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Attractant-mediated behaviour of mobile stages of *Heterodera schachtii*

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Summary – The responses of second-stage juveniles (J2) and males of *Heterodera schachtii* to root exudates and female sex pheromonal substances, respectively, at different distances from the attractant source, were studied by analysing orientation tracks left on agarose surfaces. Two classes of J2 were tested : stimulated J2, previously stimulated by root exudates, and unstimulated J2. Track lengths were quantified to differentiate between oriented and unoriented searching behaviour. The quantification of forward movements towards and away from the gradient, subsequent to each backward movement, enabled the differentiation of attractant and repellent effects. Males showed no repellent responses. The comparison of the sensory abilities between stimulated and unstimulated J2 points to the possible occurrence of " learning " processes.

Résumé – ***Influence de produits attractifs sur le comportement des stades mobiles d'*Heterodera schachtii** – La réaction des juvéniles de deuxième stade (J2) d'*Heterodera schachtii* aux exsudats radiculaires et des mâles aux phéromones produites par les femelles à des distances variées de la source d'attraction, est étudiée en analysant l'orientation des traces laissées à la surface de l'agar. Deux types de J2 ont été testés : des J2 stimulés – précédemment soumis à des exsudats radiculaires – et des J2 non stimulés. Les longueurs des traces ont été quantifiées de façon à différencier les comportements de recherche orientés et non orientés. La quantification des mouvements dirigés vers le gradient, ou à l'opposé de celui-ci, conséquence de chaque mouvement de recul, permet de différencier les effets attractifs et répulsifs. Aucun effet répulsif n'a été observé en ce qui concerne le comportement des mâles. La comparaison des capacités sensorielles entre J2 stimulés et non stimulés pourrait laisser supposer l'existence d'un processus d'apprentissage.

Key-words : Movement, searching, learning, root exudates, pheromonal substances, nematodes.

Males of amphimictic nematode species are thought to find their females by orientating themselves along sex pheromone gradients. Compared with insects, however, little is known about pheromone structures, pheromone-producing cells, pheromone receptors, and pheromone-mediated behaviour of nematodes (Haseeb & Fried, 1988).

Having hatched from eggs outside plant roots, juveniles of most plant-parasitic species search suitable roots by orientating themselves along root exudate gradients. Likewise, little is known about the structure of these attractants, nematode receptors, or root exudate-mediated behaviour (Robertson & Forrest, 1989).

The responses of nematodes to exogenous stimuli can be observed microscopically on agarose surfaces (Ward, 1973). We describe here the responses of mobile stages of *Heterodera schachtii* to root exudates and female sex pheromonal substances. Oriented and unoriented searching behaviour is characterized by the track lengths. Furthermore, the number of forward movements (after backward movements) towards and away from the attractant gradient can be used to differentiate quantitatively between attractant and repellent effects. Finally, learning processes can be studied by comparing the sensory abilities of second-stage juveniles (J2) that were previously stimulated or not stimulated by root exudates.

Materials and methods

NEMATODE CULTURES

Freshly emerged males of *H. schachtii* were obtained from monoxenic root cultures of oilradish (*Raphanus sativus* var. *oleiformis*) cv. Pegletta. In this resistant cultivar nearly all nematodes develop into males. J2 of *H. schachtii* were obtained from cysts reared under monoxenic conditions on root cultures of mustard *(Sinapis alba)* cv. Albatros (Grundler *et al.*, 1991). Excised roots of both plants were grown in the dark at 25 ± 2° C in a nutrient agar medium according to Sijmons *et al.* (1991) in 9 cm (oilradish) or 14.5 cm diameter (mustard) plastic Petri dishes.

NEMATODE ATTRACTANTS

Female substances with sex pheromone activity were obtained by transferring 100 white, living *H. schachtii* females into a microcentrifuge tube containing 1 ml of ultrapure water. They were incubated at 4-6° C and the supernatant was used 8-9 days later. Root exudates of *S. alba* cv. Albatros were produced in 1000 ml Erlenmeyer flasks under sterile conditions. The bottom of the flask

was covered with glass beads (5 mm diameter), which kept the shoots of 50 mustard seedlings (previously germinated and grown for 2 days on water agar) above the nutrient solution (the same as mentioned above but supplemented with only 1 % sucrose). The flasks were kept on a shaker (30 rpm) for 3 days at 25 °C with a photoperiod long day 16 : 8, 700 $\mu E\ m^{-2}\ s^{-1}$. The root exudate solution was then concentrated ten-fold under reduced pressure in a water bath at 38 °C. The nutrient solution served as a control and was treated in the same way.

