

Mineral nutrition, resin flow and phloem phytochemistry in loblolly pine

JEFFREY M. WARREN,¹ H. LEE ALLEN² and FITZGERALD L. BOOKER³

¹ Department of Natural Resource Sciences, Washington State University, Pullman, WA 99165, USA

² Department of Forestry, North Carolina State University, Raleigh, NC 27695, USA

³ USDA, Agricultural Research Service and Department of Crop Science, North Carolina State University, Raleigh, NC 27695, USA

Received October 31, 1997

Summary Southern pine beetles and associated pathogenic fungi represent the largest biotic threat to pine forests in the southeastern USA. The two primary defensive mechanisms of the tree to the beetle–fungal complex are the primary oleoresin flow and the concentrations of preformed and induced secondary compounds. We compared oleoresin flow and concentrations of phloem nutrients, soluble sugars, starch, total phenolics and proanthocyanidins in *Pinus taeda* L. trees in fertilized and control plots in the Sandhills region of North Carolina. Four blocks of 10 trees per treatment were sampled on five dates from May to November 1995. Phloem nitrogen and potassium concentrations were elevated in trees on fertilized plots, whereas phloem calcium concentrations were decreased. Fertilization significantly enhanced (10–20%) concentrations of phloem phenolics and proanthocyanidins. In contrast, phloem soluble sugars and starch concentrations were up to 30% lower in fertilized trees than in control trees. Increased phenolic concentrations and lower nonstructural carbohydrates should correlate with reduced tissue palatability and decreased pathogen susceptibility in fertilized trees; however, resin flows were significantly lower (30–100%) in fertilized trees compared with control trees, which may facilitate pine bark beetle establishment. Furthermore, fertilization-induced increases in phloem nitrogen concentration may be more important than tissue carbohydrate or phenolic content in determining tissue palatability.

Keywords: carbohydrates, defense, *Dendroctonus frontalis*, nitrogen, nutrition, *Ophiostoma minus*, phenolics, *Pinus taeda*, proanthocyanidins, secondary compounds, southern pine beetle.

Introduction

Improving the nutrition of coniferous forests in the southeastern USA typically results in higher productivity (Allen et al. 1990). Fertilization of loblolly pine (*Pinus taeda* L.) plantation forests in the southeastern USA has become commonplace with over 350,000 hectares fertilized in 1997 (NCSFNC 1998). The nutritional balance of these stands may be important in determining host–pathogen relations.

Of the pathogens affecting pine forests in the southeastern USA, bark beetles cause the most extensive damage to forests and productivity. The most destructive of the southeastern coniferous bark beetles, the southern pine beetle (*Dendroctonus frontalis* Zimm.), along with its fungal symbiote (*Ophiostoma minus* (Hedge.) H. and P. Sydow.), thins the pine forests of diseased or weakened trees. Under epidemic populations, however, the beetles devastate entire stands of southern pines, regardless of individual tree or stand vigor (Thatcher et al. 1980). The two primary defensive mechanisms of the tree to the beetle–fungal complex are the constitutive oleoresin system and the induced defensive response (Paine et al. 1997). Differences in the quantity or quality of these constitutive or induced secondary compounds influence pathogen success.

Mineral nutrient additions may affect tissue chemistry of conifers resulting in altered pathogen relations. Increased infection of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) by *Armillaria ostoyae* (Romagn.) Herink occurred after fertilization with nitrogen (Entry et al. 1991a). The root bark of the fertilized trees had lower concentrations of potentially inhibitory compounds (proanthocyanidins, other soluble phenolics and lignin) and higher nitrogen concentrations than thinned or control trees, resulting in a more palatable tissue (Entry et al. 1991a, Entry et al. 1991b). Additionally, conifer species with increased susceptibility to *A. ostoyae* generally have fewer phenolic compounds and more sugar in root bark (Entry et al. 1992), suggesting that the ratio of available sugars to phenolic compounds may be related to the success of the fungal infection.

Carbon is used for growth only when sufficient resources (e.g., mineral nutrients and water) exist to support the structural development and physiological activity of additional tissue, otherwise the carbon is stored or allocated to secondary metabolism (Tuomi et al. 1988). Compared with unfertilized trees, fertilized trees have larger growth sinks. Thus, more of the available carbon may be used for growth in fertilized trees, resulting in less carbon available for production of secondary metabolites than in unfertilized trees (Bryant et al. 1983, Lorio 1986, Herms and Mattson 1992, but see Iason and Hester 1993), which could consequently affect plant defense.

Nutrient availability may affect not only internal carbon relationships, but also the timing of seasonal changes in host physiology (Lorio 1993), leading to temporal changes in reproduction, growth partitioning or defensive characteristics. For example, a negative correlation was reported between resin duct density and growth rate in loblolly pine (Blanche et al. 1992). Additionally, because most ducts are formed in latewood, a delay in the transition from earlywood to latewood could result in fewer new ducts being created.

Loblolly pine has been extensively studied and widely planted across the Coastal Plain and Piedmont of North Carolina. It responds well to different silvicultural techniques, including fertilizer application and exhibits high productivity on a variety of sites (Allen et al. 1990). Addition of mineral nutrients to forests may affect tissue phytochemical composition or oleoresin production, thereby changing the phenotypic response of individual trees that could impact the success of pathogen attacks. The objective of this study was to elucidate constitutive tree resistance mechanisms to bark beetle attack. We hypothesized that biochemical and defense-related changes would occur following fertilization of loblolly pine trees. We quantified the oleoresin flow rate, phloem nutrient concentrations, and phloem nonstructural carbohydrates and phenolic compounds in fertilized and control plots of loblolly pine trees during the 1995 growing season. We discuss our results in terms of their potential importance to plant-pathogen relations.

Methods

Site description

This study forms part of a larger project designed to examine the effects of water and nutrients on various physiological and growth processes in a loblolly pine stand in the Sandhills region of southeastern NC (35° N, 79° W). The soil at the study site is a Wakulla series (sandy, siliceous, thermic Psammentic Hapludult), very infertile, well drained with a low water holding capacity: \approx 12–14 cm in a 2-m profile. Annual rainfall is \approx 121 cm and mean annual temperature is 17 °C. Mean winter and summer temperatures are 9 and 26 °C, respectively. During the study period, mean temperature was 22 °C with 89 cm of rainfall. In general, precipitation occurred throughout the growing period, although there was a three-week drought beginning in late July. The first frost (-0.3 °C) occurred in late October 1995, and the first hard freeze (-5.0 °C) occurred in early November.

