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Three dimensional study of wounded plant roots recruiting entomopathogenic nematodes with Pluronic gel as a medium

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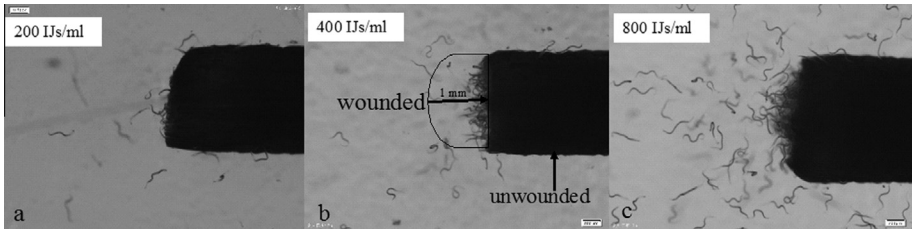
HIGHLIGHTS

- Pluronic gel system is a useful medium to study EPN host habitat finding behavior.
- Mechanically damaged roots greatly improve seeking ability of EPNs.
- Host habitat root exudates plays an important role in directing EPN localization.
- Storage duration and species/strains of EPNs influence nematode response to roots.

GRAPHICAL ABSTRACT

Attraction of *Heterorhabditis bacteriophora*-HBN (Hb-HBN) to wounded and unwounded chive root in pluronic gel with three concentrations at 4 h after assay initiation.



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ABSTRACT

Pluronic F-127 is a non-toxic and thermoreversible transparent copolymer that allows nematodes to move freely inside the gel and be observed under a microscope. Pluronic gel has previously been shown to be a useful medium for three dimensional study of plant parasitic nematode host-finding behavior, but not for host-habitat finding of entomopathogenic nematodes (EPNs). In the current study, we used Pluronic gel for the first time to investigate the foraging behavior of EPNs, which are natural enemies of root-feeding insect pests, and to investigate the effect of storage duration of EPNs on attraction to wounded roots. We first tested one isolate of EPN, *Heterorhabditis bacteriophora* HBN (Hb-HBN), from northeastern China that is known to be an effective bio-control agent against Chinese chive gnat (*Bradysia odoriphaga*). The nematodes aggregate around wounded root parts, indicating that the nematodes are attracted to compounds released from freshly wounded root tissue, where insect hosts are more likely to be present. Among species/strains of EPNs that were tested, Hb-HBN showed the strongest attraction to Chinese chive roots. Surprisingly, attraction to a Chinese chive root following storage duration of up to 30 days was much less than for Hb-HBN that were stored longer, with an optimal attraction observed for Hb-HBN that were stored 75–90 days. This might be associated with phased infectivity. This study provides a better understanding of EPN foraging behavior and introduces the Pluronic gel system as a useful medium to study EPN attraction.

1. Introduction

During the past 60 years entomopathogenic nematodes (EPN) (Rhabditida: Heterorhabditidae and Steinernematidae) have received considerable attention as biological control agents of soil insect pests (Campos-Herrera et al., 2012). EPNs are obligate insect parasites; the infective juveniles (IJs) of the third larval instar penetrate their hemocoel, release their symbiotic bacteria, and kill insects within 24–48 h (Chaston and Goodrich-Blair, 2010; Kaya and Gaugler, 1993). During the infection process, host-habitat finding (finding the habitat that favors presence of the insect host),

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might be one of the most important steps. Insect herbivore damaged plants have been shown to release chemical compounds (plant exudates) that recruit the natural enemies of their pests, and some plant exudates have been shown to attract EPNs resulting in more efficient bio-control (Ali et al., 2010, 2011; Aratchige et al., 2004; Choo et al., 1989; Gassmann et al., 2010; Hiltpold and Turlings, 2008; Jagdale et al., 2009; Rasmann et al., 2005; Turlings et al., 2012; Wang and Gaugler, 1998; Van Tol et al., 2001). Root exudates not only play a role as attractants, signal molecules, and stimulants to beneficial organisms, but also as inhibitors and repellents to soil pathogens or pests, which form mutualistic associations in the rhizosphere including root–root, root–microbe and root–insect interactions (Badri et al., 2009; Baetz and Martinoia, 2014; Bais et al., 2006). Identification and characterization of these unknown chemical compounds would help to elucidate the direct or indirect effect on plant defenses and the soil microbial community.

