Volatiles from Apple (*Malus domestica*) Eliciting Antennal Responses in Female Codling Moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae): Effect of Plant Injury and Sampling Technique

Anna-Carin Bäckman[a,*], Marie Bengtsson[a], Anna-Karin Borg-Karlsson[b], Ilme Liblikas[b] and Peter Witzgall[a]

[a] Department of Crop Science, Swedish University of Agricultural Sciences, Box 44, S-230 53 Alnarp, Sweden. Fax: (+46) 40 461991. E-mail: anna-carin.backman@vv.slu.se
[b] Department of Organic Chemistry, Royal Institute of Technology, S-100 44 Stockholm, Sweden

* Autor for correspondence and reprint requests

The antennal responses of codling moth females, *Cydia pomonella*, to volatiles from apple branches with green fruits were recorded by electroantennography coupled to gas chromatography. The antennae strongly responded to 4,8-dimethyl-1,3(*E*),7-nonatriene, linalool, β-caryophyllene, (*E*)-β-farnesene, germacrene D, (*Z,E*)-α-farnesene, (*E,E*)-α-farnesene and methyl salicylate. These compounds were all present in volatile collections on Porapak Q from both living and cut branches. Analysis by the solid phase microextraction technique (SPME) showed that the emission of some electrophysiologically active compounds increased after branches had been cut, especially 4,8-dimethyl-1,3(*E*),7-nonatriene, linalool and (*E,E*)-α-farnesene. The identification of apple volatiles eliciting antennal responses is the first step towards the identification of compounds mediating host-finding and oviposition in codling moth females.

Introduction

There is growing evidence that attraction of moths to their host plants is largely guided by volatile phytochemicals, which are perceived by specialized chemoreceptor neurons on the antenna (Wibe *et al.*, 1998; Jönsson and Anderson, 1999; Røstelien *et al.*, 2000). A first step in the identification of behaviorally active compounds is to screen host plant volatiles by electrophysiological techniques. Plants are not static dispensers of volatiles, however; the effects of photoperiod, plant damage and sampling techniques on the composition of the blend of volatiles obtained from headspace collections need to be examined.

The codling moth, *Cydia pomonella* (L.) (Lepidoptera, Tortricidae), is a major pest of apple (*Malus domestica*) worldwide. Volatiles from apple have long been known to stimulate mating and oviposition by *C. pomonella* females (Wildbolz, 1958; Wearing *et al.*, 1973; Witzgall *et al.*, 1999; Yan *et al.*, 1999), and male antennae also respond to apple odor (Skirkevicius *et al.*, 1980). An important constituent of apple odor, α-farnesene, stimulates female oviposition (Wearing and Hutchins,

1973; Hern and Dorn, 1999), and highly sensitive antennal neurons responding to this compound have been identified in males (Bäckman *et al.*, 2000).

Volatiles released by apple plants are particularly well-studied, especially floral scents (Loughrin *et al.*, 1990; Buchbauer *et al.*, 1993) and aromas emanating from ripe fruit (Dimick and Hoskin, 1983). The goal of our study was to investigate the blend of compounds emitted from branches with small green apples, which are attractive to ovipositing codling moth females. Volatiles were collected by two different techniques, both living and cut branches were used, and the collections were screened for compounds eliciting antennal responses in *C. pomonella* females.

Materials and Methods

Insect and plant material

Codling moth, *Cydia pomonella*, were reared on a semisynthetic diet (Mani *et al.*, 1978) under a photoperiod of 18:6 (L:D) h, during up to three generations. The laboratory population had been

established from wild insects collected in apple orchards in Scania (Sweden) and Bavaria (Germany). Emerging adults were separated by sex, and transferred to $20 \times 20 \times 20$ cm glass cages.

Volatile collections were made from apple branches, *Malus domestica*, (5 mm to 10 mm OD) with up to 25 leaves and eight fruits (2 to 5 cm OD) of the variety Aroma. Branches were obtained from an orchard at Alnarp (Scania, Sweden), during the seasonal flight period of codling moth.

