Identification of the Sex Pheromone of *Isoceras sibirica* Alpheraky (Lepidoptera, Cossidae)

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Z. Naturforsch. **66c**, 527–533 (2011); received December 30, 2010/April 12, 2011

We discovered that extracts of the female sex pheromone gland of the carpenterworm moth *Isoceras sibirica* Alpheraky, a pest of *Asparagus officinalis* Linn., contained (*Z*)-7-tetradecen-1-ol (Z7–14:OH), (*Z*)-9-tetradecen-1-ol (Z9–14:OH), (*Z*)-7-tetradecenyl acetate (Z7–14:Ac), (*Z*)-9-tetradecenyl acetate (Z9–14:Ac), and (*Z*)-9-hexadecadecenyl acetate (Z9–16:Ac). The average levels of the chemicals in a single sex pheromone gland of a calling moth were (0.71 ± 0.24) ng, (1.42 ± 0.44) ng, (4.36 ± 0.32) ng, (8.71 ± 0.26) ng, and (0.82 ± 0.38) ng, respectively. The electroantennography (EAG) analysis of these chemicals and their analogues demonstrated that Z9–14:Ac triggered significantly the male EAG response. Traps with rubber septa lure impregnated with Z9–14:Ac (500 μg/septum), Z7–14:Ac (250 μg/septum), and Z9–16:Ac (50 μg/septum) were more effective in catching male moths than traps with other baits or virgin females. Addition of Z7–14:OH and Z9–14:OH to rubber septa did not enhance the efficiency of the trap.

Key words: Isoceras sibirica, (*Z*)-9-Tetradecenyl Acetate, (*Z*)-7-Tetradecenyl Acetate

Introduction

The carpenterworm, *Isoceras sibirica* Alpheraky (Lepidoptera, Cossidae), is a destructive pest affecting primarily *Asparagus officinalis* Linn. It is widely distributed in the former Soviet Union (Siberia), Mongolia, and north and north-east of China.

The larvae of *I. sibirica* burrow into the root crowns of *A. officinalis*, preferentially newly planted ones, with the occurrence rate as high as over 50%. In recent years, they occurred mainly in City of Yongji and Wenxi County, Shanxi, China. Chemical insecticides are not effective enough due to the cryptic nature of *I. sibirica*. Thus, the cost of pesticides for prevention and control per hectare has lately increased by 35%. The profit from planting *Asparagus* is gravely minimized by the pest, causing losses of about 25 to 30% and subsequently posing great threat to the development of the *Asparagus* industry (Duan *et al*., 2008). Year after year, long-term heavy application of pesticides resulted in the pollution of the environment, harming human health, leading to the prohibition and restriction of pesticide application. In view of this situation, it becomes critical to seek highly efficient, nontoxic, and environmentally friendly approaches to controlling the pest.

Sex pheromones of seven related Cossidae species have been reported, *i.e*. *Prionoxystus robiniae* (Solomon *et al*., 1972), *Cossus cossus* (Capizzi *et al*., 1983), *Cossus mongolicus* (Qi *et al*., 1990), *Holcocerus insularis* Staudinger (Zhang *et al*., 2001), *Holcocerus hippophaecolus* (Fang *et al*., 2005), *Holcocerus artemisiae* (Zhang *et al*., 2009), and *Holcocerus arenicola* (Jing *et al*., 2010). Sex pheromones of *I. sibirica* have not been reported and characterized. For *I. sibirica*, although many aspects of its bionomics and integrated control technology have been studied (Zhang and Du, 2007; Du *et al*., 2007; Duan *et al*., 2008), the chemical composition of its sexual pheromone remains unknown. Therefore, the objective of the present work was to identify the components of the *I. sibirica* female pheromone, and to develop an efficient trap lure that can be used to monitor and control the pest by field trapping using blends of synthetic compounds.

Methods and Materials

Insects

Cocoons were collected from soil at 5–10 cm depth around the roots of *A. officinalis* infested

with larval *I. sibirica* in late April 2009 in Taigu, Shanxi Province, China, and were kept in an outdoor net cage (6 m × 4 m × 2 m) to allow for eclosion under natural conditions. Male moths were removed immediately after emergence from the cocoons, whereas female moths were left in the cage. The antennae of male moths were used for electroantennography (EAG) analyses. The abdominal tips of females were used for pheromone extraction and identification.

