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GLUCOSINOLATES AND TRICHOMES TRACK TISSUE VALUE IN TWO SYMPATRIC MUSTARDS

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Abstract. Glucosinolates, trichomes, nitrogen, and carbon are not distributed uniformly through the canopies of mustards. In this study, we asked whether glucosinolate concentrations and trichome densities in two sympatric mustards, *Brassica kaber* and *B. nigra*, are highest in tissues of greatest value to the plant. We also asked whether nitrogen or carbon content is the stronger predictor of tissue value, and what fraction of each resource is incorporated in glucosinolates. To quantify tissue values, we removed three equal-area fractions (lower, middle, and upper) from the canopies of *B. kaber* and *B. nigra* in the greenhouse, as well as whole canopies of naturally growing *B. nigra* in the field, at two times during growth and measured reductions in their performance relative to controls. We also measured trichome density in both experiments, as well as glucosinolate, nitrogen, and carbon concentrations for the equal-area fractions in the greenhouse. We found that upper leaves had the highest glucosinolate concentrations, trichome densities, and tissue values. Furthermore, young plants in the field had higher trichome densities and tissue values than did older plants. Collectively, these data provide strong support for optimal defense theory and are among the first such evidence for glucosinolates and for physical defenses. The positive relationship between trichome density and tissue value was strong even after we accounted for the effects of leaf expansion. While nitrogen and carbon have both received attention as currencies for trade-offs, our data suggest that nitrogen concentration is a significantly better predictor of tissue value in these two mustard species. Interestingly, $<1\%$ of the nitrogen or carbon in leaves was incorporated in glucosinolates, which may explain why glucosinolates lack a consistent response to nitrogen fertilization.

Key words: *allocation;* Brassica *spp.; defense; development; herbivory; ontogeny; resistance.*

Introduction

Leaves within a plant canopy often differ greatly in allocation to defense against herbivores (McKey 1974). Chemical defenses such as alkaloids, latex, and cardiac glycosides are typically highest in young leaves and young plants (McKey 1974, 1979, Coley 1983). Optimal defense theory (McKey 1974, 1979, Rhoades 1979) postulates that allocation to defense among plant tissues should be highest for tissues that, if attacked, would result in the greatest fitness reduction to the plant. Experimental support for this hypothesis has been observed for furanocoumarins in *Pastinaca sativa* (Berenbaum 1981, Zangerl 1986, Nitao and Zangerl 1987), cyanogenic glycosides in *Cynoglossum officinale* (van Dam et al. 1996), and nicotine in *Nicotiana sylvestris* (Ohnmeiss and Baldwin 2000).

While glucosinolates of mustards are well-studied secondary compounds that reduce damage from herbivores (Feeny 1977, Blau et al. 1978, Agrawal and Kurashige 2003), the relationship of these compounds to tissue value in leaves has received little attention (Wallace and Eigenbrode 2002, Brown et al. 2003). Physical traits, such as trichomes, constitute a major class of plant defense against herbivores, yet we are unaware of any studies that relate investment in physical defense to underlying tissue values. Simple trichomes are sharply pointed, non-glandular epidermal hairs that physically reduce access of herbivores to the leaf surface (Levin 1973, Ågren and Schemske 1993, Traw and Dawson 2002*b*). In mustards, the experimental removal of trichomes from tissues has been shown previously to increase feeding by herbivores (Lamb 1980). In the current study, we asked whether distributions of glucosinolates and trichomes in mustards are correlated positively with underlying tissue values.

Tissue value is most appropriately determined by removing plant tissues and measuring the impact on subsequent plant performance, but this approach has been rarely used (but see Nitao and Zangerl 1987, Ohmeiss and Baldwin 2000, Wallace and Eigenbrode 2002, Barto and Cipollini 2005). In several other studies, leaf nitrogen concentration has been used as a proxy for tissue value (Berenbaum 1981, Krischik and Denno 1983). Nitrogen is often a limiting element for plant growth (Chapin 1980) and leaf nitrogen concentrations are highly correlated with photosynthetic rates (Evans

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1989) and should therefore be highly correlated with tissue value. Carbon is also considered an important currency in the study of plant allocation strategies (Hilbert 1990), but its relationship to tissue value has not been examined previously. Therefore, as part of our study, we also assessed the relative importance of nitrogen and carbon as currencies of tissue value.

For vertically growing shoots, upper (i.e., younger) leaves often shade lower (i.e., older) ones, creating a gradient of decreasing light with increasing leaf age (Mooney et al. 1981, Field 1983, Traw and Ackerly 1995). Plants therefore maximize carbon gain by allocating a disproportionate amount of their photosynthetic machinery to upper leaves (Field 1983, Hirose and Werger 1987). Upper leaves are therefore expected to be the most valuable, both because they receive more light and because they contain relatively high concentrations of nitrogen (Harper 1989). A second prediction is that leaves of young plants have greater tissue value than equivalent leaves of older plants, due to the longer compounding effects of early resource acquisition (Harper 1989).

Here, we describe two experiments that assess relationships between defense and tissue value for two vertically growing mustards (*Brassica nigra* and *B. kaber*) at two times during development. In the first experiment, we studied thirds of the plant canopy for both species in the greenhouse, while in the second experiment, we examined the whole canopy of naturally growing *B. nigra* in the field. Specifically, we asked the following four questions: (1) Do upper leaves have greater tissue value and defense than do lower leaves? (2) Do whole canopies of younger plants have greater tissue value and defense than do whole canopies of older plants? (3) Which element is a better predictor of tissue value, nitrogen or carbon? (4) What percentage of canopy nitrogen and carbon is incorporated in glucosinolates?

