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Dealing with double trouble: consequences of single and double herbivory in Brassica juncea — Source link

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Abstract:	In their natural environment, plants are often attacked simultaneously by many insect species. The specificity of induced plant responses that is reported after single herbivore attacks may be compromised under double herbivory and this may influence later arriving herbivores. The present study focuses on the dynamics of induced plant responses induced by single and double herbivory, and their effects on successive herbivores. Morphological (leaf length, area and trichome density) and chemical changes (leaf alkenyl and indole glucosinolates) in Brassica juncea were evaluated four, ten, fourteen and twenty days after damage by the specialist Plutella xylostella alone, or together with the generalist Spodoptera litura. To assess the biological effect of the plant's responses, the preference and performance of both herbivores on previously induced plants were measured. We found that alkenyl glucosinolates were induced twenty days after damage by P. xylostella alone, whereas their levels were elevated as early as four days after double herbivory. Trichome density was increased in both treatments, but was higher after double herbivory. Interestingly, there was an overall decrease in indole glucosinolates and an increase in leaf size due to damage by P. xylostella, which was not observed during double damage. S. litura preferred and performed better on undamaged plants, whereas P. xylostella preferred damaged plants and performed better on plants damaged fourteen and ten days after single and double herbivory, respectively. Our results suggest that temporal studies involving single- versus multiple-attacker situations are necessary to comprehend the role of induced plant responses in plant-herbivore interactions.
Response to Reviewers:	Reviewer #1:

1. The paper is well written, but a bit lengthy, particular in the Introduction.

We have shortened the introduction from the earlier 1228 words to 927 words by deleting sentences from page 3 (lines 6-9; 13-14; 17; 19-21), page 4 (lines 6-8; 11-14; 17-19) and page 5 (lines 3-5; 16-17).

2. I am not sure, whether the Tables documenting the statistics are necessary to be included in full length in the paper. I recommend their documentation as Electronic Supplementary Materials.

The table 1 documenting the MANOVA is now included as ‘Supplementary table 1’

Reviewer #2:
3. Concerning the presentation of the data I have difficulties with the fact that the graphs in the figures start only at day 4.

As mentioned on Page 7 line 2-4, we started the period of our studies from day 4 because it is already well established that glucosinolate induction starts from third days onwards following herbivore damage (Hopkins et al., 2009; Mathur et al., 2011).

4. In figure 1b the authors interpret the data that the glucosinolate content is induced during the complete study period. But the levels remain constant after day 4.

Indeed, we meant to say that the levels in damaged plants remained higher during the entire study. We have changed the wording accordingly on page 10.

5. Even more problems arise from figure 2. The authors state that the trichome density is increasing due to herbivory. This conclusion is just based on the observation that already on day 4 the number of trichomes between treated and untreated leaves is significantly different. But if this is true the number has increased during the first 4 days of treatment and is decreasing during the remaining time of the study. This appears not very convincing. To confirm this conclusion an analysis of the trichome number within the first 4 days of the treatment would be necessary, so there are more data points to support this conclusion.

In an earlier study (Mathur et al 2011) we found that trichomes were most strongly induced 7-11 days after induction. The fast increase in this experiment was indeed surprising to us as well. The decrease in trichome densities over time is also seen in the control plants (see figure 2), thus may simply be a sign of leaf growth. Hence we can sustain the conclusion that trichome densities overall are higher in herbivore induced plants. We changed the phrasing of some sentences to more carefully describe the differences (page 10).
Further minor points:

6. Is it possible that the decrease in glucosinolate content after feeding of P. xylostella is due to the increased leaf size ("dilution effect")?

This seems highly unlikely to be the case for all glucosinolates, as aliphatic glucosinolates increased in concentration, whereas indole glucosinolates in the same samples decreased in concentration. We have no data supporting that leaf stretching comes with increases in leaf dry mass – which is the denominator for the glucosinolate concentration. We prefer not to speculate on this.

7. Introduction line 9: Please reword the sentence, as the part "?,plants respond to herbivore attack by altering their defence levels" suggests that defence is always induced after herbivory.

The sentence is now changed to “plants respond to herbivore attack by altering the levels of these traits”.

Dealing with double trouble: consequences of single and double herbivory in *Brassica juncea*

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Running head: Induced responses to single and double herbivory in Brassica juncea

Abstract

In their natural environment, plants are often attacked simultaneously by many insect species. The specificity of induced plant responses that is reported after single herbivore attacks may be compromised under double herbivory and this may influence later arriving herbivores. The present study focuses on the dynamics of induced plant responses induced by single and double herbivory, and their effects on successive herbivores. Morphological (leaf length, area and trichome density) and chemical changes (leaf alkenyl and indole glucosinolates) in *Brassica juncea* were evaluated four, ten, fourteen and twenty days after damage by the specialist *Plutella xylostella* alone, or together with the generalist *Spodoptera litura*. To assess the biological effect of the plant's responses, the preference and performance of both herbivores on previously induced plants were measured. We found that alkenyl glucosinolates were induced twenty days after damage by *P. xylostella* alone, whereas their levels were elevated as early as four days after double herbivory. Trichome density was increased in both treatments, but was higher after double herbivory. Interestingly, there was an overall decrease in indole glucosinolates and an increase in leaf size due to damage by *P. xylostella*, which was not observed during double damage. *S. litura* preferred and performed better on undamaged plants, whereas *P. xylostella* preferred damaged plants and performed better on plants damaged fourteen and ten days after single and double herbivory, respectively. Our results suggest that temporal studies involving single- versus multiple-attacker situations are necessary to comprehend the role of induced plant responses in plant-herbivore interactions.

Keywords: Brassicaceae, *Plutella xylostella*, *Spodoptera litura*, double herbivory, temporal dynamics, induced response

Introduction

Plants have to deal with various biotic and abiotic stresses in their surroundings and balance their resources to optimize growth, reproduction and defences. Insect herbivory often serves as a significant stress factor, and plants have evolved many different forms of resistance strategies to reduce or prevent it (Schoonhoven et al. 1998). Morphological structures such as trichomes, hairs, spines, and waxes and a number of secondary chemicals prevent or reduce insect herbivory. (Schoonhoven et al. 2005). In addition to these constitutive resistance mechanisms, plants respond to herbivore attack by altering the levels of these traits. These changes are known as induced responses (Karban and Baldwin 1997). The effects of these responses on insect-plant interactions can differ greatly and depend on many factors including whether the feeding herbivore is a generalist or a specialist on the plant (Karban et al. 1999).

The timing of induced changes determines which herbivores are affected. Each response may have its own temporal dynamics, which can be rapid or delayed. When there is a rapid response, the organism that causes the damage may suffer the consequences itself (De Moraes et al. 2001; van Dam et al. 2001) or a taxonomically unrelated species may bear the consequences when the response is delayed (Agrawal and Sherriffs 2001; van Dam et al. 2003; van Dam et al. 2004; Viswanathan et al. 2005). Delayed responses may also influence the attacker itself when the herbivore species has a long enough life cycle or subsequent short-lived generations (Karban and Baldwin 1997).

