Sex Pheromone of the Brazilian Apple Leafroller, *Bonagota cranaodes* Meyrick (Lepidoptera, Tortricidae)

Alvaro E. Eiras[a,*], Adalecio Kovaleski[b], Evaldo F. Vilela[c], Jean P. Chambon[d], C. Rikard Unelius[e], Anna-Karin Borg-Karlson[e], Ilme Liblikas[e], Raimondas Mozuraitis[e], Marie Bengtsson[f] and Peter Witzgall[f]

[a] Departamento de Parasitologia – ICB/UFMG, Universidade Federal de Minas Gerais, Cx.P. 486, Belo Horizonte, MG, 31270–901 Brazil. Fax: +55 31 4992970. E-mail: alvaro@mono.icb.ufmg.br
[b] Departamento de Entomologia, CNPUV/EMBRAPA, 25200–000, Vacaria, RS, Brazil
[c] Departamento de Biologia Animal, Universidade Federal de Viçosa, 36.570–000, Viçosa, MG, Brazil
[d] INRA, Station de Zoologie, 78026 Versailles Cedex, France
[e] Department of Chemistry Organic, Royal Institute of Technology, 100 44 Stockholm, Sweden
[f] Department of Plant Protection Sciences, Swedish University of Agricultural Sciences, 230 53 Alnarp, Sweden

* Author for correspondence and reprint requests

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Sex Pheromone, (3*E*,5*Z*)-3,5-dodecadienyl Acetate, *Bonagota cranaodes*, Brazilian Leaf Roller, Tortricidae

The female sex pheromone of *Bonagota (=Phthteochroa) cranaodes* (Meyrick) is a blend of (*E,Z*)-3,5-dodecadienyl acetate (*E*3,*Z*5–12:Ac) and (*Z*)-9-hexadecenyl acetate (*Z*9–16:Ac) according to analysis of pheromone – gland extracts and field trapping in apple orchards. This is the first time that *E*3,*Z*5–12:Ac has been identified as a lepidopteran sex pheromone. Traps baited with 100 µg *E*3,*Z*5–12:Ac were attractive over 15 weeks in the field and were as effective as traps baited with virgin females. Addition of *Z*9–16:Ac to *E*3,*Z*5–12:Ac at ratio of 1:10 had a significantly increase of male moths. The addition of the *Z,E* and *Z,Z* isomers to rubber septa baited with *E*3,*Z*5–12:Ac did not modify *B. cranaodes* male attraction, but 10% of *EE* enhanced trap catch.

Introduction

During the last decade, the production of apple in Southern Brazil has increased to 350,000 t/yr and to an area of 30,000 ha (Kovaleski, 1992). The Brazilian apple leaf roller, *Bonagota* (*Phtheochroa*) *cranaodes* Meyrick, is besides *Anastrepha* fruit flies the most important pest of apple in Southern Brazil and Uruguay. It accounts, despite intensive insecticide spraying, for an annual crop loss of 3 to 5%, corresponding to approx. US$ 10 million/yr. The damage is done by third- to fifth-instar larvae feeding on the fruit skin; the larvae shelter effectively between fruit and leaves and are therefore difficult to control by insecticides (Eiras *et al.,* 1992; Eiras *et al.*, 1994; Kovaleski, 1992).

There is considerable interest to develop a monitoring system based on a synthetic pheromone lure, as outbreaks of *B. cranaodes* occur throughout the growing season and this necessitates frequent insecticide sprays. Traps baited with live virgin females have been used so far, but the development of a synthetic lure is required for routine use. In view of the exposure of the work force to insecticides and their destabilizing effect on the orchard ecosystem, it is our future goal to develop safe control of *B. cranaodes* by mating disruption (Cardé and Minks, 1995; Ridgway *et al.*, 1990; Witzgall and Arn, 1997).

