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Chemical diversity in *Brassica oleracea* affects biodiversity of insect herbivores

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Abstract. Intraspecific variation in plants plays a major role in the composition and diversity of the associated insect community. Resistance traits of plants are likely candidates mediating community composition. However, it is debated whether total concentrations of chemical compounds or specific compounds determine herbivore resistance, and how chemical diversity among plant genotypes in turn affects the composition of the associated herbivore community.

To study the role of specific chemical compounds in affecting the herbivore community, we used cultivated *Brassica oleracea*. The cultivars differ qualitatively in glucosinolate profile, i.e., foliar composition of different glucosinolate compounds, and only a little in total concentration of glucosinolates, the secondary metabolites specific for the Brassicaceae family. In field and laboratory experiments, we tested whether individual compounds explained differences in herbivore community composition, and whether herbivores with a similar degree of host plant specialization responded in a similar way to variation in glucosinolate profiles.

In the field *B. oleracea* cultivars differed widely in species richness and composition of the herbivore community, as well as in the density of insects they harbored. Plants with high concentrations of the short side chain alkenyl glucosinolate, glucoiberin, harbored low herbivore diversity. Higher biodiversity was found when plants had glucosinolate profiles containing high concentrations of glucosinolates with elongated side chains, which are biosynthetically linked to glucoiberin. Although glucosinolates are known to have differential effects on generalist and specialist herbivores, all herbivore species exhibited similar responses to the intraspecific variation in foliar glucosinolate profiles of the *B. oleracea* cultivars. This observation is supported by the correspondence between oviposition preferences of the specialist herbivore *Pieris rapae* and the generalist *Mamestra brassicae* in the field and the laboratory, using the same cultivars, and may be due to the relatively low concentrations of glucosinolates in cultivars. Our results show that variation in the concentration of short side-chain glucosinolates affects the composition of the herbivore community associated with brassicaceous plants.

Key words: biodiversity; *Brassica oleracea*; common garden experiment; direct defense; glucosinolates; *Mamestra brassicae*; *Pieris rapae*; Shannon-Wiener index; species richness.

INTRODUCTION

A central issue in ecology is to understand the interactions between organisms within communities. This understanding is of even greater importance since it has been established that human-driven changes in the environment reduce the planet's biodiversity and disrupt species interactions and ecosystem functioning (Tilman 1999 and references therein). Plant–insect associations comprise most of the interactions in terrestrial ecosystems and make up a major share of the biodiversity of agro-ecosystems that today cover a substantial part of our planet (Groombridge 1992). The study of these

associations has played an important role in understanding ecological and evolutionary processes that underlie community biodiversity (Whitham et al. 2006). Not only is insect biodiversity shaped directly by the diversity of plants or their spatial distribution over the landscape (Root 1973, Di Giulio et al. 2001, Tscharntke and Brandl 2004), insect diversity is also strongly affected by intraspecific variation in plants (Fritz and Price 1988, Maddox and Root 1990). This effect of intraspecific variation at the first trophic level may even scale up to the fourth trophic level in insect communities (Bukovinszky et al. 2008) and extend to vertebrate predators (Bailey et al. 2006, Gruner and Taylor 2006). Therefore, variation in plant traits may significantly influence ecosystem biodiversity (Dungey et al. 2000, Hochwender and Fritz 2004, Wimp et al. 2005, Poelman et al. 2008c). Plant traits responsible for effects

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on higher trophic level biodiversity have been identified in a few cases only, and include plant morphology and phytochemistry (Dungey et al. 2000, Johnson and Agrawal 2005, Bailey et al. 2006, Bangert et al. 2006). Variation in phytochemistry of plant genotypes may include quantitative or qualitative differences in only a few chemical compounds, and phytochemical composition may be heritable (van Dam and Vrieling 1994, Nielsen 1997, van Leur et al. 2006).

Intraspecific differences in foliar chemical profiles, i.e., the qualitative composition of the mix of phytochemicals, may have extensive consequences for ecosystem biodiversity and especially affect species that are in close reciprocal interaction with plants, such as herbivorous insects (Johnson and Agrawal 2005). These compounds include plant-family-specific secondary metabolites that may differentially determine host plant acceptance by herbivores. In the Brassicaceae, for example, high concentrations of glucosinolates and their breakdown products deter generalist herbivores, whereas specialists exploit these compounds in host plant selection (Chew 1988, Giamoustaris and Mithen 1995, Agrawal 2000, Hopkins et al. 2009). Although glucosinolate breakdown products may also negatively affect larval performance of specialist herbivores (Agrawal and Kurashige 2003), the native glucosides are often found to function as oviposition cues or feeding stimulants for specialist herbivores (David and Gardiner 1966, van Loon et al. 1992, 2002, Renwick 2002). Total concentration of glucosinolates of a plant may, therefore, influence the rate of attack by specialist and generalist herbivores in opposite directions. However, the glucosinolates comprise a considerable molecular diversity (Fahey et al. 2001), and intraspecific natural variation in foliar glucosinolate profiles of brassicaceous species is well documented (Mithen et al. 1995, Moyes et al. 2000, Kliebenstein et al. 2002, Gols et al. 2008a, b). When plant tissue is damaged, glucosinolates are hydrolyzed by myrosinases, yielding a variety of products such as isothiocyanates, thiocyanates, nitriles, epithionitriles, and oxazolidines (Bones and Rossiter 2006). Among other factors, the type of glucosinolate determines the composition of enzymatically formed products that differ in toxicity to herbivores (Bones and Rossiter 2006).

It has been hypothesized that well-defended plants have a large number of different compounds so that they are more likely to possess defense traits against the whole suite of attacking herbivores (Jones and Firn 1991). Plants seem not to be constrained in maintenance of this diversity (Koricheva et al. 2004), although compound levels may be negatively correlated when one compound is a biosynthetic precursor for the other (Kroymann et al. 2001). When these compounds differ in the extent to which they mediate host plant acceptance by herbivores, there are trade-offs between maintaining specific compounds and converting these into others (Jaenike 1990, van der Meijden 1996, Bidart-

Bouzat and Kliebenstein 2008). The debate whether specific compounds in plant defense profiles, or the total concentration of compounds belonging to the same class, determine herbivore resistance, remains to be resolved. Furthermore, how qualitative differences in phytochemistry in turn affect the composition of the associated herbivore community has received even less experimental attention (but see Bidart-Bouzat and Kliebenstein 2008 for a study on glucosinolate variation in *Arabidopsis thaliana*).

Here, we address whether differences in foliar glucosinolate profiles in *Brassica oleracea* affect insect herbivore biodiversity and the composition of the herbivore community in the field. As a model for intraspecific phytochemical variation, we used four cultivars of *B. oleracea* that differ in glucosinolate profiles (Poelman et al. 2008b) and gene transcription profiles in response to herbivory (Broekgaarden et al. 2007). Their glucosinolates all consist of a β -thio-glucose moiety, but have a variable side chain (Bones and Rossiter 2006). Alkenyl-glucosinolates have a side chain of variable length that is controlled by genes encoding methylthioalkylmalate synthase (*MAM*) (Kroymann et al. 2001). Transcription of *MAM*-like genes results in elongation of glucosinolate side chains from three carbon atoms (C_3) to C_4 , and up to C_8 , thereby affecting the ratio of, for example, C_3 to C_4 compounds in the glucosinolate profile (Kroymann et al. 2001, Field et al. 2004). The history of cultivation of *B. oleracea* has resulted in a decrease in total glucosinolate levels compared to wild type plants (Harvey et al. 2007, Gols et al. 2008a, b). However, cultivation has also led to a diversity of chemical profiles, with cultivars having relatively similar total concentrations of glucosinolates but different mixtures of C_3 and C_4 compounds in their profile. This variation can be readily used to test the effect of different chemical profiles that are within the range of those found within the natural variation of *B. oleracea* (Mithen et al. 1995, Moyes et al. 2000). Although we cannot discard variation in other plant traits affecting the host plant selectivity of herbivorous insects such as variation in biomass, wax layers, or primary compounds, we specifically test whether qualitative differences in glucosinolate profiles are important in affecting the composition of plant-associated herbivore communities.

