

Marcy E. Litvak · John V. H. Constable  
Russell K. Monson

## Supply and demand processes as controls over needle monoterpene synthesis and concentration in Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco]

Received: 8 August 2001 / Accepted: 26 April 2002 / Published online: 25 June 2002  
© Springer-Verlag 2002

**Abstract** We measured the relative control that resource availability (as a supply-side control) and wounding (as a demand-side control) exert on patterns of monoterpene synthesis and concentration in Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] needles. While supply-side controls should alter monoterpene production due to changes in the availability of substrate (carbohydrates), demand-side controls alter the need for a defensive product. We examined these relationships by measuring constitutive (preformed) and wound-induced rates of monoterpene synthesis and pool sizes in trees grown under ambient and elevated (ambient +200  $\mu\text{mol mol}^{-1}$ )  $\text{CO}_2$ , ambient and elevated (ambient +4°C) temperature, and in trees grown under four levels of nitrogen fertilization (0, 50, 100 and 200  $\mu\text{g g}^{-1}$  N by weight). Monoterpene pool size decreased at elevated  $\text{CO}_2$ , increased at elevated temperature and did not change in response to nitrogen fertilization. Overall, we did not find that foliar nitrogen, carbon balance, or rate of monoterpene synthesis alone were consistent predictors of monoterpene concentration in current-year Douglas fir needles. In addition, despite a wound-induced decrease in monoterpene pool size, we found no evidence for induction of monoterpene synthesis in response to wounding. The influence of either resource availability or wounding on rates of monoterpene synthesis or accumulation cannot be explained by traditional supply-side or demand-side controls. We conclude that monoterpene synthesis in first-year Douglas fir needles is controlled by fairly conserva-

tive genetic mechanisms and is influenced more by past selection than by current resource state.

**Keywords** Induced defense · Supply-demand · Elevated  $\text{CO}_2$  · Elevated temperature · Nitrogen fertilization

### Introduction

Monoterpenes are ten-carbon hydrocarbons widely distributed in the resin storage structures of nearly all conifer species (Fahn 1979; Banthorpe and Charlwood 1980). Like other secondary compounds, monoterpenes serve no known physiological role in conifer tissues, but do act as toxins and/or deterrents to a variety of fungal pathogens and herbivores (Leather et al. 1987; Duncan et al. 1994; Paine and Hanlon 1994; Klepzig et al. 1996; Vourc'h et al. 2001), and as solvents to increase the mobilization and deposition of resin acids and more toxic terpenes at the wound site. In addition, monoterpenes are emitted from plant tissues in quantities large enough to affect the chemistry of the lower troposphere, resulting in increased production of organic aerosols, transformation of reactive nitrogen species, production of CO, and in the presence of adequate nitrogen oxide concentrations, increases in tropospheric ozone (Fehsenfeld et al. 1992; Litvak et al. 1999).

Dynamics in plant tissue concentration of secondary defensive compounds like monoterpenes are often predicted using the carbon-nutrient balance hypothesis (CNB, Bryant et al. 1983; Hamilton et al. 2001) and growth-differentiation balance hypothesis (GDBH, Hodges and Lorio 1975; Herms and Mattson 1992). Central to these models is the principle that only carbon that has accumulated in excess of growth requirements can be allocated to carbon-based defenses. In a review of the growth-differentiation balance model, Lerdau et al. (1994) described the relationship between carbohydrate accumulation and monoterpene production as a *supply-side* control, and distinguished it from *demand-side* controls. While supply-side controls increase the availability of substrate (carbo-

M.E. Litvak (✉) · J.V.H. Constable · R.K. Monson  
Department of Environmental,  
Population and Organismic Biology, University of Colorado,  
Boulder, CO 80309, USA

#### Present addresses:

M.E. Litvak, University of Texas-Austin,  
Section of Integrative Biology, Bio Labs 313, Austin,  
TX 78712, USA  
e-mail: mlitvak@mail.utexas.edu

J.V.H. Constable, Slippery Rock University,  
Department of Biology, Slippery Rock, PA 16057, USA

hydrate), demand-side controls increase the need for defense (monoterpene). Thus, factors that either increase the synthesis rate (e.g., elevated CO<sub>2</sub>), or decrease the consumption rate (e.g., reduced growth due to nutrient limitations) of non-structural carbohydrates should promote monoterpene synthesis through supply-side control. In contrast, factors that increase the need for defense (e.g., current herbivory, probability of attack, value of tissue to the plant, benefit of defense) would be associated with demand-side control.

Experimental tests of the resource allocation models described above and their relevance to supply-side processes have been inconclusive. Growth at elevated CO<sub>2</sub>, and/or low nutrient availability, typically increases the amount of carbon in woody plant tissues that is in excess of that required for immediate growth (Griffin et al. 1996; Kelsey et al. 1998). Yet, monoterpene pool sizes in conifer tissues either increase (Heyworth et al. 1998), decrease (Williams et al. 1994) or do not change (Roth and Lindroth 1994; Kainulainen et al. 1998; Constable et al. 1999) in response to growth at elevated CO<sub>2</sub>. Similarly, monoterpene pools in conifer needles reportedly increase (Kainulainen et al. 1996), decrease (Bjorkman et al. 1991; McCullough and Kulman 1991), or do not change (Holopainen et al. 1995) in response to reduced growth caused by nitrogen deficiency. One reason supply-side processes alone have been unable to consistently predict dynamics in monoterpene concentration is that the nitrogen requirements for enzymatic production of monoterpenes, as well as construction of resin storage structures, make it unreasonable to consider monoterpenes solely as carbon-based defenses (Lerdau and Gershenzon 1997; Haukioja et al. 1998).

One of the more obvious demand-side controls, herbivory or wounding to simulate herbivory, has been shown to induce localized monoterpene synthesis in stem tissues and needles from a variety of conifer species (Lewinsohn et al. 1991a, b; Litvak and Monson 1998). Localized induction of monoterpene synthesis was observed by monitoring the increase in activity of monoterpene cyclases (enzymes that catalyze monoterpene synthesis) in wounded conifer tissues 4–8 days after tissue damage. Once the resin structures are ruptured in wounded tissues, stored monoterpene pools are rapidly depleted until a hardened mixture of monoterpenes and diterpenoid resin acids forms at the wound site (Litvak and Monson 1998; Loreto et al. 2000; Prieme et al. 2000). Increased biosynthesis of monoterpenes to replace depleted pools in wounded tissues is consistent with the defensive roles these compounds play in conifer tissues.

Our primary objective was to determine the relative control that supply-side and demand-side processes exert on patterns of synthesis and concentration of monoterpenes in Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] needles. We examined these relationships by measuring constitutive (preformed) and wound-induced rates of monoterpene synthesis and monoterpene pool sizes in trees grown at ambient and elevated CO<sub>2</sub>, as well

as ambient and elevated temperature (experiment 1), and in trees grown at four levels of nitrogen fertilization (experiment 2).