Study design

The study was established in 1992 as a randomized complete block design consisting of four blocks with two treatments: no fertilization (control) or fertilization to maintain targeted foliar mineral concentrations (fertilized). The treatment plots were 50 \times 50 m (0.25 ha) with internal measurement plots of 30 \times 30 m (0.09 ha). For the fertilized plots, nutrient additions were based on monthly foliar nutrient analyses. Targeted winter nitrogen (N) concentrations from foliage of a terminal branch originating in the upper third of dominant trees have been

1.3–1.4%, with ratios of: phosphorus (P):N of 0.10; potassium (K):N of 0.35; calcium (Ca):N of 0.12; and magnesium (Mg):N of 0.06 (Valentine and Allen 1990). Boron (B) has also been added to maintain the estimated critical concentration of 10–12 ppm for loblolly pine (Stone 1990). Initial treatments included ground application of approximately 225 kg ha⁻¹ N, 56 kg ha⁻¹ K, 112 kg ha⁻¹ P, 135 kg ha⁻¹ Ca, 56 kg ha⁻¹ Mg and 1.7 kg ha⁻¹ B. As of May 1995, eight additional fertilizer treatments (ground and foliar) were added: 250 kg ha⁻¹ N, 78 kg ha⁻¹ P, 169 kg ha⁻¹ K, 24 kg ha⁻¹ Ca, 56 kg ha⁻¹ Mg and 1.1 kg ha⁻¹ B. The primary nutrient limitations at study establishment were believed to have been N, K and possibly B based on critical concentrations proposed by Allen (1987). A detailed description of the study site and treatments is provided in Albaugh et al. (1998).

This study was conducted during the fourth year of treatment imposition when the stand was 11 years old (1995) and had a mean density of 1260 trees ha⁻¹. In April 1995, ten dominant or codominant trees per sample plot were selected for measurement from the treated areas outside the measurement plots based on the apparent good health and diameter of the trees. Mean initial height of the study trees was 6.88 m (\pm 0.08) on the control plots and 7.35 m (\pm 0.07) on the fertilized plots. Study trees ranged in diameter from 11.5–14.5 cm (12.7 ± 0.1 , $n = 40$) for control plots and 13.5–16.5 cm (14.4 ± 0.1 , $n = 40$) for treated plots in April 1995. These diameter ranges represent the largest 20–25% of trees on each plot, excluding the largest (< 1%) of the trees. In May 1995, trees were rasped with a file at breast height (1.3 m) to remove loose outer bark and diameter was measured bimonthly within the groove. Monthly measurements consisted of resin flow rates and phloem soluble sugar, starch, total phenolic and proanthocyanidin concentrations. Phloem mineral nutrient concentrations (N, P, K, Ca and Mg) were quantified monthly by plot.

Resin flow

Resin flow was measured approximately every five weeks beginning in May 1995 and continuing through early November 1995. Resin flow rates were determined by a modification of the method described by Lorio (1994). After shaving the outer bark at a height of 0.5 m, two samples (N and S aspects) were collected from each of 10 trees per plot. A sterilized (95% ethanol) square punch (a metal tube 1.0 cm² in area), positioned so that one corner of the square pointed down, was tapped into the phloem to the xylem surface. When removed, the punch retained a 1.0-cm² square portion of the outer bark and phloem. The phloem tissue from each bark sample was peeled from the outer bark and kept for biochemical and nutritional analysis as described below. A V-shaped piece of aluminum was fastened into the bottom of each punch hole with pins inserted into the adjacent bark. A small amount of silicon was placed underneath the aluminum trough as it was fastened to the tree to prevent seepage of resin. A 14-ml centrifuge tube was attached below the trough to collect the resin. Vials were collected and weighed daily after installation. The resin was taken from the xylem-phloem interface, because

this is the area of beetle colonization and correlates closely to the actual flow realized by the pathogen (Lorio 1994). Subsequent samples were taken above, and to the right 3–8 cm (dependent on branches) to prevent disturbance from previous samples.

Biochemical analysis

The two phloem tissue samples, composited by tree, were frozen on dry ice in the field and stored at -20°C for later lyophilization. Dried samples were ground in a small electric mill to pass a 0.5-mm mesh screen and stored under vacuum at -20°C until analyzed for soluble sugars, starch, total phenolics, proanthocyanidins and nutrients.

Carbohydrates Soluble sugars were extracted from the ground phloem tissue as described by Schoeneberger et al. (National Agroforestry Center, Lincoln, NE, pers. comm.) (see also Warren 1996), as modified from Jones et al. (1977). Approximately 25 mg of the ground samples were extracted twice with 80% ethanol at 80°C for 3 min, mixed and centrifuged. The supernatant (soluble sugars) and pellet (starch) were kept at $< 0^{\circ}\text{C}$ until analysis. Phenolics and other interfering compounds were precipitated from the supernatant with a mixture of lead acetate, sodium carbonate and HCl.

Soluble sugars (primarily glucose and fructose) were quantified by means of a hexokinase assay (Schoeneberger et al. 1992) and absorbency was recorded on a Bechman spectrophotometer at 340 nm. Total soluble sugars (almost entirely sucrose, glucose and fructose) were quantified by means of a coupled hexokinase + invertase assay (Schoeneberger et al. 1992). Glucose and sucrose stock solutions were used to prepare standard curves and results of these assays were expressed as mg glucose g^{-1} dry mass of tissue (g_{dm}).

Starch was analyzed enzymatically as described by Schoeneberger et al. (pers. comm.) (see also Warren 1996). The sample pellet was hydrolyzed with KOH, digested with an amyloglucosidase solution (Schoeneberger et al. 1992), and the resulting sugars were quantified by means of the hexokinase assay. Results were expressed as mg glucose $\text{g}_{\text{dm}}^{-1}$ of phloem tissue. Addition of the calculated values of total soluble sugars and starch provided a measure of total nonstructural carbohydrates (TNC) in mg glucose $\text{g}_{\text{dm}}^{-1}$ of phloem tissue.

To maintain accuracy among runs, the phloem tissue was ground, weighed and analyzed by block. An in-house standard of finely ground pine foliage tissue was included with each run to monitor standard and enzyme uniformity among assays. Within-run variability was assessed by including replicates of 15% of the samples. Where replicates differed from the mean by more than 5%, the run was repeated.