Wind tunnel tests

Branches up to 30 cm long, with leaves and apples (2 to 3 cm OD), were placed into a 2-l Erlenmeyer flask, 1 h before the onset of tests. Air from a tank passed at 150 ml/min from the bottom of the flask, over the plant material, to the outlet of the flask (5 mm ID), which was ca. 30 cm from the ground. The same flasks were used for collection of apple volatiles (see below).

The wind tunnel has a flight section of $63 \times 90 \times 200$ cm. Air was blown by a horizontal fan (Fischbach GmbH, Neunkirchen, Germany) onto an array of 24 activated charcoal cylinders (14.5×32.5 cm; Camfil, Trosa, Sweden). The outcoming air was aspired by another fan and cleaned by 2 sets of the same charcoal filters. The wind tunnel was lit diffusely from above at 6 lux, the wind speed was 30 cm/s, and the temperature ranged from 22 to 24 °C.

Two-day-old females were mated, and kept in glass cages until the following day. They were placed individually in glass tubes (5×2.5 cm) into the wind tunnel and were allowed 15 min to respond. Additional behavioral observations were made in an apple orchard at Pålstorp (Scania, Sweden).

Volatile traps

The traps used for both laboratory and field collections of apple volatiles were made of glass tubes (4×40 mm) containing Porapak Q (25 mg, 50/80 mesh; Supelco, PA, USA) held between glass wool plugs. Before use, the traps were rinsed sequentially with 3 ml of methanol, ether, and redistilled n-hexane, between 15-min treatments in ultrasonic baths in ether and hexane, respectively. After sample collection, the traps were immediately rinsed

with 0.5 ml of hexane and sample volumes were reduced under a stream of nitrogen to $20-50$ µl. Samples were stored in glass capillary tubes at -19 °C.

Volatile collections in the laboratory

A freshly cut apple branch with leaves and apples (2 to 3 cm OD) was placed into a 2-l Erlenmeyer flask in the laboratory. The cut end of the branch was held in a 10-ml vial of water. Charcoal-filtered air was pulled through the flask and over a Porapak Q trap at 150 ml/min, for 12 h ($N = 5$). All glassware was heated to 350 °C for 10 h before use. Temperature and light were constant at 20 °C and 5 Lux.

Changes in volatile composition after cutting an apple branch were studied by the solid phase microextraction technique (SPME). A silica fibre coated with 100 µm of polydimethylsiloxane (Supelco, PA, USA) was used. The fibre was conditioned for 30 min in the injection port of a gas chromatograph (GC) at 250 °C. A cut branch with leaves and 2 to 4 apples was placed in a 1-l glass flask; the cut end was placed in a vial of water. The fibre was then inserted, through a hole in a glass lid, into the glass flask for 30 min. After sampling, the fibre was immediately desorbed for 2 min in the GC injection port and the branch transferred to a new glass flask. The sampling procedure was repeated 3.5 h after the branch was cut ($N = 6$).

Volatile collections in the field

Volatile collections were made both from intact and from cut apple branches with leaves and fruits. A 25×38 cm polyester roasting bag (Look, Terinex Ltd., England) was placed around a branch and the open end was tightened with a wire. Two traps were connected to the polyester bag with Teflon tube fittings and air was drawn through each trap at 200 ml/min. Charcoal-filtered air was pumped into the bag at 410 ml/min (CFMP3S pump, Brey, Memmingen, Germany) to keep the bag inflated and to prevent sampling of ambient orchard air. A thermometer was placed inside the bag, which was shaded during collection.

Between June 25 and July 3, 1997, samples were collected from intact branches ($N = 5$) with leaves and 3 to 6 small fruits (2 to 4 cm OD) for 2 h.

Volatile collections from each branch were repeated twice. After volatile collection, the bag was removed and the branch was cut. The cut end of the branch was placed in water, and after 1.5 hrs, a clean bag was placed around the branch for another 2-h sampling interval.

