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Research paper

Genetic effects on total phenolics, condensed tannins and non-structural carbohydrates in loblolly pine (*Pinus taeda* L.) needles

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Carbon allocation to soluble phenolics (total phenolics, proanthocyanidins (PA)) and total non-structural carbohydrates (TNC; starch and soluble sugars) in needles of widely planted, highly productive loblolly pine (*Pinus taeda* L.) genotypes could impact stand resistance to herbivory, and biogeochemical cycling in the southeastern USA. However, genetic and growth-related effects on loblolly pine needle chemistry are not well characterized. Therefore, we investigated genetic and growth-related effects on foliar concentrations of total phenolics, PA and TNC in two different field studies. The first study contained nine different genotypes representing a range of genetic homogeneity, growing in a 2-year-old plantation on the coastal plain of North Carolina (NC), USA. The second study contained eight clones with different growth potentials planted in a 9-year-old clonal trial replicated at two sites (Georgia (GA) and South Carolina (SC), USA). In the first study (NC), we found no genetic effects on total phenolics, PA and TNC, and there was no relationship between genotype size and foliar biochemistry. In the second study, there were no differences in height growth between sites, but the SC site showed greater diameter (diameter at breast height (DBH)) and volume, most likely due to greater tree mortality (lower stocking) which reduced competition for resources and increased growth of remaining trees. We found a significant site × clone effect for total phenolics with lower productivity clones showing 27–30% higher total phenolic concentrations at the GA site where DBH and volume were lower. In contrast to the predictions of growth–defense theory, clone volume was positively associated with total phenolic concentrations at the higher volume SC site, and PA concentrations at the lower volume GA site. Overall, we found no evidence of a trade-off between genotype size and defense, and genetic potential for improved growth may include increased allocation to some secondary metabolites. These results imply that deployment of more productive loblolly pine genotypes will not reduce stand resistance to herbivory, but increased production of total phenolics and PA associated with higher genotype growth potential could reduce litter decomposition rates and therefore, nutrient availability.

Keywords: carbon allocation, clone, condensed tannins, nutrient cycling, phenolics, productivity.

Introduction

Pine plantations in the southern USA cover nearly 12.2 million hectares (Conner and Hartsell 2002). The bulk (>80%) of pine plantations are composed of loblolly pine (*Pinus taeda* L.), and nearly 100% of loblolly pine plantations are established with genetically improved seedlings (McKeand et al. 2003). Genetic improvement along with intensive silvicultural practices have drastically increased loblolly pine plantation productivity, making the southern USA one of the most productive timber regions of the world (Wear and Greis 2002, McKeand et al. 2003, Stanturf et al. 2003). While several studies have investigated the physiological basis of increased productivity in genetically improved loblolly pine (Bongarten and Teskey 1987, McCrady and Jokela 1998, McGarvey et al. 2004, King et al. 2008), no studies have investigated the balance between growth and carbon allocation to secondary biochemistry in intensively selected loblolly pine genotypes. If genotype productivity and allocation to carbon-based secondary compounds (CBSC) in

loblolly pine are consistent with the framework of the growth–differentiation balance (GDB) hypothesis (Herms and Mattson 1992), where growth is mainly limited by resource availability, and differentiation to secondary metabolism is dependent upon available carbohydrates (in the absence of a photosynthate limitation), more productive genotypes may allocate more carbon to growth and less to secondary metabolism. Ultimately, genetic and growth-related effects on CBSC could have important implications for loblolly pine plantation sustainability and biogeochemical cycling.

In loblolly pine and other conifers, a significant amount of CBSC are allocated to defense compounds such as resins and monoterpenes (Litvak and Monson 1998, Lombardero et al. 2000, Klepzig et al. 2005). However, phenolic compounds also represent a substantial C cost in loblolly pine foliage (Chung and Barnes 1977). Foliar soluble phenolics, including condensed tannins, can impact herbivore deterrence, litter decomposition and nutrient cycling, soil carbon sequestration and overall productivity. For example, total phenolics and condensed tannins, in some cases, have been positively associated with plant defense against insects (Williams et al. 1994, Holopainen et al. 2006), fungal (Bahnweg et al. 2000) and microbial infestation or foliar injury (Zobel and Nighswander 1990, Booker et al. 1996, Soukupová et al. 2000). Moreover, trees that produce polyphenol-rich litterfall may limit productivity by reducing the rates of litter decomposition (Zucker 1983, Kuiters 1990, King et al. 2001), resulting in the accumulation of nutrients in the forest floor. While sequestration of N and P in unavailable pools of recalcitrant organic matter (Kuiters 1990, Northrup et al. 1995, Souto et al. 2000) can contribute to feedbacks that further diminish productivity (Chapin 1993), high concentrations of polyphenols also inhibit N leaching from the ecosystem which may maximize litter-N recovery by mycorrhizal symbionts, and improve conditions for further root growth and nutrient cycling (Northrup et al. 1997). Given the importance of CBSC in relation to growth, defense and nutrient cycling, and the lack of information on the relationship between growth and allocation to CBSC in widely planted genetically improved loblolly pine, there is a critical need for investigation of these relationships to better understand the implications for loblolly pine plantation forestry in the southeastern USA.

The objective of this study was to investigate genetic effects on production of CBSC, in this case, phenolic compounds, in two different field studies while also examining the relationship between tree volume and foliar concentrations of phenolics. Following the general framework of the GDB hypothesis (Herms and Mattson 1992), we hypothesized that individual-tree and genotype volume growth would be inversely related to foliar concentrations of total phenolics and condensed tannins (or proanthocyanidins (PA)), and that total non-structural carbohydrate (starch and soluble sugars) (TNC) concentrations would increase if sink strength and growth were constrained by genotype growth potential. To examine the relationship between genotype volume growth and production of CBSC and TNC, we measured volume growth and foliar concentrations of total phenolics, PA, starch and soluble sugars in genotypes planted in two different field studies: a mixed planting of clones, full-sib family and half-sib family genotypes in a 2-year-old plantation setting on the lower coastal plain of North Carolina (NC), and a 9-year-old clonal trial replicated among and within sites in Georgia (GA) and South Carolina (SC), USA.

