Isolation and identification of floral attractants from a nectar plant for the dried bean beetle, *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae, Bruchinae)

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Abstract

BACKGROUND: The response of virgin females of the legume pest *Acanthoscelides obtectus* (Coleoptera: Bruchidae) to headspace extracts of volatiles collected from flowers of a nectar plant, *Daucus carota*, was investigated using behaviour (four-arm olfactometry) and coupled gas chromatography–electroantennography (GC-EAG).

RESULTS: Odours from inflorescences were significantly more attractive to virgin female beetles than clean air. Similarly, a sample of volatile organic compounds (VOCs) collected by air entrainment (dynamic headspace collection) was more attractive to beetles than a solvent control. In coupled GC-EAG experiments with beetle antennae and the VOC extract, six components showed EAG activity. Using coupled GC–mass spectrometry (GC–MS) and GC peak enhancement with authentic standards, the components were identified as -pinene (*S:R* 16:1), sabinene, myrcene, limonene (*S:R* 1:3), terpinolene and (*S*)-bornyl acetate. Females preferred the synthetic blend of *D. carota* EAG-active volatiles to the solvent control in bioassays. When compared directly, odours of *D. carota* inflorescences elicited stronger positive behaviour than the synthetic blend.

CONCLUSION: This is the first report of behaviourally active volatiles linked to pollen location for *A. obtectus*, and development of the six-component blend is being pursued, which could underpin the design of semiochemical-based field management approaches against this major pest of stored products.

Keywords: *Acanthoscelides obtectus*; plant volatiles; gas chromatography – electroantennography (GC-EAG); attractant; chemical ecology; behavioural assays

1 INTRODUCTION

The dried bean beetle, *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae, Bruchinae), is a major global pest of dry beans, *Phaseolus vulgaris* L. (Fabaceae),[1] especially on smallholder farms,[2 – 5] causing 7 – 13% losses in Latin America[6]. Karel and Autrique[7] reported that, in Africa, farm storage of beans for 6 months was accompanied by about 40% loss in weight with as much as 80% of the seed being infested and unfit for human consumption, and with 23-73% yield losses. Originating in the Neotropics, *A. obtectus* has become cosmopolitan through human-mediated migrations since the domestication and dis-tribution of beans.[8] It can have several generations a year and can multiply both in the field and in granaries.[9,10] The control of *A. obtectus* using various chemical, biological, mechanical and cultural methods has met with varied success,[4,11 –18] and is still lacking a sensitive tool for the detection and monitoring of infes-tations. Also, the recent ban of methyl bromide, a broad-spectrum fumigant,[19] facilitated the search for alternatives in stored prod-uct pest management.[20,21] The use of attractive semiochemicals (pheromones and other semiochemicals, e.g. plant volatiles that act as kairomones)[22] to track the spatial and temporal population dynamics of *A. obtectus* represents a promising approach for surveillance programmes both in the field and in store houses. Surveillance in the field is crucial because harvested seeds that are already infested are a primary cause of the build-up of infestations in granaries.[10] In our earlier studies, we identified the composition of the male-produced sex pheromone of *A. obtectus*.[23,24]

Adult beetles are known to visit a range of flowering plants primarily for their pollen,[25 – 27] and laboratory feeding experi-ments with females have demonstrated that pollen consump-tion stimulates ovarian production.[28] Bruchid beetles, including *Acanthoscelides* and *Bruchus* spp.,[29,30] can be seen on members of the Apiaceae.[26,27] Of these, we chose *Daucus carota* L.[31] as the

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model plant to study floral odour detection in *A. obtectus* and hypothesized that females utilize volatile compounds to find inflo-rescences. We used behavioural (four-arm olfactometer) bioas-says and coupled gas chromatography – electroantennography (GC-EAG) to confirm that *D. carota* was a suitable source of plant-based attractants, the identification of which would under-pin the development of kairomones for use in low-cost bean beetle management strategies that are affordable for smallholder bean farmers.

