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Plant allocation to defensive compounds: interactions between elevated CO_2 and nitrogen in transgenic cotton plants

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Abstract

Plant allocation to defensive compounds in response to growth in elevated atmospheric CO_2 in combination with two levels of nitrogen was examined. The aim was to discover if allocation patterns of transgenic plants containing genes for defensive chemicals which had not evolved in the species would respond as predicted by the Carbon Nutrient Balance (CNB) hypothesis. Cotton plants (*Gossypium hirsutum* L.) were sown inside 12 environmental chambers. Six of them were maintained at an elevated CO_2 level of 900 μmol mol^{-1} and the other six at the current level of $\sim$370 μmol mol^{-1}. Half the plants in each chamber were from a transgenic line producing *Bacillus thuringiensis* (Bt) toxin and the others were from a near isogenic line without the Bt gene. The allocation to total phenolics, condensed tannins, and gossypol and related terpenoid aldehydes was measured. All the treatments were bioassayed against a non-target insect herbivore found on cotton, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). Plants had lower N concentrations and higher C : N ratios when grown in elevated CO_2. Carbon defensive compounds increased in elevated CO_2, low N availability or both. The increase in these compounds in elevated CO_2 and low N, adversely affected growth and survival of *S. exigua*. The production of the nitrogen-based toxin was affected by an interaction between CO_2 and N; elevated CO_2 decreased N allocation to Bt, but the reduction was largely alleviated by the addition of nitrogen. The CNB hypothesis accurately predicted only some of the results, and may require revision. These data indicate that for the future expected elevated CO_2 concentrations, plant allocation to defensive compounds will be affected enough to impact plant–herbivore interactions.

Key words: Carbon dioxide, CO_2, cotton, global change, *Gossypium hirsutum*, plant allocation, plant–insect interactions.

Introduction

The Carbon Nutrient Balance (CNB) hypothesis (Bryant *et al.*, 1983) predicts that the pattern of allocation to defensive compounds depends on the relative availability of carbon and nutrients as well as their relationship with the plant growth rate (Fajer, 1989; Fajer *et al.*, 1992; Lindroth *et al.*, 1993; Poorter *et al.*, 1997; Ralphs *et al.*, 1998). The basic concept is simple: increasing photosynthesis or decreasing available N should result in an increase in carbon-based defences: whereas the opposite should increase reliance on N-based defences. When insects feed on plants with a high C : N ratio, the hypothesis predicts that they will develop more slowly on such plants due to the increase in carbon defences and reduction in N per unit of food. In agreement with the predictions of this hypothesis, some shaded plants tend to decrease carbon-based defensive compounds, such as tannins (Dudt and Shure, 1994). Further, nitrogen-based defensive compounds tend to increase under both high nutrient availability and reduced incident radiation (Folgarait and Davidson, 1995). As predicted,

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low nutrient levels have been demonstrated to increase the production of tannins and other phenolics (Dudt and Shure, 1994; Peñuelas *et al.*, 1997). Most of these studies were conducted at the present atmospheric levels of CO_2. Since pre-industrial times, CO_2 concentration in the atmosphere has increased from 270–280 μmol mol^{-1} to the current value of 370 μmol mol^{-1} (Houghton *et al.*, 1996), which represents an increase of approximately 32%. Emission scenarios published in 2001 in the Third Assessment Report of the Intergovernmental Panel on Climate Change (IPCC), range from 550 up to 1000 μmol mol^{-1} by the year 2100 (IPCC, 2001). Whether the CNB hypothesis will remain valid as atmospheric CO_2 concentrations change, or as transgenic plants add new demands on plant defence allocation, is unknown. For the purposes of this study, the CNB hypothesis predicts that phenolic compounds and terpenoid aldehydes would increase in elevated CO_2. Since N plays an important role in the control of carbohydrate manufacture and allocation (Bazzaz, 1997) the CO_2 effect would be pronounced when soil nitrogen is limiting.

