Project title:	The Role of Chemicals in the Location of Host Plants by Midge Pests of UK Fruit Crops.
Project number:	CP 073
Project leader:	Prof. David R Hall (NRI) Jerry Cross (EMR)
Report:	Annual, August 2012
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Key staff:	Helen Thomas (PhD Student)
Location of project:	Natural Resources Institute, Chatham Maritime, Kent. East Malling Research, Kent.
Industry Representative:	Nigel Kitney/Tom Maynard
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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

- Chemicals have been identified from raspberry canes and blackcurrant shoots which may attract female raspberry cane midge and blackcurrant leaf midge.

Background and expected deliverables

Species of gall midge (Diptera: Cecidomyiidae) are important pests of many horticultural crops and are often very difficult to control by conventional means. NRI and EMR have made considerable progress in identification of female sex pheromones in this group of insects, and some are now in use for monitoring populations of several pest species (Hall et al., 2012). However, the female-produced sex pheromones attract only males. Attractants for the females, particularly mated females, would potentially be far more valuable for both monitoring and control of the pests. There is good evidence in several species of midge that mated females are attracted to their host plants for oviposition by specific odours from the plants. Although this has been known for over 40 years in some cases, the chemicals responsible for this attraction have not yet been identified.

This project will aim to identify the chemicals responsible for attraction of mated female midges to oviposition sites on their host crop for up to three species which are important pests of soft fruit and tree crops in the UK and where such attraction has been demonstrated previously. These are the raspberry cane midge, *Resseliella theobaldii*, the blackcurrant leaf midge, *Dasineura tetensii*, and the apple leaf midge, *D. mali*.

Summary of the project and main conclusions

Experimental work over the last growing season has focused on the raspberry cane midge and the blackcurrant leaf midge.

During the spring of 2012, collections where made form raspberry canes both before and after splits where made. In the first year it was noted that female raspberry cane midges were attracted to artificial splits made in canes in the field. The chemicals produced by raspberry primocanes before and after artificial splits were made were compared and chemicals present in larger quantities or only after splits were made were identified.

Due to the warm spring and then cold early summer, no populations of raspberry cane midge could be found in the field, so it was not possible to carry out planned laboratory bioassay and EAG work on this species. Thus subsequent work focussed on the

blackcurrant leaf midge. Volatile collections were made from young blackcurrant leaves and key chemicals produced were identified. Blackcurrant midge larvae were collected and reared to adulthood. A 4-way olfactometer bioassay was established and preliminary studies carried out on the responses of virgin and mated adult midges to the volatiles from blackcurrant shoots.

In conclusion

To date chemicals produced by two varieties of raspberry cane after splitting have been identified as potential attractants for female raspberry cane midge. Chemicals have also been identified from blackcurrant shoots as potential attractants for blackcurrant leaf midge. A laboratory bioassay for these attractants has been developed.

Financial benefits

- None to date

Action points for growers

- None have yet been identified.

SCIENCE SECTION

Introduction

The overall aim of the project is to identify chemicals responsible for attraction of mated females of up to three species of midge to their host plants: the raspberry cane midge, *Resseliella theobaldii*, the blackcurrant leaf midge, *Dasineura tetensii*, and the apple leaf midge, *D. mali*. The three target species are important pests of horticultural crops and identification of these attractants would provide a basis for development of new approaches to monitoring and control of these pests that would be compatible with both conventional IPM and organic strategies. The results will also advance our knowledge of the remarkable ability of insects to find their host plants in terms of whether a few key chemicals are involved or whether they use specific blends of several more ubiquitous chemicals.

In 2011 field observations found that raspberry cane midges where attracted to fresh splits in raspberry primocanes. This work was built upon by taking samples of volatiles before, and again after, the canes were artificially split with a mounted needle. Chemicals which were only present after the cane was split or present in larger amounts were identified. This year the analytical work was continued on two raspberry cultivars.