Pheromone extracts

After 24 h of observation of female moths in the cage, we found that 1-day-old females began calling and mating for 1 h in the morning and 2-day-old females for about 3 h after sunset. During the calling period it is optimal to conduct the extraction of pheromone. Sex pheromone glands were extruded by applying slight pressure to the females' abdominal ends to force eversion of the ovipositor, which was excised with small scissors and immersed in re-distilled *n*-hexane (*ca.* 50 μL/tip) with 1-undecanol as internal standard for 30 min at room temperature. The *n*-hexane extracts were transferred and pooled to a clean conical glass vial and kept in a freezer at –10 °C if not used immediately. Extracts were concentrated under a gentle stream of N_2 before analysis.

Chemicals

Semiochemicals (>98% purity), which were used in the analytical work, EAG analysis, and lures for field trials, were synthesized in our chemical ecology laboratory. Reagents and solvents were from Fisher Chemicals (Fair Lawn, NJ, USA).

Chemical analysis

The analytical procedure was described previously by Zhang *et al*. (2009). Briefly, GC analysis of sex pheromone gland extracts and standard compounds was performed on an Agilent 6890N gas chromatograph (Palo Alto, CA, USA) fitted with a flame ionization detector (FID) and a splitless injector. Two fused silica capillary columns, A (HP-1, 50 m × 0.22 mm × 0.33 μm film; Agilent) and B (BP10–0.5, 50 m × 0.42 mm × 0.33 μm; SGE, Pty Ltd., Rinywood, Victoria, Australia), were used with the following temperature program: 80 °C for 1 min, then 4 °C/min to 180 °C and 10 °C/min to 240 °C, isothermal for 14 min for column A; 80 °C for 2 min, then 4 °C/min to 200 °C, isothermal for 25 min for column B. Gas chromatography-mass spectrometry (GC-MS) analyses were performed on a Finnigan Trace DSQ GC-MS instrument (EI mode, 70 eV; Austin, TX, USA). Fused silica capillary columns (HP-1, 50 m × 0.22 mm × 0.33 μm) were used with the following temperature program: 80 °C/min to 280 °C, isothermal for 20 min. Mass spectral data and retention times of selected peaks on both columns were compared with the corresponding data of reference standards.

Micro-analytical reaction

Double-bond positions and geometries in unsaturated compounds were determined from dimethyldisulfide (DMDS) adducts of the insect-produced compounds (Buser *et al*., 1983; Leonhardt and Derilbiss, 1985). A mixture of 50 μL DMDS and 5 μL iodine-diethyl ether solution (0.06%) was added to an extract containing 10 FE (female equivalent) and kept at 40 °C overnight. The resulting solution was quenched with 200 μL of 5% $Na_2S_2O_3$ solution in distilled water, and the mixture was then extracted with 100 μL *n*-hexane. The resulting solution was dried over $MgSO_4$ and analysed by GC-MS on the non-polar column (HP-1).

Electroantennograms

EAG assays were performed as described previously (Roelofs *et al*., 1971; Zhang and Meng, 2000). Electrophysiological responses of antennae dissected from 1- to 2-day-old males to a series of C_{14} and C_{16} unsaturated alcohols and acetates and extracts were measured. Solvent blank puffs (filter paper and *n*-hexane) were used as the controls.

Field tests

The field trials were conducted in *Asparagus* fields in Taigu and Wenxi, Shanxi Province, China during the season of *I. sibirica* occurrence (May to June 2009). The activity of the EAG-active or identified compounds was evaluated using a trap method described previously (Zhang and Meng, 2000; Zhang *et al*., 2001), in which different chemical compositions were dissolved in petroleum ether and loaded onto green rubber septa. White

delta sticky traps were hung on wooden support about 0.5–1 m in height and in 50- to 60-m intervals in the field. The insect traps were checked every day. Virgin females of 1- to 2-day-old *I. sibirica* were put into a small cage in the centre of a trap as bait for comparison. Petroleum ether was used as control. Each formulation was repeated 6 times in a randomized block. The experiments were designed to examine the response of the pest to the different chemical compositions and the optimum dosage of each active component. Captured moths were recorded and removed daily.

