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Elevated CO_2 alters birch resistance to Lagomorpha herbivores

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Abstract

We studied the three-way interaction of elevated CO_2, nitrogen (N), and temperature (*T*), and the two-way interaction of elevated CO_2 and early-season defoliation on the secondary chemistry and resistance of Eurasian silver birch (*Betula pendula*) and North American paper birch (*B. papyrifera*) against the Eurasian hare (*Lepus timidus*) and the North American eastern cottontail rabbit (*Sylvilagus floridanus*), respectively. Elevated CO_2 decreased the palatability of winter-dormant silver and paper birch stems to both hares and rabbits, respectively. But the effect on hares was only apparent at intermediate levels of N fertilization. Elevated *T* had no effect on palatability. The effects of elevated CO_2, N, and *T* on levels of silver birch bark phenolics and terpenoids were dominated by two-way interactions between N and CO_2, and N and *T*. Generally, however, N amendments elicited a parabolic response in carbon partitioning to most biosynthetic classes of silver birch phenolics (i.e. highest concentrations occurring at intermediate N). CO_2 elevation was most enhancing at highest levels of N. On the other hand, *T* increases, more often than not, elicited reductions in phenolics, but especially so at the highest N level. In the case of *B. papyrifera*, elevated CO_2 increased carbon partitioning to Folin-Denis stem and branch phenolics and condensed tannins. Early-season defoliation, on the other hand, had no effect on phenolics and tannins but lowered both N and energy levels of branches. Elevated CO_2 substantially ameliorated the negative effects of severe defoliation on tree growth. These results support the hypothesis that continuing anthropogenic alterations of the atmosphere may trigger significant changes in plant phenotypic resistance to mammalian herbivores owing to an increasing net carbon balance between the highly vagile supply and demand capacities of plant carbon sources and sinks.

Key words: global climate change, growth-differentiation balance, phenolics, source–sink balance, tannins

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Introduction

Global mean temperatures today are warmer than at any time in the past 20 000 years and are projected to continue increasing in the future (Kattenberg *et al*., 1996; Dickson *et al*., 2000). Likewise, atmospheric carbon dioxide concentrations are higher than at anytime during the past 420 000 years and are projected to double in ca. 50–100 years (Herms 1999; Petit *et al*., 1999; Dickson *et al*., 2000). There is little doubt that impending climatic and atmospheric changes will substantially affect ecosystem processes. The myriad of effects will be both direct and indirect, and largely unpredictable because of inevitable emergent consequences in complex systems (Chapin *et al*., 2000). Some of the direct effects will be changes in abiotic factors such as available moisture, soil nutrients, heat, and light, thereby triggering changes in plant competition, community composition, distributional ranges, and historical disturbance regimes. Some important indirect effects will be mediated by phenotypic alterations in plant resistance to herbivory, and litter

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processing by a plethora of soil organisms (Hunter 2001).

The consumption of plants by herbivores and saprovores is governed by a mixture of positive (e.g. levels of primary nutrients) and negative factors (e.g. kinds and amount of secondary compounds) which altogether affect herbivore food selection, palatability, feeding, detoxication, and metabolism (Miller and Strickler 1984; Haukioja 2003). In particular, levels of secondary compounds in plants are highly responsive to environmental variation (Tuomi *et al*., 1991; Herms and Mattson 1992; Lavola and Julkunen-Tiitto 1994; Herms 2002). The growth-differentiation balance hypothesis posits that changes in the relative availability of plant carbohydrates, mediated by environmental effects on whole-plant source–sink interactions, alter carbon partitioning to secondary compounds over the course of plant ontogeny (Herms and Mattson 1992). Levels of secondary compounds tend to increase under environmental conditions that generally increase the net balance between source and sink, whether it is due to increasing source capacity (i.e. capacity to produce photoassimilates) or decreasing sink demands (i.e. capacity to utilize available photoassimilates in new growth and storage). Any environmental conditions that substantively alter the dynamic, interminable balance between growth and storage demands and photosynthesis supply can alter phenotypic plant resistance and hence ultimately the ecological trajectories of plant-herbivore interactions (Herms and Mattson 1992; Honkanen *et al*., 1994; Honkanen and Haukioja 1998).

Although there have been many studies on the effects of elevated CO_2 on the interaction between plants and their insect herbivores (see Bezemer and Jones 1998; Hunter 2001), comparable studies on mammalian herbivores are lacking altogether, even though mammals play important roles in dynamics of many ecosystems (McNaughton and Sabuni 1988; Pastor and Naiman 1992). It is likely that mammals will respond similarly though not exactly as phytophagous insects to environmentally induced changes in plant quality. For example, when confronted with typical CO_2-induced diminished plant N content, and increased allelochemical content, mammals may more readily, and more broadly than insects, seek alternative, more palatable food sources, at least until their options are exhausted. Insect larvae, being less mobile, may be obliged to directly cope *in situ* with the consequences by exercising some local microselection opportunities, increasing consumption rates, or dispersing, resulting in prolonged developmental times, and perhaps even short term developmental stasis. After food ingestion under no choice circumstances, both may be similarly physiologically challenged by the altered food stoichiometry, and secondary chemistry, causing heightened metabolism due to diet-induced thermogenesis, and elevated detoxication (Owensby *et al*., 1996; Loladze 2002; Trier and Mattson 2003).