Sufficient larvae of the raspberry cane midge could not be found for planned bioassay and electroantennogram (EAG) work, and subsequent work focussed on the blackcurrant leaf midge, *Dasineura tetensii,* which is an important pest of blackcurrant plants. Mated females lay their eggs on newly unfurling leaflets. Larval feeding caused the leaves to curl, forming a gall around the larvae within. Larval feeding reduces plant productivity and can lead to stunting in cases of severe infestation. Volatile collections from new leaves were made and analysed so that key chemicals could be identified. A laboratory bioassay was established and preliminary studies carried out on the responses of male and female blackcurrant leaf midge adults to volatiles from blackcurrant shoots.

Materials and methods

Collection of volatiles from raspberry canes

Raspberry plants of the cultivars Octavia and Glen Ample were purchased in summer 2011. Some were left out on the sandbeds at EMR over the winter while others were transferred in November 2011 to smaller 2 litre pots and put into heated glass houses. Those plants that had overwintered under glass produced primocanes early in spring 2012, thus allowing volatile collection to begin early in the 2012 season.

Plants with fresh green primocane were selected and taken to the laboratory at NRI. A cage was made from wire which was placed around the primocane. The bottom was cut off an oven bag (Sainsbury's PLC) and this was put over the cage and secured above and below with ties. A hole was made in the bag with a mounted needle and volatiles sampled with a solid-phase microextraction (SPME) fibre (blue polysiloxane/polydivinyl benzene; Supelco) pushed through the hole (Fig. 1). The fibre was then exposed for 15 minutes. Collections were made from the intact stem and immediately after making a slit in the epidermis (3 cm) with a razor blade.

Once the collection was complete the fibre was inserted into the injector of a gas chromatograph (GC; HP6890; Agilent) coupled to a mass spectrometer (MS; HP5973; Agilent) fitted with a fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µ film thickness) coated with DB5 (Supelco) . Injection was splitless (220º) with the split opening after 1 min and the oven temperature was programmed at 40ºC for 2 min then increased at a rate of 10º/min up to 250ºC. *n*-Alkane standards were run each day and retention times of compounds of interest converted to Kovats Indices (KI). Data were captured and processed with HP Chemstation. Compounds were identified by comparison of their mass spectra with those in the NIST library and of their retention indices with those in the Pherobase (El-Sayed, 2012).

Figure. 1. SPME collection from a raspberry primocane

Collection of volatiles from young blackcurrant shoots

Using the method above, volatiles were collected from new shoots on blackcurrant plants of the cultivar Ben Connan. New cages were made which fitted around blackcurrant shoot tips. An oven bag was put around the cage and secured and volatiles collected using a blue SPME fibre.

Pettersson olfactometer bioassay

Efforts were made to find a population of raspberry cane midge so that olfactometry work could be done on the adult females with the chemicals identified, as produced when the raspberry cane splits. Unfortunately no significant populations could be found in the area of Kent surrounding EMR during 2012.

As a result work was moved to the blackcurrant leaf midge which lays its eggs in new blackcurrant shoot foliage causing it to become twisted. A population of this midge was found in a polytunnel on-site at EMR. Twisted shoots were collected and stored in a ventilated Perspex box with a piece of damp blue tissue. The boxes where checked every few days and mature orange larvae removed and potted up individually in AA cups with a piece of damp filter paper. The AA cups where checked regularly and adults removed and sexed.

Initially the majority of insects tested have been virgin although attempts have been made to obtain mated females. In the current mating method a newly emerged male and female are put into a Perspex screw top jar together and left for two hours. After this period they are separated into their individual AA cups again. After thirty minutes the female is observed to see if she is displaying calling behaviour extending her ovipositor. If she is not calling it is deemed that she has mated and can be used in olfactometer work. This follows the work of Galanihe and Harris (1997)

This bioassay work used an olfactometer similar to that used by Pettersson (1970) and Vet et al (1983) where an insect is given a choice between four distinct odour fields.

Perspex olfactometers were obtained from Rothamstead (cf Birkett et al., 2004) (Fig. 2). These consisted of circular top and bottom sections and a central section with a cut–out, four-pointed star arena space. The total arena diameter was 12 cm with distance from one tip of the star to the opposite being 10 cm. The three sections screwed together with plastic screws and nuts but could be taken apart for cleaning. At the tip of each of the points was a

hole (5 mm) through which a glass arm section could be inserted. The glass arms had one wide end there plant material or filter papers could be placed and then a step down to a much narrower end which fitted into the hole in the arena.

