

Soil moisture conditions alter behavior of entomopathogenic nematodes

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Abstract

BACKGROUND: A variety of environmental factors can disrupt biotic interactions between plants, insects and soil microorganisms with consequences for agricultural management and production. Many of these belowground interactions are mediated by volatile organic compounds (VOCs) which can be used for communication under appropriate environmental conditions. Behavioral responses to these compounds may likewise be dependent on varying soil conditions which are influenced by a changing climate. To determine how changing environmental conditions may affect VOC-mediated biotic interactions, we used a belowground system where entomopathogenic nematodes (EPNs) – tiny roundworm parasitoids of soil-borne insects – respond to VOCs by moving through the soil pore matrix. Specifically, we used two genera of EPNs – *Heterorhabditis* and *Steinernema* – that are known to respond to four specific terpenes – α -pinene, linalool, D-limonene and pregeijerene – released by the roots of plants in the presence of herbivores. We assessed the response of these nematodes to these terpenes under three moisture regimes to determine whether drier conditions or inundated conditions may influence the response behavior of these nematodes.

RESULTS: Our results illustrate that the recovery rate of EPNs is positively associated with soil moisture concentration. As soil moisture concentration increases from 6% to 18%, substantially more nematodes are recovered from bioassays. In addition, we find that soil moisture influences EPN preference for VOCs, as illustrated in the variable response rates. Certain compounds shifted from acting as a repellent to acting as an attractant and vice versa depending on the soil moisture concentration.

CONCLUSION: On a broad scale, we demonstrate that soil moisture has a significant effect on EPN host-seeking behavior. EPN efficacy as biological control agents could be affected by climate change projections that predict varying soil moisture concentrations. We recommend that maintaining nematodes as biological control agents is essential for sustainable agriculture development, as they significantly contribute not only to soil health but also to efficient pest management.

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INTRODUCTION

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* are parasitic nematodes of the larval, pupal and adult stages of soil-borne insects.¹ Free-living, non-feeding infective juveniles (IJs) use a suite of volatile chemical signals to find and infect larval hosts belowground.^{1,2} Once inside their hosts, EPNs rely on symbiotic bacteria that kill their insect host and serve as food for the EPNs as they complete their lifecycle and reproduce.¹ EPNs have been shown to be effective agents of biological control of pests, as they are attracted to volatile organic compounds secreted by plants when distressed by herbivory.^{3–5} This tritrophic interaction between EPNs, plants and EPNs' insect larval hosts relies on the ability of EPNs to discern these volatiles in the complex soil medium. These volatile signals can degrade and diffuse differently depending on the type of volatile chemical compound and soil conditions.^{6,7} For example, plant volatile compounds such as limonene, linalool and

E- β -caryophyllene diffuse through the soil differently depending on factors such as soil moisture and pH.^{6,7} In order to maintain

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the efficacy and usefulness of EPNs as biological control agents, it is necessary to know how abiotic factors and soil conditions could alter their ability to recognize plant volatiles and recruit to insect hosts in the soil.

The lethal nature of these EPNs is widely sought after for biological control. Significant efforts have been made in the commercial production of various species within the *Steinernema* and *Heterorhabditis* genera,⁸ as a variety of studies show their positive impact on agricultural systems and production.^{3,9-16} Within these systems, EPNs can serve as a bioindicator for soil health and may increase plant productivity.¹⁷⁻²⁰ Further, the presence of nematodes is associated with higher biomass concentrations, microbial communities, plant growth and soil nutrients.²¹ These benefits occur as exposure to an EPN will severely limit the fitness and increase the mortality rate of agricultural pests.²² However, numerous studies have shown that EPNs are sensitive to fluctuations in environmental conditions which reduces the survival and efficacy of these organisms.²³⁻²⁵ This sensitivity becomes a major concern in the biological control of agricultural pests in regard to the unpredictability of climate change.