An important trait of induced responses is their specificity. Different herbivores can induce different phenotypic responses in the plant that may either increase resistance or susceptibility to subsequent attack (Feeny 1976; Karban and Baldwin 1997; Van Zandt and Agrawal 2004b). Plants adjust their responses specifically to the attackers by regulating their signalling pathways in order to optimize their effectiveness (Pozo et al. 2004; Beckers and Spoel 2006; Leon-Reyes et al. 2009). Particular qualities of the damage, such as feeding strategy, salivary constituents of the damaging insect, amount of leaf eaten, and/or timing and pattern of damage may be perceived differently by the plant (Bowers and Stamp 1993; Mattiacci et al. 1995; Alborn et al. 1997; Walling 2000). Even within a feeding guild, different species of herbivores may elicit different plant responses (Agrawal and Karban 1999; Voelckel and Baldwin 2004). The specificity of these responses may also depend on the number of herbivore species damaging the plant (de Boer et al. 2008).

In general, plant responses to more than one attacker may have three possible effects: 1) an additive response because of the lack of response specificity to the different attackers; 2) specificity in the plant's response with no trade-offs, whereby the plant induces a full response to each herbivore; 3) specificity in the plant's response with trade-offs, in which the plant response becomes sub-optimal (Rodriguez-Saona et al. 2010). Moreover, specificity of the induced responses should be distinguished from specificity of the effect of the responses. These may not be similar, since one induced compound can have various effects on many different herbivores, while different compounds can have similar effects (Karban and Baldwin 1997; Agrawal 2000). Thus, early herbivores inflicting damage on the plant play an important part in structuring the herbivore community that colonizes the plant later (Thaler et al. 1999; Van Zandt and Agrawal 2004a, b; Poelman et al. 2009; Poelman et al. 2011). Therefore, induced responses are an important link between induced phenotypes and herbivore community composition.

In order to address the specificity of induced plant responses under single and double herbivory, we studied the effect of *Plutella xylostella* damage alone, as well as of simultaneous damage by this specialist and the generalist *Spodoptera litura* on the induction of *Brassica juncea* resistance. We also determined the differences in behavioural responses and subsequent feeding by these two herbivores on either single or double infested plants.

B. juncea, commonly called Brown or Indian mustard is an annual herb of the family Brassicaceae and is cultivated in India and its neighbouring countries as a main source of mustard oil. This plant species possesses trichomes as a constitutive structural defence (Mathur et al. 2011). Trichomes are known to systemically increase in density or number following insect damage in different species of Brassicaceae (Agrawal 1999; Traw 2002; Traw and Dawson 2002; Mathur et al. 2011). In addition, Brassicaceous plants, including *B. juncea*, are characterized by a class of secondary compounds called glucosinolates that are hydrolysed by myrosinases upon tissue damage, resulting in the formation of toxic products such as thiocyanates, isothiocyanates, epithionitriles and nitriles (Grubb and Abel 2006; Halkier and Gershenzon 2006; Hopkins et al. 2009). When induced, glucosinolates are known to have a negative influence on herbivores and play an important role in preventing further damage to the plants (van Dam and Raaijmakers 2006; van Dam and Oomen 2008; Hopkins et al. 2009).

For the induction experiments, we used the specialist insect *Plutella xylostella*, which feeds on virtually all species of Brassicaceae including *B. juncea* (Talekar et al. 1985; Talekar and Shelton 1993). Additionally, we have used *Spodoptera litura*, which is a generalist herbivore. *S. litura* is one of the earlier infesting herbivores in *B. juncea* cultures in India. Both these insects have several generations in a year in India and can be found simultaneously on *B. juncea* due to overlapping life cycles.

With this study system, we aimed to assess the temporal dynamics of induced plant responses due a specialist herbivore and together with a generalist herbivore, and the effect of these interactions on subsequently feeding herbivores. Based on the literature, we expected that induced responses are specific for each herbivore species damaging the plant, and that this specificity may be disrupted under simultaneous attack. Moreover, plants damaged by single and double herbivory have differential effects on the performance and preference of the generalist and the specialist herbivore.

Materials and method

Plants

Seeds of *B. juncea* var. varuna were obtained from the Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi, India, and stored dry in the dark at 10°C. The experiments were conducted at Sri Venkateswara College, New Delhi, India, from October until the beginning of January, which is the natural planting season of mustard crops in India. The plants were grown in an insect-free enclosure in garden soil in earthen pots. They were treated with 0.5 Hoagland solution (Hoagland and Arnon 1950) weekly for the first 2 weeks and twice a week from the third week onwards. The concentration of potassium phosphate (KH_2PO_4) in the Hoagland solution was doubled in the third week and tripled from the fourth week onwards to avoid phosphorus deficiency. Every alternate day, five randomly chosen pots were weighed to determine the volume of solution needed to maintain the water content in the pots at 14% of the soil dry mass.

Insects

Larvae of *S. litura* were obtained from laboratory cultures maintained at Sri Venkateswara College, New Delhi since 2008 on castor bean leaves and periodically supplemented with individuals from the Division of Entomology, IARI, to avoid inbreeding. Larvae of *P. xylostella* were obtained from the Division of Entomology, IARI, and were used directly in the experiment.

Induction of plants

All the experiments were performed when the plants were approximately four weeks old and in stage 63 according to BBCH scale (Lancashire et al. 1991). For specialist induction, two fourth instar *P. xylostella* larvae were allowed to feed on the third leaf from the apex of the plant. The larvae were placed on the leaf in a clip cage. For our experiments on double damage by a generalist and a specialist herbivore, we avoided direct contact between the two species by confining the generalist and specialist on two different leaves on the plant. Hence, two fourth instar *P. xylostella* larvae were placed on the third leaf and one third instar *S. litura* larva was introduced on the fourth leaf from the apex of the plant in a clip cage. The larvae were allowed to feed for 24 h. Undamaged plants received empty clip cages. After 24 h, the larvae and clip cages were removed. Damaged and undamaged plants were placed randomly on the tables.

For measuring systemic plant responses, the second leaf from the apex of damaged and control plants was harvested at 4, 10, 14 and 20 days after introduction of the larva. We started the period of our studies on day 4 because previous studies suggested that glucosinolate induction starts from the third day after herbivore damage onwards (Hopkins et al. 2009; Mathur et al. 2011). Measurement of chemical and morphological responses were performed on separate sets of plants with equal (n = 10) number of experimental and control plants for each day of the experiment.

Glucosinolate analysis

Harvested leaves were immediately frozen and stored at -20 °C. They were freeze-dried and ground with a ball mill [Retsch ball mill (type MM301) Hann, Germany]. 50-55 mg of finely ground plant material was extracted in 1.0 ml 70% methanol (MeOH) in water (v/v) in Eppendorf tubes, vortexed and immediately boiled at 90°C for five minutes to stop remaining myrosinase activity. Tubes were placed in an ultrasonic bath for 15 minutes and centrifuged at 58 g in an eppendorf centrifuge for 10 minutes. The supernatant was transferred to a new Eppendorf tube. The extraction was repeated with the pellet once more as described above except the boiling step, and both supernatants were combined.

