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Research Article

Intraspecific geographic variation in sex pheromone of the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae)

Saeede Noorbakhsh[1], Moosa Saber[2*], Hossein Farazmand[3] and Babak HeidaryAlizadeh[3]

1. Department of Plant Protection, Faculty of Agriculture, University of Maragheh, Maragheh, Iran.
2. Department of Plant Protection, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.
3. Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran.

Abstract: The carob moth, *Ectomyelois ceratoniae* (Zeller, 1839), is the most critical pest of pomegranate in Iran. The sex pheromone components emitted by the virgin females were characterized by headspace solid-phase microextraction (HS-SPME) and subsequently analyzed by gas chromatography/mass spectrometry (GC-MS). In this research, the level of individual variation in the female sex pheromone composition of the pest was studied. By extracting pheromone glands of females from different locations, the primary component, (*Z*, *E*)-9, 11, 13-tetradecatrienal, and minor components, (*Z*, *E*)-9, 11-tetradecadienal and (*Z*)-9-tetradecenal, were identified. The following ratios were 10: 2.5: 2.1 (trienal: dienal: monoenal) in Kuhdasht, 10: 1.3: 0.7 in Tarom, 10: 1.3: 0.5 in Bajestan, 10: 1.2: 1 in Sorkheh, 10: 1.1: 0.9 in Ferdows, 10: 0.9: 1 in Neyriz, 10: 0.9: 0.9 in Khash and Meybod, 10: 0.9: 1.4 in Saveh, 10: 0.5: 1 in Behshahr, and 10: 0.45: 0.43 in Shahrreza. There was also a significant variation among the populations in response to wind tunnels and field tests. The discrepancies in these ratios show a possibility of a conspecific relationship among carob moth species in Iran. Findings led to a conclusion of the monomorphic variation in sexual communication of the species.

Keywords: *Ectomyelois ceratoniae*, GC/MS, microextraction, intraspecific, pomegranates, geographic variation

Introduction

The carob moth, *Ectomyelois ceratoniae* (Zeller, 1839) (Lepidoptera: Pyralidae), is a worldwide distributed pest on several nuts and fruits, including carobs, almonds, and dates (Gothilf, 1984; Warner, 1988; Cosse *et al*., 1994). The pomegranate is a preferred fruit for the pest in Iran. Pomegranates are grown in several regions of Iran. Up to 82540 ha of this fruit has been cultivated in recent years (Shakeri, 2015). The high yield and quality of pomegranates in Iran are important export commodities. The larvae of the pest feed inside the fruit, causing severe damage and greatly affecting fruit quality (Olyaie Torshiz *et al.*, 2017). The most recommended control method is relied on collecting and destroying the infested fruits at the end of the growing season that diminishes the overwintering population of the pest (Behdad, 1991; Shakeri, 1992). Biological control

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* Corresponding author: saber@tabrizu.ac.ir
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(Nasrollahi *et al*., 1998), stuffing the pomegranate fruit neck (Mirkarimi, 1996), and removing flags (Farazmand *et al*., 2011) are other methods used for controlling the pest. It seems that the chemical method is not effective enough against the pest. Sex pheromone is usually used for attracting males, but sometimes the commercial pheromone lures do not effectively attract male insects in the field tests (Farazmand, 2011). Three sex pheromone components of the carob moth have been isolated from female pheromone glands (Baker *et al*., 1991; Todd *et al*., 1992). The (Z, E)-9, ll, 13-tetradecatrienal identified as the major component of the carob moth sex pheromone represents a novel structure with regard to previously identified lepidopteran sex pheromone components. Two minor components have been identified: (Z, E)-9, ll-tetradecadienal, and (Z)-9-tetradecenal. Indeed, acetate, alcohol, and aldehyde are also the known sex pheromone components of the moth (Baker 1989; Baker *et al*., 1991). Geographic variation in sexual communication systems has been reported in several lepidopteran species (Toth *et al*., 1992; McElfresh and Millar, 1999; Wu *et al*., 1999; Gemeno *et al*., 2000; Groot *et al*., 2009) that may lead to speciation (Roelofs and Carde, 1974; Phelan, 1992; Baker, 2002; Smadja and Butlin, 2009). Furthermore, geographically varying sexual communication is of interest for pest management. Many lepidopteran insects are pest species that are commonly monitored, disrupted, or killed via pheromone-mediated methods (Cardé and Minks, 1995; Witzgall *et al*., 2010). Monomorphic variation occurs when geographically isolated populations of the same species use the same compounds but in different ratios. In contrast, polymorphic variation occurs when diverse populations have different compounds in their pheromone (Lofstedt 1990). Coevolution of male pheromone reception is often associated with geographic variation in pheromone chemistry (Lofstedt, 1990). Corte *et al*. (2010) demonstrated a variation in pheromone composition and covariation in haplotypes of the *Diatraea saccharalis* Fabricius populations. They suggested that *D. saccharalis* exhibits monomorphic pheromone variation. Anglade *et al*. (1984) demonstrated intraspecific sex pheromone variability in the European corn borer, *Ostrinia nubilalis* (Hubner). This variation may be subject to gradual selection and facilitate the spread of a mutant at the major locus. Communication interference is a potent environmental variable that can exert strong directional selection on the sex pheromone blend in female moths (Lofstedt, 1990, 1991, 1993). Further evidence supporting the idea of pheromone differences between populations has been provided by analysis of female-extracted pheromone components (Hansson *et al*., 1990).

This study aims at determining whether there is geographic variation in the sexual communication of *E. ceratoniae* or not. Identifying pheromone components and quantifying by comparison with all authentic components would be done for evaluating the pheromone for controlling the pest.

