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Candidate Attractants for *Bactrocera invadens* (Diptera: Tephritidae) Male Flies from G*ynandropsis gynandra* (Capparidaceae)

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Abstract
These investigations aimed at evaluating the attractiveness of *Gynandropsis gynandra* a plant that attracted male *Bactrocera invadens,* which is an invasive pest known to cause devastating losses in mangoes, citrus, guava and banana among other fruits. *B. invadens* male flies were attracted to *G. gynandra* beginning from 0630 to 1230 hrs in the field. The highest mean number of flies attracted were 65.26 ± 1.06/plant/day, showing a strong positive correlation during the day (r^2 = 0.9423). Using GC-EAD and GC-MS two compounds namely, 4-methyl-3-penten-2-one, and 4-hydroxy-4-methyl-2-pentanone were identified to elicit antennae response of the male *B. invadens*. These two compounds were identified from both *G. gynandra* and male *B. invadens* gut extracts of field collected flies. However only 4-hydroxy-4-methyl-2-pentanone was identified from gut extract of laboratory reared *B. invadens*. Results from this study has provided an insight into the interactions between a non-host plant and an invasive pest, and opened up the prospects for further investigation on the possibility of future use of 4-hydroxy-4-methyl-2-pentanone and 4-methyl-3-penten-2-one in control strategies aimed at *B. invadens*.
Key words: *Bactrocera invadens*, parapheromones, olfactometer, GC-EAD

1. Introduction

Bactrocera invadens (Diptera: Tephritidae) Drew Tsuruta and White, is regarded as one of the most destructive insects of fruits including mango, banana, citrus, guava etc (Clarke *et al* 2005). It was first reported in Kenya in 2003 and later in Tanzania (Lux *et al* 2003 and Mwatawala *et al* 2004). *B. invadens* is closely related to *B. dorsalis* (Diptera: Tephritidae) in terms of their distribution and ecology. It is considered as complex of tropical fruit flies that comprise of more than 75 species that are largely endemic to South-East Asia. The group is also arguably as one of the most important pest species in agroecosystems (Lux *et al* 2003b).

Recently, field evaluation on the host range of *B. invadens* proved that the fruit fly is capable of attacking both cultivated and wild hosts tree crops. However, of all the tree crops, mango, *Mangifera indica* L. (Anarcadiaceae), was reported as the most preferred cultivated host plant (Rwomushana *et al* 2008). Conversely, rampant infestation of *M. indica* by *B.invadens* has led to quarantine restrictions on the exportation of mango to Europe and North America from major mango exporting countries in Africa (USDA-APHIS, 2008 and EPPO Standards, 2010) and The mango export quarantine restrictions affects the well-being of farmers and foreign exchange earnings of the affected countries.

Parapheromones have been employed as one of the tools in the detection and monitoring of a number of fruit flies (Sivinski, J.M. and Calkins 1986). Flies of several economically important Tephritid flies are strongly attracted to specific chemical compounds that are referred to as parapheromones or male lures (Tan, K.H, 2000 and Tan, *et al* 2002). Most pest species of *Bactrocera* are attracted to two major natural attractants, the raspberry ketone (RK, cue-lure) and methyl eugenol (ME) (Tan, 2000 and Tan, *et al* 2002). These compounds were reported to improve the mating competitiveness of males by at least three-fold when compared to the deprived males (Shelly, 1994). Conversely, some plants produce compounds that play a role in the survival of the flies which acts as feeding deterrent to vertebrate predators (Tan, 2000).

Gynandropsis gynandra belongs to the plant family Capparidaceae that include erect, branched, somewhat hairy herbs that grow 0.4 - 1 meter high, and usually have purple stems (Hubbard and Milne-Redhead, 1964). The plant has been used for many years in African traditional medicinal practices. For example, the leaves which have a high content of Vitamin C are taken as a pot herb in soups, either fresh or dried (Ainsle, 1937; Watt, J.M. and Breyer-Brandwijk, 1962 and Kokwaro, 1976). Extracts from the leaves and stems of *G. gynandra* have antibacterial activity against *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Streptococcus faecalis,* and antifungal activity against *Candida albicans, Penicillium sp, Fusarium oxyposirum, Aspergillus flavus and Aspergillus niger* (Ajaiyeoba, 2000). In a tick-climbing repellency bioassay, the oil from *G. gynandra* was repellent to *Rhipicephalus appendiculatus,* which at the highest treatment levels was higher than that by the commercial arthropod repellent *N,N*-diethyltoluamide (Lwande *et al*

1999).
The present study investigated the attractiveness of *G gynandra*, a plant that attracted male flies of *B. invadens* in the field, and the prospects it possess as an alternative tool for monitoring and detecting this fruit fly.

