

## Phytoalexins from *Brassica* (Cruciferae) as oviposition stimulants for the cabbage root fly, *Delia radicum*

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**Summary.** Bacteria species known to induce the biosynthesis of crucifer-specific phytoalexins have earlier been shown to be associated with *Delia* flies. Eleven crucifer-specific phytoalexins and related synthetic compounds were applied on surrogate paper leaves and offered to cabbage root flies in oviposition assays. Since three of these compounds (methoxybrassinin, cyclobrassinin, brassitin) proved to be significantly stimulatory whereas the remaining metabolites had no effect, the reaction of the fly appears to be structure-specific. Inactive phytoalexins had no inhibitory effect on oviposition. 100 µg of methoxybrassinin per surrogate leaf was as stimulatory as 0.05 g (gram leaf equivalent) of a methanolic host-leaf (*Brassica oleracea*) extract. Thus the three active phytoalexins can explain only part of the activity of host-plant extracts but might induce a preference for infected plants.

**Key words.** phytoalexin – brassinin – indole – oviposition behaviour – *Brassica* – Cruciferae – *Delia radicum* – Diptera – *Erwinia carotovora* – *Pseudomonas* – Bacteria

### Introduction

Plants defend themselves against hostile organisms by means of morphological and chemical features that are either constitutive (preformed) or induced at the time of attack. Phytoalexins, antimicrobial compounds that accumulate in plants as a result of infection or stress (Kuc 1995), not only inhibit the development of pathogens but can also affect the behaviour of herbivores associated with specific plants. In the book titled “Phytoalexins” (Bailey & Mansfield 1982) Cruciferae (Brassicaceae), including the *Brassica* species, were not yet mentioned as producing stress metabolites in response to attack by microbial organisms. This picture has changed in the meantime, however, and several authors have found that a variety of wounding or stress agents can induce a specific increase of mostly indole glucosinolates or alkenyl isothiocyanates. Examples are feed-

ing damage by flea beetles (Koritsas *et al.* 1989), mechanical wounding (Koritsas *et al.* 1991), UV light, bacterial infection (Monde *et al.* 1991a), fungal attack (Doughty *et al.* 1996; Ludwig-Müller *et al.* 1997), turnip root maggot damage (Birch *et al.* 1996; Griffiths *et al.* 1994) and jasmonic acid treatments (Bodnaryk 1994; Bodnaryk & Yoshihara 1995; Doughty *et al.* 1995; Ludwig-Müller *et al.* 1997). Subsequent colonisation by insects did not always show an adapted response in the sense that insect attack was only in some cases inhibited. For example, Coleman *et al.* (1996) found no pronounced wound-induced defence against caterpillars. On the other hand, Palaniswamy & Lamb (1993) detected a wound-induced antixenotic resistance to flea beetles and Cole (1996) discovered an increased resistance in *Brassica* species to the specialist cabbage aphid *Brevicoryne brassicae*. Another example of a possible role of bacteria associated with insect behaviour has been presented by Koritsas *et al.* (1989). The authors found that bacterial isolates from the cabbage stem flea beetle, *Psylliodes chrysocephala*, increased the accumulation of indole glucosinolates during artificial wounding. In the case of the cabbage root fly *Delia radicum*, Baur *et al.* (1996) found that cruciferous plants damaged by conspecific larvae were preferred oviposition sites for the females. The mechanism of this preference has not yet been elucidated. The major oviposition stimulants for this fly are glucosinolates and non-glucosinolates (Roessingh *et al.* 1997) but their presence alone can apparently not explain the preference for plants damaged by conspecific maggots (Baur *et al.* 1996).

Host-plant selection of herbivore insects can be influenced by microorganisms associated with plants. *Erwinia carotovora* and bacteria of the genus *Pseudomonas* have been found to stimulate or synergise oviposition in two *Delia* species (*Delia platura*: Eckenrode *et al.* 1975; Hough-Goldstein & Bassler 1988; *Delia antiqua*: Hausmann & Miller 1989). In addition, other bacteria have been shown to be associated with the maggots of different *Delia* flies (cit. in Doane & Chapman 1964a,b) and to promote their development (Friend *et al.* 1959; Marshall & Eymann 1981; Schneider *et al.* 1983). In the case of *Delia radicum*, Doane & Chapman (1964a) were able to show a specific association of an *Erwinia* species with the eggs and flies.

