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Identification of volatile compounds from fungus-infected date fruit that stimulate upwind flight in female *Ectomyelois ceratoniae*

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Abstract

Four volatile compounds emitted from fungus-infected date fruit, *Phoenix dactylifera* L., were identified using coupled gas chromatographic-electroantennographic recordings, coupled gas chromatographic-mass spectrometric analysis, electroantennographic assays of synthetic standards, and wind tunnel bioassays. These compounds were ethyl hexanoate, ethanol, acetaldehyde, and 2-phenylethanol. Wind tunnel bioassays showed that ethyl hexanoate was capable of stimulating upwind flight and landing on the source by mated female carob moths, *Ectomyelois ceratoniae* (Zeller). Addition of both ethanol and acetaldehyde to ethyl hexanoate resulted in an increase in attraction to a level similar to that found for date fruits. No such effect was noted for additions of 2-phenylethanol at the dosages tested. In this study, it appears that ethyl hexanoate is a dominant olfactory stimulant and attractant for mated female carob moths, and represents a novel compound with regard to previously identified lepidopteran host odor attractants.

Introduction

Many insects use airborne volatiles emitted from plants to locate their hosts. Information on volatile chemicals that attract insects to their hosts and that stimulate oviposition upon arrival could be of benefit to pest control programs. Infestations of the carob moth, *Ectomyelois ceratoniae*, are causing serious economic losses in commercial date gardens, *Phoenix dactylifera* L., in the Coachella Valley in southern California (Warner *et al.*, 1990). If its range in this state expands northward towards the Central Valley of California, economically important nut crops such as almonds and pistachios may be threatened, as the carob moth is a pest of these crops in other parts of the world (Dhouibi, 1982; Gothilf, 1984). Female moths are attracted to ripening dates for oviposition (Warner, 1988) and olfactory stimuli may play an important role in mediating oviposition by *E. ceratoniae*. However, the volatile chemicals have not been clearly identified.

The current control method for the carob moth consists of multiple applications of malathion dust. However, the variable success of control along with an increasing public concern over widespread use of malathion has heightened the need to develop alternative practices.

In the present study, which was undertaken as part of a broader study aimed at finding alternatives to conventional insecticides, we identified several mouldy date headspace components, which elicited electroantennogram responses, and recorded upwind flight in a wind tunnel by mated female carob moths.

Materials and methods

Insects. Moths were obtained from a laboratory culture started from individuals collected in 1985 from infested dates near Indio in the Coachella Valley, California. Larvae were reared on a honey and wheat-bran

diet in 3.8 liter jars (Warner, 1988). To obtain mated females for the experiments, cages containing male and female pupae were placed in environmental chambers at 25 ± 1 °C on a reverse L14:D10 photoperiod regime. The moths had access to a 5% sucrose solution on cotton wool. Two- to six-day-old females were used for the electrophysiological experiments and 4–5 day-old females for the wind tunnel assays.

Chemical analysis of date volatiles. Date volatiles were obtained from freshly-fallen ripe fruits (variety: Deglet Noor) that had noticeable fungal infections. Dates were stored at −20 °C and thawed for 1 h prior to the start of the experiments. The collection apparatus was a modified version of the one used by Charlton & Cardé (1982), and consisted of two modified ground glass joints (B-55) forming the date-holding chamber. The lower half of the holding chamber was connected to a volatile collector trap and the upper half to an air filter (70 mm× 6.0 mm OD) containing 2 cm of Tenax GC (60–80 mesh, Alltech Assoc. Inc., Deerfield, Illinois). The collector trap, used to trap the organic volatiles, contained 20 mg of Super-Q (Alltech Assoc. Inc., Deerfield, Illinois) as the adsorbent and was constructed and treated as described by Heath & Manukian (1992). The collector trap and air filter were connected to the holding chamber with 2 cm of Teflon tubing and sealed with Teflon tape. Air was drawn over four dates at a flow rate of 175–200 ml/min for 4 h (22–25 °C). Trapped volatiles were desorbed with CS_2 (2 × 100 μl) and collected in a conical-bottomed microvial before being concentrated to 10 μl under a slow stream of N_2 . One-microliter aliquots were injected in splitless mode onto 30 m × 0.25 mm ID fused silica capillary gas chromatographic (GC) columns, coated either with DB-225 or DB-5 (J&W Scientific, Folsom, California) for analyses by GC-mass spectrometry (GC-MS) and GC-electroantennography (GC-EAG). Column conditions were as follows: He carrier gas flow of 1.5 ml/min, injector temperature 200 °C, oven temperature program 1 min delay on inlet purge, 2 min at 80 °C, then 20 °C/min to 180 °C, and flame ionization detector at 230 °C. Simultaneous GC-EAG analyses were performed, using an excised female antenna suspended between two saline-filled glass electrodes, as described by Baker *et al.* (1991) using a Varian Model 3740 GC. GC-MS analyses were performed using a Hewlett Packard 5890 gas chromatograph with a direct interface to a Hewlett Packard 5970 mass selective detector (electron impact, 70 eV). To avoid solvent peaks, purge and trap GC-MS analyses were performed interfacing the GC with a Tekmar 2000 purge and trap concentrator (Tekmar, Cincinnati, Ohio). The conditions for collecting volatiles from three dates on an active charcoal trap were as follows: 6 min purge at room temperature (He, 20 ml/min) and 4 min desorb at 180 °C with an oven temperature program of 2 min at 0 °C followed by 10 °C/min to 250 °C. GC-MS analyses were carried out with a 20 m × 0.2 mm ID Ultra-2 capillary column (Hewlett-Packard; equivalent to DB-5 in GC-EAG studies).