2 MATERIALS AND METHODS

2.1 Live material

Daucus carota inflorescences were collected from plants growing in the verge of a crop field near Rothamsted Research, Harpenden, UK. Flowers were used for experimental purposes immediately after a short transportation period between the field and the laboratory. Bruchid cultures, originating from a field infestation in Hungary, were kept on dry *P. vulgaris* 'Cannellini' beans in plastic containers, and maintained under artificial lighting at 20 °C/60% relative humidity (RH) with a 16:8 h light:dark photoperiod. To obtain virgin females, seeds were kept individually in wells of a plastic rack until emergence of adults, when sexes were separated immediately.

2.2 Collection of volatiles

Dynamic headspace collection (air entrainment)[32] was used to collect volatile compounds from *D. carota* flowers. Twenty inflorescences (umbels) were placed within a 2-L glass chamber (Biochem Glass Apparatus Ltd, Milton Keynes, UK). Air was pumped through activated charcoal filters at 600 mL min^{-1} into the chamber to provide a positive pressure of clean air. At another outlet was placed a glass tube [8 cm × 0.3 cm internal diameter (ID)] con-taining Porapak Q (50 mg) (Sigma-Aldrich, Gillingham, UK) sand-wiched between glass wool plugs. Air was drawn from the cham-ber through the tube under negative pressure at a flow rate of 500 mL min^{-1}. Volatile organic compounds (VOCs) were collected on Porapak Q traps for 6 h and were eluted with 750 L of freshly distilled diethyl ether. The extract was concentrated to 100 L under a gentle stream of nitrogen and kept at -20 °C until use.

2.3 Electrophysiology

Electroantennogram recordings from the antennae of adult female *A. obtectus* were made using Ag –AgCl glass electrodes filled with saline solution composed as in Maddrell,[33] but without glucose. An antenna was excised and suspended between the two electrodes. The tip of the terminal process of the antenna was removed to ensure a good contact. The signals were passed through a UN-06 high-impedance amplifier (Ockenfels Syntech GmbH, Kirchzarten, Germany). The coupled GC-EAG system has been described previously.[34] Separation of the collected *D. carota* volatiles was achieved on a high-resolution gas chromatograph (Agilent 6890 N; Agilent Technologies, Santa Clara, CA, USA), equipped with a cool on-column injector and a flame ionization detector (FID), using a 50 m × 0.32 mm ID HP-1 column (J & W Scientific, Folsom, CA, USA). The oven temperature was main-tained at 30 °C for 2 min and then programmed at 15 °C/min to 250 °C. The carrier gas was helium. The outputs from the EAG amplifier and the FID were monitored simultaneously and anal-ysed using a customized software package (Ockenfels Syntech GmbH). A compound was defined as EAG-active if it evoked an antennal response, distinguishable from background noise, in three or more of five coupled runs (i.e. five independent antennal preparations).

2.4 Gas chromatography analysis

The collected volatile extract was analysed by high-resolution GC using an Agilent 6890A gas chromatograph equipped with a cool on-column injector, an FID and a 50 m × 0.32 mm ID HP-1 column (J & W Scientific). The oven temperature was maintained at 30 °C for 1 min, then programmed at 5 °C/min to 150 °C and held for 0.1 min, then programmed at 10 °C/min to 250 °C and held for 20 min. The carrier gas was hydrogen. The extract was also analysed by enantioselective GC using an Agilent 6890 N gas chromatograph equipped with a cool on-column injector, an FID and a 30 m × 0.25 mm ID × 0.25 m film thickness SUPELCO® Beta DEX™ (Sigma-Aldrich, Gillingham, UK) 120 fused silica capillary column. The oven temperature was maintained at 30 °C for 1 min and then programmed at 5 °C/min to 150 °C, then at 10 °C/min to 230 °C and held for 22 min. The carrier gas was hydrogen. Quantification of compounds was achieved using the single-point external standard method with a series of C7 – C22 alkanes.