Results

Analysis of sex pheromone gland extracts

GC analysis of female sex pheromone gland extracts and a series of *Z*- and *E*-isomers of monounsaturated C_{14} and C_{16} standards was carried out on both the A and B capillary columns under different temperature conditions. In terms of the retention times (Rt), the extracts gave peaks 2 to 7 in Fig. 1, which co-chromatographed consistently with the synthetic standard compounds (*Z*)-7-tetradecen-1-ol (Z7–14:OH), (*Z*)-9-tetradecen-1-ol (Z9–14:OH), (*Z*)-7-tetradecenyl acetate (Z7–14:Ac), (*Z*)-9-tetradecenyl acetate (Z9–14:Ac), (*Z*)-9-hexadecadecenyl acetate (Z9–16:Ac), and stearic acid (18:COOH). When 0.2 μL extract and 0.2 μL blend of equal parts of 1-undecanol (internal standard 5 ng) and these compounds were injected simultaneously into column A, the peaks 2–7 increased. These results strongly suggested that Z7–14:OH, Z9–14:OH, Z7–14:Ac, Z9–14:Ac, and Z9–16:Ac were present in the sex pheromone of *I. sibirica*. Moreover, quantitative analysis by GC showed that the titer (ratio) of the compounds varied around a mean of (0.71 ± 0.24) ng, (1.42 ± 0.44 ng), (4.36 ± 0.32) ng, (8.71 ± 0.26) ng, and (0.82 ± 0.38) ng, respectively, in the sex pheromone gland of a single calling female.

The GC-MS data (Table I) obtained for the components of the gland extracts revealed that the mass spectra of peaks II and III (Fig. 2) produced common diagnostic ion fragments of *m/z* 194 [$M^+ - H_2O$], *m/z* 31 [CH_2OH^+], and *m/z* 71 [$C_4H_6OH^+$] of tetradecen-1-ol (Silverstein *et al.*, 1981) with the retention times of 23.58 and 23.89 min, agreeing with those for the same ions and retention times of a synthetic sample of (*Z*)-7-tetradecen-1-ol (Z7–14:OH) and (*Z*)-9-tetradecen-1-ol (Z9–14:OH). Peaks IV and V contained diagnostic fragments at *m/z* 194 [$M^+ - CH_3COOH$] and *m/z* 61 [$CH_3COOH_2^+$] of tetradecen-1-yl acetate (Brown *et al.*, 1988) with the retention times of 26.19 and 26.49 min, coinciding with those for synthetic (*Z*)-7-tetradecen-1-yl acetate (Z7–14:Ac) and (*Z*)-9-tetradecen-1-yl acetate (Z9–14:Ac). Peak VI showed diagnostic ions at *m/z* 222 [$M^+ - CH_3COOH$], 194 [$M^+ - CH_3COOC_2H_5$], *m/z* 61 [$CH_3COOH_2^+$], and *m/z* 43 [$O{=}CCH_3^+$], suggesting hexadecen-1-yl acetate.

