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Secondary metabolites of the leaf surface affected by sulphur fertilisation and perceived by the cabbage root fly

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Summary. Surrogate leaves treated with methanolic leaf surface extracts of *Brassica napus* L. (cv Express) plants that received three different sulphur fertilisation treatments showed even more marked differences by the oviposition choice of *Delia radicum* L. than the potted plants. This confirms that the oviposition preference of *D. radicum* is mediated by chemical compounds on the leaf surface and that the quality of host-plants in terms of their nutrition status can be perceived by the female insect.

The oviposition data were positively correlated with the content of fractionated surface extracts containing either CIF ("cabbage identification factor"; 1,2-dehydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorine-1-carboxylic acid) or glucosinolates. Electrophysiological recordings from the tarsal chemoreceptor sensilla C_5 and $D_{3,4}$ showed that receptor neurons react to glucosinolate- and CIF-fractions. We found that the chemosensory activity of specific glucosinolate- and CIF-receptor neurons corresponded with the respective behavioural activity in the oviposition choice assays. The responses of *D. radicum* to glucosinolates in the electrophysiological recordings studies corresponded to the observed oviposition preference on plants or artificial leaves characterised with an higher amount of glucosinolates on leave surfaces. The presented data suggested that CIF and glucosinolates are involved in host-plant preference of *D. radicum* and are perceived by tarsal chemoreceptors.

Key words. Sulphur plant nutritions – *Brassica napus* – *Delia radicum* – Anthomyiidae – Diptera – oviposition choice – contact chemoreception – glucosinolates – CIF

Introduction

We recently showed (Marazzi and Städler, in preparation) that sulphur (S) fertilisation of oilseed rape, *Brassica napus* (L.), influences host-plant preference in the cabbage root fly, *Delia radicum* (L.). The question arises as to which plant characters are perceived by the females leading to the observed preference. The physical and chemical stimuli that influence the oviposition behaviour of the cabbage root fly have been extensively investigated by different authors (reviewed in Städler 2002). *D. radicum* oviposition site selection is influenced by the volatile hydrolysis products of GSLs (Wallbank & Wheatley 1979; Ellis *et al.* 1980; Nottingham & Coaker 1985; Tuttle *et al.* 1988), and plant odour plays an additional role in host selection also after landing (De Jong & Städler 1999). The role of other factors, including non-volatile chemicals on the leaf surface (Roessingh *et al.* 1992b; Hurter *et al.* 1999; De Jong *et al.* 2000) as well as leaf colour (Prokopy & Roitberg 2001) have been investigated. Furthermore, certain physical characteristics, including a waxy surface, a stem, and vertical folds, increase oviposition of *D. radicum* on surrogate plants (Roessingh & Städler 1990). Roessingh *et al.* (1992a) reported that purified glucosinolates (GSLs) stimulate *D. radicum* to oviposit, but they concluded that these compounds account only partially for the stimulatory activity of the plant surface. Roessingh *et al*. (1992a), and more recently Hurter *et al.* (1999) and De Jong *et al.* (2000) were able to isolate and identify the so-called CIF compounds ("cabbage identification factor"; 1,2-dehydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorine-1-carboxylic acid and derivates) from the leaf surface of *Brassica oleracea*, one of the major cultivated host plants that induce oviposition in the cabbage root fly, *Delia radicum* (L.). Baur *et al.* (1996) found that the content of CIF in four different genotypes of two *Brassica* species (*B. rapa* L., *B. oleracea* L. var. acephala D.C.) is also related to the oviposition preference of the cabbage root fly. *D. radicum*. Contact chemoreceptor neurons in the $D_{3,4}$ sensilla chaetica (D-hairs) on the ventral side of the tarsi have been found to be sensitive to GSLs (Roessingh *et al.* 1992a). In addition, Roessingh *et al*. (1997) reported that the pair of ventro-medial C-sensilla on the fifth tarsomer contain two receptors neurons sensitive either to GSLs or CIF.

More recently, also Städler *et al.* (2002) illustrated oviposition preference in different host-plant species of the family Brassicaceae using plant compounds on the leaf surface. But only limited data are available on the role of environmental variation such as plant nutrition, in particular of sulphur (S), on the aforementioned relationship.

The influence of S plant nutrition on the behaviour of the insects has been studied before by Wolfson (1980) on *Pieris rapae* (L.), Koritsas and Garsed (1985) and Yusuf and Collins (1998) on *Brevicoryne brassicae* (L.). However, the choice and performance of insects could not always be

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attributed to the plant fertilisation, because GSLs levels are also influenced by genetical or environmental factors (Bodnaryk 1997; Hopkins *et al.* 1998). In this context, Dosdall *et al.* (2002) reported that S application rate on *B. rapa* has a significant effect on *D. radicum* egg deposition and root damage in the field, but this effect varied depending on the year and the site, indicating that environmental factors are of great importance in determining infestation levels by this pest, and the oxidation state of S in soil. In agreement with these findings, Kim *et al.* (2002) provided evidence that S and nitrogen applications strongly affected GSL content in the edible parts of *B. rapa* plants.

We investigated the effect of S plant nutrition on the oviposition behaviour and sensory perception of the cabbage root fly. *Brassica napus* plants were chosen because of their high sensitivity to S (Scherer 2001) and their economic importance. We compared GSLs and CIF production, by means of leaf surface extracts, in plants grown under three different levels of S supply and assessed the role of these compounds on *D. radicum* behavioural and chemosensory responses.

