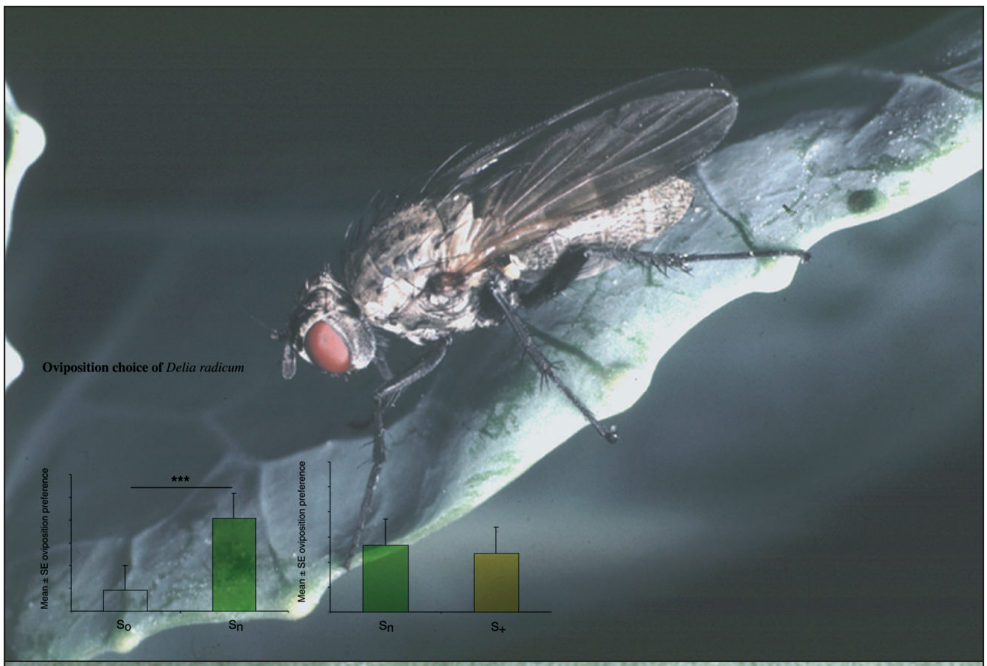
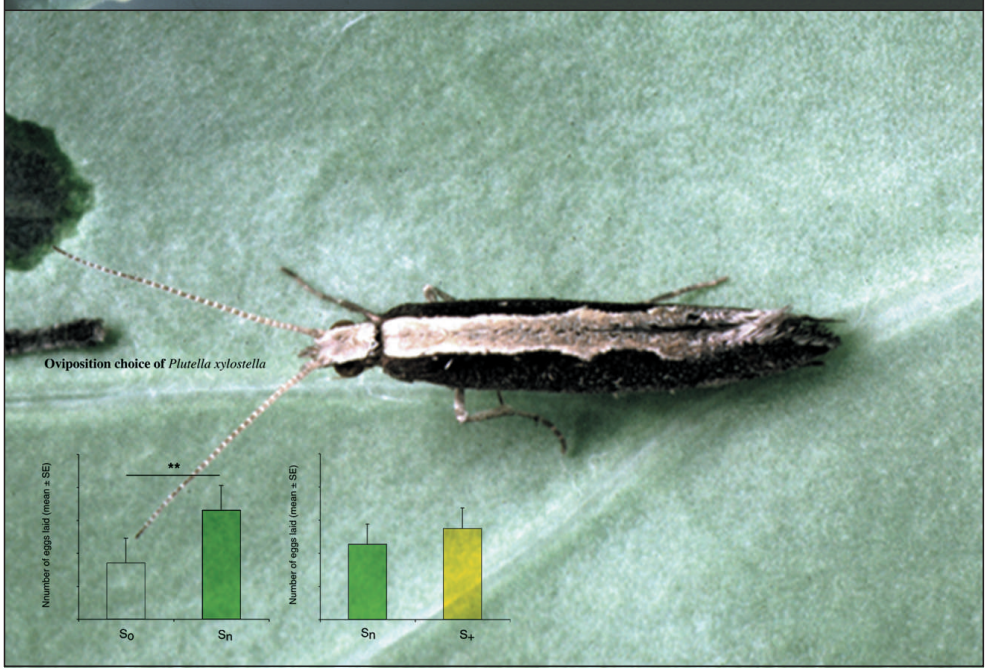
PLANT SULPHUR NUTRITION INFLUENCING HOST-PLANT SELECTION AND PERFORMANCE OF INSECT HERBIVORES





Thesis of Cristina Marazzi

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PLANT SULPHUR NUTRITION INFLUENCING HOST-PLANT SELECTION AND PERFORMANCE OF INSECT HERBIVORES

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Contents

In the last fifty years, the basic questions in the field have not changed much:

The most fundamental aspect of host selection in leaf eating species inquires whether the selection is governed (a) by the nutritional superiority of the plant or region of the plant serving as food for the insect, or (b) by the presence or absence of attractants and repellents in plants of more or less uniform food value to which the parasitic species has become adapted (Lipke & Frankel 1956).

In memory of my sweet mother

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Summary

In the past several years, sulphur (S) deficiencies in agricultural crops have been reported with increasing frequency and therefore more interest has been directed into plant nutrition and fertilisation with particular respect to this element. In this context, special attention has been given to the economically important oilseed rape crop (*Brassica napus*), because of its high S need. Oilseed rape removes between 20 and 30 kg S/ha from the soil, corresponding approximately to double the demand of cereals. Until now, the effect of different S-supplies to plants in relationship to their pests has not attracted much attention. The aim of this study was therefore to investigate the influence of S-nutrition of rape on the preferences and performance of two main crucifer pests, the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera; Plutellidae) and the cabbage root fly, *Delia radicum* (L.) (Diptera: Anthomyiidae). The larvae of *P. xylostella* attack the aerial part while *D. radicum* infest the roots of numerous wild and cultivated plants belonging to the Cruciferae family. Since the larvae of both insects have only a limited capacity to select alternative plants or roots in the soil autonomously, their survival depends largely on the host choice made by the ovipositing female.

To assess the effect of S plant nutrition on the oviposition behaviour, females of both insects were exposed to plants grown under three different S-regimes: S-free (S_0), normal-S (S_n) and S-rich (S_+) plants. In both insects the lack of S in the plant nutrient solution resulted in reduced oviposition, while differences between the two S fertilisation levels (normal and double field concentration) were smaller and not significant. In order to identify the plant characteristics influenced by S and perceived by the female insects, the ovipositional responses of the two crucifer pests were further tested by means of methanolic leaf surface extracts of the three types of *B. napus*. Using surrogate leaves treated with extracts of plants with an equivalent fresh weight, the same preferences were observed as in corresponding experiments with intact leaves, namely decreased oviposition in the absence of S.

Also in both insects, the duration of larval development, from hatching to emergence, was significantly shorter and adults were heavier on S_n than on S_0 plants. Comparing these same two parameters in S_n and S_+ plants, a somewhat shorter development time on plants rich in S was recorded, but again this trend was not statistically significant. Larval feeding preferences of the diamondback moth were tested in a dual choice assay, using leaf discs. A significantly higher number of larvae preferred leaf discs of S_n plants rather than those of S_0 plants. Further, the larvae preferred S_+ to S_n discs.

In the case of the cabbage root fly, the larval performance was evaluated using three additional intermediate sulphur levels between S_0 and S_n. The percentage pupation at the end of larval feeding ranged from 6% (S_0) to 32% (S_n), and the mean adult fresh weight of the emerging flies varied between 3.2 mg (S_0) and 8.17 mg (S_n). Both the percentage of pupation and the adult fresh weight were positively correlated with the S content of the plant nutrient solution.

Since GSLs and their volatile metabolites, the isothiocyanates, are known to stimulate oviposition in different insects, an analysis was made of the GSLs composition at the leaf surface of the three S variants. The analytical (GSL) data correlated with the host preferences of both the diamondback moth and the cabbage root fly. Furthermore, in the case of the cabbage root fly, a non-GSL compound, called CIF ("cabbage identification factor", thia-triaza-fluorenes) and known to be a powerful oviposition stimulant, was also more abundant in S fertilised plants.

The electroanntenogram recordings (EAGs) obtained from *P. xylostella* antennae confirmed that olfaction is an important modality for the stimulation of oviposition in this insect, as extracts from S fertilised plants caused more stimulation than those from S_0 plants. This might be due to the higher content of isothiocyanates. In the case of *D. radicum*, we recorded the response of the tarsal contact chemoreceptor neurons to methanolic extracts. The specific receptor neurones for CIF and GSL reacted more strongly to the extracts from the two preferred plants (S_n and S_+). This shows that the fluorene compound CIF, together with indolyl and benzyl GSLs, is involved in host acceptance and confirms the analytical results. Our findings are in agreement with earlier publications reporting that contact chemoreception is the most important modality for the stimulation of oviposition in the cabbage root fly.

In the final part of this work it was found that in dual oviposition choice bioassays leaf extracts of *Arabidopsis thaliana* plants stimulated oviposition in *D. radicum*. Both the CIF and the GSL fractions of the plant extract stimulated receptor neurons in the tarsal sensilla. This is an indication that in addition to the known GSLs, *A. thaliana* also contains CIF.

In conclusion, it appears that even a higher than optimal S fertilisation will not lead to a significantly increased accumulation or population outbreaks of the two harmful pests investigated. Further, these findings confirm that secondary plant metabolites play a crucial role in host recognition by the insect. Plant nutrient solutions not only affect directly the nature and concentration of secondary metabolites but also indirectly the host-plant relationship, modulating the host-choice and the plant suitability for bi- (herbivores) and possibly tritrophic-(parasitoids) interactions.

Zusammenfassung

In den letzten Jahren sind bei landwirtschaftlichen Kulturen vermehrt Schwefel (S) Mangelerscheinungen aufgetreten und dies hat zu einem gesteigerten Interesse an der Pflanzenernährung mit diesem Element geführt. In diesem Zusammenhang wurde dem Ölraps besondere Beachtung geschenkt, weil diese Kultur für eine optimale Ernte besonders viel Schwefel braucht und dem Boden pro Hektare 20 bis 30 kg S entzieht, was dem Doppelten des Bedarfes von Weizen entspricht. Bis heute wurde der Schwefelversorgung der Pflanzen in Bezug auf den Einfluss auf deren Schädlinge und Krankheiten wenig Beachtung geschenkt. Darum versuchte ich zu bestimmen, ob eine bessere Versorgung der Pflanzen mit S zu einer Vermehrung des Befalls und zu einer verstärkten Entwicklung der Schädlinge führt.

Für die Untersuchungen verwendete ich zwei der wichtigsten Schädlinge des Rapses, die Kohlschabe, *Plutella xylostella* (Lepidoptera, Plutellidae) und die Kohlfliege, *Delia radicum* (Diptera, Anthomyiidae). Die Larven der Kohlschabe befallen die oberirdischen Teile der Pflanze, während die Maden der Kohlfliege die Wurzeln von verschiedenen wilden und kultivierten Pflanzen der Familie der Cruciferae (Brassicaceae) befallen. Die Larven beider Insekten können nur beschränkt selbst die Wirtspflanze wählen oder wechseln, darum sind sie für ihre Entwicklung stark von der Wirtswahl der Weibchen bei der Eiablage abhängig.

Um den Effekt der S Pflanzenernährung auf das Eiablageverhalten der Weibchen zu studieren, wurden sie Pflanzen ausgesetzt, die mit unterschiedlichen S Regimen gedüngt wurden. Ich verglich die Wahl zwischen Pflanzen die ohne S (S_0), mit normaler S (S_n), oder doppelt normaler S Lösung (S_+) ernährt wurden. Bei beiden Insekten führte der S Mangel zu Pflanzen, die bei der Eibablage weniger bevorzugt wurden als die mit S gedüngten Pflanzen. Die Unterschiede zwischen den zwei S Düngungen waren dagegen viel geringer und nicht signifikant.

Auch die Dauer der Larvenentwicklung (Schlüpfen der Larven aus den Eiern bis zum Schlüpfen der Adulten aus den Puppen) beider Insekten wurde bei beiden Insekten durch die Schwefelernährung der Wirtspflanzen beeinflusst. Auf S_0 Pflanzen dauerte die Entwicklung länger und die adulten Insekten, die schlüpften waren leichter als auf den S_0 und S_n Pflanzen. Der selbe Trend zu rascherer Entwicklung und schwereren Puppen und Adulten bei Pflanzen mit besserer S Ernährung konnte auch im Vergleich zwischen den S gedüngten Pflanzen (S_n, S_+) beobachtet werden. Diese Unterschiede waren aber nicht signifikant. Bei der Kohlschabe bevorzugten die Raupen die S gedüngten Pflanzen und frassen auch mehr von den angebotenen Blattscheiben. Zudem bevorzugten die Raupen signifikant Blattscheiben von S_+ gegenüber S_n gedüngten Pflanzen. Flüchtige Stoffe der S versorgten Pflanzen ergaben stärkere Elektroantennogramme (EAG) als S_0 Pflanzen. Dies wurde vermutlich durch die höhere Konzentration von Isothiocyanaten verursacht.

Bei der Kohlfliege führte Schwefelmangel ebenfalls zu einer reduzierten Eiablage, indem die Weibchen die S gedüngten Pflanzen bevorzugten. Die Entwicklung der Larven wurde zusätzlich bei drei Pflanzengruppen mit abgestuften Schwefel Düngerlösungen zwischen S_0 and S_n getestet. Der Prozentsatz der Verpuppung am Ende der Larvenentwicklung schwankte zwischen 6 % (S0) und 32 % (Sn) und das Gewicht der schlüpfenden Adulten lag zwischen 3.2 mg bei S0 und 8.17 mg bei Sn. Die Verpuppungsrate (Anzahl Puppen pro infizierte Eier) war mit dem Lebendgewicht der Adulten mit dem S Gehalt der Nährlösung der Pflanzen positiv korreliert.

Um die Charakteren der Pflanzen zu identifizieren, welche von der S Versorgung beeinflusst werden und von den zwei untersuchten Insekten wahrgenommen werden, verglich ich auch die Oberflächenextrakte der verschieden gedüngten Pflanzen bei der Eiablage. Dazu wurden die Methanol Extrakte auf künstliche Blatt-Attrappen aufgetragen und den Fliegen zur Wahl angeboten. Die Attrappen ergaben exakt die selben Resultate wie die *B. napus* Pflanzen, die unterschiedlich gedüngte worden waren. Das heisst die Attrappen die mit Extraten von S_0 Pflanzen behandelt wurden, stimulierten viel weniger Eiablagen als jene Attrappen, welche mit Extrakten von S gedüngten Pflanzen von gleichen Gewicht herstammten. Diese Resultate zeigen, dass chemische Faktoren der Blätter für die Erkennung der besser mit S versorgten Pflanzen verantwortlich waren. Da Glucosinolate und ihre flüchtigen Metabolite (Isothiocyanate) bekannt dafür sind, dass sie die Eiabage der zwei Insekten stimulieren, analysierten wir auch den Gehalt an Glucosinolaten bei den verschieden gedüngten Pflanzen. Die Gehalte der Blattextrakte korrelierte mit den erhobenen Präferenzen der Kohlschabe und der Kohlfliege für S_n und S_+ Pflanzen. Im Falle der Kohlfliege konnten wir auch zeigen, dass die Schwefelhaltigen Inhaltsstoffe CIF (cabbage idendification factors), die die Eiablage stimulieren, aber nicht mit den Glucosinolaten verwandt sind, bei den S gedüngten Pflanzen ebenfalls stärker konzentriert waren. Es kann aber nicht ausgeschlossen werden, das noch andere Metaboliten, die auch keinen S enthalten, von der S Düngung beeinflusst wurden und die Eiablage Präferenz verschieden (positiv oder negativ).

Die Elektroantennogramme von Antennen der Kohlschabe bestätigten, dass Geruch eine wichtige Modalität für die Wirtwahl dieses Insektes ist. Die flüchtigen Substanzen der Blatt-Extrakte von S gedüngten Pflanzen waren stärkere Stimulantien als jene von S_0 Pflanzen. Im Falle der Kohlfliege untersuchten wir die Kontakt Chemorezeptoren der Tarsen und fanden, dass nicht nur die Neurone, welche auf CIF spezifisch reagieren, sondern auch die GLS Neurone auf den höheren Gehalt der Extrakte bei den S gedüngten Pflanzen reagieren. Dies bestätigt auch die chemische GS Analyse und bestärkt die Annahme, dass die durch die S Düngung beeinflussten S Inhaltsstoffe in der Tat die Präferenz der Kohlfliege erklären können.

Ich kann die Befürchtungen zerstreuen, dass eine optimale Schwefeldüngung zu einer starken Bevorzugung der besser gedüngten Pflanzen und zu Explosionen der Schädlingspopulationen führen wird. Ferner bestätigen die Resultate meiner Dissertation die Hypothese der Grundlagenforschung, dass sekundäre Pflanzenstoffe für die Wirtswahl sehr wichtig sind. Die Nährstoffversorgung der Pflanzen kann die Konzentration sekundärer Pflanzenmetabolite beeinflussen und dadurch auch die Wirtswahl und die Entwicklung der herbivoren Insekten und ihrer Parasiten beeinflussen.

Part 1: General introduction

A. Insect-plant relationships

When attempting to increase agricultural production in order to feed a world population with a present growth rate of 1.6% per year, to reduce the use of synthetic insecticides and to convert current agriculture into more sustainable systems, insights gained from insect-plant studies may appear helpful if not indispensable (Schoonhoven *et al.*, 1998).

1. Introduction

Interactions between insects and host plants will always be mediated to varying degrees by the ever-changing biotic and abiotic community (Strong *et al.*, 1984) as well as by ecological opportunities and constraints imposed by plant defensive chemistry (Feeny, 1995). For example, intraspecific variance in host-plant tissues for herbivores (Orians & Jones, 2001) may be caused by available nutrients, soil composition (Scriber, 1984a), micro-climate, individual plant genetic differences, plant tissue ontogeny (Scriber, 1984b), herbivore (or abiotic) induction responses (Tallamy & Raupp, 1991), somatic mutations (Karban & Baldwin, 1997), and/or the interplay between these factors (Städler, 1992). Insects may respond by choosing different feeding sites, by altering their consumption rates or by induction of physiological/detoxification enzymes (Scriber & Slansky, 1981; Bernays & Chapman, 1994).

2. Environmental factors influencing insect-plant interactions

The earth is undergoing rapid environmental changes due to human activities (Tilman & Lehman, 2001) changing both the players and the rules of the game in insect-plant interactions. One of the consequences of this human transformation of the earth is to alter the constraints on plant growth and thereby change the context in which insect-plant interactions operate. Environmental constraints on plant communities include resource limitation, recruitment limitation, predators and pathogens, disturbance, temperature, climate and temporal variation (Tilman & Lehman, 2001). Man's intervention is modifying these constraints in multi-dimensional and multiplicative ways, creating habitats less favourable to native residents and more favourable to exotics whose evolutionary history pre-adapts them to these changed conditions (Keane & Crawley, 2002).

Natural rates of nitrogen addition and phosphorus liberation into the terrestrial ecosystem have been doubled (Carpenter *et al.*, 1988; Vitousek, 1994; Vitousek *et al.*, 1997) . The supply of nitrogen often limits plant production in terrestrial ecosystems, while nitrogen concentration in the plants can be in many cases a limiting factor in herbivore population growth (White, 1993).

Human activities have also dramatically altered the sulphur cycle on earth (Knights *et al.*, 2001). The demand for sulphur during growth varies widely in natural and agricultural plant species. The sulphur content in natural and agricultural plant species ranges between 0.03 and 2 $\mu mol\ g^{-1}$ dry weight (Ernst, 1997; Schnug & Haneckla us, 1998). At present, sulphur deficiency appears to be one of the most common nutrient stress factors and it occurs frequently in crops throughout the world, resulting in a loss of crop production, crop resistance to pests and food quality (Schnug, 1997; Schnug & Haneckla us, 1998. One of the primary causes of sulphur deficiency appears to be the ongoing reduction of atmospheric sulphur deposits as the consequence of strict regulations on industrial sulphur emissions (Blake-Kalff *et al.*, 2000). Mineral deficiency decreases the nutritional value of a plant and therefore slows herbivore growth and performance (Fragoyiannis *et al.*, 2001; Jansson & Ekbom, 2002).

Environmental stress may alter the physiology and biochemistry of a plant in a way that alters the nutritional value (primary metabolites) for herbivores (Siemens *et al.*, 2002) but stress may also cause changes in levels of secondary metabolites that could affect the behaviour and physiology of insects (Städler, 2002). In some cases, the combined nutritional and allelochemical changes improve the quality of the host plant as a source of food, and can therefore be considered favourable to herbivorous insects (Baur *et al.*, 1998). However, there is considerable diversity in the response of different insects to environmentally induced changes such as water or fertiliser supply, as has been shown in a review by Waring & Cobb (1992). The chemical composition of the plant influenced by the environment is in many cases the most important source of information that herbivorous insects use to discriminate between host and non-host plants (Berenbaum & Zangerl, 1998). Environmental stress can therefore greatly influence plant susceptibility to herbivores and pathogens (De Bruyn *et al.*, 2002). What occurs in the period subsequent to a stress may be an underestimated aspect of such relationships (Wallin & Raffa, 2001). Tolerance of insect herbivores by plants has often been presumed to be enhanced by fertilisation, however results are equivocal (Scriber, 1984a; Mattson & Scriber, 1987; Herms & Mattson, 1992; Strauss & Agrawal, 1999; Stowe *et al.*, 2000). Contradictory results have also been reported on insect performance in connection with plant stress caused by air pollution, affecting plant susceptibility by changing its metabolism, yield, crop quality, soil fertility, temperature regimes and hydrology (Rogasik *et al.*, 2002). Plants grown in the presence of elevated CO_2 usually have higher C/N ratios than plants grown in current ambient CO_2 atmosphere. A reduced proportion of nitrogen in the plant foliage decreases growth of chewing herbivorous insects, but the few studies on the effect of elevated CO_2 levels on sucking insects such as aphids have not yielded similar consistent effects (Holopainen, 2002).

The extremely wide range of variance in environmental factors can have important and even predictable biological impacts, especially for non-linear response functions, including survival, population growth and community dynamics (Ruel & Ayres, 1999).

In summary, mankind's transformation of the earth has altered both organisms and environment, and our attitude to nature in general, including insect-plant interactions, needs to change to keep pace with this transformation.

3. Insect host-plant selection

While the ultimate causes of diet specialisation in herbivorous insects still remain largely unclear, there has been substantial progress in understanding the factors that govern host choice in feeding or ovipositing insects. Host-plant selection is mainly a behavioural process, which is primarily regulated by chemoreception (Jeremy & Szentesi, 2003). Detailed knowledge of insect behaviour is crucial to understanding the selective response of the insect to a particular plant. The

insect is attracted to or repelled by a plant due to a variety of factors such as its shape, size (Langan *et al.*, 2001), colour (Hirota & Kato, 2001), surface texture (Spencer *et al.*, 1999) and chemical constituents (Städler, 2000). The majority of phytophagous insects are monophagous or oligophagous, feeding on a very narrow range of plant species or families. Their host range is limited, in part by the chemical complexity of the plants they encounter, host plants being selected because they either contain stimulants or lack deterrents. Given that plants compounds play a major role in the selection behaviour of insects, the ability of insects to perceive and discriminate among compounds is of vital importance (Bernays, 2001). The nature of plant-derived allelochemicals or secondary metabolites involved in the different stages of insect-plant interactions, from habitat selection to host acceptance, is very complex. In the last decade our ability to isolate these plant-derived compounds and to determine their structures has greatly improved thanks to the advances in analytical phytochemical techniques, especially in the use of HPLC for non-volatiles. Consequently, the opportunities to assess the role of these compounds in insect-plant interactions, using behavioural and electrophysiological bioassays, have also improved. In particular, a large number of biological and chemical studies have been directed towards characterising secondary metabolism in plants of economic relevance.

4. Multitrophic effects

In addition to direct responses to herbivores, plants are capable of complex, indirect defence responses that involve the recruitment of a third party. For instance, volatiles phytocompounds, which were originally produced by the plant as herbivore feeding deterrents, have become cues for herbivore predators and parasites (Dicke & Van Loon, 2000). Such organisms have apparently learnt to associate these volatiles with the presence of prey or hosts, and therefore benefit by responding to the stimuli. A few recent studies (Wegner *et al.*, 2001; Mumm *et al.*, 2003) have shown that plants are able to emit volatiles also in response to herbivore's egg deposition and that these volatiles attract egg parasitoids. Studies on the mechanisms of induction of volatiles by egg deposition show several parallels to the mechanisms of induction of plant responses by feeding damage (Hilker & Meiners, 2002).

These volatiles may also be induced systemically in the damaged plant implying that responses induced by aboveground pathogens may affect belowground phytophages feeding on the same plant, and vice versa (van Dam *et al.*, 2003). Moreover, herbivores-infested plants may interact with undamaged neighbouring plants through chemical information that is exchanged in the soil.

Overall, plants profit from this multitrophic interaction, since there is an increased probability that the herbivores will be removed, so that there is still an advantage in releasing volatiles even after herbivores have evolved immunity to their toxic and deterrent properties.

