *cad-n1* may be a particularly valuable gene when deployed on a large scale in forest plantations. However, in the present study, we could not show that the phenotypic effects on wood density are solely due to the *cad-n1* allele. Further studies are needed to determine the possible influence of other closely-linked alleles on wood density. Our analyses were based on a relatively small number of trees (n=200) measured over 9 years. Additional sampling scheduled for later stages of this project should provide a more reliable determination of growth and confirmation of wood density differences for the two *cad* genotypes.

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Table 1. Significance of main effects of fertilizer treatment and *cad* genotype and their interactions from the ANOVA for growth traits at age 10 years and weighted wood density traits through 9 years (p values < 0.05 shown in **bold type**).

		Level of significance (p)					
Main effect	DF	Height (m)	DBH (cm)	Volume (m^3)	Weighted wood density (kg/m^3)	Weighted latewood (%)	Weighted earlywood dens (kg/m^3)
Fertilizer treatment (F)	1	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
cad genotype (G)	1	0.506	0.962	0.813	**0.010**	**0.029**	**0.004**
G * F	1	0.690	0.787	0.845	0.265	0.104	0.787

Table 2. Means (± standard error) of *cad* genotype and fertilizer treatments for growth traits through 10 years and weighted wood density through 9 years.

Main effect	Treatment	Height (m)	DBH(cm)	Volume (dm^3)
cad genotype	Wild–type	9.7 ± 0.09 a	11.0 ± 0.2 a	46.4 ± 1.5 a
	Heterozygote	9.8 ± 0.09 a	11.1 ± 0.2 a	47.0 ± 1.6 a
Fertilizer treatment	Control	7.9 ± 0.09 b	9.3 ± 0.2b	25.8 ± 1.5 b
	Fertilized	11.6 ± 0.09 a	12.8 ± 0.2 a	66.7 ± 1.5 a

Means within a column and main effects followed by the same letter are not significantly different at $p \leq 0.05$.

Table 3. Significance of main effects of fertilizer treatment and *cad* genotype and their interactions from the ANOVA for wood density traits through cambium ages 3 to 9 years ($p < 0.05$ shown in **bold type**).

Trait	Cambium age (years)	Fertilizer treatment (F)	*cad* genotype (G)	F x G
Ring density (kg/m^3)				
	3	0.173	0.292	0.621
	4	**0.023**	0.116	0.114
	5	**0.023**	**0.013**	0.067
	6	**0.003**	0.427	0.821
	7	**0.001**	**0.055**	0.993
	8	**0.028**	**0.011**	0.846
	9	**0.000**	**0.009**	0.297
Latewood (%)				
	3	0.134	0.097	0.455
	4	0.325	0.161	0.071
	5	0.200	0.132	**0.045**
	6	**0.004**	**0.010**	0.625
	7	0.951	0.129	0.886
	8	0.323	0.210	0.929
	9	**0.002**	**0.044**	0.153
Earlywood density (kg/m^3)				
	3	0.584	0.570	0.397
	4	0.729	0.805	0.142
	5	**0.003**	**0.011**	0.492
	6	**0.036**	0.086	0.214
	7	**0.000**	**0.022**	0.814
	8	**0.002**	0.072	0.666
	9	**0.020**	0.215	0.981
Latewood density (kg/m^3)				
	3	0.375	0.646	0.070
	4	**0.007**	0.544	0.863
	5	0.694	0.593	0.945
	6	**0.003**	0.427	0.821
	7	**0.172**	0.689	0.635
	8	0.138	0.771	0.834

	9	**0.003**	0.093	0.426

Table 4. Linear regression models to predict weighted wood density (WD) using weighted latewood percentage (LP) for heterozygous (HZ) and wild-type (WT) trees on fertilized and control plots. Regression model for wild-type trees in fertilized plots is significantly different from wild-type trees in control plots (p = 0.0072) (see Figures 4C and 4D).

Treatment	Genotype	Model R^2	Regression equation	N
Fertilized	WT	0.76	WD = 292.52 + 3.74 × LP	5
Fertilized	HZ	0.75	WD = 311.52 + 3.28 × LP	4
Control	WT	0.85	WD = 342.45 + 2.93 × LP	5
Control	HZ	0.75	WD = 335.41 + 3.21 × LP	4

1) Number of observations.
2) Regression model probability level.

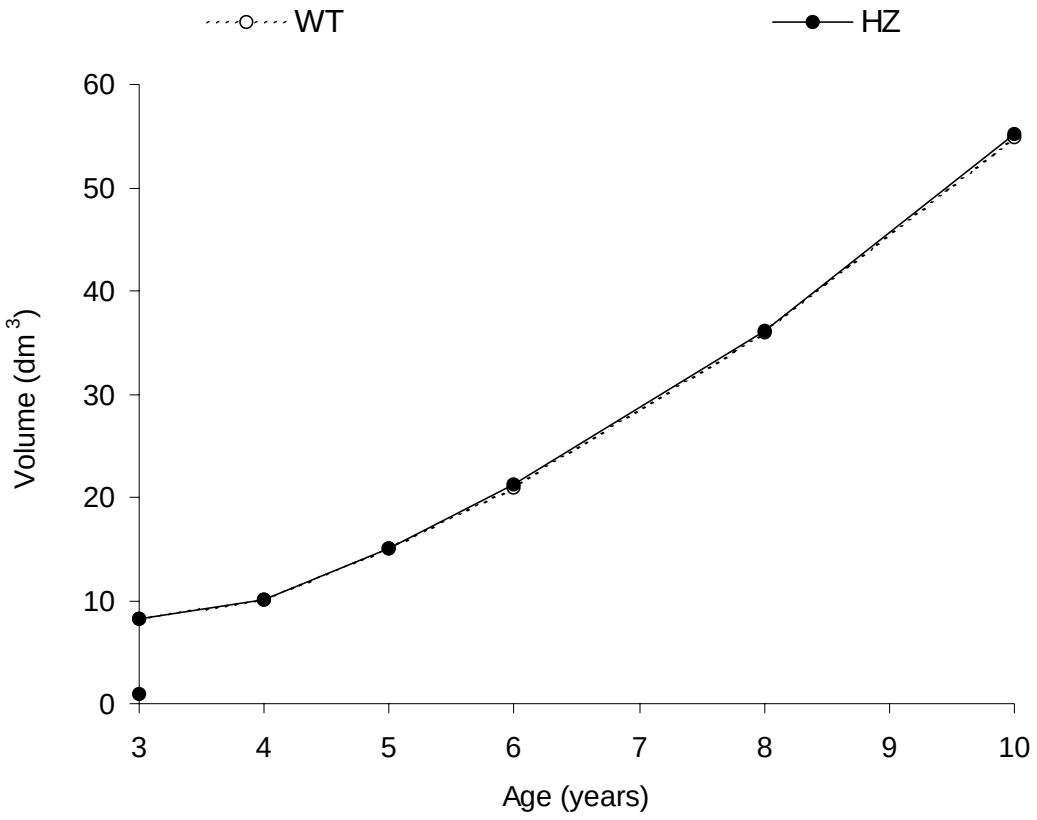


Figure 1. Mean tree volume from ages 3 to 10 yeas for heterozygous (HZ) and wild-type (WT) trees in fertilized (F) and control (C) plots. The sample sizes are 107 trees for wild-type 93 trees for heterozygous.

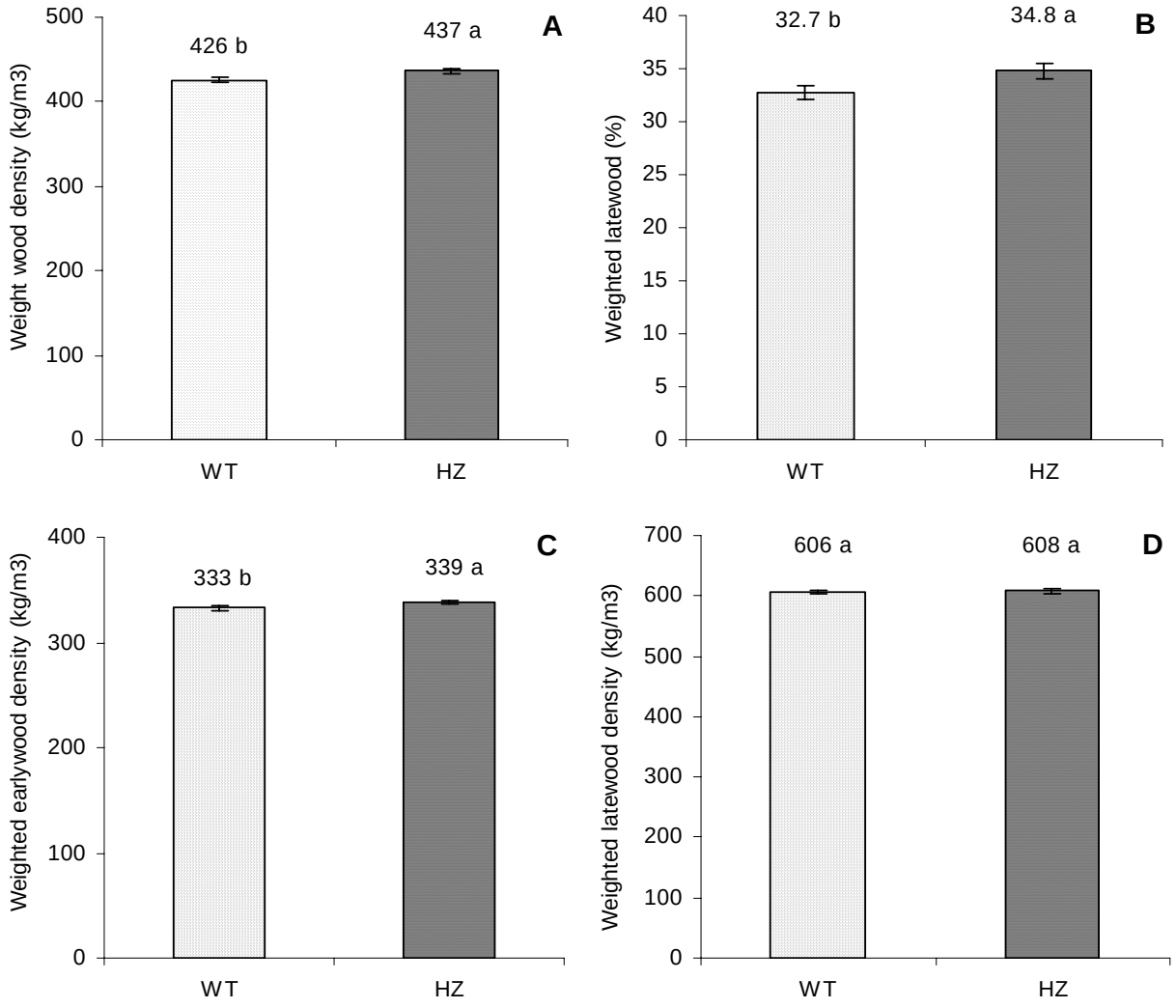
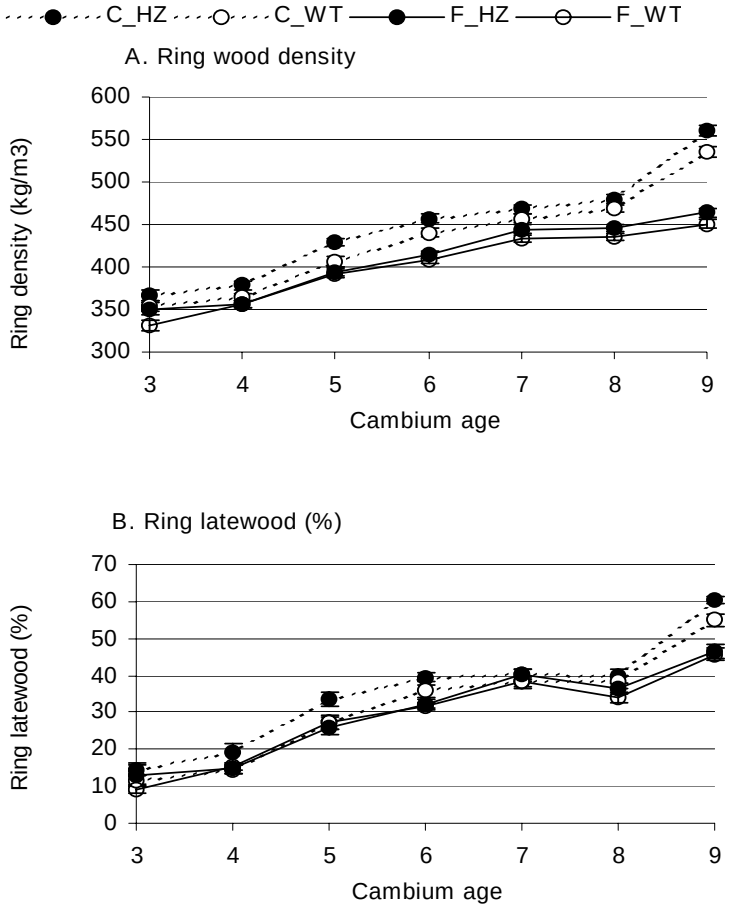


Figure 2. Comparison of wood quality traits (age 9 years) between wild-type (WT) and heterozygous trees (HZ) for all plots combined: mean of weighted wood density (A), weighted latewood percentage (B), weighted earlywood density (C), and weighted latewood density (D). Means for each trait followed by the same letter are

not significantly different at $p \leq 0.05$. The sample sizes are 107 trees for wild-type and and 93 trees for heterozygous.