2. Materials and Methods

2.1 Experimental Sites

Larvae of *B. invadens* were collected from infested mangoes in the two fields at Nguruman (01º 48' 31 S, 36 º 03' 34) and Embu (00º 29' 24 S, 37º 35' 31 E). The first site was located about 180 km South West of Nairobi and the second about 140 km from Nairobi in Eastern Kenya. The larvae were reared in the insectaries at International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya to obtain mature flies that were used for wind tunnel bioassays.

2.2 Larvae Collections

Larvae of *B. invadens* were randomly collected from infested mango fruits that were picked from mango orchards. The infested mangoes were then transferred into styroform rectangular containers (30 x 30 x 15 cm) with openings at the top that were covered by netting materials with a mesh to allow for aeration. The containers were kept in a rearing room in the insectary at *icipe*. The larvae were left to develop in the infested native mango fruits and remained in the cage until they reached the fifth instar. They were then washed out with water and transferred into clean sterile plastic bowls containing sterilized sand that mimicked the soil conditions in the field to enable pupation to take place. After a week, pupae were washed from the sand, dried and kept in Petri-dishes in a Perplex® cage (50 x 50 x 50 cm) until the emergence of adult flies. The adult fruit flies were fed on an artificial diet made up of, sugar, enzymatic yeast hydrolysate ultrapure, 3:1, USB Corporation, Cleveland, Ohio, USA, and water on pumice granules. The rearing room was maintained at a temperature of 28 ± 2 °C, relative humidity of 60-65 % and a 12L:12D photoperiod.

2.3 Response of Bactrocera invadens to Gynandopsis gynandra in the Field

Observations on the behavioural responses of *B. invadens* towards *G. gynandra* were conducted in the field at Nguruman. Four healthy plants growing in the field were selected and the numbers of *B. invadens* on them were counted at intervals of 15 minutes between 0630 to 1230 hrs local time, for 9 days. On each day, the numbers of *B. invadens* on the four plants were counted and their mean constituted one replicate. The average number of *B. invadens* on *G. gynandra* for the nine days was calculated. The relative humidity and temperatures were also recorded.

2.4 Dual Choice Olfactometric Bioassays

Behavioural observations were made in a glass dual choice flatbed wind tunnel (30 × 30 ×100 cm) equipped with a 4 inch-extractor fan on top of the mid-section of the tunnel (Plate 2.1). The extractor fan drew the air from both ends at a speed of 27.6 cm/s. The air flow rate in the working section of the tunnel was maintained at 15 ml/s. Compressed medical air (BOC gases, Kenya) from a cylinder was passed through activated charcoal and then split into two streams in order to supply air to the opposite ends (source of volatiles and control) of the wind tunnel. The source of volatiles comprised of fruits held in 2 L flasks that were connected to one end of the wind tunnel. The control was a similar empty (air) 2 L flask that was connected to the other end. Teflon® tubings (5 mm diameter) were used as connectors. The room temperature was maintained at 28 ± 2°C and relative humidity was kept between 59 and 65%. *B. invadens* used in the bioassays were kept in a Perplex® cage (25 ×25 x 25 cm) and were allowed to acclimatize to the conditions in the experimental room for four hours prior each test. Male and female flies that were 12-15 day old after emerging (DAE) were tested separately with host fruit volatiles. *B. invadens* were introduced into the wind tunnel at the central part of the working section through an aperture with a cover. Flies were left for 10 minutes to choose between the side of the tunnel with air enriched with the fruit volatiles or the opposite side which was free of volatiles (control). Responses were recorded thereafter. The flies that flew upwind up to 50% of the distance from the point of release (25 cm of each side of the wind tunnel) into the atmosphere permeated volatiles or to the control were scored as 'responders'. Flies that did not fly beyond the 25 cm mark from the point of release, were treated as 'non-responders' and were excluded from the statistical analyses. Ten flies were tested in each experiment. Each test with volatiles from a given fruit and from a particular maturity stage was replicated five times. At the end of each test, the wind tunnel was cleaned with acetone and the odour source and control were alternated for each test to account for positional biasness. All assays were conducted between 1000 and 1600 hrs local time. However, in the case of *G. gynandra*, plant parts (10 g) including leaves, stems, flowers and pods were used all together in the bioassays as the source of volatiles following the same protocol as the ones described for fruits above