Methods

Study species

Brassica kaber (DC.) Wheeler and *B. nigra* (L.) Koch are introduced mustards (Brassicaceae) that colonize disturbed soil and co-occur near Ithaca, New York, USA (M. B. Traw, *personal observation*). Both species germinate in late April, but *B. kaber* senesces in June, while *B. nigra* senesces in August (Wiegand and Eames 1924). *B*rassica *kaber* produces 8 to 10 main stem leaves on average, while *B. nigra* often produces 30 or more leaves (Traw 2002*b*). Both species are alternate leaved, lack a rosette, and do not branch until onset of flowering, and therefore permit easy tracking of leaves. Roughly 99% of glucosinolate in leaves of *B. nigra* is sinigrin (allylglucosinolate) with trace amounts of four other glucosinolates (Feeny and Rosenberry 1982, Daxenbichler et al. 1991, Traw 2002*a*). Leaves of *B. kaber* contain approximately 92% 4-hydroxybenzyl glucosinolate with small percentages of three aliphatic glucosinolates, 3-methylthiopropyl glucosinolate, 9-methylsulfonylnonyl glucosinolate, and 8-methylsulphonyloctyl glucosinolate (Daxenbichler et al. 1991).

Greenhouse leaf-removal experiment

We conducted a randomized complete-block experiment in Seeley-Mudd greenhouse at Cornell University in 1997, using seeds from the Southwest Park in Ithaca, New York (Traw 2002*a*). We sowed seeds in Deepots (Stuewe and Sons, Corvallis, Oregon, USA; 6.5 cm diameter $\times$ 25 cm depth) containing a 1:1 mixture of sterilized topsoil and fritted clay. Seedlings emerged 3 June (Day 1) and were thinned to one per pot on Day 14. Plants received 14-h days with controlled temperature (25°C day, 20°C night) and natural sunlight supplemented by halogen arc lamps (300 μmoles$\cdot$m$^{-2}\cdot$sec^{-1} at bench top). Pots were watered daily, but received no supplemental fertilizer. On Day 20, plants were divided by size into 12 blocks, with each block containing half *B. kaber* and half *B. nigra*. Within each block, one plant of each species was randomly assigned to have the lower, middle, or upper third of its leaves removed on Day 21 or Day 34, for a total of 12 treated plants per block and 144 plants in total. Four or five additional plants of each species in each block were included as controls for the damage treatments, for a total of 113 additional plants. Finally, one further plant of each species in each block was included and harvested on Day 21 and another on Day 34 for measurement of trichome density, nitrogen concentration, and carbon concentration at each leaf position, for a total of 48 additional plants and a grand experimental total of 305 plants. During treatment application, we lined up the three conspecific plants from a block and clipped leaves at the lower, middle, or upper canopy fractions until one-hird of the leaf area was removed from each plant. We evaluated the progress of this area removal for each fraction using a LI-COR 3100 leaf area meter (LI-COR, Lincoln, Nebraska, USA). The area removed was 54.1 $\pm$ 1.8cm^2 (mean $\pm$ SE) from the lower fraction, 53.8 $\pm$ 1.9 cm^2 from the middle fraction, and 45.6 $\pm$ 1.9 cm^2 from the upper fraction. Thus, while we removed statistically similar areas from lower and middle fractions, we removed roughly 16% less from upper fractions. This difference was significant by a one-way ANOVA ($F_{2,141} = 6.3$, $P = 0.002$), but would be expected to reduce our ability to detect high tissue values of the upper fraction.

Field whole-canopy removal experiment

To further compare the tissue values of younger and older plants, we conducted a randomized complete-block experiment at two ruderal sites in Tompkins County, New York, in 1997, using naturally occurring plants of *B. nigra*. The sites at van Ostrand Road and Cornell Plantations were separated by ~12 km. Plants were divided into 11 blocks on 4–5 June based on the number of leaves present. Within each block, one plant

was randomly assigned to have its entire leaf area removed on 6 June or 30 June, for a total of two treated plants per block and 22 plants total (we planned to test the effect of one third canopy removal and incude *B. kaber*, but canopy fraction treatments proved impossible to standardize in the field, and *B. kaber* lacked sufficient leaf area for the tissue removal treatment on 30 June). During treatment application, we used clippers to remove all main stem leaves >2 cm in length. One plant was included in each block as a control for the damage treatments, for a total of 11 additional plants. Finally, one further plant of each species was included and harvested on 6 June and another on 30 June in each block for measurement of trichome density at each leaf position, for a total of 22 additional plants and a grand experimental total of 55 plants.

Estimation of glucosinolate concentration

We measured glucosinolate concentrations of each tissue fraction removed from each treated plant in the greenhouse experiment (144 samples). We dried, weighed, and homogenized each tissue fraction, and then extracted a subsample of 100 mg in boiling 70% ethanol as described in Agerbirk et al. (2001). We desulfated the extracts in open columns packed with 0.1 g DEAE Sephadex A-25 (Diethylaminoethyl cellulose; Pharmacia, Piscataway, New Jersey, USA) as described in Hugentobler and Renwick (1995). We conducted high performance liquid chromatography analysis of desulfated glucosinolates using a Hewlett-Packard Model 1100 system (Santa Clara, California, USA) equipped with an autosampler, a 4.5×15 cm C-18 column (Luna, Phenomenex, Torrance, California, USA), and diode-array detector. The solvent program (1 mL/min) was water for 2 min, a linear increase to 20% acetonitrile at 5 min, 35% acetonitrile at 15 min, and 100% acetonitrile at 18 min. Identities of peaks were determined by retention time, relative to times for pure glucosinolates. Peak areas were measured at 229 nm and concentrations (micromoles per gram dry tissue mass) were determined relative to the peak area of a known concentration of internal standard, benzylglucosinolate (EM Science number GX0110-1), corrected using relative response factors (RRF = 1 for desulfosinigrin and other aliphatics, 0.95 for desulfo-4-benzylglucosinolate, 0.29 for desulfoglucobrassicin) according to Buchner (1987). Total glucosinolate concentration per mass was calculated as the sum of the individual compounds. We focused on this mass-based estimate of glucosinolates because it is more relevant in the context of defense against herbivores. However, glucosinolate allocation per area is relevant from the perspective of the plant, so we included this metric in our analysis as well. To calculate total glucosinolate concentration per area (millimole per square meter), we multiplied total glucosinolate concentration per mass by dry tissue mass and divided by leaf area.

