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SPECIES-SPECIFIC RESPONSE OF GLUCOSINOLATE CONTENT TO ELEVATED ATMOSPHERIC CO_2

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Abstract—The carbon/nutrient balance hypothesis has recently been interpreted to predict that plants grown under elevated CO_2 environments will allocate excess carbon to defense, resulting in an increase in carbon-based secondary compounds. A related prediction is that, because plant growth will be increasingly nitrogen-limited under elevated CO_2 environments, plants will allocate less nitrogen to defense, resulting in decreased levels of nitrogen-containing secondary compounds. We present the first evidence of decreased investment in nitrogen-containing secondary compounds for a plant grown under elevated CO_2. We also present evidence that plant response is species-specific and is not correlated with changes in leaf nitrogen content or leaf carbon–nitrogen ratio. When three crucifers were grown at 724 ± 8 ppm CO_2, total foliar glucosinolate content decreased significantly for mustard, but not for radish or turnip. Glucosinolate content of the second and fourth youngest mustard leaves decreased by 45% and 31%, respectively. In contrast, no significant change in total glucosinolate content was observed in turnip or radish leaves, despite significant decreases in leaf nitrogen content. Total glucosinolate content differed significantly among leaves of different age; however, the trend differed among species. For both mustard and turnip, glucosinolate content was significantly higher in older leaves, while the opposite was true for radish. No significant CO_2 × leaf age interaction was observed, suggesting that intraplant patterns of allocation to defense will not change for these species. Changes in nitrogen allocation strategy are likely to be species-specific as plants experience increasing atmospheric CO_2 levels.

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The ecological consequences of CO_2-induced changes in plant defensive investment remain to be investigated.

Key Words—Elevated CO_2, glucosinolates, crucifers, plant defense, carbon/nutrient balance hypothesis.

INTRODUCTION

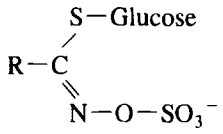
It is now clear that atmospheric carbon dioxide levels are increasing rapidly. The current average concentration of approximately 360 ppm is expected to double by the end of the twenty-first century (Houghton et al., 1990; Graedel and Crutzen, 1993; Gates, 1993). Independent of its potential effects on global climate, increasing atmospheric CO_2 may profoundly impact natural ecosystems by directly influencing plant growth and chemistry. Although plant growth is often increased under elevated CO_2 (Strain and Cure, 1985; Bazzaz, 1990; Mooney et al., 1991), leaves generally contain decreased levels of nitrogen and water and increased leaf carbon–nitrogen ratios (Lincoln et al., 1984; Bentley and Johnson, 1991; Baxter et al., 1994; Epron et al., 1996; Karowe, unpublished data). Despite increased consumption of elevated-CO_2-grown plants, insect herbivores generally exhibit reduced survivorship and/or growth (Lincoln et al., 1984; Fajer et al., 1989, 1991; Johnson and Lincoln, 1990; Bentley and Johnson, 1991; Lindroth et al., 1993; Karowe, unpublished data).

Despite the long-recognized importance of plant secondary chemistry in influencing plant–herbivore interactions (Rosenthal and Janzen, 1979; Spencer, 1988; Rosenthal and Berenbaum, 1991; Bernays, 1992; Romero et al., 1997), little information exists about the response of plant secondary chemistry to elevated atmospheric CO_2 (Fajer et al., 1992; Lindroth et al., 1993; Julkunen-Tiitto et al., 1993; Lavola and Julkunen-Tiitto, 1994). Moreover, all secondary compounds investigated thus far are carbon-based, i.e., do not contain nitrogen (Penuelas et al., 1996). Since the primary direct effect of elevated CO_2 appears to be dilution of plant nitrogen, it is plausible that elevated CO_2 may exert a particularly strong effect on nitrogen-containing secondary compounds.

The carbon/nutrient balance hypothesis (Bryant et al., 1983) predicts that the increased C:N ratio of plants grown under elevated CO_2 will result in increased production of carbon-based secondary compounds (Fajer et al., 1992; Ayres, 1993; Lindroth et al., 1993). A logical extension of this hypothesis is that under elevated CO_2, leaves will contain lower concentrations of nitrogen-based secondary compounds (Baldwin and Ohnmeiss, 1994) because increased vegetative growth will require a larger proportion of available nitrogen. To our knowledge, the response of nitrogen-based secondary compounds to elevated CO_2 has not yet been investigated.