Soil moisture contents are particularly important to consider when examining abiotic factors, as water availability in the soil is required for plants to conduct their primary metabolism, which includes regular survival mechanisms such as stomatal conductance and photosynthesis.²⁶ Since the emission of herbivore-induced plant volatiles is considered secondary metabolism in plants, while general survival mechanisms are considered the primary metabolism, the production and emission of herbivore-induced plant volatiles could be affected when plants are forced to prioritize their primary metabolism under stressful environmental conditions.²⁶ Factors that contribute to the complexity of the topic include varying degradation and diffusion patterns of volatiles under different pH and moisture levels. For instance, the plant volatile limonene diffuses more rapidly in the soil at varying soil moisture levels. Linalool has been shown to be affected by sand particles and thus does not diffuse very far in the soil.⁶ α -Pinene, another plant volatile that is a result of linalool degradation, was detected more rapidly with increasing soil moisture.⁶ α -Pinene and linalool are especially important, as they commonly attract EPNs.²⁷⁻²⁹ Other plant volatiles such as pregeijerene show opposing trends, indicating the strong variability in diffusion and degradation patterns and thus variability in detection ability.⁶

Considering that soil moisture differences could lead to potential premature diffusion of plant volatiles, EPNs could be challenged with continuing to detect these cues and could fail to aggregate to an area of herbivory, thus losing their efficacy as biological control agents. To understand the potential for this risk, we investigate the response of EPNs to several volatile organic compounds, namely pregeijerene, α -pinene, β -limonene and linalool, under different soil moisture regimes, namely 6%, 12% and 18% by volume. To do so, we expose species of EPNs to these volatile compounds using pentane as a control in two-choice olfactometers and monitor their response under the different moisture regimes.

METHODOLOGY

Nematode rearing

EPNs used for our experiment were reared from stock cultures kept at the Natural Enemy Management & Applications Lab (NEMA) at The University of North Carolina at Asheville. Stock

cultures are kept in tissue culture flasks at concentrations not exceeding 2500 IJs mL⁻¹ to ensure long-term survival of the population. The individual flasks are filled with deionized (DI) water and stored in a laboratory fridge at a temperature of 20.1 °C. In these conditions, pure EPN populations can survive for 3–6 months. To avoid potential reduction of activity and survivorship of older EPN populations, we multiplied the stock culture to ensure all EPNs used in our experiment were recently emerged and with highest viability. To achieve successful multiplication of the stock culture, *G. mellonella* larvae were inoculated with a known concentration of EPN IJs of a single species. Specifically, for each species, 15 petri dish plates were prepared with five *G. mellonella* waxworms (sourced from Speedy Worm & Minnesota Muskie Farm, MN) each, for a total of 30 plates and 150 insect hosts for EPN multiplication. Individual *G. mellonella* specimens were chosen based on their healthy appearance, which is characterized by beige coloration and active movement. Subsequently, we inoculated the plates with 1 mL of IJs at a concentration of ca 500 IJs mL⁻¹. The specimens were then left for 3–4 days in order for the EPNs to infect the larvae and begin their reproductive cycle within the body, with the help of their symbiotic bacteria. When larvae showed signs of infection, manifested as varying discoloration depending on the bacterial species, the specimens were transferred to a White trap where EPNs began exiting the host after 2–3 days.³⁰ The newly emerged IJs moved into the DI water of the petri dish where they were collected by pouring the liquid into tissue culture flasks with a clean funnel. The petri dish was then returned to the White trap setup and filled with DI water once again to allow for continued IJ collection. This procedure was repeated until there were no visible IJs left around the host or in the DI water. The resulting EPN populations were stored in tissue culture flasks filled with DI water at a temperature of 20.1 °C until needed for bioassays. Concentrations of EPN populations were held at ca 2000 IJs mL⁻¹ to ensure adequate space and oxygen for individual IJ survival. Recently emerged IJs were ideally used shortly after collection, but at latest within 28 days of collection.

Four different nematode species were used in these bioassays: *Steinernema diaprepesi*, *S. khoungi*, *S. glaseri* and *Heterorhabditis bacteriophora*. Both *S. glaseri* and *H. bacteriophora* are prevalent agents in commercial biological control applications, while *S. khoungi* and *S. diaprepesi* are common species found in natural systems near citrus trees in Florida.³¹⁻³³ *S. diaprepesi* and *S. khoungi* respond strongly to pregeijerene and two strains of each (Sd BRT, Sd HK31, Sk ARC and Sk WEB) were isolated from different locations in Florida and used in pregeijerene evaluations. *S. glaseri* and *H. bacteriophora* strains were obtained from commercial vendors (Arbico Organics) and reared in the laboratory.