Estimation of trichome distribution

We determined trichome density and total leaf area for each leaf of one plant of each species in each block on each date for a total of 48 plants and 366 leaves in the greenhouse and 22 plants and 243 leaves for *B. nigra* in the field. We removed each leaf longer than 2 cm, and determined fresh area using a LI-COR 3100 leaf area meter. We then used a hole punch to remove two disks (area = 0.29 cm^2), one from each side of the midrib as in previous experiments (Traw 2002*a*, Traw and Dawson 2002*a*, 2002*b*). We counted trichomes on both sides of the disks using a dissecting microscope and calculated leaf trichome density as the sum of top and bottom counts divided by disk area. We multiplied trichome density by leaf area to determine leaf trichome number. We determined trichome densities for lower, middle, and upper canopy fractions by summing trichome numbers represented by those leaves and dividing by the summed area.

Estimation of nitrogen and carbon distributions

We determined nitrogen and carbon concentrations for each leaf of one plant in each of two blocks harvested at each date for *B. kaber* and *B. nigra* in the greenhouse experiment, for a total of eight plants and 62 leaves. Each leaf was dried at 60°C for two weeks, weighed, and homogenized in a Wylie mill. We analyzed a 3 mg subsample of each leaf for the percentage of nitrogen and carbon using a Carlo Erba model NA1500 carbon : nitrogen analyzer (Milan, Italy). We multiplied these percentages by tissue dry mass to obtain leaf nitrogen mass (milligrams) and carbon mass (milligrams). We determined nitrogen mass and carbon mass for lower, middle, and upper canopy fractions by summing nitrogen and carbon masses represented by those leaves. We then divided those values by the summed areas to obtain nitrogen per area and carbon per area for the lower, middle, and upper canopy fractions.

Estimation of the fraction of nitrogen and carbon in glucosinolate

We estimated the percentage of nitrogen and carbon in glucosinolates for each canopy fraction on both dates for *B. kaber* and *B. nigra* in the greenhouse experiment, for a total of 12 estimates. We first multiplied concentrations of glucosinolate per mass (micromole per gram dry mass) by tissue dry mass (grams) to determine total quantity of glucosinolate (micromoles). Because each glucosinolate molecule contained one atom of nitrogen, we multiplied the quantity of glucosinolate (micromoles) by 0.014 to determine the incorporated mass of nitrogen (milligrams). Because each molecule of these glucosinolates contained ~10 atoms of carbon, we multiplied the quantity of glucosinolate (micromoles) by 0.121 to calculate the incorporated mass of carbon (milligrams). We then divided these estimates of incorporated nitrogen or

TABLE 1. Greenhouse experiment three-way ANOVA testing the effect of species (*Brassica nigra* vs. *B. kaber*), timing (Day 21 vs. 34), and canopy location (lower, middle, upper third) on glucosinolate per mass (DM, dry mass), trichome density, tissue value, and carbon and nitrogen per area.

		Glucosinolate (μmol/g DM)			Trichome density (no./cm^2)			Tissue value (% reduced growth)			Carbon per area (g/m^2)			Nitrogen per area (g/m^2)		
Source	df†	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Species (S)	1	385	7.2	**0.008**	429 145	157.5	**<0.001**	32	0.18	0.668	157	13.4	**0.003**	0.37	5.1	**0.042**
Timing (T)	1	1695	31.9	**<0.001**	2699	0.9	0.321	519	2.95	0.089	40	3.4	0.086	2.46	34.0	**<0.001**
Location (L)	2	5717	107.8	**<0.001**	163 781	60.1	**<0.001**	1078	6.12	**0.003**	109	9.3	**0.003**	2.11	29.2	**<0.001**
S × T	1	874	16.4	**<0.001**	365	0.1	0.714	92	0.53	0.470	30	2.6	0.129	0.77	10.6	**0.006**
S × L	2	99	1.8	0.156	67 620	24.8	**<0.001**	75	0.43	0.652	24	2.1	0.162	0.31	4.4	**0.036**
T × L	2	137	2.5	0.079	35 164	12.9	**<0.001**	121	0.69	0.503	17	1.4	0.270	0.13	1.9	0.192
S × T × L	2	83	1.5	0.211	28 321	10.4	**<0.001**	61	0.35	0.705	8	0.7	0.503	0.12	1.7	0.222
Covariate	1	53	2.9	0.090				144	0.82	0.367						
Residual	131	154			2723			176			11			0.07		
Total	143															

Notes: Leaf area removed (cm^2) is included as a covariate where appropriate. Significant *P* values are shown in boldface.

† Residual and total df for trichome density = 132, 143; tissue value = 125, 137; carbon per area = 11, 23; and nitrogen per area = 11, 23.

carbon mass (milligrams) by total nitrogen or carbon pool mass (milligrams) of that tissue and multiplied by 100 to determine percent total nitrogen or carbon incorporated in glucosinolates.

