Differences in wood density and growth of fertilized and nonfertilized loblolly pine associated with a mutant gene, *cad-n1*

Q. Yu, S.E. McKeand, C.D. Nelson, B. Li, J.R. Sherrill, and T.J. Mullin

Abstract: 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). Effects associated with 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 fertilizer treatment. Wood density measurements were collected from wood cores at breast height using X-ray densitometry. We found that the substitution of a *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 with 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. This 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 by increasing wood density.

Résumé : Un allèle mutant rare (*cad-n1*) du gène *cad* chez le pin à encens (*Pinus taeda* L.) entraîne une déficience de la production de l'alcool cinnamylique déshydrogénase. Les auteurs ont étudié les effets associés à cet allèle en comparant la densité du bois et les caractères de croissance des arbres hétérozygotes pour *cad-n1* avec les mêmes caractères chez des arbres sauvages. Cette comparaison a été effectuée dans un test de descendances monoparentales âgé de 10 ans et établi sous deux niveaux de fertilisation dans le comté de Scotland en Caroline du Nord. En tout, 200 arbres ont été échantillonnés à raison de 100 arbres par traitement de fertilisation. Les mesures de densité du bois ont été obtenues par densitométrie aux rayons X de carottes de bois prélevées à hauteur de poitrine. Les auteurs ont observé que la substitution de l'allèle *cad-n1* par un allèle sauvage (*Cad*) avait un effet significatif sur la densité du bois. La densité du bois était significativement plus élevée (+2,6 %) chez les hétérozygotes pour *cad-n1* comparativement aux arbres sauvages. La plus forte densité était apparemment le résultat d'un pourcentage plus élevé de bois final chez les hétérozygotes. L'effet de la fertilisation était hautement significatif à la fois pour les caractères de croissance et la densité du bois. Cette étude indique que l'allèle *cad-n1* pourrait comporter une valeur intrinsèque pour l'industrie des pâtes et papiers dans le but d'améliorer le rendement en pâte en augmentant la densité du bois.

[Traduit par la Rédaction]

Introduction

The southern 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 of 13%–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 North Carolina State University – Industry Cooperative Tree Improvement Program is the only known first-generation carrier of a rare mutant 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%–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.

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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 with 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 with 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%–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 activity (Stasolla et al. 2003). Heterozygous and homozygous *cad-n1* trees have, respectively, 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 wood density (i.e., specific gravity) of a few *cad-n1* heterozygous trees was measured in one study (Dimmel et al. 2002). Wood density 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 dry mass/m^3 (Zobel and Jett 1995). In this paper, we report on the effects associated with 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 were (1) to analyze the quantitative effects on growth and wood density associated with the mutant *cad-n1* allele and (2) to examine the magnitude of *cad* genotype by nutrient interactions on these traits.

Materials and methods

Plant materials

The field test is located in Scotland County, North Carolina, adjacent to the USDA Forest Service – North Carolina 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 m × 2.1 m spacing, with a 12-m buffer around each treatment plot to minimize the influence of adjacent fertilizer treatments. Fertilizer was applied annually to maintain an optimum supply of macro- and micronutrients as determined by foliar analysis to stimulate rapid growth in fertilized plots. 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 1.7 B, 2.0 Cu, 5.0 Fe, 5.0 Mn, and 2.0 Zn.

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 m, and every 1.2 m 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 $V = L \times (B + b)/2$, where V is the total section volume; L is the length of bolt; B is the basal area of the bolt's large end; and b is the basal area of the 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, $V = B_0 + B_1(\mathrm{DBH}^2H)$, as suggested by Spurr (1952). The explanatory variables fertilizer (X_1), $X_1 \times D^2H$, and $(D^2H)^2$ were added to the equation based on their significance ($p \leq 0.05$), to produce the following equation:

$$[1] \quad V = 4.258\,133 \times 10^{-9}(\mathrm{DBH}^2H) - 0.0204X_1 + 1.937\,33 \times 10^{-9}(X_1 \times \mathrm{DBH}^2H) - 7.9859 \times 10^{-17}(\mathrm{DBH}^2H)^2$$

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 ($p < 0.0001$).