Although great progress has been achieved toward understanding interactions among insects, EPNs and symbiotic bacteria of EPNs, relatively less is known about how plants interact with EPNs. Lack of efficient methods or tools/medium to observe nematode behaviors below ground might be a major obstacle. Previous studies were based on either two-dimensional agar plates or non-transparent sand or soil filled olfactometers. So far, a below ground six-arm olfactometer has proven to be useful in studying EPN attraction (Rasmann et al., 2005) although it remains difficult to observe nematode movement and behavior in real time. Recently, a non-toxic and thermo-reversible transparent copolymer, Pluronic F-127, widely used in medical, pharmaceutical and cosmetic fields (Barichello et al., 1999; Farrugia et al., 2014; Marsh et al., 2003; Morishita et al., 2001; Zhang et al., 2010), has received more attention and proven to be a useful medium to study interactions between plant-parasitic nematodes and plants, including nematode host-seeking behavior and nematode chemotaxis (Feng et al., 2014; Fudali et al., 2013; Sasaki-Crawley et al., 2012; Wang et al., 2009a,b, 2010). A 23% solution of Pluronic is a semisolid gel at room temperature but a liquid at 15 °C and below. A stable gradient could be formed in the Pluronic gel in the presence of roots or chemicals (Feng et al., 2014; Fudali et al., 2013; Sasaki-Crawley et al., 2012; Wang et al., 2009a,b, 2010). The transparency and semisolid states allow nematodes to move freely in the gel fostering three-dimensional assay and allowing direct and real time observation of interactions between nematodes and the root system. Clearly, the plant cues were used by plant pathogenic nematodes for direct host finding. While the plant cues were used by EPNs for host-habitat finding. Therefore, whether or not the Pluronic gel system is suitable for the study of host-habitat finding behavior is unclear.

In our group, local isolates of a species of *Heterorhabditis bacteriophora* Poinar (Hb) from Harbin, China, had been shown to tolerate relatively lower soil temperatures and to be efficient against the root gnats, *Bradysia odoriphaga* Yang et Yang (Diptera: Sciaridae) on Chinese chive (*Allium tuberosum* Rottle ex Spreng) (Asparagales: Amaryllidaceae) (Li et al., 2011, 2013), a favored vegetable of Asians. In this study, we used the Hb-HBN strain and Chinese chive root in Pluronic gel, to test the system for a host-habitat location study and then the system was used to answer some fundamental questions. Our hypothesis is that the Pluronic gel should work for the EPN-host-habitat finding study since both plant pathogenic nematodes and EPNs use chemical cues for establishing either host or host-habitat location. Our objectives were to determine (1) if Pluronic gel can be used to study EPN host habitat finding behavior, (2) if storage time has any effect on the ability of host-habitat location, (3) if the same nematode species respond the same to different plant species and (4) if different nematode species or strains respond to the same plant roots differently.

2. Materials and methods

2.1. Nematodes

H. bacteriophora-HBN (Hb-HBN) was isolated from the soil around a pine tree at the Northeast Institute of Geography and Agroecology (IGA) of the Chinese Academy of Sciences, Harbin (45°45′N/126°39′E), Heilongjiang Province, China (Li et al., 2011). Two other isolates, *H. bacteriophora*-CD-11 (CD-11) and *H. bacteriophora*-NT-82 (NT-82), were kindly provided by Dr. Shulong Chen from the Institute of Plant Protection, Hebei Academy of Agriculture and Forestry Science in China. One isolate of *H. bacteriophora*-NJ (NJ) and four species of *Steinernema carpocapsae* (Weiser) – All (Sc-all), *Steinernema glaseri* (Steiner) (Sg), *Steinernema feltiae* (Filipjev) (Sf) and *Steinernema riobrave* Cabinillas, Poinar and Raulston (Sr), were obtained from Dr. Randy Gaugler's lab (Rutgers University, USA). *Steinernema litorale* Yoshida (Sl) was obtained from Dr. Kuijun Zhao, Northeast Agricultural University, Harbin, China. All EPN species and isolates were cultured in the last-instar of the great wax moth, *Galleria mellonella* L. (Lepidoptera: Pypalididae), at room temperature for 7–10 days (Kaya and Stock, 1997). IJs emerged from insect cadavers into White traps (White, 1927) and were collected and stored in shallow water in transfer flasks at 10 °C for up to three months; the specific storage time period is referred as "storage duration". Prior to experiments, IJs taken from storage were acclimated to room temperature for 1 h and their viability on the basis of movement was checked under a stereomicroscope with a hair prob.