Estimation of tissue value

We harvested experimental plants on Days 56–58 in the greenhouse, by which time *B. nigra* had initiated flowering and *B. kaber* was setting seeds. *Brassica nigra* in the field experiment were harvested on 19–29 July. Roots were separated from shoots and washed. All plant material was dried at 60°C for two weeks, weighed, and added together to determine dry biomass at harvest. This measurement of dry biomass (grams) is highly predictive of total seed number for *B. nigra* (seed number = 211 × mass − 338, $r^2 = 0.86$, $P < 0.001$, $N = 57$) and *B. kaber* (seed number = 141 × mass − 18, $r^2 = 0.98$, $P < 0.001$, $N = 11$) in natural populations (Traw 2002*b*). For each plant, we calculated tissue value (V) as percentage of reduction in dry biomass of that treated plant (M_T) relative to average dry biomass of control plants from the same block (M_C) using the equation $V = 100 \times (M_C - M_T)/M_C$. Thus, the effect of block was accounted for in this measurement of tissue value.

Statistical analysis

Leaf glucosinolate per mass, trichome density, tissue value, nitrogen per area, and carbon per area from the greenhouse experiment were all analyzed using a three-way ANOVA containing species, timing, and location of leaf removal as fixed main effects and including all interactions. Actual leaf area removed was included as a covariate in the models for glucosinolate per area and tissue value to account for variation in treatment application. Trichome density, carbon per area, and nitrogen per area were derived from an independent set of plants, so a covariate was not necessary. Glucosinolate per mass, trichome density, nitrogen per area, and carbon per area all satisfied the assumptions of ANOVA. Six significant outliers were removed from the analysis of tissue value to improve the normality of residuals, but their inclusion did not alter the results. Simple linear regression was used to quantify relationships between defense and tissue value. Multiple regression was used to assess whether nitrogen per area or carbon per area was more closely correlated with canopy tissue value. Trichome density, tissue value, and total canopy leaf area in the field experiment were analyzed by two-way ANOVA with field site and timing as fixed main effects and including the interaction.

RESULTS

Upper leaves had significantly higher glucosinolate concentration per mass ($F_{1,131} = 215$, $P < 0.001$), trichome density ($F_{1,132} = 118$, $P < 0.001$), tissue value ($F_{1,125} = 12.1$, $P < 0.001$), carbon per area ($F_{1,12} = 18.6$, $P = 0.001$), and nitrogen per area ($F_{1,12} = 57.4$, $P < 0.001$) than did the lower leaves when averaged across the two sampling dates and species (Table 1 and Fig. 1). Middle leaves, in turn, had significantly higher glucosinolate concentration per mass ($F_{1,131} = 45.4$, $P < 0.001$), trichome density ($F_{1,132} = 18.2$, $P < 0.001$), carbon per area ($F_{1,12} = 5.14$, $P = 0.042$), and nitrogen per area ($F_{1,12} = 8.72$, $P = 0.012$) than did lower leaves when averaged across the two sampling dates and species (Fig. 1). Middle leaves had tissue values of 3% vs. −1.4% for lower leaves, but this difference was marginally nonsignificant ($F_{1,125} = 2.64$, $P = 0.106$; Fig. 1). As a covariate, the area removed from canopy fractions did not explain variation in glucosinolate per mass or tissue value (Table 1).

Differences in glucosinolate per mass among canopy fractions were not significantly influenced by species or timing (Table 1, Fig. 1) and exhibited similar patterns whether measured on a mass (Fig. 1A) or area basis (Appendix A). Differences among canopy fractions in