2.5 Solvent Extracts from Gynandropsis gynandra

Extraction of compounds from *G. gynandra* was carried out in the field at Nguruman. Plant parts (leaves, pods, flowers and stems) were cut and dipped in HPLC grade hexane (Sigma-Aldrich®) for 5 min, then decanted to obtain extracts that were stored in 8 ml glass vials. Vials containing the extracts were then wrapped with

aluminum foil and placed in a cooler box with ice. This was then quickly transported to the laboratory for analysis. The residuals were also transferred to the laboratory for weighing. The average weight of the extracted plant materials was approximately 8.9 g.

2.6 Solvent Extracts from the Gut of Male Flies, Bactrocera invadens

Compounds were extracted from the gut of laboratory-reared fruit flies that had been fed on artificial diet as well as from flies collected from the field while feeding on *G. gynandra*. The gut was removed using forceps by pulling gently from the neck while holding the abdomen. Gut materials from 10 insects were extracted using 1 ml of HPLC grade acetone (Sigma-Aldrich®), and then concentrated to remove the acetone. The extract was dissolved in HPLC grade dichloromethane (Sigma-Aldrich®) and the sample was stored at 20ºC prior to GC-MS analysis.

2.7Coupled Gas Chromatography-Electroantennographic Detection (GC-EAD) Analysis

GC-EAD analysis was used to determine the compounds in the trapped complex mixture of host fruit volatiles that stimulated antennal olfactory receptors of female and male flies.

2.7.1 Antennal Preparation

Antennal preparation for recording GC-EAD responses were performed as described by Cossé *et al* 1995 and Du and Millar 1999 with some modifications. Antennae from gravid female flies (12-15 days old) were used for the analysis. The head of an insect was cut off and a reference electrode was inserted into its base with a glass capillary tube filled with Beadle *Ephrussi Ringer* (145 mM NaC1, 1.87 mM KCI, 0.81 mM $CaC1_2$, 2.3 mM. $NaHCO_3$, 0.55 mM $NaHPO_4$). To complete the circuit, the distal end of the antenna was inserted into the tip of the recording glass capillary electrode connected by coaxial cables to a UN 05 amplifier (Syntech®) and the recording equipment.

2.7.2 Gas Chromatography-Electroantenographic Detection (GC-EAD) Analysis

For coupled GC-EAD tests, aliquots (10 µl) of volatile extracts were injected splitless into a HP 5890 Series II GC equipped with an FID detector and an appropriate capillary column (Ultra 1 cross-linked methylsilicone capillary column 25 m × 0.31 mm (i.d.) × 0.025 mm (film thickness)). Nitrogen was used as the carrier gas. The column effluent was split equally with a glass press-fit Y-tube to 2 deactivated fused silica capillaries (50 cm × 0.25 mm i.d.) with one line going to the GC detector and the other through a heated (150 °C) transfer line into a steel stimulus delivery tube delivering moistened air over the antennal preparation of *B. invadens*. The injector, in splitless mode, and flame ionization detector (GC-FID) were kept at 250°C and 270°C, respectively. The oven was programmed at 40°C and held for 3 min, then the temperature was raised at 10°C min^{-1} to a final temperature of 250°C. This was held for 8 min. The FID and EAG signals were recorded simultaneously on an EAD card (Syntech®) in a PC (Dell Optiplex GX280) and the two signals were also viewed on a monitor. Three samples of volatile collections were analysed for each host plant.