2.2. Plant root preparation

All plants were grown and maintained in the experimental field of IGA. Chinese chive (*A. tuberosum* Rottle ex Spreng) was grown for more than 2 years. Shallots (*Allium cepa* L. var. aggregatum) (Asparagales: Amaryllidaceae) were planted in the field for 2 months during the growing season, garlic (*Allium sativum* L.) (Asparagales: Amaryllidaceae), tomato (*Lycopersicon esculentum* Mill.) (Solanales: Solanaceae) cv. Qiyanaifen and soybean [*Glycine max* (L.) Merrill] (Fabales: Fabaceae) cv. Hefeng 25 were planted in the greenhouse for one month before use. Approximately 0.5 cm of washed roots, where insects dwell, were excised from 2 to 2.5 cm of the bulb base of chive, shallot and garlic and of the stem of tomato and soybean. The transverse sections of all the cut roots from each plant were approximately 0.1 cm in diameter. The segments from 6 to 6.5 cm and 12 to 12.5 cm of the bulb base of Chinese chive roots were also excised for use to compare nematode response to different parts of wounded roots.

2.3. Pluronic gel preparation

Gel preparation and attraction assays were conducted according to Wang et al. (2009b). Approximately 23% (wt/vol) Pluronic F-127 gel (NF Prill Poloxamer 407, BASF, Mt Olive, NJ, USA) in 10 mM Tris–MES (morpholino-ethanesulfonic acid) buffer (Sigma–Aldrich) was made under refrigeration at 4 °C and with continuous stirring overnight. The dissolved gel was stored at 4 °C and aliquots were dispensed for experiments.

2.4. Nematode concentration and Heterorhabditis bacteriophora-HBN response to wounded and unwounded Chinese chive roots

To optimize the system, nematode Hb-HBN attraction to Chinese chive roots was tested with variable concentrations (200, 400 and 800 IJs/mL) based on the response of root-knot nematodes

to plant roots (Wang et al., 2009b). Collected nematodes at different concentrations were washed with sterile water through centrifugation for three times. Then the total number of nematodes for each concentration were calculated and were concentrated into 1 mL water and again centrifuged. Around 900 μL of water above nematodes was removed with a pipet tip and 100 μL of nematode suspension was mixed with 10 mL Pluronic gel (depending on the number of replicates) in one beaker under constant stirring at 4 °C. About 30 min later, 3 × 5 μL gel was taken from each concentration and nematodes at the three concentrations were counted again under the dissecting microscope to make sure the actual concentration was close enough to the required concentration.

For the root attraction assay, approximately 0.5 cm of fresh root cut with a cutter blade (see Section 2.2) was placed into the center of a 3.5 cm diam. Petri dish containing 2 mL Pluronic gel with a known concentration of EPN IJs. The root was immersed into the gel and rested on the bottom of the plate. Then the Petri dishes were kept at 25 °C in an incubator. For assays over the course of time, attraction was observed and photographed with an OLYMPUS SZX-16 dissecting microscope using Cellsens Standard image software (Olympus Corporation, Japan). The number of EPNs within a 1 mm semicircle of a wounded root end and in contact with an unwounded root (root surface) (Fig. 1A) were counted at different time intervals (2, 4, and 6 h post exposure), and attraction was expressed as the percentage of nematodes within the semicircle of the total number in the Petri-dish.

2.5. Storage duration of Heterorhabditis bacteriophora-HBN relate to the response to Chinese chive roots

In order to identify the effect of the storage duration at 10 °C on nematode attraction to a fresh Chinese chive root, nine time points (1, 10, 30, 45, 60, 75, 90, 140 and 180 days) after collection of the nematodes from White traps were evaluated using Hb-HBN (400 IJs/mL). Nematode response to roots was observed 4 h after introducing them to the system and the number of nematodes within a 1 mm semicircle from both ends of the wounded roots were counted.

