Preliminary results on the analysis of volatile and non-volatile pheromones in Chilocorus nigritus by GC/MS

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Abstract

This study presents preliminary results on the analysis of the volatile and non-volatile compounds present in the scale insect Aspidiotus nerii, a notorious pest problem in crops, and the ladybird Chilocorus nigritus, a natural predator which can be used to control Aspidiotus nerii infestation. Potato tubers were used to rear Aspidiotus nerii uniparental and biparental populations. GC/MS was used to analyse volatile compounds collected from various insect cultures, as well as from control samples. Most of the volatiles observed appeared to be common to all samples, with one exception, 1,2- diethylbenzene, which was found in all samples except the controls and uniparental Aspidiotus nerii group. To investigate the presence of non-volatile compounds which could act as contact pheromones, Solid Phase Micro Extraction (SPME) samples were collected and analysed using GC/MS. Samples obtained from the ladybirds' cuticles showed no compounds of interest to pheromone research. However, direct extraction of Chilocorus nigritus haemolymph (tissue fluid) in acetone identified an alkaloid compound similar to (+-) hippodamine. Although the biological function of this compound in Chilocorus nigritus has yet to be established,

examples of the function of hippodamine in similar species suggest it could serve as an alarm pheromone or as an attractant kairomone.

Keywords Chilocorus nigritus, Aspidiotus nerii , Pheromone, Hippodamine, SPME, GC/MS

Introduction

The problems associated with conventional pesticides have inspired a search for safer, more sustainable options for crop protection. One alternative to pesticides is the use of biocontrol agents, natural predators of the target pest, which can be used to control infestations in crops of commercial significance. Scale insects are a notorious pest problem in the UK and other parts of Europe, especially on woody crops, ornamental glasshouse stock and deciduous and coniferous trees (Foldi 2000, Ülgentürk et al. 2004). Aspidiotus nerii, a species of scale insect, is mainly a pest of citrus and ornamental plants. A natural predator for A. nerii is the coccidophagous ladybird Chilocorus nigritus F., a commercially valuable ladybird which can be used to control infestations of a variety of soft and armoured scale insects (Ponsonby & Copland 2007, Omkar & Pervez 2002).

Biological control agents such as C. nigritus are costly to produce and success rates are sometimes erratic, even when conditions are apparently favourable (Ponsonby, 2009). The reasons for such failure are often unclear and much has yet to be discovered of the complex relationships that biocontrol agents have with their environments. A crucial step to enhance our understanding of the behaviour and reproduction of C. nigritus is to explore its chemical ecology. So far, the majority of research has been focused on pheromones in pest species themselves (Flint & Doane 1996). However, less work has been done on an alternative,

predator-focused research, and the complex interactions between the prey, the predator species and the host plant in which these interactions take place. Volatile compounds produced by either the host plant or the scale insect can play a role as attractant kairomones for beneficial predators. Kairomone is any substance produced and released by an organism that benefits another organism which receives it. This interaction has not yet been demonstrated in C. nigritus, but has been observed in similar species such as Coccinella septempunctata. Ninkovic et al. 2001 found that C. Septempunctata responded with increased searching behaviour where the aphid species Rhopalosiphum padi was present (or had been present recently) on barley. This particular ladybird species uses methyl salicate as one if its olfactory cues for prey location (Zhu & Park 2004).

The beneficial ladybirds C. septempunctata and Adalia bipunctata (Al Abassi, et al. 1998) are attracted to 2-isopropyl-3-methoxy pyrazine, produced by the ladybirds themselves, and previously thought only to act as an alarm pheromone. This compound could in theory be used to retain the valuable ladybird predators in particular areas, avoiding problems due to predator dispersal. Any such compound would be a great asset where C. nigritus is used, as this particular ladybird species will also disperse when the density of the prey is low (Ponsonby & Copland 2007). It is also important not to underplay the potential effects of contact pheromones, non volatile compounds found on the surface of the insects. An example is the pheromone (Z)-pentacos-12-ene found in Cheilomenes sexmaculata larvae (Klewer et al. 2007). This alkaloid causes adult female ladybirds to avoid laying sites already inhabited by members of the same species, and may therefore be of great significance in the population distribution and dynamics of these predators.