Materials and methods

Study sites, plant material and sample collection

The first study site was located at the Hofmann Forest in Onslow County, NC (34°49.4′N, 77°18.2′W) (Aspinwall et al. 2011*a*). The study site was ~19 m above sea level and was topographically uniform with very little relief. Mean annual precipitation (1971–2000) is 1435 mm, and mean temperature is 26.7 °C in July and 7.6 °C in January (National Climate Data Center, NOAA, available at http://cdo.ncdc.noaa.gov/climatenormals/clim20/nc/314144.pdf, accessed 24 March 2010). Soils are a Pantego mucky loam (fine-loamy, siliceous, semiactive, thermic Umbric Paleaquult) consisting of very poorly drained, thick loamy deposits (high organic matter) with moderate permeability (USDA, NRCS available at http://websoilsurvey.nrcs.usda.gov/, accessed 24 March 2010). A naturally regenerated pine stand had been established on the site prior to the establishment of this experiment. As is common practice for plantations in this area, drainage ditches were installed to remove excess water prior to the establishment of the previous stand (Allen and Campbell 1988, Allen et al. 1990).

In January 2006, the study was established as a randomized complete block design consisting of 20 replications of nine different genotypes from within three genetic 'groups' (clones, full-sibs and half-sibs). Therefore, the experimental unit was a single tree of each genotype randomly inter-planted within each replication. Two replications were arranged end to end along each elevated planting bed resulting in 10 rows of study plots. Between-row spacing was 6.1 m and within-row spacing was 3.05 m. Prior to planting, the site was fertilized with nitrogen (N), phosphorus (P) and boron (B) at elemental rates of 100, 40 and 1 kg ha^{-1}, respectively. In both April 2006 and 2007, competing vegetation along the elevated beds was controlled with a combined spray application of imazypyr and sulfometuron methyl at rates of 2.8 and 1.9 l ha^{-1}, respectively (Aspinwall et al. 2011*b*).

Of the nine genotypes, three were half-sib families (HS1, HS2, HS3), three were full-sib families (FS1, FS2, FS3) and three were clones (C1, C2, C3). The half-sib (open-pollinated) and full-sib (control-pollinated) family seedlings were from second-generation selections from the South Carolina–Georgia

coastal plain. The open-pollinated (half-sib) and full-sib families were all known to have excellent productivity, stem straightness and fusiform rust (caused by the fungus *Cronartium quercuum* sp. *fusiforme*) resistance. In fact, these particular full-sib and half-sib families were within the top 18 and 10%, respectively, of volume rankings of selections from the coastal plain (North Carolina State University Tree Improvement Cooperative database). Clonally propagated material originated from somatic tissue culture (somatic embryogenesis) of individuals from full-sib families. The parent of HS1 was one of the parents of both FS1 and FS3. Clone C1 originated from a full-sib family where HS1 was one of the parents, and both clones C2 and C3 originated from a single full-sib family which was unrelated to any of the genotypes in this study (Aspinwall et al. 2011*b*).

To quantify genetic differences in concentration of total soluble phenolics, PA and TNC, we collected five replications of foliage samples from all nine genotypes on 15 September 2007. One replication of HS2 had a missing tree due to mortality, and one sample of FS2 was damaged and removed from the analysis, resulting in 43 total samples. Collection of needles from each tree was standardized by selecting 5–10 fascicles from the first flush of the 2007 growing season, located on upper-crown, sun-exposed branches. Both total tree height (m) and ground-line diameter (cm) were recorded and individual-tree volume index (m^3) was calculated as the product of total tree height and ground-line diameter squared. Needle collection took place between 11:00:00 and 13:15:00 EST. Immediately following collection, needle samples were placed into liquid nitrogen and transported to the lab where they were stored at –20 °C. Samples were then freeze-dried and ground to pass a 0.5-mm mesh screen and stored in a desiccator.

The second set of genotypes used in this study was selected from a 9-year-old clonal trial replicated among and within two sites. The first site was located in Orangeburg County, SC (33°18′42.62″ N, 80°20′00.05″ W). Elevation at the site was ~27 m above sea level and the site was flat with very little variation in topography. Mean annual precipitation (1971–2000) is 1252 mm, and mean temperature is 27.6 °C in July and 8.3 °C in January (NCDC, NOAA, available at http://cdo.ncdc.noaa.gov/climatenormals/clim20/ga/096323.pdf, accessed 8 September 2010). The soils at this site are classified as Goldsboro sandy loam (fine-loamy, siliceous, subactive, thermic Aquic Paleudult) with moderately well-drained permeability (USDA, NRCS, available at http://websoilsurvey.nrcs.usda.gov/, accessed 24 March 2010). The second site was located in Screven County, GA (32°32′15.74″N, 81°35′57.67″W). Elevation at the site was ~40 m above sea level. Mean annual precipitation (1971–2000) is 1214 mm, and mean temperature is 27.6 °C in July and 8.1 °C in January (NCDC, NOAA, available at http://cdo.ncdc.noaa.gov/climatenormals/clim20/sc/384197.pdf, accessed 8 September 2010). This site was topographically uniform containing well-drained Fuquay loamy sand soils (loamy, kaolinitic, subactive, thermic Arenic Plinthic Kandiudult).

In 2001, over 200 different clones were planted at both sites in an incomplete block design, with eight blocks, and each tree was established as a single-tree plot within each block. The sites were mowed and herbicide treatments were applied prior to planting to promote clone establishment. At both sites, between-row spacing was 3.05 m and within-row spacing was 1.83 m. To ensure uniform growing conditions over time and at both sites, competing vegetation was controlled with a combined herbicide treatment of glyphosate, sulfometuron methyl and metsulfuron methyl at rates of 0.07, 0.14 and 0.07 l ha^{-1}, respectively. As of 2008, the two test sites had experienced significant differences in tree mortality; survival at the SC and GA sites was 89 and 96%, respectively ($\chi^2 = 54.7$, $P < 0.0001$). Due to higher mortality, the remaining trees at the SC site showed significantly higher diameter growth at 1.3 m (diameter at breast height (DBH)) (16.0 ± 0.08 cm) in comparison with the GA site (14.5 ± 0.07 cm). Overall basal area and stand volume were also higher at the SC site (28.1m^2 ha^{-1} and 116 m^3 ha^{-1}, respectively) relative to the GA site (26.6m^2 ha^{-1} and 111 m^3 ha^{-1}, respectively). However, the average dominant or co-dominant (non-suppressed) tree height at both sites was 11.1 ± 0.03 m, indicating that there was no difference in site quality (site index).