Between July 3 and 23, 1997, collections were made during the photophase (starting between 14 and 15 hrs) and during the scotophase (starting between 19 and 21 hrs) from intact branches ($N = 5$) with leaves and two to three fruits (3 to 5 cm OD). The temperature inside the bags varied between 19.6 and 27.5 °C during photophase and between 12.2 and 18.0 °C during scotophase.

Chemicals

Germacrene D was isolated from thermomechanical pulp (TMP) turpentine of spruce heartwood; the NMR spectrum of the purified compound was in accordance with germacrene D isolated from cubebe oil (Røstelien *et al.*, 2000). (E,E)-α-Farnesene and (E)-β-farnesene were purchased from Bedoukian Research Inc. (Danbury, CT, USA); β-caryophyllene, a racemic mixture of the enantiomers of linalool from Firmenich (Geneva, Switzerland), and (E)-4,8-dimethyl-1,3,7-nonatriene from Siber Hegner (Zurich, Switzerland).

Gas chromatography and mass spectrometry (GC-MS)

Compounds in trap eluants and SPME-desorptions were identified by comparison of retention times and mass spectra to synthetic or purified authentic compounds. Samples were analyzed on a Hewlett Packard 5970 B GC-MS instrument, with electron impact ionization (70 eV), interfaced to a Hewlett Packard 5890 GC, using a 30 m × 0.25 mm DB-Wax column (J&W Scientific, Folsom, CA, USA). The temperature program was from 50 °C (hold 5 min) at 8 °C/min to 230 °C (hold 10 min), the carrier gas was helium. Additional GC analysis was done on a Hewlett Packard 5890 instrument with a flame ionization detector (FID), using a DB-Wax column and on a nonpolar SE54 column (25 m × 0.32 mm ID, Kupper & Co., Bonaduz, Switzerland). The temperature program was from 60 °C (hold 2 min) at 10°/min to 100 °C, and 1.5°/min to 230 °C (hold 10 min).

Gas chromatography and electroantennographic detection (GC-EAD)

Laboratory and field collections were also analyzed by coupled GC-electroantennographic detection (GC-EAD; Arn *et al.*, 1975), using a Hewlett Packard 6890 GC with a HP-INNOWax column (30 m × 0.25 mm ID), which was programmed from 50 °C (hold 5 min) at 8 °C/min to 220 °C (hold 10 min). The EAD recordings were done with excised antennae of 2-day-old codling moth females. The antennae were suspended between two saline-filled wells, antennal signals were amplified (JoAc, Lund, Sweden) and recorded using GC-EAD software (Syntech, Hilversum, The Netherlands). Responses from 5 antennae to each sample were averaged (Hillbur *et al.*, 2001).

Results

Behavioral studies

Visual observations in an apple orchard showed that ovipositing codling moth females were flying upwind towards branches with green apples. They landed on leaves close to apples or on apples, where they deposited single eggs. The upwind flight behaviour of females is quite similar to male flights towards pheromone sources. In the wind tunnel, 18% of mated 3-day-old females ($N = 90$) left the holding tube by flying upwind, and landed at the outlet of a glass flask containing a freshly cut apple branch with green fruits. Females were not attracted to flasks containing a cherry branch with green fruits (*Prunus avium*) ($N = 60$). The same flasks were used for volatile collections in the laboratory (see below).