In the field we tested the hypotheses that (1) specific compounds affect herbivore biodiversity, and (2) herbivores with a similar degree of host plant specialization covary in their response to variation in glucosinolate profiles. Additionally, in the laboratory we explicitly tested whether herbivore abundance and species composition on the different cultivars in the field correspond with their host plant preference to exclude the idea that they are a result of apparent competition between herbivores or differences in predation rates. We selected a specialist (*Pieris rapae*) and a generalist herbivore (*Mamestra brassicae*; see Plate 1). Both species were

TABLE 1. Herbivore species found on *Brassica oleracea* cultivars and their degree of host plant specialization.

Species	Order	Family	Feeding type	Host specificity
<i>Pieris rapae</i>	Lepidoptera	Pieridae	leaf chewer	specialist
<i>Pieris brassicae</i>	Lepidoptera	Pieridae	leaf chewer	specialist
<i>Plutella xylostella</i>	Lepidoptera	Yponomeutidae	leaf chewer	specialist
<i>Mamestra brassicae</i>	Lepidoptera	Noctuidae	leaf chewer	generalist
<i>Autographa gamma</i>	Lepidoptera	Noctuidae	leaf chewer	generalist
<i>Brevicoryne brassicae</i>	Hemiptera	Aphididae	phloem feeder	specialist
<i>Myzus persicae</i>	Hemiptera	Aphididae	phloem feeder	generalist
<i>Phyllotreta undulata</i>	Coleoptera	Chrysomelidae	leaf chewer	specialist
<i>Phyllotreta atra</i>	Coleoptera	Chrysomelidae	leaf chewer	specialist
<i>Aleyrodes proletella</i>	Hemiptera	Aleyrodidae	phloem feeder	specialist
<i>Thrips tabaci</i>	Thysanoptera	Thripidae	cell content feeder	generalist

recorded on the cultivars in the field. By choosing a specialist and a generalist, we could analyze whether herbivores with different degrees of host plant specialization differ in their responses to intraspecific variation in phytochemistry. Finally, we discuss the ecological implications of variation in glucosinolate profiles for ecosystem biodiversity.

METHODS

Plants and insects

We used the following white cabbage cultivars (*Brassica oleracea* var. *alba* L.; donors are given in brackets): Badger Shipper, Christmas Drumhead [Centre for Genetic Resources, CGN-Wageningen, The Netherlands], Lennox and Rivera [Bejo Zaden BV, Warmenhuizen, The Netherlands]. Additionally, we used *Brassica nigra* to confirm whether herbivores observed on *B. oleracea* were also present on a wild congener, and thus, whether our model system harbored a natural herbivore community. Because of its different phenology and life history compared to *B. oleracea*, we excluded *B. nigra* from all comparative analyses. Seeds of *B. nigra* were collected from a population northwest of Wageningen, The Netherlands, and propagated by open pollination several times after collection. Seeds of all plants were germinated on peat soil (Lentse potgrond No. 4; Lentse Potgrond BV, Lent, The Netherlands), and seeds for the common garden experiment were directly sown into peat soil cubes. Trays with soil cubes containing three-week-old seedlings were placed outside during the day to acclimate plants to field conditions. The plants were transplanted with their soil cubes to the soil at the experimental site when they were five weeks old. Plants used in greenhouse experiments were transferred as two-week-old seedlings to 1.45-L pots containing the same potting soil. Pots were placed in a greenhouse, providing the plants with a 16:8 (light : dark) photoperiod with SON-T light (moles of quanta; 500 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) (Philips, Eindhoven, The Netherlands) in addition to daylight, at 18–26°C and 40–70% relative humidity. When the plants were four weeks old, they were fertilized weekly by applying 100 mL of nutrient solution (Kristalon, Nutritech System, Moscow, Russia, concentration 3 g/L, [16N:6P:20K, 3 mg]) to the soil. We

used seven-week-old plants that had 12 true leaves for herbivore preference and performance experiments.

The two Lepidoptera species studied in preference and performance experiments, *Pieris rapae* L. (Pieridae) and *Mamestra brassicae* L. (Noctuidae), originated from the respective cultures maintained at the Laboratory of Entomology, Wageningen University. The host plant used for rearing was Brussels sprouts (*Brassica oleracea* var. *gemmifera* L. cultivar Cyrus). Cultures were kept in a climatized room at 20–22°C, 50–70% relative humidity, and a 16:8 hour light : dark photoperiod. *Mamestra brassicae* moths were offered filter paper as oviposition substrate, without contact with cabbage plants.

Common garden experiment

To assess how direct defense of field-grown plants affected their associated herbivore biodiversity in the field, we established a common garden experiment in an agricultural field in the vicinity of Wageningen, The Netherlands. Forty plots (6 × 6 m), each planted with one of the four *B. oleracea* cultivars or *Brassica nigra*, were established, according to a randomized design. Five-week-old seedlings rooted in their soil cubes were transplanted to the field in week 19 (9 May) of 2005. We planted 49 plants in a square of 7 × 7 plants per plot with spacing of 75 cm between plants. Plots were isolated from each other by a strip 6 m wide with a grass mixture of *Lolium* and *Poa* species. From week 23 (6 June) until week 37 (16 September), the central nine plants of each plot were surveyed weekly for the presence of naturally colonizing herbivorous insects. Each plant was surveyed by investigating both sides of all its leaves. We found 11 species of herbivorous insects that have all been reported previously to be affiliated with *Brassica* plants (Root 1973) (Table 1). Of these 11 herbivores, we could not accurately count the number of whiteflies and thrips without damaging the plants. These herbivores were therefore excluded from further analyses. For each week, the number of individuals per species counted on the nine plants of a plot was averaged. These values were used to calculate at plot level (a) the total abundance of herbivores; (b) their species richness; and two indices of biodiversity (c) the Shannon-Wiener index (H'); and (d) Simpson's diversity index ($1 - D$). The

latter two biodiversity indices describe herbivore diversity by incorporating both the richness of species as well as the evenness of their distribution. The Simpson's index of diversity (D) presents the chance that random draws of two individuals from a plot represent individuals of the same species. The lower the value of D , the higher is the diversity of the sample. Subtraction of D from unity ($1 - D$) is therefore often presented such that higher values correspond with higher diversity. The more abundant species receive a higher weight than rare species based on this index, and the index thus estimates evenness of a sample. The Shannon-Wiener index also takes into account the species richness and the abundance of each species, but does not give an interpretation in terms of chance of draws from a population. Both unique species and higher evenness increase the value. Both indices are the most commonly used indices to describe biodiversity.

Glucosinolate composition of field plants

The foliar glucosinolate content of each cultivar was quantified at plot level, five weeks after we started the herbivore survey. In week 28, we collected two leaf disks from a total of five plants for each plot, using a cork borer (diameter of 2.3 cm). The 10 leaf disks were pooled per plot and stored on ice. Within two hours after sampling the first plant, the leaf disks were transferred to a -80°C freezer. The frozen samples were freeze-dried and ground to a fine powder. Ground leaf material (100 mg/sample) was dissolved in methanol. The extract was desulphated on a DEAE-Sephadex A25 column (GE Healthcare, Chalfont St. Giles, UK) and the glucosinolate content was assessed by high-performance liquid chromatography (HPLC), using the method described by van Dam et al. (2004). Glucosinolate detection was performed with a photodiode array (PDA) detector (200–350 nm) with 229 nm as the integration wavelength. A sinigrin (sinigrin monohydrate; Sigma-Aldrich, St. Louis, Missouri, USA) concentration series was used as external standard. We used the correction factors at 229 nm from Buchner (1987) and the EC (European Community 1990) to calculate the concentrations of the glucosinolates. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with standards kindly provided by M. Reichelt, Max Planck Institute for Chemical Ecology, Jena, Germany, and a certified rapeseed standard (Community Bureau of Reference, Brussels, Belgium, code BCR-367R).

*Preference and performance of *P. rapae* and *M. brassicae**

The behavioral response to the four cultivars was investigated in detail for two of the Lepidoptera species found in the field that had a different degree of host plant specialization, i.e., the specialist *Pieris rapae* (Pieridae) and the generalist *Mamestra brassicae* (Noctuidae). We present their abundance over the study weeks in the field and quantified their oviposition

preference and larval performance in the laboratory. Statistical models constructed for the effect of cultivars on the abundance of these species over the study weeks in the field revealed that both species were present in similar numbers on cultivars Rivera and Lennox. We therefore did not include Lennox in the preference and performance experiments.