Materials and Methods

Insect culture

Infested pomegranates, containing the pupae of *E. ceratoniae*, were collected from Shahrreza, Khash, Kuhdasht, Ferdows, Bajestan, Meybod, Sorkheh, Saveh, Behshahr, Tarom, and Neyriz in 2015 and 2016 early spring. Fruits were kept in the laboratory at 25-28 ºC, 60 ± 5% RH, and a photoperiod of (14L: 10D) for the emergence of adult moths.

Collection of pheromones by dynamic SPME

Headspace solid-phase microextraction (SPME; Supelco, Sigma-Aldrich Corporation, St. Louis, MO, United States) was used to collect pheromone compounds emitted by virgin female carob moths. The SPME fiber (70 µm polydimethylsiloxane coating) was conditioned in a GC injector at 250 °C for 10 min before use. Five virgin female moths were placed in a vial (3.0 × 2.5 cm) sealed with a cap and kept under the laboratory above conditions. The SPME fiber was located in the outlet tube of the vial to collect the emitted volatiles for three days then the loaded fiber was immediately analyzed by coupled GC/MS.

Chemical analysis

All samples were analyzed by GC/MS on a fused capillary column HP5-MS (30 m length × 0.25 mm I. D, 0.25 µm film thickness, Agilent, Technologies, Palo Alto, CA, USA) in an Agilent mod. Chemical analyses were made by an Agilent 6890 gas chromatograph (Santa Clara, USA) coupled with an Agilent 5973 mass detector (GC-MSD) (Santa Clara, USA) under the following conditions: the injector temperature was held at 250 °C; He as carrier gas at l ml/min. The sample was injected in the splitless mode, oven temperature program: 5 min isotherm at 45 °C followed by a linear temperature increase of 4 ºC/min up to 300 °C, which was held for 10 min. All chemicals and reagents were provided from Merck (Kenilworth, NJ, USA) and Sigma-Aldrich. ^{1}H-NMR spectra were measured using a Bruker 500 MHz spectrometer (Bruker, Rheinstetten, Germany), and chemical shifts were expressed as δ (ppm) with tetramethylsilane as an internal standard. The infrared (IR) spectra were obtained on a Shimadzu IRPrestige-21 (Tokyo, Japan). The purity of all compounds was confirmed by the thin-layer chromatography (TLC) using different mobile phases. The elemental analysis was performed with an Elementar Analysensysteme GmbH (Langenselbold, Germany) VarioEL in CHNS mode, which was within 0.4% of theoretical values for C, H, and N.

Chemicals

(*Z*, *E*)-9, 11, 13-tetradecatrienal was synthesized (Noorbakhsh *et al.*, 2017b) by modification of the method of Millar (1990). (*Z*, *E*)-9, 11-tetradecadienal and (*Z*)-9-tetradecenal were obtained from Agrisense-BCS Ltd (Pontypridd, UK). Tetrahydrofuran (THF) was distilled from sodium/benzophenone ketyl under N_2. Prepared solutions were dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation under reduced pressure. Crude products were purified by flash or vacuum flash chromatography on silica gel (230-400 mesh). Reactions with air- or water-sensitive reagents were done in dried glassware under N_2 atmosphere.

Wind tunnel test

Pupae of the pest were collected from Shahrreza, Khash, Kuhdasht, Ferdows, Bajestan, Meybod, Sorkheh, Saveh, Behshahr, Tarom, and Neyriz. Pupae were separated by sex and kept in a temperature room for the emergence of adults. Bioassays were conducted in a 1.5 × 0.5 × 0.5 m wind tunnel used by Miller and Roelofs (1978). Males (2-4 days old) were introduced individually into screen cages (4 cm long × 2 cm diameter) during the photophase, and each cage was covered with a Petri dish lid. Cages with males were kept in a growth chamber for one hour before introducing the wind tunnel to acclimatize males to the wind-tunnel conditions (temperature 25-28 °C, 10 lux light, 60% relative humidity, 0.4 m/sec wind velocity). Bioassays were conducted 4-6 h (the optimal sexual activity period for carob moth), and males were placed in the release site 1 m far from the odor source (Todd *et al.*, 1992). A cage containing a male was placed on the metal platform until the male took flight or until 1 min elapsed, and if males did not fly, they were checked for flight ability by forcibly dislodging them from the cage.

Field test

The experiment was conducted in a randomized complete block design with six treatments and four replications. Treatments included periods of one, two, three, and four weeks after the pheromone emission. The experiments were performed in Saveh, Khash, Meybod, and Kuhdasht regions using pheromones synthesized in Plant Protection

Research Institute and commercial pheromones provided from Spain (ECONEX). The delta-type traps were installed at 1.5 m height on the south side of trees. The traps were set at least 50 m far from each other. Traps were checked every week, and the number of trapped males in each treatment was recorded for one month.

Treatments in wind tunnel and field were:

1- Alcohol is associated with a key molecule: (*Z, E*)-9, 11, 13-OH

2- 10: 0: 0 trienal (a key molecule): dienal: monoenal

3- 10: 1: 1 trienal: dienal: monoenal

4- 10: 2: 2 trienal: dienal: monoenal

5- 10: 3: 3 trienal: dienal: monoenal

6- (*Z, E*)-7, 9, 11-Dodecatrienyl formate purchased from ECONEX Spain

7- Natural pheromone (Tree females of carob moth from the same region)

In another wind tunnel test, two additional treatments (along with the seven mentioned above) were also tested :

1- Five females of carob moth from the same region

2- Five females of carob moth from another region

1 to 3-day old virgin females each was placed in a screen cage as an odor source.

