Host Selection and Antifeedants in *Hylobius abietis* Pine Weevils

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Abstract

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We searched for antifeedant activity in predominantly non-host woody plants to find new compounds for seedling protection of Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) against feeding by pine weevil *Hylobius abietis*. In total, 38 species was tested in an assay where the insects are let to feed on the bark on a short stump of wood. Among the best candidates was linden (*Tilia cordata*). The bark was extracted and fractioned. Two fractions showed antifeedant activity in a micro feeding assay. Nonanoic acid was identified in both of these fractions. Subsequent testing in the micro feeding assay showed that nonanoic acid possessed strong antifeedant activity against *H. abietis* adults. Structure-activity and possible formulations for field application of nonanoic acid was investigated in laboratory and field by variation of the nonanoic acid molecular theme. Alkanoic acids of varying chain length from C6 to C13 and corresponding branched acids methyl-substituted in 2-position was tested. Derivatives of higher and lower volatility, restricted to straight chain C9, included 1-nonanol, nonanal, sodium nonanoate, and nonanoic anhydride were also tested. The C7 to C10 chain-length straight-chain acids were all active. Branched acids were active at +1 C higher molecular mass. Phytotoxic side effects occurred when nonanoic acid was tested in field, but an inactive wax layer, applied on the stem before the formulation, reduced the side effects. Bark extract from ten different tree species, which were earlier identified as non-preferred by the pine weevil were tested. One active extract was further fractionated. The content of active and inactive extracts was investigated by GC-MS.

Key words: feeding deterrent, *Hylobius abietis, Picea abies, Pinus sylvestris*, outer bark, phloem, pine weevil, seedling damage. Antifeedant, Curculionidae, Coleoptera, deterrent, extract, fractionation, linden, pelargonic acid, phloem, soxhlet, Tilia cordata, structure activity, formulation, , capric, heptanoic, pelargonic, octanoic, nonanoic, decanoic acid.

Contents

Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Månsson, P. E., and Schlyter, F. 2004. *Hylobius* pine weevils adult host selection and antifeedants: feeding behaviour on host and non-host woody Scandinavian plants. *Agric. Forest Entomol.* 6: 165-171.

II. Månsson, P. E., Eriksson, C., and Sjödin, K. 2005. Antifeedants against *Hylobius abietis* Pine Weevils: An active compound in extract from bark of *Tilia cordata* Linden. *J. Chem. Ecol.* 31: 000-000 (In print).

III. Månsson, Per E., Schlyter, F., Eriksson, C., 2005. Structure-activity and field performance of nonanoic acid and other long-chained carboxylic acids as antifeedants in *Hylobius abietis* pine weevils. *Manuscript for Entomologia Experimentalis & Applicata.*

IV. Eriksson, C., Månsson, P. E., Sjödin, K and Schlyter, F. 2005. Aliphatic acids and other antifeedants in non-host woody species of the pine weevil, *Hylobius abietis*. *Manuscript*

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Introduction

Behaviour-modifying compounds: From allelochemicals to antifeedants

Host plant selection

All phytophagous insects are to some extent selective in their choice of host plant for feeding, survival, and development. Selection does not only involve finding an appropriate plant habitat and species, but also the most suitable individual or plant. To select a host-plant, the insect must first be able to detect and locate it from a distance and thereafter confirm the quality of it. Plant chemicals are of great importance for host-plant selection and are classified according to their effects on insect behaviour (Dethier, et al. 1961). Attractants and repellents make the insect move towards or away from the plant, as they affect insects at a distance from the plant. Feeding/oviposition stimulants and deterrents elicit or inhibit feeding or oviposition when the insect has reached contact with plant tissue. This classification is functional and based on the biology of each insect. A chemical that functions as an attractant to one insect species may be a repellent to another. The concentration of a chemical may also affect insect behaviour: an attractant may function as a repellent when the concentration exceeds a certain threshold (Bernays and Chapman 1994). When a plant is accepted, food intake is mainly controlled by the detection of chemical stimuli. In general, primary metabolites (nutrients) stimulate feeding while secondary metabolites (non-nutrients) do not. However, some secondary metabolites are of great importance in stimulating feeding and are then termed “token stimuli” (Steiner 1984).

Definitions

Plant chemicals that changes insect feeding behaviour are a part of the allelochemicals, and are classified after their function in insect/plant interactions (Fig. 1). An allelochemical is defined as a compound used as a communication signal between different species, as between insects and plants. Terms for several kinds of allelochemicals have been coined (Box 1):

Box 1. Allelochemicals

Allomones are advantageous to the sender but not to the receiver, e.g. compounds repelling and deterring feeding of herbivorous insects (Brown 1968).

Kairomones are advantageous to the receiver but not to the sender, e.g. plant compounds attracting herbivores (Whittaker and Feeny 1971).

Synomones are advantageous to both sender and receiver, e.g. attraction of herbivores but also herbivore predators and parasites (Vet, et al. 1990).

Among allomones there are compounds that reduce or deter feeding. They are called feeding inhibitors and are subdivided after their mode of action on the herbivore when in contact (Frazier and Chyb 1995):

Box 2. Feeding inhibitors

Preingestive inhibitors affect insect orientation, searching, and host-plant selection. Those inhibitors reaches, within a few seconds or minutes, the olfactory or gustatory contact chemosensillas of an insect searching for a feeding site. A signal to the Central Nervous System (CNS) causes avoidance from further approach or feeding. Antifeedants fall under this division according to the definition used by me, see section "Antifeedants in the literature".

Ingestive inhibitors affect salivary enzymes or musculature in head, oesophagus, and foregut. The action, release or synthesis of salivary enzymes becomes blocked and the function of the food transport muscles may be disabled.

Postingestive inhibitors affect insects after feeding by blocking the physiological mechanisms involved in food storage, digestion and absorption. There are three subdivisions of postingestive inhibitors: (1) Digestive inhibitors block synthesis, release and action of digestive enzymes. (2) Feedback inhibitors disturbs the operation of the alimentary canal and, finally (3) processing inhibitors affects the interneurons of the CNS processing area so that the positive feeding signal reaches CNS intact, but the decision-making process may be disturbed.

After the orientation and host selection phase, insect food ingestion process is followed by storage in the initial portion of the alimentary canal. Digestion and absorption takes place in the mid gut, with an entry of the nutrients into the metabolic cycles that is completed with excretion of waste and undigested material (Fig. 2). Different feeding inhibitors cause different changes in the physiological mechanisms operating at each level (Box 2). A preingestive inhibitor that affects f. ex. chemosensory cells in sensillas on antennae and tarsi causes reduced palpation while a postingestive inhibitor targeting midgut muscles may cause gut movements. A reaction caused by preingestive inhibition is furthermore usually a rapid reaction while postingestive inhibition reactions take longer time and produce more chronic effects.

Antifeedants in the literature

I have found several different definitions of the term "antifeedant" in the literature. The most accepted is presented and compared to achieve an understanding of the mode of action of these compounds:

Chemical antifeedants are, according to (Munakata 1975) chemicals that inhibit feeding but do not kill the insect directly. However, the insect may remain close to the plant but will die from starvation or dehydration rather than feeding from it. Munakata also uses the terms "feeding deterrent" and "gustatory repellent".

