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Isolation, Identification and Determination of the Biological Activity of Candidate Fruit Volatile Components from *Argania spinosa L. (Sapotacea)*

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ABSTRACT: Argan, *Argania spinosa L*. (Sapotacea), is an important endemic host plant of *Ceratitis capitata* (Wied.) (Diptera: Tephritidae) in Morocco. The forest of argan covers over 700,000 ha in the SW of the country. An argan extract obtained from volatiles of artificially injured mature fruits elicited a significant response from tested adult medflies (mated and unmated males and females). Male response was higher than female response and mated females responded better than unmated females. Volatile extracts from artificially damaged yellow mature argan fruits were analyzed by capillary gas chromatography (CGC). CGC-mass spectrometry showed four peaks eluting at approximately 8-10 min retention time (early peaks) corresponding respectively to hexanal, (E)-2-hexenal, (Z)-3-hexenol, and 1-hexanol. An unidentified fifth component eluted later at 33 minutes retention time and was in approximately the same concentration as 1-hexanol. The attractiveness of synthetic argan fruit volatiles alone and in various combinations was assessed against mated and unmated males and females in the laboratory cages and in the field. No significant response was obtained with these "green leaf volatile components". However, the trimedlure and the female tri-lure deployed in parallel in the field attracted significant number of medflies. Results are discussed in terms of species specific response that argan components might elicit and the role they likely to play in developing effective trapping and monitoring systems for medflies

Key Words: Tephritidae, *Ceratitis capitata*, argan, attractant

INTRODUCTION

The argan tree (argania spinosa) grows in a harsh environment, surviving heat, drought and poor soil. It grows only in the south-west of Morocco – roughly between Essaouira and Agadir, in an area covering about 700,000-800,000 hectares. Local people collect the tree's nuts and process them into a dark brown oil. They also grind almonds with argan oil and honey to obtain a thicker mixture called amloo, which looks like peanut butter.

Medflies develop freely in argan forest which is an excellent refuge (Mazih 1992, Debouzie and Mazih, 1999). Previous studies have shown that argan fruit was highly attractive to medfly (Hadis and Bakri 1994). The objective of this study is to investigate argan semiochemicals involved in this plant-insect chemo-interaction and compare their attraction with known medfly baits (trimedlure and female-three-lures).

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MATERIAL & METHODS

Insect rearing

Ceratitis capitata (Wied.) adults tested in the laboratory were obtained as pupae which had developed from eggs and larvae in infested argan fruits. Under laboratory conditions, 24 – 25° C, 60 – 67% RH and 14L:10D photoperiod, adults were sexed just after emergence and placed inside plastic cages (30 x 30 x 30 cm). Granulated sugar, protein hydrolysate and water were provided as a diet for adults.

Argan volatile collection

Volatiles collected for bioassays were obtained by placing artificially injured argan fruits (250 g) in a tree-neck 0.5 l flask. One neck was connected to an active charcoal packed glass column (50 cm long x 3 cm ID) to introduce purified air into the flask. The medium neck was sealed. The other later neck connected to an active charcoal packed small glass column (7 cm long x 1 cm ID) for argan volatile trapping. After 24h hours, the volatile extract was eluted with 10 ml methylene chloride and concentrated under a gentle stream of nitrogen.

Argan volatiles collection for chemical analysis were conducted using the dynamic headspace (air - entrainment) technique (Heath & Manukian 1992). Briefly, the volatile collection system consists of a glass chamber (25.7 cm long and 7.6 cm ID) constructed of pyrex glass with a glass frit inlet and a ground-glass joint outlet and a single port collector base to which the collector traps were connected. Collector traps used to trap organic volatiles were made from a 4.0 cm long by 4.0 mm ID piece of glass tubing and contained 50 mg of super-Q® as the adsorbent (why super Q here but charcoal above? The first experiment was done in Morocco with charcoal while in Bob Heath laboratory super porapak Q was used, which have the same performance. The same components are expected in both extracts since the same elution solvent was used). Two stainless steel frits were used to contain the adsorbent. The collector traps were connected to stainless steel tubing using 0.64 cm unions and 0.64 cm ID Teflon® ferrules. These traps were cleaned by soxhlet extraction using methylene chloride for 24 hours and dried in a fume hood prior to initial use. Volatiles collected on the traps were eluted using 100 µl of high purity methylene chloride.

