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Bartlet, E., Kiddle, G., Martin, A. P., Williams, I. H. and Wallsgrove, R. M. 1999. Wound-induced increases in the glucosinolate content of oilseed rape and their effect on subsequent herbivory by a crucifer specialist. *Entomologia Experimentalis et Applicata.* 91 (1), pp. 163-167.

The publisher's version can be accessed at:

- https://dx.doi.org/10.1046/j.1570-7458.1999.00479.x

The output can be accessed at: https://repository.rothamsted.ac.uk/item/8811y/wound-induced-increases-in-the-glucosinolate-content-of-oilseed-rape-and-their-effect-on-subsequent-herbivory-by-a-crucifer-specialist.

Wound-induced increases in the glucosinolate content of oilseed rape and their effect on subsequent herbivory by a crucifer specialist

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Abstract

Damage to the oilseed rape plant (*Brassica napus* L.) by the cabbage stem flea beetle, *Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae) induces systemic changes to the glucosinolate profile, most noticeably an increase in the concentration of indole glucosinolates. When jasmonic acid was applied to the cotyledons of the plant, a similar effect was observed. Feeding tests with artificial substrates compared a glucosinolate fraction from jasmonic acid-treated plants with a similar fraction from untreated plants. In these tests, alterations to the glucosinolate profile increased the feeding of a crucifer-specialist feeder (*P. chrysocephala*). However, in whole plant tests, *P. chrysocephala* did not feed more on the jasmonic acid treated plants than on the controls. This implies that other aspects of the damage response are being induced by the jasmonic acid treatment and having a negative effect on subsequent herbivory.

Introduction

When an oilseed rape plant is damaged, either mechanically or by herbivores, a systemic increase in indole glucosinolate and decrease in aliphatic glucosinolate concentration occurs. The net result is usually an increase in total glucosinolates (Koritsas et al., 1991; Birch et al., 1992). Glucosinolates act as feeding deterrents for polyphagous herbivores and feeding stimulants for crucifer-specialists. For example, amongst oilseed rape pests, glucosinolates deter feeding by the polyphagous grey field slug (*Deroceras reticulatum* Müller) and stimulate feeding of the oligophagous cabbage stem flea beetle (*Psylliodes chrysocephala* L.) (Glen et al., 1990; Bartlet et al., 1994). Indole glucosinolates are known to be the most potent class of glucosinolates in stimulating several crucifer-feeding insects (Larsen et al., 1985; Städler et al., 1995; Roessingh et al. 1997; Isidoro et al. 1998). Thus, changes to the glucosinolate profile of oilseed rape following herbivory would be predicted to make the plant more palatable to crucifer specialists. Koritsas et al. (1991) even suggested that, by altering the indolyl glucosinolates, insects such as

P. chrysocephala could be altering the metabolism of oilseed rape for their own benefit. However, feeding tests with oilseed rape have reported either a reduction in the feeding of crucifer specialists on induced tissue (Bodnaryk, 1992) or no effect (Palaniswamy & Lamb, 1993; Siemens & Mitchell-Olds, 1996). This might be due to the influence of other physico-chemical factors that change after oilseed rape is wounded. Bodnaryk & Rymerson (1994) found that, in addition to altering the glucosinolate content, wounding rape seedlings or treating them with methyl jasmonate altered cotyledon toughness, protein content, cysteine proteinase inhibitor levels and viscosity of extracts.

This study is the first to investigate how induced changes to the glucosinolate profile of oilseed rape affect the feeding of a crucifer specialist (*P. chrysocephala*). To avoid effects due to herbivore cues such as frass, the damage response of oilseed rape is induced by the application of jasmonic acid, rather than by herbivory. Jasmonates are involved in the wound-induced defences of oilseed rape and other plants (Bodnaryk, 1994; Doughty et al., 1995b). Treatment of rape seedlings with methyl jasmonate or jasmonic acid induces physiological changes, including

altered glucosinolate profiles, similar to those induced by herbivore damage (Bodnaryk & Rymerson, 1994). Firstly, the effects of adult flea beetle feeding and jasmonic acid (JA) on the glucosinolate profile of oilseed rape is determined. Then the effects of the JA-altered glucosinolate profile on subsequent herbivory is tested. To determine the effects of the changes to the glucosinolate profile in the absence of other elements of the damage response, the responses of the insects to a partially purified glucosinolate fraction is also tested.

Materials and methods

Insects. Cabbage stem flea beetles were collected from crops of winter rape and kept on oilseed rape plants (cv Falcon) within nylon net bags in an outside insectary.