Glucosinolates and their derivative mustard oils are ubiquitous nitrogen-

containing secondary compounds of the order Capparales, most notably of the family Brassicaceae (Cruciferae). Nearly 100 glucosinolates have been identified among the Brassicaceae (Louda and Mole, 1991). All known glucosinolates are relatively small secondary compounds with the basic structure:



where the R group may be aromatic or aliphatic (Larsen, 1981; Chew, 1988a; Van Etten and Tookey, 1991). Glucosinolates are hydrolyzed by myrosinases to glucose, sulfate, and a mustard oil. In general, glucosinolates and myrosinases are stored separately within the plant; in commercially important crucifers, glucosinolates apparently are stored in vacuoles while myrosinases appear to be associated with cytosolic membranes (Chew, 1988a).

Glucosinolates have played a major role in the development of theories about chemically mediated plant–herbivore coevolution (Rhoades and Cates, 1976; Feeny, 1976, 1977) and are often cited as a classic "qualitative" secondary defense (sensu Feeny, 1976). Several recent reviews have concluded that, while they are not the sole determinant of crucifer–herbivore interactions, glucosinolates nonetheless have played and continue to play an integral part in the evolution of crucifer–herbivore relationships (Chew, 1988b; Louda and Mole, 1991; Renwick 1997). As nitrogen-containing secondary compounds of demonstrated ecological and evolutionary importance, glucosinolates provide an excellent system for investigating the effect of elevated atmospheric CO_2 on investment in nitrogen-containing allelochemicals. Therefore, in this study we ask: (1) Does the total glucosinolate content of crucifer species change under elevated CO_2? (2) If so, are plant responses to elevated CO_2 species-specific? (3) Are changes in within-plant nitrogen allocation consistent with those expected under the carbon/nutrient balance hypothesis?

METHODS AND MATERIALS

Seeds of mustard, *Brassica juncea* (var. Florida Broadleaf), turnip, *B. rapa* (var. Purple-top), and radish, *Raphanus sativus* (var. French Breakfast), all obtained from W. Atlee Burpee & Co. (Warminster, Pennsylvania), were sown in Hyponex potting soil and allowed to germinate in the greenhouse at the University of Michigan Biological Station in Pellston, Michigan. Seven days after sowing, plants were transplanted into 6-in. pots containing potting soil. For each species, 32 pots were planted, each containing two plants. Four pots of each species were placed into each of eight 0.5-m^3 open-topped field cham-

bers similar to those described by Drake et al. (1989). Four chambers were maintained at ambient CO_2 levels (363 $\pm$ 6 ppm) and four were maintained at elevated CO_2 levels (724 $\pm$ 8 ppm.). CO_2 content was elevated by dispensing 100% CO_2 into the inlet port of an outlet blower connected by dryer hose to the base of each elevated chamber. CO_2 levels were monitored continuously by pumping air from one ambient and all elevated chambers to a microcomputer-controlled valve manifold that directed the gas stream past an infrared gas analyzer. Levels detected by the gas analyzer were recorded to disc approximately every 5 min starting June 27. CO_2 flow to each elevated chamber was adjusted via a manual flowmeter.

Plants were watered twice daily, inspected periodically for insect herbivores and, when necessary, sprayed with Safer Soap insecticide. Plants were arranged into four blocks, each containing plants sufficient to fill one ambient and one elevated chamber. In order to minimize block effects, plant blocks were rotated among chambers such that each elevated CO_2 block spent approximately equal time within each elevated CO_2 chamber. On August 18, both plants from two pots per species per chamber were sampled for glucosinolate content. Leaf discs 0.5-in.-diam. were cut from the distal tip of the second and fourth youngest leaves from one plant per pot and from the second youngest leaf from the other. Leaf discs were weighed, immediately placed in a 1.5-ml microfuge tube containing 0.5 ml boiling distilled water, and boiled for 5 min to denature hydrolytic enzymes. After boiling, 0.5 ml of 60 mM barium lead acetate solution was added to remove myrosinase by precipitation, and the tube was placed immediately on ice. Tubes were stored at $-80°C$ for one week before shipment on dry ice to the University of Montana.