Phenolics and proanthocyanidins Phenolics were extracted from a 40-mg sample of ground phloem tissue. The tissue was extracted three times with 70% acetone; 1.25 ml for the first extraction and 1 ml for each additional extraction. Each sample was extracted for 10 min with periodic mixing each time. Insoluble material was pelleted by centrifugation; the supernatants were composited by sample and stored in the dark at 4°C until analyzed for total phenolics and proanthocyanidins (Booker et al. 1996).

Total phenolic concentrations in the phloem were determined by the Folin-Ciocalteu method (Singleton and Rossi 1965) as modified by Booker et al. (1996). After reacting the samples with Folin-Ciocalteu reagent (Sigma Chemical Co., St. Louis, MO) and NaCO_3 for 60 min, absorbency at 724 nm was measured with a Hewlett Packard Model 8452A diode array spectrophotometer (Hewlett Packard Co., Palo Alto, CA). Catechin and phenol were used to prepare standard curves and assay results were expressed as catechin equivalents or phenol equivalents.

Proanthocyanidins (condensed tannins) and their monomeric components in the phloem extracts were estimated with acidified vanillin (Broadhurst and Jones 1978). Freshly prepared vanillin solution (4% (w/v) in methanol) and concentrated HCl were added to the samples, incubated for 15 min and absorbency measured at 500 nm (Booker et al. 1996). Proanthocyanidins purified from loblolly pine phloem were used to produce a standard curve, and results were expressed as mg purified phloem proanthocyanidins $\text{g}_{\text{dm}}^{-1}$ of phloem tissue.

The procedure for the purification of proanthocyanidins from loblolly pine phloem was adapted from Czochanska et al. (1980), Karchesy and Hemingway (1980) and Booker et al. (1996). Two 5-g freeze-dried and ground composite phloem samples of fertilized and control trees from the study site were extracted three times with 25 ml of 70% acetone containing 0.1% (w/v) ascorbic acid, and the supernatants combined. After filtering (0.4 μm), the acetone was evaporated from the soluble fraction at 30°C under reduced pressure in a rotary evaporator. The remaining aqueous solution was then extracted twice with 50 ml of diethyl ether and three times with ethyl acetate. The aqueous fraction was reduced in volume in a rotary evaporator (34°C) and freeze-dried. The crude product was dissolved in 25 ml of 50% (v/v) methanol:water and 10 ml was applied to a 1.5×10 cm column of Sephadex LH-20 (Pharmacia, Sweden) previously equilibrated with 50% methanol. The adsorbed proanthocyanidins were washed with 1.9 l of 50% methanol and the polymer was eluted with 70 ml of 50% acetone. The acetone was evaporated in a rotary evaporator (33°C) and the aqueous solution freeze-dried. The final product was a fluffy tan powder.

Purified phloem proanthocyanidins conformed to the criteria for purified proanthocyanidins used by Czochanska et al. (1980). The UV spectrum for our product ($\lambda_{\text{max}} = 202, 280$ nm) was similar to those of Booker et al. (1996) and Czochanska et al. (1980). Our product yielded an extinction coefficient ($E_{1\%} = 271$) at 500 nm for the vanillin addition product in HCl, which is within the range ($E_{1\%} = 260\text{--}280$) used by Czochanska et al. (1980) for determining acceptable polymer purity. The acid vanillin assay is sensitive to proanthocyanidins as well as their phenolic monomeric components (flavan-3-ols such as catechin) and dihydrochalcones, and remains relatively specific to these compounds. The yield of 48 mg purified proanthocyanidins per g dry mass of loblolly pine phloem was similar to the 68 mg g^{-1} reported by Tiarks et al. (1989) by a slightly different method.

Total phenolic and proanthocyanidin analyses consisted of replicate assays of every sample. If the two absorbencies were

within 10% of each other then the mean of the calculated values (using the standard curve) was taken, otherwise the assay was repeated. Variability among runs was examined by including specific samples from previous assays in the current run.

Nutrient analysis

Ground phloem tissue samples were composited each month by plot and analyzed for N, P, K, Ca, and Mg. The samples were subjected to organic matter wet-digestion in a mixture of sulfuric acid and hydrogen peroxide (Parkinson and Allen 1975). Nitrogen and P were determined colorimetrically by flow injection analysis (Lachat QuickChem System IV colorimeter, Milwaukee, WI). Potassium, Ca and Mg were determined by emission or absorption spectrophotometry with a Perkin-Elmer (Norwall, CT) 560 atomic absorption spectrophotometer.

Statistical analysis

Effects of nutrient availability on phloem phytochemistry were evaluated by analysis of variance (ANOVA) using the SAS statistical software package (SAS Institute, Cary, NC). Analyses were performed for each of the measured variables by tree:

total resin flow weight, total soluble sugars, glucose, starch, total phenolics, proanthocyanidins and phloem nutrient concentrations (by plot mean) of N, P, K, Ca and Mg. A split-plot ANOVA with fertilization as a main effect and month as a subplot effect was used to test for temporal shifts in treatment effects for each of the variables. A Pearson correlation analysis was used to describe relationships among the variables through time.

Results

The ANOVA revealed that treatment, not tree size, was responsible for differences in the measured phytochemical parameters. There were no significant correlations between tree diameter and any of the measured dependent variables.

Significantly higher N and K concentrations and significantly lower Ca concentrations were found in phloem tissues of fertilized trees compared with control trees at every sampling date, whereas phloem P concentrations were significantly higher only in July and November (Table 1, Figure 1). In phloem tissues of fertilized trees, N and K concentrations were about 0.5%, whereas P concentrations were about 0.06–0.09%. The phloem Ca concentration of fertilized trees was less than 0.4% compared with a concentration of up to 0.7%

Table 1. Probability of treatment differences in phloem tissue phytochemistry in loblolly pine trees from fertilized and control plots. Analysis of variance *F*-statistics of variables tested: (a) resin flow, phloem sugars, starch, phenolics and proanthocyanidins, and (b) phloem nutrients: N, P, K, Ca and Mg. Tissues were sampled from May to November 1995 in the Sandhills region of North Carolina.

(a) Resin flow, phloem total sugars, starch, phenolics, proanthocyanidins

Source	df	Resin	Glucose ¹	Total sugars	Starch	Phenolics	Proanthocyanidins
Treatment ²	1	11.3** ³	2.94	8.64*	20.0***	38.2***	41.4***
Month	4	32.5***	440.0***	605.0***	997.0***	54.7***	54.3***
Treatment × Month	4	5.45**	1.55	0.31	8.44***	2.40	1.44
Block ²	3	0.01	0.69	1.64	4.47*	0.06	0.50
Treatment × Block ²	3	0.63	0.95	0.32	0.90	2.31	2.41
Month × Block	12	4.38***	1.35	5.04***	1.77	2.92**	8.46***
Treatment × Month × Block	12	0.47	0.19	1.33	0.84	1.47	1.47
Tree (Treatment × Block)	72	6.66***	1.18	3.78***	2.90***	5.97***	6.70***
Error	288						

¹ Values based on three months of data.