Growth rate, as a correlate of performance of *P. rapae* and *M. brassicae* caterpillars, was measured by placing single neonate caterpillars in petri dishes and feeding them *ad libitum* with leaf material of Badger Shipper, Christmas Drumhead, or Rivera. Every third day, the caterpillars were weighed on a microgram balance, and every other day the leaf material was replaced with fresh leaves of the same plant. The petri dishes were placed in a climate cabinet at $22^{\circ} \pm 2^{\circ}\text{C}$, $60\% \pm 10\%$ relative humidity, and a 16:8 hour light:dark photoperiod.

Host plant preference of *P. rapae* butterflies was tested in a three-choice situation between a plant of cultivar Badger Shipper, Christmas Drumhead, and Rivera. Freshly eclosed butterflies were placed in a cage where they were provided with a 10% sucrose solution and allowed to mate. Two days later, pairs of *P. rapae* were transferred to the experimental oviposition cages ($67 \times 50 \times 75$ cm; one male and one female butterfly per cage) that contained the three-choice situation in the same greenhouse compartment ($22^{\circ} \pm 2^{\circ}\text{C}$, $60\% \pm 10\%$ relative humidity, 16:8 hour light:dark photoperiod). During the experiment the oviposition cages were illuminated by SON-T 500-W sodium vapor lamps, in addition to natural daylight. Butterflies were allowed to oviposit on the plants from 0900 to 1400 hours. Thereafter plants were removed and eggs were counted. We repeated the experiment 14 times and established that the cultivars Badger Shipper and Rivera differed most in host plant acceptability for *P. rapae*.

The experimental setup used for *P. rapae* proved to be unsuitable for testing host plant preference of *M. brassicae* females that laid most of their egg clutches on the wood of the cage. We therefore used plastic cages (22 cm height, 13 cm diameter [Poelman et al. 2008a]) and studied the responses to the cultivars identified as extremes in the tests for *P. rapae*. For *M. brassicae*, we conducted 30 replicates of two-choice situations in plastic cages placed in a climatized room ($22^{\circ} \pm 2^{\circ}\text{C}$, $60\% \pm 10\%$ relative humidity, 16:8 hour light:dark photoperiod) by offering a choice between an excised leaf of the cultivars Badger Shipper and Rivera. Excised leaves of seven-week-old greenhouse plants were placed in glass vials containing tap water to keep the leaves turgid. After 24 h, leaves were removed and the number of egg batches as well as the number of eggs were counted.

Statistical analysis

We used repeated measurements mixed models to determine whether plants with different qualitative glucosinolate profiles harbored different herbivore

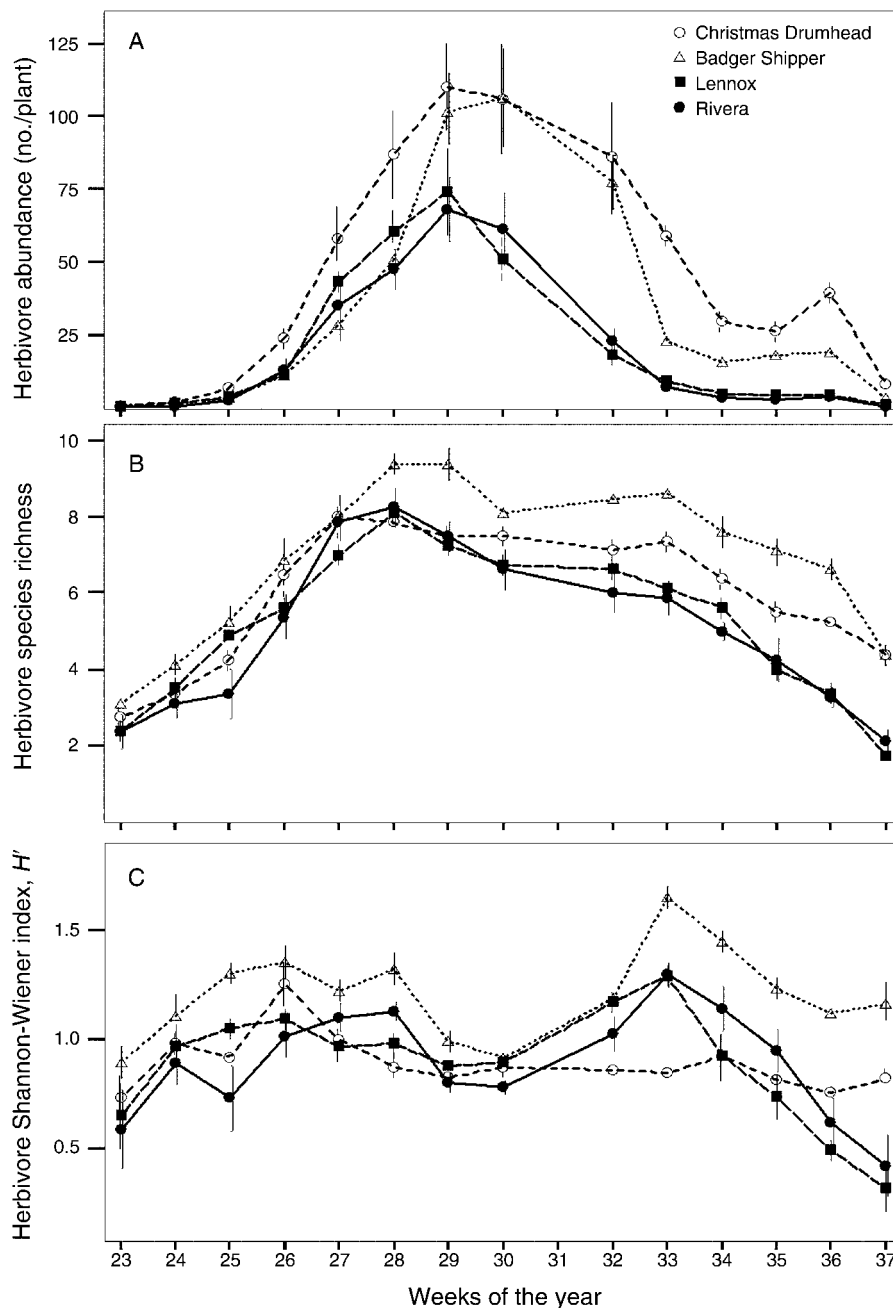


FIG. 1. Differences between cultivars of *Brassica oleracea* in (A) total herbivore abundance (number of individuals/plant), (B) species richness (number of species), and (C) Shannon-Wiener index (H') of herbivore communities in the field. Values are shown as mean \pm SE. Cultivars: Badger Shipper (open triangles), Christmas Drumhead (open circles), Lennox (solid squares), Rivera (solid circles).

communities. At the plot level, the abundance of *P. rapae*, *M. brassicae*, total number of herbivores, species richness, the Shannon-Wiener index (H') and the Simpson's diversity index ($1 - D$) were each modeled using the Proc Mixed procedure with repeated structure type AR(1) of SAS version 9.1 (SAS 2006). In each model we included the factors cultivar, time, and their interaction. We specifically addressed the question

whether differences in specific chemical compounds between cultivars could explain differences in herbivore biodiversity. We correlated phytochemistry assessed at the plot level with biodiversity in the study plots. To describe the most important qualitative differences in glucosinolate profiles between cultivars, we used principal component analysis (PCA) to analyze the multivariate chemical data, related to 10 glucosinolate

TABLE 2. Cultivar and time effects on herbivore abundance, species richness, Shannon-Wiener diversity index (H'), Simpson's diversity index ($1 - D$), and the abundance of two Lepidoptera species.