2.8 Coupled Gas Chromatography-Mass Spectrometric (GC-MS) Analysis

Gas chromatography-mass spectrometric identification of the compounds was carried out on an Agilent 7890A GC coupled to 5975 MSD. One µl of each sample of volatiles was used for the analysis. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV and emission current of 34.6 µA. The temperature of the source was held at 230 ºC (ion source), 150 ºC (Quadropole) and multiplier voltage was 1106 V. The pressure of the ion source was held at 7 $x10^{-6}$ mBar. The spectrometer had a scan cycle of 3 scans per 2 seconds. The instrument was calibrated using heptacosa (perfluorotributylamine) $[CF_3(CF_2)_3]_3N$ (Apollo scientific Ltd. UK). HP-5 MSGC capillary column, 30 m x 0.25 mm (i.d) x 0.25 µm (film thickness) supplied by J & W Scientific was used. The GC-MS was linked to a computer with MS library (NIST & WILEY). The compounds were identified by comparing their MS fragmentation patterns with library data. MS Fragmentation patterns for the active compounds were further compared with synthetic standards (Sigma-Aldrich®).

2.9 Statistical Analysis

For each test, the total numbers of flies responding in the olfactometer were pooled across replicates and then analysed using Chi-square (χ^2, $\alpha = 0.05$) (PROC FREQ, SAS Institute 1999-2000).[20] Flies that did not respond ('No response' group) were excluded from the analyses.

3. Results and Discussion

3.1 Results

3.1.1 Attraction of B. invadens Male flies to Gynandropsis gynandra in the Field

Males of *B. invadens* were fortuitously observed to visit *G. gynandra* in large numbers in the field during the day which warranted further investigations. Results showed a strong positive correlation between the number of flies visiting the plants and the time of day ($r^2 = 0.94$) for a non-linear model (Figure 1). The number of visiting flies increased gradually and reached a maximum of 65 ± 1.0 flies/plant/day between 0801 and 0815 hrs. Fly numbers then decreased gradually up to two insects per plant per day between 1216-1230 hrs. In addition, the other ambient factors recorded were temperature and relative humidity (Figure 2). The number of flies on the

plants and the relative humidity were found to decrease with an increase in ambient temperature. Thus, there was a positive correlation between the number of flies on the plants and the relative humidity ($r^2 = 0.59$). While on the plant, flies were observed to feed on the surface of the plant parts, *viz.* leaves, flowers, stems and pods (Plate 1).

3.1.2 Dual Choice Olfactometric Bioassays

Assessment in the dual choice olfactometer to evaluate the attractiveness of volatiles from *G. gynandra* indicated higher but non- *B. invadens* significant response to *G. gynandra* volatiles than the control (P> 0.05) (Figure. 3).

3.1.3 GC-MS and GC-EAD Analyses of Plant and Gut Extracts

GC-MS analyses of the hexane extracts of *G. gynandra* indicated the presence of a number of compounds including monoterpenes and diterpenes hydrocarbons, esters, alcohols, ketones, saturated as well as unsaturated hydrocarbons among others (Figure 4; Table 1). The main constituent was 4-hydroxy-4-methyl-2-pentanone at 69.7% followed by δ-3-carene (6.13 %). Among the compounds identified, ketone presented most abundant constituents constituting (70.2%) followed by terpenes at (9.66 %) (Table.1). GC-EAD analyses of antennal olfactory receptors of male flies detected two peaks in the extracts from *G. gynandra* that were identified as 4-methyl-3-penten-2-one **(1)** and 4-hydroxy-4-methyl-2-pentanone **(2)** (Figure 5). These compounds were further identified in the gut extracts removed from flies that were feeding on *G. gynandra* on the same day the extract were collected (Figure 6). On the contrary, analyses of gut extracts from laboratory-reared flies indicated the presence of only 4-hydroxy-4-methyl-2-pentanone (Figure 7). Further analysis with GC-EAD confirms the electroantenographic activities of these compounds. However, 4-methyl-3-penten-2-one exhibited a relatively weak EAG response at the dose tested.

3.2 Discussion

Parapheromones have been documented as significant lures for the management of fruit flies, in particular those of the *Bactrocera* complex of fruit flies (Shelly, 2000).

In general, the results suggest that *G. gynandra* contains potential attractants for males of *B. invadens*. This is the first report of the attractiveness of *G. gynandra* (a non-host plant) to males of *B. invadens*. The present work has discovered two compounds, which were detected by the male antenna of *B. invadens* on GC-EAD and further on GC-MS confirmed the identity of these compounds as 4-methyl-3-penten-2-one **(1)** and 4-hydroxy-4-methyl-2-pentanone **(2)**. Interestingly, the results obtained in these investigations shows behaviour related to what has been previously reported for other species of fruit flies, where many males have been reported to be attracted by compounds emitted by non-host plants (Shelly 2000 and Nishida 2004). However, attractiveness of the volatiles of *G. gynandra* under laboratory conditions was not statistically significant, which could be due to different factors including possible change in composition of volatiles from plant materials as well as artificial environmental conditions of the olfactometer used.