The isolated bacteria produced soft rot in rutabagas which was transmitted by the larvae, but Doane & Chapman (1964b) failed, probably due to experimental difficulties, to show a positive effect of the bacteria on larval development. However, Ellis *et al.* (1982) found that cabbage root flies laid up to four times as many eggs around radish seedlings grown from control seeds as on those carrying few or no microorganisms. These observations, in combination with the increased oviposition noted on larval damaged plants (Baur *et al.* 1996), are suggestive that also *Delia radicum* tend to choose plants infected by suitable bacteria, and thus phytoalexins produced by the infected plant might be providing the specific guiding signal. An induced defence metabolite that is not a glucosinolate was first isolated and identified by Takasugi *et al.* (1986) from *Brassica campestris* L. ssp. *pekinensis*. Related structures with an indole – or indole-related (oxindole) – system linked to a sulphur-containing moiety have been isolated since then. These phytoalexins apparently have multiple inhibitory or toxic effects on various organisms, including different plant pathogens, other plants (allelopathy), and mammalian cancer cells (compounds and effects reviewed by Gross 1993; Gross *et al.* 1994; Monde *et al.* 1994; Pedras & Khan 1996). Given the fact that microorganisms can induce these compounds and that cabbage root flies have a preference for damaged plants, it was of interest to examine the role of phytoalexins in the oviposition behaviour of the female fly.

## Materials and methods

### Origin of compounds and extracts:

All phytoalexins tested (Fig. 1) were isolated at Hokkaido University (Sapporo, Japan) according to previously reported procedures. The isolation of brassinin (1) and cyclobrassinin (3) is described by Takasugi *et al.* (1986, 1988) and the syntheses of (+/–)-dioxibrassinin (5) and 3-cyanomethyl-3-hydroxyoxindole (11) by Monde *et al.* (1991a). Methoxybrassinin (2) and the brassicanals A and B (7, 8) were isolated from Chinese cabbage (*B. campestris* L. ssp. *pekinensis*) inoculated with *Pseudomonas cichorii* using the methods of Takasugi *et al.* (1986, 1988), Monde *et al.* (1990) respectively. Spirobrassinin (4) was obtained from radish (*Raphanus sativus* L.) inoculated with *P. cichorii* (Takasugi *et al.* 1987). Brassitin (6) was isolated from inoculated radish (Monde *et al.* 1995). 1-methoxyindole-3-carboxaldehyde (10), obtainable from inoculated Japanese radish (Monde *et al.* 1991b), was synthesised from 1-methoxyindole using a procedure from Somei *et al.* (1992). Indole-3-carboxaldehyde (9), previously identified in inoculated Chinese cabbage (Monde *et al.* 1990), was obtained from Aldrich Chemicals Co.

Leaf surface extracts from greenhouse-grown cauliflower plants (*Brassica oleracea* L. convar *botrytis* CC Cross) at the 8–10 true leaf stage were prepared as described by Städler & Roessingh (1991) by dipping the leaves first into dichloromethane and subsequently into methanol. The methanol extract, containing the major oviposition stimulants (Städler & Roessingh 1991), was used for behavioural assays. The applied dose of extract is expressed in gle (gram leaf equivalent) per surrogate leaf, one gle representing the amount of extract obtained from 1 gram of fresh leaf material.