The supernatant was added to a DEAE-Sephadex A-25 column (5x10mm) and washed twice with 1 ml 70% MeOH, once with 1 ml MilliQ water and then twice with 1 ml 20 mM NaOAC buffer (pH 5.5). 20 µL of aryl sulfatase (Type H-1 of *Helix pomatia*; Sigma, St. Louis, IL, USA) was added to the columns and flushed down with 50 µL NaOAC buffer (pH 5.5) to break the sulfur bonds of glucosinolates. The columns were covered with aluminium foil and incubated overnight at room temperature. Thereafter, desulfoglucosinolates were eluted from the columns with two times 0.75 ml MilliQ water, and freeze-dried. The residue was dissolved in 1.0 ml of MilliQ water and stored at -20°C until further analysis.

Glucosinolate analysis was done according to van Dam et al. (2004). The desulfoglucosinolate extract was separated by means of a reversed phase C-18 column (Alltima C18 3µm, 150mm x 4.6µm) using a Dionex Ultimate 3000 HPLC with a $CH_3CN–H_2O$ gradient. Analysis was performed using a photodiodearray detector with 229 nm as the integration wavelength (Dionex, Sunnyvale, CA, USA). Desulfoglucosinolate peaks were identified by comparison of retention times and UV spectra with a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R) and authentic standards (progoitrin, gluconapin, glucoiberin, glucobrassicanapin, glucotropeaolin, gluconasturtiin, glucoraphanin, glucoerucin, glucobrassicin, sinalbin; Phytoplan, Heidelberg, Germany). Glucosinolate concentrations were calculated by using a Sinigrin calibration curve and the obtained values were divided by dry mass. Quantities of sinigrin, gluconapin and glucobrassicanapin were pooled together to obtain the total amount of alkenyl glucosinolates. Similarly, glucobrassicin and 4-methoxyglucobrassicin were combined to obtain total indole glucosinolates values.

Trichomes, leaf length and leaf area

Since trichomes were found only on the leaf veins, the number of trichomes on a 1 cm stretch was counted at four places on the adaxial as well as abaxial surface using a dissecting microscope. The average number of trichomes per cm was calculated in each individual leaf. These leaves were then scanned using a Hewlett Packard flatbed scanner (Hewlett Packard Company, Palo Alto, CA, USA). The scanned images were used to measure the length and area of that leaf using the software 'ImageJ' with a global scale set to 106 pixels per cm (Rasband 1997-2011; Abràmoff et al. 2004).

Insect behavioural and performance bioassays

Plants to be used on different time points after induction were induced on the respective days before the experiment, so that all the preference and performance experiments for each treatment group could start on the same day.

Feeding preference assays

A glass Petri dish of 20 cm diameter was lined with moist filter paper. Similarly sized leaves of undamaged plants and plants induced 4, 10, 14 and 20 days earlier were outlined with a pencil on a graph paper and placed at an equal distance from each other in a Petri dish. Feeding preference bioassays were conducted with either two fourth instar *P. xylostella* larvae or one sixth instar *S. litura* larva in one Petri dish (n = 10 per treatment). The larvae were introduced in the centre of the Petri dish and allowed to move and feed freely between the five leaves. After 24 h, the larvae were removed and damaged portions of the leaves were outlined on graph paper to measure the area that was removed.

Insect performance bioassays

For the performance experiment, the second leaf of the plants was removed, weighed and placed individually in a container lined with moist filter paper (n = 10 per treatment). Five containers were kept without larvae to determine leaf moisture loss. Newly moulted sixth instar *S. litura* or fourth instar *P. xylostella* larvae were starved for four hours. After weighing the larvae to the nearest 0.1 mg, they were introduced to the containers either with a leaf from a control plant or plants damaged 4, 10, 14 or 20 days earlier. The larvae, one *S. litura* and two *P. xylostella* per container, were allowed to feed on the leaf for 24 hours, after which they were weighed to calculate their weight gain. The unconsumed leaf was removed and weighed as well. The remaining leaf mass at the end of each experiment was subtracted from the initial mass of the leaf. An average change in mass due to moisture loss was determined from the leaves in containers without larvae and this was subtracted from the final mass of leaf remaining at the end of the experiment. Thus, we obtained the actual mass of leaf ingested by the larva. The Consumption Index (CI) was calculated as [(leaf mass ingested)/ (larval mass gain x number of days)] and the Efficiency of Conversion of Ingested food (ECI) was calculated as [(larval mass gain)/ (leaf mass ingested)] (Waldbauer 1968).

Statistical analyses

The data was analysed using SPSS 17.0 (SPSS Inc; Chicago, Illinois, USA). Normality and HOV were determined using 1-sample Kolmogorov-Smirnov test and Levene's test, respectively. For all the analyses, if the differences were found to be significant, Bonferroni post-hoc tests were conducted to correct for multiple comparisons.

The influence of 'treatment' and 'time after induction' on glucosinolates, trichome density and leaf size was analysed using MANOVA. The overall effect of damage was analysed using One-way ANOVA, and the relationship between damage and their effect at different time points was compared using univariate ANOVA. The amount of leaf area consumed of each treatment was evaluated using a non-parametric Chi-square Friedman's test. The data on larval performance (weight of leaf consumed, larval weight, consumption index and efficiency of conversion of ingested food) were analysed using One-way ANOVA when the data met the assumptions of parametric analysis. When the assumptions of parametric analysis were not met, a non-parametric Kruskal-Wallis ANOVA was used.

Results

Glucosinolates

Glucosinolate levels were significantly affected by damage due to herbivory by the specialist *P. xylostella* alone (MANOVA, Damage effect; $F_{2,68} = 6.325$, $P < 0.005$; Supplementary table 1) as well as by simultaneous damage by *P. xylostella* and *S. litura* ($F_{2,67} = 34.038$, $P < 0.001$; Supplementary table 1). However, we found a significant day effect in glucosinolate levels only when the plants were damaged by *P. xylostella* alone (MANOVA, Day effect; $F_{6,138} = 6.205$, $P < 0.001$; Supplementary table 1), but not in case of double herbivory ($F_{6,136} = 0.452$, $P = 0.843$;Supplementary table 1). Alkenyl glucosinolates, mainly gluconapin (~73%) and sinigrin (~23%), constituted about 97% of total glucosinolates. They were significantly increased due to damage by both the specialist alone (ANOVA; $F_{1,69} = 9.609$, $P < 0.005$) as well as by simultaneous herbivory by the generalist and the specialist ($F_{1,68} = 59.214$, $P < 0.001$) (Table 1). Accordingly, total glucosinolate levels were also induced by the specialist ($F_{1,69} = 9.413$, $P < 0.005$) and by the two herbivores together ($F_{1,68} = 58.128$, $P < 0.001$) (Figure 1). In contrast, indole glucosinolates differently responded to the two herbivore treatments. They decreased significantly due to specialist herbivory ($F_{1,69} = 2.330$, $P < 0.05$), but not after double herbivory ($F_{1,68} = 1.108$, $P = 0.296$) (Table 1).