Volatile collections in the laboratory

Antennae of codling moth females responded to 8 compounds in volatile collections from cut apple branches with leaves and fruitlets. The compounds identified were 4,8-dimethyl-1,3(E),7-nonatriene (1), β-linalool (2), β-caryophyllene (3), (E)-β-farnesene (4), germacrene D (5), (Z,E)- and (E,E)-α-farnesene (6, 7), and methyl salicylate (8), according to comparison of mass spectra and retention times to synthetic standards. The largest response was elicited by (E,E)-α-farnesene, which was also the most abundant compound in all samples (Fig. 1).
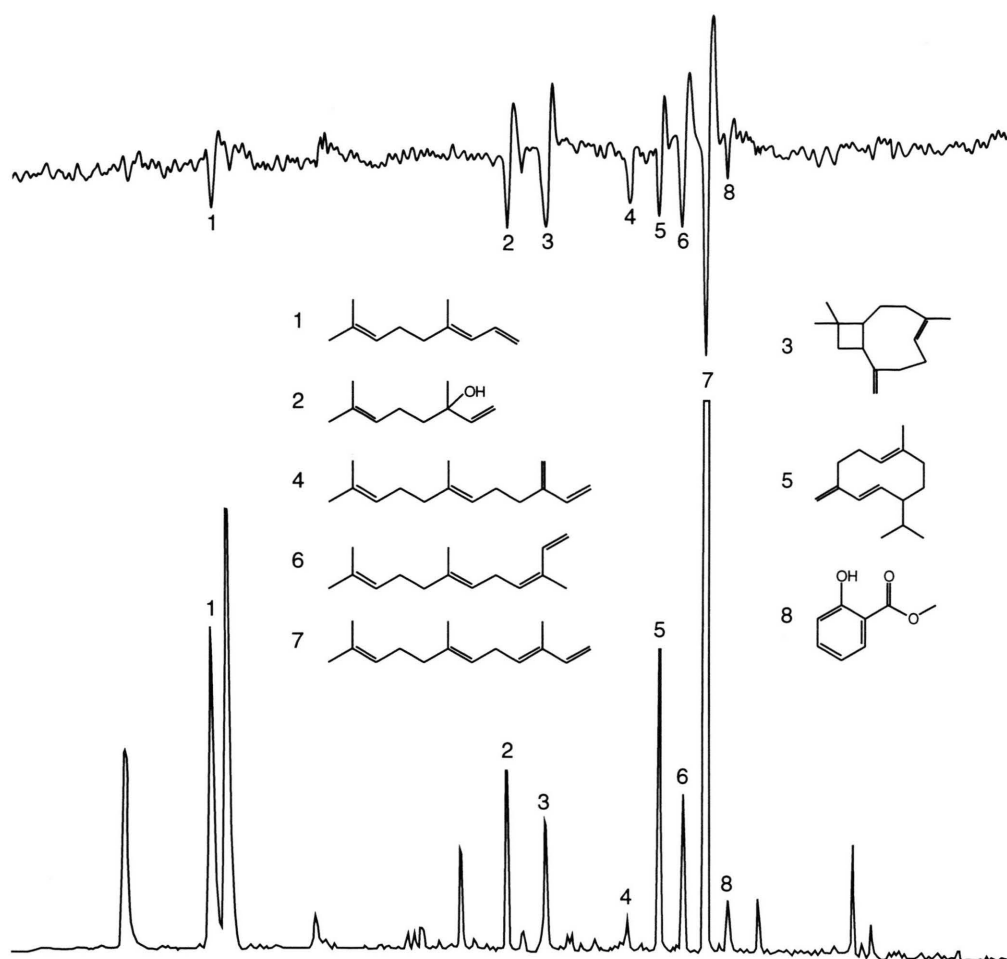
Fig. 1. Response of female codling moth antennae to volatile compounds from apple, as analyzed by coupled GC-EAD detection. The active compounds are listed in Table I. Responses of five antennae to 12-h volatile collections from apple branches with green fruit on Porapak Q were averaged.

GC-retention time and mass spectrum of peak (5) was identical to germacrene D isolated from TMP turpentine and cubebe oil. Germacrene D has previously been identified from apple (Takabayashi *et al.*, 1991). Two other terpenoids present in larger amounts, which were tentatively identified as (*E*)-β-ocimene and β-bourbonene, did not elicit an antennal response.

The composition of the blend of apple volatiles sampled from cut apple branches with Porapak traps and the SPME technique is shown in Table I. Analysis by SPME showed a change in relative release of the electrophysiologically active compounds after apple branches were cut, except for β-farnesene. The relative amounts of the other compounds increased 4 h after cutting a branch. The highest relative increase was observed for 4,8-dimethyl-1,3(*E*),7-nonatriene, followed by β-linalool and (*E,E*)-α-farnesene (Table I). Other compounds decreased during sampling. For example, the electrophysiologically inactive (*Z*)-3-hexenyl acetate decreased more than 10-fold after cutting in all replicates (data not shown).