Total glucosinolate content was determined by a protocol originally developed by Heaney and Fenwick (1981) and modified by MacGregor (unpublished) and more recently by Mitchell-Olds et al. (unpublished data) to handle larger sample sizes. Plant tissue in each tube was homogenized using a variable-speed hand drill, vortexed, and centrifuged. One milliliter of supernatant was added to a 0.3-ml DEAE Sephadex column, washed twice with 500 μl 4 M acetic acid and twice with 1 ml H_2O. One hundred microliters of 3.0 mg/ml myrosinase was added, left overnight, and eluted with 1 ml H_2O. One tenth of 1 ml eluate was assayed at 490 nm in a Biorad microplate reader, after addition of 0.1 ml glucose oxidase–peroxidase color reagent. The glucose oxidase–peroxidase color reagent is a mixture of equal volumes of color reagents 1 and 2, which are both made in imidazole buffer (0.98% imidizole, 0.03% sodium azide, and 0.42 ml glacial acetic acid brought to volume in 100 ml H_2O). Color reagent 1 contains 0.058% glucose oxidase type X-S from *Aspergillus niger* and 0.115% 4-aminoantipyrene. Color reagent 2 contains 0.009% horseradish peroxidase and 0.58% phenol. Samples were quantified by comparison to a glucose dilution series. Reagents were obtained from Sigma Chemical Co. (St. Louis, Missouri).

To determine the water and nitrogen contents of leaves, 0.5-in.-diam. leaf discs were cut from second and fourth youngest leaves of eight additional plants of each species at each CO_2 level (two from each chamber) on August 19. Each disc was weighed, dried to constant weight at 60°C, reweighed to determine water content, ground under liquid nitrogen, dried again to constant weight at 60°C, and analyzed for nitrogen, carbon, and hydrogen content with a Perkin Elmer CHN Elemental Analyzer. Within each species–CO_2–leaf age–block combination, average leaf water content was used to estimate the dry weight of leaf discs used in the glucosinolate analysis described above.

To determine the extent to which exposure to elevated CO_2 affected plant growth rates (Coleman et al., 1993), on August 19 all plants were inspected for initiation of flowering (bolting). To determine whether plant response to elevated CO_2 may have been constrained by root restriction (McConnaughay et al., 1993), all plants were subsequently removed from their pots and inspected for root crowding.

For each species, glucosinolate content, expressed as millimolar glucosinolate per gram dry leaf weight, nitrogen content, and carbon–nitrogen ratio were analyzed by three-way analysis of variance with CO_2 level, leaf age, and block as main effects.

RESULTS

Total glucosinolate content of both young and older mustard leaves was significantly lower for plants grown at elevated atmospheric CO_2 levels (Tables 1 and 2). Glucosinolate content of the second youngest mustard leaf decreased

TABLE 1 LEAF GLUCOSINOLATE CONTENT OF MUSTARD, RADISH, AND TURNIP GROWN AT AMBIENT (3 63 ± PPM) AND ELEVATED (724 ± 8 PPM) CO_2[a]

	Glucosinolate (mM/g dry wt)			
	Second youngest leaf		Fourth youngest leaf	
Species	Ambient	Elevated	Ambient	Elevated
Mustard	0 584	0 297	1 833	1 034
	(0 280)	(0 150)	(0 756)	(0 537)
Radish	0 528	0 813	1 134	1 080
	(0 358)	(0 502)	(0 296)	(0 807)
Turnip	1 628	1 496	0 833	0 593
	(0 537)	(0 893)	(0 352)	(0 425)

[a] Means are given, with standard deviations in parentheses Two way analysis of variance is presented in Table 2

TABLE 2. TWO-WAY ANALYSIS OF VARIANCE FOR TOTAL LEAF GLUCOSINOLATE CONTENT OF MUSTARD, RADISH, AND TURNIP[a]