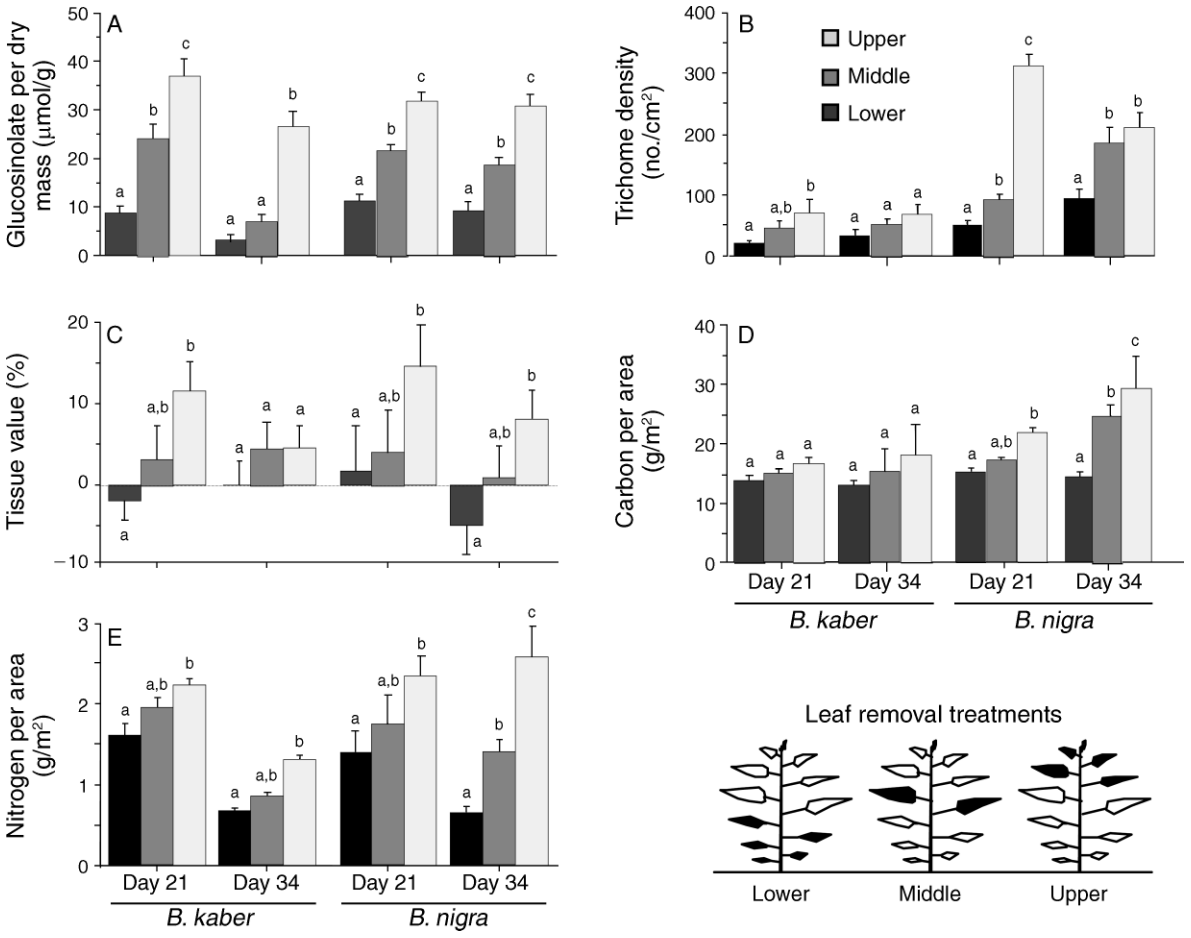

FIG. 1. Differences between lower, middle, and upper thirds of the canopy in (A) glucosinolate concentration per mass, (B) trichome density, (C) tissue value (percentage of reduction in growth relative to control), (D) carbon per area, and (E) nitrogen per area for *Brassica kaber* and *Brassica nigra* sampled at two dates during growth in the greenhouse experiment. Each bar represents the mean (+SE) of 12 replicates (glucosinolate per area, trichome density, tissue value) or two replicates (nitrogen per area, carbon per area). Within sampling date, means with different lowercase letters are significantly different (alpha = 0.05). An idealized sketch of the three removal treatments is shown.

trichome density were greater at the early sampling date, but only for *B. nigra*, resulting in a significant three-way interaction between species, timing, and location ($F_{2,132} = 10.4$, $P < 0.001$). Differences in tissue value and carbon per area among canopy fractions were not significantly influenced by species or timing. Differences in nitrogen per area among canopy fractions were significantly greater for *B. nigra* than for *B. kaber* ($F_{2,12} = 4.4$, $P = 0.036$; Table 1, Fig. 1E), and this interaction was independent of timing ($F_{2,12} = 1.7$, $P = 0.222$).

Glucosinolate per mass and trichome density both were strongly positively correlated with tissue value within the plant canopy. To quantify these relationships, we plotted both defensive traits against tissue value using the canopy fraction means and assessed the relationships by simple linear regression (Fig. 2). Squared correlation coefficients indicated that tissue value explained 63% of overall variation in glucosinolate per mass (Fig. 2A) and 33% of variation in trichome density (Fig. 2B). The slope of the regression line indicated that a 1% increase in tissue value was associated with an increase of 1.61 μmol glucosinolate/g dry mass ($P = 0.002$) and an increase of 9.2 trichomes/cm^2 ($P = 0.047$). Glucosinolate per mass was also positively correlated with nitrogen per area (Fig. 2C). Analysis of glucosinolate per area also yielded significant positive correlations with tissue value and nitrogen per area (Appendix B).

Because young leaves typically have higher tissue values than do older leaves and because a leaf's trichome allocation is determined prior to leaf expansion, some degree of correlation between tissue value and trichome density would seem to be an inevitable and fortuitous consequence of leaf ontogeny (cf. Wallace and Eigenbrode 2002). Our results indicate, however, that the initial number of trichomes allocated to a leaf varies dramatically with leaf position along the shoot, such that only part of the variation in canopy trichome