Occurrence of a female-produced sex pheromone in *B. cranaodes* was first documented by Eiras *et al.* (1993); the main pheromone component has been identified as (*E,Z*)-3,5-dodecadienyl acetate (*E*3,*Z*5–12:Ac) (Unelius *et al.*, 1996). We here report on a detailed identification of compounds produced in female sex glands and first field trapping tests.

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Methods and Materials

Insects

The laboratory population originates from apple orchards in Vacaria (RS, Brazil). Larvae were reared on a semi-synthetic diet at the Federal University of Viçosa (Eiras *et al.*, 1994; Parra *et al.*, 1995). Sexed pupae were shipped to Sweden for gland extraction and chemical analysis. The emerging adults were kept in plexiglass cages (30x30x30 cm) at 23 °C, 65% RH and a 14L:8D-photophase.

Collection of pheromone from calling female moths

Extracts of pheromone glands from 2 to 4-day-old females were made during the first hr of the calling period. Glands were excised with forceps and stored in a glass vial held in liquid air. After dissection of female glands in batches of up to 35, the vial was defrosted and 5 µl of redestilled hexane was added under 1 min. The solvent was removed with a syringe and injected on a gas chromatograph coupled to a mass-selective detector (GC/MS).

Headspace solid phase microextraction (SPME) was done according to Zhang and Pawliszyn (1993) and Borg-Karlson and Mozuraitis (1996). Prior to collection and after injection, the SPME fiber was heated in a GC injector at 225 °C for 10 min. The tip of the syringe with the cleaned SPME fiber (100 µm polydimethylsiloxane) was kept a few millimeters from the protruded abdominal gland of a virgin calling female in a glass vial (30 x 70 mm, volume 30 ml), during 2–3 hours at 18 °C. The female was then removed and hexane solutions of heptyl acetate (10 ng) and pentadecadecyl acetate (100 ng) were added to the glass vials as internal standards and adsorbed during 15 min. Peaks from non-calling and calling females were compared (N=6).

Gas chromatography and GC-mass spectrometry (GC and GC-MS)

Gland extracts were analyzed on a Varian 3400 gas chromatograph (GC) interfaced with a Finnigan SSQ 7000 mass spectrometer (MS). Two fused silica capillary columns, a DB-5 and a DB-Wax (30 m, i.d 0.25 mm, film thickness 0.25 mm; J&W, Folsom, California), were used with a temperature programme of 80 °C (1 min hold), 6 °C/min to 140 °C, and 3 °C/min to 200 °C. Mass spectral data and retention times of selected peaks on both columns were compared to corresponding data from reference standards.

Gas chromatography and electroantennographic detection (GC-EAD)

Two gland extracts were analyzed by GC-EAD. The outlet from a DB-Wax column in a Hewlett Packard 5870A GC was split between a flame ionization detector (FID) and an apparatus for recording of electroantennograms (EAG; Syntec, Hilversum, The Netherlands). Male antennae of *B. cranaodes* were used for these tests.

Synthesis of 3,5-dodecadienyl acetates

The *E,Z* and *E,E* isomers (*E*3,*Z*5–12Ac and *E*3,*E*5–12:Ac) were synthesized via a Wadsworth-Horner-Emmons condensation reaction of methyl 4-dimethylphosphonocrotonate (**2**) with octanal (Baeckström *et al.*, 1988) and subsequent deconjugation by lithium diisopropylamine and diisobutylaluminium hydride (DIBAL) reduction (Ikeda *et al.*, 1987) as the key steps (Fig. 1). The alcohols formed by reduction of the 3,5-dodecadienoates with DIBAL were acetylated and the *E,Z*- and *E,E*-isomers were separated by liquid chromatography on silver nitrate impregnated silica gel. The *Z*3,*Z*5–12Ac was synthesized by a route involving a Cadiot-Chodkiewicz coupling reaction of 3-butyn-1-ol with 1-bromo-1-octyne (**6**), and dicyclohexylborane reduction of 3,5-dodecadiyn-1-ol (**7**) as the key steps (Svirskaya and Leznoff, 1980). The *Z*3,*E*5–12Ac was prepared via a synthesis route involving the coupling reaction of 3-butyn-1-ol and (*E*)-1-octenyl iodide (**9**) in the presence of a catalytic amount of Pd(PPh3)4 under phase transfer conditions (Rossi *et al.*, 1982) followed by a (*Z*)-stereoselective reduction of the triple bond in the (*E*)-enyne acetate (**11**) using a Zn-Cu couple as the reducing agent (Sondengam *et al.*, 1980).