The aim of this study was to conduct a preliminary study of volatile and non-volatile compounds produced by C. nigritus and A. nerii. Potato tubers (cv Desiree) were used as the host plant to rear the A. nerii population. Samples from various insect cultures, as well as blank samples to be used as control, were collected and analysed using GC/MS. Solid Phase Micro Extraction (SPME) was used to collect samples from of the non-volatile areas of the insects; these samples were also analysed using GC/MS.

Experimental

Insect Cultures

C. nigritus were raised on biparental A. nerii which were in turn raised on potato tubers (cv. Desiree). A culture of uniparental A. nerii was also raised for comparison purposes. The insects used for the pheromone extractions were of specific ages where reproductive pheromones were likely to be produced. C. nigritus populations were at least 8 weeks old and contained approximately 30 beetles. A. nerii cultures were 7 weeks old.

Extraction of volatiles

The apparatus shown in Figure 1 was set up and run empty for 1 week after acid washing in HCl and multiple rinses of distilled H_2O. The extraction medium used was Poropak Q™, and approximately 500 mg were packed into each tube with glass wool. Tubes were then cleaned of unwanted volatiles by running dichloromethane through them, and drying under slow flowing nitrogen. They were then heat treated at 210-220˚C, under nitrogen flow, for 2.5 hours. Tubes were stored at room temperature and sealed with aluminium foil. Table 1 shows the experimental groups selected and a brief description of the characteristics of each group. Each of the groups was run through the extraction process for 12 days at room temperature at a flow rate of 1-2 L/min. Extraction tubes were then sealed again with aluminium foil.

Volatile Analysis

Volatiles adsorbed onto the Poropak were eluted with pesticide residue grade hexane. Samples were then analysed on a Hewlett Packard 5890 GC (using a non-polar DB-5 30m x 0.25 mm x 0.25 μm film thickness) and Carlo Erba QMD1000 Mass spectrometer (70eV). The temperature program used was 50˚C for 5 minutes then ramped up at 5˚C/minutes up to 250˚C, then raised to 300˚C at 20˚C/minutes. The injection port was held at 250˚C and 1μl of each eluted sample was injected. The results are presented in Figure 2. For comparison purposes, the same samples were also analysed on a Fisons Instruments MD800 and GC8000 containing a DB-5 (30m x 0.25 mm x 0.25 μm film thickness) at 70eV. All temperature parameters were the same as the experiments run on the HP and Carlo Erba equipment. Similar results were obtained with the Fisons Instruments apparatus.

Solid Phase Micro-Extraction (SPME)

SPME was used in two ways to explore the presence of contact (non-volatile) pheromones on the insects. On the first method, SPME fibres were used on the cuticles of C. nigritus; 100μm polydimethylsiloxane (PDMS) fibres from Sigma-Aldrich® were rubbed for 30 seconds across the elytra (protective wing case) of a single insect. The second method involved using PDMS fibres for direct sampling of C.nigritus haemolymph (tissue fluid). Haemolymph samples were prepared as follows. Male, female and mixed sex C.nigritus (the latter consisting of 4 individuals, 2 male and 2 female) were agitated to produce reflux bleeding (haemolymph). Approximately 1μl of haemolymph from each extraction was mixed with 200 μl of acetone; dichloromethane was initially used, but was subsequently discarded in favour of acetone due to solubility problems. PDMS fibres were loaded with the acetone solution and then injected into the GC port.

GC/MS analyses of the samples collected by both methods were performed on a Hewlett Packard 5890 GC (using a non-polar DB-5 30m x 0.25 mm x 0.25 μm film thickness) and Carlo Erba QMD1000 Mass spectrometer (70eV). Each fibre was heat treated in the GC port at 250˚C to desorb any contaminants prior to haemolymph sampling. The temperature program used was 50˚C for 5 minutes then ramped up at 5˚C/minutes up to 250˚C, then raised to 300˚C at 20˚C/minutes. The GC port was held at 250˚C.

Results and discussion

Volatile analysis

GC/MS results for all the samples analysed are shown in Figure 2. Most of the compounds extracted on Poropak appeared to be common to all samples including control. This suggests that there were few volatile compounds produced in any significant quantity by any of the insect (or plant) samples. However, there was one exception, 1,2- diethylbenzene (Rf 12.95, 13.25), which was found in all samples except the controls and uniparental scale group. 1,2-Diethylbenzene has also been reported in a similar pest, Hyadaphis tataricae (Honeysuckle aphid) (Hedin et al. 1999); thus, 1,2-diethylbenzene may be of interest in terms of its biological function in the C.nigritus/A.nerii relationship. However, this promising result must be taken with caution, since degradation of Poropak adsorbent produces a similar compound, 1,2-diacetylbenzene (Sturaro et al. 1991); further research will be needed to ascertain whether the compound is in fact an insect pheromone or an artifact due to the sample collection method.