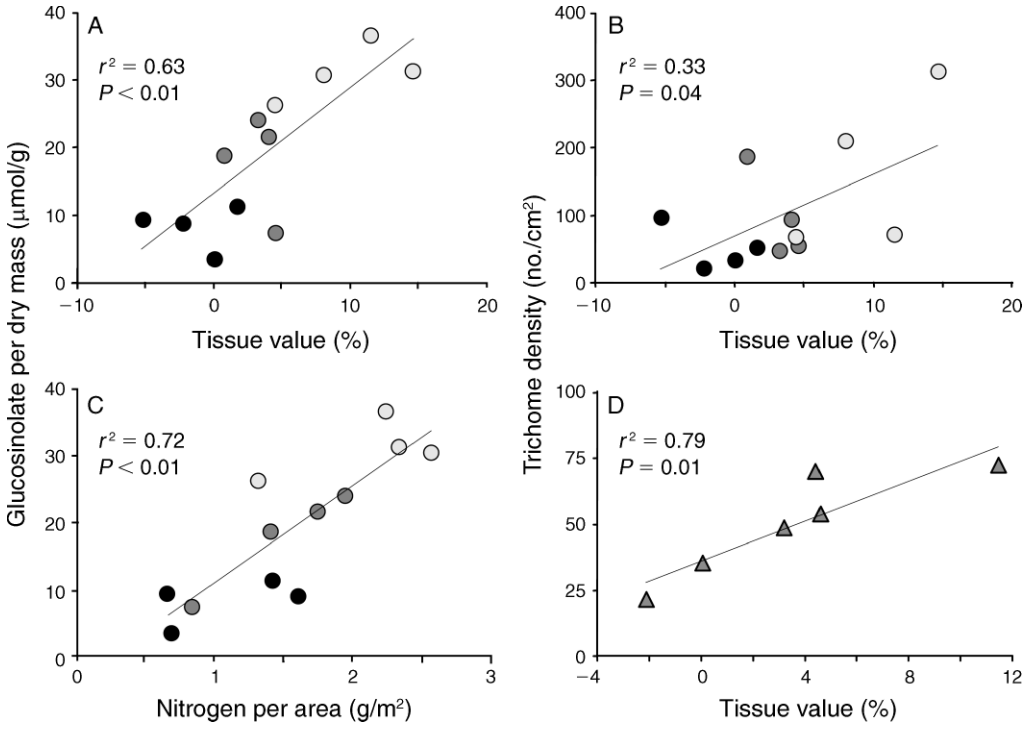

FIG. 2. Greenhouse experiment scatterplots of (A) glucosinolate concentration per mass on tissue value, (B) trichome density on tissue value (percentage of reduction in growth relative to control), (C) glucosinolate per mass on nitrogen per area, and (D) trichome density on tissue value for *B. kaber* alone. Data consist of the canopy fraction means for each trait shown in Fig. 1. Statistics are shown for least-squares regression. Upper (light gray), middle (dark gray), and lower (black) fractions of the canopy are indicated as in Fig. 1.

density can be explained simply by leaf expansion. For *B. kaber* in the greenhouse experiment, leaves had essentially completed expansion and no new leaves were added between Day 21 and 34. Yet trichome densities differed significantly at different leaf positions (Fig. 3A). Recalculation of the regression of trichome density on tissue value with *B. kaber* alone (Fig. 2D) resulted in an even stronger positive relationship (density = 3.75 × mass + 35.9, $r^2 = 0.79$, $P = 0.016$, $N = 6$; Fig. 2D).

The contribution of leaf expansion to the correlation between tissue value and trichome density was much more evident for *B. nigra* in the greenhouse experiment since these plants were actively producing new and expanding leaves on both sampling dates. On Day 21, for example, there were only eight leaves on the plants and three of them were in varying states of expansion (Fig. 3B). These three leaves were all in the upper third of the plant canopy area on that date and were responsible for the highest mean trichome density bar calculated in Fig. 1B. On Day 34, these same leaves were all in the middle fraction of canopy area and had reached a stable trichome density.

Tissue value was positively correlated with nitrogen per area within the plant canopy ($r^2 = 0.54$, $P = 0.006$, Fig. 4A), but was not correlated with carbon per area ($r^2 = 0.21$, $P = 0.132$, Fig. 4B). A multiple regression of tissue value on nitrogen per area and carbon per area explained 54% of variation in tissue value ($F_{2,9} = 5.35$, $P = 0.029$), which was no better than the simple regression with nitrogen per area. The standardized partial slope coefficients were 2.44 and 0.31 for nitrogen per area ($t = 2.55$, $P = 0.031$) and carbon per area ($t = 0.12$, $P = 0.906$), respectively.

Of the total leaf nitrogen, the percentage of nitrogen incorporated in glucosinolate was 0.45% ± 0.05% (mean ± SE), with all 12 values between 0.13% and 0.75%. Of the total leaf carbon, the average percentage of carbon incorporated in glucosinolate was 0.38% ± 0.06%, with all 12 values between 0.06% and 0.81%. These calculations were based on variables with the following ranges: tissue dry mass (126–409 mg), glucosinolate concentration (3.1–37.0 μmol/g dry mass), nitrogen concentration (1.85–5.79% dry mass), and carbon concentration (31.6–47.4% dry mass).

Naturally growing *B. nigra* on 6 June had significantly higher whole-canopy trichome densities ($F_{1,18} = 36.4$, $P < 0.001$; Fig. 5A) and tissue values ($F_{1,18} = 40.7$, $P < 0.001$; Fig. 5B) than did plants measured on 30 June. The lower tissue values on 30 June occurred in spite of the fact that the plants had nearly twice as much leaf area present at this later sampling date (Fig. 5C). The lower trichome densities on 30 June were not due to the expansion of individual leaves, because there was essentially no overlap in the leaves present at the two

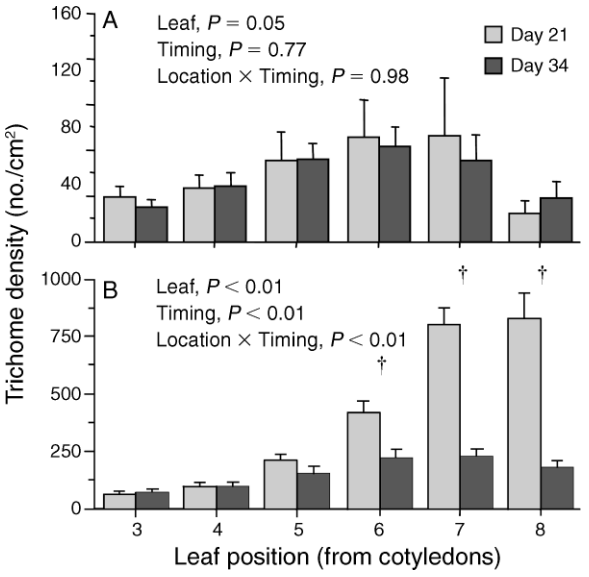

FIG. 3. Comparison of trichome density (mean ± SE) on Day 21 (light gray) and Day 34 (dark gray) for leaf positions 3 through 8 for (A) *B. kaber* and (B) *B. nigra* in the greenhouse experiment. *P* values are shown for the two-way ANOVA with leaf position and day as the main effects and including the interaction. The first and second leaves (following the cotyledons) had dropped by Day 34 on both species, so those positions are not shown. Daggers (†) indicate significant effects of sampling date at $P < 0.005$.

sampling dates (Fig. 5D). On 6 June, leaves were present only at positions 1 through 10 along the main stem, but by 30 June these leaves had all dropped off and leaves had been added at positions 11 through 25 along the main stem. Most of the newer leaves (positions 18 and above) were glabrous, i.e., lacking entirely in leaf trichomes. Plants at the van Ostrand Road and Cornell Plantations sites had similar trichome densities ($F_{1,18} = 1.2$, $P = 0.271$; Fig. 5A) and tissue values ($F_{1,18} = 4.0$, $P = 0.059$; Fig. 5A).