## Materials and methods

The Douglas fir trees used in these experiments were part of an ongoing study by the U.S. Environmental Protection Agency (EPA) on the effects of CO<sub>2</sub> and climate change on forest trees in Corvallis, Ore. (Tingey et al. 1995). Trees in experiment 1 (response to elevated CO<sub>2</sub>, elevated temperature, and wounding) were planted as bare root 2-year-old stock in sealed environmentally controlled outdoor chambers referred to as Terracosms (2 m wide × 1 m deep × 1.5 m tall) in 1993, 15 trees per chamber. These chambers received ambient light, but temperature and CO<sub>2</sub> were controlled to create four growth treatments in a full two-by-two factorial design. The two growth temperature levels were ambient and ambient +40°C. The two CO<sub>2</sub> treatments were ambient CO<sub>2</sub> and ambient + 200 μmol mol<sup>-1</sup> CO<sub>2</sub>. Twelve Terracosms total were randomly divided into the four growth treatments in 1993, three chambers per treatment. Two additional groups of 15 trees were planted as unchambered controls. For more details on the experiment see Tingey et al. (1995).

The Douglas fir trees in experiment 2 (response to variable nitrogen availability and wounding), were collected by the EPA at the same time as the trees planted in the Terracosms, but planted as bare root 2-year old stock in 13.6 l plastic pots in late June 1993. These trees were planted in the soil medium used in the chambers and remained outside adjacent to the closed-top chambers year round. The trees were randomly assigned to one of four nitrogen fertilization regimes: 0, 50, 100, and 200 μg g<sup>-1</sup> N by weight (ten trees per treatment). Nitrogen was added to the soil in each pot as 0, 2.73, 5.46 or 10.92 g NH<sub>4</sub>NO<sub>3</sub> pellets 16 months after planting, and repeated once per year for 2 years. Trees received ambient rainfall but were watered as necessary in dry periods with purified water.

### Needle measurements

The trees were 3 years old when the experiments were conducted in June 1996. On day 1 of both experiments we harvested approximately 2 g fresh mass (FM) current-year foliage from one branch per tree for pre-wound analyses from 5 trees per chamber, 3 chambers per growth treatment in experiment 1 and from 10 trees per fertilization regime in experiment 2. The foliage that was harvested for analysis of monoterpene cyclase activity and pool size concentration was separated, weighed and immediately stored in liquid nitrogen. Needles collected for total nitrogen and carbon content were dried at 70°C overnight. Foliage samples for total non-structural carbohydrate were dried at 100°C for 2 h, then overnight at 70°C.

We simulated needle damage due to insect folivores by using scissors to remove the distal half of approximately 100 needles on the same branches from which the unwounded needles were collected (Litvak and Monson 1998). After 8 days, these wounded needles were collected for monoterpene synthesis, pool size, carbon, nitrogen and starch concentration analyses as described above. We also collected unwounded needles from a similarly oriented branch on each tree to look for changes in the rates of monoterpene synthesis or pool size over the 8-day period.

### Assay for monoterpene cyclase activity

To assay monoterpene cyclase activity, we used the procedure outlined in Litvak and Monson (1998). There are multiple monoterpene cyclases that each catalyze the formation of discrete sets of monoterpenes from the substrate geranyl pyrophosphate (Croteau and Cane 1985; Savage et al. 1994). Given that radio-gas liquid

chromatography (Croteau et al. 1987) was not used in this experiment to separate and identify the labeled products, our results are indicative of the total activity of monoterpene cyclases present in the needle tissue. Protein concentrations were determined by the dye-binding method described by Bradford (1976) using the Coomassie Bio-Rad Reagent (A<sub>595</sub>).

#### Analysis of monoterpene concentration and composition

Needles for monoterpene concentration analysis were ground to a fine powder in liquid nitrogen, and stored in 20 ml of pentane to extract the monoterpenes. An internal standard, approximately 1 mg of fenchone (a terpene that is not a typical component of the Douglas fir needle monoterpene profile), was added to the pentane extractions of the tissue monoterpenes immediately after the tissue was added. Extracted monoterpenes from these liquid samples were separated on a gas chromatograph (HP5890, Series II, Hewlett-Packard) equipped with an FID detector, a split-splitless injector, and a fused silica capillary column (15 m DB-WAX, .32 mm ID, 1µm film thickness, J & W Scientific, Folsom, Calif.). Helium was used as the carrier gas at 1.5 ml/min, and the column temperature was programmed to hold at 50°C for 3 min, then increase 6°C/min to 200°C, and 15°C/min to 240°C, then held for 3 min. Injector temperature was 250°C, and detector temperature was 300°C. A 2-µl sample was injected in the split mode (80:1 split) using an autosampler (HP7673, Hewlett-Packard). Peaks were identified by comparison to the retention times of monoterpene standards (Sigma/Aldrich, St. Louis, Mo.). Peaks were quantified relative to the internal standard fenchone and expressed as milligrams terpene per milligram of needle dry weight.

#### Nonstructural carbohydrate, carbon and nitrogen analyses

Needle starch was measured on 15 mg of dried tissue following the procedure detailed in Constable et al. (1999). Carbon and ni-

trogen analyses were measured on 10–20 mg of dried tissue by the Boston University Stable Isotope Laboratory using a Heraeus carbon-nitrogen analyzer.

#### Statistical analyses

The impact of growth resource availability, wounding and their interaction on monoterpene synthesis rates and pool sizes was assessed using repeated measures ANOVA. In both experiments, growth resource availability (CO<sub>2</sub> and temperature in experiment 1 and nitrogen availability in experiment 2) was tested as the between subject source of variation and wounding as the within-subjects source. We used linear regression analysis to test for relationships between monoterpene cyclase activity and monoterpene pool size, and for relationships between both cyclase activity and pool size with carbon, nitrogen, starch, carbon/nitrogen and starch/nitrogen ratios. A general linear model procedure assessed chamber effects between the ambient temperature and CO<sub>2</sub> treatment groups (unchambered and chambered) and found no significant chamber effects on any variable. All statistics were performed on SAS Statistical Package (SAS 1991).

