Zagazig Journal of Plant Protection Research 2169-2175

Zagazig J. Agric. Res., Vol. 43 No. (6A) 2016

http:/www.journals.zu.edu.eg/journalDisplay.aspx?Journalld=1&queryType=Master

SEXUAL DIMORPHISM IN CUTICULAR HYDROCARBONS OF THE RED PALM WEEVIL, *Rhynchophorus ferrugineus* (OLIVIER) IN RELATION TO MATE CHOICE

Monzer M. Abdelrahman, M.K. Abbas*, A.M. Abdel-Ghany and H.A. Srour

Pl. Prot. Res. Inst. (PPRI), Agric. Res. Cent. (ARC), Egypt

ABSTRACT

Patterns of sexual dimorphism in the cuticular hydrocarbons of the red palm weevil, *Rhynchophorus ferrugineus* (Olivier) were analyzed using gas chromatography/mass spectrometry. A preliminary GC-MS analysis (retention time and EI mass spectrum) of dichloromethane cuticular extracts indicated that males and females share19 components above trace level. Ten of the 19 peaks found in our GC chromatographs, differed in their peak areas and relative abundance between the sexes. In mating bioassays, males attempted to mate with live and freeze-killed females, but did not respond to dead females after cuticular hydrocarbons were washed by dichloromethane, indicating that mate recognition was mediated by sex-specific contact sex pheromones. The occurrence of sexual dimorphism in cuticular hydrocarbons, together with the behavioral results supported the suggest that certain female hydrocarbons mediate mating behavior of the *R. ferrugineus* weevil and provide the first evidence of contact pheromones in *Rhynchophorus* species.

Key words: *Rhynchophorus ferrugineus* (Olivier), mating behaviour, cuticular hydrocarbons, contact sex pheromones.

INTRODUCTION

Hydrocarbons are major constituents of cuticular waxes that are found on the exoskeleton of most insects. Cuticular hydrocarbons are important for diverse functions in insects and have been studied extensively for their roles in desiccation tolerance, protection against environmental stresses, insect communication and short-range recognition of sexual partners (Monzer and Srour, 2009). Recognition of sexual partner in insects can be divided into two main phases, long-range mate location and close-range courtship. Long-range mate location is the upwind orientation and approach of one sex towards the other that mediated by volatile chemicals called sex and/or aggregation pheromones. Once males and females are in close proximity, cues such as visual, acoustic and/or close range contact pheromones are involved in triggering the courtship behavior (Guarino *et al.*, 2008).

***Corresponding author: Tel. :** +21113560621
E-mail address: mohamed.kmal55@yahoo.com

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) is the major pest of various palms in the Middle East, South and South-East Asia, North Africa and South Europe (Murphy and Briscoe, 1999). Since it had been introduced to Egypt at1992, thousands of healthy trees were damaged or lost (El-Sebaey, 2004). The larval and adult stages of this insect feed within tunnels inside the trunk of palms and this behavior frequently damages the infested tree. In infested plantations, yield has been dropped from 4.2 to 0.3 metric ton/faddan (Gush, 1997)

The newly emerged weevils leave trees and aggregate in response to an aggregation pheromone identified as 4-methyl-5-nonanol (ferrugineol), which attracts both sexes (Hallett *et al.*, 1993). Field studies conducted on pheromone traps captured *R. ferrugineus* adults have shown that the majority of adults caught were mated females (El-Sebaey, 2003), probably because of

the fact that the females had already mated inside the infested palm trunks (Hussain *et al.*, 2013). This implies that male recognize female inside the dark close space of palm trunk with mate-recognition mechanism other than visual or olfactory cues, possibly specific contact cuticle chemical (s).

The possible sexual dimorphism in surface cuticle hydrocarbons has not been studied in *R. ferrugineus*. Accordingly, we examine patterns of variation in cuticular composition between genders of *R. ferrugineus* to determine if this species cuticular composition varies between the sexes. We use analyses of gas chromatography profiles to compare individuals of the same age but different sex. The preliminary results are reported here and discussed in reference to mating behavior of *R. ferrugineus* male.