It was further interesting to observe that these compounds are the close intermediates in the synthesis of methyl–isobutyl ketone (MIBK) which is widely used in the industrial manufacture of a large number of products, such as inks, varnishes, etc (Chikán et al 1999 and Melo et al 2002). Its synthesis involves aldolization of two acetone molecules to form **(2)** followed by dehydration of this compound to give **(1)**, which is then selectively hydrogenated to give methyl isobutyl ketone (MIBK) (Chikán et al 1999 and Melo et al 2002).

Taking into account the chemistry of the two compounds, compound **1** is expected to be more stable than compound 2 since this is stabilized by the resonance of unsaturated system. On contrary the quantities of 2 are higher in both, the plant as well as insect gut extracts which suggest that there could be a mechanism that is converting compound 1 to 2. This was further evident in the gut extract of laboratory reared insects in which only compound 2 was identified indicating that there could be some other mechanisms that are employed by the insect in the synthesis of compound **1**. This requires further investigations to be elucidated.

4. Conclusions

In conclusion, this study indicated that the emission of 4-methyl-3-penten-2-one (**1**) may play a major role in the attraction of male *B. invadens* to *G. gynandra* plants in the field. This compound can also be a candidate parapheromone for the attraction of flies in the field and possible control of in the absence of its host plants. In addition, the presence of 4-hydroxy-4-methyl-2-pentanone (**2**) in the guts of male flies that were found feeding on the plant as well as in the plant extract indicates the possibility of facile hydration of compound **1** to form compound **2**. The latter may play a significant role in the biology of the flies.

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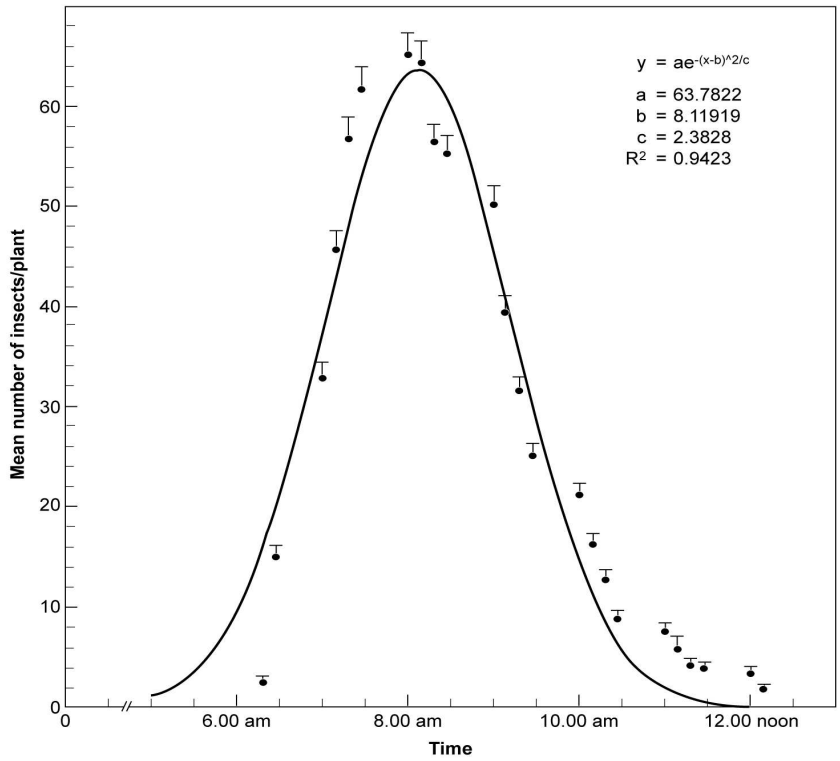

Figure 1 Mean Number of Males of *Bactrocera invadens* Per Plant Per Day Observed on *Gynandropsis gynandra*

