Fundam. appl. Nematol., 1998, **21** (2), 119-122

Gas chromatographic characterization of the female sex pheromone of *Heterodera schachtii* (Nematoda: Heteroderidae)

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Accepted for publication 12 May 1997.

Summary - It is shown that the volatile components of the sex pheromone from *Heterodera schachtii* females can be analysed directly by gas chromatography, which eliminates the need for derivatisation procedures. The pheromone was separated into two components, each with a different polarity. It may contain other, non-volatile components that could not be analysed directly by gas chromatography. © Elsevier - ORSTOM

Résumé – ***Caractérisation en chromatographie en phase gazeuse de la phéromone sexuelle femelle d'*Heterodera schachtii *(Nematoda: Heteroderidae)*** - Il est montré que la phéromone sexuelle des femelles d'*Heterodera schachtii* comprend des composés volatils pouvant être analysés directement en chromatographie en phase gazeuse sans utiliser de procédure de transformation. La phéromone a été scindée en deux composés de polarité différente. La phéromone pourrait contenir des composés non volatils qui ne peuvent être analysés directement en chromatographie en phase gazeuse. © Elsevier - ORSTOM

Keywords: attractant, attraction, beet cyst nematode, bioassay, pheromone, semiochemical.

Various reproduction mechanisms have been described in nematodes, and used to categorize amphimictic vs. parthenogenetic species. Copulation between males and females is essential for the reproduction of amphimictic species, whereas parthenogenetic females are able to reproduce without males. Females of more than thirty amphimictic species have been shown to produce sex pheromones before fertilization. The males search for such females by directed movement along sex pheromone gradients (Green, 1980; Haseeb & Fried, 1988). Production of sex pheromones may be a common trait of all of the amphimictic species. In contrast to insects where hundreds of different pheromones have been identified from many species (Tamaki, 1985; Mayer & McLaughlin, 1991), only one nematode substance – vanillic acid from the soybean cyst nematode *Hetero-dera glycines* – has been found to possess sex phero-mone activity (Jaffe *et al.*, 1989; Meyer & Huettel, 1996).

The identification of nematode sex pheromones has been hindered by several factors, *i.e.*, small size of plant-parasitic and free-living nematodes, their aqueous habitats, and lack of knowledge about which tissue or organelle produces the pheromone. Because nematodes are small and do not move over large distances in soil or plants, female nematodes may produce only small quantities of sex pheromone. Significant structural differences may have evolved between air-released insect sex pheromones and soil or plant solute-released nematode sex pheromones. Without knowing which tissues produce the pheromones, it is not possible to optimize extraction procedures.

Based on previous characterization experiments (Aumann & Hashem, 1993), we describe in this article a better characterization of the female sex pheromone of the sugar beet cyst nematode (*Heterodera schachtii*) based on the occurrence of various components characterized by gas chromatography fractionation of female extracts. A sensitive bioassay of male pheromone-mediated behaviour was also made. The results may facilitate future identification of female sex pheromones of amphimictic species of cyst nematodes.

Materials and methods

NEMATODE CULTURES

Freshly emerged males of *H. schachtii* were obtained from monoxenic root cultures of oilradish (*Raphanus sativus* var. *oleiformis*) cv. Nemex, a resistant cultivar where nearly all nematodes develop into males. Females of *H. schachtii* were grown monoxenically on turnip (*Brassica rapa* var. *rapa*). Both plants were cultivated in the dark at 25 ± 2°C in a nutrient medium according to Sijmons *et al.* (1991) in 9 cm diameter plastic Petri dishes. Decapitated single oilradish plants were cultivated in *c.* 10 ml nutrient agar medium; twelve turnip plants per Petri dish were grown in separate 1 ml nutrient agar drops. Turnip roots grew over

the bottom of the Petri dish between the nutrient medium, which means that most females developed with their posterior end outside of the medium, in the Petri dish atmosphere.