2.5 Coupled gas chromatography–mass spectrometry (GC–MS)

Electrophysiologically active GC peaks were analysed using a 50 m × 0.32 mm ID HP-1 column (J & W Scientific), equipped with a cool on-column injector, that was coupled to a Waters Autospec Ultima magnetic sector mass spectrometer (Waters Corp., Manch-ester, UK). Ionization was by electron impact at 70 eV and 220 °C. The GC oven temperature was maintained at 30 °C for 1 min, then programmed at 5 °C/min to 150 °C and held for 0.1 min, then pro-grammed at 10 °C/min to 250 °C and held for 34.9 min. Tenta-tive identifications were made by comparison of mass spectra to those contained in a library database,[35] and confirmed by GC peak enhancement using authentic samples of compounds purchased from commercial suppliers.

2.6 Chemicals

Synthetic standards required for confirmation of identity and behavioural bioassays were purchased from Sigma-Aldrich, Gillingham, UK [(*R*)- -pinene 98%, (*S*)- -pinene 97%, (*R/S*)-sabinene 75%, myrcene ≥90%, (*R*)-limonene 97% and (*S*)-limonene 96%] and Fluka (Loughborough, UK) [terpinolene ≥90% and (*S*)-bornyl acetate 99%].

2.7 Olfactometer bioassays

To determine the behavioural responses of virgin *A. obtectus* females to *D. carota* flower headspace, headspace extract and a synthetic blend of electrophysiologically active compounds, a Perspex four-arm olfactometer was used.[36] The olfactometer rested on a stand (a rubber cork; 5 cm high and 4 cm wide) and was connected to glass chambers enclosing one, fully opened, white flower head on a live *D. carota* or left empty as controls (experiment 1; see below). The connections were made using Teflon tubing, through a 3-mm-diameter hole at the end of each of the four arms. When testing the headspace extract or synthetic blend (experiments 2 and 3), glass arms (narrow part: 50 mm in length × 2.5 mm in diameter; wide part: 90 mm in length × 20 mm in diameter) were directly attached to the olfactometer. Test solutions were each applied to a piece of filter paper (ca.

2 cm^2), which was then placed into one of the glass arms of the olfactometer and tested against three control arms, except for Experiment 4, testing synthetic blend versus flower headspace, where two control arms were used. Prior to each experiment, all glassware was washed with Teepol detergent (Teepol, Orpington, UK), rinsed with acetone and distilled water and baked in an oven at 130 °C for 2 h. Perspex components were washed with Teepol solution, rinsed with 80% ethanol solution and distilled water and left to air-dry. The olfactometer was illuminated from above by diffuse uniform lighting from two 18 W/35 white fluorescent light bulbs screened with red acetate[37]. It was surrounded by black paper to remove any external visual stimuli. Charcoal-filtered air was pumped into the glass chambers at a rate of 100 mL min^{-1}, then drawn through the central hole of the olfactometer by another vacuum pump (220 –240 V AC; Charles Austen Pumps Ltd, Byfleet, UK) and thereby pulled through each of the four side arms (75 mL min^{-1}/arm), and subsequently exhausted from the room. A single beetle was introduced into the olfactometer at each test period. Each beetle (n = 10) was given 2 min to acclimatize in the olfactometer, after which the experiment was run for 16 min by rotating it 90° on the stand every 2 min to control for any directional bias (temperature 22 °C). The olfactometer was divided into five regions that corresponded to each of the four glass arms and the central compartment, and the time spent in each area was recorded using specialist software (OLFA, Udine, Italy). In order to account for the replication and areas within each replication as variance components in a split-plot design, the method of residual maximum likelihood (REML) was used to fit a linear mixed model to the time spent data, nesting the areas within each replication and testing the treatment effect using an approximate F-test. The data were analysed on the square root scale to account for some heterogeneity of variance over the treatments. Means are presented with standard error of the difference (SED) values for their comparison, and the least significant difference (LSD) at the 5% (P = 0.05) level of significance was used for separation of means when there were three treatments (experiment 4). Genstat (18th edition; VSN International Ltd, Hemel Hempstead, UK) was used for the analysis.