Antifeedants are not identical with olfactory repellents where volatile compounds repel insects before they start to feed. (Frazier and Chyb 1995) described antifeedants as a class of preingestive compounds affecting gustatory receptors and evoke rejection of plant material. (Norris 1986) defined an antifeedant as a substance that prevents or reduces feeding and that varies in volatility. Insects may contact it at sites distant from the site of chemical release. If it not only prevents feeding, but also reduces contact between plant and insect it can also be termed repellent. Norris focused on the description of volatile antifeedants but did not exclude post-feeding effects. (Messchendorp 1998) described antifeedants as compounds that inhibit feeding by sensory perception i.e. giving plant material an unpalatable taste (Jermy 1966; Wright 1967; Chapman 1974) but may also reduce feeding by toxic, postingestive effects (Berenbaum 1986; Mordue and Blackwell 1993; Frazier and Chyb 1995; Glendinning 1996). By Messchendorp, the perception of antifeedants is only described as gustation (not by olfaction), i.e. volatiles are excluded. To conclude, the definition of the term "antifeedant" varies widely.

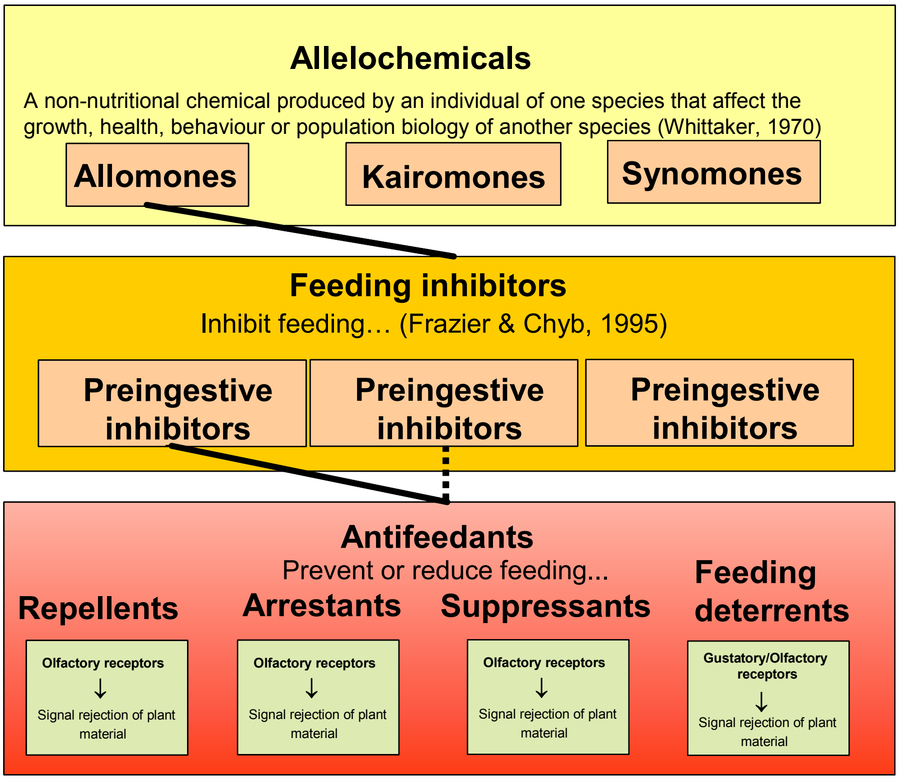

Fig. 1. Definition of the term antifeedant. Antifeedants or feeding deterrents are in this thesis classified as a subdivision of preingestive inhibitory signals causing four different types of rejection behaviours when in contact with plant material. Olfactory or gustatory receptors on tarsi, mouthparts or antennae receives plant compounds and signal rejection.

My definition of an antifeedant

Figure 1 classifies my definition of antifeedants and how the term will be used in this project. Volatile and non-volatile preingestive inhibitors will be regarded as antifeedant compounds. The reason to not include postingestive inhibitors in the antifeedant concept is that these inhibitors demands feeding during a longer period

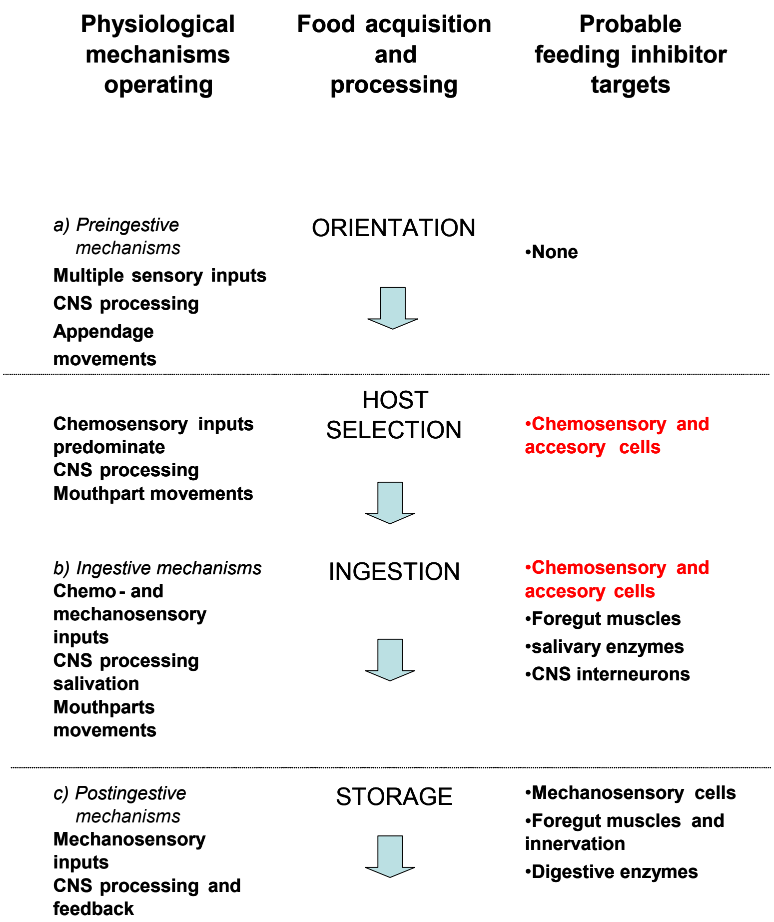

Fig. 2. Stages of food acquisition and processing in insects. Orientation is followed by host selection where chemosensory and accessory cells are the probable target cells. Antifeedants are classified as deterrents in the first, preingestive stage. However, the ingestive stage is regarded as a borderline case as the same cells are involved (Frazier & Chyb, 1995).

than preingestive inhibitors, which may already have caused significant and possibly mortal damage to the plant, when the insect finishes feeding. Such compounds may as well be directly toxic and could be considered insecticidal. The use of such plant derived compounds may be a good tactic in cases where each insect only cause minor damage on each plant, but in this project, concerning pine weevil feeding on spruce and pine saplings, one single meal causes severe damage.