Stimulation of second-stage juveniles

The J2 were stimulated by root exudates as described by Grundler *et al.* (1991), however with slight modifications : approximately 1500 J2 in a small drop of sterile water were pipetted onto an agarose surface (0.8 ml, autoclaved, 1.5 % w/v; Type I, Sigma, Deisenhofen, Germany) in sterile 3.5 cm diameter Petri dishes. The water was allowed to evaporate from the opened dishes under a sterile working bench, and after the J2 had distributed themselves homogeneously, four agarose disks (6 mm diameter) containing root exudate solution were placed onto the surface. These disks were prepared as described by Grundler *et al.* (1991). Ninety min later the agarose layer containing stimulated (aggregated and stylet-thrusting) J2 beneath the exudate disks was cut out with a cork borer (6 mm diameter) and transferred onto an agarose surface. The stimulated J2 were used immediately in the bioassay. The term " unstimulated " refers to J2 that had been randomly selected from an agarose Petri dish without root exudates.

Bioassay system

The attractiveness of female sex pheromones and root exudates for *H. schachtii* males and J2, respectively, was tested in a standardized bioassay in sterile 6 cm diameter plastic Petri dishes, coated with *ca* 1 ml of 1.5 % (w/v) autoclaved agarose (Type I, Sigma) in glass-destilled water. Five µl of the aqueous solution of the female pheromone substances or root exudates were pipetted onto a 5 mm diameter filter-paper disk (cut from paper no. 595, Schleicher & Schuell, Dassel, Germany) or agarose (1.5 % w/v) disk, respectively. The disks were placed on the agarose surface in the centre of the Petri dish. The aqueous solutions were allowed to diffuse into the agarose for 90 min at room temperature before two males or J2 were placed with a fine needle on each plate at a distance of 5, 7.5 (J2 only) or 10 mm (males only) from the disks. À 7.5-, 10- or 12.5-mm-circle with its centre in the centre of the filter-paper or agarose disk had previously been marked on the bottom of the Petri dish. Nutrient solution (J2 only) or ultrapure water (males only) served as a control. One hour later, the Petri dishes were transferred onto Kodak Kodalith Ortho type 3 films in the dark and the films were exposed and developed to record the movement tracks of the nematodes on the agarose surfaces. The tracks were evaluated under a stereo microscope at ten-fold magnification. The track lengths were measured with a distance measurement instrument for maps after enlarging the films with a photographic or slide enlarger.

The experiments were performed at least three times with 80 males and 90 to 180 J2 in total. Differences were statistically compared with the chi-square test. Petri dishes in which the tracks of individual nematodes could not be clearly differentiated were not analysed. Furthermore, in some cases (4.4 to 14.4 %) a decision between forward after backward movements towards and away from the gradient was impossible for the J2; hence these data were omitted.

Results

Males (Table 1)

The percentage of males with filter paper contact decreased with increasing initial distance from the filter disk. Except from males without filter contact in the 10 mm experiments, males performed significantly more forward (after backward) movements towards the attractant source than away from it. The mean track lengths of males with and without filter contact did not differ significantly in both female extract experiments. Tracks of males with filter contact in the presence of female extracts were significantly shorter in the 5 mm experiments than in the 10 mm treatments, whereas these differences were not significant for males without filter contact.

Second-stage juveniles (Table 2)

Stimulated J2, i.e. J2 that previously had responded to root exudates with an aggregation and exploration behaviour, showed a higher rate of contact with the root exudate disks and significantly shorter tracks (Fig. 1A) for both distances tested than unstimulated J2 (Fig. 1B) without a previous root exudate response. In addition, after each preceding backward movement, the stimulated J2 reached the disks with significantly more forward movements towards the gradient than away from it (Fig. 1C). On the other hand the reverse was true when stimulated J2 were tested against the nutrient solution in which the exudate was obtained. Most of them did not reach the disks and moved towards the edge of the Petri dish. The apparent repellent effect of the nutrient solution (Fig. 2A) was also expressed by the higher number of resumed forward movements away from the gradient. Compared with the stimulated J2 the unstimulated J2 (Fig. 2B) did not respond so drastically.