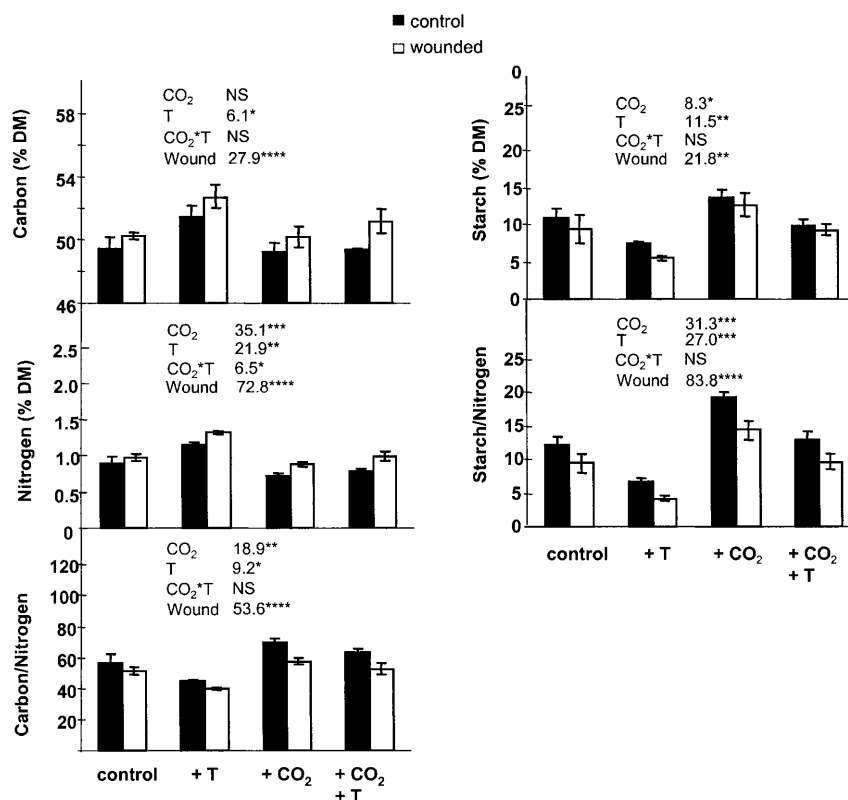
## Results

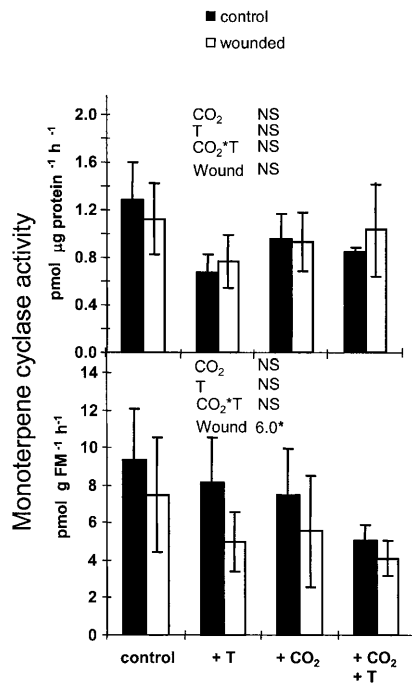
### Experiment 1 – elevated CO<sub>2</sub>, wounding and temperature

#### *Influence of CO<sub>2</sub>, temperature and wounding on needle carbon balance*

Elevated CO<sub>2</sub> and temperature both significantly altered the carbon balance of Douglas fir needles (Fig. 1). Elevated CO<sub>2</sub> alone significantly increased needle starch,

**Fig. 1** Means (± SE) of needle nitrogen, carbon and starch concentration, C/N and starch/N ratios in both un-wounded (*dark bars*) and wounded needles (*white bars*) in experiment 1. Growth treatments are ambient CO<sub>2</sub>, ambient temperature (control), ambient CO<sub>2</sub>, elevated temperature (+T), elevated CO<sub>2</sub>, ambient temperature (+CO<sub>2</sub>), and elevated CO<sub>2</sub>, elevated temperature (+CO<sub>2</sub>, +T). *F* values and significance levels from repeated measures MANOVA are given on the graph (\*= $<0.05$ , \*\*= $<0.01$ , \*\*\*= $<0.001$ , \*\*\*\*= $<0.0001$ ; *df*=1, 8 for all tests). Wound×temperature, Wound×CO<sub>2</sub>, Wound×temperature×CO<sub>2</sub> results are only given if significant





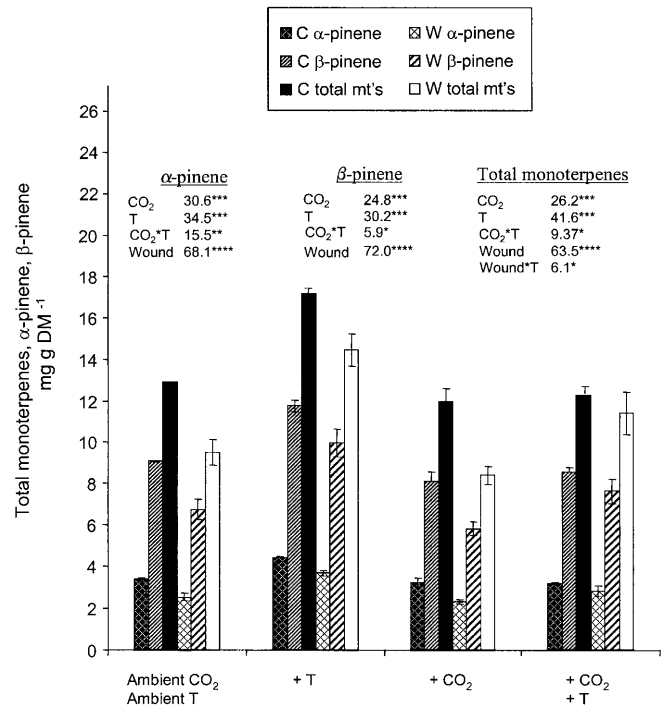
**Fig. 2** Monoterpene cyclase activity (mean values  $\pm$  SE) in both wounded (*white bars*) and unwounded needles (*dark bars*) in experiment 1 (symbols for growth treatments and significance levels explained in Fig 1 caption)

C/N ratio and starch/N, compared to needles in ambient growth conditions. In contrast, elevated temperature significantly decreased starch, C/N ratio and starch/N ratios. While needle nitrogen concentration significantly increased by 25% in elevated temperature grown trees, it significantly decreased at elevated CO<sub>2</sub>. In the combined elevated temperature and CO<sub>2</sub> chambers, elevated CO<sub>2</sub> ameliorated the increase in nitrogen observed at elevated temperature alone.

Wounding triggered an increase in C and N concentration, but a decrease in starch, C/N and starch/N (Fig. 1). Wounded needles in the elevated CO<sub>2</sub> chambers experienced the largest decrease in C/N ratios, compared to the other growth treatments. Elevated temperature did not influence any wound-induced changes in needle carbon balance.

#### *Influence of CO<sub>2</sub>, temperature and wounding on monoterpene cyclase activity*

Needle monoterpene cyclase activities ranged from 0.5 to 1.6 pmol  $\mu\text{g protein}^{-1} \text{h}^{-1}$ , and 5 to 9 pmol  $\text{g FM}^{-1} \text{h}^{-1}$ . In undamaged needles, total protein increased by 50% at elevated temperature alone ( $P=0.008$ ), but did not change in the combined elevated CO<sub>2</sub> and elevated temperature treatment (CO<sub>2</sub>:  $P=0.04$ ; CO<sub>2</sub>  $\times$  temperature interaction,  $P=0.01$ ). Despite these changes to total protein, monoterpene cyclase activity was not altered by either elevated CO<sub>2</sub> or elevated temperature (Fig. 2).



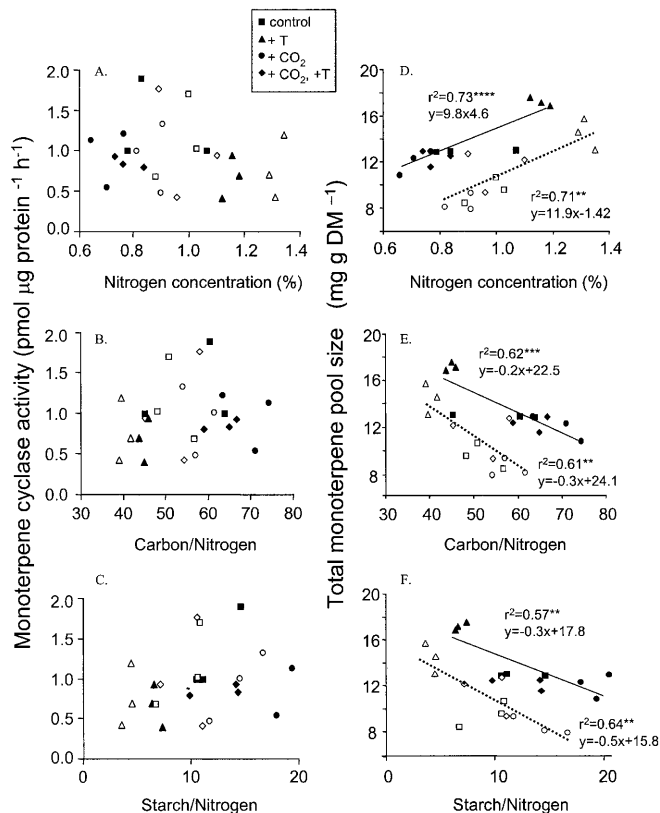
**Fig. 3** Monoterpene pool size (mean values  $\pm$  SE) in both wounded (*W*) and unwounded needles (*C*) from experiment 1 (symbols for growth treatments and significance levels explained in Fig. 1 caption)