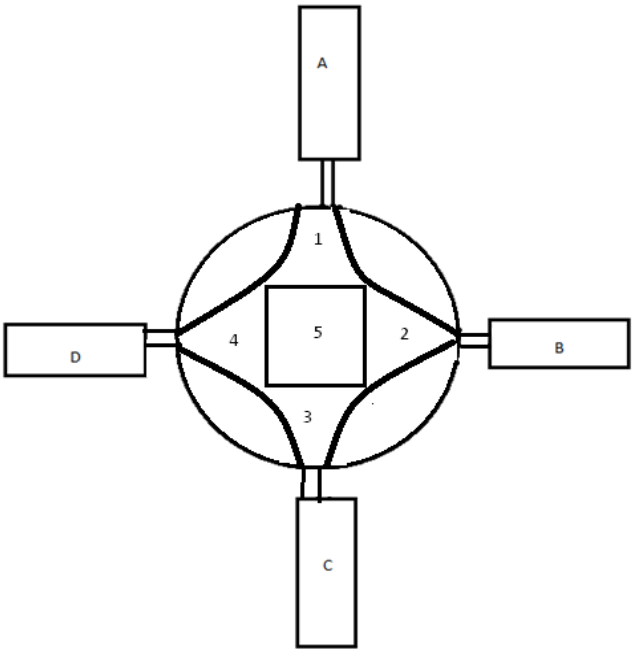

Fig. 2. Diagram of the Pettersson olfactometer (numbers represent zones of the arena and letters represent the different treatment arms).

There was also a hole (5 mm diameter) in the centre of the top plate to allow insects to be put in and a pump attached to draw air in through the glass arms and out through the top (22V, 50Hz WISA model 110). In initial trial runs it was noted that the midges could fly up through the top opening and become stuck in the pipe leading to the pump. To solve this problem a pipe connecter with one side covered in a square of net curtain secured with PTFE tape was connected to the end of the pipe entering the hole in the top of the arena. This prevented insects entering the pipe and the PTFE tape gave a good tight fit.

Small squares of muslin were put over the ends of the arms to prevent insects escaping and make a tight fit. Muslin was used as, although less robust than net curtain, it was disposable and clean (important to allow the air flowed through it to reach the insect). These muslin squares where replaced after each run.

To avoid odour contamination affecting the results a clean arena was used for each insect. To clean the arena the screws were unscrewed and each of the three sections rinsed with

distilled water and then 70% ethanol (made up with distilled water) three times. The top and bottom plates were left to dry upright on a clean incubator shelf over some blue paper (Fig. 3). The central sections could be placed on the shelf horizontally as the edge protected the central section from contamination. The glass arms were washed in the same way each time the treatment in the arm was changed. All equipment was left to air dry (preferably overnight).

Fig. 3. Cleaned components of the olfactometer left to dry in the lab.

The olfactometer was set up in a dark room at EMR. The olfactometer and pump were placed onto the bench. A flow-meter was connected between the tube joining to the top of the arena and the pump to measure the airflow in the system. For each run the airflow was set to 400 cm^3/min, meaning that 100 cm^3/min air was being drawn in through each of the arms. A block of foam was placed under the pump to absorb the vibrations so that they did not affect the arena. The set up was illuminated using a light box which contained a 22W fluorescent lamp which shone out through a sheet of opaque plastic giving a diffuse light. The light box was suspended approximately 30 cm above the bench.

The arena was divided into five sections (noted on a white piece of paper underneath the set up) – the central section, section 5 into which the midges first entered the arena through the hole; and sections 1-4 which each corresponded to a glass arm. For tests using plant material a shoot tip was placed into one of the arms and the other three left as blank controls. The insect was observed for 15 minutes and the time spent and entries into each area recorded using the computer program OLFA (Boorland International). After each run the arms where moved through 90° to eliminate any directional bias (i.e. arm 1 moved to the arm 2 position etc.).

The arena with pump attached was tested using smoke from a bee smoker to visualise the air entering the arena (Fig. 4). Although only faintly seen in the camera image, when the smoker was placed close to one of the side openings the smoke could be seen being sucked in. Clear boundaries could be seen between the four quarters of the arena with very little mixing of airflow.