GC-Mass spectrometry

Gas chromatographic analysis of the volatiles collected from artificially injured argan fruits were conducted using Hewlett Packard Model 5890A Series II gas chromatographic equipped with a cool on-column capillary injector (septum injector) and flame ionization detector. Helium was used as the carrier gas at a linear flow of 18 cm/sec. The chromatographic data was collected and processed using the Perkin-Elmer nelson Turbochrom® III software running on an IBM-type system under MS-Windows® 3.0. Capillary gas chromatography (CGC) analysis was conducted using a series of three fused silica columns (8 cm long × 0.5 mm ID, 10m long x 0.25 mm ID, 30 m long x 0.25 mm ID). The columns were connected in series using Glass Seal® connectors (Supelco Inc. Bellefonte, PA). The column temperature program used for analysis was isothermal at 40°C for 5 minutes, then programmed to 210 °C at 5°C/min. Confirmation of compound identity was obtained using mass spectroscopy. Mass spectra were obtained at using the capillary columns, operated as described above, coupled to a Finnigan Ion Trap® mass spectrometer in either electron impact (EI-ITDMS) or chemical ionization (CI-ITDMS) mode. The reagent gas used for CI was isobutane.

Traps and lures

The Tephri trap (Agro Alcoy, Alcoy, Spain) consisted of a yellow invaginated base 11 cm deep, fitted with an opaque lid (3.5cm high). The total height of the trap was 14cm and diameter at the junction of lid and base was 12 cm. Four fly entry holes, 2.1 cm in diameter, were placed 90 degrees to each other, 1 cm from the top of the trap base. Tephri traps were baited with a three-component food-based lure consisting of ammonium acetate (AA), putrescine (PUT), and trimethylamine (TMA), formulated as three separate patches backed with adhesive for securing inside the trap (Suterra LLC, Bend, OR). A small piece (1.5 cm^2) DDVP strip (Agrisense) was placed inside Tephri trap to kill insects entering the traps.

Jackson traps (Triangular cardboard) were baited with Trimedlure (TML) plugs (AgriSense BCS Ltd., MidGlamorgan, United Kingdom). The traps contained white inserts that were coated on one side with sticky insect adhesive (Tangle Trap, Tanglefoot Co., Grand Rapids, MI) to retain flies attracted to the traps.

Synthetic argan chemicals

Synthetic chemicals identified as naturally released from argan volatiles were tested in the laboratory and in the field. These components included: 1-hexanol, (Z)-3-hexenol, hexanal, (E)-2-hexenal (Aldrich Company

Inc.) and combinations of two, three and four components. Combinations were prepared from equal amount of each compound; hexanal, (E)-2-hexenal; hexanal, (E)-2-hexenal, (Z)-3-hexenol; hexanal, (E)-2-hexenal, (Z)-3-hexenol, 1-hexanol.

Laboratory Bioassays

Biological tests in the laboratory were conducted in plastic cages (30 x 30 x 30 cm) with two filter papers (3x3 cm) placed at the top corners. One filter was loaded with the argan extract (20 µl) and the other with the solvent (methylene chloride). Landing insects were recorded every minute for 10 minutes period. Six replicates were made and the position of the filter papers was changed after each replicate. Fifty flies were released in the cage one night before the test. Tested insects were classified as males and females mated or unmated.

Similar tests were conducted with the synthetic argan compounds and their combinations versus the control.