Statistical Analysis

The wind tunnel assays were designed as factorial design. Data were analyzed using SAS statistical software. The number of responded males for each treatment in the wind tunnel was subjected to a two-way analysis of variance. The means were compared by Duncan's multiple range test with a significant difference at $p < 0.05$.

Result

A blend of (*Z, E*)-9, 11, 13-tetradecatrienal (trienal), (*Z, E*)-9, 11-tetradecadienal, and (*Z*)-9-tetradecenal was identified from pheromone gland of carob moth. The ratios of the sex pheromone components in different regions are shown in Table 1. Comparison of diagnostic ions (79, 67, 55, 206, and 91) with each other indicated the peak at Retention time (RT) = 12.56 ± 0.01, belonging to (*Z, E*)-9, 11, 13-tetradecatrienal. Also, comparing the ions 67 and 55, as the base peak, for two other components resulted in the assignment of two peaks at RT = 11.45 ± 0.01 and 10.86 ± 0.01, for compounds (*Z, E*)-9, 11-tetradecadienal and (*Z*)-9-tetradecenal. As shown in Table 1, the ratio was 10: 0.45: 0.43 in the Shahrreza sample. It was 10: 1.1: 0.9 in Ferdows and 10: 0.9: 0.9 in Khash and Meybod, 10: 0.9: 1 in Neyriz, and 10: 1.2: 1 in Sorkheh. Also, the Kuhdasht sample had a different ratio of 10: 2.5: 2.1. The Bajestan and Tarom sample had 10: 1.3: 0.5 and 10: 1.3: 0.7 ratios, respectively. The Saveh and Behshahr sample had 10: 0.9: 1.4 and 10: 0.5: 1 ratios, respectively. Considering the discrepancies in the ratios, it seems that there might be a conspecific relationship between carob moth species in Iran.

Table 1 Variation in the ratio of sex pheromone components of *Ectomyelois ceratoniae* in different regions of Iran.

City	(Z, E)-9, 11, 13-tetradecatrienal	(Z, E)-9, 11-tetradecadienal	(Z)-9-tetradecenal
	Ratio	Ratio	Ratio
Kuhdasht	10.00	2.50	2.10
Tarom	10.00	1.30	0.70
Bajestan	10.00	1.30	0.50
Behshahr	10.00	0.50	1.00
Ferdows	10.00	1.10	0.90
Meybod	10.00	0.90	0.90
Neyriz	10.00	0.90	1.00
Khash	10.00	0.90	0.90
Saveh	10.00	0.90	1.40
Sorkheh	10.00	1.20	1.00
Shahrreza	10.00	0.45	0.43

In the wind tunnel bioassays, the initial results are shown in Table 2 *(Z, E)*-9, 11, 13-tetradecatrienal (Z9, *E*11, 13-14: Ald.), which is made in the laboratory, was able to evoke wing fanning and orientation toward the odor source in male moths. Males were released from their cages 1 m away from the odor source. A cage containing a male was placed on the metal platform until the male took flight or until 1 min elapsed. The males flew upwind to volatiles emitted from 47.5 ± 4.78 to 66.3 ± 3.65% and landed on the source (Table 2). After leaving the release cage, most males flew upwind zigzag towards Z9, *E*11, 13-14: aldehyde. Males flew to within 67.5 ± 5.9 - 92.5 ± 3.22 cm of the compound and hovered (remain in one place in the air) for a short time before veering (change direction suddenly) away or landing on the dispenser platform. Virgin females showed a similar pattern of the average flight distance with significantly more landing distance than synthetic pheromone (87.5 ± 5.95-95 ± 3.53: 67.5 ± 5.9 -92.5 ± 3.22 cm). However, comparison of the average time of flight(s) between the 1 mg dosage of Z9, *E*11, 13-14: Ald and virgin female indicated significantly increased upwind flight from 13 ± 0.57-29.2 ± 0.47 (s) to 22.5 ± 2.39-46.5 ± 5.20 (s) (especially in Meybod and Sorkheh). The percentage of males making contact with the source was 47.5 ± 4.78 ~ 66.3 ± 3.65, compared to virgin females 49.02 ± 6.40 ~ 62.5 ± 7.21. The strongest responses from males of *E. ceratoniae* to (Z9, *E*11, 13-14: Ald) were reported in Sorkheh (66.3 ± 3.65).

Behavioral studies and bioassays

Evaluation of synthesized pheromone in the field

The average of daily trapping was used to evaluate the performance of laboratory synthesized pheromone in the garden. The interaction of region × treatment was significantly different (F = 3.1; df = 13, 54; *P* = 0.00017). There were significant differences among the treatments (F = 6.05; df = 5, 54; *P* = 0.0002) and regions (F = 31.71; df = 3, 54; *P* = 0.0001). Aldehyde treatment showed better performance than alcohol and formate treatments in trapping the insects (Table 3).

The average daily trapping in the regions is summarized in Table 4. Kuhdasht, with a trapping of 0.35%, had the best result. The highest average of the daily trapping (0.8 ± 0.08%) was related to aldehyde 10: 2: 2 in Saveh and the non-trapping to formate in Kuhdasht (Table 5).

Bioassay and behavioral studies of carob moth in the wind tunnel

1- The males' response to synthesized pheromone in the wind tunnel

the male Carob Moth response to synthesized pheromones in the wind tunnel was investigated at 10, 50, 80, and 100 cm from the source of pheromones and pheromones contact supplier. Results showed that there were significant differences in treatments (F = 34.33; df = 6, 231; *P* = 0.0001) and different regions (F = 2.92; df = 10, 231; *P* = 0.0018) in the 10 cm distance, and the other tested distances.Only the geographical region had no significant effect at 100 cm distance (F = 0.93; df = 10, 231; *P* = 0.503). Source Contact data analysis did not show a significant difference among treatments (F = 0.83; df = 6, 231; *P* = 0.549) and among different areas (F = 1.12; df = 10, 231; *P* = 0.344).

