Project title:	Sex Pheromone Trap For Monitoring Blackberry Leaf Midge
Project number:	SF 117
Project leader:	Prof David R Hall, Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Kent ME4 4TB
Report:	Annual, March 2011
Previous report:	None
Key staff:	Dr Paul Douglas Dudley Farman Prof Jerry Cross (EMR) Mr Adrian Harris (EMR) Dr Michelle Fountain (EMR)
Location of project:	NRI/EMR
Industry Representative:	Tom Maynard
Date project commenced:	1 April 2010
Date project completed (or expected completion date):	31 March 2012

The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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

- The female sex pheromone of the blackberry leaf midge has been identified and synthesized.

Background and expected deliverables

Blackberry leaf midge (*Dasineura plicatrix*) has recently developed as a serious pest of blackberry and has now spread to raspberry in the UK and elsewhere in Europe. It attacks and kills the shoot tips of primocanes leading to growth stunting and cane branching. Growers consider that it significantly affects yield in both crops, although no crop damage assessments have yet been made. Growers currently have no method of predicting or monitoring attacks or timing sprays to control it. Timing of application is critical with midge pests as the larvae quickly become protected within the leaf rolls and it is important not to disrupt natural biocontrol mechanisms.

The female midge produces a powerful sex pheromone which attracts males. Identification of the pheromone would enable the development of sex pheromone traps for monitoring the pest and timing measures for its control. Such traps have already been developed for the raspberry cane midge and blackcurrant leaf midge. The traps will be ideal for timing application of control programmes which have been developed by ADAS in project SF102.

The most effective insecticides against midge pests of UK fruit crops vary with pest species. For raspberry cane midge for instance, chlorpyrifos is highly effective and synthetic pyrethoids have limited activity, whereas for blackcurrant leaf midge, the reverse is true. Neither are very effective against apple leaf midge. Recent work by EMR has shown that a novel systemic/translaminar selective insecticide is effective for control of blackcurrant and apple leaf midges, providing sex pheromone monitoring traps are used for timing sprays at the start of emergence of the midges. Though it is unlikely that this novel insecticide will be available for use on blackberry or raspberry, sex pheromone monitoring traps will be vital for best timing of sprays of other insecticides against blackberry leaf midge.

Summary

The female-produced sex pheromone of blackberry leaf midge was partially identified in

HDC-funded PhD project CP38 ('The Chemical Diversity of Midge Pheromones'). This project aims to complete identification of the chemical structures of the components of the pheromone, to synthesise them and develop effective lures and traps for use by growers. It is anticipated that the traps will be invaluable in monitoring the pest to assess the need for control measures and to time the application of these more effectively. Previous results indicated that the female-produced sex pheromone of the blackberry midge, D. plicatrix, consists of two components and that these were 15-carbon compounds with an acetate group at C-2 and two and one double bonds respectively. The current work provides good evidence that these are (2R,Z6,Z9)-2-acetoxy-6,9-pentadecadiene as the major component and (2R,Z6)-2-acetoxy-6-pentadecene as the minor.

When the synthetic compounds were tested in the field, the minor component alone was unattractive to male blackberry leaf midges when tested as the racemic or the separate enantiomers at two different loadings. In contrast, the (*R*)-enantiomer of the major component was highly attractive. The (*S*)-enantiomer and racemic mixture were completely unattractive, indicating that the (*S*)-enantiomer actually inhibits the attractiveness of the (*R*)-enantiomer. Moreover, adding the minor component to the major at a 1:3 ratio significantly increased the attractiveness of the major component.
Thus the two components of the female sex pheromone of the blackberry midge, *D. plicatrix*, have been identified and a blend of the synthetic compounds is highly attractive to male midges in field trapping tests. In the second year of the project the blend ratio and loading of pheromone in the lure will be optimised and the attractiveness of the most effective synthetic lure compared to that of a virgin female midge. Lures and traps will also be made available to a sample of growers for evaluation for monitoring the pest.

Financial benefits

No reliable figures are available but these will be investigated during the second year of the project.

Action points for growers

- Lures and traps for blackberry midge will be made available to selected growers for evaluation as monitoring tools in the second year of the project.

SCIENCE SECTION

Introduction

Blackberry leaf midge (*Dasineura plicatrix*) has recently developed as a serious pest of blackberry and has now spread to raspberry in the UK and elsewhere in Europe. It attacks the shoot tips killing out the terminals, stunting growth and causing branching. Growers consider that it significantly affects yield in both crops, although no crop damage assessment trials have yet been done. Growers currently have no method of predicting or monitoring the severity of attacks or of timing application of control methods. Timing of application is critical with midge pests as the larvae quickly become protected within the leaf rolls and it important not to disrupt natural biocontrol mechanisms.

The female-produced sex pheromone of blackberry leaf midge was partially identified in HDC-funded PhD project CP38 'The Chemical Diversity of Midge Pheromones', which was recently completed by Lakmali Amarawardana. The pheromone was shown to be a blend of mono-unsaturated and di-unsaturated 15-carbon mono-acetates with the acetate at C2. However, the exact positions and configurations of the double bonds in these compounds have not yet been determined.