Factor	df	Abundance		Richness		Shannon-Wiener index		Simpson's diversity index		<i>P. rapae</i> abundance		<i>M. brassicae</i> abundance	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Cultivar (<i>C</i>)	3	44.21	<0.001	28.94	<0.001	42.41	<0.001	29.30	<0.001	3.63	0.025	5.86	0.003
Time (<i>T</i>)	13	124.41	<0.001	53.46	<0.001	15.39	<0.001	9.85	<0.001	81.59	<0.001	30.40	<0.001
<i>C</i> × <i>T</i>	16	3.05	<0.001	1.59	<0.001	2.66	<0.001	2.06	<0.001	0.95	0.56	1.93	0.001

Notes: For each factor we calculated *F* statistics in mixed models. Values in boldface type indicate significant effects at $P < 0.05$.

compounds found in the cultivars. The first PC axis explained 72% of the variation in glucosinolate content with an eigenvalue of 6.47 and correlated with an increase in concentration of glucoiberin and a decrease of glucoraphanin (loading scores of glucoiberin, 0.93, and glucoraphanin, -0.19 on PC1). Other PC axes had eigenvalues lower than 1 and explained <10% of the total variation. The scores of each field plot on the first axis were used in General Linear Models as a covariate to test for a correlation with herbivore abundance, richness, and biodiversity. Cultivar identity was included as a fixed factor into these models. We included the interaction between cultivar and glucosinolate PC score into the model to test for cultivar differences in relationship between glucosinolates and diversity parameters. Per plot, the scores for herbivore abundance, richness, and biodiversity are averaged values over the 15 study weeks and were included as normally distributed dependent variables.

To determine which species in the herbivore communities were affected most by cultivar differences, we constructed principal response curves (PRC) using the CANOCO software package 4.51 (Ter Braak 1988). The PRC method uses partial redundancy analysis (RDA) and plots the first principal component of the treatment effect against time by contrasting each treatment against a preset control. We set the cultivar Rivera as a control, and thus the vertical axis of the PRC diagram shows the contrast of the other three cultivars with Rivera. The PRC method is constrained and extracts information

only from the part of the variance that is explained by the environmental factor (cultivar in our case), and implements time (weeks in our case) as a covariable. Since high abundance values influence the result of the PRC analysis more strongly than low abundance values, we log-transformed the species abundance scores. To test for significance of the principal component we used Monte Carlo permutation tests. The reported *P* value is based on 999 permutations. Associated with the PRC diagram, we present the set of species weights on the first principal component. The species weight describes the relative difference of abundance between cultivars for each herbivore species. A positive weight can be interpreted as a larger abundance of the particular species on the cultivar that also has a positive PRC score (Lepš and Šmilauer 2003). To test whether specialist and generalist herbivores differed in their response to the cultivars, we first performed a cluster analysis. Cluster analysis creates a dendrogram that depicts how herbivores covaried in abundance over the monitored field plots, with all the herbivore species individually plotted on the tips of the dendrogram. The species that link most closely with each other on the dendrogram covary most strongly in their abundance across field plots. To perform the analyses, we calculated the mean abundance of each herbivore for each of the field plots that were monitored over 15 weeks. The abundance scores were standardized so that the abundance of each herbivore species had a mean of zero and a standard deviation of one. The standardized abundance values of each species

TABLE 3. Glucosinolate profiles ($\mu\text{mol/g}$ dry mass; means with SE in parentheses) of four cultivars of *Brassica oleracea* and a wild population of *Brassica nigra*.

Species and cultivars	IBE†	SIN†	PRO†	RAPH‡	4OHGBC¶	GBN§	GBC¶
<i>Brassica oleracea</i>							
Badger Shipper	1.35 (0.26)	0.11 (0.05)	0.09 (0.01)	1.74 (0.23)	0.03 (0.01)	0.07 (0.01)	0.84 (0.11)
Christmas Drumhead	1.87 (0.22)	0.77 (0.34)	0.20 (0.04)	1.48 (0.30)	0.02 (0.01)	0.13 (0.01)	2.21 (0.24)
Lennox	5.64 (0.38)	0.67 (0.12)	0.06 (0.01)	0.45 (0.04)	0.02 (0.01)	0.10 (0.01)	1.54 (0.33)
Rivera	4.52 (0.60)	0.63 (0.12)	0.08 (0.02)	0.54 (0.09)	0.01 (0.02)	0.12 (0.02)	2.30 (0.62)
<i>Brassica nigra</i>	0.07 (0.05)	28.95 (2.60)		0.09 (0.06)		0.01 (0.01)	0.12 (0.05)

† Alkenyl C₃ glucosinolates: 3-methylsulfinylpropyl glucosinolate (glucoiberin [IBE]), 2-propenyl glucosinolate (sinigrin [SIN]), *R*-2-hydroxy-3-butenyl glucosinolate (progoitrin [PRO]).

‡ Alkenyl C₄ glucosinolates: 4-methylsulfinylbutyl glucosinolate (glucoraphanin [RAPH]).

§ Alkenyl C₅ glucosinolates 4-pentenyl glucosinolate (glucobrassicinapin [GBN]).

¶ Indolyl glucosinolates: 4-hydroxy-3-indolylmethyl glucosinolate (4-hydroxyglucobrassicin [4OHGBC]), 3-indolylmethyl glucosinolate (glucobrassicin [GBC]), 4-methoxy-3-indolylmethyl glucosinolate (4-methoxyglucobrassicin [MGBC]), 1-methoxy-3-indolylmethyl glucosinolate (Neo-glucobrassicin [NEOGBC]).

per field plot were then used in the calculation of the Pearson's correlation matrix, and we used a sequential Bonferroni correction for significance in multiple comparisons (Appendix). We used the Ward's linkage method to determine the linkage and distance between herbivore species. To statistically test whether herbivores with a similar degree of host plant specialization correlated more strongly in their abundance distribution than species that differ in degree of host plant specialization, we used ANOVA tests on the Pearson's correlation scores. We tested whether r values within generalist and specialist herbivores were higher than r values between these two herbivore groups.

The laboratory experiments to measure preference and performance were analyzed with the statistical program SPSS 12.0 (SPSS 2006). We used G statistics to analyze the oviposition preference of *P. rapae* in choice tests consisting of three cultivars. An oviposition preference of *M. brassicae* in two-choice tests was analyzed by applying Wilcoxon matched-pair signed-ranks tests for both the number of egg batches and number of eggs. The effect of cultivar on growth rate of caterpillars of both species was analyzed with repeated measurements ANOVA for the log-normalized mass of caterpillars.

RESULTS

In the field we found 11 herbivore species (Table 1) that were all attacking the wild congener *B. nigra* as well as the four cultivars of *B. oleracea*. Although most Lepidoptera species were present in much lower numbers on *B. nigra*, our model system of cultivated plants harbored an herbivore community comparable to congeneric wild-type plants. Most of the herbivore species that we recorded in our plots were found to have two or more generations over the study period. Synchronization of generations between different herbivore species resulted in highest species richness and herbivore abundance during weeks 28 and 29 (Fig. 1). Cultivars of *B. oleracea* differed substantially in the total abundance of herbivores, species richness, Shannon-Wiener index (H'), and Simpson's index of diversity

(Fig. 1, Table 2). Cultivar Badger Shipper harbored high herbivore abundance, had the highest species richness, and highest herbivore biodiversity. Cultivar Christmas Drumhead was similar to Badger Shipper in herbivore abundance, had intermediate species richness, but relatively low biodiversity. This was a result of the susceptibility of this cultivar to a particular herbivore species, the cabbage aphid *Brevicoryne brassicae*, which reached a high population size on Christmas Drumhead compared to Rivera and Lennox (Broekgaarden et al. 2008). The cultivars Rivera and Lennox did not differ significantly for any of the herbivore diversity parameters (Fig. 1). Both cultivars were characterized by having low numbers of herbivores and fewer herbivore species per plant (Fig. 1), although all herbivore species were present on these cultivars.