Experiments comprised (1) the headspace of one *D. carota* flower versus blank air; (2) one *D. carota* inflorescence-equivalent amount of headspace extract (released over ca. 20 min) ver-sus diethyl ether (10 L); (3) a synthetic blend containing iden-tified EAG-active compounds (ng/ L) in a 10- L hexane solu-tion at at similar concentration and ratio as released by one *D. carota* inflorescence/20 min [calculated from headspace samples, i.e. (*S*)- -pinene 37.6 ng, (*R*)- -pinene 2.4 ng, (*RS*)-sabinene 120 ng, myrcene 50 ng, (*S*)-limonene 32.5 ng, (*R*)-limonene 97.5 ng, terpinolene 10 ng and (*S*)-bornyl acetate 90 ng], versus hexane (10 L); (4) the headspace of one *D. carota* flower versus the syn-thetic blend (10 L) versus hexane (10 L).

3 RESULTS

In behavioural (four-arm olfactometer) assays, adult virgin female *A. obtectus* spent more time in the arm containing the odour

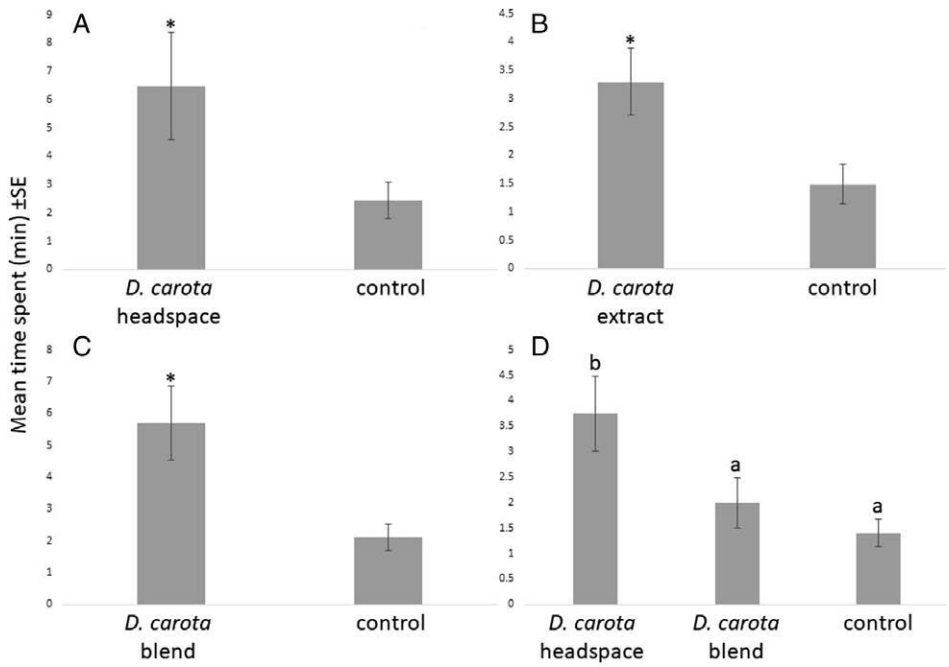

Figure 1. Behavioural response of virgin female *Acanthoscelides obtectus* to *Daucus carota* floral headspace [A; predicted means on square root scale: control = 1.199 (n = 33); floral headspace = 2.086 (n = 11); SED = 0.4038; df = 42], to a headspace extract prepared from umbels [B; predicted means on square root scale: control = 0.991 (n = 30); extract = 1.567 (n = 10); SED = 0.2880; df = 38], to a synthetic blend of electrophysiologically active compounds identified from *D. carota* headspace extracts [C; predicted means on square root scale: control = 1.226 (n = 30); synthetic blend = 2.249 (n = 10); SED = 0.2939; df = 38], and to floral headspace versus a synthetic blend [D; predicted means on square root scale: control = 0.925 (n = 32); floral headspace = 1.762 (n = 16); synthetic blend = 1.138 (n = 16); SED = 0.2827 (df = 61) for comparison to control and 0.2448 for comparison of floral headspace to synthetic blend]. The response was measured as the mean [± standard error (SE)] time spent in the arms of the olfactometer. Controls were clean air for floral headspace, diethyl ether for the air entrainment extract and hexane for the synthetic blend. *Significantly different ($P \leq 0.053$; F-test; see main text of Results for exact P-values) by the method of residual maximum likelihood (REML), used to fit a linear mixed model to the time spent data, nesting the areas within each replication and testing the treatment effect using an approximate F-test. The data were analysed on the square root scale to account for some heterogeneity of variance over the two treatments. Columns with the same letters are not significantly different ($P < 0.05$; LSD).