Field tests

Trap tests were done in apple orchard in Vacaria (RS, Brazil). White Delta sticky traps (AgriSense, Pontypridd, UK) were baited with red rubber

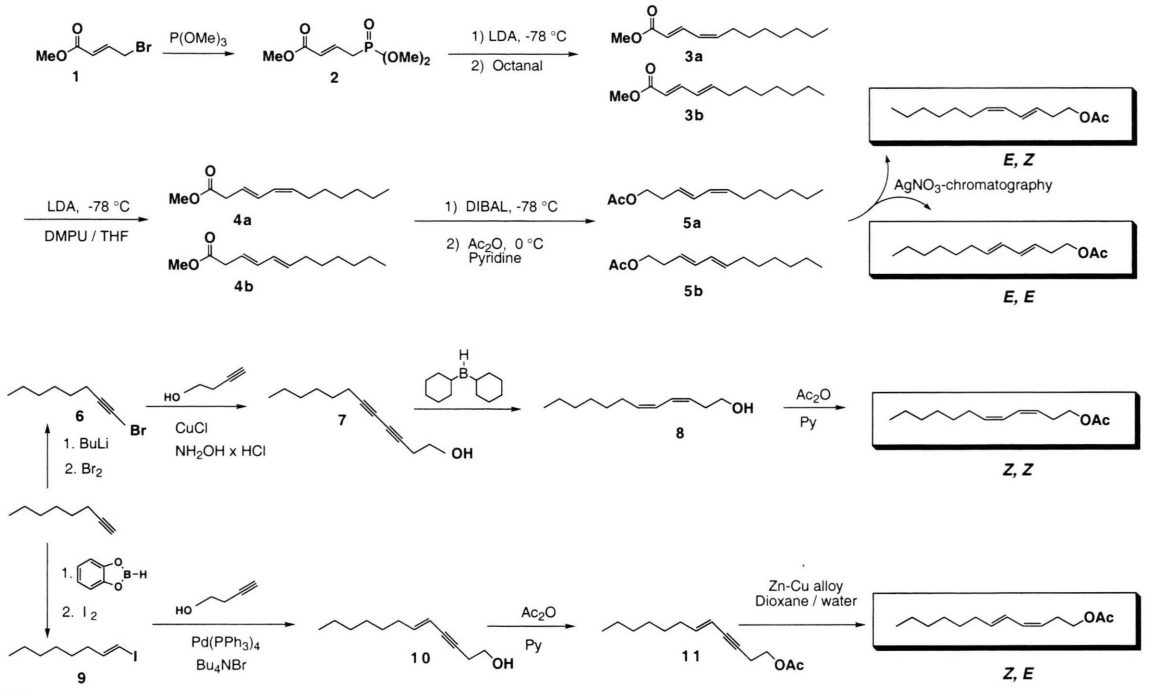

Fig. 1. Synthesis of geometric isomers of 3,5–12Ac.

septa (ABS, Dietikon, Switzerland). Test chemicals were formulated as hexane solutions. Two caged virgin female moths were used as control. The isomers of 3,5–12Ac were isomerically >98% and chemically >99% pure, the monoenic (*E*)-3-dodecenyl acetate (*E*3–12:Ac), (*Z*)-3-dodecenyl acetate (*Z*3–12:Ac), (*Z*)-5-dodecenyl acetate (*Z*5–12:Ac) and (*E*)-5-dodecenyl acetate (*E*5–12:Ac) were purchased from Dr. Simon Voerman (Wageningen Agricultural University, The Netherlands) and were isomerically >99% pure.