We selected birches (*Betula* spp.) and Lagomorpha (Leporidae) herbivores (hares and rabbits) as our experimental system because both are common and important components in boreal and north temperate ecosystems. We test the basic hypothesis that elevated CO_2 generally increases plant source capacity and may, therefore, increase plant partitioning to secondary metabolites, depending on the strength of contemporaneous, highly vagile, growth and storage (sink) demands for carbon which can vary substantially with daily fluctuations in light, moisture, nutrient availability, temperature, herbivory, and a myriad of factors (Farrar and Williams 1991; Herms and Mattson 1992; Herms 2002). However, when sink strength over the growing season is minimally diminished by daily vagries in the abiotic flux, as in glasshouses where moisture, nutrients, and herbivory are controlled, evidence suggests that increasing CO_2 causes partitioning to phenolics to rise linearly to an asymptote (Lavola and Julkunen-Tiitto 1994).

Methods

There were two separate experiments, employing two plant species, Eurasian silver birch (*Betula pendula*), and North American paper birch (*B. papyrifera*), and two Lagomorpha herbivores, the Eurasian mountain hare (*Lepus timidus*) and the North American eastern cottontail rabbit (*Sylvilagus floridanus*). In the case of *B. pendula*, we studied the three-way interactions of CO_2 (increases source capacity) with elevated temperature (*T*) (may increase both source and sink capacity) and soil nitrogen (N) availability (may increase both source and sink capacity) to test their combined effects on partitioning to secondary metabolism and phenotypic resistance. In the case of *B. papyrifera*, we studied the two-way interaction of CO_2 with severe, early-season insect defoliation (D) (decreases source and sink capacity, and may be a defense elicitor) (Honkanen and Haukioja 1998).

Experiment 1: Silver birch and Eurasian hare

We tested two levels of CO_2 (362 and 700 ppm) crossed with three levels of soil N amendments (0, 150, and 500 kg ha^{-1}), and two levels of temperature (ambient (+ 0 °C) and elevated (+ 3 °C)) on silver birch resistance to European hares. The experimental venue was the Mekrijärvi Research Station, University of Joensuu

(62°47′N, 30°58′E, 145 m a.s.l.), in eastern Finland. We employed 16 closed-top chambers that were specially designed to investigate the effects of climate change on trees. The 768 1-year-old birch seedlings were randomly divided and allocated into four treatments with four replicates: (1) ambient CO_2 and T; (2) elevated CO_2 and ambient T; (3) ambient CO_2 and elevated T; and (4) elevated CO_2 and T. The 3 °C elevated temperature treatment corresponds to predicted warming after a doubling of atmospheric CO_2 (Hänninen 1995; Kellomäki and Väisänen 1997). The position of pots in a chamber was changed every week to minimize positional effects. A detailed description of the facility and regulation of the CO_2 and temperature systems are given in Kellomäki *et al*. (2000).

The plant material consisted of 1-year-old potted birch seedlings derived from seed of *B. pendula* (63°36′N, 29°43′E, 180 m a.s.l.) from Koli, Finland. The seeds were sown on June 4, 1999 in an unheated greenhouse. On June 16, the seedlings were transferred individually into 0.3 dm^3 Enso pots containing an unfertilized, commercial peat. Seedlings were randomly allocated to three different fertilization treatments (low, medium and high) to be used in each treatment chamber. Fertilization was supplied once weekly upon watering, according to the following scheme: (1) from June 5th to July 10th Superex 9 fertilizer, containing 19.4% nitrogen (N) (7.2% NO_3-N), 5.3% phosphorus (P), 20% potassium (K), 0.2% magnesium (Mg), was used; (2) from July 11th to August 3rd Superex 5 fertilizer, containing 10.9% N (9.1% NO_3-N), 4.0% P, 25.3% K, 1.5% Mg, was used; and (3) from August 4th to September 10th Superex 7 fertilizer, containing 0 N%, 6.9% P, 31.9% K, 1% Mg was used. The fertilizer amounts correspond to (0, 149 or 497 kg N ha^{-1} yr^{-1}), (0, 64.5 or 21.5 kg P ha^{-1} yr^{-1}) and (0, 4.5 or 21.5 kg potassium ha^{-1} yr^{-1}). Micronutrients were included in all fertilization. At the lowest fertilization level, the only source of nutrients was the peat itself. To ensure winter hardening, the N level was lowered near the end of the growing season, and eventually seedlings were transferred outdoors.

In mid-winter 2000, we measured the fresh weights of stems and roots (after washing). Fresh stem biomass was converted to dry weight using a sample of each stem that was oven-dried (105 °C) for 24 h, whereas roots were oven dried and weighed.

Hare feeding experiments

Feeding trials with caged hares were carried out as multiple-choice experiments during January 2000 at the Mekrijärvi Research Station. Adult hares were trapped in Punkaharju, about 130 km SW. They were not sexed and were caged separately. For these experiments, the main stems were cut to a length of 40 cm from the top part of seedlings, weighed, and tied in random order into bundles, each containing two to four shoots. The bundles varied from 5 to 13 g depending on fertilization level, but within the experimental settings the weight variation was usually less than 1 g. In each experimental cage, consisting of a four-choice arrangement, one bundle of each treatment (elevated CO_2, elevated temperature, elevated CO_2 and elevated temperature and chamber control) was placed in a random order. The different fertilization levels were tested separately.

Each bundle was fastened individually on an automatic balance system that recorded the mass losses of bundles at 8-min intervals. The trial was repeated with new bundles over three nights, each time with four to six hares, one hare per cage. Before every trial, hares fasted for two hours. Between the experimental nights, twigs of willows, birches, aspen, commercial food pellets, and snow were offered to the hares.