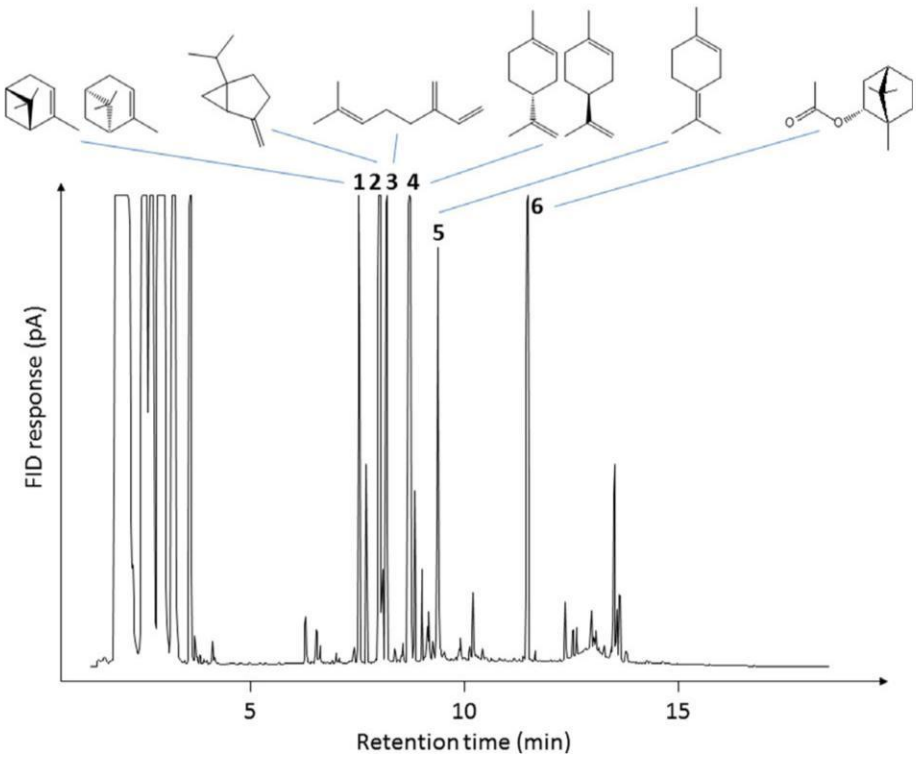

Figure 2. Gas chromatography analysis of *Daucus carota* headspace extract (50 m × 0.32 mm ID HP-1 column), highlighting EAG-active peaks. Numbers correspond to identified compounds in Table 1.

of *D. carota* inflorescences compared with control arms contain-ing clean air (*F*-test: $F = 4.83$; df = 1, 42; $P = 0.034$) (experiment 1; Fig. 1A). There was evidence of attraction to the extract of VOCs collected from *D. carota* inflorescences compared with the control arms (*F*-test: $F = 4.00$; df = 1, 38; $P = 0.053$) (experiment 2; Fig. 1B). Using coupled GC-EAG with the antennae of female *A. obtectus*, six peaks were located in the volatile extract with EAG activity (Fig. 2, Table 1 and Supporting Information Fig. S1). These were identified by coupled GC –MS and GC peak enhancement, including enantioselective (chiral) GC using authentic standards, as -pinene (*R:S* = 1:16), sabinene (stereochemistry not deter-mined), myrcene, limonene (*R:S* = 3:1), terpinolene and (*S*)-bornyl acetate, and were the most abundant constituents of the *D. carota* flower headspace, comprising 71.2% of all compounds. The ratio of compounds, based on GC peak areas, was 4:12:5:13:1:9, respectively. Females preferred the synthetic blend of *D. carota* EAG-active volatiles to the solvent control in bioassays (*F*-test: $F = 12.10$; df = 1, 38; $P = 0.001$) (experiment 3; Fig. 1C). In the final experiment, there was a significant (*F*-test: $F = 6.89$; df = 1, 61; $P = 0.011$) difference between time spent in the arms with the nat-ural and synthetic flower scents as a whole, compared with the control. Having accounted for this overall difference, there was also a difference between the two flower scent treatments (*F*-test: $F = 4.88$; df = 1, 61; $P = 0.031$), the beetles spending more time in the area of the olfactometer flushed with live flower headspace, compared with that with the synthetic blend (experiment 4; Fig. 1D).