MATERIALS AND METHODS

Insects

The field collected adults of *R. ferrugineus* were reared in the laboratory at room temperature of 25±1°C and relative humidity of 65 ±5.0% on small slices of succulent date palm wood. Obtained newly deposited eggs were collected daily and transferred into plastic cups of small slices of succulent date palm wood, covered with muslin cloth. Newly hatched larvae were immediately transferred to new similar food. The last instar larvae prior to pupation were placed in plastic boxes with similar food and parts of palm fibers were added to help in forming the cocoon. The obtained cocoons were removed from slices of succulent date palm wood, stored individually; daily inspected and emerged adults were collected soon after emergence. Newly emerged adults were separated into male and female groups. Sexing of adults was done according to the presence of a series of black hairs on the dorsal side of smout of male and their absence in the female (Abbas, 2005).

Extraction of Cuticular Hydrocarbons

Cuticular chemicals were extracted from each of 20 newly emerged freeze-killed adults for each sex. Each individual was immersed in a 5-ml aliquot of HPLC grade dichloromethane (DCM) for 5 min. Individual samples were concentrated to dryness under stream of nitrogen and dissolved in corresponding solvent to a volume of 1 ml in case of mating experiments, or to a volume of 120 µl for GC–MS analysis (Ana *et al.*, 2009).

Mating Experiments

To test the hypothesis that males of *R. ferrugineus* use contact pheromones to recognize females, we employed the bioassay methods modified from Ana *et al.* (2009) which was effective in identifying contact pheromones of Curculionidae weevil species. All female treatments were replicated three times and evaluated with 3 males. Female treatments were as follows:

1. A living female was presented to the males to study the repertoire of male mating behavior and to confirm their mating activity.
2. A living male was presented to the males.
3. A female was freeze-killed, allowed to warm to room temperature for 20 min, and presented to the male. If male attempted to mate, this would demonstrate that recognition cues were intact and that behavior of the female was not involved in mate recognition.
4. Freeze-killed females were immersed in 5 ml of DCM for 5 min just before the experiment to strip cuticular chemicals from the female body. After drying for 20 min, a solvent-washed female was presented to test whether male responded to female in any way. If male did not, one could conclude that chemical recognition cues had been eliminated.
5. A male in which cuticular chemicals were stripped was then re-coated with DCM female extract in amount equivalent to that extracted from one female, allowed the solvent to evaporate, and presented to the males to examine the effect of female cuticular chemicals in eliciting male mating behavior.
6. A female in which cuticular chemicals were stripped was then re-coated with DCM male extract in amount equivalent to that extracted from one male, allowed the solvent to evaporate and presented to the males to test whether male responded to male cuticular chemicals in any way. If male did not, one could conclude that chemicals eliciting male

mating behavior are presented in female cuticle but not male.

In most experiments, weevil behavior was observed for 15 minutes at room temperature in glass jars (5 cm height and 15 cm diameter) with covers. The 15-min observation period is adequate for observing and recording all mating activities of the *R. ferrugineus* since most of these activities occur during the first 15 minutes after the introduction of the male weevils in the test arena (Kaakeh, 1998). A trial was scored as a "attempted mating" or "responding" if the male, after antennal contact, stopped walking and mounted the female, or attempted to copulate. When males showed none of these behaviors and continued to walk after first contacting the female, the trial was recorded as "mating not attempted" or "non responding"

GC-MS Analysis

Identification of compounds in DCM extracted from *R. ferrugineus* adults was carried out with a HP 5972A mass spectrometer (MS) coupled to HP-6890 gas-chromatography (GC) equipped with a HP-5MS capillary column (30 m x 0.32 ID, 0.25 µm film thickness). Electron impact (EI) mass spectra were obtained at 70 eV and the instrument scanning from 35 to 700 amu. Helium was used as the carrier gas at a flow rate of 1 ml/min. Injector temperature was 250°C; detector temperature was 280°C, and split was 10:1. Oven temperature was programmed from 35°C (5 min) to 80°C at 10°C/min and to 250°C at 4°C/min. Integration of peaks, drawing calibration table, and standard curve were performed using HP-Chemstation software. Identification of constituents present was based on computer matching against the library spectra (Wiley275L), built up using pure substances and components of known constituents and by comparison of mass spectra and retention times with those of parent M+ ions and the corresponding molecular formulae. Compounds showed initial match quality less than 95% were considered unknown compounds.