Materials and methods

Insects

All the *Delia radicum* (Diptera, Anthomyiidae) flies originated from our continuous laboratory culture (restarted with field-collected maggots in 1996) reared according to the method of Finch and Coaker (1969). Approximately 100 adult flies were housed in cubic screen cages (65 × 65 × 65 cm) and held in a climate-controlled room (21 ± 1°C, 80 % RH and 16 h photophase). The cages were provided with a mixture of raw cane sugar, yeast hydrolysate and water (4:1:1) applied on absorbent tissue strips, supplying the flies' food. Water and 10 % sugar solution soaked into cotton-wool were offered separately. Intact cabbage plants (*B. napus* cv CC-Cross F1) at the pre-bolt stage, with a thin layer of fine grain-sized (∅ 3–5 mm) sand on the top of the soil surface were used as oviposition sites. The eggs were collected by flotation in water and transferred to swede roots or kohlrabi planted in moist sand at 20 ± 1°C, 90 % RH and 16 h photophase.

Plants

The oilseed rape (*B. napus* cv. Express) plants were grown under controlled conditions (glasshouse: 22 ± 3°C, 16:8 L:D). The seeds were arranged individually in plastic pots (Ø 9 cm) containing fine quartz sand (granules of 1.5 × 2.2 mm) covered with a surface layer of thicker granules (3.0 × 5.6 mm) to improve gaseous exchanges. The plants were watered twice a week using a modified "Hoagland" nutrient solution to provide the three different S levels of fertilisation: S_n = 1 mM of $MgSO_4$ (normal S concentration in a Swiss field, confirmed by D. Ryser, personal communication); S_+ = 2 mM of $MgSO_4$ (high sulphur level) and S_0 (S-free level), which was obtained by replacing $MgSO_4$ with $MgCl_2$ (1 mM). With the exception of sulphur and chloride, all other macro- and micronutrients were kept constant, according to the original recipe (Lemma Media, see Table 1) (Beaumont *et al.* 1976).

Oviposition choice assays

Bioassay with artificial leaves: We used the same surrogate leaves treated with leaf-surface extracts of the selected plants as previously described by Roessingh *et al.* (1992a). Oviposition bioassays were conducted in the same cages as those described for the rearing, containing approximately 100 adult moths. As for the bioassay with real plants, 4 surrogate leaves (2 for each treatment: S_n (control) vs S_0 or S_n (control) vs S_+), were arranged in a circle on the floor of the cage.

We replicated each pair-wise assay 20 times and after each count new surrogate leaves were used and the positions were changed clockwise to minimize any influence exerted by uneven light distribution. After an oviposition period of 24 hours, the eggs laid on each artificial leaf were counted and expressed as a percentage of the total number of eggs laid on all artificial leaves within one bioassay period. Thus, the resulting preference percentages for the compared treatments (S_n vs S_0 and S_n vs S_+ respectively) totalled 100 %. A Mann-Whitney U-test was performed on the percentages to determine the significant differences between treatments.

Table 1 Nutrient additions for each treatment (mM, 1M)

Treatment	Abbreviation	Element (source)	
		Sulphur ($MgSO_4$)	Chloride ($MgCl_2$)
Sulphur-free regime	S_0	0	1.0
Normal sulphur regime	S_n	1.0	0
Sulphur-rich regime	S_+	2.0	0

Chemical extraction and analysis

Leaf-surface chemicals were extracted using the same extraction procedure described by Städler and Roessingh (1991) to obtain methanolic leaf-surface extracts from S_0, S_n and S_+ plants at the 3–4 true leaf stage. Amounts and concentrations of samples were expressed in gle (gram leaf equivalent) or gle/ml, respectively. One gle represents the amount of leaf-surface extract obtained by dipping in a solvent 1 g of fresh leaf material. For the oviposition assays, these extracts were applied on the surrogate leaves. The GSL fractions of the extracts were separated from the fractions containing CIF (De Jong *et al.,* 2000) compounds using cation exchange resins (described by Baur *et al*., 1996).

In addition to the surface extract, a total leaf extract (homogenate) of each treatment was prepared and its glucosinolate content analysed qualitatively and quantitatively (Marazzi *et al.*, in preparation). We used the same extraction procedure described by Griffiths *et al.* (2001) to obtain desulfoglucosinolates. Although plant handling was particularly cautious, unwanted GSL leakage due to plant's damaging cannot be excluded but it would certainly be randomly distributed between the treatments. The glucosinolates, twenty µl aliquots (representing approximately 2 leaves), were analysed by HPLC. The analytical column used was equipped with a Lichrospher (100 RP 18, 5 µm, 4 × 250 mm). The binary mobile phase system was composed of distilled water (A) and water : acetonitrile, 80 : 20 (B). The analysis was run with the following gradient program: 0 to 45 min linear gradient 0 to 100 % B and then held for 5 min on 100 % B. The flow rate was 1 ml/min and the detection of desulfoglucosinolates was monitored with an UV/VIS detector at 230 nm.

