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SHORT COMMUNICATION

Host orientation using volatiles in the phoretic nematode *Caenorhabditis japonica*

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ABSTRACT

Host orientation is the most important step in host-searching nematodes; however, information on direct cues from hosts to evoke this behaviour is limited. *Caenorhabditis japonica* establishes a species-specific phoresy with *Parastrachia japonensis*. Dauer larvae (DL), the non-feeding and phoretic stage of *C. japonica*, are predominantly found on female phoretic hosts, but the mechanisms underlying the establishment of this phoresy remain unknown. To determine whether *C. japonica* DL are able to recognize and orient themselves to a host using a volatile cue from the host, we developed a Y-tube olfactory assay system in which *C. japonica* DL were significantly attracted to the air from *P. japonensis* but not to the air from three other insects or to CO_2. These results demonstrated that *C. japonica* DL utilize volatiles for host recognition and orientation and that the presence of a specific volatile kairomone released by the host attracts *C. japonica* DL.

KEY WORDS: Dauer, Kairomone, Nictation, Olfactometer, Navigation

INTRODUCTION

Finding a host is the most critical step for parasitic and phoretic nematodes. Host-searching nematodes respond to different types of host-related stimuli such as vibrations (Torr et al., 2004), temperature (Byers and Poinar, 1982), electric fields (Shapiro-Ilan et al., 2009) and chemical compounds (Pye and Burman, 1981; Grewal et al., 1993; Shapiro et al., 2000). However, host-searching strategies vary among species or ecological niches, and mechanisms of host finding, including host recognition and orientation, are poorly understood. Volatiles could be a useful cue for nematodes to orient, particularly those from distant locations. The recognition and importance of volatiles in host searching have been reported in entomopathogenic nematodes (EPNs). The infective juveniles (IJs) of steinernematid and heterorhabditid EPNs respond to volatiles from insects and CO_2 (Lewis et al., 1993; Campbell and Kaya, 1999; Hallem et al., 2011; Dillman et al., 2012). IJs are also attracted to herbivore-induced plant volatiles (HIPVs), which facilitates host detection, resulting in higher infection rates (Rasmann et al., 2005; Hiltpold et al., 2010; Ali et al., 2010; Ali et al., 2011).

Caenorhabditis japonica Kiontke, Hironaka and Sudhaus is a bacterial-feeding nematode that forms a species-specific and female host-biased phoresy with the burrower bug *Parastrachia japonensis* Scott (Kiontke et al., 2002; Yoshiga et al., 2013). Dauer larvae (DL), a developmentally arrested and phoretic stage of the nematode and a developmental analogue of IJs in EPNs, are predominantly found on *P. japonensis* adult females throughout the year in fields. Only the nematode benefits from this phoresy. The nematode uses the host insect to prolong its own survival (Tanaka et al., 2012) and for transport to food resources, such as the nest of the host insect where the mother insect stores fruits of *Schoepfia jasminodora* Siebold & Zuccarini for her nymphs, as well as eggs and nymphal carcasses (Okumura et al., 2013b; Yoshiga et al., 2013). Thus, the phoresy is essential only for the nematode, but the mechanisms of forming the species-specific phoresy are not well understood.

In our previous study, we demonstrated that *C. japonica* DL specifically embark on *P. japonensis* and are attracted to the hexane extracts containing body surface components of *P. japonensis* (Okumura et al., 2013a). These studies indicate the presence of a species-specific kairomone that is directly released by the host insect and attracts DL. However, hexane extracts contain not only volatiles but also non-volatiles, and it is difficult to distinguish whether *C. japonica* DL are attracted to volatiles or non-volatiles in the agar plate assay, which is commonly used in nematode chemoattraction studies (Bargmann, 2006). Thus, a new method to evaluate volatile attraction is necessary.

Because *C. japonica* DL embark on a host insect that wanders on the soil surface, DL have to detect the presence of a host on the soil surface and quickly orient themselves for embarkation on the host. Thus, it is possible that *C. japonica* DL recognize volatiles from a host and orient themselves to the host. To examine this possibility, we developed an assay system for *C. japonica* DL using a Y-tube olfactometer and demonstrated their recognition of and orientation to a host insect.

RESULTS AND DISCUSSION

Y-tube olfactometers are commonly used in experiments involving insect olfactory responses (Smith et al., 1994). Volatiles pass through one arm of the Y-tube and clean air passes through the other; this gives the possibility of choosing between two different stimuli. However, Y-tube olfactometers are not commonly used in nematode research because of some technical difficulties such as conditioning of humidity and locomotion activity. Thus, we modified a Y-tube olfactometer so that *C. japonica* DL could freely move in it (Fig. 1A,B). Unlike arthropods, nematodes require high humidity for their movements; thus, using water agar in the assay arena improved nematode movement. In addition, the assay arena was positioned vertically because *C. japonica* DL have a tendency to move upward during host searching (Okumura et al., 2013c); this improved nematode locomotion and response to volatiles (data not shown).