Solid Phase Micro Extraction (SPME) analysis

GC/MS analysis of the SPME samples obtained from the elytra of C. nigritus showed no differences in the compounds identified with respect to those found in control samples. This suggests the absence of cuticular hydrocarbons used as pheromones amongst C. nigritus individuals. However, GC/MS analysis of the haemolymph extracts suggests the presence of a compound similar to (+-) hippodamine (see Figure 3). Hippodamine has been reported in similar ladybirds such as Hippodamia convergens (Tursch et al. 1974), and Chauliognathus pulchellus (Moore & Brown 1978). In the case of Hippodamia convergens, hippodamine is thought to act as an alarm pheromone. Moreover, two other members of the Chilocorus genus species, C. renipustulatus and C. cacti, are known to produce similar alkaloids, nominally Chilocorine A, B, C and D (Laurent et al. 2002, Huang et al. 1998, Shi et al. 1995 & McCormick et al. 1994). In addition to its alarm pheromone activity, hippodamine can act as an attractant kairomone in Dinocampus coccinellae (a ladybird parasitoid), promoting oviposition (Al Abassi et al. 2001).

Conclusions and further research

Direct GC/MS analysis of the haemolymph extracts from C. nigritus individuals suggests the presence of a compound similar to (+-) hippodamine. Further analysis of synthesised hippodamine isomers, using the already published synthetic route (Newton et al. 2008) will be needed to ascertain the exact nature of this Hippodamine compound. A comparative study of the compound found in C. nigritus with those found in C. renipustulatus and C. cacti (from the same genus species as C. nigritus) could be valuable in the full identification of the C. nigritus alkaloid. The biological function of the compound found in C. nigritus has yet to be established. Examples of the function of hippodamine in similar species suggest it could serve as an alarm pheromone or as an attractant kairomone. The fact that the compound is present in the haemolymph extract may have an effect when trying to maintain healthy

populations of ladybirds for use as biocontrol agents, since their haemolymph may attract harmful parasitoids. Establishing the biological function of this compound will enhance the current understanding of the behaviour and reproduction of C. nigritus, which may help improve the use of this commercially important predator.

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Figure 1. Volatile extraction apparatus

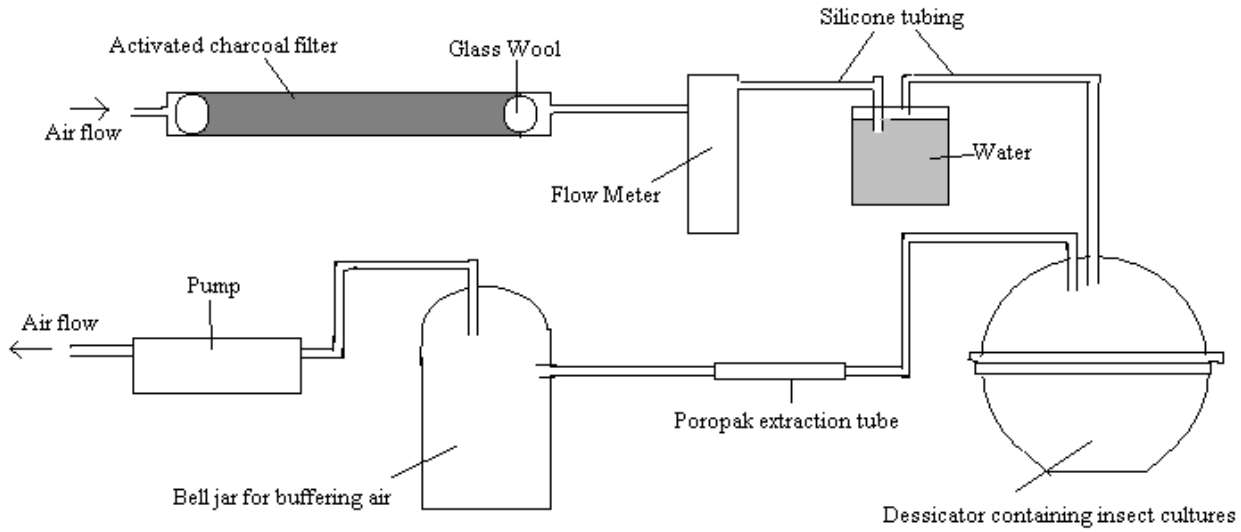

Figure 2. Chromatograms for all the sample groups.