To determine foliar concentrations of total phenolics and PA, we collected needle samples from five ramets of eight different clones at both sites ($n = 80$ trees), with each ramet collected from a different block within each site. The eight clones (A–H) selected for sampling were chosen based on height and diameter data collected in 2008 and were selected to represent a gradient of productivity from high-productivity clones to low-productivity clones (2008 clone mean height and DBH ranged from 8.7 to 12.7 (±0.3) m and 11.2 to 17.5 (±0.8) cm, respectively). Some clones were related: Clones A and C originated from the same full-sib family while Clones D and G originated from a different full-sib family. The four remaining clones were unrelated and the parents of each full-sib cross, from which the clones were selected, originated from counties along the lower coastal plain ranging from as far south as Marion County, FL, and as far north as Onslow County, NC.

Needle collection from each tree was standardized by carefully removing an upper-crown, sun-exposed branch with a pole-pruner and selecting 5–10 fascicles from the first flush of the 2009 growing season. Suppressed (i.e., diseased, dying, stressed or shaded canopy) trees were removed from sampling to ensure that differences in concentrations of phenolics and TNC were not due to sub-optimal light or visible plant health conditions. Both current-year DBH and time of needle collection were recorded. Diameter at breast height and previous years' height data were used to estimate total tree

inside-bark volume following Goebel and Warner (1966). At the SC site, needle collection occurred between the hours of 11:00:00 and 15:15:00 EST on 14 October 2009, and at the GA site, needle collection occurred between 9:15:00 and 13:15:00 EST on 15 October 2009. Needle samples were immediately placed in liquid N and were processed in the same manner as previously described.

Extraction of soluble components

Two methods of extraction were tested to determine the appropriate solvent for extraction. We compared the extraction efficiency of 70% acetone with 250 mM sodium citrate combined with 0.04% sodium bisulfite (pH 7) (Blum 1997). Needle tissue was extracted four times with 1 ml of each solvent with mixing and incubation at room temperature for 5 min. Results indicated that three extractions with 70% acetone was effective at extracting nearly 100% of the soluble fraction while three extractions with the 250 mM sodium citrate and sodium bisulfite solution was slightly less efficient (97–98%). Therefore, we chose to use 70% acetone as our solvent for extraction. Two 50-mg tissue samples from each sample tree were extracted three times with 1 ml of 70% acetone with mixing for 5 min at 25 °C. Following extraction, the insoluble material was pelleted by centrifugation (16,000*g*, 5 min), and the supernatants were pooled for each tissue sample (see Booker and Maier 2001).

Total phenolics assay

Concentrations of total phenolics were determined by the Folin–Ciocalteu method as described by Booker and Maier (2001). First, each sample supernatant was diluted 1:10 with 70% acetone, and duplicate 10-µl aliquots were mixed with 495 µl of 0.25 N Folin–Ciocalteu reagent (Sigma Chemical Co., St Louis, MO, USA) and 495 µl of 1 M Na_2CO_3. With two aliquots and two 50-mg samples extracted, a total of four samples were analyzed for each sample tree. Next, samples were inverted three times to mix, incubated at room temperature for 30 min, and solution absorbance was measured at 724 nm on a spectrophotometer (Hewlett Packard Model 8452, Palo Alto, CA, USA). A standard curve was then produced by relating catechin concentration to solution absorbance. Based on the standard curve, sample absorbance for total phenolics concentration was estimated and expressed as catechin equivalents (mg g^{-1} dry needle mass).

Proanthocyanidin assay

Following Porter et al. (1986) and Booker and Maier (2001), we determined PA concentration by oxidative depolymerization of anthocyanidins in acid butanol. The procedure involved mixing 900 µl of methanol with each of two 100-µl aliquots of sample supernatant, 6 ml of acid butanol (50 ml l^{-1} concentrated HCl in *n*-butanol) and 200 µl of 20 g l^{-1} $FeNH_4(SO_4)_2 \times 12\ H_2O$ in 2 N HCl in 15-ml polypropylene tubes. Additionally, a 100-µl aliquot of each soluble fraction was mixed with 900 µl of methanol, 6 ml of *n*-butanol and 200 µl of H_2O so that interfering substances in the extracts could be accounted for. All mixtures and solutions were incubated in a water bath at 90 °C for 40 min. After cooling to room temperature, sample absorbance was measured at 550 nm on the spectrophotometer. Results indicated that interfering substances were not present and did not affect absorbance readings. Proanthocyanidin concentration was expressed as PA equivalents (mg g^{-1} dry needle mass) using an $E_{1\%,550}$ nm value of 518 (Booker and Maier 2001).

Non-structural carbohydrates assay

Following Booker and Maier (2001), the UV method (R-Biopharm, Inc., Marshall, MI, USA) was used to enzymatically determine needle starch and soluble sugar concentrations in samples collected from the different clones, full-sibs and half-sibs planted in the NC study. To solubilize starch, duplicate 50-mg tissue samples collected from each tree were mixed with 2.4 ml of dimethylsulfoxide and 600 µl of 8 N HCl in sealed 15-ml polypropylene tubes and incubated for 60 min at 60 °C on a tube rocker. Samples were then neutralized with 600 µl of 8 N NaOH and diluted to 15 ml with 112 mM citrate buffer (pH 4). The mixtures were then filtered (Whatman No. 1) and duplicate 10-µl aliquots were prepared according to kit instructions. Sample absorbance was read at 340 nm on the spectrophotometer and starch and sugar results were expressed as D-glucose equivalents.

Statistical analysis

Prior to analysis, data were tested for homogeneity of variance and normality. For the NC study, analysis of variance (ANOVA) was performed to determine the significance of the main and interactive effects of replication, genetic group and genotype within genetic group on phenolic and TNC compounds. The linear model was written in the form

$$Y_{ijk} = \mathrm{m} + R_i + L_j + G_k(L_j) + R_iL_j + \mathbf{e}_{ijk} \quad (1)$$

where Y_{ijk} is the observed value of the trait of interest (i.e., total phenolics, PA, etc.); µ is the overall mean; R_i is the effect of the *i*th replication; L_j is the effect of the *j*th genetic group; $G_k(L_j)$ is the effect of the *k*th genotype from within the *j*th genetic group; R_iL_j is the replication × genetic group effect; and ε_{ijk} is the random error associated with the model $E(N \sim 0, \sigma^2)$. To account for size-related effects on concentrations of biochemical constituents, ground-line diameter, height and volume index were tested as covariates. Time of needle collection was not a significant covariate for total phenolics ($P = 0.82$), PA ($P = 0.46$), starch ($P = 0.31$) or soluble sugars ($P = 0.96$). To assess the level of genetic determination in the production of total

phenolics, PA and TNC, broad-sense heritabilities (H^2) were calculated from mixed-model ANOVA (Falconer and Mackay 1996). For the ANOVA in Eq. (1), variance components (σ^2) were used to calculate H^2 as

$$H^2 = \frac{\sigma^2_{\text{genotype}}}{(\sigma^2_{\text{genotype}} + \sigma^2_{\text{error}})} \quad (2)$$