² *F*-test denominator = Tree (Treat × Block); all other terms tested over MS_{Error}.

³ Asterisks: * = *P* < 0.01, ** = *P* < 0.001, *** = *P* < 0.0001.

(b) Phloem nutrients: N, P, K, Ca and Mg

Source	df	Nitrogen	Phosphorus	Potassium	Calcium	Magnesium
Treatment ¹	1	136.0** ²	0.67	72.6**	24.0*	5.49
Month	4	31.5***	1.65	8.83***	7.56***	2.11
Treatment × Month	4	4.78*	0.85	0.59	2.64	3.16*
Block ¹	3	2.77	0.58	2.14	0.83	0.17
Treatment × Block	3	2.50	2.17	3.48*	12.0**	4.18*
Error	24					

¹ *F*-test denominator = Treatment × Block; all other terms tested over MS_{Error}.

² Asterisks: * = *P* < 0.05, ** = *P* < 0.005, *** = *P* < 0.0005.

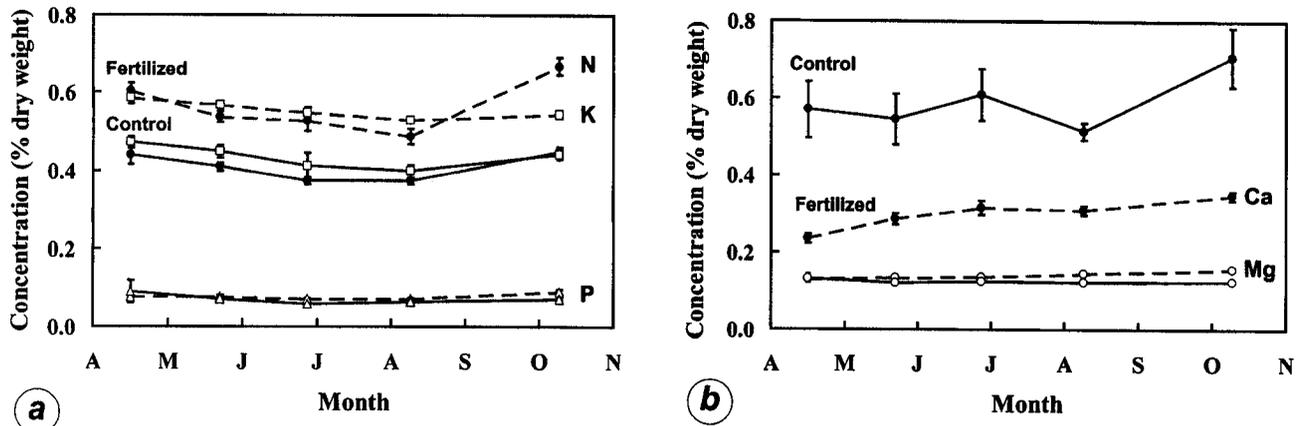


Figure 1. Nutrient concentrations in stem phloem tissues of control (solid lines) and fertilized (dashed lines) 11-year-old *Pinus taeda* L. trees: (a) nitrogen (N), potassium (K), phosphorus (P); and (b) calcium (Ca) and magnesium (Mg). Values represent means from four blocks with error bars depicting one standard error (SE) about the mean.

for control trees (Figure 1b). Phloem Mg concentrations were significantly higher in fertilized trees than in control trees on the last two sampling dates (Figure 1b). Phloem N (Figure 1a) and foliar N concentrations were positively correlated with basal area growth increment (Figure 2), which was elevated in fertilized trees.

Fertilized trees had 25–49% lower resin flow rates than control trees (Table 1, Figure 3). Resin flow rates from fertilized trees fluctuated between $0.70 \text{ g cm}^{-2} \text{ day}^{-1}$ in May to $0.85 \text{ g cm}^{-2} \text{ day}^{-1}$ in September when flows peaked (Figure 3). Resin flow rates from control trees remained constant from May to June at about $1.00 \text{ g cm}^{-2} \text{ day}^{-1}$, increased in July and peaked in September at $1.50 \text{ g cm}^{-2} \text{ day}^{-1}$ (cf. Blanche et al. 1992, Tisdale and Nebeker 1992). By November, resin flow rates in control and fertilized trees decreased to 0.46 and $0.31 \text{ g cm}^{-2} \text{ day}^{-1}$, respectively, perhaps because of increased resin viscosity at below freezing temperatures (cf. Popp et al. 1991). Resin flow in control trees was positively correlated with the concentrations of total sugars in phloem tissue (Table 2). There was also a positive correlation between resin flow and mean

temperature for the previous two weeks for both fertilized and control trees, with an especially strong relationship for the control trees.

Phloem carbohydrate concentrations varied during the study period with the fertilized and control trees following a similar seasonal pattern (Figure 4). Total nonstructural carbohydrates, the sum of total sugars and starch, declined over the season with the rate of decline diminishing in the latter months. Total soluble sugars were 3–5% lower in fertilized trees than in control trees for every sample date. In both treatments, phloem soluble sugars increased from about $120 \text{ mg glucose g}_{\text{dm}}^{-1}$ in May to $200 \text{ mg g}_{\text{dm}}^{-1}$ in September then declined to about $130 \text{ mg g}_{\text{dm}}^{-1}$ in November (cf. Hodges and Lorio 1969, Cook and Hain 1987a, Blanche et al. 1992). There were significant fertilizer effects in June and September with a month \times treatment interaction overall (Table 1).

The glucose portion of total soluble sugars was about 2% lower in fertilized trees than in control trees in May and September, and 16% lower in November. Fertilizer effects on phloem glucose concentrations were not significant (Table 1).

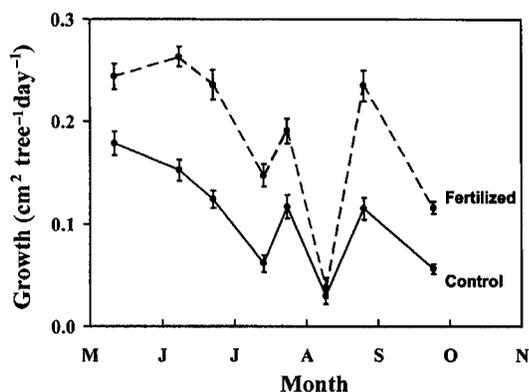


Figure 2. Basal area growth (calculated from two-week prior diameter growth) of control and fertilized *Pinus taeda* trees (\pm SE, $n = 40$).