However, ingestive inhibitors may be regarded as a borderline case as chemosensory and accessory cells are involved in both preingestive and ingestive inhibition (Fig. 2; Frazier & Chyb, 1995).

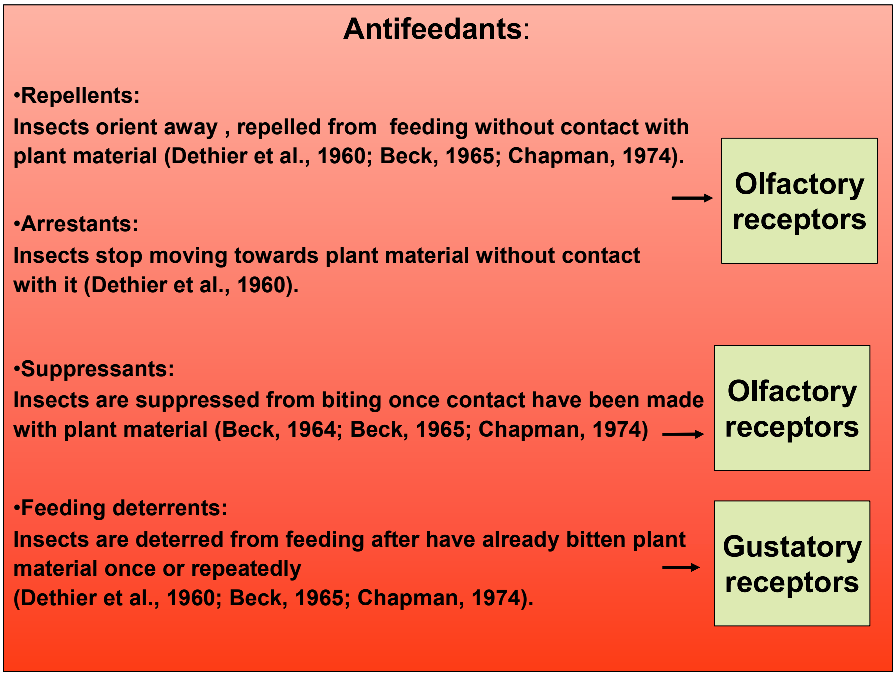

Fig. 3. The four subdivisions of Antifeedants classified on insect behaviour.

Antifeedant activity will accordingly be regarded as inhibition by olfactory and gustatory responses and not by inhibition of a later stage in insect feeding and metabolism. I will consider not only absolute antifeedants where the insect must rather die from starvation than feed from the plant, but also relative antifeedants, i.e. insects may repeatedly begin feeding but will be suppressed or deterred shortly after (Klocke, et al. 1989).

Active compounds

The actions of antifeedant compounds are commonly effective only against some particular insect species. Thus, drawing general conclusions about the relationship between antifeedant activity and chemical structure is difficult. However, there is a general rule of behavioural effect if compounds within the same group of preingestive inhibitors are compared. Compounds known as insect antifeedants usually have a more oxidised or unsaturated structure than compounds stimulating feeding. Many oxidising agents are antifeedants to one or several insects. The high antifeedant activities of the compound juglone against numerous insect species and of warburganal against *Spodoptera exempta* are examples of similar structural functions. Their oxidative characters are the key function of their antifeedant

Table 1. A selection of secondary metabolites and their feeding deterrency to insects (Frazier & Chyb, 1995).

Chemical class	**Estimated number of known structures**	**Example**
Acetylenes	750	Dihydromatricaria
Alkaloids	4500	Nicotine
Amino acids	250	Canavanine
Carotenoids	300	Fuxoxanthin
Coumarins	150	Coumarin
Cyanogenic glycisides	50	p-Hydroxymandelonitrile-glucoside
Flavonoids	1200	Quercetin
Glucosinolates	80	Sinigrin
Lignins	50	Excelsin
Phenolic acids	100	p-Hydrobenzoic acid
Quinones	200	Juglone
Terpenes	1100	Glaucolide-A

properties. Another example of this rule is the redox couple *p*-Hydroquinone and *p*-Benzoquinone, and their effect on *Spodoptera multistriatus* (Norris 1970). *p*-Benzoquinone is more unsaturated than *p*-Hydroquinone, and *p*- Benzoquinone is an antifeedant to *S. multistriatus* while *p*-Hydroquinone acts as a feeding

stimulant. However, molecular size and shape as well as functional group stereochemistry (Kubo and Ganjian 1981) also affect the antifeedant activity of a molecule.
Secondary metabolites are considered as the major cues used by insects in their rejection of a specific host plant or plant tissue. Plants may contain not only a few but complex mixtures of secondary metabolites. A possible explanation for this complexity might be that the components of the mixture act as synergists, as for example caryophyllene oxide and gossypol do in cotton (Gershenzon and Croteau 1991). Caryophyllene synergizes the growth-inhibiting effects of gossypol in the tobacco budworm *H. virescens* (Gunasena, et al. 1988). In the list of compounds (Table 1) the major chemical groups, alkaloids, flavoniods and terpenes seem to be the dominant compounds by their great number. However, less numerous classes like the quinones (e.g. Juglone) are important as well, as they deter a broad array of insect species.

Advantages and limitations of feeding inhibitors in insect control.

Feeding inhibitors have several advantages in plant protection, compared to traditional chemical methods. The host choice of generalists and to some extent, specialists may be modified when inhibitors are used. If an insect species can feed on other plants than its targeted host, it can be easier to direct away than if it is highly specialised in one host. The range of insect species targeted may be chosen by either the chemical structure of the inhibitor or by the composition of a mixture of inhibitors, if different inhibitors are active against different species within the range.
Most feeding inhibitors are less stable chemicals than traditional insecticides and will, in the case with preingestive inhibitors, act more rapidly on the insects but with lower residual activity and environmental impact. However, the inhibitor used must not be degraded too rapidly and must also not degrade to harmful residues.
Natural predators and parasites may remain unharmed by inhibitors targeting the herbivorous host insects by using feeding inhibitors (Beckage, et al. 1988). Feeding inhibition may also be achieved by combining bait containing strong attractants and a toxin to out compete the crop plants as feeding or mating sites for insects. That method could in some cases divert the attack from the crop (Fleischer and Kirk 1994). As the target sites of inhibitors are different from traditional pesticides, pesticide-resistant insect populations will still be affected by feeding inhibitors. Multiple tactics, i.e. varying between pesticides and inhibitors will also slow down the resistance development to both pesticides and to these new compounds (Holloway and McCaffery 1988). However, inhibitors used without being combined with pesticides will still reduce risks of resistance development: Because of inhibited feeding, there will be several mortality factors in insect populations, e.g. starvation and reduced resistance to diseases is obtained without using toxic compounds (Griffiths 1990).