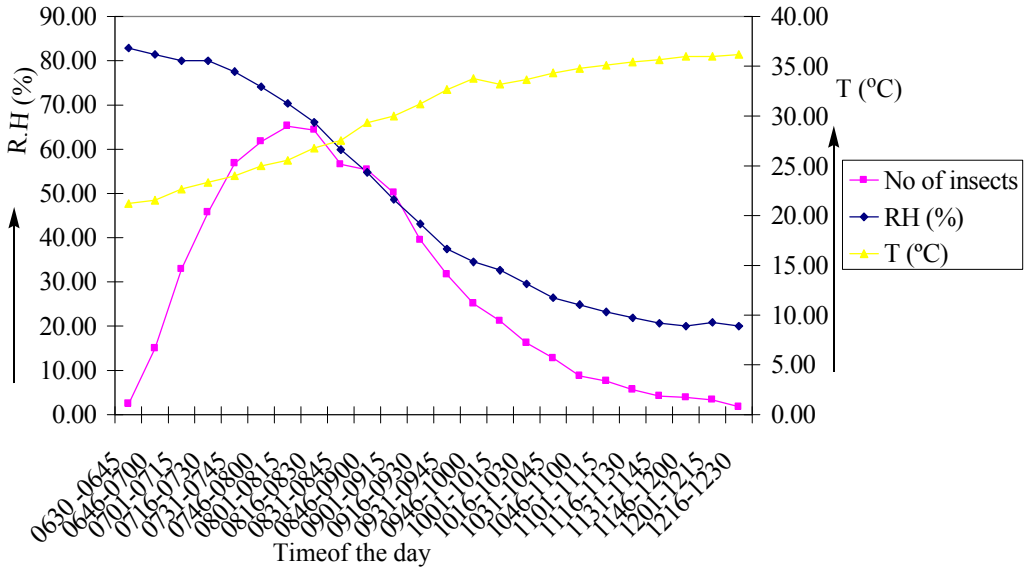

Figure 2 Changes in Temperature, Relative Humidity and Number of Flies on the Plant at Different Times of the Day

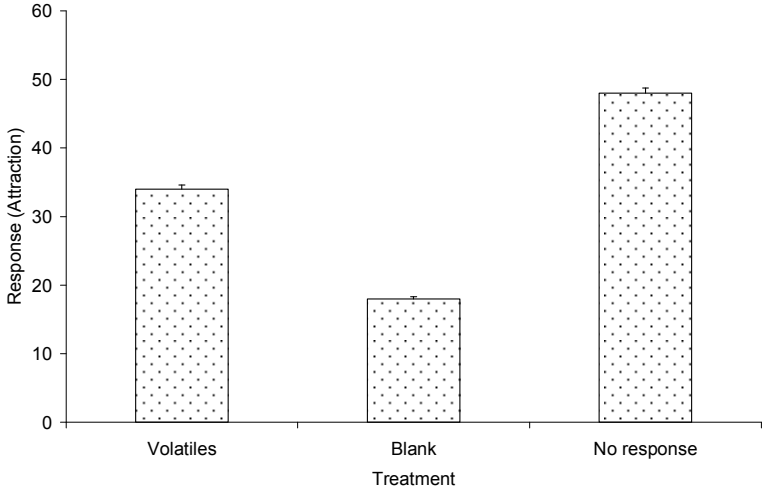

Figure 3 Mean Attraction (± S.E. %) of Male *Bactrocera invadens* to Volatiles from *Gynandropsis gynandra* in a Dual Choice Olfactometer

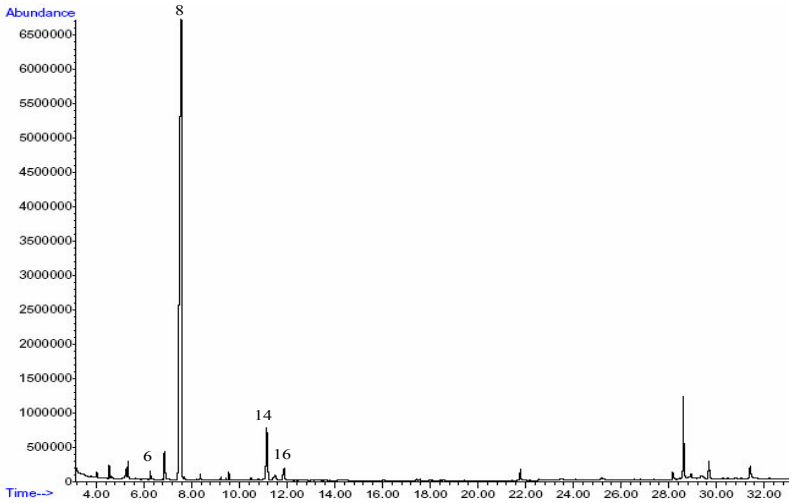

Figure 4 Representative Gas chromatogram of hexane Extracts from *Gynandropsis gynandra*.