The palatability index (I) for each treatment was calculated as the mean losses of mass from individual bundles at 8 min intervals in each night per hare:

$$I = (3(m_1 - m_i)/m_1/\mathrm{n}) \times 100,$$

where m_1 is the mass of an individual bundle before feeding, m_i is the mass of an individual bundle after feeding, i indicates the measuring time (1, 2,…, n), and n is the number of measurement times. After each night, all 8 min intervals that had no feeding on any type of bundle were omitted from the data. Each experiment lasted about 8 h, from 23:00 to 07:00 hours. During that time the less-preferred and preferred bundles were almost always totally eaten. For that reason, feeding was considered finished when about 67% of the total mass of the feeding bundles was eaten.

Chemical analyses

At the tops of the seedlings, before feeding them to hares, we measured the main putative hare defense (Laitinen *et al*., 2002) (i.e. the number of triterpene resin droplets, mainly papyriferic acid) by using 35 mm photographic slides (ratio 1 : 1) that were made on one centimeter long sections at the middle of the fourth internode from the top.

On the basal parts of the stems, which were not fed to the hares, we peeled the fresh bark (four samples/chamber/each fertilization level), dissected it into small pieces and immediately immersed it in methanol at 4 °C for 15 min, and extracted it twice for 2 min using an Ultra-Turrax (Janke and Kunkel, Staufen, Germany)

homogenizer. In the extract, we measured the concentrations of phenolic glucosides (salidroside, betuloside, betuloside-derivative, and platyfylloside), the catechin derivatives ((+)-catechin, catechin xyloside, and epicatechin) and flavonol glycosides (hyperin, quercetin-derivative 1, quercetin-derivative 2, and kaempferol-glycoside) by high-performance liquid chromatography (HPLC/DAD), according to Kuokkanen *et al*. (2001). We calculated total HPLC-phenolics by summing all the above-mentioned compounds as well as chlorogenic acid, neochlorogenic acid and 3,4′-dihydroxypropiophenone (DHPPG). Condensed tannins (proanthocyanidins) were quantified by the butanol : HCl test, using purified tannin-equivalents from *Betula nana* (Julkunen-Tiitto 1985). We also measured the concentrations of terpenoid compounds (papyriferic acid, papyriferic acid derivative, pendulic acid 1, and pendula acid 2) by capillary gas chromatography.

Statistical analyses

The birch biomass and the concentrations of secondary compounds were tested by three-way analysis of variance. The experimental design was split-plot, where CO_2 (two levels), *T* (two levels), and N (three levels) were used as fixed factors and the chamber was treated as a random factor. After verifying the assumption of normality by evaluating the residuals via the Lilliefors test, and homogeneity of the variance via Levene's test, the main effects of CO_2, temperature, and their interaction on the palatability to hares were tested by the Wilcoxon signed rank test.

Experiment 2: Paper birch and cottontail rabbit

We tested two levels of CO_2 (ca. 362 and 562 ppm) crossed with two levels of insect defoliation (ca. 10% and 75%) on paper birch resistance to cottontail rabbits. The experimental venue was Rhinelander, Wisconsin, at the Free-Air Carbon dioxide Enrichment facility (FACE) (48°47′N, 89°58′E, 445 m a.s.l.) (see Dickson *et al*., 2000 and King *et al*., 2001 for details). Open pollinated birch (*Betula papyrifera*) seedlings, started from seed on March 7, 1997 and grown in a glasshouse, were transferred to a shade house ca. 3 months later to 6 L pots containing a potting medium of peat : sand : vermiculate (2 : 1 : 1 vol.) and a slow release Osmocote fertilizer 17-6-12 ($4\,g\,L^{-1}$). On May 18, 1998, 120 vigorous seedlings were randomly assigned to one of four 30 m wide gas exposure rings, that is, ambient or elevated CO_2 (in either replication one or two) (see Dickson *et al*., 2000 for full details of the experimental design). Seedlings were watered to saturation at least twice per week until the conclusion of the growing season in mid-October. Then seedlings were measured for diameter (at 2.5 cm above root collar) and height, and over-wintered in an adjacent root cellar on site. In spring 1999, they were transferred to 16 L pots with identical potting medium, and returned to the same treatments. Approximately mid-May, one forest tent caterpillar (*Malacosoma disstria*) egg mass containing about 200 eggs was randomly assigned and attached to half of the seedlings (in each of the four exposure rings) which were fully covered with a translucent, fine mesh sleeve cages to contain the hatchling caterpillars. Approximately 1 month later, all sleeve cages and caterpillars were removed, and to achieve defoliation parity, seedlings were further defoliated by hand (removing whole leaves), if necessary, to approximately 75% defoliation. Low defoliation 'control' seedlings were not covered with sleeve cages. Background defoliation did not exceed 10–15% by fall. In early November after leaf drop, all seedlings were moved again into the 12×16 m root cellar. Seedlings were loosely grouped together in the middle of the cellar. During the winter, an unplanned experiment occurred when two to four rabbits (estimated by their local abundance at the site) found entrance to the cellar, and thereafter fed *ad libitum* (consuming the 'bark' tissues down to and scoring the wood) on the basal third of the seedlings until April 2000 when their damage was discovered. Snowshoe hares (*Lepus americanus*) were also present at the 32 ha study site but were a minor component of the winter Lagomorpha population because controlled hunts had removed nearly all of them just prior to snowfall when they were easy to find. All seedlings were measured for diameter at 2.5 cm above root collar, amount of bark surface area removed, and the number of lower branches removed by the rabbits. Stems were excised at the root collar, divided into branches, and main stem, and then chipped, immediately oven-dried at 40 °C to constant weight, and weighed to obtain dry mass. After milling to pass through a 1 mm mesh screen, tissue samples were randomly selected and analyzed for C and N content using a Carlo-Erba NA1500 Series C/N analyzer (Thermo Electron Corporation, Milan, Italy), condensed tannins (using a quebracho tannin standard) and Folin-Denis (F-D) phenolics (using a tannic acid standard) on a Rapid-Flo Analyzer (Astoria-Pacific, Clackamas, OR 97015) after the methods of Nitao *et al*. (2001), and energy content ($kJ\,g^{-1}$) using a Parr Microbomb Calorimeter (Parr Instrument Company, Moline, IL 61265, USA). Growth in 1999 was estimated by the difference between final plant mass and initial spring 1999 mass derived from a nonlinear regression of dry mass on diameter, calculated from 40 trees sacrificed at the end of the 1998 growing season.