Electroantennogram responses. To examine antennal sensitivity to identified date volatiles, as well as to functional groups and carbon chain length, electroantennograms were recorded (Roelofs, 1984) for a series of synthetic standard aldehydes, alcohols and ethyl esters obtained from commercial sources (see Table 1). On the day of each series of EAG tests, ten microliters of the standards were applied directly to filter paper (Whatman No. 1) strips, or serially diluted in redistilled HPLC grade hexane such that 100 μg were applied to filter paper in 10 μl of solvent. The filter-paper strips were placed inside Pasteur pipettes (15 cm long). The odor delivery system and recording technique were similar to those described previously (Baker *et al.*, 1991). To allow for possible deterioration of the antennal preparation, responses were calculated using a standard stimulus before and after each series of test compounds. This standard stimulus consisted of a 1.0 × 0.5 × 0.5 cm piece of fungus-infected date fruit, which was kept at −20 °C and cut off and thawed for 1 h prior to the start of a particular series of tests. EAG amplitudes were calculated by dividing the amplitude of the EAG generated from the test compound by that from the mean of the standard. Within a particular series of tests, the order in which the test compounds were presented was randomized.

Wind tunnel bioassays. Behavioral assays were conducted in a 3.5 × 1.0 × 1.0 m wind tunnel described previously by Kuenen & Baker (1982). Ten 5–6 day-old mated females were placed into screen cages (4 cm-long × 3 cm-diameter) during the photophase and each case was covered with a Petri dish lid. Cages with females were placed in the wind tunnel at least 1 h before testing to acclimate the females to the conditions in the tunnel (20–24 °C, 0.3 lux, 30–70% relative humidity, 0.5 m/s wind velocity). Bioassays were conducted 0.5–2 h into the scotophase, the optimal oviposition period for this species (Vetter, Tatevossian, and Baker, unpubl.). Treatments were loaded onto filter-

Table 1 Calculated* EAG amplitudes of female *E. ceratoniae* antennae in response to a series of alcohols, aldehydes, and ethyl esters, applied to filter-paper strips (± standard error, N = 10)

Compound (10 μl)	Chemical purity (%)[a]	Source[b]	$\overline{X}$±SE	Compound (100 μg)[c]	Chemical purity (%)[a]	Source[b]	$\overline{X}$±SE
Ethanol	100.0	A	0.5±0.1	Ethyl butyrate	99.0	C	0.5±0.2
1-Propanol	99.5	B	0.3±0.1	Ethyl hexanoate	99.0	C	3.7±0.8
2-Propanol	99.5	B	0.4±0.1	Ethyl octanoate	99.0	C	3.4±1.0
1-Butanol	99.8	B	1.0±0.2	Ethyl nonanoate	97.0	C	2.2±0.8
2-Methyl-1-propanol	99.5	B	0.7±0.1	Ethyl decanoate	99.0	C	1.9±0.5
Acetaldehyde	99.0	C	0.7±0.2	2-Phenylethanol	98.0	B	1.3±0.2
Benzaldehyde	99.0	B	0.5±0.1	Phenylacetaldehyde	90.0	C	1.3±0.4
Benzyl alcohol	99.0	B	0.7±0.2	Date standard (mV)	–	-	1.5±0.3

* EAG amplitudes were calculated by dividing the amplitude of the EAG generated from the test compound by that from the mean amplitude of the date standard

[a] label information.