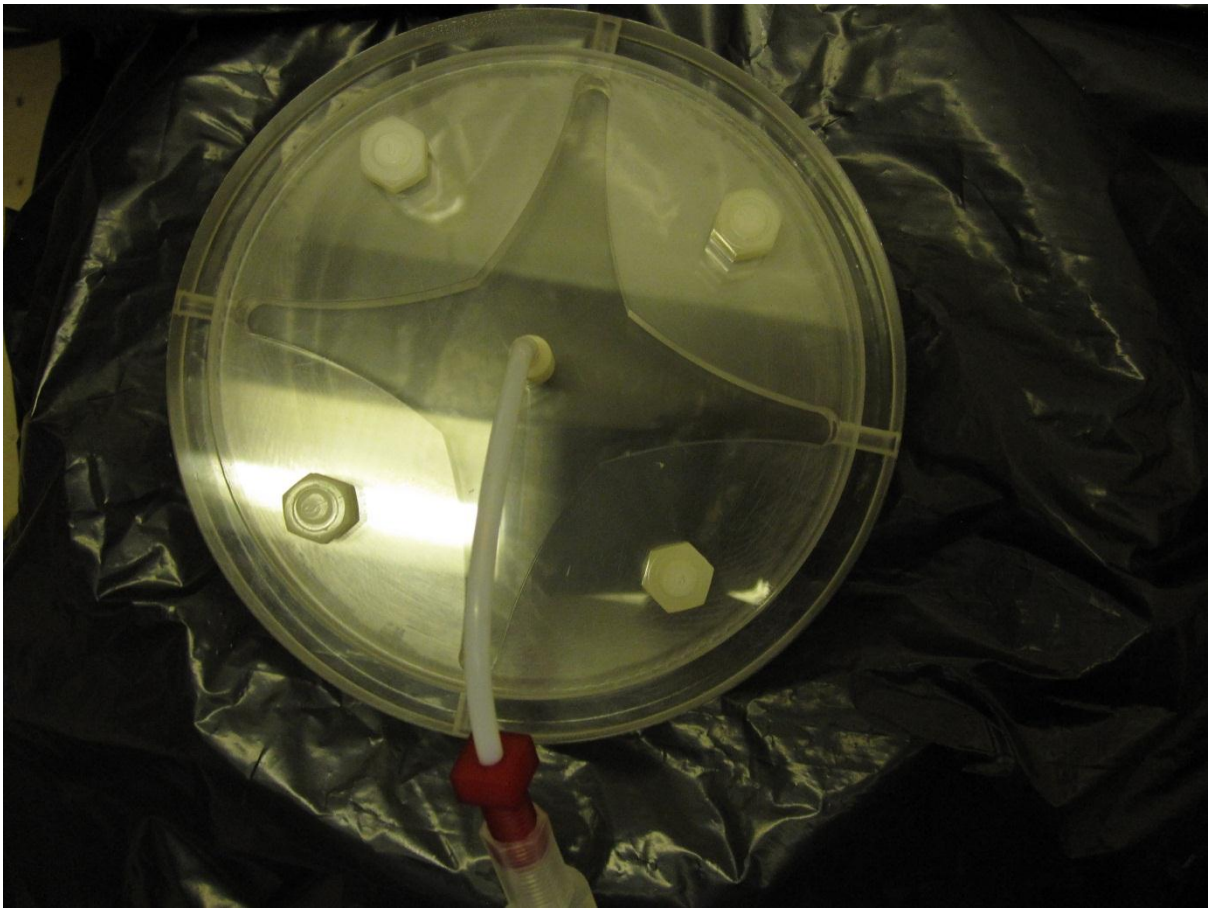

Fig. 4. Arena with pump attached and smoke to visualise air flow. The smoker was located near the leftmost opening and smoke can faintly be seen entering the arena.

Results

Volatiles from raspberry primocanes

Collections have been completed for nine plants before and after artificial splitting, including plants from both the cultivars Glen Ample and Octavia. Chemicals identified are listed in Table 1.