For the clonal study, ANOVA was also used to determine the significance of site, clone and site × clone effects on phenolic compounds. The linear model was written in the form

$$Y_{ijk} = \mu + S_i + C_j + S_iC_j + R_k(C_j) + \varepsilon_{ijk} \quad (3)$$

where Y_{ijk} is the observed value of the trait of interest (i.e., total phenolics, PA, etc.); μ is the overall mean; S_i is the effect of the ith site; C_j is the effect of the jth clone; S_iC_j is the site × clone effect; $R_k(C_j)$ is the random effect of the kth ramet from within the jth clone; and ε_{ijk} is the random error associated with the model $E(N \sim 0, \sigma^2)$. Total tree inside-bark volume, total tree height and DBH were tested as covariates in the ANOVA. Time of needle collection was not a significant covariate for total phenolics ($P = 0.16$) or PA ($P = 0.76$). Tukey's adjustment was used for pairwise comparison of genotype means. To assess the level of genetic determination in the production of phenolics concentrations considering both sites (Eq. (4)), as well as the genetic variation of phenolic concentrations in response to site (Eq. (5)), mixed-model ANOVA was used to calculate H^2 (Falconer and Mackay 1996):

$$H^2 = \frac{\sigma^2_{\text{clone}}}{(\sigma^2_{\text{clone}} + \sigma^2_{\text{site}\times\text{clone}} + \sigma^2_{\text{error}})} \quad (4)$$

$$H^2 = \frac{\sigma^2_{\text{site}\times\text{clone}}}{(\sigma^2_{\text{clone}} + \sigma^2_{\text{site}\times\text{clone}} + \sigma^2_{\text{error}})} \quad (5)$$

All analyses were conducted in SAS PROC MIXED and all tests were conducted at the $P \leq 0.05$ significance level (SAS/STAT software v9.2, SAS Institute, 2002). All P values ≤0.10 were considered marginally significant or approaching significance.

In both the NC study involving a range of clones, full-sib families and half-sib families, and the clonal study replicated within and across sites in GA and SC, linear regression was used to determine the relationship between both individual-tree and genotype productivity (volume index), and foliar phenolic compounds. All models were checked for normality and homogeneity of variance, and data points with studentized residual values >2 or less than − 2 were removed as outliers. Based on these criteria, two data points were considered outliers and were removed from the linear regression of individual-tree volume index and total phenolics in the NC study. In the same study, two data points were also removed as outliers from the linear regression of individual-tree volume and PA, and two different data points were removed as outliers from the individual-tree volume index–soluble sugars regression. All linear regression models were fitted using SAS PROC REG (SAS/STAT software v9.2, SAS Institute, 2002).

Results

Growth and total phenolic, PA and TNC concentrations across a range of genetic diversity

Among a variety of juvenile loblolly pine clones, full-sib and half-sib family genotypes, initial height at planting was a significant covariate for volume ($P = 0.002$). After adjusting for initial height at planting, there were no significant differences in volume index among genetic groups ($P = 0.47$) or genotypes ($P = 0.26$), and there was no significant replication × genotype interaction ($P = 0.23$). Across all sample trees, ground-line diameter, height and volume index were 3.76 cm (±0.8), 2.12 m (±0.4) and 0.003 m^3 (±0.002), respectively.

Individual-tree volume index was not a significant covariate for total phenolics ($P = 0.15$) or PA concentration ($P = 0.63$). We found marginally significant differences in total phenolics among replications and genotypes (Table 1). The largest differences in total phenolics occurred between full-sib genotype FS1 and half-sib family HS2 (Table 2). However, after the Tukey honestly significant difference adjustment for multiple comparisons, there were no significant pairwise differences in total phenolics among genotypes. Broad-sense heritability (H^2) of total phenolics was

Table 1. P values and degrees of freedom from ANOVA on total phenolics (mg catechin g^{-1}), PA (mg g^{-1}), starch (mg g^{-1}) and soluble sugars (mg g^{-1}) in needles of different loblolly pine genotypes (three clones, full-sibs and half-sibs, respectively) growing in a 2-year-old plantation on the coastal plain of NC. Where tree volume index was not a significant or marginally significant covariate ($P \leq 0.05$ and $P \leq 0.10$, respectively), it was removed from the analysis.

	d.f.	Total phenolics	PA	Starch	Soluble sugars
Volume index	1	ns	ns	0.04	0.09
Replication	4	0.08	0.22	0.16	0.99
Genetic group	2	0.75	0.23	0.57	0.88
Genotype (genetic group)	6	0.09	0.87	0.03	0.20
Replication × genetic group	8	0.34	0.97	0.66	0.11

Table 2. Mean (±standard error) concentrations of total phenolics, PA, starch and soluble sugars within needles of different 2-year-old loblolly pine clones (C1, C2, C3), full-sib families (FS1, FS2, FS3) and half-sib families (HS1, HS2, HS3) growing in a plantation on the coastal plain of NC.

	Total phenolics (mg catechin g^{-1})	PA (mg g^{-1})	Starch (mg g^{-1})	Soluble sugars (mg g^{-1})
C1	118.8 (6.0)	152.1 (9.3)	0.86 (0.99)	28.7 (1.94)
C2	118.5 (6.0)	151.1 (9.3)	4.66 (1.01)	27.1 (1.97)
C3	107.6 (6.0)	161.5 (9.3)	0.69 (0.98)	26.2 (1.91)
FS1	130.4 (6.0)	138.7 (9.3)	0.93 (0.96)	27.0 (1.9)
FS2	111.5 (6.0)	135.5 (9.3)	1.36 (0.94)	25.4 (1.8)
FS3	109.4 (7.0)	149.5 (10.9)	2.81 (1.10)	31.1 (2.1)
HS1	120.1 (6.0)	141.0 (9.3)	0.84 (0.95)	26.1 (1.8)
HS2	103.6 (7.0)	152.0 (10.9)	–	27.0 (2.1)
HS3	115.9 (6.0)	147.1 (9.3)	2.95 (0.94)	31.2 (1.8)
Overall	115.3 (2.3)	147.4 (3.0)	1.66 (0.41)	27.7 (0.7)

Note: Needle samples from genotype HS2 had no detectable starch.