We quantified the foliar glucosinolate content of plants grown under field conditions at the peak of herbivore diversity (week 28) to test whether foliar concentration of specific glucosinolate compounds or total glucosinolate concentration of the cultivars could be responsible for the difference in herbivore biodiversity. As expected from directional selection by humans against high concentrations of glucosinolates, cultivars differed more in the glucosinolate profile than in the total concentration of foliar glucosinolates (Table 3). Total glucosinolate concentration did not significantly affect herbivore abundance, richness, or biodiversity (GLM, $df = 1$, abundance, $\chi^2 = 2.97$, $P = 0.09$; richness, $\chi^2 = 3.18$, $P = 0.08$; Shannon-Wiener index, $\chi^2 = 0.40$, $P = 0.53$). PCA analysis on the glucosinolate profiles of plants identified that the concentration of glucoiberin (3-methylsulfinylpropylglucosinolate) that has a C_3 side chain, negatively correlated with the concentration of glucoraphanin (4-methylsulfinylbutylglucosinolate) that has a C_4 side chain. Rivera and Lennox had high concentrations of the short side chain compound glucoiberin, whereas the concentration of this compound was lower in Christmas Drumhead and even more so in Badger Shipper. The negative association between the two compounds had a high loading on the first PC axis (glucoiberin, 0.93; glucoraphanin, -0.19) that explained 72% of the variation in foliar glucosinolate content of the cultivars. For all three biodiversity parameters we found that cultivars harbored a different degree of diversity (GLM, $df = 3$, abundance, $\chi^2 = 29.221$, $P < 0.001$; richness, $\chi^2 = 31.772$, $P < 0.001$; Shannon-Wiener index, $\chi^2 = 16.192$, $P = 0.001$). The scores of field plots on the first phytochemical PC, included as a covariate into the model, correlated negatively with the total herbivore abundance and species richness of the field plots (Fig. 2A, B) (GLM, $df = 1$, abundance: $\chi^2 = 3.904$, $P = 0.048$; richness: $\chi^2 = 4.722$, $P = 0.03$). However, the glucosinolate profile of field plants did not significantly correlate with the Shannon-Wiener index (Fig. 2C) (GLM, $df = 1$, $\chi^2 = 0.018$, $P = 0.89$). The interaction between glucosinolate profiles and cultivars was not significant for each of the

TABLE 3. Extended.

MGBC¶	NEOGBC¶	Total
0.03 (0.00)	0.90 (0.15)	5.14 (0.62)
0.04 (0.00)	0.04 (0.01)	6.77 (0.70)
0.03 (0.00)	0.06 (0.01)	8.56 (0.60)
0.03 (0.01)	0.12 (0.03)	8.36 (1.47)
	0.01 (0.01)	29.25 (2.62)

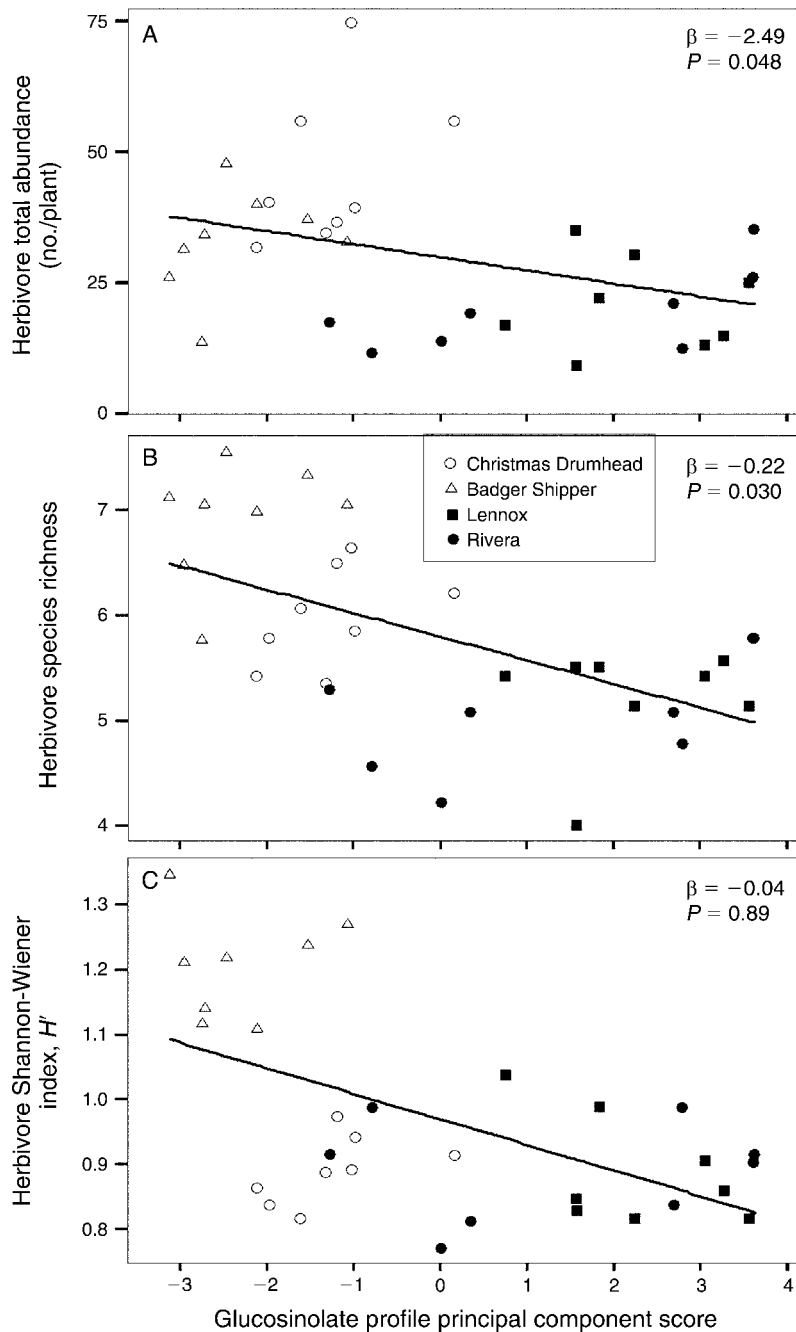


FIG. 2. Correlations between the scores of each plot on the glucosinolate first principal component and (A) total herbivore abundance, (B) species richness, and (C) Shannon-Wiener index of biodiversity of field plots. The principal component explains 72% of the variation in glucosinolate content of the four *B. oleracea* cultivars, Badger Shipper (open triangles), Christmas Drumhead (open circles), Lennox (solid squares), and Rivera (solid circles), and it correlates with increased concentrations of the glucosinolate glucoiberin and decreased concentration of glucoraphanin.

biodiversity parameters (GLM, $df = 3$, abundance, $\chi^2 = 2.610$, $P = 0.46$; richness, $\chi^2 = 0.777$, $P = 0.86$; Shannon-Wiener index, $\chi^2 = 4.771$, $P = 0.19$). This means that an increase of glucosinolate side chain length, rather than total glucosinolate concentration, was indeed associated with a higher susceptibility to herbivores and consequently enhanced herbivore biodiversity on these plants.

Response by generalist vs. specialist herbivores

To identify whether generalist or specialist herbivore species were responsible for the lower abundance and biodiversity on Rivera and Lennox compared to Badger Shipper and Christmas Drumhead plants, we contrasted herbivore communities on cultivars by principal re-