Volatile organic compounds

With the exception of pregeijerene, plant volatile compounds were purchased from ThermoFisher Scientific at close to 100% purity. Pentane (CAS: 109-66-0) was obtained at 98% purity, while linalool (CAS: 78-70-6), limonene (CAS: 5989-27-5) and α -pinene (CAS: 7785-26-4) were obtained at 97%, 96% and 98% purity, respectively. Each volatile was diluted to 10 mL of 10 ng μ L⁻¹ with pentane as the solvent. Pregeijerene was extracted from the roots of the common rue (*Ruta graveolens*) plant into pentane, quantitated using gas chromatography–mass spectrometry and diluted to 10 mL of 10 ng μ L⁻¹. The dilution was conducted as follows. First, we calculated the appropriate amounts needed of both pentane and the specific solute to arrive at our concentration of

10 ng μL^{-1} . Next, we used micropipettes to extract the calculated amount from the compound container and combined both liquids in a glass vial, performing this step rapidly to reduce potential volatile diffusion. When using these diluted compounds for experimental purposes, we utilized capillary tube pipettes to extract 10 μL from the vial and expel it onto the filter paper of the olfactometer. When switching between different compounds, we also switched to a new capillary tube pipette and additionally rinsed the tube with pentane to prevent contamination. All diluted solutions were produced at the beginning of the experiment and used consistently throughout the experimental process.

Nematode bioassays

To investigate how abiotic factors such as moisture impact EPN behavior, IJs were placed in a PVC two-choice inverted T-tube olfactometer under differing soil moisture regimes (Fig. 1).³⁴ Sand (washed, dried, screened play sand) used in the olfactometer was autoclaved and subsequently mixed with an adequate amount of deionized water to result in soil moisture mixtures of either 6%, 12% or 18% by volume. For pregeijerene trials, moisture concentrations were either 6% or 18%. After conducting the pregeijerene trials and noting the significant differences in EPN response, we opted to add an additional soil moisture level at 12% for the subsequent bioassays. To prepare the olfactometers, a small (ca 1 cm in diameter) piece of filter paper was placed into each side of the olfactometer. Next, capillary tube pipettes were used to apply an amount of 10 μL of a given chemical at a concentration of

10 ng μL^{-1} onto the filter paper (for a total of 100 ng). Immediately following this step, the olfactometer end caps containing the chemicals were filled with the appropriate specific soil moisture treatment. Soils in the endcaps were then lightly tamped and leveled before being connected to the olfactometer body. Ensuring this step was performed immediately after volatile addition minimized the potential for volatile diffusion. To allow for uniform dispersion of volatiles and to reduce the potential impact of confounding variables in volatile diffusion, the following measures were taken: volatile introduction into the olfactometer was performed by the same researcher throughout all experiments, while the olfactometer was filled by the same researcher throughout. These measures were taken to minimize potential differences in sand packing and volatile handling that could have affected volatile diffusion. Additionally, all olfactometers utilized were of the same PVC material to control for potential influence of PVC tube olfactory emissions. After filling the entirety of the olfactometer with the appropriate soil treatment, 1 mL of IJs at known concentration (between 500 and 1000 IJs mL^{-1}) was inserted into an opening in the sand in the center of the olfactometer. The exact concentration of introduced EPNs was recorded by counting the number of EPNs per 1 mL for a given flask under the microscope and averaging this value across three separate counts. For each volatile compound and controls, IJs chose between control (pentane) and treatment (α -pinene, linalool, D -limonene, pregeijerene or additional pentane control). There was a total of ten replications of each combination of moisture treatment, EPN strain and