DISCUSSION

Optimal defense theory (McKey 1974, 1979, Rhoades 1979) predicts that levels of defense will be highest in tissues of greatest value to the plant. In this study, we found that distributions of glucosinolates and trichomes in two sympatric mustard species were positively correlated with tissue value, providing strong support for this hypothesis. This is one of the first such tests for glucosinolates in the leaves of mustards (but see Wallace and Eigenbrode 2002) or for a physical defense in any plant.

The location of leaves within the plant canopy was the most important predictor of tissue value in the greenhouse experiment. Plant age did not have a significant effect on tissue value in the greenhouse, which is perhaps because the interval between measurements was only 13 days. In the field experiment, the interval between tissue removals was 24 days, and we found strong evidence that tissues of younger plants were more valuable than tissues of older plants. The tissue of the younger plants also had significantly greater levels of defense than did the tissues of older plants, as predicted by optimal defense theory. In terms of lifetime leaf production, *B. kaber* was effectively at a later ontogenetic stage on both treatment dates in the greenhouse, having accomplished 100% of lifetime leaf production by Day 21 (Fig. 3A). In contrast, *B. nigra* was still producing main stem leaves on Day 34. For this reason, we do not extensively compare responses of the two species, other than to note that their canopy distributions of tissue value and defense were generally similar in the greenhouse experiment (Fig. 1).

Our observed patterns within the canopies of plants in the greenhouse experiment are likely to reflect constitutive allocation of defense because the plants were grown in the absence of insect herbivores or pathogens. Glucosinolates and trichomes are both induced in *Brassica nigra* following attack (Traw 2002*b*, Traw and Dawson 2002*a*, *b*) and our field experiment is therefore likely to include some mixture of constitutive and induced levels of defense. Future assessment of the relationship of glucosinolate and trichome induction to plant ontogeny will certainly be of interest.

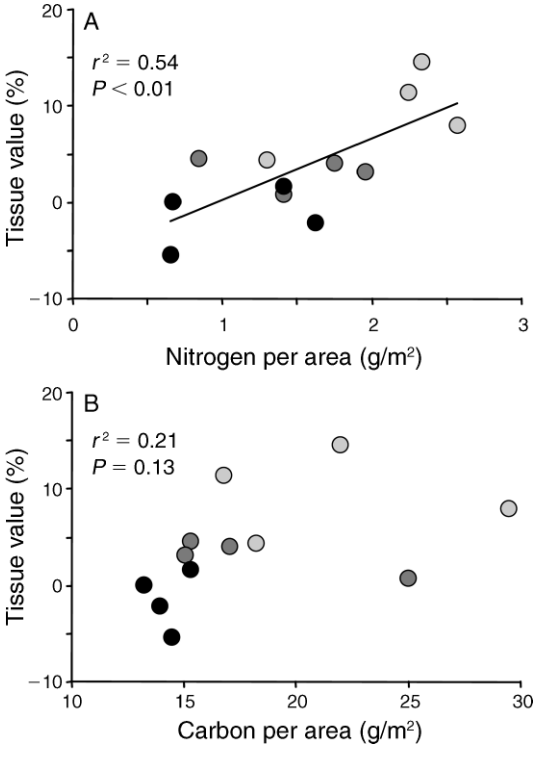

FIG. 4. Regression of tissue value on (A) nitrogen per area and (B) carbon per area. Data consist of the canopy fraction means (upper, light gray; middle, dark gray; and lower, black) for each trait in the greenhouse experiment shown in Fig. 1. Statistics are shown for least-squares regression.