Wounding decreased total protein levels at elevated temperature only (wound:  $P=0.15$ , wound  $\times$  temperature:  $P=0.05$ ). In all growth treatments, wounding significantly decreased cyclase activity per unit fresh mass, but did not influence cyclase activity per unit protein.

#### *Influence of CO<sub>2</sub>, temperature and wounding on monoterpene concentration and composition*

Monoterpenes accounted for 1–2 % of the total dry mass of current-year Douglas-fir needles. In order of decreasing abundance, the monoterpenes present in these tissues were  $\beta$ -pinene,  $\alpha$ -pinene, myrcene,  $\beta$ -phellandrene, terpinene and  $\delta$ -3-carene. The sum of  $\beta$ -pinene and  $\alpha$ -pinene accounted for over 90–95% of the total monoterpene concentration, thus these are the only individual compounds for which results are presented.

Both undamaged and damaged needles from the combined ambient CO<sub>2</sub> and elevated temperature treatment had the highest concentrations of  $\beta$ -pinene,  $\alpha$ -pinene and total monoterpenes (Fig. 3). At ambient CO<sub>2</sub> and elevated temperature, pool sizes increased by 30% in undamaged needles and by 50% in damaged needles compared to needles grown at ambient CO<sub>2</sub> and ambient temperature. Growth at elevated CO<sub>2</sub> alone caused significant decreases in  $\alpha$ -pinene,  $\beta$ -pinene and total monoterpenes by 5%, 11% and 7%, respectively, in undamaged needles, and by 8%, 12% and 14%, respectively, in damaged needles. We observed a significant temperature  $\times$



**Fig. 4** Relationships between monoterpene cyclase activity and pool size to needle nitrogen (A, D), C/N ratio (B, E) and starch/N ratio (C, F) in experiment 1. The four growth treatments are ambient CO<sub>2</sub>, ambient temperature (square), elevated temperature (triangle), elevated CO<sub>2</sub> (circle), combined elevated CO<sub>2</sub>, elevated temperature (diamond)

CO<sub>2</sub> interaction such that elevated CO<sub>2</sub> ameliorated the increase in pool size observed at elevated temperature.

Eight days after wounding, damaged needles across growth treatments had 20% lower total monoterpene,  $\alpha$ -pinene, and  $\beta$ -pinene pool sizes than undamaged needles (Fig. 3). Wounded needles in the elevated temperature chambers retained the largest fraction of pre-wound monoterpene pools. Wounding did not consistently alter pools of  $\beta$ -phellandrene or other minor constituents of the terpene pool (data not shown).

#### *Relationships between cyclase activity, monoterpene concentration, and carbon balance variables*

Monoterpene cyclase activity did not consistently respond to changes in needle nitrogen, C/N or starch/N ratio in either undamaged or damaged needles (Fig. 4). With data from all growth treatments combined, total monoterpene pool size in both undamaged and damaged needles was positively correlated with needle nitrogen concentration, and negatively correlated with C/N and starch/N ratios. Across growth treatments, we observed no relationship between the size of the total monoterpene

pool and monoterpene cyclase activity in either unwounded or wounded needles.

#### Experiment 2 – nitrogen availability

##### *Influence of nitrogen availability and wounding on needle carbon balance*

As nitrogen availability increased across fertilization treatments, total C and N concentration in current-year Douglas fir needles increased while starch, starch/N and C/N ratios decreased (Fig. 5). Eight days after needle damage, wounded needles averaged across N fertilization treatments had 10% higher nitrogen, and 7% lower C/N ratios than undamaged needles. The wound-induced change in starch and starch/N was dependent upon the level of N fertilization. Wounding decreased needle starch and starch/N in the two lowest N fertilization treatments, but increased starch and starch/N in the two highest fertilization levels. Wounding did not alter the total carbon concentration measured in these needles.

##### *Influence of nitrogen fertilization and wounding on needle monoterpene cyclase activity and pool size*

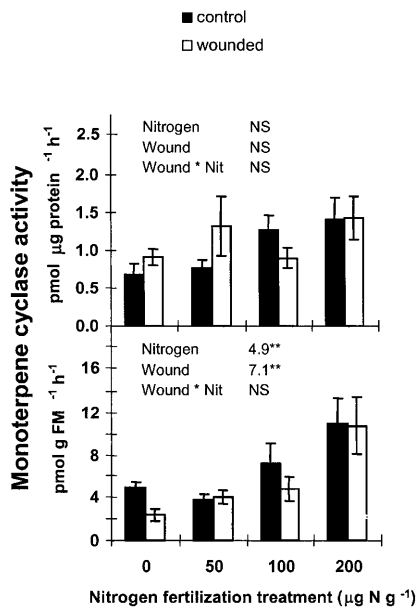
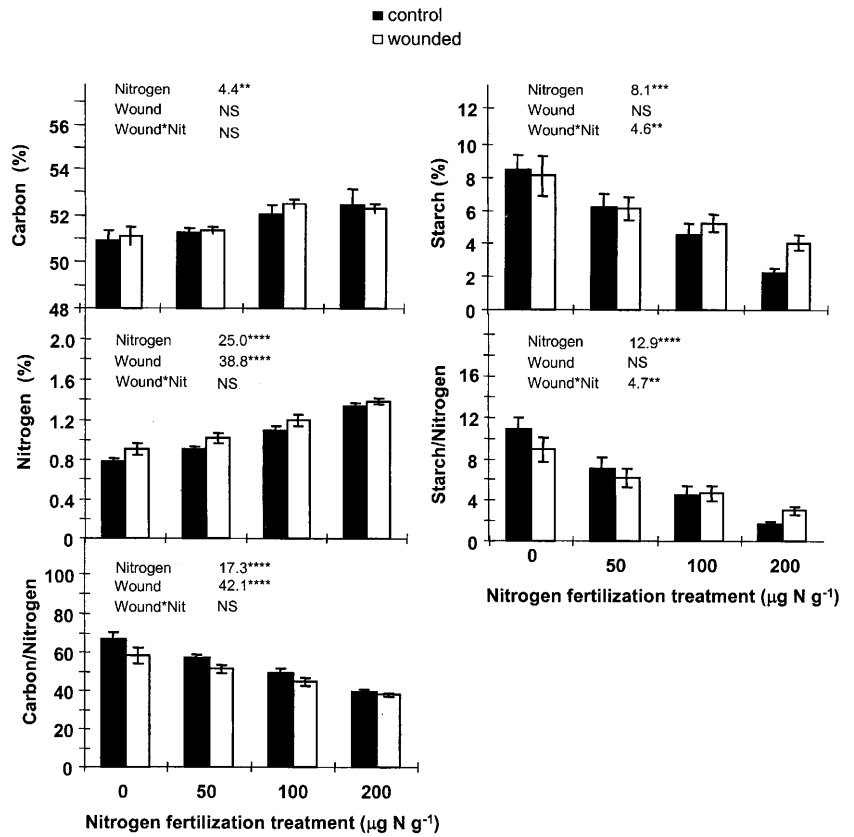
Monoterpene cyclase activity and total protein per unit fresh mass both significantly increased with N fertilization (Fig. 6; protein repeated measures ANOVA results: nitrogen  $df=3, 31, F=10.3, P<0.0001$ , wound  $df=1, 31, F=5.45, P=0.03$ , wound $\times$ nitrogen  $df=3, 31, F=1.1, P=0.4$ ). In undamaged needles cyclase activity per unit protein also significantly increased with N fertilization treatment suggesting that cyclase activity increased disproportionately more than total protein (unwounded:  $F=9.2, P=0.005, df=1, 31$ ). No significant difference in cyclase activity per unit protein was observed among the N growth treatments in wounded needles ( $F=0.8, P=0.4, df=1, 33$ ), making the overall influence of N fertilization on cyclase activity per unit protein insignificant (Fig. 6). Wounded needles across fertilization treatments had 25% lower needle cyclase activity and total protein per unit fresh mass, compared to undamaged needles (Fig. 6, see protein statistics above).