The average flight percentage of males at 10, 50, 80, and 100 cm in different treatments are presented in Table 6. The highest and lowest average flight percentage of males were recorded for the aldehyde treatment 10: 0: 0 (54.84 ± 1.71) at 10 cm distance and for the formate treatment (0.45 ± 0.45) at 100 cm, respectively.

In the percentage of flying up to 10 cm, the highest mean flying percentage was observed for aldehyde 10: 0: 0, 10: 1: 1, and natural pheromone, with weaker performance for aldehyde 10: 2: 2, 10: 3: 3, and alcohols. The formate treatment with a mean of 24.55 ± 2.01 had the lowest percentage of flight.

Table 2 Percentage of male carob moth flying in a wind tunnel in response to (*Z*, *E*)-9, 11, 13-tetradecatrienal in four regions of Iran.

Treatment	Cities	Males responding ± SE (%)[1]	The average time of flight ± SE (s)[1]	The average flight distance ± SE (cm)[1]
Z9, E11, 13-14: Ald	Saveh Saveh	47.5 ± 4.78a	13.0 ± 0.57 d	67.5 ± 5.90 b
Virgin Female	Saveh Saveh	49.0 ± 6.40 a	22.5 ± 2.39 dc	88.7 ± 5.54 ab
Z9, E11, 13-14: Ald	Meybod	60.4 ± 6.24 a	24.5 ± 1.25 dc	92.5 ± 3.22 ab
Virgin Female	Meybod	60.4 ± 6.24 a	46.5 ± 5.20 a	95.0 ± 3.53 a
Z9, E11, 13-14: Ald	Sorkheh	66.3 ± 3.65 a	29.2 ± 0.47 bc	86.2 ± 4.73 ab
Virgin Female	Sorkheh	56.2 ± 6.25 a	43.5 ± 3.06ab	90.0 ± 4.56 ab
Z9, E11, 13-14: Ald	Shahrreza	57.5 ± 6.27 a	25.5 ± 2.84 dc	81.2 ± 8.26 ab
Virgin Female	Shahrreza	62.5 ± 7.21 a	44.0 ± 4.69 a	87.5 ± 5.95 ab

[1] Means within a column followed by different letters are significantly different (Duncan's multiple range test, $P < 0.01$).

Table 3 Comparison of the average daily capturing in the field condition.

Ratio	Number	Male/trap/day (Mean ± SE) (%)[1]		
		Highest	Lowest	Mean ± SE
10: 00: 00	16	0.55	0.00	0.14 ± 0.04 a
10: 01: 01	16	0.60	0.00	0.20 ± 0.04 a
10: 02: 02	16	0.55	0.00	0.19 ± 0.05 a
10: 03: 03	16	0.50	0.00	0.17 ± 0.04 a
Z9, E11, 13-14OH	8	0.20	0.00	0.05 ± 0.02 b
Formate	16	0.32	0.00	0.02 ± 0.02 b

[1] Means within a column followed by different letters are significantly different (Duncan's multiple range test, $P < 0.01$).

Table 4 Comparison of the average daily capturing in different regions in the field.

Region	Number	Male/trap/day (Mean ± SE) (%)		
		Highest	Lowest	Mean ± SE (%)[1]
Kuhdasht	24	0.55	0.00	0.35 ± 0.04 a
Saveh	20	0.30	0.00	0.07 ± 0.02 b
Khash	20	0.60	0.00	0.05 ± 0.02 b
Meybod	24	0.32	0.00	0.10 ± 0.02 b

[1] Means within a column followed by different letters are significantly different (Duncan's multiple range test, $P < 0.01$).

Table 5 Average daily capturing rate of different ratios in the studied regions.

Region	Male/trap/day (Mean ± SE) [1]					
	10: 00: 00	10: 01: 01	10: 02: 02	10: 03: 03	Z9, E11, 13-14OH	Formate
Saveh	0.76 ± 0.04 a	0.74 ± 0.03 a	0.80 ± 0.08 a	0.76 ± 0.05 a	0.75 ± 0.04 a	0.70 ± 0.00 a
Khash	0.03 ± 0.02 a	0.16 ± 0.01 a	0.06 ± 0.04 a	0.02 ± 0.02 a	0.03 ± 0.02 a	0.02 ± 0.02 a
Kuhdasht	0.41 ± 0.04 a	0.40 ± 0.04 a	0.48 ± 0.03 a	0.45 ± 0.02 a	NT	0.00 ± 0.00 b
Meybod	0.03 ± 0.01 a	0.19 ± 0.02 a	0.08 ± 0.03 a	0.15 ± 0.05 a	NT	0.08 ± 0.08 a

NT: not tested, [1]Different letters in a row indicate a significant difference using Duncan's multiple range test ($p < 0.05$).

Table 6 Percentage of male flying in response to different ratios at distances of 10, 50, 80, and 100 cm wind tunnel.