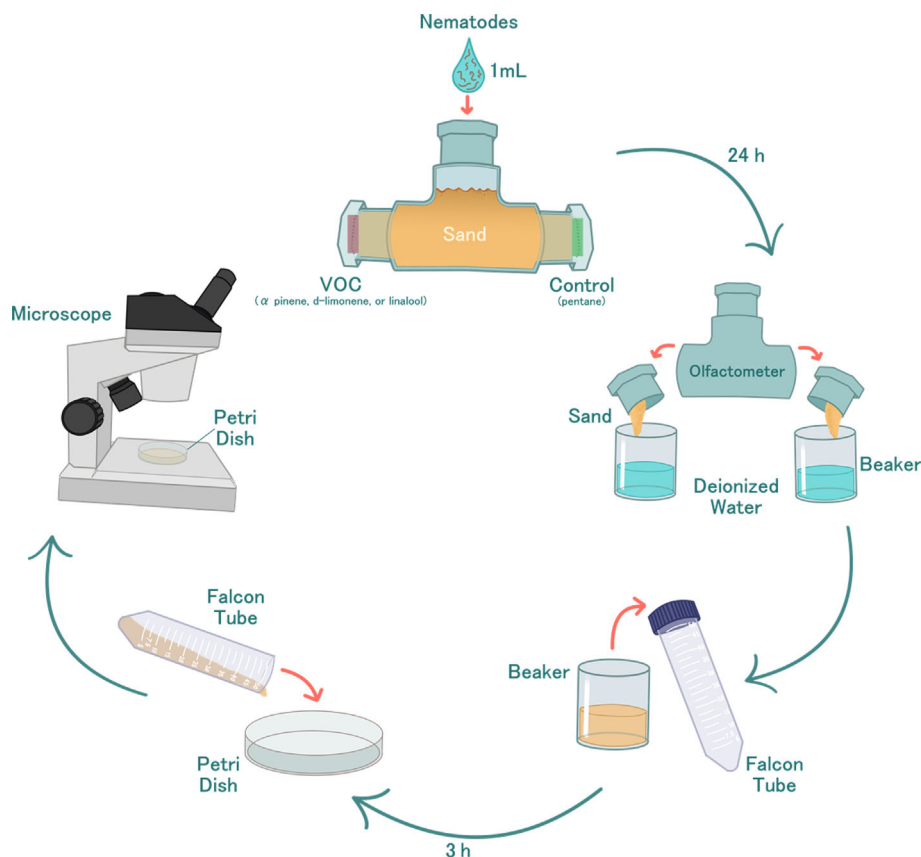


Figure 1. Visual representation of the methodology. An amount of 1 mL of EPNs (between 500 and 1000 IJs mL^{-1}) was deposited into the center of the sand-packed olfactometer (6%, 12% or 18% moisture by volume) with the control and volatile organic compound (VOC) in question on either side. After 24 h, each cap was removed and swirled with DI water to collect nematodes in a conical centrifuge tube. Subsequently, nematodes were transferred to a petri dish and counted under a microscope.

volatile treatment with directional bias reduced by randomizing or alternating the direction of treatment in bioassays. After 24 h, caps on each side of the olfactometer were removed, and the sand was placed into a beaker and swirled with deionized water to allow nematodes to detach from the sand and remain in the liquid solution. The liquid supernatant was decanted and allowed to settle for 3 h to allow nematodes to descend via gravity to the bottom of the flask. Extra liquid was removed and the remaining 10 mL volume containing the nematodes was placed into a petri dish for counting under a stereoscope (Fig. 1).

Recovery rate for each trial was calculated by summing the total number of IJs that responded to either arm of the two-choice olfactometer and dividing by the number of IJs originally inoculated into the olfactometer.

Response rate, and index of preference for the provided treatment versus a pentane blank control, was calculated by dividing the number of IJs responding to a volatile treatment by the total number of IJs responding to either arm. A response rate of 1 indicates all responding IJs responded only to the treatment. A response rate of 0 indicates that all responding IJs responded only to the pentane blank controls. A response rate of 50% indicates that responding IJs showed no preference for the treatment. A response rate of greater than 50% indicates that IJs preferred the volatile treatment; a response rate of less than 50% indicates they were deterred by the volatile treatment.

Statistical analysis

Recovery rate was evaluated using linear models and analysis of variance. Data transformations (square root) were utilized if diagnostic tests and plots indicated large discrepancies from assumptions of normality and homoscedasticity. Best-fit models were chosen after consideration of all potential interactions, residual analysis, goodness of fit metrics and likelihood ratio tests. *Post hoc* treatment comparisons were conducted using the Tukey method for pregeijerene trials or custom contrasts between the lowest and highest moisture levels with the Bonferroni correction for family-wise error rate applied for the other volatile trials. Response rate for pregeijerene trials was evaluated using mixed effects models to account for any possible effects of orientation.

Similarly, best-fit models were chosen after consideration of all potential interactions, residual analysis, goodness of fit metrics and likelihood ratio tests. *Post hoc* treatment comparisons were conducted using the Tukey method. Response rates for other volatile trials did not conform to model assumptions and differences between specific treatments (low and high moisture levels) were evaluated using permutation testing.