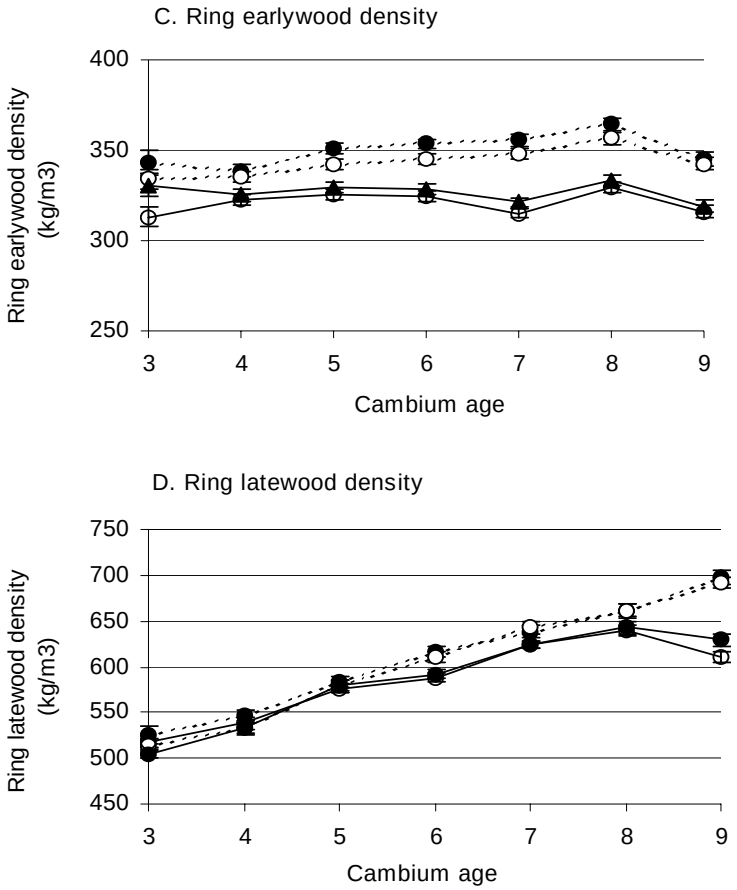
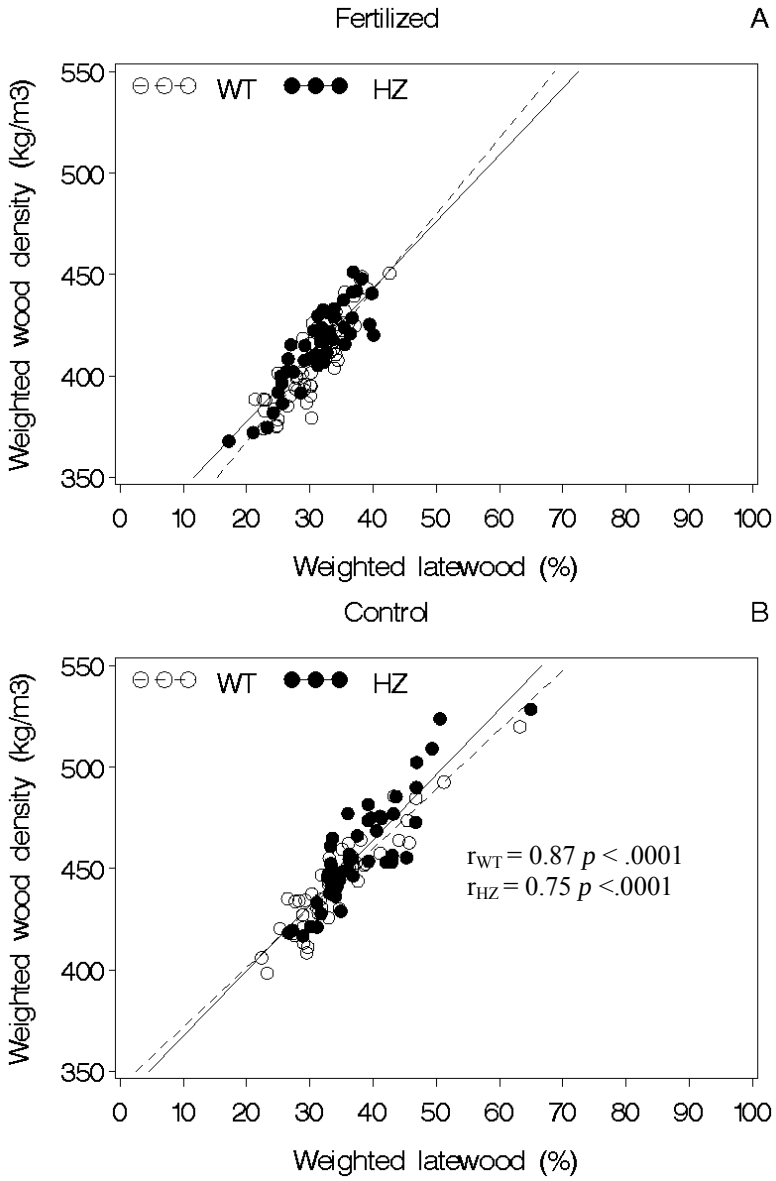


Figure 3. Ring wood density (A), ring latewood percentage (B), ring earlywood density (C) and ring latewood density (D) for heterozygous (HZ) and wild-type (WT) trees in fertilized (F) and control (C) plots for ring ages 3 to 9 years. The sample sizes were 54 and 46 trees in fertilized plots, 53 and 47 trees in control plots, for wild-type and heterozygous, respectively.



r_{WT} = 0.92 p <.0
r_{HZ} = 0.87 p <.0

Figure 4. Weighted wood density plotted against the proportion of weighted latewood in fertilized (A) and control treatments (B). The solid line indicates wild–type (WT) trees, and dashed line for heterozygous (HZ) trees. r_{WT} and r_{HZ} are correlation coefficients for wild -type and heterozygous trees, respectively. The sample sizes were 54 and 46 trees in fertilized plots, 53 and 47 trees in control plots, for wild-type and heterozygous, respectively.

ASSOCIATION OF THE *CAD-N1* ALLELE WITH INCREASED STEM GROWTH AND WOOD DENSITY IN FULL-SIB FAMILIES OF LOBLOLLY PINE

Stem growth and wood density associated with a mutant null (*cad-n1*) allele were examined in three 15-year-old loblolly pine diallel tests, established on two sites in the southern United States. In each diallel test, one or two *cad-n1* heterozygous parents were crossed with five unrelated wild-type parents, to produce five or ten full-sib families. In all, 839 trees from 20 full-sib families in four genetic backgrounds (a *cad-n1* heterozygote × 5 unrelated trees) were sampled, genotyped at the *cad* locus, and assessed for growth and wood density traits. In a combined analysis of all four genetic backgrounds, we found evidence for effects of increased wood density associated with the *cad-n1* allele at age 15 (p=0.03) years and height growth at ages 6 (p=0.03) and 15 (p=0.005). There were large differences in the *cad-n1* effects for the various growth and wood traits among the diallel tests. This variation may be due to either different genetic backgrounds among the parents of the different diallel tests, or for different growing environments at the field sites. Even though the *cad-n1* effect on growth and wood density was significant across genetic backgrounds, the effect was variable among full-sib families within backgrounds. We speculate that certain wild-type alleles from second parents specifically interact with *cad-n1* producing large positive effects. In addition, pleiotropic effects on growth and wood density appear to be associated with the *cad-n1* allele. While substantial gains are possible through deployment of trees carrying *cad-n1*, these gains may be family-specific and should be verified for each cross through field testing.

INTRODUCTION

Selection 7-56, owned by International Paper Company, is one of the best loblolly pine (*Pinus taeda L.*) parent trees in the NCSU Industry Cooperative Tree Improvement Program, producing progeny that are extremely fast growing with excellent quality traits (Li et al. 1996). 7-56 is also the only known natural carrier of a rare gene (*cad-n1*) (MacKay et al. 1995, 1997). This allele encodes a two base adenosine insertion that results in a severe deficiency in the enzyme, cinnamyl alcohol dehydrogenase (CAD) (Gill et al 2003), which catalyzes the last step in the biosynthesis of the major lignin precursors coniferyl alcohol. The extent to which this *cad- n1* allele affects the breeding value of 7-56 has been an important question in loblolly pine breeding programs. As a naturally occurring gene, acceptance of *cad*-n1 as a candidate for genetic improvement through breeding is expected to be higher than for a gene obtained from a different species introduced by genetic engineering. Before any use for marker-assisted breeding or genetic engineering can be considered, *cad-n1* must be well characterized and evaluated in harvest-age trees in a broad range of environments and in diverse genetic backgrounds.

Breeding with 7-56 produces three types of trees with respect to the *cad* locus. With self-pollination, *cad-n1* heterozygotes (*Cad/cad-n1*), wild-type homozygotes (*Cad/Cad*), and *cad-n1* homozygotes (*cad-n1/cad-n1*) are produced. With outcrossing, *cad-n1* heterozygotes and wild-type homozygotes are produced. The *cad-n1* homozygotes, originally characterized in this selfed family (7-56 × 7-56), have a distinct brown wood color and have a nearly complete absence of CAD activity in developing xylem (MacKay et al. 1995, 1997; Ralph et al. 1997, Gill et al. 2003). Dimmel et al. (2001) reported that homozygous *cad-n1* trees had poor growth after early stages and low pulp yields (due at least in part to inbreeding), compared to other genotypes, although they produced wood that was more easily delignified.

Wu et al. (1999) reported that heterozygous *cad-n1* trees produced 14% more debarked wood volume at age 4 years in an open-pollinated progeny, compared to wild-type trees. Previous studies comparing *cad-n1* heterozygotes with wild-type trees yielded inconsistent results on pulping and bleaching. In one study, kraft cooks of 4- and 6-year-old heterozygous trees resulted in kappa numbers (i.e., lignin contents) that were significantly lower than wild-type trees. Additionally, significantly less energy was required (15% to 25% lower H-factor) to pulp to a given kappa number than for wild-type trees, and the pulp of the heterozygotes was brighter and stronger (Dimmel et al. 2001). Conversely, Dimmel et al. (2002) found no apparent differences in ease of delignification or pulp yield between heterozygous and wild-type trees that were 14 years old. In a much larger sample, effects associated with this *cad-n1* allele were found by comparing wood density and growth traits of *cad-n1* heterozygous trees with wild-type trees in a 10-year-old open-pollinated family trial growing under two levels of fertilization. The substitution of *cad-n1* for a wild-type allele (*Cad*) was associated with a significant effect on wood density, but not for growth traits (Yu et al. 2005).

Prior to use of the *cad-n1* allele in tree improvement, it is necessary to estimate the direction and magnitude of the *cad-n1* effects on important traits at varying ages in different genetic backgrounds and field environments. In this paper we extend previous findings to include three *cad-n1* heterozygous selections evaluated in three half-diallel test series on two field test sites measured at two different ages. The objectives of this analysis are to: 1) quantify the association between *cad-n1* allele and tree growth and wood density in multiple genetic backgrounds, environments and ages; 2) estimate the *cad-n1* effects on growth and wood density; 3) investigate the temporal stability of the *cad-n1* effect for these traits.

MATERIAL AND METHODS

1. Plant material

Half diallel progeny tests

Three selected *cad-nl* heterozygous parents (second-generation selections that are offspring of 7-56) generating 20 full-sib families from three disconnected half-diallel progeny tests of loblolly pine were included in this study (Table 1). Each half-diallel consisted of 6 parents and 15 full-sib crosses, without selfs and reciprocal crosses. In each test, only full-sib progenies produced by *cad-n1* heterozygote parents (selections) crossed with 5 unrelated, wild-type parents were sampled and measured for growth and wood density traits (Table 2). The three diallel tests are numbered as series 1, 2 and 3, and the three *cad-n1* heterozygote selections are A, B and C. Series 1 included five crosses each for selections A and B, series 2 included five crosses with selection C, and series 3 included five crosses for selection A. Each diallel test was established in replicated trials at two sites, one in South Carolina and one in Georgia (Table 1). There are four field sites in each diallel test, but only two sites were available for this study. Each site was planted in a randomized complete block design with 6 blocks in 6-tree row plots for each cross (Li et al. 1996), but only 4 blocks were used for sampling. Genetic background is defined by a particular heterozygous selection in a specific test series, resulting in four genetic backgrounds (Table 2).

2. Growth measurements

At age 15 years, total tree height (HT, m) and diameter at breast height 1.3 m (DBH, cm) were measured in November 2003. In addition, all tests in each test series had growth measurements (HT, DBH) at age 6 years. Stem volume (VOL) was calculated using the equation of Shelton et al (1984):

[1] $VOL = 0.00748 + (0.0000353 \times DBH^2 \times HT)$

3. Genotyping and wood density measurements

Details of the genotyping and wood density measurement procedures are given in Yu et al. 2005, but a brief description follows. Inner bark tissue was collected and DNA was isolated from each of the sampled trees. Polymerase chain reaction (PCR) primers that flank the *cad-n1* specific mutation (2 bp insertion) (Gill et al. 2003) were used to amplify the *cad* locus from each DNA sample. PCR products were resolved and sized using capillary electrophoresis. Trees were scored as either heterozygous mutants (HZ) or homozygous wild-type (WT).

A 12-mm core was sampled from each tree at breast height for wood quality analyses. Cores were sectioned longitudinally to produce a strip approximately 2-mm thick. The samples were conditioned to a uniform moisture content of 8% before they were scanned. Wood density was measured using X-ray densitometry. Each strip was scanned from pith to the bark on a QMS Tree Ring Analyzer® (Model Qtrs-01x, Quintek Measurement Systems, Inc.). The last growth ring was excluded due to missing latewood on cores collected in mid-summer. For each ring scanned, the following intra-ring wood density characteristics were determined: average ring density, earlywood density, latewood density, latewood percentage, and cambial age. Weighted average wood density traits were calculated by weighting ring mean density with total ring basal area, which approximates the average density of a disk sample of wood taken at breast height.

5. Statistical analysis

For the combined analysis of all four genetic backgrounds, the following linear model was used,

[2] $Y_{hijklm} = \mu + B_h + S_{i(h)} + R_{j(hi)} + M_{k(h)} + G_l + (GM)_{kl(h)} + (GS)_{il(h)} + (GR)_{jl(hi)} + (MS)_{ik(hi)} + (MR)_{jk(hi)} + e_{hijklm}$

Where Y_{hijklm} is the m^{th} tree of the j^{th} block within the i^{th} site for the l^{th} genotype of the k^{th} mate of h^{th} genetic background; μ is the overall mean; B_h is the effect of the h^{th} genetic background; $T_{i(h)}$ is the effect of the i^{th} site within h^{th} genetic background; $R_{j(hi)}$ is the effect of the j^{th} block within the i^{th} site and h^{th} genetic background; $M_{k(h)}$ is the effect of k^{th} mate within the h^{th} genetic background; G_l is the effect of the l^{th} genotype; $(GM)_{kl(h)}$ is the effect of the interaction of the k^{th} mate and l^{th} genotype within h^{th} genetic background; $(GT)_{il(h)}$ is the effect of the interaction between l^{th} genotype and the i^{th} site within h^{th} genetic background; $(GR)_{jk(hi)}$ is the effect of interaction between l^{th} genotype within j^{th} block within i^{th} site; $(MT)_{ik(h)}$ is the interaction between the k^{th} mate and the i^{th} site within h^{th} genetic background; $(MR)_{jk(hi)}$ is the interaction between the k^{th} mate and the j^{th} block within the i^{th} site and h^{th} genetic background; e_{ijklm} is the residual random within plot error term ~ *NID N* $(0,\sigma^2_e)$. The $R_{j(hi)}$, $(GR)_{jl(hi)}$, $(MR)_{jk(hi)}$ and e_{ijklm} are considered as random effects, and the rest of terms as fixed effect. Standardized data was

used for the combined analysis, which means each observation was divided by standard deviation of each field test.

In order to evaluate the *cad-n1* allele effect in each genetic background, growth and wood density data were also analyzed separately for each background according to the following linear model:

[3] $Y_{ijklm} = \mu + S_i + R_{j(i)} + M_k + G_l + (GM)_{kl} + (GS)_{il} + (GR)_{jk(i)} + (MS)_{ik} + (MR)_{jk(i)} + e_{ijkl}$

The statistical analyses were performed by using the SAS software package (SAS Institute 2001). The genotypic effects (i.e., the difference between heterozygous *cad-n1* and wild-type trees) were estimated using PROC MIXED, and the ESTIMATE option was used to estimate the difference among the second parents in each genetic background. PROC MULTTEST provided *p*-value adjustments of significance between heterozygous and wild-type trees in each 20 full-sib families using Bon (Bonferroni) option. The Bonferroni adjustment procedure concerns the question if, in the case of doing more than one test in a particular study, the alpha level should be adjusted to consider the chance of making a Type I error. If adjusted p-value exceeds 1, it is set to 1 (SAS Institute 2001).

RESULTS

In the half-diallel progeny tests, trees within full-sib families were expected to segregate 1:1 for the two possible *cad* genotypes (*Cad/cad-n1* and *Cad/Cad*). In total, 839 trees were sampled from four genetic backgrounds; 419 were heterozygous *cad-n1* trees, and 420 were wild-type (Table 1). Chi-square analysis indicated no significant difference from the expected segregation overall and for each of the four genetic backgrounds separately (data not shown).