0.20, indicating that a small percentage of the phenotypic variance could be explained by genotype. Moreover, we found no significant differences in PA among any of the factors in the ANOVA (Table 1), and H^2 of PA was 0.00. Overall mean PA and total phenolics were 147.4 ± 3.0 mg g^{-1} and 115.3 ± 2.3 mg catechin equivalents g^{-1}, respectively (Table 2).

Individual-tree volume index was a significant and marginally significant covariate for starch and soluble sugars, respectively (Table 1). There were no significant differences in soluble sugars among genotypes; yet, starch concentrations were significantly different among genotypes. However, mean needle starch content was highly variable, with genotype mean values ranging from 0 to 4.66 ± 1.0 mg g^{-1} (Table 2). Moreover, individual-sample starch concentrations ranged from 0 to 12.9 mg g^{-1}, and out of the 43 needle tissue samples that were assayed, 14 samples had no detectable starch. Overall mean starch concentration was 1.66 ± 0.4 mg g^{-1} (Table 2), with a CV of 162.1%. Mean sugar content was 27.7 ± 0.7 mg g^{-1} (Table 2) and was more uniform with a CV of 15.9%. Broad-sense heritabilities for starch and soluble sugars were 0.31 and 0.12, respectively.

Although we found significant phenotypic relationships between individual-tree volume, total phenolics, PA and soluble sugars, we found no significant genetic relationship between tree volume and total phenolics ($P = 0.17$), PA ($P = 0.19$), starch ($P = 0.99$) and soluble sugars ($P = 0.74$). Moreover, individual-tree volume explained little of the variation in foliar biochemistry. For example, concentrations of total phenolics showed a significant negative relationship with individual-tree volume, with individual-tree volume explaining 12% of the variation in total phenolics concentration (Figure 1a). Similarly, individual-tree concentrations of PA showed a significant negative relationship with volume index (Figure 1b). There was no significant relationship between individual-tree volume and needle starch concentration ($P = 0.17$). In contrast, needle soluble sugars were positively associated with individual-tree volume (Figure 1c).

Site and genotype effects on productivity and foliar phenolic concentrations

Between the GA and SC sites, total tree height was not significantly different, but the SC site had significantly higher DBH and volume (Table 3). DBH and volume at the SC site were 16.1 ± 0.38 cm and 0.088 ± 0.005 m^3, respectively, whereas DBH and volume at the GA site were 14.4 ± 0.39 cm and 0.069 ± 0.005 m^3, respectively. Site × clone interaction was not significant for any growth trait (Table 3). As intended, our selection of clones representing a gradient in productivity resulted in highly significant differences in height, DBH and volume (Table 3, Figure 2a). Across sites, height ranged from 12.7 ± 0.32 m for Clone A, to 8.7 ± 0.32 m for Clone H. Clone A also had the highest DBH (17.5 ± 0.77 cm), while Clone H had the lowest DBH (11.1 ± 0.77 cm).

Total tree height, DBH and volume were not significant covariates for total phenolics ($P \geq 0.45$). There were significant differences in total phenolics between sites ($P < 0.001$, Table 3), with the larger volume trees at the SC site (122.1 ± 2.28 mg catechin equivalents g^{-1}) having significantly lower mean concentration of total phenolics than the smaller volume trees at the GA site (135.7 ± 2.28 mg catechin equivalents g^{-1}). The site × clone interaction was significant for total phenolics (Table 3, Figure 2c). At the SC site, the lowest volume clones (F, G and H), with the exception of clone D, tended to have the lowest concentrations of total phenolics (Figure 2c). In contrast, at the GA site, the same clones had much higher concentrations of total phenolics (Figure 2c). In fact, for Clones G and H, total phenolics increased by 27 and 30%, respectively, at the GA site (lower volume) relative to the SC site (higher volume) (Figure 2c). In contrast, larger volume clones showed no significant differences in total phenolics concentration between sites (Figure 2c). While the site × clone interaction was significant in the ANOVA, differences in total phenolics concentration between sites, for each clone, were generally not significant ($P > 0.17$) after Tukey's adjustment. Clone H was the only clone that showed a marginally significant ($P = 0.07$) difference in

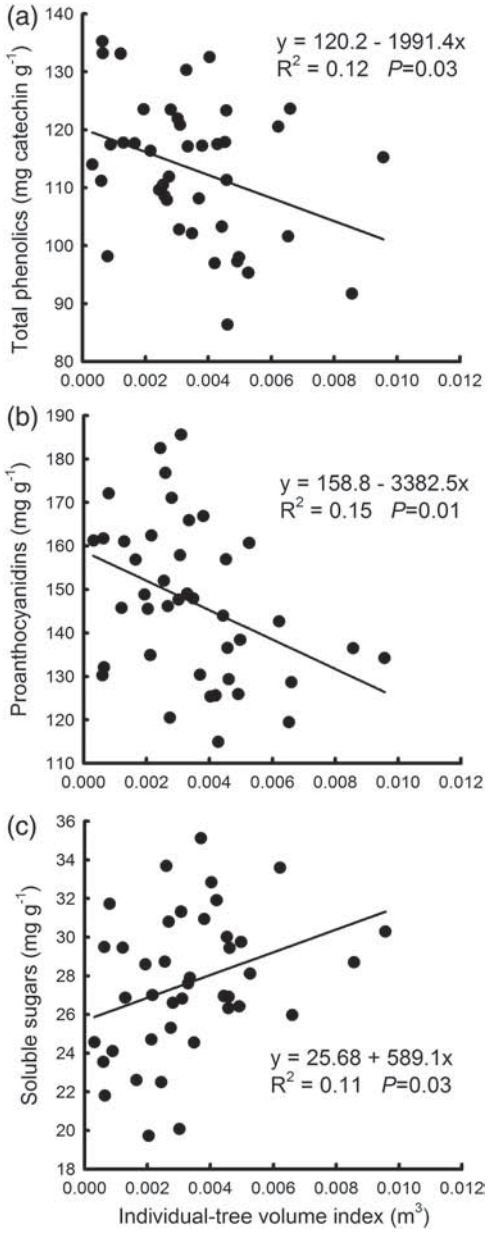

Figure 1. Linear regression models describing the relationship between individual tree-volume index and foliar concentrations of (a) total phenolics, (b) PA and (c) soluble sugars, across different 2-year-old full-sib, half-sib and clonal genotypes growing in a 2-year-old plantation on the lower coastal plain of NC. In each regression, $n = 41$.

volume between sites after Tukey's adjustment. Considering both sites, H^2 of total phenolics was 0.00; however, H^2 of total phenolics in response to site was 0.20 which corresponds to the significant site × clone interaction.