Statistical Analysis

All behavioral experiments were replicated three times and evaluated with 3 males. Percentage of male response for each treatment was calculated by dividing number of responded males for each replicate by 3 and then converted to arcsine proportion to normalize the data before statistical analysis was conducted. For the chemical data, the variation in peak area of each hydrocarbon component between both sexes was recorded and percentage concentrations for the detected hydrocarbons were calculated by dividing each peak's area by the total peak area. In this analysis, hydrocarbons with mean relative abundance exceeding 1% were considered, peaks with a mean relative abundance less than 1% were not distinguishable from noise and were not included in the analysis. GC analysis was replicated three times each for hydrocarbons extracted from 3 insects and results were recorded as mean of the three replicates ± standard deviation (SD). Both behavioral and GC data were analyzed by analysis of variance (ANOVA) at $P<0.01$ using the software package Costat (Cohort Inc, Berkeley, USA) (Costat, 1992).

RESULTS AND DISCUSSION

A series of mating experiments in Petri dishes were conducted to assess the behavior of male in the presence of male and female partner as a prelude to further investigation. Results confirmed that *R. ferrugineus* male could discriminate between female and male conspecifics by contact chemoreception. By pairing males with females (No. = 9 males vs 9 females), every male attempted to mount and mate with the female after first antennal contact. On the other hand, by pairing males with other males, only two males showed pre-mounting and mounting behavioral activities with other males, then subsequently avoided them. The proportions of males mounting live and anesthetized females differ significantly ($P<0.05$). These observations were consistent with earlier laboratory studies of Kaakeh (1998) who mentioned that unmated *R. ferrugineus* males attempted to mate with dead females and, in some cases, attempted to mount another male and recorded these observations as strange behavior of *R. ferrugineus* males. Also, our results showed that when recently freeze-killed females were presented to males, 100% of the males attempted to mate with the females, but significantly less proportion of the males (11%) attempted to mate with the dead females washed with DCM, suggesting that certain recognition cues were largely removed from female cuticle

by DCM. This conclusion could supported by the finding that 8 of the nine tested males (88%) attempted to mate with DCM-washed freeze-killed males re-coated with female extracts (Table 1). These chemicals are not present (or present in un-effective concentration) in male cuticle as about 89% of male did not show any response toward DCM-washed freeze-killed female coated with DCM male cuticular extract. The overall results confirm the aforesaid hypothesis that mate recognition in *R. ferrugineus* do not depend on visual detection, colour or behavior of the female, but on certain female cuticular chemicals that play a contact pheromonal role in the permitting copulation of this species. Our results could be confirmed by the observations of Giblin-Davis *et al.* (1996) and Said *et al.* (2003) who stated that some of the palm weevil species seem to respond to cuticle-bound courtship pheromones of conspecific females. Male of *R. palmarum* exhibits precopulatory behavior (a jerky swinging motion of the body) after antennating dead females but not dead male decoys or decoys that had been washed with hexane (Giblin-Davis *et al.*, 1996). Said *et al.* (2003) noticed that contacts of the male antennae with the female body during courtship are necessary for mate recognition, thus suggested a nonvolatile pheromone could be present on the *R. palmarum* female cuticle.

Preliminary analyses of male and female DCM cuticular extracts of *R. ferrugineus* by GC-MS indicated 19 main compounds with relative abundance more than 1% (Table 2 and Fig. 1). Although most components were present in both sexes, their percentages were different between males and females. The most abundant compounds in male extracts were Hexatriacontane and Triacontanol (30.35 and 14.24% of total surface hydrocarbons, respectively), whereas the major compounds in females were unidentified compound-1 and n-Hexacosanol (15.13 and 12.04% of total surface hydrocarbons, respectively). ANOVA showed that the peak areas of each compound differed significantly between sexes for 10 of the 19 identified compounds (Table 2). Of such 10 compounds, 4 were detected in female extracts only (unidentified compound-1, pentadecanal, hexadecane and unidentified compound-2), 3 were present in higher proportions in female cuticular extracts (Tetracosane, Hexacosanol and Octadecenoic acid methyl ester) and 3 were present in higher proportions in male cuticular extracts (2-Nonadecanone, Triacontanol and Hexatriacontane) (Table 2).

The chemical dimorphism in cuticular hydrocarbons that we observed between male and female of *R. ferrugineus*, has not been observed previously in this species or in other *Rhynchophorus* species. In contrast, Giblin-Davis *et al*. (1996) mentioned that hexane or methylene chloride cuticular extracts of *R. cruentatus* adults lack sex-specific differences when analyzed by GC-MS or thin layer chromatography, although the latter authors were the first to suggest chemical factor (contact pheromone) present on the female cuticle to be the key stimulus necessary for a copulatory attempt in different species of palm weevils (Weissling and Giblin-Davis 1993).