Flight distance	Number of males (Mean ± SE) (%)[1]						
	10: 00: 00	10: 01: 01	10: 02: 02	10: 03: 03	Z9, E11, 13-14OH	Formate	Natural pheromone
Up to 10 cm	54.84 ± 1.71a	52.86 ± 1.82a	43.80 ± 1.93b	41.54 ± 1.95b	40.62 ± 1.92b	24.55 ± 2.01c	54.65 ± 2.22a
Up to 50 cm	49.72 ± 1.42a	48.07 ± 1.60a	37.11 ± 2.00b	36.09 ± 1.76b	33.65 ± 1.79b	19.75 ± 1.76c	51.62 ± 1.75a
Up to 80 cm	23.68 ± 2.50ab	20.48 ± 2.50bc	16.80 ± 2.25c	15.57 ± 2.42c	14.11 ± 1.90c	4.54 ± 1.37d	28.26 ± 2.47a
Up to 100 cm	10.38 ± 1.97ab	9.87 ± 1.94ab	8.10 ± 1.73ab	6.70 ± 1.65b	6.79 ± 1.64b	0.45 ± 0.45c	12.67 ± 2.05a

[1] Means within a column followed by different letters are significantly different (Duncan's multiple range test, $P < 0.01$).

In the case of 50 cm flight percentage, the treatments were divided into three subgroups. The highest mean male flying rate happened for aldehyde 10: 0: 0, aldehyde 10: 1: 1, and natural pheromone. Alcohol, aldehyde 10: 2: 2, and aldehyde 10: 3: 3 were placed in the second group. Formate with the lowest average flight (19.75 ± 1.76%) was placed in the third group.

The aldehyde treatments were 10: 0: 0 and 10: 1: 1 in the second subgroup. The third group received aldehyde 10: 1: 1, 10: 2: 2, and 10: 3: 3, and finally, treatment Formate with the weakest performance was placed in the fourth subgroup. Aldehyde 10: 0: 0 and natural pheromone at 80 cm flying points had the best performance with an average of 23.68 ± 2.50 and 28.26, respectively.

Results of the mean percentages of flight from 10, 50, 80, and 100 cm for different regions are given in Table 7. The highest percentage of males flying at 100 cm distances belonged to aldehyde 10: 0: 0, 10: 1: 1, 10: 2: 2, natural pheromone, and formate with the lowest mean flight percentage (0.45 ± 0.45) were allocated to the third group. The highest average flight percentage (50.65 ± 2.71) was reported for the Sorkheh, with a 10 cm flight. While the lowest average daily trapping rate (5.03 ± 1.93) was observed for the percentage of flying 100 cm in Saveh.

2- The males' response to the female of the same region and female of the other region

According to Table 8, the differences between response of males of Ferdows region to the females of same area, relative to, the males reaction of Saveh and Khash to the Ferdows females was significant at 10 cm (F = 32.57; df = 2, 9; P = 0.0001), 50 cm (F = 10.86; df = 2, 9; *P* = 0.004), 80 cm (F = 10; df = 2, 9; *P* = 0.0052), but was not significant at 100 (F = 3.0; df = 2, 9; *P* = 0.1004).

According to Table 9, differences between response of Shahrreza males to the females of same area, relative to, the males' reaction of Bajestan, Sorkheh and Meybod to the Shahrreza females was significantly different at flight distances, 10 cm (F = 21.08; df = 3, 12; *P* = 0.0001), 50 cm (F = 9.06; df = 3, 12; *P* = 0.002), 80 cm (F = 11.58; df = 3, 12; *P* = 0.0007) and 100 cm (F = 3; df = 3, 12; *P* = 0.049).

According to Table 10, the difference in Meybod males' response to female of their region, relative to the male reaction of Neyriz and Tarom to Meybod female at flight distances, 10 cm (F = 10.29; df =2, 9; *P* = 0.004), 50 cm (F = 10.29; df = 2, 9; *P* = 0.004), 80 cm (F = 4.98; df =2, 9; *P* = 0.035) and 100 cm (F = 8.80; df = 2, 9; *P* = 0.007) were significant.

According to Table 11, differences between response of Sorkheh males to the females of same area, in comparison with the males' reaction of Shahrreza, Kuhdasht and Behshahr to the Sorkheh females was significant at flight distances, 10 cm (F = 7.08; df = 3, 12; *P* = 0.005), 50 cm (F = 6.99; df = 3, 12; *P* = 0.005), 80 cm (F = 7.83; df = 3, 12; *P* = 0.003) and 100 cm (F = 3; df = 3, 12; *P* = 0.049).

According to Tables 8-11, males of each region prefer females of the same region for mating; certainly, pheromone produced by different populations and patterns of recognition and response of males should be mutually agreeable, because otherwise a reproductive success would be prevented.

In general, the average response rate (positive response) at different distances and the time of response of male insects to pheromones treatments, regardless of geographic differences, is presented in Tables 12 and 13. According to the results, the farther the distance from the source of pheromones, the longer the reaction time and flight time.

Table 7 Average percentage of male individuals flight at distances of 10, 50, 80, and 100 cm of the wind tunnel.

Region	Number of males (Mean ± SE) (%)[1]			
	10 cm	50 cm	80 cm	100 cm
Neyriz	38.54 ± 2.78d	34.95 ± 2.79cd	20.74 ± 2.64bc	10.57 ± 2.27a
Shahrreza	50.00 ± 2.46a	44.87 ± 2.74a	19.02 ± 3.14bcd	7.65 ± 2.01a
Ferdows	43.19 ± 3.82bcd	38.70 ± 3.30abcd	18.68 ± 3.79cd	8.92 ± 2.93a
Bajestan	49.47 ± 3.55abc	40.81 ± 3.38abcd	16.17 ± 3.29cde	8.00 ± 2.10a
Kuhdasht	42.91 ± 2.91abcd	37.78 ± 3.32cd	16.36 ± 2.99cd	8.45 ± 2.38a
Saveh	40.17 ± 2.93cd	34.01 ± 2.61d	12.21 ± 2.69de	5.03 ± 1.93a
Behshahr	43.27 ± 2.41abcd	35.86 ± 2.18bcd	13.57 ± 2.30cde	5.92 ± 1.99a
Sorkheh	50.65 ± 2.71a	45.38 ± 2.91a	11.90 ± 2.07de	5.88 ± 1.58a
Khash	39.31 ± 3.13cd	35.08 ± 3.37d	8.62 ± 2.54e	5.65 ± 2.21a
Meybod	48.29 ± 3.09ab	43.53 ± 2.89ab	25.48 ± 3.25ab	8.92 ± 2.23a
Tarom	45.83 ± 3.07abcd	42.74 ± 2.78abc	31.22 ± 2.74a	11.36 ± 2.48a

[1] Means within a column followed by different letters are significantly different (Duncan's multiple range test, *P < 0.01*).