Quantifications were based on 2 GSL standard solutions (Doon Major and Dwarf), prepared and quantified at the SCRI in Dundee, Scotland. The Jasco HPLC system was equipped with Chromeleon software, which was used for data acquisition and analyses. These analytical data are used in the present paper for the correlation between oviposition and sensory data. Pure CIF-1 was isolated from *B. napus* var. *napobrassica* by De Jong *et al.* (2000).

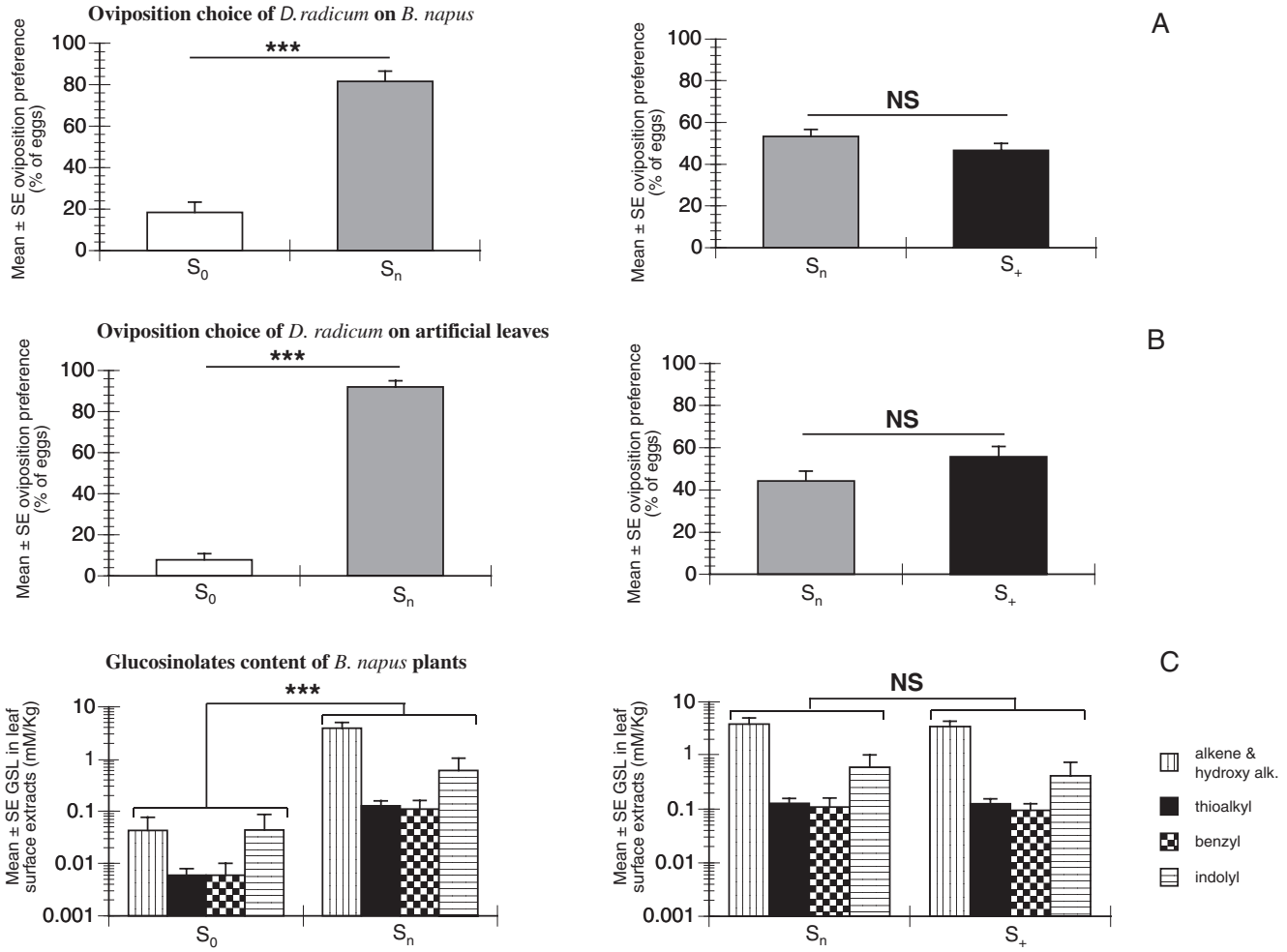

Fig. 1 Oviposition choice of *D.radicum* affected by sulphur fertilisation. (**A**) Proportions of eggs laid on S_0 (sulphur free), S_n (normal sulphur supply) and S_+ (sulphur-rich) *B. napus* plants. These data are the same as reported in Marazzi *et al*. (in preparation) and are shown for comparison. Number of replicates: 20. (**B**) Proportions of eggs laid on artificial leaves sprayed with extracts of S_0, S_n and S_+ *B. napus* at 1 gle. Number of replicates: 20. (**C**) GSL content of S_0, S_n and S_+ *B. napus* plant extracts (10 g freeze-dried plant material). These data are the same as reported in Marazzi *et al*. (in preparation) and are shown for comparison. Number of replicates: 5