5. Application of basic research in agriculture

5.1 Introduction

Plants in nature and agriculture face a diversity of challenges that includes both pathogens and herbivores. In natural ecosystems roughly 10% of a plant's resources are lost to herbivory (Kleijn *et al.*, 2001), while preharvest losses of agricultural crop production vary between 10% and 100% if insecticides are used. For reasons of mechanisation and efficiency of sowing, planting management, harvesting and processing, agricultural crops are predominantly grown as monocultures, especially in the Western world. However, such systems are known to be more prone to insect population outbreaks than the so-called natural systems (Risch, 1987).

Diversification is probably a key element in future insect control strategies in agriculture (Pimentel, 1991). For instance, to make the actual agroecosystems less favourable to natural enemies and increase vegetational diversity, a cultural strategy called intercropping, which consist in planting different crops intermingled, was developed (Parfait & Jarry, 1987). Interactions between component crops make intercropping systems more complex and frequently reduce pest attack. Another documented strategy is the trap-cropping technique (Asman, 2002). Trap crops are plants stand in the vicinity or within certain parts of a field where the principal crop is grown, attracting pest insects and preventing target crop pest infestation. Similarly, the so-called push-pull strategy consist in trapping pest insects on highly susceptible trap plants (pull) and keeping them away from the crop using repellent intercrops (push) (Kahn *et al.*, 2001).

The study of insect-plant relationships therefore constitutes, as Lipke and Frankel (1956) aptly wrote, "the very heart of agricultural entomology", and in nature, host-plant resistance and natural enemies are the two dominant factors controlling herbivorous insect populations.

5.2 Host-plant resistance

Insect-plant interactions involve cause and effect relationships between phytophagous insects and their host plants. Insect pests multiply rapidly on suitable hosts, while their populations are constrained on less suitable hosts. At the same time, unsuitable hosts and slow growth result in longer exposure to parasites and predators. Such differences in acceptability occur both among and within species of host plants and can serve as important methods of pest-control.

The various plant characteristics that help to protect crop plants from insect-pest damage are collectively known as *varietal resistance*, which was defined first by Painter (1951) as follows: "the varietal resistance is the relative amount of heritable qualities possessed by the plant, which influence the ultimate degree of damage done by the insect". The word "relative" referring to the plant's resistance is important since it is rare to find host-plant varieties, even if considered highly resistant, that are immune to insect attack. This definition, slightly modified by Kogan (1986), is still used and has been accepted as standard by the majority of workers dealing with the practical aspects of pest control in crop production. Furthermore, the concept of varietal resistance was classified into three broad categories: antibiosis (the host plant adversely affects the bionomics of the insects feeding on it), antixenosis (= non-preference: the plant's characteristics make it unattractive to insect pests for oviposition, feeding or shelter) and tolerance (the host plant undergoes only slight, non-economical injury in spite of supporting an insect population large enough to damage severely susceptible hosts) (Painter, 1951; Kogan, 1986).

Varietal resistance and antibiosis cannot be considered synonymous, because antixenosis is also an important factor. As brief infestations cause severe plant damage, such as severing of the growing parts of the plant or transmission of virus diseases, non-preference (antixenosis) may even be of greater importance than antibiosis. In field plantings non-preferred crop varieties frequently escape infestations or develop low ones as reported, for example, Ellis *et al.* (1999) for the cabbage root fly. Insects, when caged on these hosts, lay fewer eggs and develop smaller populations.

On the other hand, tolerant varieties do not inhibit insect multiplication and since they can support larger infestations while sustaining little plant damage, they may actually be more conducive to insect population build-up than susceptible crop varieties.

In this context, plant breeding exploits genetic variability within the crop species and its wild relatives and aims to enhance resistance to insect pests and diseases by prudent selection and breeding methods (Olsson & Jonasson, 1995). Present-day approaches combine (1) use of pest population growth models to explore resistance management (= antibiosis) strategies, (2) development of efficient test procedures, (3) exploitation of antixenosis as a resistance modality, and (4) evaluation of the potential of molecular biological techniques (De Ponti & Mollema, 1992). A classical example of a successful breeding program is reported by Jena & Khush (1990). In this work, high levels of resistance to two planthopper species have been transferred from *Oryza officinalis* to cultivated rice (*O. sativa*).

Traditional selective breeding can now be short-circuited by biotechnological methods (Schuler *et al.*, 1998). Genetic engineering methods permit the introduction of novel genes into crop species that render many of them resistant to insects (Murdock *et al.*, 2000; Sharma & Ortiz, 2002). For instance, introducing into a food plant genes toxic to insects may result in effective insect population control (Chen & Andreasson, 2001). Thus, genes responsible for the production of a toxin derived from the insect pathogen *Bacillus thuringensis* (Van der Salm *et al.*, 1994) have been introduced into, among others, tomato, rice, oilseed rape, cotton and spruce plants. Obviously, genetic engineering opens fascinating avenues for crop improvement and resistance to insects and has been assumed to provide the ultimate technique in agricultural production, as long as insects do not develop resistance. For instance, the diamondback moth, *Plutella xylostella*, has developed resistance to *B. thuringiensis* (Ramachandran *et al.*, 1998). As Stoner (1992) rightly remarked: "It is much too soon to abandon traditional approaches to plant resistance to insects. Researchers in the field of plant resistance to insects should take advantage of the opportunities presented by new developments in biotechnology, but should also maintain their unique focus on the behavioural, physiological, ecological, and evolutionary interactions of the insect with its host plant".

B. Introduction to the thesis

6. Oilseed rape

6.1 Importance as agricultural crop

Brassica napus is originating from the Mediterranean region (Colton & Sykes, 1992). Oilseed rape refers to several *Brassica* spp. that are cultivated in many parts of the world for the production of oil from their seeds. Oilseed rape is the source of 14% of global edible oil demand and is third in importance after soyabean and palm oil (Murphy, 1996). In Switzerland, it takes up approximately 5% of the arable farmland and satisfies 18% of the national edible oil demand (source: Swiss Department of Agriculture). Apart from their economic importance, crucifers are also interesting plant-model systems, containing the first example of a plant species with a completely sequenced genome (The *Arabidopsis* Genome Initiative, 2000), although 2002 must be remembered also as the year of rice genome sequence (Schoof & Karlowski, 2003).

6.2 Importance of Brassica metabolites in host-plant selection

6.2.1 Primary metabolites

In host-plant selection, primary plant chemicals (nutrients) were thought to have little or no role to play, since it was assumed that most nutrients are so widely distributed (Frankel, 1969). However, quantitative differences in nutrients do exist between plants, and nutrients are now recognised as having a role to play in insect host-plant selection (Slansky, 1993), as was demonstrated, for example, for the aphid *Macrosiphum euphorbiae* (Jansson & Ekbom, 2002), for the diamondback moth *Plutella xylostella* (Marazzi & Städler, 2004a) or the cabbage root fly *Delia radicum* (Marazzi & Städler, 2004b). Sugars, amino acids, proteins, vitamins, sterols and phospholipids have all been reported as feeding stimulants for insects (Chapman, 2003). Sometimes plant damage by crucifer-feeding insects in the field correlates more readily with nutrient levels than with secondary plant metabolites, such as glucosinolate levels, as reported by Louda & Mole (1991) for *Plutella xylostella* and *Phyllotreta spp*. on bittercress, *Cardamine cordifolia*. Further, the responses of insects to nutrients and secondary plant chemicals can be related.

6.2.2 Secondary metabolites

Already Thorsteinson (1960) and more recently Berenbaum (1995) suggested that secondary plant substances are important for the feeding behaviour of specialist insects because they prime insect sensitivity to nutrients.

6.2.2.1 Glucosinolates

Biosynthesis

Biosynthetically, GSLs derive from seven amino acids (alanine, leucine, isoleucine, valine, phenylalanine, tyrosine and tryptophan) and a number of chain-elongated homologues (Mithen, 2001a). Based on the structure of different amino acid precursors, GSLs are classified as aliphatic, benzyl, sulphinyl, sulphonyl and indolyl.

Readers are referred to Wittstock & Halkier (2002) or Mikkelsen *et al.* (2002) for general information on the biosynthetic pathway. Briefly, the widely accepted model for GSL biosynthesis begins with an *N*-hydroxylation of a precursor amino acid, which is followed by a decarboxylation to form an aldoxime (Fig. 1). The procedure involves then three major steps: (1) side chain elongation; (2) glucone biosynthesis and (3) side chain modification. GSL biosynthetic steps following aldoxime formation (Fig. 1) are believed to involve firstly the conversion to a thiohydroximic acid by introduction of the sulphur from cysteine, secondly the transfer of *S*-glycosyl from UDP-glucose, and finally the sulphation by the universal high energy sulphate donor, 3'-adenosine-5'-phosphosulphate [APS].

GLSs are very stable, polar water-soluble precursors of a wide range of apolar, volatile products, including isothiocyanates (Fig. 2). GSLs are typically present in fresh

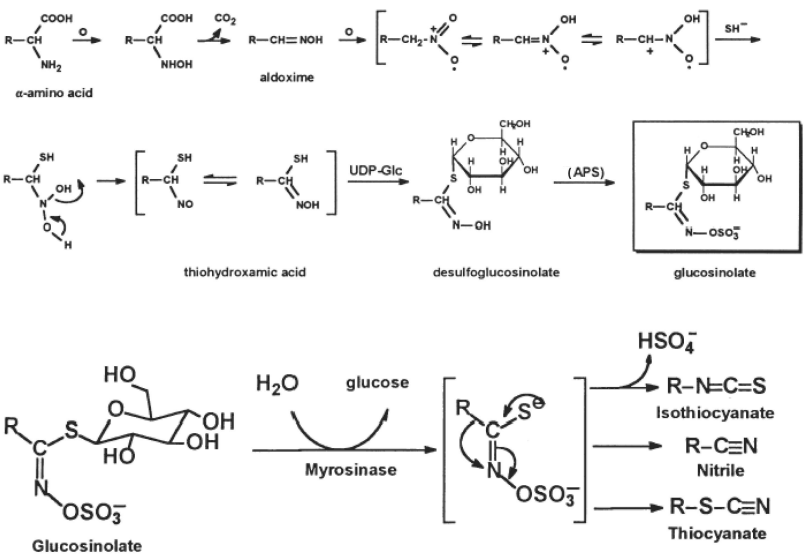

Fig. 1. Biosynthetic pathway of glucosinolates (Fahey et al., 2001)

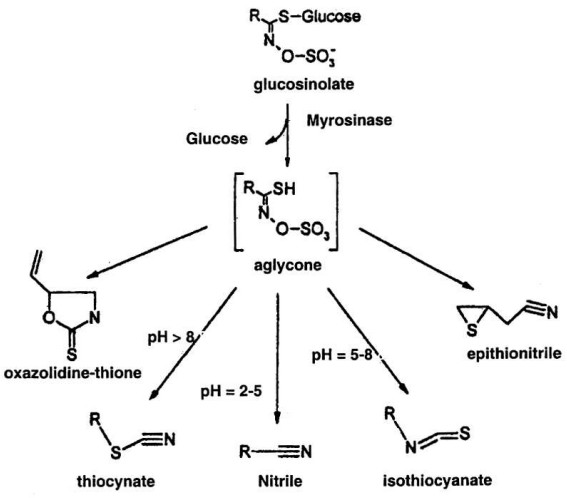

Fig. 2. Names and structures of glucosinolate degradation products (Fahey et al., 2001). Myrosinase catalyses aglycone formation while the final products are formed non-enzymatically or via an epithiospecifier protein.

plants at much higher levels than their cognate isothiocyanates (Matile, 1990). The relatively non-reactive GSLs present in the vacuoles are converted to isothiocyanates upon wounding of the plant, mastication of fresh plants (i. e. vegetables) or by tissue damage caused by bruising or freeze-thawing during cultivation, harvest, shipping or handling (Rosa *et al.*, 1997). This tissue damage releases myrosinase (EC 3.2.3.1), a glycoprotein that coexists with, but is physically segregated from its GSL substrates (Lüthy & Matile, 1994). The GSL-myrosinase system is thought to be a binary chemical defence system that is activated upon tissue damage and may be among the first chemical barriers that deter a broad spectrum of potential pathogens.

GSLs distribution in plants

Various GSLs are found in fifteen families of Brassicaceae, showing an uneven distribution pattern (Mithen, 2001a). For example, benzyl-GSLs have been found in most GSL-producing plants, whereas methyl-GSL, derived from alanine, rarely occurs outside the Capparales family. In *Brassica napus* over thirty different GSLs have been detected (Fahey *et al.*,2001); in *Arabidopsis thaliana*, 23 GSLs have been reported so far (Hogge *et al.*, 1998) and as many as 34 are present if different ecotypes are taken into account (Kliebenstein *et al.*, 2001). Up until now, more than 110 GSLs have been identified as documented on the homepage (http://robig.pmf.ukim.edu.mk/bbogdanov/glucosinolates/webG2.htm).

GSL content in plants is approximately 1% of the dry weight in young tissues of the Brassica vegetables (Rosa *et al.*, 1997), although the content is highly variable (Farnham *et al.*, 2000), and can approach 10% in the seeds of some plants, where GSLs may constitute half of the sulphur content of the seeds. Distribution of the GSLs that have been examined varies among plant organs, with both quantitative and qualitative differences between roots, leaves, stems and seeds. However, plant age is the major determinant of the qualitative and quantitative GSL composition of plants. Environmental factors such soil fertility (Booth & Walker, 1992; Fahey & Stephenson, 1999; Marazzi *et al.*, 2004a, b), pathogen challenge (Butcher *et al.*, 1974), wounding (Bodnaryk, 1992; Griffiths *et al.*, 2001) or plant growth regulators (Bodnaryk, 1994; Bodnaryk & Yoshihara, 1995) also have significant effects on levels of specific GSLs in the growing plants and may affect distribution among plant organs.

Role of GSLs in plant defence

GSLs breakdown products have been shown to be strong inhibitors or toxins and to play a role in plant defence (Fahey *et al.*, 2001). For example, naturally occurring isothiocyanates possess a range of antifungal, antibacterial and antimicrobial activities, and thus inhibit microorganisms, repel insects and molluscs (Fenwick *et al.*, 1983; Glen *et al.*, 1990), but there are very few examples of in vivo studies. Recently, Moyes *et al.* (2000) examined the patterns of herbivory and the GSL profiles of individual wild *Brassica oleracea* plants from different populations and habitats on the Dorset coast. A range of GSL profiles was determined and the data were related to the proportion of damage by different herbivores. The authors found no link between individual plant GSL profiles and herbivory by *Pieris spp*., slugs, snails, flea beetles or aphids. GSLs and their breakdown products are, however, clearly involved in host-plant recognition by specialised pests (Städler, 2000). Moyes *et al.* (2000) explained the discrepancy in their study by selection pressures from herbivores leading plants either to evolve novel chemistry to escape herbivores or maintain stable host-herbivore-parasite association. The authors found that a range of GSL profile exists in natural populations of wild cabbage, but this was only sufficient to stimulate host choice by the specialist herbivore *Selenia leplastriana* (micromoth). However, several attempts to correlate the levels of GSLs with resistance to specific pathogens have failed (Mithen, 2001a). This probably reflects the complex interplay between co-occurrence and possible co-variation of numerous other defence compounds in plants, like for example alkaloids (Macel *et al.*, 2002) or terpenoids (Langenheim, 1994; Fassbinder *et al.*, 2002), and their interacting organisms (Moyes *et al.*, 2000).

Role of GSLs in oviposition

GSLs play an important role in host recognition and host acceptance by adult insects. For example, ovipositing *Pieris* butterflies depend on GSL at the leaf surface to recognise suitable sites for their progeny (Renwick *et al.*, 1992; Van Loon *et al.*, 1992; Du *et al.*, 1995; Städler *et al.*, 1995b). Once an adult female insect has landed on a plant, she will often drum the leaf (Terofal, 1965) or walk over the leaf or stem surface (Justus & Mitchell, 1996; De Jong & Städler, 1999; De Jong *et al.*, 2000). The duration of the walk and the commencement of the next behaviour depend on what stimuli she has received from the compounds on the plant surface (Städler, 2002). This behaviour allows the contact sensilla on the tarsi and antenna to perceive compounds located in the surface waxes or in the boundary layer surrounding the leaf. Contact chemoreceptor neurons in the tarsal sensilla of adult Diptera and Lepidoptera have been shown to respond to a range of extracts and isolated compounds that stimulate oviposition through contact chemoreceptor and olfactory sensilla. In many cases the stimulatory activity of the extracts cannot be attributed to one compound alone but involves synergistic interactions between the compounds (Städler *et al.*, 2002). Attractants identified so far include volatile and non-volatile compounds. For some insects, like the cabbage root fly, non-volatile stimulants seem to be far more important than volatile compounds (Baur *et al.*, 1996), although volatiles were found to have a synergistic influence in the stimulation by non-volatiles (De Jong *et al.*, 1999).

Role of GSLs in feeding

Presently, GSLs are recognised as feeding stimulants for many species of specialist crucifer insects (Schoonhoven *et al.*, 1998). Verschaffelt (1910) was the first to demonstrate the effectiveness of GSLs as feeding stimulants for specialist crucifer-feeding insects, by showing that *Pieris brassicae* and *P. rapae* eat previously rejected plants if these plants are wetted with the juice of crucifers or the GSL sinigrin.

The close association of crucifer specialists with GSLs in their hosts has led in many cases to a sort of dependency on these chemicals. For instance, the *Pieris* butterflies hatching larvae require GSLs to initiate or continue feeding. Renwick & Lopez (1999) reported that these neonate larvae may actually feed in the absence of GSLs, but once they have experienced these compounds in their diet, they will refuse to feed unless GSLs are present.

Insect adaptation to GSLs

Despite the general effectiveness of GSLs as a barrier against herbivory, many insects have adapted to these defences and several species have become major pests of Brassica crops. Since the hydrolysis products are generally toxic for non-adapted insects, the specialists must have developed mechanisms to deal with these potentially toxic compounds. Possible adaptive mechanisms include rapid excretion of the plant products of GSLs, the isothiocyanates, hydrolysis of the glucosides, inhibition of plant GSL hydrolysis, activation of protective enzymes or sequestration of GSLs (Müller *et al.*, 2001; Renwick, 2002).
In many cases, sequestration of GSLs by insects has been suspected, but not clearly demonstrated until recently by Müller *et al.* (2001). The authors showed that the turnip sawfly, *Athalia rosae*, sequesters various GSLs from its cruciferous hosts. Sequestration of GSLs has also been demonstrated for the harlequin bug, *Margantia histrionica*, and evidence has been presented suggesting that this could result in effective protection from avian predators (Aliabadi & Whitman, 2001).

6.2.2.2 Other metabolites

In addition to the GSLs, chemical characterisation of secondary metabolites from crucifers has unravelled a remarkable array of phytoalexins (Pedras *et al.*, 2000), another plant defensive compound induced by wounding or stress. One of the most convincing argument in favour of the defensive power of phytoalexins is the genetic engineering of a phytoalexin-deficient mutant of *Arabidopsis thaliana* that showed significantly higher susceptibility to the fungus *Alternaria brassicicola* than the wild-type parental plants (Thomma *et al.*, 1999). Moreover, one of the most active compounds for the cabbage root fly, *Delia radicum*, is similar in structure to phytoalexins. In this context, Baur *et al.* (1998) tested the oviposition stimulatory effect of various compounds and found that three phytoalexins (methoxybrassicin, cyclobrassinin and brassitin) were active.

On the other hand, recent studies using electrophysiological techniques to detect stimulatory activity of plant extracts revealed the presence of additional compounds that may play an important role in host selection. Roessingh *et al.* (1997) found that contact chemoreceptors on the tarsi of *Delia radicum*, responded strongly to very low concentrations of compounds extracted from the surface of cabbage leaves. The active compound, which is a potent oviposition stimulant, was subsequently identified as CIF ("Cabbage Identification Factor"; 1,2-dehydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorine-1-carboxylic acid) (Hurter *et al.*, 1999; De Jong *et al.*, 2000) . The involvement of non-GSLs in stimulating oviposition in the turnip root fly, *Delia floralis*, has also been suggested, as a result of similar extractions and electrophysiological experiments (Hopkins *et al.*, 1997).

Furthermore, widely differing chemical compounds such as cardenolides and flavonoides can deter an insect from feeding or ovipositing, and even compounds that normally stimulate can have a deterrent effect at high concentrations (Renwick, 2001). These substances may act by stimulating specific deterrent receptors or by inhibiting stimulant receptors (Chapman, 2003). The relative importance of deterrents and stimulants in determining host-plant range is difficult to assess. The stimulatory and inhibitory effects of primary or secondary plant chemicals on the host-plant selection behaviour of herbivorous insects coexist and their balance determines the possible outcome of the decision-making process: rejection or variable degrees of acceptance, manifested as preference in choice situations (Bernays & Chapman, 1994; Renwick, 2002).

6.3 Genetic variation

In cruciferous crops there is rising interest in controlling the level of GSLs to improve pest resistance and nutritional value for farm animal and human consumption (Fahey *et al.*, 2001). Genes identified in *Arabidopsis thaliana* have provided important tools to initiate molecular strategies to modulate the quantity and quality of GSLs in a tissue-specific manner in closely related Brassica crops (Wittstock & Halkier, 2002). Most attention has been paid to reducing the GSL of seeds of oilseed rape (*Brassica napus*). This has been successfully achieved through the introgression of genes from the low seed GSL cultivar Bronowski into initially Canadian spring rape cultivars and then European winter rape cultivars (Raybould & Moyes, 2001). The reduction has only been in methionine-derived GSLs, with no effect on the level of GSLs derived from tryptophan or phenylalanine. *B. napus* cultivars were further modified to obtain double-low cultivars having a low seed content in erucic acid and in GSLs. At the seed and cotyledon level, these double-low cultivars contain 4 to 10 times less GSLs than single-low cultivars.

When low-GSL rape cultivars were introduced, increased attack from generalist phytophagous insects (in this case GSLs are antifeedant) and reduced attack from specialist crucifer-feeders (here GSLs are stimulatory) were expected (Larsen *et al.*, 1983). Increased attack from generalist feeders, such as the field slug, was indeed reported (Glen *et al.*, 1990) , but decreased attack from crucifers-specialists was not. Actual differences in GSL levels between single- and double-low cultivars may not be large enough to cause appreciable differences in feeding, probably also because "low" is mainly limited to seed content (Fahey *et al.*, 2001). Similarly, the GSL levels in the green and flowering parts of the rape plant were shown to differ little in single- as compared to double-low cultivars (Fieldsend & Milford, 1994; Mithen, 2001b).

6.4 Fertilisation

The mineral status of the soil is one of the key factors in determining crop quality (Rogasik *et al.*; 2002). One of the most common strategies aimed to improve crop yield is the employment of fertilisers. Using this approach the concentrations of the most important minerals, such as nitrogen, sulphur, phosphorous, calcium and potassium can be modified. Up to date, it has been very difficult to clearly assess the consequences of changes in single mineral

concentration on plant properties, including defence. Indeed, not only nutrient levels but also ratio of nutrients must be considered (Jansson & Ekbom, 2002). In the context of this thesis, sulphur fertilisation is of major interest.

6.4.1 Sulphur

Sulphur (S) fertilisation could be considered as an alternative approach to modulate GSLs content in the plant, since oilseed rape and Brassica species in general require S during their growth (Zhao *et al.*,1993a; Blake-Kalff *et al.*, 2000) for the synthesis of both protein and naturally occurring GSLs. It appears therefore that a lack in S nutrition leads to a decline in seed yield, while an excessive S supply can affect oil quality by increasing seed content of GSL and their toxic and goitrogenic breakdown compounds with an unpleasant taste (Rosa *et al.*, 1997). Based on these observations, an optimal S supply is required to obtain both maximal yield and good oil quality.

Dosdall *et al.* (2002) found that root maggot responses to different S treatments in the field varied with the year and the site, indicating that environmental factors are of considerable importance in determining both infestation levels by these pests and the oxidation rate of elemental S in the soil. These authors reported for the first time on the value of S in reducing crop losses caused by root maggot infestations, while in earlier studies it was shown that S is essential in maintaining oilseed rape plant vigor and in optimising seed yield (reviewed by Scherer, 2001).

6.4.2 Nitrogen

Due the particular sensitivity of oilseed rape to S deficiency, in the last 10 years significant yield responses to S application jointly with nitrogen (N) have been achieved (Zhao *et al.*, 1993; Whithers & O'Donnell, 1994; MacGrath & Zhao, 1996; Fismes *et al.*, 2000). Recently, Fismes *et al*. (2000) reported that N and S nutrition during plant growth is closely linked. Interactions between these two elements, as reflected by plant uptake, are synergistic at optimum rates and antagonistic at excessive levels of one of them. The authors demonstrated that S fertilisation is required to improve the efficiency of N-use by the plant in order to maintain an adequate oil level and fatty acid quality. On the other hand, increasing N fertiliser rates aggravates S deficiency of oilseed rape and reduces yield when available S is limited (Zhao *et al.*, 1993b). Conversely, N addition increases seed yield in conditions of S-sufficiency, and an optimum oil quality and maximum yield are obtained when the amounts of available N and S are balanced (Joshi *et al.*, 1998). Under these conditions, the content of desirable fatty acids, such as linoleic and linolenic acids, is increased (Fismes *et al.*, 2000). In contrast, no use of S fertilisers will lead to higher contents of undesirable fatty acids, such as palmitic (hypercholesterimic) and erucic (no food value) acids (Joshi *et al.*, 1998).