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 five blocks (20 trees per treatment per block). 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., Knoxville, Tennessee). The last growth ring was excluded because of missing latewood on cores collected in

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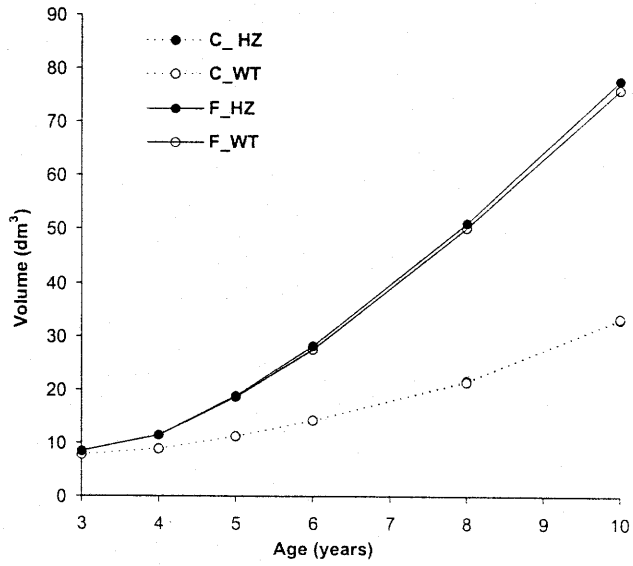
Table 1. Significance of main effects of fertilizer treatment and *cad* genotype and their interactions from the ANOVA for growth traits at age 10 years and weighted wood density traits through 9 years.

	df	Height (m)	DBH (cm)	Volume (m^3)	Weighted wood density (kg/m^3)	Weighted latewood (%)	Weighted earlywood density (kg/m^3)	Weighted latewood density (kg/m^3)
Fertilizer treatment	1	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	0.009*
cad genotype	1	0.506	0.962	0.813	0.01*	0.029*	0.004*	0.601
Fertilizer × genotype	1	0.69	0.787	0.845	0.265	0.104	0.787	0.802

Note: *, statistical significance at $p < 0.05$.

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



midsummer. For each ring scanned, the following intraring 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.

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., Valencia, California). Each DNA sample was then genotyped by polymerase chain reaction (PCR) 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., Foster City, California). Peak sizes were determined with GeneScan® software, then 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 indicate that the *cad-n1* allele contains a 2-bp insertion compared with 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 retested 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*.

Statistical analysis

The data were analyzed according to the following linear model:

$$[1] \qquad Y_{ijlk} = \mu + b_i + t_j + g_k + bt_{ij} + gt_{jk} + gbt_{ijk} + e_{ijkl}$$

where Y_{ijkl} is the observed value for the lth tree of the kth *cad* genotype in the ith block in the jth treatment; μ is the overall mean; b_i is the effect of the ith block; t_j is the effect of the jth fertilizer treatment; g_k is the effect of the kth *cad* genotype; bt_{ij} is the effect of the interaction between the ith block and the jth treatment; gt_{jk} is the effect of the interaction between the kth *cad* genotype and the jth treatment; gbt_{ijk} is the effect of the interaction between the kth *cad* genotype and ith block and the jth 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 Inc. 2001). The SAS procedures PROC CORR was 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 ($\chi^2 = 0.9800$, $p = 0.3222$).

The results of analyses of variance for growth and wood traits are given in Table 1. Volume growth responses to fertilization were large and highly significant (Tables 1 and 2; Fig. 1). 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 the *cad* genotype was not significant for any growth trait at age 10 years (Tables 1 and 2; Fig. 1). The fertilizer effect was significant for all wood properties. 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 with the controls (Table 1).