Glucosinolate levels showed considerable differences in temporal patterns between the two herbivore treatments. After *P. xylostella* damage, glucosinolate levels began to increase to higher levels from the14th day onwards, but reached a significantly higher level only by the 20th day (Figure 1a). On the other hand, when plants were damaged by the two insects together, glucosinolate levels were significantly increased compared to control plants during most of the study period (Figure 1b).

Leaf trichomes, length and area

The density of trichomes on both adaxial and abaxial surfaces increased significantly due to damage by *P. xylostella* (MANOVA, Damage effect; $F_{2,71} = 17.347$, $P < 0.001$; Supplementary table 1) as well as by simultaneous damage by the specialist and the generalist ($F_{2,71} = 35.044$, $P < 0.001$; Supplementary table 1) . Trichome density was found to be significantly higher overall on both adaxial (1-way ANOVA; $F_{1,78} = 9.859$, $P = 0.002$) and abaxial ($F_{1,78} = 14.648$, $P < 0.001$) surfaces when plants were damaged by *P. xylostella*. This difference was significant except for the 14th day after damage (Figure 2a, b). Interestingly, we also found an overall increase in leaf length (1-way ANOVA; $F_{1,78} = 5.122$, $P = 0.026$) and area ($F_{1,78} = 4.792$, $P = 0.032$). The strongest increase in length was seen on day 10 and in the leaf area on day 10 and 20 after the damage (Table 2). Thus, due to damage by this specialist herbivore, plants not only increased the trichome density, but also the size of the leaf.

When the plants were simultaneously damaged by *P. xylostella* and *S. litura*, overall trichome density was significantly higher on both adaxial (1-way ANOVA; $F_{1,78} = 19.979$, $P < 0.001$) and abaxial (1-way ANOVA; $F_{1,78} = 61.555$, $P < 0.001$) surfaces. Separate analysis on individual days showed that this difference was the greatest on day 4 and 10 after damage (Figure 2c, d). Leaf length and area were not affected due to damage by these herbivores, thus ruling out the possibility that the increase in trichome density is due to leaf contraction or decreased leaf growth (Table 2).

Feeding preference assays

When *P. xylostella* were offered a choice between leaves from undamaged plants or plants damaged by conspecific larvae at different time points, they significantly preferred leaves from plants damaged 10 and 14 days before, as compared to other treatments (Friedman test; $\chi^2(4) = 14.490$, $P = 0.006$). This preference shifted to leaves damaged 10, 14 or 20 days before when larvae were offered plants that were induced simultaneously by the generalist and the specialist herbivore ($\chi2(4) = 9.685$, $P = 0.046$) (Figure 3). In contrast, *S. litura* larvae consumed more leaf from the undamaged plants as compared to damaged plants, irrespective of whether the plants were damaged by *P. xylostella* ($\chi2(4) = 21.737$, $P < 0.001$) or by the two herbivores together ($\chi2(4) = 13.843$, $P = 0.008$) (Figure 3).

Insect performance bioassays

P. xylostella larvae fed significantly less on leaves damaged 4 days earlier by their conspecifics as compared to the other time points. However, this did not affect larval weight gain because the Efficiency of Conversion of Ingested food (ECI) was the highest for that time point. The ECI was significantly lower on leaves from undamaged plants, resulting in the lowest weight gain on these leaves. The larvae gained most weight when fed with leaves from plants damaged 14 days earlier. When larvae were fed with plants that were damaged by *S. litura* and *P. xylostella* together, their weight gain was the highest on plants damaged 10 days earlier and the lowest on undamaged plants (ANOVA with Bonferroni corrections; $P < 0.05$) (Table 3).

S. litura, on the other hand, gained the most weight on leaves from undamaged plants as compared to plants damaged by *P. xylostella* at different time points. Their ECI was significantly lower on leaves from plants damaged 10 and 20 days earlier, on which they even lost weight. When fed on leaves from plants damaged simultaneously by the two herbivores, they consumed the least amount of leaf from plants damaged 14 days earlier. Interestingly, they consumed almost similar amounts of leaf of undamaged plants and those damaged 4 days earlier, but they showed the highest weight gain on plants damaged 4 days earlier. However, their weight significantly decreased when feeding on leaves from plants damaged 14 and 20 days earlier, as compared to others leaves, and also as compared to their weight at the start of the experiment (ANOVA with Bonferroni corrections; $P < 0.05$) (Table 4).

Discussion

In the field, plants are often simultaneously exposed to more than one type of attacker. The primary objective of this study was to investigate whether *B. juncea* plants respond differently in time when attacked by a single or more than one herbivores and whether these responses have divergent effects on further herbivory. When we measured changes in glucosinolate levels at four, ten, fourteen and twenty days after damage by the specialist *P. xylostella* alone or together with the generalist *S. litura*, we found that alkenyl glucosinolates were increased as early as four days after double herbivory, but the induction was delayed until twenty days after damage by *P. xylostella* alone. Indole glucosinolates, on the other hand, significantly decreased following damage by this specialist but did not show any significant change after double herbivory. Trichome densities were increased on both adaxial and abaxial surfaces following damage by single and double herbivory, but the densities were higher when damaged by the two herbivores simultaneously. Interestingly, an overall increase in leaf size was found after damage by *P. xylostella*, but this was not observed after simultaneous damage by the generalist and specialist. In the behavioural bioassays, *S. litura* always preferred and performed better on undamaged plants, while *P. xylostella* preferred and performed better on plants that had been damaged at least ten days before.

In an earlier study, when plants were induced by the generalist *S. litura*, alkenyl glucosinolates increased significantly around seven days after induction and remained at higher levels until eleven days after induction (Mathur et al. 2011). The present study revealed that alkenyl glucosinolate levels increased to significant levels only 20 days after damage by the specialist *P. xylostella.* There is ample evidence suggesting that induced plants are more attractive to this specialist (Reddy and Guerrero 2000; Poelman et al. 2008a; Sun et al. 2009). Therefore, delaying induced responses could be a plant strategy to escape further attack by not becoming more attractive. However, when damaged by *P. xylostella* and *S. litura* simultaneously, the increase in alkenyl glucosinolates was significant from four days after damage onwards. Glucosinolate levels in *B. juncea* thus increased more quickly in response to feeding by the generalist, either when they attacked individually or together with other herbivores. This is in accordance with many studies highlighting the role of secondary metabolite induction in deterring generalists (Kliebenstein et al. 2002; Long et al. 2007; Hansen et al. 2008). Interestingly, we found a significant overall decrease in indole glucosinolates when the plant was damaged by the specialist, which was not observed after damage by the generalist (Mathur et al. 2011) or by the generalist and the specialist together. Our study is in contrast with Poelman et al. (2008b) who observed an increase in indole glucosinolates in white cabbage cultivars due to damage by *P. xylostella*. This contrast may be explained by the differences in the defence strategies of different species within the same plant family.