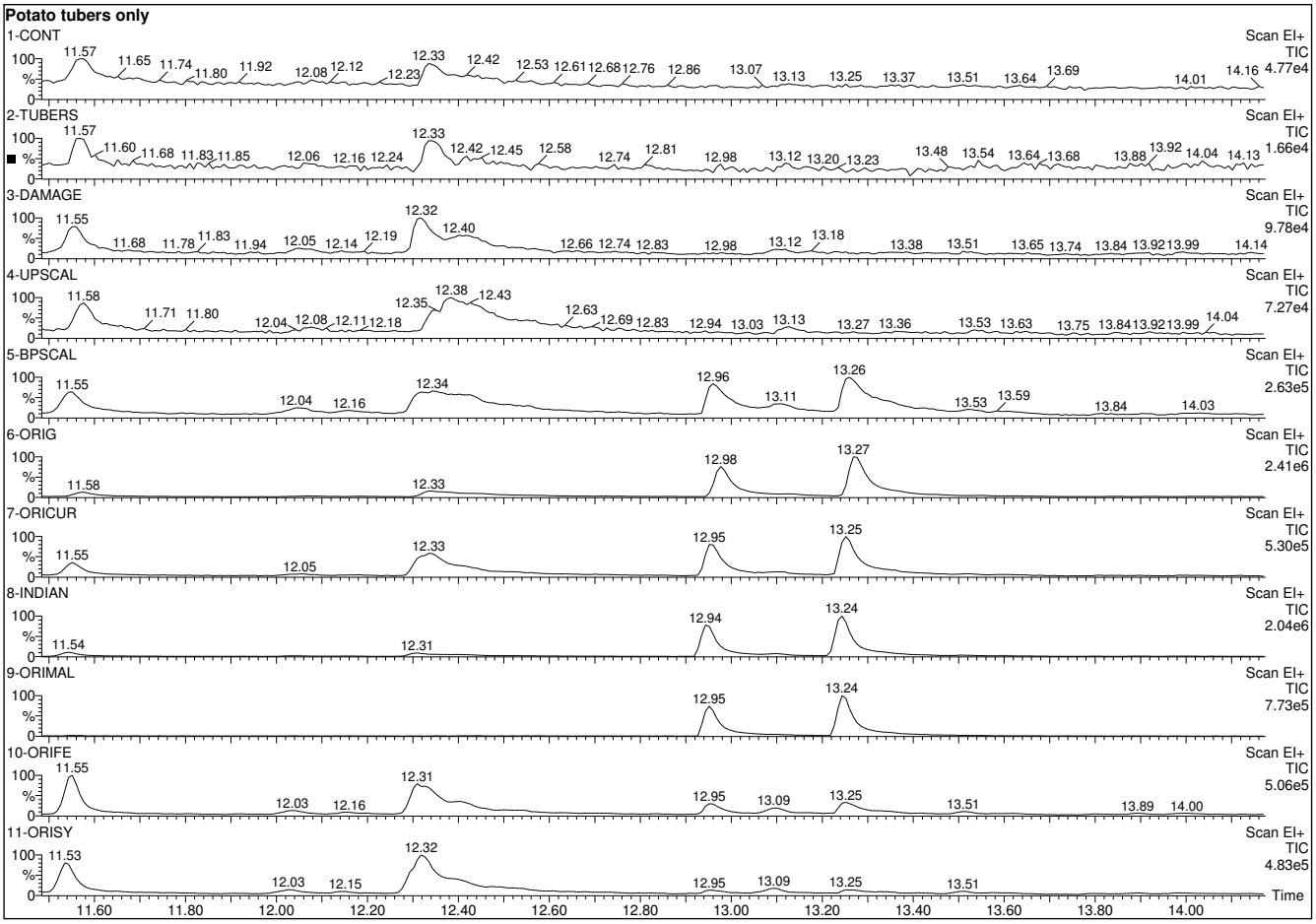

Figure 3. Mass spectra of hippodamine from mass spectrometry library (bottom) and unknown compound from C.nigritus haemolymph (top).