PHEROMONE EXTRACTION

For gas chromatographic fractionation, female sex pheromone of *H. schachtii* was obtained from vulva-secreted gelatinous matrices of females with the posterior end located above or beneath the surface of the nutrient agar medium. In these females the pheromone was expected to be concentrated in the matrices. The matrices were collected and dried overnight at room temperature, then extracted with methanol or pentane (LiChrosolv quality, Merck, Darmstadt, Germany) at a concentration of five matrices per µl solvent.

GAS CHROMATOGRAPHY

Gas chromatographic fractionation of *H. schachtii* female sex pheromone was performed with a preparative Carlo Erba GC 6000 Vega Series 2 gas chromatograph (Carlo Erba, Rodano, Italy) with capillary DB 5 column (30 m length, 0.53 mm inner diameter, 1.5 µm film thickness; J & W Scientific, Folsom, CA, USA). The gases used were H_2 (60 kPa), N_2 as make-up gas (30 kPa), synthetic air (90 kPa) and He as carrier gas (72 kPa). The detector was set at 280°C and the column orifice heater at 245°C. The column heating programme was 50°C for 3 min, 5°C/min from 50 to 80°C, 10°C/min from 80 to 200°C, and 15°C/min from 200 to 245°C. Samples of 10 µl were injected into the column and pheromone fractions were collected at the column orifice in glass tubes cooled with liquid nitrogen. Pheromone fractions were then eluted from the tubes with 200 or 300 µl pentane or methanol, respectively.

BIOASSAY

The attraction for *H. schachtii* males of the pheromone fractions separated by gas chromatography was tested in a standardized bioassay in sterile 6 cm diameter plastic Petri dishes coated with *c.* 1 ml of 1% (w/v) autoclaved agarose (Type I, Sigma, Deisenhofen, Germany) in glass-distilled water. Fifty to 100 µl of the gas chromatograph eluates were pipetted in 10 µl volumes onto a 5 mm diameter filter-paper disk (cut from paper no. 595, Schleicher und Schuell, Dassel, Germany). After solvent evaporation, one disk per Petri dish was centrally placed onto the agarose surface. Two µl of glass-distilled water were pipetted onto each disk and the test substances were allowed to diffuse into the agarose for 90 min at room temperature. Three males per Petri dish were then placed with a fine needle at a distance of 5 mm from the disk. The number of males in contact with the filter-paper was counted 1 h later.

Results

Fig. 1 shows two representative chromatograms of gas chromatography analysis of the female sex pheromone of *H. schachtii* with different pheromone solvents: pentane (Fig. 1A) and methanol (Fig. 1B). The fractionation of the pheromone extracts in conjunction with a bioassay of male nematode pheromone-mediated behaviour demonstrated the presence of one pheromone component in the 15-17 min fraction of the pentane extract, and one pheromone component in the 22-24 min fraction of the methanol extract (Table 1). In contrast to methanol, which has higher polarity, pentane solubilized several apolar compounds from the female extracts (compare Fig. 1A and B). The methanol fraction that contains the pheromone (22-24 min) did not show any detectable peak on the gas chromatogram (Fig. 1B). Only one very small peak was detected in the pentane fraction (15-17 min) that contains the pheromone (Fig. 1A).

Discussion

Most insect pheromones have been identified by gas chromatography coupled with mass spectroscopy (Tamaki, 1985). Gas chromatography can only be used with volatile compounds. Non-volatile compounds are detectable by gas chromatography only after derivatisation into volatile compounds by a number of different techniques such as methylation. The majority of insect pheromones are released into the air and thus have to be volatile. In contrast, nematode sex pheromones are released into their aqueous habitats. Volatility thus is not a prerequisite for their efficient dissemination in the environment. As we have shown here, components of the female sex pheromone of *H. schachtii* can be collected at the preparative column orifice of a gas chromatograph. Therefore, the sex pheromone of this nematode species includes volatile components. Our results confirm a similar observation of Greet *et al.* (1968) who analysed the sex pheromones of *Globodera rostochiensis* and *H. schachtii*. Vanillic acid, a substance with sex pheromone activity for males of *H. glycines* (Jaffe *et al.*, 1989), also is volatile. A possibly different component of the *G. rostochiensis* female sex pheromone was later shown to be non-volatile (Clarke *et al.*, 1976). In addition to the two volatile components, the *H. schachtii* female sex pheromone may contain non-volatile components.