Source of variation	SS	*df*	MS	*F*	*P*
Mustard					
Main effects					
CO_2 level	3.15	1	3.15	18.37	<0.001
Leaf age	10.51	1	10.51	61.37	<0.001
Interaction					
CO_2 × leaf age	0.70	1	0.70	4.10	0.0490
Error	7.54	44	0.17		
Total	21.26	47			
Radish					
Main effects					
CO_2 level	0.14	1	0.14	0.57	0.4622
Leaf age	2.03	1	2.03	8.20	0.0064
Interaction					
CO_2 × leaf age	0.31	1	0.31	1.24	0.2710
Error	10.88	44	0.25		
Total	13.57	47			
Turnip					
Main effects					
CO_2 level	0.43	1	0.43	1.14	0.2921
Leaf age	9.00	1	9.00	23.47	<0.001
Interaction					
CO_2 × leaf age	0.04	1	0.06	0.10	0.7618
Error	18.40	48	0.38		
Total	28.02	51			

[a]Corresponding means are presented in Table 1.

by 45% and that of the fourth youngest leaf decreased by 31%. In contrast, elevated CO_2 did not significantly affect the total glucosinolate content of young or old radish or turnip leaves (Tables 1 and 2).

For each plant species, glucosinolate content differed significantly between leaves of different age (Tables 1 and 2); however, the trend differed between species. For both mustard and radish, older leaves contained more glucosinolates than did younger leaves; this was true under both ambient and elevated CO_2. In contrast, older turnip leaves contained less glucosinolates than did younger leaves. For mustard only, the effect of CO_2 level depended on leaf age (significant CO_2 × leaf age interaction in Table 2). Under ambient CO_2, glucosinolate

TABLE 3. LEAF NITROGEN CONTENT AND CARBON–NITROGEN RATIO FOR MUSTARD, RADISH, AND TURNIP GROWN AT AMBIENT (363 ± 6 PPM) AND ELEVATED (724 ± 8 PPM) CO_2[a]

Species	Second youngest leaf		Fourth youngest leaf	
	Ambient	Elevated	Ambient	Elevated
Nitrogen content (% dry wt)				
Mustard	2.79	2.46	2.18	1.71
	(0.54)	(0.50)	(0.30)	(0.39)
Radish	2.94	2.83	2.49	1.83
	(0.41)	(0.44)	(0.35)	(0.23)
Turnip	3.49	2.98	2.54	2.12
	(0.83)	(0.55)	(0.26)	(0.34)
Carbon-nitrogen ratio				
Mustard	15.35	16.91	19.36	25.16
	(3.37)	(3.92)	(2.96)	(6.56)
Radish	13.67	14.08	16.40	22.15
	(1.95)	(1.92)	(2.03)	(2.59)
Turnip	12.16	13.86	15.82	19.60
	(2.91)	(2.34)	(1.53)	(3.33)

[a]Two-way analysis of variance is presented in Table 4.

content of older mustard leaves was 2.8-fold greater than for younger leaves; under elevated CO_2, the difference increased to 3.8-fold.

For each species, growth under elevated CO_2 resulted in significantly lower leaf nitrogen content and significantly higher leaf carbon-nitrogen ratio (Tables 3 and 4). When grown under elevated CO_2, nitrogen content of young leaves decreased by 3.7–14.6% and carbon–nitrogen ratios increased by 3.0–14.0%. Changes were more dramatic for old leaves. When grown under elevated CO_2, nitrogen content of old leaves decreased by 16.5–26.5%, and carbon–nitrogen ratios increased by 23.9–35.1% (Table 3). Only radish exhibited a significant CO_2 × leaf age interaction for nitrogen content and carbon–nitrogen ratio (Table 4).

Inspection of mustard plants on August 19 revealed no evidence of phenological advancement under elevated CO_2. The proportion of mustard plants that had bolted did not differ significantly between ambient (9.3%) and elevated (7.8%) CO_2 treatments ($\chi^2 = 0.03$, $P > 0.9$). No radish or turnip plants had bolted by this time in either CO_2 treatment. Inspection of all three species revealed no evidence that plants suffered from root restriction.