Nitrogen fertilization did not significantly alter monoterpene pool size in either unwounded or wounded needles (Fig. 7). Eight days after wounding, wounded needles across all fertilization treatments contained 20% less monoterpenes per unit dry mass than unwounded needles.

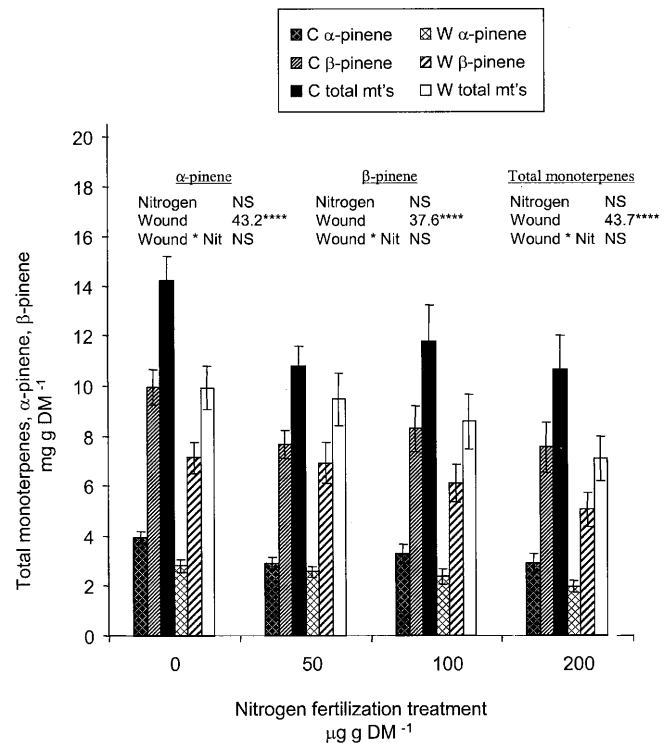
##### *Relationships between monoterpene cyclase activity, monoterpene pool size, and needle carbon balance*

Changes in total monoterpene,  $\beta$ -pinene, and  $\alpha$ -pinene concentrations in unwounded needles were independent of needle C, N, starch, C/N or starch/N changes (Fig. 8).

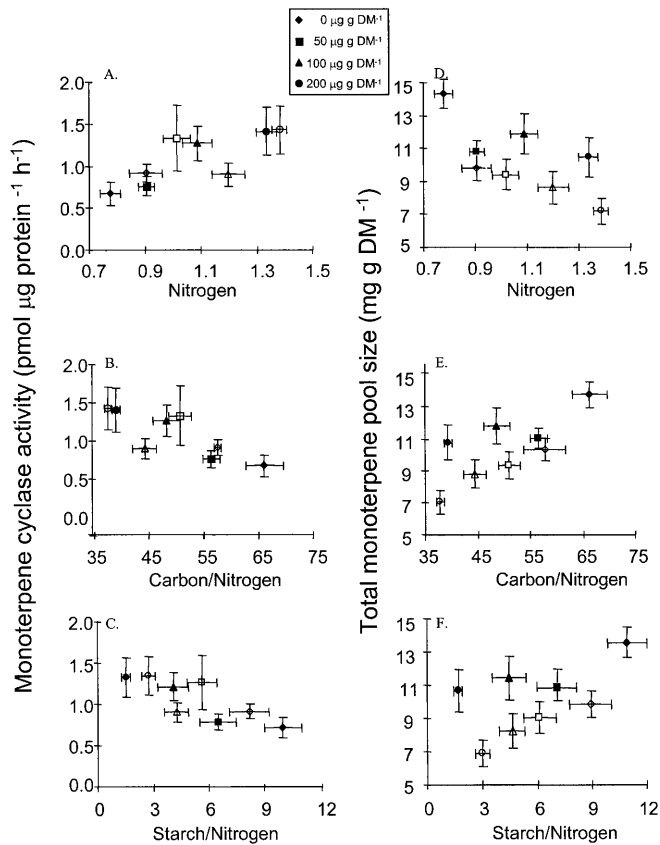
**Fig. 5** Means ( $\pm$  SE) of needle nitrogen, carbon and starch concentration, C/N and starch/N ratios in both unwounded (*dark bars*) and wounded needles (*white bars*) in experiment 2. *F*-values and statistical significance of repeated measures MANOVA analyses are reported (\*= $<0.05$ , \*\*= $<0.01$ , \*\*\*= $<0.001$ , \*\*\*\*= $<0.001$ ;  $df=3, 37$  for test of nitrogen as between subjects source of variation,  $df=1, 37$  for test of wound as within subjects source of variation, and  $df=3, 37$  for wound $\times$ nitrogen)



**Fig. 6** Monoterpene cyclase activity means ( $\pm$  SE) in current-year Douglas fir needles in both wounded (*white bars*) and unwounded needles (*dark bars*) from four different nitrogen fertilization treatments. Statistical results explained in Fig. 5 caption



**Fig. 7** Monoterpene pool size means ( $\pm$  SE) in both wounded (*W*) and unwounded needles (*C*) from four different nitrogen fertilization treatments. Statistical results explained in Fig. 5 caption

