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POTENT NATURAL EGG-LAYING STIMULANT FOR CABBAGE BUTTERFLY *Pieris rapae*

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Abstract—Solutions of glucobrassicin (3-indolylmethyl glucosinolate) purified from foliage and sinigrin (allyl glucosinolate) elicited oviposition by the cabbage butterfly, *Pieris rapae*, at threshold concentrations as low as 10^{-6} M. At higher concentrations, glucobrassicin elicited a faster oviposition rate and a stronger visual response to the substrate through associative learning. Solutions of 10^{-5} M glucobrassicin and 10^{-2} M sinigrin stimulated equally. Their enzymic hydrolysis products failed to influence oviposition. The markedly greater potency of glucobrassicin is consistent with known glucosinolate profiles of crucifers which show indolyl glucosinolates predominant in foliage.

Key Words—Plant–insect interaction, *Brassica campestris*, oviposition, 3-indolylmethyl glucosinolate, learning, reflective spectra, *Pieris rapae*, Lepidoptera, Pieridae.

INTRODUCTION

The botanical specificity of insects restricted to crucifers has long been linked with the presence in host plants of a class of involatile sulfur compounds, the glucosinolates, whose toxicity has been overcome and which now induce feeding and oviposition by the specialized insects (Feeny et al., 1970; Blau et al., 1978; Städler, 1978; Koritsas et al., 1989). The chemical ecology of insects of crucifers has the potential to be understood thoroughly, although impediments

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have been insufficient chemical analysis of glucosinolates (GSLs), and a lack of behaviorally interpretable bioassays. More than 70 GSLs are known, with 50 in the Brassicaceae, differing in a side chain derived from one of a range of amino acids (Kjaer, 1976; Fenwick, 1983). A major advance in GSL chemistry was the combination of a desulfonating enzyme with HPLC (Minchinton et al., 1982) to identify GSLs directly and more reliably than previously by inference from hydrolysis products. The new method confirmed the predominance of indolyl GSLs in brassica foliage (Sang et al., 1984). The present report seems to be the first concerning the influence of an indolyl GSL on the behavior of an insect. Nonindolyl GSLs have been compared as oviposition stimulants (Ma and Schoonhoven, 1973; Nair and McEwen, 1976) and feeding stimulants (Hicks, 1974; Nault and Styer, 1972), but no ecologically interpretable pattern emerged for different GSLs. Green cards treated with aqueous cabbage extracts elicited more oviposition by *P. rapae* than those treated with allyl GSL (sinigrin) solution (Renwick and Radke, 1983), and after further experiments with plant extracts, it was suggested that unknown substances that were not GSLs were involved (Renwick and Radke, 1988).

In field experiments, Jones (1987) recorded that a mated female *P. rapae* distributed several hundred eggs during several days by repeated, single ovipositions on host plants, in bouts of oviposition in which landings on potential host plants alternated with short flights. In the laboratory, also, flights alternated with oviposition on paper wetted with aqueous GSL solution, provided the paper was green, yellow, or pale blue. *P. rapae* learned to respond to the appearance of a chemically favorable substrate (Traynier, 1984, 1986) but did not learn to associate the presence of a chemical deterrent with the appearance of its substrate (Traynier, 1987). Interpretable bioassays of GSLs therefore required a consideration of visual responses including the influences of learning.

METHODS AND MATERIALS

In earlier experiments on oviposition by *P. rapae*, a traditional bioassay with different solutions offered on substrates of identical appearance revealed little preference for higher concentrations of sinigrin (Traynier, 1986). This lack of chemical discrimination probably resulted from butterflies learning to associate the presence of sinigrin with the appearance of the test substrate, and then responding sufficiently to visual stimuli to override chemical influences. In contrast, when butterflies were offered different solutions on disks of different but equally attractive appearance, they learned to land and oviposit on disks wetted with a 100-ppm rather than a 75-ppm solution of sinigrin. This assay method, with differently colored but equally attractive disks, was employed in the present experiments to measure oviposition by *P. rapae* elicited by different GSLs

offered at the same concentration. The insects were reared on Chinese cabbage, *Brassica campestris*.