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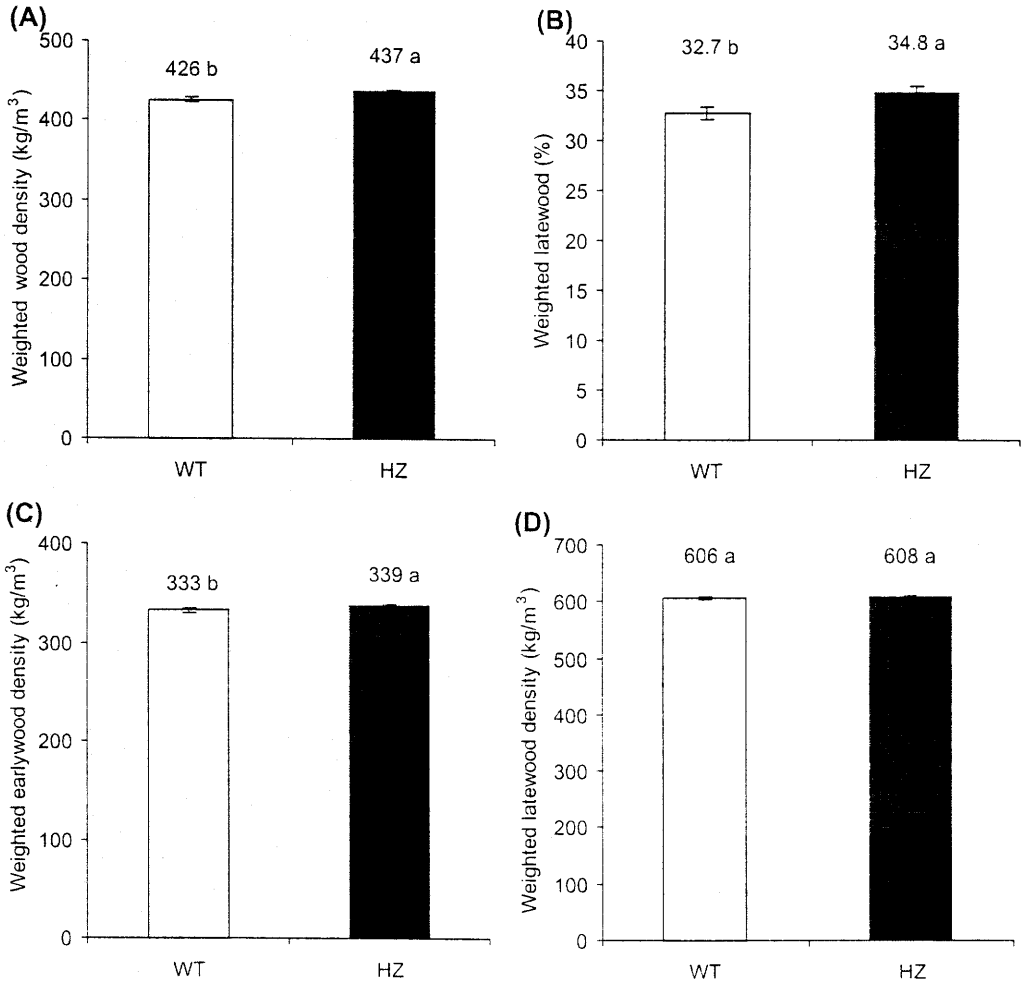
Table 2. Means (±SE) of *cad* genotype and fertilizer treatments for growth traits through 10 years.

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

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

Fig. 2. Comparison of wood-quality traits (age 9 years) between wild-type (WT) and heterozygous (HZ) trees 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 93 trees for heterozygotes.