Electrophysiology

We recorded the activity of receptor neurons of the ventro-medial C-sensillum on the fifth tarsomer (C_5) and the ventro-lateral D-sensilla on the third and fourth tarsomer ($D_{3,4}$) of 1 day-old female flies using the same technique and set up as described by De Jong *et al.* (2000). All the nerve impulses (spikes) recorded were counted in the time interval of 50–1050 ms after contact of the recording electrode with the tip of the sensillum using our spike train analysis software (STA). The spike counts were averaged for the D and C sensilla. We investigated a total of 25 C_5- and 24 $D_{3,4}$-sensilla. The preparations that gave less than 40 spikes with the standard 10 ng/ml CIF-1 or more than 20 spikes with KCl 10 mM in the initial test recordings from the C_5- sensillum were excluded. The S_0, S_n and S_+ plant extracts (GSL and CIF fractions) were examined each at 0.1 and 1 gle. The set of stimuli was tested sequentially on the sensilla in the following order: S_0, S_n and S_+, always starting with the lowest plant extract concentration. Significant differences between responses to the plant extracts were detected with the Friedman test. Comparisons among selected treatments were then performed using a Wilcoxon Signed Rank Test.

Results

Oviposition choice assays

Bioassay with artificial leaves: The choice assays with surrogate leaves sprayed with methanolic leaf surface extracts yielded the same ranking of preference described by Marazzi *et al*. (in preparation) and shown here for comparison (Fig. 1A). The surrogate leaves proved to be even more active in the oviposition choice of *D. radicum* (Fig. 1B). The number of eggs laid was significantly higher (Mann-Whitney, $n = 40$, $p < 0.001$) on artificial leaves spayed with S_n plant extracts than on artificial leaves sprayed with S_0 plant extract, representing an approximately 10-fold increase. As with real plants, no significant difference was found between the number of eggs laid on artificial leaves sprayed with S_n and S_+ plant extracts (Mann-Whitney, $n = 40$, $p < 0.3302$).

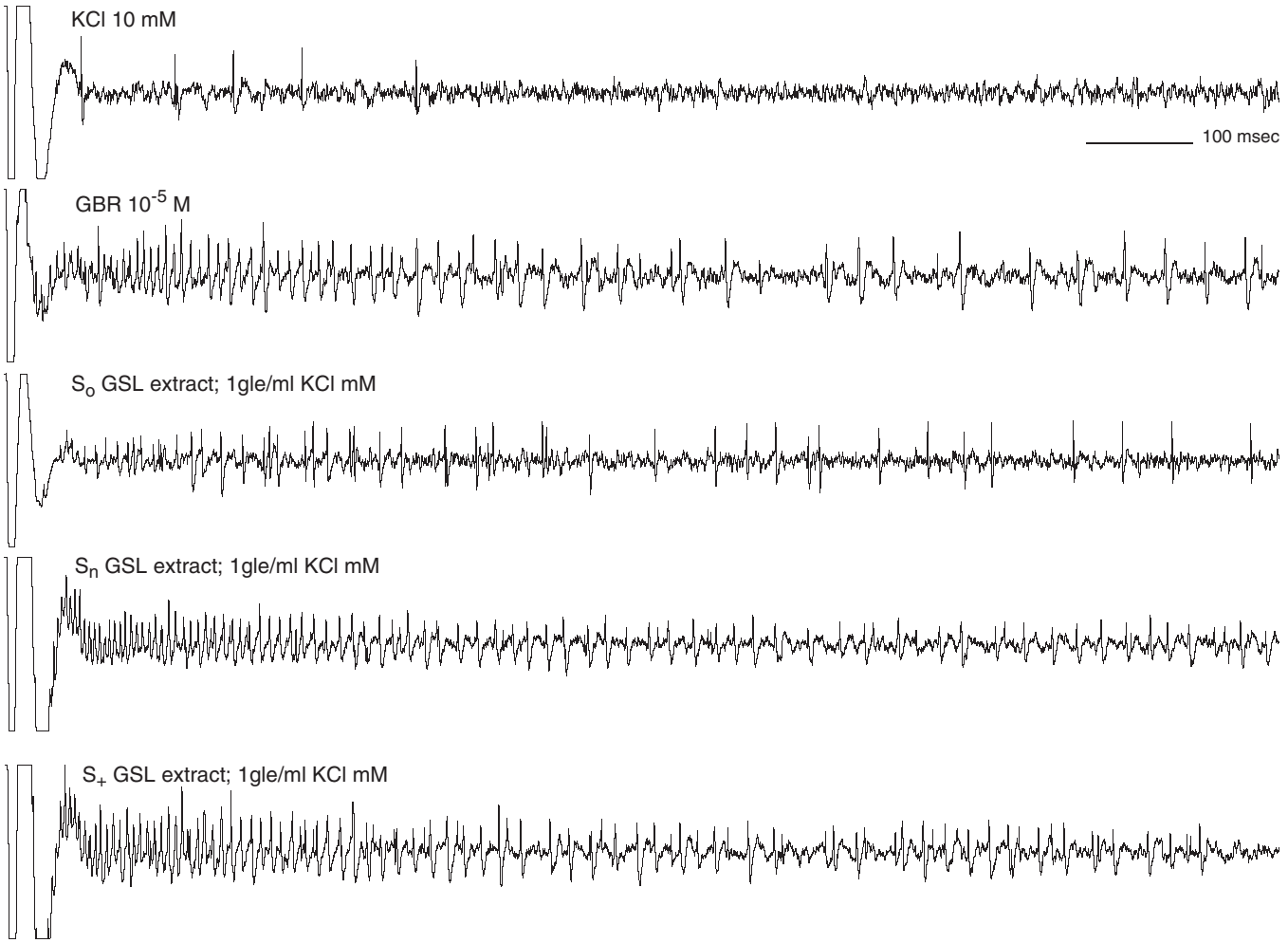

Fig. 2 Electrophysiological recordings from a D_3 sensillum stimulated with GSL fractions of S_0, S_n and S_+ *B. napus* plant extracts. Standard: glucobrassicin (GBR) 10^{-5} M, solvent: KCl 10 mM. gle/ml = gram leaf equivalent per ml. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich plant extracts