7. Pests of Brassica crops

Up to date several pests of Brassica crops have been found to have an impact. These include, the cabbage aphid *Brevicoryne brassicae* (Hughes, 1963), the cabbage root fly *Delia radicum* (Finch & Ackley, 1977), the diamondback moth *Plutella xylostella* (Harcourt, 1957), the large cabbage white butterfly *Pieris brassicae* (Davies & Gibert, 1985), the small cabbage white butterfly *Pieris rapae* (reviewed in Hern *et al.*, 1996), the cabbage moth *Mamestra brassicae* (Rojas, 1999), the garden pebble moth, *Evergestis forficalis* (Jones & Finch, 1987), the flea beetles, *Phyllotreta spp*. (Feeny *et al.*, 1970), the cabbage stem weevil, *Ceutorhynchus quadridens* (Graham & Gould, 1980), the thrips, *Thrips tabaci* (Ellis *et al.*, 1994) and the turnip sawfly *Athalia rosae* (Lee *et al.*, 1998). In this study we choose to work with *P. xylostella* and *D. radicum*, because of their large impact on this crop.

7.1 *Plutella xylostella*

This omnipresent crucifer specialist, the diamondback moth, *Plutella xylostella* (L.) is the potential cause of major losses in the Brassica crop in the United States (Buntin and Raymer, 1994) and these can occur from seedling stage to crop maturity. Larvae of this pest feed on leaves of crucifer plants during the vegetative stage of the crop, on growing tips during bolting stage, and on flowers and pods during flowering and pod developmental stages. The immature stage has poor mobility and first instars are obligatory miners, a behaviour that prevents desiccation (Justus & Mitchell, 1996).

P. xylostella females usually lay eggs singly or in groups of 2 to 8 on the upper- or under-sides of the leaves, frequently in the hollows along the vein, on the young stems or on petioles (Harcourt, 1957). After hatching, the caterpillar goes through four instars before pupation. *P. xylostella* caterpillars are sluggish and will generally remain on an acceptable host plant as long as sufficient food is available. However, the caterpillars show a behavioural change as pupation approaches. The normally placid caterpillar becomes very active and begins to wander. It is therefore relatively easy to determine when the caterpillar has completed feeding and is ready to pupate.

7.2 *Delia radicum*

The cabbage root fly, *Delia radicum*, is a pest of Brassicas of European origin (Biron *et al.*, 2000) and is now more or less evenly distributed in association with agriculture and horticulture in the temperate zone of the Holarctic region (e. g. Europe, North Africa, Canada and USA). Adults, which are dark grey flies, lay eggs on the soil surface in cracks close to the stem but some eggs may be laid on the actual plant (Finch, 1989). Each female fly lays about 100 eggs. The hatching of the eggs takes 3 to 7 days, depending on the temperature (Swailes, 1963). The larvae burrow through the soil to the plant and feed close to the tap root and tunnel into the root. Larval development takes about three weeks, then the larvae (about 8 mm long) move into the soil and pupate a few centimetres away. Pupation takes 15-35 days. The adults emerge from the ground between the end of March to the beginning of May at dates that vary from year to year and from one region to another according to the temperature (Johnsen *et al.*, 1997). The period of larval presence and therefore of evident plant damage varies according to the region from the end of April to September. The speed of development of maggots and their activity rise rapidly with temperature. On the other hand, high temperatures lead to high mortality of eggs and young larvae (Turnock & Boivin, 1997). In general, the emergence of winter rapeseed in the fall corresponds to the last insect generation (Turnock & Boivin, 1997). Seedlings not protected by insecticide are often damaged severely by *Delia* pests. In some cases, *D. radicum* infestations can destroy up to 90% of untreated Brassica crop in North America and mainland Europe (Griffiths, 1991b; Finch *et al.*, 1996). Primary plant damage is caused by larvae of the cabbage

maggot feeding on the tissue of root phloem, periderm and xylem parenchyma (McDonald & Sears, 1992). Root damage is exacerbated when feeding channels are invaded secondarily by *Fusarium* root rot fungi (Griffiths, 1986). Vigorously growing crops can support large populations of larvae without showing sign of attack. However, when the crops sustain larval damage on the part of plant used for human consumption (e.g. brussels sprouts and rutabaga), it is clear that the quality of the edible product is reduced severely (Finch *et al.*, 1996).

8. The aims

In order to understand host-plant selection, at least two main questions have to be asked: (1) Which characteristics of the plant are perceived by the pest insect? Morphology, epicuticular components, phytochemicals including secondary compounds and plant nutritional quality can influence acceptance; (2) Which behavioural and physiological characteristics does the insect possess? To exploit its host, an insect must possess abilities that enable it to find, recognise and colonise potential hosts. These include: behaviour that position the insect in a location that facilitates perception by sensory cells, sensory neurons that are sensitive to specific traits of the host (e.g. phytochemicals), and a central nervous system that integrates internal and external cues to produce coordinated motor outputs.

These two questions represent the starting point of this thesis. In spite of the large number of publications dealing with the two crucifer pests *Plutella xylostella* and *Delia radicum*, no quantitative information is available concerning some rather fundamental aspects of the insect relationship with the host plant, in particular with respect to sulphur fertilisation. It was my intention to study the effect of sulphur nutrition of oilseed rape on the preference and performance of these two major pest insects. To this end, sub- and super-normal sulphur supplies were compared with normal recommended supply. After establishing that the insects can discriminate between differently fertilised plants, the aim was to identify the plant characteristics influencing the preference behaviour. Given our current understanding of agricultural systems, it is evident that dependency on the application of pesticides is not a sustainable solution on a long term because resistance against insecticides is inevitable (Talekar & Shelton, 1993; Ramachandran *et al.*, 1998). Therefore, other possible control strategies have to be studied. Since the larval stage of both pests have poor mobility and first instars (by the cabbage root fly during the whole larval development) are obligatory miners, they are ideal candidates for prophylactic control: prevention of egg deposition by adult females or prevention of larval entry into plant tissue.

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Part 2: Sulphur plant nutrition influencing host-acceptance

9. Influence of sulphur plant nutrition on oviposition and larval performance of the diamondback moth

Influence of plant sulphur nutrition on oviposition and larval performance of the diamondback moth

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Abstract

We measured adult oviposition preference, larval growth, and feeding behaviour of the crucifer specialist *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) on plants of *Brassica napus* (L.) cv. Express (Brassicaceae), grown under three different sulphur regimes. The nutrient solutions used were the following: one sulphur-free (S_0), one normal sulphur (S_n, normal field concentration), and one sulphur-rich (S_+, double concentration of S_n). Females laid more eggs on S_n than on S_0 plants, while only a slight, non-significant difference was observed between S_n and S_+ plants. Moreover, the development time from hatching to emergence was significantly shorter, and adults were heavier on S_n than on S_0 plants. Comparing these same two parameters from S_n and S_+ plants, we found a shorter development time on plants rich in sulphur, although this trend was not statistically significant. Larval feeding preferences were tested in a dual choice assay using leaf discs. A significantly higher number of larvae preferred leaf discs of S_n plants than those of S_0 plants. Furthermore, the larvae preferred S_+ to S_n discs. An optimal supply of sulphur to oilseed rape is necessary for a good seed harvest, and it also plays an important role in acceptance by *P. xylostella* of the host plant. Maintaining higher levels of sulphur in the plant nutrient solution benefits insect performance, both at the adult and larval stage.

Introduction

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is one of the main pests of cruciferous plants throughout the world, and it has developed resistance to most of the insecticides used to control it, including *Bacillus thuringiensis* (Talekar & Shelton, 1993; Ramachandran et al., 1998). Clearly, multiple-component integrated pest management including monitoring will be the best strategy in dealing with this insect in the future. In this context, plant nutrition should also be taken into account, since it might affect the chemical stimuli mediating host plant selection and consequent population dynamics.

Water and mineral supply in the soil are among the crucial environmental factors affecting plant growth. Numerous observations relate insect growth and abundance to the soil nutrients on which their host plants grow (reviewed by Waring & Cobb, 1992).

The application of fertiliser primarily influences oilseed rape plant morphology and phenology (Justus et al., 2000), but it also alters nutrient composition (carbohydrates, fatty acids, and proteins). In addition, sulphur nutrition influences the levels of secondary plant substances (Gershenzon, 2002). Plant nutrition influences not only the total content of secondary metabolites, but also leaf surface chemistry and appearance (Eigenbrode & Pillai, 1998). The chemicals present on the surface of the plant may affect selection behaviour prior to any injury that would release cell contents. This is important for ovipositing insects that have no access to the leaf interior and that may respond differently to the differing nutritional status of the plants. An example of this is the cabbage white butterfly, *Pieris rapae*. Myers (1985) showed that ovipositing females are able to discriminate between fertilised (with ammonium nitrate, ammonium sulphate, urea, or a complete fertiliser) and unfertilised *Brassica* plants within 24 h of fertiliser application. When plants respond rapidly to the treatment, herbivorous insects may perceive these subtle differences.

Soil nitrogen is the most crucial factor for crop productivity, and nitrogen plant nutrition was shown to cause an increase in the number of eggs laid by *P. rapae* on cabbage and mustard (Wolfson, 1980; Myers, 1985). Scriber (1984) reported that in about 100 studies crop damage by pest

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insects increased with the nitrogen content of their host plants. In this context, Lower & Orians (2003) recently reported that nutrient addition led to an increase in female pupal weight, and that foliar nitrogen was positively correlated with female pupal weight and negatively correlated with female development time. In contrast, the relevance of sulphur levels has been far less studied, although indications of a relationship between plant nutrition with insect oviposition has already been reported by Gupta & Thorsteinson (1960). These authors found twice as many eggs of *P. xylostella* deposited on *Brassica nigra* plants grown in a full nutrient solution than on plants deprived of sulphur. In Europe, in recent years, two trends have contributed to changes in the sulphur supply to crops (Schnug & Haneklaus, 1998). One is the reduction in air pollution, diminishing the flow of sulphur from the atmosphere to soils, where the roots can take it up. The other is a change towards the use of more concentrated fertiliser products containing less sulphur (Scherer, 2001). Oilseed rape is very sensitive to sulphur deficiency, because of its high sulphur requirement compared to other crops. Thus, a decreased sulphur supply in agricultural soils could have important consequences for crop yield. As pointed out by Klessa & Sinclair (1989), the harvested seeds of rape remove 20–30 kg S ha^{-1} $year^{-1}$ from the soil, whereas a cereal crop removes only 5–15 kg S ha^{-1} $year^{-1}$. Furthermore, sulphur is one of the 16 elements essential for plant growth (Kim et al., 2002). According to these authors, its functions for the plant are closely linked to those of nitrogen, and the two nutrients are synergistic. Sulphur is required for plant growth in quantities equal to and sometimes exceeding those of phosphorus (Scherer, 2001). This requirement can be explained by the fact that sulphur has a variety of vital functions within the biochemistry of the plant, being a major constituent of amino acids such as cysteine and methionine. Sulphur is also essential for the synthesis of enzymes, vitamins, such as biotin and thiamine, and a variety of other important compounds in the plant, including chlorophyll (Schnug, 1997). A lack of sulphur results in reduced plant quality and growth due to lower contents and quality of proteins as well as to the accumulation of nitrate (Singh, 1994).

Sulphur-deficient plants are characteristically small and frail, with younger leaves that are light green to yellow. If the sulphur-deficiency is critical, the leaf colour may become reddish. In rape, the oil content of the seed is diminished and the maturity of the fruit is delayed in the absence of an adequate sulphur supply (Ceccotti & Messik, 1994). Therefore, the role of sulphur in plant production has increasingly been scrutinised with the aim of improving the nutritive quality of the harvest. Furthermore, sulphur-containing metabolites play a major role in plant defence mechanisms against biotic and abiotic stress, and in particular they regulate the glutathione pathway (Foyer & Rennenberg, 2000).

In brassicaceous crops, sulphur is also used in the synthesis of glucosinolates, a group of thioglucoside compounds reported to be involved in the defence mechanisms of the plant against fungi and insects (Chew, 1988). Since these compounds also act as attractants and feeding stimulants for many insects, including *P. xylostella* (Renwick, 2002), sulphur supply is an important environmental factor.

Specialist herbivores choose their host plant either as adults or as larvae. It is therefore important to consider the behaviour of both. In the majority of insects, the ovipositing female (Städler, 1992) selects the host plant, and the location of the egg determines larval survival, especially for *P. xylostella* since the first larval stages have a limited mobility (Justus & Mitchell, 1996).

Therefore, we first studied the influence of plant sulphur nutrition on oviposition preference. In addition, we examined the effects of sulphur plant nutrition on larval growth, development, and performance, all key factors in population dynamics.

Materials and methods

Insects

During the spring of 2000, a laboratory colony of *P. xylostella* (L.) was established with approximately 200 individuals. Pupae were collected in fields of broccoli and cauliflower plants (*Brassica oleracea* cultivars) in Eastern Switzerland. Even though size of the colony fluctuated, it never declined below the initial population size.

Adult moths (approximately 100 per cage) were kept in mesh cages ($50 \times 45 \times 45$ cm) containing *Brassica napus* cv. CC-Cross F1 at the pre-bolt stage, in pots of 13 cm in diameter. The moths mated and oviposited only on the leaves. Adult food was supplied by means of a filter paper soaked in 10% sugar solution, and placed in a plastic container.

After 24 h of exposure to ovipositing females, the plants were removed from the adults' cages and placed in other cages, where the larvae were allowed to emerge and develop. Defoliated plants were replaced by fresh ones. Pupae were removed from the plants and placed in separate plastic vials until emergence, and then adult moths were allowed to join the rearing population to mate and oviposit. The colonies were maintained at a constant temperature of 22 ± 0.5 °C, 60 ± 5% r.h., and L16:D8 photoperiod.

Plants

Seeds of oilseed rape (*B. napus* cv. Express) were sown individually in plastic pots (9 cm in diameter) containing

Table 1 Nutrient additions for each treatment (mM)

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

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 exchange. The pots were arranged in trays of 55 × 35 cm (20 pots per tray) and placed in a glasshouse, where daylight was supplemented with light from high-pressure sodium lamps (Philips, SON-T Plus, 400 W) (22 ± 3 °C, L16:D8). The soil was fertilised at different levels. The plants were watered twice a week with a nutrient solution (about 1 l per tray, according to seasonal conditions) based on a modified 'Hoagland' nutrient solution with the following sulphur concentrations: S_n = 1 mM of $MgSO_4$, the concentration for normal growth and harvest in a Swiss field (D. Ryser, pers. comm.); S_+ = 2 mM of $MgSO_4$, defined as sulphur-rich level. A sulphur-free level (S_0) 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 (Lemna Media, see Table 1) (Beaumont et al., 1976).

All the experiments were conducted using plants with three to four true leaves (30–35 days after planting), comparable in size, unless otherwise specified. The plants were sprayed once at the cotyledon stage with the fungicide kresoxim-methyl (Stroby® WG, Leu and Gygax AG; 0.05%) to prevent mildew attacks.

Oviposition choice assay

Four *B. napus* plants were placed randomly in two Plexiglas® cages (45 × 45 × 50 cm), i.e., two plants per cage for each treatment: S_n (control) vs. S_0, or S_n (control) vs. S_+. About 100 1–2-day-old diamondback moths were released into each cage and allowed to mate and oviposit on the plants for 24 h. The plants were then removed, placed in a climatic chamber (22 ± 0.5 °C, 60 ± 5% r.h., and L16:D8 photoperiod) and stored individually in meshed cages. We counted the total number of eggs laid on each plant after the 24-h oviposition period (n = 20, total tested plants = 160). Between replicates, the plants' arrangement was rotated clockwise by one position.

Egg counts per plant were expressed as a percentage of the total number of eggs laid on all plants within one bioassay period. Thus, the resulting preference values for the treatments compared (S_n vs. S_0 and S_n vs. S_+, respectively) totalled 100%. A Mann–Whitney U-test was performed to determine whether statistically significant differences in preference between treatments had occurred.

Larval performance assay

Three newly hatched larvae of *P. xylostella* were placed on *B. napus* plants, each with 6–7 true leaves of plants grown under one of the three fertilizer regimes (S_0, S_n, and S_+). The plants were placed individually in meshed cages (60 × 60 × 60 cm) and left in a climate-controlled room (22 ± 0.5 °C, 60 ± 5% r.h., and L16:D8) for approximately 2 weeks. The caterpillars fed on the plants until pupation. Defoliated plants were substituted with new ones, grown under the same conditions. Dead caterpillars were not replaced. Pupae were collected individually in separate plastic vials and stored until emergence.

Development time from hatching to adult emergence, as well as adult weight, were noted separately for males and females. The experiment was repeated 20 times with different cohorts of insects and plants. Each replicate comprised three larvae fed on each of the three plant samples. Differences with regard to developmental time and adult fresh weight were tested using a non-parametric Mann–Whitney U-test.

Larval feeding choice assay on leaf discs

We used *B. napus* leaf discs of 1 cm in diameter that were punched from the leaves of plants grown under the three different sulphur regimes, following the method described by Jermy et al. (1968). The discs were placed in a plastic arena (11 cm in diameter and 8 cm high) with a paraffin layer (1.5 cm thick) on the bottom. Caterpillars crawling on the floor of the arena could easily reach a disc and begin feeding on its rim. A moist sheet of filter paper was placed on the wax layer to prevent desiccation of the plant material (r.h. of the room: 70 ± 5%). Six leaf discs from each plant tested were arranged in alternating order (i.e., 12 for each arena) and direct comparisons were made in dual choice tests (S_0 vs. S_n or S_n vs. S_+). This kind of arrangement forced a larva leaving a disc to encounter the other plant before coming again to the first plant. At the beginning of the test, one last-instar larva of *P. xylostella* (L4) was placed in the middle of each arena using a fine camelhair brush.

An assay consisted of three recordings (one every 6 h) of the behavioural choice of the larva, and visual estimates of the amount of surface eaten (for example, the percentage of one disc consumed = 1/6 of the total amount of that particular treatment = ca. 17%). Each replicate consisted of five arenas tested simultaneously. The bioassay was replicated 10 times for S_n vs. S_0 (total of tested larvae = 50) and

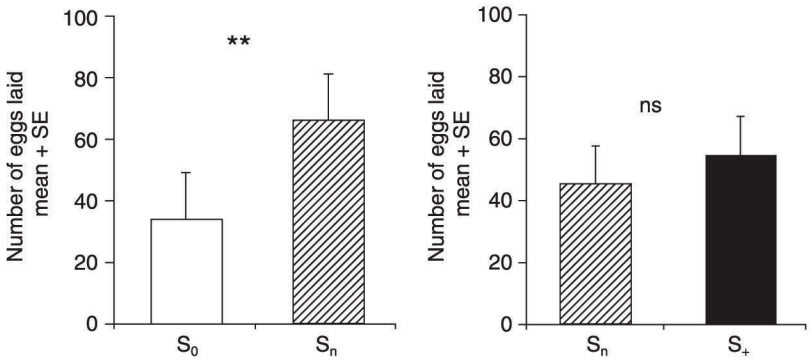

Figure 1 Effect of plant sulphur nutrition on *P. xylostella* oviposition choice: S_0 (sulphur-free), S_n (normal field-concentration of sulphur), and S_+ (sulphur-rich) *B. napus* plants were exposed to *P. xylostella* adults for a 24-h oviposition period (n = 9 for S_0/S_n and n = 13 for S_n/S_+). Symbols and abbreviations: *P<0.05, **P<0.01, ***P<0.001, ns = not significant.

20 times for S_n vs. S_+ (total of tested larvae = 100, total number of larvae making an unequivocal choice = 80).

The number of larvae choosing one of the two compared treatments was counted and compared using a contingency table and analysed with a Pearson χ^2, while the proportions of the surface consumed per treatment were compared using a non-parametric Wilcoxon signed ranks test (data not normally distributed).

Results

Effect of S-fertilisation on *Plutella xylostella* oviposition choice

The oviposition choice of *P. xylostella* was affected by the sulphur concentration of *B. napus* plants (Figure 1). The number of eggs laid was higher (n = 9, P = 0.0013) on plants with a normal sulphur nutrition (S_n), than on plants without added sulphur (S_0). Nearly twice as many eggs were found on plants grown under a normal sulphur regime. On the other hand, no significant difference was found between the number of eggs laid on S_n plants and on plants grown under a sulphur-rich regime (S_+) (n = 13, P = 0.0956). It is important to note that at the 3–4-leaf stage, there was no visible difference between the tested plants.

Larval survival and development of *Plutella xylostella*

The absence of sulphur had a negative effect on larval growth rate and performance, as the development time was longer on S_0 plants (n = 20, P<0.001) than on either S_n or S_+ plants (Figure 2). As shown in the same figure, the larval development time on S_n plants varied, and this was possibly due to the fact that the experiments were not performed simultaneously. The larvae pupated about 3 days earlier on plants fertilised with a normal amount of sulphur (average on S_0 plants: 17.17 days, SE: 2.95; average on S_n plants: 14.38 days, SE: 0.966).

The absence of sulphur also negatively affected adult insect weight (n = 10, P<0.001). As shown in Figure 3, insects fed on S_0 plants were 26% lighter than those fed on plants with normal sulphur levels. Furthermore, the lack of sulphur resulted in a reduced pupation and adult emergence rate. On S_0 plants, only 66.6% (S_n plants: 98.3%) of the larvae reached the pupal stage and 85% of the resulting pupae (S_n plants: 96.6%) emerged as adults. Comparing larval performance on S_n plants to that on S_+ plants confirmed the positive effect of sulphur, even though this difference was less pronounced (developmental time: n = 20, P = 0.0053; adult fresh weight: n = 20, P = 0.0450) than that between normal and sulphur-free plants (Figures 2

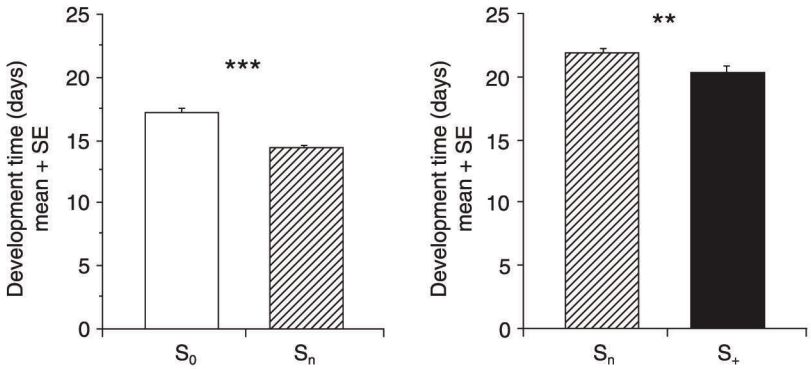

Figure 2 Effect of plant sulphur nutrition on the development time of larvae of *P. xylostella* from hatching to adult emergence: comparison of the duration of the larval stage of *P. xylostella* fed on S_0, S_n, and S_+ plants of *B. napus* (n = 20). Symbols and abbreviations as in Figure 1.

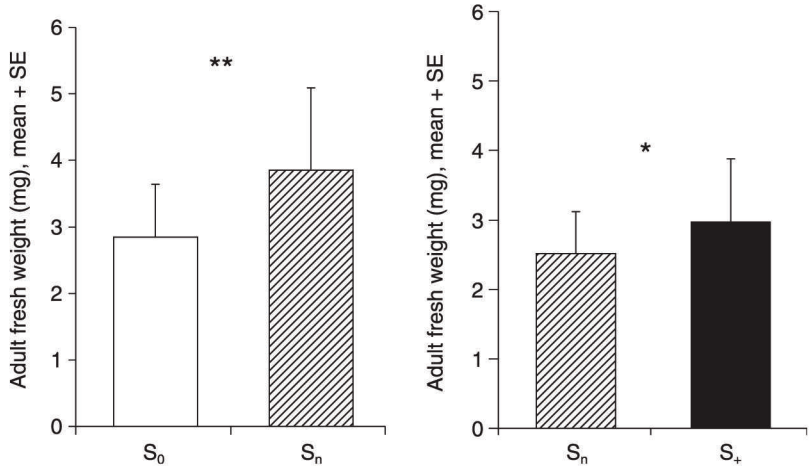

Figure 3 Effect of plant sulphur nutrition on the weight of adult of *P. xylostella*: the adults of *P. xylostella* emerging from pupae of larvae fed on S_0, S_n, and S_+ plants of *B. napus* were sexed and weighed (n = 20). Symbols and abbreviations as in Figure 1.

and 3). We found that the adults emerged approximately 1 day earlier and were 18% heavier on S_+ plants. However, the highest larval survival rate was 83.3% on S_n plants (S_+ = 76.6%), while the emergence rate was 100% on both S_n and S_+. There was also considerable variation in adult weight among the S_n-groups, again probably due to the fact that the experiments were not performed simultaneously.