We estimated least square means of growth traits and weighted wood density traits for the two *cad* genotypes (Tables 3a, 3b). In the combined analysis of the four genetic backgrounds, height, diameter and volume at ages 6 and age 15 were higher for *cad-n1* heterozygotes than for wild-type trees, and 2.4%, 2.1% and 5.4% greater at age 15, respectively, but only height at age 6 and age 15 were significant effects (Table 4a).

For the combined analysis of four genetic backgrounds for wood density traits, weighted earlywood density and latewood density were about the same between the two *cad* genotypes (Table 3b). For weighted wood density and weighted latewood percentage, heterozygous trees were 1% and 2.1 % significantly higher than wild-type trees (Tables 3b, 4b).

We did not find the interactions of *cad* genotype × site and *cad* genotype × genetic background for growth traits at either ages 6 or 15, or wood density at age 15 (Tables 4a, 4b). We also did not find *cad* genotype × age interactions for any of the growth traits (results not shown). The differences in the growth traits between the two *cad* genotypes are consistent between ages 6 and 15 (Table 3a). Genetic background, test, and mate significantly affected all study traits.

Although there were no genotype by genetic background interactions, we analyzed each of the genetic backgrounds separately to determine if the magnitude of the *cad-n1* effect was similar in each background. In the separate analyses of each genetic background (Tables 3a, 5a), age 15 volumes of A-S1, C-S2 and A-S3 were 4.3%, 5.3% and 10.8% higher for *cad-n1* heterozygotes than wild-type, respectively, however, B-S1 heterozygotes were 4.5 % lower than wild type. The effect of *cad* genotype was significant only for A in genetic background S3 (A-S3) on height

both at age 6 (p=0.04) and age 15 (p=0.02), and marginally significant for volume at ages 6 (p=0.05) and 15 (p=0.06) (Table 5a).

For wood density traits, there were large differences between A-S3 and the other three genetic backgrounds in the effect of *cad-n1* (Table 4b). In A-S3, weighted wood density and weighted latewood percentage were 2.6 % and 6.5% (p=0.03 and p=0.02) higher for heterozygotes than wild-type, respectively (Tables 3b, 5b), whereas no significant differences were found between heterozygous and wild-type trees for A-S1, B-S1 or C-S2 (Table 5b).

Within individual backgrounds, there was a significant interaction of *cad* genotype × mate for height growth at age 6 and wood density and latewood percentage at age 15 (Table 4a, 4b). The least square means for growth and wood traits for all full-sib families in each genetic background are shown in Table 6. There are large differences between *cad* genotypes for volume and density among the full-sib families in the four genetic backgrounds. For 20-full-sib families, the volume and wood density were higher for heterozygous than wild-type trees (positive effects) in 12 and 13 full-sib families, respectively. The differences of these positive effects ranged from 2.7 % to 48.3 % and from 0.4 % to 8.9 %, for volume and density respectively, whereas for the negative effect, differences ranged from -0.4 to –10.7 % and –0.4 to –3.0 %, respectively. For one particular full-sib family (BxB2), wood density was 3.0 % lower (p=0.07) for heterozygous compared to wild-type trees, whereas wood density was 8.9 % and 3.8 % higher (p= 0.005 p= 0.06) for heterozygotes compared to wild-type trees for families AxA6 and AxA9 respectively. Heterozygotes in these two families (AxA6 and Ax A9) also showed a significantly higher volume growth between ages 6 and 15 compared to their wild-type siblings. We did not find significantly lower volume growth for heterozygous trees compared to wild-type trees in any of the 20 full-sib families.

In order to test the chance of making a Type I error, Bonferroni correction was used. The alpha level of each full-sub family was adjusted downwards to ensure that overall experiment-wise risk for a number of tests remains at 0.05. The correction indicated only wood density in full-sib AxA6 was significant (p=0.002), while in this same family volume (p=0.11) was nearly significant.

Figure 1 showed the average ring density traits of full-sib A x A6 were plotted against cambium age. Ring density, latewood density and latewood percentage increased from pith to bark as expected. The *cad* heterozygotes of full-sib family AxA6 had consistently higher ring density over the years at both field trials in test series 3. Earlywood density plotted against age showed little variation and appeared to decrease with cambium age. Heterozygous trees had significantly higher ring density than wild-type trees throughout most years especially in the second field test. The higher ring density for heterozygous trees was mostly associated with higher latewood and latewood percentage compared to wild-type trees at both field trials.

DISCUSSION

Verification of the *cad-n1* effect is necessary to substantiate a biological basis for observed marker-trait associations, to provide precise estimates of the direction and magnitude of *cad-n1* effects, and to predict *cad-n1* effects at various ages in various environments and genetic backgrounds. When all three diallel test series were considered in a combined analysis, significant differences in wood density (1%), height at ages 6 (3%) and 15 (2.4%) years were

found between *cad-n1* heterozygote and wild-type trees (Tables 3a, b and Tables 4a, b). Although it was only marginally statistically significant, it appears that *cad-n1* was associated with increasing volume of about 5 % ($p = 0.09$) (Tables 4 and 5). We speculate that perhaps trees harboring the *cad-n1* allele may invest fewer resources into the production of monolignols, allowing reallocation of resource towards growth and wood density. Promotion of growth was also observed in transgenic poplar, where the lignin biosynthetic enzyme 4-coumarate: coenzyme A ligase (4CL) was down-regulated (Hu et al., 1999). Kirst et al. (2004) found that the expression of the lignin-related genes was negatively correlated with diameter growth of a Eucalyptus's hybrid. Remington and O'Malley (2000) found apparent overdominance at the *cad* locus for growth for both year 2 and year 3, although overdominance was not statistically significant. Yu et al. (2005) found that *cad-n1* heterozygous trees had greater wood density than wild-type trees, but did not find an association of *cad-n1* with growth. Wu et al. (1999) found that *cad-n1* heterozygous trees were faster growing than their wild-type siblings in a single half-sib family. Possible explanations for this discrepancy include different genetic material used (heterozygous selections and second parents), environmental differences between field trials, and differing statistical power to detect small effects.

The segregation ratio for *cad-n1* mutant and wild-type alleles was consistent with expected ratio of 1:1 (Wu et al. 1999 and Yu et al. 2005). This suggests that *cad-n1* is not strongly deleterious for survival and adaptability through age 15 in our research planting. Remington and O'Malley (2000) found that homozygotes for *cad-n1* showed no effect on survival of germination seedling after three years; however Dimmel et al. (2001) did find homozygotes to be significantly slower growing and less straight than wild-type trees at age 12 years. To date, the *cad-n1* allele has not been identified in any tree outside of the pedigree of tree 7-56.

Since 7-56 is one of the most valuable loblolly pine selections, with extremely high breeding value for growth, it is reasonable to speculate that the *cad-n1* mutation may have a major effect on its breeding value. Table 7 shows breeding values for height at age 6 years (H6BV) of 30 second-generation offspring of 7-56selected in first-generation progeny tests (NCSU-TIP, 2003). These selections were made in crosses that included 7-56 as a parent, and each cross had high breeding value for growth and quality traits. The selection criteria for the individual within each cross included the individual trees' phenotypes for height, diameter, resistance to fusiform rust (caused by *Cronartium quercuum* (Berk) Miyabe ex Shirai f. sp. *fusiforme*), and stem straightness (Li et al 1996). Of the 30 offspring selections, only 7 carried the *cad-n1* allele. Our expectation was that at least half the selections would have *cad-n1* since faster growth is associated with *cad-n1*. Based on the evaluation of progeny of these 30 selections (grandchildren of 7-56), the H6BV of the seven *cad-n1* heterozygotes ranged from -5.6 to 19.9, versus a range of 1.2 to 19.6 for the 23 wild-type trees. These results indicated that the breeding value for height at age 6 of the two *cad* genotypes is not significantly different.

It appears that the *cad-n1* allele does not have an overwhelming influence on the high breeding value of 7-56 for growth; other gene loci are likely involved. Quantitative traits such as growth and wood density in loblolly pine are assumed to be under polygenic control with relatively small effects coupled with environmental and epistatic interactions. Considering the complex nature of tree height and stem diameter, it is not surprising that *cad-n1* is not the only major allele associated with the high breeding value of 7-56.

In this study we have extended studies on the effect of *cad-n1* allele over many growing seasons in multiple genetic backgrounds, and field environments. We found large differences in the

effect of the *cad-n1* allele among genetic backgrounds on growth and wood density, although these comparisons were confounded by environmental differences at the test sites. The differences between the two *cad* genotypes on growth and wood density traits were not the same in each genetic background growing in a particular environment. However, we did not find *cad* genotype x genetic background interactions for any of the studied traits. Selection A in diallel series 3 produced heterozygous trees that averaged 10.8 % greater volume than wild-type trees, whereas in diallel series 1, selection A heterozygous trees were only 4.3 % greater than their wild-type siblings (Table 4). This may be due to either different genetic backgrounds between diallel series 1 (second parents A1-A5) and diallel series 3 (A6-A10), or different growing environments (Georgia and South Carolina, respectively). The effect of *cad-n1* allele may be larger at better sites (series 3) than poorer sites (series 1).

Overall, there was a significant genotype by mate interaction for height at age 6 years and for weighted wood density and latewood percent at age 15 (Table 4). Within individual backgrounds (Table 5), the genotype by mate interaction was significant only for height at age 6 in background C-S2 and for weighted specific gravity at age 15 in background A-S3. The 10.8 % volume and 2.6 % ($p = 0.04$) wood density increases in genetic background A-S3 for *cad-n1* heterozygotes may be due to specific wild-type alleles at the same *Cad* locus or other loci that interact with the *cad-n1* allele. In particular, two second parents (A6 and A9) may have contributed certain genetic components that specifically interacted with *cad-n1* heterozygote (selection A) to produce the large positive effect on growth and wood density. Further studies are needed to determine the genetic factors in these second parents that contribute to this interaction. In addition, there is evidence for the presence of pleiotrophy such that growth and density are associated with *cad-n1* allele but involve interactions with genes contributed from the second parents (Table 9). Bradshaw and Settler (1995) reported in poplar trees that QTLs controlling basal stem area growth and sylleptic branch habit are probably controlled by the same genes.

Forest trees experience a variety of environmental conditions over their life spans. Long-lived trees also experience different developmental stages of growth (e.g. the change from juvenile to mature wood), which are most likely controlled by different sets of regulatory factors. Several QTL studies in forestry have examined the stability of QTLs over multiple growing seasons. Sewell et al. (2002) indicated that the difference in the number of QTL between the single year and multi-year analyses suggest that possible genotype x environment interactions influence the temporal expression of different QTLs from year to year. A few of these studies repeatedly detected a subset of QTLs over time (e.g. Newcombe and Bradshaw 1996). In the present study, the effect of *cad-n1* allele on growth and wood density was consistent at age 6 and age 15 in the four genetic backgrounds (Table 4 and Table 5). There was no *cad* genotype x age interactions on growth and wood density (data not shown). These results are similar to our previous study examining *cad-n1* effects in a 10-year-old open-pollinated family trial growing under two level of fertilization (Yu et al. 2005). The *cad-n1* effects on growth and wood density that are detectable over multiple growing seasons may be most valuable in large-scale breeding programs.

For full-sib family AxA6, we found the *cad-n1* allele associated with a significant increase in wood density through 15 years (Figure 1). This increase can be attributed to higher earlywood density, latewood density, and a greater proportion of latewood in *cad-n1* heterozygotes. This result is similar to our previous study (Yu et al. 2005). In the same half-sib family (selection A open-pollinated), we found that differences in earlywood density and latewood percentage were

consistent throughout tree development in both control and fertilized treatments, which resulted in high total wood density for heterozygous trees, as compared to wild-type trees, up to age 10 years. The higher wood density was apparently due to the higher percentage of latewood in the heterozygotes.

CONCLUSION

The mechanism of high breeding value for original founder 7-56 is unknown, but our data suggest it is not exclusively associated with the *cad-n1* allele. Both wood density and height growth traits were significantly higher for *cad-n1* heterozygotes than for their wild-type sibs. Although it was not statistically significant, the same trend was apparent for stem volume growth. Within genetic backgrounds, significant differences were limited to specific full-sib families, suggesting that alleles contributed by the second parents differentially affect the phenotype of *cad-n1* heterozygous trees. These alleles could be at the *cad* locus (i.e., variation in wild-type alleles) or else where in the genome. The data also provide evidence of a pleiotrophic effect on growth and density, since *cad-n1* has an effect on both of these traits. While substantial genetic gains in growth and wood density are possible through selection and deployment of trees carrying the *cad-n1* allele, these gains are family-specific and must be verified for each new cross through field tests. In addition, further studies are needed to determine and evaluate the genetic factors that interact to cause variable phenotypic effects of *cad-n1* between full-sib families. Finally, it is clear that large, well-designed studies will be required to clearly define the main effects of the *cad-n1* allele and their interactions with other alleles and ultimately to determine the mechanisms of high breeding value in loblolly pine, especially for trees such as 7-56 and selection A.

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Table 1. Field test locations and sampling characteristics for *cad-n1* heterozygous parents.

cad-n1 selections	Diallel series	Number of sites	State	Year planted	No. of samples	No. of *cad-nl* heterozygotes	No. of wild-type
A	1	2	Georgia	1988	225	114	119
A	3	2	South Carolina	1988	194	90	100
B	1	2	Georgia	1988	233	115	108
C	2	2	South Carolina	1988	192	100	93
Total		8			839	419	420

Table 2. Parents, diallel test series, and genetic backgrounds sampled for analysis.

cad-n1 selection	Diallel series	Genetic background	Mate (second parents)				
A	S1	A-S1	A1	A2	A3	A4	A5
A	S3	A-S3	A6	A7	A8	A9	A10
B	S1	B-S1	B1	B2	B3	B4	B5
C	S2	C-S2	C1	C2	C3	C4	C5

Table 3. Least square means (±standard error) of *cad-n1* heterozygous (HZ) and wild-type (WT) trees in four genetic backgrounds. (*p* values ≤ 0.05 shown in **bold type)**.

(a) Stem growth traits at ages 6 and 15 years.

Genetic background	6 years						15 years					
	Height (m)		DBH (cm)		Volume (dm^3)		Height (m)		DBH(cm)		Volume (m^3)	
	WT	HZ	WT	HZ	WT	HZ	WT	HZ	WT	HZ	WT	HZ
A-S1†	6.0±0.1	6.1±0.1	9.4±0.2	9.6±0.2	28.0±0.9	28.6±0.9	17.5±0.1	17.8±0.1	18.4±0.3	18.8±0.3	0.23±0.01	0.24±0.01
A-S3	**6.9±0.1**	**7.2±0.1**	12.3±0.2	12.7±0.2	47.3±1.7	51.4±1.7	**19.1±0.2**	**20.1±0.2**	21.8±0.5	22.7±0.5	0.37±0.02	0.41±0.02
B-S1	5.9±0.1	5.8±0.1	9.5±0.2	9.2±0.2	28.2±0.8	27.1±0.8	16.9±0.2	16.9±0.2	18.3±0.3	18.0±0.3	0.22±0.01	0.21±0.01
C-S2	5.9±0.1	6.0±0.1	10.7±0.2	10.9±0.2	46.5±1.4	47.2±1.3	18.6±0.2	19.2±0.2	21.3±0.5	21.9±0.5	0.38±0.02	0.40±0.02
Estimated mean‡	**6.4±0.1**	**6.6±0.1**	11.0±0.2	11.2±0.2	36.5±1.2	37.5±1.2	**18.6±0.2**	**19.1±0.2**	20.3±0.2	20.8±0.2	0.30±0.01	0.31±0.01

(b) Wood density traits at age 15.