Traps were hung on apple trees at ca. 2 m height, and separated from one another by 20–30 m. Traps were deployed in a randomized block design with treatments. Traps were controlled and re-randomized within blocks every 2 to 3 days. Data were transformed to log $(x + 1)$ prior analysis of variance and means were separated using the Tukey test ($P = 0.05$). The first experiment tested the supposed main candidate pheromone component. The *E*3,*Z*5–12:Ac and those dodecenyl acetates which elicited positive EAG responses on the male antennae were loaded in red septa and tested alone (100 µg) and in combinations. The second experiment tested the two compounds found in the gland extract which elicited electrophysiological responses on the males antennae (*E*3,*Z*5–12:Ac and Z9–16:Ac) to confirm the main compound of the sex pheromone. The third experiment tested the dose-response of the main compound which caught more insect from the second experiment at 1, 10 and 100 µg/septum. The fourth experiment tested the binary combinations of the main compound (50 µg) in combination with the secondary compound at the ratios of 1:0.1, 1:1, 1:10 and 1:100 . The ratios of equivalent amount of 0; 5; 50; 500 and 5,000 µg/septa, respectively, was loaded in grey rubber septa. The fifth experiment tested the effect of isomers on the main sex pheromone compound.

Results

Taxonomy

The Brazilian apple leaf roller has been described as *Phtheochroa cranaodes* (Meyrick, 1931) and this name has been used until now by Brazilian entomologists. However, the species belongs now, according to a recent revision of neotropical Archipini, to the newly described genus *Bonagota*

(Razowski, 1986). *B. cranaodes* had been erroneously treated as *Eulia salubricola* (Meyrick, 1937) by Uruguayan entomologists. Examination of the wing pattern and genitalia of male insects collected in orchards in Uruguay (leg. Persoons) and Southern Brazil (leg. Eiras) showed that the species causing damage in apple in both countries is actually *B. cranaodes*.

Chemical analysis of gland extracts

Analysis of gland extracts by GC-MS showed the presence of a number of acetates (Table I), whereas the SPME-collection of calling females gave only one peak. A GC-EAD analysis of two female gland extracts revealed two active compounds: a very strong antennal response was obtained for *E*3,*Z*5–12Ac and weaker response for *Z*9–16Ac.

The mass spectrum of *E*3,*Z*5–12Ac had two characteristic features (Fig. 2). The fragments 79, 80 and 164 (M-60) gave very high peaks, while the molecular ion 224 was quite weak (<1%). The absence of an M+ peak indicated that the loss of the acetate group was facilitated and that the unknown acetate was homoallylic or allylic. The fragment 164 would be formed after an initial McLafferty rearrangement and a subsequent charge migration followed by a alpha cleavage. The strong characteristic peaks 79 and 80 looked like highly unsaturated molecular ions (C_6H_7 and C_6H_8) which also indicated that the double bonds were positioned close to the acetate group. With this in mind we synthesized a mixture of 2,4- and 3,5-decadienyl acetates. The mass spectra of the 2,4-isomers showed high M+ ion peaks, while the mass spectra of the 3,5-isomers were very similar to the one of our unknown acetate. Therefore, the synthesis of 3,5-dodecadienyl acetate was undertaken.

Table I. Pheromone-related compounds identified in *B. cranaodes* females by GC and GC/MS.

Compound	Short Form	ng/female
Decyl acetate	10Ac	0.8
Dodecyl acetate	12Ac	1.6
*E*3,*Z*5-Dodecadienyl acetate	*E*3,*Z*5–12Ac	7.0
Tetradecyl acetate	14Ac	1.0
Hexadecyl acetate	16Ac	11.1
*Z*9-Hexadecenyl acetate	*Z*9–16Ac	23.1
Octadecylacetate	18Ac	100