Larval choice of *Plutella xylostella*

The feeding behaviour of the larvae was also affected by the lack of sulphur nutrition. The larvae preferred the S_n leaf discs to the S_0 discs, and this was observed in each of the three periods monitored (n = 10, Pearson χ^2, P-value<0.001). On average, eight larvae out of 10 chose an S_n disc. A trend was apparent in the preference of larvae for S_+ instead of S_n discs (n = 20, Pearson χ^2, after 30 min, P = 0.039, after 6 h, P = 0.033, and after 12 h, P = 0.18). However, these differences were less pronounced than those observed between S_0 and S_n discs (Figure 4), with only six larvae out of 10 preferring the S_+ disc.

When comparing the amount of disc surface consumed, the results were very similar to those of the larval choice experiment (Figure 5). A higher percentage of S_+ leaf discs was eaten compared to that of S_n or S_0, although the difference between S_n and S_+ was not significant, with the exception of the data recorded after 6 h (n = 20, Wilcoxon signed ranks test, after 30 min, P = 0.2745, after 6 h, P = 0.0442, and after 12 h, P = 0.5936). At the end of the experiment, in the final choice estimation, the larvae fed on average 4% more on S_+ than S_n leaf discs. In the choice with S_0, they consumed significantly more of the S_n discs (n = 10, Wilcoxon signed ranks test, P = 0.001), and this was true for all three periods tested (Figure 5). It is noteworthy that in the final choice period, the amount of S_n disc surface ingested was 132% greater than that of S_0 the discs.

Discussion

Our study shows that the amount of sulphur supplied to oilseed rape plants affects the oviposition and development of *P. xylostella*. The lack of this plant nutrient

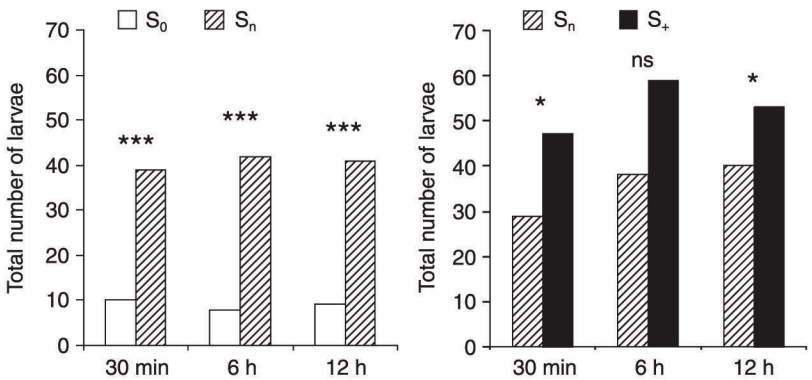

Figure 4 Effect of plant sulphur nutrition on the feeding choice of the larvae of *P. xylostella*: behavioural choice of an L4 larva (just before pupation); n = 10 for S_0/S_n and n = 20 for S_n/S_+. Time points of checking: first choice = after 30 min, intermediate choice = after 6 h, and final choice = after 12 h from the beginning of the assay. Symbols and abbreviations as in Figure 1.

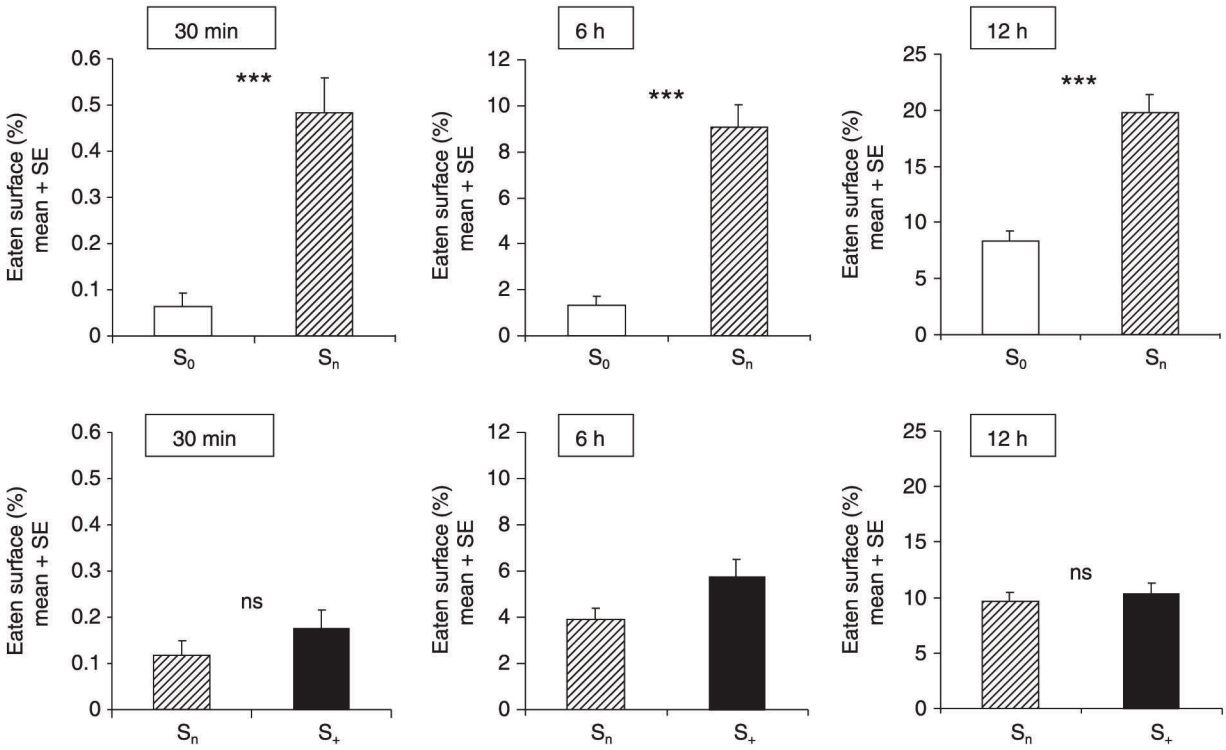

Figure 5 Effect of plant sulphur nutrition on the leaf disc consumption of the larvae of *P. xylostella*: visual estimation of leaf disc consumption of L4 *P. xylostella* larvae (n = 10 for S_0/S_n and n = 20 for S_n/S_+). Symbols and abbreviations as in Figure 1.

reduced both oviposition and larval performance, indicating that variations in sulphur availability alter the suitability of *B. napus* plants for this herbivore. This is consistent with the conclusion of Beck (1974), who asserted that the larval development of insects depends on the quality of a food resource, which must be behaviourally acceptable, non-toxic, and nutritionally adequate. Furthermore, for the first time the behavioural choices of both larvae and adults can be positively correlated to the sulphur concentration of a plant's nutrient solution.

With the exception of the study by Gupta & Thorsteinson (1960), the previous literature contains no investigations connecting the behavioural choices of *P. xylostella* with the nutrition of the host plants. However, in this context, other crucifer specialists such as *P. rapae* or *Brevicoryne brassicae* have been considered (Wolfson, 1980; Yusuf & Collins, 1998). Wolfson (1980) could show an oviposition preference of *P. rapae* for sulphur- and nitrogen-treated plants only if plant water content was considered as a covariate. Yusuf & Collins (1998) found a positive relationship between the increased sulphur content of the plants and the feeding preference of *B. brassicae*. These authors linked their findings with specific glucosinolates or derivatives, which are known to be powerful gustatory and oviposition stimulants to these pests (Cole, 1997).

Glucosinolates certainly play an important role in host recognition and larval fitness, but some aspects of the relationship between preference and performance may be a consequence of other chemical or physical factors such as leaf wax characteristics, which are believed to influence oviposition (Spencer et al., 1999). Glossy leaves have a reduced wax cover (Eigenbrode et al., 1992; Andrahennandi & Gillot, 1998), which improves the adhesiveness of the eggs (Uematsu & Sakanoshita, 1989), but reduces larval survival (Eigenbrode et al., 1992), and increases predation on *P. xylostella* (Eigenbrode & Espelie, 1995). In our study we used plants with a normal leaf wax layer, but it is possible that both plant secondary metabolites on the leaf surface and physical factors (structure of the wax layer) were affected by the mineral status of the plant, including the levels of sulphur.

There are several properties of leaf surface waxes which could at least partially be affected by the sulphur nutrition of the plant, and that could influence *P. xylostella* oviposition, especially with respect to the preference for plants grown at normal soil sulphur levels over plants grown without soil sulphur supply (the only sulphur in these plants must be originating from the seeds). These characteristics include the reflectance spectra of light, the availability of associated polar compounds that are stimulatory

to sensory receptors (e.g., glucosinolates), and the stimulation of sensory receptors by some constituents of waxes (i.e., apolar compounds) (Hughes et al., 1997). In the case of *P. xylostella*, however, spectral reflectance is unlikely to be a contributing factor in oviposition site selection by the adult females, since oviposition begins at dusk and continues throughout the scotophase in twilight or in the absence of light (Harcourt, 1957; Pivnick et al., 1990). Riggin-Bucci et al. (1998) also determined that visual stimuli are not likely to be involved in differentiating waxy from glossy plants.

Although the results of previous work on crucifer specialists indicates that host recognition for oviposition by these insects is dependent on glucosinolates (Renwick, 2002), these are not the only polar compounds present on leaf surfaces. Other compounds such as sugars and amino acids occur on the leaf surfaces (Juniper & Jeffree, 1983), and are known to stimulate the gustatory receptors of insects. Oviposition-stimulating polar compounds, such as sugars (Derridj et al., 1996), may be used by adult insects to distinguish suitable host plants, and might add to the likely basis for discrimination between sulphur-containing and sulphur-free substrates because their concentration is directly dependent on sulphur-supply in plant nutrition.

In our experiments, the larval growth and developmental parameters tested pointed to the existence in the low-sulphur *B. napus* plants of some antixenosis (= non-preference) (Kogan, 1986) or antibiosis resistance that is related to the adult preference for sulphur-containing plants in the oviposition choice assay (antixenosis). Thus, reduced feeding and larval performance on S_0 plants is correlated with oviposition choice, supporting the optimality theory, which suggests that ovipositing females tend to choose plant species that maximise larval fitness (Thompson & Pellmyr, 1991; Coleman et al., 1996). Conversely, the relative unresponsiveness of *P. xylostella* to further increases in the plant sulphur supply above the S_n level was unexpected, and indicated that saturation is reached in the dose-dependent relationship between sulphur-concentration and plant suitability around the normal field level of sulphur (S_n).

Previous reports on the leaf age/position preferences of *P. xylostella* are ambiguous, with some studies indicating a preference for the top surface of the leaf (Harcourt, 1957), and others indicating a preference for the underside of the leaf (Andrahennandi & Gillot, 1998). We regularly found the eggs on the underside of the leaf, for all the three sulphur treatment groups of *B. napus* tested, supporting the findings of Andrahennandi & Gillot (1998). A likely explanation for this is that the underside naturally provides a more sheltered environment than the upper surface. However, it is possible that where variations in wax characteristics between the two leaf surfaces have been affected by sulphur nutrition, oviposition preferences might be altered.

Comparisons between S_0 and S_n showed greater behavioural differences than those between S_n and S_+. Further studies will be required to determine the plant characteristics that are affected by sulphur nutrition and their role in attracting or repelling insects.

Our laboratory trials indicate a strong correlation between preference and performance on *B. napus* supplied with different levels of soil sulphur. The ability of *P. xylostella* to detect plant nutritional status via subtle differences in the phylloplane arouses interest in characterising the type of plant stimuli detected by the insects. This topic will be addressed in an investigation currently in progress.

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Part 2: Sulphur plant nutrition influencing host-acceptance

10. Influence of sulphur plant nutrition on oviposition and larval performance of the cabbage root fly

Influence of sulphur plant nutrition on oviposition and larval performance of the cabbage root fly

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Key words: Delia radicum, Diptera, Anthomyiidae, *Brassica napus*, Cruciferae, fertilisation, oviposition preference, larval growth rate, pupation rate

Abstract

Adult females of cabbage root flies, *Delia radicum* (Diptera: Anthomyiidae) were given a choice for 24 hours between plants of *Brassica napus* cv Express, grown under three different levels of sulphur supply: S_0 (sulphur-free) vs S_n (control = normal field concentration) and S_n vs S_+ (double the normal concentration). Flies laid over three times as many eggs on S_n than on S_0 plants that did not differ in appearance. In contrast, no significant difference was observed between S_n and S_+ plants.

The larval performance was evaluated using three additional intermediate sulphur levels between S_0 and S_n, for a total of six samples of plants. The percentage pupation at the end of larval feeding ranged from 6% (S_0) to 32% (S_n), and the mean adult fresh weight of the emerging flies was between 3.2 mg (S_0) and 8.17 mg. (S_n). The percentage of pupation and the adult fresh weight were positively correlated with the sulphur content of the plant nutrient solution.

Introduction

As pointed out by Marazzi & Städler (2004), sulphur (S) represents a key factor for plant growth in general and for oilseed rape in particular. It is therefore important to study the different effects of S fertilisation in order to avoid environmental problems and to provide the basis for sustainable agricultural practice.

The cabbage root fly, *Delia radicum*, is a pest in cultivated *Brassicas* of European origin (Griffiths, 1991) and is now widespread distributed in association with agricultural and horticultural crops in areas with temperate and moist climate (e. g. Europe, North Africa, Canada and USA). The larvae are legless maggots, which damage or destroy the root system of many *Brassicas*, including oilseed rape (Dosdall *et al.*, 2000). Feeding damage can provide routes for the infection by fungal pathogens such as *Fusarium* rot fungi and bacterial soft rot, *Erwinia carotovora* (Griffiths, 1986). The damage to the roots results in significant decreases in yield and quality. Given that *Delia radicum* is the main pest of *Brassica napus* and that sulphur fertilisation is an important factor in crop production, we studied pest behaviour and physiological responses caused by varying the amount of S in the nutrient supply. Our investigation was designed to provide the necessary data for the recommendation of an optimal S fertilisation.

Materials and Methods

Insects. All the *Delia radicum* (L) (Diptera: Anthomyiidae) required for the bioassays were taken from our laboratory colonies originating from a local population in Eastern Switzerland during the summer of 1996 and continuously reared according to the method of Finch and Coaker (1969). The adult flies (approximately 100 per cage) were held in cubic screen cages (65 x 65 x 65 cm) in a climate-controlled room (21 ± 1°C, 80% RH and 16 h photophase). 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. The flies were fed on a mixture of raw cane sugar, yeast hydrolysate and water (4:1:1) applied on absorbent tissue strips. Water and 10% sugar solution soaked into cotton-wool were offered separately.

Plants. The oilseed rape (*B. napus* cv. Express) was grown as described by Marazzi & Städler (2004). Briefly, a modified "Hoagland" nutrient solution was used to provide the three different S levels of fertilisation: S_n = 1 mM of $MgSO_4$ (normal S concentration in Swiss fields, 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).

For the larval performance assays we used three additional nutrient solutions that we define as $S_{0.1}$ containing 0.1 mM of $MgSO_4$ and 0.9 mM of $MgCl_2$, as $S_{0.2}$ containing 0.2 mM of $MgSO_4$ and 0.8 mM of $MgCl_2$ and finally as $S_{0.4}$ containing 0.4 mM of $MgSO_4$ and 0.6 mM of $MgCl_2$.

Oviposition choice assay. The bioassay was performed under the same environmental conditions as described for the rearing. Four B. napus plants were placed randomly in a screen cage (65 x 65 x 65 cm): two plants per cage for each treatment: S_n (control) vs S_0 or S_n (control) vs S_+. Each plant was provided with a top layer of sand particles (2-5 mm in a layer of about 15 mm), into which the flies oviposited. Approximately one hundred mature cabbage root flies of both sexes were released in each cage. The plants were exposed to the flies for 24 hours, after which they were removed. The eggs laid were then extracted from the sand by flotation in water and counted.

For each pair-wise assay, 20 replicates were performed and for each replicate, new plants were used and the positions of the treatments were changed clockwise to minimize any influence exerted by uneven light distribution. The egg counts per plant were expressed as a percentage of the total number of eggs laid on all plants 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.

Larval performance assay. Newly hatched larvae of *D. radicum* obtained from isolated eggs were carefully placed around the stem base of 6-7 leaf *B. napus* plants from each fertilizer level (S_0, $S_{0.1}$, $S_{0.2}$, $S_{0.4}$, and S_+). The inoculated plants were placed in meshed cages (60 x 60 x 60 cm) individually and left in a climatic room (20 ± 1°C, 90% RH

and 16:8 L:D) until pupation occurred. During this time, the maggots were allowed to feed on the roots. We collected the pupae 4 weeks after infection by washing the plant roots and the sand. The pupae were then counted and stored individually in plastic vials until emergence.

The effects of the fertilizer status of the host plant on larval performance were assessed by recording for each sex the development time from hatching to adult emergence, and the adult fresh weight.

This experiment was repeated 7 times with different cohorts of insects and plants. Each replicate consisted of three plants per treatment (total: 21 plants per treatment) and each plants was infected with ten larvae. In the first two replicates, some plants had to be discarded due to aphid attacks, resulting in an unequal number of plants per treatment (shown in legend of Figure 2).

The developmental times were not normally distributed and thus we used a non-parametric Kruskal-Wallis rank sum test to identify significant differences between the treatments. The Spearman Rank Correlation was then made to emphasise the dose-dependent relationship between developmental rates and S-levels. The adult fresh weight was analysed using a nested analysis of variance to test for differences due to treatment, sex and interactions (non-independence of the plants within the replicate nested term). Multiple comparisons were then made using a post-hoc test with Bonferroni adjustment.

Results

Effect of sulphur fertilisation on D. radicum oviposition choice. The oviposition choice of *D. radicum* was affected by the sulphur fertilisation of the *B. napus* plants (Figure 1). The number of eggs laid was about three and a half times higher on S_n, (Mann-Whitney U-test, n= 40, $p < 0.0001$) than on S_0 plants. Conversely, no significant difference was found between the number of eggs laid on S_n plants and on S_+ plants (Mann-Whitney U-test, n=40, $p = 0.2769$).

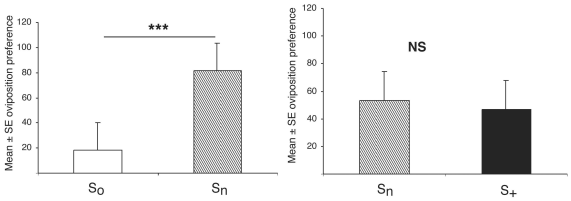

Figure 1. Oviposition choice of *D. radicum* in relation to sulphur fertilisation: number of replicates: 20. Mann-Whitney U-test, p-value (S_0-S_n) < 0.0001; p-value (S_n-S_+) = 0.2769.

Larval survival and development of D. radicum. Larval survival rate and performance were both affected by the sulphur nutrition of the plants, as demonstrated by analysis of the six treatments (Kruskal-Wallis rank sum test, n= 6, p= 0.0308). Within the individual treatments, no significant differences were found between males and females. Thus they were clustered for further analysis. On S_0 plants, only 6% of the infecting larvae reached the pupal stage and 0.5% of pupae emerged. The best larval performance was recorded on S_n plants, where the pupation rate was 32% (S_+= 31%) and the emergence rate (Figure 2) was 23.5%.(S_+= 21%). The values obtained for the three intermediate sulphur levels ranged from 14 to 25% for the pupation rate (Figure 2) and from 6% to 12.5% for the adult emergence (Figure 2). The larval pupation rate correlated with the increasing S supply

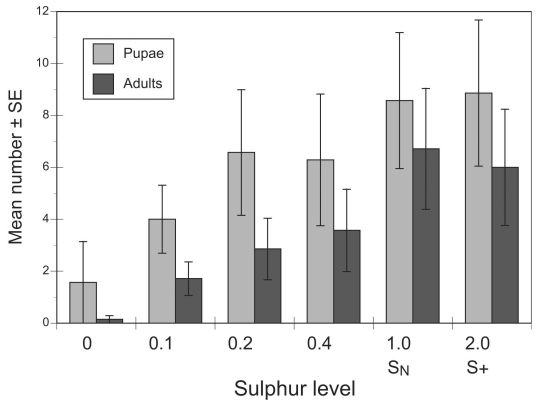

Figure 2. Larval development of *D. radicum* from hatching to adult emergence in relation to sulphur fertilisation: Number of larvae used to infect the plants = (Nr of replicates) x (plants/replicate) x 10 larvae; S_0 = 180; $S_{0,1}$ = 200; $S_{0,2}$ = 180; $S_{0,4}$ = 200; S_n = 190; S_+ = 200. The relationship between sulphur levels and the mean numbers of pupae or adults was significant (Spearman Rank Correlation, n= 70, p=0.0350).

in the nutrient solution and was maximal with S_n. This clear dose-dependent relationship was significant (Spearman Rank Correlation, n= 70, p= 0.035 for the pupation rate adult emergeance). Compared to S_n plants, on S_+ plants we observed a slight decrease in the pupation rate.

The analysis of adult insect weight (Figure 3) showed a pattern similar to that found for the pupation rate. S_0 could not be included in the analysis because of a severe aphid attack. As the weights of males and females were significantly different in all treatments (nested ANOVA, p= 0.0014), comparisons were made separately for each sex. As could be expected, the non-parametric correlation between the S-level and the weight of insects was significant for females (Spearman Rank Correlation, p= 0.0253) but not for males. Compared to the other treatments, females grown on $S_{0.1}$ and $S_{0.2}$ plants were approximately 43% and 37% lighter, respectively, while the males were 43% and 39% lighter. We also observed a significant difference between plants supplied with sulphur-free and sulphur-containing solutions in the larval development of *D. radicum*, measured by the pupation rate and adult emergence. *D. radicum* performance did not improve when plant S supply was above 1 mM of $MgSO_4$ (= S_n, Figure 2).

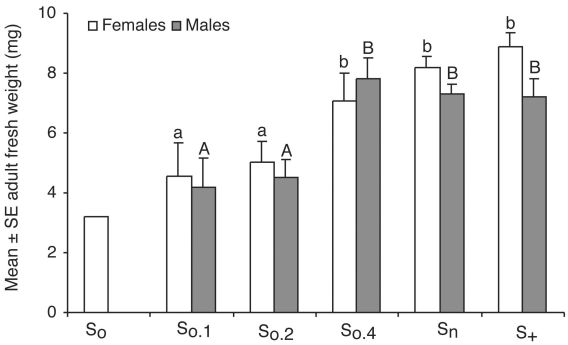

Figure 3. Adult weight of *D. radicum* in relation to sulphur fertilisation: number of replicates: 7. Nested ANOVA, source of variance: sexes: F= 10.99; p-value< 0.0001; columns followed by the same letter are not significantly different at p = 0.05. Differences between the means of females are given in lowercase and males in capital letters. Total number of females = 67; males = 49.

Discussion

The plant soil nutrients, and S in particular, affect the oilseed rape quality and this in turn has an influence on the behaviour of adults and larvae performance in *Delia radicum*. Our results indicate that increasing S supply to the plants correlates with an increase in oviposition by the adult insect. Moreover larval performance, represented by the percentage of pupation, adult emergence and adult fresh weight, was also affected positively by the S concentration of the *Brassica* nutrient solution. The study has shown for the first time that S-fertilisation affects the behavioural choice of cabbage root flies both as adults and larvae. Since glucosinolate (GSL) levels in Cruciferae are influenced by S fertilisation (Bones *et al.*, 1994), we suspected that the amount of S in the soil indirectly affects pest insect development in this plant species. In accordance with our results, Yusuf & Collins (1998) found a positive correlation between the GSL levels influenced by S fertilisation and the feeding preference of the cabbage aphid, *Brevicoryne brassicae*, on Brussels sprouts. On the other hand, an investigation carried out by Koritsas & Garsed (1985) on the effects of nitrogen and sulphur nutrition on *B. brassicae* indicated that low S nutrition increased the growth of plants receiving high nitrogen and of the aphids feeding on them. Since the authors varied the level of the two mineral nutrients simultaneously, however, a direct comparison with our results is not possible. More recently, Bodnaryk (1997) reported that the choice and performance of different insects cannot always be attributed to low GSL levels, but might be influenced by genetical or other environmental factors. The author found no relationship between GSL levels and antixenosis resistance in either *Brassica juncea* or *Sinapsis alba* against specialist herbivores such as the flea beetle (*Phyllotreta cruciferae*) or the diamondback moth (*Plutella xylostella*). The most likely explanation for the disagreement between these and our results is the fact that we used much greater differences in S fertilisation levels than other authors. The fact that we did not find relevant differences between S_n and S_+ seems to support this conclusion. Further, in those studies the likely effects of primary metabolites and water content were not considered, which could possibly explain the discrepancy with our results.