Analysis was conducted in R (4.3.1)³⁵ using RStudio as an IDE.³⁶ Supporting packages that were used for data cleaning, analysis and visualization of results include: tidyverse,³⁷ here,³⁸ cowplot,³⁹ car,⁴⁰ emmeans,⁴¹ lme4,⁴² ggfortify⁴³ and doFuture.⁴⁴

RESULTS

Recovery rate

Moisture level significantly affected the recovery rate of EPN IJs in two-choice olfactometers (Fig. 2).

More specifically, moisture ($F = 55.12$, $df = 1$, $P < 0.001$), EPN species ($F = 3.58$, $df = 3$, $P = 0.017$) and their interaction ($F = 7.49$, $df = 3$, $P < 0.001$) significantly affected recovery rate of EPNs in olfactometer trials with pregeijerene (Adj $R^2 = 0.52$, $F = 14.37$, $df = 7.79$, $P < 0.001$; Fig. 2(A)). In pregeijerene trials, increasing the moisture from 6% by volume to 18% by volume

significantly increased the recovery rate of *S. diaprepesi* BRT by $34 \pm 5\%$ (mean \pm standard deviation; $t = 7.42$, $df = 79$, $P < 0.001$), of *S. khoungi* ARC by $17 \pm 5\%$ ($t = 3.15$, $df = 79$, $P = 0.002$) and of *S. khoungi* WEB by $19 \pm 5\%$ ($t = 3.57$, $df = 79$, $P < 0.001$). No significant difference was observed for *S. diaprepesi* HK31.

Moisture ($F = 34.23$, $df = 2$, $P < 0.001$) and EPN species ($F = 10.72$, $df = 1$, $P = 0.001$) also significantly affected recovery rate of EPNs in olfactometer trials with α -pinene, linalool and δ -limonene (Adj $R^2 = 0.22$, $F = 26.4$, $df = 3.273$, $P < 0.001$; Fig. 2 (B)). Neither the interaction between moisture and EPN species, nor the volatile treatment significantly ($P > 0.9$) influenced observed recovery rate. Increasing the moisture from 6% by volume to 18% by volume significantly increased the recovery rate of *H. bacteriophora* and *S. glaseri* by $11 \pm 2\%$ (mean \pm standard deviation; $t > 5.2$, $df = 271$, $P < 0.001$).

Response rate

Moisture significantly affected EPN preference for plant volatiles as measured by their response in two-choice olfactometers (Figs 3 and 4).

Moisture ($\chi^2 = 6.68$, $df = 1$, $P = 0.0097$), EPN species ($\chi^2 = 27.01$, $df = 3$, $P < 0.001$) and their interaction ($\chi^2 = 29.53$, $df = 3$, $P < 0.001$) significantly affected the response of EPN IJs to pregeijerene (Fig. 3). Increasing the soil moisture from 6% to 18% decreased the preference of *S. diaprepesi* BRT for pregeijerene by $16 \pm 6\%$ ($t = 2.58$, $df = 54.1$, $P = 0.01$), the preference of *S. khoungi* ARC for pregeijerene by $18 \pm 8\%$ ($t = 2.33$, $df = 54.1$, $P = 0.02$) and the preference of *S. khoungi* WEB for pregeijerene by $23 \pm 8\%$ ($t = 2.91$, $df = 54.2$, $P = 0.005$). Increasing the soil moisture from 6% to 18% increased the preference of *S. diaprepesi* HK31 for pregeijerene by $21 \pm 6\%$ ($t = 3.43$, $df = 54.5$, $P = 0.001$).

Increasing soil moisture did not similarly affect EPN IJ responses to other plant volatiles for *S. glaseri*. Soil moisture level did not significantly ($P > 0.05$) affect response rate. For *H. bacteriophora*, soil moisture level did not significantly ($P > 0.05$) affect response rate for δ -limonene or α -pinene. Increasing soil moisture levels did significantly increase ($P < 0.001$) response rates to linalool for *H. bacteriophora*, however.

DISCUSSION

Our results suggest that abiotic factors can affect and change EPN behavior both in terms of their recovery from bioassays and in terms of their recruitment towards specific belowground volatile signals. Specifically, our results suggest that EPN behavior can be heavily dependent on soil moisture with ramifications for how we study these organisms, how we monitor their activity in the field and how we manage them for use in biological control.