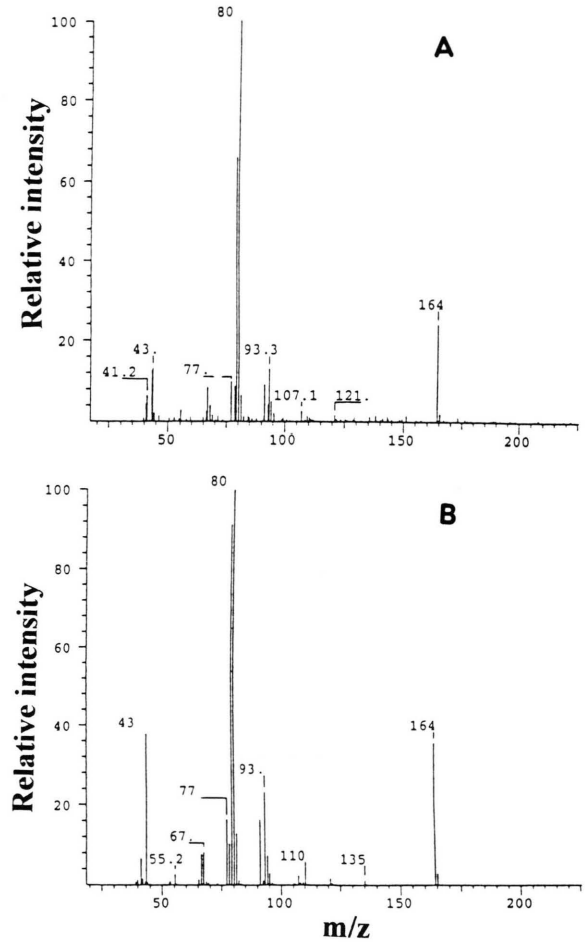

Fig. 2. Electron impact mass spectrum of *E*3,*Z*5–12Ac. (a) SPME and (b) reference *E*3,*Z*5–12Ac.

The gland extract was coinjected with an isomer mixture on two GC columns with different polarity. The unknown compound co-eluted with synthetic *E*3,*Z*5–12Ac from both columns.

Field trapping

Synthetic *E*3,*Z*5–12Ac, at a dose of 100 μg, was as attractive as traps baited with virgin females (Table II) and can thus be used for monitoring of *B. cranaodes*. The *Æ*3- and *Æ*5-monounsaturated dodecenyl acetates were completely unattractive (Table II). In another orchard, a dose-response test with 0, 1, 10 and 100 μg *E*3,*Z*5–12Ac/trap attracted 0; 7±1.4; 12.5 ± 2.4 and 7.5 ± 1.1 males/trap, respectively (N = 7), over a period of six weeks.

Table II. Field attraction of *B. cranaodes* males to virgin females, synthetic main pheromone compound and 3- or 5-unsaturated dodecenyl acetates (Tukey test, P=0.05).

Trap lure	Amount [μg]	Males caught (Mean ± s.e.)
Virgin female		39.3 ± 8.0 b
*E*3,*Z*5–12Ac	100	38.7 ± 8.0 b
*E*3–12Ac	100	0 ± 0 a
*Z*3–12Ac	100	0 ± 0 a
*E*5–12Ac	100	0 ± 0 a
*Z*5–12Ac	100	0 ± 0 a
*E*3–12Ac + *Z*3–12Ac	100 + 100	0 ± 0 a
*Z*3–12Ac + *Z*5–12Ac	100 + 100	0 ± 0 a

The traps catches of male moths were significantly increases when *Z*9–16:Ac was added to *E*3,*Z*5–12Ac in the ratio of 1:1 in the lure. No increased of catches of *B. cranaodes* was observed at any other ratio (Table III).

Dienic pheromone compounds are known to isomerize quickly on rubber septa used for field trapping (Brown and McDonough, 1986). We therefore tested the effect of the other, nonpheromonal isomers in binary blends with the main compound, *E*3,*Z*5–12Ac (Table IV). Interestingly, none of the isomers had an antagonistic effect on trap catch except the amount of 50 μg of *E*3,*E*5–12Ac. Addition of 5 μg of the *E,E*-isomer had a possible synergistic effect.