Morphological attributes of plants also change in response to herbivory. In this study, we found increases in trichome densities on the adaxial and abaxial leaf surfaces after damage. We found a similar temporal pattern of trichome induction for both the specialist and double herbivory on both surfaces. This temporal pattern is in accordance with earlier studies (Mathur et al. 2011). However, the induction in trichomes was stronger in case of double damage as compared to damage by the specialist or the generalist alone. To determine whether trichome density increased due to a decrease in leaf size, we also examined leaf length and area. Remarkably, we found a significant increase in both leaf length and area after damage by the specialist *P. xylostella*, which may indicate the activation of a tolerance mechanism by the plant after feeding by this specialist. This may partly explain the delay in glucosinolate induction as a trade-off mechanism, though further investigation is needed before drawing a firm conclusion in this respect. This increase was not observed when the plants were induced simultaneously by *P. xylostella* and *S. litura*, thus suggesting that specific responses to feeding by the specialist may be interrupted when a second, in this case a generalist, herbivore is feeding on the plant.

These different levels and patterns of responses to each treatment support earlier findings that plants have the ability to distinguish between different types of biotic challenges and respond specifically to each of them. Moreover, we found that when damaged by only their conspecifics, specialist *P. xylostella* preferred to feed the most and gained most weight on plants damaged ten and fourteen days earlier. They preferred and gained the least weight on undamaged plants. These results are in accordance with earlier studies that suggest that *P. xylostella* uses glucosinolates as cues to find its preferred host plant (Palaniswamy et al. 1986; Shiojiri et al. 2001; Sun et al. 2009). However, our results on specialist performance bioassays are in contrast with studies on wild radish by Agrawal (2000), who found that although *P. xylostella* was the least affected by induction due to other generalists and specialist insects, its growth was reduced by plants that were initially damaged by its conspecifics. *P. xylostella* is known to possess a glucosinolate sulfatase enzyme that degrades glucosinolates in their host plants before the more toxic conversion products are formed (Ratzka et al. 2002). Nevertheless, it is possible that although glucosinolates have a stimulatory effect on these specialists, various other induced phytochemicals that we have not measured may act as deterrents to them (Agerbirk et al. 2003; Hodge et al. 2006).

On the other hand, *S. litura* always chose to feed on the undamaged plants as compared to plants damaged by either the specialist alone or together with a conspecific. When fed on plants damaged by the specialist alone, between ten and twenty days earlier, these larvae even lost weight. In previous studies, *S. litura* also showed reduced preference and performance when the plants were damaged by its conspecific larvae (Mathur et al. 2011). This indicates that the damage-induced responses by a single attacker, irrespective of its species, are effective in conferring resistance against this generalist.

The importance of temporal dynamics in our induction studies was highlighted by the effect that double herbivory had on herbivore preference and performance. When *P. xylostella* were offered leaves from undamaged plants and plants damaged simultaneously by both the specialist and generalist, they preferred to feed the most on plants damaged more than a week earlier, and the least on undamaged plants and plants damaged less than a week earlier. They performed significantly better on plants damaged ten days earlier, when all the induced responses tested were the highest. Their performance was the lowest on leaves from undamaged plants, and also on plants damaged more than two weeks earlier. In contrast, *S. litura* larvae showed the highest larval weight gain on plants damaged four days earlier by the two herbivores, which was the first time point tested after damage in our studies. But they lost considerable weight when fed with plants damaged more than two weeks earlier. These results clearly demonstrate the different sensitivities of these generalist and specialist herbivores to the temporal dynamics of induced plant responses. The generalist performed well, while the specialist did not show a particular preference to the damaged plants after a short time lapse following herbivory when the induced responses were still not strong. Once these responses are enhanced, the generalist avoided these plants, while the specialist preferred and performed better on these plants. Moreover, in our current study, the damaging herbivores were allowed to feed on the plant for only 24 hours and systemic leaves were offered to the experimental herbivores, supporting the notion that the plant acts as a mediator in this ‘horizontal’ interaction between initial and subsequent herbivores.

In India, the generalist *S. litura* and the specialist *P. xylostella* have multiple generations in a year, with overlapping host plants, such as *B. juncea*. This suggests that in fields, plants gain an enhanced resistance to *S. litura* through induced resistance mechanisms, but in the process become more susceptible to *P. xylostella*. Thus, generalist pests such as *S. litura* may be combated by enhancing natural induced resistance in *B. juncea*, whereas other pest management measures, such as biological control through predators and parasitoids and cultural practices, such as crop rotation and trap cropping, should be applied to reduce damage by specialists such as *P. xylostella*.

Plant resistance mechanisms are thought to be evolutionary selected for when the attack is correlated with the risk of future attack and may be strongest when current and future attack is likely by the same organism (Karban et al. 1999). Although there are only a few previous studies on simultaneous herbivory, nevertheless they suggest that knowledge of single attacker systems may not predict the responses in a multiple-attack (Dicke et al. 2009). Moreover, most of the studies investigate these interactions only for a limited time period. The present study unravelled the intricacies behind these responses on a larger temporal scale and demonstrated the complexity of these interactions when more than one attacker is involved. Thus it supports earlier findings that induced responses determine further species interactions, even when these species are spatially and temporally separated (Poelman et al. 2011). Therefore, temporal studies involving single- versus multiple-attacker situations are necessary to comprehend the evolution of induced defence strategies and the mechanisms behind plant-herbivore interactions in complex environments.

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Figure 1: Total glucosinolates (µmoles/g dry mass) in the second leaf of *Brassica juncea* after systemic induction by (a) *Plutella xylostella* or (b) *P. xylostella* and *Spodoptera litura* together. Asterisks indicate significant induction (Univariate ANOVA; * = $P < 0.05$, ** = $P < 0.005$, *** = $P < 0.001$).

Figure 2: Trichome density after systemic induction by *Plutella xylostella* on the (a) adaxial and (b) abaxial surface and by *P. xylostella* and *Spodoptera litura* together on (c) adaxial and (d) abaxial surface of the second leaf of *Brassica juncea*. Asterisks indicate significant induction (Univariate ANOVA; * = $P < 0.05$, ** = $P < 0.005$, *** = $P < 0.001$).

Figure 3: Mean (± SE) of amount of *Brassica juncea* leaf eaten (cm^2) by (a) fourth instar *Plutella xylostella* and (b) sixth instar *Spodoptera litura* when damaged by *P. xylostella* or by *P. xylostella* and *S. litura* together on leaves from undamaged plants or plants damaged 4, 10, 14 or 20 days before. Asterisks indicate significance level (Chi- square Friedman test; * = $P < 0.01$, ** = $P < 0.005$, *** = $P < 0.001$).