Volatile collections in the field

The collections made in the orchard from cut branches showed a similar profile to the collec-

Table I. Release of volatiles from intact and cut apple branches eliciting antennal responses in codling moth females, sampled with different techniques in the laboratory and field.

Compound[a]	Mean relative amounts ($\pm$ SE)[b] collected from				
	Living branches	Cut branches			Relative increase[c]
	Porapak Q[d] (2 h)	Porapak Q[e] (2–4 h)	Porapak Q[f] (0–12 h)	SPME[g] (0–0.5 h)	SPME
4,8-Dimethyl-1,3(E),7-nonatriene (**1**)	16.5 $\pm$ 7.3	32.2 $\pm$ 6.2	20.5 $\pm$ 3.6	6.7 $\pm$ 3.3	6.93*
Linalool (**2**)	1.9 $\pm$ 1.1	4.8 $\pm$ 2.1	5.7 $\pm$ 1.5	2.1 $\pm$ 0.7	3.67*
(E)-β-Caryophyllene (**3**)	8.5 $\pm$ 4.4	4.8 $\pm$ 2.0	4 $\pm$ 1.5	8.3 $\pm$ 1.6	2.08*
(E)-β-Farnesene (**4**)	13.6 $\pm$ 6.6	8.2 $\pm$ 4.8	1 $\pm$ 0.6	6.9 $\pm$ 2.8	1.07 n.s.
Germacrene D (**5**)	12.6 $\pm$ 2.6	8.3 $\pm$ 4.2	6.9 $\pm$ 3.5	7.2 $\pm$ 1.6	1.96*
(Z,E)-α-Farnesene (**6**)	8.4 $\pm$ 0.7	3.1 $\pm$ 0.7	3.4 $\pm$ 1.7	5.6 $\pm$ 2.4	2.03*
(E,E)-α-Farnesene (**7**)	36.2 $\pm$ 4.4	35.8 $\pm$ 6.1	57.1 $\pm$ 7.1	63.2 $\pm$ 6.2	3.59*
Methyl salicylate (**8**)	2.3 $\pm$ 0.3	2.9 $\pm$ 1.8	1.5 $\pm$ 0.5	n.d. [h]	–

[a] Compounds shown in Figure 1.
[b] The average amounts of (E,E)-α-farnesene collected from cut apple branches on Porapak Q traps were 3.3 $\pm$ 2.1 µg/l at 20 °C in the laboratory ($N = 5$) and 30.8 $\pm$ 23.3 ng/l at ambient temperatures in the field ($N = 5$); and 36 $\pm$ 17 ng/SPME fibre ($N = 6$).
[c] Mean ratio between amount of volatiles collected between 0 to 0.5 h, and 3.5 to 4 h after cutting a branch; numbers followed by an asterisk show a significant increase (paired, two-sided t-test with Bonferroni adjustment; $P<0.05$).
[d] Living twigs contained in polyester bag in the orchard, collection on Porapak Q traps during 2 h ($N = 5$).
[e] Cut twigs in polyester bag in the orchard, collection on Porapak Q traps, 2–4 h after the branch was cut ($N = 5$).
[f] Cut twigs in glass jar, collection on Porapak Q traps in the laboratory during 12 h ($N = 5$).
[g] Cut twigs in glass jar, collection by SPME in the laboratory during 0.5 h ($N = 6$).
[h] not detected.

tions made in the laboratory. All compounds eliciting an antennal response were present in collections from living and cut branches (Table I). Collections from living branches corroborated presence of (E)-β-farnesene, (Z,E)-α-farnesene and methyl salicylate, which were present only in small amounts in 12-h lab collections.

Field collections from intact branches after sunset contained much lower amounts of volatile compounds than did collections from the same branches during the day. The only compounds detected in these samples were β-caryophyllene, (E)-β-farnesene and (E,E)-α-farnesene.