Variation in primary plant compounds can have profound effects on specialist insect preference and performance as reviewed by Berenbaum (1995). Acquiring a sufficient amount of water is indeed a major nutritional obstacle for most herbivorous species (Scriber, 1984). Although in conventional terms water is not a nutrient and water content of foliage varies from 45-95% of fresh weight, the amount of water in the food of many insect larvae provides a useful index of nutritional value and thus of growth performance (Scriber, 1979). In agreement, Wolfson (1980) observed that the oviposition preferences of *Pieris rapae* (L.) were always accompanied by the significantly different water content of plants grown under four nutrient treatments varying in the balance of nitrogen and sulphur. No oviposition preferences were observed when the water content in the treated plants was similar, despite the fact that the S levels differed. Besides water, other primary metabolites could affect the behavioural choices of insects. For example, Hopkins *et al.* (1999) reported that plant sugar is a good predictor of larval performance for *D. floralis*. Thus, it is very likely that *D. radicum* maggots will be sensitive to the possibly changed content of primary metabolites and water.

It has been shown that the oviposition behaviour of the cabbage root fly is based on a range of stimuli, which include colour and structure of plants (Roessingh & Städler, 1990), presence of conspecific larvae /(Baur *et al.*, 1996) and leaf surface secondary chemistry (Roessingh *et al.*, 1992a, Roessingh *et al.*, 1992b). The plants used in our experiments were at a stage (3-4 true leaves), in which visible differences due to poor S-level (small and more fragile plants, reddish leaves) were for us not yet noticeable. It may therefore be concluded that visual stimuli, such as the leaf colour or size, would probably not have influenced the insect's choice. Further, the plants designated for the oviposition dual choice bioassays were uninfected, thus excluding any possible positive or negative effects produced by feeding larvae. We can conclude that S plant nutrition affects host-plant acceptance and suitability for attack by the cabbage root fly. For further details on the plant characteristics mediating these effects in the insect see Marazzi *et al.* (2004). The objective in good agricultural practice is to achieve high yields with minimal S-leaching from the soil. The present study allows us to assess also the effect of S fertilisation on a pest insect. Fertilisation in the normal range tested here seems not to significantly affect repercussions on the host-plant choice and population dynamics of *D. radicum*.

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Part 3: Chemical plant stimuli influencing oviposition by crucifer pests

11. Secondary metabolites of the leaf surface affected by sulphur fertilsation and perceived by the diamondback moth

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CHEMOECOLOGY

Secondary metabolites of the leaf surface affected by sulphur fertilisation and perceived by the diamondback moth

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Summary. Oilseed rape, *Brassica napus* L. (cv Express), plants were grown under three different sulphur regimes: sulphur-free (S_0), normal sulphur (S_n, normal field concentration) and a sulphur-rich (S_+, 2 × concentration of S_n). We performed dual choice oviposition assays with the diamondback moth, *Plutella xylostella*, using real plants and, for the first time with this insect, artificial leaves sprayed with methanolic leaf-surface extracts. The results mirrored those of a separate study of preferences for whole plants. Females laid more eggs on surrogate leaves that were treated with S_n extracts than on S_0 plants, while only a slight, not significant, difference was observed between extracts of normal and sulphur-rich plants. This shows that chemical compounds on the leaf surface mediate the oviposition preference and that the female insect can perceive the quality of the host-plants in terms of their fertilisation status.

Since leaf volatiles are known to be oviposition stimulants, we investigated the effects of leaf-surface extracts on insect olfactory responses using electroantennograms (EAGs). In agreement with the behavioural data, we found that extracts of sulphur-treated plants yielded higher EAG amplitudes than the S_0 extracts. Since the leaf content of the volatiles isothiocyanates is influenced by sulphur nutrition, we analysed the extracts for these compounds. Above the detection threshold of our GC-MS system, no isothiocyanates were found. Thus, other compounds present in the surface extracts must be perceived by the antenna.

However, the HPLC analysis revealed 11 different glucosinolates. Progoitrin (2-Hydroxy-3-butenyl) and gluconapoleiferin (2-Hydroxy-4-pentenyl), which belong to the hydroxy-alkene class of glucosinolates, were the most abundant compounds. The total glucosinolate content sharply increased from S_0 to S_n plants, whereas it was slightly lower in S_n versus S_+ plants. Since it is known that glucosinolates can stimulate oviposition, it seems likely that the increased content we observed was influencing the insect preference in this study too.

Key words. Oilseed rape – *Brassica napus* – glucosinolates – isothiocyanates – *Plutella xylostella* – oviposition choice – EAG, HPLC, GC-MS

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Introduction

The diamondback moth, *Plutella xylostella* (L.), is a harmful pest of cruciferous crops throughout the world (Harcourt 1957). In our previous investigation of the effects of sulphur (S) plant nutrition on oviposition of the diamondback moth (Marazzi & Städler, in preparation) we found that moths clearly discriminate between S fertilised and non-fertilised *Brassica napus* oilseed plants. The question arising was which plant characteristics the moth females perceive.

In Brassicaceous plants, S is used in the synthesis of secondary metabolites like glucosinolates (GSLs) (Chew 1988), phytoalexins (Pedras *et al.* 2000) and CIF ("cabbage identification factor"; 1,2-dehydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorine-1-carboxylic acid) (Hurter *et al.* 1999). Volatile chemicals mainly produced by hydrolysis of non-volatile GSLs (Pivnick *et al.* 1994) are also known to be involved in host-plant location by different insects attacking this plant family (Blight *et al.* 1995). Gupta and Thorsteinson (1960a, b) have demonstrated that constituents of host-plants affected larval feeding and oviposition of *P. xylostella*. Later studies confirmed that host recognition and oviposition by this insect is dependent on GSLs. Reed *et al.* (1989) used plant extraction followed by myrosinase treatment to show that the stimulant activity was greatly reduced after hydrolysis of the GSLs. Individual GSLs were also active but not to the same extent as homogenised plant tissue.

The presence of GSLs in crucifers is strictly dependent on the medium in which the plant is grown (Schnug 1997). In this context, Meyer (2000) showed that soil fertility affects both the degree of defoliation and compensation for herbivory in *Brassica nigra* plants damaged by *Pieris rapae* caterpillars. Oligophagous insects such as diamondback moth larvae accept as food the leaves of only a limited number of plant species. The actual selection of the host-plant is performed by the ovipositing female (reviewed in Marazzi & Städler, in preparation), as the mobility and energy reserves of the first instars are limited and the opportunities to find a suitable host on their own are minimal (Justus & Mitchell 1996).

In the present work, S was used as a variable environmental factor, since this mineral is clearly involved in the production of secondary plant metabolites that have key roles as attractants, feeding (Renwick 2002) and oviposition stimulants (Justus & Mitchell 1996; Hughes *et al.* 1997; Spencer *et al.* 1999) for *Plutella xylostella*. Gupta and

Thorsteinson (1960a) first studied the effect of S mineral nutrition in two *Brassica* species (*B. alba* and *B. nigra*). They found that *P. xylostella* laid more eggs on the S fertilised plants than the S deficient controls. Although these differences were not significant, they seemed to be suggestive. This prompted us to study the indirect influence of S fertilisation on the behavioural and physiological responses of female diamondback moths to *Brassica napus* (L.) plant extracts hoping to find the plant characteristics related to the observed preference.

Materials and methods

Insects

All the *Plutella xylostella* (L) (Lepidoptera: Plutellidae) required for the bioassays came from our laboratory culture established with approximately 200 individuals during spring 2000 and reared as described by Marazzi & Städler (in preparation). Briefly, an equal number of male and female mature moths (approximately 100 per cage) were held in mesh cages (50 × 45 × 45 cm) in a climate-controlled room (21 ± 1°C, 70 % RH and 16 h photophase), where they were allowed to mate and oviposite only on the leaves of potted (∅ 13 cm) *B. napus* cv CC-Cross F1 at the pre-bolt stage. The moths had access to a source of water and to 10 % sugar-water.

Plants

The oilseed rape (*B. napus* cv. Express) was grown as described by Marazzi & Städler (in preparation). Briefly, a modified "Hoagland" nutrient solution was used to provide the three different S levels of fertilisation: S_n = 1 mM of $MgSO_4$ (normal S concentration in a Swiss field); 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). All the experiments were conducted using plants with 3–4 true leaves (30–35 days after planting), comparable in size.

Oviposition choice assay

Bioassay with real plants: We attempted to relate our oviposition data on real plants with the glucosinolate content of the three samples of *B. napus* plants (S_0, S_n and S_+). For this purpose we used the data concerning the oviposition choice assay with real plants from Marazzi and Städler (in preparation), where we tested 4 *B. napus* plants at a time (2 plants per cage for each treatment, i. e. S_n vs S_0 or S_n vs S_+) for a period of 24 hours, during which about one hundred 1–2 day-old diamondback moths were released into each cage and were allowed to mate and oviposit on plants (n = 20).

Bioassay with artificial leaves: We used the same extraction procedure as described by Baur *et al.* (1996) to obtain wax-free methanolic leaf-surface extracts of 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 1 g of fresh leaf material.

The oviposition substrates used in the experiments consisted of paraffin-coated green paper model leaves developed originally for the cabbage root fly by (Roessingh & Städler 1990) with a surface projection of 7 × 7 cm, 1.5 cm wide vertical folds and a flat stem of 5 × 1.5 cm. Leaves were individually inserted into hydroscopic foam (Smithers-Oasis, Germany D-67269) cylinders (8 cm in diameter, 5 cm high) and placed in plastic containers (9 cm in diameter, 5 cm high). The top of each container was covered with a black plastic layer (9 cm in diameter). Surrogate leaves were sprayed using an airbrush (Aerograph Sprite, Devilbiss) in a fume hood with 1 gle of the extract to test.

All the oviposition bioassays were conducted in the same cages as those described for the rearing (about 100 adult moths per cage). Four plants (or four surrogate leaves) were arranged in a circle on the floor of the cage (2 for each treatment: S_n (control) vs S_0 or S_n (control) vs S_+). After an oviposition period of 24 hours, the eggs laid on each plant or artificial leaf were counted and expressed as a percentage of the total number of eggs laid on all plants within one replicate. Thus, the resulting preference values for the treatments compared (S_n vs S_0 and S_n vs S_+ respectively) totalled 100 %. A Mann-Whitney U-test was performed to determine the significant differences in preference between treatments. For each pair-wise assay, 9 (S_0 vs S_n) and 13 (S_n vs S_+) repetitions were performed and after each replicate, the plant or artificial leaf positions were changed clockwise.

Chemical analysis of glucosinolates

The surface extracts of *B. napus* grown under the S_0, S_n or S_+ regimes and a total leaf extract (homogenate) of each plant group were assayed for their GSL content. For each treatment, 15 plants were removed from the pots and the sand was gently washed from the roots using the same nutrient solution to prevent contamination. Plants were then separated into roots and aerial parts, frozen in liquid nitrogen, crushed into small pieces and stored at –20°C until needed. Prior to analysis, samples of both plant parts were ground to a fine powder at –18°C with a coffee mill, 10 g weighed out, placed in 50 ml glass flasks and stored at –20°C until needed.

We used the same extraction procedure described by Griffiths *et al.* (2001) to obtain desulfoglucosinolates. 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. Reported quantifications are the means of five separate extractions and analyses. The amounts of GSLs from individual peaks were summed and these values were analysed by ANOVA to determine differences between treatments. The GSLs nomenclature is based on the identification list of Griffiths *and al.* (2001).

Chemical analysis of isothiocyanates

Isothiocyanates in the plant extracts were analysed by combined gas chromatography and mass spectrometry using a Finnigan Voyager GC-MS System. The GC was equipped with a fused silica capillary column: DB-5 (30 m, 0.25 mm, 0.25 µm coating). Each plant eluates were prepared by shaking 2 ml crude extract with 2 ml hexane. Two µl of the hexane fraction of each sample were injected splitless using an auto sampler (PAL, CTC-Analytics). Identifications and quantifications were made by external calibration, comparing obtained mass spectra and retention times with those of 4 pure isothiocyanataes (phenyl-, butenyl-, benzyl- and allyl-isothiocyanates). The detection threshold was 1.5 µg/ml crude extract.

Electrophysiology

Female diamondback moths, 1–2 days old, were cooled in a refrigerator (5.5°C) for 1–4 hours to reduce their activity. The wings and the legs of the cooled insects were amputated. The body was mounted ventral side up in the groove of a Plexiglas® holder and positioned so that the antennae were attached to a sticky wax layer, using strips of transparent tape (Scotch® 3M 810, 19 mm × 33 m). The head and scape of the antenna were fixed to the support with histology paraffin (mp 45°C) melted locally with a temperature controlled soldering iron (50°C). The recordings preparation was

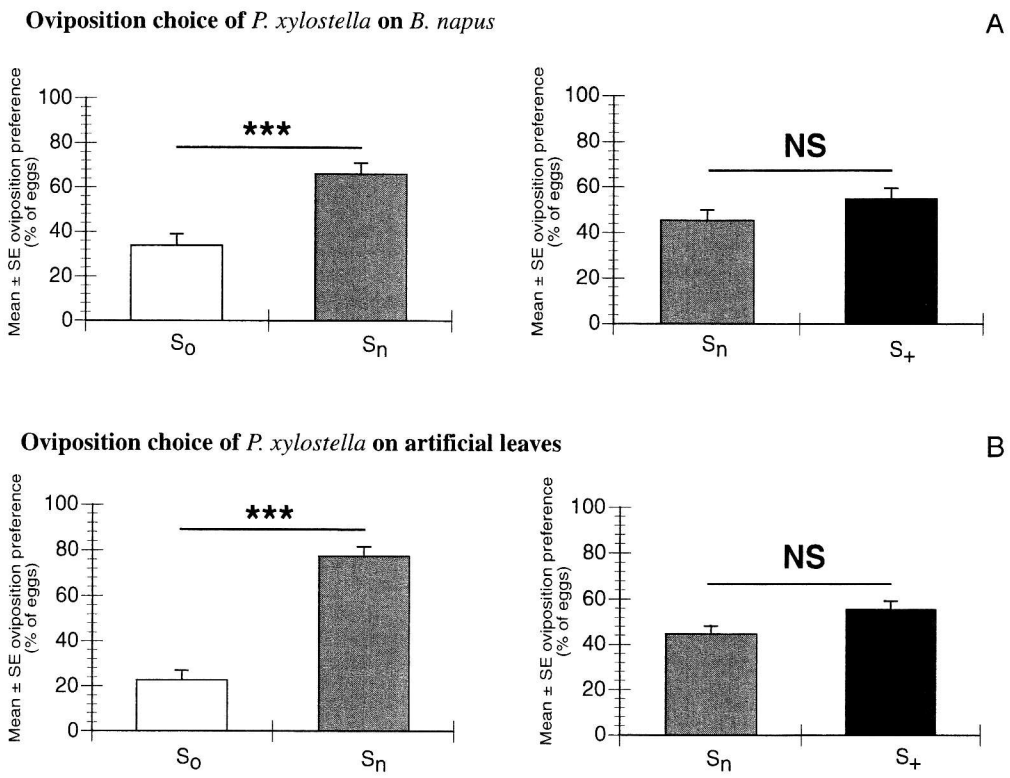

Fig. 1 Effect of sulphur fertilisation on *P. xylostella* oviposition choice. (**A**) Oviposition choice assay with *B. napus* plants. Number of replicates: n = 9 for S_0/S_n and n = 13 for S_n/S_+. These data are the same as in Marazzi & Städler (2003) and shown here for comparison. (**B**) Oviposition choice assay with leaves treated with *B. napus* plant surface extracts (1 gle). Number of replicates: n = 9 for S_0/S_n and n = 13 for S_n/S_+. S_0 = sulphur-free; S_n = normal field-concentration of sulphur; S_+ = sulphur-rich

mounted under a stereomicroscope and continuously humidified with a water-saturated air stream (1 m/s, 22 ± 3°C). We used basically the same method as described by Guerin and Visser (1980). Briefly, the airflow was split into continuous and stimulatory airstreams at a 9 : 1 ratio, which converged prior to the electrophysiological preparation. The stimulatory air stream passed through a Pasteur pipette (20 µm Ø) containing the test compound (100 µl) spread on a folded filter paper (1.6 × 5 cm, paper from Schleicher & Schuell) and upon activation of a valve, was injected into the continuous air stream. The indifferent electrode, filled with a saline solution (Kaissling 1995), was inserted in the base of the antenna and the recording electrode, containing saline plus 0.01 % (v/v) of polyvinylpyrolidone solution, was brought in contact with the antennal tip. The EAG signal was recorded using a lab-built amplifier with high input impedance (10^{13} Ω) and low bias current (< 10 pA). The signals were filtered (electronic high-pass with cornering frequency of 0.001 Hz), amplified (100 ×) and digitised using SuperScope II 3.0 software (GW Instruments, Somerville, Massachusetts) on a Macintosh computer. The EAG amplitudes were determined using PowerChrom v2.2.4 (AD Instruments, Springs, Colorado) software. The S_0, S_n and S_+ plant extracts were tested at a concentration of 0.04, 0.1 and 0.4 gle. Trans-2 hexanal (1 and 10 µg) was used as control for antennal activity at the beginning and end of a test sequence. The set of stimuli was exposed sequentially to the insect in the following order: from S_0, S_n to S_+, always starting with the lowest plant extract concentration. Responses to extracts were expressed as the mean of 5 pulses of 0.5 s with an interval of 5 s. EAG measurements were replicated on 5 female antennae.

The EAG responses to plant extracts between the 3 groups of differently fertilised plants were analysed by ANOVA with the amplitude (mV) of the EAGs as the dependent factor, type of treatment (S_0, S_n and S_+) and concentration (0.04, 0.1 and 0.4 gle) as independent factors (n = 5). We performed multiple comparisons using a Bonferroni post-hoc test within each group of plant extracts to detect differences between concentrations.

Results

Oviposition choice on artificial leaves treated with surface extracts

The *B. napus* plants differing in S fertilisation tested by Marazzi and Städler (in preparation) (Fig. 1A) were extracted and yielded the material for these experiments. As shown in Fig. 1A, the oviposition choice of *P. xylostella* on real plants was affected by S concentration (Mann-Whitney U-test, S_0 vs S_n: n = 9, p = 0.0013 and S_n vs S_+: n = 13, p = 0.0956). The wax-free methanolic leaf-surface extracts of plants fertilised with S proved to be active in the oviposition bioassays of *P. xylostella* (Fig. 1B). The number of eggs laid was significantly higher (Mann-Whitney U-test, n = 9, p = 0.0003) on artificial leaves sprayed with S_n plant extracts than on artificial leaves sprayed with S_0 plant extract, representing an increase of nearly 250 % on S_n plant extracts. Similarly, a slight but not significant difference was found between the number of eggs laid on artificial leaves sprayed with S_n and S_+ plant extracts (Mann-Whitney U-test, n = 13, p = 0.0833). Compared to S_n, S_+-sprayed artificial leaves showed a 24 % increase in oviposition.

Chemical analysis of B. napus plant extracts

Eight GSLs were consistently detected in all tissue from the three populations of *B. napus* tested (Fig. 2A). These included the alkene GSL, 3-butenyl (gluconapin), two hydroxy-alkenes GSLs, 2-hydroxy-3-butenyl (progoitrin)

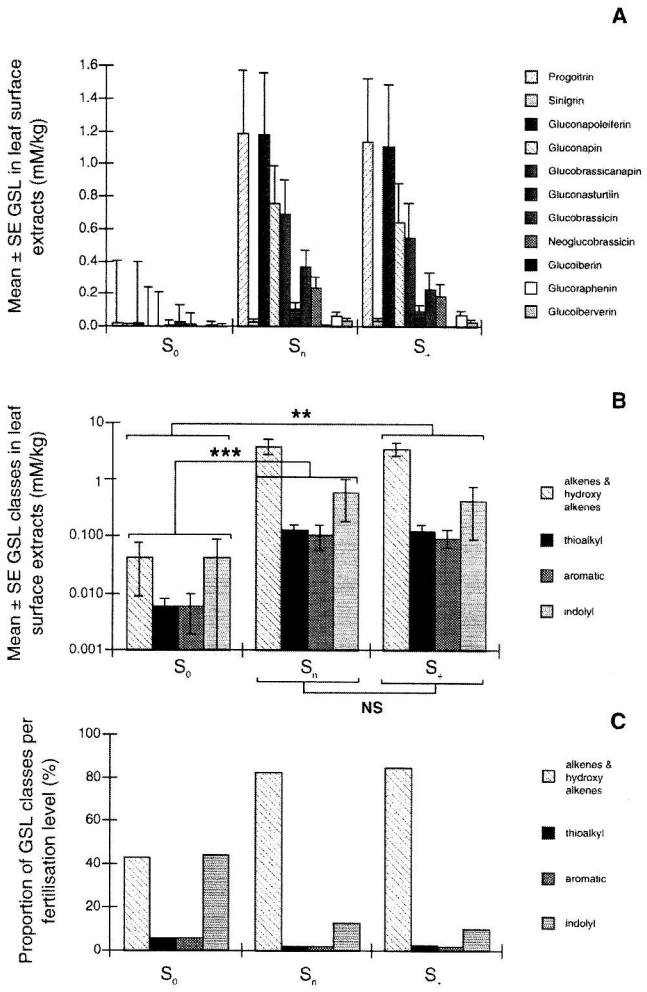

Fig. 2 Analytical data of the glucosinolates content of *B. napus* plants. (**A**) Profiles of individual glucosinolates for S_0, S_n and S_+ *B. napus* plants. Number of replicates: 5. Abbreviations: as in Fig. 1. (**B**) Profiles of glucosinolate in a logarithmic scale. Number of replicates: 5. (**C**) Proportion of the glucosinolate classes. Interaction (ANOVA) between sulphur fertilisation vs group of GSL: $p < 0.001$

and 2-hydroxy-4-pentenyl (gluconapoleiferin), two thioalkyl GSLs, 4-methylsulphinyl-3-butenyl (glucoraphenin) and 3-methylthiopropyl (glucoiberverin); two indolyl GSLs, 3-indolylmethyl (glucobrassicin) and 1-methoxy- 3-indolylmethyl (neoglucobrassicin) and the aromatic GSL 2 phenylethyl (gluconasturtiin). Moreover, we found three additional GSLs in S_n and S_+ plant extracts: two alkene GSLs, 2-propenyl (sinigrin) and 4-pentenyl (glucobrassicanapin), and a thioalkyl GLS, 3-methylsulphinylpropyl (glucoiberin) (Fig. 2A). The concentrations given in mM/kg of leaf extract of the individual GSLs detected in S_n and S_+ plant extracts were overall higher than those found in S_0 plant extracts (Fig. 2B). The proportions of individual GSLs classes varied considerably also between the plant extracts (Fig. 2C). The proportion of progoitrin observed in S_n and S_+ plant extracts (S_n = 1.188, S_+ = 1.141 mM/Kg leaf surface) was around 60 times larger than that found in S_0 plant extracts (0.019 mM/Kg leaf surface). Similarly, the proportion of gluconapoleiferin was about 50 times larger in S_n and

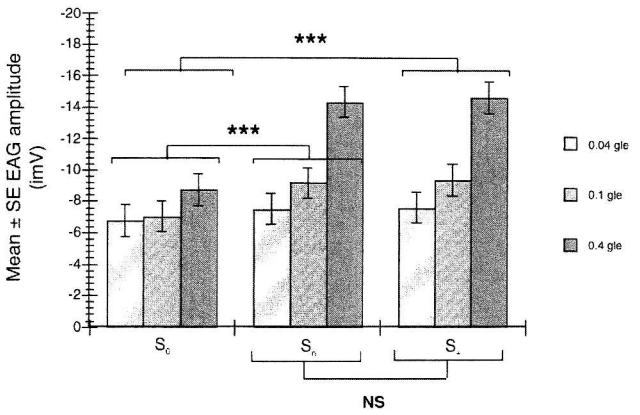

Fig. 3 Electroantennogram responses to *B. napus* surface extracts of plants differing in S nutrition. The plant extracts were tested in three different concentrations: 0.04, 0.1 and 0.4 gle (gram leaf equivalent). Values are given as means ± of 5 stimulations per antenna (insect) and per concentration. Number of replicates: 5 insects. The dose-dependent relationship between concentration and female antennal response was tested by a Bonferroni-test, n = 5, 0.04–0.1 gle: $p = 0.0029$, 0.04–0.4 gle, $p < 0.0001$, 0.1–0.4 gle: $p < 0.0001$. Abbreviations: as in Fig. 1

S_+ than in S_0 plant extracts. The greatest proportional difference was found with gluconapin, whose concentration was between 650 and 750 times higher in S_n and S_+ versus S_0 plant extracts. Quantitatively, the total GSLs detected showed differences that were between 10 and 30 times higher in S_n and S_+ versus S_0 plant extracts (Fig. 2A). The potential presence of volatile breakdown products of GSLs, the isothiocyanates, was assed by GC-MS analysis. Isothiocyanates were not present in any of the tested plant extracts (detection threshold: 1.5 µg/ml crude extract).