Discussion

Two general types of searching behaviour may be differentiated depending on the availability of information for finding resources : oriented searching occurs when information about the position of resources is

Table 1. *Movement patterns of* Heterodera schachtii *males with and without contact with filter disks wetted with female substances with sex pheromone activity. Data from a 60 min bioassay.*

Treatment and distance from source	Class of males	n (%)	Mean no. of forward after backward movements towards/away from the gradient (Std. dev.)	Mean track lengths in mm (Std. dev.)
Extract from females, 5 mm	P5+	43 (58.1)	4.05/1.28 (3.61/2.11)	13.04 (9.32)
	P5–	31 (41.9)	4.48/2.65 (3.36/2.26)	13.78 (9.26)
Water, 5 mm	W5+	12 (15.8)	–	15.99 (10.82)
	W5–	64 (84.2)	–	19.79 (11.71)
Extract from females, 10 mm	P10+	40 (50.0)	7.03/2.05 (4.53/2.54)	18.09 (8.56)
	P10–	40 (50.0)	6.20/4.20 (4.72/3.20)	21.23 (11.17)
Water, 10 mm	W10+	2 (2.5)	–	13.41 (6.34)
	W10–	78 (97.5)	–	19.99 (11.23)
Statistical intergroup comparisons		P5+ > P5–; P < 0.05 P10+ = P10–; ns	P5+; P < 0.001 P5–; P < 0.05 P10+; P < 0.001 P10–; ns	P5+ = P5–; ns P5+ < P10+; P < 0.05 P10+ = P10–; ns P5– = P10–; ns

Abbreviations : P5+, W5+, P10+, W10+, with filter contact; P5–, W5–, P10–, W10–, without filter contact; ns, not significant; Std. dev., standard deviation; –, not analysed.

available, and unoriented searching occurs when this information is not available (Bell, 1991). Our data (Table 2) indicate that the track lengths of *H. schachtii* J2 can be used to differentiate between oriented and unoriented searching behaviour. However, this parameter is obviously not applicable for *H. schachtii* males.

Attractant and repellent effects of chemicals are generally evaluated by counting nematodes at different distances from the source. Our data (Tables 1, 2) show that attractants can be differentiated from repellents by counting, subsequent to each backward movement, the number of forward movements towards and away from the gradient. For stimulated J2 and males that reached the attractant source within a definite time, the number of forward movements was always significantly higher towards the gradient than away from it. J2 that did not reach the source usually showed more forward movements away from the gradient. This was especially the case when J2, previously stimulated by root exudates, were confronted with the nutrient solution used to obtain the exudates.

In contrast to sex pheromone-mediated behaviour of males, relatively few experiments have so far been performed on the behaviour of plant-parasitic nematodes in response to their host root exudates. Recently, Grundler *et al.* (1991) described the aggregation and pre-infectional exploratory behaviour of *H. schachtii* J2 in response to mustard root exudates under *in vitro* conditions. They did not observe any nematode orientation and assumed that the J2 reached the attractant source by random movement. In contrast, one of us (C. D. Clemens, unpubl.) observed that higher concentrations of root exudate attractants induce nematode orientation. Concentration-dependent orientation has also been reported for *Heterodera avenae* (Moltmann, 1988) and *Rotylenchulus reniformis* (Riddle & Bird, 1985) toward host root exudates and chemicals, respectively. In our experiments (Table 2), the concentration of the attractive compounds in mustard root exudates at the point of inoculation did apparently not reach the threshold level at the chemoreceptors of most unstimulated J2 to trigger orientation, whereas most stimulated nematodes were