Discussion

Antennae of codling moth females strongly responded to 8 compounds sampled from apple branches with green fruits (Fig. 1). Codling moth females were attracted to such branches in the wind tunnel, and these compounds may therefore be involved in long-range attraction of codling moth females to oviposition sites. In contrast, compounds which do not elicit an antennal response

are less likely to induce a behavioural response. Screening volatiles from apple with the female antenna is the first step towards the identification of behaviorally active compounds.

The compounds eliciting antennal activity were present in dynamic headspace collections on Porapak Q from both living and cut branches. The volatile blend sampled by SPME showed a similar composition, except that methyl salicylate was not detected. The release of these compounds increased when apple branches were cut. The strongest relative increase was recorded for β-linalool and 4,8-dimethyl-1,3(E)7-nonatriene (Table I).

Infestation of apple leaves by spider mites has been shown to enhance the emission of (E,E)-α-farnesene and 4,8-dimethyl-1,3(E)7-nonatriene (Takabayashi *et al.*, 1991), and these compounds have been suggested to be induced by herbivores in many plants (Dicke, 1994). Damage of apple fruitlets by the European apple sawfly, *Hoplocampa testudinea*, has been reported to induce an increased release of both (E,E)-α-farnesene and β-linalool (Boevé *et al.*, 1996, 1999). Other known

factors which influence volatile collections from apple trees are seasonal changes (Carle *et al.*, 1987; Yahia *et al.*, 1990; Mattheis *et al.*, 1991) and drought (Ebel *et al.*, 1995), besides variation between apples of different cultivars (Kakiuchi *et al.*, 1986).

Volatile collections from intact branches contained much smaller amounts of volatiles after sunset, during the diel flight period of codling moth, when only β-caryophyllene, β-farnesene and (*E,E*)-α-farnesene could be detected during 2-h-collections. These compounds are found in a range of plants, while codling moth is specifically attracted to pome fruit trees. Ongoing behavioural studies aim at the identification of compounds which mediate host recognition in codling moth.

Arn H., Städler E. and Rauscher S. (1975), The electroantennographic detector – a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. Z. Naturforsch. **30c**, 722–725.

Bäckman A.-C., Anderson P., Bengtsson M., Löfqvist J., Unelius C. R. and Witzgall P. (2000), Antennal response of codling moth males, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), to the geometric isomers of codlemone and codlemone acetate. J. Comp. Physiol. **A 186**, 513–519.

Boevé J.-L., Lengwiler U., Tollsten L., Dorn S. and Turlings T. C. J. (1996), Volatiles emitted by apple fruitlets infested by larvae of the European sawfly. Phytochemistry **42**, 373–381.

Boevé J.-L. (1999), Chemical ecology of the European apple sawfly, *Hoplocampa testudinea*. Int. Org. Biol. Control (west-palearctic regional Section) Bulletin **22(9)**, 15–19.

Buchbauer G., Jirovetz L., Wasicky M. and Nikiforov A. (1993), Headspace and essential oil analysis of apple flowers. J. Agric. Food Chem. **41**, 116–118.

Carle S. A., Averill A. L., Rule G. S., Reissig W. H. and Roelofs W. L. (1987), Variation in host fruit volatiles attractive to apple maggot fly, *Rhagoletis pomonella*. J. Chem. Ecol. **13**, 795–805.

Dicke M. (1994), Local and systemic production of volatile herbivore-induced terpenoids: their role in plant-carnivore mutualism. J. Plant. Physiol. **143**, 456–472.

Dimick P. S. and Hoskin J. C. (1983), Review of apple flavor – state of the art. Crit. Rev. Food Sci. Nutr. **18**, 387–409.

Ebel R. C., Mattheis J. P. and Buchanan D. A. (1995), Drought stress of apple trees alters leaf emissions of volatile compounds. Physiol. Plant. **93**, 709–712.

Hern A. and Dorn S. (1999), Sexual dimorphism in the olfactory orientation of adult *Cydia pomonella* in response to α-farnesene. Entomol. Exp. Appl. **92**, 63–72.