This project aims to complete identification of the chemical structures of the components of the pheromone, to synthesise them and develop effective lures and traps for use by growers. It is anticipated that the traps will prove invaluable in monitoring the pest to assess the need for control measures and to time the application of these more effectively. Pheromone traps are now available for other bush fruit midges including the raspberry cane midge and blackcurrant leaf midge.

Materials and methods

Pheromone Identification and Synthesis

Mono- and di-unsaturated 15-carbon acetates were synthesised as candidate pheromone components and their gas chromatographic (GC) retention indices and mass spectra (MS) were determined and compared with corresponding data on the naturally-produced pheromone components obtained in HDC Studentship CP38.

Mono-unsaturated acetates and (*Z*,*E*)-2-acetoxy-10,13-pentadecadiene were synthesised from the corresponding 14-carbon alcohols which are components of many Lepidopteran sex pheromones and were available in the NRI library of compounds (Figure 1). In some cases the (*E*)-isomers were obtained by isomerisation of the corresponding (*Z*)-isomers by heating at 120°C with thiophenol.

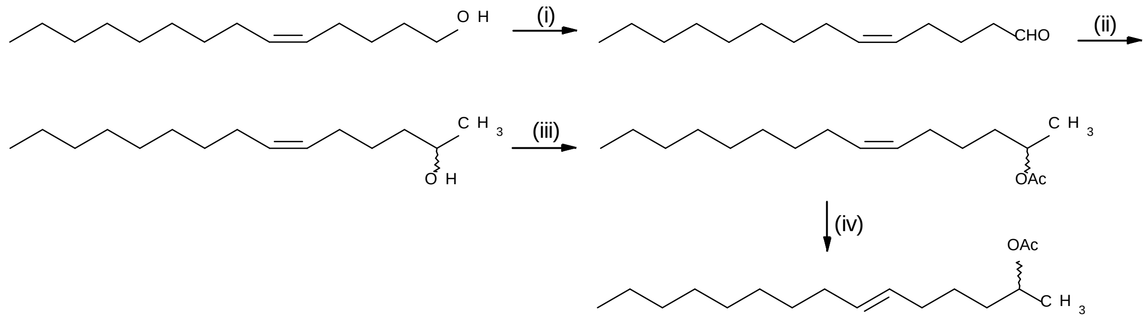


Figure 1. Synthesis of (*Z*)- and (*E*)-2-acetoxy-6-pentadecene (Reagents (i) PCC/CH_2Cl_2; (ii) MeMgI/ether; (iii) Ac_2O/pyridine; (iv) thiophenol)

(*Z,Z*)-2-Acetoxy-6,9-pentadecadiene was synthesised in six steps from 2-octyn-1-ol and 5-hexyn-1-ol as shown in Figure 2. The (*Z,E)*-isomer was synthesised by a similar route replacing the 2-octyn-1-ol with (Z)-2-octen-1-ol.