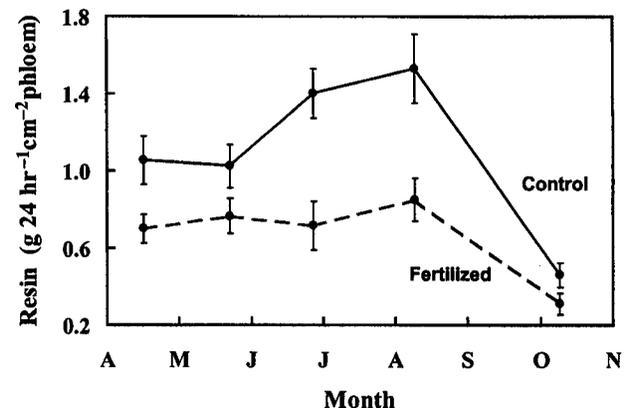


Figure 3. Oleoresin flow rate from the phloem-xylem interface in control and fertilized *Pinus taeda* trees (\pm SE, $n = 40$).

Table 2. Pearson correlation coefficients for plot means of dependent variables across treatments (total sample number is in parentheses). Similar correlations were exhibited by the variables when analyzed individually by treatment.

	Resin	Starch	Total sugars	Glucose	Phenolics
Starch	0.13 (40)	-	-	-	-
Total sugar	0.56 (40)	-0.54 (40)	-	-	-
Glucose	0.50 (16)	-0.50 (16)	0.98 (16)	-	-
Phenolics	-0.36 (40)	-0.36 (40)	-0.05 (40)	-0.16 (16)	-
Proanthocyanidins	-0.31 (40)	-0.35 (40)	-0.06 (40)	-0.24 (16)	0.91 (40)

There was a strong positive relationship between soluble sucrose and glucose concentrations (Table 2).

In both treatments, phloem starch concentrations decreased from about 150 mg glucose g_{dm}^{-1} in May to 20 mg g_{dm}^{-1} in November (Figure 4) (cf. Hodges and Lorio 1969, Blanche et al. 1992). Starch concentrations were lower in fertilized trees than in control trees from May to September (9 versus 35%), but were 4% higher in fertilized trees than in control trees in November. Significant treatment effects were found in May, June and September, and fertilizer effects were significant for the treatment \times month interaction (Table 1). Starch concentrations were negatively correlated with total soluble sugar concentrations (Table 2), with a stronger relationship in fertilized trees than in control trees.

Concentrations of total phloem phenolics were significantly higher in fertilized trees than in control trees in May and June (Table 1 and Figure 5). Concentrations ranged from about 200 to 250 mg catechin equivalents g_{dm}^{-1} (150–180 mg phenol equivalents g_{dm}^{-1}) in fertilized trees and from 170 to 200 mg catechin equivalents g_{dm}^{-1} (125–150 mg phenol equivalents g_{dm}^{-1}) in control trees. Total phloem phenolics followed a similar seasonal pattern in both treatments, although the concentrations were 13–21% higher in fertilized trees than in control trees. Lowest concentrations of phloem phenolics occurred in May, increased to a peak in July, dropped to a low in September and then increased in November. Total phenolic:total sugar ratios were higher in fertilized trees than in control trees.

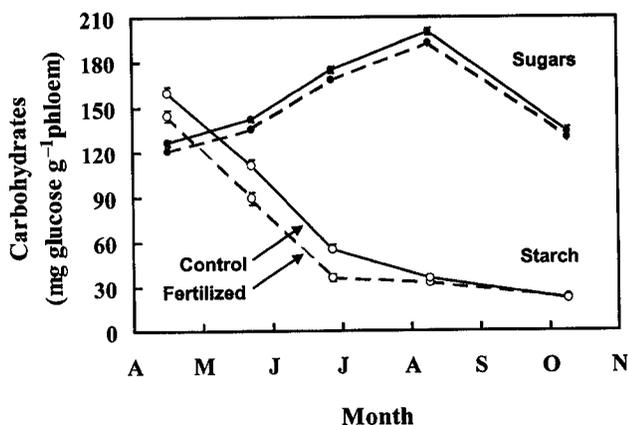


Figure 4. Carbohydrate concentrations in stem phloem tissues of control and fertilized *Pinus taeda* trees (\pm SE, $n = 40$).

Proanthocyanidins mirrored the patterns exhibited by total phenolics (Figure 5). Concentrations of phloem proanthocyanidins were 16–24% higher in fertilized trees than in control trees (190–230 versus 150–190 mg g_{dm}^{-1}), and were slightly higher than the concentrations (120 to 160 mg g_{dm}^{-1}) reported by Tiarks et al. (1989). The ratio of proanthocyanidins to total nonstructural carbohydrates was consistently higher in fertilized trees than in control trees. Treatment effects on both proanthocyanidins and total phenolics were significant with no interaction between treatment and month (Table 1). Condensed proanthocyanidins and total phenolics were highly correlated, and concentrations of both compounds exhibited slightly negative relationships with phloem starch concentrations (Table 2).

Discussion

Fertilization decreased resin flow in loblolly pine by up to 50% (Figure 3). Because resin flow is often correlated with bark beetle resistance, the fertilized trees may be more susceptible to successful penetration by beetles than the control trees. Cook and Hain (1987b) found significantly lower resin flow in shortleaf pines (*Pinus echinata* Mill.) susceptible to southern pine beetle (SPB) attack. Similarly, Hodges et al. (1979) found lower resin flows in loblolly and shortleaf pine trees exhibiting increased susceptibility to SPB. Among 36 Scots pine (*Pinus sylvestris* L.) trees attacked by the pine shoot beetle (*Tomicus piniperda* L.), the two trees that succumbed to the beetle had low vigor and the lowest resin yields (Schroeder 1990).