Statistical analyses

The Rhinelander FACE experiment is designed as a randomized block, split-plot with CO_2 main effects being tested with main plot error (Dickson *et al*., 2000). In this particular study, we employed just two CO_2 replicates because the third was inoperable at the inception of the experiment. Consequently main plot error has only one degree of freedom ((reps−1)(trts−1)) and therefore there is virtually no power for testing CO_2 effects. As an alternative, we considered each CO_2 exposure ring in each replicate a unique treatment because of the potential differences among them, thereby giving four CO_2 treatments (i.e. a completely randomized one-way ANOVA), where individual trees (20 per treatment) are the true experimental units (i.e. – 'the unit of material to which one application of a treatment is applied' (Steele and Torrie 1960). As for the four CO_2 treatments, we had also randomly assigned two defoliation treatments, thereby giving a completely randomized two-way ANOVA. Low vs. high CO_2 treatments were compared by orthogonal linear contrasts, whereas, differences among the four CO_2 treatments were compared by Tukey's HSD test (Zar 1996). All effects were considered fixed.

Results

Birch growth responses

As expected, elevated CO_2 substantially increased the above-ground woody biomass growth of both paper birch (63%) and silver birch (21%) seedlings, verifying its capacity to significantly increase net source capacity (Tables 1 and 2). In fact, elevated CO_2 stimulated paper birch to accrue ca. 31 kJ da^{-1} more than their ambient counterparts over two, ca. 140-day growing seasons. Also as expected, N fertilization robustly stimulated growth (G) of silver birch, which overall increased as power function ($G = 1/(a-b(N^c))$ of soil N amendments (Table 1, Fig. 1). However, there was a significant $CO_2 \times N$ interaction because there was a negligible growth response to elevated CO_2 at the lowest N level, and to the highest N amendment (500 kg ha^{-1}) at ambient CO_2 (Table 1). Elevated temperature had no effect on the growth of silver birch, nor did it interact significantly with either CO_2 or N (Table 1).

Severe defoliation, on the other hand, significantly reduced (ca. 23%) the biomass growth of paper birch in 1999, verifying its predicted, substantial reduction of both sink and source strengths and resulting limitations to growth (Table 2). Although there was no significant

Table 1 Mean growth (g dwt), phenolic and triterpene concentrations (mg g dwt^{-1}) and triterpene resin droplet density (no cm^{-1}) of silver birch in response to CO_2, N, and temperature (T) amendments, and the associated P values for the main effects derived from analysis of variance

Treatments					Phenolic and terpenoid compounds (mg g^{-1})						
CO_2 (ppm)	N (kg ha^{-1})	T	Growth (g)	Phen. glyc.	Catchn deriv.	Flav. glyc.	DHPPG	Total phen.	Tannin	TTR (no cm^{-1})	Terp extract.
362	0	0	1.38	25.37	4.3	0.97	0.74	31.89	48.52	21.97	7.31
362	0	3	1.98	26.19	4.1	0.39	0.78	31.45	52.17	34.38	5.88
700	0	0	1.96	29.45	5.55	0.92	0.80	36.72	63.42	29.83	12.17
700	0	3	2.14	23.73	3.73	0.39	0.63	28.49	60.31	34.43	4.83
362	150	0	3.24	32.35	5.47	0.25	0.31	38.38	100.6	28.03	1.91
362	150	3	3.11	38.31	6.49	0.13	0.41	45.34	111.2	27.83	4.62
700	150	0	4.78	32.48	5.16	0.3	0.32	38.25	126.9	33.17	2.08
700	150	3	4.67	32.43	4.82	0.17	0.24	37.66	85.57	33.15	2.24
362	500	0	3.67	18.31	4.06	0.12	0.31	22.8	58.9	32.9	2.31
362	500	3	3.06	13.26	3.27	0.06	0.35	16.94	30.9	31.62	2.3
700	500	0	5.97	26.25	6.11	0.17	0.24	32.78	106.9	38.98	2.29
700	500	3	5.56	19.25	3.86	0.07	0.23	23.4	46.14	40.88	1.56
Main effects					P values from ANOVA						
CO_2			0.02	0.50	0.75	0.74	0.15	0.56	0.17	0.01	0.67
Temperature			0.88	0.44	0.3	<0.01	0.79	0.34	0.06	0.17	0.02
N fertilization			<0.01	<0.01	0.05	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
$CO_2 \times N$			0.03	0.08	0.03	0.85	0.86	0.06	0.03	0.53	0.08
$CO_2 \times T$			0.95	0.30	0.29	0.99	0.19	0.27	0.15	0.71	<0.01
$N \times T$			0.47	0.11	0.08	<0.01	0.71	0.1	<0.01	0.03	<0.01
$CO_2 \times N \times T$			0.91	0.87	0.94	0.91	0.72	0.9	0.4	0.25	0.14