Figure 1

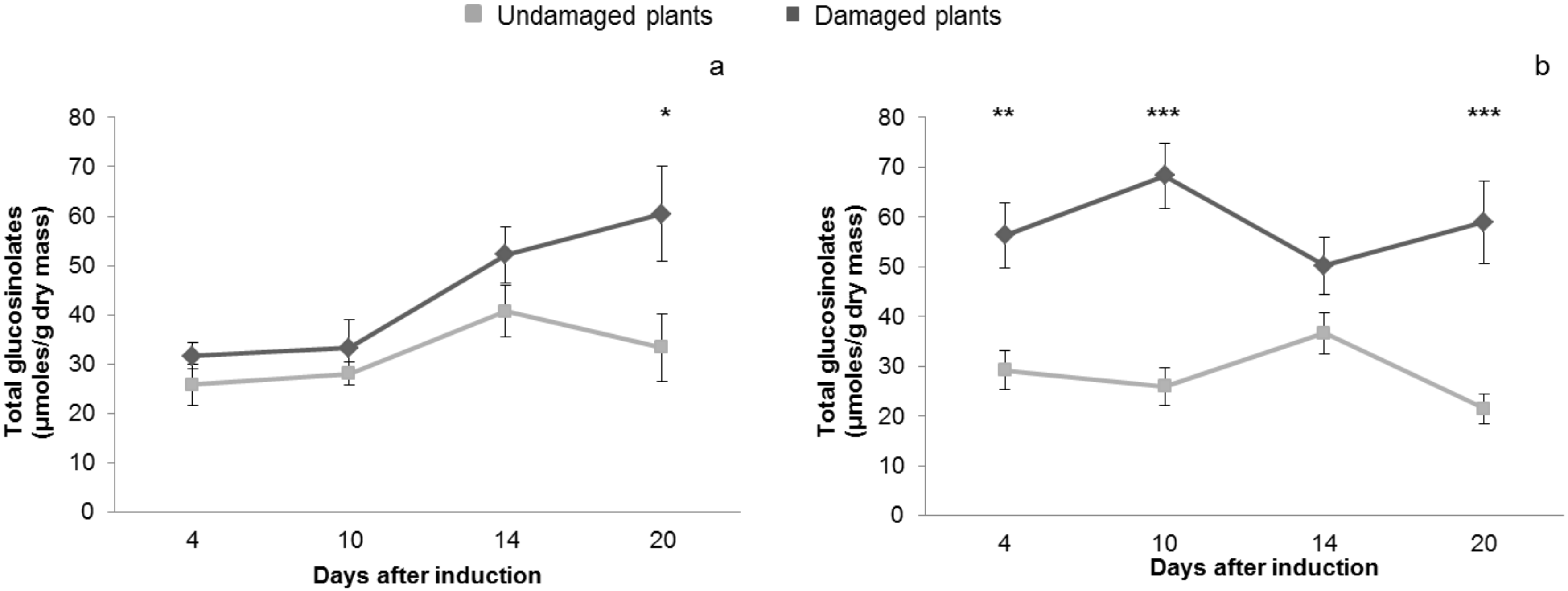

Undamaged plants
Damaged plants
a
b
Total glucosinolates (µmoles/g dry mass)
80
70
60
50
40
30
20
10
0
*
**
***
***
4
10
14
20
Days after induction

Figure 2

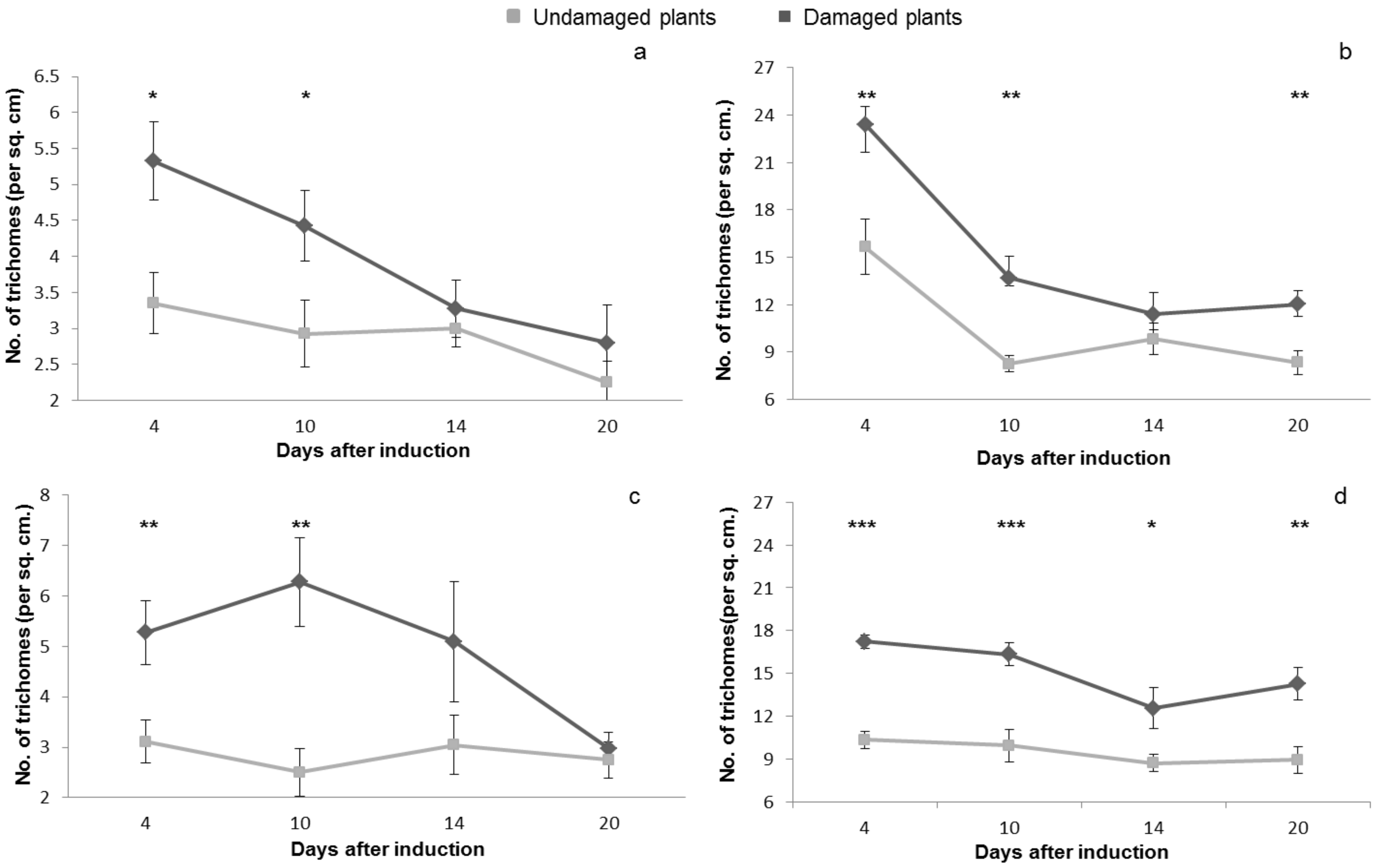
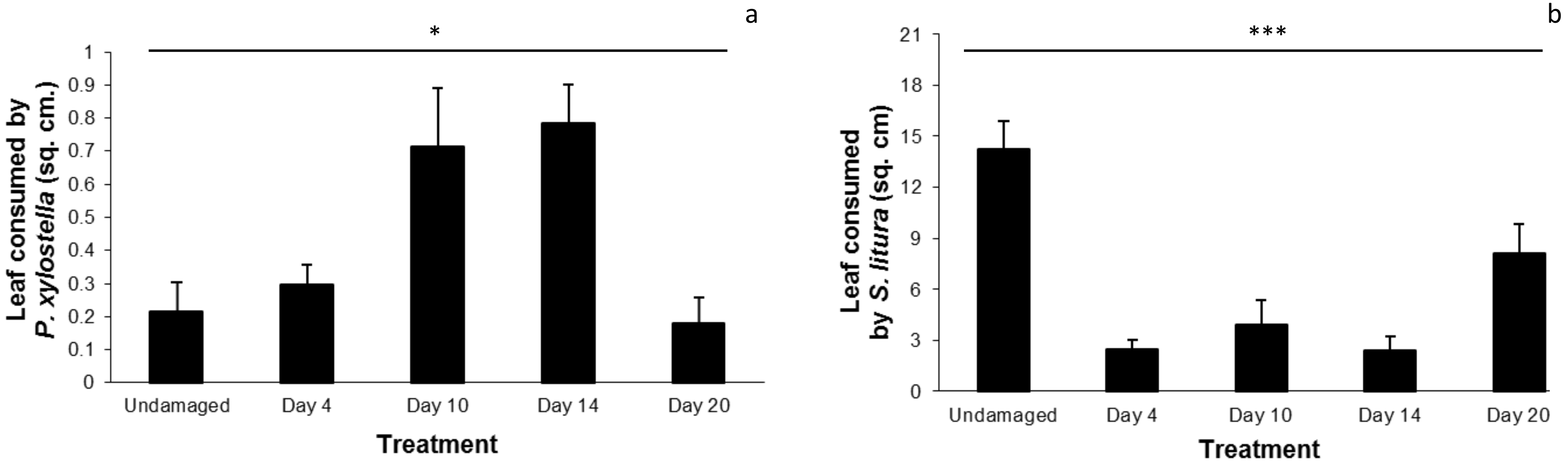