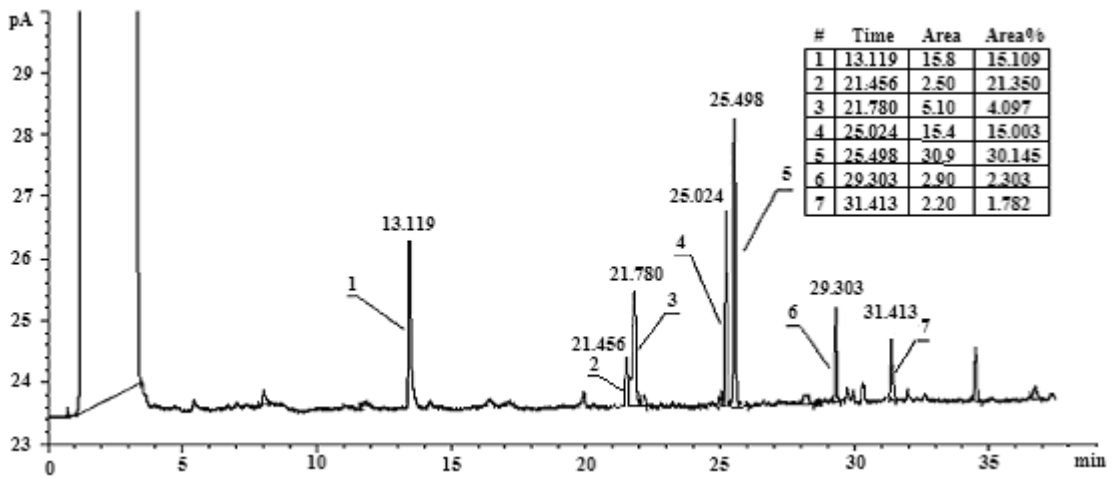

Fig. 1. Gas chromatogram (HP-1) of the pheromone gland extracts (0.2 μL) of *I. sibirica* and 1-undecanol (internal standard, 5 ng).

Table I. Mass spectral data of components of sex pheromone gland extract and standard compounds.

Component	Identity	*m/z* (relative intensity of major ions) [assignment]
II	Z7–14:OH	194(23) [$M^+ - H_2O$], 152(5), 138(13), 124(18), 123(24), 110(26), 109(34), 96(85), 95(67), 82(87), 81(57), 71(7) [$C_4H_6OH^+$], 69(40), 68(62), 67(100), 55(56), 54(40), 31(14) [CH_2OH^+]
III	Z9–14:OH	194(22) [$M^+ - H_2O$], 152(2), 138(11), 124(20), 123(23), 109(31), 96(75), 95(72), 82(68), 81(62), 71(9) [$C_4H_6OH^+$], 69(43), 68(60), 67(100), 55(68), 54(28), 31(14) [CH_2OH^+]
IV	Z7–14:Ac	194(56) [$M^+ - 60$], 152(7), 110(28), 109(37), 96(97), 95(76), 82(84), 81(78), 68(48), 67(100), 61(12) [$CH_3COOH_2^+$], 55(31), 54(26), 43(37) [$O{=}CCH_3^+$]
V	Z9–14:Ac	194(56) [$M^+ - CH_3COOH$], 152(6), 138(18), 124(32), 123(28), 110(28), 109(35), 96(97), 95(76), 82(68), 81(74), 67(100), 61(4) [$CH_3COOH_2^+$], 55(22), 43(37) [$O{=}CCH_3^+$]
VI	Z9–16:Ac	222(48) [$M^+ - CH_3COOH$], 194(3) [$M^+ - CH_3COOC_2H_5$], 166(8), 152(7), 138(16), 124(32), 110(34), 96(100), 82(63), 69(28), 67(60), 61(10) [$CH_3COOH_2^+$], 55(53), 43(81) [$O{=}CCH_3^+$]
VII	Stearic acid	284(32) [M^+], 255(7) [$M^+ - C_2H_5$], 224(2) [$M^+ - CH_3COOH$], 185(14), 171(6), 143(6), 129(28), 115(8), 97(20), 85(26), 84(14), 83(27), 73(81), 60(84) [CH_3COOH], 57(72), 43(100) [$O{=}CCH_3^+$], 29(32)

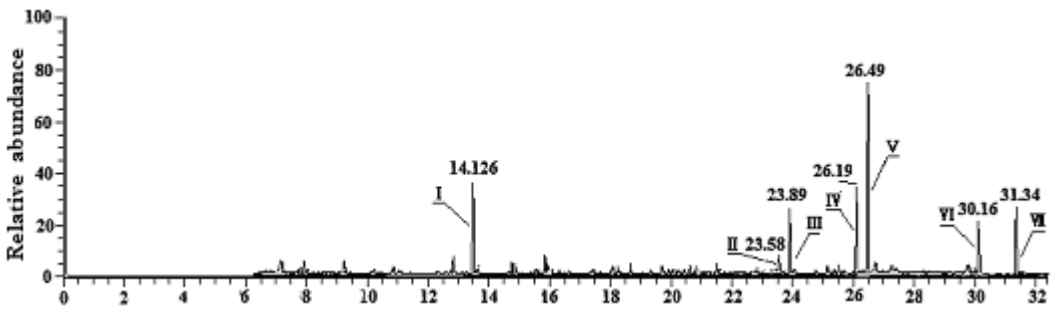

Fig. 2. EI (70 eV) mass spectrum showing the TIC of sex pheromone gland extracts of *I. sibirica*.