Table 1 Relative amounts (%) of compounds identified in hexane Extracts of *Gynandropsis gynandra*

S/N	Compound	R.T. (min)	% Composition
1	ethyl propanoate	4.03	0.34
2	3-methyl-1-butanol	4.54	0.89
3	4-methyl-2-pentene	4.63	0.20
4	ethyl isobutanoate	5.26	0.68
5	toluene	5.33	1.11
6	4-methyl-3-penten-2-one	6.29	0.48
7	isopropyl hydroperoxide	6.87	2.35
8	4-hydroxy-4-methyl-2-pentanone	7.57	69.71
9	Ethyl-2-methylbutanoate	7.7	0.34
10	3-methylbutyl acetate	8.37	0.32
11	-phellandrene	9.45	0.14
12	-pinene	9.56	0.46
13	-pinene	10.48	0.24
14	δ-3-carene	11.15	6.13
15	limonene	11.51	0.99
16	*trans*- -ocimene	11.89	1.45
17	-gurjunene	17.44	0.13
18	-caryophyllene	17.58	0.12
19	*trans*-methyl isoeugenol	21.77	0.88
20	1,13-tetradecadiene	22.55	0.14
21	hexadecanoic acid	23.47	0.20

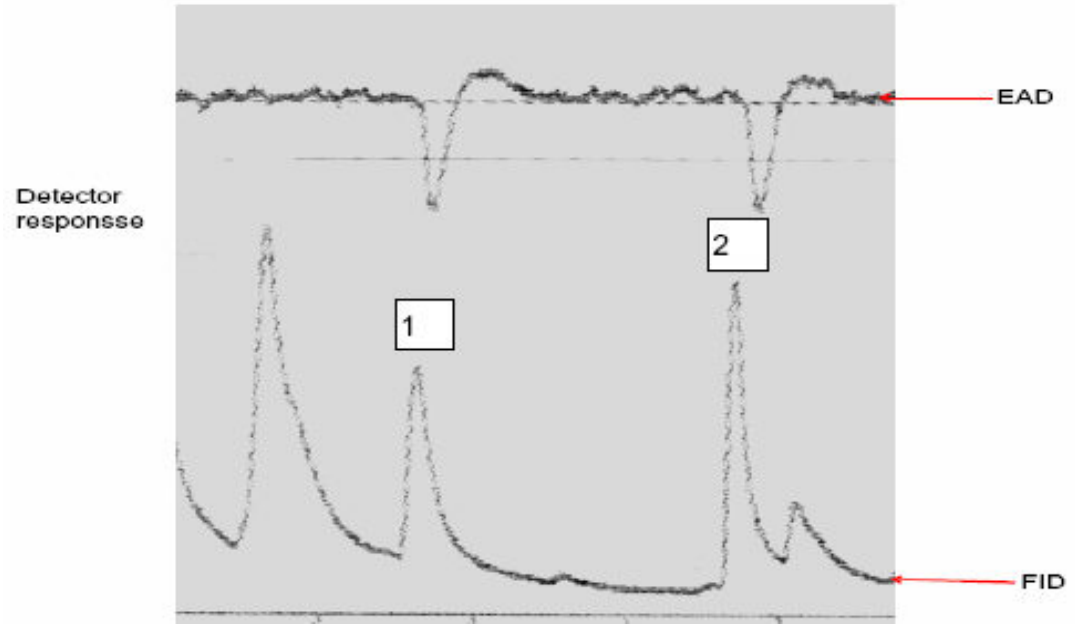

Figure 5 Representative GC-EAD Profile of Compounds in dichloromethane Extract of *Gynandropsis gynandra* Tested on the Antenna of Male *B. invadens* Showing the EAG-Active Peaks (1 and 2)