The effect of the *cad* genotype was significant for all wood properties, except for weighted latewood density (Table 1; Fig. 2). For the first 8 years, the weighted wood density for heterozygous trees was 2.6% higher than that of wild-type trees. Compared with the wild-type trees, the heterozygotes also had 6.3%, 1.5%, and 0.2% greater weighted latewood percentage, earlywood density and latewood density, respectively. The *cad* genotype × fertilizer treatment interactions were not significant for any growth trait or wood density trait. When analyzed by fertilizer treatment, these differences for weighted wood density 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 (Fig. 3). Earlywood density plotted against

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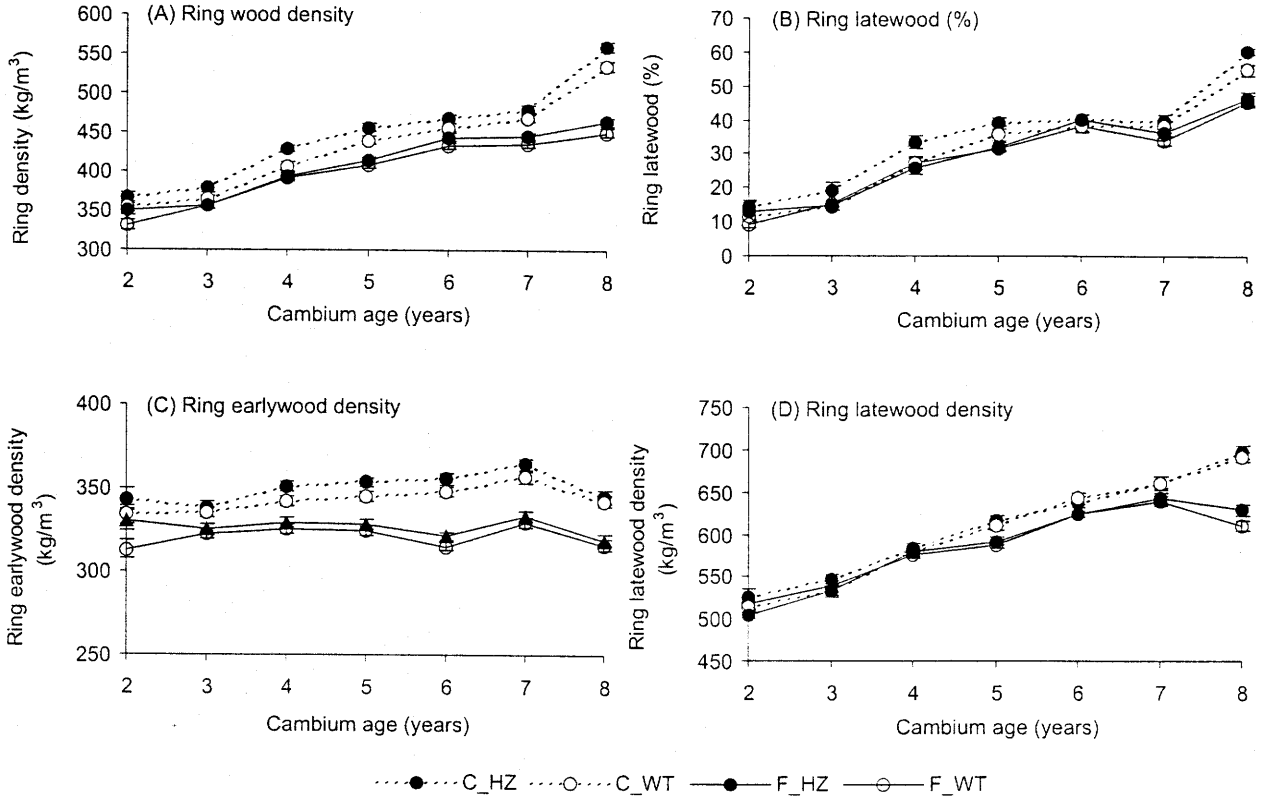
Fig. 3. Ring wood density (A), ring latewood percentage (B), ring earlywood density (C), and ring latewood density (D) for heterozygous (HZ) and wild-type (WT) trees in fertilized (F) and control (C) plots for ring ages 2–8 years. The sample sizes were 54 and 46 trees in fertilized plots and 53 and 47 trees in control plots for wild type and heterozygotes, respectively.



age showed little variation and remained more or less constant with cambium age. ANOVA results shown 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 3 years (cambium ages 6–8) and latewood percentage at cambium age 8 were significantly different between the heterozygous and wild-type trees. Fertilizer consistently lowered both ring and earlywood densities, and the effect was significant for all wood density traits at cambium age 8. *cad* genotype × 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 either fertilized or control treatments, but there were strong and highly significant correlations between weighted wood density and latewood percentage (Fig. 4).