Table 1. Chemicals identified in SPME collections made from intact and split raspberry canes (RT retention time in minutes; KI Kovats Indices; Pherobase (Kovats Indices from Pherobase)

RT (min)	KI	Compound	Pherobase
4.95	784	(*Z*)-3-hexenal	784
5.94	844	(*E*)-2-hexenal	845
6.02	848	(*Z*)-3-hexenol	857
6.18	858	(*E*)-2-hexenol	861
6.78	895	oxime?	?
6.96	905	(*E,E*)-2,4-hexadienal	909
7.80	954	5-ethyl-2-furanone	984
8.04	968	2-pentene,3-ethyl-2-methyl-	?
8.34	986	6-methyl-5-hepten-2-one	985
8.44	992	bicyclo[3.1.0]hexane,1,5-dimethyl	?
8.68	1006	(*Z*)-3-hexenyl acetate	1007
9.16	1036	limonene	1036
10.24	1104	linalool	1107
10.30	1108	nonanal	1108
11.08	1156	citronellal	1153
11.79	1201	methyl salicylate	1206
11.85	1205	myrtenal	1193
11.87	1206	decanal	1204
12.19	1229	citronellol	1228
12.83	1275	geranial	1270
14.71	1411	dodecanal	1413
15.06	1440	beta-caryophyllene	1428
15.29	1458	geranyl acetone	1453
17.05	1600	hexadecane	1600
18.18	1701	heptadecane	1700

To identify differences between the cane before and after splitting it was possible to plot the two gas chromatograph (GC) traces against each other and compare the peaks which were present in the two traces at the same time (Fig. 5). In this example from cv. Octavia, six compounds were observed to be greatly enhanced in the volatiles collected after splitting - linalool, citronellal, myrtenal, citronellol, neral and geranial.

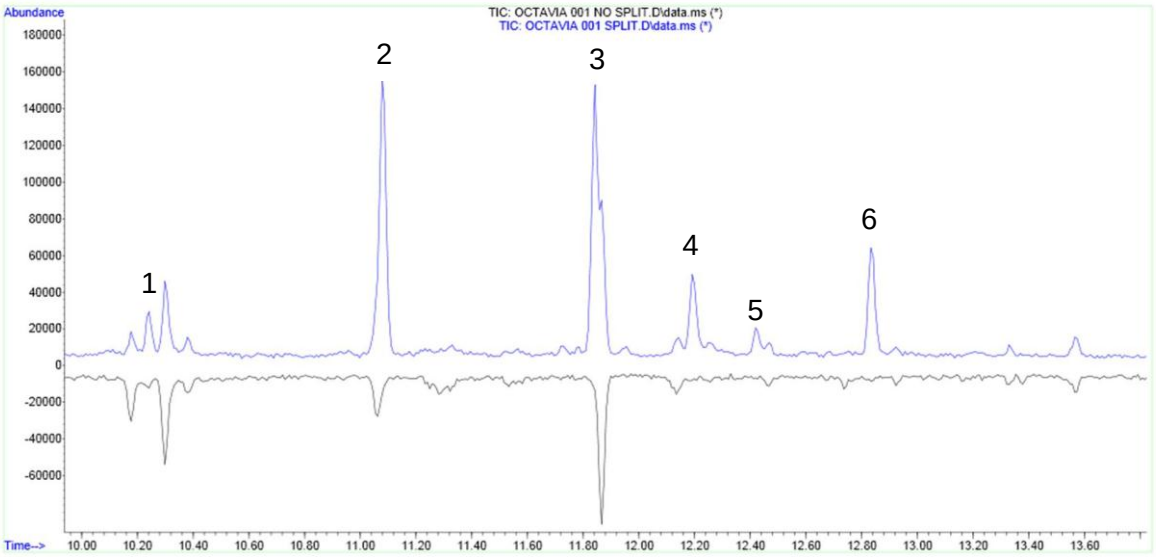

Fig. 5. GC-MS traces for SPME collections from a single cv. Octavia raspberry plant primocane before (bottom) and after (top) a split was made (1 linalool, 2 citronellal, 3 myrtenal, 4 citronellol, 5 neral, 6 geranial)

Peak areas, representing amounts of compounds present were converted to percentages of the total blend and also quantified relative to the peak area of an external standard, decyl acetate. The data are still being analysed, but the percentage composition of the volatiles before and after splitting are shown for cvs. Glen Ample (Fig. 6) and Octavia (Fig. 7).