[b] U.S. Industrial Chemicals (A), Sigma Chemical Co. (B), Aldrich Chemical Co. (C).

[c] applied in 10 μl of hexane.

paper disks (Whatman No. 1) affixed to metal clips complete with metal base (6 cm high) and placed on a 15 × 15 cm sheet metal platform 15 cm above the floor of the tunnel, and 30 cm from the tunnel's upwind end. A control treatment consisted of three freshly thawed mouldy dates placed in an open-ended screen cage (4 cm-long × 3 cm-diameter) positioned vertically on a metal platform. Females were released 10 at a time, 1 m from the odor source and 15 cm above the tunnel floor. The cages were placed on a metal platform in the plume until the females took flight or until 5 min had elapsed. Females were scored for taking flight, locking-on to the plume and progressing upwind, flying to within 5 cm of the source, contacting the source, and depositing eggs on the source. The numbers of moths performing each behavior were compared by using a χ^2 2 × 2 test of independence with Yates' correction (Steel & Torrie, 1960). Statistical significance was determined at the 0.05 level.

Results

Chemical analysis of date volatiles. Combined GC-EAG analysis of the date volatiles consistently revealed distinct EAG responses coinciding with the elution of the synthetic standards acetaldehyde, ethanol, ethyl hexanoate, and 2-phenylethanol (Fig. 1). The presence of these compounds in date volatiles was confirmed by GC-MS by comparisons with the mass spectra of the synthetic standards, followed by confirmation by matching retention times on both the DB-5 and DB-225 capillary columns. The presence of acetaldehyde, ethanol, and ethyl hexanoate was confirmed in the absence of solvent by GC-MS, through analysis of date volatiles collected by the purge and trap concentrator. The following compounds were also identified, but showed no EAG activity: acetic acid, 2-methyl-1-propanol, 3-methyl-1-butanol, and ethyl butyrate. Although attempts were not made to quantify the collected compounds, the purge and trap analysis indicated that ethanol and acetaldehyde were emitted in larger quantities (100–300 ng range) than ethyl hexanoate or 2-phenylethanol (1–5 ng range).

Electroantennogram responses. EAG responses revealed that female antennae were far more responsive to ethyl esters and to two aromatic compounds than to other molecules to which they were exposed (Table 1). Of the four GC-EAG identified compounds, ethyl hexanoate was the most active compound, generating a response 3.7 times (± 0.8 SE) as great as that to the standard date material, followed by 2-phenylethanol with a response of 1.3 (± 0.2 SE). The EAG responses from ethanol and acetaldehyde could only be recorded using 10 μl of undiluted compound and both compounds generated lower responses than the standard. Several other compounds were also tested, either because of their structural similarity to identified components or because they had been reported previously as plant volatiles. The strongest EAG responses were elicited by ethyl esters, gener-