Cyst nematode sex pheromones include different components. Several common components have been proposed to explain the interspecific attraction of female sex pheromones (Green & Plumb, 1970). We were able to fractionate the female sex pheromone of *H. schachtii* into two different components. A two-component female sex pheromone of this species was

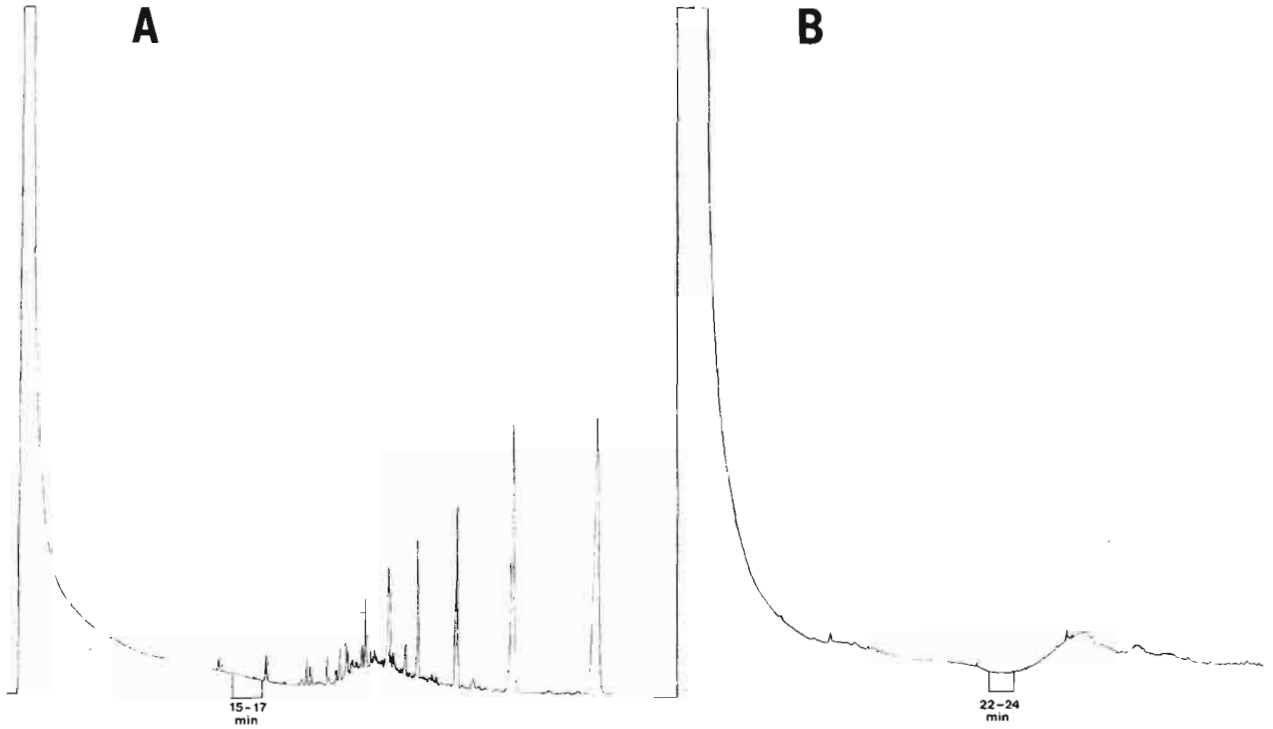

Fig. 1. *Representative gas chromatograms of the female sex pheromone of* Heterodera schachtii. *A: Pentane extract; B: Methanol extract (The fractions containing pheromone are marked).*

Table 1. *Attractiveness for males of* Heterodera schachtii *of gas chromatographically separated fractions of the female sex pheromone.*