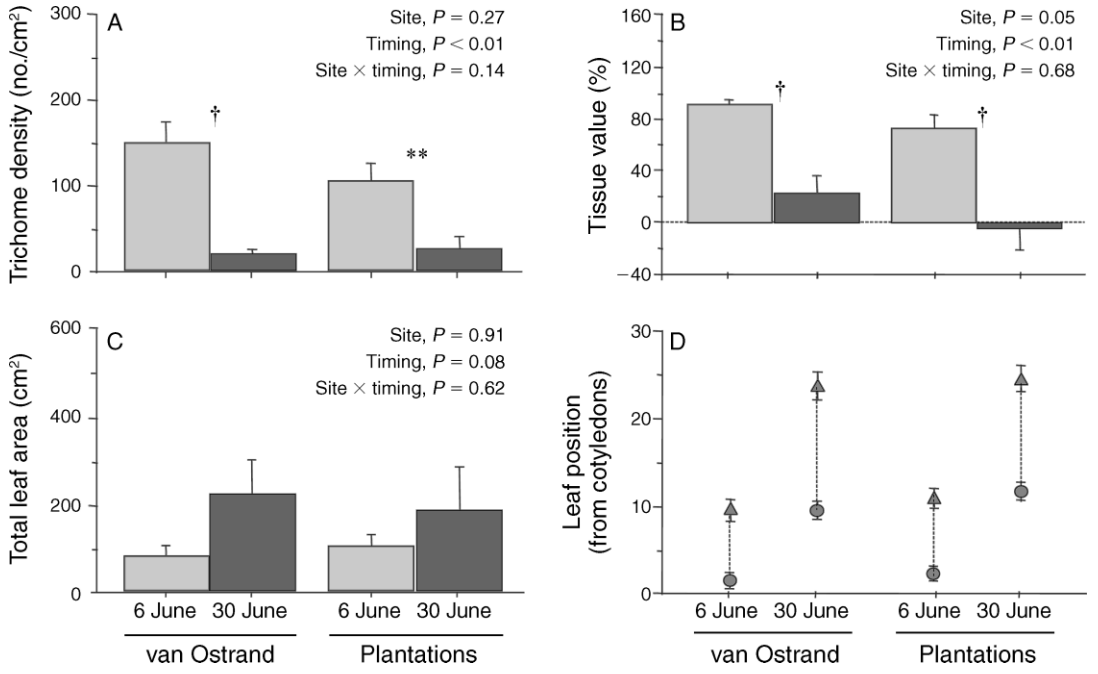

FIG. 5. Comparison of (A) trichome density, (B) tissue value (percentage reduction in growth relative to control), (C) total leaf area, and (D) leaf positions present for naturally growing *B. nigra* on 6 June and 30 June at the van Ostrand Road and Cornell Plantations populations in the field experiment. Each bar in A–C represents the mean (+SE) of 11 replicate plants. Each symbol in D represents the mean (±SE) of the lowest leaf node (circles) and highest leaf node (triangles), showing that the leaves present on 6 June had generally fallen off by 30 June. Each dotted line in D indicates the range of leaves present at that sampling date.
** $P < 0.01$; † $P < 0.005$.

Glucosinolates are mobile compounds that can be redistributed among plant tissues (Brudenell et al. 1999, Chen et al. 2001). In contrast, trichomes are allocated during leaf initiation and cannot be redistributed among tissues. Three variables therefore determine trichome densities of a leaf at any point in time: number initially allocated at a leaf position, rate of expansion of the leaf, and final leaf area. The importance of leaf area expansion to trichome densities was illustrated by *B. nigra* in the greenhouse (Fig. 3B), where trichome densities of the three highest leaves dropped precipitously between Day 21 and 34. However, leaves do typically have a static final size and they reach this full expansion rapidly (Harper 1989). For *B. nigra*, a leaf approaches its final size by the time the plant has produced three additional leaves (Fig. 3B; Traw and Dawson 2002*a*). As a result, leaves achieve a static trichome density, as can be seen for *B. kaber* leaves at the two sampling dates (Fig. 3A). When we looked only at these leaves, we found that there are significant differences in trichome densities at different leaf positions (Fig. 3A) and much of this variation can be explained by the distribution of tissue value (Fig. 2D). Based on these data, we conclude that allocation of trichomes among leaf positions is strongly associated with tissue value, independently of the process of leaf expansion.

While each individual leaf has a relatively simple trajectory of changing defense and tissue value over time, the plant as a whole is on a more complex journey through ontogenetic space. For example, the whole canopy of *B. nigra* had high trichome densities and high tissue value on 6 June, but both values were sharply lower on 30 June (Fig. 5). Interestingly, this dramatic drop in trichome density was not due to leaf expansion, because those leaves measured on 6 June had nearly all dropped from the plants by 30 June (Fig. 5D). Rather, plants produced a full new set of main stem leaves that all started out with fewer trichomes. Indeed, leaves at main stem positions 18 through 25 were glabrous. We lack sufficient resolution to interpret here the patterns at these uppermost leaf positions, but we could predict that leaves at those cauline positions would be found to have equivalent, low tissue values. However, we note that these leaves typically subtend branches of the inflorescence and may be governed under a different mandate.

Our finding that glucosinolates possess <1% of the nitrogen and carbon present in leaf tissues of both mustards is perhaps not surprising given that each molecule of glucosinolate contains only one atom of nitrogen and ~10 atoms of carbon. Glucosinolates may still be costly to produce, however, because other factors such as associated enzymes or autotoxicity of these compounds are known to incur substantial costs (Chew

and Rodman 1979, Siemens and Mitchell-Olds 1998). The low values do suggest that glucosinolate concentrations are not strictly limited by the abundance of these resources, which might explain why leaf glucosinolate concentrations don't predictably increase following nitrogen fertilization (Hugentobler and Renwick 1995, Rosen et al. 2005). Despite the small fraction of nitrogen in glucosinolates, there was still a strong relationship between glucosinolate per area and nitrogen per area (Fig. 2C), which is consistent with distribution of a defense to tissues with highest value. Our experimental evidence for a positive relationship between tissue value and nitrogen concentration is not surprising, but to our knowledge, has not been demonstrated previously. The pattern is expected based on the importance of nitrogen in plant growth and is supportive of the use of leaf nitrogen concentration as a proxy for tissue value (Chapin 1980, Evans 1989, Hilbert 1990).

In summary, glucosinolate concentration and trichome density reflected the distribution of underlying tissue values, as predicted by optimal defense theory (McKey 1974, 1979, Rhoades 1979). We observed this pattern both for leaves within the plant canopy in the greenhouse and for whole canopies sampled on 6 June and 30 June in the field. Tissue values were closely associated with nitrogen content but not carbon content, suggesting that nitrogen is a better currency for assessing trade-offs. However, less than one percent of the nitrogen or carbon in leaves was incorporated in glucosinolates, which may explain why glucosinolate concentrations do not typically increase following fertilization of plants with nitrogen.

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APPENDIX A

A figure showing glucosinolate concentration per area ($mmol/m^2$) for canopy thirds of *Brassica nigra* and *B. kaber* (*Ecological Archives* E089-042-A1).

APPENDIX B

Scatterplots of glucosinolate concentration per area ($mmol/m^2$) against tissue value and nitrogen per area (*Ecological Archives* E089-042-A2.