There are some drawbacks and limitations in this new pest management area. Feeding inhibitors may affect non-target species. Some inhibitors may affect higher animals and humans (Frazier and Chyb 1995(Mullin, et al. 1994) as well as beneficial insects depending on dose or composition (Mordue and Blackwell 1993). Even if inhibitors are a better choice than pesticides, habituation may still occur after a time and limit the results in the field (Simmonds and Blaney 1984; Raffa and Frazier 1988).

More efficiency studies of inhibitors have to be performed before any general results of their advantages and drawbacks can be documented. Mode of delivery, formulations, combinations and timing are so far not tested to give clear outlines of proper feeding inhibitor use in general.

Behavioural effects of antifeedants on insects

Studies of antifeedant effect on insect performance have principally been conducted from two points of view, with focus on either behaviour or chemistry. In the first case feeding inhibition of essential oils or plant material has been evaluated by recording changes in insect behaviour. In the second case, a new chemical analogue of an already known feeding inhibitor has been synthesized. Thereafter, the effects on insect performance have been studied.

However, the actual chemosensory regulation of insect feeding behaviour has been left out in both cases. One exception is the Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) that is well examined according to its physical reactions on feeding inhibitors. One group of alkaloids called imino sugars or polyhydroxy alkaloids (PHAs) mimic the structure of simple sugars and have the ability to inhibit glycosidases (Hartmann 1991). They function as feeding inhibitors to certain insect species. One PHA, 2-R,5-R-dihydroxymethyl-3-R,4-R-dihydroxypyrrolidine (DMDP) isolated from *Derris elliptica* Bentham (Welter, et al. 1976) act as an antifeedant against *S. littoralis* (Simmonds, et al. 1990). (Fellows, et al. 1986) conducted a series of experiments with taste sensillae on the moth parts of *S. littoralis* larvae. They could conclude that PHAs stimulated a dose-dependent neural response. When the PHAs were tested in combination with sugars there was a decrease in the total input from the sensillae. This reaction is called “peripheral interaction” (Mitchell and Sutcliffe 1984). Shortly after a sensillum had been stimulated with a PHA the response to some sugars had declined with up to 91%. The insects appeared to be blind to the phagostimulants that normally elicits feeding. Behavioural observation showed that the larvae rejected plants normally accepted when treated with PHAs. (Gonzalez-Coloma, et al. 1998) evaluated some antifeedant diterpenoid alkaloids against *S. littoralis* in *Delphinium cardiopetalum* plants, long known to be insecticidal. They found that small amounts of cardiopetamine and 15-acetylcardiopetamine (C20) strongly deterred feeding, but was not toxic to the insects. This result suggested that the tested compounds acted strictly as antifeedants against *S.littoralis*. However, the lack of toxicity and other negative postingestive effects when the compounds were orally injected to the larvae suggested that the insects may be able to detoxify these compounds.

Case studies, terpenoids in insect pests

When presented to antifeedants, insect behaviour reveals if the compound acts like a repellent, arrestant, suppressant, or feeding deterrent. In three case studies on terpenoids and how they affect different insect species, examples of all subgroups of antifeedants were found.

Terpenoids in Hylobius

(Klepzig and Schlyter 1999) found that plant-derived extracts showed strong antifeedant properties against the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae). In laboratory evaluations, only a few insects fed enough to give recordable results and that most insects did not feed at all on carvone and coumarin. This indicates that the two compounds may act as repellents, suppressants or arrestants, but not as feeding deterrents.

Terpenoids in Myzus

In a study of the green peach aphid, *Myzus persicae* (Sulzer) the behavioural effects of ten different labiate plant oils were tested (Hori 1999). Eight oils reduced the total, average, and maximum stylet penetration time, and increased the penetration frequency. However, in a choice test all 10 oils exhibited settling inhibitory activities. In no-choice tests, aphids rarely settled on diets covered with two of the oils and most of them died. Another two oils appeared to be relatively toxic. Inhibited settling indicates occurrence of repellents and arrestants in the active oils as the insects, in some cases, did not even penetrate the diets. The penetration behaviour in *M. persicae* seems to be affected by feeding deterrents (decreasing feeding time) and by suppressants and/or feeding deterrents (increasing stylet penetration frequency) in the labiate oils. Aphid stylet penetration was disrupted in a similar way in a series of experiments conducted on aphid-susceptible and aphid-resistant cowpea by (Annan, et al. 2000). The aphid behaviour, staying on the feeding site but with an altered feeding behaviour, appears to be controlled by gustatory responses.

Terpenoids in Spodoptera

(McAuslane and Alborn 1998) conducted a series of experiments were *Spodoptera exigua* larvae showed a strong preference for glandless, undamaged terminal cotton leaves, compared to glanded leaves or leaves from plants damaged from larval feeding. Terminal leaves from the damaged glanded cotton contained significantly higher concentrations of the feeding inhibitors. Gossypol works by binding the proteins in the gastrointestinal area which causes decreased protein digestion (Gershenzon and Croteau 1991). It should accordingly be classified as a digestive or postingestive inhibitor and not as an antifeedant. However, the release rate of volatiles were also measured, showing a 13-fold higher emission rate of volatile terpenes from damaged glanded cotton than from damaged glandless. Preingestive inhibition likely occurred to some extent. In this case, volatile repellents and arrestants reduced feeding in the larvae. In a similar comparison of *S. littoralis* feeding preference for damaged and undamaged terminal leaves (Månsson, unpubl.), damaged leaves were the less preferred, and had smaller punctures than undamaged leaves. The insects nibbled the damaged leaves but did not continue to eat on the same spot as they did in undamaged leaves. Instead, they moved around to find new feeding sites, causing a great number of small punctures. The result shows occurrence of suppressants and/or feeding deterrents against *S. littoralis* in cotton, induced by previous insect feeding.

In these three cases, plants are efficiently preserved from insect feeding. Certain terpenoids protect conifer seedlings from pine weevils, labiate plant oils protect plants from aphids and in cotton, feeding is decreased in the Egyptian cottonleaf worm when glanded plant types are used. Cotton is, in addition, partly protected by its induced defence system. Labiate plant oils are aromatic and may affect the smell and/or taste of the end product and do, as most aromatic compounds, evaporate relatively fast.

Why working with antifeedants?

Using naturally occurring plant compounds is a modern and environmentally friendly method to control pest insects. So far, broad-range toxics have been used with environmentally hazard as a result. Permethrin was the pyrethroid used for pine weevil control in Sweden until it got banned in 2003. Without control, 80% of the plants would be killed with severe economical loss for the forest industry (Weslien 1998). Pine and spruce saplings treated in 2003 were still allowed in reforestation during 2004. Cypermethrin is the new pesticide on the market used for treating saplings against pine weevil feeding and will be in use in Sweden from 2004. However, cypermethrin has shown even stronger toxicity to insects than permethrin (Oliveira, et al. 2002). Cypermethrin affect most insect species and is known to be acaricidal (Hashimoto, et al. 1999), toxic to most aquatic organisms (Mian and Mulla 1992; Reddy, et al. 1995; House, et al. 1997; Lutnicka, et al. 1999; Solomon, et al. 2001) as well as to most terrestrial insects (Siegfried 1993). In higher doses it severely affects the nervous system in mammals (Bainova 1979), and a number of pyrethroids have been shown to interact with human hormone receptors (Tyler, et al. 2000). There are numerous examples of pesticides that have been banned with no alternatives on the market, which probably will be the case with cypermethrin when its affects will be evaluated. The research for antifeedants is therefore of great importance.