Increasing levels of soil moisture tend to increase recovery rate of EPN IJs in two-choice olfactometers. Increasing the soil moisture from 6% to 18% increased recovery rate across multiple species of EPN and in assays with multiple plant volatiles. In addition to this consistent pattern, the increases in recovery rate can be substantial; the lowest observed increase was $11 \pm 2\%$ and the highest $34 \pm 5\%$. At first pass, this is not likely to be surprising; there is substantial documentation that moisture level influences EPN behavior specifically and nematode behavior more generally.⁴⁵⁻⁴⁸ Nematodes need water in the soil to survive – not just to prevent desiccation, although there are some adaptations to this situation – but also to move through the soil pore spaces. If

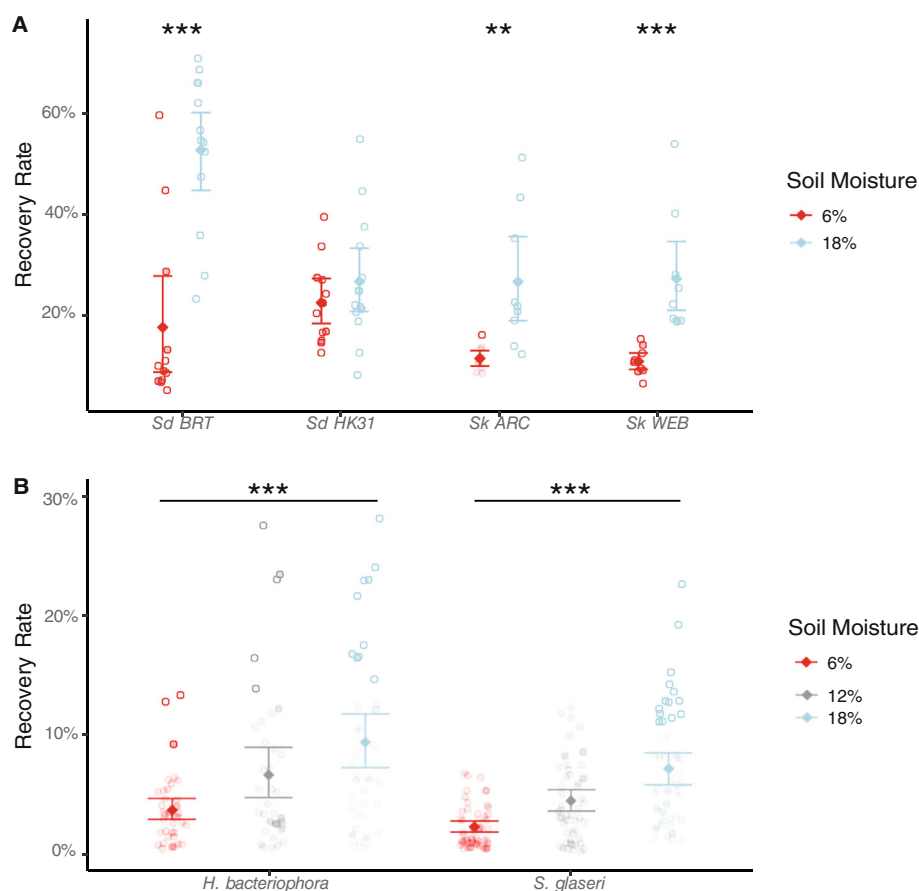


Figure 2. Recovery rate of EPN IJs from two-choice olfactometers with differing levels of soil moisture. Recovery rate is calculated as the total number of EPNs responding divided by the number of EPNs introduced to the olfactometer. (A) Recovery rate of EPNs responding to the plant volatile pregeijerene. (B) Recovery rate of EPNs responding to the plant volatiles α -pinene, linalool and β -limonene. For both plots, open circles denote actual observations. Filled diamonds and error bars denote mean and 95% confidence intervals, respectively. Asterisks denote significant differences between 18% moisture and 6% moisture at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