2.6. Heterorhabditis bacteriophora-HBN attraction to different plant roots

In order to test whether different plant species affect nematode attraction, five plant species (chive, garlic, shallot, tomato and soybean) were prepared and used as described above (Section 2.2). Approximately 0.5 cm of washed roots were excised from 2 to 2.5 cm of the bulb base of chive, shallot and garlic and the base of stem of tomato and soybean. IJs of Hb-HBN with 75–90 days of storage duration at 10 °C were assessed with respect to response to different crop roots as described above (Section 2.4). The number of nematodes within a 1 mm semicircle from both ends of the wounded roots was counted at 30 min, 2, 4 and 6 h intervals post nematode inoculation (400 IJs/mL).

2.7. Different nematode specie/strain attraction to Chinese chive roots

To observe if different nematode species/strains respond differently to the same plant roots (Chinese chive roots), the attraction assay was conducted for five nematode species of *Steinernema* and 4 strains of *H. bacteriophora* as described in Section 2.4 with the concentration of 400 IJs/mL. The number of attracted nematodes within a 1 mm semicircle of the wounded roots was counted at different time intervals (30 min, 2, 4, 24 and 48 h) post exposure.

For all experiments, there were 3–7 replicates in each treatment and each experiment was replicated 3 times.

2.8. Statistical analysis

Data were subjected to one-way analysis of variance (one-way ANOVA) using SPSS software (SAS Institute, Cary, NC, USA). Results are reported as significant or non-significant in Tukey's Honestly Significant Difference (Tukey HSD) Test ($P < 0.05$).

3. Results

3.1. Heterorhabditis bacteriophora-HBN response to wounded and unwounded Chinese chive roots

As early as 5 min after the assay, we observed that Hb-HBN quickly responded by moving toward both ends of the wounded

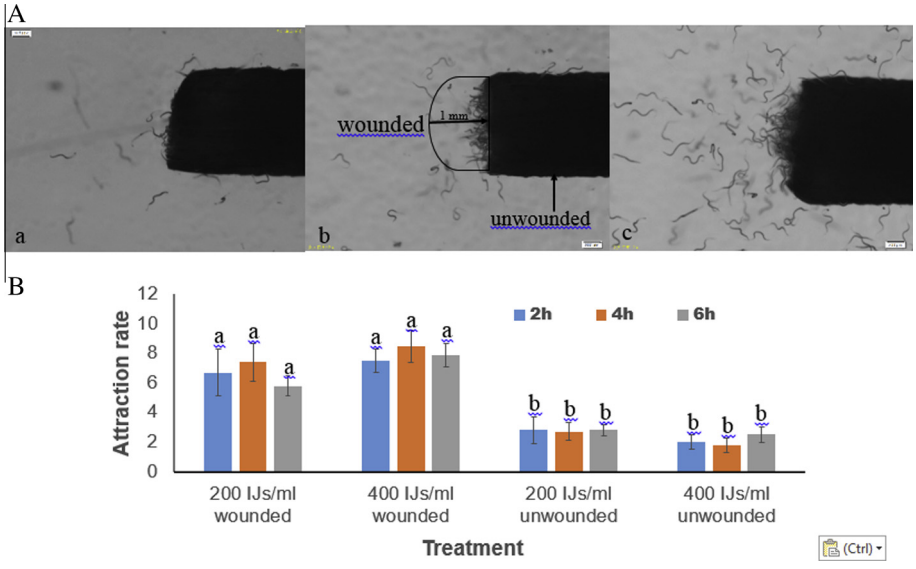

Fig. 1. Attraction of *Heterorhabditis bacteriophora*-HBN to wounded and unwounded Chinese chive roots in Pluronic gel. (A) Photographs show aggregation of the attracted nematodes with three concentrations at 4 h after assay initiation. (a) 200 IJs/mL; (b) 400 IJs/mL; (c) 800 IJs/mL (scale bar = 200 μm). (B) Percentage of nematodes attracted to wounded or non-wounded Chinese chive roots at concentrations of 200 IJs/mL and 400 IJs/mL. Bars with different letter are significantly different ($P < 0.05$) (error bars indicate standard error).