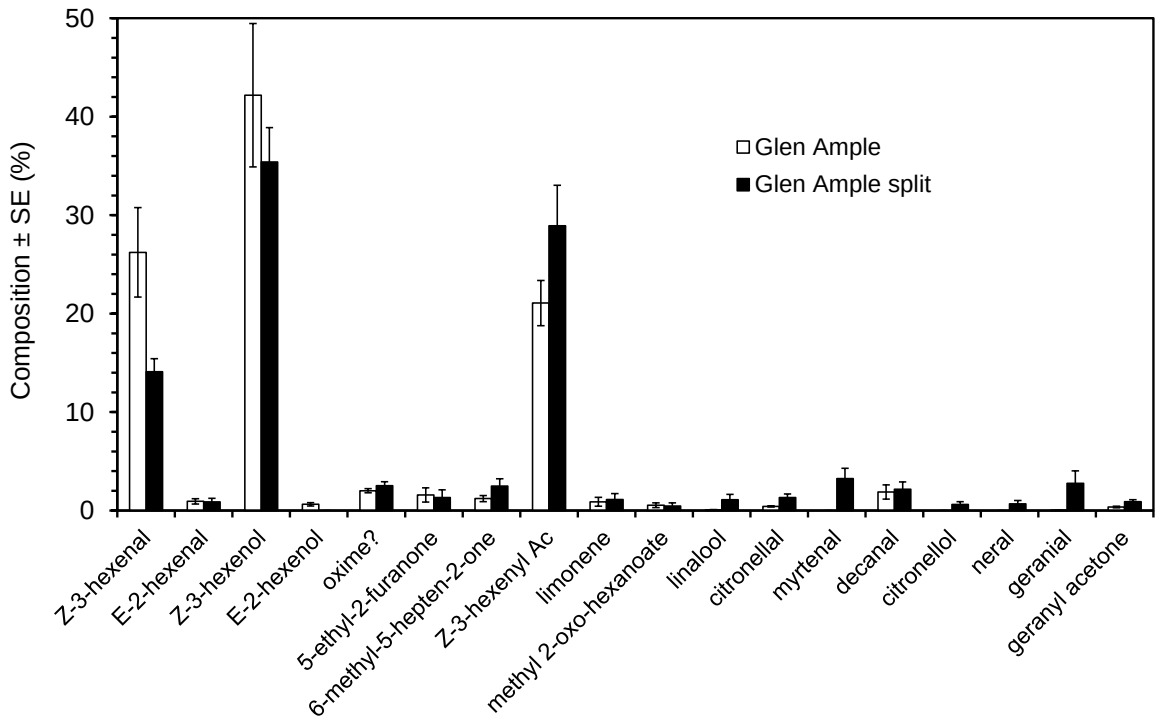

Fig 6. Comparison of volatiles produced before and after an artificial split was made in a cv. Glen Ample plant in terms of percentage of each chemical in the total volatile emission (*N* = 5).