Electroantennogram response to B. napus plant extracts

The EAG responses of female moths of *P. xylostella* to leaf surface extracts of S_0, S_n and S_+ tested at three increasing concentrations are shown in Fig. 3. Mean EAG responses elicited by the test compounds ranged from about 0.1 (at 0.04 gle, mainly S_0 plant extracts) to 3 mV (at 0.4 gle, mainly S_n and S_+ plant extracts). All concentrations of S_0 extracts tested induced significantly lower responses than those of S_n or S_+ extracts (Bonferroni, n = 5, $p < 0.001$,). Conversely, no significant difference was found between the EAG recordings for S_n- and S_+-plant extracts (Bonferroni, n = 5, $p = 0.5490$, Fig. 3A). EAG amplitudes were clearly concentration dependent and peaked at the highest dose tested (0.4 gle) for all three plant extracts tested.

Correlations between behaviour and chemical analysis

The lack of S nutrition dramatically affected the GSL content of the plants, corresponding well to the results of the behavioural assay described in Fig. 1. The significant differences in the female antennal perceptions revealed by EAG recordings were exactly mirrored by the GSL content of plant extracts.

Discussion

Oviposition preference influenced by leaf surface extracts

The acceptability of *B. napus* for *P. xylostella* was markedly affected by the S nutrient levels of the plant. The lack of S in the plant nutrient solution reduced oviposition on both real plants and artificial leaves. Surprisingly, the differences detected with sprayed surrogate leaves were more pronounced. Our study seems to be the first showing the direct role of fertilisation on plant secondary metabolites in influencing oviposition behaviour. Our results are in contrast with previous publications comparing *Brassica* species differing in GSL levels (Pivnick *et al.* 1994; Bodnaryk 1997). These authors concluded that species like *P. xylostella* are insensitive to sinigrin and suggested that its pest status on low-GSL lines would be likely to remain unchanged. Possible reasons for this disparity might be the followings: 1) "low GSL varieties" are not comparable to the effect of plant S-deficiency, 2) these plants are known to have a lower content of GSLs only in the seeds, but not necessarily in other plant parts (Fahey *et al.* 2001) and 3) in our experiments, extreme variations in the levels of S-supply were used, making the differences between the three entries more evident. It is therefore conceivable that in the behavioural choices made by the insect, only the threshold between undetectable/detectable presence of GSLs was qualitatively important and not the quantitative difference. However, other plant metabolites are certainly also influenced by the S shortage and may therefore have affected the oviposition behaviour positively or negatively. Further, some of these metabolites are not volatile and thus cannot be detected by the olfactory sensilla. In addition, the final response of an insect in accepting or rejecting a particular plant is mediated by a balance of positively and negatively interpreted sensory signals evoked by plant chemicals. In this respect, it should be noted that negative (inhibitory) effects of the plant extracts were not more specifically tested.

Non-volatiles in leaf surface extracts

In the present study, we found that the higher the S application, the higher the total GSL content. Mainly the abundance of the alkenes and the thioalkyl increased whereas the aromatic and indolyl classes were comparatively unaffected. It is interesting that S affected not only the quantities of GSLs but also the qualitative proportions (Fig. 2C). The different patterns in the four groups of GSLs in response to S can be explained by their amino acid precursors (Schnug & Hanecklaus 1994). Indolyl and aromatic GSLs are derived from tryptophan and tyrosine respectively, and their biosynthesis requires two S-containing compounds: cysteine and adenosyl-5'-phosphosulphate (APS) (Mithen 2001). In contrast the synthesis of alkenes and thioalkyl GSLs depends on three S sources: methionine, cysteine and APS. Given this difference in S requirement at the biosynthetic level, thioalkyl and alkene (synthesised via modification of the side chain of thioalkyl) GSLs might be more sensitive to the plant S status. The effect of S application on the total GSL content is in agreement with the observations of Schnug & Hanecklaus (1994) showing that the S nutritional status of the plants exerts a considerable effect on the GSL content. Similarly Mailer (1989) and Scherer (2001) reported that S application up to 100 $\mu g\ mL^{-1}$ raised the GSL concentration in the rapeseed cv. Wesbrook (*B. napus* L.). Interestingly, Mailer (1989) found that a nutrient solution provided with an excess of S (200 $\mu g\ mL^{-1}$), lowered the GSL concentration. This could explain why, in our results, S_+ values were not always higher than S_n.

After landing on a host plant, contact stimuli can influence oviposition. In the case of *P. xylostella*, individual GSLs, including sinigrin and glucobrassicin, certainly play an important role in host recognition and oviposition stimulation (Reed *et al.* 1989; Renwick 2002), as well as in larval feeding, as reported by Van Loon *et al.* (2002). This function is synergised by surface waxes, as demonstrated by Spencer *et al.* (1999), who reported only limited oviposition stimulant activity of sinigrin or cabbage homogenates in the absence of wax.

Volatiles in leaf surface extracts

In agreement with the behavioural and analytical data, antennal olfactory responses (EAGs) to the extracts were also significantly stronger for S_n and S_+ plants than for S_0 plants. Extracts of host-plant volatiles are most likely involved in host location as shown by Palaniswamy *et al.* (1986) and also enhance egg deposition rate in *P. xylostella*. Reed *et al.* (1989) identified intact glucosinolates in host-plant extracts stimulating oviposition and found that degradation with myrosinase or sulphatase largely eliminated the activity, thus excluding isothiocyanates, as active principles. Pivnick *et al.* (1994) studied attraction of the moths and found that allyl-isothiocyanates was an attractive component in homogenised plant volatiles but it was virtually absent from intact plant volatiles. The authors performed a gas chromatographic fractionation of these volatiles and found a terpene-containing fraction to be most attractive, whereas no isothiocyanates were present. Justus *et al.* (unpubl.) recorded GC-EAGs of headspace extracts from two host and non-host plants of *P. xylostella* and identified several EAG active components, but again no evidence was found that isothiocyanates matched the most stimulating compounds. All these results seem to be in agreement with our findings that isothiocyanates do not, or only in minute quantities, occur in the behaviourally active extracts. On the other hand, Renwick *et al.* (in preparation) have found convincing evidence that some isothiocyanates stimulate oviposition. These same compounds also produced matching EAG activities, so that apparently isothiocyanates do have some role to play in host-plant selection of *P. xylostella*. Renwick and Radke (1990) provided clear evidence that several chemically different compounds are involved in the stimulation of the diamondback moth oviposition. Therefore, it can be assumed that the extracts tested in our study contain also a mixture of compounds that might, individually or in combination, stimulate oviposition. The highly active plant extracts obtained in this study would be a good basis to investigate the relative importance of volatile and non-volatile compounds, because as we showed, they match the response to intact plants very well indeed.

The most important result of practical interest of this work is the fact that no significant differences between S_n and S_+ plants were noted. Consequently, as sulphur fertilisation approaches the optimal level for rape, the crop plant will have no further influence on attack or population increase rates of its pest. Within the range tested in this investigation, S fertilisers that allow optimal harvests do not lead to increased infestation by *P. xylostella*.

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Part 3: Chemical plant stimuli influencing oviposition by crucifer pests

12. Secondary metabolites of the leaf surface affected by sulphur fertilsatio and perceived by the cabbage root fly

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CHEMOECOLOGY

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).

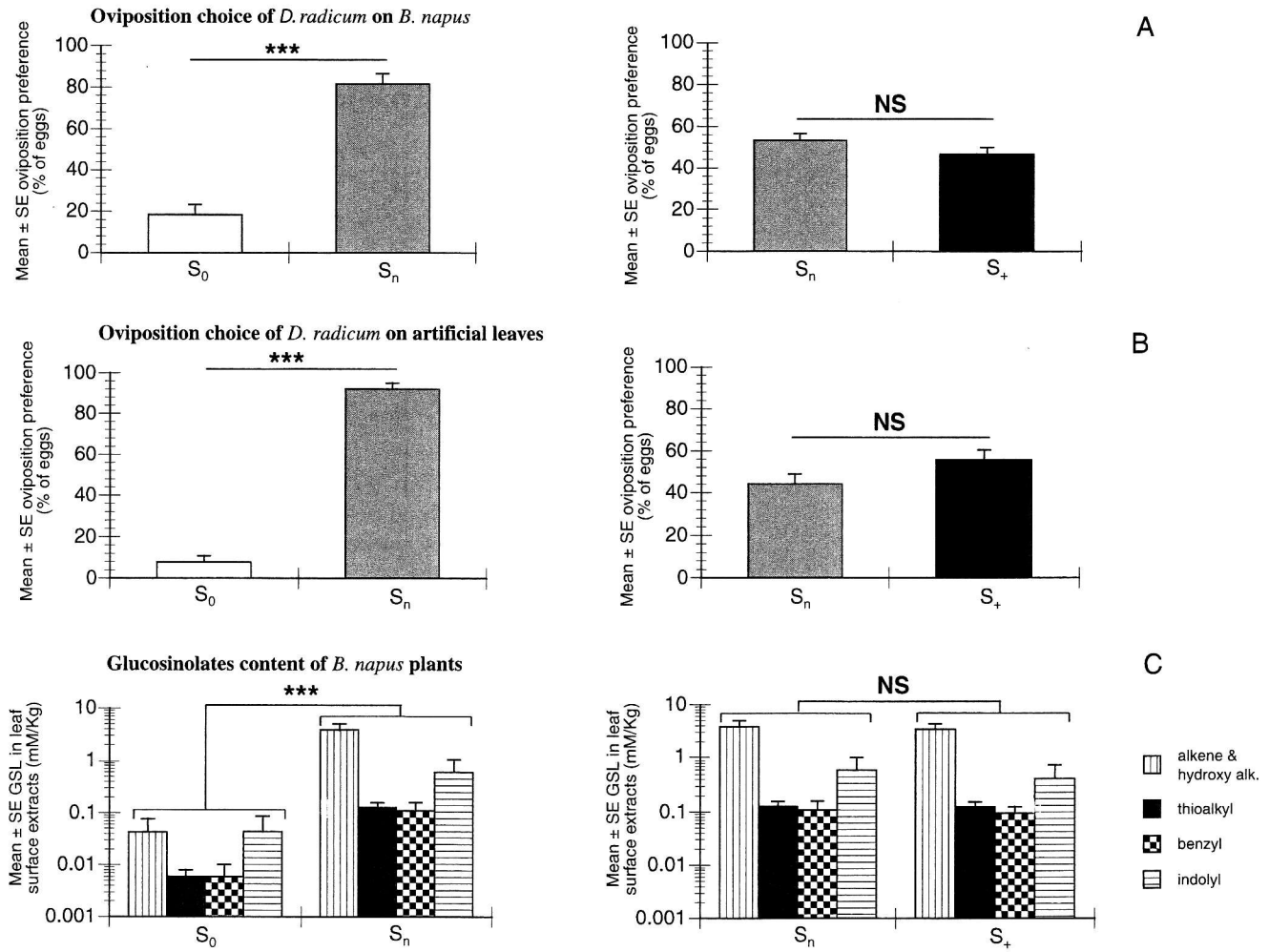

Fig. 1 Oviposition choice of *D.radicum* affected by sulphur fertilisation. (**A**) Proportions of eggs laid on S_0 (sulphur free), S_n (normal sulphur supply) and S_+ (sulphur-rich) *B. napus* plants. These data are the same as reported in Marazzi *et al.* (in preparation) and are shown for comparison. 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, $n = 40$, $p < 0.3302$).

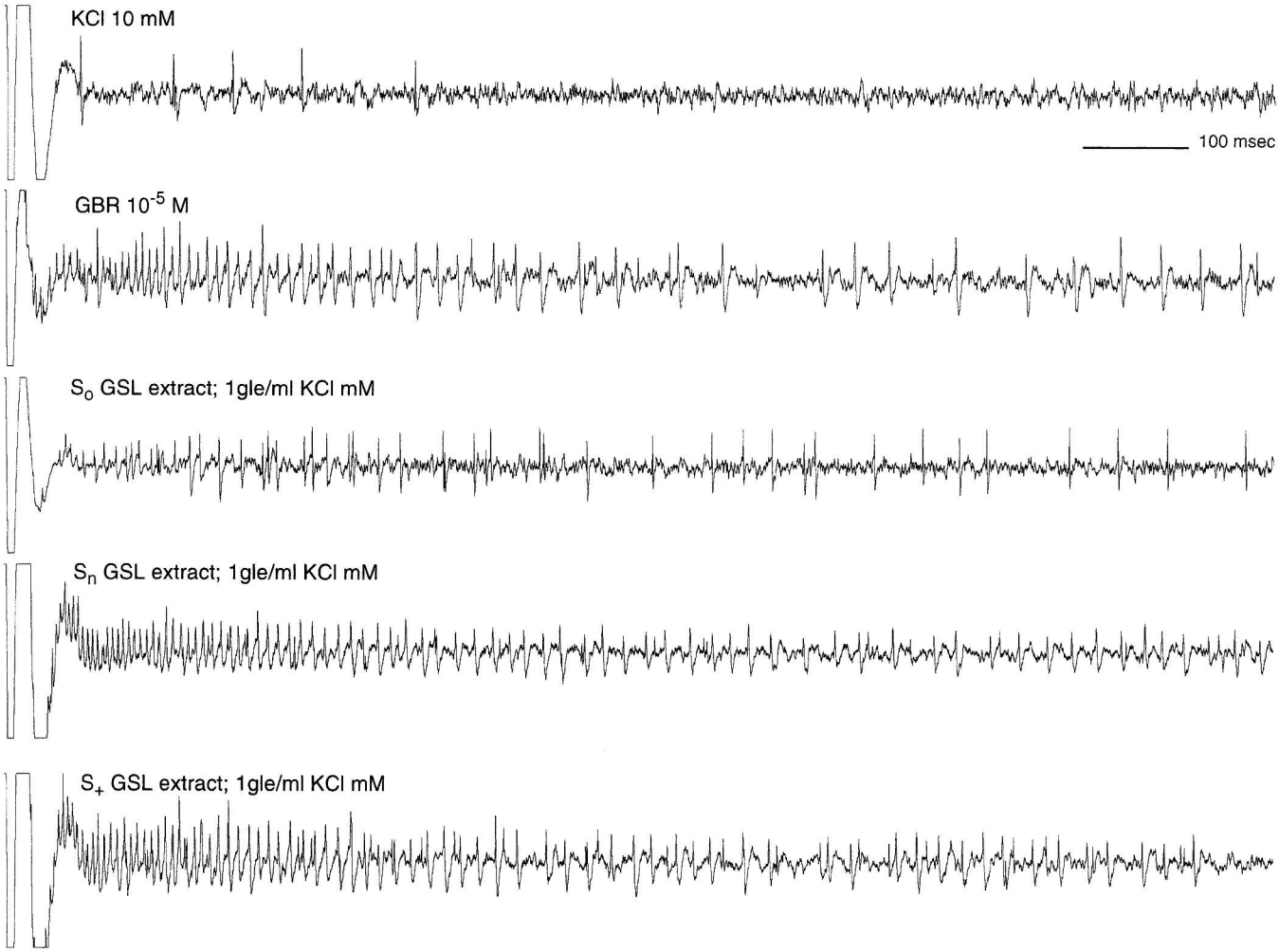

Fig. 2 Electrophysiological recordings from a D_3 sensillum stimulated with GSL fractions of S_0, S_n and S_+ *B. napus* plant extracts. Standard: glucobrassicin (GBR) 10^{-5} M, solvent: KCl 10 mM. gle/ml = gram leaf equivalent per ml. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich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

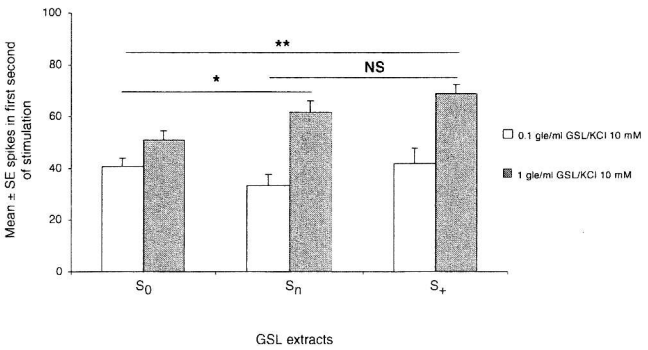

Fig. 3 $D_{3,4}$ tarsal sensilla of *D. radicum* stimulated by GSL fractions of different *B. napus* varying in S nutrition. Numbers of analysed recordings: S_0-S_n: 15; S_0-S_+: 19; S_n-S_+: 18. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich plant extracts

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.

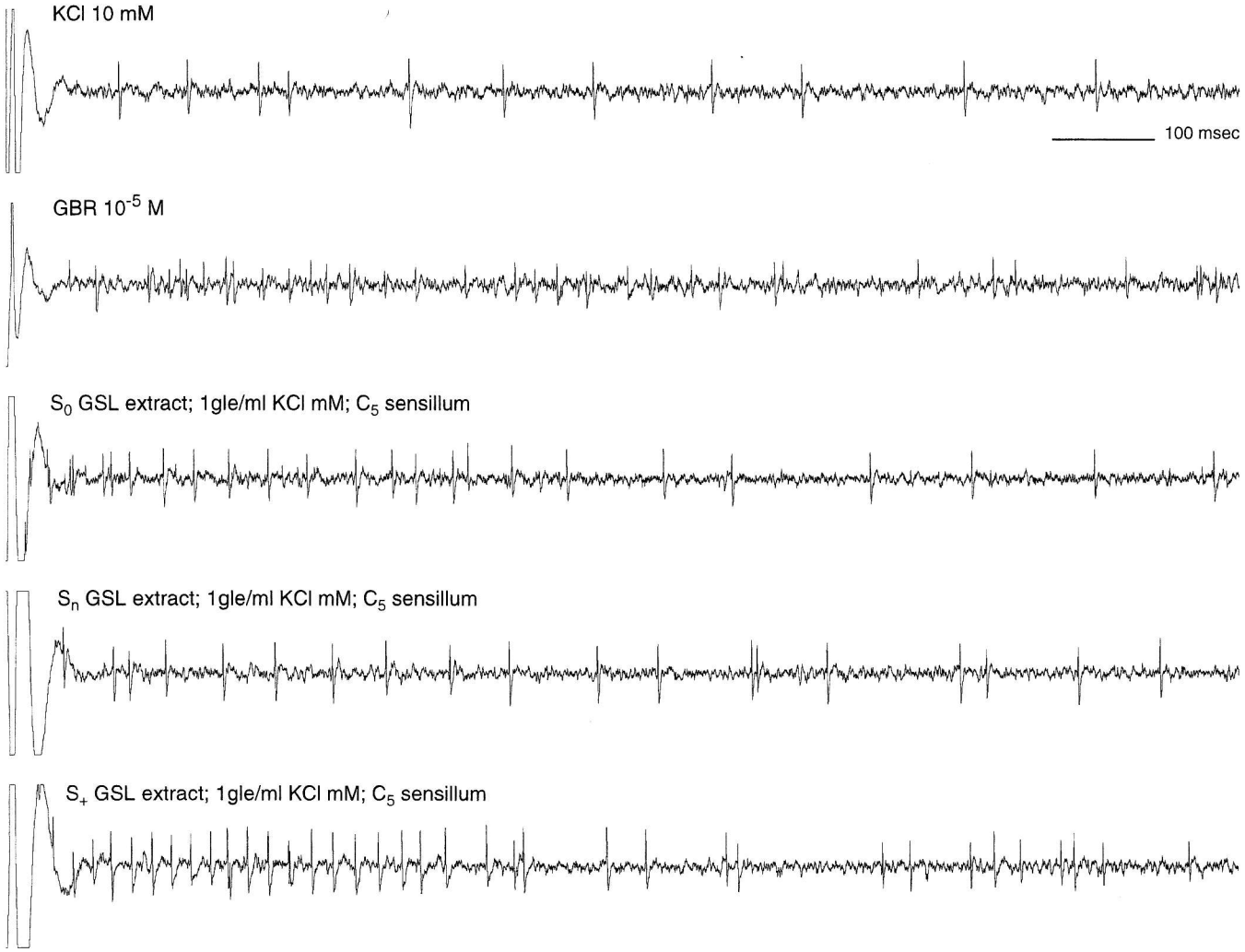

Fig. 4 Electrophysiological recordings from a C_5 sensillum stimulated with GSL fractions of S_0, S_n and S_+ *B. napus* plant extracts. Standard: glucobrassicin (GBR) 10^{-5} M, solvent: KCl 10 mM. gle/ml = gram leaf equivalent per ml. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich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

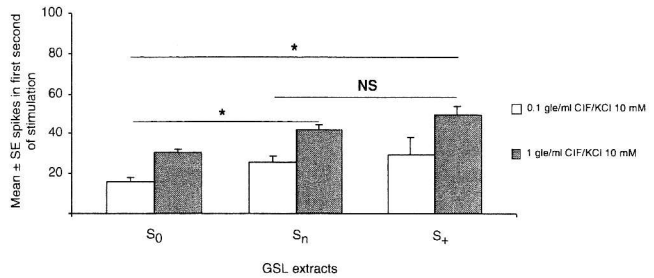

Fig. 5 C_5 tarsal sensilla neurons of *D. radicum* stimulated by GSL fractions of different *B. napus* varying in S nutrition. Numbers of analysed recordings: S_0-S_n: 15; S_0-S_+: 19; S_n-S_+: 18. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich plant extracts

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

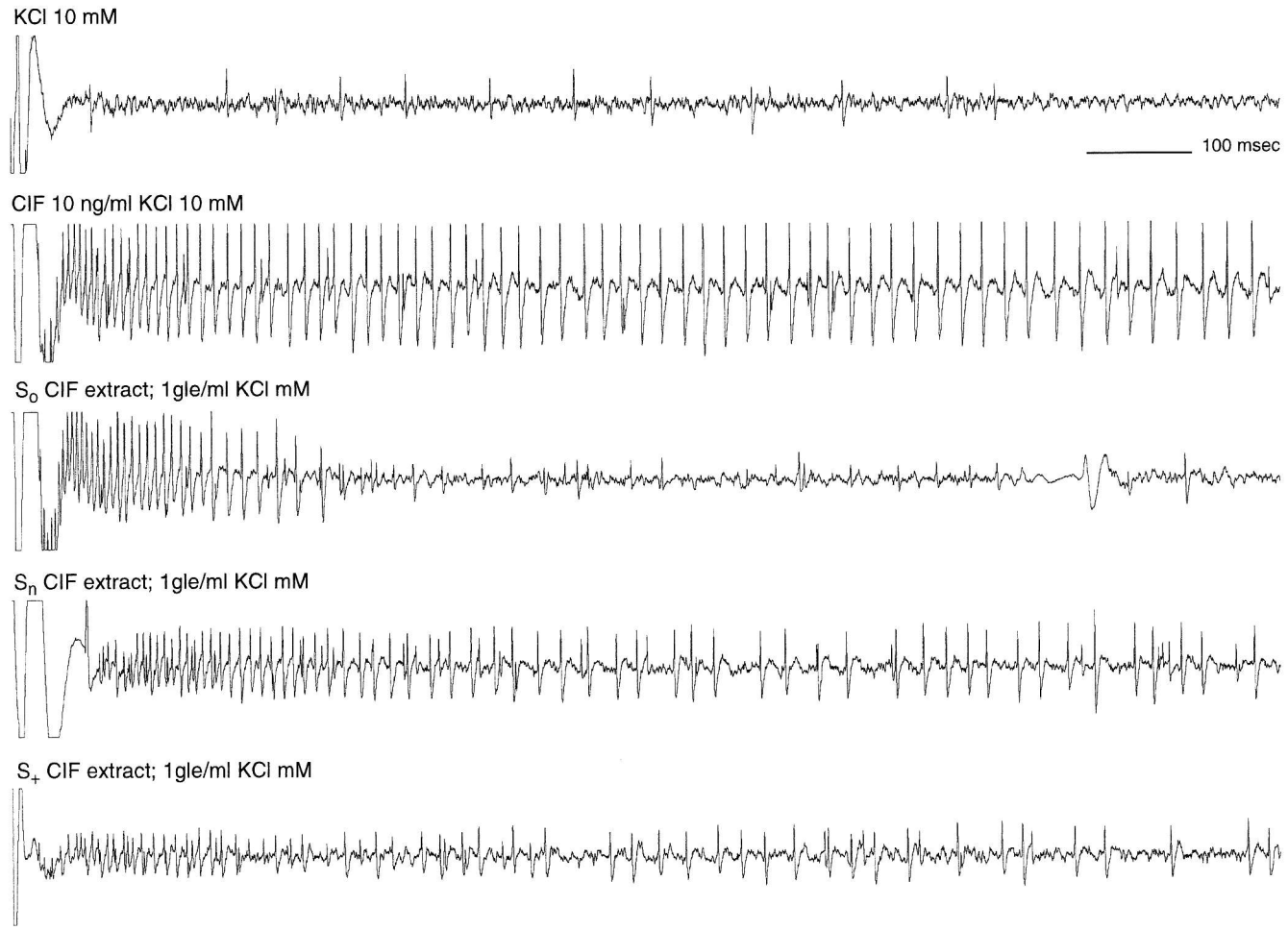

Fig. 6 Electrophysiological recordings from a C_5 sensillum stimulated with CIF fractions of S_0, S_n and S_+ *B. napus* plant extracts. Standard: CIF 10 ng/ml KCl mM, solvent: KCl 10 mM. gle/ml = gram leaf equivalent per ml. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich plant extracts

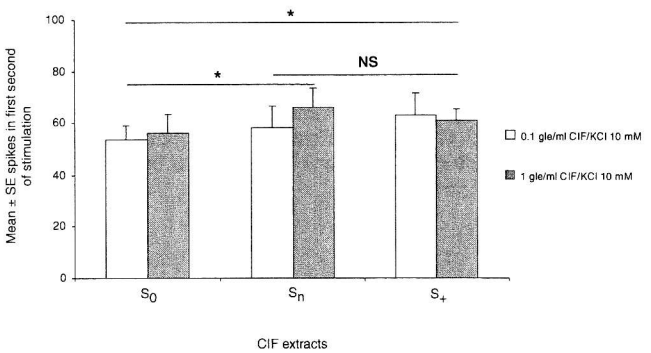

Fig. 7 C_5 tarsal sensilla neurons of *D. radicum* stimulated by CIF fractions of different *B. napus* varying in S nutrition. Numbers of analysed recordings: S_0-S_n: 15; S_0-S_+: 17; S_n-S_+: 11. S_0 = sulphur free, S_n = normal sulphur and S_+ = sulphur-rich plant extracts

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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Part 4: Host-plant suitability of Arabidopsis thaliana

13. Arabidopsis thaliana (L.) leaf-surface extracts that are detected by the cabbage root fly (Delia radicum) and stimulate oviposition

Physiological Entomology (2004) **29**, 192–198

SHORT COMMUNICATION

Arabidopsis thaliana leaf-surface extracts are detected by the cabbage root fly (*Delia radicum*) and stimulate oviposition

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Abstract. Oviposition of the cabbage root fly, *Delia radicum* (Diptera, Anthomyiidae) is stimulated by leaf-surface extracts of *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) ecotype Columbia. The leaf surface of *A. thaliana*, similar to that of many other crucifers, contains glucosinolates and CIF ('cabbage identification factor'; 1,2-dehydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorene-1-carboxylic acid). These compounds stimulate receptor neurones of the tarsal sensilla of *D. radicum* whereas additional, unknown compounds are detected by other receptor neurones.