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 can be attributed to higher earlywood density and a greater proportion of latewood in *cad-n1* heterozygotes. 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 not strongly deleterious for survival and adaptability through age 10 in our research planting, and that our sampling strategy was random. To date, the *cad-n1* allele has not been identified in any tree outside of the pedigree of the original founder parent.

Fertilization effects

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 1978), whereas P and NP treatments that were applied to planted slash pine increased wood density compared with nontreated trees (Rockwood et al. 1985; Megraw 1985; Zobel and van Buijtenen 1989).

The weighted average wood density through 8 years on fertilized plots was 9.7% less than that on control plots.

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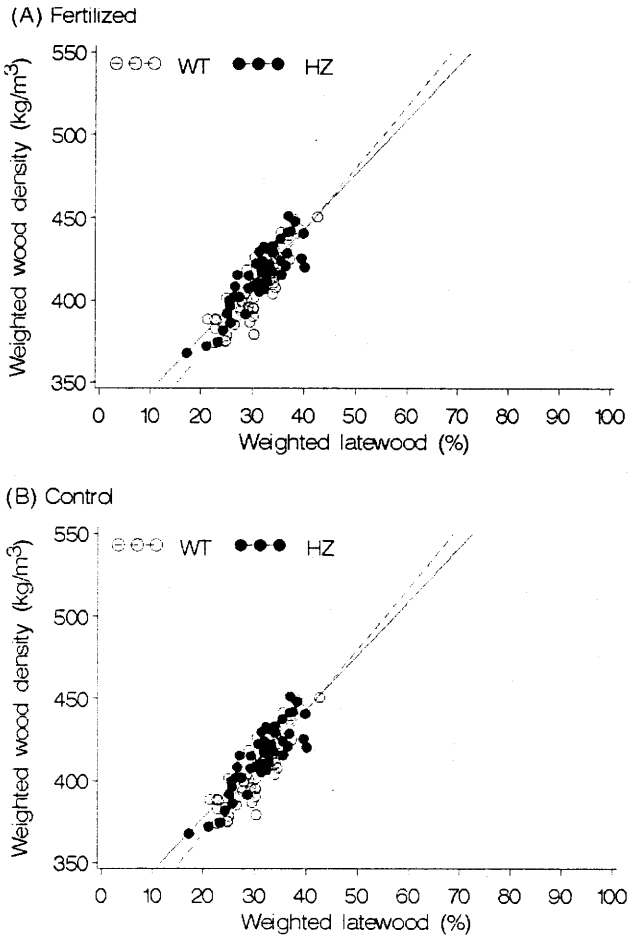
Table 3. Significance of main effects of fertilizer treatment and *cad* genotype and their interaction from the ANOVA for wood density traits through cambium ages 3–9 years.

Cambium age (years)	Fertilizer treatment	*cad* genotype	Fertilizer × genotype
Ring density (kg/m³)			
2	0.173	0.292	0.621
3	0.023*	0.116	0.114
4	0.023*	0.013*	0.067
5	0.003*	0.427	0.821
6	0.001*	0.055*	0.993
7	0.028*	0.011*	0.846
8	<0.001*	0.009*	0.297
Latewood (%)			
2	0.134	0.097	0.455
3	0.325	0.161	0.071
4	0.2	0.132	0.045*
5	0.004*	0.01*	0.625
6	0.951	0.129	0.886
7	0.323	0.21	0.929
8	0.002*	0.044*	0.153
Earlywood density (kg/m³)			
2	0.584	0.57	0.397
3	0.729	0.805	0.142
4	0.003*	0.011*	0.492
5	0.036*	0.086	0.214
6	<0.001*	0.022*	0.814
7	0.002*	0.072	0.666
8	0.02*	0.215	0.981
Latewood density (kg/m³)			
2	0.375	0.646	0.07
3	0.007*	0.544	0.863
4	0.694	0.593	0.945
5	0.003*	0.427	0.821
6	0.172*	0.689	0.635
7	0.138	0.771	0.834
8	0.003*	0.093	0.426

Note: *, statistical significance at $p < 0.05$.