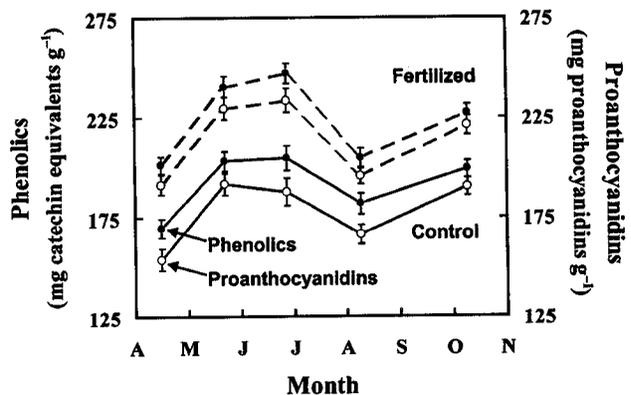


Figure 5. Concentrations of phenolics and proanthocyanidins (condensed tannins) in stem phloem tissues of control and fertilized *Pinus taeda* trees (\pm SE, $n = 40$).

Our results partially support both the growth–differentiation balance model (Lorio and Sommers 1986, Herms and Mattson 1992, Lorio 1993) and the carbon–nutrient balance model (Bryant et al. 1983, Tuomi et al. 1988). A basic premise of the growth–differentiation model is that, when growth becomes limited by moderate water stress in late summer, photosynthesis continues and excess carbohydrates are allocated to alternative carbon pools such as defensive secondary metabolism (see Ayres 1993). This hypothesis is supported by our observations of control trees which showed an increase in resin production toward the end of the growing season as precipitation declined; however, it does not explain the lack of elevated resin production in late summer in fertilized trees. Growth of the fertilized trees may not have been limited in late summer (Figure 2) if the trees experienced less water stress because of greater water-use efficiency (Laviner 1997) or uptake efficiency (even though root biomass decreased on fertilized plots (Mignano 1995, Albaugh et al. 1998)). As a result, there may have been no increase in available carbon for differentiation processes in the fertilized trees. This explanation is supported by our finding that both phloem starch and sugar concentrations appeared to be less available in fertilized trees than in control trees (Figure 4).

According to the carbon–nutrient balance model, reduced carbohydrate:nutrient ratios in the phloem of fertilized trees indicate that less carbon would be allocated to secondary compounds, as many studies have illustrated (see reviews: Herms and Mattson 1992, Ayres 1993). We found that although resin production was decreased in fertilized trees there was an increase in the production of phenolic compounds. Thus, increased nutrient availability appeared to up-regulate the shikimate acid pathway (leading to phenolics and lignin) and down-regulate the isoprenoid or mevalonic acid pathway (leading to resin and terpenes).

Because fertilization increased tree growth (Figure 2), resin duct density in cambial tissues of fertilized trees may have decreased as a result of increased cellular division, expansion and relative lack of differentiation. Blanche et al. (1992) reported significant negative correlations between both vertical and horizontal duct densities and growth rate in loblolly pine. The combined effects of fewer resin ducts and lower water or nutrient limitations may have reduced constitutive resin flow rates and buffered the seasonal pattern of resin flow in the fertilized trees.

In stems of birch (*Betula pendula* Roth.) seedlings, total soluble sugars declined in response to fertilization with N, P and K (Lavola and Julkunen-Tiitto 1994), similar to our findings in fertilized loblolly pine trees. Fungal inoculations in southern pines resulted in a reduction of inner bark soluble sugar concentrations after wounding, indicating that the fungus utilized these sugars for growth and for degradation of defensive compounds (Barras and Hodges 1969, Cook and Hain 1987a), or that the tree used this energy substrate to produce defensive compounds (Christiansen et al. 1987). Additionally, shortleaf pines susceptible to successful SPB attack contained about 10% less soluble sugars than shortleaf pines exhibiting reduced susceptibility to attack, suggesting that sugar concentrations may be related to defense (Cook and Hain

1987b). Our fertilized trees contained about 5% less available soluble sugars than control trees, possibly making them more susceptible to successful beetle attack. Lorio (1993; and citations therein) suggested that a tree's carbohydrate status determines its ability to produce compounds critical to defense against bark beetles or pathogenic fungi. In contrast, Clancy (1992) proposed that not only is host sugar a readily available form of energy for herbaceous insects, but with an adequate sugar supply, insect dietary protein can be used for growth rather than metabolized for energy.

Carbohydrates may be important for both tree defense and pathogen success. Dunn and Lorio (1993) concluded that the relationship between phloem carbohydrate status and host resistance may be more complex than simple supply and demand. The relative amounts of phenolic and isoprenoid defensive compounds, and the rate at which they can be produced from existing and translocated carbohydrates may play a critical role in the host–pathogen relationship. If the host can utilize existing carbohydrates to produce defensive compounds before the pathogen metabolizes the carbohydrates for energy, it may better survive the attack. In the fertilized trees, a stronger or energetically more expensive growth sink may account for the lower amounts of nonstructural carbohydrates initially available for production of defensive compounds (Figure 4) (Griffin et al. 1993); however, in the longer term, there may be more carbon available from other carbon pools (including new carbon acquisition) that can be allocated to a defensive role.

Fertilization increased the amount of total extractable phenolics and proanthocyanidins in loblolly pine phloem by 13–24% (Figure 5). Nebeker et al. (1993) suggests that many phenolics and tannins are less important than terpenes in the induced defensive response. The constitutive amounts of two specific stilbenes in *Picea abies* Karst. were negatively correlated with the depth of *Heterobasidion annosum* (Fr.) Bref. fungal penetration (Lindberg 1991). Although the production of these compounds may not be induced by pathogen attack (Gambiel et al. 1985), their presence may play an important role in slowing or inhibiting the beetle–pathogen complex. A similar role has been proposed for the phenolpropanoid 4-allylanisole (Hayes et al. 1994).

Fertilization affected the secondary biochemical pathways leading to decreased resin flow and increased concentrations of phenolic compounds (Figures 3 and 5). Many phenolic substances have antibiotic and allelochemical characteristics. Furthermore, many herbivores avoid consuming tissue containing phenolics, especially proanthocyanidins, in excess of their normal diet (Waterman and Mole 1994). Associated with a fertilization-induced increase in phenolics and proanthocyanidins in trees on fertilized plots, there may be a decreased energetic value and palatability of the tissue to bark beetles.

Entry et al. (1992) reported high phenolic:sugar ratios in root bark of coniferous species exhibiting reduced susceptibility to *A. ostoyae*. We found phenolic:sugar ratios in stem phloem tissue were higher in fertilized trees than in control trees (0.8–1.3 versus 0.7–1.1), perhaps indicating that the fertilized trees are more resistant to successful attack than the control trees. However, increased phloem nitrogen concentra-

tions in fertilized trees (30–50%) may compensate for the higher phenolic:sugar ratios if nitrogen is more important to herbivore success than carbohydrates or phenolic content.