Key words. Anthomyiidae, contact chemoreception, Cruciferae, *Delia radicum*, Diptera, glucosinolates, oviposition stimulants, phytoalexins, thia-triaza-fluorenes.

Introduction

Glucosinolates occur in the Capparales, which comprise several commercially important crops and many wild species. When tissue is damaged, glucosinolates are hydrolysed by plant myrosinase enzymes to produce a range of volatile and nonvolatile products depending on plant genotype and environmental conditions (Mithen, 2001). The genetic control of the concentration of glucosinolates, as well as side-chain modification, is well understood from work on *Arabidopsis* and *Brassica* crops (Fahey *et al.*, 2001). Both in the laboratory and field, many glucosinolates, or their volatile degradation products, affect the behaviour of herbivores (Renwick, 2002). It is therefore practical to study constitutive and induced defences in a model plant such as *Arabidopsis thaliana* (L.) Heynh., which has the best-studied genome.

Both glucosinolate concentration and profile appear to affect plant–insect interactions. Generalist herbivores, such as molluscs, tend to prefer to feed on plants with low concentrations of glucosinolates (Moyes *et al.*, 2000). On the other hand, herbivores that specialize on crucifers tend to prefer to feed and lay eggs on plants with high concentrations of glucosinolates (Renwick, 2002). Despite the short life cycle and early flowering of *A. thaliana*, which may make it less apparent and less susceptible to insect attack than *Brassica napus* and other crucifer crops, many insects are willing to accept it as a suitable host. For example, Kliebenstein *et al.* (2002) demonstrated that, for the generalist *Trichoplusia ni*, *A. thaliana* herbivory is greatly deterred by higher glucosinolates levels whereas, for *Plutella xylostella*, herbivory is not correlated with variation in the glucosinolate–myrosinase system. This is in agreement with evolutionary theory indicating that specialist insects may overcome host-plant chemical defences whereas generalists are inhibited by these same defences (Ratzka *et al.*, 2002). In this context, Nielsen *et al.* (2001) compared a wild-type *A. thaliana* with a transgenic one that expresses the highly stimulatory glucosinolate sinalbin (Städler, 1978). Despite the four-fold increase in content of this glucosinolate, flea beetles (*Phyllotreta* spp.) did not discriminate between wild type (control) and transgenic plants, and the authors concluded that special emphasis should be given to the effect of variations in glucosinolate profiles as well as on other plant factors that modulate insect responses.

The induced defences of plants can readily be studied in *A. thaliana* because many genes are induced by mechanical wounding (Reymond & Farmer, 1998) and water stress contributes to the regulation of a large fraction of these genes. In this context, Reymond *et al.* (2000) compared the effects of mechanical wounding with damage by feeding larvae of the cabbage butterfly, *Pieris rapae*, and obtained very different transcript profiles, suggesting that the feeding

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strategy of *P. rapae* may minimize the activation of a subset of wound-inducible, defence-related genes.

Parasitoids of specialist herbivores also respond to high isothiocyanate concentrations (Reddy *et al.*, 2002). Recently, van Poecke *et al.* (2003) showed that *Arabidopsis* plants, when infested with a herbivore that damages leaf tissue, emit a volatile blend that attracts *Cotesia rubecula* females, and the wasps can only discriminate between a host and a nonhost herbivore when the type of damage caused is different (chewing vs. piercing). When *Arabidopsis* is infested with a herbivore that hardly damages leaf tissue, such as an aphid, the parasitoids do not respond. These results may be explained by differences in the amount and type of damage resulting from larval feeding and the relative importance of different signal-transduction pathways induced by different types of herbivores.

In recent years, many efforts have been made to find a relationship between chemical plant stimuli and host acceptance by the cabbage root fly, *Delia radicum* (L.) (Städler, 2002). The systematic analysis of leaf-surface extracts of crucifers that stimulate oviposition in the cabbage root fly reveals that they contain not only glucosinolates, but also other stimulatory compounds such as phytoalexins (Baur *et al.*, 1998) and other far more stimulatory substances (Roessingh *et al.*, 1992c; Roessingh *et al.*, 1997). In this context, Hurter *et al.* (1999) and De Jong *et al.* (2000) first isolated and identified the thia-triaza-fluorene compounds (i.e. cabbage identification factors, CIFs) that are structurally related to the phytoalexins (Fig. 1). These phytochemicals are present at very low concentrations. The quantity of the major CIF compound, CIF 1, on the leaf surface is estimated at approximately 1 ng g^{-1} on cabbage leaf (*Brassica oleracea*) (Roessingh *et al.*, 1997), and the total amount of CIF is therefore considerably less than the amount of glucosinolates present at 60 μg g^{-1} (Roessingh *et al.*, 1992b). Correspondingly, in the flies, contact chemoreceptor neurones in the $D_{3,4}$ sensilla chaetica (D-hairs) on the ventral side of the tarsi were found to be sensitive to glucosinolates (Roessingh *et al.*, 1992b), whereas the receptor neurones in the tarsal C_5 sensilla are extremely sensitive to CIFs, with a threshold in the order of 10^{-11} to 10^{-12} M (Hurter *et al.*, 1999).

As a specialist herbivore, *D. radicum* uses a small number of crucifers as natural host plants (De Jong *et al.*, 2000). By contrast to *P. rapae* (Yano & Ohsaki, 1993), *A. thaliana* is not a natural host plant for *D. radicum*, probably because of its small size and early appearance in the spring before flies emerge from overwintering pupae (Finch & Collier, 1983). It was therefore of interest to investigate *A. thaliana* acceptability as a potential oviposition site for this insect and, if acceptable, to test what factors might be involved.

Materials and methods

Insects

Delia radicum required for the bioassays were originally collected in Eastern Switzerland during the summer of 1996 and continuously reared in the laboratory according to the method of Finch & Coaker (1969). The adult flies (approximately 100 flies per cage, with a sex-ratio of 1 : 1) were held in cubic screen cages (65 × 65 × 65 cm) in a climate-controlled room (21 ± 1 °C, 80% RH and LD 16 : 8 h). Intact cabbage plants (*Brassica napus* CC-Cross F_1) at the prebolt stage, with a thin layer of fine grain-sized (diameter 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 LD 16 : 8 h. The flies were fed on a mixture of raw cane sugar, yeast hydrolysate and water (4 : 1 : 1) applied on absorbent tissue strips. Water and 10% sugar solution soaked into cotton wool were offered separately.

Plants

The seeds of *A. thaliana* ecotype Columbia were obtained from the Ecology Department of the University of Lausanne, Switzerland. Plants were grown in potting soil mixed with clay (Floragard, GmbH, Oldenburg, Germany) at a density of four plants per pot (diameter 9 cm), until the cotyledon stage and were then thinned to a density of one plant per pot. The seedlings were arranged in trays of 55 × 35 cm (20 pots per tray) and incubated in the dark for 3 days at 4 °C, before transfer to a controlled-environment growth room (20 ± 3 °C, 60 ± 5% RH). The plants were watered at intervals of 4 days and grown for approximately 9–10 weeks (rosette stage with 16–18 leaves and a diameter of approximately 35 mm) under short days (LD 10 : 14 h) to suppress premature induction of flowering and maximize the size of the rosettes used for plant extractions.

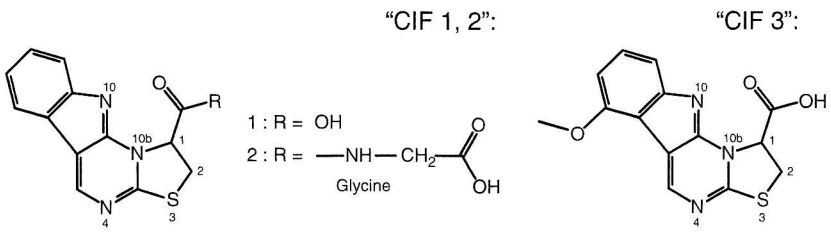

Fig. 1. Structure of 1,2-dehydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorene-carboxylic acids – cabbage identification factors CIF1, CIF2 and CIF3.

Chemical extraction and analysis

The extraction procedure described by Städler & Roessingh (1991) was used to obtain wax-free methanolic leaf-surface extracts of *A. thaliana*. Leaf-surface chemicals were extracted from plants at the 16–18 true leaf stage. Amounts and concentrations of samples were expressed in gram leaf equivalents (gle) and were sprayed on surrogate leaves. One gle represents the amount of leaf surface extract obtained by dipping 1 g of fresh leaf material in methanol.

The glucosinolate fractions of the extracts were separated from the fractions containing CIFs (De Jong *et al.*, 2000) using cation exchange resins (Baur *et al.*, 1996).

Preliminary oviposition choice assay with host and nonhost plants

Arabidopsis thaliana (16–18 true leaf stage) was compared with a nonhost plant (spider plant, *Chlorophytum comosum* (Thunb.) Liliaceae), in a dual choice assay, using the protocol below.

Oviposition choice assay with artificial leaves

Oviposition bioassays were conducted in the same cages as those described for the rearing, containing approximately 100 adult flies (sex ratio 1 : 1; 1–7-day-old flies). Eight surrogate leaves (Roessingh *et al.*, 1992b), four sprayed with 1 mL of *A. thaliana* plant extract containing 1 gle per side and four controls sprayed with 1 mL per side of pure methanol, were arranged alternately in a circle on the floor of the cage.

This pair-wise assay was replicated four times. After an oviposition period of 24 h, the eggs laid in the substrate under each artificial leaf were counted and expressed as a percentage of the total number of eggs laid within one bioassay period. Thus, the resulting percentage values for the compared treatments (*A. thaliana* extract vs. control) totalled 100%. After each egg count, new surrogate leaves were used and the positions were rotated clockwise to minimize any influence exerted by uneven light distribution. A Mann–Whitney *U*-test was performed to determine the significant differences in preference between treatments.

Electrophysiology

Electrophysiological recordings were obtained using the methodology of Roessingh *et al.* (1992b). Receptor neurones from 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 were stimulated. All the nerve action potentials (spikes) recorded were counted in the time interval from 50–1050 ms after contact of the recording electrode with the tip of the sensillum using spike train analysis software (Frazier & Hanson, 1986). The spikes were classified using the template matching method (Roessingh *et al.*, 1992a) or a manual amplitude size selection. The method was chosen according to the best result visualized in the regularity of the phasic-tonic firing pattern. A total of eight C_5 and 16 $D_{3,4}$ sensilla were investigated but preparations that gave less than 40 spikes with 10 ng ml^{-1} CIF1 or more than 20 spikes with KCl 10 mM in the first second of stimulation of C_5 sensillum were excluded. The plant extracts (CIF and glucosinolate fractions) were tested each at a concentration of 1 gle. The first compounds tested on each sensillum (C- and D-hairs) were KCl 10 mM and CIF1 (10 and 100 ng ml^{-1}), and these were used as standard for the recordings. The stimuli were applied in the following sequential order: KCl (10 mM, solvent), CIF1 (10 ng ml^{-1} KCl 10 mM, standard stimulus), *A. thaliana* extract-CIF fraction (1 gle), *A. thaliana* extract-glucosinolate fraction (1 gle) and glucobrassicin (10^{-5} M in 10 mM KCl, standard stimulus). Responses to extracts were expressed as the mean number of spikes recorded from each C_5 sensilla (CIF fractions) or $D_{3,4}$ sensilla (glucosinolate fractions).

Results and discussion

Preliminary oviposition choice assay with host and nonhost plants

The relative suitability for oviposition of *A. thaliana* was first confirmed by the preliminary bioassay ($n = 1$), were this plant was compared with a spider plant (*C. comosum*). The results showed a clear preference by the flies, which laid 641 eggs on *A. thaliana* compared to one on *C. comosum*. A comparison under normal rearing conditions resulted in between 400 and 600 eggs per cage with similar numbers of insects and time. However, when *A. thaliana* was

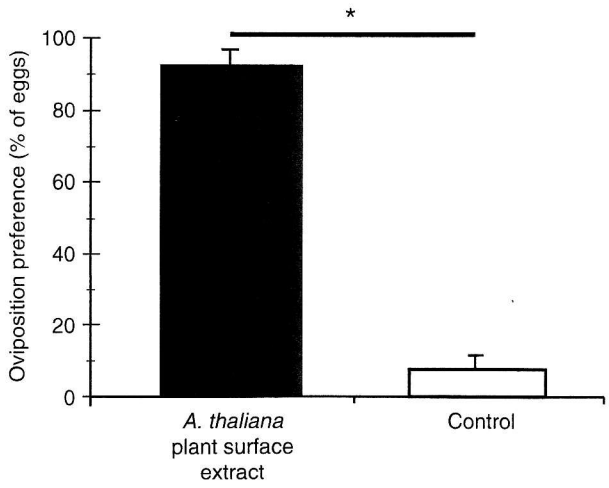

Fig. 2. Oviposition choice of *Delia radicum*. Percentage of eggs laid (mean ± SE) on artificial leaves sprayed with *Arabidopsis thaliana* plant extracts (1 gle) or pure methanol controls. Number of replicates = 4; total number of eggs laid = 632 vs. 38. *$P < 0.001$, Mann–Whitney *U*-test.

inoculated with three *D. radicum* larvae per plant at the same rosette-stage ($n = 5$), none survived. The small-sized roots were possibly either too thin or too toxic to allow larval mining.

Oviposition choice assay with artificial leaves

The mature females of *D. radicum* exhibited a clear oviposition preference for methanolic plant-surface extracts of *A. thaliana* (Fig. 2). The number of eggs laid was significantly greater (Mann–Whitney test, $n = 16$, $P < 0.001$) on artificial leaves sprayed with plant extracts than on artificial leaves sprayed with pure methanol (control). The clear preference for surrogate leaves treated with plant extracts (Fig. 2) suggests that the oviposition choice largely depends on methanol-extractable, polar chemical factors of the leaf surface.

Chemical analysis

Effective separation of the CIF from the glucosinolate fraction of *A. thaliana* plant extracts was achieved because chemical analysis showed that no glucosinolates were present in the CIF fraction (detection threshold: 1 μg glucosinolate per mL crude extract for alkenes, hydroxyalkenes, thioalkenes and benzyl-glucosinolate and 0.3 μg glucosinolate per mL crude extract for indolyl-glucosinolate).

Sensory physiology

D3, D4 sensilla (sensitive to glucosinolates, but not to CIF1). Figure 3 shows representative recordings obtained with the two fractions on the D_3 sensillum. The D_3 and D_4 sensilla are known to contain one neurone sensitive to glucosinolate and none to CIF-1 (Baur *et al.*, 1996). Both fractions induced clear phasic-tonic responses that were comparable to those obtained with pure 10^{-5} M glucobrassicin, which is the glucosinolate used as standard stimulus for the glucosinolate-sensitive neurones present in these two sensilla, whereas stimulations with 10 mM KCl induced very low activity (control) (Fig. 4). Stimulation with the glucosinolate fraction evoked spikes from additional neurones, with relatively small amplitudes and no clear phasic-tonic response. Similar to glucobrassicin, the glucosinolate fraction induced a phasic response. Within 200 ms, the sensory neurones were adapted to 40% of the starting rate.

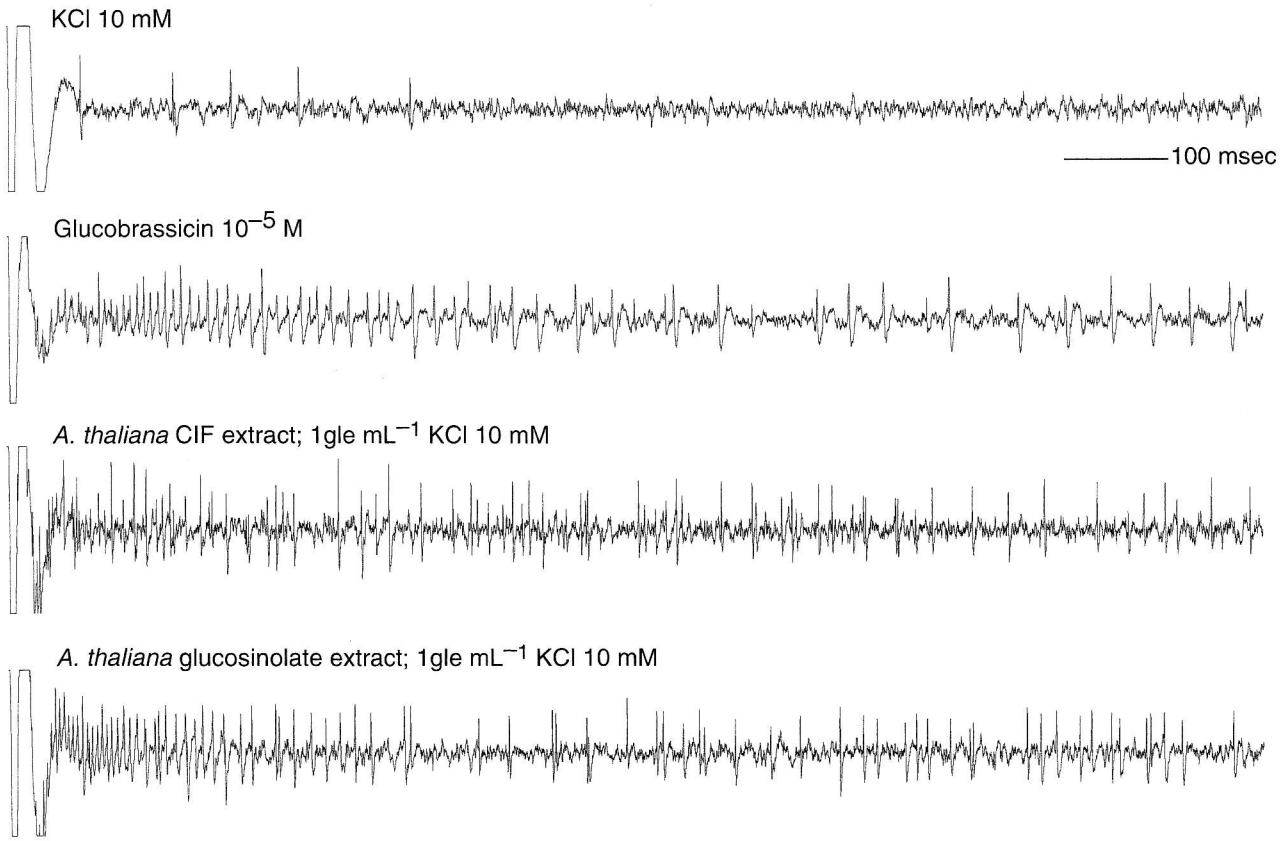

Fig. 3. Representative electrophysiological recordings from a D_3 sensillum stimulated with the glucosinolate fractions of *Arabidopsis thaliana* plant extract. All compounds were dissolved in KCl 10 mM.

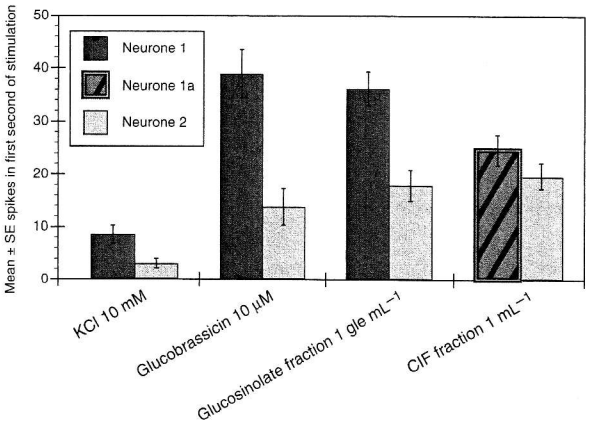

Fig. 4. $D_{3,4}$ tarsal sensilla neurones of *Delia radicum* stimulated by *Arabidopsis thaliana* plant extracts. The spikes were classified and counted in two classes of neurone 1 and 2 with the larger and smaller spike amplitude, respectively. The CIF fraction also stimulated two types of spikes, but this fraction contained no glucosinolates. The neurone producing the larger spikes was termed 'neurone 1a' (hatched column). Number analysed recordings = 16. Data are mean of spikes between 50–1050 ms after commencing stimulation.

Conversely, a more tonic pattern was obtained for the CIF fraction, where more than 1 s was necessary to reach the 40% level (approximately 45 spikes s^{-1}). Knowing that no glucosinolate contamination was detectable in the CIF-fraction and that no CIF sensitive neurones are present in these sensilla (Roessingh *et al.*, 1992c), it was assumed that other unidentified compounds responsible for the stimulatory activity are present in this fraction. The nature of these plant products and their role in insect behaviour remain to be determined.

C5 sensilla (sensitive to CIF1, glucosinolates and amino acids). As shown in Fig. 5, the chemosensory activity of the C_5-sensillum in response to the CIF fraction appeared to be stronger than that observed with the glucosinolate fraction of *A. thaliana* plant extracts. Control stimulation with 10 mM KCl induced very little activity compared with pure CIF-1, which, already at 10 ng mL^{-1}, induced on average more than 70 spikes s^{-1} in the C_5-sensillum (Fig. 6). To date, a total of four chemoreceptor neurones have been characterized in the C_5-sensillum (Isidoro *et al.*, 1994). One of these neurones is sensitive to the different CIFs, a second one to various glucosinolates (Roessingh *et al.*, 1997), a

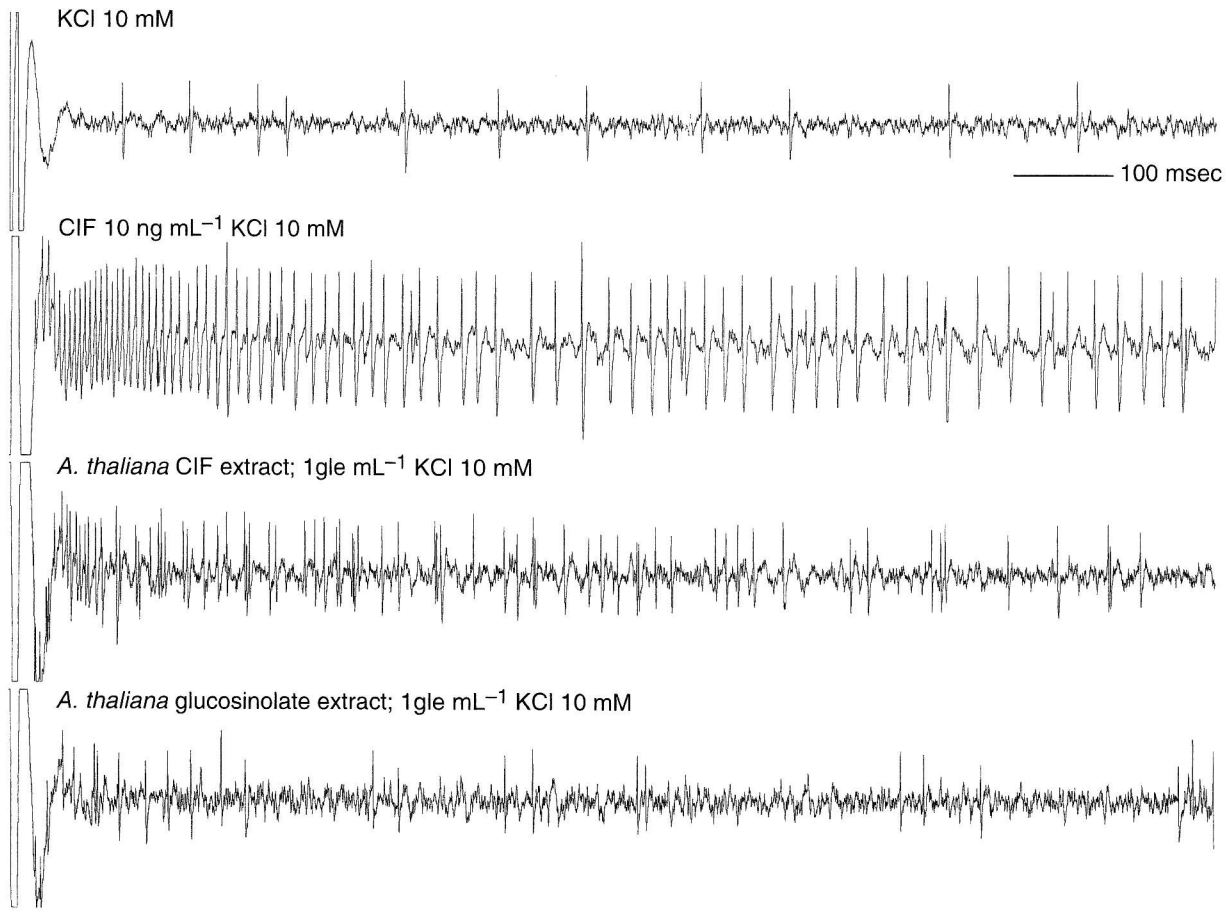

Fig. 5. Representative electrophysiological recordings from a C_5 sensillum stimulated with 1,2-dehydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorene-1-carboxylic acid (CIF1) fractions of *Arabidopsis thaliana* plant extract. All compounds were dissolved in KCl 10 mM.