Table 8 Average percentage of male flying from different regions in response to Ferdows females at different distances in the wind tunnel.

Region	Number of males (Mean ± SE) (%)[1]			
	10 cm	50 cm	80 cm	100 cm
Ferdows	68.75 ± 6.25a	62.50 ± 7.21a	37.50 ± 7.21a	12.50 ± 7.21a
Saveh	25.82 ± 2.75b	15.40 ± 5.41b	4.15 ± 4.15b	0.00 ± 0.00a
Khash	25.82 ± 2.75b	21.65 ± 2.05b	5.00 ± 5.00b	0.00 ± 0.00a

[1] Means within a column followed by different letters are significantly different (Duncan's multiple range test, *P < 0.01*).

Table 9 Average percentage of males flying from different regions in response to Shahrreza females at different distances in the wind tunnel.

Region	Number of males (Mean ± SE) (%)[1]			
	10 cm	50 cm	80 cm	100 cm
Shahrreza	62.50 ± 7.21a	56.25 ± 6.25a	43.75 ± 6.25a	12.50 ± 7.21a
Bajestan	21.25 ± 1.25b	15.00 ± 5.00 b	0.00 ± 0.00b	0.00 ± 0.00b
Sorkheh	18.70 ± 2.10b	18.70 ± 2.10b	41.15 ± 4.15ab	0.00 ± 0.00b
Meybod	26.65 ± 4.87b	21.65 ± 2.05b	10.40 ± 6.24b	0.00 ± 0.00b

[1] Means within a column followed by different letters are significantly different (Duncan's multiple range test, *P < 0.01*).

Table 10 Average percentage of males flying from different regions in response to Meybod females at different distances in the wind tunnel.

Region	Number of males (Mean ± SE) (%)[1]			
	10 cm	50 cm	80 cm	100 cm
Meybod	60.40 ± 6.24a	60.40 ± 6.24a	37.50 ± 7.21a	20.82 ± 7.21a
Neyriz	32.57 ± 4.50a	32.57 ± 4.50a	15.62 ± 9.37ab	0.00 ± 0.00b
Tarom	14.55 ± 5.23b	14.55 ± 5.23b	4.15 ± 4.15b	0.00 ± 0.00b

[1] Means within a column followed by different letters are significantly different (Duncan's multiple range test, *P < 0.01*).

Table 11 Average percentage of males flying from different regions in response to Sorkheh females at different distances in the wind tunnel.

Region	Number of males (Mean ± SE) (%)[1]			
	10 cm	50 cm	80 cm	100 cm
Sorkheh	56.25 ± 6.25a	56.25 ± 6.25a	25.00 ± 0a	12.50 ± 7.21a
Shahrreza	21.65 ± 2.05b	18.52 ± 2.64b	9.15 ± 5.32b	0.00 ± 0.00b
Kuhdasht	14.55 ± 5.23b	10.40 ± 6.24b	4.15 ± 4.15b	0.00 ± 0.00b
Behshahr	10.00 ± 5.77b	10.00 ± 5.77b	0.00 ± 0.00b	0.00 ± 0.00b

[1] Means within a column followed by different letters are significantly different (Duncan's multiple range test, $P < 0.01$).

Table 12 Average percentage of male response to different treatments in a wind tunnel.

Ratio	Average of male's response				
	10 cm	50 cm	80 cm	100 cm	Source contact
Z9, E11, 13-14OH	40.63	33.66	14.12	6.80	0.00
10: 0: 0	54.84	49.72	23.68	10.38	1.45
10: 1: 1	52.87	48.07	20.48	9.87	0.57
10: 2: 2	43.80	37.11	16.80	8.10	0.45
10: 3: 3	41.54	36.09	15.57	6.70	0.00
Formate	24.55	19.76	4.55	0.45	0.32
Nat pheromone	54.66	51.62	28.27	12.67	1.02

Table 13 Time spent to reach the source of odor by males in the wind tunnel.

Ratio	Duration of time spent (s)				
	10 cm	50 cm	80 cm	100 cm	Source contact
Z9, E11, 13-14OH	9.63	16.52	20.33	24.76	0.00
10: 0: 0	10.00	17.39	22.91	26.89	66.66
10: 1: 1	10.18	17.18	22.50	25.77	60.00
10: 2: 2	10.48	18.30	23.25	29.50	75.00
10: 3: 3	10.52	17.39	22.20	28.07	0.00
Formate	12.11	20.40	25.44	23.00	60.00
Natural pheromone	19.02	26.77	27.77	18.89	70.00

In general, aldehyde treatments were more attractive, and natural pheromones (virgin female), aldehyde 10: 0: 0, and aldehyde 10: 1: 1 showed the most attractive synthesized treatments had aldehyde comparable to the pheromones of the virgin females. In terms of attractiveness, alcohol, and formaldehyde treatments were not as good as aldehyde treatments.