Solvent	Fractions tested(min)	Number of males tested per fraction	Number of males attracted
Pentane	15-20, 20-25, 25-40	15	6, 4, 1
	15-17, 17-19, 19-25	15	3, 0, 0
Methanol	0-10, 10-20, 20-35	9	1, 3, 9
	20-25, 25-30, 30-35	15	5, 1, 0
	20-22, 22-24, 24-26	12	2, 7, 1

first postulated by Green and Plumb (1970). They also postulated a two-component pheromone for *H. glycines*, which was later confirmed by Bone (1986) using high-performance liquid chromatography separation. As shown here, the *H. schachtii* sex pheromone components have a different polarity. The first component is more soluble in pentane than in methanol, whereas the second component is nearly equally soluble in both solvents. As *i*) most substances are soluble in most solvents at least in very small quantities and *ii*) at least the second component occurred in a quantity too small to give a detectable signal on the gas chromatogram (see Fig. 1B), predictions on the function of the individual components as long- or short-distance pheromones cannot be made at present.

Identification of pheromones from *H. schachtii* and related species of cyst nematodes could be used for the development of novel control methods based on male disorientation with subsequent reduction of female fertilisation in the field.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft for financial support (Au 100/2-1 and Au 100/3-1) and the reviewers, Dr R. N. Perry and Dr. B. Jaffee, for their helpful comments on the manuscript.

References

AUMANN, J. & HASHEM, M. (1993). Studies on substances with sex pheromone activity produced by *Heterodera schachtii* females. *Fundam. appl. Nematol.*, 16: 43-46.

BONE, L. W. (1986). Fractionation of the female's pheromone of the soybean cyst nematode, *Heterodera glycines*. *Proc. helminth. Soc. Wash.*, 53: 132-134.

CLARKE, A. J., FIRTH, J. E. & GREET, D. N. (1976). The sex attractant. *Rothamsted Exp. Stn, Annual Report for 1975:* 199-200.

GREEN, C. D. (1980). Nematode sex attractants. *Helminth. Abstr., Ser. A,* 49: 327-339.

GREEN, C. D & PLUMB, S. C. (1970). The interrelationships of some *Heterodera* spp. indicated by the specificity of the male attractants emitted by their females. *Nematologica,* 16: 39-46.

GREET, D. N., GREEN, C. D. & POULTON, M. E. (1968). Extraction, standardization and assessment of the volatility of the sex attractants of *Heterodera rostochiensis* Woll. and *H. schachtii* Schm. *Ann. appl. Biol.*, 61: 511-519.

HASEEB, M. A. & FRIED, B. (1988). Chemical communication in helminths. *Adv. Parasit.*, 27: 169-207.

JAFFEE, H., HUETTEL, R. N., DEMILO, A. B., HAYES, D. K. & REBOIS, R.V. (1989). Isolation and identification of a compound from soybean cyst nematode, *Heterodera glycines,* with sex pheromone activity. *J. chem. Ecol.*, 15: 2031-2043.

MAYER, M. S. & MCLAUGHLIN, J. R. (1991). *Handbook of insect pheromones and sex attractants.* Boca Raton, FL, USA, CRC Press, 1083 p.

MEYER, S. L. F. & HUETTEL, R. N. (1996). Application of a sex pheromone, pheromone analogs, and *Verticillium lecanii* for management of *Heterodera glycines*. *J. Nematol.*, 28: 36-42.

SIJMONS, P. C., GRUNDLER, F. M. W., VON MENDE, N., BURROWS, P. R. & WYSS, U. (1991). *Arabidopsis thaliana* as a new model for plant-parasitic nematodes. *Plant J.*, 1: 245-254.

TAMAKI, Y. (1985). Sex pheromones. *In*: Kerkut, G. A. & Gilbert, L. I. (Eds). *Comprehensive insect physiology, biochemistry and pharmacology, Vol. 9, Behaviour.* Oxford, UK, Pergamon Press: 145-191.