Mated female *P. rapae*, 5–8 days old, without ovipositional experience and carrying about 100 oocytes, were tested in 60 × 60 × 60-cm cages under artificial light at 6000 lux from "daylight" tubes. Artificial blue flowers provided sucrose solution. A tray of damp peat moss in the floor of the cage humidified it to 70% relative humidity at 27°C. Experiments were made on a black bench surrounded by pink organza drapes (Sandoz dye, Nylosan Red N2RBL) within either one or a pair of cages made from a tubular frame covered by nylon mesh of the same color. Against this pink background, the green blotting papers (1) Ford's Great Green and (2) Australian Paper Manufacturers' Shoalhaven Green were accepted equally for oviposition when wetted with the same GSL solution. Butterflies oviposited on horizontal 58-mm-diam. disks made from a double layer of 0.15-mm thick celluloid supported horizontally by a central pin and covered on top with a disk of blotting paper of the same diameter. The paper was wetted with 0.8 ml of water or solution, and two disks were offered with centers 20 cm apart in tests of 20 min duration, to butterflies in groups of three, which were numbered on the hindwings for individual recognition. To start the tests, butterflies were taken in turn with their wings between the thumb and forefinger and made to walk on the oviposition sites for 10 sec to contact the test solution with their tarsal chemoreceptors and initiate oviposition behavior.

The landings and ovipositions of the butterflies were tape recorded by spoken commentary. After 10 min, 20% of the water had evaporated from the disks and was replaced around their edges with drops from a pipet. Air in the laboratory was stirred by a fan to encourage flight by the butterflies.

RESULTS

Reflectance spectra were obtained to define the oviposition substrates and cage fabric (Figure 1). Although the cage fabric was pink, it reflected a range of wavelengths including even more green and yellow (500–600 mm) than the wet green oviposition substrates. As *P. rapae* showed no ovipositional response to the pink fabric by tarsal drumming, we concluded that wavelengths additional to the green–yellow range might actively deter oviposition rather than being merely neutral.

Wet versus Dry Substrates. The most common GSL in experimental analyses of the chemical ecology of crucifers has been sinigrin (allyl glucosinolate) obtained from mustard seed. Since sinigrin and water were needed together to elicit oviposition by *P. rapae* (Traynier, 1984), we tested glucobrassicin with and without water. A green paper disk was wetted with 10^{-4} M glucobrassicin

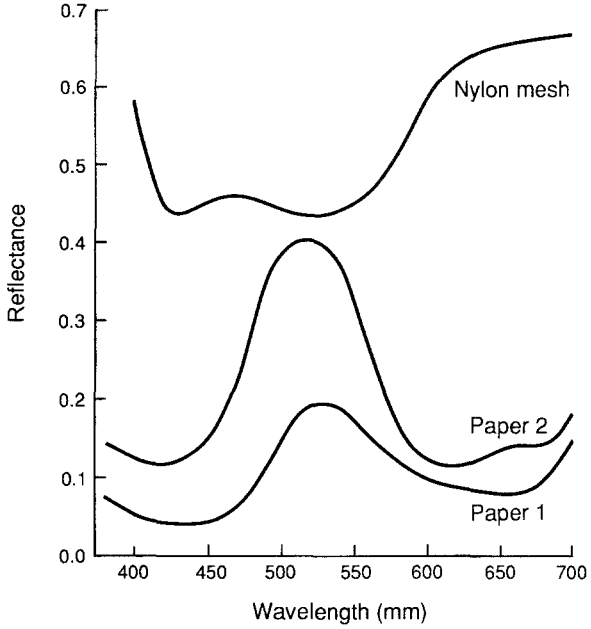

FIG. 1. Diffuse spectral reflectance from pink nylon mesh (Nylosan Red N2RBL Sandoz dye) and wetted blotting papers: (1) Ford's Great Green; (2) Australian Paper Manufacturers' Shoalhaven Green.