Hillbur Y., Bäckman A.-C., Bengtsson M., Jönsson S. (2001), Enhanced detection of weak electrophysiological responses from insect antennae by means of averaging. J. Chem. Ecol. (submitted)

Jönsson M. and Anderson P. (1999), Electrophysiological response to herbivore-induced host plant volatiles in the moth *Spodoptera littoralis*. Physiol. Entomol. **24**, 377–385.

Kakiuchi N., Moriguchi S., Fukuda H., Ichimura N., Kato Y. and Banba Y. (1986), Composition of volatile compounds of apple fruits in relation to cultivars. J. Japan Soc. Hort. Sci. **55**, 280–289.

Loughrin J. H., Hamilton-Kemp T. R., Andersen R. A. and Hildebrand D. F. (1990), Volatiles from flowers of *Nicotiana sylvestris*, *N. otophora* and *Malus × Domestica*: headspace components and day/night changes in their relative concentrations. Phytochemistry **29**, 2473–2477.

Mani E., Riggenbach W. and Mendik M. (1978), Zucht des Apfelwicklers (*Laspeyresia pomonella*) (L.) auf künstlichem Nährboden, 1968–78. Mitt. Schweiz. Entomol. Ges. **51**, 315–326.

Mattheis J. P., Fellman J. K., Chen P. M. and Petterson M. E. (1991), Changes in headspace volatiles during physiological development of Bisbee Delicious apple fruit. J. Agric. Food Chem. **39**, 1902–1906.

Røstelien T., Borg-Karlson A.-K., Fäldt J., Jacobsson U. and Mustaparta H. (2000), The plant sesquiterpene germacrene D specifically activates a major type of antennal receptor neuron of the tobacco budworm moth *Heliothes virescens*. Chem. Senses **25**, 141–148.

Skirkevicius A., Buda V., Rothschild G. H. L. and Skirkeviciene Z. (1980), The response of male codling moths (*Laspeyresia pomonella*) to naturally emitted host plant odours. Ent. Exp. Appl. **28**, 334–338.

Takabayashi J., Dicke M. and Posthumus M. A. (1991), Variation in composition of predator-attracting allelochemicals emitted by herbivore-infested plants: Relative influence of plant and herbivore. Chemoecology **2**, 1–6.

Wearing C. H. and Hutchins R. F. N. (1973), α-farnesene, a naturally occurring oviposition stimulant for the codling moth, *Lasperyesia pomonella*. J. Insect Physiol. **19**, 1251–1256.

Wearing C. H., Connor P. J. and Ambler K. D. (1973), The olfactory stimulation of oviposition and flight activity of the codling moth, *Laspeyresia pomonella* L., using apples in an automated olfactometer. New Zealand J. Sci. **16**, 697–710.

Wibe A., Borg-Karlson A.-K., Persson M., Norin T. and Mustaparta, H. (1998), Enantiomeric composition of monoterpene hydrocarbons in some conifers and receptor neuron discrimination of α-pinene and limonene enantiomers in the pine weevil, *Hylobius abietis*. J. Chem. Ecol. **24**, 273–287.

Wildbolz T. (1958), Über die Orientierung des Apfelwicklers bei der Eiablage. Mitt. Schweiz. Ent. Ges. **31**, 25–34.

Witzgall P., Bengtsson M., El-Sayed A., Bäckman A.-C., Rauscher S., Borg-Karlson A.-K., Unelius C. R. and Löfqvist J. (1999), Chemical communication in codling moth: towards environmentally safe control methods. Int. Org. Biol. Control (west-palearctic regional section) Bulletin **22(9)**, 57–65.

Yahia E. M., Acree T. E. and Liu F. W. (1990), The evolution of some odour-active volatiles during the maturation and ripening of apples on the tree. Food Sci. Technol. **23**, 488–493.

Yan F., Bengtsson M. and Witzgall P. (1999), Behavioral response of female codling moths, *Cydia pomonella*, to apple volatiles. J. Chem. Ecol. **25**, 1343–1351.