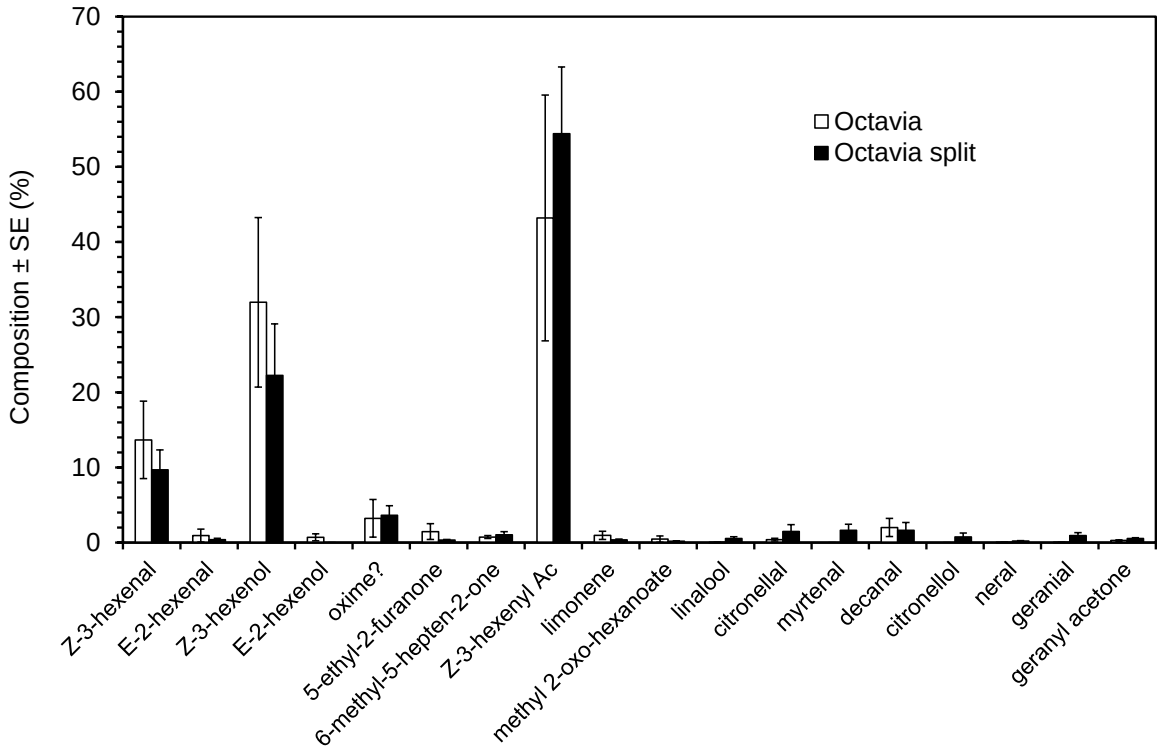

Fig 7. Comparison of volatiles produced before and after an artificial split was made in a cv. Octavia plant in terms of percentage of each chemical in the total volatile emission (*N* = 4; cf. Fig. 5).

The major components in these collections were the "green leaf volatiles", (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate. However, in both cultivars six compounds were either present only after splitting of the stem or their relative proportions were greatly increased – linalool, citronellol, myrtenal, citronellol, neral and geranial. Interestingly, 6-methyl-5-hepten-2-one and geranyl acetone, compounds often emitted by plants after wounding, were present both before and after splitting.

Volatiles from blackcurrant shoots

Analysis of this data is still ongoing. A typical trace is shown in Fig. 8 and a preliminary list of chemicals identified is shown in Table 3. The main components were the monoterpene hydrocarbons 3-carene and α-terpinolene with smaller amounts of *cis*- and *trans*-β-ocimene.