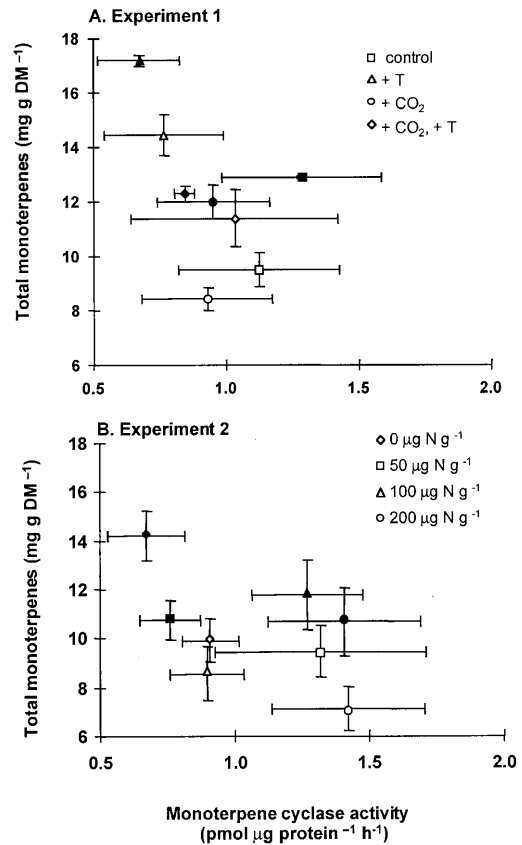


**Fig. 8.** Relationships between needle nitrogen concentration, carbon balance and monoterpene cyclase activity (A–C) and monoterpene pool size (D–F) in experiment 2. Dark symbols represent undamaged needles, and open symbols are damaged needles. Nitrogen fertilization treatments are addition of 0 (diamond), 50 (square), 100 (triangle) or 200 (circle)  $\mu\text{g g DM}^{-1}\text{ N}$

Similarly, monoterpene cyclase activity was not related to needle nitrogen or any measure of needle carbon balance (Fig. 8). We found no relationship between monoterpene pool size and cyclase activity in either unwounded or wounded needles (Fig. 9 B; unwounded:  $R^2=0.02$ ,  $P=0.46$ ; wounded:  $R^2=0.10$ ,  $P=0.51$ ).

## Discussion

The responses of monoterpene pool size to growth nitrogen availability,  $\text{CO}_2$  concentration and temperature do not support the hypothesis that carbon balance regulates monoterpene synthesis in current-year Douglas fir needles. All three growth treatments significantly altered needle starch/nitrogen ratios, an indication that these treatments were effective in altering carbon assimilation rates relative to that required for growth. As predicted and observed in other studies, starch and starch/N ratios increased at elevated  $\text{CO}_2$  (Griffin et al. 1996), but decreased at elevated temperature (Saxe et al. 2001) and with higher levels of nitrogen fertilization (Kelsey et al. 1998). If monoterpene pool size was limited solely by available carbon substrate, pool sizes should be positively



**Fig. 9** Total monoterpene pool size is not related to monoterpene cyclase activity in either experiment 1 (A, see caption for Fig. 4 for explanation of symbols) or experiment 2 (B, see caption for Fig. 8 for explanation of symbols)

correlated with starch, C/N, or starch/N ratios. While total monoterpene,  $\alpha$ -pinene and  $\beta$ -pinene concentrations decreased in response to increased C/N and starch/N ratios in experiment 1 (Fig. 4), needle monoterpene pools did not consistently respond to these measures of carbon balance in the nitrogen fertilization experiment.

Growth at elevated temperature significantly increased monoterpene pools in current-year Douglas fir needles (Fig. 3). The factor most likely to result in increased monoterpene pools is higher rates of synthesis or replacement from stored pools in other tissues relative to volatilization rates. When assayed at the same temperature, monoterpene cyclase activities were comparable between trees grown at ambient and elevated temperatures (Fig. 2). This can be thought of as a standardized rate of cyclase activity where comparable rates suggest similar concentrations of cyclase enzyme in the needle tissues. Comparable activities at the same standardized assay temperature, however, should translate into higher in situ activities for trees grown at the warmer temperature. We can predict rates of monoterpene synthesis at the higher growth temperature using Eq. 1

$$C = C_{\text{ref}} \times Q_{10}^{(T - T_{\text{ref}})/10} \quad (1)$$

where  $C_{\text{ref}}$  is the measured cyclase activity at ambient temperature  $T_{\text{ref}}$ ,  $T$  is the elevated growth temperature, and predicted  $Q_{10}$  (temperature dependence of monoterpene formation) ranges from 2–4, typical of enzyme catalyzed reactions throughout their physiological range. Using this  $Q_{10}$  range as our upper and lower limit, we calculate that rates of synthesis should increase between 32% and 74% at growth temperatures 4°C above ambient. Although monoterpene emission rates were not measured on trees grown at elevated temperature, emission rates should be 46% higher in trees grown at 4°C above ambient (Constable et al. 1999). These rough estimates of production and loss rates could explain the increased tissue monoterpene concentrations observed at elevated temperatures in the current study, depending on the actual  $Q_{10}$  for monoterpene synthesis. Increased carbon allocation to monoterpenes at elevated temperatures despite lower starch/N and C/N ratios suggests that at least under these growth conditions, starch/N and C/N ratios are not a good measure of the amount of carbon substrate available for defense. Phenotypic variation in other carbon-based secondary metabolite concentrations at elevated temperature appears to be both compound and species-specific. While elevated temperature did not affect foliar phenolic concentrations in two species of maple (Williams et al. 2000), tannin concentrations increased in English oak (*Quercus robur* L.) leaves (Dury et al. 1998). In white birch (*Betula pendula* Roth) grown at 20°C above ambient, while total foliar HPLC phenolics decreased, flavone aglycones increased (Kuokkanen et al. 2001).

Previous studies report that monoterpene pool sizes in conifer tissues either increase (Heyworth et al. 1998), decrease (Williams et al. 1994) or do not change (Roth and Lindroth 1994; Kainulainen et al. 1998) in response to growth at elevated  $\text{CO}_2$ . The reasons for the decline in monoterpene concentration in the current study at higher  $\text{CO}_2$  availability are not clear. Possible explanations include an increase in the rate of volatilization, an increase in the rate of metabolic degradation, a decrease in the rate of synthesis, or a decrease in the rate of monoterpene accumulation relative to other needle constituents. An increase in the rate of volatilization is unlikely, since Constable et al. (1999) measured monoterpene emission rates from the same trees used in this study and found no significant effect of elevated  $\text{CO}_2$ . Similarly, an increase in the rate of monoterpene degradation, or a decrease in the rate of synthesis are unlikely, since monoterpene pools in conifer needles are not subject to significant turnover (Gershenson et al. 1993), and our results reveal that monoterpene cyclase activity did not increase in response to growth at elevated  $\text{CO}_2$ . Given that elevated  $\text{CO}_2$  did not alter the dry mass per unit needle area on these trees (Constable et al. 1999), and needle starch concentrations increased, the most plausible explanation for the observed decrease in monoterpene concentration is that elevated  $\text{CO}_2$  triggered a decrease in the rate of accumulation of monoterpenes relative to other needle constituents, such as starch.

The increase in pool size that was observed at elevated temperature was cancelled by elevated  $\text{CO}_2$  in the combined elevated  $\text{CO}_2$  and temperature treatment (Fig. 3). The same trend was observed in the interactive effects of elevated  $\text{CO}_2$  and temperature on total resin droplets in white birch seedling stems (Kuokkanen et al. 2001). A 1.5–4.5°C rise in global mean temperatures is projected to accompany the anticipated doubling of atmospheric  $\text{CO}_2$  concentration in the next century (Houghton et al. 1996). Changes in plant secondary chemistry in response to combined elevated  $\text{CO}_2$  and temperature could therefore alter the nature of plant-insect interactions in the future. Although monoterpene pools responded independently to enhanced  $\text{CO}_2$  and temperature in this study, our results suggest a combined elevated  $\text{CO}_2$  and temperature environment is not likely to alter pool sizes and thus the defensive role monoterpenes play, at least not in current-year Douglas fir needles. This is consistent with other reports of no interactive effects of  $\text{CO}_2$  and temperature on foliar secondary compounds in English oak, red maple, sugar maple, and white birch (Dury et al. 1998; Williams et al. 2000; Kuokkanen et al. 2001).