The problem

A pest since 1913 but mentioned earlier

In 1758 Carl von Linné described the large pine weevil *Hylobius abietis* (Coleoptera: Curculionidae) in his “Systema Naturae”. However, the insect was not described as a conifer forest pest until 1867 by E. A. Holmgren. The pine weevil has been considered a pest ever since and is today the major insect pest in conifer forestry. The problem is a result of the methods, clear-cutting followed by reforestation, used in forestry since the early parts of the last century.

The host plant

The weevil may feed from a number of woody plants but the economic damage is limited to newly planted, 1.5 – 2 years old, saplings of spruce and pine. A single weevil may cause severe losses in growth and a number of insects may girdle the plant, which kills it. However, the insect is polyphagous and do have a great number of other feeding sites than pine and spruce. (Scott and King 1974) could conclude that the weevil feeding preference looked somewhat different from its actual damage habits. It preferred feeding sites in the order: scots pine >> silver birch >> Norway spruce >> ash > sycamore. In spite of the fact that spruce seedlings are heavily damaged, Silver birch is strongly preferred over it. In a later study, (Samuelsson 2001) could see a preference for Norway spruce > downy birch > silver birch.

Picture 1. A closer look at the large pine weevil *Hylobius abietis* (Coleoptera: Curculionidae) Photo: Fredrik Schlyter.

Modern forestry – a weevil nursery

The pine weevil is attracted by odours from newly dead wood and can fly for several kilometres to reach breeding sites. The insects are attracted by newly killed wood from storms or from clear-cuts. The in-flying adults feed mostly from the small twigs of adult trees. Feeding damages are of less economical importance as the fully grown tree can endure heavier feeding pressure than young saplings. Modern forestry provides large amounts of breeding and ovipositioning sites as the stumps are the natural egg-laying sites of the pine weevil. The odour from a clear cut attracts large amounts of insects that breeds and lay their eggs under the bark of the roots or close to them. The larvae feed and develop in the root and hatch the following season. The newly hatched adult insect walks around foraging and encounters the seedlings. It walks up on the stem and start feeding from the outer and inner bark. Young saplings are usually planted at that time and provide the insect with food. Small seedlings can be girdled and killed with high expenses for the forest industry. In northern Sweden, damage by the pine weevil is often avoided because of an extended time between clear-cutting and reforestation. No saplings are planted during the first two years after harvest, and the adult pine weevil hatches and leaves the area before reforestation. A generation time for a tree in the northern areas is so long that two more years do not cause any financial losses. In the south, where the generation time is shorter and two years extra does make an economical difference, different methods must be used.

Objectives

This thesis focuses on bark/phloem antifeedants against *Hylobius abietis* pine weevils. The insect causes damage to pine and spruce seedlings but has a wide range of alternative feeding sites on other tree species. The objectives were to find plant species that the insects do not feed from and to identify their antifeedant content. The project has been an applied work, where antifeedants found have had to be possible to test in field conditions. I.e., practical problems like sustainability over two seasons and a proper formulation have been concerned. Therefore, we hoped to find suppressants and feeding deterrents as they are, in general, heavier and consequently less volatile than repellents and arrestants. Found antifeedants would be tested in field conditions to evaluate their long term effects as well as suitable formulations. The final aim was to be able to commercialise a plant compound, effective against the feeding of the pine weevil.

The applied benefits of antifeedant studies

Outlines of the project project "Protection of conifer seedlings against Hylobius pine weevils".

The project "Protection of conifer seedlings against *Hylobius* pine weevils" is a part of the program "Pheromones and Kairomones for Control of Pest Insects" and is financed by MISTRA, Miljöstrategisk forskningsfond (The Foundation for Strategic Environmental Research). The work against pine weevil damage is based on two main sub-projects. 1) The screening of already known antifeedants from different plant-insect systems. 2) The search for antifeedants in non-host plants. The project covers the full spectrum from basic laboratory research to applied formulations in the field. I. e., the antifeedants found must be possible to commercialise and must not be classified as toxic. The focus of this work is line 2.

Methods

Twig test

The method is used in paper I and in paper III. A twig of *P. sylvestris* is cut to 12 mm of length. A filter paper is wet with tap water and put together with a standing pine twig and one pine weevil into a Petri dish. Six Petri dishes are stacked together with a rubber band to keep insects from escaping. The weevil is allowed to feed from the twig during 48 h and the eaten area is thereafter measured. The twig assay is not only a convenient method to make a first examination of weevil feeding preference, but is also a useful method to test an antifeedant compound by soaking a tree branch into it ((Klepzig and Schlyter 1999). By using pine, the natural attractants are intact and provides result closer to the applied field conditions.

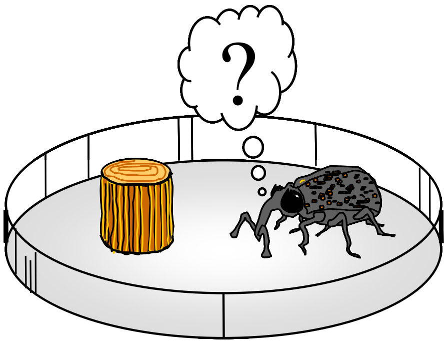

Fig. 4. The no-choice twig test. The weevil is allowed to feed from the bark of a short stump of the woody species one wants to test. After two days the weevil is removed and the eaten area is calculated. The method allows for comparison of different tree species.

Extractions (Paper II)

In order to collect compounds for further analysis, the bark and phloem were scraped off the stem. The area of bark removed was measured before extraction to quantify the bark content. A number of extraction methods were tested but few of them gave satisfying results. The extraction process is described in detail in paper II. Solvent Soxhlet extraction was used in all extractions except in one case where pressurised Soxhlet extraction using liquid, supercritical carbon dioxide was used. The only extraction that gave extracts still active after further fractioning was when dichloromethane and methanol (9:1) was used. In the solvent Soxhlet method, the sample soaked in solvent that was periodically siphoned off, distilled and returned to the sample. The temperature did not exceed 45° C in the boiler and the process was let to run for approximately four hours, where the thimble had lost all green colour. The extract was thereafter concentrated to dryness in a Rotavapor. More methanol was then added to remove the water phase in a second run in the Rotavapor. The dry extract was diluted so that the amount per area on the TLC-plate roughly corresponded to the amount per area of fresh bark. The extract was thereafter tested in a micro feeding bioassay.