roots in a sinusoidal pattern. Once nematodes reached the wounded roots, the majority of them were arrested on site within 1 mm from center of the wounded roots (Fig. 1A). With additional time, more nematodes responded to the wounded root parts relative to the unwounded part. A video showing attraction with directed and sinusoidal movement of Hb-HBN to the wounded roots at 30 min post exposure was published to YouTube (http://youtu.be/gzw8L7APUwg). For the concentration of 800 IJs/mL, more than 100 nematodes clustered together around the wounded roots at 2, 4 and 6 h post exposure, making counting hard (Fig. 1A-c). Therefore, the concentration of 800 IJs/mL was eliminated for further observation and the comparison in attraction rate to wounded roots and unwounded roots was conducted between concentrations of 200 IJs/mL and 400 IJs/mL (Fig. 1B). There were significant differences in attraction rates between wounded and unwounded parts among each of the two concentrations at 2, 4 and 6 h post-exposure (Fig. 1B) ($F = 17.6$; $df = 11, 24$; $P < 0.001$). Among the three time points, no significant differences in attraction rate to wounded roots and unwounded roots was observed respectively (Fig. 1B). [Wounded: $F = 1.1$; $df = 5, 12$; $P = 0.412$; Unwounded: $F = 0.47$; $df = 5, 12$; $P = 0.795$]. A concentration of 400 IJs/mL was used for the following experiments.

In addition, there was no significant difference in attraction rate to three parts of the wounded Chinese chive roots (2–2.5 cm, 6–6.5 cm and 12–12.5 cm from bulb base) ($F = 0.999$; $df = 2, 18$; $P = 0.388$). Since root gnats dwell close to the bulb base, a root segment of 2–2.5 cm from the bulb base was used for the following experiments.

3.2. Effect of Heterorhabditis bacteriophora-HBN storage duration on their attraction to Chinese chive roots

Initial experiments consistently showed there was almost no attraction to Chinese chive roots with newly emerged nematodes, so a series of storage periods was tested on nematode attraction to plant roots. At 30 min after the assay started, we observed nematodes migrating randomly in the gel but with little evidence of attraction to the Chinese chive roots stored for 1–30 days (1 day: 1.17 ± 0.61; 10 days: 9.5 ± 1.33; 30 days: 8.17 ± 2.54). In contrast, nematodes subjected to longer storage durations (45, 60, 75, 90, 140 and 180 days) were observed to directly migrate to the wounded roots with a higher attraction to the Chinese chive roots (45 days: 23.7 ± 3.8; 60 days: 34.7 ± 4.6; 75 days: 45.3 ± 4.1; 90 days: 46.3 ± 5.7; 140 days: 31.8 ± 3.4; 180 days: 18.2 ± 1.1) than those stored for 1–30 days ($F = 30.62$; $df = 8, 45$; $P < 0.001$). EPN attraction increased significantly and continually with storage time periods up to 90 days. Then the attractions decreased significantly for 140 days and 180 days storage durations, but resulted in significantly greater attraction than those at 1, 10 and 30 days (Fig. 2). Results suggested 75–90 days of storage duration was optimum for the attraction experiments. Therefore approximately 75–90 days of storage time was applied to the following attraction experiments.

3.3. Heterorhabditis bacteriophora-HBN response to different crop roots

The number of nematodes around wounded Chinese chive roots was significantly greater than that for shallot, garlic, tomato and soybean at the four time points considered (30 min, 2 h, 4 h, 6 h: $F = 11.28–27.54$; $df = 4, 25$; $P < 0.001$) (Table 1). Strain Hb-HBN showed greater attraction to garlic and shallot, but less attraction to soybean and tomato (garlic vs. shallot: $P = 0.808$; tomato vs. soybean: $P = 0.013$; garlic vs. tomato: $P = 0.003$) at 4 h post exposure. To each plant species, Hb-HBN did not show a significant difference in attraction to wounded roots among the four time points (Chive: $P = 0.902$; Shallot: $P = 0.618$; Garlic: $P = 0.469$; Tomato: $P = 0.800$; Soybean: $P = 0.368$). The number of nematodes contacting unwounded roots (root surface) among the five plant species was not significantly different at all equivalent time points (30 min: $P = 0.166$; 2 h: $P = 0.276$; 4 h: $P = 0.957$; 6 h: $P = 0.683$).