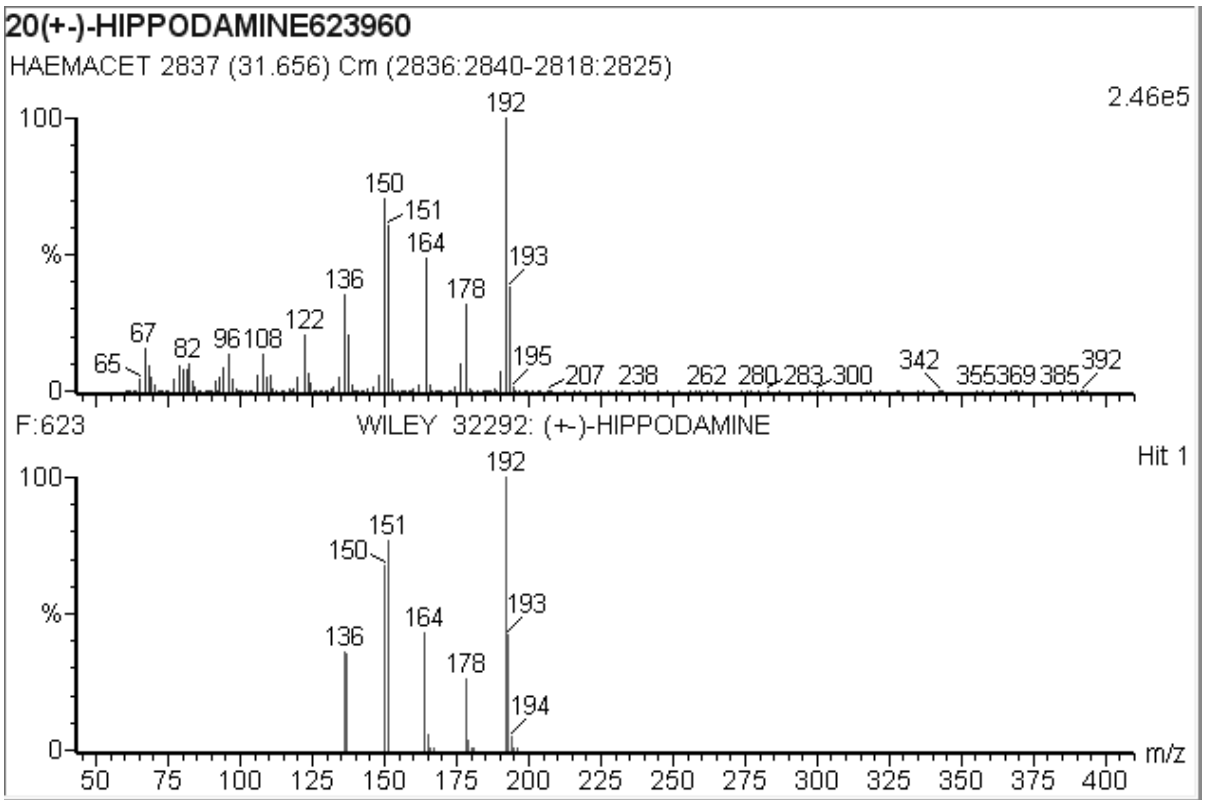

Table 1. Experimental groups (prey-predator-host plant) selected for the extraction of volatiles, including control groups.

Group	Description
1-CONT	Control – Insect chamber run empty for the whole period.
2-TUBERS	Potato tubers only.
3-DAMAGE	Potato tubers mechanically damaged with entomology pin.
4-UPSCAL	Uniparental A.nerii on tubers (25 tubers)
5-BPSCAL	Biparental A.nerii on tubers (25 tubers)
6-ORIG*	Long term captive C.nigritus from Wyebugs ltd.
7-ORICUR*	Long term captive C.nigritus from Wyebugs ltd. previously treated with tetracycline hydroxide to cure bacterial infection.
8-INDIAN*	Recently acquired wild caught C.nigritus from Rawalpindi, Pakistan.
9-ORIMAL*	Wyebugs ltd. strain C.nigritus males.
10-ORIFE *	Wyebugs ltd. strain C.nigritus females.
11-ORISY	Wyebugs ltd. mixed sex C.nigritus feeding only on syrup.

* Indicates that the group was being fed on 25 tubers infested with biparental A.nerii.