The most common plant response to increasing atmospheric CO_2 is a reduction in N per unit of mass leaf tissue (Osbrink *et al.*, 1987; Rogers *et al.*, 1996; Lawler *et al.*, 1997). The increased C : N ratios that result typically cause insect herbivores to consume more foliage (Lincoln *et al.*, 1986; Fajer, 1989; Taylor, 1989; Marks and Lincoln, 1996). It has been demonstrated that larvae increased consumption up to 80%, on leaves from high CO_2 treatments (Lincoln *et al.*, 1984, 1986). This suggests that compensatory feeding for low nitrogen may potentially increase the amount of dietary allelochemicals ingested for each unit of nitrogen consumed. Occasionally herbivores have shown reduced growth (Fajer *et al.*, 1989). In other experiments with lepidopterans, Fajer *et al.* documented that insect weight gain was positively correlated and consumption was negatively correlated with foliar nitrogen concentration (Fajer *et al.*, 1989). They also found that insects that feed on plants grown in elevated CO_2 have a reduced efficiency of conversion of ingested food to insect tissue. Thus, larvae could be prevented from completing development in climatically-limited environments with short growing seasons, and have increased exposure to their natural enemies (Fajer, 1989; Caulfield and Bunce, 1994), or both.

The published results regarding the interactions of CO_2 concentration and plant defences are often conflicting. Kinney and Lindroth found that 'dynamic metabolites' such as phenolic glycosides and simple carbohydrates do not necessarily increase in elevated CO_2 (Kinney and Lindroth, 1997). Although predicted to increase, nitrogen-based alkaloids did not increase in low light conditions when exposed to short-term shading and dark treatments (Ralphs *et al.*, 1998). However, it was found that phenolics and starch increased with exposure to elevated CO_2 (Roth and Lindroth, 1995). Other studies showed that delayed inducible resistance in relation to herbivory in birch trees was consistent with the CNB theory (Bryant *et al.*, 1983). In a study of plant herbaria specimens, an increase in C concentration as well as a decrease in N correlated with the atmospheric CO_2 increase throughout the twentieth century (Peñuelas and Estiarte, 1997). These changes were accompanied by increases in condensed tannin concentrations. Flavonoid concentration was also found to increase in elevated CO_2 (Estiarte *et al.*, 1999). By contrast, no increase in carbon-based allelochemicals was found in *Plantago lanceolata* plants grown in elevated CO_2 (Fajer *et al.*, 1992). Unfortunately, not all of these studies reported the availability of soil N.

In this paper two broad questions are asked. First, will the CNB hypothesis prove robust at different CO_2 and N concentrations? The immediate objective is to determine if changes in plant allocation in response to elevated CO_2 could, in part, be explained by nitrogen availability. Second, were the allocation patterns conserved to the degree that even transgenic plants containing genes for chemicals that had not evolved in the species would respond as predicted by the CNB hypothesis. The introduction of Bt genes into transgenic plants provides a unique opportunity for asking these questions.

Plant allocation to nitrogen and carbon-based secondary compounds under different conditions of C and N relative availability were measured using a novel system that allowed an unusually complete experimental control of the variables being tested. For this purpose, C and N concentrations were compared in cotton plants grown in elevated CO_2 with those grown at current CO_2 concentrations in combination with two levels of nitrogen availability. Normally cotton produces only carbon-based defences, but the recent introduction of transgenic cotton expressing a *Bacillus thuringiensis* (Bt) gene for a protein for a nitrogen-based toxin allowed direct comparisons within and between near isogenic lines with and without a nitrogen-based defence component. Thus, it was anticipated that the primary CO_2 effect on Bt production would be due to differences in N concentration within the plant. Because of this, any result was expected to be modulated by the nitrogen availability for the plants.