TABLE 4. TWO-WAY ANALYSIS OF VARIANCE FOR LEAF NITROGEN CONTENT AND CARBON–NITROGEN RATIO FOR MUSTARD, RADISH, AND TURNIP[a]

Source of variation	SS	*df*	MS	*F*	*P*
Mustard					
Nitrogen content					
Main effects					
CO_2 level	1.28	1	1.28	6.53	0.0163
Leaf age	3.71	1	3.71	18.90	<0.001
Interactions					
CO_2 × leaf age	0.04	1	0.04	0.19	0.6689
Error	5.50	28	0.20		
Total	10.54	31			
Carbon–nitrogen ratio					
Main effects					
CO_2 level	108.6	1	108.6	5.53	0.0259
Leaf age	300.5	1	300.5	15.32	<0.001
Interactions					
CO_2 × leaf age	36.1	1	36.1	1.84	0.1861
Error	549.9	28	19.6		
Total	995.1	31			
Radish					
Nitrogen content					
Main effects					
CO_2 level	1.21	1	1.21	8.93	0.0058
Leaf age	4.17	1	4.17	30.72	<0.001
Interactions					
CO_2 × leaf age	0.59	1	0.59	4.33	0.0467
Error	3.80	28	0.14		
Total	9.76	31			
Carbon–nitrogen ratio					
Main effects					
CO_2 level	75.9	1	75.9	16.61	<0.001
Leaf age	233.3	1	233.3	51.05	<0.001
Interactions					
CO_2 × leaf age	57.5	1	57.5	12.57	0.0014
Error	128.0	28	4.6		
Total	494.7	31			
Turnip					
Nitrogen content					
Main effects					
CO_2 level	1.71	1	1.71	5.78	0.0231
Leaf age	6.53	1	6.53	22.09	<0.001
Interactions					
CO_2 × leaf age	0.02	1	0.02	0.06	0.8166
Error	8.27	28	0.30		
Total	16.52	31			

TABLE 4. CONTINUED

Source of variation	SS	*df*	MS	*F*	*P*
Carbon-nitrogen ratio					
Main effects					
CO_2 level	60.1	1	60.1	8.76	0.0062
Leaf age	176.9	1	176.9	25.80	<0.001
Interactions					
CO_2 × leaf age	8.7	1	8.7	1.26	0.2705
Error	192.0	28	6.9		
Total	437.6	31			

[a] Corresponding means are presented in Table 3.

DISCUSSION

The results of this study suggest that, for some plants, investment in nitrogen-containing secondary compounds may change with increasing atmospheric CO_2 concentration, but that the magnitude and even direction of change are likely to be species-specific. The decrease in total glucosinolate content of mustard grown under elevated CO_2 is consistent with the prediction of the carbon/nutrient balance hypothesis (Bryant et al., 1983) that increased nitrogen limitation results in reduced allocation of nitrogen to defense. However, glucosinolate content of turnip and radish did not change significantly under elevated CO_2 despite significantly reduced leaf nitrogen contents and significantly increased leaf carbon–nitrogen ratios. Clearly, for these three crucifers, change in leaf nitrogen content is not a reliable predictor of change in leaf secondary chemistry.

Species-specific responses have also been observed with carbon-based defenses. For instance, under elevated CO_2, total volatile leaf mono- and sesquiterpenes in peppermint did not change (Lincoln and Couvet, 1989), but concentrations of the iridoid glycosides aucubin and catalpol in plantain decreased significantly (Fajer et al., 1992). Elevated CO_2 resulted in an increase in total phenolic concentration of wheat, no change in orange, and a decrease in total phenolic concentration of pine (Penuelas et al., 1996). In the most comprehensive study to date, Lindroth et al. (1993) showed that the effects of elevated CO_2 on carbon-based allelochemicals differed among three tree species and, moreover, varied among types of phenolics within species. Elevated CO_2 resulted in significant increases in foliar concentrations of salicortin (a phenolic glycoside) in trembling aspen and of ellagitannin and condensed tannin in sugar

maple. However, elevated CO_2 also resulted in a significant decrease in foliar ellagitannin in red oak, but no significant change in gallotannin levels in maple or oak or in condensed tannin levels in aspen or oak. Clearly, broad generalizations about responses of carbon- and nitrogen-based allelochemicals to elevated CO_2 are not warranted at this time.