Discussion

The sex pheromone of the Brazilian apple leaf roller, *B. cranaodes* is *E*3,*Z*5–12Ac, according to chemical analysis of female sex glands and field trapping tests with synthetic compounds. This compound has not been identified as tortricid sex pheromone before, but has been reported as sex attractant in a gelechiid species, *Chionodes lugubrella* (Reed *et al.* 1985; Arn *et al.*, 1992; 1999). *B. cranaodes* belongs to the tortricid tribe Archipini where the most common structure of sex pheromones identified consist of monounsaturated dodecenyl acetates with the double bonds in position 9 or 11. However, the Archipini tribe is polyphyletic and sex pheromones of neotropical species have not been studied.

Synthetic *E*3,*Z*5–12Ac, at a dose of 100 μg, is an effective lure (Table II) and can be used for monitoring of *B. cranaodes* populations in the field. The other isomers had very little effect on male attraction, and isomerization of *E*3,*Z*5–12Ac

Table III. Attraction of male *Bonagota cranaodes* to Delta trap baited with E3,Z5–12Ac or binary combinations with Z9–16Ac. Nova Escocia Orchard, Vacaria (RS, Brazil). Tukey test, P=0.05).

Trap lure composition 50 μg of E3,Z5–12Ac + amount of Z9–16Ac [μg]	Number of males caught (Mean ± s.e.)	Number of males caught Total	(N)
0	24.76 ± 2.95 c	619	25
5	5.52 ± 3.50 a	388	25
50	36.36 ± 3.79 d	909	25
500	19.16 ± 5.66 bc	479	25
5000	12.64 ± 2.25 b	316	25

Table IV. Field attraction of *B. cranaodes* males to virgin females, and blends of the synthetic main pheromone compound *E*3,*Z*5–12Ac with its geometric isomers *EE*, *ZE*, *ZZ* (N=30; Tukey test, P=0.05).

*E*3,*Z*5–12Ac [μg]	*E*3,*E*5–1212Ac [μg]	*Z*3,*E*5–12Ac [μg]	*Z*3,*Z*5–12Ac [μg]	Males caught (Mean ± s.e.)
50	–	–	–	25.9 ± 5.14 ab
50	5	–	–	31.9 ± 5.93 b
50	50	–	–	15.3 ± 3.98 a
50	–	5	–	27.1 ± 4.08 ab
50	–	50	–	20.4 ± 5.65 ab
50	–	–	5	24.8 ± 5.65 ab
50	–	–	50	27.6 ± 5.37 ab

in the traps will therefore not decrease the attractivity of lures. In comparison, isomerization of conjugated dienes formulated on rubber septa used for field trapping is known to deteriorate trap catch (McDonough *et al.*, 1990; Witzgall *et al.*, 1993) and monitoring traps for pea moth are therefore baited with a monoene, *E*10–12Ac, which mimics the dienic pheromone, *E*8,*E*10–12Ac (Wall, 1988). In *B. cranaodes*, the Æ3- and Æ5-monounsaturated dodecacenyl acetates were completely unattractive (Table II).

Although female glands contained about threefold amounts of *Z*9–16Ac, relative to *E*3,*Z*5–12Ac (Table I), trap catch with binary blends of these two compounds seemed to peak at a 1:10 ratio (Table III). In collections of female effluvia by the SPME technique, Z9–16Ac was not detected, whereas male antennae responded during GC-EAD analysis of gland extracts to this compound. Further studies on the sex pheromone release by *B. cranaodes* females and behavioral studies should be carried out in order to elucidate a potential behavioral role of this compound.

Synthetic *E*3,*Z*5–12Ac is currently used in experimental programs by Brazilian apple growers for monitoring the flight period and abundance of *B. cranaodes*, in order to time insecticide treatments. Experiments aiming the development of the mating disruption technique for environmentally safe control of this major pest of apple are currently carried out in orchards in Brazil. Further behavioral investigations of the effect of the geometric isomers and other, minor gland components need to be done under controlled conditions in the wind tunnel.

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