Wounding resulted in no significant stimulation of monoterpene biosynthesis in Douglas fir needles as seen in the lack of increased monoterpene cyclase activity. This result contrasts with those for ponderosa pine, lodgepole pine and white fir, in which wounding by the same method induced an increase in monoterpene cyclase activity in the remaining portion of the needle (Litvak and Monson 1998). This lack of induction in Douglas fir tissues is consistent however, with the results observed by Lewinsohn et al. (1991a, b) in a survey of species-specific patterns in the relative strength of constitutive and induced monoterpene cyclase activities in conifer stems. While wounding induced cyclase activity in the cortical tissues of other coniferous species, cyclase activity in Douglas-fir stems did not change (Lewinsohn et al. 1991a, b). The lack of induction observed in Douglas fir suggests that this species relies more on constitutive than induced monoterpenes for defense. Whether the lack of an induced response is due to a lack of machinery or an actual biochemical regulation that is prohibiting the response is not clear from this study.

Both light deprivation and water stress significantly reduced constitutive and wound-induced rates of monoterpene biosynthesis in grand fir stem tissues (Lewinsohn et al. 1993). In addition, Loreto et al. (2001) measured a decrease in monoterpene cyclase activities in holm oak (*Quercus ilex* L.) leaves grown at elevated  $\text{CO}_2$ . In the current study, in addition to the likely increase in monoterpene cyclase activity at elevated temperature, rates of synthesis as measured by cyclase activity increased with nitrogen fertilization in undamaged needles (Fig. 6). The insignificant relationship between cyclase activity and foliar nitrogen in both experiments suggests the observed increase in synthesis rates in N-fertilized trees is mediated by some factor other than foliar N. At higher growth rates typical of nitrogen fertilized trees, most enzyme activities required to support

that growth should also increase. Thus, the increase in monoterpene cyclase activity in trees grown at high N availability could be interpreted as an increase in the demand for more monoterpenes to defend new tissues (Lerdau et al. 1994). Despite the increase in monoterpene synthesis however, we did not observe a significant corresponding increase in monoterpene pool size with nitrogen fertilization. Lerdau et al. (1995) reported increases in both monoterpene pool size and emission rate with nitrogen fertilization in earlier work on current-year fully expanded Douglas fir needles. Although emission rates were not directly measured on the trees in the current study, higher emission rates coupled with higher biosynthetic rates in N-fertilized trees could explain why pool sizes in these trees did not change. Any factor that alters the diffusive resistance to monoterpene flux, for example changes in needle structure in N-fertilized trees, could explain these higher emission rates (Lerdau et al. 1995). Alternative explanations are that while nitrogen fertilization increased rates of biosynthesis in current-year Douglas fir needles, it did not increase the production of storage structures (resin canals) or availability of carbon substrate for monoterpene synthesis.

Overall, we did not find that foliar nitrogen, carbon balance, or monoterpene cyclase activity alone were good predictors of the accumulation of monoterpenes in current-year Douglas fir needles. In addition, despite a wound-induced drop in monoterpene pools in wounded needles, we found no evidence for induction of monoterpene synthesis in response to wounding. Monoterpene concentration in first-year Douglas fir needles appears to be controlled by fairly conservative genetic mechanisms. These needles rely more on constitutive versus induced levels of monoterpenes for defense that are not very responsive to environmental variability and resource availability. These results suggest rates of monoterpene synthesis and accumulation in Douglas fir needles may be better explained by considering selective factors such as the probability of attack, value of the tissue to the plant and benefit of defense (McKey 1974) than by current resource state.

**Acknowledgements** The authors wish to acknowledge insightful discussions with Manuel Lerdau, Deane Bowers and Yan Linhart concerning this research. The research was supported by a NASA Global Change Fellowship to M.E.L., and NSF Research Training Grant BIR-9413218 and a DOE-NIGEC grant administered through Subcontract TUL-032-95/96 from Tulane University and the South-Central NIGEC District, both awarded to R.K.M. The views expressed in this paper do not necessarily reflect those of the U.S. Department of Energy.

## References

- Banthorpe D, Charlwood V (1980) The terpenoids. In: Bell E, Charlwood V (eds) *Encyclopedia of plant physiology*, vol 12B. Springer, Berlin Heidelberg New York, pp 185–220
- Björkman C, Larsson S, Gref R (1991) Effects of nitrogen fertilization on pine needle chemistry and sawfly performance. *Oecologia* 86:202–209
- Bradford MM (1976) A rapid and sensitive method for the quantitation of nanogram quantities of proteins using the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Bryant JP, Chapin FS, Klein D (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368
- Constable, JVH, Litvak ME, Greenberg JP, Monson RK (1999) Monoterpene emission from coniferous trees in response to elevated CO<sub>2</sub> concentration and climate warming. *Global Change Biol* 5:255–268
- Croteau R, Cane DE (1985) Monoterpene and sesquiterpene cyclases. *Methods Enzymol* 110:383–405
- Croteau R, Gurkewitz S, Johnson MA, Fisk HJ (1987) Biochemistry of oleoresinosis. Monoterpene and diterpene biosynthesis in lodgepole pine saplings infected with *Ceratocystis clavigera* treated with carbohydrate elicitors. *Plant Physiol* 85:1123–1128
- Duncan AJ, Hartley SE, Iason GR (1994) The effect of monoterpene concentrations in Sitka spruce (*Picea sitchensis*) on the browsing behavior of red deer (*Cervus elaphus*). *Can J Zool* 72:1715–1720
- Dury SJ, Good JEG, Perrins CM, Buse A, Kaye T (1998) The effects of increasing CO<sub>2</sub> and temperature on oak leaf palatability and the implications for herbivorous insects. *Global Change Biol* 4:55–61
- Fahn A (1979) *Secretory tissues in plants*. Academic Press, London
- Fehsenfeld F, Calvert J, Fall R, Goldan P, Guenther A, Hewitt CN, Lamb B, Liu S, Trainer M, Westberg H, Zimmerman P (1992) Emissions of volatile organic compounds from vegetation and the implications for atmospheric chemistry. *Global Biogeochem Cycles* 6:389–430
- Gershenson J, Murtagh JG, Croteau R (1993) Absence of rapid terpene turnover in several diverse species of terpene-accumulating plants. *Oecologia* 96:583–592
- Griffin KL, Winner WE, Strain BR (1996) Construction cost of loblolly and ponderosa pine leaves grown with varying carbon and nitrogen availability *Plant Cell Environ* 19:729–738
- Hamilton JG, Zangerl AR, DeLucia EH, Berenbaum MR (2001) The carbon-nutrient balance hypothesis: its rise and fall. *Ecol Lett* 4:86–95
- Haukioja E, Ossipov V, Koricheva J, Honkanen T, Larsson S, Lempa K (1998) Biosynthetic origin of carbon-based secondary compounds: cause of variable responses of woody plants to fertilization? *Chemoecology* 8:133–139
- Hermis DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. *Q Rev Biol* 67:283–335
- Heyworth CJ, Iason GR, Temperton V, Jarvis PG, Duncan AJ (1998) The effect of elevated CO<sub>2</sub> concentration and nutrient supply on carbon-based plant secondary metabolites in *Pinus sylvestris* L. *Oecologia* 115:344–350
- Hodges JD, Lorio PL (1975) Moisture stress and composition of xylem oleoresin in loblolly pine. *For Sci* 21:283–290
- Holopainen J, Rikala R, Kainulainen P, Oksanen J (1995) Resource partitioning to growth, storage and defence in nitrogen-fertilized Scots pine and susceptibility of the seedlings to the tarnished plant bug *Lygus rugulipennis*. *New Phytol* 131:521–532
- Houghton JT, Meiro Filho LG, Callander BA, Harris N, Kattenberg A, Makell K (eds) (1996) *Climate change 1995: the science of climate change. Contribution of Working Group I to the Second Assessment of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge
- Kainulainen P, Holopainen JK, Palomaki V, Holopainen T (1996) Effect of nitrogen fertilization on secondary chemistry and ectomycorrhizal state of Scots pine seedlings and on growth of grey pine aphid. *J Chem Ecol* 22:617–636
- Kainulainen P, Holopainen JK, Holopainen T (1998) The influence of elevated CO<sub>2</sub> and O<sub>3</sub> concentration on Scots pine needles: changes in starch and secondary metabolites over three exposure years. *Oecologia* 114:455–460