Discussion

The mechanism by which divergence in sexual communication systems occurs and its relevance on speciation is a controversial subject of evolutionary biology (Lofstedt, 1993). The evolution of premating signals is generally shaped by intra and interspecific selection. In moths, the predominant premating signals consist of multicomponent sex pheromone blends produced by females to which only conspecific males are attracted (Lofstedt, 1990, 1993). It is important to define the role of each constituent, to delineate the direction and magnitude of selection exerted on multicomponent pheromone blends.

Pheromone composition in moths can vary drastically, especially in those species with wide distribution ranges (Carde and Haynes, 2004). This geographic dissimilarity may be genetic due to the founder effect, genetic drift, or even natural or sexual selection. However, the evolutionary basis for such divergence is unclear (Templeton, 1980; Rice, 1987). Moth sexual pheromones are widely studied as a fine-tuned system of intraspecific sexual communication that reinforces interspecific reproductive isolation (Allison and Carde, 2016)**.**

In this study, the combination of (*Z, E*)-9, 11, 13-tetradecatrienal, (*Z*, *E*)-9, 11-tetradecadienal, (*Z*)-9-tetradecenal and alcohol-related to the main molecule of *E. ceratoniae* pheromone was extracted by SPME, and the monomorphic differences in the ratio of these three molecules in different regions were found by GC/Mass method. This ratio was 10: 0.45: 0.43 in the Shahrreza sample, it was 10: 1.1: 0.9 in Ferdows and 10: 0.9: 0.9 in Khash and Meybod, 10: 0.9: 1 in Neyriz and 10: 1.2: 1 in Behshahr respectively, they are similar to the data ratio of Baker *et al*. (1991). Also, the Kuhdasht sample had a different ratio of 10: 2.5: 2.1. The Bajestan and Tarom samples had 10: 1.3: 0.5 and 10: 1.3: 0.7 ratios, respectively. Saveh and Sorkheh samples had 10: 0.9: 1.4 and 10: 0.5: 1 ratios, respectively. The ratios of Shahrreza, Ferdows, Khash, and Kuhdasht, have already been reported by Noorbakhsh *et al.* (2017a).

Based on our results, there were differences among the ratios we reported to Baker *et al*. (1991). Still, the highest difference was observed in Kuhdasht with a ratio of 10: 2.5: 2.1. These ratios lead to quantitative and qualitative differences between the pheromones of the Iranian Carob moth that occurs when different populations of the same species use the same compounds but in different amounts and ratios (Lofstedt, 1990). For example, the pheromone blend of *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae) consists of Z11–14: AC and E11–14: Ac components, so males of this species present preferential response for the ratio 97: 3 and 3: 97 for North American and European populations, respectively (Klun and Cooperators, 1975). Such variations have also been reported for other lepidopterans, such as *Agrotis segetum* (Arn *et al*., 1983; Hansson *et al*., 1990; Toth *et al*., 1992), *Hemileuca eglanterina* (McElfresh and Millar, 2001), *Agrotis ipsilon* (Gemeno *et al*., 2000; Du *et al*., 2015), *Choristoneura rosaceana* (El-Sayed *et al*., 2003), *Heliothis subflexa* (Groot *et al*., 2007) *Spodoptera frugiperda* (Unbehend *et al*., 2014) and *Ostrinia nubilalis* (Anglade *et al*. 1984).

Iranian carob moth is widespread in areas with different weather conditions and climates. Behavioral responses and individual pheromone compounds may vary geographically. Geographic differentiation between populations could occur. Understanding the evolution of pheromone communication in this context is critical because changes in population densities may result in shifts in both the pheromone blend and behavioral responses of males (Groot *et al*., 2007). However, The behavioral significance of pheromone compounds may vary geographically, and geographic variation in moth pheromone blends has been indicated in several species (e.g., Klun and Cooperators, 1975; Cardé *et al*., 1977; Guerin *et al*., 1984; Gemeno *et al*., 2000; McElfresh and Millar, 1999, 2001; Gries *et al*., 2001; El-Sayed *et al*., 2003; Groot *et al*., 2007). For instance, Mozaffarian *et al*. (2007) observed geometric and morphometric differences among Iranian carob moths due to the genetic changes in the population. These changes were considered to have resulted from natural selection and adaptation to environmental conditions (Girling and Carde 2006).

Moreover, Ziaaddini *et al*. (2010) indicated differences in foraging behaviors of males and calling behaviors of females in different populations of Carob moth in Saveh, Kerman, and Arsanjan under the same conditions. However, the differences between populations did not prevent the cross attraction, and mating was not prevented between the different populations (Gemeno *et al*., 2000; Phelan and Baker 1986). In addition, Aranzazu *et al*. (1988) studied sex pheromone of European corn borer, *Ostrinia nubilalis,* and revealed differences between laboratory and field strains.

For this reason, in this study for the whole population, the last instar larvae and pupae were collected from gardens and studied for the sex pheromone since the adults emerged. (*Z, E*)-9, 11, 13-tetradecatrienal was synthesized (Noorbakhsh *et al.,* 2017b) by modification of the method of Millar (1990). The synthesis of aldehyde molecule, which is the main component of pheromone and the alcohol-related to it, was successfully carried out in the laboratory at a shorter path and less costly, which is active in the biological studies in the wind tunnel and the garden, which attracts the males completely (Noorbakhsh *et al.*, 2017b).

The wind speed obtained was 0.4 m/s in the wind tunnel test for flying moths (Todd *et al*., 1992), and when the wind speed increased, flying to the source of pheromones and insect placement on the surface was not achieved.