Micro feeding bioassay

The micro feeding assay is used to quantify weevil feeding on control and treatment. In this method, sugar solution is used as attractant stimulating the insects to feed on cellulose plates (Schlyter et al., 2004). The advantage of this method, compared to the twig assay, was that exactly the same amount of attractant was used on each plate and the natural variation between twigs is avoided. Control solvent was added to

> Box 3.
>
> **The antifeedant index**
>
> The antifeedant index presents the quota between the area consumed on the solvent control (C) and the treatment (T).
>
> $$AFI = (C - T)/(C + T)$$
>
> The method is used on both the twig assays and in the micro feeding assays. The result is a figure between −1 and 1.
>
> AFI = −1 is indicative of the best possible feeding stimulant
> AFI = 0 is indicative of no effect
> AFI = 1 is indicative of best possible antifeedant

one plate and the same amount of extract was added to the other one. The plates were allowed to evaporate until they were dry. Sugar solution was added and one pine weevil was allowed to feed for 4 h. The insects starved for six days before

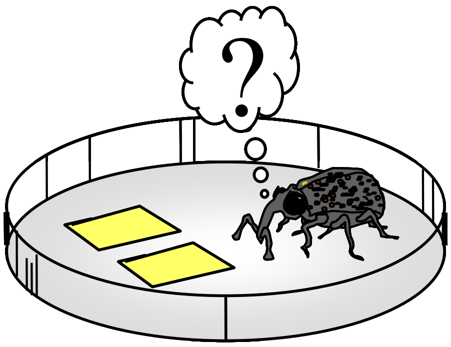

Fig. 5. The micro feeding assay. The extract to de tested is presented as a small droplet on a cellulose TL-plate. The solvent is presented on the other and after drying, sugar solution is added to both as an attractant.

each assay and were furthermore not allowed to drink during 24 h before the assay. The insect was thereafter removed and the consumed area was measured. The results were presented as an antifeedant index (see box 3), AFI, where the area consumed on the treatment (T) was compared with the area fed on the control

(C), (C-T/C+T). Positive values show antifeedant effect while negative values show feeding stimulation.

Whole plant assays

The last step in the testing process of potential antifeedant candidates was performed on whole plants in a biotron and in the field. For the field tests, compounds were dissolved at different concentrations in paraffin. The lower 15 cm of a containerized spruce seedling was then painted with warm wax. The seedlings were planted in the field and weevil damage and other damages were measured after a period.

The biotron was used to check for treatment side effects and insects were not used in that environment. A spruce seedling was planted in a pot and paraffin was prepared as in the field tests. Treated seedlings were periodically observed for treatment damages and growth.

Results and discussion

The feeding preference of the pine weevil

In order to find antifeedants for the pine weevil, we collected and tested plant species from a number of European families, large enough to cover most woody taiga plants. Some non-domestic species were also tested (paper I). In total, the food source capacity of 38 species from 25 families was tested in no-choice assays, where weevils were allowed to feed from short stumps. The area of removed bark and phloem was measured for each species and compared with the area of bark removed in Scots pine *Pinus sylvestris*. In the first assays, only bark eaten down to the xylem was considered. These assays were performed both as choice and no-choice tests. Interestingly, the outcomes differed greatly between choice and no-choice mode. When given a choice, the insects mostly fed on pine while no difference in feeding could be recorded between the other plant species (Paper I, Fig. 1). When presented to the same species in no-choice tests, a different result was obtained. The plants were possible to rank according to how much bark area that was removed and in some cases, the insects fed as much as on pine. One conclusion was that the choice of method is important and that the insects can, in lack of pine and spruce, find alternative feeding sites in the field. In a more extended assay (Fig. 6), where both outer bark and phloem was measured, three different feeding patterns were observed. In one group of tree species, the insects did not touch or feed from the twigs. Shallow nibbling occurred but there were no actual feeding or removal of the outer bark. The second group consisted of species where the outer bark of the twigs was removed or fed upon, but the insects stopped when reaching the inner bark (the phloem). The third group consisted of species where the feeding area of inner and outer bark did not significantly differ, or was greater than in pine. Species from the first and second group were considered for extraction.

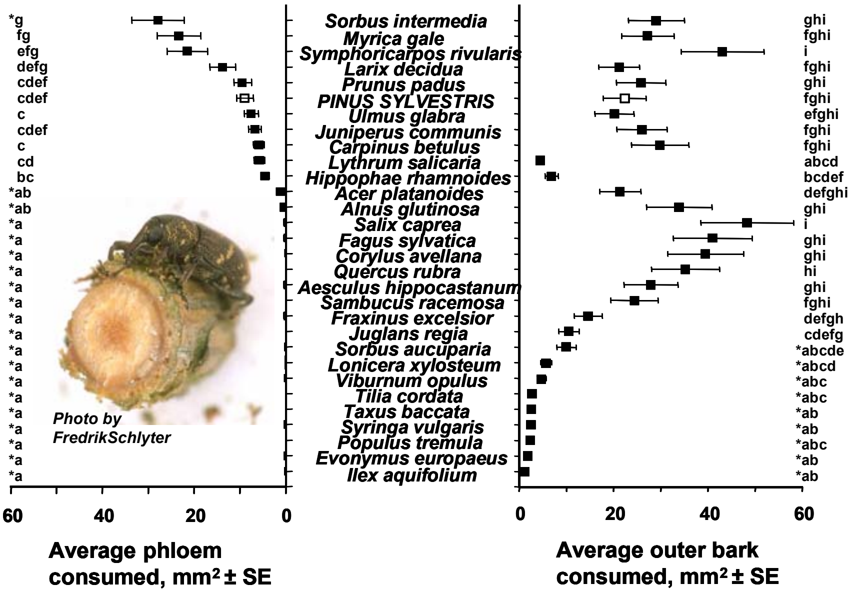

Fig. 6. No-choice twig test on 28 woody plants. Removal of outer and inner bark is quantified separately. The species are ordered by firstly, the area of outer bark removed and secondly, the area of inner bark removed.

Bark extraction and chemical content analysis

Extraction methods

The bark from the chosen species was scraped or peeled off the stem. To narrow down the number of assays, some were excluded. Bark texture and known content of toxic compounds were considered when excluding species from both the first and second group. Linden, alder, lilac, aspen, horse chestnut, ash, oak, and red elder were chosen for extractions. In the first attempt to extract the fresh bark, it was ground and shaken with solvents of different polarity like hexane, dichloromethane and methanol. However, none of the candidates gave positive results in the micro feeding assay. In the next step, solvent Soxhlet was used. Different solvents and different methods to prepare the bark, fresh or freeze-dried, were used but the results were still disappointing (Paper II). However, linden bark extracts were promising, yet not statistically positive. Later, fresh bark frozen in liquid nitrogen and immediately ground was used instead of freeze-dried bark. By measuring the area of bark removed, the concentration of the extract could be evaporated to have a similar concentration as in fresh bark when used in the micro feeding assay. The method was successful with a linden extract showing significant antifeedant properties in the micro feeding assay. The other species were tested, all extracted and presented to the insects in the micro feeding assay. Ash was the only species aside from linden that gave positive, yet not statistically significant results. The focus was set on further examination of linden bark.