In the olfactory assay using *P. japonensis* as the test insect, *C. japonica* DL moved towards the direction of air flow from *P. japonensis* soon after the initiation of the experiments, and 43%

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List of abbreviations

DL	dauer larvae
EPN	entomopathogenic nematode
GLM	generalized linear model
HIPVs	herbivore-induced plant volatile
IJ	infective juvenile

(range 13–89%) of inoculated nematodes were found in the test area while only 16% (range 0–49%) were in the control area. In contrast, more than half of the inoculated DL were recovered from the inoculation area in each assay when *Acanthocoris sordidus* Thunberg, *Acrida cinerea* Thunberg or *Oxya yezoensis* Shiraki were used as the test insect, and no attraction toward these insects was observed. The percentage of nematodes in the test area was significantly higher when *P. japonensis* was used than when other insects or CO_2 was used (Fig. 1C; $P<0.001$). CO_2 emitted by animals as a by-product of respiration attracts DL of *C. elegans* and IJs of EPNs (Hallem et al., 2011). In our study, however, CO_2 failed to attract *C. japonica* DL (Fig. 1C). The three insects used in this study, *A. sordidus*, *A. cinerea* and *O. yezoensis*, also produced CO_2 yet did not attract *C. japonica*, which suggests that CO_2 may not be a cue for *C. japonica*. These results also indicate that the movement of *C. japonica* DL towards the air from *P. japonensis* appears to be evoked by specific volatiles produced by this host insect. Our results demonstrate that *C. japonica* DL recognize volatiles from their specific host and orient toward these. To the best of our knowledge, this is the first report that demonstrates the presence of a species-specific volatile kairomone that attracts host-finding stage nematodes.

Many nematodes utilize volatiles for host searching but the utilization of volatiles could vary among species with their ecological background. Although the ecology of EPNs in the wild is not well understood (Strong, 2002), EPNs have a broad host range and can infect most insect orders (Poinar, 1979), and IJs are attracted by different types of volatiles such as HIPVs, insect volatiles and CO_2 (Lewis et al., 1993; Campbell and Kaya, 1999; Hallem et al., 2011; Dillman et al., 2012). In contrast, *C. japonica* forms a specific phoresy with *P. japonensis*, and DL are attracted only by volatiles from *P. japonensis*. The differences in response to volatiles between IJ EPNs and *C. japonica* DL may reflect the variations in host range and host-searching strategies. To effectively approach a variety of potential hosts, IJs of EPNs may utilize simple indicators of biological activities such as CO_2 as basic host stimuli along with various volatiles as additional information. In contrast, *C. japonica* DL may not utilize these simple indicators as host stimuli; they may instead exclusively depend on a specific chemical cue from the host. The decrease in response to simple indicators from biological activities and increase in the sensitivity and response to a specific kairomone may be important factors for host recognition and orientation in *C. japonica*. Analysis of the volatile components of *P. japonensis* that attract *C. japonica* DL and studies on sensory responses to known chemicals are thus necessary to better understand the mechanism and evolution of host recognition and orientation in *C. japonica*.

MATERIALS AND METHODS

Nematodes

Caenorhabditis japonica strain H1 isolated from an adult female of *P. japonensis* collected from Hinokuma Mountain Prefectural Park, Kanzaki City, Saga Prefecture, Japan, was used for the experiments. Dog food agar medium (Hara et al., 1981) seeded with *Escherichia coli* (Migula) Castellani and Chalmers OP50 was used to obtain a large number of nematodes. Nictating *C. japonica* DL were collected as described previously (Tanaka et al., 2010). In brief, a sterile yellow 200 µl pipet tip (Watson, Fukaekasei Co., Ltd, Tokyo, Japan) was vertically placed such that the tapered side was up at the centre of a 100 ml culture bottle of dog food agar medium in which *E. coli* OP50 and the nematodes were inoculated. Approximately 5 days after inoculation, masses of nictating DL at the point of the yellow tip were picked up using a fine needle and used for subsequent experiments.

Olfactometry

To determine whether volatiles from insects attract nematodes, we constructed an olfactometer that was a modified version of that used in the study of the Japanese horntail (Matsumoto and Sato, 2007). The