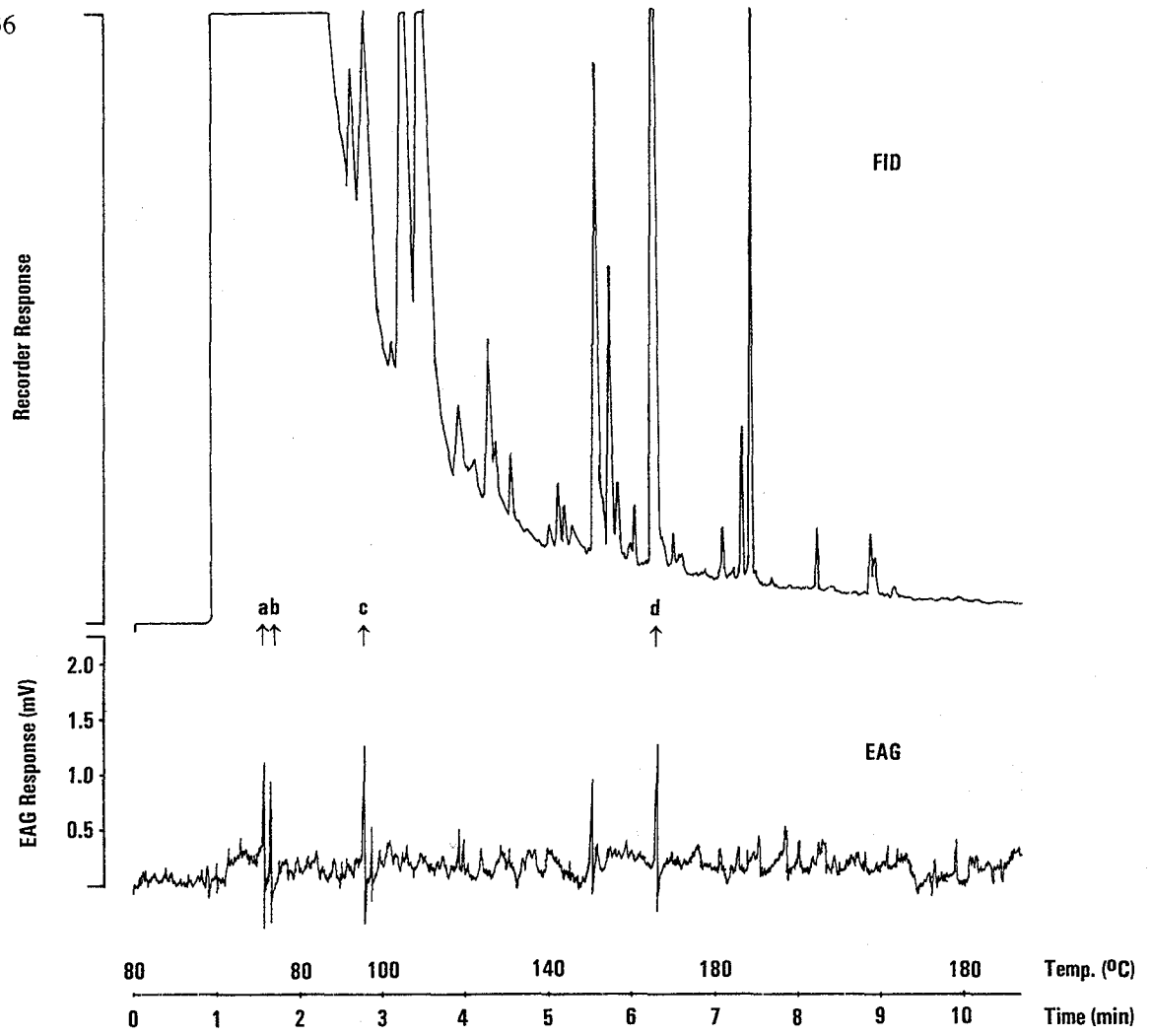

Fig 1. Simultaneously recorded gas chromatogram (FID) of trapped volatile compounds from fungus-infected date fruits, *Phoenix dactylifera*, on a DB-225 capillary column, and electroantennogram (EAG) from a female carob moth, *Ectomyelois ceratoniae*. Retention times of synthetic standards acetaldehyde (a), ethanol (b), ethyl hexanoate (c), and 2-phenylethanol (d) are indicated.

ating responses 1.9–3.4 times as great as that to the standard. 2-Phenylethanol produced an EAG response similar to that of phenylacetaldehyde. The strongest response from an undiluted compound was recorded from 1-butanol (1.0± 0.2 SE).

Wind tunnel bioassays. Mated female carob moths flew upwind to volatiles emitted from date fruits and 27% landed on the source (Table 2). After leaving the release cage, most of the females flew upwind in a zigzag pattern towards the date fruits. A few moths flew to the fruits and landed, probed the dates with their ovipositors, and deposited eggs. Others flew to within 5 cm of the date fruits and hovered for a short time before veering away or landing on the dispenser platform. Similar responses occurred to a filter paper disk treated with mixtures of volatile chemicals from date fruits. The carob moth females flew upwind and 3% landed on the source in response to 1 mg ethyl hexanoate. The responses towards 1 mg ethyl hexanoate were not altered significantly by the addition of increasing amounts of ethanol, acetaldehyde, and 2-phenylethanol, either alone or in combination. However, comparison between the 10 mg dosage of ethyl hexanoate and the 10 mg dosage of ethyl hexanoate in combination with 100 mg of both ethanol and acetaldehyde indicated significantly increased upwind flight and source location by the females to this 10:100:100 blend. Upwind flight and source contact were increased by this blend to levels not significantly different from

Table 2. Behavioral responses of female *E. ceratoniae* in a wind tunnel to different mixtures of volatile chemicals identified from dates (mg)[a] and to date fruits (N = 30)