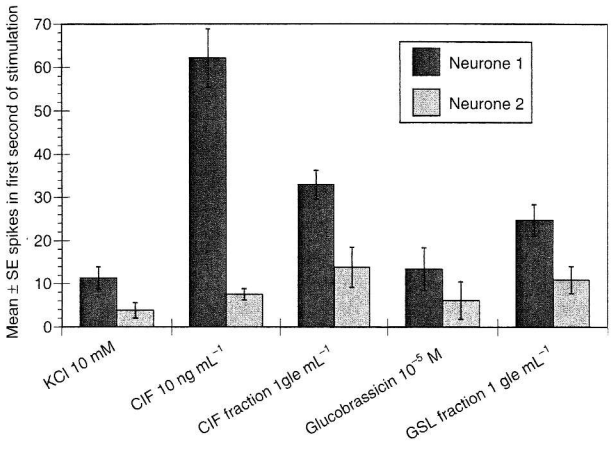

Fig. 6. C_5 tarsal sensilla neurones of *Delia radicum* stimulated by *Arabidopsis thaliana* plant extracts. The spikes were classified and counted in two classes of neurone 1 and 2 with the larger respective smaller spike amplitude. Number analysed recordings = 8. Data are mean of spikes between 50–1050 ms after commencing stimulation.

third to amino acids (Städler & Baur, unpublished observations), whereas the specificity of the remaining neurone remains unknown. Obviously, it cannot be concluded that all the activity of the C_5 sensillum neurones is linked to the presence of CIF in the stimulus solution because more than one neurone reacted to the CIF fraction (Fig. 6). Furthermore, the same fraction also exhibited some stimulatory activity in the D_3 and D_4 sensilla (Fig. 4, hatched column) that does not have CIF sensitive neurones. Because the experiments of De Jong *et al.* (2000) showed that purer CIF fractions release a stronger response in the CIF receptor neurone, the electrophysiological recordings could probably be improved with additional purification steps.

The data demonstrate that, in addition to glucosinolates, *A. thaliana* probably contains CIFs as oviposition stimulants. Further purification, or a high performance liquid chromatography–gas chromatography technique, is required to confirm the presence of CIFs and glucosinolates in the leaf surface of *A. thaliana* plants. Concerning the stimulatory properties of *A. thaliana* leaves, additional information is needed because other compounds present in the leaf extract may act as synergists or inhibitors and affect the activity of CIF and/or glucosinolate neurones. Indeed, responses to mixtures can differ widely from those to single compounds (Chapman, 2003).

In conclusion, this study provides the first evidence that compounds present on the leaf surface of *A. thaliana* stimulate oviposition in *D. radicum*. The earlier identified stimulants occurring in *Brassica* species, the thia-triaza-fluorene compounds (De Jong *et al.*, 2000) and the phytoalexins, such as methoxybrassinin, cyclobrassinin and brassitin (Baur *et al.*, 1998) are structurally related to the known phytoalexin camalexin (Pedras *et al.*, 1998) of *Arabidopsis*. This coincidence might not be accidental and may prove to be an interesting research lead in the future, especially because the cabbage root fly and its specialized receptor neurones are extremely sensitive to the thia-triaza-fluorene compounds.

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Part 5: General Discussion

14. Interactions between insects and plants

14.1 Insect-plant relationships in response to sulphur

Chapter 2 and 3 showed that the adults' oviposition choice and the larval feeding behaviour of two of the main crucifer pests, *Plutella xylostella* (Lepidoptera, Plutellidae) and *Delia radicum* (Diptera, Anthonyidae) are negatively affected by a lack of S in the plant nutrient regime. In crucifers, the S status of the plant is positively correlated with nutritional (proteins, flavour and pharmaceutical compounds) as well as morphological characters (colour, backing quality) (Schnug & Haneckl aus, 1994). Thus, a generally decreasing S supply will result in a loss of nutritive plant quality and this probably affects the oviposition choice of the pest and the larval performance. Reduction in S input will modulate pest attack as well as the palatability and nutritional quality for humans. Moreover, a decreased S availability will make plants more vulnerable to environmental stress factors and to generalist herbivores that do not normally pose a threat to crucifers. In this context, Foyer & Rennenberg (2000) showed that plants are able to deal with stress factors, such as xenobiotics and increasing ozone levels, mainly by increased glutathione metabolism, which is in turn related to the S supply of the plants (Schnug *et al.*, 1995). While the plant S supply through the air in Europe is continuously decreasing due to air pollution control of SO_2 exhaust fumes, environmental loads with xenobiotics are still high and atmospheric ozone concentrations are steadily increasing (Schnug, 1997). It is therefore important to note that our experiments (chapters 2 and 3) did not reveal any significant difference between normal- and S-rich fertilised plants, suggesting that when close to the optimal sulphur fertilisation level for rape, pest attack and multiplication rate will not further increase and affect the crop plants. Thus, with regard to *P. xylostella* and *D. radicum*, the application of fertilisers to rape doesn't need to be restricted.

14.2 The role of glucosinolates in plant-insect interactions

Plant secondary chemicals, such as glucosinolates (GSLs), are thought to be advantageous for specialists (e.g. host-recognition cues, feeding stimulants, defence against enemies) and deleterious for generalists (plant defence). Adult female insects use oviposition stimulants and avoid inhibitory compounds in order to choose plants where their offspring have a good chance of completing their development. In chapters 4, 5 and 6 we determined whether and how isothiocyanates attract and GSLs stimulate egg-laying and feeding of *P. xylostella* and *D. radicum*. Firstly, we showed a positive correlation between S-supply, GSLs (isothiocyanates) content and insect choice and damage. These findings are supported by previously published investigations relating the content of GSLs in the plant to the herbivory by *Pieris rapae* (Giamoustaris & Mithen, 1995), *Phyllotreta cruciferae*, *Plutella xylostella* (Siemens & Mitchell-Olds, 1996) and *Psylliodes chrysocephala* (Lambdon *et al.*, 1998) . We observed that insect preference may be slightly reduced at high GSLs concentrations (chapters 4 and 5). However, when GSLs concentration is not too elevated, various insects, including *Psylliodes* spp. (Pivnick *et al.*, 1992; Blight *et al.*, 1995, Isidoro *et al.*, 1998), *Ceutorhynchus* spp. (Bartlet *et al.*, 1993), *Dasineura brassicae* (Murchie *et al.*, 1997) and *Plutella xylostella* (Pivnick *et al.*, 1994; Renwick, 2002; Marazzi *et al.*, 2004) have been shown to be stimulated and attracted to volatile isothiocyanates. Moreover, insects like *Pieris rapae* , (Huang & Renwick, 1994; Städler *et al.*, 1995a) *Psylliodes chrysocephala* (Bartlet *et al.*, 1994), *Delia floralis* (Simmonds *et al.*, 1994) and *D. radicum* (Braven *et al.*, 1996) use GSLs as feeding and oviposition stimulants. However, in many of these insect-plant interactions, other compounds are also important and may act synergistically with GSL derivates. We observed that the diamondback moth and the cabbage root fly were able to discriminate between leaf extracts of plants supplied with sulphur nutrients (chapters 4 and 5). Our results support the view that stimulatory compounds, including volatiles accumulated in the boundary layer of the leaves as well as non-volatiles, are perceived by these two crucifer pests when they touch the plant surface. This concept is at the basis of the different acceptability shown by these pests for the three types of hosts (S_0, S_n and S_+ plants). We further observed that the two preferred host plants or treated surrogate leaves (S_n and S_+) in the oviposition choice contained greater amount of GSLs, suggesting that these compounds certainly play an important role in the plant acceptability. It appears, however, also from data published by Städler *et al*. (2002), that GSLs must act synergistically with other compounds present in the wax and/or boundary layer of the leaves, that remain to be identified. Investigating the olfactory responses of *P. xylostella* to leave extracts, we found that olfaction seems to be an important modality for this species in stimulating oviposition. Indeed, with increasing plant S-concentration, there was a rise in the stimulatory activity of the volatile compounds present in the headspace of the foliage extract, correlating with increased oviposition. Concerning *D. radicum*, we presented evidence that the concentration of CIF was also higher in the two preferred plants extracts S_n and S_+ than in S_0 plants. The outcome of electrophysiological recordings confirmed that both the GSLs and this non-GSL compound, CIF, which is known to be very relevant in antixenotic host-plant resistance (Baur *et al.*, 1996) were involved in *D. radicum* host acceptance. In this case, contact chemoreception seems to be the most important parameter in stimulating oviposition (Baur *et al.*, 1996; Städler, 2002), although the synergistic effect of volatiles (De Jong & Städler, 1999) cannot be discounted. As noted above, the final response of an insect in accepting or rejecting a particular plant is mediated by a balance of positive and negative sensory signals evoked by plant chemicals. Volatiles can aid the insect in finding host plants at short distance. Most Brassica insect specialists are attracted to isothiocynates, which derive from GSLs. However, when choosing an oviposition site also contact chemoreception is involved allowing the insects to discriminate between plant species that contain different kinds of GSLs (Chew & Renwick, 1995; Ebkom, 1998). Thus modifications in the GSLs chain structure have important effects in the oviposition choice. Minor chemical alterations can have major effects on the physiochemical properties of the products (Mithen, 2001b). The isothiocyanates of short-chain aliphatic GSLs may be important as precursors to volatiles used in attraction, whereas indolyl GSLs serve as stimulants for egg-laying (Giamoustaris & Mithen, 1995). Our results regarding the GSL content of the three *B. napus* plant extracts indicated that S deficiency reduced the biosynthesis of alkenes and thioalkyl GSLs. Since S-deficiency correlated with decreased oviposition, alkenes and thioalkyl GSLs seem to trigger most of this stimulatory activity. Furthermore, Giamoustaris

& Mithen (1995), Griffiths *et al.* (2001) and Städler *et al.* (2002) reported that for crucifer specialists the composition of plant GSL profiles may be more important than the total GSL concentration in mediating plant-insect interaction, and this may be particularly true when S is the limiting factor.

Finally, with regard to the effects of environmental factors on secondary metabolism, it should not be forgotten that substantial differences were observed between crop plants grown in the field and in greenhouses. The GSL content of greenhouse-grown cabbage plants reached only 10% of the levels measured in plants grown in the field (Cole, 1994). The opposite was the case, however, for tomato plants, in which the alkaloid content of greenhouse-grown plants was two to four times higher than in the field (Barbour & Kennedy, 1991). Lacking further information about the factors causing such significant differences, these examples simply show that environmental conditions may have a considerable physiological effect on the plant influencing also its allocation of resources into secondary metabolites. We should therefore be cautious in extrapolating results from greenhouse experiments to insect-plant relations in natural situations.

14.3 Human nutrition and anticarcinogenic activity

Dietary phyto-nutrients found in vegetables and fruits appear to lower the risk of cancer and cardiovascular diseases (Kaur & Kapoor, 2001). Studies on the mechanisms of chemoprotection have focused on the biological activity of plant-based phenols and polyphenols, flavonoids, isoflavones, terpenes and GSLs. In this context, the presence of GSLs in horticultural cruciferous crops is important both for flavour and potential anticarcinogenic activity. GSLs importance as flavour compounds is well established (Fahey *et al.*, 2001). Isothiocyanates provide the characteristic hot and pungent flavours of many of our cruciferous salad crops, and these and other degradation products are important flavour components of cooked cruciferous vegetable. The contribution of GSLs to flavour is, however, complex: bitterness in some cultivars of Brussel sprouts is possibly due to levels of progoitrin (2-hydroxy-3-butenyl GSL) and gluconapin (3-butenyl GSL), but the direct implication of each compound is difficult to elucidate (Fenwick *et al.*, 1983).

Of special interest has been the potential anticarcinogenic activity of GSL degradation products (Verhoeven *et al.*, 1996; Talalay, 1999). Ingestion of approximately two servings per day of cruciferous vegetables may result in as much as a 50% reduction in the relative risk for specific cancers (Fahey *et al.*, 2001). At least some of the cancer chemoprotective activity of these vegetables (reviewed by Thornalley, 2002) is widely believed to be due to their content of minor dietary components such as GSLs (Mithen, 2001a). For instance, Zhang *et al*. (1994) reported on the anticarcinogenic activities of sulphoraphane and synthetic cyclic isothiocyanate analogues in blocking mammary tumor development in rats. Similarly, numerous studies have indicated that the hydrolytic products of at least five GSLs, including 4-methylsulfinylbutyl (glucoraphanin), 2-phenylethyl (gluconasturtiin), 3-indolylmethyl (glucobrassicin), 4-methylsulphinyl-3-butenyl (sulphoraphane) and 2-propenyl (sinigrin), have anticarcinogenic activity in humans. As demonstrated in chapter 4, the lack of S dramatically affected the GSL content of the plants. Mainly, the abundance of alkenes and thioalkyl was increased, which included also the abovementioned chemopreventive sinigrin and sulphoraphane, suggesting a potential additional interest in modulating S fertilisation.

However, the greatest proportional difference between free-S and S-containing plant extracts was found with gluconapin (alkene), whose protective activity remains to be determined.

The presence of degradation products is, however, not always beneficial. Progoitrin, for instance, is responsible for goitre, the pathological enlargement of the thyroid gland (Griffiths *et al.*, 1998). It appears that high doses of GSLs have detrimental effects on both health and productivity (Chen & Andreasson, 2001). The exact magnitude and nature of these effects may depend on the type of GSLs present in the breakdown products finally encountered in the intestines and subsequently absorbed. Therefore, there is a strong interest in altering levels of specific GSLs in crop plants since certain GSLs have desirable (cancer chemoprotection) and others undesirable (goitrogenicity of rapeseed) properties (Fahey & Stephenson, 1999) .

Traditionally, the presence of anti-nutritional factors in commercial Brassica crops was considered a disadvantage and rape seeds had to be treated to destroy the toxic GSLs (Griffiths *et al.*, 1998). However, as increasing data concerning S-containing metabolites accumulates, their importance in flavour and as natural plant protectants is gradually being appreciated (Chen & Andreasson, 2001). Although the biological significance of these compounds both as plant protectants and as potential anticarcinogens remains unclear, their complete removal from seed, vegetable or fodder Brassicas can no longer be accepted as a valid breeding objective. The diversity of GSLs found among the Brassicaceae may be a distinct advantage allowing both traditional and genetic engineering techniques to be developed and used to produce, for example, vegetable cultivars with improved flavours and health-promoting characteristics (Mithen, 2001a).

14.4 *B. napus* used as green manure

The traditional use of crucifers as green manures is partly based upon the toxic nature of the GSL degradation products (mainly isothiocyanates), which serve to reduce the soil inoculum of pathogens and pests for subsequent horticultural crops. This practice is receiving renewed interest following the banning of certain soil fumigants. Moreover, it has recently been observed that the yield of wheat is enhanced following oilseed Brassica crops (Kirkegaard *et al.*, 1994). This is thought to be due to the reduction in soil borne diseases, notably “take-all”, due to the release of phenylethyl isothiocyanate from the roots of the Brassica crop, a process which has become known as biofumigation (Morra & Kirkegaard, 2002). Taking into account the results of this thesis indicating that the amount as well as the profile of GSLs released by *B. napus* are modified by S fertilisation, it can be expected that the efficiency of *B. napus* as green manures can vary depending on the plant S supply.

15. Implications and perspectives

This thesis focused on fundamental aspects of the relationship between two crucifer pests and their host-plant. The very fact that this work dealt with insect that damage economically important vegetable crops suggests that some of our results may be of practical relevance in pest control. In agronomy, various types of fertiliser are available to cover the S demand of crops (Scherer, 2001), and we have shown that even S-rich fertilisation does not affect, in the ranges tested, the undesirable *P. xylostella* and *D. radicum* multiplication. As a practical consequence, this means that growers can apply a level of S as high as necessary to guarantee an optimal yield without worrying too much about enhanced *P. xylostella*

In the last decade there has been considerable progress in the identification of the major factors that cause antixenotic or antibiotic resistance to phytophagous insects (Sharma & Ortiz, 2002). Concerning the oilseed rape, sources of antixenosis and non-preference, but not antibiosis have been identified for flea beetles (Palaniswamy *et al.*, 1997). Antibiosis, which reduces pest density, is particularly useful if the target crop is a major host of the pest, as is the case with oilseed rape (Gavoski *et al.*, 2000). However, research has rarely advanced to a point where the knowledge of the resistance mechanisms can be directly applied. Concerning the role of GSLs in oilseed rape, additional information on the genetic control of S-containing metabolite concentration and on the enzymology involved in their biosynthesis is still required to apply both the traditional plant breeding and the more modern techniques of bioengineering to alter metabolite content. In the near future, it is likely that new genes cloned from *A. thaliana* will be used to modify the GSL content of Brassica. From an ecological point of view, variation in resistance factors, including an increased production of allelochemicals, should be monitored to detect seasonal and environmental differences that could affect levels of herbivory. The positive statement that predators or parasitoids are also attracted by such indirect plant defences is important for applications in agriculture. A clear understanding of the mechanisms involved in adaptation to plant defences will be useful in order to identify insects that could be used for biological control involving the multitrophic systems.

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Curriculum vitae

Personal Data

Name:	Cristina Eliana Marazzi
Date and Place of Birth:	06 August 1968, Sorengo (TI)
Nationality:	Swiss
Languages:	Italian, French, English, German, Spanish

Education

1986-1990	High School of Lugano, Switzerland. High School scientific graduation
1990-1993	Medicine studies, University of Bern, Switzerland
1993-1998	Studies in Biology (Zoology and Botany) at the University of Lausanne, Switzerland
1999	Master in Biology, University of Lausanne, Switzerland
1999	Post grade in "Systematics and management of biodiversity", University of Lausanne, Switzerland
2000 – 2003	PhD thesis in biology (Zoology) at the Swiss Federal Research Station for fruit-growing, viticulture and horticulture of Wädenswil, Switzerland
December 2003	Doctor degree in Biology, University of Basel, Switzerland

Research Experience

1994-1995	Research training and field work at the Associazione dei Comuni Regione Malcantone, CH-6982 Agno (TI), supervisor : Engineer D. Ryser. **Theme:** Management of a riverside forest in the southern part of Switzerland.
1997	Fieldwork at the Department of Botany, Systematic and Geobotanic (IBSG), University of Lausanne, supervisor: Prof. P. Hainard. **Theme:** Plant re-colonisation of an alluvial zone in western Switzerland.
1998	Research work at the Department of Animal Ecology (L.E.A.E.), University of Neuchâtel, supervisor: Prof. M. Rowell-Rahier. **Theme:** Phytophagous insect performance on different host plants.
1998	Fieldwork at the Department of Eco – ethology of Vertebrates, University of Neuchâtel, supervisor: Prof. C. Mermod. **Theme:** Migrations, juvenile's survival and annual census of *Capra ibex* in the Swiss Alps.
1998 - 1999	Master work at the Department of Zoology and Animal Ecology (IZEA), University of Lausanne, supervisor: Prof. P. Vogel. **Theme:** Evaluation of the beaver's (*Castor fiber*) habitat in Switzerland.
1999 - 2000	Training period at the Department of Animal Ecology (L.E.A.E.). University of Neuchâtel, supervisor: Dr Ted Turlings. **Theme:** Effects of transgenic maize on the relationships between the maize parasite *Spodoptera littoralis* and its parasitoide *Microplitis rufiventris*.
2000 – 2003	PhD thesis (COST-Action 829 project) at the Swiss Federal Research Station for fruit-growing, viticulture and horticulture of Wädenswil. Supervisors: - PD Dr E. Städler, Swiss Federal Research Station for fruit-growing, viticulture and horticulture, Wädenswil - Prof. Dr B. Baur, University of Basel **Theme:** Plant sulphur nutrition influencing host-plant selection and performance of insect herbivores.

2003	Scientific collaboration with the laboratory of horticulture, Zoology Department, Swiss Federal Research Station for fruit-growing, viticulture and horticulture, Wädenswil. Supervisor: Dr R. Baur. **Theme:** Optimisation of the pheromone blend for *Contarinia nasturtii*, one of the major crucifer pests: laboratory tests (behavioural assays, wind tunnel) and field tests (specific pheromone traps).
2004	Scientific collaboration with the phytosanitary office of Canton Ticino, Department of Agriculture, Republic and Canton Ticino, Bellinzona. Supervisor: Ing. G. De Giorgi. **Theme:** Monitoring of epiphytic *Erwinia amylovora* and the incidence of fire blight in southern Switzerland (Ticino).

Continuing education

Lectures followed within the PhD framework at the University of Basel :

- « Physiologie und Verhalten der Insekten »
  PD Dr E. Städler, Eidg. Forschungsanstalt, Wädenswil (winter semester 2000/01)
- « Naturschutzbiologie »
  Prof. Dr B. Baur, PD Dr A. Erhardt (sommer semester 2000/01)
- « Modellbildung, Systemanalyse und Computersimulation in der Ökologie »
  Dr S. Zschokke (winter semester 2001/02)
- « Seminar über neue Literatur auf dem Gebiet der Naturschutzbiologie »
  Prof. Dr B. Baur (winter semesters 2001/02 and 2002/03)
- « Seminar über neue Forschungergebnisse und Methoden in der Naturschutzbiologie »
  Prof. Dr B. Baur (sommer semester 2001 - winter semester 2004)

Lectures followed within the PhD framework at the ETH of Zürich:

- Seminar in Angewandte Entomologie, Prof. S. Dorn (sommer semester 2001)
- Technical English : fundamentals 1 and communication skills, Dr J. Guess (sommer semester 2001)
- First Certificate in English, Prof. A. Brechbühl (4 semesters)
- Advanced Certificate in English, Prof. P. Kelly (winter semester 2002/03)

Professional Experience

1996 - 2000	Old people' home « Les Châtaigniers », Geneva Nurse assistant during the weekends
1997	Dept. of Botany, Systematic and Geobotanic, University of Lausanne Teaching assistant, practical course in botany
1997	Dept. of Animal Biology, University of Lausanne Teaching assistant, practical course in zoology
1999 - 2000	Botany garden, University of Neuchâtel, Visitors' guide
Since June 2000	Old people' home « Altersheim Fuhr», Wädenswil Nurse assistant during the weekends

Publications

1. **Marazzi Cristina E., Patrian Bruno and Städler Erich**. (2004). Secondary metabolites of the leaf surface influenced by sulphur fertilisation and perceived by the diamondback moth. Chemoecology 14: 81-86.

2. **Marazzi Cristina E., Patrian Bruno and Städler Erich**. (2004). Secondary metabolites of the leaf surface influenced by sulphur fertilisation and perceived by the cabbage root fly. Chemoecology 14: 87-94.

3. **Marazzi Cristina E. and Städler Erich**. (2004). Influence of sulphur plant nutrition on oviposition and larval performance: of the diamondback moth. Entomol. Exp. Appl.111: 225-232 .

4. **Marazzi Cristina E. and Städler Erich**. (2004). *Arabidopsis thaliana* (L.) leaf surface compounds that stimulate oviposition and are perceived by the cabbage root fly. Physiological Entomology 29: 192-198.

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6. **Dubuis Pierre-Henri, Cristina E. Marazzi, Erich Städler and Felix Mauch**. Sulphur deficiency causes a reduction in antimicrobial potential and leads to increased disease susceptibility of oilseed rape. Journal of Phytopathology, (submitted).