### Insects and behavioural assays:

A permanent colony of the cabbage root fly, *Delia radicum* (L.) was reared according to Finch & Coaker (1969). Oviposition preference

assays were performed with surrogate leaves as described by Roessingh & Städler (1990). Male and female flies (200–600) were released into a perspex cage and used for behavioural assays at an age between 7 and 14 days. Prior to bioassays the flies had access to a potted cauliflower plant (cv. CC-cross) for oviposition. Test compounds or leaf surface extracts were diluted in 1.5 ml methanol and were sprayed onto surrogate leaves with a surface projection of 7 × 7 cm, 1.5 cm wide vertical folds and a flat stem of 5 × 1.5 cm. The leaf area of these surrogate leaves corresponds to approximately 1.25 g of a cauliflower leaf. The leaves were positioned vertically in 10 cm diameter dishes containing paraffin wax. The wax was covered with a 1 cm layer of sand into which females could oviposit. Leaves were arranged in a circle on the floor of the cage at a maximum of 10 leaves per bioassay. After 24 h, eggs were recovered from the sand by flotation in water, then sieved and counted. The surrogate leaves were immediately re-used for a second bioassay, beginning 4 hr after the end of the first one because Košťál *et al.* (unpubl.) found that experimental leaves were apparently not marked by females during or after oviposition. For the repetitions the position of each leaf on the cage floor was rotated by at least 60°C. During the 4 hr break the flies had access to a potted cauliflower plant for oviposition to prevent a loss of selectivity due to deprivation of stimulation. The compounds which were not stimulatory were tested also in a “deterrent bioassay” by adding them to a cauliflower surface extract which was tested in a special choice assays against cauliflower surface extract alone and against the solvent control.

Egg counts were expressed per leaf as a proportion of the total number of eggs laid on all leaves in the cage within 24 h. Since a bioassay usually included in the same cage 2 or 3 replicates of each treatment, the proportions per leaf were multiplied by the number of replicates in order to obtain a total value within a set of choices of 100%. Values from replicates within a cage as well as the values from the same leaf obtained at the two consecutive days were treated as

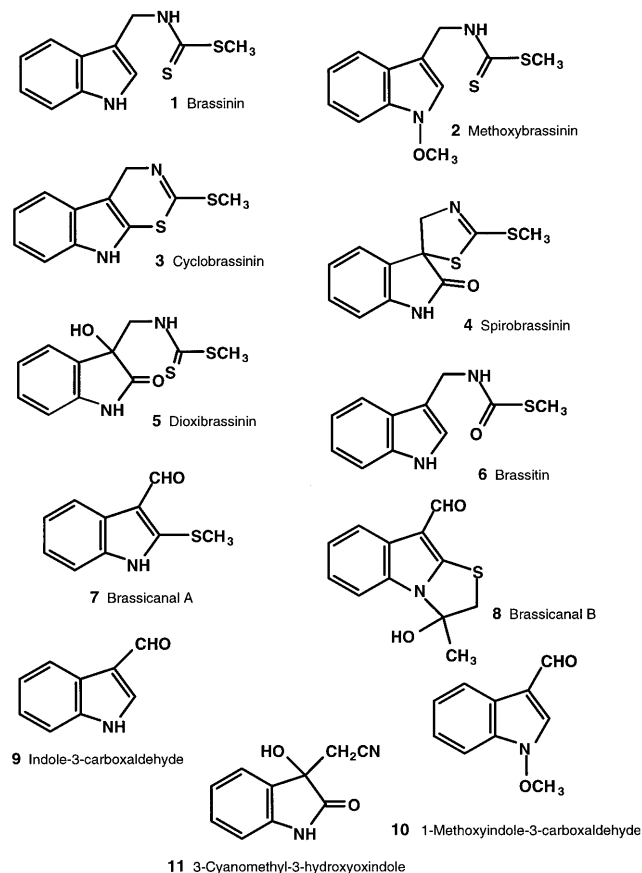


Fig. 1 Phytoalexins (1–8) and phytoalexin metabolites (9–11) isolated from *Brassica* (Cruciferae)

independent measurements and analysed statistically for differences using the Mann-Whitney test or, in the case of multiple choice assays, the Kruskal-Wallis test. If the Kruskal-Wallis test indicated significant differences, the Mann-Whitney test was used for pair-wise comparisons of treatments.