Thus, it could be concluded that encounters between the sexes in *R. ferrugineus* could depend on a close-range signal involving cuticular contact pheromones, as demonstrated in the present study, and on a long-range perception of volatile pheromonal and keromonal compounds as established long time ago (Hallett *et al.*, 1993). This conclusion could be supported by the results of microscopic and functional studies of Poorjavad *et al.* (2009) and Said *et al.* (2003) who mentioned that palm weevils antenna have two types of sensillae, the first is known as basioconic sensillae and are known to be sensitive to the aggregation pheromone. Basioconic sensillae more abundant in female than male and in palm weevils. The other type of sensillae is thick-walled with an apical pore and function as contact chemoreceptors and more abundant in male than female. In addition, Salama and Abdel Aziz (2001) mentioned that sensillae trichoidea of *R. ferrugineus* occur on the apex of the antenna club and on the ventral and dorsal sides of tarsus, and they have a dual function as contact mechano- and chemo-receptors. Therefore, authors added that this type of sensillae is much denser on the male than on the female which could reflect their function in male as contact chemoreceptors for detection of cuticle contact pheromone.

Table 1. Response of *R. ferrugineus* male to different treatments

No.	Treatment	No. of males tested*	No. of males attempted to mate	Response (%)**
1	**Live female**	9	9	100a
2	**Live male**	9	2	22b
3	**Freeze-killed female**	9	9	100a
4	**DCM-washed freeze-killed female**	9	1	11b
5	**DCM-washed freeze-killed male + female cuticular extract**	9	8	89a
6	**DCM-washed freeze-killed female + male cuticular extract**	9	1	11b

* - Total of three replicates each of observation of 3-male behaviour.

** - Percentages followed by different litters are significantly different at P < 0.01 after arcsine transformation.

Table 2. Compounds identified in the cuticle of *R. ferrugineus* (males and females), and their corresponding peak area

Peak No.	RT (min)	Relative peak area		(%) Peak area		Suggested compound
		Female	Male	Female	Male	
1	16.6	5.32±1.6	7.53± 2.1	1.75	2.57	Tetradecanal
2	16.78	3.69±1.2	10.9±2.7*	1.21	3.71	2-Nonadecanone
3	17.36	45.99±10.3	ND*	15.13	-	Unidentified compound-1
4	19.11	10.77±3.7	ND*	3.54	-	Pentadecanal
5	21.8	7.12±3.2	ND*	2.34	-	Hexadecane
6	22.93	15.76±4.8	7.9±2.3*	5.18	2.69	Tetracosane
7	23	20.3±5.7	13.37±5.2	6.68	4.56	Heneicosane
8	23.8	36.61±16.0	6.25±2.5*	12.04	2.13	n-Hexacosanol
9	25.13	7.99±3.2	ND*	2.62	-	Unidentified compound-2
10	29.83	8.94±2.9	11.89±2.2	2.94	4.05	Tetratriacontane
11	35.1	5.24±1.0	7.25±3.4	1.72	2.47	n-Nonacosane
12	35.45	3.77±2.1	5.77±2.7	1.24	1.96	unidentified compound-3
13	38.73	28.86±5.9	35.45±7.0	9.49	12.10	Henicosyl formate
14	39.36	14.41±3.9	15.1±3.4	4.74	5.15	Unidentified compound-4
15	39.83	11.03±2.4	10.09±3.1	3.62	3.44	Unidentified compound-5
16	40.34	12.65±3.3	5.07±2.3*	4.16	1.73	Octadecenoic Acid methyl ester
17	49.39	18.88±5.0	25.65±5.6	6.21	8.75	n-Nonacosane
18	52.99	23.32± 5.3	41.73±8.*	7.67	14.24	Triacontanol
19	53.54	23.21±4.1	88.92±21.2*	7.63	30.35	Hexatriacontane
	Total	303.86	292.9			

RT = Retention time. ND = Not detected. ** Significantly different at P < 0.01.