solution and allowed to dry. Ten naive, gravid female butterflies were made to walk on the dry paper to establish tarsal contact, then released. They all flew off, only one made a return flight, and none oviposited. In similar tests made with the same butterflies, and the same paper rewetted with water, nine of the 10 butterflies flew back to the disk to attempt oviposition and eight succeeded. We concluded that glucobrassicin, like sinigrin, elicited oviposition only in the presence of water. Our subsequent experiments used these GSLs in aqueous solution.

Threshold Concentrations. Previous results (Traynier, 1984) suggested a threshold concentration for sinigrin at about 10^{-6} M, and we tested sinigrin and glucobrassicin at this concentration. Gravid butterflies were made to walk on a green paper disk with a GSL solution for 10 sec to establish tarsal contact, then released. Of 30 butterflies placed on sinigrin solution, 14 made one or more return flights and nine of these laid a mean of five eggs (SE $\pm$ 2.2). Of 30 butterflies that contacted glucobrassicin, 17 returned and 16 laid a mean of nine eggs (SE $\pm$ 2.1). These differences were not significant, either between the number of butterflies landing or ovipositing (2×2 χ^2) or between mean oviposition per butterfly (Mann-Whitney). We concluded that both compounds had

closely similar threshold concentrations at about 10^{-6} M for ovipositional behaviors. Confirmatory evidence came from a comparison of 10^{-6} M and 10^{-8} M glucobrassicin, conducted as before, in which 10^{-8} M failed to elicit oviposition from any of 30 butterflies that contacted it, whereas 13 of 30 oviposited on disks with 10^{-6} M solution.

Comparisons above Threshold. To test concentrations above threshold, 10^{-4} M solutions of sinigrin and glucobrassicin were offered on different shades of green paper for 15 min. Each butterfly preferred to land and to oviposit on glucobrassicin disks ($P < 0.001$, sign test) irrespective of their shade of green (Figure 2). There were 78 ± 4% and 79 ± 4% of ovipositions by each butterfly on glucobrassicin disks of green shades 1 and 2, respectively, with respective landings of 65 ± 3% and 71 ± 3%. In contrast, when the solutions were offered in a separate experiment on disks of identical appearance (green 2), the butterflies showed no preference, with 7 ± 2 and 8 ± 2 ovipositions on sinigrin and glucobrassicin disks, respectively. The landing choice of butterflies paralleled the ovipositional choice, as in previous experiments in which butterflies learned to recognize the appearance of acceptable substrates (Traynier, 1984, 1986). We concluded that glucobrassicin was more stimulating than sinigrin at 10^{-4} M, but this influence could be overridden by visual learning with disks of the same color, as butterflies associated the presence of the most stimulating GSL with the appearance of its substrate and thereafter visual responses obscured differences between GSL solutions.

Glucobrassicin versus Water. The potency of the indolyl compound was

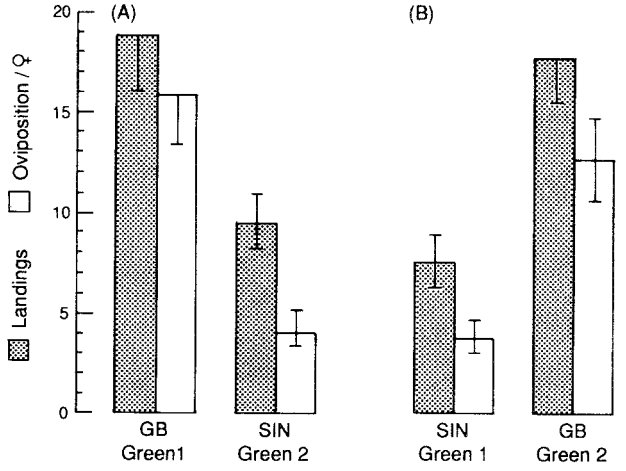

FIG. 2. Mean landings (shaded) and ovipositions (unshaded) (±SE) by 15 ovipositing *P. rapae* on a binary choice of green disks wetted with either sinigrin or glucobrassicin at 10^{-4} M, offered simultaneously in paired cages (A, B) on alternative green papers.