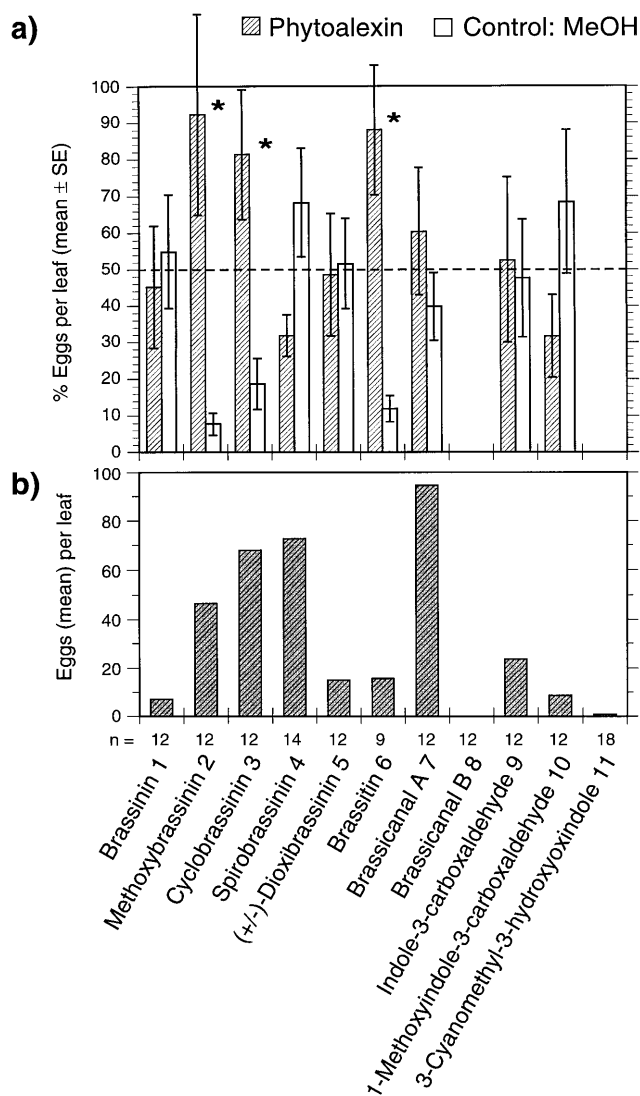
## Results

Eleven compounds (structures given in Fig. 1), which were all isolated from *Brassica* sp. and *Raphanus* sp. plants and known to be either phytoalexins or metabolically related compounds, were sprayed onto surrogate leaves and tested for their influence on oviposition behaviour of the cabbage root fly. While eight compounds did not show any activity, three compounds, methoxybrassinin (**2**), cyclobrassinin (**3**) and brassitin (**6**) significantly stimulated flies after landing on treated leaves to lay eggs into the sand around the base of the leaf models (Fig. 2a). For two compounds, brassicanal B (**8**) and 3-cyanomethyl-3-hydroxyoxindole (**11**), no preference data are given in Fig. 2a because the number of eggs laid in the respective behaviour assays was too low to allow an accurate calculation of preference values. In bioassays with these compounds 100–300 females per cage laid less than 20 eggs in total and thus it can be concluded that these compounds have no stimulatory effect on oviposition.

Considering the number of females per cage, the resulting number of eggs per model leaf was generally low and variable (Fig. 2b), indicating that even the three compounds with a significant effect did not strongly stimulate oviposition. Further evidence for this came from the observation that usually a very large number of flies (>50) oviposited within the first 20 minutes after the subsequent introduction of a potted cauliflower plant at the end of a bioassay with surrogate leaves.

The activity of methoxybrassinin (**2**) was compared to the stimulatory effect of various concentrations of a leaf surface extract from cauliflower. The highest applied concentration of this extract (0.5 g/leaf) was previously found to elicit 70–90% of the maximal oviposition activity released by the plant extract (Baur, unpublished). The stimulatory activity of methoxybrassinin (**2**) at a concentration of 100 µg/leaf corresponded to the activity of a concentration of 0.05 g/leaf of the cauliflower extract (Fig. 3).