Genetic background	Weighted wood density (kg/m^3)		Weighted earlywood density (kg/m^3)		Weighted latewood density (kg/m^3)		Weighted latewood (%)	
	WT	HZ	WT	HZ	WT	HZ	WT	HZ
A-S1†	536±3.1	536±3.13	341±1.3	341±1.3	711±3.0	709±3.1	50±0.56	50±0.58
A-S3	**498±3.8**	**511±4.03**	344±1.7	348±1.8	658±3.2	665±3.4	**46±0.83**	**49±0.88**
B-S1	529±3.2	526±3.16	331±1.5	333±1.4	701±3.0	697±3.0	50±0.62	50±0.61
C-S2	487±3.6	492±3.49	339±1.5	341±1.5	657±2.9	660±3.0	43±0.72	44±0.69
Estimated mean‡	**513±1.8**	**518±1.8**	342±1.0	341±1.0	688±1.6	690±1.6	**48±0.3**	**49±0.3**

† From model (3), estimated by REML.

‡ From model (2).

Table 4. Significance levels (*p* values) for *cad* main effects and interactions from the combined (over the four genetic backgrounds) analysis. (*p* values were estimated from model (2)).

(a) Stem growth traits at ages 6 and 15 years.

			6 years			15 years		
Effect	DF†	Den DF‡	Height (m)	DBH (cm)	Volume (dm^3)	Height (m)	DBH (cm)	Volume (m^3)
Fixed			Level of significance (*p*)					
Background	3	24	<0.001	<0.001	0.088	<0.001	<0.001	0.150
Site (B)	4	24	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Mate (B)	16	96	<0.001	<0.001	<0.001	<0.001	0.024	0.002
Genotype	1	24	0.031	0.211	0.206	0.005	0.138	0.087
B*genotype	3	24	0.253	0.283	0.133	0.179	0.572	0.472
Genotype*mate(B)	16	628	0.037	0.339	0.461	0.192	0.536	0.449
Site*genotype (B)	4	24	0.684	0.607	0.529	0.382	0.583	0.562
Site*mate(B)	16	96	0.011	0.000	0.000	0.139	0.002	0.002
Random effect			Variance component %					
Rep(B*Site)			41.2	19.5	23.9	13.2	4.8	6.6
Rep*mate(B*Site)			9.7	1.1	2.9	10.9	0	0
Rep*genotype(B*Site)			0.8	0.4	0	2.3	1.3	1.7
Residual			48.3	79	73.3	73.6	93.9	91.8

(b) Wood density traits at age 15.

Effect	DF†	Den DF‡	Weighted wood density (kg/m^3)	Weighted latewood (%)	Weighted earlywood density (kg/m^3)	Weighted latewood density (kg/m^3)
Fixed				Level of significance (*p*)		
Background	3	24	<0.000	<0.001	<0.001	<0.001
Site (B)	4	24	<0.000	<0.001	<0.001	<0.001
Mate (B)	16	96	0.004	0.004	<0.001	<0.001
Genotype	1	24	0.033	0.027	0.110	0.325
B*genotype	3	24	0.213	0.186	0.887	0.536
Genotype*mate(B)	16	628	0.043	0.046	0.150	0.198
Site*genotype (B)	4	24	0.268	0.188	0.947	0.582
Site*mate(B)	16	96	0.044	0.022	0.010	0.776
				Variance component %		
Random effect						
Rep(B*Site)			2.5	1.2	4.8	0.6
Rep*mate(B*Site)			0.0	0.0	2.3	3.6
Rep*genotype(B*Site)			0.0	0.0	3.5	0.7
Residual			97.5	98.8	89.4	95.1

† Degree of freedom of the numerator

‡ Degrees of freedom of the denominator

Table 5. Significance levels (*p* values) for *cad* main effects and interactions from the separate analyses for each genetic background. (*p* values were estimated from model (3)).

(a) Stem growth traits (Height6, DBH6, Volume6) at ages 6 and (Height, DBH, Volume) at age 15.

Genetic background	Effect	DF	Den DF	6 years			15 years		
				Height (m)	DBH (cm)	Volume (dm^3)	Height (m)	DBH (cm)	Volume (m^3)
A-S1	Site	1	6	0.181	0.734	0.559	0.431	0.000	0.000
	Mate	4	24	0.001	0.017	0.003	0.003	0.110	0.026
	Genotype	1	6	0.513	0.990	0.804	0.515	0.906	0.847
	S *G	1	6	0.292	0.526	0.550	0.113	0.154	0.167
	G*M	4	180	0.403	0.441	0.764	0.739	0.538	0.500
	T*M	4	24	0.072	0.018	0.010	0.670	0.697	0.476
	Random effect			Variance component %					
	Rep (S)			11.0	5.6	7.6	1.7	0.0	0.0
	Rep*G (S)			0.1	0.0	0.0	5.3	0.0	0.0
	Rep*M (S)			13.9	0.0	1.2	11.0	0.0	0.0
	Residual			75.0	94.4	91.1	82.1	100.0	100.0
A-S3	Site	1	6	0.496	0.075	0.501	0.001	0.002	0.000
	Mate	4	24	0.829	0.811	0.756	0.973	0.956	0.436
	Genotype	1	6	0.040	0.099	0.054	0.016	0.153	0.063
	S *G	1	6	0.998	0.215	0.173	0.716	0.385	0.153
	G*M	4	180	0.229	0.097	0.180	0.143	0.199	0.222
	T*M	4	24	0.172	0.002	0.004	0.143	0.003	0.001
	Random effect			Variance component %					
	Rep (S)			61.3	15.0	26.0	10.2	6.2	4.0
	Rep*G (S)			0.0	0.2	1.9	0.0	0.0	0.0
	Rep*M (S)			12.0	10.2	9.1	31.8	7.8	5.5
	Residual			26.6	74.7	62.9	58.0	86.0	90.5
B-S1	Site	1	6	0.016	0.024	0.053	0.019	0.016	0.029
	Mate	4	24	0.000	0.001	0.000	0.001	0.025	0.003
	Genotype	1	6	0.967	0.475	0.562	0.803	0.781	0.964
	S *G	1	6	0.978	0.772	0.844	0.377	0.933	0.883
	G*M	4	180	0.273	0.646	0.432	0.162	0.592	0.615
	T*M	4	24	0.007	0.019	0.013	0.251	0.214	0.064
	Random effect			Variance component %					
	Rep (S)			18.0	13.8	21.7	0.0	0.0	0.0
	Rep*G (S)			0.0	0.0	0.0	1.1	0.0	1.6
	Rep*M (S)			10.0	9.6	6.3	11.9	14.2	7.4
	Residual			72.0	76.6	72.0	87.0	85.8	91.0
C-S2	Site	1	6	0.000	0.000	0.000	0.000	0.000	0.000
	Mate	4	24	0.649	0.093	0.165	0.819	0.450	0.218
	Genotype	1	6	0.410	0.468	0.632	0.129	0.267	0.178
	S *G	1	6	0.494	0.729	0.757	0.628	0.861	0.673
	G*M	4	180	0.036	0.304	0.779	0.318	0.334	0.701
	T*M	4	24	0.217	0.421	0.307	0.324	0.091	0.103
	Random effect			Variance component %					
	Rep (S)			42.3	25.4	7.5	30.5	8.4	10.1
	Rep*G (S)			6.2	4.8	0.0	6.3	3.3	0.0
	Rep*M (S)			4.4	0.0	0.0	0.0	0.0	0.0
	Residual			47.2	69.8	92.5	63.2	88.3	89.9

(b) Wood density traits at age 15.

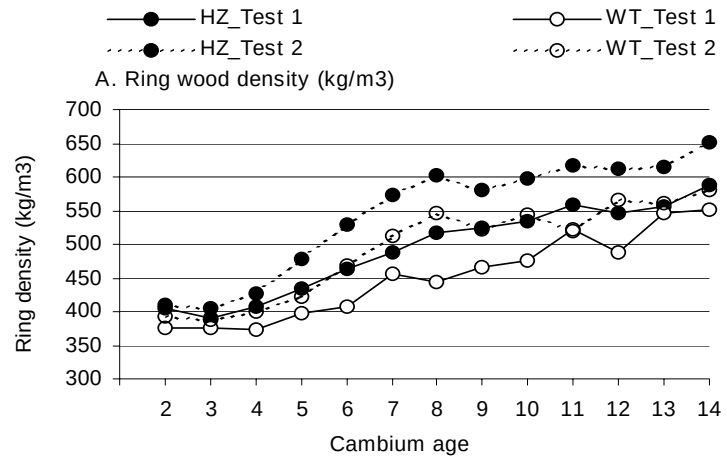
Genetic background	Effect	DF	Den DF	Wood density (kg/m^3)	Latewood (%)	Earlywood density (kg/m^3)	Latewood density (kg/m^3)
A-S1	Site	1	6	0.000	0.000	0.000	0.258
	Mate	4	24	0.216	0.242	0.706	0.001
	Genotype	1	6	0.733	0.476	0.844	0.992
	S *G	1	6	0.260	0.107	0.602	0.574
	G*M	4	180	0.428	0.203	0.331	0.590
	T*M	4	24	0.094	0.115	0.019	0.603
	Random effect			Variance component %			
	Rep(S)			3.6	0.0	0.0	5.1
	Rep*G (S)			0.0	0.2	5.6	0.7
	Rep*M (S)			0.0	0.0	0.9	0.0
	Residual			96.4	99.8	93.5	94.2
A-S3	Site	1	6	0.000	0.044	0.001	0.000
	Mate	4	24	0.391	0.695	0.043	0.121
	Genotype	1	6	0.026	0.023	0.261	0.177
	S *G	1	6	0.094	0.152	0.719	0.279
	G*M	4	180	0.035	0.081	0.105	0.335
	T*M	4	24	0.066	0.044	0.046	0.656
	Random effect			Variance component %			
	Rep(S)			0.0	0.0	0.0	0.0
	Rep*G (S)			0.0	0.0	0.0	0.0
	Rep*M (S)			5.0	0.0	0.0	17.2
	Residual			95.0	100.0	100.0	82.8
B-S1	Site	1	6	0.000	0.000	0.002	0.243
	Mate	4	24	0.015	0.052	0.012	0.001
	Genotype	1	6	0.801	0.976	0.317	0.826
	S *G	1	6	0.597	0.407	0.714	0.639
	G*M	4	180	0.261	0.464	0.319	0.024
	T*M	4	24	0.575	0.456	0.218	0.427
	Random effect			Variance component %			
	Rep(S)			0.0	1.5	6.1	0.0
	Rep*G (S)			0.0	0.0	3.0	0.9
	Rep*M (S)			0.8	0.0	8.2	0.0
	Residual			99.2	98.5	82.6	99.1
C-S2	Site	1	6	0.000	0.012	0.020	0.000
	Mate	4	24	0.043	0.009	0.000	0.211
	Genotype	1	6	0.515	0.514	0.475	0.569
	S *G	1	6	0.628	0.647	0.879	0.454
	G*M	4	180	0.164	0.087	0.233	0.926
	T*M	4	24	0.368	0.200	0.591	0.631
	Random effect				Variance component %		
	Rep(S)			4.5	3.8	17.8	0.0
	Rep*G (S)			2.4	4.2	0.0	0.0
	Rep*M (S)			0.0	0.0	0.6	4.3
	Residual			93.2	91.9	81.6	95.7

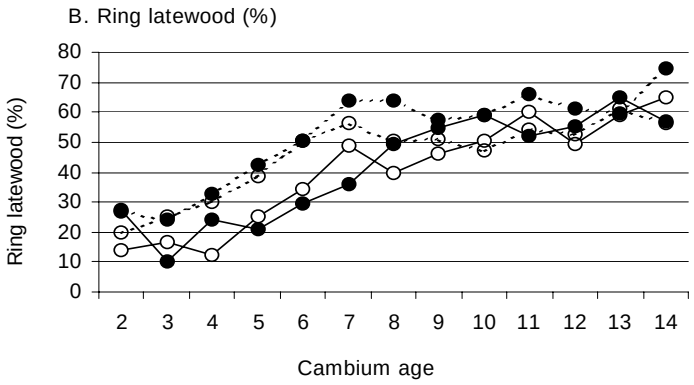
Table 6. Least square means (±standard error) of *cad-n1* heterozygous (HZ) and wild-type (WT) trees in full-sib families in four genetic backgrounds and differences (Diff. = (HZ – WT)/HZ×100) in least square means between HZ and WT within full-sib families. (*p* values and Bonferroni (Bon) adjusted *p* values were estimated using model (3))

Genetic background	Full-sib family	No. of trees		Weighted wood density (kg/m^3)					Volume (m^3)				
		WT	HZ	WT	HZ	Diff. (%)	P value	Bon	WT	HZ	Diff. (%)	P value	Bon
A-S1	AxA1	24	24	529±6.5	533±6.5	0.8	0.633	1	0.20±0.02	0.21±0.02	5.0	0.676	1
	AxA2	23	24	525±6.6	538±6.5	2.5	0.164	1	0.23±0.02	0.24±0.02	4.3	0.634	1
	AxA3	22	24	544±6.8	533±6.6	-2	0.244	1	0.28±0.02	0.25±0.02	-10.7	0.189	1
	AxA4	21	25	541±6.8	546±6.3	0.9	0.562	1	0.23±0.02	0.26±0.02	13.0	0.264	1
	AxA5	29	17	538±5.9	534±7.5	-0.7	0.663	1	0.22±0.02	0.21±0.02	-4.5	0.848	1
A-S3	AxA6	22	21	483±7.5	526±7.5	8.9	0.000	0.002	0.29±0.04	0.43±0.04	48.3	0.005	0.11
	AxA7	24	16	491±7.1	502±8.5	2.2	0.280	1	0.37±0.03	0.38±0.04	2.7	0.801	1
	AxA8	16	23	503±8.6	522±7.1	3.8	0.078	1	0.36±0.04	0.46±0.03	27.8	0.060	1
	AxA9	17	18	506±8.2	504±8.1	-0.4	0.856	1	0.36±0.04	0.38±0.04	5.6	0.672	1
	AxA10	21	12	499±7.9	501±9.9	0.4	0.873	1	0.43±0.04	0.42±0.05	-2.3	0.948	1
B-S1	BxB1	25	23	532±6.4	544±6.8	2.3	0.205	1	0.25±0.02	0.25±0.02	0.0	0.820	1
	BxB2	23	23	539±6.6	523±6.6	-3	0.080	1	0.21±0.02	0.2±0.02	-4.8	0.699	1
	BxB3	22	23	519±6.7	526±6.6	1.3	0.435	1	0.17±0.02	0.21±0.02	23.5	0.194	1
	BxB4	20	22	514±7.0	514±6.7	0	0.930	1	0.19±0.02	0.19±0.02	0.0	0.827	1
	BxB5	18	24	532±7.4	534±6.4	0.4	0.856	1	0.26±0.02	0.24±0.02	-7.7	0.457	1
C-S2	CxC1	21	21	500±7.3	495±7.4	-1	0.617	1	0.43±0.03	0.41±0.03	-4.7	0.696	1
	CxC2	22	18	482±7.2	495±8.0	2.7	0.236	1	0.32±0.03	0.39±0.04	21.9	0.113	1
	CxC3	17	22	499±8.0	486±7.3	-2.6	0.185	1	0.39±0.04	0.42±0.03	7.7	0.538	1
	CxC4	16	21	484±8.2	501±7.4	3.5	0.110	1	0.35±0.04	0.38±0.03	8.6	0.570	1
	CxC5	17	18	471±8.1	479±7.7	1.7	0.452	1	0.37±0.04	0.42±0.04	13.5	0.307	1

Table 7. Breeding values for height at age 6 years (H6BV) of 30 second-generation selections that are progeny of 7-56. The H6BV figures are % height advantage compared with an unimproved checklot.