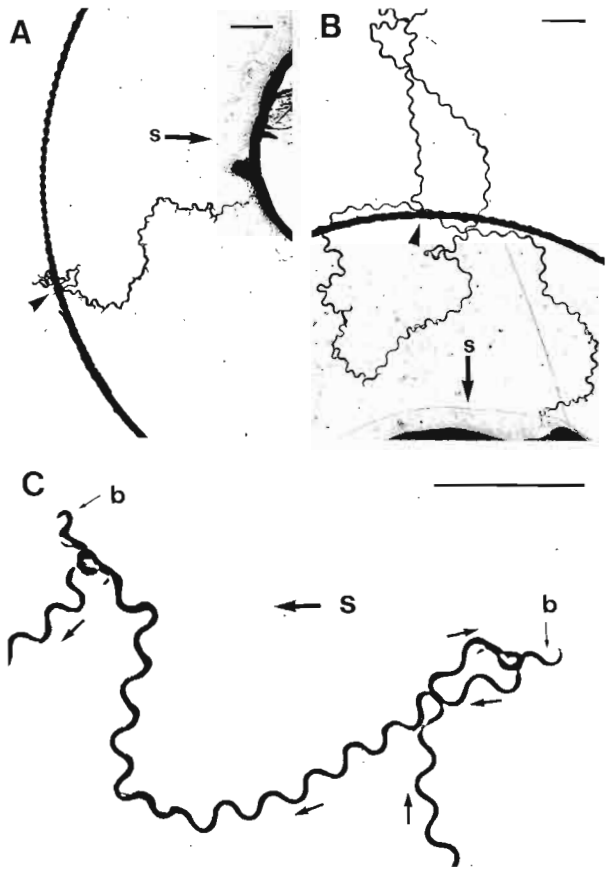

Fig. 1. *Tracks left by* Heterodera schachtii *J2 on agarose surfaces in a 60 min bioassay in the presence of root exudate (RE). A : Stimulated J2 with RE disk contact; B : Unstimulated J2 with RE disk contact; C : Typical backward movements (b), followed by forward movements. (Arrows indicate direction of movement; S with arrow = direction towards RE disk; dark rings in A and B = circles marked on the bottom of the plates.* Scale bars = 1 mm).

able to sense the exudate gradient. This remarkable behavioural change was evident from the evaluation of nematode tracks on the agarose surface.

Our results support the occurrence of two behavioural searching states in stimulated and unstimulated nematodes as previously described by Samoiloff *et al.* (1974) for *Panagrellus silusiae* males. Croll (1970) postulated that stimulation of nematodes may coincide with the release of energy quanta, followed by movement and orientation. The transition from unstimulated to stimulated behavior may be caused by external (e.g. chemicals, nutrient medium composition, temperature, light, etc.) or internal (e.g. physiological state, age, hunger, etc.) factors. The induction of chemotactic behaviour by an external factor was described by Albert and Riddle (1983) for the dauer juveniles of *Caenorhabditis elegans*. After pre-incubation with an appropriate food source, the immobile dauer juveniles became mobile and

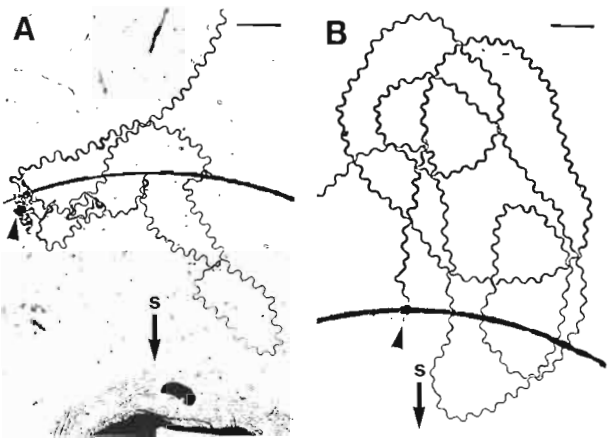

Fig. 2. *Sections of tracks left by* Heterodera schachtii *J2 on agarose surfaces in a 60 min bioassay in the presence of nutrient solution (NS). A : Stimulated J2 that moved towards and then away from the NS disk; B : Unstimulated J2 that showed no response to NS. (Arrowhead = point of nematode application; S with arrow = direction towards NS disks.* Scale bars = 1 mm).

showed chemotactic behaviour and pharyngeal pumping. In the presence of the nutrient solution used to obtain root exudates, no direct orientation or exploratory behaviour could be observed for both behavioural states of *H. schachtii* J2 (Table 2). Furthermore, in contrast to unstimulated J2, stimulated J2 appeared to be repelled by the nutrient solution, supporting the hypothesis of nematode activation by specific stimuli.