■ Undamaged plants
■ Damaged plants
a
No. of trichomes (per sq. cm)
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
*
*
4
10
14
20
Days after induction
b
No. of trichomes (per sq. cm.)
27
24
21
18
15
12
9
6
**
**
**
4
10
14
20
Days after induction
c
No. of trichomes (per sq. cm.)
8
7
6
5
4
3
2
**
**
4
10
14
20
Days after induction
d
No. of trichomes(per sq. cm.)
27
24
21
18
15
12
9
6
***
***
*
**
4
10
14
20
Days after induction

Figure 3

Damage by *P. xylostella*



Damage by *P. xylostella* and *S. litura* together

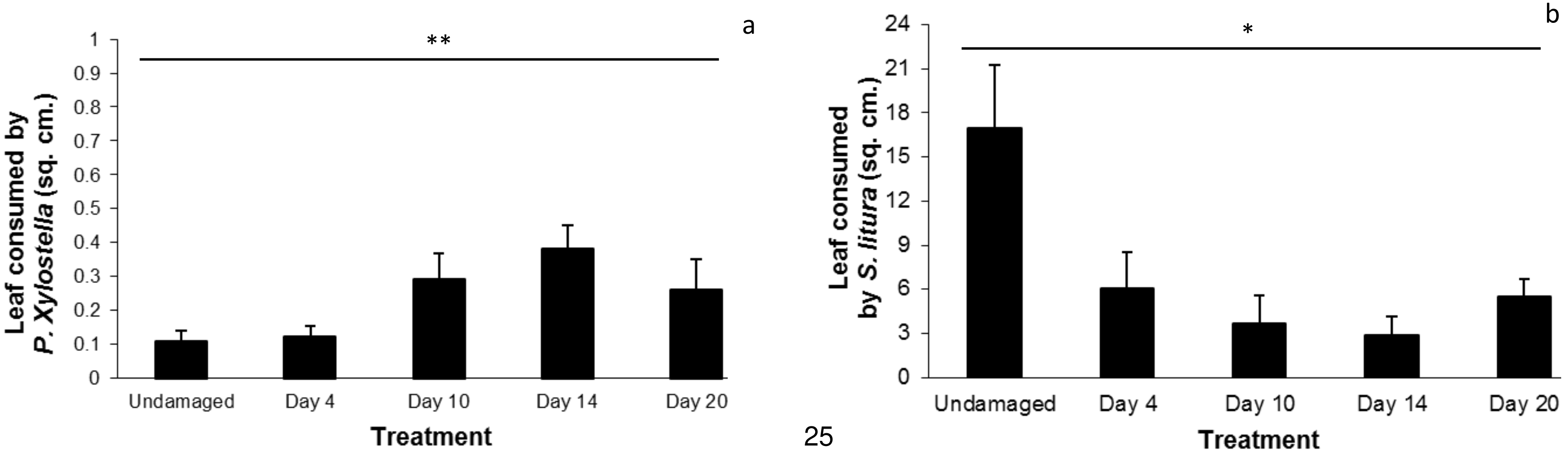


Table 1: Mean ± SE (n = 10) of alkenyl and indole glucosinolates, gluconapin and sinigrin (µmole/g dry mass) of damaged (damaged for 24 h) and undamaged second leaves of *Brassica juncea*. Plants were damaged either by the specialist *Plutella xylostella* (Specialist damage) or by *P. xylostella* and generalist *Spodoptera litura* together (Double damage). Days refer to number of days following insect damage.

Days	Treatment	Alkenyl	Gluconapin	Sinigrin	Indole
			Plutella damage		
4	Undamaged	25.10 ± 4.17	18.98 ± 3.15	5.90 ± 0.10	0.73 ± 0.07
	Damaged	30.94 ± 2.66	23.40 ± 1.95	7.22 ± 0.70	0.67 ± 0.13
10	Undamaged	26.98 ± 2.29	20.38 ± 1.78	6.34 ± 0.50	1.06 ± 0.08
	Damaged	32.27 ± 5.82	24.66 ± 4.43	7.21 ± 1.34	0.91 ± 0.11
14	Undamaged	39.82 ± 5.14	29.51 ± 3.44	9.82 ± 1.66	0.86 ± 0.10
	Damaged	51.40 ± 5.70	37.02 ± 3.58	13.67 ± 2.12	0.74 ± 0.10
20	Undamaged	32.72 ± 6.79	23.73 ± 4.94	8.63 ± 1.78	0.59 ± 0.13
	Damaged	59. 90 ± 9.62	40.14 ± 5.47	14.16 ± 2.55	0.48 ± 0.07
			Double damage		
4	Undamaged	28.29 ± 3.93	21.45 ± 3.05	6.57 ± 0.94	0.89 ± 0.14
	Damaged	55.73 ± 6.56	39.86 ± 4.52	14.94 ± 1.98	0.52 ± 0.07
10	Undamaged	25.10 ± 3.63	19.16 ± 2.94	5.63 ± 0.80	0.82 ± 0.19
	Damaged	67.62 ± 6.53	47.10 ± 4.10	19.35 ± 2.35	0.62 ± 0.07
14	Undamaged	35.85 ± 4.14	27.08 ± 2.97	8.33 ± 1.15	0.79 ± 0.08
	Damaged	49.37 ± 5.70	36.52 ± 3.86	12.02 ± 2.39	0.81 ± 0.12
20	Undamaged	20.87 ± 3.02	15.55 ± 2.27	5.17 ± 0.73	0.57 ± 0.08
	Damaged	57.93 ± 8.12	38.53 ± 5.01	18.26 ± 3.04	0.77 ± 0.14

Table 2: Mean (± SE; n = 10) leaf length (cm) and area (cm^2) of damaged (damaged for 24 h) and undamaged second leaves of *Brassica juncea*. Plants were damaged either by the specialist *Plutella xylostella* (Specialist) or by *P. xylostella* and generalist *Spodoptera litura* together (Double). Days refer to the number of days following insect damage. Asterisks indicate significant induction (Univariate ANOVA; $P < 0.05$).