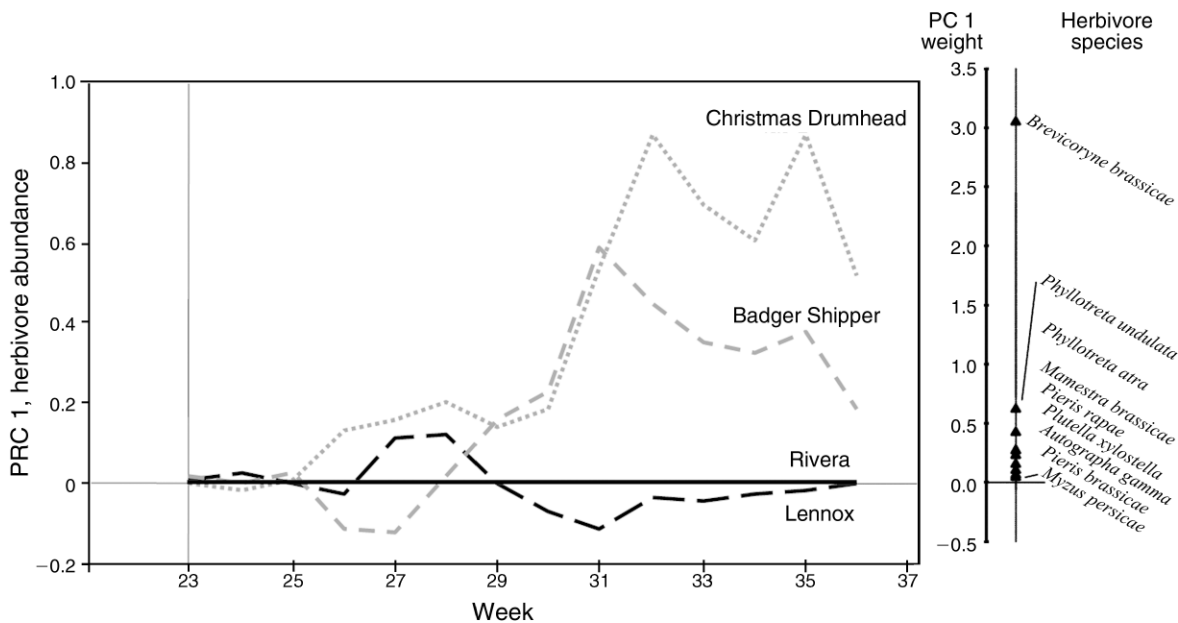


FIG. 3. Principal response curve (PRC) for herbivore species abundance over time for four cultivars of *B. oleracea*. Cultivar differences are related to the control cultivar Rivera (black solid line). Species weights on the first principal component are depicted in a score plot on the right side of the figure. Cultivar Lennox (dashed black line) is similar in species composition to the control Rivera. The cultivars Christmas Drumhead (dashed gray line) and Badger Shipper (dotted gray line) have higher abundance of all herbivore species.

sponse curves (PRC). The PRC analysis identified that all herbivore species were more abundant on Badger Shipper and Christmas Drumhead compared to Rivera and Lennox (the first RDA axis explained 9.6%, Monte Carlo permutation test $F = 193.398$, $P < 0.001$) (Fig. 3), although the magnitude of the response differed per species. The loadings of herbivore species on the first RDA axis indicated that the cabbage aphid (*B. brassicae*) showed the largest difference in abundance between cultivars, whereas the responses of other species to the cultivar differences were less strong. Cluster analysis for the covariation of herbivores in their abundance distribution over plots revealed that generalist and specialist herbivores did not significantly differ

in their response to cultivars, i.e., all herbivores positively covaried in their response (ANOVA, $df = 1, 35$, $F = 2.955$, $P = 0.10$) (Fig. 4; Appendix).

Analysis of the abundance of a specialist (*P. rapae*) and generalist (*M. brassicae*) herbivore over the study weeks in the field confirmed the similarity in response of the two types of herbivores. Both *P. rapae* and *M. brassicae* were more abundant on Badger Shipper and Christmas Drumhead than on Rivera or Lennox (Fig. 5, Table 2). These differences corresponded with differences in oviposition preference of adult butterflies and moths as recorded under controlled laboratory conditions (Fig. 6). *Pieris rapae* and *M. brassicae* both preferred Badger Shipper plants over other cultivars

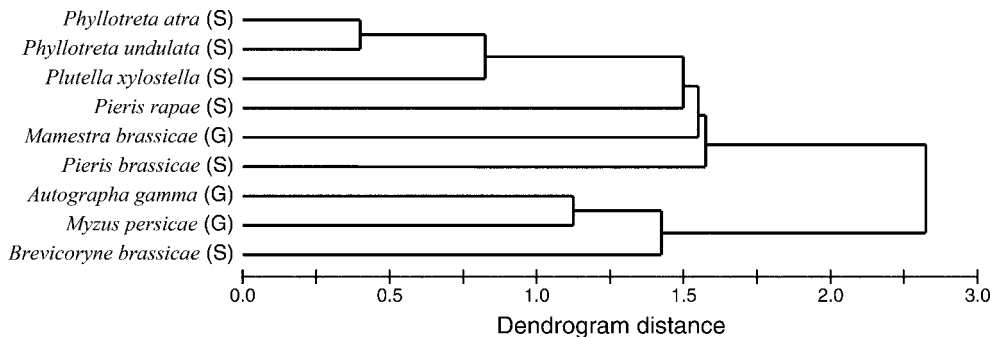


FIG. 4. Dendrogram from cluster analysis using Ward's linkage method depicting the covariation in abundance of herbivorous insects, generalists (G) and specialists (S), among field plots of four *B. oleracea* cultivars. Species that link with the lowest distance scores are most similar in their distribution over the field plots.

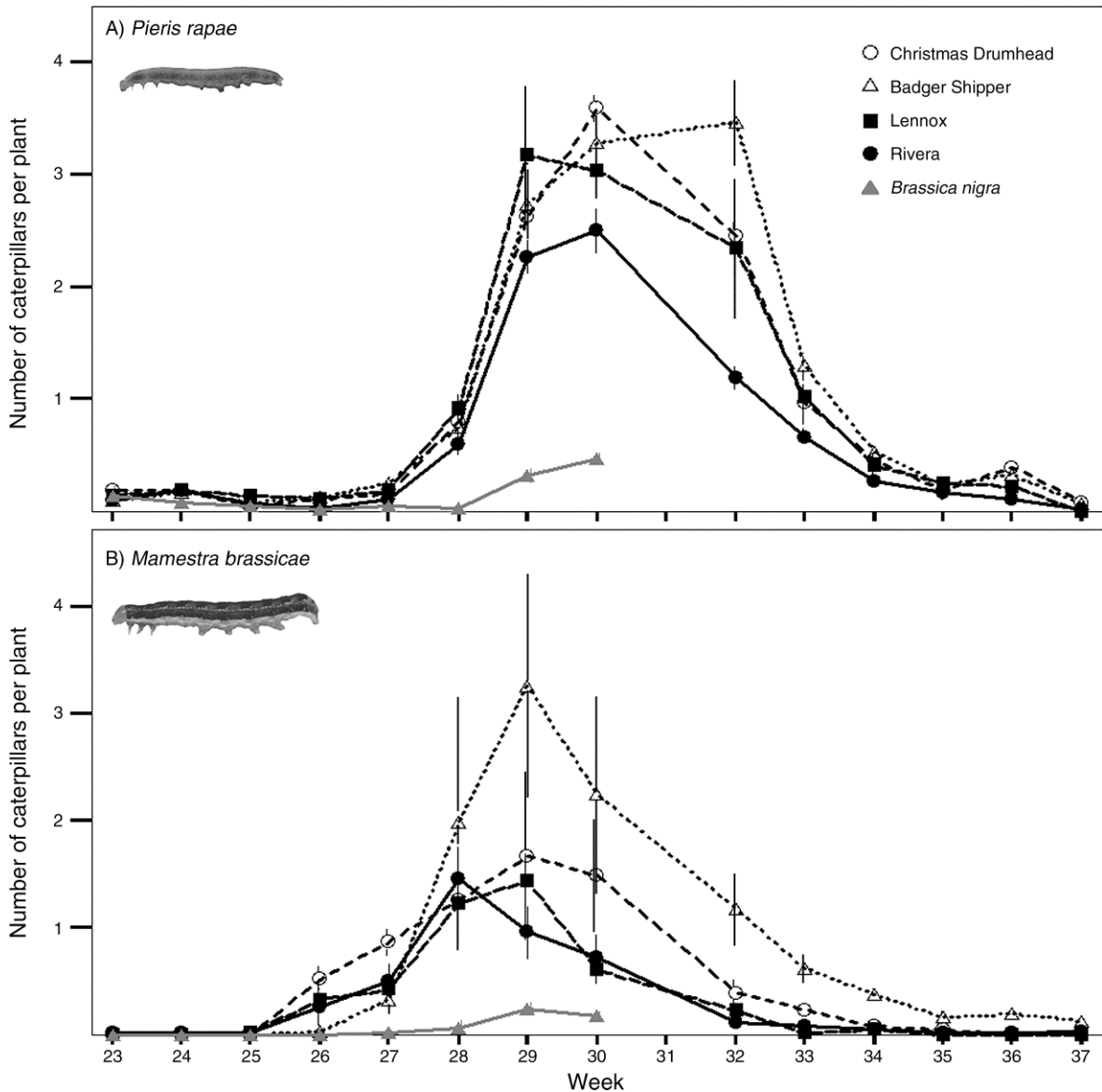


FIG. 5. Results from the common garden experiment: (A) population development of caterpillars of (A) *P. rapae* and (B) *M. brassicae* on *B. nigra* (gray line, solid triangles) and four cultivars of *B. oleracea*: Badger Shipper (open triangles), Christmas Drumhead (open circles), Lennox (solid squares), and Rivera (solid circles). Error bars indicate \pm SE (based on the field plot level).