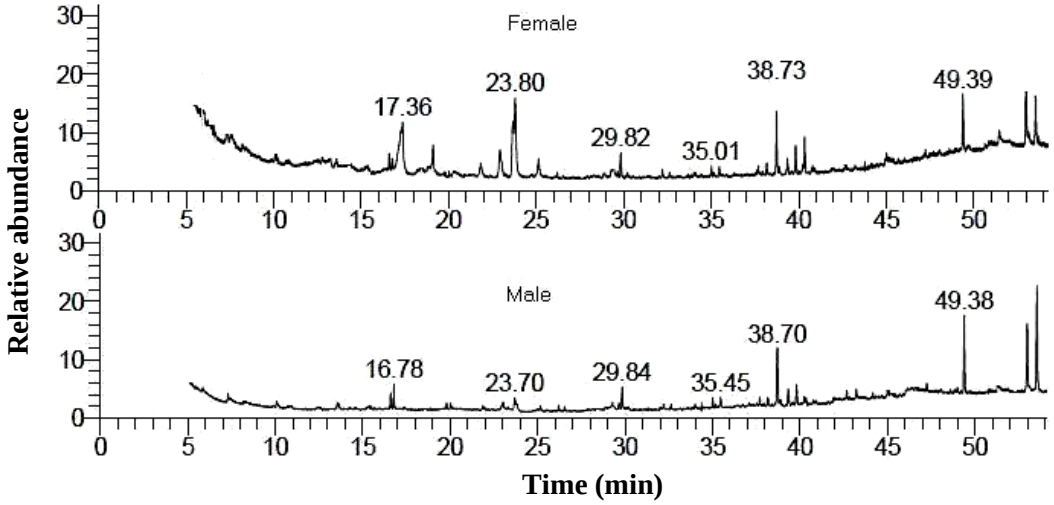

Fig. 1. Representative gas chromatograms of *R. ferrugineus* of (a) female DCM extracts and (b) male DCM extracts, major peaks are labeled with retention time given in Table 2

Although the chemical dimorphism in cuticular hydrocarbons between male and female of *R. ferrugineus* in relation to mate choice, was established by the preceding experiments, the exact identity of the cuticular component remained ambiguous because of the ambiguity of the obtained mass spectral data for several hydrocarbon components in DCM extract. Further extensive behavioral and chemical experiments to separate, identify and characterize the exact contact pheromonal component of *R. ferrugineus* cuticular hydrocarbons and determine whether a single cuticular hydrocarbon component or a combination of some components acts as a contact pheromone are in progress.

REFERENCES

Abbas, M.K. (2005). Integrated management for controlling red palm weevil. Ph.D. Thesis, Fac. Agric., Ain Shams Univ.

Ana, N., P. Leonardo, P. Rubén, P. Fernando and Q. Andrés (2009). Evidence of contact pheromone use in mating behavior of the raspberry weevil (Coleoptera: Curculionidae). Environ. Entomol., 38 (1): 192-197.

Costat, (1992). Software package, Cohort Inc., Berkeley, CA, USA, ver.1.

El-Sebaey, Y. (2003). Ecological studies on the red palm weevil *Rhynchophorus ferrugineus* Oliv., (Coleoptera: Curculionidae) in Egypt. Egypt. J. Agric. Res., 81 (2): 523-528.

El-Sebaey, Y. (2004). Field evaluation of certain insecticides against red palm weevil *Rhynchophorus ferrugineus* Oliv. (Coleoptera, Curculionidae) in Egypt. Egypt. J. Agric. Res., 82 (4): 1591-1599

Giblin-Davis, R.M., A.C. Oehlschlager, A. Perez, G. Gries, R. Gries, T.J. Weissling, C.M. Chinchilla, J.E. Peña, R.H. Hallett, H.D. Pierce Jr. and L.M. Gonzalez (1996). Chemical and behavioral ecology of palm weevils (Curculionidae: Rhynchophorinae). Florida Entomol., 79 (2): 153 -158.

Guarino, S., C. Pasquale, E. Peri, G. Alonzo and S. Colazza (2008). Role of volatile and contact pheromones in the mating behavior of *Bagrada hilaris* (Heteroptera: Pentatomidae) Eur. J. Entomol., 105: 613–617.

Gush, H. (1997). Date with disaster. The Gulf Today, 29 : 16

Hallett, R.H., G. Gries, J.H. Borden, E. Czyzewska, A.C. Oehlschlager, H.D. Pierce Jr., N.P.D. Angerilli and A. Rauf (1993). Aggregation pheromones of two Asian palm weevils, *Rhynchophorus ferrugineus* and *R. vulneratus*. Naturwissenschaften, 80 : 328-331.