Days	Treatment	Leaf length (cm)		Leaf area (cm^2)	
		Specialist	Double	Specialist	Double
4	Undamaged	7.23 ± 0.37	9.04 ± 0.28	28.78 ± 2.97	43.19 ± 2.66
	Damaged	7.38 ± 0.23	8.70 ± 0.37	26.77 ± 1.16	35.03 ± 3.19
10	Undamaged	8.81 ± 0.35	8.56 ± 0.48	39.86 ± 2.35	41.28 ± 4.70
	Damaged	10.0 ± 0.37*	9.99 ± 0.32*	48.84 ± 2.08*	47.81 ± 3.55
14	Undamaged	9.22 ± 0.33	9.26 ± 0.41	43.96 ± 2.65	49.67 ± 4.52
	Damaged	10.03 ± 0.24	9.07 ± 0.33	49.16 ± 3.65	38.11 ± 2.22*
20	Undamaged	8.99 ± 0.29	8.69 ± 0.43	35.68 ± 1.94	32.91 ± 2.75
	Damaged	9.58 ± 0.29	8.53 ± 0.28	44.67 ± 2.38*	33.19 ± 1.50

Table 3: Mean nutritional indices (± SE) of fourth instar *Plutella xylostella* larvae (n = 10). Plants were damaged either by the specialist *P. xylostella* (Specialist) or by *P. xylostella* and the generalist *Spodoptera litura* together (Double). *P*-values were calculated using One-way ANOVA for normal data and non-parametric Kruskal-Wallis ANOVA when the data were not normal.

	Treatment	Undamaged	4- day damage	10- day damage	14- day damage	20- day damage	*P*-value
Weight of the leaf consumed (mg)	Specialist	112.65 ± 7.85	27.90 ± 4.20	98.54 ± 3.00	101.98 ± 6.56	118.22 ± 8.00	$P < 0.001$ (ANOVA)
	Double	81.41 ± 14.24	75.11 ± 4.95	68.89 ± 10.97	32.00 ± 7.48	97.25 ± 7.15	$P < 0.001$ (ANOVA)
Larval weight gain ± SE (mg)	Specialist	1.18 ± 0.37	2.82 ± 0.51	3.86 ± 0.86	6.50 ± 0.97	4.00 ± 0.75	$P < 0.001$ (ANOVA)
	Double	1.72 ± 0.36	3.40 ± 0.64	7.42 ± 0.73	4.31 ± 0.75	2.60 ± 0.38	$P < 0.001$ (ANOVA)
Consumption Index ± SE (mg/mg/day)	Specialist	41.60 ± 11.48	1.97 ± 0.40	3.51 ± 0.55	2.92 ± 0.64	10.74 ± 4.64	$P < 0.001$ (Kruskal- Wallis)
	Double	8.41 ± 2.58	3.34 ± 7.78	1.09 ± 2.73	8.83 ± 2.24	4.11 ± 3.70	$P < 0.001$ (Kruskal- Wallis)
Efficiency of Conversion of Ingested food ± SE (%)	Specialist	0.06 ± 0.02	0.70 ± 0.13	0.39 ± 0.09	0.46 ± 0.07	0.20 ± 0.04	$P < 0.001$ (ANOVA)

Double	2.08 ± 1.85	4.27 ± 6.73	1.52 ± 3.78	1.91 ± 5.52	2.67 ± 3.00	$P < 0.001$ (Kruskal- Wallis)

Table 4: Mean nutritional indices (± SE) of sixth instar *Spodoptera litura* larva (n = 10). Plants were damaged either by the specialist *Plutella xylostella* (Specialist) or by *P. xylostella* and generalist *Spodoptera litura* together (Double). *P*-values were calculated using One-way ANOVA for normal data and non-parametric Kruskal-Wallis ANOVA when the data were not normal.

	Treatment	Undamaged	4- day damage	10- day damage	14- day damage	20- day damage	*P*-value
Weight of the leaf consumed (mg)	Specialist	193.64 ± 54.80	323.43 ± 89.61	92.26 ± 25.06	161.59 ± 98.61	108.48 ± 28.05	$P = 0.018$ (Kruskal-Wallis)
	Double	249.44 ± 50.62	224.80 ± 32.29	184.94 ± 23.62	25.56 ± 16.20	217.48 ± 40.06	$P < 0.001$ (ANOVA)
Larval weight gain ± SE (mg)	Specialist	85.06 ± 21.45	26.20 ± 21.33	-33.55 ± 16.33	1.54 ± 8.73	-7.94 ± 21.32	$P < 0.001$ (ANOVA)
	Double	77.06 ± 16.44	96.29 ± 14.90	53.20 ± 12.81	-61.14 ± 12.29	-22.03 ± 31.13	$P < 0.001$ (ANOVA)
Consumption Index ± SE (mg/mg/day)	Specialist	4.92 ± 1.84	1.32 ± 1.06	1.35 ± 1.20	9.92 ± 3.48	3.43 ± 1.89	$P = 0.676$ (Kruskal-Wallis)
	Double	6.65 ± 4.78	2.50 ± 2.25	1.82 ± 1.91	-6.74 ± 3.55	1.61 ± 1.82	$P = 0.014$ (Kruskal-Wallis)
Efficiency of Conversion of Ingested food ± SE (%)	Specialist	4.30 ± 1.73	8.88 ± 1.01	-1.01 ± 4.23	2.65 ± 2.56	-4.51 ± 3.58	$P = 0.007$ (ANOVA)
	Double	3.77 ± 1.18	4.32 ± 4.06	2.86 ± 5.23	-5.08 ± 2.63	-3.21 ± 1.95	$P < 0.001$ (Kruskal-Wallis)

Supplementary table 1: MANOVA table testing for the effects of damage by herbivores on the alkenyl and indole glucosinolates and trichome densities (per cm^2) of the abaxial and adaxial surface of the second leaf of *Brassica juncea* plants (n = 10). 'Specialist' represents the damage by the specialist *Plutella xylostella*; 'Double' represents the double damage by *P. xylostella* and the generalist *Spodoptera litura* together.

	Source of Variation	Pillai's Trace Value	F	Hypothesis d.f.	Error d.f.	P
			Glucosinolates			
Specialist	Day	0.425	6.205	6	138	<0.001
	Damage	0.157	6.325	2	68	0.003
Double	Day	0.039	0.452	6	136	0.843
	Damage	0.504	34.038	2	67	<0.001
			Trichome density			
Specialist	Day	0.638	11.230	6	144	<0.001
	Damage	0.328	17.347	2	71	<0.001
Double	Day	0.254	3.491	6	144	0.003
	Damage	0.497	35.044	2	71	<0.001