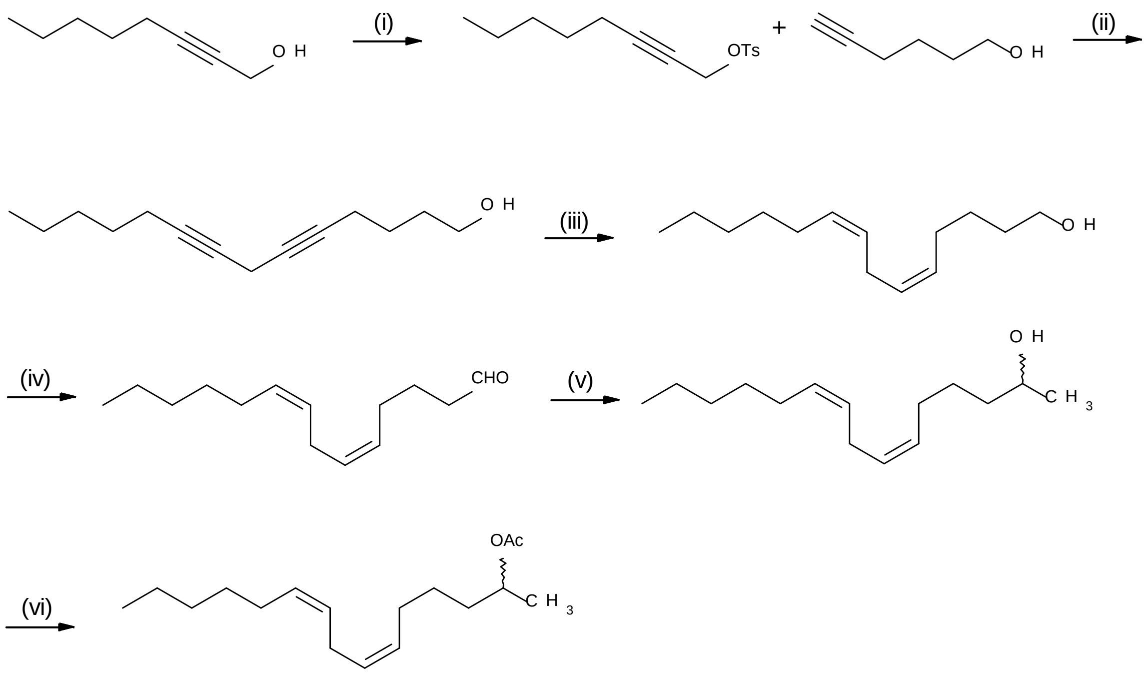


Figure 2. Synthesis of (*Z,Z*)-2-acetoxy-6,9-pentadecadiene (Reagents (i) TsCl/pyridine; (ii) EtMgBr/THF/CuI; (iii) H_2/Lindlar catalyst or P2-nickel; (iv) PCC/CH_2Cl_2; (v) MeMgI/ether; (vi) Ac_2O/pyridine)

The above syntheses gave racemic products. The corresponding enantiomers were prepared in high enantiomeric purity (>99%) by acetylating the corresponding alcohol in the presence of lipase from *Candida antarctica* which gave the (*R*)-acetate and unreacted (*S*)-alcohol (Figure 3). These were separated by liquid chromatography and the alcohol acetylated with acetic anhydride and pyridine.

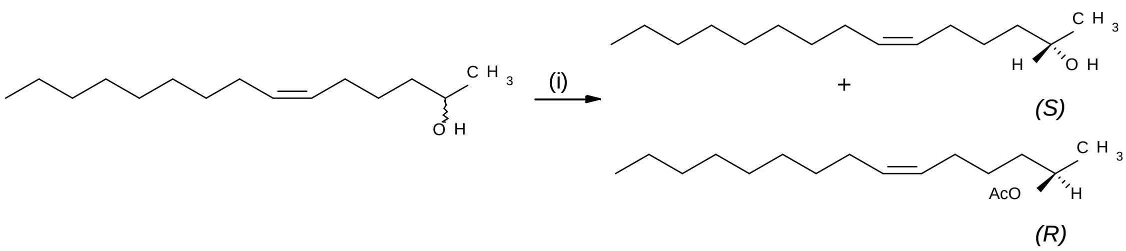


Figure 3. Enzymatic resolution of enantiomers of (Z)-6-pentadecen-1-ol (Reagent (i) lipase from *Candida antarctica*/vinyl acetate/ether)

Pheromone Formulation

Rubber septa were impregnated with the minor pheromone component, (*Z*)-2-acetoxy-6-pentadecene (100 µg) and maintained in a laboratory windtunnel at 27°C and 8 km/hr windspeed. At intervals volatiles were collected from duplicate septa onto Porapak resin and trapped volatiles quantified by GC analysis.

Field Tests

Field tests were carried out in both blackberry and raspberry fields at Belks Farm and Salmons Farm, Kent. Traps were standard white sticky delta traps positioned 0.5 m above ground level and 25 m apart. Lures were red rubber septa impregnated with 100 µg of pheromone. Traps were set out in randomised complete block designs with eight replicates and catches were recorded and discarded weekly.

Total catches in each replicate were transformed to log(x+1), subjected to analysis of variance and differences between means were tested for significance with a Least Significant (LSD) difference test (*P*<0.05).

Results

Pheromone Identification and Synthesis

In HDC Project CP38, analysis of volatiles collected from virgin female *D. plicatrix* by gas chromatography (GC) coupled to electroantennographic (EAG) recording from the antenna of a male midge showed two components eliciting an EAG response. One – the major component – was present in larger quantities than the other – the minor component – and these were assumed to be components of the female sex pheromone. Amounts of material obtained were small and the minor component could not always be detected.

The GC retention indices on polar and non-polar GC columns and the mass spectra indicated that the major component was a 15-carbon compound with an acetate function at C-2 and two non-conjugated double bonds. Data for the minor component suggested it was a similar compound with only one double bond. These proposals were further supported by showing that after catalytic hydrogenation of the collection of volatiles the two pheromone components had disappeared and 2-acetoxypentadecane was formed.

The mass spectra of the pheromone components gave no clear indication of the positions of the double bonds and the amounts present were too small for any further studies such as reaction with dimethyldisulphide, epoxidation or ozonolysis which can be used to determine the positions of double bonds.

Accordingly a number of candidate compounds were synthesised and their GC retention indices and mass spectra compared with those of the natural pheromone components.

For the minor component, mono-unsaturated 2-acetoxypentadecenes were conveniently synthesised from the corresponding tetradecen-1-ols many of which are components of Lepidopteran pheromones. Retention indices of these relative to the retention times of straight-chain acetates on both polar (DBWax) and non-polar (DB5) GC columns are shown in Table 1. The (*Z*6)-isomer had retention indices very similar to those of the minor pheromone component on both GC columns.