According to the studies, when males were ready to mate, they responded very well to the test and flew to the source. Also, attraction by natural pheromone took slightly longer than synthesized compounds, and male moths showed a faster response to synthesized substances. The main molecule was responsible for catching, and alcohol caught little.

Pheromone identification of carob moth populations from different geographical regions of the country was carried out. Differences in the ratios of three pheromone compounds were determined, synthesis of pheromone and testing its efficiency were performed in the laboratory. In addition to results of GC/MS, geographic differences and monomorphic variations were also demonstrated at various flight distances in the wind tunnel and the garden.

All compounds containing the main molecule aldehyde were effective in attracting, but the imported commercial formate was practically ineffective for Iranian populations in the wind tunnel and the garden. The males of different geographic regions preferred females of the same area because the pheromone blend produced by females of the various populations and the patterns of detection and response by males is a congruent mismatch of male and female reproductive signals. Therefore behaviors would constitute a constraint to reproductive success (McElfresh and Millar, 2001).

Lack of mutual attraction in populations may be due to lack of coordination and behavior at the time of the calling, time release of pheromones, and phase mismatch (Soofbaf et al., 2007 and 2013). on the other hand, besides the pheromones, the combination of factors such as the synchronous calling behavior in females, searching for males, the coordination of romantic behaviors effectively attracts the opposite sex (Phelan and Baker, 1986; Ziaaddini et al., 2010). Even the hairs and smells produced by the males (Hairpencil) (Hillier and Vickers, 2004) can also attract, fly, and catch. Any specific differences in these factors can lead to reproductive isolation and lack of mating.

Geographic variation in pheromone composition may be helpful to develop efficient trapping schemes for accurately monitoring this economically important insect and shed light on whether this variation is an important contributor to population divergence and potential speciation. This information can also be complemented by determining the phylogenetic history of the populations showing pheromone composition variation. In Lepidoptera, DNA sequences of the protein cytochrome oxidase II have been used successfully for determining phylogenetic relationships of populations (Sperling and Hickey, 1994).

Our studies revealed significant differences in the relative amounts of the three compounds previously reported as components of the female sex pheromone in extracts of pheromone. Our results showed geographic variations in sex pheromone composition in populations from different regions.

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تنوع جغرافیایی در فرومون جنسی کرم گلوگاه انار *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae)

سعیده نوربخش[1]، موسی صابر[2*]، حسین فرازمند[3] و بابک حیدری علیزاده[3]

۱- گروه گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه مراغه، مراغه، ایران.
۲- گروه گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه تبریز، تبریز، ایران.
۳- مؤسسه تحقیقات گیاه‌پزشکی، سازمان تحقیقات، آموزش وترویج کشاورزی، تهران، ایران.
پست الکترونیکی نویسنده مسئول مکاتبه: saber@tabrizu.ac.ir
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چکیده: شب‌پره کرم گلوگاه انار *Ectomyelois ceratoniae* (Zeller, 1839) (Lepidoptera: Pyralidae)، مهم‌ترین آفت انار در ایران و جهان است. اجزای فرومون جنسی *E. ceratoniae* که توسط ماده‌های باکره منتشر می‌شود، به‌روش میکرواستخراج فاز جامد (HS-SPME) جداسازی و سپس به‌روش گاز کروماتوگرافی- طیف‌سنجی جرمی (GC/MS) تجزیه و بررسی شد. تغییرات "مونومورفیک" فرومونی وقتی صورت می‌گیرد که به‌صورت جغرافیایی جمعیت‌های گونه‌های یکسان از هم جدا می‌شوند و ترکیبات فرومونی یکسان بوده ولی نسبت‌ها متفاوت هستند. پژوهش‌ها جهت اندازه‌گیری و تعیین سطح اختلاف افراد در اجزای فرومون جنسی کرم گلوگاه انار در سال‌های ۱۳۹۴ و ۱۳۹۵ صورت گرفت. با استخراج فرومون از غدد افراد ماده *E. ceratoniae* جمعیت‌های مختلف مناطق ایران، مولکول اصلی (Z, E)-9, 11, 13-tetradecatrienal و دو مولکول جزیی (Z, E)-9, 11-tetradecadienal و (Z)-9-tetradecenal با نسبت‌های مختلف در مناطق جغرافیایی شناسایی شدند. نسبت سه ترکیب فرومون جنسی trienal: monoenal :dienal در مناطق مختلف ایران با یک‌دیگر متفاوت است، در کوهدشت این نسبت ۲/۱ : ۲/۵: ۱۰ بود و در سایر مناطق به شرح ذیل است: طارم ۰/۷ : ۱/۳: ۱۰، بجستان ۰/۵ : ۱/۳: ۱۰، سرخه ۱ : ۱/۲: ۱۰، فردوس ۰/۹ : ۱/۱: ۱۰، نیریز ۱: ۰/۹ : ۱۰، خاش و میبد ۰/۹: ۰/۹: ۱۰، ساوه ۱/۴ : ۰/۹ : ۱۰، بهشهر ۱: ۰/۵: ۱۰، شهررضا ۰/۴۳ : ۰/۴۵ : ۱۰، با توجه به اختلافات موجود در این نسبت‌ها، به‌نظر می‌رسد که رابطه‌ای بین پروانه‌های این‌گونه در ایران وجود دارد که نشان‌دهنده تنوع مونومورفیک جنسی در این گونه است. هم‌چنین بین جمعیت‌ها در آزمایشات تونل باد و مزرعه نیز اختلاف معنی‌دار مشاهده شد.

واژگان کلیدی: *Ectomyelois ceratoniae*، GC/MS، میکرواستخراج، ارتباط درون گونه‌ای، انار، تنوع جغرافیایی