Effect of adult feeding on foliar glucosinolate levels. Oilseed rape plants (cv Bienvenue) were planted in a compost mixture (Petersfield Products) in plastic pots (4″ diam), one plant to a pot. They were grown in a heated glasshouse (15–20 °C), to the sixth true leaf stage. Ten cabbage stem flea beetles, starved for 24 h, were enclosed in a muslin bag on the third true leaf of each of eight plants. Eight control plants each had the third true leaf bagged without flea beetles. After seven days all the leaves were harvested and the glucosinolates analysed.

Feeding on whole plants after jasmonic acid induction. Oilseed rape plants (cv Bienvenue) were glasshouse-grown as above in plastic pots (4″ diam), two plants per pot. At the second true leaf stage, one plant in each pot was treated with jasmonic acid, by applying 5 nmoles of JA in 5 μl of a solution of wetting agent (Triton 0.01%) to each cotyledon, using a pipette. The other plant in each pot was treated with 5 μl of Triton (0.01%) on each cotyledon. Four days after treatment the plants in 20 pots were harvested for glucosinolate analysis and production of glucosinolate fractions. The remaining pots of rape were transferred to a controlled environment room at L12:D12, 15/5 °C. The plants in each pot were enclosed by a hurricane lamp glass, whose lower rim fitted the pot exactly. The top of each lamp glass was covered with metal gauze. Flea beetles were introduced to 20 of the lamp glass enclosures (25 beetles per pot). The remaining 20 pots were not presented for herbivory but provided an additional control for glucosinolate

analysis at the end of the experimental period. After seven hours the area (mm^2) of consumption was estimated by eye. All plants were then harvested for glucosinolate analysis.

Feeding on glucosinolate fractions after jasmonic acid induction. To produce glucosinolate fractions, a protocol similar to that of Hanley et al. (1983) was used. Lyophilised, ground plant material from the first and second true leaves was extracted in 70% methanol (3.5 ml) at 70 °C for 10 min. The extract was centrifuged at 3000 g for 10 min, after which the supernatant was stored on ice and the pellet was re-extracted in 70% methanol (3 ml) and centrifuged as before. The pellet was then discarded and the supernatants combined. As the freeze dried material absorbed liquid, the combined supernatants were made up to 5 ml in 70% methanol. DEAE Sephadex A25 (2 ml) was suspended in acetic acid (2 M) in a 1:1 ratio and loaded onto a mini column. The column was washed twice with 2 ml distilled water, the methanol extract loaded and the column washed twice with a further 2 ml distilled water. Glucosinolates were eluted from the column by washing ten times with 1 ml potassium sulphate (0.5 M). The elutes were frozen at −50 °C and freeze dried. Freeze dried material was resuspended in methanol, filtered to remove the salt and the filtrate rotary evaporated to dryness. Residue was dissolved in 1 ml ultrapure water and frozen until required. For the feeding tests, the glucosinolate fraction was stirred into agar (2%) plus sucrose (0.5%), cooled to below 50 °C. The glucosinolate fraction was made up in the same weight of agar as the fresh weight of the leaf material from which it was taken. This was poured into two circular wells (8 mm diam, 3 mm deep) cut into a perspex sheet inside a Petri dish (90 mm diameter). One well was filled with agar plus extract from JA-treated plants and the other with agar plus extract from untreated plants. Four beetles (starved for three days) were added to each dish and the dishes kept at 10 °C L12:D12 for 24 h. The number of bite marks in the agar was then counted as a measure of feeding.

Glucosinolate analysis. The cotyledons, first true leaves and second true leaves were bulked together within a treatment, weighed and harvested into separate bags. The bags were immediately immersed in liquid nitrogen, then stored in a freezer at −50 °C. After freeze drying, the tissue was reweighed and 200 mg of ground leaf material was extracted in 70% methanol (3.5 ml) which contained 600 μmoles of

Table 1. Feeding by *Psylliodes chrysocephala* on untreated and jasmonic acid-treated rape in choice tests with either plant material or glucosinolate fractions

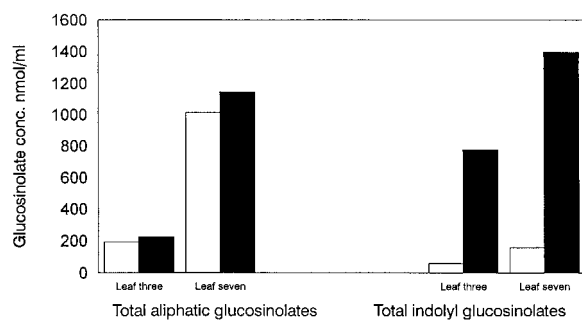| Feeding parameter	Material tested	n	Untreated plants (± s.e.)	Treated plants (± s.e.)	P*
Estimated area consumed (mm^2)	Cotyledons	20	2.20 ± 0.57	0.30 ± 0.16	0.01
	True leaves	20	5.90 ± 1.26	4.45 ± 0.87	0.60
Number of bitemarks	Agar treated with glucosinolate fraction	22	59.5 ± 10.6	92.5 ± 10.6	0.02

*Two-tailed Wilcoxon's test.