Fractioning and analysis of linden bark

We fractionated linden bark extract and collected 128 fractions. Each fraction was examined by thin layer chromatography and the ones showing similar content were merged. In this way, we could decrease the number to 17 fractions, a convenient number to test in the micro feeding assay. Two fractions showed strong antifeedant qualities and were further examined with GC-MS and NMR.

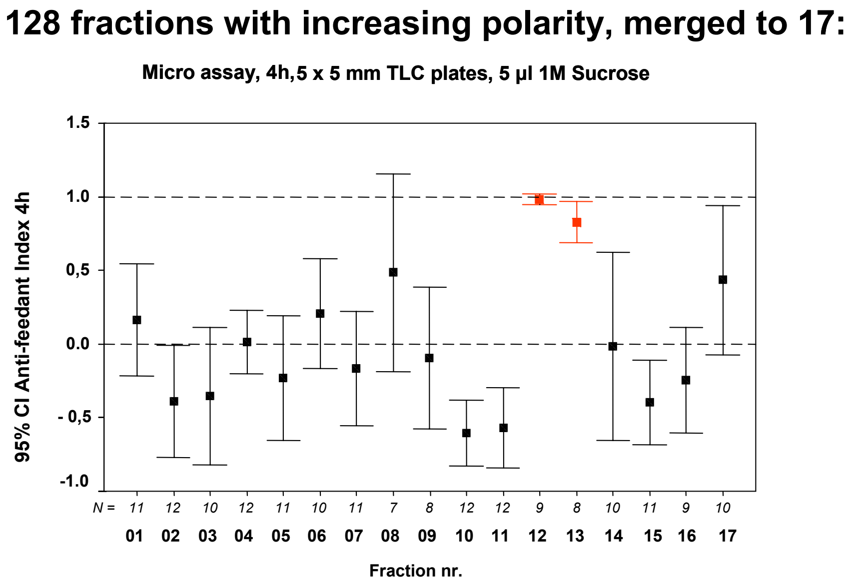

Fig. 7. The seventeen fractions from linden bark. The two red fractions where the AFI is one and close to one were analysed and nonanoic was found in both of them.

Both fractions showed to contain nonanoic acid, commonly called pelargonic acid. This compound is found in almost all species of animals (Fontan, et al. 2002) and at low levels in many of our common foods (Jirovetz, et al. 2003). Synthetic nonanoic acid was tested in micro feeding assays and showed to possess strong antifeedant qualities.

Later assays with bark from ten woody species showed four active extracts (Paper IV). By using the same methods for fractionation and analysis as in Paper II, antifeedants could be traced. Esters were found in chestnut bark extracts and showed antifeedant properties against the pine weevil.

Evaluation of nonanoic acid analogues

Nonanoic acid is a molecule with nine carbon atoms. We decided to also test the antifeedant properties of its chemical analogues (paper III). We wanted to know if acids with shorter and longer carbon chains than nonanoic acid would give similar results. Acids with chain lengths from six to thirteen carbon atoms were tested for behavioural activity in the laboratory. Carboxylic acids with six up to ten carbon atoms were found to be strongly active. As the shortest molecules were considered to be too volatile for meaningful field application, octanoic, nonanoic and decanoic acid was chosen for release tests from paraffin treatments in the field. Air was collected from treated whole plants over time. We found significant difference in release rates between octanoic and decanoic acid with lower values in the latter. The result was the expected, a shorter carbon chain is more volatile than a longer.

Modifying functional groups, and chain branching

The antifeedant properties of functional groups and the effect of chain branching were also tested in the laboratory. Some carboxylic acids were substituted with a methyl group in 2-position. Nonanoic acid sodium salt and also nonanoic anhydride in sugar solutions with pH ≈ 5 and in pH ≈ 7 were also prepared and tested. The branched chains from eight to ten carbon atoms showed an antifeedant effect but the effect was lost for longer compounds. The salt showed no effect, but the nonanoic anhydrides showed strong effect in both pH≈ 5 and in pH≈ 7. However, when tested on twigs the evaporation time was longer and the anhydrides lost their effect. This was probably due to the lack of water on the twig surface. In the micro feeding assay, the sugar solution contained water where the anhydride could form carboxylic acid during all of the experiment. On twigs, the water amount per area is smaller and a smaller amount of carboxylic acid is formed.

Side effects in field tests

The first field tests, 2003, with octanoic, nonanoic and decanoic acid in paraffin formulations on whole plants resulted in serious plant damage from the treatment after the summer period. A test run at the end of the season 2003 did not show any side effects. In the field testing the summer of 2004, a double paraffin layer, one blank inner layer and one outer layer with the treatment compound reduced the damages. Some nonanoic acid analogues were also tested in one layer of paraffin on whole plants in a biotron, with mean temperatures similar to warm summer days. However, the direct sun radiation was not mimicked. These plants did not suffer from treatment damages to the same extent as the plants in the field, so we concluded that the strong radiation on hot summer days had resulted in a too strong release of acid into the bark (Paper III).

Conclusion and perspectives

The antifeedant properties of octanoic, nonanoic and decanoic acid do to a great extent agree with the objective to find a plant derived antifeedant against the pine weevil. It acts as a suppressant/feeding deterrent but also as an arrestant (Fig. 3), preventing insect feeding from a distance to the plant. Nonanoic acid is, however, a more volatile compound than we would have preferred. It can be replaced with the heavier decanoic acid, also found in plant material (Vidal and Richard 1986) and a highly active antifeedant (paper III). Adding a methyl molecule showed to be a good tactic to reduce volatility of nonanoic acid as well as its analogues – the molecule weight of methylated decanoic acid was similar to non-methylated undecanoic acid but with intact antifeedant qualities. A field evaluation of the methylated, branched carboxylic acids remains to be done as well as an investigation of their occurrence in plant material. Are they still plant related compounds found in nature? If they are not, their affects on plants and animals have to be further evaluated. There are also practical applications in need of further development.

1) Formulation of a better carrier remains, not only to decrease the evaporation of compound into the air but also to prevent leakage into the stem of the seedling. If that problem is solved, double coating will not be necessary. Such formulation will also have to provide decreased evaporation and photo-oxidation, i.e., sustainability increases.

2) The compounds have to be cheap and efficient to produce in larger amounts and have to be able to mix with the carrier.

Future research

Using this protocol of twig test, extraction, fractionation and feeding assays in laboratory and field may be a method to screen different plant species for antifeedants. These methods will hopefully lead to discoveries of new plant derived antifeedants for both forestry and agriculture. Heavier molecules with less effect on the treated plant material and with lower volatility would be appreciated findings. Hundreds of secondary plant compound are screened against insect pests, and the number continues to grow (Nair 1994). These antifeedant compounds have to be further developed, tested and evaluated before an applied approach can be realised. When these results are obtained, the next step will be to apply them efficiently in the field. The compounds may be attached to the plants mechanically or by development of genetically modified plants, producing the active substances. New types of crop may produce the active substances in amounts high enough to protect the plants from further herbivorous damage. Still, there is a long way between successful laboratory evaluations and commercialised products, efficient in field. A raised level of one compound may not only be an antifeedant to specific insect pests, but may also act as a kairomone to another, with increased damage as a result. The new compounds may also cause auto-toxicity or changes in growth, colour and taste of the plants. Higher amounts of naturally occurring and synthetic compounds may also affect the biologic surrounding.