(*P. rapae*, G statistics, $\chi^2 = 80.24$, $P < 0.001$; *M. brassicae*, Wilcoxon matched-pair signed-ranks test, egg batches: $Z = -2.230$, $P = 0.019$; eggs, $Z = -2.983$, $P = 0.003$). Badger Shipper plants also sustained higher performance of caterpillars of both herbivores than other cultivars (repeated measurements ANOVA, *P. rapae*, cultivar, $df = 2$, 233, $F = 8.97$, $P < 0.001$; time, $df = 5$, 233, $F = 2023.51$, $P < 0.001$; time \times cultivar, $df = 10$, 233, $F = 0.035$, $P = 0.176$; *M. brassicae*, cultivar, $df = 2$, 332, $F = 17.30$, $P < 0.001$; time, $df = 8$, 332, $F = 2527.22$, $P < 0.001$; time \times cultivar, $df = 16$, 332, $F = 3.19$, $P = 0.009$). With similar rank order as oviposition preference

and abundance in the field, herbivores performed better on Badger Shipper plants (Fig. 6).

DISCUSSION

Our field data clearly show that intraspecific variation in secondary metabolite profiles of plants affects the abundance, richness, and species composition of the insect herbivore community. Rather than the total concentration of glucosinolates in cultivated plants, high concentrations of the C_3 compound glucoiberin (3-methylsulfinylpropyl glucosinolate) were negatively correlated with herbivore abundance, species richness,

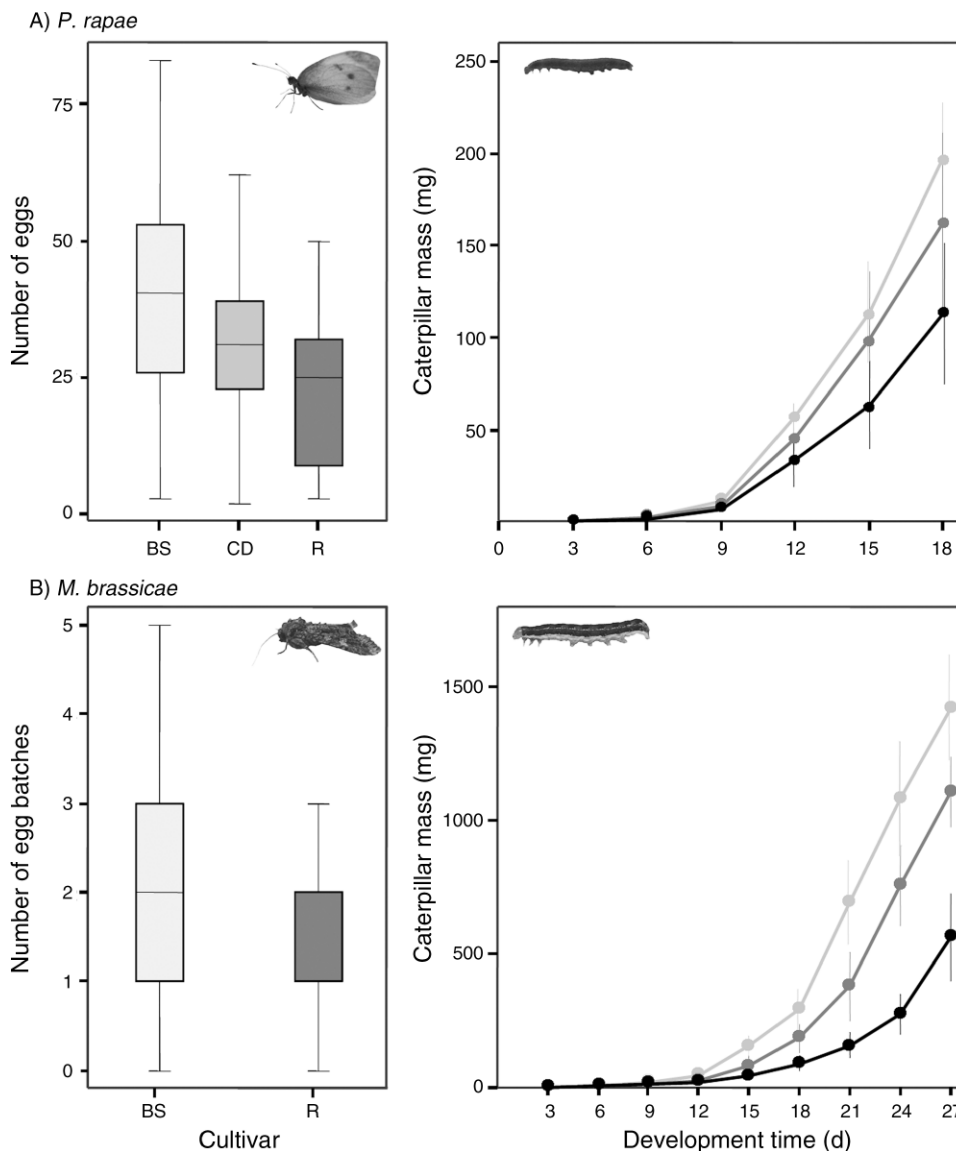


FIG. 6. Oviposition preference for cultivars (left) and caterpillar performance (right) for (A) the specialist butterfly *P. rapae*, and (B) the generalist moth *M. brassicae*. Both species show similar preference and performance differences on *B. oleracea* cultivars: Badger Shipper (BS, light gray), Christmas Drumhead (CD, medium gray), and Rivera (R, dark gray). For the graphs on the left, the height of the boxes show the first to third quartile of the interquartile range; the horizontal line within the box is the median; and the whiskers are the data minima and maxima.

and herbivore biodiversity. When glucosinolate profiles were dominated by the C₄ compound glucoraphanin (4-methylsulfinylbutyl glucosinolate), which is only one elongation step removed from the C₃ compounds, plants harbored higher herbivore biodiversity. Specialists and generalists were not differentially affected by glucosinolate profiles. The underlying mechanisms were revealed by the laboratory experiments. Both the specialist (*P. rapae*) and the generalist (*M. brassicae*) lepidopteran herbivore preferred to oviposit on plants with lower C₃ glucosinolate concentrations, and larvae had a reduced performance on plants with high concentrations of these compounds.

Herbivore community composition mediated by foliar glucosinolate profile

The effect of plant genotype on the community composition of higher trophic levels has been reported for an increasing number of plant species (Fritz and Price 1988, Maddox and Root 1990, Moyes et al. 2000, Johnson and Agrawal 2005, 2007, Bukovinszky et al. 2008). Only a few studies have identified the plant traits that mediate the diversity of a plant-associated insect community. In *Eucalyptus* trees it was shown that high terpenoid concentrations in leaves negatively correlated with arthropod biodiversity (Dungey et al. 2000). Gall

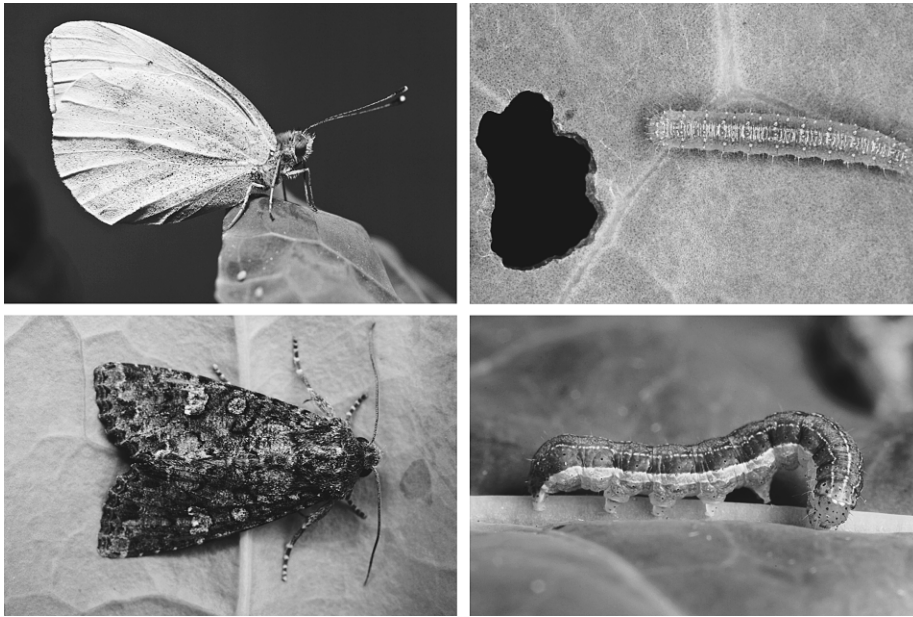


PLATE 1. Adult and larval stage of two of the common herbivores on *Brassica oleracea*: the specialist *Pieris rapae* (top) and the generalist *Mamestra brassicae* (bottom). Photo credits: Tibor Bukovinszky.