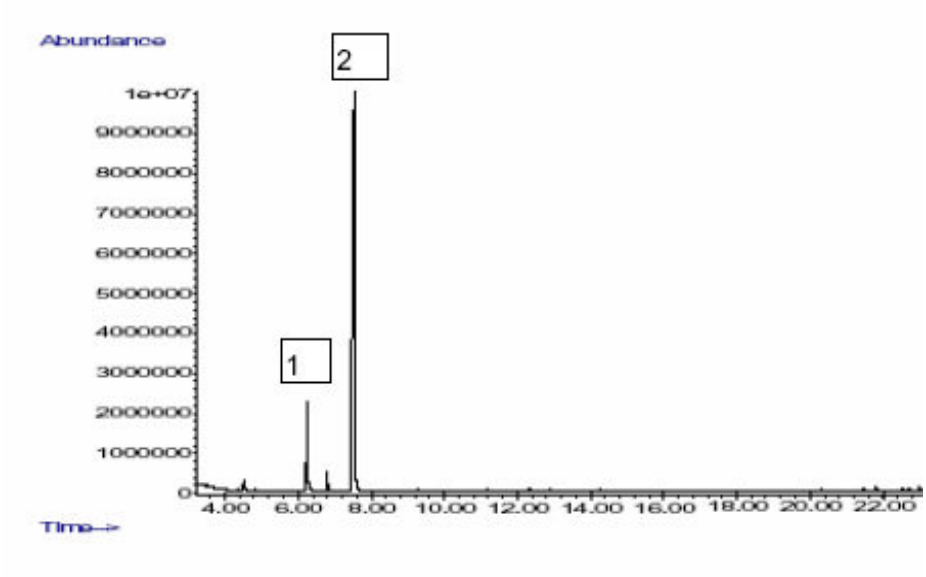

Figure 6 Representative Gas Chromatogram of Gut Extracts from Male Flies, *B. invadens* after feeding on *Gynandropsis gynandra* in the Field

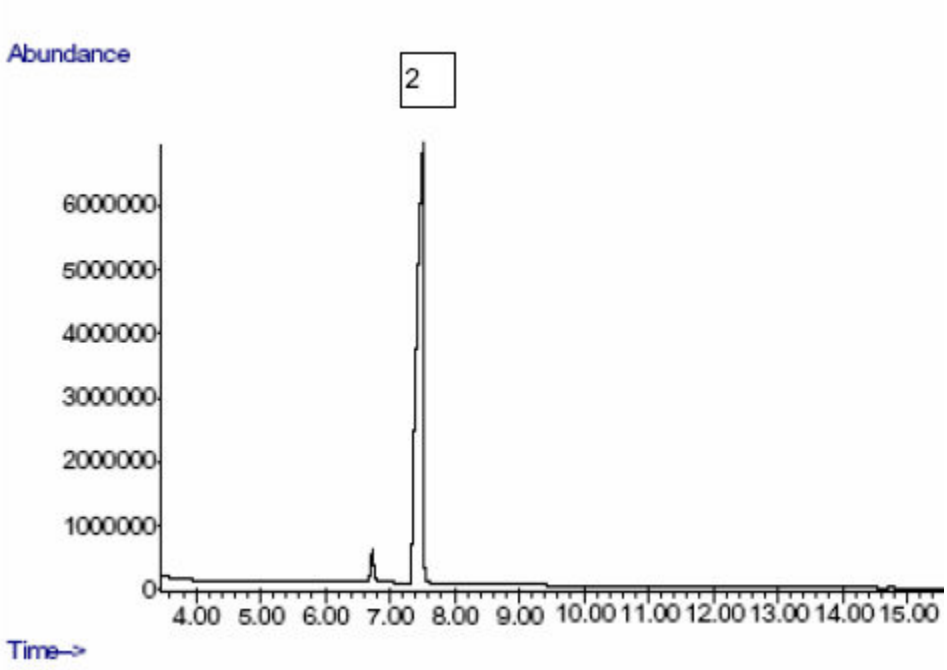

Figure 7 Representative Gas Chromatogram of Gut Extracts from Male Flies, *B. invadens* Reared on Artificial Diet in the Laboratory

Plate 1. Males of *Bactrocera invadens* on *Gynandropsis gynandra* in the Field