Biologically relevant changes in plant defensive chemistry should have measurable effects on herbivores. If conditions of increased carbon (e.g. elevated CO_2), allow plants to allocate significantly more resources to phenolics, condensed tannins, and gossypol, then insect development or survival should be reduced. Similarly, if Bt toxin production is increased by the higher foliar N concentrations associated with current CO_2 and high soil nitrogen, then insect development or survival should decrease in those circumstances. Thus, the same cotton plants used in the experiments were tested in bioassays for differential effects on an insect herbivore.

Materials and methods

Environmental chambers

The experiment was carried out inside a temperature-controlled greenhouse. Twelve environmental chambers were built inside the greenhouse and covered with Teflon® transparent film with 93.1% transmittance in the UV region (Coviella and Trumble, 2000). A fan attached to each chamber allowed for 0.5 air exchanges min^{-1}. Incoming air was filtered through activated charcoal filters before entering the chambers and exhausted outside the greenhouse directly from each of the chambers. Six of the chambers were maintained at current CO_2 (360–380 $\mu\text{mol mol}^{-1}$); the other six chambers were maintained at an average concentration of 900 $\mu\text{mol mol}^{-1}$ (ambient plus 530 ± 15 $\mu\text{mol mol}^{-1}$). The upper CO_2 concentration was an intermediate value chosen from projections of future CO_2 atmospheric equilibrium concentrations (Houghton *et al.*, 1996; IPCC, 2001). The air in each chamber was individually sampled every 3 min by a CO_2 gas analyser (Vaisala, Helsinki, Finland), and a control system injected CO_2 into the elevated CO_2 chambers as needed.

Twenty cotton plants (*Gossypium hirsutum* L.) were individually grown from seed in 3.0 l pots in each of the environmental chambers. Ten plants were of a transgenic cotton line containing the Bt gene for the production of the Cry1Ac protein (Deltapine Nuctn 33B, Delta and Pine Land Co., Casa Grande, Arizona). The remaining ten plants were of a near isogenic line without the Bt gene (Deltapine DP5415). Of the ten plants in each replicate, five received N fertilization with 130 mg N kg^{-1} soil week^{-1} ('high'), and five received 30 mg N kg^{-1} soil week^{-1} ('low') (after Rogers *et al.*, 1996).

Growing conditions

The plants were grown from seed inside the chambers. They received full sunlight and were watered to maintain the soil close to field capacity. Temperature was controlled inside the greenhouse. Four metal halide 1000 W lamps provided additional light to maintain a 14/10 h light/dark photoperiod. These lamps also added 94 $\mu\text{mol m}^{-2}\text{ s}^{-1}$ of supplementary UV light (250–400 nm) as measured on top of the chambers, to avoid the potential effects of reduced UV light levels on the production of phenolic compounds. In all the experiments, plants were between 40–45-d-old when used. Each plant was used only once and discarded.

Plant analyses

The first four fully expanded leaves were taken from all the plants from each treatment for chemical analysis at the same time as the bioassays were conducted. Leaf material was kept at −65 °C until analysis. All the plant material was analysed for total leaf carbon, total leaf nitrogen, Bt toxin, total phenolics, condensed tannins, and terpenoid aldehydes (Gossypol, Hemigossypolone, and Heliocides H1, H2, H3, and H4). For the total C and total N, leaves were taken from the plants and dried in an oven at 65 °C for 48 h. The dried leaves were analysed at the DANR Laboratory, University of California at Davis, by combustion on a Carlo-Erba elemental analyser. The Bt protein analyses were conducted using a commercially available Btk Enzyme-Linked Immunosorbent Assay (ELISA) test (Btk ELISA PathoScreen kit, Agdia Inc., Elkhart, Indiana) (Sundaram *et al.*, 1995). For the total phenolics and condensed tannins, leaf material from the same plants was taken and freeze-dried, and ground. For the total phenolics, the Folin-Denis assay was used (Waterman and Mole, 1994).