Table 1. Electrophysiologically active compounds identified in headspace extracts of *Daucus carota* inflorescences, using virgin female *Acanthoscelides obtectus* antennae ($n = 5$). Tentative identifica-tions were made by GC – MS and confirmed by GC peak enhancement with authentic standards (see Materials and Methods)

Number	Compound	Retention index[a] (non-polar)	Retention index[b] (chiral)	Concentration (ng/ L)
1	(*S*)- -Pinene	936	1026	37.6
	(*R*)- -Pinene		1033	2.4
2	Sabinene[c]	972	1054	120
3	Myrcene	987	1038	50
4	(*S*)-Limonene	1031	1104	32.5
	(*R*)-Limonene		1108	97.5
5	Terpinolene	1086	1161	10
6	(*S*)-Bornyl acetate	1286	1401	90

[a] On an Agilent HP-1 GC column.
[b] On a SUPELCO® Beta DEX™ 120 fused silica GC column. [c] Stereochemistry not determined.

4 DISCUSSION

This is the first report on the activity of flower-derived semiochemicals for the adult dried bean beetle, *A. obtectus*, a member of the Bruchinae subfamily of Chrysomelidae. The volatiles identified as EAG-active from *D. carota* flowers in this study had also been identified in headspace extracts[38] and essential oils extracted from the umbels.[39] Similarly, the essential oils and headspace extracts of the closely related *D. muricatus* L. flowers contained all six compounds identified here, but with no reference to their enantiomeric composition.[40] In Zachariae,[31] flowers of *Ranunculus arvensis* L., *Ranunculus repens* L. (Ranunculaceae), *Fragaria grandi-flora* Ehrh., *Potentilla anserina* L., *Potentilla reptans* L. (Rosaceae), *Petroselinum sativum* Hoffm., *Torilis anthriscus* Gm. and *Aethusa*

cynapium L. (Apiaceae) are reported to be visited for nectar by adult *A. obtectus*, in addition to *D. carota*. Analysis of available data on the distribution of volatiles identified in floral bouquets across the above six genera (Table S1) revealed that limonene and (*E*)-ocimene are the most widespread constituents found in *Ranunculus*, *Fragaria*, *Potentilla* and *Daucus*, including *D. carota*. (*Z*)-Ocimene, myrcene, linalool (unknown chirality), germacrene D, (*E*)-caryophyllene, methyl salicylate, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate each occur in three genera, but the latter three compounds do not occur in *Daucus*. These ubiquitous volatiles have been identified from a large number of plant species[41] with various roles in plant –arthropod interactions, including attractant properties for insects in many cases.[42 –48] Of the 39 mostly terpenoid compounds reported in the present study for *D. carota* (Table S1), 20 of them (51%) are also found in other *Daucus* species. The six EAG-active *D. carota* volatiles appear to be a unique (and perhaps characteristic) combination in the floral headspace of *Daucus* spp. -Pinene, myrcene, limonene and terpinolene are attractants of bark beetles,[44] -pinene and sabinene are suggested to contribute to the attractiveness of certain *D. carota* cultivars to *Trioza apicalis* Förster (Homoptera: Psylloidea),[38] whereas -pinene, limonene and bornyl acetate are constituents of an attractive blend for *Smicronyx fulvus* Le Conte (Coleoptera: Curculionidae).[49]