This activation may be explained by " learning " processes. Rankin *et al.* (1990) showed for the first time that a nematode is capable of learning. They demonstrated for *C. elegans* several forms of non-associative learning, like short-term habituation, dishabituation and sensitization as well as long-term retention of habituation. Habituation was defined by them " as a decrease in response amplitude due to repeated stimulation, while dishabituation is the facilitation of that decremented response by a novel or noxious stimulus. Sensitization is defined as the facilitation of non-decremented or baseline responses by a strong stimulus ". Our observations on the orientation capacity of stimulated and unstimulated *H. schachtii* J2 indicate that these nematodes might be capable of the " learning " process of sensitization as defined by Rankin *et al.* (1990).

In conclusion, our results show that relatively simple bioassays can be applied for a quantitative analysis of nematode responses to attractants and repellents. Similar results have been obtained previously with free-living (Ward, 1973; Samoiloff *et al.*, 1974) and plant-parasitic nematodes (Riddle & Bird, 1985; Aumann *et al.*, 1990). Recent experiments showed that the bioassay described here can be used for the identification of attractive components of mustard root exudates (C. D. Clemens, unpubl.).

Table 2. *Movement patterns of stimulated and non-stimulated* Heterodera schachtii *J2 with and without contact with agarose disks wetted with root exudate or nutrient solution. Data from a 60 min bioassay.*

Treatment and distance from source	Class of J2	n (%)	Mean no. of forward after backward movements towards/away from the gradient (Std. dev.)	Mean track lengths in mm (Std. dev.)
Root exudate, 5 mm, stimulated	R5/1+	63 (73.3)	5.62/2.14 (2.92/2.00)	16.82 (10.85)
	R5/1–	23 (26.7)	3.13/4.43 (2.05/2.45)	42.04 (16.41)
Root exudate, 5 mm, unstimulated	R5/2+	28 (33.3)	2.64/1.79 (2.71/2.38)	25.42 (18.15)
	R5/2–	56 (66.7)	2.14/2.98 (1.75/2.20)	56.87 (23.16)
Nutrient solution, 5 mm, stimulated	N5/1+	35 (22.9)	1.80/1.54 (1.69/1.62)	55.24 (31.64)
	N5/1–	118 (77.1)	2.08/4.84 (1.94/3.48)	67.79 (24.58)
Nutrient solution, 5 mm, unstimulated	N5/2+	31 (39.7)	2.19/1.35 (2.33/1.75)	75.06 (41.41)
	N5/2–	46 (60.3)	1.70/2.00 (1.6/1.86)	84.36 (31.62)
Root exudate, 7.5 mm, stimulated	R7.5/1+	48 (45.1)	5.15/1.96 (3.36/1.80)	35.96 (19.45)
	R7.5/1–	56 (54.9)	2.16/2.88 (2.34/2.72)	49.71 (21.18)
Root exudate, 7.5 mm, unstimulated	R7.5/2+	30 (27.0)	2.03/1.33 (1.81/1.32)	47.00 (23.35)
	R7.5/2–	81 (73.0)	1.58/2.69 (1.40/2.20)	74.60 (25.70)
Statistical intergroup comparisons		R5/1+ > R5/1–; P < 0.001 R5/2+ < R5/2–; P < 0.001 N5/1+ < N5/1–; P < 0.001 N5/2+ < N5/2–; P < 0.05 R7.5/1+ = R7.5/1–; ns R7.5/2+ < R7.5/2–; P < 0.001	R5/1+; P < 0.001 R5/1–, P < 0.01 R5/2+; ns R5/2–; ns N5/1+; ns N5 / 1–; P < 0.001 N5/2+; ns N5/2–; ns R7.5/1+; P < 0.001 R7.5/1–; ns R7.5/2+; ns R7.5/2–; ns	R5/1+ < R5/1-; P < 0.001 R5/2+ < R5/2–; P < 0.001 N5/1+ = N5/1–; ns N5/2+ = N5/2–; ns R7.5/1+ > R7.5/1–; P < 0.001 R7.5/2+ < R7.5/2–; P < 0.001

Abbreviations : R5/1+, R5/2+, N5/1+, N5/2+, R7.5/1+, R7.5/2+, with agarose disk contact; R5/1–, R5/2–, N5/1–, N5/2–, R7.5/1–, R7.5/2–, without agarose disk contact; ns, not significant; Std. dev., standard deviation.

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