Fig. 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 the broken line is 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 and 53 and 47 trees in control plots for wild-type and heterozygotes, respectively.



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 treatments can be attributed to the annual applications of fertilizer throughout the 10-year growing period.

In this study, fertilization significantly decreased all wood density traits, irrespective of *cad* genotype (Fig. 3; Table 3). The increase in radial growth under fertilization could be attributed 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 moderately strong negative correlation between earlywood width and latewood 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 or 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 (Fig. 4).

cad genotype effects

While Wu et al. (1999) found that *cad-n1* heterozygous trees had faster growth 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 with the systematic sample of 158 at one site in the earlier report. For valid estimates, further

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studies require a sufficient number of samples to be tested in additional trials, replicated in time.

Significant differences for wood density traits were observed between trees with alternative *cad* genotypes, except for latewood density (Fig. 2). The weighted wood density for heterozygotes was 2.6% higher than for 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 (a difference of 0.008 or 1.8%) for *cad-n1* 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/m^3 of dry processed kraft pulp. In the present study, the *cad-n1* heterozygotes had a significantly greater proportion of latewood, suggesting the potential for a significant impact on pulp yield. Previous studies indicated that latewood produces 2%–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 cambium age 8 years 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 × treatment interaction for any wood density or growth trait.

Wood density is determined by 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 (Fig. 3). This was expected because of the lack of a *cad* genotype by fertilizer treatment interaction effect. Heterozygotes showed consistently higher ring wood density than wild-type trees in most years through cambium age 8 (Fig. 3; Table 3). For both heterozygous and wild-type trees, latewood density increased rapidly from the pith. Megraw (1985) indicated that latewood density increases rapidly with ring number from the pith until values reach 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 can be 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 (Fig. 4).

For both heterozygous and wild-type trees, our results indicated no meaningful relationship between growth (DBH) and wood density in either control and fertilized treatments (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 *cad-n1* heterozygous loblolly pine had significantly higher weighted wood density and proportion of latewood. Differences in earlywood density and latewood percentage were consistent throughout tree development, which resulted in high total wood density for heterozygotes, as compared with wild-type trees, up to age 10 years. 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 10-year-old trees (n = 200). 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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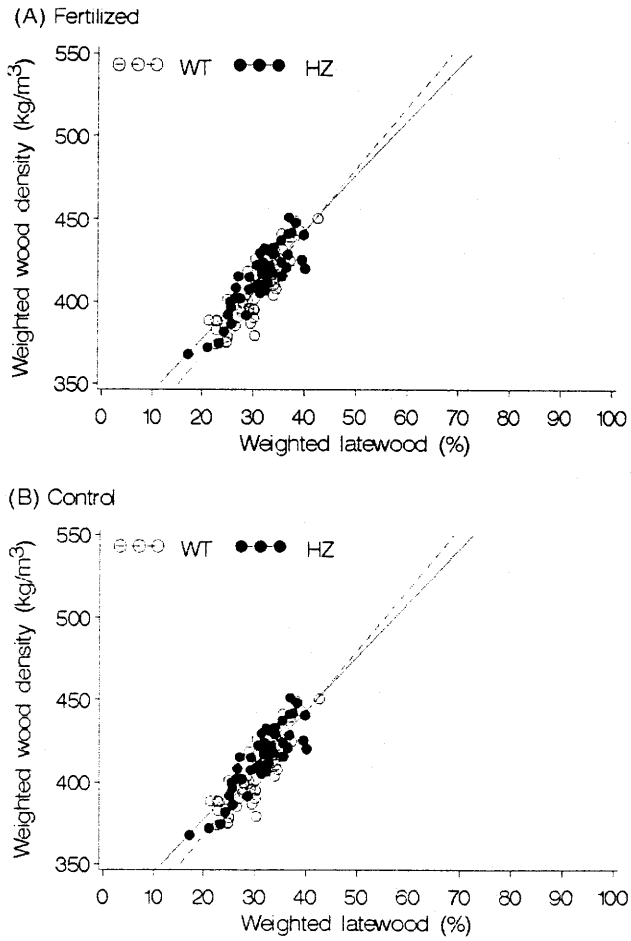
Table 3. Significance of main effects of fertilizer treatment and *cad* genotype and their interaction from the ANOVA for wood density traits through cambium ages 3–9 years.