Independent of CO_2-induced changes, total glucosinolate content also differed significantly between young and old leaves for all three crucifer species; again, the magnitude and direction of differences were species-specific. Moreover, the significant $CO_2 \times$ leaf age interaction for mustard, but not for radish or turnip, suggests that elevated atmospheric CO_2 will affect patterns of defensive allocation among leaves in some species but not in others.

Because glucosinolates affect a wide variety of herbivores both adversely and positively, the ecological consequences of changes in glucosinolate content are hard to predict. Although they do not defend crucifers against all nonadapted insect herbivores (Chew, 1988b; Louda and Mole, 1991), glucosinolates constitute an effective chemical barrier to most noncrucifer feeders (reviewed by Schoonhoven, 1972; Chew, 1988a; Louda and Mole, 1991). For instance, growth and survivorship were markedly reduced for larvae of the umbellifer specialist black swallowtail fed celery (Erickson and Feeny, 1974) or wild carrot (Blau et al., 1978) containing less than 1% allylglucosinolate. Similarly, incorporation of 1% allyl isothiocyanate into artificial diets strongly decreased larval growth and survivorship of the generalist Bertha armyworm (McCloskey and Isman, 1993). In addition, glucosinolates and their breakdown products inhibit growth of some fungi (Holley and Jones, 1985) and feeding by some mammals (Van Etten and Tookey, 1991).

In contrast, many crucifer-adapted herbivores are not adversely affected by glucosinolates and may use glucosinolates or mustard oils as attractants (Feeny et al., 1970; Schoonhoven, 1972; Pivnick et al., 1994), oviposition stimulants (David and Gardiner, 1962; Rothschild, 1987; Huang et al., 1994; Chew and Renwick, 1995), and/or larval feeding stimulants (Ma and Schoonhoven, 1973; Schoonhoven and Blom, 1988; Reed et al., 1989). It is therefore plausible that decreased glucosinolate content may result in reduced herbivory by adapted herbivores (e.g., the cabbage butterfly) that use mustard oils as attractants and/or feeding stimulants. Concomitantly, decreased glucosinolate content may result in increased herbivory by nonadapted generalists that can tolerate low glucosinolate levels (e.g., the southern armyworm; Blau et al., 1978) and may also result in increased herbivory by species that, despite feeding on crucifers, appear to be deterred by specific glucosinolates (e.g., flea beetles; Feeny, 1977; Louda and Rodman, 1983). Finally, Siemens et al. (1996) recently demonstrated that herbivory by two crucifer-adapted herbivores, flea beetles and the diamondback moth, is highest at intermediate glucosinolate levels. Clearly, since glucosino-

lates have various effects on adapted, unadapted, and potential herbivores, CO_2-induced changes in glucosinolate content are likely to have multiple, complicated effects.

Inferences about the ecological consequences of changes in glucosinolate content of crucifers are complicated by our use of a method that does not distinguish among specific glucosinolates. However, we believe this constraint would tend to obscure, rather than accentuate, the effect of elevated CO_2 on glucosinolate content. Among crucifers, glucosinolate profiles (identities and proportions of individual glucosinolates) are species- or genus-specific (Rodman and Chew, 1980; Rodman, 1981). It is possible that although elevated CO_2 did not result in different total glucosinolate content of radish or turnip, the proportions of individual glucosinolates may have changed significantly. Since individual glucosinolates differ dramatically in their biological effects (reviewed in Renwick, 1997), CO_2-induced changes in glucosinolate profiles may strongly impact herbivores, despite unaltered total glucosinolate content.

The results of this study suggest that investment of nitrogen in defensive compounds may decrease for some plants as they experience increasing atmospheric CO_2 levels. However, plant responses to elevated CO_2 are likely to be species-specific and do not appear to be predictable from changes in leaf nitrogen content or carbon-nitrogen ratio. The ecological consequences of changes in plant defensive strategy remain to be elucidated, but may be profound.

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