Figure 1. Glucosinolate levels (nmol/ml tissue water) in true leaves three and seven of oilseed rape seven days after bagging of the third true leaf with (■) and without (□) cabbage stem flea beetles.
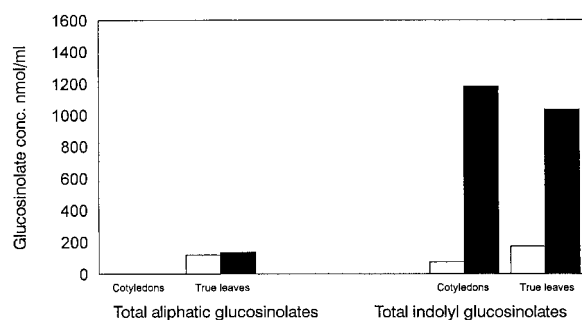

Figure 2. Glucosinolate levels (nmol/ml tissue water) in cotyledons and true leaves of oilseed rape at the beginning of the experimental period, four days after treatment of the cotyledons with wetter (□) and with wetter plus jasmonic acid (■).

Figure 3. Glucosinolate levels (nmol/ml tissue water) in oilseed rape at the end of the 7-h experimental period in plants *unexposed* to herbivory by the cabbage stem flea beetle. Plants either had cotyledons treated with wetter (□) or with wetter plus jasmonic acid (■).

Figure 4. Glucosinolate levels (nmol/ml tissue water) in oilseed rape plants at the end of the 7-h experimental period in plants *exposed* to herbivory by the cabbage stem flea beetle. Plants either had cotyledons treated with wetter (□) or with wetter plus jasmonic acid (■).

2-propenylglucosinolate as an internal standard. The extract was heated at 70 °C for 10 min and then centrifuged at 3000 rpm for 10 min to remove particulates. The supernatant was decanted and stored on ice. The pellet was then re-extracted and centrifuged as before. The supernatants were combined and made up to 5 ml. One ml of Sephadex A25/2 M acetic acid (1:1, v/v) suspension was pipetted into a mini column (0.5 ml bed volume). The column was then washed with 6 M imidazole formate (2 ml), followed by two washes in
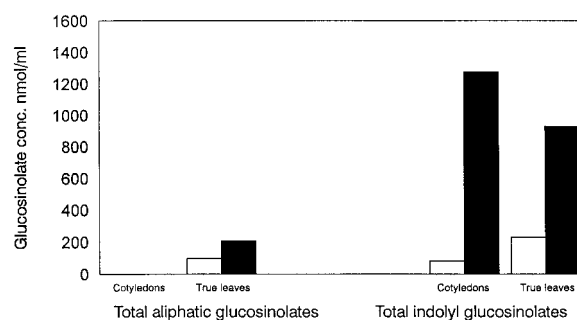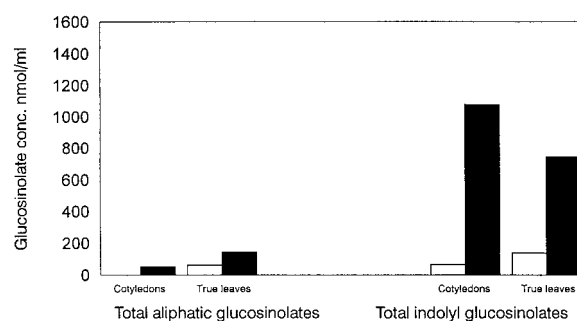
2 ml of ultrapure water. One ml of the glucosinolate extract was then loaded onto the pre-equilibrated column and unbound material removed using two, 1 ml washes of 0.1 M sodium acetate (pH 4.0). This also served to adjust the pH of the resin in preparation for desulphation of the glucosinolates. Purified sulphatase (100 μl) was loaded onto the column and digestion carried out overnight. The desulphoglucosi-

nolates were eluted with three washes with 500 μl ultra pure water and stored at $-20\,°C$ until they were analysed by HPLC. For separation by HPLC, a Waters 600LC system equipped with a 996 photodiode array detector was used. Data was collected between 200 and 300 nm to establish the class of desulphoglucosinolate. The column used was a 4.6×250 mm C_{18} column (Waters, Symmetry) with a 5 μm particle size. The mobile phase was made up of water-acetonitrile as reported in Porter et al. (1991).