Heterozygote *cad-n1*		Wild-type					
Selection	H6BV	Selection	H6BV	Selection	H6BV	Selection	H6BV
A	12.0	W1	16.7	W9	8.5	W17	12.7
B	-5.6	W2	15.6	W10	14.7	W18	12.3
C	0.4	W3	17.8	W11	12.1	W19	12.8
D	19.6	W4	19.6	W12	1.2	W20	15.3
E	19.9	W5	16.9	W13	10.8	W21	7.2
F	16.0	W6	7.4	W14	12.5	W22	2.7
G	17.5	W7	16.9	W15	9.9	W23	6.1
		W8	17.8	W16	7.7		
Average	11.4 ± 3.8						12.0 ±1.0





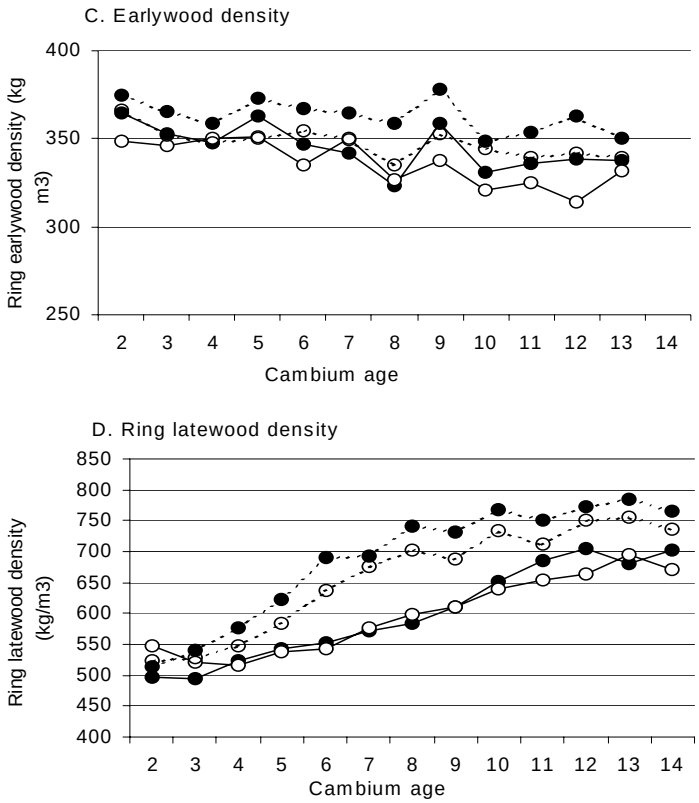


Figure 1. Ring wood density (A), ring latewood percentage (B), ring earlywood density (C) and ring latewood density (D) for heterozygous (HZ) and wild-type (WT) trees of full-sib family AxA6 in diallel test series 3.

LIGNIN (OR CHEMICAL PROPERTIES) STRUCTURAL VARIATIONS IN CAD-DEFICIENT PINE AND THEIR EFFECT ON PULPING

Incremental cores of 200 selected trees in a 10 years old open pollinated family trail growing under two nutrition treatments in Scotland County, North Carolina. In this study, we analyzed wood density, chemical and pulping properties Analysis of the samples with NIR allows estimation of lignin, α-cellulose and hemicellulose contents in each annual ring. Analysis of selected samples by quantitative 13C NMR showed almost no differences between *cad-n1* and wild MWL isolated from juvenile wood. However, there were some differences between between *cad-n1* and wild MWL isolated from mature wood. A micro technique is developing to allows isolation and comprehensive analysis of lignin using the amount of wood which can be obtained from each annual ring in an incremental core. Analysis of a fraction of crude MWL contained concentrated LC fragments allows detection and quantification of phenyl-glycoside bonds. Ester bonds between uronic acids and γ–position of lignin were detected in contrast to benzyl ester linkages.

Pulping result indicates heterozygote in the control plots give ~ 6% higher pulping yield than the wild type. ^{13}C NMR studies showed that the structure of the kraft lignin isolated from pulping of CAD-deficient pine is rather similar to that of the control. However, increased amounts of COOH and phenolic OH have been observed in the heterozygote lignin.

INTRODUCTION

The southern US produces 58% of the nation's timber, much of it grown in intensively managed plantations of genetically improved loblolly pine. One of the fastest-growing loblolly pine selections made by the NCSU Industry Cooperative Tree Improvement Program, whose progeny are widely planted, is also the only known natural carrier of a rare gene, *cad*-n1. This allele codes for deficiency in an enzyme, cinnamyl alcohol dehydrogenase (CAD), which catalyzes the last step in the biosynthesis of lignin precursors. Preliminary data for 4-year-old descendants from this fast-growing selected parent indicate that those carrying a single copy of *cad*-n1 (partially CAD-deficient) grow 14% faster and require less energy for pulping, producing higher brightness and paper strength. As a naturally occurring gene, acceptance of *cad*-n1 as a candidate for genetic engineering should be rather high. Before any such use can be considered, however, *cad*-n1 must be well characterized in harvest-age trees in a broad range of environments and in diverse genetic backgrounds. As a well-characterized candidate gene, marker-assisted selection and deployment should be possible in the breeding program. This research will enhance the sustainability of forest production in the South, where land-use pressures will limit the total area available in the future for intensively managed plantations. Furthermore, this research will provide information to establish higher-value plantation forests with more desirable wood/fiber quality traits.

The general objective of this study is to compare contents of lignin and α-cellulose, the lignin structure and pulping properties between wild type and cad-n1 heterozygous trees.

MATERIALS AND METHODS

1. Field sampling

Incremental cores of 200 selected trees in a 10 years old open pollinated family trail growing under two nutrition treatmentsin Scotland County, North Carolina. A 30-year-old F1 progeny of the *cad-n1* founder were sampled at Richmond County. In total, 21 trees were selected (10 heterozygote and 11 wild types). Average of tree height was 24 m for both heterozygote and wild type, and diameters were 232 mm for both heterozygote and wild type trees.

2. Genotyping

Samples were sent to the Southern Institute of Forest Genetics for genotyping. DNAs were isolated and PCRs were completed and analyzed by the procedures reported previously (Yu et al. 2005 in print) .

3. Evaluation of wood structure

3.1 NIR analyses (lignin, cellulose)

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 samples. Absorbance spectra totaling 32 scans were collected at 2.0 nm intervals over the range of 600-1900nm.

The increment cores extractives were removed by acetone extraction (yokoyama, 2002). The extractives-free increment cores were then soaked in deionized water overnight and cut into wafers having a thickness of 200μm using a microtome (yeh,2004). The wafers were dried over P_2O_5 under vacuum. The lignin and α-cellulose content was determined using transmittance mode Near Infra Red (NIR) spectroscopy (Yeh,2004,2005). The NIR spectrum of the 10years old trees from the two nutrition treatments, fertilized and non-fertilized fields was collect on a stack of 10 dried wafers for each ring.

The lignin content and α-cellulose content in juvenile and mature wood of the 30years old trees was estimated using transmittance NIR. From each core after extractives removal, the 2nd year ring (juvenile wood) and the combined 21st- and 22nd-year rings (mature wood) were cut and ground separately into a 60 mesh wood meals. The wood meals (75 mg each) were then pressed into pellets for NIR analyses.

The total lignin content of each set of 10 wafers and wood pellet was determined by the Klason method. The α-cellulose content of each set of 10 wafers and wood pellets were determined according with procedure of Yokoyama (Yokoyama,2002). Partial least square analysis was applied to the models, and the correlation between the lab lignin and α-cellulose values and the NIR fitted lignin values of the increment core model established.

Structure of lignin

3.2 ***NMR analysis.*** The NMR spectra were recorded on a Bruker AVANCE 300 MHz spectrometer at 300 K using DMSO-d_6 as the solvent. Chemical shifts were referenced to TMS

(0.0 ppm). 60-70 mg of lignin in 0.25 ml of DMSO-d_6 was placed in a Shigemi microtube; a 90^o pulse width, a 1.4 s acquisition time and 1.7 s relaxation delay were used. Chromium (III) acetylacetonate (0.01 M). A total of 20,000 scans were collected.

After analysis of the incremental cores for lignin content, samples with the low and the high lignin content from CAD-deficient and wild type groups, respectively have been selected to analyze differences in lignin structures. From these incremental cores, wood from annual rings 2-6 and 18+ was selected to represent juvenile and mature wood, respectively. Milled wood lignins were isolated from the selected wood using traditional Bjorkman's method (. Bjorkman, 1956) and analyzed as acetates by quantitative ^{13}C NMR (Capanema et al 2004).

RESULTS AND DISCUSSION

The ring density and α-cellulose content increase from the pith outwards on samples from both fertilized and non-fertilized plots. The *cad-n1*wood samples from fertilized and non-fertilized plots showed higher ring density for most years than the wild control wood samples (Yu, 2004a,b,c). No significant differences in lignin content were observed on rings 2 to 8. The samples from fertilized plots showed lower ring density than samples from non-fertilized plots. (**Figure-1**).

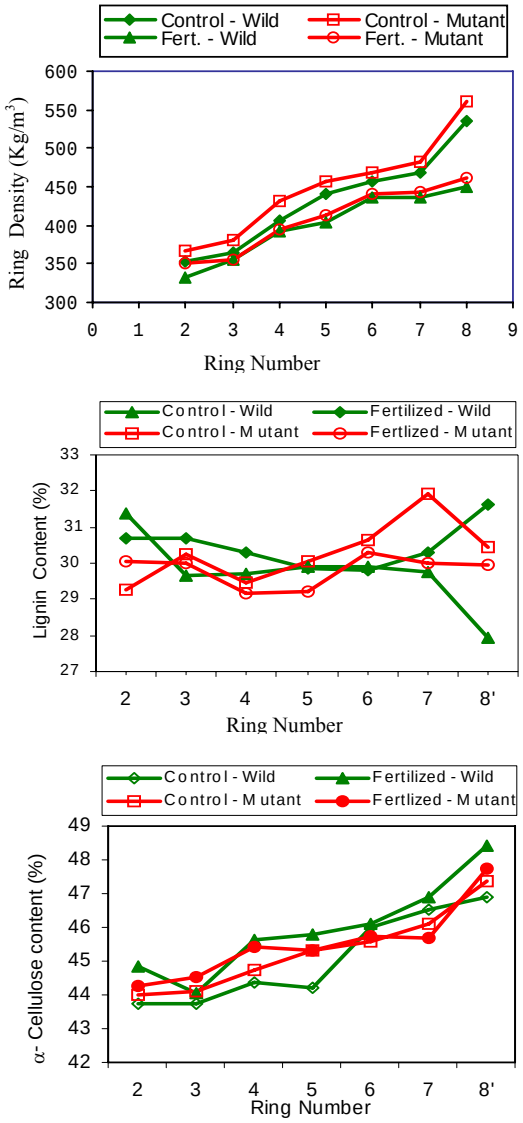


Figure 1- Ring density, lignin and α-cellulose content ring-by-ring

There are no significant differences between *cad-n1* and control wild type on lignin content and α -cellulose. The effect of fertilization is stronger than the genotype effect. In order to eliminate the effect of fertilization, on the wood traits, 30 years old wood samples grown under the same conditions were collected and analyzed for wood density, lignin and α-cellulose content. There are relatively small variations in lignin content among the four groups (Juvenile and mature woods in *cad-n1* and Wild type) on samples from Richmond County (30 years old wood samples). While Juvenile woods have higher lignin content than the mature woods, there is little difference in the lignin content between the wild type and the *cad-n1*. The α-cellulose content, on the other hand, varies considerably within the four groups.

While mature woods have considerably higher α-cellulose contents than the juvenile woods in both the *cad-n1* and the wild type, it is hard to judge if there are differences between the *cad-n1* and the wild type due to the large variations within the groups (**Figure 2**).

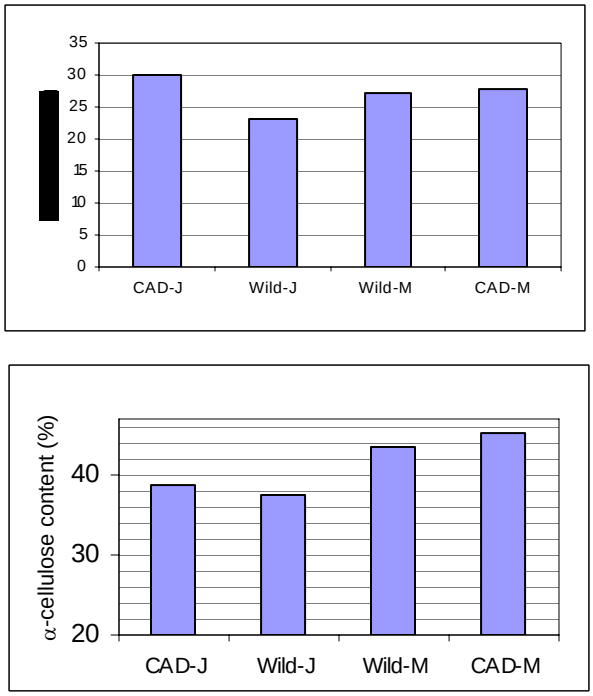


Figure 2- Lignin and α-cellulose content on juvenile and mature *cad-n1* and wild type wood samples

Lignin structure characterization using quantitative ^{13}C NMR

As we did not run NMR for non-acetylated lignins, the results are not as comprehensive as reported earlier (Capanema et al 2004). Therefore, we focused on the differences between the lignin preparations rather than on detailed comprehensive analysis. It is noteworthy that there is good correlation between related regions of the spectra. For example, the amounts of primary and secondary OH groups estimated from the corresponding acetate signals correspond well to the signals in oxygenated aliphatic regions (**Table 1**). The sum of primary, secondary and phenolic OH corresponds well to the total amount of OH groups (22-18 ppm).

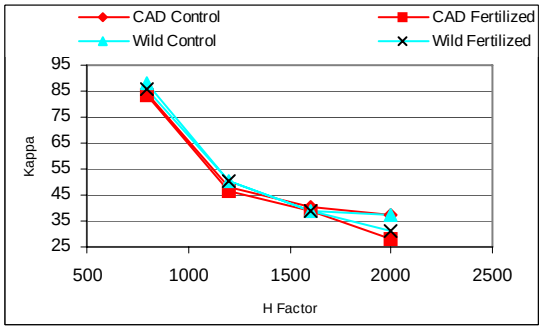


Figure 3- Quantitative ^{13}C of *cad-n1* and wild type MWL.