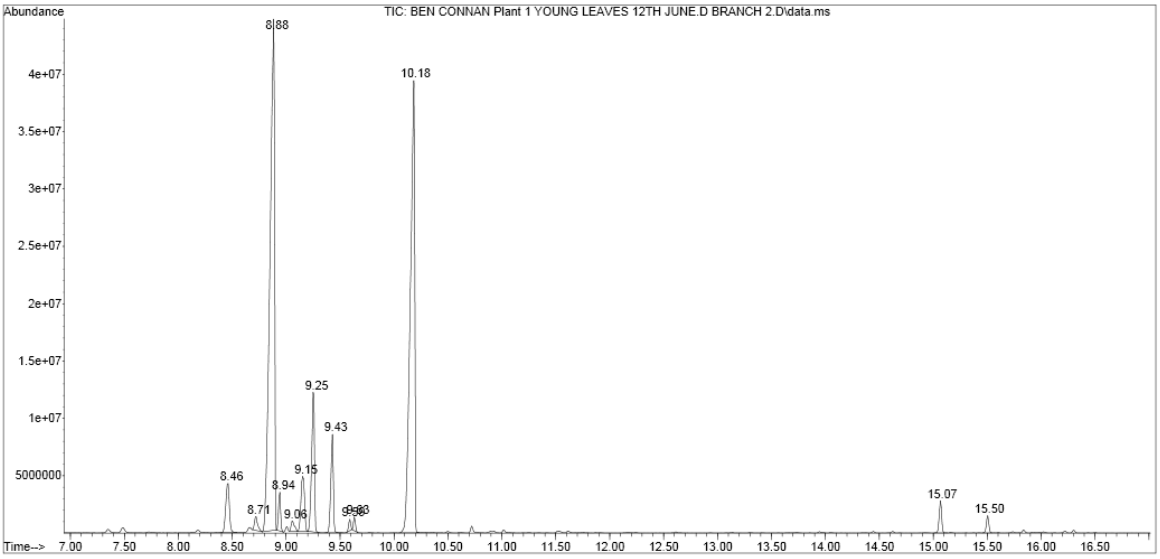

Fig. 8. GC-MS Analysis of SPME sample from shoot of cv. Ben Connan blackcurrant plant

Table 3. Chemicals identified in SPME samples taken from new leaves of cv. Ben Conan blackcurrant plants (RT retention time (mins), KI Kovats Indices, KI from Pherobase).

RT (min)	KI	Compound	Pherobase
7.34	927	α-thujene	923
7.49	936	α-pinene	939
8.46	993	myrcene	991
8.71	1008	α-phellandrene	1005
8.88	1019	3-carene	1011
8.94	1023	α-terpinene	1018
9.01	1027	*o*-cymene	1020
9.06	1030	*p*-cymene	1026
9.25	1042	*cis*-β-ocimene	1040
9.43	1053	*trans*-β-ocimene	1050
9.59	1063	γ-terpinene	1062
9.63	1066	β-terpinene	1071
10.18	1100	α-terpinolene	1088
10.72	1134	dimethyloctatriene(?)	
15.07	1436	β-caryophyllene	1418
15.51	1467	α-caryophyllene	1454

Pettersson olfactometer bioassay

The olfactometer has been set up and preliminary studies carried out with blackcurrant midge.

Initial results were possibly confounded by the fact that insects seemed to favour the fourth arm which was nearest to the pump. The pump was moved on to a separate shelf.

Discussion and conclusions

Volatiles from raspberry primocanes

SPME was found to be a convenient means to sample the rapid burst of chemicals produced when raspberry primocanes are split. Sampling before and after splitting showed that in both cvs. Octavia and Glen Ample six compounds were either present only after splitting of the stem or their relative proportions were greatly increased – linalool, citronellol, myrtenal, citronellol, neral and geranial. These compounds were also reported by Hall et al. (2011) for the Glen Moy cultivar. Interestingly, 6-methyl-5-hepten-2-one and geranyl acetone, compounds often emitted by plants after wounding, were present both before and after splitting.

Volatiles from blackcurrant shoots

Chemicals produced by young blackcurrant buds and leaves have been identified, although identification and quantification is still on-going. The chemical profile is very different from that of the raspberry canes, with only small amounts of the typical “green leaf volatiles”, and the odour has a distinct smell to the human nose, similar to that of blackcurrant fruits.

Pettersson olfactometer bioassay

The work is currently ongoing with the blackcurrant leaf midge and it is hoped that work can also begin soon with another midge, the apple leaf midge. Analysis of the results will be done in the winter months. As well as the time spent in each area, the OLFA program also records the number of entries into each arm. For future runs this data will also be recorded and entries into the arms with and without plant material can be compared. The pump will remain away from the arena so that it does not interfere with the results.

Future work

Collections of volatiles from raspberry canes will be analysed by GC coupled to EAG, recording from the antennae of both female and male raspberry cane midge, to identify compounds detected by receptors on the antennae. The laboratory bioassay will be used to assay the attractiveness of these chemicals and blends to raspberry cane midge.

Similar work will be carried out on the volatiles from blackcurrant shoots with the blackcurrant leaf midge, depending upon availability of insects.

Knowledge and Technology Transfer

Posters were presented at the HDC studentship conference in June 2012 and the Royal Entomological Society Annual Meeting at Anglia Ruskin University, Cambridge, in July 2012.

Helen Thomas, Jerry Cross and David Hall (2012). Chemical attractants for midges produced when raspberry canes split.

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