Condensed tannins were assessed with the HCl-Vanillin assay (Makkar and Becker, 1993). Gossypol and related terpenoid aldehydes were analysed with an HPLC analysis method (Stipanovic *et al.*, 1988).

Insect bioassays

In order to test whether differences in defensive chemistry were biologically meaningful, herbivore bioassays were conducted with all the treatments. These were tested against *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), an insect commonly found on cotton (Adamczyk *et al.*, 1998). The insect bioassays were conducted with cohorts of neonate *S. exigua* standardized for age. The relatively mild effect of the Bt toxin on this insect, allowed comparisons to determine if environmental changes would increase or decrease the effects of transgenic plants on insect performance. This determination would not be possible using insects targeted by the toxin.

The neonate larvae were reared individually on plant material in 30 ml plastic cups lined with agar to keep the plant material fresh. Each plant was used only once and plant material not used within 24 h was discarded. Preliminary trials showed that the level of CO_2 used did not affect the insect developmental parameters measured. Therefore, in order to minimize the effects of any possible temperature differences, the bioassays were conducted in environmental chambers at a constant temperature of 28 ± 2 °C, and 14/10 h light/dark photoperiod. Larval and pupal weights were recorded at 7 d and 10 d. From these data the relative growth rates ($RGR = \text{mg}$ biomass gained mg^{-1} of larval biomass d^{-1}) was calculated for each treatment. In addition, developmental times to pupation and adult eclosion, pupal weights, and survival/mortality were measured for all insects.

Statistical analysis

The experimental design was a split-plot arrangement with CO_2 level (current or elevated) as the whole plots, and a 2×2 factorial at the subplot level for two levels of Bt (presence or absence), and two levels of nitrogen fertilization (high and low). Treatments were assigned at random to each chamber. ANOVA (Super ANOVA, Abacus Concepts, Inc. 1993) was used to analyse these data. The same statistical approach was used for plant chemical analysis (carbon, nitrogen, total phenolics, condensed tannins, and terpenoid aldehydes) and the insect bioassays. When significant interactions were found, Least Square Means tables for all pairs (SuperANOVA, Abacus concepts Inc.) were conducted to separate treatments at the $P < 0.05$ level. Where no significant interactions were found, main effects are reported.

Results

Plant nitrogen and C : N ratios

A strong CO_2 effect on the N content in the plants was found. Plants grown in elevated CO_2 showed a 16% decrease in N content compared to plants grown in

Table 1. *Interaction effects between CO_2 concentration, N fertilization, and Bt toxin presence (+) or absence (−) on total leaf nitrogen (% N dry weight) and C : N ratios in cotton plants after 45 d of exposure*

The values are means of six replicates ±standard errors.

		Effect on total leaf N (%)		Effect on C : N	
		Low N	High N	Low N	High N
CO_2	Current	3.19±0.11	5.87±0.06	13.41±0.63	6.49±0.05
	Elevated	2.45±0.10	5.16±0.06	17.88±0.80	7.30±0.07
Bt	(−)	2.45±0.11	5.46±0.08	18.35±0.88	6.82±0.07
	(+)	3.18±0.10	5.57±0.08	13.02±0.44	6.98±0.09

Note: $CO_2 \times N$ on leaf N not significant ($P > 0.05$); all remaining interactions shown $P < 0.001$. No $CO_2 \times Bt$ interactions were significant for either parameter.

Table 2. *Interaction effects between CO_2 concentration, N fertilization, and Bt toxin presence (+) or absence (−) on allocation to total phenolics and condensed tannins (mg g^{-1} leaf dry weight) in cotton plants after 45 d of exposure*

The values are means of six replicates ±standard errors.