In conclusion, the effects of fertilizer on the secondary biochemical pathways in loblolly pine phloem were mixed. There appeared to be increases in the activities of the shikimate acid, phenolpropanoid and flavonoid pathways (leading to increased phenolics and proanthocyanidins) with increased nutrient availability (Figure 5); however, there was a decrease in activity of the isoprenoid pathway (resulting in lower resin content) (Figure 3). Brignolas et al. (1995) found that pathogen-resistant *Picea abies* clones had higher enzymatic activity in the shikimate acid pathway, leading to increased concentrations of phenolics such as flavanols and tannins. New shoots of heather (*Calluna vulgaris* L. Hull), however, showed no significant change in total phenolics or condensed tannins in response to fertilization (Iason and Hester 1993).

Although the fertilizer treatment reduced resin yield, lowered phloem Ca, raised phloem N, K and phenolic concentrations and the phenolic:carbohydrate ratio, it is not known what effects these changes have on pathogens and so the ecological implications of widespread fertilization remain unknown. Long-term effects of added nutrients on the plantation ecosystem may affect both forest vigor and biotic interactions.

Acknowledgments

We thank Dr. Kim Ludovici for guidance and use of her laboratory for the carbohydrate analysis, Tim Albaugh for extensive help with site data and Dr. Pete Lorio for comments and discussion. This research was made possible through funding of the North Carolina State Forest Nutrition Cooperative and the USDA Forest Service Southeastern Research Station, Research Triangle Park, NC.

References

- Albaugh, T.J., H.L. Allen, P.M. Dougherty, L.W. Kress and J.S. King. 1998. Leaf area and above- and belowground growth responses of loblolly pine to nutrient and water additions. *For. Sci.* 44:317–328.
- Allen, H.L. 1987. Forest fertilizers: nutrient amendment, stand productivity and environmental impact. *J. For.* 85:37–46.
- Allen, H.L., P.M. Dougherty and R.G. Campbell. 1990. Manipulation of water and nutrients—practice and opportunity in southern US pine forests. *For. Ecol. Manag.* 30:437–453.
- Ayres, M.P. 1993. Plant defense, herbivory, and climate change. In *Biotic Interactions and Global Change*. Eds. P.M. Kareiva, J.G. Kingsolver and R.B. Huey. Sinauer Associates Inc., Sunderland, MA, pp 75–94.
- Barras, S.J. and J.D. Hodges. 1969. Carbohydrates of inner bark of *Pinus taeda* as affected by *Dendroctonus frontalis* and associated microorganisms. *Can. Entomol.* 101:489–493.
- Blanche, C.A., P.L. Lorio, Jr., R.A. Sommers, J.D. Hodges and T.E. Nebeker. 1992. Seasonal cambial growth and development of loblolly pine: xylem formation, inner bark chemistry, resin ducts, and resin flow. *For. Ecol. Manag.* 49:151–165.
- Booker, F.L., S. Anttonen and A.S. Heagle. 1996. Catechin, proanthocyanidin and lignin contents of loblolly pine (*Pinus taeda* L.) needles after chronic exposure to ozone. *New Phytol.* 132:483–492.
- Brignolas, F., B. Lacroix, F. Lieutier, D. Sauvard, A. Drouet, A. Claudot, A. Yart, A.A. Berryman and E. Christiansen. 1995. Induced response in phenolic metabolism in two Norway spruce clones after wounding and inoculations with *Ophiostoma polonicum*, a bark beetle-associated fungus. *Plant Physiol.* 109:821–827.
- Broadhurst, R.B. and W.T. Jones. 1978. Analysis of condensed tannins using acidified vanillin. *J. Sci. Food Agric.* 29:788–794.
- Bryant, J.P., F.S. Chapin, III and D.R. Klein. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.
- Christiansen, E., R.H. Waring and A.A. Berryman. 1987. Resistance of conifers to bark beetle attack: searching for general relationships. *For. Ecol. Manag.* 22:89–106.
- Clancy, K.M. 1992. The role of sugars in western spruce budworm nutritional ecology. *Ecol. Entomol.* 17:189–197.
- Cook, S.P. and F.P. Hain. 1987a. Four parameters of the wound response of loblolly and shortleaf pines to inoculation with the blue-staining fungus associated with the southern pine beetle. *Can. J. Bot.* 65:2403–2409.
- Cook, S.P. and F.P. Hain. 1987b. Susceptibility of trees to southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Scolytidae). *Environ. Entomol.* 16:9–14.
- Czochanska, Z., L.Y. Foo, R.H. Newman and L.J. Porter. 1980. Polymeric proanthocyanidins: Stereochemistry, structural units, and molecular weight. *J. Chem. Soc., Perkin Transactions I. Roy. Soc. of Chem., London*, pp 2278–2286.
- Dunn, J.P. and P.L. Lorio, Jr. 1993. Modified water regimes affect photosynthesis, xylem water potential, cambial growth, and resistance of juvenile *Pinus taeda* L. to *Dendroctonus frontalis* (Coleoptera: Scolytidae). *Environ. Entomol.* 22:948–957.
- Entry, J.A., K. Cromack, Jr., R.G. Kelsey and N.E. Martin. 1991a. Response of Douglas-fir to infection by *Armillaria ostoyae* after thinning or thinning plus fertilization. *Phytopathology* 81:682–689.
- Entry, J.A., K. Cromack, Jr., E. Hansen and R. Waring. 1991b. Response of western coniferous seedlings to infection by *Armillaria ostoyae* under limited light and nitrogen. *Phytopathology* 81:89–94.
- Entry, J.A., N.E. Martin, R.G. Kelsey and K. Cromack, Jr. 1992. Chemical constituents in root bark of five species of western conifer saplings and infection by *Armillaria ostoyae*. *Phytopathology* 82:393–397.
- Gambiel, H.A., R.G. Cates, M.K. Caffey-Moquin and T.D. Paine. 1985. Variation in the chemistry of loblolly pine in relation to infection by the blue-stain fungus. In *Proc. Integrated Pest Management Research Symposium*. USDA, For. Serv. Tech Rep. SO-56, New Orleans, 383 p.
- Griffin, K.L., R.B. Thomas and B.R. Strain. 1993. Effects of nitrogen supply and elevated carbon dioxide on construction cost in leaves of *Pinus taeda* (L.) seedlings. *Oecologia* 95:575–580.
- Hayes, J.L., B.L. Strom, L.M. Roton and L.L. Ingram, Jr. 1994. Repellent properties of the host compound 4-allylanisole to the southern pine beetle. *J. Chem. Ecol.* 20:1595–1615.
- Hermes, D.A. and W.J. Mattson. 1992. The dilemma of plants: to grow or defend. *Q. Rev. Biol.* 67:283–335.
- Hodges, J.D. and P.L. Lorio, Jr. 1969. Carbohydrate and nitrogen fractions of the inner bark of loblolly pines under moisture stress. *Can. J. Bot.* 47:1651–1657.
- Hodges, J.D., W.W. Elam, W.F. Watson and T.E. Nebeker. 1979. Oleoresin characteristics and susceptibility of four southern pines to southern pine beetle (Coleoptera: Scolytidae) attacks. *Can. Entomol.* 111:889–896.
- Iason, G.R. and A.J. Hester. 1993. The response of heather (*Calluna vulgaris*) to shade and nutrients—predictions of the carbon–nutrient balance hypothesis. *J. Ecol.* 81:75–80.