Table 1- Signal assignment in the NMR spectrum of acetylated MWL

Interval (ppm)	Integral value					Assignment
	CAD-juv	CAD-mat.	Wild-juv	Wild-mat.	MWL_{ext}*.	
200-190	9	10	9	10	13	Conjugated CO
172-169	73	78	73	79	75	Primary OH
169-168	41	42	40	40	48	Secondary OH
168-165	36	37	39	35	38	Phenolic OH
162-142	178	183	179	183	180	Ar-O (w/o C_4-Oac)
142-125	178	171	179	184	186	Ar-C (and C_4-Oac)
125-103	255	256	251	244	246	Ar-H
89-86	10	11	10	10	11	β-5
86-83	12	12	10	12	10	DBDO + β-β
77-72.5	41	42	39	39	40	β-O-4 (+ minor structures)
58-53	106	107	108	104	103	OMe + β-β
90-77	73	76	72	72	75	α-O-Ar, α-O-Alk, β-O-4
77-66	82	83	78	73	86	Secondary OH, γ-O-Alk
66-58	75	83	76	80	79	Primary OH (+ some minors)
90-58	230	242	226	225	240	Oxygenated aliphatic
23-18	153	157	151	152	162	Total OH

* MWL obtained by extended milling of wild mature wood

Preliminary data show that there are very little differences between MWL isolated from ***juvenile*** *cad-n1* and wild type wood (Table 1). However, there are some differences between MWL isolated from ***mature*** wood. Particularly, the degree of condensation (calculated as DC = 300 – Ar-H) is lower for the CAD-deficient lignin than that for the wild type (44 vs 56 correspondingly). In addition, the resonance in the region of 77-66 ppm is higher for the *cad-n1* lignin. This region embodies signals of secondary OH groups and γ-O-Alk moieties. As there is no significant difference in the amount of secondary OH groups between these lignins, the difference is due to higher amount of γ-O-Alk interunit linkages. Similar speculation can be valid for the whole oxygenated aliphatic region in general: the resonance in the region of 77-66 ppm is higher and the amount of aliphatic OH is pretty close, so the difference is due to higher amounts of interunit linkages in *cad-n1* lignin.

Development of methods for isolation of lignin and LCC

As we observed variations in lignin content and structure depending on age of the tree and variations within one group of trees, it is important to develop an approach for rapid and informative analysis of lignin from rather small amounts of wood. Recently we were able to significantly decrease the amount of lignin and experimental time for comprehensive NMR analysis (Capanema et al 2004). Another crucial point is isolation of lignin with high yield. We used extended ball milling to obtain crude MWL with the yield of ca 72% of Klason lignin in wood (without correction for carbohydrate content in the MWL). About 50% of the crude MWL

was lost during the purification procedure, and the yield of the purified MWL was about 35% of the Klason lignin in wood, that is about twice higher than that in traditional procedure. It has been shown (Matsumoto et al 2004) that the structure of lignin in ***wood*** progressively changes during milling, particularly due to degradation of β–O-4 bonds. However, the structure of ***MWL*** changes much less (Matsumoto et al.), and the structure of *Eucalyptus grandis* MWLs obtained with the yields of 25 and 56% was quite similar (Capanema et al 2005). Usually, the amount of a-CO and phenolic OH increase during the milling (Chang et al 1975), therefore these werethe main changes expected in the MWL of extended milling (MWL_{ext}). However, the increase both in conjugated carbonyl content and phenolic OH groups was not high. In general, the structure of the MWL_{ext} was pretty similar to the lignin obtained by the traditional protocol (Table 1). The main difference was an increase in secondary OH group content. The detail structures of these moieties are under investigation now. Generally, these preliminary results can be considered as promising for isolation of MWL with increased yields. Additional amount of lignin can be obtained by enzymatic hydrolysis of the residue after the extraction of MWL (Chang et al 1975), and extended milling is expected to give higher yield of this preparation. Optimization of milling and enzymatic hydrolysis to produce the maximal amount of lignin with minimal changes is underway.

On the structure of LCC in wood

Studies on the structure of lignin-carbohydrate complex (LCC) in wood are very important as LCC determines many chemical properties- of wood. However, this is not an easy task because of very complex structure of the LCC. Further progress can be achieved by improvement both in isolation of representative preparations and in methods of LCC characterization. Recent studies (Balakshin et al. ISWPC-01) showed that preparations of non-purified hardwood MWL can give important information on the structure LC bonds. As purified softwood MWLs usually contain very little amounts of carbohydrates, the LC fragments detected in the crude lignins should be accumulated in the material discharged during the purification. Particularly, 30% of the crude MWL_{ext} was lost at the first purification step with AcOH. This fraction (MWL-AcOH) was analyzed with HSQC, HMBC and TOSCY correlation NMR techniques and quantitative 13C NMR. MWL-AcOH contains about 50% of carbohydrates. The HMQC spectrum (Figure) shows rather high amount of phenyl-glycoside bonds, in much higher concentration than reported earlier (Balakshin-01). The HMBC spectrum shows correlation between C-1 of carbohydrates and aromatic protons, confirming the presence of phenyl-glycoside bonds. Quantitative 13C allows an approximate estimation of these moieties as 0.10-0.15/Ar. The presence of phenyl-glycoside linkages has been suggested so far based only on different ***indirect*** data, such as increase in the phenolic hydroxyl group content after alkaline or acid treatment of LCCs, reaction of transglycosidation and some others [ref]. These methods have not allowed a reliable prove of the existence of the phenyl-glycoside linkages in wood tissues. Therefore, direct detection and quantification of phenyl-glycoside linkages in the preparations isolated wood is very important in discussion on the existence of this type of LC linkages in LCCs and can be useful in understanding of the mechanisms of biosynthesis of wood components.
Esther LC bond are one of the most abundant linkages between lignin and carbohydrates (uronic acids) in wood, and it is assumed that they are of the benzyl ester type. α–esters have a characteristical cross peak at about 75/6.2in the HSQC spectra. Surprisingly, we did not detect any signal of this type (Figure). However, there is a broad group of signals of an appreciable

intensity in the area of 62-63/4.0-4.5, which was assigned to ester bonds at γ– position of C_9-units (probably between γ–position of lignin and carboxyl group of uronic acids). Thus, surprisingly γ–ester in contrast to α–ester was identified in our preparations. This might be explained that the preparations studied are not representative for the benzyl ester bonds. If this is not the case and γ–ester really dominates, this can be due to migration of originally formed α–ester to γ–position of the lignin side chain. This reverse reaction has been described by Helm (ref.) and it has been showed that in the equilibrium γ–esters are thermodynamically preferable than the α–ester. Nevertheless, if γ-ester LC bonds are originally formed in the biosynthesis, this implies that the QM theory does not explain the biosynthesis completely and another mechanism (probably biological control (Helm)) should be also considering.

Generally, the MWL-AcOH fraction is appeared to be a very good preparation to study linkages between lignin and carbohydrates in wood.

It is noteworthy that the lignin structure in the MWL-AcOH preparation is very degraded, especially in the side chain. Thus, the signals of β–5 and β–β structures are extremely weak, the intensity of signals of β–O-4/α–OH moieties is also much lower than in the MWL. The degradation of the side chain results in high amount of conjugated CO (0.27/Ar) moieties, particularly vanillin (0.10/Ar) and the sum of cinnamaldehyde and β–O–4/α–CO structures (0.09/Ar). The amount of carboxyl and/or ester moieties (COOR) is also very high, 0.65/Ar and 0.13/Ar for the aliphatic and conjugated COOR, correspondingly. As the signals of Ac-groups (assumable those of carbohydrates) is only 0.17/Ar, the other COOR moieties belong to lignin and uronic acids. The amount of OMe groups in the MWL-AcOH preparation is quite common for pine MWL, ca 1.00/Ar indication that degradation of the aromatic ring during milling should not be significant.

3.4 Pulping

Ten trees each from the four different groups: wild type control, wild type fertilized, *cad-n1* control and *cad-n1* fertilized have been collected from Scotland County for pulping studies. Disks were obtained from each of the trees for chemical analysis. The logs were chipped separately and then classified by thickness into 0-2mm, 2-4mm, 4-6mm, 6-8, mm and 8+mm fractions. Four sets of chips have been prepared representing the four groups. The 6 and 8 mm chips were used to make up the mixed fractions. They have been pulped using the following conditions: 19%AA, 4:1 liquor to wood ratio, 170oC and 25% sulfidity. The H factor was varied from 800-1800 to produce a wide range of kappa numbers.

The results of these pulping studies are shown in Table 2 and Figures 4 and 5. As shown in Figure 1, the rates of pulping appear to be similar among the four groups except that there are some indications that the *cad*-nls (both control and fertilized) may be easier to pulp at lower H-factors. As shown in Figure 2, the pulp yield at a given kappa number is comparable between the wild and the CAD. A 2% yield difference between the control and the fertilized is observed for both the wild and the CAD. These results are verified during presently with the new set of samples.

Table 2- Pulping Results

H Factor	Total Yield		Kappa
CAD Control	800	51.2	84.5
1200	46.8		48.2
1600	45.2		40.2
2000	44.3		37.2
CAD Fertilized	800	48.7	83.8
1200	44.8		46.4
1600	43.7		38.8
2000	41.5		28.2
Wild Control	800	50.8	87.9
1200	47.7		50.1
1600	46.1		39.1
2000	44.8		37.1
Wild Fertilized	800	48.6	85.7
1200	44.8		50.7
1600	44.3		38.7
2000	42.8		31.0

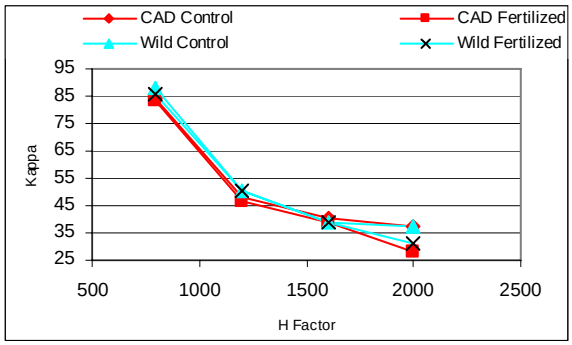


Figure 4. Rate of pulping

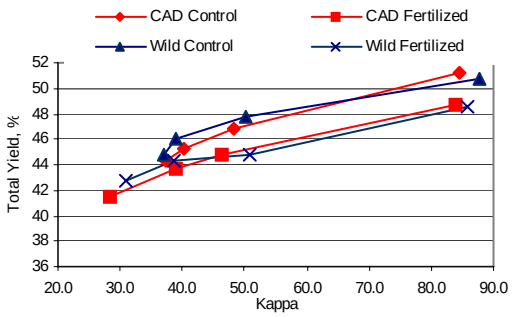


Figure 5. Pulp yield at a given kappa number

Lignin structural changes during pulping

Kraft lignins isolated from black liquor from pulping of CAD-deficient pine and the control (both non-fertilized) were comprehensively characterized using ^{13}C NMR of non-modified and acetylated samples (Table 2) . Very little differences in the structure of the kraft lignin from CAD-deficient pine and the control one have been observed.

Table 3- Characterization of kraft dissolved lignins isolated from pulping of CAD-deficient pine and control wood (both non-fertilized). Amount of moieties is expressed per 100 Ar (can be considered as mole %).

Moieites	Wild Type (control)	***Cad-n1***
Total CO	6	5
Conjugated CO	3	3
Non-Conjugated CO	3	2
Total COOH	18	16
Aliphatic COOH	16	15
Aromatic COOH	1	1
Total OH	128 (118*)	131 (121*)
Aliphatic OH	64 (54*)	65 (55*)
Primary	36	38
Secondary	28 (18*)	27 (17*)
Phenolic OH	64	66
h-units	7	7
Degree of Condensation	77	76
OMe	82	83
Oxygen. aliph. (90-58 ppm)	115 (95*)	119 (99*)
Saturated aliphatic (35-10 ppm)	76	80
Phenylcoumaran (β-5)	4	5
Pinoresinol (β - β)	2.5	2.5
β -O-4	4	4
Sugars (Xylan)	5	5

* Corrected for sugar content (as xylan)

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STRUCTURAL COMPOSITION OF NATIVE AND KRAFT LIGNIN

Composed wood samples of both juvenile and mature wood (Cad and wild type) from 10 trees selected each from first generation progenies at the Richmond County were used for pulping and solid-wood properties testing. Four pulp samples and pulping liquors obtained after kraft pulping to low kappa numbers (H-factor 2000) were selected for lignin isolation and characterization. The residual (RL) and dissolved (DL) lignins were isolated, acetylated and analyzed using 2D 1H-13C correlation NMR and quantitative 13C NMR techniques. Two-dimensional (2D) heteronuclear NMR technique is a very effective tool for the elucidation of the lignin structure. The 2D spectra did not show any significant qualitative differences between the four isolated samples. The use of non-acetylated and acetylated lignin preparations allowed quantifying various functionalities in lignin structure such as different OH group (primary and secondary aliphatic and phenolic OH), COOH and CO groups, OMe groups, oxygenated aliphatic moieties and degree of condensation. The results showed little significant difference between juvenile and mature wood in either residual or dissolved lignin.

Bleaching Results for CAD and Wild Samples

Pulps with kappa number 30 were bleached using a D(EP)D sequence to compare the bleachability of the CAD (juvenile and mature) and wild (juvenile and mature) samples. The bleaching conditions used are as follows:

Do Conditions: 10% cons., 1hr., 70C, .225kf
Ep Conditions: 10% cons., 1hr., 70C,3.4% NaOH, 0.5% H2O2

D1 Conditions: 10% cons., 3hr., 70C, 3 levels of ClO2*

The results are summarized in Tables 1-4. No difference in bleachability was observed between the CAD and the wild samples. The pulp from juvenile wood was easier to bleach for both the wild and the CAD samples. The sample bleached with 1% ClO2 in the D1 stage was also tested for strength properties.

Table 1: Bleaching Results for Mature CAD

Sample ID		**M/C**		Strength Sample
Do				
%ClO2		2.6		2.6
final pH		1.9		2.0
g/l residual		0		0
Ep				
final pH		11.8		11.9
% Iso-Brightness		59.3		59.5
D1				
% ClO2	0.5	1.0	1.5	1.0
% NaOH	0.1	0.35	0.6	0.35
final pH	4.0	3.5	3.4	3.4
% Iso-Brightness	81.4	84.5	85.0	84.4

Table 2: Bleaching Conditions for Mature Wild

Sample ID		**M/W**		Strength Sample
Do				
%ClO2		2.6		2.6
final pH		1.9		1.9
g/l residual		0		0
Ep				
final pH		11.8		11.8
% Iso-Brightness		59.4		59.1
D1				
% ClO2	0.5	1.0	1.5	1.0
% NaOH	0.1	0.35	0.6	0.35
final pH	3.6	3.6	3.6	3.5
% Iso-Brightness	80.3	84.4	86.1	84.1

Table 3: Bleaching Conditions for Juvenile CAD

Sample ID		**J/C**		Strength Sample
Do				
%ClO2		2.6		2.6
final pH		1.9		2.0
g/l residual		0		0
Ep				
final pH		11.8		11.9
% Iso-Brightness		60.3		59.5
D1				
% ClO2	0.5	1.0	1.5	1.0
% NaOH	0.1	0.35	0.6	0.35
final pH	4.0	3.5	3.7	3.5
% Iso-Brightness	80.4	86.2	87.4	86.1

Table 4: Bleaching Conditions for Juvenile Wild

Sample ID		J/W		Strength Sample
Do				
%ClO2		2.6		2.6
final pH		1.9		1.9
g/l residual		0		0
Ep				
final pH		11.8		11.8
% Iso-Brightness		62.1		59.1
D1				
% ClO2	0.5	1.0	1.5	1.0
% NaOH	0.1	0.35	0.6	0.35
final pH	3.8	3.5	3.8	3.5
% Iso-Brightness	82.9	86.6	87.2	86.3

Strength Testing of Bleached Pulps

The strength properties of the bleached pulps were measured after refining to four different refining levels. The results for tensile, burst and tear versus freeness are shown in Figures 1-3. At a freeness of 500 csf, the mature wild had a higher tensile and burst index. The strength results are being further analyzed.