		Effect on total phenolics		Effect on condensed tannins	
		Low N	High N	Low N	High N
CO_2	Current	25.4±1.71	12.28±0.53	2.68±0.35	0.75±0.09
	Elevated	31.11±1.34	12.26±0.53	4.06±0.31	0.74±0.08
Bt	(−)	30.11±1.66	11.36±0.42	3.68±0.38	0.61±0.06
	(+)	26.40±1.68	13.18±0.48	3.06±0.37	0.88±0.10

Note: Bt × N interaction on leaf N not significant ($P > 0.05$); all remaining interactions shown $P < 0.01$. No $CO_2 \times Bt$ interactions were significant for either parameter.

ambient CO_2 atmosphere ($F_{1,219} = 13.18$, $P < 0.01$). In addition, a significant Bt × N interaction effect was found for N content (Table 1; $F_{1,219} = 20.67$; $P < 0.001$). In the high N treatment, there was no difference in N content between transgenic and non-transgenic plants. However, under low nitrogen availability N content in non-transgenic plants was 23% lower (Table 1).

The reduced N affected the C : N ratio as predicted by the CNB hypothesis. A significant $CO_2 \times N$ interaction for C : N was found. The ratio was the highest in the elevated CO_2 and low nitrogen availability treatment, and lowest in the current CO_2 and high nitrogen availability treatment (Table 1; $F_{1,219} = 18.59$, $P < 0.001$). There was also a significant two-way Bt × N interaction on C : N ratios. C : N ratios were not significantly different between transgenic and non-transgenic plants when nitrogen was highly available, but the ratios increased for non-transgenic plants when grown in limiting nitrogen conditions (Table 1; $F_{1,219} = 44.25$, $P < 0.001$). Since there was no difference in carbon content, the differences were entirely due to the differences in N concentration in the plants.

Total plant phenolics

There was a significant $CO_2 \times N$ interaction effect on plant allocation to phenolic compounds (Table 2; $F_{1,38} = 7.72$, $P < 0.01$). When grown in elevated CO_2, plants in the low nitrogen treatments allocated significantly more resources to phenolics than plants grown in ambient CO_2 ($P < 0.001$). However, concentrations of C-based defences were lower and not significantly different due to CO_2 treatments when nitrogen was readily available.

A significant Bt × N interaction effect on phenolics was also observed. A reduced allocation to phenolics again was seen in the high nitrogen availability treatments (Table 2; $F_{1,38} = 7.72$, $P < 0.05$). Within this interaction, there was no significant difference in allocation to phenolics between transgenic and non-transgenic plants. However, in low nitrogen, non-transgenic plants allocated significantly more resources to phenolics than transgenic plants ($P < 0.05$).

Condensed tannins

A strong effect on allocation to condensed tannins was found (Table 2; $F_{1,38} = 8.71$, $P < 0.01$). There was a significant increase in condensed tannins from ambient to elevated CO_2 when plants were grown in low nitrogen ($P < 0.001$). However, there was no difference in allocation to condensed tannins due to CO_2 levels in the high nitrogen treatments. As previously observed for total phenolics, plants in high N treatments allocated significantly fewer resources to condensed tannins as compared to plants grown in low N treatments ($P < 0.01$). These observations are consistent with the previously stated concept that the increased production of C-based defensive compounds in low N conditions could represent an 'overflow' mechanism for carbon that could not be used for plant growth due to the limited nitrogen.

Bacillus thuringiensis *toxin production*

A strong $CO_2 \times N$ interaction effect was found on Bt toxin production (Table 3; $F_{1,91} = 4.57$, $P < 0.05$). In the high nitrogen treatments, exposure to elevated CO_2 produced lower levels of Bt toxin than in ambient CO_2. However, there was no difference in Bt production within low nitrogen treatments. As expected, when nitrogen was readily available the highest Bt toxin level was found in ambient CO_2 (Table 3).

Table 3. *Interaction effect between* CO_2 *and N fertilization on plant allocation to Cry 1Ac* Bacillus thuringiensis *toxin (ng toxin* g^{-1} *leaf fresh weight) in cotton plants after 45 d of exposure*

The values are means of three replicates ± standard errors.