Cambium age (years)	Fertilizer treatment	*cad* genotype	Fertilizer × genotype
Ring density (kg/m³)			
2	0.173	0.292	0.621
3	0.023*	0.116	0.114
4	0.023*	0.013*	0.067
5	0.003*	0.427	0.821
6	0.001*	0.055*	0.993
7	0.028*	0.011*	0.846
8	<0.001*	0.009*	0.297
Latewood (%)			
2	0.134	0.097	0.455
3	0.325	0.161	0.071
4	0.2	0.132	0.045*
5	0.004*	0.01*	0.625
6	0.951	0.129	0.886
7	0.323	0.21	0.929
8	0.002*	0.044*	0.153
Earlywood density (kg/m³)			
2	0.584	0.57	0.397
3	0.729	0.805	0.142
4	0.003*	0.011*	0.492
5	0.036*	0.086	0.214
6	<0.001*	0.022*	0.814
7	0.002*	0.072	0.666
8	0.02*	0.215	0.981
Latewood density (kg/m³)			
2	0.375	0.646	0.07
3	0.007*	0.544	0.863
4	0.694	0.593	0.945
5	0.003*	0.427	0.821
6	0.172*	0.689	0.635
7	0.138	0.771	0.834
8	0.003*	0.093	0.426

Note: *, statistical significance at $p < 0.05$.

Fig. 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 the broken line is 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 and 53 and 47 trees in control plots for wild-type and heterozygotes, respectively.



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 treatments can be attributed to the annual applications of fertilizer throughout the 10-year growing period.

In this study, fertilization significantly decreased all wood density traits, irrespective of *cad* genotype (Fig. 3; Table 3). The increase in radial growth under fertilization could be attributed 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 moderately strong negative correlation between earlywood width and latewood 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 or 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 (Fig. 4).

cad genotype effects

While Wu et al. (1999) found that *cad-n1* heterozygous trees had faster growth 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 with the systematic sample of 158 at one site in the earlier report. For valid estimates, further

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studies require a sufficient number of samples to be tested in additional trials, replicated in time.

Significant differences for wood density traits were observed between trees with alternative *cad* genotypes, except for latewood density (Fig. 2). The weighted wood density for heterozygotes was 2.6% higher than for 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 (a difference of 0.008 or 1.8%) for *cad-n1* 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/m^3 of dry processed kraft pulp. In the present study, the *cad-n1* heterozygotes had a significantly greater proportion of latewood, suggesting the potential for a significant impact on pulp yield. Previous studies indicated that latewood produces 2%–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 cambium age 8 years 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 × treatment interaction for any wood density or growth trait.

Wood density is determined by 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 (Fig. 3). This was expected because of the lack of a *cad* genotype by fertilizer treatment interaction effect. Heterozygotes showed consistently higher ring wood density than wild-type trees in most years through cambium age 8 (Fig. 3; Table 3). For both heterozygous and wild-type trees, latewood density increased rapidly from the pith. Megraw (1985) indicated that latewood density increases rapidly with ring number from the pith until values reach 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).

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