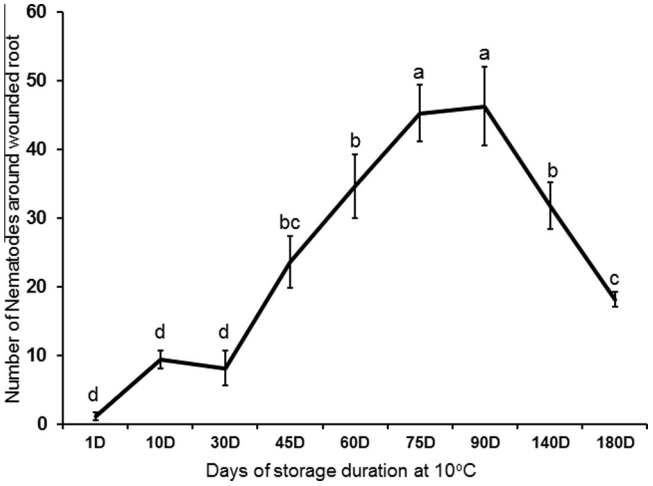

Fig. 2. Attraction of *Heterorhabditis bacteriophora*-HBN with different storage duration at 4 h post-exposure to Chinese chive roots. This experiment was carried out in diam. 3.5 cm Petri dishes containing 2 mL Pluronic gel with 400 IJs (error bars indicate standard error).

3.4. Attraction of different species and strains of entomopathogenic nematodes to Chinese chive roots

Hb-HBN showed significantly greater attraction to wounded Chinese chive roots than that of other EPN species or isolates at 30 min, 2 and 4 h ($F = 11.79–29.3$; $df = 8, 18$; $P < 0.001$) (Table 2). Whereas there fewer nematodes were found around the wounded roots during the first 4 h for *Sc*, *Sl* and *Sf*. After 4 h exposure, EPN species *Sc*, *Sl* and *Sr* began to move toward the wounded roots, with greatly increased attraction to roots at 24 and 48 h post exposure where attraction levels similar to those of HBN were reached. Response over time to wounded Chinese chive roots differed among EPN species/strains (Table 2).

4. Discussion

For the first time, we applied Pluronic F-127 gel as a medium mimicking the three-dimensional soil environment to study EPN-host-habitat finding behavior in response to environmental cues from plants and to observe other aspects of EPN behavior. The advantages of the gel were described clearly by Wang et al. (2009a,b, 2010). Simply speaking, transparency of the gel allowed close examination in real time of behavioral changes as the nematode approached and made contact with the roots. Formation of a stable gradient in the gel made the test quantitative and reproducible (Feng et al., 2014; Fudali et al., 2013; Sasaki-Crawley et al., 2012;Wang et al., 2009a,b, 2010). The gel proved to be a powerful and valuable medium to study the interaction between EPN and host-habitat plant, and potentially study tritrophic interactions of EPN, the insect host, the associated plant habitat and as well as other related aspects.

Consistently we observed that a wounded Chinese chive root (cut end) recruited significantly greater numbers of nematodes than an unwounded root (surface of root) under different treatments including storage duration and exposure time for strain Hb-HBN, but this pattern was not always true for other EPN species or strains, or other kinds of crops. Moreover, EPN showed similar

attraction to an intact Chinese chive root tip as well as to an unwounded root (unpublished data). These results indicates exudates from the wounded roots (broken roots) are major signals to direct EPN movement. Root cutting simulates damage to the plant suggestive of insect feeding in releasing chemical cues and thus increasing the chance for EPNs to locate insect hosts and increase nematode infection. Obviously, the "broken" roots exude more plant content and therefore, are more attractive. The result is in accordance with a previous report by Wang and Gaugler (1998) in which wounded grass roots were more attractive than whole roots to EPNs. Boff et al. (2002) and Van Tol et al. (2001) demonstrated that *Heterorhabditis megidis* Poinar, Jackson & Klein was attracted to thuja plant roots damaged by both larval feeding and a scissors cut, but that a root with feeding insect larvae was even more attractive. By using a two-choice assay, Boff et al. (2002) found that IJs of *H. megidis* preferred mechanically damaged strawberries as opposed to roots damaged by weevil larvae, but in the thuja root assay the nematodes were more attracted to roots damaged by larvae than by mechanical damage, indicating that attraction to different types of wounds was plant specific. Rasmann et al. (2005) reported that insect-damaged maize roots recruit more IJs of *H. megidis* than undamaged maize plants or plants with mechanically damaged roots. Further findings revealed that the first plant herbivore-induced signal, (E)-β-caryophyllene, strongly attracted EPNs, but not all maize varieties possess the signals (Rasmann et al., 2005). Later, additional studies found that EPN attraction is dependent on EPN species/strains and crop species and varieties (Ali et al., 2010, 2011; Hiltpold et al., 2010; Laznik and Trdan, 2013; Rasmann and Turlings, 2008; Turlings et al., 2012). Since we examined attraction affected by the interaction between Hb-HBN and the insects' host plant but not the host insect, it is still unknown whether root-gnats feeding upon Chinese chive roots could result in greater attraction of nematodes than a mechanically wounded plant alone. Our study demonstrated that damaged roots were able to recruit IJs of EPNs, which may benefit both the plant and the nematode.