		Effect on Bt toxin concentration	
		Low N	High N
CO_2	Current	57.19 ± 3.06	88.91 ± 5.59
	Elevated	54.13 ± 2.77	68.19 ± 4.58

Note: $CO_2 \times N$ interaction significant at $P < 0.05$.

Terpenoid aldehydes

The results showed a strong nitrogen effect on plant allocation to these compounds. Low nitrogen availability for the plants led to significantly higher levels of plant allocation to the total complex of terpenoid aldehydes (TAs) measured than found in high N treatments (Table 4; $F_{1,38} = 32.45$, $P < 0.001$). The total TAs were not affected by changes in CO_2 levels under either high or low N levels. Of the individual TAs measured only heliocide H4 showed a significant $CO_2 \times N$ two-way interaction ($P < 0.05$). Thus, data for the total TAs do not meet the predictions of the CNB hypothesis.

Insect bioassays

Both larval weights at days 7 and 10 and larval relative growth rate (*RGR*) were significantly reduced (Table 5). Developmental time was affected by a significant $CO_2 \times N$ two-way interaction. Both days to pupation ($F_{1,30} = 7.73$, $P < 0.01$) and days to adult eclosion ($F_{1,30} = 8.24$, $P < 0.01$) were increased when plants were grown with low N availability, and the longest developmental times for both parameters occurred when plants were grown in elevated CO_2 in conjunction with low N (Table 6). Insect mortality was lower by 69% in the elevated CO_2 treatments when compared with the ambient CO_2 treatments ($F_{1,30} = 10.77$, $P < 0.01$), which was consistent with the lower Bt toxin concentrations measured. Pupal weights were not significantly affected by the treatments.

Table 4. *Nitrogen fertilization main effect on allocation to terpenoid aldehydes (μg* mg^{-1} *dry mass) on cotton plants after 45 d*

The values are means of six replicates ± standard errors.

Compound	CO_2 main effect		*P*-values
	Ambient CO_2	Elevated CO_2	
Gossypol	0.05 ± 0.01	0.06 ± 0.01	NS
Hemigossypolone	0.58 ± 0.10	0.60 ± 0.10	NS
Heliocide H1	0.19 ± 0.05	0.28 ± 0.05	NS
Heliocide H2	0.58 ± 0.06	0.57 ± 0.06	NS
Heliocide H3	0.23 ± 0.03	0.23 ± 0.03	NS
Heliocide H4	0.08 ± 0.02	0.10 ± 0.02	NS
Total terpenoid aldehydes	1.71 ± 0.26	1.84 ± 0.26	NS
	N main effect		*P*-values
	Low N	High N	
Gossypol	0.08 ± 0.01	0.029 ± 0.01	< 0.001
Hemigossypolone	0.94 ± 0.09	0.253 ± 0.04	< 0.001
Heliocide H1	0.33 ± 0.05	0.140 ± 0.02	< 0.001
Heliocide H2	0.72 ± 0.07	0.433 ± 0.05	< 0.001
Heliocide H3	0.28 ± 0.02	0.175 ± 0.02	< 0.001
Heliocide H4	0.11 ± 0.01	0.075 ± 0.01	< 0.001
Total terpenoid aldehydes	2.46 ± 0.21	1.104 ± 0.13	< 0.001

Note: No interaction had a significant effect on terpenoid aldehyde production.

Table 5. *CO_2 concentration, N fertilization, and Bt toxin main effects on an insect herbivore* (S. exigua) *weight (mg), relative growth rate (RGR = mg biomass gained mg^{-1} larval biomass d) and developmental time (d)*

Insects were fed with leaf material from the same cotton plants used for the chemical analyses. The values are means of six treatments ±standard errors.