confirmed by a comparison of water and 3×10^{-4} M glucobrassicin applied to different shades of green. Glucobrassicin was markedly preferred with mean egg counts from 15 butterflies of 18 ± 3 vs. 2 ± 0.07 (respective solutions on green shades 1 and 2) and 16 ± 3 vs. 2 ± 0.05 (on shades 2 and 1). Oviposition on the water disks was 15% of the egg count in the first half of the test period, but only 3% in the second. The counts from individual butterflies showed an increase in oviposition preference between the first and second halves ($P < 0.001$, sign test). As *P. rapae* are known to learn, it seems likely they had learned to be more discriminating in the course of the test period.

Influence of Experience. In a comparison at even higher concentration (10^{-3} M) on different shades of green, glucobrassicin was again preferred to sinigrin (Figure 3). Two hours after these tests ended, the same butterflies were offered a choice of the two kinds of green disks, but wetted only with water. The butterflies tended to land initially on the same shade of green on which they had contacted glucobrassicin previously. Of 18 butterflies tested with GSLs, 17 landed later on water disks with a first landing choice of 15:2 in favor of the shade of green that mimicked the previously experienced glucobrassicin disk. Moreover, they oviposited with a preference for appearance corresponding to their earlier chemical preference ($P < 0.001$, Wilcoxon matched-pairs). Thus, these butterflies had learned a stronger response to the appearance of disks with glucobrassicin as opposed to sinigrin. In addition, a learned response to water

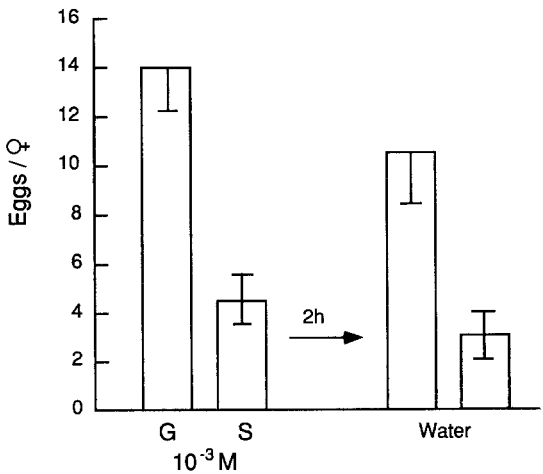

FIG. 3. *P. rapae* oviposition on alternative shades of green disks with either sinigrin or glucobrassicin at 10^{-3} M in an initial 15-min test, followed 2 hr later by oviposition on water disks of the same shade of green.

from the initial test might have contributed to the later oviposition on disks with water alone.

Equipotent Concentrations of Sinigrin and Glucobrassicin. As the responses to the two GSLs had been not significantly different at 10^{-6} M, the differences in experiments with 10^{-4}–10^{-3} M indicated a steeper dose–response curve for glucobrassicin. Oviposition equivalent to 10^{-5} M glucobrassicin was elicited by 10^{-2} M sinigrin; each induced a mean of 6 $\pm$ 1 eggs per butterfly offered these solutions on different shades of green disk.

Influence of Allyl Isothiocyanate. The question remained as to whether hydrolysis products were implicated, either singly or jointly, with GSLs. It was known that allyl isothiocyanate, derived from sinigrin, fails to elicit oviposition from *P. rapae* in the presence of water (Terofal, 1965; Traynier, 1984). As glucobrassicin and sinigrin hydrolyze to different chemical species at neutral pH, we tested the natural enzymic hydrolysis products of each in combination with the alternative GSL. We tested allyl isothiocyanate with potassium sulfate, the coproduct of enzymic hydrolysis at neutral pH, together with glucobrassicin, all three substances at 10^{-4} M in water on the one disk, against a solution of 10^{-4} M glucobrassicin on another disk of the alternative shade of green, to ensure good chemical discrimination by the butterflies. Mean oviposition by 12 butterflies given this choice yielded 7 $\pm$ 1.3 eggs on the mixture and 7 $\pm$ 1.4 eggs on the GSL with no significant preference. Clearly all oviposition had been elicited by the GSL alone.