The retention behaviour of peak VI (30.16 min) corresponded exactly with that of synthetic (*Z*)-9-hexadecen-1-yl acetate (Z9–16:Ac). Finally, based on the computation according to the fussy reasoning double-bond positional isomers method (Horiike *et al*., 1990, 1991) and the comparison of the Rt (GC) and spectral data (GC-MS) with those of authentic synthetic compounds, we concluded that peaks II to VI were Z7–14:OH, Z9–14:OH, Z7–14:Ac, Z9–14:Ac, and Z9–16:Ac, respectively.

Double-bond locations of the pheromone components were further confirmed by analyses of their DMDS derivatives. The mass spectrum of DMDS adducts derived from gland extract (diagnostic ions at *m/z* 137 [$H_3CS^+{=}CH(CH_2)_3CH_2OH$], 145 [$CH_3(CH_2)_5CH{=}SCH_3^+$], 161 [$H_3CS^+{=}CH(CH_2)_5CH_2OH$], 231 [$CH_3COO(CH_2)_8CH{=}SCH_3^+$], 203 [$CH_3COO(CH_2)_6CH{=}SCH_3^+$], 306 [$M^+$] , 348 [$M^+$] and 376 [$M^+$]) confirmed that the double bonds of the pheromone components were in the 7-position and 9-position.

Electroantennographic analyses

EAG responses of male *I. sibirica* to the gland components and their analogues varying in double-bond positions and configurations are summarized in Fig. 3. Results show that Z9–14:Ac elicited the strongest response (4.28 mV), followed by Z5–14:Ac (3.38 mV) and Z7–14:Ac (3.22 mV) among the compounds tested. The EAG response elicited from each acetate was higher than that from the corresponding alcohol. Though the EAG response of Z5–14:Ac was slightly higher than that of Z7–14:Ac, Z5–14:Ac is not detected in the gland extract.

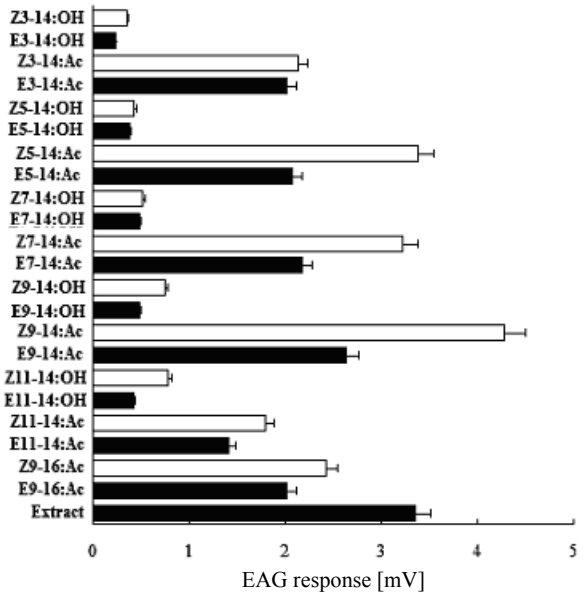

Fig. 3. EAG responses of male *I. sibirica* to synthetic compounds (1 μg) and sex pheromone extract; 6 replicates. Bars indicate mean ± SE.

Field trapping

Traps baited with a single gland component, Z7–14:OH, Z9–14:OH, Z7–14:Ac, Z9–14:Ac, or Z9–16:Ac, failed to attract male *I. sibirica* in the field. However, traps baited with the three-component blend of Z9–14:Ac, Z7–14:Ac, and Z9–16:Ac in a 10:5:1 ratio (by weight) captured more males than any other trap (Table II). The catch activity was not enhanced by combining with Z5–14:Ac.