Among all tested interactions between plant and EPN, strain Hb-HBN produced the greatest attraction to Chinese chive roots (Tables 1 and 2), indicating different root-released volatiles from different crops and that EPN response to volatiles is also species/strain dependent. Even within the same species, *H. bacteriophora*, strains showed different responses to Chinese chive roots, including changes in attraction over time (Table 2).

Surprisingly, we found that the storage duration of nematodes at 10 °C significantly affected Hb-HBN movement and attraction to Chinese chive roots. Long periods of storage duration without losing attraction would extend shelf life of some EPN species and this has important practical implications for biocontrol applications. Therefore, investigating the effect of storage duration of each species or strain on subsequent nematode attraction might be one important step to improve the efficiency of pest control. Generally for practical use, EPNs are kept at low temperatures for 2 or 3 weeks before use (Ali et al., 2010, 2011, 2013; Ennis et al., 2010; Ma et al., 2013). In the present study, we found that there was almost no response to Chinese chive roots from freshly cultured EPNs or extremely low attraction for IJs stored for less than 30 days at 10 °C. However, there was greater attraction for those stored for 75–90 days among all tested storage times. Attraction of Hb-HBN to Chinese chive roots decreased after 140 days and 180 days storage duration. These results clearly indicate that both nematode storage duration and presence/absence of a host plant affect both the type and extent of nematode migration. It may be that freshly emerged nematodes have sufficient energy reserve that they may not need to search a host such that there is no response to root volatiles. With the longer storage duration, nematode energy would be gradually depleted, such that EPNs including Hb-HBN would reactivate the response to food sources or signals, including root volatiles from the habitat of an insect host plant. Up to three months of storage time, the ability to respond to signal stimulation reached its highest point. In other words, greater attraction was due to the cost of energy depletion from the original food source. Attraction decreasing for 140 days and 180 days of storage time might be due to further declining energy (lipid) reserves as suggested by nematode bodies becoming increasingly transparent. The consumption of these reserves is thought to be associated with a decline in the ability to infect (Griffin, 2012; Menti et al., 2000). This phenomenon could be explained by phased infectivity in which infectivity is changed associated with aging (Dempsey and Griffin, 2002; Griffin, 2012; Ryder and Griffin, 2003). For example, *H. megidis* IJs with 4-week storage duration at 9 °C could increase control of vine weevil larvae in potted plants (Fitters et al., 2001). Guy et al. (2009) reported that *Steinernema* spp. increased infectivity after a storage period. Koppenhöfer et al. (2013) found virulence of *Steinernema scarabaei* Stock and Koppenhöfer remained high and infectivity even increased with storage time up to 12 weeks (84 days) at 8 °C when compared with EPNs stored at room temperature; however, presence/absence of a plant had no effect on virulence and infectivity. These results indicate that different responses of EPN subjected to different storage duration could affect tritrophic interactions among EPN species, insect and host plants. Understanding the dynamic nature of EPN infectivity will have practical applications for improving biological control of pests.

Interestingly, it was observed that *S. litorale* initially had no response to Chinese chive roots but that 4 h after exposure they were attracted to roots, and the attraction gradually increased, up to a maximum between 20 and 48 h after exposure (Table 2). The dynamics of change of nematode *S. litorale* attraction might be associated with phase infectivity. A number of species of EPNs showing a delayed peak in infectivity as reported in the present study (Table 2); this delay indicates phase infectivity that is very common but varies with EPN species/strain (Campbell et al., 1999; Hominick and Reid, 1990; O'leary et al., 1998; Ryder and Griffin, 2003). In another case, the delayed attraction peak to Chinese chive roots for *S. litorale* might be associated with risk-prone (only a few nematodes invade readily) or risk-averse (most individuals wait until the host has been invaded by others) phenomenon (Fushing et al., 2008; Griffin, 2012). It is still not clear what chemical compounds from Chinese chive roots cause attraction and further investigation using the Pluronic gel system will be conducted to distinguish long distance and short distance cues from host habitat plant to different EPN species.