Chemical analysis of B. napus plant extracts

The presence of eight GSLs was consistently detected in all tissue from the three populations of *B. napus* tested (Fig. 1C). These data are the same as presented by Marazzi *et al.* (in preparation) in connection with the study of *Plutella xylostella*. Briefly, Fig. 1C shows the differences in the GSL content of the three entries of *B. napus* plants, and reflects female oviposition preference, where the plant extracts that were favoured most, yielding the higher number of eggs per female, were those containing the higher GSLs proportion.

Sensory data

GSL-fractions: The examples of recordings from a D_3 sensilum show that the stimulation of individual D_3 or D_4 sensillum with the three samples of GSL-fractions evoked responses that were different also qualitatively (Fig. 2). Compared to S_0 extracts, we observed an increase in the number of large-sized spikes in response to S_n or S_+ GSL-fractions.

The GSL-sensitive neurons of all the D_3- and D_4-sensilla investigated responded with increased spike frequencies to the GSL fractions (1 gle/ml) in a S concentration-dependent manner (Fig. 3). The comparisons between the different fractions both at 0.1 gle/ml and 1 gle/ml revealed that the S_n and the S_+ GSL-fractions stimulated more spikes than the S_0 GSL fraction (Wilcoxon Signed Rank Test, $n = 24$, $p = 0.0397$ respectively $p = 0.0013$, Fig. 3). No significant difference was detected between spike counts of S_n and S_+ GSL fractions (Wilcoxon, $n = 24$, $p = 0.0865$, Fig. 3).

The stimulation of C-hairs with GSL-fractions evoked on average less than 40 spikes/sec and the results are shown in Fig. 4. Clearly the GSL sensitive neuron was responding too, although the difference between S_0 and S_n or S_+ was not so clear as in the D-sensilla (Fig. 5). The chemosensory responses at both concentration 0.1 gle/ml and 1 gle/ml showed a significant difference between S_0 and S_n GSL-fractions (Wilcoxon, $n = 23$, $p = 0.0337$) and between S_0 and S_+ GSL-fractions (Wilcoxon, $n = 24$, $p = 0.0133$). Conversely, no significant difference was found between responses to S_n and S_+ GSL fractions (Wilcoxon, $n = 25$, $p = 0.2194$, Fig. 5).

CIF-fractions: Fig. 6 shows representative recordings from one C_5-sensillum. Stimulation with KCl 10 mM (solvent control) caused very little activity compared to pure CIF, which

already at 10 ng/ml induced more than 70 spikes/sec in the C_5-sensilla. Recordings with the S_0 CIF-fraction evoked fewer, and more irregular spike patterns than did S_n and S_+ CIF-fractions.

The chemosensory activity of this neuron in the C_5-sensilla in response to the CIF-fractions (Fig. 7) corresponded well with the behavioural activity. The CIF-fractions of S_n and S_+ plant extracts were neurophysiologically more effective than the CIF-fraction of S_0 plant extracts. The observed activity can most likely be attributed to the CIF content, since the chemical analysis of these extracts revealed that no GSLs were present (detection threshold: 1µg GSL/ml crude extract for alkenes, hydroxyalkenes, thioalkenes and benzyl-GSL and 0.3 µg GSL/ml crude extract for indolyl-GSL). The comparison among the chemosensory responses at both concentration 0.1 gle/ml and 1 gle/ml showed a significant difference between S_0 and S_n CIF-fractions (Wilcoxon, n = 25, p = 0.0370) and between S_0 and S_+ CIF-fractions (Wilcoxon, n = 25, p = 0.0323). Conversely, no significant difference was found between responses to S_n and S_+ CIF fractions (Wilcoxon, n = 25, p = 0.9571, Fig. 7).

Correlations between behaviour and GSL analysis

The results of the behavioural assay (Figs. 1A, 1B) correlated directly with the GSLs content of the plants, which was severely affected by the lack of S in the plant nutrition (Fig. 1C). The significant differences in female chemoreception noted during the electrophysiological recordings were reflected in the results of the GSL content of plant extracts used for the tarsal stimulation.

Discussion

Our study is one of the first to show that S supply clearly affect insect behaviour and sensory physiology. So far only the effects of nitrogen fertilisation in *Brassica oleracea* crops have been shown to stimulate insect population as a result of increased consumption and higher utilisation rate (Jansson *et al.* 1991). In contrast, the influence of S nutrition on the responses of the plant to pest attacks has received little attention, and it is mainly the balance between nitrogen and S that is documented (Wolfson 1982), often in relationship with sucking insects (Koritsas and Garsed 1985). However, these studies did not relate lower insect preference and performance to nutrient-deficient plants. Even Dosdall *et al.* (2002), who specifically studied the responses of *D. radicum* to changes in S plant treatment in the field, concluded that only minor benefit may be derived from the use of S applications as a root maggot control strategy. In the present study, by utilising an extreme range in S supply to the plant, we have successfully revealed clear differences in the behaviour of the insects affected, confirming also the conclusion of Dosdall *et al.* (2002) and Kim *et al.* (2002) as well.