Possible methods to precede, a broader view

Today, only a few highly active antifeedants have been found to a limited number of insect species. This results in a lack of possibility to systemise or to predict any molecular motifs in feeding inhibition. The number of cases is simply too few to give an overview. Further studies of the synthesis and structure-activity of structurally related compounds will tell which part of the structure that plays the most important role in the interaction with the insects' olfactory and gustatory receptors (Lajide, et al. 1993). A great amount of chemicals, to date unknown, may have feeding inhibitory qualities as well as environmental persistence, but will remain unknown without improved methods to be discovered. A "mix and match" approach may be possible with different feeding inhibitors in order to create customised formulations against specific insects, thus taking advantage of natural plant defence mechanisms. The application of plant compounds may be broad and does not have to be limited to the targeted insect, e.g., azadirachtin may be used not only against insects but also against snails (Maini and Morallo-Rejesus 1993). Nor does it have to be limited to the plant parts normally fed upon or to the compounds found in the insect habitat. An example of this is betulin, a triterpene found in the bark of birch (*Betula* spp.) that acts as an antifeedant against bollworm larvae, *Heliothis zea* (Lugemwa, et al. 1990), a pest on agricultural crops.

In cotton, several insect antifeedants have been identified. To produce cotton with higher amounts of these naturally occurring defence compounds would be an attractive method to avoid herbivore attacks in the future. Glanded cotton contains more antifeedants than glandless, but the production today focuses on glandless cotton. The preference for glandless cotton is because the seeds are used as a human or non-ruminant animal food and as an oil source and high amounts of secondary metabolites may be harmful to the consumer (Cherry and Leffler 1984).

Genes, active in synthesis of deterrent chemicals, may be cloned in to vulnerable crops, thereby providing them resistance against insect damage (Gatehouse and Hilder 1988). This approach may also be coupled with other sources of unique compounds or agents (Gasser and Fraley 1989). It could result in storage of a defence chemical located within the plant, to be activated at the appropriate time. However, this strategy may cause ecological risks as well as negative changes in plant energy costs. Therefore, it has to be further evaluated before commercialisation (Griffiths 1990).

This area of applied entomology may benefit from other research areas. Pharmacology has developed a number of different controlled release strategies as delayed release, targeted release and extended release. This technique is also used and evaluated in crop protection. Micro-encapsulation, microbial pesticides and cellular methods are a few examples (Wilkins 1990). These methods may prolong the biological and chemical activity of feeding deterrents that are volatile or perishable and may also give a method for satisfactory timing of release (Griffiths 1990).

Economical aspects

Many feeding inhibitors found so far gives excellent results at the laboratory level. Field tests show that only a few of them are satisfactory alternatives to traditional pest management. However, there is a financial aspect too. The traditional chemical control is usually broad spectrum insecticides, and has to be broad. They have to sell in amounts large enough to finance development, research, and marketing. The new generation of environmentally safe pest control compounds are usually developed and tested against one, or a small group of insects, attacking a specific crop. As a compound that inhibits feeding of one species may be an attractant to another, an amount of different compounds may have to be used. Furthermore, the threat to the crop has to be big enough to create willingness in funding research and development for synthesizing and commercialisation of new protection methods. As traditional broad spectrum pesticides are replaced by much more specific semiochemicals, pest management may therefore be more expensive in the future. There are though other methods to achieve antifeedants than costly synthesises. Bentley, et al. (1988) found several citrus limonids (bitter triterpenes) with antifeedant properties for the Colorado potato beetle (*Leptinotarsa decemlineata*) in by-products of orange juice processing. There may certainly be more waste material containing valuable compounds with feeding inhibitory properties in the food industry.

Outlook

The practise of using feeding inhibition allows us to develop and exploit naturally occurring plant defence mechanisms, thereby reducing the use of traditional pest management chemicals. Most of these new methods have to be further developed before they can be commercialised, and much effort has to be put into this area. New challenges will occur. For example, compounds to date regarded as environmentally safe, may cause unexpected problems; higher concentrations of limonene in the working environment causes irritation and allergic reactions when in contact with the skin. A deeper co-operation between industrial and academic research could definitely accelerate the process, giving us new environmentally safe methods in future plant protection.

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Den svåraste biten...

Först av allt vill jag tacka min familj, det är ju på något sätt så att de ligger bakom det hela.

My dearest Karen, how can a person be so supportive at that distance? We haven't seen each other more than once a month but you have still kind of been there. Let's arrange things right so we can spend all our time together.

Jan Löfqvist, som raskt tillsatte en doktorand på linje 2 och som har stöttat delat med sig av sin kunskap under en stor del av projektet.

Fredrik, det har varit mycket harvande med baggplockning och statistik men det har varit otroligt kul. Jag har lärt mig massor under den här tiden och jag har räknat ut att jag har handplockat ungeför 20 000 baggar, en och en. Jag kommer att sakna både avdelningen och våra fältdagar i Asa (även om jag fick lappsjuka i början).

Marcus the rumspolare, så mycket roligare rumskompis kan man inte ha. Tänk så många sajter jag missat om jag inte delat med dig. Lycka till på lower Manhattan.

Peter Anderson, jomenvisst, mitt exjobb på försvarssystem i bomull var ju det som öppnade dörren.

Bill, tack för alla kul saker du har anordnat och för att du är en god styrman.

Marie, man tackar för dina historier, t. ex. om avskurna fingertoppar under pågående föreläsning, och för att jag har fått stöka runt i din labbutrustning.

Ylva och Anna-Carin, det ständiga radarparet som är mycket stämningshöjande.

Elisabeth, som ständigt vårdat baggarna ömt och som dessutom kört många, långa tunnskiktsförsök åt mig.

Martin, det blev lite stressigt på slutet, eller hur?

Feromongruppen i Lund, det hade inte blivit många Pherodays utan er. Det har varit kul att få lära känna er.

Lena på Korpamon, ja ja, jag lovar att titta in med fisk *när jag lyckas fånga en* i den där bedrövliga Bosarpasjön.

The Rothamsted team. John Pickett, Lester Wadhams, Linda Ireland, Hasse Rasmussen, I had a great time at your lab and thanks to you, the extractions finally worked!

Micke, blir det rikssvenska nu?

Åsa och Magnus, min kära syster träffade en riktig klippa. Blir det mera spika på stugorna till sommaren?

Marcus Sjöholm, the spexmaster. Glöm nu inte att bjuda in mig när det blir din tur.

Alla på försöksstationen i Asa, ni är ett mycket trevlig gäng och har alltid varit generösa och tillmötesgående då det fattats prylar.

Alla andra på avdelningen och på andra avdelningar och ni som har försvunnit härifrån. Det har varit en kul tid med er, men nu måste jag rusa till repron...