In conclusion, for the first time, Pluronic gel is demonstrated to be a powerful medium for real time observations and study of early interaction between EPNs and associated plants. The present study provides evidence in support of three points: I. Host-habitat root exudates may play an important role in directing EPN localization to an insect host-habitat to increase infectivity. II. Mechanically

Table 1
Attraction of *Heterorhabditis bacteriophora*-HBN to different plant species at specific time in Pluronic gel.

Plant	30 min	2 h	4 h	6 h
Chive	42.5 ± 3.2a*	41.0 ± 6.7a	46.4 ± 5.9a	44.3 ± 4.8a
Shallot	26.0 ± 2.9b	29.5 ± 4.2b	23.9 ± 3.9b	23.2 ± 3.5b
Garlic	20.7 ± 1.7c	17.5 ± 3.0c	25.5 ± 5.4b	19.5 ± 3.8bc
Tomato	16.7 ± 2.7c	15.7 ± 2.3c	13.2 ± 2.3c	13.2 ± 4.5cd
Soybean	8.0 ± 1.2d	7.9 ± 1.0d	5.4 ± 1.2d	7.4 ± 1.0d

* Mean number of nematodes gathering within 1 mm semicircle of wounded roots was counted at each point plus standard error. Numbers with same letter are not significantly different within each time points ($P \geqslant 0.05$).

Table 2
Attraction of different EPN species and isolates to chive root in Pluronic gel.

EPN	Abbrev	30 min	2 h	4 h	24 h	48 h
Steinernema spp.						
S. carpocapsae	Sc-all	3.0 ± 0.6d*	5.3 ± 1.2c	12.3 ± 2.7d	32.3 ± 0.9ab	24.3 ± 3.2bc
S. litorale	Sl	1.7 ± 1.2d	5.7 ± 1.2c	16.3 ± 3.8cd	40.7 ± 10.3a	51.0 ± 8.50a
S. riobrave	Sr	9.3 ± 2.4bc	16.3 ± 1.7b	15.0 ± 1.5d	41.7 ± 1.2a	36.3 ± 4.8ab
S. felitae	Sf	2.0 ± 1.0d	4.7 ± 0.3c	11.3 ± 2.7d	16.7 ± 0.9cd	15.0 ± 1.5c
S. glaseri	Sg	15.3 ± 1.5b	14.7 ± 1.3bc	16.7 ± 2.3d	28.3 ± 5.0bc	30.7 ± 9.5b
Heterorahbiditis bacteriophora strains						
	CD-11	6.3 ± 1.3cd	19.7 ± 2.0b	25.7 ± 0.3bc	10.7 ± 0.3d	4.0 ± 0.6d
	NT-82	12.3 ± 4.2bc	19.7 ± 6.0b	21.0 ± 5.5bcd	15.0 ± 5.0cd	3.7 ± 0.7d
	NJ	10.7 ± 0.3bc	22.3 ± 4.3b	27.0 ± 4.5b	23.3 ± 10.2bc	10.0 ± 2.0cd
	HBN	39.3 ± 3.4a	40.7 ± 0.9a	51.0 ± 5.1a	45.3 ± 6.4a	37.3 ± 7.8ab

* Mean number of nematodes gathering within 1 mm semicircle of wounded roots was counted at each point plus standard error. Numbers with same letter are not significantly different within each time points ($P \geqslant 0.05$).

damaged Chinese chive roots greatly improve seeking ability of EPNs, but EPNs are species/strain dependent. III. Storage duration of EPNs and EPN species/strains influence the nematode response to Chinese chive roots, and this might be associated with phase infectivity. However, many questions remain open in understanding the complicated tritrophic interactions among EPN, insect and host-habitat plant. The Pluronic gel system will not only broaden our vision of plant defense schemes but it will also facilitate the new understanding of chemicals released by roots to provide more efficient control for certain EPN-plant combination.

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