Field studies

Taps were deployed following a random design in the argan forest near Agadir city. The experiment was conducted during high population period in July. TML and the food-synthetic lures baited traps were used to assess the population level during the tests. The performance of the following traps was assessed: Tephri traps baited with the above mentioned argan synthetic components and their combinations. Twenty µl (20 µl) of synthetic argan components were loaded on a cotton dental roll and sealed with parafilm sheet to reduce the evaporation. Cotton rolls and a DDVP were placed inside the Tephri trap and suspended to argan trees. Tephri trap baited with the food-based attractant, Jackson trap baited with TML and control Tephri trap with DDVP strip alone (Same lures as bioassays or different?).

Traps were suspended on the south east side of argan trees 1.5 to 2.0 m from the ground and at least 10 m from each other. During one week, traps were checked every 24 h and rotated after each sampling period. The sticky inserts in the Jackson traps were replaced following each sampling. Male and female *Ceratitis capitata* captured were removed from traps and the total number recorded.

The longevity of the food based synthetic lures and Trimedlure is 4 and 6 weeks respectively. Dental rolls loaded with argan synthetic components were changed after each sampling.

Statistics

A student t-test was used to determine if there was significant difference between numbers of medflies choosing between argan extract and the control in laboratory bioassays. Transformed data (log (x+1)) of trap captures in the field were analyzed using one-way analysis of variance followed with least significant difference test (LSD, $P<0.05$) to separate means.

RESULTS

Volatiles collected from artificially injured fruits elicited a significant response from the tested flies - mated and unmated males and females (Table 1). Male response was higher than female response and flies responded better when they are mated.

Analysis of the extract by CGC interfaced to a mass spectrometry indicated the presence of four components in earlier eluting peaks (Fig.1). Identification by mass spectrometry indicated the peaks correspond respectively by elution order to hexanal, (E)-2-hexenal, Z, 3-hexenol, and 1-hexanol. An unidentified fifth component eluted later at 33 min retention time and had approximately the same concentration as 1-hexanol. The first three compounds were slightly more abundant.

ANOVA analysis of males and females captures in the field. Data analyzed by the least significant test ($p<0.05$) separated the means in six groups. Table 2 shows that traps baited with synthetic identified argan components or their combinations had the same or lower performance than the control (a lure-free trap). The number of males in traps baited with the food synthetic lure (AA-PUT-MAT) was also not significantly different from the control. The food synthetic lure baited traps attracted more females than males but the difference was not significant. Jackson traps baited with TML attracted the highest number of medflies (~100). Their performance to attract males was higher than the performance of the synthetic-food lure baited traps to attract females.

Similarly, tests conducted in the laboratory cages have shown that medflies were not attracted to the various synthetic argan compound tested singly or in combinations. For this reason, no statistical analysis was carried out.

DISCUSSION

Medfly is among the rare species of insects and the only fruit fly attacking argan. Previ-

Table 1: Student t-tests of the number of male and female medflies responding to (artificially injured) argan fruit volatile extract. Tests were conducted for 10 min in (30 x 30x 30 cm) cages containing 50 flies. Table shows mean ± standard error (N= 6).

Treatments	Unmated males	Unmated females	Mated males	Mated females
Extract	27.6 ±2.3	15.8 ± 1.5	38.9 ± 4.0	21.6 ± 1.8
vs				
control	3.3 ± 1.5	1.8 ± 0.6	3.8 ± 0.7	3.6 ± 0.3