Table 1. Retention indices of synthesised mono-unsaturated 2-acetoxypentadecenes and the minor pheromone component relative to the retention times of straight-chain acetates on polar (DBWax) and non-polar (DB5) GC columns.

	Retention Index	
Double Bond	Polar	Non-Polar
Z5	1384	1387
E5	1396	1400
Z6	1392	1390
E6	1398	1397
Z7	1392	1390
E7	1383	1397
Z8	1400	1394
E8	1400	1400
Z9	1403	1398
E9	1402	1401
Z10	1411	1405
E10	1407	1405
Z11	1420	1410
E11	1408	1407
Z12	1417	1415
E12	1417	1410
Minor pheromone component	1391	1388*

Comparison of the mass spectrum of (*Z*)-2-acetoxy-6-pentadecene with that of the minor pheromone component showed good agreement (Figure 4). In particular there was an ion at *m/z* 166 that was only present in the spectra of the 6-isomers and not in those of other positional isomers (e.g. Figure 5).

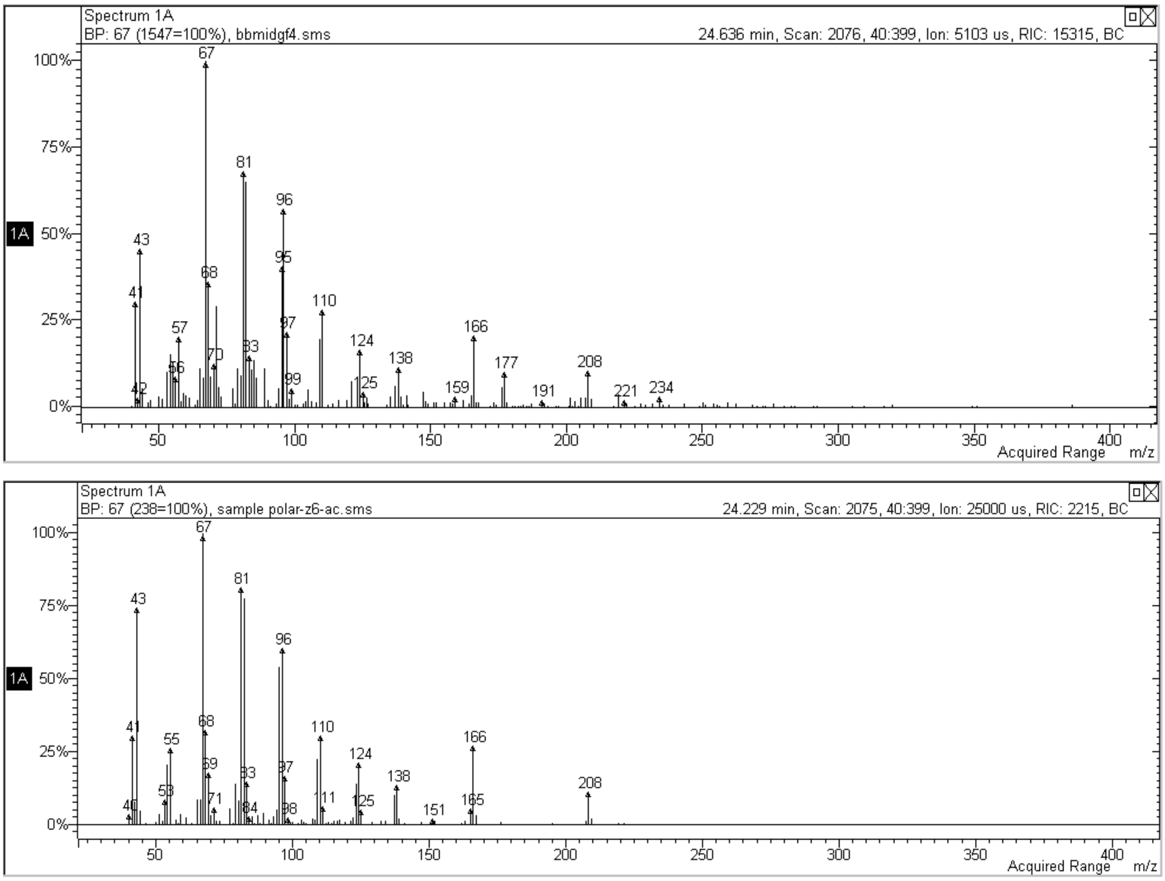


Figure 4. Mass spectra of minor pheromone component (upper) and (Z)-2-acetoxy-6-pentadecene (lower).