Oviposition choice assays with real plants and artificial leaves

An increased S supply to the plants correlated with an increase in oviposition by the adults. The lack of sulphur in the plant nutrient solution reduced oviposition on both true plants and artificial leaves. Surrogate leaves treated with methanol plant extracts showed a more pronounced preference than real plants (Figs. 1A, 1B) suggesting that the oviposition choice largely depends on chemical substances on the leaf surface that are methanol extractable and polar. In contrast, the morphological changes, such as leaf colour or plant size, were not crucial factors in the female choice, as the plants and artificial leaves used for the assays were very similar in appearance.

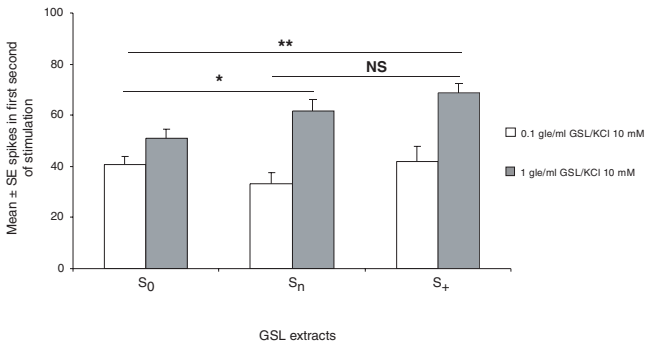

Fig. 3 $D_{3,4}$ tarsal sensilla of *D. radicum* stimulated by GSL fractions of different *B. napus* varying in S nutrition. Numbers of analysed recordings: S_o-S_n: 15; S_o-S_+: 19; S_n-S_+: 18. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich plant extracts

Chemical analysis of B. napus plant extracts

Since *D. radicum* reacts to pure GSL (Roessingh *et al.* 1992a), we expected that the acceptability of *B. napus* by *D. radicum* would be correlated by the GSL levels of the plant. So far, however, in other studies, no evidence of a correlation between the GSL content of the plants and insect preference was reported. For instance, Nair *et al*. (1976) found that the total GSL concentration in the leaves of six cruciferous plant species did not correlate with the oviposition response of *D. radicum*. The authors explained this lack of correlation by the presence or absence of plant inhibitors. But, among the different crucifers tested by Städler *et al.* (2002) and Griffiths *et al.* (2001), the authors found a clear correlation. However, it should be noted that this was only true for benzyl and indolyl GSLs, and not for aliphatic GSLs. We found that the amount of GSLs and CIF in the leaf surface determine oviposition preference in *D. radicum*, confirming the conclusions of Ellis *et al.* (1980), who showed a relationship between the amount of volatile GSLs hydrolysis products in radish extracts and the oviposition choice of the cabbage root fly. They found that radish varieties with an increased content of 4-methylthio-3-butenylisothiocyanate and 1-cyano-4 methylthio-3-butene stimulated more the oviposition.

Since volatiles can have a significant synergistic effect on oviposition (De Jong & Städler 1999), they may also be part of the stimulating effect of our extracts. However, we believe that isothiocyanates played a minor role, because our extraction procedure avoided the myrosinase enzyme reaction. A chemical analysis of the leaves clearly showed that S deficiency depressed the biosynthesis of GSLs and CIF, which reduces oviposition stimulation.