Results

The glucosinolate profile of oilseed rape was found to consist of aliphatics {mainly 2-hydroxy-3-butenyl- (progoitrin), 2-hydroxy-4-pentenyl- (gluconapoleiferin) and 4-pentenyl- (glucobrassicanapin) glucosinolates} and indolyls {3-indolylmethyl- (glucobrassicin), 1-methoxy-3-indolylmethyl-, 4-methoxy-3-indolylmethyl- and 4-hydroxy-3-indolylmethyl- glucosinolates}, with 2-phenylethyl glucosinolate (gluconasturtiin) present only in trace amounts. This profile was radically altered after herbivory by *P. chrysocephala*. Aliphatic glucosinolates did not change dramatically after induction. Some increased slightly, whilst others decreased, resulting in a slight increase in total aliphatic glucosinolate levels (Figure 1). All indolyl glucosinolates increased after the plant was damaged, leading to a dramatic increase (8.8 fold) in total indolyl glucosinolate levels. Overall, there was a 2.2 fold increase in total glucosinolates. These changes were systemic, as the profile changed, not just at the site of treatment (leaf three), but also in other true leaves (data from leaf seven are presented).

The glucosinolate profile of the plants in the JA induction experiment differed from that in the flea beetle induction experiment, probably because different leaves were sampled from plants at a different growth stage. Nevertheless, application of a low concentration of jasmonic acid to the cotyledons had a similar, systemic effect on glucosinolate levels (Figure 2). Over the plant as a whole, the concentration of indolyl glucosinolate increased 8.9 fold, whilst that of aliphatic glucosinolate did not change markedly. Overall glucosinolate concentration increased 6.4 fold in JA-treated plants. The glucosinolate profile of the plants altered little over the 7-h period of the bioassay (Figures 2 and 3). There was little difference between glucosinolate levels at the end of the experiment between plants unexposed to herbivory and those damaged by flea beetles (Figures 3 and 4), indicating that further induction of glucosinolates, due to flea beetle damage did not occur during the course of the experiment.

P. chrysocephala fed less on JA-treated plants than on untreated plants, the difference being significant for the cotyledons but not the true leaves (Table 1). However, the beetles fed significantly more on agar containing extract from JA-treated plants than on agar with extract from untreated plants.

Discussion

Systemic changes to the glucosinolate profile of oilseed rape after damage from cabbage stem flea beetle adults are similar to those that occur after damage by the larvae (Koritsas et al., 1991), although they feed in a different way (chewing the leaves, rather than mining the stems). They are also similar to the changes that are induced by the application of JA.

The glucosinolate content of JA-treated plants was still substantially greater than that of the untreated plants at the end of the 7-h bioassay period, even though feeding damage by *P. chrysocephala* would eventually be expected to induce indolyl glucosinolate production in the untreated plants. Bodnaryk (1992) demonstrated that induction of indolyl glucosinolates in oilseed rape can occur within four hours of damage (24 needle punctures to the cotyledon). Feeding on untreated plants in the first few hours of this experiment must have been insufficient to produce such rapid induction.

When glucosinolate fractions were tested, the effect of the altered profile was as predicted from our knowlege of *P. chrysocephala*'s response to glucosinolates, i.e., the combined effect of increased proportions of indolyl glucosinolates and a greater overall glucosinolate concentration stimulated more feeding. However, in whole plant tests, *P. chrysocephala* did not feed more on JA-treated plants. As mentioned above (Bodnaryk & Rymerson, 1994), other elements of rape physiology are altered by plant damage or jasmonate treatment. These must have had a deterrent effect on feeding, counteracting the stimulatory effect of the altered glucosinolate profile. This is being investigated further.

Although JA induces systemic changes, the effects may have been greater at the site of treatment than in other plant parts. If so, this would explain why feeding was significantly less on treated plants than on un-

treated ones in the case of the cotyledons, but not in the case of the true leaves.

Damage-induced changes to the glucosinolate profile may benefit the oilseed rape plant by protecting it from fungal diseases, such as downy mildew (Doughty et al., 1995a), and generalist herbivores, such as slugs (Bartlet et al., unpubl.). They do not increase the susceptibility of the plant to *P. chrysocephala* (and possibly other crucifer-specialists) because their stimulatory effect is offset by other elements of induced defence. Furthermore, although rape plants with the altered profile have a higher glucosinolate content, they are unlikely to be more attractive to crucifer specialists. This is because only indole glucosinolates (which do not produce volatile metabolites) are induced. Thus, the emission rates of compounds such as isothiocyanates and nitriles, which are important attractants for crucifer specialists (Bartlet et al., 1997), would not be expected to increase after induction.

Acknowledgements

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