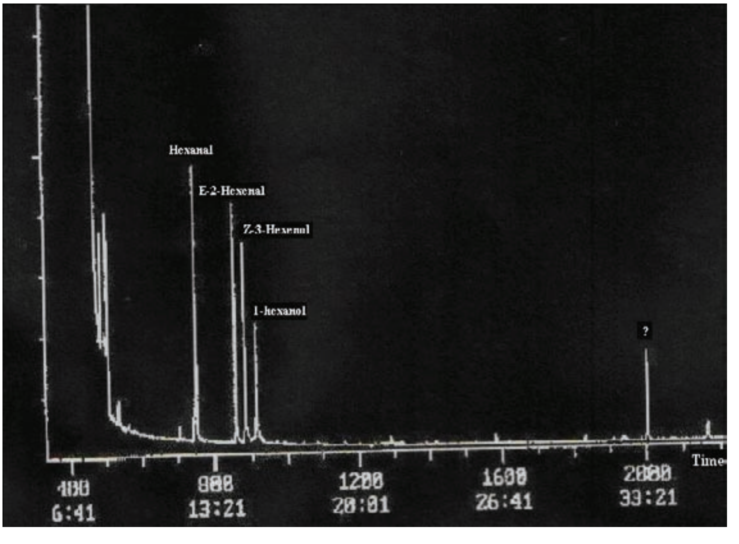

Fig.1: Chromatogram showing volatile compounds of artificially damaged yellow argan fruits. Gas chromatographic-mass spectrometry (GC- MS) was performed with a Hewlett Packard Model 5890A Series II, equipped with a serie of three fused silica columns (8 cm long × 0.5 mm ID, 10m long x 0.25 mm ID, 30 m long x 0.25 mm ID). Column temperature, isothermal at 40°C for 5 minutes, then programmed to 210 °C at 5°C/min. Mass spectra were obtained at using the capillary columns operated as described above, coupled to a Finnigan Ion Trap® mass spectrometer (EI-ITDMS or CI-ITDMS).

Table 2: Field trial captures of traps baited with synthetic argan volatile components and their combinations. Control traps, TML baited traps and AA+ PUT + TMA baited traps are used for comparison. Means in both columns followed by the same letter are not significantly different (P= 0.05; LSD mean separation test on log-transformed (x+1); non-transformed means shown).

Treatments	Mean ± SE	
	Females	Males
1) Hexanol	2.75 ± 0.16 bc	5.25 ± 0.20 bcd
2) Hexenol	2.00 ± 017 bc	4.25 ± 021 bc
3) Hexanal	3.25 ± 0.12 bc	6.50 ± 0.27 bc
4) Hexenal	1.50 ± 0.18 abc	2.50 ± 0.22 abc
5) Hexanal + Hexenal	0.75 ± 0.11 ab	3.75 ± 0.27 abc
6) Hexanal + Hexenal + Hexenol	1.25 ± 0.12 abc	3.00 ± 0.09 bc
7) Hexanal + Hexenal + Hexenol + Hexanol	1.25 ± 0.15 abc	2.50 ± 0.11 bc
8) AA + PUT + TMA	44.75 ± 0.21 ef	15.5 ± 0.16 de
9) Trimedlure	00.00 ± 00.00 a	105 ± 0.13 f
10) Control	2.25 ± 0.08 bc	4.50 ± 0.13 cd

ous studies showed strong chemo-visual attraction of medflies to argan fruits and leaves (Hadis and Bakri 1994). An extract of argan volatiles was also found to elicit more egg laying when loaded in an artificial oviposition device (Hadis et al. 1997).

This preliminary identification of argan fruit volatiles revealed the presence of only four compounds common in the so called "Green leaf volatiles" reported in plants by Dickens et al. (1990) and which are also abundant in other medfly hosts such as coffee (Warthen et al. 1997) and guava (Chyau et al. 1992, Idstein and Schreier 1985, Nishimura et al. 1989) but absent in orange (Hernandez et al. 1966) and mango volatiles (Cosse et al. 1995). Synthetic argan components of the green leaf volatile elicited very low response in laboratory cages and in the field trapping. Similarly, hexanal, (2)-E-hexenal and 1-hexanol elicited only a very low response from female medflies in a flight tunnel (Warthen et al. 1997). This suggests that green leaf volatiles in argan extract may not play a key role in attracting medflies and other longer and/or shorter chain molecular compounds might be the potential attractant candidates and their isolation and identification need still to be investigated. Based on the field results, it is clear that ammonium acetate, putrescine and trimethylamine (Heath et al. 1995, Bakri et al. 1998, Epsky et al. 1999) in association with tephri trap provide an effective system for capturing female medflies but capture about 14% of males compared to TML.

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