		Insect parameter measured				
		Weight day 7	Weight day 10	*RGR*	Days to pupa	Days to adult
CO_2	Current	0.03±0.01	0.15±0.00	0.13±0.01	12.91±0.51	23.48±0.61
	Elevated	0.04±0.01	0.18±0.01	0.14±0.01	13.30±0.51	23.58±0.61
N	Low	0.03±0.00	0.15±0.01	0.12±0.01	13.84±0.38	24.55±0.44
	High	0.04±0.01	0.18±0.01	0.15±0.01	12.20±0.25	21.88±0.30
Bt toxin	(−)	0.05±0.01	0.20±0.01	0.16±0.01	11.64±0.25	21.65±0.39
	(+)	0.02±0.00	0.12±0.01	0.10±0.01	14.52±0.34	25.41±0.58

Note: No CO_2 effect was significant ($P > 0.05$). All N and Bt toxin effects were significant at $P < 0.01$.

Table 6. *CO_2 and N fertilization interaction effects on developmental time (d) of an insect herbivore* (S. exigua)

The insects were fed with material from the same cotton plants used for all the chemical analyses and bioassays. The values are means of six replicates ±standard errors.

		Days to pupation		Days to adult	
		Low N	High N	Low N	High N
CO_2	Current	13.34±0.61	12.47±0.58	24.46±0.78	22.51±0.80
	Elevated	14.66±0.92	11.95±0.44	25.56±0.96	21.60±0.50

Note: $CO_2 \times N$ interaction effect on days to pupation and days to adult significant at $P < 0.01$.

Discussion

The patterns of plant allocation to defensive compounds found in this study only partially supported the predictions of the CNB hypothesis (Bryant *et al.*, 1983). The CNB hypothesis was best supported when nitrogen was limiting and carbon was in excess, and least predictive when nitrogen was not limiting. The fact that there was no difference in Bt toxin production within low nitrogen treatments was unexpected because it had been anticipated that in low N plants would have problems producing the N-based toxin in combination with elevated CO_2. One possible explanation is that when nitrogen is limited, nearly all available nitrogen is required for proteins and enzymes associated with growth, and therefore cannot be allocated to toxin production. In addition, the increased production of phenolic compounds in low-N conditions could simply represent an 'overflow' mechanism for carbon that could not be used for plant growth simply due to limited nitrogen. However, these data generally were consistent with the concept that plants are able to shift allocation between N-based and C-based defensive compounds depending on the relative availability of carbon and nitrogen inputs. Nonetheless, the inability of the CNB hypothesis accurately to predict some of these results, along with other recent papers on this issue (Hamilton *et al.*, 2001) suggests that a major revision of the hypothesis, or perhaps a replacement, may be in order.

At least one new hypothesis can, in part, explain the results. For total phenolics and condensed tannins, the results were consistent with the mechanism described by the Protein Competition Model of phenolic allocation (Jones and Hartley, 1999). According to this model, metabolic pathways for plant allocation to either protein or phenolics compete for phenylalanine, a common limiting resource. Thus, protein and phenolic allocation are inversely correlated; the relative allocation being regulated by the activity of the phenylalanine ammonia lyase enzyme.

These data are in agreement with recent research on plant physiology in elevated CO_2 (for a comprehensive review see Stitt and Krapp, 1999); all the results point to a strong CO_2 effect only under low nitrogen. The results suggest an overflow mechanism for carbon allocation when nitrogen is limiting. Several studies provide support for the carbon overflow mechanism. Moore *et al.* found that in elevated atmospheric CO_2 the reduction in Rubisco due to lower foliar N was more than offset by increased photosynthetic efficiency (Moore *et al.*, 1999). Further, these authors found Rubisco to be 30–55% in excess of what was required for photosynthesis in light-saturated conditions in 1000 $\mu l\ l^{-1}$ CO_2. Under such conditions, photosynthesis is limited either by electron transport capacity or the availability of inorganic phosphorus for ATP regeneration and not by Rubisco activity, despite the fact that Rubisco is substantially down-regulated under elevated CO_2 (Makino *et al.*, 2000). Thus, photosynthesis is not as limited by low nitrogen as growth. Therefore, when plants are grown in elevated CO_2 carbon will be fixed in excess of growth demands.