Individual-tree DBH was a significant covariate for PA (Table 3). We found marginally significant differences in PA among clones (Table 3, Figure 2b). After Tukey's adjustment, all comparisons of genotype mean PA were not significant ($P > 0.55$). The overall mean concentration of PA across both sites was 121.5 ± 1.30 mg g^{-1}, with a CV of 9.54%.

When data from both sites were combined, there was no significant relationship between individual-tree volume and total phenolics ($P = 0.71$) or PA ($P = 0.36$). Within the GA site, individual-tree volume and total phenolics concentration showed only a marginally significant, yet weak negative trend (slope = –101.5, $R^2 = 0.08$, $P = 0.08$). Within the SC site, individual-tree volume and total phenolics concentration showed a significant positive association (slope = 137.5, $R^2 = 0.11$, $P = 0.04$). Moreover, clone mean volume was positively associated with total phenolics within the SC site (Figure 3a). However, there was no significant relationship between clone mean volume and total phenolics within the GA site (Figure 3b) or across sites ($P = 0.60$). There was no significant relationship between clone mean volume and PA across sites ($P = 0.11$) or within the SC site (Figure 3c); however, we found a significant positive relationship between clone mean volume and PA within the lower volume GA site (Figure 3d).

Discussion

Relationship between productivity, phenolics and TNC across a gradient of genetic diversity

In the first study, our objective was to compare production of phenolics and TNC among different loblolly pine clones, full-sib families and half-sib families while examining the relationship between productivity and concentrations of these metabolites. Among these genotypes, productivity differences were not significant and we found only marginally significant differences in total phenolics and no significant differences in PA (Table 1). In loblolly pine and other southern pines, few studies have looked at genetic differences in production of

Table 3. *P* values from ANOVA on tree height (m), DBH (cm), stem volume (m^3), total phenolics (mg catechin g^{-1}) and PA concentration (mg g^{-1}) among eight different 9-year-old loblolly pine clones growing in a clonal trial across two different sites (SC and GA). DBH was tested as a covariate in the analysis of total phenolics and PA. When not significant (ns), DBH was removed from the analysis. Effects with *P* values ≤0.05 were considered significant and *P* values ≤0.10 were considered marginally significant or approaching significance.

	d.f.	Height	DBH	Volume	Total phenolics	PA
DBH	1	–	–	–	ns	0.055
Site	1	0.147	0.006	0.011	<0.001	0.105
Clone	7	<0.001	<0.001	<0.001	0.066	0.078
Site × clone	7	0.771	0.194	0.344	0.049	0.804

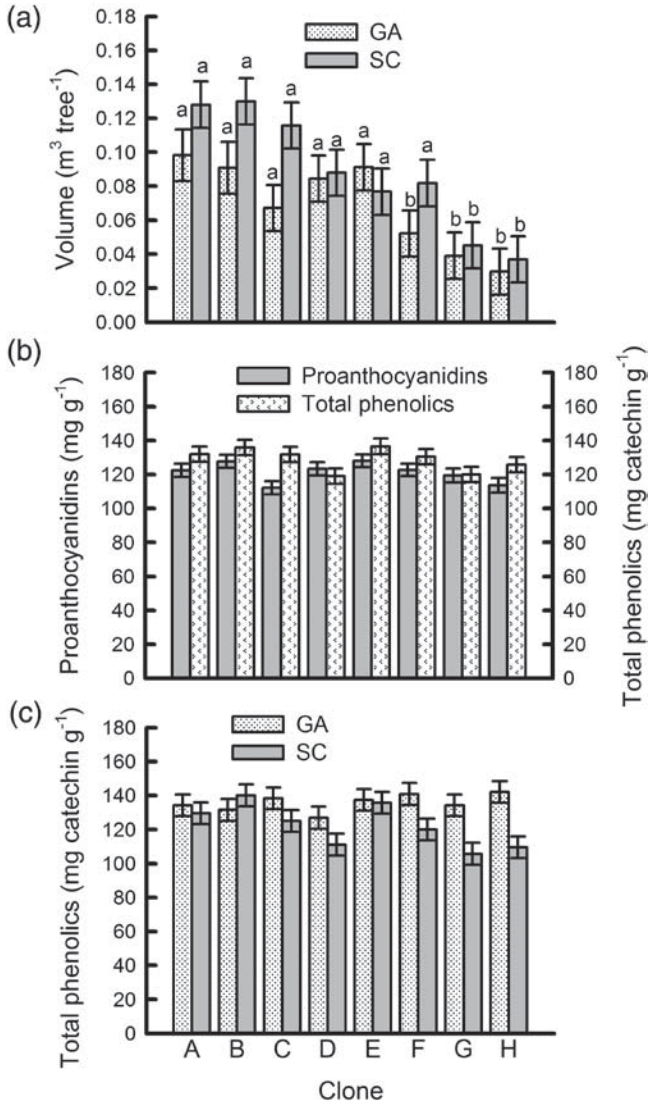

Figure 2. (a) Mean tree volume (±standard error) among eight different 9-year-old loblolly pine clones growing across sites in GA (low volume site) and SC (high volume site), USA. (b) Clone mean (±standard error) concentrations of total phenolics and PA ($n = 10$). (c) Clone mean (±standard error) concentrations of total phenolics at sites in GA (low volume site) and SC (high volume site), $n = 5$.

soluble phenolics and TNC, and those that have, conducted measurements on a limited number of genotypes (Sword et al. 1998, Saxon et al. 2004). While the paucity of information on genotypic differences in phenolics makes this study unique, our results provide little evidence of genetic variation in production of these compounds among a variety of young, plantation-grown genotypes. Moreover, in contrast to studies in other species (Hayashi et al. 2005, Donaldson and Lindroth 2007, Schweitzer et al. 2008), genotype accounted for very little of the variation in foliar biochemistry (low H^2's). The lack of volume differences among the genotypes in this study may also be linked to the generally minimal variation in soluble phenolics and TNC among genotypes.