Influence of Indolyl-3-carbinol. Enzymic hydrolysis of glucobrassicin at neutral pH, as can occur in leaves, yields indolyl-3-carbinol and the thiocyanate ion (Kjaer, 1976). Accordingly, a sinigrin solution with indolyl-3-carbinol in suspension and with potassium thiocyanate, all at 10^{-3} M, was compared with a 10^{-3} M sinigrin solution on the alternative shade of green paper. There was no significant difference. In another experiment, an aqueous suspension of indolyl-3-carbinol corresponding to 10^{-3} M was offered to *P. rapae*, together with a separate water disk, and a sinigrin solution disk, all three of green shade 2. Following contact with sinigrin, butterflies learned to oviposit on disks of the same appearance but treated with water only or with the aqueous carbinol suspension. This allowed a comparison between water with carbinol suspension for their effects on oviposition, knowing that oviposition deterrents do not contribute to associative learning. Ten butterflies oviposited 8 $\pm$ 1.2 eggs on sinigrin disks and 3 $\pm$ 0.8 eggs on both the carbinol and the water disks. Again the carbinol suspension showed no significant influence on oviposition as compared with water.

Glucobrassicin at acid pH can form indolyl-3-acetonitrile (Kjaer, 1976). This was tested like the carbinol and also showed no significant influence on oviposition.

DISCUSSION

It is known that crucifers vary in susceptibility to oviposition by *P. rapae* even after the butterfly has landed on them (Ives, 1978), and in previous attempts to analyze these effects, either total GSL content or GSL profiles without indole compounds were obtained (Nair et al., 1976; Rodman and Chew, 1980; Ahman, 1982). Although indolyl GSLs generally predominate in *Brassica* foliage, they occur in different amounts, and the oil-seed variety Zem 2 of *Brassica juncea* is unusual with sinigrin predominant in its foliage and indolyl GSLs present at low levels (Fenwick et al., 1983). From the present findings, such a plant might be expected to show a low chemical susceptibility to *P. rapae*. Another consideration, however, is that the leaf surface with which *P. rapae* makes only superficial contact (Traynier and Hines, 1987) might have a different GSL profile from the entire leaf. Taking visual responses into account, the least susceptible of all crucifers might not only lack indolyl GSLs but also be less stimulating visually and less distinguishable by *P. rapae* from other host plants by its appearance.

The finding that hydrolysis products of GSLs were ineffective oviposition stimulants for *P. rapae* differs from findings for cabbage root fly, *Delia radicum*, which oviposits in soil adjacent to the host following responses to both isothiocyanates and to GSLs (Finch, 1980; Schöni et al., 1987). The lack of responses of *P. rapae* to isothiocyanates might reflect a requirement for healthy, insect-free foliage as optimal larval food, whereas undamaged foliage might be less important to the root feeding larvae of *D. radicum*. Despite differences in responses to hydrolysis products, other insects that respond to crucifer foliage might, like *P. rapae*, be highly responsive to indolyl GSLs.

The suggestion arising from the spectral reflectance measurements that "red" wavelengths from foliage might actively deter oviposition may be of more general interest since some "red" brassicas are less preferred by other insects of crucifers as well as by ovipositing *P. rapae* (Dunn and Kempton, 1976; Rothschild and Schoonhoven, 1977; Latheef and Irwin, 1979; Myers, 1985), and it is known that additional wavelengths reflected from the sky by plastic or metal deter aphids from green foliage (Kring, 1972; Wyman et al., 1979).

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