Compared with the solvent control, spiobrassinin (**4**) and 1-methoxyindole-3-carboxaldehyde (**10**) seemed even to deter oviposition (Fig. 2a) but for both compounds differences between phytoalexin treatment and control were not significant (Mann-Whitney test for spiobrassinin (**4**):  $P = 0.14$ ; for 1-methoxyindole-3-carboxaldehyde (**10**):  $P = 0.1$ ). However, it was thought that testing the effect of a potential deterrent against a control (solvent treatment) that elicited little acceptance is unlikely to reveal pronounced detergency. Similarly, the very low egg numbers in tests with brassicanal B (**8**) and 3-

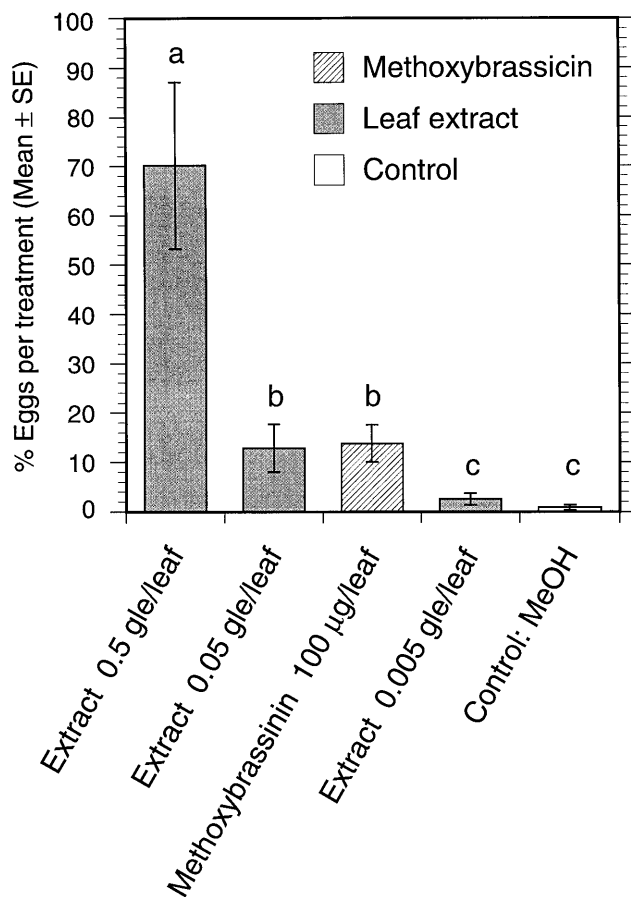


**Fig. 2** Oviposition of *Delia radicum* in dual choice assays on surrogate leaves treated with 100 µg/leaf of a phytoalexin or with solvent only. For each compound three replicates at a time were tested simultaneously ( $n$  = total number of replicates). The preference for Brassicanal B and 3-cyanomethyl-3-hydroxyoxindole was not calculated because for these treatments the mean number of eggs/surrogate leaf was less than 1. (a) Preference for either of the treatments. Values that differ significantly from 50% (=no effect) are marked with an asterisk ( $P < 0.001$ , Wilcoxon-Mann-Whitney test). (b) Mean number of eggs each treatment and control surrogate leaf received