Prior to this study, the few chemical ecology studies on host plant – Bruchinae relationships have primarily focused on lar-val host plants.[50 –52] Pouzat[53] demonstrated the EAG activity of the vapour of bean pods, as well as synthetic amyl acetate, in *A. obtectus*. Female bruchids, including *A. obtectus*,[26,28,31] however, often visit flowers to obtain pollen for their eggs to mature.[11] Examples are *Bruchus pisorum* L. on *Pisum sativum* L. (Fabaceae), where obligatory pre-copulation feeding on pollen was reported,[54] as well as nectar feeding to obtain a readily available source of energy to sustain flight,[55] and *Spermophagus sericeus* Geoffroy on *Daucus* spp.[11] Pollen has a high protein con-tent plus sugar, starch, fat, and traces of vitamins and inorganic salts, while nectar primarily consists of a solution of sugars, espe-cially glucose, fructose and sucrose.[56] *Bruchus rufimanus* Boheman is often found in flowers of *Vicia faba* L. and is attracted to a synthetic mixture of *V. faba* floral scent constituents, (*R*)-linalool, cinnamyl alcohol and cinnamaldehyde, identified from headspace extracts.[57] Ceballos *et al.*[58] identified a range of volatiles from *P. sativum* leaves, flowers and pods, and demonstrated that headspace extracts evoked positive responses from *B. pisorum* in behavioural assays. The compounds responsible for the bioactivity of the extracts are unknown. Our results provide supporting evi-dence that floral volatiles play a part in the interactions between *A. obtectus* and *D. carota*, aiding location of nectar plants. It is appre-ciated, however, that under our experimental circumstances, the bouquet of the natural floral headspace was behaviourally preferred to its synthetic mimic, indicating that there are further compounds in the floral headspace that could not be located by GC-EAG and which would increase the attractiveness of the synthetic blend, or that the blend composition, i.e. ratios and doses of constituents, requires further refinement. Optimization studies for practical uses will be carried out via trapping trials in bean fields and granaries. These will assess blend composition, dose and dispenser type to achieve maximum attractiveness.

Future blend development may also include compounds from other listed nectar plants,[31] as well as those from ripening bean pods. This approach could be particularly important for females, which directly determine the size of future generations.[57] In this way, semiochemical-focused management strategies would directly reduce future population densities locally by reducing the number of females via trapping or the recruitment of natural enemies.[59] Such research efforts should focus on field-testing synthetic blends of compounds, using trap designs developed for bruchids[57] and other chrysomelids.[60] Trap development and design for coleopteran pests need to consider the spe-cific behaviour of each species. Many chrysomelid species start climbing upwards after landing on a vertical surface. Traps for *Diabrotica v. virgifera* LeConte or *Phyllotreta* spp. take advantage of this behaviour and direct crawling insects upwards into a catch container.[60] The vertical landing surface also takes advantage of the preference of these species for a hue of yellow, the attractive effect of which is synergized by semiochemicals. Bruce *et al.*[57] used a light green-coloured cone trap to monitor *B. rufimanus* in the field, which affected the orientation behaviour of *B. rufimanus* differently from that of *A. obtectus*. The latter species typically moves upwards on vertical surfaces, suggesting that a trap design described in Tóth *et al.*[60] might be more suitable. Other floral cues may certainly be important to elicit this behaviour, including the colour of inflorescences. Attraction of flower-visiting insects can be enhanced by the combination of odour and colour stimuli,[61] whereas in other cases, colour does not synergize the effect of odour.[62] Little is known about colour preference in *A. obtectus*; Zachariae[31] lists nectar plants that all appear white or light yellow to the human eye, and traps coloured in white or yellow were found to be more attractive than other colours. Thus, trapping trials will need to assess a range of colours, including those mim-icking the reflectance spectra of common inflorescences visited by the species.

The interactions between intra- and interspecific chemical signals are regulated at the olfactory periphery and in the central nervous system,[63] and can often result in behavioural synergism.[64] Thus, the efficiency of a plant volatile-based lure for *A. obtectus* may also be enhanced by its co-application with the male-produced sex pheromone.[23] In conclusion, develop-ment of the six-component blend is being pursued, which could underpin the design of semiochemical-based field management approaches against this major pest of stored products.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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