Table 2 Mean seedling growth (grams), rabbit bark consumption (cm^2), and concentrations of N, Folin-Denis phenolics, tannins, and energy content ($kJ\,g^{-1}$, $kJ\,g_N^{-1}$) of paper birch stem and branch tissues in response to CO_2 and defoliation treatments and their associated *P* values from analysis of variance

Treatment					Stem tissues					Branch tissues				
		Growth (g)		Bark										
CO_2 (ppm)	Rep	1998–1999	1999	eaten (cm^2)	N%	FD-P ($mg\,g^{-1}$)	Tannin ($mg\,g^{-1}$)	$kJ\,g^{-1}$	$kJ\,g_N^{-1}$	N %	FD-P ($mg\,g^{-1}$)	Tannin ($mg\,g^{-1}$)	$kJ\,g^{-1}$	$kJ\,g_N^{-1}$
362	1	77.7a	47.4a	98.6a	0.72a	1.5a	12.9a	19.6a	2790a	1.39a	2.6a	20.0a	20.4ab	1498a
362	2	77.5a	48.3a	91.0a	0.73a	1.7ab	15.0ab	19.7a	2792a	1.30ab	2.8ab	21.8ab	20.4ab	1598a
562	1	103.5a	72.9a	37.3b	0.65a	1.8b	15.9b	19.7a	3109a	1.19b	2.8ab	22.0ab	20.2a	1737b
562	2	149.1b	109.0b	56.1a	0.68a	1.7ab	15.9ab	19.8b	2994a	1.26ab	3.1b	24.5b	20.6b	1644ab
Defol 10		111.4a	78.6a	63.8a	0.70a	1.7a	14.7a	19.7a	2905a	1.33a	2.8a	21.9a	20.5a	1583a
Defol 75		92.4b	60.2b	78.0a	0.69a	1.7a	15.1a	19.7a	2938a	1.24b	2.8a	22.2a	20.3b	1655a
Effects							*P* values from ANOVA							
Treatment		<0.01	<0.01	<0.01	0.06	0.04	0.03	0.07	0.09	<0.01	0.03	0.01	<0.01	<0.01
Defol		0.01	0.01	0.25	0.64	0.83	0.57	0.98	0.76	0.03	0.80	0.73	<0.01	0.15
$T \times D$		0.19	0.20	0.85	0.02	0.14	0.23	0.30	0.06	0.11	0.32	0.74	0.55	0.20
Low vs. Hi CO_2		<0.01	<0.01	<0.01	0.01	0.02	0.02	0.05	0.02	<0.01	0.02	0.01	0.52	<0.01

Furthermore, *P* values for the specific linear contrast between treatments with low and elevated CO_2 treatments are shown. Within columns, treatments sharing common letters, as determined by Tukey's HSD range test, are not significantly different.

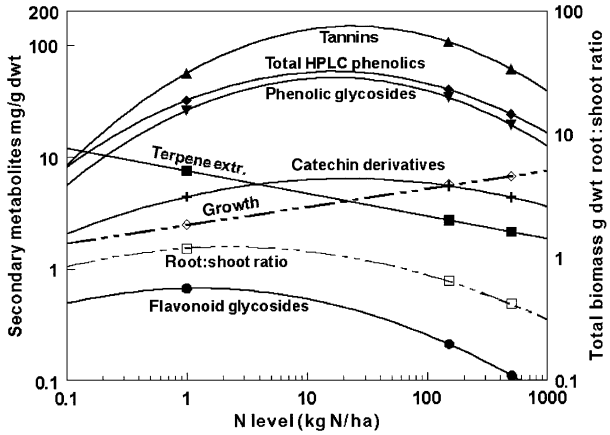

Fig. 1 Main effects of N amendments (0, 150, 500 kg N ha^{-1}) on silver birch biomass growth (g), root : shoot ratios, and concentrations of different biosynthetic classes of phenolic compounds, and extractive triterpenes ($mg\,g^{-1}$) in the bark of silver birch.

$CO_2 \times D$ interaction, severely defoliated seedlings under high CO_2 achieved nearly the same growth as their low defoliation counterparts, suggesting that elevated CO_2 almost fully ameliorated the diminished source capacity of the defoliated trees.

Partitioning to secondary metabolites

Terpenoids

High CO_2 significantly increased the triterpenoid resin (TTR) droplets on the shoot tips of silver birch at all three N fertilization levels (Table 1). There was a significant $T \times N$ effect because elevated *T* enhanced TTR at the lowest soil N level, but had no effect at the two higher levels (Table1) N fertilization stimulated a nonlinear increase in TTR, reaching an apparent maximum at the middle N level and declining slightly thereafter (Table 1).

The CO_2 effect on total extractive terpenoids in lower stem bark varied with N treatment, increasing yields at the lowest N level, but not at higher N levels (Table 1). Elevated *T*, in contrast, decreased total extractive terpene yields at the lowest N level, and then, like CO_2, had no effect at higher N levels. N fertilization effects were nonlinear and negative because extractive terpene yields decreased as a power function of N amendments (Table 1, Fig. 1).

Phenolics

The CO_2 effect on silver birch phenolics (i.e. phenolic glycosides, catechin derivatives, total HPLC phenolics and tannins) varied with the level of N fertilization (Table 1). Generally, its enhancing effect increased with N levels (Table 1, Fig. 2). N fertilization had substantial, nonlinear main effects on nearly all biosynthetic classes of silver birch bark phenolics (except DHPPG) because phenolic concentrations generally exhibited a parabolic response to N (Fig. 1), as expected from both theory and experimentation (Herms and Mattson 1992; Lavola and Julkunen-Tiitto 1994; Herms 2002). The elevated *T* effect

likewise varied with N. This interaction was mainly due to the substantial negative effect of elevated *T* at the highest, and its negligible effect at the intermediate N level (Table 1).