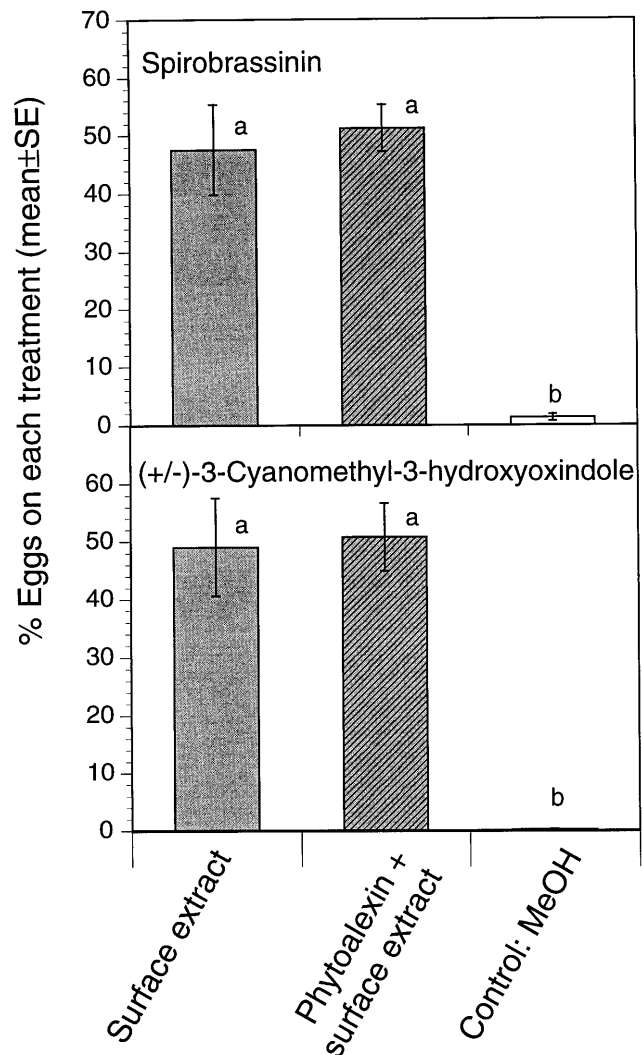
cyanomethyl-3-hydroxyoxindole (**11**) may be the result of these compounds generally inhibiting the flies' motivation to oviposit. Again, such effects would hardly be measurable with the kind of bioassay used. Consequently, spiobrassinin (**4**) and 3-cyanomethyl-3-hydroxyoxindole (**11**) were tested for deterrent effects in the "deterrent bioassay". In the given amounts, neither spiobrassinin (**4**) nor 3-cyanomethyl-3-hydroxyoxindole (**11**) significantly reduced the activity of the tested surface extract and thus both compounds are either not at all or only marginally deterrent (Fig. 4).

## Discussion

Since induced changes in plant metabolism may profoundly influence the fitness of herbivorous insects living on that plant, it can be surmised that the herbivores may have evolved the ability to detect such physiological alterations in their hosts. Karban & Baldwin (1997) cite many examples in which herbivore insects apparently make use of the detected defence signals by avoiding plants with induced protection. In many other cases it was shown that induced resistant plants have a negative effect on the life history of attacking herbivores. However, there are also examples of insects and mites which prefer or develop better on damaged plants (for references see Karban & Baldwin 1997). Yet another example has been presented recently by Stanjek *et al.* (1997). The carrot fly, *Psila rosae*, a specialist herbivore of some Umbelliferae, has been shown to prefer celery plants infiltrated with a jasmonate. In this case both the involved plant mechanisms and the behavioural response of the female insect during oviposition preference have been studied. The authors showed that jasmonates stimulated the biosynthesis of fura-



**Fig. 3** Oviposition of *D. radicum* in choice assays offering simultaneously surrogate leaves treated with various concentrations of a cauliflower leaf surface extract or with 100 µg/leaf of Methoxybrassinin. Bars labelled with different letters are significantly different (8 replicates,  $P < 0.05$ , pair-wise comparisons with Wilcoxon-Mann-Whitney test)



**Fig. 4** Potential inhibition of the oviposition behaviour of *D. radicum* by two selected compounds. Choice assays with surrogate leaves treated with either cauliflower surface extract (0.5 g/leaf), surface extract plus test compound (0.5 g/leaf + 100 µg/leaf), or solvent only. For each compound, columns topped by different letters differ significantly ( $p < 0.001$ , pair-wise comparisons with Wilcoxon-Mann-Whitney test, 12 replicates for Spirobrassinin and 6 replicates for 3-cyanomethyl-3-hydroxyoxindole)

nocoumarins (bergapten, psoralen, xanthotoxin, isopimpinellin) in the leaves, increasing up to 40-fold in relation to the controls. 20% of the total phytoalexins were found to be present on the leaf surface and leaf surface extracts stimulated oviposition in the carrot fly below surrogate leaves.