- Kelsey RG, Gladwin J, Gerson EA (1998) Ethanol synthesis, nitrogen, carbohydrates, and growth in tissues from nitrogen fertilized *Pseudotsuga menziesii* (Mirb.) Franco and *Pinus ponderosa* Dougl. Ex Laws. seedlings. *Trees* 13:103–111
- Klepzig KD, Smalley EB, Raffa KF (1996) Combined chemical defenses against an insect-fungal complex. *J Chem Ecol* 22:1367–1388
- Kuokkanen K, Julkunen-Tiito R, Keinänen M, Niemelä P, Tahvanainen J (2001) The effect of elevated CO<sub>2</sub> and temperature on the secondary chemistry of *Betula pendula* seedlings. *Trees* 15:378–384
- Leather SR, Watt AD, Forrest GI (1987) Insect-induced chemical changes in young lodgepole pine (*Pinus contorta*): the effect of previous defoliation on oviposition, growth and survival of the pine beauty moth, *Panolis flammea*. *Ecol Entomol* 12:275–281
- Lerdau M, Gershenzon J (1997) Allocation theory and chemical defense. In: Bazzaz FA, Grace J (eds) *Plant resource allocation*. Academic Press, San Diego, Calif. pp 265–277
- Lerdau MT, Litvak ME, Monson RK (1994) Supply and demand in plant chemical defense: monoterpenes and the growth-differentiation balance hypothesis. *Trends Ecol Evol* 9:58–61
- Lerdau MT, Matson P, Fall R, Monson R (1995) Ecological controls over monoterpene emissions from Douglas-fir (*Pseudotsuga menziesii*). *Ecology* 76:2640–2647
- Lewinsohn E, Gijzen M, Savage TJ, Croteau R (1991a) Defense mechanisms of conifers. Relationship of monoterpene cyclase activity to anatomical specialization and oleoresin monoterpene content. *Plant Physiol* 96:38–43
- Lewinsohn E, Gijzen M, Croteau R (1991b) Defense mechanisms of conifers. Differences in constitutive and wound-induced monoterpene biosynthesis among species. *Plant Physiol* 96:44–49
- Lewinsohn E, M Gijzen, RM Muzika, K Barton, Croteau R (1993) Oleoresinosis in grand fir (*Abies grandis*) saplings and mature trees – modulation of this wound response by light and water stresses. *Plant Physiol* 101:1021–1028
- Litvak ME, Monson RK (1998) Patterns of induced and constitutive monoterpene production in conifer needles in relation to insect herbivory. *Oecologia* 114:531–540
- Litvak ME, Madronich S, Monson RK (1999) Herbivore-induced monoterpene emissions from coniferous forests: potential impact on local tropospheric chemistry. *Ecol Appl* 9:1147–1159
- Loreto F, Nascetti P, Graverini A, Mannozi M (2000) Emission and content of monoterpenes in intact and wounded needles of the Mediterranean pine, *Pinus pinea*. *Funct Ecol* 14:589–595
- Loreto F, Fischbach RJ, Scnitzer J-P, Ciccioli P, Brancaleoni E, Calfapietra C, Seufert G (2001) Monoterpene emission and monoterpene synthase activities in the Mediterranean evergreen oak *Quercus ilex* L. grown at elevated CO<sub>2</sub> concentrations. *Global Change Biol* 7:709–717
- McCullough DG, Kulman HM (1991) Effects of nitrogen fertilization on young jack pine (*Pinus banksiana*) and on its suitability as a host for jack pine budworm (*Choristoneura pinus pinus*) Lepidoptera: Tortricidae. *Can J For Res* 21:1447–1458
- McKey D (1974) Adaptive patterns in alkaloid physiology. *Am Nat* 108:305–320
- Paine TD, Hanlon CC (1994) Influence of oleoresin constituents from *Pinus ponderosa* and *Pinus jeffreyi* on growth of mycangial fungi from *Dendroctonus ponderosae* and *Dendroctonus jeffreyi*. *J Chem Ecol* 20:2551–2563
- Priemé A, Knudsen TB, Glasius M, Christensen S (2000) Herbivory by the weevil, *Strophosoma melanogrammum*, causes several fold increase in emission of monoterpenes from young Norway spruce (*Picea abies*). *Atmos Environ* 34:711–718
- Roth SK, Lindroth RL (1994) Effects of CO<sub>2</sub>-mediated changes in paper birch and white pine chemistry on gypsy moth performance. *Oecologia* 98:133–138
- Savage TJ, Hatch MW, Croteau R (1994) Monoterpene synthases of *Pinus contorta* and related conifers: a new class of terpenoid cyclase. *J Biol Chem* 269:4010–4020
- Saxe H, MGR Cannell, O Johnsen, MG Ryan, G Vourlitis (2001) Tree and forest functioning in response to global warming. *New Phytol* 149:369–400
- Tingey DT, McVeety BD, Waschmann R, Johnson MG, Phillips DL, Rygielwicz PT, Olszyk DM (1995) A versatile sun-lit controlled-environment facility for studying plant and soil processes. *J Environ Qual* 25:614–625
- Vourc'h G, Martin J-L, Duncan P, Escarré J, Clausen TP (2001) Defensive adaptations of *Thuja plicata* to ungulate browsing: a comparative study between mainland and island populations. *Oecologia* 126:84–93
- Williams RS, Lincoln DE, Thomas RB (1994) Loblolly pine grown under elevated CO<sub>2</sub> affects early instar pine sawfly performance. *Oecologia* 98:64–71
- Williams RS, Norby RJ, Lincoln DE (2000) Effects of elevated CO<sub>2</sub> and temperature-grown red and sugar maple on gypsy moth performance. *Global Change Biol* 6:685–695