Figure 1: Tensile Index versus freeness for CAD and wild samples

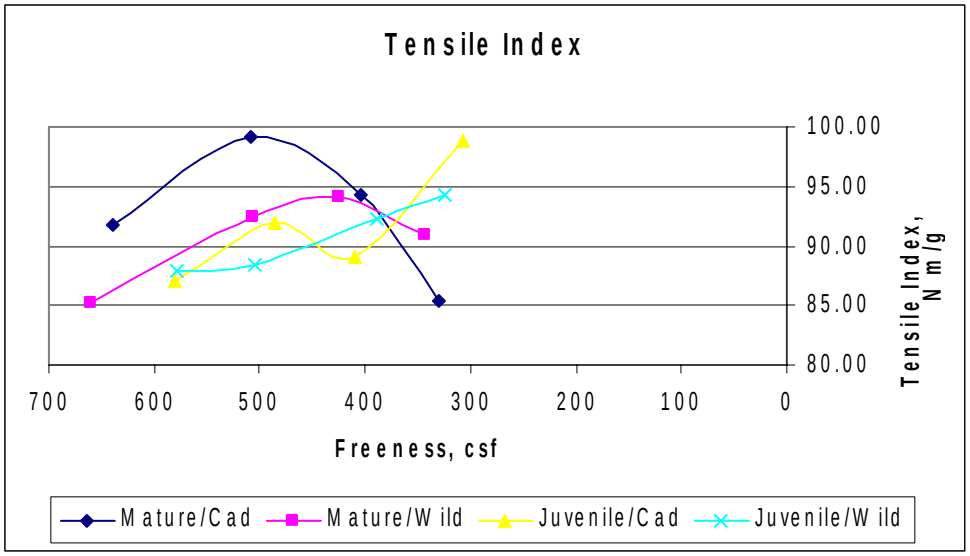


Figure 2: Burst Index versus freeness for CAD and wild samples

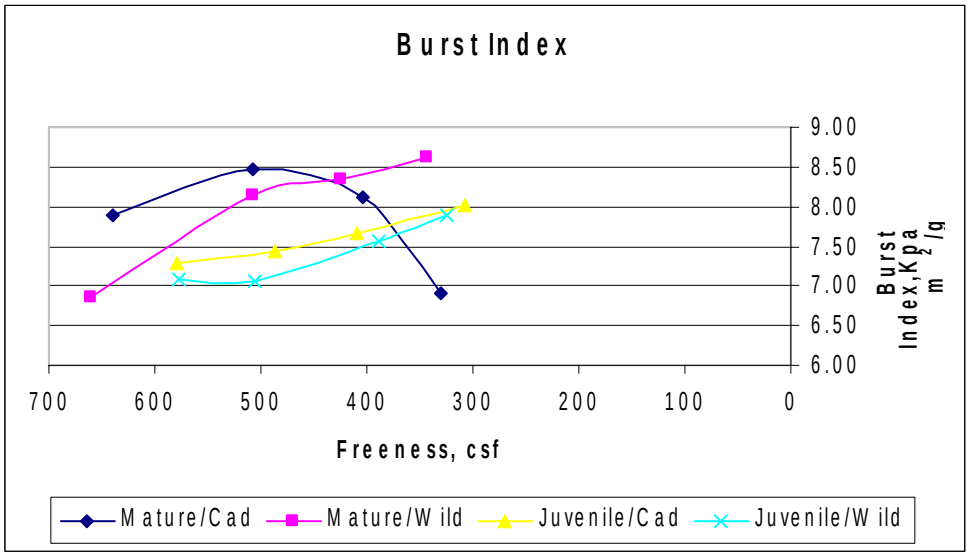
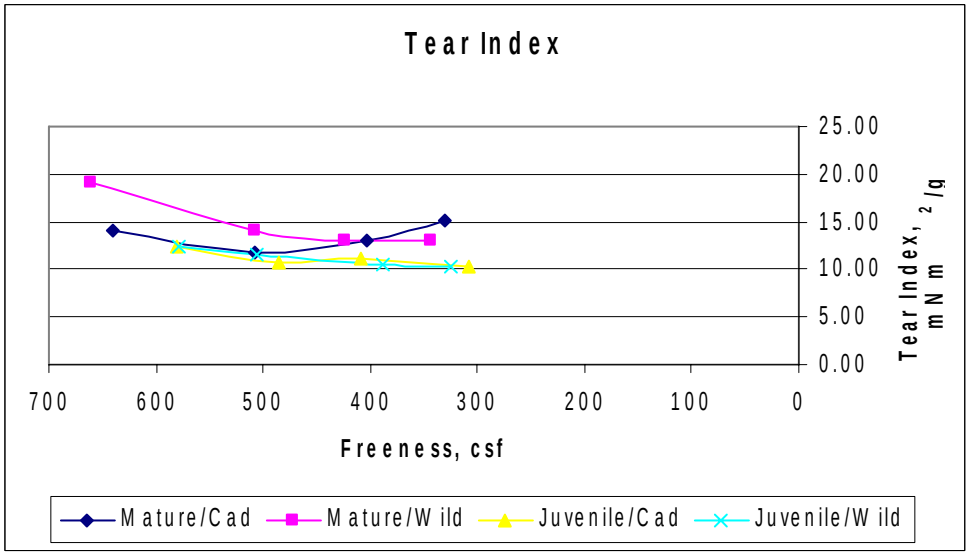


Figure 3 Tear Index versus freeness for CAD and wild samples



Strength Testing of Unbleached Pulps

The strength properties of the unbleached pulps at 100 kappa were also measured after refining to four different refining levels. The results are presently being analyzed.

The above when completed will enable for the evaluation of the strength properties of both unbleached and bleached pulps from CAD (juvenile and mature) and wild (juvenile and mature) samples.

STUDY OF POTENTIAL INTERACTION OF CAD ALLELES WITH WILD-TYPE ALLELES

Based on our analysis of diallele tests, we found high growth and wood density associated with cad-n1 allele were limited to specific crosses. There were large variations of cad-n1 effects on growth ad wood density among these 20 full families. We speculated that some wild type alleles in CAD locus from second parents interact with cad-n1 to produce the large positive effect. The working hypothesis is that one or more other *cad* alleles in the population that encode functional enzyme and are now considered "wild-type" alleles actually have an additional "interactor" phenotype, while some or most *cad* alleles that encode functional enzyme are "non-interactor" in phenotype. The "interactor" phenotype refers to the ability of a *cad* allele to confer better growth to individuals bearing the *cad*-n1 allele relative to full-sibs that do not bear the *cad*-n1 allele. The following is the summarized progress for this study.

1) Seed and needle collections

The seeds and needles of 13 selections have been collected from our industry members. In total, 23 selections will be used in this study. We are still waiting for rest of collections from one member.

2) DNA extraction

DNA was extracted with the Qiangen DNeasy procedure, and 96 samples were used (seed megagametophytes from 9 selections and cambium from 24 progenies) (Figure 4)

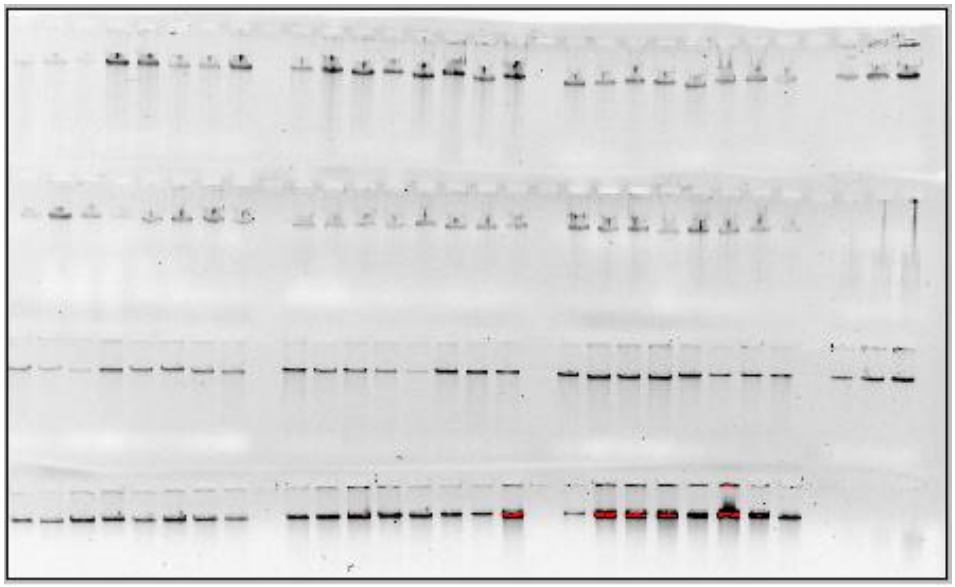


Figure 4. Results of DNA extractions were checked by gel.

3) We will use TILLING methods to look at the difference of wild type alleles among 2nd-generation selections in cad gene region. TILLING is a relatively efficient way to look for differences in those regions, because all differences in a PCR product are detectable. We have designed four pairs of PCR primers that amplify the entire *cad* gene in pieces suitable for

TILLING assays, so that the entire gene coding sequence and 5' flanking region are screened for polymorphisms (Figure 5, 6).

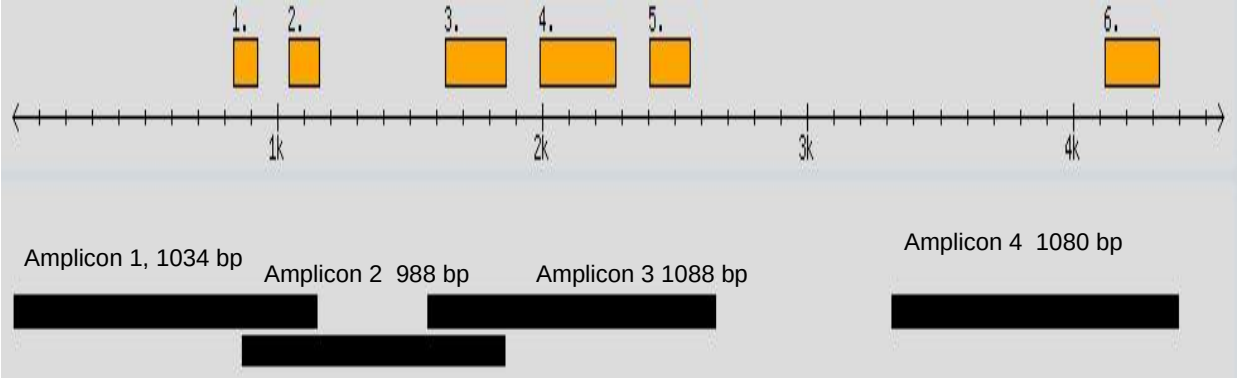


Figure 5. Design four pairs of primers that amplify the entire cad gene in pieces suitable for TILLNG assays- four Amplicons

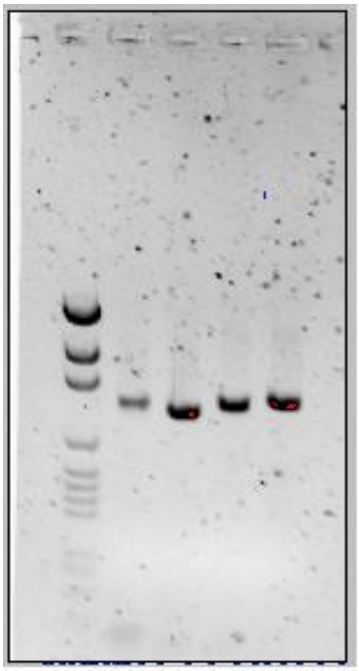


Figure 6. Produced four amplicons.

Strength Property for CAD and Wild samples

The results of refining and strength property measurements for the bleached pulps produced form 30 kappa pulps using a D(EP)D are shown in Figures 1 and 2 for the bleached pulp.

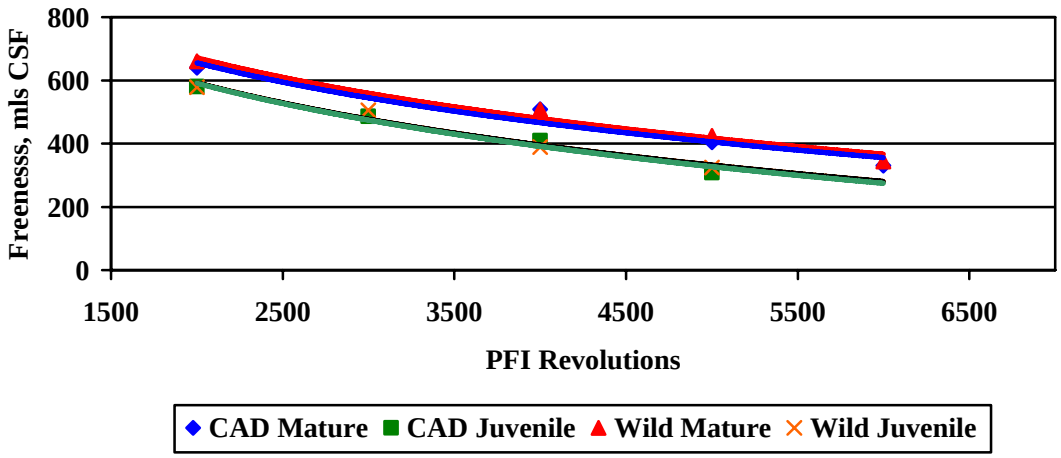


Figure 1: Refining Response of Bleached Pulps

No difference in refining energy was required between CAD and wild samples. The juvenile wood required less energy to refine than the mature wood. No significant difference in tear-tensile was measured between CAD and Wild samples but the mature wood had about ~15% higher tear at equivalent tensile.

The fiber dimensions were also measured using a Fiber Quality Analyzer (FQA) and the results are shown in Table 1. No difference was measured between the CAD and the Wild samples, but the fiber length of the juvenile samples was lower than that for the mature fibers. Interestingly enough the fiber width was the same for the juvenile and the mature samples.

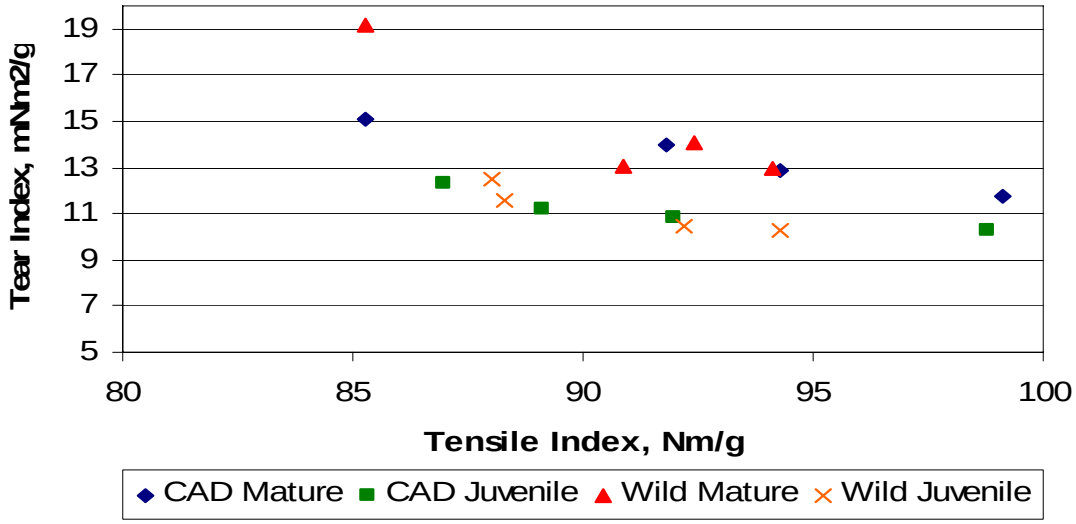


Figure 2: Tear vs Tensile for the Bleached Pulps

Table 1: Fiber properties of the bleached pulp

	Mature CAD	Mature Wild	Juvenile CAD	Juvenile Wild
Percent Fines, LW, %	15.13	19.38	13.63	11.43
Length, Length Weighted, mm	3.3	3.3	2.323	2.395
Curl Index, LW, mm	0.191	0.206	0.186	0.146
Kink Index, 1/mm	1.16	1.25	1.81	1.56
Mean Width, um	34.6	34.1	32.1	33.1

The strength properties of the high kappa liner board pulps with a kappa number of approximately 100 are shown in Figures 3 and 4 for refining energy and strength. The refining energy required for all the pulps were comparable. No significant difference between the CAD and wild samples was measured, but the mature samples had a ~20% higher tear at the same tensile than the juvenile pulps. The FQA results for the fiber properties are shown in Table 2.