While we found no genetic relationship between tree size, total phenolics, PA and TNC, we did find a negative, albeit weak, phenotypic relationship between individual-tree size and both total phenolics and PA (Figure 1a and b). Several studies in *Populus* have also found significant negative relationships between tree size and foliar CBSC (Kasola et al. 2004, Donaldson et al. 2006, Häikiö et al. 2009). We also found a positive relationship between tree size and soluble sugars (Figure 1c). The low concentrations of total phenolics and PA, and high TNC concentrations in larger trees may imply that larger trees allocated more of their available carbon to growth relative to defense. Similarly, Rühmann et al. (2002) found a negative relationship between growth and concentrations of phenolic compounds in *Malus* foliage, and sugar deficiencies caused metabolic limitations that appeared to influence phenylpropanoid concentrations in leaves.

Although genetic differences in total phenolics or PA were not apparent and only phenotypic relationships between growth and foliar biochemistry existed, our results do indicate that C allocation to total phenolics and PA in needles of juvenile loblolly pine is of a sufficient magnitude to be an important driver of variation in whole-tree growth. For instance, we used allometric equations developed from destructive harvests of the same genotypes (Aspinwall et al. 2011*b*) to estimate individual-tree foliage dry mass and whole-tree (above- and belowground) dry mass. We then scaled foliar concentrations of total phenolics to the canopy level, assumed that 50% of whole-tree dry mass is C, and determined the whole-tree C equivalent of C allocated to total phenolics at the canopy level. Our estimates indicated that trees with the highest and lowest concentrations of total phenolics (Figure 1a) allocated 21.0 and 64.2 g C, respectively, to total phenolics at the canopy level. These same trees contained roughly 171.2 and 875.7 g C (above- and belowground). Thus, C allocated to foliar total phenolics was equivalent to 12.3 and 7.3%, respectively, of whole-tree C in trees with the highest and lowest concentrations of foliar total phenolics. The difference in whole-canopy foliar total phenolics between these sample trees was 43.2 g C (>200%), while the difference in whole-tree C was of a similar magnitude (704.5 g C or >400%). Concentrations of PA were also high, and at the canopy level, C allocated to PA was equivalent to 16.4 and 9.8%, respectively, of whole-tree C in trees with the highest and lowest concentrations of foliar PA (Figure 1b). In contrast to total phenolics, the difference in whole-canopy PA and whole-tree C between these sample trees was lower: 50.1 and 11.2%, respectively. Given that the cost of foliage and phenolic production is high in loblolly pine (~1.588 and 1.919 g glucose g^{-1} dry mass, respectively; Chung and Barnes 1977), and these juvenile trees partitioned >40% of total dry mass to foliage (Aspinwall et al. 2011*b*), our results suggest that C allocation to these foliar CBSC represents an important C cost in young plantation-grown loblolly pine.

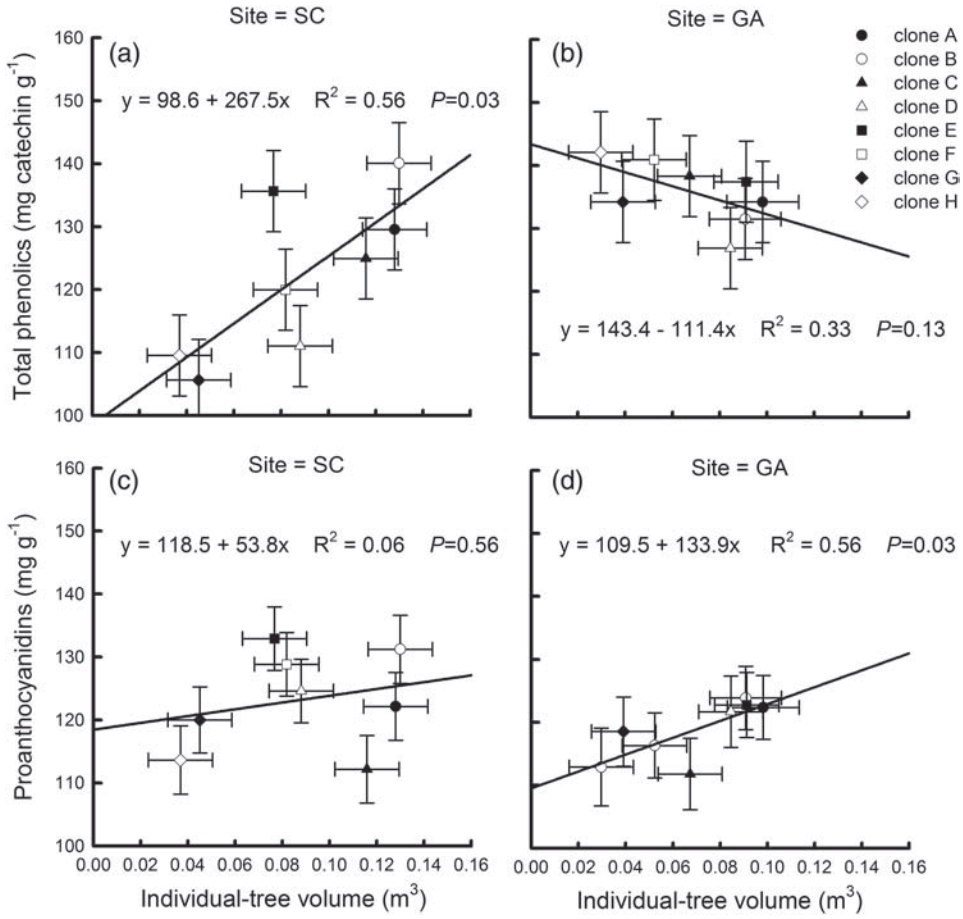

Figure 3. Linear regression models describing the relationship between clone mean (±standard error) volume and total phenolics concentrations at the SC (high volume) site (a) and the GA (low volume) site (b). (c) and (d) show the relationship between clone mean PA and volume at the SC (high volume) and GA (low volume) sites, respectively. For each regression point, $n = 5$.

There are some limitations to our study and the hypotheses set forth by the GDB hypothesis (Stamp 2003). For instance, we did not incorporate fertility-related effects into our study (Kraus et al. 2004, Matyssek et al. 2005, Holopainen et al. 2006). Genetic differences in phenotypic plasticity, localization of secondary compounds within foliage, ontogenic effects and chemical composition of different phenolic acids (Schnitzler et al. 1996, Soukupová et al. 2000, Booker and Maier 2001), or genotype photosynthetic capacity (Tyree et al. 2009) may have also contributed to variability in our data. Light environment and competitive effects have also been shown to influence production of some CBSC (Donaldson et al. 2006, Osier and Lindroth 2006).