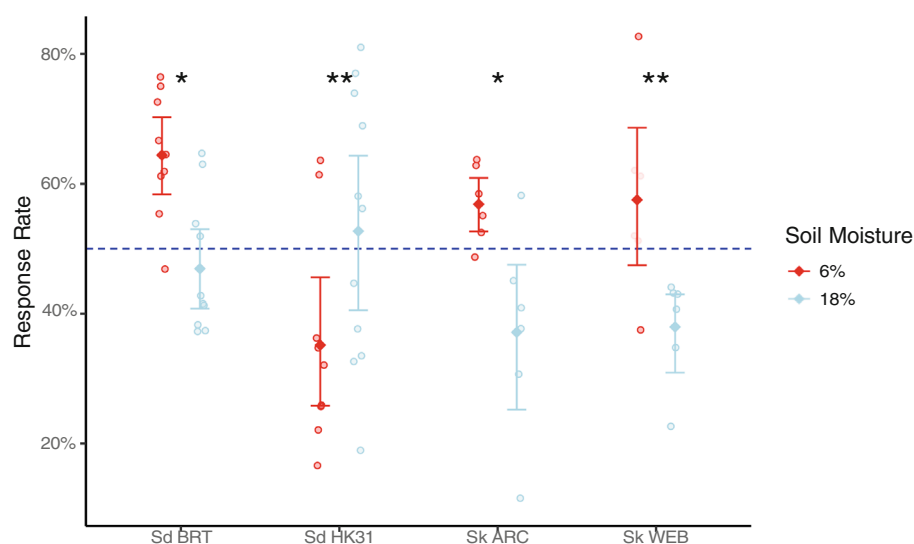


Figure 3. Response rate of EPN IJs to pregeijerene in two-choice olfactometers with differing levels of soil moisture. Response rate is the number of EPNs responding to the volatile treatment (pregeijerene) divided by the total number of EPNs responding in the olfactometer. A response rate of greater than 50% indicates that IJs preferred the volatile treatment; a response rate of less than 50% indicates they were deterred by the volatile treatment. Open circles denote actual observations. Filled diamonds and error bars denote mean and 95% confidence intervals, respectively. Asterisks denote significant differences between 18% moisture and 6% moisture at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

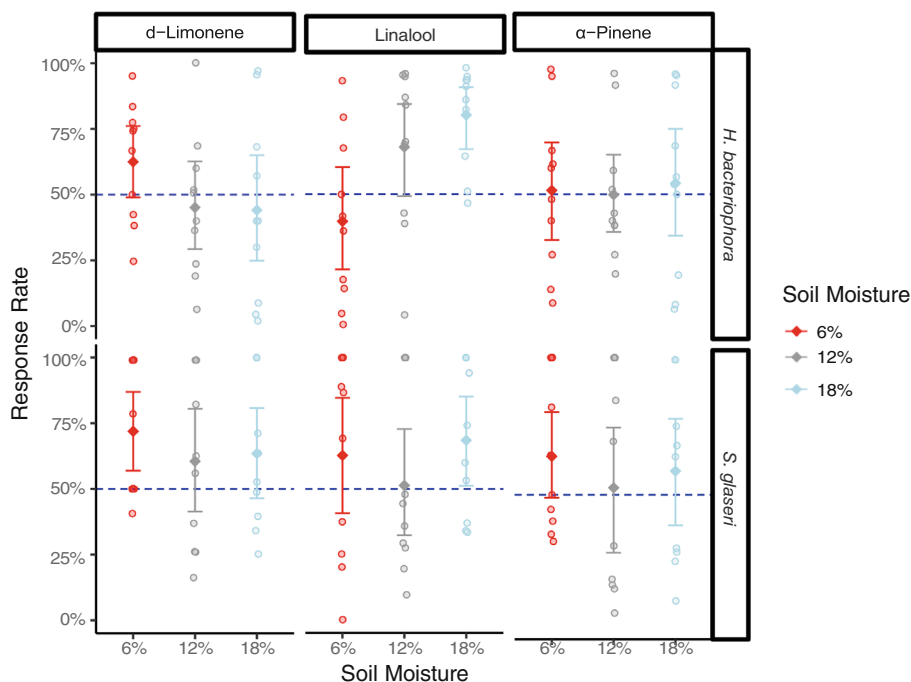


Figure 4. Response rate of two species of EPN IJs to plant volatiles belowground in two-choice olfactometers with differing levels of soil moisture. Response rate is the number of EPNs responding to the volatile treatment (pregeijerene) divided by the total number of EPNs responding in the olfactometer. A response rate of greater than 50% indicates that IJs preferred the volatile treatment; a response rate of less than 50% indicates they were deterred by the volatile treatment. Open circles denote actual observations. Filled diamonds and error bars denote mean and 95% confidence intervals, respectively. Asterisks denote significant differences between 18% moisture and 6% moisture at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

the soil is too dry, locomotion becomes an impossibility. EPNs, *H. bacteriophora* especially, are not particularly desiccation tolerant and will migrate towards moist soil rather than dry soil when given the option.⁴⁵ Alternatively, EPNs can enter into a dormant stage called anhydrobiosis, a process induced by a gradual decline in soil moisture.⁴⁵