Hussain, A., M. Rizwan-ul-Haq, A.M. Al-Jabr and H.Y. Al-Ayied (2013). Managing invasive populations of red palm weevil: A worldwide perspective. J. Food Agric. Environ., 11 (2): 456-463.

Kaakeh, W. (1998). The mating behavior of the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae). Emiron. J. Agric. Sci., 10: 24-46

Monzer, M.A. and H.A. Srour (2009). Desiccation intolerance of the red palm weevil, *Rhynchophorus ferrugineus* (Oliv.) adults in relation to their cuticular hydrocarbons. Egypt. Acad. J. Biological. Sci. (Entomol.), 2 (1): 47-53.

Murphy, S.T. and B.R. Briscoe (1999). The red palm weevil as an alien invasive: biology and the prospects for biological control as a component of IPM. Biocontrol News and Inform., 20 (1): 35-46.

Poorjavad, N., S.H. Goldansaz and A. Avand-Faghih (2009). Response of the red palm weevil *Rhynchophorus ferrugineus* to its aggregation pheromone under laboratory conditions. Bulletin of Insectol., 62 (2): 257-260.

Said, I., D. Tauban, M. Renou, K. Mori and D. Rochat (2003). Structure and function of the antennal sensillae of the palm weevil *Rhynchophorus palmarum* (Coleoptera, Curculionidae). J. Ins. Physiol., 49 : 857-872.

Salama, H.S. and S.E. Abdel Aziz (2001). Distribution of the sensillae of the red palm weevil, *Rhynchophorus ferrugineus* (Oliv.) (Coleoptera: Curculionidae). Ins. Sci. Appl., 2 (2): 179-188

Weissling, T.J. and M. Giblin-Davis (1993). Water loss dynamics and humidity preference of *Rhynchophorus cruentatus* (Coleoptera: Curculionidae) adults. Environ. Entomol., 22 (1): 93-98.

التباين بين الجنسين في الهيدروكربونات الجليدية لسوسة النخيل الحمراء، وعلاقته المحتملة باختيار التزاوج

منذر محمد عبد الرحمن - محمد كمال عباس - أحمد محمد عبد الغني - هشام أحمد سرور

معهد بحوث وقاية النباتات - مركز البحوث الزراعية - مصر

تم تحليل نمط التباين في الهيدروكربونات الجليدية لسوسة النخيل الحمراء ما بين الذكور والإناث (المرتبط بجنس الحشرة) باستخدام جهاز التحليل الكروموتوجرافي الغازي المزود بمقياس طيف الكتلة، وقد أظهرت نتائج التحليل الأولية للهيدروكربونات المستخلصة بمذيب ثنائي كلورو ميثان إلى أن ذكور وإناث سوسة النخيل الحمراء تتشارك في ١٩ مركب هيدروكربوني يمكن قياسهم بكميات محسوسة، وجد أن عشرة من هذه المركبات الـ ١٩ تختلف كميتها ما بين الجنسين اختلافاً ملحوظاً، أظهرت اختبارات التزاوج أن الذكور حاولت التزاوج مع الإناث الميتة المجمدة، ولكنها لم تحاول التزاوج مع الإناث الميتة بعد إزالة الهيدروكربونات السطحية من جليدها بمذيب ثنائي كلورو ميثان، وهو ما يشير إلى أن الذكور قد تتعرف على الإناث بواسطة فيرمونات تلامس جنسية، إن وجود ظاهرة الاختلاف الملحوظ في كميات بعض المركبات الهيدروكربونية على الجليد ما بين الذكور والإناث جنبا إلى جنب مع النتائج السلوكية تدعم الاقتراح بأن بعض الهيدروكربونات الجليدية للإناث تلعب دوراً في سلوك التزاوج لدى سوسة النخيل الحمراء وتقدم الدليل الأول عن وجود دور محتمل لفيرومونات التلامس في أنواع سوس النخيل عامة.

المحكمون :

١- أ.د. على عبدالعزيز الشيخ رئيس بحوث فسيولوجيا الحشرات المتفرغ - معهد بحوث وقاية النباتات- مركز البحوث الزراعية.

٢- أ.د. السيد مجاهـــد متولي أستاذ الحشرات الاقتصادية المتفرغ - كلية الزراعة - جامعة الزقازيق.