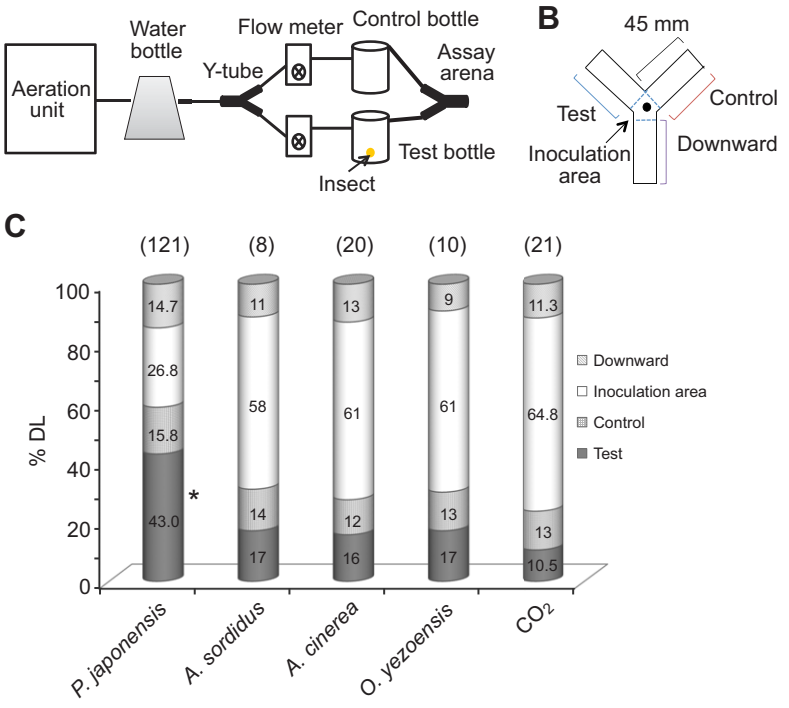

Fig. 1. Olfactory assay using *Caenorhabditis japonica* dauer larvae. (A) Olfactometer. The olfactometer consists of an air pump, a water bottle containing deionized water, a Y-shaped glass tube (inner diameter, 12 mm), two flow meters, two bottles and an assay arena (Y-shaped glass tube). Air flow rate was adjusted to 0.4 l min^{-1} using a flow meter. A faster air flow rate drifted the inoculated nematodes downward. Approximately 30–100 dauer larvae (DL) were inoculated at the centre of the inoculation area, and the assay arena was set vertically (B). When the assay arena was set horizontally, the nematodes did not move toward *P. japonensis* (data not shown).
(B) Enlargement of the assay arena. Approximately 10 min after the start of the experiment, the number of nematodes in the test, control, inoculation and downward areas was determined. The small circle in the inoculation area indicates the nematode inoculation point. (C) Olfactory response of *C. japonica* DL to CO_2 and to four insect species: *P. japonensis*, *Acanthocoris sordidus*, *Acrida cinerea* and *Oxya yezoensis*. A significantly higher number of nematodes moved towards volatiles emitted by *P. japonensis*. In contrast, more than half of the inoculated DL stayed within the inoculation area when other insects or CO_2 was used, and there was no attraction to other insects or CO_2. Data were analysed using a generalized linear model (GLM). *$P<0.001$.

Table 1. List of insects used in this study

Insects	Sampled sites and years
Hemiptera	
Acanthocaris sordidus (nymph)	The campus of Saga University, Japan, 2010
Parastrachia japonensis (adult female)	Hinokuma Mountain Prefectural Park, Japan, 2007–2009
Orthoptera	
Acrida cinerea (unsexed adult)	The campus of Saga University, Japan, 2010
Oxya yezoensis (unsexed adult)	The campus of Saga University, Japan, 2010

olfactometer used in the current study is illustrated in Fig. 1A. The aeration unit (MAU-2, Tokyo Rikakikai Co., Ltd, Tokyo, Japan) was set to obtain an air flow rate of 0.8–1.0 l min^{-1}. Air was passed through ~150 ml of distilled water in a 300 ml glass bottle to remove odours and to maintain a high humidity, and then divided into two lines by the first glass Y-shaped tube joint (Y-tube: inner diameter, 12 mm; Sansyo, Tokyo, Japan). Air flow was adjusted to a rate of 0.4 l min^{-1} using a flow meter (RK200V, Kofloc, Kyoto, Japan), which was connected to a 100 ml Pyrex test or control bottle. The test bottle contained a test insect, whereas the control bottle had no insects. The air passed through the test and control bottles connected to the assay arena (the second Y-tube), whose longitudinal half volume was filled with 1.5% water agar. Silicone tubes were used for all connections between the aeration unit, water bottles, flow meters and Y-tubes. Approximately 20 µl of the nematode suspension containing 30–100 DL was placed onto the water agar at the centre of inoculation area (Fig. 1B), the arena was set vertically, and aeration was started. Approximately 10 min after starting aeration, we disconnected the arena, and the nematodes in each area (test, control, inoculation and downward areas; Fig. 1) were counted. To test the effect of CO_2, a bottle of compressed CO_2 was connected to one of the flow meters, and the other flow meter was connected to the aeration unit. Air flow was adjusted to a rate of 0.4 l min^{-1} using a flow meter. Insects used for the olfactometry assay are listed in Table 1. The number of insect individuals of *P. japonensis*, *A. cinerea*, *O. yezoensis* and *A. sordidus* used for assay was 16, 2, 1 and 1, respectively. The number of replicates of *P. japonensis*, *A. sordidus*, *A. cinerea*, *O. yezoensis* and CO_2 experiments was 121, 8, 20, 10 and 21, respectively.

Statistical analysis

Percentages of nematodes in the test area were compared among the insects using a generalized linear model with a Gaussian distribution (StatView Ver. 4.54; Abacus Concepts, Inc., NJ, USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

E.O. performed the experiments and data analyses, and drafted the article. T.Y. contributed to the conception and design of the experiments, and the drafting and revising of the article.

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