aphid density, predator abundance, and consequently total community biodiversity were negatively correlated with high leaf tannin concentration in cottonwood, *Populus* spp. (Bailey et al. 2006, Bangert et al. 2006). In these studies only total concentration of a group of chemicals has been considered to be responsible for effects on biodiversity. However, plants may maintain a diversity of phytochemicals to confer a greater chance of resistance against all possible attackers (Jones and Firn 1991). In an evolutionary arms race with multiple attackers that adapt to specific chemical compounds, plants are under strong selection to produce novel compounds and maintain a diversity of compounds in their phytochemical profile. Specific compounds in this profile may be active against specific herbivore species, thereby shaping the herbivore community on the plant.

Using our model system of cultivars that differed widely in glucosinolate profile, but not total concentration of glucosinolates, we found that high concentrations of the C₃ compound glucoiberin in leaves of *B. oleracea* negatively affected herbivore abundance, richness, and herbivore community composition. A higher concentration of the C₃ compound glucoiberin was associated with a lower concentration of the C₄ compound glucoraphanin as identified by the first principal component (PC) in the PCA analysis. Field et al. (2004) found that the ratio of C₃ and C₄ glucosinolates is genetically determined. By overexpressing genes encoding methylthioalkylmalate synthase (*MAM*) in *Arabidopsis*, concentrations of C₃ glucosinolates decreased without changes in total glucosinolate concentration. The side chain of the C₃ glucosinolates was elongated, giving rise to C₄ compounds. The side

chain-elongation of particular compounds in the chemical profile of *B. oleracea* positively affected the abundance and diversity of herbivores on the plant. However, when controlling for differences in glucosinolate profiles that significantly correlated with herbivore diversity in the statistical model, the cultivars remained significantly different from each other in effect on herbivore diversity parameters. Thus, other differences between cultivars that were not tracked, such as surface waxes, water content, or plant architecture, also contributed to differences in herbivore diversity among cultivars. Plant size, architecture, and phenological traits have all been shown to affect insect community composition, and may even be more important than chemical defense traits, as was shown for evening primrose (*Oenothera biennis*) (Johnson and Agrawal 2005). In 2007 we conducted a field study using the same cultivars as presented in this study, but in the former study we destructively sampled the plants to assess whether plant biomass or number of leaves explained differences in herbivore abundance between the cultivars (C. Broekgaarden, E. H. Poelman, R. E. Voorrips, M. Dicke, and B. Vosman, *unpublished manuscript*). Even though cultivars differed in number of leaves and fresh mass, neither parameter correlated with herbivore abundance (C. Broekgaarden, E. H. Poelman, R. E. Voorrips, M. Dicke, and B. Vosman, *unpublished manuscript*). Therefore, these morphological traits are not likely to explain the differences in diversity parameters presented here. Natural geographic variation within brassicaceous species consists of large variation in total glucosinolate concentration and includes large differences in glucosinolate profiles that differ in ratios

of compounds with different side chain length (Mithen et al. 1995, Kliebenstein et al. 2002, Gols et al. 2008a, b). Our results suggest that these ratios feed back differentially to the herbivore community. One possible reason why this chemical variation is maintained is that herbivore species show differential responses to the different concentrations of particular compounds.

Similarity in responses of specialist and generalist herbivores

Qualitative variation in glucosinolate profiles may differentially affect herbivore performance or host plant acceptance (Bidart-Bouzat and Kliebenstein 2008, van Leur et al. 2008). Giamoustaris and Mithen (1995) found that short side chain glucosinolates, such as the C₃ compound glucoiberin, negatively affected host plant selection by generalist herbivores. At the same time, some specialist herbivores preferred plants with high concentrations of short side chain glucosinolates. However, contrary to Giamoustaris and Mithen (1995) we found that generalist and specialist species covaried in the direction of response to the glucosinolate profiles. In our field study both specialists and generalists were less abundant on plants containing high concentrations of the C₃ compound glucoiberin. *Pieris rapae* and *M. brassicae*, specialist and generalist lepidopterans, respectively, both preferred to oviposit on greenhouse-grown plants with low glucoiberin concentration under laboratory conditions. Furthermore, the performance of the specialist as well as the generalist corresponded negatively with glucoiberin concentration of plants grown in the greenhouse. Although all herbivore species responded similarly in terms of abundance on the cultivars, some species responded more strongly than others. The specialist cabbage aphid *Brevicoryne brassicae* responded more strongly to cultivar differences than most of the other species, but also avoided high concentrations of glucoiberin just as the other herbivores did. The low total glucosinolate concentrations that resulted from directional selection by humans against these pungent compounds may have reduced the concentration of glucosinolates in our cultivar model system below a threshold for natural discriminatory behavior of herbivores, and might explain the unexpected positive covariation between generalist and specialist herbivores found.

In a meta-analysis on covariation among herbivore responses, Leimu and Koricheva (2006) showed that generalist organisms covaried among themselves, as did specialists, and species that differ in degree of host plant specialization covaried less often. These patterns were, however, pronounced in mammalian herbivores and pathogens, and weaker for arthropods. Although these findings provide little support for host plant specificity of arthropods as a factor responsible for differential responses to plant genetic variation (Johnson and Agrawal 2007), support comes from studies on natural plant hybrids and their backcrosses (Whitham et al.

1999). Generalist herbivore species that are adapted to using a broad range of host plant species were found to be more common on hybrid plants that are intermediate to their parent host plants. Specialists were less common on hybrids that resembled their parental host plant less (reviewed by Whitham et al. 1999), suggesting that high concentrations of host-plant-specific chemicals could lead to negatively correlated responses of generalists and specialists. When other studies are reviewed, there is no distinct pattern in total herbivorous insect community analyses of covariation among specialist and generalist insects, or the opposite, contrasting responses, to variation in plant defense. Nevertheless, our data clearly show that variation in glucosinolate profiles of a brassicaceous plant species rather than variation in total concentration of glucosinolates affected plant-associated insect diversity.

Conclusion

Our results show that intraspecific variation in foliar glucosinolate profiles not only feed back on herbivore abundance and species richness, but may thereby affect herbivore community composition and biodiversity. More importantly, the levels of specific compounds that are biosynthetically linked offer an explanation for these observations. The degree of host plant specificity of herbivores did not affect the direction of response of herbivores to variation in plant defense in our model system of cultivars with relatively low total glucosinolate concentration. Especially in agroecosystems, glucosinolate profiles of brassicaceous plants therefore play a major role in structuring the diversity of the associated herbivore community. Biodiversity of herbivores has been identified to strongly affect higher trophic level biodiversity. And thereby monocultures in agro-ecosystems, which cover a major part of our globe, profoundly structure the entire biodiversity at the landscape level. Through identifying a role for specific compounds in a phytochemical profile of *Brassica* cultivars, it becomes a challenge to further elucidate the role of the C₃ and C₄ compound ratio in natural variation of brassicaceous plants. The regulation of alkyl glucosinolate chain elongation by a specific gene family (*MAM*-like genes) in the brassicaceous species *Arabidopsis thaliana* will allow manipulation of compound ratios in wild-type brassicaceous plants, with maintenance of the natural, high concentrations of glucosinolates, and provide opportunities to further elucidate a role for specific chemical compounds in nature.

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APPENDIX

Pearson's correlation matrix for covariation among the abundance of herbivore species on field plots of *Brassica oleracea* cultivars (*Ecological Archives* E090-129-A1).