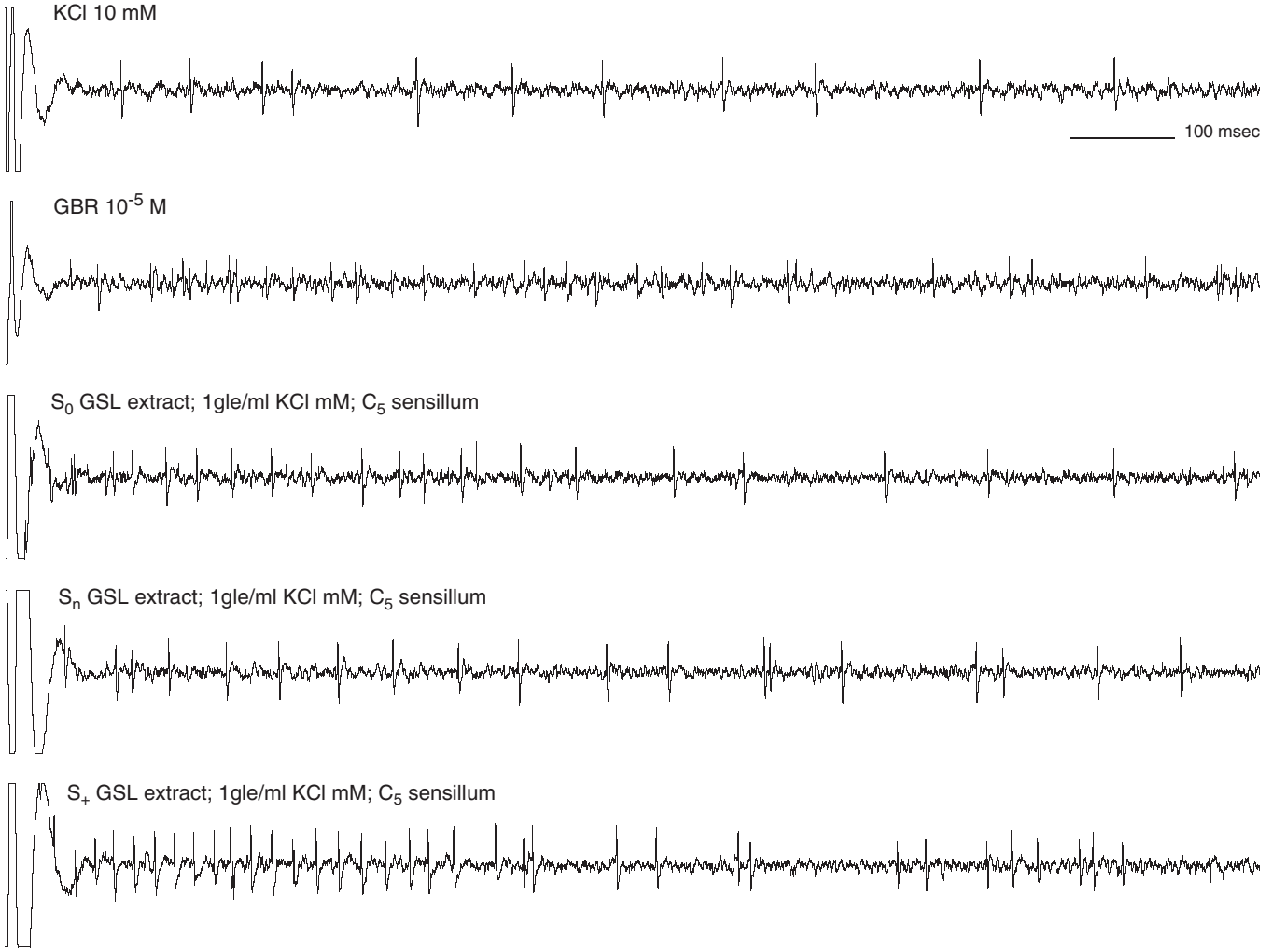

Fig. 4 Electrophysiological recordings from a C_5 sensillum stimulated with GSL fractions of S_0, S_n and S_+ *B. napus* plant extracts. Standard: glucobrassicin (GBR) 10^{-5} M, solvent: KCl 10 mM. gle/ml = gram leaf equivalent per ml. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich plant extracts

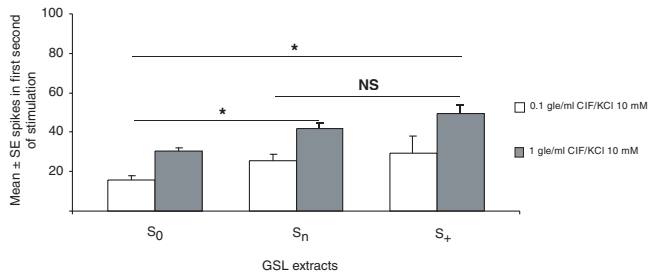

Fig. 5 C_5 tarsal sensilla neurons of *D. radicum* stimulated by GSL fractions of different *B. napus* varying in S nutrition. Numbers of analysed recordings: S_o-S_n: 15; S_o-S_+: 19; S_n-S_+: 18. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich plant extracts

Sensory physiology

In agreement with the behavioural data, the chemosensory responses of *D. radicum* were also significantly stronger for S_n and S_+ plants than for S_0 plants. Our results corroborate the view that for this insect contact chemoreception is a very important parameter for stimulation of oviposition.

The oviposition data correlated well with the CIF content of the three samples of *B. napus*, estimated by the electrophysiological recordings from C_5-sensillum and with the response to the GSL fraction. We found that the spike frequency increased depending on the S-concentration of both extracts (0.1 and 1 gle/ml), suggesting that S fertilised plants contain greater amounts of CIF than S_0 plants. A possible reason for the absence for a stronger correlation could be that other volatile or non-volatile compounds present in the extracts may affect positively or negatively the oviposition behaviour and that the GSL contribute to the sensory imput as well. Specifically, we cannot exclude that the CIF fraction contains unidentified stimulatory or inhibitory plant compounds. If present, they could be detected by one of the other two neurons (total four) (Isidoro *et al.* 1994) of the C_5-sensillum or of the many other sensilla on the tarsi and