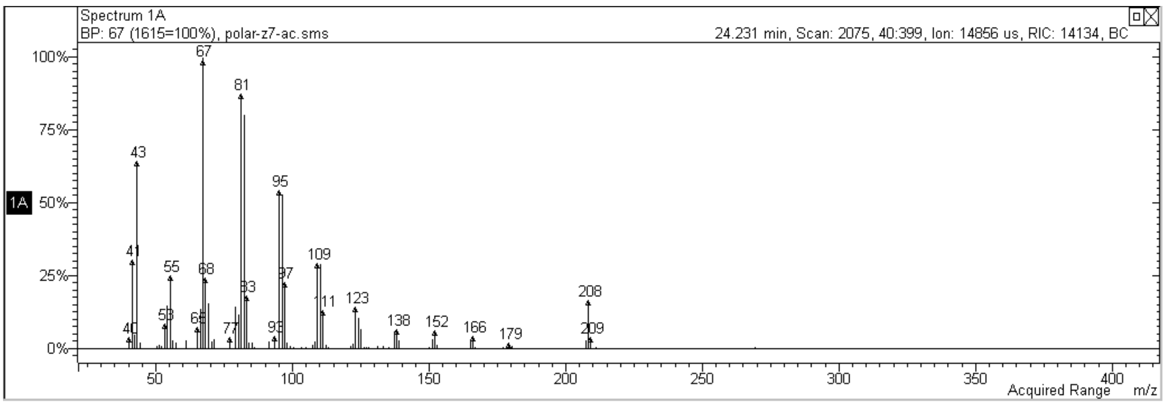


Figure 5. Mass spectrum of (Z)-2-acetoxy-7-pentadecene.

Given the structure of the minor pheromone component, it was thought likely that one of the double bonds in the major component would also be in the *Z*6-position. Calculations based on the relative shifts in retention index for double bonds in the different positions suggested the 6,9-isomer as a candidate compound. The (*Z,Z*)-2-acetoxy-6,9-pentadecadiene was synthesised and shown to have identical retention indices and mass spectrum to those of the natural major pheromone component (Table 2 and Figure 6). The retention indices were different from those for the (*Z,E*)-isomer and also those of (*Z,E*)-2-acetoxy-10,13-pentadecadiene which were also synthesised (Table 2).

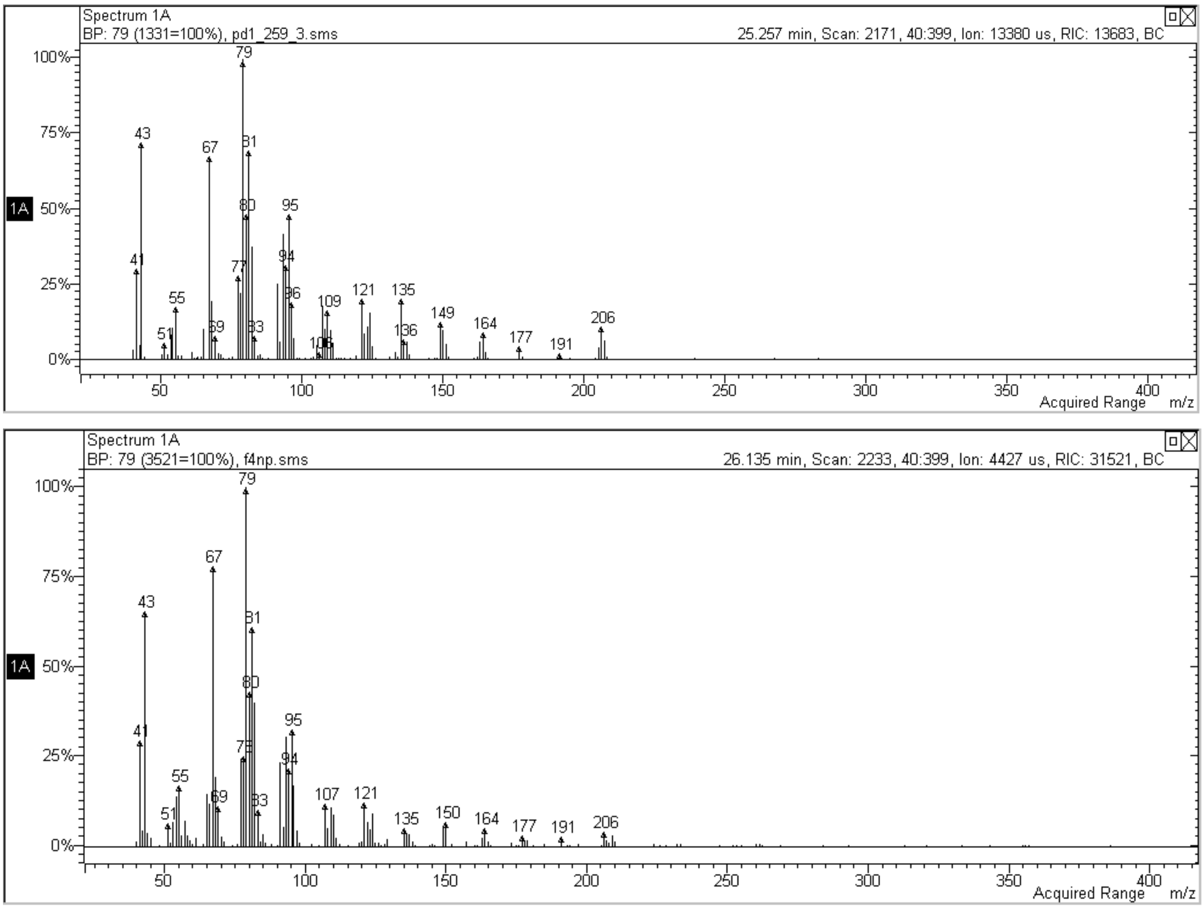
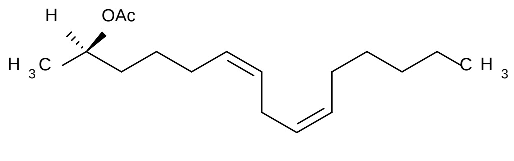


Figure 6. Mass spectra of major pheromone component (upper) and (*Z,Z*)-2-acetoxy-6,9-pentadecadiene (lower).