Although we did not study seasonal effects on foliar biochemistry, seasonal effects can influence C allocation to these compounds (Mooney 1972). In loblolly pine, Booker and Maier (2001) measured total phenolics four times over the course of a year and found that concentrations increased with time. Williams et al. (1997) also found that concentrations of another CBSC, monoterpene, were highest in current-year loblolly pine needles during the fall. Furthermore, among a range of conifer species, Hatcher (1990) found that phenolic concentrations generally increased over time and by late summer, concentrations had stabilized. Studies in deciduous forest species have also indicated that concentrations of PA increase over the growing season (Lempa et al. 2000, John King, unpublished data). In general, these studies suggest that concentrations of total phenolics and PA stabilize and reach high concentrations in the fall. Even so, as in *Populus*, genotypes may show significant seasonal variation in total phenolics and PA (Osier et al. 2000), and environmental conditions may have a significant impact on different foliar defense compounds (Mooney 1972, Osier and Lindroth 2006). The high variability in starch content may be due to seasonally low starch concentrations (Nerg et al. 1994, Ludovici et al. 2002). Nonetheless, we emphasize that there have been no studies that have investigated genetic effects on total phenolics and PA in loblolly pine and our fall sampling provides a representation of the potential differences in total phenolics and PA.

Overall, our findings provide weak support for a trade-off between growth and secondary metabolism among individual juvenile loblolly pine trees. However, because we found no

genetic differences in productivity, total phenolics or PA, and there was no relationship between genotype productivity, foliar total phenolics or PA, our results do not support the existence of a trade-off between genotype productivity and defense.

Productivity and foliar phenolic concentrations as a function of site and genotype

In the clonal study, we found no differences in height growth between sites, suggesting that site quality (site index) was not significantly different. However, greater tree mortality (lower stocking) at the SC site most likely allowed for more thorough exploitation of available resources by the remaining trees, and therefore greater diameter and volume growth. Interestingly, total phenolics concentrations were higher at the GA site in comparison with the SC site. This finding suggests that higher stand density resulted in decreased diameter and volume growth, but increased foliar concentrations of total phenolics. Studies in other forest species have found similar relationships between growth and soluble phenolics as a function of stand density (Horner et al. 1987, Thompson et al. 1989, Hall and Marchand 2009). Overall, site effects on tree volume and total phenolics suggest that within the same clones, stand characteristics can affect productivity and patterns of resource allocation.

In terms of genetic effects, clones that were less productive increased their concentrations of total phenolics at the GA site (low volume) relative to the SC site (high volume) (Figure 2c). This suggests that differences in stand structure (i.e., stocking, basal area) caused a shift in CBSC production among clones, with low-productivity clones allocating more carbon to defense compounds and less carbon to growth when competition for resources was higher. In general, studies in loblolly pine and other species have shown that when resource availability and growth are relatively low, concentrations of CBSC tend to increase (Sword et al. 1998, Glynn et al. 2003, Kasola et al. 2004). Interestingly, at the SC site, high-productivity clones showed a smaller relative decrease in total phenolics in comparison with low-productivity clones (Figure 2c). Therefore, although reduced stand density may result in enhanced diameter and volume growth, C allocation to soluble phenolics may also be higher, in relative terms, among clones with higher growth potential. This may indicate that more productive clones fix enough carbon to supply growth, maintenance, and the production and storage of secondary compounds. Moreover, stand characteristics (i.e., mortality, diameter growth, etc.) and genetic makeup might interact in ways that influence C allocation to foliar secondary compounds in unexpected ways. For instance, we found that clone mean volume and total phenolics showed a significant positive relationship at the SC site. In fact, foliar total phenolics increased by ~24% with clone volume at the higher volume SC site (Figure 3a). The null or positive associations between genotype size and both total phenolics and PA may reflect that N was not a limiting factor in tree growth at either site. For example, in *Populus*, both Donaldson et al. (2006) and Harding et al. (2009) found that the relationship between growth and defense was only negative under N-limiting conditions. Warren et al. (1999) also found that more-productive fertilized loblolly pine had significantly higher phloem phenolics and PA. Consequently, our results suggest that both genotype and stand characteristics influence the production of CBSC, and in some cases these factors directed the allocation of carbon for growth and secondary metabolism in the opposite direction of that predicted by growth–defense theory.

The positive relationship between total phenolics and clone productivity at the higher volume SC site may indicate that larger clones were more successful at competing for light resources (McCrady and Jokela 1998, Matyssek et al. 2005, Emhart et al. 2007) or had enhanced photosynthetic capacity (King et al. 2008, Tyree et al. 2009) which resulted in greater C assimilation, and therefore higher concentrations of secondary compounds. Competitive interactions between low- and high-productivity clones may have influenced this relationship, and higher volume production combined with lower competition at the SC site may partially explain the positive association between genotype size and total phenolics at the SC site. However, these trials are completely randomized and large clones are not necessarily adjacent to small clones. Additionally, if higher productivity and lower competition for resources at the SC site were causing a positive relationship between genotype size and total phenolics, we would also expect a positive relationship between genotype size and PA. However, we found no association between PA and genotype size at the SC site (Figure 3b). Furthermore, we found a positive association between genotype size and PA at the GA (low volume) site (Figure 3d) which had more likelihood for competitive effects. Therefore, we found evidence of a positive relationship between genotype size and both total phenolics and PA in stands differing in competitive status. Nonetheless, clonal differences in leaf area, crown structure, net photosynthetic rate (Emhart et al. 2007), physiological plasticity (Aspinwall et al. 2011*a*) and changes in gene expression (Day et al. 2002, Watkinson et al. 2003, Chen et al. 2009) likely influenced C assimilation and allocation to CBSC to some degree, as well as the relationship between productivity and CBSC (Vose and Allen 1988, Chmura and Tjoelker 2008, King et al. 2008).

In conclusion, our study provided the first examination of genetic and genotype-size effects on foliar defense compounds in loblolly pine. In contrast to the framework of the GDB hypothesis, genotype size and foliar secondary biochemistry showed a null or positive relationship in both studies, and genetic effects on total phenolics and PA were generally insignificant. However, we found evidence that both clone and site (in this case, stand density or competitive status) affect foliar

total phenolics, and the relationship between genotype size and both total phenolics and PA may be positive. Overall, these results suggest that enhanced productivity of some loblolly pine clones may be accompanied by higher concentrations of total phenolics and PA under different stand conditions. Moreover, these results suggest that deployment of more productive genotypes will not negatively impact stand defense against herbivory, but increased production of total phenolics and PA could result in reduced litter decomposition rates and therefore lower nutrient availability.

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