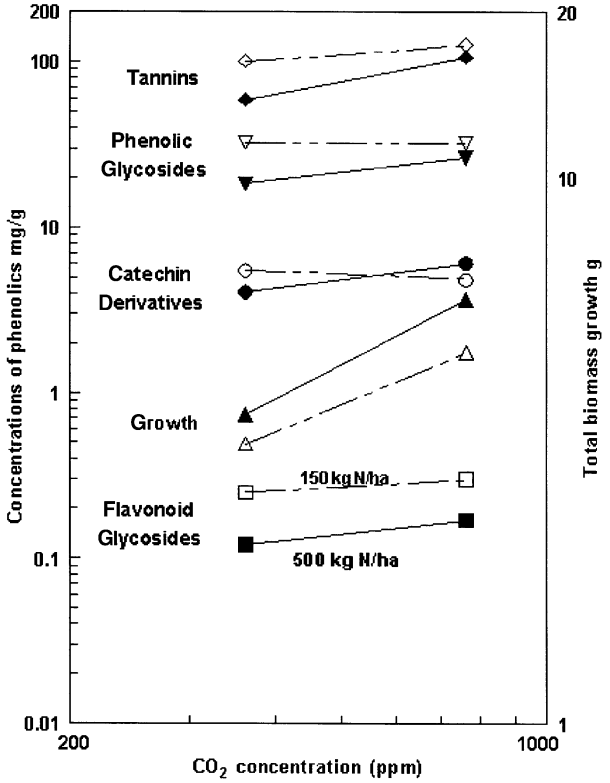

Fig. 2 Interactions between CO_2 and N amendments on concentrations of different biosynthetic classes of silver birch phenolic compounds ($mg\,g^{-1}$) and total plant biomass growth (g) at 150 (dashed lines, open markers) and 500 kg N ha^{-1} (solid lines, filled markers).

High CO_2 increased paper birch F-D phenolics (10–13%), condensed tannins (11–14%), and concomitantly reduced N concentrations (ca. 10%) in both stems and branches (Table 2). High CO_2 also increased energy content ($kJ\,g^{-1}$) of stems but not branches, and it increased energy per gram of N ($kJ\,g_N^{-1}$) in both stems and branches.

Early-season, severe defoliation, in spite of its clear, negative impact on plant source capacity, had no impact on F-D phenolic, nor tannin levels in either stems or branches. Neither did it alter N nor energy levels in stems, though it significantly lowered both N and energy levels ($kJ\,g^{-1}$) in branches. However, there was a $D \times CO_2$ effect on stem N concentrations because severe defoliation coupled to elevated CO_2 elicited no further reduction in N than either treatment alone. In brief, severe defoliation diminished N and energy levels in branches but not in the stems. High CO_2, on the other hand, increased energy levels in the stems, but not in branches.

Feeding responses

High CO_2 significantly reduced (ca. 48%) hare feeding on silver birch shoots, but only at the intermediate N level. At both the lowest and highest N levels, the CO_2 effect was insignificant (Fig. 3). Elevated temperature had no effect on hare feeding (Fig. 3), and soil N effects were not measured. High CO_2, likewise, substantially reduced (ca. 51%) rabbit feeding on winter-dormant paper birch stems (Table 2). Contrary to expectation, severe, early-summer defoliation had no significant effect on rabbit feeding, nor was there a significant $D \times CO_2$ interaction.

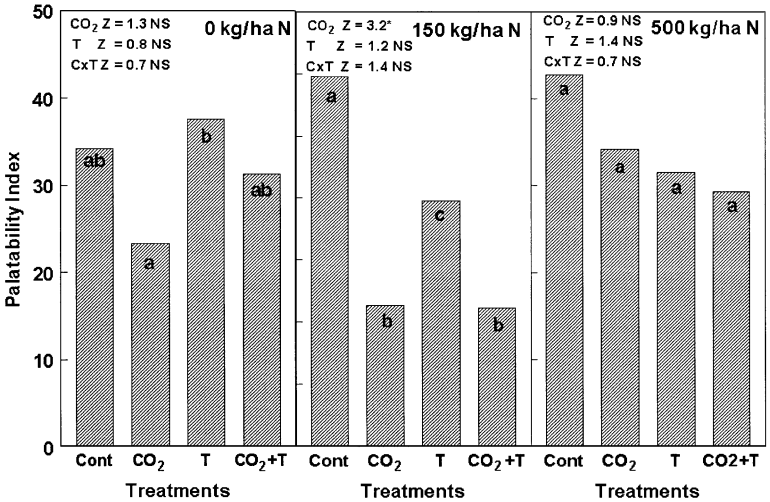

Fig. 3 European hare feeding preferences (palatability index) based on cafeteria tests, using silver birch exposed to different combinations of CO_2, temperature, and fertilizer treatments. Treatments not sharing the same letter are significantly ($P < 0.05$) different.