In situations of low soil nitrogen, plants in this study responded by increased production of gossypol and related terpenoid aldehydes. The total TA concentration was not affected by a $CO_2 \times N$ interaction, although the total phenolics and condensed tannins were. It is suspected that this difference may occur because the TAs are synthesized via the isoprenoid pathway, which is not derived from phenylalanine, and regulation of this pathway is probably controlled by different mechanisms.

The herbivore bioassays demonstrated that the changes in allocation observed in these experiments were biologically relevant. For the carbon-based compounds, a significant $CO_2 \times N$ interaction was found that increased developmental times. Any factors that increase developmental times can cause a substantial effect on insect population dynamics, including greater potential for mortality due to asynchrony with host plants, increased chances of exposure to adverse environmental conditions and the action of biological control agents (Benrey and Denno, 1997; Whittaker, 1999). The deleterious effect of the carbon-based compounds in elevated CO_2 may have been enhanced due to increased feeding. Previous studies have shown that insects eat more in elevated CO_2, probably due to the lower nitrogen concentration in their host plants (Ayres, 1993; Coviella *et al.*, 2000). Extrapolation from a laboratory study to the field should be done with caution, but it is believed that these results could have important implications for plant–insect interactions in areas of low nitrogen availability as CO_2 concentrations continue to increase during this century.

The decrease in Bt toxin production and the reduced availability of nitrogen had similar effects on the non-target insect tested. Larval weights were reduced, as was the overall growth rate (Table 5). However, direct mortality did not increase solely due to the enhanced Bt toxin production of plants grown in ambient CO_2 and high nitrogen. In high N availability, CO_2 had no differential effect.

In the elevated atmospheric CO_2 concentrations expected within the 21st century, it is anticipated many plant species will have lower nitrogen concentrations. As a result, allocation to nitrogen-based defensive compounds will probably decrease and allocation to some carbon-based compounds will increase. The relative availability of soil nitrogen, interacting with elevated CO_2 concentrations, will mediate allocation. Because patterns of allocation to defensive compounds not only regulate the interactions between herbivorous insects and their host plants but also potentially between herbivores and their natural enemies (Ohgushi, 1995; Coley, 1998), such changes are likely to be of widespread significance in both natural and agricultural systems.

Although the Bt toxin used in this study is present in transgenic plants primarily in agricultural settings typified by high soil nitrogen, the transference of Bt genes to related plants in natural systems characterized by low soil nitrogen can occur (Arriola and Ellstrand, 1996; Hancock *et al.*, 1996). Whereas the use of the transgenic plants was initially intended to test the robustness of the CNB hypothesis, these results provide an insight into the general patterns of plant allocation in natural as well as agricultural conditions. Because reduced Bt production in low N was observed even though the amount of total plant nitrogen allocated to Bt was less than 0.01%, the potential impact on plants allocating larger percentages of N to other N-based defences could be much greater (e.g. alkaloid concentrations can be 1000-fold greater). Additional research is needed to determine if other nitrogen-based defensive compounds that have been shown to be constrained by availability of nitrogen (such as alkaloids) will be affected in the same way as the Bt toxin in this study. It is anticipated that as CO_2 concentrations increase, plants growing in nutrient-poor environments will show a similar shift in allocation from nitrogen-based compounds to carbon-based defences. The observation that isoprenoid-derived compounds were not affected by changes in CO_2 levels demonstrates the complex biochemical apparatus that regulates synthesis of secondary compounds and emphasizes the need for additional research to understand fully how increased CO_2 levels will affect plant–insect interactions. If the patterns observed in this study prove broadly applicable across a range of plant and insect taxa, changes in plant–insect interactions due to elevated CO_2 are likely to be substantial.

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