Ethyl hexanoate	Ethanol	Acet-aldehyde	2-Phenyl-ethanol	Taking flight & locking-on (%)[b]	Upwind flight (%)[b]	Landing on the source (%)[b]
1	–	–	–	63bc	10cd	3c
10	–	–	–	63bc	3d	0c
1	10	–	–	70bc	0d	0c
1	–	10	–	40d	0d	0c
1	10	10	–	63bc	13cd	3c
1	100	100	–	63bc	23bc	10bc
10	100	100	–	93a	63a	37a
100	100	100	–	50bcd	13c	0c
–	–	–	1	23d	3d	0c
1	–	–	1	47cd	3d	0c
1	10	10	1	63b	13cd	0c
1	10	10	10	77ab	0d	0c
3 Dates	–	–	–	93a	47ab	27ab

[a] Applied on filter-paper disks

[b] Percentages that have no letter in common are significantly different according to χ^2 ($P<0.05$)

those stimulated by date fruits. Applying 100 mg of each of the three chemicals reduced considerably the numbers of moths that responded.

Discussion

We identified acetic acid, 2-methyl-1-propanol, 3-methyl-1-butanol, ethyl butyrate, ethyl hexanoate, ethanol, acetaldehyde, and 2-phenylethanol in the volatile chemicals released from fungus-infected date fruits. The EAG responses revealed that female carob moth antennae were more responsive to the ethyl esters than to the other test compounds. Highest EAG responses were recorded with the 8- and 10-carbon length ethyl esters. These data suggest that there are relatively specific receptors on the antennae for these molecules. The females' antennae also responded well (Table 1) to several other test compounds not found in our date samples. For example, phenylacetaldehyde, a floral compound described previously as stimulating upwind flight in *Trichoplusia ni* (Hübner) (Haynes *et al.*, 1991), elicited an EAG response similar to that to 2-phenylethanol. The strongest EAG response to an undiluted short-chain alcohol was recorded for 1-butanol, a compound identified in fungus-infected carob fruits and reported as an oviposition stimulant for *E. ceratoniae* (Gothilf *et al.*, 1975).

Ethyl hexanoate has been identified as one of the volatiles from fermenting chapote fruit that attracts adult Mexican fruit flies (*Anastrepha ludens* Loew) (Robacker *et al.*, 1990, 1992). Our wind tunnel bioassay showed that ethyl hexanoate also attracted female carob moths. Addition of both ethanol and acetaldehyde to ethyl hexanoate resulted in a level of source location similar to that found for date fruits. No such effect was noted for addition of 2-phenylethanol at the dosages tested. In our study, it appears that ethyl hexanoate elicits upwind flight in mated female carob moths, and to the best of our knowledge, represents a novel compound with regard to previously identified lepidopteran host odor attractants.

Volatile chemicals emitted from plants facilitate host-finding in a wide range of insects. In Lepidoptera such volatile chemicals enable females to locate suitable oviposition sites, but may also allow male and female adults to find food (i.e., Phelan *et al.*, 1991; Liu *et al.*, 1988; Haynes & Baker, 1989; Landolt, 1989; Haynes *et al.*, 1991; Tingle & Mitchell, 1992; Heath *et al.*, 1992). Thus far there have been fairly few identifications performed of attractants for female Lepidoptera. Cantelo & Jacobson (1979) showed that phenylacetaldehyde was the chemical responsible for attracting many species of Lepidoptera to the bladder flower, *Araujia sericofera* (Brothero). Haynes *et al.* (1991) identified four volatile compounds from flowers of *Abelia grandiflora* (André) and showed in

a wind tunnel that male and female cabbage loopers, *T. ni*, are attracted equally well to the complete blend or to the components phenylacetaldehyde and 2-phenylethanol. Heath *et al.* (1992) identified in a wind tunnel three volatile chemicals emitted from flowers of night-blooming jessamine, *Cestrum nocturnum* L., and reported that phenylacetaldehyde and to a lesser extent benzyl acetate stimulate upwind flight and source location of female *T. ni* in a wind tunnel.

Gothilf (1964) reported that female *E. ceratoniae* lay eggs on fungus-free carob fruits, although at a lower rate than on fungus-infested fruits. Similarly, female *E. ceratoniae* will oviposit on ripening date fruits or on unripe dates previously infested by insects or infected by fungi (Warner, 1988). Although we have not compared fruit volatiles between fungus-free and fungus-infested date fruits, it appears that fungal infestation in dates enhances the concentration of fruit volatiles in a manner similar to the concentrations found during the period of ripening. Expanding this investigation to other host plants for this species, including fungus-free sample materials, would likely reveal the potential of the identified date volatiles for the monitoring of the carob moth.

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