The cabbage root fly, *Delia radicum*, seems to be yet another example of a herbivore preferring already attacked and presumably physiologically altered plants. Thus it seems consistent that we found that in this fly some phytoalexins act either as a signal for host-plant detection or as a marker for optimally preconditioned plants. However, the observed effects and the amounts of phytoalexins applied to the surrogate leaves in our experiments must be compared with the concentrations that can be expected on the leaf surface. The maximal

concentrations of phytoalexins in UV-irradiation induced slices of turnip roots were 115 µg/g dry wt for cyclobrassinin (3) and 162 µg/g dry wt for spirobrassinin (4) (Monde *et al.* 1991a). We applied 100 µg onto an area that corresponds to approximately 1.25 g of a host-plant leaf. Since leaf surface extracts usually contain lower concentrations of chemicals per g than total tissue extracts, the applied concentrations in our behavioural assays are probably at the upper end of the concentration range that can be expected for phytoalexins on the leaf surface. Despite these relatively high concentrations, only 3 out of 11 phytoalexin compounds stimulated oviposition. In comparison with surface extracts at various concentrations, the most active compound, methoxybrassinin (2), had a stimulatory activity that corresponded to an approximately 20-fold diluted surface extract. Therefore, the most active of the phytoalexins tested singly stimulated oviposition only moderately.

Glucobrassicin, which increases in concentration in response to stress in some crucifers (Agrawal 1998; Griffiths *et al.* 1995), has been described as a major oviposition stimulant for the cabbage root fly (Roessingh *et al.* 1992). In inoculated turnip root tissue the induced biosynthesis of glucobrassicin was >1 mg/g dry wt and thus clearly higher than the corresponding concentrations for phytoalexins (Monde *et al.* 1991a). Roessingh *et al.* (1992) found 420 µg/g of glucobrassicin in fresh leaves of cauliflower and 29 µg/g in leaf surface extracts. The threshold at which glucobrassicin was stimulatory in oviposition assays of the same design as that described here was estimated to be 10<sup>-6</sup> M, corresponding to approximately 1 µg/surrogate leaf (Roessingh *et al.* 1992, 1997). This indicates that glucobrassicin, and some other glucosinolates, if bioassayed as individual compounds, are much more stimulatory than the tested phytoalexins.

Chemical compounds which are the result of changes in the physiological status of a host plant might also be perceived as deterrents. In our behavioural assays, very low overall oviposition activity in some assays and/or trends towards avoidance of phytoalexin-treated leaves suggested such an effect. However, a test for deterrence did not reveal positive evidence for the two candidate compounds spirobrassinin (4) and 1-methoxyindole-3-carboxaldehyde (10).

Considering that several of the tested phytoalexins and glucobrassicin share some important structural features (indole moiety), their difference in activity is remarkable. Via the common precursor tryptophan, these compounds are also biosynthetically linked (Monde *et al.* 1994). With respect to the glucosinolates, the difference in activity between phytoalexins and glucobrassicin suggests that the involved chemoreceptors of the fly are highly specific in their sensitivity. In any case, the receptor neurons sensitive to the phytoalexins remain to be identified and it would appear from preliminary electrophysiological investigations that they are not located in the tarsal sensilla. But it seems important that brassicanal C a related phytoalexin

(Monde *et al.* 1991c), not available for our assays, can be envisaged as one of the precursors of the so called "CIF compounds" which have been recently isolated and identified from *Brassica* plants (Hurter *et al.* 1998, submitted) and which are the most powerful oviposition stimulants known for the cabbage root fly (Roessingh *et al.* 1997).

Recent data from de Jong *et al.* (in preparation) indicate that bacteria associated with the maggots and the roots mediate the production of volatiles stimulating oviposition in *Delia radicum*. Further investigations are needed to elucidate the interesting interactions between plant, plant defence, biosynthesis of plant compounds, microorganisms, and associated insects, such as the cabbage root fly. The results will help to clarify the role of phytoalexins in host-plant preference by the cabbage root fly in general and will in particular be important for the evaluation of host-plant resistance in cultivated Brassicas.

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