Discussion

Carbon partitioning to phenolics

Numerous studies have shown that elevated atmospheric CO_2 often, but not always, elicits increases in carbon partitioning to carbon-based secondary plant compounds (see Bezemer and Jones 1998; Koricheva *et al*., 1998; Hartley *et al*., 2000). Our findings confirm this general pattern in silver and paper birch. Accounting for experimental treatment and concomitant environmental effects on both plant photosynthesis (supply), and growth and storage (demand), helps to explain general but not fine scale carbon partitioning to secondary compounds (Keinanen *et al*., 1999). Increasing CO_2 typically increases net photosynthesis (Curtis and Wang 1998; Gielen and Ceulemans 2001) so that if contemporaneous growth and storage demands do not fully usurp the increasingly available carbon, more may be partitioned to secondary metabolites. In this study, high CO_2 undoubtedly increased photosynthesis in paper birch because they grew 31 kJ da^{-1} more than the controls, but yet had similar leaf areas after the first growing season (Mattson *et al*., in review). Increasing soil N can, simultaneously, enhance both photoassimilate supply (>leaves and >rates of net photosynthesis) and, likewise, general growth demands. Therefore, it has the potential for both positive and negative effects on partitioning to secondary metabolites, depending ultimately on the net balance that results from the interminable source–sink dynamics over plant ontogeny (Anttonen *et al*., 2002; Herms 2002; Glynn *et al*., 2003).

In this study, increasing soil N by 150 kg ha^{-1} above the impoverished levels in the peat potting medium undoubtedly substantially enhanced total leaf area (Anttonen *et al*., 2002) and rates of net photosynthesis, as well as whole-plant growth demands, resulting in an apparently positive source–sink balance that fostered both a doubling of plant biomass and a rise in partitioning to phenolic compounds (Table 1, Fig. 2). However, further amending soil N from 150 to 500 kg ha^{-1} enhanced biomass gain about 15%, but substantially lowered partitioning to phenolics. The likely explanation is the law of diminishing returns. The 2.3-fold increment of N elicited a smaller (or negligible) increase in net photosynthesis capacity than in the coincident growth demand, thereby altering the net source–sink balance in a direction inimical to carbon partitioning to secondary metabolites. In short, source supply did not keep up with the soaring sink demands.

To further quantify the relative differences in carbon going to total growth and total HPLC phenolics, with respect to soil N in sliver birch, we calculated and plotted the relative changes in growth (dG/G) and total HPLC phenolic mass (dTP/TP) at different N levels. We estimated the total dry weight of bark phenolics by assuming that bark mass was approximately 20% of total growth, and multiplied bark mass by its total phenolic concentrations. Because growth increased as a power function of soil N, the growth increment (dG) per unit of added N, inexorably declined with soil N ($dG/dN = bc/(N^{c*}\ (a - bN^{c})^2)$, ultimately approaching zero. On the other hand, total bark phenolics increased as parabolic function of N ($TP = k + mN - nN^2$), and, therefore, changes in total phenolic content per unit increment of N ($dTP/dN = m - 2nN$) declined linearly with rising N levels, eventually becoming negative at about 280 kg N ha^{-1}. Plotting percent changes in relative growth (dG/G) and total phenolics (dTP/TP)

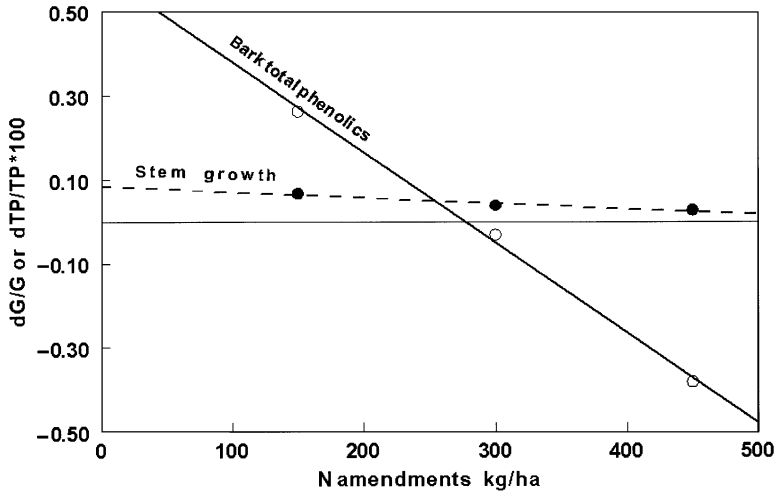

Fig. 4 Comparing the differences in carbon partitioning to total HPLC bark phenolics and overall stem growth by examining relative changes (g g^{-1}) in total phenolics (dTP/TP) and growth (dG/G) in relationship to soil N supply for silver birch seedlings.

(Fig. 4) against soil N reveals that partitioning to total phenolics declines faster than overall biomass growth as soil N increases. This is due to the fact, at low to moderate N levels, the relative increases in total phenolics are much higher than those in total growth, whereas at higher N, the opposite is true.

Changes in partitioning to phenolics were coupled to changing carbon allocation to roots and above-ground organs (Fig. 1). To test the apparent differential effects of N and CO_2 treatments on allocation to roots, we first of all removed confounded size effects due to root mass generally increasing allometrically with total plant mass (Geiger *et al*., 1996; McConnaughay and Coleman 1999). After calculating root mass predicted by plant size alone using a nonlinear regression, we then compared the differences between actual and predicted root masses (i.e. residuals) in ANOVA to discern treatment effects. At 0, 150, and 500 kg N ha^{-1} the standardized residuals were significantly ($P<0.04$) different and were, respectively, +11.1%, +4.2%, and −14.6% different from the expected root mass. Likewise, at 360 and 700 ppm CO_2, the residuals were significantly ($P<0.01$) different, being, respectively, −11.2% and +11.8% different from the expected root mass. In other words, the two lower N treatments and elevated CO_2 grew plants having more roots than expected, whereas the highest N treatment and ambient CO_2 grew plants with less roots and more shoots than expected.