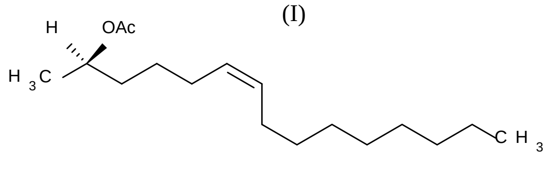
Table 2. Retention indices of synthesised di-unsaturated 2-acetoxypentadecadienes and the major pheromone component relative to the retention times of straight-chain acetates on polar (DBWax) and non-polar (DB5) GC columns.

Compound	Retention Index	
	Polar	Non-Polar
Z6,Z9-C15:2Ac	1435	1382
Unknown diene	1444	1393
Unknown diene	1452	1396
Z6,E9-C15:2Ac	1473	1379
Z10,E13-C15:2Ac	1435	1319
Major pheromone component	1434	1383

From these results, it was concluded that (*Z,Z*)-2-acetoxy-6,9-pentadecadiene and (*Z*)-2-acetoxy-6-pentadecene (Figure 7) were the most promising candidates for the major and minor pheromone components respectively. It was not possible to determine the chirality of the naturally-occurring pheromone components and the (*R*)- and (*S*)-enantiomers of both components were synthesised for field testing.



(I)



(II)

Figure 7. Proposed structures for pheromone components (2*R*,6*Z*,9*Z*)-2-acetoxy-6,9-pentadecadiene (I) and (2*R*,6*Z*)-2-acetoxy-6-pentadecene (II)

Pheromone Formulation

Release of (*Z*)-2-acetoxy-6-pentadecene from rubber septa was initially rapid but then settled down to a relatively constant rate (Figure 8). Release continued for at least 51 days even at 27°C, so lures should last for at least two months in the field under normal UK conditions.

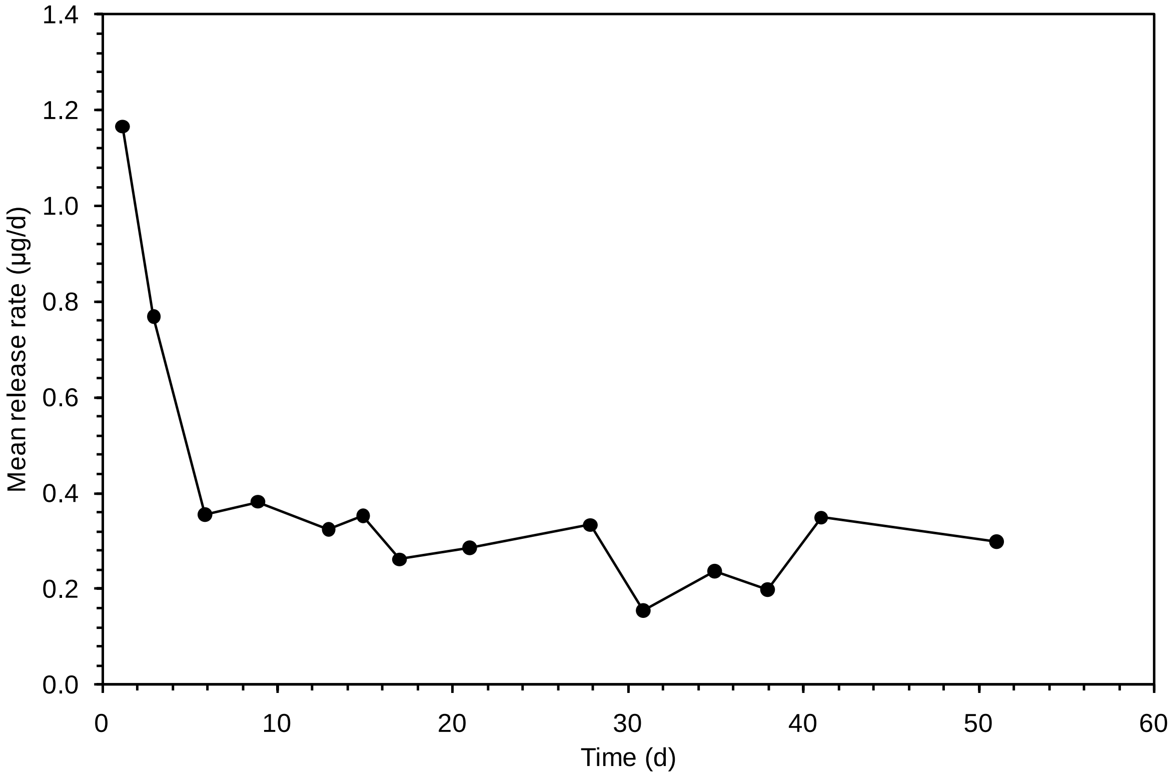


Figure 8. Release rate of (*Z*6)-2-acetoxy-6-pentadecene from rubber septa (initial loading 100 µg; 27°C, 8 km/h windspeed; mean of two replicates)