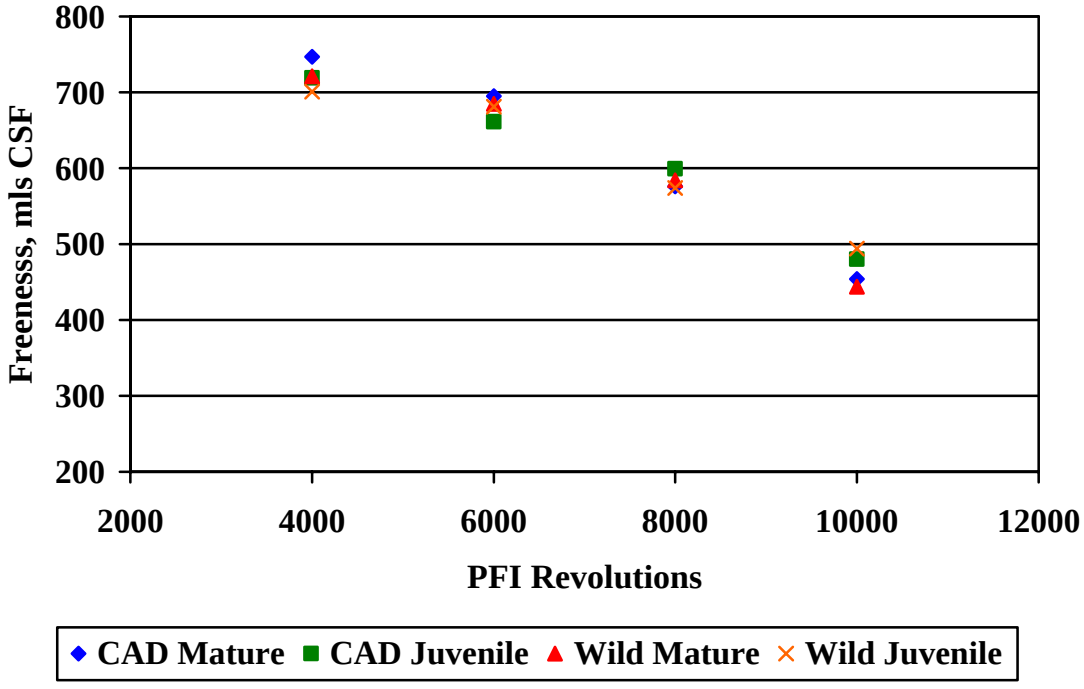


Figure 3: Refining Response of High Kappa (100) Pulps

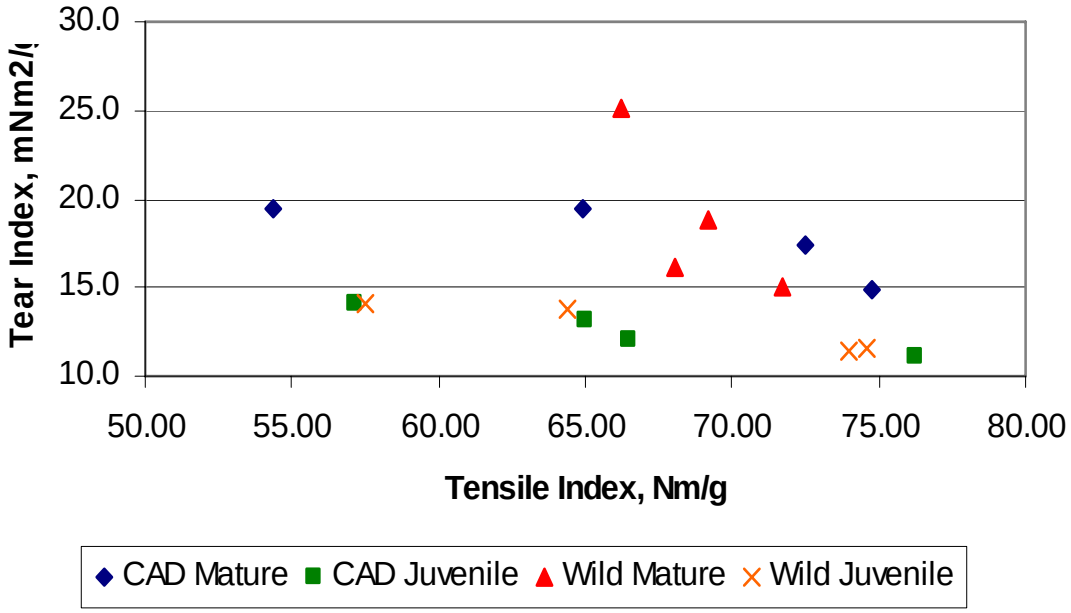


Figure 4: Tear vs Tensile for the High Kappa (100) Pulps

Table 2: Fiber properties of the High Kappa (100) pulp

	Mature CAD	Mature Wild	Juvenile CAD	Juvenile Wild
Percent Fines, LW, %	4.85	2.74	2.10	1.81
Length, Length Weighted, mm	3.249	3.352	2.491	2.410
Curl Index, LW, mm	0.143	0.181	0.074	0.079
Kink Index, 1/mm	0.69	0.81	0.71	0.81
Mean Width, um	35.5	35.7	35.9	36.9

ISOLATION AND CHARACTERIZATION OF THE DISSOLVED LIGNIN IN BLACK LIQUOR AND THE RESIDUAL LIGNIN IN UNBLEACHED PULP

Dissolved lignin in black liquor (kraft lignin) was isolated from four pulping experiments (mature CAD, mature wild, juvenile CAD and juvenile wild) cooked to bleachable grade (around 30 kappa number). Kraft lignin was precipitated by acidifying the black liquor to pH 2.5 with sulfuric acid. After washing with dilute acid solution (pH 2.5) the precipitated kraft lignin was freeze-dried in water suspension. The residual lignin in pulp was isolated from the four bleachable grade pulps obtained from the aforementioned pulping experiments. Residual lignin was isolated by first treating the pulp with cellulolytic enzyme to remove most of the carbohydrates. The resulting residue was obtained by centrifugation, freeze-dried and extract with 96% aqueous dioxane. Dioxane extract over 90% or the residual lignin, which was obtained in powder upon freeze-drying.

Both kraft lignin and residual lignin samples were characterized using quantitative C^{13} NMR spectroscopic method. The NMR spectra of kraft lignin are shown in Figures 1 and 2 for mature and juvenile pairs, respectively. As can be seen in Figure 5 and 6, there is little difference between wild and CAD for both mature and juvenile wood. Similar results were also obtained for residual lignin samples (spectra not shown). Tables 3 and 4 give data of various groups in lignin as estimated by quantitative C^{13} NMR.

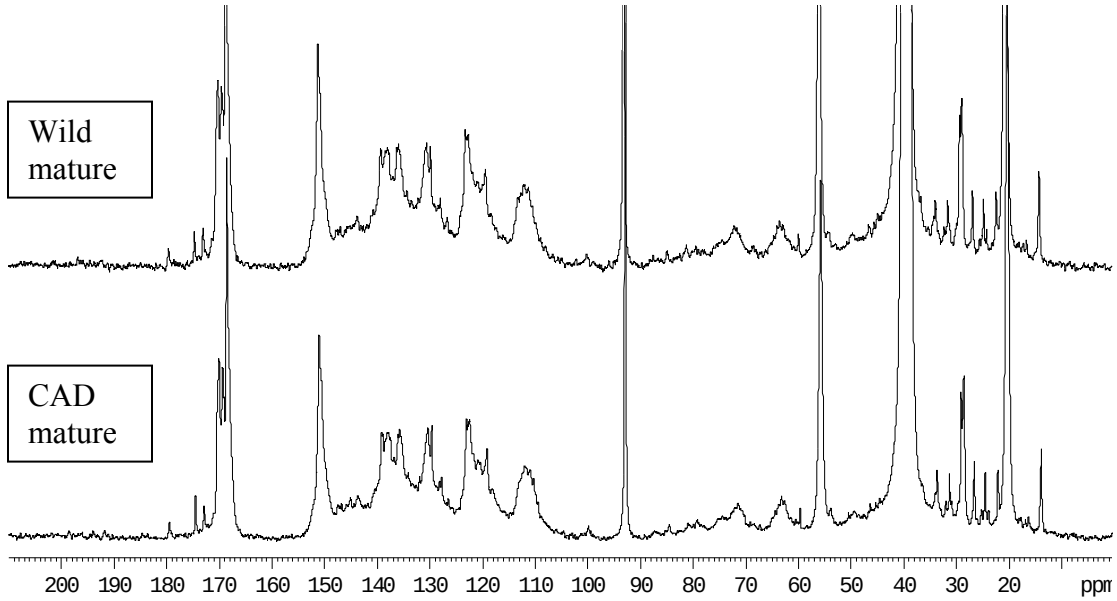


Figure 5. Quantitative C^{13} NMR spectra of kraft lignin from mature wood.

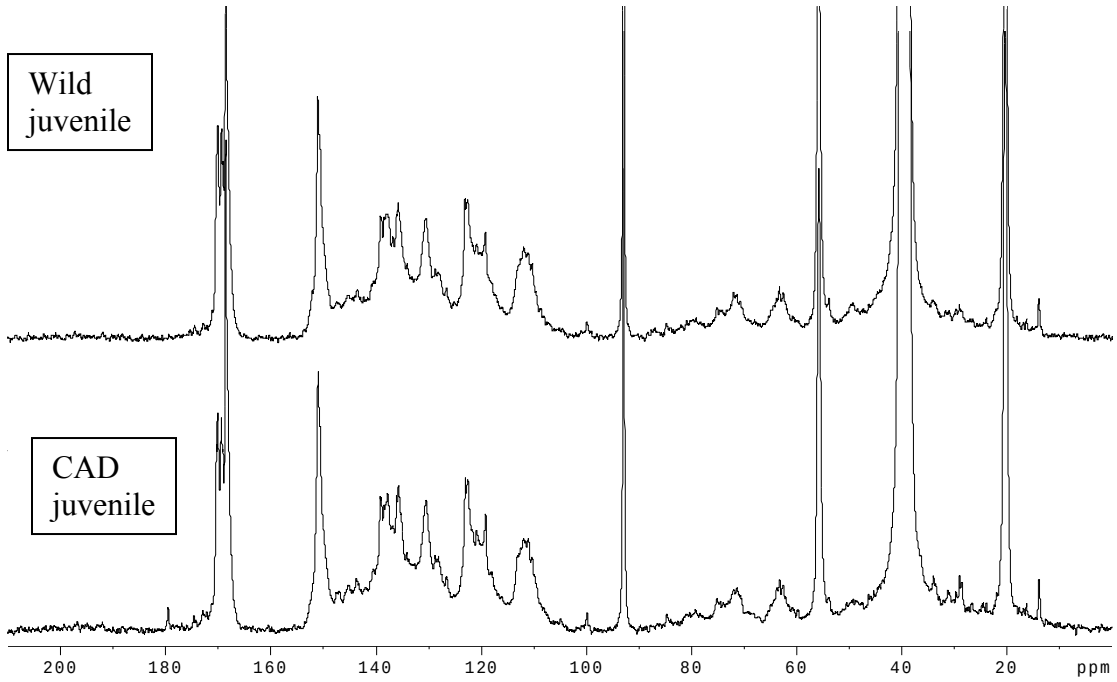


Figure 6. Quantitative C^{13} NMR spectra of kraft lignin from juvenile wood.

Table 3. Quantitative C^{13} NMR characterization of residual lignins
Amount of moieties is expressed per 100 Ar (can be considered as mole %).

Moieites	CAD-J	Wild-J	CAD-M	Wild-M
Total CO	11	12	11	11
Conjugated CO	7	8	6	7
Non-Conjugated CO	4	4	5	4
Total COOH	38	36	48	42
Aliphatic COOH	36	33	46	40
Aromatic COOH	2	3	2	2
H-units total	7	5	6	7
Ar-H	196	210	199	195
OMe	97	100	101	98
Oxygen. aliph.	175	175	179	161
90-77	33	33	34	31
77-66	98	98	101	89
66-58	44	44	44	41
Saturated aliphatic (35-10 ppm)	86	74	86	100
Sugars	11	11	…	13

Table 4. Quantitative C^{13} NMR characterization of kraft lignins
Amount of moieties is expressed per 100 Ar (can be considered as mole %).

Moieites	CAD-J	Wild-J	CAD-M	Wild-M
Total CO	9	6	11	10
Conjugated CO	6	3	6	5
Non-Conjugated CO	3	3	5	5
Total COOH	20	19	27	23
Aliphatic COOH	19	18	24	22
Aromatic COOH	1	1	3	1
Total OH	123	120	121	119
Aliphatic OH	51	49	50	49
Primary	26	23	27	24
Secondary	25	26	23	25
Phenolic OH	72	71	71	70
H-units total	4	4	5	4
H-units etherified	2	1	1	1
Ar-H	217	220	216	212
OMe	82	81	80	79
Oxygen. aliphatic (90-58 ppm)	103	105	102	106
90-77 ppm	23	24	23	22

77-66 ppm	46	46	44	47
66-58 ppm	34	35	35	37
b-5	2	3	3	4
b-b	4	4	3	3
b-O-4	3	2	3	3
Saturated aliphatic	101	88	148	149
Sugars	5	5	5	5

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PROJECT MILESTONE

ID Number	Task / Milestone Description	Planned Completion	Comments
1	***Field sampling and growth/form assessment***		
1.1	Identify candidate families and field sampling plots	01/31/04	Complete
1.2	Collect field data, cambium scrapings and cores	04/30/05	Complete
2	***Genotyping of informative crosses and individuals for CAD***		
2.1	Process parental megametophyte tissues to identify informative crosses	04/30/04	Complete
2.2	Process cambium scrapings for genotyping	04/30/05	Complete
3	***Evaluation of wood properties***		
3.1	NIR analyses (lignin, cellulose)	04/30/05	NIR calibration complete, initial sample from Scotland Co. Complete
3.2	Analyze lignin structure	04/01/06	Complete
3.3	Analyze fiber morphology	10/30/05	Complete
3.4	Pulping studies	07/31/05	Repeat samples from Scotland Co.- processed and analyzed
3.5	Analyze paper strength	04/01/06	Complete
3.6	Analyze solid wood properties	04/01/06	Density profiles complete for 1,800 trees; sample preparation of 2185 cores is Complete
4	***Quantitative analysis of dataset***	***04/01/06***	Complete
5	***Develop breeding strategies***	***04/01/06***	Complete
6	***Reports***		
6.1	***Interim report year 1***	***04/20/04***	*Peer-review conducted May 4, 2004, Atlanta, GA*
6.2	Interim report year 2	04/20/05	Meeting with DOE Project Office Team on 2005-03-10
6.3	Final report	02/28/07	Complete