If a lowered net source–sink balance at high N is a hypothesized explanation for diminished partitioning to phenolics, then one might predict that increasing atmospheric CO_2 could significantly raise levels of net photosynthesis, ameliorate the source–sink balance (Körner 2003), and avail more carbon for secondary metabolites. This was, indeed, the trend at high N; almost all biosynthetic classes of phenolics tended to increase: phenolic glycosides, flavonoid glycosides, catechin derivatives, and condensed tannins (Fig. 2). However, it was not the case for the extractive terpenoids (i.e. papyrific acids, and pendulic acids) (Table 1), suggesting that partitioning to terpenoids does not necessarily conform to the simple source–sink balance model, perhaps because of different constraints, and differential competition among different biosynthetic pathways (Gershenzon 1994; Haukioja *et al*., 1998; Koricheva *et al*., 1998; Keinanen *et al*., 1999; Herms 2002; Glynn *et al*., 2003).

Early-season defoliation, which was expected to seriously limit both sink (75% removal of growing leaves) and source capacity (Anttonen *et al*., 2002), had no significant effect on levels of phenolic compounds in winter-dormant paper birch stems and branches. Perhaps the ever vagile source–sink balance somehow remained invariant following the severe defoliation, due to sink demands falling to match reduced source capacity, and thereby maintaining the status quo in whole-plant partitioning to phenolics. There is evidence that severely defoliated plants tend to maintain their inherent root : shoot ratios, implying that root growth declines concomitantly with defoliation-diminished above-ground growth (Reichenbacker *et al*., 1996; Sanchez-Martinez and Wagner 1999; Gavloski and Lamb 2000), thereby helping to maintain an invariant net source–sink balance. However, when elevated CO_2 was coupled to severe defoliation, there was a consistent tendency for increased partitioning to phenolics, in both stems and branches because their phenolic concentrations were the equal of the simple, elevated CO_2 effect (Table 2). This and their near-equivalent biomass growth suggest that elevated CO_2 completely ameliorated the diminished source capacity of severely defoliated plants.

Increased partitioning to secondary compounds under a high net source–sink balance is predicted by the growth-differentiation balance hypothesis as extended by Herms and Mattson (1992), and does not result passively from high substrate availability but from highly regulated partitioning to various biosynthetic pathways (Keinanen *et al*., 1999; Koricheva 2002; Nitao *et al*., 2002; Glynn *et al*., 2003) depending in part on signals from glucose and sucrose receptors in the cytoplasm and on plasma membranes (Sturm and Tang 1999; Leon and Sheen 2003). Sucrose-cleaving enzymes are intimately involved and have multiple functions that regulate the shunting of carbon to growth or differentiation processes that have multipurpose survival value (Sturm and Tang 1999; Arnold and Schultz 2002). Substantial energy and resource fluctuations are the norm for plants, and therefore it is to be expected that the resulting ebbs and flows in source and sink activity have contributed to the evolution of mechanisms for managing fluxes and likewise responding to important selection pressures. The xanthophyll cycle and isoprene synthesis are two among many such well-known mechanisms responding to energy fluctuations, and offering broad fitness enhancing biochemical processes that protect leaves against free radical formation and membrane and chlorophyll degradation, herbivory, and signaling (Mulkey *et al*., 1996; Harley *et al*., 1999; Penuelas and Llusia 2003).

To our knowledge, this is one of the first experiments to demonstrate the potential effect of atmospheric changes on plant–mammalian herbivore interactions. In this study, elevated CO_2, but not temperature, nor severe, early-season defoliation affected tree resistance against Lagomorpha herbivores. In fact, the CO_2 effect was remarkably strong. In the case of paper birch that had been exposed to high CO_2 for two growing seasons,

rabbits apparently fed *ad lib* during the winter of 1999–2000, and overwhelmingly preferred ambient CO_2 plants. Although they removed about the same number of lower stem branches (ca. 7) from both ambient and high CO_2 stems, they did not eat many of those branches because they were dropped, nearly whole, on the floor of the root cellar. It was impossible to determine the exact origin of the clipped, mostly whole branches and branch parts because the various treatment trees were so closely intermingled. We suspect, however, that the rejected branches were probably mostly from high CO_2 plants. Soil N levels also significantly influenced feeding preferences because European hares were much better at discriminating between control and CO_2-treated birches at medium soil N than at either the lowest or highest soil N treatment. This may have been due to heightened levels of both TTR droplets, and tannins at medium N because both increased in response to elevated CO_2. However, changes in other key plant traits, such as sugar and N content (Lavola and Julkunen-Tiitto 1994; Haukioja 2003), as well as other nutrients may also have contributed to the superior discrimination among treatments at medium N. The data clearly suggest that the defensive biochemistry of paper birch twigs as well as the main stem were similarly altered as the result of elevated CO_2. Energy content ($kJ\,g^{-1}$) responded differently, however, increasing in the stems but not in branches, perhaps owing to stems acting as energy storage depots. CO_2-induced changes in the phenotypic resistance of plants will undoubtedly vary with nutrient availability, and the inherent phenotypic plasticity of the plant species (Herms and Mattson 1992; Herms 2002; Glynn *et al*., 2003).

Because the level of CO_2 in the atmosphere has already risen by ca. 30% (100 ppm) since the middle of the 19th century, CO_2 driven alterations in plant-herbivore-natural enemy interactions (through changes in food quality, phenology, volatile organic carbon emissions, etc.) may already be occurring, though currently going unnoticed or unrecognized as such. Such alterations may occur only gradually until thresholds are reached, and then rapid and unexplained substantive changes may occur in plant and animal community composition across landscapes, and in their population densities and cycles (Coviella and Trumble 1999; Stacey and Fellowes 2002, Teyssonneyre *et al*., 2002; Parmesan and Yohe 2003; Stiling *et al*., 2003).

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