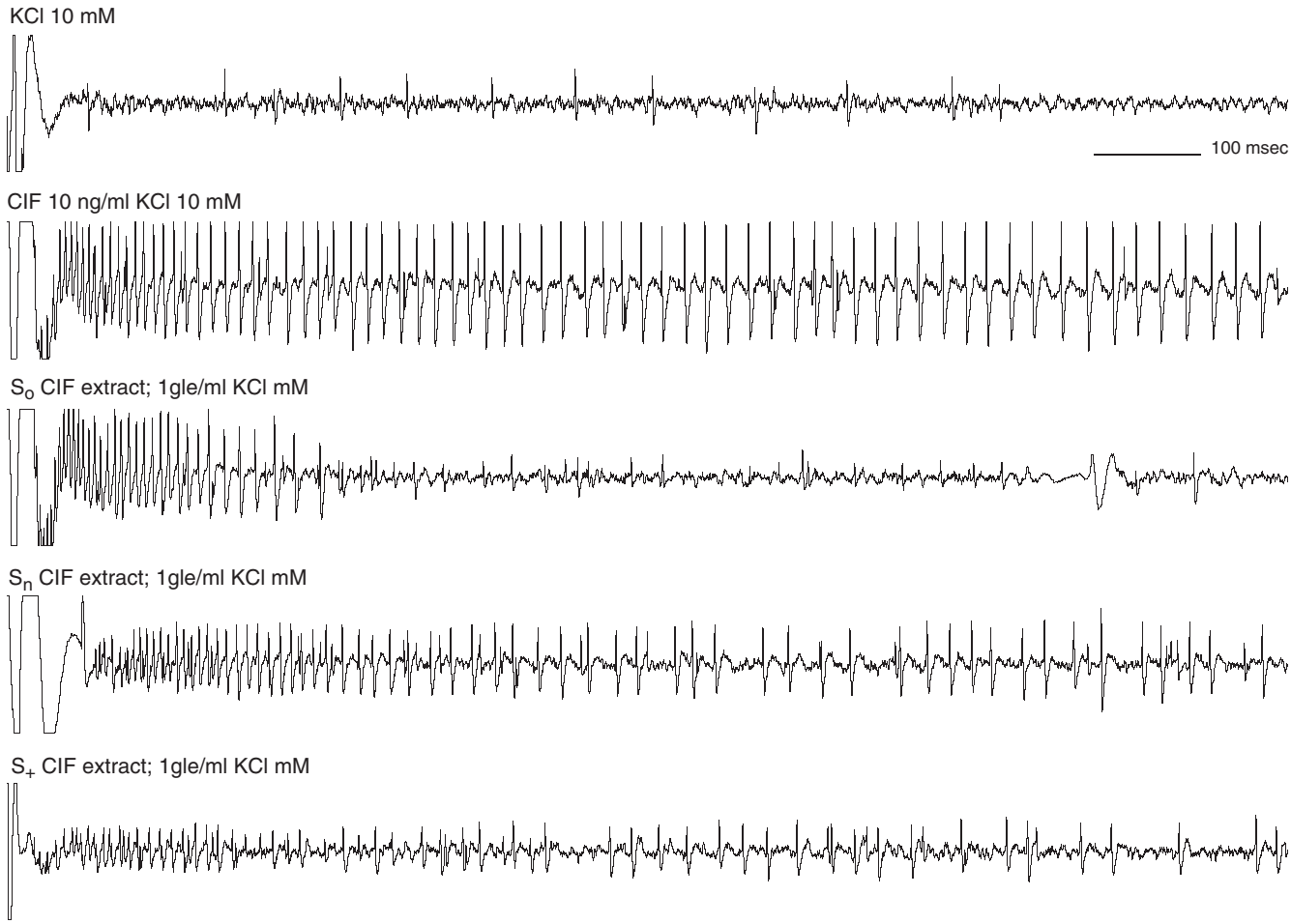

Fig. 6 Electrophysiological recordings from a C_5 sensillum stimulated with CIF fractions of S_0, S_n and S_+ *B. napus* plant extracts. Standard: CIF 10 ng/ml KCl mM, solvent: KCl 10 mM. gle/ml = gram leaf equivalent per ml. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich plant extracts

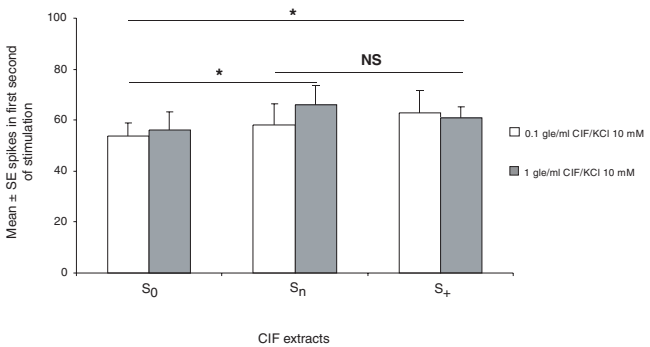

Fig. 7 C_5 tarsal sensilla neurons of *D. radicum* stimulated by CIF fractions of different *B. napus* varying in S nutrition. Numbers of analysed recordings: S_o-S_n: 15; S_o-S_+: 17; S_n-S_+: 11. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich plant extracts

proboscis and lead to an increase or reduction in spike frequency of the stimulated sensilla. Further, interacting effects that synergise or inhibit the GSL or CIF receptor neurons might be active, as reviewed recently by Chapman (2003).

In conclusion, the observed order of effectiveness in the behavioural and the electrophysiological studies correlate quite well. The two preferred host plants or surrogate leaves (S_n and S_+) in the oviposition choice contain greater amount of both GSLs and CIF, suggesting, as already pointed out by Städler *et al.* (2002), that multiple chemical stimuli are important in host acceptance.

Furthermore, the fact that no significant differences in insect behaviour were observed between S_n and S_+ plants implies that applying S amounts close to the optimal fertilisation level for oilseed rape will not cause an increase in oviposition, and this is in agreement with the conclusion of Dosdall *et al.* (2002).

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