Field Tests

In a first experiment, the minor pheromone component was tested alone as the racemic and the separate enantiomers at two different loadings. Only low numbers of blackberry midge were captured and there were no significant differences between catches with any of the treatments, including the unbaited control (Table 3). Although there was no "positive control" it was thought that blackberry midge was present and thus that the proposed minor pheromone component alone was unattractive, or the structure was wrong.

Table 3. Mean total catches of blackberry midge, *D. plicatrix*, in traps baited with (*Z*)-2-acetoxy-6-pentadecene (10 replicates at Salmons, 10 replicates at Belks; 6 May – 8 June 2011)

Compound	Loading	Mean total catch/trap
racemic	10 µg	0.38
racemic	100 µg	0.75
2S	10 µg	0.65
2S	100 µg	0.55
2R	10 µg	0.63
2R	100 µg	0.35
control	unbaited	0.40

In a second experiment, the proposed major compound was tested at one loading as the racemic and separate enantiomers. Results from blackberry plots shown in Figure 9 showed that the (2*R*,*Z*6,*Z*9)-2-acetoxy-6,9-pentadecadiene was significantly attractive to male blackberry midge but the (*S*)-enantiomer and racemic material were not.

A third field test was carried out to determine the effect of blending the two pheromone components together as their (R)-enantiomers. A 3:1 blend of the major and minor components was used as this approximated to the ratio determined by analysis of volatiles from the female midge. Results in Figure 10 confirmed the previous results that the major component alone was significantly attractive to blackberry midge males but the minor component was not. Furthermore, a 3:1 blend of the major and minor components was significantly more attractive than the major component alone.

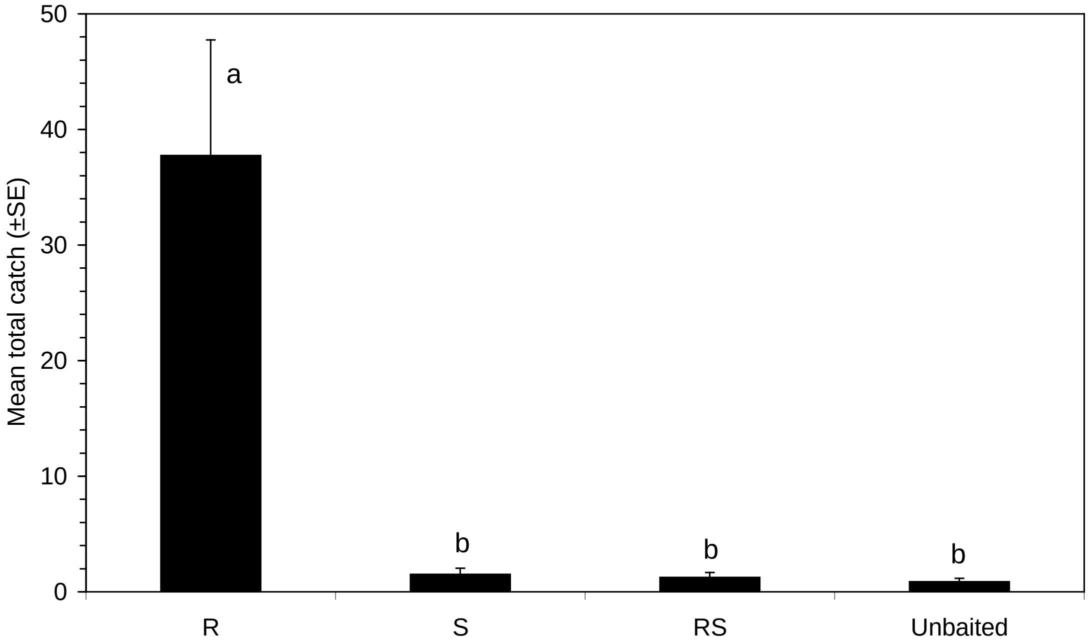


Figure 9. Trapping of *D. plicatrix* with (R)- and (S)-enantiomers and racemic (*Z*6,*Z*9)-2-acetoxy-6,9-pentadecadiene (9 June – 19 August 2010; 4 replicates at Belks, 4 replicates at Salmons; bars with different letters are significantly different after ANOVA on data transformed to log(x+1) and LSD test, $P<0.05$)