Discussion

The GC and GC-MS analyses of the extracts from the sex pheromone gland of virgin female *I. sibirica* revealed five compounds, Z7–14:OH, Z9–14:OH, Z7–14:Ac, Z9–14:Ac, and Z9–16:Ac, among which we have demonstrated by electrophysiology and field trapping tests that Z9–14:Ac and Z7–14:Ac are the major components of the sex pheromone of *I. sibirica*. The antennae of male *I. sibirica* are highly sensitive to both compounds. In field tests, traps baited with these two compounds in a 10:5 ratio (by weight) were effective (Table II). Both compounds were required for attraction. Although they had been identified as the sex pheromone of *Copitarsia decolora* (Lepidoptera: Noctuidae) and *Holcocerus hippophaecolus* (Lepidoptera: Cossidae) before (Rojas *et al.*, 2006; Fang *et al.*, 2005), this is the first time that Z9–14:Ac was identified as an active component of the sex pheromone in Cossidae. The common structures of the sex pheromone identified consist of monounsaturated decenyl acetates or tetradecenyl acetates with the double bond in positions 5 and 7 or diunsaturated tetradecadienyl acetates

Table II. Field attraction of male *I. sibirica* in traps baited with various chemicals[a].

Treatment	Composition of baits [μg]							Mean ± SE/ trap catch[b]
	Z7–14:OH	Z9–14:OH	Z7–14:Ac	Z9–14:Ac	Z9–16:Ac	Z5–14:Ac	Z5–14:OH	
1	500	-	-	-	-	-	500	0.00 a
2	-	500	-	-	-	-	-	0.00 a
3	-	-	500	-	-	-	-	0.00 a
4	-	-	-	500	-	-	-	0.00 a
5	-	-	-	-	500	-	-	0.00 a
6	-	-	-	-	-	500	-	0.00 a
7[c]	40	85	250	500	50	-	40	147 ± 3.66 b
8	40	0	250	500	50	-	0	148 ± 1.76 b
9	0	85	250	500	50	-	0	144 ± 3.28 b
11	0	0	250	500	50	-	0	148 ± 2.74 b
12	0	0	250	500	0	-	0	116 ± 3.16 c
13	0	0	250	500	50	250	0	147 ± 2.48 b
Hexane	0	0	0	0	0	0	0	0.00 a

[a] Caught in Taigu, Shanxi Province, China, May 3 – June 24, 2009.
[b] Six replicates; means marked with the same letter are not significantly different by Duncan's multiple range test ($p = 0.05$).
[c] The ratio of the components in baits were similar to that found in the pheromone gland.

with the conjugated double bond in positions 3 and 5 in Cossidae.

Although a significant amount of Z7–14:OH and Z9–14:OH was detected in the extracts from the sex pheromone gland, these two compounds produced a low-level EAG response of male moths and could hardly catch the male moths. Moreover, we did not observe any significant difference in the catches when Z7–14:OH and Z9–14:OH were added to the mixture of Z9–14:Ac and Z7–14:Ac. In contrast, when Z9–16:Ac was not included in the mixture, the catches of male moths slightly decreased. This shows that Z9–16:Ac is likely to be the indispensable subordinate. Although Z5–14:Ac showed strong EAG activity, it did not have any effect on trapping *I. sibirica* male moths.

During the course of our studies, we also found that *I. sibirica* lacks *instrumenta suctoria* described in the literature (Fang *et al*., 2005; Zhang *et al*., 2009). Therefore, adults are unable to feed during their short lifespan. They mate only once and lay eggs within their short period of adult life. These facts suggest that it may be possible to control the pest by focusing on mass-trapping or mating disruption. However, the development of mass-trapping or mating-disruption systems requires the identification of the optimum pheromone dosage in lures and determination of any other potential synergists. Currently, a triangle trap baited with the synthetic compounds Z9–14:Ac, Z7–14:Ac, and Z9–16:Ac in a 10:5:1 ratio at 800 μg/trap dosage can be used to monitor the *I. sibirica* population level and catch the males within the regions planted with *Asparagus* in China.

Acknowledgements

We are very grateful to Professor Xianzuo Meng (Institute of Zoology, Chinese Academy of Sciences, Beijing) for his help with the manuscript. This project was sponsored by the Fundamental Research Funds for the Central Universities (No. TD2010-4), Forestry Commonwealth Industry Scientific Research Plan (No. 200904029-3), and the Science and Technology Research Project (No. 20100311039) in China.

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