Less appreciated though is that soil moisture levels may influence EPN preference and recruitment toward or away from specific volatiles. In the case of *H. bacteriophora* and linalool, for example, linalool was mildly repellent at low moisture levels, but highly attractive at high moisture levels (Fig. 4). Similarly, pregeijerene was repellent to *S. diaprepesi* HK31 at low moisture levels, but less repellent at higher moisture levels (Fig. 3). Conversely, pregeijerene was highly attractive at low moisture levels, but more repellent at higher levels of moisture for other EPN strains (*S. diaprepesi* BRT, *S. khoungi* ARC and *S. khoungi* WEB) (Fig. 3). These variable trends might partly be explained by varying degradation and diffusion patterns of volatiles under different pH and moisture levels.^{6,7}

Considering these interactions between soil moisture and EPN recruitment towards volatile organic compounds, increasing levels of moisture may influence results in bioassays and in field-based biological control situations. A standard often used soil moisture level in EPN bioassays is 10%. Our results suggest that higher levels of soil moisture might result in higher recovery rates from these bioassays if desired. Similar impacts might occur in applied settings with biological control. If locomotion is diminished through lower levels of soil moisture, the number of EPNs responding may be lower.

As the United States makes the move toward sustainable and regenerative agriculture, the greater the need for more efficient integrated pest management techniques. Integrated pest

management is a holistic, multi-faceted approach to pest management, while managing the natural function of ecosystems. As drought conditions become more prevalent in the face of climate change, the effects on crop production systems may include yield reduction and changes in pest requirements.⁴⁹ Integrated pest management has been suggested as a potential mitigation strategy for the negative consequences that drought might have on agricultural production.⁴⁹ To this end, the abilities of EPNs can be leveraged for biological control purposes in agricultural settings and various species within the genera *Steinernema* and *Heterorhabditis* are being produced commercially for this exact application.⁹

The literature has documented the use of EPNs in a variety of agricultural systems⁹: they have been successfully applied in orchards,^{3,11,12,50} small fruits,^{13,14} traditional row crops (maize, vegetables and tubers) and indoor systems such as greenhouses and small-scale mushroom farms.^{15,16,51} Several field studies have shown the efficacy of the application of EPNs in reducing pest infestation, in some cases documenting up to 99% herbivore reduction.^{10,52} Consequently, agricultural yield has been shown to increase as a result of reduced pest infestation provided by EPNs.^{19,20} However, ensuring this significant success in agricultural production depends on favorable soil media, the plant species as well as the specific EPN species used.⁵³ We highlight not only that soil moisture conditions affect the ability of EPNs to move through the soil matrix, as seen in their recovery rate, but also that variable soil moisture conditions change EPN response rate to the various volatile organic compounds emitted by plants. As climate change predictions project changes in soil conditions⁵⁴⁻⁵⁸ particularly with respect to fluctuations in soil moisture levels, the efficacy of EPNs as biological control agents will likely also be affected, due to their sensitivity to soil moisture levels.

Pest management strategies may need to be tailored towards a specific geographic location under consideration of prevailing and future abiotic conditions to ensure maximum success of nematode application. Future investigations might expand on our findings by investigating similar questions in field experiments, as there likely are other variables impacting these behaviors in natural systems.

AUTHOR CONTRIBUTIONS

Dana Frankenstein: Project Formulation, Execution, Analysis and Writing. Macawan S Luu: Project Formulation, Execution, Analysis, and Writing. Jennifer Luna-Ayala: Methodology, Experimental Execution. Denis S Willett: Project Ideation, Project Design, Project Supervision, Writing. Camila C Filgueiras: Project Ideation, Project Design, Project Supervision, Writing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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