- Jones, M.G., W.H. Outlaw and O.L. Lowry. 1977. Enzymatic assay of 10^{-7} to 10^{-14} moles of sucrose in plant tissue. *Plant Physiol.* 60:379–383.
- Karchesy, J.J. and R.W. Hemingway. 1980. Loblolly pine bark polyflavanoids. *J. Agric. Food Chem.* 28:222–228.
- Laviner, M.A. 1997. Water relations and net photosynthesis of loblolly pine (*Pinus taeda* L.) in response to fertilization and irrigation. M.S. Thesis, Dept. of Forestry, North Carolina State Univ., Raleigh, NC, 31 p.
- Lavola, A. and R. Julkunen-Tiitto. 1994. The effect of elevated carbon dioxide and fertilization on primary and secondary metabolites in birch, *Betula pendula* (Roth). *Oecologia* 99:315–321.
- Lindberg, M. 1991. The resistance of *Picea abies* bark to *Heterobasidion annosum*: roles of stress, structural defense and biochemical resistance. Swedish Univ. Agric. Sci., Dept. For. Mycology and Pathology, Uppsala.
- Lorio, P.L., Jr. 1986. Growth-differentiation balance: a basis for understanding southern pine beetle–tree interactions. *For. Ecol. Manag.* 14:259–273.
- Lorio, P.L., Jr. 1993. Environmental stress and whole tree physiology. In *Beetle–Pathogen Interactions in Conifer Forests*. Eds. T.D. Schowalter and G.M. Filip. Academic Press, San Diego, CA, 252 p.
- Lorio, P.L., Jr. 1994. The relationship of oleoresin exudation pressure (or lack thereof) to flow from wounds. *J. Sustain. For.* 1:81–93.
- Lorio, P.L., Jr. and R.A. Sommers. 1986. Evidence of competition for photosynthates between growth processes and oleoresin synthesis in *Pinus taeda* L. *Tree Physiol.* 2:301–306.
- Mignano, J.R. 1995. Effects of water and nutrient availability on root biomass, necromass and production in a nine-year-old loblolly pine plantation. M.S. Thesis, Dept. of Forestry, North Carolina State Univ., Raleigh, NC, 40 p.
- NCSFNC, 1998. Twenty-seventh annual report. North Carolina State Forest Nutrition Cooperative. Department of Forestry, North Carolina State Univ. Raleigh, NC, 25 p.
- Nebeker, T.E., J.D. Hodges and C.A. Blanche. 1993. Host response to bark beetle and pathogen colonization. In *Beetle–Pathogen Interactions in Conifer Forests*. Eds. T.D. Schowalter and G.M. Filip. Academic Press, San Diego, CA, 252 p.
- Paine, T.D., K.F. Raffa and T.C. Harrington. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annu. Rev. Entomol.* 42:179–206.
- Parkinson, J.A. and S.E. Allen. 1975. A wet oxidation procedure suitable for the determination of nitrogen and mineral nutrients in biological material. *Commun. Soil Sci. Plant Anal.* 6:1–11.
- Popp, M.P., J.D. Johnson and T.L. Massey. 1991. Stimulation of resin flow in slash and loblolly pine by bark beetle vectored fungi. *Can. J. For. Res.* 21:1124–1126.
- Schroeder, L.M. 1990. Duct resin flow in Scots pine in relation to the attack of the bark beetle *Tomicus piniperda* (L.) (Col., Scolytidae). *J. Appl. Entomol.* 109:105–112.
- Singleton, V.L. and J.A. Rossi, Jr. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16:144–158.
- Stone, E.L. 1990. Boron deficiency and excess in forest trees: A review. *For. Ecol. Manag.* 37:49–76.
- Thatcher, R.C., J.L. Searcy, J.E. Coster and G.D. Hertel. 1980. The southern pine beetle. USDA, For. Serv. Tech. Bull. 1631, 266 p.
- Tiarks, A.E., J.R. Bridges, R.W. Hemingway and E. Shoulders. 1989. Condensed tannins in southern pines and their interactions with the ecosystem. In *Chemistry and Significance of Condensed Tannins*. Eds. R.W. Hemingway and J.J. Karchesy. Plenum Press, New York, NY, 553 p.
- Tisdale, R.A. and T.E. Nebeker. 1992. Resin flow as a function of height along the bole of loblolly pine. *Can. J. Bot.* 70:2509–2511.
- Tuomi, J., P. Niemelä, F.S. Chapin, III, J.P. Bryant and S. Sirén. 1988. Defensive responses of trees in relation to their carbon/nutrient balance. In *Mechanisms of Woody Plant Defenses Against Insects: Search for Pattern*. Eds. W.J. Mattson, J. Leveux and C. Bernard-Dagan. Springer-Verlag, Berlin, pp 57–72.
- Valentine, D.W. and H.L. Allen. 1990. Foliar response to fertilization identify nutrient limitation in loblolly pine. *Can. J. For. Res.* 20:144–151.
- Warren, J.M. 1996. The effect of mineral nutrition on the resin flow and phloem nonstructural carbohydrates and phenolic compounds in a loblolly pine (*Pinus taeda* L.) stand. M.S. Thesis, Dept. of Forestry, North Carolina State Univ., Raleigh, NC, 62 p.
- Waterman, P.G. and S. Mole. 1994. Analysis of plant phenolic metabolites. Blackwell Scientific Publications, New York, 238 p.