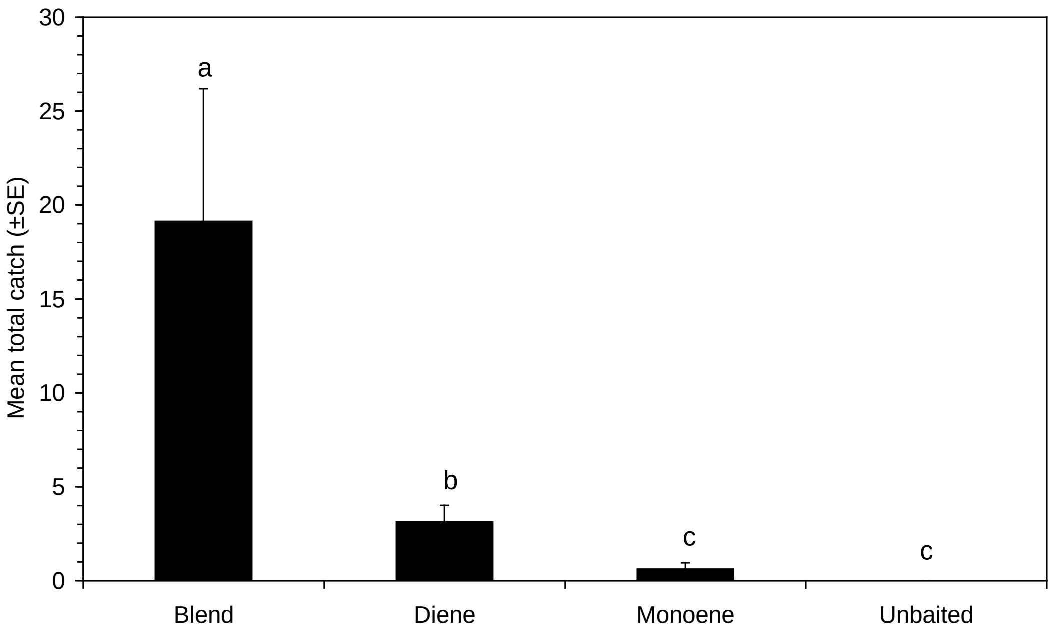


Figure 10. Trapping of *D. plicatrix* with (2*R*,Z6,Z9)-2-acetoxy-6,9-pentadecadiene (diene), (2*R*,*Z*6)-2-acetoxy-6-pentadecene (monoene) and a 3:1 blend (19 August – 17 September 2010; 8 replicates; bars with different letters are significantly different after ANOVA on data transformed to log(x+1) and LSD test, $P<0.05$).

Discussion

Previous results indicated that the female-produced sex pheromone of the blackberry midge, *D. plicatrix*, consists of two components and that these were 15-carbon compounds with an acetate group at C-2 and two and one double bonds respectively. The current work provides good evidence that these are (2*R*,*Z*6,*Z*9)-2-acetoxy-6,9-pentadecadiene as the major component and (2*R*,*Z*6)-2-acetoxy-6-pentadecene as the minor. The GC retention indices of the synthetic compounds are very similar to those of the natural pheromone components on both polar and non-polar GC columns and their mass spectra match well. In particular, the mass spectrum of the minor pheromone component has a significant ion at *m/z* 166 which was only observed in 2-acetoxy-6-pentadecene isomers and not other positional isomers.

When the synthetic compounds were tested in the field the minor component alone was unattractive to male blackberry midges when tested as the racemic or the separate enantiomers at two different loadings. In contrast, the (*R*)-enantiomer of the major component was highly attractive. The (*S*)-enantiomer and racemic mixture were completely unattractive, indicating that the (*S*)-enantiomer actually inhibits the attractiveness of the (*R*)-enantiomer. Moreover, adding the minor component to the major at a 1:3 ratio significantly increased the attractiveness of the major component. Only the (*R*)-enantiomer of the minor component was tested in the blend as it is assumed that the major and minor components have the same configuration.

The proposed structures for the pheromone components of *D. plicatrix* follow the pattern found in other midge species of having an unbranched chain of an odd number of carbon atoms with an oxygenated functionality at C-2. However, these are the only examples with 15 carbon atoms identified to date (Hall et al., in preparation). The pattern of double bonds is similar to that in the homologous (2*S*,4*Z*,7*Z*)-2-acetoxy-4,7-tridecadiene, pheromone of the Douglas fir-cone gall midge, *Contarinia oregonensis* (Griess et al., 2002).

Conclusions

Components of the female sex pheromone of the blackberry midge, *D. plicatrix*, have been identified and a blend of the synthetic compounds is highly attractive to male midges in field trapping tests. In the second year of the project the blend ratio and loading of pheromone in the lure will be optimised and the attractiveness of the most effective synthetic lure compared with that of a virgin female midge. Lures and traps will also be made available to a sample of growers for evaluation for monitoring the pest.

Knowledge and Technology Transfer

An update on the work was provided for the Soft Fruit Agronomists' Handbook 2011.

References

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